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Itaconate mediated NLRP3 inflammasome tolerance in the context of human monocyte Tenascin-C activation

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Abstract

Despite the extensive research conducted on innate immune cell memory, there are still many unknowns when it comes to the ability of endogenous danger molecules, also known as damage-associated molecular patterns (DAMPs), to influence the tolerisation of myeloid cells to repeated stimulation. This is partly due to the majority of tolerisation studies using exogenous pathogen-associated molecular patterns (PAMPs) such as Lipopolysaccharide (LPS). While understanding virulence factor-induced LPS tolerance is important for treating infectious diseases and sepsis patients, it is essential to examine cellular stimulations using disease-relevant DAMPs to fully comprehend the development of chronic inflammatory conditions such as rheumatoid arthritis. In my work, I have utilized the Toll-like receptor 4 (TLR4) activating fibrinogen-like globe domain (FBG-C) of the extracellular matrix protein tenascin-C to advance our scientific understanding of the ability of DAMPs to trigger and tolerise a critical aspect of TLR-mediated inflammation known as the NLRP3 inflammasome.

I discovered significant differences in the capacity of primary human monocytes to produce and secrete the non-inflammasome regulated cytokine tumour necrosis factor (TNF) compared to the inflammasome regulated cytokine interleukin-1 beta (IL-1ß), as well as in their capacity to secrete IL-1ß via classical NLRP3 mediated pyroptotic means versus alternative non-pyroptotic means. Additionally, I found that although the DAMP FBG-C and the PAMP LPS induce a similar cytokine response in primary human monocytes in the first 24 hours of activation, subsequent restimulation revealed fundamental differences in their capacity to induce tolerisation. Notably, this effect is specific to IL-1ß, and hence the NLRP3 inflammasome, and not due to differences in the requirement for the TLR4 co-receptor cluster of differentiation 14 (CD14).

Furthermore, I found that the inability of monocytes to tolerise the NLRP3 inflammasome following FBG-C activation is due to a delayed upregulation response of the itaconate-producing enzyme aconitate decarboxylase 1 (ACOD1). I then uncovered that ACOD1 upregulation is crucial for inhibiting the processing of the pore-forming protein Gasdermin D and thus for inhibiting pyroptosis in tolerised monocytes. Importantly, I also showed that monocytes isolated from RA patient blood have a delayed ACOD1 upregulation response following both LPS

and FBG-C TLR4 activation, which prevented the tolerisation of RA monocytes in a Gasdermin D and pyroptosis-dependent manner. Finally, I uncovered that a 2% oxygen (hypoxic) culture environment renders human monocytes unable to tolerise the NLRP3 inflammasome in an ACOD1-independent manner.

In summary, my research revealed significant differences in the way primary human monocytes interpret pathogen and damage signals, in addition to uncovering substantial changes in their ability to produce the pleiotropic proinflammatory cytokine IL-1ß over a 48-hour period. This work demonstrated that environmental stressors, endogenous triggers and chronic pathology could all trigger loss of tolerance and a prolonged NLRP3 inflammasome priming phenotype.

Table of Contents

Abstract **2**
Table of contents **4**
List of Tables **7**
List of Figures **7**
Acknowledgements **9**
Author's Declaration **10**
Definitions/Abbreviations **11**
Chapter 1 Introduction **20**
1.1 The innate immune system **20**
1.1.1 Innate immune memory and endotoxin tolerance 22
1.1.2 Monocytes as immune effector cells 27
1.1.3 Danger-associated molecular patterns (DAMPs) in health and disease 32
1.1.4 The DAMP Tenascin-C: a matrix protein with diverse immunomodulatory capabilities 37
1.2 The NLRP3 Inflammasome **48**
1.2.1 NLRP3 Inflammasome assembly and activation 49
1.2.2 NLRP3 Inflammasome dysregulation in inflammatory diseases and the implications of tolerance 54
1.3 Immunometabolism of myeloid cells **59**
1.3.1 The antimicrobial role of itaconate 62
1.3.2 The immunomodulatory properties of itaconate 64
1.3.3 The effects of hypoxia on myeloid cells in chronic inflammatory conditions 70
1.4 Rheumatoid arthritis as a chronic inflammatory disease **72**
1.4.1 Pathogenesis and cellular mediators of RA 73
1.4.2 Current RA treatment strategies 77
1.5 Hypothesis and Aims **78**
Chapter 2 Materials and Methods **81**

2.1 Materials **81**
2.2 Methods **83**
2.2.1 FBG-C protein production and purification 84
2.2.2 SDS-PAGE and Coomassie staining 85
2.2.3 Silver staining 85
2.2.4 Western blotting for FBG-C detection 86
2.2.5 Circular dichroism spectroscopy and LPS contamination testing 86
2.2.6 Monocyte isolation from human PBMCs 87
2.2.7 Monocyte activation and measurement of pyroptosis levels 88
2.2.8 Cell culture in 2% oxygen 89
2.2.9 Generation of primary human monocyte knock-out cells 89
2.2.10 Cytokine ELISA 90
2.2.11 Western blotting for the investigation of intracellular protein levels 91
2.2.12 Flow cytometry 92
2.2.13 Monocyte DNA extraction 93
2.2.14 Polymerase chain reaction and agarose gel electrophoresis 93
2.2.15 T7 endonuclease surveyor assay 94
2.2.16 Sanger DNA sequencing and assessment of editing efficiency following gene knock-out 95
2.2.17 Computational processing of publicly available RNA-seq data 95
2.2.18 Statistical analysis 96

Chapter 3 Comparing the proinflammatory properties of the DAMP tenascin-C and the PAMP LPS in human monocytes in the context of NLRP3 inflammasome activation and tolerance **97**
3.1 Introduction **97**
3.2 Results **98**
3.2.1 Production, purification and activity testing of endotoxin free FBG-C 98
3.2.2 Temporal differences of TNF and IL-1β secretion profiles in FBG-C or LPS stimulated human monocytes 104
3.2.3 Establishment of NLRP3 inflammasome tolerance by FBG-C or LPS in human monocytes 114
3.2.4 Investigating the requirement for the TLR4 co-receptor CD14 for FBG-C or LPS human monocyte activation 119
3.3 Discussion **124**

Chapter 4 Investigating the role of the immunometabolite itaconate and its enzyme ACOD1 in the establishment of NLRP3 inflammasome tolerance in human monocytes under different culture conditions and disease states **127**

4.1 Introduction **127**

4.2 Results **129**

4.2.1 Involvement of ACOD1 in the establishment of NLRP3 inflammasome tolerance in human monocytes 129

4.2.2 NLRP3 inflammasome tolerance in rheumatoid arthritis patient monocytes 140

4.2.3 Effects of hypoxia on human monocyte NLRP3 inflammasome tolerance 146

4.3 Discussion **151**

Chapter 5 General discussion **155**

5.1 Clinical relevance and future work **156**

List of References **158**

List of Tables

Table 1.1 Tenascin-C as a modulator of cell activation 39
Table 2.1 List of all reagents and resources used, along with source and identifier 81

List of Figures

Figure 1.1 Canonical and non-canonical pathways of NLRP3 inflammasome activation in human myeloid cells 53
Figure 1.2 TCA cycle remodelling following myeloid cell TLR4 activation 61
Figure 3.1 FBG-C protein preparation strategy 99
Figure 3.2 FBG-C purification protocol assessment 100
Figure 3.3 FBG-C purity control 101
Figure 3.4 FBG-C protein refolding assessment 102
Figure 3.5 FBG-C activity test on human monocytes 103
Figure 3.6 LPS titration vs FBG-C, 24-hour activation time course in primary human monocytes 105
Figure 3.7 LPS vs FBG-C activation of freshly isolated monocytes vs monocytes rested for 24 hours 107
Figure 3.8 LPS vs FBG-C activation with CASP8 inhibitor pre-treatment......... 109
Figure 3.9 LPS vs FBG-C activation followed by nigericin canonical NLRP3 inflammasome activation 111
Figure 3.10 LPS vs FBG-C activation followed by nigericin canonical NLRP3 inflammasome activation, pyroptosis images 113
Figure 3.11 LPS vs FBG-C 24-hour inflammasome tolerisation..................... 116
Figure 3.12 LPS vs FBG-C 24-hour inflammasome tolerisation, effect on pyroptosis 118
Figure 3.13 Levels of CD14 protein expression on the surface of primary human monocytes in the five days following CD14 gene Cas9 knock-out 121
Figure 3.14 Effect of CD14 knock-out on LPS and FBG-C activation mediated proinflammatory cytokine secretion in primary human monocytes 123
Figure 4.1 Effect of LPS vs FBG-C activation on ACOD1 upregulation and Gasdermin D cleavage in primary human monocytes 130

Figure 4.2 Role of ACOD1 and Gasdermin D in LPS vs FBG-C mediated inflammasome tolerisation in primary human monocytes 132
Figure 4.3 Primary human monocyte ACOD1 population-wide gene knock-out success confirmation 134
Figure 4.4 Effect of ACOD1 gene knock-out on the establishment of LPS and FBG-C mediated inflammasome tolerance in primary human monocytes 137
Figure 4.5 Effect of ACOD1 gene knock-out on Gasdermin D mediated pyroptosis 139
Figure 4.6 RA patient monocytes vs healthy monocytes inflammasome tolerisation 142
Figure 4.7 Role of ACOD1 and Gasdermin D in LPS and FBG-C mediated inflammasome tolerisation in rheumatoid arthritis patient monocytes 144
Figure 4.8 Pyroptosis levels of RA patient monocytes vs healthy monocytes in the context of LPS and FBG-C inflammasome tolerisation 145
Figure 4.9 LPS vs FBG-C mediated inflammasome tolerisation in 2% oxygen culture of primary human monocytes 148
Figure 4.10 Role of ACOD1 and Gasdermin D in LPS vs FBG-C mediated inflammasome tolerisation in 2% oxygen culture of primary human monocytes 150
Figure 5.1 Graphical summary of key findings 156

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Author’s Declaration

I declare that, unless otherwise stated, the results presented in this thesis are my own work and do not consist of work that is part of a thesis submitted or pending submission within The University of Glasgow, or elsewhere.

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Bogdan Stankov Didov

Definitions/Abbreviations

Aß – Amyloid beta

ABC - Age-associated B cell

ACOD1- Aconitate decarboxylase 1

ACPAs - Anti-citrullinated protein antibodies

AD - Alzheimer's disease

ADP - Adenosine diphosphate

AIM2 - Absent in melanoma 2

ALDOA - Aldolase A

ALRs - AIM2-like receptors

AMPAs - Anti-modified protein antibodies

ANOVA - Analysis of variance

AP-1 - Activator protein 1

APP - Amyloid precursor protein

ASC - Apoptosis-associated speck-like protein

ATP - Adenosine triphosphate

AUF1 - AU-rich elements/poly(U)-binding/degradation factor 1

BCL2 - B cell CLL/lymphoma-2

bDMARDs - Biological DMARDs

BLOC3 - Biogenesis of lysosome-related organelles complex 3

BM- Bone marrow

BMDMs - Bone marrow-derived macrophages

BNIP3 - BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

C/EBPß - CCAAT/enhancer binding protein-beta

C-22 - Cysteine 22

CAPS - Cryopyrin-associated periodic syndromes

CARD - Caspase recruitment domain

Cas9 - CRISPR associated protein 9

CASP8 - Caspase 8

CBB - Coomassie Brilliant Blue

CCL2 - C-C motif chemokine ligand 2

CCL3 - C-C motif chemokine ligand 3

CCL5 - C-C motif chemokine ligand 5

CCR2 - C-C motif chemokine receptor 2

CCR3 - C-C motif chemokine receptor 3

CCR5 - C-C motif chemokine receptor 5

CD - Circular dichroism

CD14 - Cluster of differentiation 14

CD16 - Cluster of differentiation 16

cDNA - Complementary DNA

CIA - Collagen-induced arthritis

CIRP - Cold-inducible RNA-binding protein

CLRs - C-type lectin receptors

cMoP - Common monocyte progenitors

CMP - Common myeloid progenitor

CNS - Central nervous system

CoA - Coenzyme A

COPD - Chronic obstructive pulmonary disease

CpG - Cytosine-phosphate-guanine

CRISPR - Clustered regularly interspaced short palindromic repeats

Cryo-EM - Cryogenic electron microscopy

csDMARDs - Conventional synthetic DMARDs

CX3CR1 - C-X3-C motif chemokine receptor 1

CXCR4 - C-X-C motif chemokine receptor 4

DAMPs - Damage-associated molecular patterns

DCs - Dendritic cells

DI - Dimethyl itaconate

DKK1 - Dickkopf-1

Dll1 - Delta-like 1

DMARDs - Disease-modifying antirheumatic drugs

DMF- Dimethyl fumarate

EAE - Autoimmune encephalomyelitis

eCIRP - Extracellular CIRP

ECM - Extracellular matrix

EGFL - Epidermal growth factor-like

EGFR - Epidermal growth factor receptor

ELISA - Enzyme-linked immunosorbent assay

EPS - Extracellular polysaccharides

ET - Endotoxin tolerance

FBG - Fibrinogen-like globe

FBG-C - Fibrinogen-like globe domain of Tenascin-C

FCAS - Familial cold autoinflammatory syndrome

FLS - Fibroblast-like synoviocytes

Fn-EDA - Fibronectin extra domain A

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GLUT-1 - Glucose transporter 1

GMP - Granulocyte and macrophage progenitors

GSDMD - Gasdermin D

GSDMD-NT - N-terminal domain of GSDMD

GSH - Glutathione

GSK3 - Glycogen synthesis kinase 3

H3K27me3 - Histone H3 at lysine 27 tri-methylation

H3K4me3 – Histone H3 at lysine 4 tri-methylation

H3K9me2 - Histone H3 at lysine 9 di-methylation

H4Ac - Acetylation of histone H4

HC- Healthy control

HIF - Hypoxia-inducible factor

HIF-1α - Hypoxia-inducible factor 1 alpha

HK2 - Hexokinase 2

HLA-DR1 - Human leukocyte antigen - DR1

HMGB1 - High-mobility group box protein

HMGN1 - High-mobility group nucleosome-binding protein 1

HSCs - Hematopoietic stem cells

ICL - Isocitrate lyase

IFN-α - Interferon alpha

IFN-β - Interferon beta

IgE - Immunoglobulin E

IgG - Immunoglobulin G

IL-12 - Interleukin-12

IL-13 - Interleukin-13

IL-18 - Interleukin-18

IL-1Ra - IL-1 receptor antagonist

IL-1α - Interleukin-1 alpha

IL-1β - Interleukin-1 beta

IL-21 - Interleukin-21

IL-32 - Interleukin-32

IL-4 – Interleukin-4

IL-5 - Interleukin-5

IL-6 - Interleukin-6

IL-6R - IL-6 receptor

IL-8 - Interleukin-8

iNOS - Inducible nitric oxide synthase

IRAK-M - Interleukin-1 receptor-associated kinase-M

IRF1 - Interferon regulatory factor 1

IRF3 - Interferon regulatory factor 3

IRG1 - Immune-responsive gene 1

ITGAX - Integrin subunit alpha X

JAK - Janus kinase

KCNK6 - Potassium channel subfamily K member 6

KO- Knock-out

LBP - LPS binding protein

LDHA - Lactate dehydrogenase A

LFA-1 - Lymphocyte function-associated antigen 1

LPS - Lipopolysaccharide

LRR - Leucine-rich repeat

LTA - Lipoteichoic acid

MAPK - Mitogen-activated protein kinase

MCM - Methylmalonyl-CoA mutase

MCP - Monocyte chemoattractant protein

M-CSF - Macrophage-colony stimulating factor

M-CSFR - M-CSF receptor

MD-2 - Myeloid differentiation protein-2

MDP - Macrophages and dendritic cell progenitors

MIP-1A - Macrophage inflammatory protein-1A

miRNAs - MicroRNAs

MMP-7 - Matrix metalloproteinase-7

MMP-9 - Matrix metalloproteinase-9

MMPs - Matrix-degrading metalloproteinases

MRP14 - Myeloid-related protein 14

MRP8 - Myeloid-related protein 8

MS- Multiple sclerosis

MSU - Monosodium urate

mtROS - Mitochondrial reactive oxygen species

MWS - Muckle-Wells syndrome (MWS)

MyD88 - Myeloid differentiation primary response 88

N475K -Aasparagine 745 to lysine substitution mutation

NEK7 - NIMA-related kinase 7

NETs - Neutrophil extracellular traps

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NIMA - Never in mitosis A

NLRC4 - NLR family CARD domain-containing protein 4

NLRP - Nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain containing

NLRP1 - NLR family pyrin domain containing 1

NLRP12 - NLR family pyrin domain containing 12

NLRP3 - NLR family pyrin domain containing 3

NLRP6 - NLR family pyrin domain containing 6

NLRs - NOD-like receptors

NO - Nitric oxide

NOD - Nucleotide-binding oligomerization domain

NOMID - Neonatal-onset multisystemic inflammatory disease

NR4A1 - Nuclear receptor subfamily 4 group A member 1

NR4A2 - Nuclear receptor subfamily 4 group A member 2

NR4A3 - Nuclear receptor subfamily 4 group A member 3

OD - Optical density

oxLDL - Oxidized low-density lipoprotein

P2X7 - P2X purinoceptor 7

PADs - Peptidylarginine deiminases

PAMPs - Pathogen-associated molecular patterns

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate-buffered saline

PDPN - Podoplanin

PHDs - Prolyl hydroxylases

PI3K - Phosphatidylinositol 3-kinase

PPI - Proton-pump inhibitor

PRRs - Pattern recognition receptors

PS1 - Presenilin 1

pVHN - von Hippel Lindau tumour suppressor protein

PYD - Pyrin domain

RA - Rheumatoid arthritis

RAGE - Receptor for advanced glycation endproducts

RANKL - Receptor activator of NF-κB ligand

RBPs - RNA-binding proteins

RF - Rheumatoid factor

RIG1 - Retinoic Acid-Inducible Gene 1

RLRs - RIG-like receptors

RORγT - Retinoic acid receptor-related orphan receptor gamma t

ROS - Reactive oxygen species

scRNA-seq - Single cell RNA sequencing

SCVs - Salmonella-containing vacuoles

SDH - Succinate dehydrogenase

Ser295 - Serine 295

SH2 - Src homology 2 domain

SHIP - SH2-containing inositol phosphatase

SLE - Systemic lupus erythematosus

SMAD-2 - Mothers against decapentaplegic homolog 2

SMAD-3 - Mothers against decapentaplegic homolog 3

SOCS1 - Suppressor of cytokine signalling 1

STAT - Signal transducer and activator of transcription

TBI - Traumatic brain injury

TBS- Tris-buffered saline

TCA cycle - Tricarboxylic acid cycle

Tfh – T follicular helper cells

TGF-β - Transforming growth factor beta

TGN - Trans-Golgi network

Th1 - T helper 1 cell

Th17 - T helper 17 cell

Th2 - T helper 2 cell

TIAR - T-cell-restricted intracellular antigen 1-related protein

TLR4 - Toll-like receptor 4

TLR7 - Toll-like receptor 7

TLR8 - Toll-like receptor 8

TLR9 - Toll-like receptor 9

TLRs - Toll-like receptors

TN-C - Tenascin-C

TNF - Tumour necrosis factor

TNFR1 - Tumour necrosis factor receptor 1

Treg - T regulatory cell

TRIF - Toll/IL-1R domain-containing adaptor-inducing interferon beta

tsDMARDs - Targeted synthetic DMARDs

TTP - Tristetraprolin

TWIK2 - Tandem pore domain weak inward rectifying K^+ channel 2

VEGF - Vascular endothelial growth factor

WT – Wild type

4-OI - 4-octyl itaconate

Chapter 1 Introduction

1.1 The innate immune system

The human body is under constant attack from a variety of pathogens that attempt to enter the body via barriers such as the skin and mucous membranes. To defend against this ongoing threat, our bodies have developed a complex immune system consisting of two closely connected arms: innate and adaptive immunity. While the adaptive immunity provides specific responses to combat invading pathogens, such as T cell cytotoxicity and the production of antibodies from B cells, which offer long-term protection against repeated infection, it takes time to mount these responses. Meanwhile, invading pathogens such as Escherichia coli bacteria, which are capable of rapid replication, produce millions of cells within hours (Cooper & Helmstetter, 1968). Therefore, the rapid and nonspecific mechanisms of innate immunity are crucial for providing protection during the initial phase of an infection (Medzhitov, 2007).

The outer layer of the skin and the membranes lining the respiratory, digestive, reproductive, and urinary tracts are integral parts of the innate immune system. These physical barriers act as a first line of defence, effectively separating the host system from the outside environment (Proksch et al., 2008). The epithelial tissues also produce mucous, saliva, and sweat, which help reduce pH levels and physically eliminate pathogens. Furthermore, the epithelium provides a habitat for a commensal microbiome to grow, which has a symbiotic relationship with the host. This microbiome further hinders the spread of pathogens by competing for nutrients and space and by producing antimicrobial molecules (Belkaid & Hand, 2014).

When the physical protective barriers are breached, the cells of the innate immune system spring to action. The response of the body's innate immune system is orchestrated by circulating and tissue-resident cells, such as monocytes, macrophages, neutrophils, and other granulocytes, as well as dendritic cells (DCs) and natural killer cells. In order to identify the presence of a pathogen, innate immune cells make use of pattern recognition receptors (PRRs), which are proteins with versatile structures that can be categorised into four main families: membrane-bound Toll-like receptors (TLRs), C-type lectin

receptors (CLRs), NOD and RIG-like receptors (RLRs) (Mogensen, 2009). PRRs detect molecular patterns from pathogens, collectively known as pathogen-associated molecular patterns (PAMPs). PAMPs are defined as motifs present on molecules produced only by microorganisms and not by the host and are thus recognised as non-self. These molecules are crucial for the survival of pathogens, and any changes in their structure could result in functional impairment or lethality. Hence, PAMPs are conserved among large groups of pathogens (Medzhitov, 2007). One of the most widely recognised PAMPs is lipopolysaccharide (LPS), which is the main component of the outer membrane in Gram-negative bacteria and is essential for protecting the bacterial cell from external stressors (Bertani & Ruiz, 2018). LPS is recognised by the PRR Toll-like receptor 4 (TLR4).

Recognition of PAMP ligands by PRRs initiates signalling pathways that lead to the activation of genes necessary for the antimicrobial response (Takeuchi & Akira, 2010). Upon detecting PAMPs, the cellular response of the innate immune system operates through a few different mechanisms. Namely, activated myeloid cells such as monocytes and macrophages produce cytokines and chemokines, such as tumour necrosis factor (TNF) and interleukin 6 (IL-6), which are multifunctional signalling molecules that support inflammation and facilitate immune cell infiltration into the affected tissue. Additionally, stronger activation of these cells can lead to the secretion of the pleiotropic cytokine interleukin 1 beta (IL-1ß) via the assembly of large intracellular complexes known as nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain containing (NLRP) inflammasomes, which are discussed in detail later (Section 1.2.1) (Blevins et al., 2022). Apart from secretory factors, innate immune leucocytes, particularly macrophages, neutrophils, and dendritic cells, can also eliminate pathogens via a process known as phagocytosis (Uribe-Querol & Rosales, 2020). Phagocytosis begins via the identification of complement components and opsonins on the surface of a pathogen by the phagocytic cells, leading to engulfment and internalisation of the pathogen, followed by subsequent lysis (Uribe-Querol & Rosales, 2020). Innate effector cells also provide guidance to the adaptive arm of the immune system, enabling the development of a specific immune response and long-term immune memory, a process primarily carried out by dendritic cells but also to an extent by macrophages and monocytes (Lee et

al., 2017). After capturing a pathogen, antigen-presenting cells can process and present antigens to adaptive immune lymphocytes, such as T and B cells. In addition to other functions, cytokines and immune mediators produced by the innate immune cells also enhance antigen presentation efficiency and promote lymphocyte movement and development (Lee et al., 2017).

1.1.1 Innate immune memory and endotoxin tolerance

The conventional view is that the adaptive immune system has exclusive ownership over immunological memory. Lymphocytes, the adaptive immune cells, develop specificity to antigens, and when they recognise invading pathogens, they activate, expand and then leave behind T and B memory cells, enabling the immune system to provide targeted antibody and cytotoxic responses upon subsequent infection by the same pathogen (Medzhitov, 2007). However, in approximately 97% of living organisms, there is no antibody and clonal selection-based memory system (Purvis & Hector, 2000), hence, it is hard to imagine that plants and invertebrates, which are also faced with a multitude of pathogenic microorganisms, have not developed acquired defence mechanisms to enhance clearance upon re-infection. Increasing evidence supports the concept that the innate immune system can also retain some information about recent encounters with pathogens in order to mount an adjusted response to subsequent infections.

At the start of the 20th century, neurological and psychiatric conditions, such as neurosyphilis and psychosis, were managed with a treatment known as 'fever therapy'. This treatment involved inducing fever by deliberately infecting patients with malaria (a method that received the Nobel Prize in 1927) or injecting them with other fever-inducing substances, such as the typhoid fever vaccine (Albert, 1999). It was noticed that patients who received the vaccine showed diminished responses to subsequent vaccinations over time and needed higher doses of the vaccine to produce a fever (Lehner & Hartung, 2002). This puzzling effect was studied further in rabbits, and it was found that repeated

administration of bacterial preparations led to a decrease in their ability to induce fever. The same results were seen in vivo using purified bacterial LPS, also called endotoxin, and this diminished responsive state was subsequently termed endotoxin tolerance (ET).

Additional investigation conducted in rabbits showed that multiple injections of endotoxin not only made the animals resistant to febrile responses but also made them immune to the lethal effects of LPS (Watson & Kim, 1963). Additionally, the survival of mice (Freudenberg & Galanos, 1988; Lehner et al., 2001) and rats (Sanchez-Cantu, 1989; Williams, 1985) following a lethal LPS challenge was improved by inducing endotoxin tolerance with low doses of LPS. This reduction in toxicity is attributed to the observed suppression of proinflammatory cytokine induction by LPS in tolerised animals since all three species exhibited markedly reduced serum levels of TNF, IL-1ß, and IL-6 following endotoxin administration (Erroi et al., 1993; Mengozzi et al., 1991; Sanchez-Cantu, 1989; Wakabayashi et al., 1994). A clinical trial with healthy human volunteers also reported inhibition of TNF and IL-6 release in endotoxin tolerance (Astiz et al., 1995).

The crucial role of myeloid cells in the development of ET was demonstrated in 1988 when LPS-susceptible macrophages were transferred, inducing endotoxin tolerance in LPS-resistant mice (Freudenberg & Galanos, 1988). Since then, impaired cytokine production in endotoxin-tolerised cells has been widely observed in monocytes and macrophages in both *in vitro* and *ex vivo* studies of cells isolated from both humans and different lab animals. Among all the studies that have been conducted over the past 40 years, the most significant shared feature appears to be the consistent suppression of TNF release following re-challenge with LPS, observed in murine peritoneal macrophages (Dobrovolskaia et al., 2003; Rajaiah et al., 2013; Tominaga et al., 1999), splenocytes (Julian et al., 2015), bone marrow-derived macrophages (BMDMs) (Butcher et al., 2018; O'Carroll et al., 2014) and human primary cells including peripheral blood mononuclear cells (Pena et al., 2011; Weisheit et al., 2020), monocytes (Petes et al., 2018; Widdrington et al., 2018), and monocyte-derived macrophages (Petes et al., 2018), as well as in cell lines derived from both mouse (Virca et al., 1989) and human (El Gazzar et al., 2007). In contrast to TNF, the secretion of other inflammatory mediators, such as IL-6 and IL-1ß, has been reported to be either

suppressed or augmented in endotoxin tolerance depending on the cell type and activation context (reviewed in West & Heagy, 2002). Specifically, the tolerisation of IL-1ß following a re-challenge with LPS has been the subject of debate since this cytokine is under the rigorous control of different inflammasome complexes, and it will be discussed in detail later (Sections 1.2.2 and 1.3.2). Additionally, endotoxin-tolerised human monocytes displayed an increased ability to phagocytose particles but had reduced capability to present antigens (Del Fresno et al., 2009). This indicates the complexity of ET and suggests that it is not merely a state of unresponsiveness.

The molecular processes underlying LPS tolerance have been debated since its discovery. LPS requires three additional host proteins to initiate a response via its receptor TLR4, namely, LPS binding protein (LBP), cluster of differentiation 14 (CD14), and myeloid differentiation protein-2 (MD-2). LBP carries LPS near the cell, and CD14 delivers the molecule to MD-2 bound to TLR4 (Park & Lee, 2013). The assembly of the signalling platform, consisting of two copies of the LPS-MD2-TLR4 complex, leads to the activation of a downstream signalling cascade through the adaptor protein myeloid differentiation primary response 88 (MyD88), enabling the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway activation, resulting in the production of proinflammatory cytokines. LPS-induced signalling can also trigger a MyD88-independent pathway by involving the adaptor molecule Toll/IL-1R domain-containing adaptor-inducing interferon (IFN)- ß (TRIF), which activates the interferon regulatory factor 3 (IRF3) transcription factor, leading to the upregulation of type I interferons (Kawasaki & Kawai, 2014). Initially, the tolerant state observed in cells stimulated by LPS was thought to be due to impaired signalling through its receptor, TLR4. Indeed, *in vitro* stimulation of mouse peritoneal macrophages with LPS led to a reduction in the surface expression of TLR4 within hours, and this suppression was still present after 24 hours (Nomura et al., 2000). However, additional research has shown that the TLR4 surface expression level was not decreased in peripheral blood mononuclear cells 24 hours after LPS activation (Adib-Conquy & Cavaillon, 2002), while CD14, was found to be increased (Ziegler-Heitbrock et al., 1994), and its surface expression in human THP-1 cells and mouse macrophages was either increased or remained unchanged (Martin et al., 2001; Nomura et al., 2000). Additionally, levels of MD-2 mRNA in LPS

tolerance were shown to be upregulated in human monocytes (Medvedev et al., 2002) and downregulated in peripheral blood mononuclear cells (Adib-Conquy & Cavaillon, 2002), while the TLR4 gene mRNA expression levels in human, mouse, and rat cells have been reported as decreased, unchanged, or increased (Fan & Cook, 2004). In summary, these findings do not provide enough support for the hypothesis that sequestering of TLR4 and its co-receptors from the cell surface is the primary mechanistic cause of LPS tolerance.

Following repeated exposure to LPS, cells that developed tolerance consistently exhibited impaired activation of NF-κB, as demonstrated by multiple studies (West & Heagy, 2002). This impaired activation is sometimes considered evidence of tolerance induction, along with the suppression of TNF. For example, inefficient phosphorylation and degradation of NF-κB inhibitors, which prevent the nuclear translocation of this transcription factor, was observed in mouse macrophages that had developed tolerance (Medvedev et al., 2000) and in monocytes isolated from patients with Gram-negative sepsis (Shalova et al., 2015). Additionally, several negative regulators of the MyD88-dependent signalling cascade have been identified as relevant for ET (reviewed in (Biswas & Lopez-Collazo, 2009) and may explain the reduced NF-κB activity. For instance, the SH2-containing inositol phosphatase (SHIP), which inhibits NF-κB by reducing phosphatidylinositol 3-kinase (PI3K) activity (Kalesnikoff et al., 2002), was found to be upregulated in tolerised mouse macrophages (Sly et al., 2004) and human monocytes (Piao et al., 2009). SHIP−/− bone marrow-derived macrophages were unable to undergo endotoxic tolerance (Sly et al., 2004), and knockdown of SHIP-1 using shRNA in THP-1 cells prevented them from undergoing ET as well (Pan et al., 2010). Other molecules such as Interleukin-1 receptor-associated kinase-M (IRAK-M), A20, and suppressor of cytokine signalling 1 (SOCS1) were also observed to be upregulated in ET (Nakagawa et al., 2002; Xiong & Medvedev, 2011), and their knockdown resulted in impaired tolerance in mouse macrophages (Kinjyo et al., 2002; Kobayashi et al., 2002), or in human THP-1 cells (Xiong et al., 2011). Additionally, defects in the components of the MyD88-NF-κB pathway were associated with the tolerant phenotype. A short splice variant of MyD88, which has a reduced capacity to activate downstream signalling, was upregulated in monocytes from septic patients (Adib-Conquy et al., 2006).

The modification of DNA through methylation, as well as the acetylation and methylation of histone tails, have been observed to change the structure of chromatin and its accessibility, thereby controlling gene transcription. These so-called epigenetic alterations, also known as marks, can have repressive effects, such as the example of dimethylation of histone H3 at lysine 9 (H3K9me2) and tri-methylation of H3 at lysine 27 (H3K27me3), as well as activating effects, like acetylation of H4 (H4Ac) and tri-methylation of H3 at lysine 4 (H3K4me3) (Bannister & Kouzarides, 2011). Extensive epigenetic re-modelling in the promoter and enhancer regions of LPS response genes was shown in endotoxin tolerised human monocytes, leading to changes in transcriptional activity that could account for some of the effects observed in the tolerised phenotype (Foster et al., 2007; Novakovic et al., 2016; Saeed et al., 2014). For instance, increased repressive marks such as H3K9me2 and DNA methylation and decreased phosphorylation of histone H3 at serine 10, linked with open chromatin, were identified at the TNF gene promoter in tolerised THP-1 cells (El Gazzar et al., 2007; Gazzar et al., 2008).

It is crucial to note that the production of immune mediators like TNF, IL-1ß, and IL-6 in response to LPS is regulated beyond gene transcription through post-transcriptional control mechanisms. These mechanisms involve RNA-binding proteins (RBPs) and microRNAs (miRNAs) that can bind to transcribed mRNA, control its stability, and regulate cytokine translation (Mino & Takeuchi, 2013). Additionally, RBPs and miRNAs have been found to promote the acquisition of the ET phenotype by causing mRNA decay and inhibiting the translation of tolerised effector genes. In THP-1 cells, miR-221, along with RBPs tristetraprolin (TTP) and AU-rich elements/poly(U)-binding/degradation factor 1 (AUF1), have been proven to degrade TNF transcripts, while miR-579 and miR-125b, together with T-cell-restricted intracellular antigen 1-related protein (TIAR), are responsible for halting protein synthesis (El Gazzar & McCall, 2010). Members of the miR-181 family have been shown to target both TNF and IL-6 mRNA. Inhibition of miR-181 has been demonstrated to enhance transcript half-life and restore cytokine production in human and mouse cells *in vitro* (Dan et al., 2015; Zhang et al., 2015). Conversely, it has been suggested that enhanced transcript stability and translation efficiency contribute to the sustained expression of non-tolerisable anti-inflammatory genes, such as secretory interleukin-1 receptor

antagonist, as seen in LPS tolerised THP-1 cells (Learn et al., 2000). Two miRNAs that target different downstream components of the TLR signalling cascade, miR-146a and miR-155, have also been identified as crucial in the establishment of ET (Nahid et al., 2011).

Although ET is the first identified and most extensively researched memory-like phenotype of the innate immune system, myeloid cell tolerance is not specific to LPS. Other TLR ligands, like Pam3CSK4 (TLR2 agonist), lipoteichoic acid (TLR2 agonist), poly(I:C) (TLR3 agonist), flagellin (TLR5 agonist), and R848 (TLR7/8 agonist), can also induce a hyporesponsive state (Ifrim et al., 2014; Lehner et al., 2001). Additionally, LPS and other stimuli can create cross-tolerance, providing tolerance to a different secondary stimulus (Seeley & Ghosh, 2017). These findings are supported by the presence of genome-wide epigenetic reprogramming in ET, where repressive marks hinder the reactivation of specific TLR-inducible genes, regardless of the secondary signal.

Together, these data suggest that LPS-induced tolerance goes beyond a refractory state resulting from receptor desensitisation, and instead, it emerges as a fundamental adaptation, enabling the most efficient, context-dependent response of innate immune cells when faced with repeated reactivation, protecting the host from excessive damage and unnecessary inflammation. Indeed, chronic inflammation is one of the defining features of autoimmune conditions, and a link could be made between the lack of tolerance in myeloid cells and disease pathogenesis. Hence, it is paramount to understand how ET can influence the maladaptation of tissue infiltrating myeloid cells in chronic inflammatory conditions such as rheumatoid arthritis (RA), since these cells are under constant repeated stimulation in the inflamed joint and inadequate tolerisation of monocytes to repeated endogenous triggers could lead to devasting proinflammatory cytokine production and secretion loop within the diseased tissues.

1.1.2 Monocytes as immune effector cells

Monocytes are a subset of white blood cells that travel within the bloodstream and function as immune system effector cells. They account for 5-10% of the

total white blood cell count in humans and remain in the bloodstream for a brief period before a portion of them move into tissues when needed, where they can mature into different cells such as macrophages and cells with dendritic cell like properties (Tacke & Randolph, 2006; Ziegler-Heitbrock, 2000; Zhao et al., 2023). This process has highlighted the notable characteristic of monocytes, which is their adaptable nature. Monocytes are primed to swiftly migrate in substantial numbers to various locations in the body, playing roles in either promoting inflammation or resolving it. Monocytes originate from hematopoietic stem cells (HSCs) found in the bone marrow (BM). Hematopoietic stem cells continually produce monocytes by carefully controlled population growth followed by differentiation through multiple intermediate myeloid lineage-specific precursors (Geissmann et al., 2010). HSCs generate common myeloid progenitor (CMP) cells via various multipotent precursors, which then mature into granulocyte and macrophage progenitors (GMP) dedicated to the myeloid lineage. GMPs can then produce neutrophils, and a portion of GMP cells subsequently develop into macrophages and dendritic cell progenitors (MDP). The MDPs then give rise to common monocyte progenitors (cMoP), which are committed to producing monocytes (Trzebanski & Jung, 2020).

A modified model has suggested that CMPs give rise to GMPs and MDPs through distinct pathways. Both GMPs and MDPs produce progenitors that develop into mature monocytes, but each type generates its own set of cells: MDPs give rise to classical DCs and monocyte-derived DCs, whereas GMPs produce neutrophils, neutrophil-like monocytes, and a group of atypical monocytes (Yáñez et al., 2017). Both internal and external factors influence the development of monocytes. For instance, during infection or stress, haematopoiesis can be altered to produce specific cell lineages needed for a particular systemic immune response (Yáñez et al., 2017). When monocyte chemoattractant protein (MCP) chemokines activate C-C motif chemokine receptor 2 (CCR2), monocytes exit the bone marrow and enter the bloodstream, where they respond to signals of damage, infection, or inflammation (Kuziel et al., 1997; Shi & Pamer, 2011).

Monocytes have multiple PRRs and chemokine receptors that respond to external stimuli and facilitate migration into the tissues where the signals originate. Human peripheral blood monocytes express various TLRs, including TLR2, TLR4, TLR5, and TLR9 (Cros et al., 2010). PRRs enable monocytes to recognise and

initiate an immune response against different pathogens such as viruses, bacteria, parasites and fungi (Kapellos et al., 2019). In the tissue environment, monocytes can differentiate into either macrophages or cells with DC-like properties, or they can continue mounting immune responses as monocytes until they undergo cell death (Kapellos et al., 2019).

Human circulating monocytes are heterogeneous and are primarily identified based on the presence of CD14 and CD16 proteins on their surface (Ziegler-Heitbrock et al., 2010). CD14 acts as a coreceptor for TLR4, while CD16, also known as Fc gamma RIII, is a receptor capable of binding immunoglobulin G (IgG) (Yeap et al., 2016). Based on the presence of these surface molecules, monocytes are categorised into three subsets: classical, characterised by high CD14 expression and no CD16 expression ($CD14^{++}$ $CD16^{-}$); intermediate, characterised by high CD14 expression and medium CD16 expression ($CD14^{++}CD16^{+}$); and non-classical, characterised by medium, low or negative CD14 expression and high CD16 expression ($CD14^{+/-}CD16^{++}$). The proportion of each monocyte subset is variable among healthy individuals and can be subject to further alteration in diseases such as rheumatoid arthritis (RA), where there is an increased proportion of intermediate monocytes (Cooper et al., 2012; Kawanaka et al., 2002). Classical monocytes comprise most circulating monocytes at approximately 80-95%, while intermediate monocytes represent roughly 2-8% of the population. Non-classical monocytes account for the remaining 2-10% of monocytes in the circulation (Sampath et al., 2018). Each monocyte subset exhibits distinct characteristics and functions, including differences in size, granularity, surface receptor profiles, responses to cytokines, transcriptional profiles and differentiation potential (Passlick et al., 1989; Sampath et al., 2018).

The classical and intermediate monocytes are typically considered to have inflammatory properties, while non-classical monocytes are thought to be involved in patrolling activities. Classical monocytes express the highest levels of CCR2, a receptor for chemokine ligand 2 (CCL2), which plays a role in directing cell migration to the sites of inflammation (Patel et al., 2017; Shi & Pamer, 2011). Research in mice has shown that CCR2 is necessary for the exit of monocytes out of the bone marrow. However, circulating monocytes lacking CCR2 can still migrate to the site of infection once in circulation, indicating that

the primary role of CCR2 is promoting the migration of monocytes out of the bone marrow (Serbina & Pamer, 2006). CCL2 levels increase in response to various inflammatory stimuli, which leads to an increase in the number of monocytes entering the blood from the bone marrow (Gerard & Rollins, 2001).

Intermediate monocytes have been shown to exhibit characteristics commonly associated with mature tissue macrophages (Weber et al., 2000). Compared to classical monocytes, intermediate monocytes have lower levels of CCR2 but higher levels of CCR5 (Weber et al., 2000). CCR5 is responsible for detecting macrophage inflammatory protein-1a (MIP-1a), also known as CCL3, a macrophage chemokine that is elevated in the synovial fluid and tissue of RA patients (Koch et al., 1994). In contrast to classical and intermediate monocytes, non-classical monocytes perform surveillance along the endothelium moving along the surface of the endothelium in a chemokine receptor C-X3-C motif chemokine receptor 1 (CX3CR1) and in an integrin lymphocyte function-associated antigen 1 (LFA-1) dependent manner (Auffray et al., 2007; Cros et al., 2010). Upon detecting pathogens, non-classical monocytes quickly enter tissues and mature into macrophages.

It has been shown that in humans, after classical monocytes emerge from the BM, they circulate for a day, while intermediate and non-classical monocytes circulate for approximately 4 and 7 days, respectively (Patel et al., 2017). In mice, it has been shown that the different monocyte subsets reflect their developmental status and that classical monocytes transform into non-classical monocytes over time via Notch signalling triggered by notch ligand delta-like 1 (Dll1) expressed on endothelial cells (Gamrekelashvili et al., 2016; Yona et al., 2013). Additionally, murine models have shown that in the absence of inflammation, some circulating classical monocytes return to the bone marrow in a C-X-C chemokine receptor type 4 (CXCR4)-dependent mechanism (Chong et al., 2016), where they differentiate into non-classical monocytes (Shi & Pamer, 2011). Notch2, which is highly expressed on $CD14^{+}$ human monocytes, is the preferred target for Dll1, suggesting a similar mechanism for the transition of human monocytes from classical to non-classical. The differentiation process of classical monocytes into intermediate monocytes, and ultimately into non-classical monocytes, is further supported by the observation that classical

monocytes are the only subset found in the human bone marrow (Patel et al., 2017).

Monocyte survival relies on the presence of specific growth factors present in the bloodstream. Researchers commonly use human pooled plasma or human pooled serum supplementation to mimic physiological conditions when conducting experiments using isolated monocytes. Additionally, growth factors such as macrophage-colony stimulating factor (M-CSF) are added directly into the culture medium to ensure monocyte survival. The signalling of the M-CSF receptor (M-CSFR) and its primary ligand, M-CSF (also known as CSF-1), is essential for the survival and differentiation of myeloid cells and it involves tyrosine kinase-dependent signalling cascades that activate the Phosphoinositide 3-kinase (PI3K)-Akt/Ras-Raf-MAPK- extracellular signal-regulated kinase (ERK) pathways, which also regulate cytoskeleton remodelling and cell adhesion (Mouchemore & Pixley, 2012; Pixley & Stanley, 2004).

Monocytes are commonly thought of as precursors to differentiated macrophage effector cells, however, it is now more widely appreciated that they also have their own hugely important immunological functions since they are quite efficient at producing an array of proinflammatory cytokines once they recognise a threat, as well as promoting homeostasis and repair. Each of the different monocyte subsets can respond uniquely to immune stimulants, resulting in a specific cytokine secretome when activated. For example, classical monocytes act as professional phagocytes and generate inflammatory cytokines such as TNF, IL-6 and IL-1ß in addition to reactive oxygen species (ROS) in response to microbial stimuli such as LPS (Rossol et al., 2011). On the other hand, non-classical monocytes are ineffective phagocytes and have been reported as unresponsive to ROS and cytokine production upon stimulation of cell surface TLRs. However, non-classical monocytes respond to the stimulation of intracellular TLR7/8 by viruses or immunocomplexes containing nucleic acids, producing TNF and CCL3 (Cros et al., 2010). Classical monocytes have also demonstrated their ability to enter tissues such as the skin and lungs under steady-state conditions in order to transport antigens to the draining lymph nodes without differentiating into macrophages and with minimal changes in their gene expression (Jakubzick et al., 2013).

The presence of proinflammatory stimuli results in an increased level of monocyte recruitment to peripheral tissues. Excessive levels of endogenous proinflammatory triggers present in certain chronic inflammatory conditions, such as RA, can cause monocytes to be at the centre of disease pathogenesis (Rana et al., 2018). In RA, monocytes contribute to pathogenesis through the production of proinflammatory cytokines and chemokines upon detection of disease stimuli known as damage-associated molecular patterns (DAMPs) by PRRs, which will be discussed next.

1.1.3 Danger associated molecular patterns (DAMPs) in health and disease

As previously discussed, the primary role of the innate immune system is the identification and elimination of foreign entities, however, organisms face threats to their internal balance beyond just infection. Non-microbial factors stemming from cellular stress and tissue damage can also trigger inflammatory reactions. These triggers include substances released when cells die and components of the damaged extracellular matrix, which play a crucial role in activating pathways involved in clearing cellular waste and restoring tissue integrity (Bryant et al., 2015; Schaefer, 2014). Additionally, the immune system can be stimulated by non-organic irritants like asbestos (Dostert et al., 2008), accumulations of internal molecules such as amyloid-beta (Aß) (Halle et al., 2008) and cholesterol (Duewell et al., 2010), and tumour-associated antigens (Woo et al., 2015). Collectively, these sterile triggers of the immune system have been given the term damage-associated molecular patterns. The immune response to DAMPs is triggered by pattern recognition receptors, the same receptors that identify PAMPs. For example, TLR4, the receptor that recognises bacterial LPS, also responds to several internal molecules, including biglycan (Schaefer et al., 2005), fibronectin extra domain A (Fn-EDA) (Okamura et al., 2001), and fibrinogen (Smiley et al., 2001). As DAMPs are molecules produced by the host, they must be kept separate from PRRs in healthy tissues to prevent unnecessary activation. Under normal conditions, DAMPs are segregated in specific cellular compartments, downregulated, or sequestered in the

extracellular matrix (ECM) (Schaefer, 2014). For instance, tenascin-C, a natural ligand of TLR4, which will be discussed in detail later (Section 1.1.4), is not present in healthy tissue but is produced in response to injury and inflammation (Midwood et al., 2009).

Additionally, mitochondrial DNA, which is rich in cytosine-phosphate-guanine (CpG) sites and secluded within the mitochondria, is discharged into the bloodstream by cells after an injury, where it can be absorbed and trigger TLR9 (Zhang et al., 2010). A key constituent of the ECM, namely hyaluronan fragments, accumulates during matrix degradation and prompts an immune response through TLR4 and TLR2 (Jiang et al., 2005). Although the mechanism of action of DAMPs is not entirely clear, it is known that similar to PAMPs, upon recognition by PRRs, DAMPs induce the production of proinflammatory cytokines and chemokines, leading to leukocyte recruitment to the site of damage and the promotion of inflammation (Chen & Nuñez, 2010). This immune response serves as a pre-emptive safeguard in case the injury is no longer sterile. Additionally, after being stimulated by DAMPs, activated innate immune cells become effective antigen-presenting cells and can provide co-stimulatory signals to lymphocytes when other relevant antigens emerge, such as pathogenic, tumour-associated, or altered self-antigens (Zelenay & Reis E Sousa, 2013).

Endogenous and pathogenic triggers elicit responses that partly overlap, however, while the immune system is prompted to combat invading pathogens upon detecting PAMPs, DAMPs play a critical role in stimulating tissue repair. During the initial inflammatory stage of wound healing, DAMP-induced inflammation and subsequent leukocyte infiltration are vital for clearing cellular debris. As dead tissue is removed from the injury site, immune mediators prompted by DAMPs help shift leukocytes from a proinflammatory to a repair program, facilitating the resolution of inflammation (Ellis et al., 2018). Subsequently, macrophages and other innate immune cells direct fibroblasts to produce ECM components such as collagen and promote proliferation and migration of keratinocytes. Consequently, ECM regeneration occurs, and tissue integrity is reinstated (Ellis et al., 2018; Zindel & Kubes, 2020). Thus, the immune system does not just differentiate between harmful external and harmless internal stimuli instead, it is capable of understanding the situation of the identified danger, distinguishing between internal damage signals and PAMPs,

and initiating a well-timed, appropriate reaction directed at tissue restoration rather than being restricted to immediate inflammation.

Although the induction of sterile inflammation by DAMPs enables effective tissue repair, they can also drive harmful pathological processes as they need to be promptly eliminated by phagocytes after the proinflammatory phase of healing to facilitate tissue repair completion. Failure to remove DAMPs from the inflamed tissue can result in persistent activation of immune responses, leading to unresolved inflammation and the development of chronic inflammatory conditions (Gong et al., 2020). Dysregulated inflammation induced by DAMPs has been implicated in various pathologies, including autoimmune diseases (e.g., rheumatoid arthritis (Goh & Midwood, 2012) and systemic lupus erythematosus (Liu et al., 2020)), metabolic disorders (such as diabetes (Shin et al., 2015) and atherosclerosis (Zimmer et al., 2015)), and neurodegenerative diseases (like Alzheimer's disease (Venegas & Heneka, 2017) and Parkinson's disease (Pajares et al., 2020)). Prolonged activation of inflammatory pathways by DAMPs can also lead to disordered tissue remodelling and tissue fibrosis instead of physiological regeneration. Then, the excessive fibrotic tissue, consisting of ECM components secreted by the activated fibroblasts, accumulates in the sites of inflammation and damage, leading to scarring and impaired organ function (Anders & Schaefer, 2014). Hence, while sterile inflammation is important in physiological tissue repair, failure to resolve excessive DAMP-induced inflammation can contribute to the development of pathology.

Similar to PAMPs, there is evidence suggesting that some DAMPs are capable of inducing a refractory functional state resemblant to ET. These DAMPs include high-mobility group box protein 1 (HMGB1) (Aneja et al., 2008), high-mobility group nucleosome-binding protein 1 (HMGN1) (Arts et al., 2018), cold-inducible RNA-binding protein (CIRP) (Zhou et al., 2020), and the S100 A8 and A9 proteins (Austermann et al., 2014). HMGB1 functions as a DNA-binding protein that regulates gene transcription within the cell nucleus, however, when released from the cell, it can bind to TLR4 and the receptor for advanced glycation endproducts (RAGE), triggering the production of proinflammatory cytokines (Yang et al., 2020). Research shows that priming THP-1 cells with HMGB1 reduces the release of TNF in response to LPS (Aneja et al., 2008) or lipoteichoic acid (LTA) (Robert et al., 2010) stimulation. This decrease in serum TNF levels

following LPS challenge is also observed in mice pre-treated with HMGB1 (Aneja et al., 2008). Similarly, HMGN1, a protein that binds to nucleosomal DNA and affects chromatin structure, can stimulate TLR4 signalling when present outside the cell (Yang et al., 2018). Studies have reported that HMGN1 induces reduced responsiveness to LPS in human PBMCs, leading to decreased production of IL-6 and TNF. Furthermore, HMGN1 priming creates tolerance to cell activation with Pam3CSK4 and flagellin (Arts et al., 2018). The primary function of CIRP is as an RNA chaperone that regulates translation in reaction to various forms of cellular stress. Outside the cell, however, it acts as a DAMP agonist of TLR4 (Aziz et al., 2019). It has recently been shown that extracellular CIRP (eCIRP) promotes tolerance to LPS in mouse peritoneal macrophages *in vitro* and *ex vivo*. Interestingly, this process relies on eCIRP binding to a different receptor, IL-6 receptor (IL-6R), rather than TLR4 (Zhou et al., 2020). Lastly, the two members of the S100 protein family, S100A8 (also known as myeloid-related protein 8, or MRP8) and S100A9 (MRP14), are small calcium binding proteins released in response to tissue damage, and they activate TLR4 (Wang et al., 2018). Primary human monocytes exposed to either of these proteins become tolerised to a re-challenge with the same stimulus, as well as to LPS and LTA (Austermann et al., 2014).

These examples demonstrate that not only pathogenic factors but also certain internal molecules generated in response to cell stress and injury can trigger a form of memory within the innate immune system. Recent evidence supports that both DAMP- and PAMP-based memory acquisition is linked in part to epigenetic reprogramming (Austermann et al., 2014; Bekkering et al., 2014). Apart from these recent discoveries, however, one molecule has been attributed tolerising properties since 1988 - the proinflammatory cytokine TNF (Fraker et al., 1988). It was first seen in rats and mice that TNF administration protected these animals from lethal doses of subsequent TNF administration (Fraker et al., 1988; Fraker et al., 1990). The treated mice were shielded from symptoms like fever, gastrointestinal toxicity, liver injury, anorexia, hypertension, and hypothermia induced by TNF-related inflammation (Huber et al., 2017). Additionally, TNF stimulation of monocytes and macrophages in an *in vitro* setting was found to induce tolerance to itself as well as cross-tolerance to LPS.

Subsequent investigation revealed that cells that have been exposed to TNF undergo a particular change in their transcriptome, causing a reduced production of TNF, IL-1ß, IL-6, and IL-8 upon re-stimulation (Günther et al., 2014). On the other hand, there are cytokines that cannot be TNF tolerised. Examples of such cytokines are IL-18 and IL-32 (Günther et al., 2014). The desensitisation induced by TNF is initiated by the activation of its receptor- tumour necrosis factor receptor 1 (TNFR1) (Takahashi et al., 1995). Apart from inhibition of TNFR1 in mice, inhibiting the glucocorticoid receptor also negatively affected the development of a tolerant phenotype following TNF priming (Takahashi et al., 1995). The acquisition of TNF tolerance involves two different mechanisms depending on the concentration used for priming. Low TNF doses primarily regulate a group of tolerisable genes through the control of glycogen synthesis kinase 3 (GSK3), a serine/threonine kinase. GSK3 phosphorylates interaction partners p65 and transcription factor CCAAT/enhancer binding protein (C/EBP) ß, thus repressing transcription (Günther et al., 2014; Weber et al., 2003). Higher TNF doses affect a larger part of the transcriptome, causing both absolute and induction tolerance. In addition to GSK3, the protein governing high TNF tolerance is the A20 deubiquitinase, a negative regulator of NF-κB signalling (Günther et al., 2014). In a separate study focusing on TNF/LPS cross-tolerance, GSK3 enhanced A20 expression (Park et al., 2011). Since TNF is a major mediator of inflammation induced by PAMP/DAMP sensing by PRRs, a logical question about its strong tolerising properties was asked. Is it possible that the autocrine/paracrine activity of TNF released in response to TLR4 activation by LPS is responsible for the acquisition of endotoxin tolerance? This was investigated using TNF-neutralising antibodies, as well as TNF receptor targeting antibodies: anti-TNFR1 and anti-TNFR2. Antibody blockade of TNF and TNF signalling before priming with LPS had no effect on endotoxin tolerance induction (Hunter & Loosbroock, 2014). Hence, the induction of endotoxin tolerance is not dependent on TNF production following TLR4 activation.

The existing data supports the notion that the protective memory mechanisms triggered in innate immune cells control not only disease-causing immune responses but also the immune responses driven internally. However, DAMPs encompass a diverse range of molecules, and extensive further research is

required to fully comprehend the distinct and shared aspects of cellular reprogramming specific to endogenous triggers, as well as the commonalities between DAMPs and PAMPs. Many inquiries regarding the danger model of immunity remain unresolved, particularly concerning the definition of endogenous inflammatory molecules and the mechanisms through which their activity must be closely regulated to prevent potentially devastating autoimmune damage. Addressing these inquiries will not only offer insights into a compelling immunological puzzle but may also unveil new therapeutic opportunities for treating non-infectious inflammatory diseases such as RA, where internal inflammatory stimuli are believed to instigate the pathology.

1.1.4 The DAMP Tenascin-C: a matrix protein with diverse immunomodulatory capabilities

Tenascin-C (TN-C) is a large protein made up of six subunits that is found in the extracellular matrix. It is not usually produced in healthy tissues, but it is made quickly when tissues are damaged. Typically, upon tissue injury, its expression is transient, with mRNA levels decreasing and the protein disappearing by the time tissue repair is finished (Chiquet-Ehrismann & Chiquet, 2003). However, continual presence of tenascin-C is linked to various unhealthy conditions, where it accumulates at inflammation sites in autoimmune, fibrotic and metabolic diseases and in certain types of cancer (Udalova et al., 2011). With a complex molecular structure that allows for a wide range of interactions, tenascin-C influences many different cellular processes (Table 1.1).

Tenascin-C has a specific domain structure with the N-terminus containing an assembly domain responsible for the formation of a so-called hexabrachion, a six-armed oligomer. The N-terminus is then followed by epidermal growth factor-like (EGFL) repeats, fibronectin type III-like repeats (FNIII) that are both constitutively expressed and alternatively spliced, and a C-terminal fibrinogen-like globe (FBG-C). This intricate structure allows tenascin-C to participate in a wide range of molecular interactions (reviewed in (Midwood et al., 2016)). Tenascin-C plays a crucial role in regulating the extracellular matrix by influencing its assembly and the physical properties of tissues. Its binding

partners within the extracellular matrix include collagen, fibronectin, periostin, fibrillin-2, and certain proteoglycans such as perlecan, aggrecan, neurocan, and phosphacan/receptor protein tyrosine phosphatase ß/ζ. Furthermore, tenascin-C can bind to proteins present on the cell surface, such as syndecan 4, contactin, glypican, annexin II, and CALEB/CSPG5. Importantly, Tenascin-C can directly affect internal cell signalling by binding to cell surface receptors such as epidermal growth factor receptor (EGFR), TLR4, and integrins (Midwood et al., 2016). Additionally, tenascin-C can bind to numerous growth factors, including members of the fibroblast growth factor family and the transforming growth factor beta (TGF-ß) superfamily. This extensive array of interactions makes tenascin-C a diverse regulator of cellular behaviour, influencing activities like proliferation, migration, adhesion, and differentiation, based on the cellular context and type (Midwood et al., 2016).

The inflammatory effects of tenascin-C have been attributed to three primary binding partners, namely TLR4 and integrins α9ß1 and αVß3 (Table 1.1). As discussed earlier, TLR4 is a cell surface receptor found on leukocytes and stromal cells initially identified as the PRR accountable for mediating innate immune responses to the powerful bacterial virulence factor LPS, but it also has a growing number of endogenous activators that are being discovered, including tenascin-C, biglycan, fibrinogen, and S100 proteins, among others (Yu et al., 2010). The most frequently reported effect of TLR4 activation by tenascin-C is the stimulation of proinflammatory mediators, such as IL-6, IL-8, and TNF, in macrophages (Midwood et al., 2009), (Benbow et al., 2016; Piccinini et al., 2016; Zuliani-Alvarez et al., 2017), DCs (Machino-Ohtsuka et al., 2014), fibroblasts (Midwood et al., 2009; Maqbool et al., 2016), and chondrocytes (Patel et al., 2011). Additionally, tenascin-C signalling through TLR4 has been shown to be relevant in inflammasome priming. Inflammasomes and their activation will be discussed in detail later (Section 1.2.1). Briefly, inflammasomes are multiprotein, intracellular complexes that drive specific inflammatory responses, including the release of the cytokines IL-1ß and IL-18, and the induction of pyroptosis, a type of programmed cell death (Broz & Dixit, 2016). In most immune cell types, inflammasomes require two distinct signals for activation. The first "priming" signal consists of PRR-driven pro-cytokine production and synthesis of inflammasome components; this is followed by a

second signal which drives inflammasome assembly and caspase-1-mediated maturation and release of bioactive IL-1ß and IL-18 (Jo et al., 2016). Activation of rat epicardium-derived cells with tenascin-C led to TLR4-dependent pro-IL-1ß transcription, highlighting increased tenascin-C expression in rat cardiac tissue following ischemic injury as a potential priming step for inflammasome assembly that can be followed by adenosine or adenosine triphosphate (ATP) as a second signal fully activating the inflammasome (Hesse et al., 2017). Additionally, elevated levels of secreted mature IL-1ß were detectable in the cell culture medium of mouse bone marrow-derived DCs stimulated with tenascin-C (Machino-Ohtsuka et al., 2014), suggesting that tenascin-C may be capable of driving IL-1 synthesis without an exogenous second signal in certain cell types. Nevertheless, a direct connection between tenascin-C activation of TLR4 inflammasome signalling has not been previously explored.

Table 1.1 Tenascin-C as a modulator of cell activation. TN-C Source and TN-C Domain rows were left blank if no details of source and type of used Tenascin-C were given in the reference article.

Receptor	Cell Type	TN-C Source	Response	TN-C Domain	Reference
TLR4	Primary human macrophages	Recombinant human full-length TN-C domains	↑ IL-6, IL-8 and TNF	FBG-C	(Midwood et al., 2009)
TLR4	Human synovial fibroblasts		↑ IL-6		(Midwood et al., 2009)
TLR4	Primary human chondrocytes	Full-length TN-C purified from human glioblastoma cell line	↑ IL-6, IL-8, prostaglandin E2, nitrate and ADAMTS4		(Patel et al., 2011)
TLR4	Mouse bone marrow neutrophils		↑ MMP-9		(Kuriyama et al., 2011)
TLR4	Human THP-1-derived macrophages	Recombinant human TN-C purified from mouse myeloma cell line	Macrophage differentiation into foam cells		(Liu et al., 2012)
TLR4	Mouse bone marrow-derived DCs	Full-length TN-C purified from human glioblastoma cell line	↑ IL-6, IL-1α, IL-1β and IL-17		(Machino-Ohtsuka et al., 2014)
TLR4	Human monocyte -derived macrophages	Recombinant human FBG-C domain	↑ IL-6, IL-8, TNF, IL-10, collagen, MMP-1 and MMP-14	FBG-C	(Piccinini et al., 2016)
TLR4	Human and mouse fibroblasts	Full-length TN-C purified from human glioblastoma cell line	↑ type I collagen, αSMA and TLR4		(Bhattacharyya et al., 2016)
TLR4	Human cardiac myofibroblasts	Recombinant human FBG-C domain	↑ IL-6 and MMP-3	FBG-C	(Maqbool et al., 2016)
TLR4	Mouse macrophage cell line		↑ IL-6, TNF		(Benbow et al., 2016)
TLR4	Human hepatoma cell line		↑ epithelial-mesenchymal transition		(Benbow et al., 2016)
TLR4	Primary human macrophages	Recombinant human FBG-C domain	↑ IL-6, IL-8 and TNF	FBG-C	(Zuliani-Alvarez et al., 2017)
α9	Mouse synovial macrophages	Recombinant FNIII3 domain	↑ IL-6, IL-1a, IL-1b, TNF, CCL2, CCL3, CCL4, CXCL2 and CXCL5	FNIII3	(Kanayama et al., 2009)
α9	Mouse synovial fibroblasts		↑ IL-6, IL-1a, CCL2, CCL4, CXCL5, CXCL12 and MMP-9 ↓ MMP-2		(Kanayama et al., 2009)
α9	Mouse dendritic cells	Recombinant mutant FNIII3 domain	↑ IL-6, IL-23, Th17 cell differentiation	FNIII3	(Kanayama et al., 2011)
$\alpha V\beta 3$	Mouse peritoneal macrophages		↑ IL-6, IL-1β and TNF		(Shimojo et al., 2015)

Several reports indicate that tenascin-C-mediated cytokine release also plays a crucial role in creating a conducive microenvironment for selective T-cell polarisation. DCs from mice lacking tenascin-C displayed reduced release of proinflammatory cytokines when exposed to LPS and had a diminished ability to

direct naive T cells towards T helper 17 (Th17) cell differentiation but not towards T helper 1 (Th1), T helper 2 (Th2), or T regulatory (Treg) cell differentiation (Ruhmann et al., 2012). Activation of DCs with exogenous tenascin-C enhanced their capacity to promote Th17 cells, and this effect could be nullified by using anti-IL-6 and anti-TLR4 antibodies, indicating that the synthesis of IL-6, induced by tenascin-C activating TLR4, is a critical factor in T-cell subset polarisation (Machino-Ohtsuka et al., 2014). Additionally, tenascin-C stimulated chondrocytes to secrete prostaglandin E2 (Patel et al., 2011), a complex inflammatory mediator known to be involved in Th17 cell polarisation (Boniface et al., 2009; Napolitani et al., 2009).

A more thorough examination of the effects of activating TLR4 with tenascin-C in primary human macrophages revealed functional outcomes that extend beyond cytokine production. Comparing macrophages activated by either tenascin-C or the pathogenic ligand LPS showed similar yet distinct impacts on cell behaviour. While both stimuli trigger classical NF-κB and mitogen-activated protein kinase (MAPK) signalling, LPS prompts macrophages to adopt an aggressive phenotype geared towards combating infection, characterised by high levels of proinflammatory cytokines and matrix-degrading metalloproteinases (MMPs). In contrast, tenascin-C promotes the upregulation of extracellular matrix protein expression and phosphorylation, leading to macrophage behaviour more aligned with tissue repair (Piccinini et al., 2016).

Additionally, tenascin-C has been shown to drive macrophages to become foam cells. Tenascin-C enhances oxidized low-density lipoprotein (oxLDL)-induced foam cell formation in a TLR4-dependent manner. Tenascin-C is upregulated following macrophage stimulation by oxLDL and can induce the expression of CD36, a scavenger receptor involved in LDL uptake, which suggests the presence of a positive feedback loop promoting the development of these cells crucial to atherosclerosis (Liu et al., 2012). These findings collectively demonstrate that tenascin-C significantly influences macrophage polarisation, induces a macrophage phenotype distinct from that induced by pathogenic TLR4 activation, and generates different macrophage subsets based on the environmental context.

A broader range of cell types have also been documented to react to tenascin-C through activation of TLR4. In murine bone marrow neutrophils, tenascin-C has been demonstrated to increase the expression of MMP-9 in a manner dependent on TLR4 (Kuriyama et al., 2011), and in fibroblasts, it induces high levels of IL-6 secretion without inducing IL-8 secretion, in contrast to macrophages where both cytokines are induced by tenascin-C (Midwood et al., 2009). In primary human foreskin fibroblasts, tenascin-C induces the production of type I collagen and alpha-smooth muscle actin via TLR4 (Bhattacharyya et al., 2016). These findings indicate that tenascin-C plays a role not only in initiating inflammation mediated by immune cells but also in stromally driven defence responses, suggesting that this matrix molecule contributes to both inflammatory processes right after tissue damage and also the following tissue remodelling by activating cell-type-specific responses through TLR4.

The fact that tenascin-C triggers a different type of inflammatory signalling through TLR4 compared to LPS supports the concept that the immune system is capable of distinguishing between various alarm signals using common receptors in order to activate specific responses to particular threats, such as injury versus infection. However, it is currently unclear at the molecular level how different outcomes result from the activation of the same PRR. The mechanism through which tenascin-C binds to and activates TLR4 is still not fully understood. Nevertheless, it is known that the binding site consists of three distinct epitopes within the FBG-C domain of tenascin-C and that FBG-C is the necessary and sufficient domain needed to trigger a complete TLR4 inflammatory response (Zuliani-Alvarez et al., 2017). Additionally, experimental data has suggested that the classical LPS co-receptors, myeloid differentiation protein-2 and CD14, are not necessary for TLR4 activation by tenascin-C (Midwood et al., 2009).

