UCLouvain

de Duve INSTITUTE

Gasdermin D-independent IL-1ß secretion by living human monocytic cells

Charlotte **S**ix

Novembre **2018**

Thèse présentée en vue de l'obtention du grade

de docteur en Sciences Biomédicales et Pharmaceutiques

Secteur des sciences de la santé

President Jury

Prof. Thomas Michiels
de Duve Institute
Université catholique de Louvain

Jury members

Prof. Stanislas Goriely
Université libre de Bruxelles

Prof. Sophie Lucas
de Duve Institute
Université catholique de Louvain

Prof. Stephan Constantinescu
Université catholique de Louvain
de Duve Institute
Ludwig Institute for Cancer Research

Dr. Didier Colau
Université catholique de Louvain
de Duve Institute
Ludwig Institute for Cancer Research

Supervisors

Prof. Pierre Coulie
Université catholique de Louvain
de Duve Institute

Dr. Tiphanie Gomard
Université catholique de Louvain
de Duve Institute

« None of us, acting alone,

can achieve the success. "

- *Nelson Mandela*

Je ne peux pas mettre un point final à cette thèse sans remercier de nombreuses personnes qui par leur présence, leurs conseils et leur soutien m'ont permis d'accomplir ce travail.

Je souhaite tout d'abord remercier mon promoteur, ***Pierre Coulie*** *pour ces 7 années passées au sien de votre équipe, d'abord en tant qu'étudiante et ensuite an tant que doctorante. Merci de m'avoir enseigné votre passion pour la recherche, vos connaissances, votre esprit critique et votre rigueur. Merci d'avoir eu confiance en moi et de m'avoir toujours encouragé, soutenu et poussé à aller encore plus loin !*

Je remercie également ma co-promotrice ***Tiphanie****, qui m'a guidée, conseillée et soutenue au jour le jour dans la réalisation de ce projet de thèse. Nos innombrables discussions scientifiques ont, sans aucun doute, permit d'aboutir à ce travail qui n'est que le préambule d'éminentes découvertes. Merci de m'avoir épaulée lorsque après 3 ans de thèse sur l'énigmatique GDF15, je changeais pour un tout autre sujet : l'IL-1ß. Tes encouragements et ton soutien dans les moments de doutes ont été précieux et m'ont donné la volonté d'avancer et de finaliser cette thèse en beauté.*

Je tiens également à remercier l'ensemble de mon ***jury de thèse*** *pour son soutien tout au long de mon parcours. Merci au professeur* ***S. Goriley*** *d'avoir accepté de participer à mon jury de thèse en tant que membre externe. Je remercie également le professeur* ***Emile Van Schaftingen*** *pour votre disponibilité et l'intérêt que vous portez au projet.*

Je remercie tous ceux sans qui cette thèse ne serait pas ce qu'elle est : aussi bien par les discussions que j'ai eu la chance d'avoir avec eux, leurs suggestions ou contributions. Tout d'abord, un tout grand merci à ***Nathalie****, ton enthousiasme, ta rigueur et ta volonté ont été essentiels à l'avancement de ce projet. J'ai été ravie de travailler avec toi ! Merci à* ***Catherine****, pour ta sincérité, tes blagues ;-), tes conseils et tes encouragements tout au long*

de ces années. Un grand merci pour toutes les commandes et la gestion des collections qui n'a pas toujours été de tout repos avec moi. Merci à ***Gérald*** *pour ton aide au FACS et bioplex. Merci à mon colloc de bureau,* ***Nicolas*** *pour toutes les formations FACS, les innombrables heures de tri. Merci à* ***Orian****, notre collaboration sur le projet GDF15 m'a beaucoup fait grandir scientifiquement. Ton esprit vif et inventif est un grand atout pour nous tous. Merci à* ***Kevin*** *pour ces apéros improvisés, tes encouragements à reprendre le sport me manquerons ! Merci également à* ***Alix*** *pour ces discussions (parfois) scientifiques le soir et les week-ends. Merci à* ***Marie-Sophie*** *pour ta bonne humeur contagieuse et les bons gâteaux de ta grand-mère. Un grand merci à* ***Suzanne*** *le(s) soumissions du papier et tous tes conseils pour la partie administrative. Je souhaite à Orian, Kevin, Alix et Walther une excellente suite et fin de thèse ! Bon courage et bonne chance !*

Je souhaite également remercier les différents groupes avec lesquels nous partageons le 3eme étage pour leur aide et leur amitié. Un merci particulier à ***Julie, Sara, Fanny, Rui, Délia, Joanna, Florence, Marie et Wenbin*** *pour les conversations scientifiques, les pauses café/lunchs à papoter. Merci aussi à toute l'équipe technique de l'Institut Ludwig qui nous permet de travailler dans des conditions idéales.*

Ensuite je voudrais remercier mes amis qui ont été présents et m'ont soutenue tout au long de ma thèse. D'abord les SBIMs : ***Lulu, Ed, Eli, Mati, Thom, Carole, Sara, Jona, Marc, Fanny, Marine, Eva, Sophie L.*** *et* ***Janne.*** *Merci pour les apéros les vendredi soir, les soupers/soirées improvisées, les soirées aux thermes sans oublier les inoubliables week-ends doctorants.*

Je remercie aussi ***Sandra, Fanny, Isa, Mélina*** *et* ***Nath.B*** *pour tous les moments passés au bord du bassin à Woluwe ou ailleurs. Merci pour votre soutien sans faille tant durant les compétitions d'eau libre et surtout durant ces derniers mois de thèse.*

*Merci à **Nath.D, Marie, Philou** et **Keninal.** Depuis notre rencontre sur les bancs de l'IPL, nous ne nous sommes plus quittés. Merci à **Marie, Olivia et Val** pour votre soutien.*

*Finalement, je remercie toute ma famille d'avoir été à mes côtés pendant toutes ces années ! Mes **parents** qui m'ont offert la possibilité d'arriver là aujourd'hui et qui m'ont soutenue dans les moments joyeux ou difficiles ! Merci à mes **sœurs** et les **beaufs** d'être là pour moi ! Ne changez surtout pas ! Merci à **Robin** et **Charly**, vous avez été ma bouffée d'oxygène pendant ces années !*

Table of content

Table of illustrations **13**

Abbreviations **14**

Summary **19**

Introduction **21**

1. Inflammation **22**

1.1 Definition 22

1.2 Sensors triggering inflammation 23

1.2.1 Toll-like receptors (TLRs) 24

1.2.2 Nod-like receptors (NLRs) 26

2. Interleukin-1 beta (IL-1ß) **29**

2.1 IL-1 discovery 29

2.2 IL-1ß processing 32

2.2.1 IL-1ß expression 32

2.2.2 IL-1ß maturation 35

2.2.2.1 Caspase-1 36

2.2.2.2 Inflammasomes 39

2.3 IL-1 receptors 48

2.4 Majors effects of IL-1ß 53

2.4.1 Pro-inflammatory cytokine 53

2.4.2 Pyrogenic properties 54

2.4.3 Lymphocyte Activating Factor (LAP) 54

2.4.4 B cell growth factor 55

2.4.5 Leukocytic endogenous mediator (LEM) 55

2.4.6 Mononuclear cell factor (MCF) 56

2.5 Blocking IL-1ß activity 57

2.5.1 IL-1ß antagonists 60

2.5.1.1 Anakinra (Kineret®) 60

2.5.1.2 Rilonacept (Arcalyst®) 60

2.5.1.3 Canakinumab (Ilaris®) 61

2.5.2 Inflammasome and caspase-1 inhibitors 62

3. Unconventional secretion of leaderless proteins **64**

3.1 FGF2 unconventional secretion (Type I UPS) 66

3.2 IL-1ß unconventional secretion 68

3.2.1 Secretory lysosomes, microvesicles, and exosomes 70

3.2.1.1 Secretory Lysosomes 70

3.2.1.2 Microvesicles 70

3.2.1.3 Exosomes 71

3.2.2 Secretory autophagy 71

3.2.3 Secretion across plasma membrane 72

3.2.4 Cell death 72

Aim of the study 77

Results 79

Discussion 119

1. **IL-1ß secretion and cell death in monocytes as compared to other cells** 122
2. **GSDMD-independent IL-1ß secretion** 123
3. **How many IL-1ß secretion pathways?** 125

References 131

Table of illustrations

Figure 1. Classification and protein structure of human NOD-like receptor family.

Figure 2. LPS/TLR4 signalling pathway.

Figure 3. Overview of proteolytic enzymes known to be able to process pro-IL-1ß into a biologically active form.

Figure 4. Domain structure of human caspases.

Figure 5. Inflammasome sensors and activators.

Figure 6. Canonical NLRP3 inflammasome activation.

Figure 7. Non-canonical NLRP3 inflammasome activation.

Figure 8. Alternative NLRP3 inflammasome activation

Figure 9. The formation of the MyD88/IRAK/TRAF6 signalosome in IL-1 transduction signal.

Figure 10. The IL-1 receptor complexes.

Figure 11. Regulation of IL-1ß production and current IL-1 antagonists.

Figure 12. Four types of unconventional protein secretion (UPS).

Figure 13. Unconventional secretion mechanism of FGF2.

Figure 14. Proposed mechanisms underlying the unconventional secretion of IL-1ß.

Figure 15. GSDMD executes inflammatory caspases-induced pyroptosis.

Abbreviations

AIM2: Absent **i**n **M**elanoma **2**

ASC: Apoptosis-associated Speck-like protein containing a **C**ARD

ATG: Autophagy related

ATP: Adénosine **tri**phosphate

APAF-1: Apoptotic **P**rotease **A**ctivating **F**actor **1**

BHB: Beta-**H**ydroxy**B**utyrate

BIR: Baculovirus **I**nhibitor of apoptosis protein **R**epeat

BSF-2: B-cell differentiation **F**actor**-2**

CARD: Caspase **A**ctivation and **R**ecruitment **D**omain

CAPS Cryopyrin **A**ssociated **P**eriodic **S**yndromes

CD: Custer **D**ifferentiation

CIITA: MH**C** class **II T**ranscription **A**ctivator

CLR: C-type lectin **L**ike **R**eceptor

COX-2: Cyclo **OX**ygénase**-2**

CRH: Corticotrophin **R**eleasing **H**ormone

CTNF: Ciliary **N**eurotrophic **F**actor

DAMP: Damage **A**ssociated **M**olecular **P**attern

DED: Death **E**ffector **D**omain

DHT: Di**H**ydro**T**estosterone

DNA: Deoxyribo**N**ucleic **A**cid

ELAM-1: Endothelial **L**eukocyte **A**dhesion **M**olecule **1**

ER: Endoplasmic **R**eticulum

FADD: Fas-**A**ssociated protein with **D**eath **D**omain

FCAS: **F**amilial **C**old **A**uto-inflammatory **S**yndrome

FDA: Food and **D**rug **A**dministration

FGF: Fibroblast **G**rowth **F**actor

FIIND: Function to find **D**omain

FMF: Familial **M**editerranean **F**ever

GM-CSF: Granulocyte-**M**acrophage **C**olony-**S**timulating **F**actor

HIDS: Hyper**I**mmunoglobulinemia **D** with periodic fever **S**yndrome

HIN200: Hematopoietic **I**nterferon-inducible **N**uclear proteins with a **200**-amino-acid repeat

HME-2: Ho**M**eobox protein **E**ngrailed-**2**

HMGB-1: High–**M**obility **G**roup **B**ox **1**

ICAM-1: Inter **C**ellular **A**dhesion **M**olecule-**1**

ICE: IL-1ß Converting **E**nzyme

IFI-16: Inter**f**eron-**I**nducible protein **16**

IKK: IκB Kinase

IL: Interleukin

IL1-R: Interleukin **1 R**eceptor

IL1-RaP: Interleukin **1 R**eceptor **a**ccessory **P**rotein

INF: Interferon

IRF: Interferon **R**egulatory **F**actor

IRS-1: Insulin **R**eceptor **S**ubstrate **1**

IRAK: Interleukin-1 **R**eceptor-**A**ssociated **K**inase

JNK: c-Jun **N**-terminal **K**inases

LAF: Lymphocyte **A**ctivating **F**actor

LBP: Lipopolysaccharide **B**inding **P**rotein

LEM: Leucocytic **E**ndogenous **M**ediator

LFA-1: Lymphocyte **F**unction-associated **A**ntigen-**1**

LP: Leucocyte **P**yrogen

LPS: Lipo**p**oly**s**accharide
LRR: Leucin **R**ich **R**epeats

MAPK: Mitogen-**A**ctivated **P**rotein **K**inases
MCF: Mononuclear **C**ell **F**actor
MCH II: Major **H**istocompatibility **C**omplex
MCP: Monocyte **C**hemoattractant **P**rotein
MD2 (LY96): Lymphocyte antigen 96

MDK: Mevalonate **D**eficient **K**inase
MIF: Macrophage **I**nhibitory **F**actor
MKP-1: Mitogen-activated **P**rotein **K**inase
MyD88: Myeloid **D**ifferentiation primary response **88**
MWS: Muckle-**W**ells **S**yndrome

NAIP: NLR family **A**poptosis **I**nhibitory **P**rotein
NLR: Nod **L**ike **R**eceptor
NO: Nitric **O**xide
NOD: Nucleotide **O**ligomerization **D**omain

OAF: Osteoclast **A**ctivating **F**actor
OVLT: Organum **V**asculosum of the **L**amina **T**erminalis
OX40 (TNFRSF4): Tumor **N**ecrosis **F**actor **R**eceptor **S**uper**F**amily member **4**

P2RX7: P2X Purinoceptor **7**
PAMP: Pathogen **A**ssociated **M**olecular **P**attern
PAPA: Pyogenic **A**rthritis, **P**yoderma gangrenosum and Acne
PCA: Pro**Co**agulant **A**ctivity
PGE2: Prosta**g**landin **E2**
PMA: Phorbol **M**yristate **A**cetate
PYR: Pyrin **D**omain
PSTP1P1: Proline-**S**erine-**T**hreonine **P**hosphatase interacting **P**rotein

RA: Rheumatoid **A**rthritis

RIG-1: Retinoic acid **I**nducible **G**ene **I**

RIPK1: Receptor-**I**nteracting serine/threonine-**P**rotein **K**inase **1**

RNA: Ribo **N**ucleic **A**cid

RLR: Retinoic acid-inducible I- **L**ike **R**eceptor

ROS: Reactive **O**xygen **S**pecies

T2DM: Type **2 D**iabetes **M**ellitus

TACE: TNF-Converting **E**nzyme

TANK: TRAF family member-**A**ssociated **NF-k**appa-**B** activator

TBK1: TANK-**B**inding **K**inase **1**

TIR: Toll **I**nterleukin **R**eceptor

TIRAP: Toll-**I**nterleukin **1 R**eceptor domain containing **A**daptor **P**rotein

TNFa: Tumor **N**ecrosis **F**actor **alpha**

TNF-R: Tumor **N**ecrosis **F**actor **R**eceptor

TLR: Toll **L**ike **R**eceptor

TRAF: TNF receptor **A**ssociated **F**actor

TRAM: Translocating chain-**A**ssociated **M**embrane

TRAPS: Tumor necrosis factor **R**eceptor **A**ssociated auto-inflammatory **S**yndrome

TRIF: TIR-domain-containing adapter-**I**nducing inter**f**eron-β

UPS: Unconventional **P**rotein **S**ecretion

Summary

Interleukin-1β (IL-1β), a major pro-inflammatory cytokine, is a leaderless cytosolic protein whose secretion does not follow the classical endoplasmic reticulum-to-Golgi pathway, and for which the mechanism of secretion remains to be established.

In murine macrophages, IL-1ß is known to be released through pyroptosis, a highly inflammatory cell death, mediated by gasdermin D (GSDMD) pores at the plasma membrane. Whether gasdermin D is dispensable for IL-1ß secretion in human cells was still unknown.

Here we show that human blood monocytes stimulated with LPS alone secrete mature IL-1ß without cell death and without gasdermin D cleavage, while they undergo pyroptosis with gasdermin D cleavage when stimulated with LPS and an activator of the NLRP3 inflammasome. In addition, we show that LPS-stimulated human monocytic THP-1 cells secrete IL-1ß, even after invalidation of the gasdermin D-encoding gene, pointing out to the existence of a pathway of IL-1ß secretion that is cell death- and gasdermin D-independent.

Introduction

1. Inflammation

1.1 Definition

Inflammation is a biological response of the immune system to ensure removal of harmful stimuli (Medzhitov, 2008). It is caused by several factors including pathogens, damaged cells, toxic compounds, or irradiation. At the tissue level, inflammation is characterized by five symptoms: redness, swelling, heat, pain, and loss of tissue function, which result from local immune, vascular and inflammatory cell responses to infection or injury (Takeuchi and Akira, 2010).

The innate immune system is the first line of response against invasive microbes, mediated by local and systemic inflammation. The immune system plays a crucial role in the early recognition of invading pathogens by triggering pro-inflammatory responses (Medzhitov, 2008).

1.2 Sensors triggering inflammation

The innate immune response is based on recognition of evolutionarily conserved structures on pathogens called pathogen-associated molecular patterns (PAMPs), through germ line-encoded pattern recognition receptors (PRRs) (Lemaitre et al., 1996). Additionally, in certain conditions, PRRs can also recognize host factors as "danger signals", termed as damage-associated molecular patterns (DAMPs). PAMPs are compounds exclusively expressed in pathogens and essential to their survival whereas DAMPs are host-related released compounds, including HMGB1 (High Mobility Group Box 1) or ATP, molecules released as a consequence of infection, inflammation, or other types of cellular stress (Takeuchi and Akira, 2010).

Currently, four classes of PRRs families have been identified. It includes the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). Each member being distinguished by its specific ligands, its cellular localization and the signalling pathway that it activates.

With the exception of some NLRs, the sensing of PAMPs or DAMPs by PRRs upregulates the transcription of genes involved in inflammatory responses, including pro-inflammatory cytokines (Takeuchi and Akira, 2010).

CLRs recognize mannose, fructose, and glucan carbohydrate structures present in bacterial, viral, and fungal components while RLRs recognize viral RNA in the cytoplasm, activating intrinsic anti-viral signalling pathways.

TLRs and NLRs are described in more details in the following sections.

1.2.1 Toll-like receptors (TLRs)

The Toll-like receptor family is one of the best-characterized PRR families (Akira et al., 2006). Toll was originally identified as a Drosophila gene required for ontogenesis and anti-microbial resistance (Anderson et al., 1985). In 1991, the homology between the cytosolic domain of Drosophila Toll and the IL-1R1 introduced the idea that toll might not only regulate developmental processes but also immune gene expression (Gay and Keith, 1991). It is only in 1997, that the first human homologue of toll receptor was cloned and that its implication in inflammatory responses was assessed (Medzhitov et al., 1997).

In mammalian species thirteen TLR have been identified. Humans express ten functional TLRs, whereas twelve TLRs have been identified in mice. Ligands of all TLRs have been identified except for human TLR10, mouse TLR12 and mouse TLR13 (Lee et al., 2012). TLR1, TLR2, TLR4, TLR5 and TLR6 are located at the plasma membrane and recognize molecular components at the surface of pathogens whereas TLR3, TLR7, TLR8 and TLR9 are found in endosomes, where they mediate recognition of nucleic acids (Akira et al., 2006).

TLRs are members of the Toll/interleukin-1 (IL-1) receptor (TIR) family. They are type I transmembrane proteins characterized by N-terminal leucine-rich repeats (LRRs), a transmembrane region and a cytoplasmic Toll/IL-1R homology (TIR) domain involved in recruitment of signalling adaptor molecules (O'Neill, 2000).

Most of TLRs form homodimers as a means of triggering signal. However, heterodimers have also been identified enabling differential recognition of lipopeptides. TLR2 forms heterodimers with TLR1 or TLR6, which enables recognition of triacyl lipopeptides or diacyl lipopeptides respectively (Lee et al., 2012).

Following ligand engagement, the cytoplasmic TIR domains of the TLRs recruit different signalling adaptors including MyD88, TIRAP, TRAM, and/or TRIF. Depending on the nature of the adaptor that is recruited, various kinases (IRAK4, IRAK1, IRAK2, TBK1, IKKε) and ubiquitin ligases (TRAF6 and pellino-1) are enrolled and activated. The activation of these proteins leads to the activation of NF-κB, type I interferon, p38 MAP kinase (MAPK), and JNK MAPK pathways that promote inflammatory cytokine production. Recognition of LPS by TLR4 is detailed in section 2.2.1.

1.2.2 Nod-like receptors (NLRs)

NLRs are a class of cytosolic PRRs. They are found in lymphocytes, macrophages, dendritic cells and also in non-immune cells, such as epithelial cells (Reinholz et al., 2013). They are highly conserved through evolution. Homologs have been discovered in many different animal species and also in plants demonstrating their primordial role in the host defense (Jacob et al., 2013; Martinon and Tschopp, 2005).

NLRs are multi-domain proteins composed of (Fig. 1)

- An invariant central domain: Nucleotide-binding and oligomerization (NACHT)
- A C-terminal domain: Leucine-Rich Repeat (LRR) responsible for the detection of PAMP and DAMP (expect NRLP10)
- A N-terminal domain responsible for homotypic protein-protein interaction.

There are currently more than 20 NLRs, classified into five sub-families based on their N-terminal domain: NLR**A** for Acidic trans activating domain, NLR**B** for Baculovirus Inhibitor of Apoptosis Protein Repeat (BIRs), NLR**C** for Caspase Activation and Recruitment Domain (CARD) and NLR**P** for Pyrin Domain (PYR) (Fig. 1) (Ting et al., 2008). An additional subfamily, NLR**X**, characterized by the presence of a N-terminal domain without significant homology to any known domains and by its localization to the mitochondria, has been also reported (Tattoli et al., 2008).

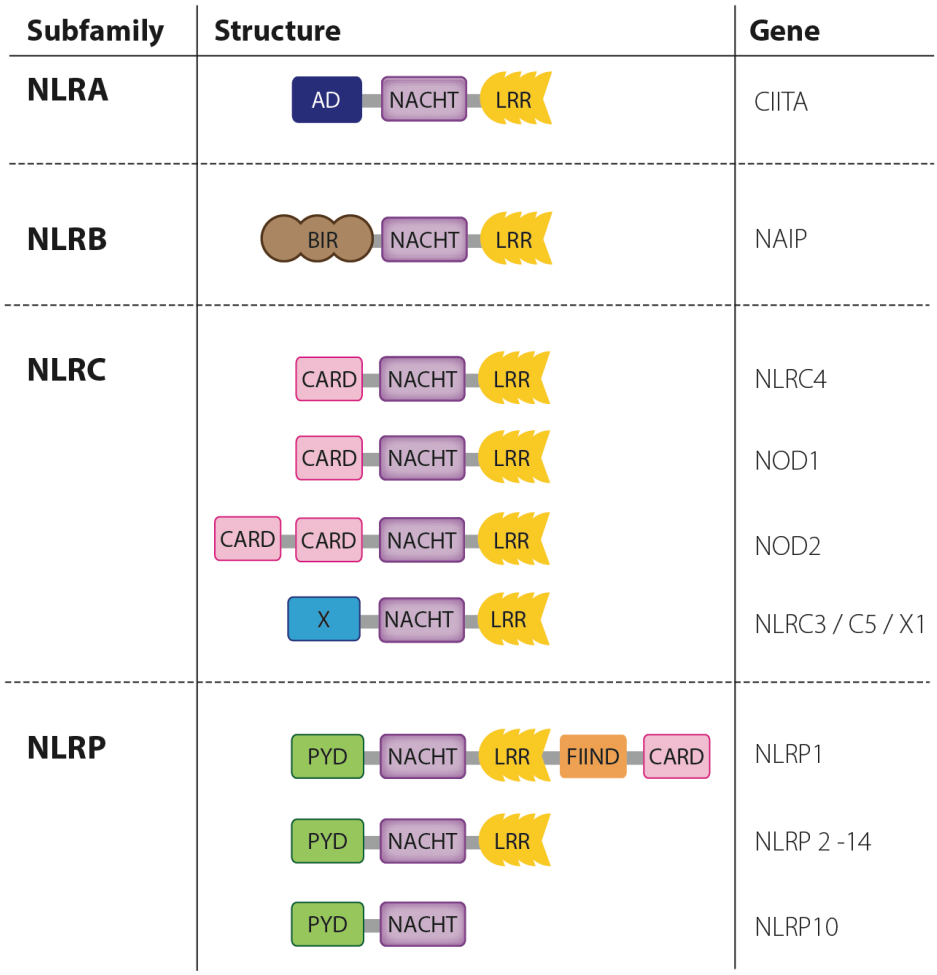


Figure 1. Classification and protein structure of human NOD-like receptor family (NLR). Adapted from "The NLR gene family: An official nomenclature" – Ward – 2008 - Immunity. AD, acidic transactivation domain; NACHT, for NAIP, CIITA, HET-T, and TP-1; BIR, baculovirus inhibitor of apoptosis repeat; CARD, caspase activation and recruitment domain; X, unidentified; PYD, pyrin domain, FIIND, function to find domain, leucine-rich repeat; NOD, nucleotide-binding and oligomerization domain.

NLRs are activated by bacterial, fungal, or viral molecules that contain PAMPs or by DAMPs released by damaged cells. NOD1 and NOD2 are two well-characterized NLRs which upon activation, homo-oligomerize and recruit signalling molecules that drive the NF-kappa B-/AP-1-dependent expression of pro-inflammatory cytokines. In addition, they stimulate the IRF3-/IRF7-dependent expression of type I interferons.

Some NLRs, including NLRP1, NLRP3, and NLRC4 respond to infections by assembling large cytosolic protein complexes in which inflammatory caspases undergo autocatalytic activation (Fig.6). These complexes, known as inflammasomes have been described for the first time by Tschoop and colleagues in 2002 (Martinon et al., 2002), as an analogy to the "apoptosome" which was already known to drive caspase-9 activation via Apaf-1 during apoptosis (Green, 1998; Zou et al., 1999).

One of the best-characterized consequence of inflammasome activation is the secretion of the pro-inflammatory cytokines such as interleukin (IL)-1β though the activation of the NLRP3/caspase-1 complex.

2. Interleukin-1 beta (IL-1ß)

2.1 IL-1 discovery

The history of IL-1 began in the 19th century with the concept that fever is associated with leukocytic infiltrates. It is only in the mid-forties that Elisha Atkins reported the existence of an endotoxin-free protein released by activated cells that cause fever (Beeson, 1948). Because most of these cells were leukocytes, the pyrogenic molecule was originally called leukocytic pyrogen (LP). Initially, little was known about the chemical nature of LP except that it was heat-labile (destroyed at 90°C), and when injected into the peripheral circulation, it acted on the hypothalamus to induce fever (Hadley et al., 1966).

It is only in 1974 that Murphy and Wood first purified LP from rabbit acute peritoneal exudates (Murphy et al., 1974). They reported the existence of two distinct molecules with pyrogenic properties characterized with the same molecular weight (15,000 Da) but with different isoelectric points, one acidic and one neutral. The same year, Dinarello and colleagues confirmed the previous observation by purifying also two LPs, the first from blood circulating monocytes and the second from blood circulating neutrophils (Dinarello et al., 1974). In addition, their purification revealed that whereas LP from activated neutrophils was constitutively synthesized *in vivo*, LP from monocytes required *in vitro* stimulation. They also discovered the existence of another form of human monocyte–derived LP with a molecular weight of 38,000. (Dinarello et al., 1974). Although there was no clear explanation for this molecular heterogeneity, these findings introduced the concept that there was more than one chemically distinct LP and that the understanding of LP complexity was at its very beginning.

Few years later, several groups reported the similarity of LP with the leukocytic endogenous mediator (LEM) (Merriman et al., 1977) and with the lymphocyte-activating factor (LAF) (Dinarello et al., 1977). Indeed, purification of LP from *in vitro* staphylococci stimulated monocytes revealed its leukocytic endogenous mediator (Kampschmidt and Upchurch, 1969) effect and its lymphocyte activating (Gery et al., 1972) biological activities, indicating that LP had other biologic properties than its ability to induce fever.

The discovery of these three distinct biological activities marked the birth of ‘‘cytokine biology,’’ with LP being the first cytokine to be described. During the second International Lymphokine meeting in 1979, the term interleukin-1 (IL-1) was introduced gathering all the biological activities previously reported for LP, although at least four different pI values and several molecular weights were already observed for LP. The term IL-1 included the originally described leucocytic pyrogen (LP) also known as endogenous pyrogen (EP), leukocytic endogenous mediator (LEM), and lymphocyte-activating factor (LAF), the mononuclear cell factor (MCF) but also the osteoclast-activating factor (OAF), and the hemopoietin-1.

It was not until the cloning of mouse IL-1 and human IL-1 in 1984 that the molecular heterogeneity was solved identifying the previous reported LP acid form as IL-1alpha (IL-1α) and the LP neutral form as IL-1beta (IL-1β) (Lomedico et al., 1984; March et al., 1985). It also revealed that the product of the IL-1ß gene was a polypeptide with a predicted mass of 31kDa, much higher than the cytokine initially purified from the blood (17 kDa). Eventually, it revealed the lack of an N-terminal signal sequence of the polypeptide. Nowadays, it is commonly accepted that the term interleukin-1 refers to two distinct proteins and not only one as it was for a long time considered. Early researches on the molecular properties of these two

cytokines revealed numerous similarities that appear to justify naming as two forms of IL-1: IL-1α and IL-1β. They are both synthetized as precursors of 31kDa, cleaved to smaller and mature forms of 17kDa which bind cell surface receptor, triggering identical biological responses (March et al., 1985). Interestingly, IL-1ß but not IL-1α deficient mice has been shown to have impaired febrile and acute-phase inflammatory responses in a turpentine-induced model of local inflammation and tissue injury, while displaying normal susceptibility to LPS challenge (Horai et al., 1998; Zheng et al., 2013).

Despites these similarities, IL-1α and IL-1ß have different amino acid sequences with only 26% identity and different processing and bioavailability. First, whereas only the mature IL-1ß is a functional cytokine, both forms of IL-1α (pro-IL-1α and mature IL-1α) are biologically active. Second, although IL-1α is biologically active as a secreted, a membrane bound and a nuclear cytokine, only secreted mature IL-1ß is functionally active (Fettelschoss et al., 2011). Third, although, IL-1ß is expressed upon cell activation in hematopoietic cells, IL-1α is constitutively expressed, in hematopoietic and non-hematopoietic cells. And finally, IL-1α is considered as an alarmin, released upon cell death whereas IL-1ß is believed to be secreted from activated cells after cleavage of its amino-terminal region by caspase-1.

Although the original IL-1 family included only IL-1α and IL-1β, it has expanded considerably since 1985. To date, there are eleven members, including 7 molecules with agonist (pro-inflammatory) activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, β, and γ), three receptor antagonists (IL-1Ra, IL-36Ra and IL-38), and an anti-inflammatory cytokine (IL-37). All of these members are synthetized without signal peptide except the IL1-Ra.

2.2 IL-1ß processing

Three major steps are involved in the release of mature IL-1β: (i) production of the biologically inactive pro-IL-1β, (ii) cleavage of pro-IL-1β by caspase-1 resulting in the production of the mature and biologically active IL-1β, and (iii) secretion of mature IL-1β.

2.2.1 IL-1ß expression

The *IL1B* gene, which encodes for pro-IL-1β precursor, is silent at homeostasis but is rapidly transcribed upon cell stimulation. The best-characterized stimulus that triggers IL-1β production in myeloid cells is the lipopolysaccharide (LPS), which binds to surface TLR-4, and activates the NFκB signalling pathway.

LPS is an important structural component of the outer membrane of Gram-negative bacteria composed of three parts: the lipid A, a core oligosaccharide, and an O side chain (Fig. 2A).

