



Institut de recherche expérimentale
et clinique

Pôle de Recherche Cardiovasculaire (CARD)
Faculté de Pharmacie et des Sciences Biomédicales

The platelet GARP/TGF- β pathway in post-myocardial infarction healing

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Thesis submitted in fulfillment of the requirements for the PhD
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Août 2024

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Publications

Articles and Review

Loss of platelet-specific GARP leads to cardiac rupture and adverse remodelling following myocardial infarction. Julie Bodart^{*}, Cécile Dufeys^{*}, Audrey Ginion, Jérôme Ambroise, Caroline Bouzin, Davide Brusa, Camille Michiels, Yotis A. Senis, Zoltan Nagy, Luc Bertrand, Christophe Beauloye, Sophie Lucas, and Sandrine Horman

**Contributed equally to the work. To be submitted*

Fibroblasts and platelets, a face-to-face dialogue at the heart of cardiac fibrosis. Dufeys Cécile^{*}, Bodart Julie^{*}, Bertrand Luc, Beauloye Christophe and Horman Sandrine.

Am J Physiol Heart Circ Physiol 2024 March.

**Contributed equally to the work.*

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Front. Med, 2022 January.

Communications in conferences

- 12-2023 **Belgian Working Group/Basic Research in Cardiology (BWG/BRC)**
Leuven, Belgium
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
*Role of platelet GARP in post-myocardial infarction inflammation and
fibrosis.* « Selected for oral presentation »
(Awarded for oral presentation)
- 09-2023 **EUPLAN International Conference (European Platelet Network)**
Bristol, England
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
*Role of platelet GARP in post-myocardial infarction inflammation and
fibrosis.* « Selected for oral presentation »
- 12-2022 **Belgian Society on Thrombosis and Hemostasis (BSTH)**
Mechelen, Belgium
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
*Role of platelet GARP in post-myocardial infarction
fibrosis.* « Selected for poster » (Awarded for the poster)
- 11-2022 **Belgian Working Group/Basic Research in Cardiology (BWG/BRC)**
Brussels, Belgium
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
*Role of platelet GARP in post-myocardial infarction
fibrosis.* « Selected for poster »

- 10-2021 **European Congress on Thrombosis and Hemostasis (ECTH)
Ghent, Belgium**
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
Role of platelet GARP in TGF- β 1 activation. « Selected for poster »
- 08-2021 **European Society of Cardiology (ESC), Online Congress**
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
Role of platelet GARP in TGF- β 1 activation. « Selected for poster »
- 07-2021 **International Society on Thrombosis and Hemostasis (ISTH),
Online Congress**
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
Role of platelet GARP in TGF- β 1 activation. « Selected for poster »
- 06-2021 **International Society for Heart Research (ISHR), Online Congress**
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
Role of platelet GARP in TGF- β 1 activation. « Selected for poster »
- 05-2021 **Printemps de la Cardiologie, Online Congress**
Julie Bodart, Cécile Dufey, Yotis A Senis, Zoltan Nagy, Luc Bertrand,
Christophe Beauloye, Sophie Lucas, and Sandrine Horman
Role of platelet GARP in TGF- β 1 activation. « Selected for oral
presentation » (*Awarded for the oral presentation*)

Remerciements

Après presque cinq années de thèse , me voilà enfin arriver à la fin de cette expérience intense et enrichissante. Il est enfin temps pour moi de remercier toutes les personnes qui ont été à mes côtés tout au long de ce parcours.

Tout d’abord, je tiens à remercier ma promotrice, la Professeure Sandrine Horman, qui m’a donné l’opportunité de réaliser ma thèse au sein du laboratoire CARD. Merci Sandrine d'avoir eu confiance en moi et d’avoir cru en mon potentiel pour mener à bien ce beau projet. Merci pour votre disponibilité, votre écoute attentive et votre soutien tout au long de ces cinq années de thèse. Vos conseils, tant sur le plan scientifique que personnel, ont été d'une grande importance pour moi. Vous avez su me pousser à donner le meilleur de moi-même et à dépasser mes limites, et pour cela, je vous en suis reconnaissante. Merci d’avoir été présente dans les moments de succès mais également dans les périodes de doute. Je tiens également à vous remercier pour les nombreuses opportunités que vous m'avez offertes, qu'il s'agisse de présenter mes travaux lors de congrès ou de m'impliquer dans des projets de recherche.

Je tiens aussi à remercier ma co-promotrice, la Professeure Sophie Lucas. Vous avez toujours été disponible pour partager votre expertise et offrir des nouvelles perspectives à mon travail, contribuant de manière importante à l'avancement du projet.

Je remercie également le Professeur Christophe Beauloye pour son soutien et son enthousiasme. Merci pour les nombreuses idées apportées lors de nos réunions, ce qui a permis d’améliorer le projet. Merci également au Professeur Luc Bertrand

pour avoir toujours été prêt à offrir des conseils et des suggestions constructives. Et aussi, merci d'avoir transformé nos pauses midis en véritables moments de débats.

Finalement, j'aimerais remercier les membres de mon comité d'encadrement ; les Professeurs Xavier Stephenne, François Huaux, et Jean-Luc Balligand pour votre bienveillance, le temps consacré au suivi de ma thèse ainsi que pour les commentaires constructifs apportés lors de nos réunions de comité. Je remercie également les Professeurs Ange Maguy et Dider Communi pour votre regard critique et externe que vous avez apporté à ma thèse.

Cécile, un grand merci pour ton investissement et ton aide dans le projet. Ce projet n'aurait pas été celui qu'il est aujourd'hui sans ton soutien.

Au cours de cette thèse, j'ai eu la très grande chance de rencontrer de nombreuses personnes qui m'ont permis de vivre une expérience incroyable. Tout d'abord, j'aimerais remercier les deux Julien(s) qui m'ont accueilli pour une courte période dans leur bureau.

Julien DP, merci d'avoir été si présent tout au long de ma thèse tant sur le plan scientifique que personnel. Merci pour ta bonne humeur constante et ton rire contagieux, qui ont apporté une touche de légèreté et de joie au sein du laboratoire. Toujours là pour offrir ton aide, et ton écoute, tu as aussi veillé à ce que je garde toujours le sourire, même lors des moments les plus stressants ou difficiles. Merci pour tous ces moments de rire et le soutien précieux que tu m'as apporté.

Julien C, merci de m'avoir accueilli avec autant de chaleur dans le bureau et le laboratoire. Merci Julien d'avoir toujours été là pour répondre à mes questions scientifiques et pour ta présence constante. Et surtout, merci d'avoir été le rassembleur du labo, toujours partant pour une sortie. Je te souhaite pleins de succès pour la fin de thèse.

Merci également à Marie. Merci Marie de m'avoir appris tous les secrets des plaquettes, mais tu m'as surtout aidé à surmonter ma peur des souris (ce qui est un véritable exploit !). Sans toi, je n'aurais pas pu réaliser tout cela aujourd'hui. Merci d'avoir toujours garder ta porte ouverte pour que je puisse venir te poser une question ou même lorsque je voulais juste faire une petite pause. Merci d'avoir apporté tant de bonne humeur au laboratoire.

J'aimerais également remercier de toute évidence Claire qui a partagé 4 années de thèse avec moi. Claire, merci de m'avoir accompagné tout au long de cette thèse dans laquelle tu m'as apporté beaucoup de rire et de joie. Sans toi, cette thèse n'aurait pas été la même. Merci pour ton soutien, ton écoute, ta générosité et ta bienveillance. Merci d'avoir toujours été là prête à écouter mes râleries et à partager aussi les moments de joies. Au-delà de la collègue que tu es, tu es devenue une véritable amie, et notre complicité a été une source importante de joie et de réconfort lors de cette thèse. Partager ensemble ces moments vont me manquer. Je te souhaite tout le succès possible pour la fin de ta thèse et pour ton retour en clinique.

Je remercie également Emma. Emma, tu es pour moi une scientifique exceptionnelle, et toujours là pour aider tout le monde (même parfois un peu trop, il faut le dire !). Au-delà de la collègue brillante que tu es, j'ai pu également te

découvrir en dehors du laboratoire grâce à nos cours de danse, qui sont devenus pour moi un véritable moment de détente et de rire. Sache que j'ai adoré partager ces moments avec toi. Je te souhaite tout le succès pour la suite de ta thèse, même si je n'en ai aucun doute sur le fait que tu réaliseras un travail incroyable. Merci pour tout et à nos futures danses ensemble!

Je tenais à remercier également Audrey, dont l'aide et le soutien ont été essentiels tout au long de mon projet. Ta persévérance et tes compétences scientifiques ont grandement contribué à l'avancement du projet. Tu es un véritable pilier du laboratoire.

Enfin, j'aimerais remercier toutes les personnes qui ont été présentes au cours de ma thèse et qui ont permis de rendre cette aventure plus joyeuse. Merci également à Laura, Laurence, Valentine, Natacha, Alice, Michele, Laurent, Jade, Gabriele, Ettore, Laurent, Marine, Charlotte, Eline, Justine, Anaïs, Mélanie, Laura F., Manu, Marine A., et Amélie. Merci d'avoir partagé avec moi cette aventure, sans vous cette expérience n'aurait pas été la même.

Un immense merci à mes amis pour votre soutien sans faille durant cette thèse, pour m'avoir aidée à me changer les idées quand ça n'allait pas mais aussi d'avoir été là pour célébrer chaque étape de cette thèse avec moi.

Je terminerai ces remerciements par les plus importants, ceux adressés à ma famille. Tout d'abord merci à ma maman de m'avoir encouragé tout au long de mon parcours. Merci de m'avoir soutenu pour aller jusqu'au bout de mes choix et merci d'avoir toujours été si fière de moi. Aussi, merci d'avoir écouté patiemment TOUTES mes présentations, et d'avoir toujours fait semblant de tout comprendre. J'espère

qu'aujourd'hui tu as enfin compris le sujet de ma thèse. Merci aussi à Eric pour avoir été un vrai et discret soutien durant toute cette période.

J'aimerais également remercier mon frère et ma sœur pour leur présence tout au long de mon parcours. Merci pour votre soutien et tous les moments partagés ensemble, et merci d'avoir toujours porté de l'intérêt à ce que je faisais. Un grand merci à mon beau-frère et ma belle-sœur pour avoir apporté une dose de joie et de bonne humeur tout au long de ma thèse. Et enfin, merci à mes neveux et nièces, Malo, Naël, Lou et Mila, de m'avoir offert des moments de légèreté et de faire de moi une Tia comblée.

Summary

After myocardial infarction (MI), the damaged heart tissue triggers an inflammatory phase, characterized by the infiltration of immune cells and upregulation of inflammatory genes. This process ultimately leads to fibrosis and adverse left ventricular remodelling. Transforming growth factor $\beta 1$ (TGF- $\beta 1$) is recognized as a pivotal regulator of both inflammation and fibrosis. Produced as a latent precursor, the mechanisms underlying latent TGF- $\beta 1$ activation after cardiac injury remain poorly understood. Platelets, which rapidly accumulate within the infarcted myocardium, are known to produce significant amounts of latent TGF- $\beta 1$. In fact, platelets contain 40-100 times more TGF- $\beta 1$ than other cell types, contributing to 40-50% of the circulating latent TGF- $\beta 1$ levels. Platelets can also produce active TGF- $\beta 1$ via a mechanism that implies Glycoprotein A Repeating Predominant (GARP) on their surface.

This thesis aimed to investigate the contribution of platelet GARP in TGF- $\beta 1$ activation, cardiac inflammation, and healing after MI. To this end, we generated a transgenic mouse strain allowing specific invalidation of the GARP gene in megakaryocyte/platelet (GpIb α -CrexGARPfl/fl). MI was induced in these mice by permanent ligation of the left anterior descending coronary artery. Our findings reveal that mice with a platelet-specific GARP deletion exhibited higher rates of myocardial rupture and exacerbated left ventricular dilatation following MI. Pathological evaluation of hearts in platelet-specific GARP knockout mice showed increased endothelial activation, neutrophil infiltration, and elevated production of pro-inflammatory cytokines, resulting in impaired healing. These results highlight the essential roles of the platelet GARP/TGF- $\beta 1$ pathway in regulating the inflammatory phase critical to myocardial healing.

Table of content

INTRODUCTION	1
CHAPTER 1. HEALING PROCESS AFTER MYOCARDIAL INFARCTION	3
1. PATHOPHYSIOLOGICAL CONTEXT	5
1.1 EPIDEMIOLOGY	5
1.2 PATHOPHYSIOLOGY	5
2. POST-MYOCARDIAL INFARCTION RESPONSE	6
2.1 NECROSIS OF CARDIOMYOCYTES	7
2.2 ACUTE INFLAMMATORY RESPONSE	8
2.2.1 <i>Cytokines involved in the inflammatory process.....</i>	<i>8</i>
2.2.2 <i>Cellular effectors of the inflammatory response.....</i>	<i>11</i>
2.2.3 <i>Targeting the inflammation.....</i>	<i>16</i>
2.2.4 <i>The extracellular matrix during the inflammatory phase</i>	<i>17</i>
2.3 RESOLUTION OF INFLAMMATION AND REPARATIVE PHASE	20
2.3.1 <i>Resolution of post-infarction inflammation.....</i>	<i>20</i>
2.3.2 <i>The cardiac fibroblast: The main effector in the reparative phase</i>	<i>26</i>
2.3.3 <i>The extracellular matrix during cardiac repair</i>	<i>27</i>
2.4 ADVERSE LEFT VENTRICULAR REMODELLING	28
2.4.1 <i>Left ventricular chamber dilatation</i>	<i>28</i>
2.4.2 <i>Eccentric hypertrophy of healthy cardiomyocytes.....</i>	<i>29</i>
2.4.3 <i>Interstitial fibrosis.....</i>	<i>30</i>
2.4.4 <i>Cardiac rupture</i>	<i>31</i>
2.5 TARGETING TGF- β SIGNALLING	32
CHAPTER 2. PLATELETS BEYOND HEMOSTASIS AND THROMBOSIS: AT THE CORE OF CARDIAC INFLAMMATION AND FIBROSIS.....	35
1. PLATELETS	37

1.1	PLATELETS IN HEMOSTASIS AND THROMBOSIS.....	37
1.1.1	<i>Platelet adhesion to endothelium</i>	38
1.1.2	<i>Platelet activation</i>	38
1.1.3	<i>Platelet aggregation and thrombus formation</i>	41
1.2	PLATELETS BEYOND HEMOSTASIS AND THROMBOSIS: AT THE CORE OF CARDIAC INFLAMMATION AND FIBROSIS	41
1.2.1	<i>Platelets as a source of TGF-β1</i>	43
1.2.2	<i>Platelets in cardiac fibrosis</i>	44
1.2.3	<i>Platelets in cardiac inflammation</i>	46
1.3	PLATELETS AS A NEW POTENTIAL THERAPEUTIC TOOL FOR CARDIAC INFLAMMATION AND FIBROSIS	48
	CHAPTER 3. THE GARP/ TGF-B1 PATHWAY	51
1.	THE GARP/TGF-B PATHWAY	53
1.1	GARP GENE AND GARP PROTEIN EXPRESSION	53
1.2	GARP BINDS AND ACTIVATES TGF-B1	53
1.2.1	<i>Role of integrins in GARP-mediated</i>	54
	<i>activation of TGF-β1</i>	54
1.3	GARP FUNCTION IN IMMUNE TOLERANCE AND CANCER	56
1.4	GARP IN PLATELETS.....	57
	AIMS OF THE WORK.....	59
	RESULTS	63
1.	ABSTRACT.....	67
2.	INTRODUCTION	68
3.	METHODS.....	71
3.1	CHEMICALS AND REAGENTS	71
3.2	EXPERIMENTAL ANIMALS.....	72
3.3	MYOCARDIAL INFARCTION PROCEDURE	73

3.4	ECHOCARDIOGRAPHIC ANALYSIS	73
3.5	ISOLATION OF PLATELETS AND PREPARATION OF PLATELET RELEASATES	74
3.6	FLOW CYTOMETRY ANALYSIS	74
3.7	MEASUREMENTS OF TGF- β 1 AND TROPONIN I	76
3.8	WESTERN BLOT ANALYSIS	76
3.9	HISTOLOGICAL ANALYSIS	77
3.10	ASSESSMENT OF CROSS-SECTIONAL CARDIOMYOCYTES AREA WITH WGA STAINING.....	78
3.11	ASSESSMENT OF CARDIAC FIBROSIS WITH PICROSIRIUS RED STAINING	78
3.12	ADULT HUMAN CARDIAC FIBROBLASTS IN CULTURE	79
3.13	RNA EXTRACTION AND MRNA EXPRESSION	79
3.14	RNA SEQUENCING.....	79
3.15	STATISTICAL ANALYSIS.....	80
4.	RESULTS	81
4.1	GENERATION AND CHARACTERIZATION OF PLATELET-SPECIFIC GARP KNOCKOUT MICE	81
4.2	PLATELET GARP DEFICIENCY DOES NOT AFFECT PLATELET REACTIVITY BUT IMPAIRS PLATELET- DERIVED TGF- β 1 ACTIVATION	83
4.3	ABSENCE OF PLATELET GARP DOES NOT ALTER INFARCT SIZE AND PLATELET INFILTRATION AFTER MI	85
4.4	PLATELET DELETION OF GARP INCREASES EARLY LV RUPTURE AND ADVERSE REMODELLING .	87
4.5	PLATELET GARP DEFICIENCY INCREASES CARDIAC INFLAMMATORY RESPONSE AFTER MI	89
4.6	IMPAIRED INFLAMMATORY RESOLUTION AND HEALING RESPONSE IN pKO MICE AFTER MI....	92
5.	SUPPLEMENTAL FIGURES AND MATERIALS	96
	DISCUSSION AND PERSPECTIVES.....	101
1.	GPIBA-CRE OR PF4-CRE MODEL?	104
2.	ANIMAL MODELS OF PERMANENT CORONARY ARTERY OCCLUSION OR ISCHEMIA- REPERFUSION INJURY?	104
3.	PLATELETS IN ACUTE MYOCARDIAL INFARCTION: THE GOOD OR THE BAD?	105

4.	SIGNIFICANCE OF PLATELET GARP IN MYOCARDIAL INFARCTION INJURY	106
5.	CLINICAL IMPLICATIONS OF THIS STUDY	109
6.	THE FUTURE.....	110
	BIBLIOGRAPHY	113
	ANNEXES	139

Figures and Tables listing

Introduction and discussion

Figure 1: Post-infarction LV remodelling.....	7
Figure 2: Timeline and cellular dynamics of the inflammatory response after myocardial injury.....	15
Figure 3: Synthesis of TGF- β 1.....	22
Figure 4: The role of TGF- β 1 in post-MI cellular response.....	24
Figure 5: Cascade of cellular and molecular events of the inflammatory phase post-MI.....	25
Figure 6: Interstitial fibrosis development post-MI.....	31
Figure 7: Granule content of platelet	40
Figure 8: Contribution of platelet-derived mediators to myocardial inflammation and/or fibrosis.....	43
Figure 9: Platelet interactions and secretory functions in post-MI inflammation.....	47
Figure 10: Integrin-mediated activation of TGF- β	56
Figure 11: Resume of the objectives of the thesis.....	62
Figure 12: Cardiac response post-MI in WT mice versus platelet GARP KO mice.....	109
Table 1: Classification and roles of MMPs in cardiac remodelling.....	19
Table 2. Preclinical <i>in vivo</i> studies using antiplatelet drugs and their effects on cardiac fibrosis.....	50
Table 3: RNA-sequencing of WT vs. pKO, 1 day post-MI.....	111

Results

Figure 1: Generation and characterization of platelet-specific GARP knockout mice.....	82
Figure 2: Platelet GARP deficiency does not affect platelet reactivity but impairs platelet-derived TGF- β 1 activation.....	84
Figure 3: Absence of platelet GARP does not alter infarct size and platelet infiltration after MI.....	86
Figure 4: Platelet deletion of GARP increases early LV rupture and adverse remodelling.....	88
Figure 5: Platelet GARP deficiency increases cardiac inflammatory response after MI.....	91
Figure 6: Impaired inflammation resolution and healing response in pKO mice after MI.....	94
Table 1: Echocardiographic measurements of WT and pKO mice at 14 days.....	89

Supplemental Figures and Tables

Figure S1: Immune cell content at 1 day post-MI in WT and pKO mice using the seq-ImmuCC webtool.....	99
Figure S2: Neutrophil and monocyte blood count in WT and pKO mice at 1 and 3 days after MI.....	100
Table S1: List of primer sequences.....	96
Table S2: Hematological parameters of WT and pKO mice at baseline.....	97
Table S3: Echocardiographic measurements of WT and pKO mice at baseline	98

Abbreviations

α SMA	alpha-smooth muscle actin
aa	amino acids
BSA	Bovine serum albumin
Ca ²⁺	Calcium
CF	Cardiac fibroblast
CRP	Collagen related peptide
DAB	Diaminobenzidine
DAMP	Damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
EC	Endothelial cell
ECM	Extracellular matrix
EF	Ejection fraction
FACS	Fluorescence activating cell sorting
FBS	Fetal bovine serum
FS	Fractional shortening
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GARP	Glycoprotein A repetition predominant
GP	Glycoprotein
GSEA	Gene set enrichment analysis
HCF	Human cardiac fibroblast
HRP	Horseradish peroxidase
I/R	Ischemia/reperfusion
ICAM	Intercellular adhesion molecule
IL1R	Interleukin-1 receptor

IL	Interleukin
KEGG	Kyoto Encyclopedia of Gene and Genomes
KO	Knockout
LAD	Left anterior descending
LAP	Latency associated peptide
LOX	Lysyl oxidase
LRR	Leucin-rich repeats
LRRC32	Leucine Rich Repeat Containing 32
LTBP	Latent TGF- β -binding protein
LV	Left ventricular
LVID	Left ventricular internal diameter
LVEDV	Left ventricle end-diastolic volume
LVESV	Left ventricle end-systolic volume
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
MI	Myocardial infarction
miRNA	MicroRNA
MK	Megakaryocyte
MMP	Matrix metalloproteinase
MPV	Mean platelet volume
MT	Metallothionein
NF-kB	Nuclear factor-kappa B
NSAIDs	Non-steroidal anti-inflammatory drugs
PAMP	Pathogen associated molecular pattern
Par4	Platelet protease-activated receptor 4
PBS	Phosphate-buffered saline solution

PCNA	Proliferating cell nuclear antigen
PD-1	Programmed cell death 1
PDGF	Platelet-Derived growth factor
PF4	Platelet factor 4
PRP	Platelet rich plasma
PRR	Pattern recognition receptor
RBC	Red blood cells
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative PCR
SDF-1	Stromal cell–derived factor-1
SEM	Standard error of the mean
Smad	Sma and Drosophila Mad proteins
STEMI	ST-segment elevation MI
TβRI/II	TGF-β receptor I/II
TBS	Tris-buffered saline
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T lymphocyte
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor
WGA	Wheat Germ Agglutinin
WHO	World Health Organization

WT

Wild-type

xGvHD

Xenogeneic Graft-Versus-Host Disease

INTRODUCTION

CHAPTER 1. HEALING PROCESS AFTER MYOCARDIAL INFARCTION

Several sections (*see 2.2.2; 2.3.1.1; and 2.3.2*) have been adapted from the review entitled “**Fibroblasts and platelets, a face-to-face dialogue at the heart of cardiac fibrosis**”, published in 2024 in the American Journal of Physiology-Heart and Circulatory Physiology, by Dufeys Cécile, Bodart Julie, Bertrand Luc, Beauloye Christophe and Horman Sandrine (*see annex 2*). Several parts have been updated, modified, extended, or removed to align with the format of this thesis.

1. PATHOPHYSIOLOGICAL CONTEXT

1.1 Epidemiology

Cardiovascular diseases refer to a group of conditions that affect the heart and blood vessels. Myocardial infarction (MI) remains a leading cause of mortality and morbidity within cardiovascular diseases. According to the World Health Organization (WHO), cardiovascular diseases caused approximately 17,9 million deaths worldwide in 2019. The WHO estimates that MI is responsible for around 9.6 million deaths worldwide each year. In Belgium, the annual incidence of MI is around 10 000 cases. The prevalence of MI varies depending on various factors such as age, sex, and lifestyle habits. In Europe, the age-standardized death rate due to MI is slightly declining, reflecting improved management of cardiovascular diseases in developed countries. Nevertheless, epidemiological data show an increasing prevalence of MI in some countries, partly due to population aging and the rising prevalence of cardiovascular risk factors (such as obesity, hypertension, and high cholesterol). However, advances in cardiovascular care, including improvements in medical treatments and surgical interventions, have contributed to a reduction in the mortality following MI. Despite the decrease in mortality, elevated patient survival increased the risk of long-term complications such as heart failure [1].

1.2 Pathophysiology

The heart pumps blood throughout the body, delivering oxygen and nutrients to organs. To sustain the contractions required for proper cardiac function, the myocardium needs a continuous supply of oxygenated blood. This supply of oxygen-rich blood is provided by coronary arteries. MI occurs when there is an obstruction in one of the coronary arteries, often due to several factors such as the

rupture of an atherosclerotic plaque or the formation of a blood clot (thrombus). Rare conditions like coronary embolism (associated with endocarditis), coronary artery dissection and arteritis may also cause occlusion of coronary arteries. Consequently, the myocardium undergoes ischemia, deprived of the vital oxygen and nutrients, necessary for its function. Prolonged ischemia initiates cellular damage, culminating in cardiomyocytes necrosis. Given the heart's limited regenerative capacity, MI triggers a repair process characterized by an initial inflammatory response, followed by the formation of scar tissue. These mechanisms contribute to structural and functional alterations in the left ventricle, collectively termed as ventricular remodelling. This remodelling involves alterations in both the size and shape of the ventricular chambers. While initially adaptative, excessive remodelling can precipitate cardiac functional decline, which can progress to heart failure. A better understanding of the cellular and molecular mechanisms underlying ventricular remodelling is imperative for the development of novel therapeutic interventions aimed at improving patient outcomes.

2. POST-MYOCARDIAL INFARCTION RESPONSE

As mentioned above, the regeneration capacity of the heart is particularly limited. Consequently, the reparative response to MI involves a complex sequence of events, starting with an inflammatory response and culminating in the formation of a scar [1-3]. The initial inflammatory phase is crucial, involving the activation of immune cells and inflammatory mediators to eliminate necrotic cells and regulate the immune response. This phase is followed by the reparative phase, marked by collagen deposition, which is essential for maintaining cardiac structural integrity, and preventing cardiac rupture. While these adaptive changes are initially compensatory and vital for cardiac function, they are concomitant with the

development of left ventricular (LV) dilatation, eccentric hypertrophy, and the development of interstitial fibrosis. These maladaptive processes are encompassed by the term “adverse LV remodelling”, strongly correlated with impaired cardiac function, and increased mortality post-MI [2, 4] (Fig. 1).

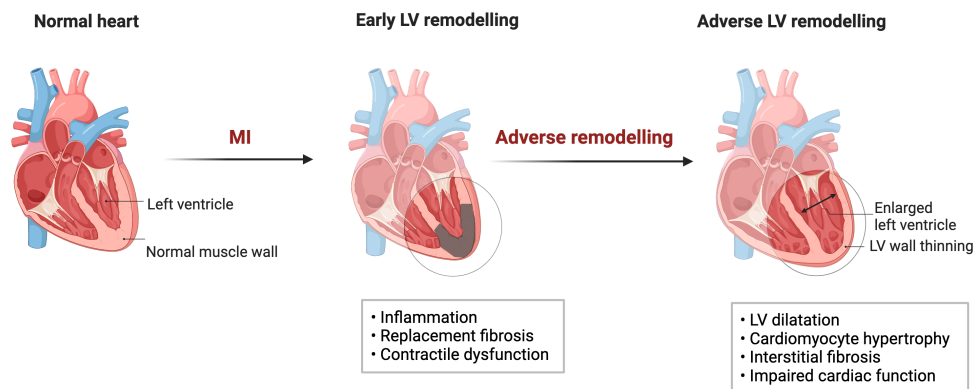


Figure 1. Post-infarction LV remodelling. Stages of post-MI tissue response. This figure illustrates the progressive changes in cardiac structure and function from a normal heart to adverse LV remodelling following MI. Early response post-MI is characterized by inflammation, replacement fibrosis, and contractile dysfunction. Over time, chronic stress leads to adverse LV remodelling, characterized by LV dilatation, cardiomyocyte hypertrophy, and thinning of the ventricular wall, leading to impaired cardiac function. More information concerning the abbreviations can be found in the text.

2.1 Necrosis of cardiomyocytes

The death of cardiomyocytes induced by ischemia is a critical event that triggers the onset of inflammation. In the acute phase following infarction, cardiomyocyte necrosis causes the release of Damage-Associated Molecular Patterns (DAMPs) including high-mobility group box 1, ATP, and heat shock proteins, into the extracellular space. These endogenous molecules act as danger signals, activating the innate immune system [5]. DAMPs interact with specific pattern recognition receptors (PRRs), notably toll-like receptors (TLRs), such as TLR2 and TLR4, expressed on both immune cells and non-immune cells. This interaction leads to

the activation of downstream signalling involving nuclear factor-kappa B (NF- κ B), and mitogen-activated protein kinases (MAPKs) [6]. Activation of these pathways induces production of pro-inflammatory cytokines and chemokines, facilitates the recruitment of immune cells, and amplifies the inflammatory response [7].

2.2 Acute inflammatory response

The post-MI inflammatory phase is characterized by a dynamic interplay among diverse immune cells, cytokines, and molecular signalling pathways. Within this phase, pro-inflammatory cytokines are secreted, orchestrating the inflammatory response, and promoting immune cell recruitment. In parallel, circulating immune cells are mobilized to the site of injury to clear cellular debris and initiate tissue repair mechanisms [8]. While this inflammatory cascade is fundamental to the healing process, its dysregulation or persistence can exacerbate myocardial injury [9-15].

2.2.1 Cytokines involved in the inflammatory process

Pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) are rapidly released following MI by various cell types such as cardiomyocytes, fibroblasts, endothelial cells, and immune cells [16]. Maintaining a delicate balance in cytokine regulation is essential to prevent adverse outcomes such as excessive inflammation or inappropriate fibrosis. This chapter will outline the role of these pro-inflammatory cytokines in the progression of post-infarction inflammation, highlighting their dual nature as crucial mediators of healing and potential contributors to pathological remodelling.

IL-1 β

IL-1 β is markedly upregulated in the infarcted heart, particularly within pro-inflammatory macrophages [17]. This cytokine acts as a pro-inflammatory mediator, facilitating the recruitment of immune cells like neutrophils and macrophages to the infarcted area [18, 19]. Furthermore, IL-1 β also exerts direct effects on cardiomyocytes, contributing to their apoptosis and impairing cardiac contractile function [20, 21]. Apart from its pro-inflammatory role, IL-1 β induces the expression of matrix metalloproteinases (MMPs) in cardiac fibroblasts (CFs) [19]. Numerous *in vivo* studies have underlined the important role of IL-1 β signalling in the pathogenesis of cardiac dysfunction and adverse cardiac remodelling. Firstly, the invalidation of IL-1 signalling receptor (IL1R1) in mice has been shown to suppress the inflammatory response and reduce the adverse remodelling post-MI [22]. Additionally, pharmacological inhibition of IL-1 pathways has demonstrated protective effects against adverse remodelling in rodent models of post-infarction heart failure, by reducing cardiomyocytes apoptosis [23]. Finally, clinical studies have revealed that elevated levels of IL-1 β are associated with a larger infarct size and an increased risk of post-MI complications [24, 25]. Recently, preliminary findings have suggested that IL-1 β blockade effectively attenuates systemic inflammatory responses in patients with ST-segment-elevation MI (STEMI). However, further research is necessary to validate these results, given the initial small size of the patient cohort [26, 27].

IL-6

IL-6 is highly expressed in various cell types including cardiomyocytes [28], mononuclear cells [28], mast cells [29], and fibroblasts [30]. This cytokine emerges as a pivotal mediator of post-MI inflammation, contributing significantly to the amplification of the immune response. Furthermore, in addition to its pro-

INTRODUCTION

inflammatory function, IL-6 has been implicated in the tissue repair phase following MI, by promoting collagen synthesis [31]. However, the role of IL-6 in regulating the post-MI inflammatory response have yielded conflicting findings. A study investigating the role of endogenous IL-6 in MI revealed that IL-6 knockout (KO) mice showed similar mortality rates, infarct sizes, and cardiac dysfunction compared to wild-type (WT) mice in non-reperfused infarction [32]. Conversely, elevated IL-6 levels have been correlated with infarct expansion and an increased risk of adverse cardiac remodelling in other studies [33]. Subsequently, a novel therapeutic approach involving pharmacological inhibition of IL-6 has demonstrated beneficial effects in attenuating post-MI inflammation and improving cardiac function in both human and murine models [34, 35].

TNF- α

TNF- α is a critical mediator of inflammation and exerts pleiotropic effects on various cell types within the infarcted myocardium [36]. Following MI, TNF- α is abundantly released by various cell types, including cardiomyocytes [37-39], macrophages [40], vascular cells [39], and mast cells [41]. It is well known that TNF- α influences numerous cellular functions. In cardiomyocytes, this inflammatory mediator has the potential to induce apoptosis by activating the cell death pathway, contributing to the loss of heart contractile function [42]. In macrophages, it stimulates the synthesis of pro-inflammatory cytokines, thereby amplifying the inflammatory response [43]. TNF- α also promotes the expression of adhesion molecules on endothelial cells, which facilitates the adhesion and transmigration of leukocytes into the myocardium [44]. Lastly, in fibroblasts, TNF- α stimulates the production of extracellular matrix (ECM) proteins, and MMPs, thereby contributing to fibrosis and structural remodelling of the cardiac ECM [45, 46]. Several lines of evidence support that TNF- α play a major role in post-MI

inflammation, and LV remodelling *in vivo*. Genetic invalidation of TNF- α in mice has demonstrated reduced infarct size, and diminished infiltration of neutrophils and macrophages following permanent ligation of the left anterior descending (LAD) coronary artery [47]. Moreover, deletion of TNF- α receptor-1 has been shown to improve survival and attenuate LV remodelling post-MI in mice [48]. Conversely, a prolonged or excessive TNF- α response after MI can be deleterious, leading to further tissue damage and contributing to the progression of cardiac remodelling [49-51].

2.2.2 Cellular effectors of the inflammatory response

Following MI, the inflammatory cascade mobilizes a multitude of cell types including resident cardiomyocytes, endothelial cells, and immune effectors. This process entails the release of cytokines and chemotactic factors, alongside the upregulation of endothelial adhesion molecules, which collectively promote the recruitment of leukocytes. Furthermore, the compromised integrity of the vascular endothelium post-MI serves as a pathway for the infiltration of leukocytes, which are crucial for the clearance of necrotic debris and the subsequent remodelling of the cardiac tissue [52]. This chapter aims to understand the relative contributions of each cell type to post-MI inflammation.

ENDOTHELIAL CELLS

Endothelial cells are rapidly activated after cardiac injury by DAMPs released by necrotic cardiomyocytes [8]. This induces upregulation of adhesion molecules (i.e. E-selectin, P-selectin and vascular cell adhesion molecule-1 (VCAM-1) on their surface, promoting leukocytes and platelets recruitment to the site of injury [53, 54]. In addition, endothelial cells are an essential source of cytokines (IL-6, IL-1 β

and TNF- α) [55] and chemokines participating in the inflammatory response [56, 57]. However, the precise role of endothelial cells in the inflammatory response post-MI remains not fully understood.

NEUTROPHILS

Neutrophils are the first immune cells to invade the ischemic myocardium after MI. They are recruited from the circulating blood to the site of injury by DAMPs released from necrotic cardiomyocytes [8, 58, 59] and inflammatory mediators secreted by resident cardiac cells [54]. The recruitment of neutrophils is critically dependent on the activation of endothelial cells. This activation is characterized by the upregulation of selectins and adhesion molecules, which interact with integrins on the surface of neutrophils. This interaction causes the neutrophils to adhere to the endothelial wall and migrate from the bloodstream into the myocardial infarcted tissue. The transmigration process is further facilitated by chemotactic factors, creating a gradient that guides neutrophils to the site of injury [60]. Once within the myocardium, neutrophils secrete large amounts of reactive oxygen species (ROS), myeloperoxidase, MMPs (MMP-8 and MMP-9), cytokines (IL-1 β , TNF- α and IL-8), and chemokines that contribute to ECM degradation and monocyte recruitment to propagate the inflammatory response [8, 29, 58, 61]. However, prolonged neutrophil activation can exacerbate inflammation [15, 62-65], impairing cardiac repair through excessive tissue degradation. This is further demonstrated by the administration of S100A8/A9, a cytosolic protein released by neutrophils, which exacerbates infarct wall thinning [66]. Furthermore, clinical studies have shown that elevated leukocyte and neutrophil counts correlate with larger infarct size and lower LV ejection fraction [62, 67]. On the other hand, neutrophils also play a critical role in the polarization of macrophages towards an anti-inflammatory and reparative phenotype, thereby facilitating the resolution of

inflammation. [68]. Neutrophil depletion has been shown to impair cardiac repair post-MI by disrupting macrophage activity, leading to reduced phagocytic capacity and impaired transition to the reparative phase [14]. Neutrophils therefore play a complex role in MI, both detrimental due to their ability to propagate inflammation and degrade tissue, but also essential in resolving inflammation by facilitating macrophage polarization.

MONOCYTES AND MACROPHAGES

Monocytes and macrophages play pivotal roles in the post-MI response, orchestrating both the inflammatory and reparative phases. Monocytes are recruited from the bloodstream to the infarcted area, where they differentiate into macrophages. This infiltration is driven by chemotactic signals, including monocyte chemoattractant protein-1 (MCP-1), released in response to tissue damage and hypoxia [69]. Different monocyte subsets are successively recruited to the site of infarction. In the first hours after MI, Ly6C^{high} classical monocytes are recruited, contributing to the inflammatory response by phagocytosing debris. Later, during the reparative phase, Ly6C^{low} non-classical monocytes predominate, supporting tissue healing [69]. In response to cytokines and growth factors, including macrophage colony-stimulating factor (M-CSF), monocytes mature and differentiate into macrophages.

Macrophages are the predominant immune cell type in the infarcted heart [57-59]. They are derived from circulating monocytes [70] and resident macrophages [8, 59]. During the early inflammatory phase, macrophages exhibit pro-inflammatory characteristics, with an increased expression of pro-inflammatory cytokines, chemokines, and proteases. Pro-inflammatory macrophages participate in inflammation by phagocytosing dead cells and ECM debris [71]. Notably, early

INTRODUCTION

macrophage depletion has demonstrated adverse effects on infarct size, LV remodelling, and cardiac function [72]. Furthermore, inhibition of macrophage infiltration within the injured myocardium has been linked to reduced LV remodelling [73]. Transitioning into the reparative phase, a crucial phenotypic switch occurs in macrophages from a pro-inflammatory to an anti-inflammatory profile. This shift is characterized by an enhanced expression of anti-inflammatory cytokines, pro-fibrotic and angiogenic factors [74]. Consequently, the release of these factors by macrophages stimulates ECM production by fibroblasts [75-78]. The dynamic modulation of macrophage phenotype constitutes a key step in mediating the progression from an inflammatory state towards tissue fibrosis and repair.

LYMPHOCYTES

Strong evidence suggests that subpopulations of lymphocytes T and B infiltrate the heart after MI and may play a critical role in regulating the inflammatory and fibrotic responses [57, 59, 79, 80]. It has been reported that distinct lymphocyte subpopulations contribute differently to the fibrotic response, both through the secretion of various inflammatory mediators and through direct adhesion and actions on CFs [57, 59, 61, 79-81]. Moreover, a growing body of evidence implicates regulatory T lymphocytes (Tregs) in modulating the immune response post-MI by exerting immunosuppressive effects [82]. *In vivo* study has demonstrated that CD4+FOXP3+ (Tregs) are essential for fostering favorable wound healing, scar formation and inflammation resolution, by modulating macrophage differentiation towards an anti-inflammatory phenotype [82, 83].

MAST CELLS

Finally, the heart contains a few resident mast cells located close to the vessels and in the epicardium. These cells store a wide range of molecules, such as TNF- α , IL-6, histamine, and proteases, which are rapidly secreted upon degranulation and function as paracrine factors [8, 29, 41, 59, 84]. Moreover, studies have noted an augmentation in the number of mast cell population within the heart following MI [41, 85]. Interestingly, resident cardiac mast cells have been identified as significant initiators of the post-infarct inflammatory response [41].

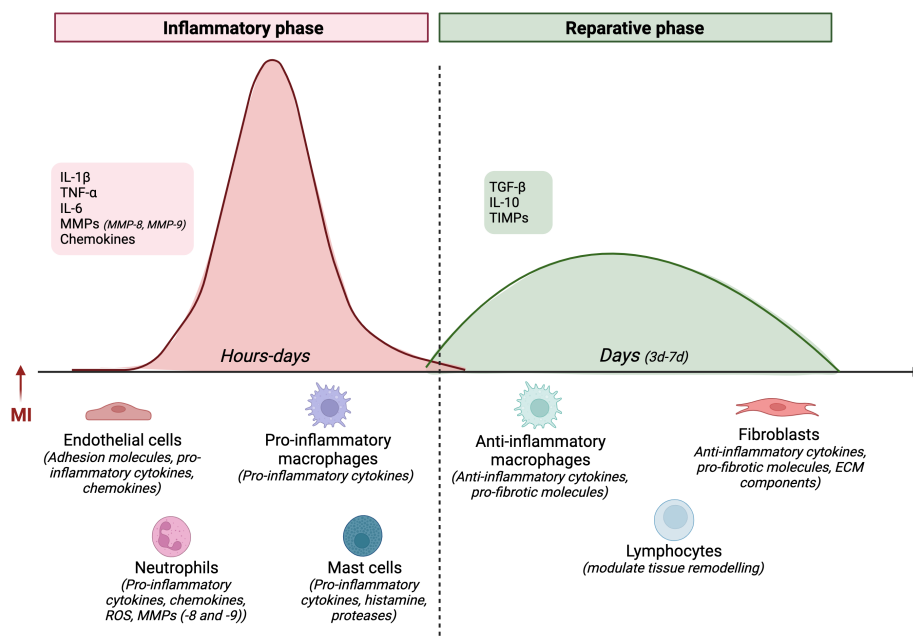


Figure 2. Timeline and cellular dynamics of the inflammatory response after myocardial injury.

