Mechanistic and therapeutic studies in cardiac inflammation

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2024

Document Version:
Publisher's PDF, also known as Version of record

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Gabriel Jakobsson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University, Sweden.

To be publicly defended on 13th of September at 13.00 in Agardh Lecture Hall, Clinical Research Center, Jan Waldenströms Gata 35, Malmö, Sweden

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Organization: LUND UNIVERSITY

Document name: DOCTORAL DISSERTATION **Date of issue:** September 13, 2024

Author(s): Gabriel Jakobsson **Sponsoring organization:**

Title and subtitle: Mechanistic and therapeutic studies in cardiac inflammation

Abstract: Cardiovascular disease (CVD) is the leading cause of death globally. Inflammatory cardiomyopathies can be life-threatening, and the underlying mechanisms are not fully understood. This thesis aimed to investigate the underlying mechanisms driving the pathogenesis of myocarditis and septic cardiomyopathy, and to explore potential treatment strategies. In particular, we focused on the pro-inflammatory neutrophil mediator S100A8/A9 and on IL-1RAP, a common accessory protein required for IL-1α, IL-1β, IL-33 and IL-36 receptor signaling.

To study the role of S100A8/A9 in sepsis-induced myocardial dysfunction (SIMD), we measured plasma S100A8/A9 levels in patients with severe sepsis. S100A8/A9 was significantly higher in patients with left ventricular dysfunction compared with patients with normal cardiac function. Further, S100A8/A9 blockade with a small-molecule inhibitor improved cardiac dysfunction and reduced systemic and cardiac inflammation in a mouse model of endotoxemia. S1009 knockout mice were similarly protected, suggesting a direct pathogenic role of S100A8/A9 in SIMD. Lastly, we found that S100A8/A9 inhibition was faster and more effective than glucocorticoids against SIMD development in endotoxemia.

In a mouse model of autoimmune myocarditis, we investigated the role of neutrophil-derived S100A8/A9 in the pathogenesis of the disease, by depleting neutrophils and blocking S100A8/A9. Neutrophil depletion prevented cardiac dysfunction, confirming their pathogenic role. S100A8/A9 blockade limited the recruitment of innate immune phagocytes into the myocardium, reduced myocardial apoptosis, and improved cardiac function. By single-cell RNA sequencing analysis, we revealed for the first time five distinct neutrophil subpopulations infiltrating the heart in myocarditis. Mechanistically, S100A8/A9 blockade polarized the infiltrating neutrophils from an inflammatory phenotype into a predominantly immunomodulatory one, preventing excessive inflammatory activation in the myocardium.

Lastly, we tested treatment with an anti-IL-1RAP monoclonal antibody, blocking IL-1α, IL-1β, IL-33 and IL-36 signaling, in two murine models of myocarditis. The anti-IL-1RAP antibody reduced the severity of both viral and autoimmune myocarditis. The treatment prevented long-term left ventricular dysfunction in mice with autoimmune myocarditis compared with isotype controls and IL-1β blockade alone. Importantly, IL-1RAP blockade potently reduced myeloid and T cell infiltration into the myocardium but did not hinder viral clearance.

We demonstrate a direct pathogenic role for S100A8/A9 in SIMD and autoimmune myocarditis, and the involvement of IL-1RAP-mediated signaling in the pathogenesis of both autoimmune and viral myocarditis. Our findings promote S100A8/A9 blockade as a potential future treatment for severe sepsis patients with SIMD. Both small-molecule S100A8/A9 blockade and antibody-mediated IL-1RAP inhibition are promising immunomodulatory therapies that warrant further development for treatment of patients with acute myocarditis.

Key words: inflammatory cardiomyopathy; sepsis; sepsis-induced myocardial dysfunction; myocarditis; neutrophils; inflammation; s100a8/a9; IL-1RAP; immunomodulation

Classification system and/or index terms (if any) Supplementary bibliographical information

Language English **Number of pages**: 79

ISSN and key title: **1652-8220**

ISBN: 978-91-8021-595-4

Recipient's notes Price Security classification

Signature  Date 2024-08-05

Mechanistic and therapeutic studies in cardiac inflammation

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LUND
UNIVERSITY

Faculty of Medicine
Department of Translational Medicine

ISBN 978-91-8021-595-4
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2024

To my family and friends

Table of Contents

Preface 10
Acknowledgements 11
Populärvetenskaplig sammanfattning 13
Original papers 15
Papers not included 16
Abbreviations 18

The immune system 20
Self-non-self and the danger theories 20
Components of the immune system 21
The innate immune system 22
Neutrophils 22
Eosinophils, Basophils and Mast cells 24
Monocytes and macrophages 25
Dendritic cells 27
Innate lymphoid cells and Natural killer cells 28
The Adaptive Immune Response 28
T cells 28
CD8+ T cells 29
CD4+ T cells 29
B cells 30
Cytokines, Chemokines and Soluble mediators 31
S100A8/A9 32
Interleukin-1 family 34

Cardiovascular disease 37
Sepsis and septic shock 38
S100A8/A9 in sepsis 39
Sepsis-induced myocardial dysfunction (SIMD) 39
Myocarditis 40

Etiology of myocarditis 41
Pathogenesis of myocarditis 41
S100A8/A9 and IL-1 signaling in myocarditis 43

Experimental methods 44

Experimental models of endotoxemia and sepsis 44

Models of myocarditis 45
Experimental autoimmune myocarditis (EAM) 45
CVB3 induced viral myocarditis 46

Treatment strategies 46
S100 inhibition 46
Anti-IL1RAP blocking antibodies 47

Echocardiography 47

Flow cytometry 48

Aim 49

Specific aims 49

Paper I 50

Therapeutic S100A8/A9 blockade inhibits myocardial and systemic inflammation and mitigates sepsis-induced myocardial dysfunction 50
Introduction, methods and results 50
Conclusion and limitations 54

Paper II 55

S100A8/A9 drives inflammatory neutrophil polarization and promotes cardiac dysfunction in autoimmune myocarditis 55
Introduction, methods and results 55
Conclusion and limitations 58

Paper III 60

IL-1 receptor accessory protein (IL1RAP) blockade with a monoclonal antibody reduces cardiac inflammation and preserves heart function in viral and autoimmune myocarditis 60
Introduction, methods and results 60
Conclusion and limitations 63

Summary and clinical perspective 64

References 67

Preface

"If I have seen further, it is by standing on the shoulders of giants."
- Isaac Newton

The progress of science is incremental, and as I now finish writing my thesis, the culmination of four years of doctoral studies and a few more years of university studies, I feel this to ring truer than ever. As is evident from the introduction chapters of this book, the corpus of knowledge that is the foundation for this work dwarfs the rather miniscule contribution that I can call my own. As our knowledge of the natural world expands, we uncover the marvelous complexity that underlies biology. But this complexity forces us as scientists, hoping to broaden the horizon of what we know, to narrow our frame of focus to ever so smaller aspects of biology. I cannot help to feel that in this quest to discern the miniscule intricacies of isolated pathways, we lose the ability to discover truths about the system as a whole. Although it leaves me slightly unfulfilled, I know that this specialization is inevitable, and is perhaps even desirable.

I have been fortunate enough to work with projects that which are very translational in nature. As I have written what feels like a million times during these last years, there is an unmet need for therapies that target pathological inflammation, but in the context of cardiovascular disease this is especially true. The biological mechanisms that I have studied could offer insights into potential therapeutic strategies, and to develop treatments for patients is a tangible goal that has given me purpose during my doctoral studies. In fact, all of the projects I have undertaken were designed to test potential therapeutics, and although I often times saw them as tools to study the underlying biological mechanisms, they could perhaps make it to patients one day.

With this in mind, I hope that this work can at least offer you the reader some insight into the immunological mechanisms of cardiac inflammation. That perhaps you can learn something, or just marvel at how cool biology is, as I have done many times throughout my studies.

Acknowledgements

Somewhere along the line I fell upon the path of doing a PhD, and I am not really sure where it started. I think I partially just thought that science was cool, and doing a doctorate where I could do my own experiments and design research questions seemed like a good opportunity to see for myself. Looking back, I am grateful that I didn't hesitate and instead jumped headfirst into something that I didn't quite grasp the consequences of. As I finish writing this book, it is clear to me that none of this would have been possible without the help and assistance from so many individuals.

Alexandru Schiopu, my main supervisor. I am grateful to you for teaching me how to do research. At the start of this journey the group was just the two of us, passionate discussions in conference rooms during late afternoons, but always fruitful, interesting and rewarding. I thank you for believing in me, entrusting me with a ridiculous degree of autonomy, and teaching me almost everything I know about cardiology. I want to thank you for always allowing me to question and argue my interpretations and for listening with an open mind, and for challenging me to convince you, forcing me to evolve and mature intellectually. These years have taught me more then I ever could've imagined, and I am forever grateful for offering me this PhD position.

Perhaps my journey towards today started during an immunology lecture… **Daniel Engelbertsen**, my co-supervisor. If you wouldn't have taken me in as a Master's student, this would've never happened. I want to thank you from the bottom of my heart for being my co-supervisor during these years. Thank you for offering me guidance and support whenever I needed it, and for always being available to me despite it being "10-minutes before your train leaves".

From classmate to fellow masters student to PhD colleague and office mate, **Megan**, I am not really sure where to begin. Thank you for being a friend and colleague throughout these years. We have shared the joys and woes of being doctorate students, and you were always there when I needed to ventilate about experiments not working, and for always being there to offer advice on how to make my emails to admin staff sound more passive aggressive.

To **Pernilla**, **Samuel** and **Anthi**, and **Bao** thank you for the fun moments, both at lab and during our AWs, they made these 4 years all the merrier.

To **Harry**, **Eva** and **Jan**, I thank you for always providing thoughtful advice during our Friday meetings and offering your insights, both regarding science and otherwise.

To the foundation of the lab, **Linda**, **Fong**, **Filiz**, **Lena**, **Mihaela, Irena**, I thank you for always humoring me when I asked for advice regarding methods and experiments, and for all the laughs we have shared.

To the whole of the CRC lab groups in the Kardiovask Forskning and CPIP umbrella, thank you for offering laughs, scientific input and providing a wonderful work environment these years. I couldn't wish for a better place to work.

To my current and past colleagues, **Jenifer**, **Greg, Laura, Neelaka,** thank you for being great colleagues, for helping me with all the experiments, and providing input during discussions and presentations.

To **Kuba, Casper, Elliot**, **Emily, djungeltrumman** and beyond, thank you for being the best of friends and offering respite from the stresses of a PhD.

To **Victoria,** thank you for supporting and believing in me. Especially during the writing of this book, which took many late nights and early mornings away from us, and I am ever grateful for you being there for me.

To my family; **Isak**, **David**, **mom** and **dad**, and **Siv** thank you for always supporting me. For believing in me and being there to offer your support when needed, for being empathetic with my sometimes strange work hours and lack of past time. As stated in the opening of this book, I dedicate this work to you.

Populärvetenskaplig sammanfattning

Hjärt-kärlsjukdomar är den främsta dödsorsaken världen över, och i Sverige lider ungefär 2 miljoner människor av någon form av hjärt-kärlsjukdom. De vanligaste formerna av hjärt-kärlsjukdom är hjärtinfarkt och stroke till följd av åderförkalkning, men en mindre känd form av hjärt-kärlsjukdomar är de inflammatoriska hjärt-sjukdomarna, som särskiljer sig genom att bland annat ofta förekomma i unga människor. Denna avhandling fokuserar på två sådana tillstånd, septisk kardiomyopati och hjärtmuskelinflammation.

Septisk kardiomyopati sker till följd av den dödliga sjukdomen sepsis, även kallat blodförgiftning. Vid sepsis överaktiveras kroppens immunförsvar till följd av en infektion och löper amok, vilket leder till en systemisk inflammation som lamslår hjärtat och andra organ. Hjärtdysfunktion i sepsis är en ledande dödsorsak, och det finns i dagsläget inga behandlingar som minskar hjärtinflammationen i sepsis. Hjärtmuskelinflammation, även kallat myokardit, är som namnet antyder en inflammation i hjärtmuskeln. Myokardit går i de flesta fall över av sig själv, men i de fallen då inflammationen består leder myokardit till akut hjärtsvikt, med hjärttransplantation som enda behandlingsalternativ. Denna avhandling har som mål att utforska de underliggande mekanismerna i dessa två sjukdomstillstånd, samt utvärdera potentiella terapeutiska strategier. Centralt för denna avhandling är fokus på att reglera immunförsvaret, och till detta ändamål har vi undersökt rollen av den lösliga molekylen S100A8/A9, samt signalering via receptorn IL-1RAP, i inflammatorisk hjärt-kärlsjukdom.

Neutrofiler, den vanligaste immuncellen i vår kropp, släpper ut stora mängder av det inflammatoriska proteinet S100A8/A9 under inflammation. Vi visade att S100A8/A9 nivåer i blodet är förhöjda i patienter, och kan särskilja sepsis patienter med eller utan hjärtsvikt. I experimentella modeller av septisk kardiomyopati och myokardit så ämnade vi att undersöka rollen av S100A8/A9, och att blockera molekylens signalering med ett läkemedel. Genom att blockera S100A8/A9 kunde vi påvisa att hjärtfunktionen blir bättre i båda sjukdomsmodeller, och att inflammationen minskade markant. Inom myokardit undersökte vi även inhibering av IL-1RAP, en receptor på immunceller som reagerar på de inflammatoriska proteinerna IL-1, IL-33 och IL-36. Proteiner som släpps ut av immunceller och bidrar till skadlig immunaktivering. Genom att blockera IL-1RAP med en antikropp, kunde vi studera dess patologiska roll i myokardit. Blockering av IL-1RAP minskade infiltrationen av immunceller, och kunde förbättra hjärtfunktionen i två modeller av experimentell myokardit. Sammantaget banar mina resultat vägen för kliniska studier där dessa två behandlingar testas mot hjärtinflammation.

Sammantaget så utforskar denna avhandling två unika strategier att bekämpa hjärtinflammation, och förhoppningsvis leder dessa resultat till framtida behandlingsstrategier som kan hjälpa patienter, då det i dagsläget saknas alternativ.

Original papers

I. **Jakobsson G**, Papareddy P, Andersson H, Mulholland M, Bhongir R, Ljungcrantz I, Engelbertsen D, Björkbacka H, Nilsson J, Manea A, Herwald H, Ruiz-Meana M, Rodríguez- Sinovas A, Chew M, Schiopu A. Therapeutic S100A8/A9 blockade inhibits myocardial and systemic inflammation and mitigates sepsis-induced myocardial dysfunction. Crit Care. 2023 Sep 29;27(1):374. doi: 10.1186/s13054-023-04652-x.

II. **Jakobsson G**, Mulholland M, Vallejo J, Tomas L, Chaffey L, Bhongir R, Ljungcrantz I, Rattik S, Manea A, Björkbacka H, Engelbertsen D, Schiopu A. S100A8/A9 drives inflammatory neutrophil polarization and promotes cardiac dysfunction in autoimmune myocarditis. *Manuscript - submitted to Cardiovascular Research*

III. Lema DA*, **Jakobsson G***, Daoud A, Elias D, Talor MV, Rattik S, Grönberg C, Kalinoski H, Jaensson Gyllenbäck E, Wang N, Liberg D, Schiopu A*, Ciháková D* IL-1 receptor accessory protein (IL1RAP) blockade with a monoclonal antibody reduces cardiac inflammation and preserves heart function in viral and autoimmune myocarditis. *Manuscript - In revision, Circulation: Heart Failure.* * Equal contribution.

Papers not included

I. Mulholland M, **Jakobsson G**, Lei Y, Sundius L, Ljungcrantz I, Rattik S, Tietge UJF, Engelbertsen D. IL-2Rβγ signalling in lymphocytes promotes systemic inflammation and reduces plasma cholesterol in atherosclerotic mice. Atherosclerosis. 2021;326:1–10.

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IV. Ding Z, Du F, V RGA, **Jakobsson G**, Rönnow C-F, Rahman M, Schiopu A, Thorlacius H. Targeting S100A9 Reduces Neutrophil Recruitment, Inflammation and Lung Damage in Abdominal Sepsis. Int J Mol Sci. 2021;22:12923.

V. Martínez MA, Ritsvall O, Bastrup JA, Celik S, **Jakobsson G**, Daoud F, Winqvist C, Aspberg A, Rippe C, Maegdefessel L, Schiopu A, Jepps TA, Holmberg J, Swärd K, Albinsson S. Vascular smooth muscle-specific YAP/TAZ deletion triggers aneurysm development in mouse aorta. JCI Insight. 2023;8:e170845.