Tenascin-C has been shown to activate two other cell surface receptors that drive inflammatory responses, namely Integrins α9β1 and αVβ3, which are transmembrane receptors capable of binding to various matrix proteins, including fibronectin and are involved in the induction of proinflammatory cytokine synthesis. The α9β1 interaction site responsible for the observed effects of tenascin-C has been pinpointed to the third FNIII repeat (Kanayama et al., 2009). Although αVβ3 is known to bind to two different sites within tenascin-C, the third FNIII repeat (Prieto et al., 1993) and the FBG-C domain (Yokoyama

et al., 2000), it is still unknown which, if any, is responsible for conferring the proinflammatory activity. In murine synovial macrophages and fibroblasts, induction of α9 integrin-mediated signalling by tenascin-C upregulates a wide array of proinflammatory molecules, including IL-6, IL-1α, CCL2, CCL4, and CXCL5 (Kanayama et al., 2009). Activation of α9 integrin by tenascin-C is also involved in Th17 cell differentiation through the induction of IL-6 and IL-23 in DCs. It was shown that the stimulation of IL-6 secretion by tenascin-C is a synergistic effect of signalling via both α9 integrin and TLR4 (Kanayama et al., 2011). The impact of tenascin-C on cytokine secretion through the activation of αVβ3 was studied in peritoneal macrophages, where it led to the upregulation of IL-6, IL-1β, and TNF in an NF-κB-dependent manner (Shimojo et al., 2015). These findings demonstrate that tenascin-C can upregulate cytokine synthesis by activating more than one receptor family. The mechanisms that mediate this interplay on the cellular level are yet to be elucidated, but the redundancy of receptors underscores the importance of tenascin-C as a danger signal.

Aside from its impact via membrane receptor signalling, tenascin-C may also indirectly control intracellular inflammation. In a human glioblastoma cell line, tenascin-C reduces the levels of the Wnt inhibitor dickkopf-1 (DKK1), leading to an increase in classical Wnt signalling by stabilising β-catenin (Ruiz et al., 2004). Tenascin-C also binds to Wnt3a, and when human adenocarcinoma cell line cells are placed on tenascin-C in the presence of Wnt3a, they show heightened classical Wnt signalling. Interestingly, the signalling was significantly reduced when cells were exposed to soluble tenascin-C (Hendaoui et al., 2014). This observation may suggest that while soluble tenascin-C could capture Wnt ligands, ECM-deposited tenascin-C is responsible for gathering ligands near the cell surface to facilitate receptor activation. Given the complex interplay between the Wnt and NF-κB pathways (Ma & Hottiger, 2016), the regulation of Wnt signalling by tenascin-C could have both positive and negative effects on the inflammatory response.

Moreover, increasing evidence supports the involvement of tenascin-C in PRR-mediated responses to pathogen invasion. In murine bone marrow-derived macrophages, rapid upregulation of tenascin-C upon LPS detection by TLR4 facilitates the maturation of the micro-RNA miR-155, while the lack of tenascin-C hinders miR-155 maturation, causing loss in the effective translation of LPS-

induced proinflammatory cytokines, including TNF (Piccinini & Midwood, 2012). Lastly, tenascin-C has been shown to interfere with HIV transmission as natural tenascin-C in human breast milk binds to the HIV-1 Envelope protein, neutralising the infectious properties of this virus in reporter cell lines and peripheral blood mononuclear cells (Mansour et al., 2016). This combined impact of microbial and endogenous alarm signals illustrates the remarkable signalling complexity within the innate immune system.

Since tenascin-C is upregulated in response to tissue damage and cellular stress, but it cannot be detected after the completion of tissue repair, an important question to answer is whether tenascin-C is actively involved in the resolution of inflammation or if its absence in tissues simply creates a less proinflammatory environment. Studies have demonstrated that tenascin-C binds to TGF-ß (De Laporte et al., 2013), which is a crucial factor in the resolution of inflammation (Kelly et al., 2017). Interactions between TGF-ß and tenascin-C can potentially elevate local concentrations or activity of this immunosuppressive factor. Furthermore, tenascin-C has been found to have immunosuppressive effects on lymphocytes as several studies have shown that tenascin-C is capable of halting T-cell activation induced by a number of stimuli (Hibino et al., 1998; Jachetti et al., 2015; Puente Navazo et al., 2001; Rüegg et al., 1989). Alternatively spliced repeats A1 and A2 within the FNIII domain of tenascin-C were shown to block anti-CD3 MAb/fibronectin-induced activation of human peripheral blood T cells via inhibition of TCR/CD3 complex internalisation (Puente Navazo et al., 2001). This data demonstrated that tenascin-C is a sophisticated regulator of inflammation, capable of conveying both pro- and anti-inflammatory responses.

In order to further study the overall effects of removing tenascin-C from a living organism, mice were genetically manipulated (Forsberg et al., 1996; Saga et al., 1992)]. Surprisingly, there were few noticeable differences between the mice without the tenascin-C gene (TNC KO) and normal mice. TNC KO mice were born healthy and able to reproduce, were of normal size, and went through the usual development stages and life expectancy. Abnormalities in mice without tenascin-C were only observed during the healing process after eye (Matsuda et al., 1999; Sumioka et al., 2013) and heart (Tamaoki et al., 2005) injuries. These differences included a reduced number of macrophages, a delay in the recruitment of myofibroblasts, and a decrease in the production of TGF-ß,

fibronectin, and collagen in TNC KO mice compared to normal mice. Combined, these factors resulted in slower wound healing.

However, in line with its proinflammatory properties observed *in vitro*, several inflammatory disease models have demonstrated that the absence of tenascin-C leads to reduced secretion of proinflammatory cytokines and subsequent diminished recruitment of inflammatory cells, thereby offering a protective effect. In an experiment involving transgenic mice overexpressing mutated amyloid precursor protein, the suppression of Alzheimer's disease-specific inflammation was observed following the removal of tenascin-C. This was accompanied by decreased amyloid deposition and improved Alzheimer's disease pathology, attributed to lower levels of proinflammatory mediators such as TNF, IL-1ß, and IL-6, and higher levels of anti-inflammatory mediators including IL-10, MRC1, and CCL2 in the central nervous system (Xie et al., 2013). Tenascin-C ablation was also found to reduce immune cell infiltration and prevent brain oedema and disruption of the blood-brain barrier in a surgical subarachnoid haemorrhage model (Fujimoto et al., 2016; Fujimoto et al., 2018).

Notably, tenascin-C deficiency also demonstrated remarkable anti-inflammatory effects in models of joint inflammation. In an acute inflammation model induced by zymosan, the resolution of synovial inflammation was significantly faster in animals lacking tenascin-C compared to those with normal levels of the protein. Additionally, tenascin-C-deficient mice were safeguarded from prolonged synovitis, cartilage, and bone destruction in a model of erosive arthritis triggered by methylated BSA (Midwood et al., 2009). During the erosive arthritis model, the absence of tenascin-C resulted in reduced cytokine synthesis in the joint, including reduced IL-6 and IL-23 expression, leading to a microenvironment unsupportive of Th17 polarisation, ultimately resulting in significantly reduced IL-17 expression (Ruhmann et al., 2012).

TNC KO mice also portrayed a decrease in the production of IL-17 by Th17 cells during experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, along with a decrease in the number of interferon-gamma-producing Th1 cells (Momčilović et al., 2017). Additionally, in ovalbumin-induced bronchial asthma, animals lacking tenascin-C exhibited significantly reduced allergic inflammation, evidenced by reduced eosinophil infiltration and decreased levels

of IgE antibodies. This attenuation of inflammation was attributed to impaired differentiation of Th2 cells, leading to a decrease in the expression of IL-5 and IL-13 (Nakahara et al., 2006). These findings support the data gathered from laboratory studies, indicating that tenascin-C can influence T-cell polarisation. Moreover, they emphasise the intricate and highly context-specific effects of tenascin-C, impacting T-cell subsets known to coordinate specific pathologies such as Th2 in asthma, Th17 in murine RA models, and Th17 and Th1 in autoimmune encephalomyelitis.

Besides worsening inflammatory responses, tenascin-C has been found to have a particularly harmful role in promoting tissue fibrosis. For instance, in the cardiovascular system, the absence of tenascin-C reduces the thickening of blood vessel walls after surgery (Sawada et al., 2007; Yamamoto et al., 2005) and improves heart function following a heart attack (Nishioka et al., 2010). Additionally, in a model of hepatitis induced by concanavalin A, a decrease in the expression and accumulation of collagen was observed in the livers of TNC KO mice. This was linked to reduced release of inflammatory cytokines such as IFN-γ, TNF, and IL-4, and lower infiltration of white blood cells into the liver, along with a significant decrease in TGF-ß expression compared to normal mice (El-Karef et al., 2007). Reduced TGF-ß signalling, with less movement of mothers against decapentaplegic homolog 2 (Smad-2) and Smad-3 into the cell nucleus, was shown to protect mice lacking tenascin-C from lung scarring in a model of acute lung injury induced by bleomycin (Carey et al., 2010). Additionally, in models of systemic sclerosis induced by bleomycin, reduction in TGF-ß and a decrease in collagen accumulation led to reduced thickening of skin and lung coverings and faster resolution of inflammation in TMC KO mice (Bhattacharyya et al., 2016). These results indicate that increased fibrosis may be partly caused by higher tissue levels of TGF-ß, allowing scarring to take place by activating fibroblasts and stimulating excessive production of the extracellular matrix (Pohlers et al., 2009).

Finally, the regeneration of liver tissue was improved in tenascin-C KO mice in a model of liver injury caused by lack of blood flow. In the absence of tenascin-C, the release of IL-6, IL-1ß, and CXCL2 was decreased, and the recruitment of white blood cells was less effective. These effects were accompanied by reduced destructive processes, lower levels of cell death markers, and reduced

expression and activation of MMP-9, an enzyme that breaks down the extracellular matrix (Kuriyama et al., 2011), suggesting that tenascin-C can affect tissue scarring by both promoting the build-up of matrix and inhibiting its removal.

Combined, these research studies validate the hypothesis that tenascin-C effectively controls inflammation and fibrosis within living organisms, however, these findings also emphasise that tenascin-C demonstrates a significant functional split. In certain scenarios, it positively impacts tissue healing by promoting tissue remodelling and safeguarding against excessive inflammation, while in other scenarios, it can drive ongoing inflammation and encourage pro-fibrotic reactions, often leading to the deterioration of the organism. The temporal tenascin-C levels within tissues may be crucial, as when its expression is reduced, inflammation and healing mediated by tenascin-C can take place in a regulated manner, while sustained expression of tenascin-C would contribute to proinflammatory and pro-fibrotic reactions. Another significant influencing factor may be the specific tissue environment, where the expression of distinct tenascin-C splicing variations, proteolytic processing, and the presence of various tenascin-C receptors on the cell surface are likely to also influence the nature of tenascin-C-mediated responses.

Additional studies on the involvement of tenascin-C in the resolution of inflammation *in vivo* demonstrated that it plays a role in the immune response to LPS-induced sepsis. TNC KO animals were shielded from the systemic inflammation following the cytokine storm caused by LPS administration, indicating a role for tenascin-C in host defence against infection *in vivo*. Interestingly, bone marrow transplants from wild-type littermates partially restored the phenotype in tenascin-C-deficient mice (Piccinini & Midwood, 2012), highlighting the participation of both myeloid and stromally derived tenascin-C in these immune responses. TNC KO animals have been reported to exhibit a decrease in cell colony–forming capacity within the bone marrow compartment without steady-state haematopoiesis being affected (Ohta et al., 1998). Nevertheless, tenascin-C-deficient mice did demonstrate impaired hematopoietic recovery following bone marrow ablation by irradiation (Nakamura-Ishizu et al., 2012). Tenascin-C has been found to regulate bone marrow–mediated angiogenesis, while another study revealed that tenascin-C

expressed in the bone marrow, but not the myocardium, was accountable for attenuating fibrosis in a mouse model of cardiac hypertrophy. TNC KO animals displayed worsened inflammation and impaired heart function following transverse aortic constriction surgery, a phenotype that was reversed by a bone marrow transplant from a wild-type donor, and conversely, transplant from a tenascin-C-null donor to a wild-type recipient resulted in declined cardiac function (Song et al., 2017).

These findings prompt questions regarding the site of tenascin-C action during inflammation, suggesting a role not only locally at the site of inflammation but also distally, impacting inflammatory cell behaviour at sites some distance from the pathology. These findings also underscore the importance of considering the background strain of mice used in studies, as different strains of mice are known to exhibit different immune compartment biases that could determine the overall impact of tenascin-C deletion *in vivo*.

Because of its unique characteristics and distinct expression pattern, tenascin-C has long been considered a promising option for therapy targeting in medical settings. Much of the current research is focused on using tenascin-C as a guiding marker for delivering treatments directly to the tumour microenvironment through aptamer or antibody technologies (reviewed in Spenlé et al., 2015). However, the possibilities of targeting the proinflammatory capacity of tenascin-C have not yet been explored. One way this could be done is by downregulating tenascin-C production at the level of mRNA transcription or protein expression, however, global inhibition of tenascin-C would eliminate not only the undesirable prolonged engagement of proinflammatory signalling, but also desirable functions such as supporting natural tissue healing and remodelling. The characteristics of the tenascin-C knockout mouse suggest some redundancy for this molecule during development, and its absence does not seem to have a significant effect on acute tissue repair, which could offer a way to treat chronic diseases without significantly affecting underlying biology.

Despite all the work characterising the effects of tenascin-C on immune regulation, it is still not fully understood how the TLR4-binding domain of tenascin-C, FBG-C, along with its ligand TLR4, drive human monocyte immune responses, specifically, how FBG-C regulates the priming of the NLR family pyrin

domain containing 3 (NLRP3) inflammasome and its capability to induce NLRP3 inflammasome tolerance.

1.2 The NLRP3 inflammasome

As discussed earlier, the identification of proinflammatory triggers through the innate immune response is heavily reliant on the recognition of pattern recognition receptors, which are categorized as receptors that are either located on the cell membrane or within the cytoplasm. The membrane-bound receptors include Toll-like receptors and C-type lectin receptors, while the cytosolic receptors include nucleotide-binding oligomerization domain-Leucine Rich Repeats (LRR)-containing receptors (NLRs), Retinoic Acid-Inducible Gene 1-like receptors (RLRs), and Absence in Melanoma 2 (AIM2)-like receptors (ALRs) (Unterholzner et al., 2010; Lamkanfi & Dixit, 2014; Amarante-Mendes et al., 2018). These receptors can detect both pathogen-associated molecular patterns and damage-associated molecular patterns (Chen & Nuñez, 2010). PAMPs are representative of the structurally conserved molecules frequently found in microorganisms, such as LPS from Gram-negative bacteria, nucleic acids from both bacteria or viruses, bacterial peptides like flagellin, and polysaccharides like ß-glucans (Mahla et al., 2013). DAMPs, on the other hand, are internal molecules released during cellular stress or damage, such as chromatin-associated proteins, heat shock proteins, uric acid, and extracellular matrix fragments such as Tenascin-C (Chen & Nuñez, 2010; Lamkanfi & Dixit, 2014; Midwood et al., 2009). In addition to classical NF-κB signalling that leads to the upregulation of inflammatory cytokines such as TNF and IL-6, activation of PRRs by PAMPs or DAMPs initiates a signalling cascade that leads to the formation of multimeric protein complexes known as inflammasomes (Lamkanfi & Dixit, 2014). These inflammasomes play a critical role in the cleavage and release of additional proinflammatory cytokines such as IL-1ß and IL-18. Over the past twenty years, extensive studies have been conducted on the molecular constituents and activation mechanism of various inflammasomes such as NLR family pyrin domain containing 1 (NLRP1), NLRP3, NLRP6, NLRP12, NLR family CARD domain-containing protein 4 (NLRC4) and absent in melanoma 2 (AIM2) (reviewed in (De Zoete et al., 2014)). Out of all the different inflammasomes,

the NLRP3 inflammasome, which is a primary subject of investigation in this thesis, garners the most attention due to its ability to be activated by a wide range of stimuli and its involvement in various inflammatory conditions such as Alzheimer's disease, multiple sclerosis and rheumatoid arthritis.

1.2.1 NLRP3 Inflammasome assembly and activation

NLRP3 is itself a cytosolic PRR protein that is produced by various cells, including neutrophils, macrophages, monocytes, microglia, lymphocytes, epithelial cells, osteoblasts, neurons, and dendritic cells (Rada et al., 2014; Zahid et al., 2019). The NLRP3 protein comprises a leucine-rich repeat (LRR) domain at the C-terminal, an ATPase-containing NACHT domain in the centre that facilitates oligomerization, and a pyrin (PYD) domain at the N-terminal, which recruits proteins for the formation of the inflammasome complex (Kelley et al., 2019). Similar to other inflammasomes, the NLRP3 inflammasome complex has a sensor - the NLRP3 protein, an adaptor - the apoptosis-associated speck-like protein (ASC), and an effector molecule - caspase-1. (de Zoete et al., 2014; Mamantopoulos et al., 2017). NLRP3 inflammasome formation occurs in two stages, namely priming and activation. During the priming stage, the transcription of the NLRP3 proteins and of proinflammatory cytokines such as pro-IL-1ß and pro-IL-18 increases following a classical PRR receptor trigger, such as LPS via TLR4 or TNF via tumour necrosis factor receptor (Liu et al., 2017). Priming leads to the activation of proteins and nuclear factors, including MyD88, NF-κB, and the activator protein 1 (AP-1), which upregulate NLRP3 and proinflammatory cytokines (Liu et al., 2017). The impact of priming on transcription factors and protein expression is widely acknowledged, however, recent research has suggested that priming also has a non-transcriptional role (Lin et al., 2014), as it also regulates the post-translational modifications of NLRP3, such as ubiquitination and phosphorylation, which play a significant role in controlling NLRP3 activation (Yang et al., 2017). In its inactive state, Adenosine diphosphate (ADP)-bound NLRP3 exists as either a monomer or oligomer. Monomeric NLRP3 is found on membranes, providing a structure for

creating a double-ring assembly with 5–8 sets of interlocking LRR domain dimers arranged to form a circular cage (Andreeva et al., 2021; Hochheiser et al., 2021) (Figure 1.1). Based on cryogenic electron microscopy (cryo-EM) studies, it is suggested that the PYD domain is positioned inside the cage to prevent unintended activation (Andreeva et al., 2021). A second stimulus then triggers the activation process to create the active inflammasome complex. Unlike classical PRRs, NLRP3 is triggered by a variety of unrelated stimuli such as solid particles (e.g., uric acid crystals, silica, asbestos), extracellular ATP, and toxins such as nigericin, as well as viral, bacterial, fungal, and protozoan pathogens (Latz et al., 2013; Jo et al., 2016). While it is not fully understood how NLRP3 can detect such different signals, it is proposed that NLRP3 detects a common cellular event caused by all stimuli rather than directly binding to the signals (Kelley et al., 2019). Backed up by a myriad of scientific investigations, this common cellular event caused by most activation stage stimuli, including ATP, Nigericin and particulate matter, is believed to be the intracellular decrease of potassium ions (K^+) (Muñoz-Planillo et al., 2013) followed by NIMA-related kinase 7 (NEK7) binding to NLRP3 (He et al., 2016) (Figure 1.1). Extracellular ATP, through its binding and activation of the non-selective cation channel receptor P2X purinoceptor 7 (P2X7), and nigericin via its effect as a K^+ ionophore directly cause the efflux of K^+, which is a necessary and sufficient signal for the activation of NLRP3 (Muñoz-Planillo et al., 2013; Katsnelson et al., 2015), while monosodium urate (MSU) crystals indirectly cause a decrease in intracellular K^+ due to an influx of water, resulting in a decrease in intracellular K^+ and leading to the activation of the NLRP3 inflammasome (Schorn et al., 2011) (Figure 1.1). Similarly to MSU, an increase in intracellular Na+ and the subsequent water influx causes the intracellular K^+ concentration drop, activating the NLRP3 inflammasome (Muñoz-Planillo et al., 2013). In a related fashion, particulate matter (e.g., cholesterol crystals, silica, aluminium salts, uric acid crystals, and asbestos) and misfolded proteins (e.g., Aß) affect K^+ efflux indirectly by inducing lysosomal damage (Murakami et al., 2012; Katsnelson et al., 2016) (Figure 1.1). It has even been shown that K^+ free medium alone is capable of activating the NLRP3 inflammasome by causing K^+ efflux due to a difference in extracellular and intracellular K^+ ions (Muñoz-Planillo et al., 2013). Extracellular Ca2$^+$ was also shown to indirectly activate NLRP3 through K^+ efflux (Muñoz-Planillo et al., 2013). For a long time, reactive

oxygen species and mitochondrial dysfunction were considered activators of the NLRP3 inflammasome, however, although it is widely accepted that these two factors play a significant role in overall inflammatory responses (Gong et al., 2018; Mahalanobish et al., 2020; Ko et al., 2021), many studies have disputed the requirement of mitochondrial dysfunction and ROS in NLRP3 activation (Muñoz-Planillo et al., 2013; Allam et al., 2014; Lawlor & Vince, 2014). Recently, it was demonstrated that deletion of the *potassium channel subfamily K member 6* (*Kcnk6)* gene encoding the tandem pore domain weak inward rectifying K^+ channel 2 (TWIK2) protein, which is a K^+ efflux channel, prevents NLRP3 activation in macrophages, highlighting the role of this family of ionic channels in inflammasome activation (Di et al., 2018). Similar to K^+ efflux, it has been shown that the breakdown of the trans-Golgi network (TGN) by various NLRP3 stimuli can attract and activate NLRP3 in a NEK7 dependent manner (Chen & Chen, 2018; Magupalli et al., 2020; Andreeva et al., 2021) (Figure 1.1). The TGN disassembly results in the localization of the NLRP3 cage on the dispersed TGN vesicle membranes, which are then transported to the microtubule-organizing centre, where NEK7 is located. Downstream of either K^+ efflux or TGN breakdown, the binding of NEK7 to NLRP3 disrupts the NLRP3 double-ring structure, leading to a structural reorganization that exposes the pyrin domains and allows for NACHT domain oligomerization (Andreeva et al., 2021). Previous research has positioned the NEK7 binding site at NLRP3's LRR domain, however, recent studies suggest that the LRR domain is not essential for NLRP3 inflammasome activation and that NEK7 may have at least one additional binding site (Hafner-Bratkovic et al., 2018; He et al., 2018). Following NACHT domain activation through ATP exchange and NEK7 binding, the PYD domain recruits the adaptor molecule ASC to form a filamentous complex known as the ASC pyroptosome or 'speck' through PYD-PYD domain interactions. The caspase recruitment domain (CARD) of ASC interacts with procaspase-1, which also possesses a CARD domain (CARD-CARD interaction). This interaction leads to the conversion of pro-caspase-1 into active caspase-1 through proximity-induced autoproteolysis (Lu et al., 2014; Lu & Wu, 2015; Malik & Kanneganti, 2017) (Figure 1.1). Caspase-1, once activated, transforms biologically inactive peptides such as pro-IL-1ß and pro-IL-18 into their active forms, IL-1ß and IL-18, respectively. It also cleaves and thereby activates Gasdermin D (GSDMD), a protein that has a defined membrane pore-forming function involved in the

programmed cell death process known as pyroptosis (Shi et al., 2015; Malik & Kanneganti, 2017) (Figure 1.1). The N-terminal domain of GSDMD (GSDMD-NT) exhibits a strong affinity for plasma membrane lipids such as cardiolipin and phosphoinositides; hence after being cleaved, GSDMD-NT forms oligomers that create openings in the cell membrane, resulting in the disturbance of the cell's osmotic balance, leading to pyroptosis and the discharge of internal contents, such as the inflammatory cytokines IL-1ß and IL-18 (Ding et al., 2016; Evavold et al., 2018) (Figure 1.1).

This type of NLRP3 inflammasome activation mediated by caspase 1 is known as the canonical pathway, as there is also another pathway termed non-canonical through which the NLRP3 inflammasome can be activated (Figure 1.1). However, activation of the NLRP3 inflammasome through either the canonical or non-canonical pathway leads to similar outcomes, albeit through different processes. In the non-canonical pathway, inflammasome formation is triggered by caspase-4 and caspase-5 in humans and by caspase-11 (also called caspase-4/11) in mice in response to Gram-negative bacterial infection (Kayagaki et al., 2015) (Figure 1.1). Similar to the canonical pathway, TLR4-MyD88 initiates the downstream signalling, leading to the translocation of NF-κB into the nucleus to increase the expression of NLRP3, pro-IL-1ß, pro-IL-18, and other inflammatory mediators (Liu et al., 2017). Additionally, downstream of TLR4 activation, TRIF triggers interferon regulatory factors (IRFs) that subsequently boost the expression of interferon (IFN)-α/ß. IFN-α/ß binds to IFN-α/ß receptors, activating caspase-4/11 expression through JAK/STAT signalling (Gurung et al., 2012; Rathinam et al., 2012). Notably, an important difference between the canonical versus non-canonical pathways of NLRP3 inflammasome activation is that recognition of extracellular LPS by TLR4 is dispensable for non-canonical inflammasome stimulation (Kayagaki et al., 2013), since phagocytosed intracellular LPS is directly detectable by caspase-4/11, which leads to oligomerization and self-cleavage of caspase-4/11 (Shi et al., 2014; Lee B. L. et al., 2018) (Figure 1.1). Cleaved and activated caspase-4/11 then promotes pyroptosis by cleaving proteins such as GSDMD and controlling the release of IL-1a (Kayagaki et al., 2015; Shi et al., 2015; Wiggins et al., 2019). Nonetheless, many of the substrates of caspase-4/11 are still not fully understood and previous research indicates that caspase-4/11 may not be able to convert pro-IL-1ß and pro-IL-18 into their

active forms, unlike caspase-1, but it can still enhance NLRP3-induced caspase-1 activation and cytokine release through different mechanisms (Kayagaki et al., 2011; Py et al., 2014; Agnew et al., 2021).

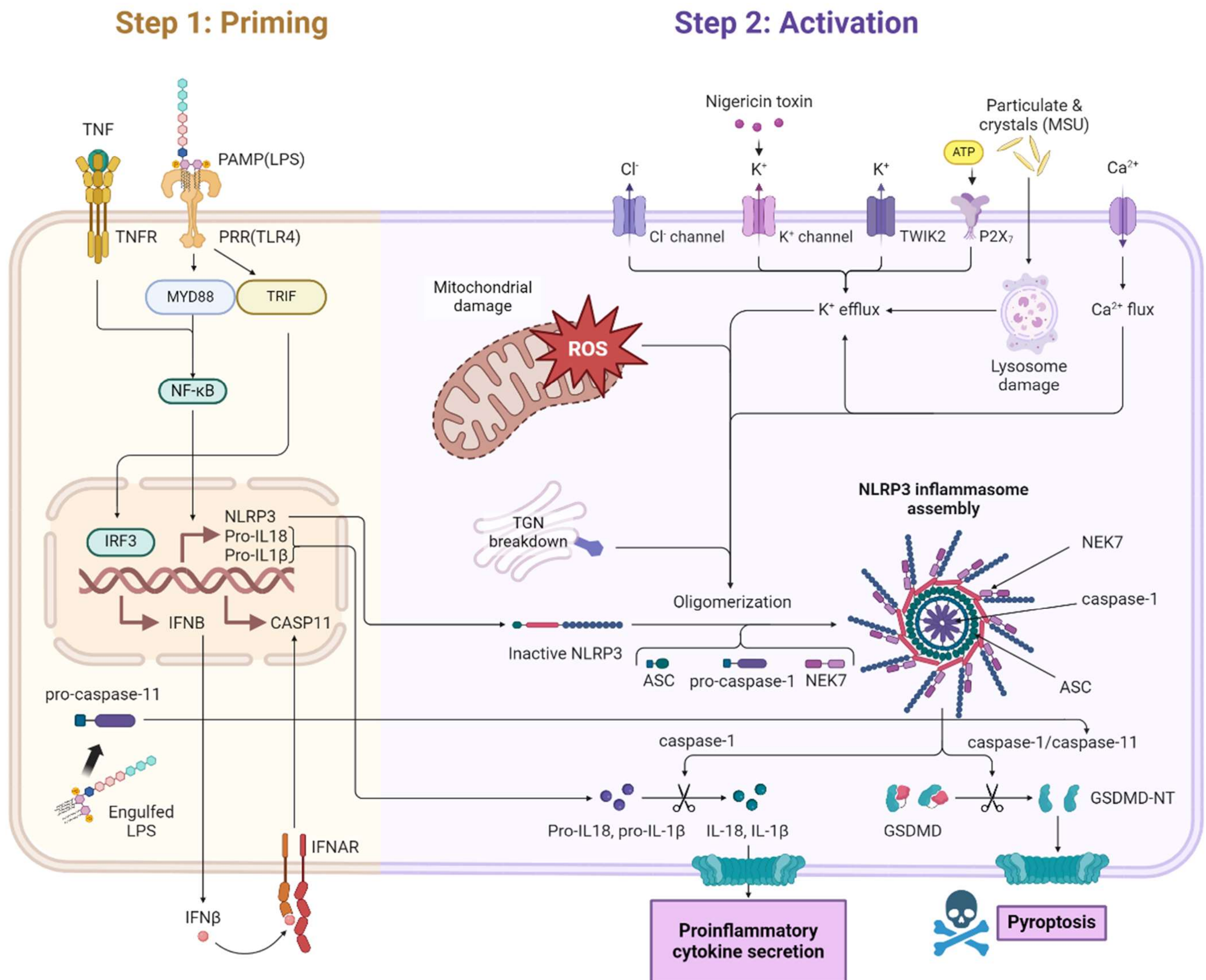


Figure 1.1 Canonical and non-canonical pathways of NLRP3 inflammasome activation in human myeloid cells. The activation of the NLRP3 inflammasome involves a two-step process in most cell types. Initially, cells need to be primed by exposure to a toll-like receptor or cytokine receptor ligand such as LPS or TNF, which activates the NF-κB pathway and leads to the transcription of NLRP3, caspase-1, pro-IL-1β and pro-IL-18. The second step can be triggered by various damage or pathogen associated events such a toxins, extracellular ATP or particulate matter, all of which ultimately cause potassium ion efflux. The NLRP3 inflammasome is then assembled from components including NLPR3, NEK7, ASC and inactive caspase 1. Once activated, the NLRP3 inflammasome facilitates the processing of caspase-1, which in turn cleaves inactive cytokines pro-IL-1β, pro-IL-18, and the pore forming protein GSDMD into their active forms. The non-

canonical NLRP3 inflammasome pathway is triggered by the sensing of cytosolic LPS by inactive caspase 11 and by upregulation of the CASP11 gene via IFN-ß auto-paracrine signalling. Activated caspase 11 then cleaves GSDMD, resulting in the formation of GSDMD-NT membrane pores, leading to potassium efflux and promoting the activation of the canonical NLRP3 inflammasome pathway. In both the canonical and non-canonical pathways cleaved GSDMD-NT fragments form membrane pores which allow mature IL-1ß and IL-18 to be secreted through the pores, ultimately resulting in pyroptotic cell death caused by osmotic imbalance. Figure was generated using BioRender.

In addition to the canonical and non-canonical pathways of NLRP3 inflammasome activation, recent studies have shown that human monocytes possess the ability to activate the inflammasome in what is termed the alternative pathway. Unlike human macrophages and murine myeloid cells, human monocytes can activate NLRP3 and secrete mature IL-1ß immediately after LPS activation via TLR4 without the need for K^+ efflux caused by a signal 2 such as ATP, nigericin or particulate matter. (Gaidt et al., 2016). It was shown by Gaidt and colleagues that the alternative inflammasome is only activated via TLR4 engagement of the TRIF signalling pathway and that it is fully dependent on caspase-8 activation for the cleavage and maturation of the NLRP3 protein complex. This phenomenon puts human monocytes in a unique position in terms of their inflammatory potential since, unlike human macrophages where the production of IL-1ß is under the rigorous control of secondary signal engagement, human monocytes could potentially produce IL-1ß immediately upon DAMP recognition by TLR4, causing a proinflammatory loop within sterile inflammatory environments such as the RA joint.

1.2.2 NLRP3 Inflammasome dysregulation in inflammatory diseases and the implications of tolerance

The important defensive function of inflammasomes in reacting to pathogens and other signals of danger also indicates that inflammasome dysfunction could be involved in human diseases caused by chronic inflammation (Schroder & Tschopp, 2010; Liu & Chan, 2014; Moossavi et al., 2018; Boxberger et al., 2019;

Eren & Özören, 2019). Different studies have demonstrated that abnormal NLRP3 activation can worsen the pathology of various diseases driven by inflammation. A good example are the cryopyrin-associated periodic syndromes (CAPS), which comprise a set of conditions resulting from gain-of-function mutations in the *nlrp3* gene. CAPS were the first autoinflammatory disorders associated with NLRP3 inflammasome dysregulation, as identified by Hoffman et al. in 2001. Inappropriate activation of NLRP3 in CAPS patients leads to recurring fever episodes, hive-like rashes, inflamed eyes, joint pain, swelling, headaches, and, if not treated, potential deafness and amyloidosis (Kuemmerle-Deschner et al., 2017). Conditions classified under the term CAPS include familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystemic inflammatory disease (NOMID). It was discovered that mutations in amino acids near serine 295 (Ser295) in human NLRP3 cause CAPS since phosphorylation of Ser295 by protein kinase A is crucial for negatively controlling NLRP3, so mutations in that region cause NLRP3 to be excessively activated (Mortimer et al., 2016). Recent research has also revealed that treating a CAPS knock-in mouse model expressing an asparagine 745 to lysine substitution mutation (N475K) of NLRP3 (corresponding to human N477K mutation) with the proton-pump inhibitor (PPI) esomeprazole can suppress IL-1ß secretion, decrease amyloid deposition, increase IL-1 receptor antagonist (IL-1Ra) production, and improve survival rates (Bertoni et al., 2020). Due to the fact that CAPS are rare genetic conditions, there are currently limited treatment options. Existing treatment methods involve IL-1 inhibitors, but the development of drugs targeting the NLRP3 inflammasome is highly sought after.

In addition to CAPS, the involvement of the NLRP3 inflammasome has been implicated in a multitude of central nervous system (CNS) diseases. For example, inflammation has been associated with promoting the advancement of Alzheimer’s disease (AD) and elevated levels of IL-1ß are discovered in the serum, cerebrospinal fluid, and brains of AD patients, where they exert neurotoxic effects on microglia and astrocytes (Rubio-Perez & Morillas-Ruiz, 2012; Parajuli et al., 2013; Saresella et al., 2016; Italiani et al., 2018; Ng et al., 2018). AD is identified by the buildup of amyloid-beta and hyperphosphorylated tau tangles. Microglial cells phagocytose the excess amyloid-beta that forms in the brain, however, the accumulation of the Aß peptide in lysosomes leads to

lysosomal swelling and destabilisation, causing the discharge of lysosomal contents, and thereby the activation of NLRP3 (Halle et al., 2008). The association between NLRP3 and AD pathology has also been demonstrated in other research, where the genetic deficiency and pharmacological inhibition of NLRP3 in mice that overexpress human amyloid precursor protein (APP) and presenilin 1 (PS1) both reduce Aß deposition and enhance cognitive functions (Heneka et al., 2013; Dempsey et al., 2017; Yin J. et al., 2018). Moreover, it has been shown that in mice, the activation of NLRP3 leads to the hyperphosphorylation of tau in a manner that is dependent on IL-1ß, suggesting that NLRP3 also operates upstream of tau (Ising et al., 2019). Due to the fact that there has been a range of unsuccessful clinical trials with drugs targeting Aß and tau tangles, NLRP3 has attracted significant interest as a new target for AD.

In addition to AD, the involvement of NLRP3 and IL-1ß in the autoinflammatory disease multiple sclerosis (MS) is well-documented, as both are known to promote CNS immune cell infiltration and excessive inflammation. Caspase-1 and IL-1ß are present in MS plaques and are upregulated in the peripheral blood mononuclear cells (PBMCs) of individuals with MS (Ming et al., 2002; Cao et al., 2015). In the experimental autoimmune encephalomyelitis (EAE) animal model for MS, NLRP3-expressing antigen-presenting cells contribute to T-cell migration to the CNS by increasing the expression of chemotaxis-related proteins (Inoue et al., 2012). Moreover, the expression of NLRP3 is elevated in the spinal cords of mice with EAE, and mice lacking NLRP3 and ASC are resistant to EAE, emphasising the significance of the inflammasome in MS (Gris et al., 2010; Inoue et al., 2012). Additionally, IFN-ß, which is one of the few therapeutic options for MS, is a known suppressor of NLRP3 inflammasome activation (Inoue et al., 2016).

Neuroinflammation is the typical reaction to traumatic brain injury (TBI), and numerous studies have provided evidence of the increase in inflammatory mediators, such as NLRP3, in the brain from hours to days after a TBI (Liu et al., 2013; Wallisch et al., 2017). mRNA for the cytokines IL-1ß and IL-1a is elevated as early as 3 hours post-TBI in rats, and pre-TBI administration of antibodies targeting these two cytokines diminishes the loss of hippocampal neurons significantly (Lu et al., 2005). Patients who have suffered severe TBI also exhibit upregulation of NLRP3 and other inflammatory mediators in the brain, and

recent research indicates that pharmacological inhibition of NLRP3 reduces cell death and prevents neurological deficits in TBI mouse models, demonstrating the potential for drug intervention targeting NLRP3 in TBI (Chen et al., 2019; Kuwar et al., 2019; Yan et al., 2020). The association between TBI and AD is well established, and some studies support the notion that there is a higher risk of neurodegenerative diseases for patients with a history of TBI (Gardner & Yaffe, 2015; Hayes et al., 2017; Ramos-Cejudo et al., 2018; Delic et al., 2020). These heightened risks are likely attributed to neuroinflammation, as post-TBI neuroinflammation leads to increased hyperphosphorylation of tau protein and Aß plaques, which are two hallmarks of AD (Johnson et al., 2010; Edwards et al., 2020).

Moving away from the CNS, a classic example of a peripheral disease caused by excessive NLRP3 inflammasome activation is gout. Gout develops due to high levels of uric acid in the blood, which over time form uric acid crystals that then build up in the joints, where they are engulfed by monocytes, macrophages and synoviocytes (Dalbeth et al., 2021; Richette & Bardin, 2010). Once engulfed, uric acid crystals disrupt lysosomes, leading to the activation of the NLRP3 inflammasome, causing the release of cytokines and the onset of inflammation and pain (Martinon et al., 2006). Due to the constant presence of uric acid in the blood of gout patients, the cycle of crystal formation and NLRP3 activation provides a mechanism for the chronic nature of the disease. Moreover, soluble uric acid has been found to trigger NLRP3 independently of crystal formation, indicating that inflammation can begin even earlier (Braga et al., 2017). Various NLRP3 inhibitors have been demonstrated to reduce the inflammation associated with gouty arthritis, underscoring the significance of targeting NLRP3 in gout.

RA has many of the same symptoms as gout, however, the pathology of these two diseases is different. While gout is caused by elevated levels of uric acid in the bloodstream, RA is a chronic inflammatory condition with pronounced autoimmune elements. Numerous studies support the involvement of NLRP3 in the pathogenesis of RA, however, the mechanism remains elusive. Some of the main characteristics of RA include persistent synovial inflammation in small diarthrodial joints and progressive cartilage and bone destruction (McInnes & Schett, 2011). Significant NLRP3 expression has been observed in the synovial proliferation and subchondral vasculitis areas in the paws of collagen-induced

arthritis (CIA) mice (Zhang et al., 2016). Additionally, although inhibition of IL-1ß as a treatment option for RA patients has not been as successful as other anti-proinflammatory cytokine therapies, pharmacological inhibition of the NLRP3 inflammasome with different inhibitors has been shown to reduce RA Pathology (Yan et al., 2012; Voon et al., 2017; Guo et al., 2018; Marchetti et al., 2018). Further research is indeed needed in order to elucidate the mechanistic link between RA and inappropriate inflammasome activation.

Much like the endotoxin tolerance in myeloid cells described previously, there has been some investigation on whether the NLRP3 inflammasome could be tolerised to repeated LPS stimulations of TLR4. From a biological standpoint, inflammasome tolerance would be logical since uncontrolled cytokine production paired with pyroptosis could be devastating for the inflamed tissue where infection occurs. Indeed, Bambouskova et al. recently showed that in mouse BMDMs, the NLRP3 inflammasome does get tolerised to repeated long (24 hours) stimulation with LPS via a mechanism involving metabolic rewiring. Specifically, Bambouskova and colleagues found that the immunometabolite itaconate accumulates in mouse BMDMs following LPS activation, reaching a critical level at 24 hours which in conjunction with accumulated nitric oxide (NO) is able to prevent the cleavage of GSDMD to its active GSDMD-NT form leading to inhibition of both pyroptosis and IL1b secretion upon TLR4 restimulation (Bambouskova et al., 2021). Considering the array of sterile inflammatory conditions described, including RA, where the accumulation of DAMPs in the affected tissue leads to the repeated activation of different immune cells, it is evident how crucial this line of investigation is since the loss of inflammasome tolerance could be causing a portion of the pathology that has not been explored yet. The importance of NLRP3 inflammasome tolerance as a mechanism becomes even more evident in cells such as human monocytes, which, as discussed above, are capable of secreting IL-1ß without undergoing pyroptosis and without the need for a second signal. Given their ability to activate the alternative inflammasome pathway, inappropriately tolerised human monocytes could potentially undergo multiple rounds of IL-1ß secretion before experiencing pyroptotic cell death.

Hence, it is of paramount importance to understand whether human monocytes can be tolerised by PAMPs and DAMPs via the immunometabolic mechanism described in mouse myeloid cells.

1.3 Immunometabolism of myeloid cells

Over the past twenty years, immunometabolism has become an increasingly important area of study with a significant impact on therapy development (Lee & Olefsky, 2021; Pålsson-McDermott & O'Neill, 2020). Immunometabolism refers to the connection between immunology and metabolism, and the interaction between these two major research areas has multiple aspects. For example, immune cells found in adipose tissue, or the liver, play a role in maintaining tissue balance and metabolic functions, such as the breakdown of fats and the effects of insulin (Daemen & Schilling, 2020; Lee & Dixit, 2020; Remmerie et al., 2020). Additionally, the consumption of nutrients like sugars, fats, or proteins and their metabolism can influence immune responses (Lee & Dixit, 2020). Inflammation, driven by cellular and molecular elements of the immune response and affecting the liver, adipose tissue or the pancreas, contributes to the development and advancement of conditions linked to non-alcoholic fatty liver disease, obesity and type 2 diabetes (Remmerie et al., 2020). For instance, the accumulation and activation of macrophages in adipose tissue and the liver are involved in the progression of metabolic disorders (Remmerie et al., 2020). Interestingly, the function of macrophages in adipose tissue and the liver during obesity is controlled by changes in their metabolism, which is prompted by the lipid-rich tissue environment. Thus, the obese adipose tissue and liver are characterised by an increase in a specific subset of macrophages with a signature related to lipid metabolism, known as "lipid-associated macrophages", as recently identified through single-cell analysis (Daemen et al., 2021; Jaitin et al., 2019).

Another significant aspect of the interaction between immunology and metabolism, which will be discussed in depth in this section, is that cellular metabolism is a central regulator of the function and activation of immune cells (Ryan & O'Neill, 2020). Different activation states of innate immune cells, particularly monocytes and macrophages, are linked to distinct cellular metabolic states; therefore, cellular metabolic adjustments support appropriate immune responses and adaptability (Ryan & O'Neill, 2020). Metabolic reprogramming of innate immune cells is also a crucial part of innate immune memory, such as trained immunity and the previously discussed endotoxin

tolerance, which means that certain stimuli can induce nonspecific memory in innate immune cells, resulting in increased preparedness for inflammation, as for example, trained innate immune cells exhibit heightened inflammatory responses upon subsequent challenges (Bekkering et al., 2021; Penkov et al., 2019). Cellular metabolism not only influences the functions of mature myeloid cells but also their production in the bone marrow, a process known as myelopoiesis (Hajishengallis et al., 2021). Changes in the metabolism of precursors of innate immune cells in the bone marrow are essential for differentiation and lineage decisions during myelopoiesis under normal conditions and in times of stress (Hajishengallis et al., 2021). Furthermore, immunometabolism has been implicated in autoimmune disorders such as multiple sclerosis, type 1 diabetes and rheumatoid arthritis, as specific alterations in the metabolism of immune cells may contribute to the loss of immunological tolerance and the emergence of autoreactive immune cells (Galgani et al., 2020; Weyand & Goronzy, 2020). Accordingly, targeting the dysfunctional interaction between immunology and metabolism is an important approach for developing new therapies not only for metabolic diseases but also for chronic inflammatory disorders.

Myeloid cells are crucial to the immune response as they react to pathogens and tissue damage, initiate inflammation and host defence, and participate in tissue repair. Recent advances in metabolomics have provided intriguing new insights into the precise and regulated metabolic changes that occur in these cells at various states of activation. Specific changes in metabolites have been associated with effector mechanisms, as glycolysis and succinate have been shown to drive inflammatory responses, while fatty acid oxidation has been linked to anti-inflammatory (Mills et al., 2017; O'Neill & Pearce, 2016).

One specific metabolite called itaconate is particularly notable for demonstrating how a regular metabolic process can be redirected to produce significant immunologic effects. Itaconate is synthesised from cis-aconitate in the tricarboxylic acid cycle (TCA cycle) in monocytes and macrophages that are activated by various factors such as TLR4 ligands and cytokines like type I and type II interferons (Michelucci et al., 2013; Shin et al., 2011; Strelko et al., 2011) (Figure 1.2). These stimuli elevate the expression of the enzyme aconitate decarboxylase 1 (ACOD1), which was originally named immune-responsive gene 1

(IRG1). Upon production of the ACOD1 enzyme, cis-aconitate is diverted from the TCA cycle and reformed into itaconate (Figure 1.2). The functional use of itaconate is likely to have existed for a long evolutionary time, as it has also been recently observed in New Zealand mussels during bacterial infection (Nguyen et al., 2018). All the evidence currently points to itaconate as being both an antimicrobial and an anti-inflammatory metabolite.

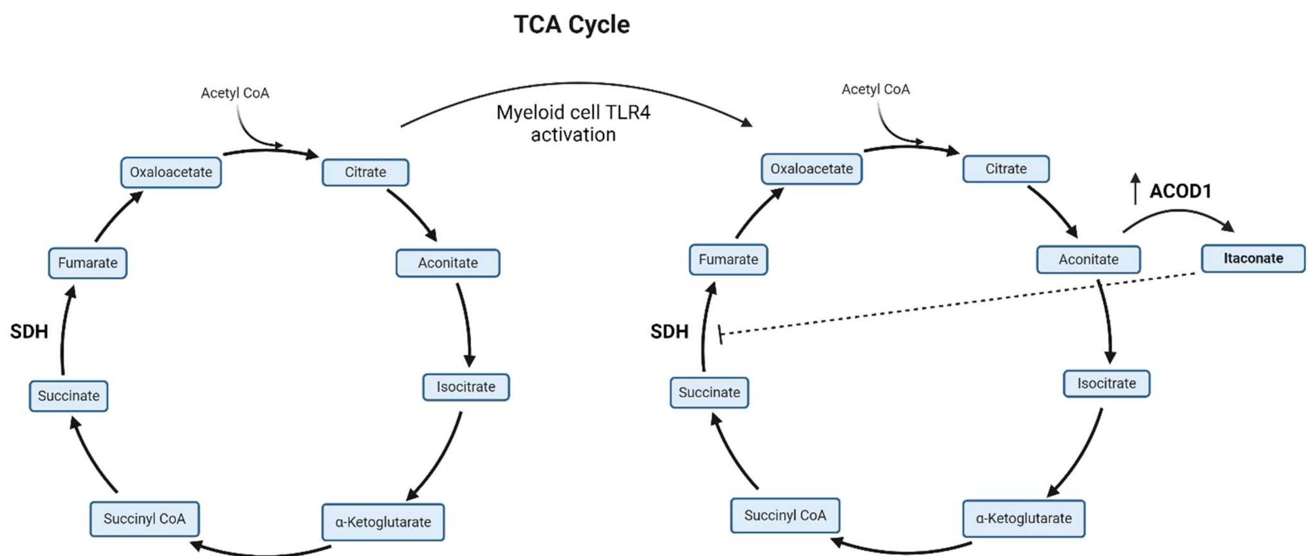


Figure 1.2 TCA cycle remodelling following myeloid cell TLR4 activation. Activation of myeloid cells through TLR4 stimulates the production of itaconate from the TCA intermediate aconitate, mediated by the increased expression of the ACOD1 enzyme. Itaconate then inhibits the succinate dehydrogenase (SDH) enzyme preventing fumarate production and causing an increase in intracellular succinate accumulation. Figure was generated using BioRender.

Gustav Crasso was the first to publish the name itaconate in 1840 as a permutation of the letters of the word aconitate since he demonstrated that itaconate is a product of the thermal decomposition of aconitate (Crasso, 1840). Later, itaconate gained attention during Hans Krebs' investigations of the TCA cycle as one of the substances he tested as a potential central metabolism intermediate in pigeon mitochondrial extracts (Holmes, 1993). He did, however, find that, unlike malate or succinate, itaconate was unable to support respiration. Subsequently, the interest in itaconate during the 20th century diminished, however, the scientific understanding of the role of this metabolite

in mammalian metabolism has evolved considerably in the 21st century. In 2011, it was reported by three different groups that itaconate production was observed in relation to mammalian immune responses. These observations were made in the lungs of mice infected with Mycobacterium tuberculosis (Shin et al., 2011), in intracellular lysates of LPS-activated RAW264.7, and in the supernatants of the LPS-activated RAW264.7 cells (Sugimoto et al., 2012). The biosynthetic pathway of itaconic acid production was then further characterised, showing that itaconate is obtained via decarboxylation of cis-aconitate, an intermediate of the TCA cycle derived from citrate. This was done using uniformly labelled 13C-glucose feeding (Strelko et al., 2011). Two years later, Michelucci et al. identified an LPS-inducible gene called *irg1* to be responsible for itaconate production in activated macrophages (Michelucci et al., 2013). The *irg1* gene was first isolated from a complementary DNA (cDNA) library of LPS-stimulated macrophages in 1995, and its function remained unknown for 18 years (Lee et al., 1995). However, the potential importance of the gene was immediately appreciated, as it was observed that it is not expressed at all in steady-state macrophages but is upregulated by almost 200-fold in activated macrophages, making it one of the most highly induced genes upon TLR4 activation, hence, it was named “immune responsive gene 1” (Lee et al., 1995). Then, in 2005 it was established that *irg1* (now called *ACOD1*) is activated by LPS in an MYD88-independent manner (Kawai & Akira, 2005), and more recently, the transcription factor interferon regulatory factor 1 (IRF1) has been suggested as the primary regulator of *irg1* on the level of mRNA (Gidon et al., 2021).

1.3.1 The antimicrobial role of itaconate

Ever since itaconate was identified as a metabolite in mammals, its function in host-pathogen and antimicrobial interactions has been the subject of prominent scientific investigation. It was shown that itaconate could suppress microbe growth by inhibiting a bacterial enzyme called isocitrate lyase (ICL) (McFadden et al., 1971; McFadden & Purohit, 1977; Nguyen et al., 2019), which is necessary for the glyoxylate shunt during bacterial infection (Hillier & Charnetzky, 1981; Lorenz & Fink, 2002; McKinney et al., 2000; Muñoz-Elías & McKinney, 2005). This inhibition restricts the growth of pathogens, like *Pseudomonas Indigofera,* that

rely on ICL activity (Naujoks et al., 2016; McFadden & Purohit, 1977; Price et al., 2019). Itaconate's breakdown product, itaconyl-Coenzyme A (CoA), has also been found to limit the growth of pathogens such as *M. tuberculosis* (Ruetz et al., 2019) since during *M. tuberculosis* infection, the metabolism of cholesterol-derived propionyl-CoA is essential for its pathogenicity (Berg et al., 2002) and itaconyl-CoA was shown to inhibit methylmalonyl-CoA mutase (MCM), an enzyme required for propionate metabolism (Ruetz et al., 2019; Savvi et al., 2008). Furthermore, itaconate reduces macrophage chemokine production and inhibits recruitment of neutrophils to the lungs of *M. tuberculosis*-infected mice, thus preventing *M. tuberculosis*–associated immunopathology (Nair et al., 2018). Recently, it was demonstrated that the Rab32 GTPase interacts with ACOD1 to help deliver itaconate into Salmonella-containing vacuoles (SCVs), thereby killing the pathogen (Chen et al., 2020). Upon further investigation, it was found that Salmonella typhimurium can bypass antimicrobial responses by using the secretion effectors SopD2 and GtgE, leading to a potential selection for itaconate-resistant strains of Salmonella (Spanò et al., 2011; Spanò et al., 2016). The absence of SopD2 and GtgE impaired Salmonella replication in hematopoietic cells but had no effect on replication in other cell types. Studies using mouse BMDMs lacking Rab32 and biogenesis of lysosome-related organelles complex 3 (BLOC3), which is necessary for Rab32 function, revealed that Rab32 plays a crucial role in delivering itaconate into specific compartments within cells (Spanò et al., 2011; Spanò et al., 2016). Furthermore, deficiencies in BLOC3 and ACOD1 led to increased replication of *S. Typhimurium*.

All these investigations highlight that itaconate is a natural antimicrobial molecule, however, some pathogens have developed sophisticated methods to counteract it during an infection. For instance, certain bacteria have the capability to decompose and utilize itaconate to produce pyruvate and acetyl-CoA. Although the breakdown of itaconate by bacteria has been studied for many years, the specific enzymes responsible and their function during infection were not clearly understood until a recent discovery that both *Yersinia pestis* and *Pseudomonas aeruginosa* contain multiple genes encoding the enzymes itaconate CoA transferase, itaconyl-CoA hydratase, and (S)-citramalyl-CoA lyase, which are essential for itaconate breakdown, and which thereby grant those pathogens increased survival within macrophages (Cooper & Kornberg, 1964; Cooper et al.,

1965; Martin et al., 1961; Sasikaran et al., 2014). Likewise, *M. tuberculosis* produces the enzyme β-hydroxyacyl-CoA lyase, which is vital for itaconate breakdown and leucine catabolism, consequently enhancing *M. tuberculosis*' ability to cause disease (Wang et al., 2019). Additionally, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two notable examples of pathogens that can utilize host-produced itaconate to support the creation of biofilms, leading to greater pathogenicity and survival (Riquelme et al., 2019; Riquelme et al., 2020). Because of its electrophilic nature, itaconate triggers a prosurvival membrane stress response in *Pseudomonas* (Juarez et al., 2017; Wongsaroj et al., 2018), and when experiencing membrane stress, *P. aeruginosa* prioritizes the synthesis of extracellular polysaccharides (EPS) over LPS, which in turn has been found to alter the metabolic processes of macrophages further, increasing itaconate production resulting in a positive feedback loop (Riquelme et al., 2020). EPS production also results in enhanced biofilm formation, creating a favourable environment for *P. aeruginosa* to survive in the lungs (Maurice et al., 2018). Similarly, itaconate has recently been discovered to play a critical role in driving biofilm formation during *S. aureus* infection (Tomlinson et al., 2021). The upregulation of itaconate caused by *S. aureus* leads to itaconate-mediated suppression of staphylococcal aldolase, causing the redirection of fructose-6-phosphate to glucosamine-6-phosphate and thereby fuelling EPS biosynthesis and subsequent biofilm formation (Tomlinson et al., 2021). From these studies, it is evident that although itaconate has antimicrobial properties, certain bacterial species have evolved mechanisms of defence against its harmful effects and can even use it to their advantage.

1.3.2 The immunomodulatory properties of itaconate

In addition to its antimicrobial function, itaconate has been shown to have a variety of anti-inflammatory properties. Itaconate possesses a five-carbon dicarboxylic acid structure with an α,β-unsaturated alkene, which renders it mildly electrophilic. Structurally, it bears resemblance to several metabolites such as succinate, malonate, phosphoenolpyruvate, and fumarate, and due to its

structural similarity to succinate and malonate, itaconate has the ability to competitively inhibit succinate dehydrogenase (SDH), preventing the oxidation of succinate to fumarate (Cordes et al., 2016; Lampropoulou et al., 2016) (Figure 1.2). Moreover, the presence of the alkene group enables itaconate to function as a Michael acceptor and react with cysteine residues akin to fumarate (Mills et al., 2018). In the past, two derivatives were commonly utilised in cell culture experiments as a surrogate for itaconate, namely 4-octyl itaconate (4-OI) and dimethyl itaconate (DI), due to their high membrane permeability. However, both 4-OI and DI are much more electrophilic compared to natural itaconate; hence, the effects they have on the modulation of immune cell effector functions have been shown to be different to that of natural itaconate (Swain et al., 2020) and observations made using these derivatives should not be considered synonymous to the effects that itaconate exerts within immune effector cells. Nonetheless, studies carried out using these itaconate derivatives have uncovered some potential anti-inflammatory functions that physiological itaconate could exert in myeloid cells.

The first ever immunoregulatory role of itaconate that was demonstrated was its ability to inhibit SDH (Cordes et al., 2016; Lampropoulou et al., 2016) (Figure 1.2), and although it was known for many years that itaconate competitively inhibits SDH due to its structural similarity to succinate (Booth et al., 1952), the physiological roles of itaconate as an SDH inhibitor remained unexplored. For example, the generation of complex-driven mitochondrial reactive oxygen (mtROS) is inhibited when SHD is blocked since the oxidation of succinate to fumarate is prevented (Chandel et al., 1998; Mills et al., 2016). The blockade of mtROS generation supports prolyl hydroxylase activity, which in turn suppresses the stabilisation of hypoxia-inducible factor (HIF), altering immune cell response to different stressors such as TLR receptor activation and hypoxia (McGettrick & O'Neill, 2020). Furthermore, it was observed that *Irg1* knockout (KO) BMDMs could not store succinate following exposure to LPS, indicating the presence of catalytically active SDH. Additionally, when faced with inflammatory triggers, *Irg1* KO cells had increased release of proinflammatory cytokines such as IL-6, IL-1ß, IL-18, and IL-12p70 compared to normal cells. TNF levels, however, were unaffected (McGettrick & O'Neill, 2020).

Although less electrophilic than the artificial derivatives 4-OI and DI, at sufficient intracellular levels, naturally produced itaconate is also able to modify cysteine residues in a process termed “itaconation”, also called 2,3-dicarboxypropylation (Bambouskova et al., 2021; Hooftman et al., 2020; Mills et al., 2018). Hence, in addition to SHD inhibition, the anti-inflammatory properties of itaconate are linked to the regulation of aerobic glycolysis since various enzymes involved in glycolysis, such as aldolase A (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and lactate dehydrogenase A (LDHA), have been found to contain alkylated cysteine residues (Liao et al., 2019; Mills et al., 2018; Qin et al., 2019). It has been reported that itaconate reduces ALDOA enzyme activity, glucose consumption, and lactate production, with *Irg1* KO macrophages showing increased ALDOA activity (Qin et al., 2019). Liao et al. demonstrated that 4-OI alkylates cysteine 22 (C-22) of GAPDH, thus inhibiting its enzymatic activity, lactate production, and extracellular acidification rate (ECAR), aligning with glycolytic inhibition phenotypes (Liao et al., 2019). Additionally, the impact of 4-OI on the expression of IL-1ß, TNF and inducible nitric oxide synthase (iNOS) mirrored the effects of the known GAPDH inhibitor heptelidic acid (Liao et al., 2019). Cells expressing mutant GAPDH with alanine-substituted C-22 showed reduced IL-1ß production, indicating the functional importance of C-22 in maintaining GAPDH activity and also demonstrating why itaconation of this part of the protein can influence its function (Liao et al., 2019). Since most of the investigation was carried out using the highly electrophilic 4-OI, *irg1* KO was done in order to investigate the role of endogenous itaconate in regulating GAPDH. It was found that $Irg1^{-/-}$ BMDMs exhibited increased GAPDH enzyme activity, lactate production, and ECAR (Liao et al., 2019). Notably, GAPDH has also been shown to be a target of dimethyl fumarate (DMF) (Kornberg et al., 2018). Screens for cysteine succination in fumarate hydratase-deficient cells, which accumulate endogenous fumarate, have revealed that both GAPDH and LDHA are succinated by fumarate (Ternette et al., 2013), hence, it appears that both itaconate and fumarate can control glycolysis in myeloid cells. Furthermore, itaconate has been demonstrated to act as an inhibitor of fructose-6-phosphate 2-kinase, functioning similarly to the itaconate analogue phosphoenolpyruvate, which is an intermediate in glycolysis, leading to a reduction in the synthesis of fructose-2,6-biphosphate (Sakai et al., 2004; Van Schaftingen et al., 1981). Given that fructose-2,6-biphosphate

stimulates the activity of phosphofructokinase, it is plausible to suggest that a decrease in fructose-2,6-biphosphate levels due to itaconate inhibition could contribute to the inhibition of glycolysis in LPS-activated macrophages.

Recently, the significance of confirming mechanistic studies conducted with derivatives such as 4-OI and DI by using underivatised itaconate and Irg1$^{-/-}$ macrophages has been demonstrated by Swain et al. (Swain et al., 2020). There was previous uncertainty about the cell permeability of underivatised itaconate, but Swain and colleagues showed that BMDMs could take up exogenous itaconate, as evidenced by an increase in both intracellular itaconate and succinate levels upon exogenous itaconate administration. In fact, neither DI nor 4-OI were able to raise intracellular itaconate levels and were ineffective in causing succinate buildup in *Irg1* deficient macrophages, suggesting that the immunomodulatory effects of highly electrophilic itaconate derivatives are unrelated to SDH inhibition (Swain et al., 2020). Furthermore, it was observed that DI and 4-OI notably decreased glutathione (GSH) levels, while exogenous itaconate did not, demonstrating a clear consequence of the enhanced electrophilicity of the derivatives and emphasising the necessity of confirming the physiological roles of itaconate in *Irg1* KO cells or with exogenous underivatised itaconate (Swain et al., 2020). This study also reported a significant distinction between itaconate and its derivatives in the control of type I IFNs (Swain et al., 2020). Type I IFNs, which are released by activated monocytes and macrophages, play various roles in defending against infections (McNab et al., 2015). Initially, itaconate was believed to hinder type I IFN responses based on studies using 4-OI and DI, which showed a substantial reduction in type I IFN-related genes in LPS-activated macrophages (Bambouskova et al., 2021; Mills et al., 2016). However, Swain and colleagues showed that the diminished induction of type I IFNs in response to LPS in *Irg1* KO BMDMs was restored to normal levels upon administration of extracellular itaconate, indicating that itaconate is involved in the upregulation and not the inhibition of type I IFNs in macrophages. However, the exact mechanism by which the derivatives reduce type I IFNs or how itaconate enhances type I IFN production is currently unclear.

An intriguing emerging function of itaconate is its ability to dampen the activation of the NLRP3 inflammasome, which, as discussed earlier, is an innate

immune process that stimulates the secretion of proinflammatory cytokines upon detection of a priming signal such as microbial products (e.g., LPS) and a second activating signal such as cellular damage or toxins (e.g., ATP/nigericin) (Swanson et al., 2019). Initially, an increase in IL-1ß and IL-18 release was observed in Irg1$^{-/-}$ BMDMs stimulated with LPS and ATP (Lampropoulou et al., 2016); however, the precise mechanism through which itaconate inhibited inflammasome activation and IL-1ß/IL-18 release remained unclear. Swain et al. later demonstrated that exogenous itaconate specifically reduced IL-1ß release without affecting the transcription of pro-IL-1ß, indicating a direct influence on NLRP3 activity and the potential targeting at the level of signal 2 for NLRP3 activation (Swain et al., 2020).

Two recent research studies have outlined detailed mechanisms explaining how itaconate could downregulate the activation of NLRP3 (Bambouskova et al., 2021; Hooftman et al., 2020). According to Hooftman et al., the effects on the suppression of NLRP3 activation by itaconate are attributed to the alkylation of cysteine 548 (C-548) on NLRP3, thus preventing its interaction with NEK7 (Hooftman et al., 2020). In this study, after the initial LPS stimulation, 4-OI was introduced to specifically explore the effects of itaconate on signal 2. To rule out potential effects of 4-OI on LPS-mediated signal 1, no impact on pro-IL-1ß expression was observed (Hooftman et al., 2020). 4-OI was found to inhibit lactate dehydrogenase (LDH) release, IL-18 release, ASC speck formation, Gasdermin D and IL-1ß processing, all of which are indicators of NLRP3 activation. Additionally, 4-OI had no impact on the NLRC4 or AIM2 inflammasomes and the effects observed using 4-OI were confirmed by exogenous itaconate and Irg1-deficient BMDMs. Lastly, in a system where the murine inflammasome was reconstituted in HEK293 cells, overexpressing Irg1 inhibited IL-1ß release (Hooftman et al., 2020).

On the other hand, Bambouskova and colleagues proposed a mechanism in which itaconate and iNOS work together to induce tolerance to late NLRP3 inflammasome activation (Bambouskova et al., 2021). The experimental model that was used involved two LPS activations with a 24-hour interval followed by ATP as signal 2. The idea behind this experimental design is that primary LPS activation tolerises the cells, presumably due to upregulation of ACOD1 and accumulation of endogenously produced itaconate. To prove this hypothesis,

they showed that while LPS tolerised wild-type BMDMs were unable to process pro-IL-1ß and secrete mature IL-1ß, LPS tolerised *irg1* KO BMDMs showed normal levels of pro-IL-1ß processing and mature IL-1ß secretion (Bambouskova et al., 2021). In contrast to the findings of Hooftman et al., this study suggested that itaconate acts downstream of ASC speck formation, as wild-type BMDMs activated classically exhibited levels of ASC speck formation like those in tolerised BMDMs (Bambouskova et al., 2021). Furthermore, GSDMD cleavage and pyroptosis were inhibited entirely in wild-type LPS tolerised BMDMs, while *Irg1* KO BMDMs were able to cleave GSDMD into its active GSDMD-NT form and thereby undergo pyroptosis and IL-1ß secretion, showing their inability to become tolerised by primary LPS stimulation. Notably, the addition of cell-permeable glutathione ethyl ester to wild-type BMDMs restored IL-1ß secretion upon late inflammasome activation, indicating that thiol reactivity may be responsible for inflammasome tolerance induced by itaconate. To further understand the mechanism, a proteomic screen was conducted, identifying numerous potential targets for itaconate during late inflammasome activation. Importantly, using mass-spec analysis, itaconated GSDMD at cysteine 77 was shown in WT macrophages, specifically at late time points of LPS stimulation when a sufficient intracellular accumulation of itaconate could occur, allowing itaconate, albeit mildly electrophilic, to function as a Michael acceptor and react with cysteine residues. This GSDMD modification was also found in *Irg1* KO macrophages reconstituted with the addition of 1mM exogenous itaconate but not in unreconstituted KO cells (Bambouskova et al., 2021). Although GSDMD is traditionally considered downstream of caspase-1, caspase-1 activity upon late inflammasome activation was shown to be largely dependent on GSDMD, suggesting that GSDMD may be necessary for late inflammasome activation and caspase-1 processing (Figure 1.1). Thus, it is reasonable to consider the alkylation of GSDMD by itaconate as a potential mechanism of NLRP3 inhibition by itaconate.

It is still unknown, however, if itaconate-mediated tolerisation of the NLRP3 inflammasome also occurs in primary human monocytes and whether the tolerisation mechanism is dependent on the cleavage of GSDMD to its active GSDMD-NT. Additionally, in order to fully understand inflammasome tolerance in human myeloid cells, it is important to consider other immunometabolic factors,

such as hypoxia, for example, that have been shown to influence the responsiveness to different stimuli and the inflammatory state of these cells.

