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Investigating the role of human peroxiredoxins as damage-associated molecular patterns

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Abstract

Sterile inflammation occurs when endogenous molecules termed damage-associated molecular patterns (DAMPs) are released into the extracellular environment and activate immune receptors to induce the secretion of pro-inflammatory cytokines. As this phenomenon contributes to the pathogenesis of many diseases such as ischemia-reperfusion injury, understanding how DAMPs are able to induce a pro-inflammatory response is of crucial importance for the development of new therapeutic strategies. Recently, human peroxiredoxin (PRDX) family proteins have been reported to act as DAMPs. However, the molecular mechanisms allowing PRDXs to induce a pro-inflammatory response remain to be clarified, starting from the early step of PRDX recognition (*i.e.* binding to receptors) to the late events triggered within the cell (*i.e.* cytokine secretion).

In this PhD thesis, we used human PRDX2 and PRDX5 to investigate the role of (i) Toll-like receptor 4 (TLR4), one of the major innate immune receptors involved in pro-inflammatory responses, (ii) TLR4 accessory molecules MD-2 and CD14, and (iii) the redox state of PRDX cysteine residues, in the PRDX pro-inflammatory activity. To this end, we used a multidisciplinary approach combining biomolecular and biophysical techniques to gain insights into the binding properties of PRDX2 and PRDX5 towards TLR4 and its accessory molecules, as well as interleukin-8 secretion from cells exposed to PRDX2 and PRDX5.

Using these approaches, we confirmed the role of TLR4 in the pro-inflammatory activity of PRDX2 and PRDX5 and further demonstrated that both PRDXs are able to interact directly with TLR4. Moreover, we showed that disulfide PRDX5 triggers potent pro-inflammatory response compared to reduced or hyperoxidized forms of the protein, and that the catalytic cysteine is required for the PRDX5 pro-inflammatory activity. We next observed that MD-2 is required for the TLR4-dependent cytokine-inducing ability of PRDX2 and PRDX5. In addition, we discovered that both PRDXs are able to induce a TLR4-independent signaling pathway involving CD14. We finally reported that PRDX2 and PRDX5 are able to interact directly with both MD-2 and CD14.

Altogether, our results highlight key roles of TLR4, MD-2 and CD14 as well as the importance of the redox state in the pro-inflammatory activity of human PRDXs. This work therefore provides a basis for further studies aiming at understanding the finer modulation of pro-inflammatory responses induced by human PRDXs.

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Preface

When this thesis project started at the end of 2017, it was part of a research project that aimed at investigating the role of extracellular human peroxiredoxins (PRDXs) as mediators of inflammation. This project was a collaborative work involving two laboratories with different expertise: the lab of Prof. Bernard Knoops, specialized in animal cell biology and peroxiredoxins, and the lab of Prof. David Alsteens, specialized in the biophysics of biomolecular interactions taking place at the cell surface.

Initially, this project involved two PhD candidates: one attached to the lab of Prof. B. Knoops, and myself, attached to the lab of Prof. D. Alsteens, supervisor of my thesis project. The objective of the two PhD projects were to understand the mechanisms involved in peroxiredoxin-mediated inflammation, using different approaches: a molecular biology approach within the lab of Prof. B. Knoops, and a biophysics approach in the lab of Prof. D. Alsteens. This would allow each PhD candidate to benefit of knowledge and results of the other, providing a guide to fine tune their research strategy. In particular, the original objectives of my PhD project (as defined in my FRIA grant application) were restrained to the use of atomic force microscopy (AFM). These objectives include: (i) probing the binding of human PRDXs to the surface of living mammalian cells, (ii) probing the direct binding of human PRDXs with their presumed Toll-like receptors in order to characterize the kinetic parameters of the interactions, and (iii) probing the surface of living cells to examine the release of fluorescently-labeled cytokines using an AFM coupled to a confocal microscope.

Unfortunately, the project of the other PhD candidate stopped after two years due to lack of funding. Therefore, the research strategy of my PhD project had to be modified as evidence from a molecular biology approach were also needed to obtain valuable insights into the mechanisms under investigation. As a consequence, the objectives were revised accordingly. Thereby, one of the challenges of this PhD project was to determine which tools and techniques would be best to use and combine (by one researcher) to obtain the most valuable data, and to implement these techniques. As my project shifted to a combined biomolecular-biophysics approach within one PhD thesis, Prof. Bernard Knoops became co-supervisor of my thesis project. In the beginning of 2022, Prof. David Alsteens withdrew from the project and Prof. Bernard Knoops became the unique supervisor.

Given the multidisciplinary aspect of this thesis, technical features are thoroughly presented and discussed within this thesis, together with the studied biological processes.

List of abbreviations

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
ACN	Acetonitrile
AFM	Atomic force microscopy
AIM2	Absent in melanoma 2
ALI	Acute liver injury
ALR	AIM2-like receptor
AP	Alkaline phosphatase
APTES	3-aminopropyl triethoxysilane
AP-1	Activation protein-1
Arg	Arginine
AR2G	Amine reactive second generation
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BF	Binding frequency
BLI	Biolayer interferometry
BSA	Bovine serum albumin
BMDC	Bone marrow-derived dendritic cell
CAM	2-chloroacetamide
CD14	Cluster of differentiation 14
cGAS	Cyclic GMP-AMP synthase
CHO	Chinese hamster ovary
CLSM	Confocal laser scanning microscopy
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
C _P	Peroxidatic cysteine
C _R	Resolving cysteine
Cys	Cysteine
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DFS	Dynamic force spectroscopy
DTT	Dithiothreitol
eCIRP	Extracellular cold-inducible RNA binding protein
EDC	1-ethyl-3-(3-dimethylaminopropyl)
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESI	Electrospray ionization
EV	Extracellular vesicle
FC	Flow cytometry
FD	Force-distance
FF	Fully folded
FITC	Fluorescein isothiocyanate

F _R	Rupture force
GPCR	G-protein-coupled receptor
GRP94	Glucose-regulated protein of 94 kDa
GSDMD	Gasdermin-D
GSH	Glutathione
HA	Hyaluronan
HAMP	Homeostasis-altering molecular process
HEK	Human embryotic kidney
His	Histidine
HMGB1	High-mobility group box 1
HMW	High molecular weight
HRP	Horseradish peroxidase
HSP	Heat shock protein
iBMDC	Immature BMDC
IAM	Iodoacetamide
ICAM-1	Intracellular adhesion molecule-1
IF	Immunofluorescence
IKK	I κ B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP ₃	Inositol-triphosphate
IRF	Interferon response factor
IRI	Ischemia/reperfusion injury
I κ B	Inhibitor of NF- κ B
K _D	Affinity constant
k _{eff}	Effective spring constant
k _{off}	Dissociate rate constant (kinetic off-rate)
k _{on}	Association rate constant (kinetic on-rate)
LBP	LPS-binding protein
LC	Liquid chromatography
Leu	Leucine
LPCAT	Lysophospholipid acyl transferase
LPS	Lipopolysaccharide
LR	Loading rate
LRR	Leucine-rich repeat
LU	Locally unfolded
Lys	Lysine
MARCO	Macrophage receptor with collagenous structure
MAC	Membrane attack complex
MALS	Multiangle light scattering
M-CSF	Macrophage colony-stimulating factor
MD-2	Myeloid differentiation factor-2
MS	Mass spectrometry
MVB	Multivesicular body
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate

NEM	N-ethylmaleimide
NFAT	Nuclear factor of activated T-cells
NF- κ B	Nuclear factor- κ B
NHS	N-hydroxysuccinimide
NLR	NOD-like receptor
NLRC4	NRL containing a caspase recruitment domain 4
NLRP3	NLR family pyrin-domain-containing protein 3
NOD	Nucleotide-binding oligomerization domain
NTA	Nitrilotriacetic acid
oxLDL	Oxidized low-density lipoprotein
PAMP	Pathogen-associated molecular pattern
PDF	Probability density function
PDGF	Platelet-derived growth factor
PEG	Poly(ethylene glycol)
Phe	Phenylalanine
PIP ₂	Phosphatidylinositol bisphosphate
PK	Proteinase K
PLA ₂	Phospholipase A ₂
PLC γ 2	Phospholipase C γ 2
PMA	Phorbol 12-myristate 13-acetate
PMB	Polymyxin B
PMSF	Phenylmethylsulfonyl fluoride
PRAT4A	Protein associated with TLR4
PRDX	Peroxiredoxin
PTS1	Peroxisomal targeting signal type 1
Pro	Proline
PRR	Pattern recognition receptor
PTM	Post-translational modifications
RAGE	Receptor for advanced glycation end products
RIG-I	Retinoic acid inducible gene I
RLR	RIG-I-like receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAMP	Stress-associated molecular pattern
SARM	Sterile α - and Armadillo-motif-containing protein
SEC	Size-exclusion chromatography
Ser	Serine
SFK	Src-family kinase
SMFS	Single-molecule force spectroscopy
Srx	Sulfiredoxin
TCEP	tris(2-carboxyethyl)phosphine
TDM	Trehalose 6,6'-dimycolate
TEAB	Triethylammonium bicarbonate
Thr	Threonine
Th2	T helper type 2
TIR	Toll/interleukin receptor

TIRAP	TIR domain-containing adaptor protein
TG	Thioglycolate
TGF- β 1	Transforming growth factor- β 1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TREM	Triggering receptors expressed by myeloid cells
TRIF	TIR domain-containing adaptor inducing interferon- β
Trx	Thioredoxin
Tyr	Tyrosine
UNC93B1	Uncoordinated 93 homolog B1
6xHis	Hexa-histidine
π GST	π isoform of the glutathione-S-transferase

Chapter I – Introduction

The present thesis project is focused on the role of human peroxiredoxins (PRDXs) as damage-associated molecular patterns (DAMPs). This first chapter will therefore introduce these two topics. Firstly, an overview of PRDXs will be presented. As more than 8000 validated PRDX sequences have been identified so far [1], an emphasis will be made on mammalian PRDXs, expressed as six isoforms (PRDX1–6), encoded by six different genes [2]. Secondly, the role of DAMPs as inducers of inflammation will be discussed. Finally, the activity of mammalian PRDXs as DAMPs will be introduced, bridging the gap between the two topics.

1.1. Overview of the peroxiredoxin family

PRDXs represent a large family of proteins that are unique for several reasons: (i) they are ubiquitous since they are present in all biological kingdoms, in virtually all cell types, in most cellular compartments, and as multiple isoforms (*e.g.* three in *Escherichia coli*, five in *Saccharomyces cerevisiae*, six in *Homo sapiens*, nine in *Arabidopsis thaliana*), (ii) they are highly conserved, (iii) they have a high cellular abundance, which reflects their physiological importance, and (iv) they display various functions, both within and outside the cell [3–5]. These functions will first be summarized before presenting the PRDX classification systems, catalytic reaction mechanisms, structures and conformations. Finally, as this research project mainly addressed human PRDX2 and PRDX5, the key features of these two PRDXs will be outlined.

1.1.1. Functions of peroxiredoxins

All PRDXs possess an active site cysteine (Cys) that allows them to function as peroxidases. This peroxidase activity confers PRDXs different physiological roles; they act as cytoprotective antioxidant enzymes and as modulators of redox signaling. They also show other functions both inside and outside the cell, some of which are restricted to specific PRDXs.

Peroxidase function

PRDXs were first discovered in the yeast *Saccharomyces cerevisiae* as a thiol-specific antioxidant enzyme [6]. They were then broadly characterized as thiol-dependent peroxidases able to reduce hydrogen peroxide (H_2O_2) [3], alkyl hydroperoxides and peroxynitrite [7]. Unlike enzymes that require a prosthetic group, the peroxidase activity of PRDXs relies on a catalytic mechanism that implies conserved redox-active Cys residues. Their peroxidase function was first considered to confer PRDXs only a protective role. By scavenging peroxides, PRDXs are able to

protect the cell from deleterious effects caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) during oxidative stress [8–13].

However, this role evolved with the emergence of the notion that H₂O₂ is an important messenger in signal transduction, which is involved in the maintenance of cellular homeostasis by mediating the regulation of various cell functions [14,15]. The role of PRDXs in redox signaling was first thought to be only indirect. They would serve as regulators of local H₂O₂ concentration by undergoing spatially confined reversible inactivation through hyperoxidation [3,5,16] or phosphorylation at tyrosine (Tyr) or threonine (Thr) residues [17,18]. This reversible inactivation allows the generation of H₂O₂ gradients in specific regions of the cell containing target proteins of H₂O₂ signaling [17,19], but also the limitation of H₂O₂ accumulation to toxic levels and disturbance of global redox potential. This mechanism plays important physiological roles in steroidogenesis [20], circadian rhythmicity [21], growth factor signaling [17], and cell cycle progression [22].

More recently, however, it has been shown that PRDXs have a more direct role in redox signaling by acting as sensors and transducers of H₂O₂ signaling. PRDXs indeed participate in redox relays where oxidative equivalents are transferred from the PRDXs to bound effector proteins that are less susceptible to H₂O₂ oxidation, by thiol-disulfide exchange [23–31].

Additional functions

In addition, PRDXs exhibit other intracellular functions, including a molecular chaperone activity [32–34], a phospholipidase A₂ (PLA₂) activity [35], and a lysophospholipid acyl transferase (LPCAT) activity [36]. Whereas the chaperone activity is common to several PRDXs, PLA₂ and LPCAT activities are restricted to PRDX6 [37]. More recently, PRDXs were also shown to function outside the cell. PRDXs were indeed reported to perform their peroxidase function extracellularly by acting as antioxidant enzymes [38,39]. In addition, PRDXs released extracellularly are able to act as pathogen-associated molecular patterns (PAMPs) or DAMPs, triggering an inflammatory response through activation of innate immune receptors [40,41]. The role of PRDXs as DAMPs will be detailed in **section 1.3**.

Factors influencing peroxiredoxin functions

The functions of PRDXs are governed by a variety of factors that are interconnected and dependent of each other, including subcellular localization, oligomeric state, protein motifs, redox state, and post-translational modifications (PTMs) [42]. These factors will be discussed in **sections 1.1.3** and **1.1.4**.

1.1.2. Classification of peroxiredoxins

Two different systems of classification exist for PRDXs. The first one is based on the conservation of PRDX Cys residues involved in the catalytic mechanism, whereas the second one is based on a bioinformatic approach.

Mechanistic classification

With the identification and characterization of an increasing number of PRDXs, it was realized that they all possess an absolutely conserved N-terminal Cys residue, and that some of them have a second conserved Cys residue [43]. This observation led to the establishment of the first classification system. Based on the presence or location of this additional Cys residue, PRDXs were designated as 1-Cys PRDXs or 2-Cys PRDXs. As the details of their catalytic mechanism were explored, it was evidenced that both Cys residues exhibit catalytic properties. The absolutely conserved Cys residue serves as the site of oxidation by peroxides, with the sulfur attacking the peroxide substrate, and was therefore termed peroxidatic Cys (C_P) [44,45]. When present, the second residue undergoes a condensation reaction with the sulfenic acid generated after the sulfur attack of the peroxide substrate, forming a disulfide bond, and was named resolving Cys (C_R).

Initially, the C_R on the known PRDXs at the time was located in the C-terminus of a partner subunit. But it was later discovered that Cys residues located in other parts of the PRDX protein could also serve as resolving Cys (e.g. the C_R of mammalian PRDX5 is located on the C-terminal domain of the same subunit as the C_P). Therefore, PRDXs were historically divided into three classes: typical 2-Cys PRDXs, which include mammalian PRDX1–4, atypical 2-Cys PRDXs, including mammalian PRDX5, and 1-Cys PRDXs, including mammalian PRDX6 [46].

Global evolutionary classification

As more PRDXs were discovered, it became clear that this mechanistic classification had limitations. More recently, a second classification system emerged, based on bioinformatic analysis of crystal structures and sequences [4,46,47]. This global evolutionary classification divide PRDXs into six subfamilies that are denoted based on the name of isoform archetypes: AhpC/Prx1 (abbreviated Prx1), Prx5, Prx6, Tpx, BCP/PrxQ (abbreviated PrxQ), and AhpE. These subfamilies vary in oligomeric states and interfaces between subunits, as well as in the locations of the C_R [47]. Mammalian PRDX1–4 are included in the Prx1 subgroup, while mammalian PRDX5 and PRDX6 belong to the Prx5 and Prx6 subfamilies, respectively. Mammalian cells do not express isoforms of the Tpx and PrxQ subfamilies.

1.1.3. Peroxidase reaction mechanism of peroxiredoxins

Most, if not all, PRDX functions are related to the redox state of their redox-active Cys residues and, therefore, their peroxidase activity. The catalytic mechanisms for mammalian PRDXs are shown schematically in **Figure 1**. It consists of three steps: (i) peroxidative reduction, (ii) resolution, and (iii) regeneration.

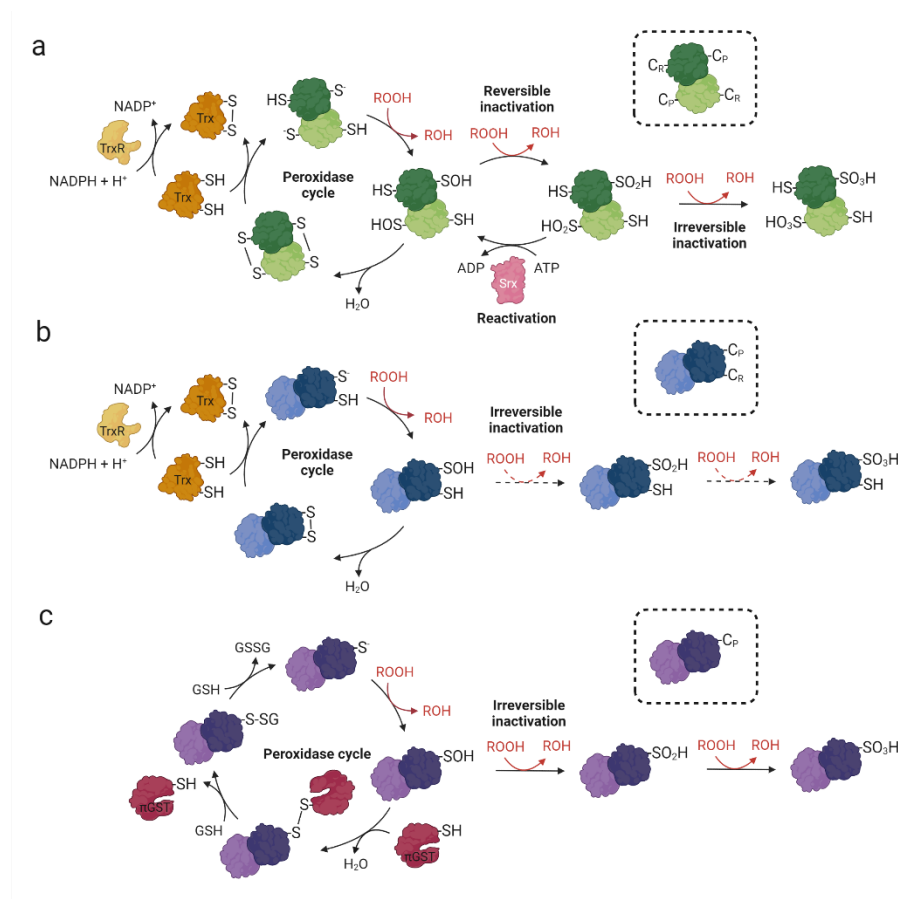


Figure 1. Reaction mechanisms of mammalian PRDXs¹. The mechanisms of **(a)** mammalian PRDX1–4 (2-Cys PRDXs), **(b)** mammalian PRDX5 (atypical 2-Cys PRDX), and **(c)** mammalian PRDX6 (1-Cys PRDX) are shown. Position of the peroxidatic Cys (C_P) and the resolving Cys (C_R) is schematically shown on the PRDXs in the dashed frames. The first step of the catalytic cycle, which consists in the oxidation of the C_P thiolate ($-S^-$) to sulfenic acid ($-SOH$), is common for the three subgroups. The next step, *i.e.* disulfide formation, and the final step, *i.e.* regeneration, of the catalytic cycle, varies depending on the subgroup. **(a)** For 2-Cys PRDXs, the C_P -SOH of one subunit of the homodimer undergoes a condensation reaction with the C_R located in the second subunit. The disulfide bond is reduced by the thioredoxin (Trx) system, which consists of Trx, thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide

¹ Although PRDXs can oligomerize, they were depicted as dimers for more clarity.

phosphate (NADPH). 2-Cys PRDXs can be hyperoxidized to sulfinic acid ($-\text{SO}_2\text{H}$), resulting in PRDX catalytic inactivation. The sulfinic acid ($-\text{SO}_2\text{H}$) can be reduced by the adenosine triphosphate (ATP)-dependent activity of sulfiredoxin (Srx). 2-Cys PRDXs can also undergo irreversible hyperoxidation to sulfonic acid ($-\text{SO}_3\text{H}$). **(b)** In PRDX5, formation of the disulfide bond occurs within the same subunit and is subsequently reduced by the Trx system. Hyperoxidation of PRDX5 has been observed on the recombinant protein. **(c)** As PRDX6 lacks a C_R residue, the C_P sulfinic acid ($-\text{SOH}$) is resolved by a Cys thiol provided by the π isoform of glutathione-S-transferase (πGST), thereby leading to a heterodimeric disulfide, the latter being reduced by the successive action of two glutathione (GSH) molecules that are converted to oxidized glutathione (GSSG). Hyperoxidation of PRDX6 can occur and is irreversible. Created with BioRender.com.

Catalytic mechanism of peroxiredoxin enzymes

The first step of the PRDX catalytic mechanism consists in the oxidation of the PRDX C_P thiol ($-\text{SH}$) by a peroxide substrate to generate a sulfinic acid ($-\text{SOH}$) [48]. In the second step, depending on the PRDX subfamily, the sulfinic acid undergoes a condensation reaction with: (i) a thiol on an adjacent subunit to form an inter-subunit disulfide in the 2-Cys subfamily (mammalian PRDX1–4) [49] (**Figure 1a**), (ii) a thiol on the same subunit to form an intra-subunit disulfide in the atypical 2-Cys subfamily (mammalian PRDX5) [50] (**Figure 1b**), or (iii) the π isoform of the glutathione-S-transferase (πGST) to form a heterodimeric disulfide in the 1-Cys subfamily (mammalian PRDX6) [51] (**Figure 1c**). Finally, the last step corresponds to the reduction of the disulfide by a reductant protein. In the case of mammalian PRDX1–5, this role is fulfilled by a thioredoxin (Trx) [52], while glutathione (GSH) is the reductant protein for mammalian PRDX6 [53].

Catalytic power of peroxiredoxins

The C_P has an exceptional reactivity towards peroxides as it is oxidized very rapidly with rate constants for the reduction of H_2O_2 ranging from 1×10^6 to $10^8 \text{ M}^{-1}\text{s}^{-1}$ [54], which are 4 to 8 orders of magnitude faster than the same reaction with a low-molecular weight thiol as reductant [55]. Two structural features account for the catalytic power of PRDXs: (i) the C_P sulfur atom is surrounded by basic amino acid residues, and (ii) an extensive hydrogen bond network is established in the active site in the transition state (**Figure 2**) [13].

On one hand, the basic amino acid residues, namely arginine (Arg) and histidine (His), confer a positively charged microenvironment to the C_P thiol that lowers its pK_a by stabilizing the anionic thiolate, and thereby facilitates nucleophilic attack on the peroxide substrate. As the reference pK_a for Cys thiol is ~ 8.3 [56], *i.e.* above neutral pH, most protein sulfhydryl groups in biological systems are in their protonated (thiol) form. In contrast, the pK_a of PRDX C_P thiol is much lower, with values between 5.1 and 6.3 [57–62].

On the other hand, all PRDXs contain a conserved active site conformation in which the C_P is surrounded by three additional conserved residues, namely proline (Pro), Thr, and Arg. The Pro, Thr, and C_P residues are within a conserved PXXTXXC_P sequence motif (where X is any amino acid, and T is replaced by a serine (Ser) residue in rare cases). The Arg residue is distant from the C_P in the primary structure (amino acid sequence) but located in the active site in the tertiary structure (three-dimensional fold). In the transition state, these residues (C_P, Pro, Thr, Arg) are involved in a hydrogen bond network that (i) provides a binding site for the peroxide substrate, allowing its tight binding, proper orientation (*i.e.* alignment) and polarization for attack by the C_P sulfur atom, and (ii) lowers the activation energy of the reaction (*i.e.* bond-breaking and bond-forming process) by stabilizing the transition state intermediate [63]. Mammalian PRDX1–4 and PRDX6, but not PRDX5, contain an additional conserved Arg residue that participates in the hydrogen bonding network [64].

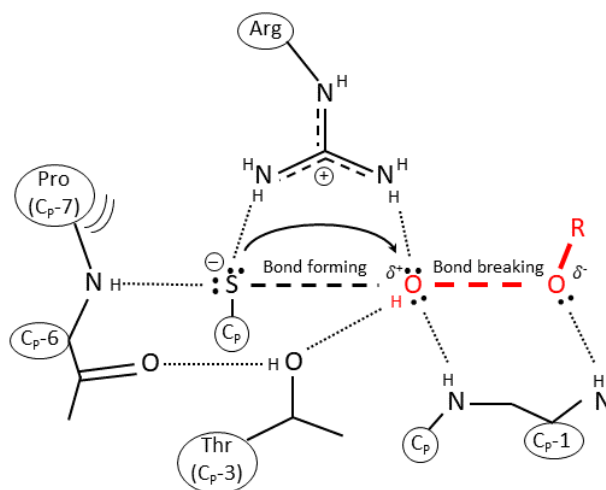


Figure 2. Proposed active site organization of PRDXs in the transition state. The low pK_a of the C_P and the extensive hydrogen bond network account for the catalytic power of PRDXs. Thr (C_P – 3) and Pro (C_P – 7) residues are located within a conserved PXXTXXC_P active site motif, and a conserved Arg residue is present in the active site. The peroxide substrate is shown in red. Adapted from [63,65].

Catalytic inactivation of peroxiredoxins through hyperoxidation

Hyperoxidation can also occur *in vivo* for typical 2-Cys PRDXs, resulting in catalytic inactivation. The sulfenic acid (-SOH) can be oxidized to a sulfinic acid (-SO₂H) [66], which is reduced by the adenosine triphosphate (ATP)-dependent enzyme sulfiredoxin (Srx) [67]. In addition, 2-Cys PRDXs can undergo irreversible hyperoxidation to sulfonic acid (-SO₃H) [68] (**Figure 1**). PRDX6 is also able to undergo

irreversible hyperoxidation to sulfinic or sulfonic acid in cells treated with H_2O_2 [69,70]. Whereas recombinant PRDX5 can be hyperoxidized with a high concentration of H_2O_2 , hyperoxidized PRDX5 could barely be detected in lysates of H_2O_2 -treated cells [69].

The sensitivity of 2-Cys PRDXs to hyperoxidation varies widely within organisms, with eukaryotic PRDXs being more sensitive than their prokaryotic counterparts. There is also an additional stratification of hyperoxidation sensitivity among PRDXs of the same class. For example, human PRDX3 is significantly more resistant to hyperoxidation than human PRDX1 and PRDX2 [71,72], and human PRDX1 is more susceptible to inactivation by H_2O_2 than human PRDX2 [73].

This differential sensitivity can be explained by (i) a conformational model [3] (detailed in **section 1.1.4.**), and (ii) conserved motifs [3,74]. Many eukaryotic PRDXs, but no prokaryotic PRDXs possess two motifs that render them sensitive to hyperoxidation: a glycine-glycine-leucine-glycine (GGLG) and a C-terminal tyrosine-phenylalanine (YF) motifs [3], located near the active site (**Figure 3a**).

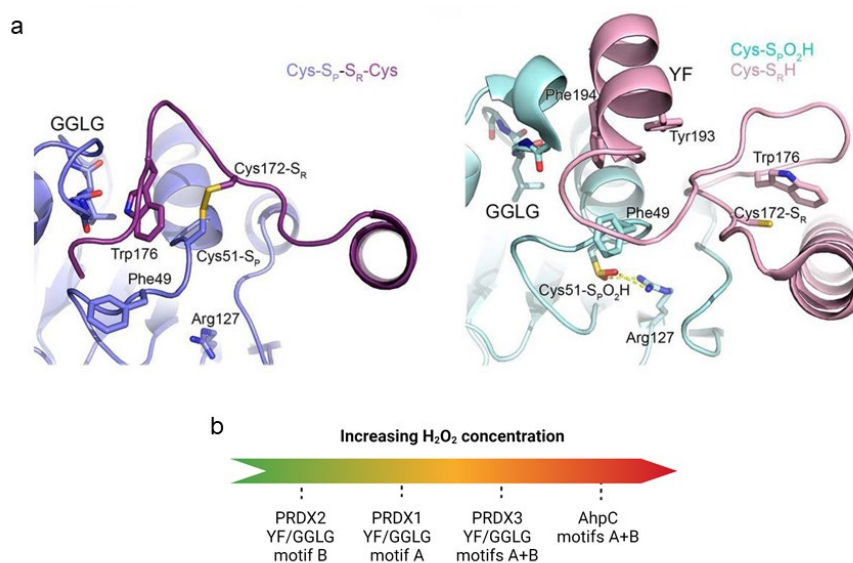


Figure 3. Hyperoxidation of PRDXs. (a) Crystal structure of active site structure of PRDX2 underlying GGLG and YF motifs involved in hyperoxidation. Disulfide PRDX2 (Cys-S_P-S_R-Cys, left) and hyperoxidized PRDX2 (Cys-S_PO₂H, Cys-S_R-H, right) are represented. In hyperoxidized PRDX2, the YF motif (residues 193 and 194) is located in a helix that packs against the GGLG motif, thereby hindering the C_P. PDB ID: 1QMV [75]. Adapted from [74]. **(b)** Model of the stratified PRDX susceptibility to hyperoxidation. Each PRDX is inactivated at a different H_2O_2 concentration threshold, accounting for the diversity of the cellular responses. Inspired from [74].

In addition, two resistance motifs were discovered more recently, referred to as motifs A and B [74]. Motif A is $DX_8(N/G)X_{10}HX_{27}(S/G)$, and motif B is $TX_3(S/T)$ [74]. For instance, human PRDX1 only possesses motif A, whereas human PRDX2 lacks motif A and has motif B, and human PRDX3 own both [74]. These motifs confer PRDXs with a stratified susceptibility to hyperoxidation, so that they are inactivated at different H_2O_2 threshold levels (**Figure 3b**), enabling diverse cellular responses.

1.1.4. Structure and conformation of mammalian peroxiredoxins

Tertiary structure

PRDXs have a compact, globular tertiary structure based on a Trx fold [76] with the common core being composed of seven β -strands ($\beta 1$ – $\beta 7$) and five α -helices ($\alpha 1$ – $\alpha 5$). Five β -strands ($\beta 5$, $\beta 4$, $\beta 3$, $\beta 6$, $\beta 7$) form a central twisted β -sheet that is covered by $\beta 1$, $\beta 2$, $\alpha 1$ and $\alpha 4$ on one face, and by $\alpha 2$, $\alpha 3$ and $\alpha 5$ on the other face [77] (**Figure 4a**).

Oligomeric states

All mammalian PRDXs are obligatory dimers [13]. They all form homodimers, but their dimerization interface varies depending on the subgroup (**Figure 4a**). Mammalian 2-Cys PRDX1–4 and mammalian 1-Cys PRDX6 dimers are formed through interactions at the edges of their central β -sheets, called the B-type interface, to form an extended β -sheet, whereas mammalian atypical 2-Cys PRDX5 dimerizes through interactions at an alternative interface, called the A-type interface (denoting ‘alternate’ or ‘ancestral’), near the $\alpha 3$ helix.

Mammalian 2-Cys PRDXs dimers can form a decameric (pentamer of dimers) or a dodecameric (hexamer of dimers) toroid structure by establishing interactions through their A interfaces (*i.e.* interaction of five or six B-type dimers) [75,78–81]. These toroid structures can further oligomerize by stacking with one another to form high molecular weight (HMW) structures [82–84]. Reduced 2-Cys PRDXs exist in a dynamic equilibrium between dimer and decamer/dodecamer, with the decamer/dodecamer being favored [85] (**Figure 4b**). Upon oxidation, the decamer/dodecamer state is destabilized, leading to its dissociation into dimers [86]. Hyperoxidation favors the decamer and promotes stacking of the decamers to form HMW structures [68,87–89]. On the contrary to typical 2-Cys PRDXs, mammalian atypical 2-Cys PRDX5 exists as a dimer irrespective of the redox state [90]. Oxidation of mammalian 1-Cys PRDX6 has been shown to destabilize the dimer, leading to the formation of monomeric species [51,91–93].

The oligomeric state of PRDXs is influenced by various other factors than the redox state, including pH, PTMs, ionic strength, presence of affinity tag, and protein concentration [78,89,94–100].

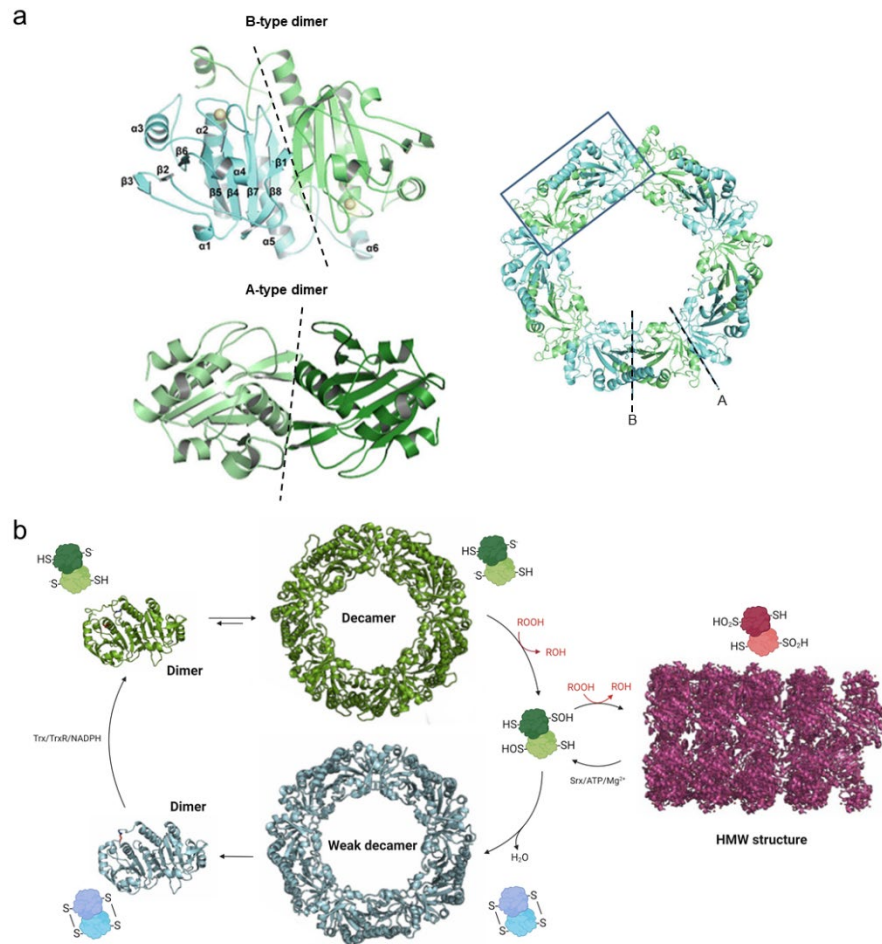


Figure 4. Crystal structures representative of mammalian PRDX quaternary structures. (a) PRDX dimers are formed either through a B-type interface (left, mammalian PRDX1–4 and PRDX6) or through an A-type interface (middle, mammalian PRDX5). Decameric structures (right) are formed through interaction of five B-type dimers (inset) *via* the A-type dimer interface. PDB ID: 4OWY (A-type, Ahp1 from *Saccharomyces cerevisiae*), 3TJB (B-type and decamer, human PRDX4). Adapted from [82,101]. **(b)** Structures representative of the oligomeric states of mammalian PRDXs. There is a dynamic equilibrium between reduced PRDX dimers and reduced PRDX decamers, with the decameric state being favored. Oxidation loosens the decamers, leading to its breakdown into dimers. Hyperoxidation favors the decamer and promotes stacking of the decamers to form high molecular weight (HMW) structures. The structure depicted in green and blue is *Salmonella typhimurium* AhpC. PDB ID: 4MA9 (reduced form, green), 1EYP (oxidized form, blue), and 5JCG (HMW structure, purple, human PRDX3). Adapted from [42].

Conformational states

Regarding their conformation, PRDXs can switch from a fully folded (FF) to a locally unfolded (LU) conformational state [3] (**Figure 5**). The FF conformation is required for the formation of the peroxide binding pocket, leading to the oxidation of the C_P thiolate to sulfenic acid (C_P-SOH). On the other hand, the formation of the inter- or intramolecular disulfide requires a structural rearrangement that generates the LU conformation. This conformational change consists in the local unfolding of the active site in order for the C_P-SOH to be exposed and react with another thiol. There is a dynamic equilibrium between the two conformers until disulfide formation, and the FF conformation is restored when the disulfide is reduced, completing the catalytic cycle.

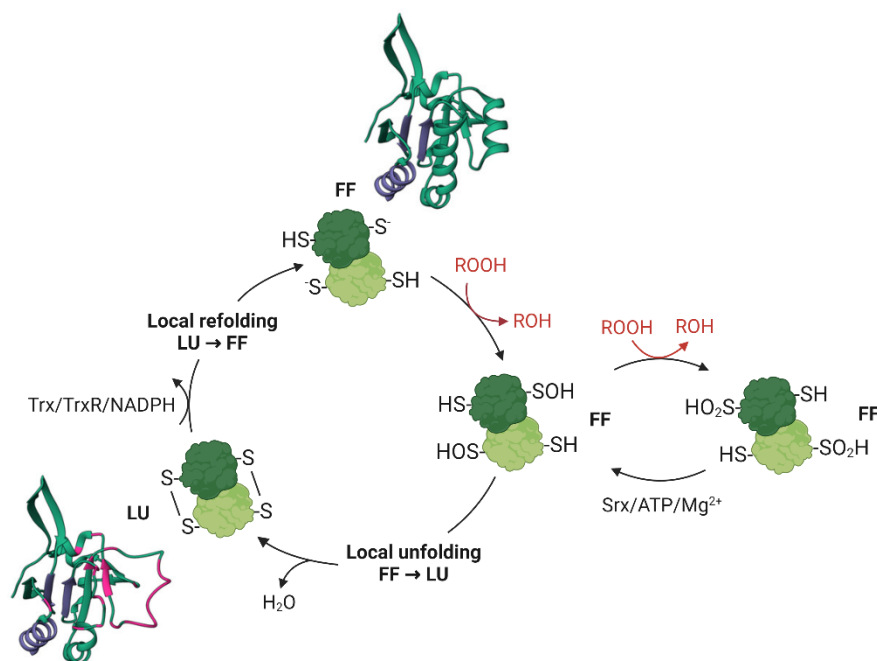


Figure 5. Conformational states of PRDXs. PRDXs exist in both fully folded (FF) and locally unfolded (LU) conformational states. Reaction of PRDX C_P with a peroxide substrate requires the FF conformation in which the peroxide binding pocket is formed. The formation of the disulfide bond requires a structural rearrangement that consists in the local unfolding of the active site to generate a LU conformation in which C_P-SOH is exposed and reacts with C_R-SH. The resulting disulfide bond is reduced by the Trx system to restore the FF state, completing the peroxidase cycle. The C_P sulfenic acid (-SOH) in the FF state can enter another pathway resulting in hyperoxidation in sulfinic acid (-SO₂H). The crystal structure of a representative PRDX (PDB ID: 5IIZ and 5IOX, *Xanthomonas campestris* PrxQ) is shown to represent the local unfolding (adapted from [102]). Created with BioRender.com.

As mentioned earlier, the conformational state accounts for the differential susceptibility of PRDXs to hyperoxidation. Once formed, the C_P sulfinic acid can either form a disulfide with another thiolate, which requires the LU conformation, or react with a second peroxide to form C_P sulfinic acid (-SO₂H) as the FF conformation has an intact peroxide binding site. Therefore, the sensitivity of PRDXs to hyperoxidation depends on the competition between the hyperoxidation rate and the rate of disulfide formation. PRDXs in which the local unfolding step is favorable will be more resistant to hyperoxidation. The rate of unfolding is controlled by the two sequences motifs discussed above, namely GGLC and YF. These motifs actually promote hyperoxidation by allowing the C-terminus to cover the active site in the FF conformation, thereby hindering the local unfolding required for disulfide formation [3,4,79].

1.1.5. Human peroxiredoxin-2 and peroxiredoxin-5

As this research project involved human PRDX2 and PRDX5, this section will summarize the key features of these two PRDXs, namely their structures, oligomeric states, redox status, PTMs and main functions.

Human peroxiredoxin-2

Human PRDX2 is a highly abundant ~22 kDa-protein located in the cytosol, nucleus and plasma membrane [65,103,104]. In addition to the two conserved catalytic Cys residues (C51 and C172), it contains a third Cys residue (C70). PRDX2 exists in an equilibrium of dimers and decamers in solution [105,106]. Disulfide PRDX2 is preferentially present as dimers, whereas reduced or hyperoxidized PRDX2 tends to form decamers (**Figure 6a**). Upon oxidation, it was observed that intermolecular disulfide bond formation in one or two dimers within the decamer was sufficient to trigger complete decamer dissociation into dimers [86]. In the hyperoxidized form, a salt bridge between the C_P sulfinic acid group and Arg¹²⁷ may further stabilize the decamer [3,4]. Whereas the PRDX2 sulfinic form may undergo reversible reduction by Srx, a persistent state of elevated H₂O₂ levels leads to an irreversible hyperoxidized form, *i.e.* sulfonic acid. In addition, high H₂O₂ levels and hyperoxidation promote decamer stacking into HMW structures [86].

Human PRDX2 is regulated *via* PTMs other than oxidation, including phosphorylation (on Thr⁸⁹, Ser¹¹², and Thr¹⁸²), acetylation (on Ala² and Lys¹⁹⁶), S-glutathionylation (on Cys⁵¹ and Cys¹⁷²), and S-nitrosylation (on Cys¹⁵¹ and Cys¹⁷²) [42,107]. Acetylated PRDX2 has been shown to have an increased peroxidase activity, a higher resistance to hyperoxidation, and is less prone to transition to HMW complexes [108]. N-terminal acetylation of PRDX2 was also reported to prevent irreversible hyperoxidation [109]. S-nitrosylation and phosphorylation of PRDX2

Thr⁸⁹ are associated with an inhibition of the peroxidase activity [107]. In particular, S-nitrosylation of PRDX2 in Parkinson's disease was suggested to contribute to the neurodegeneration process by inhibiting its peroxidase activity, hence worsening oxidative stress [110]. Phosphorylation of Thr⁸⁹ also enhances PRDX2 decamer formation and chaperone activity [107]. Glutathionylated PRDX2 was found to be released by macrophages treated with lipopolysaccharide (LPS) [111] and was observed to protect the enzyme against hyperoxidation [107].

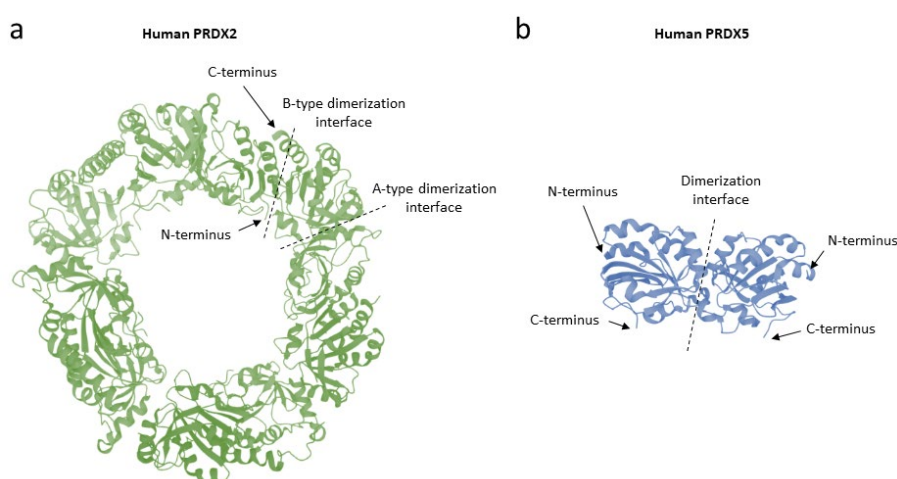


Figure 6. Crystal structure of human PRDX2 and PRDX5. (a) Decameric human PRDX2. The location of A-type and B-type interfaces is depicted. PDB ID: 5IJT. Adapted from [74]. **(b)** Dimeric PRDX5. PDB ID: 1HD2. Adapted from [50]. **(a,b)** The location of the N-terminus and C-terminus is shown.

Human PRDX2 is highly reactive with various peroxides. It reacts with H₂O₂ with a rate constant of $1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ [112]. Besides its role as cytoprotective antioxidant enzyme [113,114], human PRDX2 has been shown to regulate locally H₂O₂ concentration, hence to modulate signaling pathways, such as platelet-derived growth factor (PDGF) signaling [115]. Human PRDX2 was also demonstrated to act as a H₂O₂ signal sensor and transmitter in redox signaling. Notably, PRDX2 was reported to form a redox relay with the transcription factor STAT3, allowing the transfer of oxidative equivalents from PRDX2 to STAT3, leading to the generation of disulfide-linked STAT3 oligomers with attenuated transcriptional activity [24]. In addition, human PRDX2, together with PRDX1, has been shown to transfer its disulfide oxidation state to high-mobility group box 1 (HMGB1), allowing HMGB1 disulfide formation, which is essential for nucleocytoplasmic translocation and secretion of the protein [31]. The redox-dependent conformational change of PRDX2 to HMW structures assigns PRDX2 a molecular chaperone function [33]. It was notably shown that the chaperone function of human PRDX2 inhibits HeLa cell death

induced by H₂O₂ [33]. Finally, extracellular PRDX2 is able to induce secretion of pro-inflammatory cytokines, thereby acting as DAMP [116,117]. This role will be discussed in **section 1.3**.

Human peroxiredoxin-5

Human PRDX5, a ~17-kDa protein, is the only mammalian atypical 2-Cys PRDX, and has the most divergent amino acid sequence among mammalian PRDXs [118,119]. It is constitutively expressed in virtually all tissues at different levels [52,120,121]. It also exhibits a wider subcellular distribution compared with the other mammalian PRDXs, being localized in the cytosol, nucleus, mitochondria, and peroxisomes [52,120,122–124]. The subcellular targeting of human PRDX5 is the consequence of the existence of two protein isoforms, a long form (L-PRDX5) and a short form (S-PRDX5), that are produced by alternative transcription start sites and two alternative in-frame translation initiation sites [90,120,125]. L-PRDX5 contains a mitochondrial targeting sequence (MTS) or presequence at the N-terminal part, that is cleaved after mitochondrial import, converting the precursor protein to a mature mitochondrial form that cannot be distinguished from S-PRDX5 [120,125]. Both forms possess a weak C-terminal peroxisomal targeting signal type 1 (PTS1) [120,122].

Human PRDX5 contains three Cys residues (C47, C72 and C151), among which two of them (C47 and C151) are involved in the catalytic mechanism [126]. PRDX5 exists as an homodimer in solution [79] (**Figure 6b**), irrespective of the redox state of its Cys residues [52,90]. Unlike typical 2-Cys PRDXs, PRDX5 dimers are not antiparallel and dimerization occurs through an alternative interface [4,90]. As human PRDX5 lacks the GGLC and the YF motifs, it appears to be more resistant to hyperoxidation than are typical 2-Cys human PRDXs [69,127]. Accordingly, it was also reported that Srx does not interact with PRDX5 [128]. Regarding other PTMs than oxidation, human PRDX5 has been shown to be regulated by covalent attachment of coenzyme A (CoAlation) to C47 in cellular response to oxidative and metabolic stress [129]. In addition, PRDX5 Ser, Thr, Asn, or Lys residues may be modified through phosphorylation, acetylation, ubiquitination, glutathionylation [130], or succinylation [42,131]. However, the functional significance of such PTMs remains to be investigated.

Human PRDX5 preferentially reduces alkyl hydroperoxides and peroxynitrite (rate constants in the range of 10^6 to 10^7 M⁻¹s⁻¹) over hydrogen peroxide (rate constant of $\sim 10^5$ M⁻¹s⁻¹) [57,132]. The cytoprotective antioxidant function of human PRDX5 has been extensively studied [2,52,133–142]. In addition, human PRDX5 was reported to interact with signaling proteins, including JAK2 to modulate the JAK2-STAT5 pathway [143], and dihydrolipoamide branched-chain transacylase E2 [144].

Although these interactions were shown to depend on the presence of the C_P, it remains unclear whether PRDX5 is involved in a redox relay with these proteins [25]. In addition, human PRDX5 has been demonstrated to act as DAMP when released extracellularly [116], as detailed in **section 1.3**.

1.2. Damage-associated molecular pattern-induced inflammation

To fully understand how PRDXs act as DAMPs, an overview of innate immunity and inflammation is required. This second section will therefore introduce these two concepts before focusing on the definition, release mechanisms and main sensors of DAMPs. An emphasis will be made on Toll-like receptors (TLRs) as they are the main receptors involved in the regulation of innate immune responses. As TLR4 and its accessory molecules myeloid differentiation factor-2 (MD-2) and cluster of differentiation 14 (CD14) were central to the present study, this section will end with a detailed presentation of these proteins.

1.2.1. Overview of innate immunity and inflammation

The immune system is a host defense system comprising complex cellular and molecular mechanisms that aims at protecting the body from foreign organisms or injury in order to maintain or restore homeostasis. The immune system can be divided into innate (or native) and adaptive (or acquired) immune systems, that are intimately interconnected, but differ notably in terms of response timeline, specificity, mechanisms, and duration [145]. Innate immunity, which consists of the first line of defense, is immediate, antigen-independent (*i.e.* the same response is mounted to a wide variety of agents), and has no immunological memory (*i.e.* responds the same way to repeated exposures) [145]. On the other hand, adaptive immunity develops over several days, is antigen-specific, and has the ability to respond with increased magnitude, rapidity and effectiveness to repeated exposures (memory) [145]. As DAMPs are components of the innate immune system, capable of inducing a pro-inflammatory response, this section will first introduce the concepts of innate immunity and inflammation.

Innate immune responses

The two major types of responses by which the innate immune system deals with infection and tissue injury are inflammation and antiviral defense [146,147]. Inflammation is a multi-step, coordinated process of vascularized tissue which is triggered within seconds to minutes after detection of infection or tissue injury [148]. It consists in the recruitment of circulating cellular components and soluble factors from the blood to the sites of infection or injury to eliminate the causative agent and repair the damage [149]. Antiviral defense consists of changes in the cells, *i.e.* synthesis or activation of proteins, to block infection, prevent virus replication, and destroy or inhibit virus-infected cells [150].

In addition to inflammation and antiviral response, the innate immune system involves physical and chemical barriers (*e.g.* skin, mucous membranes of the respiratory system, acidic pH of the stomach) [149], as well as several soluble components, such as the proteins of the complement system [151]. The complement system consists in an assembly of proteins that form a proteolytic cascade. Complement activation results in the generation of effectors molecules that target various cells involved in the immune response [151].

Inflammation

Inflammation is induced by different stimuli that can be broadly categorized as infectious (*e.g.* virus, bacteria, protozoa), physical (*e.g.* trauma, radiation), chemical (*e.g.* toxins, osmolarity), and metabolic (*e.g.* ischemia, hypoxia) [152,153] (**Figure 7**). Inflammation is termed (i) infectious when it is mediated by pathogenic microorganisms, and (ii) sterile when it is initiated by non-microbial stimuli such as mechanical trauma or ischemia [154]. The physiological role of inflammation is protective as it aims at controlling infection and promoting tissue repair to restore homeostasis.

Cellular components involved in inflammation include leukocytes (*e.g.* monocytes, lymphocytes, neutrophils), platelets, mast cells, endothelial cells, and fibroblasts [145]. Typically, neutrophils are the first to arrive at the site of infection or injury and are the most abundant leukocytes in early inflammation. On the other hand, monocytes, the precursors of tissue macrophages, are prominent in the late inflammatory response [148]. Soluble factors that play a role in inflammation comprise complement proteins (*e.g.* C1q, C3), acute-phase reactants (*i.e.* pentraxins), collectins and ficolins [155]. The delivery of cells and soluble factors to the inflammatory sites is dependent on changes in blood vessels in the infected or damaged tissue induced by mediators initially derived from resident cells in the tissue [145]. These reversible changes include increased blood flow due to arteriolar dilatation, increased adhesiveness of circulating leukocytes to the endothelial lining of venules, and increased permeability of the capillaries and venules to plasma proteins and fluid.

Inflammation can be classified as either acute or chronic [149]. Acute inflammation is a short-term response, taking place during a few days and ended by a process called resolution. However, persistent or excessive inflammatory stimuli can lead to prolonged inflammation, known as chronic inflammation, which is deleterious for the host. Chronic inflammation has been linked to many pathologies and diseases, including cancer, autoimmune diseases, and cardiovascular diseases [156].

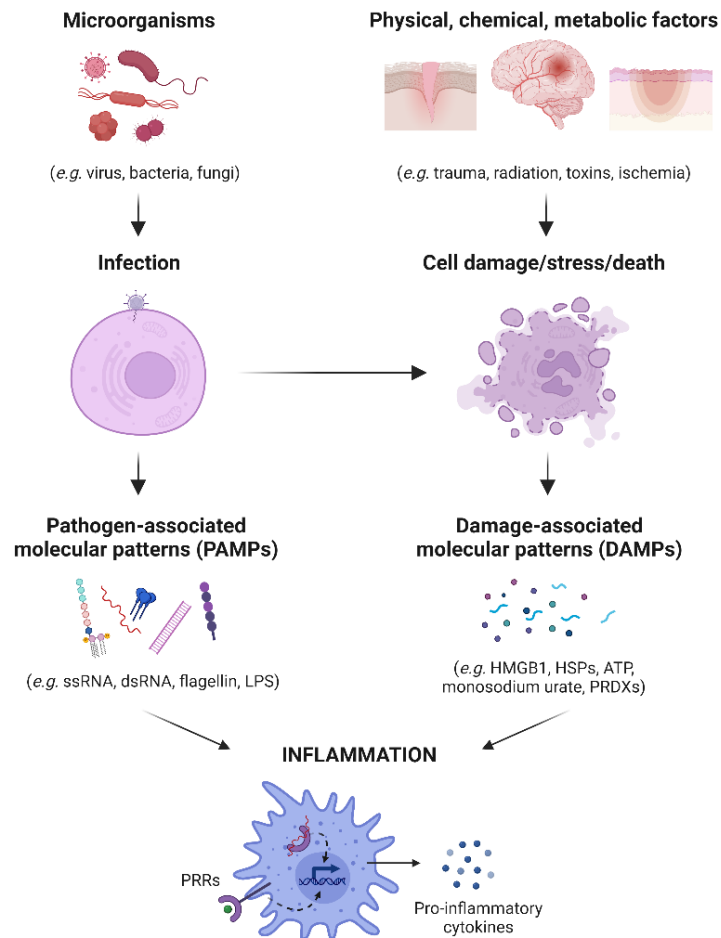


Figure 7. Simplified overview of the various inducers of inflammation. Various threats exist to host homeostasis, including pathogenic microorganisms, as well as physical, chemical, and metabolic factors. Infection-induced inflammation is triggered by molecular structures produced by microbial pathogens, referred to as pathogen-associated molecular patterns (PAMPs). They notably include single-stranded (ss) RNA, double-stranded (ds) RNA, flagellin, and lipopolysaccharides (LPS). On the other hand, cell damage, stress or death leads to the release of endogenous molecular structures that stimulate inflammation, called damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), ATP and PRDXs. Infection also results in the release of DAMPs. By binding to pattern recognition receptors (PRRs) and other cellular sensors of innate immune cells, PAMPs and DAMPs trigger inflammation, notably through the release of pro-inflammatory mediators called cytokines. Created with BioRender.com.

1.2.2. Damage-associated molecular patterns as mediators of inflammation

Infection-induced inflammatory response is triggered by molecular structures that are shared by groups of related microbes, called PAMPs [157]. On the other hand, sterile inflammation is mediated by endogenous molecules that are produced by or released from damaged, stressed and dying cells, known as DAMPs [158] (**Figure 7**). In addition, as infection causes, per definition, cell stress and tissue injury, the presence of PAMPs is always associated with the release of DAMPs [159–161].

DAMPs are highly diverse in terms of original localization, chemical structure and function [160], as detailed below. Examples of DAMPs include high-mobility group box 1 (HMGB1), ATP, heat shock proteins (HSPs), monosodium urate, extracellular cold-inducible RNA-binding protein (eCIRP), and PRDXs.

Definition of DAMPs

The definition of DAMPs is still controversial [162–164]. Purists stick to the original definition that directly associates DAMPs with tissue damage (*i.e.* damaged or dying cells) [162], and distinguish them from other groups, such as ‘stress-associated molecular patterns’ (SAMPs) or ‘homeostasis-altering molecular processes’ (HAMPs) [165,166].

On the contrary, some researchers have broadened the definition by including almost any endogenous molecule that is linked to an inflammatory pathway by virtue of the existence of a cognate sensing/recognizing molecule [160]. In this scenario, DAMPs are separated into two categories, namely ‘constitutive’ DAMPs and ‘inducible’ or ‘conditional’ DAMPs [167–169], and the terms DAMPs, ‘danger-associated molecular patterns’, ‘danger signals’, or ‘alarmins’ are interchangeably used to refer to these endogenous molecules. To further complicate the matters, some researchers even extended the definition to include exogenous DAMPs, which refer to all dangerous molecules or components which are not encoded by the host genome but affect an organism from outside (*e.g.* metal allergens, silica particles) [160]. However, this tends to blur the distinction between PAMPs and DAMPs, so that some researchers even consider PAMPs as a subgroup of DAMPs [164]. A classification of DAMPs based on this broad definition is shown in **Figure 8**. This classification, although not consensual, has the merit of presenting the diversity of the inducers of inflammation regarding initial locations, release mechanisms and chemical natures.

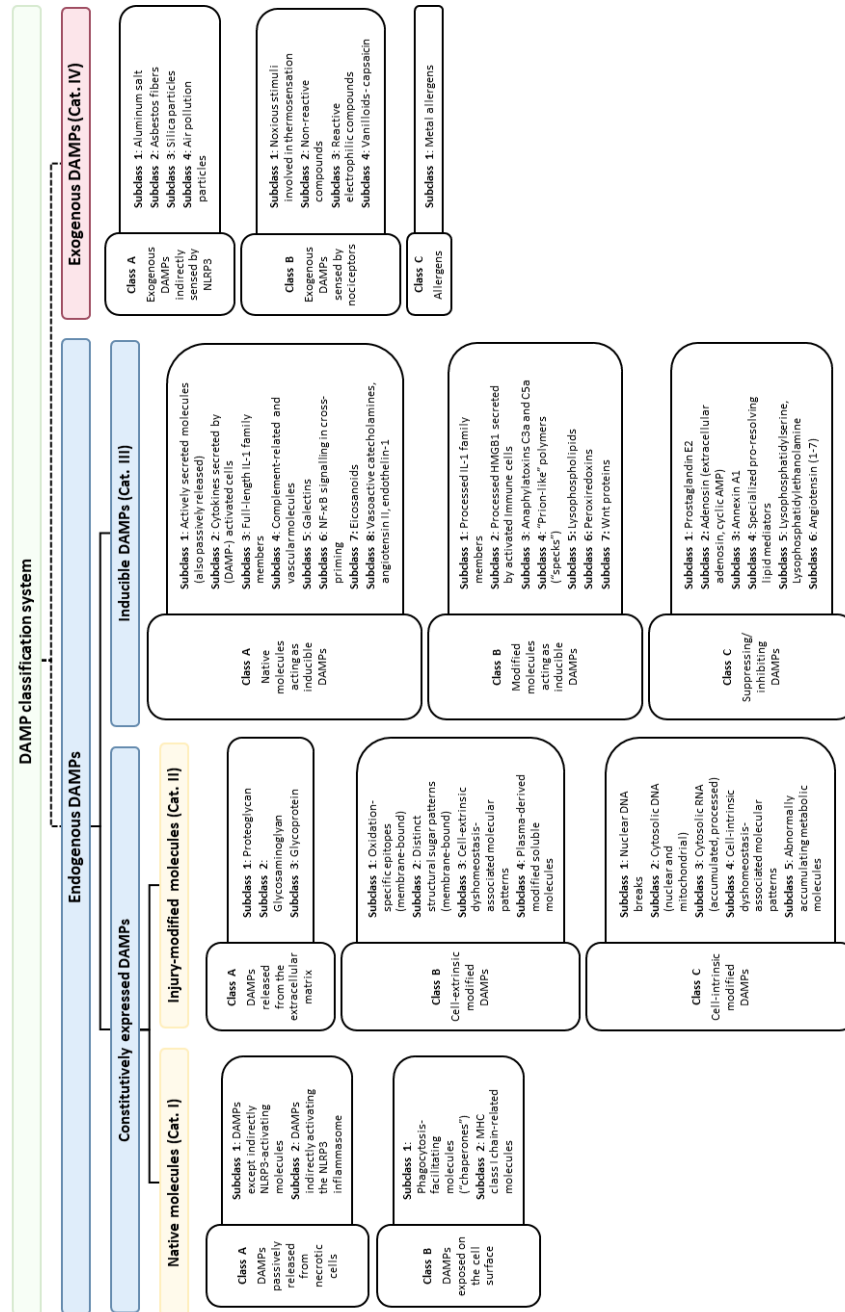


Figure 8. Example of classification system of DAMPs. DAMPs can be broadly classified as endogenous and exogenous DAMPs, and endogenous DAMPs can be further divided as constitutively expressed DAMPs and inducible DAMPs. Constitutively expressed DAMPs can be ultimately separated between those that are native molecules and those that are injury-modified molecules. This diagram is adapted from Table 3.1 published in [170].

Role of DAMPs in inflammation-related diseases

Although the physiological role of DAMPs is to alert the organism of a threat, DAMPs have also been shown to contribute the pathogenesis of many diseases. Circulating DAMPs (*e.g.* HMGB1, eCIRP, histones) have been reported to correlate with sepsis severity [161,171]. In cancer, DAMPs can also promote the development and progression of tumors, as well as metastasis [172]. Furthermore, DAMPs are linked to autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis [173]. Finally, DAMPs are known to be involved in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

Modes of release of DAMPs

DAMPs can be emitted from cells in five ways: (i) passive release following membrane rupture, (ii) intracellular active translocation to the plasma membrane and subsequent exposure on the surface of a stressed or dying cell, (iii) proteolytic release of sequestered extracellular matrix components, (iv) intracellular dislocation from the nucleus into the cytosol and intranuclear DNA changes, and (v) active secretion by live cells [170]. Regulated cell deaths leading to the emission of DAMPs include necroptosis, apoptosis, pyroptosis, ferroptosis and NETosis [153]. Regarding active secretion, many DAMPs lack the N-terminal signal sequence that allows proteins to be secreted through the classical secretory pathway, *i.e.* translocation into the ER, transport to the Golgi apparatus, and packing into secretory or transport vesicles that fuse with the plasma membrane to allow protein extracellular release. Therefore, active release of DAMPs from live cells occurs through unconventional secretory routes, including exocytosis of secretory lysosomes or exosomes, ectosomes, and activation of cell membrane channel pores [153]. The common mechanisms of DAMP release are depicted in **Figure 9**.

A given DAMP can be released through various ways [153]. For example, HMGB1 can be passively released by cells due to accidental necrosis, but also actively secreted by innate immune cells in response to infection [174,175]. However, a certain type of DAMP will not particularly be released in the same form (*e.g.* presence of PTMs, different redox states) through different release mechanisms [153]. For example, HMGB1 released by pyroptosis *via* inflammasome activation is hyperacetylated, which is not the case when it is released following necrosis or apoptosis [176]. In addition, HMGB1 is in the disulfide form when released from pyroptotic cells, in the fully reduced or disulfide form after necrosis, and in the fully oxidized form (sulfonyl HMGB1) after apoptosis [177]. Furthermore, whereas certain mechanisms are common to most if not all DAMPs (*e.g.* passive release due to necrosis), others are rather specific to certain types of DAMPs (*e.g.* pores of connexin

and pannexin hemichannels for ATP) [153]. Furthermore, different DAMPs can be released at different stages of the emission process [153]. For example, during apoptosis, ATP was shown to be released at the pre-apoptotic stage, while HMGB1 is released at the late stage [178,179].

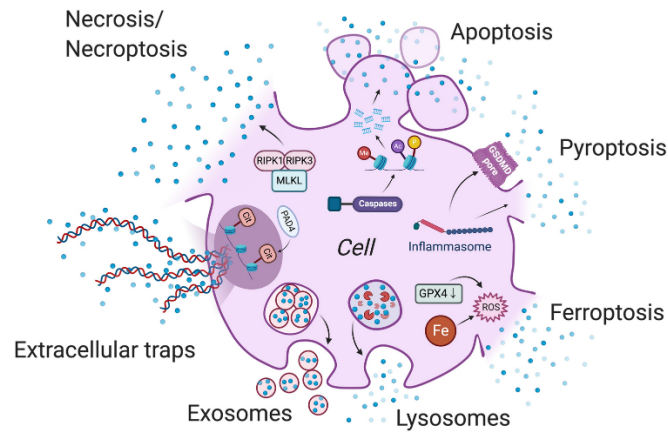


Figure 9. Common mechanisms of DAMP release. DAMPs can be passively released from necrotic cells, as well as emitted *via* regulated cell deaths such as necroptosis, apoptosis, pyroptosis, ferroptosis, and NETosis (*i.e.* formation of neutrophil extracellular traps, NETs). DAMPs can also be actively secreted by live cells through unconventional (non-classical) vesicular pathways including exocytosis of secretory lysosomes or exosomes, ectosomes, as well as activation of cell membrane channel pores such as gasdermin D (GSDMD) pores. Reproduced from [153].

1.2.3. Toll-like receptors as sensors of innate immunity

Both PAMPs and DAMPs are recognized by germline-encoded cellular receptors called pattern recognition receptors (PRRs). PRRs are expressed in immune cells (*e.g.* neutrophils, macrophages, dendritic cells) as well as non-immune cells (*e.g.* epithelial cells, endothelial cells, fibroblasts) [180]. Distinct classes have been identified: membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and absent in melanoma 2 (AIM2)-like receptors (ALRs), as well as intracellular DNA sensors such as cyclic GMP-AMP synthase (cGAS) [181,182] (**Figure 10**).

In addition, DAMPs can be sensed by several other non-PRR receptors, including receptor for advanced glycation end products (RAGE) [183], triggering receptors expressed by myeloid cells (TREMs) [184], G-protein-coupled receptors (GPCRs) [185], and ion channels [186] (**Figure 10**). Recognition of PAMPs and DAMPs leads to intracellular signal transduction pathways that activate various cellular responses, including the production of pro-inflammatory molecules.

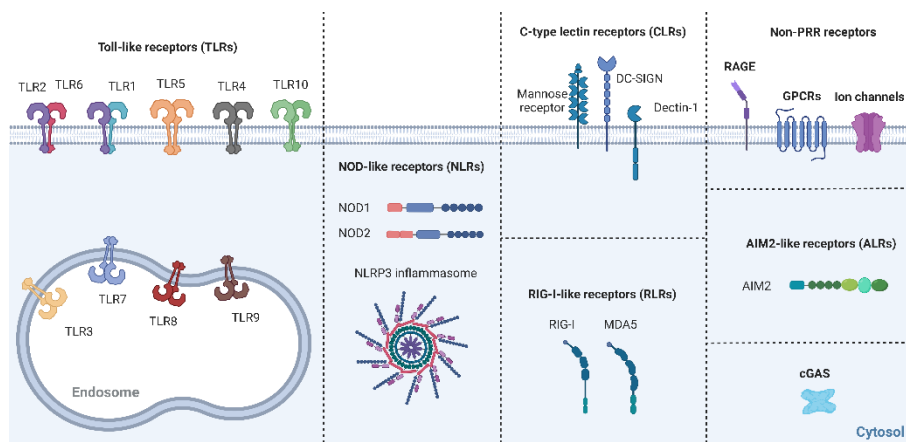


Figure 10. Pattern recognition receptors (PRRs) and other sensors of innate immunity. Receptors expressed on the cell surface include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and non-PRR receptors such as receptor for advanced glycation end products (RAGE), G-protein-coupled receptors (GPCRs), and ion channels. Other TLRs are expressed on endosomal membranes. Cytosolic sensors include NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and cytosolic DNA sensors such as cyclic GMP-AMP synthase (cGAS). Created with BioRender.com.

In addition to membrane-bound and intracellular receptors, several kinds of molecules that recognize pathogens and altered endogenous molecules exist in soluble form in the blood and extracellular fluids, among which the complement system [187]. The complement system consists of several plasma proteins (*e.g.* C1q, C3, C5) involved in proteolytic cascades that result in the generation of large amounts of products that perform effector functions. The complement system can be activated through three pathways: classical, alternative, and lectin [187].

Toll-like receptor family

Among PRRs, TLRs were the first to be identified and are the best characterized. TLRs are type I integral membrane glycoproteins, *i.e.* membrane proteins with a single transmembrane domain. They contain (i) an ectodomain composed of leucine-rich repeats (LRRs) flanked by characteristic Cys-rich motifs, involved in ligand binding, (ii) a transmembrane domain consisting of a single transmembrane helix, and (iii) a Toll/interleukin-1 (IL-1) receptor (TIR) homology endodomain, which is essential for downstream signal transduction [188,189] (**Figure 11a**). In humans, ten TLRs have been identified (TLR1–10) [181]. TLRs can be divided into two subgroups based on their cellular localization, *i.e.* cell surface TLRs and intracellular TLRs. Whereas TLR1, TLR2, TLR4–6, and TLR10 are expressed on the plasma membrane, TLR3 and TLR7–9 are expressed in the membrane of endoplasmic reticulum (ER) vesicles, endosomes, lysosomes and endolysosomes [188,190,191] (**Figure 10**).

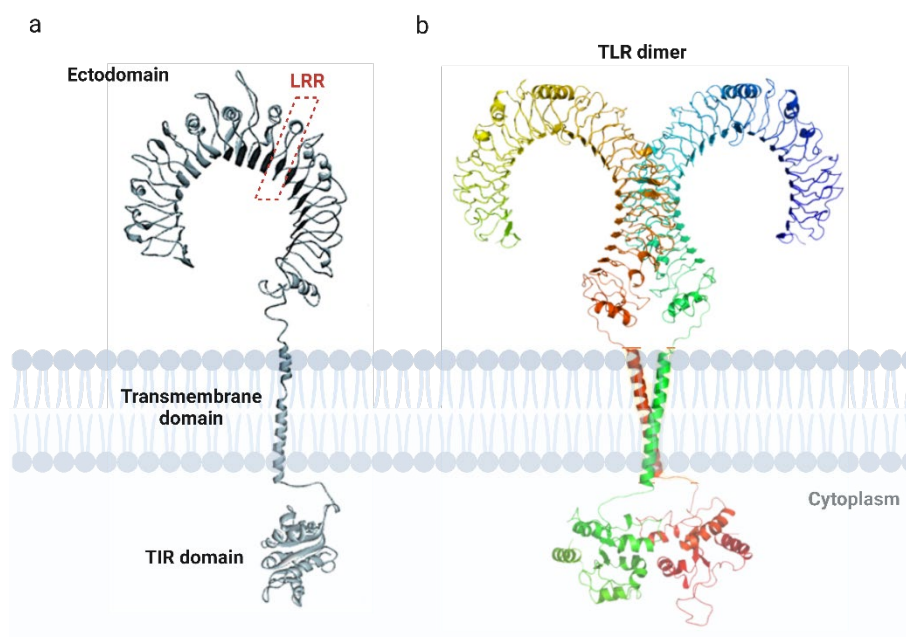


Figure 11. Structure of TLRs. (a) The crystal structure of TLR2 is shown as representative TLR structure. TLRs are composed of an ectodomain consisting in leucine-rich repeat (LRR) motifs, a transmembrane domain, and a TIR endodomain. Adapted from [192]. (b) Crystal structure of TLR4 dimer. TLRs homo- or heterodimerize, bringing the TIR domains in close proximity for downstream signaling. Adapted from [193].

The diversity of TLRs allows the recognition of several exogenous and endogenous ligands. Examples of exogenous ligands include single-stranded and double-stranded RNA from viruses, unmethylated CpG DNA sequences of bacteria, pilin and flagellin from bacteria, and LPS of Gram-negative bacteria. Endogenous ligands include proteins and peptides, polysaccharides and proteoglycan, nucleic acids, phospholipids, and crystals [194]. A non-exhaustive list of endogenous TLR ligands is presented in **Table 1**.

1.2.4. Factors involved in TLR ligand discrimination

Specificities of the TLRs regarding ligand binding notably reside in (i) their subcellular localization, (ii) the difference in the number and composition of amino acids of the LRRs that lead to variations in TLR structural conformation, (iii) their ability to heterodimerize with one another, and (iv) their association with co-receptors and accessory molecules that are involved in ligand delivery and recognition.

Subcellular localization

The TLR subcellular localization (**Figure 10**) is required for ligand recognition, as it corresponds to some extent to the compartments in which their respective ligands are found. For examples, TLR4 recognizes bacterial LPS [195], while TLR3 binds to dsRNA produced during viral replication [196]. In addition, intracellular TLR localization is important to prevent TLRs from coming into contact with self-nucleic acids, leading to autoimmunity [181]. Localization to the appropriate subcellular compartment is ensured by various TLR accessory molecules (*e.g.* GRP94 or PRAT4A) [197].

Table 1. Endogenous TLR ligands categorized based on their chemical nature. Adapted from [194,198].

Chemical nature	TLR ligands and references
Proteins, peptides	β -defensin 2 [199,200], β -defensin 3 [201], β -amyloid [202], fibrinogen [203,204], extra domain A (EDA) of fibronectin [205,206], HMGB1 [207,208], HSPs (HSP22 [209], HSP60 [210], HSP70 [211], HSP72 [212], HSP90B1 [213]), S100A8 and S100A9 proteins [214,215], cardiac myosin [216], resistin [217,218], surfactant protein A [219], surfactant protein D [220], tenascin-C [221], eosinophil-derived neurotoxin [222], antiphospholipid antibodies [223–225], neutrophil elastase [226], lactoferrin [227]
Proteoglycans, glycosaminoglycans	Biglycan [228], heparan sulphate [229,230], hyaluronan [231–233]
Nucleic acids, protein-nucleic acids complexes	DNA [234–236], RNA [237–239], mRNA [240,241] and small interfering RNA (siRNA) [242]
Fatty acids, lipoproteins	Serum amyloid A [243–245], oxidized low-density lipoprotein (oxLDL) [246–248], saturated fatty acids [249,250]
Crystals	Monosodium urate crystals [251,252]

Leucine-rich repeats

The ectodomain of TLRs notably varies in the number of LRR motifs, their amino acid composition, the curvature, as well as the extent of N-linked glycosylation [253]. TLR ectodomains possess characteristic horseshoe-shaped solenoid structure comprising 19–25 tandem copies of the LRR module [189,254] (**Figure 11a**). Each LRR module is typically composed of 20–30 amino acids and can be divided into a highly conserved segment and a variable segment [255]. The highly conserved part consists of the consensus LXXLXXN sequence [256,257]. The LRR three-dimensional structure consists in a loop that contains three residues in the β -strand configuration. Within the tertiary structure, these β -strands align in the concave surface of the horseshoe to form a hydrogen-bonded parallel β -sheet [257].

An example of the role of LRRs in ligand discrimination is given by TLR1 and TLR6, that are involved with TLR2 in the recognition of triacylated and diacylated

lipopeptides, respectively [258,259]. The TLR2/TLR1 heterodimer recognizes Pam₃CSK₄ (**Figure 12a**), whereas the TLR2/TLR6 heterodimer binds Pam₂CSK₄ (**Figure 12b**). In both dimers, two lipid chains are buried into the TLR2 hydrophobic binding pocket. Whereas TLR1 also possesses a hydrophobic channel that can accommodate the third lipopeptide lipid chain, the side chains of two phenylalanine (Phe) residues in TLR6 restrict this binding channel, so that it is less than half the length of that of TLR1 [259].

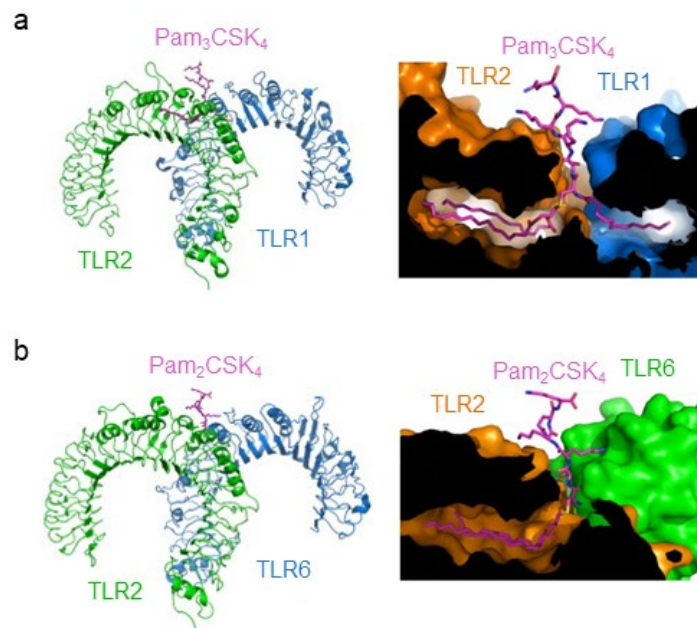


Figure 12. TLR2/TLR1/Pam₃CSK₄ and TLR2/TLR6/Pam₂CSK₄ complexes. Crystal structure (left) and cross-section (right) through the molecular surface of (a) the TLR2/TLR1/Pam₃CSK₄ complex (PDB ID: 2Z7X), and (b) the TLR2/TLR6/Pam₂CSK₄ complex (PDB ID: 3A79). On the contrary to TLR6, TLR1 possesses a hydrophobic channel that can accommodate a lipid chain. Adapted from [253].

TLR homo- and heterodimerization

TLRs are able to discriminate between different ligands by functioning in a combinatorial repertoire [260], *i.e.* they are able to homo- and/or heterodimerize (**Figure 10**, **Figure 11b** and **Figure 12**). Whereas TLR1–6 dimerize upon ligand binding, TLR7–9 exist as preformed inactive dimers that undergo conformational rearrangement upon ligand binding [190]. As mentioned above, TLR2 forms heterodimers with TLR1 or TLR6, and both receptors contain structural differences in their dimerization region with TLR2 [253]. In addition, TLR4 has been shown to form a complex with TLR5, promoting inducible nitric oxide synthase (iNOS)

production after flagellin exposure [261] or lung injury after LPS, hyaluronan (HA) or ozone exposure [262]. In addition, TLR4 in complex with TLR1 was demonstrated to inhibit LPS-induced TLR4 signaling [263]. Furthermore, TLR4 associates with TLR6 to recognize oxidized low-density lipoprotein (oxLDL) and β -amyloid [264].

Co-receptors and accessory molecules

In the present thesis, a TLR accessory molecule will refer to any molecule involved in TLR biosynthesis and activation (*i.e.* ligand-recognition and delivery cofactors, chaperones and trafficking proteins) [197], while a TLR co-receptor will be defined as a cell surface receptor that binds a ligand together with a TLR to support or stabilize the formation of a signaling complex [265]. Co-receptors are therefore a defined subset of accessory molecules. Whereas co-receptors are membrane-bound, accessory proteins can also be cytoplasmic or extracellular soluble proteins. A large variety of co-receptors and accessory molecules are involved in TLR ligand delivery and recognition.

TLRs notably cooperate with other families of innate immune receptors, including scavenger receptors, CLRs, and ion channels [266]. Examples include CD36, a transmembrane glycoprotein of the scavenger receptor class B family, that functions as co-receptor for the TLR2/TLR6 or TLR4/TLR6 complex in the recognition of diacylated lipopeptides [267] or oxLDL and β -amyloid [264], respectively. TLR2 collaborates with the CLR dectin-1 to bind and internalize fungus β -glucan [268,269]. TLR2 can also associate with the macrophage receptor with collagenous structure (MARCO), a class A scavenger receptor, to respond to trehalose 6,6'-dimycolate (TDM), a cell wall glycolipid from *Mycobacterium tuberculosis* [270]. Furthermore, biglycan induces inflammasome activation through activation of TLR2/TLR4 and purinergic P2X4/P2X7 receptors, which are ATP-gated ion channels [271].

Other accessory molecules involved in ligand delivery and discrimination include MD-2 and CD14 [197,198]. MD-2 is a soluble protein that is required for TLR4-dependent responses to certain PAMPs and DAMPs, including HMGB1 [165], HA [233], and LPS [164]. HA is recognized by a complex composed of TLR4, the glycoprotein CD44, and MD-2 [233]. LPS first binds the LPS-binding protein (LBP), after which it is presented to CD14, which transfers it to the TLR4/MD-2 complex [272]. MD-2 also discriminate between HMGB1 redox isoforms, binding specifically the disulfide isoform to the exclusion of others, in a complex with TLR4 [273]. CD14 is a glycoprotein expressed as a glycosylphosphatidylinositol (GPI)-anchored receptor as well as secreted in a soluble form [274]. CD14 acts as a co-receptor of several TLRs by binding various ligands, including Pam₃CSK₄ *via* the TLR2/TLR1 complex [275] and CpG DNA with TLR9 [276]. Details about the role of MD-2 and CD14 in TLR4-dependent signaling are presented in **section 1.2.6**.

1.2.5. Overview of Toll-like receptor signaling

Upon ligand binding, TLRs homo- or heterodimerize, or change their conformation in case of pre-existing dimers, to bring the TIR domains of each receptor close to one another and activate downstream signaling pathways [190] (**Figure 11b**). The TIR domains act as a scaffold for the recruitment of TIR-domain containing adaptor proteins, and this is followed by the recruitment and activation of various kinases, leading to the activation of transcription factors, and ultimately, to the induction of genes for the production of inflammatory cytokines and other mediators [115]. Various combinations of adaptor proteins and signaling intermediates are used by different TLRs, accounting for the specificity of their downstream effects [97]. In addition, a certain ligand can induce multiple signaling pathways.

Adaptor proteins and transcription factors

In mammals, five types of TIR domain-containing adaptor proteins can be recruited: (i) myeloid differentiation primary response 88 (MyD88), (ii) TIR domain-containing adaptor protein (TIRAP or MAL), (iii) TIR domain-containing adaptor inducing interferon- β (TRIF), (iv) TRIF-related adaptor molecule (TRAM), and (v) sterile α - and Armadillo-motif-containing protein (SARM) [254,277]. The major transcription factors involved in TLR signaling are nuclear factor- κ B (NF- κ B), interferon response factors (IRFs), as well as activation protein-1 (AP-1) [278].

Signaling pathways

There are two major TLR intracellular signaling pathways: the MyD88-dependent pathway and the TRIF-dependent pathway [181]. The MyD88 signaling pathway is activated by all TLRs with the exception of TLR3, while the TRIF signaling pathway is activated upon stimulation of TLR3 or TLR4 [279,280]. Both pathways are able to activate the transcription factor NF- κ B. Given the wide variety of TLR-induced signaling pathways, and as NF- κ B signaling is one of the most important in inflammation, this section will be restricted to the description of the classical NF- κ B activation (**Figure 13**).

Functional NF- κ B requires homo- or heterodimerization of five subunits of the NF- κ B family: p65 (RelA), RelB, c-Rel, p105/p50 (NF- κ B1) and p100/p52 (NF- κ B2) [281]. Among these, p65:p50 is ubiquitously expressed and is the primary mediator of inflammation [282]. NF- κ B dimers reside in an inactive state in the cytoplasm, sequestered by NF- κ B inhibitor proteins (I κ Bs). The mechanism of NF- κ B activation involves the I κ B kinase (IKK) complex, which is a trimeric enzyme complex composed of the catalytic subunits IKK α and IKK β , and the regulatory subunit IKK γ (also called NEMO) [283]. Upon activation (stimulus), the IKK signalosome phosphorylates I κ B α

at specific sites, triggering its dissociation from NF- κ B and its ubiquitin-dependent degradation in the proteasome, thereby liberating NF- κ B dimers to translocate to the nucleus and regulate target gene transcription [284].

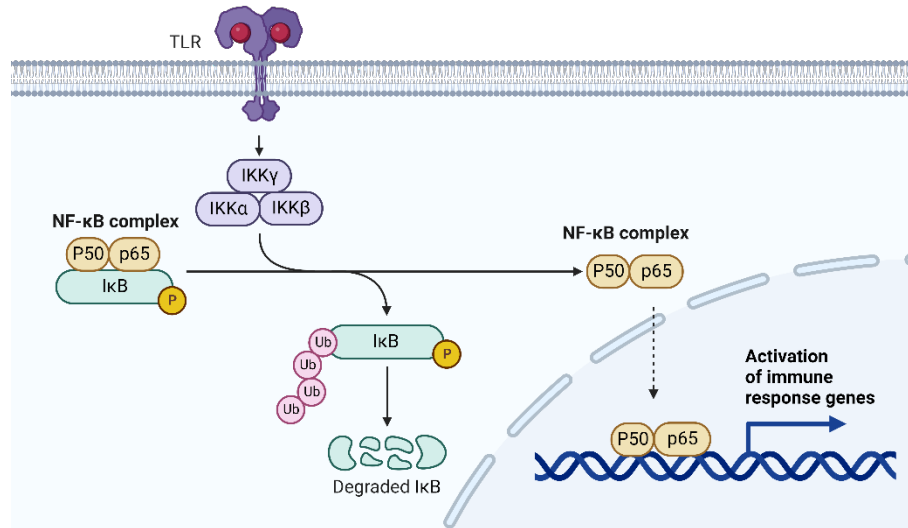


Figure 13. Classical NF- κ B signaling pathway. Upon TLR stimulation by a DAMP, the IKK complex (IKK α , IKK β and IKK γ) phosphorylates the NF- κ B inhibitor protein I κ B, leading to its ubiquitin-dependent degradation, and thereby liberating the NF- κ B dimer (p65:p50) for translocation to the nucleus. In the nucleus, NF- κ B binds specific DNA sequences. Created with BioRender.com.

Furthermore, TLR signaling can prime other innate immune receptor signalings, such as the NLR family pyrin-domain-containing protein 3 (NLRP3) [285]. NLRPs are NLRs that respond to various PAMPs and DAMPs by forming multimeric signaling complexes called inflammasomes. Among these, the NLRP3 inflammasome is one of the most studied [285]. It consists of sensor proteins (NLRP3), adaptor proteins (apoptosis-associated speck-like protein containing a caspase-recruitment domain, ASC), and effector proteins (caspase-1) [119,120].

Pro-inflammatory mediators

Whereas NF- κ B, AP-1 and IRF5 induce the expression of genes encoding molecules required for inflammatory responses, including pro-inflammatory cytokines (e.g. tumor necrosis factor (TNF)- α , IL-1 β) and chemokines (e.g. IL-2 and IL-8), IRF3 and IRF7 control the expression of type I interferons (IFN) and IFN-inducible genes, which are important for antiviral defense. Other molecules that are synthesized include endothelial adhesion molecules (e.g. E-selectin), growth factors, tissue-degrading enzymes such as metalloproteinases, and enzymes that generate inflammatory mediators such as cyclo-oxygenase 2 and iNOS [198].

1.2.6. Toll-like receptor 4 and its accessory molecules

As the present thesis is focused on the role of TLR4 and its accessory molecules MD-2 and CD14 in the DAMP function of PRDXs, this section will describe the structures and functions of these three proteins in more details. We previously mentioned that DAMPs were involved in the pathogenesis of several diseases, notably related to sterile inflammation. The role of TLR4 in such diseases will also be briefly mentioned.

Toll-like receptor 4

TLR4 is one of the most studied TLRs. Human TLR4 consists of an extracellular domain composed of 624 amino acids (residues 1–624), a transmembrane domain composed of 33 amino acids (residues 625–658), a proximal cytoplasmic domain composed of 159 amino acids (residue 659–818), and a distal cytoplasmic domain of 19 amino acids (residues 819–838) [286]. As in all TLRs, the extracellular portion has a horseshoe-shaped solenoid structure (**Figure 14**). TLR4 was initially studied for its role in bacterial LPS-induced inflammation [287,288], but it has been widely demonstrated that it is involved in the recognition of various PAMPs and DAMPs. A list of DAMPs that activate a TLR4-dependent pathway is presented in **Table 2**. For several DAMPs, TLR4 activation and function require the presence of accessory molecules, including CD36, MD-2 and CD14. Details about MD-2 and CD14 with respect to their functional interaction with TLR4 are discussed below. TLR4 can recruit four adaptor proteins to signal, namely MyD88, TIRAP (or MAL), TRIF and TRAM, and stimulus activates both MyD88- and TRIF-dependent intracellular signaling pathways from two different organelles [289–291]. MyD88-dependent signaling is activated from the plasma membrane and involves recruitment of MyD88 and TIRAP. After internalization in the endosomal compartment, a MyD88-independent pathway is activated, involving the recruitment of TRIF and TRAM [292,293].

Myeloid differentiation factor-2

MD-2 is a ~20-kDa glycosylated soluble protein with a β -cup fold structure composed of two antiparallel β -sheets that form a large hydrophobic binding pocket. MD-2 is able to associate with the extracellular domain of TLR4 through two chemically distinct regions, namely A and B patches, that are located in the N-terminal and the central domains of TLR4 [294,295]. Although MD-2 and TLR4 are assembled in the Golgi apparatus when coexpressed [296], the proper TLR4 localization on the plasma membrane does not depend on MD-2 [297].

MD-2 is essential for TLR4 binding of certain ligands, including LPS and HMGB1 [273,298] (**Table 2**). The interaction of LPS with the TLR4/MD-2 complex has been

widely studied [272,295,299]. LPS is a glycolipid composed of three structural domains: a hydrophobic lipid A component, an hydrophilic core oligosaccharide, and an hydrophilic O-antigen (also known as O-specific polysaccharides) [300,301]. The receptor multimer consists of two TLR4/MD-2/LPS complexes arranged in a symmetrical fashion [295] (**Figure 14**). LPS lipid A chains first bind the hydrophobic pocket of MD-2, already bound itself to TLR4 through a primary interface [295] (**Figure 14a**). Upon LPS binding, two TLR4/MD-2/LPS complexes dimerize [295]. Within the multimer, MD-2 and LPS from one TLR4/MD-2/LPS complex form interactions with TLR4 from the other complex [295] (**Figure 14a**). The dimerization interface between MD-2 and TLR4 is located on the opposite side of the primary interface and involved LRR modules 15–17 of the TLR4 C-terminal domain [295] (**Figure 14b**). Regarding HMGB1 binding to the TLR4/MD-2 complex, MD-2 was shown to discriminate between HMGB1 redox forms, binding specifically to the disulfide isoform of HMGB1, with the exclusion of other isoforms [273,302].

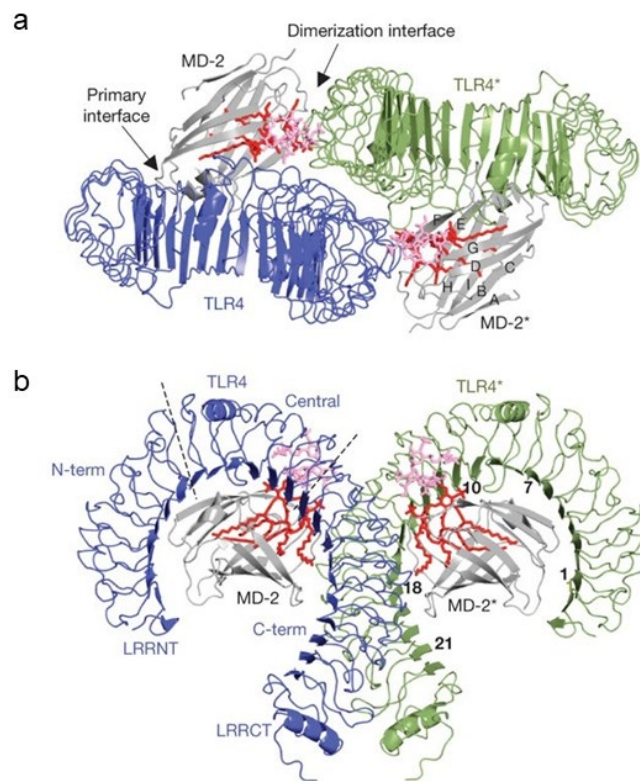


Figure 14. Crystal structure of the TLR4/MD-2/LPS complex. Top view (**a**) and side view (**b**) of the complex. (**a**) Binding of LPS induces the formation of a symmetrical dimer composed of two TLR4/MD-2/LPS complexes. The primary interface between TLR4 and MD-2 is formed prior to LPS binding, whereas the dimerization interface is induced upon LPS binding. (**b**) Five

of the six lipid chains of the lipid A component of LPS (red) are completely inserted within the hydrophobic pocket of MD-2 (grey) and the sixth forms a hydrophobic interaction with the TLR4 molecule of the other TLR4/MD-2/LPS complex. LPS bound to MD-2 therefore directly bridges the two TLR4 molecules. PDB ID: 3FXI. Adapted from [295].

In addition to its role in ligand binding, MD-2 is essential for TLR4 dimerization upon binding of certain ligands [295,303]. Furthermore, MD-2 is required for TLR4 endocytosis following LPS recognition [304].

Cluster of differentiation 14

CD14 is a ~40-kDa glycoprotein that belongs to the LRR family and exhibits a curved solenoid structure [305,306]. As previously mentioned, CD14 is present both as a secreted, soluble protein, and as a GPI-anchored membrane protein. CD14 interacts with multiple ligands, but it was first shown to be involved in TLR4-mediated immune responses, and TLR4-dependent LPS-induced responses in particular. CD14 was shown to deliver LPS to the TLR4–MD-2 complex [307] and to mediate LPS-induced endocytosis of TLR4 [308]. Therefore, CD14 is required for both LPS-induced MyD88-dependent signaling and TRIF-dependent signaling [308,309]. CD14 displays a N-terminal hydrophobic pocket as well as a secondary hydrophobic cluster outside the entrance to this cavity, which are the principal components of the LPS binding site [305,306]. Upon LPS exposure, CD14 and the TLR4/MD-2 complex are brought in close proximity within lipid rafts [310,311]. TLR4 in the plasma membrane then activates the TIRAP-MyD88-dependent pathway, which leads to the early phase of NF- κ B activation [312]. Subsequently, the receptor complex (including CD14) is internalized [308] and redirected to the endosome, from which starts the activation of the TRAM-TRIF pathway [292]. However, the CD14 dependence of TLR4 endocytosis was reported to display ligand specificity [313]. CD14 was shown to be required for the TLR4-dependent signaling of various DAMPs, as illustrated in **Table 2**. Furthermore, CD14 has been reported to have a TLR4-independent signal transduction capacity [314].