VI. **Jakobsson G**, Andersson H, Chew M, Schiopu A. Reply to "Potential confounders in linking elevated S100A8/A9 to left ventricular dysfunction in septic shock patients." Crit Care. 2024;28:9.

VII. Mulholland M, Depuydt MAC, **Jakobsson G**, Ljungcrantz I, Grentzmann A, To F, Bengtsson E, Gyllenbäck EJ, Grönberg C, Rattik S, Liberg D, Schiopu A, Björkbacka H, Kuiper J, Bot I, Slütter B, Engelbertsen D. Interleukin-1 receptor accessory protein blockade limits the development of atherosclerosis and reduces plaque inflammation. Cardiovasc Res. 2024;120:581–595.

VIII. Razvan Gheorghita Mares; Viorel Iulian Suica; Elena Uyy; Raluca Maria Boteanu; Luminita Ivan; Iuliu Gabriel Cocuz; Adrian Horatiu Sabau; Vikas Yadav; Istvan Adorjan Szabo; Ovidiu Simion Cotoi; Mihaela Elena Tomut; **Gabriel Jakobsson**; Maya Simionescu; Felicia Antohe; Alexandru Schiopu. Short-term S100A8/A9 blockade promotes cardiac neovascularization after myocardial infarction. *J. of Cardiovasc. Trans. Res.* (2024)

Abbreviations

aMHC	α-myosin heavy chain
APC	Antigen-presenting cell
ASC	apoptosis-associated speck-like protein containing a CARD
BCR	B cell receptor
cDC	Classical dendritic cell
CFA	Complete Freund's Adjuvant
CLP	Common lymphoid progenitor
CLP	Cecal ligation and puncture
CMP	Common myeloid progenitor
CVB3	Coxsackievirus B3
CVD	Cardiovascular disease
DAMPS	Damage associated molecular patterns
DC	Dendritic cell
DCM	Dilated cardiomyopathy
DIC	Disseminated intravascular coagulation
EAM	Experimental autoimmune myocarditis
G-CSF	Granulocyte colony-stimulating factor
IBD	Inflammatory bowel disease
IgE	Immunoglobulin E
IL-17	Interleukin-17
IL-1R1	IL-1 Receptor Type I
IL-1R2	IL-1 Receptor Type II
IL-1RAP	IL-1 Receptor Accessory Protein
IL-1β	Interleukin-1β
ILC	Innate lymphoid cell
IRAK	IL-1 receptor-associated kinases

LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LVEF	Left ventricular ejection fraction
M-CSF	Monocyte colony-stimulating factor
MAPK	Mitogen-activated protein kinase
mDC	Monocyte derived dendritic cell
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response 88
NETs	Neutrophil extracellular traps
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
PAMPS	Pathogen associated molecular patterns
pDC	Plasmacytoid dendritic cells
PRR	Pattern recognition receptors
PVB19	Parvovirus B19
RAGE	Receptors for advanced glycation end products
scRNA-seq	Single-cell RNA sequencing
SIMD	Sepsis induced myocardial dysfunction
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th cells	T helper cells
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TRAF6	Tumor necrosis factor receptor-associated factor 6

The immune system

The immune system plays an essential role in human physiology, being responsible for protecting the body against pathogens, as well as facilitating tissue healing and repair. The immune system is comprised of a complex network of cells, tissues, and organs that work together to defend the body against harm. Harm can be external in the form of pathogens such as bacteria, viruses, fungi, and parasites, but it can also be of an internal origin in the form of tissue injury, cell death and cancer[1]. The immune system continuously monitors the body for pathogens, foreign substances and signals of tissue damage, and is equipped with receptors to identify molecular structures associated with these signals. Upon detection of a pathogen, the immune system mounts a response to eliminate it. This involves the recruitment and activation of immune cells, production of antibodies, and release of various signaling molecules that coordinate the attack on the pathogen. After an infection, the immune system retains memory of the pathogen, enabling a faster and stronger response if the same pathogen is encountered again in the future. The immune system is vital for survival, providing defense against infectious agents and maintaining overall health. Conversely, intense or prolonged activation of immune responses in certain pathological scenarios may cause or extend tissue damage, leading to immune-mediated or autoimmune diseases. Understanding the components and functions of the immune system is essential for developing treatments against these diseases[1,2].

Self-non-self and the danger theories

The threats posed to the body come in many forms, and the immune system needs to be aptly equipped to deal with a range of external pathogenic stimuli, as well as eliminating cancerous tissues and orchestrating healing responses to injury. The immune system is thus not only tasked with eliminating foreign invaders, but also with combating endogenous pathogenic processes. The ability of the immune system to efficiently perform both tasks depends on the underlying processes that governs its functions. An early theory of the function of the immune system that gained widespread traction in immunology was the “Self-non-self theory”, proposed by Frank Macfarlane

Burnet in the 1950s[3,4]. This theory postulates that the primary function of the immune systems is to differentiate between self (the body's own cells), and non-self (foreign entities and cancer), attacking the latter while tolerating the former. This theory was furthered by Charles A. Janeway Jr. who suggested that the innate immune system does so by recognizing pathogen-associated molecular patterns (PAMPs) with the help of pattern recognition receptors (PRR), leading to pathogen elimination[5]. In contrast, the danger theory, introduced by Polly Matzinger in the 1990s, suggests that the immune system also responds to danger signals from damaged or stressed cells, rather than just the presence of non-self antigens[6,7]. This theory emphasizes the role of context and is underpinned by the recognition of so-called damage-associated molecular patterns (DAMPs). The danger theory reconciles the fact that microbes such as the commensal bacteria live in our gut without excessive inflammation, and offers insights into autoimmune diseases and immune reactions to transplanted tissues. While the Self-non-self theory centers on a binary distinction of antigens, the danger theory focuses on contextual danger signals as crucial triggers for immune responses, collectively enhancing our understanding of immune system function. The danger theory also provides a framework for understanding why sterile (the absence of pathogens) inflammation such as in the case of a myocardial infarction or auto-immune disorders cause extensive immune activation and inflammation[4].

Components of the immune system

The components of the immune system can be divided two main types: the innate and adaptive immune systems. The innate immune system provides the first line of defense through physical barriers like the skin and mucous membranes, as well as cellular components such as phagocytes (macrophages and neutrophils), natural killer cells, and dendritic cells, and others which respond to pathogens non-specifically. It also involves chemical mediators like complement proteins and cytokines that enhance pathogen clearance and trigger recruitment and activation of immune cells. In contrast, the adaptive immune system features lymphocytes, specifically B cells and T cells. B cells produce antibodies, while T cells perform various roles, including killing infected cells and regulating immune responses. The adaptive immune system is characterized by its ability to recognize and remember specific pathogens, leading to a more effective response upon subsequent exposures, which forms the basis of immunological memory and vaccination. In the next section I will delve further into specific cells of the immune system with particular importance for this work[1,8].

The innate immune system

Neutrophils

Neutrophils are a type of granulocyte, characterized by the abundance of vesicular bodies in the cytoplasm as well as a multilobular nucleus. It is the most numerous leukocyte type, comprising around 50% – 70% of all immune cells in the human body, and constitutes a key component of the innate immune system[9]. Their primary function is to defend against infections, particularly bacterial and fungal infections, but are equally active in the response to sterile injury[10,11] . Neutrophils play a critical role in the early stages of the immune response, providing a rapid and effective defense against infections and injury. Their ability to quickly mobilize to infection sites and destroy pathogens is essential for maintaining the health and preventing the spread of infectious agents[12]. Neutrophils are short lived in circulation, only living for hours to at most days, and there is a large need for continuous neutrophil production[10]. Neutrophils are produced in the bone marrow by a process known as granulocytopoiesis. During steady-state, approximately 10^{11} neutrophils are generated per day in a normal adult human[11]. During infection this number can increase 10-fold, and granulocytopoiesis is stimulated by a range of growth factors, of which Granulocyte colony-stimulating factor (G-CSF) is the most well studied[10]. During the maturation of neutrophils, they are kept in the bone marrow by expression of integrin $\alpha 4\beta 1$ and activation of CXCR4 on neutrophil surface by CXCL12 produced locally in the bone marrow[9]. Upon maturation, downregulation of CXCR4 and signaling through the chemotactic receptor CXCR2 by CXCL1 and CXCL2, among others, recruit the mature neutrophils into circulation. Once in the circulation, neutrophils roll along the endothelium of blood vessels surveying the body for pathogens and injury (Figure 1). Upon tissue injury, release of neutrophil attracting factors such as CXCL1, Leukotriene B4 (LTB4), and DAMPs such as S100A8/A9 direct neutrophils to the site by a process called chemotaxis. Once neutrophils arrive at the site of injury, stimuli such as tumor necrosis factor-alpha (TNF-α), Interleukin-1β (IL-1β) and Interleukin-17 (IL-17) facilitate the upregulation and exposure of adhesion molecules E-selectin, P-selectin and integrins on the endothelium, which facilitate initial neutrophil attachment to the endothelium through L-selectin and PSGL1 among others[11]. Initial attachment induces integrin extension slowing down the rolling process, and also involves tethers and slings, which facilitates firm adhesion to the endothelium through Beta-2 integrins expressed on neutrophils[10]. Following firm adhesion, the neutrophils transmigrate into the tissue where they follow chemotactic gradients to the site where they perform their effector functions[11].

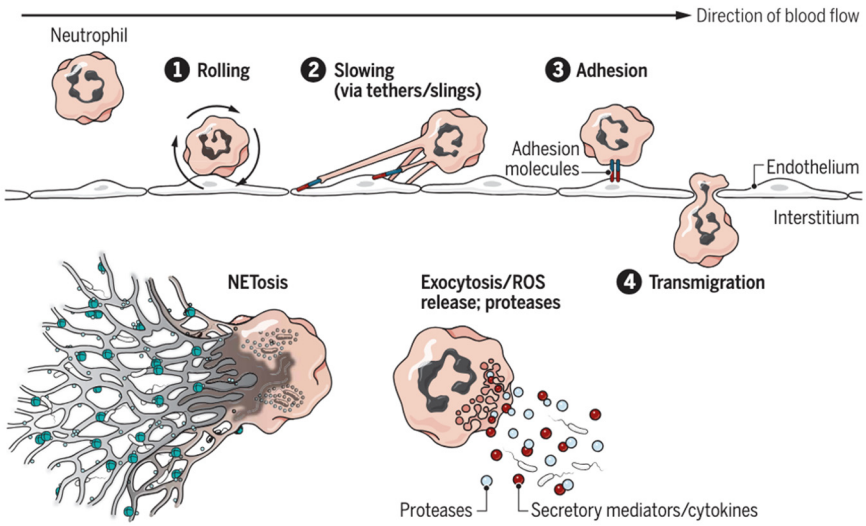

Figure 1 – Neutrophil migration and effector functions. Reproduced from Ley K, Hoffman HM, Kubes P, Cassatella MA, Zychlinsky A, Hedrick CC, Catz SD. Neutrophils: New insights and open questions. Sci Immunol. 2018;3. Reprinted with permission from AAAS.[10]

A main effector function of neutrophils is to engulf and digest pathogens (such as bacteria and fungi) and debris through a process called phagocytosis. They ingest invading microorganisms and use enzymes and reactive oxygen species within their lysosomes to destroy them. Another key mechanism is the process of degranulation. Neutrophils contain granules filled with ready-made antimicrobial proteins and enzymes, such as proteases, defensins, and myeloperoxidase[13]. Upon encountering pathogens, they release these granules into the extracellular space to kill and digest microbes. However, in the process of sterilizing the tissue from microbes, it also damages the surrounding tissues[12]. Neutrophils can also undergo a unique form of cell death known as NETosis, during which they release web-like structures composed of DNA, cytoplasmic and granular proteins, called neutrophil extracellular traps (NETs)[14–16]. The surface of the NETs is covered in negatively charged histones, as well as proteolytic enzymes such as neutrophil elastase (NE) and the immune activating protein S100A8/A9[16]. Due to electrostatic interaction between the negatively charged NETs and positively charged bacterial outer layer, bacteria are trapped and neutralized by NETs[17]. Neutrophils influence the activity of other immune cells, such as macrophages and lymphocytes, by releasing cytokines and other signaling molecules in the extracellular milieu, helping to coordinate the overall immune response[15]. After performing their functions, neutrophils undergo programmed cell death (apoptosis)

and are cleared by macrophages and other phagocytes. This process helps to resolve inflammation and prevent excessive tissue damage caused by prolonged neutrophil activity[18].

Eosinophils, Basophils and Mast cells

Eosinophils are another cell type of the granulocyte family, owing its name due to its arginine-rich basic granules staining bright pink in contact with the histological dye eosin. The cell is relatively rare in the circulation and only comprises around 6% of the circulating immune cells in healthy individuals[1]. Eosinophils are traditionally associated with the immune response to allergy and parasites, but are also involved in cardiovascular disease in the context of eosinophilic myocarditis[19]. Due to their role in fighting parasites and allergy, eosinophils are found in the highest amounts in mucosal tissues such as the gut and lungs as well as in the skin. Similarly to neutrophils, the cytoplasm of eosinophils is filled with granules containing enzymes, toxic radicals and cytokines, and their main effector function is to release these contents in the extracellular space to combat pathogens. However, due to the highly toxic content of their granules, eosinophil degranulation in the context of allergy also damages the host tissue, and needs to be tightly regulated. The production of eosinophils is controlled in the bone marrow by interleukin-3 and interleukin-5, and the recruitment and activation of eosinophils involves a family of cytokines known as eotaxins, which signal through the CCR3 receptor[20].

The basophil is another member of the granulocyte family. Basophils are normally present in very low numbers in the circulation but perform important functions in the response to parasites and allergy[1]. On their surface, they have the high-affinity receptor for immunoglobulin E (IgE) known as FcεRI, which binds antibodies produced during the immune response to allergy and parasites. Upon bound IgE encountering their antigen, it induces degranulation which releases histamines as well as production of interleukin-4 and interleukin-13[8].

Mast cells, the final member of the granulocyte family, share a close resemblance with basophils. Mast cells are found in large numbers in mucosal tissues such as the lung and are rare in the circulation. Critically involved in the response to allergy and parasites, mast cells express the high affinity IgE receptor FcεRI, and are covered with IgE which arms them to respond during hypersensitivity (allergy) as well as during immune responses to parasites[1]. Akin to the basophil, upon FcεRI-bound IgE binding an antigen, the mast cells release large amounts of histamines and other mediators from their granules, as well as lipid mediators (leukotrienes and prostaglandins), which

together stimulate inflammation. Akin to basophils, mast cells can also release high amounts of IL-4, which is important in the induction of allergic responses.

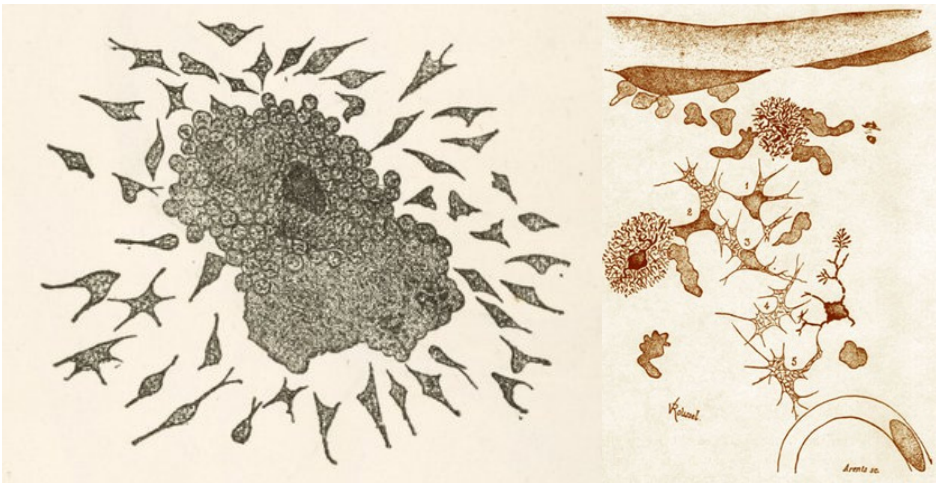

Figure 2 – Metchnikoff's drawings of phagocytes. Drawing of accumulation of phagocytes around a splinter and phagocytes at a site of inflammation. Reproduced from Tauber 2003 and Hallett 2020[21],[152]

Monocytes and macrophages

In 1882 the Russian zoologist Ilya Metchnikoff observed and described cells which had the capacity to engulf and digest microbes, a process he called phagocytosis, and the cell he observed doing so he aptly named the macrophage (Figure 2)[21]. Aside from neutrophils, the main classes of phagocytic cells in the innate immune system are macrophages and their progenitors, the monocytes[1]. Macrophages are the main resident phagocyte population in most normal tissues during steady state, where they phagocytose pathogens, apoptotic cells and debris, playing a key role both in infections and in tissue homeostasis. During early embryonic development, macrophage progenitors from the yolk sac and fetal liver infiltrate tissues. These macrophages remain in tissues throughout adult life and comprise a self-renewing population, known as tissue resident macrophages[22]. Although tissue resident macrophages dominate during homeostasis, upon infection or injury there is a greater need for phagocytosis of pathogens and dead cells, which is met by influx of monocytes from circulation.

