Inflammasome and cell death pathways in tissue tolerance and disease

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ABSTRACT

Innate immunity and cell death are two interconnected processes that play vital roles in host defence. However, inappropriate activation of these pathways can have detrimental effects, leading to inflammatory disorders. These ailments can be initiated by the recognition of cellular damage and stress by germline encoded pattern recognition receptors (PRRs). The inflammasome is an intracellular multi-protein complex capable of recognizing both foreign and host derived danger signals. Its activation leads to the induction of inflammation and the initiation of cell death. It is now well known that the inflammasome plays an important role in the development of multiple diseases. In this thesis we assess how the inflammasome and cell death contribute to metabolic disease and inflammatory bowel disease. Caspase-12 is a reported inhibitor of the inflammasome and is associated with susceptibility to bacterial infection in mice. We determined that mice lacking caspase-12 develop obesity, insulin resistance, and adipose tissue inflammation. Patients deficient in caspase-12 expression also had elevated inflammatory markers within the adipose tissue. To further investigate the role of the inflammasome in metabolic disease, we generated mice that express a hyperactive Nlrp3 allele exclusively in adipocytes. These mice were observed to develop glucose intolerance, implicating a role for the inflammasome in adipocytes. Finally, we examined the role of necroptotic cell death in inflammatory bowel disease. Mice deficient in the necroptosis effector Mlkl displayed increased resistance to an experimental model of colitis. The colons of these mice were characterized by increased cell death and elevated secretion of reparative cytokines. This thesis furthers our understanding of metabolic disease and inflammatory bowel disease and implicates new proteins and pathways in their pathogenesis.

RÉSUMÉ

L'immunité innée et la mort cellulaire sont deux processus interconnectés qui jouent un rôle vital dans la défense de l'hôte face aux dangers et aux infections. Cependant, une activation inappropriée de ces mécanismes peut avoir des effets néfastes, conduisant à des troubles inflammatoires. Ces dispositifs de défense peuvent être initiées par la reconnaissance de dommages cellulaires ou de stress par des récepteurs capables de reconnaître des motifs moléculaires caractéristiques de ces conditions. L'inflammasome est un complexe multi-protéique intracellulaire capable de reconnaître des signaux de danger issus de pathogènes ou de l'hôte. Son activation va alors conduire à l'induction de l'inflammation et à l'initiation de la mort cellulaire. Il est maintenant bien connu que la l'inflammasome joue un rôle important dans le développement de plusieurs maladies. Dans cette thèse, nous évaluons comment l'inflammasome et la mort cellulaire contribuent aux maladies métaboliques et aux maladies inflammatoires de l'intestin. La Caspase-12 est un inhibiteur connu de l'Inflammasome et est associée à une susceptibilité aux infections bactériennes chez la souris. Nous avons déterminé que les souris dépourvues de Caspase-12 développaient une obésité, une résistance à l'insuline et une plus forte inflammation du tissu adipeux que des souris sauvages. Les patients déficients pour l'expression de la Caspase-12 présentent également des niveaux élevés des marqueurs inflammatoires dans le tissu adipeux. Afin d'étudier davantage le rôle de l'inflammasome dans les maladies métaboliques, nous avons généré des souris exprimant un allèle NLRP3 hyperactif exclusivement dans les adipocytes. Ces souris développent également une intolérance au glucose, ce qui implique un rôle pour l'inflammasome dans les adipocytes. Enfin, nous avons examiné le rôle de la nécroptose, un type de mort inflammatoire, dans les maladies inflammatoire de l'intestin. Les souris déficientes pour l'expression de la proteine effectrice de la nécroptose Mlkl ont montré une résistance accrue à un modèle expérimental de colite. Une augmentation de la mort cellulaire et de la sécrétion de cytokines réparatrices a été observée dans ces souris. Cette thèse renforce notre compréhension des maladies métaboliques et des maladies inflammatoires de l'intestin et implique de nouvelles protéines et de voies de signalisation dans leur pathogenèse.

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PREFACE AND CONTRIBUTION OF THE AUTHORS

This thesis is composed of 5 chapters, containing a literature review, three original manuscripts, and a discussion. One manuscript has been published, while the other two are in preparation for publication.

Chapter 2 - Caspase-12, but Not Caspase-11, Inhibits Obesity and Insulin Resistance

The results of this study were generated by myself and several collaborators. I performed all *in vitro* and mouse experiments and analyzed data contained within figures 1 to 5. Dr. Alexandre Morizot and Todd Douglas assisted in the dissection of mice, provided technical help, and experimental advice during the course of the study. Dr. Nicola Santoro, Dr. Romy Kursaw, Dr. Sonia Caprio, and Dr. Wajahat Mehal were responsible for Figure 6 of the manuscript and all data concerning the Yale Pediatric cohort. Dr. Julia Kozlitina performed statistical analysis of the Dallas Heart Study data and was responsible for Table I. I wrote, and edited the manuscript along with Dr. Maya Saleh.

Chapter 3 - Adipocyte specific Nlrp3 inflammasome activity contributes to glucose intolerance

The results of this study were generated solely by myself. I wrote, and edited the manuscript along with Dr. Maya Saleh.

Chapter 4 - The necroptosis effector MLKL mediates erosive colitis

The results of this study were generated by myself and several collaborators. I performed experiments, analyzed the results, and generated data for all figures except for figure 2B. Maryse Dagenais assisted in dissection of the mice and provided experimental advice. Claudia Champagne assisted in the dissections, provided experimental advice, and is responsible for the immunohistochemistry and analysis of figure 2B. Dr. Alexandre Morizot performed part of the qPCR analysis of Figure 4H. I wrote, and edited the manuscript along with Dr. Maya Saleh.

My thesis supervisor, Dr. Maya Saleh, supervised each project, and was responsible for experimental design, analysis of the data, and writing and editing of the manuscripts.

The work of this thesis has been published or prepared for publication as follows:

Chapter 1- Introduction

Parts of section 1.2 were adapted from the following published manuscript:

Skeldon A, Saleh M. "The inflammasomes: molecular effectors of host resistance against bacterial, viral, parasitic, and fungal infections." Front Microbiol. 2011 Feb 17;2:15

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Chapter 2 - Caspase-12, but Not Caspase-11, Inhibits Obesity and Insulin Resistance

Skeldon AM, Morizot A, Douglas T, Santoro N, Kursawe R, Kozlitina J, Caprio S, Mehal WZ, Saleh M. "Caspase-12, but Not Caspase-11, Inhibits Obesity and Insulin Resistance." J Immunol. 2016 Jan 1;196(1):437-47.

Chapter 3 – Adipocyte specific Nlrp3 inflammasome activity contributes to glucose intolerance

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Chapter 4 - The necroptosis effector MLKL mediates erosive colitis

Skeldon AM, Dagenais M., Champagne C., Morizot A., Saleh M. "The necroptosis effector MLKL mediates erosive colitis.**"** *Manuscript in preparation*

RATIONALE AND OBJECTIVES

The innate immune system and cell death pathways are a vital component of our defence against foreign pathogens. However, these mechanisms can also have detrimental effects when they are activated aberrantly. The increasing incidence of metabolic disease and inflammatory bowel disease over the last half century has facilitated an increasing necessity to more fully understand their pathogenesis. While these diseases are complex in nature and develop from a combination of multiple factors, unregulated inflammation is a strong contributor to both. A better understanding of the intricacies of innate immune and cell death pathways and their involvement in disease is crucial for the development of better treatment and therapies.

Beginning in 2009, multiple studies have reported a role for caspase-1 and the inflammasome as a positive contributor to metabolic disease in humans and mice (1). Despite this involvement, little is known regarding the role of inflammasome regulators and their respective functions in obesity and insulin resistance. Caspase-12 has been characterized as an inhibitor of the inflammasome and other inflammatory pathways. Interestingly, caspase-12 is not expressed in the majority of the human population due to a single nucleotide polymorphism (SNP) causing a premature stop codon. However, the full length ancestral form is found in approximately 20% of people of African descent. Given these findings and the role of the inflammasome in insulin resistance, **in chapter 2 of this thesis we investigated if caspase-12 contributes to metabolic disease.** We determined that mice deficient in caspase-12 developed obesity, insulin resistance, and inflamed adipose tissue compared to controls. Loss of caspase-11 had no bearing on disease outcome. This phenotype was dependent on loss of caspase-12 in the radioresistant compartment and could be rescued by co-deletion of Nlrp3. Finally, caspase-12 was determined to also impair adipose tissue inflammation in obese humans.

The findings of chapter 2 suggested that inflammatory protein expression in the radioresistant compartment was a factor in metabolic disease. While there is a multitude of studies detailing the involvement of immune cells in insulin resistance, there are comparatively little studying inflammatory genes in other cell types. While a small number of studies have also identified the inflammasome as a metabolic regulator in the radioresistant compartment (2, 3), there is little

insight to its cellular origin. *In vitro* studies suggest that the Nlrp3 inflammasome may also play a role in adipocytes (4), though it is unknown how this could factor into disease pathogenesis. **In chapter 3, we sought to address these questions through the study of a mouse expressing a hyperactive Nlrp3 allele in adipocytes**. Analysis of these mice revealed that adipocyte expression of Nlrp3 correlated with reduced glucose tolerance and suggests that the inflamamsome may have an *in vivo* function in adipocytes, in addition to its role in immune cells.

Cell death is an important response of the immune system and impedes pathogen infection and replication. Work in multiple labs has linked necroptosis, a regulated mode of necrotic cell death, to the development of inflammatory bowel disease (5-9). While various genetic mouse models have confirmed this role, necroptosis has not been studied in isolation from other inflammatory or cell death pathways. Mlkl is the most downstream effector of necroptosis and is not currently known to possess alternate functions. Therefore Mlkl deficient mice can be utilized to specifically study necroptosis without confounding effects. **In chapter 4, we determined the role of Mlkl in experimental colitis.** These studies revealed that loss of Mlkl protected mice from colitis, suggesting a detrimental role for necroptosis in disease. While the colons of *Mlkl*$^{-/-}$ mice had reduced cell death, there were also striking signs of epithelial repair. This finding coincided with elevated levels of IL-22 and IL-17, cytokines associated with the induction of repair of the intestinal barrier.

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LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AIM2	Absent in Melanoma 2
ALPS	Autoimmune Lymphoproliferative Syndrome
AMP	Antimicrobial peptide
APAF-1	Apoptosis protease-activating factor 1
APC	Antigen presenting cell
ASC	Apoptosis-associated Speck-like protein containing a CARD
BIR	Baculovirus IAP repeat
BMDM	Bone marrow–derived macrophage
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase recruitment domain
CD	Crohn's disease
CEDS	Caspase-8-deficient state
cFLIP	cellular FLICE-like inhibitory protein
cIAP	Cellular inhibitor of apoptosis
CLR	C-type lectin receptor
COP	CARD-only protein
CRP	C-reactive protein
CVD	Cardiovascular diseases
DAMP	Danger-associated molecular patterns
DC	Dendritic cell
DD	Death domain
DED	Death effector domains
DHA	Docosahexaenoic acid
Dhx15	DEAH (Asp-Glu-Ala-His) box helicase 15
DIO	Diet induced obesity
DSS	Dextran sodium sulfate
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ES	Embryonic stem
FADD	FAS-associated death domain
FCAS	Familial cold autoinflammatory syndrome
FMF	Familial Mediterranean fever
GALT	Gut-associated lymphoid tissue
GBP5	Guanylate binding protein 5
GI	Gastrointestinal
GWAS	Genome-wide association studies
HFD	High fat diet
HIN-200	Hematopoietic interferon-inducible nuclear protein
IAPP	Islet amyloid polypeptide
IEC	Intestinal epithelial cell
IFN	Interferon
IKK	IκB kinase
IKKi	IKK-related kinase
IL	Interleukin

IL-18BP	IL-18 binding protein
IL-1Ra	IL-1 receptor antagonist
IL-1RAcP	IL‑1R accessory protein
IL-1RI	IL-1 receptor I
ILC	Innate lymphoid cell
iNKT	Invariant natural killer t
IRS-1	Insulin receptor substrate-1
LBP	LPS-binding protein
LeTx	Lethal Toxin
LF	Lethal Factor
LRR	Leucine-rich repeat
LUBAC	Linear ubiquitin chain assembly complex
M cell	Microfold cell
MAVS	Mitochondrial antiviral signaling protein
MCMV	Murine cytomegalovirus
MCP-1	Monocyte chemoattractant protein-1
MDP	Muramyl dipeptide
MOMP	Mitochondrial outer membrane permeabilization
MWS	Muckle-Wells syndrome
MYD88	Myeloid differentiation primary response gene 88
NAIP	Neuronal apoptosis inhibitory protein
Nec-1	Necrostatin-1
NK	Natural Killer
NLR	Nod-like receptor
NMD	Nonsense mediated decay
NOD	Nucleotide binding and oligomerization domain
NOMID	Neonatal-onset multisystem inflammatory disease
PA	Protective antigen
PAMP	Pathogen-associated molecular pattern
PARP	ADP-ribose polymerase
PKR	Protein kinase R
POP	PYD-only protein
PP2A	Protein phosphatase 2
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PYD	Pyrin domain
RIG-I	Retinoic acid inducible gene-I
RIP/RIPK	Receptor-interacting serine/threonine-protein kinase
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SH2	Src homology 2
SHP	Small heterodimer partner
T2D	Type 2 Diabetes
T3SS	Type 3 secretion system
T_H	T helper cell
TIR	Toll–interleukin 1 receptor

TLR	Toll-like Receptor
TRAPS	TNF receptor–associated periodic syndrome
Tri-DAP	L-Ala-g-D-Glu-mesoDAP
TRIF	TIR domain-containing adaptor inducing IFNβ
Ub	Ubiquitin
UC	Ulcerative colitis
UPR	Unfolded protein response
WAT	White adipose tissue
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 The Immune System

The immune system is composed of numerous cells, tissues and organs that protect us from an immense variety of pathogens that we are exposed to within the environment. These defences are found in evolutionary primitive organisms such as bacteria and in all species of the animal and plant kingdoms, and have co-evolved with hazardous microbes in an ever advancing arms race. The distinction between “self” and "non-self" is a principal property of immunity that requires a complex network of recognition mechanisms and regulatory processes. Besides "non-self", the immune system can also recognize endogenous danger signals including cancerous cells or metabolic perturbations, referred to as "altered-self", and elicit important physiological responses to restore homeostasis.

The immune system can be subdivided into the innate and adaptive branches. Together, they work synergistically to protect the organism through a wide range of mechanisms. The innate immune system offers an initial line of defence that is either constitutive or quickly inducible through broad recognition of highly conserved molecular patterns found on foreign agents or sterile danger signals. Constitutive innate immunity is conferred through physical barriers, such as the skin and internal epithelial layers, the microbiota, and soluble factors such as the complement system. Inducible innate immunity is governed by pattern recognition receptors (PRRs) following sensing of noxious triggers, and includes the secretion of immunologically active compounds such as antimicrobial peptides (AMPs), cytokines and chemokines, phagocytosis, autophagy and many other processes. In addition to these defences, the innate immune system is also required to prime the evolutionarily more recent adaptive immune system through the presentation of antigens. This allows for a specific response through cell mediated or antibody driven humoral immunity. The outcome of this activation leads to immunological memory and future protection against a similar threat.

Unfortunately, immunological protection is imperfect and can lead to various diseases when proper regulation fails. Allergies, autoimmune diseases, and chronic inflammatory disorders are a growing concern due to their increasing global incidence. A broader knowledge and

understanding of the underlying immune mechanisms is warranted to advance medical treatments and therapeutic discoveries.

1.2 Pattern Recognition Receptors (PRRs)

The initial step in orchestrating an innate immune response is the recognition of foreign or dangerous entities by the innate immune system. Host cells, including among others cells of the myeloid and epithelial lineages, express a number of germline-encoded PRRs that recognize conserved protein, lipid, polysaccharide, or nucleic acid motifs. The concept of PRRs was first proposed by Charles Janeway in 1989 as a mechanism for innate immunity to interface with the adaptive immune system (10). His theory was proven in 1996 with the discovery of the first Toll-like receptor (TLR), TLR4 (11-13). It is now well known that the mammalian innate immune system is equipped with an ever expanding number of PRR families (trans-membrane TLRs, C-type lectin receptors (CLRs), cytosolic Nod-like receptors (NLRs), Retinoic acid inducible gene-I (RIG-I)-like helicases and HIN200 proteins (14, 15)) able to recognize specific motifs of foreign or endogenous origin. These motifs are known as PAMPs (Pathogen-associated molecular patterns) or DAMPs (Danger-associated molecular patterns). DAMPs are sterile danger signals arising in the host in response to stress, tissue injury or cell death, or encountered in the environment such as asbestos or silica crystals. PAMP and DAMP recognition by PRRs activates downstream transcriptional or post-translational pathways to induce an innate immune response such as inflammation, cell death, phagocytosis, and autophagy.

1.2.1 TLRs

TLRs were the first PRRs to be discovered and have sparked our understanding of how the innate immune system responds to specific triggers. This system was first identified in *Drosophila melanogaster* with the discovery of the antifungal protein, Toll, the namesake of the TLR family (11). Since this finding, 13 mammalian TLRs were identified including TLR1-10 in humans and TLR1-9 and TLR11-13 in mice. TLRs primarily recognize PAMPs including LPS (TLR4), flagellin (TLR5), and microbial nucleic acid motifs such as CpG DNA (TLR9) and dsRNA (TLR3). Notably, nucleic acid-sensing TLRs, i.e.TLR3, 7, 8, and 9, are all localized to the endosomal membranes whereas the remainder, i.e. TLR1, 2, 4, 5, 6, 10, 11 and 13, are found on the cell surface.

TLRs are transmembrane proteins consisting of an ectodomain containing leucine-rich repeats (LRR) for ligand recognition, a transmembrane domain, and a cytoplasmic intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain that propagates signalling. The TIR domain is essential in recruiting downstream adaptor molecules to initiate an inflammatory response. All TLRs except TLR3 recruit Myeloid differentiation primary response gene 88 (MYD88) to induce NF-κB and MAPK activation. TLR3 and TLR4 interact with TIR domain-containing adaptor inducing IFNβ (TRIF), leading to NF-κB activation as well as a type I interferon antiviral response via IRF3. TLRs typically function as homodimers but can form heterodimers for differential ligand specificity. TLR2 can dimerize with TLR1 to recognize Gram-negative lipopeptides or TLR6 to bind Gram-positive lipopeptides. In addition, co-receptors and co-factors may also be required for recognition. For example, TLR4 mediated recognition of LPS requires LPS-binding protein (LBP), MD2 and CD14 (16).

1.2.2 NLRs

The membrane bound TLRs are complemented by the cytosolic NLR family of receptors (Figure 1). To date, there are over 20 human NLRs that have been identified. NLRs are related to disease resistance R proteins in plants (17) and to Apoptosis protease-activating factor 1 (APAF-1) (18). They share a tripartite structure consisting of an N-terminal protein-protein interaction module (e.g. a pyrin domain (PYD), a caspase recruitment domain (CARD), or a baculovirus IAP repeat (BIR)), a central nucleotide binding and oligomerization domain (NOD) and a C-terminal agonist-sensing domain consisting of LRRs, as in TLRs.

1.2.2.1 NOD1 and NOD2

The first identified NLRs, NOD1 and NOD2, were determined to recognize bacterial peptidoglycan moieties (19-21). Specifically, NOD1 detects an L-Ala-g-D-Glu-mesoDAP (Tri-DAP) motif while NOD2 binds to muramyl dipeptide (MDP). MDP is internalized into endo-lysosomal organelles before the SLC15A3 and SLC15A5 transporters mediate its release to the cytosol (22). Upon ligand recognition, NOD1 and NOD2 self-oligomerize and interact with the kinase Receptor-interacting serine/threonine-protein kinase 2 (RIP2 or RIPK2) through a

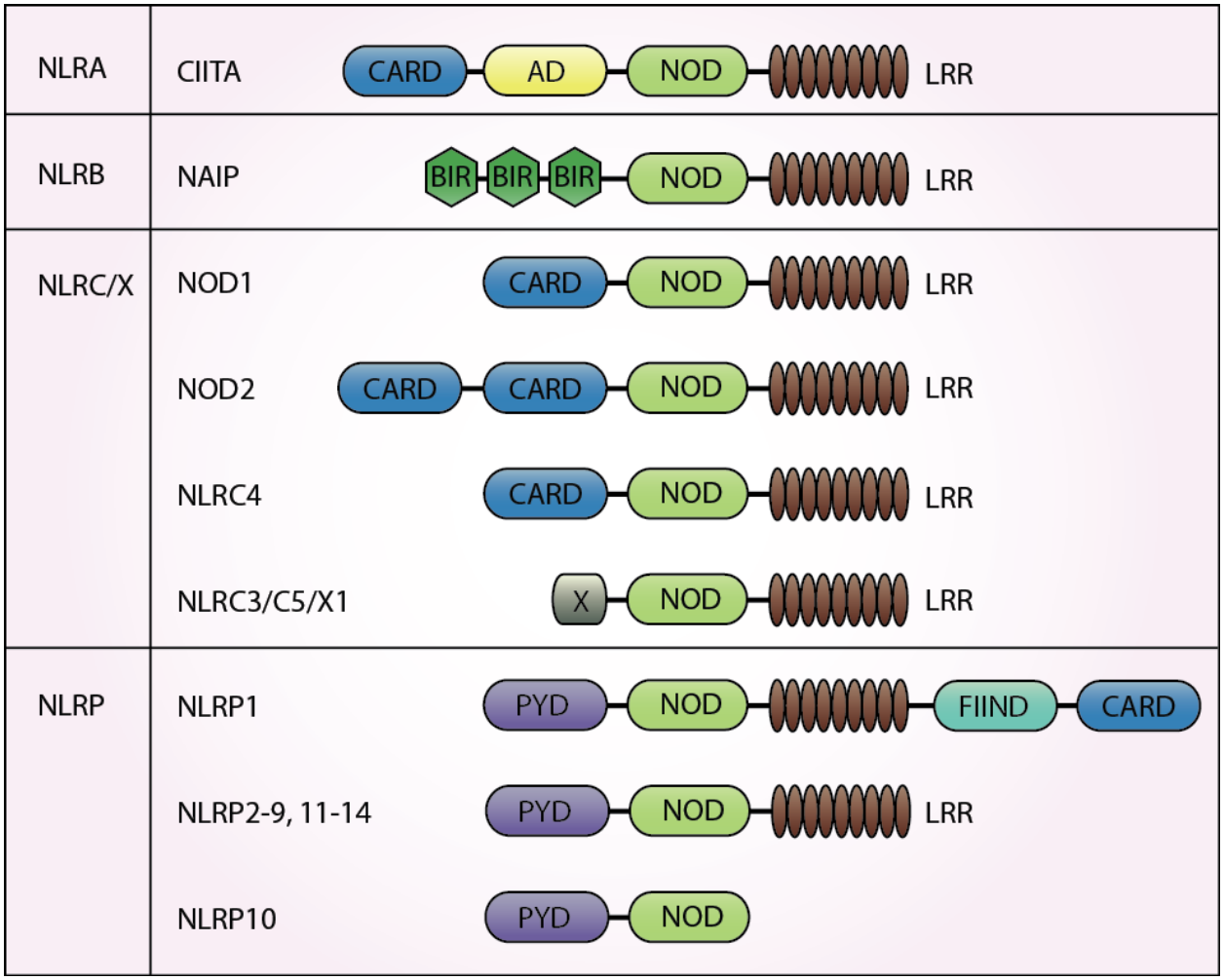

Figure 1. The NLR family

The NLR family of proteins share a common nucleotide binding and oligomerization domain (NOD) and are classified into four different subfamilies based on their N-terminal domain. CIITA proteins of the NLRA family contain an acidic trans-activating domain (AD). NAIP contains baculovirus inhibitor of apoptosis protein repeat (BIR) domains on the N-terminus. The NLRC/X subfamily possesses a caspase activation and recruitment domain (CARD) or an undefined domain (X). NLRP members share an N-terminal *pyrin* domain (PYD). Additional NLR domains include leucine rich repeats (LRRs) and a *function to* find domain (FIND).

homotypic CARD-CARD interaction. RIPK2 recruits and activates TAK1 and the IKK complex to initiate NF-κB and MAP kinase signalling (23, 24).

Enzymatic post-translational modifications are important regulators of cell processes and fine-tune both cell death and inflammatory pathways. For example, ubiquitination of proteins can promote interactions and the formation of a scaffolding network to form signalling complexes. The NOD-RIPK2 signalling platform is regulated in this manner by E3 ubiquitin (Ub) ligases including X-linked inhibitor of apoptosis protein (XIAP) (25), cellular inhibitor of apoptosis (cIAP)1 and cIAP2 (26), ITCH (27), and Pellino3 (28) through the ubiquitination of RIPK2. These Ub chains allow the recruitment of further signal modulators, such as linear ubiquitin chain assembly complex (LUBAC), which enhances NF-κB activation (29). The stimulation of NF-κB and MAP kinase pathways by NOD1 and NOD2 illustrates a redundancy with TLR signalling, as bacterial pathogens could potentially activate both sets of sensors. However, the importance of these NLRs is evident in murine models of infection where loss of Nod1 or Nod2 confers susceptibility to bacterial pathogens, such as *Helicobacter pylori*, *Mycobacterium tuberculosis*, or *Listeria monocytogenes* (30-32). Intestinal infections may be of particular relevance due to the expression patterns of the NLRs. NOD1 is expressed in numerous cells including epithelial and stromal cells while NOD1 is expressed in numerous cells including epithelial and stromal cells while NOD2 is primarily expressed in hematopoietic cells as well as Paneth cells in the small intestine (23, 33). Interestingly, hyperactivation of NOD signalling can also have detrimental affects upon bacterial infection. Viral PAMPs or interferon (IFN)β induces Nod1 and Nod2 expression that increases mouse susceptibility to secondary bacterial infections (34).

In addition to the activation of innate inflammatory pathways, NOD1 and NOD2 are also capable of priming the adaptive immune system as their activation leads to T helper cell (T_H)2 polarization (35, 36). Evidence also exists for a link between NOD1 and NOD2 and the cellular recycling autophagy pathway (37-40). NOD1 and NOD2 recruit the autophagy protein ATG16L1 to the site of bacterial entry along the cell membrane to regulate bacterial degradation in autolysosomes. In addition to its role in autophagy, ATG16L1 also inhibits NOD2 signalling by interfering with RIPK2 ubiquitination (40).

1.2.2.2 The Inflammasome

NLR family members and other PRRs are capable of forming intracellular multi-protein complexes, termed inflammasomes (Figure 2). Multiple inflammasomes have been characterized based on the nature of the scaffolding PRR, leading to the recognition of various pathogenic microbes and host danger signals. These platforms recruit and activate caspase-1 directly or through the adaptor protein Apoptosis-associated Speck-like protein containing a CARD (ASC). This discovery importantly led to the link between pattern recognition and activation of caspase-1 and its associated IL-1 family cytokines (41). Inflammasome assembly is mediated through CARD-CARD or PYD-PYD homotypic domain interactions. Imaging of ASC dependent inflammasome activation determined that ASC oligomers form a highly condensed speck within a cell (42). This is the result of stimulated PRRs nucleating ASC to undergo prion-like polymerization and form filamentous chains (43, 44). ASC can then induce caspase-1 activation "by proximity".

Activated caspase-1 is capable of processing a number of cellular substrates, which is a pre-requisite to the induction of an inflammatory response. Most notably, caspase-1 converts the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into their mature biologically active forms (45-47). In addition to its pro-inflammatory effects, excessive activation of caspase-1 leads to a form of cell death called pyroptosis, with characteristics of both apoptosis and necrosis (48, 49). The major features of pyroptotic cell death include plasma membrane rupture, allowing cellular contents such as processed cytokines and other alarmins, to leak out into the extracellular environment, along with nuclear condensation and DNA fragmentation (50). These alarmins act as immunostimulants to induce innate and adaptive immunity (51). Upon pyroptosis, ASC specks are also released extracellularly where they can further propagate an inflammatory response as their phagocytosis by macrophages induces inflammasome activation in these cells (52). The serum of autoimmune disease patients contains ASC speck reactive autoantibodies that may contribute to pathogenesis. These antibodies appear capable of opsonising ASC for uptake by phagocytes to propagate inflammation.

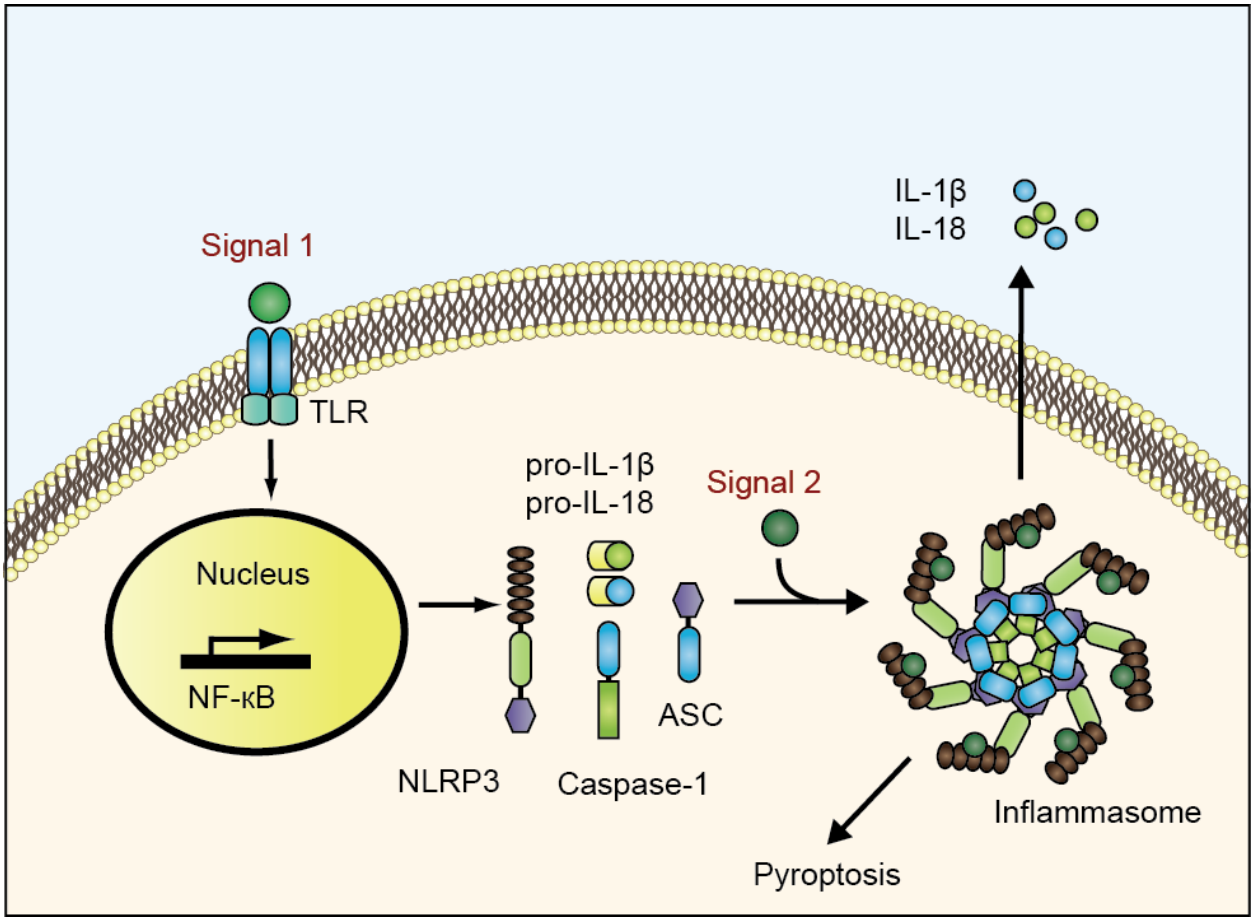

Figure 2. The inflammasome
The inflammasome is composed of a PRR, such as NLRP3, caspase-1 and ASC. Activation requires a two step process. Signal 1 induces NF-κB transcription of the inflammasome components. Signal 2 can be an exogenous or endogenous ligand that is recognized by the PRR and induces assembly of the complex and the activation of caspase-1. Caspase-1 can then process its substrates, such as the immature pro-IL-1β and pro-IL-18 cytokines into their bioactive forms and induce pyroptosis.

1.2.2.2.1 Inflammasome Regulation

Due to the potentially severe effects of caspase-1 activation during disease and infection, regulation of the inflammasome pathway is tightly controlled through a number of checkpoints (Figure 3). Firstly, inflammasome activation depends on a two-step process, whereby signal 1 "primes" the inflammasome via transcriptional induction of inflammasome components and immature cytokines (usually mediated by NF-κB downstream of PRRs or cytokine receptors) and signal 2, which "activates" the inflammasome by complex oligomerization and activation of caspase-1 by proximity. Regulation can occur at the levels of both signal 1 and signal 2. For example, aryl hydrocarbon receptor (AhR) negatively regulates the inflammasome at the transcriptional level through binding the Nlrp3 promoter to inhibit mRNA synthesis (53). Many proteins and pathways have been implicated that specifically target the second phase of activation, acting as positive or negative regulators. Two groups of proteins termed COPs (CARD-only proteins) and POPs (PYD-only proteins) form CARD-CARD or PYD-PYD interactions respectively with inflammasome components to inhibit assembly (54). Other cellular regulators can bind to inflammasome components to enhance activation. For example, NEK7 binds to the LRR of NLRP3 while guanylate binding protein 5 (GBP5) binds to the PYD domain of NLRP3 to augment activation (55, 56).

The inflammasome can be further regulated by post-translational modifications. The Ub ligases cIAP1 and 2 amplify inflammasome activation through K63-linked polyubiquitination of caspase-1 (57). LUBAC, consisting of the proteins HOIL-1L, HOIP, and SHARPIN, is a known regulator of NF-κB signalling and also modifies ASC. This linear ubiquitination of ASC is essential for NLRP3 inflammasome activation (58, 59). NLRP3 is also a target of these post-translational modifications and requires deubiquitination by BRCC3 to become active (60). Together these examples illustrate the complex cross-regulation that occurs to fine tune the inflammatory response.

Phosphorylation and dephosphorylation of key residues are also crucial in controlling cell processes. The kinases Syk and JNK were demonstrated to phosphorylate Asc at tyrosine 144 in mice to induce inflammasome activation (61). IκB kinase (IKK)α negatively regulates

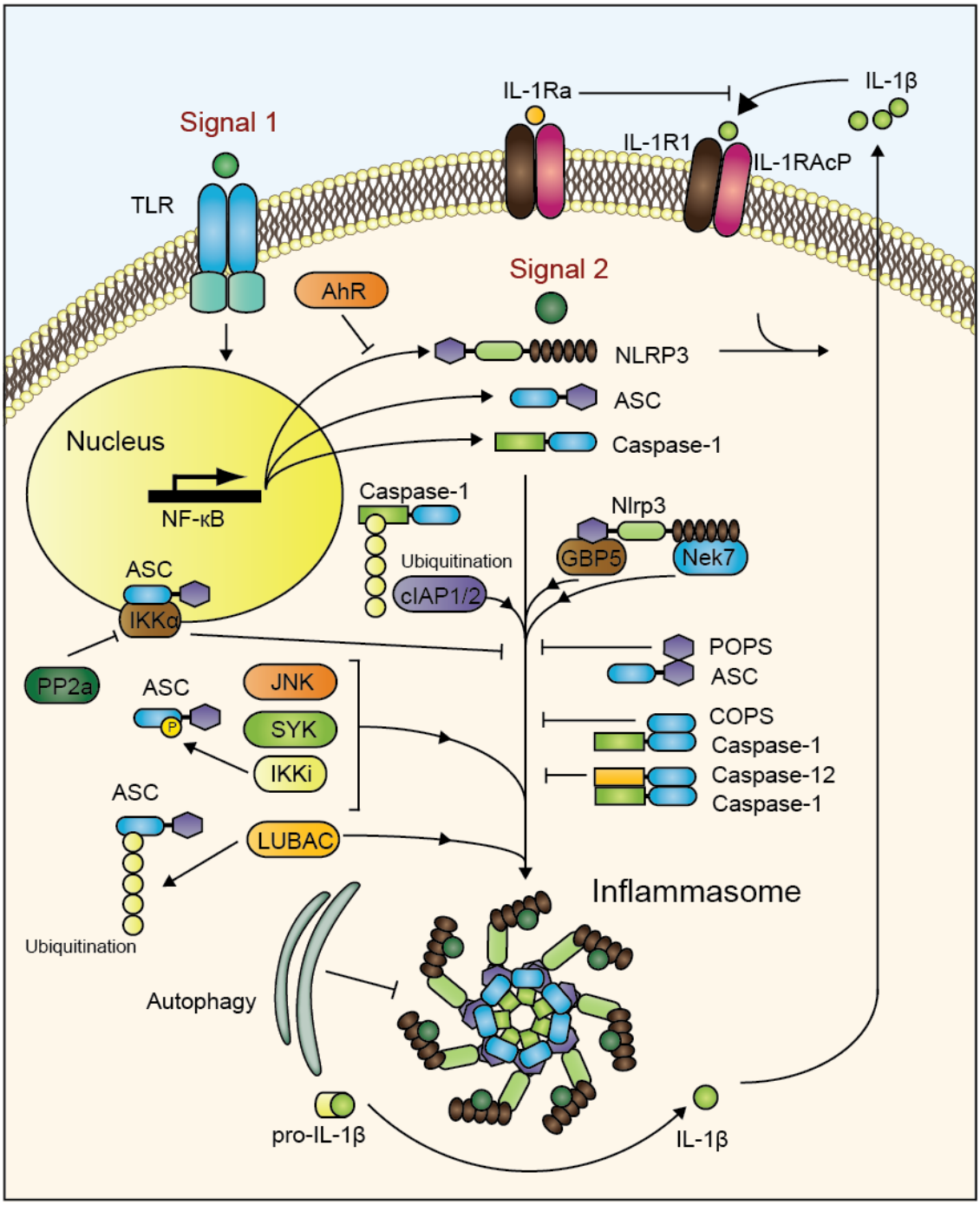

Figure 3. Regulation of the NLRP3 inflammasome

The NLRP3 inflammasome is highly regulated at multiple steps to prevent unwarranted activation. Inhibitors such as pyrin only proteins (POPs) and CARD only proteins (COPs) can bind to inflammasome components to prevent complex assembly. Conversely, other inflammasome binding partners, such as GBP5 and Nek7, and post-translational modifications, such as ubiquitination by LUBAC and cIAP1/2, are required for activation. Autophagy is thought to play a major role in terminating inflammasome activation.

inflammasome activation by sequestering ASC in the nucleus. Activation requires recruitment of protein phosphatase 2 (PP2A) to inhibit IKKα and release Asc. This permits phosphorylation by IKK-related kinase (IKKi) on Serine 58 of ASC (62). The Nlrc4 inflammasome also requires phosphorylation for its activation that is induced by bacterial flagellin, independently of other inflammasome components (63, 64).

An additional layer of regulation is applied to the processed cytokines downstream of inflammasome activation. Secreted IL-1β binds to IL-1 receptor I (IL-1RI) and recruits the IL‑1R accessory protein (IL-1RAcP), which is required for signalling (65). The dominant negative receptor IL-1RII and soluble IL-1 receptor antagonist (IL-1Ra) are able to inhibit this process (65).