In mammalian cells, LPS is mainly recognized by TLR4, assisted by two accessory proteins: MD-2 and CD14 molecules (Fig. 2B). Additionally, the LPS binding protein (LBP) has been reported to facilitate LPS transfer on CD14-TLR4-MD2 complexes (Schumann, 1992). MD-2 is a soluble protein which non-covalently associates with TLR4 for LPS recognition. CD14 is a glycosylphosphatidylinositol-anchored protein, which facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and modulates its recognition. The crystal structure of a complex comprising TLR4, MD2, and LPS revealed that two complexes of TLR4-MD2-LPS interact symmetrically to form a homodimer and induce signalling (Park et al., 2009)

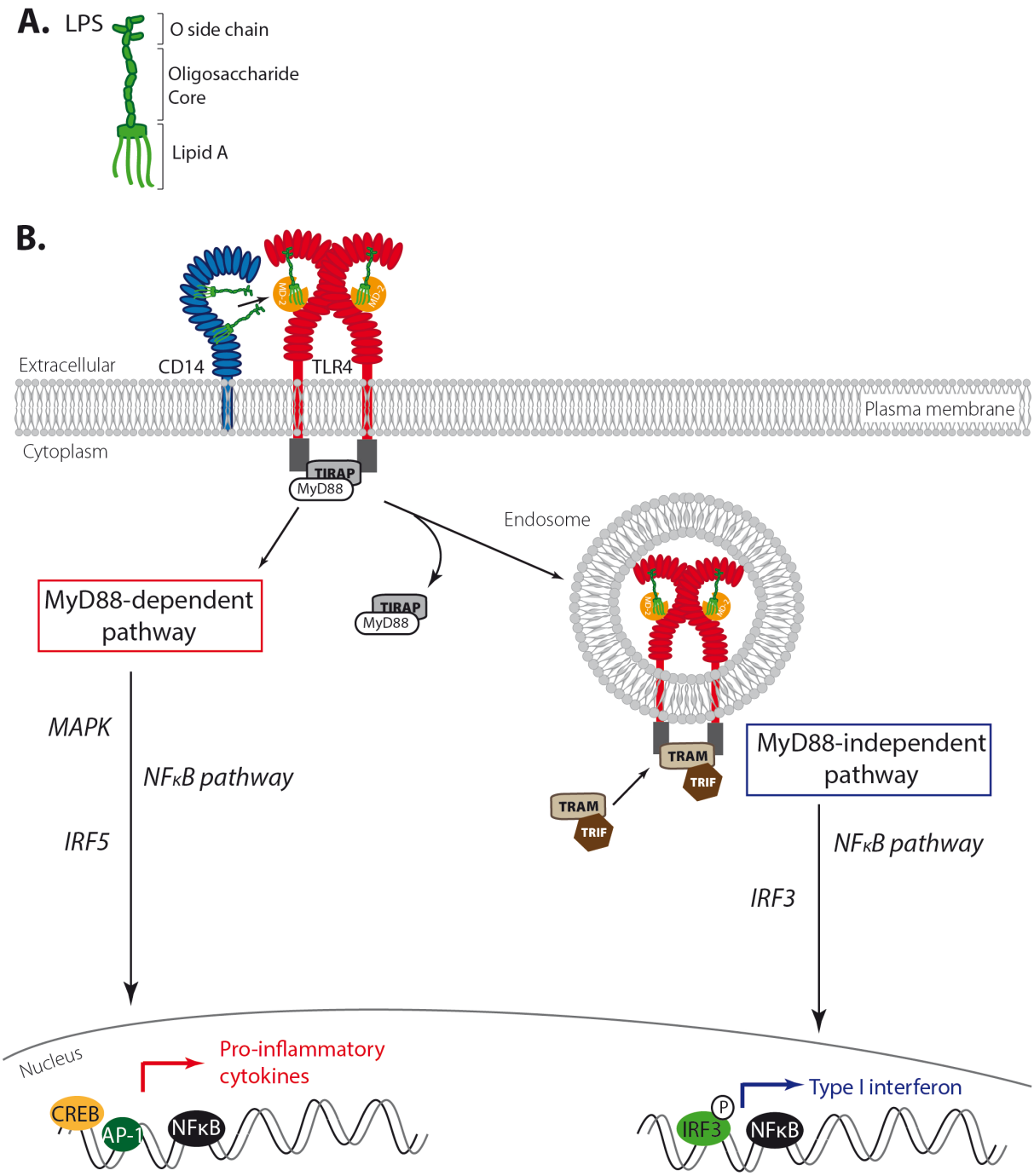


Figure 2. LPS/TLR4 signalling pathway. Adapted from "LPS/TLR4 signal transduction pathway" Ohashi – 2008 - Cytokine A. LPS composition **B.** LPS recognition is facilitated by CD14, and is mediated by TLR4/MD-2 receptor complex. LPS/ TLR4 signalling can be separated into MyD88-dependent (plasma membrane TLR4) and MyD88-independent (endosome TLR4) pathways, which mediate the activation of pro-inflammatory cytokine and type I interferon genes.

Upon LPS binding, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with its TIR domains. TLR4 is known to induce two independent signalling pathways that are regulated by the TIRAP-MyD88 and TRAM-TRIF adaptor pairs (Fig. 2B). The TIRAP-MyD88–dependent signaling pathway induces the activation of serine-threonine kinases such as p38 and IKKβ, whereas the TRAM-TRIF pathway induces the activation of interferon-regulatory factors such as IRF3 (Fig. 2B) (Kagan et al., 2008; Lu et al., 2008).

For long time, it was believed that the two signalling pathways were induced simultaneously from the plasma membrane. In 2008, Kagan *et al.* made a side-by-side comparison of the activity of these two pathways and found that whereas the TIRAP-MyD88-dendepend signalling happened at the plasma membrane, the TRAM-TRIF–dependent signalling occurred in endosomes, after LPS-induced TLR4 endocytosis (Kagan et al., 2008). They and others suggested that the switch between the two pathways may be caused by depletion of phosphatidylinositol-4,5-bisphosphate from the membrane during TLR4 endocytosis, which causes the release of the TIRAP-MyD88 complex, thereby enabling TRAM–TRIF complex to interact with TLR4 in endosomes (Botelho et al., 2000; Murphy et al., 2009).

2.2.2 IL-1ß maturation

Studies revealed that full length pro-IL-1ß lacked biologic activity (Mosley et al., 1987) and needed to be converted into the mature 17-kDa form to become biologically active. Subsequently, a lot of work was devoted to the identification of the enzyme that cleaves the IL-1ß precursor into the active cytokine. In 1992, caspase-1 was identified as the main IL-1ß converting enzyme (ICE) (Cerretti et al., 1992) processing IL-1ß at two distinct sites (Fig. 3), producing a minor 26-kDa product, the function of which is unknown, and a mature 17-kDa form corresponding to IL-1ß biologically active form (Fig.3). Both cleavage have been shown to occur after an aspartic acid (D) usually followed by a small amino acid such as glycine (G), serine (S) or alanine (A) (Agard et al., 2010).

Caspase-8 has also been reported to directly cleave pro–IL-1β in response to TLR3 or TLR4 stimulation (Maelfait et al., 2008). Moreover, fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* have been shown to activate capsase-8 which cleaves pro–IL-1β at the same sites as recombinant caspase-1 and produce similar mature IL-1β (Fig. 3) (Gringhuis et al., 2012).

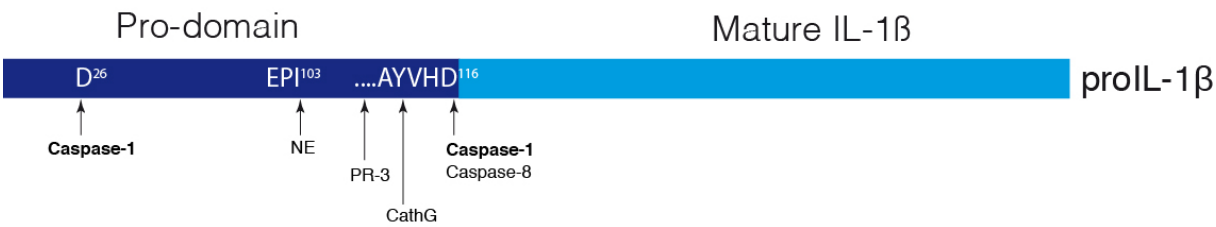


Figure 3. Overview of proteolytic enzymes known to be able to process pro-IL-1ß into a biologically active form. Adapted from "Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme" – Beyaert - 2015– Immunity review. Schematic representation of proIL-1ß showing the cleavage sites of different proteases (NE, neutrophil elastase; CathG, cathepsin G; PR-3, proteinase-3). Numbering is according to the primary amino acid sequence of the human IL-1ß protein. D: Acide aspartique; E: Acide glutamique ; P : Proline ; I : Isoleucine ; A: Alanine; Y: tyrosine; V: Valine, H: Threonine.

Additionally, *in vitro* experiments have identified other proteases able to cleave extracellular pro-IL-1ß (Afonina et al., 2015). It includes chymotrypsin-like serine protease chymase derived from mast cells (Mizutani et al., 1991), neutrophil elastase, cathepsin G (Hazuda et al., 1990) and proteinase-3 (PR-3) (Coeshott et al., 1999) which process IL-1ß just a few amino acids upstream of the caspase-1 cleavage site (Fig. 3). The proteolysis of pro-IL1-ß at these sites generates a mature cytokine that is biologically active but with much weaker activity as compared to the one resulted from caspase-1 cleavage (Black et al., 1988; Hazuda et al., 1990).

The following section will focus on the pro-IL-1ß maturation by caspase-1.

2.2.2.1 Caspase-1

Caspases for (Cysteine protease after aspartic acid residues) are a family of 12 proteases playing essential roles in programmed cell death and inflammation (Alnemri et al., 1996). They were originally identified for their role in apoptosis (Thornberry, 1997) but are now identified to also play roles in pyroptosis, necroptosis and inflammation.

Caspases are divided in two main groups, the inflammatory caspases including the caspase-1, 4 and 5 and the apoptotic caspases which is subcategorized into initiator caspases (2, 8, 9 and 10) and executioner caspases (3, 6 and 7) (Pop and Salvesen, 2009). They are expressed as inactive precursors (pro-caspase) with an amino terminal pro-domain containing either death effector domain (DED) or caspase activation and recruitment domain (CARD) domain followed by a catalytic domain, composed of large (p20) and small (p10) subunits (McIlwain et al., 2015) (Fig. 4A).

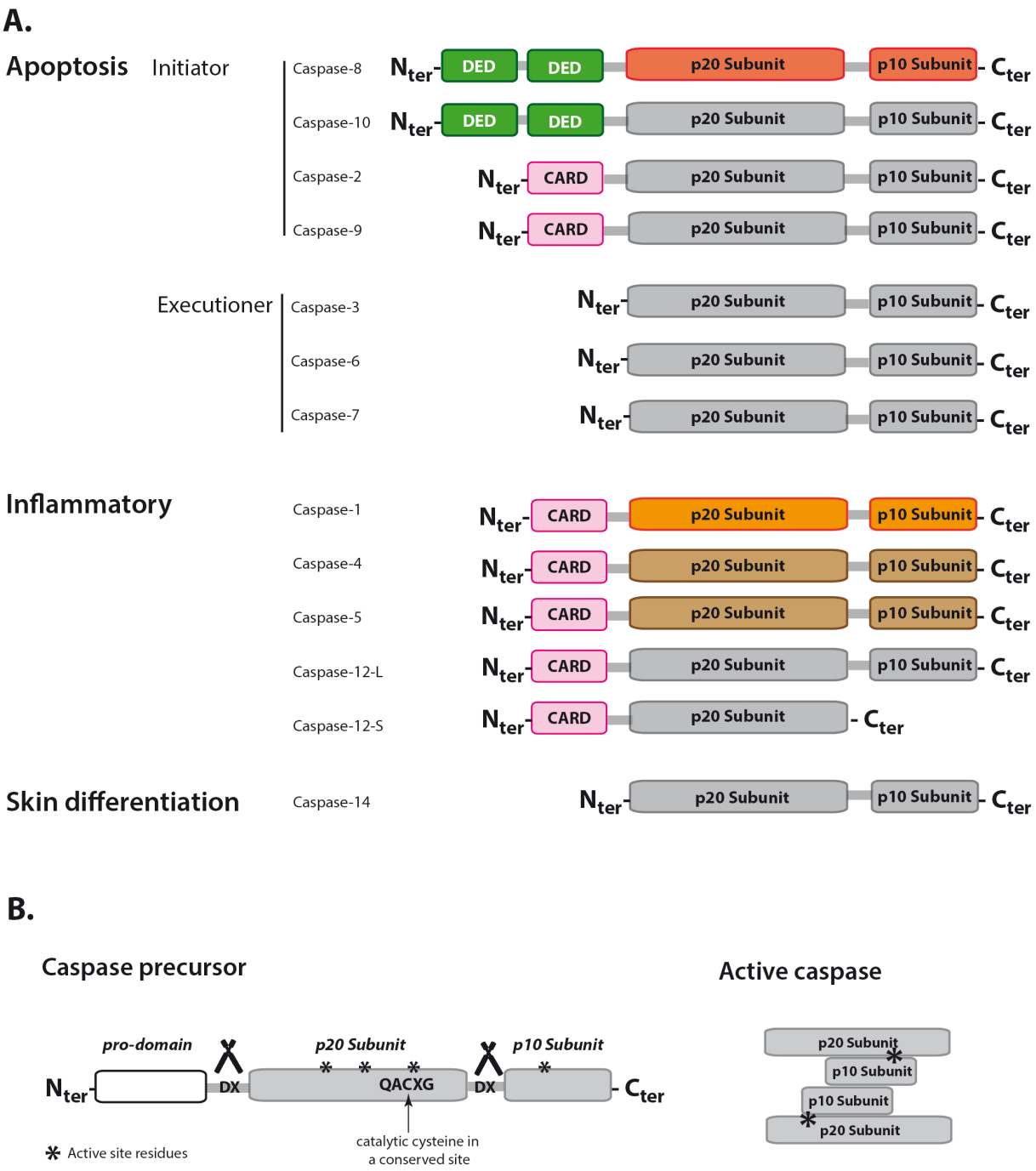


Figure 4. Domain structure of human caspases. Adapted from "Caspase Functions in Cell Death and Disease" – Mark – 2013 - Cold Spring Harbor Perspectives in Biology. (A). Classification and schematic representation of structural features of mammalian caspases. **(B).** Caspases are synthesized as inactive pro-enzymes with an N-terminal prodomain, a large (p20) subunit and small (p10) subunit. The prodomain and the p20 and p10 subunits are separated by a linker peptide that is proteolytically cleaved, yielding an active caspase tetramer constituted of two p20 and two p10 subunits. DED: Death Effector Domain; CARD: Caspase Activation and recruitment Domain, D: Acide aspartique; Q: Glutamine; A: Alanine; C: cysteine; G: Glycine, X: any amino acid.

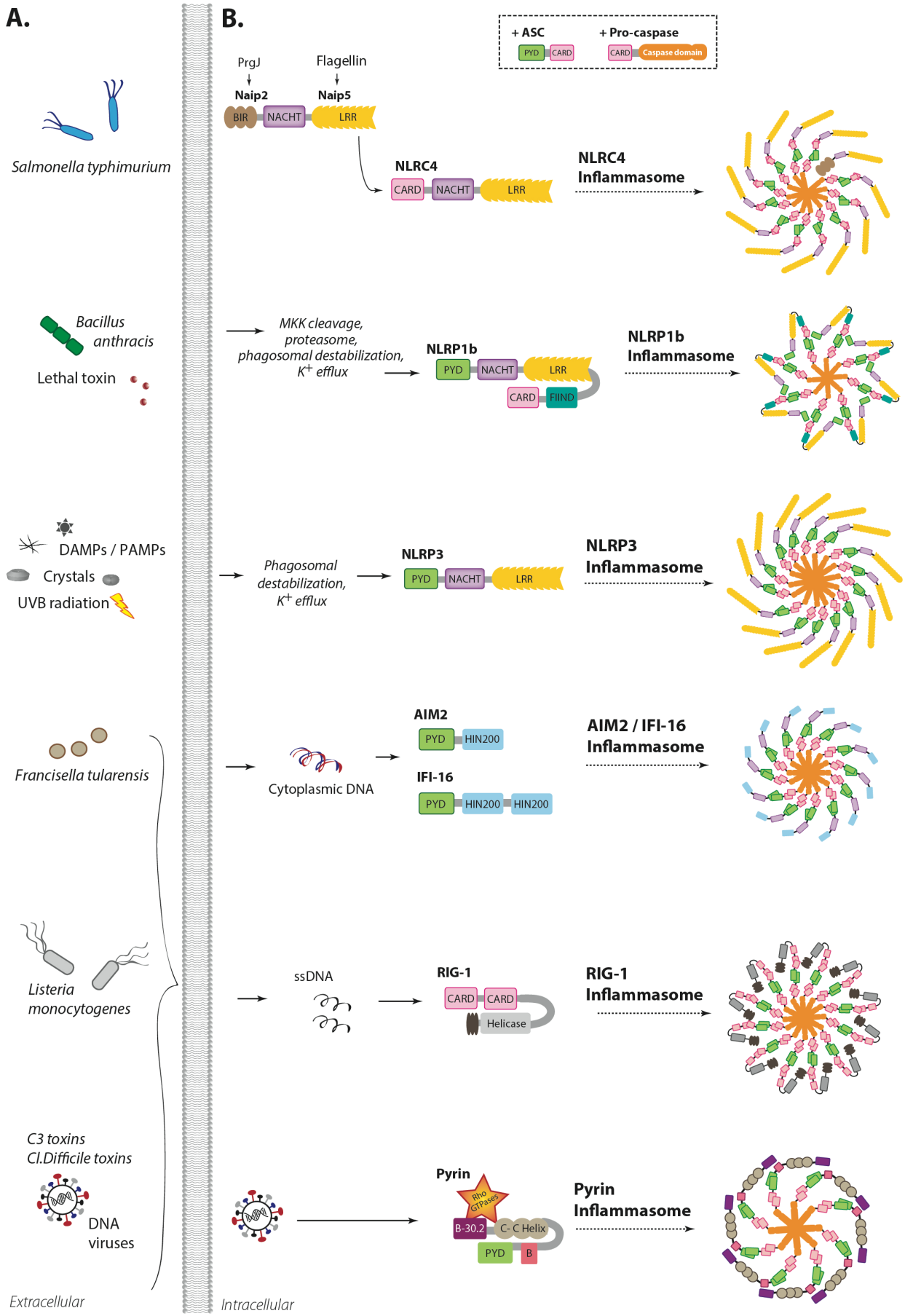
Caspase-1 is the best-characterized inflammatory caspase, originally identified as the main protease responsible for the processing of pro-IL-1ß (Thornberry et al., 1992). Today, capsase-1 is also known to be the key activator of other IL-1 family cytokines such as IL-18 and IL-37 (Lamkanfi and Dixit, 2012) but also in gasdermin D cleavage, the effector molecule of the pyroptotic cell death (Kayagaki et al., 2015). Mice lacking caspase-1 are reported to be highly resistant to lipopolysaccharide (LPS)-induced endotoxic shock and to have decreased serum amounts of IL-1ß (Kuida et al., 1995; Li et al., 1995) indicating that caspase-1 is an essential protease in IL-1ß processing.

Similar to pro-IL-1ß, caspase-1 is also present in cells as an inactive precursor that needs to be activated by cleavage. Following its recruitment into inflammasome multi-protein signaling complex (detailed in the following section), full-length caspase-1 undergoes self-processing within the two linker peptides (Fig. 4B) generating p20 and p10 proteins. Two heterodimers of p20 and p10 subunits assemble and form the catalytically active caspase-1 (Fig. 4B) enable to cleave its specific substrates.

2.2.2.2 Inflammasomes

Inflammasomes are multiprotein complexes responsible for the activation of pro-inflammatory responses. They are generally composed of an NLR receptor (Fig. 1), an ASC adapter protein (Apoptosis-associated Speck-like containing CARD domain) and an inactive precursor of caspase. Additionally, proteins other than NLR can also form functional inflammasomes including AIM2 protein (like absent in melanoma 2), IFI-16 (interferon-inducible protein 16), RIG-1 (retinoic acid-inducible gene I), and pyrin (Fig. 5).

Inflammasomes are not assembled at the basal state. Once NLRs sense danger signals (PAMPs or DAMPs), they recruit the adapter protein ASC into helically structured filaments forming a high-molecular weight complex termed as inflammasome (Fig. 5). In turn, pro-caspase-1 is recruited to the complex, enabling its proximity-induced trans/auto-cleavage and subsequent activation. Once activated, mature caspase-1 can cleave its substrates including IL-1ß, IL-18 and GSDMD.


A.
B.
+ ASC
PYD
CARD
+ Pro-caspase
CARD
Caspase domain
Salmonella typhimurium
PrgJ
Flagellin
Naip2
Naip5
BIR
NACHT
LRR
NLRC4
CARD
NACHT
LRR
NLRC4 Inflammasome
Bacillus anthracis
Lethal toxin
MKK cleavage, proteasome, phagosomal destabilization, K+ efflux
NLRP1b
PYD
NACHT
LRR
CARD
FIIND
NLRP1b Inflammasome
DAMPs / PAMPs
Crystals
UVB radiation
Phagosomal destabilization, K+ efflux
NLRP3
PYD
NACHT
LRR
NLRP3 Inflammasome
Francisella tularensis
Cytoplasmic DNA
AIM2
PYD
HIN200
IFI-16
PYD
HIN200
HIN200
AIM2 / IFI-16 Inflammasome
Listeria monocytogenes
ssDNA
RIG-1
CARD
CARD
Helicase
RIG-1 Inflammasome
C3 toxins
Cl.Difficile toxins
DNA viruses
Pyrin
Rho GTPases
B-30.2
C- C Helix
PYD
B
Pyrin Inflammasome
Extracellular
Intracellular

Various inflammasome complexes have been distinguished, generally named after the sensor protein that initiates the signalling (Fig. 5). The assembly and activation of inflammasomes proceed in a signal-specific manner. The BIR-containing NLRs NAIP2 and NAIP5 induce NLRC4 activation through the recognition of two bacterial components: the flagellin and the PrgJ basal body of bacterial type III secretion systems (Duncan and Canna, 2018). NLRP1b is activated by cytosolic Bacillus anthracis lethal toxin. AIM2 and interferon-inducible protein 16 (IFI-16) sense dsDNA through their HIN-200 domains, whereas retinoic acid-inducible gene I (RIG-I) activates caspase-1 after it detects single stranded RNA (Wu and Hur, 2015). Pyrin inflammasome is induced by bacterial toxins that modify RhoA GTPase (Jamilloux et al., 2018) whereas NLRP3 is activated by a broad spectrum of PAMPs including parasites, bacterial, fungal and viral pathogens but also by several DAMPs such as ATP, uric acid crystals, amyloid-β fibrils, alum and silica (Lamkanfi and Dixit, 2012).

Among the inflammasomes, the NLRP3 inflammasome has been the most widely studied given its involvement in multiple human diseases including cancer (Wei et al., 2014), Alzheimer disease (Heneka et al., 2013) Parkinson disease, and prion diseases (Shi et al., 2015a), type 2 diabetes (Masters et al., 2010), gout (Martinon et al., 2006) and auto-inflammatory diseases (Duewell et al., 2010; Zhong et al., 2016).

Figure 5. Inflammasome sensors and activators. Adapted from "The NLRP3 and Pyrin Inflammasomes: Implications in the Pathophysiology of Autoinflammatory Diseases" Pelegrin – 2012 – Frontiers of Immunology. **(A)** A wide variety of pathogenic ligands and **(B)** intracellular mediators are involved in inflammasome assembly. The BIR-containing NLRs NAIP2 and NAIP5 induce NLRC4 activation through the flagellin and the PrgJ basal recognition. NLRP1b is activated by cytosolic Bacillus anthracis lethal toxin. NLRP3 is activated by a various PAMPs. AIM2 and IFI-16 sense dsDNA through their HIN-200 domains. RIG-I activates caspase-1 after it detects single stranded RNA. Pyrin inflammasome is induced by bacterial toxins that modify RhoA GTPase.

In contrast to NLRP1 and NLRC4, NLRP3 is not constitutively expressed and needs a two-step activation process: (1) priming and (2) inflammasome assembly. The first signal is provided by microbial or endogenous molecules that induce *NLRP3* expression through activation of the NF-κB-pathway, whereas the second signal leads to NLRP3 inflammasome oligomerization and activation (Fig. 6 - Fig. 7).

Several molecular mechanisms have been suggested for NLRP3 activation. It includes the canonical inflammasome activation (Fig. 6) and the non-canonical caspase-11-dependent inflammasome activation (Fig. 7).

The canonical NLRP3 activation is mediated by a wide array of molecules including asbestos, silica (Dostert et al., 2008), aluminum adjuvants ("Alum") (Eisenbarth et al., 2008), ATP (Mariathasan et al., 2006), viral infections (Ichinohe et al., 2009), fungus products (Gross et al., 2009), RNAs or bacterial DNA (Muruve et al., 2008) and bacterial toxins (Mariathasan et al., 2006). Although the molecular mechanisms leading to the canonical activation of NLRP3 inflammasome remains unclear, several mechanisms are currently proposed including K^+ efflux (Franchi et al., 2007; Perregaux and Gabel, 1994), Ca^{2+} signalling, reactive oxygen species (ROS) (Zhou et al., 2011), mitochondrial dysfunction, and lysosomal rupture (Hentze et al., 2003) (Fig. 6). Additionally, we cannot exclude de possibility that an intermediate adaptor may be required for transmitting the cellular signals to the NLRP3-inflammasome (Fig. 6).

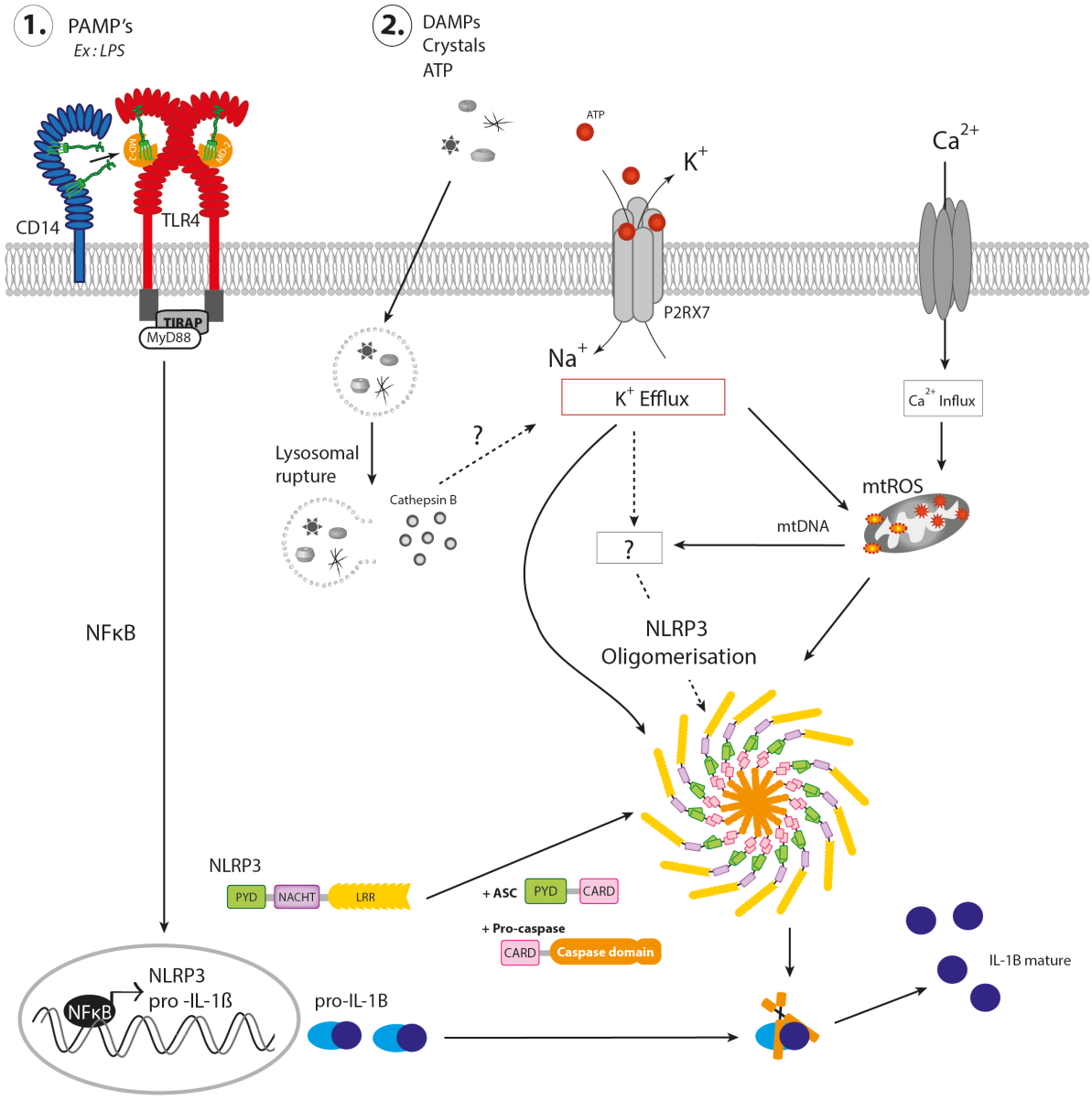


Figure 6. Canonical NLRP3 inflammasome activation. Adapted From « NOD-Like Receptors: Master Regulators of Inflammation and Cancer"- Saxena – 2014 - Frontiers of Immunolology. (1.) Priming through TLRs–MyD88 activate pro-IL-1β and NLRP3 transcription via the NF-κB pathway. **(2.)** Extracellular ATP, crystals or DAMPs, activate NLRP3 oligomerization through potassium efflux, ROS, cathepsin. NLRP3 oligomerisation activates capsase-1 into mature active caspase-1 which cleaves GSDMD and pro-IL-1ß into mature IL-1ß.

Besides the canonical NLRP3 inflammasome activation, a non-canonical caspase-11-dependent NLRP3 activation has been characterized (Ding and Shao, 2017). In this case, priming is triggered by Gram-negative bacteria i.e. *Salmonella typhimurium, Citrobacter rodentium*, *Escherichia coli*, and *Vibrio cholera* which lead to caspase-11 (murine orthologue of caspase-4 and -5) expression through IFN-α/β signalling (Fig. 7). Activation of caspase-11 is thought to be triggered by the recognition of intracellular LPS and/or a-yet-unidentified scaffold proteins but the exact mechanism for the initial caspase-11 activation step remains unclear. It has been proposed that the lipid A moiety of LPS binds directly to the caspase-11 CARD domain and leads to the oligomerization and its proximity-induced activation, an event likely due to the aggregation behaviour of LPS. Caspase-11 activation leads to massive intracellular ATP release through pore forming proteins including pannexin-1 or gasdermin D (see page 66). Released ATP promotes the activation of the NLRP3-ASC-caspase-1 pathway through P2RX7 receptor binding (Fig. 7) (Shi et al., 2014).

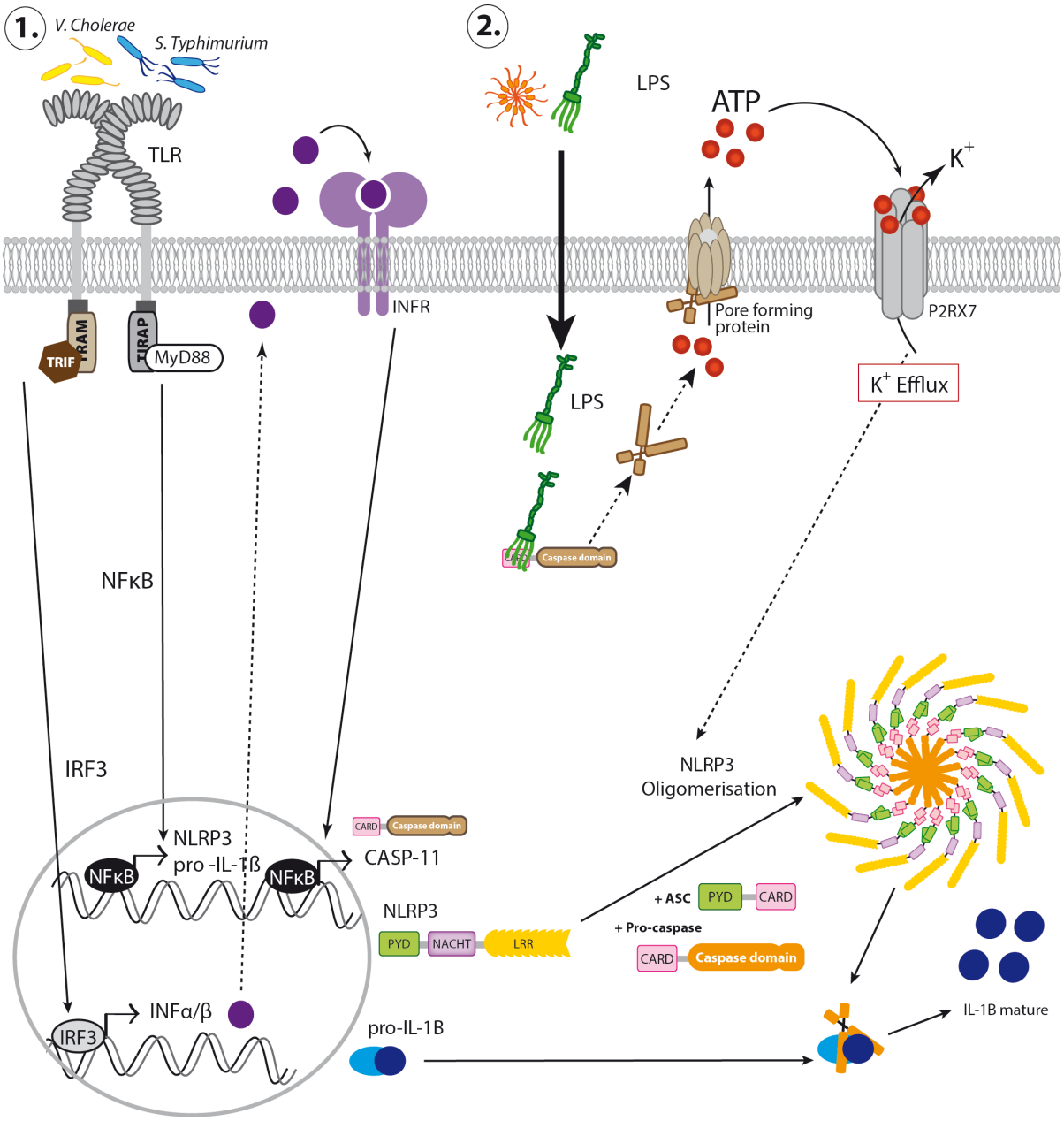


Figure 7. Non-canonical NLRP3 inflammasome activation. Adapted From « NOD-Like Receptors: Master Regulators of Inflammation and Cancer"- Saxena – 2014 - Frontiers of Immunolology. (1.) Gram-negative bacteria activate the TLR4–MyD88 and TRIF pathways inducing nuclear translocation of NF-κB, which promotes the transcription of IL-1β and NLRP3 as well as IRF-3 and IRF7 genes. The IRF3–IRF7 complex elicits the expression of IFN-α/β that binds the IFNAR1/IFNAR2 receptor, leading to activation of the JAK/STAT pathway and transcription of caspase-11 gene. **(2.)** Intracellular LPS and yet unidentified scaffold proteins cleave and activate caspase-11, which in turn cleaves pannexin-1 leading to intracellular ATP exit. ATP induces NLRP3 inflammasome oligomerisation that activates pro-capsase-1 into mature active caspase-1 which cleaves its specific substrates e.g: IL-1ß.