Monocytes are normally produced in the bone marrow, originating from a common progenitor shared with neutrophils, known as the common myeloid progenitor (CMP) (Figure 3). The production and survival of monocytes in the bone marrow is critically dependent on the release of monocyte colony-stimulating factor (M-CSF), which

signals through its receptor CSF1R (also known as CD115). The deficiency of either, results in marked monocytopenia[23]. Monocytes comprise 5 – 10% of the total amount of leukocytes in circulation during homeostasis[24]. During inflammation, monocytes can also be produced in large numbers in the spleen from hematopoietic stem cells that have relocated from the bone marrow, in a process known as extramedullary hematopoiesis[23,25]. Circulating monocytes are divided according to expression of chemokine receptors and other surface molecules. In mice, the most common subset of monocytes expresses high levels of the surface molecule Ly-6C as well as the chemokine receptor CCR2 and are referred to as inflammatory monocytes or Ly-6C^{high} monocytes. CCR2 binds the chemokine CCL2, which is produced at sites of infection and injury, leading to rapid recruitment of monocytes to sites of inflammation. Conversely, monocytes expressing low levels of Ly-6C also express low levels of CCR2. These monocytes migrate along small blood vessels surveying for vessel and tissue integrity and are known as patrolling monocytes or Ly-6C^{low} monocytes[23,25–27]. The human circulation contains analogous subsets of monocytes, identified by their expression of CD14 and CD16, where CD14^{high}CD16^{low} monocytes represent the inflammatory subset and the CD14^{low}CD16^{high} monocytes are patrolling, with the addition of an intermediary CD14^{high}CD16^{high} subset which has been shown to be inflammatory[24,25].

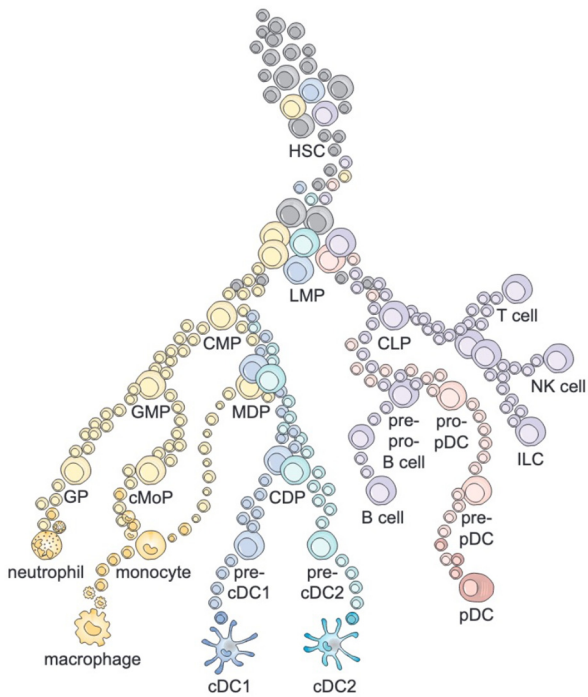

Figure 3 – Hematopoietic lineages. Reproduced from Ginhoux 2018[22]

During injury or infection, inflammatory Ly-6C^{high} monocytes are attracted to sites of inflammation through CCR2 signaling, and upon transmigration into the tissue they start differentiating into macrophages. This transition involves profound changes in the cells including increase in diameter, changes in metabolism and expression of antigen-presenting molecules, reflecting their change in function[28]. In the tissue, inflammatory macrophages phagocytose pathogens and debris, and release inflammatory mediators such as TNF-α, interleukin-1 and nitric oxide[27]. Additionally, macrophages function as antigen presenting cells (APCs) displaying antigens on major histocompatibility complex (MHC) molecules to activate T cells, and promote the adaptive immune response by producing T cell stimulating cytokines such as interleukin-12 and interleukin-23. As the inflammatory response shifts into a repair and recovery phase, macrophages shift into a predominantly reparatory phenotype. Reparatory macrophages clear debris, suppress inflammation and stimulate collagen deposition by fibroblasts. These reparatory macrophages are characterized by high expression of the efferocytosis receptor MerTK and phagocytic receptor CD206. The transition from inflammatory Ly6C^{high} monocytes into reparatory Ly6C^{low} macrophages is governed by the transcription factor Nr4a1 (known as Nur77)[29].

Dendritic cells

Dendritic cells (DCs) are the professional antigen-presenting cells (APC), and their role in the immune response lies in their ability to present antigens to adaptive immune cells to initiate and regulate T cell responses [1]. Dendritic cells can be of myeloid origin in the case of classical dendritic cells (cDCs) and monocyte derived dendritic cells (mDCs), as well as a distinct lymphoid lineage known as plasmacytoid dendritic cells (pDCs, Figure 3)[30]. Dendritic cells take up antigens and travel to secondary lymph organs where they prime naïve T cells, functioning as a bridge between the innate and adaptive immune response. DCs orchestrate immune responses by various effector mechanisms. Recognition of PAMPs and DAMPs produced during infection and injury are recognized by PRRs and results in upregulation of costimulatory molecules and MHC. Similarly, DCs ingest antigens by a form of phagocytosis, and process them for presentation on MHC molecules[31]. DCs then migrate to secondary lymphoid organs, where they present antigen:MHC complexes to naive T cells in conjunction with costimulatory molecules such as CD80, CD86 and CD40[30]. The recognition of antigen:MHC complexes in the presence of co-stimulation primes naïve T cells specific for the respective antigen and stimulate their expansion. During activation of T cells, cytokines released by DCs control which flavor of T cell is induced. For instance, T cell priming in the presence of IL-12, IL-6 or IL-4 produces Th1, Th17 and Th2 T cells,

respectively[1]. The tight regulation of T cells by dendritic cells highlights their role in mediating adaptive immune responses.

Innate lymphoid cells and Natural killer cells

The other main branch of the immune system other than the myeloid cell lineage are known as lymphocytes. The lymphocyte lineage gives rise to antigen-specific T and B cells, but also to innate cells lacking antigen specific receptors known as innate lymphoid cells (ILC), which arise from a common progenitor known as the common lymphoid progenitor (CLP)[1]. ILCs are classified into ILC1, ILC2 and ILC3, identified by their distinct cytokine expression and capacity to amplify innate and adaptive immune responses. Natural Killer cells (NK cells) are similar to the ILC1 subset, but differ from the other ILCs by their direct cytotoxic effects. NK cells can kill other cells by recognizing certain surface receptors that are upregulated in virus infected and cancerous cells.

The Adaptive Immune Response

The innate immune response has evolved to respond rapidly to infections and injuries, and has the capacity to recognize a large variety of pathogens and damage associated molecular signatures. However, the capacity to mobilize swift responses comes with the cost of mounting similar responses to a range of insults, resulting in a non-specific immune response. In contrast, the adaptive immune response has evolved the capacity to generate specific responses, as well as establishing life-long cellular memory to pathogens, enabling quick and efficient mobilization of a upon secondary exposure. The main cells of the adaptive immune system are T and B cells, of which the next sections will elaborate upon.

T cells

Originating from the same CLP as ILCs, T cells are generated in the bone marrow, and upon egression from the bone marrow they travel to the thymus. Once in the thymus, they mature as they migrate through the various compartments of the thymus, as they rearrange their T cell receptor (TCR), are positively selected for recognition of MHC molecules, and are negatively selected for recognition of self[1]. Upon exiting the thymus, T cells are either CD4+ T cells restricted to recognizing peptides displayed on MHC-II molecules, or CD8+ T cells recognizing peptides displayed on MHC-I. Additionally,

T cells that recognize self-peptides have either been removed or differentiated into regulatory T cells (T regs) with the capacity to prevent autoimmunity.

Mature T cells exit from the thymus as naïve T cells and require priming by professional APCs in secondary lymphoid organs before they can exert their effector functions. T cells circulate throughout the body to lymphoid organs until they encounter an APC displaying their cognate antigen on the correct MHC, and in the presence of co-stimulation (CD28 on T cells binding CD80 and CD86 on APCs, as well as CD40L binding CD40 on APCs). The antigen recognition and co-stimulation initiates a complex signaling cascade ultimately resulting in proliferation, known as clonal expansion[32]. This process generates a large number of clones from a small number of precursor cells, giving rise to both effector and memory T cells recognizing the same antigen.

CD8+ T cells

CD8+ T cells are also known as cytotoxic T cells, and recognize peptides presented on MHC-I molecules. MHC-I antigens are of intracellular origin, and DCs have the capacity to engulf extracellular material (such as virus infected cells) and display on MHC-I by a process called cross-presentation in order to instruct CD8+ T cells[1]. Once primed, CD8+ T cells execute their effector function by killing cells infected or malignant cells expressing MHC-I:antigen complexes directly, by releasing cytotoxins such as perforin and granzyme B[1,33].

CD4+ T cells

In contrast to CD8+ T cells, CD4+ T cells perform their effector functions by releasing cytokines which modulate the function of other immune cells, and are called T helper cells (Th cells). The cytokine milieu present during the initial T cell activation influences the differentiation pathway of CD4+ T cells, whereby specific cytokines promote the development of Th cell subsets by activating distinct transcription factors[1,33,34]. **Th1 cells** produce IFN-γ, which activates macrophages, enhances microbial killing, and promotes cell-mediated immunity. They are crucial for combating intracellular pathogens like viruses and certain bacteria. Th1 cells are induced by IL-12 and IFN-γ, which activate the transcription factor T-bet (T-box expressed in T cells), which is the master regulator for Th1 cells. **Th2 cells** produce IL-4, IL-5, and IL-13, which are important for humoral immunity and allergic responses. Th2 cells help activate B cells to produce antibodies and are involved in the response against extracellular pathogens, such as helminths and. The primary cytokine involved

in driving Th2 cell polarization is IL-4, which activates the transcription factor GATA3 which regulates Th2 cell differentiation. **Th17 cells** produce IL-17 and IL-22, which are involved in inflammation and defense against extracellular bacteria and fungi. They play a role in autoimmune diseases and chronic inflammatory conditions. Th17 cells are induced by the cytokines IL-6, transforming growth factor-beta (TGF-β) and IL-23, and the transcription factor RORγt controls Th17 transcriptional programming. **Treg cells** produce IL-10 and TGF-β, which are involved in suppressing immune responses and maintaining tolerance to self-antigens, thereby preventing autoimmunity. The primary cytokine promoting Treg differentiation is TGF-β, inducing the transcription factor FoxP3, which controls Treg development. Follicular Helper T cells (**Tfh cells**) differ from the other subsets due to their distinct role in helping B cells in the germinal centers of lymphoid organs. They are a key player in the generation of B cell responses by promoting B cell differentiation, isotype switching, and formation of high-affinity antibodies. The cytokines involved in Tfh differentiation are IL-6 and IL-21, which engage transcriptional programming controlled by the transcription factor Bcl-6. Following priming and activation of a T cells response, some activated T cells become **memory T cells**, which persist long-term in secondary lymphoid organs[32]. Memory T cells respond more quickly and robustly upon re-exposure to the same antigen, which is important for effective and persistent immunity.

B cells

B cells are vital components of the adaptive immune system, responsible for antibody production, antigen presentation, and immunological memory. Their development is a highly regulated process involving genetic rearrangements, cytokine signaling, and selection mechanisms to ensure a highly specific, diverse and self-tolerant B cell repertoire[1]. Similarly to ILCs and T cells, B cells originate from the CLP in bone marrow. During maturation, they pass through a range of steps in which their B cell receptor (BCR) undergoes recombination of both heavy and light chains. Upon successful rearrangement, the cell expresses a complete BCR (IgM) on its surface which is tested for autoreactivity. Those strongly reactive to self-antigens are eliminated or undergo receptor editing to change their specificity[35]. Cells that pass negative selection exit the bone marrow as mature naïve B cells expressing both IgM and IgD BCRs, and circulate through the blood and home to secondary lymphoid organs (e.g., lymph nodes, spleen). In secondary lymph nodes, B cell activation occurs in response to antigen encounter and involves several critical steps. When a B cell encounters its specific antigen, the antigen binds to the BCR, triggering the first activation signal. Antigen binding causes cross-linking of BCRs, leading to clustering and initiation of

signaling cascades leading to activation of several downstream pathways and internalization of the BCR-antigen complex[1]. Peptide fragments are processed and loaded onto MHC class II molecules and presented on the cell surface. B cells then require the interaction with Tfh cells, where the B cell presents the antigen:MHC class II complex to Tfh cells[33]. Interaction between CD40 on the B cell and CD40L on the T cell provides necessary co-stimulatory signals. Tfh cytokines (e.g., IL-4, IL-5, IL-6) further stimulate B cell activation, proliferation, and differentiation. Activated B cells undergo clonal expansion, producing a large number of progenies with the same antigen specificity. Some B cells migrate to germinal centers in secondary lymphoid organs, where they undergo somatic hypermutation and class-switch recombination, a process by which their BCR is further refined by random mutations and affinity selection[35]. Activated B cells differentiate into either plasma cells that secrete high amounts of antibodies into the circulation, as well as memory B cells which persist throughout life and provide lifelong immunological memory, providing a rapid response upon subsequent exposure to the same antigen.

Cytokines, Chemokines and Soluble mediators

Having described the cellular components of the immune system, we turn our attention to the soluble mediators which mediate many of the effector functions of immune cells. These mediators are small soluble proteins mainly produced in immune cells and secreted by a range of mechanisms. Once released, they signal through receptors on the surface of cells and modulate inflammation and cellular immune responses.

Cytokines are essential for regulating immune responses, inflammation, cell growth, and differentiation. They act as signaling molecules to coordinate the activity of immune cells and maintain homeostasis. **Chemokines** are specialized cytokines that primarily regulate cell migration (chemotaxis). They are crucial for directing the movement of immune cells to sites of infection, injury, and inflammation, as well as for maintaining the organization and function of lymphoid tissues. Together, cytokines and chemokines orchestrate the immune response, ensuring that it is efficient, targeted, and regulated to prevent excessive tissue damage[1].

Another type of soluble mediators are known as DAMPs or **alarmins**. Alarmins are a subset of endogenous molecules that are released by cells in response to tissue damage, stress, or infection and act as danger signals to alert the immune system to the presence of damage. Alarmins are often released through non-classical secretion pathways, such as during cell death or through active secretion in response to stress signals. Some examples of alarmins released during damage are S100 proteins, HMGB1, heat-shock

proteins, interleukin-1 alpha and ATP[36–39]. Much like cytokines and chemokines, they rapidly activate the innate immune response to initiate inflammation and repair processes, and to mobilize other immune cells to the site of injury or infection. Alarmins bind to PRRs on immune cells, such as Toll-like receptors (TLRs) and receptors for advanced glycation end products (RAGE), leading to the activation of inflammatory signaling pathways. This results in cell recruitment and production of pro-inflammatory cytokines and chemokines that in turn recruit and activate immune cells.