The repair response after cardiac injury encompasses the inflammatory, and the reparative phase. The initial inflammatory phase is characterized by the activation of endothelial cells, which then secrete pro-inflammatory cytokines and chemokines, and upregulate adhesion molecules, all of which are essential for recruiting immune cells to the site of injury. The infiltration of neutrophils contributes to the inflammatory environment through the release of ROS, MMPs, and pro-inflammatory cytokines. This leads to the recruitment of pro-inflammatory macrophages which release pro-inflammatory mediators. During the reparative phase, there is a shift towards an anti-inflammatory environment. Anti-inflammatory macrophages take

control, releasing cytokines and fibrotic molecules that facilitate tissue repair. Concurrently, lymphocytes, including T and B cells, begin to modulate tissue remodelling. Figure adapted from [86]. More information concerning the abbreviations can be found in the text.

2.2.3 Targeting the inflammation

The inflammatory response plays a crucial role in clearing necrotic tissue and initiating repair processes following MI. However, excessive or prolonged inflammation can exacerbate tissue damage and contribute to adverse cardiac remodelling. Consequently, targeting post-MI inflammation has become a central focus of therapeutic strategies aimed at improving outcomes after MI. One of the primary strategies involves the use of anti-inflammatory drugs. Initially, non-steroidal anti-inflammatory drugs (NSAIDs) were considered due to their potent anti-inflammatory effects. Early studies in animal models suggested that NSAIDs may have protective effects following MI, by mitigating adverse remodelling, and reducing cardiomyocyte apoptosis [87, 88]. Conversely, some experimental research demonstrated that NSAIDs could negatively impact cardiac healing, leading to scar thinning and worsened systolic function [89, 90]. Furthermore, clinical studies revealed that NSAIDs increase the risk of adverse cardiovascular events, including heart failure in post-MI patients [91]. Additionally, cytokine inhibitors have emerged as a promising therapeutic approach. Firstly, the inhibition of IL-1 significantly reduced the incidence of recurrent cardiovascular events in MI patients with high levels of C-reactive protein [26, 27]. Similarly, targeting IL-6 with monoclonal antibodies has shown encouraging results in pre-clinical studies by reducing systemic inflammation [35, 92]. Despite these results, several challenges remain in targeting post-MI inflammation. A major challenge is achieving a balance between reducing harmful inflammation and preserving the beneficial aspects of the inflammatory response. Excessive suppression of inflammation can hinder

wound healing and increase the risk of infection [93]. Furthermore, the inflammatory response can vary widely among individuals, adding another layer of complexity to therapeutic interventions [93].

2.2.4 The extracellular matrix during the inflammatory phase

The cardiac ECM is a complex network of fibrillar and non-fibrillar components that provides structural support and regulates cellular activity within cardiac tissue [94]. It is predominantly composed of fibrillar proteins such as collagen which confer tensile strength and elasticity to the heart [95, 96]. In addition to collagens, the cardiac ECM also comprises fibronectin, proteoglycans and glycosaminoglycans, among other constituents. Beyond its structural function, the ECM serves as a reservoir for growth factors, and cytokines, modulating the cellular responses to environmental changes [95, 97-99].

During the post-infarction inflammatory phase, the ECM undergoes remodelling mediated by MMPs, a group of proteolytic enzymes that degrade ECM components. The enzymatic breakdown of the ECM by MMPs generates bioactive fragments known as matrikines, crucial for regulating inflammatory, fibrogenic and angiogenic processes in injured hearts [100, 101]. Moreover, ECM fragments influence the migration and differentiation of immune cells, vascular cells, and fibroblasts, contributing to cardiac fibrosis formation [100, 102, 103]. Additionally, the ECM experiences dynamic changes, leading to the formation of a provisional matrix that acts as a scaffold for tissue repair. Indeed, increased vascular permeability facilitates the extravasation of plasma proteins such as fibrin, fibrinogen [104] and fibronectin into the infarcted area. These proteins, integrated

into a fibrin-based provisional matrix, form a dynamic network that facilitates the migration of immune cells and regulates inflammatory cascades [95].

2.2.4.1 The matrix metalloproteinases

At the core of ECM regulation lie the MMPs, zinc-dependent endopeptidases, classified into five main groups: collagenases (i.e., MMP-8 and MMP-13), gelatinases (i.e., MMP-2 and MMP-9), stromelysins (i.e., MMP-3), matrylisins, and membrane type MMPs (i.e., MMP-14). These zinc-dependent enzymes exert a significant influence on the ECM, shaping to the dynamics of the inflammatory and healing phases. The initial inflammatory response triggers the expression and activation of MMPs, pivotal in modulating ECM composition. MMPs are produced by multiple cell types, including cardiomyocytes, fibroblasts, endothelial cells, and leukocytes [105, 106]. Among the MMPs involved in the pathophysiology of MI, MMP-2 and MMP-9 stand out for their importance [107-109]. Extensive research has linked elevated levels of these gelatinases to increased risk of LV remodelling and compromised cardiac function in humans [110, 111]. Moreover, murine models have revealed that the absence of MMP-2 and MMP-9 can prevent cardiac rupture and is associated with reduced LV dilatation [112-115]. Additionally, MMP-8, primarily produced by neutrophils, plays a pivotal role in neutrophils migration [116], with elevated levels serving as a significant predictor of LV remodelling and cardiac rupture [117, 118]. Alongside, MMP-1 and MMP-3 levels correlate with LV dysfunction and dilatation in MI patients [119, 120]. While the impact of MMP-7 on LV dilatation appears modest, it may influence cardiac conduction through interaction with connexin-43, affecting post-MI survival [121]. Lastly, MMP-14 is noted for its substantial role in inducing fibrosis and altering LV function [122]. However, contradictory results in mice and humans underscore the complex interactions between MMPs and post-MI response [109, 123] (Table 1). In addition,

INTRODUCTION

it's important to note that there are 25 known MMPs, and this chapter covers only a subset of them. Overall, MMPs play a critical role not only degrade the cardiac ECM, facilitating leukocyte recruitment to the injured area, but also actively participate in the immune response.

Finally, MMPs activity is tightly regulated by the endogenous tissue inhibitors of metalloproteinases (TIMPs), including TIMP-1,-2,-3 and -4, which counteract matrix degradation. Studies indicate that mice lacking TIMP-1 display an exaggerated healing response which is marked by increased end-diastolic pressure and LV volume 14 days post-MI [124]. Similarly, the loss of TIMP-3 in mice results in spontaneous LV dilatation, cardiomyocyte hypertrophy, and impaired contractile function post-MI [125]. Thus, imbalance between MMP and TIMP levels contributes to adverse ECM remodelling [126].

Class	Number	Cellular sources	Cardiac role	References
Collagenases				
Interstitial collagenase	MMP-1	Fibroblasts, leukocytes and EC	Associated with LV dysfunction and dilatation in MI patients	[120]
Collagenase 2	MMP-8	Neutrophils	Predictor of LV remodelling, cardiac rupture and heart failure. ↑ neutrophils infiltration	[116-118]
Collagenase 3	MMP-13	Fibroblasts and macrophages	Activates MMP-9	[127]
Gelatinases				
Gelatinase 1	MMP-2	Macrophages, fibroblasts, EC, and cardiomyocytes	↑ macrophages infiltration, LV rupture. ↓ survival	[107-109], [114-115]
Gelatinase 2	MMP-9	Neutrophils, macrophages, cardiomyocytes and EC	Activation of TGF-β1. Effects are dependent of cell sources and timing of expression	[107-110], [112-113]
Stromelysins				
Stromelysin-1	MMP-3	Cardiomyocytes, fibroblasts, and macrophages	Associated with LV dysfunction and adverse LV remodelling in patients	[119]
Matrylisin	MMP-7	Cardiomyocytes and macrophages	↓ survival	[121]
Membrane-type MMP				
MT1-MMP	MMP-14	Fibroblasts, macrophages, VSMC, and cardiomyocytes	↑ fibrosis, ↓ survival and LV function	[122]

Table 1. Classification and roles of MMPs in cardiac remodelling. This table provides a comprehensive overview of various classes and subtypes of MMPs, detailing their cellular sources, and implications in the context of MI. Table adapted from [127]. MMP, metalloproteinase; EC, endothelial cells; VSMC, vascular smooth muscle cells.

2.3 Resolution of inflammation and reparative phase

The transition from the inflammatory phase to the reparative phase following MI signifies a pivotal stage in cardiac healing. After the inflammatory phase, the reparative phase assumes dominance, marking a complex and tightly regulated process encompassing the resolution of inflammation, activation of anti-inflammatory pathways, and initiation of tissue repair. The following chapters will define the cellular and molecular mechanisms underlying effective cardiac repair.

2.3.1 Resolution of post-infarction inflammation

To ensure tissue repair after myocardial injury, it is imperative to activate endogenous signals that not only limit the inflammatory cascade but also promote repair [13]. These signals play a fundamental role in the context of cardiac injury, as an uncontrolled inflammatory response can lead to detrimental outcomes, including extensive matrix degradation, maladaptive ventricular remodelling, and progressive development of heart failure [1, 12]. The resolution of inflammation heavily relies on the removal of dead cells and matrix debris from the infarcted myocardium, thereby eliminating the primary stimulus for inflammatory activation [1]. Neutrophils also play a crucial role in resolution as they are programmed to undergo apoptosis, and their clearance by phagocytes via efferocytosis actively contributes to limit inflammation. [128]. Indeed, the ingestion of apoptotic neutrophils by macrophages triggers an anti-inflammatory response, inducing the synthesis and release of transforming growth factor- β 1 (TGF- β 1), IL-10, as well as pro-resolving lipid mediators [129-131]. Notably, disruption of efferocytosis of apoptotic cells has been associated with a prolongation of the pro-inflammatory phase, underlining its importance. Moreover, mediators such as annexin A1 and lactoferrin are released by apoptotic neutrophils further inhibiting the recruitment

of additional neutrophils [132, 133]. Overall, the resolution of inflammation fosters an environment favorable to transitioning to the fibrotic phase, characterized by the controlled synthesis of collagen and other ECM components.

2.3.1.1 Cytokines involved in the resolution of inflammation

TGF- β 1

TGF- β expression, activation and signalling

There are three TGF- β isoforms, including TGF- β 1, - β 2, and - β 3, which are encoded by distinct genes. Although all three isoforms are expressed in the mammalian heart, their specific actions have not yet been studied *in vivo*, with most studies focusing on the role of TGF- β 1. While CFs, cardiomyocytes, leukocytes, and platelets actively produce TGF- β *de novo*, the relative contribution of each cardiac cell type to TGF- β secretion in the pathological heart remains unclear and warrants further investigation. TGF- β is initially secreted as a dimer, forming a complex with the latency associated peptide (LAP), which maintains TGF- β molecules into a latent non-active form. These TGF- β /LAP complexes also bind to latent TGF- β binding protein (LTBP), retaining the secreted TGF- β within the ECM [134]. Consequently, the cardiac ECM contains a significant amount of latent TGF- β /LAP/LTBP complexes that can be rapidly activated following injury. Additionally, TGF- β can also bind to a transmembrane protein called Glycoprotein A Repetitions Predominant (GARP), forming latent complexes on the cell surface [135] (Fig. 3). The latent complexes can be activated by several factors including proteases (MMPs, cathepsins, serine proteases, and calpain), integrins, ROS, thrombospondin-1, and acidic environment [134, 136-138]. Once activated, TGF- β binds to its constitutively active type II receptor (T β RII) on the surface of various target cells, initiating the dimerization and phosphorylation of TGF- β type I receptor (T β RI), and activating downstream

intracellular signalling pathways. The canonical TGF- β -mediated pathway involves the transcriptional co-factors Smads. TGF- β can also activate non-canonical cascades, including the MAPK, NADPH oxidase, phosphatidylinositol 3-kinase, and small GTPases pathways.

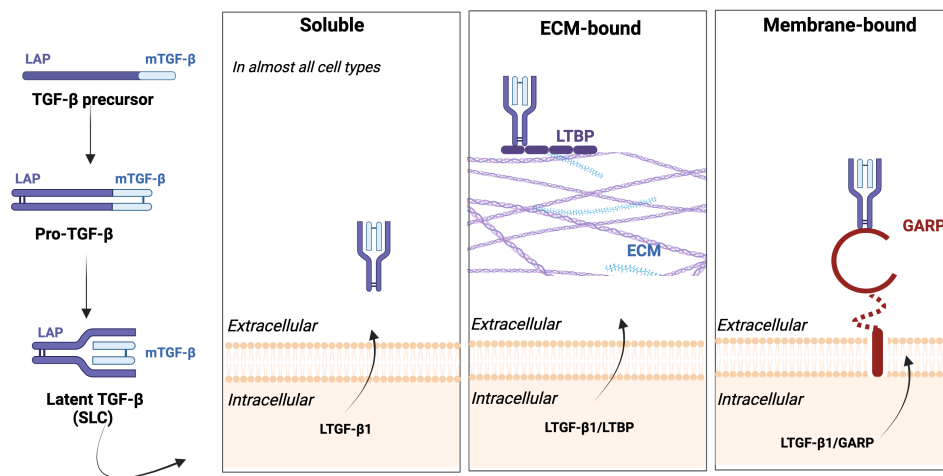


Figure 3. Synthesis of TGF- β 1. TGF- β is synthesized as a precursor molecule that is cleaved to form pro-TGF- β . Further cleavage releases mature TGF- β (mTGF- β) while remaining non-covalently associated with latency-associated peptide LAP, forming latent TGF- β (also known as the small latent complex, SLC). This latent complex can be anchored to the ECM via binding to LTBP, forming a pool of latent TGF- β in the ECM. Alternatively, the latent TGF- β complex can bind to membrane-bound GARP on the surface of certain cells like Tregs and platelets. LTGF, latent TGF- β .

TGF- β 1 in the infarcted heart

TGF- β 1 expression is significantly upregulated in animal model of MI or pressure overload, as well as in patients with heart failure [134]. TGF- β 1 exerts pleiotropic effects in the injured heart, influencing immune-cell response, angiogenesis, cardiomyocyte survival and CF activation [134, 139]. Regarding the immune response, TGF- β 1 may exert a pro- or anti-inflammatory role, depending on its concentration, the cellular environment, and the type of injury [134]. Several lines

INTRODUCTION

of evidence suggest an anti-inflammatory role of TGF- β after MI. For instance, TGF- β 1 has been demonstrated to reduce neutrophil infiltration into the heart submitted to ischemia-reperfusion, by decreasing endothelial cell-leukocyte interaction [140]. Moreover, early systemic inhibition of TGF- β 1, through overexpression of soluble T β RII, was shown to promote neutrophil recruitment and expression of pro-inflammatory cytokines, exacerbating ventricular dilatation in a mouse model of ischemia-reperfusion [141]. Finally, myeloid cell-specific invalidation of Smad3 results in a defective transition of macrophages towards an anti-inflammatory phenotype after LAD coronary artery permanent ligation [142]. In contrast, specific deletion of TGF- β receptors in cardiomyocytes was found to attenuate neutrophil infiltration and MMP-9 activation after LAD permanent ligation, leading to a reduced mortality due to wall rupture [143]. According to these studies, inhibition of TGF- β may have either protective or detrimental effects, depending on the timing of the inhibition. Furthermore, it is well established that TGF- β drives the transition of macrophages from the pro-inflammatory phenotype to the reparative phenotype, supporting inflammation resolution after MI [142, 144, 145]. TGF- β is also described to regulate T cells functions [146]. Although it has been shown to induce an anti-inflammatory environment in atherosclerotic plaques by modulating Tregs activation [147], the relative contribution of TGF- β in the regulation of these cells in the injured heart remains unknown. Lastly, *in vitro* evidence suggests that TGF- β reduces endothelial expression of pro-inflammatory chemokines and adhesion molecules, thereby inhibiting leukocyte recruitment [148]. However, TGF- β has been demonstrated to induce pro-inflammatory signalling in endothelial cells in a mouse model of atherosclerosis [149]. The extent to which TGF- β contributes to angiogenesis and neovascularization *in vivo*, after MI or pressure overload, remains unknown. Besides its role in inflammation, TGF- β is a key player in the modulation of post-infarction fibrosis. It induces the

differentiation of fibroblasts into myofibroblasts, promoting the synthesis of a collagen-rich ECM [134, 150, 151]. Clearly, activation of TGF- β signalling affects phenotype and function of all cells involved in cardiac repair, exerting both protective and detrimental effects (Fig. 4).

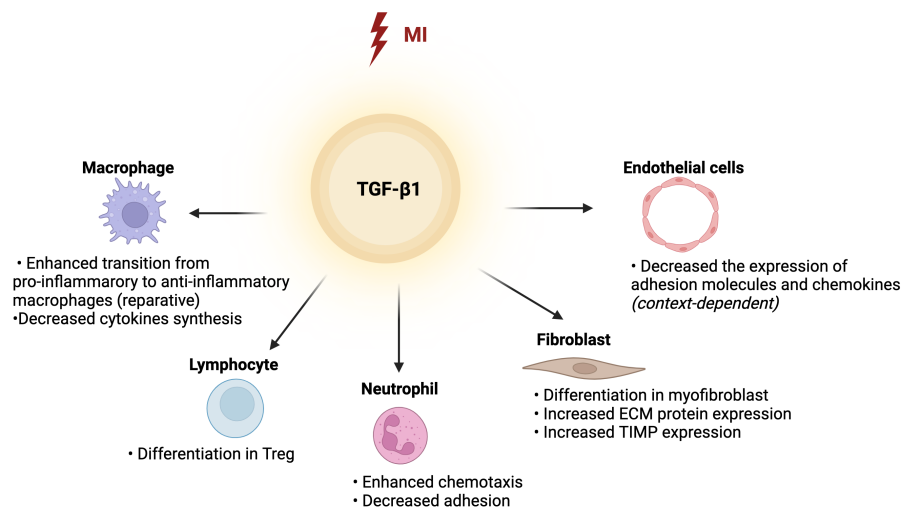


Figure 4. The role of TGF- β 1 in post-MI cellular response. Following MI, TGF- β 1 mediates the transition of macrophages from a pro-inflammatory phenotype to a reparative and anti-inflammatory phenotype, which is associated with reduced pro-inflammatory cytokine synthesis. It also promotes the differentiation of lymphocytes into Tregs, which are crucial for modulating the immune response. Neutrophils exhibit enhanced chemotaxis and reduced adhesion under the influence of TGF- β 1. In fibroblasts, TGF- β 1 triggers differentiation into myofibroblasts, increased ECM protein expression, and elevated TIMP expression. Endothelial cells respond to TGF- β 1 by reducing the expression of adhesion molecules and chemokines, although this effect is context-dependent. More information concerning the abbreviations can be found in the text. Figure adapted from [152].

IL-10

IL-10 also plays a pivotal role during the resolution of inflammation. Predominantly expressed by activated T cells and macrophages [153], IL-10 has potent anti-inflammatory actions. IL-10 exerts its influence by modulating various immune cells, particularly macrophages, favoring their polarization towards an anti-

inflammatory phenotype [154]. This action contributes to the elimination of cellular debris and the promotion of tissue repair. For instance, in a mouse model of permanent ligation of LAD, treatment with exogenous IL-10 was found to attenuate inflammation, improve LV function, and influence macrophage polarization [154, 155]. Furthermore, Yang *et al.* observed that the absence of IL-10 resulted in increased mortality, accentuated neutrophil recruitment and higher levels of pro-inflammatory cytokines in a mouse model of ischemia-reperfusion. This study demonstrated that endogenous IL-10 plays a crucial role in inhibiting the production of inflammatory mediators, such as TNF- α , and in suppressing neutrophil recruitment, thereby protecting the ischemic and reperfused myocardium [156].

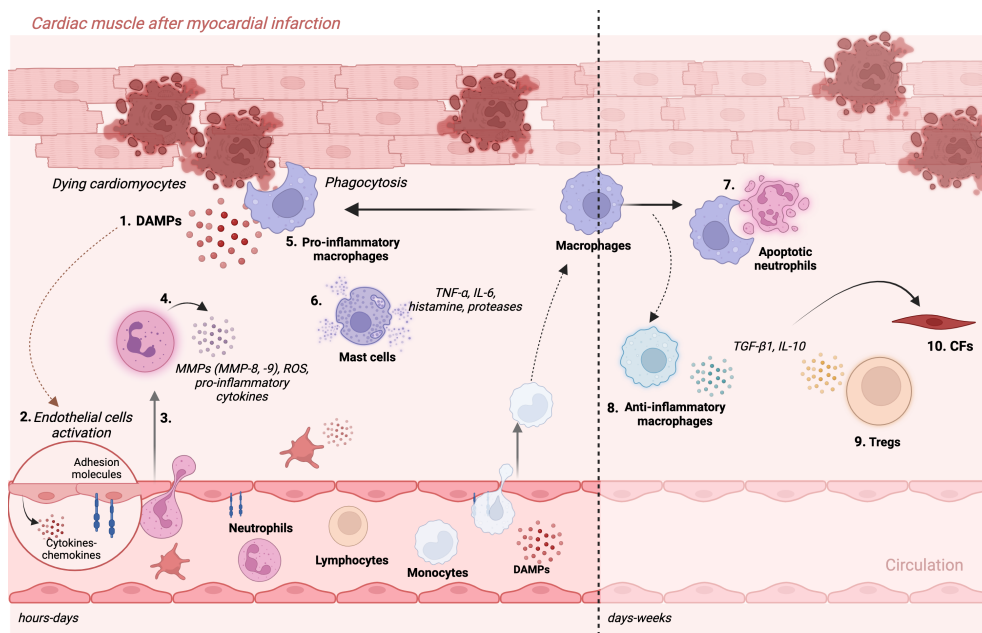


Figure 5. Cascade of cellular and molecular events of the inflammatory phase and its resolution post-MI. (1) Dying cardiomyocytes release DAMPs, initiating an immune response. (2) DAMPs activate endothelial cells, which in turn attract immune cells, such as neutrophils. (3) Infiltration of neutrophils into the injured heart is observed. (4) Neutrophils are the first cells to infiltrate the infarcted myocardium and produce a range of cytokines, chemokines, and other mediators that contribute to inflammation and monocytes recruitment. (5) Pro-inflammatory macrophages

participate in clearing cell debris through phagocytosis. (6) Mast cells also contribute to the inflammatory milieu by releasing histamine, pro-inflammatory cytokines, and proteases. (7) During the resolution of inflammation, macrophages phagocytose apoptotic neutrophils, which induces a phenotypic switch in macrophages from a pro-inflammatory to an anti-inflammatory phenotype. (8) Transition to a reparative phase is marked by the release of anti-inflammatory cytokines such as TGF- β , IL-10 by anti-inflammatory macrophages. (9) Tregs aid in resolving inflammation and promoting fibrosis. (10) TGF- β and IL-10 leads to the activation of CFs. More information concerning the abbreviations can be found in the text.

2.3.2 The cardiac fibroblast: The main effector in the reparative phase

Cardiac fibroblasts are described as the main players in the synthesis of cardiac ECM proteins and the development of fibrosis in response to heart injury. CFs are found within the cardiac tissue, surrounding cardiomyocytes, and represent one of the most predominant cell populations in the mammalian heart [157-159]. In a healthy heart, CFs remain quiescent, and maintain the cardiac ECM homeostasis [157, 160]. Through the secretion of structural ECM proteins such as collagen and elastin, CFs provide a scaffold for cardiomyocytes and other cardiac cells, but also a storage network for latent growth factors such as TGF- β [150]. In addition, CFs secrete enzymes involved in ECM turnover, such as MMPs and TIMPs, which regulate the balance between ECM synthesis and degradation [53]. In response to inflammatory and stress signals, CFs are activated and their proliferation and differentiation into myofibroblasts are promoted. Myofibroblasts have a cellular phenotype characterized by the expression of alpha-smooth muscle actin (α SMA) stress fibers [150, 157, 161, 162]. Recent studies have pointed toward a spectrum of CF phenotypes, which are probably more complex than a simple binary state between activated and quiescent fibroblasts [157]. Multiple cell types, such as endothelial cells [151], pericytes [163] and bone marrow-derived myeloid cells [164] have been suggested as potential sources of activated CFs. However, recent studies using

lineage tracing strategies have revealed that most activated CFs within the injured heart are derived from resident CFs [165, 166]. Activated CFs display enhanced synthetic properties, and are the main source of structural ECM proteins, matricellular proteins, and enzymes involved in the ECM processing. They therefore contribute primarily to the development of fibrosis [162, 167-169]. CF activation is triggered by a large variety of molecules, such as growth factors (TGF- β , platelet-derived growth factor, [PDGF]), cytokines (IL-6, IL-1 β , TNF- α), hormones (angiotensin II, aldosterone, adrenalin, noradrenalin), and neuropeptides (endothelin-1). These molecules are highly present in the pathological heart due to inflammation and adaptive responses [150, 161, 170, 171].

2.3.3 The extracellular matrix during cardiac repair

In the reparative phase, the ECM undergoes a transition from a provisional matrix, rich in fibrin and fibronectin, to a more stable structure composed predominantly of collagen types I and III. This process is driven by growth factors (TGF- β) secreted by mononuclear cells, macrophages, and platelets which activate myofibroblasts to deposit significant amounts of structural ECM proteins that maintain cardiac integrity [172]. Alongside these structural proteins, myofibroblasts also release fibronectin, and matricellular proteins including thrombospondin, tenascin C, osteopontin, and periostin. These matricellular proteins, unlike structural ECM components, primarily regulate cell activity, facilitate matrix formation, and protect the myocardium against detrimental remodelling [94, 95]. Finally, the transition from the reparative phase to the maturation phase involves a decrease in cellular content, as myofibroblasts undergo apoptosis [3, 173]. The maturation phase is further characterized by increased collagen synthesis, accompanied by an upregulation in lysyl oxidase (LOX) enzymes within the myocardium. LOX enzymes catalyze the cross-linking of collagen fibers, strengthening the scar [174]. Although

the development of fibrosis is indispensable to maintain the cardiac structure, it also leads to heart stiffening, electrical signalling impairment and subsequent heart failure [175, 176] (*see 2.4.3*).

2.4 Adverse left ventricular remodelling

Adverse LV remodelling typically evidences around one month after the ischemic event, and is characterized by eccentric hypertrophy of healthy cardiomyocytes, LV dilatation and development of interstitial fibrosis [2, 177]. These changes collectively contribute to the decline in cardiac function. The underlying pathophysiological mechanisms are complex, involving chronic neurohormonal activation, persistent inflammatory responses, fibrosis as well as cardiomyocyte apoptosis or necrosis [178]. It is associated with an elevated risk of heart failure and mortality, making it a critical focus for therapeutic intervention and patient prognosis [4].

2.4.1 Left ventricular chamber dilatation

Left ventricular dilatation following MI is a hallmark of adverse cardiac remodelling and is closely associated with poor clinical outcomes [179]. The loss of myocardial tissue due to MI leads to a compensatory response aimed at maintaining cardiac output, which often results in LV enlargement. This process involves complex molecular and cellular mechanisms, including neurohormonal activation, as well as inflammatory response. Initially, LV dilatation serves to preserve stroke volume. However, chronic dilatation can lead to geometric alterations in the LV chamber, compromising cardiac function. Mechanistically, LV dilatation is characterized by increased LV end-diastolic (LVEDV) and end-systolic volumes (LVESV), thinning of the LV wall, and alterations in LV geometry [4]. Moreover, infarct expansion,

occurring shortly after MI, significantly impacts LV dilatation [180, 181]. Infarct expansion is characterized by the thinning and lengthening of the infarcted segment. This process includes the rearrangement (*slippage*) and elongation of cardiomyocytes, as well as the breakdown of the cardiac ECM. These mechanisms collectively contribute to the thinning and stretching of the LV wall, potentially fostering LV dilatation [1]. In addition, the replacement of dead cardiomyocytes with a thin scar further contributes to an increase in LV volume. Finally, according to Laplace's law, LV dilatation augments wall stress, promoting cardiomyocyte hypertrophy and further LV dilatation [182-184]. Clinically, LV dilatation is a strong predictor of adverse cardiovascular events, including heart failure, arrhythmias, and sudden cardiac death [179, 185].

2.4.2 Eccentric hypertrophy of healthy cardiomyocytes

Hypertrophy of healthy cardiomyocytes is a crucial aspect of the complex cardiac remodelling process post-MI. One significant factor contributing to hypertrophy is the need for the remaining healthy myocardium to compensate for the loss of contractile tissue. This process involves an increase in the size of healthy cardiomyocytes. In the context of MI, hypertrophy refers to the lengthening of healthy cardiomyocytes, caused by the addition of sarcomeres in series. This type of hypertrophy, termed eccentric hypertrophy, is characterized by an increase in LV volume with reduced wall thickness. Mechanical stress and volume overload signals resulting from the loss of functional myocardial tissue after MI initiate the development of eccentric hypertrophy. Additionally, growth factors such as angiotensin II and endothelin-1, released in response to cardiac remodelling, also participate in transmitting these signals to cardiomyocytes, further contributing to the development of eccentric hypertrophy [186]. Initially, eccentric hypertrophy is considered a compensatory mechanism to maintain cardiac function by increasing

the heart's pumping capacity. However, excessive hypertrophy can exacerbate cardiac dysfunction and remodelling, contributing to the progression of heart failure [187].

2.4.3 Interstitial fibrosis

Interstitial fibrosis is a pathological process characterized by the excessive accumulation of collagen within the healthy non-infarcted tissue. This excessive accumulation of collagen alters the normal structure, and the function of the heart [53, 171]. Interstitial fibrosis drastically reduces myocardial compliance, increases passive stiffness, and may lead to diastolic dysfunction [188]. In addition, interstitial fibrosis is also associated with electrical conduction disturbance in the heart, which can increase the risk of potentially serious arrhythmias [175, 189]. Finally, collagen accumulation around the small coronary arteries and arterioles alters the oxygen supply to the remaining cardiomyocytes, impacting their survival [190] (Fig. 6). Overall, interstitial fibrosis compromises the contractile function of the heart, and increases the progression to heart failure and is strongly correlated with adverse outcome [191].

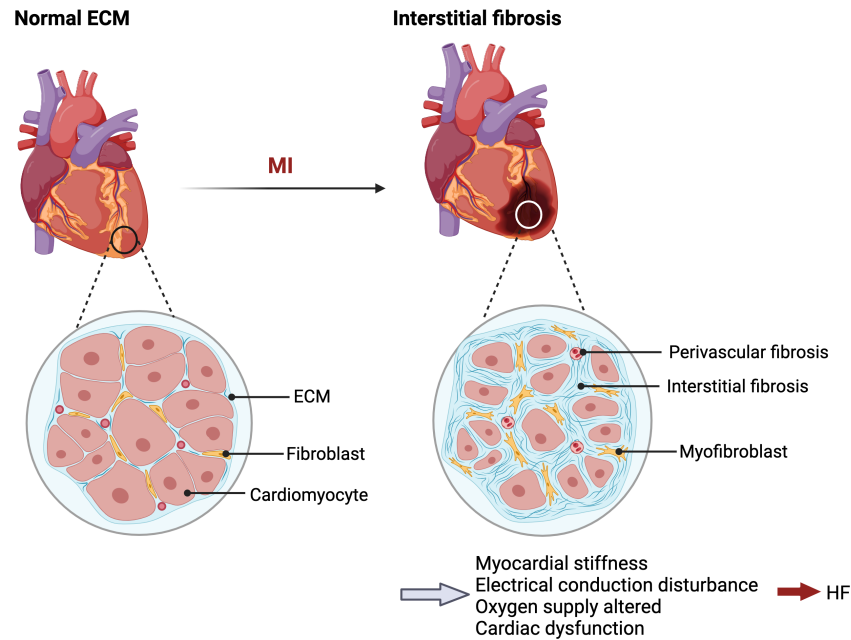


Figure 6. Interstitial fibrosis development post-MI. Interstitial fibrosis is characterized by the accumulation of collagen in the healthy myocardium. The consequences of these changes are an increased myocardial stiffness, disruption of electrical conduction, and altered oxygen supply, ultimately contributing to cardiac dysfunction and the progression to heart failure. HF, heart failure; ECM, extracellular matrix.

2.4.4 Cardiac rupture

Cardiac rupture is a serious complication that can occur after MI, often resulting in sudden death. This event is characterized by the tearing of the myocardial wall, leading to the leakage of blood into the pericardial cavity. The primary mechanism behind cardiac rupture is the structural weakening of the myocardium, associated with alterations in the ECM composition [1, 192]. The ECM of the myocardium provides structural support and mechanical integrity to the heart tissue. Following MI, ischemic injury leads to a cascade of events that disrupt ECM homeostasis. This disruption is characterized by increased degradation of ECM components by MMPs,

and an imbalance in the synthesis of ECM components in the infarcted area. This degradation of the ECM, and the disorganized structure of the scar creates a weakened zone within the infarcted myocardium, predisposing it to rupture [1, 192, 193]. Another contributing factor to cardiac rupture is the mechanical stress on the infarcted myocardial wall. During the healing process post-MI, the infarcted area undergoes remodelling, which includes thinning and dilatation of the myocardial wall. This remodelling increases wall stress, further compromising the weakened area and increasing the risk of rupture [2]. Therefore, cardiac rupture post-MI results from the interplay of inflammatory degradation of the ECM, mechanical stress, and adverse remodelling of the myocardial wall. These processes collectively weaken the structural integrity of the infarcted myocardial tissue, making it susceptible to myocardial rupture.

2.5 Targeting TGF- β signalling

Because of its critical involvement in the cardiac fibrotic response, TGF- β signalling remains an attractive therapeutic target. To date, various types of TGF- β inhibitors have been tested in preclinical studies for the treatment of cardiac fibrosis. Neutralizing anti-TGF- β antibodies were used in rodent models of MI and hypertrophic cardiomyopathy. These studies have been inconclusive, with conflicting results depending on the timing and dose of administration [194-197]. In addition, cardiovascular toxicities of anti-TGF- β antibodies have been demonstrated [198]. The soluble T β RII, which acts as a competitive inhibitor of TGF- β , has shown promising effects when injected on days 3–4 after MI, whereas it has been ineffective when injected a few weeks later [199, 200]. On the contrary, Ikeuchi *et al.* have demonstrated that soluble T β RII overexpression during the early stage after MI exacerbates ventricular dilatation and increases mice mortality,

INTRODUCTION

whereas the inhibition of TGF- β signalling during the later stage after MI alleviates cardiac remodelling [141]. Interestingly, adenoviral overexpression of decorin, a TGF- β antagonist, has been shown to inhibit hypertension-induced cardiac fibrosis in rats [201]. GW788388 is a TGF- β receptor I (ALK5) inhibitor that has been demonstrated to reduce fibrosis after MI in rats [202]. It has also been found to inhibit cardiac fibrosis in an experimental mouse model of Chagas' heart disease [203, 204]. Although GW788388 appears to have beneficial antifibrotic effects, these preclinical data need to be confirmed. Indeed, SM16, another ALK5 inhibitor, has been shown to induce beneficial effects on pressure overload-induced cardiac fibrosis while increasing LV dilatation and mortality in mice [205]. In addition, ALK5 inhibitors have been found to induce heart valve damage in rats [206]. Pirfenidone is an antifibrotic drug currently approved for the treatment of idiopathic pulmonary fibrosis. Although its exact mechanism of action is not fully understood, it is thought to exert its effects by inhibiting TGF- β expression. The beneficial effects of Pirfenidone on cardiac fibrosis have been demonstrated in several animal models [207-212]. The PIROUETTE clinical trial demonstrated that administration of pirfenidone is associated with a significant reduction in cardiac fibrosis whereas it unfortunately does not improve diastolic function [213]. Finally, tranilast is used for the treatment of allergic disorders. Although its mechanism of action is not fully understood, tranilast inhibits the expression and activity of TGF- β and has been shown to reduce cardiac fibrosis in rodents. In summary, although some anti-TGF- β therapies have shown encouraging results, the pleiotropic effects as well as the context and time-dependent actions of TGF- β after cardiac injury make anti-TGF- β therapy challenging. Therefore, more specific agents should be developed to safely and robustly inhibit TGF- β signalling.

INTRODUCTION

CHAPTER 2. PLATELETS BEYOND HEMOSTASIS AND THROMBOSIS: AT THE CORE OF CARDIAC INFLAMMATION AND FIBROSIS

Based on:

Fibroblasts and platelets, a face-to-face dialogue at the heart of cardiac fibrosis.

Dufeys Cécile*, Bodart Julie*, Bertrand Luc, Beauloye Christophe and Horman Sandrine. *Am J Physiol Heart Circ Physiol* 2024 March.

**Contributed equally to the work.*

INTRODUCTION

1. PLATELETS

Platelets are small anucleate blood cells of 2 to 4 μm diameter, which are derived from the hematopoietic lineage by fragmentation of megakaryocytes. This process, called thrombopoiesis, occurs continuously in the bone marrow or as shown more recently, in the spleen and lungs [214]. Thrombopoiesis results in the release into the circulation of 5,000 to 10,000 platelets per megakaryocyte. Platelets survive between 8 and 12 days in human blood, compared with 3–4 days in mice, before being removed from vessels by neutrophils and macrophages, and transported to the spleen and liver for elimination. They are the most prevalent blood components after red blood cells (RBC) and are typically known to be central players in hemostasis and thrombosis. However, platelet functions extend far beyond hemostasis and are currently involved in the regulation of many physiological and pathological processes [214-216].

1.1 Platelets in hemostasis and thrombosis

The primary physiological function of platelets is hemostasis, where they contribute to the formation of a thrombus at a damaged vascular site to limit blood loss. Under physiological conditions, platelets circulate into bloodstream in a resting state, without interacting with the sub-endothelium. However, in response to vascular injury caused by erosion or atherosclerotic plaque rupture, the integrity of the endothelial layer is disrupted, exposing the subendothelial ECM to blood components. This event triggers platelet adhesion to the injured endothelium, followed by platelet activation, aggregation and ultimately, the formation of a thrombus. It is important to note that while hemostasis is a normal physiological process, thrombosis represents a pathological condition. In thrombosis, a

hemostatic clot forms within the lumen of a blood vessel, obstructing blood flow and leading to ischemia [214, 217].

1.1.1 Platelet adhesion to endothelium

The initial anchoring results from the interaction between the endothelial von Willebrand factor (vWF) binding protein, attached to soluble vWF, and the platelet membrane glycoprotein (GP)Ib-IX-V, which leads to platelet rolling. This process enables a direct association between exposed collagen fibres and the platelet collagen receptors, GPIIb/IIIa and $\alpha_2\beta_1$ integrin, which stabilize platelet adhesion. Other adhesive proteins, including fibronectin, laminin, and thrombospondin, are also involved in the adhesion process by binding to their specific receptors at the platelet surface [215, 218, 219].

1.1.2 Platelet activation

Platelet adhesion to the injured endothelium induces the activation of various platelet-intracellular signalling pathways leading to an increase in cytosolic calcium (Ca^{2+}), with subsequent platelet activation [220]. It results in the secretion of granules and the release of secondary agonists which further potentiates thrombus growth by recruiting and activating additional platelets at the site of injury. Platelets contain two major types of secretory granules: alpha- (α -) granules and dense granules. The α -granules, ranging in diameter from 200 to 500 nm and numbering between 50 and 80 per platelets, constitute the most abundant secretory organelles in platelets [221]. Upon platelet activation, they release a diverse array of soluble proteins into the circulation. This includes chemokines, growth factors, pro- angiogenic and anti-angiogenic factors, coagulation factors, and immune mediators [221-223]. In addition, α -granules contain adhesive

INTRODUCTION

proteins crucial for platelet-platelet and platelet-endothelial cells interactions. Among these granules are membrane-bound proteins, such as P-selectin and CD40L, which become expressed on the platelet surface, contributing to interactions with immune cells [215, 221, 222]. Proteins within α -granules play essential roles in various physiological functions, including hemostasis, thrombosis, inflammation, angiogenesis, wound repair, and antimicrobial host defense. Dense granules, approximately 150 nm in diameter with 4 to 6 per platelet, contain over 200 nonprotein molecules, including cations, nucleotides, polyphosphates, and bioactive amines [221, 222]. These molecules predominantly participate in the secondary aggregation response which is crucial for amplifying and sustaining platelet activation through positive feed-back mechanisms [215, 220-222, 224]. In addition to granules, activated platelets release lysosomes containing enzymes, such as acid hydrolases, involved in the degradation of proteins, lipids and carbohydrates [225]. The exact role of platelet lysosomes remains not fully understood. Furthermore, platelets secrete microvesicles (100-1,000 nm) that carry growth and angiogenic factors, coagulation factors, enzymes, adhesion molecules, chemokines, and cytokines, complement proteins, apoptosis regulators, bioactive lipids, and microRNAs [216, 226] (Fig. 7). Platelet-derived microvesicles are abundant in blood and are now recognized as important mediators of intercellular communication [227]. Platelet activation also results in the exposure of negatively charged phospholipids (phosphatidylserine) on platelet surface, supporting the binding and activation of coagulation factors [222]. Activated platelets secrete thromboxane A_2 , which is synthesized from platelet membrane phospholipids, and acts as a secondary agonist by stimulating and recruiting additional platelets. Finally, platelet activation leads to a conformational change of the $\alpha 2b\beta 3$ membrane receptor, from a low- to a high- affinity state for fibrinogen,

resulting in aggregation through the formation of interplatelet bridges and the formation of platelet plug at the site of injury [217, 228].