Recently an alternative NLRP3 activation pathway has been described to be activated in human monocytes in response to LPS (Gaidt et al., 2016; Gaidt and Hornung, 2017). In this pathway potassium efflux is not required, which contrasts with NLRP3 activation induced by ATP, pore-forming toxins, or particulate matter. Thus, this pathway is defined as an alternative NLRP3 inflammasome pathway. Mechanistically, Gaidt *et al.* showed that RIPK1, FADD, and caspase-8 molecules act downstream of TLR4–TRIF signalling pathway (Fig. 8) to activate NLRP3 after LPS treatment (Gaidt et al., 2016; Gaidt and Hornung, 2017). This particular inflammasome activation has also been described in mouse bone marrow-derived dendritic cells in response to TLR ligands alone (He et al., 2013), through an ATP–P2RX7-independent pathway (Fig. 8).

The main well-known effect of NLRP3 inflammasome activation is the maturation of IL-1ß through capsase-1 cleavage activity. In addition to the NLRP3 inflammasome that has been extensively studied, other inflammasome have been reported to be implicated in IL-1ß maturation. Indeed, activated NLRC4 has been reported to drive IL-1ß production and secretion in neutrophils, macrophages and dendritic cells during acute *Salmonella typhimurium* infection (Chen et al., 2014). Keratinocytes have been also reported to express a functional AIM2 which triggers a strong IL-1ß release in response to cytosolic dsDNA (Kopfnagel et al., 2011). NLRP1 has also been identified to induce IL-1ß maturation and release upon anthrax lethal toxin exposure in macrophages (Thomas et al., 2011).

Once mature IL-1ß is generated, it is secreted into the extracellular space where it can bind to specific receptors.

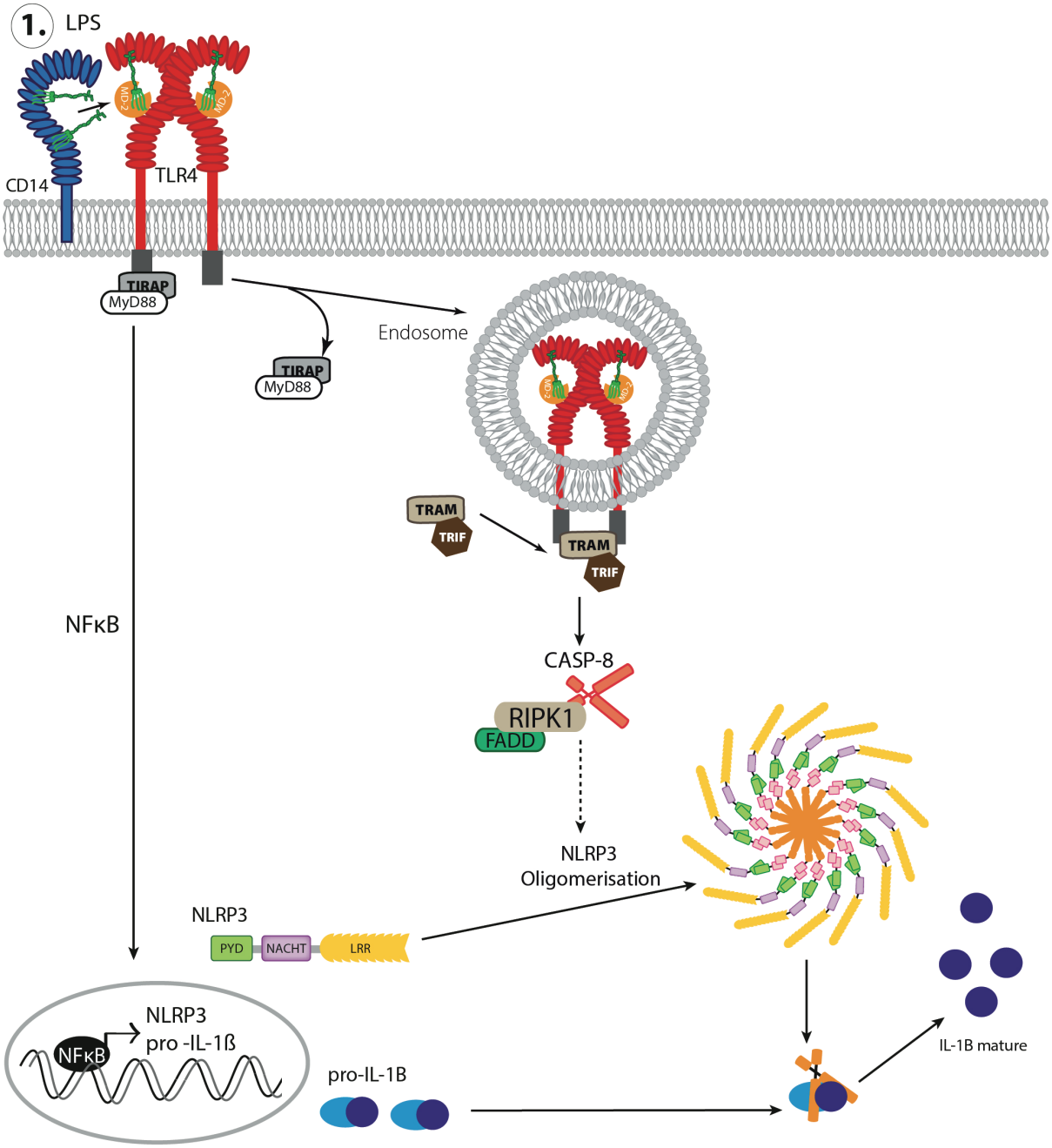


Figure 8. Alternative NLRP3 inflammasome activation. Adapted From « Human Monocytes Engage an Alternative Inflammasome Pathway"- Hornung – 2016 - Immunity. LPS binding to surface TLR4 induces pro-IL-1β and NLRP3 transcription via the TIRAP-MyD88-NF-κB pathway. Endocytose of LPS/TLR4 complexes induces RIPK1/FADD-CASP-8 complex through the TRAM-TRIF pathway. The latter complex activates NLRP3 inflammasome oligomerization thought a still unknown mechanism.

2.3 IL-1 receptors

The IL-1R family comprises 10 type I transmembrane proteins with a similar architecture (Fig. 9): an extracellular portion that generally contains three extracellular immunoglobulin (Ig)-like domains, usually responsible of ligand binding, a, and an intracellular portion containing the TIR domain, essential to initiate signal transduction.

IL-1 receptors are active in heterodimeric complexes composed of a ligand-binding chain (IL-1R1, IL-1R2, IL-1R4, IL-1R5 and IL-1R6) that, upon the binding with the agonist cytokine, recruits an accessory chain (IL-1RaP and IL-1R7) and initiate the signalling pathway (Boraschi and Tagliabue, 2013). Importantly, both TIR domains of receptors and co-receptors need to be present in the heterodimeric receptor complexes to generate signalling (Fig. 9). IL-1R1 is the ligand-binding receptor of IL-1α and IL-1β, thereby mediating IL-1-dependent activation. Whereas IL-1α and IL-1β activate signalling pathways, IL-1 receptor antagonist (IL-1Ra) which can also bind to IL-1R1, prevent its activation by IL-1α and IL-1β (Symons et al., 1995).

After IL-1ß binding to IL1-R1 and formation of the heterodimeric receptor complex (IL1-R1/IL-1RaP), the two TIR domains associate creating novel scaffolds, allowing the interaction of the adapter proteins myeloid differentiation primary response 88 (MyD88) with these TIR domains (Fig. 9). This interaction leads to the translocation of IRAK-4, an IL-1R1 associated serine/threonine-specific protein kinase, to the active receptor complex and its interaction with MyD88. Additionally, upon IL-1 binding, the Tollip/IRAK-1 complex is translocating to the active IL-1R complex bringing the death domains of IRAK-1 and MyD88 into close contact.

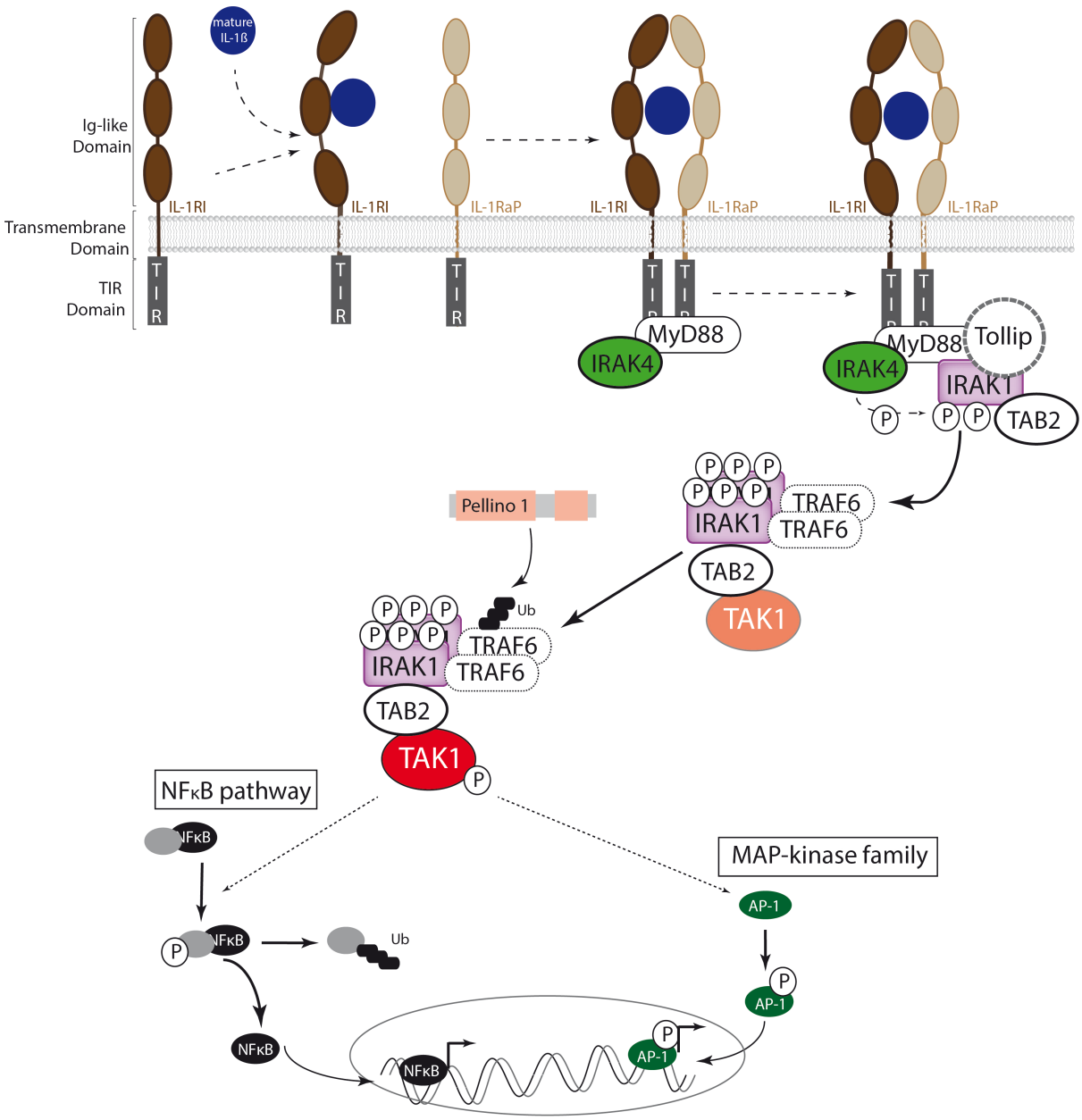


Figure 9. The formation of the MyD88/IRAK/TRAF6 signalosome in IL-1 transduction signal. Adapted from "Summary and comparison of the signalling mechanisms of the Toll / interleukin-1 receptor family"– Wesche - 2002. The IL-1R family comprises three parts: an Ig-like extracellular domain, a transmembrane domain and an intracellular TIR domain. Binding of IL-1α and IL-1β on IL1-R1 induce the recruitment of the IL-1RaP to initiate the signalling. The proximity of each receptor TIR domains creates a scaffold that induce the recruitment of MyD88 and subsequent IRAK-4 at the receptor complex. After its recruitment at the receptor complex, IRAK-1 is phosphorylated by IRAK-4. Hyperphosphorylated IRAK-1 dissociates from the receptor complex and dimerizes. Dimers of IRAK-1 allow dimerization of TRAF6 and its subsequent poly-ubiquitinylation by pellino. TAK1 meets TAB2 at the signalosome complex. Poly-ubiquitinylated TRAF6 that triggers TAK1 autophosphorylation, which subsequently phosphorylates regulatory kinases in different downstream signalling pathways: NFκB and MAPK.

Very rapidly, IRAK1 undergoes cross- (by IRAK4) and/or auto- phosphorylation at the active receptor complex resulting in its hyper-phosphorylation. Hyper-phosphorylated IRAK-1 dissociates from the receptor complex, dimerize in the cytoplasm and interacts with the tumour necrosis factor–associated factor 6 (TRAF6). This interaction activates TRAF6, which becomes polyubiquitinylated TRAF6 (Ub-TRAF6) by Pellino and triggers the phosphorylation of TAK1. Phosphorylated TAK1 subsequently phosphorylates regulatory kinases in different downstream signalling pathways leading to transcriptional modulation of canonical IL-1 target genes such as IL-6, IL-8, MCP-1, COX-2, IκBα, IL-1α, IL-1ß and MKP-1 (Weber et al., 2010).

Three central downstream signalling pathways are activated by IL-1ß on the MyD88/IRAK/TRAF6 signalosome. These are: the activation of the IKK complex resulting in the phosphorylation and degradation of IκB and translocation of NFκB to the nucleus, the activation of the JNK pathway resulting in the phosphorylation of AP-1 complexes, and the activation of the p38 pathway, which is involved in regulating mRNA stability (Martin and Wesche, 2002) (Fig. 9).

IL-1ß is a major orchestrator of the inflammatory responses and it is also involved in many inflammatory and autoimmune diseases, thus mechanisms modulating IL-1ß biological activity is crucial. IL-1Ra is not the only physiologically inhibiting mechanism (Fig. 10). IL-1R1 and IL-1R2 can undergo metalloproteinase-dependent cleavage of the extracellular domain, giving rise to a soluble receptor that acts as a soluble decoy, capturing IL-1 agonists and preventing their interactions with membrane receptors (Fig. 10). Additionally, IL-1ß binding to membrane IL-1R2 results not only in IL-1ß blocking activity but it also sequestrates IL-1RaP, preventing its availability for other activating receptor complexes.

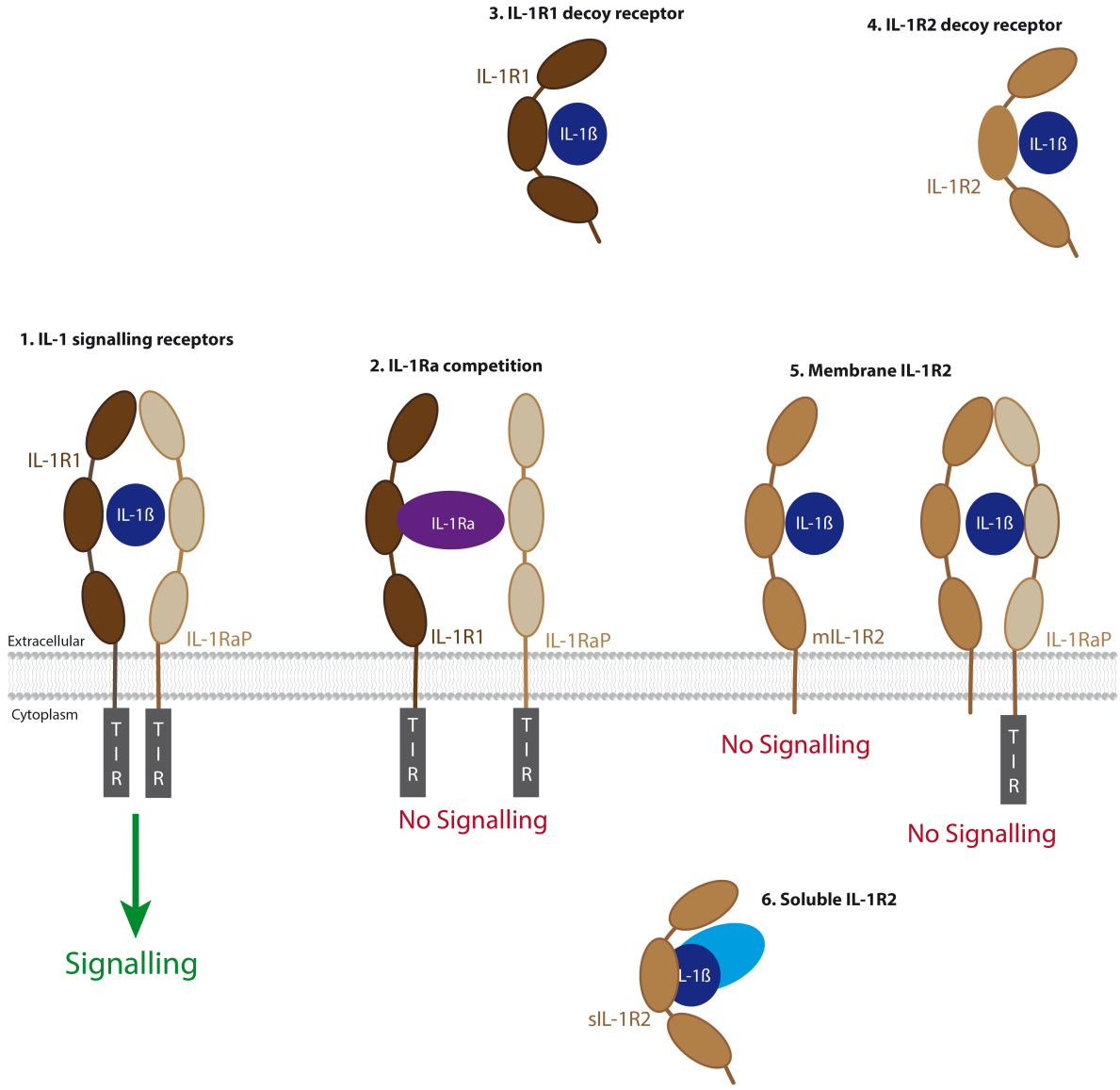


Figure 10. The IL-1 receptor complexes. Adapted from "The interleukin-1 receptor family" – Tagliabue - 2013- Seminars in Immunology. The IL-1 receptor complexes. **(1.)** The binding of IL-1ß to the receptor IL-1R1 induces a conformational change that allows the recruitment of the accessory chain IL-1RaP. Proximity of the intracellular TIR domains of the two chains initiates signalling. **(2.)** IL-1Ra binds to IL-1R1 which does not induce conformational changes nor recruitment of IL-1RaP, thus, signalling does not take place. **(3-4.)** Soluble IL1R1 and IL1R2 present in the extracellular space retains IL-1ß preventing its binding to its signalling receptor **(5.)** Binding of IL-1ß to membrane-IL-1R2 results in its sequestration as there is no TIR domain, thus no signalling can occur. **(6.)** The intracellular form of sIL-1R2 (sIL-1R2) can bind proIL-1ß and protect it from proteolytic cleavage and generation of the mature form.

Eventually, the intracellular form of sIL-1R2 (sIL-1R2) is able to bind the precursor of IL-1ß and prevent its proteolytic cleavage by caspase-1 (Fig. 10) (Boraschi and Tagliabue, 2013).

Due to the wide spectrum of IL-1ß biological functions, efforts have been made to map the distribution of IL-1R1. So far, IL-1R1 was found to be expressed in all cells of innate immune system including macrophages, neutrophils, eosinophils, basophils, mast cells, as well on several T cell populations (Bebes et al., 2014). More recently, IL-1R1 expression has been reported in endothelial cells within the dorsal root ganglia (DRG), spinal cord, pancreas, colon, muscles fibroblastic reticular cells and endothelial cells (Song et al., 2018).

2.4 Majors effects of IL-1ß

More than fifty years were needed to discover the wide spectrum of biological activities of the interleukin-1 beta. Here, a non-exhaustive list of its major biological effects.

2.4.1 Pro-inflammatory cytokine

One of the most well-known feature of IL-1ß is its ability to induce the inflammatory response by inducing locally an inflammatory exudate, constituted from plasma proteins and leukocytes (Medzhitov, 2008). It increases the expression of selectins at the surface of the blood vessel endothelium, allowing selective extravasation of leukocytes at the site of infection. Working on the initiation of coagulation and the modulation of leukocyte-vessel wall adhesion, Bevilacqua and colleagues were the first to report that IL-1ß treatment induced the expression of endothelial-leukocyte adhesion molecule-1 (ELAM-1) and the intracellular adhesion molecule-1 (ICAM-1) (Bevilacqua et al., 1985). Two years later, Marlin and Springer provided evidence that ICAM-1 was a ligand of the lymphocyte function-associated antigen 1 (LFA-1), a molecule at the surface of leukocytes (Marlin and Springer, 1987), confirming the hypothesis that IL-1ß could promote localized adhesion of blood leukocytes at the site of inflammation (Bevilacqua et al., 1985).

Nowadays, it is believed that IL-1β, promotes the recruitment of macrophages, which is the second phase of the inflammatory recruitment, whereas IL-1α, released from dying cells at the beginning of the tissue damage, initiates inflammation by inducing recruitment of neutrophils (Rider et al., 2011). Additionally, IL-1ß has been reported to act on blood vessels to inhibit the contraction of vascular smooth muscle (Beasley et al., 1989) contributing to the hypotension of the septic shock.

2.4.2 Pyrogenic properties

IL-1ß was first reported as the endogenous pyrogen due to its ability to cause fever (Atkins, 1960). Fever is referred as an increase of body temperature that occurs in response to infection, inflammation or trauma. The interaction of IL-1ß as well as IL-6 and TNFα, with the organum vasculosum of the lamina terminalis (OVLT), part of the anterior hypothalamus leads to the synthesis of prostanoids such as prostaglandin E2 (PGE2). PGE2 then acts the hypothalamus and induces fever (Walter et al., 2016). Of note, in addition to the production of pro-inflammatory cytokines, exogenous molecules such as LPS have also been reported to induce a more rapid onset of the fever by direct stimulation of PGE2 peripheral production from hepatic Kupffer cells (Steiner et al., 2006).

2.4.3 Lymphocyte Activating Factor (LAP)

IL-1ß also functions as lymphocyte activating factor (LAF). It stimulates the production of several cytokines including IL-2 but also to increase the expression of the receptor of IL-2 on T cells (Lowenthal et al., 1986; Williams et al., 1985), both required for optimal T-cell activation and proliferation. More recently, IL-1ß has been shown to promote the differentiation of naïve CD4 T cells into Th17 cells (Acosta-Rodriguez et al., 2007) and to promote the expansion of the IL-17-secreting memory CD4 T cells (Chung et al., 2009; Wilson et al., 2007). Additionally, it has been reported that IL-1R1 signal was required for the conversion of Foxp3+ T cells into IL-17-producing cells. IL-1 also allowed Th17 cells to maintain their cytokine secretion profile, after polarization (Chung et al., 2009). Eventually, IL-1ß has been reported to act directly on Th2 CD4 T cells to enhance their antigen-driven expansion (Ben-Sasson et al., 2009).

2.4.4 B cell growth factor

IL-1ß has been described to increase B-cell proliferation and immunoglobulin synthesis. It induces the expression of CD40L and OX40 costimulatory molecules at the surface of follicular helper T cells (T_{FH}) (Nakae et al., 2001). Subsequent, CD40L-CD40 interaction is essential to promote B cell maturation as it induces isotype switching, somatic hyper-mutation, and immunoglobulins production (Fuleihan, 1998). CD40L is also required for formation of B memory cells and germinal centers, and signalling through CD40 prevents apoptosis of germinal center B cells (Laman et al., 2017). In contrast, OX40-OX40L interaction has been reported to be essential for the differentiation of activated B cells into highly Ig-producing cells (Stuber and Strober, 1996).

2.4.5 Leukocytic endogenous mediator (LEM)

IL-1ß is known to induce the synthesis of other inflammatory cytokines, including TNFα, IL-6, GM-CSF, as a positive feedback mechanism, additionally to itself (Eskan et al., 2008; Tosato and Jones, 1990). By inducing the expression of IL-6, IL-1ß also stimulates the synthesis of acute phase response peptides such as amyloid peptide, C-reactive peptide, and the C3 of the complement (Ridker, 2016).

2.4.6 Mononuclear cell factor (MCF)

Previously known as the mononuclear cell factor, IL-1ß was shown to stimulate the production of collagenase and prostaglandin E2 (PGE), by fibroblastoid synovial cells (Krane et al., 1985; Poubelle et al., 1985), two molecules implicated in synovial cell proliferation, cartilage and bone resorption, as well as collagen deposition. High level of collagenase and PGE, present within the synovium has been related to the destruction of the connective tissue and bone resorption which are pathological features of chronic inflammatory diseases such as rheumatoid arthritis (Krane et al., 1985).

Given the wide array of its biological activities, IL-1ß is not only a key mediator of the innate immunity but is also important in modulating adaptive immune responses.

2.5 Blocking IL-1ß activity

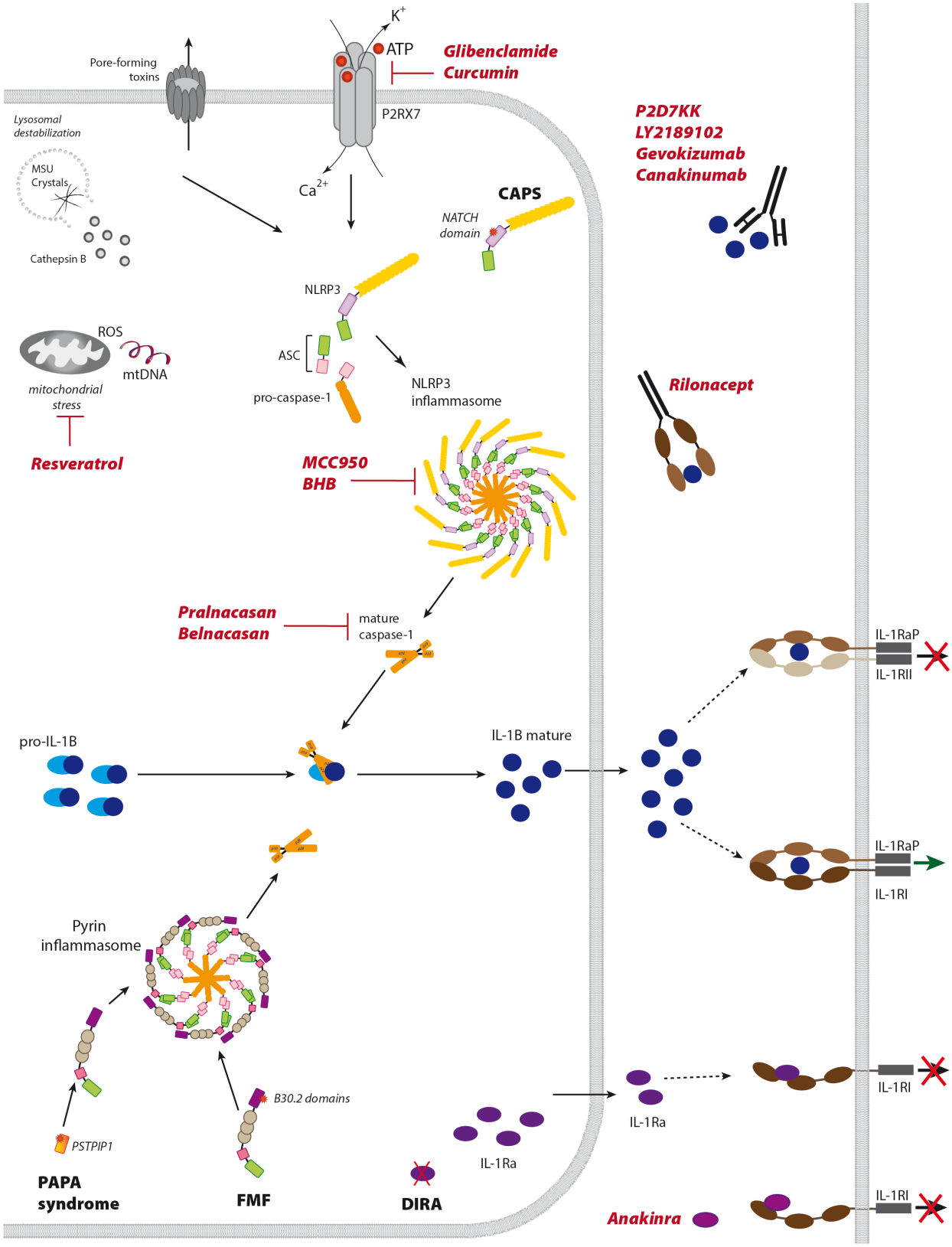
Despites it's numerous beneficial functions, dysregulated production of IL-1ß is implicated in many chronic diseases including rheumatoid arthritis, inflammatory bowel disease, graft-vs-host disease and auto-inflammatory syndromes (Dinarello, 2000).

Auto-inflammatory syndromes comprise a group of genetically inherited disorders characterized by amplified innate immune responses leading to recurrent febrile episodes of fever accompanied by inflammatory cutaneous, mucosal, serosal, and osteoarticular manifestations (Cush, 2013). Many of these diseases are unified by a genetically driven dysregulated innate immune response with excess of inflammasome activation, usually NLRP3, and cytokine release. Theses includes the periodic syndromes like familial Mediterranean fever (FMF), hyperimmunoglobulinemia D with periodic fever syndrome (HIDS), tumor necrosis factor receptor associated auto-inflammatory syndrome (TRAPS), cryopyrin associated periodic syndromes (CAPS), Blau syndrome and pyogenic sterile arthritis pyoderma gangrenosum and acne syndrome (PAPA). In some auto-inflammatory syndromes, excessive IL-1ß signalling is directly linked to pathogenesis whereas in others this relationship seems less clear. However, the development of IL-1ß-targeting agents constituted a major hope for these patients (Cush, 2013).

During these past 20 years, researches focused on blocking IL-1ß or reducing its activity by specific strategies. It involved neutralizing antibodies, soluble receptors, receptor antagonist, and inhibitors of proteases that convert inactive precursors to active molecules. Blocking IL-1ß has constituted revolutionary advances in the treatment of several chronic diseases. In the following figure (Fig. 11) you will find a summary of the IL-1ß-targeting agents that have

been developed until now. Some of them are currently used in the clinics, but for most of them the efficiency has not been fully proven yet.

Figure 11. Regulation of IL-1ß production and current IL-1 antagonists. Adapted from "Potential of IL-1, IL-18 and Inflammasome Inhibition for the Treatment of Inflammatory Skin Diseases" – French – 2017 – Frontiers in pharmacology. Formation of a functional inflammasome occurs when PAMPs and DAMPs induce lysosomal destabilization with cathepsin B release, mitochondrial stress with release of oxidized mtDNA and ROS, and changes in $Ca^{2+}$and K^{+} intracellular concentration. Mutations of NACHT-PYD domains of NLRP3 in CAPS lead also to NLRP3 inflammasome activation. Mutations of B30.2 domains in FMF and of PSTPIP1 in PAPA syndrome lead to activation of the pyrin inflammasome. Activated inflammasomes recruit procaspase-1 which induces trans/auto-cleavage and caspase-1 activation that process IL-1ß. The secreted cytokine binds to the IL-1 receptor. Soluble IL-1R1 and IL-1R2 block IL-1ß signalling. Pralnacasan and Belnacasan regulate caspase-1 activity. Glibenclamide and curcumin inhibit/downregulate the P2RX7. Resveratrol increase autophagy and inhibits mitochondrial stress. MCC950 and BHB are specific NLRP3 inhibitors. Anakinra and IL-1Ra compete with IL-1ß for binding to its receptor. Rilonacept acts as a soluble decoy binding IL-1ß. The recombinant antibodies Canakinumab, Gevokizumab, LY2189102, and P2D7KK specifically target and neutralize IL-1ß.