Table 2. DAMPs signaling through TLR4 together with the known co-receptors or accessory molecules.

Biochemical nature	TLR4 agonist ligand		Accessory molecules	Refs
Proteins, peptides	HMGB1		MD-2, CD14	[207,208,336]
	β -amyloid		MD-2, CD14	[202,327,337]
	Fibronectin EDA		MD-2, CD14	[205,338]
	Fibrinogen			[203]
	Tenascin-C (FBG)			[221]
	α -synuclein			[339]
	Surfactant proteins	Surfactant protein A Surfactant proteins D	CD14	[219,220,340]
	β -defensin-2			[199]
	HSPs	HSP60	MD-2, CD14	[209,211–213,219,341]
		HSP70 (HSP70, HSP70L1)	MD-2, CD14	
		HSP72		
		HSP22		
		HSP90B1 (gp96)	MD-2	
	S100 proteins	S100A8 (MRP8)	MD-2, CD14	[214,342]
		S100A9 (MRP14)		
	Neutrophil elastase			[226]
	Antiphospholipid antibodies			[224,225]
	Lactoferrin		CD14	[227,343]
	Resistin		CD14	[217,218]
	PRDXs	PRDX1	MD-2, CD14	[116,117,344,345]
		PRDX2		
		PRDX4		
		PRDX5		
		PRDX6		
	DJ-1			[346]
Lipoproteins, fatty acids, phospholipids, glycolipids	Serum amyloid A proteins, serum amyloid P component		MD-2, CD14	[245,347–349]
	OxLDL (OxPAPC)		MD-2, CD14	[246–248,350]
	Saturated fatty acids		MD-2, CD14	[249,250,351,352]
	Sulfatides		MD-2	[353]
Proteoglycans, glycosaminoglycans	Biglycan		MD-2, CD14, P2X4/P2X7	[228]
	Heparan sulfate fragments			[229]
	Hyaluronic acid fragments		MD-2, CD44	[231]
Crystals	Monosodium urate crystals		CD14	[251,354]

Role of TLR4 in sterile inflammatory-related diseases

TLR4 signaling is involved in the pathogenesis of sterile inflammation-related diseases, including ischemia/reperfusion injury (IRI) as well as neurodegenerative and neurological diseases [162,290].

Ischemia occurs due to an insufficient blood supply, resulting in deficiencies in oxygen, glucose and other components required for metabolism. Although the

restoration of blood supply, known as reperfusion, is essential, it paradoxically also leads to further damage. IRI occurs in a wide range of organs, including the heart, brain, kidney, lung, gut, and skeletal muscle [315]. Several studies in animal models demonstrated that TLR4 plays a key role in the promotion of tissue injury in response to IRI, being the receptor of several DAMPs [316–321]. Many studies used TLR4 mutated (C3H/HeJ) or TLR4-deficient mice. Among the various DAMPs that are involved in the activation of TLR4 signaling in IRI, HMGB1 and PRDXs were reported to be the most important [116,322,323].

Neuroinflammation, *i.e.* inflammation of the central nervous system (CNS), is common to several neurodegenerative and neurological diseases [324,325]. TLR4 has notably been shown to be linked to neuroinflammation in animal models and patients of Alzheimer's disease [326–328], Parkinson's disease [329–332], and amyotrophic lateral sclerosis [333–335].

Therefore, understanding the role of TLR4 in DAMP-induced pro-inflammatory responses is of crucial importance for the development of therapeutic drug targets.

1.3. Mammalian peroxiredoxins as damage-associated molecular patterns

PRDXs have been reported to be key players in inflammatory processes, notably by acting as PAMPs and DAMPs [40,41].

Several studies reported the case of exogenous PRDXs acting as PAMPs. For example, PRDXs secreted by parasitic helminths trigger the activation of macrophages and a T helper type 2 (Th2) response with the production of high levels of IL-10 and prostaglandin E2 [355,356]. A 2-Cys malarial PRDX from *Plasmodium berghei* is able to interact with TLR4 on macrophages to trigger the production of pro-inflammatory cytokines [357]. Furthermore, a PRDX from *Toxoplasma gondii* (TgPrx) was shown to reduce levels of ROS, caspase-1 activity and IL-1 β secretion in macrophages, whilst simultaneously enhancing IL-10 secretion and promoting parasite replication [358]. Finally, PRDXs from *Entamoeba histolytica* were shown to activate TLR4-dependent NLRP3 inflammasome in macrophages [359].

On the other hand, mammalian PRDXs have been found to act as DAMPs. According to the classification of **Figure 8**, PRDXs belongs to the class of endogenous modified molecules acting as inducible DAMPs. However, as they were also reported to be released from necrotic cells, they also fall into the class of endogenous constitutively expressed native molecules released passively from necrotic cells. This section will provide state-of-the-art information about what is known on how mammalian PRDXs are able to act as DAMPs, which is the central focus of the present thesis.

1.3.1. Overview

Mammalian PRDXs were shown to act as DAMPs *in vitro* using various cell lines. PRDX1 and PRDX2 were originally identified as cytosolic factors released from erythrocytes that enhanced the cytotoxic activity of natural killer cells [360,361]. Since then, extracellular PRDX1 has been shown to induce secretion of TNF- α and IL-6 in thioglycolate (TG)-elicited murine macrophages and immature bone marrow-derived dendritic cells (iBMDCs), as well as dendritic cell maturation [344]. Recombinant PRDX1 was also reported to induce the expression of pro-IL-1 β , IL-6 and TNF- α in peritoneal macrophages isolated from mouse [362]. Similarly, recombinant human PRDX2 was demonstrated to induce TNF- α release from RAW264.7 cells and human primary macrophages [111]. Secreted PRDX4 has been reported to activate NF- κ B and to up-regulate the expression of NF- κ B-dependent genes, *i.e.* intracellular adhesion molecule-1 (ICAM-1) and iNOS, in astrocyte cells [363]. The role of human PRDX1, PRDX2, PRDX4, PRDX5 and PRDX6 as DAMPs was also demonstrated by Shichita *et al.* [116] and Zhao *et al.* [345]. PRDX1, PRDX2,

PRDX5 and PRDX6 released from necrotic brain cells were shown to induce expression of pro-inflammatory cytokines, including IL-23, in macrophages [116]. In addition, stimulation of murine RAW264.7 cells by PRDX1, PRDX2 and PRDX4 was reported to induce IL-6 and TNF- α secretion [345]. The function of PRDXs as DAMPs has also been studied *in vivo* in the context of sterile inflammatory-related diseases such as acute liver injury (ALI) and cerebral IRI [116,362,364–366].

1.3.2. Modes of release of peroxiredoxins into the extracellular space

Among mammalian PRDXs, PRDX4 is the only member to possess a hydrophobic N-terminal signal sequence, which drives its translocation into the ER [367,368]. Although PRDX4 has long been believed to be a secreted protein [368], it was later found to be retained within the ER in complex with ERp44 [369,370]. Therefore, mammalian PRDXs are referred to as unconventionally (non-classically) secreted proteins [371]. PRDXs have been shown to be released by various cell lines upon exposure to inflammatory stimuli such as LPS, TNF- α , and transforming growth factor- β 1 (TGF- β 1) [104,111,372].

Release mechanisms

Mammalian PRDXs have been reported to be (i) passively released from cells following necrosis [116], (ii) actively secreted by exocytosis of exosomes [104] following sorting into multivesicular bodies (MVBs) [373], and (iii) actively secreted through cell membrane channel pore formation [374] (**Figure 15**). Human PRDX1 and PRDX2 have been shown to be released in exosomes from human embryonic kidney (HEK) 293 cells following TNF- α stimulation, and from THP-1 cells stimulated with LPS [104]. Furthermore, mammalian PRDX1, PRDX2, PRDX5 and PRDX6 were found to be secreted by J774A.1 cells following NLRP3 inflammasome activation in a caspase-1 dependent manner [374]. NOD-like receptor containing a caspase recruitment domain 4 (NLRC4) and AIM2 inflammasome activation were also shown to induce PRDX1 and PRDX2 secretion in a caspase-1 dependent manner [374]. Furthermore, release of PRDX1 and PRDX2 was shown to rely on gasdermin D (GSDMD) pore formation on the plasma membrane [374]. PRDX4 was also found to be secreted with inflammasome components (including caspase-1) in extracellular vesicles most likely originating from MVBs, upon inflammasome-activation of macrophages following LPS treatment [373]. Finally, PRDX1, PRDX2, PRDX5 and PRDX6 were shown to be released passively from necrotic brain cells [116]. Although insights regarding the release modes of mammalian PRDXs are emerging, the diversity of the mechanisms remains to be elucidated and the molecular and cellular details fully investigated.

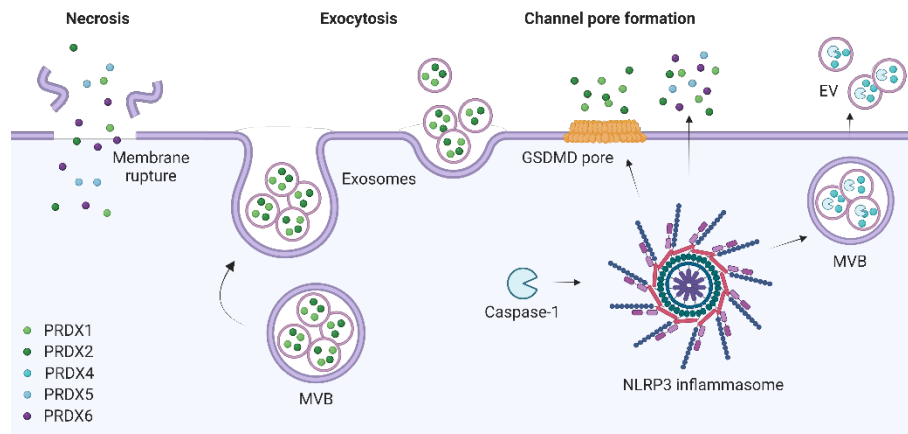


Figure 15. Release mechanisms of mammalian PRDXs. Necrotic brain cells release PRDX1, PRDX2, PRDX5 and PRDX6. PRDX1 and PRDX2 are released in exosomes after sorting into multivesicular bodies (MVBs) following macrophage activation. In addition, caspase-1-dependent NLRP3 inflammasome activation triggers secretion of PRDX1, PRDX2, PRDX5 and PRDX6, and both PRDX1 and PRDX2 are secreted through GSDMD pore formation. Activation of other caspase-1-dependent inflammasomes, including NLRC4 and AIM2, is linked with release of PRDX1 and PRDX2 (not shown). Activated macrophages release extracellular vesicles (EVs) containing PRDX4 and caspase-1 after sorting into MVBs. Created with BioRender.com.

Influence of the PRDX redox state

Circulating PRDX1 and PRDX2 were found to be present exclusively as disulfide-linked homodimers [104]. In addition, upon TNF- α stimulation, HEK293 cells were shown to release PRDX1 and PRDX2 in their disulfide, dimeric form [104]. Mutation of Cys residues involved in disulfide bond formation, *i.e.* C51 or C172 (but not C70) for PRDX2, and C52 or C173 (but not C71 nor C83) for PRDX1, by Ser residues was found to prevent their TNF- α -induced release from HEK293T cells [104]. These results indicate that oxidation of catalytic Cys residues in PRDX1 and PRDX2 is required for their secretion in response to TNF- α . However, whether different PRDX redox states are associated with different release mechanisms, as it is the case for the DAMP HMGB1 [177], remains to be investigated.

1.3.3. Sensors involved in extracellular PRDX recognition

Among the mammalian PRDXs able to act as DAMPs, many were shown to signal through TLR2, among which PRDX1, PRDX2, PRDX5 and PRDX6, as well as through TLR4, including PRDX1, PRDX2, PRDX4, PRDX5, and PRDX6. In addition, PRDX1 and PRDX2 were more recently linked to the complement system. The immune sensors of PRDXs are depicted in **Figure 16**.

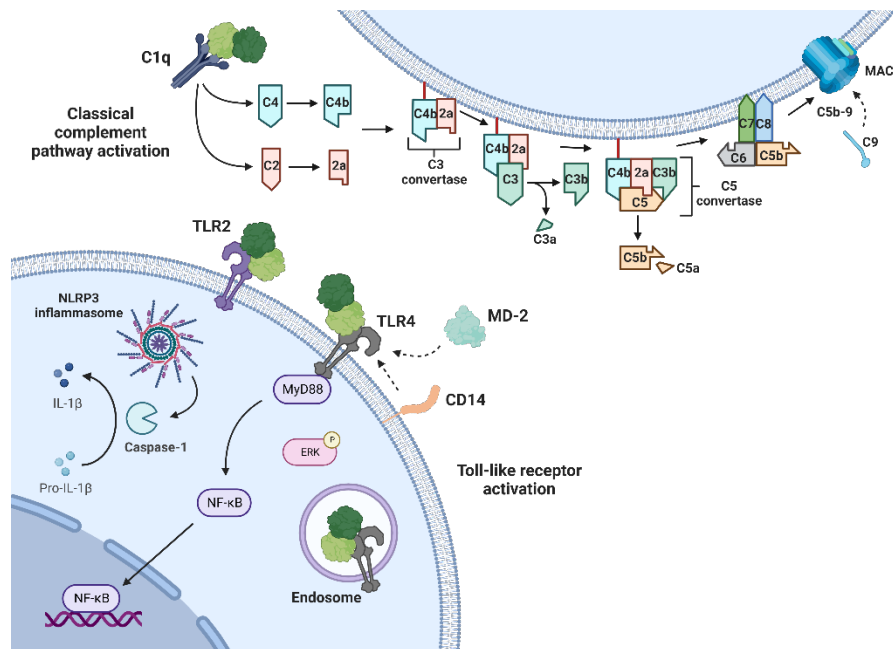


Figure 16. Extracellular PRDX recognition and signaling. PRDXs are recognized by TLRs, including TLR2 and TLR4. TLR4 accessory molecules, MD-2 and CD14, were shown to be involved in PRDX1 recognition. PRDXs induce a TLR4/MyD88/NF- κ B pathway, as well as NLRP3 inflammasome activation, the latter resulting in caspase-1 activation and production of mature IL-1 β . PRDX1/TLR4 complex has also been shown to undergo a clathrin-dependent endocytosis. Furthermore, PRDX1 and PRDX2 are able to bind to the complement protein C1q. Following C1q binding, PRDX2 can initiate an antibody-independent classical complement pathway. The classical pathway of complement activation consists in the cleavage of C4, following by the binding of C4b to cell surface. Cleavage of C2 is followed by the formation of the C4b2a complex (C3 convertase), which allows the cleavage of C3 and the subsequent binding of C5 to the C4b2aC3b complex (C5 convertase). Cleavage of C5 leads to initiation of the late steps of complement activation, consisting in the subsequent binding of C6 and C7 to C5b, following by C5b,6,7 complex insertion into the plasma membrane, and finally insertion of C8. Several C9 molecules are ultimately recruited and polymerize around the complex to form the membrane attack complex (MAC), which creates pores in the membrane. Created with BioRender.com.

TLR2 and TLR4 activation upon PRDX recognition

The involvement of TLR2 and TLR4 in PRDX signaling as DAMPs has been shown both *in vitro* and *in vivo* with different techniques and using various cell lines. Induction of IL-23p19 expression by recombinant PRDX1, PRDX2, PRDX5 and PRDX6 was shown to be lower in TLR2- and TLR4-deficient BMDCs as compared to WT BMDCs, and completely absent in doubly-deficient BMDCs [116]. In addition, the expression levels of IL-23p19, TNF- α and IL-1 β in infiltrated mononuclear cells collected from the ischemic brain of mice after stroke were significantly reduced in mice deficient in TLR2 or TLR4, and even more severely reduced in TLR2 and TLR4

double-knockout mice [116]. The TLR4-dependence of PRDX1 was also shown with iBMDCs and TG-elicited macrophages isolated from mice, with a largely reduced PRDX1-induced IL-6 secretion in TLR4-deficient mice [344]. In addition, injection of recombinant PRDX1 in TLR4-deficient mice resulted in no significant systemic IL-6 production levels [344]. TLR4 expression in RAW264.7 macrophages detected by immunocytochemistry was higher in PRDX1-, PRDX2-, and PRDX4-treated cells compared to untreated cells [345]. In addition, TLR4-knockout microglia were found to be resistant to the PRDX2-induced neuronal cytotoxicity induced by oxyhemoglobin [117].

Interaction of PRDXs with TLR2 and TLR4

In addition to studies showing that mammalian PRDXs were able to activate TLR2- and TLR4-dependent pathways, a direct interaction between PRDXs and both TLR2 and TLR4 was demonstrated using immunoprecipitation or pull-down assays. Notably, PRDX1 was shown to interact with TLR4, but not with TLR2, by immunoprecipitation using TG-elicited macrophages and anti-TLR antibodies [344]. Similarly, immunoprecipitation using HA-tagged PRDX1 and FLAG-tagged TLR4 overexpressed in HEK293T cells revealed that PRDX1 co-immunoprecipitated with TLR4 and *vice versa* [365]. In addition, direct interaction between endogenous murine PRDX1 and both TLR2 and TLR4 was shown by co-immunoprecipitation using RAW264.7 cells transfected with FLAG-tagged human TLR2 or TLR4 [375]. A physical interaction between PRDX1 and TLR4 was further confirmed by pull-down assay using recombinant 6xHis-tagged PRDX1 and RAW264.7 macrophages [375]. Likewise, GST-tagged PRDX1 was shown to effectively pull down TLR4, and GST-tagged TLR4 could conversely pull down PRDX1 [365].

Other sensors involved in PRDX recognition

In addition to TLRs, the complement system was recently linked to PRDX recognition. The classical pathway of the complement system involves the plasma protein C1q to detect IgM or IgG in immune complexes, PAMPs, as well as apoptotic cellular debris [376,377]. Park *et al.* reported that both murine and human PRDX1 and PRDX2 were able to bind to C1q, and the binding of PRDX2, but not PRDX1, to C1q was found to activate the classical complement pathway [374] (**Figure 16**).

1.3.4. Molecular mechanisms of PRDX recognition

Although TLRs have been shown to be involved in extracellular PRDX-induced pro-inflammatory response and physical interactions could be demonstrated, the molecular mechanisms underlying TLR2 and TLR4 activation by PRDXs remains to be clarified. Indeed, no evidence has yet been provided regarding the specific PRDX region(s) involved in TLR binding. The only information available on the molecular

mechanism related to the DAMP function of PRDXs concerns the region allowing the IL-23-inducing activity of PRDXs. Shichita *et al.* demonstrated that the conserved region of PRDXs is essential for IL-23p19 induction in BMDCs [116]. This region is mainly located on the surface of PRDXs and contains β_4 sheet and α_3 helix regions that are well conserved among PRDXs and among various species [48–50] (**Figure 17**). However, whether such region is involved in the interaction with TLRs (and/or complement system components) remains to be investigated.

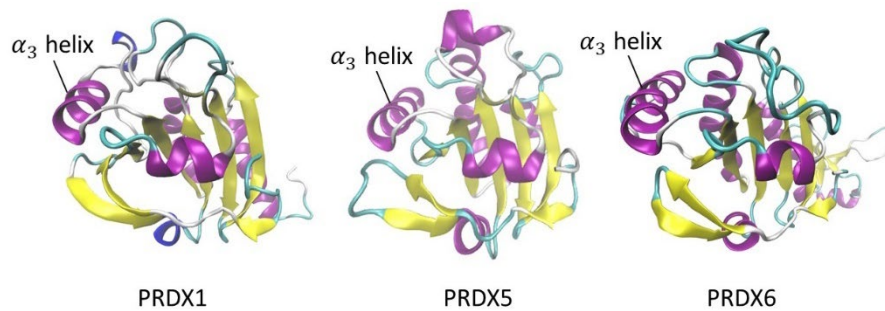


Figure 17. Crystal structure of PRDX1, PRDX5 and PRDX6 underlying the conserved region essential for IL-23p19 induction in BMDCs. Rat homologue of PRDX1 (Hbp23, MMDB ID:11298, left), human PRDX5 (MMDB ID:26393, middle), and human homologue of PRDX6 (hORF6, MMDB ID: 7978, right). α_3 helix of PRDXs, which is sequestered on the protein surface, is shown. Adapted from [116].

In this section, different hypotheses regarding the molecular mechanisms of PRDX-induced TLR activation will be presented.

Role of TLR accessory molecules

The role of TLR accessory molecules in the recognition of PRDXs by TLRs has not been extensively studied as it was only evidenced for PRDX1. Riddell *et al.* reported a reduced expression of IL-6 by TG-elicited macrophages following PRDX1 incubation in the absence of serum, which suggested that serum proteins may contribute to optimal PRDX1–TLR4 interaction [344]. Incubating cells in the presence of blocking antibodies to MD-2 and CD14 significantly inhibited the ability of PRDX1 to trigger IL-6 secretion, confirming that CD14 and MD-2 enhance cytokine secretion induced by PRDX1 [344]. The physical interaction of PRDX1 with the TLR4/MD-2/CD14 complex was further demonstrated by co-immunoprecipitation using TG-elicited macrophage cell lysates and antibodies specific for TLR4 [344]. The TLR4/PRDX1 complexes isolated from treated cells were shown to contain both MD-2 and CD14. A link between PRDX1 and CD14 has also been found in the context of sterile inflammation following pediatric cardiopulmonary bypass [375,378].

These results suggest that MD-2 and CD14 may be involved in the recognition of PRDXs by TLR4 (**Figure 16**). Their involvement for other PRDXs than PRDX1 needs to be investigated and the exact role of both MD-2 and CD14 remains to be determined. In the present thesis, we attempted to answer these questions.

Putative role of cargo molecules in receptor activation

Some researchers suggested the possibility that it is the PRDX cargo rather than the PRDX itself that is being recognized by TLR4 [344,379,380]. Indeed, proteins that are recognized by TLR4, including HMGB1, HSP70, and PRDX1, do not appear to have significant structural similarity, nor to share homology with LPS. However, they do share a common function, *i.e.* they act as intracellular molecular chaperones [73,381,382], which indicates that they are all able to bind cargo molecules. Furthermore, it was shown for HMGB1 that the binding to PRRs (including TLR4, TLR2, TLR9, IL-1R and RAGE) is mediated through complex formation with partner pro-inflammatory molecules, including DNA [383], RNA [384], LPS [385,386], CXCL12 [387], IL-1 α and IL-1 β [386,388], Pam₃CSK₄ [389], as well as additional factors [390].

Oxidoreduction of membrane receptor thiols/disulfide

On the other hand, Salzano *et al.* proposed a mechanism of immune signaling (**Figure 18**) based on the discovery that Trx1 was released along with PRDX2 by activated macrophages [111], that are also known to release H₂O₂ [391]. They suggest that secreted Trx1, in combination with PRDX2, might modify the redox state of cell surface receptors, enabling the induction of inflammatory responses [111]. Although Trx1 is often viewed only as a reducing enzyme, various studies have shown that it can oxidize protein thiols in an oxidizing environment and in the absence of TrxR [392,393]. Secreted Trx1, along with PRDX2, might therefore oxidize or reduce membrane receptors depending on the presence and concentrations of extracellular thiol antioxidants and H₂O₂, thereby inducing receptor dimerization or disassembly, and thus causing their activation or inhibition [111].

This mechanism suggests another hypothesis that PRDX2 might act as a DAMP not only through the ligation of PRRs, but also by participating in the Trx-dependent redox regulation of TLRs or other cytokine receptors. However, the extracellular role of PRDX2-Trx1 as an oxidoreductase remains to be experimentally confirmed.

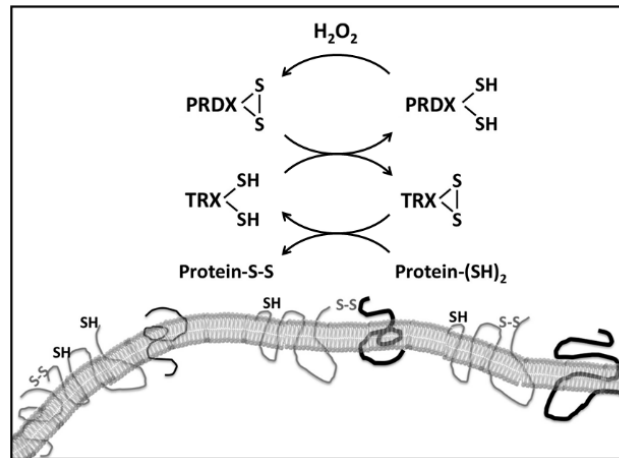


Figure 18. Proposed mechanism of receptor activation by Trx1 in combination with PRDX2. Both PRDX2 and Trx1 are released extracellularly from macrophages following LPS stimulation. PRDX2 uses reduced Trx1 as the electron donor in the reduction of H₂O₂. Given that Trx is an oxidoreductase, it might modify the redox state of membrane receptors (e.g. by oxidizing protein thiols), thereby enabling induction of inflammatory responses. Adapted from [111].

1.3.5. Influence of the redox state, oligomeric state, and post-translational modifications

Many factors have been shown to influence the role of mammalian PRDXs as DAMPs, among which their structure, oligomeric state, redox state, and other PTMs. As redox and oligomeric states are intimately linked in PRDXs, these two factors will be discussed together.

Redox and oligomeric states of PRDXs

The redox and/or oligomeric states of PRDXs were shown to be altered by mutation of their Cys residues or by chemically-induced redox modifications, leading to modulation of their functions (**Figure 19**).

The PRDX1C52S mutant retains its ability to form decamer and its associated chaperone activity, but lacks peroxidase activity, while the PRDX1C83S mutant exists mainly as a dimer, has an intact peroxidase activity but a reduced chaperone activity [73,344,375]. It was shown that PRDX1C52S was able to induce IL-6 secretion from TG-elicited macrophages to the same extent as WT PRDX1, while PRDX1C83S mediated a significantly reduced cytokine secretion [344]. This result was supported by Liu *et al.* who showed that both PRDX1C83S and dithiothreitol (DTT)-treated, monomeric, PRDX1 significantly inhibited TNF- α and IL-10 release from RAW264.7 cells compared to WT PRDX1 [375]. Reduced levels were also observed with phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 cells exposed to PRDX1C83S

[375]. On the contrary to PRDX1C52S, PRDX1C83S binding propensity to TG-elicited macrophages was shown to be significantly reduced compared to WT PRDX1 [344,375]. Furthermore, DTT pre-treatment of PRDX1 was found to prevent binding of the protein to RAW264.7 macrophages and its subsequent internalization [375]. These results suggest that the decameric state of PRDX1 is required for the protein to bind macrophages and induce cytokine secretion.

PRDX1 C52 C71 C83 C173					
	Structure	Peroxidase activity	Chaperone activity	Pro-inflammatory activity	Binding to TLR4
WT PRDX1				 TNF-α secretion ↗ IL-6 secretion ↗ IL-10 secretion ↗	
PRDX1C52S				 TNF-α secretion ↗ IL-6 secretion ↗	
PRDX1C83S				 TNF-α secretion ↘ IL-6 secretion ↘ IL-10 secretion ↘	
DTT-treated PRDX1				 IL-10 secretion ↘	

Figure 19. Influence of the PRDX1 redox and oligomeric states on the pro-inflammatory activity of the protein. PRDX1 has four Cys residues, with two of them being involved in the catalytic cycle (C52 and C173, in red). The PRDX1C52S mutant lacks a peroxidase activity but retains a decameric structure and associated chaperone activity. On the contrary, the PRDX1C83S mutant is present mainly as a dimer, keeps the peroxidase activity but lacks the chaperone activity. Whereas PRDX1C52S is able to induce cell secretion of pro-inflammatory cytokines and to bind to the surface of TLR4-expressing cells to the same extent as WT PRDX1, PRDX1C83S shows a reduced pro-inflammatory activity and a reduced binding to TLR4-expressing cells. DTT-reduced PRDX1 is in a monomeric form, lacks both peroxidase and chaperone activities, and shows decreased pro-inflammatory activity and reduced binding to the surface of TLR4-expressing cells. Created with BioRender.com.

However, as previously mentioned, circulating PRDX1 and PRDX2, as well as PRDX1 and PRDX2 released *in vitro* following inflammatory stimuli, were shown to be present exclusively as disulfide-linked homodimers [104]. Further studies are therefore required to elucidate whether PRDXs can be emitted in various redox forms, as well as the link between these redox forms and the associated immunological or other biological functions. In addition, whether the redox state of other PRDXs than PRDX1 influences their role as DAMPs needs to be investigated. In the present thesis, we notably examined the influence of the redox state of human PRDX5 Cys residues on its function as DAMP.

Post-translational modifications (PTMs)

Although a plethora of PTMs have been identified for mammalian PRDXs using both low- and high-throughput methods [42], how they regulate the function of PRDXs as DAMPs has not been extensively studied. The only study linking the DAMP function of PRDXs with PTMs was performed by Salzano *et al.* who showed that LPS-stimulated RAW264.7 macrophages release glutathionylated, disulfide-linked, PRDX2 homodimers [111]. However, whether this protein has a pro-inflammatory activity remains to be evaluated.

1.3.6. Peroxiredoxin-induced signaling pathways

PRDX1-mediated IL-6 secretion from TG-elicited macrophages was reported to depend on the MyD88-dependent signaling pathway, and stimulation with PRDX1 was shown to result in the TLR4-dependent nuclear translocation of NF- κ B [344] (**Figure 16**). In the context of prostate cancer, PRDX1 stimulation of 2H-11 endothelial cells was also associated with increased NF- κ B activity, as well as with extracellular signal-regulated kinase (ERK) phosphorylation [394] (**Figure 16**). In agreement, PRDX1 stimulation of peritoneal macrophages isolated from mice leads to the NF- κ B-dependent secretion of IL-6, TNF- α , and pro-IL-1 β [362]. Furthermore, PRDX1 stimulation also induces NLRP3 upregulation in primary mouse macrophages, which was shown to facilitate PRDX1-mediated activation of the NLRP3 inflammasome complex through a positive feedback loop, resulting in caspase-1 activation and production of mature IL-1 β [362] (**Figure 16**). Similarly to PRDX1, PRDX2 was shown to activate microglia through TLR4/MyD88/NF- κ B signaling pathway [117]. Zhao *et al.* observed that PRDX1, PRDX2 and PRDX4 induce nuclear translocation of NF- κ B p65 subunit [345].

Extracellular PRDXs have also been reported to be internalized. Indeed, Liu *et al.* demonstrated that PRDX1 binding to TLR4 subsequently results in complex internalization, which plays a crucial role in IL-10 secretion from PMA-differentiated

THP-1 cells [375]. They also showed with RAW264.7 macrophages that PRDX1 endocytosis was clathrin-dependent [375].

1.3.7. Pathological significance of extracellular peroxiredoxins in inflammatory diseases

By acting as DAMP and thereby inducing inflammation, PRDXs play a key role in the pathogenesis of human diseases, including metabolic disorders, autoimmune diseases, neurodegenerative diseases, and cancer.

PRDXs were found to contribute to the pathogenicity of sepsis, which is a dysregulated immune response to infection associated with life-threatening organ dysfunction [395]. Circulating PRDX1 was reported to aggravates LPS-induced septic shock [396]. PRDXs are also associated with various sterile inflammatory-related diseases, including intracerebral hemorrhage (hemorrhagic stroke), subarachnoid hemorrhage, IRI, ALI, and cancer. Circulating PRDX1 was shown to contribute to ALI through promoting inflammation [362]. As the third most abundant protein present in erythrocytes [114], PRDX2 release following hemolysis has been shown to be implicated in intracerebral hemorrhage-induced brain injury [366]. Notably, injection of recombinant PRDX2 in rats resulted in blood-brain barrier disruption, brain swelling, neutrophil infiltration, microglia activation, and neuronal death [366]. In addition, Lu *et al.* revealed that TLR4-knockout microglia lose the ability to induce neuronal apoptosis caused by extracellular PRDX2 after subarachnoid hemorrhage [13]. The role PRDX1, PRDX2, PRDX5, and PRDX6 as DAMPs was also emphasized in brain IRI [11], as depicted on **Figure 20**.

Therefore, understanding how extracellular PRDXs are able to promote inflammation by acting as DAMPs is required for the development of therapeutic strategies related to such diseases.

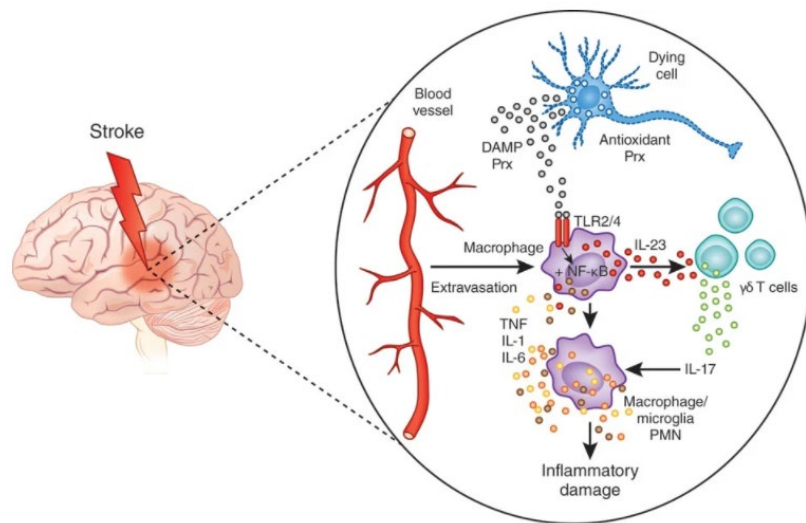


Figure 20. PRDXs are involved in the pathogenesis of post-ischemic inflammation in the brain. Release of PRDXs from necrotic brain cells promotes post-ischemic inflammation after stroke by activating TLR2 and TLR4 on infiltrating macrophages, leading to NF- κ B-dependent release of IL-23. IL-23 aggravates the damage through IL-17-producing $\gamma\delta$ T cells that further enhance the inflammatory response. Reproduced from [397].

Chapter II – Objectives and strategy

2.1. Research aim and objectives

2.1.1. Main goal

The main goal of the present thesis was to investigate the role of human PRDXs as DAMPs. Elucidating how extracellular PRDXs are involved in the initiation of inflammatory processes, and more specifically, in the development of several inflammatory-related conditions (*e.g.* IRI), would allow opening the door for new or better approaches for diagnostics and treatments.

The discovery that mammalian PRDXs are able to trigger a pro-inflammatory response is quite recent, and as a consequence, only few studies have addressed this matter. Furthermore, PRDXs are unique and complex proteins in the sense that they can modify their structural conformation, oligomeric and redox states together, in addition to being subjected to PTMs for their regulation, to have several interacting partners, and to be involved in numerous cellular processes. PRDXs could also play key roles in the modulation of inflammation. Therefore, the role of PRDXs as DAMPs raises a plethora of questions that will need to be investigated in the coming years. What are the release mechanisms of PRDXs into the extracellular milieu? What are the associated PRDX redox forms, oligomeric states and PTMs? To which extent are PRDXs able to activate various cellular receptors and other innate immune sensors? What are the activation mechanisms of these receptors? Which are the signaling pathways involved and is there crosstalk between them? Which inflammatory mediators are secreted and what are the downstream effects triggered locally, but also within the organism?

Among all these questions, studying the molecular events that occur at the cell surface to allow PRDX recognition constitutes an important research topic. The large majority of the studies on the role of PRDXs as DAMPs have investigated the activation of TLR4 since this receptor is one of the most well-known receptors involved in innate immunity and inflammation. However, the molecular mechanisms underlying TLR4 activation mediated by PRDXs are still unclear. Are PRDXs able to bind directly to TLR4 to activate it? Do PRDXs act by binding cargo molecules? Are accessory molecules involved in PRDX recognition by TLR4? Are specific redox forms required to trigger TLR4 activation?

Therefore, this project focuses on how certain human PRDXs are recognized by TLR4 and trigger its activation. As some of these questions have mostly been addressed previously for human PRDX1, we focused this project on two other PRDXs,

namely 2-Cys PRDX2 and atypical 2-Cys PRDX5. These proteins could be produced in sufficient quantity and relatively good purity compared to the others (see **section 2.3.1., Figure 22**). Both PRDXs have been shown to act as DAMPs in the context of sterile inflammatory-related diseases, such as brain IRI or subarachnoid hemorrhage [116,117]. As they belong to two different subfamilies, mechanistic and structural differences (e.g. conformational state, oligomeric state, location of C_R) could be considered within this project.

2.1.2. Specific objectives

In particular, we wanted to address three questions (**Figure 21**). Are human PRDX2 and PRDX5 able to bind directly to TLR4? Do TLR4 accessory molecules MD-2 and CD14 play a role in TLR4 activation and subsequent cytokine secretion induced by PRDX2 and PRDX5? Is the redox state of human PRDX5 Cys residues important for the recognition by TLR4 and subsequent cytokine secretion?

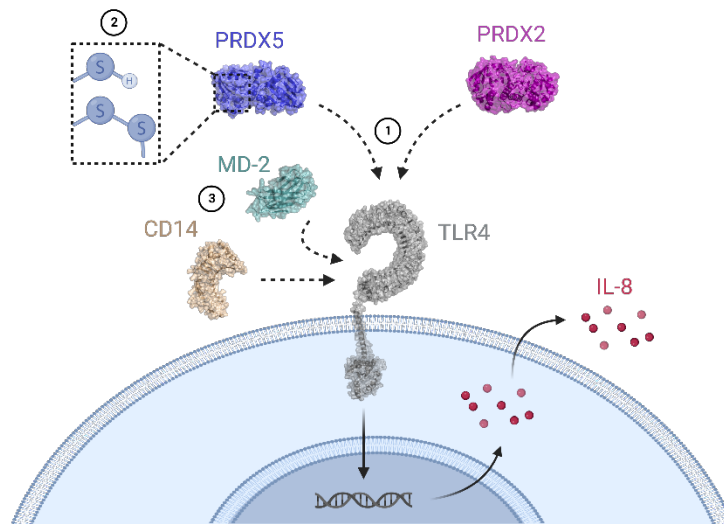


Figure 21. Main objectives of the thesis project. The objectives consisted in probing the direct interaction of human PRDX2 and PRDX5 with TLR4 (1), investigating the influence of PRDX5 redox state on the binding of the protein to TLR4 and its IL-8 secretion-inducing activity (2), and evaluating the role of TLR4 accessory molecules MD-2 and CD14 in PRDX2 and PRDX5 delivery, recognition and subsequent IL-8 secretion. Created with BioRender.com.

More precisely, the objectives were:

- (i) Validating the TLR4-dependent pro-inflammatory activity of human PRDX2 and PRDX5;
- (ii) Analyzing the influence of the redox state of PRDX5 Cys residues (*i.e.* disulfide, reduced, hyperoxidized, and C47S-mutated PRDX5) on the

- ability of PRDX5 to induce a TLR4-dependent secretion of pro-inflammatory cytokines;
- (iii) Probing the direct binding of PRDX2 and PRDX5 with TLR4 and characterizing the interactions in terms of kinetic parameters (*i.e.* dissociation rate constant and distance to the unbinding transition state);
 - (iv) Evaluating the influence of the redox state of PRDX5 Cys residues (*i.e.* disulfide, reduced, hyperoxidized, and C47S-mutated PRDX5) on the binding with TLR4;
 - (v) Elucidating whether the TLR4 accessory molecules MD-2 and CD14 are involved in the PRDX2- and PRDX5-induced TLR4-dependent secretion of pro-inflammatory cytokines;
 - (vi) Probing and characterizing the kinetic parameters of the interactions of PRDX2 and PRDX5 with MD-2 and CD14.

2.2. Experimental strategy

To validate the TLR4-dependent pro-inflammatory activity of PRDX2 and PRDX5, we performed enzyme-linked immunosorbent assay (ELISA) to measure IL-8 secretion levels using a model cell line commonly used in immunology-related studies, namely HEK293 cell line. This cell line lacks the typical TLRs involved in inflammation, including cell surface TLR2 and TLR4 [398]. We therefore used a 'gain-of-function' approach to study the role of TLR4 in the ability of PRDX2 and PRDX5 to trigger cytokine secretion. To do so, we stably transfected MD-2- and CD14-expressing HEK293 cells with a TLR4-coding vector (HEK293 MD2-CD14-TLR4 cells), and compared the IL-8 secretion levels produced with those of HEK293 control cells (lacking TLR4, MD-2 and CD14).

To assess the influence of the redox state of PRDX Cys residues on the ability of PRDXs to induce a TLR4-dependent secretion of IL-8, we focused on human PRDX5 as it represents the simplest model in terms of oligomeric profile. Indeed, human PRDX5 exists as a dimer irrespective of its oxidation state [90]. We used the wild-type PRDX5 and a PRDX5 mutant in which the C_P was replaced by a Ser residue (PRDX5^{C47S}), that was previously characterized [399]. In addition, we chemically treated the wild-type PRDX5 with DTT and iodoacetamide (IAM) to reduce and alkylate the Cys residues, respectively, as well as with DTT and H₂O₂ to obtain an hyperoxidized form of the protein. We characterized the four redox species with Western blot (WB), mass spectrometry (MS) and size-exclusion chromatography (SEC)-multiangle light scattering (MALS). To evaluate the TLR4-dependent pro-inflammatory activity of the different PRDX5 redox forms, we used ELISA to measure IL-8 secretion levels from HEK293 MD2-CD14-TLR4 cells.

To probe the binding between PRDX2 or PRDX5 and TLR4, we used atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) and biolayer interferometry (BLI). We worked with recombinant TLR4 molecules, either fixed on a biosensor in the case of BLI, or on an inert surface for AFM-based SMFS. Whereas BLI allows bulk measurements with PRDX molecules in solution, AFM-based SMFS consists in probing single-molecule interactions by means of a sharp tip carrying PRDX molecules. Both techniques allow to gain insights into the kinetics of the interactions. To determine whether the redox state of PRDX5 Cys residues influences the binding of the protein with TLR4, we probed the interaction between the various PRDX5 redox forms and TLR4 using AFM-based SMFS. Furthermore, to examine the putative effect of the 6xHis tag on the interaction with TLR4, we performed AFM-based SMFS with an untagged PRDX2. In a cellular context, we performed live-cell fluorescence imaging using confocal laser scanning microscopy (CLSM) to observe the binding of PRDX5 at the surface of HEK293 MD2-CD14-TLR4 cells. Furthermore,

we evaluated and compared the binding levels of the PRDX5 redox forms on the surface of living HEK293 MD2-CD14-TLR4 cells using flow cytometry (FC).

To investigate the role of the TLR4 accessory molecules MD-2 and CD14 on the DAMP activity of PRDX2 and PRDX5, we used various engineered HEK293 cell lines expressing combinations of TLR4, MD-2 and CD14. TLR4 and CD14 expression levels in the various cell lines were assessed with FC and Western blot. To determine whether MD-2 and/or CD14 are involved in the ability of PRDX2 and PRDX5 to induce cytokine secretion, we performed ELISA to quantify IL-8 levels produced by the different cell lines following exposure to PRDX2 and PRDX5. Moreover, we probed the binding of PRDX2 and PRDX5 to both recombinant MD-2 and CD14 using AFM-based SMFS. We also started to set up BLI experiments with the aim of examining the role of MD-2 in the binding of PRDXs to TLR4. To approach the physiological conditions, we evaluated the binding of PRDX5 to HEK293 MD2-CD14-TLR4 cells and HEK293 CD14 cells with FC. Furthermore, we tried to identify whether MD-2 and/or CD14 were contained within the PRDX5/TLR4 complex using pull-down assays.

Finally, we attempted to characterize the dynamics of NF- κ B activation following cell stimulation by PRDXs using CLSM.

2.3. Detailed presentation of the techniques used

The project is multidisciplinary, involving various techniques from molecular and cellular biology as well as biophysics. As the readers of this thesis have different fields of expertise, a detailed explanation of the principles of the various techniques used to fulfill the objectives will be provided. These might be unnecessary for the experienced reader. The precise protocols used will be described in the 'Material and Methods' sections of the next chapter (**Chapter III – Results and Discussion**).

2.3.1. Endotoxin removal following protein production

We used endotoxin-purified recombinant human PRDX2 and PRDX5 with a N-terminal hexa-histidine (6xHis) tag that were produced in *E. coli*. The proteins were produced and purified by J. Glibert and A. Clippe from the group of Prof. B. Knoop (LIBST, UClouvain). A representative SDS-PAGE (with Coomassie blue staining) of all the recombinant human PRDX productions (PRDX1–6) is shown on **Figure 22**. Among them, PRDX2 and PRDX5 represent the best candidates for this project in terms of purity and quantity.

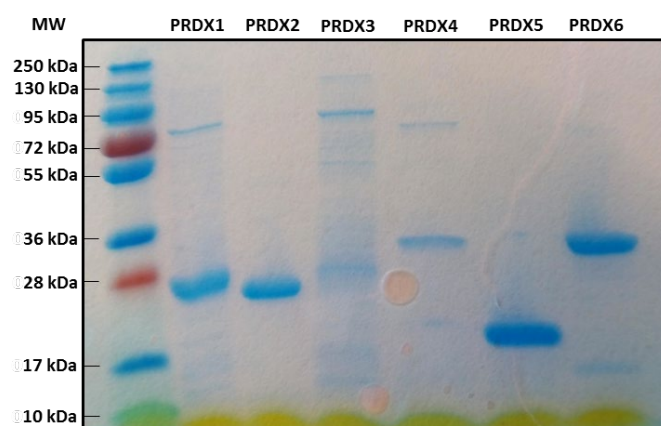


Figure 22. SDS-PAGE analysis of recombinant PRDX1–6 productions. SDS-PAGE was performed under reducing conditions. Representative of all SDS-PAGE analyses performed on PRDX productions used in this thesis project. Data from J. Glibert.

The presence of endotoxins (*i.e.* LPS) within recombinant protein preparations can generate misleading experimental results when studying DAMP-induced inflammation. Indeed, LPS is known to induce a pro-inflammatory response by interacting with the TLR4/MD-2 complex [195,295,298]. Therefore, when recombinant proteins are produced in Gram-negative bacteria such as *E. coli*, care must be taken to remove the putative LPS. Several techniques exist for the downstream removal of endotoxins, including ultrafiltration, solvent extraction, ion

exchange chromatography, affinity chromatography, and membrane adsorption [400]. We used two different endotoxin removal kits in the course of this project: (i) Pierce High Capacity Endotoxin Removal Spin Columns (Thermo Scientific), based on affinity chromatography, for large volumes of protein solution (>4 mL), and (ii) ToxOut Phase Extraction Endotoxin Removal Kit (BioVision), based on solvent extraction, for small volumes (<0.5 mL). The first column system contains a matrix composed of porous cellulose beads covered with covalently attached, modified ϵ -poly-L-lysine, which has a high affinity for endotoxins. This system has a maximum endotoxin binding capacity of 2×10^6 Endotoxin Unit (EU)·mL⁻¹, eliminating >99% of endotoxins, and a protein recovery of $\geq 85\%$. On the other hand, the columns based on phase extraction use Triton X-114 as solvent. The column has a maximum endotoxin binding capacity of 1×10^7 EU·mL⁻¹, removing up to 99.99% of endotoxins, and the protein recovery is >90% (typically >95%).

Different techniques allow endotoxin detection to control the efficiency of removal, including biological detection techniques (e.g. Limulus amoebocyte lysate assay), monocyte activation test, biosensor technique, as well as chemical, optical, fluorescence and luminescence techniques [400]. We quantified endotoxins before and after endotoxin removal, using the Pierce Chromogenic Endotoxin Quant Kit (Thermo Scientific). This detection kit is a chromogenic detection method based on the amoebocyte lysate assay, which measures endotoxins through their interaction with the proenzyme Factor C found in circulating amoebocytes of the horseshoe crab. The enzymatic sensitivity range to endotoxins is 0.01–1 EU·mL⁻¹.

2.3.2. Characterization of PRDX redox and oligomeric status

Prior to the assessment of the influence of the redox state of PRDX5 Cys residues, we characterized the four redox forms in terms of redox and oligomeric statuses using MS and SEC-MALS, respectively.

Mass spectrometry to identify Cys modifications

MS measures the mass-to-charge ratio (m/z) of ionized analytes. MS approaches can be divided into 'top-down', which involves analysis of intact proteins and their fragmentation within the mass spectrometer, and 'bottom-up', which requires protein sample to be digested enzymatically to a peptide mixture before being introduced to the instrument [401]. In this project, we used both approaches to investigate the redox status of the PRDX5 redox forms. As redox modifications change the protein chemical composition, they change the m/z ratio of the intact protein, allowing their detection by MS [401].

We performed intact protein analysis using electrospray ionization (ESI)-MS to determine the mass of the intact PRDX5 redox forms. Changes in mass were

indicative of modifications to the protein residues. However, this approach does not inform on the site(s) of modifications. Therefore, we used a ‘bottom-up’ approach by conducting liquid chromatography (LC)-MS/MS to confirm that the redox-active C47 was modified.

SEC-MALS to identify the oligomeric forms

SEC-MALS is commonly used to analyze the molecular weight of native proteins in solution, and more particularly, to determine the oligomeric state [402]. This method is very useful following protein production as incorrect oligomeric form or the presence of non-native aggregates may create artifacts or inaccuracies when using techniques that quantify interactions, such as SMFS. First, SEC is used to separate the various species in solution based in their hydrodynamic size and diffusion properties [403]. The SEC column will impact how well the different species present in the sample are resolved, but the retention time will not be used to determine molecular weight. Instead, MALS, combined with UV and differential refractive index detectors, allows absolute molecular weight determination. The MALS detector measures the proportion of light scattered by an analyte into multiple angles relative to the incident laser beam. Both the UV and differential refractive index detectors determine protein concentration, the first one *via* absorbance at a wavelength of 280 nm and the second one based on the change in solution refractive index due to the presence of the analyte. The molecular weight is determined from first principles (**Equation 1**) [404].

$$M = \frac{R(0)}{Kc \left(\frac{dn}{dc}\right)^2}$$

Equation 1. Absolute molecular weight determination by SEC-MALS. M is the molecular weight of the analyte, $R(0)$ the reduced Rayleigh ratio (*i.e.* the amount of light scattered by the analyte relative to the laser intensity) determined by the MALS detector and extrapolate to angle zero, c the weight concentration determined by the UV or differential refractive index detector, dn/dc the refractive index increment of the analyte (essentially the difference between the refractive index of the analyte and the buffer), and K a system constant.

In this project, we used SEC-MALS to determine the oligomeric state of the four PRDX5 redox forms.

2.3.3. Binding of individual PRDXs to immobilized receptors

Several tools exist to probe and characterize biomolecular interactions, among which AFM, optical tweezers, BLI, and surface plasmon resonance. Among these, AFM has the advantage of giving insights into the interaction at the single-molecule level and to provide valuable information on the kinetics of interactions. As AFM is

not a commonly used technique in molecular biology, this technique will be more thoroughly explained compared to the other techniques used.

AFM components and basic principle

In biophysics, AFM is a technique mainly used to image sample topography, map sample mechanical properties and probe biomolecular interactions. The basic principle consists in recording local interaction forces between a probe and a scanned surface (**Figure 23a**). To reconstruct the sample topography, the probe is raster-scanned across the sample surface in the X-Y directions. In addition, the probe can perform single approach and retract movements in the Z direction at each point on the surface, enabling the quantitative characterization of the interaction forces. Furthermore, the ability to functionalize both AFM probes and inert surfaces with biomolecules or cells led to the possibility of studying the biophysical properties of interacting biological systems. AFM can be operated both in air or in liquid, at a broad range of temperatures, and reaches a lateral resolution of $\sim 1\text{-}50\text{ nm}$ for imaging [405] and down to the piconewton (pN) range for measuring interaction forces [406].

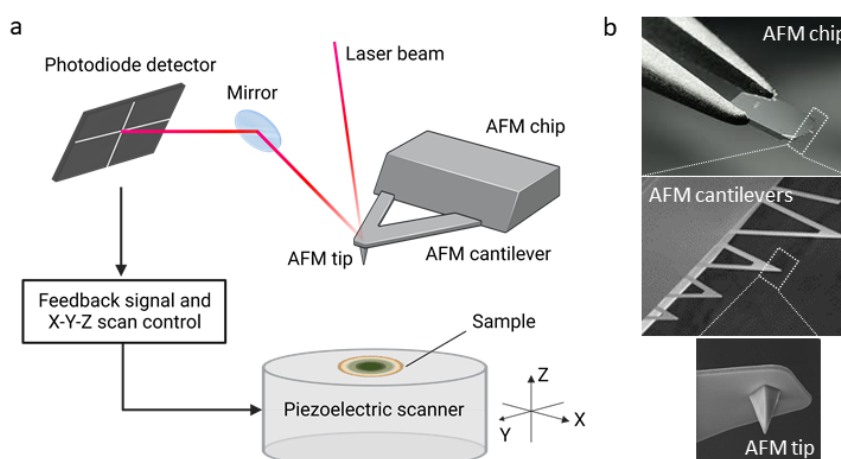


Figure 23. AFM components. (a) The main components are the probe which consists of a chip bearing cantilever(s) with a sharp tip at the extremity, the sample stage, the optical detection system composed of a laser beam and a photodiode, the piezoelectric scanner which allows precise movement of the sample (or the tip) in the X-Y-Z directions, and the computer-based feedback system made up of a controller that provides appropriate feedback signal to the scanner based on the output signal from the photodiode. Created with BioRender.com. (b) Picture of a tweezer carrying a chip (up), and electron microscopy images of rectangular and triangular cantilevers (middle), and tip (down). Images from [407,408].

The AFM probe consists of a chip generally made of silicon (Si) or silicon nitride (Si_3N_4), bearing one or more cantilever(s) of tens to hundreds of μm long, at the

extremity of which is attached a sharp tip (**Figure 23b**). The tip is $\sim 5\text{-}15\ \mu\text{m}$ long and has a conical or pyramidal apex with a radius of curvature of $1\text{-}70\ \text{nm}$. The chip is supported by a holder and placed above the sample stage. The interaction forces are measured by an optical detection system composed of a laser beam and a photodetector. The laser beam is projected on the upper surface of the metal-coated cantilever, close to the tip fixation point. The reflected beam is then deviated by a mirror into a four-segmented, position-sensitive photodiode. Either the chip or the sample is mounted on a piezoelectric scanner that allows precise three-dimensional positioning and movement. By virtue of their composition, piezoelectric materials can deform mechanically when subjected to an electrical voltage, and therefore expand or contract in the X-Y-Z directions. A computer-based feedback system made up of a controller collects and processes the data to provide appropriate feedback signal to the piezoelectric scanner based on the output signal from the photodiode.

When the tip is brought close to the sample surface, the interaction forces between the tip and the sample leads to a vertical deflection (*i.e.* bending) of the cantilever. This deflection is monitored through a shift in the position of the laser beam into the photodiode, leading to a voltage difference. The photodiode signal can be directly related to the extent of cantilever deflection, thereby providing a measure of the force experienced by the tip: a higher voltage change indicates a larger cantilever deflection and thereby a higher force exerted on the tip within its close environment, and conversely.

AFM-based force spectroscopy

Force spectroscopy consists in measuring the magnitude of the interaction forces (attractive or repulsive) arising between the tip and the sample surface while performing cycles of approach and retraction movement. The result of the measurement from an approach and retraction cycle is initially a measure of the photodiode signal (in V) *versus* position of the piezoelectric scanner normal to the surface (in m). To obtain a measure of the force (F , in N) *versus* the distance separating the tip from the sample (d , in m), the voltage difference and the position of the piezoelectric scanner must be converted into a force and a separation distance between the tip and the sample surface, respectively. Multiplying the voltage difference (in V) by the photodiode sensitivity (in $\text{m}\cdot\text{V}^{-1}$), a factor inherent to each experiment, results in the deflection (δ in m) of the cantilever. Since the photodiode sensitivity varies notably with the set-up used, the medium refractive index, and the position of the laser beam onto the cantilever, it must be calibrated for each experiment. Then, as a cantilever can roughly be approximated as a damped spring with a spring constant k (in $\text{N}\cdot\text{m}^{-1}$), the interaction force (F) can be considered proportional to the deflection (δ) of the spring-like cantilever, according to Hooke's law (**Equation 2**). The tip-sample distance (d) is calculated by subtracting the

cantilever deflection (in m) from the position of the piezoelectric scanner (in m). The final result consists in a force *versus* distance (FD) curve, generated for each up-and-down cycle of the AFM tip towards the sample (**Figure 24**).

$$F = -k \cdot \delta$$

Equation 2. Hooke's law. Considering the cantilever as a Hookean spring with a spring constant k (in $\text{N}\cdot\text{m}^{-1}$), the deflection of the cantilever δ (in m) gives the measurement of the interaction force F (in N).

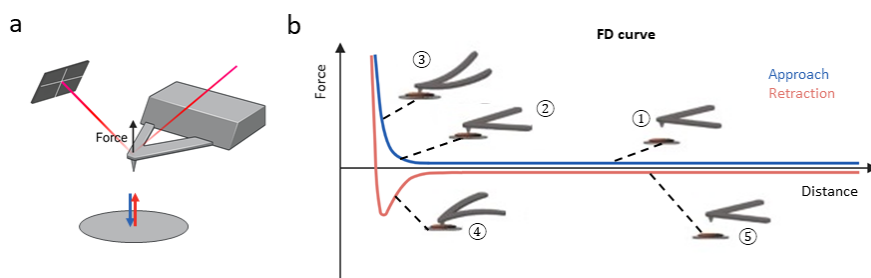


Figure 24. Principle of force spectroscopy. (a) In force spectroscopy, the tip is approached and retracted from the surface in the Z direction. Created with BioRender.com. (b) Typical force-distance (FD) curve. During the approach of the cantilever towards the surface, there is no force acting on the cantilever (1). When the tip touches the surface (2), the cantilever senses an increasing force and is deflected (3). Upon retraction of the tip, adhesion force between the tip and the surface may occur, leading to the bending of the cantilever downwards (4), before the interaction ruptures. The cantilever is further retracted (5) until its initial position away from the sample. Inspired from [409].

Coating the AFM tip and/or a sample surface with chemical functional groups, biological entities or particles opened up new possibilities to study intramolecular interactions, as well as intermolecular interactions between such entities.

AFM-based single-molecule force spectroscopy

Advanced functionalization strategies allowing the controlled grafting of biomolecules on AFM tips and on sample surfaces led to the possibility of performing SMFS studies to probe the characteristics of biomolecular interactions. SMFS mapping consists in raster scanning a sample surface while performing single approach and retraction cycles in discrete points on the sample surface to record FD curves (**Figure 25**). To perform SFMS experiments, several requirements must be fulfilled: (i) the strength of the chemical link between the tip and the ligand, as well as between the receptor and the surface, must be greater than that of the probed ligand-receptor interaction, (ii) ligands and receptors must be grafted at a sufficiently low density to reduce the occurrence of multiple molecular interactions during a

single up-and-down cycle of the tip, and (iii) the ligand must have sufficient mobility to adopt proper orientation enabling the binding with its receptor.

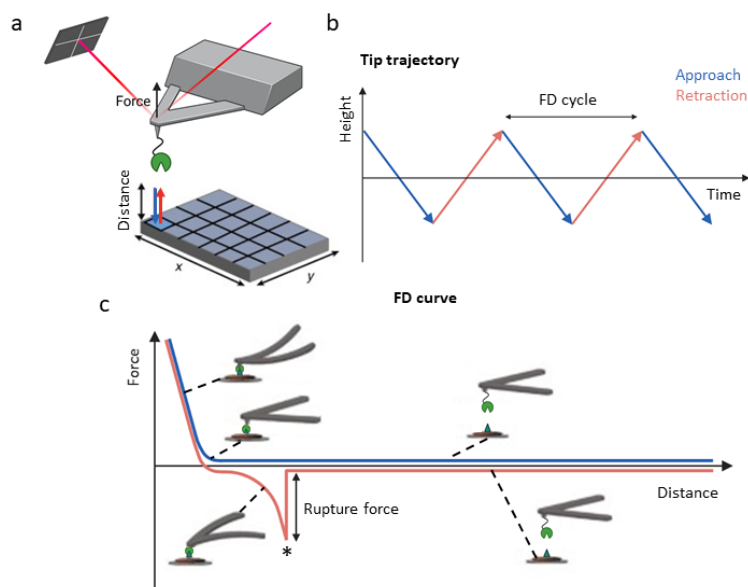


Figure 25. Principle of single-molecule force spectroscopy (SMFS). (a) The AFM tip, represented here with a ligand, raster scans the sample surface while performing approach and retraction cycles towards the sample surface. Created with BioRender.com. (b) One FD cycle corresponds to the combination of an approach and retraction movement of the AFM tip towards the sample surface. (c) Typical FD curve showing an adhesive event obtained when probing a biomolecular interaction between a ligand and its receptor. Retraction of the tip pulled one of the interaction partners, leading to the deflection of the cantilever, before the interaction ruptures (*). The rupture force corresponds to the force required to break the interaction and can be extracted directly from the FD curve. The rupture distance is the distance between the point at which the tip contacts the sample surface and the point at which the interaction ruptures. Inspired from [409,410].

Several functionalization chemistry strategies have been developed, that rely either on bare Si/Si₃N₄ tips or on gold-coated tips. With Si/Si₃N₄ tips, the first step consists in introducing amino groups on the tip surface, either by treatment with silanes (*e.g.* using (3-aminopropyl)triethoxysilane, APTES), or by esterification with ethanolamine hydrochloride [411]. The second step involves the use of a heterobifunctional flexible polymeric linker such as poly(ethylene glycol) (PEG) chains carrying an *N*-hydroxysuccinimide (NHS) ester group at one extremity and another chemical reactive group at the other end, free to bind the ligand (Figure 26) [412,413].

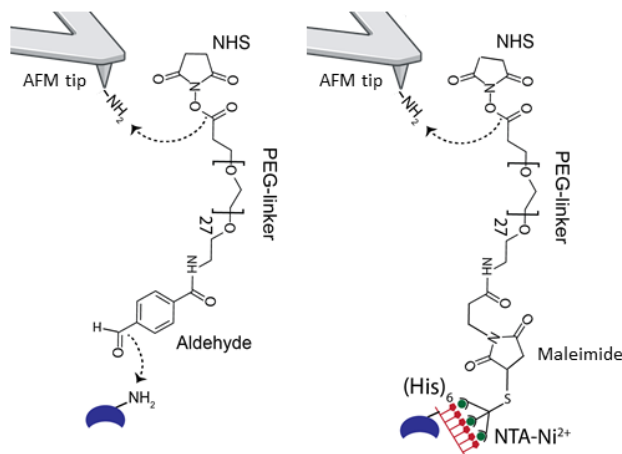


Figure 26. Examples of functionalization strategies based on the use of a heterobifunctional PEG linker. The PEG linker is composed of an NHS group at one extremity and another reactive chemical group to bind the protein at the other extremity. Aldehyde group allows grafting a protein through any of its free amino groups (left), whereas maleimide group allows grafting a protein through any of its free thiol groups (not shown), or a His-tagged protein by using disulfide-*tris*-NTA molecules and Ni^{2+} (right). Adapted from [414].

The NHS group activates a carboxylic group from the linker, allowing reaction with the tip surface amino groups (**Figure 26**). Numerous reactive groups are available, among which aldehyde/acetal and maleimide groups that allow the grafting of proteins through their free amino or thiol groups, respectively (**Figure 26**). Another example consists in the use of disulfide-nitrilotriacetic acid (NTA) or disulfide-*tris*-NTA molecules that can react with the maleimide moiety of the linker to allow the coupling of His-tagged proteins through chelation with metal cations (typically, Ni^{2+}) (**Figure 26**). Therefore, the use of a specific functionalization chemistry depends on the features of the ligand to be grafted to the AFM tip.

The use of a PEG linker (i) confers mobility to the ligand, and (ii) allows detecting the adhesive events arising from its elongation. Indeed, the elongation of the linker gives rise to a characteristic pattern and a higher rupture distance (*i.e.* distance between the point at which the tip contacts the sample surface and the point at which the interaction ruptures) on the FD curves (**Figure 25c**). This allows neglecting unspecific binding events occurring between the tip itself and the surface.

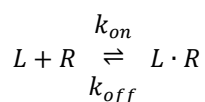
Gold-coated tips can be functionalized by chemisorption with a self-assembled monolayer (SAM) of alkanethiol molecules, taking advantage of the high affinity of thiol groups with gold. To control the density of the ligand, a mix of alkanethiols with two different terminal groups, *i.e.* one inert and the other one reactive toward the ligand, can be used in various proportions (*e.g.* 99%-1%) [415]. For example, tips can

be functionalized with a mix of carboxy- and hydroxy-terminated alkanethiols [416]. While carboxy-terminated alkanethiols are activated by NHS/1-ethyl-3-(3-dimethylaminopropyl) (EDC) to allow covalent attachment of proteins through their free amino groups, hydroxy-terminated alkanethiols remain inert. Another example consists in the use of NTA-terminated alkanethiols for the attachment of recombinant His-tagged proteins by forming a complex using metal cations [417]. Gold-coated tips may also be functionalized by PEG linkers carrying a thiol group at one end, by mixing low amount of linkers with an NTA molecule at the other end with a higher amount of linkers without NTA molecule [418].

The sample surface consists in a silicon wafer coated with a 5-nm thick chromium layer using electron beam thermal evaporation followed by a 30-nm thick gold layer. This surface can be functionalized with similar chemistries as gold-coated tips.

Dynamic force spectroscopy and biomolecular recognition

Biomolecular recognition consists in the reversible association of two biological entities with high specificity and affinity, through the formation of a combination of non-covalent interactions (ionic/electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic effects) to form a specific complex [419]. Biomolecular interactions between a ligand (L) and its receptor (R) can be characterized in terms of binding kinetics and thermodynamics (*e.g.* association and dissociation rate constants, binding free energy) (**Equation 3**). These characteristics can be pictured on the free energy profile of the biomolecular partners, which describes the variation of the free energy of the molecules within all their physical and configurational states. The binding free energy landscape of a biomolecular interaction represents the binding free energy profile of the interacting biomolecules as a function of their separation along their preferred association-dissociation pathway (**Figure 27**). Most biomolecular interactions can be modeled as ideal bonds, in which the energy landscape contains a free energy minimum corresponding to the bound state of the two partners, separated by a single energy barrier from another free energy minimum, corresponding to the unbound state. The binding free energy (ΔG_{bu}) then corresponds to the difference between the two energy minima.



Equation 3. Binding model for a biomolecular interaction. This binding model assumes a simple 1:1 interaction, where one ligand molecule interacts with one receptor molecule. The rate of complex formation ($L \cdot R$) is notably dictated by an association rate constant (k_{on} , in $M^{-1} \cdot s^{-1}$). The dissociation of the complex occurs at a rate that is notably function of the dissociation rate constant (k_{off} , in s^{-1}), which measures the stability of the complex, or the fraction of complex that decay per second.

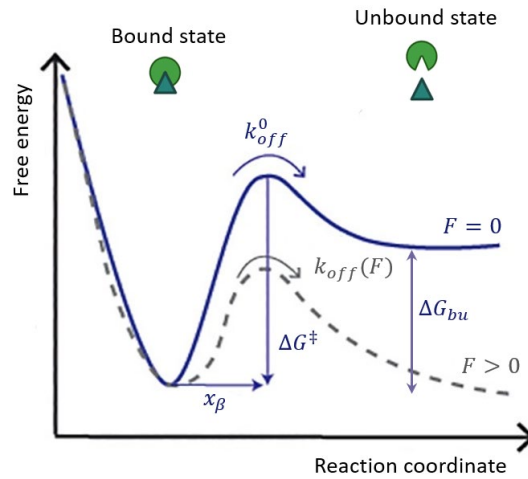


Figure 27. Free energy landscape of a biomolecular interaction. Free-energy potential of biomolecular partners in their natural environment (blue) or submitted to an externally applied force F (grey). $\Delta G^\ddagger$ is the activation energy of unbinding, ΔG_{bu} is the free energy difference between the bound and unbound states, and x_β represents the distance between the bound state and the unbound state. In equilibrium conditions ($F = 0$), the energy barrier is spontaneously crossed with a dissociation rate constant k_{off}^0 . The application of an external force to the interaction leads to a modification of the energy landscape by reducing the height of the energy barrier towards dissociation, thereby reducing the dissociation rate constant, and as a result, the interaction lifetime. Adapted from [420].

In their natural environment, biomolecular interactions are subjected to thermal fluctuations. Spontaneous dissociation of a biomolecular complex can occur when thermal impulses stochastically reach the activation energy required to disrupt the interactions holding the two partners together. Each biomolecular interaction has therefore a finite lifetime, which corresponds to the average time during which the two partners interact, before thermal energy disrupts the biomolecular complex. The dissociation rate constant, or kinetic off-rate (k_{off}), represents the average frequency at which the biomolecular complex ($L \cdot R$) is thermally disrupted, and corresponds to the inverse of the complex lifetime. Ideal bonds are characterized by a single kinetic off-rate, which depends on the energy barrier height and is therefore unique for each biomolecular interaction [421].

Out-of-equilibrium single-molecule experiments such as SMFS have been extensively used to determine the kinetic off-rate of biomolecular interactions in order to characterize their inherent stability [422–424]. Applying SMFS to biomolecular systems consists in pulling one of the interacting partners to mechanically break the complex probed. This externally applied force interferes with the natural dissociation process of the two partners as it provides a certain amount

of energy towards the disruption of the complex. The height of the energy barrier that has to be overcome by thermal energy is thereby reduced, which increases the probability to break the interaction (**Figure 27**). An externally applied constant pulling force (F) thereby reduces the natural lifetime of a biomolecular interaction (**Equation 4**).

$$k_{off}(F) = k_{off}^0 \exp\left(\frac{Fx}{k_b T}\right)$$

Equation 4. Dissociation rate constant of a biomolecular interaction submitted to an externally applied force. Upon loading (*i.e.* application of an external force), the dissociation rate constant of a biomolecular interaction increases as the applied force diminishes the energy barrier towards dissociation. F (in N) is the externally applied force, k_{off}^0 (in s^{-1}) is the dissociation rate constant when no external force is applied, x (in m) is the reaction coordinate, and $k_b T$ (in J) is the thermal energy (with k_b the Boltzmann's constant).

Upon pulling on biomolecular partners, disruption of the complex is correlated with a certain amount of force required to break the interaction. This rupture force (F_R) depends on the time during which the external load is applied, *i.e.* the velocity at which the partners are pulled apart. At high pulling velocity, there is less time available for thermal disruption of the complex so that a higher mechanical energy is required to break it, and the rupture force measured is therefore high. Conversely, at low pulling velocity, the contribution of thermal energy is higher so that a lower mechanical rupture force is required, and the rupture force measured is low.

Practically, the rupture force of a biomolecular interaction depends on the loading rate (LR), which represents the increase of force applied over time ($LR = \Delta F / \Delta t$, in $N \cdot s^{-1}$) and is proportional to the pulling velocity ($LR = v \cdot k_{eff}$, where v , in $m \cdot s^{-1}$, is the tip retraction speed, and k_{eff} , in $N \cdot m^{-1}$, is the effective spring constant of the cantilever-linker-ligand system). The dependency of the rupture force of a biomolecular system on the LR was subject of various theoretical and experimental studies [425–427]. The Bell-Evans model, which is the first to have emerged, describes the dissociation probability of a biomolecular interaction subjected to an increasing external force [423,428–430]. According to this model, far from equilibrium, the most probable rupture force of a biomolecular complex increases linearly with the LR with a dependency on two kinetic parameters, namely the kinetic off-rate and the distance to the transition state (or width of the energy barrier, x_β), the latter corresponding to the minimum distance required between the two partners to consider them as separated (**Equation 5**). Therefore, by force-probing biomolecular interactions at a wide range of LRs, and by measuring the corresponding rupture forces, the Bell-Evans model allows extracting the dissociation rate constant of biomolecular complexes [423].

$$F_R = \frac{k_b T}{x_\beta} \ln \left(\frac{LR x_\beta}{k_b T k_{off}} \right)$$

Equation 5. Bell-Evans model. The rupture force F_R (in N) of a biomolecular complex increases linearly with the logarithm of the loading rate (LR, in $\text{N}\cdot\text{s}^{-1}$). x_β (in m) is the width of the energy barrier (or distance to the transition state).

More recently, the Dudko-Hummer-Szabo model was developed to account for deviations from the behavior predicted by the Bell-Evans model observed in DFS experiments spanning over an extremely wide range of LRs [426,431]. This model integrates the possibility to expand the calculation of the natural lifetime of a biomolecular interaction for different shapes of energy landscapes, through the use of an arbitrary exponential parameter α . This parameter allows materializing the various shapes of energy potentials arising from the influence of molecular linkers, which can display non-perfect spring behaviors. These include the cusp-shaped potential ($\alpha = 1/2$), the linear cubic potential ($\alpha = 2/3$), or the harmonic potential ($\alpha = 1$, which recovers the Bell-Evans model). Other experiments showed that DFS data tend to level out for rupture forces measured at low LRs. This was accounted for by the Friddle-Noy-de Yoreo model [425]. This model states that at low LRs, the biomolecular complex is probed in a near-equilibrium regime, within which the confinement of the two partners by the AFM tip prevents the spontaneous dissociation of the complex, yielding a minimum required rupture force. Measuring the equilibrium rupture force allows gaining insights into thermodynamic parameters of the biomolecular interaction such as the equilibrium free-energy difference between the bound and unbound states. A comparison of the three models is presented on **Figure 28**.

In SMFS experiments, even if surface density is controlled through functionalization strategies to favor the occurrence of single molecular interactions during a single tip-sample interaction, FD curves containing traces of simultaneously rupturing multiple interactions are often observed. A model was developed by Williams and Evans [433] showing that, for multiple parallel uncorrelated interactions rupturing simultaneously, the most probable rupture force of the system scales with the number of bonds engaged (**Equation 6**). This model allows predicting the rupture force-LR dependency of multivalent complexes based on the kinetic parameters of the monovalent interaction.

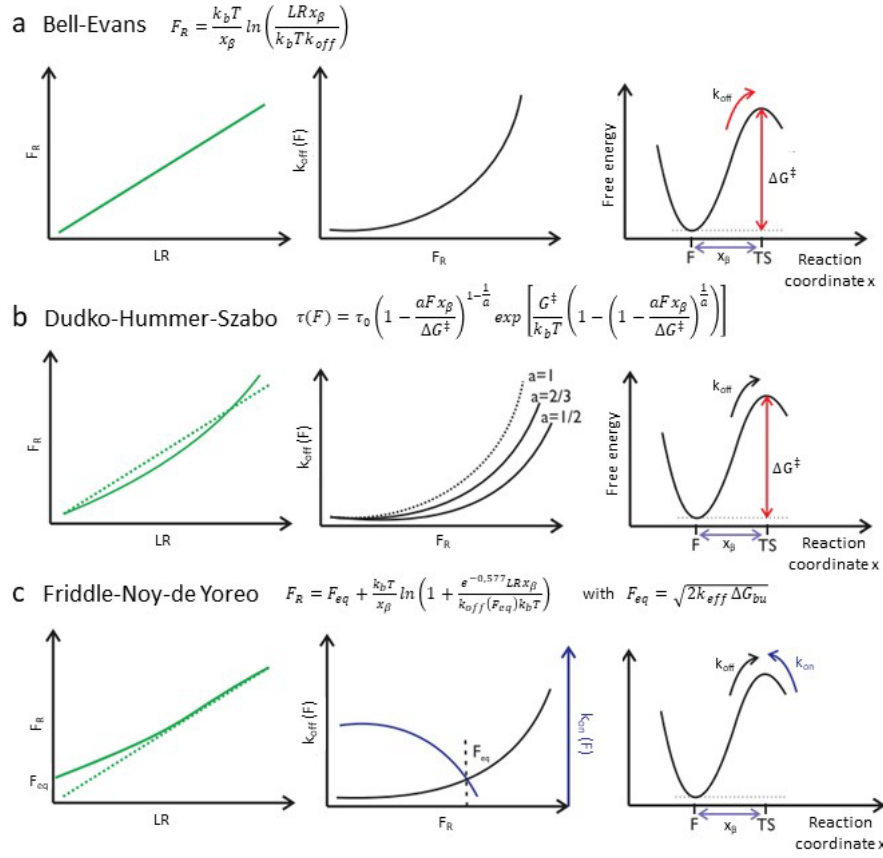


Figure 28. Comparison of the three main DFS models. (a) Bell-Evans model. (b) Dudko-Hummer-Szabo model. (c) Friddle-Noy-de Yoreo model. Schematic diagrams illustrating the dependence of the rupture force on the LR (left), the dependence of the rupture force on the kinetic off-rate (middle), and the energy landscape (right). F stands for ‘folded’, and TS for ‘transition state’. Adapted from [432].

$$\frac{1}{LR} = \frac{x_\beta}{k_{off} k_b T} \sum_{n=1}^N \frac{1}{n^2} \exp \left(\frac{-F_R x_\beta}{n k_b T} \right)$$

Equation 6. Williams-Evans model. N is the number of partners available for dissociation, k_{off} and x_β are the kinetic parameters of the single interaction obtained with the Bell-Evans model.

AFM-based SMFS to probe and characterize PRDX binding to TLR4, MD-2 and CD14

In this thesis project, we used AFM-based SMFS to probe and characterize the binding kinetics of (i) human PRDX2 and PRDX5 to TLR4, (ii) PRDX5 redox forms to TLR4, (iii) PRDX2 and PRDX5 to MD-2, and (iv) PRDX2 and PRDX5 to CD14. The

strategy involves selecting appropriate (i) AFM probe and calibration method for quantitative measurements, (ii) tip and surface functionalization chemistries, (iii) environmental factors (buffer composition, pH, temperature), (iv) controls for the specificity of the interactions, and (v) data analysis procedure.

AFM probe, calibration method and environmental conditions

When choosing an AFM probe, the most important features to consider are the spring constant of the cantilever and the tip sharpness. Biomolecular interactions between proteins are usually in the pN range. To be able to detect such small forces, very soft cantilevers must be used ($k < 0.1 \text{ N}\cdot\text{m}^{-1}$). Indeed, for an identical force applied to the AFM tip, a soft cantilever will undergo a larger deflection compared to a stiffer one (e.g. a force of 10 pN would cause a deflection of 1 nm for a cantilever with a spring constant of $0.01 \text{ N}\cdot\text{m}^{-1}$ whereas the deflection would be 0.1 nm for a cantilever of $0.1 \text{ N}\cdot\text{m}^{-1}$). We used cantilevers with a nominal spring constant of $0.01 \text{ N}\cdot\text{m}^{-1}$ and a nominal tip radius of 10 nm.

For quantitative measurements, the optical (photodiode) sensitivity and cantilever spring constant must be calibrated. Both parameters were calibrated after the recording of the AFM maps. The sensitivity was calibrated by performing a set of FD curves on a hard mica surface. In the contact region of the tip with the surface, the cantilever deflection corresponds to the displacement of the piezoelectric scanner. This allows extracting the relationship between the signal within the photodiode and the cantilever deflection, which corresponds to the sensitivity. The cantilever spring constant was calibrated using the thermal noise method [434], which is based on the measure and analysis of the cantilever movements due to thermal fluctuations.

Regarding the environmental conditions, we worked in liquid (within a drop of $\sim 100 \text{ }\mu\text{L}$) using phosphate-buffered saline (PBS, pH 7.4), and at room temperature (RT).

Functionalization chemistries of tips and surfaces

To probe the binding between PRDX2 and TLR4, we chose the functionalization strategies based on the presence of a His tag on both proteins. Indeed, recombinant TLR4 used in the experiments consists in the C-terminal 10xHis-tagged extracellular and transmembrane portion of the receptor. AFM tips were functionalized with 6xHis-tagged PRDX2 using an NHS-PEG₂₇-maleimide linker in combination with disulfide-*tris*-NTA molecules and Ni^{2+} . This strategy has the advantage of grafting the protein in a constant and oriented manner, *i.e.* through its N-terminus. Surfaces were functionalized with NH_2 -*tris*-NTA-molecules bound to the carboxyl-terminated alkanethiols of a mixed SAM of carboxyl- and hydroxyl-terminated alkanethiols (in a

1:99 ratio) using NHS/EDC chemistry. This allowed the grafting of TLR4 through its 10xHis tag by using Ni^{2+} . To ensure that the PRDX2 orientation on the AFM tip does not affect the binding of the protein to TLR4, we also used an untagged PRDX2 produced in *E. coli*. Untagged PRDX2 was covalently attached to the AFM tip through any of its free amino groups using an NHS-PEG₂₇-acetal linker. This functionalization strategy has the advantage of a covalent binding between the tip and the protein, but may result in alternative binding orientations.

As the functionalization strategies resulted in the presence of nonspecific binding, most likely due to the presence of *tris*-NTA- Ni^{2+} , we changed our approach for the use of covalent functionalization chemistries to probe further interactions. These interactions include the PRDX5 redox forms with TLR4, and PRDX2 or PRDX5 with both recombinant MD-2 and CD14. PRDX molecules were covalently grafted onto the AFM tips through any of their free amino groups by means of an NHS-PEG₂₄-aldehyde linker. TLR4, MD-2 and CD14 were covalently attached to the carboxyl-terminated alkanethiols of a mixed SAM using NHS/EDC chemistry.

Controls for the specificity of the interactions probed

To assess the specificity of the interactions, two types of controls can be used: (i) one of the partners of the complex is either removed from the set-up or replaced by a nonrelated molecule, and (ii) either one of the partners, or a blocking antibody directed against one of the partners, is injected at saturating concentration in the solution to block the interaction [435]. By examining the effect of such controls on the binding frequency (BF), the specificity of the interactions probed may be evaluated. The BF is given by the ratio between the number of specific events and that of total recorded events. In this project, we performed both types of controls. We removed PRDX2 from the tip or TLR4 from the surface, and we injected antibodies (*i.e.* neutralizing anti-CD14, anti-MD-2, and anti-TLR4 antibodies or anti-PRDX5 and anti-PRDX2 sera [436]).

DFS data treatment

To obtain the kinetic parameters of the interaction, an extensive analysis of the raw data after physical measurements is required. FD curves must first be sorted since most curves recorded do not display an adhesive event. Next, the 'specific' adhesive FD curves must be sorted from the 'nonspecific' adhesive curves. 'Nonspecific' interactions concern all kind of unwanted physical or chemical interactions, such as the tip-functionalized ligand with the molecules used to saturate the gold-coated substrate (*i.e.* alkanethiols in this project), or the PEG linker atoms with atoms from the surface-grafted receptor [435]. The features that are currently considered for the selection of specific FD curves rely on the extension of the PEG linker. As previously mentioned, the extension of the linker leads to (i) a

characteristic extension pattern (*i.e.* signature) on the adhesive event that can be modeled by classical polymer extension models such as the worm-like chain model² [437], and (ii) a longer rupture distance. For practical reasons (*i.e.* no available algorithm on the analysis software), we did not use any elasticity model to fit the unbinding event in the retraction curve, but rather relied on the human eye to recognize the linker signature. We also selected specific FD curves showing an unbinding event having a rupture distance falling within the maximum theoretical length of the systems under study (**Figure 29**).

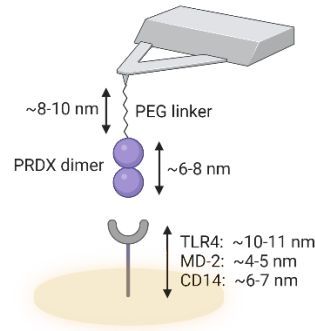


Figure 29. Calculation of the maximum theoretical length of the systems under AFM study. The theoretical length of the PEG linker combined with the biological complex under study is ~29 nm, ~23 nm and ~25 nm in the case of PRDX–TLR4, PRDX–MD-2 and PRDX–CD14, respectively. Created with BioRender.com.

The next step in the data analysis consists in extracting the rupture forces and LRs from the curves. As previously mentioned, rupture forces are extracted from the adhesive peaks of the FD curves (**Figure 25c**). LRs are practically extracted by fitting linearly the force-time curve just before rupture to get the slope, which corresponds to the LR. Once the rupture forces and the LRs have been extracted for each single specific adhesive curve, all values are pooled into the so-called dynamic force spectroscopy (DFS) plot, which displays rupture forces as a function of the logarithm of the LRs. Practically, the DFS plot appears as a cloud of data points (**Figure 30a** and **Figure 31a**) due to two or, in some cases, three phenomena: (i) the stochastic behavior of biomolecular rupture events, (ii) the presence of a polymer linker, and (iii) when applicable, the multivalency of interactions.

² The worm-like chain model is used to estimate the contour length L_c (*i.e.* length at maximum physically possible extension) of the complex tethered to the AFM tip, by considering it as a homogeneous stiff elastic rod without discrete structure, characterized by a persistence length l_p and a stretch modulus ϕ :

$$F(x) = \frac{k_B T}{l_p} \left[\frac{1}{4 \left(1 - \frac{x}{L_c} + \frac{F(x)}{\phi} \right)^2} - \frac{F(x)}{\phi} - \frac{1}{4} + \frac{x}{L_c} \right]$$

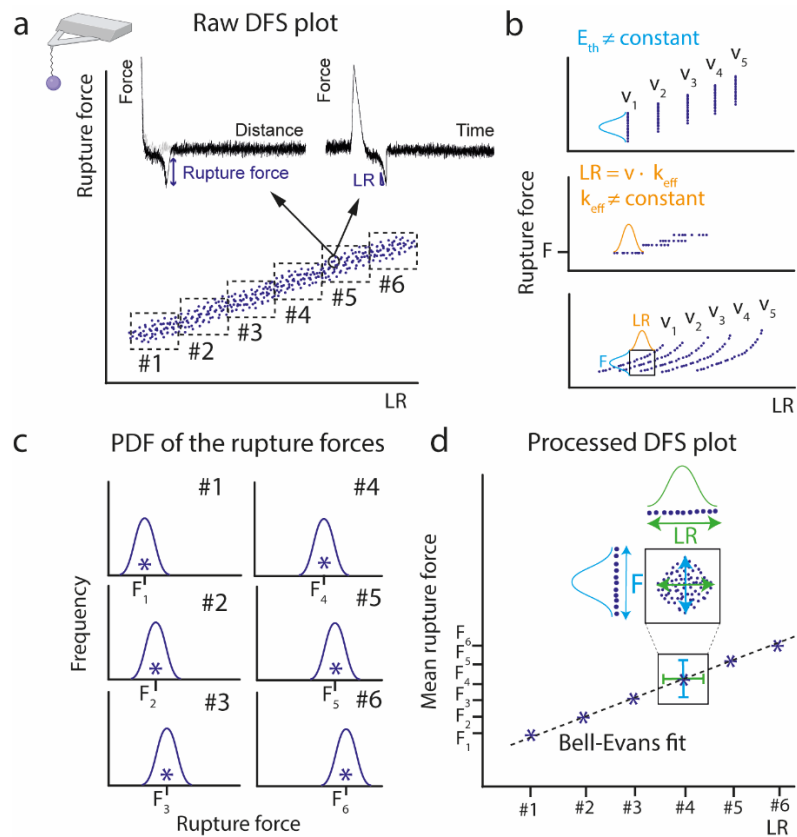


Figure 30. Theoretical illustrations explaining DFS data treatment when probing single interactions. (a) Rupture forces and LRs are extracted from specific FD and force *versus* time curves, respectively, and are displayed in a raw DFS plot. Each dot represents a single ligand–receptor interaction (*i.e.* rupture event). (b) For a single tip retraction speed (v), a distribution of the rupture forces arises from variations in the thermal energy available for dissociation between the rupture events probed (up). For each tip retraction speed (v), a single rupture force F value can correspond to various LRs as the effective stiffness of the cantilever–linker–biomolecular system varies for each interaction probed (middle). The DFS data cloud actually results from the combination of these two phenomena (down). (c) Rupture forces are pooled within discrete ranges (#1 to #6 in the raw DFS plot) for which a probability density function (PDF) allowing extraction of the most probable rupture force (*) is generated. (d) The most probable rupture forces obtained at each LR range are then plotted on the DFS plot, and the Bell-Evans model is used to fit them, allowing determination of kinetic parameters of the interaction. Inspired from [414].

First, the stochastic nature of bond rupture must be considered in the analysis. The rupture force associated with a single LR is indeed not a single value, –and would not be a single value even if SMFS experiments were performed without experimental error–, but instead follow a statistical distribution. Indeed, as mentioned above, individual bond rupture in a biomolecular complex is a stochastic

event as the thermal energy available to break the bond is subjected to spatial and temporal fluctuations. As the contribution of thermal energy is not identical for each rupture event, the mechanical energy required to add up to the thermal energy contribution in order to dissociate the complex is not identical either, giving rise to a distribution of the rupture forces. As a consequence, the most probable rupture force for each LR value, *i.e.* the mode of the rupture force distribution, must be determined (**Figure 30b**). These distributions are usually fitted by gaussian or Poisson functions [435]. In this project, we extracted the most probable rupture forces from gaussian function maxima.

Secondly, the presence of a PEG linker tethering the ligand has an impact on the LR [438]. Indeed, at fixed tip retraction speed (v), the LR at which the complex is probed varies since the effective stiffness of the cantilever-linker-ligand system (k_{eff}) changes for each rupture event. As a consequence, the presence of the linker leads to a distribution of the LRs (**Figure 30b**).

Thirdly, in some cases, another consideration must be taken in the analysis. Despite careful tip and surface functionalization strategies performed as to obtain ideal SMFS experiments that probe single interactions only, a percentage of the rupture events detect multiple interactions [439]. On one hand, a few molecules can be present at the tip apex, leading to the possible probing of multiple interactions. On the other hand, the binding of a ligand to its receptor can be more complex, for instance if the ligand or the receptor consists of a dimer or a higher oligomeric structure, giving rise to multiple interactions. As a consequence, more than one most probable rupture force may exist for each LR, corresponding to the rupture of one or multiple interactions disrupting simultaneously. Ideally, the contribution of single and multiple interactions should be determined for each individual LR value. In practice, as there is an insufficient number of rupture force values recorded for each LR value, a strategy to overcome this issue consists in separating the spectrum of LRs probed into discrete ranges, *i.e.* pooling the rupture forces corresponding to various LRs within a same range (*e.g.* 1000–3500 pN·s⁻¹). The choice of the width of the LR ranges is arbitrary, and balance between two opposite considerations. The range should be sufficiently narrow so that all rupture forces within that range may be considered to correspond to the same LR value without generating too much error, but large enough in order to contain sufficient data values in order to estimate the distribution, *i.e.* probability density function (PDF), of the rupture forces. Taking into account these considerations, we divided the spectrum of LRs probed (~100–315,000 pN·s⁻¹) into six or seven ranges of equal width (**Figure 30a** and **Figure 31a**).

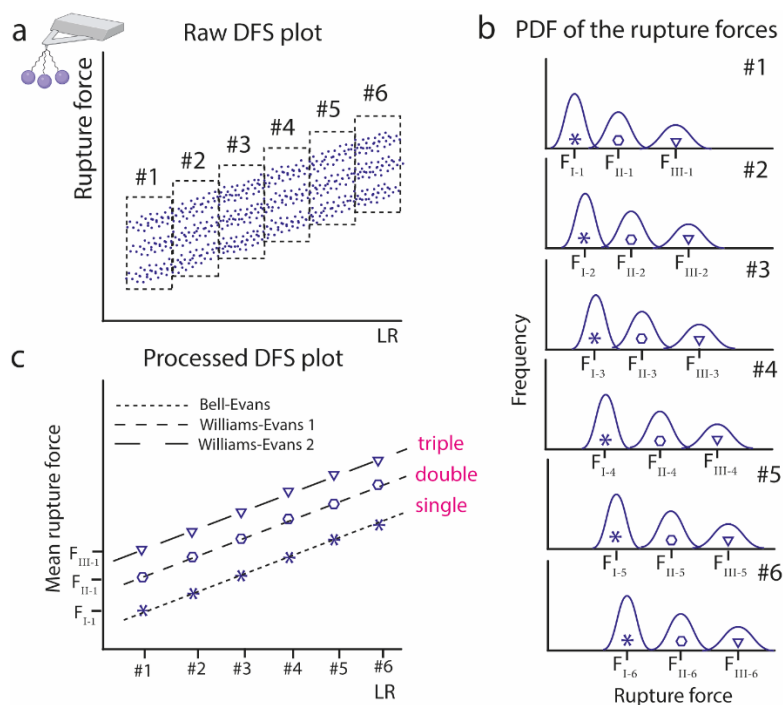


Figure 31. Theoretical illustrations explaining DFS data treatment when probing multiple interactions simultaneously. (a) Rupture forces and LR are displayed in a raw DFS plot. Each dot represents a single ligand–receptor interaction (*i.e.* rupture event). Rupture forces are pooled within discrete ranges (#1 to #6). (b) A probability density function (PDF) of the rupture forces is generated and is deconvoluted to determine the contribution of single and multiple interactions. (c) The most probable rupture forces determined for single (*) and multiple (o and ▽) interactions are then plotted on the DFS plot. The Bell-Evans model is used to fit the most probable rupture forces corresponding to the monovalent interaction, allowing extraction of kinetic parameters of the interaction. The Williams-Evans model is used to predict the rupture force-LR dependency of multivalent complexes based on the kinetic parameters obtained with the Bell-Evans model. Inspired from [414].

To estimate the PDF of the rupture forces, different methods are used. Among these, histograms showing the binding frequency of the rupture forces for discrete classes are widely used (*e.g.* [440–442]). In the present thesis, we used another approach based on the use of a kernel distribution to estimate the PDF of the rupture forces, as performed elsewhere (*e.g.* [443–446]). This method presents several advantages compared to the commonly used histograms [447]. A kernel distribution is a nonparametric representation of the PDF of a random variable and is defined by a smoothing function (*e.g.* triangular, gaussian, Epanechnikov), which defines the shape of the curve used to generate the PDF, and a bandwidth value, which controls the smoothness of the resulting density curve (**Figure 32a**). Unlike a histogram, which places the values into discrete bins, a kernel distribution creates an individual probability density curve for each data value, then sums the component smoothing

functions for each data value to produce a smooth, continuous probability curve (Figure 32b).