S100A8/A9

An important example of endogenous alarmins are the S100 family of calcium-binding proteins. They are a family of small (10 – 13 kDa) proteins containing EF-hand domains, capable of binding bivalent cations such as Ca^{2+} and Zn^{2+}. The proteins S100A8 and S100A9 (also known as MRP-8 and MRP-14, respectively) are two prominent members of the S100 protein family. Like other S100 proteins, S100A8 and S100A9 have the capacity to form homo- and heterodimers, and in-vivo they preferentially form the heterodimer **S100A8/A9** (Figure 4)[40]. The binding of Ca^{2+} results in conformational changes which facilitate interactions with other proteins. The heterodimers are additionally stabilized by the binding of Zn^{2+}, upon which they can form heterotetrameric structures[40]. Upon tetramer formation, the signaling capacity of S100A8/A9 heterodimers is partially lost, limiting inflammation locally and preventing systemic inflammation[41,42]. S100A8/A9 is primarily expressed in myeloid cells. In neutrophils they comprise up to 40% of the soluble cytoplasmic protein content, and can also be induced in monocytes upon inflammation, although S100A8/A9 is not produced in tissue resident macrophages[40]. S100A8/A9 plays a crucial role in the regulation of inflammatory processes and immune responses. Upon cellular activation or tissue damage, S100A8/A9 is released into the extracellular space by neutrophils, where it acts as an alarmin, thereby initiating and propagating inflammatory responses. The specific mechanism by which neutrophil-derived S100A8/A9 is secreted has long been unknown, but a recent publication by Pruenster et al showed that it is dependent on E-selectin mediated NLRP3 inflammasome activation[43]. Specifically, endothelial cell bound E-selectin triggers NLRP3 inflammasome priming, and upon inflammasome activation by K^+ efflux gasdermin D pores are formed in the cell membrane, releasing S100A8/A9 into the extracellular space. Importantly, gasdermin D pore formation is transient and is counteracted by the ESCRT III membrane repair machinery, avoiding cell lysis[43].

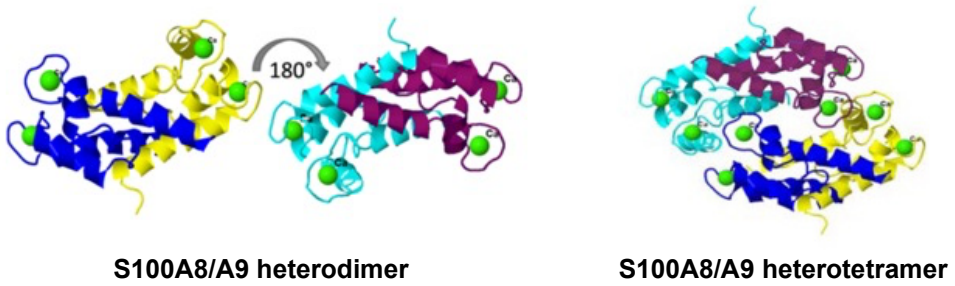

Figure 4. Tertiary structure of S100A8 and S100A9 homodimers, S100A8/A9 heterodimer and S100A8/A9 tetramers. Ca2+ ions are shown by green spheres. Modified from[40].

Once secreted in the extracellular space, S100A8/A9 mainly binds to TLR4 and RAGE on immune cells[44,45]. This binding triggers downstream signaling pathways that lead to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinase (MAPK), and these signaling events result in the production of pro-inflammatory cytokines and chemokines, amplifying the inflammatory response. Binding of S100A8/A9 to TLR4 and RAGE can induce NLRP3 inflammasome priming through ROS production (Figure 5). Subsequent activation promotes assembly of the NLRP3 inflammasome complex leading to the processing and secretion of mature IL-1β and IL-18[46–48]. Aside from TLR4 and RAGE, studies have also shown that S100A8/A9 can bind to a range of other receptors such as TLR-2, CD69, CD36, as well as GPIbα on platelets[42,47,49,50]

Upon release and receptor binding, S100A8/A9 functions as a critical mediator in the inflammatory response, exerting its effects through several mechanisms. S100A8/A9 acts as a chemoattractant for neutrophils and monocytes, guiding them to sites of infection or injury, thus contributing to the rapid deployment of the body's first line of defense[42,51,52]. S100A8/A9 binding to TLR-4 induces CD11b and β2-integrin upregulation and L-selectin shedding in neutrophils, as well as increased ICAM-1 and VCAM-1 expression in endothelial cells[51–53]. Together this results in decreased rolling, facilitating firm adhesion and transmigration (Figure 1).

S100A8/A9 modulates the activity of leukocytes, enhancing the production of reactive oxygen species (ROS) in neutrophils, thereby increasing their microbicidal activity[46]. S100A8/A9 also promotes the secretion of key pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6[38,53]. Given its central role in inflammation, dysregulation of S100A8/A9 is associated with various inflammatory and autoimmune diseases. S100A8/A9 plays a pathogenic role in a multitude of inflammatory diseases such inflammatory bowel disease (IBD), ulcerative colitis, rheumatoid arthritis, cancer,

infection and sepsis. The role of S100A8/A9 in cardiovascular disease has also been studied extensively, and the protein has been found to play a key role in mediating inflammation in atherosclerosis, ischemic heart disease and inflammatory cardiovascular disease[38,53–56]. As such, S100A8/A9 is a pivotal immune mediator that bridges innate and adaptive immunity, playing a central role in the promotion of inflammatory responses. Its pathogenic involvement in a wide range of diseases highlights its importance as a potential target for therapeutic intervention.

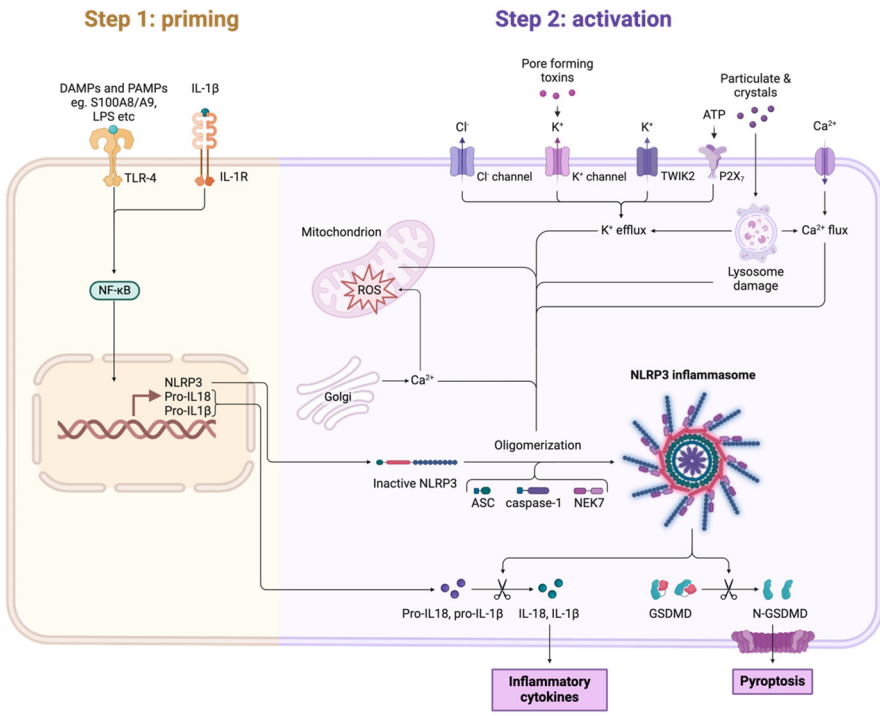

Figure 5 - Process of NLRP3 Inflammasome priming and activation. **Created with Biorender.**

Interleukin-1 family

The main function of the interleukin-1 (IL-1) family of cytokines is to mediate pro-inflammatory reactions triggered by PAMPs during infection and DAMPs produced by tissue injury (such as S100A8/A9, HMGB1, heat-shock proteins and histones)[57]. Interleukin-1 is a major mediator of innate immune reactions and is mainly released by innate immune cells such as neutrophils, monocytes and macrophages, although endothelial cells and fibroblasts also have the capacity to produce IL-1[58]. IL-1α is anchored in the membrane of cells, and is released upon damage, functioning akin to a

DAMP or alarmin. IL-1β, however, is tightly regulated by the NLRP3 inflammasome, both at the level of production and secretion (Figure 5)[57]. The inflammasome is a multiprotein complex within the cell's cytoplasm that regulates the activation of inflammatory responses, and serves as a platform for the activation of caspase-1, an enzyme which mediates the processing and release of pro-inflammatory cytokines interleukin-1β and interleukin-18[1]. The inflammasome works by a 'priming' step followed by a second signal 'activation' by a sensor such as NLRP3. A multitude of different inflammasomes have been described, but the NLRP3 is the most well studied in the context of immune response and inflammation[59]. Upon recognition of PAMPs or DAMPs by PRRs such as TLRs, NFκB and MAPK signaling pathways are activated. This leads to expression of several key components of the inflammasome complex such as NLRP3, pro-caspase-1, and pro-IL-1β, during a process termed the 'priming' of the inflammasome. Upon a second signal such as K+ efflux, reactive oxygen species (ROS) production, lysosomal damage or mitochondrial dysfunction, the inflammasome is 'activated', resulting in oligomerization of NLRP3 (or another sensor), and the apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein, and recruitment of pro-caspase-1 into a multimeric protein structure which then in turn oligomerizes into a large structure[59]. This large complex cleaves pro-caspase-1 into its active form, caspase-1 which in turn cleaves pro-IL-1β and others into their active form ready to be secreted. The exact mechanism of IL-1β secretion is not fully understood but involves non-classical pathways, potentially including vesicle-mediated transport or direct release through membrane pores such as gasdermin D.

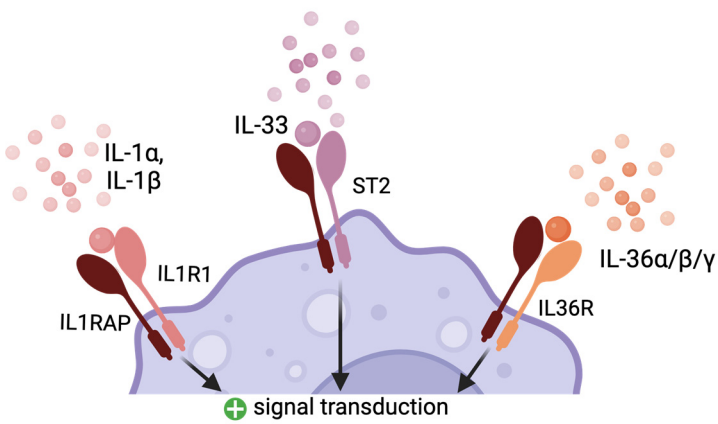

Figure 6 – Interleukin-1 family receptors. Created with Biorender, courtesy of Dr. Megan Mulholland.

Once secreted, IL-1 can bind to either an activating receptor comprised of IL-1 Receptor Type I (IL-1R1) and IL-1 Receptor Accessory Protein (**IL-1RAP**), the primary signaling receptor for IL-1, or IL-1 Receptor Type II (IL-1R2) which functions as a decoy receptor to inhibit IL-1 signaling, lacking intracellular signaling domains[58]. Upon ligand binding, IL-1R1 undergoes a conformational change allowing it to recruit IL-1RAP, initiating signal transduction (Figure 6). Formation of the receptor complex activates several intracellular signaling pathways, primarily myeloid differentiation primary response 88 (MyD88). MyD88 recruits IL-1 receptor-associated kinases (IRAKs), leading to their phosphorylation and activation which in turn interact with tumor necrosis factor receptor-associated factor 6 (TRAF6), leading to its ubiquitination and activation. TRAF6 activation leads to subsequent activation of NF-kB which transclocates to the nucleus acting as a transcription factor[60]. TRAF6 also activates MAPKs, which phosphorylate various transcription factors (e.g., AP-1), leading to the expression of inflammatory cytokines, chemokines, and other mediators[58,60]. Activation of NFκB and MAPKs leads to the transcription of genes involved in inflammation, immune response, and cell survival. This results in the production of various cytokines, chemokines, adhesion molecules, and enzymes that mediate inflammation and immune responses. The downstream effects of IL-1 signaling include inducing fever, production of acute-phase proteins by the liver, activation and recruitment of immune cells, and enhancing the expression of adhesion molecules on endothelial cells to facilitate leukocyte migration.

Two additional members of the IL-1 family are IL-33 and IL-36. IL-33 is released upon cellular damage or stress, and plays a role in promoting Th2 immune responses[61]. Upon release, IL-33 binds to the IL-33 receptor, a heterodimer comprised of the subunits ST-2 and IL-1RAP, and signals through MyD88 similarly to IL-1 (Figure 5). Its functions include activation of mast cells, eosinophils and basophils, contributing to allergic inflammation in conditions such as asthma[61]. Additionally, it plays a role in promoting tissue repair and maintaining barrier function in epithelial tissues[62]. IL-36 has several isoforms including IL-36α, IL-36β, IL-36γ[63]. IL-36 cytokines have been well described in skin inflammation and psoriasis, and their functions include production of pro-inflammatory cytokines, activation of epithelial cells and dendritic cells[63–65]. The receptor for IL-36, similar to other cytokine members of the IL-1 family, engage a heterodimeric receptor comprised by the IL-36R and IL-1RAP, and their signaling activates MyD88 leading to NFκB activation[65].

Cardiovascular disease

Cardiovascular disease (CVD) refers to a broad range of disorders affecting the heart and blood vessels. It is the leading cause of death globally, posing significant health and economic burden on societies worldwide. CVD encompasses various conditions, the most important ones being coronary artery disease, stroke and peripheral artery disease. In its turn, coronary artery disease can lead to severe complications such as myocardial infarction, chronic ischemic heart disease, heart failure and arrhythmias. These conditions can lead to long-term disabilities, affecting the overall health and quality of life of the patient. CVD is the leading cause of death globally. In 2019, it accounted for an estimated 17.9 million deaths, representing 32% of all global deaths. Of these deaths, 85% were due to ischemic disease such as myocardial infarction and stroke[66]. The underlying pathology of a large proportion of cardiovascular disease is atherosclerosis. Atherosclerosis involves lipid infiltration within arterial walls, triggering a chronic inflammatory process upon attempted clearance of these lipids by the immune system, in particular macrophages[67]. This leads to development of atherosclerotic plaques into the arterial wall, involving cells of both the adaptive and innate immune response. As plaques grow, they can progressively narrow the arterial lumen and restrict blood flow. Atherosclerotic plaques may also rupture, leading to acute thrombotic complications such as myocardial infarction and ischemic stroke. Aside from atherosclerosis-related complications, another important pathogenic entity of CVD is inflammatory heart disease[68,69]. Patients with severe systemic infections, known as sepsis, may develop a condition known as **sepsis induced myocardial dysfunction** (SIMD), leading to acute heart failure and circulatory collapse[70]. Another important pathogenic entity is **myocarditis**, acute inflammation of the heart muscle (myocardium), which can result from viral or bacterial infections, autoimmune diseases, or exposure to toxins[68,71]. Inflammation during myocarditis damages the cardiomyocytes and leading in severe cases to heart failure, arrhythmias, or sudden cardiac death[72]. In the next section, I will elaborate further on these two types of inflammatory cardiomyopathy in more detail.

Sepsis and septic shock

Sepsis is a life-threatening condition that arises when the body's response to an infection causes widespread systemic inflammation, leading to tissue damage, organ failure, and potentially death. Sepsis is one of the leading causes of mortality worldwide. In 2017, an estimated 48.9 million cases of sepsis were recorded worldwide, and 11 million sepsis-related deaths were reported representing 20% of all global deaths[73]. Sepsis is most commonly, although not exclusively, caused by bacterial infections, the most common of which being pneumonia, abdominal infection and urinary tract infection[74,75]. The underlying pathophysiology of sepsis involves a dysregulated immune response, resulting in the release of a cascade of inflammatory mediators with subsequent systemic effects. The primary cause of death in sepsis patients is cardiovascular collapse, which results in multiple organ failure[76]. The cardiovascular collapse in sepsis is caused by leakage of fluids into tissues through a dysfunctional endothelium and a significant reduction in vascular tone, in combination with impaired cardiac function. Together, this leads to hypotension and insufficient perfusion to peripheral tissues.

The immune response in sepsis involves both innate and adaptive immune mechanisms, and it can be divided into an initial pro-inflammatory phase and subsequent anti-inflammatory phase[74,75]. The response begins with the recognition of pathogen-related PAMPs by PRRs on the surface of immune cells, triggering the activation of neutrophils, monocytes and macrophages. The activation of innate immune cells leads to potent release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and interferons[74,77]. This leads to a "cytokine storm," causing widespread inflammation and recruitment of additional immune cells to the site of infection[78]. Neutrophils are the first immune cells to arrive, followed by monocytes and other innate and adaptive immune cells[15,79]. Inflammation and immune cell activation leads to endothelial dysfunction, resulting in increased vascular permeability, edema, and impaired tissue perfusion. This leads to septic shock, characterized by severe hypotension and organ failure[74,75]. The immune response in sepsis also triggers the coagulation system. Pro-inflammatory cytokines activate the coagulation cascade, leading to the formation of microthrombi in small blood vessels. Disseminated intravascular coagulation (DIC) can occur, where widespread clotting depletes clotting factors and platelets, increasing the risk of bleeding and further complicating sepsis management[75]. Following the initial hyper-inflammatory response, the immune system attempts to regulate and resolve inflammation. Anti-inflammatory cytokines such as IL-10 and TGF-β are released to counteract the pro-inflammatory response. In some cases, the immune system shifts towards an immunosuppressive state, characterized by

reduced cytokine production and impaired function of immune cells[74]. This phase, often referred to as "immune paralysis," increases the risk of secondary infections and poor outcomes.