As inflammasome activation can lead to pyroptosis, the ability to disable the complex and prevent premature cell death can be advantageous. Evidence suggests that this can be accomplished through autophagy. PRR stimulation can also independently activate the autophagy pathway where K63-ubiquitnation of inflammasome components promotes their engulfment by an autophagosome (66). Mitophagy, an autophagic mechanism targeting mitochondria, also plays a role in limiting inflammasome activation (67, 68).

Given the widespread activation of the inflammasomes in response to various microbes, it is not surprising that pathogens have devised multiple strategies to alter inflammasome function and downstream signalling. Bacteria utilize effector molecules, injected through secretion systems into the host cytosol, for this purpose. For instance, *Y. enterocolitica* YopE and YopT (69), *Y. pseudotuberculosis* YopK (70), and *P. aeruginosa* ExoU (71) have been reported to blunt inflammasome activation. Viruses also encode proteins that target this pathway including Influenza NS1 (72), Myxoma virus M13L-PYD, and Shope fibroma virus gp013L (73, 74) that act as POPs. The active inhibition of the inflammasome by various pathogens supports the notion that its pro-inflammatory effects together with the induction of pyroptosis are deleterious to infectious microbes.

1.2.2.2.2 NLRP1

The NLRP1 inflammasome represents the first described caspase-1-activating complex scaffolded by a PRR. Thus, this exciting discovery marked the beginning of inflammasome research (Figure 4) (41). There is one human NLRP1 gene while gene duplication has led to three murine isoforms (Nlrp1a-c). Human NLRP1 contains a PYD, NOD, LRR, function to find domain (FIIND) and CARD domain (Figure 1). The CARD domain can directly interact with caspase-1 without ASC, however ASC speck formation leads to increased activation (75, 76). Nlrp1b is the best characterized mouse isoform and was first determined to be activated by *Bacillus anthracis* Lethal Toxin (LeTx) (77). A functional Nlrp1b gene is required for murine resistance to *B. anthracis* infection (78, 79). The bacteria secrete LeTx which is composed of the Protective antigen (PA) and the zinc-dependent metalloprotease Lethal Factor (LF) (80). Mechanistically, LeTx cleaves the N-terminus of Nlrp1b to initiate inflammasome activation (81-83). This mode of activation is not specific to LeTX however, as ectopic proteolysis can also activate Nlrp1b (83). *Toxoplasma gondii* has also been implicated in murine and rat Nlrp1 activation, however the underlying mechanism is not well understood (84-86). Unlike LeTx stimulus, Nlrp1 cleavage is not required for *T. gondii* signalling. MDP is also capable of activating the Nlrp1b inflammasome, however bacterial products are not the sole ligands for this inflammasome, as reduced ATP levels also lead to activation, suggesting that ATP may be a negative regulator of activation (87, 88). Aside from Nlrp1b, the other Nlrp1 isoforms are less well described. Nlrp1a was demonstrated to play a role in hematopoietic stress, where a hyperactive mutant induces pyroptosis in hematopoietic progenitor cells (89).

1.2.2.2.3 NLRP3

NLRP3 is the most studied inflammasome due to its ability to recognize a vast array of structurally unrelated stimuli including both PAMPs and DAMPs (Figure 5). These include elevated concentrations of ATP (90), pore forming toxins (90), ER stress (91), host-derived metabolites (68), bacterial mRNA (92), UVB irradiation, and particulate matter such as crystalline forms of monosodium urate (MSU) (93), asbestos and silica (94-96), and amyloid β aggregates (97). Due to the unrelated nature of these agonists, it has been hypothesized that they likely converge on one downstream signal that is recognized by NLRP3, though there is no

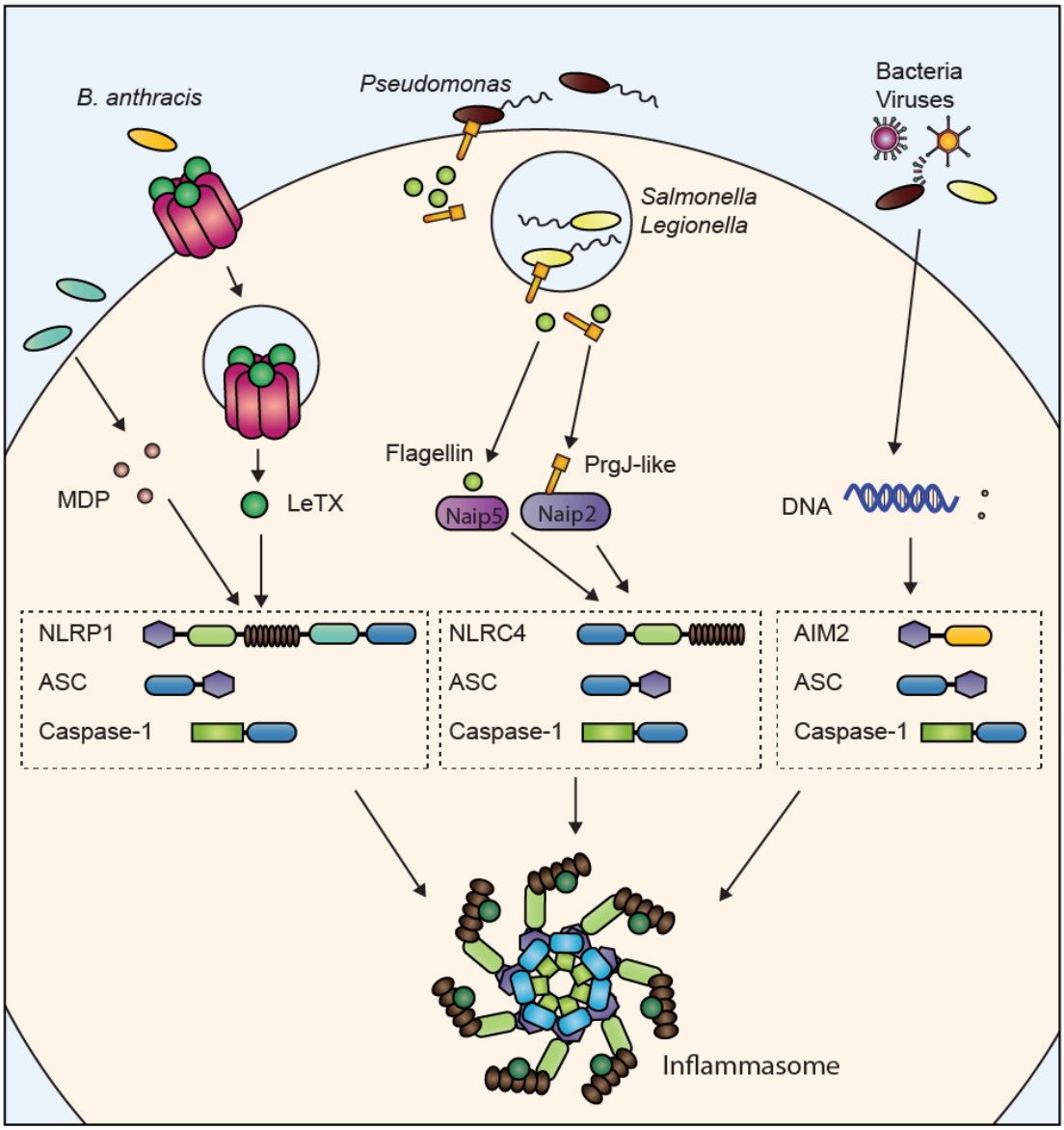

Figure 4. Activation of the NLRP1, NLRC4, and AIM2 inflammasomes
Different intracellular PRRs permit the recognition of a multitude of ligands to initiate inflammasome activation. The NLRP1 inflammasome is activated by bacterial muramyl dipeptide (MDP) or *B. anthracis* lethal toxin (LeTX). The NLRC4 inflammasome senses bacterial flagellin and PrgJ-like proteins of the type 3 secretion system. These are first bound by Naip5 and Naip2 respectively to induce inflammasome assembly. ASC is not required for NLRC4 activation, but can enhance its effects. AIM2 senses intracellular viral or bacterial DNA to induce inflammasome activation.

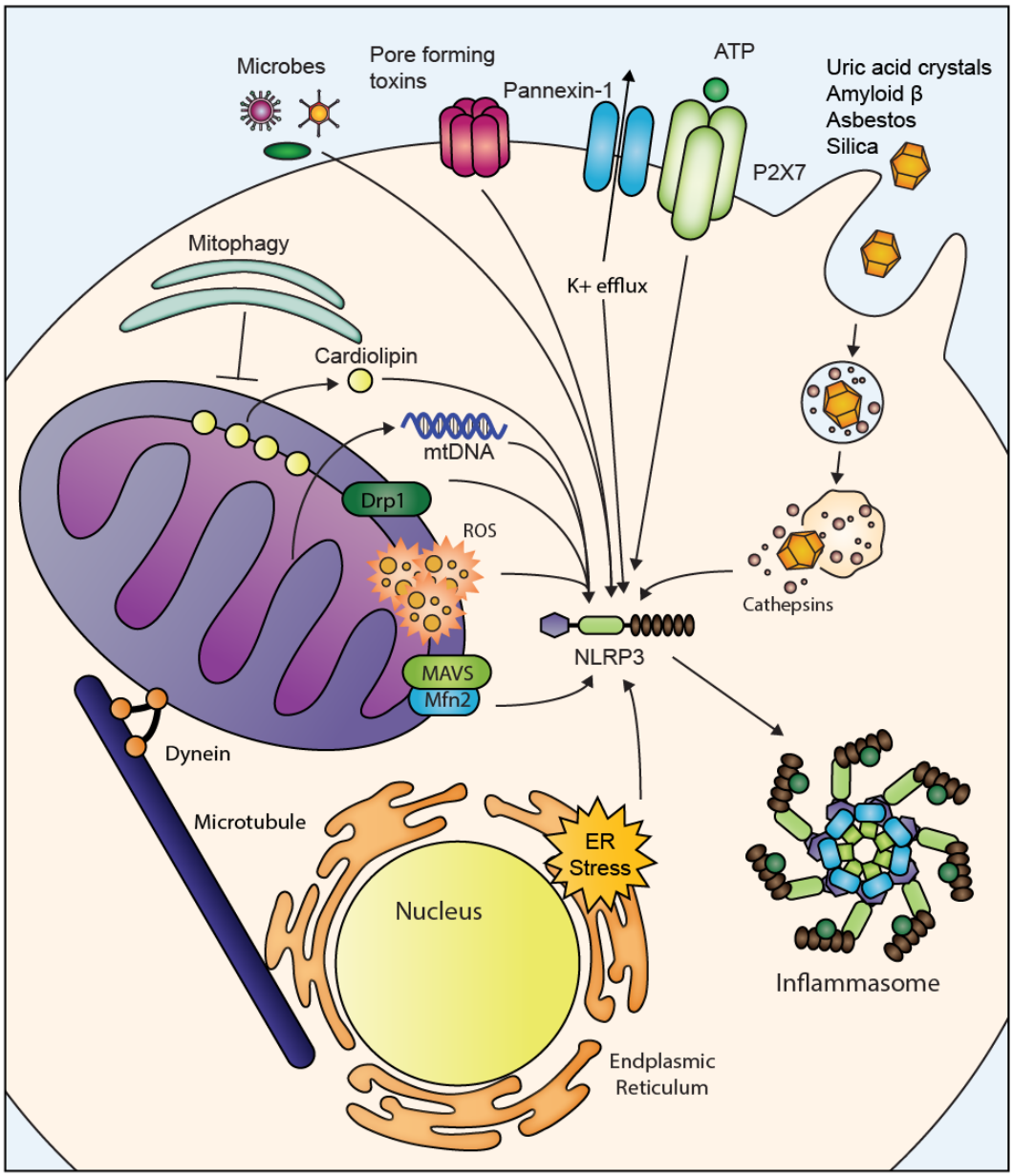

Figure 5. Activation of the "canonical" NLRP3 inflammasome

The NLRP3 inflammasome senses multiple ligands of host or foreign origin. It has been hypothesized that these ligands converge on a common signal for recognition, however there is no consensus on its identity. Potassium efflux from the cell is a well known activator of the inflammasome. The phagocytosis of crystalline substances, such as asbestos and silica, can result in phagosome rupture and the release of cathepsins that stimulate NLRP3. The mitochondria are also the source of many activating ligands, including mitochondrial DNA (mtDNA), reactive oxygen species (ROS), and cardiolipin. Mitophagy and removal of damaged mitochondria can inhibit activation by these pathways. The transport of mitochondria by dynein via microtubules has also been linked to NLRP3 activation. Adapted from reference (98)

consensus over its identity. Currently there are multiple potential theories over what signal is detected and some ligands may stimulate more than one of these pathways.

Potassium efflux from the cell is a requirement for inflammasome activation driven by ATP, pore forming toxins, the K+ ionophore nigericin, and phagocytosed particulate matter (99, 100). Culturing cells in low K+ media can induce spontaneous inflammasome activity while high concentrations of extracellular K+ can inhibit ligand induced activation. The discovery that elevated levels of intracellular calcium can also activate the NLRP3 inflammasome further highlights the importance of cellular ion concentrations as a potential broad signal downstream of multiple agonists (101, 102).

Mitochondrial dysfunction and reactive oxygen species (ROS) have also been proposed as a necessity for NLRP3 activation by multiple agonists. Early studies indicated that phagocytosis of asbestos and silica inflammasome activation was dependent on NADPH oxidase driven ROS (94). The mitochondria are a second source of cellular ROS that is released upon damage or depolarization to directly activate NLRP3 (103). Consequently, inhibition of mitophagy, also leads to inflammasome activation. Inflammasome activation may in fact damage the mitochondria, further propagating its own signal. This was shown with the demonstration that Nlrp3 activation by ER stress resulted in caspase-2-mediated processing of Bid, which damages the mitochondria as in intrinsic apoptosis (104). Caspase-1-mediated cleavage of Parkin, a regulator of mitophagy, also prevents the clearance of damaged mitochondria (105).

The cellular localization of NLRP3 inflammasome components at the mitochondria and associated ER further highlights a role for mitochondria in inflammasome regulation (103). Upon agonist stimulation, microtubule dynamics are required for the transport of ASC found in the proximity of mitochondria to Nlrp3 at the ER surface to permit complex assembly (106). Multiple mitochondrial proteins have also been determined to co-localize with these components. MAVS (mitochondrial antiviral signaling protein) interaction with the N-terminus of NLRP3 is required for its recruitment to the mitochondria upon activation (107). MAVS and NLRP3 also associate with mitofusin (Mfn2), a protein involved in mitochondrial fusion (108). Mfn2 and mitochondrial membrane potential are required for Nlrp3 activation following RNA viral

infection. A second protein involved in mitochondrial dynamics, DRP1, was also found to be necessary for NLRP3 inflammasome activation by RNA viruses (109). Finally, small heterodimer partner (SHP), a protein localized to the mitochondria was found to negatively regulate this inflammasome (110).

Aside from ROS, other mitochondrial associated ligands activate NLRP3. Oxidized mitochondrial DNA released from damaged mitochondria during apoptosis was demonstrated to bind to and activate NLRP3 (111). Cardiolipin, a phospholipid of the mitochondrial inner membrane, can also bind NLRP3 to induce inflammasome activation (112).

In the case of particulate agonists, disruption of the lysosomal membrane after phagocytosis leads to intracellular release of lysosomal contents and NLRP3 inflammasome activation. Intracellular chemical inhibition of cathepsin B, cathepsin B-deficiency, or treatment of cells with inhibitors of the vacuolar H^+ ATPase results in reduced caspase-1 activation (96, 97).

1.2.2.2.4 NLRC4

The NLRC4 inflammasome specifically recognizes bacterial flagellin and PrgJ, the inner rod protein of the type 3 secretion system (T3SS), complementing TLR5 and serving as an intracellular platform for detection (Figure 4). Ligand binding is initiated by the NLR family Neuronal apoptosis inhibitory proteins (NAIP) family of proteins. Murine Naip5 and 6 are required for detection of flagellin and Naip1 and 2 interact with the T3SS rod protein (113-116). The sole human NAIP recognizes the T3SS as well (114).

Activation of the Nlrc4 inflammasome is regulated by a biphasic mechanism, requiring Nlrc4 phosphorylation on Serine 533, followed by Naip binding (63, 64). Crystal structures have determined that one PrgJ-bound Naip2 protein can engage 9-11 Nlrc4 monomers to assemble a wheel shaped structure (117, 118). NLRC4 does not require ASC to interact with caspase-1, due to its own CARD permitting CARD-CARD interactions. However NLCR4 is not prohibited from forming ASC containing inflammasomes, which allow for the amplification of IL-1β and IL-18 secretion (119-123).

Activation of Nlrc4 invokes an immune response to wide range of bacterial pathogens such as *Salmonella typhimurium* (122), *Pseudomonas aeruginosa* (71, 124), and *Shigella flexneri* (125) among others. Nlrc4 activation in intestinal mononuclear phagocytes can aid in the distinction and elimination of enteric pathogens from the gut (126). These cells have repressed TLR signalling to inhibit a response to the commensal microbiota, however their expression of Nlrc4 permits recognition of infectious bacteria such as *Salmonella*. Importantly, the Nlrp3 inflammasome is also activated by systemic *Salmonella* infection, demonstrating that multiple inflammasomes play a role in mounting a host immune response (121).

1.2.2.2.5 NLRP6

NLRP6 has been hypothesized to form an inflammasome with ASC, however very little is known regarding its activation (127). Early *in vivo* studies reported that *Nlrp6*$^{-/-}$ mice are susceptible to colitis and enteric infections, similarly to mice deficient in Asc (128, 129). Interestingly, loss of Nlrp6 led to dysbiosis of the microbiome, and transfer of this microbiota to wild-type (WT) mice through co-housing conferred susceptibility to disease. Defective autophagy in intestinal goblet cells and reduced mucin secretion was also observed in *Nlrp6*$^{-/-}$ mice and correlated with susceptibility to enteric infection (129).

Nlrp6 is also involved in inflammasome independent functions and can recognize intestinal viruses through interaction with DEAH (Asp-Glu-Ala-His) box helicase 15 (Dhx15) (130). Dhx15 is a RNA helicase that binds viral RNA and utilizes Nlrp6 to initiate IFN production and an antiviral response. Nlrp6 was reported to also impair inflammation through the inhibition of NF-κB activation (131). Loss of Nlrp6 in mice was associated with resistance to various bacterial pathogens when injected intraperitoneally. This contrasts with the susceptibility to enteric infection and suggests Nlrp6 expression can have alternate effects in different tissues.

1.2.2.2.6 Other NLR-forming inflammasomes

In addition to above-described NLR-forming inflammasomes, there is early evidence documenting that other NLRs might also function through inflammasome signalling. NLRP7 forms an ASC containing inflammasome that recognizes bacterial acylated lipopeptides, a ligand

also recognized by TLR2 (132). Nlrp12 was demonstrated to confer protection to *Yersinia pestis* infection in mice through inflammasome activation and IL-18 secretion (133).

1.2.2.2.7 Non-NLR inflammasomes

In addition to NLRs, other PRRs are capable of forming inflammasomes. Mutations in the PYRIN encoding *MEFV* gene can lead to Familial Mediterranean fever (FMF), an inflammatory disease (134, 135). *Mefv* gain of function mutations in mice similarly result in autoinflammation and aberrant IL-1β production (136). These disorders have been linked to the ability of PYRIN to form an inflammasome with ASC and caspase-1 (137, 138). Mechanistically, PYRIN has been reported to associate with microtubules and actin (139) and may in fact serve as a sensor to cytoskeleton destabilization. The Pyrin inflammasome can recognize modifications to Rho GTPases by bacterial pathogens targeting the actin cytoskeleton and consequently, *Mefv* deficient mice have increased susceptibility to infection by *Burkholderia cenocepacia* (140, 141). Hypomorphic mutations to the Wdr1 gene in mice result in alterations to actin dynamics and the development of a Pyrin and IL-18 dependent inflammatory disease (142).

Early studies examining infection with *Francisella tularensis*, an intracellular bacterium responsible for tularaemia in humans, demonstrated that caspase-1 and Asc, but not Nlrp3 and Nlrc4, were essential for macrophage cell death, bacterial clearance *in vitro* and *in vivo* and mouse survival (90, 143). This suggested that caspase-1 activation was mediated by a different inflammasome that required Asc. The unknown sensor was later determined to be Absent in Melanoma 2 (AIM2), a member of the hematopoietic interferon-inducible nuclear protein (HIN-200) family that senses cytosolic DNA (Figure 4) (144-148). Lysosomal activation was shown to be required to stimulate this inflammasome during infection, possibly due to lysis of bacteria, which allows the release of DNA into the cytosol (148, 149). DNA viruses, such as vaccinia virus and mouse cytomegalovirus (MCMV), are also capable of activating caspase-1 through the Aim2 inflammasome (150). Similarly to its response to bacterial DNA, Aim2 requires Asc, but not Nlrp3 (146, 150). MCMV-infected Aim2 deficient mice have reduced IL-18 serum levels that correlate with a reduced population of $IFN\gamma^+$ Natural Killer (NK) cells. IL-18 is a known activator of IFNγ production in NK cells (151, 152), which play an important role in eliminating

tumour or virus-infected cells, and is likely one of the primary contributors to the higher viral titres found in the spleen of Aim2-deficient mice (150).

The RNA virus Vesicular stomatitis virus (VSV) is recognized by the intracellular PRR RIG-I. RIG-I detects 5′ triphosphate RNA as a viral signature and induces type I IFN production and NF-κB activation through recruitment of the adaptor protein MAVS (153). Interestingly, infection with VSV also triggers IL-1β secretion in a RIG-I-dependent manner (154). ASC interacts with RIG-I and is required for VSV-induced caspase-1 activation through potassium efflux, but not Type I IFN. These results indicate that RIG-I serves multiple roles during the innate immune response to viruses, as it triggers Type I IFN expression and activates the NF-κB and caspase-1 pro-inflammatory pathways.

1.2.2.2.8 Inflammasome linked diseases

The importance in proper regulation of the inflammasome is evident by the discovery that genetic mutations in *NLRP3* can lead to auto-inflammatory disorders. These hereditary diseases have been named cryopyrin-associated periodic syndromes (CAPS), and include familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease (NOMID) (155, 156). Mutations associated with CAPS are associated with spontaneous IL-1β release due to hyperactivation of the NLRP3 inflammasome. Consequently, inhibition of IL-1β has proven to have therapeutic benefit in affected patients (157, 158).

Other inflammasome forming PRRs have also been genetically linked to disorders. Mutations in the NOD domain of *NLRP1* are associated with vitiligo and other autoimmune diseases (159). *NLRP7* and *NLRP12* mutations are linked to familial Hydatidiform mole formation during pregnancy (160) and CAPS –like autoinflammation respectively (161). FMF is linked to *MEFV* mutations (134, 135) and multiple case reports indicate symptom resolution upon treatment with the recombinant IL-1Ra anakinra (162, 163).

1.2.3 The "Inflammatory" Caspases

Caspases are a family of proteases that play crucial roles in mammalian physiology (164). Structurally, they share a critical cysteine residue that is involved in catalytic activity and allows substrate cleavage at specific aspartic acid residues. Caspases are translated as inactive zymogens that require activation by oligomerization and/or processing. They can be further subdivided based on their functions in cell death and inflammation into the apoptotic and inflammatory caspase subfamilies (Figure 6). The inflammatory subgroup includes murine caspase-1, -11, -12 and -14 and human caspase-1, -4, -5, -12 and -14. While this subgroup was originally named based on the pro-inflammatory role of caspase-1, it is now clear that a) other 'apoptotic' caspases, primarily caspase-8, are also inflammatory; b) the inflammatory caspases are also capable of triggering a form of cell death termed pyroptosis; c) caspase-12, a member of the 'inflammatory caspases' is anti-inflammatory; and d) these enzymes may be involved in additional host processes unrelated to inflammation or cell death. Structurally, the caspases are characterized by an N-terminal CARD domain, capable of forming homotypic interactions, and C-terminal catalytic domain.

1.2.3.1 Caspase-1

Caspase-1 was the first inflammatory caspase discovered and has been the most extensively studied. It was initially identified due to its ability to cleave the pro-inflammatory cytokine pro-IL-1β into its mature biologically active form and accordingly named IL-1β-converting enzyme (ICE) (165). Caspase-1 was later determined to also process and activate pro-IL-18 (166), and more recently to cleave Gasdermin D to induce pyroptotic cell death (167, 168). In addition to these pro-inflammatory functions, proteomic screens have also identified numerous other substrates including glycolysis enzymes and caspase-7, suggesting that caspase-1 activation may regulate a diverse range of cell processes in addition to inflammation (169, 170). Caspase-1 is also responsible for the secretion of proteins from the cell beyond its proteolytic substrates (171)

Structurally, caspase-1 is synthesized as a 45 kDa (p45) precursor protein that is incapable of IL-1β processing (172). Oligomerization induced activation and autoproteolysis result in the cleavage and release of the CARD domain and a 20 kDa (p20) and 10 kDa (p10) fragment (173, 174). Two p20 and p10 heterodimers form a tetrameric active enzyme complex. The active site

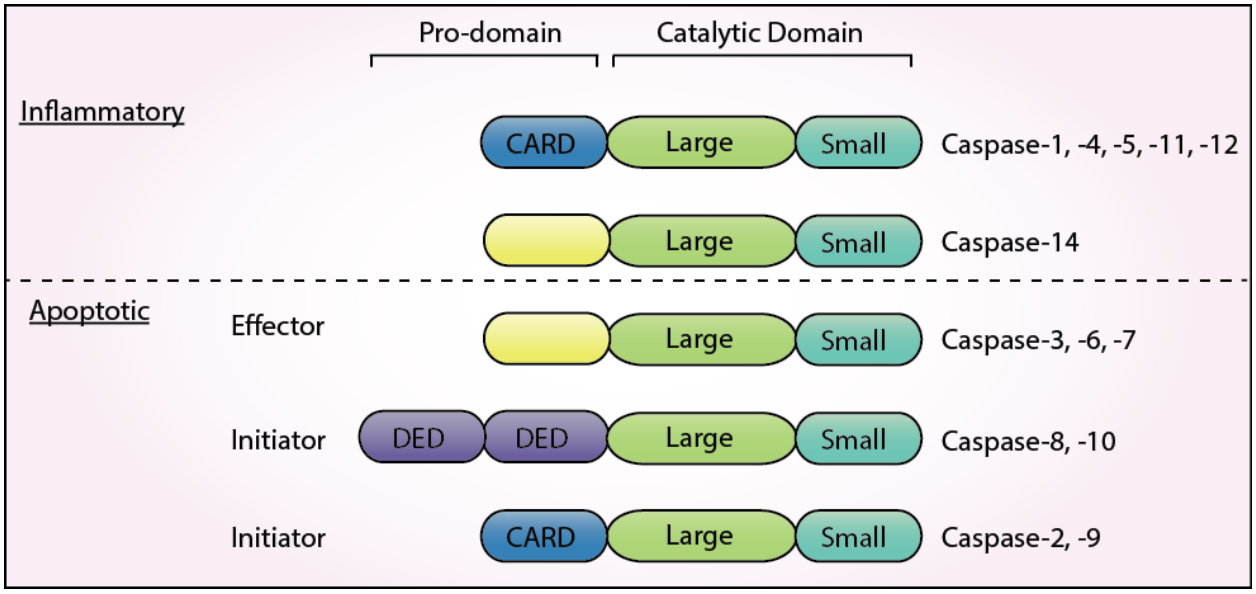

Figure 6. The Mammalian Caspases

The mammalian caspases can be divided into the inflammatory and apoptotic families. All caspases are synthesized in an inactive zymogen form and contain a pro-domain as well as a catalytic domain. The prodomain permits the recruitment to receptors or intracellular signaling complexes through homotypic domain interactions. Activation requires proteolytic processing and release of the large and small domains. Caspase-4 and -5 are the human orthologs of murine caspase-11.

containing the catalytic cysteine residue is in the p20 fragment while the p10 is required for substrate specificity. Caspase-1 autoproteolysis is not mandatory for enzymatic activity and pyroptosis, as was demonstrated following Nlrp1b activation (75, 120)

1.2.3.2 Caspase-8

Caspase-8 is an apoptotic caspase that is instrumental in regulating cell death pathways, however recent evidence has demonstrated that it also displays inflammatory functions (Figure 7). It has been linked to regulation of the Nlrp3 inflammasome, as well as possessing the ability to directly process IL-1β (175). Loss of caspase-8 in macrophages impairs induction of pro-IL-1β in response to TLR agonists or bacterial infection, though it is unknown precisely how this is accomplished (176, 177). Fungal pathogens such as *Candida albicans* activate the dectin-1 receptor and induce Syk signaling. This leads to caspase-8 dependent cleavage of IL-1β through recruitment into a "non-canonical" inflammasome complex containing CARD9, Bcl-10, MALT1, and ASC (178). Other stimuli such as ER stress, CD95 ligand (CD95L), and chemotherapeutic drugs can also induce caspase-8 processing of IL-1β independently of ASC or caspase-1 (179-181). It is unknown what additional proteins may associate with caspase-8 during these responses.

Caspase-8 has also been implicated in the regulation of the Nlrp3 inflammasome. Loss of caspase-8 results in reduced IL-1β secretion and caspase-1 activation following stimulation by Nlrp3 agonists (177). Mechanistically, caspase-8 is recruited to the Nlrp3 inflammasome complex allowing it to cleave and process caspase-1. However, other studies have reported an inhibitory role for caspase-8, observing hyperactive inflammasome activation in *Casp8*$^{-/-}$ cells in response to LPS alone, an effect dependent on Ripk3, Ripk1, and mixed lineage kinase domain-like (Mlkl) (182). Further work is required to decipher how caspase-8 can differentially regulate inflammation. Overall, caspase-8 can be recruited to multiple complexes by various stimuli to play a role in inflammation or cell death.

1.2.3.3 Caspase-11

Murine caspase-11, and its human orthologs caspases-4 and -5, were originally discovered due to

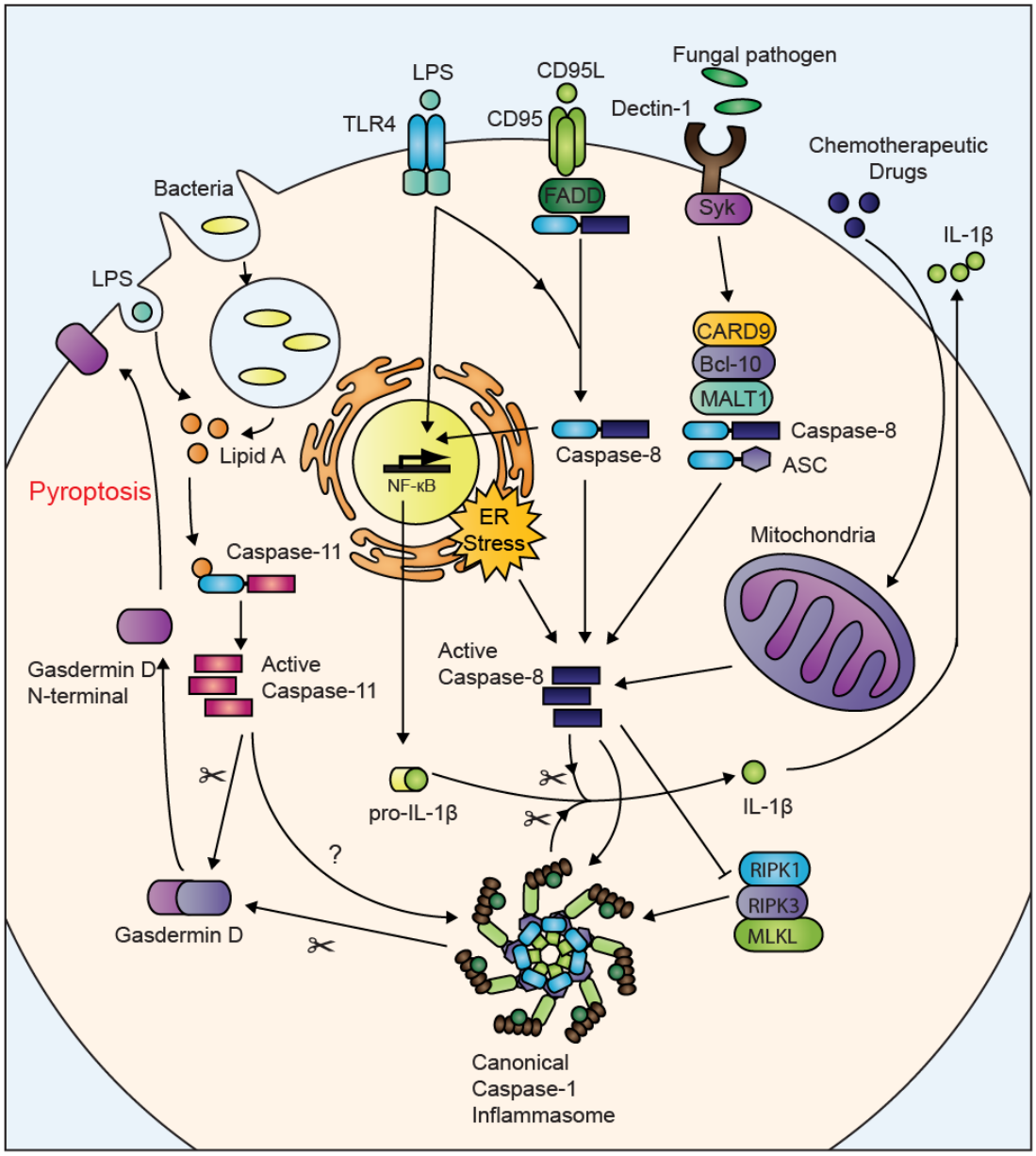

Figure 7. "Non-canonical" inflammasomes

Inflammasome formation and IL-1β processing can be initiated by caspases other than caspase-1 to form "non-canonical" inflammasomes. The Lipid A moiety of LPS binds to the CARD domain of caspase-11 leading to its activation and the formation of an inflammasome. Caspase-11 can cleave Gasdermin D, resulting in the excised N-terminus translocating to the plasma membrane to initiate pyroptotic cell death. Active caspase-11 can also initiate "canonical" NLRP3 inflammasome activation by an unknown mechanism to induce IL-1β secretion from the cell. In addition to its apoptotic functions, caspase-8 has also been reported to induce transcription of pro-IL-1β and process the cytokine to its active form. This can occur in response to fungal pathogens, CD95 ligand (CD95L) stimulation, chemotherapeutic drugs, or endoplasmic reticulum (ER) stress. Caspase-8 can also enhance NLRP3 inflammasome activity and inhibit RIPK1-RIPK3-MLKL induced activation.

their homology to caspase-1 (183). Over expression of these proteins was demonstrated to induce cell death while mice deficient in *Casp11* were resistant to LPS endotoxemia, similarly to *Casp1*$^{-/-}$ mice (183, 184). In contrast to caspase-1 however, caspase-11 poorly processes pro-IL-1β to its mature form (183). The two proteases were thought to play similar inflammatory roles until it was discovered that previously generated caspase-1 knockout mice also carried a null mutation in *Casp11*, rendering the mice deficient in both proteins (Figure 8) (185). This is the result of a 5 base pair deletion in exon 7 of the *Casp11* gene in the 129 strain, embryonic stem (ES) cells of which were used in developing KO mice. Backcrossing of caspase-1-deficient mice did not result in a rescuing recombination event, as this event is extremely unlikely due to the close proximity of the genes. By expressing a transgenic caspase-11 in *Casp1*$^{-/-}$*Casp11*$^{129/129}$ mice, the authors were able to distinguish their effects (185). Caspase-11 was determined to form a "non-canonical" inflammasome that recognizes cholera toxin B and Gram-negative bacteria, but not ATP which activates the caspase-1 canonical inflammasome. Non-canonical inflammasome activation leads to cell death and downstream caspase-1 processing and IL-1β release.

While the caspase-1 canonical inflammasome is activated by numerous triggers, caspase-11 is solely activated by the lipid A moiety of LPS from Gram-negative bacteria (Figure 7) (186, 187). Detection of LPS occurs intracellularly and is independent of TLR4. Caspase-11 does not require a PRR to sense LPS, but is activated by direct LPS binding to its CARD domain (187). Downstream of this activation, caspase- 11 cleaves Gasdermin D into an N-terminal fragment that is required for pyroptosis (167, 168). Caspase-11-dependent cleavage of the pannexin-1 channel and subsequent ATP release was also reported to be required for non-canonical inflammasome mediated cell death (188). Further work is required to elucidate the precise mechanism by which the N-terminal fragment of Gasdermin D leads to pyroptosis.

1.2.3.4 Caspase-12

In contrast to the other members of the inflammatory caspase family, caspase-12 seems to exert anti-inflammatory functions. Expression of caspase-12 leads to reduced IL-1β release and caspase-1 activity (189). Caspase-12 can bind to caspase-1, suggesting this interaction may be important for its inhibitory activity. While murine caspase-12 is proteolytically active, it is only

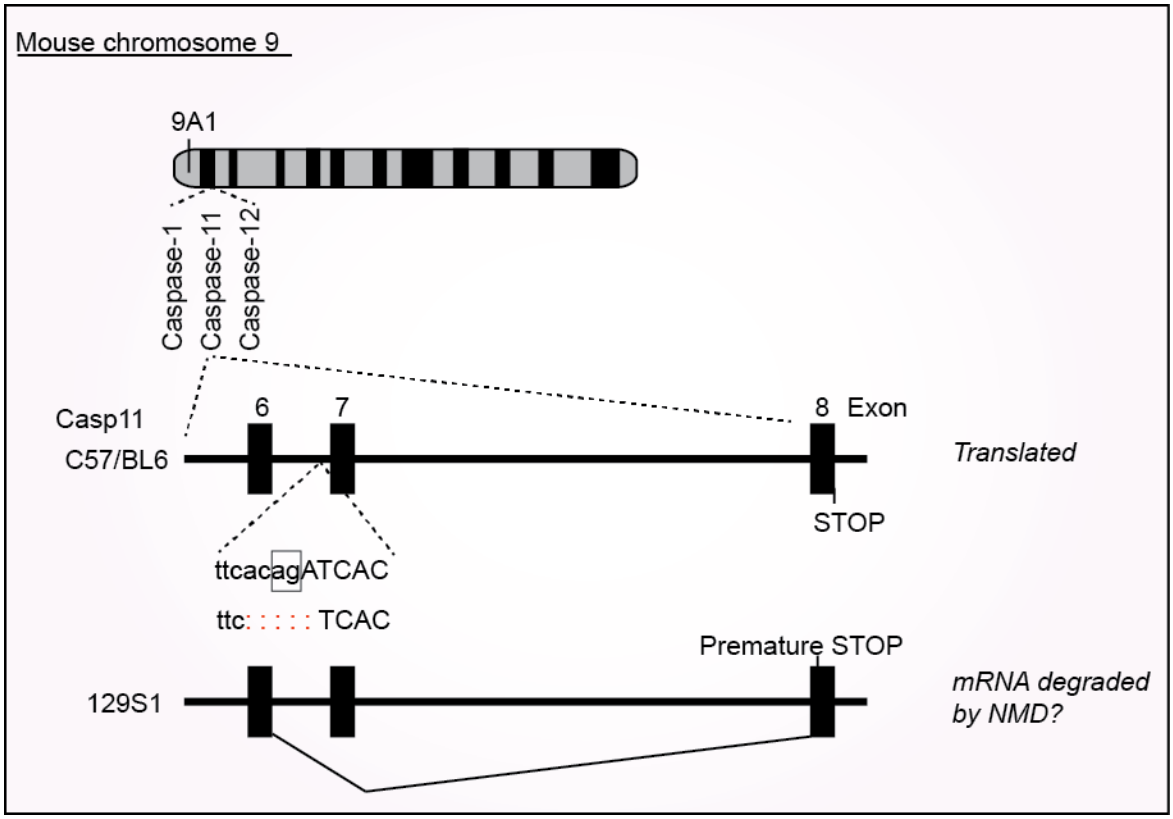

Figure 8. Murine Caspase-11 is not expressed in 129S1 mice

The 129S1 mouse strain *Casp11* gene contains a 5 base pair deletion in the splice acceptor site of exon 7. This results in a transcript containing a premature stop codon that is most likely degraded by nonsense mediated decay (NMD). In contrast the C57/BL6 strain of mice expresses a functional caspase-11. Knockout mouse lines generated on the 129S1 strain may consequently lack expression of caspase-11 despite significant backcrossing to a C57/BL6 background if the targeted mutation lies in close proximity to *Casp11*. Figure adapted from reference (184).

capable of autoproteolysis (190), and its protease activity is not required to restrict caspase-1 activity (189). Further studies have also indicated that caspase-12 can diminish numerous inflammatory pathways, including Ripk2 signalling through displacement of TRAF6 from the complex, and NF-κB activation by competitive binding with NEMO (191, 192). In response to West Nile virus, caspase-12 is required for type 1 IFN production through modulating RIG-I ubiquitination (193).