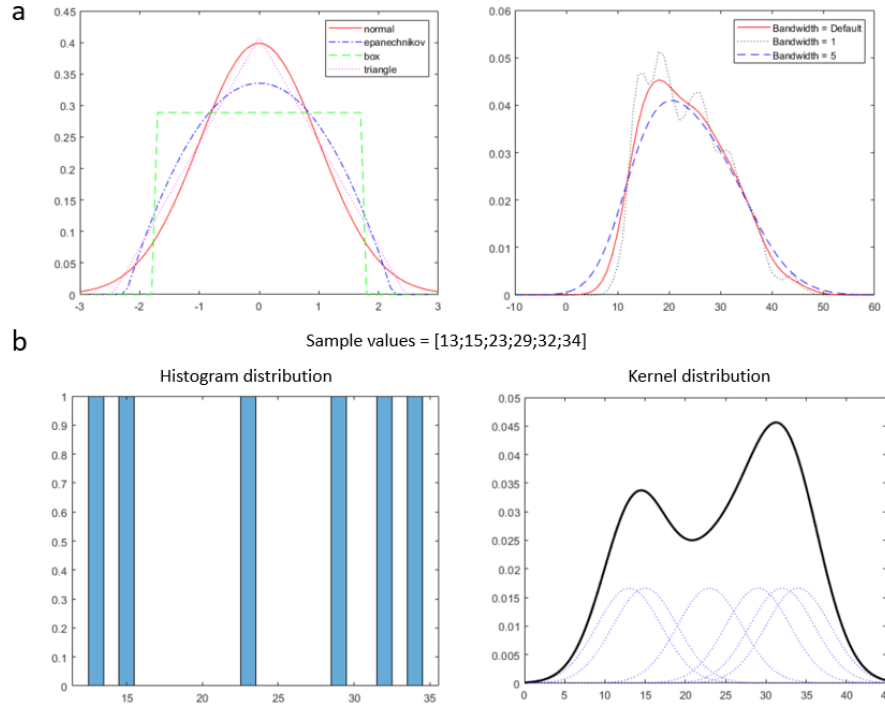


Figure 32. Principle of kernel distribution. (a) Examples of kernel smoothing functions (left) and bandwidth (right) used to generate the PDF. A too small bandwidth may reveal insignificant information, whereas a too large bandwidth may hide information. **(b)** Comparison of histogram and kernel distributions. The probability density of sample values, 13;15;23;29;32;34 in the example, can be represented in a histogram (left) or with a kernel distribution (right). For the histogram, bins are established and each data value is placed in the appropriate bin, thereby producing a discrete PDF. The kernel distribution builds the PDF by summing the individual probability density curves generated for each data value, thereby producing a smooth, continuous PDF of the data. Adapted from [448].

The probability density $p(x)$ is estimated by $\hat{p}(x)$ (Equation 7). A too small bandwidth produces a very rough curve and may cause the estimator to show insignificant details (e.g. noise), while a too large bandwidth causes oversmoothing of the information contained in the sample and may obscure potentially important features of the data (e.g. multimodality) [447]. The choice of the optimal bandwidth is based on formulas that minimize the discrepancy between the kernel density estimator $\hat{p}(x)$ and the true density $p(x)$ [449–454].

$$\hat{p}(x) = \frac{1}{nh} \sum_{i=1}^n K\left(\frac{x - x_i}{h}\right)$$

Equation 7. Kernel density estimator. The kernel density estimator for any value x is the estimated PDF of a random variable. n is the sample size, h is the bandwidth, x_i are random sample values from a unknown distribution, and K is the kernel smoothing function.

In the present thesis, we assume the kernel distribution and the true distribution as normal, and therefore used the kernel smoothing function $ksdensity(F)$ in **Equation 8** to estimate the probability density of rupture forces.

$$ksdensity(F) = \frac{1}{n} \sum_{i=1}^n \frac{1}{\sqrt{2\pi}h} \exp\left(-\frac{(F - F_i)^2}{2h^2}\right)$$

Equation 8. Kernel smooth function to estimate the probability density of the rupture forces. F_i are the rupture force values, n is the sample size, and h is the bandwidth.

When the kernel function is symmetrical, the method of choice for the determination of the bandwidth is the Silverman's rule of thumb [455] (**Equation 9**), which is based on the asymptotic mean integrated square error as the measure chosen to estimate the difference between the true density function $p(x)$ and its estimator $\hat{p}(x)$. We used this method for the determination of the bandwidth.

$$h = 0.9 \cdot \min\left(\hat{\sigma}, \frac{IQR}{1.349}\right) \cdot n^{-1/5}$$

Equation 9. Silverman's rule of thumb for the determination of the optimal bandwidth. h is the bandwidth, $\hat{\sigma}$ is the sample standard deviation, IQR is the sample interquartile range, and n is the sample size.

To determine the most probable rupture forces for single and multiple interactions (**Figure 30c** and **Figure 31b**), one of the strategies used in the case of histogram distribution involves an iterative fitting using multiple gaussian functions to deconvolute the distribution into its multiple components [440,441,456]. The number of peaks, exact position (*i.e.* peak center), width and area are adjusted by the operator based on these two main assumptions: (i) the probability to observe rupture events involving N simultaneously disrupting complexes should decrease as N increases, so that the height of each gaussian distribution should decrease with the interaction valency, (ii) the variations of measured rupture forces around the most probable rupture force increases with the number of simultaneously disrupting interactions (N), so that the standard deviation associated to each gaussian distribution (σ) should increase with N (following $\sim N \cdot \sigma$), leading to broaden curves. However, in practice, we cannot verify the biophysical characteristics of the interactions probed (*i.e.* multivalency), despite optimizing the functionalization

protocols in order to favor single interactions. In addition, interactions involving dimers or oligomers may give rise to more complex binding than a simple 1:1 binding, no matter the functionalization strategies used.

In this thesis, we therefore used a novel approach based on a computational method ('Peak Analyzer' tool on OriginPro software), allowing detecting the peaks and performing non-linear fitting to the peaks in the spectrum data. For the detection of the peaks, we used the second derivative of the kernel smoothing function and restricted the detection to positive peaks only. This method allows to search for hidden peaks. We filtered the peaks by height, choosing a threshold sufficiently high to avoid peaks arising from noise and sufficiently low to detect multimodality. With a few exceptions, this threshold height was set at 20% (which is the default value). Peaks were fitted with gaussian functions using the iterative Levenberg-Marquardt algorithm in order for the cumulative gaussian function to converge to the kernel smoothing function. In some cases, area under the first peak was modified.

Regardless of the strategy used, the most probable rupture forces associated with each gaussian functions (hence, with single and multiple interactions) are then extracted for each LR range, and plotted as a function of the logarithm of the mean LR of the range in the DFS plot. The final step then involves the use of biophysical model such as the Bell-Evans model (**Equation 5**) to fit the most probable rupture forces in order to extract kinetic parameters describing the interactions probed (**Figure 30d** and **Figure 31c**).

2.3.4. Binding of in-solution PRDXs to immobilized receptors

AFM-based SMFS is time-consuming as both the experiment itself and the subsequent data analysis require several days. In this sense, BLI has the advantage of obtaining valuable information in less time as far as optimized conditions are found. However, it has a lower sensitivity.

Bio-layer interferometry principle

BLI is a biosensor-based technique that relies on the principles of optical interferometry (*i.e.* interaction of light waves) and allows the analysis of the kinetics of biomolecular interaction (**Figure 33**). BLI involves the use a fiber-optic biosensor to immobilize one interactant, called ligand, while the other one, called analyte, remains in solution. The biosensor tip has an internal optical reference layer and is coated with a biocompatible matrix layer. White light is directed down the biosensor and is reflected from (i) the interface between the glass fiber and the optical layer, and (ii) the interface between the biocompatible layer and the solution. The reflected beams interfere in a constructive or destructive manner at different

wavelengths in the visible spectrum, and the interference patterns produced at each wavelength are detected, analyzed, and plotted together to derive an interferometric profile.

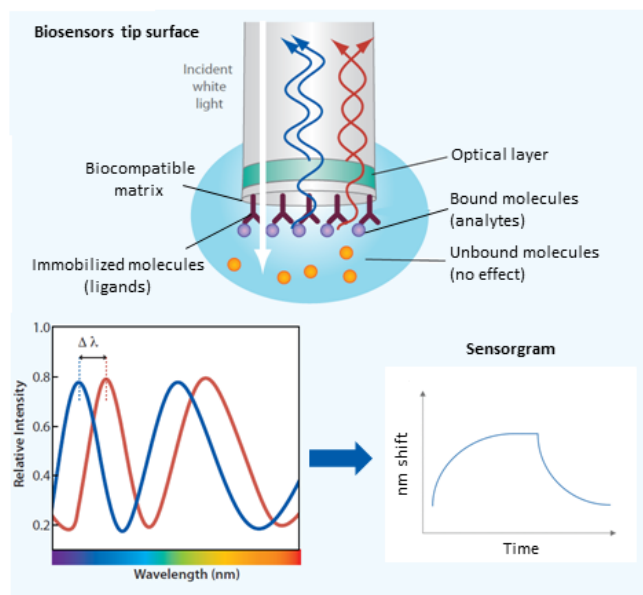


Figure 33. BLI biosensor and principle. The biosensor tip is coated with a biocompatible layer, allowing immobilization of the ligand, and under which lies an optical reference layer. The biosensor is dipped into the analyte solution. White light is directed onto the biosensor tip and reflected from two different interfaces, generating an interference pattern. When analyte molecules bind to the immobilized ligands, a shift in the interference patterns is created, which is a direct measure of the change in thickness of the biological layer. Association and dissociation of molecules from the biosensor change the biolayer thickness, allowing a real-time monitoring of biomolecular interactions, which is displayed on a sensorgram (nm shift *versus* time). Adapted from [457].

When analyte molecules bind to the biosensor tip surface, they form a layer of a certain thickness, which increases as more molecules bind. This causes the path length of the reflection from the interface between the biolayer and the solution to increase while that of the reflection from the other interface remains the same. As a consequence, the interference patterns of the reflected light change for all wavelengths. Plotting the different contribution of all the wavelengths results in a new interferometric profile that is shifted from the original profile. Therefore, the spectral pattern of the reflected light varies as a function of the optical thickness of the molecular layer, *i.e.* the number of molecules bound to the biosensor tip. When the magnitude of the spectral shift (in nm) is plotted as a function of time, it results in a sensorgram that shows a typical association/dissociation curve. A positive shift corresponds to an increase in biolayer thickness, reflecting more association,

whereas a negative shift (*i.e.* a shift towards the initial position) implies a decrease in thickness, and therefore more dissociation.

When setting up a BLI experiment, the strategy involves (i) choosing an appropriate biosensor chemistry depending on the features of the probed system (*e.g.* presence of an affinity tag on the ligand), (ii) optimizing the kinetic buffer to avoid nonspecific interactions with the biosensor surface, (iii) testing various ligand concentrations to obtain a proper level of binding on the biosensor surface, (iv) varying the analyte concentration to be able to extract kinetic parameters of the interaction, and (v) optimizing the time required for each step. Various biosensor chemistries are available, including, but not limited to, anti-human IgG Fc-coated biosensors (for immobilization of human IgG or other human Fc-containing proteins), anti-FLAG-coated biosensors (for capture of FLAG-tagged recombinant proteins), amine reactive biosensors coated with carboxy groups (for covalent coupling of proteins *via* NHS/EDC-mediated amide bond formation), Ni-NTA biosensors coated with *tris*-NTA molecules (for binding of His-tagged recombinant proteins) and streptavidin-coated biosensors (for immobilization of biotinylated molecules).

Binding kinetics

As AFM-based SMFS, BLI also relies on the simplest model used to describe a biomolecular interaction (**Equation 3**). This binding model assumes that binding is independent and of equal strength for all binding sites. The formation of a complex follows pseudo-first order kinetics. In a 1:1 biomolecular interaction, both the association and the dissociation phases display time-dependent responses that are described by single exponential functions. The association curve shows an exponential increase in signal followed by a leveling off to plateau as the binding reaches equilibrium (**Equation 10**), whereas the dissociation curve follows a single exponential decay with signal eventually returning to baseline (**Equation 11**). The equilibrium dissociation constant (or affinity constant, K_D) is given by the ratio between the dissociation rate constant and the association rate constant ($K_D = k_{off}/k_{on}$) and corresponds to the concentration of analyte at which half of ligand binding sites are occupied at equilibrium. Therefore, a smaller affinity constant indicates a greater affinity (hence, tighter interaction).

$$Y = Y_0 + R_{eq}(1 - e^{-k_{obs}t})$$

Equation 10. Partial association phase. Y is the signal (in nm), indicating the binding level as a wavelength shift, t is time (in s), Y_0 is the initial Y binding level of the fitted association curve (noise), R_{eq} is the fitted binding response value when the binding interaction reaches equilibrium for a given analyte concentration and is given by **Equation 12**, and k_{obs} is the observed rate constant, given by **Equation 13**.

$$Y = Y_e + Y_{\Delta}e^{-k_{off}t}$$

Equation 11. Partial dissociation phase. Y_e is the fitted value that the exponential decay curve will eventually approach to, Y_{Δ} is the wavelength shift difference between the first data point of the fitted dissociation curve and Y_e , and k_{off} is the dissociation rate constant.

$$R_{eq} = R_{max} \frac{[analyte]}{[analyte] + K_D}$$

Equation 12. Binding response value at equilibrium for a given analyte concentration. R_{max} is the calculated maximum achievable binding for an analyte to a given level of immobilized ligand on the biosensor surface, and K_D is the equilibrium dissociation constant.

$$k_{obs} = k_{on}[analyte] + k_{off}$$

Equation 13. Expression of the observed rate constant. It reflects the overall rate of the combined association and dissociation of the interaction partners.

BLI to characterize bulk PRDX binding to TLR4 and TLR4/MD-2 complex

We tested two different biosensor chemistries: amino-reactive (amine reactive second-generation, AR2G) and Ni-NTA biosensors (**Figure 34**). The assay using amino-reactive biosensors consists of seven steps: (i) equilibration (kinetic buffer), (ii) activation of the carboxyl groups with NHS/EDC to generate highly reactive NHS esters, (iii) immobilization (loading) of the ligand through primary amines, (iv) quenching of the unreactive esters with ethanolamine hydrochloride (pH 8.5), (v) baseline (kinetic buffer), (vi) association with the analyte diluted in kinetic buffer (binding), and (vii) dissociation (kinetic buffer). On the other hand, the assay based on Ni-NTA biosensors involves five steps: (i) equilibration with kinetic buffer, (ii) loading to capture the His-tagged ligand onto the biosensor surface, (iii) baseline with kinetic buffer, (iv) association with the analyte diluted in kinetic buffer (binding), and (v) dissociation of the analyte from the ligand in kinetic buffer. These steps may be followed by additional steps to regenerate the biosensor for additional uses, consisting of cycling the biosensor surface between 10 mM glycine (pH 1.7) and kinetic buffer a few times, and then re-charging with 10 mM NiCl₂. In both cases, recombinant TLR4 or TLR4/MD-2 complex serves as ligand (immobilized onto the biosensors), and PRDX2 or PRDX5 was used as analyte (in solution).

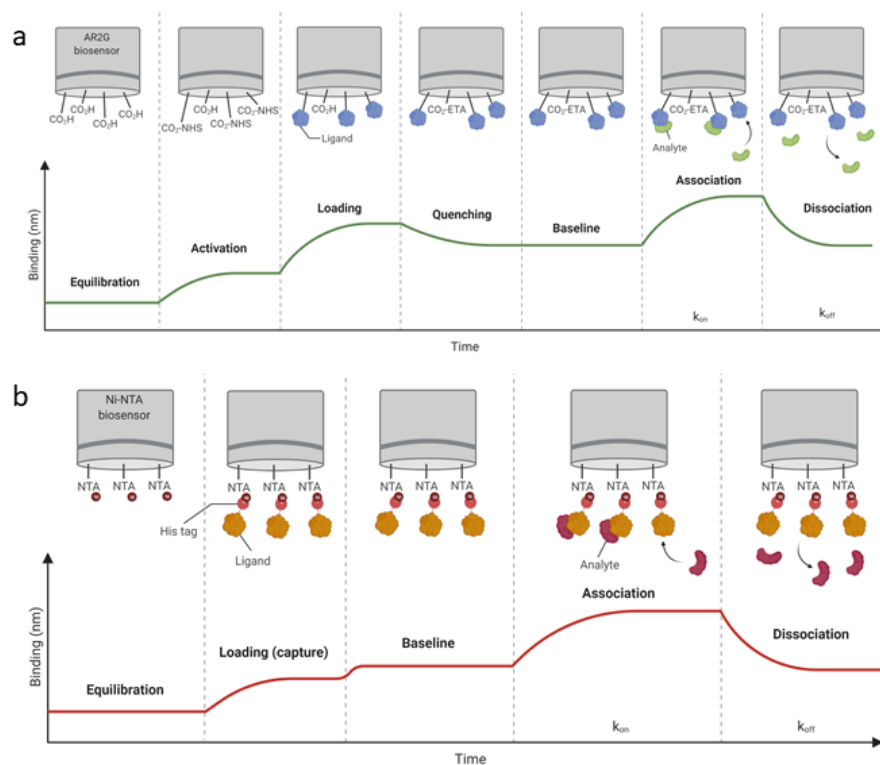


Figure 34. BLI assays based on the use of amino-reactive or Ni-NTA biosensors. (a) The surface of amine reactive second-generation (AR2G) biosensor consists of carboxylic acids that are activated by NHS/EDC to form highly reactive esters, after an equilibration step. The ester groups can react with amino groups from proteins to form amide bond, resulting in the covalent attachment of the proteins onto the biosensor surface (loading). Unreacted binding sites are quenched with ethanolamine hydrochloride to avoid nonspecific binding of the analyte onto the biosensor surface. The biosensor is then dipped into the analyte solution, allowing the binding of the analyte to the ligand onto the biosensor surface (association). Finally, the biosensor is dipped into buffer for dissociation of the complex. **(b)** The surface of Ni-NTA biosensors is covered with *tris*-NTA groups charged with Ni^{2+} , allowing the attachment of His-tagged proteins. After an equilibration step, the biosensor is dipped into the 6xHis-tagged ligand solution for capture onto the biosensor surface (loading). Association and dissociation steps are performed by dipping the biosensor into the analyte solution following by buffer. **(a,b)** Typical sensorgrams are shown for each type of biosensors. The association and dissociation steps allow determination of the k_{on} and k_{off} , respectively. Created with BioRender.com.

2.3.5. Binding of PRDXs to receptors at the surface of living cells

In addition to the characterization of the binding of recombinant PRDXs to recombinant receptors, we used FC and immunofluorescence to analyze the binding of PRDXs at the surface of living cells expressing TLR4. Furthermore, we performed pull-down assays with the aim of identifying whether MD-2 and/or CD14 are physical partners contained within the PRDX5/TLR4 complex.

Live-cell fluorescence imaging to observe the binding of PRDX to the cell surface

Live-cell imaging with CLSM shows the advantage of allowing the observation of dynamic cellular processes. In our case, we investigated the binding of human PRDX5 at the surface of living HEK293 cells expressing TLR4 fused to enhanced yellow fluorescent protein (eYFP), MD-2 and CD14. To this end, we labeled the 6xHis-tagged PRDX5 protein with NTA-fluorescein isothiocyanate (FITC) using Ni^{2+} .

Flow cytometry binding assay

We used a FC binding assay to assess the binding level of (i) the PRDX5 redox forms to the surface of living HEK293 cells expressing TLR4 along with its accessory molecules MD-2 and CD14, as well as (ii) PRDX5 to the surface of living HEK293 cells expressing CD14. As HEK293 cells are loosely adherent, they do not withstand extensive washing steps. FC allows overcoming this issue as it requires cells in suspension. Cells were exposed to PRDX5, and PRDX5 molecules bound to the cell surface were labeled with an anti-PRDX5 antibody followed by a fluorescently-labeled secondary antibody. The fluorescence intensity of the secondary antibody provides a direct measurement of the amount of PRDX5 molecules bound to the cell surface. To prevent endocytosis, cells were manipulated on ice. To control for the nonspecific binding of the antibodies, unexposed cells were similarly labeled with the antibodies.

Co-immunoprecipitation and pull-down assay to identify the binding partners

Co-immunoprecipitation (co-IP) and pull-down assays are common methods used to identify protein-protein interactions. Whereas co-IP relies on the use of specific antibodies to indirectly capture protein complexes, pull-down assays use a fusion-tagged protein instead of an antibody. In co-IP, the protein complex is incubated with an antibody-coupled support in which the antibody recognizes a specific protein within the complex. In a pull-down assay, a protein that has an affinity tag, or 'bait' protein, is immobilized on a support carrying an affinity ligand specific for the tag, thereby leading to the isolation of the partner proteins, or 'prey' proteins, involved in the complex.

We tested two strategies: (i) pull-down assays with 6xHis-tagged PRDX5 as bait protein and Ni^{2+} -NTA agarose resin beads as support, and (ii) co-IP with GFP nanobody/V_HH coupled to agarose beads to bind to eYFP-fused TLR4. HEK293 cells expressing TLR4-eYFP, MD-2 and CD14 were exposed to PRDX5, and cell lysates were then incubated with Ni^{2+} -NTA agarose beads to isolate the complex through the PRDX5 6xHis tag, or with GFP nanobody/V_HH coupled to agarose beads to isolate the complex through the eYFP fusion of TLR4. Finally, Western blot was performed to

identify the binding partners, using an anti-GFP antibody to detect TLR4-eYFP, as well as anti-PRDX5, anti-MD-2 and anti-CD14 antibodies.

2.3.6. Dynamics of NF- κ B activation

With the aim of elucidating the downstream events following PRDX binding, we attempted to characterize the dynamics of NF- κ B activation following cell stimulation by PRDXs using CLSM. Initially, the idea consisted in probing the cell surface with a PRDX-functionalized tip or with a PRDX-functionalized bead glued to the extremity of an AFM cantilever, to determine whether a single PRDX or multiple PRDX proteins, respectively, are required to activate intracellular signaling following binding. Furthermore, cell stimulation can be achieved either by performing multiple cycles of approach and retraction of the AFM probe in the Z direction, hence probing limited number of cell surface receptors on a localized area onto the cell, or by raster scanning in the X-Y direction to activate several receptors sequentially.

2.3.7. PRDX-induced secretion of pro-inflammatory cytokines

ELISA is one of the most commonly used methods in immunology. In this project, the use of this technique served two purposes: (i) validating the DAMP function of human PRDX2 and PRDX5, *i.e.* their ability to induce a pro-inflammatory response, and (ii) correlating this function to receptor activation.

ELISA principle

ELISA is a microplate-based assay technique designed for detecting and quantifying a specific protein in a complex mixture. The basic principle consists in immobilizing the antigen (target molecule) on a solid surface (microplate well), before complexing it with an antibody that is linked to a reporter enzyme or a fluorophore for detection. The signal observed is proportional to the amount of antigen bound in the sample.

ELISA to quantify IL-8 secretion levels from THP-1 and HEK293 cell lines

We performed ELISA to quantify IL-8 secretion levels from PMA-differentiated THP-1 cells and from various engineered HEK293 cell lines expressing TLR4, MD-2 or CD14 alone or in combinations, after exposure to human PRDX2 or PRDX5. As the PRDX proteins are dialyzed against PBS after production, we used PBS as negative control. As positive control, both the TLR4 PAMP ligand LPS and the TLR4 DAMP ligand HMGB1 were used. We also used neutralizing antibodies to confirm the involvement of TLR4, MD-2 and CD14 in the PRDX-induced cytokine secretion. We performed the capture (or 'sandwich') assay, in which the antigen is immobilized on the wells of the microplate *via* a specific antibody (**Figure 35**). Detection was

performed using a biotinylated anti-human IL-8 antibody by addition of a streptavidin-horseradish peroxidase conjugate and its colorimetric substrate TMB.

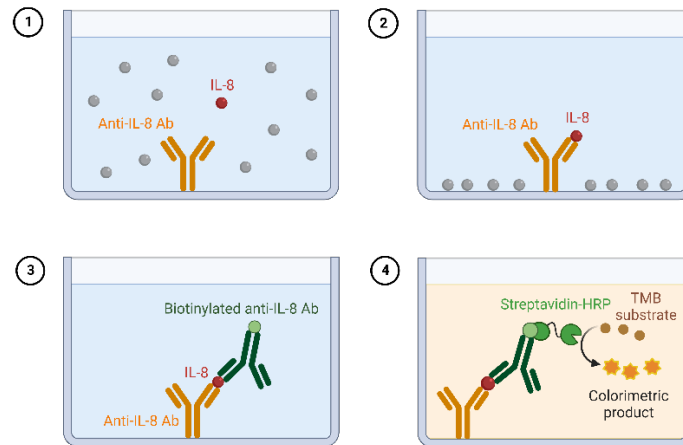


Figure 35. ELISA to measure IL-8 secretion levels. Wells of a 96-well plate are coated with a monoclonal antibody specific for human IL-8 (capture antibody) and supernatants are added to the wells (1). Any IL-8 present binds the immobilized capture antibody (2). The wells are washed and biotinylated anti-human IL-8 monoclonal antibody (detection antibody) is added and binds the bound IL-8, producing an antibody-antigen-antibody “sandwich” (3). Streptavidin-HRP conjugate binds to biotin and catalyzes an enzymatic color reaction upon addition of TMB substrate (4). The color is in direct proportion to the amount of IL-8 present in the sample. Created with BioRender.com.

To obtain statistically robust results with ELISA, several sources of variation need to be considered. In this project, one experiment consisted in: (i) seeding cells in wells of 96-well plates, (ii) exposing the cells to the various treatments (PBS, LPS, PRDXs, etc), (iii) collecting the cell supernatants, and (iv) performing the ELISA itself. To account for the biological variation within one experiment, each treatment should be applied to at least two different wells of cells (biological replicates). In addition, to account for the technical variation of the ELISA itself (well-to-well intra-assay variation), the cell supernatant collected from one well should be ran at least two times, *i.e.* divided and quantified in at least two different wells of the ELISA plate (technical replicates). In this project, within one experiment, each treatment was applied to two (or three) different wells of cells, leading to duplicates (or triplicates) for each treatment (biological replicates). The cell supernatant from one well of cells was next ran one time in the ELISA itself (one well in the ELISA plate). In this way, the technical variation was not assessed independently. However, as one given treatment was run into two different ELISA wells, both technical and biological variation were assessed simultaneously. Doing so, the data obtained from one experiment contained all the possible intra-assay experimental variation.

Furthermore, to include the inter-assay variation, we performed at least two independent experiments for each condition. In addition, for the ELISA involving the PRDX5 redox forms, protein redox modifications (*i.e.* reduction and alkylation, or hyperoxidation) were performed independently before each experiment. Finally, when comparing IL-8 secretion from various engineered HEK293 cell lines, HEK293 MD2-CD14-TLR4 cells were systematically used as control in the experiments.

Chapter III – Results and Discussion

This chapter contains the detailed materials and methods, as well as the results and discussions related to the main studies performed in this work. Three studies are presented in the form of scientific publications. In addition, other experiments that complete the previous studies or lead to preliminary results are presented.

Preliminary results: *Specific interactions measured by AFM on living cells between peroxiredoxin-5 and TLR4: Relevance for mechanisms of innate immunity*

This study evaluates the binding of human PRDX5 to TLR4 and was performed before I started my PhD thesis. Therefore, it is considered as preliminary results for my project. For this study, I performed AFM experiments for the reviewing process. The results from this study constitute an important basis for my PhD work. However, as my contribution to this study was minor, the results will not be further discussed in the next Chapter (**Chapter IV – General discussion**).

Study 1: *Role of the redox state of human peroxiredoxin-5 on its TLR4-activating DAMP function*

This study concerns the role of the redox state of human PRDX5 Cys residues on the ability of the protein to trigger cytokine secretion and to bind to TLR4.

Study 2: *Role of Toll-like receptor accessory molecules MD-2 and CD14 in peroxiredoxin-mediated pro-inflammatory response*

This study focuses on human PRDX2 and PRDX5 to evaluate the role of the TLR4 accessory molecules MD-2 and CD14 in the pro-inflammatory activity of both PRDXs.

Additional Results

This section summarizes the main additional experiments that were performed. The results were either preliminary, requiring further experimental optimization, or complete the previous studies.

3.1. Preliminary results – Specific interactions measured by AFM on living cells between peroxiredoxin-5 and TLR4: Relevance for mechanisms of innate immunity

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*Adapted from Knoop et al. “Specific interactions measured by AFM on living cells between peroxiredoxin-5 and TLR4: Relevance for mechanisms of innate immunity” **Cell Chemical Biology** 25(5), 550-559, May 17, 2018*

The original (published) paper is presented in **Annex 1**.

Personal contribution: I performed the AFM experiments of Figure S3B and I realized the graphical abstract.

3.1.1 Abstract

Inflammation is a pathophysiological response of innate immunity to infection or tissue damage. This response is among others triggered by factors released by damaged or dying cells, termed damage-associated molecular pattern (DAMP) molecules that act as danger signals. DAMPs interact with pattern recognition receptors (PRRs) to contribute to the induction of inflammation. However, how released peroxiredoxins (PRDXs) are able to activate PRRs, such as Toll-like receptors (TLRs), remains elusive. Here, we used force-distance curve-based atomic force microscopy to investigate the molecular mechanisms by which extracellular human PRDX5 can activate a pro-inflammatory response (**Figure 36**). Single-molecule experiments demonstrated that PRDX5 binds to purified TLR4 receptors, on macrophage-differentiated THP-1 cells, and on human TLR4-transfected CHO cells. These findings suggest that extracellular PRDX5 can specifically trigger a pro-inflammatory response. Moreover, our work also revealed that PRDX5 binding induces a cellular mechano-response. Collectively, this study provides insights into the role of extracellular PRDX5 in innate immunity.

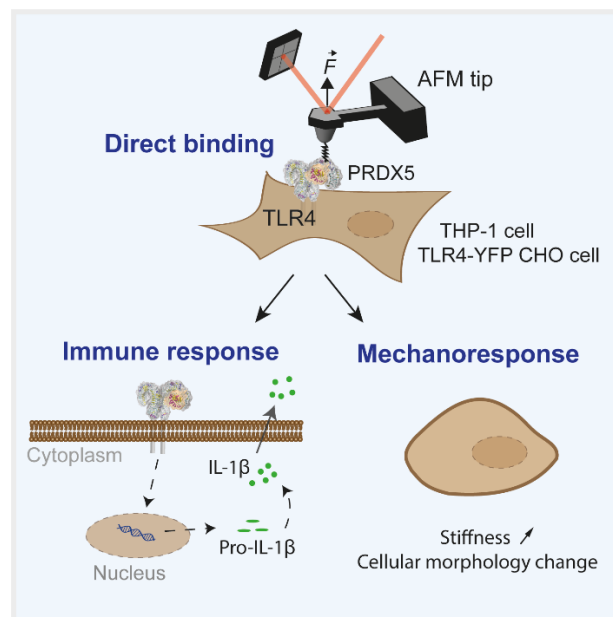


Figure 36. Graphical abstract. Knoops *et al.* used force-distance curve-based atomic force microscopy to investigate the molecular mechanisms by which extracellular human PRDX5 can activate a pro-inflammatory response. Single-molecule experiments demonstrate specific binding of PRDX5 to TLR4 on purified receptors and living cells.

3.1.2 Introduction

Inflammation is an essential component of the innate immune response to infection or tissue damage, which has the physiological role of restoring tissue homeostasis. Inflammation is initiated and perpetuated by pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules. While PAMPs are derived products of pathogens, DAMPs are host-derived nuclear or cytosolic proteins with defined intracellular functions. Following tissue injury, DAMPs are released extracellularly and move from a reducing to an oxidizing milieu. PAMPs and DAMPs are recognized by membrane-bound pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), the NOD-like receptors, and RIG-I-like receptors. So, PRRs are activated and promote intracellular cascades that lead to an inflammatory response [180,458,459].

The superfamily of peroxidases named peroxiredoxins (PRDXs), has been reported to play important roles in innate immunity (for reviews, see [41,460,461]). PRDXs are a family of thiol-dependent peroxidases that are able to reduce peroxides (hydrogen peroxide, alkyl hydroperoxides, and peroxynitrite) and are constitutively expressed by virtually all cell types in mammals [13]. It has been reported that, intracellularly, PRDXs may act as cytoprotective antioxidant enzymes, protein chaperones, modulators of peroxide signaling, and redox relays [65]. Interestingly, PRDXs were also shown to play a role in immune modulation as PAMPs or DAMPs upon their release into the extracellular milieu [40,116,344]. Studies conducted either after brain injury in rats [462] or after acute ischemic stroke in humans [463], demonstrated that PRDXs are released into extracellular fluids and could function as DAMPs under conditions of sterile inflammation. Moreover, Shichita *et al.* have shown that post-ischemic brain inflammation can be mediated through PRDXs and is dependent on TLR2 and TLR4 [116]. In particular, it was shown that, among PRDXs, PRDX5 highly contributed to induce the expression and the release of inflammatory cytokines [116]. However, the molecular mechanisms underlying TLR2 and TLR4 activation through PRDXs still remained to be clarified (**Figure 37A**).

Atomic force microscopy (AFM), invented to image the topography of samples at high resolution and in native conditions [464], evolved rapidly allowing the investigation of living mammalian cells and the probing of specific interactions under physiological conditions [465,466]. Force-distance curve-based AFM (FD-based AFM) enables us to contour the surface of biological systems with simultaneous mapping of their physical properties at (sub-)nanometer resolution [467]. A tip positioned at the very end of a soft cantilever is oscillated and scanned across a cell while performing a single approach and retract movement at each discrete point of the surface. During the approach and retraction cycle, the tip interacts with the cell

surface and is deflected depending on the force exerted on it, which in turn deviates the path of a laser, reflected from the cantilever onto a position-sensitive detector. This deflection correlates to the interaction force between cantilever tip and cell surface, and can be displayed as force-time and FD curves. Analysis of the FD curves allows the extraction of parameters including height, adhesion, contact force, sample deformation, energy dissipation, and Young's modulus [467]. As the parameters are determined for each point of the cell surface, parametric maps can be generated and directly correlated to the AFM image. Recently, we have introduced FD-based AFM to image human G-protein-coupled receptors in proteoliposomes at high resolution (<5 nm) [409,467,468], and we quantified the kinetic and thermodynamic parameters that describe the binding of ligands [410]. In a second study, we introduced a chamber to keep mammalian cells in incubator-like conditions and maintain homeostasis for days [440]. This set-up allowed us to quantify and topographically map the binding of single EnvA-RABV(DG) viruses at high resolution (<50 nm) on MDCK cells. Functionalized AFM tips with single viruses enabled us to detect the binding events of their receptors, and we observed a stepwise formation of virus-cell surface receptor bonds occurring with a positive allosteric modulation. Here, we examined how PRDX5 released from cells into the extracellular milieu can function as a DAMP, and can activate a cell response through direct specific binding to TLR4 receptor on living cells. To investigate the molecular mechanisms by which PRDX5 interacts with TLR4, we used AFM as a unique and novel approach in immunology to probe and map single-molecule interactions on purified receptors and on individual living cells. This single-cell approach allowed us to provide insights into fundamental processes related to the molecular cell biology of innate immunity.

Our results show that human PRDX5 is able to specifically bind to TLR4. First, we probed specific interactions at the single-molecule level between the tip derivatized with PRDX5 and TLR4 receptors immobilized on a surface. Next, we combined FD-based AFM with optical microscopy to image the binding sites of PRDX5 on macrophage-differentiated THP-1 cells, and confirmed the specific interaction with TLR4 receptors using transfected Chinese hamster ovary (CHO) cells expressing fluorescently (YFP) labeled human TLR4. Moreover, our experimental data were supported by TLR4-PRDX5 docking simulations.

3.1.3 Materials and Methods

Cell lines

Chinese Hamster Ovary (CHO-K1) cells (ATCC) were cultured in Dulbecco's modified eagle medium (DMEM) (Life Technologies) with 10% FBS (Lonza), with 100 mg·mL⁻¹ penicillin and 100 mg·mL⁻¹ streptomycin (Life Technologies) at 37°C in

humidified atmosphere supplemented with 5% CO₂. THP-1 cells (ATCC), an acute monocytic leukemia cell line from a 1-year old male infant, were cultured in RPMI-1640-GlutaMAX (Life Technologies) supplemented with 10% FBS (Lonza), and 100 mg·mL⁻¹ penicillin and 100 mg·mL⁻¹ streptomycin (Life Technologies) at 37°C in humidified atmosphere supplemented with 5% CO₂.

TLR4-YFP expressing CHO cells (CHO-TLR4-YFP)

CHO cells (provided by Prof. Y-J Schneider, UCLouvain, Belgium) were plated at a density of 0.5 x 10⁶ cells in 40-mm culture dishes with 2 mL of DMEM – high glucose (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (DMEM/High-10), 100 mg·mL⁻¹ penicillin and 100 mg·mL⁻¹ streptomycin (Life Technologies) at 37°C in humidified atmosphere supplemented with 5% CO₂. Cells were transiently transfected with 3.25 mg of pcDNA3-TLR4-YFP (Addgene, plasmid #13018, gift of Dr. D. Golenbock, University of Massachusetts Medical School, USA) using 6.5 mL Lipofectamine 2000 (ThermoFisher) in 325 mL of OptiMEM (Gibco). Culture medium was changed after 24 h and Petri dishes were used for AFM experiments after 48-72 h.

Production and purification of recombinant human PRDX5

The expression and purification of recombinant N-terminal hexahistidine (6xHis)-tagged human PRDX5 were described previously [50]. First strand cDNA was obtained with Moloney leukemia virus reverse transcriptase (Superscript II, Life Technologies) from 2 mg of human lung RNA using oligo(dT)25 as primer according to the manufacturer's instructions. Human PRDX5 cDNA was PCR amplified using forward primer 5'-GCTGCAGGATCCGCCCAATCAAGGTGGGAG-3' (BamHI site underlined) and reverse primer 5'-GGCCCAAAGCTTCAGAGCTGTGAGATGATA-3' (HindIII site underlined). The PCR product was digested with BamHI and HindIII, and ligated into the pQE-30 expression vector (Qiagen). The insert was sequenced and the N-terminal fusion with the 6xHis tag was confirmed. The resulting vector was used to transform *E. coli* strain M15 (pRep4). *E. coli* were grown at 37°C in LB medium containing 1 mM IPTG. Pelleted cells were lysed in 10 mM imidazole, 50 mM phosphate, 300 mM NaCl (pH 8) by sonication and clarified by centrifugation. The supernatant containing 6xHis-tagged PRDX5 was loaded onto a Ni²⁺-NTA column (Qiagen). The column was washed and the protein was eluted with 50 mM phosphate, 300 mM NaCl, 250 mM imidazole (pH 8). Eluted protein was then dialysed against PBS (pH 7.2). Putative contaminating endotoxins were removed using EndoTrap HD kit (Hyglos) and the recombinant protein was collected in PBS at 0.9 mg·mL⁻¹.

Differentiation of THP-1 cells into macrophages

The human monocytic cell line THP-1 (provided by Prof. C. Remacle, UCLouvain, Belgium) was cultured in RPMI-1640, GlutaMAX (Life Technologies) supplemented with 10% FBS (Lonza), 100 U·mL⁻¹ streptomycin and 100 U·mL⁻¹ penicillin (Life Technologies). THP-1 cells were maintained at 37°C in 5% CO₂. THP-1 cells were differentiated into macrophages *via* exposure to 8 nM PMA (Sigma-Aldrich) in 96-well plates or 40 mm culture dishes in, respectively, 100 µl or 3 mL of RPMI-1640-GlutaMAX (Life Technologies) supplemented with 10% FBS (Lonza) and 100 U·mL⁻¹ streptomycin and 100 U·mL⁻¹ penicillin (Life Technologies) for 48 h according to Park *et al.* [469].

Quantification of human IL-1β

Macrophage-differentiated THP-1 cells cultured in 96-well plates were exposed to 2 mM of purified recombinant human PRDX5 [50], 1/10 antiserum directed to human PRDX5 [436], 30 mg·mL⁻¹ polymyxin B and 1 µg·mL⁻¹ LPS (from *E. coli* O111:B4, Sigma-Aldrich) in RPMI-1640-GlutaMAX (Life Technologies) supplemented with 100 U·mL⁻¹ streptomycin and 100 U·mL⁻¹ penicillin (Life Technologies) without FBS. Culture medium was harvested 48 h after cell exposure and IL-1β was measured, using Quantikine ELISA human IL-1β (R&D Systems).

Functionalization of AFM tips

To functionalize AFM tips we used NHS-PEG₂₇-acetal linkers [470]. The cantilevers were immersed in 1 mL of citric acid (1%, w/v) for 10 min. After washing the cantilevers three times for 5 min in MilliQ water, they were dried with N₂. The cantilevers were immersed in a 100 µL droplet of the amino-*tris*-NTA molecule (aS,a'S,a''S)-11-(6-amino-1-oxohexyl)-a,a',a''-tris[bis(carboxymethyl)amino]-d,d',d''-trioxo-1,4,8,11 tetraazacyclotetra-decane-1,4,8-tripentanoic acid, Toronto Research Chemicals, Toronto, Ontario, Canada) (2 mM in PBS). 2 µL of a 1 M cyanoborohydride solution were added and mixed carefully. The cantilevers were incubated for 2 h. The reaction was quenched by adding 5 µL of 1 M ethanolamine (pH 8.0). After 10 min, the cantilevers were washed in PBS three times for 5 min and immersed in 5 mM NiCl₂ for 10 min. Then, the *tris*-NTA-Ni²⁺ AFM tips were incubated for 2 h in a solution of 6xHis-PRDX5 or 6xHis-HMGB1 at 0.2 mg·mL⁻¹. Finally, the cantilevers were washed in PBS three times for 5 min and transferred to a multiwell plate filled with PBS buffer. Cantilevers were used in AFM experiments the same day as the last step of the functionalization protocol.

Single-molecule force spectroscopy on model surfaces

SMFS measurements were performed using the PRDX5-functionalized tips (see above mentioned protocol) using MSCT tips ($k = 0.01 \text{ N}\cdot\text{m}^{-1}$) (Bruker Corporation, Santa Barbara, CA) and substrates functionalized with TLR4 at room temperature (20°C) in PBS buffer using a Nanoscope VIII Multimode AFM (Bruker Corporation, Santa Barbara, CA). Recombinant TLR4 was immobilized onto gold substrates using *N*-hydroxysuccinimide (NHS) (Sigma-Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as described previously [416]. The activated surfaces were then incubated with $100 \text{ mg}\cdot\text{mL}^{-1}$ TLR4 (R&D Systems) in PBS. Multiple force curves were recorded at 0 ms contact time, with a maximum applied force of 250 pN, and using a constant approach speed of $1,000 \text{ nm}\cdot\text{s}^{-1}$ and retraction speeds of $100 \text{ nm}\cdot\text{s}^{-1}$, $200 \text{ nm}\cdot\text{s}^{-1}$, $500 \text{ nm}\cdot\text{s}^{-1}$, $1,000 \text{ nm}\cdot\text{s}^{-1}$, $2,000 \text{ nm}\cdot\text{s}^{-1}$ and $10,000 \text{ nm}\cdot\text{s}^{-1}$. For each experiment, these speeds were recorded in different order. Cantilevers were first calibrated on glass using thermal noise method [434], yielding values ranging from $0.011\text{--}0.023 \text{ N}\cdot\text{m}^{-1}$.

Combined FD-based AFM and optical microscopy

Correlative microscopy was acquired on an AFM (Bioscope Catalyst, Bruker Corporation, Santa Barbara, CA) operated in the PeakForce QNM mode (Nanoscope software v9.1) to conduct FD-based AFM and coupled on an inverted epifluorescence microscope (Axio observer Z1, Zeiss). The AFM was equipped with a 150-mm piezoelectric scanner. Overview images ($40 \times 40 \text{ mm}^2$) were recorded at imaging forces of 350–750 pN using PeakForce QNM-Live Cell (PFQNM-LC) cantilevers (Bruker Corporation, Santa Barbara, CA) having tip lengths of 17 mm, tip radii of 65 nm and opening angles of 15° . All microscopy and FD-based AFM imaging was performed at cell culture conditions using the combined AFM and optical microscopy chamber at 37°C in medium supplemented with 10% FBS. A gas mixture of synthetic air supplemented with 5% CO_2 at 95% relative humidity using a gas humidifier membrane (PermSelect silicone) was blown at $0.1 \text{ L}\cdot\text{min}^{-1}$ into the microscopy chamber. The humidity was controlled using a humidity sensor (Sensirion). The cantilevers were first calibrated on glass using thermal noise method [434], yielding values ranging from $0.061\text{--}0.102 \text{ N}\cdot\text{m}^{-1}$. The AFM tip was oscillated vertically at 0.25 kHz applying 300–1,000 nm amplitudes in the PeakForce QNM mode. The sample was scanned using a frequency of 0.125 Hz and 256 pixels per line (256 lines). AFM images and force curves were analyzed using the Nanoscope analysis software (v1.8, Bruker).

Analysis of force curves

Individual force curves detecting unbinding events of the protein were analyzed using the Nanoscope analysis software. The baseline of the retraction curve was corrected using a linear fit on the last 30% of the retraction curve. Using the force-time curve, the loading rate (slope) of each rupture event was approached by dividing the force segment ΔF through the time segment Δt (**Figure 38F**).

Extraction of kinetic parameters

Origin software (OriginLab) was used to fit the loading rate-dependent interaction forces using a linear iterative fitting algorithm (Levenberg-Marquardt) along with the Bell-Evans model [471]. Each iteration computed a chi-square value (χ^2) after which the parameter values were adjusted to reduce χ^2 . When the difference between two consecutive χ^2 values computed in two successive iterations was small enough (tolerance 10^{-15}), the fitting procedure converged. We fixed the maximum number of iterations to 200. To evaluate the validity of the fit, we also checked if the adjusted R^2 (coefficient of determination) was >0.75 and looked at the residual plot and the Q-Q plot.

Molecular docking

The oxidized form of PRDX5 (PDB ID: 2VL3) was docked into TLR4 receptor (PDB ID: 3FXI) using ClusPro webserver (<https://cluspro.bu.edu/login.php>) [472] using custom settings. For each docking experiment, the ClusPro generated 20 most-probable docking poses. The most representative poses of the docking experiments are represented in **Figure 41** and **Figure S5**.

Quantification and statistical analysis

All experiments were performed in at least triplicate ($n = 3$, experimental replication). For each experiment the number of replicates is provided in the figure caption. All data are expressed as the mean $\pm$ standard deviation (SD) and the differences between groups were compared using paired sample Student's t -test. Significance is indicated within figures as *** signifying $p < 0.001$.

3.1.4 Results

PRDX5 induces release of IL-1 β by THP-1 cells

Firstly, THP-1 cells were used to assess the role of PRDX5 as a DAMP, and to evaluate THP-1 pro-inflammatory cytokine release after exposure to extracellular PRDX5. THP-1 cells in the monocyte state were first differentiated into macrophage-like cells using phorbol-12-myristate-13-acetate (PMA) at 8 nM for 48 h. Macrophage-differentiated THP-1 cells were kept for 3 h in culture media without

PMA to increase macrophage phenotype [469]. Exposure of macrophage-differentiated THP-1 cells to recombinant human PRDX5 resulted in the secretion of interleukin-1 β (IL-1 β) significantly greater than that observed in control unexposed cells but lower than after LPS exposure (**Figure S1A**). Preincubation of PRDX5 with blocking antibodies directed to PRDX5 significantly decreased IL-1 β level release (**Figure 37A** and **Figure S1A**). Surprisingly, IL-1 β levels even increased after preincubation with PRDX5 and polymyxin B used to neutralize putative residual contamination by LPS (**Figure S1A**). It must be noted that similar potentiation of a PRDX by polymyxin B has been already observed after exposure of macrophages to PRDX1 in presence of polymyxin B [344].

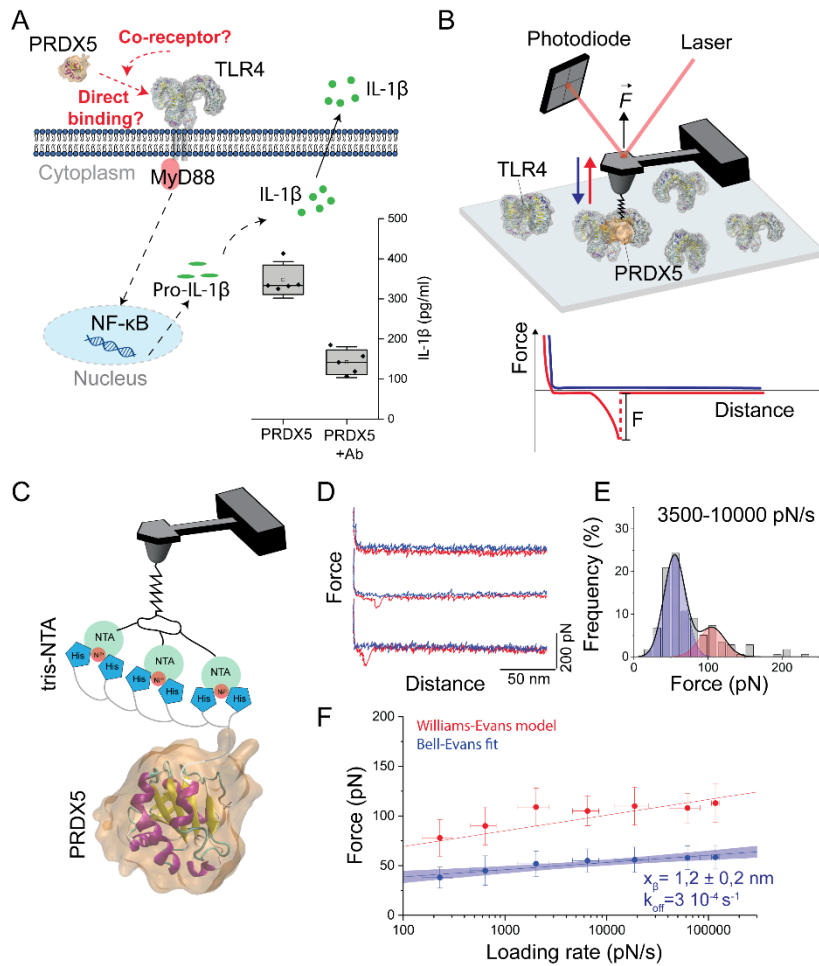


Figure 37. PRDX5 induces release of IL-1 β and interacts specifically with immobilized TLR4. (**A**) IL-1 β secretion from macrophage-differentiated THP-1 cells is induced after stimulation with PRDX5. THP-1 cells were incubated with 2 mM PRDX5 and further blocked using 1/10

blocking antiserum against PRDX5 (Ab) (see also **Figure S1**). Experiments were reproduced five times ($n = 5$; each data point is an independent experiment). Error bars denote SD. **(B)** Schematic of the force spectroscopy experiments performed between PRDX5 grafted on the AFM tip and a TLR4-derivatized surface. The AFM tip is approached (blue arrow) and retracted (red arrow) from the model surface and the force is monitored using a laser focused on the AFM cantilever and deflected into a photodiode resulting in a force *versus* distance curve (FD curve) from which the binding force (F) is extracted. **(C)** Schematic of the surface chemistry used to functionalize AFM tips with PRDX5 using *tris*-NTA-Ni²⁺. **(D)** Representative FD curves recorded showing non-adhesive curve (upper curve) or curves with specific adhesive event. Forces were extracted along with the loading rate from each individual adhesive curve (**Figure S2**). **(E and F)** Small loading rate ranges nos. 1–7 (see also **Figure S2**) are binned and the distributions of the rupture forces are plotted as histograms **(E)**. This classification reveals multiple force peaks with average values corresponding to single (blue) or double (red) simultaneously established ligand-receptor interactions. The average values of the force distributions are extracted and plotted on the dynamic force spectroscopy plot **(F)**, enabling their analysis using the Bell-Evans fit (single interactions) or the Williams-Evans model (double interactions) **(F)**. Error bars denote SD. To approximate the distance to the transition state (x_u) and the unbinding rate (k_{off}) of the bonds, the data describing the rupture of single and multiple simultaneously occurring bonds are fitted (solid lines) by the Bell-Evans model within 95% confidence intervals (colored shaded regions). Experiments were reproduced four times independently and data were merged to extract the dynamic force spectroscopy plot.

PRDX5 interacts specifically with TLR4 in vitro in an acellular model

To determine whether PRDX5 could function as a DAMP by direct interaction with the cell surface receptor TLR4, we used AFM to probe the interaction between PRDX5 and TLR4 (**Figure 37B**). To this end, an AFM tip was functionalized with *tris*-nitrilotriacetic acids (*tris*-NTA), which shows subnanomolar affinity toward the hexahistidine (6xHis)-tagged molecule [473]. The *tris*-NTA-Ni²⁺ probe was then incubated with 6xHis-PRDX5 (**Figure 37C**). TLR4 was covalently attached to COOH-derivatized gold substrate using *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride chemistry. To evaluate PRDX5 binding with TLR4, an array of 32 by 32 FD curves was recorded over a 1 mm² area on the TLR4-derivatized substrate. Some FD curves detected specific adhesion events (**Figure 37D**) at rupture distances >5 nm, and with extension patterns that can be fitted with the worm-like chain model, a biophysical model that fits the extension of proteins under an applied force.

To further determine whether these binding events were specific, we performed independent controls using (i) a tip functionalized with the *tris*-NTA-Ni²⁺ but without PRDX5 (**Figure S1B**), (ii) the functionalized tip after blocking with the anti-PRDX5 antibody (**Figure S1C**), and (iii) preincubation of PRDX5 with the endotoxin inactivator polymyxin B (**Figure S1C**). These controls showed that (i) *tris*-NTA-Ni²⁺ did not interact specifically with TLR4, (ii) the antibody against PRDX5 significantly reduced the interaction frequency, and (iii) interactions did not involve endotoxin.

This confirmed that the PRDX5 ligand linked to the tip could undergo specific interactions with TLR4.

For each adhesive curve, the force (height of the adhesive peak), as well as the loading rate (*i.e.* the increase of the force over time applied on the bond), were extracted. For each unbinding event, the loading rate was determined using the slope of the adhesion event measured on the force-time curve just before the rupture. The experiments were performed at six different piezo retraction speeds (**Figure S2A**), and all the results were sorted into seven loading rate ranges and plotted as frequency *versus* force histograms (**Figure 37E** and **Figure S2B**). The histograms revealed bi-Gaussian distributions, with the second peak centered at the double of the first one, supporting the hypothesis that the first peak is a single interaction while the second peak corresponds to double interactions. For each peak the mean and the SD were extracted and displayed in a dynamic force spectroscopy plot. The dynamic force spectroscopy plot showed that single PRDX5-TLR4 bond ruptured at forces ranging from 30 to 60 pN for loading rates between 200 and 200,000 pN·s⁻¹ (**Figure 37F**). The unbinding forces of single and multiple receptor bonds were directly proportional to the logarithmic loading rates. According to the Bell-Evans model [423,429], such a linear increase of the unbinding force is expected when crossing a single free-energy barrier during mechanical pulling of a single ligand-receptor bond to the unbound state (**Figure 37F**). Using the Bell-Evans model, we extracted the separation of bond to the transition state toward the unbound state $x_u = 1.2 \pm 0.2$ nm and the kinetic off-rate $k_{off} = 3 \times 10^{-4}$ s⁻¹ for the single PRDX5-TLR4 bond. Remarkably, the analysis of the multiple bond rupture using the Williams-Evans model [433] for multiple uncorrelated bonds loaded in parallel nicely described our experimental observations (**Figure 37F**). This behavior has already been observed for multiple antibody-antigen interactions [474] or virus-receptor bonds [440,475,476].

Imaging and detecting PRDX5 binding events on macrophage-differentiated THP-1 cells

Using AFM tips functionalized with PRDX5, we imaged macrophage-differentiated THP-1 cells expressing TLR4 under cell culture conditions (**Figure 38A**). Guided by wide-field microscopy (**Figure 38B**), we chose an area where multiple cells were in close proximity, which facilitated FD-based AFM topographical imaging since height differences are limited. In such an area, we recorded a height image (**Figure 38B**, inset), reconstructed from FD curves recorded for each topographic pixel (**Figure 38C**). Simultaneously, FD curves could also be analyzed and the specific adhesion events from the retraction curves were used to reconstruct the adhesion map (**Figure 38D** and **Figure S3A**). Invisible to the naked eye, adhesive pixels were enlarged by a factor of 2, by contouring each gray pixel with two brown pixels in each

direction. The specific adhesion events, with adhesion forces ranging from 80 to 500 pN, occurred at distances >50 nm separating the tip from the cell surface. The distribution of the binding events on the cell surface was homogenously distributed.

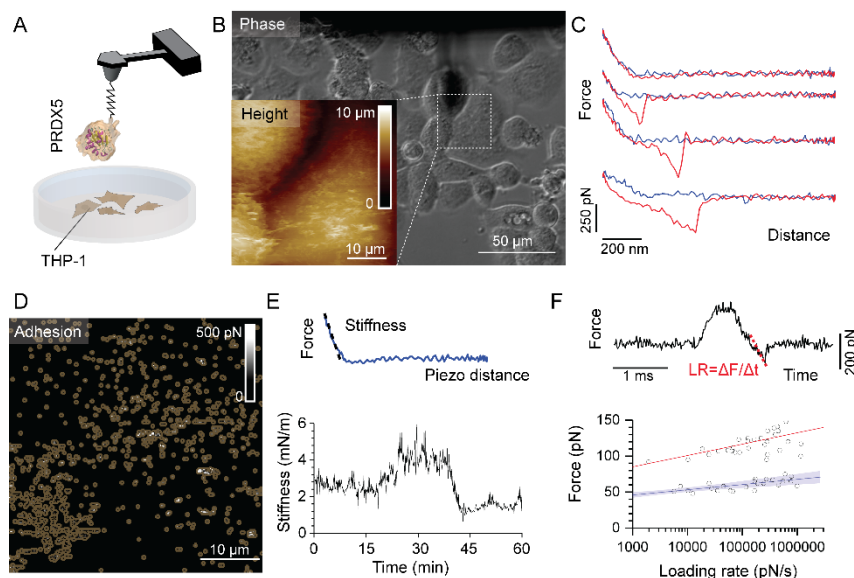


Figure 38. Mapping PRDX5 binding to macrophage-differentiated THP-1 cells using correlative wide-field microscopy and FD-based AFM. (A) Schematic of the FD-based AFM using an AFM tip bearing a PRDX5 molecule and living THP-1 cells. AFM experiments were performed in cell culture conditions (37°C, RPMI-1640 medium, and humidified atmosphere supplemented with 5% CO₂). (B) Phase image showing THP-1 cells and the AFM tip placed above (see shadow). The height image recorded in the dashed square is shown in inset. AFM images were acquired using an oscillation frequency of 0.25 kHz and an amplitude of 500 nm. (C) Representative FD curves extracted from FD-based AFM images. (D) Corresponding adhesion channel simultaneously obtained with the height image, inset in (B). Vertical scales are given in upper right corners. Gray pixels were enlarged by a factor 2 (brown contour). (E) Force versus piezo-distance curve showing the linear fit in the contact area used to extract the stiffness. Plot showing the evolution of the stiffness over time. Stiffness is extracted from the slope of the approach curve in the contact area. (F) Extraction of the rupture forces separating the PRDX5 from the cell surface as well as the loading rate $LR = \Delta F / \Delta t$. For each rupture force (hollow circle, in the dynamic force spectroscopy plot below) the loading rate is extracted from the force-time curve as shown in the curve. Rupture forces (hollow circles) determined were plotted together with the fits obtained on the model surface (Figure 37F). Experiments were reproduced at least five times and other examples are shown in Figure S3.

We also looked at the stiffness and, surprisingly, we observed that the cells became stiffer after 15–30 min while probed with the PRDX5-derivatized tip (Figure 38E). Stiffness was extracted using the slope of the retraction curves during cell surface indentation (see also Table S1). This observation suggests that the specific interactions between PRDX5 and THP-1 cell surface receptors induce a mechano-response. As control experiments, we first probed THP-1 cells with a bare tip

revealing a constant stiffness within 2 h of AFM probing (**Figure S3B**). Next, we probed the stiffness with a bare tip while injecting free PRDX5 at 10 and 15 μM (**Figure S3B**). We observed a concentration-dependent mechano-response strongly suggesting a direct relationship between PRDX5 and cell stiffening. Altogether these observations are in good agreement with a recent study performed by Preira *et al.* in which stiffening of THP-1 cells was observed in presence of cytokine IL-1 β [477].

Further analysis of the adhesion force was performed to evaluate whether they are similar to the forces observed on purified TLR4. We selected FD curves with adhesion forces <150 pN, corresponding to single and double interactions *in vitro*. For each adhesive peak of these curves we extracted the adhesion force together with the loading rate (obtained from the slope before rupture, see **Figure 38F**). We then superimposed these values (see circles in **Figure 38F**) on the dynamic force spectroscopy plot obtained on the purified systems (blue and red curves for, respectively, single and double interactions, see **Figure 37F**), and we observed that these points very well aligned with the fits. This observation suggests that the interactions measured on living THP-1 cells are most likely similar to the PRDX5-TLR4 model system.

TLR4 is a specific receptor for PRDX5 in vitro in a cellular model

Next, we wanted to test whether TLR4 serves as a specific receptor of PRDX5 in the cellular context. CHO cells were transiently transfected with a construct coding for human TLR4, in fusion with the YFP, addressed to the plasma membrane, and functionally preserved [478]. Using the wide-field and fluorescence signal, we chose areas in which both cell types (transfected and not transfected) were in close proximity to one another (**Figure 39A** and **Figure 39B**). Having both CHO and CHO-TLR4-YFP cell types in a single AFM image area (40 x 40 mm) served as a direct control to evaluate whether interactions measured by the PRDX5-functionalized AFM tip were indeed due to specific binding to TLR4 receptors, and directly evaluate the extent of unspecific interactions and/or interactions with other cell surface receptors. The AFM was centered on the fluorescent cell and then the area was imaged using FD-based AFM. The topographic image (**Figure 39C**) and corresponding adhesion map (**Figure 39D**) were reconstructed from the individuals FD curves recorded. To help the visualization of the adhesion events, pixels were enlarged as mentioned above (**Figure 39E**). Retraction curves showed specific adhesion events mainly on CHO cells expressing the TLR4-YFP receptors (adhesive pixels are observed with a frequency >5%, $n > 10,000$), with adhesion forces ranging from 100 to 600 pN (**Figure 39F**). These specific adhesion events occurred at distances of >50 nm, corresponding to the extension of the PEG₂₇ spacer, the size of the PRDX5 molecule grafted on the tip, the size of the cell surface receptor, and the cellular plasticity. FD

curves recorded on non-fluorescent cells less frequently showed adhesion events ($<0.25\%$, $n > 40,000$; **Figure 39E**).

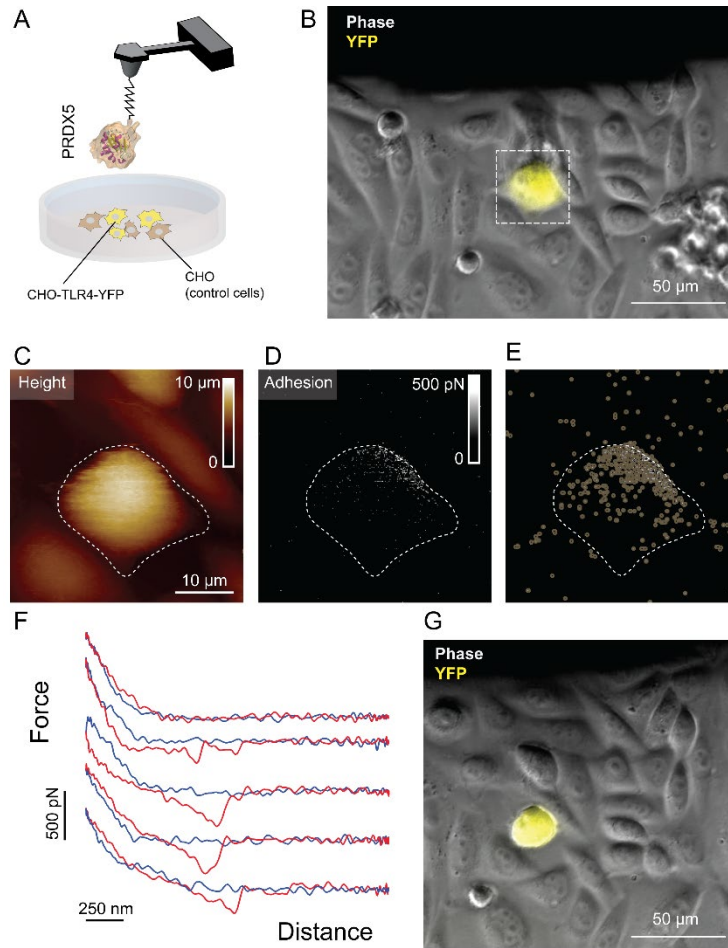


Figure 39. Mapping PRDX5 binding to CHO-TLR4-YFP cells using correlative fluorescence microscopy and FD-based AFM. (A) Wild-type CHO cells were grown for 3 days before transfection with TLR4-YFP plasmid producing CHO-TLR4-YFP cells expressing TLR4-YFP (yellow). (B) Superimposition of phase image and YFP channel showing a confluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. (C) AFM height image recorded in the dashed square shown in (B). (D and E) Adhesion image with adhesive pixels enlarged in (E). Vertical color scales are given in the upper right corner. (F) Representative FD curves recorded from CHO-TLR4-YFP cell. (G) Superimposition of phase image and YFP channel recorded at the end of the FD-based AFM imaging. Experiments were reproduced ten times.

As a control, we investigated a well-known ligand of TLR4 to ensure that the TLR receptors are well expressed at the surface of transfected CHO cells and properly folded. To this end, we functionalized the AFM tip with the high-mobility group box 1 nuclear protein (HMGB1; recombinant human 6xHis-HMGB1) and similarly

mapped the CHO-TLR4-YFP cells. Overall, adhesion events were localized on CHO-TLR4-YFP cells, while control CHO cells showed only rare events (**Figure S4**).

We concluded from the above experiments that the specific adhesion events measured on CHO-TLR4-YFP cells correspond to specific binding events of the PRDX5 ligand tethered to the AFM tip and the TLR4 receptors expressed at the cell surface. Surprisingly, we also observed that CHO-TLR4-YFP cells became round during AFM probing, while non-transfected cells remain spread, suggesting that the specific interaction between PRDX5 and TLR4 is causing this phenomenon (**Figure 39G**).

TLR4 binding induces cellular stiffening

To evaluate the change of cellular morphology during FD-based AFM using a PRDX5-derivatized tip, we scanned a small area of the cell surface in the nuclear region (100 x 100 nm, see red star in **Figure 40A**) for more than 1 h and simultaneously looked at the cell morphology using optical microscopy. The size area was intentionally maintained small to always probe the same area (the nuclear region), so that the cellular heterogeneous stiffness has only a small influence. As shown in **Figure 40**, the stiffness of the cell was extracted using the slope of the extended curve in the contact region and measured over time. We observed that the cell progressively became stiffer during the first 50 min (**Figure 40B** and **Figure 40C**).

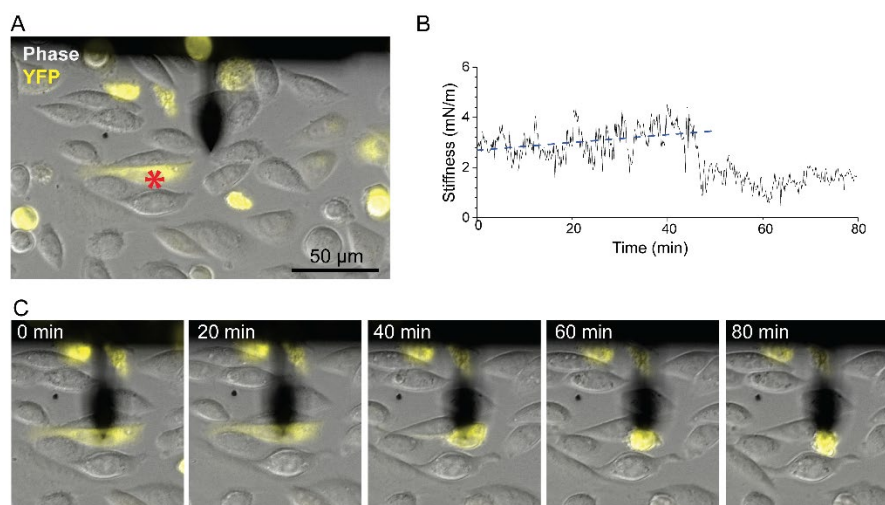


Figure 40. TLR4 binding induces cellular stiffening. Probing cellular stiffness evolution upon PRDX5-TLR4 interactions on CHO-TLR4-YFP cells. **(A)** Superimposition of phase image and YFP channel showing a subconfluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. FD-curves were recorded on a 100 nm x 100 nm area shown by the red asterisk. **(B and C)** Plot showing the evolution of the stiffness over time. Stiffness is extracted from the slope of the approach curve in the contact area. **(C)** Time-lapse evolution of the cellular morphology during FD-based AFM imaging showing that the cell rounds up (see also **Movie S1**).

Simultaneously the cell changed its morphology from a spread state to a rounder state (**Figure 40C**). The cell phenotype of CHO-TLR4-YFP was very similar to that observed on THP-1 cells (**Figure 39G**), as well as the extracted stiffness values (**Table S1**). In the context of endothelial inflammation, this observation is in good agreement with a recent study showing that DAMP ligands are released by pulmonary artery endothelial cells (PAECs) under stress and activate downstream PAEC inflammation through TLR2/NF- κ B, resulting in the stiffening of pulmonary artery [479]³.

Molecular docking study of TLR4-PRDX5 interaction

AFM results obtained here highly support a direct interaction between PRDX5 and TLR4. To further gain insights into PRDX5-TLR4 binding, we used molecular docking simulations.

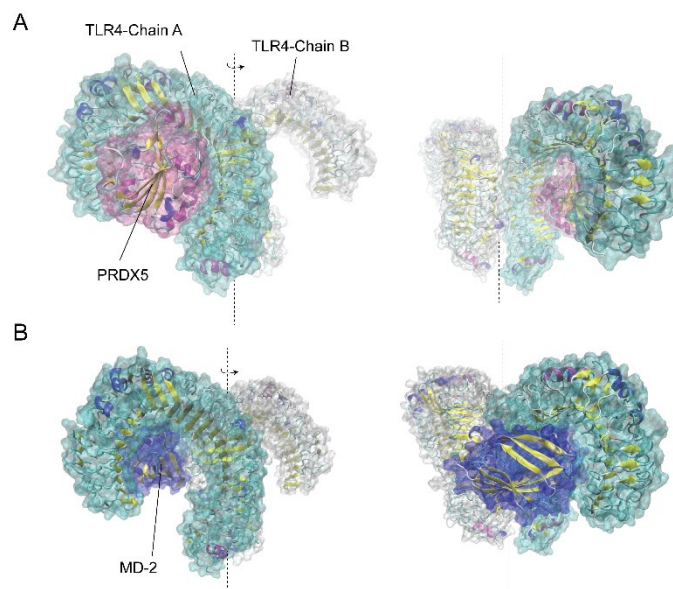


Figure 41. Docking of PRDX5 to TLR4 structure. (A) Front view (left) and side view (right) of PRDX5 (pink colored) binding to a single chain of TLR4 (cyan-colored). (B) Comparison with MD-2 associated to an LPS molecule bound to TLR4. For PRDX5 the simulated interaction occurs within a single TLR chain without contact to the second TLR4 chain forming the dimer. For other plausible docking positions please see **Figure S5**.

The docking was performed using ClusPro webserver [472] using the structure of TLR4 obtained in association with the myeloid differentiation factor-2 (MD-2) bound to LPS (PDB ID: 3FXI) and the oxidized form of the PDRX5 (PDB ID: 2VL3), which is the expected form in the oxidizing extracellular milieu [480]. Both TLR4 chains were used

³ This article has been retracted.

as center of mass and remain fixed at the origin of the coordinate, and possible rotational and translational positions of the ligand PRDX5 were evaluated at the position sampled on a sphere-based grid centered on the receptor center of mass. A Piper docking algorithm allowed evaluating the interaction energy between the two proteins taking into account van der Waals, electrostatic, and hydrophobic contributions. Docking experiment results suggest that PRDX5 predominantly interacts in the center of TLR4 crook similarly to MD-2 (**Figure 41** and **Figure S5**). However, due to its smaller size in comparison with MD-2, PRDX5 would only bind to a single TLR4 monomer. It must be noted that this is also experimentally supported by the AFM experiments on model surfaces where TLR4 was grafted as monomer.

3.1.5 Discussion

Using *in vitro* acellular and cellular models, we unraveled by AFM the mechanisms by which human PRDX5 can act as a DAMP, binding to TLR4 and activating TLR4 to induce pro-inflammatory cytokine IL-1 β release. To our knowledge, it is the first time that AFM has been used to measure DAMP interactions on the surface of living cells. Although most PRDXs have been described mainly as intracellular thiol-dependent peroxidases or protein chaperones, the role of PRDXs as PAMPs or DAMPs after their release into the extracellular milieu is now well documented in the literature. Indeed, PRDXs secreted by parasitic helminths or released by malarial *Plasmodium berghei* have been shown to act as PAMPs by, respectively, triggering the recruitment of macrophages and a Th2 response in mice, or inducing a pro-inflammatory response by binding to TLR4 receptors [355–357]. Also, before its complete characterization as a peroxidase, PRDX1 was initially described as a soluble protein released from lysed cells and exhibiting immune modulating functions able to enhance the cytotoxic activity of natural killer cells [360]. More recently, mammalian PRDX1, PRDX2, PRDX4, PRDX5, and PRDX6 have been proposed to act as DAMPs through activation of TLR2 and TLR4 [104,111,116,344,345,481]. Although mammalian PRDX5 was mainly studied so far for its role as an intracellular cytoprotective antioxidant enzyme or a redox modulator, according to its large subcellular distribution [90], PRDX5 was also shown to play a major role as DAMP when released extracellularly from necrotic brain cells to initiate post-ischemic inflammation through activation of macrophages *via* TLR2 and TLR4 [116]. Therefore, in a first step, we used purified PRDX5 and TLR4 to mechanistically examine whether these receptor-ligand pairs could interact through specific binding. Using PRDX5-derivatized AFM tips and TLR4-functionalized surfaces, we observed that specific bonds are established with a rupture force ranging from 30 to 60 pN, a typical force measured for other single receptor-ligand interactions, such as RGD peptide with integrin [482]. Extraction of kinetic parameters using the

Bell-Evans model revealed a highly stable interaction (very low off-rate) with an average bond lifetime of 38 min, therefore leading to a more stable interaction than TLR4-LPS interaction [483], which has a bond lifetime of 15 s, while TLR4 presents an intermediate bond lifetime with MD-2 (5 min) [484]. Binding specificity was assessed by control experiments including blocking antibodies or endotoxin inactivator polymyxin B.

Using FD-based AFM directly on living cells allowed us to demonstrate that such specific binding events similarly occur in the cellular context. Using macrophage-differentiated THP-1 cells, as a model for mimicking the function of macrophages, we probed PRDX5 binding to cell surface receptors. We furthermore observed specific interactions that shared kinetic characteristics with the model TLR4-PRDX5 system. A TLR4 role in PRDX5 binding in the cellular context was further demonstrated using CHO-TLR4-YFP cells mixed with control cells. Whether the redox status of PRDX5 may affect PRDX5-TLR4 binding would necessitate more in-depth investigation although under our experimental conditions the oxidized form of PRDX5 is expected to be the major form [480].

FD-based AFM interactions observed between PRDX5 and TLR4 on purified receptors revealed that specific interactions occurred within either a monomeric TLR or within a single chain of a TLR4 dimer (**Figure 37**). Experiments on living cells provided evidence that the binding site properties are very similar to *in vitro* data, suggesting that the interaction within a single TLR4 chain site is also established on living cells. These experimental findings were further reinforced by *in silico* simulations (**Figure 41**). Together these data suggest that the TLR4 activation by PRDX5 is most likely very different from the TLR4 activation by LPS binding that induces receptor dimerization by contact point with the two TLR4 chains.

Surprisingly, PRDX5 binding to living cells resulted in a rapid cellular phenotype. Cells probed with a single PRDX5 molecule, even on small areas ($<0.01 \text{ mm}^2$), became stiffer. To the best of our knowledge this is the first direct evidence that DAMP binding through TLR4 induces a mechano-response by the cells, and is particularly relevant in the context of inflammation. Increasing evidence shows that, for example, artery stiffening or reduced arterial compliance is a prominent feature of systemic hypertension, pulmonary hypertension, and other vascular-related diseases [477]. However, so far, the mechanism underlying such stiffening remains elusive. Our study illustrates a new potential mechanism for how cellular mechanical stiffening is triggered downstream of the inflammation site by direct specific binding to TLR4.

In the future, the combined fluorescence-nanoscopic approach used in this work could help to characterize more deeply the role of different ligands binding to

immune receptors in living cells. Moreover, our quantitative method could not only help to uncover the receptors involved in the triggering of the immune response, but could also provide a tool to quantify the effects of molecules interfering with receptor activation and therefore aid the development of novel drugs modulating the inflammatory response.

3.1.6 Significance

Inflammation is induced when innate immune cells, such as macrophages, detect danger molecules during infection or tissue injury. Among such molecules, PRDXs were recently shown to act as factors released by damaged or dying cells, termed DAMPs. However, the molecular mechanisms of how extracellular PRDXs act as DAMPs, interact with pattern recognition receptors, and contribute to the induction of inflammation by modulating pro-inflammatory cytokine release are still unclear. Moreover, a better understanding of these mechanisms could help developing new therapeutic approaches for inflammation-related diseases. Here, we examined at the nanoscopic scale the interactions between PRDX5, previously shown to act as a DAMP, and TLR4, using AFM in acellular and cellular models. We demonstrated that PRDX5 specifically binds to TLR4. Surprisingly, this binding also triggers a cellular mechano-response, suggesting that PRDX5-TLR4 interactions not only modulate pro-inflammatory cytokine release but also cell stiffening, which could be highly relevant in the inflammatory response. Indeed, increasing evidence shows that, for example, artery stiffening or reduced arterial compliance is a prominent feature of systemic hypertension, pulmonary hypertension, and other vascular diseases with an inflammatory component. However, so far, the mechanism underlying such coupling remained elusive. Our study illustrates a new potential mechanism of how cellular mechanical stiffening is triggered during inflammation by a direct specific binding to TLR4. Our study demonstrates also the potential of AFM as a multifunctional approach to simultaneously characterize both ligand binding and mechanical properties.

3.1.7 Supplementary information

Table S1. Related to **Figure 38** and **Figure 40**. Cell stiffening by PRDX5.

Cell type	Initial stiffness	Stiffening	Final
THP-1	$2.94 \pm 0.25 \text{ mN}\cdot\text{m}^{-1}$ * ($n = 5,120$)	$3.63 \pm 0.48 \text{ mN}\cdot\text{m}^{-1}$ ($n = 11,008$)	$1.65 \pm 0.17 \text{ mN}\cdot\text{m}^{-1}$ ($n = 9,472$)
CHO-TLR4	$2.57 \pm 0.34 \text{ mN}\cdot\text{m}^{-1}$ ($n = 65,536$)	$3.84 \pm 0.67 \text{ mN}\cdot\text{m}^{-1}$ ($n = 60,416$)	$1.50 \pm 0.88 \text{ mN}\cdot\text{m}^{-1}$ ($n = 64,000$)

* Stiffness: average $\pm$ standard deviation (n = number of FD curves)

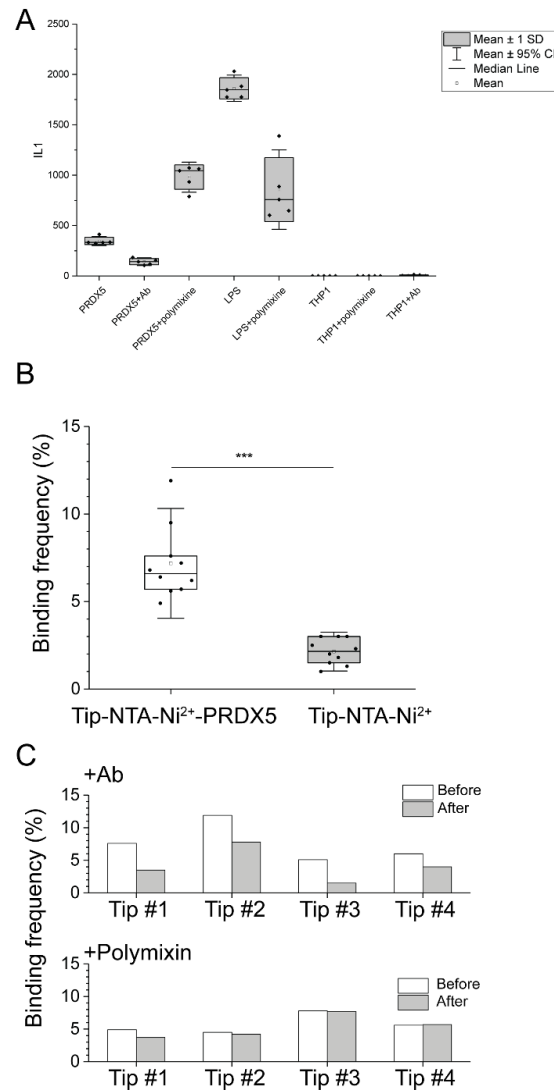


Figure S1. Related to Figure 37. IL-1 β secretion from macrophage-differentiated THP-1 cells after stimulation and validation of PRDX5-functionalized AFM tips specificity. (A) THP-1 cells were incubated with stimulants individually or mixed at concentration of 2 μ M PRDX5, 1/10 blocking antiserum against PRDX5 (Ab), 30 μ g $\cdot$ ml⁻¹ polymyxin B and 1 μ g $\cdot$ ml⁻¹ LPS in medium without fetal calf serum. Supernatants were harvested 48 h after cell exposure and human IL-1 β levels were measured ($n = 5$ for each condition). Asterisks (***) indicate a p -value ≤ 0.001 using paired sample Student's t -test. Data correspond to a representative experiment of five independent experiments. **(B)** Comparison of binding frequency using an AFM tip functionalized with or without the PRDX5 molecule. Experiments were reproduced 10 times ($n = 10$; 10 different functionalized surfaces and AFM tips). **(C)** Comparison of binding frequency observed before and after injection of anti-PRDX5 antibody (Ab; upper) and polymyxin B (lower). Asterisks (***) indicate a p -value ≤ 0.001 using paired sample Student's t -test. Experiments were reproduced 4 times.

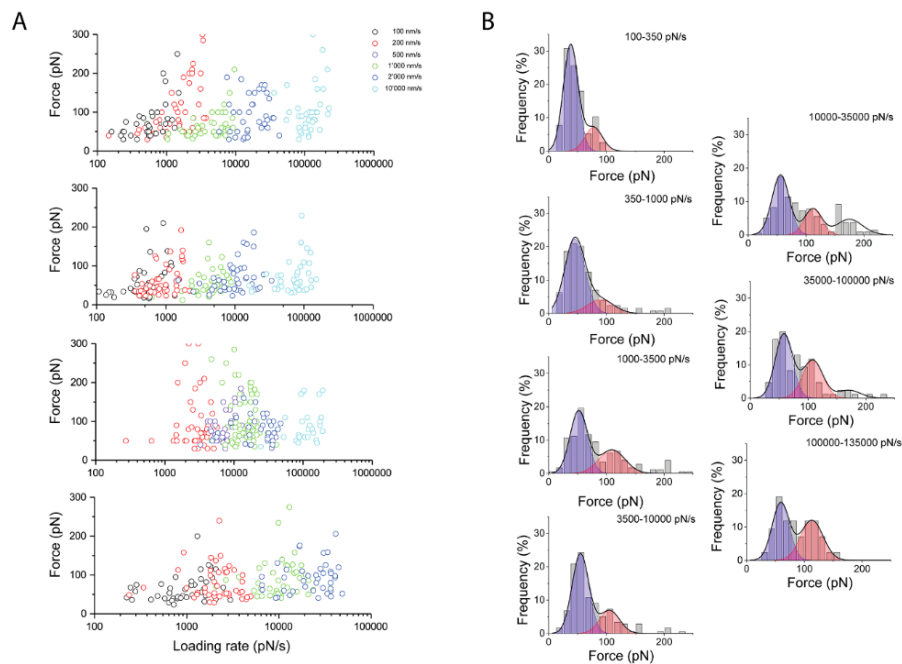


Figure S2. Related to Figure 37. Extraction of forces with loading rates and average rupture forces. (A) Rupture forces were measured with the loading rate (measured as the slope of the unbinding event just before the rupture on the force *versus* time curve). The force-distance curves are collected at different retraction speeds ($100 - 10'000 \text{ nm}\cdot\text{s}^{-1}$) and displayed as different colors. **(B)** Distribution of rupture forces was sorted in seven LR ranges and the distributions of the rupture forces are plotted as histograms. This classification reveals multiple force peaks with average values corresponding to single (blue), double (red) simultaneously established PRDX5-TLR4 interactions. The average values of the force distributions for single interactions were extracted and plotted on the dynamic force spectroscopy plot (**Figure 37F**), enabling their analysis using the Bell-Evans model. Data are representatives from four independent experiments; meaning different samples and AFM tips.

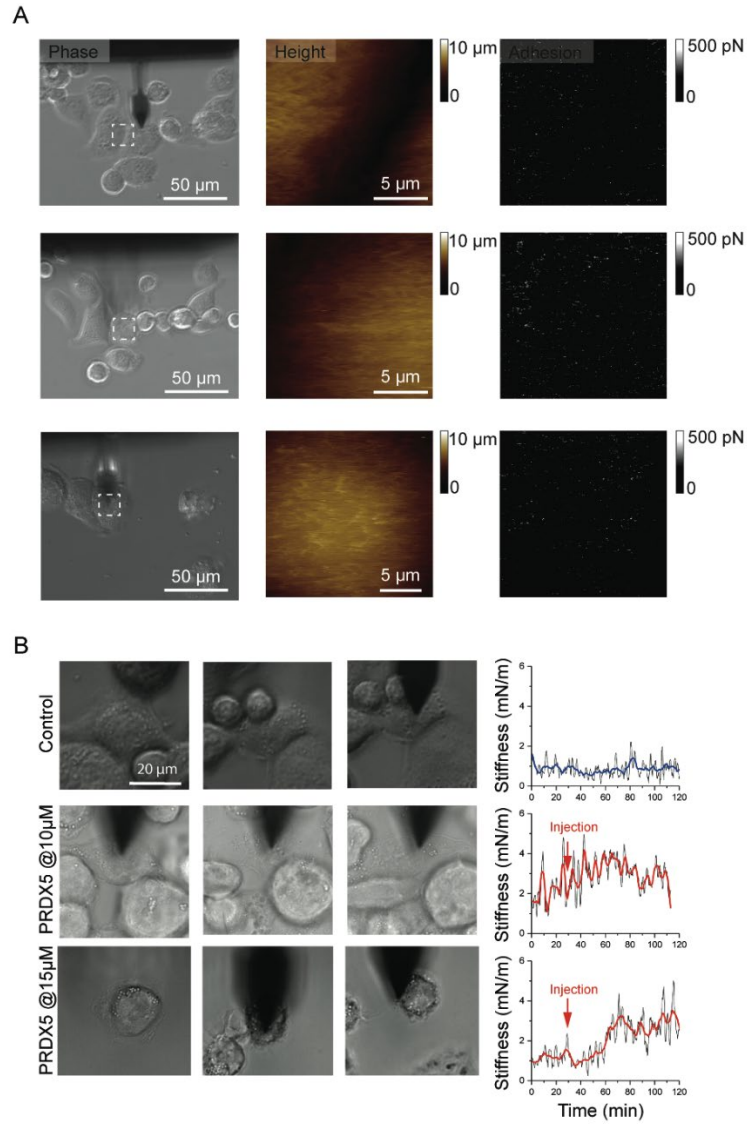


Figure S3. Related to Figure 38. Mapping PRDX5 binding to macrophage-differentiated THP-1 cells and extracting stiffness. (A) Experiments were performed in cell culture conditions (37°C, DMEM medium, and humidified atmosphere supplemented with 5% CO₂, see Methods). First column shows phase images of THP-1 cells with the AFM tip placed above (see shadow). The second column is the AFM height image recorded in the dashed square shown in the corresponding phase image. AFM images were acquired using an oscillation frequency of 0.25 kHz and amplitude of 500 nm. Corresponding adhesion channel is shown in the right column and is simultaneously obtained with the height image. Vertical scales are given in upper right corners. **(B)** Phase image showing THP-1 cells and the AFM tip placed above (see shadow) and plot showing the evolution of the stiffness over time for control cell (1st line) or upon incubation with free PRDX5 at 10 μ M (2nd line) or 15 μ M (3rd line). Stiffness is extracted from the slope of the approach curve in the contact area.

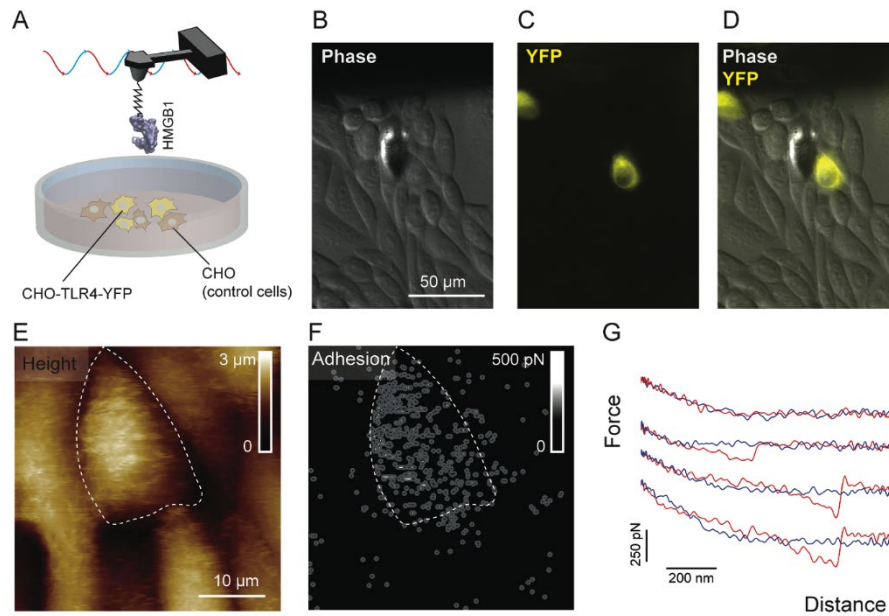


Figure S4. Related to Figure 39. Mapping HMGB1 binding to CHO-TLR4-YFP cells using correlative epifluorescence microscopy and FD-based AFM. (A) Wild-type CHO cells were grown for 3 days before transfection with TLR4-YFP plasmid producing CHO-TLR4-YFP cells expressing TLR4-YFP (yellow). (B) Phase image, (C) YFP fluorescence channel and (D) superimposition of phase image and YFP channel showing a confluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. (E) AFM height image recorded in the center of (D). The dashed line encircles the fluorescent cell. (F) Adhesion image with adhesive pixels enlarged by a factor 2. Vertical color scales are given in the upper right corners of the height and adhesion images. (G) Representative FD curves recorded from CHO-TLR4-YFP cell. Experiments were reproduced three times.

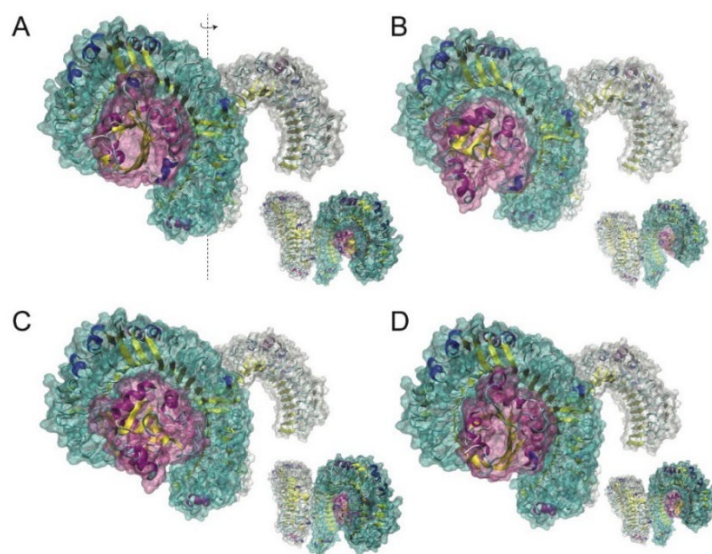


Figure S5. Related to Figure 41. Further examples of docking positions of the PRDX5 to the TLR4 structure. **(A-D)** Front view and side view of PRDX5 (pink colored) binding to a single chain of TLR4 (cyan-colored) showing that interaction may occur within a single TLR chain.

[https://www.cell.com/cell-chemical-biology/fulltext/S2451-9456\(18\)30070-9?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS2451945618300709%3Fshowall%3Dtrue#supplementaryMaterial](https://www.cell.com/cell-chemical-biology/fulltext/S2451-9456(18)30070-9?_returnURL=https%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS2451945618300709%3Fshowall%3Dtrue#supplementaryMaterial)

Movie S1. Related to Figure 40. Time-lapse evolution of the cellular morphology during FD-based AFM imaging showing that the cell rounds up. Superimposition of phase image and YFP channel showing a sub-confluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. FD-curves were recorded on a 100 nm × 100 nm on the center of the CHO-TLR4-YFP cell. Probed cell rounds up over time. Movie relates to **Figure 40C**.

3.1.8 General comments

As this study was performed before I started my own thesis project, I will briefly address some issues that I raised together with the changes I made accordingly in the next parts of the project.

First, the method of extracting the most probable rupture forces is missing. In particular, the parameters that were used to obtain the histograms (*i.e.* bin size and bounds in terms of peak center, peak width and peak area) are not specified. During my project, I tried to get more objective results by searching for a method allowing peak detection without arbitrary choices. Comparison of the DFS analyses that were performed in this study and the following ones will be discussed in **Chapter IV – General discussion** (see **section 4.1.3.**).

Next, statistical information regarding SMFS experiments is missing or insufficient. For example, it is stated that the experiments leading to **Figure 37F** (and its associated **Figure S2**) were performed four times independently. However, these results were actually obtained from solely two different AFM tip and surface functionalization procedures, with two different AFM tips in each case. The same question applies for SMFS on living cells. Does “experiments were reproduced at least X times” mean that at least X independent functionalization procedures were performed or that at least X different cells were probed (with a certain number of AFM tips)? During my project, I tried to get more robust statistics by increasing the number of independent experiments and the number of data values, *i.e.* by increasing the number independent AFM tip and surface functionalization procedures, the number of AFM tips and surfaces used within one experiment, and the number of AFM maps recorded with one AFM tip-surface system. I also specified this statistical information.

As control for the specificity of the interaction probed in SMFS, the interactions between a tip functionalized with *tris*-NTA-Ni²⁺ and the TLR4-functionalized surface were probed (**Figure S1**). As the recombinant TLR4 portion that was grafted on the surface contains a 10xHis tag, specific interactions might occur between *tris*-NTA-Ni²⁺ complex tethered on the AFM tip and the TLR4 His tag. Therefore, how can we explain that a decrease in the BF was observed? In my project, I tried to optimize the functionalization strategies and I compared them.