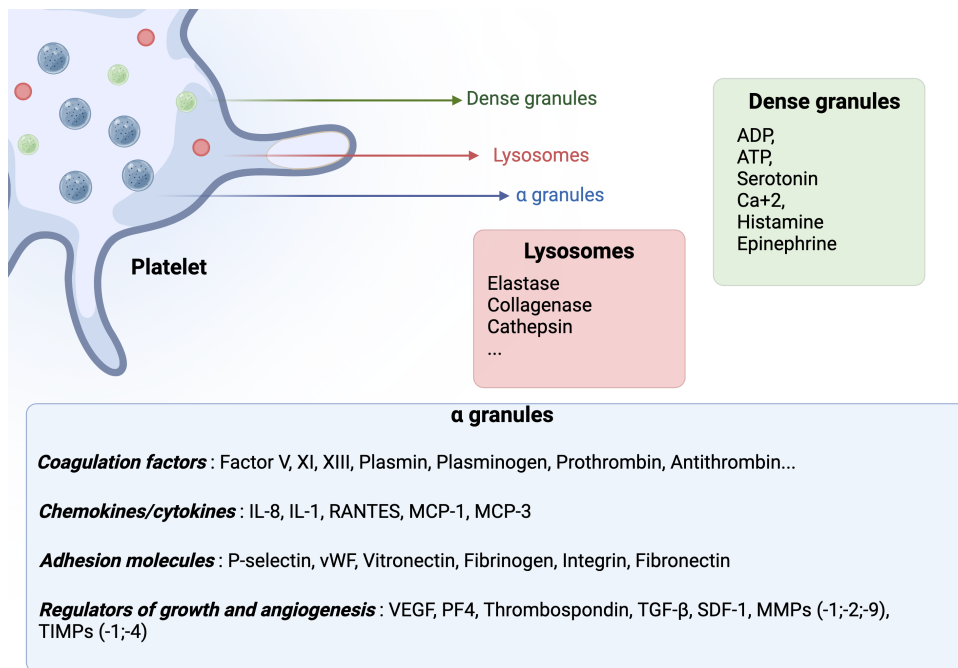


Figure 7. Granule content of platelets. Platelet contains three types of granules: dense granules, alpha granules, and lysosomes. Dense granules release ADP, serotonin, calcium ions, histamine, and epinephrine, which are important for platelet aggregation. Lysosomes contain enzymes like elastase, collagenase, and cathepsin, crucial for ECM degradation and remodelling. α-granules are rich in coagulation factors, including factor V, XI, XIII, plasminogen, and antithrombin, which are essential for clot formation and stability. Additionally, α-granules contain chemokines/cytokines which mediate inflammatory responses; adhesion molecules like P-selectin and integrins that facilitate cell-cell interactions; and regulators of growth and angiogenesis including VEGF, PF4, and TGF-β, which promote healing and new blood vessel formation post-injury. MCP-1/3, monocyte chemotactic protein; VEGF, vascular endothelial growth factor; PF4, platelet factor 4; SDF-1, stromal cell-derived factor 1. Figure adapted from [229].

1.1.3 Platelet aggregation and thrombus formation

The thrombus formation is the result of a well-coordinated series of events. It is initiated by the activation of the coagulation cascade which is a series of enzymatic reactions, involving serine proteases called coagulation factors, and results in the conversion of pro-thrombin to thrombin. Thrombin ultimately converts fibrinogen to fibrin, which polymerizes into an insoluble fibrin network creating a fibrous blood clot to seal the injured area. Thrombin is also the most potent platelet agonist that strongly contributes to platelet activation and amplifies the coagulation cascade [217, 230]. The coagulation cascade is a powerful response which is finely regulated by a complex inhibitory system called the fibrinolysis and mediated by the plasmin [231].

1.2 Platelets beyond hemostasis and thrombosis: at the core of cardiac inflammation and fibrosis

A growing body of evidence supports that platelet functions extend far beyond hemostasis and thrombosis. Indeed, they exhibit the capability to release a wide variety of biomolecules which are functionally involved in a large number of physiological or pathological responses, such as angiogenesis [232], wound healing [233-235], inflammation and fibrosis [236-239] (Fig. 8). In addition to endothelial injury, various other stimuli can prompt platelet activation and recruitment into the heart. Platelets express PRRs (TLRs, C-type Lectin Receptors, and Nucleotide-binding oligomerization domain-like Receptors) enabling them to recognize DAMPs released by necrotic cardiomyocytes or pathogen-associated molecular patterns (PAMPs) expressed by pathogens [223, 240]. Moreover, in case of vascular inflammation, activated endothelial cells express several factors responsible for platelet activation, including vWF, tissue factor, ROS and others [222, 223]. Finally,

INTRODUCTION

angiotensin II and oxidative stress observed in conditions like hypertension and/or metabolic syndrome may also activate platelets [239, 241-243]. Hence, platelets have been shown to infiltrate both the vasculature and the interstitial myocardium following ischemia [244-249] induced injury. Notably, in the ischemic mouse myocardium, platelet recruitment begins as early as 2 minutes after reperfusion, progressively increasing up to 60 minutes [247]. This rapid recruitment underscores platelets as among the first blood cells to respond to ischemia-reperfusion injury, as the appearance of neutrophils occurs more slowly, starting from 30 minutes of reperfusion [247]. Magnetic resonance and PET-scan imaging have confirmed the very early recruitment of platelets after ischemia-reperfusion, showing their presence in the ischemic myocardium reaching peak accumulation at 2 h after reperfusion, followed by a decline by 4 hours [248, 249]. During this period, platelets predominantly colocalize with leukocytes [246]. Moreover, Xu *et al.* demonstrated that platelet activation and recruitment into the ischemic heart are dependent on the duration of the ischemia [247]. In a mouse model of LAD coronary artery permanent ligation, platelets were observed to accumulate into the heart as early as 6 hours after MI, becoming more prominent by 72 hours. This study revealed that platelets constitute one of the initial waves of inflammatory cells accumulating within the infarcted myocardium, colocalizing with leukocytes at 6 hours post-MI [246]. Although platelets were still present within the ischemic myocardium at 1 day and 7 days after ischemia/reperfusion [244], the question of whether platelets are cleared from the site of injury or persist in the late stages of cardiac fibrosis development after MI remains to be investigated.

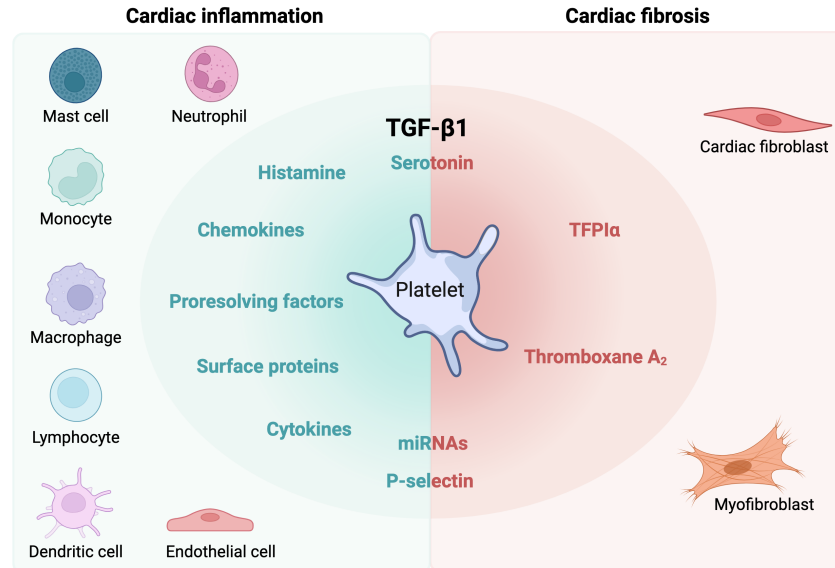


Figure 8. Contribution of platelet-derived mediators to myocardial inflammation and/or fibrosis. TFPI α , tissue factor pathway inhibitor- α ; TGF- β 1, transforming growth factor- β 1. Figure adapted from [250].

1.2.1 Platelets as a source of TGF- β 1

Platelets constitute a major source of TGF- β 1, as they contain 40-100 times more TGF- β 1 than other cell types [236-238, 251]. Therefore, platelets are the leading regulator of TGF- β 1 concentrations in plasma. Indeed, it has been demonstrated that total TGF- β 1 levels are reduced by approximately 45% in the plasma of mice with a platelet-specific deletion of TGF- β 1. Furthermore, profound thrombocytopenia induced by injecting monoclonal anti- α IIb β 3 antibodies in mice, reduces plasma levels of total TGF- β 1 [237]. Platelet TGF- β 1 is contained in α -granules and rapidly released upon platelet activation. Latent TGF- β 1 is present in two different pools in platelets. The first pool, representing 95% of total platelet TGF- β 1, corresponds to the TGF- β 1 associated with LTBP and LAP. The second pool corresponds to the TGF- β 1 complexed with LAP, without LTBP. Recent results

suggest that the TGF- β 1/LAP/LTBP pool is released into plasma in a single step, whereas the TGF- β 1/LAP-containing complex is trapped on platelet surface before being released into plasma upon activation [252]. Indeed, it has been found that activated platelets express TGF- β 1 on their extracellular surface through its binding to the GARP protein [135, 253]. However, the mechanism by which active TGF- β 1 is generated from latent TGF- β 1/GARP complexes on platelets remains poorly understood (*see chapter 4.2.1*).

1.2.2 Platelets in cardiac fibrosis

Platelet TGF- β 1 also modulate cardiac fibrosis. In this context, Meyer *et al.* provided the first evidence that TGF- β 1 originating from platelets contributes to cardiac fibrosis. Using a platelet specific TGF- β 1 deletion transgenic mouse model, these authors demonstrated that mice lacking platelet TGF- β 1 developed less interstitial and perivascular fibrosis following pressure overload generated by transverse aorta constriction. These mice were partially protected from cardiac hypertrophy and systolic dysfunction [237]. A few years later, Varshney *et al.* demonstrated that aortic stenosis progression could be delayed in the absence of platelet TGF- β 1. This was associated with a significant decrease in fibroblast myodifferentiation and fibrosis in aortic valve tissue [236]. More recently, platelet-derived TGF- β 1 was found to promote angiotensin II-induced atrial fibrosis and atrial fibrillation in mice. In this study, TGF- β 1 from platelets was shown to directly promote myodifferentiation of CFs [239].

In addition to TGF- β 1, numerous mediators of fibroblast activation are contained in platelet granules and microvesicles, and some of them have been shown to be involved in the regulation of cardiac fibrosis. Indeed, serotonin released by platelets

has been described to directly affect neonatal rat CFs by promoting their migration and myodifferentiation and by increasing the secretion of TGF- β 1 and MMPs [254]. Furthermore, activated platelets have been found to stimulate cardiac fibrosis in a mouse model of angiotensin-II hypertension by secreting P-selectin [255]. Then, platelet thromboxane A2 was shown to stimulate CF myodifferentiation, profibrotic gene expression, and consequently cardiac fibrosis [256]. Platelet-derived tissue factor pathway inhibitor- α (TFPI α) was reported to prevent cardiac fibrosis by reducing thrombin generation and thrombin-mediated platelet activation in mice [257]. Recently, platelets have emerged as a rich source of microRNAs (miRNAs), acting as paracrine mediators that tightly regulate surrounding cells, including CFs [201-204, 244]. Notably, despite lacking nuclei, platelets contain mRNAs, miRNAs, and the necessary enzymes for the conversion of precursor miRNAs (pre-miRNA) into mature miRNAs [205]. Intriguingly, studies have revealed that platelet-derived miRNAs play a crucial role in modulating cardiac fibrosis following ischemia-reperfusion. In a genetic mouse model featuring megakaryocyte/platelet-specific invalidation of the pre-miRNA processing ribonuclease Dicer, inducing a disruption in miRNA processing in platelets, researchers observed worsened myocardial remodelling and exacerbated cardiac fibrosis in platelet-specific Dicer knockout mice subjected to transient left coronary artery ligation [244]. Other molecules influencing CFs such as thrombospondin-1 and PDGF are highly enriched in platelet granules [53, 227, 258]. However, it is not clear whether these platelet-derived molecules directly contribute to cardiac fibrosis. Although the correlation between platelet count in the bloodstream and liver fibrosis is well established [207, 210], there is currently a lack of studies investigating a similar correlation in the context of the heart.

1.2.3 Platelets in cardiac inflammation

Given the high interconnectedness between the inflammatory and fibrotic processes, platelets may regulate cardiac fibrosis indirectly by modulating inflammation. In recent years, the role of platelets in inflammatory reactions and immune responses modulation has emerged and they are now considered as full-blown immune cells [216, 222, 223, 259]. Platelets secrete a wide range of immunomodulatory factors (chemokines, cytokines, adhesive proteins, miRNAs,...) contained within their α -granules and microvesicles. Furthermore, they also directly interact with leukocytes through their surface proteins (P-selectin, $\alpha 2b\beta 3$ integrin, GpIb α), contributing to leukocyte recruitment, trans-endothelial migration, and activation [246, 247, 259]. Circulating platelets have been shown to interact with monocytes, promoting P-selectin-mediated delivery of platelet-derived pro-inflammatory factors, and ultimately contributing to the formation of atherosclerotic lesions in apolipoprotein-E-deficient mice [260]. Moreover, platelets induce a pro-inflammatory phenotype in monocytes [261]. Recently, Reusswig *et al.* demonstrated that in a model of ischemia-reperfusion, acute thrombocytopenia reduced inflammatory response and improved cardiac remodelling, highlighting the role of platelets in modulating inflammation post-MI [262]. In addition, platelet-derived microvesicles have been implicated in cardiac inflammation following ischemia-reperfusion injury through their microRNAs [244]. Finally, platelet-derived serotonin was also shown to induce neutrophil degranulation, enhancing post-MI inflammation [263]. However, platelets have also been described to exert anti-inflammatory effects. Indeed, they release an abundance of anti-inflammatory cytokines, such as TGF- $\beta 1$ and other proresolving mediators including lipoxin A4, maresin 1, and resolvin E1, which attenuate neutrophil trafficking and promote their apoptosis [259, 264, 265]. Moreover,

platelets play a role in modulating macrophage phenotype towards their reparative function [234, 259, 266]. Notably, platelets were shown to drive the differentiation of profibrotic macrophages by secreting CXCL4 [267]. Altogether, these findings underscore the intricate and multifaceted role of platelets in inflammation and its resolution (Fig. 9).

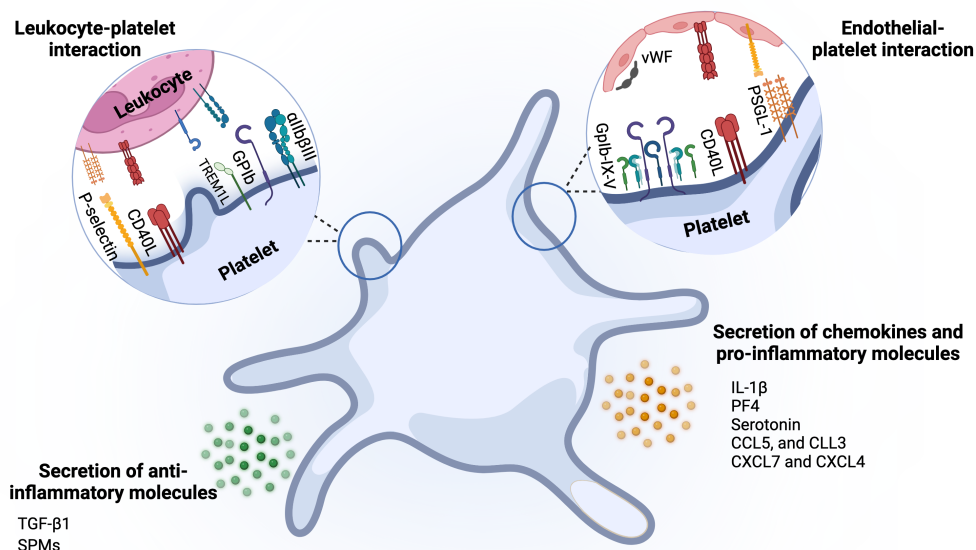


Figure 9. Platelet interactions and secretory functions in post-MI inflammation. Platelets are shown to interact with leukocytes and endothelial cells through various surface proteins (P-selectin, α 2 β 3 integrin, GPIb α and CD40L). Platelets also secrete a wide range of pro-inflammatory factors (chemokines, cytokines, adhesive proteins) contained in their α -granules and microvesicles. Platelets have also been described to exert anti-inflammatory effects. They release an abundance of anti-inflammatory cytokines, such as TGF- β 1 and proresolving mediators including lipoxin A4, maresin 1, and resolvin E1. Collectively, this figure summarizes the complex contributions of platelets to both the promotion and resolution of inflammation in the context of cardiac injury. SPMs, specialized pro-resolvin mediators.

1.3 Platelets as a new potential therapeutic tool for cardiac inflammation and fibrosis

Given the key role of platelets in inflammatory and fibrotic processes, platelet-targeting may be a promising therapeutic strategy (Table 2). The use of antiplatelet drugs has already shown encouraging beneficial effects in the treatment of liver and pulmonary fibrosis [268-270]. In the cardiovascular field, platelet depletion with an anti-GPIIb/IIIa antibody strongly reduces thrombus fibrosis and adverse vein wall remodelling in a mouse model of inferior vena cava stenosis, indicating that platelets are a major contributor to thrombus fibrosis [271]. Several studies have found that treatment with antiplatelet drugs significantly reduces fibrosis in different models of cardiac injury. First, the inhibition of platelet activation by the irreversible ADP receptor blocker clopidogrel has been shown to prevent cardiac inflammation and fibrosis in a mouse model of angiotensin II-induced hypertension [272]. More recently, Liu *et al.* [239] demonstrated that clopidogrel reduces angiotensin II-induced platelet accumulation, inflammation, and fibrosis in the atrium. Second, ticagrelor, a reversible ADP receptor blocker, has been described to decrease collagen expression and fibrosis after ischemia-reperfusion injury. Interestingly, despite a similar degree of platelet inhibition, clopidogrel had no effect on cardiac fibrosis in this study [273]. Inhibition of cyclooxygenase-1 by aspirin has been shown to decrease interstitial fibrosis in infarcted rat hearts [274] and attenuate pressure overload-induced cardiac fibrosis by inhibiting ERK1/2 and Akt/ β -catenin signalling pathways in mice [275]. Moreover, ticagrelor and aspirin have been found to reduce collagen expression in a rat model of ischemia-reperfusion [276]. A recent study showed that different anti-platelet drugs, namely aspirin, clopidogrel, ticagrelor and prasugrel, significantly reduce inflammatory cell infiltration and cardiac fibrosis in a mouse model of MI [277].

INTRODUCTION

Nonetheless, TGF- β is well established as a key regulator of cardiac fibrosis, and platelets have been identified as a significant source of TGF- β 1 [194, 264, 278, 279]. The specific targeting of platelet-derived TGF- β could prove to be a promising therapeutic avenue. In this regard, the GARP protein emerges as a potential candidate. GARP associates with latent TGF- β on the platelet surface, triggering its activation [280, 281]. While GARP expression has been identified in Tregs, platelets, and some endothelial and fibroblast cell lines, only Tregs and platelets have been demonstrated to generate active TGF- β 1 from GARP/latent TGF- β 1 complexes [195, 280, 282]. Therefore, investigating the role of platelet GARP in active TGF- β 1 production following heart injury holds significant interest.

INTRODUCTION

Dose	Administration	Treatment duration	Animal	Model	Major Findings	Reference
Clopidogrel 50 mg/kg/day	ig	For 7 days	Mouse	ANG II	↓ Inflammation ↓ Fibrosis	[272]
Clopidogrel Loading: 50 mg/kg/day Maintenance: 5 mg/kg/day	ig	For 3 wk	Mouse	ANG II	↓ Inflammation ↓ Fibrosis ↓ Atrial fibrillation	[239]
Ticagrelor Acute: 10 or 30 mg/kg Chronic: 300 mg/kg/day	Acute: ip Chronic: in diet	Acute: 5 min before reperfusion Chronic: Starting 1d after I/R, for 4w	Rat	I/R	↓ Infarct size ↓ Apoptosis ↓ Fibrosis Improve cardiac function	[273]
Clopidogrel Acute: 12.5 mg/kg Chronic: 62.5 mg/kg/day					No effect	
Aspirin 25 mg/kg/day	ip	From 2 days before to 21 days after LAD	Rat	LAD	↓ Fibrosis	[274]
Aspirin 10 mg/kg/day	ig	From 4 days before to 4 wk after TAC	Mouse	TAC	↓ Fibrosis	[275]
Ticagrelor 300 mg/kg/day Aspirin 20 mg/kg/day	Chow	From 1 wk after I/R, for 1 wk or 3 wk	Rat	I/R	Improve cardiac function ↓ Fibrosis ↑ Regenerative cells	[276]
Prasugrel 15 mg/kg/day Ticagrelor + Aspirin 300 and 20 mg/kg/day					No effect	
Aspirin 15 mg/kg Clopidogrel 11 mg/kg Ticagrelor 27 mg/kg Prasugrel 1.5 mg/kg	ig	1 week before LAD or From 1 wk before to 1 wk after LAD or 1 wk after LAD	Mouse	LAD	↓ Inflammation ↓ Fibrosis Improve cardiac function when treating before surgery	[277]

Table 2. Preclinical *in vivo* studies using antiplatelet drugs and their effects on cardiac fibrosis.
ig, intragastrically; ip, intraperitoneal; ANG II, angiotensin II; TAC, pressure overload generated by transaortic constriction; LAD, myocardial infarction induced by permanent ligation of left anterior descending coronary artery; I/R, ischemia-reperfusion.

CHAPTER 3. THE GARP/ TGF-B1 PATHWAY

INTRODUCTION

1. The GARP/TGF- β pathway

1.1 GARP gene and GARP protein expression

GARP, also identified as *LRRC32* (Leucin Rich Repeats Containin protein 32) was initially described in 1992 by Ollendorf *et al.* [283]. The human *GARP* gene is located on chromosome 11q14, and its murine counterpart is located on chromosome 7 [283, 284]. Both the mouse and human GARP genes exhibit high homology, sharing approximately 80% identity in nucleotide sequence [281]. The *GARP* gene encodes a type I transmembrane protein of 662 amino acids (aa) [284]. Its extracellular domain (608 aa) consists of two series of 10 leucine-rich repeats (LRR) motifs, each composed of a highly conserved region with a consensus sequence of LxxLxLxxNxL or LxxLxLxxCxxL [285]. The cytoplasmic domain of *GARP* (14 aa) includes a PDZ-like domain, suggested to regulate GARP trafficking to the cell surface, although it lacks known signalling activity [286]. GARP mRNA expression has been detected in various tissues, including placenta, lungs, kidneys, as well as to a lesser extent in the heart, liver, pancreas, and skeletal muscle. Additionally, GARP expression has been described in megakaryocytes [285, 287, 288], platelets [135, 287, 289], endothelial cells [289-291], Tregs [135, 292-294], B cells [295, 296], hepatic stellate cells [297], mesenchymal stem cells [291, 298], and fibroblasts [299].

1.2 GARP binds and activates TGF- β 1

GARP is essential for the surface presentation of latent TGF- β 1. It binds to latent TGF- β 1 through disulfide bonds, specifically between Cys33 of LAP and Cys192 and 331 of GARP. For latent TGF- β 1 to become biologically active and initiate its downstream signalling pathways, it must be activated, a process that GARP

facilitates on the cell surface. While many cells can produce latent TGF- β 1, only a few possess the ability to activate it in response to specific stimulation, a process that involves cell-type specific mechanisms. Recent research, particularly focused on Tregs, has shed light on the importance of GARP in TGF- β activation. Studies by Sophie Lucas' group have demonstrated that inhibition of GARP using monoclonal antibodies prevents TGF- β activation in Tregs. Incubating stimulated human Tregs with these antibodies *in vitro* resulted in the inhibition of Smad2 phosphorylation, a downstream signalling event of TGF- β activation [280]. Additionally, silencing GARP expression in human Tregs through siRNA reduced their suppressive capacity *in vitro* [135]. However, the presence of membrane-bound GARP alone is not sufficient to activate latent TGF- β . Co-transfection of GARP and latent TGF- β in T helper cells resulted in the surface display of latent TGF- β without initiating its activation [135, 293, 300]. Integrins have emerged as a promising candidate for TGF- β activation, although further research is needed to elucidate their precise role in this process.

1.2.1 Role of integrins in GARP-mediated activation of TGF- β 1

Integrins are heterodimeric transmembrane receptors composed of an α and a β subunits and mediating cell adhesion to the ECM and linking the ECM to the cytoskeleton [301]. Recent studies by Wang *et al.* have identified integrins α v β 6 and α v β 8 as key players in the activation of TGF- β from GARP/latent TGF- β complexes on the cell surface [300]. They demonstrated that membrane anchoring is essential for TGF- β activation, as soluble GARP/latent TGF- β complexes cannot be activated by integrins alone [300]. The mechanism of TGF- β activation requires integrin interaction with LAP through the RGD (Arg-Gly-Asp) motif, inducing mechanical forces that facilitate the release of active TGF- β [281, 300] (Fig. 10).

INTRODUCTION

Interestingly, integrin $\alpha\beta6$ is exclusively expressed in epithelial cells, where GARP expression is not observed. Conversely, Tregs lack expression of the $\beta6$ gene, indicating that $\alpha\beta6$ -mediated activation of TGF- β by Tregs likely occurs in trans, with GARP/latent TGF- β complexes on Tregs being activated by $\alpha\beta6$ on another cell [281]. However, studies have demonstrated TGF- $\beta1$ activation by Tregs alone, implying the involvement of a protein distinct from $\alpha\beta6$. Therefore, $\alpha\beta8$ -mediated mechanical activation of TGF- β has been proposed, as its genetic deletion in mouse Tregs impaired the induction of Th17 cells [302]. This process, dependent on TGF- β , was further validated by blocking anti-human $\alpha\beta8$ antibodies, which inhibited GARP-dependent TGF- $\beta1$ activation by Tregs [282]. These findings highlight the critical involvement of $\alpha\beta8$ in the active release of TGF- β from Tregs. Alternatively, latent TGF- β can be activated by protease-dependent mechanisms including the recruitment of MMPs and serine proteases, which cleave the LAP fragment to release mature TGF- $\beta1$ [134, 136-138].

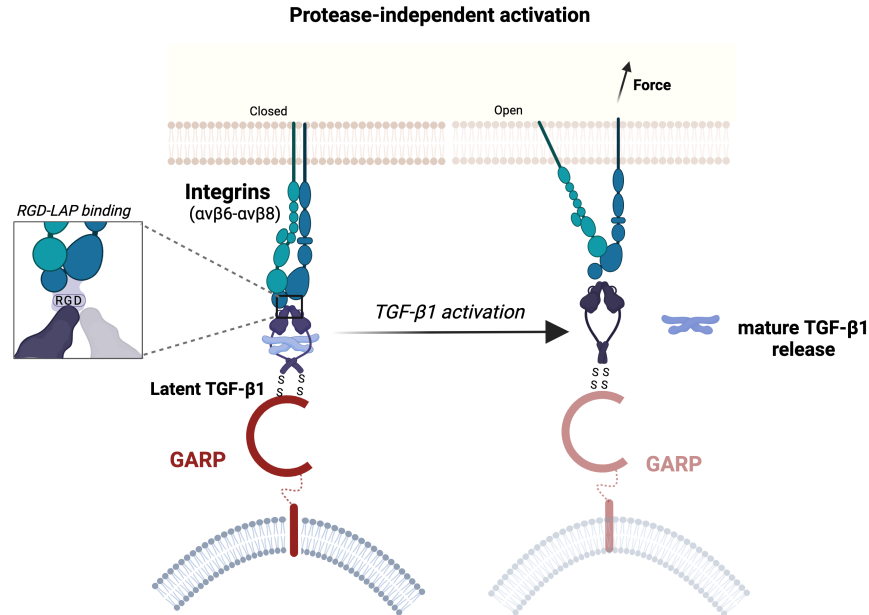


Figure 10. Integrin-mediated activation of TGF-β. Latent TGF-β1 is bound to GARP on the cell surface, forming a latent complex which is inactive. Integrins, specifically αvβ6 and αvβ8, bind to the RGD motif in LAP. The application of mechanical force by the integrins induces a conformational change. This conformational change allows the release of mature TGF-β1 from the LAP, making it available for binding to its receptors and initiating downstream signalling pathways. Figure adapted from [303].

1.3 GARP function in immune tolerance and cancer

As previously mentioned, GARP is predominantly expressed on Tregs, playing a crucial role in the activation of latent TGF-β1, a potent immunosuppressive cytokine. Its activation by GARP-expressing Tregs contributes to immune homeostasis by suppressing excessive immune responses and preventing autoimmunity. The interaction between GARP and latent TGF-β on the Treg cell surface enables a regulated release of active TGF-β, which, in turn, modulates the activity of various immune cells, promoting an anti-inflammatory microenvironment. Intriguingly, mice with T-cell specific GARP deletion do not exhibit severe spontaneous autoimmunity, suggesting a nuanced role of TGF-β

produced by Tregs in the maintenance of immune tolerance [304]. However, *in vivo* experiment using xenogeneic graft-versus-host (xGvHD) disease model in mice has demonstrated that monoclonal GARP/latent TGF- β 1 antibodies can prevent TGF- β 1 activation by human Tregs, inhibiting their immunosuppressive activity and thereby intensifying the alloreactive immune response [280].

In the context of cancer, GARP has emerged as a potential therapeutic target due to its role in tumor immune evasion and metastasis. Tumor-infiltrating Tregs often express high levels of GARP, promoting an immunosuppressive tumor microenvironment through TGF- β 1 activation. Moreover, GARP expression has been detected in certain tumor cells, suggesting its potential role in tumor progression [281, 305]. Therefore, strategies aimed at targeting GARP have shown promise in preclinical studies by enhancing anti-tumor immune responses and inhibiting tumor growth. For instance, specific blockade of TGF- β 1 production by Tregs using antibodies targeting GARP/TGF- β 1 complexes has been effective in improving anti-tumor immunity, particularly in tumors initially resistant to anti-PD-1 (Programmed cell death 1) immunotherapy. Administration of GARP/TGF- β 1 antibodies alone did not exhibit anti-tumor activity as monotherapy in solid tumors, whereas it was sufficient to reduce tumor burden in mouse models of blood cancers (myeloproliferative neoplasms) [304, 306, 307].

1.4 GARP in platelets

Although initially discovered on activated Tregs and platelets, GARP's role in platelet has received little attention until recently. Early studies in zebrafish models revealed GARP's involvement in thrombus formation and hemostasis, as evidenced by defective thrombus growth and increased spontaneous bleeding events in GARP-deficient mice [288]. However, contrasting findings emerged from studies in

INTRODUCTION

mice with a conditional deletion of GARP in platelets (Pf4-Cre x GARP flox/flox), where platelet function remained unaffected in *ex vivo* analysis. Furthermore, murine *in vivo* thrombosis model demonstrated normal tail bleeding and thrombus formation occlusion time in platelet GARP-deficient mice [289]. More recent research using the same genetic mouse model revealed a drastic reduction in active TGF- β 1 levels in the serum of platelet-specific GARP KO mice, indicating that platelets GARP is required for the activation of systemic TGF- β 1 [253]. Moreover, this study evaluated the contribution of platelet GARP to anticancer immunity, showing that platelet-specific deletion of GARP enhances immune defense against both murine models of melanoma and colon cancer, suggesting that platelet-derived GARP has immunosuppressive properties [253]. Currently, research on the role of the platelet GARP protein is primarily focused on cancer, elucidating its implications in tumor progression and immune evasion. However, the involvement of GARP in platelets, particularly in conditions where TGF- β 1 plays a crucial role such as cardiovascular diseases, remains less understood. Therefore, further investigation is needed to define the specific functions of platelet-derived GARP, shedding light on its potential contributions to various physiological and pathological processes beyond cancer biology.

AIMS OF THE WORK

Platelets play a direct role in the pathogenesis of MI, not only through thrombus formation in epicardial coronary arteries but also by contributing to post-MI myocardial inflammation and fibrosis. They serve as a significant source of TGF- β 1, and the presence of GARP on their surface suggests that they might play a crucial role in activating TGF- β 1 within the infarcted heart. However, nothing is known about the role of platelet GARP in TGF- β 1-dependent cellular processes related to post-MI inflammation, repair, and fibrogenesis. Therefore, the overall aims of this thesis are to investigate the effects of the platelet GARP-TGF- β 1 pathway on the early inflammatory response initiated after MI, and its subsequent impact on LV fibrosis and remodelling.

Specific Aims

Aim 1. Characterization of the platelet-specific GARP KO mouse model.

This aim involves generating platelet GARP-deficient mice and exploring how platelet GARP deletion affects platelet functions and TGF- β 1 activation.

Aim 2. Study of the influence of platelet GARP deficiency on cardiac injury, remodelling and LV function after MI.

Using a mouse model of permanent LAD occlusion, we aim to assess how platelet GARP deficiency affects survival, LV remodelling and LV function post-MI, providing insights into the contribution of the platelet GARP/TGF- β pathway to structural and functional alterations in the infarcted heart.

Aim 3. Exploration of the effects of platelet GARP deletion on the early inflammatory response after MI, and elucidation of potential underlying mechanism(s).

We will conduct RNA-sequencing to obtain unbiased profiling of the infarcted heart transcriptome and examine how this relates to neutrophil and macrophage infiltration, as well as cytokine expression.

Aim 4. Investigation into whether platelet GARP deficiency influences the resolution of inflammation and cardiac healing post-MI.

We will further explore whether the acute effects of platelet GARP deletion observed during the early inflammatory response after MI lead to altered myocardial healing processes.

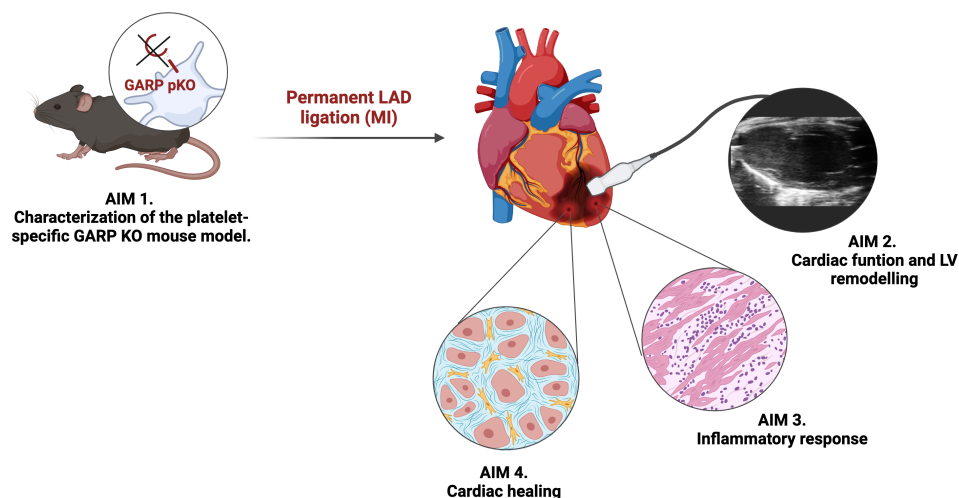


Figure 11. Resume of the objectives of the thesis. Using a mouse model or permanent LAD occlusion, we aim to assess how platelet GARP deficiency affects survival, LV remodelling, LV function, inflammatory response, and cardiac healing post-MI.

RESULTS

RESULTS

Loss of platelet-specific GARP leads to cardiac rupture and adverse remodelling following myocardial infarction

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Authors' contributions

J.B., C.D. designed research studies, conducted experiments, acquired data, analysed data, wrote, and edited the manuscript. A.G. conducted experiments and acquired data. J.A. acquired data and analysed data. Y.S., Z.N. provided Gplb-Cre mice. Ca.B., D.B. acquired data and analysed data. L.B. secured funding and edited the manuscript. Ch.B. designed research studies, secured funding, and edited the manuscript. S.L. provided GARP^{fl/fl} mice, acquired data, analysed data, and edited the manuscript. S.H. designed research studies, secured funding, analysed data, wrote, and edited the manuscript. All authors read and approved the final manuscript prior to submission.

1. ABSTRACT

After myocardial infarction (MI), necrotic cardiomyocytes trigger an inflammatory phase crucial for infarct healing and ventricular remodelling, characterized by the infiltration of inflammatory cells and upregulation of inflammatory genes. Transforming growth factor- β (TGF- β) is pivotal in regulating inflammation and cardiac repair post-MI. Platelets infiltrate the infarcted myocardium early, producing significant amounts of TGF- β 1. Moreover, they express Glycoprotein A Reiterations Predominant (GARP) on their surface, indicating their capacity to both produce and activate TGF- β 1. In this study, using a mouse model of permanent coronary artery ligation, we investigated the role of platelet GARP in TGF- β 1 activation and subsequent regulation of post-MI processes. Platelet-specific GARP knockout (pKO) mice showed higher rates of myocardial rupture and exacerbated left ventricular dilatation after MI compared to wild-type mice. Pathological evaluation of pKO hearts revealed heightened endothelial activation, neutrophil and macrophage infiltration, and increased production of pro-inflammatory cytokines, resulting in delayed inflammation resolution and impaired healing. These findings highlight the essential roles of the platelet GARP/ TGF- β 1 pathway in regulating the inflammatory phase and inflammation resolution during myocardial healing. They also emphasize the potential risks associated with GARP inhibition therapy, especially during the acute phases of ischemic insult.

2. INTRODUCTION

The occurrence of cardiac rupture following acute myocardial infarction (MI) represents a fatal complication associated with high mortality rates. Extensive clinical and experimental studies have highlighted the significant role of exaggerated inflammation and degradation of the extracellular matrix (ECM) in triggering cardiac rupture during the early stages of MI [118, 308]. However, the intricate molecular mechanisms governing this sequence of events remain largely unclear. A deeper understanding of the fundamental cellular and molecular mechanisms involved in the response to cardiac injury has the potential to uncover novel therapeutic targets for the treatment of heart disease [309]. Cardiac repair following MI begins with a robust inflammatory response and infiltration of immune cells, marking the onset of the inflammatory phase [11, 310]. This phase is initiated by the death of cardiomyocytes, which triggers the release of Danger-Associated Molecular Patterns (DAMPs) [310]. The recognition of these molecular signals by Pattern Recognition Receptors (PRRs), which are found in both immune and non-immune cells within the cardiac tissue, activates a signalling cascade resulting in the secretion of pro-inflammatory cytokines such as $\text{TNF}\alpha$, $\text{IL-1}\beta$, and IL-6 [11, 310]. These cytokines activate endothelial cells, causing increased expression of adhesion molecules of E-selectin, Vascular cell adhesion molecule-1 (VCAM-1), or intercellular adhesion molecule-1 (ICAM-1) on their surface [311]. Consequently, this facilitates the recruitment of leukocytes from the bone marrow and the spleen into the infarct site [11, 127, 311]. Neutrophils are recruited to the site of damage within 6 to 48 hours after MI [11, 250, 310, 311]. They release pro-inflammatory cytokines that escalate cardiac inflammation and secrete proteases like myeloperoxidase, neutrophil elastase, and matrix metalloproteinases-8 (MMP-8), which degrade the ECM, aiding in the clearance of necrotic material [11, 127]. By 48-72 hours post-infarction, pro-inflammatory monocytes accumulate at the

injury site and differentiate into pro-inflammatory macrophages, further facilitating the removal of necrotic cells and ECM debris [127, 310]. Between 4-7 days post-MI, macrophages undergo a phenotypic shift towards an anti-inflammatory phenotype triggered by the phagocytosis of apoptotic neutrophils [127, 310]. This shift is characterized by the release of anti-inflammatory cytokines (IL10, etc.), growth factors (TGF- β , VEGF, etc.), and ECM components (collagens, etc.) stimulating cardiac fibroblast (CF) proliferation, neovascularization, and fostering cardiac healing and collagen-based scar formation [250, 310, 311]. Platelets play an important role in MI, contributing to the thrombotic occlusion of coronary vessels. Beyond their primary function in hemostasis and thrombosis, platelets are involved in a broad spectrum of physiological and pathological processes [216, 250]. Notably, platelets have been observed to infiltrate the infarcted myocardium, prior to the appearance of neutrophils [246, 247]. In this context, they play a critical role in cardiac repair by releasing a plethora of immunomodulatory factors [250]. Platelets are particularly rich in TGF- β 1 [237, 312]. Furthermore, they possess the ability to secrete this factor in its active form, thanks to the presence of Glycoprotein A Repetitions Predominant (GARP) on their surface [253], although the mechanism underlying TGF- β 1 activation by GARP in platelets has not yet been described. Given the pivotal role of TGF- β 1 in multiple post-infarction processes, including leukocyte recruitment to endothelial surface, inflammation resolution, and fibrous scar development [134], exploring the influence of platelet GARP on TGF- β 1 activation and implications for these events warrants further investigation. In the present study, we report that platelet GARP is a critical regulator of latent TGF- β 1 activation. Platelet GARP-deficient mice show impaired cardiac healing, leading to increased rates of myocardial rupture and mortality, or exacerbated LV dilatation following MI, in contrast to control mice. These deleterious outcomes seem to be associated with heightened endothelial activation, augmented

RESULTS

leukocyte recruitment, intensified production of pro-inflammatory cytokines, and a delayed resolution of inflammation.