S100A8/A9 in sepsis

During sepsis, the potent neutrophil mobilization and activation results in large amounts of S100A8/A9 to be released. Several clinical studies have shown that plasma levels of S100A8/A9 are increased in sepsis patients, and are associated with mortality and poor prognosis[80–85]. The role of S100A8/A9 mediated inflammation in sepsis has also been studied experimentally. A study in *Escheria coli*-induced abdominal sepsis showed that mice lacking S100A8/A9 were protected from sepsis-induced mortality and showed diminished NFκB-mediated TLR4 signaling, leading to lower levels of IL-1 and TNF-α[44]. Additionally, in previous studies using the cecal ligation and puncture (CLP) mouse model of multi-bacterial abdominal sepsis, our group has demonstrated that S100A8/A9 binding to TLR4 and RAGE potently stimulates formation of NETs, and that S100A8/A9 blockade inhibits neutrophil recruitment and lung damage[86,87]. These findings suggest that S100A8/A9 may play a causal role in the development of severe sepsis and could be a potential therapeutic target to reduce disease severity.

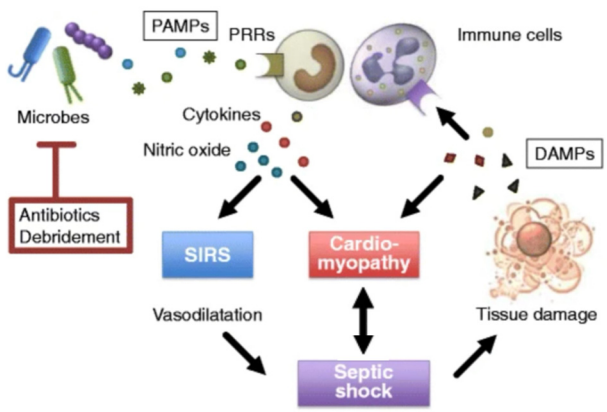

Figure 7 – Pathogenesis of SIMD[153]

Sepsis-induced myocardial dysfunction (SIMD)

A significant number of patients with severe sepsis experience impaired myocardial contractility, known as sepsis-induced myocardial dysfunction (SIMD, Figure 7)[70,88,89]. This condition was initially described by Calvin et al. in the 1980s, and is characterized

by an acute and reversible global cardiac dysfunction with decreased contractility[90,91]. It is often accompanied by left ventricular (LV) dilation, reduced response to fluid resuscitation and catecholamines, in the absence of acute coronary syndrome[92,93]. Reduction in myocardial strain has been found to be associated with increased mortality in sepsis patients, suggesting that the presence of SIMD might contribute to a poor prognosis[88]. The mechanisms underlying cardiac dysfunction in sepsis include impaired myocardial circulation, mitochondrial dysfunction, downregulation of beta-adrenoceptors, and direct myocardial depressants acting through receptors on cardiomyocytes[76,94–97]. Indeed, a so called "myocardial depressant factor" has been proposed to be the causative agent, but it has so far remained elusive[92]. The mechanisms are not yet fully understood, and currently no therapies can reverse the myocardial dysfunction in severe sepsis. Inflammatory cytokines such as IL-1β and TNF-α have been suggested to play a role in SIMD, but randomized controlled trials targeting these cytokines in sepsis patients have failed to improve cardiac function or reduce mortality[89]. The role of S100A8/A9 during SIMD in the clinical setting has so far been sparsely studied, although there have been some experimental studies supporting a link. Experimental work has demonstrated that overexpression of S100A8/A9 aggravates myocardial dysfunction during endotoxemia, suggesting a link between elevated levels and impaired contractility[98]. Additionally, cardiac delivery of S100A9-silencing RNA during endotoxemia attenuated cardiac dysfunction, suggesting that cardiac S100A8/A9 might be involved in SIMD[98]. Additionally, genetic deficiency of the S100A8/A9 receptors TLR4 and RAGE in cardiomyocytes has been shown to protect against SIMD during endotoxemia, and S100A8/A9-TLR4 signaling has been shown to be cardio-depressant in myocardial infarction studies[95,99–102] Taken together, this suggests a potential pathogenic involvement of S100A8/A9 in cardiac dysfunction during sepsis.

Myocarditis

Myocarditis is an inflammatory condition of the myocardium, and is a prominent cause of heart failure, arrhythmias and sudden death. The prevalence of myocarditis is challenging to determine accurately, due to its heterogeneous presentation and diagnostic challenges. Many cases are unrecognized or are misclassified as other forms of cardiomyopathy or heart failure. The incidence of myocarditis varies globally, partially due to heterogenous distribution of causative agents and lack of proper diagnostic tools in low-income countries, but global incidence is estimated to be around 22 per 100,000 individuals[19]. Unlike most other CVD pathologies, particularly

atherosclerosis-related, it is more common in younger people, with the highest incidence of myocarditis occurring in males aged 20 – 40[103]. Myocarditis can be especially dangerous in young patients, and some studies have found the prevalence of myocarditis to be as high as 42% in young individuals who suffered sudden death[104].

Etiology of myocarditis

The causes for myocarditis are heterogeneous, and it can be triggered by infectious agents such as viruses and parasites, autoimmune mechanisms, vaccinations and cancer immunotherapy, or be idiopathic (of unknown cause)[19,71,72]. Globally, the most common cause of myocarditis is parasitic. The protozoa *Trypanosoma cruzi*, endemic to Central and South America, causes Chagas disease and associated myocarditis[68,71,105]. In the western world however, the most common cause for myocarditis is believed to be viral, especially in children, and a range of viruses have been found to be associated with myocarditis. Endomyocardial biopsies from patients have identified enteroviruses such as Coxsackievirus B3 (CVB3), adenoviruses, parvovirus B19 (PVB19), Influenza A virus, Epstein-Barr virus, cytomegalovirus, hepatitis C, HIV and SARS-CoV-2, where PVB19 is by far the most commonly occurring[19,71,72,106]. Apart from Coxsackievirus B3, which preferentially infects cardiomyocytes, it is unclear if other viral infections can cause direct cardiomyocyte damage, or they induce injury by initiating pathological immune-mediated mechanisms[106,107]. There is ample evidence for auto-immune processes in patients with myocarditis, such as the presence of cardiac autoantibodies[72,107,108]. Symptoms of myocarditis are varied, including chest pain, shortness of breath, fatigue and decreased exercise tolerance. Due to the myocardial destruction, myocarditis can lead to arrhythmias and reduced left ventricular ejection fraction (LVEF)[19,71,103]. In most patients, as high as 87%, acute myocarditis is self-resolving, but in some cases, it can cause sudden death or progress to dilated cardiomyopathy (DCM), with patients requiring cardiac transplantation[19,103]. Due to the heterogeneity of myocarditis, there is a lack of treatment options that can prevent disease progression, and therapies for myocarditis are mostly supportive. For certain virus-negative myocarditis cases, as well as giant cell myocarditis, the use of immunosuppressive therapy has been successful in small clinical trials, but despite this there is a need for therapies that target the immunological drivers of myocarditis[109].

Pathogenesis of myocarditis

The pathogenesis of myocarditis involves direct injury to cardiomyocytes followed by immune activation and subsequent injury exacerbated by invading leukocytes. Most of what is known about the pathogenesis of myocarditis comes from studying

autoimmune mechanisms and CVB3 infection in animal models, where there is evidence of a direct CVB3 mediated myocardial injury followed by a pathological immune response against the heart[19,68,71]. Following direct injury, viral particles as well as DAMPs and cardiac myosin are released by damaged cardiomyocytes, engage PRRs such as TLR2 and TLR4 on immune cells, initiating an innate immune response[110]. Circulating immune cells are recruited to the heart and form an inflammatory infiltrate involving neutrophils, mast cells, dendritic cells, monocytes and macrophages, the latter being the most prominent cardiac cell type in experimental and human myocarditis[111]. Neutrophils arrive first and exacerbate inflammation, and the amount of neutrophil infiltrate is associated with disease severity in patients[112]. Additionally, experimental work has shown that depletion of neutrophils in CVB3 induced myocarditis was beneficial[113,114]. Inflammatory NETosis has also been shown to be a pathogenic driver in an experimental model of autoimmune myocarditis[115]. Following the rapid influx of neutrophils, monocytes are also mobilized to the heart. Ly6Chigh monocytes expressing CCR2, produced in the bone marrow, are recruited into the myocardium and differentiate into inflammatory macrophages, producing cytokines such as IL-6 and TNF-α. Studies silencing CCR2 during autoimmune myocarditis have shown lower numbers of monocytes/macrophages in the immune infiltrates and improved cardiac function, highlighting the pathogenic role of monocytes[72,116].

Following the influx of innate immune cells during myocarditis, an adaptive immune response is mounted. T and B cells recognizing viral peptides as well as cardiac-specific antigens are generated, exacerbating cardiac inflammation[71,72]. There has been substantial evidence for cardiac myosin-specific antibodies in patients and experimental models, but the process by which cardiac-specific immune responses were initiated was for a long time unclear[19,68,108,110]. A proposed mechanism is molecular mimicry, where viral and parasitic structures and endogenous cardiac peptides share common structures[68,107]. This theory is supported by the finding that cardiac myosin is not presented to T cells during thymic development and myosin-specific T cells are not deleted, providing the basis for myosin-reactive T cells aggravating myocarditis[117]. Hence, peptides resembling cardiac myosin as well as myosin released from damaged cardiomyocytes have the capacity to induce T and B cell responses in myocarditis. CD4+ T cells have been shown to have a detrimental role in both viral and autoimmune myocarditis, whereas CD8+ T cells have a beneficial role in viral myocarditis, assisting with viral clearance[72,114,118]. The role of specific CD4 T cell subsets has also been investigated, where IL-17+ Th17 cells have been shown to contribute to the development of fibrosis and DCM[119,120]. Conversely, IFNγ+ Th1 T cells have been shown to be pathogenic in the initiation of myocarditis but protective in the later stages, by polarizing the T cell response away from the more pathogenic IL-17+ Th 17

cells[72,119,121]. The role of B cells in myocarditis seems to be pathogenic, as evident by the detection of antibodies recognizing cardiac myosin in both experimental and clinical studies[71,72].

S100A8/A9 and IL-1 signaling in myocarditis

S100A8/A9 is a primarily neutrophil-derived immune mediator, and the evidence for neutrophil contribution of neutrophils indicate a role of S100A8/A9. Plasma levels of S100A8/A9 have been shown to be elevated in patients with myocarditis and correlate with disease severity[122]. In this study, plasma S100A8/A9 correlated with S100 mRNA levels from endomyocardial biopsies, as well as with circulating leukocyte levels, indicating that S100A8/A9 is a potential biomarker for myocarditis. Experimental work in CVB3 viral myocarditis using mice deficient for S100A8/A9 showed decreased inflammation and improved cardiac function in knockout mice[123]. Taken together, the existing evidence suggests a pathogenic role of S100A8/A9 in myocarditis and points to a potential therapeutic role for S100A8/A9 inhibition.

Similarly, Interleukin-1 family signaling in myocarditis has recently gathered attention as a potential pathogenic mechanism[124]. This can in part be attributed to the success of IL-1 blockade during inflammatory and cardiovascular diseases in recent times, as well as evidence for a role of IL-1 cytokines in the pathogenesis of myocarditis[124]. An experimental study found that blocking of IL-1β in viral myocarditis ameliorated disease by reducing cardiac inflammation and fibrosis, while also preventing viral replication[125]. Likewise, expression of the IL-1 neutralizing protein IL-1Ra improved survival and reduced fibrosis build-up in a CVB3 model of myocarditis[126]. Investigation the role of IL-1 family cytokine IL-33 showed that administration of recombinant IL-33 during myocarditis increased acute perimyocarditis, eosinophilia, and impaired cardiac function, while IL-33-neutralizing recombinant ST2 treatment ameliorated disease[127]. Less is known regarding the role of IL-36, but one study showed that indirect promotion of IL-36 caused by blocking IL-38 was detrimental, suggesting that IL-36 blockade might be beneficial[128]. On the background of experimental work targeting IL-1, case reports have shown beneficial effects of the IL-1 blocking therapeutic Anakinra in severe disease. This led to the initiation of the ARAMIS trial, in acute myocarditis patients were treated with Anakinra. The results of this trial were neutral, indicating that IL-1 inhibition alone might not be sufficient to reduce disease pathogenesis[129,130].

Experimental methods

Experimental models of endotoxemia and sepsis

Sepsis is most commonly caused by gram-negative, and their bacterial products are the initial trigger that activates the immune system[75]. A main component of the gram-negative bacterial wall is lipopolysaccharide (LPS), which binds to TLR4 on immune cells and endothelial cells. A common way to emulate gram-negative sepsis in experimental models is to administer LPS to mice, resulting in a sterile inflammation known as endotoxemia. This mimics the clinical symptoms of sepsis, resulting in cytokine storm, weight-loss, cardiac dysfunction and death[131,132]. Mice quickly become hypothermic and exhibit lethargy and reduced intake of water and food, rapidly losing body weight. Cardiac dysfunction can be observed as soon as 2 hours after injection, and gradually recovers spontaneously in survivors by 72 hours post injection[132]. By use of echocardiography, cardiac function can be continuously monitored, and offers a good model system to study SIMD in animals. Male mice exhibit stronger inflammatory responses to LPS-induced endotoxemia and higher mortality, and it is preferable to use both genders when possible[133]. Alternatively to using LPS, live-bacteria models of sepsis are also available, of which injection of live *Escheria coli* bacteria best recaptures the effects of endotoxemia. This model, however, only displays the clinical symptoms of cytokine storm after some time in which the bacteria have multiplied and released sufficient levels of products into the circulation. Since in the clinical setting patients receive high doses of antibiotics to combat the bacterial infection, it constitutes a less clinically relevant model in some regards. Another common model is the cecal ligation and puncture (CLP) model, where the cecum is ligated and punctured by surgery, releasing fecal bacteria into the peritoneum and causing polymicrobial sepsis. This model is reminiscent of intestinal perforation and peritonitis, and is induced by the release of a wide range of bacteria and their products, making it unclear which mechanisms are responsible for the observed syndrome[131].

Models of myocarditis

Experimental autoimmune myocarditis (EAM)

Although the origin of myocarditis can be heterogeneous, the elicited immune responses generally involve auto-reactive immune mechanisms to some extent[72,107,108]. Induction of autoimmune myocarditis in mice is performed by vaccination, in which fragments of cardiac peptides are emulsified in a strong adjuvant and administered to susceptible mice, causing autoimmune inflammation in the heart, a model known as experimental autoimmune myocarditis (EAM). Several peptides can induce myocarditis, such as troponins and myosin, but the most commonly used is the α-myosin heavy chain (aMHC) fragment 614 – 631 (RSLKLMATLFSTYASADR). The aMHC fragment is emulsified in Complete Freund's Adjuvant (CFA), a mixture of mineral oils and heat-killed *Mycobacterium tuberculosis* of the avirulent strain H37Ra[134]. BALB/c mice are vaccinated on day 0 and day 7, and develop robust cardiac inflammation involving both innate and adaptive immune cells by day 14 – 21, of which monocytes and macrophages are the most prominent similar to what is seen in patients (Figure 8). Concomitantly, mice develop cardiac dysfunction and fibrotic deposition in the heart, and if followed long enough also develop DCM.