A correlation is made between the interactions probed at the surface of living PMA-differentiated THP-1 cells and the interactions measured on model surfaces functionalized with isolated TLR4 molecules. However, THP-1 cells are also known to express at least another receptor believed to interact with PRDX5, namely TLR2. According to me, a control assessing the specificity of the interactions probed (*i.e.*

whether they actually arise from the probing of the PRDX5–TLR4 complex) is missing. To overcome this issue, an AFM tip carrying an unrelated ligand should be used to demonstrate that the data points obtained from the interactions measured at the surface of PMA-differentiated THP-1 cells do not align with the fits obtained on model surfaces.

To assess whether cell surface TLR4 was a specific receptor for PRDX5, the CHO cells overexpressing TLR4 were probed with PRDX5-functionalized tips. However, CHO cells are known to express endogenous TLR4. As human PRDX5 might interact with murine TLR4, I oriented the choice of the cell line in order to eliminate such uncertainty. I opted for HEK293 cells, known to lack endogenous TLR2 and TLR4.

Regarding the docking experiments, the PRDX5 monomeric form was used, despite that PRDX5 is known to exist exclusively as a homodimer. According to me, whether such conformations are actually relevant should be discussed. In particular, whether the PRDX5 portion that interacts with the TLR4 monomer is at the opposite side of the dimer interface should be specified. Furthermore, it is stated that this result is supported by the AFM experiments on model surfaces. However, it was most likely a dimer that was grafted onto the AFM tip. As a consequence, such conclusion cannot be drawn. During my thesis, I considered the dimeric structure of PRDX5 and I tried to investigate more deeply the oligomeric state of the proteins under study.

Finally, it is stated that AFM was used to unravel how human PRDX5 can activate TLR4 to induce a pro-inflammatory IL-1 β release. However, as PMA-differentiated THP-1 macrophages express a wide range of receptors, of which at least two (TLR2 and TLR4) are known to be involved in PRDX5-induced cytokine secretion, this conclusion cannot be drawn. No direct correlation was made between PRDX5 binding to TLR4 and IL-1 β release.

3.2. Study 1 – Role of the redox state of human peroxiredoxin-5 on its TLR4-activating DAMP function

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The original (published) paper is presented in **Annex 2**.

Personal contribution: I performed all experiments, with the exception of MS, SEC-MALS, and CLSM.

3.2.1. Abstract

Human peroxiredoxin-5 is a unique redox-sensitive protein that plays a dual role in brain ischemia-reperfusion injury. While intracellular PRDX5 has been reported to act as a neuroprotective antioxidative enzyme by scavenging peroxides, once released extracellularly from necrotic brain cells, the protein aggravates neural cell death by inducing expression of pro-inflammatory cytokines in macrophages through activation of Toll-like receptor 2 (TLR2) and TLR4. Although recent evidence showed that PRDX5 was able to interact directly with TLR4, little is known regarding the role of the cysteine redox state of PRDX5 on its DAMP function. To gain insight into the role of PRDX5 redox-active cysteine residues in the TLR4-dependent pro-inflammatory activity of the protein, we used a recombinant human PRDX5 in the disulfide (oxidized) form and a mutant version lacking the peroxidatic cysteine, as well as chemically reduced and hyperoxidized PRDX5 proteins. We first analyzed the oxidation state and oligomerization profile by Western blot, mass spectrometry, and SEC-MALS. Using ELISA, we demonstrate that the disulfide bridge between the enzymatic cysteines is required to allow improved TLR4-dependent IL-8 secretion. Moreover, single-molecule force spectroscopy experiments revealed that TLR4 alone is not sufficient to discriminate the different PRDX5 redox forms. Finally, flow cytometry binding assay show that disulfide PRDX5 has a higher propensity to bind to the surface of living TLR4-expressing cells than the mutant protein. Taken together, these results demonstrate the importance of the redox state of PRDX5 cysteine residues on TLR4-induced inflammation.

3.2.2. Introduction

Peroxiredoxins (PRDXs) constitute a large superfamily of highly diverse and multifunctional peroxidases, expressed as six isoforms in mammals (PRDX1-6). Their intracellular functions are related to the redox state of their catalytic cysteine residues (Cys), of which there are one in PRDX6 and two in PRDX1-5. Intracellularly, PRDXs act as cytoprotective peroxidases against oxidative stress by reducing peroxides (hydrogen peroxide (H₂O₂), alkyl hydroperoxides, and peroxyxynitrite), as modulators of redox signaling, and as chaperones [27,33,360]. Unlike prosthetic group-dependent enzymes, the PRDX catalytic cycle relies on a conserved Cys, so-called peroxydatic Cys, located in the N-terminal part of the protein. When the thiol (-SH) of this peroxydatic Cys is oxidized by peroxide, this results in a reactive sulfenic acid (-SOH) [48]. Depending on the PRDX subfamily, the sulfenic acid undergoes a condensation reaction with (i) a thiol on an adjacent subunit to form an inter-subunit disulfide in 2-Cys subfamily (PRDX1-4) [49], (ii) a thiol on the same subunit to form an intra-subunit disulfide in atypical 2-Cys subfamily (PRDX5) [50], or (iii) the π isoform of glutathione-S-transferase to form an heterodimeric disulfide in 1-Cys

subfamily (PRDX6) [51]. The disulfide is reduced *in vivo* by a thioredoxin (Trx) for mammalian PRDX1-5 [52], and by glutathione for mammalian PRDX6 [53]. Hyperoxidation can also occur *in vivo* for typical 2-Cys PRDXs, with the sulfenic acid being oxidized to a sulfinic acid (-SO₂H) [66], the latter being reduced by the sulfiredoxin system [67], or further oxidized to an irreversible sulfonic acid (-SO₃H) [68].

Mammalian PRDXs have also been shown to play key roles extracellularly by acting as damage-associated molecular patterns (DAMPs), triggering the production of cytokines in macrophages [116,117,344,362,485]. In humans, extracellular PRDXs released from necrotic brain cells have notably been shown to worsen brain ischemia-reperfusion injury by inducing expression of pro-inflammatory cytokines through activation of Toll-like receptor 2 (TLR2) and TLR4 [116]. Among the PRDXs, PRDX5 in particular was shown to be a strong TLR activator [486]. The molecular mechanism underlying this TLR activation remains unclear, although we have recently shown using atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) that recombinant human PRDX5 was able to specifically bind to TLR4 [487]. However, very few studies have focused on the importance of the redox state of catalytic PRDX Cys in their DAMP function [116,344].

Here, we combine an array of interdisciplinary techniques including AFM-based SMFS, size-exclusion chromatography – multiangle light scattering (SEC-MALS), mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA), flow cytometry (FC), and confocal laser scanning microscopy (CLSM), to investigate the importance of the redox state of human PRDX5 redox-active Cys in the TLR4-dependent pro-inflammatory activity of the protein. By disrupting the C47-C151 disulfide bridge, either through C47 point mutation or dithiothreitol (DTT)/iodoacetamide (IAM) reduction/alkylation treatment, or *via* hyperoxidation with H₂O₂, we demonstrate the role of the C47-C151 disulfide bridge in the PRDX5 interleukin (IL)-8-inducing ability. To determine whether TLR4 is able to discriminate between the PRDX5 redox forms, we used SMFS to evaluate the binding kinetics of reduced, oxidized (disulfide) and hyperoxidized PRDX5 to TLR4. Our results indicate that all redox forms bind TLR4 with a similar kinetic dissociation rate (k_{off}), with the exception of hyperoxidized PRDX5 that is not able to establish a specific interaction with the receptor. We further show with a flow cytometry binding assay that the binding propensity of disulfide PRDX5 to the surface of living cells expressing TLR4 is higher than that of the mutant protein. Overall, these results provide the basis for better understanding how the redox state of catalytic redox-sensitive Cys allows PRDXs to promote inflammation in inflammatory-related diseases, which is of crucial importance for the development of new therapeutic strategies related to ischemia-reperfusion injury.

3.2.3. Materials and Methods

Production and purification of recombinant human PRDX5 and C47S mutant PRDX5

The expression and purification of recombinant wild-type (dsPRDX5) and C47S mutant (PRDX5^{C47S}) N-terminal 6xHis-tagged human PRDX5 are described elsewhere [50,142,487]. Briefly, pQE-30 expression vector (Qiagen) coding for wild-type and C47S mutant human PRDX5 were used to transform *Escherichia coli* (*E. coli*) strain M15 (pRep4). *E. coli* were grown at 37°C in lysogeny broth (LB) medium containing 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Pelleted cells were lysed in 10 mM imidazole, 50 mM phosphate, 300 mM NaCl (pH 8) by sonication and clarified by centrifugation. The supernatant containing 6xHis-tagged PRDX5 was loaded onto a Ni²⁺-nitrilotriacetic acid (NTA) column (Qiagen). The column was washed and the protein was eluted with 50 mM phosphate, 300 mM NaCl, 250 mM imidazole (pH 8). Eluted protein was then dialyzed against phosphate-buffered saline (PBS) (pH 7.2).

Preparation of the PRDX5 redox forms

To generate the chemically reduced or hyperoxidized forms, recombinant human PRDX5 (dsPRDX5) was first exposed to 10 mM dithiothreitol (DTT, Sigma-Aldrich) at room temperature (RT) for 1 h to reduce the disulfide bonds. To block the free cysteine thiols in order to maintain the protein in a reduced state, DTT-reduced PRDX5 was subsequently alkylated using 55 mM iodoacetamide [116] (IAM, Sigma-Aldrich) in a dark chamber at RT for 1 h (r/aPRDX5), as described previously. To oxidize cysteine residues to sulfinic or sulfonic acids, DTT-reduced PRDX5 was incubated with 5 mM H₂O₂ (Carl Roth) [69] at RT for 1 h (s/sPRDX5).

dsPRDX5, PRDX5^{C47S}, r/aPRDX5, and s/sPRDX5 solutions were ultra-filtered using Amicon Ultra-0.5 centrifugal filter devices (Merck). First, 500 μ L of protein samples were concentrated by spinning the device at 14,000 x g for 30 minutes. Then, the filtrate was discarded, and the concentrate was reconstituted to the original sample volume (500 μ L) with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich). This process was repeated six times so that the concentrations of the residual DTT and IAM or H₂O₂ were sufficiently reduced ($\sim 10^6$ times). To recover the concentrated proteins, the filter device was placed upside down in a clean microcentrifuge tube and spun for 2 minutes at 1000 x g to transfer the sample from the device to the tube. The sample was then reconstituted to the original volume (500 μ L).

Protein samples were quantified either using the NanoDrop™ One/One^C Microvolume UV-Vis spectrophotometer (ThermoFisher) (in MS, SEC-MALS, SMFS and FC experiments) or with the Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Scientific) (in ELISA and Western blot experiments).

Western blotting for detection of hyperoxidized PRDX5

100 ng of each redox form was loaded on two SDS-polyacrylamide gels (Any kD Mini-PROTEAN TGX Precast Protein gel, Bio-Rad) and SDS-PAGE was performed under non-reducing conditions. Proteins were then transferred to nitrocellulose membranes (Amersham Protan 0.45 μ m NC, GE Healthcare Life Sciences). After transfer, proteins were detected using either an anti-PRDX5 serum (1/200) [436] or an anti-PRDX5-SO₃ antibody (1/2000) (gift from Prof. Sue Goo Rhee) [69]. Polyclonal goat anti-rabbit IgG/HRP (1/2000) (Agilent) was used as the secondary antibody, and BM Chemiluminescence Blotting Substrate (Roche) was used to visualize the protein bands on an Amersham Imager 600 (GE Healthcare Life Sciences).

Mass spectrometric characterization of the PRDX5 cysteine redox state

Blocking of cysteine redox states

Prior to MS analysis, buffer exchange was accomplished using Amicon Ultra-0.5 centrifugal filter devices (Merck) by concentrating the protein samples, discarding the filtrates, and reconstituting the concentrate protein samples to the original volume with 100 mM triethylammonium bicarbonate (pH 8.5) buffer (TEAB, Sigma-Aldrich). Potent cysteine sulfenic acids were trapped with 5 mM 5,5-dimethyl-1,3-cyclo-hexanedione (dimedone, Sigma-Aldrich) for 30 min at RT [488,489], and thiols remaining after redox treatments were subsequently blocked with 55 mM 2-chloroacetamide (CAM) for 30 min at RT in the dark. To be able to determine thioethers formed after alkylation with IAM from those formed after alkylation with CAM, MS analysis of r/aPRDX5 was performed with and without CAM treatment.

Sample preparation

1 μ g of samples was acidified with 1% (v/v) trifluoroacetic acid (TFA), desalted and concentrated with C4 ZipTip (Millipore) according to the manufacturer's protocol. Samples were then eluted with 10 μ L of 50% (v/v) acetonitrile, 0.1% formic acid.

ESI-Q-TOF analysis of intact proteins by direct infusion

Intact protein analysis was done on a SYNAPT G2-Si high definition mass spectrometer (Waters) equipped with a NanoLockSpray dual electrospray ion source (Waters). Coated fused silica PicoTip™ Econo12 Emitters for nanoelectrospray, outer diameters: 1 μ m Tip (New Objective, USA) were filled with 5 μ L of samples and placed on the Universal NanoFlow™ Sprayer (Waters). The eluent was sprayed at a spray voltage of 1.2 kV. The source temperature was set to 120°C. The cone gas flow was 20 liters·h⁻¹ with a nanoflow gas pressure of 0.3 bar. MS spectra were acquired and processed with MassLynx software (Waters).

Maximum entropy deconvolution

MaxEnt1 deconvolution algorithm was used to extract the exact masses with the following parameters: ranges from 17,000 Da to 39,000 Da, resolution of 0.5 Da/channel, Uniform Gaussian peak width at half height of 0.4 Da. A centering process was performed using the centroid method to obtain accurate average molecular masses. The minimum peak width at half height was set to 2, and the fraction of the resolved portion of the peak used to calculate the centroid was set to 80%. The height of a bar represents the sum of intensities of the points across the peak in the continuum trace.

SEC-MALS analysis of the oligomeric state

SEC-MALS experiments were performed using an LC-20 Prominence BioInert HPLC system (Shimadzu), coupled to a miniDAWN TREOS-II MALS instrument (Wyatt Technology), a Refractive Index Detector RID-20A (Shimadzu) and a UV-VIS Detectors SPD-20A (Shimadzu). For chromatographic separation, 50 μ L of sample were loaded alternately onto a Superdex 200 Increase 10/300 GL (Cytiva) and a Superdex 75 Increase 10/300 GL (Cytiva) (data not shown) equilibrated in the PBS running buffer (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , NaCl 137 mM, KCl 2.7 mM pH 7.4). Data were analyzed with ASTRA 7.3 software (Wyatt Technology) and molecular mass was calculated using a Zimm fit model.

Relative quantification of the absolute molar masses of PRDX5 redox form species was performed by taking the ratio of the calculated mass of the selected peak over the total calculated mass of all peaks of interest, expressed as a percentage.

Generation and culture of HEK293 cell line expressing TLR4

HEK293 cells expressing human MD-2 and CD14 (HEK293 MD2-CD14) (Invivogen) were plated at a density of $\sim 2 \times 10^6$ cells in 100 mm culture dishes and stably transfected with 10 μ g pcDNA3-TLR4-YFP (Addgene, plasmid #13018) using 25 μ L Lipofectamine LTX (ThermoFisher) and 10 μ L PLUS Reagent (ThermoFisher) in 1 mL of Opti-MEM (ThermoFisher), when cells reached $\sim 70\%$ confluency. Stable transfectants (HEK293 MD2-CD14 TLR4) were first selected using the antibiotic Geneticin (G418) (Invivogen). To control TLR4 expression at the cell surface, cells were further sorted based on the fluorescence of an APC-conjugated anti-TLR4 antibody (ThermoFisher) using fluorescence-activated cell sorting (FACS). Single cells were then isolated to produce stable cell lines.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX (ThermoFisher) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Lonza), 100 units $\cdot$ mL $^{-1}$ streptomycin and 100 units $\cdot$ mL $^{-1}$ penicillin (Sigma-

Aldrich), 100 mg·mL⁻¹ of Normocin (InvivoGen) and 0.5 mg·mL⁻¹ G418 (InvivoGen). Cells were grown at 37°C in a humidified atmosphere supplemented with 5% CO₂.

Quantification of human IL-8 secretion

Prior to performing ELISAs, endotoxins were removed from the PRDX5 productions using the Pierce High Capacity Endotoxin Removal Spin Columns (ThermoFisher), following manufacturer's instructions. For each experiment (ELISA), a new redox treatment for r/aPBS, r/aPRDX5, s/sPBS, and s/sPRDX5 was performed, and two independent PRDX5^{C47S} production batches were used.

HEK293 MD2-CD14 TLR4 cells were counted and cultured in 96-well plates with complete growth medium for 48 hours before being exposed to 2 μM of the different PRDX5 redox forms, 2 μM HMGB1, and 100 ng·mL⁻¹ LPS, or to increased concentrations (1, 2, 5, 10, 15, and 20 μM) of the different PRDX5 redox forms, in DMEM supplemented with 100 units·mL⁻¹ streptomycin and 100 units·mL⁻¹ penicillin without FBS. Culture medium was harvested 16 h after cell exposure and IL-8 was measured using ELISA kit (BD Biosciences), following manufacturer's instructions.

Functionalization of AFM tips

PRDX5 molecules were grafted onto the AFM tips (MST probes, Bruker) using (3-aminopropyl) triethoxysilane (APTES) coating and a heterobifunctional polyethylene glycol (PEG₂₄) linker composed of an aldehyde molecule at one end and an *N*-hydroxy succinimide (NHS) molecule at the other end. The protocol was adapted from Professor H. J. Gruber (Johannes Kepler Universität, Institute of Biophysics, Linz).

Amino-functionalization

Probes were first washed three times in chloroform, dried with a gentle stream of nitrogen and cleaned for 15 min in an ultraviolet radiation and ozone (UV-O₃) cleaner (Jelight). Probes were placed for 2 h in a hermetic desiccator filled with argon, in which were placed a tray containing 30 μL of APTES and another tray containing 10 μL of triethylamine. Finally, the trays were removed and probes were kept for 2 more days in the desiccator filled with argon.

Coupling of aldehyde-PEG₂₄-NHS linker

The amino-functionalized probes were first immersed for 2 h in a solution containing 1 mg of aldehyde-PEG₂₄-NHS linker dissolved in 0.5 mL of chloroform, and 30 μL of triethylamine. Probes were then washed three times in chloroform, and dried with a gentle stream of nitrogen. The probes were immersed in 100 μL of 0.2 mg·mL⁻¹ PRDX5 solution and 2 μL of a freshly prepared solution of 1 M NaCNBH₃ (with 20 mM NaOH) for 1 h. The reaction was quenched by adding 5 μL of 1 M

ethanolamine solution (pH 8). After 10 min, the tips were rinsed three times with phosphate buffered saline (PBS) and stored in PBS. Experiments were conducted within 48 h following protein coupling.

Preparation of TLR4-coated surfaces

Recombinant human TLR4 (R&D Systems) was covalently attached to a mixed self-assembled monolayer (SAM) of carboxyl (COOH)- and hydroxyl (OH)-terminated alkanethiols immobilized onto model surfaces *via* NHS/*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) chemistry.

Alkanethiol SAM formation

Gold-coated silicon surfaces of ~1 cm x 1 cm were first washed with ethanol, dried with a gentle flow of nitrogen, and cleaned for 15 min by UV-O₃ treatment. Surfaces were then immersed overnight in an ethanol solution containing a 1 mM mixture (1:99 mol/mol) of 16-mercaptohexadecanoic acid (HS(CH₂)₁₆COOH) (Sigma-Aldrich) and 11-mercapto-1-undecanol (HS(CH₂)₁₁OH) (Sigma-Aldrich).

NHS/EDC chemistry

After sonicating briefly to remove alkanethiol aggregates that may be adsorbed, surfaces were rinsed with ethanol and dried with nitrogen. Next, surfaces were immersed for 30 min in a solution of 175 mM NHS (Sigma-Aldrich) and 260 mM EDC (Sigma-Aldrich) in ultrapure water and rinsed 10 times with PBS. The activated surfaces were finally incubated with 0.1 mg·mL⁻¹ TLR4 in PBS for 2 h and rinsed three times in PBS. Experiments were conducted within 48 h following surface functionalization.

FD-curve based AFM on TLR4-coated surfaces

SMFS on model surfaces was operated with a NanoScope 8 Multimode AFM (Bruker) and with the NanoScope 9.2 software (Bruker), using PRDX5-functionalized MSCT tips (C triangular cantilevers, Bruker) and TLR4-functionalized gold surfaces in PBS at ~25°C. Using Force Volume in contact mode, FD curves were recorded on 5 μm x 5 μm arrays of 32 x 32 curves. Constant approach speed was set at 1 μm·s⁻¹ and retraction speeds were varied (100 nm·s⁻¹, 200 nm·s⁻¹, 500 nm·s⁻¹, 1 μm·s⁻¹, 2 μm·s⁻¹, 10 μm·s⁻¹). A ramp size of 200 nm and a force threshold of 300 pN were used. Cantilever spring constants were calibrated on mica surfaces with the thermal noise method [434].

Force curves were analyzed using the Nanoscope Analysis 1.8 software (Bruker). FD curves were first extracted from individual adhesion maps and sorted to keep the curves displaying specific adhesion event(s). For each tip retraction speed, the BF

was calculated by dividing the number of specific adhesive curves by the total number of FD curves recorded for each map. For the DFS analysis, the baseline of the retraction curves was first corrected using a linear fit to remove tilt and offset. Rupture forces (expressed in pN) were extracted by measuring the height of the adhesive peak(s), and LRs (expressed in $\text{pN}\cdot\text{s}^{-1}$) were extracted by fitting the adhesive peak on the force-time curve just before rupture with a linear model. Determination of the kinetic parameters of the interactions was performed on OriginPro 9.8.0.200 software. Spectrum of LRs was divided into 5 or 6 discrete ranges (LR#1: 315-1000 $\text{pN}\cdot\text{s}^{-1}$; LR#2: 1000-3150 $\text{pN}\cdot\text{s}^{-1}$; LR#3: 3150-10,000 $\text{pN}\cdot\text{s}^{-1}$; LR#4: 10,000-31,500 $\text{pN}\cdot\text{s}^{-1}$; LR#5: 31,500-100,000 $\text{pN}\cdot\text{s}^{-1}$; LR#6: 100,000-315,000 $\text{pN}\cdot\text{s}^{-1}$), depending on the redox form. For each LR range, a kernel distribution was used to represent the probability density function (PDF) of the rupture forces, based on the following function:

$$ksdensity(x, Vx, w) = \frac{1}{n} \sum_{i=1}^n \frac{1}{\sqrt{2\pi}w} e^{-\frac{(x-Vx_i)^2}{2w^2}}$$

This function returns the kernel density at x for a given vector Vx of size n with a bandwidth w , calculated as follow:

$$w = 0.9 \min\left(\hat{\sigma}, \frac{IQR}{1.349}\right) n^{-1/5}$$

Where $\hat{\sigma}$ and IQR are the standard deviation and interquartile range of Vx , respectively. The second derivative of the kernel Smooth function was used to search for hidden positive peaks, by choosing the lowest peak filtering threshold allowing the fit to converge. Peaks were then fitted with Gaussian functions using the Levenberg-Marquardt algorithm. The peak centers determined the most probable rupture forces for single and multiple interactions. The Bell-Evans model [429] (**Equation 5**) was fitted to most probable rupture forces corresponding to single interactions to determine the dissociation rate constant k_{off} and the distance to the transition state x_{β} . The predicted rupture forces for two ($n = 2$) and three ($n = 3$) simultaneous uncorrelated interactions were determined using Williams-Evans prediction [433] (**Equation 6**).

Confocal microscopy analysis of PRDX5 binding to the cell surface

Prior to imaging, HEK293 MD2-CD14 TLR4 cells were seeded onto polyethylenimine (PEI)-coated glass bottom μ -dishes and left overnight to grow to confluence. 100 μM NTA-FITC (Santa Cruz Biotechnology) were incubated with 500 μM NiCl_2 for 1 h at RT, and 10 μM dsPRDX5 was then added for 1 h. Unbound probe was then removed using a PD SpinTrap G-25 column (Cytiva), by following manufacturer's instructions. Cells were imaged using a Zeiss 980 confocal laser

scanning microscope (Zeiss GmbH, Germany). This microscope is equipped with a 405 diode and Helium-Neon lasers, GaAsP detectors, and additional modifications to allow for long term live cell imaging. These modifications include a chamber that maintains cells at 37°C, with 5% CO₂ in air, and a relative humidity of >95%. Excitation and emission settings were determined using YFP positive cells, prior to the addition of PRDX5-NTA-FITC, to reduce cross-talk between the channels. Excitation for FITC and YFP were at 488 and 514 nm respectively, with the key collection bands being between 495-522 nm for FITC and 565-648 nm, with the channels being collected sequentially.

Flow cytometry binding assay

Cells were first harvested with Accutase solution (Sigma-Aldrich) prior to performing cell count and viability analysis. Cells were resuspended in ice-cold medium and $\sim 5 \times 10^5$ cells·mL⁻¹ were exposed to 1 μ M of the PRDX5 redox forms or to increase concentration of dsPRDX5 in DMEM for 40 min on ice to prevent endocytosis. As negative control, cells were incubated with medium only. Cells were centrifuged at 500 x g for 5 min at 4°C and then incubated with PBS containing 0.2% BSA for 15 min on ice. Cells were resuspended in anti-PRDX5 serum (1/200) for 40 min on ice, before being washed three times with PBS/0.2% BSA. Cells were finally labeled for 40 min on ice with goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 647 Plus (1/250) (ThermoFisher), washed three times, and resuspended in PBS. Samples were acquired on the Guava easyCyte System (Merck) using GuavaSoft software. Gates were set to select living Alexa Fluor 647 Plus-positive cells. Median fluorescence intensity (MFI) of control cells was subtracted from MFI of PRDX5-exposed cells.

Statistical analysis

JMP Pro 15.2.0 software was used to perform the statistical tests. For ELISAs, FC experiments and SMFS BF measurements, a mixed model was used with the conditions (either exposition agents in ELISAs, or PRDX5 redox forms in FC and SMFS) as fixed independent variables and replicates as a random variable defining a random intercept. When the condition was significant (p -value <5%), pairwise comparisons were computed with Tukey HSD test. To fulfill the assumptions of the model, a logarithmic transformation ($\log_{10}$) of the response (IL-8 concentration) was performed in ELISAs. Bell-Evans fitting results were compared with OriginPro 9.8.0.200 software. F-test was used to determine whether pairwise datasets were significantly different from each other. A p -value was then calculated based on the F value.

3.2.4. Results and Discussion

Production and characterization of PRDX5 redox forms

To evaluate the influence of the redox state of human PRDX5 catalytic cysteine residues (Cys) on its DAMP function, we used four different redox forms (**Figure 42**).

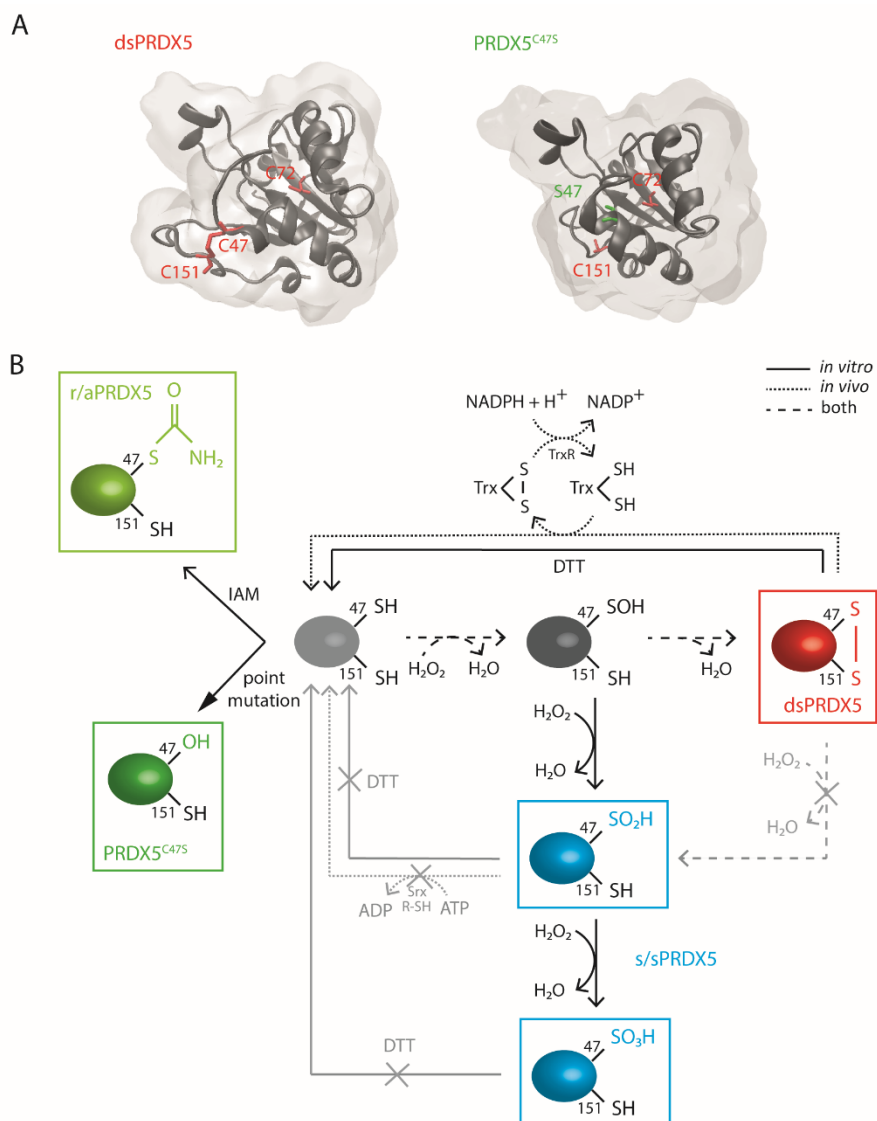


Figure 42. Structural and redox state insights of PRDX5. (A) Crystal structures of recombinant human PRDX5 (upper, PDB ID: 2vl3) and mutated C47S PRDX5 (lower, PDB ID: 1urm), showing that changes in Cys redox state is accompanied by conformational changes. dsPRDX5 contains three Cys (C47, C72, C151), with two of them being catalytically active (C47 and C151). **(B)**

Schematic of the PRDX5 C47 redox modifications⁴. The PRDX5 C47 thiol (-SH) can be oxidized *in vitro* or *in vivo* to an intermediate sulfenic acid (-SOH) by H₂O₂ or other peroxides. The reactive -SOH either engages into an intramolecular disulfide bond with C151 (dsPRDX5, red) or is hyperoxidized *in vitro* to sulfinic acid (-SO₂H) or further to sulfonic acid (-SO₃H) (s/sPRDX5, blue). *In vivo*, the disulfide bridge is reduced by thioredoxin (Trx). Unlike typical 2-Cys PRDXs, the PRDX5 sulfinic form cannot be reduced by the sulfiredoxin system. Both Cys sulfinic and sulfonic acids are therefore irreversible. *In vitro*, DTT can be used to reduce the disulfide form, but not the hyperoxidized forms. To maintain the protein in a reduced state, the thiol can be alkylated by iodoacetamide (IAM) to prevent re-oxidation (r/aPRDX5, light green), or the protein can be point-mutated to replace the C47 by a serine (PRDX5^{C47S}, dark green).

Purified recombinant human 6xHis-tagged PRDX5 produced in *Escherichia coli* (*E. coli*) has a disulfide bridge between the C47 and the C151 [52,126] (dsPRDX5) (**Figure 42A, Figure 42B**). A mutated form of the protein was obtained by replacing the C47 by a serine (S), which maintains the protein in a reduced state by preventing the formation of the C47-C151 disulfide bridge (PRDX5^{C47S}) (**Figure 42A, Figure 42B**). Previous studies showed that the 6xHis tag does not alter the enzymatic activity of the protein, which remains active [2,55,62,129,132,490,491]. To alter PRDX5 redox state without modifying the structure, purified PRDX5 proteins were also chemically treated with dithiothreitol (DTT) to reduce disulfide bonds. Pre-reduced proteins were then exposed either to the alkylating agent iodoacetamide (IAM) to prevent their re-oxidation (r/aPRDX5, **Figure 42B**), or to a high concentration of H₂O₂ to form Cys sulfinic and/or sulfonic acids (s/sPRDX5) (**Figure 42B**). It is important to note that PRDX5 is known to be highly resistant to hyperoxidation compared to typical 2-Cys PRDXs, and that, to our knowledge, hyperoxidized PRDX5 has never been detected *in vivo* [69]. Therefore, the implication of PRDX5 hyperoxidized forms in physiological or pathophysiological mechanisms is very unlikely, however serve here as an important comparison. We pre-treated PRDX5 with DTT before hyperoxidation, as per Yang *et al.* [66], as H₂O₂ alone is not sufficient for disulfide disruption.

Next, we characterized the PRDX5 redox forms using various approaches (**Figure 43**). Western blot analysis (**Figure 43A**) first revealed that dsPRDX5 showed similar bands before and after ultrafiltration, indicating that the ultrafiltration process used to remove the residual agents after the chemical treatments does not alter the proteins. We then confirmed that DTT/H₂O₂ treatment led to at least partial hyperoxidation of PRDX5 Cys by comparing similar blots probed either with an antiserum raised against recombinant human PRDX5 or an antibody highly specific for the sulfonated PRDX5 form [69].

⁴ Redox modifications were only shown on the C47 for more clarity. Note however that they can occur on all Cys residues.

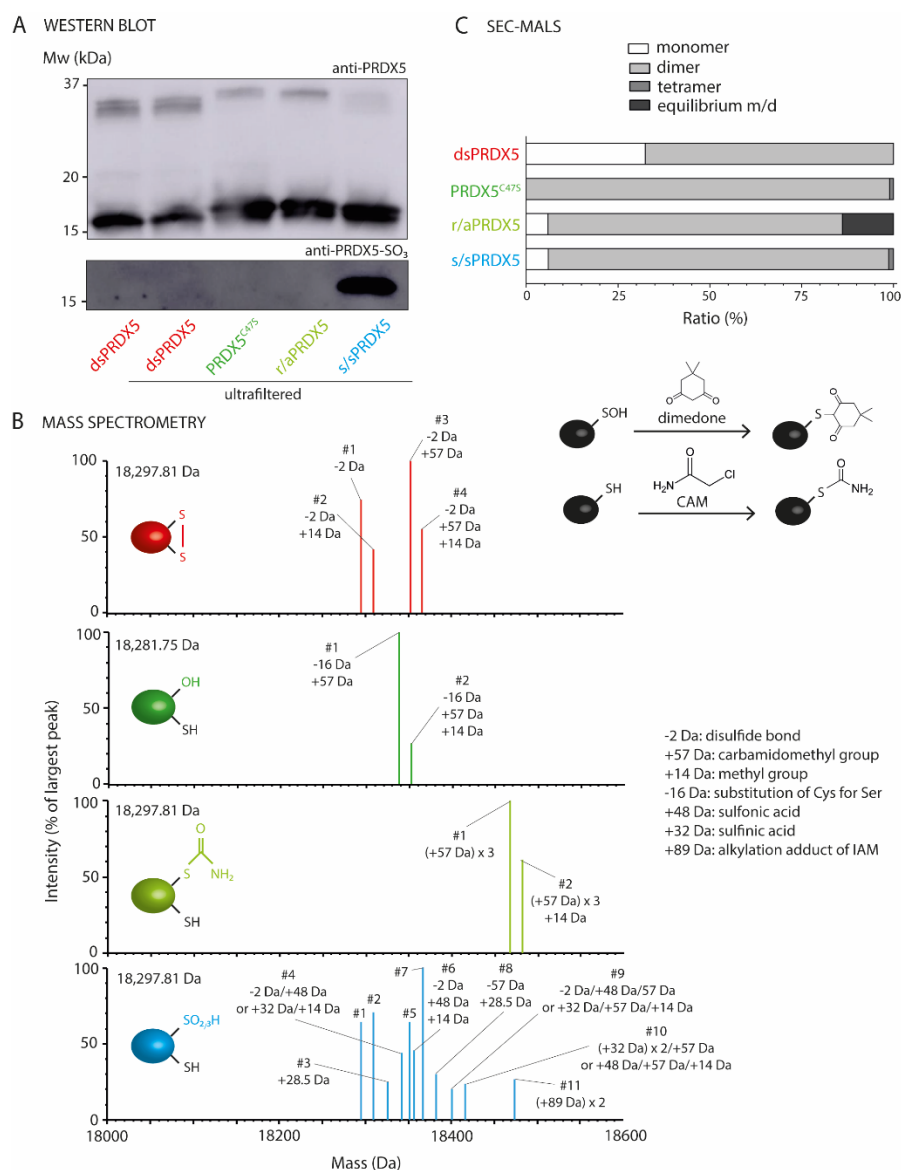


Figure 43. Characterization of the redox and oligomeric statuses of the PRDX5 redox forms. **(A)** Western blot analysis of PRDX5 redox forms performed under non-reducing conditions. Western blotting against PRDX5 reveals the presence of monomers and dimers (upper) and presence of sulfonic acids was confirmed by Western blotting against PRDX5-SO₃ and was only detected in s/sPRDX5 sample. dsPRDX5 was probed before and after ultrafiltration. Representative of three independent experiments. **(B)** Centroid spectrum showing monomeric species of the different redox forms (dimeric species are shown in Figure S7), generated after MaxEnt1 deconvolution of the multiply-charged spectrum obtained by direct infusion ESI-Q-TOF MS analysis on intact proteins. An intensity threshold of 20% normalized to the largest peak was applied to display the major peaks. dsPRDX5, r/aPRDX5, and s/sPRDX5 contains three Cys, while PRDX5^{C47S} has two remaining Cys. Prior to MS analysis, potent Cys

sulfenic acids were trapped with dimedone, and remaining Cys thiols were blocked with 2-chloroacetamide (CAM). Identification of each peak is presented in **Table S2**. **(C)** Quantification of the monomeric and oligomeric states using SEC-MALS. Source data are provided in **Figure S6** (blots), in **Figure S7** (MS multiply-charged spectra), and in **Figure S9** (SEC-MALS spectra).

We further characterized the redox state of the PRDX5 redox forms using direct infusion electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) on intact proteins (**Figure 43B**). After redox treatments and prior to MS analysis, potent Cys sulfenic acids were trapped with dimedone, and thiols were subsequently blocked by alkylation with 2-chloroacetamide (CAM). Raw multiply-charged electrospray spectra were processed with the maximum entropy method [492–494] to produce true molecular mass spectra (**Figure S7**).

Based on the amino acid sequence of dsPRDX5 and PRDX5^{C47S}, the calculated mass of the proteins with reduced Cys thiols are 18,297.81 and 18,281.75 Da, respectively. The deconvoluted mass spectra revealed major monomeric and minor dimeric species for each redox form (**Figure S7**). The centroid spectrum of dsPRDX5 shows four major peaks related to the monomeric species (**Figure 43B**, **Figure S7**), of which mass and identification are presented in **Table S2**. Peaks #1 and #3 correspond to the protein with a disulfide bond (mass shift of -2 Da), without or with an additional carbamidomethyl group (mass shift of +57 Da), respectively. The two secondary peaks (#2 and #4) indicate a mass increase of 14 Da compared to the parent peaks, and are most likely due to a N-terminal methylation [495]. This phenomenon has been observed by other researchers who performed similar intact-protein ESI-MS analysis on the recombinant N-terminal 6xHis-tagged Z domain of protein A [496]. The deconvoluted mass spectrum of PRDX5^{C47S} reveals two major peaks for the monomeric species (**Figure 43B**, **Table S2**, **Figure S7**). The parent peak (#1) corresponds to the mutant protein with a serine residue (Ser) replacing the Cys and a carbamidomethyl group, and the secondary peak (#2) indicates an additional mass of 14 Da, as for dsPRDX5. In the case of r/aPRDX5, the two major peaks detected correspond to the protein with three carbamidomethyl groups, either with or without a 14-Da increase in mass (**Figure 43B**, **Table S2**, **Figure S7**). As both IAM and CAM modifications result in the covalent addition of a carbamidomethyl group (mass shift of +57 Da), we performed the analysis without CAM alkylation in order to determine which adducts were formed after IAM redox treatment (insets in **Figure S7**). The analysis performed without CAM reveals identical peaks with a mass shift of -57 Da each, indicating that alkylating the protein with IAM at pH 7.2 more likely forms thioethers on the two more reactive Cys, *i.e.* the ones involved in the disulfide bond. Lastly, the major peaks resolved on the deconvoluted mass spectrum of s/sPRDX5 (peaks #1, #2, #5, and #7) are identical to those of dsPRDX5 (**Figure 43B**, **Table S2**, **Figure S7**). However, several other peaks (#3, #4, #6, #8, #9, #10, and #11)

were detected and indicate that hyperoxidizing the DTT-reduced protein with H₂O₂ results in a mix of species composed of Cys sulfinic acid (-SO₂H), sulfonic acids (-SO₃H), and carbamidomethyl groups. Summing the peak intensities corresponding to these species gives a proportion of ~42%, but the disulfide bond remains in many of these species, which is in agreement with the fact that PRDX5 is resistant to hyperoxidation by H₂O₂ [69]. The identification of these peaks is presented in **Table S3**. No sulfenic intermediate were identified (mass shift of +138 Da). Moreover, analysis of the fragments corresponding to peptides containing C47 using LC-Q-TOF-MS/MS revealed the presence of the redox modifications (disulfide bridge, carbamidomethyl group, sulfinic acid, sulfonic acid) on the Cys (**Figure S8**). Overall, these results demonstrate that dsPRDX5, PRDX5^{C47S}, and r/aPRDX5 are largely homogenous redox forms and confirm the efficiency of the reducing/alkylating treatment. However, s/sPRDX5 is only partially hyperoxidized, with a proportion of species having a disulfide bridge.

While the oligomeric conformation of 2-Cys PRDX subfamily directly links redox state to function [4,87], atypical 2-Cys PRDX5 is thought to exist as a dimer irrespective of the redox state of the cysteine residues [52,90]. Western blot performed under non-reducing conditions revealed the presence of monomers, but also of dimers (**Figure 43A**), and to a lesser extent, of oligomers (**Figure S6**). Although these results are consistent with previous studies [129,480], whether covalent dimers (and oligomers) linked by intramolecular disulfide bridges are physiologically relevant remains to be determined [90]. We further refined the oligomeric state picture of PRDX5 for all redox states in native conditions using size-exclusion chromatography – multiangle light scattering (SEC-MALS) (**Figure 43C**, see spectra in **Figure S9**). The main quaternary structure detected for all conditions was the dimeric form. In dsPRDX5, however, a relatively high fraction (~30%) of monomers could also be identified, and r/aPRDX5 shows ~15% of proteins in a monomer-dimer equilibrium. This is consistent with previous results showing that there is a rapid equilibrium between a major dimer and a minor monomer species [480]. A very small fraction of tetramers was observed for PRDX5^{C47S} and s/sPRDX5. Although the covalent or non-covalent nature of such dimers and oligomers cannot be determined in SEC-MALS, MS analysis of intact proteins revealed the presence of non-covalent dimers for PRDX5^{C47S} and r/aPRDX5 (**Figure S7**, **Table S3**). This suggests that such non-covalent dimers are also present in dsPRDX5 and s/sPRDX5. Overall, these data indicate that the redox state of PRDX5 does not alter its oligomeric conformation, facilitating further analysis and interpretation.

The PRDX5 disulfide bond allows a stronger cytokine-stimulating activity

Our previous study showed that dsPRDX5 was able to stimulate IL-1 β secretion from PMA-differentiated THP-1 cells [487]. To demonstrate the direct role of TLR4 in

PRDX5-triggered cytokine secretion, we examined the IL-8 production from HEK293 MD2-CD14 transfected with TLR4 cells using ELISA (**Figure 44**). We first used FC to validate the stable transfection and to confirm that the receptor was expressed at the cell surface (**Figure 44A**). We also established that the receptor was functional by exposing the cells to LPS, which requires both MD-2 and CD14 to trigger cytokine secretion [298,497,498] (**Figure 44C**). LPS exposure resulted in a secretion of $14,085 \pm 2401 \text{ pg} \cdot \text{mL}^{-1}$ (mean $\pm$ SD_{pooled}, $N = 3$).

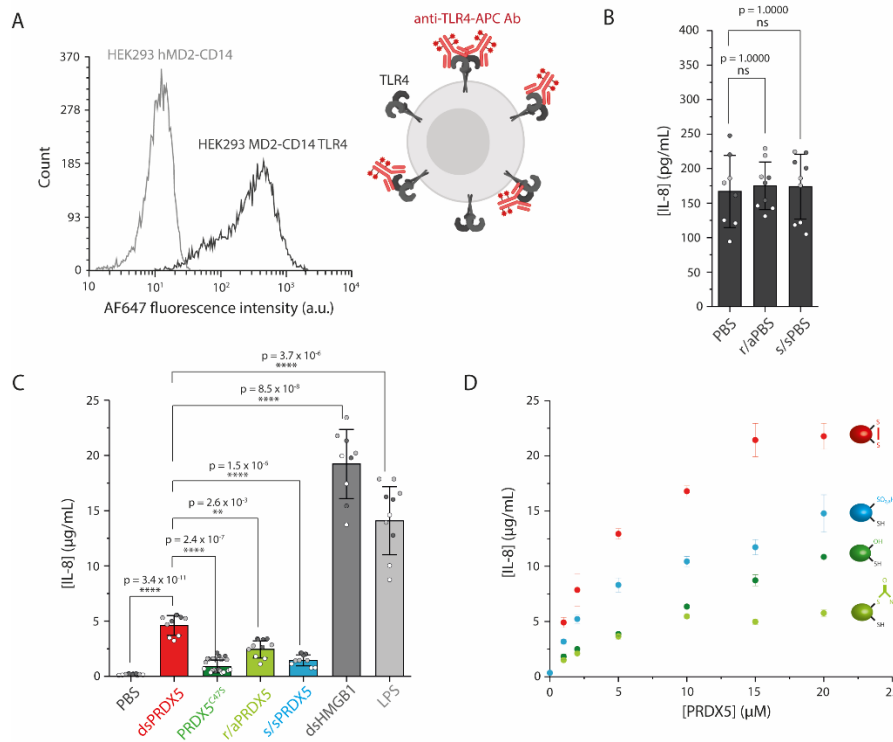


Figure 44. Disulfide PRDX5 triggers potent IL-8 secretion. (A) Stable transfection of TLR4 in HEK293 MD2-CD14 cells was validated by flow cytometry (FC). Schematic depicting the labeling strategy used to validate the correct TLR4 expression at the cell surface. (B) Control experiment showing IL-8 production levels from HEK293 MD2-CD14 TLR4 cells treated with ultra-filtered PBS, “reduced/alkylated” PBS (r/aPBS) or “hyperoxidized” PBS (s/sPBS), quantified by ELISA. Three independent experiments (white, light grey and dark grey points) with three replicates per condition for each experiment. (C) IL-8 secretion from HEK293 MD2-CD14 TLR4 cells induced after stimulation with the PRDX5 redox forms, quantified by ELISA. LPS was used as a control to validate that TLR4 was functional, and HMGB1 was used as a DAMP control. Three independent experiments (white, light grey and dark grey points) with three replicates per condition for each experiment and two independent PRDX5^{C47S} protein productions. (D) Concentration-dependence of IL-8 secretion from HEK293 MD2-CD14 TLR4 cells for the redox forms. Data are representative of three replicates. Data are mean $\pm$ S.D. ns, not significant ($p > 0.05$). ** $p \leq 0.01$, *** $p \leq 0.0001$ (Tukey HSD test).

We then verified that the residual DTT, IAM and H₂O₂ remaining after protein redox treatments had been efficiently removed by ultrafiltration and thus would not impact cell viability or interfere with IL-8 secretion. To do so, we treated PBS with the same reducing and hyperoxidizing agents that we used for dsPRDX5, and we ultra-filtrated the samples. As shown in **Figure 44B**, exposing cells to r/aPBS or s/sPBS resulted in similar IL-8 secretion levels (175 ± 17 and 174 ± 17 pg·mL⁻¹, respectively, mean $\pm$ SD_{pooled}, *N* = 3) as with PBS (187 ± 61 pg·mL⁻¹, mean $\pm$ SD_{pooled}, *N* = 3), confirming that the further results would not be on account of residual treatment agents.

We next assessed the IL-8 production from HEK293 MD2-CD14 TLR4 cells mediated by exposure to the PRDX5 redox forms (**Figure 44C**). We observed that dsPRDX5 yielded higher IL-8 stimulating activity with 4608 ± 288 pg·mL⁻¹ (mean $\pm$ SD_{pooled}, *N* = 3) compared to the other redox forms. The C47S point mutation led to significantly reduced IL-8 secretion level (893 ± 142 pg·mL⁻¹, mean $\pm$ SD_{pooled}, *N* = 3). The same tendency could be observed with the chemically reduced/alkylated protein, albeit less prominent (2437 ± 343 pg·mL⁻¹, mean $\pm$ SD_{pooled}, *N* = 3). Similarly, hyperoxidizing the protein *in vitro* with H₂O₂ resulted in a significant decrease in IL-8 secretion (1447 ± 136 pg·mL⁻¹, mean $\pm$ SD_{pooled}, *N* = 3) compared to dsPRDX5. As control, we used another DAMP protein that can signal through TLR4, namely high-mobility group box 1 (HMGB1) [499], which led to an IL-8 concentration of $19,227 \pm 2695$ pg·mL⁻¹ (mean $\pm$ SD_{pooled}, *N* = 3). We also demonstrated that the magnitude of IL-8 secretion was dependent on the concentration of the redox forms (**Figure 44D**).

Our results highlight the importance of the C47-C151 disulfide bond in PRDX5-mediated cytokine production through TLR4, and is in line with other studies showing that the activity of HMGB1 strongly depends on the redox state of its three Cys [500,501]. Nevertheless, our results are in contradiction with a previous study showing that the disruption of PRDX5 Cys by point mutations or IAM treatment did not affect the IL-23p19 mRNA expression level in bone marrow-derived dendritic (BMDC) cells [116]. However, their conditions differ from ours: (i) PRDX5 was reduced with only 1 mM DTT, while several studies use 10 mM [69,132]; (ii) they investigated the mRNA level, although they showed for PRDX1 and PRDX2 that IL-23p19 mRNA expression level does not correlate with IL-23 expression; (iii) BMDC cells were exposed for 1 h, while we treated our cells for 16 h, (iv) BMDC cells have a wider TLR expression profile [502], making it difficult to interpret the contribution of TLR4. The effect of the Cys redox state has also been studied for PRDXs from the typical 2-Cys subfamily, with conflicting results. While Riddell *et al.* showed that human PRDX1 interaction with TLR4 was independent of its peroxidase activity [344], López *et al.* recently demonstrated that pro-inflammatory properties of the cytosolic

tryparedoxin peroxidase from *Trypanosoma cruzi* depend on the presence of peroxidatic cysteine [503].

Disulfide, mutant and reduced PRDX5 bind to TLR4 with similar kinetic off-rates

We previously demonstrated with AFM-based SMFS that dsPRDX5 was able to specifically interact with TLR4 [487]. To determine whether the difference in the cytokine-inducing capability of the PRDX5 redox forms could originate from differential binding properties to TLR4, we used AFM-based SMFS to probe the interaction between the PRDX5 redox forms and TLR4 (**Figure 45A**). To this end, we covalently grafted the PRDX5 redox forms to AFM tips by means of a flexible polyethylene glycol (PEG₂₄) linker, and we covalently attached TLR4 molecules to a mixed self-assembled monolayer of carboxyl- and hydroxyl-terminated alkanethiols immobilized onto an inert gold surface using *N*-hydroxysuccinimide (NHS)/*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) chemistry. We then used force-distance (FD) curve-based AFM to investigate the binding kinetics of the PRDX5 redox forms (**Figure 45B; Figure S10A**). We recorded multiple maps (30 for dsPRDX5, PRDX5^{C47S} and r/aPRDX5; 35 for s/sPRDX5) at different tip retraction speeds (100 nm·s⁻¹, 200 nm·s⁻¹, 500 nm·s⁻¹, 1 μm·s⁻¹, 2 μm·s⁻¹, and 10 μm·s⁻¹).

We first assessed the binding frequency (BF) by examining the FD curves showing specific adhesive events between the tip-bound PRDX5 and surface-immobilized TLR4 (*i.e.* whose extension patterns could be fitted with the worm-like chain model) (**Figure 45C, Figure S10B**). The BF is given by the ratio between the number of specific events and that of total recorded events [435]. There was no significant difference between the different redox forms, except for the r/aPRDX5 that showed a higher BF.

To investigate the binding kinetics of PRDX5-TLR4 interactions for the different redox forms, we performed a dynamic force spectroscopy (DFS) analysis. For each specific adhesive curve, we extracted the rupture force (F) and the loading rate (LR), which corresponds to the force applied over time (**Figure 45B**). We divided the spectrum of LRs probed into discrete ranges (LR#1-6) as performed in previous studies [440,487,504], and for each of them, we used a kernel distribution of the rupture forces to define the most probable forces for single and multiples interactions (**Figure S11**). The second derivative of the density function allowed finding hidden peaks, which were fitted with Gaussian functions (**Figure 45D, Figure S11**). The resulting Gaussian function overlapped very well the kernel distribution.

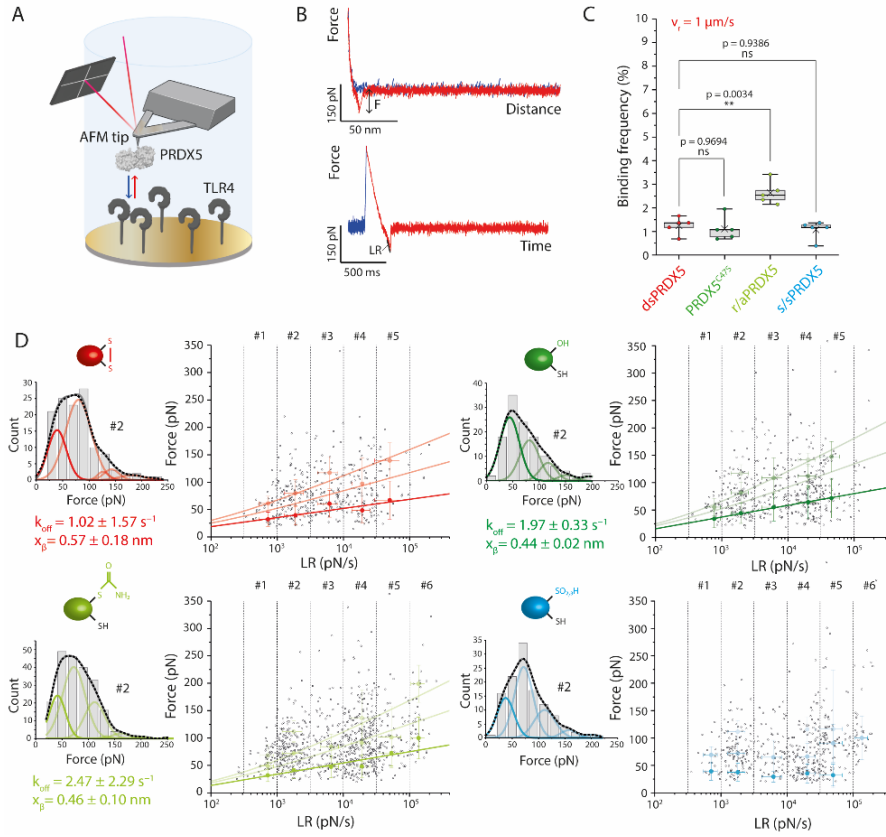


Figure 45. PRDX5 redox forms bind surface-immobilized TLR4 with similar k_{off} . (A) Cartoon showing the experimental set-up used to probe the interaction between the PRDX5 redox forms and TLR4. (B) Representative specific force-distance (FD) and force-time curves. Examples for all redox forms are shown in Figure S10. Rupture force (F) corresponds to the height of the adhesive peak on the FD curve, and the loading rate (LR) is calculated by fitting the adhesive peak on the force-time curve just before rupture with a linear model. (C) Box plot showing the binding frequencies (BF) obtained at a tip retraction speed of $1 \mu\text{m}\cdot\text{s}^{-1}$. Number of AFM maps, $n = 5$ for each redox form (dots). Box plot shows mean ($\times$), median ($-$), 25th and 75th percentiles (boxes), and ranges from min to max (whiskers). ns, not significant ($p > 0.05$). $**p \leq 0.01$ (Tukey HSD test). (D) Dynamic force spectroscopy (DFS) analysis for the PRDX5 redox forms. Spectrum of LR s was divided into discrete ranges ($LR\#1-6$). For each LR range, a kernel distribution of the rupture forces (grey line) was generated, as shown on the histogram for $LR\#2$, which allows to determine hidden peaks based on the second derivative. Peaks were fitted with Gaussian functions (colored lines), revealing the most probable rupture forces for single (1st peak) and multiple (following peaks) interactions. These forces were reported on the DFS plot (full colored data points). The Bell-Evans model was fitted to data corresponding to single interactions (solid line), and the predicted rupture forces for two and three simultaneous uncorrelated interactions were calculated using Williams-Evans prediction (dashed lines). From the Bell-Evans model, the k_{off} and x_β values were extracted. The most probable rupture forces (full colored data points) are peak center $\pm$ S.D.

With the exception of s/sPRDX5, all the DFS plots showed a linear dependency of the most probable rupture forces with the LR for single interactions, which is frequently observed for ligand-receptor interactions and is explained by a single free-energy barrier being crossed during the mechanical pulling [505]. The Bell-Evans model [429] was used to fit to the most probable forces measured for the single interaction at the different LR ranges. From the fit (**Figure 45D**, dark line), we extracted the dissociation rate constant (k_{off}), which corresponds to the inverse of the interaction lifetime, as well as the distance to the transition state (x_β), which provides useful information related to the chemistry of interaction. The k_{off} values obtained were $1.02 \pm 1.57 \text{ s}^{-1}$, $1.97 \pm 0.33 \text{ s}^{-1}$, and $2.47 \pm 2.29 \text{ s}^{-1}$ for dsPRDX5, PRDX5^{C47S}, and r/aPRDX5, respectively. Regarding s/sPRDX5, the DFS plot reveals no increase in the binding force with the LR, suggesting an unspecific interaction. A similar x_β was observed for dsPRDX5 ($0.57 \pm 0.18 \text{ nm}$), PRDX5^{C47S} ($0.44 \pm 0.02 \text{ nm}$) et r/aPRDX5 ($0.46 \pm 0.10 \text{ nm}$), indicating a similar binding conformation. Using these parameters, we applied the Williams-Evans prediction [433] to infer the interaction forces of multiple uncorrelated interactions breaking simultaneously (**Figure 45D**, light lines). These predictions are in good agreement with the mean rupture forces observed for double and triple interactions. By performing a F-test on the Bell-Evans fittings, we could determine that dsPRDX5 and PRDX5^{C47S}, as well as dsPRDX5 and r/aPRDX5, were not statistically distinct, with 0.05 significance level (p -value of 0.50492 and 0.63374, respectively). This can be visualized by superimposing the DFS plots of the different redox forms, as shown in **Figure S12**. The residual dsPRDX5 present in the hyperoxidized sample can explain the higher rupture forces observed, which probably result from the interaction between dsPRDX5 and TLR4. Overall, these results indicate that the differences observed in the IL-8-stimulating activity of the different redox forms cannot be attributable to differential interaction lifetime with TLR4. Further work is needed to investigate the associate rate constant (k_{on}) in order to determine whether TLR4 alone is sufficient to discriminate between the redox forms.

PRDX5^{C47S} shows a lower propensity to bind to the cell surface

We consequently turned to living HEK293 cells expressing TLR4 to investigate the binding of PRDX5 molecules in a cellular context. We first performed a colocalization experiment to visually confirm that dsPRDX5 binding to TLR4 is responsible for the inflammatory response (**Figure 46A**). To this end, we incubated HEK293 MD2-CD14 TLR4 cells with PRDX5 labeled with NTA-FITC using Ni^{2+} (PRDX5-NTA-FITC). Shortly after the addition of PRDX5-NTA-FITC, a membrane staining motif began to emerge, with discrete pockets of FITC fluorescence present at the cell membrane. Of particular note, fluorescence signals present in filopodia and focal adhesions were commonly co-localized. This observation was subsequently followed by FITC positive

vesicles gathering at the cell periphery and trafficking within the cell interior. Together these staining behaviors indicate binding and internalization. To ensure that the fluorescence observed was not merely cross-talk, λ -scans were performed to assess whether the fluorescent species present included the earlier emission peak from the FITC dye and fluorescence intensities across the membrane examined using a simple Line of Response (**Figure S13**).

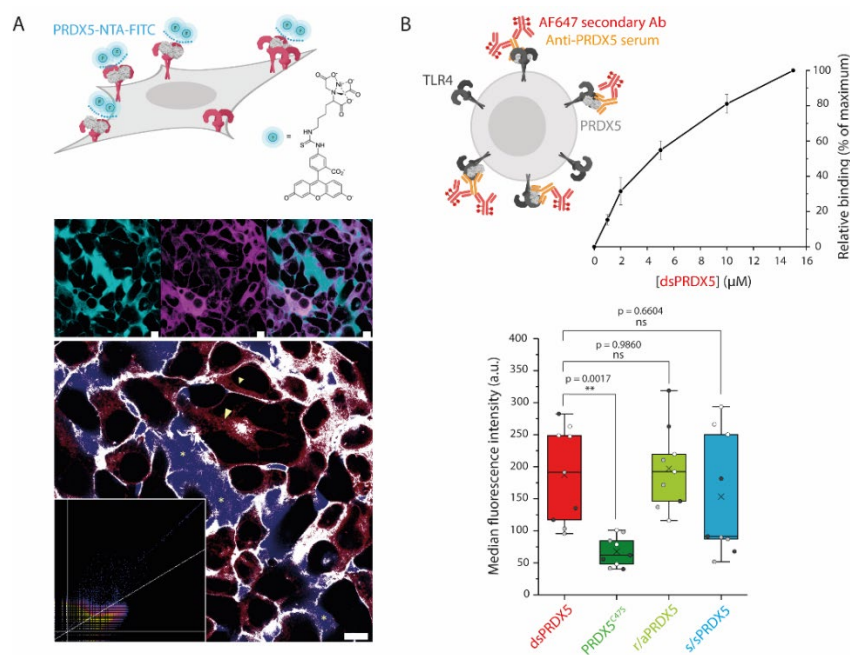


Figure 46. Presence of C47 is required for PRDX5 binding to the cell surface. Binding of PRDX5 to the surface of HEK293 MD2-CD14 TLR4 cells monitored by fluorescence. **(A)** Cartoon (upper) showing the protocol used to detect PRDX5 binding to TLR4 using confocal. Confocal images of, left to right, PRDX5-NTA-FITC (cyan), TLR4-YFP (magenta), fluorescence overlay. Co-localization (white) of FITC (blue) and YFP (red) signals predominantly at the plasma membrane and in the endomembranous region. There is a lack of co-localization in the extracellular medium (*) and in YFP exclusive regions within cells (arrowhead). Co-localization map and scatterplot (inset). The cross-talk is represented by unique perfectly co-localized pixels. Scale bars are 10 μ m. **(B)** Cartoon (upper) showing the protocol used to detect PRDX5 binding to living cells using flow cytometry (FC). Graph from FC binding assay showing the concentration dependence of dsPRDX5 binding to the surface of HEK293 MD2-CD14 TLR4 cells. Binding was normalized to the signal of untreated cells and is expressed relatively (in %) to the fluorescence intensity observed with 15 μ M PRDX5. Three independent experiments with two replicates in each. Box blot (lower) from FC binding assay showing the median fluorescence intensity of PRDX5-treated cells. The fluorescence intensity was normalized to the untreated cells. Seven experiments with three independent protein treatments (white, light grey and dark grey points) or two protein productions in the case of PRDX5^{C47S}. Three replicates for each redox form in each treatment. Box plot shows mean (x), median (—), 25th and 75th percentiles (boxes), and ranges from min to max (whiskers). ns, not significant ($p > 0.05$). ** $p \leq 0.01$ (Tukey HSD test).

We further investigated the binding level of redox PRDX5 isoforms to the surface of HEK293 MD2-CD14 TLR4 cells using a FC binding assay. To prevent endocytosis, cells were incubated with PRDX5 on ice, and labelled with an antiserum against PRDX5. As a control, we also labelled untreated cells. We first showed that dsPRDX5 binding to the surface of cells expressing TLR4 was concentration-dependent (**Figure 46B**). These results confirm the role of TLR4 in the pro-inflammatory activity of PRDX5, and demonstrate that LPS is not responsible for the concentration-dependent increase in IL-8 secretion following exposure to PRDX5 (**Figure 44D**). At identical concentration, PRDX5^{C47S} shows a ~3-fold decrease in binding propensity compared to dsPRDX5 (**Figure 46B**). However, this result was not observed for r/aPRDX5 and s/sPRDX5. This could be explained by the fact that r/aPRDX5 and s/sPRDX5 might be able to bind other cell surface receptors that are not related to a pro-inflammatory process resulting in IL-8 secretion. This phenomenon is observed with HMGB1 which binds other receptors than TLRs, including CXCR4, RAGE, and TIM-3 [506,507]. As the difference observed between dsPRDX5 and the mutant protein was not observed with SMFS, this result could indicate either that TLR4 conformation (*i.e.* dimer) and/or stabilization in the plasma membrane is important for the binding, or that the TLR4 accessory molecules MD-2 and/or CD14 might allow discriminating between redox forms. Further investigation is needed to understand the role of TLR4 accessory molecules in this context.

3.2.5. Conclusion

Human PRDX5 has been shown to enhance brain ischemia-reperfusion injury through activation of TLR2- and TLR4-mediated inflammation. Although our previous findings reported that PRDX5 was able to directly interact with TLR4, the role of the redox state of PRDX5 cysteine residues remained to be investigated. Indeed, PRDX5 is a redox-sensitive protein that act as a peroxidase inside the cell. Here, we investigated the impact of the PRDX5 redox state on its TLR4-dependent pro-inflammatory activity by modifying the redox state of PRDX5 cysteine residues, either by point-mutation or by chemical treatments. Using molecular biology and biophysics approaches, we provide evidence that the disulfide bridge between the two PRDX5 enzymatic cysteines (C47 and C151) is required to allow a strong TLR4-dependent IL-8 secretion. Although the interaction lifetime between TLR4 and oxidized or reduced PRDX5 is not different, binding assays performed on living cells expressing TLR4 demonstrated that disulfide PRDX5 has a higher propensity to bind the cell surface than the mutant protein lacking the peroxidatic cysteine. Taken together, these results shed new light on the importance of the protein redox biology in inflammation, which is of crucial importance for the development of therapeutic treatments to administer after a stroke.

3.2.6. Supplementary information

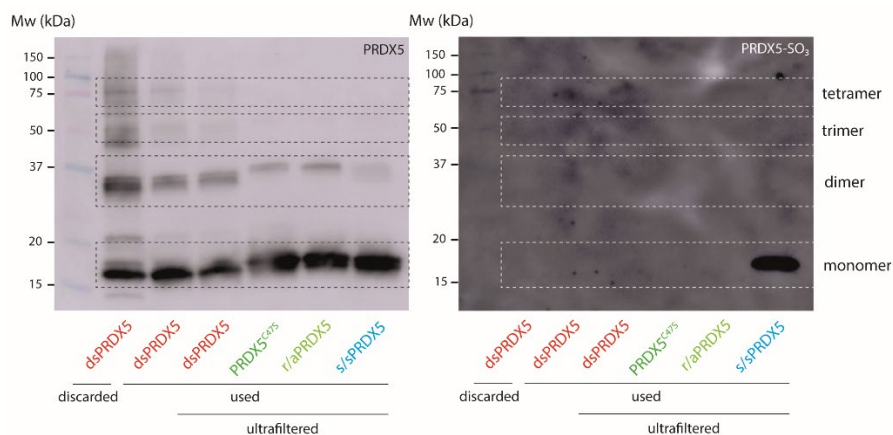


Figure S6. Western blot analysis of the PRDX5 redox forms. Western blot was performed under non-reducing conditions against PRDX5 (left) or against PRDX5-SO₃ (right). Monomers, dimers, and to a lesser extent, oligomers are present within the samples. dsPRDX5 probed before and after ultrafiltration indicates that the protein is not altered by the ultrafiltration process used to remove residual chemical agents. PRDX5 is at least partially hyperoxidized following DTT/H₂O₂ treatment (s/sPRDX5). Protein samples that were stored for >1 week at 4°C were not used in the experiments (discarded). Representative of three independent experiments.

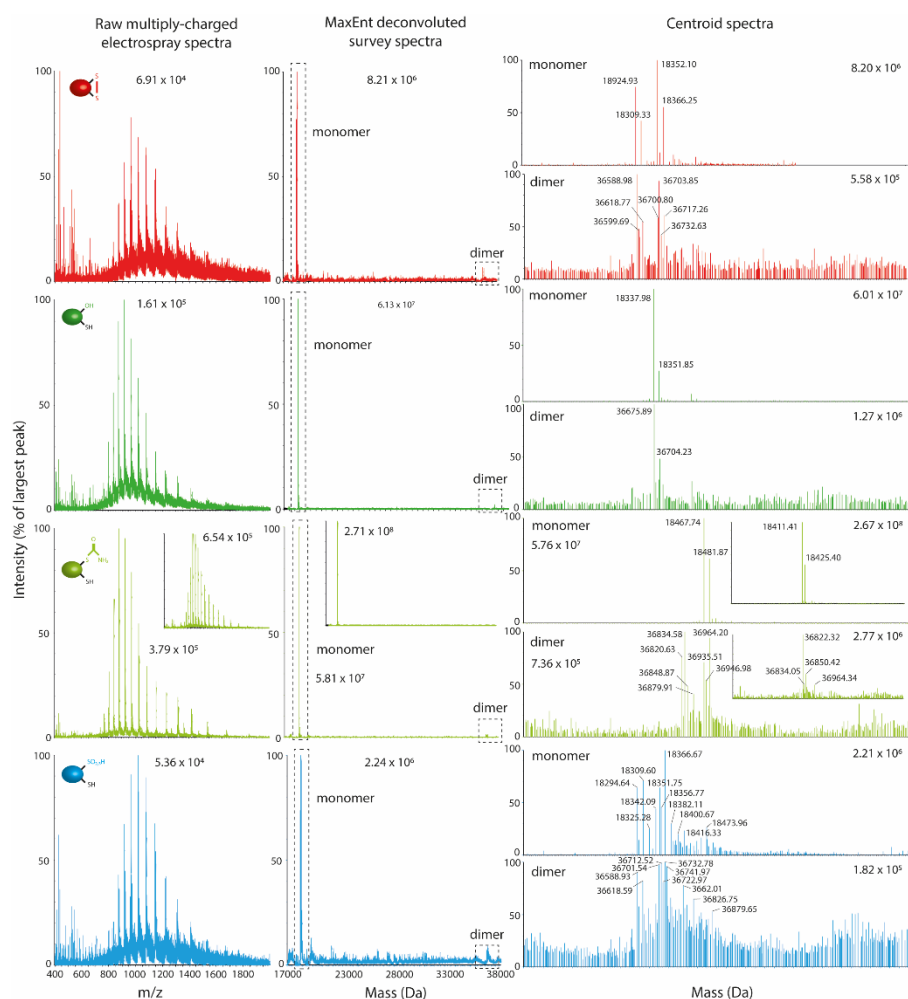


Figure S7. Identification of the monomeric and dimeric redox species in samples of the PRDX5 redox forms using direct-infusion ESI-Q-TOF analysis. Raw multiply-charged electrospray spectra (m/z 400-2000, left) were processed with MaxEnt1 to produce true molecular mass spectra (middle), from which were generated centroid spectra (right). Ranges of centroid spectra are 18,000-18,600 Da for peaks related to monomeric species (right, upper), and 36,200-37,200 Da for peaks related to dimeric species (right, lower). Potent Cys sulfenic acids were trapped with dimedone and remaining thiols were blocked with 2-chloroacetamide (CAM). Identification of Cys alkylated with iodoacetamide (IAM) in r/aPRDX5 was performed without using CAM prior to MS analysis (insets). Intensities and masses of the major peaks ($\geq 20\%$ and $\geq 40\%$ in intensity relative to the largest peak of monomeric or dimeric species, respectively) are detailed in **Table S1** (monomeric species) and **Table S2** (dimeric species).

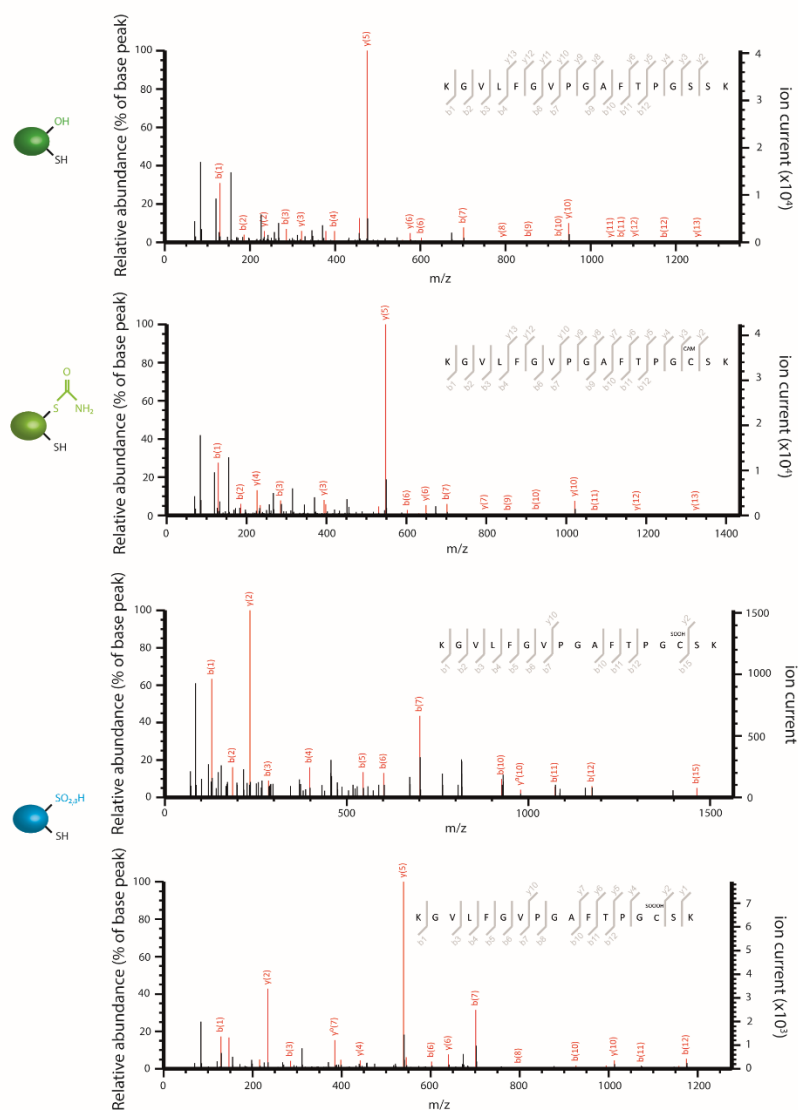


Figure S8. Representative spectra of fragments corresponding to peptides containing the 47th residue. Spectra showing the point mutation (C47S) in PRDX5^{C47S}, or the Cys redox modifications (*i.e.* carbamidomethyl group in r/aPRDX5, or sulfinic acid and sulfonic acid in s/sPRDX5). Representative of three independent protein treatments. See **Supplementary Materials and Methods**.

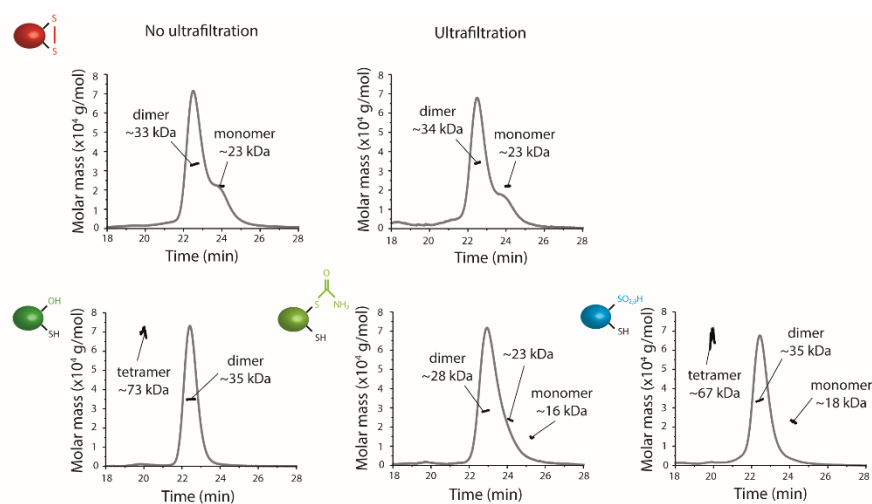
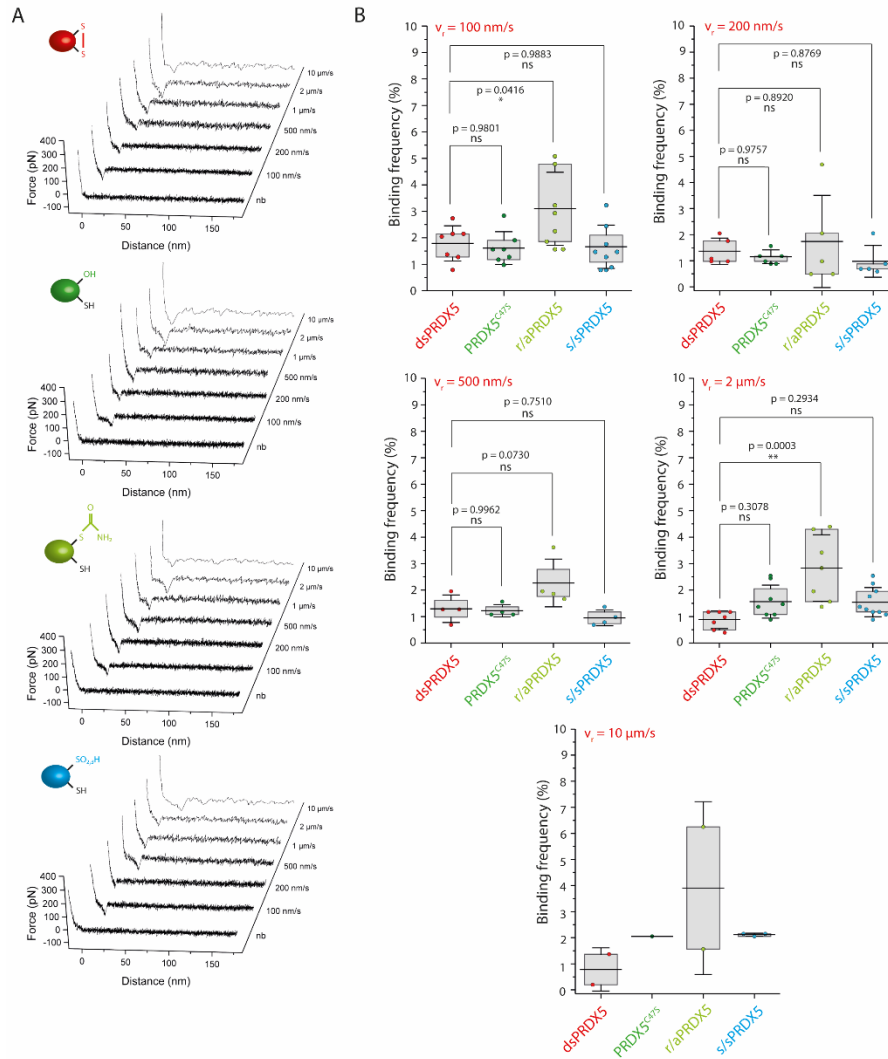


Figure S9. Analysis of the oligomeric status of the PRDX5 redox forms with SEC-MALS. Light scattering spectrum (grey line) of dsPRDX5 before (upper left) and after (upper right) ultrafiltration, and PRDX5^{C47S} lower (left), r/aPRDX5 (lower middle), and s/sPRDX5 (lower right) allowing molar mass determination (black line) of the monomers and oligomers.



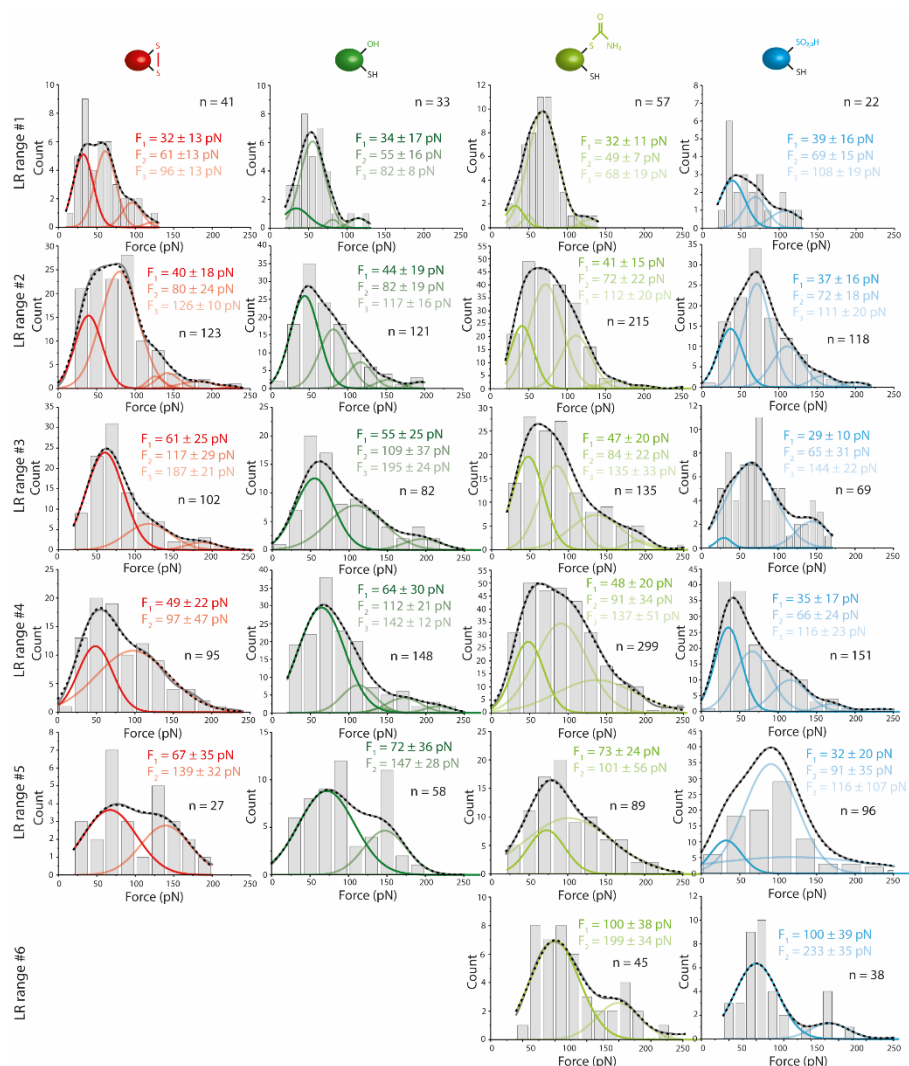


Figure S11. Kernel density function of the rupture forces obtained at the different loading rate (LR) ranges for the PRDX5 redox forms. Rupture forces were divided into discrete LR ranges (LR#1: 315-1000 pN·s⁻¹; LR#2: 1000-3150 pN·s⁻¹; LR#3: 3150-10,000 pN·s⁻¹; LR#4: 10,000-31,500 pN·s⁻¹; LR#5: 31,500-100,000 pN·s⁻¹; LR#6: 100,000-315,000 pN·s⁻¹). Kernel density function (grey line) of the rupture forces (which is superimposed on force histograms for more clarity) was then used to determine the hidden peaks based on the second derivative. The peak centers determined with the kernel density function were then used to fit a Gaussian function (colored lines), from which the most probable rupture forces for single (1st peak) and multiple interactions (following peaks) were determined by taking peak centers (F₁, F₂, F₃ for single, double and triple interactions, respectively). The cumulative peak fit (black dashed line) overlap very well with the kernel density function. n, number of data points.

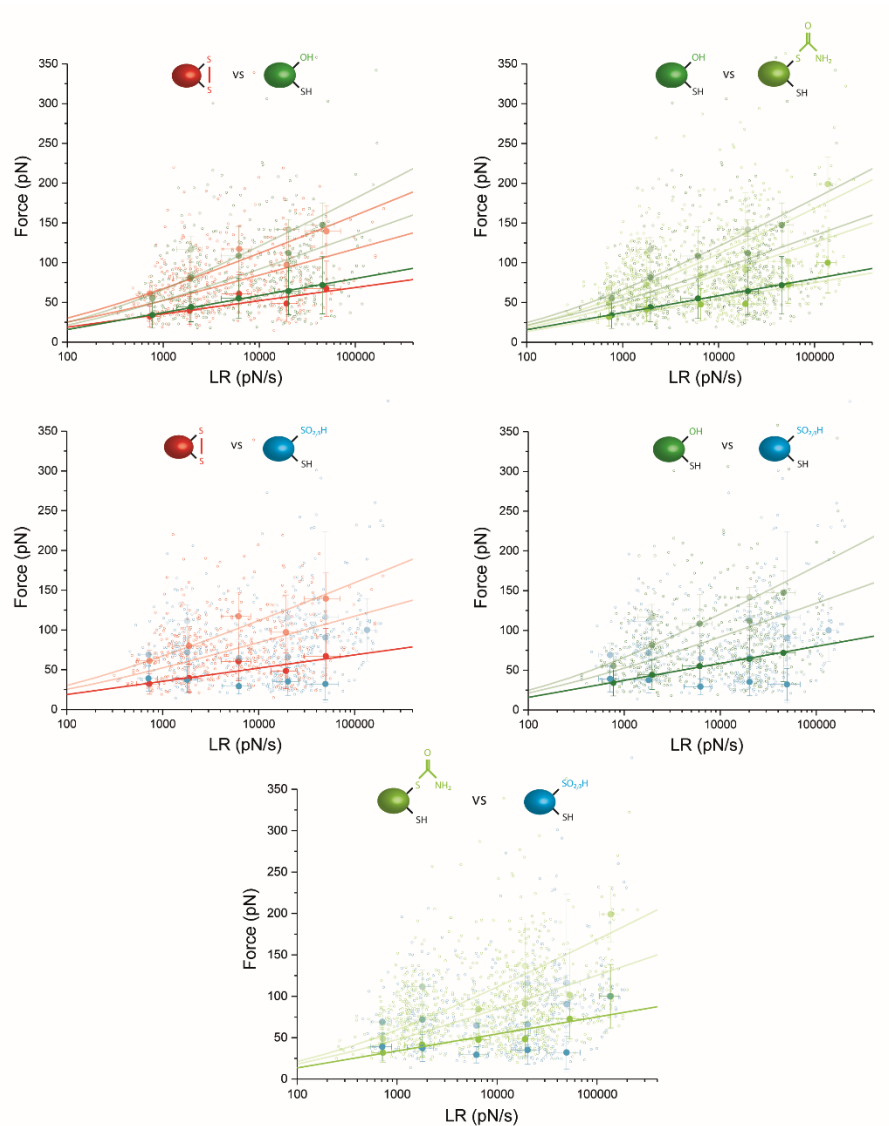


Figure S12. Superimposition of dynamic force spectroscopy (DFS) plots. PRDX^{C47S} DFS plot was superimposed with dsPRDX5, r/aPRDX5, and s/sPRDX5 DFS plots. s/sPRDX5 DFS plot was superimposed with dsPRDX5, and r/aPRDX5 DFS plots. The mean rupture forces obtained for dsPRDX5, PRDX^{C47S}, and r/aPRDX5 are not significantly different.

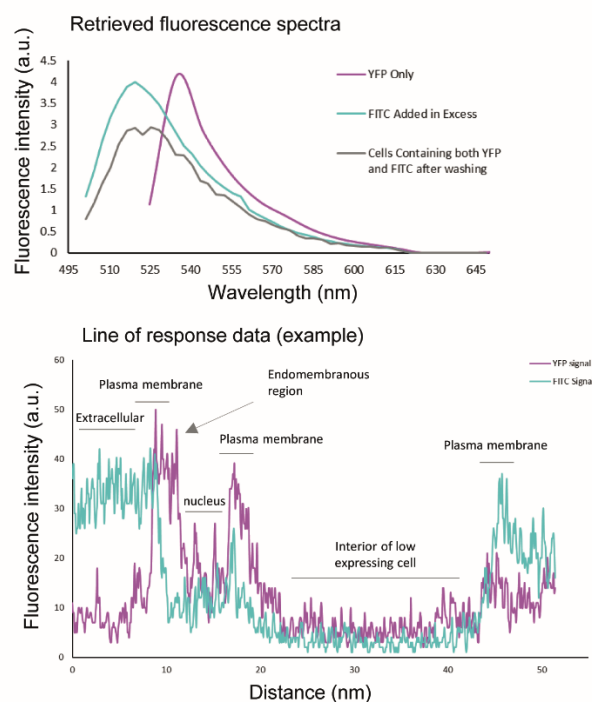


Figure S13. Additional fluorescence data from confocal laser scanning microscopy (CLSM). Spectral scan data retrieved from YFP (magenta) cells (upper), PRDX5-NTA-FITC (cyan) added in excess to dominate the spectra, and after YFP positive cells had been exposed and washed (gray). The shifting dual peak in the latter is indicative of more than one fluorescent species being present, although the PRDX5-NTA-FITC is the most common. Line of Response (LoR) through two adjacent cells, showing local distribution of the two fluorophores (FITC represented by cyan, YFP by magenta). FITC fluorescence intensity is highest at the edge of the plasma membrane and in the surrounding extracellular medium, whereas YFP predominates at the plasma membrane and in the endomembranous region (target site and manufacturing locale respectively). The co-occurring peaks support the co-localization analysis presented in the main manuscript.

Table S2. Masses and intensities of the peaks corresponding to monomeric species detected by ESI-Q-TOF MS on intact proteins. Peaks $\geq 20\%$ in intensity relatively to the largest peak were considered and taken into account for the calculation of the relative intensity. The most probable Cys modification(s) corresponding to the mass shifts are given.

	Peak #	Intensity	Relative int. (%)	Mass (Da)	Identification	Mass shift (Da)
dsPRDX5	1	6.11×10^9	27	18,294.93	Disulfide Cys-S-S-Cys	-2
	2	3.46×10^9	16	18,309.33	Disulfide Cys-S-S-Cys Methylation	-2 +14
	3	8.20×10^9	37	18,352.10	Disulfide Cys-S-S-Cys Carbamidomethyl	-2 +57
	4	4.53×10^9	20	18,366.25	Disulfide Cys-S-S-Cys Carbamidomethyl Methylation	-2 +57 +14
PRDX5^{C47S}	1	6.01×10^{10}	79	18,337.98	Cys $\rightarrow$ Ser substitution Carbamidomethyl	-16 +57
	2	1.63×10^{10}	21	18,351.85	Cys $\rightarrow$ Ser substitution Carbamidomethyl Methylation	-16 +57 +14
r/aPRDX5	1	5.76×10^{10}	62	18,467.74	Carbamidomethyl x 3	+57 x 3
	2	3.53×10^{10}	38	18,481.87	Carbamidomethyl x 3 Methylation	+57 x 3 +14
s/sPRDX5	1	1.43×10^9	13	18,294.64	Disulfide Cys-S-S-Cys	-2
	2	1.57×10^9	14	18,309.60	Disulfide Cys-S-S-Cys Methylation	-2 +14
	3	5.46×10^8	5	18,325.28	?	+28.5
	4	9.73×10^8	9	18,342.09	Disulfide Cys-S-S-Cys Sulfonic acid Cys-SO ₃ H or Sulfinic acid Cys-SO ₂ H Methylation	-2 +48 or +32 +14
	5	1.43×10^9	13	18,351.75	Disulfide Cys-S-S-Cys Carbamidomethyl	-2 +57
	6	1.01×10^9	9	18,356.77	Disulfide Cys-S-S-Cys Sulfonic acid Cys-SO ₃ H Methylation	-2 +48 +14
	7	2.21×10^9	19	18,366.67	Disulfide Cys-S-S-Cys Carbamidomethyl Methylation	-2 +57 +14
	8	6.60×10^8	6	18,382.21	Carbamidomethyl ?	+57 +28.5
	9	4.44×10^8	4	18,400.67	Disulfide Cys-S-S-Cys Sulfonic acid Cys-SO ₃ H Carbamidomethyl or Sulfinic acid Cys-SO ₂ H Carbamidomethyl Methylation	-2 +48 +57 or +32 +57 +14
	10	5.17×10^8	5	18,416.33	Sulfinic acid Cys-SO ₂ H x 2 Carbamidomethyl or Sulfonic acid Cys-SO ₃ H Carbamidomethyl Methylation	+32 x 2 +57 or +48 +57 +14

	11	5.82×10^8	5	18,473.96	Cys-SO ₂ H alkylation adduct of IAM x 2	+89 x 2
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Table S3. Masses and intensities of the peaks corresponding to dimeric species detected by ESI-Q-TOF MS on intact proteins. Peaks $\geq 40\%$ in intensity relatively to the largest peak were considered. The most probable Cys modification(s) corresponding to the mass shifts are given. s/sPRDX5 is not shown given the high number of possible combinations.

	Peak #	Intensity	Mass (Da)	Identification	Mass shift (Da)
dsPRDX5	1	5.58×10^8	36,588.98	Disulfide Cys-S-S-Cys x 2	-2 x 2
	2	2.65×10^8	36,599.69	?	+6
	3	3.08×10^8	36,618.77	Disulfide Cys-S-S-Cys x 2 Methylation x 2	-2 x 2 +14 x 2
	4	3.24×10^8	36,700.80	Carbamidomethyl x 2	+57 x 2
	5	5.21×10^8	36,703.85	Disulfide Cys-S-S-Cys x 2 Carbamidomethyl x 2	-2 x 2 +57 x 2
	6	2.32×10^8	36,717.26	Disulfide Cys-S-S-Cys x 2 Carbamidomethyl x 2 Methylation	-2 x 2 +57 x 2 +14
	7	3.38×10^8	36,732.63	Disulfide Cys-S-S-Cys x 2 Carbamidomethyl x 2 Methylation x 2	-2 x 2 +57 x 2 +14 x 2
PRDX5^{C47S}	1	1.27×10^9	36,675.89	Cys → Ser substitution x 2 Carbamidomethyl x 2	-16 x 2 +57 x 2
	2	6.09×10^8	36,704.23	Cys → Ser substitution x 2 Carbamidomethyl x 2 Methylation x 2	-16 x 2 +57 x 2 +14 x 2
r/aPRDX5	1	5.57×10^8	36,820.63	Carbamidomethyl x 4	+57 x 4
	2	7.36×10^8	36,834.58	Carbamidomethyl x 4 Methylation	+57 x 4 +14
	3	3.56×10^8	36,848.87	Carbamidomethyl x 4 Methylation x 2	+57 x 4 +14 x 2
	4	3.00×10^8	36,879.91	Carbamidomethyl x 5	+57 x 5
	5	5.20×10^8	36,935.51	Carbamidomethyl x 6	+57 x 6
	6	3.96×10^8	36,945.73	?	+351
	7	3.13×10^8	36,946.98	?	+354
	8	6.95×10^8	36,964.20	Carbamidomethyl x 6 Methylation x 2	+ 57 x 6 +14 x 2

Supplementary Materials and Methods

LC-Q-TOF-MS/MS characterization of the PRDX5 cysteine redox state

Blocking of cysteine redox states

Redox states of PRDX5^{C47} in dsPRDX5 and s/sPRDX5 were blocked with 55 mM IAM (Sigma-Aldrich) for 10 min at RT in the dark to alkylate the thiols, and 5 mM 5,5-Dimethyl-1,3-cyclo-hexanedione (dimedone, Sigma-Aldrich) for 5 min at RT to trap sulfenic acids [488,489].