3. METHODS

3.1 Chemicals and reagents

Collagen-related peptide (CRP-XL) was obtained from Cambcol laboratories. Thrombin from bovine plasma (#T6634), apyrase (#A6132), acid citrate-dextrose solution (#C3821), bovine serum albumin (BSA) (#3854.4), and phosphomolybdic acid (#4440.3) were purchased from Roth. Phycoerythrin (PE)-conjugated anti-active $\alpha\text{IIb}\beta\text{3}$ (JON/A) (#MO23-2) and PE-conjugated IgG (#P190-2) antibodies were purchased from Emfret analytics. Fluorescein isothiocyanate (FITC)-conjugated anti-CD62P (#553744), FITC-conjugated IgG1 λ (#553995), and anti-Ly6g (#551459) antibodies were from BD pharmingen. PE-conjugated IgG2b (#400607), APC-conjugated anti-LAP (#TW7-16B4), APC-conjugated IgG1 (#141406), FITC anti-CD8a (#100706), BV650 anti-B220 (#103241), PE-Cy7 anti-CD4 (#100422), and anti-CD16/32 (#101320) antibodies were purchased from Biolegend. Ketamine (Nimatek) was purchased from Dechra. Xylazine (Rompun) was from Bayer. Rhodamine labeled wheat germ agglutinin (WGA) (#RL1022) and Vectashield mounting medium (#H1000) were purchased from Vector Laboratories. EnVision system-HRP labelled polymer anti-rabbit (#K4003), Dako Liquid DAB+ Substrate Chromogen System (#K3468), Dako Automation Hematoxylin Histological Staining Reagent (#S3301), and Dako fluorescence mounting medium (#S3023) were obtained from Agilent. Human cardiac fibroblasts (HCFs) were obtained from ScienCell Research Laboratories (#6300). HCF basal medium (#315500) and HCF growth supplement (#316GS) were purchased from Cell applications. PE-conjugated anti-GARP (#12989182), alexa fluor-488 (#B40953) or -555 (#B40955) tyramide reagent, Hoechst 33342 (#62249), Halt protease and phosphatase inhibitor cocktail (#78446), RBC lysis buffer (#00433357), Dyabeads mouse T-activator (#11456D), RPMI 1640 medium (#11875093), GlutaMAX supplement (#35050061), and DMEM high glucose GlutaMAX supplement pyruvate

RESULTS

(#31966021) were sourced from Thermo Fischer Scientific. The iScript cDNA synthesis kit (#1708891) came from Bio-Rad. The qPCR core kit for SYBR Green I (#RTSN1005NR) was obtained from Eurogentec. Anti-GAPDH (#5174), anti-PCNA (#13110), and anti-F480 (#78588) antibodies were purchased from Cell Signaling. Anti-vimentin (#ab92547), anti-CD41 (#ab181582), anti-pSmad3 (#ab52903) anti- α SMA (#ab124964) antibodies were obtained from Abcam. Anti-GARP (#AF6229) antibody and mouse TGF-beta 1 DuoSet ELISA were from R&D Systems. RNeasy mini kit (#74104) and DNase (#79254) were purchased from Qiagen. Tribromoethanol (#T48402) was from Sigma. Mouse cardiac troponin-I ELISA (#CTNI1US) comes from Life Diagnostics. F(ab')₂ fragment anti-mouse IgM (#115006075) was purchased from Jackson ImmunoResearch. FOXP3 Transcription Factor Staining Buffer (#00512343), mouse IL-21 (#14821162), viability dye (#65086514), and APC anti-FOXP3 antibody (#17577382) came from eBioscience. CpG (#ODN2395) was from InvivoGen.

3.2 Experimental animals

We generated a Cre recombinase transgenic mouse strain allowing megakaryocyte (MK)/platelet-specific invalidation of GARP. In this model, the Cre recombinase is driven by the mouse glycoprotein (GP)Ib α promoter [313]. GpIb α -Cre^{+/–} mice were bred with GARP^{fl/fl} mice [314] to generate MK/platelet-specific GARP knockout mice (pKO). GpIb α -Cre^{–/–} x GARP^{fl/fl} littermates were used as the controls. Ten- to twelve-week-old male and female mice were used in this study. Animal procedures and protocols were approved by local authorities (Comité d'éthique facultaire pour l'expérimentation animale, 2021/UCL/MD/013) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication, revised 2011). All animals were housed under a 12-h/12-h light/dark cycle, with the dark cycle occurring from

6.00 p.m. to 6.00 a.m., and a relative humidity of between 50 and 70%. Mice were observed daily with free access to water and standard chow.

3.3 Myocardial infarction procedure

MI was induced via permanent ligation of the left anterior descending (LAD) coronary artery and performed by an experienced operator. Animals were pre-treated with buprenorphine hydrochloride (0.1 mg/kg, i.p.) 30 minutes before being anesthetized with tribromoethanol (310mg/kg, i.p.). Their tracheas were intubated, a left-sided thoracotomy was performed at the fourth intercostal space and the LAD coronary artery was ligated approximately 2 mm below the base of the left auricle using 8.0 polyamide silk. MI was confirmed by visualization of ventricle cyanosis. The heart was subsequently replaced into the thoracic cavity and the chest was closed. Sham animals underwent the same surgical procedure, without LAD coronary ligation. Mice were randomly assigned to sham or MI. Animals were euthanized with a combination of ketamine and xylazine hydrochloride (150mg/kg and 10mg/kg, respectively, i.p.) followed by cervical translocation on days 1, 3, 7 and 14 post-surgeries.

3.4 Echocardiographic analysis

Cardiac function was assessed by non-invasive transthoracic echocardiography on anesthetized mice (2% of isoflurane for induction and 1-2% for maintenance, in 100% oxygen) using a Vevo 3100 Imaging System (FUJIFILM VisualSonics, Toronto, Canada) equipped with a MX transducer 550D. To ensure the accuracy of all measurements, physiological parameters were monitored and recorded at a heart rate of 450 to 550 beats per minute. All measurements and analysis were performed by the same experienced operator who was blinded to the experimental groups. Cardiac parameters were measured in 2-dimensional (2D) B-mode and M-

mode long axis and short axis. Infarct extent was determined by analysing regional LV wall motion at 3 days post-MI, based on adjustments for human heart imaging [18]. Echocardiographic measurement of cardiac structure and function was performed at baseline (1-3 days before surgery) and 14 days post-MI. LV volumes were measured using B-mode parasternal long-axis view, at end-systole and end-diastole, from which ejection fraction (EF %) was deduced. LV mass was calculated from LV long-axis measurements and normalized by tibia length.

3.5 Isolation of platelets and preparation of platelet releasates

Platelets. Mice were anesthetized with a combination of ketamine and xylazine (150mg/kg and 10mg/kg, respectively, i.p.). Blood was collected via the retro-orbital plexus in 1/6 acid-citrate-dextrose solution containing 1 U/mL apyrase. Platelet-rich-plasma (PRP) was obtained by centrifugation at 800 g for 5 seconds, followed by 5 minutes at 100 g. PRP was washed with 2 volumes of acid-citrate-dextrose with 1 U/mL apyrase. Platelets were pelleted by centrifugation at 400 g for 5 minutes and resuspended in modified Tyrode's buffer (135 mM NaCl, 12 mM NaHCO₃, 3 mM KCl, 0.3 mM Na₂HPO₄, 1 mM MgCl₂, 5 mM D-glucose, 10 mM Hepes, 0.35 % BSA, pH 7.4) at a final concentration of 250x10³ platelets/ μ L. Platelets were counted using the Cell-Dyn Emerald (Abbott).

Platelet releasates. Platelets were stimulated with CRP (0.3 μ g/mL) for 5 minutes at 37°C, and releasates were collected as supernatants after centrifuging the samples at 13,000 g for 15 seconds.

3.6 Flow cytometry analysis

Spleens. Spleens were harvested and minced in 2 mL ID supplemented with 5% fetal bovine serum (FBS) and AJ. Spleen homogenates were filtered through a 70 μ m nylon filter and red blood cells (RBC) were lysed in RBC lysis buffer for 5 minutes at

RESULTS

room temperature. Cells were filtered through 40 μ M nylon filter and resuspended in x-vivo medium supplemented with Glutamax. Splenocytes were stimulated for 24 hours using Dynabeads mouse T-activator CD3/CD28 for T cells expansion and activation, whereas B cells were stimulated with a mix containing 25 μ g/mL F(ab')₂ fragment anti-mouse IgM, 10 ng/mL mouse IL-21, 20 μ g/mL anti-CD40 and 1 μ M CpG oligonucleotides. After 24 hours, cells were incubated for 10 minutes at 4°C with anti-CD16/32 antibodies (1:100) to block Fc receptors. Samples were incubated with the following fluorescently conjugated antibodies: PE-Cy7 anti-CD4 (1:200), PE anti-GARP (1:50), BV650 anti-B220 (1:100) and FITC anti-CD8a (1:200) in the presence of a viability dye (1:1,000) for 30 minutes at 4°C. For intra-cellular staining, cells were fixed and permeabilized using FOXP3 Transcription Factor Staining Buffer during 20h at 4°C, then stained with APC anti-FOXP3 (1:100) in the presence of anti-CD16/32 (1:100). Analyses were performed on a FACS LSR Fortessa flow cytometer (BD Biosciences), and data were computed using the FlowJo software (Tree Star).

Washed platelets. Washed platelets ($250 \times 10^3/\mu$ L) were incubated for 20 minutes at room temperature with the following fluorescently conjugated antibodies: PE anti-GARP (1:12.5), APC anti-LAP (1:12.5), FITC anti-CD62p (1:12.5) or PE anti-Jon/A (1:12.5) or the corresponding isotype control antibody. Platelets were washed and stimulated with different concentrations of thrombin or CRP at 37°C for 8 or 5 minutes, respectively, in the presence of 2 mM Ca²⁺. Paraformaldehyde 4 % was added to platelets to stop the reaction and samples were acquired on a BD FACSCanto™ II (BD Biosciences). Data were computed using the FlowJo software (Tree Star).

3.7 Measurements of TGF- β 1 and Troponin I

Active and total TGF- β 1. Blood was collected from mice without anticoagulants by retro-orbital bleeding and was incubated at room temperature for 2 hours. Blood was then centrifugated at 2,000 g for 20 minutes at 4°C and the supernatant (serum) was collected and stored at -80°C. Active and total TGF- β 1 were measured using the DuoSet ELISA assay according to the manufacturer's instructions. Active TGF- β 1 levels were measured without acidic treatment.

Troponin I. Troponin I levels were measured in plasma according to the manufacturer's recommendations.

3.8 Western Blot analysis

Cells were lysed in cold buffer containing 50 mM Tris-HCl pH7.5, 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 1 % (v/v) triton X-100, 20 mM glycerol-2-phosphate disodium, 50 mM NaF, 5 mM Na₄P₂O₇·10H₂O, 1 mM dithiothreitol, 1x proteases and phosphatases inhibitor cocktail. Lysates were centrifuged at 15,000 g for 10 minutes and protein concentration in the supernatant was measured by the Bradford method. Equal amounts of proteins resuspended in a modified Laemmli's buffer containing 50 mM Tris pH 6.8, 10 % glycerol, 2 % sodium dodecyl sulfate (SDS), 0.5 mM EDTA, 25 mM dithiothreitol, and 0.01 % bromophenol blue were separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride membrane. Membranes were blocked with Tris-buffer saline (TBS) / 0.01 % Tween 20 / 5 % BSA and probed overnight at 4°C with antibodies targeting pSMAD3 (1:2,000), GARP (1:1,000) or GAPDH (1:50,000). After incubation with the appropriate secondary antibody (1:20,000) for 1 hour at room temperature, proteins were visualized with chemiluminescence HRP substrate. Band intensities were quantified by Image J software (version 1.8, National Institutes of Health, Bethesda, MD, USA). GAPDH was used as a loading control.

Band intensities were normalized relative to those of loading controls on the same gel and results are reported as densitometric relative expression.

3.9 Histological analysis

Tissue processing. Hearts were excised, cleared of blood with ice-cold phosphate buffer saline (PBS) and fixed with 4 % paraformaldehyde for 48 hours at room temperature. Fixed tissues were paraffin embedded with Tissue-Tek VIP6 (Sakura) and 5 μ m thick serial sections were made. Before each staining or immunochemistry, slices were deparaffinized and rehydrated.

Immunohistochemistry. Heart sections were incubated for 20 minutes with 3% H₂O₂ to inhibit endogenous peroxidases and antigens were retrieved by microwave heating for 15 minutes in citrate buffer pH 5.7, EDTA pH8, or Tris-EDTA buffer pH 9 (for anti-F4/80, anti-Ly6G, or anti-CD41 staining respectively). Heart sections were permeabilized in TBS / 0.1 % Tween 20 and blocked for 30 min with 5 % BSA solution. Infiltrated platelets and neutrophils were assessed using anti-CD41 (1:250) or anti-Ly6g (1:2,000) antibodies, respectively. Primary antibodies were incubated overnight at 4°C. Slides were washed with TBS / 0.1% Tween 20, incubated with DAB substrate for 5 minutes, washed and counterstained with haematoxylin for 5 minutes. Slides were then mounted. Omission of the primary antibody served as negative control. Staining was digitized by the Panoramic SCANII slide scanner (3DHitech). Automatic analysis was performed using the Visiopharm software.

Immunohistofluorescence. Heart sections were incubated for 20 minutes with 3% H₂O₂ to inhibit endogenous peroxidases and antigens were retrieved by microwave heating for 15 minutes in citrate buffer pH 5.7. Heart sections were permeabilized in TBS / 0.1 % Tween 20 and blocked for 30 min with 5 % BSA solution. Cardiac fibroblast proliferation and myodifferentiation were assessed by

double fluorescent staining with anti-PCNA (1:800), or anti- α SMA (1:5000) respectively, as well as anti-vimentin (1:10,000) antibodies. Primary antibodies were incubated for 90 minutes at room temperature. Slides were washed with TBS / 0.1% Tween 20 and incubated for 40 minutes with HRP labelled polymer anti-rabbit at room temperature. After washing, slides were incubated with either 488 or 555 tyramide reagent (1:200) for 10 minutes, washed and nuclei were stained with 10 μ g/ml Hoechst. Slides were then mounted. Omission of the primary antibody served as negative control. Staining was digitized by the scanner Axio Scan.Z1 (Carl Zeiss). Automatic analysis was performed using the Visiopharm software.

3.10 Assessment of cross-sectional cardiomyocytes area with WGA staining

Heart sections were washed with PBS and incubated for 2 hours with WGA-rhodamin (1:150 in PBS). After washing in PBS, slides were mounted in vectashield mounting medium. Pictures were acquired with an Axio Imager microscope (Carl Zeiss) with a 40X objective. Cell size (>500 cells per sample) was determined using Visiopharm software.

3.11 Assessment of cardiac fibrosis with Picrosirius red staining

Heart sections were treated with 1.5 % phosphomolybdic acid for 2 minutes, washed with H₂O and incubated in 0.1 % picrosirius red solution for 2 hours at room temperature. Sections were then quickly rinsed in 0.01 M hydrochloric acid, washed with H₂O, and mounted. Stainings were digitized using a Panoramic SCANII slide scanner (3DHitech). Scar circumference was assessed by measuring the largest circumference of serial sections from apex to base. Scar thickness was

defined as the mean of ten to twenty thickness all along the scar. Scar circumference and thickness were determined using Cytomine software.

3.12 Adult human cardiac fibroblasts in culture

Human cardiac fibroblasts (HCFs) were seeded at 20,000/cm² and cultured for 24 hours in HCF basal medium 10 % serum / 0.1 % antibiotics according to the manufacturer's recommendations. Cells were serum-starved in RPMI 1640 medium for 4 hours prior to treatment. HCFs were incubated with serum, plasma, or platelet releasate for 30 minutes and lysed for western blot analysis.

3.13 RNA extraction and mRNA expression

Total RNA was isolated from infarcted heart areas or sham LV with RNeasy mini kit using chloroform/phenol lysis. Samples were treated with DNase according to the manufacturer's instructions. RNA was quantified with NanoDrop (Thermo Scientific) and was reverse transcribed with 0.75 µg of RNA using the iScript™ cDNA synthesis kit. Real-time quantitative PCR was performed using SYBR Green on CFX Connect (Biorad). RPL32 served as housekeeping gene. Primer sequences are presented in Supplementary Table S1.

3.14 RNA sequencing

Total RNA was isolated from infarcted heart areas and treated with DNase according to the manufacturer's instructions. RNA quality test, libraries preparation and sequencing were carried out by Biomarker Technologies BMK GENE (Münster, Germany) according to their pipeline. HISAT2 [315] was used to align the NGS sequences (reads) of each sample to the reference genome (Mus musculus.GRCm39.genome.fa) and to estimate the gene expression count matrix. From this matrix, the determination of differential expression was performed using

models based on the negative binomial distribution with the Deseq2 v.1.40.2 [316] Bioconductor package. In this package, fold-changes associated with the genotype (pKO vs WT), were adjusted for mouse sex. Differences in gene expression levels between pKO and WT were considered statistically significant when adjusted p-value (i.e. FDR computed with Benjamini-Hochberg correction) was lower than 0.05. Based on the resulting gene expression modulations, gene set enrichment analysis (GSEA) was performed using the fgsea v1.24.0 Bioconductor package on hallmark gene sets of the MSigDB collection [317] and on the Kyoto Encyclopedia of Gene and Genomes (KEGG) [318] database. Raw and processed RNA-sequencing (RNA-seq) data were deposited and made publicly available on the Gene Expression Omnibus.

In addition, the immune microenvironment was analysed using the seq-ImmuCC; bioinformatic tool (<https://guolab.wchscu.cn/ImmuCellAI-mouse//#!/>). By employing a deconvolution model, seq-ImmuCC estimates the relative proportions of principal immune cell types within a sample.

3.15 Statistical analysis

Data are expressed as means $\pm$ SEM. Normal distribution of continuous variables was tested according to the Shapiro–Wilk method and equality of variances. Statistical significance was evaluated using unpaired Student T-test for comparing two groups, when they had passed the normality test. The non-parametric Mann-Whitney test was applied to compare two groups that failed the normality test. Either one-way or two-way ANOVA were employed for multiple comparisons. Post-hoc pairwise comparisons were carried out using the Sidak method. Survival analysis was conducted by means of the log rank test. Data were analysed using GraphPad Prism 9 statistical software.

4. RESULTS

4.1 Generation and characterization of platelet-specific GARP knockout mice

To explore the influence of platelet GARP on post-MI responses, we generated pKO utilizing the Cre-loxP recombination system driven by the *Gplb α* promoter [313]. *Gplb α* encodes the glycoprotein (GP)Ib α subunit of the GPIb-IX-V complex, the von Willebrand factor receptor [319]. To produce pKO mice, *Gplb α -Cre $^{+/-}$* mice were crossed with *GARP $^{fl/fl}$* mice [314]. *Gplb α -Cre $^{-/-}$* x *GARP $^{fl/fl}$* littermates were used as WT controls. The pKO progeny exhibited normal viability, fertility, and Mendelian ratios, with no phenotypic differences. As expected, GARP protein was expressed in platelets from WT mice, while its expression was completely abolished in platelets isolated from pKO mice (Fig. 1a). Flow cytometric analysis revealed low expression of GARP and latency-associated peptide (LAP), reflecting latent TGF- β 1, on the surface of resting WT platelets, with increased externalization upon thrombin or collagen related peptide (CRP) stimulation (Fig. 1b and 1c). Conversely, platelets from pKO mice showed no expression of GARP/latent TGF- β 1 complexes, either at baseline or after stimulation (Fig. 1b and 1c). Furthermore, flow cytometry data demonstrated induction of GARP expression on the surfaces of stimulated regulatory T lymphocytes (Fig. 1d and 1e) and B lymphocytes (Fig. 1f and 1g). Importantly, GARP expression in regulatory T lymphocytes and B lymphocytes isolated from pKO mice remained unchanged compared to that from WT, thus validating the platelet specificity of the *Gplb α -Cre $^{+/-}$* mouse model. There were no significant differences in hemoglobin levels, hematocrit, or blood cell counts between WT and pKO mice, except for a 10% increase in mean platelet volume (MPV) observed in pKO mice compared to WT littermates (Supplementary Table S2).

RESULTS

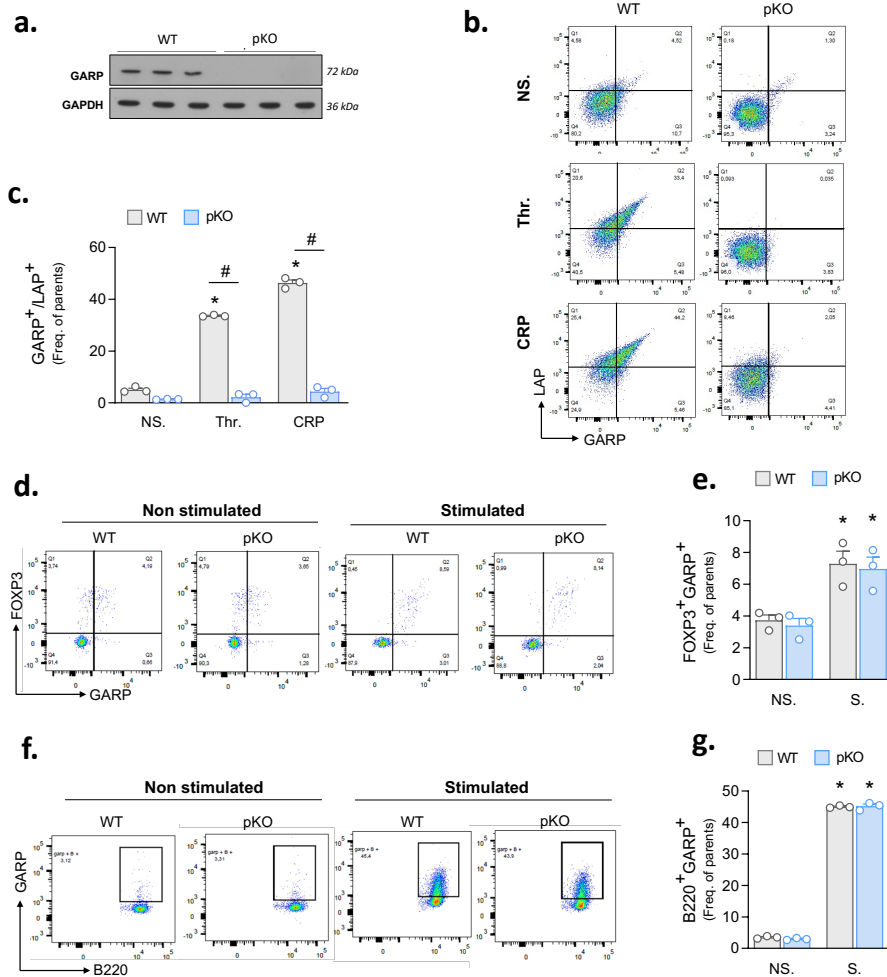


Figure 1. Generation and characterization of platelet-specific GARP knockout mice. (a) Western blot analysis of GARP expression in WT and pKO platelet lysates. GAPDH was used as loading control. (b, c) GARP and LAP exposure on the surface of WT and pKO platelets in response to growing concentrations of thrombin (mU/mL) or collagen-related peptide (CRP) (ng/mL). GARP and LAP expression was measured by flow cytometry. Results are expressed as percentage of parent. (d, e) GARP expression on the surface of resting and activated T regulatory lymphocytes. The expression of GARP was measured by flow cytometry on Foxp3 positive live cells. Results are expressed as percentage of parent. (f, g) GARP expression on the surface of resting and activated B lymphocytes. The expression of GARP was measured by flow cytometry on B220 positive live cells. Results are expressed as percentage of parent. Dot plots represent data from individual mice (n=3 mice/group), as well as mean±SEM. Statistical significance was determined by two-

way ANOVA followed by Sidak's multiple comparisons test. * $p < 0.05$ compared to the non-stimulated condition. # $p < 0.05$ compared to the WT condition.

4.2 Platelet GARP deficiency does not affect platelet reactivity but impairs platelet-derived TGF- β 1 activation

To assess the impact of platelet GARP deficiency on platelet reactivity, we examined platelet activation *in vitro* by flow cytometry analysis of activated integrin α IIb β 3 (Jon/A) and P-selectin (CD62p) externalization on the platelet surface in response to increasing concentrations of thrombin or CRP. Both WT and pKO platelets exhibited integrin α IIb β 3 activation (Fig. 2a) and P-selectin exposure (Fig. 2b) upon stimulation with these agonists, indicating comparable reactivity between the two groups. To investigate the implication of platelet GARP in TGF- β 1 activation and release, we quantified active and total TGF- β 1 levels in the serum of WT and pKO mice using ELISA. We observed a 50% reduction in active TGF- β 1 concentration in the serum of pKO mice compared to WT (Fig. 2c), while total TGF- β 1 levels remained constant between the two genotypes (Fig. 2d). To corroborate this finding, HCFs, highly responsive to active TGF- β , were stimulated with serum from WT or pKO mice, followed by western blot analysis of Smad3 phosphorylation, a downstream effector of TGF- β receptor activation (Fig. 2e). Stimulation of HCFs with serum from WT mice significantly increased Smad3 phosphorylation, whereas this response was markedly reduced in HCFs treated with serum from pKO mice (Fig. 2f). Furthermore, to demonstrate the capacity of platelets for releasing active TGF- β 1 in a GARP-dependent manner, we exposed HCFs to the releasates of both WT and pKO platelets, whether activated or not by CRP treatment (Fig. 2g). We observed a significant increase in Smad3 phosphorylation in HCFs exposed to releasates from activated WT platelets compared to those from resting WT platelets, indicating active TGF- β 1 release upon platelet activation (Fig. 2h). In contrast, releasates from activated pKO platelets failed to induce an increase in

RESULTS

Smad3 phosphorylation in HCFs (Fig. 2h). Altogether, these findings demonstrate that activated platelets can release active TGF- β 1 from GARP/latent TGF- β 1 complexes.

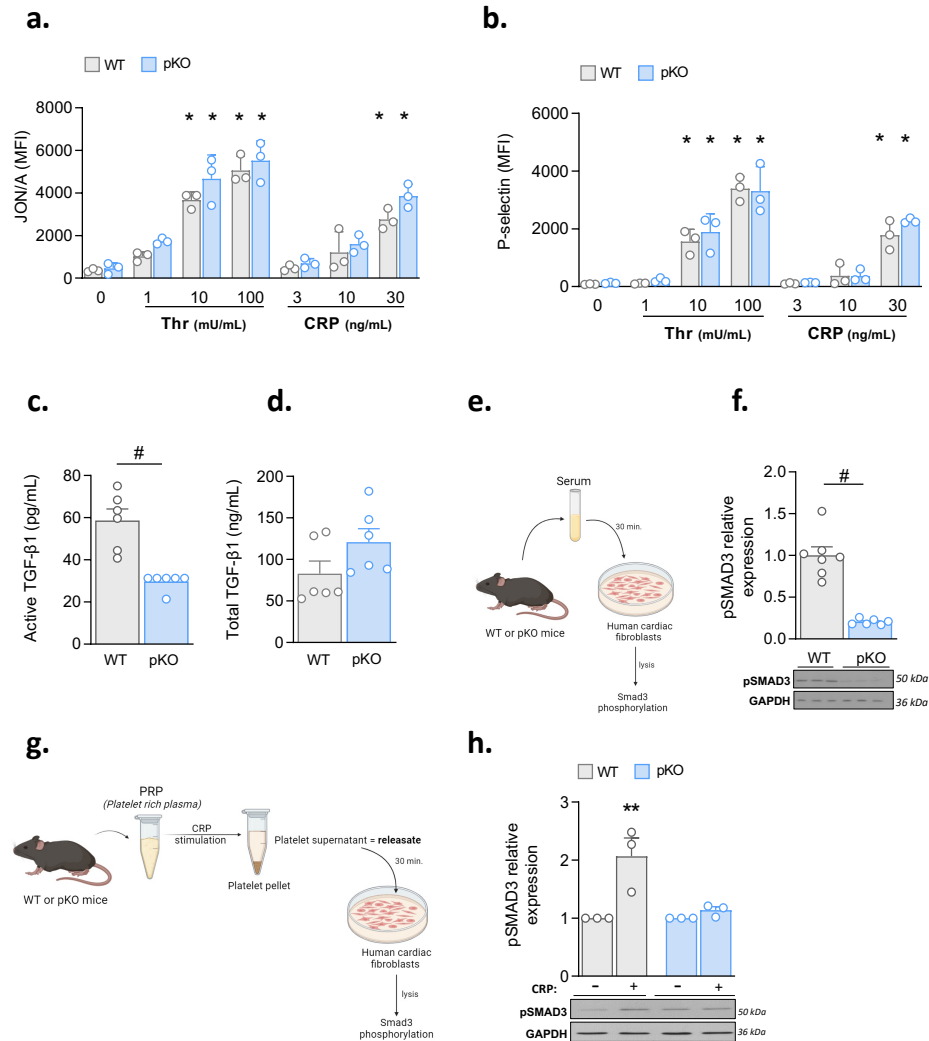


Figure 2. Platelet GARP deficiency does not affect platelet reactivity but impairs platelet-derived TGF- β 1 activation. (a, b) JON/A (activated α IIb β 3) (a) and P-selectin (CD62Ps) (b) expression on the surface of WT and pKO platelets in response to growing concentrations of thrombin (mU/mL) or collagen-related peptide (CRP) (ng/mL). JON/A and P-selectin expression was measured by flow cytometry. Results are expressed as mean fluorescence intensity (MFI) (n=3 mice/group). (c, d) Serum concentration of active TGF- β 1 (c) and total TGF- β 1 (d) in WT and

RESULTS

pKO mice at baseline (n=6 mice/group). **(e, f)** Human cardiac fibroblasts (HCFs) were incubated for 30 minutes with serum from WT or pKO mice and Smad3 phosphorylation in HCFs was assessed by Western blot (n=6 mice/group). **(g, h)** HCFs were incubated for 30 minutes with platelet releasates from WT or pKO mice, whether activated or not by CRP, and Smad3 phosphorylation was assessed by Western blot (n=3 mice/group). Dot plots represent data from individual mice, as well as mean $\pm$ SEM. Statistical significance was determined by unpaired t-test **(c, d, and f)** or two-way ANOVA followed by Sidak's multiple comparisons test **(a, b, and h)**. *p<0.05 compared to the non-stimulated condition. #p<0.05 compared to the WT condition.

4.3 Absence of platelet GARP does not alter infarct size and platelet infiltration after MI

To investigate the structural and functional consequences associated with platelet-specific GARP invalidation in the infarcted heart, pKO mice and their WT controls underwent permanent ligation of the LAD coronary artery. Infarct size in WT and pKO mice was assessed by echocardiographic evaluation of regional LV wall motion at 3 days post-MI. The extent of infarcted myocardium did not differ between WT and pKO mice (Fig. 3a). Consistently, plasma troponin I levels at 1 day post-MI were not different between both genotypes (Fig. 3b), indicating that platelet-specific GARP deficiency does not influence cardiomyocyte death. We next examined the impact of platelet GARP deficiency on platelet infiltration within the infarcted myocardium by immunohistochemistry using anti-CD41 antibody, at 1 and 3 days after surgery. As previously observed [246, 247], platelets infiltrated the infarcted heart as early as at 1 day post-MI and remained present at 3 days (Fig. 3c-e). Importantly, platelet accumulation was similar between WT and pKO infarcted hearts at both time points (Fig. 3c-e).

RESULTS

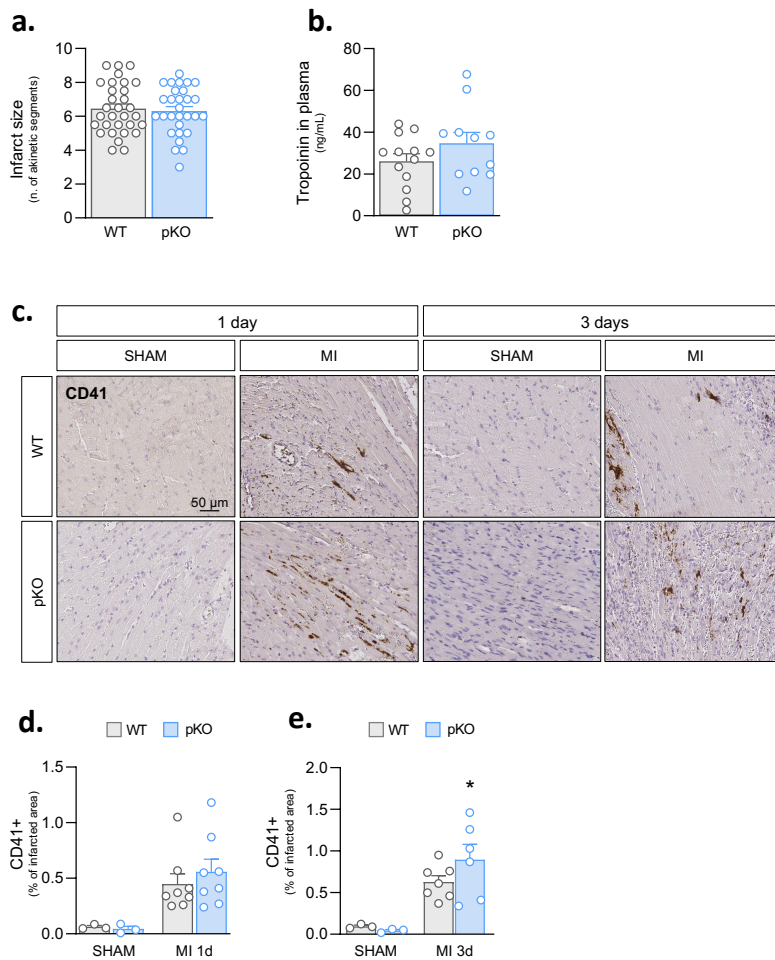


Figure 3. Absence of platelet GARP does not alter infarct size and platelet infiltration after MI. **(a)** Echocardiographic evaluation of infarct size at 3 days after MI (n=29-31 mice/group). **(b)** ELISA measurement of plasma troponin I in WT and pKO mice, at 1 day post-MI (n=11-13 mice/group). **(c)** Representative images of CD41 staining (platelets) in the heart of WT and pKO mice, at 1 and 3 days post-MI. Scale bar = 50µm. **(d, e)** Quantification of CD41 positive area in the infarct of WT or pKO mice, at 1 day **(d)** and 3 days **(e)** post-MI (n=3–8 mice/group). Dot plots represent data from individual mice, as well as mean±SEM. Statistical significance was determined by unpaired t-test **(a, b)** or two-way ANOVA followed by Sidak's multiple comparisons test **(d, e)**. *p<0.05 compared to the corresponding SHAM.

4.4 Platelet deletion of GARP increases early LV rupture and adverse remodelling

Although peri-operative mortality (within 24 hours after surgery) was similar between both groups (WT: 13.11% vs pKO: 15.07%, n=122/126), we observed an increased mortality among pKO mice within the first week following MI, compared to WT littermates (Fig. 4a). Necropsy post-MI revealed a greater incidence of fatal cardiac rupture of the LV free wall in pKO animals (WT: 11.11% vs pKO: 54.55%, n=9/11), characterized by visible slit-like myocardial rupture and blood-filled chest cavity (Fig. 4b and 4c). Baseline echocardiographic analysis demonstrated similar LV function and dimensions between both genotypes (Supplementary Table S3). However, pKO mice surviving until 14 days after MI exhibited a substantial increase in LV end-diastolic (LVEDV) (Fig. 4f) and systolic (LVESV) (Fig. 4g) volumes compared to WT littermates, while there was a trend towards increased diastolic (Fig. 4h) and systolic (Fig. 4i) LV internal dimensions, and reduced LV ejection fraction (EF) (Fig. 4j) and fractional shortening (FS) (Fig. 4k) in pKO mice. Additionally, interventricular septum and posterior wall thickness remained similar between genotypes (Table 1), while LV mass normalized to tibia length showed no significant difference between WT and pKO mice (Table 1). In line with these results, histological analyses of cardiomyocyte cross-sectional area in the non-infarcted myocardium revealed no disparity between WT and pKO mice (Fig. 4l and 4m).

RESULTS

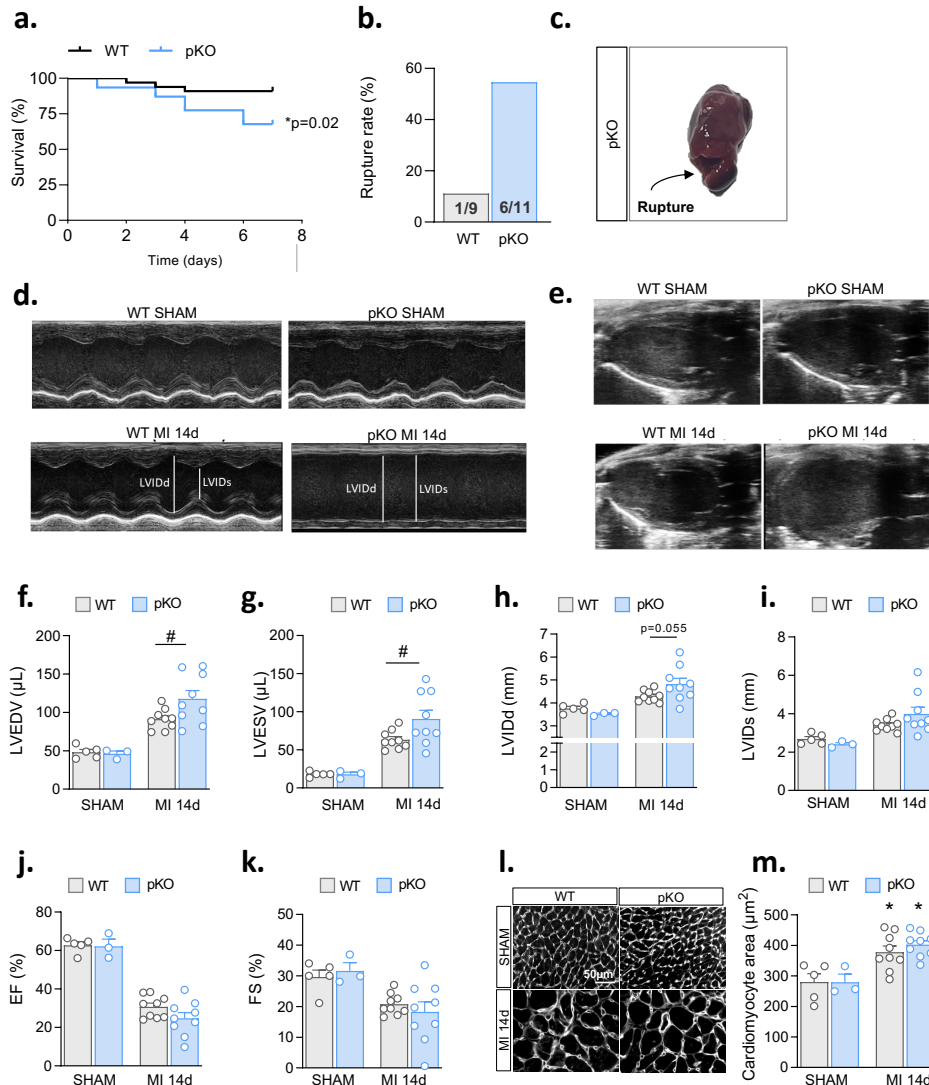


Figure 4. Platelet deletion of GARP increases early LV rupture and adverse remodelling. (a) Kaplan-Meier survival curves of WT and pKO mice until 7 days after MI. Differences between groups were determined by the log-rank test, $p=0.03$ ($n=33-31$ /group) **(b)** Incidence of LV wall rupture in WT and pKO mice. **(c)** Representative image of LV rupture in pKO mice. **(d, e)** Representative images of M-mode **(d)** and B-mode **(e)**. **(f-k)** Echocardiographic analyses. Left ventricular end diastolic volume (LVED) **(f)**, left ventricular end systolic volume (LVESV) **(g)**, left ventricular internal diameter in diastole **(h)** and systole **(i)**, ejection fraction (EF) **(j)** and fractional shortening (FS) **(k)** of WT and pKO mice measured by transthoracic echocardiography, at 14 days post-MI. ($n=3-9$ mice/group). **(l)** Representative images of wheat-germ agglutinin (WGA) in the remote area of pKO and WT mice, at 14 days post MI. Scale=50 μ m. **(m)** Quantification of

RESULTS

cardiomyocyte hypertrophy in the remote area of WT and pKO mice, at 14 days post-MI (n=3-9 mice/group). Dot plots represent data from individual mouse hearts as well as mean±SEM. Statistical significance was determined by two-way ANOVA followed by Sidak's comparisons test. #p<0.05 compared to the WT condition.

Table 1 Echocardiographic measurements of pKO and WT mice at 14 days after MI

Variable	SHAM 14 days		MI 14 days	
	WT (n=5)	pKO (n=3)	WT (n=9)	pKO (n=9)
M-mode				
End-diastolic interventricular septal thickness (mm)	0.80 ± 0.03	0.81 ± 0.03	0.91 ± 0.03	0.93 ± 0.06
End-systolic interventricular septal thickness (mm)	1.18 ± 0.05	1.11 ± 0.05	1.16 ± 0.04	1.13 ± 0.09
End-diastolic left ventricular internal diameter (mm)	3.76 ± 0.09	3.52 ± 0.04	4.29 ± 0.08**	4.83 ± 0.25**
End-systolic left ventricular internal diameter (mm)	2.69 ± 0.12	2.40 ± 0.09	3.41 ± 0.11**	3.99 ± 0.35**
End-diastolic left ventricular posterior wall thickness (mm)	0.61 ± 0.01	0.61 ± 0.04	0.77 ± 0.04**	0.74 ± 0.03**
End-systolic left ventricular posterior wall thickness (mm)	0.90 ± 0.09	0.96 ± 0.04	1.03 ± 0.04	0.97 ± 0.08
Fractional shortening (%)	29.59 ± 2.26	31.64 ± 2.69	20.76 ± 1.15**	18.74 ± 3.30**
2D parasternal long axis				
Left ventricular end-diastolic volume (μL)	48.20 ± 3.61	46.13 ± 3.56	91.19 ± 4.44****	117.6 ± 10.79****
Left ventricular end-systolic volume (μL)	18.05 ± 1.51	17.83 ± 2.89	63.31 ± 4.05****	90.61 ± 11.42****
Ejection fraction (%)	62.74 ± 1.77	62.22 ± 3.71	30.73 ± 1.93****	24.75 ± 3.01****
LV mass/tibia length (mg/mm)	4.81 ± 0.25	4.50 ± 0.43	5.63 ± 0.26*	5.75 ± 0.28*
Heart rate (bpm)	461.60 ± 14.17	464.70 ± 26.43	456.00 ± 11.26	454.40 ± 6.49

Table 1. Echocardiographic measurements of WT and pKO mice at 14 days post-MI. Cardiac function was quantified by transthoracic echocardiography 14 days after myocardial infarction (MI) or sham surgery. Data are reported as mean±SEM. Statistical significance was determined by two-way ANOVA followed by Sidak's multiple comparisons test.

4.5 Platelet GARP deficiency increases cardiac inflammatory response after MI

To elucidate potential pathogenic mechanisms associated with cardiac rupture and LV dilatation in pKO mice, we performed RNA-seq analysis on infarcted LV tissue extracts from eight WT and six pKO mice, at 1 day post-MI. Our transcriptomic analysis revealed a significant upregulation of the hallmark inflammatory response in pKO infarcts compared to WT (Fig. 5a and 5b). Additionally, pathway analysis with the KEGG Pathway Database demonstrated enrichment of genes involved in the inflammatory response, leukocyte transendothelial migration, and cell adhesion (Fig. 5c). We validated the profiling data by RT-qPCR for E-selectin, VCAM, IL-6, and IL-1β, and confirmed significant upregulation in pKO infarcted hearts (Fig.