1.3.3 The effects of hypoxia on myeloid cells in chronic inflammatory conditions

Many chronic inflammatory diseases, like RA and chronic obstructive pulmonary disease (COPD), commonly exhibit hypoxia within the tissue microenvironment. The presence of hypoxia in the synovial fluid of the RA joint was initially documented in the 1970s (Lund-Olesen, 1970) and is gaining recognition in COPD (Kent et al., 2011). Hypoxia in an inflammatory setting occurs when the demand for oxygen surpasses the supply, and in the context of inflammation, this occurrence is linked to the heightened infiltration and proliferation of immune cells, which primarily rely on oxygen for metabolic processes in order to function effectively. In the case of RA, it is also thought that tissue overgrowth, accumulation of synovial fluid, and movement of the injured joint can disrupt the capillary network and block blood flow to the tissue (Jawed et al., 1997). As a result of the excessive oxygen demand, neovascularisation takes place, however, these newly generated structures are relatively dysfunctional and inadequately organised, thus contributing even further to hypoxia within the tissue (Kennedy et al., 2010; Strehl et al., 2014). Consequently, immune cells such as blood monocytes face environmental challenges when recruited to the inflammatory tissue, as it is widely acknowledged that to be able to adapt to hypoxic microenvironments and maintain survival and functionality, immune cells adjust via the expression of the transcription factor hypoxia-inducible factor 1 alpha (HIF-1α) (Semenza & G. L.Wang, 1992). This master regulator has been reported to control various processes, including cell metabolism, angiogenesis, migration, and inflammatory cascades (Semenza, 2009). HIFs typically consist of heterodimers, comprising an oxygen-regulated alpha subunit and a stable beta subunit (Semenza & G. L. Wang 1992). Under normal oxygen levels, prolyl hydroxylases (PHDs) hydroxylate the alpha subunits, which are then targeted for degradation by an E3 ubiquitin ligase complex known as von Hippel Lindau tumour suppressor protein (pVHL). The polyubiquitinated alpha subunits

are subsequently degraded by the proteasome (Berra et al., 2003). In hypoxic conditions, the enzymatic activity of PHDs is inhibited due to the lack of oxygen, which is essential for their activity, leading to the stabilisation of the alpha subunits and the formation of the heterodimer (Epstein et al., 2001; Ivan et al., 2001). Subsequently, the stabilised HIFs can translocate into the nucleus to regulate the transcription of genes responsible for the adaptation to hypoxic conditions (Fangradt et al., 2012; Staples et al., 2011). Although this mechanism is well established in macrophages, it is debated in human monocytes. Despite the stabilisation of HIF-1α under hypoxic conditions in monocytes, it has been suggested that NF-κB may be responsible for the necessary transcriptional processes, as HIF-1α seems to be absent from the nucleus (Fangradt et al., 2012). Recent studies have also proposed that mitochondrial complex II may regulate hypoxia adaptation in monocytes (Sharma et al., 2017). These studies illustrate the need for further investigation into the precise mechanism of monocyte adaptation to hypoxia.

Regardless of the mechanism, however, when faced with an environmental challenge like hypoxia, it is believed that myeloid cells change their functional characteristics. To understand how hypoxia affects the phenotype of human monocytes, Bosco et al. conducted a comprehensive study of hypoxia-cultured monocytes (Bosco et al., 2006). It was revealed that monocytes increase the expression of several genes linked to typical hypoxia responses, including vascular endothelial growth factor (VEGF) -responsible for angiogenesis, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) – responsible for apoptosis, glucose transporter 1 (GLUT-1) – responsible for glucose transport, hexokinase 2 (HK2) – responsible for glycolytic metabolism, and chemokine-related genes -CCL15, CCL2 and CCR2. Monocytes displayed elevated levels of CCL20 gene and protein expression when exposed to low oxygen levels, suggesting that hypoxia could enhance the migration of other immune cells to the inflamed area (Bosco et al., 2006). Later studies identified CD300a as a surface receptor induced by hypoxia that can regulate CCL20 and VEGF expression (Raggi et al., 2014). The significance of hypoxia in mediating chemotactic responses in monocytes was further emphasised by the upregulation of CXCR4 receptor expression (Schioppa et al., 2003). Additionally, CCL20 production has also been linked to the activation of the p50 subunit of NF-κB

(Battaglia et al., 2008). The action of this subunit supports the notion that NF-κB is crucial for hypoxia adaptation in human monocytes (Battaglia et al., 2008; Fangradt et al., 2012). Interestingly, decreased O2 levels in the joint have been associated with increased canonical NF-κB signalling in the synovial fluids of active RA patients (Oliver et al., 2009). Hypoxic conditions *in vitro* have also been demonstrated to enhance the survival rate of both monocytes and macrophages, believed to occur through the stimulation of glycolytic metabolism (Roiniotis et al., 2009) and upon encountering hypoxic sites, both monocytes and macrophages have been shown to decrease their migratory properties (L. Turner et al., 1999; Grimshaw & Balkwill, 2001). Furthermore, macrophages are believed to alter their proteolytic capacity under hypoxic conditions by increasing MMP-7 gene expression (Burke et al., 2003). In palmitate-activated macrophages, hypoxia is believed to enhance the production of IL-6 and IL-1ß (Snodgrass et al., 2016).

Overall, this evidence indicates that hypoxia may encourage proinflammatory properties in myeloid cells, and its presence in chronic inflammatory tissue such as the RA joint exacerbates disease severity. It is, however, still unknown whether RA joint hypoxia in tandem with continuous stimulation by DAMPs, such as tenascin-C, could alter and break the NLRP3 tolerisation phenotype of human monocytes, leading to disease chronicity due to dysregulated secretion of the proinflammatory cytokine IL-1ß.

1.4 Rheumatoid arthritis as a chronic inflammatory disease

It is currently estimated that RA impacts 17.6 million individuals globally, with an estimated increase to 31.7 million RA patients by 2050 (Black et al., 2023). RA is currently recognised as a chronic inflammatory disease characterised by persistent uncontrollable synovitis, as well as pannus and bone erosion (Gravallese & Firestein, 2023; Komatsu & Takayanagi, 2022). Typically, RA presents as symmetrical polyarthritis, predominantly affecting small joints in the extremities (Grassi et al., 1998; Sokolova et al., 2021;). Generally, there are two

subdivisions of RA patients, namely those who manifest disease with anti-citrullinated protein antibodies (ACPAs) and/or autoantibodies to immunoglobulin G, termed rheumatoid factor (RF), and those that are both ACPA and RF-negative (Sokolova et al., 2021). The two groups of patients are termed seropositive and seronegative, respectively. About 80% of RA patients are seropositive; hence, the majority of research is focused on seropositive RA (Bizzaro et al., 2013). ACPA production has been associated with both genetic and environmental factors. The strongest genetic links are associated with the human leukocyte antigen (HLA)-DRB1 (Gonzalez-Gay et al., 2002), and the strongest environmental triggers include exposure to toxic agents, such as cigarette smoke and silica dust (Stolt et al., 2010), and *Porphyromonas gingivalis* infection (Wegner et al., 2010). ACPAs can be present in the blood before clinical disease manifestation; hence, in some instances, they can serve as a predictive tool for early RA diagnosis (Bizzaro et al., 2013). However, it must be noted that not all individuals with ACPAs progress to RA. In RA, articular symptoms may be accompanied by systemic complications, such as pulmonary interstitial fibrosis and cardiovascular disease (Akiyama & Kaneko, 2022; Figus et al., 2021; Weber et al., 2023), hence individuals who receive inadequate or delayed treatment experience joint destruction, disability, and even death in the years following initial symptoms. Therefore, although there is currently no cure for RA, adequate and timely treatment is paramount for the successful management of RA symptoms.

1.4.1 Pathogenesis and cellular mediators of RA

Autoantibodies can be identified in some individuals with RA several years before they show symptoms (Bizzaro et al., 2013), however, it is still under investigation whether they are actively involved in disease pathogenesis or simply a byproduct of an already present diseased cellular state. The most widely investigated autoantibodies are those against citrullinated proteins. Citrullination is the enzymatic conversion of specific polypeptide arginine amino acids to the amino acid citrulline, a reaction catalysed by peptidylarginine deiminases (PADs). Citrullination changes some of the properties of the polypeptide as citrulline is a neutral amino acid while arginine is a positively

charged amino acid. Citrullinated proteins have a higher affinity for the antigen-binding groove of HLA-DRB1 (Van Der Helm-van Mil et al., 2006) and are more immunogenic compared to natural proteins (Ge & Holmdahl, 2019). Neutrophil extracellular traps (NETs) are a significant source of citrullinated proteins, and recent research has revealed that more NETs are present in the circulation and synovial fluids of RA patients compared to healthy individuals, with the formation of NETs leading to a sustained inflammatory microenvironment in the joints (Apel et al., 2018). Additionally, studies have shown that citrullinated proteins from NETs can be internalised through the receptor for advanced glycation end-products pathway, leading to an inflammatory phenotype in fibroblast-like synoviocytes (FLS) (Carmona-Rivera et al., 2017).

It has been found that a subset of autoantibodies recognizing a citrullinated epitope in the FBG-C domain of tenascin-C are detectable years before any disease manifestation and thus could help in early diagnosis (Schwenzer et al., 2016), as well as in disease prediction among people presenting with early synovitis (Raza et al., 2016). Additionally, the levels of activated $CD4^+$ T cells specific for five different citrullinated tenascin-C epitopes were shown to be elevated in the peripheral blood and synovial fluid of RA patients, and two of these epitopes were also recognized by antibodies within the serum and synovial fluid of RA patients (Song et al., 2021). These findings highlight the possibility that coinciding citrullinated Tenascin-C epitopes recognized by both T and B cells have the potential to increase autoimmunity and promote RA disease progression.

Chronic inflammation caused by inappropriately activated immune cells plays a crucial role in the development of RA. Rheumatoid joints are mainly infiltrated by T cells, with $CD4^+$ T cells outnumbering $CD8^+$ T cells in most patients (Kondo et al., 2018). T cells in the synovium exhibit an activated phenotype and behave similarly to cytokine-stimulated T cells rather than T cell receptor-stimulated T cells (Brennan et al., 2002), with specific cytokines present in inflamed joints leading to the predominant expression of distinct T cell transcription factors, such as T-bet and retinoic acid receptor-related orphan receptor gamma t (RORγT), resulting in an imbalance in T cell differentiation, favouring T helper 1 cells over Th2, and Th17 over regulatory T cells (Kondo et al., 2018). The activated Th1 cells then contribute to the activation of infiltrating myeloid cells,

perpetuating joint inflammation. In addition to $CD4^{+}$ T cells, recent reports have indicated that Granzyme K^{+} $CD8^{+}$ T cells in the synovium of RA patients produce high levels of IFN-γ, amplifying the proinflammatory microenvironment in the joint (Jonsson et al., 2022). Furthermore, a recent study using mass cytometry to analyse activated T cells in joint tissue from RA patients identified a subset of $CD4^{+}$ $PD1^{+}$ $CXCR5^{-}$ follicular helper T cells (Tfh) located near B cells, whose main function is to produce interleukin IL-21 to support the proliferation and differentiation of B cells (Rao et al., 2017).

Although B cells make up only a small proportion of cells in the synovium, they are considered important contributors to the start and continuation of RA pathology. Activated B cells perform various effector functions, such as releasing cytokines, creating abnormal germinal centres, activating T cells through antigen presentation, and transforming into antibody-producing cells (Wu et al., 2021). B cells and plasma cells make and secrete RF and anti-modified-protein antibodies (AMPAs), including ACPAs, anti-carbamylated protein antibodies, and anti-acetylated-protein antibodies. Evidence suggests that most RA patients have at least two types of AMPAs, indicating that a variety of antigens could potentially activate B cells, both during their initial development in lymph nodes and in the synovium, where they may encounter different modified antigens (Scherer et al., 2022). Recent research data has revealed that a B cell population characterised by the co-expression of nuclear receptor subfamily 4 group A member 1 (NR4A1), NR4A2, and NR4A3 is highly concentrated in RA synovial tissue where it helps produce and maintain synovial germinal centres by releasing IL-6, lymphotoxin α, lymphotoxin β (Meednu et al., 2022). Additionally, age-associated B cells (ABC) with high expression levels of integrin subunit alpha X (ITGAX), T-bet, and activation-induced cytidine deaminase, originally found in systemic lupus erythematosus (SLE), have been shown in the synovium and peripheral blood of RA patients where they contributed to the development of RA by inducing activation of FLS via TNF-mediated ERK1/2 and JAK-STAT1 pathways (Qin et al., 2022).

RA is characterised by the presence of persistent, severe synovitis with significant enlargement of the synovial lining and sublining layers. FLS present in human synovial tissue originate from mesenchymal stem cells and can demonstrate characteristics resembling those of tumours, as these cells not only

proliferate extensively in RA but also invade and degrade the cartilage (Nygaard & Firestein, 2020). The use of single cell RNA sequencing (scRNA-seq) technology has led to the identification of several new types of pathogenic FLS. Recently, a subset of $CD34^{-}$ $CD90^{+}$ FLS originating from vascular endothelial cells has been discovered through scRNA-seq analysis of synovial tissue organoids, and the differentiation of this subset was shown to be crucial for the development of inflammatory arthritis (Wei et al., 2020). Additionally, longitudinal transcriptomics approaches combined with scRNA-seq have revealed $CD45^{-}$ $CD31^{-}$ Podoplanin $(PDPN)^{+}$ cells in the peripheral blood of RA patients, which have FLS characteristics (Orange et al., 2020). These cells were termed PRIME cells and were shown to become activated by B cells several weeks before RA relapse and to migrate to the synovial tissue and cause local inflammation (Orange et al., 2020).

In addition to FLS, in human synovial tissue, two main synovial tissue macrophage populations have been identified based on their expression of the tyrosine kinase receptor MerTK and their expression of the C-type lectin mannose receptor CD206 (Kurowska-Stolarska & Alivernini, 2022). Using different single-cell examination methodologies, these synovial tissue macrophages have been shown to contain distinct clusters that become enriched or diminished at different stages of RA development and progression (Alivernini et al., 2020). For example, in healthy synovium, the predominant subtype is the $MerTK^{+}$ $CD206^{+}$ subtype (Alivernini et al., 2020; Culemann et al., 2019). Additionally, it was found that remission RA synovium is characterised by higher levels of $MerTK^{+}$ $CD206^{+}$ synovial tissue macrophages (Alivernini et al., 2020), and the ratio of $MerTK^{+}$ $CD206^{+}$ to $MerTK^{-}$ $CD206^{-}$ cells can predict the persistence of remission when medications are discontinued in RA patients (Alivernini et al., 2020).

In RA, the majority of proinflammatory cytokines are produced by joint infiltrating monocytes, macrophages, and FLS. These cytokines include TNF, IL-1β, IL-6, and others. TNF has been shown to enhance inflammation by activating the NF-κB pathway, increasing TNF receptor 2 levels, and promoting the secretion of receptor activator of NF-κB ligand (RANKL) for osteoclast formation, among other proinflammatory functions (Jang et al., 2021; Marahleh et al., 2019). In a similar fashion, IL-1β has been shown to trigger rapid and strong proinflammatory responses, such as encouraging the proliferation of FLS and the

production of IL-6, IL-8, GM-CSF, collagenase, and prostaglandins, as well as promoting the expression of adhesion molecules in FLS and endothelial cells (Gabay et al., 2010). Both TNF and IL-1ß are highly produced by human monocytes following activation, and the distribution of circulating monocyte populations has been shown to change in RA, leading to an increase in intermediate monocytes, primarily due to higher CD16 expression across all monocyte subtypes (Lacerte et al., 2016). It has been shown that in both early and long-standing RA patients, $CD14^{++}$ classical monocytes show elevated CD16 expression (Cooper et al., 2012). Notably, variations in monocyte populations are evident when comparing active disease and remission in RA, with a decrease in intermediate monocyte populations during remission (Radwan et al., 2016). Given that CD16 functions as a coreceptor to TLR4, the increased expression of CD16 likely results in an enhanced induction of the proinflammatory cytokine cascade upon TLR4 activation. This proposition is supported by prior studies indicating $CD16^{+}$ monocytes as the main producers of TNF in response to LPS (Cros et al., 2010). Additionally, intermediate monocytes exhibit higher TLR2 expression compared to classical monocytes (Iwahashi et al., 2004). The increase in TLR2 expression can then contribute to heightened TNF responses to endogenous ligands in the joint (Iwahashi et al., 2004). Changes in receptor expression occur alongside distinct alterations in gene expression in RA monocytes. The modified transcriptional profile is often linked to TNF (Smiljanovic et al., 2012). Several changes in monocytes are also observed in signalling pathways, notably in the PI3K-mammalian target of rapamycin (mTOR) pathway. The significance of this pathway in RA was demonstrated through the inhibition of mTOR in monocytes isolated from RA patients, resulting in reduced osteoclast production and, hence, reduced bone erosion (Cejka et al., 2010).

1.4.2 Current RA treatment strategies

If left untreated, RA is an extremely debilitating disease with a drastic decrease in the quality of life of patients, leading to chronic disabilities. There is no cure for RA, however, early diagnosis and initiation of treatment has been shown to reduce joint destruction and disability and to promote a higher chance for clinical remission to occur (Moura et al., 2015). It has been shown that patients

receiving delayed therapy are at a higher risk of bony erosions (Finckh et al., 2006), with poorly controlled RA patients presenting with erosive changes predictive of poorer functional outcomes within two years of initial symptoms. Based on these observations, the established treatment principles for RA involve immediate symptomatic management and disease treatment modification, commonly known as the treat-to-target approach. In this widely used treatment methodology, disease progress is measured rigorously, and therapy is administered as appropriate until remission is achieved (Klarenbeek et al., 2011). As our scientific understanding of RA pathogenesis has increased, new and effective therapeutic drugs have been developed over the last three decades. These drugs are termed disease-modifying antirheumatic drugs (DMARDs), and they have significantly reduced joint inflammation and bone destruction in RA patients (Smolen et al., 2023). The current DMARDs mainly fall into three categories: conventional synthetic DMARDs (csDMARDs), biological DMARDs (bDMARDs), and targeted synthetic DMARDs (tsDMARDs) (Smolen et al., 2023). Conventional synthetic DMARDs include anti-inflammatory drugs such as Methotrexate, Sulfasalazine and Hydroxychloroquine; biological DMARDS include antibodies targeting prominent proinflammatory cytokines such as TNF and IL-6; targeted synthetic DMARDs include inhibitors of the Janus kinase (JAK)- Signal transducer and activator of transcription (STAT) signalling pathway.

As a first line of treatment upon RA diagnosis, a combination of methotrexate and glucocorticoids successfully leads to remission in 25% of patients within 6 months, a success rate which isn’t surpassed by using biological DMARDs and methotrexate instead (Nam et al., 2014; Smolen et al., 2017). This observation has so far led to the consensus that currently, all newly diagnosed RA patients are started on the methotrexate plus glucocorticoid treatment before considering biological DMARDs or targeted synthetic DMARDs due to an unwarranted increased price of treatment without any increased benefit in disease modification (Kavanaugh et al., 2013) and the possibility of overtreatment in 25% of newly diagnosed patients. Albeit the undeniable and crucial success of DMARD treatment, genetic and environmental factors are thought to contribute to resistance to DMARD therapy in between 30% to 50% of patients (Smolen et al., 2018) hence, even with a continuous treat-to-target approach, about half of treatment responders suffer disease relapse shortly after

treatment termination (Smolen et al., 2018). This highlights the need for a better understanding of RA pathology and disease progression in order for better standalone and/or complementary RA therapies to be developed.

1.5 Hypothesis and Aims

Despite the extensive research that has been carried out on innate immune memory, a lot remains unknown about the DAMP-mediated tolerisation of myeloid cells since the majority of tolerisation experiments are carried out using the PAMP LPS. Although understanding LPS-mediated endotoxin tolerance is crucial for infectious disease treatment and sepsis patients, to fully understand the pathogenesis of chronic inflammatory conditions such as RA, cellular stimulations need to be examined using disease-relevant DAMPs instead. For example, most research on a crucial aspect of TLR-mediated chronic inflammation, namely the NLRP3 inflammasome, is carried out exclusively using PAMPs. In order to further the scientific understanding of endogenous inflammatory triggers crucial to the development of autoimmunity, I have used the proinflammatory TLR4 activating domain, FBG-C, of the RA-relevant ECM protein tenascin-C. Specifically, I have hypothesised that the DAMP FBG-C is capable of activating the NLRP3 inflammasome in human monocytes, but that, unlike the PAMP LPS, its ability to tolerise the secretion of the proinflammatory cytokine IL-1ß is altered due to a delayed upregulation of the metabolite itaconate. I have also hypothesised that additional environmental stressors, such as joint hypoxia, can alter the inflammasome tolerance phenotypes conferred by both DAMPs and PAMPs, contributing to RA disease pathogenesis.

This work specifically aims to:

- Characterise the temporal differences between the secretion profiles of the proinflammatory cytokines TNF and IL-1ß in human monocytes when activated by FBG-C or LPS.
- Investigate the ability of FBG-C to activate the alternative inflammasome pathway in human monocytes and whether caspase 8 inhibition can block its activation.

- Investigate the ability of FBG-C to prime the canonical NLRP3 inflammasome in human monocytes and examine the requirement for the TLR4 co-receptor CD14 when cells are primed with either FBG-C or LPS.
- Determine whether FBG-C and LPS can tolerise the NLRP3 inflammasome to repeated TLR4 activation in human monocytes and whether the tolerisation is dependent on the immunometabolite itaconate and its enzyme ACOD1.
- Investigate the role of Gasdermin D and pyroptosis in the establishment of itaconate mediated NLRP3 inflammasome tolerance.
- Investigate the effects of hypoxia on NLRP3 inflammasome activation and tolerisation in human monocytes activated by either FBG-C or LPS.
- Explore whether monocytes from RA patients exhibit a reduced ability to tolerise the NLRP3 inflammasome compared to those from healthy donors and investigate the potential involvement of itaconate.

Chapter 2 Materials and Methods

2.1 Materials

All materials used in the experiments presented in this thesis are shown in Table 2.1.

Table 2.1 List of all reagents and resources used, along with source and identifier.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Tetra·His Antibody, BSA-free	Qiagen	Cat# 34670; **RRID:AB_2571551**
Goat anti-Mouse IgG (H + L)-HRP Conjugate	Bio-Rad	Cat# 1706516; **RRID:AB_11125547**
PE/Cyanine5 mouse anti-human CD3 Antibody	BioLegend	Cat# 317356; **RRID:AB_2904343**
Brilliant Violet 605™ mouse anti-human CD14 Antibody	BioLegend	Cat# 301834; **RRID:AB_2563798**
PE mouse anti-human CD16 Antibody	BioLegend	Cat# 360704; **RRID:AB_2562749**
Brilliant Violet 510™ mouse anti-human CD19 Antibody	BioLegend	Cat# 302242; **RRID:AB_2561668**
Brilliant Violet 510™ mouse anti-human CD56 (NCAM) Antibody	BioLegend	Cat# 318340; **RRID:AB_2561944**
FITC mouse anti-human CD45 Antibody	BD Biosciences	Cat# 555482; **RRID:AB_395874**
Rabbit anti-human IRG1 (D6H2Y) Antibody	Cell Signaling Technology	Cat# 77510; **RRID:AB_2799901**
Rabbit anti-β-Actin (13E5) Antibody (HRPConjugate)	Cell Signaling Technology	Cat# 5125; **RRID:AB_1903890**
Rabbit anti-human Gasdermin D (E9S1X) Antibody	Cell Signaling Technology	Cat# 39754; **RRID:AB_2916333**
Rabbit anti-human IRF-1 (D5E4) Antibody	Cell Signaling Technology	Cat# 8478; **RRID:AB_10949108**
Goat Anti-Rabbit Immunoglobulins/HRP	Agilent	Cat# P0448; **RRID:AB_2617138**
Biological samples		
Human leucocyte cones	NHS Blood and Transplant, Newcastle	N/A
Fresh human blood from healthy individuals	Sir Greame Davies Building, Glasgow	N/A
Fresh human blood from indviduals with RA	Gartnavel General Hospital, Glasgow	N/A
Commercial assays		
PyroGene™ Recombinant Factor C Endpoint Fluorescent Assay	Lonza	Cat# 50-658U
EasySep™ Human Monocyte Isolation Kit	Stemcell Technologies	Cat# 19359
LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells	Invitrogen	Cat# L3224
Human TNF alpha Matched Antibody Pair ELISA kit	Invitrogen	Cat# CHC1753
Human IL-1 beta Matched Antibody Pair ELISA kit	Invitrogen	Cat# CHC1213
Griess Reagent System	Promega	Cat# G2930
PureLink™ Genomic DNA Mini Kit	Invitrogen	Cat# K182001
T7 Endonuclease I surveyor kit	New England Biolabs	Cat# M0302S
Mix2Seq Kit NightXpress	Eurofins Genomics	N/A
Chemicals, pepties, recombinant proteins and culture media		
Recombinant Human M-CSF	PeproTech	Cat# 300-25
Lysogeny broth powder (Luria Broth Base)	Invitrogen	Cat# 12795084
Carbenicillin disodium salt	Sigma-Aldrich	Cat# C1389
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	Cat# I5502
Urea powder	Sigma-Aldrich	Cat# U5378
Tris	Sigma-Aldrich	Cat# T3253
HisPur™ Ni-NTA Superflow Agarose	Thermo Scientific	Cat# 25215
Triton™ X-114	Invitrogen	Cat# AC422365000
Imidazole	Sigma-Aldrich	Cat# I202
Cystamine dihydrochloride	Thermo Scientific Chemicals	Cat# B22873.22
β-mercaeptoethanol	Gibco	Cat# 31350010
2-hydroxyethyldisulphide	Sigma-Aldrich	Cat# 380474
Precision Plus Protein Dual Color Standards	Bio-Rad	Cat# 1610374
Coomassie Brilliant Blue	Thermo Scientific	Cat# 20279
Sodium chloride crystals	Thermo Scientific Chemicals	Cat# 194090010
Acrylamide/Bis-acrylamide	Sigma-Aldrich	Cat# A3699
Ammonium persulfate	Sigma-Aldrich	Cat# 248614
Tetramethylethylenediamine (TEMED)	Thermo Scientific	Cat# 17919
Sodium dodecyl sulfate	Sigma-Aldrich	Cat# L4509
Glycine	Sigma-Aldrich	Cat# G7126

Glycerol	Sigma-Aldrich	Cat# G5516
Bromophenol Blue	Sigma-Aldrich	Cat# B0126
Methanol	Sigma-Aldrich	Cat# 5895961000
Acetic acid	Sigma-Aldrich	Cat# A6283
Tween 20	Sigma-Aldrich	Cat# P1379
Sodium thiosulfate	Sigma-Aldrich	Cat# 217263
Silver nitrate	Sigma-Aldrich	Cat# S6506
Sodium carbonate	Sigma-Aldrich	Cat# 223530
Formalin solution	Sigma-Aldrich	Cat# HT501128
Bovine Serum Albumin	Sigma-Aldrich	Cat# A9418
Ficoll-Paque PLUS density gradient media	Cytiva	Cat# 17144003
RBC Lysis Buffer	Invitrogen	Cat# 00-4333-57
EDTA (0.5 M), pH 8.0, RNase-free	Invitrogen	Cat# AM9260G
Fetal Bovine Serum	Sigma-Aldrich	Cat# F9665
Trypan Blue Solution, 0.4%	Gibco	Cat# 15250061
RPMI 1640 Medium (Dutch modification)	Gibco	Cat# 22409015
Penicillin-Streptomycin	Gibco	Cat# 15070063
GlutaMAX™ Supplement	Gibco	Cat# 35050061
Sodium Pyruvate	Gibco	Cat# 11360070
Lipopolysaccharides from Escherichia coli O55:B5	Sigma-Aldrich	Cat# L4524
Polymyxin B solution	Sigma-Aldrich	Cat# 92283
RIPA Lysis and Extraction Buffer	Thermo Scientific	Cat# 89900
Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free	Thermo Scientific	Cat# 78441
Nigericin sodium salt	InvivoGen	Cat# tlrl-nig
Caspase-8 Inhibitor II	Sigma-Aldrich	Cat# 218759
TMB Solution (1X)	Invitrogen	Cat# 00-4201-56
Pierce™ Bradford Protein Assay Kit	Thermo Scientific	Cat# 23200
NuPAGE™ Bis-Tris Mini Protein Gels, 4–12%, 1.0–1.5 mm	Invitrogen	Cat# NP0321BOX
iBlot™ 2 Transfer Stacks, PVDF, regular size	Invitrogen	Cat# IB24001
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific	Cat# 34579
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific	Cat# 34094
NuPAGE™ Sample Reducing Agent	Invitrogen	Cat# NP0004
Gel Loading Buffer II	Invitrogen	Cat# AM8547
NuPAGE™ MOPS SDS Running Buffer	Invitrogen	Cat# NP0001
FcR Blocking Reagent, human	Miltenyi Biotec	Cat# 130-059-901
Fixable Viability Dye eFluor™ 780	Invitrogen	Cat# 65-0865-14
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	BD Biosciences	Cat# 552843
Cytofix™ Fixation Buffer	BD Biosciences	Cat# 554655
DreamTaq Green PCR Master Mix	Thermo Scientific	Cat# K1081
TAE (10X), RNase-free	Invitrogen	Cat# AM9869
SYBR™ Safe DNA Gel Stain	Invitrogen	Cat# S33102
Software and algorithms		
FlowJo™ v10.10	FlowJo	https://www.flowjo.com/
Prism v10.2.3	GraphPad	https://www.graphpad.com/
BD FACSDiva™ Software v9.0	BD Biosciences	https://www.bdbiosciences.com/
Biorender	Biorender	https://www.biorender.com/

2.2 Methods

In-house made buffers were prepared as follows:

TN buffer: 50 mM Tris, 150 mM NaCl, pH 8.0

Urea-Tris buffer: 8 M urea, 20 mM Tris, pH 8.0

4% Stacking gel: 125 mM Tris, 4% acrylamide/bis-acrylamide, 0.1% (w/v) ammonium persulfate, 0.1% (v/v) Tetramethylethylenediamine (TEMED), pH 6.8

12% Separating gel: 375 mM Tris, 12% acrylamide/bis-acrylamide, 0.1% (w/v) ammonium persulfate, 0.1% (v/v) TEMED, pH 8.8

4x Separating gel buffer: 1.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8

4x Stacking gel buffer: 0.5 M Tris-HCl, 0.4% (w/v) SDS, pH 6.8

10x Running buffer: 248 mM Tris, 1.92 M glycine, 1% (w/v) SDS

2x Loading buffer: 125 mM Tris, 4.1% (w/v) SDS, 20% (v/v) glycerol, 5% (v/v)

mercaeptoethanol, 0.001% (w/v) bromophenol blue, pH 6.8

CBB staining solution: 0.1% (w/v) Coomassie Brilliant Blue G-250, 50% (v/v) methanol, 20% (v/v) acetic acid

De-staining solution: 30% (v/v) methanol, 1% (v/v) formic acid

TBST: 1x TBS, 0.1% (v/v) Tween 20

FC buffer: 1x PBS, 0.5% (w/v) bovine serum albumin, 2mM Ethylenediaminetetraacetic acid (EDTA)

Cell separation buffer: 1x PBS, 2mM EDTA, 1% (v/v) heat Inactivated Foetal Bovine Serum

Assay buffer: 1x PBS, 0.1% (v/v) Tween 20, 0.5% (w/v) bovine serum albumin

Wash Buffer: 1x PBS, 0.05% (v/v) Tween 20

2.2.1 FBG-C protein production and purification

Carbenicillin resistant E. coli strain BL21 expressing human His-tagged FBG-C was used to inoculate 10 mL of Lysogeny Broth (LB) (Invitrogen) with 250 mg/mL of carbenicillin (Merk). The culture was left to incubate overnight at 37°C with shaking at 200 RPM. The next day, the overnight culture was added to 1 L of LB/carbenicillin and incubated at 37°C with shaking at 200 RPM until the OD600nm reached 0.6 AU (measured using a 1 mL sample in a quartz glass cuvette). Isopropyl ß-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) at a final concentration of 1 mM was added to induce FBG-C expression for 4 hours at 37°C with shaking at 200 RPM. The bacterial culture was subsequently centrifuged at 3500 RPM for 15 minutes; the pellet was collected, washed with 20 mL of protein buffer (referred to as Tenascin-C buffer - TN buffer), repleted by 3500 RPM for 15 minutes centrifugation and stored at -20°C for further processing. The bacterial pellet from a 0.5 L culture was thawed, re-suspended in 20 mL of TN buffer, and subjected to probe sonication using Q125 Sonicator Ultrasonic Homogenizer (four times for 30 seconds, amplitude 30%). The insoluble fraction was then collected by centrifugation at 10000 RPM for 15 minutes at 4°C. The pellet was re-suspended in 40 mL of urea-Tris buffer through brief sonication (as described above) and then left to incubate overnight at 4°C with constant rotation. The following day, the solution was centrifuged again, passed through a 0.8 µm syringe filter, and then passed twice through a column containing 5 mL of nickel beads (Life Technologies) that had been equilibrated with 50 mL of urea-Tris buffer. The flow-through was collected and the beads were washed with 50 mL of urea-Tris buffer, followed by ice-cold 125 mL urea-Tris with 0.1% (v/v) Triton X-114 (Invitrogen), and finally 50 mL of ice-cold urea-Tris buffer. The protein was eluted from the column with urea-Tris containing 300 mM imidazole (Sigma-Aldrich). Five fractions of 5 mL were collected, and the protein concentration was determined by measuring absorbance at 280 nm using a NanoDrop Microvolume Spectrophotometer (Thermo Scientific). Fractions containing protein were combined and diluted to 0.5 L with urea-Tris buffer. To achieve appropriate protein refolding Cystamine (Thermo Scientific Chemicals) was added to a concentration of 20 mM, and the protein was dialyzed at 4°C against 2 L of TN buffer supplemented with 5 mM ß-mercaeptoethanol (Gibco) and 1 mM 2-hydroxyethyldisulphide (Sigma-Aldrich) for two days using cellulose

dialysis tubing retaining proteins with a molecular weight higher than 12 kDa, followed by 2 L of pure TN buffer for another two days with daily buffer changes. The refolded protein was then applied to 1.2 mL of nickel beads in a column that had been equilibrated with 50 mL of TN buffer. The beads were washed with 125 mL of ice-cold TN buffer with 0.1% (v/v) Triton X-114, and then with 100 mL of ice-cold TN buffer. The protein was eluted with TN buffer supplemented with 300 mM imidazole. Five fractions of 1 mL were collected, and the protein concentration was again measured using NanoDrop (as described above). The efficiency of purification was assessed using SDS-PAGE. Fractions containing the protein were dialyzed twice against 2 L of TN buffer for 2 hours and overnight at 4°C to remove imidazole using cellulose dialysis tubing retaining proteins with a molecular weight higher than 12 kDa. The purified protein was then stored at -80°C.

2.2.2 SDS-PAGE and Coomassie staining

Proteins were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis on custom-made 12% polyacrylamide gels containing 10 wells. The gels were inserted into a gel tank (ATTO) filled with running buffer. The protein samples were combined with an equal volume of 2x Loading buffer and then heated and denatured at 95°C for 5 minutes. Each well was loaded with either 10-20 µL of protein samples or 6 µL of pre-stained protein ladder (Bio-Rad). Electrophoresis was conducted at 130 V until the visible bromophenol blue line reached the bottom of the gel. To visualize protein bands post SDS-PAGE, the gels were exposed to a Coomassie Brilliant Blue staining solution for 30 minutes at room temperature with moderate agitation. To remove background staining, the gels were treated with de-staining solution for 5 minutes.

2.2.3 Silver staining

1 µg of purified FBG-C was separated via SDS-PAGE. The gels were fixed with a solution of 50% methanol and 5% acetic acid for 1 hour at room temperature, followed by overnight incubation at 4°C. Subsequently, the gels underwent a 10-

minute wash in 50% methanol and two 10-minute washes in water. The gels were treated with 0.02% (w/v) sodium thiosulfate (Sigma-Aldrich) for 1.5 minutes and washed twice in water for 1 minute. Silver staining was performed by immersing the gels in 0.1% (w/v) silver nitrate (Sigma-Aldrich) for 1 hour at 4°C. After a 1-minute wash in water, the gels were developed by incubating in 2% (w/v) sodium carbonate (Sigma-Aldrich) and 0.04% (v/v) formalin (Sigma-Aldrich) until bands were clearly visible. The reaction was stopped by incubating in 5% acetic acid for 5 minutes.

2.2.4 Western blotting for FBG-C detection

1 µg of purified FBG-C was separated via SDS-PAGE and then transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (BioRad) as per the manufacturer's guidelines. The membrane was blocked for 1 hour with 5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) in TBST and subsequently incubated overnight at 4°C with mouse Tetra-His antibody (Quiagen) at a 1:10,000 dilution in 2% (w/v) BSA in TBST. The membrane was then washed three times with TBST for 10 minutes and incubated with anti-mouse IgG-HRP antibody (Bio-Rad) at a 1:20,000 dilution in 2% (w/v) BSA in TBST for 1 hour, followed by three 10-minute TBST washes. All incubation and washing steps were carried out with vigorous agitation of the membrane. Chemiluminescence was used to visualize protein bands with Amersham enhanced chemiluminescence (Merk) and the bands were captured on X-ray film (Thermo Scientific).

2.2.5 Circular dichroism spectroscopy and LPS contamination testing

J-815 Circular Dichroism (CD) Spectrometer (Jasco) was employed to analyse the secondary structure of the refolded FBG-C protein following production and purification. Spectra were obtained in the 190 nm to 250 nm range at 20ºC. Each sample was measured 5 times, and the averages were plotted using GraphPad Prism software v10.2.3. LPS contamination of produced FBG-C was assessed using

the Recombinant Factor C Assay (Lonza) following the manufacturer's instructions. Only samples with less than 10 pg/mL of LPS were used in subsequent experiments.

2.2.6 Monocyte isolation from human PBMCs

Human monocytes were isolated from healthy donor or RA patient donor blood in accordance with the terms of the informed consents under an approved protocol, or from leukocyte cones obtained from NHS Blood and Transplant, Newcastle. Peripheral blood from patients with RA was collected at the Gartnavel General Hospital Rheumatology unit Glasgow, while healthy controls were obtained from volunteers at the University of Glasgow. RA patient blood sample use was under the following ethics approval: West of Scotland REC 4 approval 14/WS/1035, project reference: CG_2021_17_A. Healthy donor blood samples were used under approval from The College of MVLS Ethics Committee. All donor information was treated anonymously. Peripheral blood mononuclear cells (PBMCs) were separated from human blood by density gradient centrifugation using Ficoll Paque Plus (Cytiva). Leukocyte cone samples were diluted at a 1:1 ratio with PBS, while fresh blood was used without dilution. 25 mL of blood was layered onto 20 mL of Ficoll-Paque PLUS in 50 mL centrifuge tubes (Corning). The layered blood samples were centrifuged at 400g for 30 minutes without brake at room temperature. Using a Pasteur pipette, the PBMC layer was transferred into a fresh 50 mL centrifuge tube. To eliminate residual red blood cell (RBC) contamination of the PBMCs, RBC lysis was performed by resuspending the cells in 1x RBC lysis buffer (Invitrogen) for 3 minutes. Following RBC lysis, the PBMCs were washed twice with 50 mL of sterile PBS. The cells were then resuspended in 10 mL of cell separation buffer. 5 µl of the resuspended PBMCs were mixed with 45 µl of Trypan Blue (Gibco), and 10 µl of the mixture was added to a haemocytometer and counted using a light microscope. To achieve the desired concentration, a final wash of the PBMCs was done with 50 mL of sterile PBS to allow resuspension of the cells. The cells were resuspended at the desired concentration in cell separation buffer, ready for $CD14^+$ monocyte isolation. Human monocytes were isolated from donor PBMCs using human monocyte negative isolation kit with CD16 depletion (EasySep™

Human Monocyte Isolation Kit - StemCell technologies) following the manufacturer's instructions. The isolated $CD14^+$ monocytes were resuspended in RPMI 1640 - Dutch modification (Gibco) medium supplemented with 1% Penicillin-Streptomycin (Gibco), 2 mM L-Glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 10% Heat Inactivated Foetal Bovine Serum (Sigma-Aldrich) and, for culture experiments longer than 2 days, 25 ng/mL recombinant human M-CSF (PeproTech) at 1×10^5 cells/mL. Cells were plated in a TC treated 96 well plate (Corning) and maintained in a humidified incubator at 37°C, 5% CO2 awaiting activation. In all cell culture experiments, three wells were used as technical replicates for each treatment condition. The number of independent donor samples used (biological replicates) was different depending on the experimental design and is therefore specified where appropriate in the figure legends of the figures presented in the results chapters.

2.2.7 Monocyte activation and measurement of pyroptosis levels

Human monocytes were rested for 1-hour post-isolation before activation with LPS (E. coli O55:B5, Sigma – Aldrich) at different concentrations as specified in each experiment, or 1 µM FBG-C. In all cases of FBG-C stimulation, the stimuli were pre-incubated for 30 min with 10 µg/mL polymyxin B (PMB) on ice, as a control for a possible LPS contamination. Following monocyte activation, after a specified amount of time depending on the experimental design, medium was collected and stored at -80°C for subsequent investigation. In certain experiments, cells were lysed using RIPA buffer (ThermoFisher) with a protease/phosphatase inhibitor cocktail (10ul/mL) (ThermoFisher) and lysates were stored at -20°C for subsequent investigation. For tolerisation experiments, following primary activation, cells were washed and then rested in fresh media for 1 hour before re-stimulation with LPS O55:B5 at 10 ng/mL. After a specified amount of time of secondary stimulation dependent on the experimental design, culture medium was again collected and stored at -80°C, or cells were triggered for NLRP3 inflammasome activation with 5 mM nigericin (InvivoGen) for 45 minutes before media collection and RIPA buffer cell lysis. In specific

experiments instead of cell lysis, pyroptosis was measured using cell staining LIVE/DEAD Viability/Cytotoxicity kit (invitrogen) as per manufacturer's instructions and pyroptosis was observed using a fluorescence microscope (EVOS M7000 Imaging System).

2.2.8 Cell culture in 2% oxygen

For the induction of 2% O_2, an incubator with the ability to regulate and maintain O_2 levels was used (Fisher scientific). To reduce the level of oxygen in the incubator, a steady stream of nitrogen gas from a nitrogen cylinder (BOC) was pumped into the incubator's atmosphere at a carefully monitored rate until a 2% O_2 environment was achieved. Cell culture plates were placed inside the incubator for a variable amount of time dependent on the specific experiment.

2.2.9 Generation of primary human monocyte knock-out cells

To form guide-Cas9 Ribonucleoproteins (RNPs), the RNA guides with the following sequence were synthesised by and purchased from Synthego:

Gene: ACOD1

single guide RNA Sequences:

UUUCAAGCCAUGGAUUGCUG

GAGGAUGAUUCUAGACACUC

CAUAGCCAGCCAAUAUAGCA

Gene: CD14

single guide RNA Sequences:

CCUCUACUGCAGACACACAC

CGGAGAAGUUGCAGACGCAG

GUCGCAGAGACGUGCACCAG

The guides were mixed with Cas9 (SpCas9 2NLS Nuclease; Synthego) at a molar ratio of 5:1 (guide:Cas9) in an RNase-free low bind tube and incubated at room temperature for 30 minutes for the RNPs to complex. At the end of the 30 minutes incubation, electroporation buffer (Lonza) was mixed with the RNPs. The electroporation/RNPs buffer mix was used to resuspend a human monocyte cell pellet generated immediately after cell isolation via centrifugation at 500g for 5 minutes. The cell suspension was then immediately transferred to a 100 µl electroporation cuvette (Lonza). Induction of the RNPs into the primary human monocytes was achieved by electroporation using the Amaxa 3D Nucleofector (Lonza). The cells were electroporated using the CM137 pulse code in the Amaxa 3D Nucleofector. After the successful electroporation, cells were immediately transferred in culture medium for plating and incubation.

2.2.10 Cytokine ELISA

After monocyte activation, cell culture plates underwent centrifugation at 300 g for 5 minutes at room temperature to settle cells and debris at the bottom of the plate. The supernatants were carefully transferred to a clean 96-well plate, sealed with Parafilm, and stored at -80°C until needed. For single cytokine analysis, Enzyme-Linked Immunosorbent Assay (ELISA) kits were used to detect levels of TNF and IL-1ß (Invitrogen) in cultured samples. Recombinant human cytokines were employed to create standard curves, with a lower detection limit of 15.6 pg/mL. To analyse the cytokines, samples were diluted using assay buffer. The ELISAs were performed according to the manufacturer’s instructions specific to the cytokine being analysed. In brief, flat bottom high-binding plates (Corning) were coated with 50 µl of capture antibody per well diluted in PBS and incubated overnight at 4°C. The ELISA plates were washed with 300 µl of wash per well and then blocked with 150 µl of assay buffer for 1 hour at room

temperature. Samples and standard dilutions were prepared and 50 µl of each were added per well, with both standards and samples replicated in duplicates. The samples were incubated with 25 µl of detection antibody per well diluted in assay buffer for 2 hours at room temperature with continual shaking. The ELISA plate was washed 5 times with wash buffer before 50 µl of streptavidin-HRP solution diluted in assay buffer was added per well and incubated for 30 minutes at room temperature with continual shaking. After washing the ELISA plate 5 times with wash buffer, 50 µl of TMB substrate (Invitrogen) was added and incubated for up to 30 minutes at room temperature with continual shaking until the standard curve had developed. Once the standard curve had developed or 30 minutes had passed, the reaction was stopped using stop solution (2N sulfuric acid). The absorbance was measured at 450nm on an ELISA plate reader within 15 minutes after adding the stop solution. The concentration of the cytokine was obtained from the standard curve produced by the machine software.

2.2.11 Western blotting for the investigation of intracellular protein levels

Following cell culture and activation, cells were lysed on ice using RIPA buffer (Thermo Scientific) with a protease/phosphatase inhibitor cocktail (10ul/mL). Protein quantification was then carried out using a Bradford assay (Thermo Scientific) to normalise protein concentration between the samples. The normalised protein samples were then mixed with gel loading buffer (Invitrogen) and sample reducing agent (Invitrogen) at the appropriate concertation and denatured at 95ºC for 5 minutes. The samples were loaded into NuPAGE pre-cast 4-12% Bis-Tris gels (Invitrogen) equilibrated with NuPAGE® MOPS SDS running buffer (Invitrogen) and placed into a gel tank. Electrophoresis was conducted at 120 V until samples migrated to the bottom of the gel. Proteins were transferred to Polyvinylidene fluoride (PVDF) membranes (Invitrogen) using the iBlot 2 dry transfer system (Invitrogen) as per manufacturer's instructions and subsequently blocked with TBST containing 5% (w/v) non-fat dried milk for one hour and then incubated overnight at 4ºC with antibodies specific for the protein of interest or housekeeping control (namely IRG1 (Cell Signalling Technology), ß-actin (Cell

Signalling Technology), GSDMD (Cell Signalling Technology) or IRF1 (Cell Signalling Technology)) at a 1:1,000 dilution in TBST containing 5% (w/v) non-fat dried milk. On the next day the membranes were washed in TBST and incubated with an HRP-labelled secondary antibody (Dako) at a 1:2,000 dilution in TBST containing 5% (w/v) non-fat dried milk. After subsequent washing in TBST, membranes were incubated in West Pico substrate solution (Thermo Scientific) or West Femto substrate solution (Thermo Scientific) for 2 minutes and protein bands were visualised using an Azure Biosystems c500 Western Blot imaging system.

2.2.12 Flow cytometry

Flow cytometry was used to immunophenotype the isolated human monocytes immediately post-isolation. Cells were counted and plated into 96-well V-bottom polypropylene microtiter plates (Greiner). Cells were washed twice in PBS and stained with fixable live/dead dye (Invitrogen) for 10 minutes. Cells were washed twice in flow cytometry (FC) buffer and incubated with 2 µL of Fc Blocking Reagent (Miltenyi Biotec) per 10^6 cells for 10 minutes at RT. Samples and controls were then stained for extracellular markers with the appropriate surface antibody stains and fluorescence minus one (FMO) controls by adding anti-human CD3, CD14, CD16, CD19, CD45 and CD56 antibodies (BioLegend) (Table 2.1) directly to the cell suspensions at the manufacturers' recommended concentration. The cells were incubated with the antibody stains for 20 minutes at 4° in the dark before being washed twice in FC buffer. Following the last FC buffer wash, samples were resuspended in 100 µL fixation buffer (BD Biosciences) and incubated for 20 minutes at room temperature. Samples were washed once in FC buffer and resuspended in a final volume of 200 µL FC buffer until acquisition on the following day. On the day of acquisition fixed samples were acquired using the BD LSRFortessa™ cell analyzer (BD Biosciences), collecting 50,000 singlet events in each instance. Compensation matrices were generated using single-stained cells or compensation beads (BD Biosciences) and applied as required. Samples were visualised using dot plots and were gated on viable, single events, excluding cellular debris, doublets and dead cells. Positive gates for each marker were set using the appropriate FMO controls. Data were

batch analysed on the BD FACSDiva™ software v9.0 and exported as flow cytometry standard (FCS) files for further analysis on the FlowJo software v10.10.

2.2.13 Monocyte DNA extraction

DNA extraction and purification was performed using PureLink Genomic DNA Mini Kit extraction columns as per manufacturer's instructions (Invitrogen). Following extraction and purification, the DNA was quantified Using Nanodrop 2000, using the Nucleic Acid DNA programme, and conetrations were recorded in ng/µL.

2.2.14 Polymerase chain reaction and agarose gel electrophoresis

The PCR for *ACOD1* gene amplification was performed in a 50 µL reaction mix, comprising of: 25 µL DreamTaq Green PCR Master Mix (Thermo Scientific), 0.5 µL *ACOD1* forward Primer, 0.5 µL *ACOD1* reverse Primer, 19 µl of nuclease free water and 5 µL of the sample DNA template

Primers used in the PCR were customised and ordered from Integrated DNA Technologies (IDT) were with the following sequence:

Forward Primer: GCAACTGGGTTTCTTGTAGTGG

Reverse Primer: CTGTGCCTCACTGTGTATGGT

The PCR reaction was run on a thermocycler as follows:

Start-Up: 95 °C for 3 minutes

35 Cycles of: Denaturation 95 °C for 30 sec

Annealing 60 °C for 30 sec

Elongation 72 °C for 1 minute

Elongation Step: 72 °C for 1 minute

Expected amplicon size was 497 base pairs and following PCR 5 µL of the reactions was used to visualise the amplicons on 2% agarose gel. 2% agarose gel was prepared by adding 2 grams of agarose in 100 mL of 1X TAE buffer (Invitrogen), with 5 µl of SYBR™ Safe DNA Gel Stain (Invitrogen). 5 µL of the reaction suspension was added into wells of the 2% Agarose gel and electrophoresis was run at 140V for 40 minutes. The DNA bands were then visualised by exposure to UV light in a Universal Hood II Gel Doc System (BioRad).

2.2.15 T7 endonuclease surveyor assay

T7 endonuclease assay was performed on DNA from *ACOD1* KO and control monocytes using the T7 Endonuclease I kit from New England Biolabs as per manufacturer's instructions. The previously purified DNA was equalised to 200 ng per sample for the random annealing stage and master mixes were prepared as follows:

***ACOD1* control**: 7.8 µL of purified DNA (200ng DNA) + 2 µL of reaction buffer + 9.2 µL of nuclease free water (total of 19 µL annealing suspension)

***ACOD1* KO**: 16 µL of DNA purified (200 ng DNA) + 2 µL of reaction buffer + 1 µL of nuclease free water (total of 19 µL annealing suspension)

Random Annealing was then performed in a thermocycler as follows:

STEP	TEMPERATURE	RAMP RATE	TIME
Initial Denaturation	95°C		5 minutes
Annealing	95-85°C	-2°C/second	
	85-25°C	-0.1°C/second	
Hold	4°C		Hold

After the random annealing, 1 µL of the T7 endonuclease (New England Biolabs) was added to each sample, and digestion reaction was run by incubating the samples at 37 ºC for 15 minutes. Following T7 endonuclease digestion of the amplified sample DNA the fragments were run on a 2% agarose gel and visualised as described in the previous section (see section 2.2.14).

2.2.16 Sanger DNA sequencing and assessment of editing efficiency following gene knock-out

Following DNA extraction and purification, DNA samples from *ACOD1* KO and control monocytes were sent for Sanger sequencing to Eurofins Scientific, using the NightXpress Mix2Seq Kit barcoded tubes (Eurofins Genomics). The sequencing mix in each tube comprised of 2 µL of each purified DNA, 3 µL of nuclease free water and 5 µL of the sequencing primer. The *ACOD1* sequencing primer used had the following sequence: GTTTCTTGTAGTGGAGCAAAACTCTTTATG

Following Sanger sequencing, assessment of gene editing efficiency was done using Synthego's CRISPR analysis tool - Inference of CRISPR Edits analysis (ICE) following the instructions on:
https://www.synthego.com/products/bioinformatics/crispr-analysis

2.2.17 Computational processing of publicly available RNA-Seq data

RNA sequencing data for primary human monocytes left in culture for 24 hours without activation was obtained from a publicly available dataset published on the webpage of the National Center for Biotechnology Information (NCBI):
https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165998. The methodology of how this dataset was generated is outlined in the scientific publication from Hussain et al., 2022.

The Bioconductor R package DESeq2 (Love et al., 2014) was then used to normalise read counts and identify differentially expressed genes between sample groups. The normalised data was then presented using the ggplot2 R package. Statistical analysis, carried out using the DESeq2 R package, comparing

each culture condition to the control is done using the Wald test and the obtained p values are adjusted using the Benjamini and Hochberg method to account for multiple testing. An adjusted p value (padj) less than 0.05 was considered statistically significant.

2.2.18 Statistical analysis

Data analysis was performed using GraphPad Prism software v10.2.3. Multiple group datasets were analysed by one-way analysis of variance (ANOVA) followed by Tukey's Honestly-Significant Difference post-hoc test. Two-tailed paired or unpaired T-test was used for comparisons involving only two groups. P values lower than 0.05 were considered significant. For RNA sequencing data, statistical analysis was done using the R package DEseq2 which utilises the Wald test to obtain p values which are then adjusted using the Benjamini and Hochberg method to account for multiple testing. P adjusted values lower than 0.05 were considered significant.

Chapter 3 Comparing the proinflammatory properties of the DAMP tenascin-C and the PAMP LPS in human monocytes in the context of NLRP3 inflammasome activation and tolerance

3.1 Introduction

Within the innate immune system, several virulence factors have been shown to trigger memory phenotypes, which are modified responses of myeloid cells to subsequent stimuli after being exposed to an initial trigger such as bacterial LPS (see section 1.1.1). LPS is a large glycolipid that acts as a crucial component of the outer membrane in Gram-negative bacteria, comprises lipid A, core oligosaccharide, and O antigen. In the environment bacterial cells normally reside in, it forms a barrier, protecting them from harmful molecules such as antibiotics (Bertani & Ruiz, 2018). Lipid A is the most conserved region, and it strongly activates the host immune response via the surface receptor TLR4 and its co-receptor CD14 in human myeloid cells. Following TLR4 activation, immune cells release mediators such as the proinflammatory cytokines TNF and IL-6 (Alexander & Rietschel, 2001). Additionally, TLR4 activation primes myeloid cells for inflammasome activation by upregulating the production of different inflammasome components, as well as the immature forms of the cytokines IL-1ß and IL-18 (Liu et al., 2017). It was established many decades ago that repeated injection of LPS in rabbits desensitises these animals, characterised by a gradual decrease in febrile response to the re-challenge (Beeson & Roberts, 1947). Likewise, patients recovering from infectious diseases and volunteers exposed to pathogens showed reduced fever due to LPS administration (Cavaillon & Adib-Conquy, 2006). Furthermore, a notable decrease in cytokine production was observed in immune cells that were primed and then re-stimulated with LPS *in*

vitro, leading to a refractory state known as LPS or endotoxin tolerance (Butcher et al., 2018; Lehner & Hartung, 2002; O'Carroll et al., 2014)

While tolerance induced by pathogenic stimuli has been widely researched, much less is known about innate immune memory triggered by non-infectious stimuli such as DAMPs (see sections 1.1.3 and 1.1.4). Recently generated unpublished data from the Midwood lab has shown that the TLR4 activating domain FBG of the extracellular matrix protein tenascin-C is capable of tolerising the proinflammatory cytokines IL-6 and TNF in human myeloid cells. However, there are no studies to date on the effects of FBG-C on the production and secretion of the inflammasome regulated immune response cytokine IL-1ß in primary human monocytes. Moreover, the tolerisation of the primary effector complex responsible for IL-1ß maturation and secretion, namely the NLRP3 inflammasome, by either PAMPs or DAMPs in the context of primary human myeloid cells has largely remained unexplored (see section 1.2.2).

In this first part of my thesis, I explored the differences between the TNF and IL-1ß secretion profiles of primary human monocytes stimulated by either FBG-C or LPS over the course of a 24-to-48-hour period, typical for the lifespan of activated classical human monocytes. Additionally, I investigated whether FBG-C and LPS are capable of tolerising the NLRP3 inflammasome dependent secretion of IL-1ß in primary human monocytes.

3.2 Results

3.2.1 Production, purification and activity testing of endotoxin free FBG-C

Neither whole tenascin-C, nor its TLR4 binding domain FBG-C were commercially available for purchase when I initiated my studies, hence production of bulk quantities of FBG-C was required. The Midwood lab at the Kennedy institute of Rheumatology at the University of Oxford has established expertise in the production and validation of endotoxin free FBG-C (Midwood et al., 2009; Piccinini et al., 2016). Thus, I spent 9 months at the Kennedy institute working

with different members of the Midwood group to acquire the skill to produce and purify research grade FBG-C.

The sequence of the FBG-C domain of Tenascin-C had previously been cloned into an expression vector incorporating an N-terminal hexa-histidine tag and over-expressed in *E. coli* (Midwood et al., 2009). This clone was used for the expression of recombinant FBG-C that was subsequently purified from the inclusion body fraction of *E. coli* in a three-step protocol using immobilized metal affinity chromatography with nickel resin (Figure 3.1; methods section 2.2.1) (Midwood et al., 2009). All chromatography steps included a Triton X-114 wash to remove LPS contamination (Zimmerman et al., 2006). To ensure produced FBG-C quality, purity and functionality, quality control testing was carried out at each step of the purification and after each separate protein preparation, followed by a functional activity test and a recombinant factor C endotoxin contamination test (see methods chapters 2.2.1 to 2.2.5).

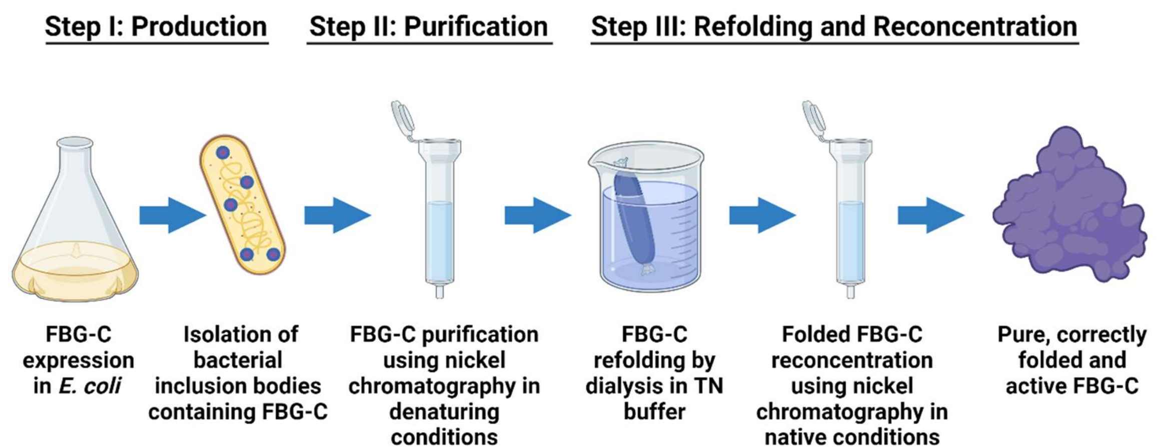


Figure 3.1 FBG-C protein preparation strategy. FBG-C domains containing hexa-histidine tags were expressed in *E. coli* strain BL21, bacterial pellet was sonicated, and inclusion body fraction of the cells was isolated by centrifugation (Step I). Next, inclusion bodies were dissolved in urea buffer and FBG-C was purified using nickel chromatography in denaturing conditions (Step II). Eluted FBG-C was then diluted and refolded by dialysis in TN buffer. Finally, the produced FBG-C was reconcentrated using nickel bead chromatography in TN buffer, yielding pure and active protein (Step III). Figure was generated using BioRender.

The first quality control step requires the assessment of the efficiency of FBG-C purification at the two stages of nickel column binding, namely following

recombinant protein expression and bacterial lysis, and following protein refolding and reconcentration. The assessment of FBG-C purification showed that the hexa-histidine tagged protein was adequately binding to the nickel bead column, as evident by the lack of FBG-C in any of the samples taken from the urea or native buffer washes, followed by a strong band at the correct FBG-C size of 25 kDa in samples taken at the stage of imidazole elution (Figure 3.2).

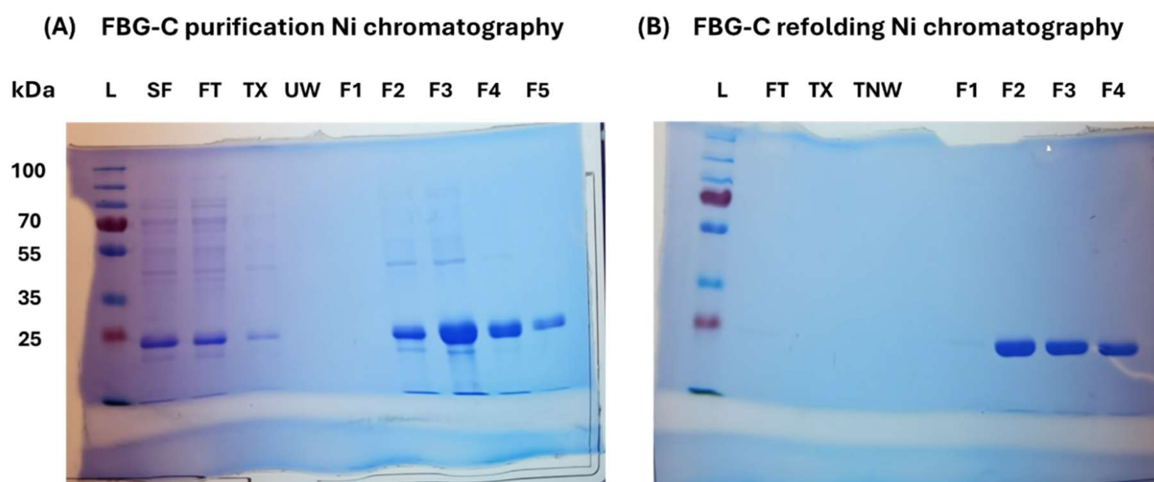


Figure 3.2 FBG-C purification protocol assessment. The efficiency of the FBG-C Nickel purification **(A)** and refolding **(B)** chromatography protocol was assessed using SDS-PAGE and subsequent Coomassie staining of 10 µL samples taken from the soluble lysate fraction (SF), column flow through (FT), urea wash (UW), Triton X-114 wash (TX) and Tris-NaCl wash (TNW). L stands for known size protein ladder mix. The designated imidazole FBG-C elution fractions are given the abbreviations F1 to F5 (fraction 1 to 5). FBG-C is detected at the size of 25 kDa.

The next quality control step aimed to investigate the purity of the expressed recombinant FBG-C protein. Protein purity was tested using a highly sensitive silver stain assay (see methods section 2.2.3) and the protein identity was confirmed through western blotting using an anti-histidine antibody (Figure 3.3). The silver stain showed no detectable contamination in any of the protein samples taken from the different protein preparations, as the only visible bands appeared at the correct FBG-C monomer size of 25 kDa and at the FBG-C homodimer size of 55 kDa. Similarly western blotting with an anti-histidine antibody further confirmed the identity of the produced protein (Figure 3.3).

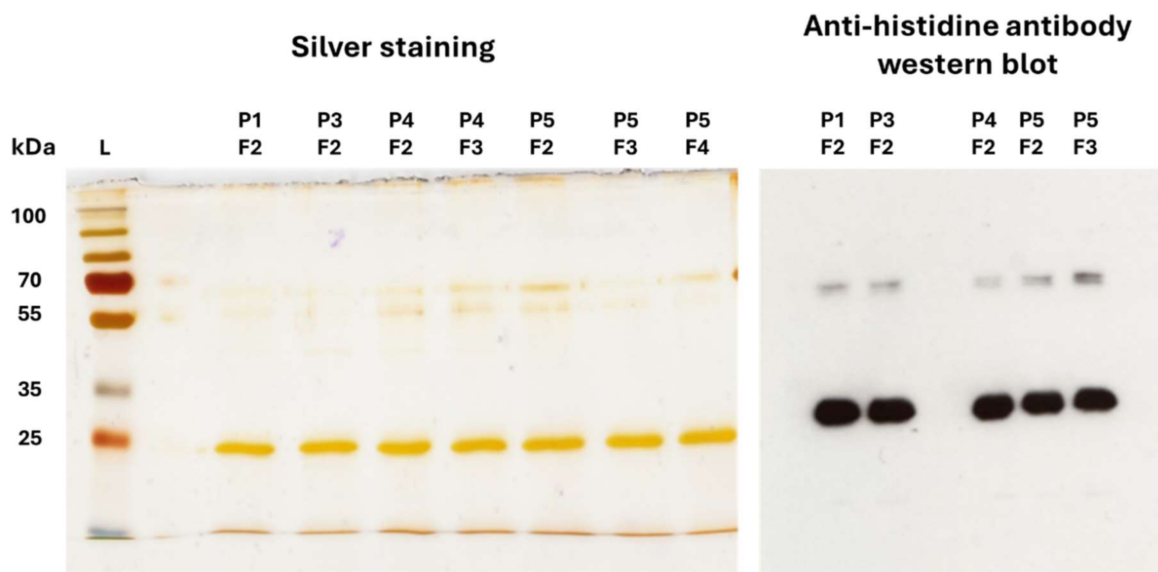


Figure 3.3 FBG-C purity control. The purity of the obtained recombinant FBG-C domain from each protein preparation experiment (denoted as P1 to P5) and column elution fractions (denoted as F2 to F4) was assessed by silver staining and western blotting with anti-histidine antibody. L stands for known size protein ladder mix. FBG-C monomer was detected at the size of 25 kDa and FBG-C homodimer was detected at 55 kDa.

To test whether the newly produced FBG-C domains were refolded correctly after purification and dialysis, circular dichroism (CD) was utilised (see methods section 2.2.5). Complex asymmetric molecules, such as proteins, interact with light beams and differentially absorb left- and right- circularly polarized light, producing distinct spectra. These CD spectra are dependent on the secondary structure composition and polypeptide chain conformation, therefore can be used to survey protein folding. CD spectra were obtained for the newly produced FBG-C domain and were comparable to previously obtained active FBG-C protein preparations produced at the Midwood lab (Zuliani-Alvarez et al., 2017), indicating correct protein refolding following purification (Figure 3.4). Additionally, Thermal denaturation at 95 degrees Celsius abolished the characteristic CD spectra.