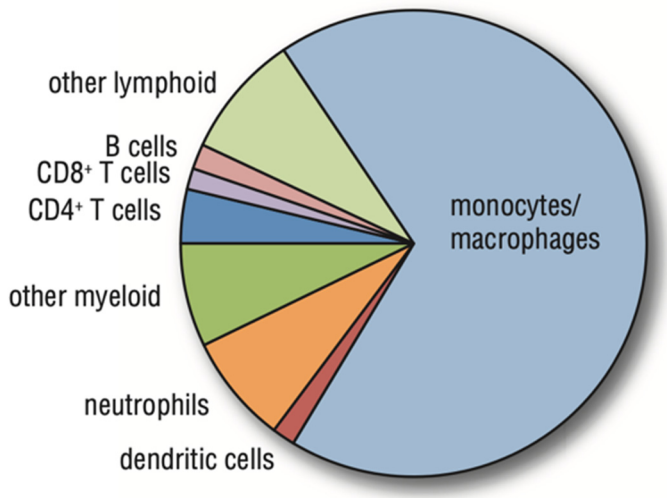

Figure 8 - Immune infiltrate during EAM. Modified from Rose 2013[134]

CVB3 induced viral myocarditis

An alternative model of myocarditis is the coxsackievirus B3 induced viral myocarditis model. This model features two stages of inflammation, where at first cardiomyocytes are infected by the CVB3 virus, producing a viral inflammation in the myocardium which is then followed by an autoimmune-driven inflammation akin to the one seen in EAM models[134,135]. However, since CVB3 is a BSL-2 classified pathogen capable of infecting humans, the method offers some complications with respect to work safety and disposal of reagents. To induce CVB3 myocarditis, CVB3 virus is first injected into a C57Bl/6 mouse, the hearts are harvested and cardiotropic CVB3 virus is isolated from heart tissue. Cardiotropic CVB3 is then injected into C57Bl/6 mice, who develop acute myocarditis 5 – 14 days post induction, which transitions into chronic myocarditis with autoimmune characteristics and complete viral clearance 21 – 28 days post induction[135]. During the acute phase, CVB3 myocarditis involves immune cell infiltration, containing monocytes and macrophages, neutrophils, CD4+ and CD8+ T cells, NK cells and eosinophils. The model also captures key features of human myocarditis such as T cell infiltration, reduced cardiac function, generation of cardiac autoantibodies and in the most severe cases death due to heart failure.

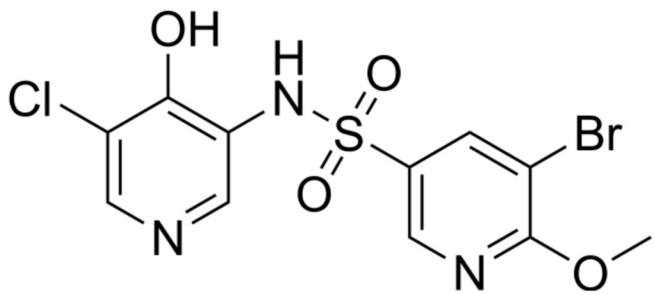

Figure 9 - Chemical structure of ABR-238901

Treatment strategies

S100 inhibition

ABR-238901 is a small < 400 g/mol water-soluble heteroaryl-sulfonamide derivative (Figure 9). ABR-238901 binds to S100A8/A9 and inhibits its binding and signaling to TLR-4 and RAGE[136]. ABR-238901 has a half-life in circulation of ~4 hours, and is orally tolerable and non-toxic. Work by our group and others have utilized ABR-

238901 and similar compounds in a range of inflammatory and cardiovascular diseases, showing the anti-inflammatory effect of inhibiting S100A8/A9 signaling[86,87,137–140].We utilized ABR-238901 as an S100A8/A9 inhibitor in this work. In sepsis studies, 30mg/kg ABR-238901 was administered by intraperitoneal injection at 0h and 6h post LPS administration. In longitudinal myocarditis studies, ABR-238901 was administered through drinking water at dose of 30mg/kg/day.

Anti-IL1RAP blocking antibodies

In order to investigate the role of IL-1RAP signaling in myocarditis, we utilized an IL-1RAP blocking antibody (mCan10), provided by Cantargia AB, Lund, Sweden. mCAN10 is a monoclonal mIgG2a anti-mouse IL1RAP antibody with L234A, L235A and P329G [LALA-PG] mutations leading to decreased Fc/FcγR effector function, which is not capable of facilitating antibody-dependent cellular cytotoxicity[141]. mCAN10 was administered through intraperitoneal injection with a loading dose (20mg/kg) on the first injection, followed by bi-weekly injections (10mg/kg). Treatment with two injections a week with an initial loading dose saturates soluble IL1RAP, and gives stable mCAN10 levels in plasma, a regimen found to have the best effect against heart function deterioration in a preliminary study.

Echocardiography

In order to assess cardiac function, we have utilized echocardiography, the measurement of cardiac function by use of ultrasound. Echocardiography is a non-invasive imaging technique that uses ultrasound waves to create detailed images of the heart. It provides information about the size, structure, and function of the heart and its various components. By using the Vevo 3100 system from Visualsonics and a small probe suitable for rodents, we performed transthoracic echocardiography to measure left ventricular function in mice in both myocarditis and sepsis models. Images were taken in the parasternal long axis, and ventricular volumes were calculated by tracing the ventricle in diastole and systole by Simpson method using VevoLAB (Visualsonics).

Flow cytometry

One of the main techniques used in this thesis for the purpose of characterizing immune cells is flow cytometry. Flow cytometry utilizes the staining of cells with fluorescently tagged antibodies specific for surface and intracellular markers, after which an instrument known as a flow cytometer detects the presence of fluorescent signal on individual cells. Specifically, we utilize antibodies specific for surface proteins expressed on immune cells such as cytokine receptors, cell adhesion molecules and growth factor receptors. The antibodies are covalently bound to proteins and synthetic molecules that emit fluorescent signals upon excitation with certain wavelengths known as fluorochromes. The cells are stained and prepared in a single cell suspension which is loaded into a flow cytometer. The flow cytometer uses a set of fluidics to hydroscopically focus the single cell suspension into a thin stream of individual cells. High power lasers of specific wavelengths are directed at the cells, and the light emitted is detected in a series of detectors consisting of photodetectors and filters, where specific light signatures correspond to specific fluorochromes. Taken together, this provides a high throughput method for detecting cell surface proteins on a large number of cells, which allows us to phenotypically differentiate various types of immune cells from tissues and blood.

Aim

The overarching objective of this doctoral project was to develop immunomodulatory treatments for sepsis-induced myocardial dysfunction (SIMD) and myocarditis based on S100A8/A9 and IL-1RAP blockade, and to dissect the underlying mechanisms of action.

Specific aims

Investigate the efficacy of S100A8/A9 blockade as a potential new treatment in SIMD (**Paper I**)

Explore the role of S100A8/A9 in the neutrophil-driven pathogenesis of autoimmune myocarditis and develop an immunomodulatory treatment based on S100A8/A9 blockade (**Paper II**)

Investigate antibody-mediated IL1RAP inhibition as a potential therapeutic strategy in viral and autoimmune myocarditis (**Paper III**)

Paper I

Therapeutic S100A8/A9 blockade inhibits myocardial and systemic inflammation and mitigates sepsis-induced myocardial dysfunction

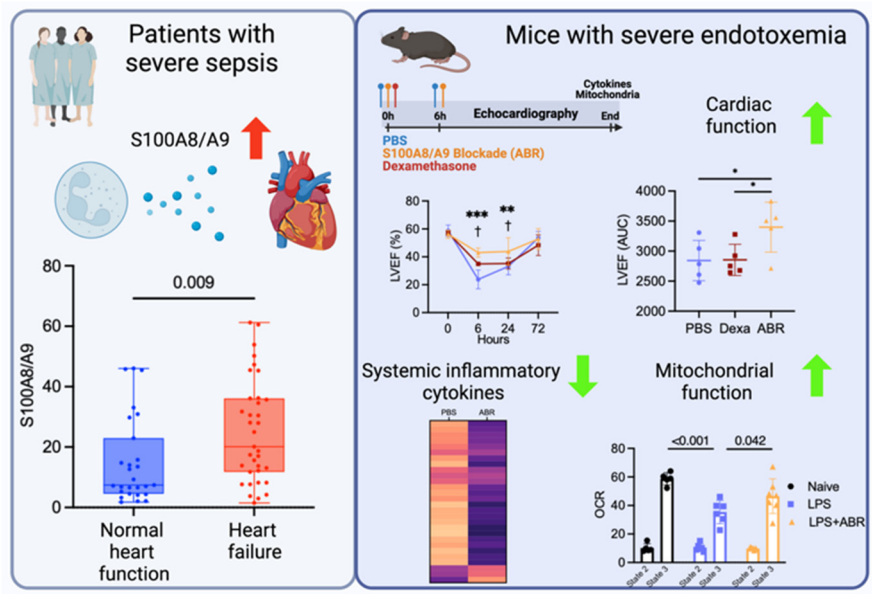

Figure 10 - Graphical abstract Paper I

Introduction, methods and results

Currently, there are no efficient treatments able to prevent or ameliorate the development of SIMD in sepsis. In this work, we sought to investigate the role of S100A8/A9 as a potential pathogenic factor in sepsis and SIMD, and to test whether blockade of S100A8/A9 with ABR-238901 could constitute a potential therapeutic strategy.

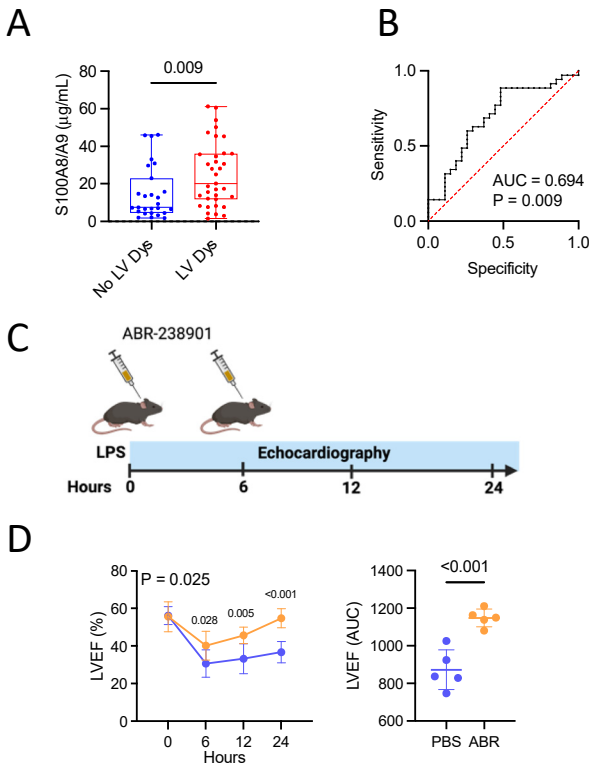

Figure 11 – S100A8/A9 is associated with SIMD

We measured S100A8/A9 levels in a cohort of patients with severe sepsis and examined the relationship between S100A8/A9 plasma levels and cardiac dysfunction. Using a mouse model of endotoxemia, we investigated the effects of S100A8/A9 blockade on cardiac function and inflammation. We then evaluated the therapeutic efficacy of S100A8/A9 inhibition, both by administering a delayed treatment at a later timepoint and by comparing the effects with a broad anti-inflammatory therapy used in sepsis patients.

In a cohort of 62 patients with severe sepsis, we investigated the association between plasma S100A8/A9 at admission and left ventricular (LV) dysfunction. Plasma S100A8/A9 was significantly higher in patients with LV dysfunction (P = 0.009, Fig. 11A). Receiver operating characteristic (ROC) analysis revealed that plasma S100A8/A9 levels can identify sepsis patients with LV dysfunction (Fig. 11B, c-statistic = 0.6942, P = 0.009). Next, we investigated if S100A8/A9 inhibition can prevent SIMD in an experimental model of endotoxemia. LV systolic function was significantly

improved by the treatment over the course of the experiment (2-way ANOVA, P = 0.025; area under-the-curve (AUC) comparison, P < 0.001). The post-hoc analysis revealed higher LVEF at 6h (P = 0.028), 12h (P = 0.005) and 24 hours (P < 0.001) in mice receiving S100A8/A9 blockade compared to PBS treated controls (Fig. 11C-D). These results suggests that S100A8/A9 is directly involved in the pathogenesis of SIMD, and that S100A8/A9 blockade can ameliorate cardiac dysfunction during endotoxemia.

Since endotoxemia is associated with a dysregulated immune response, we next investigated the anti-inflammatory effects of S100A8/A9 blockade. Inhibition of S100A8/A9 signaling resulted in a broad reduction of systemic inflammatory cytokine levels (Fig. 12A). S100A8/A9 blockade reduced circulating levels of the inflammatory cytokines IL-1β, IFN-γ, TNF-α, and of the chemokines MCP-1, MIP-2α and CXCL5, among others. To investigate the effects of ABR-238901 on myocardial inflammation, we performed qPCR analysis of cardiac tissue 12 hours post-LPS (Fig. 12B). The treatment significantly reduced cardiac expression of TNF-α and of the inflammasome components NLRP3, caspase-1, IL-1β and IL-18. We observed changes in immune cell composition between naïve and endotoxemic mice, but S100A8/A9 blockade did not change immune cell infiltration into the heart, indicating that the treatment effects are not mediated through changes in cardiac immune cell composition (Fig. 12C).

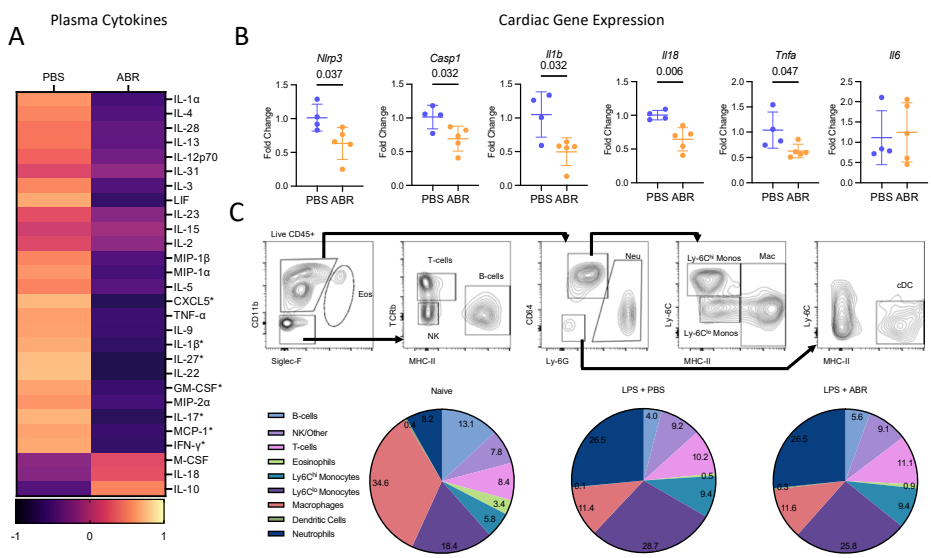

Figure 12 – Anti-inflammatory effects of S100A8/A9 blockade during endotoxemia. (A) Plasma cytokines. (B) Cardiac gene expression. (C) Cardiac flow cytometry.

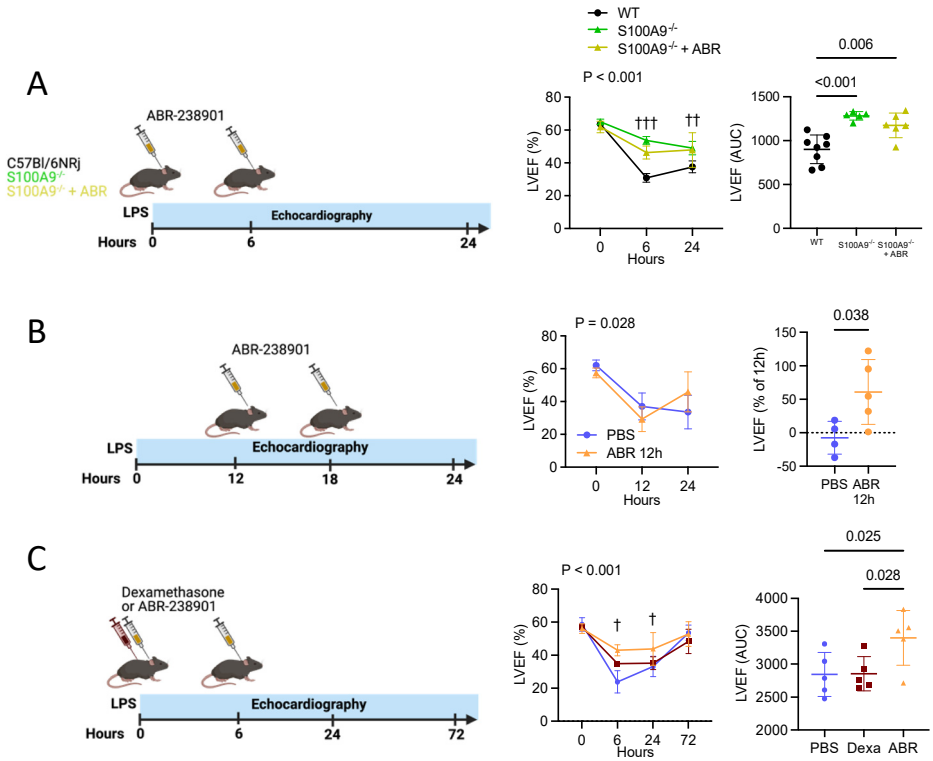

Figure 13 – S100A9 deficient mice are protected from cardiac dysfunction and therapeutic efficacy of S100A8/A9 blockade. (A–C) Experimental layouts and quantification of left-ventricular ejection fraction (LVEF).