RESULTS

5d-g). This supports the notion that endothelial activation and inflammation are heightened in the absence of platelet GARP-dependent TGF- β production. Given the known actions of TGF- β on the endothelium, which include reducing leukocyte adhesion and inhibiting neutrophil migration across the endothelial layer, we evaluated neutrophil and macrophage infiltration into the infarcted myocardium at 1 day and 3 days post-MI. As expected, sham-operated WT and pKO mice displayed minimal or no Ly6G-positive neutrophils and F4/80 macrophages in the infarcted myocardium, while substantial Ly6G and F4/80 positive staining was observed at 1 and 3 days post-MI injury (Fig. 5h-j and 5l-n). Notably, there was a marked and significant increase in neutrophil infiltration in the infarcted area of pKO mice, compared to WT, at 3 days post-MI (Fig. 5h and 5j). This finding was further supported by the upregulation of MMP8 gene expression, a major component of neutrophil granules, in the pKO infarcted hearts at 3 days post-MI compared to WT (Fig. 5k). Additionally, at 3 days, there was also a significant increase in macrophage F4/80 infiltration in the LV of pKO mice compared to WT (Fig. 5l and 5n). Finally, we utilized RNA-seq data with the seq-immuCC bioinformatics software to define signature gene sets for differential immune cells types and identify them based on the enrichment scores of the signature genes. This analysis corroborates our histological observations by revealing increased neutrophil and monocyte content in pKO mice at 1 day post-MI (Supplementary Fig. S1a). In summary, our results suggest that platelet-specific invalidation of GARP leads to heightened endothelial cell activation and increased expression of pro-inflammatory cytokines. These factors may contribute to enhanced neutrophil and monocyte recruitment and infiltration into the infarcted heart, escalating the inflammatory response following MI

RESULTS

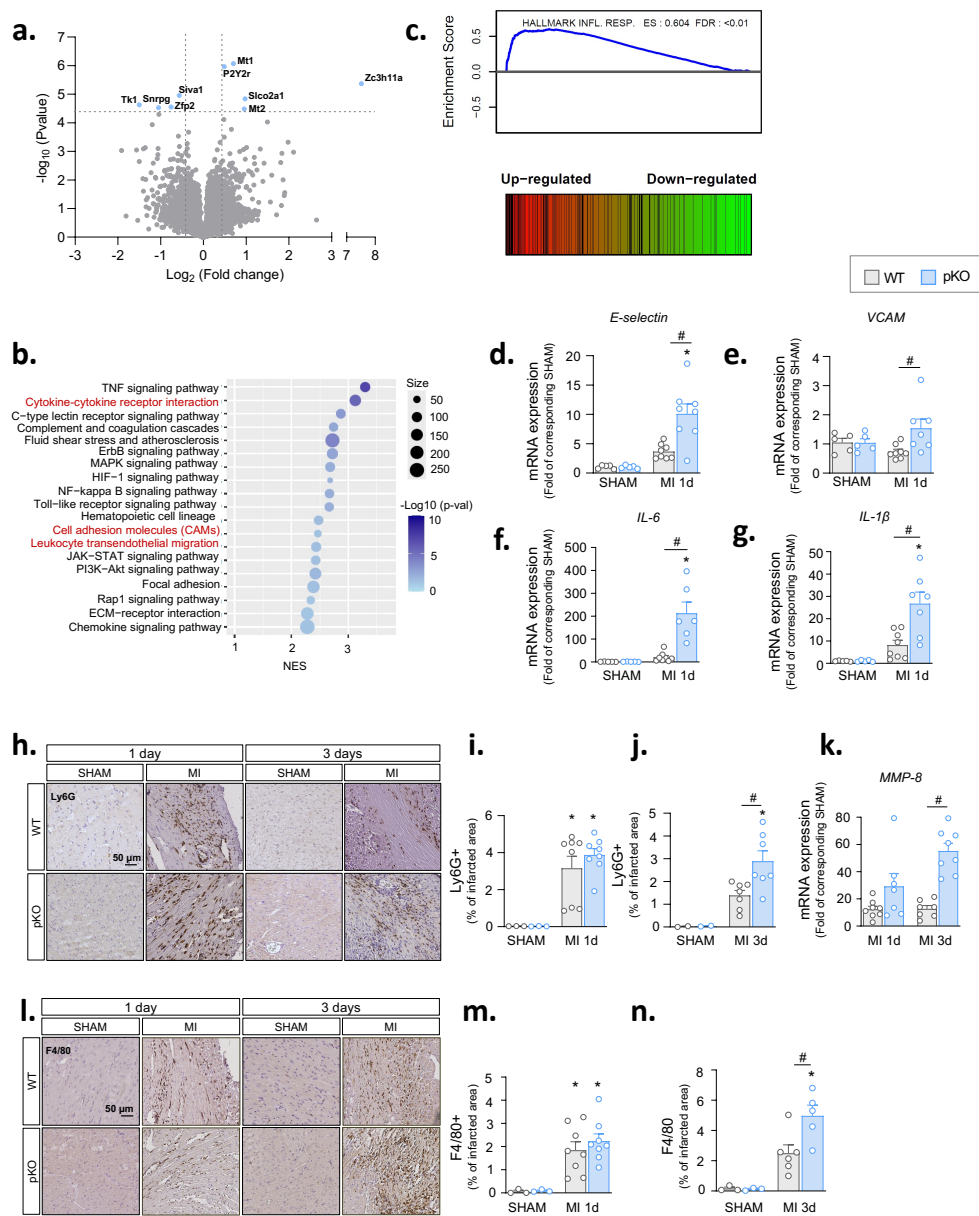


Figure 5. Platelet GARP deficiency increases cardiac inflammatory response after MI. (a-c) RNA sequencing analysis in the infarcted myocardium of WT and pKO mice, at 1 day post-MI. **(a)** GSEA hallmark for the inflammatory response. **(b)** Volcano plot representation of differentially expressed genes. Blue dots represent inflammatory genes. **(c)** KEGG pathway enrichment analysis shows significant differential regulation of biological processes and pathways involved in inflammation. The bubble size indicates the number of genes. The color bar indicates the

adjusted p-value. **(d-g)** Quantitative RT-PCR measurement of E-Selectin **(d)**, VCAM **(e)**, IL-6 **(f)** and IL-1 β **(g)** in the infarcted myocardium of WT and pKO mice, at 1-day post-MI (n=4-8 mice/group). **(h)** Representative images of Ly6G staining (neutrophils) in the heart of WT and pKO mice, at 1 and 3 days post-MI. Scale bar = 50 μ m. **(i and j)** Quantification of Ly6G positive area in the infarcts of WT and pKO mice, at 1 day **(i)** and 3 days **(j)** post-MI. (n=3-8 mice/group). **(k)** Quantitative RT-PCR measurement of MMP-8 in the infarcted myocardium of WT and pKO mice, at 1 day and 3 days post-MI (n=7-8 mice/group). **(l)** Representative images of F4/80 staining (macrophages) in the heart of WT and pKO mice, 1 and 3 days post-MI. Scale bar = 50 μ m. **(m-n)** Quantification of F4/80 positive area in the infarcts of WT and pKO mice, at 1 day **(m)** and 3 days **(n)** post-MI. (n=3-8 mice/group). Dot plots represent data from individual mouse hearts as well as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA followed by Sidak's multiple comparisons test. *p<0.05 compared to the corresponding SHAM. #p<0.05 Compared to the WT condition.

4.6 Impaired inflammatory resolution and healing response in pKO mice after MI.

We hypothesized that the observed increase of neutrophils and macrophages within the infarcted area could result from enhanced recruitment, and/or prolonged accumulation due to reduced clearance. Given the crucial role of neutrophil clearance in inflammation resolution, we next examined whether mRNA expression levels of genes associated with the transition to anti-inflammatory and reparative phases in WT and pKO infarcted hearts. Our analysis revealed significant reduced expression of IL-10 (Fig. 6a) and TGF- β 1 (Fig. 6b) in pKO infarcts at 3 days post-MI. Additionally, there was a notable decline in MMP-2 (Fig. 6c), and MMP-14 (Fig. 6d), two key proteases essential for the reparative process [310]. As alterations in the reparative phase can impact the fibrogenic response, we assessed the expression levels of collagen I, collagen III, and periostin, a marker of myofibroblasts that serves as a primary source of ECM protein synthesis in the infarcted myocardium. Expression levels of these fibrotic markers were reduced in pKO infarcts compared to WT, at both 3 and 7 days post-MI (Fig. 6e-g). To next explore CF proliferation, we used fluorescent immunohistochemistry to label proliferating cell nuclear antigen (PCNA) and vimentin, a marker for interstitial cells that

RESULTS

demarcates the infarcted area (Fig. 6h and 6i). At 7 days post-MI, immunohistochemistry indicated a trend toward decreased proliferation in pKO infarcts, as compared to WT hearts (Fig. 6h and 6i). In addition, staining for α SMA (Fig. 6j and 6k) confirmed that myofibroblasts were less abundant in the pKO infarct area at 7 days post-MI, although the difference between genotypes was not significant (Fig. 6j and 6k). This impaired fibrogenic process ultimately led to thinning of the infarcted area (Fig. 6l) and a larger scar circumference (Fig. 6m), as evidenced by picrosirius red staining of pKO infarcts compared to WT (Fig. 6n). In conclusion, our data collectively indicate impaired anti-inflammatory and healing responses following MI in pKO mice, compared to their WT littermates.

RESULTS

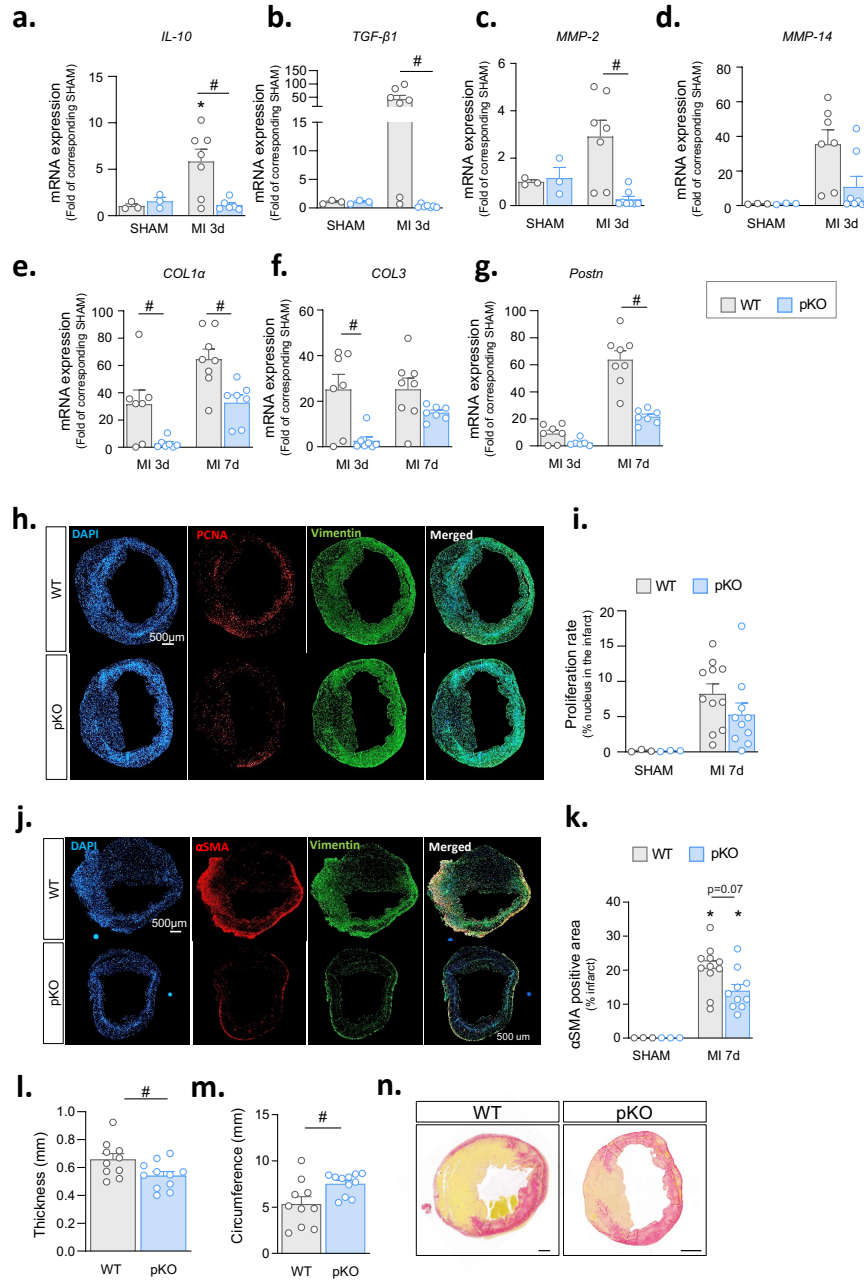


Figure 6. Impaired inflammation resolution and healing response in pKO mice after MI. (a-e) Quantitative RT-PCR measurement of IL-10 (a), TGF- β 1 (b), MMP-2 (c), and MMP-14 (d) in the infarcted myocardium of WT and pKO mice, at 3 days post-MI (n=3-7 mice/group). (e-g) Quantitative RT-PCR measurement of collagen I (COL1 α) (e), collagen III (COL3 α) (f), and periostin (Postn) (g), in the infarcted myocardium of WT and pKO mice, at 3 and 7 days post-MI

RESULTS

(n=3-8 mice/group). **(h)** Representative images of co-immunofluorescent staining of proliferative cardiac fibroblasts (PCNA-positive: red fluorescence; vimentin: green fluorescence) in the heart of WT and pKO mice, at 7 days post-MI. Scale bar=500 μ m. **(i)** Quantification of PCNA positive cardiac fibroblasts in the infarcted myocardium of WT and pKO mice, at 7 days post-MI (n=3-7 mice/group). **(j)** Representative images of co-immunofluorescent staining of myofibroblasts (α SMA-positive: red fluorescence; vimentin-positive: green fluorescence) in the heart of WT and pKO mice, at 7 days post-MI. Scale bar=500 μ m. **(k)** Quantification of α SMA positive cardiac fibroblasts in the infarcted myocardium of WT and pKO mice, at 7 days. Scar thickness **(l-n)** and scar circumference **(m-n)** in WT and pKO mice, at 7 days post-MI. The scar was measured from heart sections stained with picosirius red (n=11-12 mice/group). **(n)** Representative images of picosirius red staining of WT and pKO infarcted hearts, at 7 days post-MI. Dot plots represent data from individual mouse hearts as well as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA followed by Sidak's multiple comparisons test. *p<0.05 compared to the corresponding SHAM. #p<0.05 compared to the WT condition.

5. SUPPLEMENTAL FIGURES AND MATERIALS

Supplementary Table S1. List of primer sequences.

Gene	Direction	Sequence
RPL32	Forward	GGCACCAGTCAGACCGATAT
	Reverse	CAGGATCTGGCCCTTGAAC
IL-1 β	Forward	GCCACCTTTTGACAGTGATGAG
	Reverse	GACAGCCCAGGTCAAAGGTT
IL6	Forward	AAGAGTTGTGCAATGGCAATTCT
	Reverse	AAATTTTCAATAGGCAAATTCCTGAT
TGF- β 1	Forward	TTGCTTCAGCTCCACAGAGA
	Reverse	TGGTTGTAGAGGGCAAGGAC
MMP2	Forward	GTCGCCCCTAAAACAGACAA
	Reverse	GGTCTCGATGGTGTTCTGGT
MMP8	Forward	ACGTGTCCTGCCCAGACTTT
	Reverse	GCATTACGAACCAGGGACG
MMP13	Forward	GATGGCACTGCTGACATCAT
	Reverse	CCCACCATAGTTTGGTCCAG
MMP14	Forward	CCGCCATGCAAAAGTTCTAT
	Reverse	GCCACCTTAGGGGTGTAAT
Col1 α	Forward	AGACGTGGAAACCCGAGGTA
	Reverse	TGGGTCCCTCGACTCCTA
Col3	Forward	GTCCACGAGGTGACAAAGGT
	Reverse	GATGCCCACTTGTTCCATCT
Post	Forward	ATGTTTATGGCACGCTGGGC
	Reverse	CAGGTTCTCCCAAGCCTCGT
α -SMA	Forward	CTGACAGAGGCACCACTGAA
	Reverse	CATCTCCAGAGTCCAGCACA
E-selectin	Forward	AGAAAGCAAAGAAATTTGTTCTGC
	Reverse	CCCACGATGCATTTGTGTTCTT
VCAM	Forward	TGTGAAGATGGTCGCGGTCT
	Reverse	GGGCCATGGAGTCACCGATT

Supplementary Table S2. Hematological parameters of WT and pKO mice at baseline.

Table S2 Hematological parameters in WT and pKO mice at baseline

	pGARP WT (n=7) (mean $\pm$ SEM)	pGARP KO (n=6) (mean $\pm$ SEM)	
WBC ($\times 10^3$ cells/ μ L)	3.68 $\pm$ 0.31	5.08 $\pm$ 1.06	n.s.
Lym. ($\times 10^3$ cells/ μ L)	86.00 $\pm$ 1.09	86.80 $\pm$ 0.76	n.s.
Mid. ($\times 10^3$ cells/ μ L)	8.47 $\pm$ 1.00	8.00 $\pm$ 0.76	n.s.
Gra. ($\times 10^3$ cells/ μ L)	5.53 $\pm$ 0.10	5.20 $\pm$ 0.60	n.s.
RBC ($\times 10^3$ cells/ μ L)	7.01 $\pm$ 0.17	7.22 $\pm$ 0.13	n.s.
HGB (g/L)	11.23 $\pm$ 0.32	11.65 $\pm$ 0.21	n.s.
HCT (%)	38.15 $\pm$ 0.64	39.55 $\pm$ 1.13	n.s.
MCV (fL)	54.52 $\pm$ 0.82	54.78 $\pm$ 1.14	n.s.
Platelet ($\times 10^3$ cells/ μ L)	674.70 $\pm$ 21.30	672.70 $\pm$ 14.10	n.s.
MPV (fL)	5.03 $\pm$ 0.07	5.55 $\pm$ 0.15	**p<0.01

Supplementary Table 2. Hematological parameters of WT and pKO mice at baseline.

Hematological parameters were analysed with Cell-Dyn Emerald. Data are reported as mean $\pm$ SEM. Statistical significance was determined by unpaired t-test. WBC, White blood cells; Lym, Lymphocytes; Mid, Mid cells; Gra, Granulocytes; RBC, Red blood cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean cell volume; MPV, Mean platelet volume.

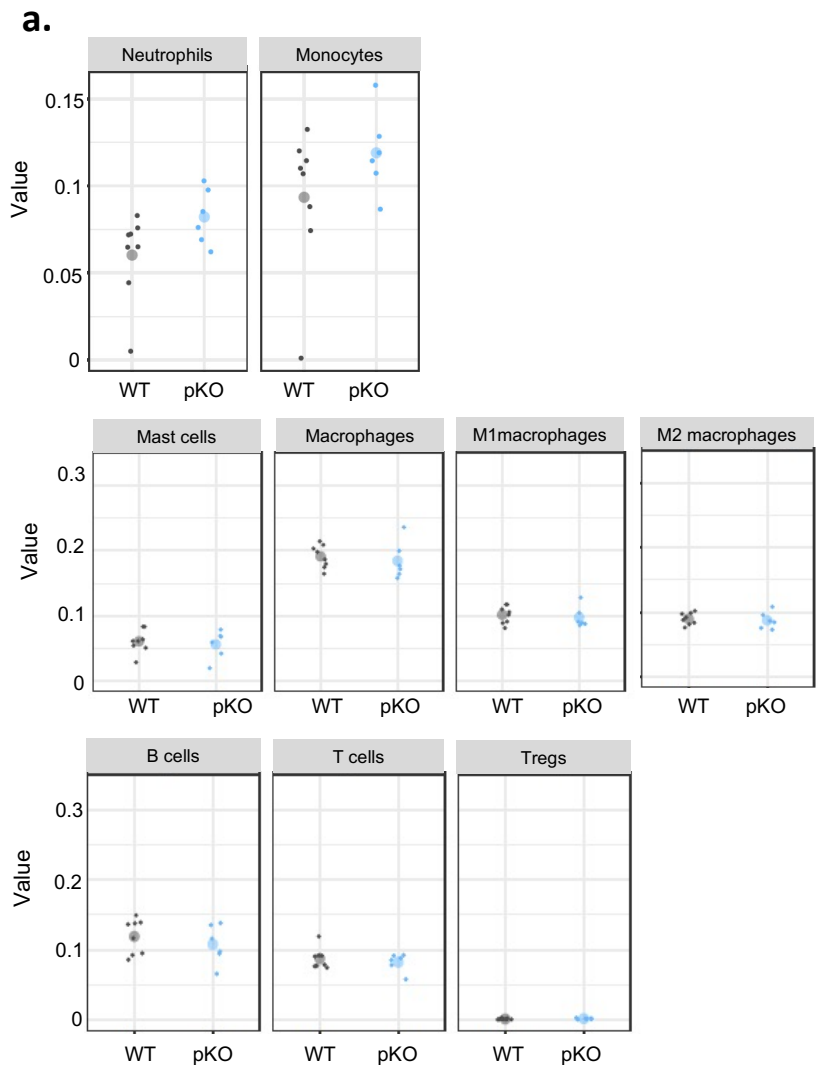
Supplementary Table S3. Echocardiographic measurements of pKO and WT mice at baseline.

Table S3 Echocardiographic measurements of pKO and WT mice at baseline

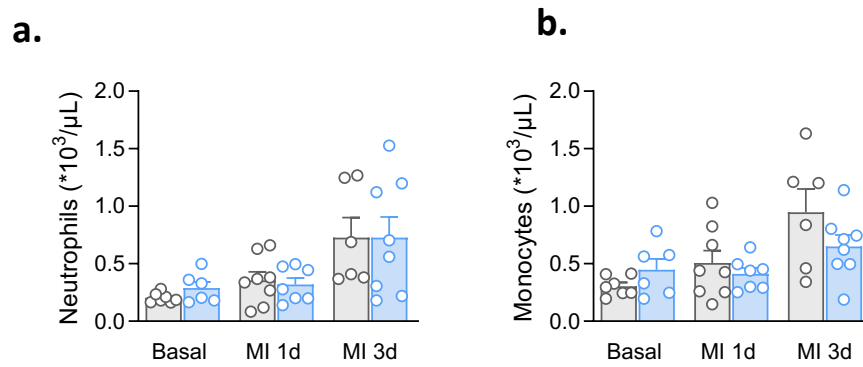
Variable	Baseline 14 days	
	WT (<i>n</i> =5)	
M-mode		
End-diastolic interventricular septal thickness (mm)	0.71 ± 0.02	0.78 ± 0.02
End-systolic interventricular septal thickness (mm)	1.03 ± 0.05	1.11 ± 0.02
End-diastolic left ventricular internal diameter (mm)	3.49 ± 0.10	3.64 ± 0.09
End-systolic left ventricular internal diameter (mm)	2.53 ± 0.12	2.57 ± 0.10
End-diastolic left ventricular posterior wall thickness (mm)	0.61 ± 0.02	0.68 ± 0.02
End-systolic left ventricular posterior wall thickness (mm)	0.91 ± 0.03	0.97 ± 0.02
Fractional shortening (%)	30.50 ± 3.43	30.06 ± 1.96
2D parasternal long axis		
Left ventricular end-diastolic volume (μL)	50.95 ± 3.77	50.43 ± 4.34
Left ventricular end-systolic volume (μL)	21.03 ± 2.24	20.68 ± 2.86
Ejection fraction (%)	51.63 ± 2.40	60.31 ± 3.20
LV mass/tibia length (mg/mm)	4.66 ± 0.17	4.64 ± 0.71
Heart rate (bpm)	459.30 ± 9.99	475.30 ± 10.68

Supplementary Table 3. Echocardiographic measurements of WT and pKO mice at baseline. Cardiac function was quantified by transthoracic echocardiography 2 to 3 days before the surgery. Data are reported as mean±SEM. Statistical significance was determined by unpaired t-test.

Supplementary Fig. S1.



Supplementary figure S1. Immune cell content at 1 day post-MI in WT and pKO mice using the seq-ImmuCC webtool. (a) The immune cell subpopulations were characterized by seq-ImmuCC.

Supplementary Fig. S2.

Supplementary figure S2. Neutrophil and monocyte blood count in WT and pKO mice at 1 and 3 days after MI. (a) Blood count of neutrophils and (b) monocytes in WT and pKO mice were measured with Cell-Dyn Emerald at indicated time points after MI (n=6-8 mice/group). Data are reported as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA followed by Sidak's multiple comparisons test.

DISCUSSION AND PERSPECTIVES

DISCUSSION AND PERSPECTIVES

DISCUSSION AND PERSPECTIVES

Myocardial infarction begins with disrupted blood flow to a part of the heart, leading to ischemic injury and subsequent tissue damage. Yet, its consequences extend beyond the initial event, involving a cascade of responses that determine survival, cardiac remodelling, and functional recovery. By unraveling these mechanisms, researchers have identified key processes such as inflammation and fibrosis, which shape post-infarction remodelling. Additionally, interactions among different cardiac cell types, including cardiomyocytes, fibroblasts, endothelial cells, immune cells, and platelets reveal the intricate cellular network governing cardiac repair.

In this thesis, we elucidate for the first time the role of platelet GARP in post-MI responses using a mouse genetic model. Our results identify the platelet GARP-TGF- β 1 pathway as a key regulator of cardiac inflammation and healing after MI. Platelet GARP-deficient mice presented significantly increased mortality and cardiac rupture rates. Surviving mice also showed LV dilatation after chronic MI. These phenotypes were associated with an exacerbated inflammatory response and delayed resolution of inflammation. These findings are in line with experimental and clinical observations demonstrating the importance of temporal and spatial containment of the early inflammatory response for organized wound healing and prevention of LV rupture or adverse remodelling. Furthermore, they support the somewhat counterintuitive notion that platelets may exert protective effects in the MI pathogenesis by activating specific mechanisms that likely contribute to improved outcomes in MI resolution.

1. Gplb α -Cre or PF4-Cre model?

We chose to utilize the Gplb α gene, which is predominantly expressed in MK and platelets, to generate a deleter mouse for selective targeting of GARP in these cell types [313]. While the platelet factor 4 (PF4)-Cre mouse is commonly used to create MK/platelet-targeted conditional KO models, it has limitations. One major drawback is its ectopic expression of Cre in leukocytes due to PF4 expression in other hematopoietic cells [320, 321]. This unintended Cre expression can lead to off-target gene ablation in leukocytes and macrophages. In contrast, the Gplb α -Cre mouse model we employed has the Cre insert integrated into the native Gplb α gene, ensuring Cre expression is limited to the MK lineage. This Cre expression, driven by the Gplb α locus, likely explains the slight increase in MPV observed in pKO mice compared to WT mice [313]. Importantly, this alteration does not compromise platelet function, which remains intact in both genotypes.

2. Animal models of permanent coronary artery occlusion or ischemia-reperfusion injury?

Numerous studies have explored the role of platelets in post-MI responses, predominantly using cardiac ischemia/reperfusion (I/R) models [247, 322-325]. These studies often emphasize the detrimental impact of platelets, exacerbating cardiac injury and dysfunction through increased inflammation, secretion of vasoconstrictors, or microthrombi formation in the vasculature [262, 263, 326, 327]. To minimize the confounding effects of vascular thrombosis, we chose a mouse model of MI with permanent coronary artery occlusion over an I/R model. While I/R may be considered more representative of human MI, it is crucial to note that approximately 30% of acute MI patients in clinical practice do not undergo reperfusion due to factors such as delayed presentation, diagnosis, or lack of

success in reperfusion [328]. Moreover, among patients receiving reperfusion therapy, up to 50% can experience the 'no-reflow' phenomenon, characterized by myocardial hypoperfusion despite a patent epicardial coronary artery [329, 330]. The permanent occlusion model we employed mimics the phenotype of those non-reperfused or 'no-reflow' patients, while also generating more consistent and larger infarct sizes, which facilitates the study of early tissue injury and repair responses [331]. Considering the significant modulation of both the dynamics and magnitude of the inflammatory response by the presence or absence of reperfusion, it would be interesting to explore the role of platelet GARP in the I/R model to validate our findings.

3. Platelets in acute myocardial infarction: the good or the bad?

Large myocardial infarctions with extensive tissue damage are frequently associated with an elevated acute inflammatory response, preceding a rise in systemic markers of inflammation such as C-reactive protein. The peak level of C-reactive protein correlates with post-MI complications, including cardiac rupture, mortality, LV remodelling and increased risk of heart failure. Several small-scale trials have been conducted to address this early burst of excessive inflammation, with a focus on pathways related to the IL-1 and IL-6 cytokine families [17, 92]. However, despite encouraging findings, these studies have yet to yield clinical success.

The permanent occlusion model has been previously used to investigate the role of platelets in post-MI inflammation, LV rupture pathogenesis, and remodelling [15, 246, 277]. These studies highlight the cardiac protection offered by antiplatelet interventions that attenuate inflammatory responses, while the risk of bleeding and hemorrhagic infarction is increased [246, 277]. Interestingly, emerging evidence

suggests that platelet-derived factors may also confer beneficial effects during or after ischemia. For instance, Cheng *et al.* demonstrated improved cardiac function and reduced cardiac remodelling in infarcted rats treated with platelet gel [332]. Furthermore, Walsh and Poole found that stromal cell–derived factor 1 α (SDF-1 α) and TGF- β , released from α -granules of activated platelets, protect cardiomyocytes from cell death during ischemia [227]. Reduced TGF- β 1 expression in platelets has also been linked to increased mortality and incidence of STEMI [333]. Therefore, the overall impact of platelets during the acute phase of post-MI likely represents a delicate balance between their detrimental and beneficial effects, with TGF- β 1 potentially playing a protective role. In this study, we specifically evaluated the role of platelet GARP in TGF- β 1-dependent cellular processes related to post-MI inflammation, repair, and fibrogenesis.

4. Significance of platelet GARP in myocardial infarction injury

A key finding from our study is that platelet GARP deficiency increased mortality and cardiac rupture rates during post-MI recovery. Given that LV rupture often results from severe regional inflammation [334, 335], we aimed to uncover the mechanisms driving the post-MI proinflammatory response in pKO mice. RNA-sequencing analysis revealed that pKO infarcts were marked by pro-inflammatory molecular signatures. These findings were further corroborated by a significant increase in E-selectin and VCAM-1 expression levels, coupled with the enhanced accumulation of neutrophils and monocytes/macrophages within the infarct region of these mice. Enhanced neutrophil accumulation was further supported by elevated MMP-8 expression, primarily produced by these cells, and released from granules upon inflammatory activation [64]. Collectively, the presence of neutrophils and macrophages, in addition to removing cell debris, contributes to collateral cardiac injury through the release of pro-inflammatory cytokines and

DISCUSSION AND PERSPECTIVES

chemokines, reactive oxygen species (ROS), MMPs, or extracellular traps [336, 337]. Our study is the first to link platelet GARP to inflammation and cardiac rupture after MI, which is in line with previous reports showing a close relationship between elevated levels of these inflammatory cells, incidences of LV ruptures, and exacerbated thinning of the infarct wall [66, 338, 339]. We postulate that the platelet GARP-TGF- β pathway protects against LV rupture by mitigating leukocyte adhesion and transendothelial migration within the infarcted tissue, as well as by decreasing cytokine and chemokine production by endothelial cells. However, we cannot exclude the possibility that endothelial activation and leukocyte infiltration result from heightened cytokine production by other cardiac cells, notably mast cells, pivotal in initiating the inflammatory response. Determining whether platelet GARP can influence mast cell degranulation via TGF- β production and activation requires further investigation.

Interestingly, the role of platelet GARP in modulating TGF- β -dependent inflammation has previously been explored in the context of cancer. Metelli *et al.* reported that reducing GARP-dependent production of TGF- β from platelets blocked its deposition in the tumor bed and the stroma of colon carcinoma, leading to an increase in CD8 T cells, natural killer cells, and neutrophils within the tumor microenvironment [340]. In this context, the maturation of TGF- β 1 from the platelet GARP-TGF- β 1 complex appears to involve the proteolytic cleavage of GARP by thrombin at the plasma membrane. Our study extends these findings, revealing that active TGF- β 1 can be produced from platelets activated with CRP. Additionally, in Tregs, TGF- β 1 production occurs from GARP-TGF- β 1 complexes in a manner dependent on the α V β 8 integrin [281, 282]. We acknowledge that other proteases and/or integrins may also be involved in TGF- β 1 activation by platelet GARP in the context of MI.

DISCUSSION AND PERSPECTIVES

Finally, it is essential to note that the heightened and/or prolonged presence of neutrophils in the infarcted hearts of pKO mice can not only result from increased infiltration but also from impaired clearance. The sustained high inflammatory signal and the compromised clearance of apoptotic neutrophils by macrophages through efferocytosis can independently affect the repair phase after MI, leading to tissue damage and chronic inflammation [66, 338, 339]. To assess inflammation resolution, we measured the expression levels of pro-repair mediators IL-10 and TGF- β 1, finding them significantly reduced in pKO infarcts compared to WT. It is recognized that circulating platelets contribute to the anti-inflammatory polarization of macrophages by releasing pro-resolution mediators like lipotoxin A4, lipolytic E1, and annexin A1, which facilitate inflammation regression [227, 341, 342]. Investigating whether platelet GARP deficiency influences the secretion of these resolving mediators could provide valuable insights. Additionally, macrophage MMPs also play a crucial role in resolution regulation. Inhibition or reduced activity of MMP-2 exacerbates and prolongs inflammation, affecting not only the injured heart but also non-cardiac organs such as the liver and adipose tissue [343]. This exacerbation results notably from an increased bioavailability of pro-inflammatory cytokines typically cleaved and inactivated by MMP-2. Furthermore, macrophages deficient in MMP-14 exhibit elevated pro-inflammatory gene expression, leading to an overly aggressive inflammatory response in acute inflammation models [344, 345]. In our study, the significant decrease in MMP-2 and MMP-14 expression levels, along with reduced IL-10 and TGF- β 1, indicates a disrupted transition towards the post-MI reparative phase in pKO infarcted hearts. This is logically associated with impaired collagens and periostin expression, likely due to a reduced accumulation of myofibroblasts. Collectively, these alterations

contribute to subsequent LV thinning, frequent cardiac ruptures, and poor prognosis observed in platelet GARP-deficient mice.

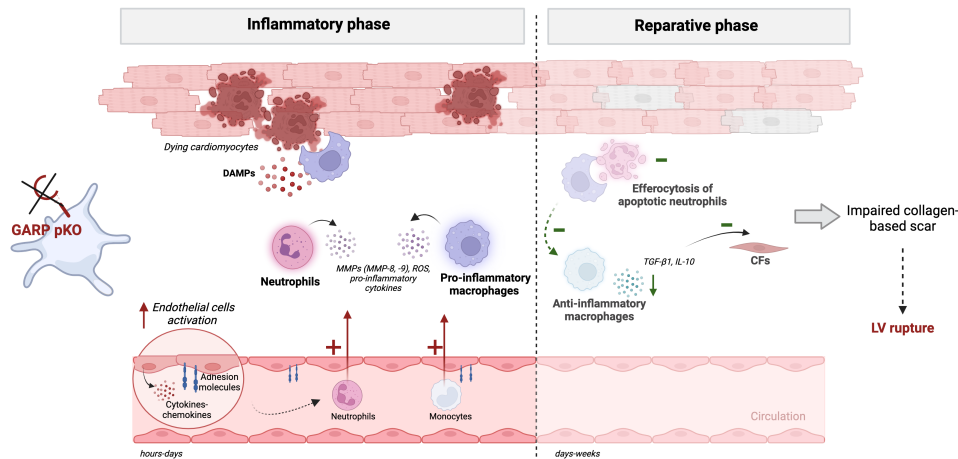


Figure 12. Cardiac response post-MI in WT mice versus platelet GARP KO mice. The absence of platelet GARP results in an exacerbated inflammatory response associated with increased endothelial cells activation, and markedly increased levels of $\text{TNF-}\alpha$, IL-6, IL-1 β , and MMP-8, as well as higher neutrophil and macrophage accumulation. This unmitigated inflammation results in a more severe degree of adverse LV remodelling, leading to a higher risk of cardiac rupture. This study highlights the crucial role of GARP in modulating inflammatory and reparative processes following MI.

5. Clinical implications of this study

Our experimental findings are scientifically and clinically important. The identification of platelet GARP as a novel and central player in regulating the early inflammatory response post-MI deepens our understanding of this complex process. Our results support a beneficial role of platelet GARP in shaping the inflammatory landscape after MI, suggesting that therapeutic amplification of this pathway could offer a novel approach for inflammation-targeted therapy following MI. The current challenge lies in determining whether this therapeutic amplification could effectively mitigate the inflammatory cascade triggered by the initial burst of excessive inflammation, while preserving the protective aspects of the healing

process. Moreover, it is crucial to acknowledge that targeting inflammation may also pose the risk of compromising host defense mechanisms and increasing susceptibility to infections. Therefore, further investigation is warranted to fully elucidate the therapeutic potential of this pathway.

Our work also has significant clinical implications, especially regarding the safety of targeting the GARP-TGF- β 1 pathway in cancer immunotherapy. Blocking this pathway could potentially amplify inflammatory responses, thereby increasing the risk of cardiac events in patients with pre-existing cardiovascular conditions or those at high risk for MI. This raises particular concern given the high prevalence of cardiovascular diseases among cancer patients, attributed to both the disease itself and the cardiotoxic effects of certain cancer treatments. Consequently, future research is needed to determine the impact of anti-GARP-TGF- β 1 monoclonal antibodies on cardiac function and integrity.

6. The Future

Despite the interesting discoveries presented in this thesis, numerous questions remain unanswered, highlighting the need for further investigation into the influence of platelet-derived GARP on myocardial healing following LAD permanent ligation.

Currently, the precise mechanism underlying the alteration of the repair process remains elusive. Platelets, by rapidly releasing active TGF- β 1, can affect various cell types within the heart post-MI, including endothelial cells, resident macrophages, mast cells and cardiomyocytes. Therefore, investigating, *in vitro* and *in vivo*, the impact of platelet-derived GARP on these diverse cell populations could yield valuable insight. Moreover, given the critical role of inflammation in post-MI

DISCUSSION AND PERSPECTIVES

healing, a closer look at how platelet GARP influences the resolution and repair phases is of particular interest. This entails studying processes such as neutrophil apoptosis and the switch of macrophages from a pro-inflammatory to an anti-inflammatory phenotype, both critical for halting inflammation and facilitating tissue repair.

Furthermore, our study employed RNA-sequencing to evaluate the effect of platelet GARP deficiency on the inflammatory response post-MI (Fig. 5a). This analysis revealed differential expression of 10 genes, with half down-regulated and half up-regulated ($p < 0.05$) (Table 3). Notably, the up-regulated genes predominantly participate in inflammatory processes.

Genes	Function	Log2(FC)	Adjusted P-val.
P2ry2	Purinergic receptor	0.488	0.00469
Mt1	Metallothionein 1. Homeostatic control of metal in the cell	0.703	0.00469
Slco2a1	Prostaglandin transporter	0.976	0.03159
Mt2	Metallothionein 2. Homeostatic control of metal in the cell	0.954	0.4276
Siva1	Apoptotic pathway	-0.566	0.02876
Snrgp	Small Nuclear Ribonucleoprotein G playing a role in pre-mRNA splicing	-1.050	0.04242
Zfp2	Zinc finger protein involved in regulation of transcription	-0.756	0.04242
Tk1	Thymidine Kinase 1: Cell-cycle-regulated enzyme	-1.499	0.04242

Table 3. RNA-sequencing of WT vs. pKO, 1 day post-MI. Table shows 8 differentially expressed genes (adjusted p -val. <0.05). P2ry2, P2Y2 receptor; MT1, metallothionein 1; Slco2a1, Solute Carrier Organic Anion Transporter Family Member 2A1; Snrgp, Small Nuclear Ribonucleoprotein Polypeptide G; Zfp2, Zinc finger protein 2; Tk1, Thymidine kinase 1; FC, Fold Change.

Of particular interest is the P2Y2 receptor, given its established link to cardiovascular diseases. The P2Y2 receptor, member of the purinergic family of G protein-coupled receptors, responds to extracellular stress signals such as ATP and

DISCUSSION AND PERSPECTIVES

UTP. It is widely expressed in cardiovascular-relevant cells, including endothelial cells, cardiomyocytes, and immune cell types like mast cells, macrophages, and neutrophils [346-349]. Studies have linked increased P2Y2 receptor activity to endothelial dysfunction and a pro-inflammatory state [348, 350, 351], influencing the expression of cell adhesion molecules, and exacerbating inflammation [348, 350, 351]. Additionally, P2Y2 receptors on myeloid cells play a crucial role in acute inflammatory responses [352]. Conversely, P2Y2 receptor deficiency has been associated with reduced inflammatory indicators in conditions like atherosclerosis [348, 350, 351]. These findings underscore the necessity for further research to delineate the impact of platelet GARP deletion on P2Y2 receptor, shedding light on its significance in post-MI inflammation.

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ANNEXES

1. Articles

During my thesis, the unexpected arrival of the COVID-19 pandemic brought many challenges and necessitated a temporary reorientation of my main thesis work. However, this period also presented unique opportunities for scientific contribution and learning. During the pandemic, I was closely involved in two pivotal studies, which were published in 2022 and 2023, respectively:

Dechamps M, De Poortere J, Martin M, Gatto L, Daumerie A, Bouzin C, Octave M, Ginion A, Robaux V, Pirotton L, **Bodart J**, Gerard L, Montiel V, Champion A, Gruson D, Van Dievoet MA, Doux fils J, Haguët H, Morimont L, Derive M, Jolly L, Bertrand L, Dumoutier L. Inflammation-Induced Coagulopathy Substantially Differs Between COVID-19 and Septic Shock: A Prospective Observational Study. *Front. Med*, 2022 January.