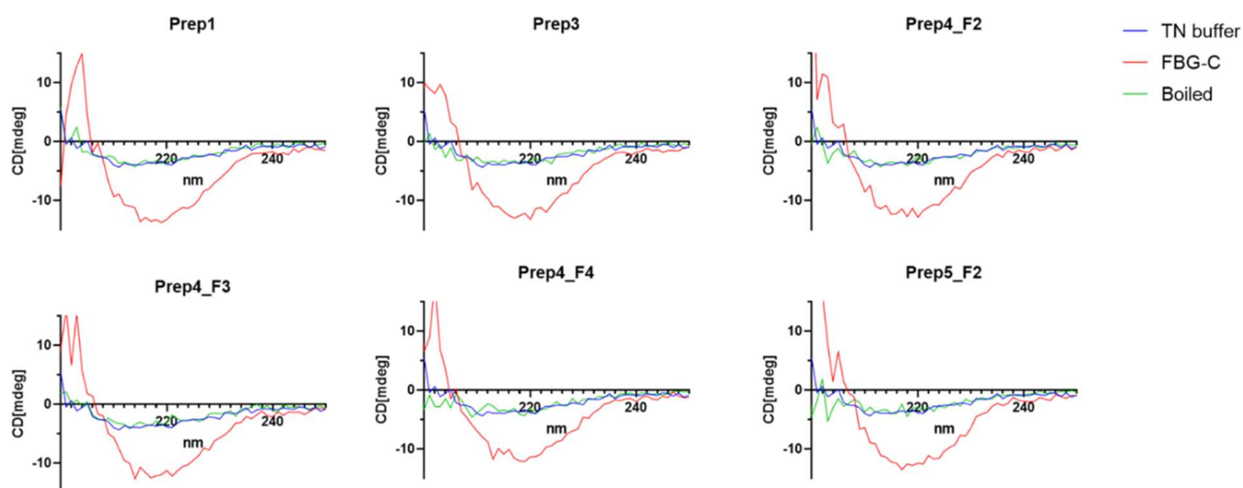


Figure 3.4 FBG-C protein refolding assessment. Correct refolding of the purified FBG-C peptide from each preparation (denoted as Prep1 to Prep5) and column elution fractions (denoted as F1 to F4) was assessed by circular dichroism. Correctly refolded FBG-C domains generate a distinct molar ellipticity negative peak measured in millidegrees [mdeg] (y axis) between 210 and 230 nanometres [nm] of light wavelength absorption (x axis). Red line: protein sample; Green line: protein sample boiled; Blue line: buffer.

An endotoxin test kit was used to examine the levels of endotoxin contamination after each protein preparation (see methods section 2.2.5). The conducted endotoxin tests concluded that all preparations were consistently clean, with LPS levels below 10 pg/mL. To further exclude potential contamination during sample storage and handling, FBG-C was pre-incubated with polymyxin B, an LPS neutralizing agent, prior to all cell activation experiments (Zavascki et al., 2007). Upon activation of TLR4 in human myeloid cells, downstream signalling cascades are activated that result in the production of proinflammatory cytokines, such as IL-6 and TNF. The induction of these cytokines in human primary monocytes has been previously used to confirm the activity of the different tenascin family members (Midwood et al., 2009). Here, the same method was utilised to evaluate the activity of purified recombinant FBG-C domains *in vitro*. Previous work at the Midwood lab has demonstrated that a 1 µM FBG-C concentration is sufficient to activate myeloid cells in a comparable manner to LPS in the concentration range of 10 ng/mL to 100 ng/mL. To assess the activity of the prepared FBG-C, all successful protein preparations were tested on primary human monocytes isolated from healthy donors (Figure 3.5). FBG-C was able to induce comparable levels of IL-6 and TNF to LPS at 24 hours

following activation, confirming the functional ability of the purified protein to activate TLR4. Boiling the protein at 95ºC for one hour completely abolished its activity, thus confirming the results of the endotoxin test and ensuring FBG-C cell activation is not due to LPS contamination (Figure 3.5). Simultaneously, addition of polymyxin B abolished the activity of LPS yet it had no effect on FBG-C, further establishing the robustness of the functional effect on the primary human monocytes exercised by FBG-C (Figure 3.5).

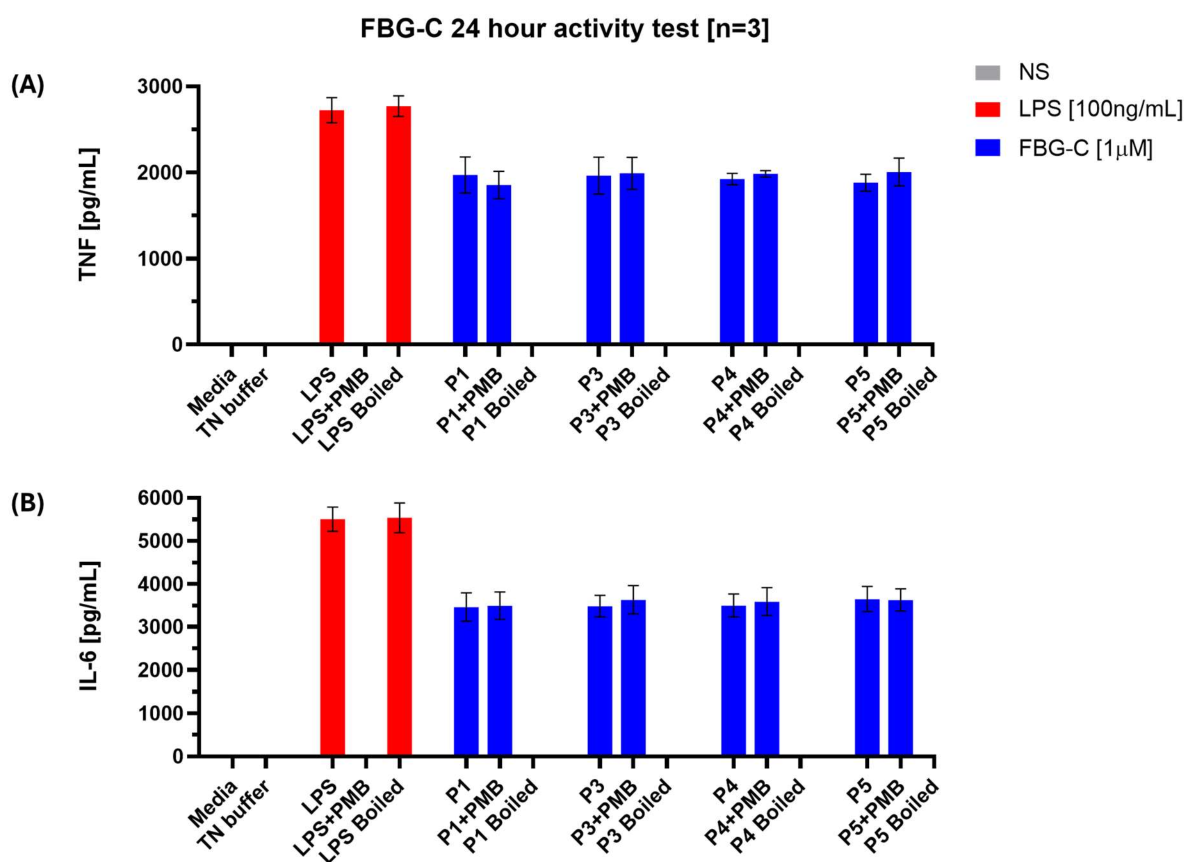


Figure 3.5 FBG-C activity test on human monocytes. Primary human monocytes were activated with either LPS at a concentration of 100 ng/mL or FBG-C at a 1µM concentration from each FBG-C protein preparation (denoted as P1 to P5). Non-activated control cells were either left in culture medium or in culture medium with TN protein buffer (denoted as Media or TN buffer). Boiled controls were heated at 95 ºC for 1 hour. PMB controls were pre-incubated with 10 µg/mL Polymyxin B for 30 min before cell activation. In panel **(A)** are shown TNF levels in culture supernatant at 24 hours after activation, measured by ELISA. In panel **(B)** are shown IL-6 levels in culture supernatant at 24 hours after activation, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors.

Overall, four out of the five protein preparations successfully passed all the necessary quality control steps and were deemed functionally active when used on primary human monocytes, hence these FBG-C peptide preparations were sent to the University of Glasgow for the use in all subsequent FBG-C experiments.

3.2.2 Temporal differences of TNF and IL-1β secretion profiles in FBG-C or LPS stimulated human monocytes

To conduct experiments of biological and functional relevance that accurately compare the activation of primary human monocytes by either FBG-C or LPS, activation strength needs to be carefully considered. Historically, LPS as an *in vitro* or *in vivo* stimulus of myeloid cells has been used at a wide range of concentrations, ranging from 1 ng/mL to 100 µg/mL, depending on the end goal and experimental design. With a physiological relevance in mind, it has been established that *in vitro* concentrations higher than 100 ng/mL are more akin to a sepsis model, while concentrations lower than 1 ng/mL are often undetectable by primary human myeloid cells (Honda & Inagawa, 2023). Additionally, previous experimental comparisons between LPS and FBG-C have been conducted at an LPS concertation range between 1 ng/mL and 100 ng/mL, and FBG-C at a 1µM concentration (Midwood et al., 2009; Piccinini et al., 2016). These comparisons did not consider the kinetics of activation, as they only examined a single time point at which functional outcomes were measured. Using this methodology, underlying signal strength differences might overpower a true biological and functional difference between the stimuli, hence in my experimental work, I sought to find a concertation at which LPS signal strength is functionally comparable to FBG-C at a 1 µM concertation in an activation timespan of 24 hours. To achieve this, primary human monocytes were isolated from healthy donor blood and activated by LPS at the commonly utilised concentrations of 1 ng/mL, 10 ng/mL and 100 ng/mL, and by FBG-C at a 1 µM concentration. At 2-,

4-,16- and 24- hours following activation, culture supernatants were taken and TNF and IL-1ß cytokine levels were measured (Figure 3.6). One-way ANOVA statistical testing followed by Tukey's Honestly-Significant Difference post-hoc test between the different LPS and FBG-C activation conditions, comparing the cytokine secretion profiles of both TNF and IL-1ß over the whole duration of the time course, revealed that an LPS concentration of 10 ng/mL matched the kinetics of FBG-C activation at a 1µM concertation in primary human monocytes since there was a substantial statistical difference between the kinetics of LPS and FBG-C activation when LPS concentrations of 1 ng/ml or 100 ng/mL were used (Figure 3.6). Given these results, in all future experiments comparing the two stimuli, an LPS concentration of 10 ng/mL was used as a functionally relevant control to 1µM FBG-C stimulation, while an LPS concentration of 100 ng/mL was used as an overstimulation positive control.

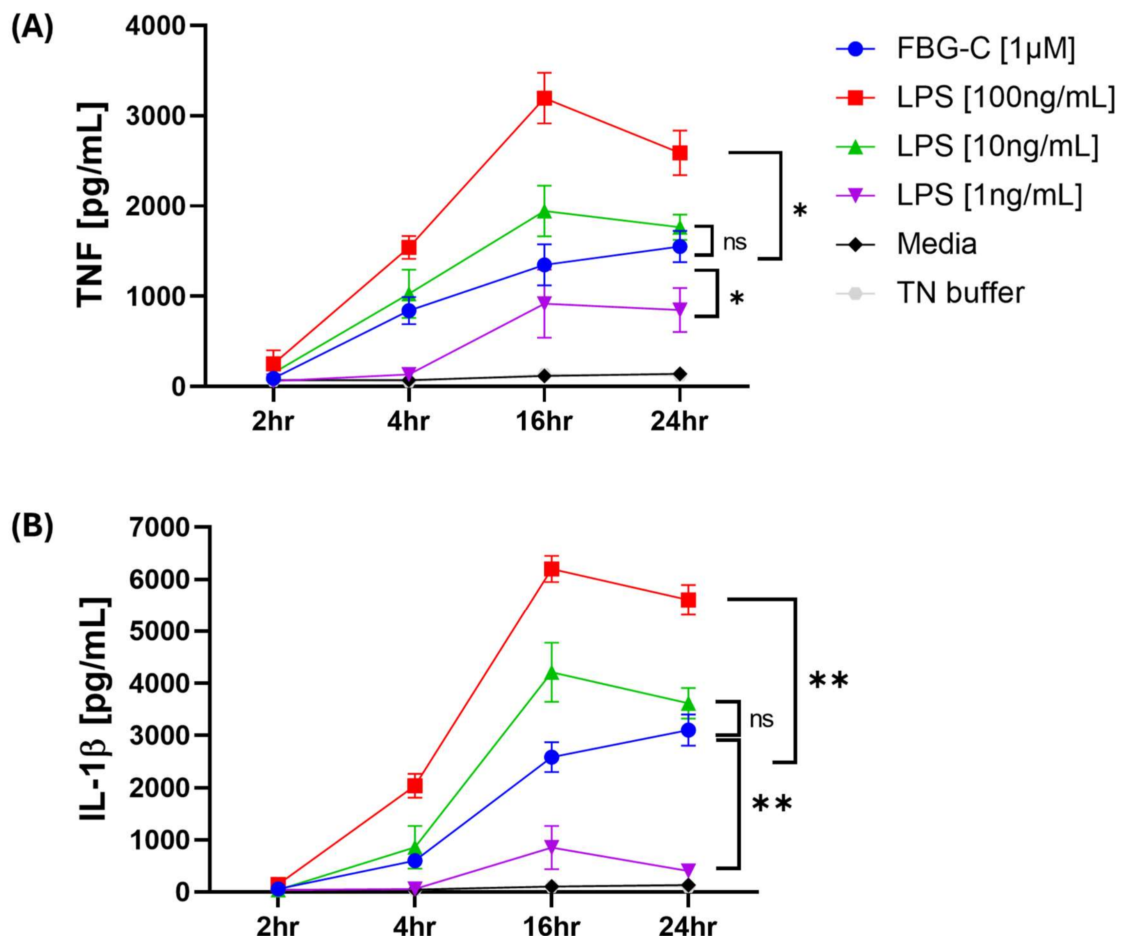


Figure 3.6 LPS titration vs FBG-C, 24-hour activation time course in primary human monocytes. Primary human monocytes were isolated and activated for 24 hours with either LPS at three different concentrations (100ng/mL; 10ng/mL; 1ng/mL) or FBG-C at a 1µM concentration. Non-activated cells were either left without activation in culture medium or in culture medium with TN protein buffer (denoted as Media or TN buffer). In panel **(A)** are shown TNF levels in culture supernatant at 2, 4, 16 and 24 hours after activation, measured by ELISA. In panel **(B)** are shown IL-1ß levels in culture supernatant at 2, 4, 16 and 24 hours after activation, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors. Data was analysed using one-way ANOVA followed by Tukey's Honestly-Significant Difference post-hoc test between different activation conditions over the whole duration of the time course, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Unlike human macrophages and murine myeloid cells, human monocytes can activate the NLRP3 inflammasome and secrete mature IL-1ß immediately after TLR4 activation without the need for K+ efflux caused by a second signal such as ATP, nigericin or particulate matter (Figure 3.6 B) (Gaidt et al., 2016). However, to examine inflammasome tolerance to repeated activation following 24 hours of primary activation, cells must possess the ability to activate the alternative inflammasome to a similar extent after a 24-hour period, as they do fresh out of blood isolation. To test whether primary human monocytes can secrete IL-1ß via alternative inflammasome activation after 24 hours of *in vitro* culture, primary human monocytes were isolated from healthy donor blood and either activated by LPS or FBG-C immediately following isolation or after 24 hours of rest in culture medium. The data from this experiment revealed that both freshly isolated and 24-hour rested monocytes were able to produce and secrete similar levels of TNF, but only freshly isolated monocytes had the ability to secrete mature IL-1ß via alternative inflammasome activation following both LPS and FBG-C activation (Figure 3.7 A and B). To find a possible explanation for this phenomenon, I investigated an RNA sequencing dataset generated using primary human monocytes left in culture for 24 hours without activation (Hussain et al., 2022) obtained from the publicly available webpage of the National Center for Biotechnology Information (see methods section 2.2.17). In 2016 Gaidt and colleagues had demonstrated that RIPK1, caspase 8 and FADD are crucial mediators of alternative inflammasome activation in primary human monocytes, hence I used the RNA sequencing data to compare the RNA levels of these inflammasome activation mediators at 0 hours of cell culture and after 24 hours

of rest in culture media. Bioinformatic analysis revealed that from the three mediators, only the *CASP8* gene was downregulated at 24 hours of culture when compared to freshly isolated monocytes (Figure 3.7 C). This downregulation points to a possible *CASP8* inhibition mechanism that could be preventing alternative inflammasome activation of 24-hour rested monocytes.

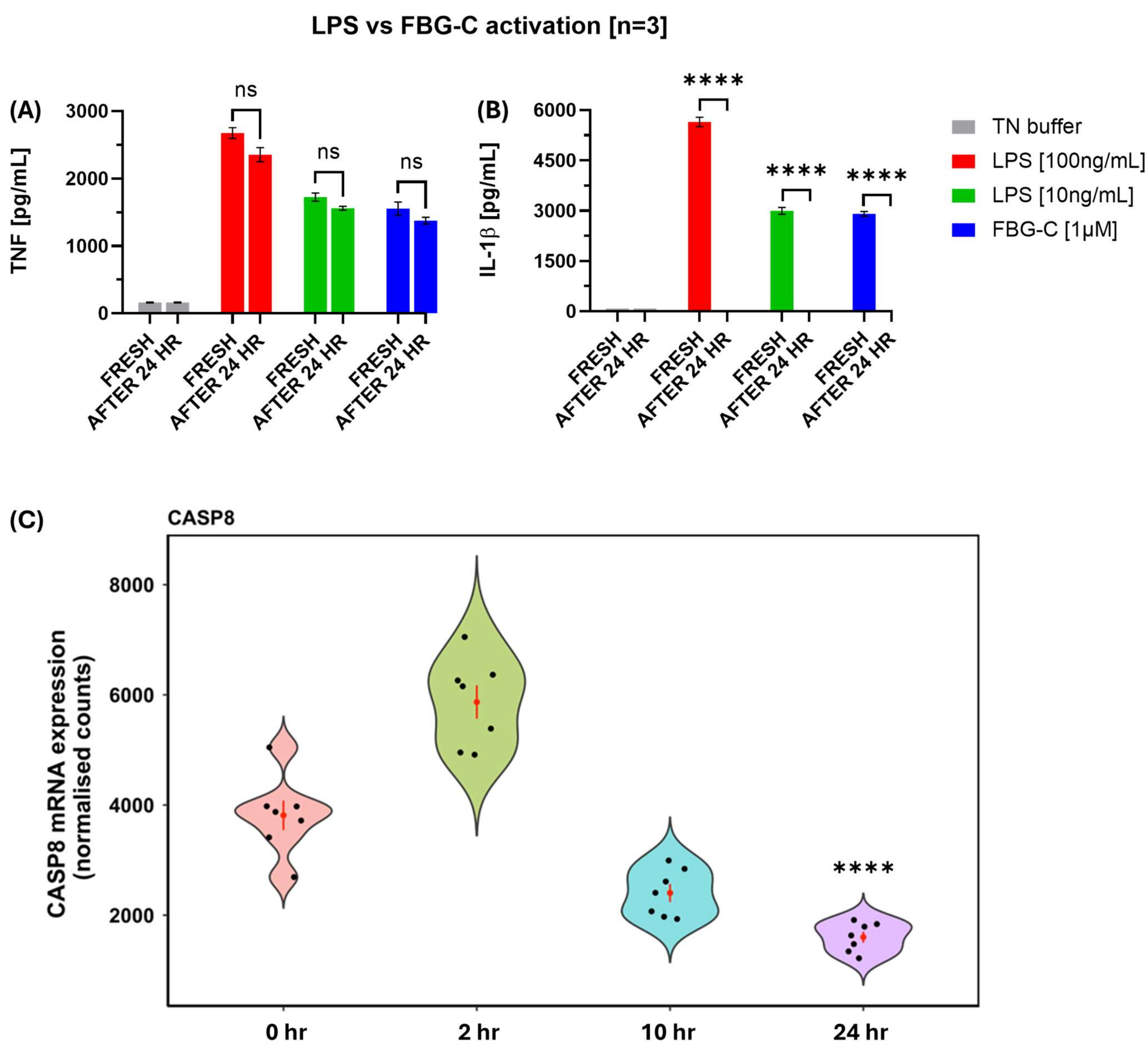


Figure 3.7 LPS vs FBG-C activation of freshly isolated monocytes vs monocytes rested for 24 hours. Primary human monocytes were isolated and either activated immediately or after 24 hours of rest in culture medium. Freshly isolated or 24-hour rested monocytes were activated for 24 hours with either LPS at two different concentrations (100ng/mL; 10ng/mL) or FBG-C at a 1μM concentration. Non-activated control cells were left in culture medium with TN protein buffer (denoted as TN buffer). In panel **(A)** are shown TNF levels in culture supernatant at 24 hours

after activation, measured by ELISA. In panel **(B)** are shown IL-1ß levels in culture supernatant at 24 hours after activation, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors. Data was analysed using unpaired t-test between fresh and 24 hour rested cells, *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001. In panel **(C)** are shown violin plots of Caspase 8 (CASP8) normalised read counts from RNA sequencing of non-activated primary human monocytes either sequenced immediately after isolation from healthy blood (0 hr) or after being left in culture for 2, 10 or 24 hours (2 hr, 10 hr, 24 hr). Each individual dot represents CASP8 normalised read counts of an independent donor sample. Mean ± SD from seven independent healthy donors is shown as a red dot (mean) with a vertical line (± SD). Statistical analysis comparing each time point to the 0-hour timepoint was done using the R package DEseq2 which utilises the Wald test to obtain p values which are then adjusted using the Benjamini and Hochberg method to account for multiple testing, *padj < 0.05, **padj < 0.01, *** padj < 0.001, ****padj < 0,0001.

RNA sequencing data was obtained from a publicly available dataset published on the webpage of the National Center for Biotechnology Information: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165998 (Hussain et al., 2022)

To confirm the requirement for active caspase 8 for the secretion of mature IL-1ß following TLR4 activation without a potassium efflux triggering secondary signal, I conducted an experiment in which primary human monocytes were isolated from healthy donor blood and incubated for one hour with the caspase 8 inhibitor Z-IE(OMe)TD(OMe)-FMK before LPS or FBG-C activation. It has previously been shown that in addition to inflammasome activation, caspase 8 has a second function as a necroptosis mediator when completely inhibited prior to monocyte activation, hence administration of Z-IE(OMe)TD(OMe)-FMK at a concentration higher than 5 µM has been shown to cause mature IL-1ß secretion via necroptosis (Gaidt et al., 2016). In contrast, 5 µM and lower levels of the inhibitor have been shown to prevent alternative inflammasome activation without triggering necroptosis mediated IL-1ß secretion (Gaidt et al., 2016). To examine the role of caspase 8 for both necroptosis and alternative inflammasome activation, in my experimental procedure, cells were incubated with Z-IE(OMe)TD(OMe)-FMK at a range of different concentrations, both higher and lower than 5 µM, for one hour before activation (Figure 3.8). The experimental data from three independent healthy donors revealed that contrary to previous reports, 5 µM and 1 µM concentrations of Z-IE(OMe)TD(OMe)-FMK triggered necroptotic secretion of mature IL-1ß, and only a

concentration of 0.1 µM was able to inhibit alternative inflammasome activation, and thereby IL-1ß secretion, without triggering necroptosis following activation by LPS and FBG-C (Figure 3.8 A). As expected, caspase 8 inhibition had no effect on TNF secretion following activation, showcasing the specificity of its function as an alternative inflammasome activation mediator (Figure 3.8 B).

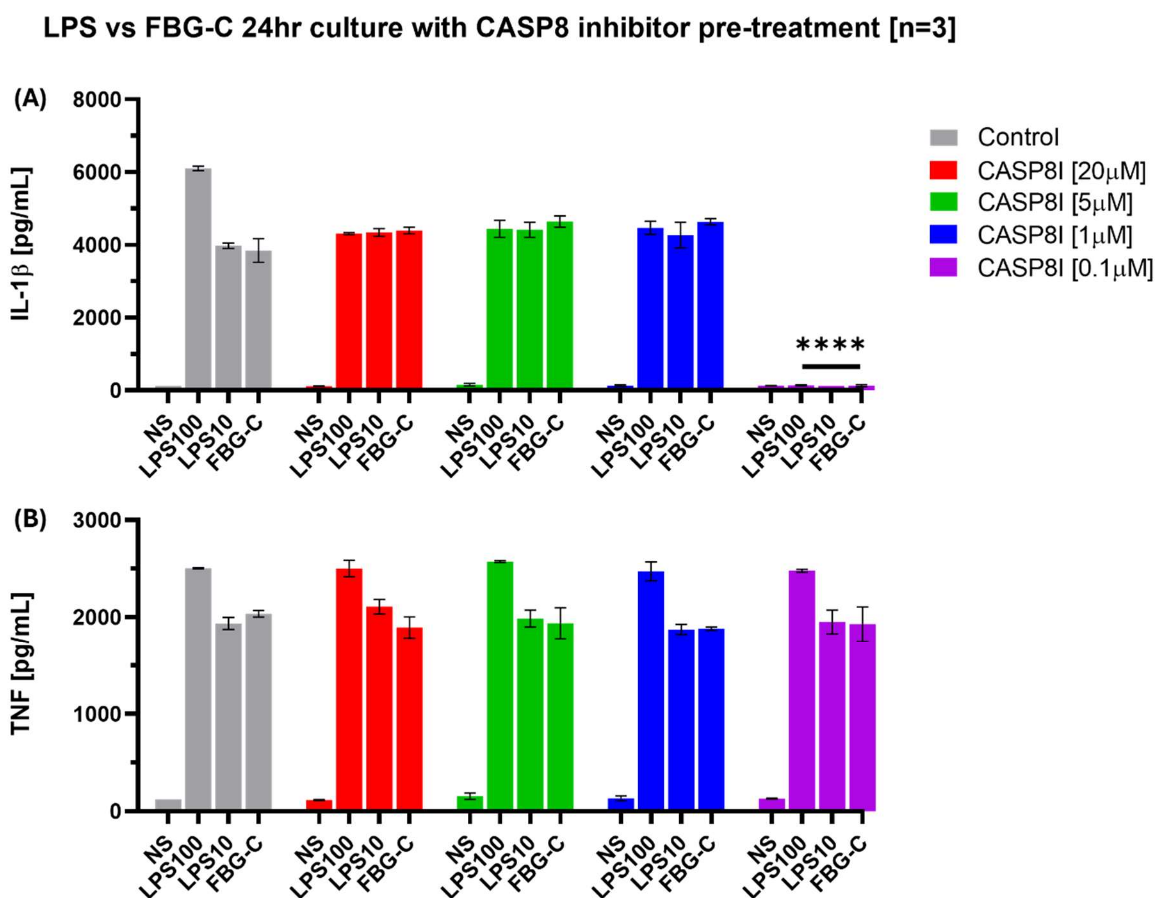


Figure 3.8 LPS vs FBG-C activation with CASP8 inhibitor pre-treatment. Primary human monocytes were isolated and incubated with different concentrations of the caspase 8 inhibitor (CASP8I) Z-IE(OMe)TD(OMe)-FMK (20 µM, 5 µM, 1 µM, 0.1 µM) for one hour. Control cells (Control) were incubated with the CASP8I vehicle dimethyl sulfoxide (DMSO) instead of CASP8 inhibitor for one hour before activation. After 1 hour of incubation, cells were either left without activation (NS) or activated for 24 hours with either LPS at two different concentrations (LPS100 – 100 ng/mL and LPS10 – 10 ng/mL) or FBG-C at a 1µM concentration (FBG-C). In panel **(A)** are shown TNF levels in culture supernatant at 24 hours after activation, measured by ELISA. In panel **(B)** are shown IL-1ß levels in culture supernatant at 24 hours after activation, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors. Data was analysed using

unpaired t-test between control cells and cells incubated with 0.1µM CASP8 inhibitor, *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001.

These data also confirmed the requirement for active caspase 8 for the secretion of mature IL-1ß without a second signal in primary human monocytes, thereby highlighting that *CASP8* downregulation in monocytes after 24 hours in culture (shown in figure 3.7 C) is a possible mechanism for the inability of the cells to secrete IL-1ß via the alternative inflammasome.

Since primary human monocytes were unable to activate the alternative inflammasome after 24 hours of culture, before conducting any tolerisation experiments, I examined whether the cells still possess the ability to activate the canonical NLRP3 inflammasome after a 24-hour rest. Primary human monocytes were isolated from healthy blood and either activated immediately or after 24 hours. Both freshly isolated and 24-hour rested monocytes were activated for 3 hours with either LPS or FBG-C. After 3 hours of TLR4 activation, nigericin toxin was added as a second signal, triggering potassium efflux and canonical NLRP3 assembly and activation (Muñoz-Planillo et al., 2013). Nigericin was added for 45 minutes at three different commonly used concentrations in order to select a concentration for future experiments that was potent enough to trigger robust NLRP3 activation without over activating the cells and thereby masking functional differences between the different primary stimuli. Data from three independent healthy donors revealed that 24-hour rested primary human monocytes retain their ability to activate the canonical inflammasome when stimulated with 5 and 10 µM nigericin, albeit to a lower extent compared to freshly isolated monocytes (Figure 3.9 A and B). Additionally, the data showed that 1 µM nigericin concentration is not sufficient to trigger canonical inflammasome assembly and activation in primary human monocytes following LPS or FBG-C activation, since cells stimulated with 1µM nigericin secreted the same levels of mature IL-1ß as cells incubated with the nigericin vehicle buffer (Figure 3.9 A). 24-hour rested monocytes did not secrete any IL-1ß when cultured with 1 µM nigericin as these cells had lost the ability to secrete IL-1ß directly following primary activation via the alternative inflammasome, and 1 µM nigericin was not potent enough to activate the canonical inflammasome activation pathway (Figure 3.9 B). Since there was no difference between the

levels of IL-1β secretion when cells were subjected to either 5 or 10 μM nigericin, the lower concentration of 5μM was selected for use in future inflammasome activation experiments, as it showed to be sufficiently potent to trigger robust canonical inflammasome activation. As expected, regardless of the concertation used, nigericin had no effect on TNF secretion both in freshly isolated and in 24-hour rested monocytes following TLR4 activation, demonstrating the specificity of its function as a canonical inflammasome activator (Figure 3.9 C and D).

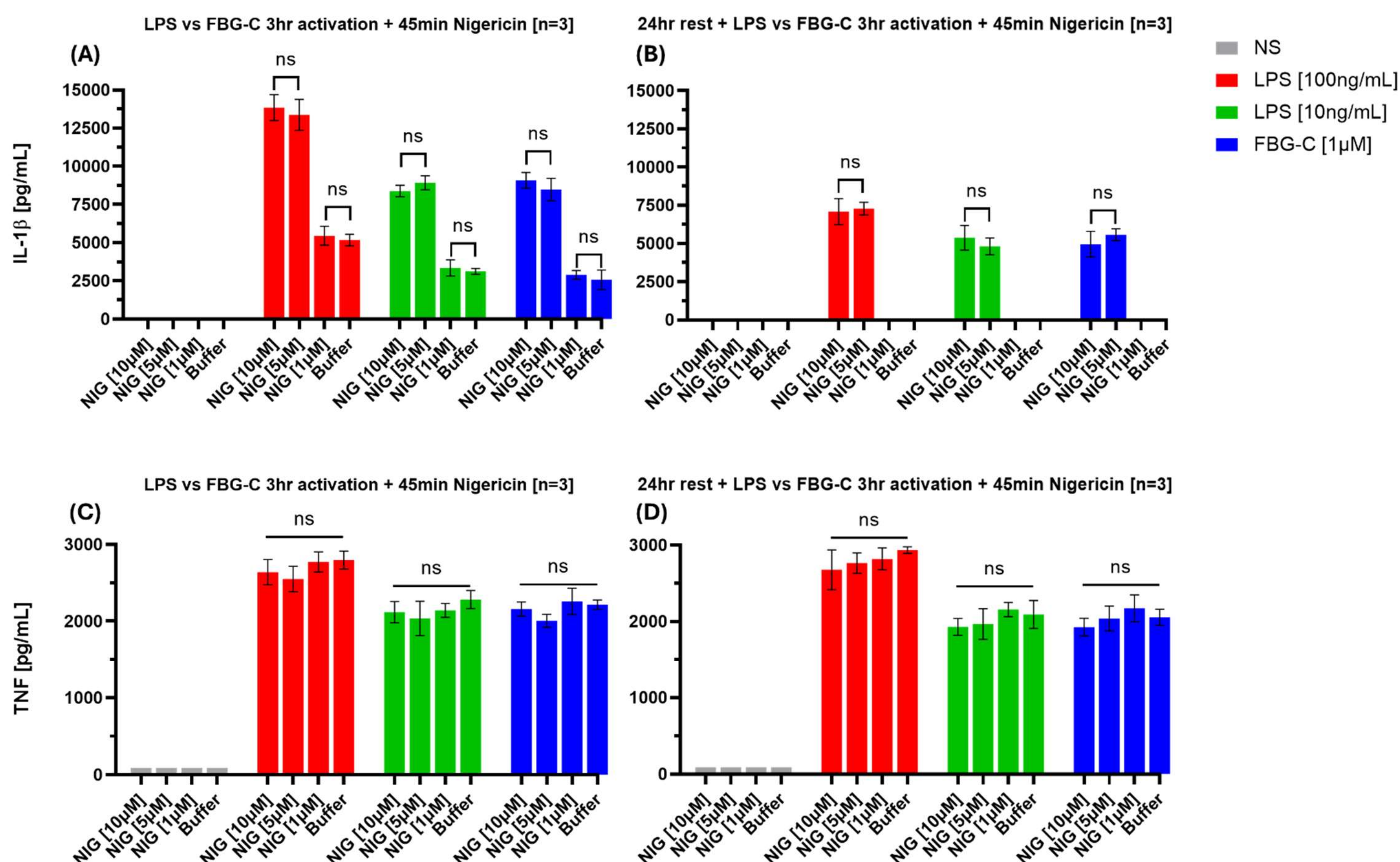


Figure 3.9 LPS vs FBG-C activation followed by nigericin canonical NLRP3 inflammasome activation. Primary human monocytes were isolated and either activated immediately or after 24 hours of rest in culture medium. Freshly isolated or 24-hour rested monocytes were either left without activation in culture media (NS) or activated for 3 hours with either LPS at two different concentrations (100ng/mL; 10ng/mL) or FBG-C at a 1μM concentration. After 3 hours of activation, vehicle control (Buffer) or nigericin (NIG) at different concentrations (10 μM, 5 μM, 1 μM) were added to the cell cultures and cells were left to incubate for another 45 minutes before culture supernatant was taken. In panel **(A)** are shown IL-1β levels in culture supernatant at end of culture of freshly activated following isolation monocytes, measured by ELISA. In panel **(B)** are shown IL-1β levels in culture supernatant at end of culture of 24-hour rested before

activation monocytes, measured by ELISA. In panel **(C)** are shown TNF levels in culture supernatant at end of culture of freshly activated following isolation monocytes, measured by ELISA. In panel **(D)** are shown TNF levels in culture supernatant at end of culture of 24-hour rested before activation monocytes, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors. Data was analysed using unpaired t-tests between different nigericin concentration conditions, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Previous reports have described the alternative inflammasome as a non-pyroptotic mechanism of IL-1ß secretion, allowing human monocytes to keep functioning after secreting mature IL-1ß following primary TLR4 activation (Gaidt et al., 2016). In contrast, canonical NLRP3 inflammasome mediated secretion of IL-1ß has been shown to occur strictly via pyroptosis since triggers of potassium efflux, such as nigericin and extracellular ATP, following primary TLR4 activation stimulate myeloid cells to enter a state of controlled Gasdermin D pore formation and cell death allowing them to secrete exceptionally high levels of mature IL-1ß (Ding et al., 2016; Evavold et al., 2018). To confirm the pyroptotic nature of IL-1ß secretion by the 5 and 10 µM nigericin activated monocytes, light microscopy images were taken at the end of the culture. These images revealed that in both freshly activated and 24-hour rested cells, 5 and 10 µM nigericin stimulation triggered pyroptosis, which can be observed as a pronounced loss of plasma membrane integrity and subsequent cell rupturing (Figure 3.10 A to D and I to L) (Ding et al., 2016; Labbé & Saleh, 2011). In contrast, 1 µM nigericin and buffer incubated cells showed no signs of pyroptosis (Figure 3.10 E to H and M to P). These images thereby confirm that 5 and 10 µM nigericin concentrations trigger canonical pyroptotic inflammasome activation (Figure 3.10 A to D and I to L), while freshly isolated monocytes subjected to 1 µM nigericin or vehicle buffer secrete IL-1ß via the non-pyroptotic alternative inflammasome (Figure 3.10 E to H).

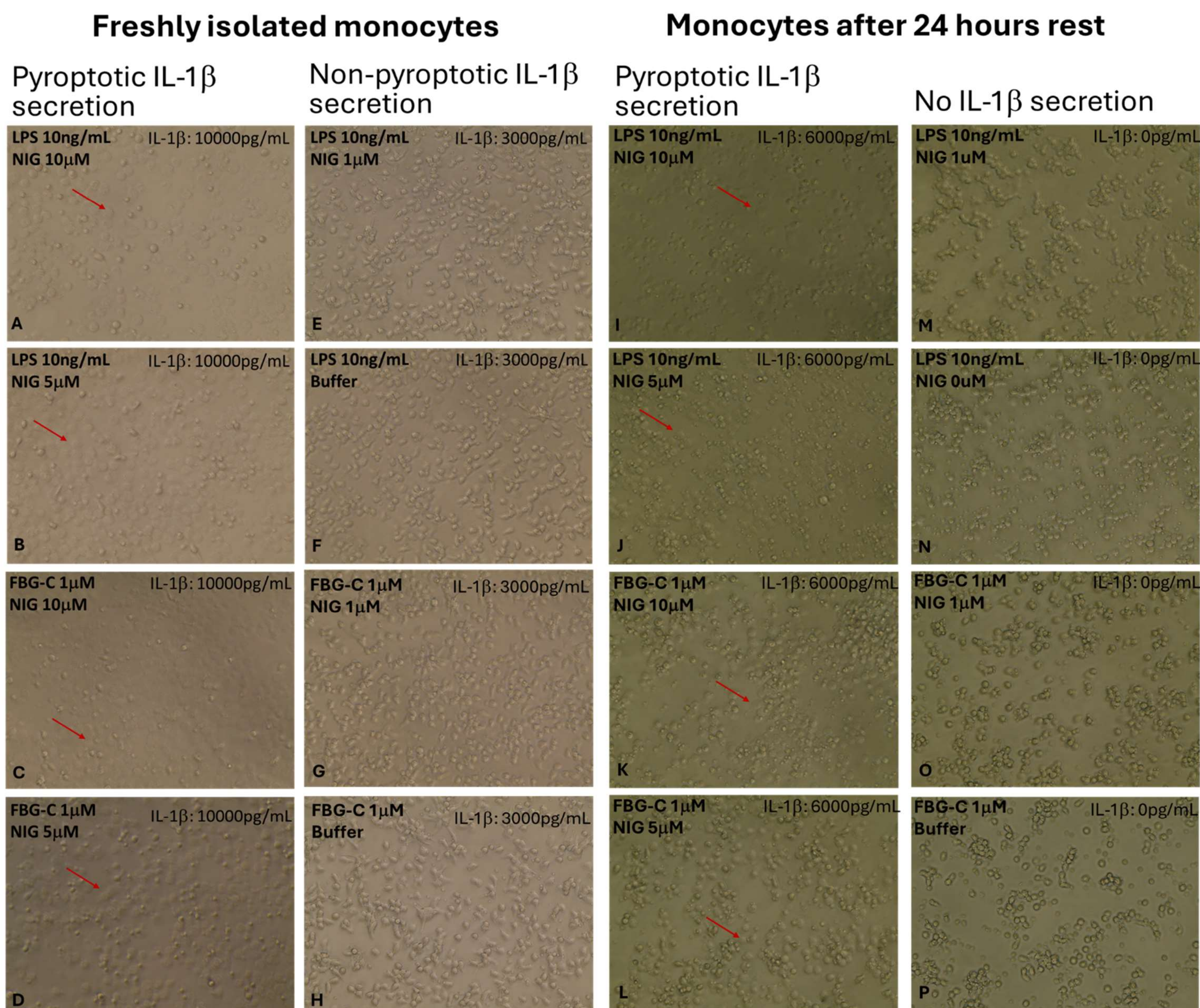


Figure 3.10 LPS vs FBG-C activation followed by nigericin canonical NLRP3 inflammasome activation, pyroptosis images. Primary human monocytes were isolated and either activated immediately or after 24 hours of rest in culture medium. Freshly isolated or 24-hour rested monocytes were either left without activation in culture media (NS) or activated for 3 hours with either LPS at two different concentrations (100ng/mL; 10ng/mL) or FBG-C at a 1µM concentration. After 3 hours of activation, vehicle control (Buffer) or nigericin (NIG) at different concentrations (10 µM, 5 µM, 1 µM) were added to the cell cultures and cells were left to incubate for another 45 minutes before. At the end of nigericin activation, images of the cells were taken using a digital light microscope. In panels **A to D** are shown images of freshly activated following isolation monocytes, secreting IL-1β via pyroptotic means when incubated with nigericin at a 10 µM or a 5 µM concentration. The red arrow points to an example of a pyroptotic cell. In panels **E to H** are shown images of freshly activated following isolation monocytes, secreting IL-1β via non-pyroptotic means when incubated with vehicle buffer or

nigericin at a 1 μM concentration. In panels **I to L** are shown images of 24-hour rested before activation monocytes, secreting IL-1ß via pyroptotic means when incubated with nigericin at a 10 μM or a 5 μM concentration. The red arrow points to an example of a pyroptotic cell. In panels **M to P** are shown images of 24-hour rested before activation monocytes, unable to secrete IL-1ß via either pyroptotic or non-pyroptotic means when incubated with vehicle buffer or nigericin at a 1 μM concentration.

3.2.3 Establishment of NLRP3 inflammasome tolerance by FBG-C or LPS in human monocytes

In section 3.2.2, it was demonstrated that primary human monocytes lose their ability to activate the alternative inflammasome after 24 hours of rest in culture medium, potentially due to loss of *CASP8* gene transcript. In contrast, monocytes were still able to robustly activate the canonical NLRP3 inflammasome pathway even after 24 hours of rest. Following these observations, I examined 24-hour canonical inflammasome tolerance, namely whether primary human monocytes that had been previously stimulated by either LPS or FBG-C were able to activate the canonical NLRP3 inflammasome following a second TLR4 stimulation 24 hours after the first stimulation. To be able to specifically investigate the effect of the primary stimulus, LPS vs FBG-C, on the generation of inflammasome tolerance, restimulation was always conducted using LPS at a 10 ng/mL concentration. Using this restimulation normalisation methodology, differences in functional outcomes can be directly attributed to differences between the FBG-C and LPS primary stimulation. Human monocytes were isolated from healthy donor blood and either left in culture without activation as non-tolerised controls or activated with LPS or FBG-C for 24 hours. After 24 hours, culture media was replaced with fresh media, and cells were left to rest for one hour before either experiencing re-activation with 10 ng/mL LPS or being left as non-reactivated controls (Figure 3.11 A). Following 3 hours of re-activation, nigericin at a 5 μM concentration was added to the cell culture for 45 minutes to trigger canonical inflammasome activation (Figure 3.11 A). Data from six independent healthy donors revealed that primary LPS activation tolerised both the TNF and IL-1ß responses 24 hours after primary activation since

restimulated cells produced very low levels of these cytokines (Figure 3.11 C and E). In contrast to LPS, however, FBG-C activation was unable to tolerise IL-1ß secretion to restimulation 24 hours after primary activation since restimulated cells produced close to normal levels of IL-1ß (Figure 3.11 E). Interestingly, this effect was specific to the tolerisation of the NLRP3 inflammasome activation since FBG-C activation was still able to tolerise the secretion of TNF in the same way as LPS (Figure 3.11 C).

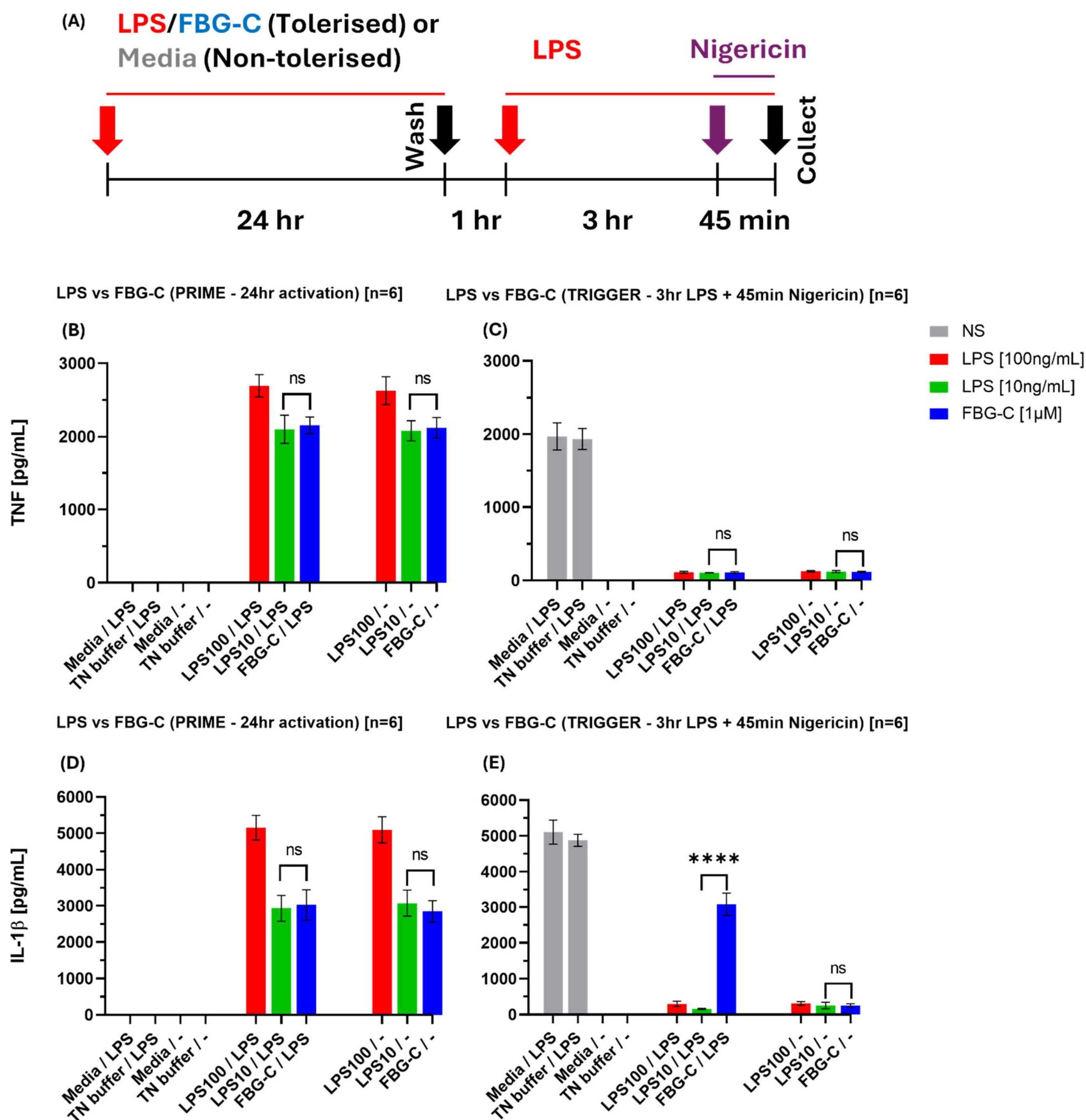


Figure 3.11 LPS vs FBG-C 24-hour inflammasome tolerisation. In panel **(A)** is shown the design of the inflammasome tolerance investigation experiment. Primary human monocytes were isolated and either left in culture without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (100 ng/mL, 10 ng/mL) or FBG-C at a 1μM concentration for 24 hours (tolerised). After 24 hours, culture media was replaced with fresh media, and cells were left to rest for one hour before either experiencing re-activation by LPS at a 10 ng/mL concentration or being left as non-reactivated controls. Following 3 hours of re-activation, nigericin at a 5 μM concentration was added to the cell culture for 45 minutes to trigger inflammasome activation. In panel **(B)** are shown TNF levels in culture supernatant at end

of the 24-hour primary activation (PRIME), measured by ELISA. In panel **(C)** are shown TNF levels in culture supernatant following 3 hours of re-activation (TRIGGER) and 45 minutes of nigericin inflammasome activation, measured by ELISA. In panel **(D)** are shown IL-1β levels in culture supernatant at end of the 24-hour primary activation (PRIME), measured by ELISA. In panel **(E)** are shown IL-1β levels in culture supernatant following 3 hours of re-activation (TRIGGER) and 45 minutes of nigericin inflammasome activation, measured by ELISA. Data are shown as mean ± SD from six independent healthy donors. Data was analysed using unpaired t-tests between LPS and FBG-C activated conditions, $*p < 0.05$, $**p < 0.01$, $*** p < 0.001$, $**** p < 0.0001$.

To assess whether 24-hour LPS and FBG-C tolerised monocytes underwent pyroptosis or whether they remained viable following 3 hours of restimulation and 45 minutes of nigericin activation, cells were stained with a fluorescence viability kit at the end of culture comprising Calcein-AM (green fluorescence), Ethidium homodimer-1 (red fluorescence) and Hoechst (blue fluorescence). Cells visible in the green fluorescence channel had their extracellular membrane intact, while cells visible in the red and blue fluorescence channels had their DNA and nucleus exposed and thus were in the process of undergoing pyroptosis (Figure 3.12). Fluorescence images taken at the end of cell culture showed that LPS tolerised cells did not undergo pyroptosis following restimulation and nigericin activation (Figure 3.12 C), indicating that the tolerisation mechanism interferes with the canonical inflammasome pyroptosis pathway. In contrast, FBG-C stimulation for 24 hours did not interfere with the ability of the cells to undergo pyroptosis to the same extent following restimulation based on the high pyroptosis levels observed in the fluorescence images of FBG-C-activated cells (Figure 3.12 D), similar to those observed in non-tolerised control monocytes (Figure 3.12 A and B). These differences in pyroptosis inhibition following LPS and FBG-C activation provide an explanation for the different IL-1β tolerisation phenotypes observed following 24 hours of primary human monocyte TLR4 activation (Figure 3.11 E)

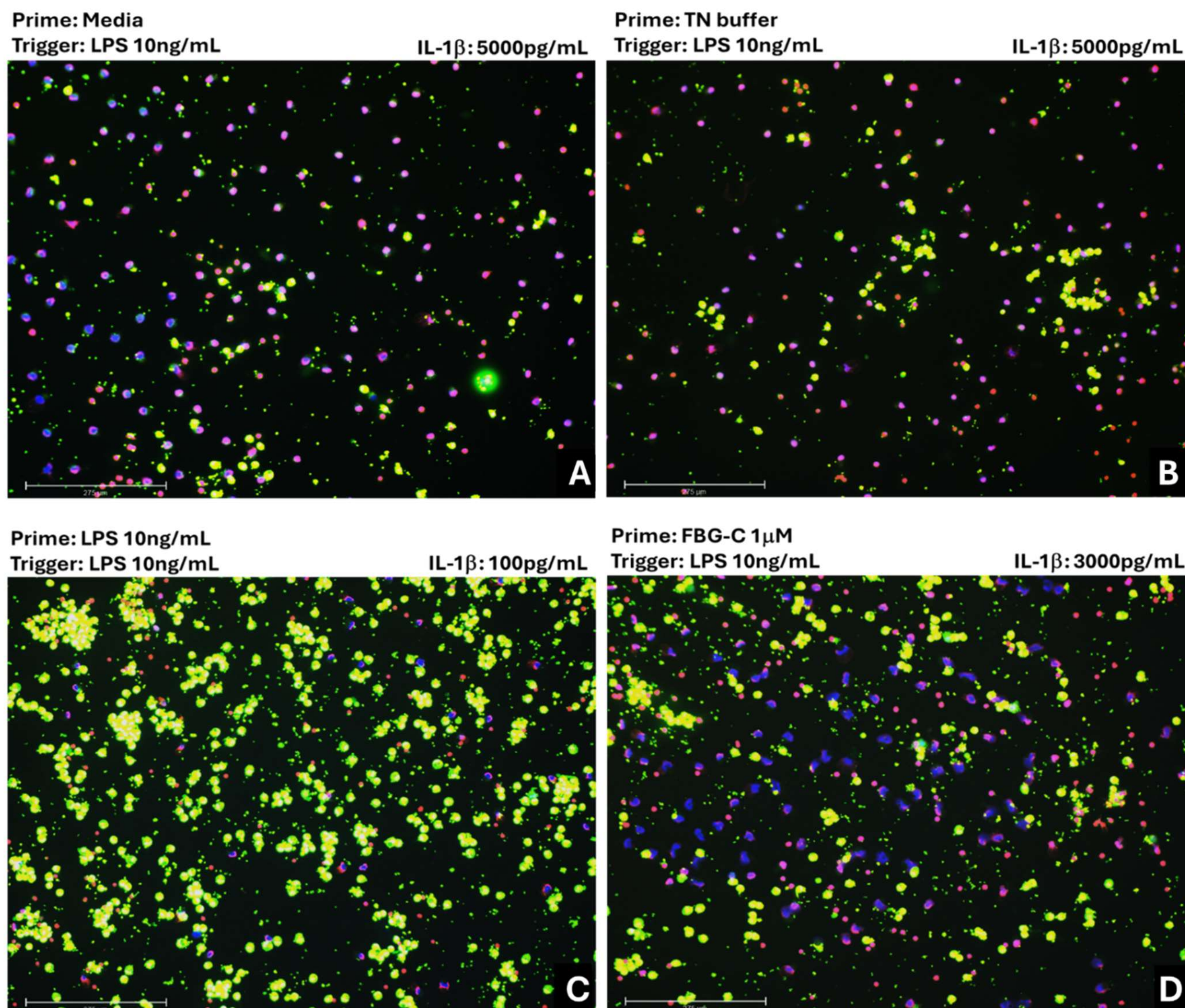


Figure 3.12 LPS vs FBG-C 24-hour inflammasome tolerisation, effect on pyroptosis. Primary human monocytes were isolated and either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (100 ng/mL, 10 ng/mL) or FBG-C at a 1μM concentration for 24 hours (tolerised). After 24 hours, culture media was replaced with fresh media, and cells were left to rest for one hour before either experiencing re-activation by LPS at a 10 ng/mL concentration or being left as non-reactivated controls. Following 3 hours of re-activation, nigericin at a 5 μM concentration was added to the cell culture for 45 minutes to trigger inflammasome activation. At the end of nigericin activation cells were stained with Calcein-AM (Green), Ethidium homodimer-1 (red) and Hoechst (blue) to assess pyroptosis levels via fluorescence microscopy. In panels **A and B** are shown fluorescence microscopy images of non-tolerised control monocytes, cultured in media alone or in media with TN buffer for 24

hours, undergoing pyroptosis upon 3 hours of 10 ng/mL LPS activation followed by 45 minutes of 5 µM nigericin inflammasome activation. In panel **C** is shown a fluorescence microscopy image of LPS tolerised monocytes, activated with 10 ng/mL LPS for 24 hours, unable to undergo pyroptosis upon 3 hours of 10 ng/mL LPS re-activation followed by 45 minutes of 5 µM nigericin inflammasome activation. In panel **D** is shown a fluorescence microscopy image of FBG-C tolerised monocytes, activated with FBG-C at a 1 µM concentration for 24 hours, undergoing pyroptosis upon 3 hours of 10 ng/mL LPS re-activation followed by 45 minutes of 5 µM nigericin inflammasome activation.

3.2.4 Investigating the requirement for the TLR4 co-receptor CD14 for FBG-C or LPS human monocyte activation

In section 3.2.2, it was demonstrated that 24-hour primary activation leads to identical TNF and IL-1ß secretion profiles between LPS and FBG-C stimulated primary human monocytes (Figure 3.6; Figure 3.9). However, subsequent experiments aimed at investigating NLRP3 inflammasome tolerance revealed a fundamental difference between LPS and FBG-C activation (Figure 3.11). Since LPS and FBG-C are structurally very different, it is conceivable that their binding interaction with the TLR4 receptor is also different, which in turn can lead to changes in downstream signalling. Over the last decade, it has been demonstrated that for the robust activation of TLR4, the co-receptor CD14 is required in human myeloid cells (Na et al., 2023). One of the primary functions of CD14 has been described as a ligand carrier, since when CD14 binds to a TLR4 ligand such as LPS in the extracellular microenvironment, it moves along the cell membrane until it finds TLR4, thus bringing the ligand to the receptor and stabilising their interaction by increasing the affinity of TLR4 for its ligand (Ryu et al., 2017). The TLR4-LPS transfer capabilities of CD14 have been described in detail, and although previous work using anti-CD14 antibodies has suggested that CD14 is not required for FBG-C signalling (Midwood et al., 2009) it is still not fully understood whether FBG-C requires the same type of CD14 trafficking to cause a robust activation response. To investigate whether primary human monocytes can detect FBG-C without the CD14 co-receptor, I generated primary human *CD14* knock-out cells via a mechanism utilising *CD14*-guided

Streptococcus pyogenes Cas9 endonuclease (see methods section 2.2.9). Although different studies have described the mechanisms through which CD14 gets trafficked to and from the cell surface of myeloid cells, there are few reports on how long cells can keep CD14 before it is targeted for degradation, and even less is known about the timespan of CD14 re-trafficking to the cell surface after internalisation. Therefore, to ensure that there is no CD14 receptor remaining on the surface of *CD14* KO cells before they are subjected to FBG-C and LPS activation, I examined the surface levels of CD14 using flow cytometry for a period of 5 days following knock-out (Figure 3.13). Mock KO cells were used as a negative control to show the wild-type surface expression levels of CD14 for 5 days following the start of cell culture (Figure 3.13). To keep the cells alive throughout the 5-day culture period, recombinant human M-CSF was added to the culture media at a 25 ng/mL concentration. The flow cytometric analysis revealed that after 3 days of culture, 48% of the *CD14* KO cell population still maintained CD14 receptor on their surface (Figure 3.13). Interestingly, by day 5 following KO, the surface expression of CD14 had finally been reduced to less than 1% of the *CD14* KO cell population (Figure 3.13). A fixable viability dye was used in the flow cytometric analysis to ensure the decrease in CD14 was not due to a drop in cell viability. Together with T7 endonuclease surveyor assay data (data not shown), which demonstrated a population-wide *CD14* knock-out by 2 hours following the KO procedure, the 5-day flow cytometric analysis data revealed an exceptionally long maintenance period of CD14 protein expression on the surface of primary human myeloid cells.

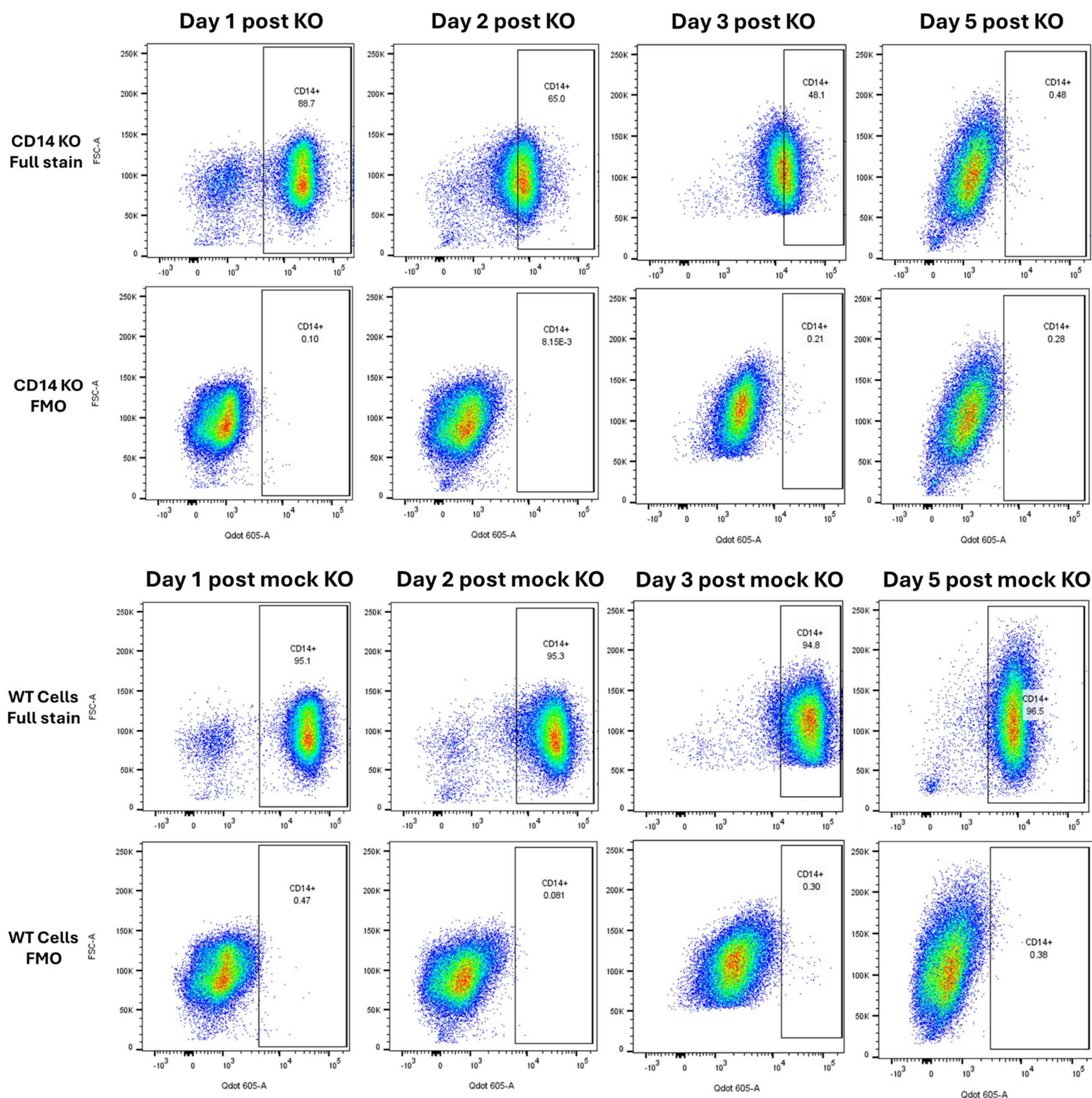
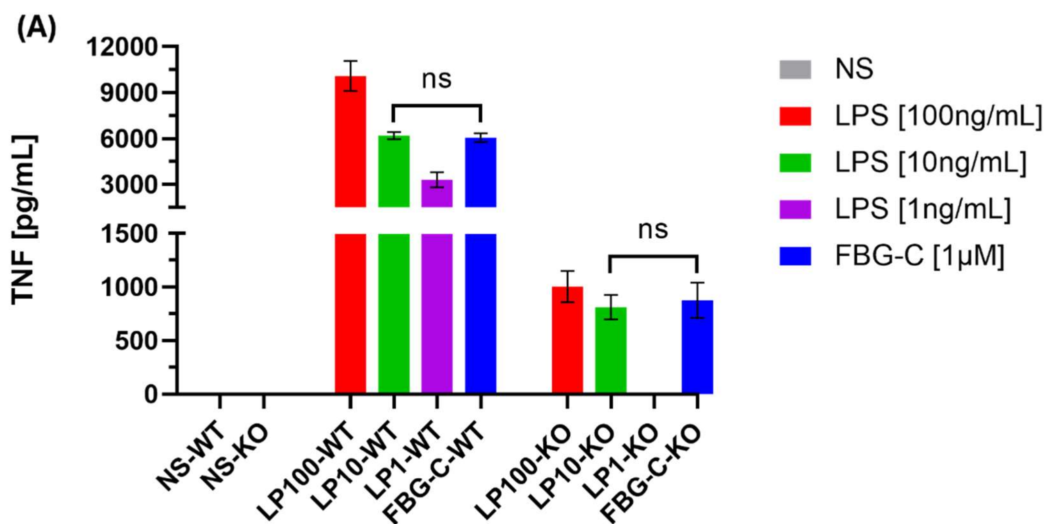


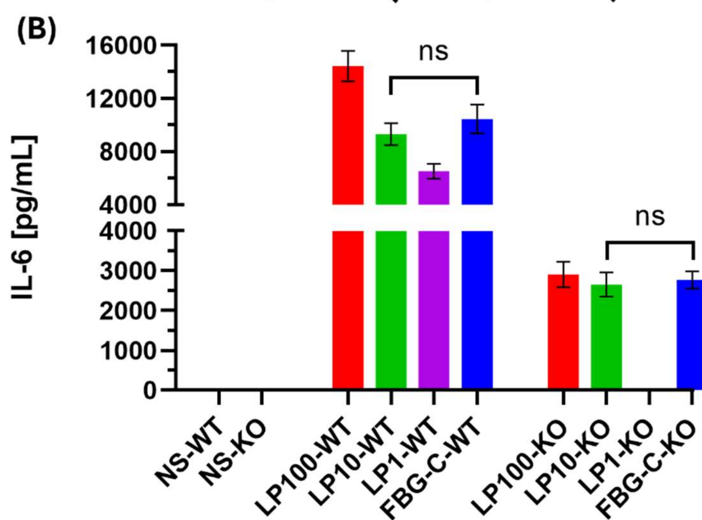
Figure 3.13 Levels of CD14 protein expression on the surface of primary human monocytes in the five days following *CD14* gene Cas9 knock-out. Primary human monocytes were isolated and the *CD14* gene was knocked out via a mechanism utilising *CD14* guided *Streptococcus pyogenes* Cas9 endonuclease (see methods section 2.2.9). Following the *CD14* gene KO, the expression of the CD14 protein on the surface of KO monocytes was measured via flow cytometry. To achieve this, on days 1, 2, 3 and 5 post KO, cells were stained with Brilliant Violet 605™ mouse anti-human CD14 Antibody (fluorescence channel: Qdot 605) and processed on a BD LSRFortessa™ cell analyser. Mock KO control cells (WT cells) underwent the same procedure as KO cells utilising a non-guided

Streptococcus pyogenes Cas9 endonuclease instead, and their surface expression levels of CD14 protein were measured in the same manner as KO cells. Density plots of forward scatter area (FSC-A) against Brilliant Violet 605™ fluorescence intensity (Qdot 605-A) are shown for days 1, 2, 3 and 5 for both KO cells and mock KO control cells. For all time points and conditions fluorescence minus one control (FMO) is used to set the CD14 positive gate. Each density plot is showing 50,000 live, singlet cellular events. Percentage of cells expressing CD14 protein on their surface is shown on each plot.

The *CD14* KO experiment was repeated three times using monocytes isolated from three independent healthy donors and in all experiments, 5 days were necessary for the CD14 receptor surface expression to be reduced to less than 1% of the *CD14* KO cell population. On day 5 of each experiment the cells were activated with LPS or FBG-C for 24 hours to examine whether primary human myeloid cells require CD14 to detect FBG-C to the same extent as they do for LPS (Figure 3.14). Not surprisingly, *CD14* KO cells could not mount a robust TNF and IL-6 cytokine response to LPS as compared to mock KO control cells (Figure 3.14 A and B). However, the cells were still able to detect LPS at 10 and 100 ng/mL concentrations even without CD14, albeit with a 10-fold reduced cytokine response (Figure 3.14 A and B). At a concentration of 1 ng/mL, LPS was completely undetectable by *CD14* KO cells based on both TNF and IL-6 secretion. Importantly, FBG-C demonstrated the exact same requirement for CD14 as did LPS, as there was a 10-fold reduction in the production of both TNF and IL-6 by *CD14* KO cells stimulated with FBG-C compared to mock KO control cells stimulated with FBG-C (Figure 3.14 A and B). To ensure any differences in cytokine production between KO and WT cells at the end of the 24-hour activation were not due to loss of viability, cells were stained with a fluorescence viability kit comprising Calcein-AM (green fluorescence) and Ethidium homodimer-1 (red fluorescence). Cells visible in the green fluorescence channel had their extracellular membrane intact, while cells visible in the red fluorescence channels had their DNA exposed and thus were in the process of undergoing cell death. No loss of viability was observed in *CD14* KO cells following 24 hours of activation (Figure 3.14 C). Based on the data shown here, any functional differences between the tolerance responses triggered by FBG-C and LPS cannot be attributed to differences in CD14 co-receptor signalling requirement.