K+
ATP
Glibenclamide
Curcumin
Pore-forming toxins
P2RX7
Lysosomal destabilization
MSU Crystals
Cathepsin B
Ca2+
CAPS
NATCH domain
P2D7KK
LY2189102
Gevokizumab
Canakinumab
NLRP3
ROS
mtDNA
ASC
pro-caspase-1
NLRP3 inflammasome
mitochondrial stress
Resveratrol
Rilonacept
MCC950
BHB
Pralnacasan
Belnacasan
mature caspase-1
IL-1RaP
IL-1RII
pro-IL-1B
IL-1B mature
IL-1RaP
IL-1RI
Pyrin inflammasome
B30.2 domains
PSTPIP1
IL-1Ra
IL-1RI
IL-1Ra
PAPA syndrome
FMF
DIRA
Anakinra
IL-1RI

2.5.1 IL-1ß antagonists

2.5.1.1 Anakinra (Kineret®)

The first successful drug developed was a recombinant homolog of IL-1Ra that competes with IL-1 agonists for the binding to their receptor, preventing their signalling (Fig. 11). This drug is known as **Anakinra** (Kineret®). It was first developed for sepsis, but studies in this severe systemic and fatal disease were challenging and no significant effect of the drug could be observed. In 2001, Anakinra was finally approved by the Food and Drug Administration (FDA) for the treatment of rheumatoid arthritis (RA), even if it demonstrated modest efficacy. A decade later, its use was extended to cryopyrin-associated periodic syndromes (CAPS). However, its short half-life (4 to 6 hours) requiring daily dosing, modest efficacy in RA treatment and painful injection site reactions have encouraged the development of new IL-1ß targeted drugs.

Two additional IL-1ß-targeted drugs with longer half-lives and fewer injection site reactions have been developed and marketed. The Rilonacept (Arcalyst®) also known as “IL-1ß TRAP” and the monoclonal antibody Canakinumab.

2.5.1.2 Rilonacept (Arcalyst®)

Rilonacept is a long-acting dimeric fusion protein consisting of portions of IL-1R1 and IL-1RaP linked to the Fc portion of human immunoglobulin G1 (IgG1). It works as a soluble decoy receptor trapping IL-1 preventing its interaction with membrane IL-1R1 (Fig. 11). Rilonacept has a half-life of approximately 7 days which allows a weekly subcutaneous administration of 320 mg (loading dose) followed by weekly injections of half the loading dose (Fenini et al., 2017). It was approved in 2008 by the FDA for the treatment of cryopyrin associated periodic syndromes. Eighty-four percent of patients treated with rilonacept

experienced a statistically significant improvement in overall symptom compared with patient treated with placebo (Gillespie et al., 2010).

2.5.1.3 Canakinumab (Ilaris®)

Canakinumab is a fully humanized monoclonal antibody (IgG1/k isotype) against IL-1ß (Fig. 11). It has an approximate half-life of 25 days meaning it can be administered only once every 2 months. Canakinumab was approved by the FDA in 2009 for the treatment of CAPS and active systemic juvenile idiopathic arthritis. In 2016, it received the FDA approval as first-line treatment for TNF receptor associated periodic syndrome (TRAPS), Mevalonate kinase deficiency (MKD) and familial Mediterranean fever (FMF) (Savic and McDermott, 2009).

Additional IL-1ß-targeted drugs have been developed and tested in clinical trials, however their efficiency on these diseases has not been fully proven.

Gevokizumab, another humanized anti-IL-1β antibody (Fig. 11) was developed and tested in clinic. Unfortunately, the clinical development of this antibody was interrupted in 2016 after a phase 3 on uveitis in patients with Behçet's disease as it did not meet the primary endpoint criteria (Tugal-Tutkun et al., 2018). **LY2189102**, anti-IL-1β humanized monoclonal immunoglobulin G4 (Fig. 11), was tested in patients suffering from type 2 diabetes mellitus (T2DM) but did not show considerable results (Sloan-Lancaster et al., 2013). **P2D7KK** is another neutralizing monoclonal antibody against IL-1β (Fig. 11) developed by A-STAR in Singapore (Goh et al., 2014). The antibody was reported to bind IL-1β with more than 10 times higher than the marketed antibody canakinumab. However, it has not been evaluated in human subjects yet but cross-reacts with mouse and monkey IL-1β, hence facilitating preclinical studies and development.

2.5.2 Inflammasome and caspase-1 inhibitors

Other possibilities to block IL-1β include to target molecules implicated in its processing such as the NLRP3 inflammasome or the caspase-1 protease. NLRP3 inhibitors include MCC950, β-hydroxybutyrate (BHB) and glibenclamide whereas Pralnacasan and Belnacasan are referred to as caspase-1 inhibitors.

MCC950 is a small-molecule able to specifically inhibit NLRP3-ASC oligomerization (Fig. 11). It has been shown to be effective for the treatment of CAPS with activating *Nlrp3* mutations. Additionally, MCC950 has been shown to be active in *ex vivo* samples from individuals with Muckle-Wells syndrome (Coll et al., 2015).

BHB specifically inhibits NLRP3 inflammasome by preventing K+ efflux and reducing ASC oligomerization and speck formation (Fig. 11). It has been shown to be effective in in murine models of familial cold auto-inflammatory syndrome (FCAS) and Muckle-Wells syndrome (Youm et al., 2015).

Glibenclamide, an anti-diabetic drug that inhibits the ATP-sensitive K+ channel, was shown to block the NLRP3 inflammasome activation induced by LPS and ATP (Fig. 11). It may be considered for the treatment of arthritis, silicosis, and Alzheimer's, in which excessive Cryopyrin-dependent IL-1ß production was proposed as a major cause of pathology (Lamkanfi et al., 2009).

Resveratrol and **curcumin** are natural polyphenols found in several plants and are able to block IL-1β secretion. Resveratrol was shown to directly bind and block COX-2 activity (Zykova et al., 2008) which is known to be involved in NLRP3 activation (Fig. 11). Curcumin has been shown to impair IL-1β secretion in PMA-treated macrophages by downregulating P2X7 receptor (Fig.11) (Kong et al., 2016).

Finally, two caspase-1 inhibitors have been developed, namely **Pralnacasan** and **Belnacasan** (Fig. 11). They are synthetized as prodrugs, which are then converted into the active principle after oral absorption. Pralnacasan has been evaluated in clinical trials for the treatment of Rheumatoid arthritis (RA) and osteoarthritis but due to safety reasons its development has been disrupted (Braddock and Quinn, 2004). Belnacasan was shown to induce anti-inflammatory effects in a mouse model of delayed-type hypersensitivity (DTH) (Wannamaker et al., 2007) and it has been evaluated in phase 1 and 2a clinical trials in the setting of epilepsy and psoriasis.

3. Unconventional secretion of leaderless proteins

In eukaryotic cells, proteins are generally secreted through the conventional endoplasmic reticulum (ER)–Golgi pathway. These proteins carry a N-terminal signal peptide that directs their entry into the ER compartment from where they exit via COPII-coated vesicles to reach the Golgi apparatus and the plasma membrane (Lopez-Castejon and Brough, 2011).

Beyond this well-established mechanism of protein secretion, a number of extracellular proteins are known to be secreted in a signal peptide recognition independent manner. Such processes have been termed 'unconventional secretory pathways' (UPS) (Fig. 12) (Rabouille, 2017). Four UPS have currently been described: Type I, or pore-mediated translocation across the plasma membrane; Type II, or ABC transporter-based secretion, Type III or vesicles-based secretion, including autophagosomes, exosomes, microvesicles and autophagy cargos and Type IV, or Golgi-bypass pathway (Fig.12) (Rabouille, 2017).

Interleukin-1ß is one of those secreted ‘leaderless’ proteins which use unconventional secretory pathways. It is not the only one. Few other proteins are also concerned including all members of IL-1 family (IL-1α, IL-18, IL-33, IL-36 (αβγ), IL-37, IL-38), with the exception of IL-1Ra, the family of extracellular matrix lectins: the galectins (Hughes, 1999; Popa et al., 2018), the members of annexin family (Popa et al., 2018), the ciliary neurotrophic factor (CTNF), the fibroblast growth factor -1 and 2 (FGF-1 & FGF-2), the glutathione synthetase (HME2), the high–mobility group box 1 (HMGB1) and the macrophage inhibitory factor (MIF). For the moment, there is no evidence of a common UPS for all of the leaderless proteins. The way these proteins export into the extracellular space is not well defined except for FGF-2 (Fig. 12).

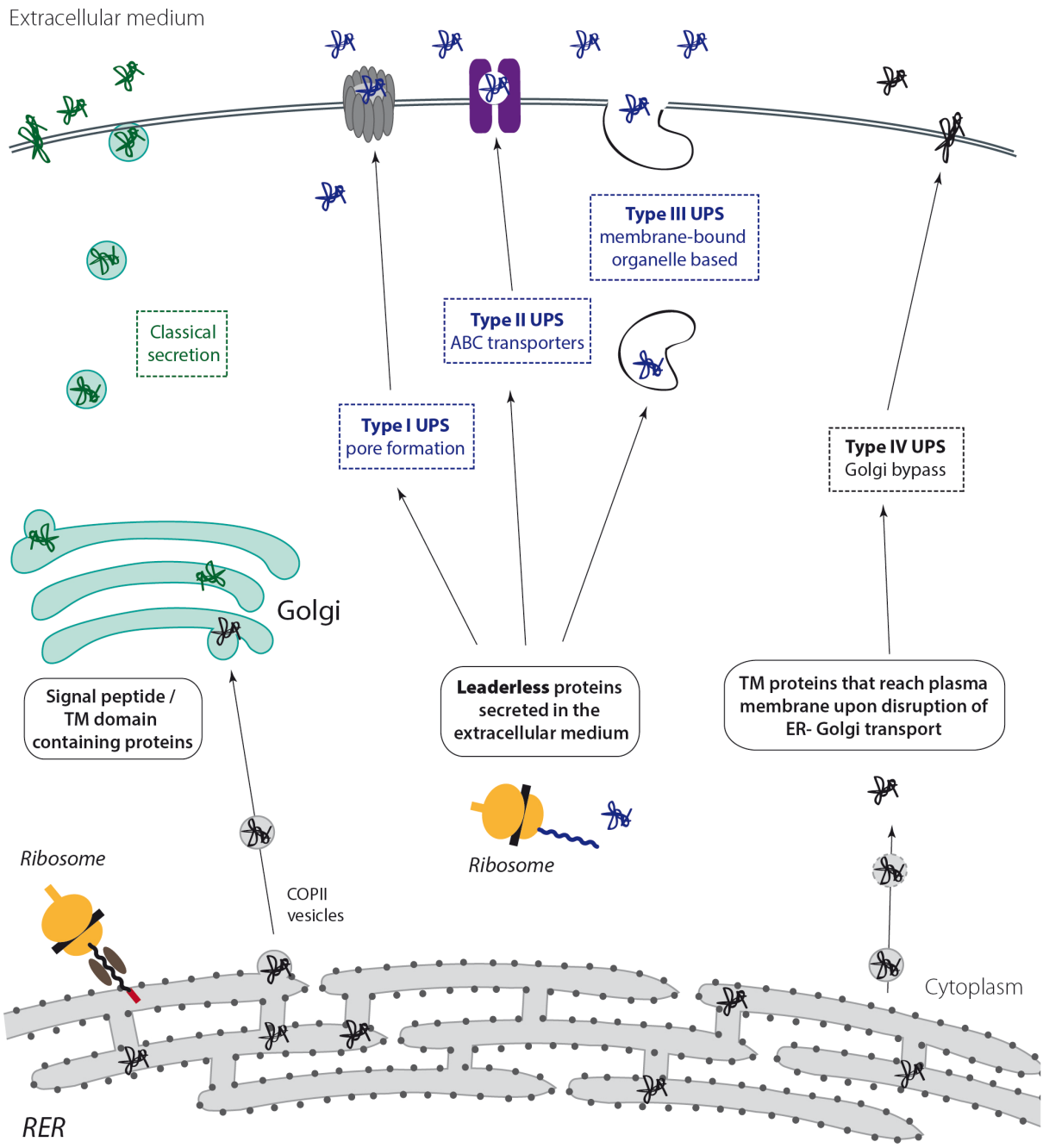


Figure 12. Four types of unconventional protein secretion (UPS). Adapted from "Pathways of Unconventional Protein Secretion" – Rabouille – 2017 – Trends in Cell biology. Leaderless proteins (blue) can translocate across the plasma membrane through a pore (Type I UPS), via ABC transporter-based secretion (Type II UPS) or via a membrane-bound organelle (Type III UPS). Transmembrane proteins (black) can be transported to the plasma membrane using the classical secretory pathway (green) or the Golgi-bypass pathway (Type IV UPS).

3.1 FGF2 unconventional secretion (Type I UPS)

As mentioned above, the only mechanism of unconventional secretion mechanism that has been completely deciphered is that used by FGF-2, which forms a self-made lipidic pore that allows its translocation across the plasma membrane to reach the extracellular space (Fig. 13) (Steringer et al., 2012; Steringer and Nickel, 2018; Zacherl et al., 2015).

FGF2 secretion comprises five steps (Fig. 13): (i) recruitment to the cytoplasmic leaflet of the plasma membrane mediated by ATP1A1 and interaction with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) (Zacherl et al., 2015), (ii) phosphorylation by Tec kinase, (iii) PI(4,5)P_2-induced self-oligomerization at the plasma membrane, (iv) membrane insertion and pore formation with a toroidal architecture (Steringer et al., 2012), and eventually (iv) extracellular dissociation of monomeric FGF2 which is then trapped by surface heparan sulfate proteoglycans (Steringer and Nickel, 2018).

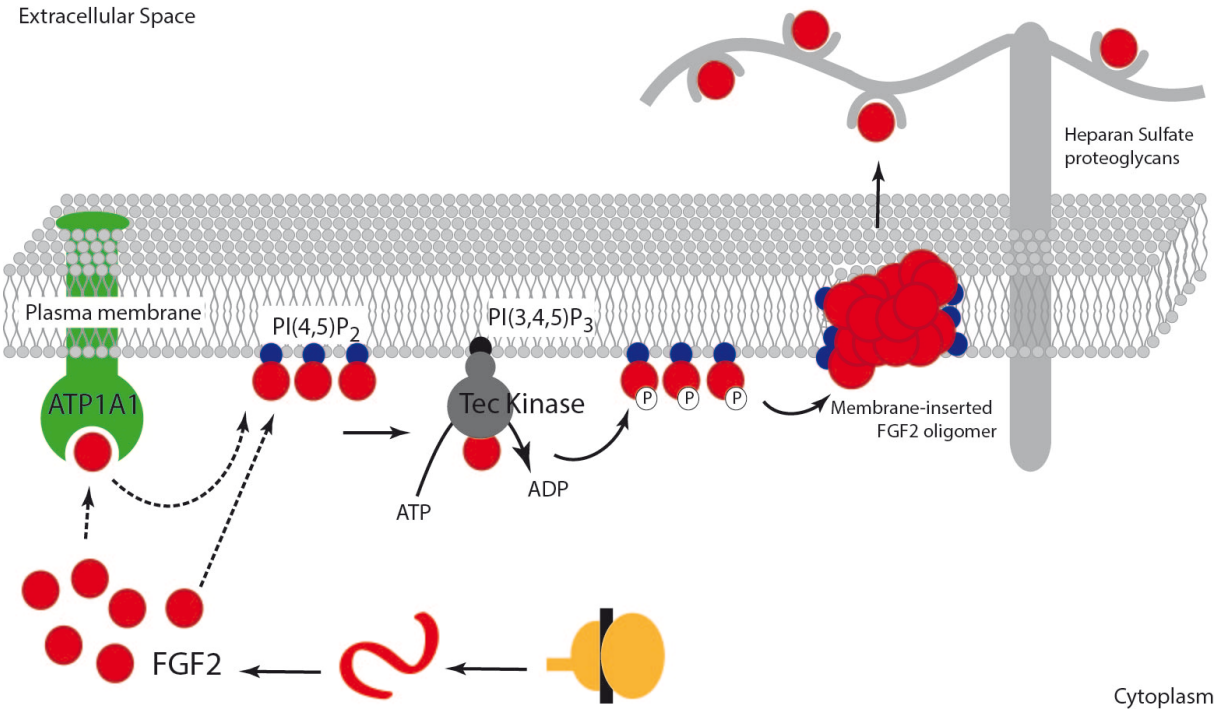


Figure 13. Unconventional secretion mechanism of FGF2. Adapted from "A Direct Role for ATP1A1 in Unconventional Secretion of Fibroblast Growth Factor 2" – Nickel – 2014 - Journal of Biological Chemistry. FGF2 is recruited at the inner leaflet of the plasma membrane through its interaction with ATP1A1 (the α subunit of the Na/K ATPase), the phosphoinositide $PI(4,5)P_2$. Once at the plasma membrane FGF2 is phosphorylated by the Tec kinase and form membrane-inserted oligomers. These oligomers form a pore allowing their translocation as monomers into the extracellular space. Finally, monomers of FGF2 are trapped on heparan sulfate cell surface.

3.2 IL-1ß unconventional secretion

Concerning IL-1ß, multiple mechanisms have been proposed to facilitate its cellular exit (Fig.14). It includes cargo delivery to the extracellular space via secretory lysosomes, microvesicle shedding, exosome release, secretory autophagy (Fig. 12 - Type III UPS), export through the plasma membrane by direct translocation (Fig. 12 - Type I UPS), or through a membrane transporter (Fig. 12 - Type II UPS), and finally passive release during cell lysis.

However, it remains unclear whether all these mechanisms are physiologically relevant and if it's the case, why multiple mechanisms of IL-1ß release exist for the secretion of only one cytokine.

Here, I will briefly review the various mechanisms that have been proposed for IL-1ß secretion by monocytic cells during this past thirty years (Fig. 14).

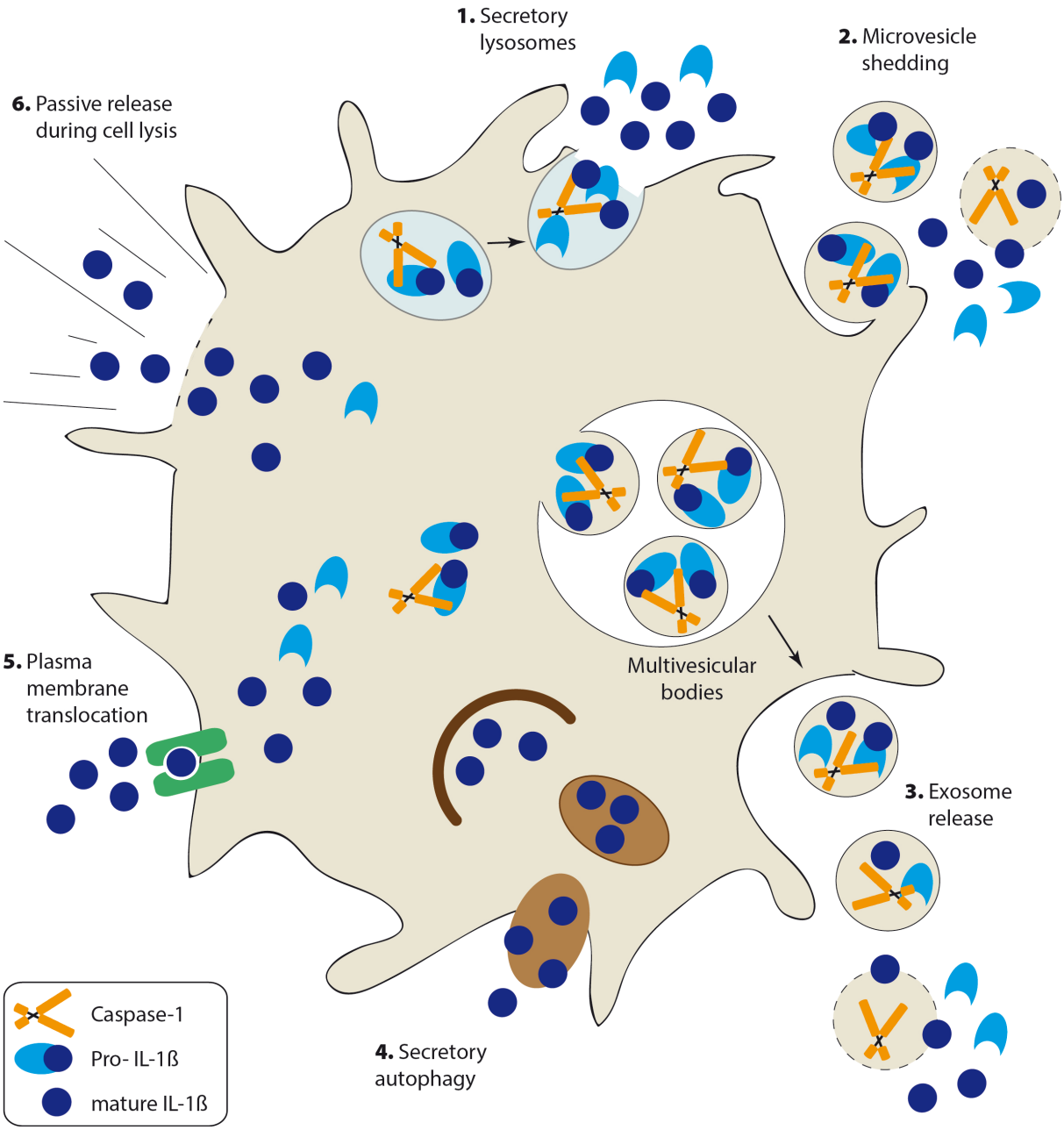


Figure 14. Proposed mechanisms underlying the unconventional secretion of IL-1ß. Adapted from "Mechanisms of unconventional secretion of IL-1 family cytokines" – Schroder – 2015 – Cytokine. Representation of the many pathways proposed to mediate unconventional secretion of IL-1ß: **(1)** Pro-caspase-1 and pro-IL-1ß are translocated into secretory lysosomes, in which pro-IL-1ß is cleaved by caspase-1 and released by lysosome exocytosis. **(2)** Pro-IL-1ß and pro-caspase-1 are packed into microvesicles, which are shed from the plasma membrane. Caspase-1 then becomes activated and cleaves IL-1ß, which is delivered to the extracellular space upon the lysis of the microvesicle. **(3)** Pro-IL-1ß and pro-caspase-1 are packed into multivesicular bodies, and released by the cell within exosomes. **(4)** IL-1ß translocate into autophagy vesicle via an unkown mechanism and delivered to the extracellular space. **(5)** Mature IL-1ß pass through the the plasma membrane via an unknown plasma membrane transporter. **(6)** Mature IL-1ß is passively released upon caspase-1-dependent cell lysis (pyroptosis).

3.2.1 Secretory lysosomes, microvesicles, and exosomes

3.2.1.1 Secretory Lysosomes

The secretory lysosome-dependent pathway was proposed by Rubartelli and colleagues (Andrei et al., 1999) (Fig. 14-1). In LPS-activated primary human monocytes, they observed a co-localization of pro-IL-1ß and caspase-1 with the endo-lysosomal hydrolase cathepsin D and the lysosomal membrane protein Lamp-1, suggesting endo-lysosomal vesicles IL-1ß localization. They also observed that the release of mature IL-1ß from ATP treated monocytes occurred with cathepsin D but without pro-IL-1ß secretion (Andrei et al., 1999). Additionally, they reported that disruption of microtubules with nocodazole impaired IL-1ß release from ATP-stimulated monocytes, indicating that functional microtubule network is essential for IL-1ß secretion (Carta et al., 2006). All their data support the hypothesis that both pro-IL-1ß and caspase-1 are transported into secretory lysosomes, in which mature IL-1ß was generated before lysosome exocytosis and IL-1ß release (Sitia and Rubartelli, 2018).

3.2.1.2 Microvesicles

Extracellular vesicles have been proposed to mediate ATP-dependent IL-1ß secretion (Fig. 14-2). Studies on THP-1 monocytes (MacKenzie et al., 2001) microglia (Bianco et al., 2005) and human dendritic cells (Pizzirani et al., 2007), reported that ATP induced IL-1ß containing microvesicles shedding. MacKenzie and co-workers show that THP-1 shed microvesicles from their plasma membrane within 2–5 sec of P2X7 receptors activation. Additionally, two minutes after such stimulation, the released microvesicles contained bioactive IL-1ß, which was detectable in the supernatants only several minutes after microvesicles shedding and subsequent lysis (MacKenzie et al., 2001). The mechanism by which mature IL-1ß is released

from theses microvesicles is still controversial either via an unknown Ca^{2+} dependent mechanism (Bianco et al., 2005) or followed by ATP-induced lysis of microvesicles (Pizzirani et al., 2007).

3.2.1.3 Exosomes

An alternative vesicular model of IL-1ß secretion was proposed in 2007 involving exosomes (Fig. 14-3) (Qu et al., 2007). This model proposes that the P2X7R stimulation induces formation of multivesicular bodies that contain exosomes with entrapped IL-1ß, caspase-1, and other inflammasome components. Because exosomes released from immune cells were previously reported to contain MHC-II (Fevrier and Raposo, 2004), the co-secretion of IL-1ß with MHC class II molecules suggested that IL-1ß may use another type of vesicular delivery than those previously described (Qu et al., 2007).

3.2.2 Secretory autophagy

Autophagy is generally defined as a catabolic process that sequesters cytoplasmic material in specific structures, called autophagosomes, targeting it for lysosomal degradation. Recently, the involvement of autophagy in the process of secretion of the leaderless IL-1ß protein has been extensively studied. However, it remains unclear whether autophagy promotes or inhibits IL-1ß secretion.

Studies suggested that autophagy is essential for IL-1ß secretion (Fig. 14-4). Indeed, siRNA-mediated ATG5 knockdown in neutrophil-differentiated PLB985 cells markedly reduced IL-1β secretion(Iula et al., 2018). Additionally, Dupont et al. showed that starvation strongly enhanced IL-1ß secretion (Dupont et al., 2011). Eventually, in a 293T reconstituted model for

IL-1ß secretion, Schekman and co-workers showed that IL-1ß translocate into an autophagy-mediated vesicle before its export in the extracellular space (Zhang et al., 2015).

In contrast, it has been shown that inhibition of autophagy in macrophages by knock down of Atg7 Atg16L1 promoted the secretion of IL-1ß in response to LPS (Saitoh et al., 2008). Further work showed that pro-IL-1ß was specifically sequestered into autophagosomes for degradation (Harris et al., 2011).

3.2.3 Secretion across plasma membrane

One of the earliest hypothesis was that IL-1ß may directly cross the plasma membrane, either alone or via a ABC membrane transporters (Fig. 14-5) (Singer et al., 1995). Glyburide which is known to block the anion exchanger function of the ABC1 transporter was reported to inhibit IL-1ß secretion, suggesting a role of ABC1 transporter in the export of the cytokine (Hamon et al., 1997b) . However, further experiments showed that glyburide inhibited the processing of pro-IL-1ß explaining its effect the amount of secreted mature IL-1ß (Lamkanfi et al., 2009).

3.2.4 Cell death

A number of studies have highlighted the strong correlation between caspase-1 activation, IL-1ß secretion and cell lysis favouring the hypothesis that IL-1ß is passively released during cell lysis (Fig. 14-6) (He et al., 2015; Hogquist et al., 1991; Liu et al., 2016; Shi et al., 2015a; Shirasaki et al., 2014; Zychlinsky et al., 1994; Zychlinsky et al., 1992). Cytokine secretion at the single cell level was monitored in real-time by fluorescence microscopy and revealed the

tight temporal coupling events of loss of plasma membrane integrity and IL-1ß release (Shirasaki et al., 2014), indicating that all IL-1ß producing monocyte/macrophages finally died. However, these studies do not demonstrate that the loss of plasma membrane integrity is required for IL-1ß secretion, suggesting that IL-1ß secretion and cell lysis might not be concomitant but successive events.

Recently, several groups revealed a mechanism of IL-1ß release which involved plasma membrane pores formed by cleaved gasdermin-D, associated to pyroptotic cell death (He et al., 2015; Shi et al., 2015b). In this setting, it is not clear whether IL-1ß is secreted through the pores of the gasdermin D pore or in the course of plasma membrane rupture (Evavold et al., 2018).

Pyroptosis or caspase-1 dependent cell death is a highly inflammatory form of programmed cell death resulting from inflammatory caspase (caspase-1, -4, -5 and -11) activation. The term pyroptosis from the Greek roots 'Pyro', meaning fire or fever, reflects the inflammatory nature of this form of cell death, and 'ptosis', meaning falling, matches the terms used for other forms of programmed cell death (Cookson and Brennan, 2001).

Pyroptosis is a mechanism of defense against intracellular infection. It eliminates the infected cell, by removing the protective niche of the pathogen, and simultaneously eliciting the host's inflammatory response. During pyroptosis, the intracellular bacteria is not killed but trapped within the corpse of the pyroptotic cell structure, called the pore-induced intracellular trap (PIT). The PIT jails the bacteria and coordinates innate immune responses via complement and scavenger receptors to drive recruitment of neutrophils that engulf and kill the trapped bacteria when they efferocytose the PIT (Jorgensen et al., 2016).

Pyroptosis involves pore formation in the plasma membrane, cell swelling resulting in rupture of the membrane and the release of cytosolic contents into the extracellular space (Fig. 15) (Bergsbaken et al., 2009). Whereas pyroptosis was described for the first time in 1992 (Hogquist et al., 1991), little was known about the mediators driving this lytic form of cell death. It is only in 2015 that two independent groups identified gasdermin D as the link between activation of inflammatory caspases and the pyroptotic cell death. Gasdermin D was found to be a direct target of the inflammatory caspase-1 and the executioner of pyroptotic cell death (Kayagaki et al., 2015; Shi et al., 2015b).

Gasdermin D (GSDMD) is a cytosolic protein containing two domains, namely the N-terminal domain, also termed as the pore forming domain, GSDMD-N and the repressor C-terminal domain. The N- and C-terminal domains are separated by a linker peptide. Upon proteolytic cleavage of the linker peptide by the inflammatory caspases (caspase-1, -4, -5, -11), the GSDMD-N integrates into the cell membrane, where an estimated 16 monomers oligomerize to form a large 10–15-nm diameter pore (Fig. 15) (Aglietti et al., 2016; Ding et al., 2016; Sborgi et al., 2016). Following permabilization of the plasma membrane by the pore of GSDMD, ions and water rush into the cell causing cell swelling and cell lysis, resulting in the release of cytosolic contents (Kayagaki et al., 2015).

Reconstitution studies using liposomes showed that GSDMD-N displays a preferential affinity with cardiolipin, phosphatidylinositol (PI) 4-phosphate and PI 4,5-bisphosphate (Liu et al., 2016; Sborgi et al., 2016). As a result, GSDMD-N forms pores in the plasma membrane only when exposed to the cytosolic PI-containing inner leaflet, and not when exposed to the extracellular outer leaflet, which lacks PI. This unique feature implies that neighbouring cells are protected from gasdermin D arising from adjacent dying cells.

In murine macrophages, the prevailing view is that IL-1ß is release during pyroptotic cell death (He et al., 2015). Whether it is secreted before, thought the gasdermin D pores, or in the course of plasma membrane rupture remains to be elucidated (Fig. 15). Most studies reported the absolute necessity of the gasdermin D for IL-1ß secretion (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015b), suggesting that pyroptotic cell lysis is the ultimate fate of IL-1ß producing cells.

Recently, Evavold et al. reported IL-1ß secretion in living murine macrophages (Evavold et al., 2018). In their settings, gasdermin D is essential for IL-1ß secretion but does not induce pyroptotic cell lysis, suggesting a non-pyroptotic role of gasdermin D consisting of transient conduit for IL-1ß export only (Fig. 15).