Mélanie Dechamps, Julien Depoortere, Marie Octave, Audrey Ginion, Valentine Robaux, Pirotton Laurence, **Julie Bodart**, Damien Gruson, Marie-Astrid Van Dievoet, Jonathan Doux fils, Hélène Haguët, Laure Morimont, Marc Derive, Lucie Jolly, Luc Bertrand, Pierre-François Laterre, Sandrine Horman, Christophe Beauloye. Dexamethasone modulates the cytokines response but not COVID-19-induced coagulopathy in critically ill. *Int J Mol Sci*. 2023 April.

My specific role in these studies focused on measuring platelet activation parameters in patients with COVID-19 as well as in septic patients, which led to a better understanding of the pathophysiology of these diseases. Moreover, this experience highlighted the critical interaction between platelets and immune response, enriching my thesis with a broader perspective on the importance of platelets in inflammation, and their role as an immune cell.



OPEN ACCESS

Edited by:

W. Conrad Liles,
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Specialty section:

This article was submitted to
Intensive Care Medicine and
Anesthesiology,
a section of the journal
Frontiers in Medicine

Received: 21 September 2021

Accepted: 24 December 2021

Published: 17 January 2022

Citation:

Dechamps M, De Poortere J,
Martin M, Gatto L, Daumerie A,
Bouzin C, Octave M, Ginion A,
Robaux V, Pirotton L, Bodart J,
Gerard L, Montiel V, Campion A,
Gruson D, Van Dievoet M-A,
Douxflis J, Haguet H, Morimont L,
Derive M, Jolly L, Bertrand L,
Dumoutier L, Castanares-Zapatero D,
Laterre P-F, Horman S and
Beauloye C (2022)
Inflammation-Induced Coagulopathy
Substantially Differs Between
COVID-19 and Septic Shock: A
Prospective Observational Study.
Front. Med. 8:780750.
doi: 10.3389/fmed.2021.780750

Inflammation-Induced Coagulopathy Substantially Differs Between COVID-19 and Septic Shock: A Prospective Observational Study

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Critical COVID-19, like septic shock, is related to a dysregulated systemic inflammatory reaction and is associated with a high incidence of thrombosis and microthrombosis. Improving the understanding of the underlying pathophysiology of critical COVID-19 could help in finding new therapeutic targets already explored in the treatment of septic shock. The current study prospectively compared 48 patients with septic shock and 22 patients with critical COVID-19 regarding their clinical characteristics and outcomes, as well as key plasmatic soluble biomarkers of inflammation, coagulation, endothelial activation, platelet activation, and NETosis. Forty-eight patients with matched age, gender, and co-morbidities were used as controls. Critical COVID-19 patients exhibited less organ failure but a prolonged ICU length-of-stay due to a prolonged respiratory failure. Inflammatory reaction of critical COVID-19 was distinguished by very high levels of interleukin (IL)-1 β and T lymphocyte activation (including IL-7 and CD40L), whereas septic shock displays higher levels of IL-6, IL-8, and a more significant elevation of myeloid response biomarkers, including Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) and IL-1ra. Subsequent inflammation-induced coagulopathy of COVID-19 also differed from sepsis-induced coagulopathy (SIC) and was characterized by a marked increase in soluble tissue factor (TF) but less platelets, antithrombin, and fibrinogen

consumption, and less fibrinolysis alteration. In conclusion, COVID-19 inflammation-induced coagulopathy substantially differs from SIC. Modulating TF release and activity should be evaluated in critical COVID-19 patients.

Keywords: COVID-19, septic shock, inflammation, coagulopathy, platelet, NETosis, endothelium

INTRODUCTION

The coronavirus disease 2019 (COVID-19) varies from asymptomatic to a severe form with acute respiratory distress syndrome (ARDS) and the pathophysiology of the severe form has yet to be fully elucidated. The variability of host immune responses suggests that dysregulated systemic inflammation, classically called a “cytokine storm,” could contribute to the pathogenesis of severe cases (1, 2). In addition, COVID-19 is associated with a high incidence of arterial and deep venous thrombosis leading to pulmonary embolism (3, 4) and pulmonary microthrombosis (5).

Several mechanisms underlying the pathophysiology of COVID-19 have been proposed. First, ribonucleic acid (RNA) viruses like SARS-CoV-2 usually initiate innate immunity through their detection by pattern recognition receptors (PRRs), triggering subsequent inflammatory and immune responses via the secretion of various cytokines (6). Supporting this hypothesis, critical COVID-19 has been associated with high levels of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), numerous interleukins (ILs), macrophage inflammatory proteins (MIPs), and IFN γ -induced protein 10 (IP-10) (1, 7, 8). Second, internalization of SARS-CoV-2 within endothelial cells after binding to angiotensin-converting enzyme 2 (ACE2) contributes to endotheliitis (9). Both direct injury and activation of the endothelium by dysregulated inflammation may lead to loss of endothelial barrier integrity and to the occurrence of a prothrombotic phenotype (10). Indeed, plasmatic level of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) (11), plasminogen activator inhibitor-1 (PAI-1) (12), tissue factor (TF) (13), and von Willebrand factor (vWF) (4, 14) antigens and activities are all elevated in severe COVID-19 cases. However, a simultaneous increase in tissue plasminogen activator (tPA) or tissue factor pathway inhibitor (TFPI) could mitigate thrombosis formation (15).

Critical COVID-19-associated coagulopathy is characterized by high D-dimers due to increased thrombin generation and increased fibrinolysis (14, 16). In addition, platelets are hyperactivated (8, 17) and cooperate with the endothelium and immune cells to promote pulmonary microthrombosis through neutrophil extracellular trap (NET) formation. Dysregulated NETosis contributes to hypercoagulability and thrombosis (18). Accordingly, NET-containing microthrombi have been detected in COVID-19 pulmonary autopsies, and increased NET formation correlates with COVID-19-related ARDS and disease severity (19). Moreover, the interaction of platelets with monocytes triggers TF expression in severe COVID-19 patients, further exacerbating the hypercoagulable state (20).

Interestingly, sepsis and septic shock are well-known models of cytokine storms and coagulopathy (21). Like infection by RNA viruses, sepsis is initiated by activation of the innate immune system through recognition of pathogen-associated molecular patterns and damage-associated molecular patterns by PRRs. This inflammatory reaction enhances endothelial dysfunction and contributes to the procoagulant state with microvascular thrombi formation and, eventually, organ damage (22). The procoagulant state of sepsis is further amplified by NETs (23). Sepsis-induced coagulopathy (SIC) occurs as a continuum, progressing to disseminated intravascular coagulopathy (DIC) if the underlying etiology of the sepsis is not resolved. The clinical characteristics of critical COVID-19 and septic shock patients are extensively discussed in the literature although the entities have never been systematically compared in a prospective clinical setting including control patients with matched age, gender, and co-morbidities (24–26).

The current study prospectively compared patients with septic shock and those with critical COVID-19 on admission to the ICU regarding their clinical characteristics and outcomes, as well as key plasmatic soluble biomarkers of inflammation, coagulation, endothelial activation, platelet activation, and NETosis. Here, we show both coagulopathy and inflammatory pattern substantially differ between patients with septic shock and critical COVID-19, highlighting the specific actors involved in their respective pathogenesis.

METHODS

Aim, Design, and Setting of the Study

This study comparing clinical outcomes, inflammatory reaction, and coagulopathy between critical COVID-19 and septic shock was a monocenter, prospective, translational observational study. Adult patients were systematically included between February 1, 2019, and June 1, 2020. The ethics committee approved the study protocol, and all patients signed their informed consent (B403201938590, NCT04107402). Protocol amendment was done to include COVID-19 patients in the ongoing study. All authors had full access to primary clinical data.

Population

Patients with critical COVID-19 were those admitted to the ICU for moderate or severe ARDS due to SARS-Cov-2 infection; they were included within 5 days of admission. Acute respiratory distress syndrome was diagnosed according to the Berlin definition (27), and SARS-Cov-2 infection was demonstrated by real-time reverse transcription PCR on nasopharyngeal swabs. Septic shock was defined according to the Sepsis-3 definition as sepsis with vasopressor therapy needed to elevate the mean arterial pressure ≥ 65 mmHg and lactate levels > 2 mmol/L

despite adequate fluid resuscitation of 30 ml/kg of intravenous crystalloid within 6 h (28). A similar protective ventilation strategy (including positive end expiratory pressure above 5 cmH₂O, maximum tidal volume of 6 ml/kg, and maximal plateau pressure of 30 cmH₂O) was applied in both COVID-19 and septic shock patients with ARDS. Prone positioning and inhaled nitric oxide were used for severe ARDS, and venovenous extracorporeal membrane oxygenation (VV-ECMO) for refractory hypoxemia despite optimal treatment (29). Venovenous extracorporeal membrane oxygenation was never used in septic shock patients. Patients with septic shock admitted to the ICU were included within 2 days of admission. Control patients with matched age, gender, and co-morbidities were recruited at a central laboratory consultation. Similar exclusion criteria were applied to all groups: therapeutic anticoagulation (oral or parenteral, including heparins, fondaparinux, vitamin K antagonists, and direct oral anticoagulants), recent (within <1 month) chemotherapy, active inflammatory disease, hemophilia and other coagulopathies, previous history of thrombocytopenia (<100,000 platelets/mm³), cirrhosis (Child–Pugh >A), recent (within <48 h) major surgery (behalf, for septic shock, for infection source control), cardiac arrest during ICU stay, and decision of care limitation. All septic and COVID-19 cases received thromboprophylaxis using low-molecular-weight heparin (LMWH; nadroparin 3,800 IU/days subcutaneously). Sampling was performed at least 6 h after LMWH injection. For the patients with COVID-19, patients on antibiotics for any suspected or confirmed bacterial coinfections were formally excluded.

Clinical Outcomes

Patient baseline characteristics and clinical outcomes were compared. Patient prognosis was assessed using acute physiologic assessment and chronic health evaluation II (APACHE II) (30) and sequential organ failure assessment (SOFA) (31) scores. Moreover, disseminated intravascular coagulation (DIC) and SIC were diagnosed using the International Society of Thrombosis and Hemostasis scoring at inclusion (32, 33). Data were collected from central medical records, including biological datasets that were routinely performed in patients admitted in ICU such as platelet count, CRP (C-reactive protein) level, coagulation assessment, renal function, and liver enzymology. Clinical outcomes were assessed 30 days after ICU admission. Bleeding complications were assessed with Thrombolysis in Myocardial Infarction (TIMI) bleeding criteria, frequently used for cardiovascular trials (34). A major bleeding is defined by the following criteria: any intracranial bleeding, clinically overt signs of hemorrhage associated with a drop in hemoglobin of ≥5 g/dl or a ≥15% absolute decrease in haematocrit and fatal bleeding.

Sampling

Blood samples were collected through the central venous catheter in all ICU patients and by venous puncture in the control group. Venous blood was collected using vacutainer tubes containing CPDA. After two centrifugation runs enabling platelet isolation, plasma was collected, apportioned into 1 ml aliquots and stored at –80°C until use.

Measurement of Biomarkers

Soluble biomarkers of inflammation, coagulation, endothelial and platelet activation, and NETosis were measured using enzyme-linked immunosorbent assay (ELISA) or suspension array sandwich immunoassays according to regulatory requirements for commercially available research use only ELISA assays. Frozen platelet poor plasma was thawed at room temperature the day of the experiment. The details of each markers analyzed are listed in the **Supplementary Table 1**. Each analytical run was performed in duplicate. Methods respected their respective validated lower limit of quantification and upper limit of quantification. Cytokines and chemokines were measured using Bio-Plex Pro Human Cytokine 27-Plex Panel (27-Plex) and Bio-Plex Human ICAM-VCAM (hICAM-hVCAM) following the manufacturer's protocol.

Lung Biopsies and Autopsies

Lung biopsies and autopsies were obtained from the biolibrary of Cliniques Universitaires Saint-Luc. A “Human Material Transfer Agreement for research purpose” was elaborated between CUSL and UCLouvain. Use of Residual Human Body Material was approved by the local Ethic committee (B403201938590). Control patients, patients with ARDS from septic shock, and COVID-19 were compared. COVID-19 specimens were lung autopsies obtained from patients who died from respiratory failure at intensive care unit (ICU). Septic shock samples were biopsies taken owing to ARDS due to bacterial pulmonary or extra-pulmonary infection. Control specimens were archived tissues from patients who underwent lung surgery. Details about the methods and analyses are provided in the **Supplementary Material (Supplementary Methods)**.

Statistical Analyses

The analyses were conducted using GraphPad Prism Version 9 (GraphPad Software, San Diego, California). Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables were expressed as number and percentage. The data were subjected to the Kolmogorov–Smirnov normality test and Bartlett's test for homogeneity of variance. Log transformations were performed when appropriate. The categorical variables were analyzed using the Chi-squared test or Fisher's exact test, and the continuous variables using Tukey's ordinary one-way ANOVA or an unpaired Student's *t*-test, as appropriate. A log-rank test was applied to compare ICU length of stay and ventilation duration. All *p*-values were two-sided, and *p* < 0.05 was considered statistically significant.

A principal component analysis (PCA) was conducted in R (35), based on the patients with COVID-19 or septic shock and study outcomes. As an exploratory multivariate analysis, PCA provides a condensed overview of the main sources of variability in a dataset composed of individuals (here, the patients) with a large number of variables measured (here, the study outcomes). The core idea is to reduce data dimensionality by building new latent variables—called the principal components (PCs)—from the measured ones. The PCs, which are orthogonal between each other, capture the main sources of data variability in a decreasing manner (PC1 captures more variability than PC2,

etc.). Each original variable contributes with a certain weight (called a loading) to the construction of these PCs. Thereafter, the individuals are projected onto these PCs, with these projections called the scores. From this graphical representation, one can inspect the data structure with respect to the main sources of variability. This technique enables to explore the variables that seek to recover the grouped structure of the patients from the measured variables that are segregating the patients and the relationship between the variables of interest. Prior to PCA, the data were standardized, then imputed with the missMDA package (36), so that imputed values would not impact the factorial analysis results.

RESULTS

Baseline Characteristics and Clinical Outcomes of Critical COVID-19 and Septic Shock

Overall, 118 patients were enrolled, including 48 with septic shock, 22 with COVID-19, and 48 controls matched for age, gender, and main co-morbidities (flowchart, **Figure 1**). Type of infection and culture in septic shock patients are detailed in **Supplementary Table 2**. The baseline characteristics of all groups, as well as the clinical outcomes of septic shock and COVID-19 patients are detailed in **Table 1**.

The demographic characteristics and past medical history were similar among the three groups, except that the COVID-19 group included less smokers and oncologic patients. Before inclusion into the study, most COVID-19 patients had been treated with hydroxychloroquine ($n = 18$, 82%) but only one had received corticosteroids (methylprednisolone) as compared with five septic shock patients receiving low-dose hydrocortisone. At the end of the ICU stay, five COVID-19 patients (19%) and 22 septic shock patients (46%) had been treated with corticosteroids. The time delay between symptom onset and ICU admission was longer for COVID-19 compared with septic shock (2.6 ± 2.4 days and 7.3 ± 3.2 days, respectively, $p < 0.01$). The patients with septic shock displayed worse severity scores due to multiple organ failure. By contrast, the COVID-19 patients presented with more severe respiratory failure, as indicated by a lower $\text{PaO}_2/\text{FiO}_2$ (arterial oxygen partial pressure/fractional inspired oxygen) ratio, a higher rate of mechanically ventilated patients, longer ventilation duration and ICU length of stay. The 30-day mortality did not differ between both groups.

Coagulopathies in Critical COVID-19 and Septic Shock Patients

The critical COVID-19 and septic shock cases were compared with the matched controls considered as a reference. Circulating levels of ICAM-1, reflecting endothelial dysfunction, were similarly increased in both COVID-19 and septic shock patients, compared to matched controls (**Figure 2A**). Other endothelial biomarkers differed between COVID-19 and septic shock. Circulating TF was higher in COVID-19 patients than septic shock patients, while TFPI was similarly elevated in both groups (**Figures 2B,C**). By contrast, vWF, PAI-1, and tPA were

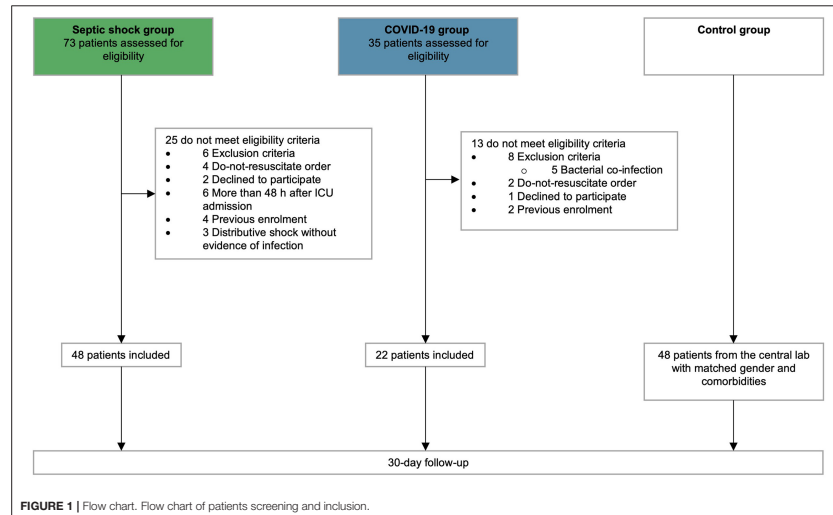
predominant in septic shock (**Figures 2D–F**). The difference in TF levels did not impact thrombin generation, as reflected by the thrombin–antithrombin complex (TAT), which was similarly increased in critical COVID-19 and septic shock, compared to matched controls (**Figure 2G**). Both critical conditions led to increased international normalized ratio (INR) and antithrombin consumption, while these changes were more pronounced in septic shock (**Figures 2H,I**). Interestingly, platelet and fibrinogen consumption mainly occurred in septic shock (**Figures 2J,K**). Accordingly, SIC and DIC were diagnosed in 24 and 16% of septic shock cases, respectively, but in none of the critical COVID-19 cases ($p < 0.05$). D-dimers were significantly elevated in both COVID-19 and septic shock patients compared with matched controls, but further in septic shock compared with COVID-19 patients (**Figure 2L**). Of interest, the levels of TAT and D-dimers that were measured in several control patients with co-morbidities were like those found in some critically ill patients, whether they had COVID-19 or not.

Platelet Activation and NETosis in Critical COVID-19 and Septic Shock Patients

Platelet activation soluble biomarkers P-selectin (sCD62P) and triggering receptor expressed on myeloid cells (TREM)-like transcript-1 (sTLT-1) were significantly increased in septic shock, but not in COVID-19 (**Figures 3A,B**). Levels of circulating NE and Cit-H3, reflecting neutrophil activation and NETosis, respectively, were increased in both diseases compared to matched controls but there were no differences between septic shock and COVID-19 (**Figures 3C,D**). This result was supported by the histopathological analysis of lung sections from control patients and patients with critical COVID-19- and septic shock-induced ARDS (**Figure 3E**). The baseline characteristics of patients included in the histopathological analysis are described in the **Supplementary Table 3**. As already described (37–39), vascular immunothrombosis containing platelets and neutrophils was observed in the lungs of patients with COVID-19 and septic shock, but not in the controls.

Inflammation and Immune Response in Critical COVID-19 and Septic Shock Patients

The inflammation and immune response substantially differed between COVID-19 and septic shock cases. The CRP elevation and peak were similar in both groups (**Figure 4A**; **Table 1**). The septic shock group exhibited a higher neutrophil count, whereas the lymphocyte count at inclusion or nadir during follow-up was similar in both groups (**Table 1**). Ubiquitous proinflammatory cytokines like TNF- α , IL-1 β , IL-6, and IL-8 were discrepantly increased in both groups (**Figures 4B–E**). Septic shock was characterized by a higher level of myeloid-derived cytokines including IL-1 receptor antagonists (IL-1ra), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), and the soluble form of TREM-1 (**Figures 4F–I**). In contrast, COVID-19 was distinguished by a lymphocyte T cytokine response (40), as reflected by an increase in IL-2, IL-4, IL-5, IL-7, IL-13, IL-17, and



soluble CD40L (sCD40L) (Figures 4J–P). Of note, IFN- γ , IL-10, and IP-10, although elevated, did not differ between COVID-19 and sepsis (Figures 4Q–S).

Exploratory Multivariate Analysis

A PCA was done on the basis of 53 variables to assess the main relations between study outcomes (correlations), patients (similarities among patients from the same group), and between the outcomes and patients. The first two PCs of the PCA captured 39.65% of the total data variability and inflammatory and immune response biomarkers as well as SOFA, PAI-1, NE, and antithrombin ranked among their main contributors (Supplementary Figures 1A–C). The first two PCs allowed the discrimination of COVID-19 and septic shock patients (Figure 5A), consolidating results of the univariate analysis. COVID-19 patients were characterized by an increased IL1 β , increased lymphoid activation biomarkers, increased TF, and higher fibrinogen, antithrombin, and platelet count. Septic shock patients were characterized by an increased IL-6 and IL-8, increased myeloid activation biomarkers, endothelial activation biomarkers (except TF), a prolonged coagulation, increased D-Dimers, and platelets activation biomarkers (Figures 5B–G).

DISCUSSION

For the first time, the inflammation-related coagulopathy of critical COVID-19 and septic shock was prospectively

and extensively compared. This study overcame two major limitations of the literature, given that bacterial coinfections were formally excluded from the COVID-19 cohort, while pathological changes were assessed using a control group matched for age, gender, and comorbidities instead of healthy volunteers.

This study highlighted essential clinical differences between critical COVID-19 and septic shock cases. The patients with COVID-19 displayed a longer time delay between symptom onset and ICU admission, and a much longer ICU length of stay, reflecting an extended disease course. Critical COVID-19 patients, in the absence of any bacterial coinfection, rarely presented with hypotension or organ failure. These results contrast with previous observational studies that did not exclude coinfection, demonstrating a higher rate of vasopressor use (41, 42).

Inflammation-induced coagulopathy remarkably differed between COVID-19 and septic shock patients. For instance, COVID-19 was characterized by high circulating TF levels, no platelet or fibrinogen consumption, and lower levels of PAI-1, tPA, and D-dimers, suggesting less fibrinolysis activation. Although TF is recognized as one of the main actors in the inflammatory response during sepsis (43), an increase in circulating TF has never been described outside the DIC context (44). However, an increase in extracellular vesicle TF activity has been shown in COVID-19 (13, 45) and moreover, other viral diseases including human dengue infection and HIV

TABLE 1 | Baseline characteristics of prospective cohort of patients.

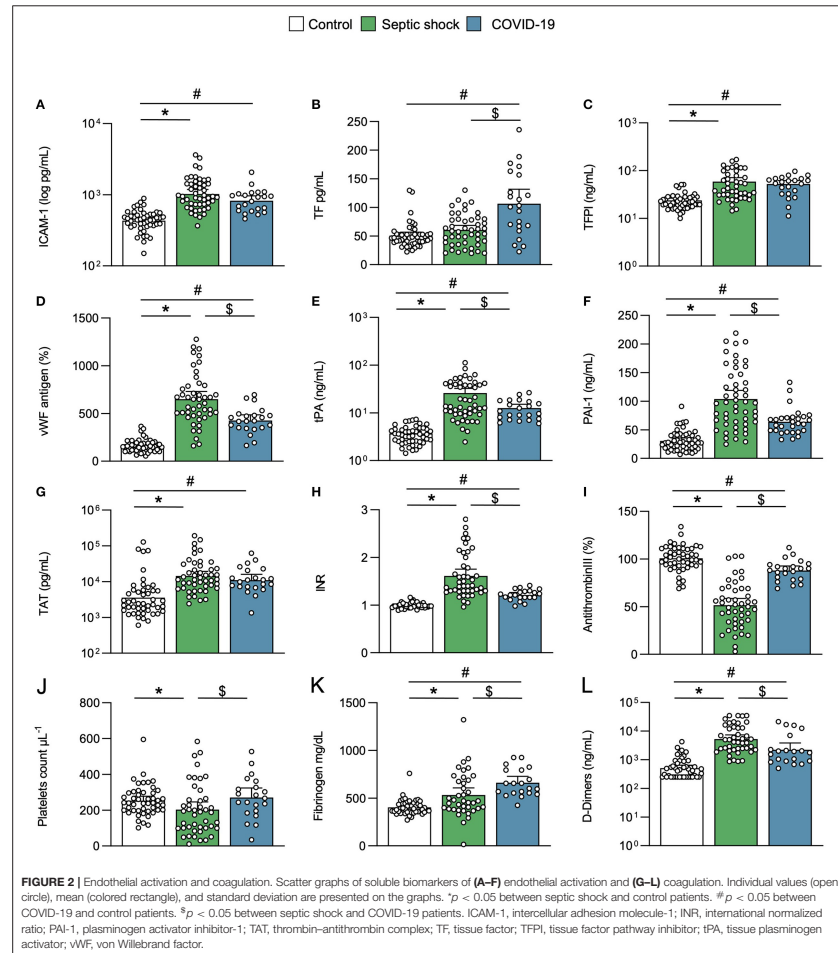
	Control <i>n</i> = 48	COVID-19 <i>n</i> = 22	Septic shock <i>n</i> = 48	<i>p</i> -Value
Demographics				
Men	26 (54)	15 (68)	24 (50)	0.36
Women	22 (46)	7 (32)	24 (50)	
Age (years)	61.9 ± 14.5	59.9 ± 10.3	65.0 ± 14.2	0.53
Medical history				
Hypertension	20 (42)	12 (56)	25 (52)	0.48
BMI >25	26 (58)	14 (74)	26 (54)	0.34
Diabetes	11 (23)	8 (36)	5 (10)	0.71
History of smoking	10 (21)	1 (5)	15 (31)	0.04
COPD	4 (8)	3 (14)	5 (10)	0.75
CKD	9 (19)	0 (0)	10 (21)	0.07
Cancer	15 (31)	0 (0)	9 (19)	0.01
Delay symptoms-ICU admission (days)		7.3 ± 3.2	2.6 ± 2.4	<0.01
Routine laboratory testing				
Highest CRP (mg/dl)		323 ± 119	313 ± 122	0.75
Creatinine (mg/dl)		0.91 ± 0.59	2.19 ± 1.91	<0.01
Hemoglobin (g/dl)		11.62 ± 1.90	10.34 ± 2.05	0.02
Neutrophils (10 ³ /μl)		8.0 ± 3.0	15.5 ± 10.2	<0.01
Lymphocytes (/μl)		893 ± 319	779 ± 521	0.33
Lowest lymphocytes (10 ³ /μl)		484 ± 335	469 ± 310	0.86
Platelet count (10 ³ /μl)		271 ± 117	203 ± 143	0.04
Organ failure, severity scores, and complications				
PaO ₂ /FIO ₂		103 ± 37	225 ± 119	<0.01
Mechanical ventilation		17 (77)	25 (52)	0.06
VV ECMO		5 (23)	0 (0)	<0.01
Norepinephrine (μg/kg/min)		0.049 ± 0.105	0.330 ± 0.350	<0.01
Norepinephrine duration (days)		1.2 ± 3.4	4.8 ± 6.1	<0.01
Renal replacement therapy		1 (5)	13 (27)	0.04
Apache II score		15 ± 4	20 ± 7	<0.01
SOFA score		4 ± 1	9 ± 3	<0.01
SIC		0 (0)	11 (24)	0.01
DIC		0 (0)	7 (16)	0.09
Thromboembolic events		6 (27)	4 (8)	0.06
TIMI major bleeding events		5 (23)	1 (2)	0.01
Outcome				
30-day mortality		6 (27)	22 (46)	0.45
Ventilation duration (days)		27 ± 24	4 ± 7	<0.01
ICU length of stay (days)		29 ± 30	8 ± 9	<0.01

Values are numbers (percentages) or mean ± standard deviation. Major bleeding complications have been defined according to the TIMI definition. All bleeding complications in COVID-19 group occurred in ECMO-treated patients. APACHE, acute physiology and chronic health evaluation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CRP, C-reactive protein; DIC, disseminated intravascular coagulopathy; ICU, intensive care unit; PaO₂/FIO₂, arterial oxygen partial pressure/fractional inspired oxygen; SIC, sepsis-induced coagulopathy; SOFA, sepsis-related organ failure assessment; VV ECMO, venovenous extracorporeal membrane oxygenation.

are characterized by an increased soluble TF release (46, 47). Altogether, these results indicate that coagulation abnormalities of COVID-19-associated coagulopathy promote a procoagulant state. In contrast, SIC and DIC are septic shock features, in which the coagulation imbalance can be either pro- or anti-coagulant (48). Therefore, the current study formally supports that COVID-19-associated coagulopathy should not be assimilated to SIC or DIC (49). Given the prolonged disease course, a

procoagulant state may explain the high rate of thrombotic complications observed in COVID-19 patients (3, 4).

The histopathological data on COVID-19- and septic-shock-induced ARDS confirmed the presence of lung microthrombi in both diseases. These microthrombi contain numerous Cit-H3⁺-NE⁺ neutrophils at early stages of NET formation, as reported by others (37–39). Neutrophil extracellular traps represent a mechanism by which neutrophils



participate to thrombus formation in COVID-19 (19), possibly leading to organ dysfunction (50) and lung injury (51). The similar level of NETosis formation in ARDS due to COVID-19 vs. septic shock suggests that this parameter could be equally involved in the pathophysiology of both

diseases. This hypothesis was reinforced by the plasmatic measurements in this clinical cohort revealing identical levels of circulating Cit-H3.

The secretion of soluble markers of platelet activation, namely CD62P and TLT1, was increased in septic shock but unaltered in

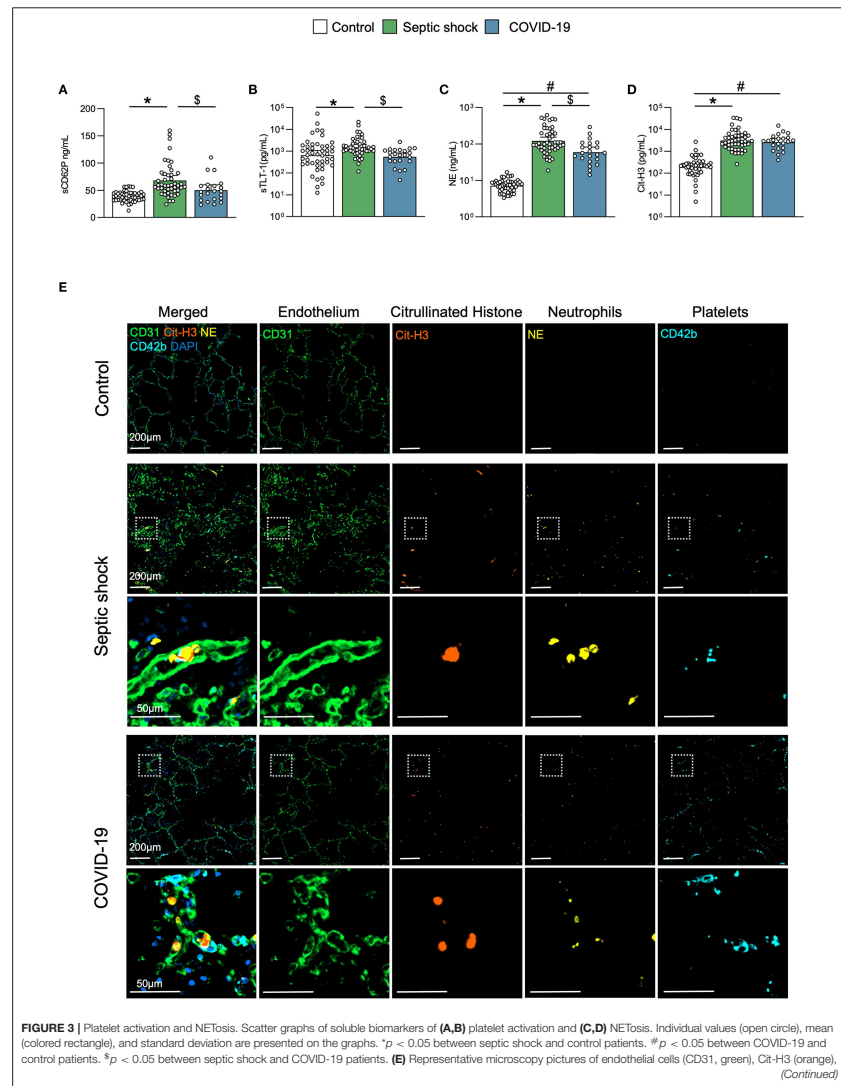


FIGURE 3 | neutrophils (NE, yellow), platelets (CD42b, light blue), nucleus (DAPI, dark blue), and merged staining, from control, septic shock, and COVID-19 lungs. sCD62P, soluble p-selectin; Cit-H3, citrullinated histone 3; NE, neutrophil elastase; NET, neutrophil extracellular traps; MPO, myeloperoxidase; sTLT-1, soluble triggering receptor expressed on myeloid cells (TREM) like transcript-1.

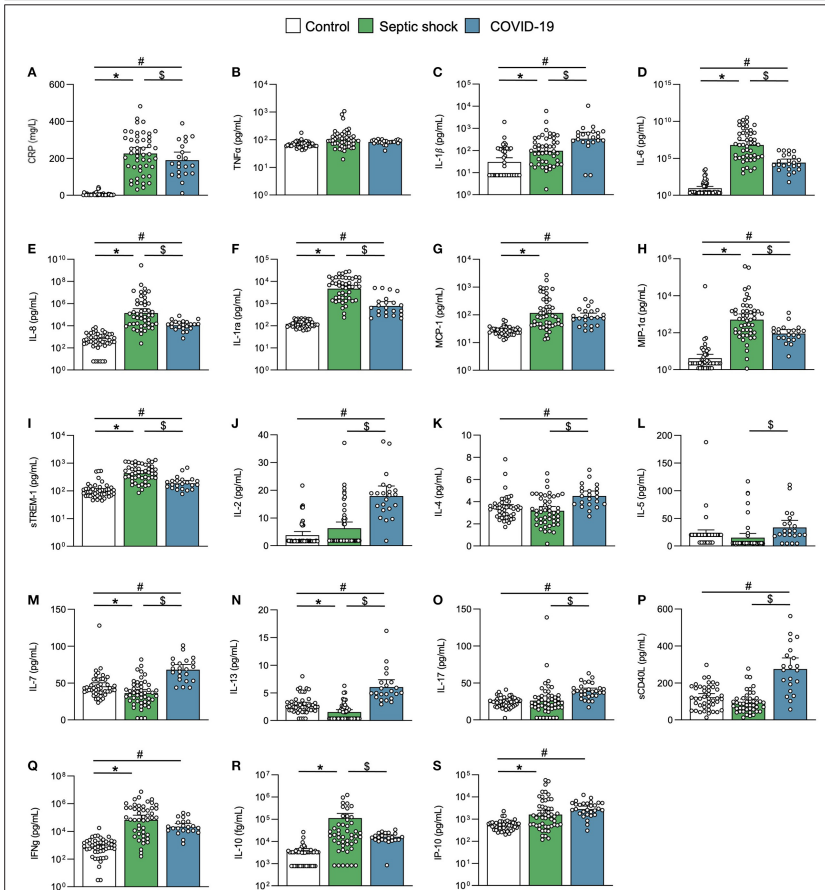


FIGURE 4 | Inflammation and immune response. Scatter graphs of (A–E) ubiquitous proinflammatory cytokines, (F–I) myeloid inflammatory cytokines, and (J–S) lymphoid inflammatory cytokines. Individual values (open circle), mean (colored rectangle), and standard deviation are presented on the graphs. * $p < 0.05$ between septic shock and control patients. # $p < 0.05$ between COVID-19 and control patients. \$ $p < 0.05$ between septic shock and COVID-19 patients. CD40L, CD40 ligand; CRP, C-reactive protein; IFNγ, interferon gamma; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1α, Macrophage inflammatory protein-1α; sTREM-1, soluble triggering receptor expressed on myeloid cells 1.

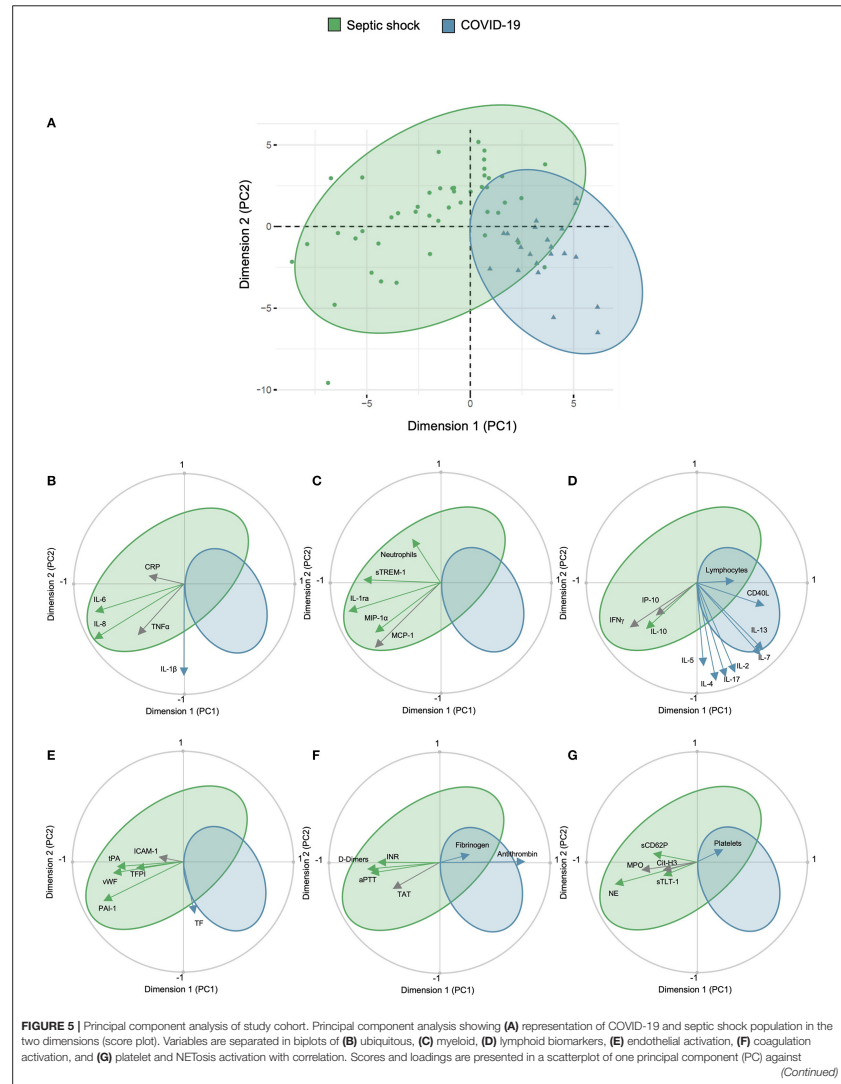


FIGURE 5 | another. The loadings are represented in a circle of correlations: the closer the arrow of a loading is to the circle, the more the variable is well-represented in the space of the two plotted PCs and contributed to the building of these PCs. This graph also indicates the positive or negative links between the variables and groupings of variables. Arrow colors correspond to the between-group comparison as mentioned in the scatter graphs. CD40L, CD40 ligand; TCell-H3, circulating histone H3; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule-1; IFN γ , interferon gamma; IL, interleukin; IL-1ra, IL-1 receptor antagonist; INR, international normalized ratio; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1 α , macrophage inflammatory protein-1 α ; PCA, principal component analysis; MPO, myeloperoxidase; NE, neutrophil elastase; PAI-1, plasminogen activator inhibitor-1; TAT, thrombin-antithrombin complex; TF, tissue factor; TFPI, tissue factor pathway inhibitor; sTLT-1, soluble TREM like transcript-1; tPA, tissue plasminogen activator; sTREM-1, soluble triggering receptor expressed on myeloid cells 1; TT, thrombin time; VCAM-1, vascular cell adhesion protein 1; vWF, von Willebrand factor; WBC, white blood cells.

COVID-19 cases, thereby corroborating previous findings (17). However, this observation remains controversial in the literature (52, 53). The discrepancies could be explained by a small sample size or the possible presence of bacterial coinfections in the critical COVID-19 cohort. Nevertheless, platelets from COVID-19 patients have been reported to display a hyperactive phenotype upon agonist stimulation (8, 17) and it must be noted that soluble CD62P does not inform on the presence of the protein on platelet surface neither on platelet reactivity.

The immune response was characterized by lymphocyte T activation in critical COVID-19 cases, whereas those with septic shock exhibited a greater activation of myeloid cells. Variables related to the immune and inflammatory responses were the most discriminating in distinguishing COVID-19 from septic shock at ICU admission. It has already been shown for patients infected with SARS-CoV-1 that elevation of T helper Type 2 (Th2) cytokines (IL-4, IL-5, IL-10) is correlated with disease severity (54). Interestingly, here, sCD40L also differentiated critical COVID-19 from septic shock patients. sCD40L is expressed by cells of the immune system, especially by activated CD4⁺ T lymphocytes and activated platelets (55). In COVID-19, the elevation of sCD40L was dissociated from the raise of the other platelet activation biomarkers sCD62P and sTLT1, suggesting that its source could be the T lymphocytes. A similar result was recently found in another study (56) and corroborates the elevation of other lymphocyte T-derived ILs. Finally, sCD40L–CD40 interaction promoted endothelial cell and monocyte TF expression (57, 58), linking these two COVID-19 specific observations.

The study had limitations. First, the COVID-19 group was relatively small, so the results must probably be validated in a larger confirmation cohort. However, prospective and systematic enrollment of patients with either COVID-19 or septic shock within a closed time period has limited inclusion bias. Second, the patients with COVID-19 were included during the first wave of the pandemic, when the systematic use of corticosteroids was not yet recommended. It is therefore possible that the study findings would differ in patients that were systematically treated with dexamethasone. Finally, the observations were based on a single time point, namely early after ICU admission. A longitudinal assessment of these specific biomarkers could better define the dynamic changes in inflammatory and coagulation processes over time, which remain to be clarified, particularly in the COVID-19 context.