CD14 KO vs WT 24 hour activation [n=3]





(C) Cell Viability 24 hours post activation

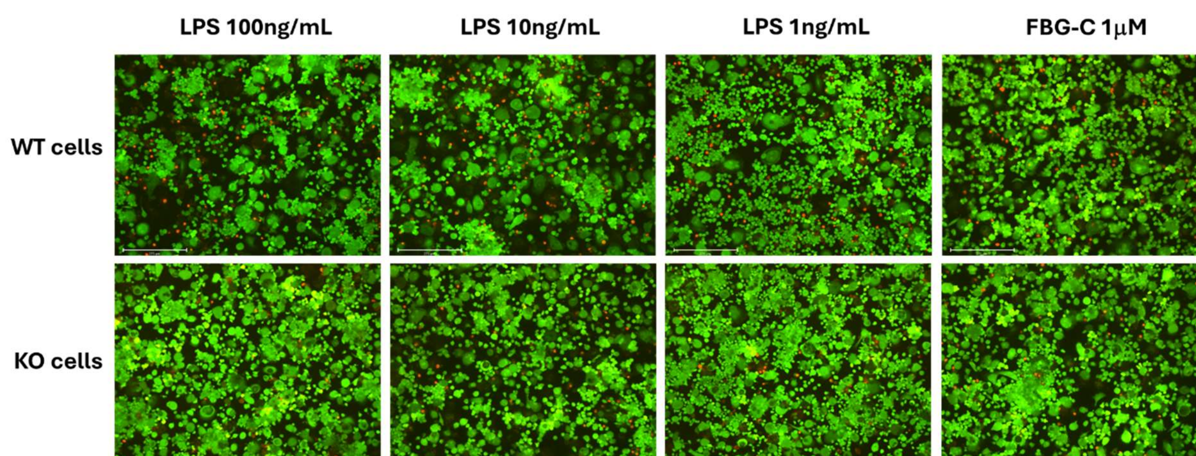


Figure 3.14 Effect of *CD14* knock-out on LPS and FBG-C activation mediated proinflammatory cytokine secretion in primary human monocytes. Primary human monocytes were isolated and the *CD14* gene was knocked out via a mechanism utilising *CD14* guided *Streptococcus pyogenes* Cas9 endonuclease (see methods section 2.2.9). Mock KO control cells (WT cells) underwent the same procedure as KO cells utilising a non-guided *Streptococcus pyogenes* Cas9 endonuclease instead. 5 days following KO, cells were either left without activation in media with TN buffer or activated for 24 hours with LPS at three different concentrations (LP100 - 100 ng/mL; LP10 - 10 ng/mL; LP1 - 1 ng/mL) or FBG-C at a 1μM concentration. In panel **(A)** are shown TNF levels in culture supernatant at end of the 24-hour culture, measured by ELISA. In panel **(B)** are shown IL-6 levels in culture supernatant at end of the 24-hour culture, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors. Data was analysed using unpaired t-tests between LPS and FBG-C activated conditions, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. In panel **(C)** are shown fluorescence microscopy viability stain images of KO and WT cells at end of the 24-hour activation culture. Green: Calcein-AM; Red: Ethidium homodimer-1.

3.3 Discussion

In this chapter, it was demonstrated that to adequately compare myeloid cell functional responses to PAMPs versus DAMPs *in vitro*, it is paramount that experimental design takes into account the influence of signal strength on the kinetics of cell activation. For example, without a functional normalisation for signal strength, LPS and FBG-C would appear to have distinct TNF and IL-1ß secretion profiles over the first 24 hours of activation. However, using functionally equivalent activation strength, it was shown that both stimuli can activate the alternative and the canonical NLRP3 inflammasome to the same extent in human monocytes, while true functional differences only appeared following subsequent restimulation.

Unexpectedly, human monocytes were unable to produce IL1ß directly after TLR4 activation via the alternative inflammasome 24 hours following isolation from healthy donor blood, potentially due to *CASP8* gene inhibition. While it has been established that human macrophages require a signal two, such as extracellular ATP or nigericin, to secrete mature IL-1ß (Perregaux & Gabel, 1994; Swanson et al., 2019), until now, it was not known at which stage of their differentiation towards macrophages human monocytes lose their ability to produce IL-1ß following primary activation. Based on the data presented here, it appears that even without a differentiation factor, such as M-CSF, classical

monocytes already adopt an inhibitory mechanism after 24 hours in culture to prevent sporadic IL-1ß production following TLR4 activation. The involvement of caspase 8 in regulating this mechanism appears to be crucial since caspase 8 inhibition using a small molecule inhibitor led to the inability of cells to produce mature IL-1ß without a second signal. A recent study in human monocyte derived macrophages showed that cigarette smoke inhibits the activation of the canonical NLRP3 inflammasome whilst simultaneously promoting caspase 8 upregulation via TRIF pathway activation, thereby allowing for pathological chronic IL-1ß secretion in the lungs of smokers following primary LPS stimulation without the need for a signal two (Buscetta et al., 2020). This intriguing newly discovered function of caspase 8 demonstrates that in certain pathological states, a mechanism resembling the monocyte alternative inflammasome can cause chronic IL-1ß secretion by macrophages, which also sheds some light on why longer-lived cells, such as macrophages, possess a more stringent regulation on mature IL-1ß production and secretion compared to short-lived cells such as monocytes.

It was thus quite intriguing to observe the lack of IL-1ß tolerisation, shown in this chapter, when monocytes were subjected to 24-hour primary activation with FBG-C, as it effectively allowed those cells to produce double the amount of IL-1ß compared to LPS-activated cells. When activated with LPS, cells produced IL-1ß directly via the alternative inflammasome but were then tolerant to further LPS stimulation even when triggered for canonical NLRP3 activation by nigericin. In contrast, FBG-C-activated cells did not acquire such tolerance, and after secretion of IL-1ß directly following TLR4 activation, they were still able to activate the canonical inflammasome 24 hours later. Since FBG-C has been shown as a crucial requirement for the generation of certain murine RA models (Midwood et al., 2009), it is possible to see how dysregulation of tenascin-c expression following joint damage could exacerbate the severity of the proinflammatory microenvironment within the joint by causing infiltrating blood monocytes to secrete abnormal levels of IL-1ß following repeated activation by FBG-C and other damage signals such as extracellular ATP.

To understand the differences between LPS and FBG-C activation that led to this distinct tolerisation phenotype, the specifics of TLR4 signalling following ligand binding must be considered. The expression of proinflammatory cytokine genes

after LPS activation of TLR4 is a result of signalling through the MyD88-dependent pathway which leads to NF-kB activation (Lu et al., 2008). Detection of LPS by TLR4 also initiates the MyD88-independent pathway via the adaptor protein TRIF and signalling through TRIF results in IRF3-induced expression of type I interferons (Sakai et al., 2017). It has been shown that similarly to LPS, the induction of cytokine synthesis in human and mouse fibroblasts by FBG-C requires MyD88 engagement (Midwood et al., 2009); however, it is unclear if the TRIF-dependent pathway is also activated by FBG-C. Furthermore, both FBG-C and LPS have been shown to activate NF-kB and MAPK pathways upon recognition by TLR4, but they elicit two distinct phenotypes in human monocyte-derived macrophages (Piccinini et al., 2016), suggesting different signalling outcomes via signalling through the same receptor. It can then be speculated that the generation of canonical NLRP3 inflammasome tolerisation by LPS depends on the activation of specific components of the TLR4 pathway (e.g. the TRIF pathway) that are not triggered by FBG-C. Additionally, in contrast to previous findings showing that the CD14 co-receptor involved in LPS signalling via TLR4 is not necessary for FBG-C activation of human myeloid cells (Midwood et al., 2009), my work demonstrated that FBG-C could not induce robust cytokine production in *CD14* KO monocytes. Therefore, differences in CD14 requirement cannot explain the lack of inflammasome tolerance after FBG-C activation. It is possible, however, that another currently unknown co-receptor is required for TLR4 activation by FBG-C, which might inhibit part of the signal transduction cascade that is responsible for the induction of inflammasome tolerance.

Overall, in this chapter of my work, I uncovered key differences in the way primary human monocytes perceive pathogen versus damage signals, in addition to revealing some profound changes in their readiness to produce the pleiotropic proinflammatory cytokine IL-1ß over a 24-hour period. Both of these findings can be used to inform the way human monocytes are viewed in their function as immune effector cells and in their role in inflammatory disease commencement and pathogenesis.

Chapter 4 Investigating the role of the immunometabolite itaconate and its enzyme ACOD1 in the establishment of NLRP3 inflammasome tolerance in human monocytes under different culture conditions and disease states.

4.1 Introduction

Recent studies have examined the role of the immunomodulatory metabolite itaconate and its enzyme ACOD1 in regulating the NLRP3 inflammasome (Bambouskova et al., 2021; Hooftman et al., 2020). In the study conducted by Hooftman and colleagues, high concentrations of the cell-permeable itaconate derivative, 4-octyl itaconate, were added to BMDMs following LPS stimulation, 45 minutes before the addition of nigericin, to examine the effects of exogenous itaconate on canonical inflammasome activation. They found that the addition of 4-OI at that crucial activation stage inhibited NLRP3 inflammasome activation but not the AIM2 or NLRC4 inflammasomes. Specifically, 4-OI was found to inhibit pyroptosis, ASC speck formation and both Gasdermin D and pro-IL-1ß cleavage, which are all downstream consequences of NLRP3 inflammasome assembly and activation. The study then demonstrated that due to its electrophilic properties, 4-OI directly modifies cysteine 548 on NLRP3 via dicarboxypropylation (also known as itaconation), which prevents its interaction with the essential inflammasome modulator NEK7, thereby inhibiting inflammasome assembly. Lastly, 4-OI also inhibited NLRP3-dependent IL-1ß release from PBMCs isolated from patients with cryopyrin-associated periodic syndrome (CAPS).

Using a different approach, Bambouskova and colleagues studied the effects of natural endogenous itaconate on NLRP3 inflammasome activation by comparing WT BMDMs to BMDMs from an ACOD1 KO mouse model. Even though ACOD1 is one

of the most highly upregulated genes following TLR4 activation, they found that a physiological level of natural itaconate accumulated over 3 hours of LPS activation was not able to inhibit NLRP3 inflammasome activation. This inability is thought to be due to the decreased electrophilic properties of natural itaconate in comparison to the artificial derivative 4-OI. However, the study was able to demonstrate that if given sufficient time to accumulate intracellularly, natural itaconate can also become a potent inhibitor of inflammasome activation. Specifically, it was demonstrated that 24 hours of LPS activation in BMDMs is long enough for endogenous itaconate to reach a critical intracellular concentration that enables it to modify cysteine residues on Gasdermin D, thereby preventing Gasdermin D cleavage to its active pore-forming N-terminal domain and thus inhibiting pyroptosis mediated IL-1ß secretion. These experimental observations are quite intriguing, as they suggest that although natural itaconate cannot suppress canonical inflammasome activation immediately after TLR4 activation, the upregulation of ACOD1 caused by the TLR4 signalling serves as a delayed negative feedback tolerisation mechanism, which leads to the prevention of further inflammasome activation upon restimulation 24 hours after primary activation.

Based on my experimental observations shown in section 3.2.3, which uncovered that human monocytes exhibit inflammasome tolerance following 24 hours of LPS activation (Figure 3.11 E), I hypothesised that, similarly to BMDMs, human cells could utilise ACOD1 upregulation and itaconate accumulation as a mechanism for NLRP3 inflammasome desensitisation. Moreover, I hypothesised that this mechanism of intracellular itaconate accumulation is insufficiently promoted by FBG-C activation, which could explain the inability of FBG-C to induce inflammasome tolerance. To test this hypothesis, I examined the intracellular expression levels of the ACOD1 enzyme following both LPS and FBG-C monocyte activation. I also investigated the involvement of the pore-forming protein Gasdermin D in the establishment of inflammasome tolerance in primary human cells and whether additional environmental stressors, such as low oxygen availability, could impact the regulation of IL-1ß secretion and tolerance. Finally, I explored the clinical relevance of my findings by applying the different tolerisation assays to primary human monocytes isolated from the blood of active RA patients.

4.2 Results

4.2.1 Involvement of ACOD1 in the establishment of NLRP3 inflammasome tolerance in human monocytes

As shown in section 3.2.3, 24 hours of LPS but not FBG-C activation was able to tolerise IL-1ß secretion in primary human monocytes (Figure 3.11 E). To investigate whether the inability of FBG-C to tolerise IL-1ß secretion was due to a delay in ACOD1 upregulation and subsequent delay in itaconate accumulation, the effect of LPS and FBG-C monocyte activation on the upregulation of ACOD1 was examined. Primary human monocytes were isolated from healthy donor blood and activated using either stimulus for 36 hours. Cells were lysed at 2, 4, 16, 24, and 36 hours following activation and lysates were used for ACOD 1 western blotting (Figure 4.1 A). Data from three independent donors revealed that LPS-activated monocytes produce ACOD1 at 2 hours following activation, while FBG-C-activated cells did not show any signs of ACOD1 production even after 4 hours of activation (Figure 4.1 A). To investigate the effect of delayed ACOD1 upregulation on the cleavage and activation of the pyroptosis gatekeeper Gasdermin D, 24 and 36 hours following primary TLR4 activation, a portion of the cells were restimulated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 μM nigericin activation. At the end of the reactivation period, cells were lysed, and lysates were used for Gasdermin D western blotting (Figure 4.1 B). Data from three independent healthy donors revealed that 24-hour LPS activation prevented the cleavage of Gasdermin D to its active form upon restimulation and nigericin activation (Figure 4.1 B). In contrast, 24-hour FBG-C activation was unable to achieve tolerisation of Gasdermin D cleavage (Figure 4.1 B). Interestingly, 36-hour FBG-C activated cells were able to tolerise Gasdermin D cleavage, which aligns with the observation that FBG-C triggered ACOD1 upregulation occurred 4 to 16 hours later than in LPS activated cells (Figure 4.1 B). Based on these observations, it can be hypothesised that due to the delayed ACOD1 upregulation, 24-hour FBG-C activation was not sufficient to allow itaconate to reach the sufficient intracellular concertation, allowing it to modify Gasdermin D and to prevent its cleavage.

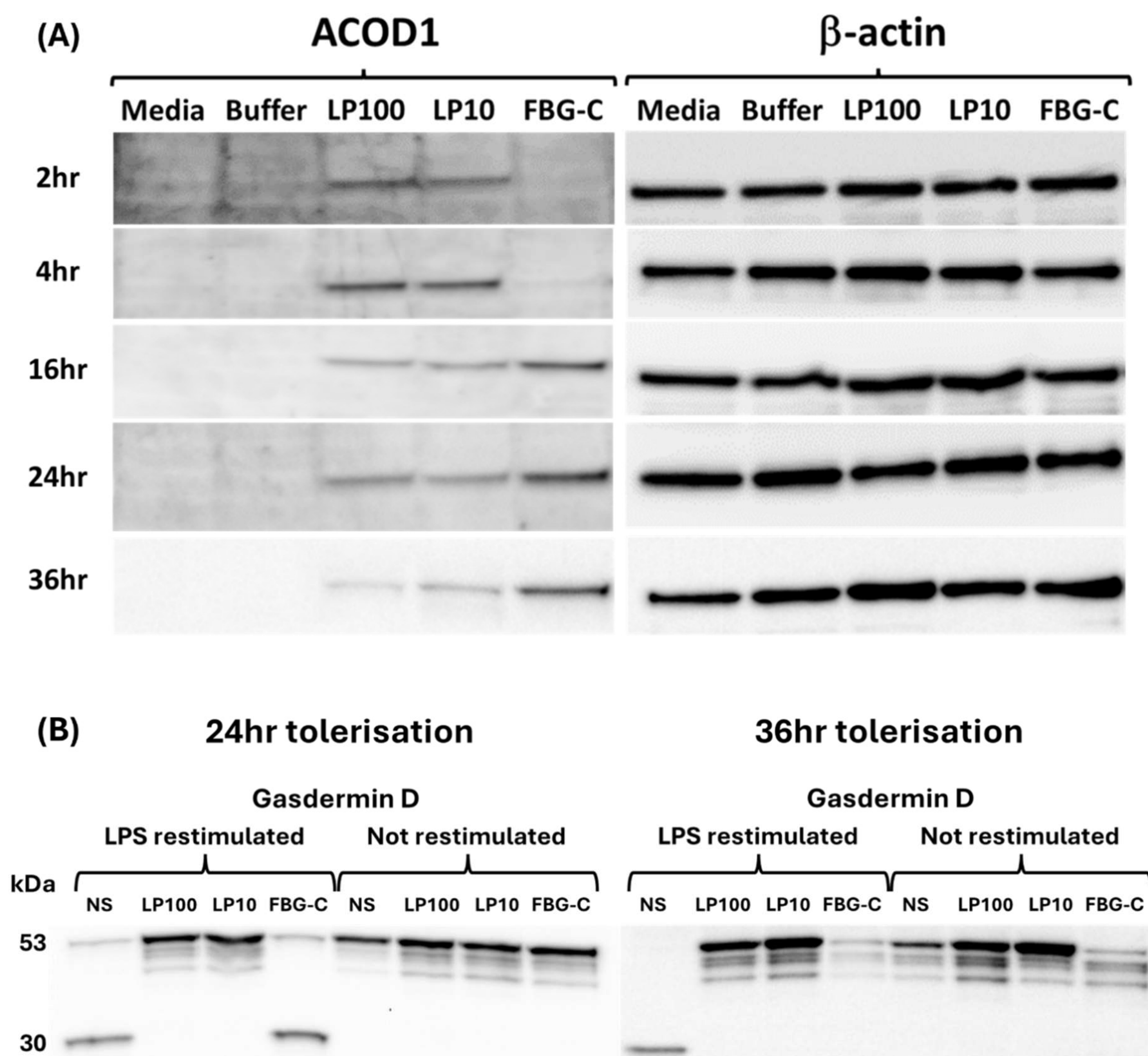


Figure 4.1 Effect of LPS vs FBG-C activation on ACOD1 upregulation and Gasdermin D cleavage in primary human monocytes. Primary human monocytes were isolated and either left without activation in media alone or in media with TN buffer (Media/Buffer: non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1 μM concentration for 24 or 36 hours (24hr tolerised/36hr tolerised). Cells were lysed at 2, 4, 16, 24 and 36 hours following activation and lysates were used for western blotting for the itaconate-producing enzyme ACOD1. At 24 and 36 hours a portion of the cells were also restimulated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 μM nigericin. At the end of the reactivation period, cells were lysed, and lysates were used for western blotting for the pyroptosis gatekeeper Gasdermin D. In panel **(A)** are shown ACOD1 western blots at the different time points following LPS vs FBG-C activation, along with β-actin used as a housekeeper control. In panel **(B)** are shown Gasdermin D western blots of cells which were either not restimulated or restimulated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 μM nigericin, after 24 or 36 hours of primary LPS or FBG-C activation.

In order to compare the IL-1ß tolerisation profiles of 24- and 36-hour FBG-C activated monocytes and to confirm that Gasdermin D cleavage tolerisation is responsible for IL-1ß secretion tolerisation, at the end of the 3-hour LPS and 45 minutes nigericin reactivation period, culture supernatants were taken, and cytokine levels were measured by ELISA (Figure 4.2). These data revealed that, unlike 24 hours of FBG-C activation, 36 hours of FBG-C activation was able to tolerise canonical NLRP3 mediated IL-1ß secretion (Figure 4.2 B). This suggests that in this experimental setting, canonical NLRP3-mediated IL-1ß tolerisation of human monocytes is dependent on Gasdermin D cleavage tolerisation. As expected, differences in Gasdermin D cleavage had no effect on TNF tolerisation (Figure 4.2 D). Additionally, the data demonstrated that there was no additional increase in the absolute amount of TNF or IL-1ß in the culture media after 24 hours of primary LPS or FBG-C activation, potentially because the cells did not produce any additional TNF or IL-1ß after 24 hours of activation (Figure 4.2 A and C).

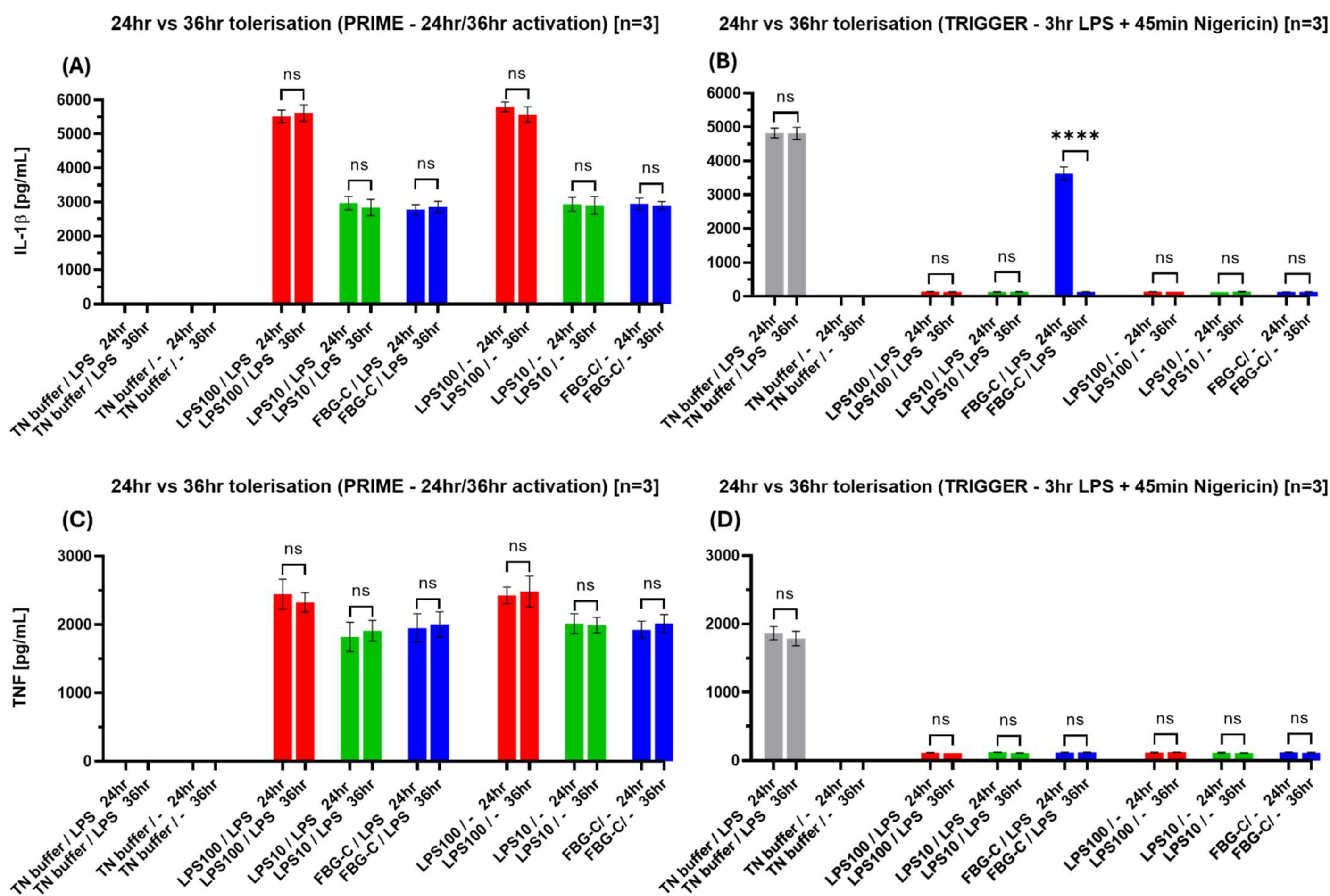


Figure 4.2 Role of ACOD1 and Gasdermin D in LPS vs FBG-C mediated inflammasome tolerisation in primary human monocytes. Primary human monocytes were isolated and either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1 µM concentration for 24 or 36 hours (24hr tolerised/36hr tolerised). At 24 and 36 hours, culture media was replaced with fresh media, and cells were restimulated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 µM nigericin. At the end of the reactivation with LPS and nigericin, supernatants were taken and processed by cytokine ELISA. In panel **(A)** are shown IL-1β levels in culture supernatant at the end of the 24- and 36-hour primary activation cultures (PRIME), measured by ELISA. In panel **(B)** are shown IL-1β levels in culture supernatant following 3 hours of reactivation (TRIGGER) and 45 minutes of nigericin inflammasome activation, measured by ELISA. In panel **(C)** are shown TNF levels in culture supernatant at the end of the 24- and 36-hour primary activation cultures (PRIME), measured by ELISA. In panel **(D)** are shown TNF levels in culture supernatant following 3 hours of reactivation (TRIGGER) and 45 minutes of nigericin inflammasome activation, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors. Data was analysed using paired t-tests between 24- and 36-hour activated conditions, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

To confirm the hypothesis that Gasdermin D cleavage inhibition is dependent on ACOD1 protein expression in the context of 24-hour inflammasome tolerance in primary human monocytes, I generated primary human *ACOD1* knock-out cells via a mechanism utilising *ACOD1*-guided Streptococcus pyogenes Cas9 endonuclease (see methods section 2.2.9). Mock KO control cells (WT) underwent the same procedure as KO cells, utilising a non-guided Streptococcus pyogenes Cas9 endonuclease instead. Two hours following the *ACOD1* gene knock-out procedure, population-wide KO success was assessed by *ACOD1* PCR on genomic DNA (Figure 4.3 B), T7 digestion surveyor assay (Figure 4.3 B), and Sanger sequencing of KO vs mock KO cell population genomic DNA (Figure 4.3 C). All three of these genomic DNA KO quality control tests revealed a complete *ACOD1* gene population-wide KO two hours following the KO procedure. Importantly, the intracellular expression of the ACOD1 protein in KO vs mock KO cells following LPS or FBG-C activation was examined by ACOD1 western blotting of cell lysates from 24-hour activated KO vs mock KO monocytes (Figure 4.3 A). ACOD1 western blotting confirmed the genomic DNA KO quality control results since KO cells did not produce any ACOD1 protein 24 hours following LPS or FBG-C activation.

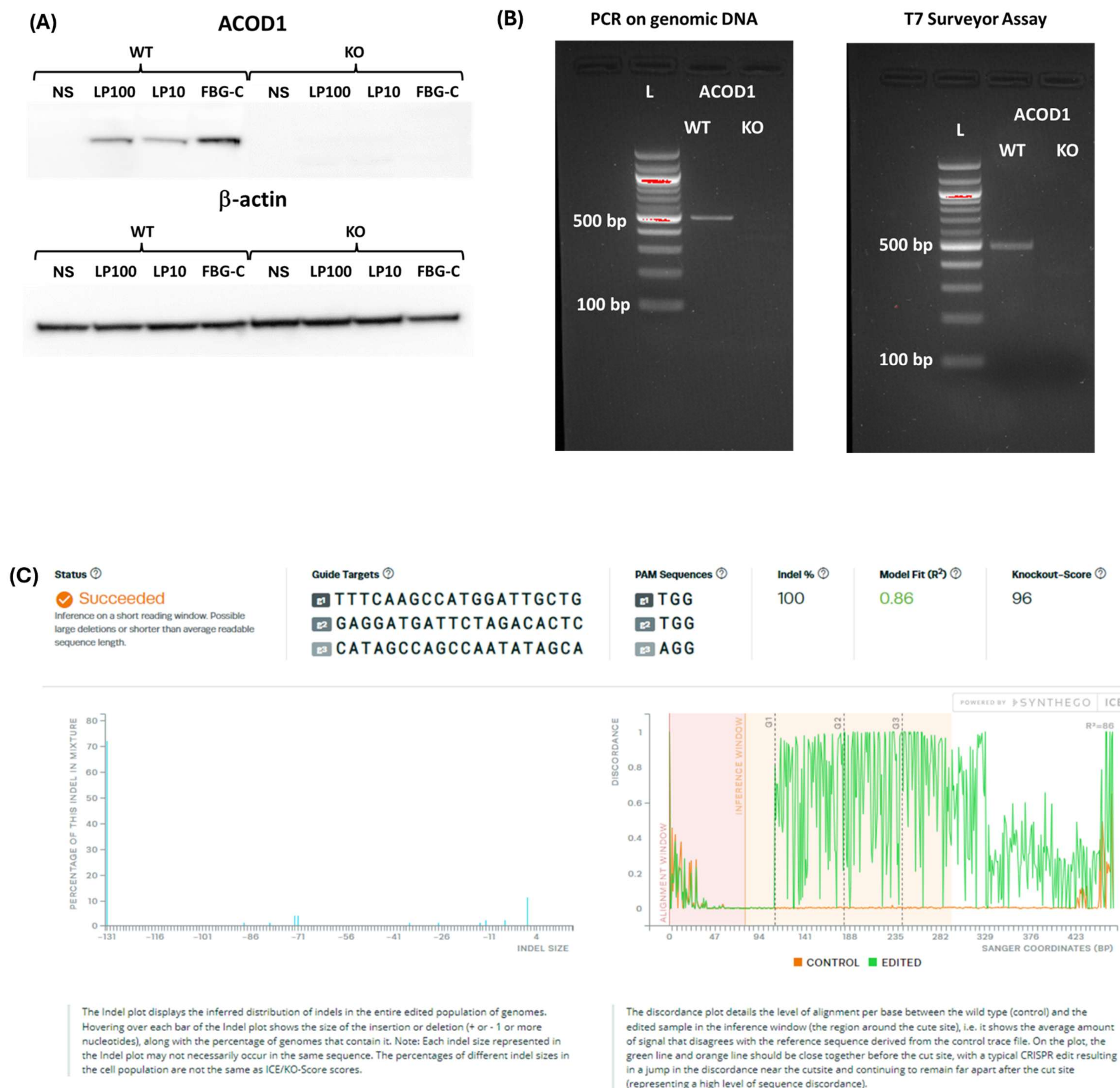


Figure 4.3 Primary human monocyte *ACOD1* population-wide gene knock-out success confirmation. Primary human monocytes were isolated, and the *ACOD1* gene was knocked out via a mechanism utilising *ACOD1*-guided Streptococcus pyogenes Cas9 endonuclease (see methods section 2.2.9). Mock KO control cells (WT cells) underwent the same procedure as KO cells utilising a non-guided Streptococcus pyogenes Cas9 endonuclease instead. Two hours following the *ACOD1* gene knock-out (KO) procedure, population-wide KO success was assessed by *ACOD1* PCR on KO vs mock KO cell population genomic DNA, T7 digestion surveyor assay and Sanger sequencing of KO vs mock KO cell population genomic DNA. Additionally, the intracellular

expression of the ACOD1 protein in KO vs mock KO cells following a 24-hour LPS or FBG-C activation was examined by ACOD1 western blotting of cell lysates from 24-hour activated KO vs mock KO monocytes. The results from the ACOD1 protein western blot are shown in panel **(A)** and the results from the *ACOD1* gene PCR and T7 surveyor assay are shown in panel **(B)**. The assessment of the gene editing efficiency, conducted using the ICE CRISPR Analysis Tool (see methods section 2.2.16), following the Sanger sequencing of KO vs mock KO monocytes, is presented in panel **(C)**.

Since both genomic DNA and protein level examination of *ACOD1* KO monocytes revealed that KO cells are ready for experimental activation 2 hours following the KO procedure, inflammasome tolerisation experiments involving *ACOD1* KO cells were conducted exactly 2 hours following the KO procedure to minimise the effects of extended cell culture described in section 3.2.2. To examine the effect of *ACOD1* gene knock-out on the establishment of LPS and FBG-C mediated inflammasome tolerance, primary human monocytes were isolated and the *ACOD1* gene was knocked out. Exactly 2 hours after the KO procedure, cells were activated with LPS or FBG-C for 24 hours. After 24 hours, cells were either left as non-reactivated controls or reactivated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 µM nigericin. At the end of the reactivation culture, supernatant was taken, and cells were lysed. Supernatants were then processed by cytokine ELISA, and lysates were used for Gasdermin D western blotting. Data from three independent healthy donors revealed that *ACOD1* KO monocytes entirely lost their ability to inhibit Gasdermin D cleavage when primed for 24 hours by either LPS or FBG-C and triggered by 45 minutes 5 µM nigericin, even without a 3-hour LPS restimulation (Figure 4.4 A). The inability of the cells to inhibit Gasdermin D cleavage completely deprived them of their ability to tolerise the canonical NLRP3 inflammasome mediated secretion of IL-1ß when activated for 24 hours by LPS followed by 45 minutes of 5 µM nigericin stimulation, even without a 3-hour LPS restimulation (Figure 4.4 C). This data highlights the crucial importance of itaconate in the regulation of IL-1ß secretion and demonstrates that lack of ACOD1 leaves human monocytes in a primed state for nigericin inflammasome activation over an extended period. As described earlier, FBG-C stimulation over 24 hours was unable to tolerise the canonical NLRP3 inflammasome mediated secretion of IL-1ß, and the absence of the *ACOD1* gene did not lead to a further increase of IL-1ß secretion (Figure 4.4 C),

supporting the hypothesis that the delay in ACOD1 upregulation following primary FBG-C activation is responsible for the inability of FBG-C to tolerise the NLRP3 inflammasome in human monocytes after 24 hours of activation. It is important to note that *ACOD1* KO cells produced the same amount of IL-1β over the first 24-hours of activation as WT cells, indicating that endogenous itaconate has no direct effect on alternative inflammasome activation (Figure 4.4 B). Additionally, as expected, the absence of ACOD1 did not perturb either the 24-hour primary secretion or the tolerisation of TNF (Figure 4.4 D and E).

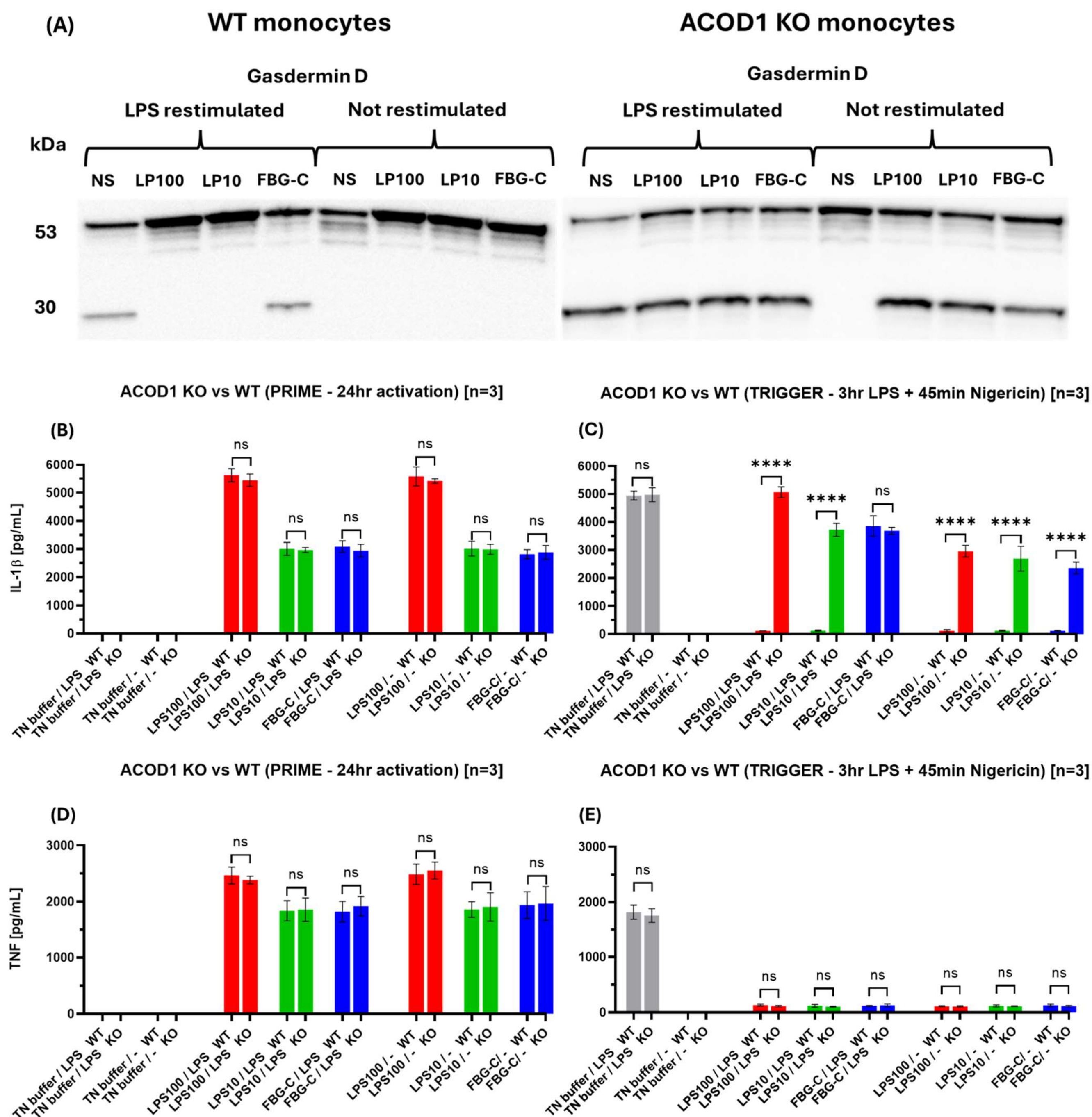


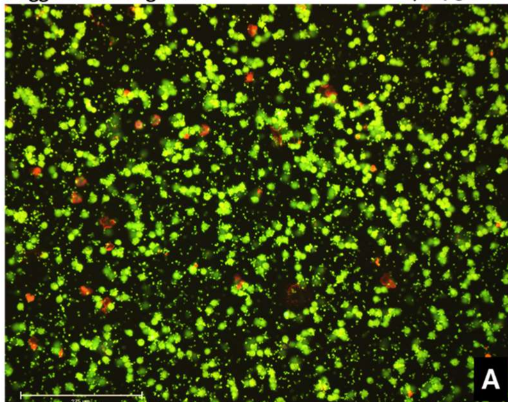
Figure 4.4 Effect of *ACOD1* gene knock-out on the establishment of LPS and FBG-C mediated inflammasome tolerance in primary human monocytes. Primary human monocytes were isolated, and the *ACOD1* gene was knocked out via a mechanism utilising *ACOD1*-guided Streptococcus pyogenes Cas9 endonuclease (see methods section 2.2.9). Mock KO control cells (WT cells) underwent the same procedure as KO cells, utilising a non-guided Streptococcus pyogenes Cas9 endonuclease instead. Two hours following the *ACOD1* gene knock-out (KO) procedure, cells were either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1μM concentration for 24 hours (tolerised). After 24 hours, culture media was replaced with fresh media, and cells were left to rest for one hour before either experiencing reactivation with 10 ng/mL LPS or being left as non-reactivated controls. After 3 hours, nigericin at a 5 μM

concentration was added to the cell culture for 45 minutes to trigger inflammasome activation. At the end of the reactivation culture, supernatant was taken, and cells were lysed. Supernatants were then processed by cytokine ELISA and lysates were used for western blotting for the pyroptosis gatekeeper Gasdermin D. In panel **(A)** are shown Gasdermin D western blots of 3-hour LPS restimulated and non-restimulated KO and mock KO (WT monocytes) cells following 24 hours of primary LPS or FBG-C activation and 45 minutes of 5 μM nigericin activation. In panel **(B)** are shown IL-1ß levels in culture supernatant at the end of the 24-hour primary activation (PRIME) of KO and WT cells, measured by ELISA. In panel **(C)** are shown IL-1b levels in culture supernatant following 3 hours of reactivation (TRIGGER) and 45 minutes of nigericin inflammasome activation of KO and WT cells, measured by ELISA. In panel **(D)** are shown TNF levels in culture supernatant at the end of the 24-hour primary activation (PRIME) of KO and WT cells, measured by ELISA. In panel **(E)** are shown TNF levels in culture supernatant following 3 hours of re-activation (TRIGGER) and 45 minutes of nigericin inflammasome activation of KO and WT cells, measured by ELISA. Data are shown as mean ± SD from three independent healthy donors. Data was analysed using unpaired t-tests between KO and WT activation conditions, *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001.

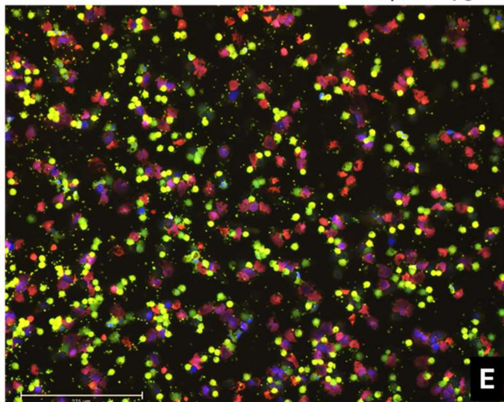
To investigate whether the *ACOD1* KO mediated inhibition of Gasdermin D cleavage promotes lack of IL-1ß secretion tolerance via the induction of pyroptosis, KO and mock KO cells were stained with a fluorescence viability kit at the end of culture comprising Calcein-AM (green fluorescence), Ethidium homodimer-1 (red fluorescence) and Hoechst (blue fluorescence). Cells visible in the green fluorescence channel had their extracellular membrane intact, while cells visible in the red and blue fluorescence channels had their DNA and nucleus exposed and thus were in the process of undergoing pyroptosis (Figure 4.5). The pyroptosis fluorescence imaging data revealed that in all *ACOD1* KO conditions in which Gasdermin D cleavage was not inhibited, cells underwent high levels of pyroptosis (Figure 4.5 E, F, G and H). Additionally, as previously described, 24-hour FBG-C activation was unable to promote Gasdermin D cleavage inhibition in WT monocytes, which was reflected by similar pyroptosis levels in WT and KO FBG-C-activated monocytes (Figure 4.5 B and F).

WT monocytes | **ACOD1 KO monocytes**

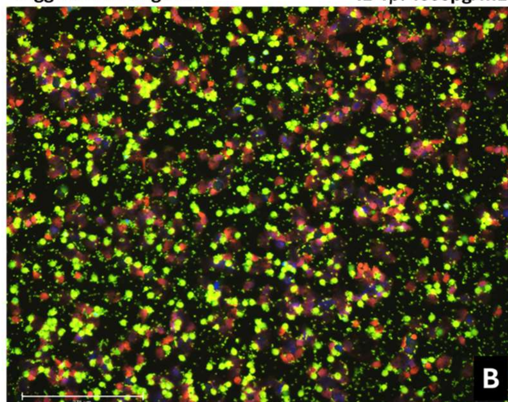
Prime: LPS 10ng/mL
Trigger: LPS 10ng/mL
IL-1β: 0pg/mL



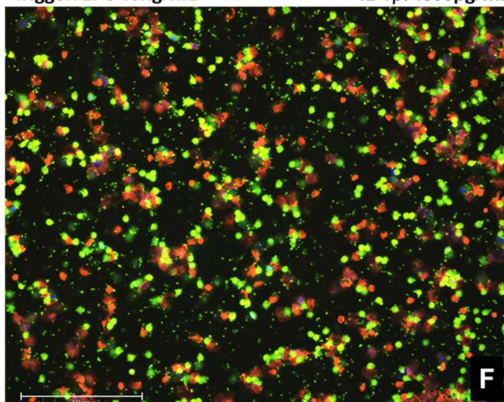
Prime: LPS 10ng/mL
Trigger: LPS 10ng/mL
IL-1β: 4000pg/mL



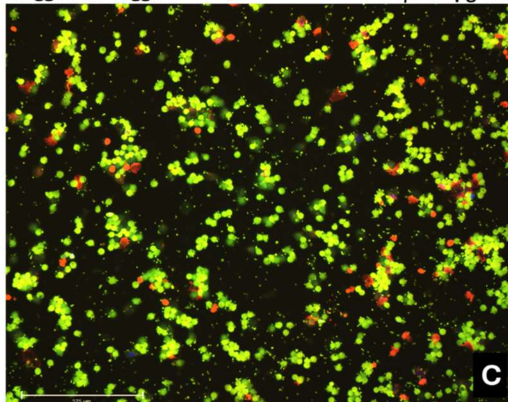
Prime: FBG-C 1μM
Trigger: LPS 10ng/mL
IL-1β: 4000pg/mL



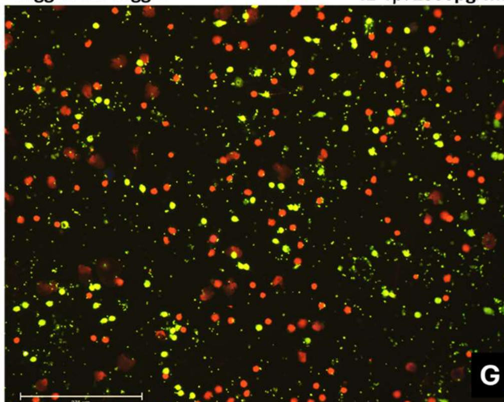
Prime: FBG-C 1μM
Trigger: LPS 10ng/mL
IL-1β: 4000pg/mL



Prime: LPS 10ng/mL
Trigger: No trigger
IL-1β: 100pg/mL



Prime: LPS 10ng/mL
Trigger: No trigger
IL-1β: 2500pg/mL



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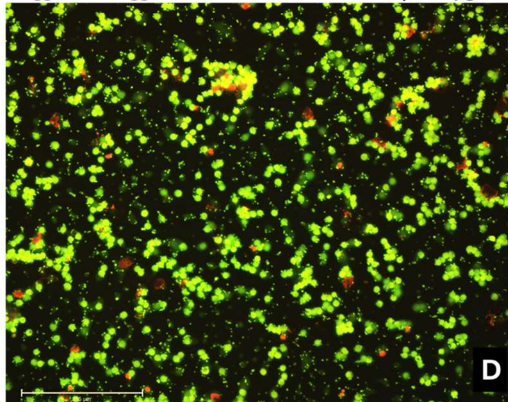
Trigger: No trigger
IL-1β: 100pg/mL



Prime: FBG-C 1μM
Trigger: No trigger
IL-1β: 2500pg/mL

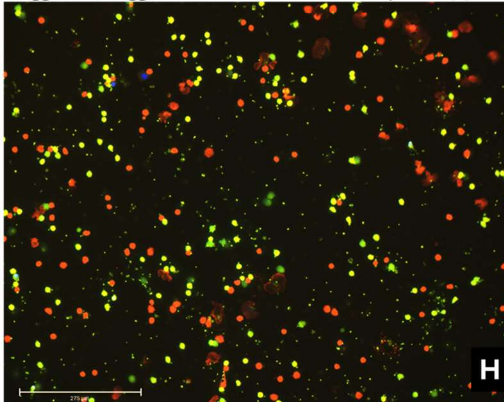


Figure 4.5 Effect of ACOD1 gene knock-out on Gasdermin D mediated pyroptosis. Primary human monocytes were isolated, and the *ACOD1* gene was knocked out via a mechanism utilising *ACOD1*-guided Streptococcus pyogenes Cas9 endonuclease (see methods section 2.2.9). Mock KO control cells (WT cells) underwent the same procedure as KO cells, utilising a non-guided Streptococcus pyogenes Cas9 endonuclease instead. Two hours following the *ACOD1* gene knock-out (KO) procedure, cells were either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1 µM concentration for 24 hours (tolerised). After 24 hours, culture media was replaced with fresh media, and cells were left to rest for one hour before either experiencing reactivation with 10 ng/mL LPS or being left as non-reactivated controls. After 3 hours, nigericin at a 5 µM concentration was added to the cell culture for 45 minutes to trigger inflammasome activation. At the end of the reactivation culture, cells were stained with viability markers designed to show cells undergoing cell death, such as pyroptosis (Green: Calcein-AM; Red: Ethidium homodimer-1; Blue: Hoechst). In panels **A to D** are shown WT monocyte fluorescence microscopy viability/pyroptosis images. In panels **E to H** are shown ACOD1 KO monocyte fluorescence microscopy viability/pyroptosis images of the equivalent activation conditions shown for WT cells. Representative images are shown from three independent healthy donors.

4.2.2 NLRP3 inflammasome tolerance in rheumatoid arthritis patient monocytes

Previous reports have shown that monocytes taken from the blood of RA patients have an altered metabolic state, which primes them for excessive inflammatory responses (McGarry et al., 2021). To investigate whether RA patient monocytes behave differently from healthy monocytes in terms of the immunometabolic itaconate production-mediated NLRP3 inflammasome tolerance, I utilised the tolerance assay on monocytes freshly isolated from active RA patient donor blood in accordance with the terms of the informed consent. Primary monocytes were isolated from the blood of three active RA patients and three age-matched healthy individuals and either left without activation or activated with LPS or FBG-C for 24 hours. After 24 hours, cells were either left as non-reactivated controls or reactivated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 µM nigericin. At the end of the reactivation with LPS and nigericin, supernatants were taken and processed by cytokine ELISA. Indeed, the data revealed that, unlike healthy monocytes, 24-hour LPS-activated RA monocytes

were unable to tolerise IL-1ß secretion to repeated LPS activation (Figure 4.6 B). As previously described, 24-hour FBG-C activation of healthy monocytes is unable to tolerise IL-1ß secretion, and importantly there was no difference in FBG-C tolerisation capacity between healthy and RA monocytes (Figure 4.6 B). Interestingly, unlike *ACOD1* KO monocytes, which were able to activate the NLRP3 inflammasome 24 hours following primary activation without the need for LPS restimulation, RA monocytes had not completely lost their ability to regulate inflammasome activation and hence, similarly to healthy monocytes, RA monocytes did require LPS restimulation in order secrete IL-1ß after 24-hours of primary stimulation (Figure 4.6 B). Additionally, RA monocytes produced the same levels of IL-1ß and TNF in the first 24 hours of LPS and FBG-C activation, indicating that in terms of these two cytokines, the cells did not demonstrate a more aggressive proinflammatory phenotype compared to healthy cells following primary TLR4 activation (Figure 4.6 A and C). Finally, the data revealed that the loss of tolerance phenotype observed is specific for the NLRP3 inflammasome since RA monocytes were still able to tolerise the secretion of TNF after 24-hours of primary TLR4 activation (Figure 4.6 D).

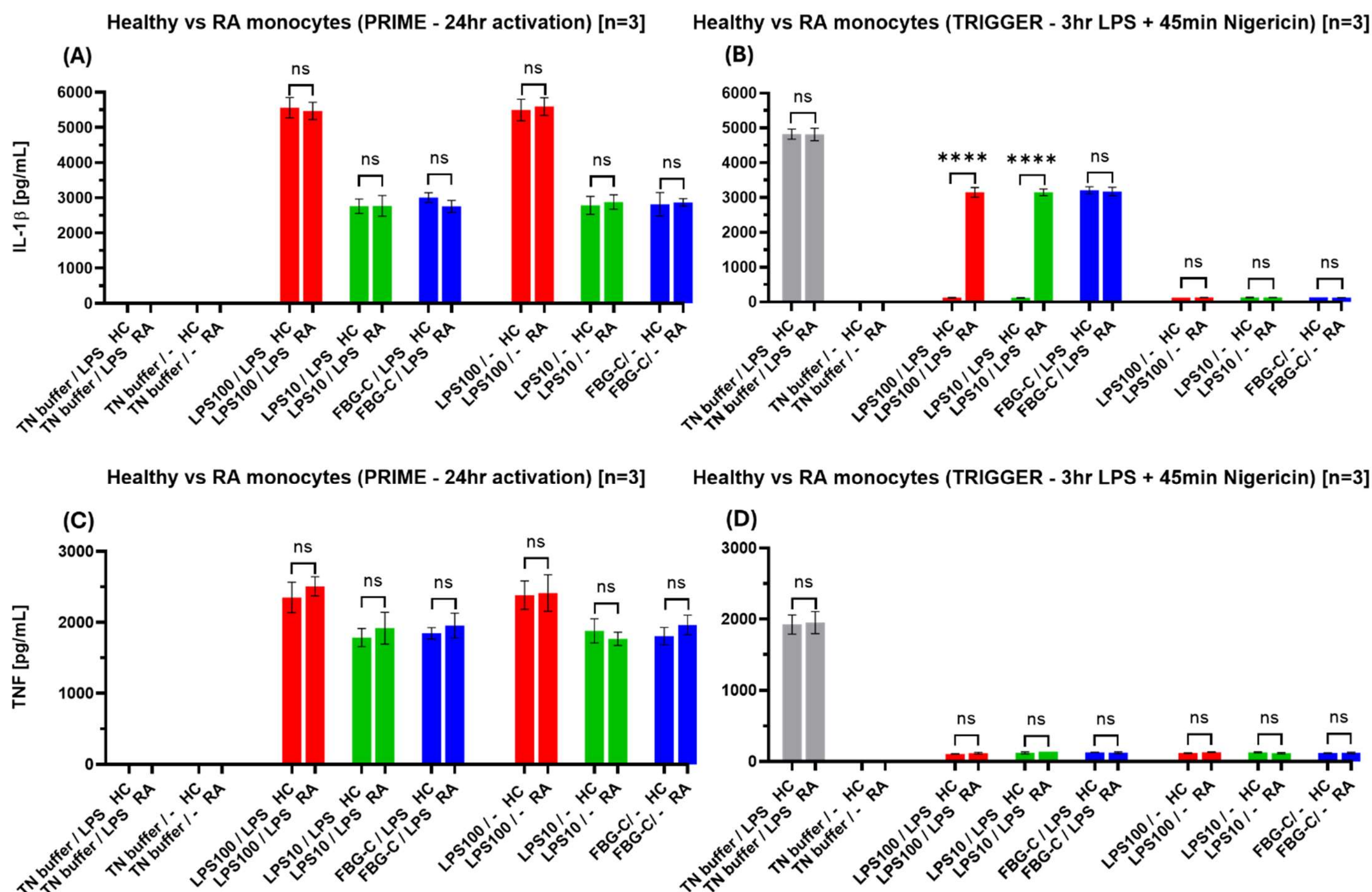


Figure 4.6 RA patient monocytes vs healthy monocytes inflammasome tolerisation. Primary human monocytes were isolated from the blood of three active RA patient donors (RA) and three age-matched healthy donors (HC) and either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1 µM concentration for 24 hours (tolerised). After 24 hours, culture media was replaced with fresh media, and cells were left to rest for one hour before either experiencing reactivation with LPS or being left as non-reactivated controls. Following 3 hours of reactivation, nigericin at a 5 µM concentration was added to the cell culture for 45 minutes to trigger inflammasome activation. At the end of the culture, supernatants were taken and processed by cytokine ELISA. In panel **(A)** are shown IL-1β levels in culture supernatant at the end of the 24-hour primary activation (PRIME) of RA and healthy monocytes, measured by ELISA. In panel **(B)** are shown IL-1β levels in culture supernatant following 3 hours of 10 ng/mL LPS reactivation (TRIGGER) and 45 minutes of nigericin inflammasome activation of RA patient and healthy monocytes, measured by ELISA. In panel **(C)** are shown TNF levels in culture supernatant at the end of the 24-hour primary activation (PRIME) of RA patient and healthy monocytes, measured by ELISA. In panel **(D)** are shown TNF levels in culture supernatant following 3 hours of 10 ng/mL LPS reactivation (TRIGGER) and 45 minutes of nigericin inflammasome activation of RA patient and healthy monocytes, measured by ELISA. Data are shown as mean ± SD from three independent RA patient and age matched healthy donors. Data was analysed using unpaired t-

tests between RA patient and healthy monocyte activation conditions, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To investigate whether the observed lack of IL-1ß tolerisation in RA monocytes following TLR4 activation is mediated by inappropriate Gasdermin D cleavage, caused by a delayed ACOD1 upregulation response, a portion of the activated cells were lysed at 2, 4 and 16 hours following activation and lysates were used for ACOD1 western blotting. Then, at the end of the LPS and nigericin reactivation period, a portion of the cells were lysed, and lysates were used for western blotting for the pyroptosis gatekeeper Gasdermin D, while the rest of the cells were stained with the previously described fluorescence viability kit for the measurement of pyroptosis levels. The generated data revealed that unlike healthy monocytes, which already have intracellular ACOD1 enzyme present at 2 hours following LPS activation (Figure 4.1 A), RA patient monocytes do not produce any ACOD1 until 4 hours after LPS activation (Figure 4.7 A), which was then shown to render the cells unable to inhibit Gasdermin D cleavage following LPS restimulation (Figure 4.7B), providing an explanation for the lack of IL-1ß tolerance demonstrated in figure 4.6 B. The pyroptosis fluorescence imaging then confirmed that the abnormal IL-1ß secretion, caused by the inability of RA patient monocytes to inhibit Gasdermin D processing, was mediated by high levels of pyroptosis in LPS primed and restimulated RA patient monocytes compared to LPS primed and restimulated healthy monocytes (Figure 4.8 A and C). As expected, there was no difference in FBG-C-activated healthy and RA monocyte pyroptosis (Figure 4.8 B and D) since FBG-C stimulated healthy monocytes already have a breach of inflammasome tolerance due to the previously described delay in ACOD1 upregulation (Figure 4.1 A).

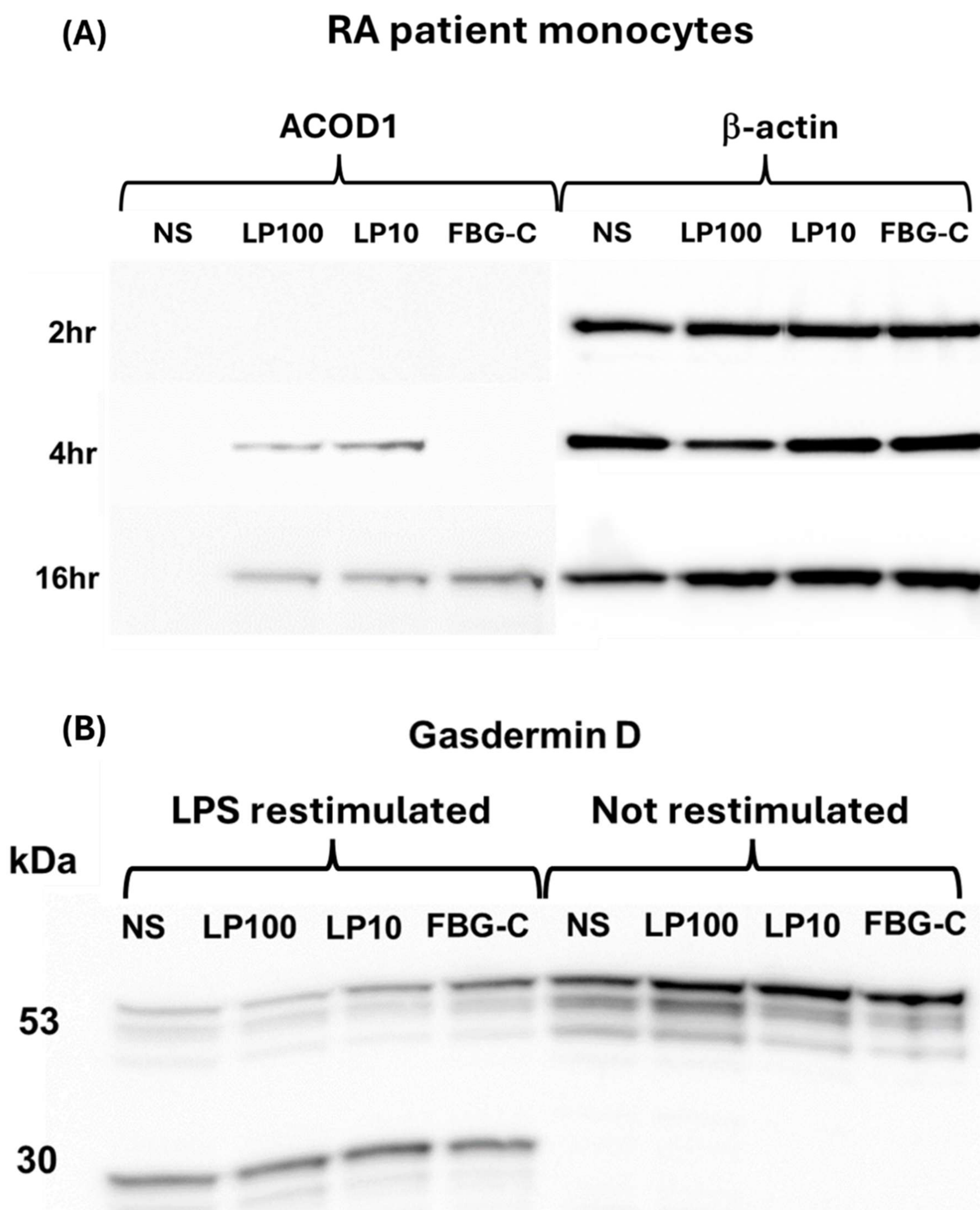
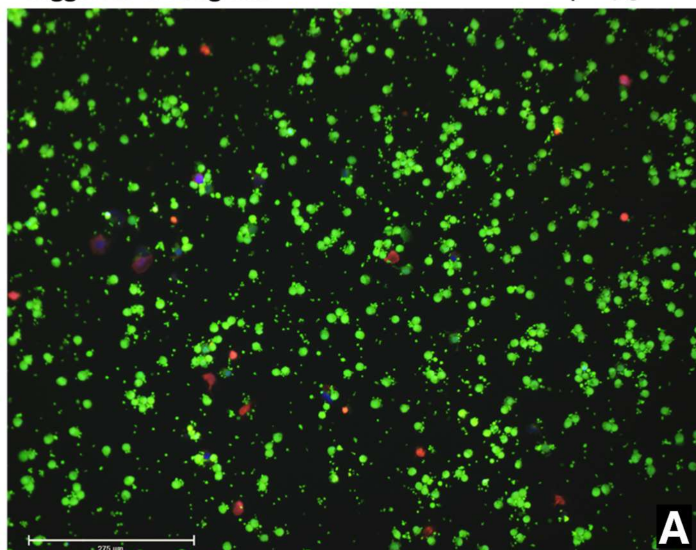


Figure 4.7 Role of ACOD1 and Gasdermin D in LPS and FBG-C mediated inflammasome tolerisation in rheumatoid arthritis patient monocytes. Primary monocytes were isolated from the blood of three active RA patients and either left without activation in media with TN buffer (NS: non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1µM concentration for 24 hours (tolerised). Cells were lysed at 2, 4 and 16 hours following activation and lysates were used for western blotting for the itaconate-producing enzyme ACOD1. At 24 hours, a portion of the cells were restimulated for 3 hours using

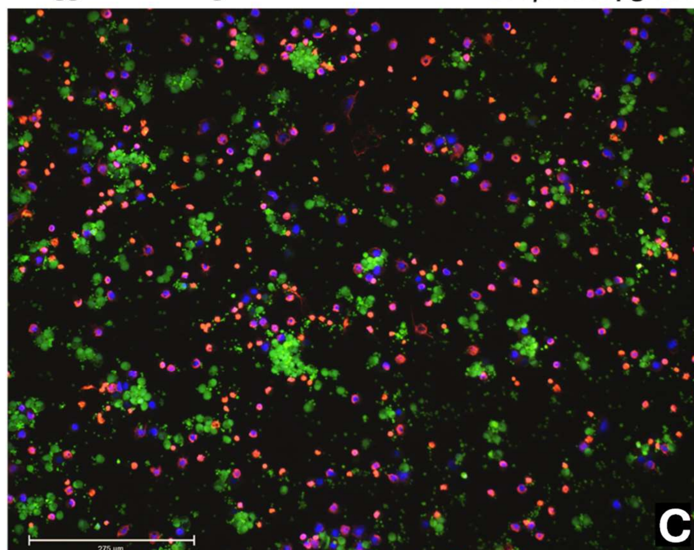
10 ng/mL LPS, followed by 45 minutes of 5 µM nigericin. At the end of the reactivation period, cells were lysed, and lysates were used for western blotting for the pyroptosis gatekeeper Gasdermin D. In panel **(A)** are shown ACOD1 western blots at the different time points following LPS vs FBG-C activation, along with β-actin used as a housekeeper control. In panel **(B)** are shown Gasdermin D western blots of 3-hour LPS restimulated and non-restimulated cells after 24 hours of primary LPS or FBG-C activation. Representative images are shown from three independent RA patient donors.

Healthy monocytes | **RA patient monocytes**

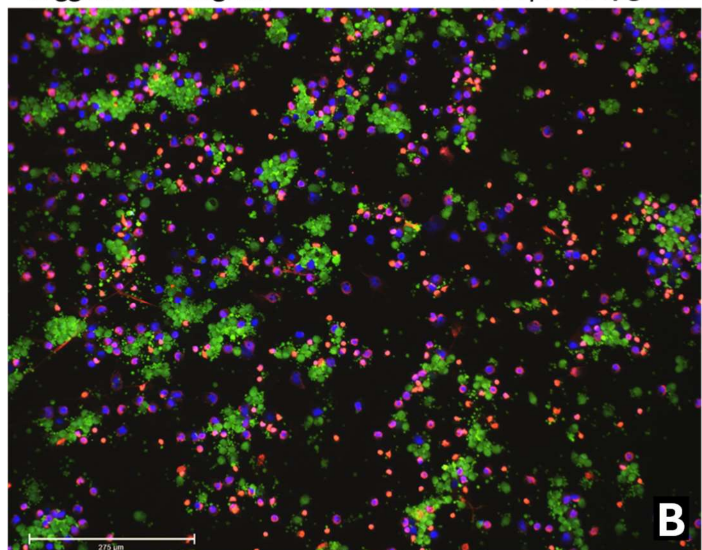
Prime: LPS 10ng/mL
Trigger: LPS 10ng/mL **IL-1β: 0pg/mL**



Prime: LPS 10ng/mL
Trigger: LPS 10ng/mL **IL-1β: 3500pg/mL**



Prime: FBG-C 1µM
Trigger: LPS 10ng/mL **IL-1β: 3500pg/mL**



Prime: FBG-C 1µM
Trigger: LPS 10ng/mL **IL-1β: 3500pg/mL**

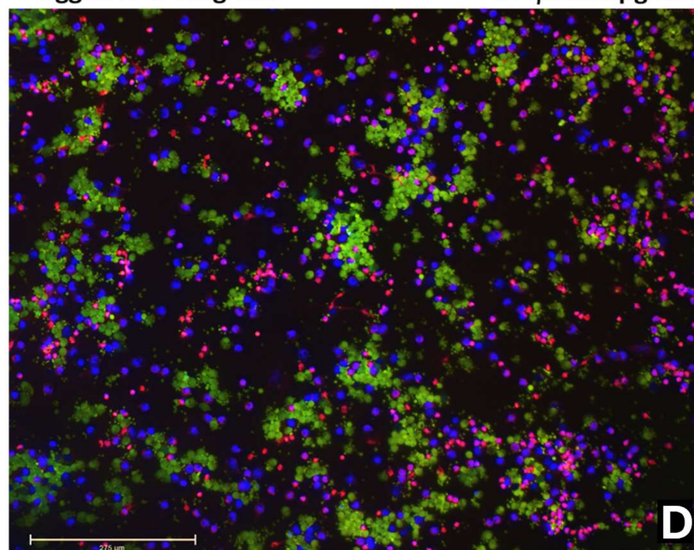


Figure 4.8 Pyroptosis levels of RA patient monocytes vs healthy monocytes in the context of LPS and FBG-C inflammasome tolerisation. Primary monocytes were isolated from the blood of three active RA patients and three age-matched healthy individuals and either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-Cat a 1 µM concentration for 24 hours (tolerised). After 24 hours, culture media was replaced with fresh media, and cells were left to rest for one hour before either experiencing reactivation with 10 ng/mL LPS or being left as non-reactivated controls. Following 3 hours of re-activation, nigericin at a 5 µM concentration was added to the cell culture for 45 minutes to trigger inflammasome activation. At the end of the re-activation culture, cells were stained with viability markers designed to show cells undergoing cell death, such as pyroptosis (Green: Calcein-AM; Red: Ethidium homodimer-1; Blue: Hoechst). In panels **A and B** are shown healthy monocyte fluorescence microscopy viability/pyroptosis images. In panels **C and D** are shown RA patient monocyte fluorescence microscopy viability/pyroptosis images of the equivalent activation conditions shown for healthy cells. Representative images are shown from three independent RA patient and age matched healthy donors.