To confirm the pathogenic role of S100A8/A9 in SIMD, we used mice genetically deficient in S100A9, which also lack S100A8 and the S100A8/A9 heterodimer. S100A9$^{-/-}$ mice developed a lower degree of LV dysfunction during endotoxemia (6h LVEF $P < 0.001$, AUC $P < 0.001$, Fig 13A). Additionally, we examined if delayed treatment was able to rescue LV function, by initiating S100A8/A9 blockade at 12h post-LPS. Delayed S100A8/A9 blockade efficiently rescued LVEF at 24h compared to PBS ($P = 0.036$, AUC $P = 0.038$, Fig 13B). Next, we compared S100A8/A9 blockade to anti-inflammatory glucocorticoid treatment with dexamethasone. Administration of dexamethasone improved LVEF at 6h post-LPS ($P = 0.003$) while ABR-238901 improved LVEF at 6 hours post-LPS, both compared to PBS ($P < 0.001$) and to dexamethasone ($P = 0.026$, Fig 13C).

Conclusion and limitations

In **paper I**, we conclude that S100A8/A9 blockade reduces inflammation and cardiac dysfunction in endotoxemia. We show that S100A8/A9 levels are associated with SIMD in patients with severe sepsis, and that therapeutic inhibition of S100A8/A9 can ameliorate cardiac dysfunction in endotoxemia. S100A8/A9 blockade reversed established LV dysfunction, and outperformed dexamethasone with regard to ameliorating heart failure in the murine model of endotoxemia. Our results suggest that S100A8/A9 is a viable marker for cardiac dysfunction in sepsis and highlight S100A8/A9 blockade as a potential therapeutic strategy against SIMD.

Our study has a few limitations which are important to consider. LPS-induced endotoxemia is a model of gram-negative sepsis, but it remains to be determined if S100A8/A9 blockade has similar effects in gram-positive sepsis. Although we have previously demonstrated the anti-inflammatory effects of ABR-238901 in a model of polymicrobial sepsis, the effects on cardiac function in bacterial models of sepsis remain to be determined[86,87]. Cardiac dysfunction in sepsis patients is heterogeneous, where depressed LVEF can be accompanied by LV dilatation and endothelial dysfunction, or occur in the presence of right ventricular (RV) dysfunction[70]. Due to these differences, the results should be viewed in the context of the experimental setting, and caution should be exercised when extrapolating to the clinical scenario.

Paper II

S100A8/A9 drives inflammatory neutrophil polarization and promotes cardiac dysfunction in autoimmune myocarditis

Introduction, methods and results

Neutrophil infiltration into the heart has been shown to correlate with the severity of myocarditis, and depletion of neutrophils in viral myocarditis has been shown to ameliorate disease[112,113]. In **paper II**, we hypothesized that neutrophil-derived S100A8/A9 contributes to disease development during autoimmune myocarditis, and that pharmacologic inhibition of S100A8/A9 could be a potential therapeutic strategy

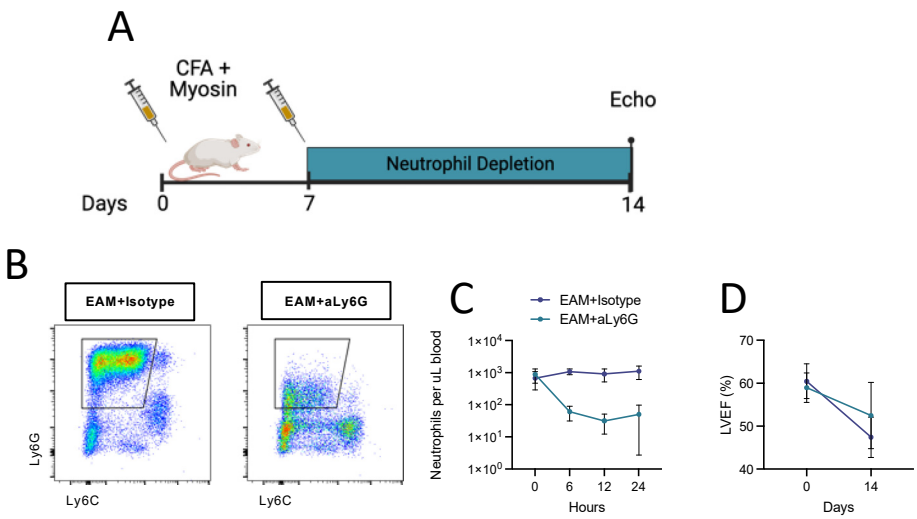

Figure 14 – Neutrophil depletion during EAM. (A) Experimental layout. (B-C) Neutrophil flow cytometry. (D) Left ventricular ejection fraction (LVEF) quantification.

We induced EAM in mice, and assessed the role of neutrophil driven inflammation in cardiac dysfunction by depleting neutrophils, or by inhibiting neutrophil-derived S100A8/A9 with ABR-238901. Using flow cytometry, we evaluated immune cell infiltration into the heart, and measured cellular apoptosis by TUNEL immunofluorescent staining of heart sections. Lastly, we interrogated the effects of the treatment on cardiac neutrophil phenotypes by single-cell RNA sequencing (scRNA-seq).

We confirmed efficient neutrophil depletion by flow cytometry (Fig 14B-C). Neutrophil depletion for 1 week improved cardiac function compared to controls, confirming the pathogenic role of neutrophils during EAM (P = 0.009, Fig 14D). As neutrophil depletion is not feasible in patients, we then focused on targeting the neutrophil-derived inflammatory mediator S100A8/A9 as a potential therapeutic strategy in myocarditis.

We found highly elevated plasma levels of S100A8/A9 on day 21 in EAM mice compared with controls receiving CFA alone (P= 0.01, Fig 15A). We administered ABR-238901 in drinking water to inhibit S100A8/A9 throughout the duration of the experiment, and observed an improvement in cardiac function compared to vehicle-treated mice (Fig 15A-B). At day 42, mice receiving S100A8/A9 inhibition developed significantly less cardiac fibrosis (P = 0.031, Fig 15C). The cardiac functional improvement was accompanied by reduced number of TUNEL+ apoptotic cells in the myocardium on day 21 (Fig. 15D, P = 0.004).

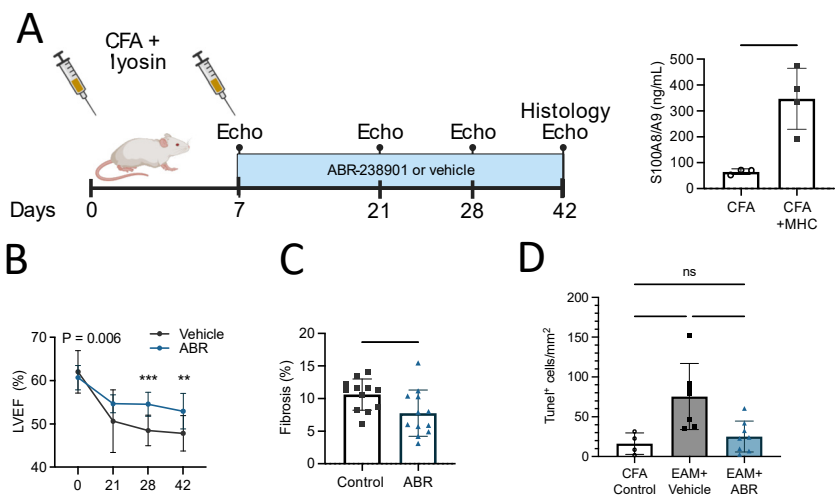

Figure 15 – S100A8/A9 inhibition improved function during EAM. (A) Experimental layout and S100A8/A9 plasma levels. (B-C) Quantification of left ventricular ejection fraction (LVEF). (D) Cardiac apoptosis

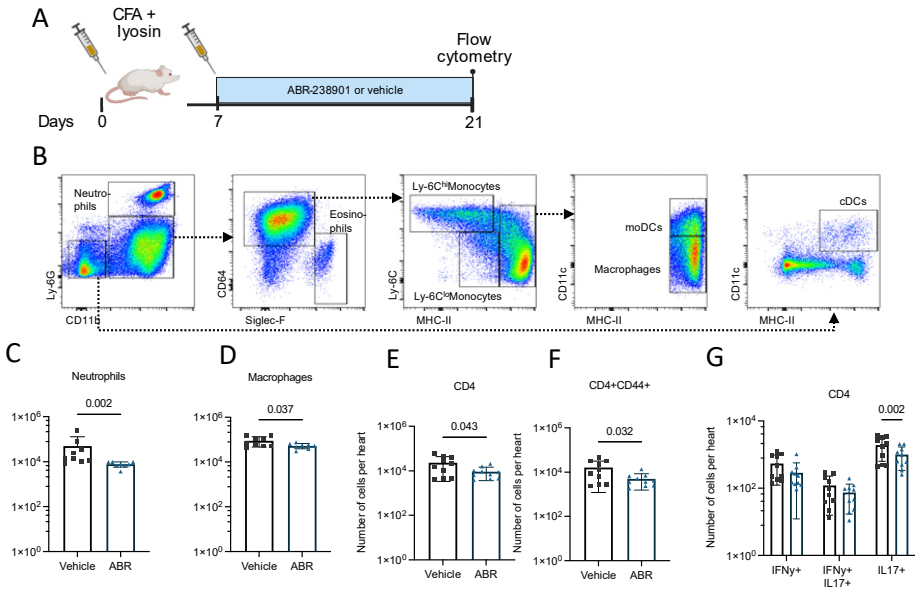

Figure 16 – Cardiac immune infiltration during EAM. (A) Experimental layout. (B-G) Cardiac flow cytometry.

Myocardial neutrophil infiltration was markedly reduced in mice receiving S100A8/A9 blockade, and macrophage content was also lower (Fig 16 A-D). We also characterized the impact on T cell responses, and found fewer CD4+ T cells and activated CD44+ T cells, as well as significantly fewer IL-17-producing CD4 cells in the hearts of mice receiving S100A8/A9 inhibition (Fig. 16E-G).

To investigate the cardiac neutrophil phenotype, we performed scRNA-seq on cardiac CD45+ leukocytes isolated on day 21. Neutrophils were re-clustered, resulting in the identification of 5 sub-populations (Fig. 17A-B). We identified a cluster of newly recruited neutrophils (Cluster 1), two clusters of neutrophils with a pro-inflammatory gene signature (Cluster 2-3), and a cluster with immune modulatory gene expression (Cluster 4, Fig 17B-D). When analyzing neutrophils by treatment group, we found that the majority of cardiac-infiltrating neutrophils in control mice belonged to the inflammatory-activated clusters. ABR-238901 treatment decreased the proportion of inflammatory-activated neutrophils, and polarized neutrophils towards the immunomodulatory cluster (Fig 17E). Taken together, we show a remarkable heterogeneity of cardiac neutrophils during EAM, dominated by inflammatory-activated neutrophil subsets. S100A8/A9 blockade shifts myocardial neutrophil polarization towards a phenotype predominantly expressing immunoregulatory genes,

suggesting that S100A8/A9 is a central switch in the inflammatory pathogenesis of myocarditis.

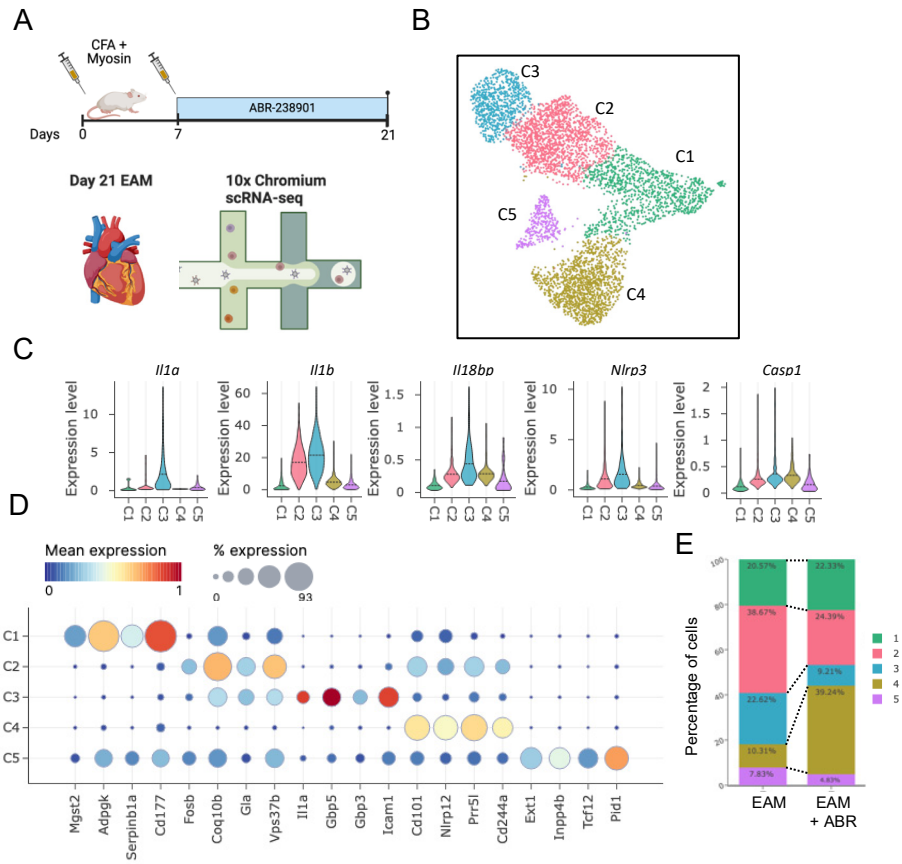

Figure 17 – Neutrophil heterogeneity during EAM. (A) Experimental layout. (B) UMAP of neutrophil subclusters. (C-D) Neutrophil gene expression. (E) Frequency of neutrophil populations.

Conclusion and limitations

In **paper II**, we investigated the pathogenic role, diversity and therapeutic targeting of neutrophils in autoimmune myocarditis. We show that neutrophil depletion improves cardiac function in EAM. Inhibition of S100A8/A9 function mitigated myocardial apoptosis and fibrosis, and improved cardiac function. The treatment lowered cardiac neutrophil infiltration and had potent immunomodulatory effects on cardiac neutrophil environment. S100A8/A9 blockade induced an important shift from a pro-inflammatory to an immunomodulatory neutrophil phenotype. Our data identify

S100A8/A9 as a central driver of neutrophil recruitment and inflammatory activation in the myocardium, and promote S100A8/A9 blockade as a novel immunomodulatory therapy in myocarditis.

Our study has a few limitations to be considered. Firstly, while the EAM model mirrors the autoreactive immune processes during myocarditis, the viral CVB3 model better reproduces the clinical setting where myocarditis is triggered by a direct cardiac viral infection. It this remains to be determined if S100A8/A9 blockade modulates neutrophils in a similar fashion in viral myocarditis. Secondly, although neutrophils were successfully depleted in the short term, they were still present at sacrifice, suggesting incomplete depletion over a long time. Neutrophil depletion in inflammatory settings is difficult, and a potential "rebound" effect might have compromised depletion over time[142,143].

Paper III

IL-1 receptor accessory protein (IL1RAP) blockade with a monoclonal antibody reduces cardiac inflammation and preserves heart function in viral and autoimmune myocarditis

Introduction, methods and results

Pre-clinical studies have demonstrated that blocking individual components of the IL-1 signaling pathway or neutralizing IL-33 by a soluble decoy receptor may hold therapeutic promise in treating myocarditis. We hypothesized that blockade of IL1RAP might be an efficient therapeutic approach in myocarditis. In **Paper III**, we evaluated the efficacy of blocking IL-1RAP signaling for the treatment of viral and autoimmune myocarditis. We used an IL1RAP blocking antibody developed by Cantargia AB, Lund, Sweden. The studies were conducted in collaboration with the group of Prof. Daniela Čiháková at Johns Hopkins University, Baltimore, USA.