In conclusion, coagulopathy and inflammation patterns of critical COVID-19 substantially differ from septic shock at ICU

admission. Critical COVID-19 is distinguished by very high levels of IL-1 β and T lymphocyte activation (including IL-7), whereas septic shock displays higher levels of IL-6, IL-8 and a more significant myeloid response (including TREM-1 and IL-1ra). Moreover, COVID-19-associated coagulopathy is formally different from SIC and DIC, with a marked increase in soluble TF, yet less platelet, antithrombin, and fibrinogen consumption, and less fibrinolysis alteration. Hence, coagulation imbalance of severe COVID-19 almost exclusively tends toward a procoagulant state, and in the context of a prolonged disease course, this could explain the very high rate of thrombo-embolic complications of the disease. A better understanding of critical COVID-19 pathophysiology suggests potential therapeutic strategies, in particular recombinant IL-1ra and recombinant TFPI could modulate these two over-expressed pathways.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'Ethique Hospitalo-Facultaire Saint-Luc-ULouvain. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH and CBe: had full access to all of the study data and takes responsibility for the data integrity and the accuracy of data analysis. MDec, DC-Z, P-FL, SH, and CBe: concept and design. CBo, AD, SH, LGe, MDec, JDP, MO, LP, VR, AG, JB, DG, M-AVD, JD, HH, LM, MDer, and LJ: acquisition, analysis, or interpretation of data. MDec, JDP, P-FL, SH, and CBe: drafting of the manuscript. VM, LD, and LB: critical revision of the manuscript for important intellectual content. MDec, JDP, MM, and LGa: statistical analysis. MDec, P-FL, LB, SH, and CBe: obtained funding. P-FL and CBe: administrative, technical, or material support. P-FL, SH, and CBe: supervision. MDec, JDP, P-FL, AC, and VM: other—monitoring of the study progress, supporting patient recruitment, data clarifications, and data

entry. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the *Fondation Saint-Luc* (Brussels, Belgium). The Division of Cardiology at Cliniques Universitaires Saint-Luc, Belgium, has received unrestricted research grants from AstraZeneca (Belgium). MDec is Clinical Master Specialist Applicant to a Ph.D. at the *Fonds National de la Recherche Scientifique et Médicale* (FNRS, Belgium). JDP was supported by a grant from the Salus Sanguinis Foundation (UCLouvain, Belgium). MO, LP, and JB are supported by Fund for Research Training in Industry and Agriculture (FRIA, FNRS). VM is post-doctorate Clinical Master Specialist at the FNRS. LD and SH are senior research associates at FNRS. QUALiblood s.a. offered the analyses for determining the cytokine profile.

ACKNOWLEDGMENTS

The authors would like to thank all the patients for their participation in this study, as well as Fatima Laarbaui, Olivier Van Caenegem, Sophie Pierard, Luc Jaquet, Xavier Wittebole, Philippe Hantson, Christine Collienne, and Mathieu Luyckx for their help to enroll patients, Suzanne Renard, Caroline Berghe, Marie-France Dujardin, Leslie Gieslen, Florence Sinnaeve, Brice Lambert, all ICU nurses, and central lab nurses for their help at collecting samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmed.2021.780750/full#supplementary-material>

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Conflict of Interest: JD is the CEO and founder of QUALIblood s.a., a Belgian Contract Research Organization. LM and HH were employed by the company QUALIblood s.a. and authors MDer and LJ were employed by the company Inotrem s.a.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that this study received funding from Foundation Saint-Luc.

The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

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Article

Dexamethasone Modulates the Cytokine Response but Not COVID-19-Induced Coagulopathy in Critically Ill

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Citation: Dechamps, M.; De Poortere, J.; Octave, M.; Ginion, A.; Robaux, V.; Piroton, L.; Bodart, J.; Gruson, D.; Van Dievoet, M.-A.; Douxfils, J.; et al. Dexamethasone Modulates the Cytokine Response but Not COVID-19-Induced Coagulopathy in Critically Ill. *Int. J. Mol. Sci.* **2023**, *24*, 7278. <https://doi.org/10.3390/ijms24087278>

Academic Editor: Toshiyuki Kaji

Received: 2 March 2023

Revised: 29 March 2023

Accepted: 5 April 2023

Published: 14 April 2023



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Abstract: Severe forms of coronavirus 2019 (COVID-19) disease are caused by an exaggerated systemic inflammatory response and subsequent inflammation-related coagulopathy. Anti-inflammatory treatment with low dose dexamethasone has been shown to reduce mortality in COVID-19 patients requiring oxygen therapy. However, the mechanisms of action of corticosteroids have not been extensively studied in critically ill patients in the context of COVID-19. Plasma biomarkers of inflammatory and immune responses, endothelial and platelet activation, neutrophil extracellular trap formation, and coagulopathy were compared between patients treated or not by systemic dexamethasone for severe forms of COVID-19. Dexamethasone treatment significantly reduced the inflammatory and lymphoid immune response in critical COVID-19 patients but had little effect on the myeloid immune response and no effect on endothelial activation, platelet activation, neutrophil extracellular trap formation, and coagulopathy. The benefits of low dose dexamethasone on outcome in critical COVID-19 can be partially explained by a modulation of the inflammatory response but not by reduction of coagulopathy. Future studies should explore the impact of combining dexamethasone with other immunomodulatory or anticoagulant drugs in severe COVID-19.

Keywords: COVID-19; low dose dexamethasone; inflammation; coagulopathy; cytokine storm

1. Introduction

Coronavirus disease 2019 (COVID-19), is an airborne viral infection due to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). SARS-CoV-2 enters organs by binding its spike glycoprotein to the angiotensin-converting enzyme 2 receptor. The local immune response triggers a progressive systemic inflammatory reaction, implicating endothelial cells, monocytes, macrophages, neutrophils, and lymphocytes. Severe forms of COVID-19 are driven by a dysregulated inflammatory reaction, also called “cytokine storm”, characterized by high systemic levels of cytokines, including tumor necrosis factor α (TNF α), interleukine-1 (IL-1), interleukine-6 (IL-6), and interferon γ (IFN γ) [1–7].

Inflammatory reactions, immune system and endothelial cell activation and dysfunction lead to an activation of the coagulation system in a phenomenon called “immunothrombosis” involving the formation of Neutrophil Extracellular Traps (NETs) [5,8–15]. NETs formation is an innate immune response that neutrophils deploy in addition to phagocytosis to constrain the spread of microorganisms. They are made of large networks of extracellular DNA fibers that are assembled with cytosolic and granule proteins such as histones [16]. NETs are known to be pro-coagulant due to their intrinsic capacity to activate platelets and bind coagulation factors [17]. Accordingly, severe forms of COVID-19 can develop a coagulopathy called COVID-19-associated coagulopathy (CAC), leading to deep venous thrombosis, pulmonary embolisms, arterial thrombosis, and an increased mortality [18–20]. CAC had been initially assimilated to a disseminated intravascular coagulopathy (DIC) but is currently recognized to be a specific coagulopathy characterized by elevated plasmatic levels of von Willebrand factor (vWF), soluble P-selectin (sP-selectin), tissue factor (TF), tissue factor pathway inhibitor (TFPI), and thrombin–antithrombin complexes (TAT). As opposed to sepsis, lower platelet, fibrinogen and antithrombin consumption, and less fibrinolysis alteration is seen [4,11].

Considering the pathophysiology of critical COVID-19, several anti-inflammatory, immunomodulatory or anticoagulant medications have been explored as a treatment [21]. Therapeutic anticoagulation, compared with standard low- or intermediate-dose thromboprophylaxis, has shown conflicting results, and seems to be beneficial for hospitalized but not critically ill patients [22–25]. Among anti-inflammatory medications, low-dose dexamethasone (DXM) is the only treatment that was shown to reduce the risk of mortality for the patients with respiratory insufficiency requiring oxygen supplementation [26–31].

Steroids are known to bind to the glucocorticoid receptors present in the cytoplasm of almost all cells and travel to the nucleus, directly or indirectly modulating the transcription of many genes implicated in the inflammatory response. The main expected effect is the inhibition of the synthesis of inflammatory mediators such as pro-inflammatory ILs, TNF α , and IFN γ . At the same time, steroids usually promote the biosynthesis of anti-inflammatory cytokines, including IL-10 [32], inhibiting neutrophil adhesion to endothelial cells and macrophage activation [33,34]. In severely ill COVID-19 patients, DXM administration has been shown to reduce plasma levels of several cytokines at day seven after intensive care unit (ICU) admission [35]. However, changes in coagulopathy following steroid administration in severe COVID-19 have not been explored. One meta-analysis found a higher likelihood of COVID-19 patients presenting with venous thromboembolism when administered corticosteroids [36], suggesting the medication could worsen the coagulopathy. Furthermore, the use of steroids has significant potential adverse effects, mainly their immunosuppressive action that could promote secondary bacterial infection in COVID-19 ventilated patients [29,37]. Therefore, it would be of major importance to determine the underlying changes driving DXM effects in severe COVID-19, looking for the opportunity to explore targeted anti-inflammatory, immunomodulatory, or anticoagulant medications as alternatives with less side effects or as adjunct therapies to increase efficacy.

The present study aimed to prospectively compare plasma inflammation and coagulation biomarkers in patients admitted to the ICU for critical forms of COVID-19 treated with or without DXM.

2. Results

2.1. Patients Baseline Characteristics

Overall, 22 patients were included in the no DXM group and 24 in the DXM group. The mean age was 59.9 ± 10.4 years and 64.5 ± 8.9 years, respectively ($p = 0.12$). There were no significant differences in the main comorbidities between the two groups. The patients included in the DXM group had more severe respiratory failure and, accordingly, a higher sequential organ failure assessment (SOFA) score. None of the patients met the criteria for sepsis-induced coagulopathy (SIC) nor DIC. The delay between symptom onset and inclusion was similar in both groups (10 ± 5 days in the no DXM group and 10 ± 4 in

the DXM group). The duration of treatment with DXM before inclusion was 3.0 ± 2.6 days. Throughout the inclusion period, the SARS-CoV-2 variant was the initial Alpha variant.

2.2. Clinical Outcome

The mean ventilation duration and ICU length of stay were longer for the patients in the DXM group. The 30-day and 1-year mortality rates were 27% and 36% for the no DXM group and 37% and 50% for the DXM group. The patients' baseline characteristics and clinical outcome are described in Table 1.

Table 1. Baseline characteristics and clinical outcomes of patients treated or not with DXM.

	No DXM Group n = 22	DXM Group n = 24	p Value
DEMOGRAPHICS			
Men	15 (68)	19 (79)	0.51
Women	7 (32)	5 (21)	
Age (years)	59.9 ± 10.4	64.45 ± 8.9	0.12
MEDICAL HISTORY			
Hypertension	13 (59)	17 (71)	0.54
BMI > 25	15 (68)	17 (71)	>0.99
Diabetes	5 (23)	10 (42)	0.22
History of smoking	2 (9)	1 (4)	0.60
COPD	3 (14)	1 (4)	0.34
CKD	0 (0)	2 (8)	0.49
Cancer	0 (0)	4 (17)	0.11
ROUTINE LABORATORY TESTING			
CRP (mg/dL)	199.3 ± 88.9	118.8 ± 76.6	<0.01 *
Highest CRP (mg/dL)	315.0 ± 126.4	285.8 ± 104.6	0.40
Creatinine (mg/dL)	0.92 ± 0.60	1.46 ± 2.44	0.40
Hemoglobin (g/dL)	11.2 ± 2.1	12.4 ± 2.2	0.07
WBCs (/μL)	9497 ± 3563	$10,739 \pm 4167$	0.29
Neutrophils (/μL)	7803 ± 3170	9073 ± 3762	0.22
Lymphocytes (/μL)	894 ± 329	802 ± 425	0.27
Lowest lymphocyte count (/μL)	459 ± 323	504 ± 294	0.64
ORGAN FAILURE AND SEVERITY SCORES			
PaO ₂ /FiO ₂	103 ± 4	86 ± 3	0.04 *
Apache II score	15 ± 4	14 ± 4	0.49
SOFA Score	4 ± 1	6 ± 2	<0.01 *
SIC score	0 (0)	0 (0)	>0.99
DIC score	0 (0)	0 (0)	>0.99
TREATMENT BEFORE INCLUSION			
Delay symptoms—inclusion (days)	10 ± 5	10 ± 4	0.87
Delay steroids—inclusion (days)	1.0	3.0 ± 2.6	NA
Hydroxychloroquine	19 (86)	0 (0)	<0.01
OUTCOME			
30-day mortality	6 (27)	9 (37)	0.54
1-year mortality	8 (36)	12 (50)	0.39
ICU length of stay (days)	27 ± 26	38 ± 42	<0.05 *
Ventilation duration (days)	21 ± 24	30 ± 35	0.08
Thrombo-embolic event	6 (27)	3 (13)	0.28

Values are numbers (percentages) or mean $\pm$ standard deviation. * means p value < 0.05. APACHE, acute physiology and chronic health evaluation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CRP, C-reactive protein; DIC, disseminated intravascular coagulopathy; ICU, intensive care unit; PaO₂/FiO₂, arterial oxygen partial pressure/fractional inspired oxygen; SIC, sepsis-induced coagulopathy; SOFA, sepsis-related organ failure assessment; WBCs, white blood cells.

2.3. Endothelial Activation and Coagulopathy (Figure 1)

The study showed no difference in the level of plasmatic endothelial and coagulopathy biomarkers between the two groups (intercellular adhesion molecule [ICAM], vascular cell adhesion molecule [VCAM], TF, TFPI, vWF, tissue plasminogen activator [tPA], plasminogen activator inhibitor-1 [PAI-1], thrombin–antithrombin complex [TAT], international normalized ratio [INR], antithrombin, D-Dimers, and Fibrinogen) except for VCAM which was more elevated in the DXM group. INR was slightly prolonged in both groups (1.23 ± 0.10 and 1.22 ± 0.13) and there was no antithrombin consumption ($87 \pm 11\%$ and $96 \pm 21\%$).

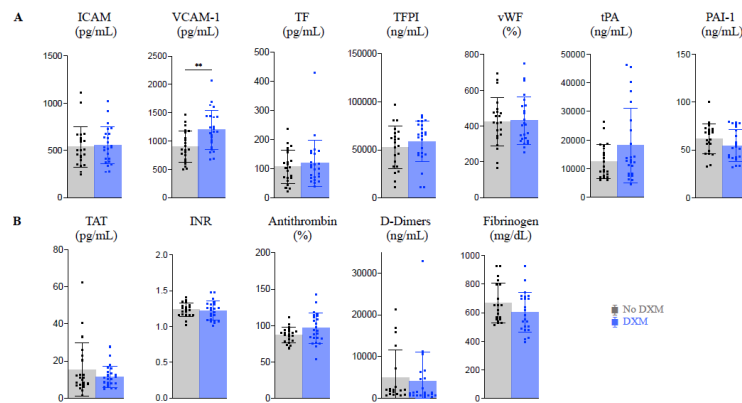


Figure 1. Corticosteroid effects on endothelial activation (A) and coagulation (B) in critical COVID-19 patients. Scatter graphs of soluble biomarkers of endothelial activation and coagulation. Individual values (dots), mean (colored rectangle), and standard deviation are presented on the graphs. ICAM, intercellular adhesion molecule; INR, international normalized ratio; PAI-1, plasminogen activator inhibitor-1; TAT, thrombin–antithrombin complex; TF, tissue factor; TFPI, tissue factor pathway inhibitor; tPA, tissue plasminogen activator; VCAM, vascular cell adhesion molecule; vWF, von Willebrand factor. ** $p < 0.01$.

2.4. Platelet Activation and NETosis (Figure 2)

On average, the platelet count was in the normal range in both groups ($278,000 \pm 115,000/\mu\text{L}$ and $277,000/\mu\text{L} \pm 104,000/\mu\text{L}$) with similar levels of sP-selectin (50 ± 22 and $43 \pm 21 \text{ ng/mL}$) as a biomarker of platelet activation. NETosis was assessed using citrullinated histone 3 (Cit-H3), neutrophil elastase (NE), and myeloperoxidase (MPO). Cit-H3 and NE remained unchanged but MPO was significantly reduced in the DXM group ($405.8 \pm 343.9 \text{ ng/mL}$ and $196.8 \pm 111.1 \text{ ng/mL}$).

2.5. Inflammatory Reaction and Immune Response

Ubiquitous inflammation biomarkers C-reactive protein (CRP), IL-1 β , IL-6, IL-8, and TNF α were all significantly reduced in the DXM group (Figure 3). White blood cell and neutrophil counts at ICU admission were similarly elevated in both groups. Among the plasmatic biomarkers of myeloid immune response activation, IL-1ra, MCP-1, MIP-1 α , MIP-1 β , and TREM-1, only MCP-1 was significantly reduced in the DXM-treated group (Figure 4). The lymphocyte count was unchanged between the groups. Among the plasmatic biomarkers of lymphoid immune response activation, INF γ , IL-7, IL-12, IL-13,

IL-17, and sCD40L were all significantly reduced in the DXM group whereas the levels of IL-2, IL-4, IL-10, and interferon gamma-induced protein 10 (IP10) were similar (Figure 5).

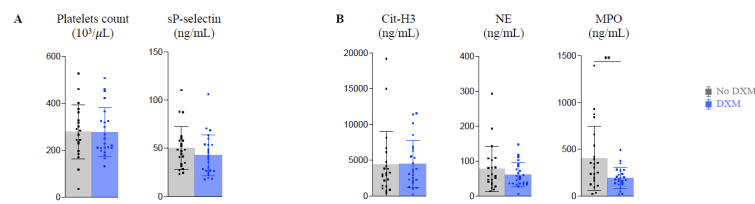


Figure 2. Corticosteroid effects on platelet activation (A) and NETosis (B) in critical COVID-19 patients. Scatter graphs of soluble biomarkers of platelet activation and NETosis. Individual values (dots), mean (colored rectangle), and standard deviation are presented on the graphs. ** $p < 0.01$ between the no DXM group and the DXM group. sP-selectin, soluble p-selectin; Cit-H3, citrullinated histone 3; MPO, myeloperoxidase; NE, neutrophil elastase.

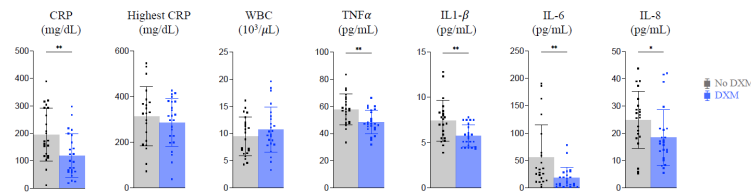


Figure 3. Corticosteroid effects on ubiquitous inflammatory reaction in critical COVID-19 patients. Scatter graphs of ubiquitous proinflammatory cytokines. Individual values (dots), mean (colored rectangle), and standard deviation are presented on the graphs. * $p < 0.05$, ** $p < 0.01$ between the no DXM group and the DXM group. CRP, C-reactive protein; IL, interleukin; TNF α , tumor necrosis factor α ; WBCs, white blood cells.

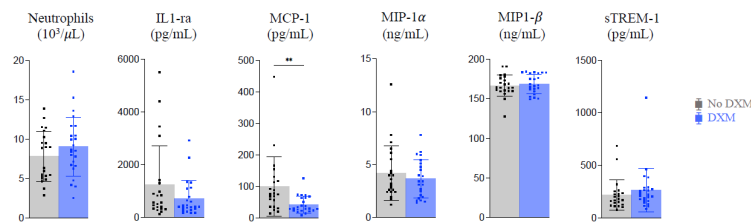


Figure 4. Corticosteroid effects on myeloid immune response in critical COVID-19 patients. Scatter graphs of myeloid inflammatory cytokines. Individual values (dots), mean (colored rectangle), and standard deviation are presented on the graphs. ** $p < 0.01$ between the no DXM group and the DXM group. IL, interleukin; IL-1-ra, IL-1 receptor antagonist; MCP-1, monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein-1 α ; sTREM-1, soluble triggering receptor expressed on myeloid cells 1.

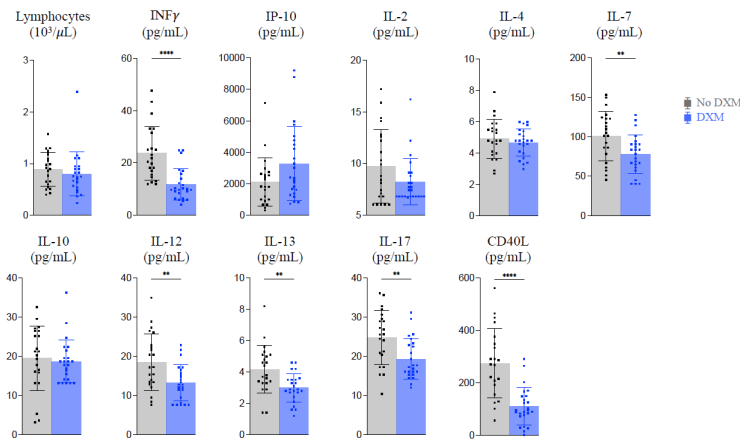


Figure 5. Corticosteroid effects on lymphoid immune response in critical COVID-19 patients. Scatter graphs of lymphoid inflammatory cytokines. Individual values (dots), mean (colored rectangle), and standard deviation are presented on the graphs. ** $p < 0.01$, **** $p < 0.0001$ between the no DXM group and the DXM group. CD40L, CD40 ligand; IFN γ , interferon gamma; IL, interleukin; IP-10, interferon gamma-induced protein 10.

Figure 6 provides an overview of corticosteroid effects on the inflammatory and immune responses, endothelial and platelet activation, NETosis, and coagulopathy in patients with severe COVID-19.

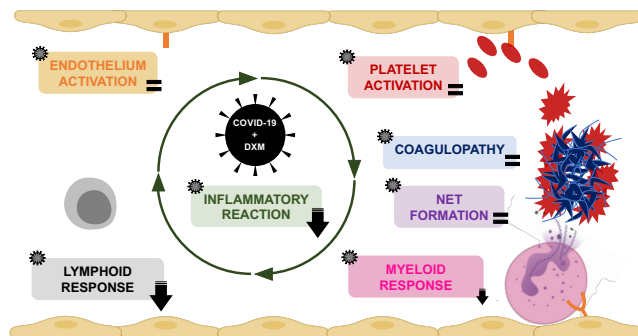


Figure 6. Overview of corticosteroid effects on the inflammatory and immune responses, endothelial and platelet activation, NETosis, and coagulopathy in patients with severe COVID-19.

3. Discussion

In this study, low dose DXM systemic administration did not influence CAC in critical COVID-19 patients but, as expected, reduced the plasma levels of circulating pro-

inflammatory cytokines [32]. With regards to the immune response, DXM mostly affected the lymphoid immune response but had several impacts on the myeloid immune response. This observation could be explained by the fact that the immune response during severe forms of COVID-19 is characterized by an important lymphoid immune response, similar to SARS-CoV1 infection and as opposed to severe bacterial infections [4,38]. The effects of steroids may, therefore, be more marked on the most important changes in the inflammatory response that are normally caused by the virus. Of note, DXM did not increase the level of the anti-inflammatory interleukins IL1ra and IL10.

However, although the occurrence of the CAC is related to severe inflammatory and immune responses [10–12], the reduction of the latter using low dose DXM did not affect the coagulopathy. In line with this, endothelial activation and NETosis formation, which are also closely related to coagulopathy [11], were not affected by DXM treatment. Among plasma biomarkers of endothelial activation, the levels were similar between the two patient cohorts and even higher in corticosteroid-treated patients with respect to VCAM. Endothelial dysfunction in COVID-19 is not only related to the inflammatory response, but also because SARS-CoV-2 directly infects vascular endothelial cells, leading to cell damage and apoptosis and reducing their normal anti-thrombotic activity [39,40]. Regarding NETs formation, MPO was significantly reduced in corticosteroid-treated patients in contrast to the Cit-H3 level, which remained elevated and is a better biomarker of actual NETs formation [41]. The decrease in MPO is, therefore, probably related to the reduction of myeloid activation and not to a reduction in NETs formation. As previously described [4], critical COVID-19 patients had a very high level of circulating TF, unaltered by steroid treatment, and TF is known to be a key player in the initiation of CAC. Hence, the outcome improvement that was observed on DXM for COVID-19 patients requiring oxygen [26–30] is not related to a decrease in the coagulopathy. The present study reinforces data that prove that CAC is a distinct entity from SIC and DIC, without platelet consumption, without AT consumption, only a slight prolongation of the INR, and a high fibrinogen and D-Dimer level. Accordingly, none of the patients met the criteria for SIC nor DIC diagnosis [42,43]. As CAC is associated with a worse prognosis for patients [18], efforts should be made to find a specific treatment for CAC to use in addition to DXM.

Our study is in line with previous descriptions of the role of steroids on sepsis-induced coagulopathy. In healthy volunteers exposed to LPS, prednisolone reduced cytokines release of TNF-alpha and IL-6 but had no impact on LPS-induced coagulation activation. Soluble TF plasma levels were not modified by prednisolone [44]. With regard to COVID-19, two studies showed results that were consistent with our study. In the first, for patients admitted to the general ward with lower severity scores than in our study, DXM that was administered within 19 h of collection reduced plasma IL-6 and IL-1ra levels but did not influence COVID-19 coagulation disorders [45]. In the second, for patients admitted to intensive care, DXM decreased plasma levels of cytokines (IL-1, IL-2, IL-6, IL-8, IL-10, IL-17, IFN γ , and MIP-1) and endothelial markers but biomarkers of coagulopathy were not measured [35]. A third study reported that DXM use in severe COVID-19 patients was associated with a decrease of both proinflammatory and procoagulant profiles, however, the influence of DXM on coagulation disorders was indirectly estimated by a reduction in the need for heparin over time but no data on procoagulant or anticoagulant markers were provided [46].

Of note, hydroxychloroquine (HCQ) was given to the vast majority of patients in the no DXM group. This drug was indeed part of the usual treatment regimen during the first months of the pandemic. HCQ has immunomodulatory actions including inhibition of immune complex-mediated Toll-like receptors 7 and 9 preventing downstream type-1 interferon transcription. HCQ also decreases T-cell- and B-cell-mediated cytokine release [47]. Therefore, it may be argued that plasma cytokine levels could have been modulated by this drug in the no DXM group, decreasing the between-group differences with the DXM group. Yet, there is no published study comparing the level of the inflammatory biomarkers in COVID-19 patients that were treated or not with HCQ. In a cohort of seven patients with

mild respiratory symptoms, the impact of HCQ on cytokine kinetics remained unclear [48]. In our cohort, the three patients in the no DXM group who did not receive HCQ had cytokine levels similar to the overall group and did not influence the study results. Moreover, HCQ has no known effect on coagulation disorders.

In this study, patients included in the DXM group had more severe respiratory failure and a higher mortality rate than patients in the no DXM group. Indeed, the present study was not designed to show an impact on clinical outcome and the anti-inflammatory effect of DXM may have been underestimated by the higher severity of patients in the DXM group.

This study has several limitations. Firstly, the number of patients that were recruited was limited and the findings would need to be confirmed in a larger population. Changes in SARS-CoV2 variants, the decrease in COVID-19 severity, and the corresponding decrease in ICU admissions prevented further inclusion of patients in our study. Standard DXM treatment will of course prevent the recruitment of additional patients not treated with DXM. Secondly, all biomarkers were determined in a single sampling within the first days of ICU admission and their time course was not assessed. Finally, plasma biomarkers do not assess cellular reactivity and do not fully reflect the inflammatory and immune response since many effectors are located on the cell surface.

4. Materials and Methods

4.1. Design and Setting of the Study

This before and after intervention analysis comparing clinical outcomes, inflammatory reaction, and coagulopathy between critical COVID-19 patients that were treated or not with DXM was a sub-study of a monocenter, prospective, translational observational study. Adult patients were systematically included between 30 March 2020 and 11 November 2020.

4.2. Population

Patients with critical COVID-19 were those admitted to the ICU for moderate or severe ARDS due to SARS-CoV-2 infection. They were included within five days of admission. ARDS was diagnosed according to the Berlin definition [49], and SARS-CoV-2 infection was demonstrated by real-time reverse transcription PCR on nasopharyngeal swabs. The exclusion criteria were patients on antibiotics for any suspected or confirmed bacterial coinfections, therapeutic anticoagulation (oral or parenteral, including heparins, fondaparinux, vitamin K antagonists, and direct oral anticoagulants), recent (within less than one month) chemotherapy, active inflammatory disease, hemophilia and other coagulopathies, previous history of thrombocytopenia ($<100,000$ platelets/ mm^3), cirrhosis (Child–Pugh $> A$), recent (within less than 48 h) major surgery, cardiac arrest during ICU stay, and decision of care limitation. All patients received thromboprophylaxis using low-molecular-weight heparin (LMWH; nadroparin 3800 IU/days subcutaneously) and sampling was performed at least six hours after LMWH injection. Patients included in the first wave of COVID-19 (1 March to 30 June 2020) were compared with patients included in the second wave of COVID-19 (1 October to 30 December 2020), the latter having been routinely treated with low dose DXM on admission to hospital with oxygen supplementation. The treatment consisted of 6 milligrams of DXM per day, administered orally, for a period of 10 days.

4.3. Clinical Outcomes

Patient baseline characteristics and clinical outcomes were compared. Patient prognosis was assessed using acute physiologic assessment and chronic health evaluation II (APACHE II) [50] and SOFA [51] scores. Moreover, DIC and SIC were diagnosed using the International Society of Thrombosis and Haemostasis scoring at inclusion [42,43]. Data were collected from central medical records, including biological datasets that were routinely performed in patients that are admitted to the ICU such as platelet count, CRP level, coagulation assessment, renal function, and liver enzymology. Clinical outcomes were assessed 30 days and 1 year after ICU admission.

4.4. Sampling

Blood samples were collected through the central venous catheter using vacutainer tubes containing CPDA. After two centrifugation runs enabling platelet isolation, plasma was collected, apportioned into 1 mL aliquots, and stored at -80°C until use.

4.5. Measurement of Biomarkers

Soluble biomarkers of inflammation, coagulation, endothelial and platelet activation, and NETosis were measured using an enzyme-linked immunosorbent assay (ELISA) or suspension array sandwich immunoassays according to regulatory requirements for commercially available research use only. Frozen platelet poor plasma was thawed at room temperature the day of the experiment. The details of each marker that was analyzed are listed in the Supplemental Table S1. Each analytical run was performed in duplicate. The methods respected their respective validated lower limit of quantification and upper limit of quantification. Cytokines and chemokines were measured using Bio-Plex Pro Human Cytokine 27-Plex Panel (27-Plex) and Bio-Plex Human ICAM-VCAM (hICAM-hVCAM) (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol.

4.6. Statistics

The analyses were conducted using GraphPad Prism Version 9 (GraphPad Software, San Diego, CA, USA). Continuous variables were expressed as the mean $\pm$ standard deviation and categorical variables were expressed as number and percentage. The data were subjected to the Kolmogorov–Smirnov normality test and Bartlett's test for homogeneity of variance. Log transformations were performed when appropriate. The categorical variables were analyzed using the Chi-squared test or Fisher's exact test, and the continuous variables using unpaired Student's *t*-test as appropriate. A log-rank test was applied to compare the ICU length of stay and ventilation duration. All *p*-values were two-sided, and $p < 0.05$ was considered statistically significant.

5. Conclusions

In conclusion, treatment of critical COVID-19 patients with low dose DXM resulted in a reduction of the inflammatory and lymphoid immune response but had little impact on the myeloid immune response, and no effect on endothelial activation, NETosis formation, and COVID-19-induced coagulopathy with persistent elevated levels of circulating TF.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms24087278/s1>.

Author Contributions: Conceptualization, M.D. (Melanie Dechamps); Formal analysis, J.D.P., M.O., A.G., V.R., L.P., J.B., D.G., M.-A.V.D., J.D., H.H., L.M., M.D. (Marc Derive), L.J., L.B., S.H. and C.B.; Writing—review and editing, J.D.P. and P.-F.L. All authors have read and agreed to the published version of the manuscript.

Funding: The study was supported by a grant from the “Fondation Saint-Luc” (Brussels, Belgium). The funders had no role in study design, data collection and analysis, interpretation, or writing of the manuscript.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethics committee (B403201938590, NCT04107402).

Informed Consent Statement: Written informed consent has been obtained from the patients or their relatives to publish this paper.

Data Availability Statement: The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors would like to thank all the patients for their participation in this study, as well as Fatima Laarbaui, Olivier Van Caenegem, Sophie Pierard, Luc Jaquet, Philippe Hantson, and Christine Collienne for their help to enroll patients and Suzanne Renard, Caroline Berghe, Marie-France Dujardin, Leslie Gieslen, Brice Lambert, and all ICU nurses for their help at collecting samples.

Conflicts of Interest: This work was supported by grants from the Foundation Saint-Luc (Brussels, Belgium). The Division of Cardiology at Cliniques Universitaires Saint-Luc, Belgium, has received unrestricted research grants from AstraZeneca (Belgium). MD is Clinical Master Specialist Applicant to a Ph.D. at the Fonds National de la Recherche Scientifique et Médicale (FNRS, Belgium). JDP was supported by a grant from the Salus Sanguinis Foundation (UCLouvain, Belgium). MO, LP, and JB are supported by Fund for Research Training in Industry and Agriculture (FRIA, FNRS). LB and SH are senior research associates at FNRS. QUALIBlood s.a. offered the analyses for determining the cytokine profile.

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2. Review

Fibroblasts and platelets, a face-to-face dialogue at the heart of cardiac fibrosis.

Dufeys Cécile*, Bodart Julie*, Bertrand Luc, Beauloye Christophe and Horman Sandrine. *Am J Physiol Heart Circ Physiol* 2024 March.

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REVIEW

Cardiac Fibroblasts

Fibroblasts and platelets: a face-to-face dialogue at the heart of cardiac fibrosis

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Abstract

Myocardial fibrosis is a feature found in most cardiac diseases and a key element contributing to heart failure and its progression. It has therefore become a subject of particular interest in cardiac research. Mechanisms leading to pathological cardiac remodeling and heart failure are diverse, including effects on cardiac fibroblasts, the main players in cardiac extracellular matrix synthesis, but also on cardiomyocytes, immune cells, endothelial cells, and more recently, platelets. Although transforming growth factor- β (TGF- β) is a primary regulator of fibrosis development, the cellular and molecular mechanisms that trigger its activation after cardiac injury remain poorly understood. Different types of anti-TGF- β drugs have been tested for the treatment of cardiac fibrosis and have been associated with side effects. Therefore, a better understanding of these mechanisms is of great clinical relevance and could allow us to identify new therapeutic targets. Interestingly, it has been shown that platelets infiltrate the myocardium at an early stage after cardiac injury, producing large amounts of cytokines and growth factors. These molecules can directly or indirectly regulate cells involved in the fibrotic response, including cardiac fibroblasts and immune cells. In particular, platelets are known to be a major source of TGF- β . In this review, we have provided an overview of the classical cellular effectors involved in the pathogenesis of cardiac fibrosis, focusing on the emergent role of platelets, while discussing opportunities for novel therapeutic interventions.

antifibrotic drugs; antiplatelet drugs; cardiac fibroblast; platelets; TGF- β

CARDIAC FIBROSIS

Fibrosis is characterized primarily by excessive deposition of collagen and other extracellular matrix (ECM) proteins, leading to their accumulation in a tissue. This phenomenon is typically induced by an acute or chronic tissue injury and constitutes a major cause of organ dysfunction (1–3). Although cardiac dysfunction results from many etiologies including myocardial infarction, valvular diseases, hypertension, or myocarditis, they are almost all characterized by fibrous tissue formation. Indeed, as the heart has no regenerative capacity, the loss or damage of cardiomyocytes induces an inflammatory phase, which involves immune cell infiltration and upregulation of inflammatory genes leading to fibrosis (2–6). The type and the extent of fibrosis are closely related to the etiology of cardiac conditions (2, 7, 8). After a myocardial infarction, cardiac fibrosis is initially reparative, reflecting the replacement of dead cardiomyocytes by a collagen-based scar, called cicatricial or replacement fibrosis (2, 3, 6, 8, 9). The latter is therefore essential for survival in the early post-myocardial infarction stages. However, fibrosis

extends into the healthy noninfarcted tissue, known as interstitial fibrosis, inducing deleterious effects on cardiac structure and function (2, 7). Thus, fibrosis drastically alters myocardial compliance, increasing passive stiffness, and contributing to diastolic dysfunction (10). Small changes in collagen content have been shown to exert significant effects on the passive mechanical properties of the heart (11, 12). In addition, fibrosis disturbs cardiac electrical conduction, leading to a predisposition to arrhythmia (13–15). Finally, collagen accumulation around the small coronary arteries and arterioles can lead to microvascular dysfunction and impair the diffusion of oxygen from the vessels to the cardiomyocytes (16, 17). In other cardiac diseases, such as left ventricular pressure overload caused by aortic stenosis or hypertension, metabolic syndrome, and myocarditis, fibrosis is mainly interstitial as it develops in the absence of significant cardiomyocyte loss, increasing myocardial stiffness and causing diastolic dysfunction (2, 7–9). Ultimately, fibrosis accelerates the progression to heart failure and is strongly correlated with adverse outcome (11, 18–21). Therefore, myocardial fibrosis is a complex process that has become a prime focus of cardiac research.

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Submitted 8 September 2023 / Revised 2 January 2024 / Accepted 10 January 2024

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CLASSICAL CELLULAR EFFECTORS OF CARDIAC FIBROSIS

Cardiac fibroblasts are described as the main players in the synthesis of cardiac ECM proteins and the development of fibrosis in response to heart injury. However, there is increasing evidence that other cell types present in the injured heart, including immune cells, endothelial cells, and cardiomyocytes, also contribute significantly to fibrosis (6, 7). In recent years, several studies have suggested that blood platelets, by infiltrating the infarcted (22–27) or hypertensive myocardium (28–31), and by recognizing pathogens responsible for myocarditis (32, 33) are involved in the pathogenesis of cardiac fibrosis. The present review highlights this newly recognized role of platelets.

Cardiac Fibroblasts and Myofibroblasts

Cardiac fibroblasts are found within the cardiac tissue, surrounding cardiomyocytes, and are one of the largest cell populations in the mammalian heart (34–36). In a healthy heart, cardiac fibroblasts remain quiescent and maintain the cardiac ECM homeostasis (34, 37). By secreting structural ECM proteins such as collagen and elastin, cardiac fibroblasts provide a scaffold for cardiomyocytes and other cardiac cells, but also a storage network for latent growth factors, such as transforming growth factor- β (TGF- β) (8, 37). In addition, cardiac fibroblasts secrete enzymes involved in ECM turnover, such as matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), which regulate the balance between ECM synthesis and degradation (7, 38). Following cardiac injury, cardiac fibroblasts are activated and their proliferation and differentiation into myofibroblasts are promoted. Myofibroblasts have a cellular phenotype characterized by the expression of α -smooth muscle actin (α SMA) stress fibers (1, 3, 8, 34, 37). Interestingly, recent studies have pointed toward a spectrum of cardiac fibroblast phenotypes, which are probably more complex than a simple binary state between activated and quiescent fibroblasts (34). Multiple cell types, such as endothelial cells (39), pericytes (40), and bone marrow-derived myeloid cells, (41) have been suggested as potential sources of activated cardiac fibroblasts (1, 34). However, recent studies using lineage tracing strategies have revealed that most activated cardiac fibroblasts within the injured heart are derived from resident cardiac fibroblasts (42, 43). Activated cardiac fibroblasts display enhanced synthetic properties, and are the main source of structural ECM proteins, matricellular proteins, and enzymes involved in the ECM processing. They therefore contribute primarily to the development of fibrosis (3, 44–46). Cardiac fibroblast activation is triggered by a large variety of molecules, such as growth factors [TGF- β , platelet-derived growth factor (PDGF)], cytokines [interleukine-6 (IL-6), IL-1 β , tumor necrosis factor- α (TNF- α)], hormones (angiotensin II, aldosterone, epinephrine, norepinephrine), and neuropeptides (endothelin-1). These molecules are highly present in the pathological heart due to inflammation and adaptive responses (1, 2, 4, 8). The increased mechanical stress observed in the pathological heart also activates cardiac fibroblasts through mechanosensitive pathways, involving focal adhesions, integrins, stretch-activated channels, and transient receptor potential channels (1, 7, 47–49).

Immune Cells

Immune cells are key regulators of cardiac fibrosis as they orchestrate the inflammatory phase that precedes and triggers the fibrotic process. Neutrophils are recruited from the circulating blood to the site of injury by danger-associated molecular patterns (DAMPs) released from necrotic cardiomyocytes (38, 50, 51) and inflammatory mediators secreted by resident cardiac cells (52). Neutrophils are the first immune cells to invade the injured heart after myocardial infarction (38, 50, 51) or pressure overload (53). Infiltrated neutrophils secrete large amounts of ROS, myeloperoxidase, MMPs, cytokines, and chemokines that are involved in ECM degradation and monocytes recruitment to propagate the inflammatory response (38, 51, 54, 55). Interestingly, neutrophils play a critical role in the polarization of macrophages toward an anti-inflammatory and reparative phenotype. Therefore, neutrophil depletion in the infarcted mouse heart has been shown to enhance cardiac fibroblast activation and fibrosis (56).

Macrophages are the predominant immune cell type in the infarcted (50, 51, 57) and hypertensive heart (53). They are derived from circulating monocytes (58) and resident macrophages (38, 50, 51, 53). Their main role is to phagocytose dead cells and ECM debris. Macrophages also secrete cytokines, growth factors, proteases, and matricellular proteins participating in cardiac ECM remodeling (7, 51, 54, 55, 59, 60). Macrophages have been shown to regulate the activity of neutrophils by secreting paracrine factors (61). Moreover, macrophages and monocytes were found to directly regulate cardiac fibroblast activation by secreting fibrotic mediators (62–64). Finally, their phenotypic change during the process is a key step in the transition from inflammation to fibrosis (50, 55, 65).

The heart contains a number of resident mast cells located close to the vessels and in the epicardium (53). These cells store a wide range of molecules, such as TNF- α , histamine, and proteases, that are rapidly secreted upon degranulation and act as paracrine factors (38, 50, 53, 66, 67). Studies have shown an increase in the number of mast cells in the heart following myocardial infarction (67, 68) or pressure overload (53). Interestingly, resident cardiac mast cells have been described as important initiators of the postinfarct inflammatory response (67) and a major source of IL-6 in the heart following transaortic constriction (TAC) (53).