In mice, caspase-12 is expressed constitutively in the muscle, brain, and heart among other tissues, but is absent from macrophages under basal conditions (194). Its expression can be induced by IFNγ, LPS, and TNF-α. Interestingly, the majority of humans do not express caspase-12 due to a premature stop codon (195, 196) leading to an aberrant transcript that is likely degraded by NMD (Figure 9) (197). However, a $T^{125}C$ SNP found in a percentage of people of African descent restores expression of the full length protein and was associated with protection from sepsis (196). It is unclear to what extent the immune modulating effects of murine caspase-12 translate to the human protein as other studies have reported little effect of caspase-12 expression in response to pneumonia, candidemia, or rheumatoid arthritis (198-200). Unlike its mouse counterpart, human caspase-12 lacks a functional catalytic domain though it is unknown if this may be a contributing factor (195).

1.3 Cell Death

Cell death is an important process for the survival of complex organisms. It plays a vital role in the immune response, the removal of damaged or cancerous cells, and is crucial for host development. Multiple pathways have evolved to allow the cell to properly respond to a wide range of stimuli and circumstances. These cell death pathways can be group into programmed or passive forms. In the latter, a cell that is exposed to stressful conditions may die through a process termed necrosis. This may be the result of mechanical or physiochemical stress, ischemia, or extreme temperature exposure. Due to these suffered injuries, the cell lyses and releases its contents in an uncontrolled manner. DAMPs entering the extracellular environment can promote an inflammatory response. Aside from passive or accidental cell death, multiple pathways have evolved using precise molecular machinery to induce cell death in a regulated

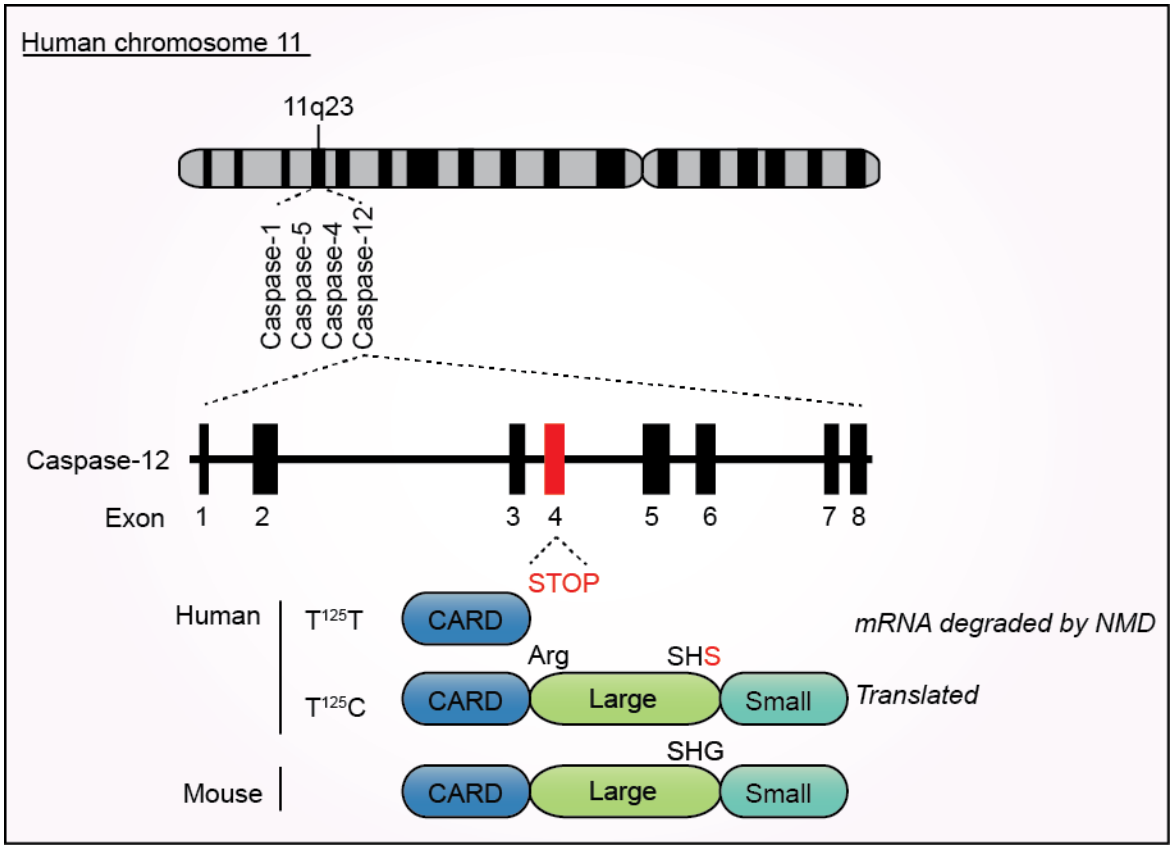

Figure 9. Mammalian Caspase-12

The majority of humans lack expression of caspase-12 due to a premature stop codon in exon 4. This leads to the degradation of the mRNA transcript by nonsense mediated decay (NMD). Approximately 20% of individuals of African descent have a $T^{125}C$ SNP that allows expression of the full length protein. The proteolytic domain of human caspase-12 has a mutation to the SHG group that renders it inactive. Mouse caspase-12 is expressed and catalytically active. Figure adapted from reference (189).

fashion. Regulated cell death pathways include apoptosis, pyroptosis, and necroptosis. These multiple mechanisms permit cellular suicide in response to various conditions.

1.3.1 Apoptosis

Apoptosis was the first described method of programmed cell death and is an essential function in development and physiological processes. It is morphologically characterized by cell membrane blebbing, chromatin condensation, and nuclear fragmentation (201). In contrast to necrosis, apoptosis is immunologically inert as phagocytes are attracted to consume the dying cells, preventing the release of DAMPs. A variety of signals can trigger apoptosis, including DNA damage, ER stress, developmental cues, or the stimulation of cytokine and cell surface death-receptors.

Apoptosis can proceed through two major pathways: the mitochondria induced intrinsic pathway and through external cell signalling mediating the extrinsic pathway (Figure 10). These are carried out by the 'apoptotic' caspases, which can be further subdivided into initiator caspases (-2, -9, -8 and -10) or executioner caspases (-3, -6, and -7). The initiator caspases are the first to respond to apoptotic stimuli and in turn cleave and activate the executioner caspases. The executioner caspases have hundreds of substrates that are processed to induce apoptosis.

Various stimuli can promote apoptosis through the intrinsic pathway, including nutrient deprivation, endoplasmic reticulum (ER) stress, developmental signals, and DNA damage. This ultimately results in mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome C, an activator of caspase-9. In a healthy cell, the mitochondrial membrane prevents its contents from entering the cytoplasm. During intrinsic apoptosis, the Bcl2-family proteins Bax and Bak form oligomers on the mitochondrial outer membrane leading to MOMP and release of intermembrane proteins and molecules. This process is regulated by pro-apoptotic BH3 family proteins (Bid, Bim, Bad, and Noxa) and anti-apoptotic proteins (Bcl2, Bcl-xL, and Mcl1). Cytoplasmic cytochrome C binds to APAF-1 forming a complex called an apoptosome. The apoptosome acts as a platform for caspase-9 auto-activation.

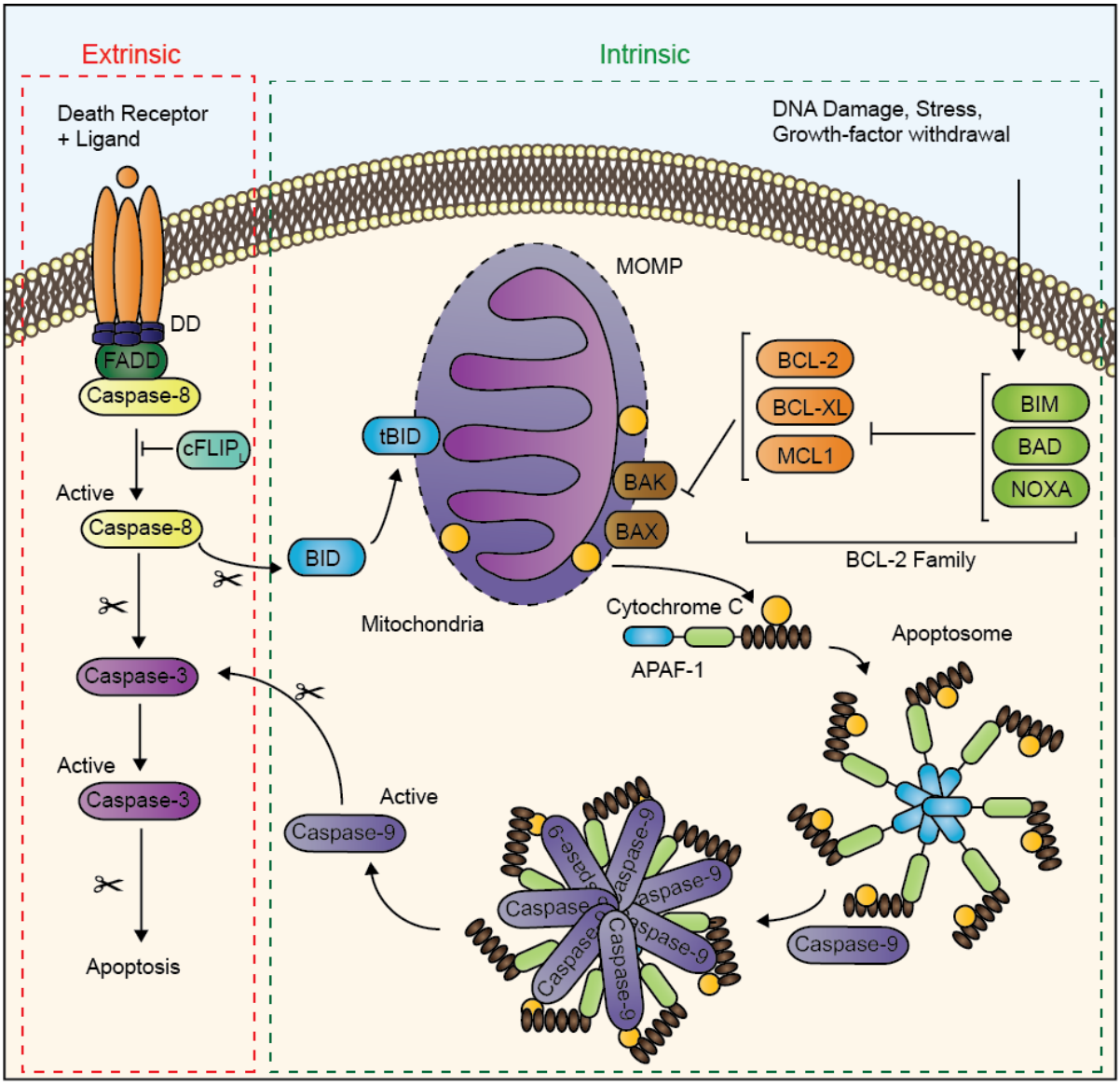

Figure 10. Apoptosis

Apoptosis can be initiated through two distinct pathways. Death receptor activation induces the extrinsic pathway, leading to death domain (DD) binding of FADD to the receptor. Caspase-8 is recruited to FADD and is activated, allowing it to cleave the executioner caspase-3 to begin apoptosis. Other apoptotic inducers such as DNA damage, cellular stress, or growth-factor withdrawal proceed by the intrinsic pathway. These signals affect the balance of anti-apoptotic and pro-apoptotic BH3 family members that regulate BAK and BAX. BAK and BAX form pores in the mitochondria, leading to mitochondria outer membrane permeabilization (MOMP) and the release of cytochrome C. Intracellular cytochrome C binds to APAF-1 to initiate apoptosome complex formation and the activation of caspase-9. Caspase-9 is then capable of activating caspase-3 to drive apoptosis. Cross-talk does exist between the intrinsic and extrinsic pathways as caspase-8 is capable of cleaving BID, liberating the C-terminal fragment (tBID) to insert into the outer mitochondrial membrane and promote BAX or BAK dependent MOMP.

Cells can also be instructed to die through the binding of death ligands to cell surface receptors, such as TNFR1, TRAIL-R1/2, and FAS. This signalling leads to the formation of a death-inducing signalling complex (DISC) on the cytoplasmic receptor tail beginning with death domain (DD)-mediated recruitment of FAS-associated death domain (FADD). FADD can in turn bind to caspase-8/-10 through their respective death effector domains (DED), leading to caspase activation and autoprocessing.

1.3.2 Pyroptosis

Pyroptosis occurs downstream of caspase-1 or caspase-11 activation and has unique morphological characteristics (202). In contrast to apoptosis, this mechanism of cell death results in membrane pore formation leading to osmotic lysis and rupture of the cell (203). DNA fragmentation is common to both cell death forms, though pyroptosis lacks apoptotic nuclear condensation. Finally, ADP-ribose polymerase (PARP), an NAD+ consuming enzyme that is cleaved in apoptosis, remains intact in caspase-1 dependent cell death.

Physiologically, pyroptosis has been demonstrated to be an integral defence against pathogenic bacteria. During *in vivo Salmonella* infection, Nlrc4 induces pyroptosis of infected macrophages, leading to bacterial release and phagocytic ROS-dependent killing by neutrophils (204). *Listeria monocytogenes* may be eradicated by a similar mechanism as the bacteria are eliminated from mice by the Nlrc4 inflammasome, independently of IL-1β or IL-18 cytokines (205). It is currently poorly understood how IL-1β is released from the cell after inflammasome activation and if the mechanism occurs through an active or passive process. Pyroptosis provides one explanation as it permits the release of processed mature cytokines from the cell.

1.3.3 Necroptosis

Necrosis was long considered as an unregulated mode of cell death independent of cellular signalling pathways. However, the discovery of a TNF-driven non-apoptotic, caspase-independent, cell death that had characteristics similar to necrosis suggested that necrotic cell death can be regulated. This ‘regulated necrosis’ or necroptosis is blocked by Necrostatin (Nec)-1, an inhibitor of RIP1 (or RIPK1) activity (206). It is now established that necroptosis is

initiated by a protein complex, composed of RIPK1, RIPK3 and MLKL, termed the necrosome (Figure 11) (207).

Necroptosis can be initiated by multiple pathways of which signalling through the TNFα receptor, TNFR1, was the first to be described. The outcome of TNFR1 stimulation by TNFα is determined by the activity of downstream signalling events. Under basal conditions, TNFα leads to inflammation and cell survival. This is initiated through the recruitment of RIPK1 and TRADD to TNFR1 (208). TRADD recruits the E3 Ub ligases cIAP1 and cIAP2 that conjugate K63-linked poly-Ub chains on RIPK1, and this Ub scaffold engages LUBAC, NEMO-IKK1-IKK2, and TAK1-TAB1-TAB2 complexes to activate NF-κB and MAPK pathways (209-211). LUBAC linear Ub of RIPK1 further promotes NEMO recruitment and TAK1 phosphorylation and activation of the IKK complex, leading to NF-κB signalling and proinflammatory cytokine production and cell survival. This signalling platform has been termed complex I.

Aside from its pro-inflammatory effects, TNFα is also cytotoxic and was named for its ability to kill tumour cells (212). Depletion of the cIAPs, loss of LUBAC, or the induction of CYLD-mediated deubiquitination of RIPK1 lead to the formation of an apoptosis-inducing DISC, termed complex II (213-215). In addition to RIPK1, complex II also includes RIPK3, FADD, caspase-8 and cFLIP (cellular FLICE-like inhibitory protein). Active caspase-8 initiates apoptosis while also cleaving RIPK1 and RIPK3 to prevent necroptosis (216, 217). Complex II activation is negatively regulated by the long isoform of cFLIP ($cFLIP_L$), an inactive caspase-8 homolog, which forms heterodimers with caspase-8 to inhibit its full activity and prevent apoptosis (218). Interestingly, caspase-8 expresses partial activity in this state, allowing it to process RIPK1 and block necroptosis (219). cFLIP can also be expressed as a short isoform ($cFLIP_S$) that binds to caspase-8, but lacks the ability to partially activate it (220). This serves to inhibit apoptosis but promotes a shift to necroptosis. Therefore relative concentrations of the cFLIP isoforms can influence these two cell death pathways.

Complex II inhibition (e.g. through ablation of caspase-8 or FADD) leads to necrosome engagement. RIPK1 and RIPK3 interact through their respective RHIM domains, are cross-

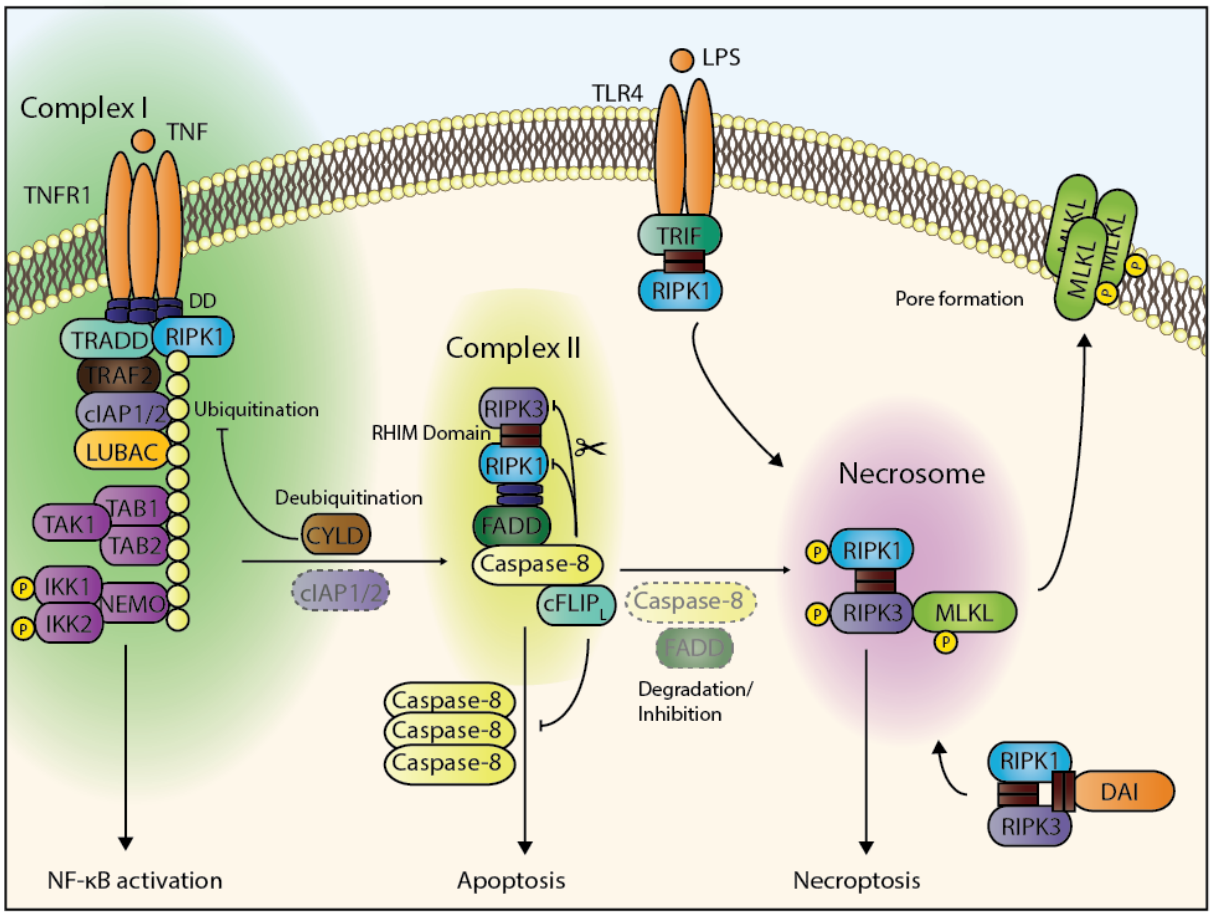

Figure 11. Necroptosis

TNF signaling at the TNF receptor (TNFR1) initiates the formation of complex I. TRADD and RIPK1 are recruited to TNFR1 by their Death domains (DD) and TRADD serves as an adaptor for the recruitment of TRAF2 and the ubiquitin ligases cIAP1/2 and LUBAC. LUBAC and cIAP1/2 add ubiquitin chains to RIPK1, which act as scaffolding for TAB1-TAB2-TAK1 and IKK1-IKK2-NEMO complexes to initiate NF-κB signaling. Loss of cIAP1/2 or deubiquitination of RIPK1 by CYLD results in the formation of complex II composed of RIPK3, RIPK1, FADD, caspase-8, and cFLIP$_L$. RIPK3 and RIPK1 associate via their RHIM domains while RIPK1 binds FADD through DD interactions. Caspase-8 recruitment to complex II leads to its activation and the initiation of apoptosis. cFLIP$_L$ inhibits this process through binding to caspase-8 to repress its activity. Loss or inhibition of caspase-8 and FADD permits formation of a third complex, the necrosome, comprised of RIPK1, RIPK3, and MLKL. This complex is inhibited by caspase-8 cleavage of RIPK3 and RIPK1. Necrosome formation results in the phosphorylation of each component and MLKL oligomerization and translocation to the plasma membrane to induce necroptotic cell death. In addition to TNF and other death receptors, TLR4-TRIF mediated signaling or recognition of viral DNA by DAI can also induce necroptosis.

activated by phosphorylation and assemble into an amyloid-like aggregate (221-224). The search for RIPK3 substrates led to the identification of MLKL (225, 226). MLKL is the most downstream effector of necroptosis and is absolutely required for this mode of cell death. Its phosphorylation by RIPK3 within the necrosome leads to a conformational change (227), promoting its translocation to the plasma membrane where it forms pores to trigger osmotic lysis (228-231).

A role for necroptosis in inflammatory disease pathogenesis has been demonstrated *in vivo* through the study of knockout mouse lines. While mice deficient in the necrosome components *Ripk*3 and *Mlkl* survive to adulthood and appear phenotypically healthy, *Casp8*$^{-/-}$ mice die embryonically (232), but can be rescued with the ablation of *Ripk*3 and *Mlkl*, demonstrating a requirement for caspase-8 to inhibit necroptosis (219, 233). Similar studies have shown that FADD-deficient mice also exhibit an embryonic lethality phenotype that can be rescued with loss of *Ripk*3, due to FADD's ability to induce caspase-8 activation and inhibit necroptosis (234-236). *Ripk1*$^{-/-}$ mice are also embryonically lethal, but ablation of *Ripk*3 or *Mlkl* only results in partial rescue. Full survival of Ripk1-deficient mice is only achieved in *Rikp1*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ triple KO mice (237, 238). This finding demonstrates that Ripk1 plays an important regulatory role in caspase-8 dependent apoptosis as well as necroptosis. Survival is independent of Ripk1 kinase activity as kinase dead mutants D138N and K45A live to adulthood, indicating that it is the scaffolding role for Ripk1 that is required to inhibit cell death (239, 240).

Cell death is an important anti-viral host defence strategy and consequently many viruses express proteins to block apoptosis. Since necroptosis occurs when key apoptotic components, such as caspase-8, are inhibited, it has been hypothesized that necroptosis has evolved to provide an alternative mode of cell death to combat viral infection (241). In particular, necroptosis has been demonstrated to play a key role in providing immunity to MCMV and herpes simplex virus (242, 243). Aside from protection against these viral pathogens, necroptosis has also been implicated in pulmonary tissue damage in influenza infection, neurodegeneration, atherosclerosis, skin inflammation, kidney injury, and liver disease (244, 245).

In addition to classical TNFR1-induced necroptosis, other death receptors and PRRs converge on necrosome activation. These include recognition of viruses by the DNA sensor DAI (243, 246), TLR3/4/TRIF (247), and IFN-mediated PKR signalling (248).

1.4 The innate immune system and factors contributing to chronic inflammatory disease

Chronic inflammation is an important pathogenic component of many illnesses. A thorough understanding of the involved pathways and affected genes can provide crucial insight into potential therapies. Inappropriate regulation of the innate immunity is now well understood to result in auto-inflammatory disease. Pathogenesis is independent of the adaptive immune response, as patients lack high-titer autoantibodies and antigen-specific T cells. Instead, these diseases are driven by mutations in genes of the innate immune system, including PRRs, cytokines, and immune cell regulators. An example includes the previously discussed inflammasomopathies, such as CAPS and FMF. TNF signalling can also become deleterious due to mutations in the TNFR1 encoding gene *TNFRSF1A*, leading to TNF receptor–associated periodic syndrome (TRAPS) (249). These mutations result in ligand-independent receptor activation, causing recurrent prolonged fevers, arthritis, skin rash, and abdominal pain in patients.

Mutations to cell death protein encoding genes are also causative for inflammatory disease. Caspase-8-deficient state (CEDS) results from *CASP8* mutations leading to lymphadenoapthy, splenomegaly and immunodeficiency (250). Mutations to *FAS*, *FASLG*, and *CASP10* result in a disorder with multiple overlapping symptoms called Autoimmune Lymphoproliferative Syndrome (ALPS) (251).

Innate immunity and cell death can also contribute to the development of more complex diseases, which are dependent on more than one initiating factor. Along with host genetics, the intestinal microbiota, and environmental factors, the innate immune system is also involved in the etiology of inflammatory bowel disease (IBD) and metabolic disease. For example, mutations to *NOD2* represent the greatest known risk for the development of Crohn's Disease (CD) (252, 253) and anti-TNF therapy can ameliorate symptoms in IBD patients by preventing epithelial

cell death (254). Elevated inflammatory markers and PRR activation within the adipose tissue of obese subjects are highly correlative with the development of insulin resistance (255).

1.4.1 The intestinal microbiota and chronic inflammation

The importance and influence of the intestinal microbiota in modulating various diseases is now well known. The gastrointestinal tract (GI) harbours a vast variety of commensal microbiota, including bacteria, viruses and fungi, that exist in a symbiotic relationship with the host. The role of the bacterial flora has been the most heavily studied. It is estimated that the human GI tract microbiota may include up to 10-100 trillion bacteria, approximately 10 times greater than the number of host cells. The host and commensal flora have coevolved to share a symbiotic relationship that is required for healthy intestinal physiological processes. The luminal microbes aid in the digestion and absorption of nutrients, produce essential vitamins, prevent the colonization of pathogenic bacteria, and also play an important role in the education and priming of the immune system from an early age to induce tolerance and protective immunity (256). Technical advances in high throughput sequencing and the utilization of germ-free mice have provided important tools to dissect the precise mechanisms involved in microbiome regulation of host processes. The importance of the gut microbiome is clearly evident through studies of germ-free mice. The absence of microbiota colonization results in alterations to the gut-associated lymphoid tissue (GALT), dampened antibody production, reduced mucous layer thickness, an altered immune system architecture, and changes to intestinal epithelial cell (IEC) microvillus formation (257).Consequently, germ-free mice are more susceptible to enteric infection by pathogenic bacteria. Specific types of bacteria are also known to directly modulate the immune response. For example, it was determined that the presence of segmented filamentous bacteria can induce Th17 T cell differentiation (258).

The immunoregulatory properties of the microbiota can affect various inflammatory diseases. Human and animal studies have linked the composition of the intestinal flora to IBD pathogenesis (259). Interestingly, tissues distal from the gut are also affected by the microbiota. Fecal bacteria composition correlates with atopic eczema and its incidence can be decreased by the administration of probiotics (260, 261).Metabolic disease and obesity driven inflammation are also affected by the microbiome (262). Further analysis of the human microbiota and its

influence on disease may provide important insight to the treatment of multiple diseases and modulation of the immune system.

1.5 Metabolic Disease

Obesity is a rapidly growing problem worldwide in both developed and developing countries (263). It is currently estimated that in the United States, approximately 66% of adults are overweight and 32% are obese (264). This leads to an enormous healthcare burden as obesity drastically increases the risk of developing various conditions and diseases such as insulin resistance, Type 2 Diabetes (T2D), cardiovascular disease (CVD), fatty liver, and numerous cancers (265). Over 370 million people have diabetes worldwide (266) and CVD is the primary cause of death globally and in diabetic subjects in particular. Factors like genetics, aging, inflammation, an unhealthy life style and obesity are known modulators of these complex diseases.

1.5.1 Insulin resistance

Insulin resistance occurs when the metabolic tissues of the body, namely the adipose tissue, liver, and muscle, no longer respond effectively to insulin. Insulin is required to increase glucose uptake into the muscle, inhibit hepatic gluconeogenesis, and inhibit adipose tissue lipolysis (267). Together these processes allow the body to properly regulate its blood glucose levels. In order to maintain normal glycemia, insulin secretion increases to compensate for insulin resistance. However over time, and in conjunction with pancreatic inflammation, β-cells undergo exhaustion, apoptosis and reduced insulin secretion, leading to hyperglycemia and progression to T2D (268). The inability to properly manage ones blood glucose levels can lead to cardiovascular complications, diabetic retinopathy, kidney failure, and death.

1.5.2 Inflammation and metabolic disease

While acute inflammation is crucial to innate immunity and host resistance to infection or injury, obesity-associated chronic inflammation in peripheral tissues is an established instigator of T2D and associated CVD (269-271). An enormous body of evidence has now linked the development of insulin resistance to obesity driven inflammation. The first evidence to support this was the discovery that the adipose tissue of obese mice and humans has much higher levels of the

proinflammatory cytokine TNFα (272, 273). This landmark discovery promoted a great undertaking to further characterize the mechanisms and role of inflammation in obesity and T2D. Our current understanding of the disease suggests that the excess nutrients in obese individuals initiates inflammation in the adipose tissue and through the effects of various intracellular inflammatory pathways, cytokines, and immune cells, results in a low level of chronic inflammation and insulin resistance (Figure 12).

One of the most relevant tissues in relation to the promotion of systemic inflammation and metabolic abnormalities is the white adipose tissue (WAT) (255). WAT is located throughout the body in visceral or subcutaneous depots and primarily functions to store energy in the form of triglyceride lipid droplets. The WAT also plays a significant role in the sensing and modulation of host metabolism and is a highly secretory endocrine organ. These functions can be compromised during obesity and result in deregulated secretion of adipokines that normally control much of the autocrine, paracrine and endocrine functions of the WAT (274). Inflammation of the adipose tissue is characterized by increased macrophage infiltration, particularly the M1 or "classically activated" macrophages, and increased synthesis and secretion of pro-inflammatory cytokines including IL-1β, IL-6, TNFα, and monocyte chemoattractant protein-1 (MCP-1) (255). These cytokines act in an autocrine and endocrine manner, further upregulating WAT and systemic inflammation and suppressing insulin secretion and signalling. Adipose depot location also affects its contribution to disease. The size and inflammatory status of visceral WAT is most predictive of metabolic disease (275). Surgical removal and transplantation studies provide further evidence of the importance of depot location with metabolic perturbation (276).

Interestingly, adipocyte secreted adipokines can also regulate the inflammatory state of the tissue. Leptin was originally discovered for its ability to suppress appetite by acting on the central nervous system (277). Recent work has also demonstrated it can signal immune cells, such as macrophages, to express pro-inflammatory cytokines (278). Resistin is an adipokine that is also associated with insulin resistance and similarly induces an inflammatory phenotype on myeloid cells (279). Conversely, adiponectin has anti-inflammatory effects and is downregulated during obesity and insulin resistance (274). Stimulation of macrophages with this adipokine can

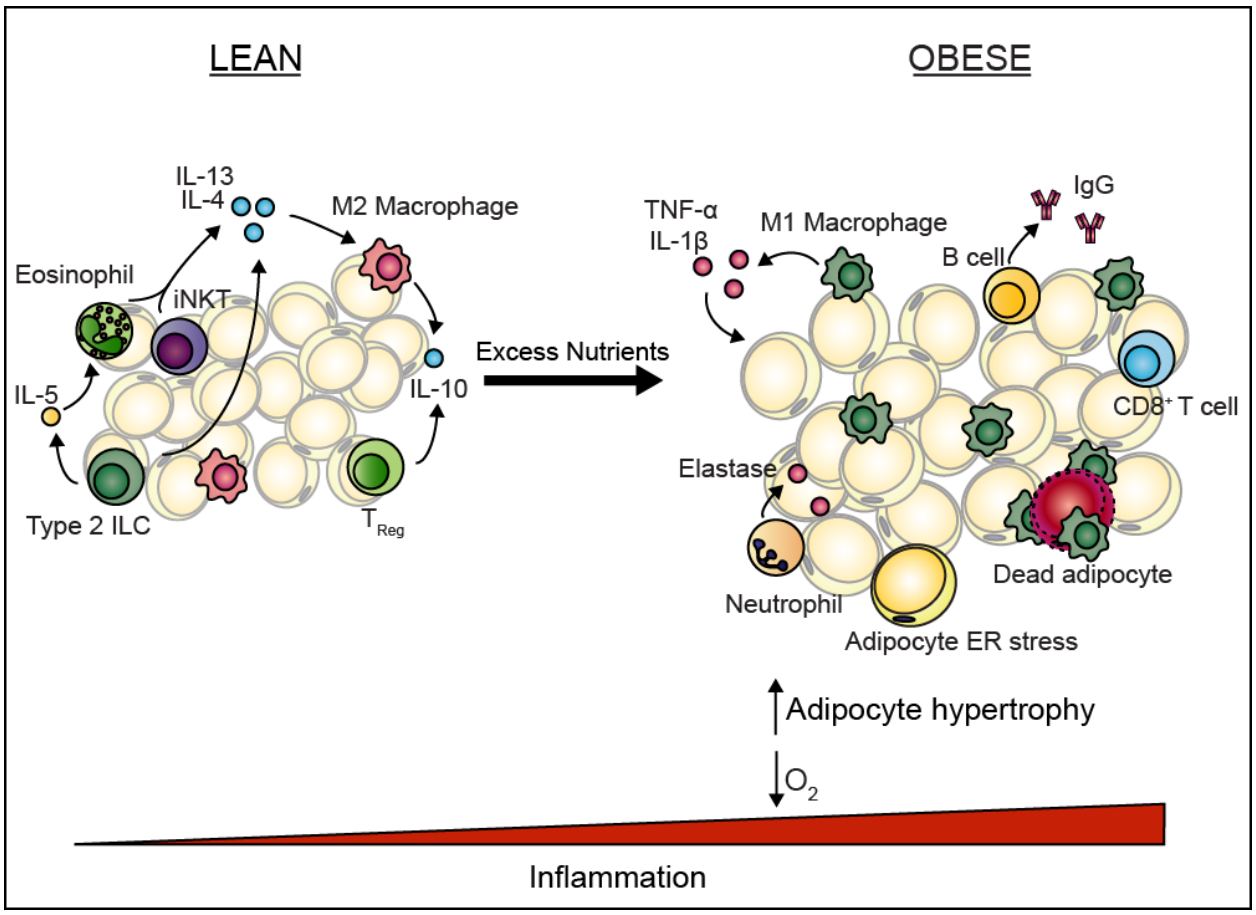

Figure 12. Adipose tissue inflammation

Inflammation of the adipose tissue is now a well known consequence of obesity. In the lean state, the adipose tissue contains resident anti-inflammatory M2 polarized macrophages, iNKT cells, Type 2 ILC cells, eosinophils, and regulatory T cells (T_{Reg}). These cells secrete anti-inflammatory cytokines, such as IL-10, or IL-4, IL-5, and IL-13 to induce a T_H2 polarizing environment. During the development of obesity, excess energy is stored within the adipocytes as lipids and leads to hypertrophy. Rapid expansion of the adipose tissue can result in hypoxic conditions or the induction of ER stress in adipocytes, promoting inflammation and the recruitment of circulating immune cells. The immune cells in the adipose tissue of obese individuals are highly inflammatory and include inflammatory M1 macrophages, $CD8^+$ T cells, neutrophils, and mature B cells. The secretion of pro-inflammatory cytokines, elastase, and IgG antibodies impair cell function and lead to insulin resistance.

promote secretion of the immunosuppressive cytokine IL-10 (280).

1.5.2.1 Initiation of inflammation in the adipose tissue

The initiation of inflammation in obese individuals begins within the metabolic tissues, namely the adipose tissue, liver, and muscle. In obese individuals, the adipocytes undergo hypertrophy in order to properly store the available excess nutrients. The rapidly expanding adipose tissue is prone to hypoxia (281, 282), where the growth of tissue proceeds at a faster rate than proper vascularisation, similar to what is seen in tumours. Hypoxia results in the induction of hypoxia-inducible factor 1α (HIF-1α) and proinflammatory gene transcription (281). Mice with adipocyte specific deletion of HIF-1α are protected from obesity and insulin resistance, demonstrating the detrimental effects of this pathway (283).

Expanding adipocytes also enter a state of increased protein translation in order to proliferate and undergo the architectural changes required to meet the demands for increased lipid storage. This can lead to a build-up of unfolded proteins within the ER, leading to ER stress and triggering of the Unfolded Protein Response (UPR). The UPR attempts to return the ER back to a state of equilibrium by inducing the expression of genes to reduce protein synthesis, aid in the folding and modification of the proteins, or to begin to degrade them. However ER stress also leads to an inflammatory response, which is seen in the adipose tissue of obese rodents and humans (284, 285). This leads to the production of proinflammatory cytokines, such as IL-6 and TNFα, chemokines such as CCL2, and activation of the kinases c-jun N-terminal kinase (JNK) and IKK, which can initiate inflammatory gene expression (284, 286). In support of the role of ER stress as a contributing factor in T2D, chemical chaperones that reduce ER stress were shown to alleviate insulin resistance in a diet induced obesity (DIO) mouse model (287).

The effects of adipose tissue hypoxia and ER stress result in localized inflammation that can lead to adipocyte death and further induce the release of various DAMPs, chemokines and cytokines (288). This environment promotes the attraction of immune cells into the adipose tissue and consequently the adipose tissue of obese individuals contains numerous dead cells, surrounded by infiltrated macrophages, called ‘crown-like structures’ (288).

1.5.2.2 Infiltrating immune cells

Inflammation of the adipose tissue results in the production of cytokines and chemokines that promote vascular permeabilization and the recruitment of infiltrating immune cells. These cells become activated within the tissue upon exposure to the inflammatory environment and are then capable of propagating the inflammation in a positive feedback loop. Various lymphoid and myeloid cells have been detected in the adipose tissue and there is a positive correlation between obesity and the number and type of infiltrating immune cells (289).