Preparation for in solution samples

Chloroform-methanol precipitation. 20 µg of each sample were transferred to 0.5 mL polypropylene Protein LoBind tubes (Eppendorf) and precipitated with chloroform-methanol method [508].

Solubilization before trypsin digestion. Samples were dried under vacuum with a Savant SpeedVac Concentrator (ThermoFisher). 20 µL of 100 mM triethylammonium bicarbonate (TEAB), pH 8.5 were added to the pellets for solubilization and vortexed for 30 min at RT.

In-solution trypsin digestion. Proteolysis was performed with 1 µg of sequencing grade trypsin (Promega, USA) and allowed to continue overnight at 37°C. Each sample was dried under vacuum with a Savant SpeedVac Concentrator (Thermo Fisher).

Peptide separation using nanoUPLC

Before peptide separation, the samples were dissolved in 20 µL of 0.1 % (v/v) formic acid and 2% (v/v) acetonitrile (ACN). The resulting peptide mixture was separated by reverse phase chromatography on a NanoACQUITY UPLC MClass system (Waters) working with MassLynx V4.1 (Waters) software. 200 ng of digested proteins were injected on a C18 trap column, 100 Å, 5 µm, 180 µm x 20 mm (Waters), and desalted using isocratic conditions at a flow rate of 15 µL·min⁻¹ using a 99% formic acid and 1% (v/v) ACN buffer for 3 min. Peptide mixture was subjected to reverse phase chromatography on a PepMap C18 column, 100 Å, 1.8 µm, 75 µm x 150 mm (Waters) for 130 min at 35°C at a flow rate of 300 nL·min⁻¹ using a two part linear gradient from 1% (v/v) ACN, 0.1 % formic acid to 35 % (v/v) ACN, 0.1 % formic acid for 90 min, and from 35% (v/v) ACN, 0.1 % formic acid to 85 % (v/v) ACN, 0.1 % formic acid for 10 min. The column was re-equilibrated at initial conditions after washing for 30 min at 85% (v/v) ACN, 0.1 % formic acid at a flow rate of 300 nL·min⁻¹. For online LC-MS analysis, the nanoUPLC was coupled to the mass spectrometer through a nano-electrospray ionization (nanoESI) source emitter.

LC-Q-TOF-MS/MS Analysis (DDA)

Data Dependent Analysis (DDA) was performed on an SYNAPT G2-Si high definition mass spectrometer (Waters) equipped with a NanoLockSpray dual electrospray ion source (Waters). Precut fused silica PicoTipR Emitters for nanoelectrospray (outer diameters: 360 μm ; inner diameter: 20 μm ; 10 μm tip; 2.5" length) (Waters) were used for samples and Precut fused silica TicoTip Emitters for nanoelectrospray (outer diameters: 360 μm , inner diameter: 20 μm , 2.5" length) (Waters) were used for the lock mass solution. The eluent was sprayed at a spray voltage of 2.8 kV with a sampling cone voltage of 25 V and a source offset of 30 V. The source temperature was set to 80°C. The cone gas flow was 20 $\text{L}\cdot\text{h}^{-1}$ with a nanoflow gas pressure of 0.4 bar, and the purge gas was turned off. The SYNAPT G2Si instrument was operated in DDA (data-dependent mode), automatically switching between MS and MS2. Full scan MS and MS2 spectra (m/z 400 – 2000) were acquired from 2 min after injection to 30 min in resolution mode (20,000 resolution FWHM at m/z 400) with a scan time of 0.1 sec. Tandem mass spectra of up to 10 precursors were generated in the trapping region of the ion mobility cell by using a collision energy ramp from 17/19 V (low mass, start/end) to up to 65/75 V (high mass, start/end). Charged ions (+2, +3, +4) were selected to be submitted to the MSMS fragmentation over the m/z range from 50 to 2000 with a scan time of 0.25 sec. For the post-acquisition lock mass correction of the data in the MS method, the doubly charged monoisotopic ion of [Glu1]-fibrinopeptide B was used at 100 $\text{fmol}\cdot\mu\text{L}^{-1}$ using the reference sprayer of the nanoESI source with a frequency of 30 s at 0.5 $\mu\text{L}\cdot\text{min}^{-1}$ into the mass spectrometer.

ESI-QTOF data processing

Data were processed with UNIFI (Waters) using the known PRDX5 sequence. Dimedone, carbamidomethylation, sulfenic acid, sulfinic acid, and sulfonic acid as the variable cysteine modifications, oxidation as the variable methionine modification, and trypsin as the digestion enzyme, were selected, and one miss cleavage was allowed.

3.3. Study 2 – Role of Toll-like receptor accessory molecules MD-2 and CD14 in peroxiredoxin-mediated pro-inflammatory response

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Personal contribution: I performed all experiments, with the exception of AFM-based SMFS experiments involving CD14.

3.3.1. Abstract

Extracellular human peroxiredoxins (PRDXs) can act as damage-associated molecular patterns (DAMPs) by activating Toll-like receptor 4 (TLR4). However, the role of TLR4 accessory molecules MD-2 and CD14 in PRDX-dependent inflammation remained to be determined. Here, we combined biomolecular (ELISA and flow cytometry) and single-molecule (atomic force microscopy) approaches to investigate whether MD-2 and CD14 are involved in PRDX2 and PRDX5 recognition and signaling. In particular, we demonstrate that PRDX2 is able to directly bind to TLR4, as previously shown for PRDX5, and that both PRDXs are able to signal through a TLR4-dependent signaling pathway. Whereas cells expressing TLR4 alone are not able to trigger interleukin-8 (IL-8) secretion following PRDX exposure, they become responsive when TLR4 is expressed along with MD-2. Interestingly, HEK293 cells expressing CD14 and devoid of TLR4 secrete IL-8 when exposed to PRDX2 or PRDX5, suggesting an alternative pathway in PRDX-mediated pro-inflammatory response. We further show that both PRDX2 and PRDX5 are able to directly bind to recombinant MD-2 and CD14, and to CD14 expressed at the surface of living cells. Our results show for the first time that accessory molecules play a key role in TLR-dependent and -independent signaling of PRDXs, as it has previously been shown for other pro-inflammatory molecules such as LPS and HMGB1.

3.3.2. Introduction

Peroxiredoxins (PRDXs) are a unique set of proteins given their ubiquity, their high cellular abundance, and their diverse functions [3,4]. As redox-sensitive proteins, they were first characterized as antioxidant peroxidases [43,46], protecting the cell by scavenging peroxides. However, their role as peroxidases was also found to serve in redox signaling, both indirectly by locally regulating H_2O_2 concentration, or more directly by sensing and transducing H_2O_2 signaling through their involvement in redox relays [16,24]. Moreover, they exhibit other intracellular functions, including molecular chaperone [32,34] and phospholipidase [35] activities. Expressed as six isoforms in mammals (PRDX1-6), they can be classified into three subfamilies based on the number and location of redox-active cysteine residues (Cys) involved in the catalytic mechanism: typical 2-Cys PRDXs (PRDX1-4), atypical 2-Cys PRDXs (PRDX5), and 1-Cys PRDXs (PRDX6) [46].

Mammalian PRDXs were recently shown to act also as damage-associated molecular patterns (DAMPs) when released extracellularly [111,116,117,344,362,364,481,485,509]. They are indeed able to induce pro-inflammatory responses under diverse pathological conditions (*e.g.* ischemia-reperfusion injury, acute liver injury), notably through activation of Toll-like receptor (TLR) family members, and TLR2 and TLR4 in particular. Tumor-cell released PRDX1

was shown to trigger TLR4-dependent secretion of tumor necrosis factor (TNF)- α and interleukin (IL)-6 from thioglycolate-elicited murine macrophages or immature BMDCs [344]. Similar TLR4 activation by PRDX1 was also demonstrated in the context of intracerebral hemorrhage [481]. In addition, Lu *et al.* revealed that TLR4-knockout microglia lose the ability to induce neuronal apoptosis caused by extracellular PRDX2 after subarachnoid hemorrhage [117]. The role of PRDX1, PRDX2, PRDX5, and PRDX6 as DAMPs was also emphasized in post-ischemic inflammation in the brain [116]. In particular, Shichita *et al.* illustrated that PRDXs released from necrotic brain cells induce expression of pro-inflammatory cytokines (including IL-23) in macrophages through activation of TLR2 and TLR4, thereby promoting neural cell death [116]. This observation was also supported by other researchers who demonstrated the protective effect of therapeutic agents against ischemic brain injury through the TLR4-dependent signaling of PRDX6 [364] or PRDX1, PRDX2 and PRDX4 [509], respectively.

To gain mechanistic insights into TLR4 activation, we previously investigated PRDX5 binding to TLR4, and showed that recombinant human PRDX5 was able to directly bind to TLR4 using atomic force microscopy (AFM)-based single molecule force spectroscopy (SMFS) [487]. In addition, other works reported a direct interaction between PRDX1 and TLR4 using co-immunoprecipitation and pull-down assays [344,365,375]. However, the role of TLR4 accessory molecules myeloid differentiation factor 2 (MD-2) and cluster of differentiation 14 (CD14) in PRDX-elicited pro-inflammatory response was not studied so far.

Here, we combined ELISA, flow cytometry (FC), and AFM-based SMFS to investigate whether MD-2 and/or CD14 are involved in PRDX recognition and/or signaling, using the typical 2-Cys PRDX2 and the atypical 2-Cys PRDX5. We first show that both recombinant PRDX2 and PRDX5 trigger IL-8 secretion from PMA-differentiated THP-1 cells, confirming their role as DAMPs. We also report increased IL-8 production from human embryonic kidney (HEK) 293 cells stably transfected with MD-2-, CD14-, and TLR4-coding vectors following exposure to PRDX2 or PRDX5 compared to HEK293 control cells, and that IL-8 levels significantly decreased in the presence of a neutralizing anti-TLR4 antibody. As for PRDX5, we also demonstrate that PRDX2 is able to bind directly to TLR4. Although these results confirm a TLR4-dependent signaling pathway of PRDXs, HEK293 cells expressing solely TLR4 are unresponsive, indicating that MD-2 and/or CD14 are required for functional PRDX-induced TLR4-dependent signaling. We further observe that MD-2 expression along with TLR4 in HEK293 cells lead to IL-8 secretion after exposure to PRDX2 and PRDX5. In addition, the role of MD-2 in the TLR4-dependent signaling of PRDXs was emphasized with a neutralizing anti-MD-2 antibody. In an attempt to decipher the role of CD14 in the PRDX-induced TLR4-dependent signaling, we quantified IL-8

secretion from HEK293 cells expressing CD14 along with TLR4, and compared IL-8 levels to those of HEK293 cells solely expressing CD14. Surprisingly, both PRDX2 and PRDX5 are able to induce IL-8 secretion through a TLR4-independent pathway involving CD14. The role of CD14 in PRDX-induced pro-inflammatory response was further confirmed with a neutralizing anti-CD14 antibody. In addition, we show that PRDX2 and PRDX5 are able to bind directly to both recombinant MD-2 and CD14, and to CD14 expressed at the surface of living cells. Taken together, these results demonstrate that TLR accessory molecules MD-2 and CD14 play key roles in TLR4-dependent and -independent pro-inflammatory response triggered by PRDXs.

3.3.3. Materials and Methods

Production and purification of recombinant human PRDX2 and PRDX5

The expression and purification of recombinant N-terminal 6xHis-tagged human PRDX5 is extensively described elsewhere [50,142,487]. N-terminal 6xHis-tagged human PRDX2 was expressed and purified similarly. Briefly, pQE-30 expression vector (Qiagen, Hilden, Germany) coding for wild-type human PRDX2 or PRDX5 was used to transform *Escherichia coli* (*E. coli*) strain M15 (pRep4). *E. coli* were grown at 37°C in lysogeny broth (LB) medium containing 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Pelleted cells were lysed in 10 mM imidazole, 50 mM phosphate, 300 mM NaCl (pH 8) by sonication and clarified by centrifugation. The supernatant containing 6xHis-tagged PRDX2 or PRDX5 was loaded onto a Ni²⁺-nitrilotriacetic acid (NTA) column (Qiagen). The column was washed and the protein was eluted with 50 mM phosphate, 300 mM NaCl, 250 mM imidazole (pH 8). Eluted proteins were then dialyzed against phosphate-buffered saline (PBS) (pH 7.2).

Cell lines

The human monocytic cell line THP-1 (provided by Prof. C. Remacle, UCLouvain, Belgium) was cultured in RPMI-1640 GlutaMAX (Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 100 units·mL⁻¹ streptomycin and 100 units·mL⁻¹ penicillin (Sigma-Aldrich, St. Louis, MO, USA) and were maintained at 37°C in a humidified atmosphere supplemented with 5% CO₂. THP-1 cells were differentiated into macrophage-like cells by exposure to 25 nM phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) in 96-well plates for 48 h [510], followed by a recovery period of 3 h in the absence of PMA.

Human embryonic kidney (HEK) 293 cells and HEK293 cells stably expressing human MD-2 and CD14 (HEK293 MD2-CD14) were purchased from InvivoGen (San Diego, CA, USA) (293/null and 293/MD2-CD14, respectively). HEK293 cells stably expressing CD14 (HEK293 CD14) were a gift from Prof. D. Golenbock (University of

Massachusetts Medical Center, Worcester, MA). HEK293 cells and HEK293 MD2-CD14 cells were transfected with pcDNA3-TLR4-YFP (Addgene, Watertown, MA, USA, plasmid #13018), as described previously [511]. Cells were plated at a density of $\sim 2 \times 10^6$ cells in 100 mm culture dishes and stably transfected with 10 μg pcDNA3-TLR4-YFP using 25 μL Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA, USA) and 10 μL PLUS Reagent (Thermo Fisher Scientific) in 1 mL Opti-MEM (Thermo Fisher Scientific), when cells reached $\sim 70\%$ confluency. Stable transfectants (HEK293 TLR4 cells and HEK293 MD2-CD14 TLR4 cells) were first selected using the antibiotic Geniticin (G418) (InvivoGen). To control TLR4 expression at the cell surface, cells were further sorted based on the fluorescence of an APC-conjugated anti-TLR4 antibody (Thermo Fisher Scientific) using fluorescence-activated cell sorting (FACS). Single cells were then isolated to produce stable cell lines. For transient transfections of pcDNA3-TLR4-YFP, pcDNA3-CD14 (Addgene, plasmid #13645), and pUNO1-hMD2a (InvivoGen), $\sim 15 \times 10^3$ cells were plated in each well of 96-well plates for 48 h, before being transfected with 100 ng of plasmid using 0.15 μL Lipofectamine 3000 (Thermo Fisher Scientific) and 0.2 μL P3000 Reagent (Thermo Fisher Scientific) in 10 μL Opti-MEM. All HEK293 cell lines were cultured in DMEM GlutaMAX (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Lonza), 100 units $\cdot\text{mL}^{-1}$ streptomycin and 100 units $\cdot\text{mL}^{-1}$ penicillin (Sigma-Aldrich), and 100 mg $\cdot\text{mL}^{-1}$ of Normocin (InvivoGen), at 37°C in a humidified atmosphere supplemented with 5% CO₂.

Flow cytometry analysis of receptor cell surface expression

Cells were first harvested with Accutase solution (Sigma-Aldrich) prior to performing cell count and viability analysis. Cells were washed with ice-cold DMEM, centrifuged at 500 x g for 3 min at 4°C, and then resuspended at a concentration of $\sim 2.5 \times 10^5$ cells $\cdot\text{mL}^{-1}$ in ice-cold PBS containing 0.5% BSA. After a 15-min incubation, cells were resuspended in PBS/0.5% BSA in the presence of anti-TLR4 antibody (Thermo Fisher Scientific, 1/20) or anti-CD14-APC antibody (Thermo Fisher Scientific, 1/20), and were incubated for 40 min on ice. As negative control, cells were incubated with PBS/0.5% BSA without antibody. Cells were finally washed two times and resuspended in PBS. Samples were run on the Guava easyCyte System (Merck, Darmstadt, Germany) using GuavaSoft software. Gates were set based on the YFP and APC signals for TLR4 expression, or on the APC signal only for CD14 expression. Median fluorescence intensity (MFI) of control cells was subtracted from MFI of labeled cells.

Quantification of human IL-8 secretion

Prior to performing ELISAs, endotoxins were removed from the PRDX productions using the ToxOut Phase Extraction Endotoxin Removal kit (BioVision, Milpitas, CA, USA), following manufacturer's instructions.

HEK293 cells were counted and cultured in 96-well plates with complete growth medium for 48 hours before being exposed to PBS, 100 ng·mL⁻¹ smooth lipopolysaccharide (LPS, from *E. coli* O111:B4, Sigma-Aldrich), 0.2 μM or 2 μM HMGB1, 2 μM PRDX5, and 2 μM PRDX2, in DMEM (Life Technologies) supplemented with 100 units·mL⁻¹ streptomycin and 100 units·mL⁻¹ penicillin (Sigma-Aldrich), without FBS. Cells were also exposed to the various treatments, either in the presence of 20 μg·mL⁻¹ anti-TLR4 antibody (InvivoGen), anti-CD14 antibody (InvivoGen) or anti-MD-2 antibody (Genetex, Irvine, CA, USA), or in the presence of 20 μg·mL⁻¹ isotype control antibody, namely mouse IgG1 isotype control (InvivoGen), anti-β-Gal-hlgG1 (InvivoGen), and mouse IgG2b isotype control (Genetex), respectively. Culture medium was harvested 16 h after cell exposure and IL-8 secretion was measured using ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA), following manufacturer's instructions.

As control for endotoxin contamination, PBS, PRDX5, PRDX2 and LPS were incubated with 150 μg·mL⁻¹ proteinase K (PK, from *Tritirachium album*, Sigma-Aldrich), an endopeptidase with broad specificity and very high specific activity, for 2 h at 37°C and overnight at 4°C, followed by the addition of 0.25 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Thermo Fisher Scientific) for 1 h at RT to degrade cytotoxic PK before cell exposure. HEK293 MD2-CD14-TLR4 cells were exposed to these mixtures for 16 h and IL-8 secretion was measured. To control for the putative pro-inflammatory of the PRDX 6xHis-tag, HEK293 MD2-CD14-TLR4 cells were exposed to a synthetic Cys-His₆ peptide (GenScript, Piscataway, NJ, USA) for 16 h and IL-8 secretion was measured.

Western blot analysis of total and soluble CD14

HEK293 CD14 cells, HEK293 MD2-CD14-TLR4 cells, HEK293 MD2-CD14 cells, and HEK293 cells were seeded in six-well plates (3 x 10⁵ cells/well). The next day, complete medium was replaced by serum-free medium and cells were incubated for 16 hours. Supernatants were then collected and cells were washed with ice-cold PBS and lysed with RIPA buffer (Thermo Fisher Scientific) completed with protease inhibitors (Thermo Fisher Scientific). Sample protein levels were quantified, and equal amount of each sample was boiled at 95°C for 5 min and subsequently loaded on SDS-polyacrylamide gels (Any kD and 4-20% Mini-PROTEAN TGX Precast Protein gel, Bio-Rad, Hercules, CA, USA). SDS-PAGE was performed under reducing

conditions. Proteins were then transferred to nitrocellulose membranes (Amersham Protan 0.45 μm NC, GE Healthcare Life Sciences, Chicago, IL, USA). After transfer, proteins were detected using a polyclonal anti-CD14 antibody (1/1000) (Thermo Fisher Scientific) followed by an anti-rabbit IgG/HRP secondary antibody (1/2000) (Argilent, Santa Clara, CA, USA). For cell lysates, a monoclonal anti- α -tubulin (Sigma) was used as loading control, followed by an anti-mouse IgG/HRP secondary antibody (1/1500) (Argilent). A similar control was not possible for cell culture supernatants, and the membrane was therefore stained with Ponceau S. The only visible band is shown as loading control. Blots were developed with BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland) using an Amersham Imager 600 (GE Healthcare Life Sciences).

Functionalization of AFM tips

PRDX2 or PRDX5 molecules were grafted onto the AFM tips (MSCT probes, Bruker, Billerica, MA, USA) using (3-aminopropyl) triethoxysilane (APTES) coating and a heterobifunctional polyethylene glycol linker composed of a *N*-hydroxy succinimide (NHS) at one end, and either a maleimide molecule or an aldehyde molecule at the other end (NHS-PEG₂₇-maleimide and NHS-PEG₂₄-aldehyde, respectively). The protocol was adapted from Professor H. J. Gruber (Johannes Kepler University, Institute of Biophysics, Linz).

Amino-functionalization

Probes were first washed three times in chloroform, dried with a gentle stream of nitrogen and cleaned for 15 min in an ultraviolet radiation and ozone (UV-O₃) cleaner (Jelight, Irvine, CA, USA). Probes were placed for 2 h in a hermetic desiccator filled with argon, in the presence of 30 μL of APTES and 10 μL of triethylamine. Finally, probes were kept for two more days in the desiccator filled with argon.

Coupling of NHS-PEG₂₇-maleimide/NHS-PEG₂₄-aldehyde linker

The amino-functionalized probes were first immersed for 2 h in a solution containing 1 mg of PEG linker dissolved in 0.5 mL of chloroform, and 30 μL of triethylamine. Probes were then washed three times in chloroform, and dried with a gentle stream of nitrogen. In the case of the PEG₂₄-aldehyde linker, the probes were immersed in 100 μL of 0.2 $\text{mg}\cdot\text{mL}^{-1}$ PRDX5 or PRDX2 solution and 2 μL of a freshly prepared solution of 1 M NaCNBH₃ (with 20 mM NaOH) for 1 h. The reaction was quenched by adding 5 μL of 1 M ethanolamine solution (pH 8). After 10 min, the probes were rinsed three times with phosphate buffered saline (PBS) and stored in PBS. In the case of the PEG₂₇-maleimide linker, the probes were immersed in a mix solution containing 1 mM disulfide-*tris*-NTA, 100 mM EDTA (pH 7.5), 1 M Hepes (pH 7.5), 100 mM TCEP hydrochloride and 1 M Hepes (pH 9.6) for 2 h. Probes were

washed and immersed in 100 μL of 0.2 $\text{mg}\cdot\text{mL}^{-1}$ PRDX2 or PRDX5 solution with 5 mM NiCl_2 for 90 min. The probes were then rinsed three times with PBS and stored in PBS. Experiments were conducted within 48 h following protein coupling.

Preparation of TLR4-, MD-2- and CD14-coated surfaces

Recombinant human TLR4 (R&D Systems, Minneapolis, MN, USA), MD-2 (R&D Systems) and CD14 (R&D Systems) were covalently attached to carboxyl groups of a mixed self-assembled monolayer (SAM) of carboxyl (COOH)- and hydroxyl (OH)-terminated alkanethiols immobilized onto inert surfaces using NHS/*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) chemistry.

Alkanethiol SAM formation

Gold-coated silicon surfaces were first washed with ethanol, dried with a gentle flow of nitrogen, and cleaned for 15 min by UV- O_3 treatment. Surfaces were then immersed overnight in an ethanol solution containing a 1 mM mixture (1:99 mol/mol) of 16-mercaptohexadecanoic acid ($\text{HS}(\text{CH}_2)_{16}\text{COOH}$) (Sigma-Aldrich) and 11-mercapto-1-undecanol ($\text{HS}(\text{CH}_2)_{11}\text{OH}$) (Sigma-Aldrich).

NHS/EDC chemistry

After sonicating briefly to remove alkanethiol aggregates that may be adsorbed, surfaces were rinsed with ethanol and dried with nitrogen. Next, surfaces were stuck to Teflon and ~ 50 μL of a solution composed of 175 mM NHS (Sigma-Aldrich) and 260 mM EDC (Sigma-Aldrich) in ultrapure water was added onto the surfaces for 30 min. Activated surfaces were then rinsed 10 times with PBS, before being incubated with 0.1 $\text{mg}\cdot\text{mL}^{-1}$ TLR4, 0.15 $\text{mg}\cdot\text{mL}^{-1}$ MD-2 or 0.1 $\text{mg}\cdot\text{mL}^{-1}$ CD14 in PBS for 90 min, and finally rinsed again three times with PBS. Experiments were conducted within 48 h following surface functionalization.

FD-curve based AFM on model surfaces

SMFS on model surfaces was operated with a NanoScope 8 Multimode AFM (Bruker) equipped with the NanoScope 9.2 software (Bruker), or a Force Robot 300 AFM (JPK, Berlin, Germany) equipped with Control Software v6.1.144 (JPK), using PRDX5- or PRDX2-functionalized MSCOT tips (C triangular cantilevers, Bruker) and TLR4-, CD14-, or MD-2-functionalized gold surfaces in PBS at $\sim 25^\circ\text{C}$. For controls, tips functionalized without PRDX, surface functionalized without receptor, as well as anti-PRDX2 or anti-PRDX5 sera [436] (0.5 μL) and anti-TLR4 or anti-CD14 antibodies (50 $\mu\text{g}\cdot\text{mL}^{-1}$) (InvivoGen), were used. Using Force Volume in contact mode, FD curves were recorded on 5 μm x 5 μm arrays of 32 x 32 curves. Constant approach speed was set at 1 $\mu\text{m}\cdot\text{s}^{-1}$ and retraction speeds were varied (100 $\text{nm}\cdot\text{s}^{-1}$, 200 $\text{nm}\cdot\text{s}^{-1}$, 500 $\text{nm}\cdot\text{s}^{-1}$, 1 $\mu\text{m}\cdot\text{s}^{-1}$, 2 $\mu\text{m}\cdot\text{s}^{-1}$, and 10 $\mu\text{m}\cdot\text{s}^{-1}$, with additional 20 $\text{nm}\cdot\text{s}^{-1}$ and 5 $\mu\text{m}\cdot\text{s}^{-1}$ for

TLR4- and CD14-coated surfaces, respectively). A ramp size of 200 nm and a force threshold of 300 pN were used. Cantilever spring constants were calibrated on mica surfaces with the thermal noise method [434].

Force curves were analyzed using the Nanoscope Analysis 1.8 software (Bruker) or the JPK Data Analysis Software v7.1.14+ (JPK). FD curves were first extracted from individual adhesion maps and sorted to keep the curves displaying specific adhesion event(s). For each tip retraction speed, the binding frequency (BF) was calculated by dividing the number of specific adhesive curves by the total number of FD curves recorded for each map. For the DFS analysis, the baseline of the retraction curves was first corrected using a linear fit to remove tilt and offset. Rupture forces (expressed in pN) were extracted by measuring the height of the adhesive peak(s), and LRs (expressed in $\text{pN}\cdot\text{s}^{-1}$) were extracted by fitting the adhesive peak on the force-time curve just before rupture with a linear model. Determination of the kinetic parameters of the interactions was performed on OriginPro 9.8.0.200 software (OriginLab Corporation, Northampton, MA, USA). Spectrum of LRs was divided into six or seven discrete ranges (100–315 $\text{pN}\cdot\text{s}^{-1}$; 315–1000 $\text{pN}\cdot\text{s}^{-1}$; 1000–3150 $\text{pN}\cdot\text{s}^{-1}$; 3150–10,000 $\text{pN}\cdot\text{s}^{-1}$; 10,000–31,500 $\text{pN}\cdot\text{s}^{-1}$; 31,500–100,000 $\text{pN}\cdot\text{s}^{-1}$; 100,000–315,000 $\text{pN}\cdot\text{s}^{-1}$), depending on the receptor. Analysis of the rupture forces was performed as previously [511]. Briefly, a kernel distribution was generated to represent the probability density function (PDF) of the rupture forces, using a normal kernel smoothing function. The second derivative of this function was used to search for hidden positive peak, by choosing a threshold of 20% in height in most cases. Peaks were fitted with gaussian functions using the Levenberg-Marquardt algorithm, of which centers allow determination of the most probable rupture forces for single and multiple interactions. The Bell-Evans model [429] (**Equation 5**) was fitted to most probable rupture forces corresponding to single interactions to determine the dissociation rate constant k_{off} and the distance to the transition state x_β . The predicted rupture forces for two ($n = 2$) simultaneous uncorrelated interactions were determined using Williams-Evans prediction [433] (**Equation 6**).

Flow cytometry binding assay

Cells were first harvested with Accutase solution (Sigma-Aldrich) prior to performing cell count and viability analysis. Cells were resuspended in ice-cold DMEM (Life Technologies) and $\sim 5 \times 10^5$ cells·mL⁻¹ were exposed to increased concentrations of PRDX5 (1 μM , 2 μM , 5 μM , 10 μM , and 15 μM) in DMEM (Life Technologies) for 40 min on ice to prevent endocytosis. As negative control, cells were incubated with medium only. Cells were centrifuged at 500 x g for 5 min at 4°C and then incubated with PBS containing 0.5% BSA for 15 min on ice. Cells were resuspended in anti-PRDX5 serum (1/200) [436] for 40 min on ice, before being

washed three times with PBS/0.5% BSA. Cells were finally labeled for 40 min on ice with goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 647 Plus (1/250) (Thermo Fisher Scientific), washed three times, and resuspended in PBS. Samples were acquired on the Guava easyCyte System (Merck) using GuavaSoft software. Gates were set to select living Alexa Fluor 647 Plus-positive cells. Median fluorescence intensity (MFI) of control cells was subtracted from MFI of PRDX5-exposed cells.

Statistical analysis

JMP Pro 16.0.0 software (SAS Institute, Cary, NC, USA) was used to perform the statistical tests. Student's *t*-test, Dunnett's test, and Tukey HSD test were used for pairwise comparisons, comparisons to a reference group (PBS), or multiple pairwise comparisons, respectively. To fulfil the assumptions of the model, a logarithmic transformation ($\log_{10}$) of the IL-8 concentration values was performed for ELISA data. Statistical differences were considered significant at $p < 0.05$.

3.3.4. Results and Discussion

PRDX2 and PRDX5 induce IL-8 secretion from PMA-differentiated THP-1 cells

We first investigated the pro-inflammatory activity of recombinant human PRDX2 and PRDX5 using THP-1 cells in order to confirm their role as DAMPs. As human PRDXs were produced in *Escherichia coli*, endotoxins were removed from the preparation before their use with living cells. Prior to exposure, THP-1 cells were differentiated with 25 nM phorbol 12-myristate 13-acetate (PMA) for 48 h to induce a macrophage-like phenotype [510]. PMA-differentiated THP-1 cells were exposed for 16 h to either PBS (negative control), smooth lipopolysaccharide (LPS, 100 ng·mL⁻¹) (positive control), recombinant HMGB1 (2 μM) (positive DAMP control), recombinant PRDX2 (2 μM), or recombinant PRDX5 (2 μM). Interleukin-8 (IL-8) levels from supernatants were measured by ELISA. All conditions induced significantly increased IL-8 levels compared to the PBS control (**Figure 47a**).

PRDX2- and PRDX5-induced IL-8 release is TLR4-dependent

To evaluate the direct role of TLR4 in PRDX-induced IL-8 secretion, we examined IL-8 production from human embryonic kidney (HEK) 293 cells, that lack both TLR2 and TLR4 [398]. Exposure of HEK293 cells to PBS, LPS (100 ng·mL⁻¹), HMGB1 (0.2 μM), PRDX2 (2 μM) or PRDX5 (2 μM) for 16 h lead to a very low IL-8 secretion level (<0.1 ng·mL⁻¹) for cells exposed to PRDXs (**Figure 47b**). To elucidate the role of TLR4 in PRDX-dependent inflammatory response, we stably transfected HEK293 cells expressing myeloid differentiation factor 2 (MD-2) and cluster of differentiation 14 (CD14) with TLR4-eYFP plasmid (HEK293 MD2-CD14-TLR4 cells) (**Figure 47c**). We first

performed flow cytometry (FC) to confirm that the receptor was properly expressed at the cell surface (**Figure 47d**). We then exposed HEK293 MD2-CD14-TLR4 cells to PBS, LPS (100 ng·mL⁻¹), HMGB1 (0.2 and 2 μM), PRDX2 (2 μM) or PRDX5 (2 μM) for 16 h, and quantified IL-8 secretion levels (**Figure 47e**). Smooth LPS serves here as a control to establish that the receptor complex is functional as it requires MD-2, CD14 and TLR4 to trigger cytokine secretion [309]. On the contrary to HEK293 cells, LPS induced IL-8 release in HEK293 MD2-CD14-TLR4 cells. A similar result was obtained for HMGB1, even at a low concentration of 0.2 μM, as well as for PRDX2 and PRDX5. This observation is consistent with a previous study demonstrating that PRDX5 activates nuclear factor-κB in HEK293 cells transfected with TLR4-, MD-2- and CD14-coding vectors [116]. We also demonstrated that recombinant PRDX2 increases IL-8 release from HEK293 MD2-CD14-TLR4 cells in a dose-dependent manner (**Figure 47f**), as we previously reported for PRDX5 [511].

To further validate the role of TLR4 in the PRDX-dependent IL-8 secretion, HEK293 MD2-CD14-TLR4 cells were exposed to the various treatments in presence of a neutralizing anti-TLR4 antibody (**Figure 47g**). Quantification of IL-8 levels compared to the isotype control antibody lead to a 1.2-fold decrease for LPS, 1.3-fold decrease for HMGB1, 3.1-fold decrease for PRDX5, and 4.2-fold decrease for PRDX2, confirming that PRDX-induced IL-8 secretion occurs through a TLR4-dependent signaling.

As expression of MD-2, CD14 and TLR4 together restored LPS responsiveness to HEK293 cells, we used this cell line to demonstrate that putative endotoxin contamination did not account for the IL-8 secretion induced by PRDX2 and PRDX5. IL-8 secretion levels were measured before and after endotoxin removal (**Figure S14a**). Before endotoxin removal, IL-8 levels were significantly higher, indicating LPS contamination. To verify that no LPS remains after the endotoxin removal process, PRDX productions were incubated with proteinase K [512], a serine protease, following by the subsequent addition of the protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) before cell exposure in order to suppress the cytotoxic effect of the protease. Proteinase K treatment significantly inhibited IL-8 production from PRDX2- and PRDX5-treated cells, but not from LPS-treated cells (**Figure S14a**), indicating that PRDXs, but not the presence of putative contaminant LPS, is responsible for the pro-inflammatory activity observed. These results underly once again the importance of removing contaminant LPS from recombinant proteins produced in bacteria prior to study DAMP-induced inflammation.

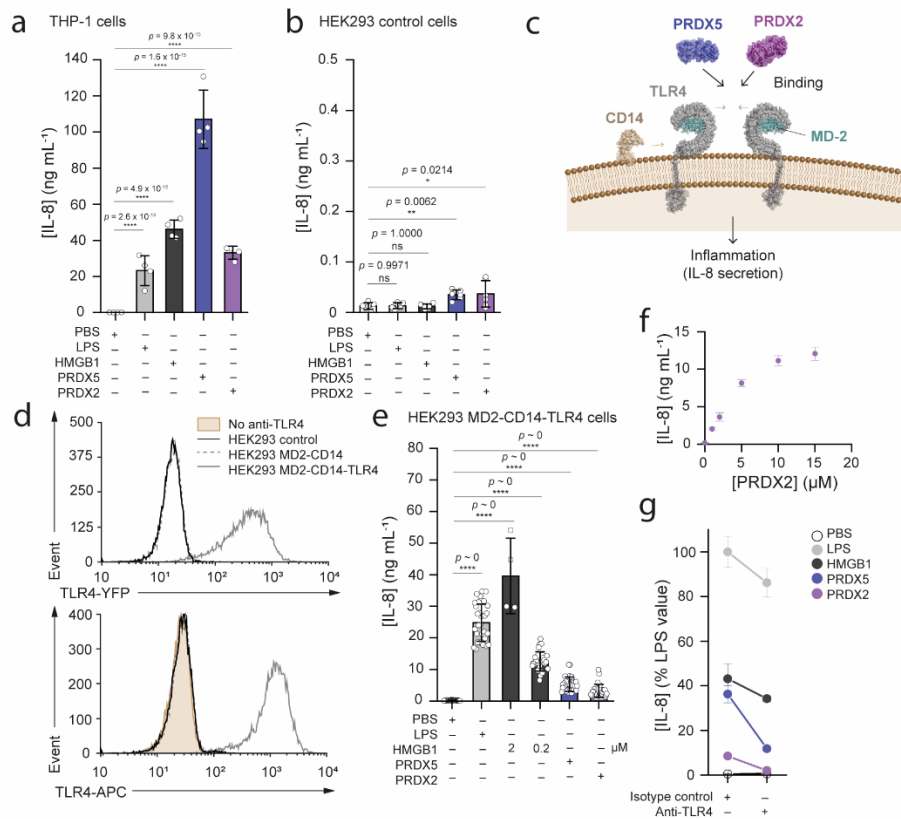


Figure 47. PRDX2- and PRDX5-induced IL-8 secretion is TLR4-dependent. IL-8 secretion levels produced by **(a)** PMA-differentiated THP-1 cells or **(b)** HEK293 cells, after stimulation with PBS, LPS (100 ng·mL⁻¹), HMGB1 (2 μM for THP-1 cells and 0.2 μM for HEK293 cells), PRDX2 (2 μM) or PRDX5 (2 μM) for 16 h. Data in **(a,b)** are mean ± S.D. Number of independent experiments, *N* = 2 with duplicates in each. **(c)** To assess the role of TLR4 in the PRDX-induced IL-8 secretion, HEK293 cells expressing the TLR4 complex (TLR4, MD-2 and CD14) were used. **(d)** Flow cytometry (FC) analysis of total (up) and cell surface (down) TLR4 staining on HEK293 MD2-CD14-TLR4 cells compared to HEK293 MD2-CD14 and HEK293 control cells. Representative of *N* = 3 independent experiments, with duplicates in each. **(e)** IL-8 secretion levels from HEK293 MD2-CD14-TLR4 cells after exposure to PBS, LPS (100 ng·mL⁻¹), HMGB1 (0.2 and 2 μM), PRDX2 (2 μM) or PRDX5 (2 μM) for 16 h. Data are mean ± S.D. Number of independent experiments, *N* = 14 (*N* = 2 for the 2 μM HMGB1 condition), with duplicates in each. **(f)** IL-8 secretion from HEK293 MD2-CD14-TLR4 cells is PRDX2-concentration dependent. Data are mean ± S.D. Each data point corresponds to triplicates. **(g)** Stimulation of HEK293 MD2-CD14-TLR4 cells with PBS (control), LPS, HMGB1, PRDX2 and PRDX5 in presence of isotype control antibody or neutralizing anti-TLR4 antibody. Data are mean ± S.D. Number of independent experiments, *N* = 2, with duplicates in each. **(a,b,e)** ns, not significant (*p* > 0.05), * *p* ≤ 0.05, ** *p* ≤ 0.01, **** *p* ≤ 0.0001 (Dunnett's test with PBS as control condition).

As both recombinant PRDXs possess a N-terminal 6xHis tag, we also validated that such tag did not induce IL-8 secretion by itself, using a synthetic peptide composed of a cysteine residue and six histidine residues (Cys-His₆) (**Figure S14b**).

As PRDX5, PRDX2 can bind directly to immobilized TLR4

Using AFM-based SMFS, we previously demonstrated that human PRDX5 was able to directly and specifically interact with immobilized TLR4 [487,511]. Here, we employed a similar strategy to probe the interactions between recombinant human PRDX2 and TLR4 (**Figure 48**). AFM tips were functionalized by taking advantage of the PRDX2 6xHis tag to graft the molecule in an oriented manner, *i.e.* through its N-terminus. PRDX2 molecules were attached to maleimide-terminated flexible polyethylene glycol (PEG₂₇) linkers by means of *tris*-nitrilotriacetic acid (*tris*-NTA) molecules, that binds 6xHis tag with a very high affinity through complex formation with Ni²⁺ (**Figure 48a**). For the surface bearing TLR4, *tris*-NTA molecules were covalently attached to the carboxyl groups of a mixed self-assembled monolayer (SAM) of carboxyl- and hydroxyl-terminated alkanethiols immobilized onto an inert gold surface. Recombinant 10xHis-tagged human TLR4 molecules lacking the intracellular domain portion were then complexed with the *tris*-NTA molecules using Ni²⁺ (**Figure 48a**).

Using force-distance (FD) curve-based AFM, we first recorded multiple 25-μm²-maps at a piezo retraction speed of 1 μm·s⁻¹. As some FD curves detected adhesion events, we performed controls to verify the specificity of the events, using either tips lacking PRDX2 (functionalized solely with *tris*-NTA-Ni²⁺), or surfaces lacking TLR4 (functionalized solely with *tris*-NTA-Ni²⁺), or by injecting neutralizing anti-TLR4 antibody or anti-PRDX2 serum. We assessed the binding frequency (BF) by taking the ratio between the number of specific adhesive FD curves and that of total recorded FD curves. In all cases, the BF significantly decreases, confirming that PRDX2 ligand could undergo specific interactions with TLR4 (**Figure 48b**).

We next investigated the binding kinetics of the PRDX2-TLR4 interaction by performing a dynamic force spectroscopy (DFS) analysis. We recorded multiple maps at different piezo retraction speed (**Figure 48c**). For each specific adhesive curve, we extracted the rupture force (F) and the loading rate (LR), which corresponds to the force applied over time (**Figure 48c**). The spectrum of LRs probed was divided into discrete ranges (LR#1-7) as previously established [440,504]. For each of them, we generated a kernel distribution of the rupture forces using a normal kernel smoothing function, allowing the determination of the most probable rupture forces for single and double interactions, as performed previously [511] (**Figure 48d** and **Figure S15**). The forces corresponding to single interactions showed a linear increase with the LR, and the Bell-Evans model [429] was therefore fitted to these data, resulting in a dissociation rate constant (k_{off}) of 0.0052 ± 0.0050 s⁻¹, and a distance to the transition state (x_{β}) of 0.762 ± 0.072 nm (**Figure 48e**). As the k_{off} corresponds to the inverse of the interaction lifetime, this result means that the PRDX2-TLR4

complex is more stable than the PRDX5–TLR4 complex [511]. Using these parameters, we employed the Williams-Evans prediction [433] to model the rupture forces of double uncorrelated bonds which would simultaneously disrupt. The most probable rupture forces obtained for double interaction align well with the prediction (**Figure 48e**). Overall, these results indicate that PRDX2 direct binding to TLR4 might be associated to its DAMP function, *i.e.* that IL-8 secretion might result from the binding of PRDX2 to TLR4.

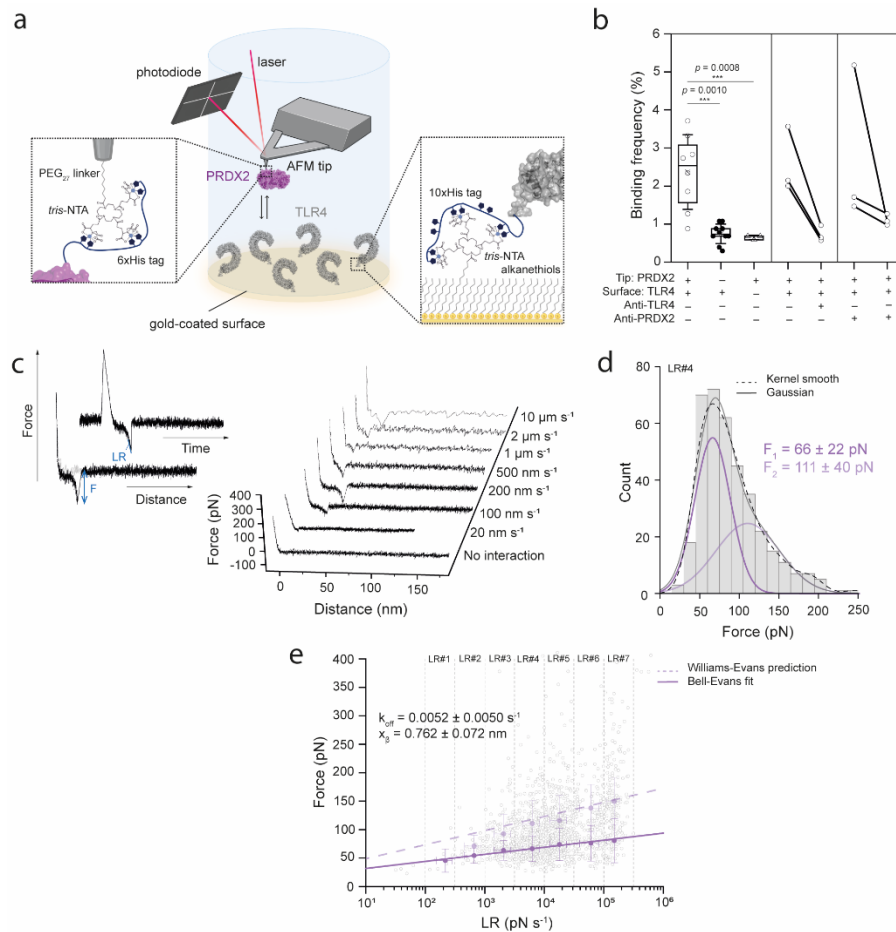


Figure 48. Recombinant PRDX2 is able to bind directly to immobilized TLR4. (a) Atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) was used to probe the interaction between PRDX2 and TLR4. PRDX2 and TLR4 were attached by means of their histidine tag by forming a complex with *tris*-NTA-Ni²⁺, either to a PEG linker-functionalized AFM tip, or to carboxyl groups of a mixed self-assembled monolayer (SAM) of alkanethiols on a gold surface, respectively. Created with BioRender.com. (b) Box plot showing the binding frequencies (BFs) obtained at a piezo retraction speed of 1 $\mu\text{m}\cdot\text{s}^{-1}$ with PRDX2-functionalized tips and TLR4-grafted surfaces (○), tips functionalized with *tris*-NTA-Ni²⁺ but without PRDX2 (●), and PRDX2-functionalized tips and surfaces functionalized with *tris*-NTA-Ni²⁺ but without

TLR4 (Δ). Box plot shows mean ($\times$), median ($-$), 25th and 75th percentiles (boxes), and S.D. (whiskers). Number of AFM maps, $n = 8, 10$, and 3 , respectively. BFs obtained before and after injection of a neutralizing anti-TLR4 antibody or anti-PRDX2 serum. Number of AFM maps, $n = 2$ for each data points. **(c)** Representative specific force-distance (FD) curve (up) and force-time curve (down) showing an adhesive event. Rupture force corresponds to the height of the adhesive peak, and the loading rate (LR) is obtained with a linear fitting of the force-time curve just before rupture. *** $p \leq 0.001$ (Student's t -test). **(c)** Representative FD curves showing no interaction or adhesive events recorded at the seven piezo retraction speeds used. **(d,e)** Dynamic force spectroscopy (DFS) analysis of PRDX2-TLR4 interaction. Spectrum of LRs was divided into discrete LR ranges (LR#1–7). For each LR range, a kernel distribution of the rupture forces was generated, as shown on the histogram for LR#4 **(d)** (see **Figure S15** for other LR ranges), which was fitted with multiple Gaussian functions to determine the most probable rupture forces (full colored data points) for single (1st peak) or double (2nd peak) interactions. These forces were reported on the DFS plot **(e)**. The Bell-Evans model was fitted to most probable rupture forces corresponding to single interactions (solid line), and the predicted rupture forces for two simultaneous uncorrelated interactions were calculated using Williams-Evans prediction (dashed line). From the Bell-Evans model, the k_{off} and x_β values were extracted. The most probable rupture forces are peak center $\pm$ S.D. Number of independent experiments, $N = 8$, number of AFM maps, $n = 100$, obtained with 25 different tips.

TLR4 alone is not able to induce PRDX2- and PRDX5-dependent IL-8 secretion

As both PRDX2 and PRDX5 are able to bind to TLR4 and to induce signaling in a TLR4-dependent manner, we next assess whether TLR4 was sufficient to trigger IL-8 secretion by itself. We quantified IL-8 production from HEK293 cells expressing TLR4 (HEK293 TLR4 cells) exposed to PBS, LPS ($100 \text{ ng}\cdot\text{mL}^{-1}$), HMGB1 ($0.2 \text{ }\mu\text{M}$), PRDX2 ($2 \text{ }\mu\text{M}$) or PRDX5 ($2 \text{ }\mu\text{M}$) for 16 h. Cell surface expression of TLR4 after stable gene transfection into HEK293 cells was validated using FC (**Figure 49a**), and revealed a similar TLR4 expression level compared to HEK293 MD2-CD14-TLR4 cells. For all conditions (except LPS), IL-8 levels were not significantly higher than the control condition (PBS), indicating that TLR4 alone is not sufficient to trigger IL-8 secretion from HEK293 cells (**Figure 49b**). As this result is not the consequence of an insufficient number nor an incorrect cell surface expression of TLR4 [297], it indicates that TLR4 accessory molecules are required for PRDX-induced IL-8 secretion. The irresponsiveness of HEK293 TLR4 cell line could arise from the absence of MD-2 as it was reported to assist TLR4 for several ligands, such as HMGB1, the extra domain A of fibronectin, or oxidized low density lipoprotein [205,273,303], and/or from the absence of CD14 which is known to assist TLR4 in ligand delivery and recognition on cell surface [513,514], as well as internalization [514,515].

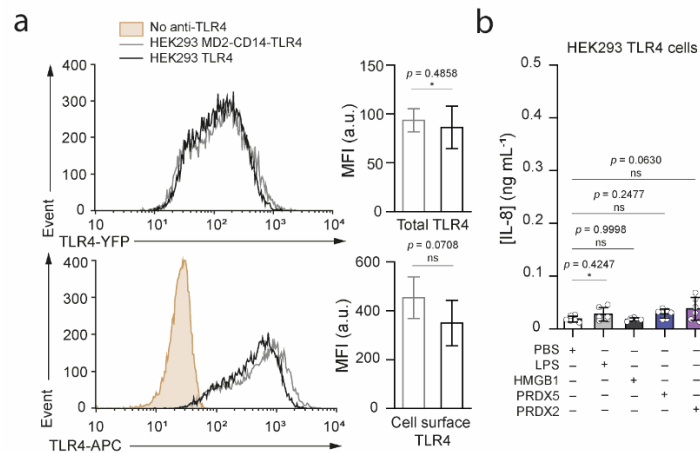


Figure 49. TLR4 alone is not able to induce a PRDX2- and PRDX5-dependent signaling. (a) FC analysis of total (up) and cell surface (down) TLR4 staining on HEK293 TLR4 cells compared to HEK293 MD2-CD14-TLR4 cells. Bars represent median fluorescence intensity (MFI) $\pm$ S.D. Number of independent experiments, $N = 3$, with duplicates in each. ns, not significant ($p > 0.05$), * $p \leq 0.5$ (Student's t -test). **(b)** IL-8 secretion levels from HEK293 TLR4 cells after exposure to PBS, LPS (100 ng·mL⁻¹), HMGB1 (0.2 μ M), PRDX2 (2 μ M) or PRDX5 (2 μ M) for 16 h. Data are mean $\pm$ S.D. Number of independent experiments, $N = 3$, with duplicates in each. ns, not significant ($p > 0.05$), * $p \leq 0.5$ (Dunnett's test with PBS as control condition).

MD-2 assists TLR4 in the recognition of PRDX2 and PRDX5

To evidence the role of MD-2 in the TLR4-dependent signaling of PRDX2 and PRDX5, we expressed MD-2 and TLR4 together in HEK293 cells. Transient transfection of both genes was functionally validated by observing whether responsiveness of HEK293 cells was restored upon transient expression of MD-2, CD14 and TLR4 following by exposure to LPS (100 ng·mL⁻¹), HMGB1 (0.2 μ M), PRDX2 (2 μ M) or PRDX5 (2 μ M) (**Figure S14c**). Exposing HEK293 MD2-TLR4 cells to PRDX2 or PRDX5 resulted in a significant higher IL-8 secretion compared to the control PBS condition (**Figure 50a**), indicating that MD-2 is required for TLR4-dependent signaling of both PRDXs. The role of MD-2 was further validated by exposing HEK293 MD2-CD14-TLR4 cells to the various treatments in presence of a neutralizing anti-MD-2 antibody (**Figure 50b**). Quantification of IL-8 levels compared to the isotype control antibody resulted in a 1.5-fold decrease for LPS, 6.3-fold decrease for HMGB1, 3.7-fold decrease for PRDX5 and 5.9-fold decrease for PRDX2, confirming that PRDX signaling through TLR4 involves MD-2.

Further, to investigate the role of MD-2 in the TLR4-dependent recognition of PRDX2 and PRDX5, we performed AFM-based SMFS using recombinant MD-2, assuming that PRDX binding may occur simultaneously on binding sites on both TLR4 and MD-2, just like LPS and HMGB1. PRDX molecules were covalently grafted to AFM

tips using an aldehyde-terminated PEG₂₄ linker, and recombinant MD-2 molecules were covalently attached to carboxyl groups of a mixed SAM of carboxyl- and hydroxyl-terminated alkanethiols immobilized onto an inert gold surface (**Figure 50c**). We performed FD curve-based AFM to extract the BF_s and DFS plots for both interactions (PRDX2–MD-2 and PRDX5–MD-2) (**Figure 50d,e,f** and **Figure S16**). As controls, we injected anti-PRDX2 or anti-PRDX5 sera, leading to decreased BF_s. DFS analysis resulted in k_{off} and x_{β} values of $0.1825 \pm 0.0774 \text{ s}^{-1}$ and $0.451 \pm 0.029 \text{ nm}$ for PRDX5–MD-2 interaction, and $0.0644 \pm 0.0593 \text{ s}^{-1}$ and $0.457 \pm 0.051 \text{ nm}$ for PRDX2–MD-2 interaction (**Figure 50e,f**). These results mean that the interaction lifetime of MD-2 with PRDX5 is lower than with PRDX2. In addition, the k_{off} value obtained for PRDX2–MD-2 interaction is higher than that of PRDX2–TLR4 interaction, indicating that PRDX2 form a more stable complex with TLR4.

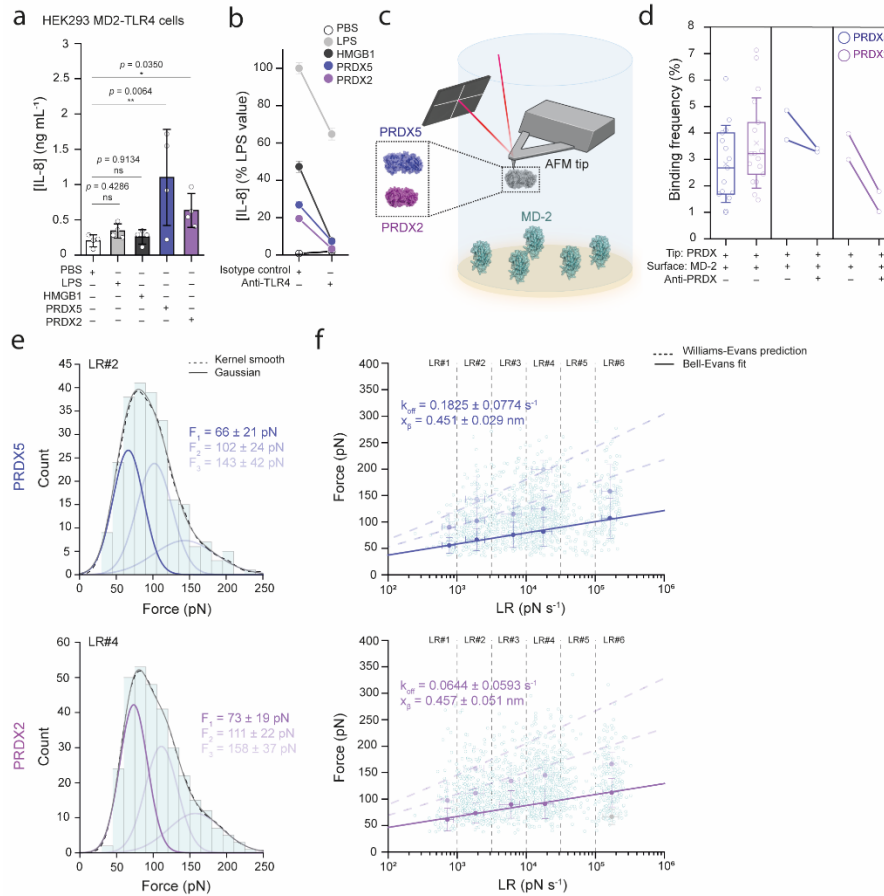


Figure 50. MD-2 is required for PRDX2 and PRDX5 to induce a TLR4-dependent IL-8 secretion. (a) IL-8 secretion levels produced by HEK293 cells transiently expressing MD-2 and TLR4, after stimulation with PBS, LPS (100 ng·mL⁻¹), HMGB1 (0.2 μ M), PRDX2 (2 μ M) or PRDX5 (2 μ M) for

16 h. Data are mean $\pm$ S.D. Number of independent experiments, $N = 2$ with duplicates in each. ns, not significant ($p > 0.05$), * $p \leq 0.05$, ** $p \leq 0.01$ (Dunnett's test with PBS as control condition). **(b)** Stimulation of HEK293 MD2-CD14-TLR4 cells with PBS (control), LPS, HMGB1, PRDX2 and PRDX5 in the presence of isotype control antibody or neutralizing anti-MD-2 antibody. Number of independent experiments, $N = 2$, with duplicates in each. **(c,d,e,f)** PRDX2 and PRDX5 are able to bind directly to recombinant MD-2. **(c)** AFM-based SMFS set-up used to probe PRDX2–MD-2 and PRDX5–MD-2 interactions. PRDXs were attached to PEG linker-functionalized AFM tips, and MD-2 molecules were grafted to carboxyl groups of a mixed SAM of alkanethiols on the surface, both through free amino groups. Created with BioRender.com. **(d)** Box plot showing the BFs obtained at a piezo retraction speed of $1 \mu\text{m}\cdot\text{s}^{-1}$ with PRDX-functionalized tips and MD-2-grafted surfaces. Box plot shows mean ($\times$), median (–), 25th and 75th percentiles (boxes), and S.D. (whiskers). Number of AFM maps, $n = 15$ and 17 for PRDX5 and PRDX2, respectively. BFs obtained before and after injection of anti-PRDX serum. Number of AFM maps, $n = 3$ for each data points. **(e,f)** DFS analysis of PRDX5–MD-2 (up) and PRDX2–MD-2 (down) interactions. Histograms **(e)** showing the kernel density function and the gaussian functions (see **Figure S3** for other LR ranges), and DFS plots **(f)** showing the most probable rupture forces for single and multiple interactions. The most probable rupture forces are peak center $\pm$ S.D. Number of independent experiments, $N = 3$ and 2 , number of AFM maps, $n = 54$ and 61 , for PRDX5 and PRDX2, respectively, obtained with 11 different tips in each case.

PRDX2 and PRDX5 can induce a TLR4-independent signaling involving CD14

To assess the role of CD14 in PRDX5- and PRDX2-induced TLR4-dependent signaling, we exposed HEK293 MD2-CD14-TLR4 cells to PBS, LPS ($100 \text{ ng}\cdot\text{mL}^{-1}$), HMGB1 ($0.2 \mu\text{M}$), PRDX2 ($2 \mu\text{M}$), and PRDX5 ($2 \mu\text{M}$) in the presence of a neutralizing anti-CD14 antibody. Compared to the conditions with the isotype control antibody, IL-8 secretion shows a 9.4-fold decrease for LPS, a 14.2-fold decrease for HMGB1, a 2.2-fold decrease for PRDX5 and a 1.8-fold decrease for PRDX2, in the presence of anti-CD14 antibody (**Figure 51a**).

Furthermore, we transiently transfected HEK293 cells stably expressing CD14 (HEK293 CD14 cells) with the TLR4-coding vector, and we measured IL-8 secretion following stimulation with PBS, LPS ($100 \text{ ng}\cdot\text{mL}^{-1}$), HMGB1 ($0.2 \mu\text{M}$), PRDX2 ($2 \mu\text{M}$), and PRDX5 ($2 \mu\text{M}$) for 16 h (**Figure 51b**). Transient transfection with TLR4 plasmid was functionally validated by evaluating whether HEK293 CD14 cells transiently expressing both MD-2 and TLR4 become responsive again to LPS and HMGB1 (**Figure S14d**). As expected, expression of TLR4 in HEK293 CD14 cells revealed no substantial IL-8 secretion from cells exposed to PBS, LPS, and HMGB1, as both LPS and HMGB1 require MD-2 together with CD14 [273,309,516]. On the other hand, PRDX2- and PRDX5-treated cells significantly increased IL-8 secretion. As control, we analyzed IL-8 secretion from HEK293 CD14 cells. Surprisingly, stimulation with PRDX2 and PRDX5 elicited an IL-8 secretion whereas no IL-8 secretion was observed for cells exposed to PBS, LPS or HMGB1 (even at $2 \mu\text{M}$) (**Figure 51c**). Similar results were obtained with HEK293 cells stably transfected with MD-2 and CD14 (**Figure 51d**), as well as with HEK293 cells transiently expressing CD14 (**Figure S14e**). Using FC, we assessed CD14

surface expression of HEK293 CD14 cells, HEK293 MD2-CD14 cells, and HEK293 MD2-CD14-TLR4 cells. CD14 expression was similar at the surface of HEK293 CD14 cells and HEK293 MD2-CD14-TLR4 cells, whereas HEK293 MD2-CD14 cells showed a slightly increased expression (**Figure 51e**). However, Western blot analysis of total (*i.e.* membrane-bound and intracellular) CD14 expression from lysates of HEK293 CD14 cells revealed a higher level than that of HEK293 MD2-CD14 cells (**Figure 51f**). As controls, we probed total CD14 expression from lysates of HEK293 MD2-CD14-TLR4 cells and from HEK293 cells. HEK293 MD2-CD14-TLR4 cell line revealed a similar level of total CD14 expression than that of HEK293 MD2-CD14 cells, which is consistent with the fact that HEK293 MD2-CD14-TLR4 cell line is derived from the HEK293 MD2-CD14 cell line. In addition, HEK293 cells showed no detectable CD14 expression, as expected [517]. As CD14 is known to be associated with a constitutive endocytosis pathway that is accelerated upon ligand exposure such as LPS [304], the higher amount of total CD14 in HEK293 CD14 cells could explain the higher rate of PRDX internalization, hence the higher IL-8 secretion level from HEK293 CD14 cells compared to HEK293 MD2-CD14 cells.

Altogether, these results indicate that both PRDX2 and PRDX5 can trigger a TLR4-independent signaling involving CD14. In addition, they also corroborate the fact that putative LPS contamination is not responsible for the pro-inflammatory effect observed with the recombinant PRDXs. However, the extent of CD14 involvement in PRDX2- and PRDX5-induced TLR4-dependent pro-inflammatory response should be further explored.

Soluble CD14 is not involved in PRDX2- and PRDX5-dependent IL-8 secretion

By probing soluble CD14 (sCD14) expression levels from supernatants of HEK293 CD14 cells and HEK293 MD2-CD14 cells using Western blot, we reported a higher expression from HEK293 CD14 cells (**Figure 51f**). Therefore, the higher IL-8 secretion levels from HEK293 CD14 cells compared to HEK293 MD2-CD14 cells could be also attributed to the presence of a large number of sCD14 molecules allowing more PRDXs to be recognized in a given time. To test this hypothesis, we assessed whether sCD14 could increase responsiveness of HEK293 cells co-expressing MD-2 and TLR4 following exposure to PRDX2 and PRDX5 by quantifying IL-8 levels following exposure to PBS, LPS ($100 \text{ ng}\cdot\text{mL}^{-1}$), HMGB1 ($0.2 \text{ }\mu\text{M}$), PRDX2 ($2 \text{ }\mu\text{M}$), and PRDX5 ($2 \text{ }\mu\text{M}$), in the presence of $4 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$ recombinant human CD14 for 16 h. As shown in **Figure 50a** and **Figure 51g**, cells were more responsive to LPS and HMGB1 upon incubation with sCD14, as expected [513,518]. However, increased IL-8 production was not observed for PRDX2 nor PRDX5, which indicates that sCD14 is not involved in PRDX-dependent IL-8 secretion.

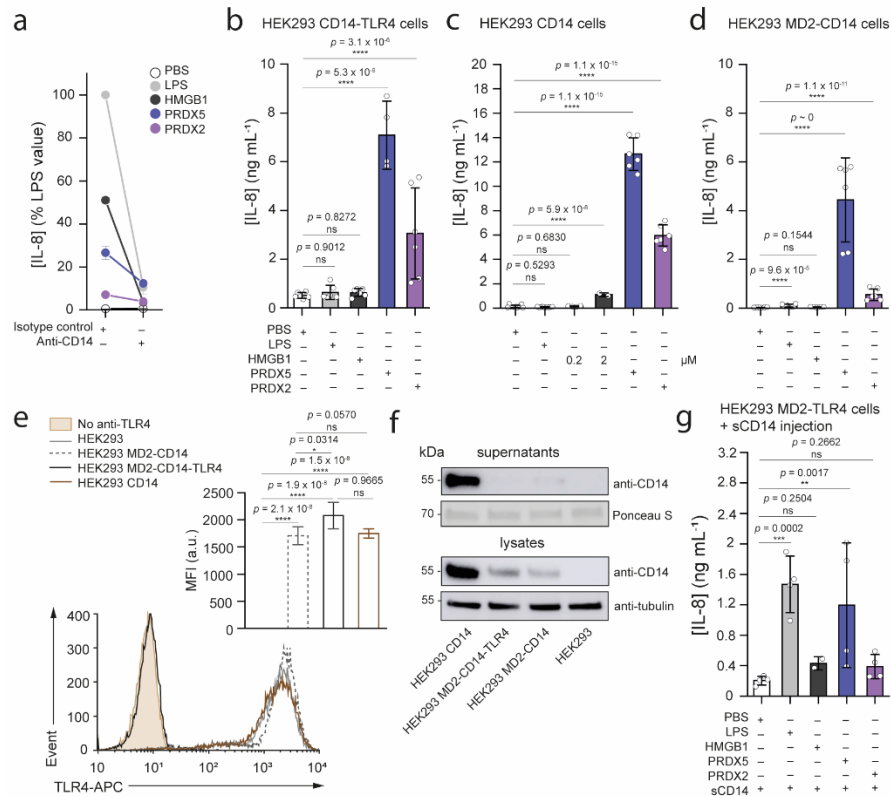


Figure S1. PRDX2 and PRDX5 can induce a TLR4-independent signaling involving CD14. (a) Stimulation of HEK293 MD2-CD14-TLR4 cells with PBS (control), LPS, HMGB1, PRDX2 and PRDX5 in presence of isotype control antibody or neutralizing anti-CD14 antibody. Data are mean $\pm$ S.D. Number of independent experiments, $N = 2$, with duplicates in each. IL-8 secretion levels secreted from (b) HEK293 CD14-TLR4 cells, (c) HEK293 CD14 cells, or (d) HEK293 MD2-CD14 cells, after stimulation with PBS, LPS (100 ng·mL⁻¹), HMGB1 (0.2 μ M, and 0.2 or 2 μ M for HEK293 CD14 cells), PRDX2 (2 μ M) or PRDX5 (2 μ M) for 16 h. Data are mean $\pm$ S.D. Number of independent experiments, $N = 3$ with duplicates in each. (e) FC analysis of cell surface CD14 staining on HEK293 CD14 cells, HEK293 MD2-CD14-TLR4 cells, and HEK293 MD2-CD14 cells compared to HEK293 control cells. Bars represent median fluorescence intensity (MFI) $\pm$ S.D. Number of independent experiments, $N = 2$, with duplicates in each. ns, not significant ($p > 0.05$), * $p \leq 0.05$, **** $p \leq 0.0001$ (Tukey HSD test). (f) Western blot analysis of soluble CD14 from cell supernatants (up) and of total (intracellular and membrane-bound) CD14 from cell lysates (down), from HEK293 CD14 cells, HEK293 MD2-CD14-TLR4 cells, HEK293 MD2-CD14 cells, and HEK293 cells. Western blot with anti-CD14 antibody (upper bands) and with Ponceau red staining or anti-tubulin antibody (lower bands). Source data are provided in Figure S17. Representative from two independent experiments. (g) IL-8 secretion levels produced by HEK293 cells transiently expressing MD-2 and TLR4, after stimulation with PBS, LPS (100 ng·mL⁻¹), HMGB1 (0.2 μ M), PRDX2 (2 μ M) or PRDX5 (2 μ M) in the presence of soluble recombinant CD14 for 16 h. Data are mean $\pm$ S.D. Number of independent experiments, $N = 2$ with duplicates in each. (b,c,d,g) ns, not significant ($p > 0.05$), ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Dunnett's test with PBS as control condition).

PRDX2 and PRDX5 are able to bind directly to CD14

We next used AFM-based SMFS to determine whether human PRDX2 and PRDX5 were able to interact directly with CD14 (**Figure 52a**). Similar functionalization strategies as for MD-2 were used. We performed FD curve-based AFM to extract the BFs and DFS plot for both interactions (PRDX2–CD14 and PRDX5–CD14) (**Figure 52b**). As controls, we injected neutralizing anti-CD14 antibody and anti-PRDX2 or anti-PRDX5 sera. While anti-PRDX sera lead to a decrease in the BFs, no substantial decrease could be observed with anti-CD14 antibody. This could be explained by the fact that this monoclonal antibody was selected for its ability to efficiently block the biological activity of TLR4. Therefore, the CD14 antibody paratope that recognizes the specific TLR4 epitope might be at a different location than the binding site of PRDX2 and PRDX5 on CD14⁵. DFS analysis resulted in k_{off} and x_{β} values of $0.0049 \pm 0.0057 \text{ s}^{-1}$ and $0.938 \pm 0.107 \text{ nm}$ for PRDX5–CD14 interaction, and $0.0059 \pm 0.0071 \text{ s}^{-1}$ and $0.913 \pm 0.112 \text{ nm}$ for PRDX2–CD14 interaction (**Figure 52c,d** and **Figure S18**). These results suggest that PRDX2 interaction lifetime with CD14 is very similar to that of PRDX5. In addition, the k_{off} value obtained for PRDX2–CD14 interaction is similar to that of PRDX2–TLR4 interaction, indicating that the stability of the PRDX2–CD14 complex is similar to that of the PRDX2–TLR4 complex. This observation is consistent with the structural similarity between CD14 and the extracellular domain of TLR4, containing both leucine-rich repeats assembled in a characteristic horseshoe-shaped solenoid structure [519]. However, the x_{β} values are different, which may indicate a different binding geometry.

To further investigate the binding of PRDX2 and PRDX5 to CD14, we assessed the binding of PRDX5 at the surface of living HEK293 MD2-CD14-TLR4 cells and HEK293 CD14 cells using a FC binding assay as per prior experiments [511]. As shown in **Figure 52e**, PRDX5 is able to bind the surface of HEK293 cells expressing CD14 and lacking TLR4.

⁵ To test whether the anti-CD14 antibody is able to block the biological activity of CD14 independently of the presence of TLR4, IL-8 secretion levels from HEK293 CD14 cells stimulated with PBS (control), LPS, HMGB1, PRDX2 and PRDX5 in presence of the isotype control antibody or the anti-CD14 antibody could be assessed and compared.

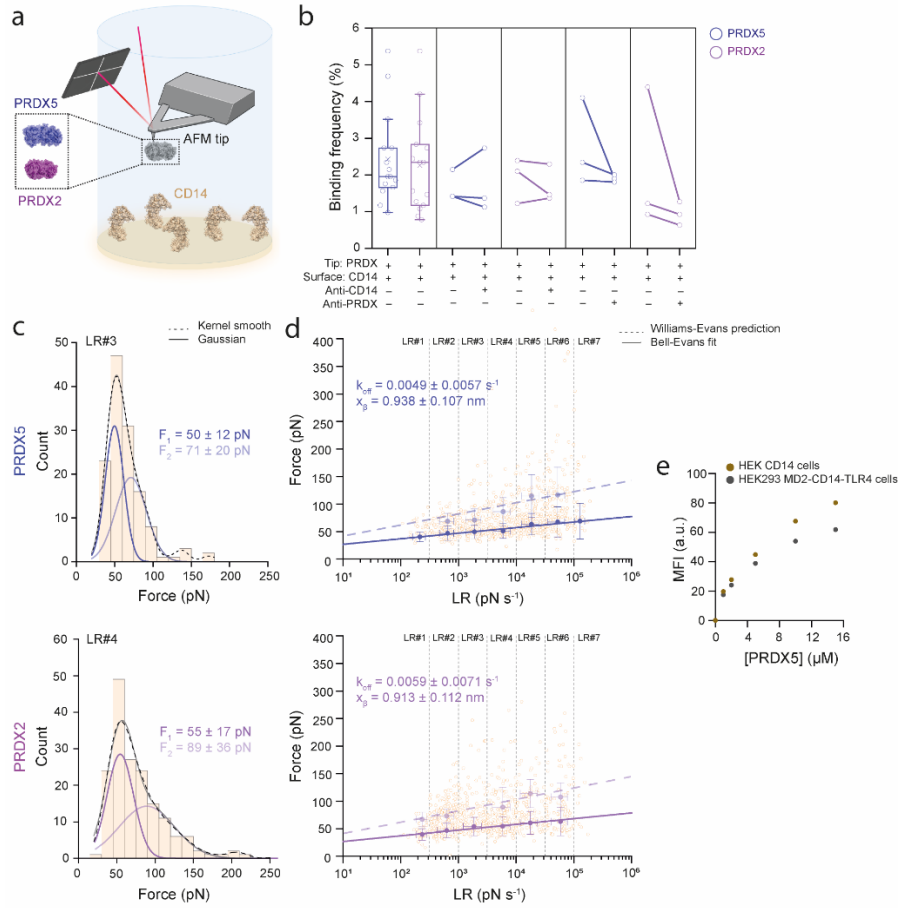


Figure 52. PRDX2 and PRDX5 are able to bind directly to recombinant CD14. (a) AFM-based SMFS set-up used to probe PRDX2-CD14 and PRDX5-CD14 interactions. PRDXs were attached to PEG linker-functionalized AFM tips, and CD14 were grafted to carboxyl groups of a mixed SAM of alkanethiols on the surface, both through free amino groups. Created with BioRender.com. (b) Box plot showing the BF_s obtained at a piezo retraction speed of $1 \mu\text{m}\cdot\text{s}^{-1}$ with PRDX-functionalized tips and CD14-grafted surfaces. Box plot shows mean (x), median (—), 25th and 75th percentiles (boxes), and S.D. (whiskers). Number of AFM maps, $n = 15$. BF_s obtained before and after injection of a neutralizing anti-CD14 antibody or anti-PRDX serum. Number of AFM maps, $n = 2$ for each data points. (c,d) DFS analysis of PRDX5-CD14 (up) and PRDX2-CD14 (down) interactions. Histograms (left) showing the kernel density function and the gaussian functions (see Figure S18 for other LR ranges), and DFS plots (right) showing the most probable rupture forces for single and double interactions. The most probable rupture forces are peak center $\pm$ S.D. Number of independent experiments, $N = 2$, number of AFM maps, $n = 27$ and 21 for PRDX5 and PRDX2, respectively, obtained with 11 different tips in each case. (e) Graph from FC binding assay showing PRDX5 binding at the surface of HEK293 CD14 cells compared to HEK293 MD2-CD14-TLR4 cells. Binding corresponds to the MFI and was normalized to the signal of untreated cells. Each data point corresponds to duplicates.

3.3.5. General Discussion

Understanding how DAMPs are able to induce a pro-inflammatory response is of crucial importance to unveil new drug targets to control inflammatory response, as highlighted in the recent COVID-19 pandemic [520,521]. Although TLR4 was shown to be a key receptor involved in human PRDX-dependent pro-inflammatory responses [116,117,344,365,487], the role of its accessory molecules MD-2 and CD14 remained unclear. In this context, we investigated whether TLR4-dependent pro-inflammatory response induced by human typical and atypical 2-Cys PRDXs, and PRDX2 and PRDX5 in particular, requires MD-2 and/or CD14.

We previously showed that human PRDX5 was able to induce IL-1 β secretion from PMA-differentiated THP-1 cells [487]. Here, we report that both recombinant human PRDX2 and PRDX5 are able to trigger IL-8 secretion from PMA-treated THP-1 cells, confirming their role as DAMPs [41]. The measurement of IL-8 levels to investigate the effect of PRDXs as DAMPs is relevant as IL-8 is known to play a key role in inflammatory-related pathologies such as ischemia-reperfusion injury [522]. Although we have previously demonstrated the importance of the disulfide bond in the pro-inflammatory activity of PRDX5 [511], little is known about how PRDX2 redox state and oligomeric conformation could affect its pro-inflammatory activity. Further work would be required to elucidate the redox state in which PRDX2 is secreted/released to be able to induce cytokine secretion.

To assess the role of MD-2 and CD14, we quantified PRDX2- and PRDX5-induced IL-8 production from HEK293 cells engineered to express combinations of TLR4, MD-2 and CD14. Such cell systems have been extensively used for the study of DAMP-induced TLR4-dependent cytokine secretion, including HMGB1 [513], the partially unfolded III-1 domain of fibronectin (Fn III-1c) [338], hyaluronic acid [233], calcineurin B (CnB) [514], heat shock proteins such as Hsp70-like protein 1 [523], as well as S1008A and S1009A proteins [214,524]. We showed that HEK293 cells stably transfected with TLR4, MD-2 and CD14 vectors secreted IL-8 after exposure to PRDX2 or PRDX5, and that addition of neutralizing anti-TLR4 antibody significantly reduce IL-8 levels, confirming the involvement of TLR4 and validating the use of the cell line. We also illustrated that recombinant PRDX2 was able to bind directly to TLR4 using AFM-based SMFS, as previously shown for PRDX5 [487,511]. Although all these results confirm a TLR4-dependent signaling induced by PRDX2 and PRDX5, HEK293 cells transfected solely with TLR4 were not responsive, indicating that MD-2 and/or CD14 are required for PRDX-induced functional TLR4-dependent cytokine secretion. While MD-2 was thought to be required for TLR4 to undergo glycosylation that is essential for its translocation to the cell surface [299,525], it was later evidenced with HEK293 cells that neither TLR4 function nor TLR4 surface targeting require MD-

2 co-expression [297]. The involvement of MD-2 and/or CD14 in recognition and signaling has been reported for other DAMPs. MD-2 has been shown to discriminate between HMGB1 redox isoforms, with the disulfide form being the only one recognized by the TLR4/MD-2 complex [273], and efficient recognition was reported to require CD14 [513]. FnIII-1c-mediated pro-inflammatory response requires both MD-2 and CD14, and is strictly dependent on membrane-associated CD14 [338]. Moreover, CD14 has been demonstrated to have a direct role in endocytosis of the DAMP protein S100A9 [515], whereas recognition and internalization of CnB is MD-2- and CD14-dependent [514]. Furthermore, the response of microglia to fibrillar β -amyloid (fA β) was found to be reliant upon CD14, which act together with TLR4 and TLR2 to bind fA β and to activate intracellular signaling [337]. Also, biglycan-mediated TLR2/TLR4-dependent inflammatory response in macrophages was reported to require CD14 [526]. Therefore, elucidating whether MD-2 and/or CD14 are involved in the TLR4-dependent recognition of PRDXs and subsequent cytokine secretion was of relevant interest.

With the aim of deciphering the role of MD-2 in the TLR4-dependent cytokine secretion induced by PRDX2 and PRDX5, we quantified IL-8 secretion from HEK293 cells co-expressing MD-2 and TLR4, which confirmed the involvement of MD-2. The role of MD-2 was further demonstrated with the use of a neutralizing anti-MD-2 antibody that lead to decreased IL-8 secretion levels. Furthermore, a direct interaction between both PRDXs and recombinant MD-2 was observed with AFM-based SMFS, which most likely indicates that MD-2 is involved in the complex formed by PRDX2 or PRDX5 and TLR4. We previously showed that the disulfide form of PRDX5 was required to induce a strong IL-8 secretion [511], but whether MD-2 is able to discriminate between PRDX redox forms, as is the case for HMGB1 [273], remains to be determined.

For the purpose of deciphering the role of CD14, we quantified IL-8 secretion from HEK293 cells co-expressing CD14 and TLR4, and compared the levels secreted to those of HEK293 cells solely transfected with the CD14 gene. Surprisingly, both PRDX2 and PRDX5 were able to induce a TLR4-independent IL-8 secretion involving CD14. This result is in line with another study showing a TLR4-independent internalization of S100A9 in TLR4-knockout BMDCs [515], and with the observation of Riddell *et al.* reporting the binding of recombinant human PRDX1 to macrophages isolated from TLR4^{-/-} (B10ScNJ) mice [344]. Therefore, these results may indicate that other receptor(s) than TLR4 are involved in PRDX recognition and signaling. The role of CD14 in PRDX2- and PRDX5-induced inflammation was further confirmed with the use of a neutralizing anti-CD14 antibody that decreased IL-8 levels. We showed with AFM-based SMFS that PRDX2 and PRDX5 were able to bind directly to recombinant CD14 immobilized on model surfaces, and these results were

confirmed for PRDX5 at the surface of living cells with FC. However, whether TLR4-independent PRDX-mediated signaling through CD14 involves another cell surface transmembrane receptor or leads to endocytosis and subsequent intracellular signaling remains to be elucidated. In addition, further investigation is needed to gain insights into the role of CD14 in PRDX-induced TLR4-dependent binding and cytokine secretion.

Altogether, our results are consistent with previous works on endogenous PRDXs acting as DAMPs or exogenous PRDXs from other species acting as pathogen-associated molecular patterns (PAMPs) showing the involvement of MD-2 and/or CD14. In particular, for exogenous PRDXs, Futura *et al.* showed that 2-Cys *Plasmodium berghei* ANKA (PbA) PRDX induces innate immunity through TLR4/MD-2 [357]. In addition, Riddell *et al.* demonstrated that recombinant human PRDX1 interacts with TLR4 in a complex containing both MD-2 and CD14 [344]. Here, we demonstrated that both PRDX2 and PRDX5 are able to directly bind to MD-2 and that they mediate a TLR4-dependent cytokine secretion requiring MD-2. Moreover, our results show for the first time a TLR4-independent signaling of PRDXs through CD14, underlying a finer modulation of the pro-inflammatory response triggered by PRDXs than initially believed.

3.3.6. Supplementary information

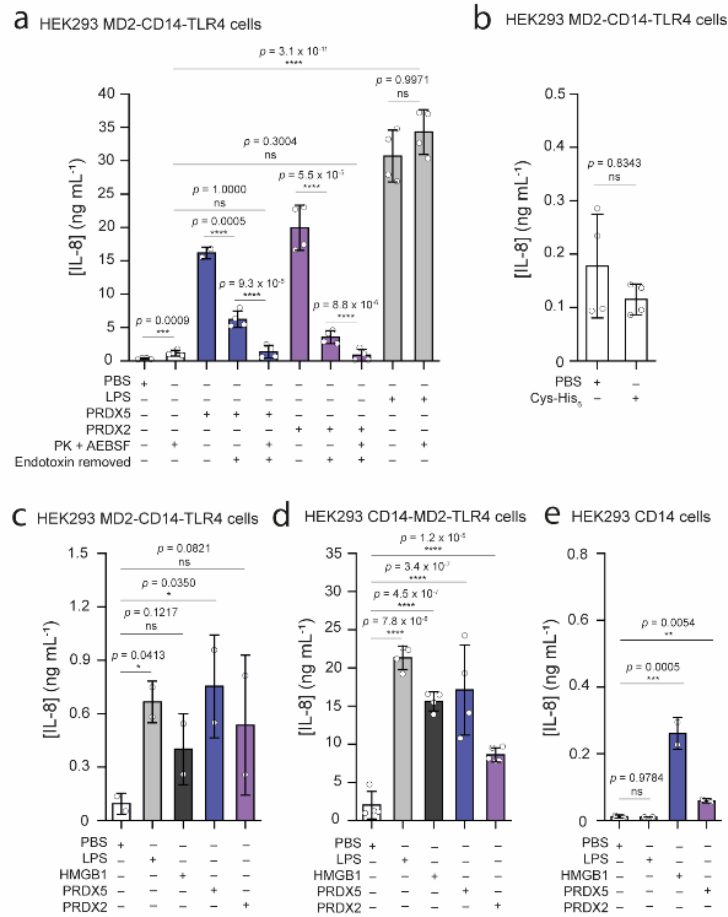


Figure S14. Controls for putative endotoxin contamination, presence of the 6xHis tag, and transient transfections. (a) IL-8 secretion levels produced by HEK293 MD2-CD14-TLR4 cells after stimulation with PBS, proteinase K (PK, 150 $\mu\text{g}\cdot\text{mL}^{-1}$) and AEBSF (0.25 mM), PRDX5 (2 μM) before and after endotoxin removal, PRDX5 (2 μM) incubated in presence of PK (150 $\mu\text{g}\cdot\text{mL}^{-1}$) and AEBSF (0.25 mM), PRDX2 (2 μM) before and after endotoxin removal, PRDX2 (2 μM) incubated in presence of PK (150 $\mu\text{g}\cdot\text{mL}^{-1}$) and AEBSF (0.25 mM), LPS (100 $\text{ng}\cdot\text{mL}^{-1}$), and LPS (100 $\text{ng}\cdot\text{mL}^{-1}$) incubated in presence of PK (150 $\mu\text{g}\cdot\text{mL}^{-1}$) and AEBSF (0.25 mM), for 16 h. ns, not significant ($p > 0.05$), *** $p \leq 0.001$, **** $p \leq 0.0001$ (Tukey HSD test). (b) IL-8 secretion levels produced by HEK293 MD2-CD14-TLR4 cells after stimulation with PBS or Cys-His₆ peptide (2 μM) for 16 h. ns, not significant ($p > 0.05$) (Student's *t*-test). (c,d,e) IL-8 secretion levels produced by HEK293 cells transiently expressing MD-2, CD14 and TLR4 (c), HEK293 CD14 cells transiently expressing MD-2 and TLR4 (d) and HEK293 cells transiently expressing CD14 (e) after stimulation with PBS, LPS (100 $\text{ng}\cdot\text{mL}^{-1}$), HMGB1 (0.2 μM), PRDX5 (2 μM) or PRDX2 (2 μM) for 16 h. All data in are mean $\pm$ S.D. Number of independent experiments, $N = 2$ (a,b,d) or $N = 1$ (c,e) with duplicates in each. ns, not significant ($p > 0.05$), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (Dunnett's test with PBS as control condition).

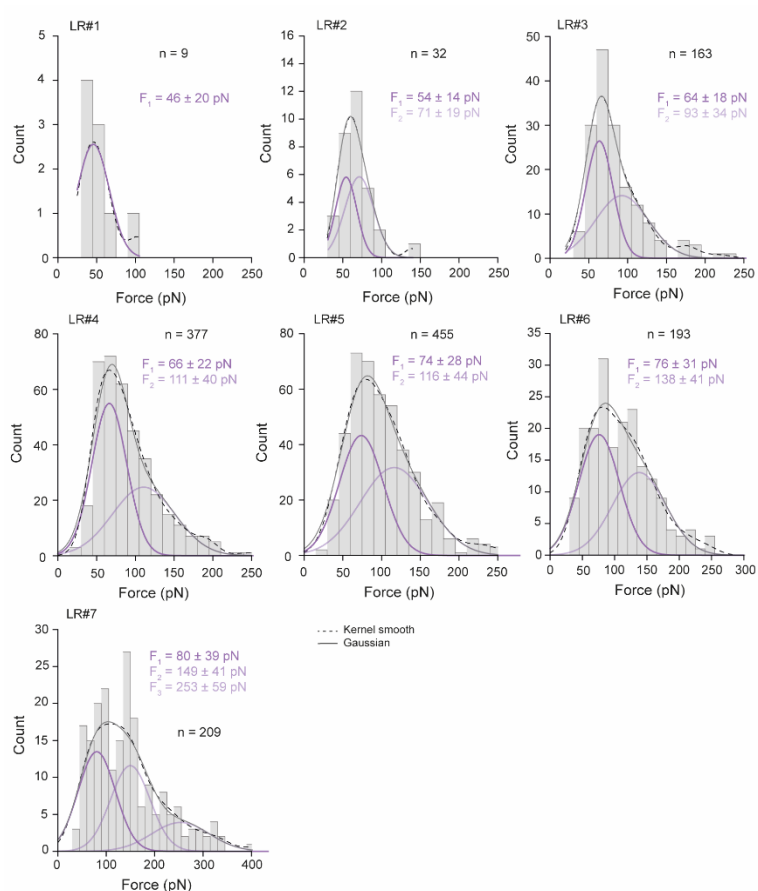


Figure S15. Kernel density function and gaussian functions of the rupture forces obtained at different loading rate (LR) ranges for the PRDX2-TLR4 interaction. Rupture forces were divided into seven discrete LR ranges (LR#1: 100–315 pN·s⁻¹; LR#2: 315–1000 pN·s⁻¹; LR#3: 1000–3150 pN·s⁻¹; LR#4: 3150–10,000 pN·s⁻¹; LR#5: 10,000–31,500 pN·s⁻¹; LR#6: 31,500–100,000 pN·s⁻¹; LR#7: 100,000–315,000 pN·s⁻¹). Kernel smooth function (dashed black line) of the rupture forces (superimposed on force histograms for more clarity) was used to determine the hidden peaks based on its second derivative. Iterative fitting with gaussian functions (magenta lines) was performed so that the cumulative function (grey line) converges to the kernel smooth function. The peaks centers of the gaussian functions were used to determine the most probable rupture forces for single (1st peak) and multiple (following peaks) interactions (F₁, F₂ and F₃ for single and double and triple interactions, respectively). Number of independent experiments, *N* = 8 (comprising 100 AFM maps obtained with 25 different tips). *n*, number of data points.

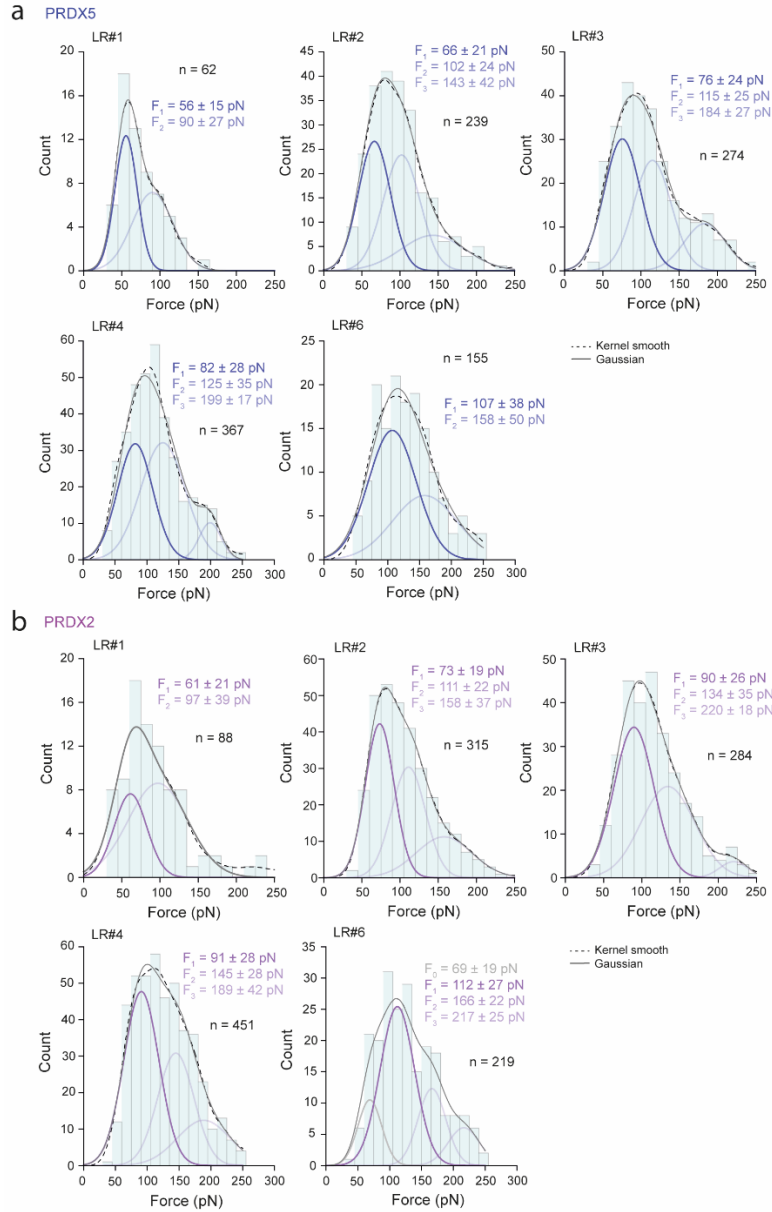


Figure S16. Kernel density function and gaussian functions of the rupture forces obtained at different loading rate (LR) ranges for the PRDX2–MD-2 and PRDX5–MD-2 interactions. Rupture forces of the PRDX5–MD-2 (**a**) and PRDX2–MD-2 (**b**) interactions were divided into six discrete LR ranges (LR#1: 315–1000 pN·s⁻¹; LR#2: 1000–3150 pN·s⁻¹; LR#3: 3150–10,000 pN·s⁻¹; LR#4: 10,000–31,500 pN·s⁻¹; LR#5: 31,500–100,000 pN·s⁻¹; LR#6: 100,000–315,000 pN·s⁻¹). Kernel smooth function (dashed black line) of the rupture forces (superimposed on force histograms for more clarity) was used to determine the hidden peaks based on its second derivative. Iterative fitting with gaussian functions (blue or magenta lines) was performed so that the cumulative function (grey line) converges to the kernel smooth function. The peaks

centers of the gaussian functions were used to determine the most probable rupture forces for single (1st peak) and multiple (following peaks) interactions (F_1 , F_2 and F_3 for single, double and triple interactions, respectively). The total BF (*i.e.* FD curves with specific and nonspecific adhesive events) obtained with MD-2 was higher than that obtained with TLR4 and CD14, which could reflect the presence of dimers or higher molecular weight structures of MD-2 on the surface, leading to a higher frequency of multiple interactions. Note that since there is more noise at higher tip retraction speed (*i.e.* LR) due to thermal fluctuations of the cantilever, some events detected on FD curves that seem to be specific might actually arise from noise. For this reason, the first peak of the LR#6 was considered as arising from noise since it did not align with the first peak of the other LR ranges. Number of independent experiments, $N = 3$ and 2 for PRDX5 and PRDX2, respectively (comprising 54 and 61 AFM maps, respectively, obtained with 11 different tips in each case). n , number of data points.