There is strong evidence to suggest that subpopulations of lymphocytes T and B infiltrate the heart after myocardial infarction (9, 50, 57, 69) or pressure overload (53) and may play a critical role in regulating the inflammatory and fibrotic responses. It has been reported that different lymphocyte subpopulations exert distinct roles in the fibrotic response, by both secreting various inflammatory mediators and by direct adhesion and actions on cardiac fibroblasts (7, 9, 50, 54, 57, 69).

Finally, dendritic cells have also been shown to infiltrate the infarcted (50, 70, 71) and hypertensive (53) heart where they regulate the inflammation and the angiogenesis.

The resolution of inflammation is a complex process crucial for fibrosis development, involving several mechanisms: the release of proresolving factors that promote inflammation resolution, the apoptosis of neutrophils, and their phagocytosis

by macrophages, as well as the transition of macrophages from a proinflammatory to an anti-inflammatory phenotype (38). Insufficient inflammatory response, as well as persistent inflammation, can promote further tissue damage (38, 56, 72).

Endothelial Cells

Endothelial cells are the most abundant nonmuscular cell type in the adult mammal heart and have been shown to contribute to cardiac fibrosis in several ways (36). First, endothelial cells are activated after cardiac injury, which induces upregulation of adhesion molecules on their surface, while promoting leukocyte recruitment to the site of injury (7, 52). In addition, endothelial cells are an essential source of cytokines and chemokines (57). Finally, endothelial cells can undergo endothelial-to-mesenchymal transition, thus contributing to the pool of activated cardiac fibroblasts (73). Although previous results reported that approximately 30% of activated cardiac fibroblasts are derived from endothelial-to-mesenchymal transition (39), a recent study using robust lineage tracing demonstrated that there are in fact very few endothelium-derived cardiac fibroblasts (42), indicating that the endothelial-to-mesenchymal transition plays only a limited role in cardiac fibrosis.

Cardiomyocytes

Following cardiac injury, necrotic cardiomyocytes release a wide variety of molecules (DAMPs) which are the main stimulus for the induction of the inflammatory response preceding fibrosis development (7, 8). Therefore, massive cardiomyocyte death after infarction promotes a significant inflammatory reaction followed by a considerable fibrotic response, whereas this phenomenon is less evident in other cardiac fibrosis models. Moreover, stressed cardiomyocytes have been reported to secrete cytokines, such as IL-6 and IL-11, influencing inflammatory cells (53) and cardiac fibroblasts (74, 75).

TRANSFORMING GROWTH FACTOR- β

TGF- β Expression, Activation, and Signaling

There are three TGF- β isoforms, including TGF- β 1, - β 2, and - β 3, which are encoded by distinct genes. While all three isoforms are expressed in the mammalian heart, their specific actions have not yet been studied *in vivo*, and the majority of studies have focused on the role of TGF- β 1. While cardiac fibroblasts, cardiomyocytes, leukocytes, and platelets actively produce TGF- β *de novo*, the relative contribution of each cardiac cell type to TGF- β secretion in the pathological heart remains unclear and therefore requires further investigation. TGF- β is initially secreted as a dimer, forming a complex with the latency-associated peptide (LAP), which maintains TGF- β molecules into a latent nonactive form. TGF- β /LAP complexes also bind to latent TGF- β binding protein (LTBP), keeping the secreted TGF- β within the ECM (76). Therefore, the cardiac ECM contains a large amount of latent TGF- β /LAP/LTBP that can be rapidly activated following injury. The latent complexes can be activated by several factors including proteases (MMPs, cathepsins, serine proteases, and calpain), integrins, reactive oxygen species (ROS), thrombospondin-1, and acidic environment (76–79). Once activated,

TGF- β binds to its constitutively active type II receptor (T β RII) on the surface of various target cells. It triggers the dimerization and phosphorylation of TGF- β type I receptor (T β RI), along with the activation of downstream intracellular signaling pathways. The canonical TGF- β -mediated pathway involves the transcriptional cofactors Smads. TGF- β can also activate noncanonical cascades, including the mitogen-activated protein kinases (MAPK), NADPH oxidase (Nox), phosphatidylinositol 3-kinase (PI3K), and small GTPases (47, 80).

TGF- β in the Injured Heart

TGF- β expression is significantly upregulated in animal models of myocardial infarction or pressure overload, as well as in patients with heart failure (76). The action of TGF- β depends on its concentration, specific characteristics of the cellular environment, and the type of injury (76). Consequently, it exerts pleiotropic effects in the injured heart, influencing immune-cell response, angiogenesis, cardiomyocyte survival, and cardiac fibroblast activation (4, 76, 80). Primarily, TGF- β serves as a key regulator of the immune response. Various lines of evidence suggest an anti-inflammatory role of TGF- β following myocardial infarction. Indeed, TGF- β has been shown to reduce neutrophil infiltration into the ischemia-reperfusion-subjected heart by decreasing endothelial cell-leukocyte interactions (81). In addition, systemic inhibition of TGF- β has been demonstrated to promote neutrophil recruitment and the expression of proinflammatory cytokines in a mouse model of ischemia-reperfusion (82). Finally, the myeloid cell-specific invalidation of Smad3 results in a defective transition of macrophages toward an anti-inflammatory phenotype after left anterior descending (LAD) coronary artery permanent ligation (83). Contrastingly, TGF- β has been reported to contribute to monocyte invasiveness into the infarcted heart (84). The specific deletion of TGF- β receptors in cardiomyocytes was found to attenuate neutrophil infiltration and MMP-9 activation after LAD permanent ligation, ultimately leading to reduced mortality due to wall rupture (85). Although TGF- β is well described in regulating T cell functions (86), its relative contribution to the regulation of these cells in the injured heart remains unknown despite evidence of inducing an anti-inflammatory environment in atherosclerotic plaques by modulating regulatory T cell activation (87). *In vitro* evidence suggests that TGF- β reduces endothelial expression of proinflammatory chemokines and adhesion molecules, thereby inhibiting leukocyte recruitment (88). However, TGF- β has been demonstrated to induce proinflammatory signaling in endothelial cells in a mouse model of atherosclerosis (89). The extent to which TGF- β contributes to angiogenesis and neovascularization *in vivo*, after myocardial infarction or pressure overload, remains unknown. The effect of TGF- β on cardiomyocytes is also controversial. It has been described to exert an antiapoptotic effect on cardiomyocytes through p42/p44 MAPK activation (90). Conversely, the cardiomyocyte-specific invalidation of Smad3 was shown to reduce cardiomyocyte apoptosis in rat and mouse models of ischemia-reperfusion (91). Adding to the complexity, mouse models of pressure overload have produced conflicting results, with reports of both prohypertrophic (92, 93) and antihypertrophic effects of TGF- β on cardiomyocytes (94). On the

contrary, the critical role of TGF- β in cardiac fibroblast activation and differentiation remains strongly established, irrespective of the specific pathological injury (1, 3, 8, 76).

PLATELETS

Platelets are small anucleate blood cells of 2- to 4- μ m diameter, which are derived from the hematopoietic lineage by fragmentation of megakaryocytes. This process, called thrombopoiesis, occurs continuously in the bone marrow or, as shown more recently, in the spleen and lungs (81). Thrombopoiesis results in the release into the circulation of 5,000 to 10,000 platelets per megakaryocyte. Platelets survive between 8 and 12 days in human blood, compared with 3–4 days in mice, before being removed from vessels by neutrophils and macrophages, and transported to the spleen and liver for elimination. They are the most prevalent blood components after red blood cells and are typically known to be central players in hemostasis and thrombosis. However, platelet functions extend far beyond hemostasis and are currently involved in the regulation of many physiological and pathological processes (81–83).

Platelets in Hemostasis and Thrombosis

The primary physiological function of platelets is hemostasis, where they contribute to the formation of a thrombus at a damaged vascular site to limit blood loss. Under physiological conditions, platelets circulate in the bloodstream in a resting state, without interacting with the subendothelium. However, in response to vascular injury caused by erosion or atherosclerotic plaque rupture, the integrity of the endothelial layer is disrupted, exposing the subendothelial extracellular matrix (ECM) to blood components. This event triggers platelet adhesion to the injured endothelium, followed by platelet activation, aggregation, and ultimately, the formation of a thrombus. It is important to note that while hemostasis is a normal physiological process, thrombosis represents a pathological condition. In thrombosis, a hemostatic clot forms within the lumen of a blood vessel, obstructing blood flow and leading to ischemia (95, 96).

Platelet adhesion to endothelium.

The initial anchoring results from the interaction between the endothelial von Willebrand factor (vWF) binding protein, attached to soluble vWF, and the platelet membrane glycoprotein (GP)Ib-IX-V, which leads to platelet rolling. This process enables a direct association between exposed collagen fibers and the platelet collagen receptors, GPVI and α 2 β 1 integrin, which stabilize platelet adhesion. Other adhesive proteins, including fibronectin, laminin, and thrombospondin, are also involved in the adhesion process by binding to their specific receptors at the platelet surface (83–85).

Platelet activation.

Platelet adhesion to the injured endothelium induces the activation of various platelet intracellular signaling pathways leading to an increase in cytosolic calcium (Ca^{2+}), with subsequent platelet activation (86). It results in the secretion of granules and the release of secondary agonists which further potentiates thrombus growth by recruiting and activating additional platelets at the site of injury.

Platelets contain two major types of secretory granules: α -granules and dense granules. The α -granules, ranging in diameter from 200 to 500 nm and numbering between 50 and 80 per platelet (97), constitute the most abundant secretory organelles in platelets. Upon platelet activation, they release a diverse array of soluble proteins into the circulation. This includes chemokines, growth factors, proangiogenic and antiangiogenic factors, coagulation factors, and immune mediators, as detailed in Table 1 (95, 97, 98). In addition, α -granules house adhesive proteins crucial for platelet-platelet and platelet-endothelial cell interactions. Among these granules are membrane-bound proteins, such as P-selectin and CD40L, which become expressed on the platelet surface, contributing to interactions with immune cells (95, 97, 99). Proteins within α -granules play essential roles in various physiological functions, encompassing hemostasis, thrombosis, inflammation, angiogenesis, wound repair, and antimicrobial host defense. Dense granules, approximately 150 nm in diameter with four to six per platelet, contain over 200 nonprotein molecules, including cations, nucleotides, polyphosphates, and bioactive amines (Table 1) (95, 97). These molecules predominantly participate in the secondary aggregation response, crucial for amplifying and sustaining platelet activation through positive feedback mechanisms (95, 97, 99–101). In addition to granules, activated platelets release lysosomes containing enzymes, such as acid hydrolases, involved in the degradation of proteins, lipids, and carbohydrates (102). The exact role of platelet lysosomes remains not fully understood. Furthermore, platelets secrete microvesicles (100–1,000 nm) that carry growth and angiogenic factors, coagulation factors, enzymes, adhesion molecules, chemokines, and cytokines, complement proteins, apoptosis regulators, bioactive lipids, and microRNAs (Table 1) (103, 104). Platelet-derived microvesicles are abundant in blood and are now recognized as important mediators of intercellular communication (104).

Platelets activation also results in the exposure of negatively charged phospholipids (phosphatidylserine) on platelet surface, supporting the binding and activation of coagulation factors (81). Activated platelets secrete thromboxane A_2 , which is synthesized from platelet membrane phospholipids, and acts as a secondary agonist by stimulating and recruiting additional platelets. Finally, platelet activation leads to a conformational change of the α 2 β 3 membrane receptor, from a low- to a high-affinity state for fibrinogen, resulting in aggregation through the formation of interplatelet bridges and the formation of platelet plug at the site of injury (87, 88).

Platelet aggregation and thrombus formation.

The thrombus formation is the result of a well-coordinated series of events. It is initiated by the activation of the coagulation cascade which is a series of enzymatic reactions, involving serine proteases called coagulation factors, and results in the conversion of prothrombin to thrombin. Thrombin ultimately converts fibrinogen to fibrin, which polymerizes into an insoluble fibrin network creating a fibrous blood clot to seal the injured area. Thrombin is also the most potent platelet agonist that strongly contributes to platelet activation and amplifies the coagulation cascade (88, 89). The coagulation cascade is a powerful response which is

Table 1. Examples of platelet α -granule and dense granule content

	Chemokines	Growth and Angiogenic Factors	Pro-/Anticoagulant Factors and Fibrinolytic Proteins		Membrane-Bound and Adhesive Proteins
			Immune Mediators		
α -Granules	I-309 (CCL1)	TGF- β 1	Factor V	Complement C3 precursor	P-selectin
	MCP-1 (CCL2)	VEGF	Factor XI		CD40L
	MIP-1 α (CCL3)	bFGF	Factor XIII	Complement C4 precursor	GP1b-IX-V
	RANTES (CCL5)	HGF	Prothrombin		GARP
	MCP-3 (CCL7)	IGF-1	α ₂ -macroglobulin	β IIH globulin	GPVI
	TARC (CCL17)	PDGF	Factor D		Fc receptors
	GRO- α (CXCL1)	BDNF	Plasmin	Factor H	PECAM
	PF-4 (CXCL4)	EGF	PAI-1	C1 inhibitor	vWF
	(CXCL4L1)	CTGF	TFPI	IgG	Vitronectin
	ENA78 (CXCL5)	Angiopoietin-1	Antithrombin	Thymosin- β 4	Fibronectin
	NAP-2, PBP, CTAPIII, β -thromboglobulin (CXCL7 and precursors)	MMP-1, -2, -9 TIMP-1, -4 BMP-2, -4, -6	Protein S Plasminogen	Thrombocidins	Fibrinogen Thrombospondin Fibrocystin
	IL-8 (CXCL8)	Angiostatin			Integrins
	SDF-1 α (CXCL12)	Thrombospondin Endostatin			Tetraspanins Glut-3 CD36 Scamp2 CD109
	Cations		Nucleotides		
	Ca ²⁺	Bioactive Amines			
	Mg ²⁺	Serotonin	ATP		
	K ⁺	Histamine	ADP		
	Polyphosphate				
	Pyrophosphate				
Dense granules					

MCP-1 and -3, monocyte chemoattractant protein-1 and -3; MIP-1 α , macrophage inflammatory protein-1 α ; TARC, thymus- and activation-regulated chemokine; PF-4, platelet factor-4; ENA-78, epithelial neutrophil-activating protein-78; NAP-2, neutrophil-activating peptide-2; PBP, platelet basic protein; CTAPIII, connective tissue-activating protein III; SDF-1 α , stromal cell-derived factor-1; TGF- β 1, transforming growth factor- β ; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HGF, hepatic growth factor; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; BDNF, brain-derived neurotrophic factor; EGF, epidermal growth factor; CTGF, connective tissue growth factor; MMP, matrix metalloproteinase; BMP, bone morphogenetic protein; PAI-1, plasminogen activator inhibitor-1; TFPI, tissue factor pathway inhibitor; GARP, glycoprotein a repetitions predominant protein; PECAM, platelet endothelial cell adhesion molecule; vWF, von Willebrand factor; Scamp2, secretory carrier membrane protein 2.

finely regulated by a complex inhibitory system called the fibrinolysis and mediated by the plasmin (90).

Platelets Beyond Hemostasis and Thrombosis: At the Core of Cardiac Fibrosis and Inflammation

A growing body of evidence supports that platelet functions extend far beyond hemostasis and thrombosis. Indeed, as previously explained, they exhibit the capability to release a wide variety of biomolecules that are functionally involved in a large number of physiological or pathological responses, such as angiogenesis (91), wound healing (92–94), inflammation, and fibrosis (Fig. 1) (29, 95, 99, 103).

In addition to endothelial injury, various other stimuli can prompt platelet activation and recruitment into the heart (Fig. 2). Platelets express pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and nucleotide-binding oligomerization domain-like receptors (NLRs), enabling them to recognize damage-associated molecular patterns (DAMPs) released by necrotic cardiomyocytes or pathogen-associated molecular patterns (PAMPs) expressed by pathogens (98, 105). Furthermore, in instances of vascular inflammation (such as systemic inflammation or atherosclerosis), activated endothelial cells express various factors responsible for platelet activation, including von Willebrand factor (vWF), tissue factor, reactive oxygen species

(ROS), and others (95, 98). Lastly, angiotensin II and oxidative stress observed in conditions like hypertension and/or metabolic syndrome may also activate platelets (29, 106–108).

Hence, platelets have been demonstrated to infiltrate both the vasculature and the interstitial myocardium following ischemia (22–27) or pressure overload (28–31)-induced injuries. Notably, in the ischemic mouse myocardium, platelet recruitment begins as early as 2 min after reperfusion, progressively increasing up to 60 min (25). This rapid recruitment underscores platelets as among the first blood cells to respond to ischemia-reperfusion injury, as the appearance of neutrophils occurs more slowly, starting from 30 min of reperfusion (25). Magnetic resonance and PET-scan imaging have confirmed the very early recruitment of platelets after ischemia-reperfusion, showing their presence in the ischemic myocardium reaching peak accumulation at 2 h after reperfusion, followed by a decline by 4 h (26, 27). During this period, platelets predominantly colocalize with leukocytes (27). In addition, Xu et al. (25) demonstrated that the activation and recruitment of platelets into the ischemic heart are dependent on the duration of ischemia. In a mouse model of LAD coronary artery permanent ligation, platelets were observed to accumulate in the heart as early as 6 h after myocardial infarction, becoming more prominent by 72 h. This study revealed that platelets constitute one of the initial waves of inflammatory cells accumulating within the

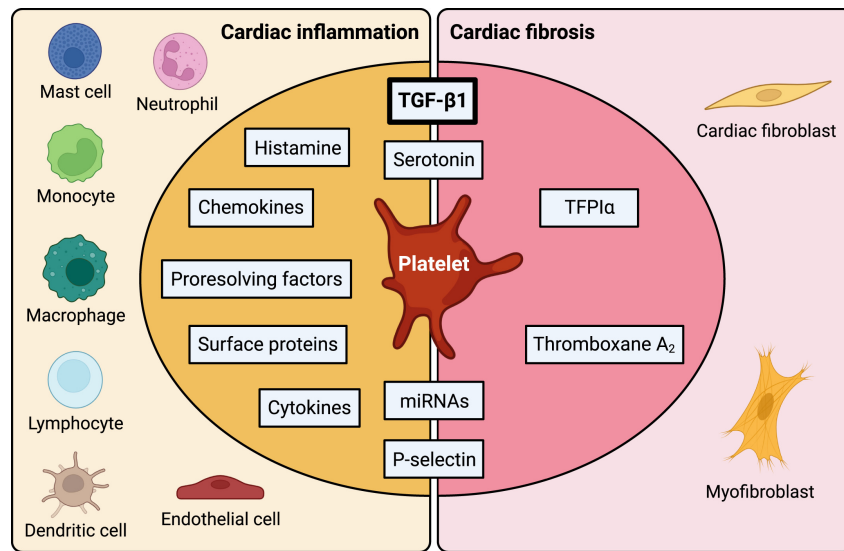


Figure 1. Contribution of platelet-derived mediators to myocardial inflammation and/or fibrosis. TFPI α , tissue factor pathway inhibitor- α ; TGF- β 1, transforming growth factor- β 1. Created with a licensed version of BioRender.com.

infarcted myocardium, colocalizing with leukocytes at 6 h post-MI (24). Although platelets were still present within the ischemic myocardium at 1 day and 7 days after ischemia-reperfusion (22), the question of whether platelets are cleared from the site of injury or persist in the late stages of cardiac fibrosis development after myocardial infarction remains to be investigated.

In the hypertensive heart, platelets have been observed after 7 days of angiotensin II infusion (28). However, in response to transverse aortic constriction (TAC), platelet recruitment was initiated as early as 3 days postsurgery. In this model, platelet accumulation reached its peak at 7 days after TAC, with a subsequent decline observed by 1 mo (30, 31). Furthermore, an elevation in circulating platelet activation was noted in mice at 3 days after TAC compared with the sham group, gradually decreasing at 1 wk and 4 wk postsurgery (30). The persistence of platelets within the heart at later stages of pressure overload-induced injury remains unknown. Interestingly, platelets were identified in the atrium of patients with hypertension suffering from atrial fibrillation. In addition, these patients with hypertension exhibited increased circulating platelet activity compared with normotensive individuals (29).

More recently, platelets have been demonstrated to accumulate and play a pivotal role in cardiac infectious diseases. Notably, they were implicated in a mouse model

of autoimmune myocarditis (32) as well as in human endomyocardial specimens with inflammatory myocarditis (33). In these investigations, platelets were observed to infiltrate the myocardium at 2 days following the induction of autoimmune myocarditis in mice, with their numbers significantly reduced after 2 wk and 1 mo (32, 33). Interestingly, it was revealed that the peak number of platelets occurred before immune cell infiltration, suggesting that platelets serve as a first-line defense against infection (32).

Platelets in cardiac fibrosis.

Platelets as a source of TGF- β . As previously explained, TGF- β is the most important factor in cardiac fibroblast activation and fibrosis development (1, 8, 76). Platelets constitute a major source of TGF- β 1, as they contain 40–100 times more TGF- β 1 than other cell types (95,96, 99, 103). Therefore, platelets are the leading regulator of TGF- β 1 concentrations in plasma. Indeed, it has been demonstrated that total TGF- β 1 levels are reduced by approximately 45% in the plasma of mice with a platelet-specific deletion of TGF- β 1. Furthermore, profound thrombocytopenia induced by injecting monoclonal anti- α IIb β 3 antibodies in mice, reduces plasma levels of total TGF- β 1 (103). Platelet TGF- β 1 is contained in α -granules and rapidly released upon

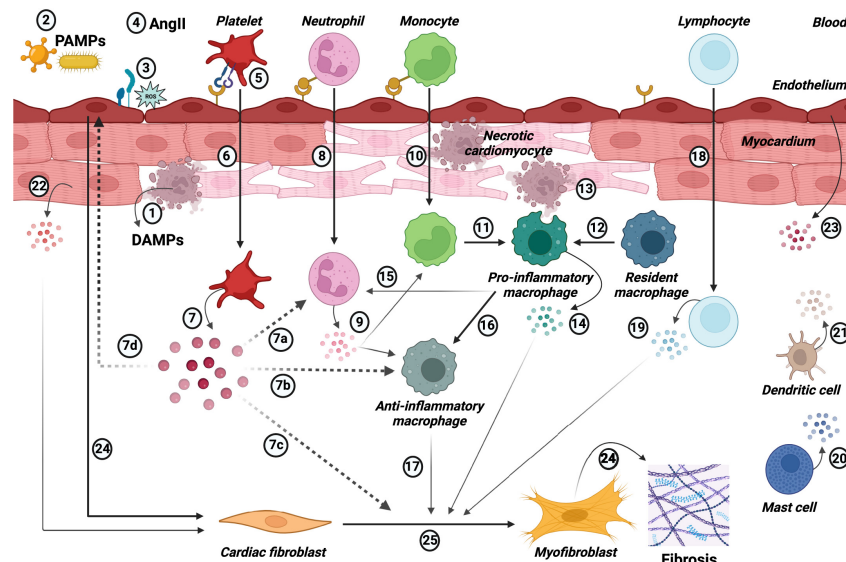


Figure 2. Platelets are at the core of cardiac inflammation and fibrosis. 1: necrotic cardiomyocytes release danger-associated molecular patterns (DAMPs), which are recognized by platelet-surface receptors, inducing platelet activation. 2: pathogens express pathogen-associated molecular patterns (PAMPs), which are recognized by platelet-surface receptors, inducing platelet activation. 3: activated endothelial cells express von Willebrand factor, tissue factor and reactive oxygen species (ROS) responsible for platelet activation. 4: hypertension induces angiotensin II (ANG II) expression responsible for platelet activation. 5: upregulation of adhesion molecules on endothelial cells surface promoting platelet and leukocyte recruitment to the site of injury. 6: infiltration of platelets into the injured myocardium. 7: platelets release factors influencing the inflammatory and fibrotic responses. 7a: platelets influence neutrophil recruitment. 7b: platelets influence differentiation of proinflammatory macrophages toward an anti-inflammatory phenotype. 7c: platelets influence cardiac fibroblast activation. 7d: platelets influence angiogenesis. 8: infiltration of neutrophils into the injured myocardium. 9: infiltrated neutrophils secrete factors, which are involved in extracellular matrix degradation, monocyte recruitment and polarization of macrophages toward an anti-inflammatory phenotype. 10: infiltration of monocytes into the injured myocardium. 11: infiltrated monocytes differentiate into proinflammatory macrophages. 12: activation of resident macrophages. 13: proinflammatory macrophages phagocytose dead cardiomyocytes and extracellular matrix debris. 14: proinflammatory macrophages secrete factors influencing neutrophil activity, extracellular matrix remodeling and cardiac fibroblast activation. 15: differentiation of macrophages from a proinflammatory toward an anti-inflammatory phenotype. 16: anti-inflammatory macrophages influence cardiac fibroblast activation. 17: infiltration of different subpopulations of lymphocytes into the injured myocardium. 18: infiltrated lymphocytes secrete factors influencing the inflammatory and fibrotic responses. 19: degranulation of resident mast cells influencing the inflammatory response. 20: dendritic cells secrete factors influencing inflammation and angiogenesis. 21: surviving cardiomyocytes secrete factors influencing immune cells and cardiac fibroblasts. 22: endothelial cells secrete factors influencing inflammatory and fibrotic responses. 23: endothelial-to-mesenchymal transition. 24: activation of cardiac fibroblasts and differentiation into myofibroblasts. 25: myofibroblasts secrete large amounts of extracellular matrix proteins inducing fibrosis. Created with a licensed version of BioRender.com.

platelet activation. Latent TGF- β 1 is present in two different pools in platelets. The first pool, representing 95% of total platelet TGF- β 1, corresponds to the TGF- β 1 associated with LTBP and LAP. The second pool corresponds to the TGF- β 1 complexed with LAP, without LTBP. Recent results suggest that the TGF- β 1/LAP/LTBP pool is released into plasma in a single step, whereas the TGF- β 1/LAP-containing complex is trapped on platelet surface before being released into plasma upon activation (109). Indeed, it has been found that activated platelets express TGF- β 1 on their extracellular surface through its binding to the glycoprotein A repetitions predominant protein (GARP) (100, 110). However, the

mechanism by which active TGF- β 1 is generated from latent TGF- β 1/GARP complexes on platelets remains poorly understood. Platelet TGF- β 1 was shown to modulate a number of biological processes including cardiac fibrosis. In this context, Meyer et al. provided the first evidence that TGF- β 1 originating from platelets contributes to cardiac fibrosis. Using a platelet specific TGF- β 1 deletion transgenic mouse model, these authors demonstrated that mice lacking platelet TGF- β 1 developed less interstitial and perivascular fibrosis following pressure overload generated by transverse aorta constriction. These mice were partially protected from cardiac hypertrophy and systolic dysfunction (103). A

few years later, Varshney et al. demonstrated that aortic stenosis progression could be delayed in the absence of platelet TGF- β 1. This was associated with a significant decrease in fibroblast myodifferentiation and fibrosis in aortic valve tissue (95). More recently, platelet-derived TGF- β 1 was found to promote angiotensin II-induced atrial fibrosis and atrial fibrillation in mice. In this study, TGF- β 1 from platelets was shown to directly promote myodifferentiation of cardiac fibroblasts (29).

Platelets as a source of other fibrotic molecules. In addition to TGF- β 1, numerous mediators of fibroblast activation are contained in platelet granules and microvesicles, and some of them have been shown to be involved in the regulation of cardiac fibrosis. Indeed, serotonin released by platelets has been described to directly affect neonatal rat cardiac fibroblasts by promoting their migration and myodifferentiation and by increasing the secretion of TGF- β 1 and MMPs (97). Furthermore, activated platelets have been found to stimulate cardiac fibrosis in a mouse model of angiotensin-II hypertension by secreting P-selectin (98). Then, platelet thromboxane A₂ was shown to stimulate cardiac fibroblast myodifferentiation, profibrotic gene expression, and consequently cardiac fibrosis (101). Platelet-derived tissue factor pathway inhibitor- α (TFPI α) was reported to prevent cardiac fibrosis by reducing thrombin generation and thrombin-mediated platelet activation in mice (102). Recently, platelets have emerged as a rich source of microRNAs (miRNAs), acting as paracrine mediators that tightly regulate surrounding cells, including cardiac fibroblasts (22, 111–114). Notably, despite lacking nuclei, platelets contain mRNAs, miRNAs, and the necessary enzymes for the conversion of precursor miRNAs (pre-miRNA) into mature miRNAs (115). Intriguingly, studies have revealed that platelet-derived miRNAs play a crucial role in modulating cardiac fibrosis following ischemia-reperfusion. In a genetic mouse model featuring megakaryocyte/platelet-specific inactivation of the pre-miRNA processing ribonuclease Dicer, inducing a disruption in miRNA processing in platelets, researchers observed worsened myocardial remodeling and exacerbated cardiac fibrosis in platelet-specific Dicer knockout mice subjected to transient left coronary artery ligation (22). Other molecules influencing cardiac fibroblasts such as thrombospondin-1 and PDGF are highly enriched in platelet granules (2, 104, 116). However, it is not clear whether these platelet-derived molecules directly contribute to cardiac fibrosis.

Although the correlation between platelet count in the bloodstream and liver fibrosis is well established (117, 118), there is currently a lack of studies investigating a similar correlation in the context of the heart. Interestingly, the mean platelet volume (MPV) has been identified as a prognostic marker in heart failure, predicting adverse cardiovascular events and associated with high mortality rates (119–123). However, the connection between MPV and cardiac fibrosis remains unexplored.

Platelets in cardiac inflammation.

Given the high interconnectedness between the inflammatory and fibrotic processes, platelets may regulate cardiac fibrosis indirectly by modulating inflammation. Indeed, in recent years, the role of platelets in inflammatory reactions

and immune response modulation has emerged and they are now considered as full-blown immune cells (81, 82, 124, 125). Platelets secrete a wide range of immunomodulatory factors (chemokines, cytokines, adhesive proteins, miRNA, etc.) containing in their α -granules and microvesicles. They also directly interact with leukocytes through their surface proteins (P-selectin, α 2b β 3 integrin, Gplb α), contributing to leukocyte recruitment, trans-endothelial migration, and activation (24, 25, 125). Therefore, circulating platelets have been shown to interact with monocytes, thereby promoting P-selectin-mediated delivery of platelet-derived proinflammatory factors, and ultimately the formation of atherosclerotic lesions in apolipoprotein-E-deficient mice (126). Moreover, platelets have been described to induce a proinflammatory phenotype in monocytes (127). Recently, platelets were reported to induce upregulation of osteopontin in macrophages, inducing inflammation into the abdominal aortic wall (128). In addition, platelet microvesicles-derived microRNAs were demonstrated to play an important role in cardiac inflammation following ischemia-reperfusion injury (22). Finally, platelet-derived serotonin was shown to induce neutrophil degranulation, enhancing post-myocardial infarction inflammation (129).

Nonetheless, platelets have also been described to exert anti-inflammatory effects. Indeed, they release an abundance of anti-inflammatory cytokines, such as TGF- β 1 and proresolving mediators including lipoxin A₄, maresin 1, and resolvin E1, which attenuate neutrophil trafficking and promote their apoptosis (125, 130, 131). Moreover, platelets were implicated in the modulation of macrophage phenotype toward their reparative function (93, 125, 132). Interestingly, platelets were shown to drive the differentiation of profibrotic macrophages by secreting CXCL4 (105). Altogether, these data reveal the complexity of platelets' contribution to inflammation and its resolution. Additional studies are needed to further clarify this process, which is challenging but might lead to attractive new therapeutic targets for the management of cardiac fibrosis.

ANTIFIBROTIC THERAPIES

Although cardiac fibrosis represents a major medical issue, current antifibrotic drugs have limited efficiency. Moreover, they are insufficient to inhibit fibrotic remodeling and avoid its progression to heart failure (4, 5, 80, 106). Therefore, the development of novel and effective antifibrotic therapeutic strategies for heart diseases is crucial. A wide range of studies have targeted classical signaling pathways involved in fibroblast activation and inflammation following injury (5, 7, 80, 106, 107). Numerous compounds are currently in clinical trials for the treatment of fibrosis from different etiologies. However, few have so far proved sufficient beneficial effects in the context of cardiac fibrosis (108). Indeed, while some of these led to promising results in reducing cardiac fibrosis in animal models, several major challenges for translating them into human clinical practice still remain.

Targeting TGF- β Signaling

Because of its critical involvement in the cardiac fibrotic response, TGF- β signaling remains an attractive therapeutic

target. To date, various types of TGF- β inhibitors have been tested in preclinical studies for the treatment of cardiac fibrosis. Neutralizing anti-TGF- β antibodies were used in rodent models of myocardial infarction and hypertrophic cardiomyopathy. These studies have been inconclusive, with conflicting results depending on the timing and dose of administration (133–136). In addition, cardiovascular toxicities of anti-TGF- β antibodies have been demonstrated (137). The soluble T β RII, which acts as a competitive inhibitor of TGF- β , has shown promising effects when injected on days 3–4 after myocardial infarction, whereas it has been ineffective when injected a few weeks later (138, 139). On the contrary, Ikeuchi et al. have demonstrated that soluble T β RII overexpression during the early stage after myocardial infarction exacerbates ventricular dilatation and increases mice mortality, whereas the inhibition of TGF- β signaling during the later stage after myocardial infarction alleviates cardiac remodeling (140). Interestingly, adenoviral overexpression of decorin, a TGF- β antagonist, has been shown to inhibit hypertension-induced cardiac fibrosis in rats (111). GW788388 is a TGF- β receptor I (ALK5) inhibitor that has been demonstrated to reduce fibrosis after myocardial

infarction in rats (112). It has also been found to inhibit cardiac fibrosis in an experimental mouse model of Chagas' heart disease (113, 114). Although GW788388 appears to have beneficial antifibrotic effects, these preclinical data need to be confirmed. Indeed, SM16, another ALK5 inhibitor, has been shown to induce beneficial effects on pressure overload-induced cardiac fibrosis while increasing left ventricular dilatation and mortality in mice (115). In addition, ALK5 inhibitors have been found to induce heart valve damage in rats (141). Pirfenidone is an antifibrotic drug currently approved for the treatment of idiopathic pulmonary fibrosis. Although its exact mechanism of action is not fully understood, it is thought to exert its effects by inhibiting TGF- β expression. The beneficial effects of Pirfenidone on cardiac fibrosis have been demonstrated in several animal models (117–121, 142). The PIROUETTE clinical trial demonstrated that administration of pirfenidone is associated with a significant reduction in cardiac fibrosis whereas it unfortunately does not improve diastolic function (122). Finally, tranilast is used for the treatment of allergic disorders. Although its mechanism of action is not fully understood, tranilast inhibits the expression and activity of TGF- β . It has been shown to

Table 2. Preclinical *in vivo* studies using antiplatelet drugs and their effects on cardiac fibrosis

Dose	Administration	Treatment Duration	Animal	Model	Major Findings	Reference
Clopidogrel 50 mg/kg/day	ig	For 7 days	Mouse	ANG II	↓ Inflammation ↓ Fibrosis	(28)
Clopidogrel Loading: 15 mg/kg/day Maintenance: 5 mg/kg/day	ig	For 3 wk	Mouse	ANG II	↓ inflammation ↓ fibrosis ↓ atrial fibrillation	(29)
Ticagrelor Acute: 10 or 30 mg/kg Chronic: 300 mg/kg/day	Acute: ip Chronic: in diet	Acute: 5 min before reperfusion Chronic: Starting 1 day after I/R, for 4 wk	Rat	I/R	↓ infarct size ↓ apoptosis ↓ fibrosis Improve cardiac function	(146)
Clopidogrel Acute: 12.5 mg/kg Chronic: 62.5 mg/kg/day					No effect	
Aspirin 25 mg/kg/day	ip	From 2 days before to 21 days after LAD	Rat	LAD	↓ fibrosis	(147)
Aspirin 10 mg/kg/day	ig	From 4 days before to 4 wk after TAC	Mouse	TAC	↓ fibrosis	(148)
Ticagrelor 300 mg/kg/day Aspirin 20 mg/kg/day Prasugrel 15 mg/kg/day Ticagrelor + Aspirin 300 and 20 mg/kg/day	Chow	From 1 wk after I/R, for 1 wk or 3 wk	Rat	I/R	Improve cardiac function ↓ fibrosis ↑ regenerative cells No effect	(149)
Aspirin 15 mg/kg Clopidogrel 11 mg/kg Ticagrelor 27 mg/kg Prasugrel 1.5 mg/kg	ig	1 wk before LAD or From 1 wk before to 1 wk after LAD or 1 wk after LAD	Mouse	LAD	↓ inflammation ↓ fibrosis Improve cardiac function when treating before surgery	(150)
Cilostazol 0.1% wt/wt	Chow	From 7 days before to 28 days after ANG II	Mouse	ANG II	↓ hypertrophy ↓ fibrosis	(151)

ig, intragastrically; ip, intraperitoneal; ANG II, angiotensin II; TAC, pressure overload generated by transaortic constriction; LAD, myocardial infarction induced by permanent ligation of left anterior descending coronary artery; I/R, ischemia-reperfusion.

reduce cardiac fibrosis in rodents. In summary, although some anti-TGF- β therapies have shown encouraging results, the pleiotropic effects as well as the context and time-dependent actions of TGF- β after cardiac injury make anti-TGF- β therapy challenging. Therefore, more specific agents should be developed to safely and robustly inhibit TGF- β signaling.

Platelets as a New Potential Therapeutic Tool for Cardiac Fibrosis

Given the key role of platelets in inflammatory and fibrotic processes, platelet-targeting may be a promising therapeutic strategy (Table 2). The use of antiplatelet drugs has already shown encouraging beneficial effects in the treatment of liver and pulmonary fibrosis (123, 143, 144). In the cardiovascular field, platelet depletion with an anti-GPIIb/IIIa antibody strongly reduces thrombus fibrosis and adverse vein wall remodeling in a mouse model of inferior vena cava stenosis, indicating that platelets are a major contributor to thrombus fibrosis (145). Several studies have found that treatment with antiplatelet drugs significantly reduces fibrosis in different models of cardiac injury. First, the inhibition of platelet activation by the irreversible ADP receptor blocker clopidogrel has been shown to prevent cardiac inflammation and fibrosis in a mouse model of angiotensin II-induced hypertension (28). More recently, Liu et al. (29) demonstrated that clopidogrel reduces angiotensin II-induced platelet accumulation, inflammation, and fibrosis in the atrium. Second, ticagrelor, a reversible ADP receptor blocker, has been described to decrease collagen expression and fibrosis after ischemia-reperfusion injury. Interestingly, despite a similar degree of platelet inhibition, clopidogrel had no effect on cardiac fibrosis in this study (146). Inhibition of cyclooxygenase-1 by aspirin has been shown to decrease interstitial fibrosis in infarcted rat hearts (147) and attenuate pressure overload-induced cardiac fibrosis by inhibiting ERK1/2 and Akt/ β -catenin signaling pathways in mice (148). Moreover, ticagrelor and aspirin have been found to reduce collagen expression in a rat model of ischemia-reperfusion (149). A recent study showed that different anti-platelet drugs, namely aspirin, clopidogrel, ticagrelor and prasugrel, significantly reduce inflammatory cell infiltration and cardiac fibrosis in a mouse model of myocardial infarction (150). Finally, Cilostazol has been found to reduce angiotensin II-induced perivascular and interstitial fibrosis in mice through the activation of the cyclic AMP-protein kinase A pathway (151).

CONCLUSIONS

Although current antifibrotic therapies have proven to slow the progression of heart failure, they remain insufficient. It is therefore essential to find new therapeutic strategies targeting cardiac fibrosis. Ongoing research is focusing on novel therapeutic strategies based on cell transplantation or reprogramming, biomaterials engineering, noncoding RNAs, and epigenetic modifiers (4, 106, 107). However, the complexity of the cell types and signaling pathways involved in the fibrotic process makes these approaches rather difficult. Platelets are classically known for their role in hemostasis and thrombosis, but it is now well described that their

function extends far beyond these processes. They have been shown to infiltrate the myocardium in the very acute phase after myocardial infarction (22–27), pressure overload (28–31), and myocarditis (32, 33), where they secrete large amounts of molecules, therefore directly or indirectly regulating cells involved in the fibrotic response, including cardiac fibroblasts and immune cells. Therefore, platelets represent an interesting candidate for the management of cardiac fibrosis. Indeed, several studies have demonstrated the beneficial effects of antiplatelet therapies on the cardiac fibrotic response (28, 29, 145–151). However, the exact molecular and cellular mechanisms involved are still poorly understood, requiring further research. As platelets store a large number of molecules, it would be interesting to study the relative contribution of platelet-derived profibrotic factors to myocardial fibrosis. In this regard, the use of transgenic mice with invalidation of megakaryocyte-specific genes would provide additional knowledge.

Nonetheless, TGF- β is well established as a key regulator of cardiac fibrosis, and platelets have been identified as a significant source of TGF- β 1 (130–133). The specific targeting of platelet-derived TGF- β could prove to be a promising therapeutic avenue. In this regard, the GARP protein emerges as a potential candidate. GARP associates with latent TGF- β 1 on the platelet surface, triggering its activation (152, 153). While GARP expression has been identified in T-regulatory lymphocytes, platelets, and some endothelial and fibroblast cell lines, only T-regulatory lymphocytes and platelets have been demonstrated to generate active TGF- β 1 from GARP/latent TGF- β 1 complexes (135, 152, 153). Therefore, investigating the role of platelet GARP in active TGF- β 1 production following heart injury holds significant interest. GARP represents a promising target that could play a crucial role in TGF- β 1 activation in the injured heart.

GRANTS

C.D. and J.B. are supported by a fellowship from the Fonds National de la Recherche Scientifique et Médicale (FNRS, Belgium) and the Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA, FNRS, Belgium), respectively. L.B. is supported by a grant from Walloon Excellence Research Institute WELRI-WELBIO (Belgium). S.H. works as a senior research associate at FNRS, Belgium.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.D. prepared figures; C.D., J.B., and S.H. drafted manuscript; C.D., J.B., L.B., C.B., and S.H. edited and revised manuscript; C.D., J.B., L.B., C.B., and S.H. approved final version of manuscript.

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