Macrophages infiltrate the fat tissue where they proliferate locally (290). Their presence was first demonstrated by two groups using genetically obese mice and a DIO mouse model that the adipose tissue had a large number of macrophage specific transcripts that positively correlated with obesity (291, 292). The presence of macrophages in the tissue was verified through histological examination and it was also demonstrated that these macrophages were the cell types primarily responsible for the expression of TNFα and IL-6 in the adipose tissue.

Further examination of the adipose tissue macrophage population revealed the presence of two different subsets. Macrophages can be induced by environmental stimuli to differentiate into different classes, either proinflammatory classically activated M1 or anti-inflammatory alternatively activated M2 macrophages (293). M1 macrophages produce proinflammatory cytokines and secrete ROS, while M2 macrophages generate anti-inflammatory cytokines such as IL-10. The adipose tissue of healthy lean mice contains predominantly M2 macrophages. The adipose tissue of obese mice however has a much higher percentage of M1 polarized macrophages, contributing to the development of increased inflammation and insulin resistance. Depletion of the M1 macrophage subset in mice results in insulin sensitization (294). Further evidence suggesting the importance of M2 polarization to insulin sensitivity is seen in mice lacking the nuclear receptor peroxisome proliferator-activated receptor δ (PPARδ), which is required for M2 activation. These mice have increased insulin resistance and reconstitution of obese mice with PPARδ-deficient myeloid cells leads to increased pathogenesis in comparison to reconstitution with WT cells (295, 296). This M1/M2 model has been further refined to suggest that adipose macrophages may develop a “metabolically activated” phenotype due to exposure to fatty acids, insulin, and glucose (297). In addition to pro-inflammatory cytokines secretion, this

polarization induces the expression of unique cell surface markers and lipid metabolism genes that differ from M1 macrophages in other tissues.

Since the discovery of inflammatory macrophages within the adipose tissue, it has been demonstrated that numerous cells of the immune system also play key roles in obesity and insulin resistance. Interestingly, many of these cell populations undergo a shift from an anti-inflammatory phenotype to a pro-inflammatory program during the course of obesity. Lean individuals have increased numbers of eosinophils, Type 2 innate lymphoid cells (ILCs), and invariant natural killer T (iNKT) cells that produce IL-4 and IL-13, a requirement for M2 macrophage polarization (298-300). Type 2 ILCs also secrete IL-5 to maintain VAT eosinophil populations and support this anti-inflammatory axis (299). All three cell types have also been associated with reduced body weight and improved insulin sensitivity in mice.

The development of obesity induces a flux of pro-inflammatory immune cells. Neutrophils enter the adipose tissue and induce inflammation through the secretion of the proteolytic enzyme elastase (301). T cells are found to infiltrate the adipose tissue prior to macrophages and their differentiation into specific subsets is also vital in determining their contribution to insulin resistance. Cytotoxic $CD8^+$ T cells are found at higher proportion than $CD4^+$ T cells in the fat pads of obese compared to lean mice and were found to promote macrophage infiltration (302). Thus, depletion of $CD8^+$ T cells has a beneficial effect on insulin sensitivity. $CD4^+$ T cells also have an important role in controlling insulin resistance. They can differentiate into different subsets, including T_H1, T_H2, and regulatory T (T_{Reg}) cells. T_H1 cells produce the proinflammatory cytokines TNFα and IFNγ to activate macrophages, while T_H2 cells secrete type 2 cytokines such as IL-4, IL-5, and IL-13 to stimulate the humoral immune system (303). In the context of obesity, T_H2 $CD4^+$ T cells are capable of reducing insulin resistance, demonstrated through adoptive transfer into insulin resistant Rag1 null mice, which lack an adaptive immune system (304). In contrast, T_H1 cells are pathogenic and consequently, this is the subset that is most predominant in obese individuals (304). It has been shown that there is a reduction in the number of T_{Reg} cells in the adipose tissue of obese humans and mice (305). The decrease in T_{Regs} may be due to an increase in leptin in obese individuals, which is a negative regulator of T_{Reg} development (306). T_{Regs} depletion in mice was also found to lead to an increase in insulin

resistance, their beneficial function being possibly linked to the secretion of the cytokine IL-10 (305). IL-10 treatment of the 3T3-L1 mouse adipocyte cell line induces protection to TNFα mediated upregulation of proinflammatory mediators and insulin resistance.

Finally, B cells, the antibody producing cells of the body, have been demonstrated to have a detrimental effect in the development of insulin resistance (307). In a DIO mouse model, B cells were found to localize to the adipose tissue where they promote activation of $CD8^+$ T cells and macrophages. The IgG antibodies produced by B cells are also pathogenic, as the transfer of purified IgG from DIO mice induces insulin resistance whereas IgG from control mice does not. Mice lacking functional B cells are more insulin sensitive on a high fat diet (HFD), and B cell depletion from WT mice ameliorates insulin resistance. Interestingly, B cell subsets that secrete IL-10 can also have a beneficial anti-inflammatory effect in DIO mice (308, 309).

1.5.2.3 Inhibition of insulin signalling through IRS-1 phosphorylation

Insulin receptor substrate-1 (IRS-1) lies downstream of the insulin receptor (IR) and is an important modulator of metabolism (310). Upon insulin binding, the IR tyrosine kinase phosphorylates IRS-1 to generate Src homology 2 (SH2) bindings sites, permitting downstream signalling. This results in the activation of various MAPK, such as AKT, and associated gene expression programs. Insulin signalling can be positively or negative regulated through serine/threonine phosphorylation of IRS-1.

It was not clear initially how adipose tissue inflammation could lead to insulin resistance at the mechanistic level. Analysis of the intracellular insulin signal transduction pathway revealed that proinflammatory kinases, including JNK, IKK, and protein kinase R (PKR), engaged downstream of cytokine receptors and PRRs are capable of directly inhibiting insulin signalling (311). Concordantly, the activation of these kinases is increased in the adipose tissue of obese subjects (312-314). Besides triggering NF-κB and AP-1 transcriptional programs (315), these kinases can phosphorylate and inhibit IRS-1 on serine S307 and S3012 of the human protein (S302 and S307 in mice) (312-314). Mice deficient in JNK, IKK, or PKR are insulin sensitive in a DIO mouse model, and have reduced inhibitory phosphorylation of IRS-1. Tissue specific depletion and bone marrow transfer experiments have demonstrated that these kinases exert their

effects on insulin resistance by acting both in metabolic tissues as well as myeloid cells (316, 317).

1.5.3 PRRs and obesity

PRR expression is not restricted to immune cells, as metabolic cells including adiopocytes, also express a battery of these receptors. Although PRRs were initially characterized for their role in detecting foreign microbial motifs, more recently they have been implicated in recognition of metabolic danger signals arising in metabolic disease (318). TLR4 for instance detects both bacterial LPS (319) and the fatty acid palmitate (320, 321). Mice deficient in the *Tlr4* gene are thus protected from DIO insulin resistance (320, 322). In this context, Tlr-4 mediated its effects by acting primarily in myeloid cells, as suggested by bone marrow reconstitution experiments (323).

During obesity, increased intestinal permeability leads to dissemination of microbial ligands in the systemic circulation, which is referred to as "metabolic endotoxemia" (324). Recognition of these microbial triggers by PRRs has been proposed to promote insulin resistance. Loss of the intracellular NLRs Nod1 and Nod2 in mice correlates with insulin sensitivity and reduced weight gain on a HFD (325). This is mediated through circulating bacterial peptidoglycans derived from the host microbiota stimulating the Nod1/2 pathways.

1.5.3.1 The inflammasome and metabolic disease

Earlier work had demonstrated a role for IL-1β in the development of insulin resistance, and studies linking the inflammasome to this process soon followed. The use of mice deficient in inflammasome components, including caspase-1, Nlrp3, and Asc, has unravelled a metabolic role of this pathway *in vivo*.

The first direct implication of the inflammasome stemmed from the finding that NLRP3 binds to thioredoxin-interacting protein (TXNIP), a protein previously linked to pancreatic β-cell death and insulin resistance (326). TXNIP inhibits thioredoxin (TRX), a protein involved in reducing oxidative stress and limiting ROS, a common activator of the NLRP3 inflammasome pathway. Bone marrow–derived macrophages (BMDMs) from *Txnip*$^{-/-}$ mice have reduced caspase-1

activation and IL-1β secretion after stimulation with NLRP3 agonists. Interestingly, *Nlrp3*$^{-/-}$ mice on a HFD phenocopy mice ablated for Txnip and were determined to be insulin sensitive compared to WT controls (326). This groundbreaking study sparked a quick succession of work further characterizing the role of the NLRP3 inflammasome in obesity and metabolic disease.

Using a DIO model, it was first reported that mice ablated for caspase-1 had a leaner phenotype than WT mice when placed on a HFD for 10 weeks (4). Metabolic analysis of these mice detected no differences in food intake, stool output, or energy expenditure; however, an increased rate of fatty acid oxidation compared to carbohydrate utilization, was measured in *Casp1*$^{-/-}$ mice through indirect calorimetry. A similar lean phenotype was observed for *Casp1*$^{-/-}$, *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice in a later DIO study (327). Collectively, these two earlier studies suggested that *Casp1*$^{-/-}$ mice are resistant to obesity. However, such resistance is not observed universally. For instance, two recent reports found that *Casp1*$^{-/-}$ mice were contradictorily more obese than WT mice, developing increased fat mass on a HFD, or by 40 weeks of age when fed a chow diet (2, 328). Other studies have reported that *Nlrp3*$^{-/-}$ mice are not protected from DIO and instead have equivalent total body weight compared to WT mice at 6 weeks, 4 months and 7 months irrespective of diet type (68, 329).

The contradictory reports on the role of the inflammasome in obesity are difficult to rationalize and suggest that environmental factors could be a factor. An altered microbiota composition in different animal colonies might underlie the reported variability. The inflammasome-IL-18 pathway has been implicated in intestinal microbiota regulation (128). Indeed, loss of inflammasome signalling resulted in dysbiosis and enhanced susceptibility to colitis (128) and non-alcoholic steatohepatitis (NASH) (330). Bone marrow transplantation studies revealed that the inflammasome is required in the radio-resistant compartment for its effects on microbial regulation, arguably through the maintenance of intestinal epithelial barrier integrity. Consistently, the NASH phenotype involved sensing of microbial products by TLR4 and TLR9 and induction of TNFα-dependent inflammation.

1.5.3.2.1 Inflammasomes and insulin resistance

Given that obesity can promote insulin resistance through numerous pathways, the role of the inflammasome can most directly be determined in mice with equivalent body weight. DIO *Nlrp3*$^{-/-}$ mice equivalent in body weight to WT controls have reduced inflammation. This is characterized by reduced caspase-1 activation and IL-1β production in the WAT and liver, and reduced circulating levels of IL-18 (329). Expression of inflammasome components in the adipose tissue was attributed primarily to infiltrating macrophages, as opposed to adipocytes. A second study investigating Asc-deficient bone marrow chimeric mice demonstrated that inflammasome activation within myeloid cell populations was responsible for insulin resistance (68). Multiple studies have observed a similar insulin sensitive phenotype in *Casp1*$^{-/-}$, *Nlrp3*$^{-/-}$or *Asc*$^{-/-}$ mice on a HFD, independent of body weight differences (4, 68, 327).

Insulin resistance can be successfully compensated for by increased insulin secretion, however as the disease progresses, this compensation fails and results in elevated blood glucose levels and diabetes. This is often the result of pancreatic decline and β-cell failure. Inflammation of the pancreas, by IL-1β and other inflammatory mediators, has been strongly linked to apoptosis and reduced function (331). Pancreatic islets express Nlrp3, Asc, caspase-1 and secrete IL-1β in response to high glucose concentrations (326). Compared to WT controls, Nlrp3 and Asc deficient mice have increased serum insulin after 1 year on HFD, although they are more insulin sensitive compared to WT mice (332). Closer examination of the pancreas in the mutant mice revealed reduced IL-1β expression in infiltrating macrophages, reduced pancreatic cell death, and increased islet size. In a Zucker diabetic fatty rat model of T2D, it was demonstrated that inhibition of endocannabinoid signalling through macrophage CB_1 receptors is able to block the progression of diabetes (333). In human macrophages, endocannabinoid treatment can stimulate transcription of ASC and NLRP3 mRNA and lead to increased IL-1β and IL-18 secretion.

Islet amyloid polypeptide (IAPP) is a 37 amino acid peptide hormone that is secreted by β-cells along with insulin and its deposition in human islets has been strongly linked to the development of T2D and inflammation (334). It is capable of activating the Nlrp3 inflammasome in macrophages and dendritic cells (DCs) while transgenic mice expressing human IAPP have

elevated pancreatic IL-1β (335-337). In cells undergoing substantial protein translation, such as β-cells, failure to properly process and fold proteins results in ER stress triggering a UPR. The UPR is characterized by the activation of IRE1α, PERK, and ATF6. Recent work has linked PERK and IRE1 to the induction of TXNIP, and downregulation of microRNA miR-17 that decreases TXNIP mRNA stability. TXNIP upregulation in turn results in increased inflammasome activation and IL-1β secretion in islets and INS-1 832/13 rat Insulinoma cells (338, 339). Overall, the inflammasome appears to play a role in multiple tissues during the progression of insulin resistance, primarily mediated by its activation in immune cells and to a lesser extent in metabolic cells.

The inflammasome has also been implicated in human insulin resistance. There is a positive correlation between NLRP3 expression in subcutaneous WAT and fasting glucose levels after patients underwent a 1 year weight loss intervention (329). Moreover, recent studies have similarly shown that WAT and macrophage expression of NLRP3 inflammasome components is higher in obese subjects with impaired-glucose tolerance or diabetes compared to healthy controls (340, 341), and that obese individuals have higher plasma concentrations of IL-1β and IL-18 (340). Further supporting a correlation between inflammasome activation and clinical metabolic outcomes is the finding that the WAT macrophages of metabolically unhealthy obese individuals have increased activation of caspase-1 in comparison to macrophages from metabolically healthy obese patients (342).

While the Nlrp3 inflammasome has been the most commonly studied regarding metabolic disease, other inflammasomes may contribute as well. Nlrp1 was demonstrated to play a protective role in murine models of obesity (3). Activation of this NLR leads to increased IL-18 production, a protective factor against increased adiposity. In contrast, the Nlrc4 inflammasome which recognizes bacterial flagellin and components of the type 3 secretion system has been tested and found not to play a role in metabolic disease (330).

1.5.3.2.2 Metabolic activators of the Nlrp3 inflammasome

Inflammasome activation during metabolic disease can occur in the absence of pathogens, indicating that host danger signals can activate this pathway (Figure 13). Circulating levels of

free fatty acids are elevated during obesity (343). Besides recognition by TLR4, the saturated fatty acid palmitate also activates the Nlrp3 inflammasome (68). Treatment of macrophages with palmitate reduces phosphorylation and activation of AMPK, an important regulator of cellular metabolism and ATP levels. Constitutively active AMPKα1 represses palmitate driven inflammasome activation by autophagy induction and reduction in mitochondrial ROS, an NLRP3 activator. Ceramides, lipids composed of sphingosine and a fatty acid, are heavily linked to insulin resistance (344) and have been found to activate the Nlrp3 inflammasome in murine BMDM and WAT (329).

The progression of insulin resistance to T2D leads to elevated blood glucose levels. High glucose concentrations have been reported to activate Nlrp3 in pancreatic islets (326) and human WAT (345). Crystalline compounds engulfed by phagocytic cells, which lead to disruption of the phagolysosome and the release of cathepsins, are also potent activators of the Nlrp3 inflammasome (93, 94, 96). IAPP crystals thus activate Nlrp3, which can be inhibited by bafilomycin A, APDC, and CA-074 Me, inhibitors of the vacuolar ATPase, ROS, and cathepsin-B, respectively (337). Cholesterol crystals also induced IL-1β secretion from macrophages, in an Nlrp3 and cathepsin-dependent manner.

1.5.3.2.3 IL-1β in metabolic disease

A relationship between IL-1β, obesity and insulin resistance was recognized in studies predating those linking the inflammasome to metabolic disease. IL-1β was identified to be toxic to pancreatic β-cells and is involved in the pathogenesis of Type 1 Diabetes (331). Recent work on the inflammasome has implicated IL-1β as one of the primary mechanisms for caspase-1 driven metabolic phenotypes. IL-1β is increased in the WAT of mice on a HFD and in *db/db* mice (4, 329). Treatment of the 3T3-L1 murine adipocyte cell-line with recombinant IL-1β induces insulin resistance (346). In differentiating human SGBS adipocytes, IL-1β represses PPARγ, GLUT4, and adiponectin expression, and inhibits differentiation (4). The opposite effect is observed when adipocytes are treated with IL-1Ra or IL-1β neutralizing antibodies. IL-1β signalling leads to activation of IKK, which phosphorylates IRS-1, preventing insulin-dependent signalling and contributing to insulin resistance (347).

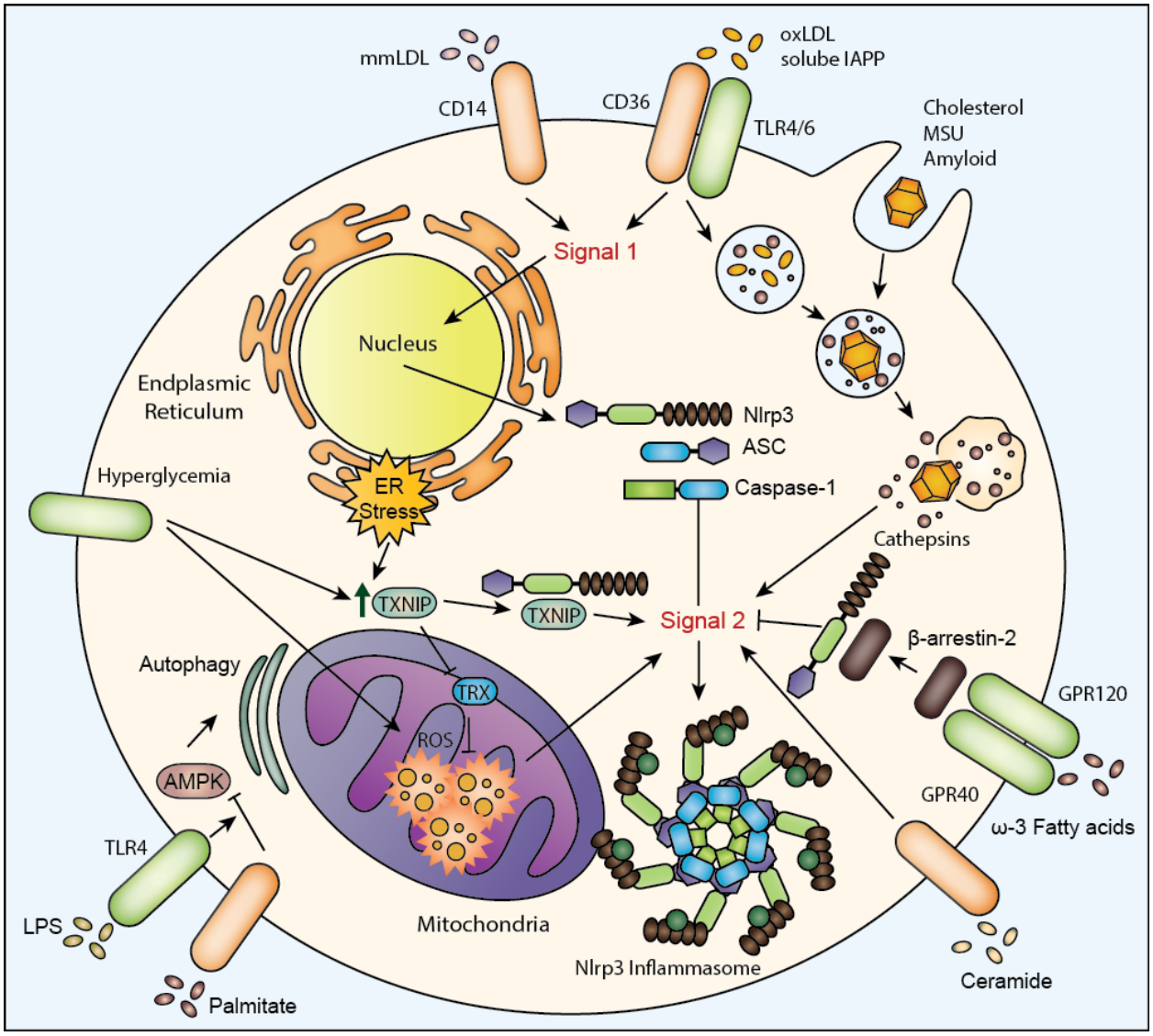

Figure 13. Metabolic activators of the NLRP3 inflammasome

The NLRP3 inflammasome can be activated by various metabolites and signals that are elevated in states of metabolic disease. Signal 1 and transcription of the inflammasome components NLRP3, ASC and caspase-1 and can be induced by minimally modified LDL or oxLDL, mediated through NF-kB. The second activation signal can be induced by cholesterol, monosodium urate and amyloid fibers that are directly phagocytosed into the cell or form through the nucleation of soluble ligands upon internalization through CD36. These crystals induce lysosomal destabilization and the release of cathepsins into the cytosol, stimulating activation. Reactive oxygen species (ROS) are potent activators of the Nlrp3 inflammasome and are induced by multiple NLRP3 agonists. ROS are negatively regulated by thioredoxin, which is in turn inhibited by TXNIP. Elevated glucose levels as well as endoplasmic reticulum (ER) stress have been linked to the induction of TXNIP. TXNIP is also a binding partner of NLRP3 and is required for its activation. Numerous lipids also influence inflammasome activation. Palmitate, a saturated fatty acid, inhibits AMPK in LPS-treated cells, resulting in reduced autophagy and increased ROS. ω-3 fatty acids have the opposite effect through β-arrestin-2 binding and inhibition of NLRP3. Adapted from reference (98).

The use of mouse models corroborates the deleterious effects of IL-1β on insulin resistance. Mice deficient in IL-1β or IL-1R1 are insulin sensitive on a HFD and injection of recombinant cytokine can induce insulin resistance (4, 68, 348). *Il1r1*$^{-/-}$ mice have reduced WAT inflammation (348). Interestingly, mice lacking IL-1Ra have a lean phenotype and were reported to have increased energy expenditure (349, 350).

1.5.3.2.4 IL-18 in metabolic disease

IL-18 plays an important role in innate and adaptive immunity. It was initially discovered due to its ability to stimulate IFNγ production by immune cells. Unlike pro-IL-1β, pro-IL-18 is constitutively expressed, but also requires processing by caspase-1 for activity. Surprisingly, in contrast to IL-1β, IL-18 appears to have a beneficial role in mice with respect to obesity and insulin resistance. Early work demonstrated that *Il18*$^{-/-}$ mice develop spontaneous obesity on a normal chow diet due to hyperphagia, a phenotype that could be reversed through intracerebral injection of recombinant IL-18 (351, 352). *Il18*$^{-/-}$ mice were also insulin resistant, which could be reversed by acute intraperitoneal injection of recombinant IL-18. This phenotype was shared by *Il18r*$^{-/-}$ mice and mice over-expressing IL-18-binding protein (IL-18BP). Mechanistically, the liver of IL-18-deficient mice had increased expression of the gluconeogenic enzyme PEPCK and increased STAT-3 phosphorylation.

A more recent study has expanded on these initial findings to determine that mice lacking IL-18R were not hyperphagic, despite also developing spontaneous obesity and insulin resistance (353). Instead, the authors demonstrated that *Il18r*$^{-/-}$ mice had reduced phosphorylation of ACCβ, a downstream target of AMPK. Further *in vitro* and *in vivo* studies demonstrated that exogenous IL-18 induced AMPK mediated skeletal muscle lipid oxidation to prevent DIO. Nlrp1 inflammasome induced IL-18 production from the radioresistant compartment has also been demonstrated to confer protection in DIO mice (3).

This beneficial role of IL-18 is paradoxical considering IL-18's proinflammatory effects. Serum IL-18 is elevated in DIO mice and correlates with metabolic syndrome in humans (354-356). IL-18 may also promote effector memory T cell differentiation in murine WAT through IFNγ (329). It has been hypothesized that similar to leptin, obese individuals may develop resistance to IL-

18. It is also speculated that in the metabolic syndrome, IL-18's beneficial effects may be overweighed by its inflammatory function.

1.5.4 The intestinal microbiota in obesity and insulin resistance

The composition of microbiota is heavily dependent on the diet and metabolic state of the host. Changes in abundance of the major phyla Bacteroides and Firmicutes is observed in mouse studies along with a decrease in species diversity, while conflicting results have been observed in humans (357-359). Interestingly, while there is high bacterial variation observed in humans at the phylogenetic species level, there may be common sets of genes encoded by the bacteria (360). The expression profile of the microbiome can have a profound impact on metabolic health. A landmark study suggested that specific bacterial phyla may contribute to host obesity due to an increased ability to extract energy from consumed food (357). Consequently, the transplantation of microbiota from obese mice into gnobiotic mice transferred this obesity phenotype when compared to bacteria transferred from lean mice. This effect was similarly observed when transplanting the microbiome from obese versus lean human twins into mice (361).

The intestinal flora is also capable of producing short-chain fatty acids (SCFAs) that are derived from the fermentation of complex carbohydrates. The most abundant SCFAs are butyrate, acetate, and propionate and recent work has demonstrated a beneficial effect of SCFAs on metabolic tissues by increasing insulin sensitivity and AMPK activation (362).

Modulation of the microbiome is an intriguing target for the therapeutic intervention of metabolic disease. Early studies have suggested that antibiotics or microbial transfer may have potential benefits in humans (363, 364).

1.5.4.1 PRR modulation of the microbiota and obesity

The immune system and PRRs can influence the composition of the intestinal flora and in turn impact metabolic disease (365). TLR5 recognizes bacterial flagellin and is expressed on the basal lateral surface of intestinal epithelial cells (319). It was demonstrated that littermate mice deficient in TLR5, have an altered microbiome which correlates to increased weight gain and

insulin resistance (366). Treatment of the mice with antibiotics rendered them equivalent to WT mice and importantly, transfer of the TLR5 microbiota into germ free mice conferred the phenotype to them. Nod2 deficient mice are also insulin resistant due to an altered microbiome (367).These studies represent important contributions to the field, though it remains to be seen if mutations or SNPs in PRR genes in humans contribute to obesity through the microbiota.

The effect of the inflammasome on metabolic disease may also be dependent on the microbiota as susceptibility to obesity and NASH was shown to be transferrable from inflammasome-deficient mice to WT animals through co-housing, which permits microbiota transfer by coprophagy (330). *Db/db* obese leptin receptor mutant or DIO WT mice cohoused with *Asc*$^{-/-}$ mice developed increased weight gain compared to those cohoused with WT mice. Concordantly, antibiotic treatment with ciprofloxacin and metronidazole for 3 weeks before the start of HFD reduced the obesity phenotype of *Asc*$^{-/-}$ mice.

1.5.5 Immunotherapy of metabolic disease

The rising incidence of metabolic disease around the globe has placed an increasing demand for the development of novel therapeutics. Targeting inflammatory pathways may provide a promising route for the treatment of insulin resistance and there is preliminary evidence from mouse models and clinical studies that support this inclination.

Neutralization of TNFα was proven to be effective in rodents in reducing insulin resistance (273), although clinical trials involving etanercept, a drug capable of blocking TNFα in humans, were ineffective in promoting insulin sensitivity in obese patients (368, 369). In contrast, Salsalate, a drug hypothesized to inhibit IKK activity, has been shown to improve insulin sensitivity in patients (370, 371).

Curtailing IL-1β signalling has been the goal of multiple therapeutic interventions. A groundbreaking clinical study demonstrated the successful utilization of the IL-1Ra anakinra to treat insulin resistance in a blinded, placebo-controlled, randomized clinical trial (372). Compared to placebo controls, treatment of patients with daily injections for 13 weeks reduced glycated hemoglobin levels, increased pancreatic function, and reduced inflammation.

Preclinical studies in mice have also provided preliminary evidence about the therapeutic benefits of targeting the inflammasome. *Ob/ob* mice treated with the caspase-1 inhibitor Pralnacasan had reduced weight gain and improved insulin sensitivity (4). Interestingly, multiple anti-inflammatory compounds used to treat inflammatory disorders and metabolic diseases have now been demonstrated to inhibit the inflammasome. Fish oil and polyunsaturated ω-3 fatty acids found in fish oil (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are believed to be anti-inflammatory (373). ω-3 fatty acids were shown to inhibit Nlrp3 and Nlrp1b activation signalling through the receptors GPR120 and GPR40 in macrophages, partially through β-arrestin-2 (374). *In vivo*, the treatment of DIO mice with DHA was capable of increasing insulin sensitization in an Nlrp3 dependent manner; however, the role of ω-3 fatty acids in diabetes in humans is controversial.

1.6 Inflammatory Bowel Disease (IBD)

IBD is a chronic inflammatory disorder of the GI tract comprised of two major diseases: CD and Ulcerative Colitis (UC). Despite similarities between the two, there are distinct differences, the most telling being the regions of the GI tract they affect. CD can develop throughout the GI tract, from the mouth to the anus, with affected areas interspersed with healthy tissue. In contrast UC is restricted to the colon in a continuous region. Symptoms for both can include bleeding, abdominal pain, and diarrhea.

Interestingly, incidences of IBD have increased over the past few decades, most strikingly in developed countries. While IBD etiology is unknown, it has been established that genetic determinants, environmental factors, the immune system, and microbiome are all contributing factors. Of primary importance lies the balance between tolerating the commensal flora while maintaining protection against enteric pathogens. Disruption of the anti-inflammatory pathways that regulate the immune response to pathogens can lead to IBD. A better understanding of these interactions may yield important therapeutic targets for IBD treatment.

1.6.1 The intestinal microbiota and IBD

Within a healthy individual, the host immune system is fully capable of responding to enteric pathogens while suppressing a chronic inflammatory response to the resident microbiota. An imbalance or inability to perform this task can result in IBD. The composition of the intestinal bacterial flora is dynamic and constantly in flux depending on the environmental factors and diet. Interestingly, the gut microbiota of IBD patients is altered and lacks the diversity of healthy controls (375, 376). Genetic loci associated with CD are also known to affect microbiota composition (377). Antibiotics administered at an early age to children, have also been linked as a potential contributor to disease (378). These findings suggest that the acquisition of a healthy microbiota may have therapeutic potential in IBD patients. Recent studies have investigated this possibility and demonstrated that fecal microbiota transfer can induce remission in UC patients (379).

1.6.2 The genetics of IBD

Advances in genetic sequencing technologies such as Genome-wide association studies (GWAS) have revealed ~200 susceptibility loci for IBD. Many of these loci encompass genes associated with the immune response and uncontrolled inflammation. The majority of currently identified loci are associated with both CD and UC, though some are exclusive to only one disorder (380). However, the currently identified loci are estimated to account for less than 25% of predicted heritability (381). The concordance rate for monozygotic twins in CD is ~30% compared to ~15% in UC, suggesting genetic factors have a greater impact in the former (382).

In humans, *NOD2*/*CARD15* polymorphisms are associated with the greatest risk of developing CD (252, 253). It is unknown how these inactivating mutations lead to disease, though multiple theories have been proposed. These include: reduced MDP dependent inflammation leading to defective clearance of bacteria, reduced barrier function, impaired T_H2 cell polarization, diminished AMP secretion, and dampened autophagy (36, 38, 39, 383, 384). The presence of *NOD2* mutations alone does not confer disease, indicating that environmental factors are required. In line with this theory, Nod2 deficient mice or those carrying CD associated polymorphisms do not develop spontaneous colitis (31, 385). IBD linkage studies and GWAS

have also implicated other proteins involved in the regulation of NOD signalling. Mutations in *XIAP*, an E3 Ub ligase that modifies RIPK2, are associated with CD (386). Genes encoding factors involved in autophagy, such as *ATG16L1* and *IRGM* have been both implicated in Crohn's Disease (387, 388).

Other immune pathways are also risk factors associated with IBD. IL-23 is a cytokine that induces the differentiation of immune cell subsets. GWAS studies have identified that SNPs in the gene encoding the cognate receptor, *IL-23R*, correlate with IBD risk (389, 390).

1.6.3 The immune response in IBD

The intestinal immune system plays a vital role in maintaining tolerance to food antigens and the microflora while being capable of mounting a response to eliminate pathogens. An imbalance of anti-inflammatory and inflammatory cytokines or inappropriate cellular activation by both innate and adaptive immune systems contribute to disease. Both the adaptive and innate branches are required for intestinal homeostasis and the efficient regulation of the gut microbiota within the GI tract (391).

1.6.3.1 The GI tract and barrier defences

To enhance its absorptive capabilities, the GI tract has evolved to maximize its surface area, and is covered with protruding villi and invaginations called crypts (Figure 14). However this also vastly increases the organ's exposure to the environment. Consequently it is equipped with numerous defences to segregate and restrict the luminal flora from initiating an inflammatory reaction. These mechanisms include physical barriers and a tightly regulated immune response.

The initial layer of defence separating the host cells from the flora is a mucous layer. The mucous layer is secreted by goblet cells and consists primarily of the highly glycosylated protein MUC2. The mucous concentrates AMPs and IgA antibodies at the epithelial surface to limit direct bacterial contact with the underlying host cells. The AMP RegIIIγ was similarly determined to be essential in segregating bacteria from the IECs (392). Besides providing a physical barrier, Muc2 was also reported to provide immunoregulatory signals to DCs (393). The importance of the mucous layer in humans is evident by the association of *MUC3* mutations and

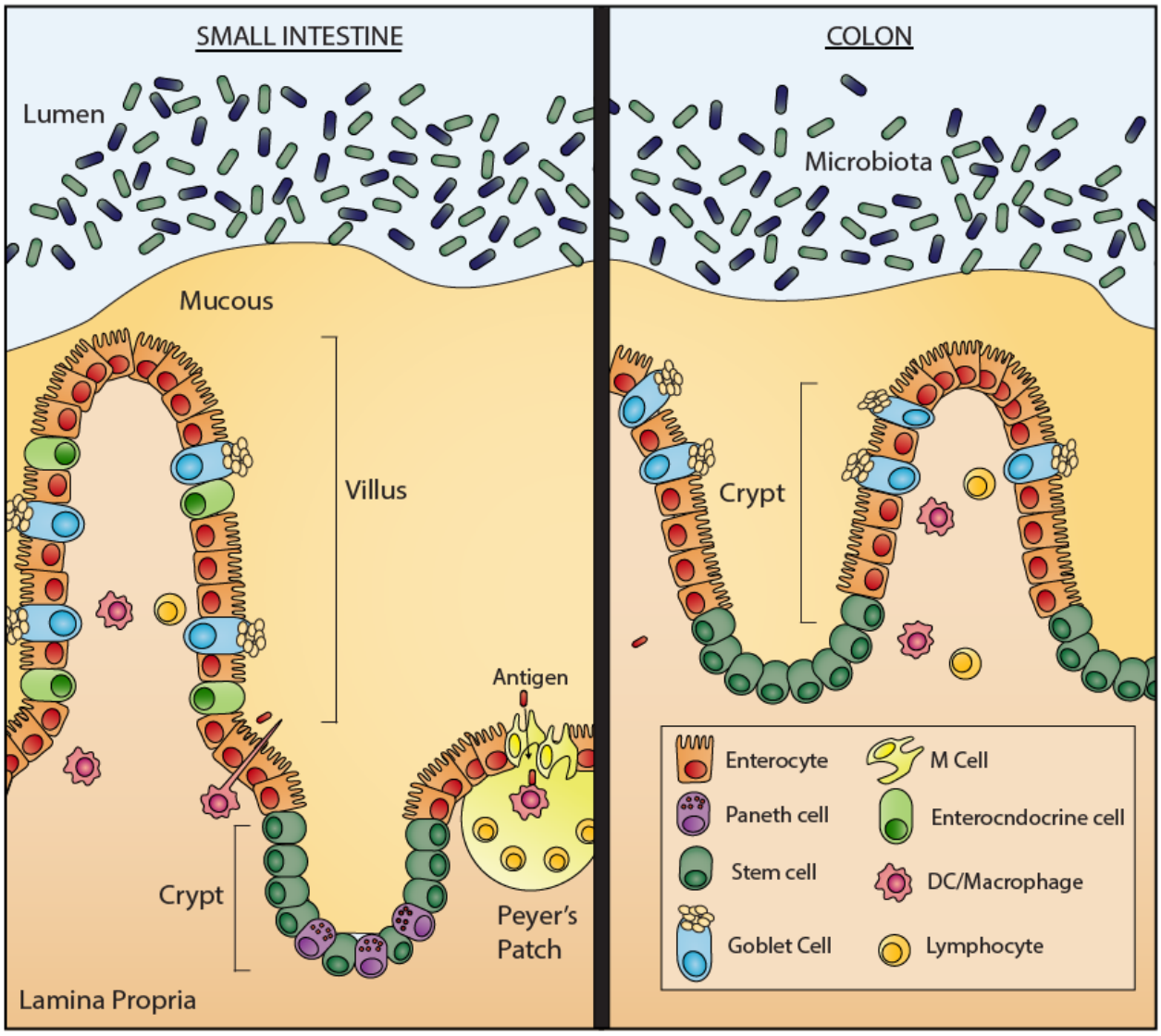

Figure 14. The GI tract

The GI tract is comprised of multiple cell types with unique functions and serves to separate the host from the external environment and luminal microbiota. The small intestine contains protruding villi and crypts, while the former are absent in the colon. This structure is designed to maximize surface area to increase absorption of nutrients and energy. At the base of the crypts lie the stem cells and progenitors that differentiate as they transit towards the tips of the villi. Paneth cells also reside within the crypts of the small intestine and produce AMPs. M cells permit the translocation of antigens across the epithelial barrier to antigen presenting cells (APCs). These APCs can prime lymphocytes within the Peyer's patches. Other cell types include mucous secreting goblet cells, enteroendocrine cells, and absorptive enterocytes. Only stem cells, enterocytes, and goblet cells are present in the colon.

IBD (394, 395). Mouse models suggest a similar significance, as animals lacking the Muc2 gene develop spontaneous colitis (396).

The epithelial layer of the intestine serves multiple physiological purposes including the absorption of nutrients, secretory functions, digestion of food, and as a barrier to separate the body from the gut microbiota (397). These functions are performed by a number of specialized IEC types including goblet cells, microfold cells (M cells), enteroendocrine cells that produce hormones, Paneth cells that secrete AMPs and enterocytes that are involved in absorption. These epithelial cells are in a constant flux of renewal and death. Proliferating pluripotent intestinal epithelial stem cells from the base of the crypts migrate to the top of the villi or crypts where they are sloughed off into the lumen and die. This ensures a healthy epithelium that is completely renewed every 4-5 days and is essential to maintain barrier integrity in the intestine (398). This barrier is maintained by tight junctions connecting adjacent IECs. Destabilization of this epithelial layer can lead to increased intestinal permeability, a phenotype associated with IBD (399).

Paneth cells reside in the small intestine at the base of the crypts of Lieberkühn. They contain granules storing AMPs and other proteins that are secreted into the intestinal environment. Paneth cell defects are associated with susceptibility to colitis in animal models and human disease. CD patients have reduced expression of α-defensin AMPs (384, 400). Interestingly, loci associated with IBD, have also been determined to impact Paneth cell function. Mice expressing a hypomorphic variant of *Atg16l1* and infected with murine norovirus have defects in Paneth cell granule morphology and increased susceptibility to dextran sodium sulfate (DSS) induced colitis (401, 402). DSS is a chemically induced acute model of colitis that damages intestinal enterocytes.