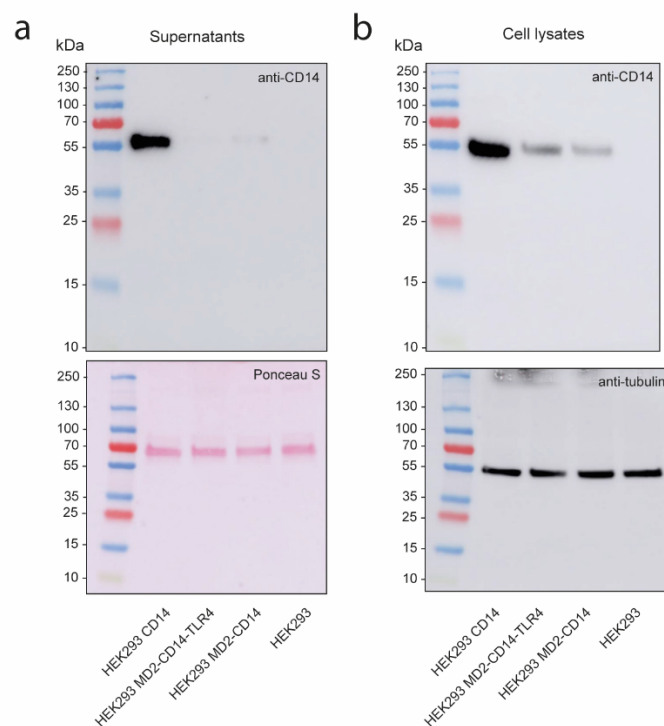
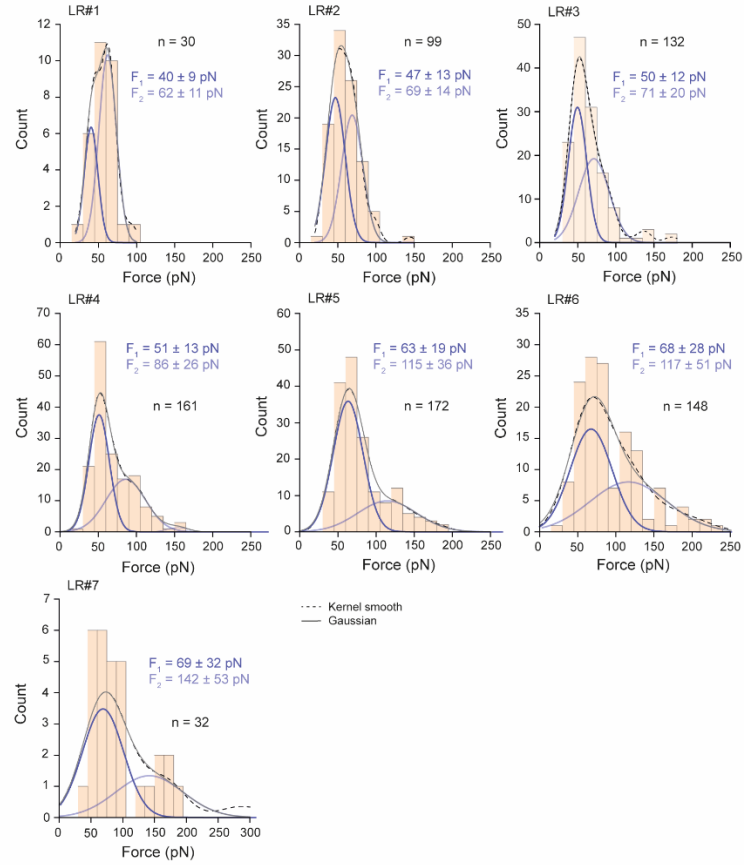


Figure S17. Western blot analysis of CD14 expression. Soluble CD14 **(a)** and total (*i.e.* intracellular and membrane-bound) CD14 **(b)** expression was analyzed by Western blot. **(a)** Cell supernatants containing soluble CD14 were collected after cell incubation in serum-free medium for 16 h. Blot with anti-CD14 antibody (up) and Ponceau S staining (down). **(b)** Total CD14 was analyzed from cell lysates. Blot with anti-CD14 antibody (up) and anti-tubulin antibody as loading control (down). **(a,b)** Representative of two independent experiments.

a PRDX5



b PRDX2

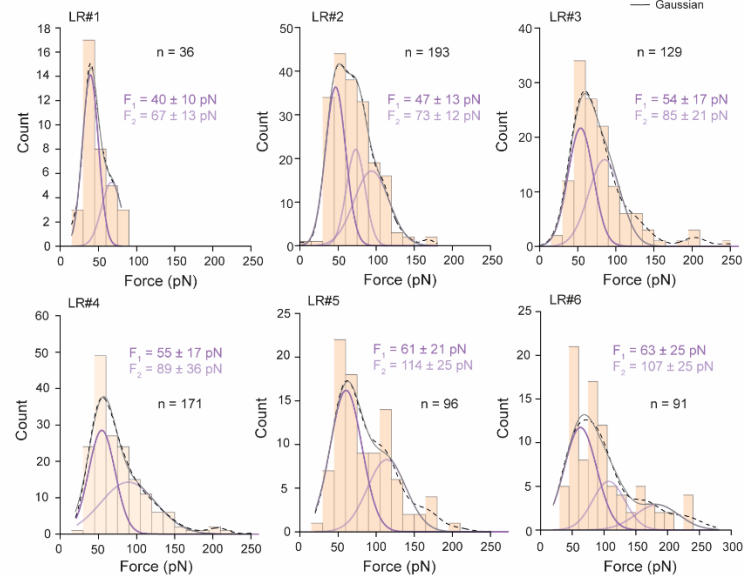


Figure S18. Kernel density function and gaussian functions of the rupture forces obtained at different loading rate (LR) ranges for the PRDX2–CD14 and PRDX5–CD14 interactions. Rupture forces of the PRDX5–CD14 (**a**) and PRDX2–CD14 (**b**) interactions were divided into six or seven discrete LR ranges (LR#1: 100–315 pN·s⁻¹; LR#2: 315–1000 pN·s⁻¹; LR#3: 1000–3150 pN·s⁻¹; LR#4: 3150–10,000 pN·s⁻¹; LR#5: 10,000–31,500 pN·s⁻¹; LR#6: 31,500–100,000 pN·s⁻¹; LR#7: 100,000–315,000 pN·s⁻¹). Kernel smooth function (dashed black line) of the rupture forces (superimposed on force histograms for more clarity) was used to determine the hidden peaks based on its second derivative. Iterative fitting with gaussian functions (blue or magenta lines) was performed so that the cumulative function (grey line) converges to the kernel smooth function. The peaks centers of the gaussian functions were used to determine the most probable rupture forces for single (1st peak) and multiple (following peaks) interactions (F_1 , F_2 and F_3 for single, double and triple interactions, respectively). Number of independent experiments, $N = 2$ (comprising 27 and 21 AFM maps for PRDX5 and PRDX2, respectively, obtained with 11 different tips in each case). n , number of data points.

3.4. Additional results

During the course of the project, we tried to combine various techniques to get a broad understanding of the PRDX-induced inflammatory response, from the early events triggered by cell stimulation with PRDXs (*i.e.* receptor binding) to the late events (*i.e.* transcription factor activation and cytokine secretion). We used label-based and label-free techniques, as well as single-molecule and bulk measurements. However, some of the experiments performed led to results that are not exploitable as such, requiring further developments. This section will summarize the main additional experiments that were performed.

3.4.1. Introduction

In the previous sections, we mainly presented results obtained using SMFS and FC to decipher PRDX binding to TLR4 and its accessory proteins, as well as ELISA to quantify cytokine secretion. To complete the picture of PRDX binding to TLR4 and accessory proteins, we also wanted to use the BLI technique, a “dip-and-read” method that does not require immobilization of the analyte, to investigate the binding of PRDXs to the TLR4/MD-2 complex. In other studies [302,500], valuable insights into the interaction of HMGB1 with the TLR4/MD-2 complex immobilized on a sensor chip were gained using surface plasmon resonance (SPR). As the functioning principle of SPR is close to that of BLI, we wanted to test a similar strategy using BLI to investigate the binding of PRDXs to the TLR4/MD-2 complex immobilized on the biosensor. Furthermore, we tried to isolate the complex formed by PRDX5 and TLR4, with the aim of elucidating whether MD-2 and/or CD14 are physically involved. To this end, we started to set up pull-down and co-IP assays. These experiments were performed in collaboration with D. Marchese from the group of Prof. R. Rezsöházy (LIBST, UCLouvain). Moreover, we attempted to characterize the dynamics of NF- κ B activation following cell stimulation by PRDXs using CLSM. However, all of these techniques have led to rather inconclusive results. Further optimization is certainly necessary to get interpretable results.

Several additional questions were also raised during the course of the project, such as whether the 6xHis tag has an impact on the PRDX activity as DAMP or whether the additional peaks observed on the MS spectra are indeed due to a methylation of the N-terminal Met residue. In this section, we present results aiming at answering these questions. To evaluate the impact of the 6xHis tag on PRDX binding to TLR4, we performed AFM-based SMFS experiments using a PRDX2 protein without affinity tag (also produced in *E. coli* for consistency with the 6xHis-tagged PRDX2). Furthermore, we performed LC-Q-TOF-MS/MS analysis to confirm the hypothesis that some PRDX5 N-terminal Met residues are methylated. These experiments were performed by H. Degand (LIBST, UCLouvain).

In this section, we will first briefly introduce the material and methods used to perform all these experiments, before presenting the preliminary results obtained. In each case, we will suggest lines of improvement.

3.4.2. Material and Methods

LC-Q-TOF-MS/MS to detect N-terminal Met methylation of PRDX5

Preparation for in-solution sample

20 µg of PRDX5^{C47S} and r/aPRDX5 samples were transferred to 0.5 mL polypropylene Protein LoBind tubes (Eppendorf) and precipitated with chloroform-methanol method [508]. The samples were dried under vacuum with a Savant SpeedVac Concentrator (ThermoFisher). 20 µL of 50 mM ammonium bicarbonate (TRIS) pH 8 were added to the pellets for solubilization and vortexed for 30 min at RT. Proteolysis was performed with 1 µg of Mass Spec Grade endo Lys-C (Promega) and allowed to continue overnight at 37°C. The samples were dried under vacuum with the Savant SpeedVac Concentrator.

Peptide separation using nanoUPLC

Before peptide separation, the samples were dissolved in 20 µL of 0.1 % (v/v) formic acid and 2% (v/v) acetonitrile (ACN). The resulting peptide mixtures were separated by reverse phase chromatography on a NanoACQUITY UPLC M-Class system (Waters) working with MassLynx V4.1 (Waters) software. 200 ng of digested proteins were injected on a C18 trap column, 100 Å, 5 µm, 180 µm x 20 mm (Waters), and desalted using isocratic conditions at a flow rate of 15 µL·min⁻¹ using a 99% formic acid and 1% (v/v) ACN buffer for 3 min. Peptide mixtures were subjected to reverse phase chromatography on a PepMap C18 column, 100 Å, 1.8 µm, 75 µm x 150 mm (Waters) for 130 min at 35°C at a flow rate of 300 nL·min⁻¹ using a two part linear gradient from 1% (v/v) ACN, 0.1 % formic acid to 35 % (v/v) ACN, 0.1 % formic acid for 90 min, and from 35% (v/v) ACN, 0.1 % formic acid to 85 % (v/v) ACN, 0.1 % formic acid for 10 min. The column was re-equilibrated at initial conditions after washing for 30 min at 85% (v/v) ACN, 0.1 % formic acid at a flow rate of 300 nL·min⁻¹. For online LC-MS analysis, the nanoUPLC was coupled to the mass spectrometer through a nano-electrospray ionization (nanoESI) source emitter.

LC-Q-TOF-MS/MS Analysis (DDA)

Data-dependent analysis (DDA) was performed on an SYNAPT G2-Si high definition mass spectrometer (Waters) equipped with a NanoLockSpray dual electrospray ion source (Waters). Precut fused silica PicoTipR Emitters for nanoelectrospray (outer diameters: 360 µm; inner diameter: 20 µm; 10 µm tip; 2.5" length) (Waters) were used for samples and Precut fused silica TicoTip Emitters for

nanoelectrospray (outer diameters: 360 μm , inner diameter: 20 μm , 2.5" length) (Waters) were used for the lock mass solutions. The eluent was sprayed at a spray voltage of 2.8 kV with a sampling cone voltage of 25 V and a source offset of 30 V. The source temperature was set to 80°C. The cone gas flow was 20 $\text{L}\cdot\text{h}^{-1}$ with a nanoflow gas pressure of 0.4 bar, and the purge gas was turned off. The SYNAPT G2Si instrument was operated in DDA (data-dependent mode), automatically switching between MS and MS2. Full scan MS and MS2 spectra (m/z 400 to 2000) were acquired from 2 min after injection to 30 min in resolution mode (20,000 resolution FWHM at m/z 400) with a scan time of 0.1 sec. Tandem mass spectra of up to 10 precursors were generated in the trapping region of the ion mobility cell by using a collision energy ramp from 17/19 V (low mass, start/end) to up to 65/75 V (high mass, start/end). Charged ions (+2, +3, +4, +5) were selected to be submitted to the MS/MS fragmentation over the m/z range from 50 to 2000 with a scan time of 0.25 sec. For the post-acquisition lock mass correction of the data in the MS method, the doubly charged monoisotopic ion of [Glu1]-fibrinopeptide B was used at 100 $\text{fmol}\cdot\mu\text{L}^{-1}$ using the reference sprayer of the nanoESI source with a frequency of 30 s at 0.5 $\mu\text{L}\cdot\text{min}^{-1}$ into the mass spectrometer.

ESI-QTOF data processing

Data were processed with UNIFI (Waters) using the known PRDX5 and PRDX5^{C47S} sequence. Methylation as the variable methionine modification and endo-K as the digestion enzyme were selected, and one miss cleavage was allowed.

Pull-down assays and co-IP to identify PRDX5 binding partners

HEK293 MD2-CD14-TLR4 cells were seeded into wells of a 6-well plate at a density of 1×10^6 cells per well. The next day, cells were incubated with recombinant 6xHis-tagged PRDX5 in ice-cold medium for 30 min. As control, cells were incubated with ice-cold medium only. Cells were washed with ice-cold PBS and lysed with ice-cold HEPES lysis buffer (200 mM HEPES, 3 M NaCl, 50% glycerol and 1/200 (v/v) detergent, pH 7.8) for 30 min with gentle agitation. Two detergent were tested for optimization, namely n-dodecyl-B-D-maltoside (DDM) and NP-40. In a first approach, Ni^{2+} -NTA agarose beads (Qiagen) were used to capture the complex *via* 6xHis-tagged PRDX5. Cell lysates were incubated with Ni^{2+} -NTA agarose beads in sodium phosphate elution buffer (20 mM sodium phosphate pH 7.8, 1 M NaCl, imidazole, and in some cases, 8 M urea) at 4°C overnight with gentle agitation. Various imidazole concentrations were tested for optimization. After washing, captured proteins on the beads were separated by SDS-PAGE and analyzed by Western blot using antibodies recognizing YFP (Thermo Fisher Scientific), PRDX5 (Novus Biologicals), MD-2 (R&D Systems) and CD14 (Thermo Fisher Scientific).

As alternative strategy, GFP-Trap agarose beads (Chromotek) were used to immunoprecipitate TLR4-eYFP from cell lysate, together with the other proteins involved in the complex. This system consists of an anti-GFP single domain antibody fragment (V_HH, nanobody) that specifically binds to most common GFP derivatives, including eYFP. Beads were diluted into 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 0.5 mM EDTA. The wash buffer consisted of 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.05% NP-40, and 0.5 mM EDTA.

AFM-based SMFS using untagged PRDX2

Untagged PRDX2 (Sigma) was grafted onto the AFM tips (MSCT probes, Bruker) using APTES coating and a heterobifunctional NHS-PEG₂₇-acetal linker. The protocol was adapted from Professor H. J. Gruber (Johannes Kepler Universität, Institute of Biophysics, Linz). Recombinant human TLR4 (R&D Systems) was grafted through its 10xHis tag by chelation with Ni²⁺-*tris*-NTA molecules covalently attached *via* NHS/EDC to carboxyl groups of a mixed SAM of carboxyl- and hydroxyl-terminated alkanethiols immobilized onto gold surfaces. Tip and surface functionalization, FD-based AFM and data analysis were performed as previously (see **sections 3.2. and 3.3.**).

BLI to analyze binding kinetics of PRDX2 and PRDX5 to TLR4, MD-2 and TLR4/MD-2 complex

BLI experiments were performed with a BLItz instrument (ForteBio) and the BLItz Pro 1.2.1.5 software, using the Advanced Kinetics mode. Two types of biosensors were used, namely amine reactive second generation (AR2G) biosensors functionalized with carboxylic acids, allowing covalent coupling of proteins *via* NHS/EDC-mediated amide bond formation, and Ni-NTA biosensors that have a surface charged with *tris*-NTA groups showing a strong affinity towards His-tagged recombinant proteins. All biosensors were hydrated for at least 10 min prior to use. The recombinant 10xHis-tagged extracellular portion of TLR4 (R&D systems), 10xHis-tagged MD-2 (R&D Systems), and recombinant 10xHis-tagged TLR4/MD-2 complex (R&D Systems) were used as ligands, and 6xHis-tagged PRDX5, 6xHis-tagged PRDX2 and untagged PRDX2 (Sigma) were used as analytes.

ARG2 biosensors were dipped sequentially into kinetic buffer for equilibration, a solution containing 10 mM NHS and 20 mM EDC for activation, the ligand solution (either TLR4, MD-2 or TLR4/MD-2) for loading, and 1 M ethanolamine hydrochloride (pH 8.5) for quenching. For analyte (PRDX) binding, biosensors were sequentially dipped into kinetic buffer for baseline, PRDX solution for association of the analyte with the ligand, and finally kinetic buffer for dissociation of the analyte from the ligand. Three kinetic buffers were tested for optimization: PBS, PBS with 0.1% BSA

and 0.02% Tween 20, and the latter with additional 20 mM imidazole. As for Ni-NTA biosensors, they were dipped sequentially into PBS for equilibration, the ligand solution (His-tagged TLR4, MD-2 or TLR4/MD-2), PBS for baseline, analyte (PRDX) solution for association, and finally PBS for dissociation. For regeneration, biosensors were alternately dipped three times into 10 mM glycine (pH 1.5) for 5 s and PBS for 5 s, before being re-charged with 10 mM NiCl₂ for 1 min. We tested various durations for the loading step so that the signal resulted in a gradual increase without reaching saturation.

Data were processed and analyzed using BLItz Pro 1.2.1.5 software. Step correction was applied at the start of both association and dissociation to correct misalignment resulting from assay artifacts. Data from the reference biosensor were subtracted from data from the sample biosensor, followed by a local fit with a 1:1 binding model, assuming that the interaction between ligand and analyte follows pseudo-first-order kinetics. Fits were evaluated by confirming that χ^2 was below 3 and R² over 0.95.

Confocal microscopy to observe NF- κ B activation

HEK293 MD2-CD14-TLR4-eYFP cells were plated at a density of $\sim 3 \times 10^5$ cells into glass-bottomed dishes of 50 x 7 mm (WillCo). When they reach 70-90% confluency, cells were transfected with 2.5 μ g pCMV3-OFPSpark-RelA (Sino Biological) using 3.75 μ L Lipofectamine 3000 (ThermoFisher) and 5 μ L P3000 Reagent (ThermoFisher) in 250 μ L Opti-MEM (ThermoFisher). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 48 h before testing for NF- κ B translocation following cell stimulation.

For stimulation, cells were exposed to 10, 50 or 100 ng·min⁻¹ of the positive control TNF- α (Invivogen) for several hours. Cells were imaged using a Zeiss 980 confocal laser scanning microscope (Zeiss GmbH), equipped with a 405 diode and Helium-Neon lasers, GaAsP detectors, and additional modifications to allow for long term live cell imaging, *i.e.* a chamber that maintains cells at 37°C, with 5% CO₂ in air and a relative humidity of >95%. Excitation for OFP and YFP were at 547 nm and 508 nm, respectively, with the channels being collected sequentially.

3.4.3. Results and Discussion

PRDX5 exhibits methylated N-terminal Met residues

The MS experiments performed to identify PRDX5 oxidation states lead to the fortuitous discovery of a potential methylation site on PRDX5 proteins. On the deconvoluted mass spectra of PRDX5 and PRDX5^{C47S}, we observed the presence of secondary peaks corresponding to a mass increase of 14 Da compared to the parent

peaks associated with specific redox states (see **section 3.2.**). This increase in mass could be attributed neither to oxidation nor to a mix of oxidation and alkylation. Furthermore, a +14-Da mass shift is typically associated with the presence of methylation [527]. We therefore hypothesize that both PRDX5 and PRDX5^{C47S} protein samples contain a mixture of methylated and unmethylated proteins.

A previous study showed that bacterial proteins undergoing N-terminal methylation have either (i) a characteristic basic residue, namely Lys or Arg, in the second position, or (ii) a Pro residue at position 4, and that such methylation could be triggered by recombinant sequences of proteins produced in *E. coli* [495]. The vector that we used for the production of 6xHis-tagged PRDXs encodes the RGS·His epitope (RGS·HHHH), initially developed to allow the recognition of the proteins by anti-RGS·His antibody [528]. In addition, a linker consisting of three residues (GSG) was inserted between the tag and the protein sequences. The whole sequence portion before the protein itself therefore contains 13 residues when considering the additional Met required to initiate synthesis: MRGSHHHHHHGSG. As a consequence, an Arg residue is located in the second position of the sequence, thereby indicating a methylation signal for *E. coli*.

To confirm the hypothesis of a methylation on the N-terminal Met residue, we performed LC-Q-TOF-MS/MS analysis on PRDX5 redox forms. Analysis of the fragments corresponding to peptides containing the N-terminal Met revealed a mass shift of +14 Da in PRDX5^{C47S} and r/aPRDX5, indicating the presence of a methylation (**Figure 53**). This phenomenon has been observed by other researchers who performed a similar intact-protein ESI-MS analysis on the recombinant 6xHis-tagged Z domain of protein A [496]. The 6xHis tag used in their study also consists in MRGSHHHHHHGSG.

These results confirm that the additional peaks can indeed be attributed to the presence of methylated Met residues, thereby dispelling any doubt related to the redox state of the PRDX5 redox forms.

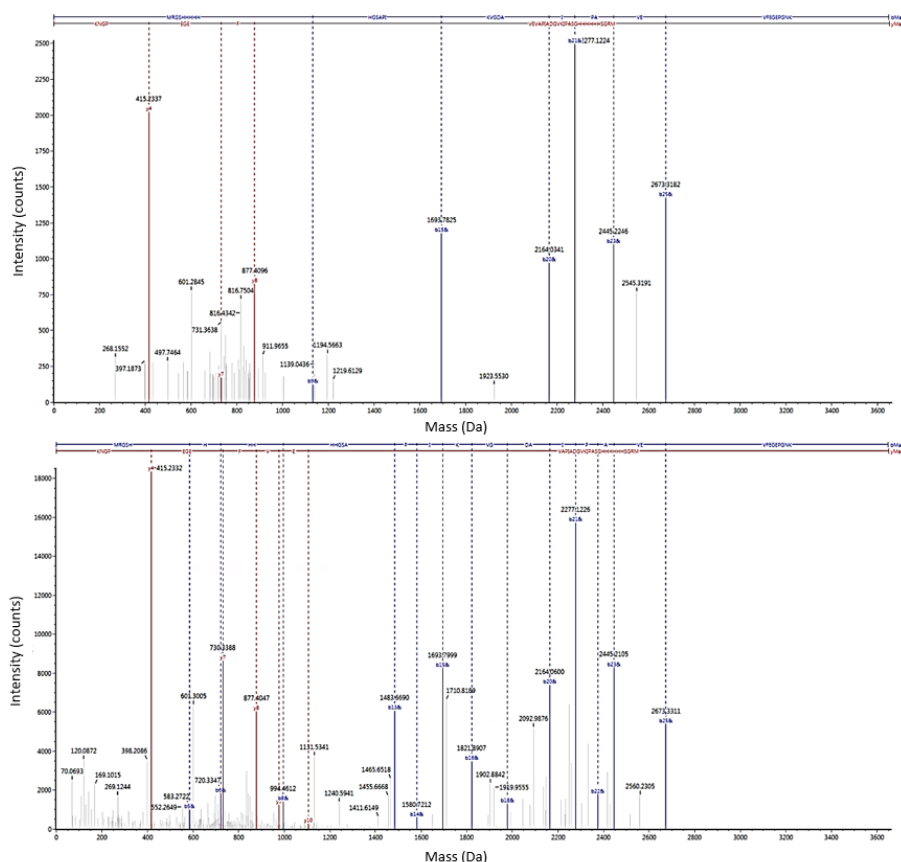


Figure 53. Representative MS/MS spectra of fragments corresponding to peptides containing the N-terminal methionine residue. MS/MS spectrum of PRDX5^{C47S} (up) and r/aPRDX5 (down). Mass spectrometry analysis of a +5 charged parent ion at 730.55 m/z. The Met residue is modified with a methylation, which produces a +14 Da mass increment; PRDX5^{C47S} (up) gives a b5 ion with a m/z of 1131.51 (sequence: M*RGSH) and r/aPRDX5 (down) gives a b9 ion with a m/z of 584.34 (sequence: M*RGSHHHH) allowing exact localization of the modified Met.

Isolating the binding complex with pull-down assays

In an attempt to isolate the binding complex formed by PRDX5 and TLR4, with the aim of evidencing the presence of other binding partners (*i.e.* MD-2 and CD14), we performed pull-down assays following exposure of HEK293 MD2-CD14-TLR4 cells to 6xHis-tagged PRDX5, using Ni²⁺-NTA agarose beads to isolate the complex *via* PRDX5 ('bait' protein) (**Figure 54**).

We first tried different lysis buffer compositions to determine the appropriate detergent (DDM or NP-40) allowing extraction of TLR4 from the plasma membrane (**Figure 54a**). The two bands around 130 kDa observed for TLR4 correspond to

different glycosylation states [525]. As the use of NP-40 resulted in a higher fraction of TLR4 in the cell lysate (*i.e.* soluble fraction) compared to the insoluble fraction (*i.e.* pellet), we performed further experiments using NP-40 as detergent in the lysis buffer. As this experiment was performed with a high PRDX5 concentration of 10 μ M, we analyzed whether PRDX5 concentration could be decreased to 1 μ M (**Figure 54b**). However, a lower PRDX5 concentration yielded a lower signal from the bound proteins on the beads (*i.e.* pull-down), resulting in a too low signal in the blot. We noticed that the amount of pull-down TLR4 was similar no matter the PRDX5 concentration used, as a band of similar intensity could be detected in the blot with the control condition (no cell exposure to PRDX5). As the bands were faint, we performed another pull-down assay with 10 μ M PRDX5 (**Figure 54c**), which resulted in a higher signal for the bands related to TLR4 detection (anti-GFP antibody). However, the band intensity for the negative control (–) was similar to the band obtained for cells incubated with PRDX5 (see red arrow in **Figure 54c**). This nonspecific binding could be due to the presence of five His residues in the TLR4 dimerization site [529], which may result in unwanted binding of TLR4 to the Ni²⁺-NTA agarose beads. We therefore tested various imidazole concentration in the elution buffer, as imidazole is known to compete with His residue for the binding of Ni²⁺-NTA. We performed a pull-down assay with cells that were not exposed to PRDX5 to detect the imidazole concentration that would result in reduced nonspecific TLR4 binding onto the beads (**Figure 54d**). We chose to use an imidazole concentration of 45 mM, which is a good compromise between a sufficiently high concentration to eliminate nonspecific TLR4 binding onto the beads, and a sufficiently low concentration to prevent disruption of 6xHis-tagged PRDX5 binding on the beads. However, the pull-down assay performed with these optimized conditions resulted in similar band for the positive (cell incubation with 6xHis-tagged PRDX5) and the control (no PRDX5 exposure) conditions (see red arrow in **Figure 54e**), yet fainter than previously obtained (**Figure 54c**) due to the higher imidazole concentration.

We therefore turned to an alternative approach to isolate the binding complex by taking advantage of the eYFP fusion of TLR4. To this end, we used agarose beads consisting of the anti-GFP single domain antibody fragment (VHH, nanobody). However, this approach proved to be unsuccessful in isolating TLR4-eYFP in the conditions used (*data not shown*). As we were unsuccessful to isolate the PRDX5–TLR4 complex, we did not go further in the analysis of TLR accessory molecules MD-2 and CD14.

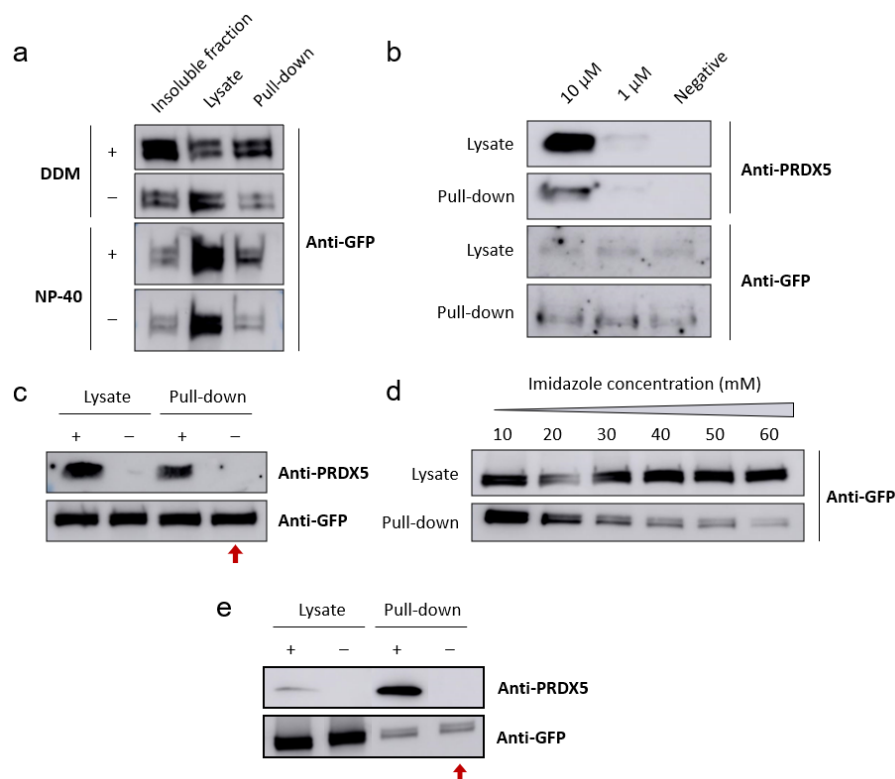


Figure 54. Pull-down assays to isolate the binding complex formed by PRDX5 and TLR4. 6xHis-tagged PRDX5 was used to pull down TLR4 from HEK293 MD2-CD14-TLR4 cells. **(a)** Optimization of the detergent (DDM or NP-40) used in the lysis buffer in order to extract transmembrane TLR4. Cells were incubated in the presence of PRDX5 or not (negative control) before being lysed. The pellets, cell lysates (input), and the proteins bound on the beads (pull-down) were analyzed using an anti-GFP antibody that recognizes the YFP tag of TLR4. **(b)** Optimization of PRDX5 concentration (10 μ M or 1 μ M) used to expose the cells. **(c)** Pull-down assay performed with 10 μ M PRDX5 revealing the presence of nonspecific TLR4 binding on the beads. **(d)** Optimization of the imidazole concentration used in the elution buffer to avoid nonspecific interaction of TLR4 with the beads. **(e)** Pull-down assay performed with 45 mM imidazole in the elution buffer.

These approaches were both based on the incubation of living cells with extracellular PRDX5. This might result in insufficient amount of PRDX5 molecules bound to the cell surface, thereby impeding detection. In addition, upon cell lysis, intracellular endogenous PRDX5 might compete with 6xHis-tagged PRDX5 molecules for the TLR4 complex, thereby preventing capture of the complex with Ni-NTA agarose beads. Further directions of improvement would therefore consist in using another method that does not rely on cell exposure to extracellular recombinant PRDXs. For instance, affinity-tagged PRDX5 and TLR4 could be overexpressed in a cell

system, as previously performed for PRDX1 [365]. This would allow a higher amount of proteins to form a complex, thereby facilitating the detection.

Binding of untagged PRDX2 to TLR4 using AFM-based SMFS

To ensure that PRDX2 orientation on the AFM tip (*i.e.* attached through its N-terminus) does not affect the binding of the protein to TLR4, we performed AFM-based SMFS with an untagged PRDX2, commercially produced in *E. coli*. The AFM tip functionalization strategy relied on the use of an NHS-PEG₂₇-acetal linker to covalently attach untagged PRDX2 through any of its free amino groups available. The same functionalization strategy as for the 6xHis-tagged PRDX2 was used to graft TLR4 molecules to gold surfaces (see **section 3.3.**). AFM-based SMFS was performed in the same conditions and the same way as for the 6xHis-tagged PRDX2 (**Figure 55a**).

As control of the specific binding, we recorded FD curves with a functionalized tip lacking the protein, at a tip retraction speed of $1 \mu\text{m}\cdot\text{s}^{-1}$ (**Figure 55b**). Based on the number of adhesive curves showing specific adhesive event(s), we calculated the BF for several maps obtained with different AFM tips (**Figure 55b**). The BFs obtained with the PRDX2-functionalized tip shows a large heterogeneity that could be attributed to the various orientations of the protein on the different AFM tips used, if different free amino groups are available. On the contrary, the BFs obtained with the control tips displays a narrower distribution. The mean BFs obtained with PRDX2-functionalized tips and linker-functionalized tips are 3.0% and 2.0%, respectively, and are not statistically different (Student's *t*-test with $\alpha = 0.05$).

As for the 6xHis-tagged PRDX2 and PRDX5, we performed similar DFS analysis by recording FD curves at various tip retraction speeds (**Figure 55c**). The spectrum of LRs probed was divided into seven discrete LR ranges ($100\text{--}315 \text{ pN}\cdot\text{s}^{-1}$; $315\text{--}1000 \text{ pN}\cdot\text{s}^{-1}$; $1000\text{--}3150 \text{ pN}\cdot\text{s}^{-1}$; $3150\text{--}10,000 \text{ pN}\cdot\text{s}^{-1}$; $10,000\text{--}31,500 \text{ pN}\cdot\text{s}^{-1}$; $31,500\text{--}100,000 \text{ pN}\cdot\text{s}^{-1}$; $100,000\text{--}315,000 \text{ pN}\cdot\text{s}^{-1}$). For each of them, a kernel distribution of the rupture forces was generated to determine the most probable rupture forces for single, and for some LR ranges, multiple interactions when enough data points allowed it (**Figure 56a**). Using the Bell-Evans model to fit the most probable rupture forces for the single interaction resulted in k_{off} and x_β values of $0.0004 \pm 0.0014 \text{ s}^{-1}$ and $1.128 \pm 0.274 \text{ nm}$, respectively (**Figure 56b**). With these parameters, we used the Williams-Evans prediction to model the rupture forces of double uncorrelated bonds which would simultaneously disrupt. The most probable rupture forces obtained for double interaction align well with the prediction (**Figure 56b**).

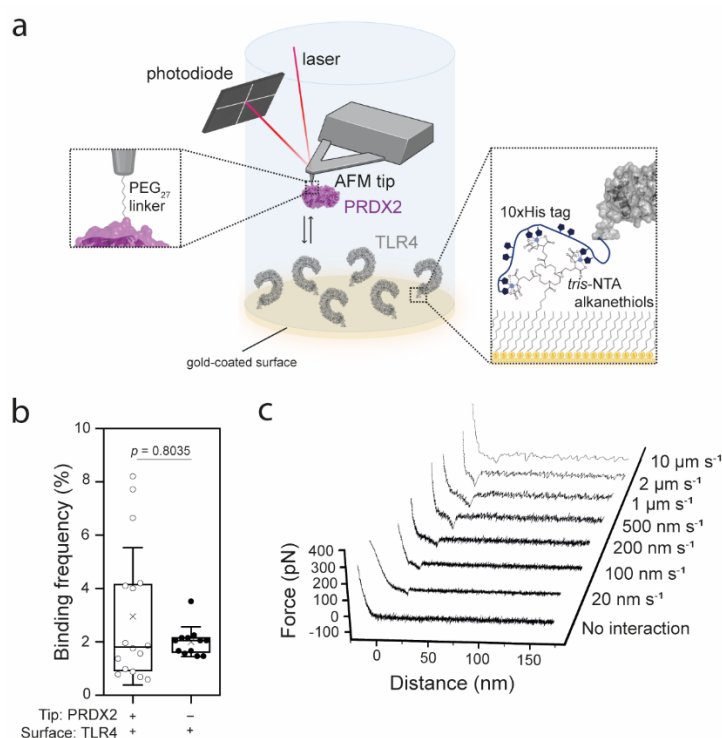


Figure 55. AFM-based SMFS performed with untagged PRDX2. (a) AFM-based SMFS was used to probe the interaction between recombinant untagged PRDX2 and recombinant TLR4. Untagged PRDX2 was covalently attached on the AFM tip through any of its free amino groups, and TLR4 was attached on an alkanethiol-grafted gold surface by means of its His tag through complex formation with *tris*-NTA- Ni^{2+} . Created with BioRender.com. (b) Box plot showing the BF obtained at a tip retraction speed of $1 \mu\text{m}\cdot\text{s}^{-1}$ with PRDX2-functionalized tips (○) or tips functionalized with the linker only (●). Box plot shows mean (×), median (—), 25th and 75th percentiles (boxes), and whiskers show S.D. Number of AFM maps, $n = 16$ and 12 , respectively. (c) Examples of FD curves showing either no interaction or adhesive events recorded at the seven tip retraction speeds used.

We used an F-test (with $\alpha = 0.05$) to compare whether the most probable rupture forces obtained with the untagged PRDX2 (**Figure 56**) and with the 6xHis-tagged PRDX2 (see **section 3.3.**, **Figure 48e** and **Figure S15**) were significantly different from each other. The two datasets were statistically different (p -value of 3×10^{-13}). This difference could be explained by (i) a different structural conformation due to the presence of the 6xHis tag, (ii) the different functionalization strategies used, or (iii) the data analysis. Given that we wanted to compare the binding of PRDX2 with that of PRDX5, or the binding with other receptors (e.g. MD-2 and CD14), we had to choose the same protein system for all experiments. For practical reasons (availability of in-lab productions of 6xHis-tagged proteins), we worked with recombinant 6xHis-tagged PRDXs for all DFS analyses.

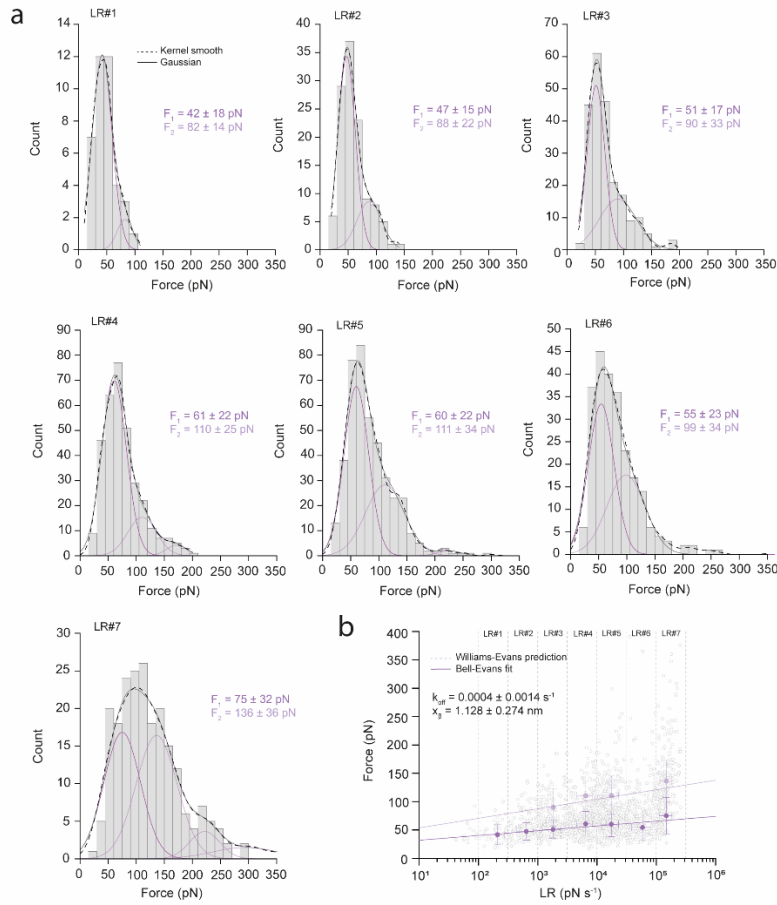


Figure 56. DFS analysis of the interaction between untagged PRDX2 and TLR4. (a) Spectrum of LRs was divided into seven discrete LR ranges (LR#1–7). For each LR range, a kernel distribution of the rupture forces was generated, which was fitted with multiple gaussian functions to determine the most probable rupture forces for single (1st peak), double (2nd peak) or multiple (following peaks) interactions. **(b)** DFS plot showing the most probable rupture forces for single or double interactions. The Bell-Evans model was fitted to the most probable rupture forces corresponding to single interactions (solid line), and the predicted rupture forces for two simultaneously-rupturing uncorrelated interactions were calculated using the Williams-Evans prediction (dashed line). The k_{off} and x_β values were extracted from the Bell-Evans model. Number of independent experiments, $N = 4$, number of AFM maps, $n = 65$.

Probing the interactions between PRDXs and TLR4, MD-2 and TLR4/MD-2 with BLI

AFM has the advantage of probing interactions at the single-molecule level. However, data analysis is time-consuming and interactions can only be kinetically characterized between two partners of a protein complex only. To overcome these

issues, we used BLI with the aim of elucidating the role of TLR accessory molecule MD-2 in the complex formed by TLR4 and PRDX2 or PRDX5.

We first tried to use amino-reactive (amine reactive second-generation, AR2G) biosensors. Indeed, as both the ligand (TLR4 extracellular portion, MD-2 or TLR4/MD-2 complex) and the analyte (PRDX5 or PRDX2) possess His tags, the use of Ni-NTA biosensors could result in unwanted binding of the analyte to free *tris*-NTA sites on the biosensor surface, resulting in a false positive binding. The surface of ARG2 biosensors is coated with a high density of carboxylic acids that allow covalent coupling of proteins *via* NHS/EDC-mediated amide bond formation, similar to the strategy that we used in AFM-based SMFS experiments.

In a first attempt, we used $0.05 \text{ mg}\cdot\text{mL}^{-1}$ recombinant TLR4 (extracellular portion) as ligand for immobilization onto the biosensor surface, and $0.2 \text{ mg}\cdot\text{mL}^{-1}$ recombinant 6xHis-tagged PRDX5 as analyte, with PBS as kinetic buffer (**Figure 57a**). As controls, we ran an assay with a reference biosensor loaded with TLR4 using a buffer blank (*i.e.* kinetic buffer without analyte) for the association step. As shown in **Figure 57a**, 6xHis-tagged PRDX5 binds the biosensor surface in a larger extent than the nm shift associated to the control biosensor. This signal can be subtracted from the signal obtained with the 6xHis-tagged protein. Moreover, we ran an additional control consisting of a biosensor without ligand and 6xHis-tagged PRDX5 as analyte. The association step (**Figure 57a**) showed a large increase in signal, revealing nonspecific binding of the analyte onto the biosensor surface. As consequence, the binding observed with 6xHis-tagged PRDX5 may display a nonspecific contribution, compromising the proper interpretation of the results.

To determine whether the nonspecific binding was due to the presence of the PRDX5 His tag, we ran a similar experiment using $0.2 \text{ mg}\cdot\text{mL}^{-1}$ untagged PRDX2 as analyte (**Figure 57b**). Although the signal obtained with the TLR4-immobilized biosensor was larger than that of the uncoated biosensor, we could still observe nonspecific binding. These results may indicate that the biosensor surface still possesses reactive ester groups even after the quenching step, leading to unwanted binding of the analyte to the biosensor surface.

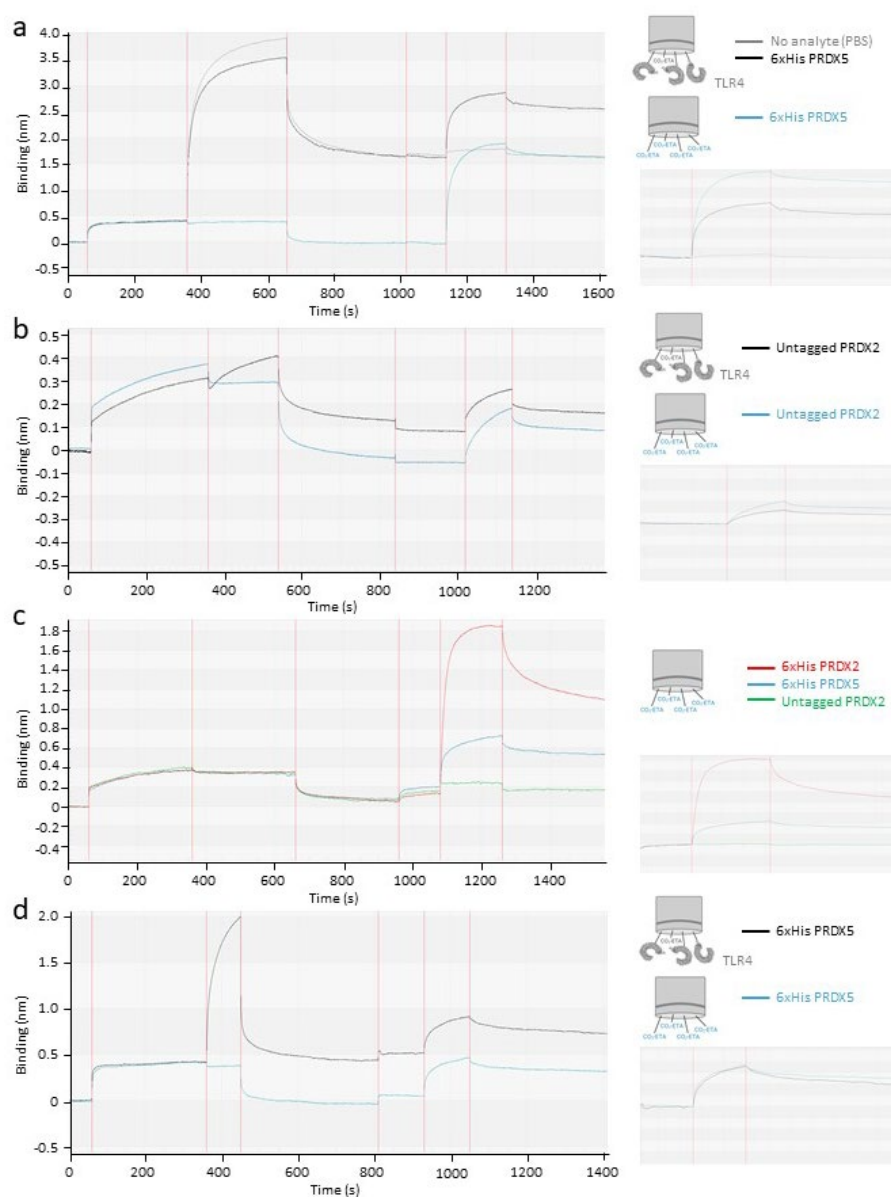


Figure 57. Probing PRDX-TLR4 complexes with BLI using AR2G biosensors. Full sensorgrams (left) or sensorgrams showing the association and dissociation steps (right), obtained with 6xHis-tagged PRDX5 in PBS (**a**), untagged PRDX2 in PBS (**b**), 6xHis-tagged PRDX5, 6xHis-tagged PRDX2 or untagged PRDX2 in PBS with 0.1% BSA and 0.02% Tween 20 (**c**), and 6xHis-tagged PRDX5 in PBS with 0.1% BSA, 0.02% Tween 20, and 20 mM imidazole (**d**).

We therefore tried to optimize the composition of the kinetic buffer in order to avoid nonspecific interactions of the analyte. We used a kinetic buffer consisting of PBS with 0.1% BSA and 0.02% Tween 20. Running uncoated biosensors with 6xHis-

tagged PRDX2 and 6xHis-tagged PRDX5 resulted in a large increase in signal in the association step, indicating that nonspecific binding was still occurring (**Figure 57c**). However, the biosensor ran with the untagged PRDX2 showed no binding in the association step (**Figure 57c**), indicating that the kinetic buffer used allowed suppressing undesirable nonspecific binding. This result indicates that the 6xHis tag of the proteins most likely contributes to these nonspecific interactions. In an attempt to eliminate the nonspecific binding due to the presence of the 6xHis tag, we used the previous kinetic buffer in which 20 mM imidazole was added. However, 6xHis-tagged PRDX5 could still bind in absence of TLR4 immobilized onto the biosensor surface (**Figure 57d**). Increasing the imidazole concentration up to 250 mM did not result in a different sensorgram (*data not shown*).

Taken together, these preliminary results indicate that further experiments should therefore involve the use of untagged PRDX2 as analyte and kinetic buffer with 0.1% BSA and 0.02% Tween 20.

In the meantime, we also ran assays using another biosensor chemistry, namely Ni-NTA biosensors (**Figure 34b**), to take advantage of the 10xHis-tag of the recombinant extracellular portion of TLR4. To avoid unwanted binding of His-tagged analyte to the biosensor surface, we used the untagged PRDX2. The Ni-NTA biosensor surface is covered with Ni²⁺-charged *tris*-NTA groups, allowing His-tagged proteins to bind, in a similar fashion as in AFM-based SMFS. We performed assays with 12.5 µg·mL⁻¹ TLR4 (extracellular portion) (**Figure 58a**), 12.5 µg·mL⁻¹ recombinant MD-2 (**Figure 58b**), or 12.5 µg·mL⁻¹ recombinant TLR4/MD-2 complex (**Figure 58c**) immobilized onto the biosensor surface through their His tag, and 0.5 mg·mL⁻¹ untagged PRDX2 as analyte. As kinetic buffer, we used PBS. Given that some His-tagged ligands may dissociate from the biosensor surface, a reference biosensor was used to correct for the resulting drift. The reference biosensor was loaded with the His-tagged ligand and ran with a buffer blank (*i.e.* kinetic buffer without analyte) for the association step.

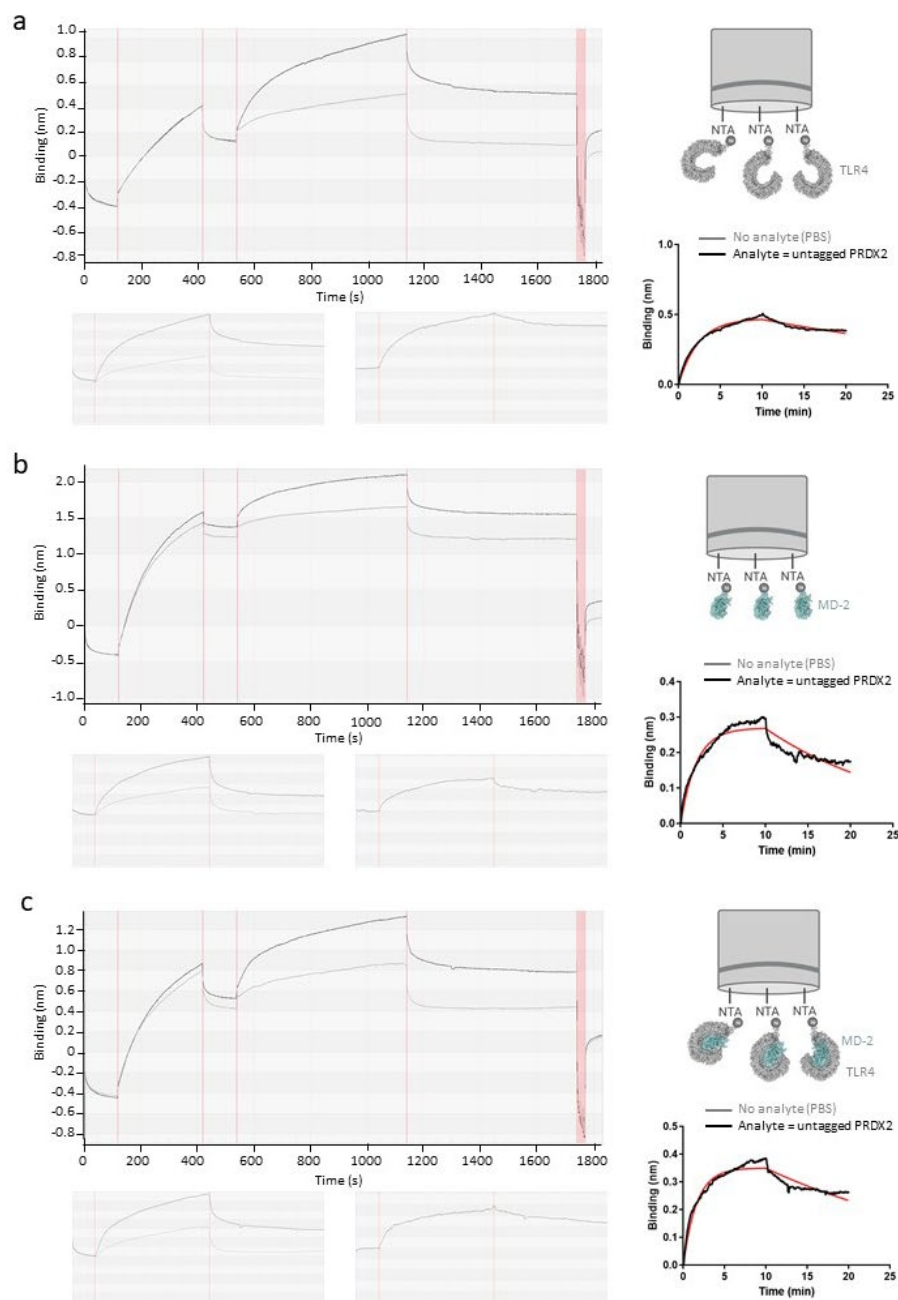


Figure 58. Probing untagged PRDX2 binding to TLR4, MD-2 or TLR4/MD-2 using Ni-NTA biosensors. Full sensorgrams (up) or sensorgrams showing the association and dissociation steps (down), before biosensor reference subtraction (down, left) or after (down, middle and right), obtained with untagged PRDX2 and either TLR4 (**a**), MD-2 (**b**) or the TLR4/MD-2 complex (**c**). Association and dissociation curves were fitted with a 1:1 binding model to obtain the k_{on} and k_{off} values, shown in Table 3.

The values obtained for the association rate constant (k_{on}) and dissociation rate constant (k_{off}), as well as the resulting dissociation constant (K_D), are presented in **Table 3**. These results must be interpreted with care as no control biosensor was used to determine the extent of nonspecific binding of the analyte (untagged PRDX2) in the absence of ligand bound to the biosensor surface. The k_{on} value obtained with BLI is in the same order as magnitude as the value obtained with AFM-based SMFS with untagged PRDX2 (**Figure 56b**). The k_{on} and K_D values obtained with MD-2 as receptor (immobilized onto the biosensor surface) are higher, indicating that the PRDX2–MD-2 complex is less stable and has a shorter lifetime than the PRDX2–TLR4 complex. This is consistent with the DFS analysis on PRDX2–TLR4 and PRDX2–MD-2 interactions performed with AFM-based SMFS, which revealed that the PRDX2–TLR4 complex was more stable than the PRDX2–MD-2 complex. Regarding the binding with the TLR4/MD-2 complex immobilized onto the biosensor surface, three identical runs were performed (**Table 3**). The results have to be carefully interpreted as it is unknown how the TLR4/MD-2 complex binds the biosensor surface. Indeed, as both proteins possess a 10xHis tag, it is conceivable that the biosensor surface resulted in a combination of TLR4 proteins, MD-2 proteins, and TLR4 proteins with bound MD-2.

For all these experiments, we measured binding for a single analyte concentration, which is not sufficient to get accurate kinetic constant values. Therefore, further experiments would be required to be able to properly interpret the data.

Table 3. Association rate constant (k_{on}), dissociation rate constant (k_{off}) and dissociation constant (K_D) obtained with untagged PRDX2 as analyte and Ni-NTA biosensors. The biosensor surface was functionalized either with TLR4, MD-2 or TLR4/MD-2 complex as ligand onto the biosensor surface. Triplicate measurements were performed for the TLR4/MD-2 complex.

Ligand	K_D (M) (local fit)	k_{on} (M ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	R_{max}	χ^2	R^2
TLR4	3.4×10^{-6}	2.3×10^2	7.8×10^{-4}	0.7418	0.1789	0.9883
MD-2	3.6×10^{-5}	7.9×10^1	2.8×10^{-3}	1.355	0.2395	0.9593
TLR4/MD-2	5.1×10^{-7}	1.3×10^3	6.7×10^{-4}	0.4889	0.1988	0.9633
	1.6×10^{-6}	8.4×10^2	1.3×10^{-3}	0.5582	0.2652	0.9672
	7.4×10^{-6}	2.5×10^2	1.8×10^{-3}	0.7074	0.2061	0.9732

NF- κ B activation following PRDX5 binding

The downstream events following PRDX binding include a signaling cascade eventually leading to activation of transcription factors. As the NF- κ B dimer p65:p50 (or RelA:p50) is the primary mediator of inflammation [282] and was shown to be involved in PRDX5-dependent pro-inflammatory response [117,344,345,362,394], we attempted to characterize the dynamics of p65:p50 activation following cell stimulation by PRDXs using CLSM. To this end, we transfected HEK293 MD2-CD14

TLR4 cells with a plasmid encoding the p65 gene fused to the fluorescent OFPspark protein, allowing the observation of the dimer translocation from the cytosol to the nucleus by fluorescence.

As dynamics of NF- κ B has been extensively studied following stimulation by TNF- α [530–534], we first wanted to validate our system using TNF- α instead of PRDX proteins. However, addition of increasing concentrations of TNF- α and observation during several hours did not result in fluorescence signal migration from the cytoplasm to the nucleus, as representatively shown in **Figure 59**. After TNF- α exposure, cells eventually became rounder and were dying, but no NF- κ B translocation from the cytosol to the nucleus was observed.

As the issue seems to arise from the cell line itself, a future approach would be to test the same experiment with another cell line to confirm that transfection with pCMV3-OFPSpark-RELA is functionally effective and that it was the HEK293 cell line itself that was problematic.

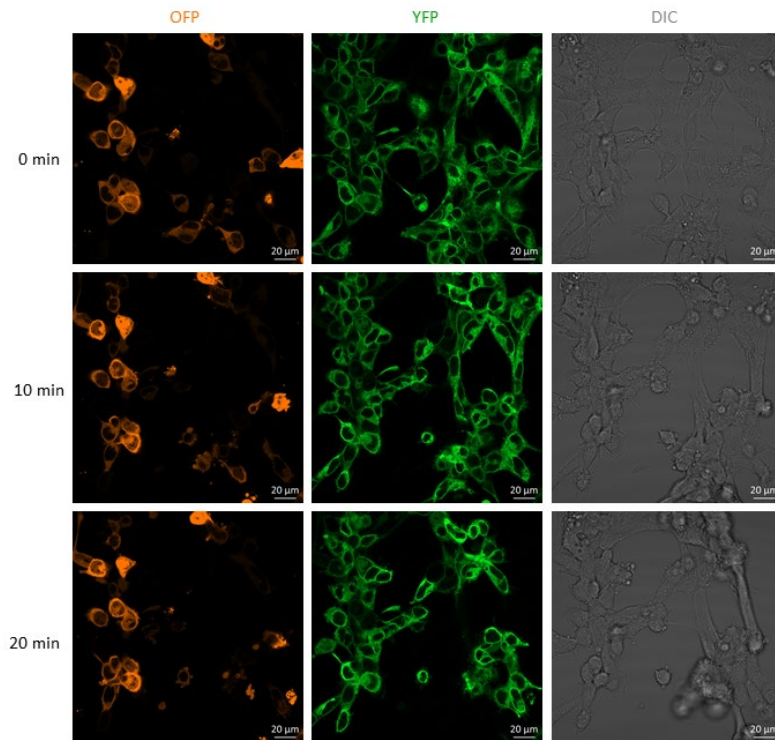


Figure 59. NF- κ B activation and translocation from the cytosol to the nucleus following TNF- α binding. Confocal images of OFP (orange, left), YFP (green, middle), and DIC (right) of HEK293 cells expressing MD-2, CD14 and TLR4-eYFP, transiently transfected with pCMV3-OFPSpark-RelA, before (up) or after (middle and down) TNF- α injection. Representative of three independent experiments.

3.4.4. Summary

In summary, this section reviewed the various additional experiments that we performed with the aim of going further in the understanding of the PRDX function as DAMPs. These include investigating the binding of PRDXs with TLR4, MD-2 and TLR4/MD-2 with BLI, trying to isolate the PRDX5/TLR4 complex along with the accessory molecules MD-2 and/or CD14, and examining the dynamics of NF- κ B activation following PRDX stimulation with CLSM. However, all of these experiments require further optimization in order to obtain conclusive results. In addition, we also tried to answer two main questions that were raised during the course of the project. To elucidate whether the 6xHis tag could impact the DAMP function of PRDXs, we focused on the binding kinetics of the interaction with TLR4 to determine whether the tag affects the measurements. Our results showed that the rupture forces for the PRDX2–TLR4 interaction are significantly different when the protein exhibits a 6xHis tag. Furthermore, we confirmed using LC-Q-TOF-MS/MS analysis on PRDX5 that the additional peaks observed on the MS spectra were not attributed to another oxidation state, but were rather due to the presence of a N-terminal Met methylation.

Chapter IV – General discussion

In this thesis, we used biomolecular (ELISA, FC, MS) and single-molecule (AFM-based SMFS) approaches to gain insights into the extracellular DAMP function of PRDXs, and the role of TLR4 in PRDX-mediated inflammation in particular. In this section, the experimental set-up (recombinant proteins, model cell lines, and main techniques) will first be discussed to highlight the strengths and limitations of our study. The main biological insights will then be presented in a broader context. Further directions and suggestions of improvement will be approached in the last Chapter (**Chapter V – Conclusion and Perspectives**).

4.1. Analysis of the experimental set-up used

4.1.1. Production and modification of recombinant PRDX proteins

Our work focused on two human PRDXs: the typical 2-Cys PRDX2 and the atypical 2-Cys PRDX5. These PRDXs have previously been reported to act as DAMPs in the context of sterile inflammatory-related diseases, such as brain IRI or subarachnoid hemorrhage [116,117]. Furthermore, they belong to two different subgroups, allowing to consider mechanistic and structural differences (*e.g.* conformational state, oligomeric state, location of C_R) in the study.

The recombinant human PRDXs that were used, namely PRDX5, PRDX5^{C47S}, and PRDX2, were all produced in *E. coli* and possess a N-terminal 6xHis tag. The implications of such production system and of the presence of the tag will first be discussed. As PRDX5 was subjected to chemical modifications, the efficiency and site selectivity of such reactions will next be analyzed.

Quality control of PRDX productions

Quality control of purified recombinant protein samples is the final and critical check-point during protein production. In this project, this step was somewhat overlooked, mainly due to the context of the thesis (see **Preface**). However, to be able to correctly interpret biological and biophysical characterization experiments, essential protein properties need to be assessed, including purity, integrity, homogeneity, and activity [535]. In addition, protein concentration must be determined precisely [535].

Regarding the assessment of purity and integrity, SDS-PAGE associated with Coomassie blue staining was performed following protein production. As shown in **Figure 22**, no potential contaminants or degradation products were detected in recombinant PRDX2 and PRDX5 samples. However, this technique shows limitations

as low amount of impurities and proteolysis events could go unnoticed. In addition to SDS-PAGE, MS can be used for detailed analysis of protein integrity as it can detect the presence of proteolysis events affecting just a few amino acids and the overall state of modification of the proteins (*i.e.* chemical alterations, PTMs) [535]. We performed MS analyses on PRDX5 samples, both on the WT and on the redox-modified forms of the protein (**Figure 43B**, **Figure S7**, **Figure S8**, and **Figure 53**).

Regarding the purity of the samples, PRDX production in *E. coli* can sometimes result in the formation of a heterodimeric protein composed of a recombinant human monomer associated to a bacterial monomer⁶. However, MS analysis conducted on intact proteins did not reveal the presence of peaks corresponding to bacterial PRDXs (*e.g.* AhpC, molecular weight of 20,761 Da; BCP, molecular weight of 17,634 Da; Tpx, molecular weight of 17,835 Da). Furthermore, we used MS analysis after trypsin digestion to assess the protein contaminants in the PRDX5 production. Any peptide sequences detected were matched against the protein database Uniprot. To avoid false positive protein identification, a threshold of >2 unique peptides identified within a single protein was applied [536]. We also restricted protein identification to the species of interest, namely *E. coli*. The resulting proteins identified are shown in **Table 4**. Although human PRDX5 is by far the most abundant protein in the sample, about 15 contaminants were identified. Among these, many are native proteins from *E. coli* commonly purified by metal affinity chromatography, including Fur, CRP, SlyD, EF-Tu 1, and Can [537–539]. Some proteins that are rich in His residues, but not known as common contaminants, were most likely also retained on the Ni²⁺-NTA matrix, such as purine nucleoside phosphoramidase which contains the His triad motif (HXHXHXX, in which W is a hydrophobic amino acid) [540]. Furthermore, due to the leakage of Ni²⁺ from the purification column, free carboxyl groups are formed, which could interact nonspecifically with strongly basic proteins with high isoelectric point values (pI >9) [541]. This could explain the presence of ribosomal proteins, such as RsuA and 30S ribosomal protein S10 [541]. Besides the endogenous proteins, some accessory proteins that are specifically expressed by some expression plasmids to facilitate recombinant protein expression are also known to become contaminants, including antibiotic resistance proteins, repressors/activators, chaperones, RNA polymerases, and proteases [539]. This could explain the presence of chloramphenicol acetyltransferase as the vector used to produce the human recombinant PRDX2 and PRDX5, namely pQE-30, contains the gene coding for this protein. Finally, the target protein can be co-purified with binding partners [542]. AhpF, the major contaminant, is the specialized reductant for AhpC [543]. As human PRDX5 share ~30% sequence identity with AhpC from *E. coli*, AhpF might bind human PRDX5, and thereby, be co-purified with PRDX5. Regarding

⁶ Unpublished data: personal communication from Prof. J. Messens (VUB, Belgium).

the integrity of the recombinant protein samples, MS on intact protein reveals that the major form in dsPRDX5 was the intact protein (**Figure 43** and **Figure S7**), indicating that PRDX5 is most likely not (or barely) degraded. Similar MS experiments should have been performed to assess the purity and the integrity of the recombinant PRDX2 production.

Table 4. Protein contaminants in recombinant human PRDX5 production. Protein identification was restrained to *E. coli* and a threshold of >2 unique peptides identified within a single protein was applied.

#	Protein identification	Peptide count	Unique peptides	Confidence score	Abundance
1	PRDX5 (<i>Homo sapiens</i>)	33 40	8 16	240,4458 238,0017	2640416,67 3080100,15
2	Alkyl hydroperoxide reductase subunit F (AhpF)	14	14	144,5751	16186,0162
3	cAMP-activated global transcriptional regulator CRP (CRP)	10	10	86,4939	39674,8857
4	Ferric uptake regulator protein (Fur)	5	5	56,5347	30421,4884
5	Chloramphenicol acetyltransferase (CAT)	6	6	47,0087	30816,1013
6	Ribosomal small subunit pseudouridine synthase A (RsuA)	6	6	46,7967	12235,1158
7	30S ribosomal protein S10	5	5	36,0725	6544,97973
8	NADH-quinone oxidoreductase subunit E (NuoE)	6	6	35,7653	10416,8538
9	Purine nucleoside phosphoramidase	3	3	27,2245	6999,45396
10	Bifunctional chorismate mutase/prephenate dehydratase	4	4	23,9542	3028,83874
11	50S ribosomal protein L2	4	4	23,6716	5805,02426
12	tRNA (guanine-N(7)-)-methyltransferase	3	3	22,8276	5430,35951
13	Carbonic anhydrase 2	4	4	21,4256	3289,27464
14	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD (FKBP-type PPlase SlyD)	3	3	21,1403	28114,3012
15	Elongation factor Tu 1 (EF-Tu 1)	3	3	18,0892	53038,5481
16	Histidine biosynthesis bifunctional protein HisB	3	3	15,773	2436,2758

To ensure that the protein production is homogenous, dynamic light scattering is a convenient method as it allows the characterization the size of biomolecules in solution [544]. It can be used to distinguish between a homogenous monodisperse and an aggregated sample (including soluble high-order assemblies and aggregates) [544]. However, the resolution of dynamic light scattering is not sufficient to resolve close quaternary structure (e.g. monomers from dimers and small-order oligomers) [535]. To assess the homogeneity of a protein sample in terms of oligomerization, SEC-MALS is proving to be a technique of choice [402]. We used SEC-MALS to characterize the oligomeric state of the PRDX5 redox forms (**Figure 43C**). However,

these experiments revealed that the samples were not completely homogenous in terms of oligomeric status. This result poses a few issues, in particular for the SMFS experiments in which the characterization of the protein structure is essential for correct interpretation of the results. Here again, similar experiments were not performed with the recombinant PRDX2 productions. Therefore, we cannot attest that we were constantly working with the dimeric form of the PRDX proteins, so that SMFS results in particular should be assessed carefully.

Furthermore, recombinant protein samples need to be investigated to determine whether the protein is active and functional. The enzymatic activity of PRDX2 and PRDX5 productions is discussed below.

To determine protein concentration, we used two different methods: bicinchoninic acid (BCA) assay and UV-visible absorbance measurement. The BCA assay has the disadvantage of using a standard for calibration, which can lead to quantification errors as the composition of the protein of interest may not necessarily match that of the protein standard [545]. On the other hand, the use of UV-visible absorbance measurements for concentration determination needs prior extinction coefficient determination or calculation [545,546]. Furthermore, it is possible to determine protein concentration only if the protein of interest contains the two main light-absorbing amino acids, *i.e.* tryptophan and tyrosine. In the present thesis, we consistently used the same method for all the experiments of the same type (*e.g.* ELISA, SMFS, FC). As we were interested in the relative concentration rather than in an absolute value, both methods were proven to be suitable.

Importance of endotoxin removal in the study of DAMPs

A single *E. coli* bacterium contains $\sim 2 \times 10^6$ LPS molecules [547]. During protein production, bacteria lysis generates the release of large amounts of LPS, so that recombinant protein preparations are contaminated with the presence of endotoxins (*i.e.* LPS). As a consequence, the use of such protein productions will generate misleading experimental results in the context of DAMP-mediated inflammation studies. Indeed, LPS is known to induce a pro-inflammatory response by interacting with the TLR4/MD-2 complex [195,295,298]. Therefore, when recombinant proteins are produced in Gram-negative bacteria such as *E. coli*, care must be taken to remove the putative LPS.

Prior to use, we removed endotoxins from PRDX samples and quantified the endotoxin levels before and after the process using Limulus amoebocyte lysate assay (see **section 2.3.1.**). Whereas endotoxin levels reached $\sim 6000 \text{ EU}\cdot\text{mL}^{-1}$ and $\sim 550 \text{ EU}\cdot\text{mL}^{-1}$ after PRDX5 and PRDX2 production, respectively, it decreased down to $\sim 0.2 \text{ EU}\cdot\text{mL}^{-1}$ and $\sim 0.1 \text{ EU}\cdot\text{mL}^{-1}$ after chromatography-based endotoxin removal,

confirming the efficiency of the procedure. Given the fact that protein productions were diluted prior to their use in experiments, the endotoxin levels were further diminished.

Impact of the presence of a 6xHis tag on PRDX expression, structure and function

The 6xHis tag of recombinant PRDXs presented several advantages in the context of the present thesis. It allowed both recombinant PRDX purification on metal ion affinity chromatography using Ni^{2+} -loaded NTA-agarose matrix, and oriented immobilization of recombinant PRDX molecules on AFM tips using a PEG linker with a maleimide group, disulfide-*tris*-NTA molecules and Ni^{2+} . Given its relatively small size (~ 2.5 kDa), the 6xHis tag was initially not believed to interfere with the structure and function of recombinant proteins [539,548]. However, several studies have now demonstrated that this assumption may be false. The incorporation of a 6xHis tag was shown to notably affect oligomeric states, function and enzymatic activity [549–554], including for PRDXs [95,555].

Nevertheless, previous studies showed that enzymatic activity of the recombinant N-terminal 6xHis-tagged human PRDX5 that we used in this thesis project was retained [2,55,62,129,132,490,491]. By comparing the Trx-dependent peroxidase activity obtained by Dubuisson *et al.* [132] and Baković *et al.* [129] for the 6xHis-tagged PRDX5 with that of the wild-type PRDX5 obtained by Seo *et al.* [52], we can observe that 6xHis-tagged PRDX5 ($\sim 2.3\text{--}2.5 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) displays similar specific activity as the wild-type PRDX5 ($\sim 3.8 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$). The location of the tag on the three-dimensional structure of PRDX5 might explained its relatively low impact. Indeed, PRDX5 exists as a dimer irrespective of its redox state, with a dimerization that occurs through an alternative interface compared to typical 2-Cys PRDXs [10,11]. Within the dimer, the N-terminal parts of the two subunits are located at the opposite of the dimerization interface (**Figure 6b**).

Regarding recombinant 6xHis-tagged PRDX2, a recent study reported that the tag alters both the peroxidase activity of the protein and its ability to interact with partner proteins (*e.g.* STAT3), but not its ability to oligomerize into decamers and to form disulfide-linked dimers upon reaction with H_2O_2 [555]. In this project, we showed that the most probable rupture forces upon breaking interaction with TLR4 for the LR range $100\text{--}315,000 \text{ pN}\cdot\text{s}^{-1}$ of the 6xHis-tagged PRDX2 were significantly different from that of untagged PRDX2 (see **section 3.4.3.**), and that this difference might be attributed to the presence of the 6xHis tag. Furthermore, BLI experiments resulted in nonspecific binding of the 6xHis-tagged PRDX2 onto the biosensor surface, most likely due to the presence of the His tag. These observations indicate that the presence of the tag might influence the results.

The experiments performed to identify PRDX5 redox states using MS revealed that a certain proportion of proteins possess a N-terminal methylated methionine. This PTM is the consequence of the presence of the RGS-His epitope (RGSHHHH) after the first Met residue in the vector used for the production of recombinant PRDXs. The Arg located in the second position indicates a methylation site for *E. coli*. As mammalian PRDXs are known to be subjected to PTMs that regulate their functions [42,107], this discovery may raise the question whether such PTM could influence the oligomeric state and/or conformation and/or function of PRDX5, and its pro-inflammatory activity in particular. Indeed, a previous study showed that post-translational mono-methylation of HMGB1 at Lys⁴² regulates its relocalization from the nucleus to the cytoplasm by changing its conformation [556]. Regarding human PRDXs more specifically, no methylation has been detected through low-throughput or high-throughput methods [42], which may indicate that such PTM is not involved in the regulation of the protein functions. In addition, as the methylation site is located at the extremity of the 6xHis tag and on a residue that is absent from the wild-type PRDX5, it is unlikely that the presence of this PTM affects the function of PRDX5.

Chemical modification of the Cys redox state

Among the well-characterized PTMs of human PRDXs, different types of oxidation (SOH, SS, SO₂, SO₃) of their Cys residues are known to have an influence on their structure, oligomeric state and intracellular functions (see **section 1.1.3. and 1.1.4.**). The redox state of Cys residues can be modified *in vitro* either through (i) point mutation, (ii) peroxidase reaction (as occurs physiologically), or (iii) chemical treatment. Cys mutation, typically with Ser or alanine (Ala), lead to 'redox-dead' mutants [557]. The peroxidase reaction consists in mixing PRDX with the other components of the catalytic cycle, namely Trx, TrxR, NADPH and H₂O₂, to mimic the way in which the reaction occurs *in vivo*. This system has been widely adopted to study the peroxidase activity of human PRDXs [52,129,132]. Finally, chemical treatment with reducing or oxidizing agents has also been used to alter the PRDX redox state [69,127,375]. A large variety of chemicals are available, including DTT, β -mercaptoethanol, and tris(2-carboxyethyl)phosphine (TCEP) for reduction [558], and H₂O₂, peroxynitrite, hypochlorous acid (HOCl), and monochloramine for oxidation [559]. In addition, an alkylating step can be performed after reduction in order to protect the Cys thiol groups from re-oxidation. Iodoacetamide (IAM), 2-chloroacetamide (CAM), *N*-ethylmaleimide (NEM) and iodoacetic acid are among the alkylating agents that are commonly used [560].

To study the role of the redox state of PRDX5 Cys residues on its pro-inflammatory activity, we chose to use a PRDX5 mutant in which the C_P was replaced by a Ser (PRDX5^{C47S}), as well as chemically oxidized and reduced PRDX5 proteins. The

protein mutation prevents the formation of the intramolecular disulfide bridge involved in the protein catalytic cycle, maintaining the protein in a reduced state. We chose the use of chemicals over the peroxidase reaction because chemicals can be easily removed from the solution after redox treatment using ultrafiltration, on the contrary of proteins with similar molecular weight as PRDX5 (*e.g.* Trx). This step is indeed required prior to the use of treated PRDXs in experiments such as ELISA or AFM-based SMFS. To reduce the disulfide bond in PRDX5, the protein was exposed to 10 mM DTT at RT for 1 hour. This concentration of DTT is typically used to reduce proteins, notably in MS studies [561], and a 20-fold molar excess of DTT over protein was shown to be sufficient to completely reduce PRDXs [69,562]. DTT-reduced PRDX5 was subsequently alkylated with 55 mM IAM at RT for 1 h in the dark as IAM is light sensitive. The IAM concentration of 55 mM is commonly chosen in MS studies involving protein alkylation [561,563]. Furthermore, Shichita *et al.* previously worked with this IAM concentration to alkylate human PRDX5 [116]. For PRDX5 hyperoxidation, the protein was first reduced with 10 mM DTT prior to the addition of 5 mM H₂O₂ at RT for 1 h. This protocol, consisting in a single addition of H₂O₂, has been used in other studies [71,112], and the use of 5 mM is based on a previous investigation of the hyperoxidation of human PRDXs, including PRDX5 [69]. This concentration is higher than the concentration required for typical 2-Cys human PRDXs, as PRDX5, which lack the GGLC and YF motifs, is more resistant to hyperoxidation (see **section 1.1.3.**) [62,69].

The disadvantages of using chemical agents is the incompleteness of the reactions and the occurrence of off-target labeling and side reactions. As mentioned in **section 1.3.3.**, the sensitivity of a given PRDX depends on the competition between the rate of hyperoxidation and the rate of disulfide formation [65]. As PRDX5 is known to be highly resistant to hyperoxidation, a more efficient way to hyperoxidize the Cys residues would have been to promote cycling of the protein, for instance by making alternate additions of H₂O₂ and DTT, as it occurs in the peroxidase reaction mixtures by virtue of the presence of the Trx system (*i.e.* Trx, TrxR and NADPH). In addition, the use of peroxyxynitrite instead of H₂O₂ might have been a better choice as PRDX5 oxidation is more efficient with peroxyxynitrite [57]. Regarding alkylation, IAM can result in off-target labeling of other amino acid and side reactions [560,564,565]. In particular, IAM was shown to artifactually alkylate the N-terminal residue of proteins, along with Asp, Glu, His, Lys, Met, Ser, Thr, and Tyr [566–570]. Therefore, to block remaining Cys thiol groups after chemical redox treatments and prior to MS analysis, we used CAM instead of IAM, as CAM shows a high degree of specificity toward Cys residue [568].

4.1.2. Cell line choice and engineering

We investigated binding of PRDX5 to the cell surface and cytokine secretion following PRDX2 or PRDX5 exposure using living cells. We worked with human cells for consistency with the use of human PRDXs. As immune cell model, we used the THP-1 human monocytic cell line and differentiated the cells with PMA. To study the role of TLR4 more specifically, we used a 'gain-of-function' approach based on HEK293 cells as they lack the two major plasma membrane TLRs involved in DAMP recognition, namely TLR2 and TLR4 [398].

PMA-differentiated THP-1 cells to confirm the DAMP function of PRDXs

Macrophages are crucial in the initiation of inflammation, notably through immunomodulation *via* production of various cytokines and growth factors following DAMP recognition [571]. Macrophages therefore represent appropriate cells in the study of the DAMP function of proteins. The THP-1 cell line has been widely used as a model to study human macrophage biology and function [510,572,573], and was therefore the cell line we chose to validate the DAMP function of the recombinant human PRDXs used in this project. THP-1 monocytes can be differentiated into macrophage-like cells following exposure to PMA, 1 α ,25-dihydroxyvitamin D3, or macrophage colony-stimulating factor (M-CSF) [510]. Here, we used 25 nM PMA to induce THP-1 differentiation for 48 h, followed by a rest of 3 h. The concentration of 25 nM was previously shown to confer satisfying cytokine-inducing ability while limiting the inflammatory response caused by PMA exposure *per se* [510].

HEK293 cells to study the role of TLR4 and its accessory molecules

To investigate the role of TLR4 and its accessory molecules MD-2 and CD14 in the recognition of human PRDX5 and PRDX2, we chose a 'gain-of-function' approach using HEK293 cells given that these cells lack both TLR2 and TLR4 [398], the two main plasma membrane TLRs involved in the recognition of extracellular DAMPs [194,198]. Such cell system was first used in the context of the pro-inflammatory response triggered by LPS. Indeed, whereas HEK293 cells are unresponsive to stimulation with LPS, they become capable of cytokine secretion upon transfection with plasmids encoding TLR4, MD-2 and CD14 [574]. HEK293 cells have now been extensively used for the study of DAMP-induced TLR4-dependent cytokine secretion [214,233,338,513,514,523,524].

4.1.3. Main techniques used for the study of the DAMP function of PRDXs

In the study of the function of PRDXs as DAMPs, we mainly investigated two mechanisms, namely the binding of PRDXs and the subsequent pro-inflammatory

cytokine secretion. The two major techniques used were AFM-based SMFS and ELISA, respectively. Some aspects of these techniques will be discussed in this section.

ELISA for the study of cytokine secretion

To study cytokine secretion following PRDX exposure, we used ELISA, a technique that cannot be overlooked in the field of immunology. The use of this technique serves two purposes: (i) confirming the DAMP function of PRDXs, *i.e.* their ability to induce cytokine secretion, and (ii) correlating this function to receptor activation. We measured the secretion of IL-8, a cytokine that is known to have chemotactic activity and to activate neutrophils in the early inflammatory response [575]. Furthermore, the deleterious effects of IL-8 have been emphasized in ischemic injury [522,576,577], and this cytokine has been extensively quantified in inflammation-related studies involving the use of HEK293 cells expressing various TLRs, as well as THP-1 cells [578–581].

Controls for putative contamination by endotoxins

We previously discussed the importance of endotoxin removal (**section 4.1.1**). In this project, endotoxin removal was particularly important in the context of IL-8 quantification. Indeed, the putative presence of LPS in the recombinant protein productions would trigger the release of IL-8 [582,583], thereby distorting the results. To account for this possibility, different types of controls can be included in the ELISA itself. The recombinant proteins can be (i) combined with the LPS inhibitor polymyxin B (PMB) [584], (ii) digested with the endopeptidase proteinase K (PK) [512,585], or (iii) or heat-inactivated [586], prior to cell exposure. PMB is a cyclic lipopeptide with a high affinity for LPS which is known to inhibit various biological activities induced by LPS, including its ability to stimulate cytokine secretion [587,588]. Therefore, combining PMB with recombinant proteins prior to their addition to cell medium would inhibit the pro-inflammatory activity of the putative LPS contamination. However, we observed an increase in IL-1 β levels secreted by PMA-differentiated THP-1 cells after preincubation of PRDX5 with 30 $\mu\text{g}\cdot\text{mL}^{-1}$ PMB (**Figure S1**). PMB is known to sensitize cells to extracellular ATP by modulating the function of the pore-forming P2X7 receptor [589], and ATP is a well-known DAMP able to trigger IL-1 β release *via* activation of P2XRs [589,590]. Therefore, one hypothesis that might explain such increase in IL-1 β secretion levels from PMA-differentiated THP-1 cells, is whether cell exposure to PRDX somehow lead to the extracellular release of ATP.

We therefore decided to use PK as control for the LPS-induced cytokine secretion. PK is an endopeptidase with broad specificity and very high specific activity [591]. As PK is cytotoxic, we next added the serine protease inhibitor AEBSF

to degrade PK before cell incubation. We chose AEBSF over phenylmethylsulfonyl fluoride (PMSF) as AEBSF is soluble in water and is less toxic than PMSF [592]. PK and AEBSF treatment of both PRDX2 and PRDX5 resulted in significantly decreased IL-8 levels, whereas it had no effect on the ability of LPS to stimulate IL-8 release. These results demonstrate (i) the efficiency of the endotoxin removal method, (ii) the proper choice of PK in combination with AEBSF as endotoxin control strategy in the ELISA, and (iii) the intrinsic pro-inflammatory activity of PRDX2 and PRDX5, that is not attributed to LPS contamination of the protein productions.

Negative and positive controls

We used two different positive controls in the assays, namely smooth LPS (*i.e.* LPS with long O-polysaccharide chains) and HMGB1, both known to induce a TLR4-dependent pro-inflammatory response [287,499]. LPS signaling through TLR4/MD-2/CD14 is a process fully characterized as the transfer cascade events of LPS [272] and the crystal structure of the LPS/MD-2/TLR4 complex [295] were determined (**Figure 14**). Disulfide HMGB1 is known to bind TLR4/MD-2 to induce a pro-inflammatory response [273], and CD14 was shown to be required for optimal HMGB1-dependent TLR4 activation [513]. Regarding cell transfection with TLR4-, MD-2 or CD-14 coding vectors, an additional control consisting in the use of an empty vector would have enabled maintaining all cells in the same conditions, thereby allowing comparison of the IL-8 secretion levels between cell lines (*e.g.* between HEK293 cells and HEK293 cells transiently transfected with a CD14-coding vector).

AFM-based SMFS for the study of PRDXs binding towards recombinant receptors

To probe the binding of PRDXs towards recombinant receptors and characterize the kinetics of the interactions, we performed AFM-based SMFS. In the present thesis, we investigated the kinetic parameters of various interactions. As discussed in **section 2.3.3.**, the strategy of AFM-based DFS study involves selecting appropriate (i) AFM probe and calibration method for quantitative measurements, (ii) tip and surface functionalization chemistries, (iii) environmental factors (buffer composition, pH, temperature), (iv) controls for the specificity of the interactions, and (v) data analysis procedure. Some of these aspects are discussed below.

AFM probe, functionalization chemistries and environmental conditions

For all AFM experiments, we used the softest cantilever available (cantilever C of MSCT probe). Cantilevers calibrated by the thermal tune method in this study displayed spring constants ranging from 0.0181 to 0.0352 N·m⁻¹. Softer cantilevers allow detecting smaller forces, but have larger thermal fluctuations, so that the force

resolution is usually limited by the thermal noise of the cantilever. Ideally, combination of cantilevers of different stiffness should be used.

We used two approaches to graft the PRDX proteins and the partners probed (*i.e.* TLR4, MD-2 and CD14) on AFM tips and surfaces, respectively. Either we took advantage of the protein His tag by using *tris*-NTA groups with Ni^{2+} , or we covalently attach the proteins through any of their free amino group. In a first approach, PRDX2 and TLR4 were grafted *via* their His tag. However, as PRDX2 exists as a dimer or decamer, only the tag of one subunit is bound to the AFM tip. Therefore, this approach may lead to nonspecific binding arising from interactions between the 6xHis tag of other subunits and unbound *tris*-NTA groups on the surface. For this reason, and for the purpose of verifying that the oriented attachment of PRDX2 did not impede the binding, we performed similar experiments using an untagged PRDX2 covalently grafted through the AFM tip. Although the most probable rupture forces obtained upon breaking interactions with TLR4 were not the same, whether this difference can be attributed to the orientation of the protein on the tip remains to be determined.

The experiments involving both PRDXs and TLR4 were performed in PBS. Despite the presence of PO_4^{3-} that can precipitate Ni^{2+} in solution, whether chelated Ni^{2+} will precipitate cannot be determined based on the solubility product since it is immobilized onto a support. In practice, phosphate buffers are often used in protein purification for the binding of the proteins using Ni-NTA matrixes [593]. As proteins are retained on the matrix, it is unlikely that chelated Ni^{2+} precipitate from the AFM tips. Although *tris*-NTA reach a nanomolar and subnanomolar affinity towards 6xHis and 10xHis tags, respectively [473], we chose to covalently graft the proteins on both tips and surfaces *via* their free amino groups for the following experiments to avoid by any chance that PRDXs and probed partners detach from the tip and surface during the course of the experiments (~2 days). These experiments include the binding of the PRDX5 redox forms with TLR4, the binding of PRDX5 with MD-2 and CD14, as well as the binding of PRDX2 with MD-2 and CD14. It is worth noting that no quenching step (*e.g.* with ethanolamine hydrochloride, pH 8.5) is currently performed during surface functionalization [416,487,594], on the contrary of tip functionalization. Therefore, unbound reactive NHS ester groups on the surface could bind the AFM tip-functionalized ligand, giving rise to nonspecific binding.

Controls for the specificity of the interactions probed

As explained in **section 2.3.3.**, the specificity of the interactions probed is evaluated by assessing the BF before and after injecting either a blocking antibody or one of the partners of the interaction, as well as by removing or replacing one of the partners (either on the tip or on the surface). In this project, we removed PRDX2

from the tip or TLR4 from the surface, and we injected antibodies (*i.e.* neutralizing anti-CD14, anti-MD-2, and anti-TLR4 antibodies or anti-PRDX5 and anti-PRDX2 sera). However, the use of these controls shows limitations. Removing one of the partners from the set-up may give rise to nonspecific interactions, for instance, between *tris*-NTA groups on the AFM tip and the coated surface, as shown on **Figure 60**.

The control consisting in injecting a saturating concentration of one of the partners does not constitute a suitable control when the ligand or the receptor is able to dimerize (or further oligomerize). As PRDXs, TLR4, CD14 and MD-2 are able to dimerize or oligomerize, injecting one of the two partners would not impede specific interactions from occurring. For instance, when probing PRDX2–TLR4 interaction, we could control the specificity by injecting free PRDX2 molecules in the solution. However, as PRDX2 subunits are able to interact with each other, rupture events could arise from the unbinding of PRDX2–PRDX2 interactions between PRDX2 molecules bound to surface-coated TLR4 and tip-grafted PRDX2 molecules. As a consequence, the BF would remain falsely constant before and after injection.

Finally, the use of an antibody requires (i) prior characterization of its specificity by performing similar experiments with an isotype control antibody, and (ii) validation of its specific blocking ability regarding the binding. Indeed, monoclonal antibodies may recognize an epitope on a receptor that is different from the ligand binding site to that receptor. In this project, we showed that the neutralizing anti-CD14 antibody could inhibit the cytokine-inducing ability of PRDX2 and PRDX5 but was not able to block the binding of both PRDXs to CD14. This may be explained by the fact that the anti-CD14 antibody used was selected for its ability to efficiently neutralize the CD14-mediated biological activity of TLR4. Therefore, if the binding site of both PRDXs on CD14 is different from the paratope recognized by the anti-CD14 antibody on TLR4, PRDXs would still be able to bind to CD14.

It is worth mentioning that our BFs were very low (*i.e.* <5%) for all the interactions probed. Low BFs complicate observation of the effect of the controls. To increase the occurrence of binding events, we could have modified the functionalization strategies (*e.g.* increase the ratio of carboxyl- and hydroxyl-terminated alkanethiols on the surface) or increased the contact time of the tip on the surface. However, increasing the contact time often give rise to more nonspecific binding.

Data treatment

During the course of this project, the method used to perform DFS data analysis evolved slightly. The similarities and variations between the different result sections (see **sections 3.1., 3.2. and 3.3.**) are summarized in **Table 5**, and the main points are discussed below.

Table 5. Comparison of the strategies employed to analyze DFS data of SMFS experiments in sections 3.1., 3.2. and 3.3. The main similarities and divergences are presented.

Steps	Procedure	Section 3.1.	Section 3.2.	Section 3.3.
1	Sorting and selection of specific FD curves	Manually (one by one), with the human eye, by looking at the extension pattern of the PEG linker and the rupture distance (≤ 30 nm)		
2	Extraction of the rupture forces and LRs	Manually F = height of adhesive peak; LR = linear fit (slope) of the F versus time curve just before rupture		
3	Analysis of the rupture force distribution	Separation of the spectrum of LRs into discrete ranges of equal width Estimation of the PDF with histograms	Estimation of the PDF with a kernel distribution	
4	Analysis of the contribution of multiple interactions	Normal (gaussian) distribution(s) - Iterative fitting using multiple gaussian functions to deconvolute the distribution into its multiple components so that the cumulative gaussian function to converge to the kernel smoothing function - Number, exact position and width of the peaks determined with a computational method, using the second derivative of the kernel smoothing function and restricting the detection to positive peaks only	- Iterative fitting using multiple gaussian functions to deconvolute the distribution into its multiple components so that the cumulative gaussian function to converge to the kernel smoothing function - Number, exact position and width of the peaks determined with a computational method, using the second derivative of the kernel smoothing function and restricting the detection to positive peaks only	Peak filtering threshold height of 20% in height (default value)
5	Extraction of the kinetic parameters with a biophysical model	Bell-Evans model, using the most probable rupture forces and the mean LR for each LR range		

The first step of the data analysis consists in sorting the specific FD curves. Despite current efforts towards the development of automated software [595],

specific FD curves are often selected with the human eye, thereby introducing a first operator bias in the analysis. Furthermore, a FD curve showing a typical extension pattern could arise from the interaction between a bare linker on the AFM tip and the coated surface. With the objective of investigating the extent of the forces of these nonspecific interactions, we plotted the rupture forces obtained with a tip without 6xHis-tagged PRDX2 (**Figure 60a**) or a surface without TLR4 (**Figure 60b**) on the DFS plot obtained for the 6xHis-tagged PRDX2–TLR4 interaction. As illustrated in **Figure 60**, nonspecific interactions form a data cloud that overlap with the data obtained by probing the interactions with both PRDX2-functionalized tips and TLR4-coated surfaces. In the present case, it is most likely the 6xHis tag that interacts with Ni^{2+} -NTA. As a consequence, the mere consideration of the extension pattern of the PEG linker to select specific FC curves is not sufficient since it does not necessarily guarantee the formation of the biomolecular complex under study. In short, the extent of nonspecific interactions cannot currently be fully evaluated in DFS analysis.