4.2.3 Effects of hypoxia on human monocyte NLRP3 inflammasome tolerance

The diseased joints of rheumatoid arthritis patients have been shown to possess limited oxygen availability for infiltrating cells (Quiñonez-Flores et al., 2016), so when recruited from the blood into the joint, in addition to other stimuli, RA patient monocytes face the challenge of dealing with different levels of hypoxia. Over the last decade, hypoxia has been shown to have many effects on immune cells, a portion of which are strongly proinflammatory (i.e., the upregulation of NF-kB and STAT signalling in macrophages) (Castillo-Rodríguez et al., 2022). To investigate whether hypoxia could also influence the induction of NLRP3 inflammasome tolerance in human monocytes, I conducted tolerisation experiments in which cells were cultured under 2% oxygen conditions. Primary human monocytes were isolated and either left without activation or activated with LPS or FBG-C. Cells were then immediately placed in an incubator which had its oxygen levels already reduced to 2% (see methods section 2.2.8). To test the effects of hypoxia exclusively on the key inflammasome tolerisation stage of primary 24-hour TLR4 activation, after 24 hours of 2% oxygen culture, all cells were transferred to an incubator with atmospheric oxygen levels (normoxia),

and after 1-hour rest a portion of the cells were restimulated in normoxic conditions for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 μM nigericin. At the end of the reactivation with LPS and nigericin, supernatants were taken and processed by cytokine ELISA. Results from three independent healthy donors revealed staggering levels of IL-1β secretion following nigericin stimulation after 24 hours of primary TLR4 activation in hypoxia, both with and without LPS restimulation (Figure 4.9 B). These results demonstrated that 24-hour hypoxia culture was able to reprogram the ability of cells to downmodulate NLRP3 inflammasome activation, allowing the monocytes to remain primed for inflammasome activation for a period of 24 hours following the initial TLR4 stimulus and abolishing all the inflammasome tolerance effects observed in normoxia cultured cells (Figure 4.9 B). Interestingly, the hypoxic culture environment did not cause an increase in the levels of IL-1β production following primary TLR4 activation in the first 24- hours of culture, highlighting that alternative inflammasome activation is not regulated by oxygen availability (Figure 4.9 A). Surprisingly, hypoxia did not affect TNF 24-hour secretion or TNF tolerisation to repeated stimulation, as hypoxia-cultured cells exhibited the same levels of TNF secretion as normoxia-cultured cells (Figure 4.9 C and D).

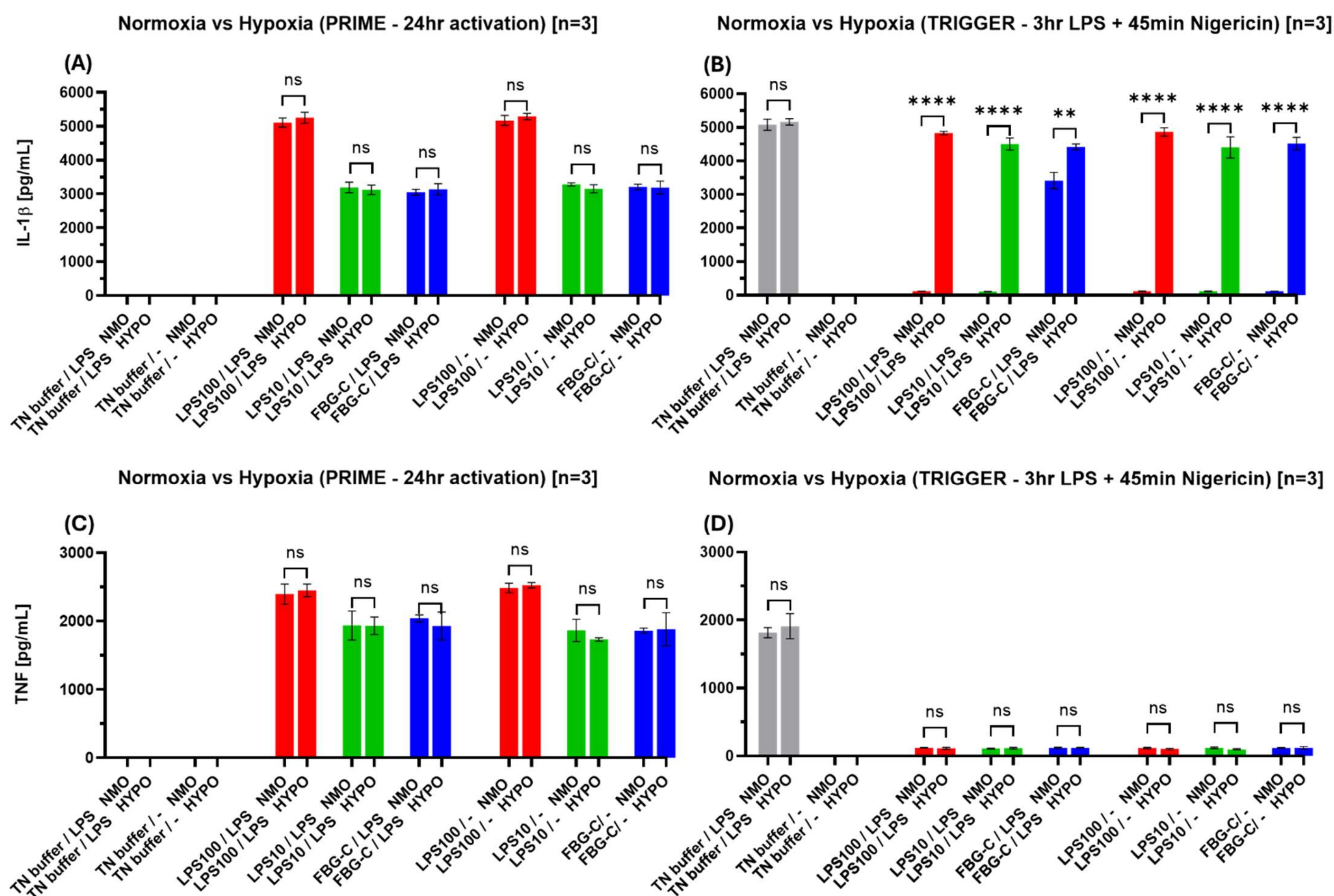
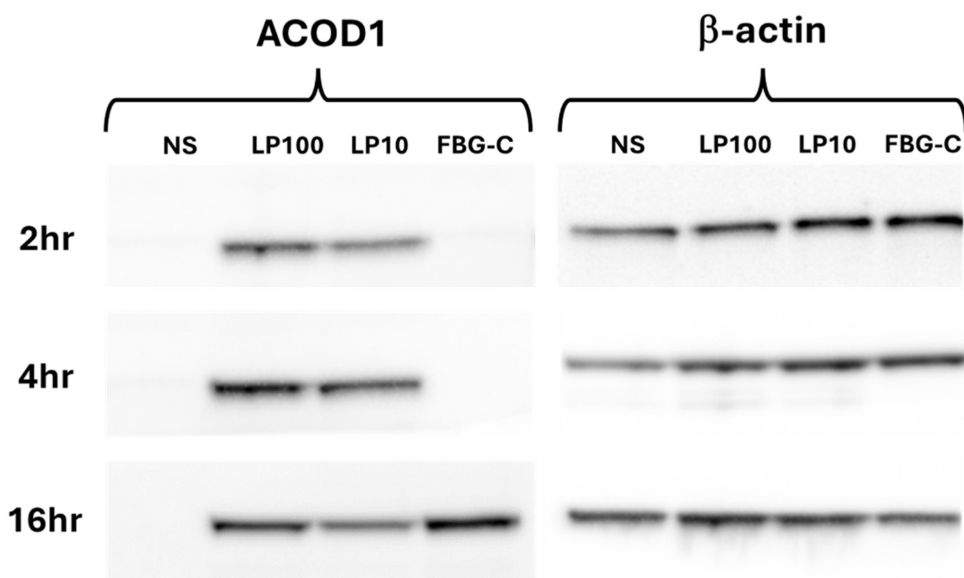


Figure 4.9 LPS vs FBG-C mediated inflammasome tolerisation in 2% oxygen culture of primary human monocytes. Primary human monocytes were isolated and either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1 μM concentration (tolerised). Cells were then immediately placed in an incubator which had its oxygen levels already reduced to 2% (see methods section 2.2.8). After 24 hours of 2% oxygen culture, culture media was replaced with fresh media and all cells were transferred to an incubator with atmospheric oxygen levels (normoxia); after a 1-hour rest, a portion of the cells were restimulated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 μM nigericin. At the end of the reactivation with LPS and nigericin, supernatants were taken and processed by cytokine ELISA. In panel **(A)** are shown IL-1ß levels in culture supernatant at the end of the 24-hour primary activation (PRIME) of normoxic (NMO) and hypoxic (HYPO) cultured monocytes, measured by ELISA. In panel **(B)** are shown IL-1ß levels in culture supernatant following 3 hours of reactivation (TRIGGER) and 45 minutes of nigericin inflammasome activation of normoxic (NMO) and hypoxic (HYPO) cultured monocytes, measured by ELISA. In panel **(C)** are shown TNF levels in culture supernatant at the end of the 24-hour primary activation (PRIME) of normoxic (NMO) and hypoxic (HYPO) cultured monocytes, measured by ELISA. In panel **(D)** are shown levels in culture supernatant following 3 hours of reactivation (TRIGGER) and 45 minutes of nigericin inflammasome activation of normoxic (NMO) and hypoxic (HYPO) cultured monocytes, measured by ELISA. Data are shown as mean ± SD from

three independent donors. Data was analysed using unpaired t-tests between normoxic and hypoxic monocyte activation conditions, $*p < 0.05$, $**p < 0.01$, $*** p < 0.001$, $**** p < 0.0001$.

To investigate whether the hypoxia-mediated reprogramming of NLRP3 inflammasome priming and tolerisation operates via ACOD1 downmodulation and subsequent increase in Gasdermin D cleavage, at 2, 4 and 16 hours of 2% oxygen culture, a portion of the cells were lysed, and lysates were used for ACOD1 western blotting. Additionally, at the end of the LPS and nigericin restimulation period, a portion of the cells were lysed, and lysates were used for Gasdermin D western blotting. Data from three independent healthy donors revealed that hypoxic culture promoted Gasdermin D cleavage in both LPS restimulated and non-restimulated cells following initial 24-hour TLR4 activation (Figure 4.10 B), a Gasdermin D processing phenotype only previously observed in *ACOD1* KO monocytes (Figure 4.4 A). In this experimental setting, however, the enhanced inflammasome activation phenotype was not under the control of ACOD1 downmodulation since hypoxia-cultured monocytes demonstrated the same ACOD1 protein levels following TLR4 activation as normoxia-cultured cells at all investigated time points (Figure 4.10 A). These data highlight that Gasdermin D serves a crucial role as an NLRP3 inflammasome priming and tolerisation gatekeeper, both under the control of itaconate and ACOD1, but also under the control of yet unknown regulation mechanisms capable of sensing different environmental triggers, such as oxygen availability.



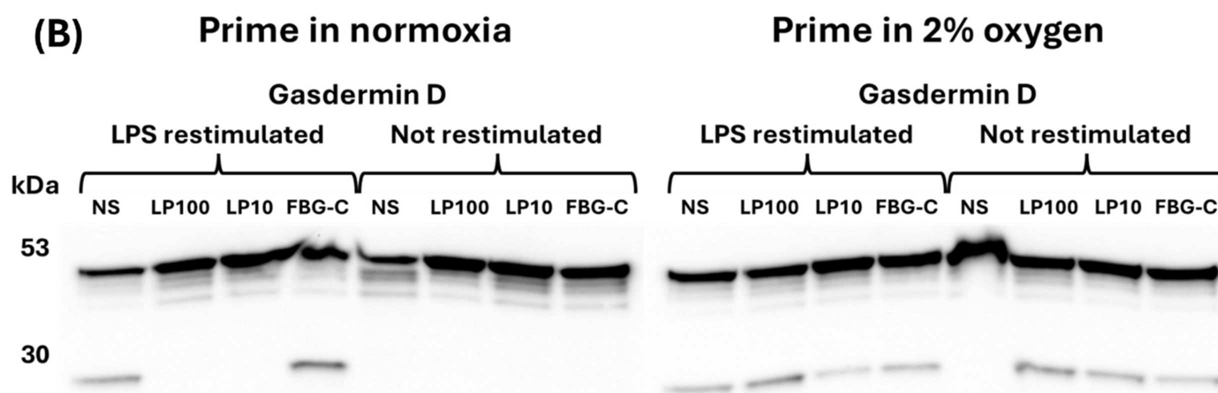


Figure 4.10 Role of ACOD1 and Gasdermin D in LPS vs FBG-C mediated inflammasome tolerisation in 2% oxygen culture of primary human monocytes. Primary human monocytes were isolated and either left without activation in media with TN buffer (non-tolerised) or activated with LPS at two different concentrations (LP100 - 100 ng/mL, LP10 - 10 ng/mL) or FBG-C at a 1 μM concentration (tolerised). Cells were then immediately placed in an incubator which had its oxygen levels already reduced to 2% (see methods section 2.2.8). At 2, 4 and 16 hours of 2% oxygen culture, cells were lysed, and lysates were used for western blotting for the itaconate-producing enzyme ACOD1. After 24 hours of 2% oxygen culture, all cells were transferred to an incubator with atmospheric oxygen levels (normoxia); after a 1-hour rest, a portion of the cells was restimulated for 3 hours using 10 ng/mL LPS followed by 45 minutes of 5 μM nigericin. At the end of the restimulation period, cells were lysed, and lysates were used for western blotting for the pyroptosis gatekeeper Gasdermin D. In panel **(A)** are shown ACOD1 western blots at the different time points following LPS vs FBG-C activation in 2% oxygen, along with β-actin used as a housekeeper control. In panel **(B)** are shown Gasdermin D western blots of

3-hour LPS restimulated and non-restimulated cells after 24 hours of primary LPS or FBG-C activation in 2% oxygen. Representative images are shown from three independent healthy donors.

4.3 Discussion

Previous studies that explored the role of itaconate as an NLRP3 inflammasome regulator in murine macrophages demonstrated that if given enough time to accumulate intracellularly, natural itaconate can become a potent inhibitor of inflammasome activation (Bambouskova et al., 2021). In this chapter, I demonstrated that in primary human monocytes, ACOD1 upregulation following TLR4 activation serves as a timekeeper for NLRP3 inflammasome priming and tolerisation.

Initially, based on data shown in section 3.2.3, I demonstrated that FBG-C was unable to tolerise NLRP3 inflammasome activation in the same way that LPS did after 24 hours of activation (Figure 3.11 E). However, further investigation, presented in this current chapter, revealed that there is a delay in ACOD1 upregulation following FBG-C monocyte activation (Figure 4.1 A), which is responsible for the delay in the ability of FBG-C to tolerise the NLRP3 inflammasome mediated secretion of IL-1ß. Hence FBG-C is indeed able to tolerise the inflammasome, however, after 36 instead of 24 hours of activation (Figure 4.2 B). In terms of RA disease pathogenesis and autoinflammatory chronicity, this 5-to-10-hour delay in ACOD1 upregulation and inflammasome tolerisation following DAMP activation of TLR4 can become catastrophic since monocytes are short-lived cells which experience a range of triggers when recruited to the damaged joint. Repeated activation can occur rapidly and while the PAMP LPS triggers a quick upregulation of ACOD1, and thus NLRP3 inflammasome inhibition, the damage signal FBG-C can trigger multiple events of IL-1ß secretion, first through alternative and then through canonical NLRP3 inflammasome activation. Additionally, the experimental data I presented showed that in monocytes isolated from the blood of active RA patients, there was a general delay in ACOD1 upregulation following not just FBG-C but also LPS TLR4 activation, which caused a subsequent inability of RA monocytes to tolerise IL-1ß secretion. Based on this observation, it can be hypothesised that long-term

RA alters TLR4 signalling responses in monocytes, such that they can no longer rapidly promote the increase of negative feedback mediators of inflammation, such as ACOD1, while producing normal or increased levels of proinflammatory cytokines.

Previous studies conducted using *ACOD1* KO cell lines or mouse models have shown the importance of natural itaconate for the regulation of inflammasome activation (Bambouskova et al., 2021; Hooftman et al., 2020), but at the time of writing this thesis, there have not been any reports of investigations in which primary human *ACOD1* KO cells have been generated. In my research, I used cutting-edge guided Cas9 technology to generate primary human monocyte *ACOD1* KO cells for the first time. This advancement in gene editing technology has allowed me to explore the effects of complete ACOD1 absence directly in primary human cells. I showed that although there is no difference in the first 24-hour cytokine production in *ACOD1* KO cells compared to control cells, drastic differences appeared in their ability to prime and tolerise the NLRP3 inflammasome. Without ACOD1, and thus without itaconate, monocytes were able to remain primed for NLRP3 inflammasome activation substantially longer than control cells and they completely lost their ability to tolerise IL-1ß secretion. *ACOD1* gene KO experiments also allowed me to demonstrate that itaconate exercises its regulatory activity on the NLRP3 inflammasome, at least in part, by preventing the pyroptosis gatekeeper Gasdermin D from being cleaved into its active pore-forming N-terminal domain. I demonstrated that in the absence of ACOD1, Gasdermin D cleavage occurs even after 24 hours following the initial TRL4 trigger when nigericin is added to the culture, without the need for TLR4 restimulation. A similar Gasdermin D processing phenotype was observed following FBG-C activation; however, FBG-C activated cells still required TLR4 restimulation 24 hours after primary TLR4 activation to induce pyroptosis and IL-1ß secretion, as in those cells ACOD1 upregulation was only delayed but not completely abolished.

Additionally, in this chapter, I showed that although ACOD1 does have a crucial role in NLRP3 inflammasome regulation, it is not the sole mechanism responsible for said modulation. The availability of oxygen in the atmosphere of *in vitro* cultured monocytes proved to be a remarkably potent regulator of NLRP3 inflammasome priming and tolerisation. Cells cultured in hypoxic conditions

behaved in a similar manner to *ACOD1* KO cells since they were able to promote Gasdermin D cleavage even after 24 hours following the initial TRL4 stimulation without the need for TLR4 restimulation. Interestingly, this effect was independent of ACOD1 upregulation, as hypoxia did not influence the ability of the cells to rapidly upregulate ACOD1 following primary LPS activation. This effect can in part be attributed to HIF-1α and NF-kB upregulation since both transcription factors have been shown to become upregulated when myeloid cells encounter hypoxic conditions (Fangradt et al., 2012; Quiñonez-Flores et al., 2016). Since NF-kB is responsible for upregulating both the *IL-1*ß and *GSDMD* genes (Liu et al., 2017), it can be hypothesised that higher availability of Gasdemin D and pro-IL-1ß can promote a more sustained NLRP3 inflammasome priming phenotype leading to the observed increased levels of IL-1ß following nigericin activation. Additionally, previous work has shown that nitric oxide can downmodulate NLRP3 inflammasome priming and activation in conjunction with itaconate (Bambouskova et al., 2021). However, in my work, I was unable to see any nitric oxide production in primary human monocytes following TLR4 activation. Very recent studies have also shown that Gasdermin D cleavage can be promoted without the involvement of the NLRP3 inflammasome via a caspase 8 mediated caspase 1 cleavage (Buscetta et al., 2022; Cristaldi et al., 2023), so it is also possible that hypoxic conditions influence Gasdermin D cleavage via NLRP3 inflammasome independent mechanisms.

In the RA joint there are many different triggers such as DAMPs and extracellular ATP from broken down cells, so adding the layer of reduced oxygen availability can lead to catastrophic levels of inflammasome mediated inflammation. As I have demonstrated, both DAMP TLR4 activation and hypoxic culture promote higher levels of Gasdermin D cleavage and pyroptosis-mediated IL-1ß secretion compared to PAMP TLR4 stimulation and normoxic culture conditions. Taken together these observations highlight how both join damage, and diseased joint environmental triggers can, in conjunction or independently, prime monocytes for increased levels of inflammasome activation and IL-1ß secretion.

Overall, in this chapter of my work, I confirmed the hypothesis that ACOD1 and itaconate are drivers of NLRP3 inflammasome tolerance in human monocytes and that the functional effect of itaconate-mediated tolerisation is mediated by Gasdermin D cleavage and pyroptotic pore-formation. I also showed that RA

patient monocytes have a delayed ACOD1-mediated tolerance response to TLR4 activation monocytes, and I demonstrated that major environmental stressors such as hypoxia can trigger loss of tolerance and extended inflammasome priming by yet unknown ACOD1-independent mechanisms.

Chapter 5 General discussion

The danger theory was proposed approximately thirty years ago (Matzinger, 1994), and now the concept of an internally driven immune response is widely accepted and continues to be extensively researched, yet there are still numerous unanswered biological questions regarding the nature of internal immune triggers. My research aimed to enhance our comprehension of the molecular basis of danger and to investigate the role of DAMPs beyond the initial activation of myeloid cells, extending into their involvement in prolonged NLRP3 inflammasome activation, priming, and tolerance. Furthermore, this study examined the regulatory mechanisms governing DAMP-induced inflammation in the context of the chronic inflammatory condition RA, and compared them to the inflammasome responses induced by the PAMP LPS (Figure 5.1). Specifically, tenascin-C was investigated as an example DAMP, previously shown to bind TLR4 and activate immune responses through a positively charged ridge on the surface of the C-terminal FBG domain. I discovered that the FBG-C domain of Tenascin-C induces innate immune memory in human monocytes, with some similarities but also major differences to the PAMP LPS response (Figure 5.1). The data obtained during this investigation contributes to the evidence that the human immune system can sense and respond to infection versus injury differently since, although both pathogenic and internal triggers can activate the same downstream signalling cascades, they are fundamentally signals of very different threats, which require tailored responses to restore the system to its natural state. Specifically, I uncovered substantial differences in the ability of FBG-C stimulated primary human monocytes to tolerise the secretion of the pleiotropic inflammasome regulated cytokine IL-1ß compared to LPS stimulated cells (Figure 5.1). Through the generation of primary human *ACOD1* KO monocytes, I then showed that the immunometabolite itaconate and its enzyme ACOD1 drive NLRP3 inflammasome tolerance in human monocytes following both DAMP and PAMP activation (Figure 5.1). Additionally, I found that the functional impact of ACOD1-induced tolerance is achieved through the inhibition of Gasdermin D cleavage and pyroptotic pore formation (Figure 5.1).

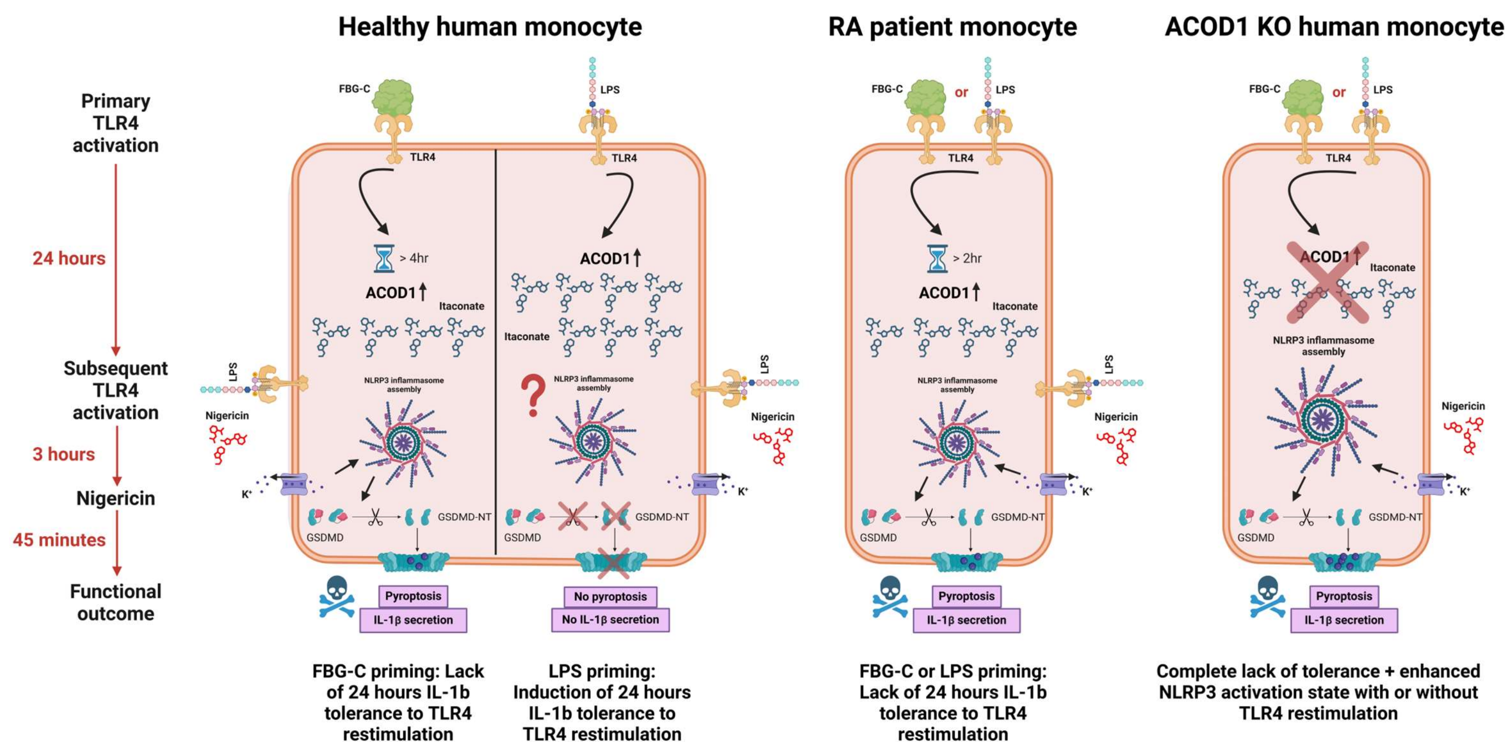


Figure 5.1 Graphical summary of key findings. A visual summary of the key findings presented and discussed in this thesis.

5.1 Clinical relevance and future work

With clinical relevance in mind, my research demonstrated that monocytes from rheumatoid arthritis patients exhibit a delayed ACOD1-mediated tolerance response to TLR4 activation, which for the first time revealed a break of an inflammatory tolerance response in human RA monocytes caused by the DAMP tenascin-C. Modern RA therapies enable effective disease management, and sustained clinical remission can be achieved in 20-40% of patients (Nagy & Van Vollenhoven, 2015); however, even with this life-changing success of DMARD treatment, resistance to DMARD therapy eventually occurs in between 30% to 50% of patients (Smolen et al., 2018), and the achievement of drug-free remission still remains elusive, with up to 50% of patients experiencing flare within the first year if DMARD therapy is discontinued (Kubo et al., 2023). Therefore, there is a high demand for novel RA therapeutics that can complement the existing treatment strategies. Despite our current knowledge of genetic and environmental risk factors, there is still limited understanding of the precise sequence of events that

lead to inflammation dysregulation in the joint and the onset of RA. Molecular and cellular culprits, as well as impaired regulatory mechanisms contributing to the intensified immune processes triggering the initiation and perpetuation of inflammation in early arthritis, are yet to be identified. Tenascin-C is a protein with a highly specific expression pattern, generally absent in most healthy adult tissues but locally upregulated in response to injury and inflammation (Midwood et al., 2016). In the context of RA, levels of tenascin-C are elevated in the blood of patients and are associated with joint erosion (Page et al., 2012). Furthermore, in the inflamed joint, the expression of tenascin-C persists, and the protein accumulates in the tissue (Chevalier et al., 1994; Salter, 1993). In an RA mouse model, the absence of tenascin-C protected the animals from erosive arthritis, and the administration of FBG-C induced joint damage (Midwood et al., 2009). Additionally, in rats, monoclonal anti-FBG-C antibodies that neutralise the activation of TLR4 by the protein prevented the progression of experimental arthritis (Aungier et al., 2019). The translational research findings presented in this thesis demonstrate how potential dysregulation of tenascin-C expression in predisposed individuals could lead to a proinflammatory inflammasome activation cascade where endogenous FBG-C can trigger repeated cycles of IL-1ß secretion due to an ACOD1 and itaconate dependent delay of tolerance. This in turn can lead to the proliferation of FLS in the joint and the production of further proinflammatory cytokines by different IL-1ß activated cells. Therefore, tenascin-C and immunometabolites such as itaconate are promising candidates for innovative treatments for RA. Due to the short lifespan of activated human monocytes, it is difficult to assess to what extent their inability to induce short term inflammasome tolerance following FBG-C activation can contribute to RA pathology. However, recent studies have demonstrated that, based on synovial tissue examination, in a portion of patients infiltrating monocytes might play a crucial role in RA pathogenesis (Zhang et al., 2023). Specifically, cell-state composition was analysed using single-cell RNA-sequencing, surface protein data and histology of synovial tissue from a clinically diverse set of 79 patients with active RA. This led to the stratification of RA tissues into six groups, which were referred to as cell-type abundance phenotypes (CTAPs), each characterized by selectively enriched cell states (Zhang et al., 2023). These CTAPs showed that some samples were enriched for T and B cells, but there were also those mostly lacking lymphocytes. For example, some samples predominantly contained

myeloid cells such as the previously described *IL1B*$^{+}$ pro-inflammatory subset of infiltrating monocytes (Zhang et al., 2019; Zhang et al., 2023). These findings highlight the importance of further research into the RA pathogenesis contribution of monocytes and inflammasome activation in the portion of patients with myeloid cell driven disease.

Since I also demonstrated that strong environmental stressors such as joint hypoxia can trigger the loss of tolerance and prolonged inflammasome priming through mechanisms independent of ACOD1, further extensive research is necessary to confirm and characterise the *in vitro* observed loss of inflammasome tolerance in myeloid cells. However, it is evident that prevention of this phenotype can help limit the excessive production of proinflammatory cytokines, regain control over chronic inflammation, and influence autoinflammatory disease progression.

In terms of future work, several important questions emerged which could not be answered within the scope of this research. These questions include:

1. What are the exact means through which itaconate is able to prevent Gasdermin D cleavage?
2. What are the complementary mechanisms to ACOD1 upregulation which establish Gasdermin D and pyroptosis mediated inflammasome tolerance and how are these mechanisms perturbed by hypoxia?
3. Are other inflammasomes susceptible to tolerisation by DAMPs and PAMPs in a similar manner to the NLRP3 inflammasome in primary human myeloid cells?
4. What is the exact mode of TLR4 activation by FBG-C, and what are the specific co-receptor requirements and downstream signalling cascades employed to elicit immune responses different from those provoked by LPS activation?
5. Could the FBG-C-mediated delay in NLRP3 inflammasome tolerance be recapitulated *in vivo*?

List of References

Accelerating Medicines Partnership Rheumatoid Arthritis and Systemic Lupus Erythematosus (AMP RA/SLE) Consortium, Zhang, F., Wei, K., Slowikowski, K., Fonseka, C. Y., Rao, D. A., Kelly, S., Goodman, S. M., Tabechian, D., Hughes, L. B., Salomon-Escoto, K., Watts, G. F. M., Jonsson, A. H., Rangel-Moreno, J., Meednu, N., Rozo, C., Apruzzese, W., Eisenhaure, T. M., Lieb, D. J., … Raychaudhuri, S. (2019). Defining inflammatory cell states in rheumatoid arthritis joint synovial tissues by integrating single-cell transcriptomics and mass cytometry. *Nature Immunology*, *20*(7), 928–942. https://doi.org/10.1038/s41590-019-0378-1

Adib-Conquy, M., Adrie, C., Fitting, C., Gattolliat, O., Beyaert, R., & Cavaillon, J.-M. (2006). Up-regulation of MyD88s and SIGIRR, molecules inhibiting Toll-like receptor signaling, in monocytes from septic patients*: *Critical Care Medicine*, *34*(9), 2377–2385. https://doi.org/10.1097/01.CCM.0000233875.93866.88

Adib-Conquy, M., & Cavaillon, J.-M. (2002). Gamma interferon and granulocyte/monocyte colony-stimulating factor prevent endotoxin tolerance in human monocytes by promoting interleukin-1 receptor-associated kinase expression and its association to myd88 and not by modulating tlr4 expression. *Journal of Biological Chemistry*, *277*(31), 27927–27934. https://doi.org/10.1074/jbc.M200705200

Agnew, A., Nulty, C., & Creagh, E. M. (2021). Regulation, activation and function of caspase-11 during health and disease. *International Journal of Molecular Sciences*, *22*(4), 1506. https://doi.org/10.3390/ijms22041506

Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J., & Tang, X. (2017). Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1863*(2), 585–597. https://doi.org/10.1016/j.bbadis.2016.11.005

Akiyama, M., & Kaneko, Y. (2022). Pathogenesis, clinical features, and treatment strategy for rheumatoid arthritis-associated interstitial lung disease. *Autoimmunity Reviews*, *21*(5), 103056. https://doi.org/10.1016/j.autrev.2022.103056

Albert, M. R. (1999). Fever therapy for general paresis. *International Journal of Dermatology*, *38*(8), 633–637. https://doi.org/10.1046/j.1365-4362.1999.00659.x

Alexander, C., & Rietschel, E. T. (2001). Bacterial lipopolysaccharides and innate immunity. *Journal of Endotoxin Research*, *7*(3), 167–202. https://doi.org/10.1179/096805101101532675

Alivernini, S., MacDonald, L., Elmesmari, A., Finlay, S., Tolusso, B., Gigante, M. R., Petricca, L., Di Mario, C., Bui, L., Perniola, S., Attar, M., Gessi, M., Fedele, A. L., Chilaka, S., Somma, D., Sansom, S. N., Filer, A., McSharry, C., Millar, N. L., … Kurowska-Stolarska, M. (2020). Distinct synovial tissue macrophage subsets

regulate inflammation and remission in rheumatoid arthritis. *Nature Medicine*, *26*(8), 1295–1306. https://doi.org/10.1038/s41591-020-0939-8

Alivernini, S., Tolusso, B., Ferraccioli, G., Gremese, E., Kurowska-Stolarska, M., & McInnes, I. B. (2018). Driving chronicity in rheumatoid arthritis: Perpetuating role of myeloid cells. *Clinical and Experimental Immunology*, *193*(1), 13–23. https://doi.org/10.1111/cei.13098

Allam, R., Lawlor, K. E., Yu, E. C., Mildenhall, A. L., Moujalled, D. M., Lewis, R. S., Ke, F., Mason, K. D., White, M. J., Stacey, K. J., Strasser, A., O'Reilly, L. A., Alexander, W., Kile, B. T., Vaux, D. L., & Vince, J. E. (2014). Mitochondrial apoptosis is dispensable for NLRP 3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. *EMBO Reports*, *15*(9), 982–990. https://doi.org/10.15252/embr.201438463

Amarante-Mendes, G. P., Adjemian, S., Branco, L. M., Zanetti, L. C., Weinlich, R., & Bortoluci, K. R. (2018). Pattern recognition receptors and the host cell death molecular machinery. *Frontiers in Immunology*, *9*, 2379. https://doi.org/10.3389/fimmu.2018.02379

Anders, H.-J., & Schaefer, L. (2014). Beyond tissue injury—Damage-associated molecular patterns, toll-like receptors, and inflammasomes also drive regeneration and fibrosis. *Journal of the American Society of Nephrology*, *25*(7), 1387–1400. https://doi.org/10.1681/ASN.2014010117

Andreeva, L., David, L., Rawson, S., Shen, C., Pasricha, T., Pelegrin, P., & Wu, H. (2021). NLRP3 cages revealed by full-length mouse NLRP3 structure control pathway activation. *Cell*, *184*(26), 6299-6312.e22. https://doi.org/10.1016/j.cell.2021.11.011

Aneja, R. K., Tsung, A., Sjodin, H., Gefter, J. V., Delude, R. L., Billiar, T. R., & Fink, M. P. (2008). Preconditioning with high mobility group box 1 (Hmgb1) induces lipopolysaccharide (Lps) tolerance. *Journal of Leukocyte Biology*, *84*(5), 1326–1334. https://doi.org/10.1189/jlb.0108030

Apel, F., Zychlinsky, A., & Kenny, E. F. (2018). The role of neutrophil extracellular traps in rheumatic diseases. *Nature Reviews Rheumatology*, *14*(8), 467–475. https://doi.org/10.1038/s41584-018-0039-z

Arts, R. J. W., Huang, P.-K., Yang, D., Joosten, L. A. B., Van Der Meer, J. W. M., Oppenheim, J. J., Netea, M. G., & Cheng, S.-C. (2018). High-mobility group nucleosome-binding protein 1 as endogenous ligand induces innate immune tolerance in a tlr4-sirtuin-1 dependent manner in human blood peripheral mononuclear cells. *Frontiers in Immunology*, *9*, 526. https://doi.org/10.3389/fimmu.2018.00526

Astiz, M. E., Rackow, E. C., Still, J. G., Howell, S. T., Cato, A., B. Von Eschen, K., Ulrich, J. T., Rudbach, J. A., McMahon, G., Vargas, R., & Stern, W. (1995). Pretreatment of normal humans with monophosphoryl lipid A induces tolerance to endotoxin: A prospective, double-blind, randomized, controlled trial. *Critical Care Medicine*, *23*(1), 9–17. https://doi.org/10.1097/00003246-199501000-00006

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., & Geissmann, F. (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*, *317*(5838), 666-670. https://doi.org/10.1126/science.1142883

Aungier, S. R., Cartwright, A. J., Schwenzer, A., Marshall, J. L., Dyson, M. R., Slavny, P., Parthiban, K., Karatt-Vellatt, A., Sahbudin, I., Culbert, E., Hextall, P., Clanchy, F. I., Williams, R., Marsden, B. D., Raza, K., Filer, A., Buckley, C. D., McCafferty, J., & Midwood, K. S. (2019). Targeting early changes in the synovial microenvironment: A new class of immunomodulatory therapy? *Annals of the Rheumatic Diseases*, *78*(2), 186–191. https://doi.org/10.1136/annrheumdis-2018-214294

Austermann, J., Friesenhagen, J., Fassl, S. K., Ortkras, T., Burgmann, J., Barczyk-Kahlert, K., Faist, E., Zedler, S., Pirr, S., Rohde, C., Müller-Tidow, C., von Köckritz-Blickwede, M., von Kaisenberg, C. S., Flohé, S. B., Ulas, T., Schultze, J. L., Roth, J., Vogl, T., & Viemann, D. (2014). Alarmins mrp8 and mrp14 induce stress tolerance in phagocytes under sterile inflammatory conditions. *Cell Reports*, *9*(6), 2112–2123. https://doi.org/10.1016/j.celrep.2014.11.020

Aziz, M., Brenner, M., & Wang, P. (2019). Extracellular CIRP (Ecirp) and inflammation. *Journal of Leukocyte Biology*, *106*(1), 133–146. https://doi.org/10.1002/JLB.3MIR1118-443R

Bae, S., Kim, H., Lee, N., Won, C., Kim, H.-R., Hwang, Y., Song, Y. W., Kang, J. S., & Lee, W. J. (2012). A-enolase expressed on the surfaces of monocytes and macrophages induces robust synovial inflammation in rheumatoid arthritis. *The Journal of Immunology*, *189*(1), 365–372. https://doi.org/10.4049/jimmunol.1102073

Bambouskova, M., Gorvel, L., Lampropoulou, V., Sergushichev, A., Loginicheva, E., Johnson, K., Korenfeld, D., Mathyer, M. E., Kim, H., Huang, L.-H., Duncan, D., Bregman, H., Keskin, A., Santeford, A., Apte, R. S., Sehgal, R., Johnson, B., Amarasinghe, G. K., Soares, M. P., … Artyomov, M. N. (2018). Electrophilic properties of itaconate and derivatives regulate the IκBζ–ATF3 inflammatory axis. *Nature*, *556*(7702), 501–504. https://doi.org/10.1038/s41586-018-0052-z

Bambouskova, M., Potuckova, L., Paulenda, T., Kerndl, M., Mogilenko, D. A., Lizotte, K., Swain, A., Hayes, S., Sheldon, R. D., Kim, H., Kapadnis, U., Ellis, A. E., Isaguirre, C., Burdess, S., Laha, A., Amarasinghe, G. K., Chubukov, V., Roddy, T. P., Diamond, M. S., … Artyomov, M. N. (2021). Itaconate confers tolerance to late NLRP3 inflammasome activation. *Cell Reports*, *34*(10), 108756. https://doi.org/10.1016/j.celrep.2021.108756

Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Research*, *21*(3), 381–395. https://doi.org/10.1038/cr.2011.22

Battaglia, F., Delfino, S., Merello, E., Puppo, M., Piva, R., Varesio, L., & Bosco, M. C. (2008). Hypoxia transcriptionally induces macrophage-inflammatory protein-3α/CCL-20 in primary human mononuclear phagocytes through nuclear

factor (Nf)-κB. *Journal of Leukocyte Biology*, *83*(3), 648–662. https://doi.org/10.1189/jlb.0607349

Beeson, P. B. & With the Technical Assistance of Elizabeth Roberts. (1947). Tolerance to bacterial pyrogens. *Journal of Experimental Medicine, 86*(1), 29–38. https://doi.org/10.1084/jem.86.1.29

Bekkering, S., Domínguez-Andrés, J., Joosten, L. A. B., Riksen, N. P., & Netea, M. G. (2021). Trained immunity: Reprogramming innate immunity in health and disease. *Annual Review of Immunology*, *39*(1), 667–693. https://doi.org/10.1146/annurev-immunol-102119-073855

Bekkering, S., Quintin, J., Joosten, L. A. B., Van Der Meer, J. W. M., Netea, M. G., & Riksen, N. P. (2014). Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *34*(8), 1731–1738. https://doi.org/10.1161/ATVBAHA.114.303887

Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. *Cell*, *157*(1), 121–141. https://doi.org/10.1016/j.cell.2014.03.011

Benbow, J. H., Thompson, K. J., Cope, H. L., Brandon-Warner, E., Culberson, C. R., Bossi, K. L., Li, T., Russo, M. W., Gersin, K. S., McKillop, I. H., deLemos, A. S., & Schrum, L. W. (2016). Diet-induced obesity enhances progression of hepatocellular carcinoma through tenascin-c/toll-like receptor 4 signaling. *The American Journal of Pathology*, *186*(1), 145–158. https://doi.org/10.1016/j.ajpath.2015.09.015

Berg, I. A., Filatova, L. V., & Ivanovsky, R. N. (2002). Inhibition of acetate and propionate assimilation by itaconate via propionyl-CoA carboxylase in isocitrate lyase-negative purple bacterium *Rhodospirillum rubrum*. *FEMS Microbiology Letters*, *216*(1), 49–54. https://doi.org/10.1111/j.1574-6968.2002.tb11413.x

Berra, E. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1 in normoxia. *The EMBO Journal*, *22*(16), 4082–4090. https://doi.org/10.1093/emboj/cdg392

Bertani, B., & Ruiz, N. (2018). Function and biogenesis of lipopolysaccharides. *EcoSal Plus*, *8*(1), 10.1128/ecosalplus.ESP-0001–2018. https://doi.org/10.1128/ecosalplus.esp-0001-2018

Berthelot, J.-M., Bataille, R., Maugars, Y., & Prost, A. (1996). Rheumatoid arthritis as a bone marrow disorder. *Seminars in Arthritis and Rheumatism*, *26*(2), 505–514. https://doi.org/10.1016/S0049-0172(96)80039-4

Bertoni, A., Carta, S., Baldovini, C., Penco, F., Balza, E., Borghini, S., Duca, M. D., Ognio, E., Nozza, P., Schena, F., Castellani, P., Pastorino, C., Perrone, C., Obici, L., Martini, A., Ceccherini, I., Gattorno, M., Rubartelli, A., & Chiesa, S. (2019). Op0106 a novel knock-in mouse model of caps that develops amyloidosis: Therapeutic efficacy of proton pump inhibitors. *Oral Presentations*, 126–126. https://doi.org/10.1136/annrheumdis-2019-eular.5727

Bhattacharyya, S., Wang, W., Morales-Nebreda, L., Feng, G., Wu, M., Zhou, X., Lafyatis, R., Lee, J., Hinchcliff, M., Feghali-Bostwick, C., Lakota, K., Budinger, G. R. S., Raparia, K., Tamaki, Z., & Varga, J. (2016). Tenascin-C drives persistence of organ fibrosis. *Nature Communications*, *7*(1), 11703. https://doi.org/10.1038/ncomms11703

Biswas, S. K., & Lopez-Collazo, E. (2009). Endotoxin tolerance: New mechanisms, molecules and clinical significance. *Trends in Immunology*, *30*(10), 475–487. https://doi.org/10.1016/j.it.2009.07.009

Bizzaro, N., Bartoloni, E., Morozzi, G., Manganelli, S., Riccieri, V., Sabatini, P., Filippini, M., Tampoia, M., Afeltra, A., Sebastiani, G., Alpini, C., Bini, V., Bistoni, O., Alunno, A., Gerli, R., & the Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni (FIRMA Group). (2013). Anti-cyclic citrullinated peptide antibody titer predicts time to rheumatoid arthritis onset in patients with undifferentiated arthritis: Results from a 2-year prospective study. *Arthritis Research & Therapy*, *15*(1), R16. https://doi.org/10.1186/ar4148

Black, R. J., Cross, M., Haile, L. M., Culbreth, G. T., Steinmetz, J. D., Hagins, H., Kopec, J. A., Brooks, P. M., Woolf, A. D., Ong, K. L., Kopansky-Giles, D. R., Dreinhoefer, K. E., Betteridge, N., Aali, A., Abbasifard, M., Abbasi-Kangevari, M., Abdurehman, A. M., Abedi, A., Abidi, H., … March, L. M. (2023). Global, regional, and national burden of rheumatoid arthritis, 1990–2020, and projections to 2050: A systematic analysis of the Global Burden of Disease Study 2021. *The Lancet Rheumatology*, *5*(10), e594–e610. https://doi.org/10.1016/S2665-9913(23)00211-4

Blevins, H. M., Xu, Y., Biby, S., & Zhang, S. (2022). The nlrp3 inflammasome pathway: A review of mechanisms and inhibitors for the treatment of inflammatory diseases. *Frontiers in Aging Neuroscience*, *14*, 879021. https://doi.org/10.3389/fnagi.2022.879021

Boniface, K., Bak-Jensen, K. S., Li, Y., Blumenschein, W. M., McGeachy, M. J., McClanahan, T. K., McKenzie, B. S., Kastelein, R. A., Cua, D. J., & De Waal Malefyt, R. (2009). Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *Journal of Experimental Medicine*, *206*(3), 535–548. https://doi.org/10.1084/jem.20082293

Booth, A. N., Taylor, Joan., Wilson, R. H., & DeEds, Floyd. (1952). The inhibitory effects of itaconic acid in vitro and in vivo. *Journal of Biological Chemistry*, *195*(2), 697–702. https://doi.org/10.1016/S0021-9258(18)55778-1

Borrero, C. G., Mountz, J. M., & Mountz, J. D. (2011). Emerging MRI methods in rheumatoid arthritis. *Nature Reviews Rheumatology*, *7*(2), 85–95. https://doi.org/10.1038/nrrheum.2010.173

Bosco, M. C., Puppo, M., Santangelo, C., Anfosso, L., Pfeffer, U., Fardin, P., Battaglia, F., & Varesio, L. (2006). Hypoxia modifies the transcriptome of primary human monocytes: Modulation of novel immune-related genes and identification of cc-chemokine ligand 20 as a new hypoxia-inducible gene. *The Journal of Immunology*, *177*(3), 1941–1955. https://doi.org/10.4049/jimmunol.177.3.1941

Boxberger, N., Hecker, M., & Zettl, U. K. (2019). Dysregulation of inflammasome priming and activation by micrornas in human immune-mediated diseases. *The Journal of Immunology*, *202*(8), 2177–2187. https://doi.org/10.4049/jimmunol.1801416

Braga, T. T., Forni, M. F., Correa-Costa, M., Ramos, R. N., Barbuto, J. A., Branco, P., Castoldi, A., Hiyane, M. I., Davanso, M. R., Latz, E., Franklin, B. S., Kowaltowski, A. J., & Camara, N. O. S. (2017). Soluble uric acid activates the nlrp3 inflammasome. *Scientific Reports*, *7*(1), 39884. https://doi.org/10.1038/srep39884

Brennan, F. M., Hayes, A. L., Ciesielski, C. J., Green, P., Foxwell, B. M. J., & Feldmann, M. (2002). Evidence that rheumatoid arthritis synovial T cells are similar to cytokine-activated T cells: Involvement of phosphatidylinositol 3-kinase and nuclear factor ?B pathways in tumor necrosis factor ? production in rheumatoid arthritis. *Arthritis & Rheumatism*, *46*(1), 31–41. https://doi.org/10.1002/1529-0131(200201)46:1<31::AID-ART10029>3.0.CO;2-5

Broz, P., & Dixit, V. M. (2016). Inflammasomes: Mechanism of assembly, regulation and signalling. *Nature Reviews Immunology*, *16*(7), 407–420. https://doi.org/10.1038/nri.2016.58

Bryant, C. E., Gay, N. J., Heymans, S., Sacre, S., Schaefer, L., & Midwood, K. S. (2015). Advances in Toll-like receptor biology: Modes of activation by diverse stimuli. *Critical Reviews in Biochemistry and Molecular Biology*, *50*(5), 359–379. https://doi.org/10.3109/10409238.2015.1033511

Burke, B., Giannoudis, A., Corke, K. P., Gill, D., Wells, M., Ziegler-Heitbrock, L., & Lewis, C. E. (2003). Hypoxia-induced gene expression in human macrophages. *The American Journal of Pathology*, *163*(4), 1233–1243. https://doi.org/10.1016/S0002-9440(10)63483-9

Burmester, G. R., Dimitriu-Bona, A., Waters, S. J., & Winchester, R. J. (1983). Identification of three major synovial lining cell populations by monoclonal antibodies directed to ia antigens and antigens associated with monocytes/macrophages and fibroblasts. *Scandinavian Journal of Immunology*, *17*(1), 69–82. https://doi.org/10.1111/j.1365-3083.1983.tb00767.x

Burmester, G. R., Panaccione, R., Gordon, K. B., McIlraith, M. J., & Lacerda, A. P. M. (2013). Adalimumab: Long-term safety in 23 458 patients from global clinical trials in rheumatoid arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis and Crohn’s disease. *Annals of the Rheumatic Diseases*, *72*(4), 517–524. https://doi.org/10.1136/annrheumdis-2011-201244

Buscetta, M., Cristaldi, M., Cimino, M., La Mensa, A., Dino, P., Bucchieri, F., Rappa, F., Amato, S., Aronica, T. S., Pace, E., Bertani, A., & Cipollina, C. (2022). Cigarette smoke promotes inflammasome-independent activation of caspase-1 and -4 leading to gasdermin D cleavage in human macrophages. *The FASEB Journal*, *36*(9), e22525. https://doi.org/10.1096/fj.202200837R

Buscetta, M., Di Vincenzo, S., Miele, M., Badami, E., Pace, E., & Cipollina, C. (2020). Cigarette smoke inhibits the NLRP3 inflammasome and leads to caspase-1 activation via the TLR4-TRIF-caspase-8 axis in human macrophages. *The FASEB Journal*, *34*(1), 1819–1832. https://doi.org/10.1096/fj.201901239R

Butcher, S. K., O'Carroll, C. E., Wells, C. A., & Carmody, R. J. (2018). Toll-like receptors drive specific patterns of tolerance and training on restimulation of macrophages. *Frontiers in Immunology*, *9*, 933. https://doi.org/10.3389/fimmu.2018.00933

CAN-AIM, Moura, C. S., Abrahamowicz, M., Beauchamp, M.-E., Lacaille, D., Wang, Y., Boire, G., Fortin, P. R., Bessette, L., Bombardier, C., Widdifield, J., Hanly, J. G., Feldman, D., Maksymowych, W., Peschken, C., Barnabe, C., Edworthy, S., & Bernatsky, S. (2015). Early medication use in new-onset rheumatoid arthritis may delay joint replacement: Results of a large population-based study. *Arthritis Research & Therapy*, *17*(1), 197. https://doi.org/10.1186/s13075-015-0713-3

Cao, Y., Goods, B. A., Raddassi, K., Nepom, G. T., Kwok, W. W., Love, J. C., & Hafler, D. A. (2015). Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Science Translational Medicine*, *7*(287). https://doi.org/10.1126/scitranslmed.aaa8038

Carey, W. A., Taylor, G. D., Dean, W. B., & Bristow, J. D. (2010). Tenascin-C deficiency attenuates TGF-ß-mediated fibrosis following murine lung injury. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *299*(6), L785–L793. https://doi.org/10.1152/ajplung.00385.2009

Carmona-Rivera, C., Carlucci, P. M., Moore, E., Lingampalli, N., Uchtenhagen, H., James, E., Liu, Y., Bicker, K. L., Wahamaa, H., Hoffmann, V., Catrina, A. I., Thompson, P. R., Buckner, J. H., Robinson, W. H., Fox, D. A., & Kaplan, M. J. (2017). Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Science Immunology*, *2*(10), eaag3358. https://doi.org/10.1126/sciimmunol.aag3358

Castillo-Rodríguez, R. A., Trejo-Solís, C., Cabrera-Cano, A., Gómez-Manzo, S., & Dávila-Borja, V. M. (2022). Hypoxia as a modulator of inflammation and immune response in cancer. *Cancers*, *14*(9), 2291. https://doi.org/10.3390/cancers14092291

Cauli, A., Yanni, G., & Panayi, G. S. (1997). Interleukin-1, interleukin-1 receptor antagonist and macrophage populations in rheumatoid arthritis synovial membrane. *Rheumatology*, *36*(9), 935–940. https://doi.org/10.1093/rheumatology/36.9.935

Cavaillon, J.-M., & Adib-Conquy, M. (2006). [No title found]. *Critical Care*, *10*(5), 233. https://doi.org/10.1186/cc5055

Cejka, D., Hayer, S., Niederreiter, B., Sieghart, W., Fuereder, T., Zwerina, J., & Schett, G. (2010). Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from

patients with rheumatoid arthritis. *Arthritis & Rheumatism*, *62*(8), 2294–2302. https://doi.org/10.1002/art.27504

Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., & Schumacker, P. T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences*, *95*(20), 11715–11720. https://doi.org/10.1073/pnas.95.20.11715

Chatzidionysiou, K., Emamikia, S., Nam, J., Ramiro, S., Smolen, J., Van Der Heijde, D., Dougados, M., Bijlsma, J., Burmester, G., Scholte, M., Van Vollenhoven, R., & Landewé, R. (2017). Efficacy of glucocorticoids, conventional and targeted synthetic disease-modifying antirheumatic drugs: A systematic literature review informing the 2016 update of the EULAR recommendations for the management of rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *76*(6), 1102–1107. https://doi.org/10.1136/annrheumdis-2016-210711

Chen, G. Y., & Nuñez, G. (2010). Sterile inflammation: Sensing and reacting to damage. *Nature Reviews Immunology*, *10*(12), 826–837. https://doi.org/10.1038/nri2873

Chen, J., & Chen, Z. J. (2018). PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. *Nature*, *564*(7734), 71–76. https://doi.org/10.1038/s41586-018-0761-3

Chen, M., Sun, H., Boot, M., Shao, L., Chang, S.-J., Wang, W., Lam, T. T., Lara-Tejero, M., Rego, E. H., & Galán, J. E. (2020). Itaconate is an effector of a Rab GTPase cell-autonomous host defense pathway against *Salmonella*. *Science*, *369*(6502), 450–455. https://doi.org/10.1126/science.aaz1333

Chen, Y., Chang, Y., Wang, C., & Wu, C. (2011). The risk of cancer in patients with rheumatoid arthritis: A nationwide cohort study in Taiwan. *Arthritis & Rheumatism*, *63*(2), 352–358. https://doi.org/10.1002/art.30134

Chen, Y., Meng, J., Xu, Q., Long, T., Bi, F., Chang, C., & Liu, W. (2019). Rapamycin improves the neuroprotection effect of inhibition of NLRP3 inflammasome activation after TBI. *Brain Research*, *1710*, 163–172. https://doi.org/10.1016/j.brainres.2019.01.005

Chevalier, X., Groult, N., Larget-Piet, B., Zardi, L., & Hornebeck, W. (1994). Tenascin distribution in articular cartilage from normal subjects and from patients with osteoarthritis and rheumatoid arthritis. *Arthritis & Rheumatism*, *37*(7), 1013–1022. https://doi.org/10.1002/art.1780370706

Chiquet-Ehrismann, R., & Chiquet, M. (2003). Tenascins: Regulation and putative functions during pathological stress. *The Journal of Pathology*, *200*(4), 488–499. https://doi.org/10.1002/path.1415

Chong, S. Z., Evrard, M., Devi, S., Chen, J., Lim, J. Y., See, P., Zhang, Y., Adrover, J. M., Lee, B., Tan, L., Li, J. L. Y., Liong, K. H., Phua, C., Balachander, A., Boey, A., Liebl, D., Tan, S. M., Chan, J. K. Y., Balabanian, K., … Ng, L. G. (2016). CXCR4 identifies transitional bone marrow premonocytes that replenish

the mature monocyte pool for peripheral responses. *Journal of Experimental Medicine*, *213*(11), 2293–2314. https://doi.org/10.1084/jem.20160800

Chu, C. Q., Field, M., Allard, S., Abney, E., Feldmann, M., & Maini, R. N. (1992). Detection of cytokines at the cartilage/pannus junction in patients with rheumatoid arthritis: Implications for the role of cytokines in cartilage destruction and repair. *Rheumatology*, *31*(10), 653–661. https://doi.org/10.1093/rheumatology/31.10.653

Cooper, D. L., Martin, S. G., Robinson, J. I., Mackie, S. L., Charles, C. J., Nam, J., Consortium, Y., Isaacs, J. D., Emery, P., & Morgan, A. W. (2012). Fcγriiia expression on monocytes in rheumatoid arthritis: Role in immune-complex stimulated tnf production and non-response to methotrexate therapy. *PLoS ONE*, *7*(1), e28918. https://doi.org/10.1371/journal.pone.0028918

Cooper, R., Itiaba, K., & Kornberg, H. (1965). The utilization of aconate and itaconate by *micrococcus* sp. *Biochemical Journal*, *94*(1), 25–31. https://doi.org/10.1042/bj0940025

Cooper, R., & Kornberg, H. (1964). The utilization of itaconate by *Pseudomonas* sp. *Biochemical Journal*, *91*(1), 82–91. https://doi.org/10.1042/bj0910082

Cooper, S., & Helmstetter, C. E. (1968). Chromosome replication and the division cycle of Escherichia coli. *Journal of Molecular Biology*, *31*(3), 519–540. https://doi.org/10.1016/0022-2836(68)90425-7

Cordes, T., Wallace, M., Michelucci, A., Divakaruni, A. S., Sapcariu, S. C., Sousa, C., Koseki, H., Cabrales, P., Murphy, A. N., Hiller, K., & Metallo, C. M. (2016). Immunoresponsive gene 1 and itaconate inhibit succinate dehydrogenase to modulate intracellular succinate levels. *Journal of Biological Chemistry*, *291*(27), 14274–14284. https://doi.org/10.1074/jbc.M115.685792

Crasso, Gust. Ludw. (1840). Untersuchungen über das Verhalten der Citronsäure in höherer Temperatur und die daraus hervorgehenden Produkte. *Justus Liebigs Annalen Der Chemie*, *34*(1), 53–84. https://doi.org/10.1002/jlac.18400340104

Cristaldi, M., Buscetta, M., Cimino, M., La Mensa, A., Giuffrè, M. R., Fiore, L., Carcione, C., Bucchieri, F., Rappa, F., Coronnello, C., Sciaraffa, N., Amato, S., Aronica, T. S., Lo Iacono, G., Bertani, A., Pace, E., & Cipollina, C. (2023). Caspase-8 activation by cigarette smoke induces proinflammatory cell death of human macrophages exposed to lipopolysaccharide. *Cell Death & Disease*, *14*(11), 773. https://doi.org/10.1038/s41419-023-06318-6

Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S.-Y., Senechal, B., Puel, A., Biswas, S. K., Moshous, D., Picard, C., Jais, J.-P., D'Cruz, D., Casanova, J.-L., Trouillet, C., & Geissmann, F. (2010). Human cd14dim monocytes patrol and sense nucleic acids and viruses via tlr7 and tlr8 receptors. *Immunity*, *33*(3), 375–386. https://doi.org/10.1016/j.immuni.2010.08.012

Crowson, C. S., Liao, K. P., Davis, J. M., Solomon, D. H., Matteson, E. L., Knutson, K. L., Hlatky, M. A., & Gabriel, S. E. (2013). Rheumatoid arthritis and

cardiovascular disease. *American Heart Journal*, *166*(4), 622-628.e1. https://doi.org/10.1016/j.ahj.2013.07.010

Culemann, S., Grüneboom, A., Nicolás-Ávila, J. Á., Weidner, D., Lämmle, K. F., Rothe, T., Quintana, J. A., Kirchner, P., Krljanac, B., Eberhardt, M., Ferrazzi, F., Kretzschmar, E., Schicht, M., Fischer, K., Gelse, K., Faas, M., Pfeifle, R., Ackermann, J. A., Pachowsky, M., … Krönke, G. (2019). Locally renewing resident synovial macrophages provide a protective barrier for the joint. *Nature*, *572*(7771), 670–675. https://doi.org/10.1038/s41586-019-1471-1

Daemen, S., Gainullina, A., Kalugotla, G., He, L., Chan, M. M., Beals, J. W., Liss, K. H., Klein, S., Feldstein, A. E., Finck, B. N., Artyomov, M. N., & Schilling, J. D. (2021). Dynamic shifts in the composition of resident and recruited macrophages influence tissue remodeling in nash. *Cell Reports*, *34*(2), 108626. https://doi.org/10.1016/j.celrep.2020.108626

Daemen, S., & Schilling, J. D. (2020). The interplay between tissue niche and macrophage cellular metabolism in obesity. *Frontiers in Immunology*, *10*, 3133. https://doi.org/10.3389/fimmu.2019.03133

Dalbeth, N., Gosling, A. L., Gaffo, A., & Abhishek, A. (2021). Gout. *The Lancet*, *397*(10287), 1843–1855. https://doi.org/10.1016/S0140-6736(21)00569-9

Dan, C., Jinjun, B., Zi-Chun, H., Lin, M., Wei, C., Xu, Z., Ri, Z., Shun, C., Wen-Zhu, S., Qing-Cai, J., & Wu, Y. (2015). Modulation of TNF -α mRNA stability by human antigen R and miR181s in sepsis-induced immunoparalysis. *EMBO Molecular Medicine*, *7*(2), 140–157. https://doi.org/10.15252/emmm.201404797

Davignon, J.-L., Hayder, M., Baron, M., Boyer, J.-F., Constantin, A., Apparailly, F., Poupot, R., & Cantagrel, A. (2013). Targeting monocytes/macrophages in the treatment of rheumatoid arthritis. *Rheumatology*, *52*(4), 590–598. https://doi.org/10.1093/rheumatology/kes304

De Laporte, L., Rice, J. J., Tortelli, F., & Hubbell, J. A. (2013). Tenascin c promiscuously binds growth factors via its fifth fibronectin type iii-like domain. *PLoS ONE*, *8*(4), e62076. https://doi.org/10.1371/journal.pone.0062076

De Zoete, M. R., Palm, N. W., Zhu, S., & Flavell, R. A. (2014). Inflammasomes. *Cold Spring Harbor Perspectives in Biology*, *6*(12), a016287–a016287. https://doi.org/10.1101/cshperspect.a016287

Del Fresno, C., García-Rio, F., Gómez-Piña, V., Soares-Schanoski, A., Fernández-Ruíz, I., Jurado, T., Kajiji, T., Shu, C., Marín, E., Gutierrez Del Arroyo, A., Prados, C., Arnalich, F., Fuentes-Prior, P., Biswas, S. K., & López-Collazo, E. (2009). Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: Demonstration in isolated monocytes from cystic fibrosis patients. *The Journal of Immunology*, *182*(10), 6494–6507. https://doi.org/10.4049/jimmunol.0803350

Delic, V., Beck, K. D., Pang, K. C. H., & Citron, B. A. (2020). Biological links between traumatic brain injury and Parkinson’s disease. *Acta Neuropathologica Communications*, *8*(1), 45. https://doi.org/10.1186/s40478-020-00924-7

Di, A., Xiong, S., Ye, Z., Malireddi, R. K. S., Kometani, S., Zhong, M., Mittal, M., Hong, Z., Kanneganti, T.-D., Rehman, J., & Malik, A. B. (2018). The twik2 potassium efflux channel in macrophages mediates nlrp3 inflammasome-induced inflammation. *Immunity*, *49*(1), 56-65.e4. https://doi.org/10.1016/j.immuni.2018.04.032

Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., Sun, H., Wang, D.-C., & Shao, F. (2016). Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature*, *535*(7610), 111–116. https://doi.org/10.1038/nature18590

Dobrovolskaia, M. A., Medvedev, A. E., Thomas, K. E., Cuesta, N., Toshchakov, V., Ren, T., Cody, M. J., Michalek, S. M., Rice, N. R., & Vogel, S. N. (2003). Induction of in vitro reprogramming by toll-like receptor (Tlr)2 and tlr4 agonists in murine macrophages: Effects of tlr “homotolerance” versus “heterotolerance” on nf-κb signaling pathway components. *The Journal of Immunology*, *170*(1), 508–519. https://doi.org/10.4049/jimmunol.170.1.508

Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T., & Tschopp, J. (2008). Innate immune activation through nalp3 inflammasome sensing of asbestos and silica. *Science*, *320*(5876), 674–677. https://doi.org/10.1126/science.1156995

Duewell, P., Kono, H., Rayner, K. J., Sirois, C. M., Vladimer, G., Bauernfeind, F. G., Abela, G. S., Franchi, L., Nuñez, G., Schnurr, M., Espevik, T., Lien, E., Fitzgerald, K. A., Rock, K. L., Moore, K. J., Wright, S. D., Hornung, V., & Latz, E. (2010). NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*, *464*(7293), 1357–1361. https://doi.org/10.1038/nature08938

Edwards, G., Zhao, J., Dash, P. K., Soto, C., & Moreno-Gonzalez, I. (2020). Traumatic brain injury induces tau aggregation and spreading. *Journal of Neurotrauma*, *37*(1), 80–92. https://doi.org/10.1089/neu.2018.6348

El Gazzar, M., & McCall, C. E. (2010). Micrornas distinguish translational from transcriptional silencing during endotoxin tolerance. *Journal of Biological Chemistry*, *285*(27), 20940–20951. https://doi.org/10.1074/jbc.M110.115063

El Gazzar, M., Yoza, B. K., Hu, J. Y.-Q., Cousart, S. L., & McCall, C. E. (2007). Epigenetic silencing of tumor necrosis factor α during endotoxin tolerance. *Journal of Biological Chemistry*, *282*(37), 26857–26864. https://doi.org/10.1074/jbc.M704584200

El-Karef, A., Yoshida, T., Gabazza, E., Nishioka, T., Inada, H., Sakakura, T., & Imanaka-Yoshida, K. (2007). Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice. *The Journal of Pathology*, *211*(1), 86–94. https://doi.org/10.1002/path.2099

Ellis, S., Lin, E. J., & Tartar, D. (2018). Immunology of wound healing. *Current Dermatology Reports*, *7*(4), 350–358. https://doi.org/10.1007/s13671-018-0234-9

Epstein, A. C. R., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y.-M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., & Ratcliffe, P. J. (2001). C. Elegans egl-9 and mammalian homologs define a family of dioxygenases that regulate hif by prolyl hydroxylation. *Cell*, *107*(1), 43–54. https://doi.org/10.1016/S0092-8674(01)00507-4

Eren, E., & Özören, N. (2019). The NLRP3 inflammasome: A new player in neurological diseases. *TURKISH JOURNAL OF BIOLOGY*, *43*(6), 349–359. https://doi.org/10.3906/biy-1909-31

Erroi, A., Fantuzzi, G., Mengozzi, M., Sironi, M., Orencole, S. F., Clark, B. D., Dinarello, C. A., Isetta, A., Gnocchi, P., & Giovarelli, M. (1993). Differential regulation of cytokine production in lipopolysaccharide tolerance in mice. *Infection and Immunity*, *61*(10), 4356–4359. https://doi.org/10.1128/iai.61.10.4356-4359.1993

Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., & Kagan, J. C. (2018). The pore-forming protein gasdermin d regulates interleukin-1 secretion from living macrophages. *Immunity*, *48*(1), 35-44.e6. https://doi.org/10.1016/j.immuni.2017.11.013

Fan, H., & Cook, J. A. (2004). Molecular mechanisms of endotoxin tolerance. *Journal of Endotoxin Research*, *10*(2), 71–84. https://doi.org/10.1179/096805104225003997

Fangradt, M., Hahne, M., Gaber, T., Strehl, C., Rauch, R., Hoff, P., Löhning, M., Burmester, G.-R., & Buttgereit, F. (2012). Human monocytes and macrophages differ in their mechanisms of adaptation to hypoxia. *Arthritis Research & Therapy*, *14*(4), R181. https://doi.org/10.1186/ar4011

Figus, F. A., Piga, M., Azzolin, I., McConnell, R., & Iagnocco, A. (2021). Rheumatoid arthritis: Extra-articular manifestations and comorbidities. *Autoimmunity Reviews*, *20*(4), 102776. https://doi.org/10.1016/j.autrev.2021.102776

Filer, A., Parsonage, G., Smith, E., Osborne, C., Thomas, A. M. C., Curnow, S. J., Rainger, G. E., Raza, K., Nash, G. B., Lord, J., Salmon, M., & Buckley, C. D. (2006). Differential survival of leukocyte subsets mediated by synovial, bone marrow, and skin fibroblasts: Site-specific versus activation-dependent survival of T cells and neutrophils. *Arthritis & Rheumatism*, *54*(7), 2096–2108. https://doi.org/10.1002/art.21930

Finckh, A., Liang, M. H., Van Herckenrode, C. M., & De Pablo, P. (2006). Long-term impact of early treatment on radiographic progression in rheumatoid arthritis: A meta-analysis. *Arthritis Care & Research*, *55*(6), 864–872. https://doi.org/10.1002/art.22353

Fitzgerald, K. A., Rowe, D. C., & Golenbock, D. T. (2004). Endotoxin recognition and signal transduction by the TLR4/MD2-complex. *Microbes and Infection*, *6*(15), 1361–1367. https://doi.org/10.1016/j.micinf.2004.08.015

Fleischmann, R., Mysler, E., Hall, S., Kivitz, A. J., Moots, R. J., Luo, Z., DeMasi, R., Soma, K., Zhang, R., Takiya, L., Tatulych, S., Mojcik, C., Krishnaswami, S., Menon, S., Smolen, J. S., Adams, L., Ally, M. M., Du Plooy, M. C., Louw, I. C., … Pena, R. (2017). Efficacy and safety of tofacitinib monotherapy, tofacitinib with methotrexate, and adalimumab with methotrexate in patients with rheumatoid arthritis (Oral strategy): A phase 3b/4, double-blind, head-to-head, randomised controlled trial. *The Lancet*, *390*(10093), 457–468. https://doi.org/10.1016/S0140-6736(17)31618-5

Forsberg, E., Hirsch, E., Fröhlich, L., Meyer, M., Ekblom, P., Aszodi, A., Werner, S., & Fässler, R. (1996). Skin wounds and severed nerves heal normally in mice lacking tenascin-C. *Proceedings of the National Academy of Sciences*, *93*(13), 6594–6599. https://doi.org/10.1073/pnas.93.13.6594

Foster, S. L., Hargreaves, D. C., & Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature*, *447*(7147), 972–978. https://doi.org/10.1038/nature05836

Fraker, D. L., Sheppard, B. C., & Norton, J. A. (1990). Impact of tolerance on antitumor efficacy of tumor necrosis factor in mice. *Cancer Research*, *50*(8), 2261–2267.