1.6.3.2 PRRs of the GI tract

PRRs are expressed in IECs and immune cell populations in the gut and are essential for immunotolerance. PRR activation in the gut epithelium needs to be carefully regulated. In addition to the previously discussed NOD2, other TLRs and NLRs are also involved. TLR expression is reduced on the apical side of IECs, limiting activation by the microflora.

Interestingly, membrane localization of the receptor can lead to different outcomes. TLR9 expression on the basolateral membrane of IECs leads to NF-κB activation while receptor engagement on the apical surface results in a tolerogenic signal (403).

PRR activation and downstream signalling is also inhibited through multiple immunosuppressive pathways that are essential for intestinal homeostasis. For example, the TLR and IL-1R inhibitor SIGIRR in epithelial cells represses inflammatory signalling, and mice deficient in SIGIRR have increased susceptibility to DSS-induced colitis (404). Despite the importance of limiting PRR signalling, PRRs also provide important survival and regulatory functions in the gut. Myd88 signalling is required for regulation of mucin production, AMP secretion, and IgA translocation (405). This signalling pathway also provides basal signals required for proper intestinal maintenance including the production of anti-apoptotic factors, cell proliferation and the stabilization of tight junctions. Consequently, mice deficient in Myd88 and therefore lacking most TLR and IL-1 receptor family signalling, are also susceptible to chemically-induced colitis (406).

1.6.3.2.1 The Inflammasome and IBD

The inflammasome and its associated PRRs have been implicated in the pathogenesis of IBD. Deficiency in the inflammasome components caspase-1, Asc or Nlrp3, confers susceptibility to mice treated with DSS (407-409). These inflammasome knockout animals lack caspase-1-dependent IL-18 processing and secretion and can be rescued by administration of the recombinant cytokine (408, 409). Despite its classification as a pro-inflammatory cytokine, in these contexts IL-18 induces proliferation and repair of the intestinal epithelium. Another laboratory has also demonstrated that *Nlrp6*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$, and *Il18*$^{-/-}$ mice have reduced tolerance to DSS, although the phenotype was dependent on microbiota dysbiosis in the mutant mice (128). Co-housing of WT mice with the affected KO strains resulted in colitis through transfer of the microbiota that correlated with the abundance of *Prevotellaceae* bacteria. Disease incidence required microbiota induction of the chemokine CCL5 that presumably attracted neutrophils to initiate colitis.

The caspase-11 non-canonical inflammasome was also found to play a role in colitis. Mice lacking caspase-11 are susceptible to DSS suggesting that its ligand, microbiota released LPS, provides protection in this context (410-412). Mechanistically, two studies reported a reduction in IL-18 in the knockout mice as a possible explanation for the phenotype, while the third observed equivalent levels of this cytokine but suggested a protective role of IEC pyroptosis in countering colitis.

1.6.3.3 Innate Lymphoid Cells

Innate lymphoid cells are a recently identified subset of immune cells found at barrier surfaces that have now been implicated in tissue homeostasis, inflammation and numerous diseases (413). They differentiate from a common lymphoid progenitor, have a lymphoid morphology, but lack a B or T cell antigen receptor. There are currently 3 ILC subsets that parallel helper T cells based on their secreted cytokines and induced transcription factors (Figure 15) (414). ILC1s express T-bet and secrete IFNγ, ILC2s express GATA-3 and secrete IL-5, and IL-13, and ILC3s express RORγt and secrete IL-17A and IL-22.

Group 2 ILCs have been demonstrated to play a protective role in the gut during helminth infection (415). ILC3s have specifically been linked to protective functions in the gut due to the production of the repair cytokine IL-22 (416, 417).

1.6.3.4 Adaptive immunity in IBD

The adaptive immune system also plays a role in the pathogenesis of IBD. T and B cells can adapt and respond to numerous stimuli by differentiating into multiple phenotypes upon activation by antigen presenting cells (APCs) and extracellular signals in the local environment and extracellular signals. Intestinal DCs are APCs that reside in the lamina propria underlying the epithelium, or in lymphoid tissue such as Peyer's patches. Specialized epithelial cells called M cells permit the of transport luminal antigens to the intestinal immune system. These antigens are delivered to DCs within the subepithelial dome of the Peyer's patches where they can then engage $CD4^+$ T cells. In addition to M cell mediated antigen acquisition, $CD11c^+CX_3CR1^+$ macrophages act as sentinels, sampling the lumen for antigens using transepithelial dendrites (418). They then transfer the antigen to migratory CD103+ DCs that move to the Peyer's patches

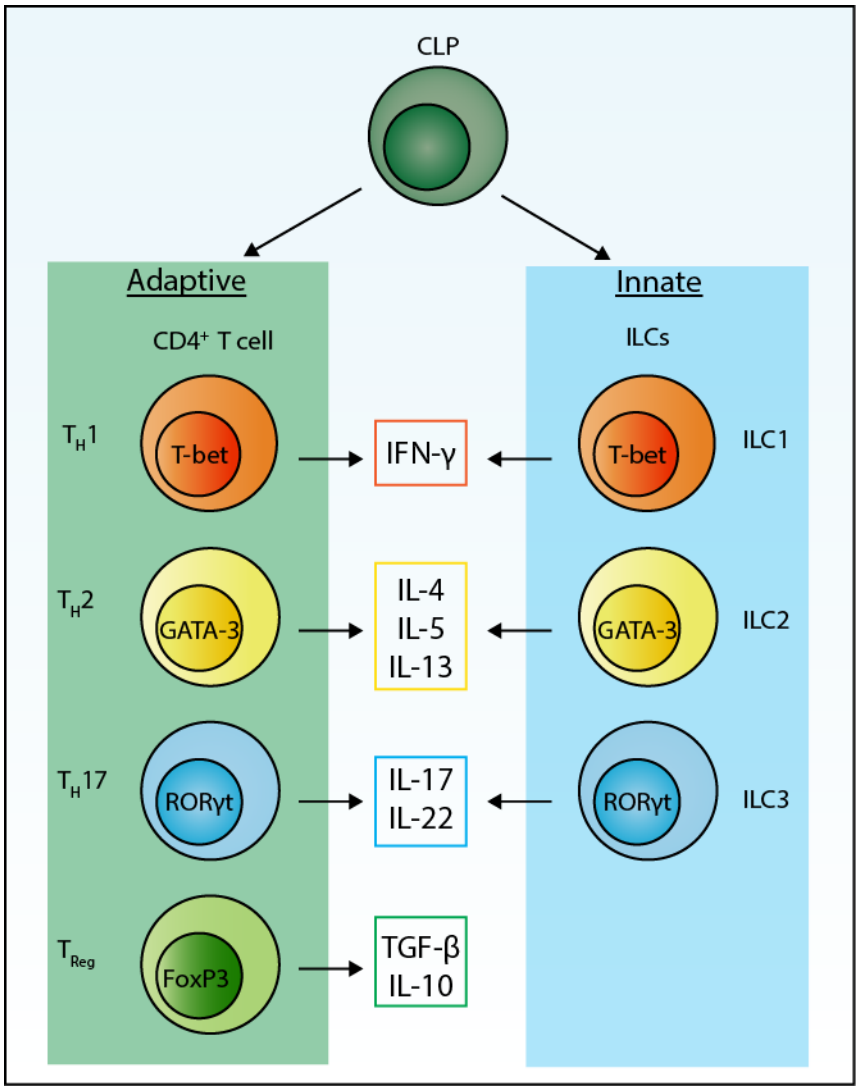

Figure 15. CD4$^+$ T cell and Innate Lymphoid Cell (ILC) differentiation
CD4+ T cells and ILCs both differentiate from a common lymphoid progenitor (CLP) though function as part of the adaptive or innate immune systems respectively. Exposure to polarizing cytokines activates specific transcription factors and induces differentiation into cell types with specialized functions. ILC1s and T_H1 cells express T-bet and produce IFNγ. GATA-3 induces T_H2 and ILC2 differentiation and the secretion of IL4, IL-5, and IL-13. The RORγt transcription factor induces IL-17 and IL-22 producing T_H17 and ILC3 cells. Finally, CD4$^+$ T cells can also develop into tolerogenic regulatory T cells (T_{Regs}) that secrete TGF-β and IL-10.

or mesynteric lymph nodes (419). Under basal conditions, DCs are polarized to a tolerogenic phenotype driven by IEC mediated secretion of TSLP, TGFβ, and retinoic acid (420). Naive CD4+T helper cells (T_H0) can be polarized to a T_H1 phenotype by IL-12, characterized by IFNγ, TNFα, and IL-12 secretion and upregulation of the transcription factor T-bet (Figure 15). T_H2 cells are induced by IL-4 and characterized by IL-4, IL-5 and IL-13 secretion and GATA-3 expression. T_H1 cells are important in the eradication of intracellular pathogens while T_H2 cells restrict parasitic growth. In IBD, initial studies suggested that T_H1 T cells were thought to drive CD while T_H2 polarization was implicated in UC (421). Cells from CD patients were determined to secrete high levels of IFNγ (422), while cells from UC patients secrete enhanced T_H2 cytokines (423). The discovery of T_H17 and T_{Reg} cells reveal that this T_H1/T_H2 paradigm is more complex than initially thought.

1.6.3.4.1 Regulatory T cells

$FoxP3^+$ T_{Regs} and $FoxP3^-$ Tr1 IL-10 secreting cells have a vital role in suppressing inappropriate immune responses and are required for intestinal homeostasis. Under basal conditions and in the absence of inflammatory signals, presentation of antigen to T cells leads to an anergic phenotype or differentiation into T_{Regs} (424). The secretion of TGFβ and retinoic acid by DCs leads to a regulatory phenotype (425). The importance of this population is evident in murine adaptive T-cell transfer models, where the colitis inducing transfer of $CD4^+$ $CD45RB^{Hi}$ naive T cells to a host deficient in lymphocytes, can be rescued by the co-transfer of T_{Reg} progenitors (426-428).

1.6.3.4.2 T_H17 Cells

T_H17 cells were discovered due to their role in experimental autoimmune encephalomyelitis and collagen-induced arthritis murine models. In addition to autoimmune diseases, this T cell subset is also required for resistance to bacterial, viral, and fungal pathogens (429). T_H17 cells have been implicated in human IBD as IL-17 is elevated in the serum and colonic tissue (430). Interestingly however, monoclonal antibodies targeting IL-17 are ineffective in treating CD and in one case increased disease severity (431, 432). Neutralization of IL-17 in mice produced similar results, suggesting that this cytokine may have beneficial properties (433).

T_H17 differentiation is induced by IL-23, a heterodimer cytokine composed of the IL-23p19 subunit and the p40 subunit that is shared with IL-12. Antibodies targeting the p40 subunit have been found to offer protection in mouse models of colitis, effects originally attributed to IL-12 (434). However, it is now known that IL-23 is responsible for disease pathogenesis (435). Targeting IL-23 may also have therapeutic potential as the use of Ustekinumab, a human monoclonal antibody against IL-12 and IL-23, resulted in increased rates of clinical remission (436). It was originally hypothesized that these results were due to downstream effects of IL-23 on T_H17 differentiation. However, closer analysis has identified distinct roles for the IL-23 and IL-17 with respect to IBD. Neutralization of IL-17 leads to exacerbated disease in animal models while IL-17 deficient mice are protected from DSS-induced colitis (433, 437). Mechanistically, IL-17 was determined to confer protection by reducing gut permeability and increasing IEC barrier function (438, 439) and may also inhibit CD4+ T_H1 cell differentiation and activation (440).

In addition to T_H17 cells, IL-17A is produced by numerous cell types, including γδT cells, NKT cells, and ILC3 cells. IL-17 signals through an IL17RA and IL-17RC heterodimer receptor that is found on numerous immune and non-immune cell types.

Interestingly, T_H cell differentiation is not terminal and instead exhibits plasticity. This can result in heterogeneous subsets that express markers of multiple lineages. A transition of T_H17 cells to T_H1-like cells expressing the T_H1 signature cytokine IFNγ is seen in the colon of IBD patients and contributes to colitis pathogenesis in mice (441, 442). Intestinal T_H17 cells have also been reported to differentiate into Tr1-like IL-10 expressing cells as determined by murine fate mapping studies (443). This T_H cell plasticity hints at the possibility that pharmacological conversion of T cells may be beneficial in restoring intestinal tolerance in IBD patients.

1.6.3.4.3 IgA antibodies

An important arm of the adaptive immune response is the production of immunoglobulin by plasma cells. IgA is the dominant isotype expressed at mucosal surfaces including the gut (444). Naïve B cells are activated by CD4+ T cells in the Peyer's patches or mesenteric lymph nodes to generate IgA secreting plasma cells. Plasma cells within the GALT and lamina propria secrete

IgA that is transcytosed from the basolateral surface of the IECs to the lumen (445). These antibodies can bind to commensal and pathogenic microbes to restrict motility and adherence or invasion on epithelial cells. Humoral immunity has been linked to IBD, however it is unclear how antibodies against host or bacterial antigens affect pathogenesis. It has been observed that elevated titers of antibacterial antibodies correlate with CD severity (446). Antibodies targeting the host protein tropomyosin have been detected in the colonic mucosa of UC but not CD patients (447), although the significance of this finding is unknown.

1.6.3.6 Cytokines and intestinal inflammation

The GI tract of IBD patients is aberrantly inflamed due to inappropriate activation of the immune system. A major contributor is the imbalance between pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines such as IL-1β and TNFα are elevated in the disease state (Figure 16) (448). Consequently, these cytokines may provide a suitable therapeutic target. Monoclonal antibodies generated against TNFα induce remission in a proportion of IBD patients (254, 449, 450).

Anti-inflammatory cytokines are imperative to dull the immune response and permit tolerance. TGFβ, a cytokine with anti-inflammatory properties, plays a vital role in suppressing inflammatory T cell function in mouse models of colitis (451, 452). Interestingly, TGFβ is upregulated in IBD, however its immunosuppressive functions are muted due to the upregulation of Smad7, which inhibits downstream receptor signalling mediated by Smad2, Smad3, and Smad4 (453). An early phase II clinical trial indicates that targeting Smad7 may have therapeutic potential (454).

IL-10 is an anti-inflammatory cytokine secreted by various subsets of leukocytes (455). It signals through the IL-10Rα and IL-10Rβ heterodimer receptor to initiate STAT3 activation through Jak1 and Tyk2. The regulatory properties of IL-10 are protective for colitis as evident in animal models. IL-10 deficient mice develop spontaneous colitis that is dependent on the microbiota (456, 457). Within the GI tract, IL-10 is produced by multiple immune cell types, including T_{Reg} cells, Tr1 cells, macrophages and B cells (458, 459). The IL-10 receptor is expressed on various immune cell populations and plays an important regulatory function in $CD4^+$ T cells. In murine

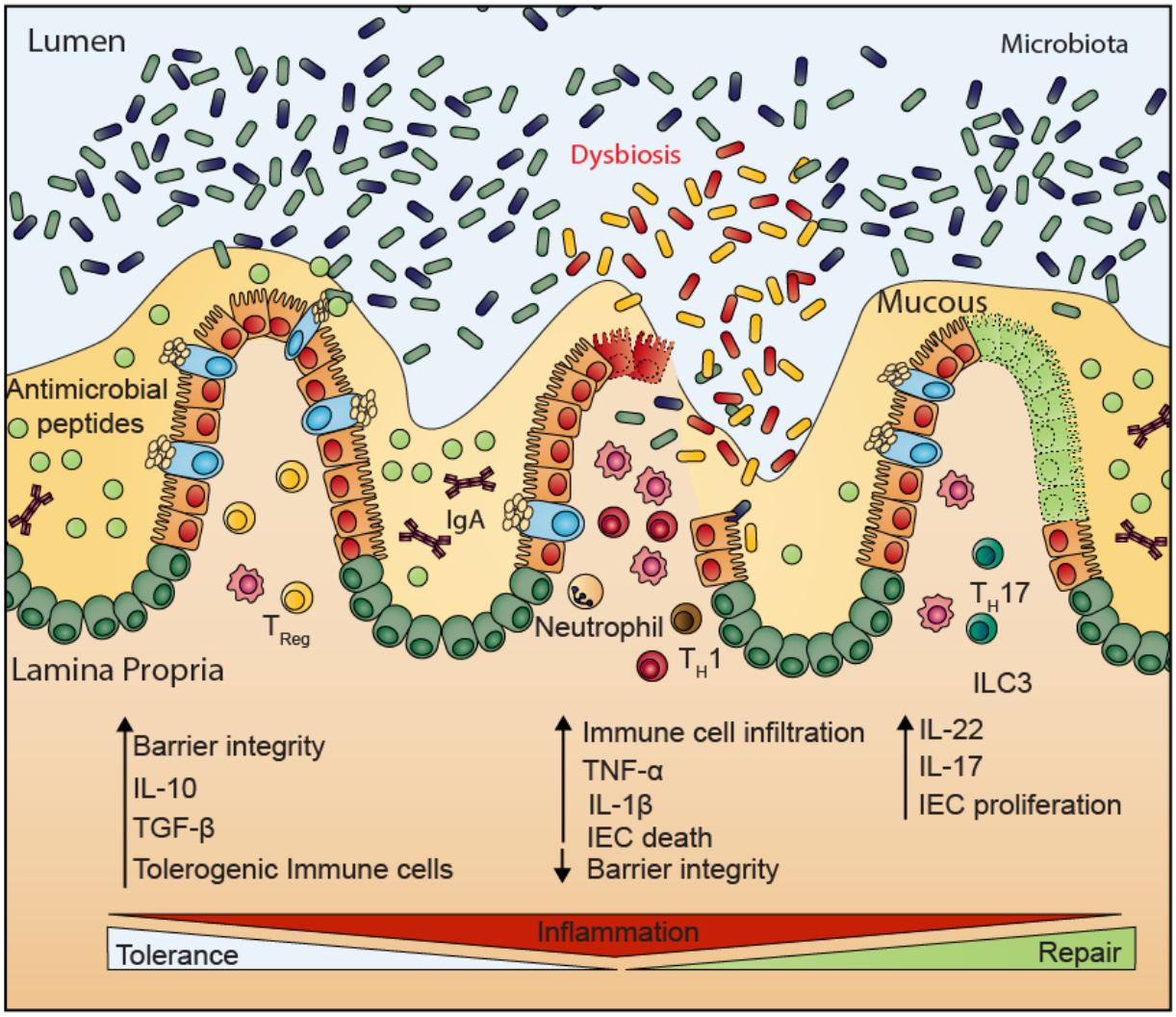

Figure 16. IBD development and remission
A healthy intestine requires a state of tolerance between host cells and the microbiota within the GI tract. This is promoted by intestinal barrier integrity, the differentiation of tolerogenic immune cells, such as regulatory T cells, and the secretion of anti-inflammatory cytokines, such as IL-10 and TGFβ. Microbial dysbiosis, a breakdown in barrier integrity, or IEC death can lead to chronic inflammation. The infiltration and activation of immune cells by microbial motifs will induce a pro-inflammatory polarization and the secretion of inflammatory cytokines, such as IL-1β and TNF-α. Remission and repair of inflammatory colitis is initiated by restoration of the intestinal barrier. IL-22 secreted by Type 3 ILC cells induces IEC proliferation while IL-17 is linked to barrier integrity.

colitis models, IL-10 signalling is required to repress T_H17 effector T cell activity and boost T_{Reg} function (460, 461). The IL-10 signalling pathway is also involved in human disease, as mutations in the *IL-10* or *IL-10R* genes have been found in IBD (462, 463).

Repair of the intestinal epithelium after insult or chronic injury is stimulated by multiple signals (Figure 16). Interestingly, this process can be mediated by cytokines that also have a pro-inflammatory function in other contexts. IL-22 is a member of the IL-10 family of cytokines that is produced by numerous cell types including ILC3 (464-468), neutrophils (469), $CD4^+$ T_H17 and T_H22 cells(470, 471), and NKT cells (472). Essential to IL-22 expression is the transcription factor RORγt in ILC3 and aryl hydrocarbon receptor (AhR) and T-bet in $CD4^+$ T cells (471, 473). The IL-22 receptor is comprised of a heterodimer of IL-22RA1 and IL-10R2 (474-476). IL-22RA1 expression is confined primarily to cells of epithelial origin and is not found on immune cells (477). Cytokine binding initiates Jak1 and Tyk2 signalling, leading primarily to STAT3 phosphorylation and activation (478). STAT3 activation is crucial for IL-22-induced intestinal tissue repair (479). IL-22 also induces AMP production including hBD2 and hBD3.

IL-22RA1 expression is confined primarily to cells of epithelial origin and is not found on immune cells (477). Cytokine binding initiates Jak1 and Tyk2 signalling, leading primarily to STAT3 phosphorylation and activation (478). STAT3 activation is crucial for IL-22-induced intestinal tissue repair (479). IL-22 also induces AMP production including hBD2 and hBD3 (477), and RegIIIβ and RegIIIγ (480). Similarly to IL-17, IL-22 production is induced primarily by IL-23, which is secreted by DCs, macrophages, and epithelial cells (480-482). Administration of IL-22 confers protection in murine models of colitis (483). IL-22 is also required for barrier defence against enteric pathogens such as *Clostridium difficile* and *Citrobacter rodentium* (480, 484). In humans, IL-22 positive cells are reduced in inflamed colonic tissue of UC patients but elevated in CD (485).

PREFACE TO CHAPTER 2

Inflammation is a crucial component of obesity driven insulin resistance. Recent studies have implicated the inflammasome as an important contributor to the development of metabolic disease through the use of mouse models and clinical experiments. Caspase-12 was previously identified by our lab as an inhibitor of caspase-1 activity, as well as a repressor of other inflammatory pathways. Mice deficient in caspase-12 are resistant to sepsis and infection by microbial pathogens. To determine if caspase-12 also plays a role in metabolic disease we investigated its effects through the use of mouse models and the analysis of patient data.

CHAPTER 2

Caspase-12, but not caspase-11, inhibits obesity and insulin resistance

2.1 ABSTRACT

Inflammation is well established to significantly impact metabolic diseases. The inflammatory protease caspase-1 has been implicated in metabolic dysfunction, however a potential role for the related inflammatory caspases is currently unknown. In this study, we investigated a role for caspase-11 and caspase-12 in obesity and insulin resistance. Loss of caspase-12 in two independently generated mouse strains predisposed mice to develop obesity, metabolic inflammation and insulin resistance, while loss of caspase-11 had no effect. The use of bone marrow chimeras determined that deletion of caspase-12 in the radio-resistant compartment was responsible for this metabolic phenotype. The Nlrp3 inflammasome pathway mediated the metabolic syndrome of caspase-12-deficient mice as ablation of *Nlrp3* reversed *Casp12*$^{-/-}$ mice obesity phenotype. While the majority of people lack a functional caspase-12 due to a T^{125} single nucleotide polymorphism (SNP) that introduces a premature stop codon, a fraction of African descendents express full-length caspase-12. Expression of caspase-12 was linked to decreased systemic and adipose tissue inflammation in a cohort of African-American obese children. However, analysis of the Dallas Heart Study African American cohort indicated that the coding $T^{125}C$ SNP was not associated with metabolic parameters in humans, suggesting that host specific differences mediate the expressivity of metabolic disease.

2.2 INTRODUCTION

Metabolic diseases such as Type 2 Diabetes (T2D) and the metabolic syndrome have quickly become a major global health concern. The increasing prevalence of obesity has led to the development of these diseases in adults as well as children. While lifestyle is a large contributor to this problem, the underlying genetic factors are less well understood, and their elucidation may thus aid in the identification of therapeutic targets. A rapidly expanding number of studies have implicated the immune system in playing a pivotal role in the development of metabolic diseases.

Obesity results from an energy imbalance, caused by excess caloric intake that exceeds metabolic requirements. This energy surplus is stored as lipids, leading to overexpansion of the adipose tissue, and consequently local inflammation, possibly initiated by hypoxia, ER stress, and/or pattern recognition receptor (PRR) detection of endogenous metabolic 'danger' signals (486). The onset of obesity correlates with chronic inflammation of metabolic tissues, characterized by high pro-inflammatory cytokine secretion and elevated levels of infiltrating immune cells including macrophages, neutrophils, T cells, and B cells (486). Macrophages in particular may constitute up to 40% of the cells found in the adipose tissue of obese patients (292). Genetic studies or antibody depletion of immune cells or chemokines have implicated numerous inflammatory mediators in metabolic disease. Inflammation can lead to insulin resistance by acting directly on insulin responsive cells, partially through the action of the inflammatory kinases JNK and IKK that phosphorylate and inhibit Insulin Receptor Substrate-1 (IRS-1) (314, 347).

The inflammatory caspases are a family of cysteine proteases, comprised of caspases-1, -4, -5 and -12 in humans and -1, -11 and 12 in mice. Caspase-1, along with a PRR and the adaptor molecule ASC, is capable of forming a cytosolic multi-protein complex termed the inflammasome. Detection of exogenous or endogenous danger signals by the PRR promotes the activation of caspase-1, which triggers an inflammatory response that is primarily characterized by IL-1β and IL-18 secretion (487). Mounting evidence suggests a critical role for the Nlrp3 inflammasome as a major regulator of inflammation in metabolic diseases. In response to several

metabolic danger signals, including saturated fatty acids, ceramide, Islet Amyloid *Polypeptide (IAPP)* and hyperglycemia (68, 329, 337), Nlrp3 assembles an inflammasome, which activates the pro-inflammatory protease caspase-1. Implementation of the diet-induced obesity (DIO) experimental model to mice deficient in various inflammasome components, such as *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, or *Ice*$^{-/-}$ mice (deficient in caspase-1 and carrying a null mutation in caspase-11 (184, 488)), has demonstrated that inflammasome signalling alters mouse susceptibility to high fat diet (HFD)-induced insulin resistance. Whereas many studies have demonstrated that caspase-1-dependent IL-1β production is the triggering pathological mechanism in insulin resistance, it is debated whether inflammasome signalling regulates obesity *per se* and how. For instance, while some reported that the weight of *Nlrp3*$^{-/-}$ mice was comparable to that of WT controls in the DIO model (68, 329), others described *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Ice*$^{-/-}$ mice leaner in phenotype (4, 327), possibly mediated by decreased intestinal lipid absorption (489) or increased lipid oxidation (4).

Furthermore, it was shown that the inflammasome pathway might affect obesity indirectly through effects on the host microbiota (330). While the inflammasome is primarily studied in cells of hematopoietic origin, it may play a role in both immune cells and stromal cells in the context of metabolic disease. The radio-sensitive compartment was determined to be important for the insulin sensitivity observed in *Asc*$^{-/-}$ mice (68). However, bone marrow chimera experiments have also indicated that caspase-1 is active in radio-resistant cells, where it mediates host lipid metabolism (2). The precise roles of the inflammasome and its regulation of obesity and metabolic disease have not yet been fully determined.

Unlike Caspase-1, little is known about how the remaining inflammatory caspases affect metabolic disease. Caspase-11 has recently been established to recognize intracellular LPS and Gram-negative bacterial pathogens, leading to activation of a non-canonical inflammasome (184). A role of caspase-12, as an immunomodulatory factor, has been primarily studied in the context of infections and exposure to microbial ligands (189, 191-193), however little is known regarding its function in sterile inflammation, such as that elicited in obesity. While Caspase-1 can cleave a variety of substrates (169), the only known substrate of rodent Caspase-12 is itself (190), though this proteolytic activity was demonstrated to be unnecessary for its regulation of innate immune pathways (189). Human Caspase-12 likely lacks this enzymatic activity, due to a SHG (Ser-His-Gly motif) to SHS (Ser-His-Ser) mutation in the catalytic domain (490).

Interestingly, a premature stop codon in human Caspase-12 prevents its expression in the majority of the population with the resulting truncated transcript degraded due to nonsense-mediated mRNA decay (197). However, a single nucleotide polymorphism (SNP) in the human *CASP12* gene at amino acid position 125 permits expression of the full length protein in a fraction of African descendants (196). It is currently unknown what evolutionary pressures have resulted in the loss of a functional caspase-12 allele from the majority of the human population or its continued maintenance.

Given the importance of the contribution of innate immunity to metabolic diseases (486), we investigated the role of caspase-11 and caspase-12 in obesity and insulin resistance in mice and humans. In this study we demonstrate that mice ablated for caspase-12 develop spontaneous obesity and insulin resistance. This is dependent on the Nlrp3 inflammasome, though interestingly independent of the radio-sensitive compartment. Analysis of the effect of the *CASP12* $T^{125}C$ SNP in the Dallas Heart Study African-American cohort (491) however, suggested that a functional caspase-12 allele does not correlate with improved metabolic parameters in humans, although in a small cohort of African American obese children, expression of caspase-12 was associated with dampened inflammatory markers.

2.3 MATERIALS and METHODS

Animal experiments

Mice were housed at room temperature with a 12 hour light/dark cycle with food and water provided ad libitum. The mice were fed either a standard chow diet (LFD) (2020x Teklad Rodent Diet; 16% calories from fat; 3.1 kcal/g) or a HFD (Research Diet D12451; 45% kcal from fat; 4.73 kcal/g). HFD feeding was initiated in mice at 6 weeks of age.

Mouse strains

Casp11$^{-/-}$, *Casp12*$^{-/-(129)}$, *Ice*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Ripk2*$^{-/-}$ mice have been previously described (90, 184, 189, 488, 492). *Casp12*$^{-/-(129)}$ are from Deltagen though are not direct descendants of the Merck *Casp12*$^{-/-(129)}$ mice used in reference 18. *Casp12*$^{-/-}$*Ripk2*$^{-/-}$ and *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ were generated for this study. *Casp12*$^{-/-(B6)}$ (Casp12$^{tm1a(KOMP)Wtsi}$) mice were generated on a B6 background by the Wellcome Trust Sanger Institute. All experiments were performed under guidelines of the animal ethics committee of McGill University (Canada).

GTT and ITT

Age matched male mice were fasted for 6h before i.p. injection with 2g/kg dextrose (LFD and HFD) or human recombinant insulin (Humulin, Eli Lilly) 0.75 mU/g (LFD) or 2.0 mU/g (HFD). Blood glucose levels were measured from the tail vein using a Onetouch ultra 2 glucometer.

Western Blots

Tissues were lysed in buffer B150 (20 mM Tris-HCl pH 8.0, 150 mM KCl, 10% glycerol, 5 mM MgCl2, and 0.1% Nonidet P-40) supplemented with Complete-mini protease inhibitors (Roche Applied Science, Cat# 11836153001) and phosphatase inhibitors (Sigma Cat# S7920, 71768, G6376). Protein lysates were separated on SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with antibodies against Caspase-1 p20 (Genentech), Caspase-12 (Sigma, Cat# C7611), Caspase-11 (Sigma, Cat# C1354), β-actin (Sigma, Cat# A1978), β -tubulin (sc-9104), AKT (Cell signalling #4691), and P473 AKT (Cell signalling #4060). Densitometry was performed using ImageJ (NIH).

ELISAs and serum analysis

Cytokines were determined using the following ELISA kits: IL-6 (R&D, Cat# DY406), IL-18 (MBL International, Cat# 1625), KC (R&D, Cat#DY453), MCP-1 (R&D, Cat#DY479). Serum ALT and cholesterol were determined by a Vitros 250/350 machine.

Dual-energy X-ray absorptiometry Scan

Fat and lean mass were calculated using a GE Lunar PIXImus machine.

Hepatic Triglyceride Analysis

Hepatic lipids were extracted using a modified Bligh-Dyer extraction protocol. Approximately 200 mg of liver tissue was homogenized in a 1/2.5/1.25 (vol/vol) mixture of 0.5M acetic acid/methanol/chloroform. The mixture was shaken and 1.25 volumes of chloroform were added. After overnight shaking, 1.25 volumes of 0.5M acetic acid were added and the samples were spun at 1,500 x g. The organic phase was collected, dried, and resuspended in Isopropanol. Triglycerides were determined using a Serum Triglyceride Determination kit (Sigma TR0100) and normalized to liver weight.

Bone marrow Chimera

Age matched male mice were irradiated and reconstituted with bone marrow cells from donor mice. Genotype was verified by PCR analysis of blood. Mice were placed on antibiotics (Trimethoprim 0.2 g/L, Sulfamethoxazole 1 g/L), beginning 3 days prior to lethal irradiation, for 3 weeks. Mice were allowed to recover for 8 weeks before being fed a HFD.

Flow Cytometry

Epididymal adipose tissue was excised from 26w old HFD mice, minced, and incubated in 1mg/ml Type 2 Collagenase (Sigma) for 1 hour at 37°C. Folllowing RBC lysis, the stromal vascular fraction cells were counted and stained. Data were acquired on a Canto instrument (BD Biosciences) equipped for the detection of 8 fluorescent parameters. The following antibodies were used for flow cytometry analysis: anti-CD3-PerCPCy5.5 (145-2C11), anti-CD11b eFluor450 (M1/70), anti-B220-APC (RA3-6B2), anti-CD45-PECy7 (30-F11) (all from eBioscience); anti-CD19-PECy7 (1D3), anti-GR1-APCCy7 (RB6-8C5), (all from BD Biosciences); anti-F4/80-PerCPCy5.5 (BM8.1) (Tonbo Biosciences).

Indirect Calorimetry

Mice were placed in Oxymax-CLAMS system (Columbus Instruments) metabolic cages housed with a 12 hour light/dark cycle and free access to water and food. Animals were allowed to acclimatize for 48 hours before readings were taken for a 24 hour period.

Immunohistochemistry

Subcutaneous biopsies from 15 subjects were used for immunohistochemical staining and CD68 was used as a marker for macrophages. Staining was performed using a standard protocol on sections from formalin-fixed paraffin-embedded tissue blocks. Serial sections were deparaffinized, rehydrated and treated with 10 mM citrate buffer (pH 6.0) in a steamer and then endogeneous peroxidase was blocked with 3% H2O2. The sections were then incubated for 1 h at room temperature with primary antibodies, mouse monoclonal anti-CD68 (Ab-3 clone KP1, Thermo Fisher Scientific, Fremont, CA). After rinsing in TBS buffer containing 0.25% Triton X-100 (pH 7.2), sections were incubated with ENVISION+ (K4007 or K4011, DAKO, Carpinteria, CA) followed by visualization with 3.3'-diaminobenzidine tetrachloride (DAKO, Carpinteria, CA). All sections were counterstained with GILL III Hematoxylin, dehydrated and coverslipped with a resinous mounting media. For each subject, the number of macrophages (identified as CD68+ cells) within 10 regions of interest (ROI) were counted by two independent observers using a light microscope and normalized to the number of counted adipocytes.

Quantitative real-time PCR of human samples

Total RNA was isolated using TRIzol reagent and was further purified using an RNeasy kit (Qiagen, Valencia, CA). The quantification of IL-6 and TNF-a by real-time RT-PCR was performed using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The nucleotide sequences of the primers and PCR conditions can be provided upon request. For each run, samples were run in duplicates for both the gene of interest and 18S. Quantitative analysis was determined by $\Delta/\Delta CT$ method normalized to both a control and 18S message.

Yale Pediatric Cohort

The Yale Pediatric Obesity cohort is a multiethnic cohort of obese children and adolescents carefully phenotyped in regard to glucose and lipids metabolism, liver function as well as to fat partitioning. As of today the cohort consists of 1109 obese children and adolescents from New Haven area (New Haven, CT) recruited through the Yale Pediatric Obesity Clinic. For the purpose of this study we genotyped 256 obese African American children and adolescents with a mean age of 14.1+/-3.7 and a mean z-score BMI of 2.33+/-0.52 who underwent an oral glucose tolerance test, and the measure of plasma CRP and IL-6 as described before (493), a subgroup of 15 of them (mean age 14.8+/- 3.4 and mean z-score BMI 2.0 +/-0.4) underwent a subcutaneous fat biopsy (494). All the patients were genotyped for the $T^{125}C$ variant by automatic sequencing using the following primers 5'-ATATAATTCCTATAATATCATAC-3' and 5'-GTCTAAACTCTCCACCACCT-3' (TA 55 ºC).

Dallas Heart Study Analysis

Dallas Heart Study (DHS) is a longitudinal, multiethnic population-based probability sample of Dallas County residents. African Americans were oversampled to comprise approximately 50% of the population. Details of the study design and recruitment procedures have been previously described (491). The study was approved by the institutional review board of the University of Texas Southwestern Medical Center, and all participants provided written informed consent. The present investigation includes all African American participants of the DHS who provided fasting blood samples during the initial enrollment (2000-2002) or the follow-up examination (2007-2009) (n=2,360). During each examination, participants completed a detailed staff-administered survey, which included questions about demographics, socioeconomic status, medical history, and current medication use, and underwent a health evaluation that involved measurement of blood pressure, anthropometry, blood and urine sample collection, and imaging studies. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Blood lipid and lipoprotein levels were measured using standard enzymatic methods. Insulin resistance was quantified from fasting blood glucose and insulin levels using homeostatic model assessment (HOMA-IR). Diabetes was defined as a self-reported physician diagnosis of diabetes, use of glucose-lowering medication, or fasting glucose ≥ 126 mg/dL. Hepatic triglyceride content was measured using ^{1}H-MRS in a subset of 1,106 African

Americans participants, who completed the initial clinic visit (495). Genotyping was performed using Illumina Infinium Human Exome-12 v1_A BeadChip. Genotype calling and quality control have been previously described (496). *CASP12* genotypes were in Hardy-Weinberg equilibrium ($p = 0.80$). The association between *CASP12* $T^{125}C$ genotype and clinical phenotypes was tested using linear regression adjusted for age, gender, BMI and type-2 diabetes mellitus, where necessary. We applied a logarithm transformation to BMI, HOMA-IR, and triglycerides (TG) and a power transformation to hepatic TG content prior to analysis to achieve approximate normality of the residuals. Diabetic individuals were excluded from selected analyses as indicated.