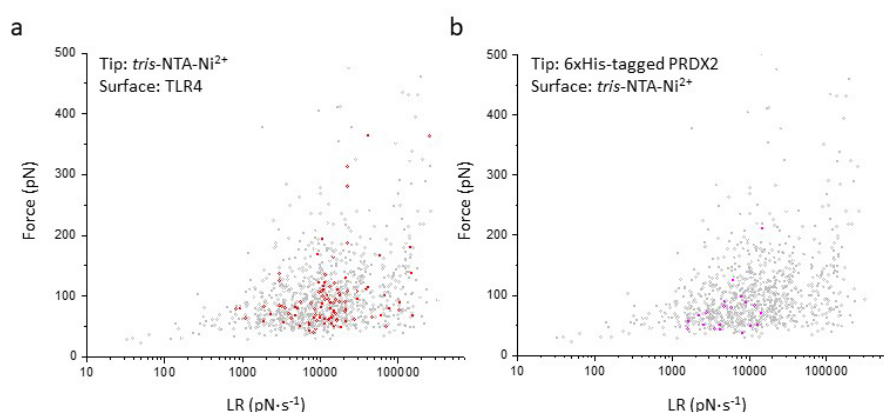


Figure 60. Rupture forces and LR of nonspecific interactions plotted on the DFS plot of the PRDX2–TLR4 interaction. Data obtained by probing the interactions between 6xHis-tagged PRDX2 and TLR4 are shown as grey circles. **(a)** The TLR4-coated surface was probed with tips carrying *tris*-NTA-Ni²⁺ (on a narrower range of LR) and the values of the rupture forces and the LR were plotted (red circles). **(b)** AFM tips functionalized with 6xHis-tagged PRDX2 were used to probe model surfaces functionalized with *tris*-NTA-Ni²⁺ but lacking TLR4 (on a narrower range of LR). The rupture forces and the LR were plotted (pink circles).

The next step in the data analysis consists in extracting the rupture forces and LR from the curves. This analysis is also performed with the human eye, introducing another operator bias in the analysis. As previously mentioned, rupture forces are extracted from the adhesive peaks of the FD curves and LR are practically extracted by fitting linearly the force-time curve just before rupture. However, no consensus exists on the definition of the curve portion to be considered as being ‘just before rupture’, leading to variations in the LR value depending on the selected portion, as

depicted in **Figure 61**. However, as LR values are eventually expressed as their logarithms and are pooled within ranges, the influence of such variations remains limited.

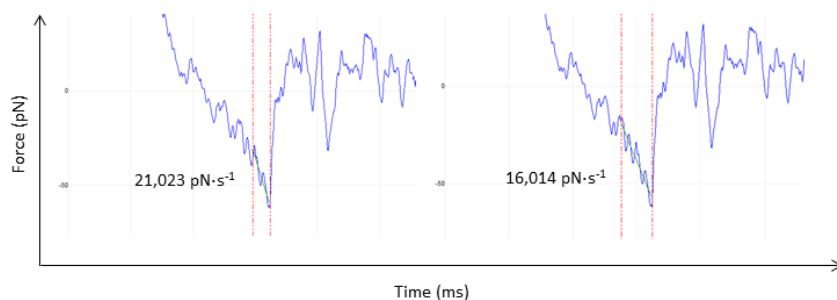


Figure 61. Force-time curve of an adhesive event between untagged PRDX2 and TLR4. The portion of the curve fitted with a linear model is used to determine the LR value. Depending on the portion considered for the analysis, the LR value varies. LR = 21,023 pN·s⁻¹ (left) and LR = 16,014 pN·s⁻¹ (right).

Once the rupture forces and the LR values have been extracted for each single specific adhesive curve, all values are pooled into the DFS plot, which displays rupture forces as a function of the logarithm of the LR values. Depending on the system under study, the cloud of data points on the DFS plot should be assessed on whether it results from the introduction of a PEG linker between the AFM tip and the ligand or from multiple interactions. As mentioned in **section 2.3.3.**, SMFS experiments can lead to the probing of multiple interactions arising between multiple PRDX-carrying linkers on the AFM tip apex and one or multiple receptors on the surface, despite the fact that tip and surface functionalization strategies were developed in such a way as to limit the occurrence of such interactions (**Figure 62**). In addition, multiple interactions may also occur due to oligomeric nature of the biological systems studied in this project (**Figure 62**). Indeed, both PRDX2 and PRDX5, tethered on the AFM tips, can form dimers, and even decamers in the case of PRDX2 [90,106]. Similarly, TLR4 and MD-2 (but not CD14), immobilized onto AFM surfaces, are also known to form dimers, and even HMW structures in the case of MD-2 [306,596,597]. As a consequence, we considered that the DFS plot most likely contains a mix of single and multiple interactions. However, determining their contribution, *i.e.* the number of peaks or populations, is the main issue in DFS analysis. In our studies, we first pooled the LR values into discrete ranges of equal width. So far, there is no consensus on the minimum number of data points required within a single LR range to achieve statistical significance. However, in some LR ranges, the number of points is very low, so that the results provided by these LR ranges should be used and interpreted carefully. Ideally, they should not have been considered further in the analysis.

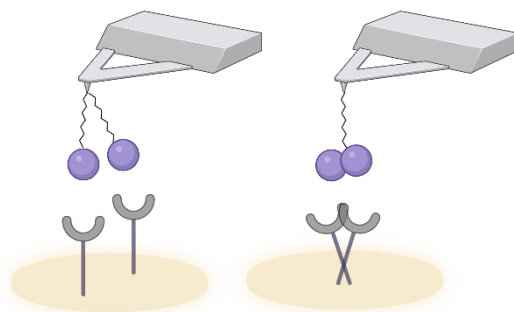


Figure 62. Schematics depicting the possible causes for the probing of multiple interactions in SMFS experiments. Multiple interactions can arise due to multiple linkers on the AFM tip interacting with multiple receptors on the surface (left) or a dimeric/oligomeric ligand interacting with a dimeric/oligomeric receptor (right). Created with BioRender.com.

Next, the PDF of the rupture forces must be determined. As mentioned in **section 2.3.3.**, histograms showing the frequency of the rupture forces for discrete classes are widely used [440–442]. This procedure is simple and intuitive, but shows several disadvantages: (i) the choice of bins (bin width and bin edge) is arbitrary, despite attempts to identify a method to determine the optimal number of bins [598–602], (ii) the histogram shows jump discontinuities at the bin edges, producing a discrete PDF, and is therefore a poor estimate for continuously differentiable probability densities, (iii) the histogram displays bumps that may have been observed only due to noise [603]. This approach was performed in the preliminary study (see **section 3.1.**). In the present thesis (see **sections 3.2. and 3.3.**), we therefore used another method to estimate the PDF of the rupture forces, namely a kernel estimation method [604]. The advantages of the kernel density estimator compared to the commonly used histograms have been summarized by Węglarczyk [447]: “(i) *It is a smooth curve and thus it better exhibits the details of the PDF, suggesting in some cases non-unimodality; (ii) It uses all sample points' locations, so, therefore, it better reveals the information contained in the sample; (iii) It more convincingly suggests multimodality; (iv) The bias of the kernel estimator is of one order better than that of a histogram estimator* [605]. [...]”.

When using histograms, the strategy commonly used involves an iterative fitting using multiple gaussian functions to deconvolute the distribution into its multiple components. In practice, however, the number of peaks, exact position (*i.e.* peak center), gaussian width or area are adjusted arbitrarily by the operator, introducing an additional bias in the analysis [435]. Indeed, since we cannot verify the biophysical characteristics (*i.e.* multivalency) of the interactions probed, it is not possible to know the number of peaks nor the frequency of single or multiple interactions probed. This approach was performed in **section 3.1.**, using identical bin sizes for all

the LR ranges, and lead to the fitting of a first, a second, and for some LR ranges, a third population (*i.e.* peak), corresponding to single, double, and triple interactions. However, given that some parameters (*e.g.* peak width) were chosen, whether cumulative gaussian function matches the histogram distribution is debatable. Furthermore, the value of the error (*i.e.* standard deviation) on the most probable rupture forces impacts the kinetic off-rate value given by the fit. As the standard deviation associated with the gaussian function is proportional to the width (**Equation 14**), modifying arbitrarily the peak widths changes the associated errors, and thereby influences the value of the kinetic off-rate.

$$\sigma = \frac{w}{\sqrt{8 \ln 2}}$$

Equation 14. Standard deviation (σ) associated with the gaussian function. w is the full width at half maximum of the gaussian function.

In the present thesis (see **sections 3.2. and 3.3.**), we innovated by using a computational method ('Peak Analyzer' tool on OriginPro software), allowing peak detection and non-linear fitting to the peaks. Our method, based on a kernel distribution and the 'Peak Analyzer' software, has the merit of leading to more reproducible results compared to the method based on histograms and fitting with the human eye. However, depending on the peak filtering threshold used, the number of populations (*i.e.* peaks) that are detected varies. In the first study (see **section 3.2.**), we set the lowest threshold allowing the fit (cumulative gaussian function) to converge to the kernel smoothing function. However, this resulted in some populations arising from noise. In the second study (see **section 3.3.**), we chose a threshold of 20% of the maximum peak height so that the populations arising from noise were not considered. This approach resulted in the fitting of one, two or three populations (*i.e.* peaks), indicating the probing of single, double, or triple interactions depending on the biological partners and LR range. To compare the impact of the peak filtering threshold value, we performed again the DFS analysis of the interactions between two redox forms of the first study (see **section 3.2.**) and TLR4, by setting the threshold at 20% as performed in the second study (**Figure 63**).

The comparison of the most probable rupture forces for the first population (*i.e.* 1st peak) and kinetic parameters for all strategies (**sections 3.1., 3.2. and 3.3.**) are shown in **Table 6**. The values of the most probable rupture forces differ between **section 3.1.** and **section 3.2.**, but are very similar regardless of the threshold value, which indicates that the first population in the first study (see **section 3.2.**) is most likely biophysically relevant in all LR ranges, thereby that the first peak does not arise from noise. In addition, these results suggest that the value of the kinetic parameters is influenced by the method used to generate the PDF of the rupture forces, on the

contrary of the peak filtering threshold chosen. Note that the Bayesian information criterion (BIC) and Akaike information criterion (AIC) are tools that can provide statistically valuable information regarding the number of gaussian functions to use during deconvolution [606].

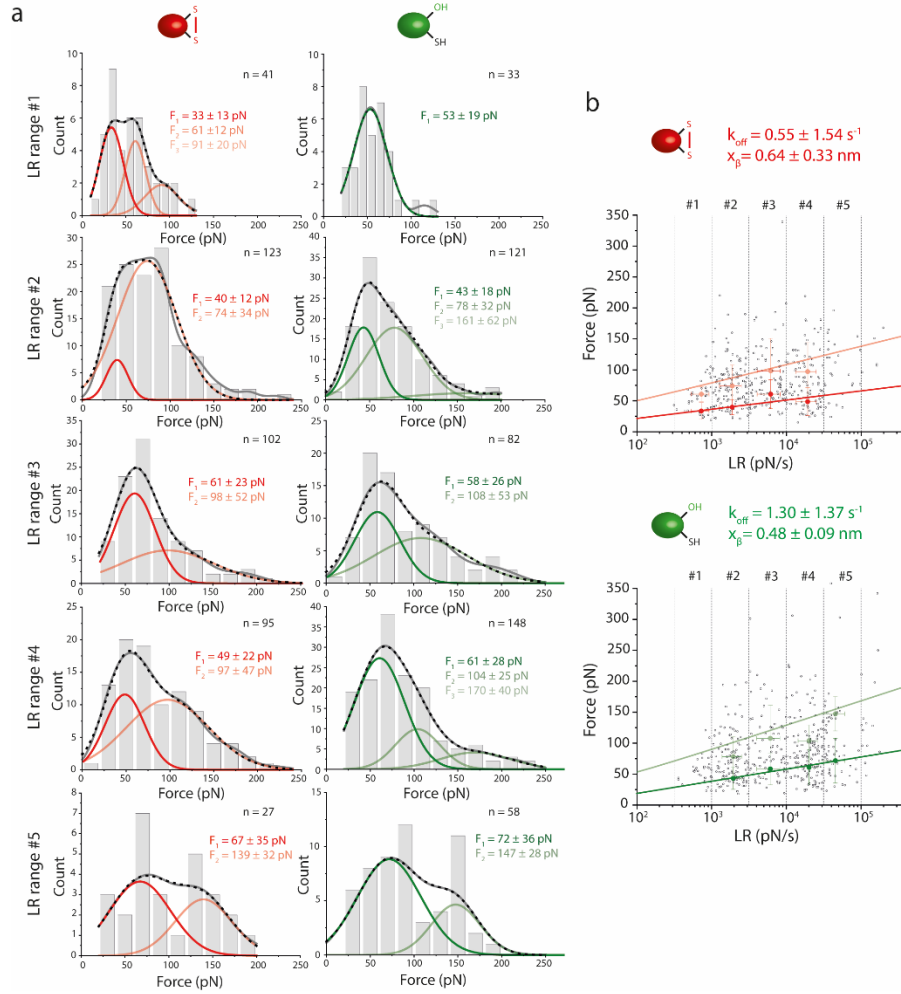


Figure 63. DFS analysis of the interactions between dsPRDX5 or PRDX5^{C47S} and TLR4 using a peak filtering threshold of 20% in height. (a) Kernel density function and multi-gaussian analysis of the rupture forces obtained at the different LR ranges for dsPRDX5 (left) and PRDX5^{C47S} (right). A filtering threshold of 20% in peak height was set for peak detection. (b) DFS plots of dsPRDX5 (up) and PRDX5^{C47S} (down). LR ranges with a number of points ≥ 40 were considered for the analysis. The k_{off} and x_β values were extracted from the Bell-Evans fit.

Table 6. Comparison of the most probable rupture forces and kinetic parameters obtained for dsPRDX5 and PRDX5^{C47S} depending on the strategy used for DFS analysis. In the DFS analysis of **section 3.1.**, the rupture forces were binned into histograms, while in the DFS analysis of **sections 3.2. and 3.3.**, a kernel distribution was used to represent the PDF of the rupture forces. In **section 3.2.**, the lowest threshold allowing the fit to converge was used, while in **section 3.3.**, the threshold was set at 20% in peak height. The k_{off} and x_β values are derived from the first population (F_1), which corresponds to a single interaction. Note that the k_{off} and x_β values of the first study were recalculated by rejecting from the analysis the LR ranges having a number of data points ≤ 40 (shown in *italics*).

		Section 3.1.	Section 3.2.	
		Histograms	Lowest threshold	Threshold of 20%
dsPRDX5	LR#1	$F_1 = 45 \pm 15$ pN	$F_1 = 32 \pm 13$ pN	$F_1 = 33 \pm 13$ pN
	LR#2	$F_1 = 52 \pm 13$ pN	$F_1 = 40 \pm 18$ pN	$F_1 = 40 \pm 12$ pN
	LR#3	$F_1 = 55 \pm 12$ pN	$F_1 = 61 \pm 25$ pN	$F_1 = 61 \pm 23$ pN
	LR#4	$F_1 = 56 \pm 13$ pN	$F_1 = 49 \pm 22$ pN	$F_1 = 49 \pm 22$ pN
	LR#5	$F_1 = 58 \pm 12$ pN	$F_1 = 67 \pm 35$ pN	$F_1 = 67 \pm 35$ pN
	k_{off}	0.0003 ± 0.0004 s ⁻¹	0.74 ± 1.82 s ⁻¹	0.55 ± 1.54 s ⁻¹
	x_β	1.2 ± 0.2 nm	0.61 ± 0.29 nm	0.64 ± 0.33 nm
PRDX5 ^{C47S}	LR#1	n/a	$F_1 = 34 \pm 17$ pN	$F_1 = 53 \pm 19$ pN
	LR#2	n/a	$F_1 = 44 \pm 19$ pN	$F_1 = 43 \pm 18$ pN
	LR#3	n/a	$F_1 = 55 \pm 25$ pN	$F_1 = 58 \pm 26$ pN
	LR#4	n/a	$F_1 = 64 \pm 30$ pN	$F_1 = 61 \pm 28$ pN
	LR#5	n/a	$F_1 = 72 \pm 36$ pN	$F_1 = 72 \pm 36$ pN
	k_{off}	n/a	1.36 ± 0.24 s ⁻¹	1.30 ± 1.37 s ⁻¹
	x_β	n/a	0.47 ± 0.02 nm	0.48 ± 0.09 nm

Finally, the last step in SMFS data treatment consists in the use of a biophysical model to fit the most probable rupture forces corresponding to the monovalent interaction in order to extract the kinetic parameters of the interaction. For all the DFS analyses of this thesis, the Bell-Evans model was used since we observed a linear dependency of the rupture force on the logarithmic LR. This dependence can take into account the nonlinear stretching of the PEG linker [438,607]. However, this model has some limitations, as discussed in **section 2.3.3**. As the choice of the model is always based on assumptions, some researchers carried out experiments to compare kinetic parameters extracted from different models [608]. However, no clear-cut evidence was gained to rule out the parameters extracted from one of the models, and the authors concluded that: *“Given the variance between SMFS experiments conducted under identical conditions, we suggest that the scientific value of SMFS is not to obtain absolute kinetic and thermodynamic parameters of a given system, but rather serves as a tool to compare two or more different experiments or how a variable affects these parameters.”* Therefore, comparing kinetic parameters obtained from DFS analysis should be limited to values obtained with similar fitting processes and biophysical models, hence the k_{off} extraction procedure should always be specified together with the obtained value.

4.2. Biological insights of the main achievements

4.2.1. Human PRDX2 and PRDX5 induce a TLR4-dependent pro-inflammatory response

The discovery that extracellular PRDXs are capable of triggering a TLR4-dependent pro-inflammatory response was first illustrated for the malarial PRDX1 homolog (from *Plasmodium berghei* ANKA), PbA [357]. This pioneering work served as the basis for the preliminary investigation on whether extracellular/secreted human PRDXs could function as DAMPs through TLR4 activation. Firstly demonstrated for human PRDX1 [344] and extensively studied for that protein since then [111,362,365,375], it was later shown that TLR4 also plays a key role in the extracellular activity of PRDX2, PRDX5 and PRDX6 [116,117,345].

In this work, we examined the TLR4-dependent cytokine secretion triggered by human PRDX2 and PRDX5. We exposed HEK293 cells stably expressing TLR4 along with its accessory molecules MD-2 and CD14 to PRDXs and we measured the IL-8 secretion levels. Both PRDX2 and PRDX5 significantly induce IL-8 secretion from cells compared to the control condition (PBS). We further confirmed the role of TLR4 in the pro-inflammatory response triggered by PRDX2 and PRDX5 with a neutralizing antibody against TLR4. Both PRDX2 and PRDX5 induced IL-8 secretion to a lesser extent compared to LPS or HMGB1, even with a 10-times lower concentration of the latter. Another study however showed that PRDX5 and PRDX6 had potent IL-23p19-inducing activity compared to HMGB1 [116]. This difference might be explained by the fact that different cytokines display different effective functions, and measurements of additional cytokines than IL-8 would allow to gain a broader view of the pro-inflammatory effects triggered by PRDXs.

Although IL-8 secretion levels from HEK293 cells expressing TLR4, MD-2 and CD14 were lower for PRDX2 and PRDX5 compared to HMGB1, PMA-differentiated THP-1 cells produced higher IL-8 level upon exposure to PRDX5 and similar level with PRDX2 (**Figure 41a,e**), indicating that other receptors (including TLR2) are most likely involved in the PRDX-induced pro-inflammatory activity. The involvement of TLR2 has indeed been reported for PRDX5 [116]. Moreover, we observed that CD14 plays a key role in the pro-inflammatory response induced by PRDX2 and PRDX5 independently of TLR4 (see **section 4.2.5**).

Finally, we demonstrated that human PRDX5 exhibits a more potent cytokine-inducing activity compared to PRDX2. Whereas PRDX5 is mainly present as dimers in solution, PRDX2 exists in an equilibrium between dimers and decamers. Therefore, the PRDX2 productions used in our study could contain both oligomeric forms, which might show differential ability to induce cytokine secretion. In particular, it was

shown that circulating PRDX2 was present exclusively as disulfide-linked homodimers [104]. In addition, HEK293 cells stimulated with TNF- α were reported to release PRDX2 in its disulfide, dimeric form [104]. These results might indicate that solely the dimeric form is able to induce cytokine secretion. Further investigation is therefore required to elucidate the influence of the oligomeric state of PRDX2 on its cytokine-inducing activity.

4.2.2. Disulfide PRDX5 triggers potent pro-inflammatory cytokine release

The redox and/or oligomeric state of PRDXs is altered by redox modifications or mutation of their Cys residues, leading to modulation of their functions [42]. We therefore investigated whether the redox status of PRDX Cys residues influences the pro-inflammatory activity of the extracellular protein. Among human PRDXs, PRDX5 represents the simplest model regarding the interconnection between redox and oligomeric states, as it exists as a dimer irrespective of its oxidation status [90]. We validated with SEC-MALS that PRDX5 was mainly present as dimers in solution, regardless of the redox state. As discussed in **section 4.1.1.**, we compared the 6xHis-tagged PRDX5, which is mainly oxidized with a disulfide between C47 and C171 with the PRDX5^{C47S} mutant, as well as chemically reduced/alkylated and hyperoxidized PRDX5.

We evaluated whether the redox state of the PRDX5 Cys residues plays a role in the IL-8 secretion induced by PRDX5 by exposing HEK293 cells expressing TLR4 along with its accessory proteins MD-2 and CD14 to the various PRDX5 redox forms. We found that disulfide PRDX5 has potent pro-inflammatory activity compared to the other redox forms. In particular, DTT and IAM treatments inhibited the cytokine-inducing capacity of PRDX5. These results are in line with the observation that DTT-treated PRDX1 leads to significantly reduced secretion levels of IL-6 from TG-elicited macrophages as well as TNF- α from RAW264.7 macrophages [344]. However, our results are in contradiction with a previous study showing that IAM treatment of PRDX5 did not affect the IL-23p19 mRNA expression level produced by BMDCs [116]. We previously explained that our conditions largely differ (see **section 3.2.4.**), which may explain the discrepancies between the studies.

Moreover, the IL-8 level resulting from PRDX5^{C47S} exposure was lower than that of the other redox forms, indicating that the presence of the absolutely conserved catalytic Cys is required for the ability of PRDX5 to induce cytokine secretion. These results are in contradiction with other studies evaluating the influence of the PRDX redox state [116,344,503]. Firstly, Shichita *et al.* showed that disruption of PRDX5 Cys residues by point mutations did not affect the IL-23p19 mRNA expression level produced by BMDCs [116]. However, as just mentioned, the conditions used for our

respective assays may explain the divergent results. Secondly, mutation of the catalytic C52 in human PRDX1 was not found to impact the IL-6 secretion from TG-elicited macrophages compared to wild-type PRDX1 [344]. Thirdly, mutation of the catalytic C52 in the PAMP cytosolic tryparedoxin peroxidase from *Trypanosoma cruzi* was evidenced to induce similar IL-8 production from HEK293 cells overexpressing murine TLR4 compared to the wild-type protein [503]. On the other hand, the presence of the catalytic Cys in the tryparedoxin peroxidase from *Trypanosoma cruzi* was demonstrated to be required for inducing the recruitment of IL-12/23p40-producing innate antigen-presenting cells and promoting a strong specific Th1 immune response [503]. Altogether, these observations underly a finer modulation of the pro-inflammatory response triggered by PRDXs, that most likely depends on specific features of each PRDX subgroup. Therefore, further investigation is needed to fully understand the role of the catalytic Cys residue in the modulation of the pro-inflammatory response and the link with the oligomeric status of the protein.

4.2.3. Human PRDX2 and PRDX5 bind to TLR4

Although most human PRDXs have been shown to activate TLR4, a physical interaction with TLR4 has only been reported for PRDX1 using immunoprecipitation and pull-down assays [344,365,375]. As both PRDX2 and PRDX5 were able to induce cytokine secretion through a TLR4-dependent pathway, we investigated a direct interaction between the partners by probing the binding between both PRDXs and TLR4 using AFM-based SMFS. In particular, we investigated the binding of 6xHis-tagged PRDX5 redox forms (dsPRDX5, PRDX5^{C47S}, r/aPRDX5 and s/sPRDX5) as well as 6xHis-tagged and untagged PRDX2, with TLR4. We also probed the binding of untagged PRDX2 with TLR4 using BLI.

With the exception of hyperoxidized PRDX5 (s/sPRDX5), we demonstrated that all PRDXs were able to directly bind to TLR4. The binding kinetic parameters (*i.e.* k_{off} and x_{β}) obtained are summarized in **Table 7**. Our results illustrate that the PRDX2–TLR4 complex is more stable than the PRDX5–TLR4 complex. This observation might be explained by the differential conformation of both PRDXs (**Figure 6**). Indeed, Shichita *et al.* [116] evidenced that the region essential for IL-23p19 induction in BMDCs was mainly located on the PRDX surface and contains β_4 sheet and α_3 helix regions that are well conserved among PRDXs and among various species [48–50] (**Figure 17**). Whereas such region is present near the dimerization interface in PRDX5, PRDX2 subunits dimerizes at a different interface, leading to exposed α_3 helix. Therefore, access to this conserved region in PRDX2 might be facilitated compared to PRDX5. However, whether such conserved region involved in IL-23p19 induction corresponds to the binding site of PRDXs with TLR4 remains to be investigated.

Furthermore, we found that TLR4 could not discriminate between the redox states of PRDX5 regarding the dissociation rate constant and the distance to the transition state. This is not a generality since it was shown for the well-studied DAMP HMGB1 that TLR4 binds disulfide and DTT-reduced HMGB1 with different dissociation rate constants [302]. In more details, the authors observed in a first study that HMGB1 was not capable of binding TLR4 directly in the absence of MD-2 [273]. However, by refining the experimental conditions, they found that disulfide HMGB1 does bind to TLR4, and further showed that both disulfide and DTT-reduced HMGB1 bind TLR4 with similar equilibrium dissociation constant, but that disulfide HMGB1 shows a 2-fold lower k_{off} value compared with reduced HMGB1 [302] (Table 7).

Table 7. Comparison⁷ of kinetic parameters describing interactions of PRDX2, PRDX5 and HMGB1 with TLR4.

Ligand	Feature	Technique	k_{off} (s ⁻¹)	x_β (nm)	Ref
PRDX5	Disulfide	AFM-based SMFS	1.02 ± 1.57	0.57 ± 0.18	[511]
	C47S	AFM-based SMFS	1.97 ± 0.33	0.44 ± 0.02	[511]
	DTT-reduced, IAM-alkylated	AFM-based SMFS	2.47 ± 2.29	0.46 ± 0.10	[511]
PRDX2	6xHis tag	AFM-based SMFS	$5.2 \times 10^{-3} \pm 5.0 \times 10^{-3}$	0.76 ± 0.07	
	Untagged	AFM-based SMFS BLI	$4 \times 10^{-4} \pm 1.4 \times 10^{-3}$ 8×10^{-4}	1.13 ± 0.27	
HMGB1	Disulfide	BLI	No binding		[513]
		SPR	No binding		[273]
		SPR	0.128 ± 0.002		[302]
	DTT-reduced	SPR	0.203 ± 0.001		[302]

In the cellular context, we demonstrated that the binding extent of the PRDX5^{C47S} mutant to the surface of living HEK293 cells expressing TLR4, MD-2 and CD14 was limited compared to disulfide, reduced/alkylated and hyperoxidized PRDX5, indicating that the C47 is required for proper binding to the surface of cells. This is surprising since the conserved region involved in the IL-23p19 induction in BMDCs is almost on the opposite side of the peroxide binding site [116]. In addition, the observations that PRDX5 redox forms bind differentially the surface of living cells but interact with a similar dissociation rate constant with TLR4 might indicate that either TLR4 conformation (*i.e.* dimer) and/or stabilization in the plasma membrane are important factors involved in the binding, or that the accessory molecules MD-2 and CD14 allow discriminating between the redox forms.

⁷ Note that the kinetic off-rate values obtained with different techniques should be compared with caution, as discussed in **section 4.1.3**.

4.2.4. The TLR4-dependent pro-inflammatory activity of PRDX2 and PRDX5 is dependent on MD-2

As developed in **section 1.2.6.**, TLR4 is often associated in a complex with MD-2 for the recognition of a large variety of DAMP ligands, including HMGB1, HSPs and β -amyloid. We therefore examined whether MD-2 was involved in the TLR4-dependent pro-inflammatory activity of PRDX2 and PRDX5. We previously reported that PRDX2 and PRDX5 induced IL-8 secretion from HEK293 cells stably expressing TLR4 along with both MD-2 and CD14. However, HEK293 cells stably expressing TLR4 alone produced much lower IL-8 levels, albeit significantly higher than the control PBS condition, indicating that the mere presence of TLR4 is not sufficient to trigger potent cytokine secretion following PRDX exposure. Co-transfecting both TLR4- and MD-2-coding vectors in HEK293 cells induced higher levels of IL-8 following exposure to PRDX2 or PRDX5, pinpointing the importance of MD-2 in the TLR4-dependent cytokine-inducing ability of PRDXs. The role of MD-2 was further confirmed with a neutralizing antibody against MD-2. The involvement of MD-2 in the TLR4-dependent pro-inflammatory activity of PRDXs has previously been shown for human PRDX1 [344]. Indeed, addition of a blocking antibody to MD-2 significantly inhibited the ability of PRDX1 to stimulate IL-6 secretion from TG-elicited macrophages, and MD-2 was found to be contained in the TLR4/PRDX1 complex immunoprecipitated from lysates of TG-elicited macrophages [344]. These results therefore suggest that PRDXs are recognized by the TLR4/MD-2 complex to induce cytokine secretion.

4.2.5. Human PRDX2 and PRDX5 activate a TLR4-independent pro-inflammatory response involving CD14

Most studies on CD14 concern its role as co-receptor of TLR4, facilitating notably cellular responses to LPS or to various DAMPs, as highlighted in **section 1.2.6.** We therefore questioned whether CD14 was involved in the TLR4-dependent pro-inflammatory activity of PRDX2 and PRDX5 by assessing IL-8 levels produced by HEK293 cells co-expressing TLR4 and CD14. Whereas cells were unresponsive to both LPS and HMGB1 as expected, exposure to PRDX2 and PRDX5 significantly induced IL-8 secretion. As control, we measured IL-8 levels from HEK293 cells stably expressing CD14 solely. Surprisingly, the mere presence of CD14 lead to potent cytokine secretion upon cell exposure to PRDX2 or PRDX5. This result was further confirmed with HEK293 cells stably co-expressing MD-2 and CD14, and by transiently transfecting HEK293 cells with a CD14-coding vector allowing to examine whether such effect could be evidenced in cells expressing lower amount of CD14. The involvement of CD14 in the PRDX-dependent cytokine secretion was further confirmed with a neutralizing antibody to CD14. Although the involvement of CD14

was previously shown for human PRDX1 in the complex formed by TLR4 and MD-2 [344], our results show for the first time a TLR4-independent pathway involving CD14 for PRDXs.

Could it be that CD14 displays signaling capacities regarding PRDXs? From what is known, CD14 was identified before TLR4, as a marker of monocytes [609], and was initially proposed to be a PRR [610]. However, given the absence of an intracellular domain allowing to transduce intracellular signaling, it seemed unlikely to function as a PRR [274]. Most studies on CD14 at the time were therefore focused on its role as TLR co-receptor [276,611,612], and a large majority were related to its role in mediating cellular response to LPS [307,613,614]. In particular, CD14 was demonstrated to be involved in (i) controlling LPS presentation to the TLR4/MD-2 complex, thereby facilitating the cellular responses to low LPS concentrations, and (ii) regulating the re-localization of the LPS receptor complex (LPS/TLR4/MD-2) to the endosome, irrespective of the LPS concentration, leading to the activation of the TRAM-TRIF pathway and type-I IFN expression [292,308]. Some studies have now confirmed the role of CD14 as PRR [308,314], being capable of transducing a TLR4-independent signaling pathway following LPS exposure, leading to the activation of nuclear factor of activated T-cells (NFAT) transcription factor family members. Following LPS stimulation of dendritic cells (DCs), CD14 induces activation of Src-family kinase (SFK) and phospholipase $\text{C}\gamma 2$ (PLC $\gamma 2$) [314]. PLC $\gamma 2$, in turn, hydrolyzes membrane phosphatidylinositol bisphosphate (PIP $_2$), generating inositol-triphosphate (IP $_3$) and diacylglycerol [314]. This leads to an influx of extracellular Ca^{2+} and calcineurin-dependent nuclear NFAT translocation [314]. This pathway was shown to lead to the production of IL-2 and the apoptotic death of terminally differentiated DCs [314]. Whether human PRDXs mediate similar CD14-dependent pathway is conceivable. Indeed, the calcineurin/NFAT signaling pathway has been reported to regulate IL-8 expression [615,616]. Moreover, it is worth mentioning that both IL-2 and IL-8 are chemokines involved in the regulation of T-cell responses [617,618].

Furthermore, soluble CD14 was found unable to restore extracellular Ca^{2+} influx in CD14-deficient DCs [314]. In our work, we demonstrated that the presence of soluble CD14 did not lead to increased IL-8 levels produced by HEK293 co-expressing MD-2 and TLR4 following exposure to PRDX2 or PRDX5, on the contrary to LPS. This result underlies the requirement of GPI-anchored CD14 in the pro-inflammatory response.

To summarize, our results reveal novel aspects of the cellular responses to PRDXs as DAMPs, providing insights into the finer modulation of the pro-inflammatory response than initially believed. Whether PRDXs induce a similar pathway as LPS remains to be investigated.

4.2.6. Human PRDX2 and PRDX5 bind directly to MD-2 and CD14

To determine whether PRDX2 and PRDX5 are recognized by the TLR4/MD-2 complex through direct interaction with both proteins, we performed AFM-based SMFS to probe the interactions of PRDX2 and PRDX5 with MD-2. In addition, as we showed that both PRDXs were capable of inducing cytokine secretion through a TLR4-independent pathway involving CD14, we also examined the interactions of PRDX2 and PRDX5 with CD14. The kinetic parameters obtained for the interactions of both PRDXs with MD-2 and CD14 are shown in **Table 8**, respectively. The PRDX5–MD-2 interaction is less stable than the PRDX2–MD-2 interaction, as was also observed for the complexes formed with TLR4. Although comparison with another measurement system should be made with caution, the k_{off} values for PRDX2 and PRDX5 are close to the k_{off} value obtained for the HMGB1–MD-2 interaction using SPR [302] (**Table 8**).

Table 8. Comparison⁸ of kinetic parameters describing interactions with MD-2 and CD14.

System probed	Feature	Technique	k_{off} (s ⁻¹)	x_β (nm)	Ref
MD-2	PRDX5	6xHis tag	AFM-based SMFS	0.1825 ± 0.0774	0.45 ± 0.03
	PRDX2	6xHis tag	AFM-based SMFS	0.0644 ± 0.0593	0.48 ± 0.05
	HMGB1	Disulfide	SPR	0.110 ± 0.004	[302]
CD14	PRDX5	6xHis tag	AFM-based SMFS	0.0049 ± 0.0057	0.94 ± 0.11
	PRDX2	6xHis tag	AFM-based SMFS	0.0059 ± 0.0071	0.91 ± 0.11

⁸ Note that the kinetic off-rate values obtained with different techniques should be compared with caution, as discussed in **section 4.1.3**.

4.3. Proposition of a model for the action of PRDXs as DAMPs

Taken altogether, these results indicate that human PRDX2 and PRDX5 are able to act as DAMPs by inducing a TLR4-dependent pro-inflammatory response that requires MD-2. Both PRDXs most likely interact in a complex with TLR4 and MD-2. Furthermore, the disulfide in PRDX5 is required for potent pro-inflammatory response. In addition, both PRDX2 and PRDX5 can induce a TLR4-independent signaling pathway *via* GPI-anchored CD14 and are able to physically interact with CD14. These main biological achievements allow the proposition of a model for the molecular mechanism of action of PRDXs as DAMPs, depicted in **Figure 64**.

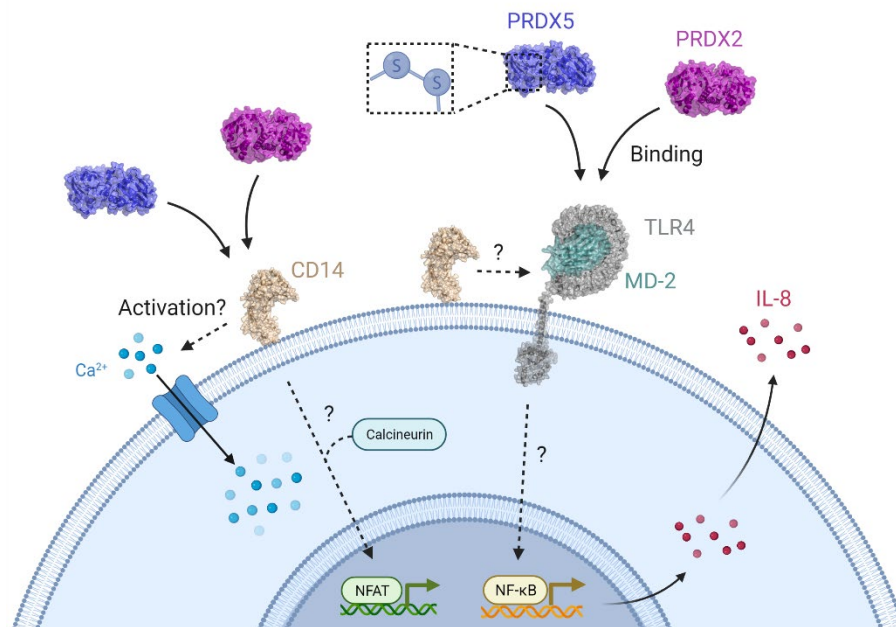


Figure 64. Main biological achievements and future perspectives. Human PRDX2 and PRDX5 are able to induce two different pro-inflammatory pathways leading to the secretion of IL-8. In the TLR4-dependent pathway, PRDX2 and PRDX5 most likely bind to the TLR4/MD-2 complex. The role of CD14 in the TLR4-dependent pro-inflammatory activity of PRDX2 and PRDX5 remains to be clarified. Moreover, whether the TLR4-dependent pathway induced by PRDX2 and PRDX5 leads to NF-κB activation needs further investigation. In the TLR4-independent pathway involving CD14, PRDX2 and PRDX5 might bind directly to CD14. Whether this pathway leads to Ca²⁺-calcineurin-dependent NFAT activation needs to be addressed. Created with BioRender.com.

Chapter V – Conclusion and Perspectives

The present thesis aimed at investigating the role of human PRDXs as DAMPs. The discovery that PRDXs are able to trigger a pro-inflammatory response is quite recent, and as a consequence, only few studies have addressed this matter. We focused on PRDXs from two different subgroups, namely the typical 2-Cys PRDX2 and the atypical 2-Cys PRDX5, to grasp current immunological questions related to the function of PRDXs as DAMPs that are still a major focus of present research. In particular, we investigated the role of (i) TLR4, one of the major innate immune receptors involved in pro-inflammatory responses, (ii) TLR4 accessory molecules MD-2 and CD14, and (iii) the redox state of PRDX Cys residues, in the pro-inflammatory activity of PRDX2 and PRDX5. To this end, we used a multidisciplinary approach allowing to address both the early step of PRDX recognition (*i.e.* binding to receptors) to the late events triggered within the cell (*i.e.* cytokine secretion). More specifically, we combined biomolecular (ELISA, FC) and biophysical (AFM-based SMFS) approaches to gain insights into extracellular DAMP function of PRDX2 and PRDX5.

Using these approaches, we first confirmed that both PRDX2 and PRDX5 were able to induce a pro-inflammatory response by triggering IL-8 secretion from PMA-differentiated THP-1 cells. We also highlighted the role of TLR4 in the PRDX pro-inflammatory activity by exposing HEK293 cells expressing TLR4 along with its accessory molecules MD-2 and CD14 to both PRDX2 and PRDX5. Neutralizing the activity of TLR4 with a specific antibody inhibited the cytokine-inducing ability of PRDX2 and PRDX5, confirming the involvement of TLR4 in the PRDX-induced cytokine secretion. We further demonstrated with AFM-based SMFS that both PRDXs were able to interact directly with TLR4.

To investigate whether the oxidation status of PRDX Cys residues influence the pro-inflammatory activity of PRDXs, we focused on human PRDX5 as it represents the simplest model in terms of oligomeric state, existing exclusively as a dimer in solution. We compared oxidized (disulfide) PRDX5 with chemically reduced/alkylated and hyperoxidized forms obtained using DTT/IAM or DTT/H₂O₂ treatment, respectively. The redox status of these PRDX5 forms were analyzed using mass spectrometry on intact proteins. Disulfide PRDX5 triggered potent IL-8 secretion from HEK293 cells expressing TLR4, MD-2 and CD14, compared to reduced/alkylated and hyperoxidized PRDX5. We also analyzed the role of the catalytic C47 by using a PRDX5 mutant in which the C47 was replaced by a Ser residue. IL-8 secretion level induced by PRDX5^{C47S} was significantly lower compared to the other redox forms, underlying the importance of the presence of the catalytic

Cys. We further confirmed this result by assessing the binding of the PRDX5 redox forms to the surface of HEK293 cells expressing TLR4, MD-2 and CD14 using FC. The binding of PRDX5^{C47S} was significantly reduced compared to the other PRDX5 redox forms. Finally, we found that all PRDX5 redox forms bind to TLR4 with similar kinetic parameters, highlighting the potential role of the TLR4 accessory molecules to allow discriminating the PRDX5 redox forms.

In that sense, we next addressed the role of TLR4 accessory molecules MD-2 and CD14 in the pro-inflammatory activity of human PRDX2 and PRDX5. Whereas HEK293 cells expressing solely TLR4 were almost completely unresponsive to both PRDXs, MD-2 co-expression restored IL-8 secretion. The role of MD-2 in the TLR4-dependent cytokine-inducing ability of PRDX2 and PRDX5 was further confirmed with a neutralizing antibody specific for MD-2. Surprisingly, potent IL-8 secretion was observed with HEK293 cells expressing solely CD14 upon stimulation with PRDX2 and PRDX5. Moreover, neutralizing antibody specific to CD14 significantly inhibited cytokine secretion, thereby confirming a role for CD14 in the pro-inflammatory activity of both PRDXs. To our knowledge, this is the first time that a TLR4-independent signaling pathway is discovered for extracellular PRDXs.

Altogether, these results report key roles for TLR4, MD-2, and CD14 in the extracellular DAMP function of PRDX2 and PRDX5. Furthermore, our work illustrated the importance of PRDX5 redox state on the pro-inflammatory activity of the protein, underlying once more the interplay between the redox status of PRDXs and modulation of their functions. We believe that our work contributes greatly to the understanding of how human PRDXs are capable of inducing a pro-inflammatory response. Of course, several questions remain unanswered.

In particular, the release mechanisms of human PRDXs into the extracellular milieu are still unclear, along with the associated redox/oligomeric states. Indeed, the redox status of HMGB1, one of the most studied DAMPs, was shown to differ depending on the mode of release [153]. HMGB1 is released in the disulfide form after pyroptosis, in the fully reduced or disulfide form after necrosis, and in the hyperoxidized (sulfonyl) form after apoptosis [177]. Regarding the oligomeric state, both circulating human PRDX1 and PRDX2 were previously shown to be present exclusively as disulfide-linked homodimers, and both proteins are released in this form upon stimulation with TNF- α [111]. Although these observations are in line with our study on the PRDX5 redox state, whether the pro-inflammatory activity of human PRDXs that are able to oligomerize (including PRDX2) is restricted to the disulfide-linked dimeric form needs to be explored.

Furthermore, our results using PMA-differentiated THP-1 cells emphasized the involvement of other receptors than TLR4 in the pro-inflammatory activity of PRDX2

and PRDX5. The extent of cellular receptors and other sensors able to recognize human PRDXs remains to be investigated. In this work, we provide a first step in answering this question by underlying the role of CD14, which is able to trigger a PRDX-dependent pro-inflammatory response independently of TLR4. The differential PRDX modulation of the pro-inflammatory response through TLR4 and CD14 requires further work to be fully understood. In addition, the precise mechanism of activation of TLR4 (and other receptors) needs to be evidenced. By evidencing a role for MD-2, we provided pieces of information related to the recognition mechanism, but the determination of the crystal structure would allow gaining valuable insights. In particular, whether MD-2 allows discriminating between various redox forms, as it is the case for HMGB1 [273], seems of crucial importance to us. Moreover, the role of CD14 in the TLR4-dependent pro-inflammatory activity of PRDX2 and PRDX5 remains unclear.

Other unanswered questions are related to the signaling pathways involved and their crosstalk. In particular, whether human PRDXs are able to activate a CD14-dependent calcium/NFAT pathway, such as LPS, seems to constitute an interesting research question to us. In this work, we focused on the secretion of IL-8. However, the diversity of inflammatory mediators that are secreted and their downstream effects, both locally and within the organism, constitute a large field of research studies that needs to be investigated in the case of human PRDXs.

On the other hand, various avenues for improving this work have been identified, both in methodological and technical terms. In the present thesis, we mainly emphasized the importance of endotoxin removal, the putative impact of the 6xHis tag, the technical considerations regarding ELISA, and the limitations of the DFS analysis in AFM-based SMFS. In particular, the issue arising from protein production contamination with endotoxins could be avoided by using another expression system, such as the ClearColi® [619] or the baculovirus-insect cell systems. Moreover, tag removal could be performed subsequently to recombinant protein expressing, for instance by placing a protease site between the tag and the target protein [620]. Regarding the need to quantify a larger diversity of inflammatory mediators, multiplex fluorescent immunoassay seems an interesting approach [621,622]. Furthermore, the use of a 'loss-of-function' approach would complete the picture that we initiated based on a 'gain-of-function' approach with HEK293 cells. Finally, we proposed an innovative method to analyze the DFS data in the present thesis based on a kernel distribution and the 'Peak Analyzer' tool on OriginPro software. We believe that this approach is more reproducible than the method based on histograms and manual peak fitting. It seems to us that improving the DFS data analysis to get more objective, reproducible and robust results is the prerequisite to be able to fully consider and compare DFS data results. Such

improvement would require the collaboration of biophysicists together with statisticians. In this sense, we wonder whether a bivariate kernel approach could help discarding the arbitrary choices that are currently made in the analysis.

Annexes

This section includes the two scientific papers that were published in peer-reviewed journals (see **sections 3.1. and 3.2.**).

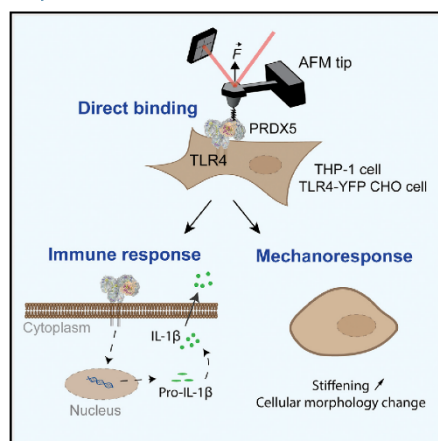
Annex 1

Article

Cell Chemical Biology

Specific Interactions Measured by AFM on Living Cells between Peroxiredoxin-5 and TLR4: Relevance for Mechanisms of Innate Immunity

Graphical Abstract



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In Brief

Knoops et al. used force-distance curve-based atomic force microscopy to investigate the molecular mechanisms by which extracellular human PRDX5 can activate a proinflammatory response. Single-molecule experiments demonstrate specific binding of PRDX5 to TLR4 on purified receptors and living cells.

Highlights

- Single-molecule experiments demonstrate that PRDX5 specifically binds to TLR4
- Specific interaction observed on THP-1 cells and CHO cells overexpressing TLR4-YFP
- PRDX5 binding induces a cellular mechanoresponse



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CellPress

Specific Interactions Measured by AFM on Living Cells between Peroxiredoxin-5 and TLR4: Relevance for Mechanisms of Innate Immunity

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SUMMARY

Inflammation is a pathophysiological response of innate immunity to infection or tissue damage. This response is among others triggered by factors released by damaged or dying cells, termed damage-associated molecular pattern (DAMP) molecules that act as danger signals. DAMPs interact with pattern recognition receptors (PRRs) to contribute to the induction of inflammation. However, how released peroxiredoxins (PRDXs) are able to activate PRRs, such as Toll-like receptors (TLRs), remains elusive. Here, we used force-distance curve-based atomic force microscopy to investigate the molecular mechanisms by which extracellular human PRDX5 can activate a proinflammatory response. Single-molecule experiments demonstrated that PRDX5 binds to purified TLR4 receptors, on macrophage-differentiated THP-1 cells, and on human TLR4-transfected CHO cells. These findings suggest that extracellular PRDX5 can specifically trigger a proinflammatory response. Moreover, our work also revealed that PRDX5 binding induces a cellular mechanoreponse. Collectively, this study provides insights into the role of extracellular PRDX5 in innate immunity.

INTRODUCTION

Inflammation is an essential component of the innate immune response to infection or tissue damage, which has the physiological role of restoring tissue homeostasis. Inflammation is initiated and perpetuated by pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules. While PAMPs are derived products of pathogens, DAMPs are host-derived nuclear or cytosolic proteins with defined intracellular functions. Following tissue injury, DAMPs are released extracellularly and move from a reducing to an oxidizing milieu. PAMPs and DAMPs are recognized by membrane-bound pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), the NOD-like receptors, and RIG-I-

like receptors. So, PRRs are activated and promote intracellular cascades that lead to an inflammatory response (Chen and Nunez, 2010; Kono and Rock, 2008; Kumar et al., 2013).

The superfamily of peroxidases named peroxiredoxins (PRDXs), has been reported to play important roles in innate immunity (for reviews, see Ishii et al., 2012; Knoops et al., 2016; Park et al., 2016). PRDXs are a family of thiol-dependent peroxidases that are able to reduce peroxides (hydrogen peroxide, alkyl hydroperoxides, and peroxynitrite) and are constitutively expressed by virtually all cell types in mammals (Perkins et al., 2015). It has been reported that, intracellularly, PRDXs may act as cytoprotective antioxidant enzymes, protein chaperones, modulators of peroxide signaling, and redox relays (Rhee and Kil, 2017). Interestingly, PRDXs were also shown to play a role in immune modulation as PAMPs or DAMPs upon their release into the extracellular milieu (Riddell et al., 2010b; Robinson et al., 2010; Shichita et al., 2012). Studies conducted either after brain injury in rats (Chou et al., 2011) or after acute ischemic stroke in humans (Dayon et al., 2011), demonstrated that PRDXs are released into extracellular fluids and could function as DAMPs under conditions of sterile inflammation. Moreover, Shichita et al. (2012) have shown that post-ischemic brain inflammation can be mediated through PRDXs and is dependent on TLR2 and TLR4. In particular, it was shown that, among PRDXs, PRDX5 highly contributed to induce the expression and the release of inflammatory cytokines (Shichita et al., 2012). However, the molecular mechanisms underlying TLR2 and TLR4 activation through PRDXs still remained to be clarified (Figure 1A).

Atomic force microscopy (AFM), invented to image the topography of samples at high resolution and in native conditions (Binig et al., 1986), evolved rapidly allowing the investigation of living mammalian cells and the probing of specific interactions under physiological conditions (Alsteens et al., 2017a; Dufrene et al., 2017). Force-distance curve-based AFM (FD-based AFM) enables us to contour the surface of biological systems with simultaneous mapping of their physical properties at (sub-) nanometer resolution (Dufrene et al., 2013). A tip positioned at the very end of a soft cantilever is oscillated and scanned across a cell while performing a single approach and retract movement at each discrete point of the surface. During the approach and retraction cycle, the tip interacts with the cell surface and is deflected depending on the force exerted on it, which in turn deviates the path of a laser, reflected from the cantilever onto a position-sensitive detector. This deflection



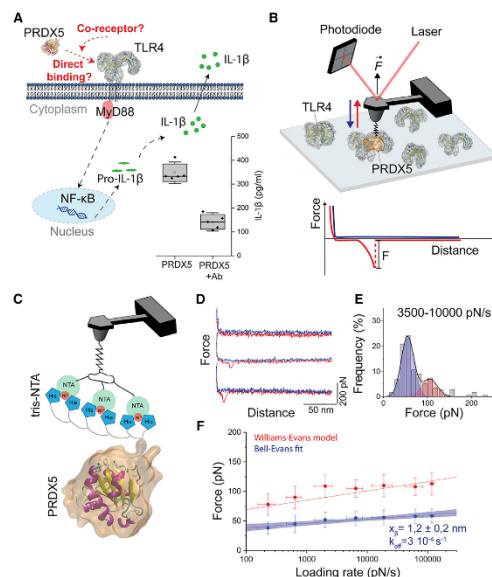


Figure 1. Studying PRDX5/TLR4 Interaction

(A) IL-1 β secretion from macrophage-differentiated THP-1 cells is induced after stimulation with PRDX5. THP-1 cells were incubated with 2 μ M PRDX5 and further blocked using 1/10 blocking antiserum against PRDX5 (Ab) (see also Figure S1). Experiments were reproduced five times ($n = 5$; each data point is an independent experiment). Error bars denote SD.

(B) Schematic of the force spectroscopy experiments performed between PRDX5 grafted on the AFM tip and a TLR4-derivatized surface. The AFM tip is approached (blue arrow) and retracted (red arrow) from the model surface and the force is monitored using a laser focused on the AFM cantilever and deflected into a photodiode resulting in a force versus distance curve (FD curve) from which the binding force (F) is extracted.

(C) Schematics of the surface chemistry used to functionalize AFM tips with PRDX5 using tris-NTA-Ni $^{2+}$.

(D) Representative FD curves recorded showing non-adhesive curve (upper curve) or curves with specific adhesive event. Forces were extracted along with the loading rate from each individual adhesive curves (Figure S2).

(E and F) Small loading rate ranges nos. 1–7 (see also Figure S2) are binned and the distributions of the rupture forces are plotted as histograms (E). This classification reveals multiple force peaks with average values corresponding to single (blue) or double (red) simultaneously established ligand-receptor interactions. The average values of the force distributions are extracted and plotted on the dynamic force spectroscopy plot (F), enabling their analysis using the Bell-Evans fit (single interactions) or the Williams-Evans model (double interactions) (F). Error bars denote SD. To approximate the distance to the transition state (x_s) and the unbinding rate (k_{off}) of the bonds, the data describing the rupture of single and multiple

simultaneously occurring bonds are fitted (solid lines) by the Bell-Evans model within 95% confidence intervals (colored shaded regions). Experiments were reproduced four times independently and data were merged to extract the dynamic force spectroscopy plot.

correlates to the interaction force between cantilever tip and cell surface, and can be displayed as force-time and FD curves. Analysis of the FD curves allows the extraction of parameters including height, adhesion, contact force, sample deformation, energy dissipation, and Young's modulus (Dufrene et al., 2013). As the parameters are determined for each point of the cell surface, parametric maps can be generated and directly correlated to the AFM image. Recently, we have introduced FD-based AFM to image human G-protein-coupled receptors in proteoliposomes at high resolution (<5 nm) (Alsteens et al., 2013; Dufrene et al., 2013; Preunnschuh et al., 2014), and we quantified the kinetic and thermodynamic parameters that describe the binding of ligands (Alsteens et al., 2015). In a second study, we introduced a chamber to keep mammalian cells in incubator-like conditions and maintain homeostasis for days (Alsteens et al., 2017b). This setup allowed us to quantify and topographically map the binding of single EnvA-RABV(Δ G) viruses at high resolution (<50 nm) on MDCK cells. Functionalized AFM tips with single viruses enabled us to detect the binding events of their receptors, and we observed a stepwise formation

of virus-cell surface receptor bonds occurring with a positive allosteric modulation. Here, we examined how PRDX5 released from cells into the extracellular milieu can function as a DAMP, and can activate a cell response through direct specific binding to TLR4 receptor on living cells. To investigate the molecular mechanisms by which PRDX5 interacts with TLR4, we used AFM as a unique and novel approach in immunology to probe and map single-molecule interactions on purified receptors and on individual living cells. This single-cell approach allowed us to provide insights into fundamental processes related to the molecular cell biology of innate immunity.

Our results show that human PRDX5 is able to specifically bind to TLR4. First, we probed specific interactions at the single-molecule level between the tip derivatized with PRDX5 and TLR4 receptors immobilized on a surface. Next, we combined FD-based AFM with optical microscopy to image the binding sites of PRDX5 on macrophage-differentiated THP-1 cells, and confirmed the specific interaction with TLR4 receptors using transfected Chinese hamster ovary (CHO) cells expressing fluorescently (YFP) labeled human TLR4. Moreover,

our experimental data were supported by TLR4-PRDX5 docking simulations.

RESULTS

PRDX5 Induces Release of IL-1 β by THP-1 Cells

Firstly, THP-1 cells were used to assess the role of PRDX5 as a DAMP, and to evaluate THP-1 proinflammatory cytokine release after exposure to extracellular PRDX5. THP-1 cells in the monocyte state were first differentiated into macrophage-like cells using phorbol-12-myristate-13-acetate (PMA) at 8 nM for 48 hr. Macrophage-differentiated THP-1 cells were kept for 3 hr in culture media without PMA to increase macrophage phenotype (Park et al., 2007). Exposure of macrophage-differentiated THP-1 cells to recombinant human PRDX5 resulted in the secretion of interleukin-1 β (IL-1 β) significantly greater than that observed in control unexposed cells but lower than after LPS exposure (Figure S1A). Preincubation of PRDX5 with blocking antibodies directed to PRDX5 significantly decreased IL-1 β level release (Figures 1A and S1A). Surprisingly, IL-1 β levels even increased after preincubation with PRDX5 and polymyxin B used to neutralize putative residual contamination by LPS (Figure S1A). It must be noted that similar potentiation of a PRDX5 by polymyxin B has been already observed after exposure of macrophages to PRDX1 in presence of polymyxin B (Riddell et al., 2010a).

PRDX5 Interacts Specifically with TLR4 *In Vitro* in an Acellular Model

To determine whether PRDX5 could function as a DAMP by direct interaction with the cell surface receptor TLR4, we used AFM to probe the interaction between PRDX5 and TLR4 (Figure 1B). To this end, an AFM tip was functionalized with *tris*-nitroliacetic acids (*tris*-NTA), which shows subnanomolar affinity toward the hexahistidine (6xHis)-tagged molecule (Lata et al., 2005). The *tris*-NTA-Ni²⁺ probe was then incubated with 6xHis-PRDX5 (Figure 1C). TLR4 was covalently attached to COOH derivatized gold substrate using *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride chemistry. To evaluate PRDX5 binding with TLR4, an array of 32 by 32 FD curves was recorded over a 1 μm^2 area on the TLR4-derivatized substrate. Some FD curves detected specific adhesion events (Figure 1D) at rupture distances >5 nm, and with extension patterns that can be fitted with the worm-like chain model, a biophysical model that fits the extension of proteins under an applied force.

To further determine whether these binding events were specific, we performed independent controls using (1) a tip functionalized with the *tris*-NTA-Ni²⁺ but without PRDX5 (Figure S1B), (2) the functionalized tip after blocking with the anti-PRDX5 antibody (Figure S1C), and (3) preincubation of PRDX5 with the endotoxin inactivator polymyxin B (Figure S1C). These controls showed that (1) *tris*-NTA-Ni²⁺ did not interact specifically with TLR4, (2) the antibody against PRDX5 significantly reduced the interaction frequency, and (3) interactions did not involve endotoxin. This confirmed that the PRDX5 ligand linked to the tip could undergo specific interactions with TLR4.

For each adhesive curve, the force (height of the adhesive peak), as well as the loading rate (i.e., the increase of the force over time applied on the bond), were extracted. For each unbind-

ing event, the loading rate was determined using the slope of the adhesion event measured on the force-time curve just before the rupture. The experiments were performed at six different piezo retraction speeds (Figure S2A), and all the results were sorted into seven loading rate ranges and plotted as frequency versus force histograms (Figures 1E and S2B). The histograms revealed biGaussian distributions, with the second peak centered at the double of the first one, supporting the hypothesis that the first peak is a single interaction while the second peak corresponds to double interactions. For each peak the mean and the SD were extracted and displayed in a dynamic force spectroscopy plot. The dynamic force spectroscopy plot showed that single PRDX5-TLR4 bond ruptured at forces ranging from 30 to 60 pN for loading rates between 200 and 200,000 pN s⁻¹ (Figure 1F). The unbinding forces of single and multiple receptor bonds were directly proportional to the logarithmic loading rates. According to the Bell-Evans model (Evans and Ritchie, 1997; Merkel et al., 1999), such a linear increase of the unbinding force is expected when crossing a single free-energy barrier during mechanical pulling of a single ligand-receptor bond to the unbound state (Figure 1F). Using the Bell-Evans model, we extracted the separation of bond to the transition state toward the unbound state $x_u = 1.2 \pm 0.2$ nm and the kinetic off-rate $k_{off} = 3 \times 10^{-4}$ s⁻¹ for the single PRDX5-TLR4 bond. Remarkably, the analysis of the multiple bond rupture using the Williams-Evans model (Evans and Williams, 2002) for multiple uncorrelated bonds loaded in parallel nicely described our experimental observations (Figure 2F). This behavior has already been observed for multiple antibody-antigen interactions (Sulchek et al., 2006) or virus-receptor bonds (Alsteens et al., 2017b; Rankl et al., 2008; Sieben et al., 2012).

Imaging and Detecting PRDX5 Binding Events on Macrophage-Differentiated THP-1 Cells

Using AFM tips functionalized with PRDX5, we imaged macrophage-differentiated THP-1 cells expressing TLR4 under cell culture conditions (Figure 2A). Guided by wide-field microscopy (Figure 2B), we chose an area where multiple cells were in close proximity, which facilitated FD-based AFM topographical imaging since height differences are limited. In such an area, we recorded a height image (Figure 2B, inset), reconstructed from FD curves recorded for each topographic pixel (Figure 2C). Simultaneously, FD curves could also be analyzed and the specific adhesion events from the retraction curves were used to reconstruct the adhesion map (Figures 2D and S3A). Invisible to the naked eye, adhesive pixels were enlarged by a factor of 2, by contouring each gray pixel with two brown pixels in each direction. The specific adhesion events, with adhesion forces ranging from 80 to 500 pN, occurred at distances >50 nm separating the tip from the cell surface. The distribution of the binding events on the cell surface was homogeneously distributed.

We also looked at the stiffness and, surprisingly, we observed that the cells became stiffer after 15–30 min while probed with the PRDX5-derivatized tip (Figure 2E). Stiffness was extracted using the slope of the retraction curves during cell surface indentation (see also Table S1). This observation suggests that the specific interactions between PRDX5 and THP-1 cell surface receptors induce a mechano-response. As control experiments,

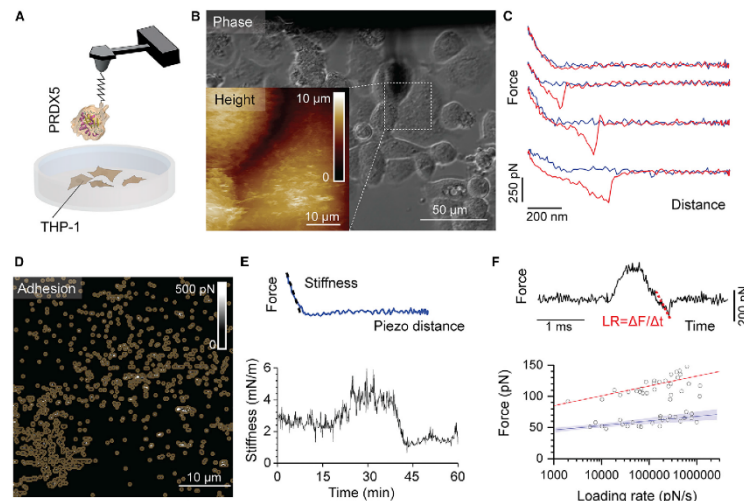


Figure 2. Mapping PRDX5 Binding to Macrophage-Differentiated THP-1 Cells Using Correlative Wide-Field Microscopy and FD-Based AFM
 (A) Schematic of the FD-based AFM using an AFM tip bearing a PRDX5 molecule and living THP-1 cells. AFM experiments were performed in cell culture conditions (37°C, RPMI-1640 medium, and humidified atmosphere supplemented with 5% CO₂; see STAR Methods).
 (B) Phase image showing THP-1 cells and the AFM tip placed above (see shadow). The height image recorded in the dashed square is shown in inset. AFM images were acquired using an oscillation frequency of 0.25 kHz and an amplitude of 500 nm.
 (C) Representative FD curves extracted from FD-based AFM images.
 (D) Corresponding adhesion channel simultaneously obtained with the height image, inset in (B). Vertical scales are given in upper right corners. Gray pixels were enlarged by a factor 2 (brown contour).
 (E) Force versus piezo-distance curve showing the linear fit in the contact area used to extract the stiffness. Plot showing the evolution of the stiffness over time. Stiffness is extracted from the slope of the approach curve in the contact area.
 (F) Extraction of the rupture forces separating the PRDX5 from the cell surface as well as the loading rate $LR = \Delta F / \Delta t$. For each rupture force (hollow circle, in the dynamic force spectroscopy plot below) the loading rate is extracted from the force-time curve as shown in the curve. Rupture forces (hollow circles) determined were plotted together with the fits obtained on the model surface (Figure 1F). Experiments were reproduced at least five times and other examples are shown in Figure S3.

we first probed THP-1 cells with a bare tip revealing a constant stiffness within 2 hr of AFM probing (Figure S3B). Next we probed the stiffness with a bare tip while injecting free PRDX5 at 10 and 15 μ M (Figure S3B). We observed a concentration-dependent mechano response strongly suggesting a direct relationship between PRDX5 and cell stiffening. Altogether these observations are in good agreement with a recent study performed by [Freira et al. \(2015\)](#) in which stiffening of THP-1 cells was observed in presence of cytokine IL-1 β .

Further analysis of the adhesion force was performed to evaluate whether they are similar to the forces observed on purified TLR4. We selected FD curves with adhesion forces <150 pN, corresponding to single and double interactions *in vitro*. For each adhesive peak of these curves we extracted the adhesion

force together with the loading rate (obtained from the slope before rupture, see Figure 2F). We then superimposed these values (see circles in Figure 2F) on the dynamic force spectroscopy plot obtained on the purified systems (blue and red curves for, respectively, single and double interactions, see Figure 1F), and we observed that these points very well aligned with the fits. This observation suggests that the interactions measured on living THP-1 cells are most likely similar to the PRDX5-TLR4 model system.

TLR4 Is a Specific Receptor for PRDX5 *In Vitro* in a Cellular Model

Next, we wanted to test whether TLR4 serves as a specific receptor of PRDX5 in the cellular context. CHO cells were

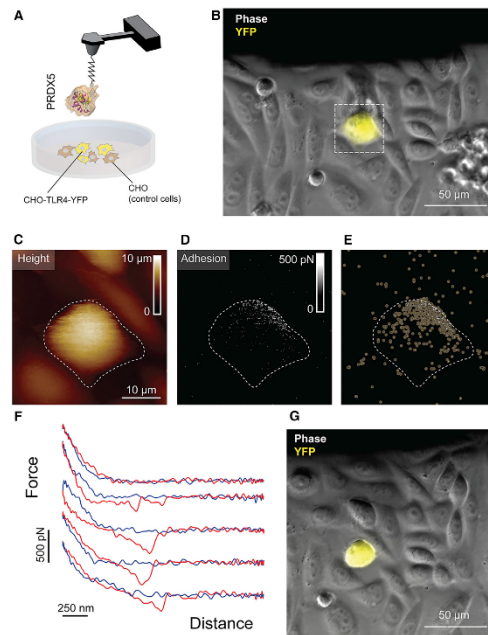


Figure 3. Mapping PRDX5 Binding to CHO-TLR4-YFP Cells Using Correlative Fluorescence Microscopy and FD-Based AFM
(A) Wild-type CHO cells were grown for 3 days before transfection with TLR4-YFP plasmid producing CHO-TLR4-YFP cells expressing TLR4-YFP (yellow). (B) Superimposition of phase image and YFP channel showing a confluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. (C) AFM height image recorded in the dashed square shown in (B). (D and E) Adhesion image with adhesive pixels enlarged in (E). Vertical color scales are given in the upper right corner. (F) Representative FD curves recorded from CHO-TLR4-YFP cell. (G) Superimposition of phase image and YFP channel recorded at the end of the FD-based AFM imaging. Experiments were reproduced ten times.

are observed with a frequency >5%, $n > 10,000$), with adhesion forces ranging from 100 to 600 pN (Figure 3F). These specific adhesion events occurred at distances of >50 nm, corresponding to the extension of the PEG₂₇ spacer, the size of the PRDX5 molecule grafted on the tip, the size of the cell surface receptor, and the cellular plasticity. FD curves recorded on non-fluorescent cells less frequently showed adhesion events (<0.25%, $n > 40,000$; Figure 3E).

As a control, we investigated a well-known ligand of TLR4 to ensure that the TLR receptors are well expressed at the surface of transfected CHO cells and properly folded. To this end, we functionalized the AFM tip with the high-mobility group box 1 nuclear protein (HMGB1; recombinant human 6xHis-HMGB1, Sigma) and similarly mapped the CHO-TLR4-YFP cells. Overall, adhesion events were localized on CHO-TLR4-YFP cells, while control CHO cells showed only rare events (Figure S4).

We concluded from the above experiments that the specific adhesion events measured on CHO-TLR4-YFP cells correspond to specific binding events of the PRDX5 ligand tethered to the AFM tip and the TLR4 receptors expressed at the cell surface. Surprisingly, we also observed that CHO-TLR4-YFP cells became round during AFM probing, while non-transfected cells remain spread, suggesting that the specific interaction between PRDX5 and TLR4 is causing this phenomenon (Figure 3G).

TLR4 Binding Induces Cellular Stiffening

To evaluate the change of cellular morphology during FD-based AFM using a PRDX5-derivatized tip, we scanned a small area of the cell surface in the nuclear region (100 × 100 nm, see red star in Figure 4A) for more than 1 hr and simultaneously looked at the cell morphology using optical microscopy. The size area was

transiently transfected with a construct coding for human TLR4, in fusion with the YFP, addressed to the plasma membrane, and functionally preserved (Latz et al., 2002). Using the wide-field and fluorescence signal, we chose areas in which both cell types (transfected and not transfected) were in close proximity to one another (Figures 3A and 3B). Having both CHO and CHO-TLR4-YFP cell types in a single AFM image area (40 × 40 μm) served as a direct control to evaluate whether interactions measured by the PRDX5-functionalized AFM tip were indeed due to specific binding to TLR4 receptors, and directly evaluate the extent of unspecific interactions and/or interactions with other cell surface receptors. The AFM was centered on the fluorescent cell and then the area was imaged using FD-based AFM. The topographic image (Figure 3C) and corresponding adhesion map (Figure 3D) were reconstructed from the individual FD curves recorded. To help the visualization of the adhesion events, pixels were enlarged as mentioned above (Figure 3E). Retraction curves showed specific adhesion events mainly on CHO cells expressing the TLR4-YFP receptors (adhesive pixels

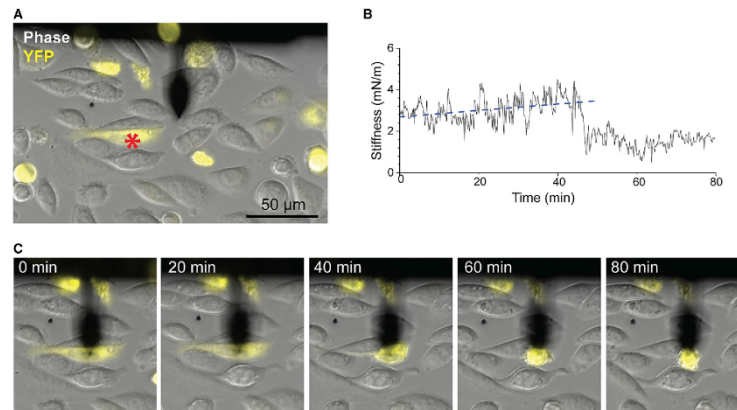


Figure 4. Probing Cellular Stiffness Evolution upon PRDX5-TLR4 Interactions on CHO-TLR4-YFP Cells
(A) Superimposition of Phase image and YFP channel showing a subconfluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. FD-curves were recorded on a $100 \times 100 \text{ nm}^2$ area shown by the red asterisk. (B and C) Plot showing the evolution of the stiffness over time. Stiffness is extracted from the slope of the approach curve in the contact area. (C) Time-lapse evolution of the cellular morphology during FD-based AFM imaging showing that the cell rounds up (see also Movie S1).

intentionally maintained small to always probe the same area (the nuclear region), so that the cellular heterogeneous stiffness has only a small influence. As shown in Figure 4, the stiffness of the cell was extracted using the slope of the extended curve in the contact region and measured over time. We observed that the cell progressively became stiffer during the first 50 min (Figures 4B and 4C). Simultaneously the cell changed its morphology from a spread state to a more round state (Figure 4C). The cell phenotype of CHO-TLR4-YFP was very similar to that observed on THP-1 cells (Figure 3G), as well as the extracted stiffness values (Table S1). In the context of endothelial inflammation, this observation is in good agreement with a recent study showing that DAMP ligands are released by pulmonary artery endothelial cells (PAECs) under stress and activate downstream PAEC inflammation through TLR2/NF- κ B, resulting in the stiffening of pulmonary artery (Tan et al., 2014).

Molecular Docking Study of TLR4-PRDX5 Interaction

AFM results obtained here highly support a direct interaction between PRDX5 and TLR4. To further gain insights into PRDX5-TLR4 binding, we used molecular docking simulations. The docking was performed using ClusPro webserver (Kozakov et al., 2017) using the structure of TLR4 obtained in association with the myeloid differentiation factor 2 (MD-2) bound to LPS (PDB: 3FXI) and the oxidized form of the PRDX5 (PDB: 2VL3), which is the expected form in the oxidizing extracellular milieu (Evrard et al., 2004). Both TLR4 chains were used as center of

mass and remain fixed at the origin of the coordinate, and possible rotational and translational positions of the ligand PRDX5 were evaluated at the position sampled on a sphere-based grid centered on the receptor center of mass. A Piper docking algorithm allowed evaluating the interaction energy between the two proteins taking into account van der Waals, electrostatic, and hydrophobic contributions. Docking experiment results suggest that PRDX5 predominantly interacts in the center of TLR4 crook similarly to MD-2 (Figures 5 and S5). However, due to its smaller size in comparison with MD-2, PRDX5 would only bind to a single TLR4 monomer. It must be noted that this is also experimentally supported by the AFM experiments on model surfaces where TLR4 was grafted as monomer.

DISCUSSION

Using *in vitro* acellular and cellular models, we unraveled by AFM the mechanisms by which human PRDX5 can act as a DAMP, binding to TLR4 and activating TLR4 to induce proinflammatory cytokine IL-13 release. To our knowledge, it is the first time that AFM has been used to measure DAMP interactions on the surface of living cells. Although most PRDXs have been described mainly as intracellular thiol-dependent peroxidases or protein chaperones, the role of PRDXs as PAMPs or DAMPs after their release into the extracellular milieu is now well documented in the literature. Indeed, PRDXs secreted by parasitic helminths or released by malarial *Plasmodium berghei* have been shown

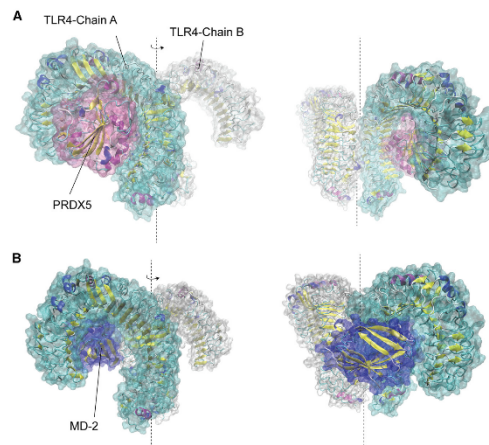


Figure 5. Docking of PRDX5 to TLR4 Structure

(A) Front view (left) and side view (right) of PRDX5 (pink colored) binding to a single chain of TLR4 (cyan-colored). (B) Comparison with MD-2 associated to an LPS molecule bound to TLR4. For PRDX5 the simulated interaction occurs within a single TLR chain without contact to the second TLR chain forming the dimer. For other plausible docking positions please see Figure S5.

interaction (Shin et al., 2007), which has a bond lifetime of ~15 s, while TLR4 presents an intermediate bond lifetime with myeloid differentiation protein 2 (~5 min) (Lian et al., 2012). Binding specificity was assessed by control experiments including blocking antibodies or endotoxin inactivator polymyxin B.

Using FD-based AFM directly on living cells allowed us to demonstrate that such specific binding events similarly occur in the cellular context. Using macrophage-differentiated THP-1 cells, as a model for mimicking the function of macrophages, we probed PRDX5 binding to cell surface receptors. We further-

to act as PAMPs by, respectively, triggering the recruitment of macrophages and a Th2 response in mice, or inducing a proinflammatory response by binding to TLR4 receptors (Donnelly et al., 2005, 2008; Furuta et al., 2008). Also, before its complete characterization as a peroxidase, PRDX1 was initially described as a soluble protein released from lysed cells and exhibiting immune modulating functions able to enhance the cytotoxic activity of natural killer cells (Shau et al., 1993). More recently, mammalian PRDX1, PRDX2, PRDX4, PRDX5, and PRDX6 have been proposed to act as DAMPs through activation of TLR2 and TLR4 (Liu et al., 2016; Mullen et al., 2015; Riddell et al., 2010b; Salzano et al., 2014; Shichita et al., 2012; Zhao et al., 2016). Although mammalian PRDX5 was mainly studied so far for its role as an intracellular cytoprotective antioxidant enzyme or a redox modulator, according to its large subcellular distribution (Knoops et al., 2011), PRDX5 was also shown to play a major role as DAMP when released extracellularly from necrotic brain cells to initiate post-ischemic inflammation through activation of macrophages via TLR2 and TLR4 (Shichita et al., 2012). Therefore, in a first step, we used purified PRDX5 and TLR4 to mechanistically examine whether these receptor-ligand pairs could interact through specific binding. Using PRDX5-derivatized AFM tips and TLR4-functionalized surfaces, we observed that specific bonds are established with a rupture force ranging from 30 to 60 pN, a typical force measured for other single receptor-ligand interactions, such as RGD peptide with integrin (Lehenkari and Horton, 1999). Extraction of kinetic parameters using the Bell-Evans model revealed a highly stable interaction (very low off-rate) with an average bond lifetime of ~38 min, therefore leading to a more stable interaction than TLR4-LPS

more observed specific interactions that shared kinetic characteristics with the model TLR4-PRDX5 system. A TLR4 role in PRDX5 binding in the cellular context was further demonstrated using CHO-TLR4-YFP cells mixed with control cells. Whether the redox status of PRDX5 may affect PRDX5-TLR4 binding would necessitate more in-depth investigation although under our experimental conditions the oxidized form of PRDX5 is expected to be the major form (Evvard et al., 2004).

FD-based AFM interactions observed between PRDX5 and TLR4 on purified receptors revealed that specific interactions occurred within either a monomeric TLR or within a single chain of a TLR4 dimer (Figure 1). Experiments on living cells provided evidence that the binding site properties are very similar to *in vitro* data, suggesting that the interaction within a single TLR4 chain site is also established on living cells. These experimental findings were further reinforced by *in silico* simulations (Figure 5). Together these data suggest that the TLR4 activation by PRDX5 is most likely very different from the TLR4 activation by LPS binding that induces receptor dimerization by contact point with the two TLR4 chains.

Surprisingly, PRDX5 binding to living cells resulted in a rapid cellular phenotype. Cells probed with a single PRDX5 molecule, even on small areas (<0.01 μm^2), became stiffer. To the best of our knowledge this is the first direct evidence that DAMPs binding through TLR4 induces a mechano-response by the cells, and is particularly relevant in the context of inflammation. Increasing evidence shows that, for example, artery stiffening or reduced arterial compliance is a prominent feature of systemic hypertension, pulmonary hypertension, and other vascular-related diseases (Freira et al., 2015). However, so far, the mechanism

underlying such stiffening remains elusive. Our study illustrates a new potential mechanism for how cellular mechanical stiffening is triggered downstream of the inflammation site by direct specific binding to TLR4.

In the future, the combined fluorescence-nanoscope approach used in this work could help to characterize more deeply the role of different ligands binding to immune receptors in living cells. Moreover, our quantitative method could not only help to uncover the receptors involved in the triggering of the immune response, but could also provide a tool to quantify the effects of molecules interfering with receptor activation and therefore aid the development of novel drugs modulating the inflammatory response.

SIGNIFICANCE

Inflammation is induced when innate immune cells, such as macrophages, detect danger molecules during infection or tissue injury. Among such molecules, PRDXs were shown recently to act as factors released by damaged or dying cells, termed DAMPs. However, the molecular mechanisms of how extracellular PRDXs act as DAMPs, interact with pattern-recognition receptors, and contribute to the induction of inflammation by modulating proinflammatory cytokine release are still unclear. Moreover, a better understanding of these mechanisms could help developing new therapeutic approaches for inflammation-related diseases. Here, we examined at the nanoscopic scale the interactions between PRDX5, previously shown to act as a DAMP, and TLR4, using AFM in acellular and cellular models. We demonstrated that PRDX5 specifically binds to TLR4. Surprisingly, this binding triggers also a cellular mechanoreponse, suggesting that PRDX5-TLR4 interactions not only modulate proinflammatory cytokine release but also cell stiffening, which could be highly relevant in the inflammatory response. Indeed, increasing evidence shows that, for example, artery stiffening or reduced arterial compliance is a prominent feature of systemic hypertension, pulmonary hypertension, and other vascular diseases with an inflammatory component. However, so far, the mechanism underlying such coupling remained elusive. Our study illustrates a new potential mechanism of how cellular mechanical stiffening is triggered during inflammation by a direct specific binding to TLR4. Our study demonstrates also the potential of AFM as a multifunctional approach to simultaneously characterize both ligand binding and mechanical properties.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and one movie and can be found with this article online at <https://doi.org/10.1016/j.chembiol.2018.02.006>.

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AUTHOR CONTRIBUTIONS

D.A. and B.K. designed the experiments. A.C. produced PRDX5 and performed IL-1 β assays. A.C., J.G., and S.B. transfected CHO cells. S.B. provided differentiated THP-1 cells. D.A., S.D., and M.A.P. performed AFM experiments. D.A., S.D., and M.A.P. co-analyzed the experimental and calculated data. All authors wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
LPS from <i>Escherichia coli</i> O111:B4	Sigma-Aldrich	Cat#L2630
PRDX5, N-terminal 6xHis-tagged, human	Declercq et al., 2001	N/A
6xHis-HMGB1	Sigma-Aldrich	Cat#H4652
Lipofectamine 2000	ThermoFisher	Cat#11668000
OptiMEM	Gibco	Cat#31985-062
EndoTrap HD kit	Hyglos	Cat#800053
DMEM high glucose	Life Technologies	Cat#61965-026
Fetal Bovine Serum	Lonza	Cat#DE14-802F
Penicillin-Streptomycin	Life Technologies	Cat#15140-122
RPMI-1640 GlutaMAX	Life Technologies	Cat#61870-010
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich	Cat#P8139
Antiserum directed to human PRDX5	Goemaere and Knoops, 2012	N/A
Polymixin B	Sigma-Aldrich	Cat#61271
NHS-PEG ₂₇ -acetal linker	Wilding et al., 2011	N/A
Citric acid	Sigma-Aldrich	Cat#251275
tris-NTA Trifluoroacetic Acid Salt	Toronto Research Chemicals	Cat#N825005
Cyanoborohydride	Sigma-Aldrich	Cat#156159
Ethanolamine hydrochloride	Sigma-Aldrich	Cat#E6133
NI ₂	Sigma-Aldrich	Cat#338350
Critical Commercial Assays		
Quantikine ELISA human IL-1 β	R&D Systems	Cat#DLB50
Experimental Models: Cell Lines		
THP-1	ATCC	ATCC TIB-202
CHO (CHO-K1)	ATCC	ATCC CRL-9618
Recombinant DNA		
pcDNA3-TLR4-YFP	Latz et al., 2002	Addgene Plasmid #13018
Software and Algorithms		
Nanoscope Analysis software	Bruker	N/A
Origin	OriginLab	N/A
Other		
MSCT AFM tips	Bruker	Cat#MSCT
Peak-Force QNM-Live cells AFM tips	Bruker	Cat#PF-QNM-Lc

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David Alsteens (david.alsteens@uclouvain.be).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Chinese Hamster Ovary (CHO-K1) cells (ATCC) were cultured in DMEM (Life Technologies) with 10% FBS (Lonza), with 100 $\mu\text{g mL}^{-1}$ penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin (Life Technologies) at 37°C in humidified atmosphere supplemented with 5% CO₂. THP-1

cells (ATCC), an acute monocytic leukemia cell line from a 1 year old male infant, were cultured in RPMI-1640-GlutaMAX (Life Technologies) supplemented with 10% FBS (Lonza), and 100 $\mu\text{g mL}^{-1}$ penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin (Life Technologies) at 37°C in humidified atmosphere supplemented with 5% CO_2 .

METHODS DETAILS

TLR4-YFP Expressing CHO Cells (CHO-TLR4-YFP)

CHO cells (provided by Prof. Y.-J. Schneider, UCL, Belgium) were plated at a density of $\sim 0.5 \times 10^6$ cells in 40 mm culture dishes with 2 mL of Dulbecco's modified eagle medium-high glucose (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (DMEM/High-10), 100 $\mu\text{g mL}^{-1}$ penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin (Life Technologies) at 37°C in humidified atmosphere supplemented with 5% CO_2 . Cells were transiently transfected with 3.25 μg of pcDNA3-TLR4-YFP (Addgene, plasmid #13018, gift of Dr. D. Golenbock, University of Massachusetts Medical School, USA) using 6.5 μL Lipofectamine 2000 (ThermoFisher) in 325 μL of OptiMEM (Gibco). Culture medium was changed after 24h and Petri dishes were used for AFM experiments after 48–72h.

Production and Purification of Recombinant Human PRDX5

The expression and purification of recombinant N-terminal 6xHis-tagged human PRDX5 were described previously (Declercq et al., 2001). First strand cDNA was obtained with Moloney leukemia virus reverse transcriptase (Superscript II, Life Technologies) from 2 μg of human lung RNA using oligo(dT)25 as primer according to the manufacturer's instructions. Human PRDX5 cDNA was PCR amplified using forward primer 5'-GCTGCAGGATCCGCCCAATCAAGGTGGGAG-3' (*Bam*HI site underlined) and reverse primer 5'-GGCCCAAGCTTCAGAGCTGTGAGATGATA-3' (*Hind*III site underlined). The PCR product was digested with *Bam*HI and *Hind*III, and ligated into the pQE-30 expression vector (Qiagen). The insert was sequenced and the N-terminal fusion with the hexahistidine (6xHis) tag was confirmed. The resulting vector was used to transform *E. coli* strain M15 (pRep4). *E. coli* were grown at 37°C in LB medium containing 1 mM IPTG. Pelleted cells were lysed in 10 mM imidazole, 50 mM phosphate, 300 mM NaCl (pH 8) by sonication and clarified by centrifugation. The supernatant containing 6xHis-tagged PRDX5 was loaded onto a Ni^{2+} -NTA column (Qiagen). The column was washed and the protein was eluted with 50 mM phosphate, 300 mM NaCl, 250 mM imidazole (pH 8). Eluted protein was then dialysed against PBS (pH 7.2). Putative contaminating endotoxins were removed using EndoTrap HD kit (Hyglos) and the recombinant protein was collected in PBS at 0.9 mg/mL.

Differentiation of THP-1 Cells into Macrophages

The human monocytic cell line THP-1 (provided by Prof. C. Remacle, UCL, Belgium) was cultured in RPMI-1640, GlutaMAX (Life Technologies) supplemented with 10% FBS (Lonza), 100 U/ml streptomycin and 100 U/ml penicillin (Life Technologies). THP-1 cells were maintained at 37°C in 5% CO_2 . THP-1 cells were differentiated into macrophages via exposure to 8 nM PMA (Sigma-Aldrich) in 96-well plates or 40 mm culture dishes in, respectively, 100 μL or 3 mL of RPMI-1640-GlutaMAX (Life Technologies) supplemented with 10% FBS (Lonza) and 100 U/ml streptomycin and 100 U/ml penicillin (Life Technologies) for 48h according to (Park et al., 2007).

Quantification of Human IL-1 β

Macrophage-differentiated THP-1 cells cultured in 96-well plates were exposed to 2 μM of purified recombinant human PRDX5 (Declercq et al., 2001), 1/10 antiserum directed to human PRDX5 (Goemaere and Knoops, 2012), 30 $\mu\text{g/mL}$ polymyxin B and 1 $\mu\text{g/mL}$ LPS (from *Escherichia coli* O111:B4, Sigma-Aldrich) in RPMI-1640-GlutaMAX (Life Technologies) supplemented with 100 U/ml streptomycin and 100 U/ml penicillin (Life Technologies) without FBS. Culture medium was harvested 48h after cell exposure and IL-1 β was measured, using Quantikine ELISA human IL-1 β (R&D Systems).

Functionalization of AFM Tips

To functionalize AFM tips we used NHS-PEG₂₇-acetal linkers (Wilding et al., 2011). The cantilevers were immersed in 1 mL of citric acid (1%, w/v) for 10 min. After washing the cantilevers three times for 5 min in MilliQ water, they were dried with N_2 . The cantilevers were immersed in a 100 μL droplet of the amino-*tris*-NTA molecule ($\alpha\text{S}, \alpha'\text{S}, \alpha''\text{S}$)-11-(6-amino-1-oxohexyl)- $\alpha, \alpha', \alpha''$ -*tris*[bis(carboxymethylamino)]-5,5',5''-trioxo-1,4,8,11-tetraazacyclotetra-decane-1,4,8-tripentanolic acid, Toronto Research Chemicals, Toronto, Ontario, Canada) (2 mM in PBS). 2 μL of a 1 M cyanoborohydride solution were added and mixed carefully. The cantilevers were incubated for 2 h. The reaction was quenched by adding 5 μL of 1 M ethanolamine (pH 8.0). After 10 min, the cantilevers were washed in PBS three times for 5 min and immersed in 5 mM NiCl_2 for 10 min. Then, the *tris*-NTA- Ni^{2+} AFM tips were incubated for 2h in a solution of His₆-PRDX5 or 6xHis-HMGB1 at 0.2 mg/mL. Finally the cantilevers were washed in PBS three times for 5 min and transferred to a multiwell plate filled with PBS buffer. Cantilevers were used in AFM experiments the same day as the last step of the functionalization protocol.

Single-Molecule Force Spectroscopy on Modal Surfaces

SMFS measurements were performed using the PRDX5 functionalized tips (see above mentioned protocol) using MSCT tips ($k=0.01$ N/m) (Bruker Corporation, Santa Barbara, CA) and substrates functionalized with TLR4 at room temperature (20°C) in PBS buffer using a Nanoscope VIII Multimode AFM (Bruker Corporation, Santa Barbara, CA). Recombinant TLR4 was immobilized onto gold substrates using N-hydroxysuccinimide (NHS) (Sigma-Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)

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as described previously (Verbelen et al., 2009). The activated surfaces were then incubated with 100 µg/mL TLR4 (R&D Systems) in PBS. Multiple force curves were recorded at 0 ms contact time, with a maximum applied force of 250 pN, and using a constant approach speed of 1,000 nm/s and retraction speeds of 100 nm/s, 200 nm/s, 500 nm/s, 1,000 nm/s, 2,000 nm/s and 10,000 nm/s. For each experiment, these speeds were recorded in different order. Cantilevers were first calibrated on glass using thermal noise method (Butt and Jaschke, 1995), yielding values ranging from 0.011–0.023 N m⁻¹.

Combined FD-Based AFM and Optical Microscopy

Correlative microscopy was acquired on an AFM (Bioscope Catalyst, Bruker Corporation, Santa Barbara, CA) operated in the PeakForce QNM mode (Nanoscope software v9.1) to conduct FD-based AFM and coupled on an inverted epifluorescence microscope (Axio observer Z1, Zeiss). The AFM was equipped with a 150 µm piezoelectric scanner. Overview images (~40 x 40 µm²) were recorded at imaging forces of ~350–750 pN using PeakForce QNM-Live Cell (PFQNM-LC) cantilevers (Bruker Corporation, Santa Barbara, CA) having tip lengths of 17 µm, tip radii of 65 nm and opening angles of 15°. All microscopy and FD-based AFM imaging was performed at cell culture conditions using the combined AFM and optical microscopy chamber at 37°C in medium supplemented with 10% FBS. A gas mixture of synthetic air supplemented with 5% CO₂ at 95% relative humidity using a gas humidifier membrane (PermSelect silicone) was blown at 0.1 L min⁻¹ into the microscopy chamber. The humidity was controlled using a humidity sensor (Sensirion). The Cantilevers were first calibrated on glass using thermal noise method (Butt and Jaschke, 1995), yielding values ranging from 0.061–0.102 N m⁻¹. The AFM tip was oscillated vertically at 0.25 kHz applying 300–1,000 nm amplitudes in the PeakForce QNM mode. The sample was scanned using a frequency of 0.125 Hz and 256 pixels per line (256 lines). AFM images and force curves were analyzed using the Nanoscope analysis software (v1.8, Bruker).

Analysis of Force Curves

Individual force curves detecting unbinding events of the protein were analysed using the Nanoscope analysis software. The base line of the retraction curve was corrected using a linear fit on the last 30% of the retraction curve. Using the force-time curve the loading rate (slope) of each rupture event was approached by dividing the force segment ΔF through the time segment Δt (Figure 2F).

Extraction of Kinetic Parameters

Origin software (OriginLab) was used to fit the loading rate-dependent interaction forces using a linear iterative fitting algorithm (Levenberg Marquardt) along with the Bell-Evans model (Evans and Williams, 2002). Each iteration computed a chi-square value (χ^2) after which the parameter values were adjusted to reduce χ^2 . When the difference between two consecutive χ^2 values computed in two successive iterations was small enough (tolerance 10⁻¹⁵), the fitting procedure converged. We fixed the maximum number of iterations to 200. To evaluate the validity of the fit, we also checked if the adjusted R² (coefficient of determination) was >0.75 and looked at the residual plot and the Q-Q plot.

Molecular Docking

The oxidized form of PRDX5 (PDB id: 2VL3) was docked into TLR4 receptor (PDB id: 3FXI) using ClusPro webserver (<https://cluspro.bu.edu/login.php>) (Kozakov et al., 2017) using custom settings. For each docking experiment, the ClusPro generated 20 most probable docking poses. The most representative poses of the docking experiments are represented in Figures 5 and S5.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were performed in at least triplicate (n=3, experimental replication). For each experiment the number of replicate is provided in the figure caption. All data are expressed as the mean ± standard deviation (SD) and the differences between groups were compared using paired sample Student t-test. Significance is indicated within figures as *** signifying p<0.001.