Fraker, D. L., Stovroff, M. C., Merino, M. J., & Norton, J. A. (1988). Tolerance to tumor necrosis factor in rats and the relationship to endotoxin tolerance and toxicity. *The Journal of Experimental Medicine*, *168*(1), 95–105. https://doi.org/10.1084/jem.168.1.95

Freudenberg, M. A., & Galanos, C. (1988). Induction of tolerance to lipopolysaccharide (Lps)-D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. *Infection and Immunity*, *56*(5), 1352–1357. https://doi.org/10.1128/iai.56.5.1352-1357.1988

Fujimoto, M., Shiba, M., Kawakita, F., Liu, L., Shimojo, N., Imanaka-Yoshida, K., Yoshida, T., & Suzuki, H. (2016). Deficiency of tenascin-C and attenuation of blood-brain barrier disruption following experimental subarachnoid hemorrhage in mice. *Journal of Neurosurgery*, *124*(6), 1693–1702. https://doi.org/10.3171/2015.4.JNS15484

Fujimoto, M., Shiba, M., Kawakita, F., Liu, L., Shimojo, N., Imanaka-Yoshida, K., Yoshida, T., & Suzuki, H. (2018). Effects of tenascin-c knockout on cerebral vasospasm after experimental subarachnoid hemorrhage in mice. *Molecular Neurobiology*, *55*(3), 1951–1958. https://doi.org/10.1007/s12035-017-0466-x

Fukui, S., Iwamoto, N., Takatani, A., Igawa, T., Shimizu, T., Umeda, M., Nishino, A., Horai, Y., Hirai, Y., Koga, T., Kawashiri, S., Tamai, M., Ichinose, K., Nakamura, H., Origuchi, T., Masuyama, R., Kosai, K., Yanagihara, K., & Kawakami, A. (2018). M1 and m2 monocytes in rheumatoid arthritis: A contribution of imbalance of m1/m2 monocytes to osteoclastogenesis. *Frontiers in Immunology*, *8*, 1958. https://doi.org/10.3389/fimmu.2017.01958

Gabay, C., Lamacchia, C., & Palmer, G. (2010). IL-1 pathways in inflammation and human diseases. *Nature Reviews Rheumatology*, *6*(4), 232–241. https://doi.org/10.1038/nrrheum.2010.4

Gaidt, M. M., Ebert, T. S., Chauhan, D., Schmidt, T., Schmid-Burgk, J. L., Rapino, F., Robertson, A. A. B., Cooper, M. A., Graf, T., & Hornung, V. (2016). Human monocytes engage an alternative inflammasome pathway. *Immunity*, *44*(4), 833–846. https://doi.org/10.1016/j.immuni.2016.01.012

Galgani, M., Bruzzaniti, S., & Matarese, G. (2020). Immunometabolism and autoimmunity. *Current Opinion in Immunology*, *67*, 10–17. https://doi.org/10.1016/j.coi.2020.07.002

Gamrekelashvili, J., Giagnorio, R., Jussofie, J., Soehnlein, O., Duchene, J., Briseño, C. G., Ramasamy, S. K., Krishnasamy, K., Limbourg, A., Häger, C., Kapanadze, T., Ishifune, C., Hinkel, R., Radtke, F., Strobl, L. J., Zimber-Strobl, U., Napp, L. C., Bauersachs, J., Haller, H., … Limbourg, F. P. (2016). Regulation of monocyte cell fate by blood vessels mediated by Notch signalling. *Nature Communications*, *7*(1), 12597. https://doi.org/10.1038/ncomms12597

Gardner, R. C., & Yaffe, K. (2015). Epidemiology of mild traumatic brain injury and neurodegenerative disease. *Molecular and Cellular Neuroscience*, *66*, 75–80. https://doi.org/10.1016/j.mcn.2015.03.001

Gazzar, M. E., Yoza, B. K., Chen, X., Hu, J., Hawkins, G. A., & McCall, C. E. (2008). G9a and hp1 couple histone and dna methylation to tnfα transcription silencing during endotoxin tolerance. *Journal of Biological Chemistry*, *283*(47), 32198–32208. https://doi.org/10.1074/jbc.M803446200

Ge, C., & Holmdahl, R. (2019). The structure, specificity and function of anti-citrullinated protein antibodies. *Nature Reviews Rheumatology*, *15*(8), 503–508. https://doi.org/10.1038/s41584-019-0244-4

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science*, *327*(5966), 656–661. https://doi.org/10.1126/science.1178331

Gerard, C., & Rollins, B. J. (2001). Chemokines and disease. *Nature Immunology*, *2*(2), 108–115. https://doi.org/10.1038/84209

Gidon, A., Louet, C., Røst, L. M., Bruheim, P., & Flo, T. H. (2021). The tumor necrosis factor alpha and interleukin 6 auto-paracrine signaling loop controls mycobacterium avium infection via induction of irf1/irg1 in human primary macrophages. *mBio*, *12*(5), e02121-21. https://doi.org/10.1128/mBio.02121-21

Ginhoux, F., & Guilliams, M. (2016). Tissue-resident macrophage ontogeny and homeostasis. *Immunity*, *44*(3), 439–449. https://doi.org/10.1016/j.immuni.2016.02.024

Goh, F. G., & Midwood, K. S. (2012). Intrinsic danger: Activation of Toll-like receptors in rheumatoid arthritis. *Rheumatology*, *51*(1), 7–23. https://doi.org/10.1093/rheumatology/ker257

Gong, T., Liu, L., Jiang, W., & Zhou, R. (2020). DAMP-sensing receptors in sterile inflammation and inflammatory diseases. *Nature Reviews Immunology*, *20*(2), 95–112. https://doi.org/10.1038/s41577-019-0215-7

Gong, Z., Pan, J., Shen, Q., Li, M., & Peng, Y. (2018). Mitochondrial dysfunction induces NLRP3 inflammasome activation during cerebral ischemia/reperfusion injury. *Journal of Neuroinflammation*, *15*(1), 242. https://doi.org/10.1186/s12974-018-1282-6

Gonzalez-Gay, M. A., Garcia-Porrua, C., & Hajeer, A. H. (2002). Influence of human leukocyte antigen-DRB1 on the susceptibility and severity of rheumatoid arthritis. *Seminars in Arthritis and Rheumatism*, *31*(6), 355–360. https://doi.org/10.1053/sarh.2002.32552

Grassi, W., De Angelis, R., Lamanna, G., & Cervini, C. (1998). The clinical features of rheumatoid arthritis. *European Journal of Radiology*, *27*, S18–S24. https://doi.org/10.1016/S0720-048X(98)00038-2

Gravallese, E. M., & Firestein, G. S. (2023). Rheumatoid arthritis—Common origins, divergent mechanisms. *New England Journal of Medicine*, *388*(6), 529–542. https://doi.org/10.1056/NEJMra2103726

Grennan, D. M. (2001). Methotrexate and early postoperative complications in patients with rheumatoid arthritis undergoing elective orthopaedic surgery. *Annals of the Rheumatic Diseases*, *60*(3), 214–217. https://doi.org/10.1136/ard.60.3.214

Grimshaw, M. J., & Balkwill, F. R. (2001). Inhibition of monocyte and macrophage chemotaxis by hypoxia and inflammation—A potential mechanism. *European Journal of Immunology*, *31*(2), 480–489. https://doi.org/10.1002/1521-4141(200102)31:2<480::AID-IMMU480>3.0.CO;2-L

Gris, D., Ye, Z., Iocca, H. A., Wen, H., Craven, R. R., Gris, P., Huang, M., Schneider, M., Miller, S. D., & Ting, J. P.-Y. (2010). Nlrp3 plays a critical role in the development of experimental autoimmune encephalomyelitis by mediating th1 and th17 responses. *The Journal of Immunology*, *185*(2), 974–981. https://doi.org/10.4049/jimmunol.0904145

Günther, J., Vogt, N., Hampel, K., Bikker, R., Page, S., Müller, B., Kandemir, J., Kracht, M., Dittrich-Breiholz, O., Huber, R., & Brand, K. (2014). Identification of two forms of tnf tolerance in human monocytes: Differential inhibition of nf-κb/ap-1– and pp1-associated signaling. *The Journal of Immunology*, *192*(7), 3143–3155. https://doi.org/10.4049/jimmunol.1301610

Guo, C., Fu, R., Wang, S., Huang, Y., Li, X., Zhou, M., Zhao, J., & Yang, N. (2018). NLRP3 inflammasome activation contributes to the pathogenesis of rheumatoid arthritis. *Clinical and Experimental Immunology*, *194*(2), 231–243. https://doi.org/10.1111/cei.13167

Gurung, P., Malireddi, R. K. S., Anand, P. K., Demon, D., Walle, L. V., Liu, Z., Vogel, P., Lamkanfi, M., & Kanneganti, T.-D. (2012). Toll or interleukin-1 receptor (Tir) domain-containing adaptor inducing interferon-β (Trif)-mediated caspase-11

protease production integrates toll-like receptor 4 (Tlr4) protein- and nlrp3 inflammasome-mediated host defense against enteropathogens. *Journal of Biological Chemistry*, *287*(41), 34474–34483. https://doi.org/10.1074/jbc.M112.401406

Hafner-Bratkovič, I., Sušjan, P., Lainšček, D., Tapia-Abellán, A., Cerović, K., Kadunc, L., Angosto-Bazarra, D., Pelegrin, P., & Jerala, R. (2018). NLRP3 lacking the leucine-rich repeat domain can be fully activated via the canonical inflammasome pathway. *Nature Communications*, *9*(1), 5182. https://doi.org/10.1038/s41467-018-07573-4

Hajishengallis, G., Li, X., & Chavakis, T. (2021). Immunometabolic control of hematopoiesis. *Molecular Aspects of Medicine*, *77*, 100923. https://doi.org/10.1016/j.mam.2020.100923

Halle, A., Hornung, V., Petzold, G. C., Stewart, C. R., Monks, B. G., Reinheckel, T., Fitzgerald, K. A., Latz, E., Moore, K. J., & Golenbock, D. T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-β. *Nature Immunology*, *9*(8), 857–865. https://doi.org/10.1038/ni.1636

Harre, U., Georgess, D., Bang, H., Bozec, A., Axmann, R., Ossipova, E., Jakobsson, P.-J., Baum, W., Nimmerjahn, F., Szarka, E., Sarmay, G., Krumbholz, G., Neumann, E., Toes, R., Scherer, H.-U., Catrina, A. I., Klareskog, L., Jurdic, P., & Schett, G. (2012). Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. *Journal of Clinical Investigation*, *122*(5), 1791–1802. https://doi.org/10.1172/JCI60975

Hayes, J. D., & Dinkova-Kostova, A. T. (2014). The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends in Biochemical Sciences*, *39*(4), 199–218. https://doi.org/10.1016/j.tibs.2014.02.002

Hayes, J. P., Logue, M. W., Sadeh, N., Spielberg, J. M., Verfaellie, M., Hayes, S. M., Reagan, A., Salat, D. H., Wolf, E. J., McGlinchey, R. E., Milberg, W. P., Stone, A., Schichman, S. A., & Miller, M. W. (2017). Mild traumatic brain injury is associated with reduced cortical thickness in those at risk for Alzheimer's disease. *Brain*, aww344. https://doi.org/10.1093/brain/aww344

He, H., Jiang, H., Chen, Y., Ye, J., Wang, A., Wang, C., Liu, Q., Liang, G., Deng, X., Jiang, W., & Zhou, R. (2018). Oridonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity. *Nature Communications*, *9*(1), 2550. https://doi.org/10.1038/s41467-018-04947-6

He, Y., Zeng, M. Y., Yang, D., Motro, B., & Núñez, G. (2016). NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature*, *530*(7590), 354–357. https://doi.org/10.1038/nature16959

Hendaoui, I., Tucker, R. P., Zingg, D., Bichet, S., Schittny, J., & Chiquet-Ehrismann, R. (2014). Tenascin-C is required for normal Wnt/β-catenin signaling in the whisker follicle stem cell niche. *Matrix Biology*, *40*, 46–53. https://doi.org/10.1016/j.matbio.2014.08.017

Heneka, M. T., Kummer, M. P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., Griep, A., Axt, D., Remus, A., Tzeng, T.-C., Gelpi, E., Halle, A., Korte, M., Latz, E., & Golenbock, D. T. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature*, *493*(7434), 674–678. https://doi.org/10.1038/nature11729

Hesse, J., Leberling, S., Boden, E., Friebe, D., Schmidt, T., Ding, Z., Dieterich, P., Deussen, A., Roderigo, C., Rose, C. R., Floss, D. M., Scheller, J., & Schrader, J. (2017). CD73-derived adenosine and tenascin-C control cytokine production by epicardium-derived cells formed after myocardial infarction. *The FASEB Journal*, *31*(7), 3040–3053. https://doi.org/10.1096/fj.201601307R

Hibino, S., Kato, K., Kudoh, S., Yagita, H., & Okumura, K. (1998). Tenascin suppresses cd3-mediated t cell activation. *Biochemical and Biophysical Research Communications*, *250*(1), 119–124. https://doi.org/10.1006/bbrc.1998.9258

Hillier, S., & Charnetzky, W. T. (1981). Glyoxylate bypass enzymes in Yersinia species and multiple forms of isocitrate lyase in Yersinia pestis. *Journal of Bacteriology*, *145*(1), 452–458. https://doi.org/10.1128/jb.145.1.452-458.1981

Hochheiser, I. V., Pilsl, M., Hagelueken, G., Moecking, J., Marleaux, M., Brinkschulte, R., Latz, E., Engel, C., & Geyer, M. (2022). Structure of the NLRP3 decamer bound to the cytokine release inhibitor CRID3. *Nature*, *604*(7904), 184–189. https://doi.org/10.1038/s41586-022-04467-w

Hoffman, H. M., Mueller, J. L., Broide, D. H., Wanderer, A. A., & Kolodner, R. D. (2001). Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle–Wells syndrome. *Nature Genetics*, *29*(3), 301–305. https://doi.org/10.1038/ng756

Holmes, F. L. (1993). *Hans krebs: Architect of intermediary metabolism 1933-1937*. Oxford University PressNew York, NY. https://doi.org/10.1093/oso/9780195076578.001.0001

Honda, T., & Inagawa, H. (2023). Utility of in vitro cellular models of low-dose lipopolysaccharide in elucidating the mechanisms of anti-inflammatory and wound-healing-promoting effects of lipopolysaccharide administration in vivo. *International Journal of Molecular Sciences*, *24*(18), 14387. https://doi.org/10.3390/ijms241814387

Hooftman, A., Angiari, S., Hester, S., Corcoran, S. E., Runtsch, M. C., Ling, C., Ruzek, M. C., Slivka, P. F., McGettrick, A. F., Banahan, K., Hughes, M. M., Irvine, A. D., Fischer, R., & O'Neill, L. A. J. (2020). The immunomodulatory metabolite itaconate modifies nlrp3 and inhibits inflammasome activation. *Cell Metabolism*, *32*(3), 468-478.e7. https://doi.org/10.1016/j.cmet.2020.07.016

Hotamisligil, G. S. (2017). Inflammation, metaflammation and immunometabolic disorders. *Nature*, *542*(7640), 177–185. https://doi.org/10.1038/nature21363

Huber, R., Bikker, R., Welz, B., Christmann, M., & Brand, K. (2017). Tnf tolerance in monocytes and macrophages: Characteristics and molecular

mechanisms. *Journal of Immunology Research*, *2017*, 1–9. https://doi.org/10.1155/2017/9570129

Hunter, K., & Loosbroock, C. (2014). Inhibiting TNF-α signaling does not attenuate induction of endotoxin tolerance. *Journal of Inflammation Research*, 159. https://doi.org/10.2147/JIR.S75037

Ifrim, D. C., Quintin, J., Joosten, L. A. B., Jacobs, C., Jansen, T., Jacobs, L., Gow, N. A. R., Williams, D. L., Van Der Meer, J. W. M., & Netea, M. G. (2014). Trained immunity or tolerance: Opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. *Clinical and Vaccine Immunology*, *21*(4), 534–545. https://doi.org/10.1128/CVI.00688-13

Inoue, M., Chen, P., Siecinski, S., Li, Q., Liu, C., Steinman, L., Gregory, S. G., Benner, E., & Shinohara, M. L. (2016). An interferon-β-resistant and NLRP3 inflammasome–independent subtype of EAE with neuronal damage. *Nature Neuroscience*, *19*(12), 1599–1609. https://doi.org/10.1038/nn.4421

Inoue, M., Williams, K. L., Gunn, M. D., & Shinohara, M. L. (2012). NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences*, *109*(26), 10480–10485. https://doi.org/10.1073/pnas.1201836109

Italiani, P., Puxeddu, I., Napoletano, S., Scala, E., Melillo, D., Manocchio, S., Angiolillo, A., Migliorini, P., Boraschi, D., Vitale, E., & Di Costanzo, A. (2018). Circulating levels of IL-1 family cytokines and receptors in Alzheimer's disease: New markers of disease progression? *Journal of Neuroinflammation*, *15*(1), 342. https://doi.org/10.1186/s12974-018-1376-1

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., & Kaelin Jr., W. G. (2001). Hifα targeted for vhl-mediated destruction by proline hydroxylation: Implications for o_2 sensing. *Science*, *292*(5516), 464–468. https://doi.org/10.1126/science.1059817

Iwahashi, M., Yamamura, M., Aita, T., Okamoto, A., Ueno, A., Ogawa, N., Akashi, S., Miyake, K., Godowski, P. J., & Makino, H. (2004). Expression of toll-like receptor 2 on CD16+ blood monocytes and synovial tissue macrophages in rheumatoid arthritis. *Arthritis & Rheumatism*, *50*(5), 1457–1467. https://doi.org/10.1002/art.20219

Jachetti, E., Caputo, S., Mazzoleni, S., Brambillasca, C. S., Parigi, S. M., Grioni, M., Piras, I. S., Restuccia, U., Calcinotto, A., Freschi, M., Bachi, A., Galli, R., & Bellone, M. (2015). Tenascin-c protects cancer stem-like cells from immune surveillance by arresting t-cell activation. *Cancer Research*, *75*(10), 2095–2108. https://doi.org/10.1158/0008-5472.CAN-14-2346

Jaitin, D. A., Adlung, L., Thaiss, C. A., Weiner, A., Li, B., Descamps, H., Lundgren, P., Bleriot, C., Liu, Z., Deczkowska, A., Keren-Shaul, H., David, E., Zmora, N., Eldar, S. M., Lubezky, N., Shibolet, O., Hill, D. A., Lazar, M. A., Colonna, M., … Amit, I. (2019). Lipid-associated macrophages control metabolic

homeostasis in a trem2-dependent manner. *Cell*, *178*(3), 686-698.e14. https://doi.org/10.1016/j.cell.2019.05.054

Jakubzick, C., Gautier, E. L., Gibbings, S. L., Sojka, D. K., Schlitzer, A., Johnson, T. E., Ivanov, S., Duan, Q., Bala, S., Condon, T., van Rooijen, N., Grainger, J. R., Belkaid, Y., Ma'ayan, A., Riches, D. W. H., Yokoyama, W. M., Ginhoux, F., Henson, P. M., & Randolph, G. J. (2013). Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity*, *39*(3), 599–610. https://doi.org/10.1016/j.immuni.2013.08.007

Jang, D., Lee, A.-H., Shin, H.-Y., Song, H.-R., Park, J.-H., Kang, T.-B., Lee, S.-R., & Yang, S.-H. (2021). The role of tumor necrosis factor alpha (Tnf-α) in autoimmune disease and current tnf-α inhibitors in therapeutics. *International Journal of Molecular Sciences*, *22*(5), 2719. https://doi.org/10.3390/ijms22052719

Jawed, S., Gaffney, K., & Blake, D. R. (1997). Intra-articular pressure profile of the knee joint in a spectrum of inflammatory arthropathies. *Annals of the Rheumatic Diseases*, *56*(11), 686–689. https://doi.org/10.1136/ard.56.11.686

Jha, A. K., Huang, S. C.-C., Sergushichev, A., Lampropoulou, V., Ivanova, Y., Loginicheva, E., Chmielewski, K., Stewart, K. M., Ashall, J., Everts, B., Pearce, E. J., Driggers, E. M., & Artyomov, M. N. (2015). Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity*, *42*(3), 419–430. https://doi.org/10.1016/j.immuni.2015.02.005

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., Prestwich, G. D., Mascarenhas, M. M., Garg, H. G., Quinn, D. A., Homer, R. J., Goldstein, D. R., Bucala, R., Lee, P. J., Medzhitov, R., & Noble, P. W. (2005). Regulation of lung injury and repair by Toll-like receptors and hyaluronan. *Nature Medicine*, *11*(11), 1173–1179. https://doi.org/10.1038/nm1315

Jo, E.-K., Kim, J. K., Shin, D.-M., & Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. *Cellular & Molecular Immunology*, *13*(2), 148–159. https://doi.org/10.1038/cmi.2015.95

Johnson, V. E., Stewart, W., & Smith, D. H. (2010). Traumatic brain injury and amyloid-ß pathology: A link to Alzheimer's disease? *Nature Reviews Neuroscience*, *11*(5), 361–370. https://doi.org/10.1038/nrn2808

Jonsson, A. H., Zhang, F., Dunlap, G., Gomez-Rivas, E., Watts, G. F. M., Faust, H. J., Rupani, K. V., Mears, J. R., Meednu, N., Wang, R., Keras, G., Coblyn, J. S., Massarotti, E. M., Todd, D. J., Anolik, J. H., McDavid, A., Accelerating Medicines Partnership RA/SLE Network, Wei, K., Rao, D. A., … Brenner, M. B. (2022). Granzyme K $^{+}$ CD8 T cells form a core population in inflamed human tissue. *Science Translational Medicine*, *14*(649), eabo0686. https://doi.org/10.1126/scitranslmed.abo0686

Juarez, P., Jeannot, K., Plésiat, P., & Llanes, C. (2017). Toxic electrophiles induce expression of the multidrug efflux pump mexef-oprn in pseudomonas

aeruginosa through a novel transcriptional regulator, cmra. *Antimicrobial Agents and Chemotherapy*, *61*(8), e00585-17. https://doi.org/10.1128/AAC.00585-17

Julian, M. W., Strange, H. R., Ballinger, M. N., Hotchkiss, R. S., Papenfuss, T. L., & Crouser, E. D. (2015). Tolerance and cross-tolerance following toll-like receptor (Tlr)-4 and -9 activation are mediated by irak-m and modulated by il-7 in murine splenocytes. *PLOS ONE*, *10*(7), e0132921. https://doi.org/10.1371/journal.pone.0132921

Kalesnikoff, J., Baur, N., Leitges, M., Hughes, M. R., Damen, J. E., Huber, M., & Krystal, G. (2002). Ship negatively regulates ige + antigen-induced il-6 production in mast cells by inhibiting nf-κb activity. *The Journal of Immunology*, *168*(9), 4737–4746. https://doi.org/10.4049/jimmunol.168.9.4737

Kanayama, M., Kurotaki, D., Morimoto, J., Asano, T., Matsui, Y., Nakayama, Y., Saito, Y., Ito, K., Kimura, C., Iwasaki, N., Suzuki, K., Harada, T., Li, H. M., Uehara, J., Miyazaki, T., Minami, A., Kon, S., & Uede, T. (2009). A9 integrin and its ligands constitute critical joint microenvironments for development of autoimmune arthritis. *The Journal of Immunology*, *182*(12), 8015–8025. https://doi.org/10.4049/jimmunol.0900725

Kanayama, M., Morimoto, J., Matsui, Y., Ikesue, M., Danzaki, K., Kurotaki, D., Ito, K., Yoshida, T., & Uede, T. (2011). Α9β1 integrin-mediated signaling serves as an intrinsic regulator of pathogenic th17 cell generation. *The Journal of Immunology*, *187*(11), 5851–5864. https://doi.org/10.4049/jimmunol.1101524

Kapellos, T. S., Bonaguro, L., Gemünd, I., Reusch, N., Saglam, A., Hinkley, E. R., & Schultze, J. L. (2019). Human monocyte subsets and phenotypes in major chronic inflammatory diseases. *Frontiers in Immunology*, *10*, 2035. https://doi.org/10.3389/fimmu.2019.02035

Katsnelson, M. A., Lozada-Soto, K. M., Russo, H. M., Miller, B. A., & Dubyak, G. R. (2016). NLRP3 inflammasome signaling is activated by low-level lysosome disruption but inhibited by extensive lysosome disruption: Roles for K^{+} efflux and Ca^{2+} influx. *American Journal of Physiology-Cell Physiology*, *311*(1), C83–C100. https://doi.org/10.1152/ajpcell.00298.2015

Katsnelson, M. A., Rucker, L. G., Russo, H. M., & Dubyak, G. R. (2015). K+ efflux agonists induce nlrp3 inflammasome activation independently of ca2+ signaling. *The Journal of Immunology*, *194*(8), 3937–3952. https://doi.org/10.4049/jimmunol.1402658

Kavanaugh, A., Fleischmann, R. M., Emery, P., Kupper, H., Redden, L., Guerette, B., Santra, S., & Smolen, J. S. (2013). Clinical, functional and radiographic consequences of achieving stable low disease activity and remission with adalimumab plus methotrexate or methotrexate alone in early rheumatoid arthritis: 26-week results from the randomised, controlled OPTIMA study. *Annals of the Rheumatic Diseases*, *72*(1), 64–71. https://doi.org/10.1136/annrheumdis-2011-201247

Kawai, T., & Akira, S. (2005). Toll-like receptor downstream signaling. *Arthritis Research & Therapy*, *7*(1), 12. https://doi.org/10.1186/ar1469

Kawanaka, N., Yamamura, M., Aita, T., Morita, Y., Okamoto, A., Kawashima, M., Iwahashi, M., Ueno, A., Ohmoto, Y., & Makino, H. (2002). CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis & Rheumatism*, *46*(10), 2578–2586. https://doi.org/10.1002/art.10545

Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. *Frontiers in Immunology*, *5*. https://doi.org/10.3389/fimmu.2014.00461

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., Cuellar, T., Haley, B., Roose-Girma, M., Phung, Q. T., Liu, P. S., Lill, J. R., Li, H., Wu, J., Kummerfeld, S., Zhang, J., Lee, W. P., Snipas, S. J., Salvesen, G. S., … Dixit, V. M. (2015). Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*, *526*(7575), 666–671. https://doi.org/10.1038/nature15541

Kayagaki, N., Warming, S., Lamkanfi, M., Walle, L. V., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., Zhang, J., Lee, W. P., Roose-Girma, M., & Dixit, V. M. (2011). Non-canonical inflammasome activation targets caspase-11. *Nature*, *479*(7371), 117–121. https://doi.org/10.1038/nature10558

Kayagaki, N., Wong, M. T., Stowe, I. B., Ramani, S. R., Gonzalez, L. C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W. P., Muszyński, A., Forsberg, L. S., Carlson, R. W., & Dixit, V. M. (2013). Noncanonical inflammasome activation by intracellular lps independent of tlr4. *Science*, *341*(6151), 1246–1249. https://doi.org/10.1126/science.1240248

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The nlrp3 inflammasome: An overview of mechanisms of activation and regulation. *International Journal of Molecular Sciences*, *20*(13), 3328. https://doi.org/10.3390/ijms20133328

Kelly, A., Houston, S. A., Sherwood, E., Casulli, J., & Travis, M. A. (2017). Regulation of innate and adaptive immunity by tgfß. In *Advances in Immunology* (Vol. 134, pp. 137–233). Elsevier. https://doi.org/10.1016/bs.ai.2017.01.001

Kennedy, A., Ng, C. T., Biniecka, M., Saber, T., Taylor, C., O'Sullivan, J., Veale, D. J., & Fearon, U. (2010). Angiogenesis and blood vessel stability in inflammatory arthritis. *Arthritis & Rheumatism*, *62*(3), 711–721. https://doi.org/10.1002/art.27287

Kinjyo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., Yoshida, H., Kubo, M., & Yoshimura, A. (2002). Socs1/jab is a negative regulator of lps-induced macrophage activation. *Immunity*, *17*(5), 583–591. https://doi.org/10.1016/S1074-7613(02)00446-6

Kinloch, A., Lundberg, K., Wait, R., Wegner, N., Lim, N. H., Zendman, A. J. W., Saxne, T., Malmstr, V., & Venables, P. J. (2008). Synovial fluid is a site of citrullination of autoantigens in inflammatory arthritis. *Arthritis & Rheumatism*, *58*(8), 2287–2295. https://doi.org/10.1002/art.23618

Kiyota, T., Ingraham, K. L., Swan, R. J., Jacobsen, M. T., Andrews, S. J., & Ikezu, T. (2012). AAV serotype 2/1-mediated gene delivery of anti-inflammatory

interleukin-10 enhances neurogenesis and cognitive function in APP+PS1 mice. *Gene Therapy*, *19*(7), 724–733. https://doi.org/10.1038/gt.2011.126

Klarenbeek, N. B., Güler-Yüksel, M., Van Der Kooij, S. M., Han, K. H., Ronday, H. K., Kerstens, P. J. S. M., Seys, P. E. H., Huizinga, T. W. J., Dijkmans, B. A. C., & Allaart, C. F. (2011). The impact of four dynamic, goal-steered treatment strategies on the 5-year outcomes of rheumatoid arthritis patients in the BeSt study. *Annals of the Rheumatic Diseases*, *70*(6), 1039–1046. https://doi.org/10.1136/ard.2010.141234

Ko, M. S., Yun, J. Y., Baek, I.-J., Jang, J. E., Hwang, J. J., Lee, S. E., Heo, S.-H., Bader, D. A., Lee, C.-H., Han, J., Moon, J.-S., Lee, J. M., Hong, E.-G., Lee, I.-K., Kim, S. W., Park, J. Y., Hartig, S. M., Kang, U. J., Moore, D. D., … Lee, K. (2021). Mitophagy deficiency increases NLRP3 to induce brown fat dysfunction in mice. *Autophagy*, *17*(5), 1205–1221. https://doi.org/10.1080/15548627.2020.1753002

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., Tanaka, N., Moriguchi, T., Motohashi, H., Nakayama, K., & Yamamoto, M. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nature Communications*, *7*(1), 11624. https://doi.org/10.1038/ncomms11624

Kobayashi, K., Hernandez, L. D., Galán, J. E., Janeway, C. A., Medzhitov, R., & Flavell, R. A. (2002). Irak-m is a negative regulator of toll-like receptor signaling. *Cell*, *110*(2), 191–202. https://doi.org/10.1016/S0092-8674(02)00827-9

Koch, A. E., Kunkel, S. L., Harlow, L. A., Mazarakis, D. D., Haines, G. K., Burdick, M. D., Pope, R. M., & Strieter, R. M. (1994). Macrophage inflammatory protein-1 alpha. A novel chemotactic cytokine for macrophages in rheumatoid arthritis. *Journal of Clinical Investigation*, *93*(3), 921–928. https://doi.org/10.1172/JCI117097

Komatsu, N., & Takayanagi, H. (2022). Mechanisms of joint destruction in rheumatoid arthritis—Immune cell–fibroblast–bone interactions. *Nature Reviews Rheumatology*, *18*(7), 415–429. https://doi.org/10.1038/s41584-022-00793-5

Kondo, Y., Yokosawa, M., Kaneko, S., Furuyama, K., Segawa, S., Tsuboi, H., Matsumoto, I., & Sumida, T. (2018). Review: Transcriptional regulation of cd 4+ t cell differentiation in experimentally induced arthritis and rheumatoid arthritis. *Arthritis & Rheumatology*, *70*(5), 653–661. https://doi.org/10.1002/art.40398

Kornberg, M. D., Bhargava, P., Kim, P. M., Putluri, V., Snowman, A. M., Putluri, N., Calabresi, P. A., & Snyder, S. H. (2018). Dimethyl fumarate targets GAPDH and aerobic glycolysis to modulate immunity. *Science*, *360*(6387), 449–453. https://doi.org/10.1126/science.aan4665

Krishnamurthy, A., Joshua, V., Haj Hensvold, A., Jin, T., Sun, M., Vivar, N., Ytterberg, A. J., Engström, M., Fernandes-Cerqueira, C., Amara, K., Magnusson, M., Wigerblad, G., Kato, J., Jiménez-Andrade, J. M., Tyson, K., Rapecki, S., Lundberg, K., Catrina, S.-B., Jakobsson, P.-J., … Catrina, A. I. (2016). Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss. *Annals of the*

Rheumatic Diseases, *75*(4), 721–729. https://doi.org/10.1136/annrheumdis-2015-208093

Kubo, S., Miyazaki, Y., Amano, K., Matsui, K., Kameda, H., Inoue, Y., Nakayamada, S., Ogura, T., Kaneko, Y., Yamaoka, K., & Tanaka, Y. (2023). Sustained remission following the discontinuation of tofacitinib in patients with rheumatoid arthritis (XANADU study): An open-label randomised study. *RMD Open*, *9*(2), e003029. https://doi.org/10.1136/rmdopen-2023-003029

Kuemmerle-Deschner, J. B., Ozen, S., Tyrrell, P. N., Kone-Paut, I., Goldbach-Mansky, R., Lachmann, H., Blank, N., Hoffman, H. M., Weissbarth-Riedel, E., Hugle, B., Kallinich, T., Gattorno, M., Gul, A., Ter Haar, N., Oswald, M., Dedeoglu, F., Cantarini, L., & Benseler, S. M. (2017). Diagnostic criteria for cryopyrin-associated periodic syndrome (Caps). *Annals of the Rheumatic Diseases*, *76*(6), 942–947. https://doi.org/10.1136/annrheumdis-2016-209686

Kuriyama, N., Duarte, S., Hamada, T., Busuttil, R. W., & Coito, A. J. (2011). Tenascin-C: A novel mediator of hepatic ischemia and reperfusion injury. *Hepatology*, *54*(6), 2125–2136. https://doi.org/10.1002/hep.24639

Kurowska, W., & Kuca-Warnawin, E. (2016). New evidence for a role of bone marrow in the pathogenesis of rheumatoid arthritis. *Rheumatology*, *54*(5), 215–216. https://doi.org/10.5114/reum.2016.63660

Kurowska-Stolarska, M., & Alivernini, S. (2022). Synovial tissue macrophages in joint homeostasis, rheumatoid arthritis and disease remission. *Nature Reviews Rheumatology*, *18*(7), 384–397. https://doi.org/10.1038/s41584-022-00790-8

Kuwar, R., Rolfe, A., Di, L., Xu, H., He, L., Jiang, Y., Zhang, S., & Sun, D. (2019). A novel small molecular NLRP3 inflammasome inhibitor alleviates neuroinflammatory response following traumatic brain injury. *Journal of Neuroinflammation*, *16*(1), 81. https://doi.org/10.1186/s12974-019-1471-y

Kuziel, W. A., Morgan, S. J., Dawson, T. C., Griffin, S., Smithies, O., Ley, K., & Maeda, N. (1997). Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proceedings of the National Academy of Sciences*, *94*(22), 12053–12058. https://doi.org/10.1073/pnas.94.22.12053

Labbé, K., & Saleh, M. (2011). Pyroptosis: A caspase-1-dependent programmed cell death and a barrier to infection. In I. Couillin, V. Pétrilli, & F. Martinon (Eds.), *The Inflammasomes* (pp. 17–36). Springer Basel. https://doi.org/10.1007/978-3-0348-0148-5_2

Lacerte, P., Brunet, A., Egarnes, B., Duchêne, B., Brown, J. P., & Gosselin, J. (2016). Overexpression of TLR2 and TLR9 on monocyte subsets of active rheumatoid arthritis patients contributes to enhance responsiveness to TLR agonists. *Arthritis Research & Therapy*, *18*(1), 10. https://doi.org/10.1186/s13075-015-0901-1

Lamkanfi, M., & Dixit, V. M. (2014). Mechanisms and functions of inflammasomes. *Cell*, *157*(5), 1013–1022. https://doi.org/10.1016/j.cell.2014.04.007

Lampropoulou, V., Sergushichev, A., Bambouskova, M., Nair, S., Vincent, E. E., Loginicheva, E., Cervantes-Barragan, L., Ma, X., Huang, S. C.-C., Griss, T., Weinheimer, C. J., Khader, S., Randolph, G. J., Pearce, E. J., Jones, R. G., Diwan, A., Diamond, M. S., & Artyomov, M. N. (2016). Itaconate links inhibition of succinate dehydrogenase with macrophage metabolic remodeling and regulation of inflammation. *Cell Metabolism*, *24*(1), 158–166. https://doi.org/10.1016/j.cmet.2016.06.004

Latz, E., Xiao, T. S., & Stutz, A. (2013). Activation and regulation of the inflammasomes. *Nature Reviews Immunology*, *13*(6), 397–411. https://doi.org/10.1038/nri3452

Lawlor, K. E., & Vince, J. E. (2014). Ambiguities in NLRP3 inflammasome regulation: Is there a role for mitochondria? *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1840*(4), 1433–1440. https://doi.org/10.1016/j.bbagen.2013.08.014

Learn, C. A., Mizel, S. B., & McCall, C. E. (2000). Mrna and protein stability regulate the differential expression of pro- and anti-inflammatory genes in endotoxin-tolerant thp-1 cells. *Journal of Biological Chemistry*, *275*(16), 12185–12193. https://doi.org/10.1074/jbc.275.16.12185

Lee, A. H., & Dixit, V. D. (2020). Dietary regulation of immunity. *Immunity*, *53*(3), 510–523. https://doi.org/10.1016/j.immuni.2020.08.013

Lee, CarolineG. L., Jenkins, NancyA., Gilbert, DebraJ., Copeland, NealG., & O'Brien, WilliamE. (1995). Cloning and analysis of gene regulation of a novel LPS-inducible cDNA. *Immunogenetics*, *41*(5). https://doi.org/10.1007/BF00172150

Lee, J., Tam, H., Adler, L., Ilstad-Minnihan, A., Macaubas, C., & Mellins, E. D. (2017). The MHC class II antigen presentation pathway in human monocytes differs by subset and is regulated by cytokines. *PLOS ONE*, *12*(8), e0183594. https://doi.org/10.1371/journal.pone.0183594

Lee, K.-M., Kang, J. H., Yun, M., & Lee, S.-B. (2018). Quercetin inhibits the poly(Da:dt)-induced secretion of IL-18 via down-regulation of the expressions of AIM2 and pro-caspase-1 by inhibiting the JAK2/STAT1 pathway in IFN-γ-primed human keratinocytes. *Biochemical and Biophysical Research Communications*, *503*(1), 116–122. https://doi.org/10.1016/j.bbrc.2018.05.191

Lee, Y. S., & Olefsky, J. (2021). Chronic tissue inflammation and metabolic disease. *Genes & Development*, *35*(5–6), 307–328. https://doi.org/10.1101/gad.346312.120

Lehner, M. D., & Hartung, T. (2002). Endotoxin tolerance—Mechanisms and beneficial effects in bacterial infection. In *Reviews of Physiology, Biochemistry and Pharmacology* (Vol. 144, pp. 95–141). Springer Berlin Heidelberg. https://doi.org/10.1007/BFb0116586

Lehner, M. D., Ittner, J., Bundschuh, D. S., Van Rooijen, N., Wendel, A., & Hartung, T. (2001). Improved innate immunity of endotoxin-tolerant mice increases resistance to *salmonella enterica* serovar typhimurium infection despite attenuated cytokine response. *Infection and Immunity*, *69*(1), 463–471. https://doi.org/10.1128/IAI.69.1.463-471.2001

Lehner, M. D., Morath, S., Michelsen, K. S., Schumann, R. R., & Hartung, T. (2001). Induction of cross-tolerance by lipopolysaccharide and highly purified lipoteichoic acid via different toll-like receptors independent of paracrine mediators. *The Journal of Immunology*, *166*(8), 5161–5167. https://doi.org/10.4049/jimmunol.166.8.5161

Liao, S.-T., Han, C., Xu, D.-Q., Fu, X.-W., Wang, J.-S., & Kong, L.-Y. (2019). 4-Octyl itaconate inhibits aerobic glycolysis by targeting GAPDH to exert anti-inflammatory effects. *Nature Communications*, *10*(1), 5091. https://doi.org/10.1038/s41467-019-13078-5

Lin, K.-M., Hu, W., Troutman, T. D., Jennings, M., Brewer, T., Li, X., Nanda, S., Cohen, P., Thomas, J. A., & Pasare, C. (2014). IRAK-1 bypasses priming and directly links TLRs to rapid NLRP3 inflammasome activation. *Proceedings of the National Academy of Sciences*, *111*(2), 775–780. https://doi.org/10.1073/pnas.1320294111

Liu, H.-D., Li, W., Chen, Z.-R., Hu, Y.-C., Zhang, D.-D., Shen, W., Zhou, M.-L., Zhu, L., & Hang, C.-H. (2013). Expression of the nlrp3 inflammasome in cerebral cortex after traumatic brain injury in a rat model. *Neurochemical Research*, *38*(10), 2072–2083. https://doi.org/10.1007/s11064-013-1115-z

Liu, L., & Chan, C. (2014). The role of inflammasome in Alzheimer’s disease. *Ageing Research Reviews*, *15*, 6–15. https://doi.org/10.1016/j.arr.2013.12.007

Liu, R., He, Y., Li, B., Liu, J., Ren, Y., Han, W., Wang, X., & Zhang, L. (2012). Tenascin-c produced by oxidized ldl-stimulated macrophages increases foam cell formation through toll-like receptor-4. *Molecules and Cells*, *34*(1), 35–42. https://doi.org/10.1007/s10059-012-0054-x

Liu, T., Son, M., & Diamond, B. (2020). Hmgb1 in systemic lupus erythematosus. *Frontiers in Immunology*, *11*, 1057. https://doi.org/10.3389/fimmu.2020.01057

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, *2*(1), 17023. https://doi.org/10.1038/sigtrans.2017.23

Lorenz, M. C., & Fink, G. R. (2002). Life and death in a macrophage: Role of the glyoxylate cycle in virulence. *Eukaryotic Cell*, *1*(5), 657–662. https://doi.org/10.1128/EC.1.5.657-662.2002

Lu, A., Magupalli, V. G., Ruan, J., Yin, Q., Atianand, M. K., Vos, M. R., Schröder, G. F., Fitzgerald, K. A., Wu, H., & Egelman, E. H. (2014). Unified polymerization mechanism for the assembly of asc-dependent inflammasomes. *Cell*, *156*(6), 1193–1206. https://doi.org/10.1016/j.cell.2014.02.008

Lu, A., & Wu, H. (2015). Structural mechanisms of inflammasome assembly. *The FEBS Journal*, *282*(3), 435–444. https://doi.org/10.1111/febs.13133

Lu, K.-T., Wang, Y.-W., Yang, J.-T., Yang, Y.-L., & Chen, H.-I. (2005). Effect of interleukin-1 on traumatic brain injury–induced damage to hippocampal neurons. *Journal of Neurotrauma*, *22*(8), 885–895. https://doi.org/10.1089/neu.2005.22.885

Lu, M., Lai, N., Yu, H., Huang, H., Hsieh, S., & Yu, C. (2010). Anti–citrullinated protein antibodies bind surface-expressed citrullinated Grp78 on monocyte/macrophages and stimulate tumor necrosis factor α production. *Arthritis & Rheumatism*, *62*(5), 1213–1223. https://doi.org/10.1002/art.27386

Lu, Y.-C., Yeh, W.-C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, *42*(2), 145–151. https://doi.org/10.1016/j.cyto.2008.01.006

Lund-Olesen, K. (1970). Oxygen tension in synovial fluids. *Arthritis & Rheumatism*, *13*(6), 769–776. https://doi.org/10.1002/art.1780130606

Ma, B., & Hottiger, M. O. (2016). Crosstalk between wnt/β-catenin and nf-κb signaling pathway during inflammation. *Frontiers in Immunology*, *7*. https://doi.org/10.3389/fimmu.2016.00378

Machino-Ohtsuka, T., Tajiri, K., Kimura, T., Sakai, S., Sato, A., Yoshida, T., Hiroe, M., Yasutomi, Y., Aonuma, K., & Imanaka-Yoshida, K. (2014). Tenascin-c aggravates autoimmune myocarditis via dendritic cell activation and th17 cell differentiation. *Journal of the American Heart Association*, *3*(6), e001052. https://doi.org/10.1161/JAHA.114.001052

Magupalli, V. G., Negro, R., Tian, Y., Hauenstein, A. V., Di Caprio, G., Skillern, W., Deng, Q., Orning, P., Alam, H. B., Maliga, Z., Sharif, H., Hu, J. J., Evavold, C. L., Kagan, J. C., Schmidt, F. I., Fitzgerald, K. A., Kirchhausen, T., Li, Y., & Wu, H. (2020). HDAC6 mediates an aggresome-like mechanism for NLRP3 and pyrin inflammasome activation. *Science*, *369*(6510), eaas8995. https://doi.org/10.1126/science.aas8995

Mahalanobish, S., Dutta, S., Saha, S., & Sil, P. C. (2020). Melatonin induced suppression of ER stress and mitochondrial dysfunction inhibited NLRP3 inflammasome activation in COPD mice. *Food and Chemical Toxicology*, *144*, 111588. https://doi.org/10.1016/j.fct.2020.111588

Mahla, R. S. (2013). Sweeten PAMPs: Role of sugar complexed PAMPs in innate immunity and vaccine biology. *Frontiers in Immunology*, *4*. https://doi.org/10.3389/fimmu.2013.00248

Malik, A., & Kanneganti, T.-D. (2017). Inflammasome activation and assembly at a glance. *Journal of Cell Science*, *130*(23), 3955–3963. https://doi.org/10.1242/jcs.207365

Malmström, V., Catrina, A. I., & Klareskog, L. (2017). The immunopathogenesis of seropositive rheumatoid arthritis: From triggering to targeting. *Nature Reviews Immunology*, *17*(1), 60–75. https://doi.org/10.1038/nri.2016.124

Mamantopoulos, M., Ronchi, F., Van Hauwermeiren, F., Vieira-Silva, S., Yilmaz, B., Martens, L., Saeys, Y., Drexler, S. K., Yazdi, A. S., Raes, J., Lamkanfi, M., McCoy, K. D., & Wullaert, A. (2017). Nlrp6- and asc-dependent inflammasomes do not shape the commensal gut microbiota composition. *Immunity*, *47*(2), 339-348.e4. https://doi.org/10.1016/j.immuni.2017.07.011

Mansour, R. G., Stamper, L., Jaeger, F., McGuire, E., Fouda, G., Amos, J., Barbas, K., Ohashi, T., Alam, S. M., Erickson, H., & Permar, S. R. (2016). The presence and anti-hiv-1 function of tenascin c in breast milk and genital fluids. *PLOS ONE*, *11*(5), e0155261. https://doi.org/10.1371/journal.pone.0155261

Maqbool, A., Spary, E. J., Manfield, I. W., Ruhmann, M., Zuliani-Alvarez, L., Gamboa-Esteves, F. O., Porter, K. E., Drinkhill, M. J., Midwood, K. S., & Turner, N. A. (2016). Tenascin C upregulates interleukin-6 expression in human cardiac myofibroblasts *via* toll-like receptor 4. *World Journal of Cardiology*, *8*(5), 340. https://doi.org/10.4330/wjc.v8.i5.340

Marahleh, A., Kitaura, H., Ohori, F., Kishikawa, A., Ogawa, S., Shen, W.-R., Qi, J., Noguchi, T., Nara, Y., & Mizoguchi, I. (2019). Tnf-α directly enhances osteocyte rankl expression and promotes osteoclast formation. *Frontiers in Immunology*, *10*, 2925. https://doi.org/10.3389/fimmu.2019.02925

Marchetti, C., Swartzwelter, B., Gamboni, F., Neff, C. P., Richter, K., Azam, T., Carta, S., Tengesdal, I., Nemkov, T., D'Alessandro, A., Henry, C., Jones, G. S., Goodrich, S. A., St. Laurent, J. P., Jones, T. M., Scribner, C. L., Barrow, R. B., Altman, R. D., Skouras, D. B., … Dinarello, C. A. (2018). OLT1177, a β-sulfonyl nitrile compound, safe in humans, inhibits the NLRP3 inflammasome and reverses the metabolic cost of inflammation. *Proceedings of the National Academy of Sciences*, *115*(7). https://doi.org/10.1073/pnas.1716095115

Martin, M., Katz, J., Vogel, S. N., & Michalek, S. M. (2001). Differential Induction of Endotoxin Tolerance by Lipopolysaccharides Derived from *Porphyromonas gingivalis* and *Escherichia coli*. *The Journal of Immunology*, *167*(9), 5278–5285. https://doi.org/10.4049/jimmunol.167.9.5278

Martin, W. R., Frigan, F., & Bergman, E. H. (1961). Noninductive metabolism of itaconic acid by *pseudomonas* and *salmonella* species. *Journal of Bacteriology*, *82*(6), 905–908. https://doi.org/10.1128/jb.82.6.905-908.1961

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., & Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*, *440*(7081), 237–241. https://doi.org/10.1038/nature04516

Matsuda , A., Yoshiki, A., Tagawa, Y., Matsuda, H., & Kusakabe, M. (1999). Corneal wound healing in tenascin knockout mouse. *Investigative Ophthalmology & Visual Science*, *40*(6), 1071–1080.

Matzinger, P. (1994). Tolerance, danger, and the extended family. *Annual Review of Immunology*, *12*(1), 991–1045. https://doi.org/10.1146/annurev.iy.12.040194.005015

Maurice, N. M., Bedi, B., & Sadikot, R. T. (2018). *pseudomonas aeruginosa* biofilms: Host response and clinical implications in lung infections. *American Journal of Respiratory Cell and Molecular Biology*, *58*(4), 428–439. https://doi.org/10.1165/rcmb.2017-0321TR

McFadden, B. A., & Purohit, S. (1977). Itaconate, an isocitrate lyase-directed inhibitor in Pseudomonas indigofera. *Journal of Bacteriology*, *131*(1), 136–144. https://doi.org/10.1128/jb.131.1.136-144.1977

McFadden, B. A., Williams, J. O., & Roche, T. E. (1971). Mechanism of action of isocitrate lyase from Pseudomonas indigofera. *Biochemistry*, *10*(8), 1384–1390. https://doi.org/10.1021/bi00784a017

McGarry, T., Hanlon, M. M., Marzaioli, V., Cunningham, C. C., Krishna, V., Murray, K., Hurson, C., Gallagher, P., Nagpal, S., Veale, D. J., & Fearon, U. (2021). Rheumatoid arthritis CD14 $^{+}$ monocytes display metabolic and inflammatory dysfunction, a phenotype that precedes clinical manifestation of disease. *Clinical & Translational Immunology*, *10*(1), e1237. https://doi.org/10.1002/cti2.1237

McGettrick, A. F., & O'Neill, L. A. J. (2020). The role of hif in immunity and inflammation. *Cell Metabolism*, *32*(4), 524–536. https://doi.org/10.1016/j.cmet.2020.08.002

McInnes, I. B., & Schett, G. (2011). The pathogenesis of rheumatoid arthritis. *New England Journal of Medicine*, *365*(23), 2205–2219. https://doi.org/10.1056/NEJMra1004965

McInnes, I. B., & Schett, G. (2017). Pathogenetic insights from the treatment of rheumatoid arthritis. *The Lancet*, *389*(10086), 2328–2337. https://doi.org/10.1016/S0140-6736(17)31472-1

McKinney, J. D., Zu Bentrup, K. H., Muñoz-Elías, E. J., Miczak, A., Chen, B., Chan, W.-T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., & Russell, D. G. (2000). Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*, *406*(6797), 735–738. https://doi.org/10.1038/35021074

McNab, F., Mayer-Barber, K., Sher, A., Wack, A., & O'Garra, A. (2015). Type I interferons in infectious disease. *Nature Reviews Immunology*, *15*(2), 87–103. https://doi.org/10.1038/nri3787

McNicholas, W., Kent, & Mitchell. (2011). Hypoxemia in patients with COPD: Cause, effects, and disease progression. *International Journal of Chronic Obstructive Pulmonary Disease*, 199. https://doi.org/10.2147/COPD.S10611

Mease, P. J. (2007). Adalimumab in the treatment of arthritis. *Therapeutics and Clinical Risk Management*, *3*(1), 133–148. https://doi.org/10.2147/tcrm.2007.3.1.133

Medvedev, A. E., Kopydlowski, K. M., & Vogel, S. N. (2000). Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: Dysregulation of cytokine, chemokine, and toll-like receptor 2 and

4 gene expression. *The Journal of Immunology*, *164*(11), 5564–5574. https://doi.org/10.4049/jimmunol.164.11.5564

Medvedev, A. E., Lentschat, A., Wahl, L. M., Golenbock, D. T., & Vogel, S. N. (2002). Dysregulation of lps-induced toll-like receptor 4-myd88 complex formation and il-1 receptor-associated kinase 1 activation in endotoxin-tolerant cells. *The Journal of Immunology*, *169*(9), 5209–5216. https://doi.org/10.4049/jimmunol.169.9.5209

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature*, *449*(7164), 819–826. https://doi.org/10.1038/nature06246

Medzhitov, R., & Janeway, C. A. (2002). Decoding the patterns of self and nonself by the innate immune system. *Science*, *296*(5566), 298–300. https://doi.org/10.1126/science.1068883

Meednu, N., Rangel-Moreno, J., Zhang, F., Escalera-Rivera, K., Corsiero, E., Prediletto, E., DiCarlo, E., Goodman, S., Donlin, L. T., Raychauduri, S., Bombardieri, M., Pitzalis, C., Orange, D. E., McDavid, A., & Anolik, J. H. (2022). Dynamic spectrum of ectopic lymphoid B cell activation and hypermutation in the RA synovium characterized by NR4A nuclear receptor expression. *Cell Reports*, *39*(5), 110766. https://doi.org/10.1016/j.celrep.2022.110766

Mengozzi, M., Sironi, M., Gadina, M., & Ghezzi, P. (1991). Reversal of defective IL-6 production in lipopolysaccharide-tolerant mice by phorbol myristate acetate. *The Journal of Immunology*, *147*(3), 899–902. https://doi.org/10.4049/jimmunol.147.3.899

Michelucci, A., Cordes, T., Ghelfi, J., Pailot, A., Reiling, N., Goldmann, O., Binz, T., Wegner, A., Tallam, A., Rausell, A., Buttini, M., Linster, C. L., Medina, E., Balling, R., & Hiller, K. (2013). Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proceedings of the National Academy of Sciences*, *110*(19), 7820–7825. https://doi.org/10.1073/pnas.1218599110

Midwood, K. S., Chiquet, M., Tucker, R. P., & Orend, G. (2016). Tenascin-C at a glance. *Journal of Cell Science*, *129*(23), 4321–4327. https://doi.org/10.1242/jcs.190546

Midwood, K. S., & Orend, G. (2009). The role of tenascin-C in tissue injury and tumorigenesis. *Journal of Cell Communication and Signaling*, *3*(3–4), 287–310. https://doi.org/10.1007/s12079-009-0075-1

Midwood, K., Sacre, S., Piccinini, A. M., Inglis, J., Trebaul, A., Chan, E., Drexler, S., Sofat, N., Kashiwagi, M., Orend, G., Brennan, F., & Foxwell, B. (2009). Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nature Medicine*, *15*(7), 774–780. https://doi.org/10.1038/nm.1987

Mills, E. L., Kelly, B., Logan, A., Costa, A. S. H., Varma, M., Bryant, C. E., Tourlomousis, P., Däbritz, J. H. M., Gottlieb, E., Latorre, I., Corr, S. C.,

McManus, G., Ryan, D., Jacobs, H. T., Szibor, M., Xavier, R. J., Braun, T., Frezza, C., Murphy, M. P., & O'Neill, L. A. (2016). Succinate dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. *Cell*, *167*(2), 457-470.e13. https://doi.org/10.1016/j.cell.2016.08.064

Mills, E. L., Kelly, B., & O'Neill, L. A. J. (2017). Mitochondria are the powerhouses of immunity. *Nature Immunology*, *18*(5), 488–498. https://doi.org/10.1038/ni.3704

Mills, E. L., Ryan, D. G., Prag, H. A., Dikovskaya, D., Menon, D., Zaslona, Z., Jedrychowski, M. P., Costa, A. S. H., Higgins, M., Hams, E., Szpyt, J., Runtsch, M. C., King, M. S., McGouran, J. F., Fischer, R., Kessler, B. M., McGettrick, A. F., Hughes, M. M., Carroll, R. G., … O'Neill, L. A. (2018). Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature*, *556*(7699), 113–117. https://doi.org/10.1038/nature25986

Ming, X., Li, W., Maeda, Y., Blumberg, B., Raval, S., Cook, S. D., & Dowling, P. C. (2002). Caspase-1 expression in multiple sclerosis plaques and cultured glial cells. *Journal of the Neurological Sciences*, *197*(1–2), 9–18. https://doi.org/10.1016/S0022-510X(02)00030-8

Mino, T., & Takeuchi, O. (2013). Post-transcriptional regulation of cytokine mRNA controls the initiation and resolution of inflammation. *Biotechnology and Genetic Engineering Reviews*, *29*(1), 49–60. https://doi.org/10.1080/02648725.2013.801236

Mogensen, T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews*, *22*(2), 240–273. https://doi.org/10.1128/CMR.00046-08

Momčilović, M., Stamenković, V., Jovanović, M., Andjus, P. R., Jakovčevski, I., Schachner, M., & Miljković, Đ. (2017). Tenascin-C deficiency protects mice from experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology*, *302*, 1–6. https://doi.org/10.1016/j.jneuroim.2016.12.001

Moossavi, M., Parsamanesh, N., Bahrami, A., Atkin, S. L., & Sahebkar, A. (2018). Role of the NLRP3 inflammasome in cancer. *Molecular Cancer*, *17*(1), 158. https://doi.org/10.1186/s12943-018-0900-3

Mortimer, L., Moreau, F., MacDonald, J. A., & Chadee, K. (2016). NLRP3 inflammasome inhibition is disrupted in a group of auto-inflammatory disease CAPS mutations. *Nature Immunology*, *17*(10), 1176–1186. https://doi.org/10.1038/ni.3538

Mouchemore, K. A., & Pixley, F. J. (2012). CSF-1 signaling in macrophages: Pleiotrophy through phosphotyrosine-based signaling pathways. *Critical Reviews in Clinical Laboratory Sciences*, *49*(2), 49–61. https://doi.org/10.3109/10408363.2012.666845

Muñoz-Elías, E. J., & McKinney, J. D. (2005). Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nature Medicine*, *11*(6), 638–644. https://doi.org/10.1038/nm1252

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., & Núñez, G. (2013). K+ efflux is the common trigger of nlrp3 inflammasome activation by bacterial toxins and particulate matter. *Immunity*, *38*(6), 1142–1153. https://doi.org/10.1016/j.immuni.2013.05.016

Murakami, T., Ockinger, J., Yu, J., Byles, V., McColl, A., Hofer, A. M., & Horng, T. (2012). Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proceedings of the National Academy of Sciences*, *109*(28), 11282–11287. https://doi.org/10.1073/pnas.1117765109

Mysler, E., Caubet, M., & Lizarraga, A. (2021). Current and emerging dmards for the treatment of rheumatoid arthritis. *Open Access Rheumatology: Research and Reviews, Volume 13*, 139–152. https://doi.org/10.2147/OARRR.S282627

Na, K., Oh, B.-C., & Jung, Y. (2023). Multifaceted role of CD14 in innate immunity and tissue homeostasis. *Cytokine & Growth Factor Reviews*, *74*, 100–107. https://doi.org/10.1016/j.cytogfr.2023.08.008

Nagy, G., & Van Vollenhoven, R. F. (2015). Sustained biologic-free and drug-free remission in rheumatoid arthritis, where are we now? *Arthritis Research & Therapy*, *17*(1). https://doi.org/10.1186/s13075-015-0707-1

Nahid, M. A., Satoh, M., & Chan, E. K. (2011). MicroRNA in TLR signaling and endotoxin tolerance. *Cellular & Molecular Immunology*, *8*(5), 388–403. https://doi.org/10.1038/cmi.2011.26

Nair, S., Huynh, J. P., Lampropoulou, V., Loginicheva, E., Esaulova, E., Gounder, A. P., Boon, A. C. M., Schwarzkopf, E. A., Bradstreet, T. R., Edelson, B. T., Artyomov, M. N., Stallings, C. L., & Diamond, M. S. (2018). *irg1* expression in myeloid cells prevents immunopathology during *m. Tuberculosis* infection. *Journal of Experimental Medicine*, *215*(4), 1035–1045. https://doi.org/10.1084/jem.20180118

Nakagawa, R., Naka, T., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., Seki, E., Sato, S., Takeuchi, O., Takeda, K., Akira, S., Yamanishi, K., Kawase, I., Nakanishi, K., & Kishimoto, T. (2002). Socs-1 participates in negative regulation of lps responses. *Immunity*, *17*(5), 677–687. https://doi.org/10.1016/S1074-7613(02)00449-1

Nakahara, H., Gabazza, E. C., Fujimoto, H., Nishii, Y., D'Alessandro-Gabazza, C. N., Bruno, N. E., Takagi, T., Hayashi, T., Maruyama, J., Maruyama, K., Imanaka-Yoshida, K., Suzuki, K., Yoshida, T., Adachi, Y., & Taguchi, O. (2006). Deficiency of tenascin C attenuates allergen-induced bronchial asthma in the mouse. *European Journal of Immunology*, *36*(12), 3334–3345. https://doi.org/10.1002/eji.200636271

Nakamura-Ishizu, A., Okuno, Y., Omatsu, Y., Okabe, K., Morimoto, J., Uede, T., Nagasawa, T., Suda, T., & Kubota, Y. (2012). Extracellular matrix protein tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. *Blood*, *119*(23), 5429–5437. https://doi.org/10.1182/blood-2011-11-393645

Nam, J. L., Takase-Minegishi, K., Ramiro, S., Chatzidionysiou, K., Smolen, J. S., Van Der Heijde, D., Bijlsma, J. W., Burmester, G. R., Dougados, M., Scholte-Voshaar, M., Van Vollenhoven, R., & Landewé, R. (2017). Efficacy of biological disease-modifying antirheumatic drugs: A systematic literature review informing the 2016 update of the EULAR recommendations for the management of rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *76*(6), 1113–1136. https://doi.org/10.1136/annrheumdis-2016-210713

Nam, J. L., Villeneuve, E., Hensor, E. M. A., Conaghan, P. G., Keen, H. I., Buch, M. H., Gough, A. K., Green, M. J., Helliwell, P. S., Keenan, A. M., Morgan, A. W., Quinn, M., Reece, R., Van Der Heijde, D. M., Wakefield, R. J., & Emery, P. (2014). Remission induction comparing infliximab and high-dose intravenous steroid, followed by treat-to-target: A double-blind, randomised, controlled trial in new-onset, treatment-naive, rheumatoid arthritis (The idea study). *Annals of the Rheumatic Diseases*, *73*(1), 75–85. https://doi.org/10.1136/annrheumdis-2013-203440

Napolitani, G., Acosta-Rodriguez, E. V., Lanzavecchia, A., & Sallusto, F. (2009). Prostaglandin E2 enhances Th17 responses *via* modulation of IL-17 and IFN-γ production by memory CD4 $^{+}$ T cells. *European Journal of Immunology*, *39*(5), 1301–1312. https://doi.org/10.1002/eji.200838969

Naujoks, J., Tabeling, C., Dill, B. D., Hoffmann, C., Brown, A. S., Kunze, M., Kempa, S., Peter, A., Mollenkopf, H.-J., Dorhoi, A., Kershaw, O., Gruber, A. D., Sander, L. E., Witzenrath, M., Herold, S., Nerlich, A., Hocke, A. C., Van Driel, I., Suttorp, N., … Opitz, B. (2016). Ifns modify the proteome of legionella-containing vacuoles and restrict infection via irg1-derived itaconic acid. *PLOS Pathogens*, *12*(2), e1005408. https://doi.org/10.1371/journal.ppat.1005408

Ng, A., Tam, W. W., Zhang, M. W., Ho, C. S., Husain, S. F., McIntyre, R. S., & Ho, R. C. (2018). Il-1β, il-6, tnf- α and crp in elderly patients with depression or alzheimer's disease: Systematic review and meta-analysis. *Scientific Reports*, *8*(1), 12050. https://doi.org/10.1038/s41598-018-30487-6

Ng, C. T., Biniecka, M., Kennedy, A., McCormick, J., FitzGerald, O., Bresnihan, B., Buggy, D., Taylor, C. T., O'Sullivan, J., Fearon, U., & Veale, D. J. (2010). Synovial tissue hypoxia and inflammation in vivo. *Annals of the Rheumatic Diseases*, *69*(7), 1389–1395. https://doi.org/10.1136/ard.2009.119776

Nguyen, T. V., Alfaro, A. C., Merien, F., Young, T., & Grandiosa, R. (2018). Metabolic and immunological responses of male and female new Zealand Greenshell™ mussels (Perna canaliculus) infected with Vibrio sp. *Journal of Invertebrate Pathology*, *157*, 80–89. https://doi.org/10.1016/j.jip.2018.08.008

Nguyen, T. V., Alfaro, A. C., Young, T., Green, S., Zarate, E., & Merien, F. (2019). Itaconic acid inhibits growth of a pathogenic marine Vibrio strain: A metabolomics approach. *Scientific Reports*, *9*(1), 5937. https://doi.org/10.1038/s41598-019-42315-6

Nishioka, T., Onishi, K., Shimojo, N., Nagano, Y., Matsusaka, H., Ikeuchi, M., Ide, T., Tsutsui, H., Hiroe, M., Yoshida, T., & Imanaka-Yoshida, K. (2010). Tenascin-C may aggravate left ventricular remodeling and function after myocardial

infarction in mice. *American Journal of Physiology-Heart and Circulatory Physiology*, *298*(3), H1072–H1078. https://doi.org/10.1152/ajpheart.00255.2009

Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., & Akira, S. (2000). Cutting edge: Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *The Journal of Immunology*, *164*(7), 3476–3479. https://doi.org/10.4049/jimmunol.164.7.3476

Novakovic, B., Habibi, E., Wang, S.-Y., Arts, R. J. W., Davar, R., Megchelenbrink, W., Kim, B., Kuznetsova, T., Kox, M., Zwaag, J., Matarese, F., Van Heeringen, S. J., Janssen-Megens, E. M., Sharifi, N., Wang, C., Keramati, F., Schoonenberg, V., Flicek, P., Clarke, L., … Stunnenberg, H. G. (2016). Β-glucan reverses the epigenetic state of lps-induced immunological tolerance. *Cell*, *167*(5), 1354-1368.e14. https://doi.org/10.1016/j.cell.2016.09.034

Nygaard, G., & Firestein, G. S. (2020). Restoring synovial homeostasis in rheumatoid arthritis by targeting fibroblast-like synoviocytes. *Nature Reviews Rheumatology*, *16*(6), 316–333. https://doi.org/10.1038/s41584-020-0413-5

O'Carroll, C., Fagan, A., Shanahan, F., & Carmody, R. J. (2014). Identification of a unique hybrid macrophage-polarization state following recovery from lipopolysaccharide tolerance. *The Journal of Immunology*, *192*(1), 427–436. https://doi.org/10.4049/jimmunol.1301722

Ohta, M., Sakai, T., Saga, Y., Aizawa, S., & Saito, M. (1998). Suppression of hematopoietic activity in tenascin-C-deficient mice. *Blood*, *91*(11), 4074–4083.