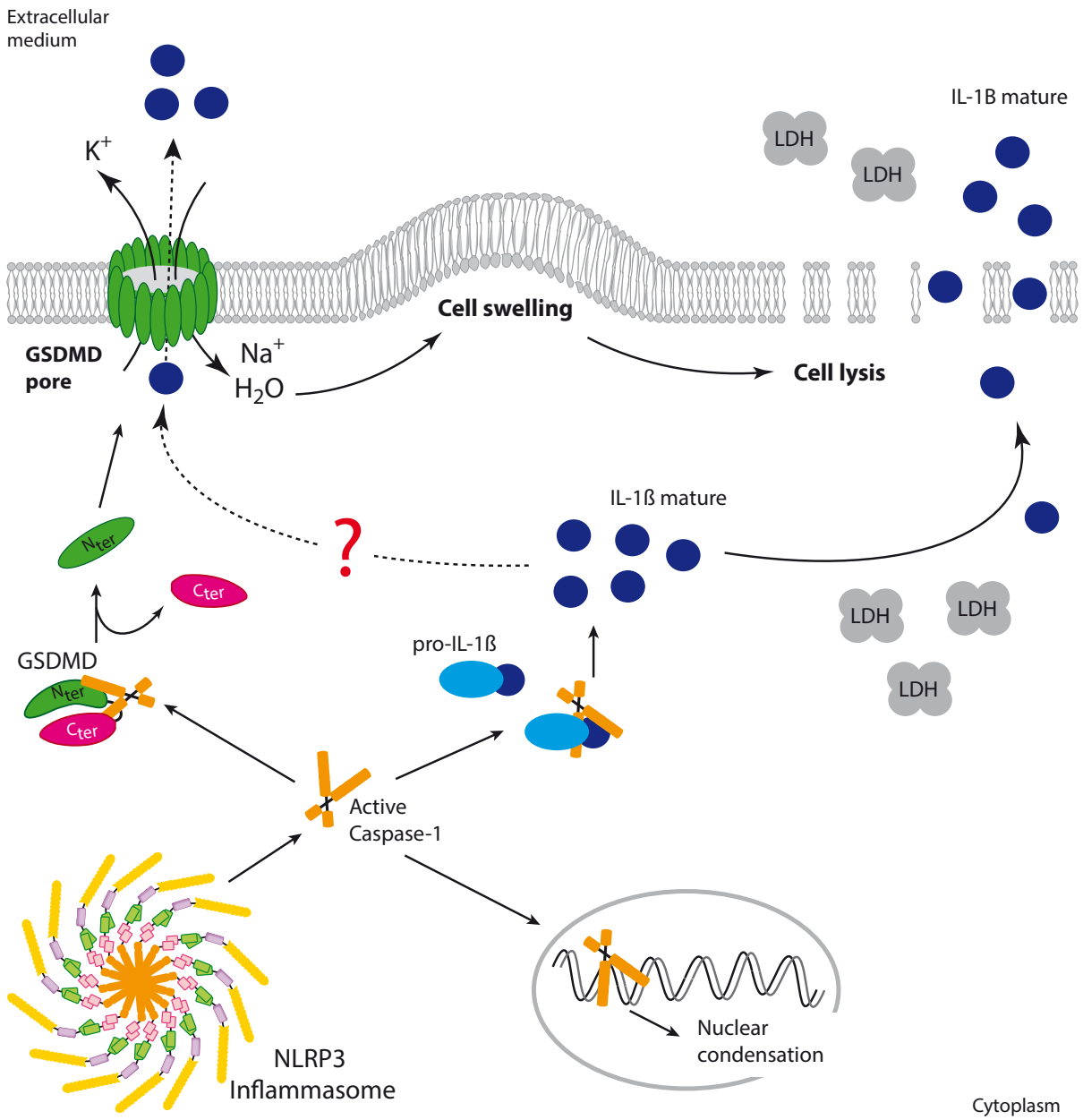


Figure 15. GSDMD executes inflammatory caspases-induced pyroptosis. Adapted from "Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death" - Shao – 2017 – Cell Press. NLRP3 inflamamsome oligomerization activates caspase-1 which subsequently cleave pro-IL-1ß into mature IL-1ß but also cleave GSDMD within the linker between its N-terminal gasdermin-N (green) and C-terminal gasdermin-C (pink) domains to break the auto-inhibitory of the C-terminal domain on the N-terminal domain. The released gasdermin-N domain binds to phosphoinositides in the plasma membrane and oligomerizes to generate 16 monomer membrane pores. The formation of the pores disrupts the osmotic potential, resulting in cell swelling and cell lysis. IL-1ß is released upon cell lysis as well as other cytoplasmic proteins such as LDH. IL-1ß might eventually be secreted thought the GSDMD pores.

Aim of the study

The mechanisms implicated in IL-1ß processing have been extensively studied. It is now well established that IL-1ß is produced first as the inactive 31 kDa precursor pro-IL-1β which after inflammasome activation is cleaved by caspase-1 into the immediately secreted mature IL-1β.

How the leaderless mature IL-1β is secreted remains an active research topic. In 2015, an important progress was made when IL-1ß was shown to be released by cells dying from the inflammatory cell death called pyroptosis, which is triggered by gasdermin D, itself activated by the inflammasome. However, it became clear that IL-1ß can also be secreted by living cells, suggesting that there are at least two distinct secretion pathways for IL-1ß.

The aim of my work was to understand the mechanism of IL-1ß secretion by human monocytic cells stimulated by LPS alone, i.e under conditions in which cells do not undergo pyroptosis. I investigated the role of gasdermin D in IL-1ß secretion by freshly isolated human monocytes and by the human monocytic cell line THP-1, stimulated with LPS alone or with LPS and an inflammasome activator that triggers pyroptosis.

Results

Gasdermin D-Independent IL-1ß Secretion by Living Human Monocytic Cells.

Charlotte Six[1], Nathalie Remy[1], Orian Bricard[1], Emile Van Schaftingen[1], Pierre G. Coulie[1,2,3,4,*], and Tiphanie Gomard[1, 3].

Manuscript submitted in **eLife**

[1] de Duve Institute, Université catholique de Louvain; 1200 Brussels; Belgium.
[2] WELBIO, Université catholique de Louvain; 1200 Brussels; Belgium.
[3] These authors contributed equally
[4] Lead Contact
*Corresponding author: pierre.coulie@uclouvain.be

Abstract

IL-1ß is a leaderless cytosolic protein whose secretion does not follow the classical endoplasmic reticulum-to-Golgi pathway. It is released during pyroptosis, a caspase-dependent inflammatory cell death initiated by gasdermin D cleavage and pore formation at the plasma membrane. Up to now it has been unknown whether gasdermin D is dispensable for IL-1ß secretion, particularly in human cells. Here we report that human blood monocytes stimulated with LPS alone secrete mature IL-1ß without cell death and without gasdermin D cleavage, while they undergo pyroptosis with gasdermin D cleavage when stimulated with LPS and an activator of the NLRP3 inflammasome. In addition, we show that LPS-stimulated human monocytic THP-1 cells secrete IL-1ß without cell death, even after invalidation of the gasdermin D-encoding gene. These findings point to the existence of a pathway of IL-1ß secretion that is both cell death-independent and gasdermin D-independent.

Keywords

IL-1ß secretion, gasdermin D, human monocytes, pyroptosis, THP-1

Introduction

Interleukin (IL)-1ß is a highly inflammatory cytokine that plays a key role in immune homeostasis by orchestrating innate and adaptive immune responses to pathogens. IL-1ß secretion is observed also in auto-inflammatory diseases such as atherosclerosis, rheumatoid arthritis, gout or type 2 diabetes (Dinarello, 2011). IL-1ß is mainly secreted by myeloid cells such as monocytes, macrophages or dendritic cells, through a tightly regulated multistep process. A first signal provided by microbial or danger-associated molecules induces the expression of gene *IL1B*, which encodes pro-IL-1ß (31 kDa), the inactive precursor of IL-1ß. The pro-IL-1ß N-terminal propeptide needs to be cleaved by caspase-1 to generate the mature and biologically active IL-1ß (17 kDa) (Martinon and Tschopp, 2004). This maturation of IL-1ß requires a second signal, which induces the formation of inflammasome complexes and subsequent caspase-1 activation. Mature IL-1ß is then secreted into the extracellular space where it exerts pro-inflammatory functions by binding to ubiquitously expressed high affinity receptors (Dinarello, 2009).

The inflammasomes that are important for IL-1ß maturation are cytosolic protein complexes composed of a Nod-like receptor (NLR), the adaptor protein ASC (PYCARD) and the effector protein pro-caspase-1(Martinon et al., 2002). Inflammasome assembly and activation occur upon NLR sensing danger signals, which differ according to the NLR. The most extensively studied inflammasome contains the protein NLRP3. After activation, assembled NLRP3 are bridged to pro-caspase-1 by ASC, leading to caspase-1 trans-autocleavage and activation (Lu et al., 2014). NLRP3 inflammasomes are activated by a variety of microbial or sterile cellular stress signals, including high concentrations of extracellular ATP (Mariathasan

et al., 2006), bacterial pore-forming toxins such as the ionophore nigericin (Mariathasan et al., 2006) or vaccine adjuvants (Eisenbarth et al., 2008; Kool et al., 2008).

IL-1ß has no signal peptide and therefore cannot transit through the ER-Golgi conventional secretory pathway. Several mechanisms have been proposed for the unconventional secretion pathway of mature IL-1ß. They include secretory lysosomes (Andrei et al., 1999; Andrei et al., 2004), multivesicular bodies and exosomes (Denzer et al., 2000; Hamon et al., 1997a), shedded microvesicles (Bianco et al., 2005), secretory autophagy (Dupont et al., 2011), direct translocation through a membrane transporter (Hamon et al., 1997a), passive release during apoptosis (Hogquist et al., 1991) and, more recently, release during pyroptosis (Kayagaki et al., 2015). Pyroptosis is a lytic programmed cell death initiated by inflammasomes. It involves pore formation in the plasma membrane, cell swelling, rupture of the plasma membrane and release of cytosolic contents into the surrounding media. The only known effector of pyroptosis is the protein gasdermin D, which acquires pore-forming activity after its cleavage by caspase-1. This cleavage releases the C-terminal repressor domain from the p30 N-terminal pore forming domain. The latter has a high affinity for negatively charged lipids such as phosphatidylinositol phosphates and phosphatidylserine which are restricted to the plasma membrane inner leaflet (Liu et al., 2016). In membranes, the pore-forming domain of gasdermin D oligomerizes to form pores with an inner diameter of about 13 nm (Gaidt et al., 2016; Liu et al., 2016). It is not yet totally clear whether mature IL-1ß, with a diameter of 4.5 nm (Finzel et al., 1989; Priestle et al., 1989), can be secreted by cells through the gasdermin D pores or is only released after plasma membrane rupture during pyroptosis (Heilig et al., 2018).

Our interest in IL-1ß stems from its role in the recruitment of immune cells, including T lymphocytes, into tumors. Many current cancer immunotherapies consist in injecting, inducing or increasing the activity of tumor-specific T cells. While these treatments are remarkably efficient in sizeable proportions of patients with various types of cancer, they are inefficient in most patients with 'cold' tumors, i.e. that do not contain immune cells prior to therapy. Thus, we set out to examine IL-1ß secretion by human myeloid cells, namely blood monocytes. While the involvement of inflammasomes to generate mature IL-1ß in stimulated human monocytes is well established, the pathway of IL-1ß secretion by these cells is unknown. Like other investigators but with different stimulation conditions, we realized that human monocytes could secrete large amounts of IL-1ß without undergoing cell death during or after this secretion. Such results challenge the consensus view based on studies carried out with murine cells, that the main pathway for IL-1ß secretion is gasdermin D-mediated pyroptosis. We set out to study the role of gasdermin D in IL-1ß secretion by living human monocytic cells.

Results

Stimulation of human monocytes with LPS alone leads to inflammasome activation, pro-IL-1ß cleavage by caspase-1 and secretion of mature IL-1ß (Gaidt et al., 2016; Netea et al., 2009; Piccini et al., 2008; Vigano et al., 2015). But it is different with murine cells: IL-1ß secretion is not obtained with LPS alone but requires that a second signal activates the inflammasome. Most studies of IL-1ß secretion have been conducted in murine cells stimulated with LPS and inflammasome activators. It is under these conditions that the role of gasdermin D in pyroptosis was discovered. It is intriguing that, while murine cells undergo pyroptosis when secreting IL-1ß after LPS and inflammasome activation, human monocytes stimulated with LPS alone can secrete IL-1ß without massive cell death. In this context, we conducted a careful side by side comparison of primary human monocytes stimulated with LPS either alone or with an additional inflammasome activator, for IL-1ß secretion, gasdermin D cleavage and cell death.

Low concentrations of LPS induce *IL1B* expression in primary human monocytes

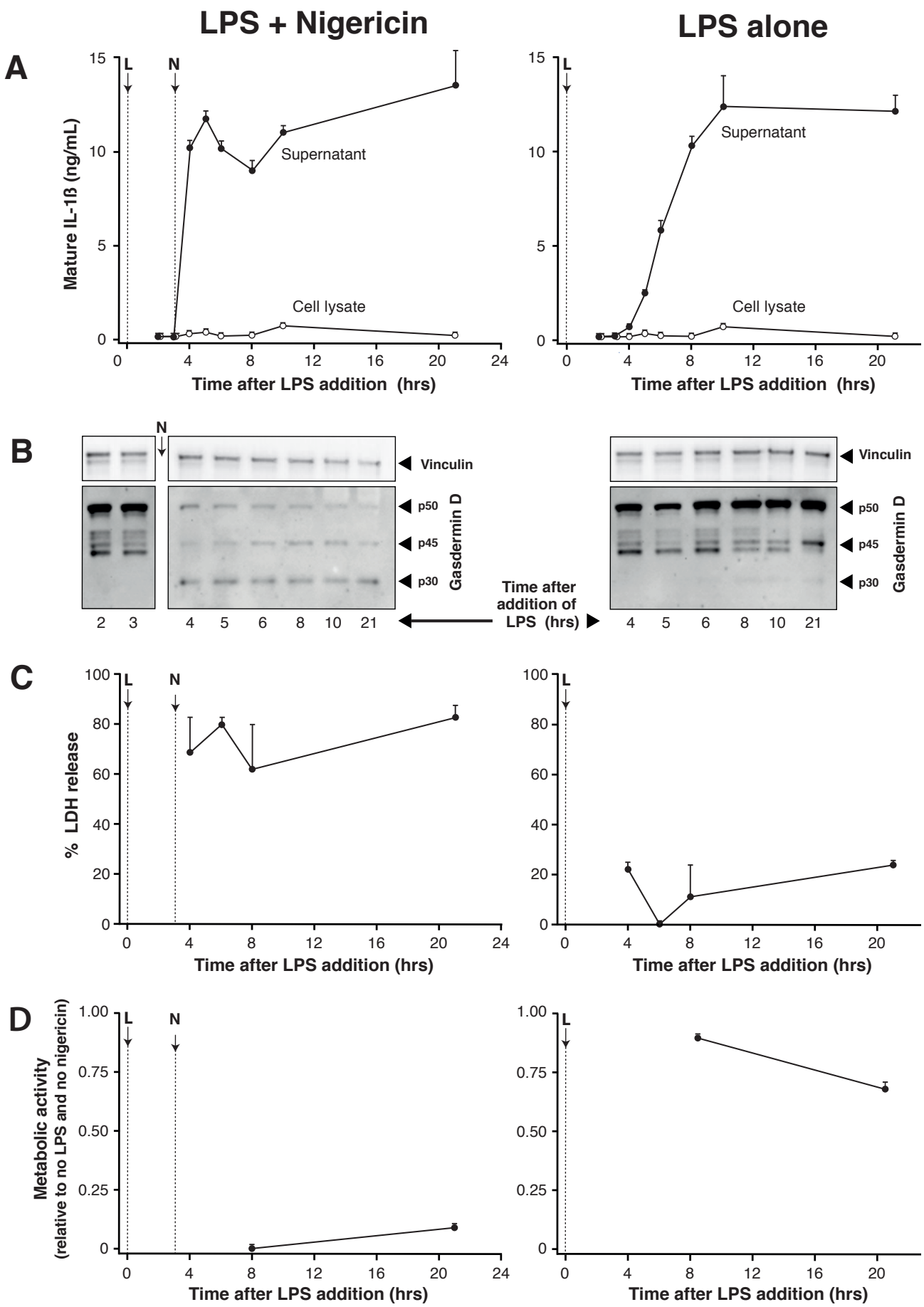
The human monocytes that we stimulated with LPS were enriched from blood using column-free negative selection. In our hands, commonly used positive selection with magnetic microbeads often lead to detectable cell death and IL-1ß release or secretion in control 'unstimulated' conditions. LPS binds to the surface Toll-like receptor 4 (TLR4)-MD2 complex which triggers *IL1B* expression. Moreover, it is known that LPS introduced into the cytoplasm of murine or human cells triggers pyroptosis by directly activating caspase-11 or its human ortholog caspase-4 (Kayagaki et al., 2013; Shi et al., 2014). To minimize monocyte death resulting from aberrant LPS transfer into the cytoplasm we titrated down ultrapure LPS and observed that with 10 ng/mL the kinetics and levels of *IL1B* expression were similar to those obtained with the more classically used concentration of 1 µg/mL (Fig. S1). Noteworthy, 10 ng/mL of LPS were inefficient in serum-free medium unless LPS- Binding Protein (LBP) was added (data not shown). LBP forms a high affinity complex with LPS and facilitates its transfer to the co-receptor CD14, which then transfers the LPS to TLR4-MD2 (Ryu et al., 2017). In medium supplemented with serum, similar *IL1B* inductions were obtained with or without LBP (Fig. S1). For all experiments of this report 'LPS' stands for 10 ng/mL LPS + 10 ng/mL LBP.

Human monocytes stimulated with LPS, with or without an inflammasome activator, secrete mature IL-1ß

Monocytes were stimulated by LPS followed or not 3h later by nigericin, a bacterial exotoxin and ionophore. Nigericin induces a potassium efflux that activates the NLRP3 inflammasome (Munoz-Planillo et al., 2013). We used also extracellular ATP, another strong activator of the NLRP3 inflammasome. We obtained similar results with either of these activators and only show here the nigericin results.

With monocytes stimulated with LPS followed by nigericin, no mature IL-1ß was secreted prior to nigericin addition (Fig. 1A, left panel), even though *IL1B* was transcribed at a high level (Fig. S1). A large amount of mature IL-1ß was secreted immediately after the addition of nigericin (Fig. 1A). One hour after nigericin addition, the concentration of IL-1ß in the supernatant reached a plateau, maintained for at least 20 h (Fig. 1A). To assess the efficiency of the secretion process we measured the concentrations of mature IL-1ß present inside the cells (Fig. 1A). In order to be able to directly compare the amounts of secreted and intracellular mature IL-1ß, the cells were harvested and lysed in the same volume of medium as that used during secretion.

Figure 1. Human blood monocytes can either release IL-1ß during pyroptosis or secrete IL- 1ß in the absence of pyroptosis and of GSDMD cleavage. Freshly isolated human blood monocytes were stimulated with LPS (L) for 3 hours, and then treated or not with nigericin (N) over 18 hours. **(A)** Mature IL-1ß was measured in supernatants and in cell lysates by ELISA. **(B)** GSDMD cleavage was analyzed by immunoblot at different time points after LPS stimulation with or without nigericin. **(C)** LDH released in the supernatants was quantified with an enzymatic assay. **(D)** Metabolic activity was assessed with a MTS/PMS assay and expressed relative to that of unstimulated (neither with LPS nor with nigericin) cells. Data are represented as means of biological triplicates ± SEM. Similar results were obtained with monocytes of two other donors (Fig. S2 and S3).


LPS + Nigericin
LPS alone
A
Mature IL-1ß (ng/mL)
L
N
Supernatant
Cell lysate
Time after LPS addition (hrs)
B
Vinculin
p50
p45
p30
Gasdermin D
Time after addition of LPS (hrs)
2 3 4 5 6 8 10 21
4 5 6 8 10 21
C
% LDH release
Time after LPS addition (hrs)
D
Metabolic activity
(relative to no LPS and no nigericin)
Time after LPS addition (hrs)

Before nigericin addition, no mature IL-1ß was produced in the cells. Two hours after nigericin addition, we measured 11.7 ng/mL of IL-1ß in the medium and 0.4 ng/mL in cell lysates. Thus, virtually all the intracellular mature IL-1ß had been secreted, namely 97% of 24.2 ng per million cells. These results indicated that all the molecules of mature IL-1ß produced by the inflammasome were subsequently secreted. As expected, while pro-IL-1ß was readily measured with ELISA in the lysates of the LPS-stimulated monocytes before the addition of nigericin, it became undetectable 2 h later (data not shown). We concluded that the maturation of IL-1ß by caspase-1 and the secretion of mature IL-1ß were two very efficient processes in monocytes stimulated with LPS and nigericin.

With monocytes stimulated with LPS alone, the total amount of secreted mature IL-1ß was similar to that obtained with LPS+nigericin (Fig. 1A, right panel). A plateau of about 20 ng of IL-1ß secreted by 10^6 cells was reached 10 h after stimulation (Fig. 1A). The efficiency of the secretion process was also similar to that observed with LPS+nigericin, with more than 95% of mature IL-1ß secreted within 10 h (Fig. 1A).

These results indicated that the quantities of IL-1ß secreted by human monocytes stimulated either with LPS alone or with LPS and an inflammasome activator were identical. However, the durations of secretion were different: less than one hour with LPS+nigericin and about 7h with LPS alone.

In contrast to monocytes stimulated with LPS and an inflammasome activator, monocytes stimulated with LPS alone secrete IL-1ß without gasdermin D cleavage and without undergoing cell death

Next, we analysed gasdermin D cleavage and cell death. Gasdermin D cleavage was assessed with Western blot, with the detection of the non-cleaved gasdermin D (p50) and of the cleaved N-terminal pore-forming domain (p30). For cell death, we measured lactate dehydrogenase (LDH) activity in the supernatants and assessed metabolic activities of the cells with a tetrazolium reduction assay. When monocytes were stimulated with LPS and nigericin, gasdermin D cleavage was clearly detected, with the presence of the p30 fragment and decrease of the p50 protein (Fig. 1B, left panel). Gasdermin D cleavage occurred only after the addition of nigericin. The pore-forming domain (p30) was detected already 1h after nigericin addition, and consistently at later time points (Fig. 1B). Massive LDH release was detected also 1h after nigericin addition (Fig. 1C, left panel). Cell death was confirmed with null or very low metabolic activities (Fig. 1D, left panel). Similar results were obtained with the monocytes of two other donors (Fig. S2 and S3).

When monocytes were stimulated with LPS alone, there was no detectable gasdermin D p30 pore-forming domain and the amounts of gasdermin D p50 were unchanged throughout the experiment (Fig. 1B, right panel). LDH release in the medium was minimal and in the range of the limit of detection of the enzymatic assay (Fig. 1C, right panel). The metabolic activities of the cells remained similar to those of unstimulated cells: 70-90% for the cells analysed in Fig. 1D (right panel) and close to 100% for the monocytes of the two other donors tested (Fig. S2 and S3).

We concluded that human monocytes stimulated with LPS and nigericin release mature IL-1ß while dying from pyroptosis. Even though gasdermin D cleavage has not been documented in human monocytes, these results are in line with what has been shown in murine cells. On the contrary, we observed neither gasdermin D cleavage nor cell death with monocytes stimulated by LPS alone. Thus, in addition to pyroptosis, primary human monocytes have a cell death-independent and gasdermin D-independent pathway of IL-1ß secretion. Remarkably, the amounts of IL-1ß released or secreted by both pathways are identical.

As a first step in the analysis of this other pathway of unconventional secretion of IL-1ß, we wished to explore its independency from gasdermin D. First, because gasdermin D could possibly be involved in IL-1ß maturation or secretion otherwise than through pore formation by the p30 fragments. Second, because low p30 levels undetected in Western blots could suffice for IL-1ß secretion. Indeed, several authors have discussed the possibility that mature IL-1ß could exit the cells through the gasdermin D pores themselves and that the latter could be transient or rare and not necessarily cause pyroptosis (Kovacs and Miao, 2017). Thus, we set out to inhibit *GSDMD* gene expression in primary human monocytes. However, we realized that when these cells were cultured for 21 h or more prior to stimulation with LPS alone, their IL-1ß secretion plummeted to less than 2% of that obtained with freshly isolated cells (Table S1). Moreover, the levels of intracellular mature IL-1ß were also very low (Table S1) and nigericin added 3 h after LPS did stimulate secretion, indicating that pro-IL-1ß was present. We concluded that LPS alone did not anymore activate the inflammasome in cultured primary human monocytes. We obtained similar results with various culture media and plasticware, including non-adherent conditions (Table S1).

To the best of our knowledge the reason for the dramatic drop of inflammasome activation by LPS alone between freshly isolated and overnight cultured human monocytes is not known. We considered that using siRNAs or CRISPR/Cas9 to explore the importance of gasdermin D on this residual IL-1ß secretion by primary monocytes was inappropriate. We resorted to using a human monocytic cell line to carry out CRISPR/Cas9 *GSDMD* invalidations.

PMA-differentiated THP-1 cells stimulated with LPS alone secrete IL-1ß

The spontaneously immortalized cell line THP-1, derived from the blood of a patient with acute monocytic leukemia, is the most studied human monocytic cell line (Tsuchiya et al., 1980). However, the process of IL-1ß secretion by THP-1 stimulated with LPS alone has not been studied and for some investigators THP-1 is not a good model for such a work (Gaidt et al., 2016). When we stimulated THP-1 under the same conditions as our primary monocytes, namely with LPS at 10 ng/ml and LBP at 10 ng/ml, the level of *IL1B* transcription was very low, about 50-100-fold lower than in monocytes (Fig. 2A and Fig. S1).

The response of THP-1 to LPS is usually studied after a treatment with PMA, which induces a differentiation into adherent macrophage-like cells (Park et al., 2007). Among the numerous differentiation protocols that have been described we selected 3 h of PMA at 300 ng/mL followed by 18 h of culture in fresh medium before stimulation with LPS/LBP over 24 h (Grahames et al., 1999). In our hands, this procedure lead to minimal cell death, a key consideration in our study. This PMA treatment of THP-1 induced *IL1B* transcription (Fig. 2A) and gene *IL1B* was actually one of the most induced and most expressed of a set of genes encoding proteins involved in IL-1 family response to LPS (Fig. 2B). Noteworthy, no mature IL-1ß was detected in our PMA-treated THP-1 cells or their supernatants (Fig. 3A, 24h, 'Without LPS'), indicating the absence of inflammasome activation. When the PMA-differentiated THP-1 cells were stimulated with LPS alone, mature IL-1ß was secreted (Fig. 3A). The comparison of the amounts of mature IL-1ß either secreted or still present inside the cells indicated that, like in primary monocytes stimulated with LPS, the efficiency of the IL-1ß secretion pathway was very high (Fig. 3A).

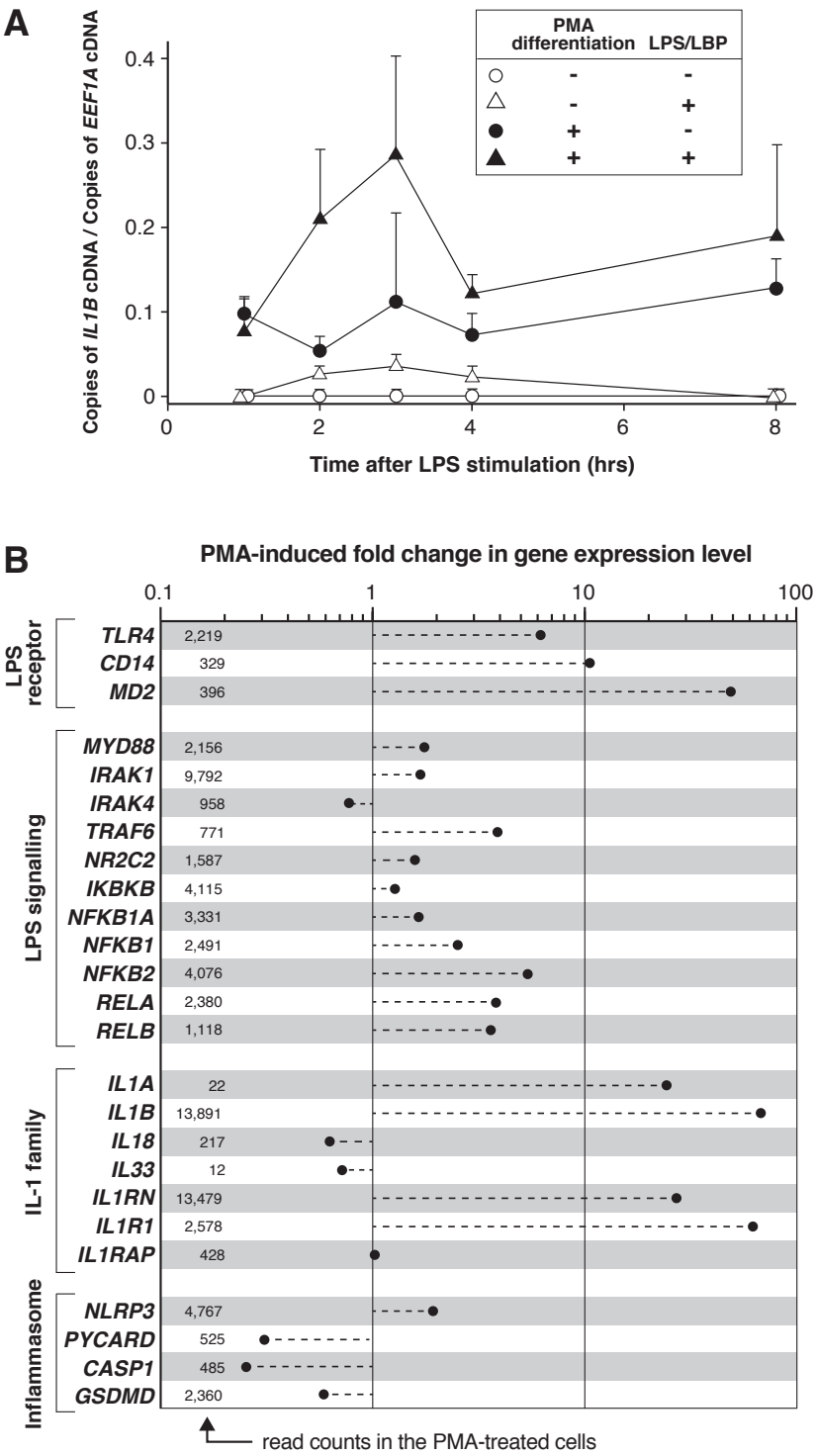


Figure 2. IL1B transcription in PMA-treated THP-1 cells. (A) THP-1 cells were treated or not with PMA for 3 hours, washed, further differentiated for 18 hours and then treated or not with LPS/LBP over 8 hours. IL1B expression was measured by RT- qPCR and normalized to EEF1A1 expression. Data are represented as means of biological triplicates ± SEM. This panel is a representative experiment of at least 3 repeats. **(B)** THP-1 cells were treated or not with PMA for 3 hours, washed, further differentiated for 18 hours and incubated for 24 hours without LPS. Gene expression profiles were analyzed by RNASeq. Levels of expression (read counts) in the PMA-treated cells are indicated to the right of the gene names.

We concluded that, at least under these experimental conditions, a pathway of IL-1ß secretion can be studied in THP-1 cells treated with PMA and stimulated with LPS and no other inflammasome activator. In this model PMA induced *IL1B* transcription but did not activate the inflammasome while LPS did little to *IL1B* transcription but activated the inflammasome.

PMA-treated and LPS-stimulated THP-1 secrete IL-1ß without undergoing cell death

In these THP-1 cells, treated with PMA and stimulated with LPS, IL-1ß secretion was not accompanied by a detectable cleavage of gasdermin D (Fig. 3B) or by LDH release (Fig. 3C). The metabolic activity of the stimulated THP-1 cells remained intact 24 h after LPS stimulation (Fig. 3C), excluding that a sizeable fraction of the cells had died without releasing LDH. Thus, like primary human monocytes, THP-1 can secrete IL-1ß without undergoing pyroptosis or another form of cell death.

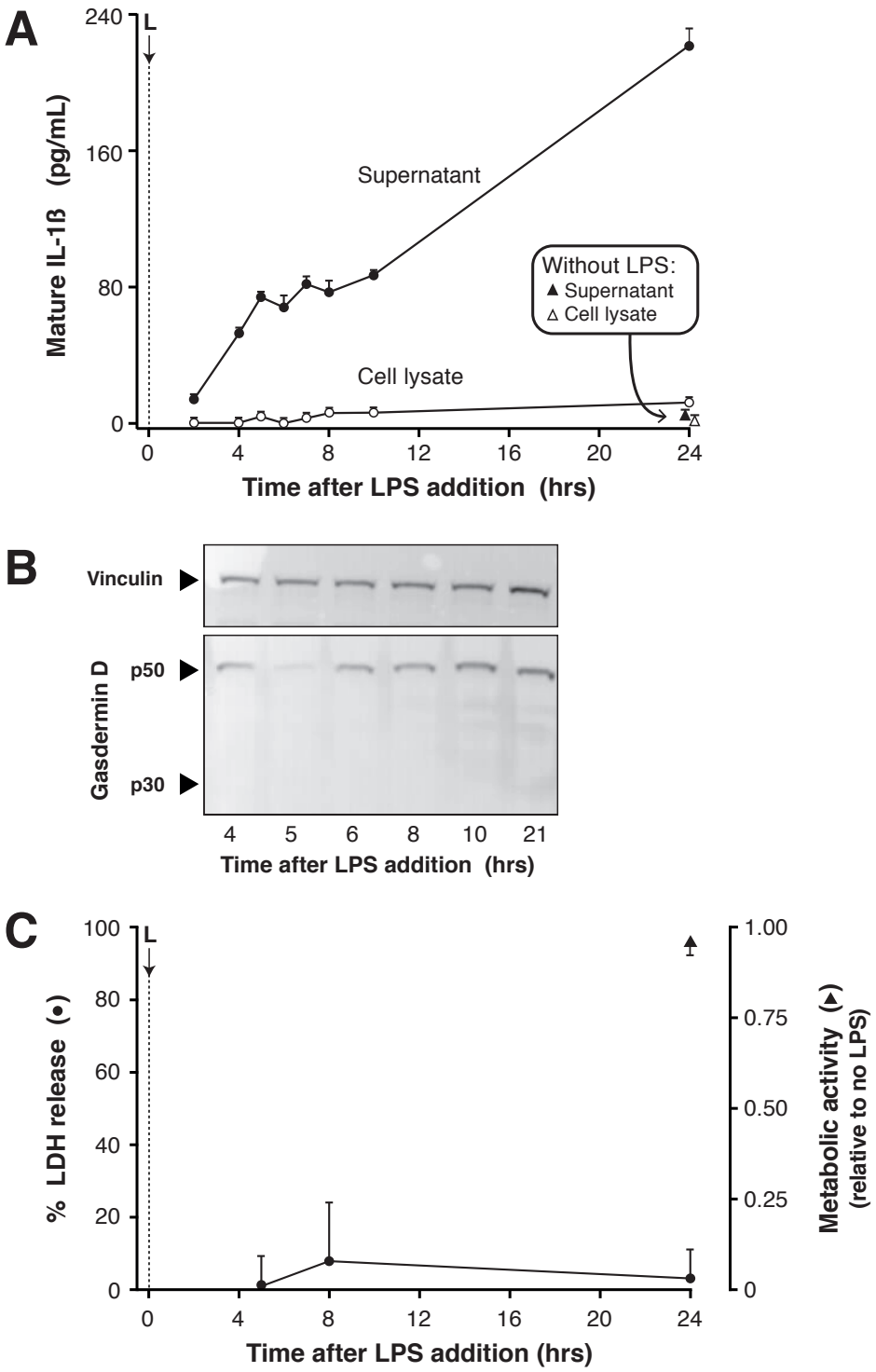


Figure 3. PMA-differentiated THP-1 can secrete IL-1ß in the absence of pyroptosis and of GSDMD cleavage. PMA-differentiated THP-1 were stimulated with LPS (L) over 24 hours. **(A)** Mature IL-1ß was measured in supernatants and in cell lysates by ELISA. **(B)** GSDMD cleavage was analysed by immunoblot at different time points after LPS stimulation. **(C)** LDH released in the extracellular media was quantified with an enzymatic assay. Metabolic activity was assessed with a MTS/PMS assay and expressed relative to that of PMA-treated cells not stimulated with LPS. Data are represented as means of biological triplicates ± SEM. Each panel is a representative experiment of at least 3 repeats.