DATA AND SOFTWARE AVAILABILITY

The software for the docking analysis is available under ClusPro webserver (<https://cluspro.bu.edu/login.php>).

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Supplemental Information

Specific Interactions Measured by AFM on Living

Cells between Peroxiredoxin-5 and TLR4:

Relevance for Mechanisms of Innate Immunity

Bernard Knoops, Sarah Becker, Mégane Anne Poncin, Julien Glibert, Sylvie Derclaye, André Clippe, and David Alsteens

Supplementary files

Specific interactions measured by AFM on living cells between peroxiredoxin-5 and TLR4: relevance for mechanisms of innate immunity

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This file includes:

Table S1
Figure S1 to S5
Caption for Movie S1

Other Supplementary Information for this manuscript includes the following:

Movie S1

Table S1. Related to Figure 2 and Figure 4. Cell stiffening by PRDX5

Cell type	Initial stiffness	Stiffening	Final
THP1	2.94 ± 0.25 mN/m* (N= 5'120)	3.63 ± 0.48 mN/m (N= 11'008)	1.65 ± 0.17 mN/m (N= 9'472)
CHO-TLR4	2.57 ± 0.34 mN/m (N= 65'536)	3.84 ± 0.67 mN/m (N= 60'416)	1.50 ± 0.88 mN/m (N= 64'000)

*Stiffness: average ± standard deviation (N= number of FD curves)

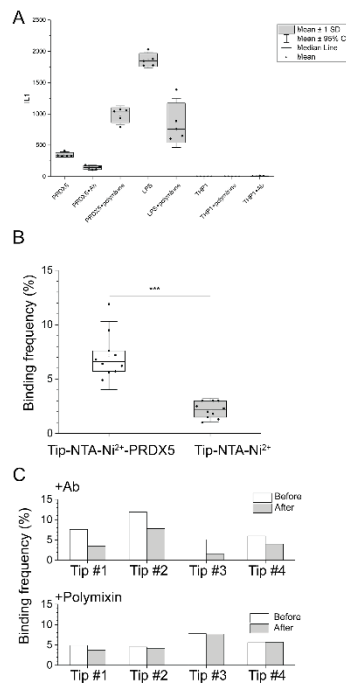


Figure S1. Related to Figure 1. IL-1 β secretion from macrophage-differentiated THP-1 cells after stimulation and validation of PRDX5-functionalized AFM tips specificity. (A) THP-1 cells were incubated with stimulants individually or mixed at concentration of 2 μ M PRDX5, 1/10 blocking antiserum against PRDX5 (Ab), 30 μ g/ml polymyxin B and 1 μ g/ml LPS in medium without fetal calf serum. Supernatants were harvested 48h after cell exposure and human IL-1 β levels were measured (n=5 for each condition). Asterisks (***) indicate a P value $\leq$ 0.001 using paired sample Student t-test. Data correspond to a representative experiment of four independent experiments. (B) Comparison of binding frequency using an AFM tip functionalized with or without the PRDX5 molecule. Experiments were reproduced 10 times (n=10: 10 different functionalized surfaces and AFM tips) (C) Comparison of binding frequency observed before and after injection of anti-PRDX5 antibody (Ab; upper) and polymyxin B (lower). Asterisks (***) indicate a P value $\leq$ 0.001 using paired sample Student t-test. Experiments were reproduced 4 times.

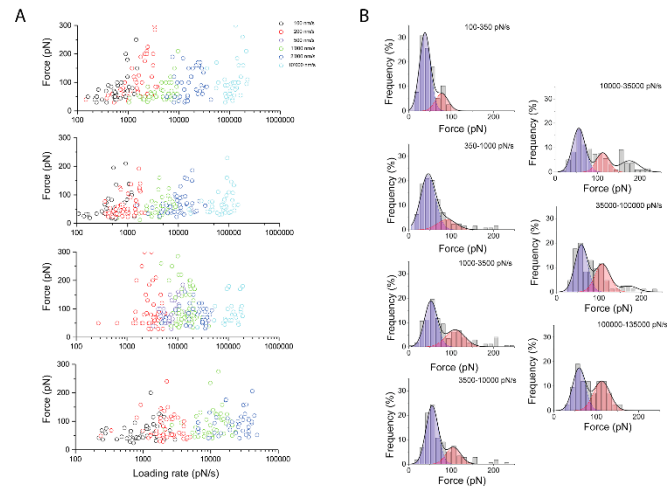


Figure S2. Related to Figure 1. Extraction of forces with loading rates and average rupture forces. (A) Rupture forces were measured with the loading rate (measured as the slope of the unbinding event just before the rupture on the force vs time curve). The force-distance curves are collected at different retraction speeds (100 – 10'000 nm/s) and displayed as different colors. (B) Distribution of rupture forces was sorted in seven *LR* ranges and the distributions of the rupture forces are plotted as histograms. This classification reveals multiple force peaks with average values corresponding to single (blue), double (red) simultaneously established PRDX5-TLR4 interactions. The average values of the force distributions for single interactions were extracted and plotted on the DFS plot (Figure 1F), enabling their analysis using the Bell-Evans model. Data are representatives from 4 independent experiments; meaning different samples and AFM tips.

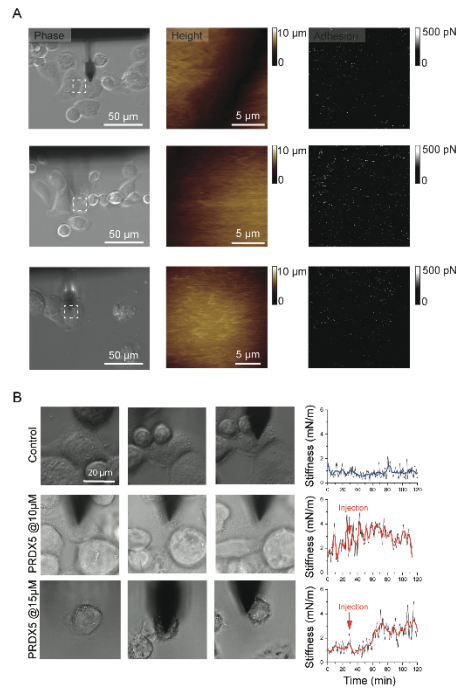


Figure S3. Related to Figure 2. Mapping PRDX5 binding to macrophage-differentiated THP-1 cells and extracting stiffness. (A) Experiments were performed in cell culture conditions (37°C, DMEM medium, and humidified atmosphere supplemented with 5% CO₂, see Methods). First column shows phase images of THP-1 cells with the AFM tip placed above (see shadow). The second column is the AFM height image recorded in the dashed square shown in the corresponding phase image. AFM images were acquired using an oscillation frequency of 0.25 kHz and amplitude of 500 nm. Corresponding adhesion channel is shown in the right column and is simultaneously obtained with the height image. Vertical scales are given in upper right corners. (B) Phase image showing THP-1 cells and the AFM tip placed above (see shadow) and plot showing the evolution of the stiffness over time for control cell (1st line) or upon incubation with free PRDX5 at 10µM (2nd line) or 15 µM (3rd line). Stiffness is extracted from the slope of the approach curve in the contact area.

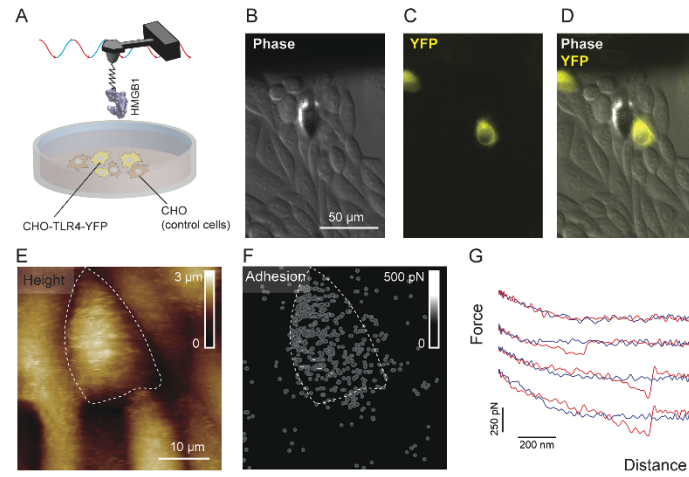


Figure S4. Related to Figure 3. Mapping IIMGB1 binding to CHO-TLR4-YFP cells using correlative epifluorescence microscopy and FD-based AFM. (A) Wild-type CHO cells were grown for 3 days before transfection with TLR4-YFP plasmid producing CHO-TLR4-YFP cells expressing TLR4-YFP (yellow). (B) Phase image, (C) YFP fluorescence channel and (D) superimposition of phase image and YFP channel showing a confluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. (E) AFM height image recorded in the center of D. The dashed line encircles the fluorescent cell. (F) Adhesion image with adhesive pixels enlarged by a factor 2. Vertical color scales are given in the upper right corners of the height and adhesion images. (G) Representative FD curves recorded from CHO-TLR4-YFP cell. Experiments were reproduced three times.

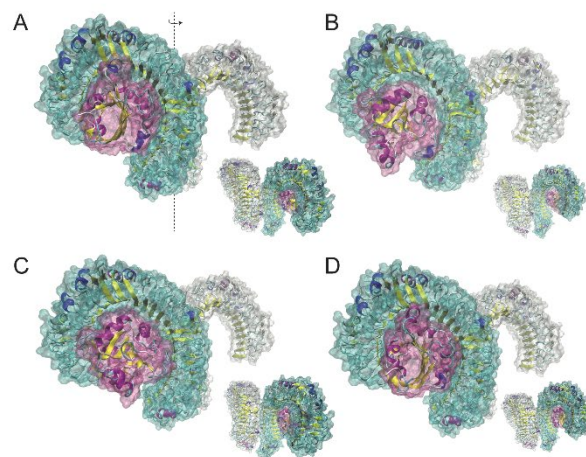


Figure S5 Related to Figure 5. Further examples of docking positions of the PRDX5 to the TLR4 structure. **(A-D)** Front view and side view of PRDX5 (pink colored) binding to a single chain of TLR4 (cyan-colored) showing that interaction may occur within a single TLR chain.

Movie S1. Related to Figure 4. Time-lapse evolution of the cellular morphology during FD-based AFM imaging showing that the cell rounds up. Superimposition of phase image and YFP channel showing a sub-confluent layer of CHO cells with sparsely distributed CHO-TLR4-YFP cells. FD-curves were recorded on a $100 \times 100 \text{ nm}^2$ on the center of the CHO-TLR4-YFP cell. Probed cell rounds up over time. Movie relates to Figure 4C



Article

Role of the Redox State of Human Peroxiredoxin-5 on Its TLR4-Activating DAMP Function

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Abstract: Human peroxiredoxin-5 (PRDX5) is a unique redox-sensitive protein that plays a dual role in brain ischemia-reperfusion injury. While intracellular PRDX5 has been reported to act as a neuroprotective antioxidant enzyme by scavenging peroxides, once released extracellularly from necrotic brain cells, the protein aggravates neural cell death by inducing expression of proinflammatory cytokines in macrophages through activation of Toll-like receptor (TLR) 2 (TLR2) and 4 (TLR4). Although recent evidence showed that PRDX5 was able to interact directly with TLR4, little is known regarding the role of the cysteine redox state of PRDX5 on its DAMP function. To gain insights into the role of PRDX5 redox-active cysteine residues in the TLR4-dependent proinflammatory activity of the protein, we used a recombinant human PRDX5 in the disulfide (oxidized) form and a mutant version lacking the peroxidatic cysteine, as well as chemically reduced and hyperoxidized PRDX5 proteins. We first analyzed the oxidation state and oligomerization profile by Western blot, mass spectrometry, and SEC-MALS. Using ELISA, we demonstrate that the disulfide bridge between the enzymatic cysteines is required to allow improved TLR4-dependent IL-8 secretion. Moreover, single-molecule force spectroscopy experiments revealed that TLR4 alone is not sufficient to discriminate the different PRDX5 redox forms. Finally, flow cytometry binding assays show that disulfide PRDX5 has a higher propensity to bind to the surface of living TLR4-expressing cells than the mutant protein. Taken together, these results demonstrate the importance of the redox state of PRDX5 cysteine residues on TLR4-induced inflammation.

Keywords: peroxiredoxins; PRDX5; Toll-like receptor; TLR4; DAMP; redox; cysteine residue; atomic force microscopy; SMFS

1. Introduction

Peroxiredoxins (PRDXs) constitute a large superfamily of highly diverse and multi-functional peroxidases, expressed as six isoforms in mammals (PRDX1–6). Their intracellular functions are related to the redox state of their catalytic cysteine residues (Cys), of which there are one in PRDX6 and two in PRDX1–5. Intracellularly, PRDXs act as cytoprotective peroxidases against oxidative stress by reducing peroxides (hydrogen peroxide (H₂O₂), alkyl hydroperoxides, and peroxynitrite), as modulators of redox signaling, and as chaperones [1–3]. Unlike prosthetic group-dependent enzymes, the PRDX catalytic cycle relies on a conserved Cys, so-called peroxidatic Cys, located in the N-terminal part of the protein. When the thiol (-SH) of this peroxidatic Cys is oxidized by peroxide, this results in a reactive sulfenic acid (-SOH) [4]. Depending on the PRDX subfamily, the

sulfenic acid undergoes a condensation reaction with: (i) a thiol on an adjacent subunit to form an inter-subunit disulfide in 2-Cys subfamily (PRDX1–4) [5], (ii) a thiol on the same subunit to form an intra-subunit disulfide in atypical 2-Cys subfamily (PRDX5) [6], or (iii) the π isoform of glutathione-S-transferase to form a heterodimeric disulfide in 1-Cys subfamily (PRDX6) [7]. The disulfide is reduced in vivo by a thioredoxin (Trx) for mammalian PRDX1–5 [8], and by glutathione for mammalian PRDX6 [9]. Hyperoxidation can also occur in vivo for typical 2-Cys PRDXs, with the sulfenic acid being oxidized to a sulfinic acid ($-\text{SO}_2\text{H}$) [10], the latter being reduced by the sulfiredoxin system [11], or to an irreversible sulfonic acid ($-\text{SO}_3\text{H}$) [12].

Mammalian PRDXs have also been shown to play key roles extracellularly by acting as damage-associated molecular patterns (DAMPs), triggering the production of cytokines in macrophages [13–17]. In humans, extracellular PRDXs released from necrotic brain cells have notably been shown to worsen brain ischemia-reperfusion injury by inducing expression of proinflammatory cytokines through activation of Toll-like receptor (TLR) 2 (TLR2) and 4 (TLR4) [14]. Among the PRDXs, PRDX5 in particular was shown to be a strong TLR activator [18]. The molecular mechanism underlying this TLR activation remains unclear, although we have recently shown using atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) that recombinant human PRDX5 was able to specifically bind to TLR4 [19]. However, very few studies have focused on the importance of the redox state of catalytic PRDX Cys in their DAMP function [13,14].

Here, we combine an array of interdisciplinary techniques including AFM-based SMFS, size-exclusion chromatography–multiangle light scattering (SEC-MALS), mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA), flow cytometry (FC), and confocal laser scanning microscopy (CLSM), to investigate the importance of the redox state of human PRDX5 redox-active Cys in the TLR4-dependent pro-inflammatory activity of the protein. By disrupting the C47–C151 disulfide bridge, either through C47 point mutation or dithiothreitol (DTT)/iodoacetamide (IAM) reduction/alkylation treatment, or via hyperoxidation with H_2O_2 , we demonstrate the role of the C47–C151 disulfide bridge in the PRDX5 interleukin (IL)-8-inducing ability. To determine whether TLR4 is able to discriminate between the PRDX5 redox forms, we used SMFS to evaluate the binding kinetics of reduced, oxidized (disulfide) and hyperoxidized PRDX5 to TLR4. Our results indicate that all redox forms bind TLR4 with a similar kinetic dissociation rate (k_{off}), with the exception of hyperoxidized PRDX5 that is not able to establish a specific interaction with the receptor. We further show with a flow cytometry binding assay that the binding propensity of disulfide PRDX5 to the surface of living cells expressing TLR4 is higher than that of the mutant protein. Overall, these results provide the basis for better understanding how the redox state of catalytic redox-sensitive Cys allows PRDXs to promote inflammation in inflammatory-related diseases, which is of crucial importance for the development of new therapeutic strategies related to ischemia-reperfusion injury.

2. Materials and Methods

2.1. Production and Purification of Recombinant Human PRDX5 and C47S Mutant PRDX5

The expression and purification of recombinant wild-type (dsPRDX5) and C47S mutant (PRDX5^{C47S}) N-terminal 6xHis-tagged human PRDX5 are described elsewhere [6,19,20]. Briefly, pQE-30 expression vector (Qiagen, Hilden, Germany) coding for wild-type and C47S mutant human PRDX5 were used to transform *Escherichia coli* (*E. coli*) strain M15 (pRep4). *E. coli* were grown at 37 °C in lysogeny broth (LB) medium containing 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Pelleted cells were lysed in 10 mM imidazole, 50 mM phosphate, 300 mM NaCl (pH 8) by sonication and clarified by centrifugation. The supernatant containing 6xHis-tagged PRDX5 was loaded onto a Ni^{2+} -nitrilotriacetic acid (NTA) column (Qiagen). The column was washed and the protein was eluted with 50 mM phosphate, 300 mM NaCl, 250 mM imidazole (pH 8). Eluted protein was then dialyzed against phosphate-buffered saline (PBS) (pH 7.2).

2.2. Preparation of the PRDX5 Redox Forms

To generate the chemically reduced or hyperoxidized forms, recombinant human PRDX5 (dsPRDX5) was first exposed to 10 mM dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO, USA) at room temperature (RT) for 1 h to reduce the disulfide bonds. To block the free cysteine thiols in order to maintain the protein in a reduced state, DTT-reduced PRDX5 was subsequently alkylated using 55 mM iodoacetamide [14] (IAM, Sigma-Aldrich) in a dark chamber at RT for 1 h (r/aPRDX5), as described previously. To oxidize cysteine residues to sulfinic or sulfonic acids, DTT-reduced PRDX5 was incubated with 5 mM H₂O₂ (Carl Roth, Karlsruhe, Germany) [21] at RT for 1 h (s/sPRDX5).

dsPRDX5, PRDX5^{C47S}, r/aPRDX5, and s/sPRDX5 solutions were ultra-filtered using Amicon Ultra-0.5 centrifugal filter devices (Merck, Darmstadt, Germany). First, 500 µL of protein samples were concentrated by spinning the device at 14,000 × *g* for 30 min. Then, the filtrate was discarded, and the concentrate was reconstituted to the original sample volume (500 µL) with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich). This process was repeated six times so that the concentrations of the residual DTT and IAM or H₂O₂ were sufficiently reduced (~10⁶ times). To recover the concentrated proteins, the filter device was placed upside down in a clean microcentrifuge tube and spinned for 2 min at 1000 × *g* to transfer the sample from the device to the tube. The sample was then reconstituted to the original volume (500 µL).

Protein samples were quantified either using the NanoDropTM One/One^C Microvolume UV-Vis spectrophotometer (ThermoFisher, Waltham, MA, USA) (in MS, SEC-MALS, SMFS and FC experiments) or with the PierceTM Rapid Gold BCA Protein Assay Kit (Thermo Scientific) (in ELISA and Western blot experiments).

2.3. Western Blotting for Detection of Hyperoxidized PRDX5

100 ng of each redox form was loaded on two SDS-polyacrylamide gels (Any kD Mini-PROTEAN TGX Precast Protein gel, Bio-Rad, Hercules, CA, USA) and SDS-PAGE was performed under non-reducing conditions. Proteins were then transferred to nitrocellulose membranes (Amersham Protran 0.45 µm NC, GE Healthcare Life Sciences, Chicago, IL, USA). After transfer, proteins were detected using either an anti-PRDX5 serum (1/200) [22] or an anti-PRDX5-SO₃ antibody (1/2000) (gift from Prof. Sue Goo Rhee, Yonsei University College of Medicine, Seoul, South Korea) [21]. Polyclonal goat anti-rabbit IgG/HRP (1/2000) (Agilent, Santa Clara, CA, USA) was used as the secondary antibody, and BM Chemiluminescence Blotting Substrate (Roche, Basel, Switzerland) was used to visualize the protein bands on an Amersham Imager 600 (GE Healthcare Life Sciences).

2.4. Mass Spectrometric Characterization of the PRDX5 Cysteine Redox State

2.4.1. Blocking of Cysteine Redox States

Prior to MS analysis, buffer exchange was accomplished using Amicon Ultra-0.5 centrifugal filter devices (Merck, Darmstadt, Germany) by concentrating the protein samples, discarding the filtrates, and reconstituting the concentrate protein samples to the original volume with 100 mM triethylammonium bicarbonate (pH 8.5) buffer (TEAB, Sigma-Aldrich). Potent cysteine sulfenic acids were trapped with 5 mM 5,5-dimethyl-1,3-cyclohexanedione (dimedone, Sigma-Aldrich) for 30 min at RT [23,24], and thiols remaining after redox treatments were subsequently blocked with 55 mM 2-chloroacetamide (CAM) for 30 min at RT in the dark. To be able to determine thioethers formed after alkylation with IAM from those formed after alkylation with CAM, MS analysis of r/aPRDX5 was performed with and without CAM treatment.

2.4.2. Sample Preparation

1 µg of samples was acidified with 1% (*v/v*) trifluoroacetic acid (TFA), desalted and concentrated with C4 ZipTip (Millipore, Burlington, MA, USA) according to the manufacturer's protocol. Samples were then eluted with 10 µL of 50% (*v/v*) acetonitrile, 0.1% formic acid.

2.4.3. ESI-Q-TOF Analysis of Intact Proteins by Direct Infusion

Intact protein analysis was done on a SYNAPT G2-Si high definition mass spectrometer (Waters) equipped with a NanoLockSpray dual electrospray ion source (Waters, Milford, MA, USA). Coated fused silica PicoTip™ Econo12 Emitters for nanoelectrospray, outer diameters: 1 µm Tip (New Objective, Littleton, MA, USA) were filled with 5 µL of samples and placed on the Universal NanoFlow™ Sprayer (Waters). The eluent was sprayed at a spray voltage of 1.2 kV. The source temperature was set to 120 °C. The cone gas flow was 20 liters·h⁻¹ with a nanoflow gas pressure of 0.3 bar. MS spectra were acquired and processed with MassLynx software (Waters).

2.4.4. Maximum Entropy Deconvolution

MaxEnt1 deconvolution algorithm was used to extract the exact masses with the following parameters: ranges from 17,000 Da to 39,000 Da, resolution of 0.5 Da/channel, Uniform Gaussian peak width at half height of 0.4 Da. A centering process was performed using the centroid method to obtain accurate average molecular masses. The minimum peak width at half height was set to 2, and the fraction of the resolved portion of the peak used to calculate the centroid was set to 80%. The height of a bar represents the sum of intensities of the points across the peak in the continuum trace.

2.5. SEC-MALS Analysis of the Oligomeric State

SEC-MALS experiments were performed using an LC-20 Prominence BioInert HPLC system (Shimadzu, Kyoto, Japan), coupled to a miniDAWN TREOS-II MALS instrument (Wyatt Technology, Santa Barbara, CA, USA), a RID-20A refractive index detector (Shimadzu) and an SPD-20A UV-VIS detector (Shimadzu). For chromatographic separation, 50 µL of sample were loaded alternately onto a Superdex 200 Increase 10/300 GL (Cytiva, Marlborough, MA, USA) and a Superdex 75 Increase 10/300 GL (Cytiva) (data not shown) equilibrated in the PBS running buffer (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, NaCl 137 mM, KCl 2.7 mM pH 7.4). Data were analyzed with ASTRA 7.3 software (Wyatt Technology) and molecular mass was calculated using a Zimm fit model.

Relative quantification of the absolute molar masses of PRDX5 redox form species was performed by taking the ratio of the calculated mass of the selected peak over the total calculated mass of all peaks of interest, expressed as a percentage.

2.6. Generation and Culture of HEK293 Cell Line Expressing TLR4

HEK293 cells expressing human MD-2 and CD14 (HEK293 MD2-CD14) (InvivoGen, San Diego, CA, USA) were plated at a density of $\sim 2 \times 10^6$ cells in 100 mm culture dishes and stably transfected with 10 µg pcDNA3-TLR4-YFP (Addgene, Watertown, MA, USA, plasmid #13018) using 25 µL Lipofectamine LTX (ThermoFisher) and 10 µL PLUS Reagent (ThermoFisher) in 1 mL of Opti-MEM (ThermoFisher), when cells reached $\sim 70\%$ confluency. Stable transfectants (HEK293 MD2-CD14 TLR4) were first selected using the antibiotic Geneticin (G418) (InvivoGen). To control TLR4 expression at the cell surface, cells were further sorted based on the fluorescence of an APC-conjugated anti-TLR4 antibody (ThermoFisher) using fluorescence-activated cell sorting (FACS). Single cells were then isolated to produce stable cell lines.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX (ThermoFisher) supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (FBS) (Lonza), 100 units·mL⁻¹ streptomycin and 100 units·mL⁻¹ penicillin (Sigma-Aldrich), 100 mg·mL⁻¹ of Normocin (InvivoGen) and 0.5 mg·mL⁻¹ G418 (InvivoGen). Cells were grown at 37 °C in a humidified atmosphere supplemented with 5% CO₂.

2.7. Quantification of Human IL-8 Secretion

Prior to performing ELISAs, endotoxins were removed from the PRDX5 productions using the Pierce High Capacity Endotoxin Removal Spin Columns (ThermoFisher), following the manufacturer's instructions. For each experiment (ELISA), a new redox treatment

for r/aPBS, r/aPRDX5, s/sPBS, and s/sPRDX5 was performed, and two independent PRDX5^{C47S} production batches were used.

HEK293 MD2-CD14 TLR4 cells were counted and cultured in 96-well plates with complete growth medium for 48 h before being exposed to 2 μ M of the different PRDX5 redox forms, 2 μ M HMGB1, and 100 ng mL⁻¹ LPS, or to increased concentrations (1, 2, 5, 10, 15, and 20 μ M) of the different PRDX5 redox forms, in DMEM supplemented with 100 units mL⁻¹ streptomycin and 100 units mL⁻¹ penicillin without FBS. Culture medium was harvested 16 h after cell exposure and IL-8 was measured using ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA), following manufacturer's instructions.

2.8. Functionalization of AFM Tips

PRDX5 molecules were grafted onto the AFM tips (MST probes, Bruker, Billerica, MA, USA) using (3-aminopropyl) triethoxysilane (APTES) coating and a heterobifunctional polyethylene glycol (PEG₂₄) linker composed of an aldehyde molecule at one end and an *N*-hydroxy succinimide (NHS) molecule at the other end. The protocol was adapted from Professor H. J. Gruber (Johannes Kepler University, Institute of Biophysics, Linz, Austria).

2.8.1. Amino-Functionalization

Probes were first washed three times in chloroform, dried with a gentle stream of nitrogen and cleaned for 15 min in an ultraviolet radiation and ozone (UV-O₃) cleaner (JeLight, Irvine, CA, USA). Probes were placed for 2 h in a hermetic desiccator filled with argon, in which were placed a tray containing 30 μ L of APTES and another tray containing 10 μ L of triethylamine. Finally, the trays were removed and probes were kept for 2 more days in the desiccator filled with argon.

2.8.2. Coupling of Aldehyde-PEG₂₄-NHS Linker

The amino-functionalized probes were first immersed for 2 h in a solution containing 1 mg of aldehyde-PEG₂₄-NHS linker dissolved in 0.5 mL of chloroform, and 30 μ L of triethylamine. Probes were then washed three times in chloroform and dried with a gentle stream of nitrogen. The probes were immersed in 100 μ L of 0.2 mg mL⁻¹ PRDX5 solution and 2 μ L of a freshly prepared solution of 1 M NaCNBH₃ (with 20 mM NaOH) for 1 h. The reaction was quenched by adding 5 μ L of 1 M ethanolamine solution (pH 8). After 10 min, the tips were rinsed three times with phosphate buffered saline (PBS) and stored in PBS. Experiments were conducted within 48 h following protein coupling.

2.9. Preparation of TLR4-Coated Surfaces

Recombinant human TLR4 (R&D Systems, Minneapolis, MN, USA) was covalently attached to a mixed self-assembled monolayer (SAM) of carboxyl (COOH)- and hydroxyl (OH)-terminated alkanethiols immobilized onto model surfaces using NHS/*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) chemistry.

2.9.1. Alkanethiol SAM Formation

Gold-coated silicon surfaces of $\sim 1 \times 1$ cm were first washed with ethanol, dried with a gentle flow of nitrogen, and cleaned for 15 min by UV-O₃ treatment. Surfaces were then immersed overnight in an ethanol solution containing a 1 mM mixture (1:99 mol/mol) of 16-mercaptohexadecanoic acid (HS(CH₂)₁₆COOH) (Sigma-Aldrich) and 11-mercapto-1-undecanol (HS(CH₂)₁₁OH; Sigma-Aldrich).

2.9.2. NHS/EDC Chemistry

After sonicating briefly to remove alkanethiol aggregates that may be adsorbed, surfaces were rinsed with ethanol and dried with nitrogen. Next, surfaces were immersed for 30 min in a solution of 175 mM NHS (Sigma-Aldrich) and 260 mM EDC (Sigma-Aldrich) in ultrapure water and rinsed 10 times with PBS. The activated surfaces were

finally incubated with 0.1 mg·mL^{−1} TLR4 in PBS for 2 h and rinsed three times in PBS. Experiments were conducted within 48 h following surface functionalization.

2.10. FD Curve Based AFM on TLR4 Coated Surfaces

SMFS on model surfaces was operated with a NanoScope 8 Multimode AFM (Bruker) and with the NanoScope 9.2 software (Bruker), using PRDX5-functionalized MSCCT tips (C triangular cantilevers, Bruker) and TLR4-functionalized gold surfaces in PBS at ~25 °C. Using Force Volume in contact mode, FD curves were recorded on 5 × 5 μm² arrays of 32 × 32 curves. Constant approach speed was set at 1 μm·s^{−1} and retraction speeds were varied (100 nm·s^{−1}, 200 nm·s^{−1}, 500 nm·s^{−1}, 1 μm·s^{−1}, 2 μm·s^{−1}, 10 μm·s^{−1}). A ramp size of 200 nm and a force threshold of 300 pN were used. Cantilever spring constants were calibrated on mica surfaces with the thermal noise method [25].

Force curves were analyzed using the Nanoscope Analysis 1.8 software (Bruker). FD curves were first extracted from individual adhesion maps and sorted to keep the curves displaying specific adhesion event(s). For each tip retraction speed, the binding frequency (BF) was calculated by dividing the number of specific adhesive curves by the total number of FD curves recorded for each map. For the DFS analysis, the baseline of the retraction curves was first corrected using a linear fit to remove tilt and offset. Rupture forces (expressed in pN) were extracted by measuring the height of the adhesive peak(s), and LR (expressed in pN·s^{−1}) were extracted by fitting the adhesive peak on the force-time curve just before rupture with a linear model. Determination of the kinetic parameters of the interactions was performed on OriginPro 9.8.0.200 software (OriginLab Corporation, Northampton, MA, USA). The spectra of LR were divided into five or six discrete ranges (LR#1: 315–1000 pN·s^{−1}; LR#2: 1000–3150 pN·s^{−1}; LR#3: 3150–10,000 pN·s^{−1}; LR#4: 10,000–31,500 pN·s^{−1}; LR#5: 31,500–100,000 pN·s^{−1}; LR#6: 100,000–315,000 pN·s^{−1}), depending on the redox form. For each LR range, a Kernel distribution was used to represent the probability density function (pdf) of the rupture forces, based on the following function:

$$ksdensity(x, Vx, w) = \frac{1}{n} \sum_{i=1}^n \frac{1}{\sqrt{2\pi}w} e^{-\frac{(x - \bar{x}_i)^2}{2w^2}}$$

This function returns the kernel density at x for a given vector Vx of size n with a bandwidth w , calculated as follows:

$$w = 0.9 \min\left(\hat{\sigma}, \frac{IQR}{1.349}\right) n^{-1/5}$$

where $\hat{\sigma}$ and IQR are the standard deviation and interquartile range of Vx , respectively. The second derivative of the Kernel Smooth function was used to search for hidden positive peaks, by choosing the lower peak filtering threshold allowing the fit to converge. Peaks were then fitted with Gaussian functions using the Levenberg-Marquardt algorithm. The peak centers determined the most probable rupture forces for single and multiple interactions. The Bell-Evans model [26] was fitted to most probable rupture forces corresponding to single interactions to determine the dissociation rate constant k_{off} and the distance to the transition state x_{β} :

$$F = \frac{k_B T}{x_{\beta}} \left(\ln(LR) + \ln\left(\frac{x_{\beta}}{k_B T k_{off}}\right) \right)$$

where F is the rupture force, $k_B T$ is the thermal energy, and LR is the loading rate. The predicted rupture forces for two ($n = 2$) and three ($n = 3$) simultaneous uncorrelated interactions were determined using Williams-Evans prediction [27]:

$$LR = k_{off} \frac{k_B T}{x_{\beta}} \left(\sum_{n=1}^N \frac{1}{n^2} \exp\left(\frac{-F x_{\beta}}{n k_B T}\right) \right)^{-1}$$

2.11. Confocal Microscopy Analysis of PRDX5 Binding to the Cell Surface

Prior to imaging, HEK293 MD2-CD14 TLR4 cells were seeded onto polyethylenimine (PEI)-coated glass bottom μ -dishes and left overnight to grow to confluence. 100 μ M NTA-FITC (Santa Cruz Biotechnology, Dallas, TX, USA) were incubated with 500 μ M NiCl₂ for 1 h at RT, and 10 μ M dsPRDX5 was then added for 1 h. Unbound probe was then removed using a PD SpinTrap G-25 column (Cytiva), by following manufacturer's instructions. Cells were imaged using a Zeiss 980 confocal laser scanning microscope (Zeiss GmbH, Oberkochen, Germany). This microscope is equipped with a 405 diode and Helium-Neon lasers, GaAsP detectors, and additional modifications to allow for long term live cell imaging. These modifications include a chamber that maintains cells at 37 °C, with 5% CO₂ in air, and a relative humidity of >95%. Excitation and emission settings were determined using YFP positive cells, prior to the addition of PRDX5-NTA-FITC, to reduce crosstalk between the channels. Excitation for FITC and YFP were at 488 and 514 nm respectively, with the key collection bands being between 495–522 nm for FITC and 565–648 nm, with the channels being collected sequentially.

2.12. Flow Cytometry Binding Assay

Cells were first harvested with Accutase solution (Sigma-Aldrich) prior to performing cell count and viability analysis. Cells were resuspended in ice-cold medium and $\sim 5 \times 10^5$ cells·mL⁻¹ were exposed to 1 μ M of the PRDX5 redox forms or to increase concentration of dsPRDX5 in DMEM for 40 min on ice to prevent endocytosis. As negative control, cells were incubated with medium only. Cells were centrifuged at $500 \times g$ for 5 min at 4 °C and then incubated with PBS containing 0.2% BSA for 15 min on ice. Cells were resuspended in anti-PRDX5 serum (1/200) for 40 min on ice, before being washed three times with PBS/0.2% BSA. Cells were finally labeled for 40 min on ice with goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 647 Plus (1/250) (ThermoFisher), washed three times, and resuspended in PBS. Samples were acquired on the Guava easyCyte System (Merck) using GuavaSoft software. Gates were set to select living Alexa Fluor 647 Plus-positive cells. Median fluorescence intensity (MFI) of control cells was subtracted from MFI of PRDX5-exposed cells.

2.13. Statistical Analysis

JMP Pro 15.2.0 software (SAS Institute, Cary, NC, USA) was used to perform the statistical tests. For ELISAs, FC experiments and SMFS BF measurements, a mixed model was used with the conditions (either exposition agents in ELISAs, or PRDX5 redox forms in FC and SMFS) as fixed independent variables and replicates as a random variable defining a random intercept. When the condition was significant (p -value < 5%), pairwise comparisons were computed with a Tukey HSD test. To fulfill the assumptions of the model, a logarithmic transformation ($\log_{10}$) of the response (IL-8 concentration) was performed in ELISAs.

Bell-Evans fitting results were compared with OriginPro 9.8.0.200 software. F-test was used to determine whether pairwise datasets were significantly different from each other. A p -value was then calculated based on the F value.

3. Results and Discussion

3.1. Production and Characterization of PRDX5 Redox Forms

To evaluate the influence of the redox state of human PRDX5 catalytic cysteine residues (Cys) on its DAMP function, we used four different redox forms (Figure 1). Purified recombinant human 6xHis-tagged PRDX5 produced in *Escherichia coli* (*E. coli*) has a disulfide bridge between the C47 and the C151 [8,28] (dsPRDX5) (Figure 1A,B). A mutated form of the protein was obtained by replacing the C47 by a serine (S), which maintains the protein in a reduced state by preventing the formation of the C47-C151 disulfide bridge (PRDX5^{C47S}) (Figure 1A,B). Previous studies showed that the 6xHis tag does not alter the enzymatic activity of the protein, which remains active [29–35]. To alter PRDX5 redox state

without modifying the structure, purified PRDX5 proteins were also chemically treated with dithiothreitol (DTT) to reduce disulfide bonds. Pre-reduced proteins were then exposed either to the alkylating agent iodoacetamide (IAM) to prevent their re-oxidation (r/aPRDX5, Figure 1B), or to a high concentration of H_2O_2 to form Cys sulfenic and/or sulfonic acids (s/sPRDX5) (Figure 1B). It is important to note that PRDX5 is known to be highly resistant to hyperoxidation compared to typical 2-Cys PRDXs, and that, to our knowledge, hyperoxidized PRDX5 has never been detected in vivo [21]. Therefore, the implication of PRDX5 hyperoxidized forms in physiological or pathophysiological mechanisms is very unlikely, however s/sPRDX5 serves here as an important comparison. We pre-treated PRDX5 with DTT before hyperoxidation, as per Yang et al. [10], as H_2O_2 alone is not sufficient for disulfide disruption.

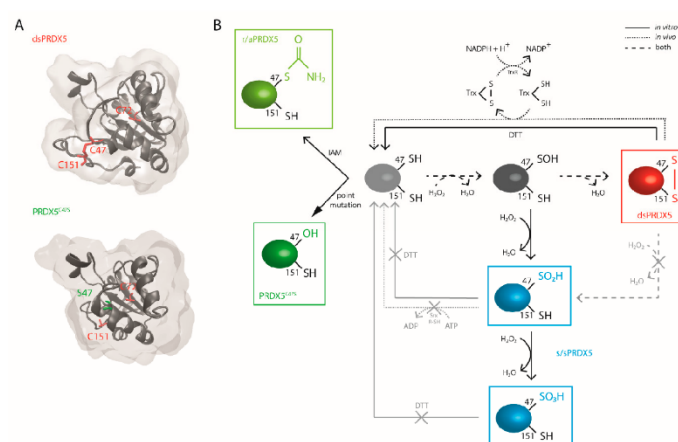


Figure 1. Structural and redox state insights of PRDX5. (A) Crystal structures of recombinant human PRDX5 (upper, PDB ID: 2vl3) and mutated C47S PRDX5 (lower, PDB ID: 1ur1n), showing that changes in Cys redox state is accompanied by conformational changes. dsPRDX5 contains three Cys (C47, C72, C151), with two of them being catalytically active (C45 and C151). (B) Schematic of the PRDX5 C47 redox modifications. The PRDX5 C47 thiol (-SH) can be oxidized in vitro or in vivo to an intermediate sulfenic acid (-SOH) by H_2O_2 or other peroxides. The reactive -SOH either engages into an intramolecular disulfide bond with C151 (dsPRDX5, red) or is hyperoxidized in vitro to sulfinic acid (-SO₂H) or further to sulfonic acid (-SO₃H) (s/sPRDX5, blue). In vivo, the disulfide bridge is reduced by thioredoxin (Trx). Unlike typical 2-Cys PRDXs, the PRDX5 sulfenic form cannot be reduced by the sulfiredoxin system. Both Cys sulfenic and sulfonic acids are therefore irreversible. In vitro, DTT can be used to reduce the disulfide form, but not the hyperoxidized forms. To maintain the protein in a reduce state, the thiol can be alkylated by iodoacetamide (IAM) to prevent re-oxidation (r/aPRDX5, light green), or the protein can be point-mutated to replace the C47 by a serine (PRDX5^{C47S}, dark green).

Next, we characterized the PRDX5 redox forms using various approaches (Figure 2). Western blot analysis (Figure 2A) first revealed that dsPRDX5 showed similar bands before and after ultrafiltration, indicating that the ultrafiltration process used to remove the residual agents after the chemical treatments does not alter the proteins. We then confirmed that DTT/ H_2O_2 treatment led to at least partial hyperoxidation of PRDX5 Cys by comparing similar blots probed either with an antiserum raised against recombinant human PRDX5 or an antibody highly specific for the sulfonated PRDX5 form [21].

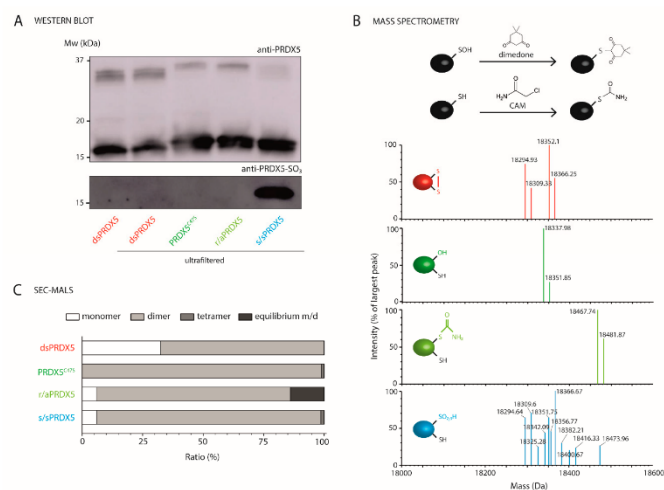


Figure 2. Characterization of the PRDX5 redox forms. (A) Western blot analysis of PRDX5 redox forms performed under non-reducing conditions. Western blotting against PRDX5 reveals the presence of monomers and dimers (upper) and presence of sulfonic acids was confirmed by Western blotting against PRDX5-SO₃ and was only detected in s/sPRDX5 sample. dsPRDX5 was probed before and after ultrafiltration. Representative of three independent experiments. (B) Centroid spectrum showing monomeric species of the different redox forms (dimeric species are shown in Supplementary Figure S2), generated after MaxEnt1 deconvolution of the multiply-charged spectrum obtained by direct infusion ESI-Q-TOF MS analysis on intact proteins. An intensity threshold of 20% normalized to the largest peak was applied to display the major peaks. dsPRDX5, r/aPRDX5, and s/sPRDX5 contains three Cys, while PRDX5^{C47S} has two remaining Cys. Prior to MS analysis, potent Cys sulfenic acids were trapped with dimedone, and remaining Cys thiols were blocked with 2-chloroacetamide (CAM). Identification of each peak is presented in Supplementary Table S1. (C) Quantification of the monomeric and oligomeric states using SEC-MALS. Source data are provided in Supplementary Figure S1 (blots), in Supplementary Figure S2 (MS multiply-charged spectra), and in Supplementary Figure S4 (SEC-MALS spectra).

We further characterized the redox state of the PRDX5 redox forms using direct infusion electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) on intact proteins (Figure 2B). After redox treatments and prior to MS analysis, potent Cys sulfenic acids were trapped with dimedone, and thiols were subsequently blocked by alkylation with 2-chloroacetamide (CAM). Raw multiply-charged electrospray spectra were processed with the maximum entropy method [36–38] to produce true molecular mass spectra (Supplementary Figure S2).

Based on the amino acid sequence of dsPRDX5 and PRDX5^{C47S}, the calculated mass of the proteins with reduced Cys thiols are 18,297.81 and 18,281.75 Da, respectively. The deconvoluted mass spectra revealed major monomeric and minor dimeric species for each redox form (Supplementary Figure S2). The centroid spectrum of dsPRDX5 shows four major peaks related to the monomeric species (Figure 2B, Supplementary Figure S2), of which mass and identification are presented in Supplementary Table S1. Peaks #1 and #3 correspond to the protein with a disulfide bond (mass shift of -2 Da), without or with an additional carbamidomethyl group (mass shift of +57 Da), respectively. The two

secondary peaks (#2 and #4) indicate a mass increase of 14 Da compared to the parent peaks, and are most likely due to a *N*-terminal methylation [39]. This phenomenon has been observed by other researchers who performed similar intact-protein ESI-MS analysis on the recombinant *N*-terminal 6xHis-tagged Z domain of protein A [40]. The deconvoluted mass spectrum of PRDX5^{C47S} reveals two major peaks for the monomeric species (Figure 2B, Supplementary Table S1 and Figure S2). The parent peak (#1) corresponds to the mutant protein with a serine residue (Ser) replacing the Cys and a carbamidomethyl group, and the secondary peak (#2) indicates an additional mass of 14 Da, as for dsPRDX5. In the case of *r/a*PRDX5, the two major peaks detected correspond to the protein with three carbamidomethyl groups, either with or without a 14-Da increase in mass (Figure 2B, Supplementary Table S1 and Figure S2). As both IAM and CAM modifications result in the covalent addition of a carbamidomethyl group (mass shift of +57 Da), we performed the analysis without CAM alkylation in order to determine which adducts were formed after IAM redox treatment (insets in Supplementary Figure S2). The analysis performed without CAM reveals identical peaks with a mass shift of −57 Da each, indicating that alkylating the protein with IAM at pH 7.2 more likely forms thioethers on the two more reactive Cys, i.e., the ones involved in the disulfide bond. Lastly, the major peaks resolved on the deconvoluted mass spectrum of *s/s*PRDX5 (peaks #1, #2, #5, and #7) are identical to those of dsPRDX5 (Figure 2B, Supplementary Table S1 and Figure S2). However, several other peaks (#3, #4, #6, #8, #9, #10, and #11) were detected and indicate that hyperoxidizing the DTT-reduced protein with H₂O₂ results in a mix of species composed of Cys sulfinic acid (−SO₂H), sulfonic acids (−SO₃H), and carbamidomethyl groups. Summing the peak intensities corresponding to these species gives a proportion of ~42%, but the disulfide bond remains in many of these species, which is in agreement with the fact that PRDX5 is resistant to hyperoxidation by H₂O₂ [21]. The identification of these peaks is presented in Supplementary Table S2. No sulfinic intermediate was identified (mass shift of +138 Da). Moreover, analysis of the fragments corresponding to peptides containing C47 using LC-Q-TOF-MS/MS revealed the presence of the redox modifications (disulfide bridge, carbamidomethyl group, sulfinic acid, sulfonic acid) on the Cys (Supplementary Figure S3). Overall, these results demonstrate that dsPRDX5, PRDX5^{C47S}, and *r/a*PRDX5 are largely homogenous redox forms and confirm the efficiency of the reducing/alkylating treatment. However, *s/s*PRDX5 is only partially hyperoxidized, with a proportion of species having a disulfide bridge.

While the oligomeric conformation of 2-Cys PRDX subfamily directly links redox state to function [41,42], atypical 2-Cys PRDX5 is thought to exist as a dimer irrespective of the redox state of the cysteine residues [8,43]. Western blot performed under non-reducing conditions revealed the presence of monomers, but also of dimers (Figure 2A), and to a lesser extent, of oligomers (Supplementary Figure S1). Although these results are consistent with previous studies [35,44], whether covalent dimers (and oligomers) linked by intramolecular disulfide bridges are physiologically relevant remains to be determined [43]. We further refined the oligomeric state picture of PRDX5 for all redox states in native conditions using size-exclusion chromatography–multiangle light scattering (SEC-MALS) (Figure 2C, see spectra in Supplementary Figure S4). The main quaternary structure detected for all conditions was the dimeric form. In dsPRDX5, however, a relatively high fraction (~30%) of monomers could also be identified, and *r/a*PRDX5 shows ~15% of proteins in a monomer-dimer equilibrium. This is consistent with previous results showing that there is a rapid equilibrium between a major dimer and a minor monomer species [44]. A very small fraction of tetramers was observed for PRDX5^{C47S} and *s/s*PRDX5. Although the covalent or non-covalent nature of such dimers and oligomers cannot be determined in SEC-MALS, MS analysis of intact proteins revealed the presence of non-covalent dimers for PRDX5^{C47S} and *r/a*PRDX5 (Supplementary Figure S2 and Table S2). This suggests that such non-covalent dimers are also present in dsPRDX5 and *s/s*PRDX5. Overall, these data indicate that the redox state of PRDX5 does not alter its oligomeric conformation, facilitating further analysis and interpretation.

3.2. The PRDX5 Disulfide Bond Allows a Stronger Cytokine-Stimulating Activity

Our previous study showed that dsPRDX5 was able to stimulate IL-1 β secretion from PMA-differentiated THP-1 cells [19]. To demonstrate the direct role of TLR4 in PRDX5-triggered cytokine secretion, we examined the IL-8 production from HEK293 MD2-CD14 transfected with TLR4 cells using ELISA (Figure 3). We first used FC to validate the stable transfection and to confirm that the receptor was expressed at the cell surface (Figure 3A). We also established that the receptor was functional by exposing the cells to LPS, which requires both MD-2 and CD14 to trigger cytokine secretion [45–47] (Figure 3C). LPS exposure resulted in a secretion of $14,085 \pm 2401$ pg mL⁻¹ (mean $\pm$ SD_{pooled}, N = 3).

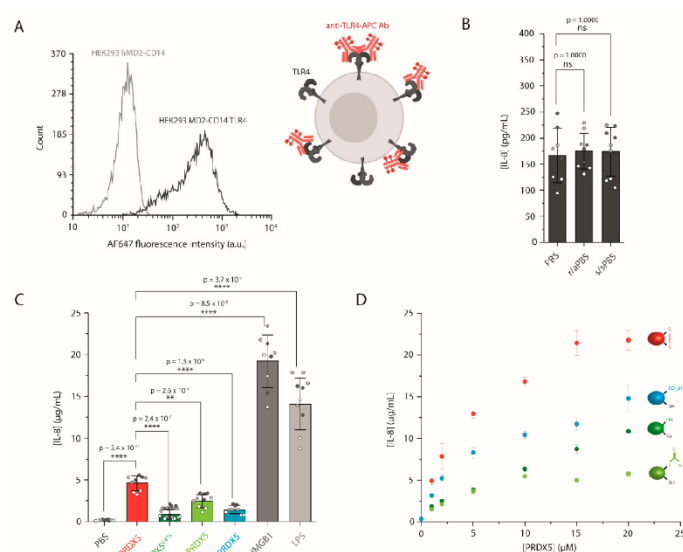


Figure 3. PRDX5 redox-dependent IL-8 secretion from HEK293 MD2-CD14 TLR4 cells. **(A)** Stable transfection of TLR4 in HEK293 MD2-CD14 cells was validated by flow cytometry (FC). Schematic depicting the labeling strategy used to validate the correct TLR4 expression at the cell surface. **(B)** Control experiment showing IL-8 production levels from HEK293 MD2-CD14 TLR4 cells treated with ultra-filtered PBS, “reduced/alkylated” PBS (r/aPBS) or “hyperoxidized” PBS (s/sPBS), quantified by ELISA. Three independent experiments (white, light grey and dark grey points) with three replicates per condition for each experiment. **(C)** IL-8 secretion from HEK293 MD2-CD14 TLR4 cells induced after stimulation with the PRDX5 redox forms, quantified by ELISA. LPS was used as a control to validate that TLR4 was functional, and HMGB1 was used as a DAMP control. Three independent experiments (white, light grey and dark grey points) with three replicates per condition for each experiment and two independent PRDX5^{C47S} protein productions. **(D)** Concentration-dependence of IL-8 secretion from HEK293 MD2-CD14 TLR4 cells for the redox forms. Data are representative of three replicates. Data are mean $\pm$ S.D. ns, not significant ($p > 0.05$). ** $p < 0.01$, *** $p < 0.0001$ (Tukey HSD test).

We then verified that the residual DTT, IAM and H₂O₂ remaining after protein redox treatments had been efficiently removed by ultrafiltration and thus would not impact cell

viability or interfere with IL-8 secretion. To do so, we treated PBS with the same reducing and hyperoxidizing agents that we used for dsPRDX5, and we ultra-filtrated the samples. As shown in Figure 3B, exposing cells to r/aPBS or s/sPBS resulted in similar IL-8 secretion levels (175 ± 17 and 174 ± 17 pg·mL⁻¹, respectively, mean $\pm$ SD_{pooled}, $N = 3$) as with PBS (187 ± 61 pg·mL⁻¹, mean $\pm$ SD_{pooled}, $N = 3$), confirming that the further results would not be on account of residual treatment agents.

We next assessed the IL-8 production from HEK293 MD2-CD14 TLR4 cells mediated by exposure to the PRDX5 redox forms (Figure 3C). We observed that dsPRDX5 yielded higher IL-8 stimulating activity with 4608 ± 288 pg·mL⁻¹ (mean $\pm$ SD_{pooled}, $N = 3$) compared to the other redox forms. The C47S point mutation led to significantly reduced IL-8 secretion level (893 ± 142 pg·mL⁻¹, mean $\pm$ SD_{pooled}, $N = 3$). The same tendency could be observed with the chemically reduced/alkylated protein, albeit less prominent (2437 ± 343 pg·mL⁻¹, mean $\pm$ SD_{pooled}, $N = 3$). Similarly, hyperoxidizing the protein in vitro with H₂O₂ resulted in a significant decrease in IL-8 secretion (1447 ± 136 pg·mL⁻¹, mean $\pm$ SD_{pooled}, $N = 3$) compared to dsPRDX5. As control, we used another DAMP protein that can signal through TLR4, namely high-mobility group box 1 (HMGB1) [48], which led to an IL-8 concentration of $19,227 \pm 2695$ pg·mL⁻¹ (mean $\pm$ SD_{pooled}, $N = 3$). We also demonstrated that the magnitude of IL-8 secretion was dependent on the concentration of the redox forms (Figure 3D). Our results highlight the importance of the C47-C151 disulfide bond in PRDX5-mediated cytokine production through TLR4, and is in line with other studies showing that the activity of HMGB1 strongly depends on the redox state of its three Cys [49,50]. Nevertheless, our results are in contradiction with a previous study showing that the disruption of PRDX5 Cys by point mutations or IAM treatment did not affect the IL-23p19 mRNA expression level in bone marrow-derived dendritic (BMDC) cells [14]. However, their conditions differ from ours: (i) PRDX5 was reduced with only 1 mM DTT, while several studies use 10 mM [21,29]; (ii) they investigated the mRNA level, although they showed for PRDX1 and PRDX2 that IL-23p19 mRNA expression level does not correlate with IL-23 expression; (iii) BMDC cells were exposed for 1 h, while we treated our cells for 16 h, (iv) BMDC cells have a wider TLR expression profile [51], making it difficult to interpret the contribution of TLR4. The effect of the Cys redox state has also been studied for PRDXs from the typical 2-Cys subfamily, with conflicting results. While Riddell et al. showed that human PRDX1 interaction with TLR4 was independent of its peroxidase activity [13], López et al. recently demonstrated that pro-inflammatory properties of the cytosolic trypanoxin peroxidase from *Trypanosoma cruzi* depend on the presence of peroxidatic cysteine [52].

3.3. Disulfide, Mutant and Reduced PRDX5 Bind to TLR4 with Similar Kinetic Off-Rates

We previously demonstrated with AFM-based SMFS that dsPRDX5 was able to specifically interact with TLR4 [19]. To determine whether the difference in the cytokine-inducing capability of the PRDX5 redox forms could originate from differential binding properties to TLR4, we used AFM-based SMFS to probe the interaction between the PRDX5 redox forms and TLR4 (Figure 4A). To this end, we covalently grafted the PRDX5 redox forms to AFM tips by means of a flexible polyethylene glycol (PEG₂₄) linker, and we covalently attached TLR4 molecules to a mixed self-assembled monolayer of carboxyl- and hydroxyl-terminated alkanethiols immobilized onto an inert gold surface using *N*-hydroxysuccinimide (NHS)/*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) chemistry. We then used force-distance (FD) curve-based AFM to investigate the binding kinetics of the PRDX5 redox forms (Figure 4B, Supplementary Figure S5A). We recorded multiple maps (30 for dsPRDX5, PRDX5^{C47S} and r/aPRDX5; 35 for s/sPRDX5) at different tip retraction speeds (100 nm·s⁻¹, 200 nm·s⁻¹, 500 nm·s⁻¹, 1 µm·s⁻¹, 2 µm·s⁻¹, and 10 µm·s⁻¹).

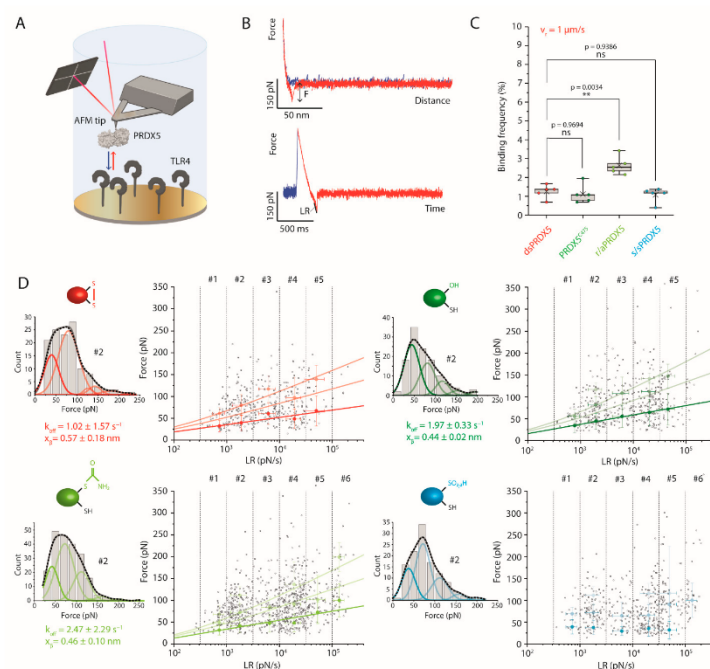


Figure 4. PRDX5 redox forms bind surface-immobilized TLR4 with similar k_{off} . (A) Cartoon showing the experimental set-up used to probe the interaction between the PRDX5 redox forms and TLR4. (B) Representative specific force-distance (FD) and force-time curves. Examples for all redox forms are shown in Supplementary Figure S5. Rupture force (F) corresponds to the height of the adhesive peak on the FD curve, and the loading rate (LR) is calculated by fitting the adhesive peak on the force-time curve just before rupture with a linear model. (C) Box plot showing the binding frequencies (BF) obtained at a tip retraction speed of $1 \mu\text{m}\cdot\text{s}^{-1}$. Number of AFM maps, $n = 5$ for each redox form (dots). Box plot shows mean ($\times$), median ($-$), 25th and 75th percentiles (boxes), and ranges from min to max (whiskers). ns, not significant ($p > 0.05$). ** $p \leq 0.01$ (Tukey HSD test). (D) Dynamic force spectroscopy (DFS) analysis for the PRDX5 redox forms. Spectrum of LR was divided into discrete ranges (LR#1–6). For each LR range, a Kernel distribution of the rupture forces (grey line) was generated, as shown on the histogram for LR#2, which allows to determine hidden peaks based on the second derivative. Peaks were fitted with Gaussian functions (colored lines), revealing the most probable rupture forces for single (1st peak) and multiple (following peaks) interactions. These forces were reported on the DFS plot (full colored data points). The Bell-Evans model was fitted to data corresponding to single interactions (solid line), and the predicted rupture forces for two and three simultaneous uncorrelated interactions were calculated using Williams-Evans prediction (dashed lines). From the Bell-Evans model, the k_{off} and x_p values were extracted. The most probable rupture forces (full colored data points) are peak center $\pm$ S.D.

We first assessed the binding frequency (BF) by examining the FD curves showing specific adhesive events between the tip-bound PRDX5 and surface-immobilized TLR4

(i.e., whose extension patterns could be fitted with the worm-like chain model) (Figure 4C, Supplementary Figure S5B). The BF is given by the ratio between the number of specific events and that of total recorded events [53]. There was no significant difference between the different redox forms, except for the r/aPRDX5 that showed a higher BF.

To investigate the binding kinetics of PRDX5-TLR4 interactions for the different redox forms, we performed a dynamic force spectroscopy (DFS) analysis. For each specific adhesive curve, we extracted the rupture force (F) and the loading rate (LR), which corresponds to the force applied over time (Figure 4B). We divided the spectrum of LR's probed into discrete ranges (LR#1–6) as performed in previous studies [19,54,55], and for each of them, we used a Kernel distribution of the rupture forces to define the most probable forces for single and multiples interactions (Supplementary Figure S6). The second derivative of the density function allowed finding hidden peaks, which were fitted with Gaussian functions (Figure 4D, Supplementary Figure S6). The resulting Gaussian function overlapped very well the Kernel distribution.

With the exception of s/sPRDX5, all the DFS plots showed a linear dependency of the most probable rupture forces with the LR for single interactions, which is frequently observed for ligand-receptor interactions and is explained by a single free-energy barrier being crossed during the mechanical pulling [56]. The Bell-Evans model [26] was used to fit to the most probable forces measured for the single interaction at the different LR ranges. From the fit (Figure 4D, dark line), we extracted the dissociation rate constant (k_{off}), which corresponds to the inverse of the interaction lifetime, as well as the distance to the transition state (x_β), which provides useful information related to the chemistry of interaction. The k_{off} values obtained were $1.02 \pm 1.57 \text{ s}^{-1}$, $1.97 \pm 0.33 \text{ s}^{-1}$, and $2.47 \pm 2.29 \text{ s}^{-1}$ for dsPRDX5, PRDX5^{C47S}, and r/aPRDX5, respectively. Regarding s/sPRDX5, the DFS plot reveals no increase in the binding force with the LR, suggesting an unspecific interaction. A similar x_β was observed for dsPRDX5 ($0.57 \pm 0.18 \text{ nm}$), PRDX5^{C47S} ($0.44 \pm 0.02 \text{ nm}$) et r/aPRDX5 ($0.46 \pm 0.10 \text{ nm}$), indicating a similar binding conformation. Using these parameters, we applied the Williams-Evans prediction [57] to infer the interaction forces of multiple uncorrelated interactions breaking simultaneously (Figure 4D, light lines). These predictions are in good agreement with the mean rupture forces observed for double and triple interactions. By performing a F-test on the Bell-Evans fittings, we could determine that dsPRDX5 and PRDX5^{C47S}, as well as dsPRDX5 and r/aPRDX5, were not statistically distinct, with 0.05 significance level (*p*-value of 0.50492 and 0.63374, respectively). This can be visualized by superimposing the DFS plots of the different redox forms, as shown in Supplementary Figure S7. The residual dsPRDX5 present in the hyperoxidized sample can explain the higher rupture forces observed, which probably result from the interaction between dsPRDX5 and TLR4. Overall, these results indicate that the differences observed in the IL-8-stimulating activity of the different redox forms cannot be attributable to differential interaction lifetime with TLR4. Further work is needed to investigate the associate rate constant (k_{on}) in order to determine whether TLR4 alone is sufficient to discriminate between the redox forms.

3.4. PRDX5^{C47S} Shows a Lower Propensity to Bind to the Cell Surface

We consequently turned to living HEK293 cells expressing TLR4 to investigate the binding of PRDX5 molecules in a cellular context. We first performed a colocalization experiment to visually confirm that dsPRDX5 binding to TLR4 is responsible for the inflammatory response (Figure 5A). To this end, we incubated HEK293 MD2-CD14 TLR4 cells with PRDX5 labeled with NTA-FITC using Ni^{2+} (PRDX5-NTA-FITC). Shortly after the addition of PRDX5-NTA-FITC, a membrane staining motif began to emerge, with discrete pockets of FITC fluorescence present at the cell membrane. Of particular note, fluorescence signals present in filopodia and focal adhesions were commonly co-localized. This observation was subsequently followed by FITC positive vesicles gathering at the cell periphery and trafficking within the cell interior. Together these staining behaviors indicate binding and internalization. To ensure that the fluorescence observed was not

merely crosstalk, λ -scans were performed to assess whether the fluorescent species present included the earlier emission peak from the FITC dye and fluorescence intensities across the membrane examined using a simple Line of Response (Supplementary Figure S8).

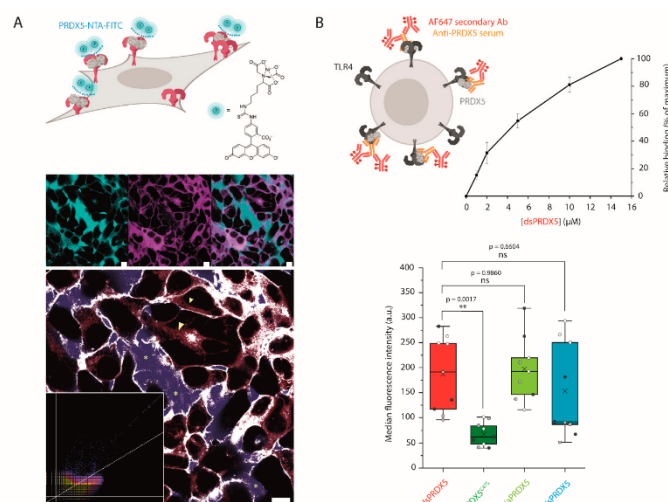


Figure 5. Binding of PRDX5 to the surface of HEK293 MD2-CD14 TLR4 cells monitored by fluorescence. (A) Cartoon (upper) showing the protocol used to detect PRDX5 binding to TLR4 using confocal. Confocal images of, left to right, PRDX5-NTA-FITC (cyan), TLR4-YFP (magenta), fluorescence overlay. Co-localization (white) of FITC (blue) and YFP (red) signals predominantly at the plasma membrane and in the endomembranous region. There is a lack of co-localization in the extracellular medium (*) and in YFP exclusive regions within cells (arrowhead). Co-localization map and scatterplot (inset). The cross-talk is represented by unique perfectly co-localized pixels. Scale bars are 10 μ m. (B) Cartoon (upper) showing the protocol used to detect PRDX5 binding to living cells using flow cytometry (FC). Graph from FC binding assay showing the concentration dependence of dsPRDX5 binding to the surface of HEK293 MD2-CD14 TLR4 cells. Binding was normalized to the signal of untreated cells and is expressed relatively (in %) to the fluorescence intensity observed with 15 μ M PRDX5. Three independent experiments with two replicates in each. Box blot (lower) from FC binding assay showing the median fluorescence intensity of PRDX5-treated cells. The fluorescence intensity was normalized to the untreated cells. Seven experiments with three independent protein treatments (white, light grey and dark grey points) or two protein productions in the case of PRDX5^{C17S}. Three replicates for each redox form in each treatment. Box plot shows mean ($\times$), median ($-$), 25th and 75th percentiles (boxes), and ranges from min to max (whiskers). ns, not significant ($p > 0.05$). ** $p \leq 0.01$ (Tukey HSD test).

We further investigated the binding level of redox PRDX5 isoforms to the surface of HEK293 MD2-CD14 TLR4 cells using a FC binding assay. To prevent endocytosis, cells were incubated with PRDX5 on ice, and labelled with an antiserum against PRDX5. As a control, we also labelled untreated cells. We first showed that dsPRDX5 binding to the surface of cells expressing TLR4 was concentration-dependent (Figure 5B). These results confirm the role of TLR4 in the proinflammatory activity of PRDX5 and demonstrate that LPS is not responsible for the concentration-dependent increase in IL-8 secretion following

exposure to PRDX5 (Figure 3D). At identical concentration, PRDX5^{C47S} shows a ~3-fold decrease in binding propensity compared to dsPRDX5 (Figure 5B). However, this result was not observed for r/aPRDX5 and s/sPRDX5. As the difference observed between dsPRDX5 and the mutant protein was not observed with SMFS, this result could indicate either that TLR4 conformation (i.e., dimer) and/or stabilization in the plasma membrane is important for the binding, or that the TLR4 accessory molecules MD-2 and/or CD14 might allow discriminating between redox forms. Further investigation is needed to understand the role of TLR4 accessory molecules in this context.

4. Conclusions

Human PRDX5 has been shown to enhance brain ischemia-reperfusion injury through activation of TLR2- and TLR4-mediated inflammation. Although our previous findings reported that PRDX5 was able to directly interact with TLR4, the role of the redox state of PRDX5 cysteine residues remained to be investigated. Indeed, PRDX5 is a redox-sensitive protein that act as a peroxidase inside the cell. Here, we investigated the impact of the PRDX5 redox state on its TLR4-dependent proinflammatory activity by modifying the redox state of PRDX5 cysteine residues, either by point-mutation or by chemical treatments. Using molecular biology and biophysics approaches, we provide evidence that the disulfide bridge between PRDX5 enzymatic cysteine is required to allow a strong TLR4-dependent IL-8 secretion. Although the interaction lifetime between TLR4 and oxidized or reduced PRDX5 is not different, binding assays performed on living cells expressing TLR4 demonstrated that disulfide PRDX5 has a higher propensity to bind the cell surface than the mutant protein lacking the peroxidatic cysteine. Taken together, these results shed new light on the importance of the protein redox biology in inflammation, which is of crucial importance for the development of therapeutic treatments to administer after a stroke.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/antiox10121902/s1>: Figure S1: Western blot analysis of the PRDX5 redox forms performed under non-reducing conditions; Figure S2: Direct-infusion ESI-Q-TOF analysis of the PRDX5 redox forms; Figure S3: Representative spectra of fragments corresponding to peptides containing the 47th residue; Figure S4: SEC-MALS analysis of the PRDX5 redox forms; Figure S5: SMFS analysis of PRDX5 redox form binding to surface-immobilized TLR4; Figure S6: Kernel density function of the rupture forces obtained at the different loading rate (LR) ranges for the PRDX5 redox forms; Figure S7: Superimposition of dynamic force spectroscopy (DFS) plots; Figure S8: Additional fluorescence data from confocal laser scanning microscopy (CLSM); Table S1: Masses and intensities of the peaks corresponding to monomeric species detected by ESI-Q-TOF MS on intact proteins; Table S2: Masses and intensities of the peaks corresponding to dimeric species detected by ESI-Q-TOF MS on intact proteins.

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Supplementary Information for

Role of the redox state of human peroxiredoxin-5

on its TLR4-activating DAMP function

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Supplementary Figures 1 to 8
Supplementary Tables 1 and 2
Supplementary Materials and Methods

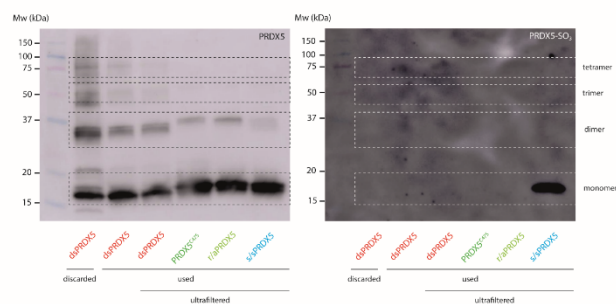


Figure S1. Western blot analysis of the PRDX5 redox forms performed under non-reducing conditions. Western blotting against PRDX5 (left) or against PRDX5-SO₂ (right). dsPRDX5 was probed before and after ultrafiltration. Representative of three independent experiments.

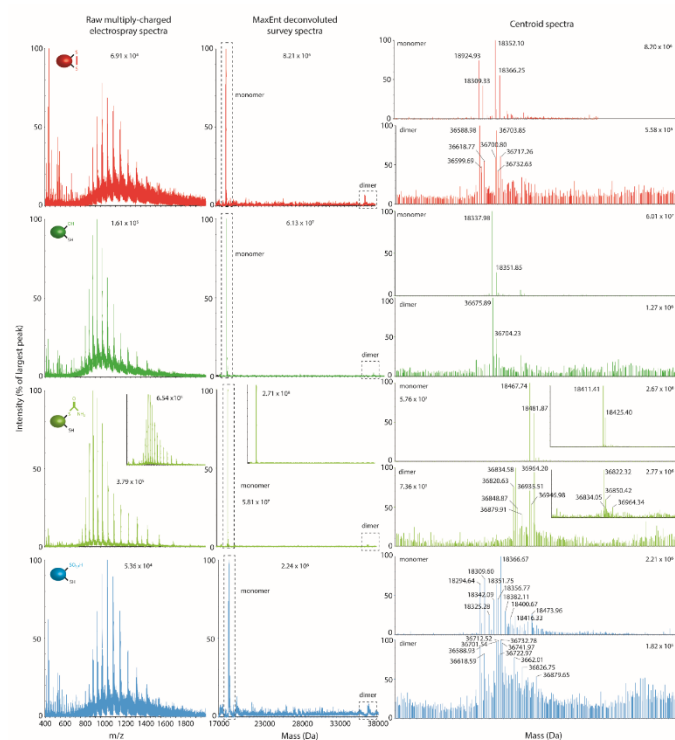


Figure S2. Direct-infusion ESI-Q-TOF analysis of the PRDX5 redox forms. Raw multiply-charged electrospray spectra (m/z 400-2000, left) were processed with MaxEnt1 to produce true molecular mass spectra (middle), from which were generated centroid spectra (right). Ranges of centroid spectra are 18,000-18,600 Da for peaks related to monomeric species (right, upper), and 36,200-37,200 Da for peaks related to dimeric species (right, lower). Potent Cys sulfenic acids were trapped with dimedone and remaining thiols were blocked with 2-chloroacetamide (CAM). Identification of Cys alkylated with iodoacetamide (IAM) in *r/a*PRDX5 was performed without using CAM prior to MS analysis (insets). Intensities and masses of the major peaks ($\geq 20\%$ and $\geq 40\%$ in intensity relative to the largest peak of monomeric or dimeric species, respectively) are detailed in **Suppl. Table S1** (monomeric species) and **Suppl. Table S2** (dimeric species).

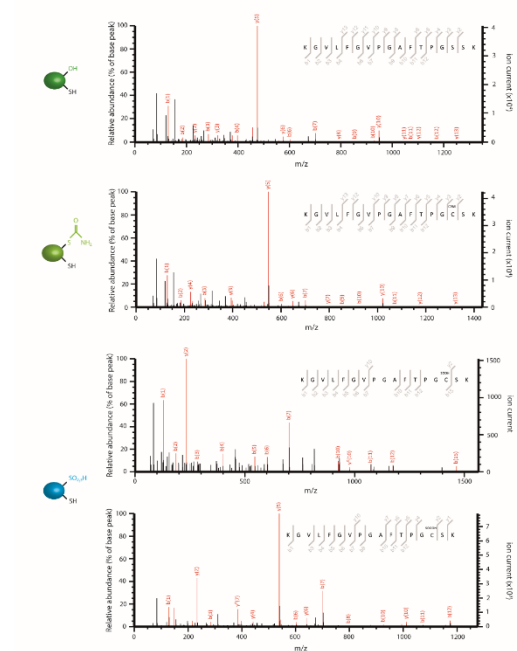


Figure S3. Representative spectra of fragments corresponding to peptides containing the 47th residue. Spectra showing the point mutation (C47S) in PRDX5^{C47S}, or the Cys redox modifications (*i.e.*, carbamidomethyl group in r/aPRDX5, or sulfinic acid and sulfonic acid in s/sPRDX5). Representative of three independent protein treatments. See **Supplementary Materials and Methods**.

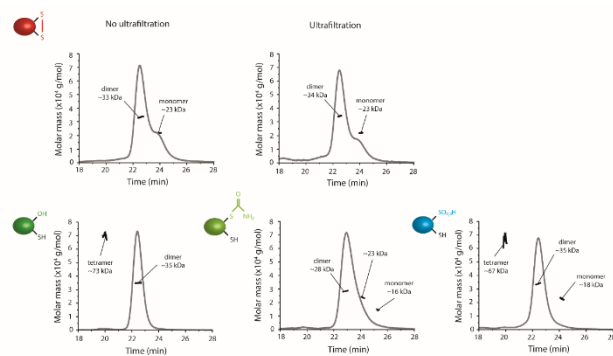


Figure S4. SEC-MALS analysis of the PRDX5 redox forms. Light scattering spectrum (grey line) of dsPRDX5 before (upper left) and after (upper right) ultrafiltration, and PRDX5_{C47S} lower (left), r/aPRDX5 (lower middle), and s/sPRDX5 (lower right) allowing molar mass determination (black line) of the monomers and oligomers.

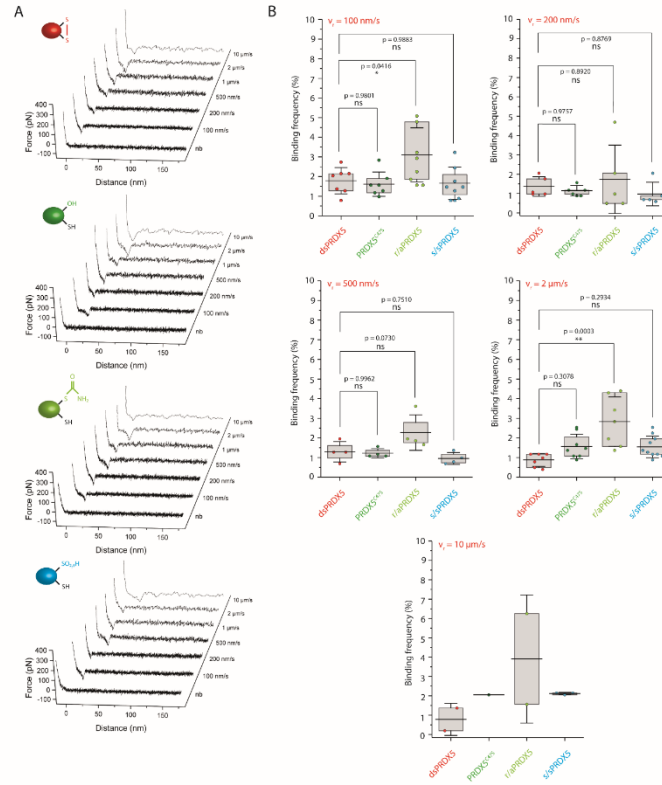


Figure S5. SMFS analysis of PRDX5 redox form binding to surface-immobilized TLR4. (A) Representative non-adhesive force-distance (FD) curve (nb, no binding) or FD curves showing a specific adhesive event, at the 6 tip retraction speeds, for all the PRDX5 redox forms. **(B)** Box plots showing the binding frequencies (BF) obtained at tip retraction speeds of 100 nm·s⁻¹, 200 nm·s⁻¹, 500 nm·s⁻¹, 2 μm·s⁻¹, and 10 μm·s⁻¹. Number of AFM maps (circles). Box plots show mean (x), median (—), 25th and 75th percentiles (boxes), and range from min to max (whiskers). ns, not significant ($p > 0.05$). * $p \leq 0.05$, ** $p \leq 0.01$ (Tukey HSD test).

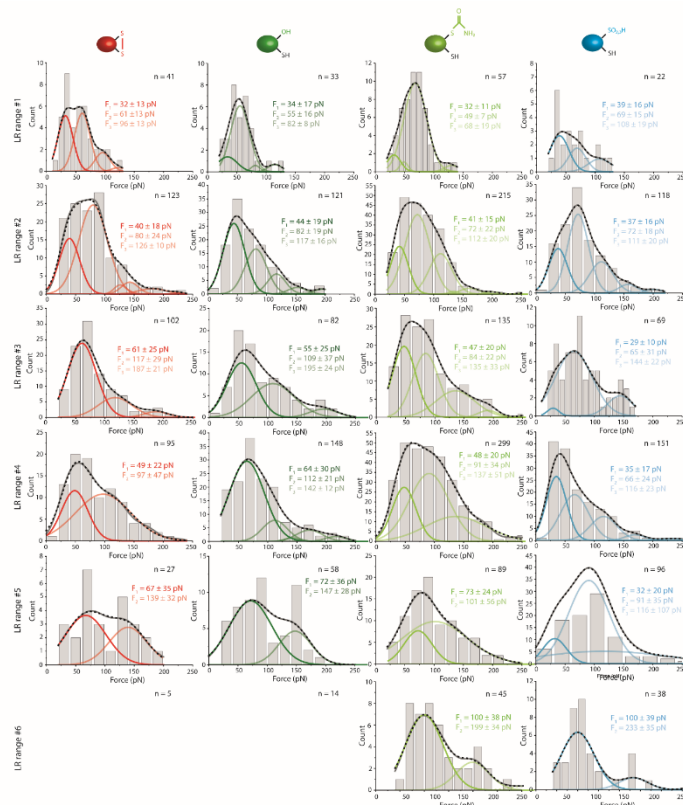


Figure S6. Kernel density function of the rupture forces obtained at the different loading rate (LR) ranges for the PRDX5 redox forms. Rupture forces were divided into discrete LR ranges (LR#1: 315-1000 pN·s⁻¹; LR#2: 1000-3150 pN·s⁻¹; LR#3: 3150-10,000 pN·s⁻¹; LR#4: 10,000-31,500 pN·s⁻¹; LR#5: 31,500-100,000 pN·s⁻¹; LR#6: 100,000-315,000 pN·s⁻¹). Kernel density function (grey line) of the rupture forces (which is superimposed on force histograms for more clarity) was then used to determine the hidden peaks based on the second derivative. The peak centers determined with the Kernel density function were then used to fit a Gaussian function (colored lines), from which the most probable rupture forces for single (1st peak) and multiple interactions (following peaks) were determined by taking peak centers (F₁, F₂, F₃ for single, double and triple interactions, respectively). The cumulative peak fit (black dashed line) overlap very well with the Kernel density function.

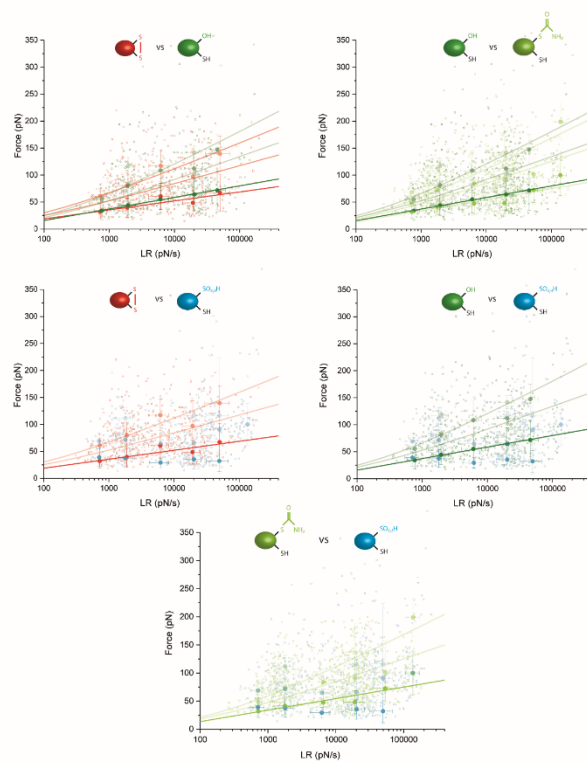


Figure S7. Superimposition of dynamic force spectroscopy (DFS) plots. PRDX^{C47S} DFS plot was superimposed with dsPRDX5, r/aPRDX5, and s/sPRDX5 DFS plots. s/sPRDX5 DFS plot was superimposed with dsPRDX5, and r/aPRDX5 DFS plots.

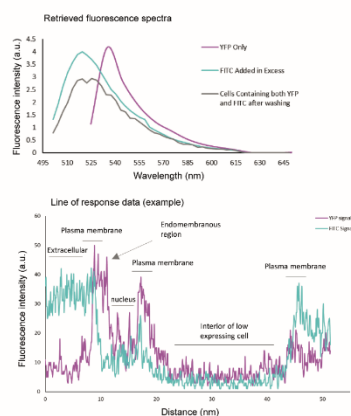


Figure S8. Additional fluorescence data from confocal laser scanning microscopy (CLSM). Spectral scan data retrieved from YFP (magenta) cells (upper), PRDX5-NTA-FITC (cyan) added in excess to dominate the spectra, and after YFP positive cells had been exposed and washed (gray). The shifting dual peak in the latter is indicative of more than one fluorescent species being present, although the PRDX5-NTA-FITC is the most common. Line of Response (LoR) through two adjacent cells, showing local distribution of the two fluorophores (FITC represented by cyan, YFP by magenta). FITC fluorescence intensity is highest at the edge of the plasma membrane and in the surrounding extracellular medium, whereas YFP predominates at the plasma membrane and in the endomembranous region (target site and manufacturing locale respectively). The co-occurring peaks support the co-localization analysis presented in the main manuscript.

Table S1. Masses and intensities of the peaks corresponding to monomeric species detected by ESI-Q-TOF MS on intact proteins. Peaks $\geq 20\%$ in intensity relatively to the largest peak were considered and taken into account for the calculation of the relative intensity. The most probable Cys modification(s) corresponding to the mass shifts are given.

	Peak #	Intensity	Relative intensity (%)	Mass (Da)	Identification	Mass shift (Da)
dsPRDX5	1	6.11×10^9	27	18,294.93	Disulfide Cys-S-S-Cys	-2
	2	3.46×10^9	16	18,309.33	Disulfide Cys-S-S-Cys Methylation	-2 +14
	3	8.20×10^9	37	18,352.10	Disulfide Cys-S-S-Cys Carbamidomethyl	-2 +57
	4	4.53×10^9	20	18,366.25	Disulfide Cys-S-S-Cys Carbamidomethyl Methylation	-2 +57 +14
PRDX5 ^{C47S}	1	6.01×10^{10}	79	18,337.98	Cys $\rightarrow$ Ser substitution Carbamidomethyl	-16 +57
	2	1.63×10^{10}	21	18,351.85	Cys $\rightarrow$ Ser substitution Carbamidomethyl Methylation	-16 +57 +14
r/aPRDX5	1	5.76×10^{10}	62	18,467.74	Carbamidomethyl x 3	+57 x 3
	2	3.53×10^{10}	38	18,481.87	Carbamidomethyl x 3 Methylation	+57 x 3 +14
s/sPRDX5	1	1.43×10^9	13	18,294.64	Disulfide Cys-S-S-Cys	-2
	2	1.57×10^9	14	18,309.60	Disulfide Cys-S-S-Cys Methylation	-2 +14
	3	5.46×10^8	5	18,325.28	?	+28.5
	4	9.73×10^8	9	18,342.09	Disulfide Cys-S-S-Cys Sulfonic acid Cys-SO ₃ H or Sulfinic acid Cys-SO ₂ H Methylation	-2 +48 or +32 +14
	5	1.43×10^9	13	18,351.75	Disulfide Cys-S-S-Cys Carbamidomethyl	-2 +57
	6	1.01×10^9	9	18,356.77	Disulfide Cys-S-S-Cys Sulfonic acid Cys-SO ₃ H Methylation	-2 +48 +14
	7	2.21×10^9	19	18,366.67	Disulfide Cys-S-S-Cys Carbamidomethyl Methylation	-2 +57 +14
	8	6.60×10^8	6	18,382.21	Carbamidomethyl ?	+57 +28.5
	9	4.44×10^8	4	18,400.67	Disulfide Cys-S-S-Cys Sulfonic acid Cys-SO ₃ H Carbamidomethyl or Sulfinic acid Cys-SO ₂ H Carbamidomethyl Methylation	-2 +48 +57 or +32 +57 +14
	10	5.17×10^8	5	18,416.33	Sulfinic acid Cys-SO ₂ H x 2 Carbamidomethyl or Sulfonic acid Cys-SO ₃ H Carbamidomethyl Methylation	+32 x 2 +57 or +48 +57 +14
	11	5.82×10^8	5	18,473.96	Cys-SO ₂ H alkylation adduct of IAM x 2	+89 x 2

Table S2. Masses and intensities of the peaks corresponding to dimeric species detected by ESI-Q-TOF MS on intact proteins. Peaks $\geq 40\%$ in intensity relatively to the largest peak were considered. The most probable Cys modification(s) corresponding to the mass shifts are given. s/sPRDX5 is not shown given the high number of possible combinations.

	Peak #	Intensity	Mass (Da)	Identification	Mass shift (Da)
dsPRDX5	1	5.58×10^8	36,588.98	Disulfide Cys-S-S-Cys x 2	-2 x 2
	2	2.65×10^8	36,599.69	?	+6
	3	3.08×10^8	36,618.77	Disulfide Cys-S-S-Cys x 2 Methylation x 2	-2 x 2 +14 x 2
	4	3.24×10^8	36,700.80	Carbamidomethyl x 2	+57 x 2
	5	5.21×10^8	36,703.85	Disulfide Cys-S-S-Cys x 2 Carbamidomethyl x 2	-2 x 2 +57 x 2
	6	2.32×10^8	36,717.26	Disulfide Cys-S-S-Cys x 2 Carbamidomethyl x 2 Methylation	-2 x 2 +57 x 2 +14
	7	3.38×10^8	36,732.63	Disulfide Cys-S-S-Cys x 2 Carbamidomethyl x 2 Methylation x 2	-2 x 2 +57 x 2 +14 x 2
PRDX5 ^{C47S}	1	1.27×10^9	36,675.89	Cys $\rightarrow$ Ser substitution x 2 Carbamidomethyl x 2	-16 x 2 +57 x 2
	2	6.09×10^8	36,704.23	Cys $\rightarrow$ Ser substitution x 2 Carbamidomethyl x 2 Methylation x 2	-16 x 2 +57 x 2 +14 x 2
r/aPRDX5	1	5.57×10^8	36,820.63	Carbamidomethyl x 4	+57 x 4
	2	7.36×10^8	36,834.58	Carbamidomethyl x 4 Methylation	+57 x 4 +14
	3	3.56×10^8	36,848.87	Carbamidomethyl x 4 Methylation x 2	+57 x 4 +14 x 2
	4	3.00×10^8	36,879.91	Carbamidomethyl x 5	+57 x 5
	5	5.20×10^8	36,935.51	Carbamidomethyl x 6	+57 x 6
	6	3.96×10^8	36,945.73	?	+351
	7	3.13×10^8	36,946.98	?	+354
	8	6.95×10^8	36,964.20	Carbamidomethyl x 6 Methylation x 2	+57 x 6 +14 x 2

Supplementary Materials and Methods

LC-Q-TOF-MS/MS characterization of the PRDX5 cysteine redox state

Blocking of cysteine redox states

Redox states of PRDX5 C47 in dsPRDX5 and s/sPRDX5 were blocked with 55 mM IAM (Sigma-Aldrich) for 10 min at RT in the dark to alkylate the thiols, and 5 mM 5,5-Dimethyl-1,3-cyclo-hexanedione (dimedone, Sigma-Aldrich) for 5 min at RT to trap sulfenic acids [1,2].

Preparation for in solution samples

Chloroform-methanol precipitation. 20 µg of each sample were transferred to 0.5 mL polypropylene Protein LoBind tubes (Eppendorf) and precipitated with chloroform-methanol method [3].

Solubilization before trypsin digestion. Samples were dried under vacuum with a Savant SpeedVac Concentrator (ThermoFisher). 20 µL of 100 mM triethylammonium bicarbonate (TEAB), pH 8.5 were added to the pellets for solubilization and vortexed for 30 min at RT.

In-solution trypsin digestion. Proteolysis was performed with 1 µg of sequencing grade trypsin (Promega, USA) and allowed to continue overnight at 37°C. Each sample was dried under vacuum with a Savant SpeedVac Concentrator (Thermo Fisher).

Peptide separation using nanoUPLC

Before peptide separation, the samples were dissolved in 20 µL of 0.1 % (v/v) formic acid and 2% (v/v) acetonitrile (ACN). The resulting peptide mixture was separated by reverse phase chromatography on a NanoACQUITY UPLC MClass system (Waters) working with MassLynx V4.1 (Waters) software. 200 ng of digested proteins were injected on a C18 trap column, 100Å, 5 µm, 180 µm x 20 mm (Waters), and desalted using isocratic conditions with at a flow rate of 15 µL·min⁻¹ using a 99% formic acid and 1% (v/v) ACN buffer for 3 min. Peptide mixture was subjected to reverse phase chromatography on a PepMap C18 column, 100Å, 1.8 µm, 75 µm x 150 mm (Waters) for 130 min at 35°C at a flow rate of 300 nL·min⁻¹ using a two part linear gradient from 1% (v/v) ACN, 0.1 % formic acid to 35 % (v/v) ACN, 0.1 % formic acid for 90 min, and from 35% (v/v) ACN, 0.1 % formic acid to 85 % (v/v) ACN, 0.1 % formic acid for 10 min. The column was re-equilibrated at initial conditions after washing for 30 min at 85% (v/v) ACN, 0.1 % formic acid at a flow rate of 300 nL·min⁻¹. For online LC-MS analysis, the nanoUPLC was coupled to the mass spectrometer through a nano-electrospray ionization (nanoESI) source emitter.

LC-Q-TOF-MS/MS Analysis (DDA)

Data Dependent Analysis (DDA) was performed on an SYNAPT G2-Si high definition mass spectrometer (Waters) equipped with a NanoLockSpray dual electrospray ion source (Waters). Precut fused silica PicoTipR Emitters for nanoelectrospray, outer diameters: 360 µm; inner diameter: 20 µm; 10 µm tip; 2.5" length (Waters) were used for samples and Precut fused silica TicoTip Emitters for nanoelectrospray (outer diameters: 360 µm, inner diameter: 20 µm, 2.5" length) (Waters) were used for the lock mass solution. The eluent was sprayed at a spray voltage of 2.8 kV with a sampling cone voltage of 25 V and a source offset of 30 V. The source temperature was set to 80°C. The cone gas flow was 20 liters·h⁻¹ with a nanoflow gas pressure of 0.4 bar, and the purge gas was turned off. The SYNAPT G2Si instrument was operated in DDA (data-dependent mode), automatically switching between MS and MS2. Full scan MS and MS2 spectra (m/z 400 - 2000) were acquired from 2 min after injection to 30 min in resolution mode (20,000 resolution FWHM at m/z 400) with a scan time of 0.1 sec. Tandem mass spectra of up to 10 precursors were generated in the trapping region of the ion mobility cell by using a collision energy ramp from 17/19 V (low mass, start/end) to up to 65/75 V (high mass,

start/end). Charged ions (+2, +3, +4) were selected to be submitted to the MSMS fragmentation over the m/z range from 50 to 2000 with a scan time of 0.25 sec. For the post-acquisition lock mass correction of the data in the MS method, the doubly charged monoisotopic ion of [Glu1]-fibrinopeptide B was used at $100 \text{ fmol} \cdot \mu\text{L}^{-1}$ using the reference sprayer of the nanoESI source with a frequency of 30 s at $0.5 \mu\text{L} \cdot \text{min}^{-1}$ into the mass spectrometer.

ESI-QTOF data processing

Data were processed with UNIFI (Waters) using the known PRDX5 sequence. Dimedone, carbamidomethylation, sulfenic acid, sulfinic acid, and sulfonic acid as the variable cysteine modifications, oxidation as the variable methionine modification, trypsin as the digestion enzyme were selected, and one miss cleavage was allowed.

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