Okamoto, K., Nakashima, T., Shinohara, M., Negishi-Koga, T., Komatsu, N., Terashima, A., Sawa, S., Nitta, T., & Takayanagi, H. (2017). Osteoimmunology: The conceptual framework unifying the immune and skeletal systems. *Physiological Reviews*, *97*(4), 1295–1349. https://doi.org/10.1152/physrev.00036.2016

Okamura, Y., Watari, M., Jerud, E. S., Young, D. W., Ishizaka, S. T., Rose, J., Chow, J. C., & Strauss, J. F. (2001). The extra domain a of fibronectin activates toll-like receptor 4. *Journal of Biological Chemistry*, *276*(13), 10229–10233. https://doi.org/10.1074/jbc.M100099200

Oliver, K. M., Garvey, J. F., Ng, C. T., Veale, D. J., Fearon, U., Cummins, E. P., & Taylor, C. T. (2009). Hypoxia activates nf-κb-dependent gene expression through the canonical signaling pathway. *Antioxidants & Redox Signaling*, *11*(9), 2057–2064. https://doi.org/10.1089/ars.2008.2400

O'Neill, L. A. J., Kishton, R. J., & Rathmell, J. (2016). A guide to immunometabolism for immunologists. *Nature Reviews Immunology*, *16*(9), 553–565. https://doi.org/10.1038/nri.2016.70

O'Neill, L. A. J., & Pearce, E. J. (2016). Immunometabolism governs dendritic cell and macrophage function. *Journal of Experimental Medicine*, *213*(1), 15–23. https://doi.org/10.1084/jem.20151570

Orange, D. E., Yao, V., Sawicka, K., Fak, J., Frank, M. O., Parveen, S., Blachere, N. E., Hale, C., Zhang, F., Raychaudhuri, S., Troyanskaya, O. G., & Darnell, R. B. (2020). Rna identification of prime cells predicting rheumatoid arthritis flares. *New England Journal of Medicine*, *383*(3), 218–228. https://doi.org/10.1056/NEJMoa2004114

Ospelt, C. (2017). Synovial fibroblasts in 2017. *RMD Open*, *3*(2), e000471. https://doi.org/10.1136/rmdopen-2017-000471

Page, T. H., Charles, P. J., Piccinini, A. M., Nicolaidou, V., Taylor, P. C., & Midwood, K. S. (2012). Raised circulating tenascin-C in rheumatoid arthritis. *Arthritis Research & Therapy*, *14*(6), R260. https://doi.org/10.1186/ar4105

Pajares, M., I. Rojo, A., Manda, G., Boscá, L., & Cuadrado, A. (2020). Inflammation in parkinson's disease: Mechanisms and therapeutic implications. *Cells*, *9*(7), 1687. https://doi.org/10.3390/cells9071687

Pålsson-McDermott, E. M., & O'Neill, L. A. J. (2020). Targeting immunometabolism as an anti-inflammatory strategy. *Cell Research*, *30*(4), 300–314. https://doi.org/10.1038/s41422-020-0291-z

Pan, H., Ding, E., Hu, M., Lagoo, A. S., Datto, M. B., & Lagoo-Deenadayalan, S. A. (2010). Smad4 is required for development of maximal endotoxin tolerance. *The Journal of Immunology*, *184*(10), 5502–5509. https://doi.org/10.4049/jimmunol.0901601

Pap, T., & Korb-Pap, A. (2015). Cartilage damage in osteoarthritis and rheumatoid arthritis—Two unequal siblings. *Nature Reviews Rheumatology*, *11*(10), 606–615. https://doi.org/10.1038/nrrheum.2015.95

Parajuli, B., Sonobe, Y., Horiuchi, H., Takeuchi, H., Mizuno, T., & Suzumura, A. (2013). Oligomeric amyloid β induces IL-1β processing via production of ROS: Implication in Alzheimer's disease. *Cell Death & Disease*, *4*(12), e975–e975. https://doi.org/10.1038/cddis.2013.503

Park, B. S., & Lee, J.-O. (2013). Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental & Molecular Medicine*, *45*(12), e66–e66. https://doi.org/10.1038/emm.2013.97

Park, S. H., Park-Min, K.-H., Chen, J., Hu, X., & Ivashkiv, L. B. (2011). Tumor necrosis factor induces GSK3 kinase-mediated cross-tolerance to endotoxin in macrophages. *Nature Immunology*, *12*(7), 607–615. https://doi.org/10.1038/ni.2043

Passlick, B., Flieger, D., & Ziegler-Heitbrock, H. (1989). Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*, *74*(7), 2527–2534. https://doi.org/10.1182/blood.V74.7.2527.2527

Patel, A. A., Zhang, Y., Fullerton, J. N., Boelen, L., Rongvaux, A., Maini, A. A., Bigley, V., Flavell, R. A., Gilroy, D. W., Asquith, B., Macallan, D., & Yona, S. (2017). The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *Journal of Experimental Medicine*, *214*(7), 1913–1923. https://doi.org/10.1084/jem.20170355

Patel, L., Sun, W., Glasson, S. S., Morris, E. A., Flannery, C. R., & Chockalingam, P. S. (2011). Tenascin-C induces inflammatory mediators and matrix degradation in osteoarthritic cartilage. *BMC Musculoskeletal Disorders*, *12*(1), 164. https://doi.org/10.1186/1471-2474-12-164

Pena, O. M., Pistolic, J., Raj, D., Fjell, C. D., & Hancock, R. E. W. (2011). Endotoxin tolerance represents a distinctive state of alternative polarization (M2) in human mononuclear cells. *The Journal of Immunology*, *186*(12), 7243–7254. https://doi.org/10.4049/jimmunol.1001952

Penkov, S., Mitroulis, I., Hajishengallis, G., & Chavakis, T. (2019). Immunometabolic crosstalk: An ancestral principle of trained immunity? *Trends in Immunology*, *40*(1), 1–11. https://doi.org/10.1016/j.it.2018.11.002

Perregaux, D., & Gabel, C. A. (1994). Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *Journal of Biological Chemistry*, *269*(21), 15195–15203. https://doi.org/10.1016/S0021-9258(17)36591-2

Petes, C., Mintsopoulos, V., Finnen, R. L., Banfield, B. W., & Gee, K. (2018). The effects of CD14 and IL-27 on induction of endotoxin tolerance in human monocytes and macrophages. *Journal of Biological Chemistry*, *293*(45), 17631–17645. https://doi.org/10.1074/jbc.RA118.003501

Piao, W., Song, C., Chen, H., Quevedo Diaz, M. A., Wahl, L. M., Fitzgerald, K. A., Li, L., & Medvedev, A. E. (2009). Endotoxin tolerance dysregulates MyD88- and Toll/IL-1R domain-containing adapter inducing IFN-β-dependent pathways and increases expression of negative regulators of TLR signaling. *Journal of Leukocyte Biology*, *86*(4), 863–875. https://doi.org/10.1189/jlb.0309189

Piccinini, A. M., & Midwood, K. S. (2012). Endogenous control of immunity against infection: Tenascin-c regulates tlr4-mediated inflammation via microrna-155. *Cell Reports*, *2*(4), 914–926. https://doi.org/10.1016/j.celrep.2012.09.005

Piccinini, A. M., Zuliani-Alvarez, L., Lim, J. M. P., & Midwood, K. S. (2016). Distinct microenvironmental cues stimulate divergent TLR4-mediated signaling pathways in macrophages. *Science Signaling*, *9*(443). https://doi.org/10.1126/scisignal.aaf3596

Pixley, F. J., & Stanley, E. R. (2004). CSF-1 regulation of the wandering macrophage: Complexity in action. *Trends in Cell Biology*, *14*(11), 628–638. https://doi.org/10.1016/j.tcb.2004.09.016

Pohlers, D., Brenmoehl, J., Löffler, I., Müller, C. K., Leipner, C., Schultze-Mosgau, S., Stallmach, A., Kinne, R. W., & Wolf, G. (2009). TGF-β and fibrosis in different organs—Molecular pathway imprints. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1792*(8), 746–756. https://doi.org/10.1016/j.bbadis.2009.06.004

Porta, C., Rimoldi, M., Raes, G., Brys, L., Ghezzi, P., Di Liberto, D., Dieli, F., Ghisletti, S., Natoli, G., De Baetselier, P., Mantovani, A., & Sica, A. (2009).

Tolerance and M2 (Alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κB. *Proceedings of the National Academy of Sciences*, *106*(35), 14978–14983. https://doi.org/10.1073/pnas.0809784106

Price, J. V., Russo, D., Ji, D. X., Chavez, R. A., DiPeso, L., Lee, A. Y.-F., Coers, J., & Vance, R. E. (2019). Irg1 and inducible nitric oxide synthase act redundantly with other interferon-gamma-induced factors to restrict intracellular replication of legionella pneumophila. *mBio*, *10*(6), e02629-19. https://doi.org/10.1128/mBio.02629-19

Prieto, A. L., Edelman, G. M., & Crossin, K. L. (1993). Multiple integrins mediate cell attachment to cytotactin/tenascin. *Proceedings of the National Academy of Sciences*, *90*(21), 10154–10158. https://doi.org/10.1073/pnas.90.21.10154

Proksch, E., Brandner, J. M., & Jensen, J. (2008). The skin: An indispensable barrier. *Experimental Dermatology*, *17*(12), 1063–1072. https://doi.org/10.1111/j.1600-0625.2008.00786.x

Puente Navazo, M. D., Valmori, D., & Rüegg, C. (2001). The alternatively spliced domain tnfniii a1a2 of the extracellular matrix protein tenascin-c suppresses activation-induced t lymphocyte proliferation and cytokine production. *The Journal of Immunology*, *167*(11), 6431–6440. https://doi.org/10.4049/jimmunol.167.11.6431

Purvis, A., & Hector, A. (2000). Getting the measure of biodiversity. *Nature*, *405*(6783), 212–219. https://doi.org/10.1038/35012221

Py, B. F., Jin, M., Desai, B. N., Penumaka, A., Zhu, H., Kober, M., Dietrich, A., Lipinski, M. M., Henry, T., Clapham, D. E., & Yuan, J. (2014). Caspase-11 controls interleukin-1ß release through degradation of trpc1. *Cell Reports*, *6*(6), 1122–1128. https://doi.org/10.1016/j.celrep.2014.02.015

Qin, W., Qin, K., Zhang, Y., Jia, W., Chen, Y., Cheng, B., Peng, L., Chen, N., Liu, Y., Zhou, W., Wang, Y.-L., Chen, X., & Wang, C. (2019). S-glycosylation-based cysteine profiling reveals regulation of glycolysis by itaconate. *Nature Chemical Biology*, *15*(10), 983–991. https://doi.org/10.1038/s41589-019-0323-5

Qin, Y., Cai, M.-L., Jin, H.-Z., Huang, W., Zhu, C., Bozec, A., Huang, J., & Chen, Z. (2022). Age-associated B cells contribute to the pathogenesis of rheumatoid arthritis by inducing activation of fibroblast-like synoviocytes via TNF-α-mediated ERK1/2 and JAK-STAT1 pathways. *Annals of the Rheumatic Diseases*, *81*(11), 1504–1514. https://doi.org/10.1136/ard-2022-222605

Quiñonez-Flores, C. M., González-Chávez, S. A., & Pacheco-Tena, C. (2016). Hypoxia and its implications in rheumatoid arthritis. *Journal of Biomedical Science*, *23*(1), 62. https://doi.org/10.1186/s12929-016-0281-0

Rada, B., Park, J. J., Sil, P., Geiszt, M., & Leto, T. L. (2014). NLRP3 inflammasome activation and interleukin-1ß release in macrophages require calcium but are independent of calcium-activated NADPH oxidases. *Inflammation Research*, *63*(10), 821–830. https://doi.org/10.1007/s00011-014-0756-y

Radwan, W. M., Khalifa, K. A., Esaily, H. A., & Lashin, N. A. (2016). CD14++CD16+ monocyte subset expansion in rheumatoid arthritis patients: Relation to disease activity and interleukin-17. *The Egyptian Rheumatologist*, *38*(3), 161–169. https://doi.org/10.1016/j.ejr.2015.12.002

Raggi, F., Blengio, F., Eva, A., Pende, D., Varesio, L., & Bosco, M. C. (2014). Identification of CD300a as a new hypoxia-inducible gene and a regulator of CCL20 and VEGF production by human monocytes and macrophages. *Innate Immunity*, *20*(7), 721–734. https://doi.org/10.1177/1753425913507095

Rajaiah, R., Perkins, D. J., Polumuri, S. K., Zhao, A., Keegan, A. D., & Vogel, S. N. (2013). Dissociation of endotoxin tolerance and differentiation of alternatively activated macrophages. *The Journal of Immunology*, *190*(9), 4763–4772. https://doi.org/10.4049/jimmunol.1202407

Ramos-Cejudo, J., Wisniewski, T., Marmar, C., Zetterberg, H., Blennow, K., De Leon, M. J., & Fossati, S. (2018). Traumatic brain injury and alzheimer's disease: The cerebrovascular link. *EBioMedicine*, *28*, 21–30. https://doi.org/10.1016/j.ebiom.2018.01.021

Rana, A. K., Li, Y., Dang, Q., & Yang, F. (2018). Monocytes in rheumatoid arthritis: Circulating precursors of macrophages and osteoclasts and, their heterogeneity and plasticity role in RA pathogenesis. *International Immunopharmacology*, *65*, 348–359. https://doi.org/10.1016/j.intimp.2018.10.016

Rao, D. A., Gurish, M. F., Marshall, J. L., Slowikowski, K., Fonseka, C. Y., Liu, Y., Donlin, L. T., Henderson, L. A., Wei, K., Mizoguchi, F., Teslovich, N. C., Weinblatt, M. E., Massarotti, E. M., Coblyn, J. S., Helfgott, S. M., Lee, Y. C., Todd, D. J., Bykerk, V. P., Goodman, S. M., … Brenner, M. B. (2017). Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. *Nature*, *542*(7639), 110–114. https://doi.org/10.1038/nature20810

Rathinam, V. A. K., Vanaja, S. K., Waggoner, L., Sokolovska, A., Becker, C., Stuart, L. M., Leong, J. M., & Fitzgerald, K. A. (2012). Trif licenses caspase-11-dependent nlrp3 inflammasome activation by gram-negative bacteria. *Cell*, *150*(3), 606–619. https://doi.org/10.1016/j.cell.2012.07.007

Raychaudhuri, S., Sandor, C., Stahl, E. A., Freudenberg, J., Lee, H.-S., Jia, X., Alfredsson, L., Padyukov, L., Klareskog, L., Worthington, J., Siminovitch, K. A., Bae, S.-C., Plenge, R. M., Gregersen, P. K., & De Bakker, P. I. W. (2012). Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. *Nature Genetics*, *44*(3), 291–296. https://doi.org/10.1038/ng.1076

Raza, K., Schwenzer, A., Juarez, M., Venables, P., Filer, A., Buckley, C. D., & Midwood, K. S. (2016). Detection of antibodies to citrullinated tenascin-C in patients with early synovitis is associated with the development of rheumatoid arthritis. *RMD Open*, *2*(2), e000318. https://doi.org/10.1136/rmdopen-2016-000318

Remmerie, A., Martens, L., & Scott, C. L. (2020). Macrophage subsets in obesity, aligning the liver and adipose tissue. *Frontiers in Endocrinology*, *11*, 259. https://doi.org/10.3389/fendo.2020.00259

Riquelme, S. A., Liimatta, K., Wong Fok Lung, T., Fields, B., Ahn, D., Chen, D., Lozano, C., Sáenz, Y., Uhlemann, A.-C., Kahl, B. C., Britto, C. J., DiMango, E., & Prince, A. (2020). Pseudomonas aeruginosa utilizes host-derived itaconate to redirect its metabolism to promote biofilm formation. *Cell Metabolism*, *31*(6), 1091-1106.e6. https://doi.org/10.1016/j.cmet.2020.04.017

Riquelme, S. A., Lozano, C., Moustafa, A. M., Liimatta, K., Tomlinson, K. L., Britto, C., Khanal, S., Gill, S. K., Narechania, A., Azcona-Gutiérrez, J. M., DiMango, E., Saénz, Y., Planet, P., & Prince, A. (2019). CFTR-PTEN–dependent mitochondrial metabolic dysfunction promotes *Pseudomonas aeruginosa* airway infection. *Science Translational Medicine*, *11*(499), eaav4634. https://doi.org/10.1126/scitranslmed.aav4634

Robert, S. M., Sjodin, H., Fink, M. P., & Aneja, R. K. (2010). Preconditioning with high mobility group box 1 (Hmgb1) induces lipoteichoic acid (Lta) tolerance. *Journal of Immunotherapy*, *33*(7), 663–671. https://doi.org/10.1097/CJI.0b013e3181dcd111

Roberts, A. W., Lee, B. L., Deguine, J., John, S., Shlomchik, M. J., & Barton, G. M. (2017). Tissue-resident macrophages are locally programmed for silent clearance of apoptotic cells. *Immunity*, *47*(5), 913-927.e6. https://doi.org/10.1016/j.immuni.2017.10.006

Roiniotis, J., Dinh, H., Masendycz, P., Turner, A., Elsegood, C. L., Scholz, G. M., & Hamilton, J. A. (2009). Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions. *The Journal of Immunology*, *182*(12), 7974–7981. https://doi.org/10.4049/jimmunol.0804216

Rossol, M., Heine, H., Meusch, U., Quandt, D., Klein, C., Sweet, M. J., & Hauschildt, S. (2011). Lps-induced cytokine production in human monocytes and macrophages. *Critical Reviews™ in Immunology*, *31*(5), 379–446. https://doi.org/10.1615/CritRevImmunol.v31.i5.20

Rubio-Perez, J. M., & Morillas-Ruiz, J. M. (2012). A review: Inflammatory process in alzheimer's disease, role of cytokines. *The Scientific World Journal*, *2012*, 1–15. https://doi.org/10.1100/2012/756357

Rüegg, C. R., Chiquet-Ehrismann, R., & Alkan, S. S. (1989). Tenascin, an extracellular matrix protein, exerts immunomodulatory activities. *Proceedings of the National Academy of Sciences*, *86*(19), 7437–7441. https://doi.org/10.1073/pnas.86.19.7437

Ruetz, M., Campanello, G. C., Purchal, M., Shen, H., McDevitt, L., Gouda, H., Wakabayashi, S., Zhu, J., Rubin, E. J., Warncke, K., Mootha, V. K., Koutmos, M., & Banerjee, R. (2019). Itaconyl-CoA forms a stable biradical in methylmalonyl-CoA mutase and derails its activity and repair. *Science*, *366*(6465), 589–593. https://doi.org/10.1126/science.aay0934

Ruhmann, M., Piccinini, A. M., Kong, P. L., & Midwood, K. S. (2012). Endogenous activation of adaptive immunity: Tenascin-C drives interleukin-17 synthesis in murine arthritic joint disease. *Arthritis & Rheumatism*, *64*(7), 2179–2190. https://doi.org/10.1002/art.34401

Ruiz, C., Huang, W., Hegi, M. E., Lange, K., Hamou, M.-F., Fluri, E., Oakeley, E. J., Chiquet-Ehrismann, R., & Orend, G. (2004). Differential gene expression analysis reveals activation of growth promoting signaling pathways by tenascin-c. *Cancer Research*, *64*(20), 7377–7385. https://doi.org/10.1158/0008-5472.CAN-04-1234

Ryan, D. G., & O'Neill, L. A. J. (2020). Krebs cycle reborn in macrophage immunometabolism. *Annual Review of Immunology*, *38*(1), 289–313. https://doi.org/10.1146/annurev-immunol-081619-104850

Ryu, J.-K., Kim, S. J., Rah, S.-H., Kang, J. I., Jung, H. E., Lee, D., Lee, H. K., Lee, J.-O., Park, B. S., Yoon, T.-Y., & Kim, H. M. (2017). Reconstruction of lps transfer cascade reveals structural determinants within lbp, cd14, and tlr4-md2 for efficient lps recognition and transfer. *Immunity*, *46*(1), 38–50. https://doi.org/10.1016/j.immuni.2016.11.007

Sabeh, F., Fox, D., & Weiss, S. J. (2010). Membrane-type i matrix metalloproteinase-dependent regulation of rheumatoid arthritis synoviocyte function. *The Journal of Immunology*, *184*(11), 6396–6406. https://doi.org/10.4049/jimmunol.0904068

Saeed, S., Quintin, J., Kerstens, H. H. D., Rao, N. A., Aghajanirefah, A., Matarese, F., Cheng, S.-C., Ratter, J., Berentsen, K., Van Der Ent, M. A., Sharifi, N., Janssen-Megens, E. M., Ter Huurne, M., Mandoli, A., Van Schaik, T., Ng, A., Burden, F., Downes, K., Frontini, M., … Stunnenberg, H. G. (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*, *345*(6204), 1251086. https://doi.org/10.1126/science.1251086

Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T., & Aizawa, S. (1992). Mice develop normally without tenascin. *Genes & Development*, *6*(10), 1821–1831. https://doi.org/10.1101/gad.6.10.1821

Sakai, A., Kusumoto, A., Kiso, Y., & Furuya, E. (2004). Itaconate reduces visceral fat by inhibiting fructose 2,6-bisphosphate synthesis in rat liver. *Nutrition*, *20*(11–12), 997–1002. https://doi.org/10.1016/j.nut.2004.08.007

Sakai, J., Cammarota, E., Wright, J. A., Cicuta, P., Gottschalk, R. A., Li, N., Fraser, I. D. C., & Bryant, C. E. (2017). Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Scientific Reports*, *7*(1), 1428. https://doi.org/10.1038/s41598-017-01600-y

Salter, D. M. (1993). Tenascin is increased in cartilage and synovium from arthritic knees. *Rheumatology*, *32*(9), 780–786. https://doi.org/10.1093/rheumatology/32.9.780

Sampath, P., Moideen, K., Ranganathan, U. D., & Bethunaickan, R. (2018). Monocyte subsets: Phenotypes and function in tuberculosis infection. *Frontiers in Immunology*, *9*, 1726. https://doi.org/10.3389/fimmu.2018.01726

Sanchez-Cantu, L. (1989). Endotoxin tolerance is associated with reduced secretion of tumor necrosis factor. *Archives of Surgery*, *124*(12), 1432. https://doi.org/10.1001/archsurg.1989.01410120082016

Saresella, M., La Rosa, F., Piancone, F., Zoppis, M., Marventano, I., Calabrese, E., Rainone, V., Nemni, R., Mancuso, R., & Clerici, M. (2016). The NLRP3 and NLRP1 inflammasomes are activated in Alzheimer's disease. *Molecular Neurodegeneration*, *11*(1), 23. https://doi.org/10.1186/s13024-016-0088-1

Sasikaran, J., Ziemski, M., Zadora, P. K., Fleig, A., & Berg, I. A. (2014). Bacterial itaconate degradation promotes pathogenicity. *Nature Chemical Biology*, *10*(5), 371–377. https://doi.org/10.1038/nchembio.1482

Savvi, S., Warner, D. F., Kana, B. D., McKinney, J. D., Mizrahi, V., & Dawes, S. S. (2008). Functional characterization of a vitamin b $_{12}$ -dependent methylmalonyl pathway in *mycobacterium tuberculosis*: Implications for propionate metabolism during growth on fatty acids. *Journal of Bacteriology*, *190*(11), 3886–3895. https://doi.org/10.1128/JB.01767-07

Sawada, Y., Onoda, K., Imanakayoshida, K., Maruyama, J., Yamamoto, K., Yoshida, T., & Shimpo, H. (2007). Tenascin-C synthesized in both donor grafts and recipients accelerates artery graft stenosis. *Cardiovascular Research*, *74*(3), 366–376. https://doi.org/10.1016/j.cardiores.2007.02.028

Schaefer, L. (2014). Complexity of danger: The diverse nature of damage-associated molecular patterns. *Journal of Biological Chemistry*, *289*(51), 35237–35245. https://doi.org/10.1074/jbc.R114.619304

Schaefer, L., Babelova, A., Kiss, E., Hausser, H.-J., Baliova, M., Krzyzankova, M., Marsche, G., Young, M. F., Mihalik, D., Götte, M., Malle, E., Schaefer, R. M., & Gröne, H.-J. (2005). The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. *Journal of Clinical Investigation*, *115*(8), 2223–2233. https://doi.org/10.1172/JCI23755

Scherer, H. U., Van Der Woude, D., & Toes, R. E. M. (2022). From risk to chronicity: Evolution of autoreactive B cell and antibody responses in rheumatoid arthritis. *Nature Reviews Rheumatology*, *18*(7), 371–383. https://doi.org/10.1038/s41584-022-00786-4

Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S. K., Doni, A., Rapisarda, A., Bernasconi, S., Saccani, S., Nebuloni, M., Vago, L., Mantovani, A., Melillo, G., & Sica, A. (2003). Regulation of the chemokine receptor cxcr4 by hypoxia. *The Journal of Experimental Medicine*, *198*(9), 1391–1402. https://doi.org/10.1084/jem.20030267

Schorn, C., Frey, B., Lauber, K., Janko, C., Strysio, M., Keppeler, H., Gaipl, U. S., Voll, R. E., Springer, E., Munoz, L. E., Schett, G., & Herrmann, M. (2011).

Sodium overload and water influx activate the nalp3 inflammasome. *Journal of Biological Chemistry*, *286*(1), 35–41. https://doi.org/10.1074/jbc.M110.139048

Schroder, K., & Tschopp, J. (2010). The inflammasomes. *Cell*, *140*(6), 821–832. https://doi.org/10.1016/j.cell.2010.01.040

Schwenzer, A., Jiang, X., Mikuls, T. R., Payne, J. B., Sayles, H. R., Quirke, A.-M., Kessler, B. M., Fischer, R., Venables, P. J., Lundberg, K., & Midwood, K. S. (2016). Identification of an immunodominant peptide from citrullinated tenascin-C as a major target for autoantibodies in rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *75*(10), 1876–1883. https://doi.org/10.1136/annrheumdis-2015-208495

Seeley, J. J., & Ghosh, S. (2017). Molecular mechanisms of innate memory and tolerance to LPS. *Journal of Leukocyte Biology*, *101*(1), 107–119. https://doi.org/10.1189/jlb.3MR0316-118RR

Semenza, G. L. (2009). Regulation of cancer cell metabolism by hypoxia-inducible factor 1. *Seminars in Cancer Biology*, *19*(1), 12–16. https://doi.org/10.1016/j.semcancer.2008.11.009

Semenza, G. L., & Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and Cellular Biology*, *12*(12), 5447–5454. https://doi.org/10.1128/MCB.12.12.5447

Serbina, N. V., & Pamer, E. G. (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nature Immunology*, *7*(3), 311–317. https://doi.org/10.1038/ni1309

Shalova, I. N., Lim, J. Y., Chittezhath, M., Zinkernagel, A. S., Beasley, F., Hernández-Jiménez, E., Toledano, V., Cubillos-Zapata, C., Rapisarda, A., Chen, J., Duan, K., Yang, H., Poidinger, M., Melillo, G., Nizet, V., Arnalich, F., López-Collazo, E., & Biswas, S. K. (2015). Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1α. *Immunity*, *42*(3), 484–498. https://doi.org/10.1016/j.immuni.2015.02.001

Sharma, S., Wang, J., Cortes Gomez, E., Taggart, R. T., & Baysal, B. E. (2017). Mitochondrial complex II regulates a distinct oxygen sensing mechanism in monocytes. *Human Molecular Genetics*, *26*(7), 1328–1339. https://doi.org/10.1093/hmg/ddx041

Shi, C., & Pamer, E. G. (2011). Monocyte recruitment during infection and inflammation. *Nature Reviews Immunology*, *11*(11), 762–774. https://doi.org/10.1038/nri3070

Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y., Cai, T., Wang, F., & Shao, F. (2015). Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature*, *526*(7575), 660–665. https://doi.org/10.1038/nature15514

Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., Hu, L., & Shao, F. (2014). Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature*, *514*(7521), 187–192. https://doi.org/10.1038/nature13683

Shimojo, N., Hashizume, R., Kanayama, K., Hara, M., Suzuki, Y., Nishioka, T., Hiroe, M., Yoshida, T., & Imanaka-Yoshida, K. (2015). Tenascin-c may accelerate cardiac fibrosis by activating macrophages via the integrin αvβ3/nuclear factor-κb/interleukin-6 axis. *Hypertension*, *66*(4), 757–766. https://doi.org/10.1161/HYPERTENSIONAHA.115.06004

Shin, J. J., Lee, E. K., Park, T. J., & Kim, W. (2015). Damage-associated molecular patterns and their pathological relevance in diabetes mellitus. *Ageing Research Reviews*, *24*, 66–76. https://doi.org/10.1016/j.arr.2015.06.004

Shin, J.-H., Yang, J.-Y., Jeon, B.-Y., Yoon, Y. J., Cho, S.-N., Kang, Y.-H., Ryu, D. H., & Hwang, G.-S. (2011). ^{1} h nmr-based metabolomic profiling in mice infected with *mycobacterium tuberculosis*. *Journal of Proteome Research*, *10*(5), 2238–2247. https://doi.org/10.1021/pr101054m

Sly, L. M., Rauh, M. J., Kalesnikoff, J., Song, C. H., & Krystal, G. (2004). Lps-induced upregulation of ship is essential for endotoxin tolerance. *Immunity*, *21*(2), 227–239. https://doi.org/10.1016/j.immuni.2004.07.010

Smiley, S. T., King, J. A., & Hancock, W. W. (2001). Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *The Journal of Immunology*, *167*(5), 2887–2894. https://doi.org/10.4049/jimmunol.167.5.2887

Smiljanovic, B., Grün, J. R., Biesen, R., Schulte-Wrede, U., Baumgrass, R., Stuhlmüller, B., Maslinski, W., Hiepe, F., Burmester, G.-R., Radbruch, A., Häupl, T., & Grützkau, A. (2012). The multifaceted balance of TNF-α and type I/II interferon responses in SLE and RA: How monocytes manage the impact of cytokines. *Journal of Molecular Medicine*, *90*(11), 1295–1309. https://doi.org/10.1007/s00109-012-0907-y

Smiljanovic, B., Radzikowska, A., Kuca-Warnawin, E., Kurowska, W., Grün, J. R., Stuhlmüller, B., Bonin, M., Schulte-Wrede, U., Sörensen, T., Kyogoku, C., Bruns, A., Hermann, S., Ohrndorf, S., Aupperle, K., Backhaus, M., Burmester, G. R., Radbruch, A., Grützkau, A., Maslinski, W., & Häupl, T. (2018). Monocyte alterations in rheumatoid arthritis are dominated by preterm release from bone marrow and prominent triggering in the joint. *Annals of the Rheumatic Diseases*, *77*(2), 300–308. https://doi.org/10.1136/annrheumdis-2017-211649

Smolen, J. S., Aletaha, D., Barton, A., Burmester, G. R., Emery, P., Firestein, G. S., Kavanaugh, A., McInnes, I. B., Solomon, D. H., Strand, V., & Yamamoto, K. (2018). Rheumatoid arthritis. *Nature Reviews Disease Primers*, *4*(1), 18001. https://doi.org/10.1038/nrdp.2018.1

Smolen, J. S., Landewé, R. B. M., Bergstra, S. A., Kerschbaumer, A., Sepriano, A., Aletaha, D., Caporali, R., Edwards, C. J., Hyrich, K. L., Pope, J. E., De Souza, S., Stamm, T. A., Takeuchi, T., Verschueren, P., Winthrop, K. L., Balsa, A., Bathon, J. M., Buch, M. H., Burmester, G. R., … Van Der Heijde, D. (2023). EULAR recommendations for the management of rheumatoid arthritis with

synthetic and biological disease-modifying antirheumatic drugs: 2022 update. *Annals of the Rheumatic Diseases*, *82*(1), 3–18. https://doi.org/10.1136/ard-2022-223356

Smolen, J. S., Landewé, R., Bijlsma, J., Burmester, G., Chatzidionysiou, K., Dougados, M., Nam, J., Ramiro, S., Voshaar, M., Van Vollenhoven, R., Aletaha, D., Aringer, M., Boers, M., Buckley, C. D., Buttgereit, F., Bykerk, V., Cardiel, M., Combe, B., Cutolo, M., … Van Der Heijde, D. (2017). EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2016 update. *Annals of the Rheumatic Diseases*, *76*(6), 960–977. https://doi.org/10.1136/annrheumdis-2016-210715

Snodgrass, R. G., Boß, M., Zezina, E., Weigert, A., Dehne, N., Fleming, I., Brüne, B., & Namgaladze, D. (2016). Hypoxia potentiates palmitate-induced proinflammatory activation of primary human macrophages. *Journal of Biological Chemistry*, *291*(1), 413–424. https://doi.org/10.1074/jbc.M115.686709

Sokolova, M. V., Schett, G., & Steffen, U. (2021). Autoantibodies in rheumatoid arthritis: Historical background and novel findings. *Clinical Reviews in Allergy & Immunology*, *63*(2), 138–151. https://doi.org/10.1007/s12016-021-08890-1

Song, J., Schwenzer, A., Wong, A., Turcinov, S., Rims, C., Martinez, L. R., Arribas-Layton, D., Gerstner, C., Muir, V. S., Midwood, K. S., Malmström, V., James, E. A., & Buckner, J. H. (2021). Shared recognition of citrullinated tenascin-C peptides by T and B cells in rheumatoid arthritis. *JCI Insight*, *6*(5), e145217. https://doi.org/10.1172/jci.insight.145217

Song, L., Wang, L., Li, F., Yukht, A., Qin, M., Ruther, H., Yang, M., Chaux, A., Shah, P. K., & Sharifi, B. G. (2017). Bone marrow-derived tenascin-c attenuates cardiac hypertrophy by controlling inflammation. *Journal of the American College of Cardiology*, *70*(13), 1601–1615. https://doi.org/10.1016/j.jacc.2017.07.789

Spanò, S., Gao, X., Hannemann, S., Lara-Tejero, M., & Galán, J. E. (2016). A bacterial pathogen targets a host rab-family gtpase defense pathway with a gap. *Cell Host & Microbe*, *19*(2), 216–226. https://doi.org/10.1016/j.chom.2016.01.004

Spanò, S., Liu, X., & Galán, J. E. (2011). Proteolytic targeting of Rab29 by an effector protein distinguishes the intracellular compartments of human-adapted and broad-host *Salmonella*. *Proceedings of the National Academy of Sciences*, *108*(45), 18418–18423. https://doi.org/10.1073/pnas.1111959108

Spenlé, C., Saupe, F., Midwood, K., Burckel, H., Noel, G., & Orend, G. (2015). Tenascin-C: Exploitation and collateral damage in cancer management. *Cell Adhesion & Migration*, *9*(1–2), 141–153. https://doi.org/10.1080/19336918.2014.1000074

Staples, K. J., Sotoodehnejadnematalahi, F., Pearson, H., Frankenberger, M., Francescut, L., Ziegler-Heitbrock, L., & Burke, B. (2011). Monocyte-derived macrophages matured under prolonged hypoxia transcriptionally up-regulate

HIF-1α mRNA. *Immunobiology*, *216*(7), 832–839. https://doi.org/10.1016/j.imbio.2010.12.005

Stolt, P., Yahya, A., Bengtsson, C., Källberg, H., Rönnelid, J., Lundberg, I., Klareskog, L., Alfredsson, L., & the EIRA Study Group. (2010). Silica exposure among male current smokers is associated with a high risk of developing ACPA-positive rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *69*(6), 1072–1076. https://doi.org/10.1136/ard.2009.114694

Strehl, C., Fangradt, M., Fearon, U., Gaber, T., Buttgereit, F., & Veale, D. J. (2013). Hypoxia: How does the monocyte-macrophage system respond to changes in oxygen availability? *Journal of Leukocyte Biology*, *95*(2), 233–241. https://doi.org/10.1189/jlb.1212627

Strelko, C. L., Lu, W., Dufort, F. J., Seyfried, T. N., Chiles, T. C., Rabinowitz, J. D., & Roberts, M. F. (2011). Itaconic acid is a mammalian metabolite induced during macrophage activation. *Journal of the American Chemical Society*, *133*(41), 16386–16389. https://doi.org/10.1021/ja2070889

Sugimoto, M., Sakagami, H., Yokote, Y., Onuma, H., Kaneko, M., Mori, M., Sakaguchi, Y., Soga, T., & Tomita, M. (2012). Non-targeted metabolite profiling in activated macrophage secretion. *Metabolomics*, *8*(4), 624–633. https://doi.org/10.1007/s11306-011-0353-9

Sumioka, T., Kitano, A., Flanders, K. C., Okada, Y., Yamanaka, O., Fujita, N., Iwanishi, H., Kao, W. W.-Y., & Saika, S. (2013). Impaired cornea wound healing in a tenascin C-deficient mouse model. *Laboratory Investigation*, *93*(2), 207–217. https://doi.org/10.1038/labinvest.2012.157

Swain, A., Bambouskova, M., Kim, H., Andhey, P. S., Duncan, D., Auclair, K., Chubukov, V., Simons, D. M., Roddy, T. P., Stewart, K. M., & Artyomov, M. N. (2020). Comparative evaluation of itaconate and its derivatives reveals divergent inflammasome and type I interferon regulation in macrophages. *Nature Metabolism*, *2*(7), 594–602. https://doi.org/10.1038/s42255-020-0210-0

Swanson, K. V., Deng, M., & Ting, J. P.-Y. (2019). The NLRP3 inflammasome: Molecular activation and regulation to therapeutics. *Nature Reviews Immunology*, *19*(8), 477–489. https://doi.org/10.1038/s41577-019-0165-0

Tacke, F., & Randolph, G. J. (2006). Migratory fate and differentiation of blood monocyte subsets. *Immunobiology*, *211*(6–8), 609–618. https://doi.org/10.1016/j.imbio.2006.05.025

Takahashi, N., Brouckaert, P., & Fiers, W. (1995). Mechanism of tolerance to tumor necrosis factor: Receptor-specific pathway and selectivity. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *269*(2), R398–R405. https://doi.org/10.1152/ajpregu.1995.269.2.R398

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, *140*(6), 805–820. https://doi.org/10.1016/j.cell.2010.01.022

Tamaoki, M., Imanaka-Yoshida, K., Yokoyama, K., Nishioka, T., Inada, H., Hiroe, M., Sakakura, T., & Yoshida, T. (2005). Tenascin-c regulates recruitment of

myofibroblasts during tissue repair after myocardial injury. *The American Journal of Pathology*, *167*(1), 71–80. https://doi.org/10.1016/S0002-9440(10)62954-9

Tannahill, G. M., Curtis, A. M., Adamik, J., Palsson-McDermott, E. M., McGettrick, A. F., Goel, G., Frezza, C., Bernard, N. J., Kelly, B., Foley, N. H., Zheng, L., Gardet, A., Tong, Z., Jany, S. S., Corr, S. C., Haneklaus, M., Caffrey, B. E., Pierce, K., Walmsley, S., … O'Neill, L. A. J. (2013). Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature*, *496*(7444), 238–242. https://doi.org/10.1038/nature11986

Ternette, N., Yang, M., Laroyia, M., Kitagawa, M., O'Flaherty, L., Wolhulter, K., Igarashi, K., Saito, K., Kato, K., Fischer, R., Berquand, A., Kessler, B. M., Lappin, T., Frizzell, N., Soga, T., Adam, J., & Pollard, P. J. (2013). Inhibition of mitochondrial aconitase by succination in fumarate hydratase deficiency. *Cell Reports*, *3*(3), 689–700. https://doi.org/10.1016/j.celrep.2013.02.013

Tominaga, K., Saito, S., Matsuura, M., & Nakano, M. (1999). Lipopolysaccharide tolerance in murine peritoneal macrophages induces downregulation of the lipopolysaccharide signal transduction pathway through mitogen-activated protein kinase and nuclear factor-κB cascades, but not lipopolysaccharide-incorporation steps. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1450*(2), 130–144. https://doi.org/10.1016/S0167-4889(99)00037-3

Tomlinson, K. L., Lung, T. W. F., Dach, F., Annavajhala, M. K., Gabryszewski, S. J., Groves, R. A., Drikic, M., Francoeur, N. J., Sridhar, S. H., Smith, M. L., Khanal, S., Britto, C. J., Sebra, R., Lewis, I., Uhlemann, A.-C., Kahl, B. C., Prince, A. S., & Riquelme, S. A. (2021). Staphylococcus aureus induces an itaconate-dominated immunometabolic response that drives biofilm formation. *Nature Communications*, *12*(1), 1399. https://doi.org/10.1038/s41467-021-21718-y

Trzebanski, S., & Jung, S. (2020). Plasticity of monocyte development and monocyte fates. *Immunology Letters*, *227*, 66–78. https://doi.org/10.1016/j.imlet.2020.07.007

Tsai, C., Diaz, L. A., Singer, N. G., Li, L. L., Kirsch, A. H., Mitra, R., Nickoloff, B. J., Crofford, L. J., & Fox, D. A. (1996). Responsiveness of human T lymphocytes to bacterial superantigens presented by cultured rheumatoid arthritis synoviocytes. *Arthritis & Rheumatism*, *39*(1), 125–136. https://doi.org/10.1002/art.1780390117

Tsoi, L. C., Spain, S. L., Ellinghaus, E., Stuart, P. E., Capon, F., Knight, J., Tejasvi, T., Kang, H. M., Allen, M. H., Lambert, S., Stoll, S. W., Weidinger, S., Gudjonsson, J. E., Koks, S., Kingo, K., Esko, T., Das, S., Metspalu, A., Weichenthal, M., … Elder, J. T. (2015). Enhanced meta-analysis and replication studies identify five new psoriasis susceptibility loci. *Nature Communications*, *6*(1), 7001. https://doi.org/10.1038/ncomms8001

Turner, L., Scotton, C., Negus, R., & Balkwill, F. (1999). Hypoxia inhibits macrophage migration. *European Journal of Immunology*, *29*(7), 2280–2287.

https://doi.org/10.1002/(SICI)1521-4141(199907)29:07<2280::AID-IMMU2280>3.0.CO;2-C

Tutturen, A. E. V., Fleckenstein, B., & De Souza, G. A. (2014). Assessing the citrullinome in rheumatoid arthritis synovial fluid with and without enrichment of citrullinated peptides. *Journal of Proteome Research*, *13*(6), 2867–2873. https://doi.org/10.1021/pr500030x

Udalova, I. A., Ruhmann, M., Thomson, S. J. P., & Midwood, K. S. (2011). Expression and immune function of tenascin-c. *Critical Reviews™ in Immunology*, *31*(2), 115–145. https://doi.org/10.1615/CritRevImmunol.v31.i2.30

Unterholzner, L., Keating, S. E., Baran, M., Horan, K. A., Jensen, S. B., Sharma, S., Sirois, C. M., Jin, T., Latz, E., Xiao, T. S., Fitzgerald, K. A., Paludan, S. R., & Bowie, A. G. (2010). IFI16 is an innate immune sensor for intracellular DNA. *Nature Immunology*, *11*(11), 997–1004. https://doi.org/10.1038/ni.1932

Uribe-Querol, E., & Rosales, C. (2020). Phagocytosis: Our current understanding of a universal biological process. *Frontiers in Immunology*, *11*, 1066. https://doi.org/10.3389/fimmu.2020.01066

Van Der Helm-van Mil, A. H. M., Verpoort, K. N., Breedveld, F. C., Huizinga, T. W. J., Toes, R. E. M., & De Vries, R. R. P. (2006). The HLA–DRB1 shared epitope alleles are primarily a risk factor for anti–cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. *Arthritis & Rheumatism*, *54*(4), 1117–1121. https://doi.org/10.1002/art.21739

Van Der Woude, D., Rantapaa-Dahlqvist, S., Ioan-Facsinay, A., Onnekink, C., Schwarte, C. M., Verpoort, K. N., Drijfhout, J. W., Huizinga, T. W. J., Toes, R. E. M., & Pruijn, G. J. M. (2010). Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. *Annals of the Rheumatic Diseases*, *69*(8), 1554–1561. https://doi.org/10.1136/ard.2009.124537

Van Schaftingen, E., Davies, D. R., & Hers, H.-G. (1981). Inactivation of phosphofructokinase 2 by cyclic AMP-dependent protein kinase. *Biochemical and Biophysical Research Communications*, *103*(1), 362–368. https://doi.org/10.1016/0006-291X(81)91701-0

Van Steenbergen, H. W., Tsonaka, R., Huizinga, T. W. J., Boonen, A., & Van Der Helm-van Mil, A. H. M. (2015). Fatigue in rheumatoid arthritis; a persistent problem: A large longitudinal study. *RMD Open*, *1*(1), e000041. https://doi.org/10.1136/rmdopen-2014-000041

Venegas, C., & Heneka, M. T. (2017). Danger-associated molecular patterns in Alzheimer's disease. *Journal of Leukocyte Biology*, *101*(1), 87–98. https://doi.org/10.1189/jlb.3MR0416-204R

Virca, G. D., Kim, S. Y., Glaser, K. B., & Ulevitch, R. J. (1989). Lipopolysaccharide induces hyporesponsiveness to its own action in raw 264. 7 cells. *Journal of Biological Chemistry*, *264*(36), 21951–21956. https://doi.org/10.1016/S0021-9258(20)88277-5

Voon, F.-L., Sulaiman, M. R., Akhtar, M. N., Idris, M. F., Akira, A., Perimal, E. K., Israf, D. A., & Ming-Tatt, L. (2017). Cardamonin (2′,4′-dihydroxy-6′-methoxychalcone) isolated from Boesenbergia rotunda (L.) Mansf. Inhibits CFA-induced rheumatoid arthritis in rats. *European Journal of Pharmacology*, *794*, 127–134. https://doi.org/10.1016/j.ejphar.2016.11.009

Wakabayashi, G., Cannon, J. G., Gelfand, J. A., Clark, B. D., Aiura, K., Burke, J. F., Wolff, S. M., & Dinarello, C. A. (1994). Altered interleukin-1 and tumor necrosis factor production and secretion during pyrogenic tolerance to LPS in rabbits. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *267*(1), R329–R336. https://doi.org/10.1152/ajpregu.1994.267.1.R329

Wallisch, J. S., Simon, D. W., Bayır, H., Bell, M. J., Kochanek, P. M., & Clark, R. S. B. (2017). Cerebrospinal fluid nlrp3 is increased after severe traumatic brain injury in infants and children. *Neurocritical Care*, *27*(1), 44–50. https://doi.org/10.1007/s12028-017-0378-7

Wang, H., Fedorov, A. A., Fedorov, E. V., Hunt, D. M., Rodgers, A., Douglas, H. L., Garza-Garcia, A., Bonanno, J. B., Almo, S. C., & De Carvalho, L. P. S. (2019). An essential bifunctional enzyme in *Mycobacterium tuberculosis* for itaconate dissimilation and leucine catabolism. *Proceedings of the National Academy of Sciences*, *116*(32), 15907–15913. https://doi.org/10.1073/pnas.1906606116

Wang, S., Song, R., Wang, Z., Jing, Z., Wang, S., & Ma, J. (2018). S100a8/a9 in inflammation. *Frontiers in Immunology*, *9*, 1298. https://doi.org/10.3389/fimmu.2018.01298

Watson, D. W., & Kim, Y. B. (1963). Modification of host responses to bacterial endotoxins. *The Journal of Experimental Medicine*, *118*(3), 425–446. https://doi.org/10.1084/jem.118.3.425

Weber, B. N., Giles, J. T., & Liao, K. P. (2023). Shared inflammatory pathways of rheumatoid arthritis and atherosclerotic cardiovascular disease. *Nature Reviews Rheumatology*, *19*(7), 417–428. https://doi.org/10.1038/s41584-023-00969-7

Weber, C., Belge, K.-U., Von Hundelshausen, P., Draude, G., Steppich, B., Mack, M., Frankenberger, M., Weber, K. S. C., & Ziegler-Heitbrock, H. W. L. (2000). Differential chemokine receptor expression and function in human monocyte subpopulations. *Journal of Leukocyte Biology*, *67*(5), 699–704. https://doi.org/10.1002/jlb.67.5.699

Weber, M., Sydlik, C., Quirling, M., Nothdurfter, C., Zwergal, A., Heiss, P., Bell, S., Neumeier, D., Ziegler-Heitbrock, H. W. L., & Brand, K. (2003). Transcriptional inhibition of interleukin-8 expression in tumor necrosis factor-tolerant cells. *Journal of Biological Chemistry*, *278*(26), 23586–23593. https://doi.org/10.1074/jbc.M211646200

Wegner, N., Lundberg, K., Kinloch, A., Fisher, B., Malmström, V., Feldmann, M., & Venables, P. J. (2010). Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. *Immunological Reviews*, *233*(1), 34–54. https://doi.org/10.1111/j.0105-2896.2009.00850.x

Wegner, N., Wait, R., Sroka, A., Eick, S., Nguyen, K., Lundberg, K., Kinloch, A., Culshaw, S., Potempa, J., & Venables, P. J. (2010). Peptidylarginine deiminase from *Porphyromonas gingivalis* citrullinates human fibrinogen and α-enolase: Implications for autoimmunity in rheumatoid arthritis. *Arthritis & Rheumatism*, *62*(9), 2662–2672. https://doi.org/10.1002/art.27552

Wei, K., Korsunsky, I., Marshall, J. L., Gao, A., Watts, G. F. M., Major, T., Croft, A. P., Watts, J., Blazar, P. E., Lange, J. K., Thornhill, T. S., Filer, A., Raza, K., Donlin, L. T., Accelerating Medicines Partnership Rheumatoid Arthritis & Systemic Lupus Erythematosus (AMP RA/SLE) Consortium, Albrecht, J., Anolik, J. H., Apruzzese, W., Boyce, B. F., … Brenner, M. B. (2020). Notch signalling drives synovial fibroblast identity and arthritis pathology. *Nature*, *582*(7811), 259–264. https://doi.org/10.1038/s41586-020-2222-z

Weisheit, C. K., Klüners, A., Wild, L., Casalter, A., Heilmann-Heimbach, S., Sivalingam, S., Kleiner, J. L., Ehrentraut, S. F., Hoeft, A., Frede, S., & Ehrentraut, H. (2020). Sustained immunoparalysis in endotoxin-tolerized monocytic cells. *Mediators of Inflammation*, *2020*, 1–10. https://doi.org/10.1155/2020/8294342

West, M. A., & Heagy, W. (2002). Endotoxin tolerance: A review: *Critical Care Medicine*, *30*(Suppl.), S64–S73. https://doi.org/10.1097/00003246-200201001-00009

Weyand, C. M., & Goronzy, J. J. (2020). Immunometabolism in the development of rheumatoid arthritis. *Immunological Reviews*, *294*(1), 177–187. https://doi.org/10.1111/imr.12838

Widdrington, J. D., Gomez-Duran, A., Pyle, A., Ruchaud-Sparagano, M.-H., Scott, J., Baudouin, S. V., Rostron, A. J., Lovat, P. E., Chinnery, P. F., & Simpson, A. J. (2018). Exposure of monocytic cells to lipopolysaccharide induces coordinated endotoxin tolerance, mitochondrial biogenesis, mitophagy, and antioxidant defenses. *Frontiers in Immunology*, *9*, 2217. https://doi.org/10.3389/fimmu.2018.02217

Wigerblad, G., Bas, D. B., Fernades-Cerqueira, C., Krishnamurthy, A., Nandakumar, K. S., Rogoz, K., Kato, J., Sandor, K., Su, J., Jimenez-Andrade, J. M., Finn, A., Bersellini Farinotti, A., Amara, K., Lundberg, K., Holmdahl, R., Jakobsson, P.-J., Malmström, V., Catrina, A. I., Klareskog, L., & Svensson, C. I. (2016). Autoantibodies to citrullinated proteins may induce joint pain independent of inflammation. *Annals of the Rheumatic Diseases*, *75*(4), 730–738. https://doi.org/10.1136/annrheumdis-2015-208094

Wiggins, K. A., Parry, A. J., Cassidy, L. D., Humphry, M., Webster, S. J., Goodall, J. C., Narita, M., & Clarke, M. C. H. (2019). IL-1α cleavage by inflammatory caspases of the noncanonical inflammasome controls the senescence-associated secretory phenotype. *Aging Cell*, *18*(3), e12946. https://doi.org/10.1111/acel.12946

Williams, J. F. (1985). Induction of tolerance in mice and rats to the effect of endotoxin to decrease the hepatic microsomal mixed-function oxidase system. Evidence for a possible macrophage-derived factor in the endotoxin effect.

International Journal of Immunopharmacology, *7*(4), 501–509. https://doi.org/10.1016/0192-0561(85)90069-4

Wongsaroj, L., Saninjuk, K., Romsang, A., Duang-nkern, J., Trinachartvanit, W., Vattanaviboon, P., & Mongkolsuk, S. (2018). Pseudomonas aeruginosa glutathione biosynthesis genes play multiple roles in stress protection, bacterial virulence and biofilm formation. *PLOS ONE*, *13*(10), e0205815. https://doi.org/10.1371/journal.pone.0205815

Woo, S.-R., Corrales, L., & Gajewski, T. F. (2015). Innate immune recognition of cancer. *Annual Review of Immunology*, *33*(1), 445–474. https://doi.org/10.1146/annurev-immunol-032414-112043

Wu, F., Gao, J., Kang, J., Wang, X., Niu, Q., Liu, J., & Zhang, L. (2021). B cells in rheumatoid arthritis：pathogenic mechanisms and treatment prospects. *Frontiers in Immunology*, *12*, 750753. https://doi.org/10.3389/fimmu.2021.750753

Xie, K., Liu, Y., Hao, W., Walter, S., Penke, B., Hartmann, T., Schachner, M., & Fassbender, K. (2013). Tenascin-C deficiency ameliorates Alzheimer's disease-related pathology in mice. *Neurobiology of Aging*, *34*(10), 2389–2398. https://doi.org/10.1016/j.neurobiolaging.2013.04.013

Xiong, Y., & Medvedev, A. E. (2011). Induction of endotoxin tolerance in vivo inhibits activation of IRAK4 and increases negative regulators IRAK-M, SHIP-1, and A20. *Journal of Leukocyte Biology*, *90*(6), 1141–1148. https://doi.org/10.1189/jlb.0611273

Xiong, Y., Qiu, F., Piao, W., Song, C., Wahl, L. M., & Medvedev, A. E. (2011). Endotoxin tolerance impairs il-1 receptor-associated kinase (Irak) 4 and tgf-β-activated kinase 1 activation, k63-linked polyubiquitination and assembly of irak1, tnf receptor-associated factor 6, and iκb kinase γ and increases a20 expression. *Journal of Biological Chemistry*, *286*(10), 7905–7916. https://doi.org/10.1074/jbc.M110.182873

Yamamoto, K., Onoda, K., Sawada, Y., Fujinaga, K., Imanakayoshida, K., Shimpo, H., Yoshida, T., & Yada, I. (2005). Tenascin-C is an essential factor for neointimal hyperplasia after aortotomy in mice. *Cardiovascular Research*, *65*(3), 737–742. https://doi.org/10.1016/j.cardiores.2004.10.034

Yan, C., Yan, H., Mao, J., Liu, Y., Xu, L., Zhao, H., Shen, J., Cao, Y., Gao, Y., Li, K., & Jin, W. (2020). Neuroprotective effect of oridonin on traumatic brain injury via inhibiting nlrp3 inflammasome in experimental mice. *Frontiers in Neuroscience*, *14*, 557170. https://doi.org/10.3389/fnins.2020.557170

Yan, J., Chen, Y., He, C., Yang, Z., Lü, C., & Chen, X. (2012). Andrographolide induces cell cycle arrest and apoptosis in human rheumatoid arthritis fibroblast-like synoviocytes. *Cell Biology and Toxicology*, *28*(1), 47–56. https://doi.org/10.1007/s10565-011-9204-8

Yáñez, A., Coetzee, S. G., Olsson, A., Muench, D. E., Berman, B. P., Hazelett, D. J., Salomonis, N., Grimes, H. L., & Goodridge, H. S. (2017). Granulocyte-

monocyte progenitors and monocyte-dendritic cell progenitors independently produce functionally distinct monocytes. *Immunity*, *47*(5), 890-902.e4. https://doi.org/10.1016/j.immuni.2017.10.021

Yang, D., Han, Z., Alam, M. M., & Oppenheim, J. J. (2018). High-mobility group nucleosome binding domain 1 (Hmgn1) functions as a Th1-polarizing alarmin. *Seminars in Immunology*, *38*, 49–53. https://doi.org/10.1016/j.smim.2018.02.012

Yang, H., Wang, H., & Andersson, U. (2020). Targeting inflammation driven by hmgb1. *Frontiers in Immunology*, *11*, 484. https://doi.org/10.3389/fimmu.2020.00484

Yang, J., Liu, Z., & Xiao, T. S. (2017). Post-translational regulation of inflammasomes. *Cellular & Molecular Immunology*, *14*(1), 65–79. https://doi.org/10.1038/cmi.2016.29

Yeap, W. H., Wong, K. L., Shimasaki, N., Teo, E. C. Y., Quek, J. K. S., Yong, H. X., Diong, C. P., Bertoletti, A., Linn, Y. C., & Wong, S. C. (2016). CD16 is indispensable for antibody-dependent cellular cytotoxicity by human monocytes. *Scientific Reports*, *6*(1), 34310. https://doi.org/10.1038/srep34310

Yeo, L., Adlard, N., Biehl, M., Juarez, M., Smallie, T., Snow, M., Buckley, C. D., Raza, K., Filer, A., & Scheel-Toellner, D. (2016). Expression of chemokines CXCL4 and CXCL7 by synovial macrophages defines an early stage of rheumatoid arthritis. *Annals of the Rheumatic Diseases*, *75*(4), 763–771. https://doi.org/10.1136/annrheumdis-2014-206921

Yin, J., Zhao, F., Chojnacki, J. E., Fulp, J., Klein, W. L., Zhang, S., & Zhu, X. (2018). Nlrp3 inflammasome inhibitor ameliorates amyloid pathology in a mouse model of alzheimer's disease. *Molecular Neurobiology*, *55*(3), 1977–1987. https://doi.org/10.1007/s12035-017-0467-9

Yokoyama, K., Erickson, H. P., Ikeda, Y., & Takada, Y. (2000). Identification of amino acid sequences in fibrinogen γ-chain and tenascin c c-terminal domains critical for binding to integrin αvβ3. *Journal of Biological Chemistry*, *275*(22), 16891–16898. https://doi.org/10.1074/jbc.M000610200

Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M., Misharin, A., Hume, D. A., Perlman, H., Malissen, B., Zelzer, E., & Jung, S. (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*, *38*(1), 79–91. https://doi.org/10.1016/j.immuni.2012.12.001

Yu, L., Wang, L., & Chen, S. (2010). Endogenous toll-like receptor ligands and their biological significance. *Journal of Cellular and Molecular Medicine*, *14*(11), 2592–2603. https://doi.org/10.1111/j.1582-4934.2010.01127.x

Zahid, A., Li, B., Kombe, A. J. K., Jin, T., & Tao, J. (2019). Pharmacological inhibitors of the nlrp3 inflammasome. *Frontiers in Immunology*, *10*, 2538. https://doi.org/10.3389/fimmu.2019.02538

Zavascki, A. P., Goldani, L. Z., Li, J., & Nation, R. L. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: A critical review. *Journal of Antimicrobial Chemotherapy*, *60*(6), 1206–1215. https://doi.org/10.1093/jac/dkm357

Zelenay, S., & Reis E Sousa, C. (2013). Adaptive immunity after cell death. *Trends in Immunology*, *34*(7), 329–335. https://doi.org/10.1016/j.it.2013.03.005

Zeng, L., Yang, K., Zhang, T., Zhu, X., Hao, W., Chen, H., & Ge, J. (2022). Research progress of single-cell transcriptome sequencing in autoimmune diseases and autoinflammatory disease: A review. *Journal of Autoimmunity*, *133*, 102919. https://doi.org/10.1016/j.jaut.2022.102919

Zhang, F., Jonsson, A. H., Nathan, A., Millard, N., Curtis, M., Xiao, Q., Gutierrez-Arcelus, M., Apruzzese, W., Watts, G. F. M., Weisenfeld, D., Nayar, S., Rangel-Moreno, J., Meednu, N., Marks, K. E., Mantel, I., Kang, J. B., Rumker, L., Mears, J., Slowikowski, K., … Raychaudhuri, S. (2023). Deconstruction of rheumatoid arthritis synovium defines inflammatory subtypes. *Nature*, *623*(7987), 616–624. https://doi.org/10.1038/s41586-023-06708-y

Zhang, Q., Raoof, M., Chen, Y., Sumi, Y., Sursal, T., Junger, W., Brohi, K., Itagaki, K., & Hauser, C. J. (2010). Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature*, *464*(7285), 104–107. https://doi.org/10.1038/nature08780

Zhang, W., Shen, X., Xie, L., Chu, M., & Ma, Y. (2015). MicroRNA-181b regulates endotoxin tolerance by targeting IL-6 in macrophage RAW264.7 cells. *Journal of Inflammation*, *12*(1), 18. https://doi.org/10.1186/s12950-015-0061-8

Zhang, Y., Zheng, Y., & Li, H. (2016). Nlrp3 inflammasome plays an important role in the pathogenesis of collagen-induced arthritis. *Mediators of Inflammation*, *2016*, 1–9. https://doi.org/10.1155/2016/9656270

Zhao, Y., Gao, C., Liu, L., Wang, L., & Song, Z. (2023). The development and function of human monocyte-derived dendritic cells regulated by metabolic reprogramming. *Journal of Leukocyte Biology*, *114*(3), 212–222. https://doi.org/10.1093/jleuko/qiad062

Zhou, M., Aziz, M., Denning, N.-L., Yen, H.-T., Ma, G., & Wang, P. (2020). Extracellular CIRP induces macrophage endotoxin tolerance through IL-6R-mediated STAT3 activation. *JCI Insight*, *5*(5), e133715. https://doi.org/10.1172/jci.insight.133715

Ziegler-Heitbrock, H. W. L. (2000). Definition of human blood monocytes. *Journal of Leukocyte Biology*, *67*(5), 603–606. https://doi.org/10.1002/jlb.67.5.603

Ziegler-Heitbrock, H. W., Wedel, A., Schraut, W., Ströbel, M., Wendelgass, P., Sternsdorf, T., Bäuerle, P. A., Haas, J. G., & Riethmüller, G. (1994). Tolerance to lipopolysaccharide involves mobilization of nuclear factor kappa B with predominance of p50 homodimers. *Journal of Biological Chemistry*, *269*(25), 17001–17004. https://doi.org/10.1016/S0021-9258(17)32510-3

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., Leenen, P. J. M., Liu, Y.-J., MacPherson, G., Randolph, G. J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J. M., & Lutz, M. B. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood*, *116*(16), e74–e80. https://doi.org/10.1182/blood-2010-02-258558

Zimmer, S., Grebe, A., & Latz, E. (2015). Danger signaling in atherosclerosis. *Circulation Research*, *116*(2), 323–340. https://doi.org/10.1161/CIRCRESAHA.116.301135

Zimmerman, T., Petit Frère, C., Satzger, M., Raba, M., Weisbach, M., Döhn, K., Popp, A., & Donzeau, M. (2006). Simultaneous metal chelate affinity purification and endotoxin clearance of recombinant antibody fragments. *Journal of Immunological Methods*, *314*(1–2), 67–73. https://doi.org/10.1016/j.jim.2006.05.012

Zindel, J., & Kubes, P. (2020). Damps, pamps, and lamps in immunity and sterile inflammation. *Annual Review of Pathology: Mechanisms of Disease*, *15*(1), 493–518. https://doi.org/10.1146/annurev-pathmechdis-012419-032847

Zuliani-Alvarez, L., Marzeda, A. M., Deligne, C., Schwenzer, A., McCann, F. E., Marsden, B. D., Piccinini, A. M., & Midwood, K. S. (2017). Mapping tenascin-C interaction with toll-like receptor 4 reveals a new subset of endogenous inflammatory triggers. *Nature Communications*, *8*(1), 1595. https://doi.org/10.1038/s41467-017-01718-7

Zvaifler, N. J., Steinman, R. M., Kaplan, G., Lau, L. L., & Rivelis, M. (1985). Identification of immunostimulatory dendritic cells in the synovial effusions of patients with rheumatoid arthritis. *Journal of Clinical Investigation*, *76*(2), 789–800. https://doi.org/10.1172/JCI112036