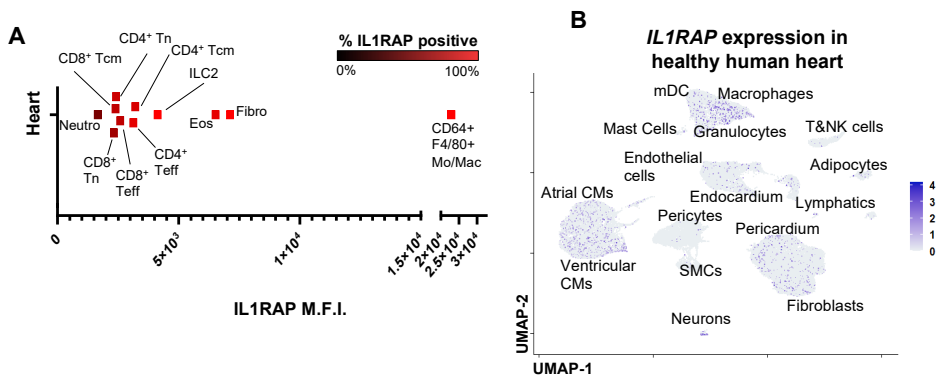

Figure 18 – IL1RAP expression during CVB3 infection and in healthy human hearts. (A) Expression of IL1RAP in mouse hearts during viral myocarditis. (B) IL1RAP expression in healthy human hearts.

We induced coxsackievirus B3 (CVB3)-mediated or experimental autoimmune myocarditis (EAM) in BALB/c mice, followed by treatment with an Fc-modified IL1RAP-blocking monoclonal antibody (mCAN10) that does not induce cell lysis. Myocarditis severity and immune infiltration were assessed by histology and flow cytometry, and cardiac function was measured by echocardiography.

IL1RAP was highly expressed in CD64+F4/80+ monocytes/macrophages, fibroblasts and eosinophils in the hearts of naïve or CVB3-infected mice (Fig. 18A). To examine if IL1RAP is also expressed in human cells, we used a publicly available single-cell RNA dataset from healthy human heart samples, where IL1RAP was predominantly expressed in macrophages and granulocytes (Fig 18B).

Mice with CVB3 myocarditis were treated with mCAN10 or IL-1Ra (which only blocks IL-1), and disease severity at day 10 was evaluated (Fig 19A). The mCAN10-treated animals displayed significantly reduced myocarditis severity, as evaluated by histology, compared to the isotype and PBS groups (P = 0.010) (Fig. 19B-C). Viral persistence in the heart was similar among groups (Fig. 19D). This suggests that IL1RAP blockade reduces CVB3 myocarditis severity without affecting viral clearance.

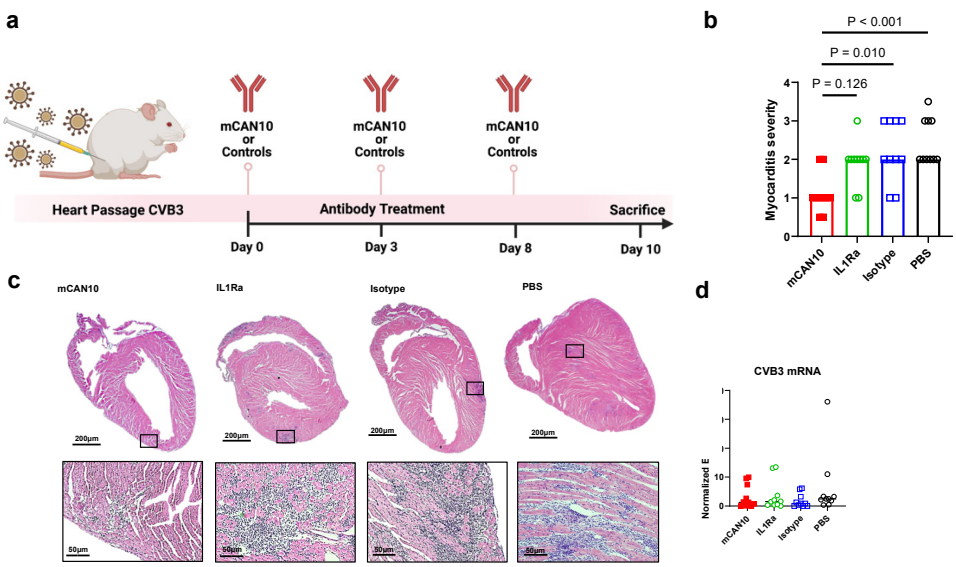

Figure 19 – IL-1RAP inhibition during CVB3 myocarditis. (A) Experimental layout. (B-C) Myocarditis severity scoring. (D) Viral mRNA levels.

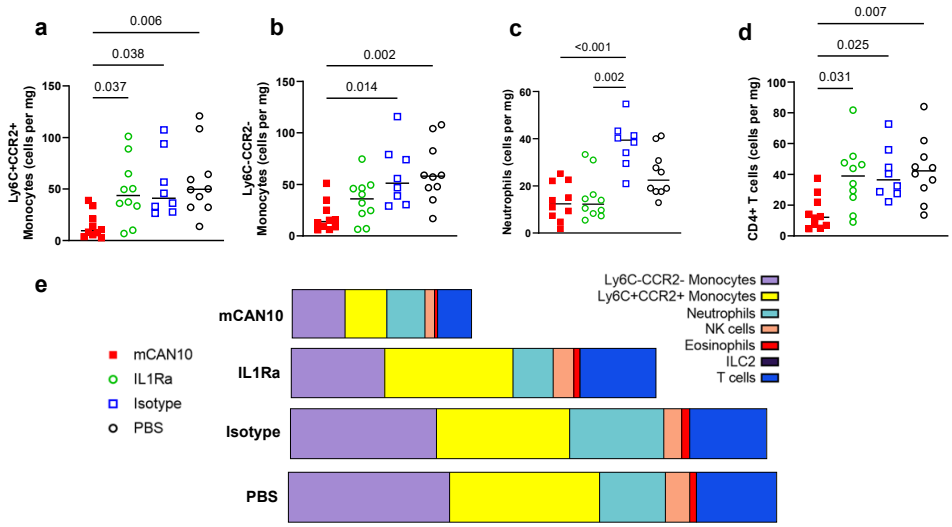

Figure 20 – mCAN10 reduced immune cell infiltration during CVB3 myocarditis. (A-E) Cardiac immune cell populations during viral myocarditis.

mCAN10 potently reduced the numbers of CD45+ immune cells infiltrating the heart compared to its isotype and PBS controls, as assessed by flow cytometry (Fig. 20). Several immune populations in the hearts of the mCAN10 group were reduced compared to isotype, particularly the numbers of Ly6C+CCR2+ monocytes (P = 0.038), macrophages (P = 0.014) and neutrophils (P = 0.001, Fig 20A-C). Total CD4+ T cells were also decreased by the mCAN10 treatment (P = 0.025, Fig. 20D).

To investigate the therapeutic potential of mCAN10 in ameliorating long-term cardiac dysfunction, we used a model of autoimmune myocarditis (Fig 21A). mCAN10 improved cardiac function on day 28 and day 42 compared to the isotype control (Fig 21B). We quantified the presence of monocytes/macrophages and neutrophils on day 21 by immunohistochemistry, and found fewer CD68+ macrophages and Ly6G+ neutrophils in the hearts of mCAN10-treated animals (Fig 21C-D).

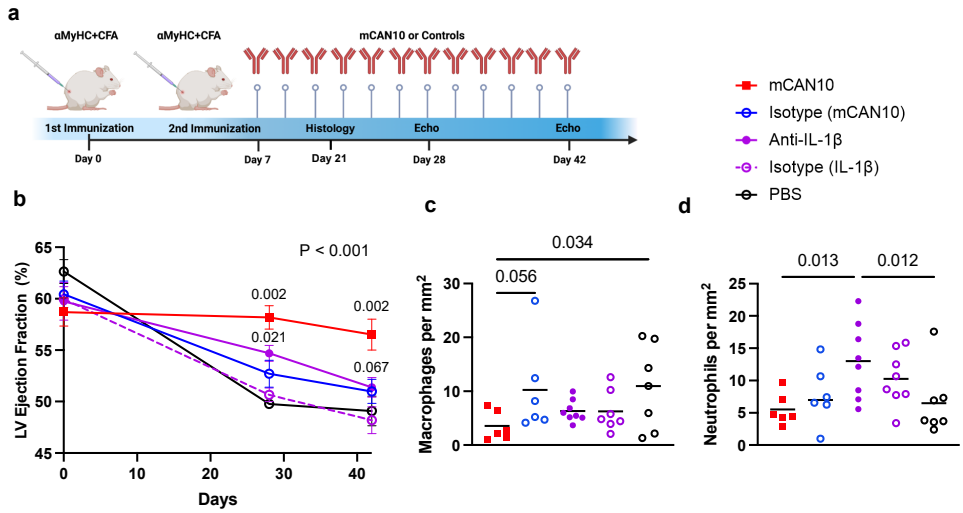

Figure 21 – IL1RAP blockade during autoimmune myocarditis. (A) Experimental layout. (B) Left ventricular ejection fraction. (C-D) Cardiac histology of macrophage and neutrophil content.

Conclusion and limitations

In Paper III, we show that blocking IL1RAP reduces the severity of acute CVB3-myocarditis and EAM, and ameliorates long-term cardiac dysfunction in EAM. We conclude that IL1RAP blockade is a potential therapeutic strategy in viral and autoimmune myocarditis. These findings underscore the potential translational benefit of IL1RAP blockade to treat cardiac inflammatory disease.

The study is not without limitations. Firstly, we used different mouse models to test treatment efficacy in acute and chronic myocarditis. The EAM model was chosen for chronic myocarditis in order to avoid potential bias induced by the death of the sickest animals in the CVB3 model. As the acute CVB3 myocarditis experiments were terminated on day 10, potential long-term beneficial effects of delayed mCAN10 treatment remains to be investigated.

Summary and clinical perspective

SIMD and myocarditis are inflammatory cardiomyopathies. The studies presented in this thesis focus on the development of possible new immunomodulatory therapeutic approaches targeting the pathogenic immune responses in the respective diseases. In sepsis, we still don't completely understand the underlying mechanisms and there is a lack of specific anti-inflammatory strategies that can mitigate myocardial dysfunction. Myocarditis is a heterogeneous disease without clinically approved therapies. We hypothesize that targeting common pathogenic immune pathways might prove to be a viable strategy for both autoimmune and viral myocarditis.

In **Paper I**, we showed that S100A8/A9 is associated with cardiac dysfunction in sepsis patients, and that blockade of S100A8/A9 could present a novel therapeutic strategy. Previous studies have identified elevated S100A8/A9 levels in patients with sepsis and indicated that high plasma S100A8/A9 is associated with increased mortality[80,82–84,144,145]. Experimental work has highlighted the relationship between S100A8/A9 and mortality in experimental sepsis, where S100A9$^{-/-}$ mice displayed lower inflammation and mortality, which could be aggravated by administration of recombinant S100A8/A9[44]. However, the causal relationship between S100A8/A9 and mortality remains poorly understood. SIMD is an underappreciated aspect of sepsis in patients, and our results offer a potential mechanistic link between elevated S100A8/A9 and mortality. Assays for measuring S100A8/A9 are already used in the clinic to detect exacerbations of inflammatory bowel disease, facilitating the use of S100A8/A9 levels as a biomarker in other disease contexts. The finding that S100A8/A9 levels in SIMD can function both as a biomarker and potential treatment target opens the possibility of using S100A8/A9 levels as a measure of treatment efficacy, and underlines its importance in the pathogenesis of SIMD. In **Paper I** and **Paper II**, we evaluated the efficacy of the S100A8/A9 blocker ABR-238901 as a potential anti-inflammatory strategy in sepsis and myocarditis. Earlier generations of S100A8/A9 blockers laquinimod (ABR-215062), tasquinimod (ABR-215050) and paquinimod (ABR-215757), have been tested in clinical trials for inflammatory conditions such as multiple sclerosis, Crohn's disease and systemic sclerosis, and multiple myeloma (NCT04405167)[136,146–148]. In previous trials, the ABR family of S100A8/A9 blockers were well tolerated by patients, and our findings in **Paper I** and **Paper II** open the way

for future clinical testing and re-purposing of these S100A8/A9 blockers for treatment of patients with sepsis and myocarditis.

In **Paper II** and **Paper III**, we evaluated S100A8/A9 inhibition and IL-1RAP blockade as anti-inflammatory therapies in myocarditis. Viral myocarditis patients that develop cardiac dysfunction and fibrosis have a 50% 10-year survival rate[149]. Treatments are primarily supportive, and patients with end-stage disease often require cardiac transplantation, with the exception of giant cell myocarditis where immunosuppressive therapy has been shown to have some benefit[150]. However, specific immunomodulatory treatments that target the pathogenic immune mechanisms driving pathogenesis are missing. In **Paper II** and **Paper III**, we explore S100A8/A9 inhibition and IL-1RAP blockade as potential anti-inflammatory therapeutics in this disease. There are important differences between the two strategies, one being a small-molecule inhibitor and the other an antibody-mediated blockade, with important considerations. ABR-238901 has a half-life around 4 hours, while mCAN10 and other antibody-mediated strategies have a half-life in the magnitude of days to weeks. Although necessitating continuous dosing, a short half-life allows for the possibility of a shorter treatment targeting excessive acute inflammation, a strategy shown to be especially beneficial in experimental myocardial infarction, where long-term immune inhibition potentially impact cardiac repair[137,138]. In myocarditis, cardiac injury is exclusively immune-driven and lasts for weeks, which favors the use of an antibody-based therapy where one dose is likely to cover the entire therapeutic window. On the other hand, as shown in our sepsis study, S100A8/A9 blockade with ABR-238901 has a very broad immunosuppressive profile, as S100A8/A9 is one of the most important NLRP3 inflammasome triggers early on during the immune response. In contrast, mCAN10 only blocks the activity of IL-1, IL-33 and IL-36 and might thus have lower potency. It remains to be determined, in a direct comparison or in future clinical trials, which of the two strategies generates the best protective effects and the lowest complication rates.

Excessive immunosuppression comes with increased risk of secondary infections, as seen with the antibody-mediated IL-1b blockade by Canakinumab tested in the CANTOS trial. Defining the optimal therapeutic window is an important aspect of all anti-inflammatory therapies[151]. We only tested S100A8/A9 inhibition in chronic autoimmune myocarditis, while IL-RAP blockade was investigated in both acute viral and chronic autoimmune disease. It remains to be determined how S100A8/A9 inhibition impacts viral clearance.

As highlighted above, ABR blockers have been used previously in anti-inflammatory conditions, and further clinical testing might pave the way for re-purposing as treatments for myocarditis and sepsis. A humanized anti-IL1RAP antibody (CAN10, Cantargia AB, Lund, Sweden) is under development for treatment of inflammatory and

fibrotic diseases and is currently undergoing a phase 1 clinical trial in healthy subjects and in patients with plaque psoriasis (NCT06143371). Thus, our finding in **Paper III** showing the anti-inflammatory ability of mCAN10 in myocarditis underscores the potential translational benefit of IL-1RAP blockade for cardiovascular inflammation. IL-1 inhibition has previously been reported to have a beneficial effect in myocarditis. In the ARAMIS trial, patients with acute myocarditis were treated with the IL-1 inhibitor Anakinra[124,129,130]. The results of this small trial were neutral due to low incidence of complications, and larger trials are needed to adequately explore the potential of IL-1 inhibition in myocarditis. Taken together, both anti-inflammatory strategies employed in **Paper II** and **Paper III** have translational potential. S100A8/A9 inhibition by ABR compounds as well as IL-1RAP inhibition are currently being tested in other inflammatory diseases, and favorable outcomes could pave the way for application in patients with cardiac inflammation.

The goals of future studies will be to more closely investigate the pathogenic signaling mechanisms of S100A8/A9 such as relative contribution of TLR4 and RAGE receptors, as well as the relative contribution of immune versus non-immune signaling. In myocarditis, S100A8/A9 inhibition and IL-1RAP blockade both show promise, and clinical trials are needed to further explore their use potential therapies for cardiac inflammation.

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Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

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Lund University, Faculty of Medicine
Doctoral Dissertation Series 2024:99
ISBN 978-91-8021-595-4
ISSN 1652-8220