2.4 RESULTS

Caspase-12 deficient mice develop obesity and insulin resistance on a HFD

Following the extensive evidence linking innate immunity and the inflammasome-caspase-1 pathway to metabolic disease, we sought to investigate a possible role of the related inflammatory caspases-12 and -11 in metabolic regulation. We examined two independent *Casp12*$^{-/-}$ mutant mouse lines, the first generated with 129 embryonic stem (ES) cells then backcrossed to a B6 background (referred to as *Casp12*$^{-/-(129)}$ mice) and the second generated using B6 ES cells (referred to *Casp12*$^{-/-(B6)}$). Kayagaki *et al.* recently identified that the 129 strain of mice harbored a null mutation in the *Casp11* gene (184), raising the possibility that knockout mice of genes neighboring *Casp11* generated with 129 ES cells might also be deficient in caspase-11. Indeed, *Casp1*$^{-/-}$ mice (herein referred to as *Ice*$^{-/-}$ mice) also carry the 129S-derived *Casp11* null mutation (184). Genotyping for the passenger mutation in *Casp11* indicated that the Casp12$^{-/-(129)}$ mice in our facility are also deficient in caspase-11, while caspase-11 is expressed in *Casp12*$^{-/-(B6)}$ mice (Figure S1A). Interestingly, when placed on a HFD (45% kcal fat) both *Casp12*$^{-/-(B6)}$ and *Casp12*$^{-/-(129)}$ strains both became obese compared to WT mice (Figure 1A). *Casp11*$^{-/-}$ and *Ice*$^{-/-}$ mice were similar to controls, suggesting that loss of caspase-11 had little effect on the weight gain of the *Casp12*$^{-/-(129)}$ mice. After 16 weeks of HFD, caspase-12 deficient strains had increased inguinal (iFAT), mesenteric (mFAT), and perirenal (pFAT) adipose depot weight compared to WT mice (Figure 1B). *Ice*$^{-/-}$ mice had increased epididymal (eFAT) fat pad weight in relation to all other strains, however in our studies, eFAT weight from DIO mice did not directly correlate with total body weight or the weight of other fat pads (data not shown). *Casp11*$^{-/-}$ mice were similar to controls. Liver weight was increased in *Casp12*$^{-/-(129)}$ mice, which correlated with their overall heavier bodyweight, while reduced in *Ice*$^{-/-}$ mice compared to WT controls. Histological analysis of the eFAT revealed no differences in adipocyte hypertrophy (Figure 1C), while the liver of caspase-12 deficient mice had increased lipid droplet formation (Figure 1C). *Casp12*$^{-/-(129)}$ mice also had increased serum cholesterol compared to WT mice (Figure 1D) and both caspase-12-deficient strains exhibited increased liver triglycerides (Figure 1E). Interestingly all strains tested had slightly elevated alanine transaminase (ALT) serum levels (Figure 1F), indicative of liver damage, with the highest levels detected in *Casp12*$^{-/-(129)}$ mice,

which correlated with increased liver weight (Figure 1B, C). Overall these results suggest that caspase-12 plays a role in inhibiting obesity in DIO mice.

Casp12$^{-/-(129)}$ mice develop spontaneous obesity and insulin resistance even on low fat diet

To further characterize the role of caspase-12 in obesity, we assessed the metabolic phenotype of *Casp12*$^{-/-(129)}$ mice fed a normal chow diet (LFD). Similarly to the HFD phenotype, loss of caspase-12 also led to obesity on LFD, however this response was confined to male but not female mice (Figure 2A, B and S1B, C). The difference in body weight gain between LFD fed *Casp12*$^{-/-(129)}$ mice and WT controls was evident starting at 12 weeks of age (Figure 2A, B). The increased weight gain in *Casp12*$^{-/-(129)}$ mice corresponded to increased adiposity, as determined by dual-energy X-ray absorptiometry scan (Figure S1D), increased adipose depot weight of the inguinal (iFAT), epididymal (eFAT), and mesenteric (mFAT) fat pads (Figure 2C), and increased eFAT adipocyte hypertrophy in LFD fed mice (Figure 2D, F, G). Although differences in liver weight and liver injury, as determined by serum ALT levels, were only apparent on HFD (Figure 2C, H), both diets resulted in increased hepatic lipid deposition and triglycerides in *Casp12*$^{-/-(129)}$ mice compared to WT controls (Figure 2E, I). Additionally, serum cholesterol was more elevated in *Casp12*$^{-/-(129)}$ mice when fed a HFD (Figure 1D and 2J). To determine if there were metabolic abnormalities associated with caspase-12 ablation, we first determined food intake in LFD-fed mice and found that it was similar between genotypes (Figure S2A). Next, we performed indirect calorimetry experiments on 8 week old WT and *Casp12*$^{-/-(129)}$ mice fed a LFD. We selected this time point as it is prior to the onset of differential body weight gains in the two genotypes to assess causative rather than consequential effects of obesity (Figure S2B). We observed no significant differences in respiration (VO_2 or CO_2), energy expenditure, or movement in the cages (Figure S2C-H). However, after HFD, *Casp12*$^{-/-(129)}$ mice had reduced respiration and movement (Figure S2I-O), suggesting that after the onset of obesity, metabolic abnormalities may begin to contribute to disease. Altogether, these results indicate that loss of caspase-12 in mice results in spontaneous obesity that is exacerbated with HFD feeding leading to fatty liver disease. This phenotype, however, is not a result of drastic intrinsic metabolic abnormalities in *Casp12*$^{-/-(129)}$ mice.

Caspase-12 deficient mice develop glucose intolerance and insulin resistance

There is a significant correlation between obesity and metabolic diseases in humans and rodent models, particularly between obesity-induced metabolic inflammation and insulin resistance. To determine whether obese *Casp12*$^{-/-(129)}$ mice became insulin resistant, we performed glucose tolerance tests (GTT) and insulin tolerance tests (ITT). This analysis demonstrated that at approximately 20 weeks of age *Casp12*$^{-/-(129)}$ mice developed glucose intolerance and insulin resistance both when fed a LFD (Figure 3A, B) or a HFD (Figure 3C, D). HFD fed *Casp12*$^{-/-(B6)}$ mice also had impaired tolerance to glucose compared to WT controls (Figure 3E), while loss of caspase-11 or caspase-1 did not have an effect at this time point (Figure 3F). Analysis of the epididymal fat revealed reduced expression of the insulin sensitive genes Adiponectin and Ppar-γ in caspase-12-deficient mouse strains but not in *Casp11*$^{-/-}$ mice (Figure 3G). Binding of insulin to its receptor leads to a downstream signalling cascade, resulting in phosphorylation of AKT. To assess insulin signalling in metabolic tissues, HFD-fed mice were injected intraperitoneally (i.p.) with a 5.0 U/kg bolus of insulin and levels of phosphorylated AKT in white adipose tissue (WAT), muscle and liver were analyzed 10 minutes later by immunoblot analysis. Figure 3H shows that *Casp12*$^{-/-(129)}$ mice exhibited reduced insulin signalling in all three metabolic tissues.

Ablation of Caspase-12 in the radio-resistant compartment leads to obesity

To further delineate how caspase-12 may be contributing to the development of obesity, we generated bone marrow chimeras. Interestingly, HFD-fed *Casp12*$^{-/-(129)}$ recipient mice developed increased body weight, adiposity, and liver weight irrespective of whether they were transplanted with bone marrow from WT or *Casp12*$^{-/-(129)}$ donors (Figure 4A-B). Thus, the genotype of the transplanted hematopoietic cells had little bearing on the obesity phenotype of *Casp12*$^{-/-(129)}$ mice. In agreement with this finding, Caspase-12 could not be detected in the radio-sensitive compartment of adipose tissue (Figure S3A). Similarly loss of caspase-12 in the radio-resistant compartment was responsible for mediating the glucose intolerance phenotype (Figure 4C). These results suggested that both obesity and insulin resistance in *Casp12*$^{-/-(129)}$ mice are likely independent of bone marrow-derived immune cells but instead may be linked to the function of caspase-12 in radio-resistant cells.

Caspase-12 deficiency leads to increased WAT inflammation

Given our previous findings that caspase-12 can regulate various inflammatory pathways, including caspase-1, NOD and NF-κB Signalling (189, 191, 192), we examined adipose tissue inflammation in DIO WT and *Casp12*$^{-/-(129)}$ mice. Overnight organ culture of epididymal adipose tissue from *Casp12*$^{-/-(129)}$ mice after HFD revealed enhanced secretion of IL-6, and to a lesser extent KC (Figure 4D). Similarly, *Casp12*$^{-/-(129)}$ mice had significantly more serum IL-18 cytokine, but not MCP1, compared to WT mice (Figure 4E). Consistent with increased IL-18 levels, *Casp12*$^{-/-(129)}$ and *Casp12*$^{-/-(B6)}$ mice also exhibited increased activation of caspase-1 in the epididymal adipose tissue, evident by elevated levels of the active p20 fragment, as detected by western blot (Figure 4F and S3B). The epididymal adipose tissue of *Casp12*$^{-/-(129)}$ and *Casp12*$^{-/-(B6)}$ had increased expression of CD45 suggestive of increased immune cell infiltration (Figure 4G). We next determined levels of immune cell infiltrates in the epididymal adipose tissue stromal vascular fraction (SVF) from DIO mice by flow cytometry. Quantification of the numbers of neutrophils, myeloid cells, NK cells, and T and B lymphocytes revealed a general trend of increased immune cell infiltration in the WAT of *Casp12*$^{-/-(129)}$ mice compared to WT controls with macrophages being most significantly increased in the absence of caspase-12 (Figure 4H and S3C).

Ablation of *Nlrp3* but not *Ripk2* reverses the obesity and insulin resistance phenotype of *Casp12*$^{-/-(129)}$ mice

To define the mechanism of caspase-12 action in metabolism and metabolic inflammation and determine whether the inflammasome or Nod1/2 pathways (189, 191) were involved, we bred *Casp12*$^{-/-(129)}$ mice with *Nlrp3*$^{-/-}$ mice or mice deficient in the Nod1/2 pathways central adaptor Receptor-interacting protein kinase (Ripk)2 (497) to generate *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ and *Casp12*$^{-/-}$ *Ripk2*$^{-/-}$ double-knockout (DKO) mice. Deletion of *Ripk2* did not modify the *Casp12*$^{-/-}$ mouse obesity phenotype; *Casp12*$^{-/-}$*Ripk2*$^{-/-}$ mice were similar in weight to *Casp12*$^{-/-}$ mice, both being significantly heavier than *Ripk2*$^{-/-}$ and WT mice (Figure 5A). Interestingly, in contrast to *Casp12*$^{-/-}$ and *Casp12*$^{-/-}$*Ripk2*$^{-/-}$ mice, *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ mice did not develop increased bodyweight gain and were similar to *Nlrp3*$^{-/-}$ mice and WT controls (Figure 5A). These results suggest that the Nlrp3 inflammasome may promote obesity in *Casp12*$^{-/-}$ mice. As expected from body weight differences, *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ mice were similar in adiposity and liver weight to *Nlrp3*$^{-/-}$ and WT

mice, which are significantly reduced than in *Casp12*$^{-/-}$ mice (Figure 5B). Histopathological examination of the liver revealed that both *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice had similar levels of steatosis and liver triglycerides compared to WT mice, which is significantly attenuated compared to *Casp12*$^{-/-}$ mice, though there was no difference in serum ALT (Figure 5C-D). Serum cholesterol of the DKO mice was similarly reduced compared to *Casp12*$^{-/-}$ mice (Figure 5E). *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ DKO mice were also analyzed on a HFD, and as for the LFD, these mice were leaner and more glucose tolerant compared to *Casp12*$^{-/-}$ mice (Figure 5F, G). While the Nlrp3 inflammasome is commonly studied in macrophages, there is little data regarding its role in adipocytes. Caspase-1 is expressed during adipogenesis (4) and we were able to detect inducible Nlrp3 expression in primary differentiated adipocytes (Figure S3D). Thus, adipocytes represent a potential radio-resistant cell type that expresses both Caspase-12 (Figure S1A) and Nlrp3.

The human *CASP12* T^{125} allele is associated with elevated metabolic inflammation but not with metabolic disease.

A SNP in exon 4 of the human *CASP12* gene at amino acid position 125 (T^{125}) introduces a premature stop codon and precludes expression of caspase-12 from the majority of the human population. In contrast, a fraction of people of African descent carry a functional allele due to a T^{125}C SNP (nucleotide substitution c.373C>T) (196). To assess if the expression of human caspase-12 plays a role in modulating metabolic inflammation, we examined African American obese children of the Yale Pediatric Obesity cohort for inflammatory markers in both the serum and subcutaneous adipose tissue. ELISA measurements revealed decreased levels of C-reactive protein (CRP) (p=0.04) and IL-6 (p=0.002) in the serum of carriers of the function C allele. This was most significant in boys (p=0.02 for CRP and p=0.006 for IL-6) compared to girls (p=0.95 for CRP and p=0.11 for IL-6) (Figure 6A-F). Consistently, quantitative PCR (qPCR) analysis of the subcutaneous adipose tissue indicated decreased levels of IL-6 and TNF expression in carriers of the C allele as well as a lower macrophage count revealed by immunohistochemistry using a CD68-antibody (Figure 6G-I). Next, to determine whether the *CASP12* polymorphism is associated with metabolic disease parameters, we examined the African American cohort of the Dallas Heart Study (491). Association of the c.373T>C SNP with BMI, fasting blood glucose, HOMA-IR, hepatic triglycerides, and serum triglycerides revealed no protective effect in carriers of the C allele (Table 1). Together, these results suggest that in humans, loss of caspase-12 may

not lead to obesity, in contrast to the effects observed in mice, but may contribute to inflammation associated with metabolic disease.

2.5 DISCUSSION

It is now well known that inflammation and metabolism are tightly interwoven processes, and fluctuations in one can have detrimental consequences in the other, potentially leading to disease. Studies in mice and humans has implicated various inflammatory pathways, including caspase-1 and the inflammasome, to obesity and diabetes (498). In this study we investigated the role of the two related inflammatory caspases, caspase-11 and caspase-12 in a DIO mouse model. Interestingly, two mouse strains deficient in caspase-12 developed obesity on a HFD while *Casp11*$^{-/-}$ or *Ice*$^{-/-}$ mice did not. The increased weight gain and adiposity of caspase-12-deficient mice was also associated with glucose intolerance and insulin resistance. As *Casp11*$^{-/-}$ mice were equivalent to WT mice, deficiency in caspase-11 is unlikely to mediate the phenotype of *Casp12*$^{-/-(129)}$ mice. This was confirmed in *Casp12*$^{-/-(B6)}$ mice that are sufficient for caspase-11. Caspase-11 is known to recognize intracellular LPS and intracellular bacterial pathogens (499). Obesity has been linked to increased gut permeability, resulting in elevated levels of circulating microbial products and LPS (324). While studies have linked numerous PRRs that detect bacterial motifs, such as Nod1/Nod2 (325) and TLR4 (320) to metabolic disease, caspase-11 may not play a similar role. However, since we observed slight differences between the two Caspase-12 deficient strains, we cannot rule out that Caspase-11 may become involved in the absence of Caspase-12. It has been observed that loss of Caspase-11 impacts *Salmonella* infection only in the absence of Caspase-1 (500), and it is possible that Caspase-12 deficiency could result in a small Caspase-11 dependent effect in our model.

In contrast with males, female *Casp12*$^{-/-(129)}$ mice were found to be equivalent in weight to WT mice when fed a LFD. Female mice are known to be protected from obesity due to the inhibitory role of estrogen on adipogenesis (501) and it is possible that the increased weight gain driven by loss of Caspase-12 could not compensate for this effect.

Caspase-12 has previously been demonstrated to have anti-inflammatory properties. While the protease is catalytically active, currently its only known substrate is itself (190) and autoproteolysis was determined to not be required for its ability to inhibit both caspase-1 and NF-κB activity (189, 192). This suggests that caspase-12 primarily functions through modulating

various signalling pathways through its ability to form CARD-CARD interactions. These include binding to caspase-1 (189), Ripk2 (191), NF-κB (192) and RIG-I (193), stearically hindering protein interactions and leading to attenuation of inflammation. Concordant with these functions, in our DIO mice we observed increased inflammation in the visceral adipose tissue, characterized by elevated inflammasome activation and immune cell infiltration. However, it is difficult to determine if the increased inflammation is a consequence of obesity, rather than an underlying cause. Within the adipose tissue Caspase-12 was not detectable in the radio-sensitive compartment and is likely absent in infiltrating myeloid cells. Therefore the robust immune cell driven inflammation observed in the adipose tissue at later stages of obesity may be independent of Caspase-12 activity.

It is unknown how caspase-12 may be affecting host metabolism, leading to the obese state of the mice. Indirect calorimetry and food intake measurements revealed no influence conferred by caspase-12 ablation. However the obesity phenotype was dependent on loss of caspase-12 in the radio-resistant compartment. Caspase-12 is expressed in the muscle, liver and fat (data not shown), tissues that are highly associated with metabolic health. Tissue specific knockouts are required to determine precisely where the protease is required to suppress obesity. Interestingly, when *Nlrp3*, but not *Ripk2*, was deleted in the caspase-12 null background, the mice were protected from increased adiposity and weight gain. The Nlrp3 inflammasome and caspase-1 have been previously linked to the regulation of metabolic health in humans and animal models, though there is some discrepancy in how it may regulate obesity in rodents. Caspase-1, Nlrp3, and ASC deficient mice have been reported to be leaner than WT controls (4, 327), equivalent in body weight (68, 329) or have increased adiposity (2, 328, 330). Obesity in these mice is also correlated with reduced glucose tolerance, suggesting that any beneficial effects on metabolic health that loss of the inflammasome may have, are not significant enough to counteract other pathways influenced by obesity. Differences in adipogenesis (4), cleavage of SIRT1 (502), and alteration of lipid metabolism (2, 489) are a few of the proposed mechanisms by which caspase-1 may regulated metabolic processes. In the context of obesity, caspase-12 might impact caspase-1 directly or indirectly. Loss of Nlrp3 may antagonize direct effects of caspase-12 on caspase-1, conferring a lean phenotype. Nlrp3 and Caspase-12 are both expressed in adipocytes (503) (Figure S1A, S3D), which represents one potential radio-resistant cell type where an interaction

between the two proteins may take place. Alternatively, caspase-12 may affect a parallel metabolic pathway that is reversed by loss of Nlrp3. For example, the inflammasome has been linked to gut microbiota dysbiosis, which affects obesity (330). Potential microbial dysbiosis in caspase-12 deficient mice may be altered upon removal of Nlrp3. Further work is required to determine how caspase-12 may function in radio-resistant cells, and the use of tissue-specific knockouts may aid in examining how these potential interactions may drive metabolic health in mice.

While the majority of people lack a functional caspase-12, the $T^{125}C$ SNP has persisted in a small percent of people of African descent. Many studies have examined why the functional caspase-12 allele has been maintained, including examining a potential role in protection against bacterial sepsis (196, 198), candidemia (199), and rheumatoid arthritis (200). Our previous work has implicated that the $T^{125}C$ SNP may confer susceptibility to sepsis (196), however a second study assessing community-acquired pneumonia, did not report similar findings (198). There is no clear indication what role caspase-12 may play in people and given how the loss of caspase-12 led to obesity in mice, we investigated if it may have similar properties in the human population. In the Yale Pediatric Cohort, the caspase-12 SNP was associated with reduced inflammatory parameters in the serum and adipose tissue. These results correlate with previous findings implicating Caspase-12 playing an anti-inflammatory role. However, genotyping the Dallas Heart Study cohort of African descent for the $T^{125}C$ SNP indicated that there was no association with improved metabolic parameters. These results are at odds with our findings in mice, suggesting that host specific differences are involved. It is possible that caspase-12 is differentially regulated in mice and humans. For example, we have previously reported that human caspase-12 is repressed by estrogen, an effect not observed in the murine system (197). Therefore in humans, loss of caspase-12 appears to recapitulate the pro-inflammatory effects observed in mice during obesity though may not be involved with the development of an altered metabolic state. Further analyses of other populations are required to further clarify its role.

2.6 ACKNOWLEDGEMENTS

We thank the Wellcome Trust Sanger Institute for providing *Casp12*$^{-/-(B6)}$ (Casp12$^{tm1a(KOMP)Wtsi}$) mice; V. Dixit, Genentech, for providing *Casp11*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice; R. Flavell, Yale University, for providing *Ice*$^{-/-}$ and *Ripk2*$^{-/-}$ mice; Helen Hobbs, University of Texas Southwestern Medical Center, and the Dallas Heart Study investigators; J. Rinz, McGill University, and G. Perrault, McGill University, for animal husbandry. Anna Kinio for technical assistance

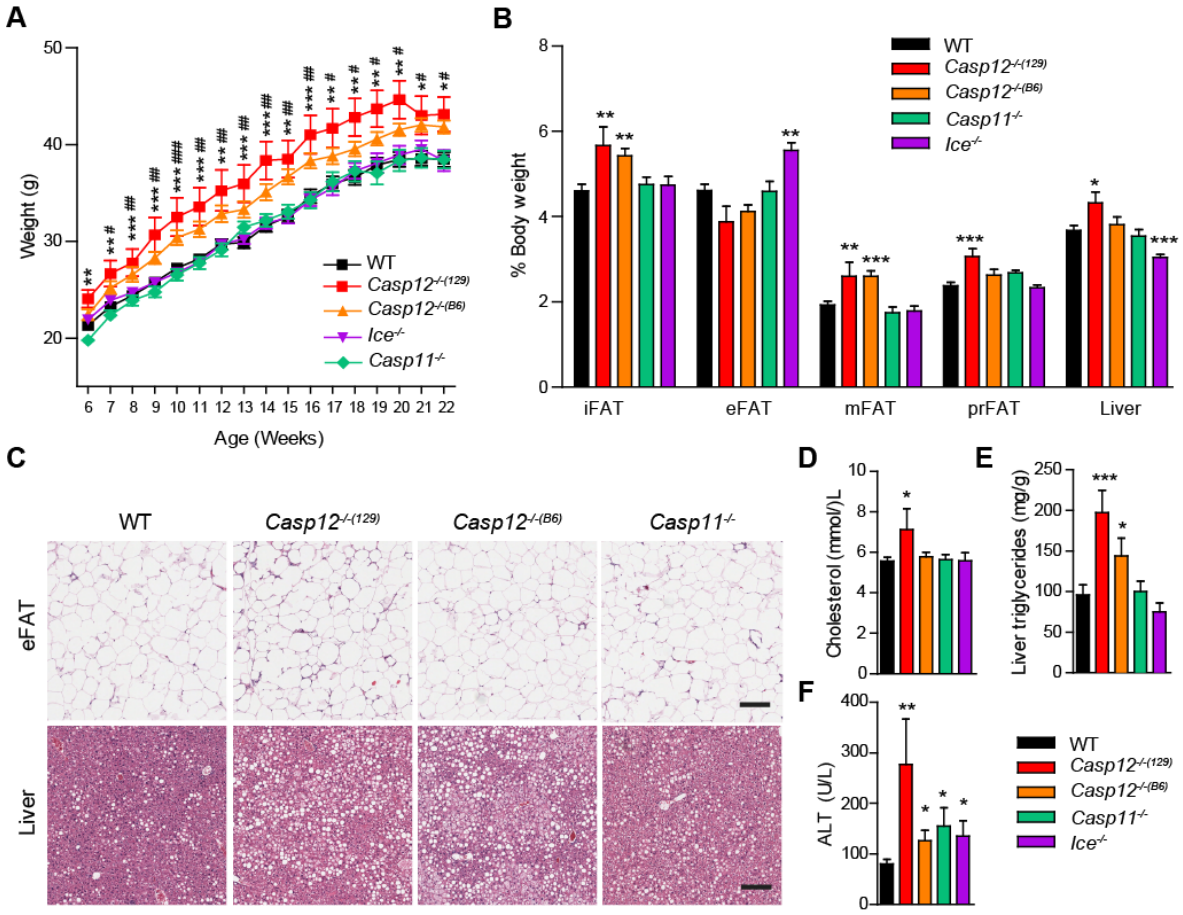

Figure 1. Caspase-12 deficient mice develop obesity on a HFD

A) Body weight curve of male mice fed a HFD started at 6 weeks of age, WT n=31, *Casp12*$^{-/-(129)}$ n= 10, *Casp12*$^{-/-(B6)}$ n=13, *Ice*$^{-/-}$ n=15, *Casp11*$^{-/-}$ n=12. B) Relative weight of adipose depots and liver after 16 weeks of HFD. C) H&E staining of epididymal adipose (eFAT) and liver sections after 16 weeks of HFD. Scale bar is 200 µM. D) Serum cholesterol. E) Liver triglycerides. F) Serum ALT. Data represent the mean +/- SEM. Statistical analysis was performed using Student's t-test. Statistical significance is presented as follows: # P<0.05 ## $P < 0.01$, ### P<0.001 for *Casp12*$^{-/-(B6)}$ vs WT; * P<0.05 ** $P < .01$; *** P <0.001 for *Casp12*$^{-/-(129)}$ vs WT in A).

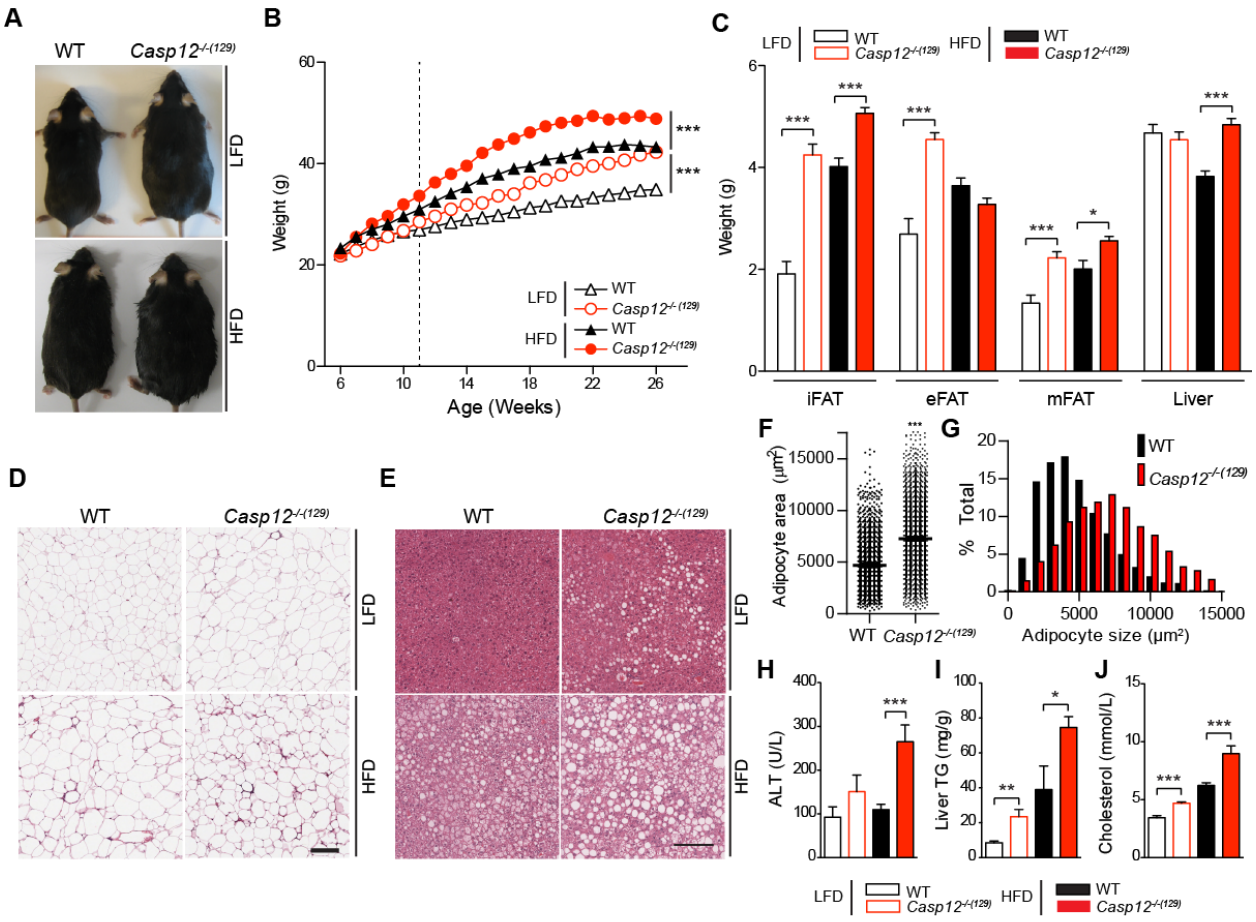

Figure 2. *Casp12*$^{-/-(129)}$ mice develop spontaneous obesity on a LFD

A) Photos of male C57BL/6 WT mice and *Casp12*$^{-/-(129)}$ at 30 weeks of age, fed a LFD or HFD. B) Body weight curve of male mice. HFD started at 6 weeks of age, n=24-39 mice per group. C) Relative weight of adipose depots and liver at 26 weeks of age (n≥15 mice per group). D) H&E staining of epididymal adipose tissue at 26 weeks of age. Scale bar is 200 µM. E) H&E staining of liver tissue from 26 week old mice. Scale bar is 200 µM. F-G) Average epididymal adipocyte area from LFD fed mice. H) Serum ALT (LFD WT n=13, LFD *Casp12*$^{-/-(129)}$ n= 12, HFD WT n=12, HFD *Casp12*$^{-/-(129)}$ n=10). I) Hepatic triglycerides. (LFD WT n=14, LFD *Casp12*$^{-/-(129)}$ n= 13, HFD WT n=6, HFD *Casp12*$^{-/-(129)}$ n=6). J) Serum cholesterol. Data represent the mean +/- SEM. Statistical analysis was performed using Student's t-test. Statistical significance is presented as follows: ** $P < .01$; *** $P < 0.001$.

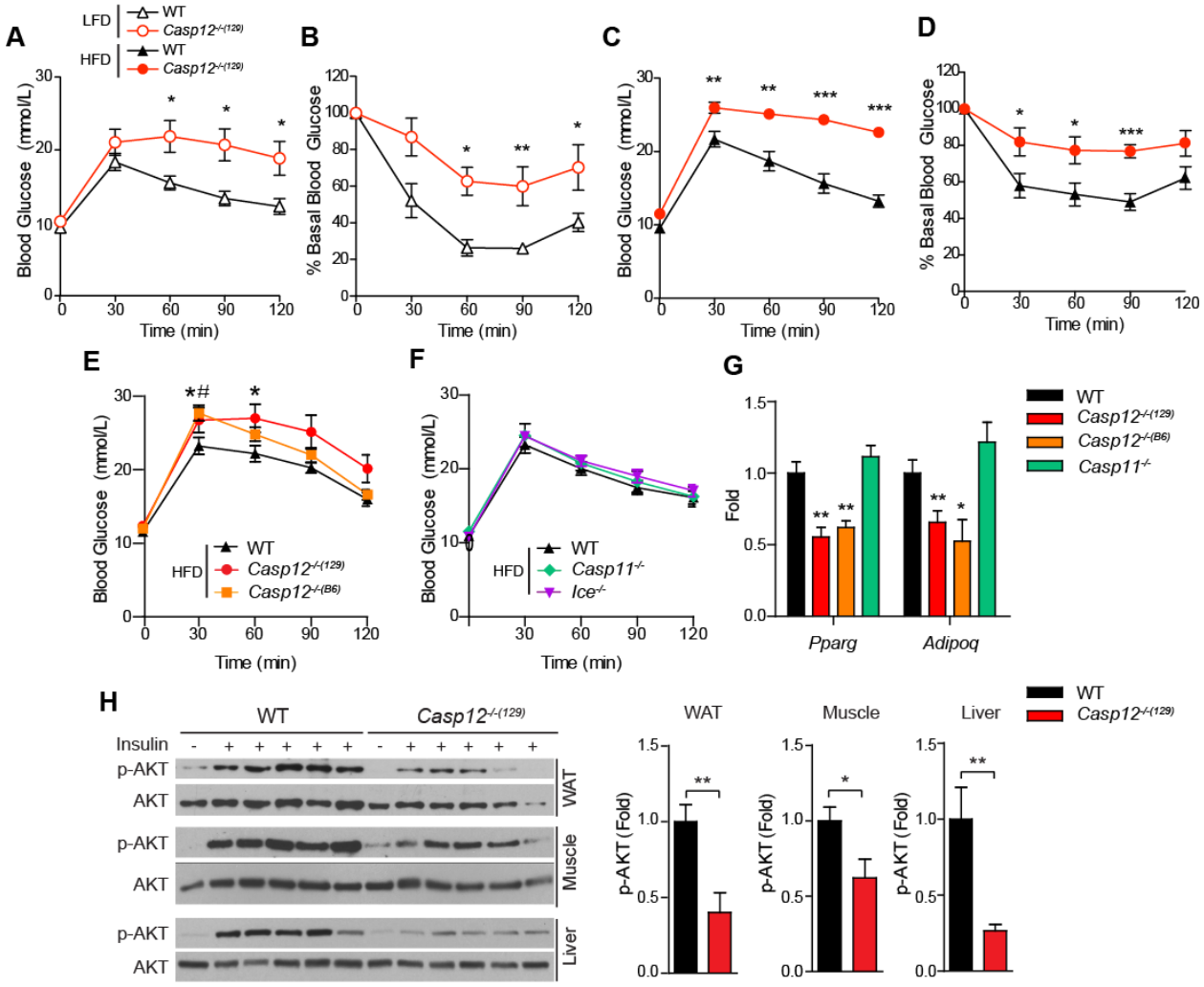

Figure 3. *Casp12*$^{-/-}$ mice are glucose intolerant and insulin resistant

A) LFD fed C57BL/6 WT and *Casp12*$^{-/-(129)}$ mice were injected i.p. with 2.0 mg/g dextrose at 20 weeks of age (n=5). B) LFD WT and *Casp12*$^{-/-(129)}$ mice were injected i.p. with 0.75 U/kg insulin at 20 weeks of age (n=4). C) HFD fed WT and *Casp12*$^{-/-(129)}$ mice were injected i.p. with 2.0 mg/g dextrose at 20 weeks old (n=6-7). D) HFD fed mice were injected with 2.0 U/kg of insulin at 25 weeks of age (n=6). E) GTT of WT, *Casp12*$^{-/-(129)}$ and *Casp12*$^{-/-(B6)}$ mice at 20 weeks of age (n=8). F) GTT of WT, *Casp11*$^{-/-}$ and *Ice*$^{-/-}$ mice at 20 weeks of age (n=8). G) qPCR analysis of epididymal adipose tissue in HFD mice (n=8-16). H) WT and *Casp12*$^{-/-(129)}$ mice on HFD were injected i.p. with 5.0 U/kg insulin and sacrificed 10 minutes later. Tissues were frozen and immunoblotted for Ser-473 phospho-AKT and total AKT. Ratio of phospho-AKT to total AKT was calculated using ImageJ. Data represent the mean +/- SEM. Determined by student's t-test. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT). For 2E, *p < 0.05 for Casp12-/-(129) vs WT and #p < 0.05 for Casp12-/-(B6) vs WT.

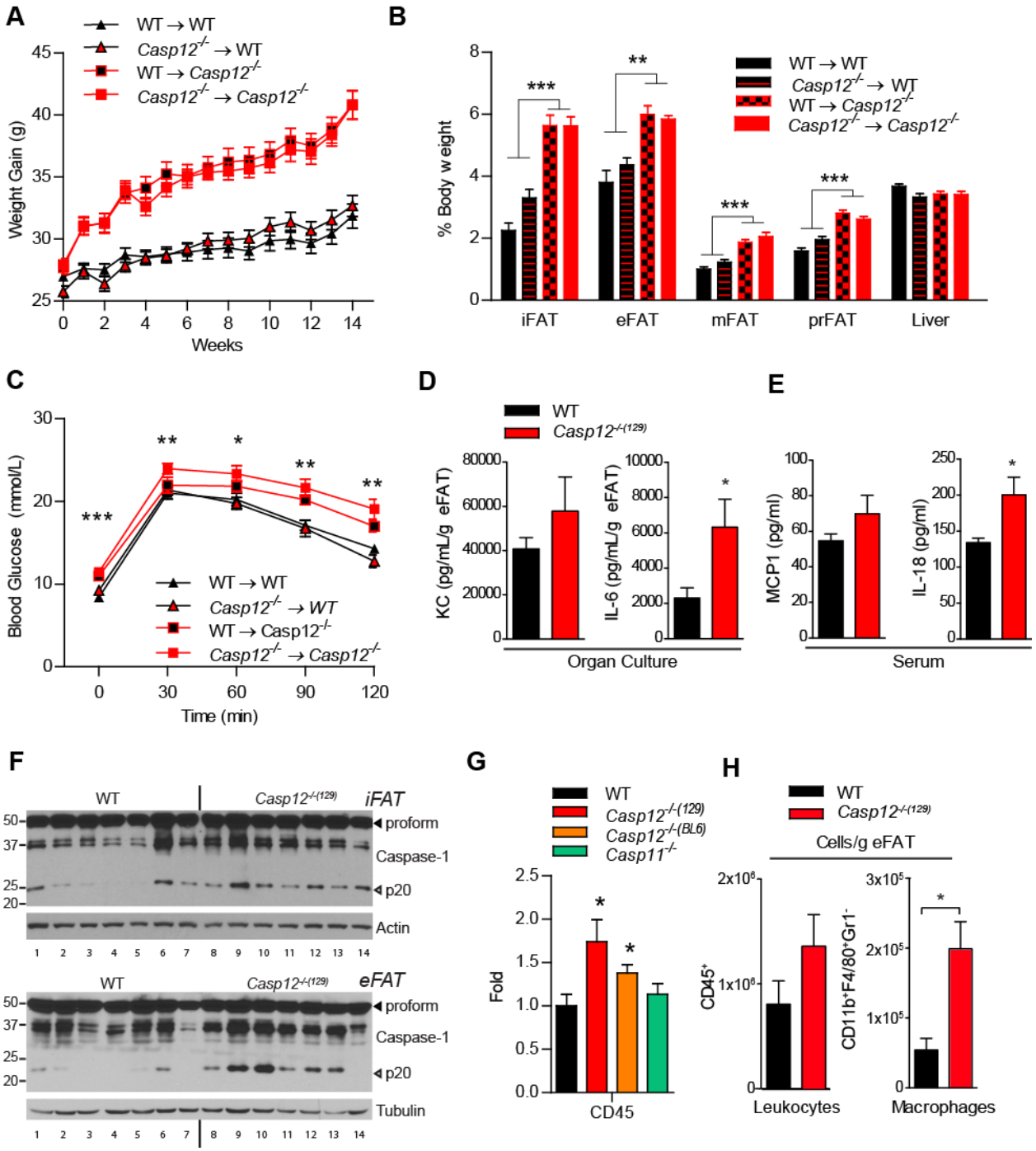

Figure 4. Loss of caspase-12 in the radioresistant compartment results in obesity and glucose intolerance

Figure 4. Loss of caspase-12 in the radioresistant compartment results in obesity and glucose intolerance

Age matched male C57BL/6 WT and *Casp12*$^{-/-(129)}$ recipient mice were irradiated and reconstituted with bone marrow from WT and *Casp12*$^{-/-(129)}$ donor mice. The mice were then fed a HFD. A) Body weight curve. (n=12-13) B) Relative weight of adipose depots and liver after 14 weeks of HFD. C) GTT after 14 weeks of HFD injected i.p. with 2.0 mg/g dextrose. Data represent the mean +/- SEM. Statistical analysis was performed using Student's t-test. Statistical significance is presented as follows: * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ for WT>WT vs Casp12>Casp12. ## $P < 0.01$, ### $P < 0.001$ for WT>WT vs WT>Casp12. D) Organ culture of epididymal adipose tissue excised from HFD mice and incubated overnight in media. IL-6 and KC levels were analyzed by ELISA and normalized to adipose tissue mass. E) Serum ELISA of HFD mice. F) Epididymal (eFAT) adipose depots were excised from 26 week old HFD mice and immunobloted for Caspase-1. G) qPCR analysis of eFAT from HFD fed mice. (n=7-16 per genotype). H) Flow cytometry analysis of eFAT infiltrating immune cells after HFD (n=5 per genotype).