IL-1ß secretion by PMA-treated and LPS-stimulated THP-1 does not require gasdermin D

Next, we targeted *GSDMD* in THP-1 with lentiviral CRISPR/Cas9 constructs and selected three clones with undetectable gasdermin D on immunoblots and with *GSDMD* gene invalidations demonstrated with RNAseq data (Fig. S4). We pooled these 3 clones for our experiments. As controls we produced THP-1 cells with invalidations of the gene encoding ß2-microglobulin. Immunoblotting indicated that the *B2M* knockout cells contained similar amounts of gasdermin D as the parental cells (data not shown).

The *B2M* and *GSDMD* knockout cells were differentiated with PMA and stimulated with LPS. The *B2M* knockout cells secreted IL-1ß without signs of death (Fig. 4A and 4B, left panels), like the parental THP-1. The *GSDMD* knockout THP-1 did similarly secrete IL-1ß without LDH release or loss of metabolic activity (Fig. 4A and 4B, right panels). These results demonstrated unambiguously that THP-1 secreted mature IL-1ß through a gasdermin D-independent and cell death-independent pathway.

Noteworthy, the amounts of mature IL-1ß secreted by the *GSDMD* knockout THP-1 were consistently higher than those secreted by the *B2M* knockout THP-1 (Fig. 4A). In addition, we consistently observed an initial accumulation of mature IL-1ß inside the *GSDMD* knockout cells prior to a decrease concomitant with secretion (Fig. 4A, right panel). We have never observed such an accumulation in primary monocytes or control THP-1. The simplest explanation is a more active inflammasome in the absence of gasdermin D, together with a pathway of IL-1ß secretion that after LPS is not immediately active or initially saturated. This pathway is yet to be defined

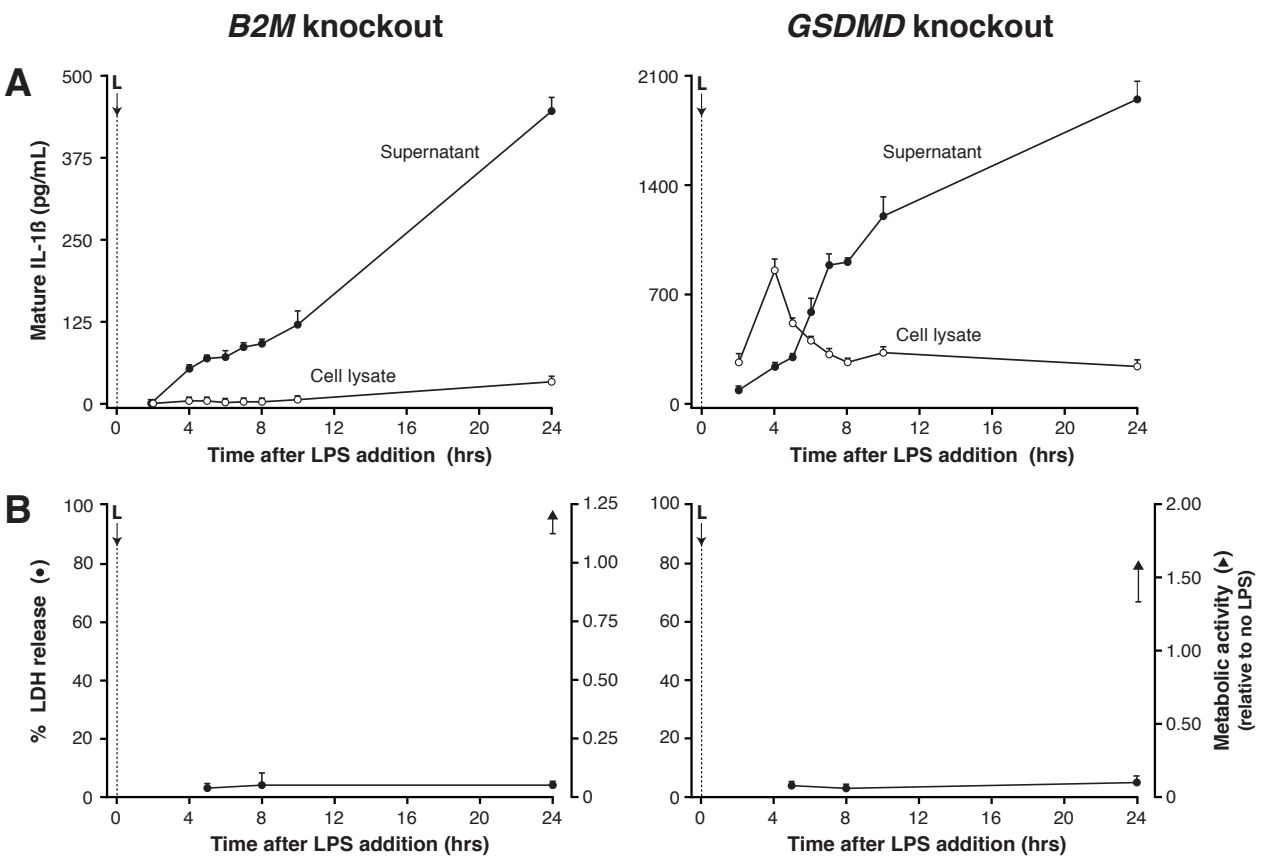


Figure 4. IL-1ß secretion by PMA-treated and LPS-stimulated THP-1 does not require gasdermin D. PMA-differentiated B2M knockout or GSDMD knockout (pool of 3 clones) THP-1 were stimulated with LPS (L) over 24 hours. **(A)** Mature IL-1ß was measured in supernatants or in cell lysates by ELISA. **(B)** LDH released in the culture medium was quantified with an enzymatic assay. Metabolic activity was assessed with a MTS/PMS assay and expressed relative to that of PMA-treated cells not stimulated with LPS. Data are represented as means of biological triplicates ± SEM. Each panel is a representative experiment of at least 3 repeats.

Discussion

It is well established that innate and adaptive immune responses start with the detection of PAMPs of microbes or DAMPs of damaged cells by an array of sensors (Iwasaki and Medzhitov, 2015). It is followed by the secretion of inflammatory cytokines, notably IL-1ß produced mostly by myeloid cells that play a pivotal role in adaptive immunity through antigen capture, antigen processing and antigen presentation to prime or restimulate T lymphocytes. The prevailing view is that mature IL-1ß is released during pyroptosis, an inflammatory cell death characterized by the activation of caspase-1. This suicidal process of inflammatory cytokine secretion may be advantageous e.g. by limiting dissemination of intracellular pathogens or in the presence of potentially oncogenic genotoxicity.

Several groups have documented IL-1ß secretion without cell death by different cell types: human monocytes stimulated with LPS (Gaidt et al., 2016; Vigano et al., 2015), LPS-activated transdifferentiated human Burkitt lymphoma cells BLaER1 as a model for human macrophages (Gaidt et al., 2016; Gaidt et al., 2018b; Rapino et al., 2013), human neutrophils stimulated with LPS (Iula et al., 2018), murine fibroblasts transduced with an activatable caspase-1 (Conos et al., 2016), murine dendritic cells 'hyperactivated' with LPS and oxidized phospholipids (Zanoni et al., 2016), and similarly 'hyperactivated' murine macrophages (Evavold et al., 2018). Here, with our conditions of stimulation, we also observed an IL-1ß secretion by living human primary monocytes. However, we showed in addition that the amounts of secreted IL-1ß were identical when the cells did undergo pyroptosis (LPS+nigericin) or did not (LPS alone). To the best of our knowledge such a comparison has not been made. In several works, the amounts of IL-1ß secreted during pyroptosis were much higher than those secreted without pyroptosis (Evavold et al., 2018; Gaidt et al., 2016). It contributed to the concept that, as compared to pyroptosis, IL-1ß secretion by living

monocytes is a minor, less effective pathway (Sitia and Rubartelli, 2018). Our results rather suggest that two major pathways of unconventional secretion of IL-1ß are present in human monocytes: pyroptosis and a cell death-independent pathway. More work, and a molecular characterization of the cell-death independent pathway, will be required to assess the relative *in vivo* contributions of these two pathways in various diseases and inflammatory contexts.

Our main contribution is the demonstration that in human monocytic cells stimulated with LPS alone, gasdermin D is not required for IL-1ß secretion. This requirement has never been addressed in human cells. We have two experimental evidences for gasdermin D dispensability in this context. First, in LPS-stimulated primary blood monocytes gasdermin D was not detectably cleaved, even though the presence of mature IL-1ß indicated that caspase-1 was activated. Second, in THP-1 cells invalidated for the *GSDMD* gene with CRISPR/Cas9, IL-1ß was still secreted after LPS stimulation.

The gasdermin D p30 pores, in addition to triggering pyroptosis, were proposed to allow the outflow of mature IL-1ß from the cytosol (Heilig et al., 2018; Kovacs and Miao, 2017). Direct imaging of the pores in liposomal membranes by electron microscopy indicated an average inner diameter of ± 13 nm (Aglietti et al., 2016; Ding et al., 2016; Liu et al., 2016), larger than IL-1ß. In the 'hyperactivated' murine immortalized bone marrow-derived macrophages that secrete IL-1ß without pyroptosis, gasdermin D was shown to be required for IL-1ß secretion, suggesting that gasdermin D pores act as a conduit for the cytokine (Evavold et al., 2018). While our results do not exclude that in given cell types or experimental conditions IL-1ß could exit cells through gasdermin D pores, they demonstrate that in living human monocytes and THP-1 cells there is also a gasdermin D-independent pathway of IL-1ß secretion.

It will be important to understand why gasdermin D is not cleaved by caspase-1 in monocytes or THP-1 stimulated only with LPS. Caspase-1 activity is clearly present, as indicated by the presence of mature IL-1ß. We found similar total amounts of mature IL-1ß in the cells that were stimulated with LPS alone or with LPS and nigericin. However, caspase-1 activity measured in intact cells with a bioluminescent substrate was 3-fold lower after 4 h of LPS alone than after 4h of LPS and 1h of nigericin (data not shown). Thus, the results could be explained by a higher activity of caspase-1 on pro-IL-1ß than on gasdermin D, or by an unknown regulation of gasdermin D cleavage by caspase-1. Our observations are compatible with those of Hornung and coll., who document the coexistence in human monocytes of two NLRP3 inflammasome activation pathways, an all-or-nothing response leading to IL-1ß secretion and pyroptosis and a more gradual response leading to IL-1ß secretion without pyroptosis (Gaidt et al., 2016). It would be very interesting to study gasdermin D under their experimental conditions.

While we were finalizing the submission of this manuscript, Monteleone et al. showed that *GSDMD* knockout murine neutrophils could secrete IL-1ß (Monteleone et al., 2018). It is the first description of a gasdermin D-independent IL-1ß secretion. From their results, it is not totally clear that murine *GSDMD* knockout macrophages can also secrete IL-1ß. Human cells were not studied.

The nature of the gasdermin D-independent IL-1ß secretion pathway that we have studied in living human cells and its mode of activation remain to be defined. THP-1 cells might be useful in this endeavor. To date, THP-1 appears to be the only murine or human cell line that secretes IL-1ß without cell death and without gasdermin D. The secretion pathway is clearly unconventional and distinct from a passive release by damaged cells destined to die.

Importantly, most unconventional IL-1ß secretion pathways described to date, such as secretory autophagy or secretory lysosomes, were studied in cells stimulated with inflammasome activators, thus under conditions conducive to pyroptosis immediately after inflammasome activation or during the following hours. A reappraisal of these IL-1ß secretion pathways in living cells is warranted.

That myeloid cells such as primary monocytes, and possibly macrophages, can raise the alert through IL-1ß without dying could be very important, e.g. during chronic low-grade inflammatory processes or in tumors where PAMPs and DAMPs are rare and the role of local inflammation pivotal as illustrated recently by the clinical results of various immunotherapies, highly dependent on intra- or peritumoral inflammatory infiltrates (Chen and Mellman, 2017).

Material and Methods

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pierre G. Coulie (pierre.coulie@uclouvain.be).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines, transfection and retroviral transduction

THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 Medium supplemented with L-glutamine, HEPES, 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin (Pen+Strep) and 10% (v/v) FBS. HEK293T were cultured in DMEM, Low Glucose supplemented with L-glutamine, sodium pyruvate, Pen+Strep and 10% (v/v) FBS. Cells were detached with 2 mM EDTA in PBS. For lentiviral mediated CRISPR/Cas9 editing, lentiviral particles were produced by transfecting HEK293T cells with plasmids psPAX2 (Addgene, #12260), pCMV-VSV-G (Addgene, #8454) and pLentiCRISPRv2 modified to express the EFS-hSpCas9-IRES-CD90.1 cassette (Dr Jingjing Zhu). Plasmids were transfected into HEK293T cells in 10 cm dishes at a confluency of 70% with TransIT-LT1 (Mirus, MIR-2304) according to the provider's protocol. Viral supernatants were collected 48h post-transfection and fresh media was added. Viral supernatants were collected again 24 h later and pooled with the first viral supernatants. Supernatants were passed through a 0,45 µm PVDF filter via a syringe and viruses concentrated 25 times using Vivaspin 20 centrifugal concentrators (Sigma- Aldrich, Z614637), aliquoted and stored in liquid nitrogen.

PMA-differentiation of THP-1 cells

THP-1 at 7 x 10^5 cells per mL were differentiated during 3 hours with 300 ng/ml phorbol 12-myristate 13-acetate (PMA, InvivoGen, tlrl-pma), washed three times with warm medium

and plated at 10^5 cells per well in flat-bottom 96 well TC microplate. Cells were incubated 18 hours in fresh medium before LPS stimulation. To limit background in LDH release assays, cells were stimulated as indicated in RPMI 1640 Medium without red phenol, supplemented with L-glutamine, 2-mercaptoethanol, Pen+Strep and 5% (v/v) FBS.

Generation of *GSDMD*-deficient THP-1 cells

THP-1 were plated in TC 48-wells at 375,000 cells per well and transduced the same day in a final volume of 400 µL with lentiviruses expressing a *GSDMD*-specific guide RNA (TCTCCGGACTACCCGCTCAAAGG). All transductions were performed in a specific laboratory with biosafety level 3. At 48h post-transduction, cells were washed 3 times with warm medium to remove excess of viruses, plated in a TC 24-wells and further experiments were performed in laboratories with biosafety level 2. After 24 h, cells were labelled for CD90.1 expression (Miltenyi Biotec, Cat# 130-112-875) and the CD90.1-positive cells were cloned (1 cell per well in M96 TC micro wells) with the BD FACS AriaIII cell sorter. Growing clones were expanded and screened by western blot for GSDMD expression. Using RNA-Seq transcriptome analysis, we confirmed that in clones without detectable GSDMD expression we detected neither wild type nor in-frame indel sequences. To limit inter-clone diversity in the IL-1ß secretions assays we pooled 3 independent Gasdermin D deficient clones to reconstitute a *GSDMD*-deficient THP-1 cell population. As control, THP-1 deficient for ß2 microglobulin were also generated. THP-1 were transduced with lentiviruses expressing *B2M*-specific guide RNA (CCGGGCCGAGATGTCTCGCTCCG) with the same procedure as above. CD90.1-positive and HLA-A,B,C-negative cells were FACS-sorted with the BD FACSAria III cell sorter. Of note, ß2 microglobulin is required for the surface expression of HLA class I molecules.

Purification of primary human monocytes

PBMCs were isolated from blood from hemochromatosis patients. Experiments with human cells were approved by our Institution's ethics committee (Commission d'Ethique Biomédicale Hospitalo-Facultaire de l'Université catholique de Louvain), under registration number B403201110966. Written informed consent for the use of blood samples was not always obtained, in accordance with the Belgian law of 19 December 2008 which states that, in the absence of written opposition by the patient, consent is considered given for residual body material. This applies to blood samples from hemochromatosis patients. No patient opposed the use of blood samples. Data obtained from blood samples were analyzed anonymously.

Citrate-treated whole blood was centrifuged 20 min at 4°C at 3,700 g and the PBMC were isolated from the resulting buffy coat by density gradient centrifugation. In 50 mL polypropylene tubes, 25 mL of the buffy coat were carefully layered on 15 mL of Lymphoprep and centrifuged at 930 g for 15 minutes at room temperature in a swinging-bucket rotor without brake. PBMC were collected on the Lymphoprep and washed 3 times in PBS-EDTA 2mM + 1%FBS. Human primary monocytes were enriched from PBMC by negative selection using the EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion (StemCell, #19058), following the manufacturer’s protocol. Monocyte purity was assessed by flow cytometry using a FITC anti-human CD14 antibody (Biolegend, #325604) and routinely reached 80-90%.

METHOD DETAILS

Reagents to be reconstituted

Ultrapure Lipopolydaccharide (LPS) from *E.coli*, serotype 055:B5 was purchased from InvivoGen (tlrl-pb5lps) and suspended in sterile endotoxin-free water at 5 mg/mL. LPS was used in assays at a concentration of 10 ng/mL. Recombinant human LBP protein, purchased from R&D (870-LP-025/CF), was suspended at 100 µg/mL in sterile PBS and used at 10 ng/mL. Nigericin from InvivoGen (tlrl-nig) was resuspended at 6,7 mM in sterile ethanol and used at 10 µM.

Cell stimulation

Both primary human monocytes and PMA-differentiated THP-1 were seeded at 10^5 cells in 200 µL in flat-bottom 96 well TC-treated plates. Cells were stimulated with 10 ng/ml ultrapure LPS from *E. coli* and 10 ng/ml recombinant human LBP over 21 hours for monocytes or 24 hours for PMA-differentiated THP-1. To induce pyroptosis in human primary monocytes, after 3 hours of LPS stimulation, Nigericin (or its control ethanol) was added to a final concentration of 10 µM. To limit background in LDH release assays, cells were stimulated as indicated in RPMI 1640 Medium without red phenol supplemented with L-glutamine, 2-mercaptoethanol, Pen+Strep and 5% (v/v) FBS. For delayed stimulation of primary human monocytes, after enrichment, cells were seeded at 10^5 cells in 200 µL in flat-bottom 96 well TC-treated plates and incubated 21 hours at 37°C with 5% CO_2. After removing the medium, cells were stimulated with 10 ng/ml ultrapure LPS from *E. coli* and 10 ng/ml recombinant human LBP over 21 hours with or without nigericin addition after 3 hours of LPS stimulation.

Real-time quantitative PCR

RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, #740955) according to manufacturer's instructions. Total RNA was reverse transcribed with the RevertAid Reverse Transcriptase from ThermoFischer (#EP0441) in a final volume of 20 µL. cDNA was diluted 5 times in water and qPCR amplifications were carried out in a final volume of 20µL with the Takyon Master Mix (Eurogentech, UF-RPMT-B0701) in a StreponePlus Real-Time PCR System (Applied Biosystems) under standard conditions: 95°C for 3 min, 45 cycles of 95°C for 10 sec and 60°C for 1 min. Quantitative real-time PCR was performed in triplicate with the following primer pairs and probes :

IL1B forward 5'-GAGCAACAAGTGGTGTTCTCC-3'

reverse 5'-AACACGCAGGACAGGTACAG-3'

probe FAM-TAMRA 5'-ACCTGTGGCCTTGGGCCTCA-3'

EEF1 forward 5'- GCTTCACTGCTCAGGTGAT -3'

reverse 5'-GCCGTGTGGCAATCCAAT-3'

probe FAM-TAMRA 5'-AAATAAGCGCCGGCTATGCCCCTG -3'

ELISA

Supernatants were collected at indicated times in non-TC treated 96-well plates and centrifuged to eliminate residual cells. Supernatants were then transferred to a new plate and kept at -20°C. To compare the amounts of secreted and intracellular mature IL-1ß, after the collection of culture supernatants the cells were resuspended in 200 µL of medium (same volume as supernatants) and lysed by one cycle of freeze/thaw at -20°C. Human mature IL-1ß was quantified in supernatants and inside cells with the IL-1ß Human Matched Antibody Pair ELISA kit from ThermoFisher Scientific (#CHC1213) according to the provider's protocol. We verified that this kit recognizes mature human IL-1ß and that pro-IL-1ß is not detected.

Lactate deshydrogenase (LDH) release assay

Supernatants (100 µL out of 200 µL) were collected from the cultures of stimulated cells and transferred in 96-well microplates of white polystyrene. To generate the maximum LDH release controls, 2 µl of lysis solution were added to all the remaining cells, in 100 µL of residual supernatants, incubated 10 min at room temperature and transferred to the white 96-well plate. LDH activity was measured with the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega, #G5421) according to the manufacturer's instructions. Fluorescence was measured using the Glomax Discover device. The percentage of LDH release was 100 x (Experimental – Culture Medium Background) / (Maximum – Culture Medium Background), with Experimental = 2 x Value of supernatant, Maximum = Value of Maximum LDH release Control – Value of supernatant. Of note, other LDH assays can be inhibited by ethanol, in which nigericin was resuspended, which is why we used this kit from Promega.

Cell viability assay

Cell viability was measured with the CellTiter 96 Aqueous Non-radioactive cell proliferation assay from Promega (#G5421) according to the manufacturer’s protocol. At indicated times, supernatants were discarded and replaced by 100 µL of warm medium plus 20 µL of MTS/PMS mix. After a 2 hours-incubation at 37°C 5% CO_2, light absorbance was recorded at 490 nm. Results were expressed as a ratio of metabolic activities of treated as compared to control cells (neither LPS-stimulated nor nigericin-treated).

Immunoblotting

Total cell lysates were prepared using Laemmli buffer containing ß-mercaptoethanol, denaturated for 10 min at 95°C, separated by Bis-Tris denaturing SDS-PAGE and transferred

onto nitrocellulose membranes (ThermoFisher Scientific). Membranes were blocked with 5% non-fat dry milk in PBS with 0,1% Tween 20 (PBST) for 1h at RT, and incubated overnight at 4°C with indicated primary antibodies diluted in 5% BSA/PBST at 1:200 for anti-GSDMD-C1 (Santa-Cruz Biotechnology, sc-81868) and 1:3000 for anti-vinculin (Sigma-Aldrich, #V9131). After washes, membranes were incubated 1h at RT with anti-mouse-HRP (R&D Systems, #HAF007). Chemoluminescent signals were recorded with a CCD-camera (Fusion Solo S, Vilber).

Cell staining for flow cytometry

For surface staining, human primary monocytes or THP-1 cells were washed in HBSS supplemented with 1% human serum to block unspecific bindings. Surface labeling was then performed for 15 min at 4°C with antibodies conjugated directly to fluorochromes. Stained cells were acquired on FACS Fortessa and analysed with the FlowJo software.

Library preparation for RNA seq

RNA was isolated using the NucleoSpin RNA II kit according to manufacturer's instructions (Macherey-Nagel, #740955). Libraries were prepared by Integragen Genomics with TruSeq Stranded mRNA kit protocol according to the supplier's recommendations. Briefly, purification of Poly-A containing mRNA molecules using poly-T coated magnetic beads from 1µg of total RNA, fragmentation using divalent cations under elevated temperature to obtain approximately 300 bp fragments, double strand cDNA synthesis, Illumina adapters ligation, cDNA library PCR amplification and 100 bp paired-end sequencing on Illumina HiSeq4000.

RNAseq analysis

Alignment to the GRCh38 reference genome was done using STAR. Bam files were sorted with Samtools 1.6. Gene counts were obtained using HTSeq 0.9.1 htseq-count (union mode). Count normalization was performed with DESeq2_1.18.1.

DATA AND SOFTWARE AVAILABILITY

The RNAseq data are deposited at NCBI GEO with accession number GSE117162 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE117162).

Supplementary Information

Supplementary 1

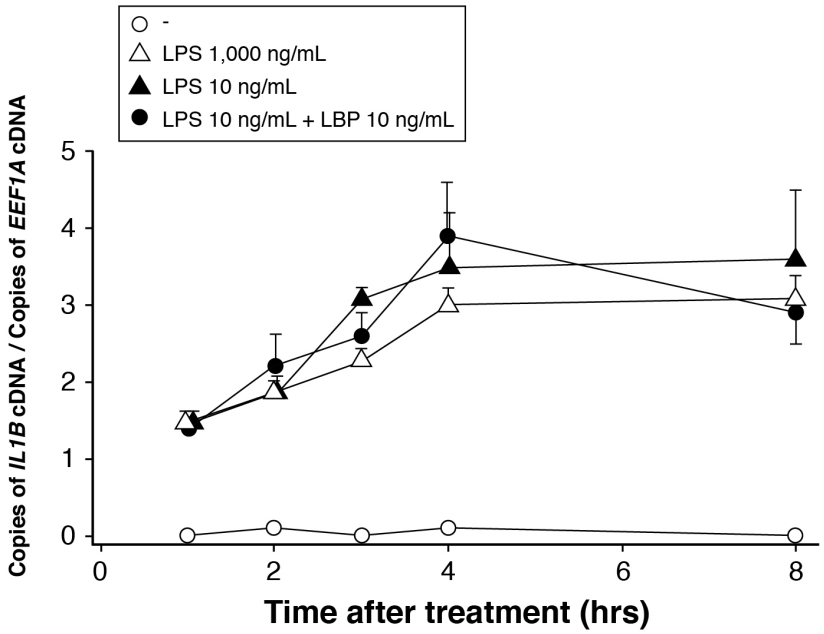
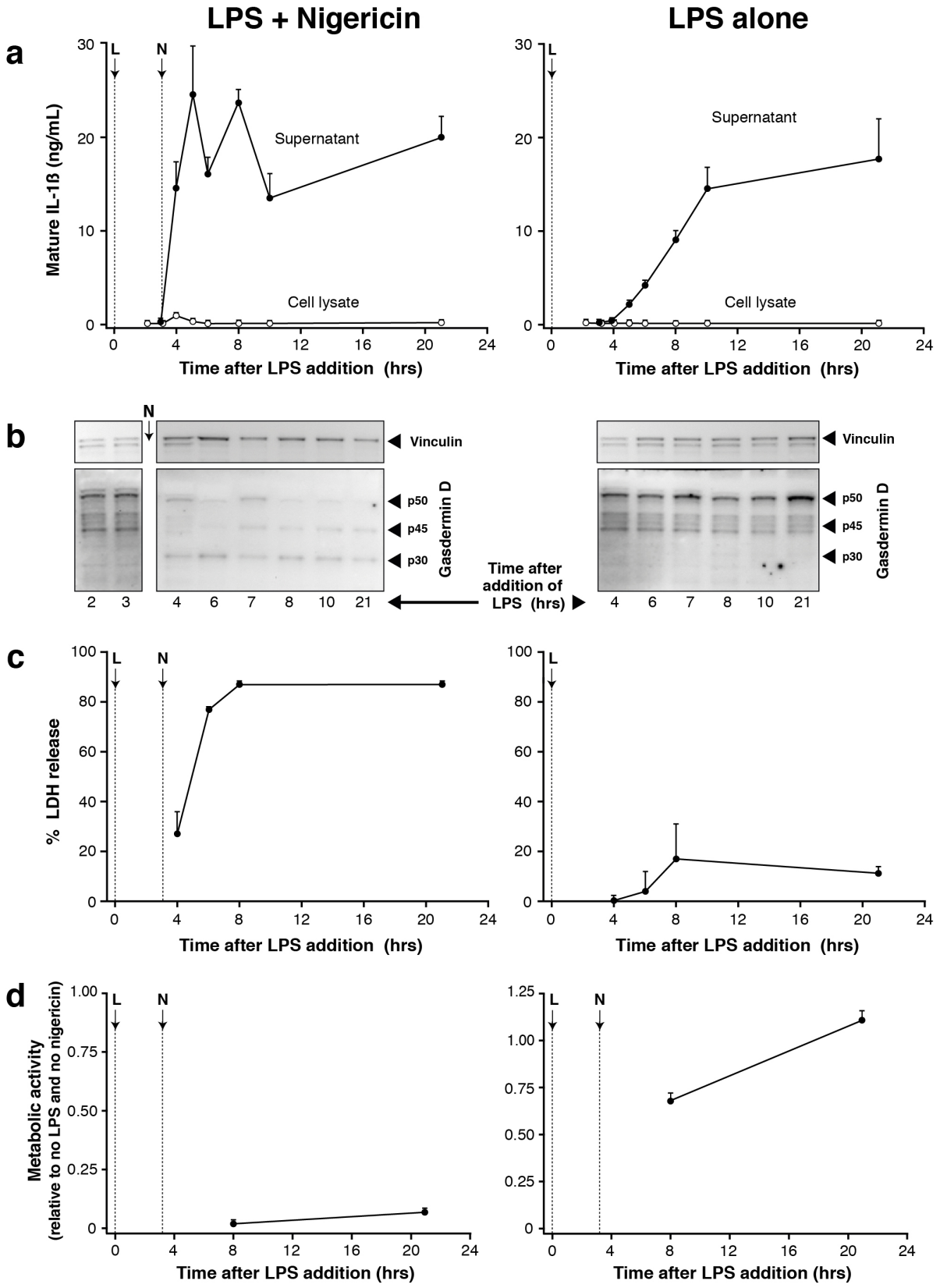


Figure S1. Low concentrations of LPS induce IL1B expression in primary human monocytes. Freshly isolated human blood monocytes were stimulated with the indicated concentrations of ultrapure LPS, alone or with LBP, in medium containing bovine serum. IL1B expression was measured by RT-qPCR and normalized to EEF1A1 expression. Data are represented as means of biological triplicates ± SEM. Similar results were obtained with monocytes of two other donors.

Figure S2. Human blood monocytes can either release IL-1ß during pyroptosis or secrete IL-1ß in the absence of pyroptosis and of GSDMD cleavage (donor 2). Freshly isolated human blood monocytes were stimulated with LPS (L) for 3 hours, and then treated or not with nigericin (N) over 18 hours. **(A)** Mature IL-1ß was measured in supernatants or in cell lysates with ELISA. **(B)** GSDMD cleavage was analysed by immunoblot at different time points after LPS stimulation with or without nigericin. **(C)** LDH released in the supernatants was quantified with an enzymatic assay. **(D)** Metabolic activity was assessed with a MTS/PMS assay and expressed relative to that of unstimulated (neither LPS nor nigericin) cells. Data are represented as means of biological triplicates ± SEM.