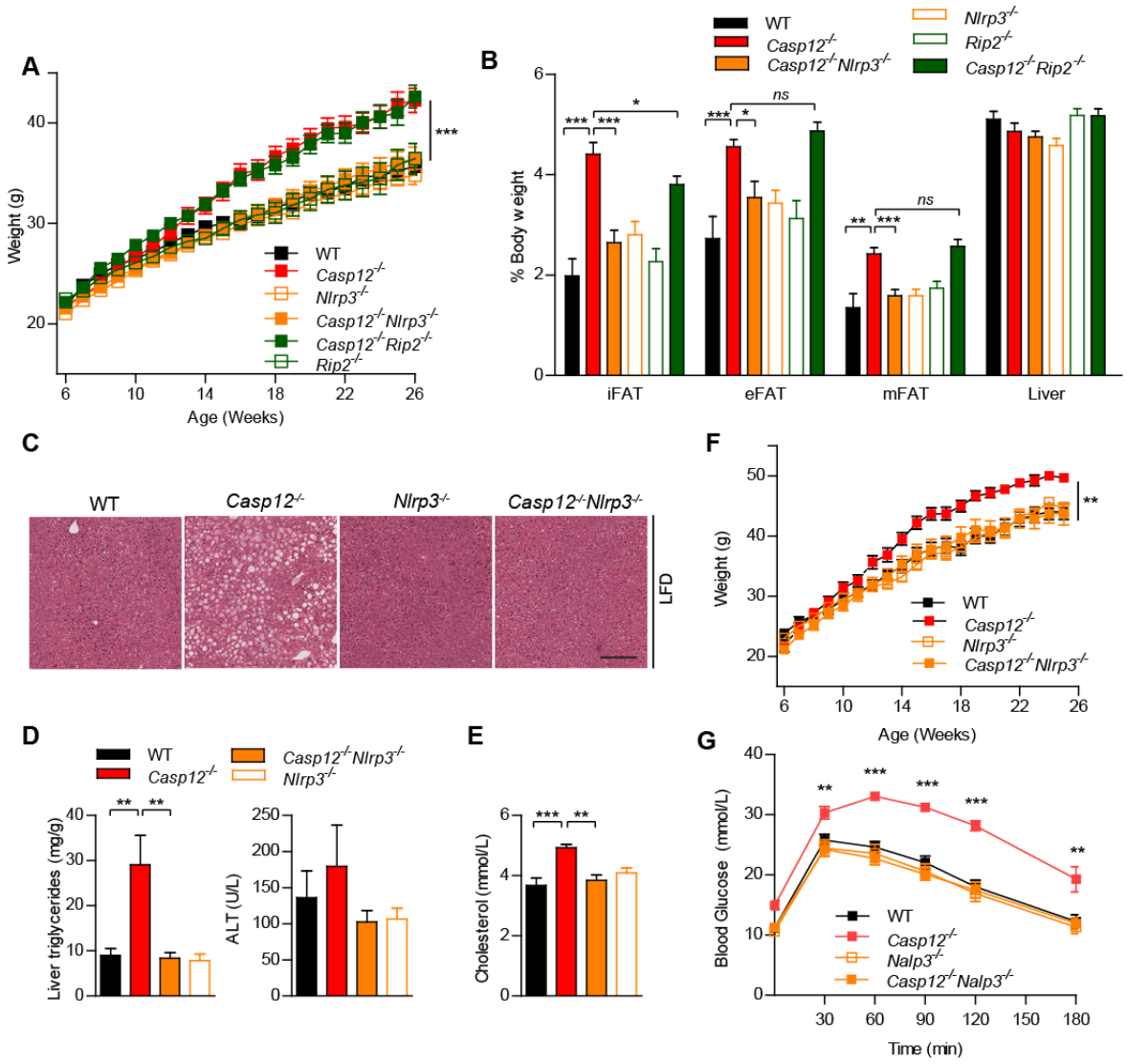

Figure 5. *Casp12*$^{-/-}$ *Nlrp3*$^{-/-}$ mice are protected from obesity and glucose intolerance

Casp12$^{-/-(129)}$ mice were crossed with *Ripk2*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice to generate double knock-out strains *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ *and Casp12*$^{-/-}$*Ripk2*$^{-/-}$. A) Body weight curve of male mice fed a LFD. B) Relative weight of adipose depots and liver at 26 weeks of age. Statistical analysis was performed using Student's t-test. Statistical significance is presented as follows: * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$ vs the *Casp12*$^{-/-}$ group. C) H&E staining of liver tissue of 26 week old mice. Scale bar is 200 μM. D) Hepatic triglycerides and serum ALT. E) Serum cholesterol. F) Body weight curve of male mice fed a HFD. G) GTT of HFD mice at 22 weeks of age injected i.p. with 2.0 mg/g dextrose. Data represent the mean +/- SEM. Determined by student's t-test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. WT).

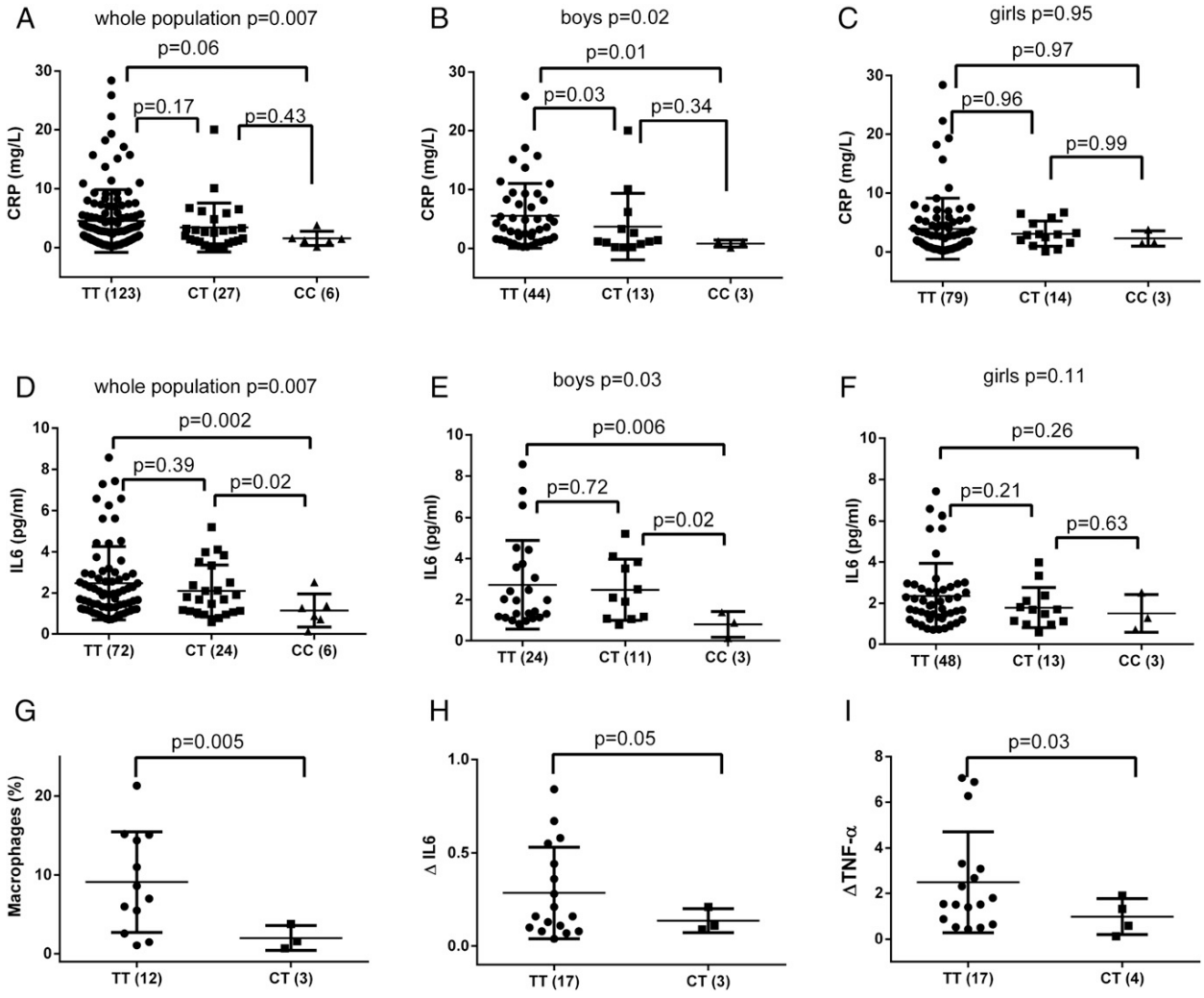

Figure 6. Human caspase-12 dampens metabolic inflammation.

Subjects carrying the risk allele for the $T^{125}C$ SNP showed lower CRP (A-C) and IL-6 (D-F) levels than the non-carriers. This phenomenon seemed to be more marked in males than in females. In the subgroup of subjects who underwent an adipose tissue biopsy, the $T^{125}C$ allele was associated with a lower percent of macrophages by IHC, and a lower expression of CD68, IL-6 and TNF-alpha by qPCR (G-I).

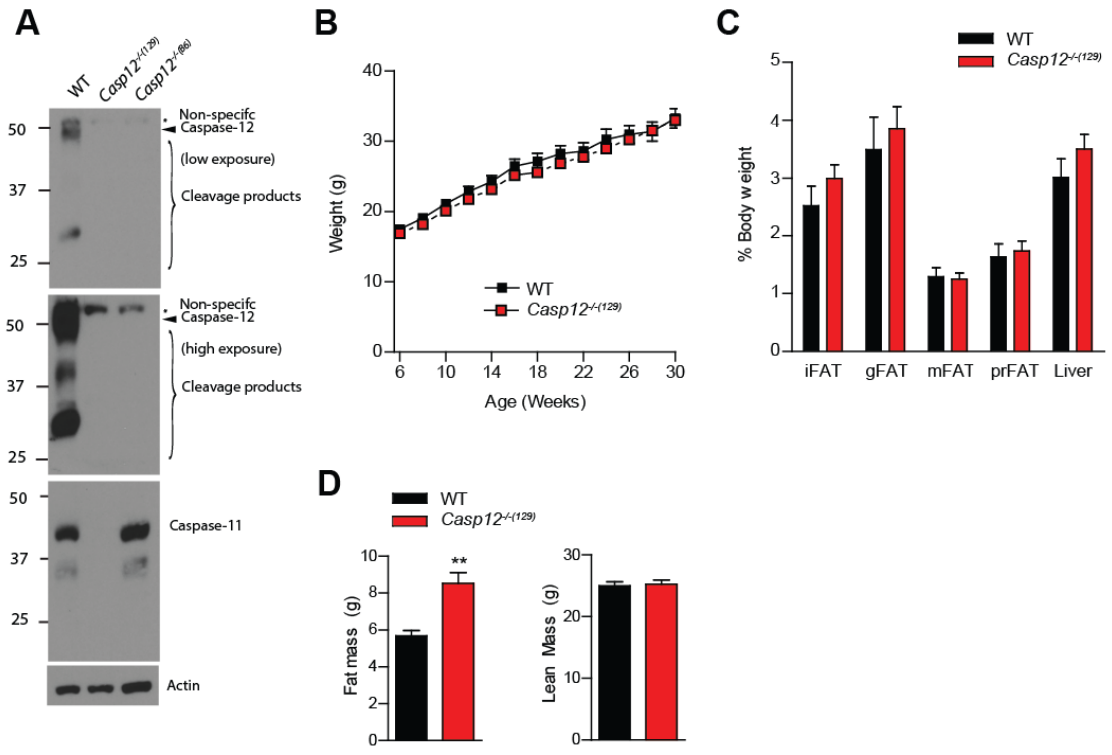

Figure S1. *Casp12*$^{-/-(129)}$ mice develop spontaneous obesity

A) Western blot of primary differentiated adipocytes treated overnight with 100 ng/mL LPS. B) Weight curve of female WT and *Casp12*$^{-/-}$ mice fed a LFD (n=14-19). C) Relative weight of adipose depots and liver of female mice at 30 weeks of age. D) DEXA scan of 20 week old WT and *Casp12*$^{-/-}$ mice. Data represent the mean +/- SEM. Determined by student's t-test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. WT).

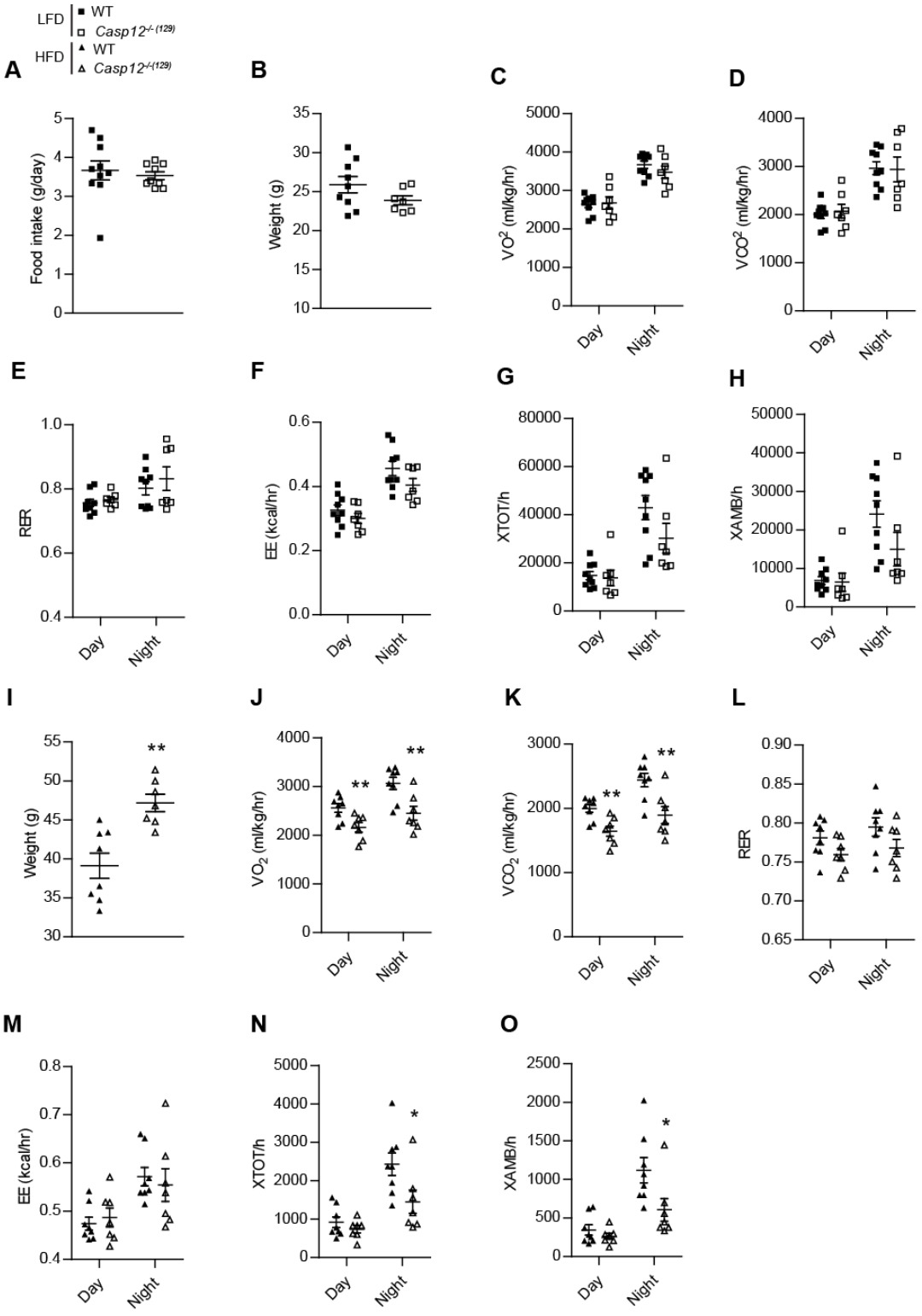

Figure S2. Indirect calorimetry of WT and *Casp12*$^{-/-(129)}$ mice.

Figure S2. Indirect calorimetry of WT and *Casp12*$^{-/-(129)}$ mice.
A-H) LFD fed 8 week old mice were allowed to acclimatize for 48h in Oxymax-CLAMS system metabolic cages and readings were taken over a 24h period. A) Food intake. B) Initial weight. C) VO_2. D) VCO_2. E) RER (respiratory exchange ratio). F) Energy Expenditure. G) Total movements. H) Ambulatory movements (consecutive beam breaks). I-O) Mice fed a HFD for 20 weeks. I) Weight. J) VO_2. K) VCO_2. L) RER. M) Energy Expenditure. N) Total movements. O) Ambulatory movements (consecutive beam breaks).

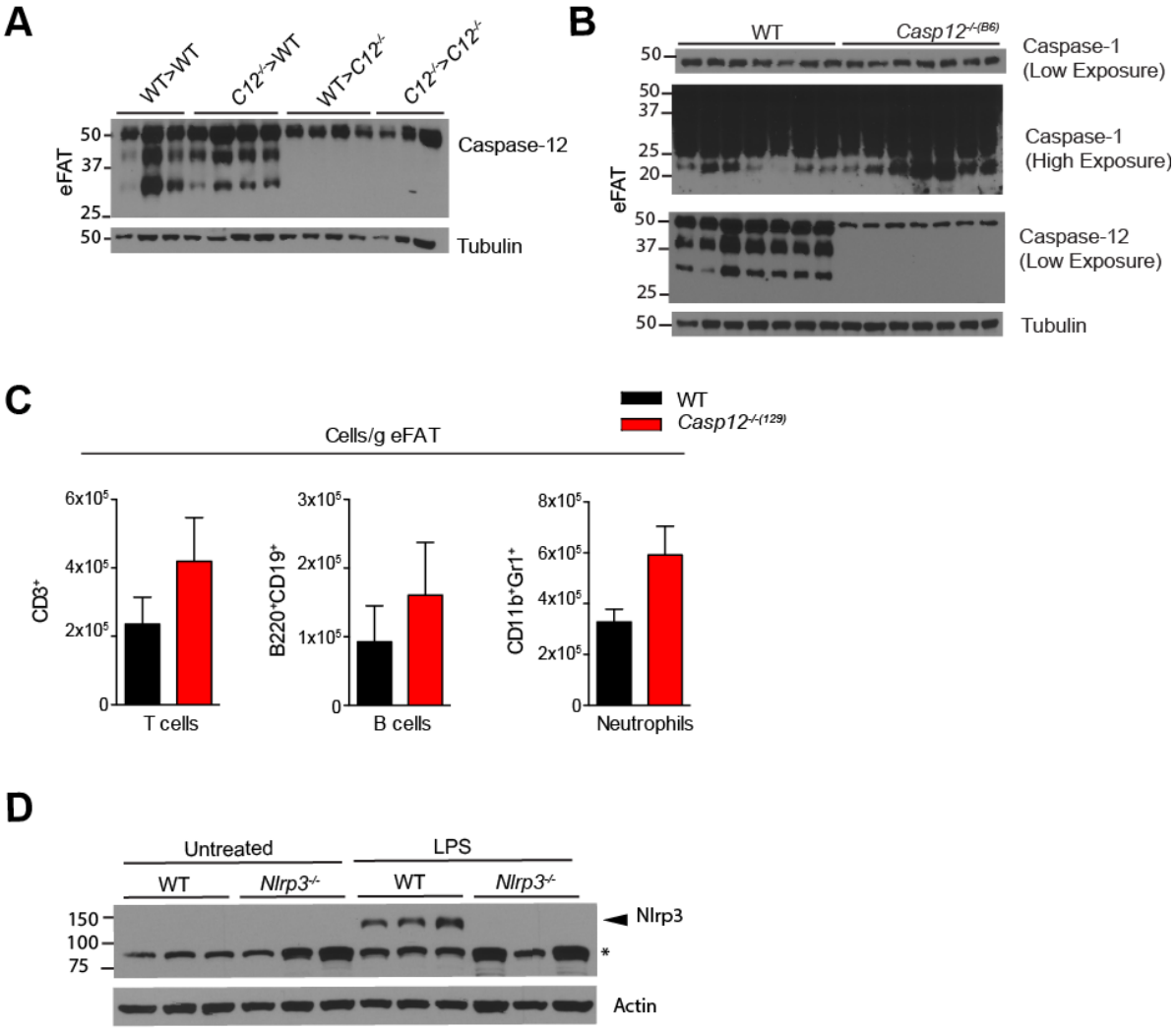

Figure S3. *Casp12*$^{-/-}$ mice have increased adipose inflammation

A) Epididymal adipose tissue of bone marrow chimera mice immunoblotted for Caspase-12. B) Epididymal adipose tissue of 22 week old HFD fed WT and *Casp12*$^{-/-(BL6)}$ mice. C) Flow cytometry analysis of eFAT infiltrating immune cells after HFD (n=5 per genotype). D) Primary differentiated adipocytes from WT or *Nlrp3*$^{-/-}$ mice were treated with or without LPS overnight and immunoblotted for Nlrp3. Data represent the mean +/- SEM

Characteristic	Female (N=1,412)			P-value	Male (N=955)			P-value	Total (N=2,367)			P-value
	TT (N=1042)	TC (N=345)	CC (N=25)		TT (N=696)	TC (N=238)	CC (N=21)		TT (N=1738)	TC (N=583)	CC (N=46)	
Age, years (N=2,367)	46±11	44±11	45±8	0.14	45±11	44±11	47±12	0.87	45±11	44±11	46±10	0.22
BMI, kg/m^2 (N=2,349)	32.2±8.3	32.6±7.7	35.3±6.9	0.038	28.6±6.4	29.4±7.1	29.6±7.3	0.13	30.7±7.8	31.3±7.6	32.6±7.6	0.011
Glucose, mg/dL[a] (N=2,003)	90±12	91±12	93±11	0.29	92±12	93±15	92±11	0.23	91±12	92±14	93±11	0.11
HOMA-IR, U[b] (N=2,031)	4.5±4.4	4.8±4.1	5.2±3.3	0.37	4.3±5.0	4.6±5.8	5.7±7.7	0.37	4.5±4.6	4.7±4.9	5.4±5.7	0.92
TG, mg/dL[b] (N=2,365)	83 (61-114)	78 (61-116)	73 (63-87)	0.76	91 (65-133)	92 (66-134)	85 (60-148)	0.68	86 (63-122)	84 (63-122)	75 (60-120)	0.60
HTGC, %[b] (N=1,065)	3.3 (1.9-5.1)	3.3 (2.0-6.3)	3.1 (1.6-3.8)	0.47	3.1 (2.0-5.3)	3.4 (2.0-5.3)	2.4 (1.4-3.7)	0.44	3.2 (2.0-5.2)	3.3 (2.0-5.8)	2.9 (1.1-3.9)	0.96
CRP (N=1,761)	4.5 (1.8-9.6)	5 (2.1-10.4)	8.9 (5.7-12.8)	0.056	2.2 (1.0-5.5)	2.4 (1.1-6.7)	2.0 (1.1-4.0)	0.46	3.5 (1.4-8.1)	3.9 (1.5-9.2)	5.5 (2.0-10.0)	0.058

Table 1. Demographic, anthropometric and clinical characteristics of African American participants of the Dallas Heart Study stratified by gender and *CASP12* R125X (rs497116) genotype.

Analysis of T125C SNP in African American cohort of DHS. The DHS population was genotyped for the Caspase-12 T125C SNP using the Illumina Human Exome chip, as described in Materials and Methods. The analysis was performed using linear regression, adjusted for age and BMI and stratified by sex. Values are mean 6 SD or median (interquartile range). Comparisons were made using linear regression models, adjusted for sex, age, and BMI, as necessary. The numbers in parentheses indicate the number of individuals with available data for each phenotype.

[a]Diabetic individuals excluded.

[b]Analysis adjusted for diabetes in addition to other covariates.

HTGC, hepatic TG content

PREFACE TO CHAPTER 3

The results of chapter 2 suggested that loss of caspase-12 in the radioresistant compartment led to obesity in mice. This effect was dependent on the Nlrp3 inflammasome. While inflammasome function is well characterized in immune cells, its potential role in other cell types is less well understood. Preliminary data suggests that the inflammasome plays a role in the radioresistant compartment in regulating metabolism and may affect adipocyte function, as shown in *in vitro* studies. However, it is unknown how inflammasome activation in adipocytes *in vivo* may contribute to disease. To investigate this possibility, we utilized mice expressing a hyperactive Nlrp3 allele specifically in adipocytes and assessed their development of obesity and insulin resistance.

CHAPTER 3

Adipocyte specific Nlrp3 inflammasome activity contributes to glucose intolerance

3.1 ABSTRACT

The inflammasomes exert important functions in innate immunity and tissue tolerance but are also key determinants of several human diseases. Specifically, the Nlrp3 inflammasome has been implicated in obesity and metabolic disease but its tissue-specific roles in these conditions remain unclear. To examine the role of the Nlrp3 inflammasome in adipocytes we generated knock-in (KI) mice that express the hyperactive Nlrp3 D301N mutant allele in adiponectin-expressing cells. Using a diet induced obesity (DIO) model, we show that while body weight was unaffected in these mutant mice, they had reduced glucose tolerance. This evidence points to an adipocyte-specific role for the Nlrp3 inflammasome in contributing to insulin resistance and metabolic disease.

3.2 INTRODUCTION

Obesity and its associated metabolic diseases are becoming an immense problem worldwide. Consequently, a better understanding of the biological processes contributing to the development of these disorders is rapidly growing in importance. It is now well established that the immune system plays a significant role in the development of insulin resistance and Type 2 Diabetes (255). Numerous immune cell types, cytokines, and inflammatory signalling pathways have been identified as causative factors leading to metabolic disease.

Recent work has implicated the inflammasome as an important source of metabolic inflammation, arising during metabolic disease (1). The inflammasome is a cytosolic complex composed of an intracellular pattern recognition receptor (PRR), the adaptor molecule Asc, and caspase-1 (487). Of the multiple pattern recognition receptors determined to form inflammasomes, several studies have suggested that Nlrp3 is primarily responsible for inflammasome activation during metabolic dysfunction. Various metabolites that are elevated during obesity, such as cholesterol crystals, palmitate, and ceramide act as endogenous activators of the Nlrp3 inflammasome.

Inflammasome activation leads to caspase-1 mediated cleavage of its downstream substrates, including the pro-inflammatory cytokines IL-1β and IL-18 and Gasdermin D, which leads to pyroptotic cell death. IL-1β has been linked to insulin resistance (68, 331, 504) and has been a target of therapeutic intervention in clinical trials (372, 505). In contrast, IL-18 is reported to have beneficial effects in murine models of obesity due to its appetite suppressant properties and ability to activate AMPK (351-353). However collectively, the inflammasome appears to produce a detrimental effect with respect to metabolic disease. Multiple studies have demonstrated that loss of caspase-1, Asc, or Nlrp3 activity leads to insulin sensitivity (4, 68, 326, 327, 329). Additionally, the inflammasome might affect obesity and insulin resistance indirectly through regulation of the intestinal microbiota (330). In humans, expression of inflammasome components is induced in the adipose tissue of obese patients and correlates with insulin resistance (329, 341, 506).

Inflammasome function has been most widely studied in myeloid cell lineages, and comparatively little data exist for roles in non-immune cell types. The use of tissue specific knockout mice of inflammasome components has been under-utilized and currently bone marrow chimeras have offered the best, albeit crude, tool to dissect a role of the inflammasome in different compartments. Radiosensitive cells were shown to mediate insulin resistance in Asc-deficient mice, which agrees with the well established role of the inflammasome in macrophages (68). However, other studies also employing bone marrow chimeras have pointed to alternative inflammasome functions in the radio-resistant compartment. For instance, the Nlrp1 inflammasome was determined to inhibit obesity through its actions in radio-resistant cells (3). Moreover, triglyceride clearance was shown to require caspase-1 and Nlrp3 actions in non-hematopoietic cells, independently of IL-1β and IL-18 (2). In both cases, further studies are required to identify the specific cell type involved in these processes.

A role of inflammasome activity in adipocytes as a potential determinant of metabolic disease has recently gained added interest. Caspase-1 is induced in adipogenesis and its ablation correlates with increased differentiation and insulin sensitivity (4). Nlrp3 expression is also induced during obesity or after glucose exposure in purified adipocytes from humans and mice (345, 503). SIRT1, a metabolic regulator, was reported to be a caspase-1 substrate *in vitro* (502). Adipocyte-specific deletion of SIRT1 in mice is associated with obesity and insulin resistance, suggesting that cleavage by Caspase-1 could confer a similar phenotype. However, despite this mounting evidence, the *in vivo* contribution of adipocyte-specific inflammasome activation remains unclear.

To determine the role of adipocyte-specific inflammasome activation in metabolic disease, we generated KI mice expressing a hyperactive $Nlrp3^{D301N}$ mutant allele, specifically in adipocytes. D301N mutation in murine Nlrp3 corresponds to D303N mutation in human NLRP3 that is associated with Cryopyrin-Associated Periodic Syndromes (CAPS) (507, 508). Preliminary studies indicate that adipocyte-specific expression of this hyperactive Nlrp3 results in a reduction in glucose tolerance, suggesting that the inflammasome might play a role in adipocytes to influence metabolic disease outcome.

3.3 MATERIALS AND METHODS

Animal experiments

Mice were housed at room temperature with a 12 hour light/dark cycle with food and water provided ad libitum. The mice were fed either a standard chow diet (LFD) (2020x Teklad Rodent Diet; 16% calories from fat; 3.1 kcal/g) or a HFD (Research Diet D12451; 45% kcal from fat; 4.73 kcal/g). HFD feeding was initiated in mice at 6 weeks of age.

Mouse strains

Nlrp3$^{D301N/D301N}$ (Nlrp3$^{tm3.1Hhf}$) and *adipoq*-Cre (B6;FVB-Tg(Adipoq-cre)1Evdr/J) mouse strains were purchased from The Jackson Laboratory. Expression of the *Nlrp3*^{D301N} mutation requires Cre mediated excision of a neomycin resistance cassette. The *Nlrp3*^{D301N} allele is expressed by the natural *Nlrp3* promoter. All experiments were performed under guidelines of the animal ethics committee of McGill University (Canada).

GTT

Age matched male mice were fasted for 6h before i.p. injection with 2g/kg dextrose and blood glucose levels were measured from the tail vein using a Onetouch ultra 2 glucometer.

Genotyping

To genotype the D301N Nlrp3 allele, the following primers were used: Mutant forward, 5' GCTACTTCCATTTGTCACGTCC 3'. Wild type Forward, 5' CACCCTGCATTTTGTTGTTG 3'. Common Reverse primer, 5' CGTGTAGCGACTGTTGAGGT 3'. To genotype the *adipoq*-Cre expression the following primers were used: Transgene Forward, 5' GGATGTGCCATGTGAGTCTG 3'. Transgene Reverse, 5' ACGGACAGAAGCATTTTCCA 3'. Internal Positive Control Forward, 5' CTAGGCCACAGAATTGAAAGATCT 3'. Internal Positive Control Reverse, 5' GTAGGTGGAAATTCTAGCATCATCC 3'.

Western Blots

Cells were lysed in 2X laemmli sample buffer and separated on SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with antibodies against caspase-1 p20 (Genentech), Nlrp3 (Adipogen Cryo-2), ASC (SantaCruz SC-22514) and β-actin (Sigma, Cat# A1978).

Primary adipocyte culture

The inguinal adipose tissue was removed from age-matched male mice, minced and incubated in 1mg/ml Type 2 Collagenase for 1 hour at 37°C. The stromal vascular fraction was resuspended and cultured following standard protocols. Differentiation was induced using 850 nM insulin, 125 nM indomethacin, 500 uM IBMX, 1 μM rosiglitazone and 1 μM dexamethasone for the first 2 days. On day 2, media was replaced with 850 nM insulin and 1 μM rosiglitazone for the following 4 days. Cells were harvested on day 6.

3.4 RESULTS

To determine the function of the Nlrp3 inflammasome in adipocytes, we generated mice expressing the hyperactive *Nlrp3*^{D301N} gene by *adipoq*-Cre expression (Figure 1A). This mutation in the nucleotide binding domain has been reported to lead to a constitutively active inflammasome due to a conformational change in the protein (509). Mice expressing the *Nlrp3*^{D301N} mutation globally or in myeloid cells develop inflammatory disease and die prematurely (510, 511).

To detect expression of the mutant *Nlrp3*^{D301N} allele, we cultured primary differentiated adipocytes that were homozygous for *Nlrp3*^{D301N} with or without *adipoq*-Cre expression (Figure 1B). The cells were treated with or without LPS to induce expression of Nlrp3. Immunoblot of cell lysates determined that Nlrp3^{D301N} is expressed in adipocytes (Figure 1C). Expression levels were elevated in response to LPS, as expected. Mice lacking Cre expression and heterozygous for the WT protein had much higher levels of Nlrp3. This may be a consequence of contaminating cells, such as immune cells, that may express comparatively higher levels of the protein or inefficient Cre expression. Caspase-1 and Asc were also present in the differentiated adipocytes suggesting that all components for a functional inflammasome are present in adipocytes.

To determine if adipocyte expression of the *Nlrp3*^{D301N} allele has an effect *in vivo*, we fed mice a 45% kcal lipid based HFD. As Nlrp3 expression is induced by NF-κB activation (Figure 1C), the excess lipids and adipose inflammation that result from a HFD model should ensure expression in the adipocytes. WT, *Nlrp3*$^{D301N/+}$ Cre-, and *Nlrp3*$^{D301N/+}$ Cre+ mice were placed on HFD beginning at 6 weeks of age and were subsequently weighed weekly (Figure 2A). The latter expresses both the *Nlrp3*^{D301N} and WT *Nlrp3* alleles. No differences in body weight were observed between the three genotypes. To assess adiposity, the inguinal (iFAT), epididymal (eFAT), mysenteric (mFAT), and perirenal (prFAT) adipose depots were weighed after 20 weeks of HFD (Figure 2B). While *Nlrp3*$^{D301N/+}$ Cre+ mice had slightly larger mFAT depots compared to control mice, there were no differences in the other fat pads, suggesting adiposity was not

greatly affected by expression of the hyperactive Nlrp3 gene. No differences in liver weight were observed.

A glucose tolerance test was also conducted to assess metabolic disease in the mice after 13 weeks of HFD (Figure 2C). Glucose at a dose of 2.0 g/kg was administered intraperitoneally to the mice and blood glucose was determined every 30 minutes. Interestingly, *Nlrp3*$^{D301N/+}$ Cre+ mice had elevated blood glucose levels and were glucose intolerant compared to controls. This result suggests that Nlrp3 expression specifically in the fat cells negatively impacts the metabolic health of the mice.

3.5 DISCUSSION

The Nlrp3 inflammasome has been implicated as an important contributor of metabolic disease. The function of this complex is well defined in immune cells however its role in metabolic cell types has only been suggested and there is a lack of *in vivo* evidence to confirm its importance. To determine if the Nlrp3 inflammasome plays a role in adipocytes during obesity, we expressed the $D^{301}N$ constitutively active Nlrp3 mutant specifically in these cells. Expression was confirmed in primary adipocytes along with the inflammasome components caspase-1 and Asc.

Nlrp3$^{D301N/+}$ Cre+ mice were equivalent in bodyweight to Cre negative controls, suggesting that Nlrp3 expression in the adipose tissue does not affect body weight or adiposity. The glucose intolerance observed in Cre expressing mice suggests that Nlrp3 is negatively impacting metabolic function within the adipocytes, in a manner that is independent of differential obesity. Further work is required to determine the functions of Nlrp3 in adipocytes. As adiponectin is expressed not only in white adipocytes, but also the brown and beige adipocyte cell-types, $D^{301}N$ Nlrp3 mutant is expected to be also induced in these cells (512). Brown and beige adipocytes play an important role in energy balance through the induction of adaptive thermogenesis and affect metabolic health (513). Further insight into activation of the inflammasome in these adipocytes may be required for a full understanding of its role in fat tissue.

Inflammasome activation can lead to the secretion of IL-1β and IL-18, pyroptosis, as well as the cleavage of other caspase-1 substrates. Whether adipocytes are capable of secreting inflammasome derived pro-inflammatory cytokines *in vivo* is unclear. Immature pro-IL-1β has been detected in the lysates of human adipocytes, but it is not clear if it is secreted (345). Some studies have detected IL-1β secretion by the murine 3T3-L1 adipocyte cell-line (514), while others have been unable to (515). Our own studies could not detect pro-IL-1β expression by Western blot in 3T3-L1 cells (Data not shown). In the obese state it is likely that adipose tissue macrophages would represent the predominant source of secreted cytokines as adipose tissue macrophages have higher expression of inflammasome components relative to adipocytes (329).

Inflammasome-dependent death of adipocytes could be another contributing factor to glucose intolerance, as adipocyte cell death has been found to play a role in insulin resistance (516). Cell death morphologically resembling pyroptosis has been observed in adipocytes (517), however a more precise readout, such as Gasdermin D cleavage, is required to verify this claim (167, 168). Caspase-1-dependent cleavage of substrates such as SIRT1 may also impact host metabolism (502). While screens have identified novel caspase-1 targets (169), these studies have not been performed in adipocytes, which may be required to detect cell-specific protein activity. The requirement of caspase-1 for sterol regulatory element binding proteins (SREBP) activation could also be involved (518). SREBPs are transcription factors that stimulate cholesterol and fatty acid biosynthesis and are required for adipocyte differentiation (519). Finally, Nlrp3 may have functions that are independent of inflammasome activation. It has been reported that Nlrp3 can act as a transcription factor in T_H2 cells (520). It is unknown if it may function similarly in other cell types such as adipocytes.

Overall, these results suggest that a full understanding of the inflammasome in metabolic disease requires examination of its role in specific tissues. The study of the inflammasome in adipocytes provides insight into disease pathogenesis and may reveal future targets for therapeutic intervention.

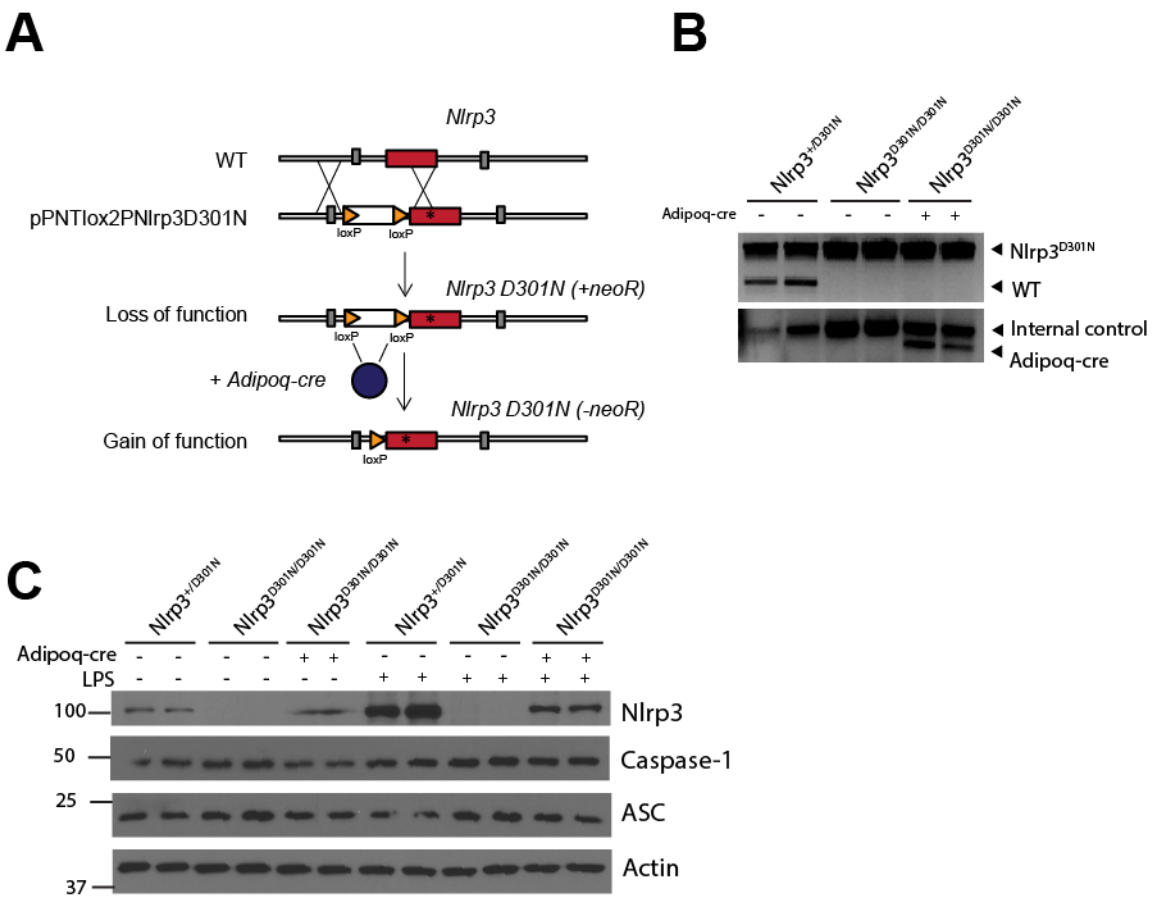

Figure 1. *Nlrp3*^{D301N} is expressed in adipocytes.
A) Diagram detailing the *Nlrp3*^{D301N} construct and Cre recombination. B) PCR Genotyping of transgenic mice. C) Primary adipocytes were differentiated *in vitro* and stimulated overnight with or without 100ng/mL LPS overnight. Cells were harvested and immunoblot performed on lysates.