Supplementary 2



Supplementary 3

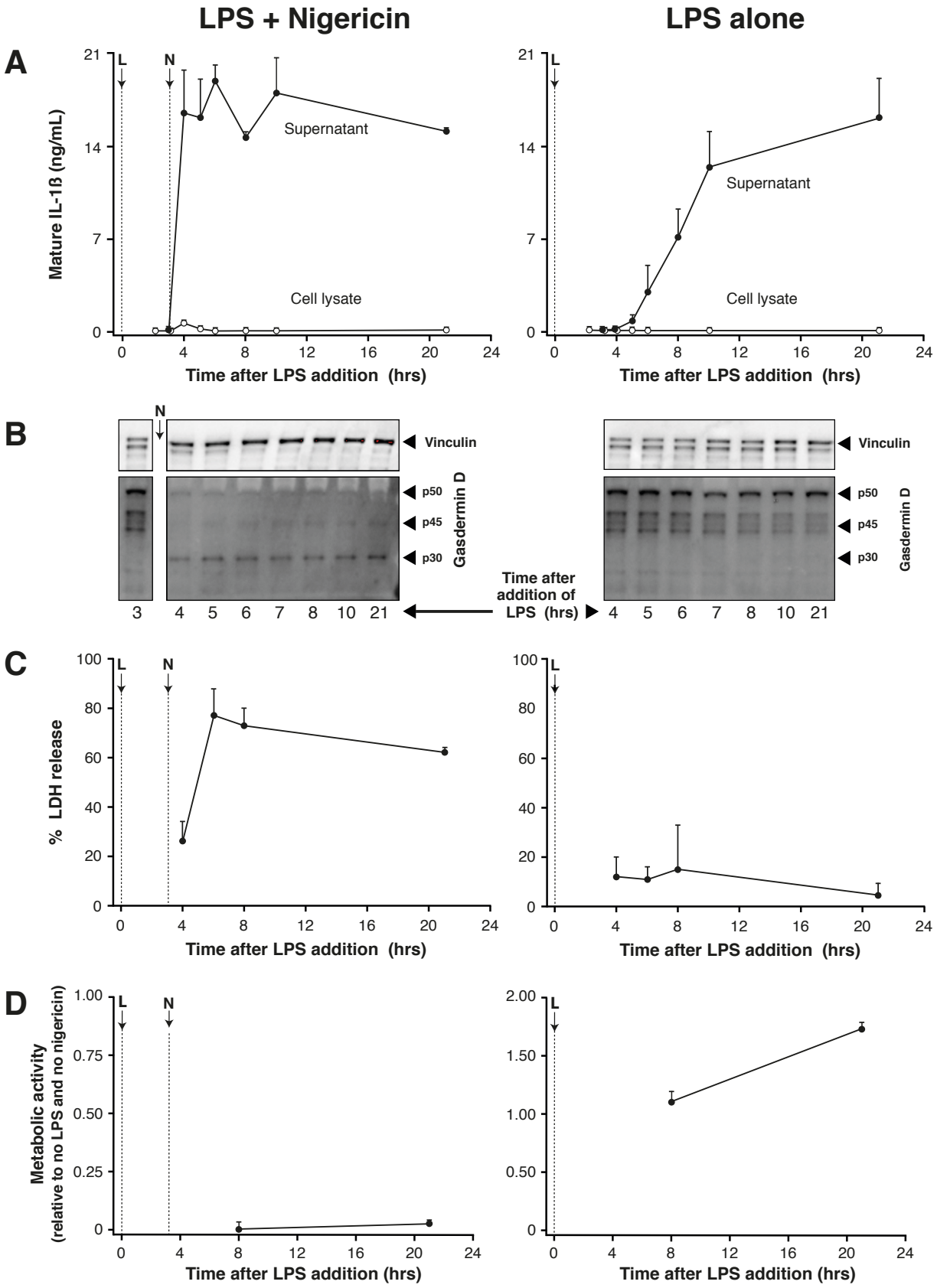


Figure S3. Human blood monocytes can either release IL-1ß during pyroptosis or secrete IL-1ß in the absence of pyroptosis and of GSDMD cleavage (donor 2). Freshly isolated human blood monocytes were stimulated with LPS (L) for 3 hours, and then treated or not with nigericin (N) over 18 hours. **(A)** Mature IL-1ß was measured in supernatants or in cell lysates with ELISA. **(B)** GSDMD cleavage was analysed by immunoblot at different time points after LPS stimulation with or without nigericin. **(C)** LDH released in the supernatants was quantified with an enzymatic assay. **(D)** Metabolic activity was assessed with a MTS/PMS assay and expressed relative to that of unstimulated (neither LPS nor nigericin) cells. Data are represented as means of biological triplicates ± SEM

Supplementary 4

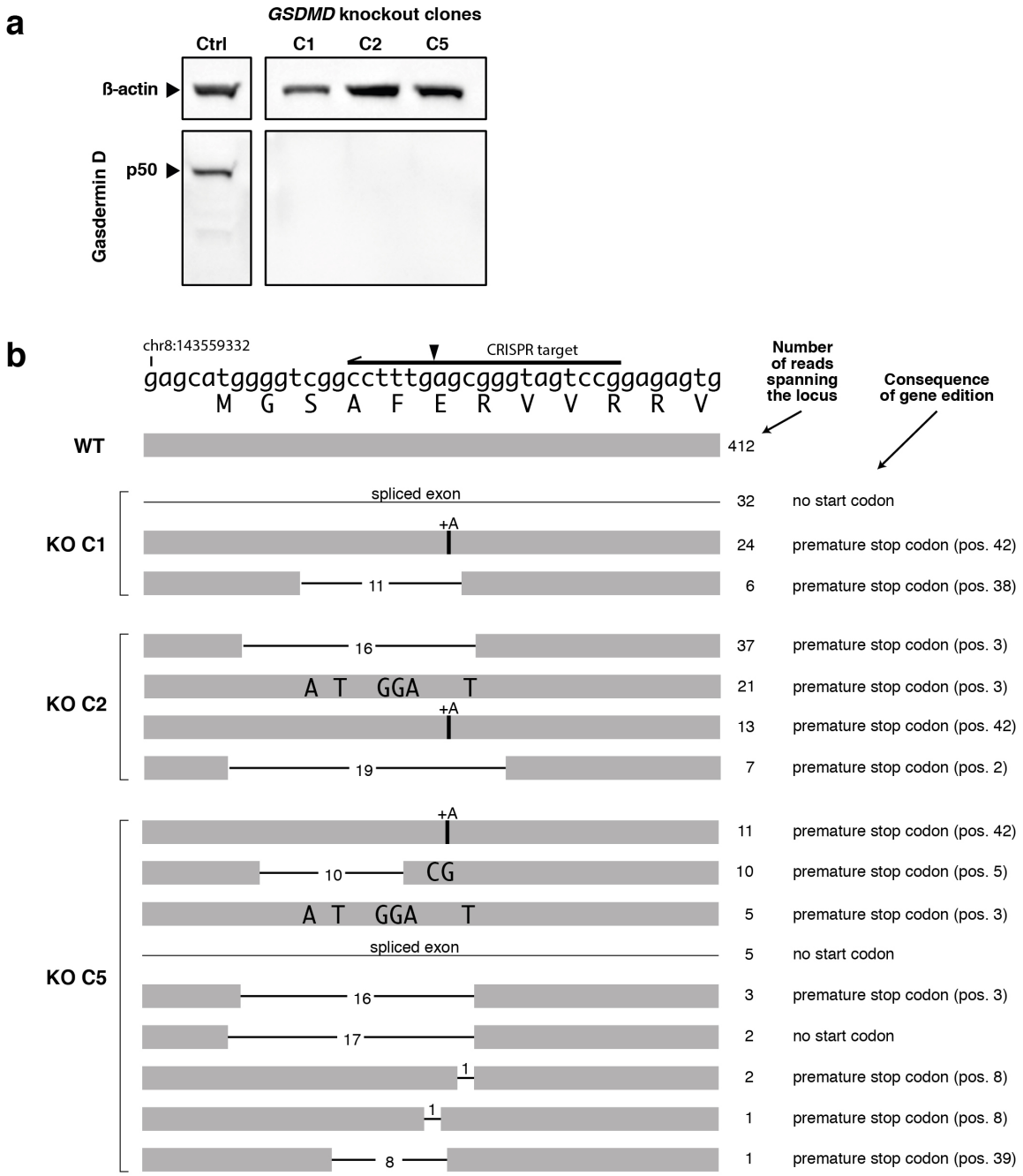


Figure S4. GSDMD knockout cells were validated by immunoblotting and RNAseq analysis. THP-1 cells were transduced with a CRISPR-Cas9 lentivirus encoding a GSDMD-specific guide RNA targeting the 5' end of the coding sequence, in exon 2. After 72 hours cells were cloned and growing clones were expanded. **(A)** Gasdermin D expression in clones C1, C2 and C5 was analyzed by immunoblotting. **(B)** Gene editing in clones C1, C2 and C5 was analysed by RNAseq. Reads in a region spanning 40 nucleotides around the CRISPR-cutting site were piled up. For each of the 3 clones we obtained more than two different sequences, which is likely explained by the presence of 3 copies of chromosome 8 in THP-1 and by the persistence of Cas9 activity after cloning.

Table supplementary 1

			IL-1ß concentrations (pg/ml)			
	Cell stimulation		Supernatants		Cell lysates	
	LPS	Nigericin	Immediate stimulation	Delayed stimulation	Immediate stimulation	Delayed stimulation
Tissue culture plate	4 h		539 ± 178	11 ± 4	72 ± 4	1 ± 0
	21 h		7655 ± 510	18 ± 4	63 ± 6	2 ± 2
	3 h	+ 1 h	9003 ± 218	1355 ± 48	429 ± 120	218 ± 9
Low binding plate	4 h		1138 ± 68	15 ± 1	111 ± 10	1 ± 0
	21 h		7008 ± 364	21 ± 2	85 ± 30	1 ± 0
	3 h	+ 1 h	9017 ± 226	2430 ± 122	429 ± 36	217 ±1

Table S1. Mature IL-1ß produced by human primary monocytes stimulated by LPS with or without Nigericin. Freshly isolated human blood monocytes were seeded at 10^5 cells in 200 µL in flat-bottom 96 well TC-treated plates and stimulated either immediately or 24 hours later. Stimuli included LPS alone for 4 or 21 hours and LPS for 3 hours followed by nigericin for 1 hour. Mature IL-1ß was measured in supernatants and cell lysates by ELISA. Data are represented as means of biological triplicates ± SEM. Similar results were obtained with monocytes of two other donors.

Discussion

While most secreted proteins are transported through the ER-Golgi pathway, to which they are directed by their signal sequence, a few secreted proteins do not contain a signal sequence and therefore reach the extracellular space using pathways conveniently described as 'unconventional secretion pathways' (Rabouille, 2017). This is the case for all members of the IL-1 family (IL-1ß, IL-18, IL-33, IL-36 (αβγ), IL-37, IL-38) with the exception of IL-1ra and IL-1α. It is worth noting that in cellular biology a secretion process is the exit of cellular products from living cells. The release of cellular proteins from dying or dead cells is not a secretion. Thus IL-1α, which is a alarmin released by cells undergoing cell death, is actually not secreted through an unconventional secretion pathway (Kim et al., 2013). The only mechanism of unconventional secretion that has been completely deciphered is that used by FGF-2, which translocate across the plasma membrane by direct penetration (Steringer et al., 2012; Steringer and Nickel, 2018). There is no evidence for a common mechanism of unconventional secretion. For IL-1ß, several mechanisms have been proposed but their physiological relevance still needs to be evaluated.

In 2015, two groups discovered the implication of the pore forming protein gasdermin D in pyroptotic cell death and IL-1ß secretion by murine myeloid cells (Kayagaki et al., 2015; Shi et al., 2015b). Subsequently, a lot of work was devoted to the mechanisms of gasdermin D pore-forming activity and to the possibility that IL-1ß exits cells through these pores rather than, or prior to, the massive release from disrupted cells (Evavold et al., 2018). Several groups have reported that some cell types, such as neutrophils, or cells stimulated under certain conditions, such as monocytes stimulated with LPS alone, secreted IL-1ß but did not die. After the discovery of gasdermin D/pyroptosis axis, it is clear that completely excluding cell death is mandatory to describe another unconventional secretion pathway. As a matter of fact, this work is ongoing as in most publications cell death was not fully evaluated and IL-1ß

secretion was triggered with inflammasome activators that are now known to trigger pyroptosis. Our main interest was the mechanism of IL-1ß secretion by human cells and the pyroptosis results indicated that the experimental conditions under which IL-1ß secretion is studied are crucially important as they might or might not lead to cell death, itself a mechanism of IL-1ß release.

1. IL-1ß secretion and cell death in monocytes as compared to other cells

As far as primary human cells are concerned, IL-1ß secretion is mainly investigated in monocytes freshly isolated from blood. The main limitation is the difficulty to genetically manipulate the cells. For this reason, most studies are conducted on macrophages (Dupont et al., 2011; Evavold et al., 2018; Harris et al., 2011; Qu et al., 2007; Saitoh et al., 2008). One group works with murine bone marrow derived neutrophils (Chen et al., 2014; Monteleone et al., 2018). Murine monocytes are never used ought to their low number. Among these cells, only human monocytes stimulated with LPS alone and human or murine neutrophils stimulated with LPS and an inflammasome activator are known to secrete IL-1ß without undergoing cell death (Chen et al., 2014; Gaidt et al., 2016; Iula et al., 2018).

Very few human cell lines can be used to study IL-1ß secretion in response to LPS alone, thus without pyroptosis. The spontaneously immortalized cell line THP-1, the most studied human monocytic cell line, has never been used to study IL-1ß secretion in response to LPS alone. Recently, Gaidt and colleagues developed the transdifferentiated human Burkitt lymphoma cell line BLaER1, with a phenotype resembling primary human monocytes and a response to LPS alone (Gaidt and Hornung, 2017; Gaidt et al., 2018a). We established conditions under which we could analyze the mechanism of IL-1ß secretion induced by LPS alone in THP-1. A short incubation with a low dose of PMA provided cells that responded to LPS alone without undergoing cell death, very much like primary monocytes.

2. GSDMD-independent IL-1ß secretion

Gasdermin D is essential for IL-1ß pyroptotic secretion (Kayagaki et al., 2015; Shi et al., 2015b). However, whether IL-1ß is already secreted through the gasdermin D pores at the plasma membrane or only later during the pyroptotic release of the cytoplasmic content is still controversial. Recently, Evavold et al. reported that murine macrophages activated with LPS and an inflamasomme activator could secrete IL-1ß while maintaining their viability (Evavold et al., 2018). No IL-1ß secretion was observed in gasdermin D knockout macrophages, suggesting that IL-1ß could be secreted through the gasdermin D pores. In human cells, the role of gasdermin D in the non-pyroptotic IL-1ß secretion had not been evaluated.

I showed that in THP-1, knocking out *GSDMD* did not affect the LPS-induced IL-1ß secretion, indicating that in human monocytic cells gasdermin D is not always required for IL-1ß secretion. Unexpectedly, the amounts of IL-1ß produced and secreted in *GSDMD*$^{-/-}$ THP-1 cells were 5-fold-higher than in control THP-1. Further analysis revealed higher levels of *IL1B* gene expression and of inflammasome activity in the *GSDMD*$^{-/-}$ THP-1. I observed the same increase in THP-1 cells that were nucleofected with the Cas9 protein and guide RNAs. RNAseq analysis of the GSDMD$^{-/-}$ cells has not yet provided a clear explanation but we noted that 500 genes had a ≥5-fold change in expression. CD93, a transmembrane glycoprotein known to enhance TLR responses (Jeon et al., 2010), was expressed at much higher levels (6,700 vs 270 RPKM) in the GSDMD$^{-/-}$ cells. It might explain the increased *IL1B* gene expression and inflammasome activity in these cells due to increase of LPS responsiveness. Another potentially interesting observation made with the *GSDMD*$^{-/-}$ THP-1 cells is a delayed IL-1ß secretion concomitant with an intracellular accumulation. It suggests that gasdermin D could be involved in facilitating the first steps of the secretion process. In any case the analysis of the IL-1ß secretion process and transcriptomic analysis of the

GSDMD knockout human cells suggest that the roles of gasdermin D extend much beyond what is known today.

In August 2018, Monteleone et al. showed a gasdermin D independent IL-1ß secretion in murine neutrophils (Monteleone et al., 2018). The same group had shown that contrary to murine macrophages, neutrophils could secrete IL-1ß after LPS and inflammasome activators without undergoing pyroptosis (Chen et al., 2014). They now showed that this secretion still occurred in absence of GSDMD. These results are perfectly in line with our observations with human cells. To go further in the mechanisms of IL-1ß secretion, the authors transduced mature IL-1ß in macrophages derived from the bone marrow or in the RAW 264.7 cell line. They show that IL-1ß maturation is sufficient for mature IL-1ß secretion as it enables its relocation of the mature IL-1ß from the cytosol to the plasma membrane by unmasking polybasic residues. The experiments supporting this hypothesis are however not very convincing. First, they were conducted on murine macrophages for which they/others have shown that they do not secrete IL-1ß in a GSDMD independent and non-pyroptotic manner, as neutrophils do. Second, their studies were conducted under conditions which leads to pyroptotic cell death. Third, their hypothesis relies on the amount of mature IL-1ß detected by western blot in supernatants and cell lysate fractions, for which loaded proportion is not provided. Thus, we do not believe that the secretion mechanisms deciphered in their paper is the GSDMD independent and non-pyroptotic independent secretion that occurs in murine neutrophils and in our human monocytes stimulated with LPS alone.

3. How many IL-1ß secretion pathways?

Many mechanisms have been proposed for the unconventional secretion pathway of IL-1ß. Understanding IL-1ß secretion, with new potential targets for anti-inflammatory drugs, is of great interest. Until recently, it was unclear whether several mechanisms were present in the same or in different cell types, or whether a single mechanism could account for all the generated experimental data. We know now that there are at least two mechanisms of IL-1ß secretion: pyroptosis and another pathway. And we believe that the same unknown mechanism could explain all the other observations. What follows are the main reasons for this proposal.

Gasdermin D-driven pyroptosis is the first elucidated mechanism of IL-1ß secretion. In our view, it is more a release than a secretion. LDH is detected in the culture medium together with IL-1ß and nobody mentions a 'secretion' for the exit of this cytoplasmic enzyme from the dying cells. Noteworthy we have observed that LDH release was a very poorly sensitive assay to detect the cell death of primary monocytes or of THP-1 cells. It proved very difficult if not impossible to detect a 10% death rate, while doing so for other cell types was not a problem. We have not explored the reason for this observation but we believe that many investigators underestimate cell death of monocytic cells because they used classical LDH release assays. In our hands, the CytoTox-ONE Homogeneous Membrane Integrity Assay proposed by Promega, based also on LDH release, was more sensitive than the other tests that we tried. The possibility remains that part of the IL-1ß that exits cells during pyroptosis is not released but really secreted through the gasdermin D pores. While the size of the gasdermin D pores in liposomes is compatible with a transfer of IL-1ß (Sborgi et al., 2016), whether this applies also to intact cell remains unexplored. Some authors argue that gasdermin D-dependent IL-1ß

secretion without cell death indicates a transfer through the pores (Evavold et al., 2018; Kovacs and Miao, 2017) but in our view, such a claim requires a careful control of the absence of death of even a small proportion of the cells, which was not done. Thus, a true secretion of IL-1ß through the gasdermin D pores of pyroptosis remains purely hypothetical.

The suicide of the IL-1ß-releasing cells during pyroptosis can be considered as a part of innate immunity, considering that intracellular pathogens can trigger the process. In this respect, it is very interesting to note that sepsis is almost abolished in *GSDMD* knockout mice challenged with LPS (Kayagaki 2015). Thus, it is almost certain that the pyroptotic release of IL-1ß is relevant to human pathophysiology in the context of severe infections. Non-mammalian vertebrates lack GSDMD yet exhibit a pyroptotic cell death, thus with activated inflammatory caspases and pores at the plasma membrane, with massive IL-1ß release. It suggests that the latter is an important defense mechanism conserved throughout evolution (Angosto et al., 2012; Kayagaki et al., 2015).

An element that is often put forward is the 'quick and massive' IL-1ß production during pyroptosis, contrasting with a low and slow secretion through several other mechanisms. It is even proposed that all the other mechanisms of IL-1ß secretion are useful because they allow the production of very different amounts of cytokine, tuned according to the intensity of the stimulatory signals (Evavold et al., 2018; Sitia and Rubartelli, 2018).

Four mechanisms have been proposed that can account for unconventional IL-1ß secretion without cell death.

A first mechanism is secretory lysosomes, studied since the nineties by the group of A. Rubartelli in Genoa. They showed that in human monocytes stimulated with LPS and ATP, proIL-1ß and procaspase-1 are colocalized with cathepsin D and LAMP1, which are markers of endolysosomes (Andrei et al., 1999; Andrei et al., 2004). How proIL-1ß and procaspase-1 reach the lysosomal compartment is unknown. Only when ATP is used in addition to LPS, mature IL-1ß is found in the supernatant. They never observed mature IL-1ß in the cytoplasm. IL-1ß secretion is inhibited by the microtubule inhibitor nocodazole (Carta et al., 2006) and by inhibitors of PC-PLC and $cPLA_2$ (Andrei et al., 2004), which are required for the fusion of secretory vesicles with the plasma membrane (Ramoni et al., 2001). Altogether, the hypotheses are that mature IL-1ß is generated in the lysosomes and exits the cells through secretory lysosomes (Andrei et al., 1999; Carta et al., 2006; Sitia and Rubartelli, 2018). The authors propose that after LPS/ATP, secretory lysosomes first secrete low amounts of IL-1ß before the onset of pyroptosis and the massive IL-1ß release (Sitia and Rubartelli, 2018). Several questions remain however unanswered in the model. How do procaspase-1 and proIL-1ß reach lysosomes? How can caspsase-1 exert its enzymatic activity in the acidic environment of lysosomes and without inflammasomes? PLC inhibitors were recently shown to inhibit the infllammasome, which could explain the results without a requirement for secretory lysosomes (Franchi et al., 2009). The absolute requirement of ATP to induce an IL-1ß secretion through secretory lysosomes is an important confounding factor, as ATP is known to induce cell death in human monocytes. Finally, we have tested a few elements of the model: we did not observe the absolute requirement for ATP, as even the addition of apyrase or of the inhibitor of P2X7R A437980 to primary monocytes stimulated with LPS alone did not prevent secretion, and a microtubule inhibitor had no effect. Our tentative conclusion is that while it is certainly possible that some (pro)IL-1ß can be present in

lysosomes, and as indicated below one possible reason is autophagy, we are very far from a demonstration that lysosomes are the secretory pathway of IL-1ß in living cells.

A second proposed mechanism is autophagy, notably with the work of the authoritative group of Randy Shekman (Zhang et al., 2015). The main elements in favour of autophagy are the following. First, the colocalization of IL-1ß and markers of autophagosomes such as LC3. This colocalization is observed in murine macrophages (Dupont et al., 2011) and neutrophils (Iula et al., 2018) and in the human cells 293 (Zhang et al., 2015). In the latter case the cells have to be transfected with, and thus overexpress, genes encoding proIL-1ß and pro-caspase-1. Second, decreased or abolished expression of the autophagy gene *ATG5* decreases IL-1ß secretion in the human neutrophil-differentiated cell line PLB985 (Iula et al., 2018) and in murine macrophages (Dupont et al., 2011). However, while it is clear that IL-1ß can be present in autophagosomes, whether this participates in a secretion process remains controversial. In some systems knocking down autophagy genes increases IL-1ß secretion (Saitoh et al., 2008). Autophagy has been shown to decrease instead of increase IL-1ß secretion, by targeting pro-IL-1ß or inflammasomes for degradation (Harris et al., 2011; Saitoh et al., 2008). Increasing autophagy by starvation appears to activate the inflammasome and therefore could increase IL-1ß secretion through any mechanism (Zhang et al., 2015). Finally, we invalidated *ATG5* in THP-1 with CRISPR-Cas9 and did not observe a decrease in IL-1ß secretion after LPS alone and treated primary monocytes with chloroquine or wortmanin without modifying IL-1ß secretion. In conclusion, a role of autophagy in regulating IL-1ß secretion is certainly possible but it appears unlikely that autophagy is the mechanism of IL-1ß by living human monocytic cells.

Third, a few reports have proposed that other vesicles were involved in IL-1ß secretion. A correlation was found between IL-1ß secretion and the release of membrane MHC class II molecules, a marker of plasma membranes, recycling endosomes, multivesicular bodies, and released exosomes (Qu et al., 2007). The experiments were conducted in murine macrophages stimulated with high doses of ATP, which we now know leads to pyroptosis. Another group observed that within seconds of ATP addition to LPS-primed THP-1 cells, blebs formed at the cell surface and microvesicles were shed while no mature IL-1ß could be detected in the surrounding medium or in the vesicles. IL-1ß was detected two minutes later within shed microvesicles and later in the vesicle-free supernatant (MacKenzie et al., 2001). It is however unclear whether this microvesicle-associated and pre-pyroptotic IL-1ß secretion is also responsible for IL-1ß secretion without cell death.

Finally, the unanticipated participation of a pore-forming protein to pyroptosis points to the possibility of a transmembrane transport of IL-1ß. Could another transporter or pore-forming protein be the main pathway of IL-1ß secretion by living cells? Actually, my work on IL-1ß secretion began with the idea that IL-1ß could be conveyed by a transmembrane transporter similar to those that transfer folded cytosolic proteins into the peroxysome, namely PEX1 and PEX6 (Kim and Hettema, 2015). Searching databases for transporters with ATP-binding domain sequences similar to those of PEX1 and PEX6 yielded SPATA5, SPATA5L1, NVL and VCP. Knocking out the corresponding genes, including *PEX1* and *PEX6*, in THP-1 killed the cells. With siRNAs, I obtained THP-1 with decreased levels of PEX6, SPATA5, NVL or VCP but did not observe significant decreases of IL-1ß secretion. Another candidate is MLKL, a pore-forming protein involved in necroptosis (Dhuriya and Sharma, 2018; Yoon et al., 2017). However, I observed that THP-1 knocked-out for *MLKL* secrete IL-1ß like the parental cells. Obvious other candidates are the other gasdermin proteins: GSDMA, GSDMB,

GSDMC and GSMDE, which are all know to form pores. *GSDMA*, *GSDMB* and *GSMDE* are expressed in THP-1, though at much lower levels than *GSDMD*. Invalidating these genes is underway. Finally, pannexin 1 could be involved in IL-1ß secretion. Pannexin 1 is an oligomeric membrane protein cleaved by casp4/5 during the non-canonical inflammasome activation (Yang et al., 2015). Cleaved pannexins 1 form a pore through which intracellular ATP exits cells (He et al., 2016; Yang et al., 2015). Here also, I will invalidate the *PANX1* gene in THP-1.

References

Acosta-Rodriguez, E.V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8:942-949.

Afonina, I.S., C. Muller, S.J. Martin, and R. Beyaert. 2015. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. *Immunity* 42:991-1004.

Agard, N.J., D. Maltby, and J.A. Wells. 2010. Inflammatory stimuli regulate caspase substrate profiles. *Mol Cell Proteomics* 9:880-893.

Aglietti, R.A., A. Estevez, A. Gupta, M.G. Ramirez, P.S. Liu, N. Kayagaki, C. Ciferri, V.M. Dixit, and E.C. Dueber. 2016. GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. *Proc Natl Acad Sci U S A* 113:7858-7863.

Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.

Alnemri, E.S., D.J. Livingston, D.W. Nicholson, G. Salvesen, N.A. Thornberry, W.W. Wong, and J. Yuan. 1996. Human ICE/CED-3 protease nomenclature. *Cell* 87:171.

Anderson, K.V., G. Jurgens, and C. Nusslein-Volhard. 1985. Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. *Cell* 42:779-789.

Andrei, C., C. Dazzi, L. Lotti, M.R. Torrisi, G. Chimini, and A. Rubartelli. 1999. The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol Biol Cell* 10:1463-1475.

Andrei, C., P. Margiocco, A. Poggi, L.V. Lotti, M.R. Torrisi, and A. Rubartelli. 2004. Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. *Proc Natl Acad Sci U S A* 101:9745-9750.

Angosto, D., G. Lopez-Castejon, A. Lopez-Munoz, M.P. Sepulcre, M. Arizcun, J. Meseguer, and V. Mulero. 2012. Evolution of inflammasome functions in vertebrates: Inflammasome and caspase-1 trigger fish macrophage cell death but are dispensable for the processing of IL-1beta. *Innate Immun* 18:815-824.

Atkins, E. 1960. Pathogenesis of fever. *Physiol Rev* 40:580-646.

Beasley, D., R.A. Cohen, and N.G. Levinsky. 1989. Interleukin 1 inhibits contraction of vascular smooth muscle. *J Clin Invest* 83:331-335.

Bebes, A., F. Kovacs-Solyom, J. Prihoda, R. Kui, L. Kemeny, and R. Gyulai. 2014. Interleukin-1 receptors are differentially expressed in normal and psoriatic T cells. *Mediators Inflamm* 2014:472625.

Beeson, P.B. 1948. Temperature-elevating effect of a substance obtained from polymorphonuclear leucocytes. *J Clin Invest* 27:524.

Ben-Sasson, S.Z., J. Hu-Li, J. Quiel, S. Cauchetaux, M. Ratner, I. Shapira, C.A. Dinarello, and W.E. Paul. 2009. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A* 106:7119-7124.

Bergsbaken, T., S.L. Fink, and B.T. Cookson. 2009. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* 7:99-109.

Bevilacqua, M.P., J.S. Pober, M.E. Wheeler, R.S. Cotran, and M.A. Gimbrone, Jr. 1985. Interleukin-1 activation of vascular endothelium. Effects on procoagulant activity and leukocyte adhesion. *Am J Pathol* 121:394-403.

Bianco, F., E. Pravettoni, A. Colombo, U. Schenk, T. Moller, M. Matteoli, and C. Verderio. 2005. Astrocyte-derived ATP induces vesicle shedding and IL-1 release from microglia. *J. Immunol.* 174:7268-7277.

Black, R.A., S.R. Kronheim, M. Cantrell, M.C. Deeley, C.J. March, K.S. Prickett, J. Wignall, P.J. Conlon, D. Cosman, T.P. Hopp, and et al. 1988. Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *J Biol Chem* 263:9437-9442.

Boraschi, D., and A. Tagliabue. 2013. The interleukin-1 receptor family. *Semin Immunol* 25:394-407.

Botelho, R.J., M. Teruel, R. Dierckman, R. Anderson, A. Wells, J.D. York, T. Meyer, and S. Grinstein. 2000. Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. *J Cell Biol* 151:1353-1368.

Braddock, M., and A. Quinn. 2004. Targeting IL-1 in inflammatory disease: new opportunities for therapeutic intervention. *Nat Rev Drug Discov* 3:330-339.

Carta, S., S. Tassi, C. Semino, G. Fossati, P. Mascagni, C.A. Dinarello, and A. Rubartelli. 2006. Histone deacetylase inhibitors prevent exocytosis of interleukin-1beta-containing secretory lysosomes: role of microtubules. *Blood* 108:1618-1626.

Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, K. Van Ness, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Cannizzaro, and et al. 1992. Molecular cloning of the interleukin-1 beta converting enzyme. *Science* 256:97-100.

Chen, D.S., and I. Mellman. 2017. Elements of cancer immunity and the cancer-immune set point. *Nature* 541:321-330.

Chen, K.W., C.J. Gross, F.V. Sotomayor, K.J. Stacey, J. Tschopp, M.J. Sweet, and K. Schroder. 2014. The neutrophil NLRC4 inflammasome selectively promotes IL-1beta maturation without pyroptosis during acute Salmonella challenge. *Cell Rep* 8:570-582.

Chung, Y., S.H. Chang, G.J. Martinez, X.O. Yang, R. Nurieva, H.S. Kang, L. Ma, S.S. Watowich, A.M. Jetten, Q. Tian, and C. Dong. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 30:576-587.

Coeshott, C., C. Ohnemus, A. Pilyavskaya, S. Ross, M. Wieczorek, H. Kroona, A.H. Leimer, and J. Cheronis. 1999. Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci U S A* 96:6261-6266.

Coll, R.C., A.A. Robertson, J.J. Chae, S.C. Higgins, R. Munoz-Planillo, M.C. Inserra, I. Vetter, L.S. Dungan, B.G. Monks, A. Stutz, D.E. Croker, M.S. Butler, M. Haneklaus, C.E. Sutton, G. Nunez, E. Latz, D.L. Kastner, K.H. Mills, S.L. Masters, K. Schroder, M.A. Cooper, and L.A. O'Neill. 2015. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med* 21:248-255.

Conos, S.A., K.E. Lawlor, D.L. Vaux, J.E. Vince, and L.M. Lindqvist. 2016. Cell death is not essential for caspase-1-mediated interleukin-1ß activation and secretion. *Cell Death Differ.* 23:1827-1838.

Cookson, B.T., and M.A. Brennan. 2001. Pro-inflammatory programmed cell death. *Trends Microbiol* 9:113-114.

Cush, J.J. 2013. Autoinflammatory syndromes. *Dermatol Clin* 31:471-480.

Denzer, K., M.J. Kleijmeer, H.F. Heijnen, W. Stoorvogel, and H.J. Geuze. 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell. Sci.* 113 Pt 19:3365-3374.

Dhuriya, Y.K., and D. Sharma. 2018. Necroptosis: a regulated inflammatory mode of cell death. *J Neuroinflammation* 15:199.

Dinarello, C.A. 2000. Proinflammatory cytokines. *Chest* 118:503-508.

Dinarello, C.A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 27:519-550.

Dinarello, C.A. 2011. A clinical perspective of IL-1beta as the gatekeeper of inflammation. *Eur J Immunol* 41:1203-1217.

Dinarello, C.A., N.P. Goldin, and S.M. Wolff. 1974. Demonstration and characterization of two distinct human leukocytic pyrogens. *J Exp Med* 139:1369-1381.

Ding, J., and F. Shao. 2017. SnapShot: The Noncanonical Inflammasome. *Cell* 168:544-544 e541.

Ding, J., K. Wang, W. Liu, Y. She, Q. Sun, J. Shi, H. Sun, D.C. Wang, and F. Shao. 2016. Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* 535:111-116.

Dostert, C., V. Petrilli, R. Van Bruggen, C. Steele, B.T. Mossman, and J. Tschopp. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320:674-677.

Duewell, P., H. Kono, K.J. Rayner, C.M. Sirois, G. Vladimer, F.G. Bauernfeind, G.S. Abela, L. Franchi, G. Nunez, M. Schnurr, T. Espevik, E. Lien, K.A. Fitzgerald, K.L. Rock, K.J. Moore, S.D. Wright, V. Hornung, and E. Latz. 2010. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464:1357-1361.

Duncan, J.A., and S.W. Canna. 2018. The NLRC4 Inflammasome. *Immunol Rev* 281:115-123.

Dupont, N., S. Jiang, M. Pilli, W. Ornatowski, D. Bhattacharya, and V. Deretic. 2011. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. *EMBO J* 30:4701-4711.

Eisenbarth, S.C., O.R. Colegio, W. O'Connor, F.S. Sutterwala, and R.A. Flavell. 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453:1122-1126.

Eskan, M.A., M.R. Benakanakere, B.G. Rose, P. Zhang, J. Zhao, P. Stathopoulou, D. Fujioka, and D.F. Kinane. 2008. Interleukin-1beta modulates proinflammatory cytokine production in human epithelial cells. *Infect Immun* 76:2080-2089.

Evavold, C.L., J. Ruan, Y. Tan, S. Xia, H. Wu, and J.C. Kagan. 2018. The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. *Immunity* 48:35-44 e36.

Fenini, G., E. Contassot, and L.E. French. 2017. Potential of IL-1, IL-18 and Inflammasome Inhibition for the Treatment of Inflammatory Skin Diseases. *Front Pharmacol* 8:278.

Fettelschoss, A., M. Kistowska, S. LeibundGut-Landmann, H.D. Beer, P. Johansen, G. Senti, E. Contassot, M.F. Bachmann, L.E. French, A. Oxenius, and T.M. Kundig. 2011. Inflammasome activation and IL-1beta target IL-1alpha for secretion as opposed to surface expression. *Proc Natl Acad Sci U S A* 108:18055-18060.

Fevrier, B., and G. Raposo. 2004. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol* 16:415-421.

Finzel, B.C., L.L. Clancy, D.R. Holland, S.W. Muchmore, K.D. Watenpaugh, and H.M. Einspahr. 1989. Crystal structure of recombinant human interleukin-1ß at 2.0 Å resolution. *J. Mol. Biol.* 209:779-791.

Franchi, L., G. Chen, N. Marina-Garcia, A. Abe, Y. Qu, S. Bao, J.A. Shayman, J. Turk, G.R. Dubyak, and G. Nunez. 2009. Calcium-independent phospholipase A2 beta is dispensable in inflammasome activation and its inhibition by bromoenol lactone. *J Innate Immun* 1:607-617.

Franchi, L., T.D. Kanneganti, G.R. Dubyak, and G. Nunez. 2007. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J Biol Chem* 282:18810-18818.

Fuleihan, R.L. 1998. The X-linked hyperimmunoglobulin M syndrome. *Semin Hematol* 35:321-331.

Gaidt, M.M., T.S. Ebert, D. Chauhan, T. Schmidt, J.L. Schmid-Burgk, F. Rapino, A.A. Robertson, M.A. Cooper, T. Graf, and V. Hornung. 2016. Human Monocytes Engage an Alternative Inflammasome Pathway. *Immunity* 44:833-846.

Gaidt, M.M., and V. Hornung. 2017. Alternative inflammasome activation enables IL-1beta release from living cells. *Curr Opin Immunol* 44:7-13.

Gaidt, M.M., F. Rapino, T. Graf, and V. Hornung. 2018a. Modeling Primary Human Monocytes with the Trans-Differentiation Cell Line BLaER1. *Methods Mol Biol* 1714:57-66.

Gaidt, M.M., F. Rapino, T. Graf, and V. Hornung. 2018b. Modeling Primary Human Monocytes with the Trans-Differentiation Cell Line BLaER1. *Methods Mol. Biol.* 1714:57-66.

Gay, N.J., and F.J. Keith. 1991. Drosophila Toll and IL-1 receptor. *Nature* 351:355-356.

Gillespie, J., R. Mathews, and M.F. McDermott. 2010. Rilonacept in the management of cryopyrin-associated periodic syndromes (CAPS). *J Inflamm Res* 3:1-8.

Goh, A.X., S. Bertin-Maghit, S. Ping Yeo, A.W. Ho, H. Derks, A. Mortellaro, and C.I. Wang. 2014. A novel human anti-interleukin-1beta neutralizing monoclonal antibody showing in vivo efficacy. *MAbs* 6:765-773.

Grahames, C.B., A.D. Michel, I.P. Chessell, and P.P. Humphrey. 1999. Pharmacological characterization of ATP- and LPS-induced IL-1ß release in human monocytes. *Br. J. Pharmacol.* 127:1915-1921.

Green, D.R. 1998. Apoptotic pathways: the roads to ruin. *Cell* 94:695-698.

Gringhuis, S.I., T.M. Kaptein, B.A. Wevers, B. Theelen, M. van der Vlist, T. Boekhout, and T.B. Geijtenbeek. 2012. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. *Nat Immunol* 13:246-254.

Gross, O., H. Poeck, M. Bscheider, C. Dostert, N. Hannesschlager, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, and J. Ruland. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459:433-436.

Hadley, W.K., J. O'Rourke, and E. Atkins. 1966. Purification and characterization of an endogenous pyrogen. *Yale J Biol Med* 38:339-354.

Hamon, Y., M.F. Luciani, F. Becq, B. Verrier, A. Rubartelli, and G. Chimini. 1997a. Interleukin-1beta secretion is impaired by inhibitors of the Atp binding cassette transporter, ABC1. *Blood* 90:2911-2915.

Hamon, Y., M.F. Luciani, F. Becq, B. Verrier, A. Rubartelli, and G. Chimini. 1997b. Interleukin-1ß secretion is impaired by inhibitors of the ATP binding cassette transporter, ABC1. *Blood* 90:2911-2915.

Harris, J., M. Hartman, C. Roche, S.G. Zeng, A. O'Shea, F.A. Sharp, E.M. Lambe, E.M. Creagh, D.T. Golenbock, J. Tschopp, H. Kornfeld, K.A. Fitzgerald, and E.C. Lavelle. 2011. Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. *J Biol Chem* 286:9587-9597.

Hazuda, D.J., J. Strickler, F. Kueppers, P.L. Simon, and P.R. Young. 1990. Processing of precursor interleukin 1 beta and inflammatory disease. *J Biol Chem* 265:6318-6322.

He, W.T., H. Wan, L. Hu, P. Chen, X. Wang, Z. Huang, Z.H. Yang, C.Q. Zhong, and J. Han. 2015. Gasdermin D is an executor of pyroptosis and required for interleukin-1beta secretion. *Cell Res* 25:1285-1298.

He, Y., L. Franchi, and G. Nunez. 2013. TLR agonists stimulate Nlrp3-dependent IL-1beta production independently of the purinergic P2X7 receptor in dendritic cells and in vivo. *J Immunol* 190:334-339.

He, Y., H. Hara, and G. Nunez. 2016. Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends Biochem Sci* 41:1012-1021.

Heilig, R., M.S. Dick, L. Sborgi, E. Meunier, S. Hiller, and P. Broz. 2018. The Gasdermin-D pore acts as a conduit for IL-1ß secretion in mice. *Eur. J. Immunol.* 48:584-592.

Heneka, M.T., M.P. Kummer, A. Stutz, A. Delekate, S. Schwartz, A. Vieira-Saecker, A. Griep, D. Axt, A. Remus, T.C. Tzeng, E. Gelpi, A. Halle, M. Korte, E. Latz, and D.T. Golenbock. 2013. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* 493:674-678.

Hentze, H., X.Y. Lin, M.S. Choi, and A.G. Porter. 2003. Critical role for cathepsin B in mediating caspase-1-dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial toxin nigericin. *Cell Death Differ* 10:956-968.

Hogquist, K.A., M.A. Nett, E.R. Unanue, and D.D. Chaplin. 1991. Interleukin 1 is processed and released during apoptosis. *Proc Natl Acad Sci U S A* 88:8485-8489.

Horai, R., M. Asano, K. Sudo, H. Kanuka, M. Suzuki, M. Nishihara, M. Takahashi, and Y. Iwakura. 1998. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in

turpentine-induced fever development and glucocorticoid secretion. *J Exp Med* 187:1463-1475.

Hughes, R.C. 1999. Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim Biophys Acta* 1473:172-185.

Ichinohe, T., H.K. Lee, Y. Ogura, R. Flavell, and A. Iwasaki. 2009. Inflammasome recognition of influenza virus is essential for adaptive immune responses. *J Exp Med* 206:79-87.

Iula, L., I.A. Keitelman, F. Sabbione, F. Fuentes, M. Guzman, J.G. Galletti, P.P. Gerber, M. Ostrowski, J.R. Geffner, C.C. Jancic, and A.S. Trevani. 2018. Autophagy Mediates Interleukin-1beta Secretion in Human Neutrophils. *Front Immunol* 9:269.

Iwasaki, A., and R. Medzhitov. 2015. Control of adaptive immunity by the innate immune system. *Nat. Immunol.* 16:343-353.

Jacob, F., S. Vernaldi, and T. Maekawa. 2013. Evolution and Conservation of Plant NLR Functions. *Front Immunol* 4:297.

Jamilloux, Y., F. Magnotti, A. Belot, and T. Henry. 2018. The pyrin inflammasome: from sensing RhoA GTPases-inhibiting toxins to triggering autoinflammatory syndromes. *Pathog Dis* 76:

Jeon, J.W., J.G. Jung, E.C. Shin, H.I. Choi, H.Y. Kim, M.L. Cho, S.W. Kim, Y.S. Jang, M.H. Sohn, J.H. Moon, Y.H. Cho, K.L. Hoe, Y.S. Seo, and Y.W. Park. 2010. Soluble CD93 induces differentiation of monocytes and enhances TLR responses. *J Immunol* 185:4921-4927.

Jorgensen, I., Y. Zhang, B.A. Krantz, and E.A. Miao. 2016. Pyroptosis triggers pore-induced intracellular traps (PITs) that capture bacteria and lead to their clearance by efferocytosis. *J Exp Med* 213:2113-2128.

Kagan, J.C., T. Su, T. Horng, A. Chow, S. Akira, and R. Medzhitov. 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* 9:361-368.

Kayagaki, N., I.B. Stowe, B.L. Lee, K. O'Rourke, K. Anderson, S. Warming, T. Cuellar, B. Haley, M. Roose-Girma, Q.T. Phung, P.S. Liu, J.R. Lill, H. Li, J. Wu, S. Kummerfeld, J. Zhang, W.P. Lee, S.J. Snipas, G.S. Salvesen, L.X. Morris, L. Fitzgerald, Y. Zhang, E.M. Bertram, C.C. Goodnow, and V.M. Dixit. 2015. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 526:666-671.

Kayagaki, N., M.T. Wong, I.B. Stowe, S.R. Ramani, L.C. Gonzalez, S. Akashi-Takamura, K. Miyake, J. Zhang, W.P. Lee, A. Muszynski, L.S. Forsberg, R.W. Carlson, and V.M. Dixit. 2013. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 341:1246-1249.

Kim, B., Y. Lee, E. Kim, A. Kwak, S. Ryoo, S.H. Bae, T. Azam, S. Kim, and C.A. Dinarello. 2013. The Interleukin-1alpha Precursor is Biologically Active and is Likely a Key Alarmin in the IL-1 Family of Cytokines. *Front Immunol* 4:391.

Kim, P.K., and E.H. Hettema. 2015. Multiple pathways for protein transport to peroxisomes. *J Mol Biol* 427:1176-1190.

Kong, F., B. Ye, J. Cao, X. Cai, L. Lin, S. Huang, W. Huang, and Z. Huang. 2016. Curcumin Represses NLRP3 Inflammasome Activation via TLR4/MyD88/NF-kappaB and P2X7R Signaling in PMA-Induced Macrophages. *Front Pharmacol* 7:369.

Kool, M., V. Petrilli, T. De Smedt, A. Rolaz, H. Hammad, M. van Nimwegen, I.M. Bergen, R. Castillo, B.N. Lambrecht, and J. Tschopp. 2008. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. *J. Immunol.* 181:3755-3759.

Kopfnagel, V., M. Wittmann, and T. Werfel. 2011. Human keratinocytes express AIM2 and respond to dsDNA with IL-1beta secretion. *Exp Dermatol* 20:1027-1029.

Kovacs, S.B., and E.A. Miao. 2017. Gasdermins: Effectors of Pyroptosis. *Trends Cell Biol* 27:673-684.

Krane, S.M., J.M. Dayer, L.S. Simon, and M.S. Byrne. 1985. Mononuclear cell-conditioned medium containing mononuclear cell factor (MCF), homologous with interleukin 1, stimulates collagen and fibronectin synthesis by adherent rheumatoid synovial cells: effects of prostaglandin E2 and indomethacin. *Coll Relat Res* 5:99-117.

Kuida, K., J.A. Lippke, G. Ku, M.W. Harding, D.J. Livingston, M.S. Su, and R.A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* 267:2000-2003.

Laman, J.D., E. Claassen, and R.J. Noelle. 2017. Functions of CD40 and Its Ligand, gp39 (CD40L). *Crit Rev Immunol* 37:371-420.

Lamkanfi, M., and V.M. Dixit. 2012. Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol* 28:137-161.

Lamkanfi, M., J.L. Mueller, A.C. Vitari, S. Misaghi, A. Fedorova, K. Deshayes, W.P. Lee, H.M. Hoffman, and V.M. Dixit. 2009. Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *J Cell Biol* 187:61-70.

Lee, C.C., A.M. Avalos, and H.L. Ploegh. 2012. Accessory molecules for Toll-like receptors and their function. *Nat Rev Immunol* 12:168-179.

Lemaitre, B., E. Nicolas, L. Michaut, J.M. Reichhart, and J.A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86:973-983.

Li, P., H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, and et al. 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* 80:401-411.

Liu, X., Z. Zhang, J. Ruan, Y. Pan, V.G. Magupalli, H. Wu, and J. Lieberman. 2016. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* 535:153-158.

Lomedico, P.T., U. Gubler, C.P. Hellmann, M. Dukovich, J.G. Giri, Y.C. Pan, K. Collier, R. Semionow, A.O. Chua, and S.B. Mizel. 1984. Cloning and expression of murine interleukin-1 cDNA in Escherichia coli. *Nature* 312:458-462.

Lopez-Castejon, G., and D. Brough. 2011. Understanding the mechanism of IL-1beta secretion. *Cytokine Growth Factor Rev* 22:189-195.

Lowenthal, J.W., J.C. Cerottini, and H.R. MacDonald. 1986. Interleukin 1-dependent induction of both interleukin 2 secretion and interleukin 2 receptor expression by thymoma cells. *J Immunol* 137:1226-1231.

Lu, A., V.G. Magupalli, J. Ruan, Q. Yin, M.K. Atianand, M.R. Vos, G.F. Schroder, K.A. Fitzgerald, H. Wu, and E.H. Egelman. 2014. Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes. *Cell* 156:1193-1206.

Lu, Y.C., W.C. Yeh, and P.S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. *Cytokine* 42:145-151.

MacKenzie, A., H.L. Wilson, E. Kiss-Toth, S.K. Dower, R.A. North, and A. Surprenant. 2001. Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 15:825-835.

Maelfait, J., E. Vercammen, S. Janssens, P. Schotte, M. Haegman, S. Magez, and R. Beyaert. 2008. Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta maturation by caspase-8. *J Exp Med* 205:1967-1973.

March, C.J., B. Mosley, A. Larsen, D.P. Cerretti, G. Braedt, V. Price, S. Gillis, C.S. Henney, S.R. Kronheim, K. Grabstein, and et al. 1985. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* 315:641-647.

Mariathasan, S., D.S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W.P. Lee, Y. Weinrauch, D.M. Monack, and V.M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.

Marlin, S.D., and T.A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* 51:813-819.

Martin, M.U., and H. Wesche. 2002. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochim Biophys Acta* 1592:265-280.

Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10:417-426.

Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.

Martinon, F., and J. Tschopp. 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 117:561-574.

Martinon, F., and J. Tschopp. 2005. NLRs join TLRs as innate sensors of pathogens. *Trends Immunol* 26:447-454.

Masters, S.L., A. Dunne, S.L. Subramanian, R.L. Hull, G.M. Tannahill, F.A. Sharp, C. Becker, L. Franchi, E. Yoshihara, Z. Chen, N. Mullooly, L.A. Mielke, J. Harris, R.C. Coll, K.H. Mills, K.H. Mok, P. Newsholme, G. Nunez, J. Yodoi, S.E. Kahn, E.C. Lavelle, and L.A. O'Neill. 2010. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol* 11:897-904.

McIlwain, D.R., T. Berger, and T.W. Mak. 2015. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* 7:

Medzhitov, R. 2008. Origin and physiological roles of inflammation. *Nature* 454:428-435.

Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388:394-397.

Mizutani, H., N. Schechter, G. Lazarus, R.A. Black, and T.S. Kupper. 1991. Rapid and specific conversion of precursor interleukin 1 beta (IL-1 beta) to an active IL-1 species by human mast cell chymase. *J Exp Med* 174:821-825.

Monteleone, M., A.C. Stanley, K.W. Chen, D.L. Brown, J.S. Bezbradica, J.B. von Pein, C.L. Holley, D. Boucher, M.R. Shakespear, R. Kapetanovic, V. Rolfes, M.J. Sweet, J.L. Stow, and K. Schroder. 2018. Interleukin-1beta Maturation Triggers Its Relocation to the Plasma Membrane for Gasdermin-D-Dependent and -Independent Secretion. *Cell Rep* 24:1425-1433.

Mosley, B., D.L. Urdal, K.S. Prickett, A. Larsen, D. Cosman, P.J. Conlon, S. Gillis, and S.K. Dower. 1987. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. *J Biol Chem* 262:2941-2944.

Munoz-Planillo, R., P. Kuffa, G. Martinez-Colon, B.L. Smith, T.M. Rajendiran, and G. Nunez. 2013. K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38:1142-1153.

Murphy, J.E., B.E. Padilla, B. Hasdemir, G.S. Cottrell, and N.W. Bunnett. 2009. Endosomes: a legitimate platform for the signaling train. *Proc Natl Acad Sci U S A* 106:17615-17622.

Murphy, P.A., P.J. Chesney, and W.B. Wood, Jr. 1974. Further purification of rabbit leukocyte pyrogen. *J Lab Clin Med* 83:310-322.

Muruve, D.A., V. Petrilli, A.K. Zaiss, L.R. White, S.A. Clark, P.J. Ross, R.J. Parks, and J. Tschopp. 2008. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 452:103-107.

Nakae, S., M. Asano, R. Horai, N. Sakaguchi, and Y. Iwakura. 2001. IL-1 enhances T cell-dependent antibody production through induction of CD40 ligand and OX40 on T cells. *J Immunol* 167:90-97.

Netea, M.G., C.A. Nold-Petry, M.F. Nold, L.A. Joosten, B. Opitz, J.H. van der Meer, F.L. van de Veerdonk, G. Ferwerda, B. Heinhuis, I. Devesa, C.J. Funk, R.J. Mason, B.J. Kullberg, A. Rubartelli, J.W. van der Meer, and C.A. Dinarello. 2009. Differential requirement for the activation of the inflammasome for processing and release of IL-1ß in monocytes and macrophages. *Blood* 113:2324-2335.

O'Neill, L.A. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE* 2000:re1.

Park, B.S., D.H. Song, H.M. Kim, B.S. Choi, H. Lee, and J.O. Lee. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458:1191-1195.

Park, E.K., H.S. Jung, H.I. Yang, M.C. Yoo, C. Kim, and K.S. Kim. 2007. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm. Res.* 56:45-50.

Perregaux, D., and C.A. Gabel. 1994. Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem* 269:15195-15203.

Piccini, A., S. Carta, S. Tassi, D. Lasiglie, G. Fossati, and A. Rubartelli. 2008. ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1ß and IL-18 secretion in an autocrine way. *Proc. Natl. Acad. Sci. U S A* 105:8067-8072.

Pizzirani, C., D. Ferrari, P. Chiozzi, E. Adinolfi, D. Sandona, E. Savaglio, and F. Di Virgilio. 2007. Stimulation of P2 receptors causes release of IL-1beta-loaded microvesicles from human dendritic cells. *Blood* 109:3856-3864.

Pop, C., and G.S. Salvesen. 2009. Human caspases: activation, specificity, and regulation. *J Biol Chem* 284:21777-21781.

Popa, S.J., S.E. Stewart, and K. Moreau. 2018. Unconventional secretion of annexins and galectins. *Semin Cell Dev Biol*

Poubelle, P., M. Damon, F. Blotman, and J.M. Dayer. 1985. Production of mononuclear cell factor by mononuclear phagocytes from rheumatoid synovial fluid. *J Rheumatol* 12:412-417.

Priestle, J.P., H.P. Schar, and M.G. Grutter. 1989. Crystallographic refinement of interleukin 1ß at 2.0 Å resolution. *Proc. Natl. Acad. Sci. U S A* 86:9667-9671.

Qu, Y., L. Franchi, G. Nunez, and G.R. Dubyak. 2007. Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J Immunol* 179:1913-1925.

Rabouille, C. 2017. Pathways of Unconventional Protein Secretion. *Trends Cell Biol* 27:230-240.

Ramoni, C., F. Spadaro, M. Menegon, and F. Podo. 2001. Cellular localization and functional role of phosphatidylcholine-specific phospholipase C in NK cells. *J Immunol* 167:2642-2650.

Rapino, F., E.F. Robles, J.A. Richter-Larrea, E.M. Kallin, J.A. Martinez-Climent, and T. Graf. 2013. C/EBPα induces highly efficient macrophage transdifferentiation of B lymphoma and leukemia cell lines and impairs their tumorigenicity. *Cell Rep* 3:1153-1163.

Reinholz, M., Y. Kawakami, S. Salzer, A. Kreuter, Y. Dombrowski, S. Koglin, S. Kresse, T. Ruzicka, and J. Schauber. 2013. HPV16 activates the AIM2 inflammasome in keratinocytes. *Arch Dermatol Res* 305:723-732.

Rider, P., Y. Carmi, O. Guttman, A. Braiman, I. Cohen, E. Voronov, M.R. White, C.A. Dinarello, and R.N. Apte. 2011. IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. *J Immunol* 187:4835-4843.

Ridker, P.M. 2016. From C-Reactive Protein to Interleukin-6 to Interleukin-1: Moving Upstream To Identify Novel Targets for Atheroprotection. *Circ Res* 118:145-156.

Ryu, J.K., S.J. Kim, S.H. Rah, J.I. Kang, H.E. Jung, D. Lee, H.K. Lee, J.O. Lee, B.S. Park, T.Y. Yoon, and H.M. Kim. 2017. Reconstruction of LPS transfer cascade reveals structural determinants within LBP, CD14, and TLR4-MD2 for efficient LPS recognition and transfer. *Immunity* 46:38-50.

Saitoh, T., N. Fujita, M.H. Jang, S. Uematsu, B.G. Yang, T. Satoh, H. Omori, T. Noda, N. Yamamoto, M. Komatsu, K. Tanaka, T. Kawai, T. Tsujimura, O. Takeuchi, T.

Yoshimori, and S. Akira. 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 456:264-268.

Savic, S., and M.F. McDermott. 2009. Inflammation: canakinumab for the cryopyrin-associated periodic syndromes. *Nat Rev Rheumatol* 5:529-530.

Sborgi, L., S. Ruhl, E. Mulvihill, J. Pipercevic, R. Heilig, H. Stahlberg, C.J. Farady, D.J. Muller, P. Broz, and S. Hiller. 2016. GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death. *EMBO J* 35:1766-1778.

Schumann, R.R. 1992. Function of lipopolysaccharide (LPS)-binding protein (LBP) and CD14, the receptor for LPS/LBP complexes: a short review. *Res Immunol* 143:11-15.

Shi, F., M. Kouadir, and Y. Yang. 2015a. NALP3 inflammasome activation in protein misfolding diseases. *Life Sci* 135:9-14.

Shi, J., Y. Zhao, K. Wang, X. Shi, Y. Wang, H. Huang, Y. Zhuang, T. Cai, F. Wang, and F. Shao. 2015b. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 526:660-665.

Shi, J., Y. Zhao, Y. Wang, W. Gao, J. Ding, P. Li, L. Hu, and F. Shao. 2014. Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514:187-192.

Shirasaki, Y., M. Yamagishi, N. Suzuki, K. Izawa, A. Nakahara, J. Mizuno, S. Shoji, T. Heike, Y. Harada, R. Nishikomori, and O. Ohara. 2014. Real-time single-cell imaging of protein secretion. *Sci Rep* 4:4736.

Singer, II, S. Scott, J. Chin, E.K. Bayne, G. Limjuco, J. Weidner, D.K. Miller, K. Chapman, and M.J. Kostura. 1995. The interleukin-1 beta-converting enzyme (ICE) is localized on the external cell surface membranes and in the cytoplasmic ground substance of human monocytes by immuno-electron microscopy. *J Exp Med* 182:1447-1459.

Sitia, R., and A. Rubartelli. 2018. The unconventional secretion of IL-1beta: Handling a dangerous weapon to optimize inflammatory responses. *Semin Cell Dev Biol*

Sloan-Lancaster, J., E. Abu-Raddad, J. Polzer, J.W. Miller, J.C. Scherer, A. De Gaetano, J.K. Berg, and W.H. Landschulz. 2013. Double-blind, randomized study evaluating the glycemic and anti-inflammatory effects of subcutaneous LY2189102, a neutralizing IL-1beta antibody, in patients with type 2 diabetes. *Diabetes Care* 36:2239-2246.

Song, A., L. Zhu, G. Gorantla, O. Berdysz, S.A. Amici, M. Guerau-de-Arellano, K.M. Madalena, J.K. Lerch, X. Liu, and N. Quan. 2018. Salient type 1 interleukin 1 receptor expression in peripheral non-immune cells. *Sci Rep* 8:723.

Steiner, A.A., A.I. Ivanov, J. Serrats, H. Hosokawa, A.N. Phayre, J.R. Robbins, J.L. Roberts, S. Kobayashi, K. Matsumura, P.E. Sawchenko, and A.A. Romanovsky. 2006. Cellular and molecular bases of the initiation of fever. *PLoS Biol* 4:e284.

Steringer, J.P., S. Bleicken, H. Andreas, S. Zacherl, M. Laussmann, K. Temmerman, F.X. Contreras, T.A. Bharat, J. Lechner, H.M. Muller, J.A. Briggs, A.J. Garcia-Saez, and W. Nickel. 2012. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-dependent oligomerization of fibroblast growth factor 2 (FGF2) triggers the formation of a lipidic membrane pore implicated in unconventional secretion. *J Biol Chem* 287:27659-27669.

Steringer, J.P., and W. Nickel. 2018. A direct gateway into the extracellular space: Unconventional secretion of FGF2 through self-sustained plasma membrane pores. *Semin Cell Dev Biol*

Stuber, E., and W. Strober. 1996. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. *J Exp Med* 183:979-989.

Symons, J.A., P.R. Young, and G.W. Duff. 1995. Soluble type II interleukin 1 (IL-1) receptor binds and blocks processing of IL-1 beta precursor and loses affinity for IL-1 receptor antagonist. *Proc Natl Acad Sci U S A* 92:1714-1718.

Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. *Cell* 140:805-820.

Tattoli, I., L.A. Carneiro, M. Jehanno, J.G. Magalhaes, Y. Shu, D.J. Philpott, D. Arnoult, and S.E. Girardin. 2008. NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production. *EMBO Rep* 9:293-300.

Thomas, J., Y. Epshtein, A. Chopra, B. Ordog, M. Ghassemi, J.W. Christman, S. Nattel, J.L. Cook, and I. Levitan. 2011. Anthrax lethal factor activates K(+) channels to induce IL-1beta secretion in macrophages. *J Immunol* 186:5236-5243.

Thornberry, N.A. 1997. The caspase family of cysteine proteases. *Br Med Bull* 53:478-490.

Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins, and et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356:768-774.

Ting, J.P., R.C. Lovering, E.S. Alnemri, J. Bertin, J.M. Boss, B.K. Davis, R.A. Flavell, S.E. Girardin, A. Godzik, J.A. Harton, H.M. Hoffman, J.P. Hugot, N. Inohara, A. Mackenzie, L.J. Maltais, G. Nunez, Y. Ogura, L.A. Otten, D. Philpott, J.C. Reed, W. Reith, S. Schreiber, V. Steimle, and P.A. Ward. 2008. The NLR gene family: a standard nomenclature. *Immunity* 28:285-287.

Tosato, G., and K.D. Jones. 1990. Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. *Blood* 75:1305-1310.

Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* 26:171-176.

Tugal-Tutkun, I., C. Pavesio, A. De Cordoue, O. Bernard-Poenaru, and A. Gul. 2018. Use of Gevokizumab in Patients with Behcet's Disease Uveitis: An International, Randomized, Double-Masked, Placebo-Controlled Study and Open-Label Extension Study. *Ocul Immunol Inflamm* 1-11.

Vigano, E., C.E. Diamond, R. Spreafico, A. Balachander, R.M. Sobota, and A. Mortellaro. 2015. Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nat. Commun.* 6:8761.

Walter, E.J., S. Hanna-Jumma, M. Carraretto, and L. Forni. 2016. The pathophysiological basis and consequences of fever. *Crit Care* 20:200.

Wannamaker, W., R. Davies, M. Namchuk, J. Pollard, P. Ford, G. Ku, C. Decker, P. Charifson, P. Weber, U.A. Germann, K. Kuida, and J.C. Randle. 2007. (S)-1-((S)-2-{[1-(4-amino-3-chloro-phenyl)-methanoyl]-amino}-3,3-dimethyl-butanoy l)-pyrrolidine-2-carboxylic acid ((2R,3S)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide (VX-765), an orally available selective interleukin (IL)-converting enzyme/caspase-1

inhibitor, exhibits potent anti-inflammatory activities by inhibiting the release of IL-1beta and IL-18. *J Pharmacol Exp Ther* 321:509-516.

Weber, A., P. Wasiliew, and M. Kracht. 2010. Interleukin-1 (IL-1) pathway. *Sci Signal* 3:cm1.

Wei, Q., K. Mu, T. Li, Y. Zhang, Z. Yang, X. Jia, W. Zhao, W. Huai, P. Guo, and L. Han. 2014. Deregulation of the NLRP3 inflammasome in hepatic parenchymal cells during liver cancer progression. *Lab Invest* 94:52-62.

Williams, J.M., D. Deloria, J.A. Hansen, C.A. Dinarello, R. Loertscher, H.M. Shapiro, and T.B. Strom. 1985. The events of primary T cell activation can be staged by use of Sepharose-bound anti-T3 (64.1) monoclonal antibody and purified interleukin 1. *J Immunol* 135:2249-2255.

Wilson, N.J., K. Boniface, J.R. Chan, B.S. McKenzie, W.M. Blumenschein, J.D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, J.C. Lecron, R.A. Kastelein, D.J. Cua, T.K. McClanahan, E.P. Bowman, and R. de Waal Malefyt. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 8:950-957.

Wu, B., and S. Hur. 2015. How RIG-I like receptors activate MAVS. *Curr Opin Virol* 12:91-98.

Yang, D., Y. He, R. Munoz-Planillo, Q. Liu, and G. Nunez. 2015. Caspase-11 Requires the Pannexin-1 Channel and the Purinergic P2X7 Pore to Mediate Pyroptosis and Endotoxic Shock. *Immunity* 43:923-932.

Yoon, S., A. Kovalenko, K. Bogdanov, and D. Wallach. 2017. MLKL, the Protein that Mediates Necroptosis, Also Regulates Endosomal Trafficking and Extracellular Vesicle Generation. *Immunity* 47:51-65 e57.

Youm, Y.H., K.Y. Nguyen, R.W. Grant, E.L. Goldberg, M. Bodogai, D. Kim, D. D'Agostino, N. Planavsky, C. Lupfer, T.D. Kanneganti, S. Kang, T.L. Horvath, T.M. Fahmy, P.A. Crawford, A. Biragyn, E. Alnemri, and V.D. Dixit. 2015. The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med* 21:263-269.

Zacherl, S., G. La Venuta, H.M. Muller, S. Wegehingel, E. Dimou, P. Sehr, J.D. Lewis, H. Erfle, R. Pepperkok, and W. Nickel. 2015. A direct role for ATP1A1 in unconventional secretion of fibroblast growth factor 2. *J Biol Chem* 290:3654-3665.

Zanoni, I., Y. Tan, M. Di Gioia, A. Broggi, J. Ruan, J. Shi, C.A. Donado, F. Shao, H. Wu, J.R. Springstead, and J.C. Kagan. 2016. An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. *Science* 352:1232-1236.

Zhang, M., S.J. Kenny, L. Ge, K. Xu, and R. Schekman. 2015. Translocation of interleukin-1beta into a vesicle intermediate in autophagy-mediated secretion. *Elife* 4:

Zheng, Y., M. Humphry, J.J. Maguire, M.R. Bennett, and M.C. Clarke. 2013. Intracellular interleukin-1 receptor 2 binding prevents cleavage and activity of interleukin-1alpha, controlling necrosis-induced sterile inflammation. *Immunity* 38:285-295.

Zhong, Z., E. Sanchez-Lopez, and M. Karin. 2016. Autophagy, NLRP3 inflammasome and auto-inflammatory/immune diseases. *Clin Exp Rheumatol* 34:12-16.

Zhou, R., A.S. Yazdi, P. Menu, and J. Tschopp. 2011. A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469:221-225.

Zou, H., Y. Li, X. Liu, and X. Wang. 1999. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 274:11549-11556.

Zychlinsky, A., C. Fitting, J.M. Cavaillon, and P.J. Sansonetti. 1994. Interleukin 1 is released by murine macrophages during apoptosis induced by Shigella flexneri. *J Clin Invest* 94:1328-1332.

Zychlinsky, A., M.C. Prevost, and P.J. Sansonetti. 1992. Shigella flexneri induces apoptosis in infected macrophages. *Nature* 358:167-169.

Zykova, T.A., F. Zhu, X. Zhai, W.Y. Ma, S.P. Ermakova, K.W. Lee, A.M. Bode, and Z. Dong. 2008. Resveratrol directly targets COX-2 to inhibit carcinogenesis. *Mol Carcinog* 47:797-805.