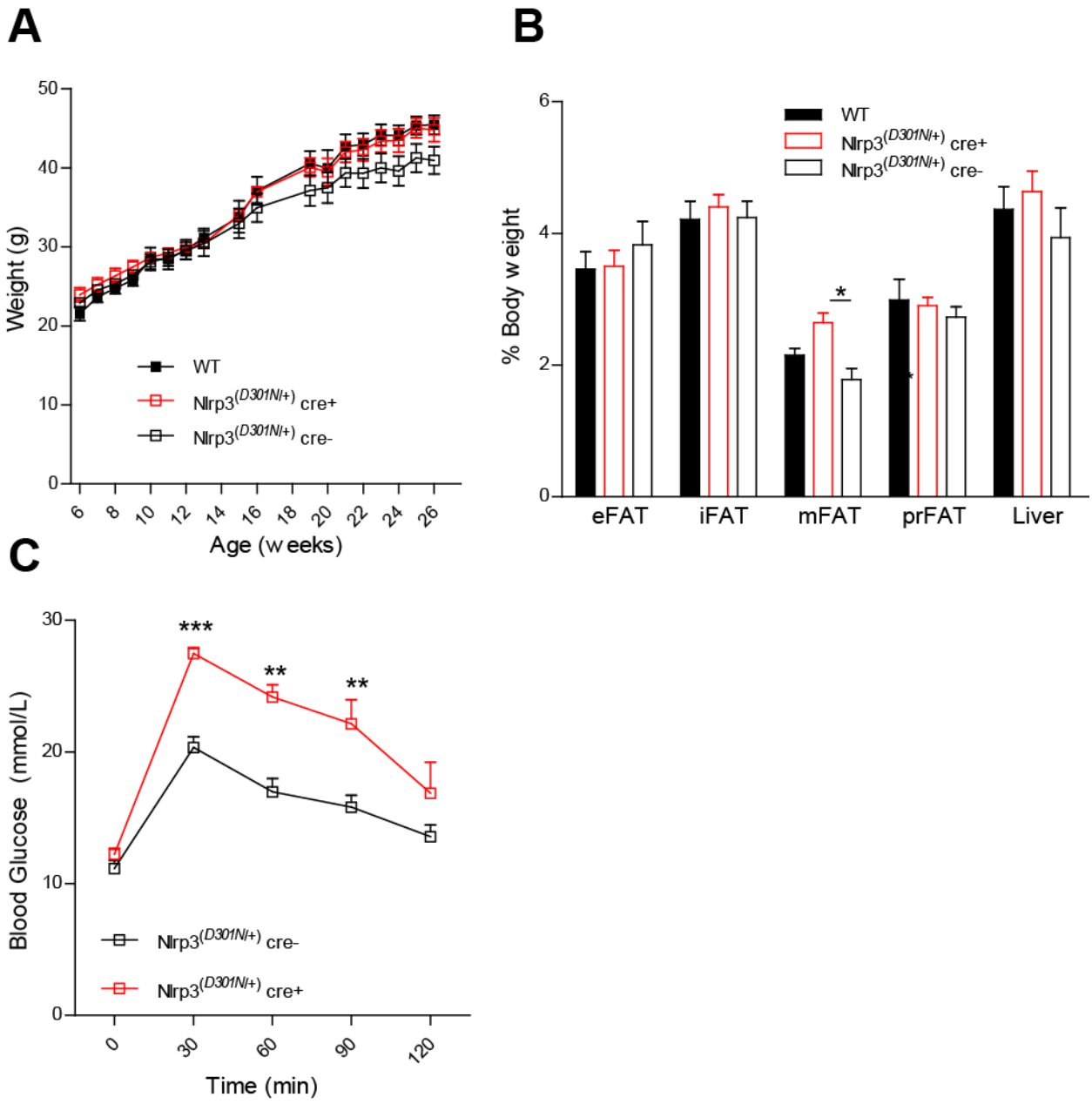

Figure 2. *Nlrp3*$^{D301N/+}$ Cre+ mice are glucose intolerant.
A) WT, *Nlrp3*$^{D301N/+}$ Cre+ and *Nlrp3*$^{D301N/+}$ Cre- mice were fed a 45% kcal lipid HFD beginning at 6 weeks of age. Weight was recorded for 20 weeks of HFD. B) The inguinal (iFAT), epididymal (eFAT), mysenteric (mFAT), and perirenal (prFAT) adipose depots and Liver were weighed after 20 weeks of HFD. C) After 13 weeks of HFD, mice were fasted for 6 hours before i.p. injection with 2g/kg glucose. Blood glucose was recorded every 30 minutes. Data represent the mean +/- SEM. Significance was determined by student's t-test. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

PREFACE TO CHAPTER 4

Cell death and inflammation are interconnected processes that play pivotal roles in disease. Previous studies in our lab have examined how these pathways contribute to inflammatory bowel disease using mouse models. Necroptosis is a recently defined regulated mode of necrotic cell death that has been indirectly linked to the development of colitis. To specifically examine a direct role of necroptosis in the pathogenesis of colitis, we investigated how absence of the central necroptosis effector Mlkl affects experimental colitis in mice.

CHAPTER 4

The necroptosis effector MLKL mediates erosive colitis

4.1 ABSTRACT

A breach in intestinal barrier integrity is a key initiating event in inflammatory bowel disease (IBD). Necroptosis of intestinal epithelial cells (IECs) has been previously described, but whether necroptosis contributes to IBD pathogenesis and how is not fully understood. To more precisely determine the role of necroptosis in IBD, we applied the dextran sulfate sodium (DSS) erosive colitis model to mice lacking Mlkl, the most downstream effector of this cell death pathway. *Mlkl*$^{-/-}$ mice were highly resistant to colitis, had reduced cell death in the colon elevated IEC proliferation. Pro-inflammatory cytokines secreted by the colons of *Mlkl*$^{-/-}$ mice were equivalent to control animals, however loss of Mlkl resulted in increased secretion of the repair cytokines IL-22 and IL-17. These results indicate that Mlkl-dependent necroptosis promotes colitis and that its inhibition may have therapeutic potential in IBD.

4.2 INTRODUCTION

Necroptosis is a regulated form of necrotic cell death that has been linked to the development of numerous diseases, affecting multiple tissues and organs (245). Activation requires assembly of the necrosome, comprised of the kinases Receptor-Interacting Protein Kinase (RIPK)1 and RIPK3 and the downstream effector Mixed Lineage Kinase Like (MLKL). This process can be triggered through various pathways such as TNFα signalling, death ligand stimulation, TLR3 or TLR4 activation of TRIF, and IFN pathways (521).

Necroptosis activation is initiated by RIPK3 recruitment to RIPK1 through RHIM domain binding, permitting the phosphorylation of RIPK3 and its activation. RIPK3 dependent phosphorylation of the pseudokinase domain of MLKL leads to a conformation change, permitting the N-terminal executioner 4 Helix Bundle (4HB) domain to be released for oligomerization (522). MLKL oligomers proceed to form pores in the plasma membrane leading to osmotic lysis.

The induction of necroptosis typically occurs following inhibition or loss of the pro-apoptotic proteins caspase-8 or FADD. Caspase-8 can cleave RIPK1 and RIPK3 to block necroptosis induction (216, 217). Mice deficient in caspase-8 or FADD have long been known to die embryonically, but can be rescued through ablation of RIPK3-dependent necroptosis (219, 233, 236). Recent studies have expanded on these results to implicate necroptosis in the development of IBD. Mice lacking caspase-8 in IECs and therefore more susceptible to RIPK3-dependent necroptosis, have basal ileal pathology, loss of Paneth cells, and are also susceptible to chemically induced colitis (5). Comparable results were observed in $FADD^{IEC\text{-}KO}$ mice which could also be rescued by ablation of the *Ripk3* gene (6).

Despite the proposed pathogenic role of necroptosis in these IEC specific models, it is important to note that genetic deletion of necrosome components not only affects necroptosis but also perturbs other signalling pathways, thus could contribute to the development of colitis indirectly. For instance, loss of *Ripk1* in IECs results in intestinal inflammation, mediated by caspase-8/FADD-dependent apoptosis (7, 9). Indeed, $Ripk1^{IEC\text{-}KO}FADD^{IEC\text{-}KO}$ compound mice are rescued

from apoptosis. However, they become susceptible to necroptotic cell death and require ablation of *Ripk3* to completely restore intestinal health (7). Mice globally ablated for the necrosome component *Ripk3* were surprisingly more susceptible to DSS-induced colitis. This phenotype was ascribed to a novel cell death-independent function of Ripk3 in dendritic cells (DCs), where it controls NF-κB-mediated production of inflammatory cytokines including IL-23 (8). Other studies have similarly identified necroptosis-independent functions of Ripk3, such as a role in inflammasome activation (523, 524), apoptosis (239, 525), and proinflammatory cytokine secretion (526). Ripk1 is also involved in multiple pathways and has similarly been linked to apoptosis and NF-κB and ERK activation (207, 527).

Preliminary evidence has suggested a potential role for necroptosis in human IBD. RIPK3 and MLKL expression are increased while caspase-8 is decreased in the intestine of patients, suggesting an association between necrosome components and intestinal inflammation (528). Biopsies also indicate RIPK3 induction may specifically correlate with Paneth cell death (5). Inhibition of the necroptosis inducing cytokine TNFα by the monoclonal antibody Infliximab has demonstrated positive therapeutic outcomes and is approved by the FDA for CD patients (529).

While these reports have suggested a role of necroptosis in colitis in mice and IBD in humans, the results are difficult to interpret because of necroptosis-independent roles of RIPK1 and RIPK3, namely in apoptosis, NF-κB activation, and potentially others pathways. To study necroptosis in the absence of these confounding factors, mice deficient in *Mlkl* represent a more suitable model, based on our current understanding of its necroptosis-restricted functions.

In this study, we identify an important role for Mlkl in mediating the development of colitis in mice. *Mlkl*$^{-/-}$ mice exhibited reduced cell death in the ileum and colon coupled with elevated production of the repair cytokines IL-22 and IL-17. Consequently, increased IEC proliferation was observed in the colon of *Mlkl*$^{-/-}$ mice. These results specifically implicate necroptosis as an important regulator of colitis.

4.3 MATERIALS AND METHODS

Animal experiments

Mice were housed at room temperature with a 12 hour light/dark cycle with food and water provided *ad libitum*. Only male mice, aged 6-12 weeks were used. *Mlkl*$^{-/-}$ mice were obtained from Jiahuai Han, Xiamen University, under MTA (530). WT C57/BL6 mice and *Mlkl*$^{-/-}$ mice were maintained separately bred colonies. All experiments were performed under guidelines of the animal ethics committee of McGill University (Canada).

DSS Colitis model

To induce experimental colitis in mice, DSS (55 000 kDa, MPBiomedical Cat# 160110) was added to the mouse drinking water at a concentration of 5% (w/v). After 5 days of DSS treatment, mice were either sacrificed or administered normal drinking water for another 2 days before sacrifice. The animals were monitored and weighed daily during treatment.

Western Blots

Tissues were lysed in buffer B150 (20 mM Tris-HCl pH 8.0, 150 mM KCl, 10% glycerol, 5 mM MgCl2, and 0.1% NP40) supplemented with Complete-mini protease inhibitors (Roche Applied Science, Cat# 11836153001) and phosphatase inhibitors (Sigma Cat# S7920, 71768, G6376). Protein lysates were separated on SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with antibodies against Ripk1 (BD #610459), Ripk3 (ProSci #2283), MLKL (Abcam #172868), cleaved caspase-3 (Cell Signaling #9661), cleaved caspase-8 (Cell Signaling #9429), B-actin (Sigma, Cat# A1978), cyclin D1 (Thermo Fisher Scientific #SP4), and GAPDH (Santa Cruz sc-25778).

Colon organ culture and ELISAs

Approximately 0.5 cm^2 sections of colon tissue were weighed and incubated overnight in OPTI-MEM (ThermoFisher) culture media. Cytokines were determined using the following ELISA kits: IL-6 (R&D, Cat# DY406), IL-1β (R&D, Cat# DY401), IL-23 (R&D, Cat# DY1887), IL-22 (R&D, Cat# DY582), IL-11 (R&D, Cat# DY418), IL-17 (R&D, Cat# DY421).

Dendritic cell culture

Bone marrow was obtained by flushing the femurs and tibias of WT and *Mlkl*$^{-/-}$ mice. Cells were cultured in non-tissue culture-treated dishes and differentiated into dendritic cells at 37°C 5% CO_2 for 10 days in RPMI-1640 media (containing 10% heat-inactivated FBS, 2 mM glutamine, 100 μg/ml penicillin/streptomycin, 50 μM 2-mercaptoethanol). 20 ng/mL GM-CSF (Peprotech #AF-315-03) was added to the media on Days 0, 3, 6 and cells were treated on Day 10 with LPS (5 ng/mL).

Hematoxylin and eosin staining

Colons were fixed in 10% buffered formalin overnight, paraffin embedded, and cut and processed for hematoxylin and eosin staining. Sections were digitally scanned using a ScanScope XT digital scanner (Leica). ImageScope software (Leica) was used to quantify colon pathology. The length of healthy, damaged, or fully eroded crypts was measured to assess colon damage.

Immunohistochemistry

Paraffin embedded slides were dewaxed with xylene and rehydrated in ethanol beginning at 100%, followed by 90%, 70%, and 50% ethanol for 5 min each. The slides were then incubated in water for 5 min before a 95°C incubation for 15 min in a 0.1 M citrate buffer (pH 6.0) for antigen retrieval. Slides were permeabilized with 0.25% Triton X-100 in PBS for 20 min at room temperature. Click-iT TUNEL Alexa Fluor 647 (Life Technologies, Cat no. C10247) staining was performed according to the manufacturer's instructions. Slides were then blocked (10% fetal bovine serum and 3% bovine serum albumin) for 30 min at 37°C and tissues were incubated with primary antibodies in PBS containing 3% bovine serum albumin overnight at room temperature. The following antibodies were used: PCNA (Abcam, Cat no. AB2426), active caspase-3 (R&D, Cat no. AF835), and E-cadherin (BD Bioscience, Cat no. 610182). Slides were then washed and incubated with secondary antibodies coupled with AlexaFluor 488, 594, or 647 (Invitrogen). Finally, nuclei were stained with Hoechst 33342 (Invitrogen, Cat# H3570). Slides were imaged on a Zeiss Axioskop upright wide-field microscope. Analysis of images was performed using Volocity 6.0 software (Quorum Technologies). For PCNA analysis, the E-cadherin positive crypt area was selected and the total number of PCNA+ nuclei calculated as a percentage of total nuclei. For each colon, 2-6 fields of view were analyzed, containing greater than 500 nuclei each.

For quantification of cell death, apoptosis (caspase-3+) and TUNEL (late apoptosis/non-apoptotic death) cells were quantified. A minimum of 3 fields of view were analyzed per colon.

4.4 RESULTS

Mlkl$^{-/-}$ mice are resistant to DSS-induced colitis

Recent evidence has implicated necroptosis in the pathogenesis of colitis, but the genetic models used are difficult to interpret as the effect of necroptosis is confounded by functions of RIPK1 and RIPK3 in other pathways. We aimed to specifically study necroptosis in the development of colitis through the use of Mlkl deficient mice. *Mlkl*$^{-/-}$ and WT mice were administered 5% (w/v) DSS in the drinking water for 5 days to induce erosive colitis followed by 2 days of normal drinking water for recovery. DSS-treated *Mlkl*$^{-/-}$ mice had reduced bodyweight loss and increased survival compared to WT controls (Figure 1A, B). Interestingly, this phenotype was independent of differences in colon length or cecum weight, indicators commonly associated with intestinal inflammation, both on day 7 (Figure 1C, D) and day 5 (Figure S1).

Mlkl$^{-/-}$ mice have reduced colon pathology and cell death

H&E stained colon tissue sections from WT and *Mlkl*$^{-/-}$ mice were assessed for tissue damage and crypt erosion. *Mlkl*$^{-/-}$ mice had relatively healthier tissue with fewer fully eroded crypts on Day 7 compared to WT mice (Figure 2A). As the DSS model is associated with cell death in the colon, colon tissue sections were next stained for the cell death markers active caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (Figure 2B). Cell death was assessed on experimental day 5, as we have previously shown that this time point is the cell death peak in this model (531). Caspase-3 activation is indicative of apoptotic cell death, while TUNEL staining will reveal late apoptosis as well as necrosis. In contrast to colon tissue sections from WT mice, those from *Mlkl*$^{-/-}$ mice exhibited decreased cell death staining including decreased numbers of TUNEL positive cells, potentially indicating reduced necroptosis.

Absence of Mlkl leads to accumulation of Ripk1 and Ripk3 in the colon

To further understand why *Mlkl*$^{-/-}$ mice were resistant to DSS colitis, we analyzed colon lysates of DSS-treated animals by immunoblot. There were no differences in apoptosis markers, namely cleaved caspase-3 or caspase-8, on Day 7 post-DSS (Figure S2). In contrast, we observed increased levels of the necrosome components Ripk3 and Ripk1 in the colon of *Mlkl*$^{-/-}$ mice

(Figure 3A). Elevated expression of these proteins was not observed in the ileum, consistent with lack of DSS-induced pathology outside of the colon (Figure 3B) (532).

Secretion of IL-17 and IL-22 is elevated in the colon of *Mlkl*$^{-/-}$ mice

To assess the extent of colonic inflammation post DSS, we performed colon organ culture and analyzed pro-inflammatory cytokines secretion (Figure 4A-G). DSS treatment induced increased cytokine secretion compared to untreated conditions, however there were no significant differences in the levels of IL-6, KC, IL-1β, IL-23, or IL-11 between genotypes, as determined by ELISA. In contrast, we did observe heightened production of the repair cytokines IL-17 and IL-22 (438, 439, 479, 483) in colon organ cultures from *Mlkl*$^{-/-}$ mice compared to WT controls. Augmented production of IL-22 in the colon of *Mlkl*$^{-/-}$ mice was confirmed by qPCR analysis and was independent of differential expression of the IL-22 antagonist IL-22BP or its inducer IL-23 (Figure 4H). To exclude a necroptosis-independent function of Mlkl in cytokine production, as was recently demonstrated for Ripk3 (8), we cultured primary bone-marrow derived dendritic cells (BMDCs) and treated them with LPS with or without the Nlrp3 inflammasome agonist nigericin to induce IL-1β secretion (Figure S3). No differences were observed between genotypes in IL-1β, IL-23, or IL-6 cytokine production. This result combined with the equivalent levels of these cytokines in the colon (Figure 4A-D) suggests that factors inducing IL-22 or IL-17 production are not equivalently production, independently of Mlkl expression.

***Mlkl*$^{-/-}$ mice have increased colonic epithelium repair**

Given that IL-17 and IL-22 are linked to intestinal regeneration, we determined if tissue repair may play a role in the increased resistance to DSS of *Mlkl*$^{-/-}$ mice. Colon tissue sections stained for the mitotic marker proliferating cellular nuclear antigen (PCNA) revealed increased proliferating cells in the crypts of *Mlkl*$^{-/-}$ mice compared to WT controls (Figure 5A). Cyclin D1, an additional marker of cellular proliferation, was also elevated in colon lysates of *Mlkl*$^{-/-}$ mice, as evaluated by immunoblot, further suggesting that epithelial repair was enhanced in the absence of Mlkl. Taken together, these results suggest that Mlkl-induced cell death, which is associated with reduced levels of intestinal protective cytokines, is pathogenic in DSS colitis.

4.5 DISCUSSION

The incidence of inflammatory bowel disease has increased since the second half of the 20th century and there is a growing demand for improved therapies. Experimental mouse models provide an informative tool to study the function of potential pathways of pathogenesis and therapeutic targets. Recent studies have indirectly implicated necroptosis in IBD. In this study, we assessed the role of Mlkl in a murine experimental model of erosive colitis. We present evidence that show that ablation of this restricted pro-necroptotic factor rendered mice highly resistant to DSS-induced colitis.

The induction of compensatory proliferation is essential for intestinal tissue repair following DSS-induced damage. Among the primary repair cytokines in the colon, IL-22 induces epithelial proliferation through STAT3 activation (479, 483), while IL-17 induces the expression of tight junction proteins, reducing barrier permeability (438, 439). The elevated levels of these two cytokines in the colon of *Mlkl*$^{-/-}$ mice correlated with the increased number of proliferating IECs. IL-22 and IL-17 are primarily secreted by Retinoic-acid-receptor-related orphan receptor gamma t (RORγt)-positive immune cells including type 3 ILCs and T helper 17 (T_H17) cells (470, 533, 534). ILC3s are thought to be the primary source of IL-22 in the colon (417, 438, 535), while γδ T cells are mainly responsible for IL-17 production (438). While DCs from *Ripk3*$^{-/-}$ mice were reported to have defects in NF-κB-mediated production of IL-1β and IL-23, these responses were unaffected in DCs from *Mlkl*$^{-/-}$ mice. Bone marrow derived macrophages (BMDMs) from these mice also have a normal NF-κB response, confirming the proposed restriction of Mlkl function to necroptosis (530). As no differences were observed between WT and of in *Mlkl*$^{-/-}$ mice in IL-1β or IL-23, which act upstream of IL-22 and IL-17 induction, the heightened production of the latter in *Mlkl*$^{-/-}$ mice may instead be the result of decreased necroptosis of immune cells involved in their production.

Immunoblot analysis of the colon revealed elevated expression of Ripk1 and Ripk3 in *Mlkl*$^{-/-}$ mice. Induction of these kinases is associated with necroptosis, and overexpression of Ripk3 alone can directly induce this cell death modality (223). Cells potentially primed for death by this pathway may now persist due to the absence of Mlkl. Reduced markers of cell death were also

detected in the colon of *Mlkl*$^{-/-}$ mice, including fewer TUNEL positive cells, potentially representing cells dying of necroptosis. Consistently, T cells were shown to be susceptible to this mode of cell death (536, 537). The recent generation of an antibody that recognizes the phosphorylated active form of murine MLKL combined with immune cell markers may represent the best method to detect necroptosis in these cell types.

Necroptosis inhibitors have shown considerable therapeutic potential in animal models of various human diseases (245). Inhibitors targeting Ripk1 or Ripk3 kinase activity have been developed, but have yet to proceed beyond preclinical testing. It is now predicted that targeting these kinases may lead to unintended consequences, given their recently uncovered pleiotropic functions. For example, Ripk3 inhibitors were reported to induce apoptosis (525), and as mentioned earlier might neutralize the function of Ripk3 in intestinal tissue repair (8).

Our results point to MLKL as a potential therapeutic target for IBD. MLKL inhibitors have not been reported to perturb necroptosis-independent pathways. Thus, these compounds, may provide more viable options for human therapy (538).

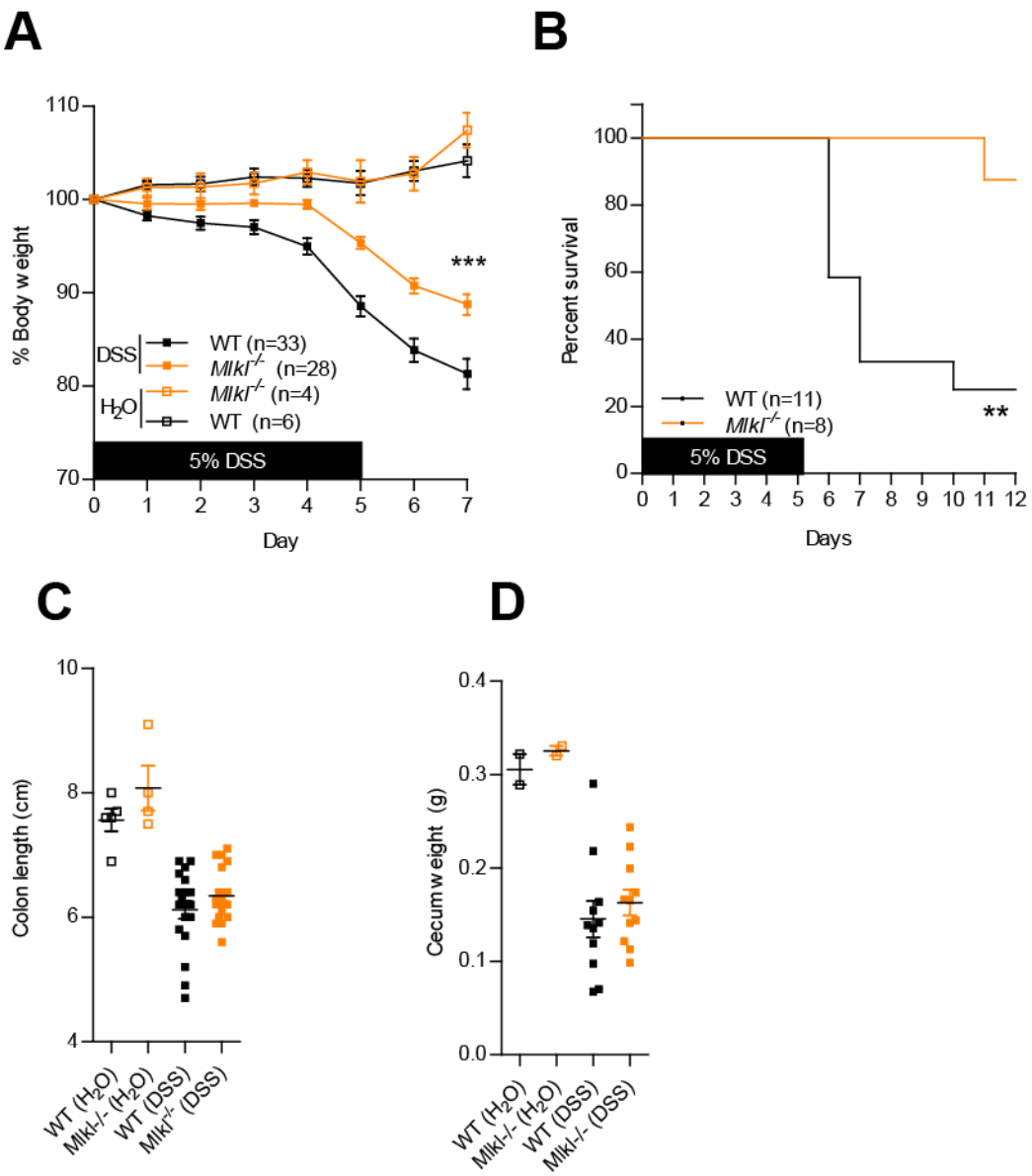

Figure 1. *Mlkl*$^{-/-}$ mice are resistant to DSS-induced colitis.
A) WT and *Mlkl*$^{-/-}$ mice were administered 5% DSS in the drinking water for 5 days followed by a 2 day recovery period. Results are pooled from 3 independent experiments. B) Kaplan-Meier survival analysis of DSS treated mice followed by 7 day recovery period. C) Colon length of mice sacrificed on experimental Day 7. D) Cecum weight of mice sacrificed on experimental Day 7. Data represent the mean +/- SEM. Significance was determined by student's t-test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

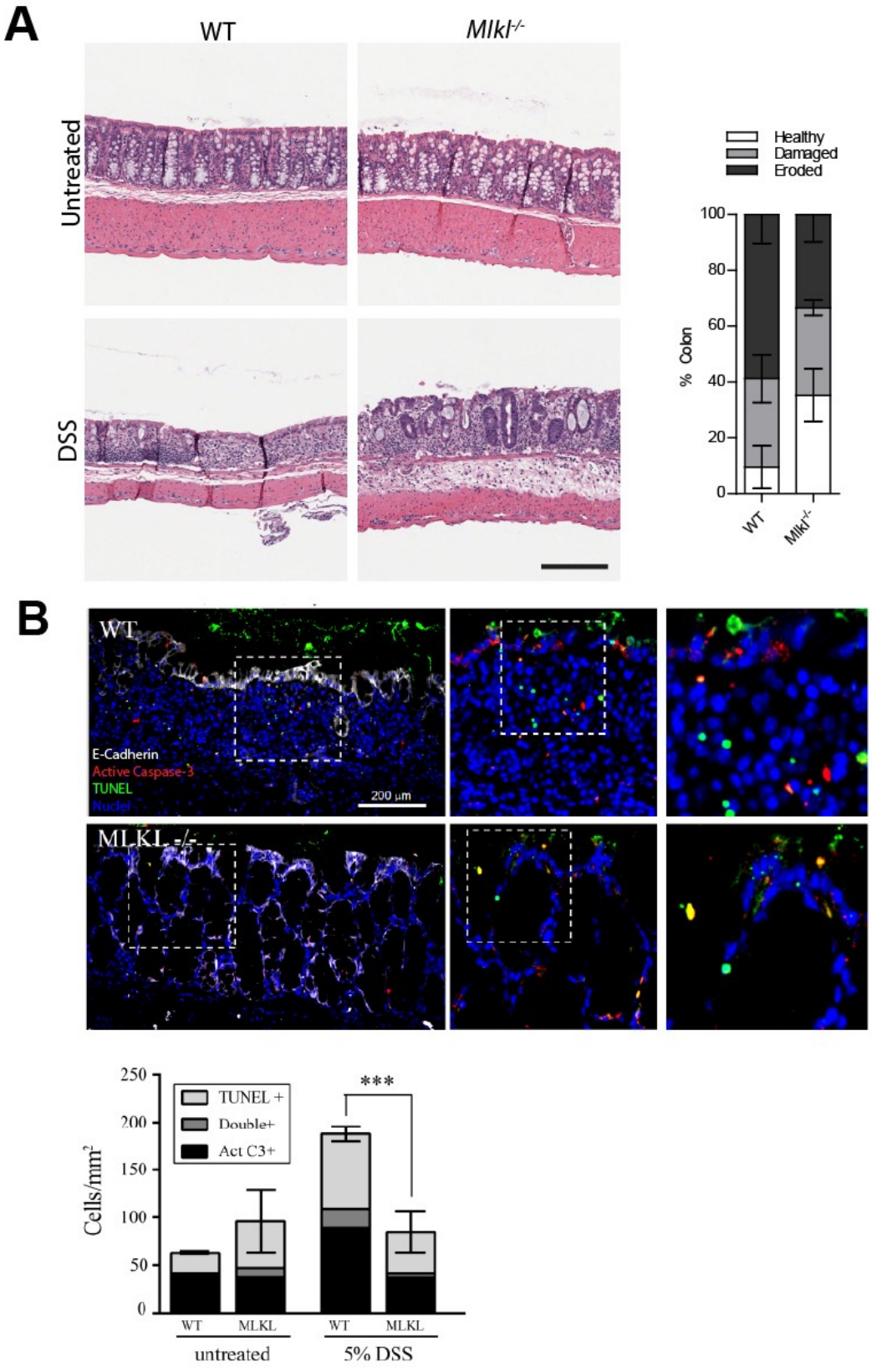

Figure 2. The colons of *Mlkl*$^{-/-}$ mice have reduced pathology and cell death.

Figure 2. The colons of *Mlkl*$^{-/-}$ mice have reduced pathology and cell death.
A) Colon sections from WT and *Mlkl*$^{-/-}$ mice on experimental Day 7 were fixed and H&E stained. The percentage of healthy, damaged, and eroded crypts for each colon was quantified. Scale bar represents 200 μm. B) Colon sections from WT and *Mlkl*$^{-/-}$ mice on experimental Day 5 were stained for E-cadherin (White), active caspase-3 (Green), TUNEL (Red), and Hoeschst (Blue). The total number of active caspase-3, TUNEL, and active caspase-3/TUNEL double positive cells was quantified in 3 fields of view per mouse. n=3-4 for untreated mice and n=7-8 for DSS treated mice. Data represent the mean +/- SEM. Significance was determined by student's t-test. ($^{***}p < 0.001$).

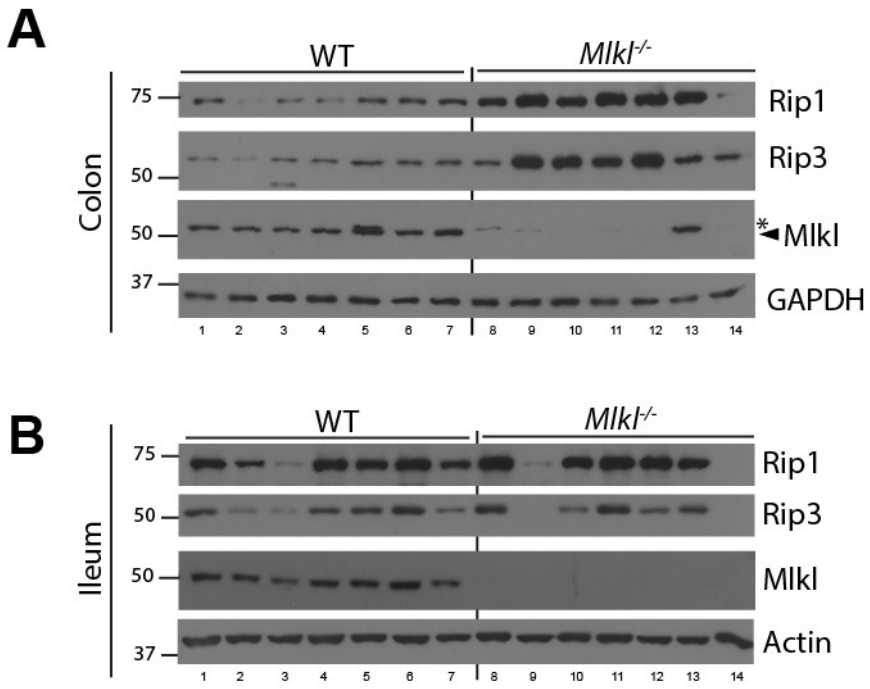

Figure 3. Ripk1 and Ripk3 are induced in the colon of *Mlkl*$^{-/-}$ mice after DSS.
A) Colon lysates from experimental Day 7 mice were immunoblotted for necrosome proteins. B) Ileum lysates from Day 7 mice were immunoblotted for necrosome proteins. * indicates a non-specific band.

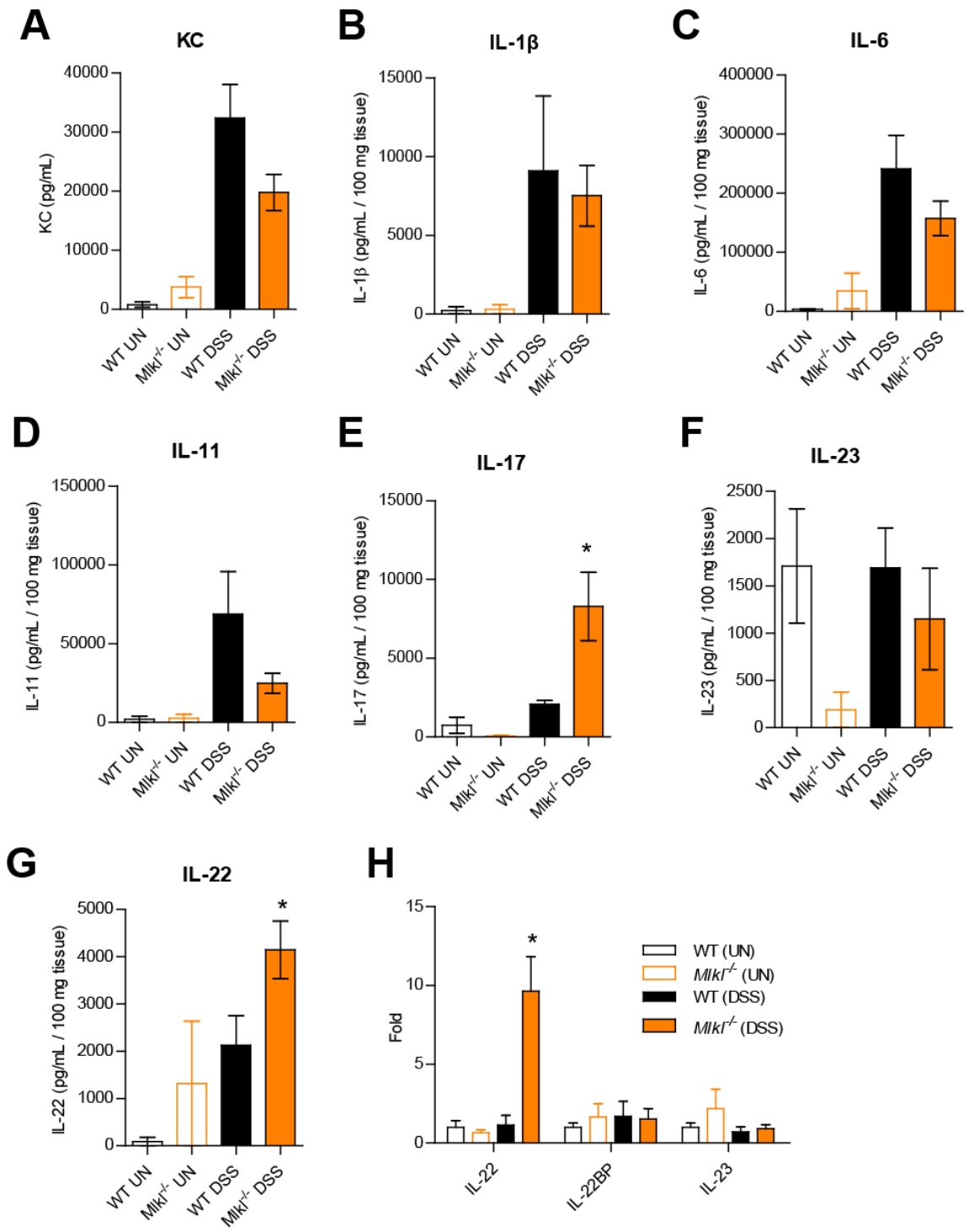

Figure 4. Augmented production of IL-22 and IL-17 in the colon of *Mlkl*$^{-/-}$ mice.
A-G) A ~0.5cm^2 section of the distal colon from Day 7 mice was cultured overnight in media and secreted cytokines were quantified by ELISA. H) qPCR analysis of colon lysates from Day 7 mice. Data represent the mean +/- SEM. Significance was determined by student's t-test. ($^*p < 0.05$).

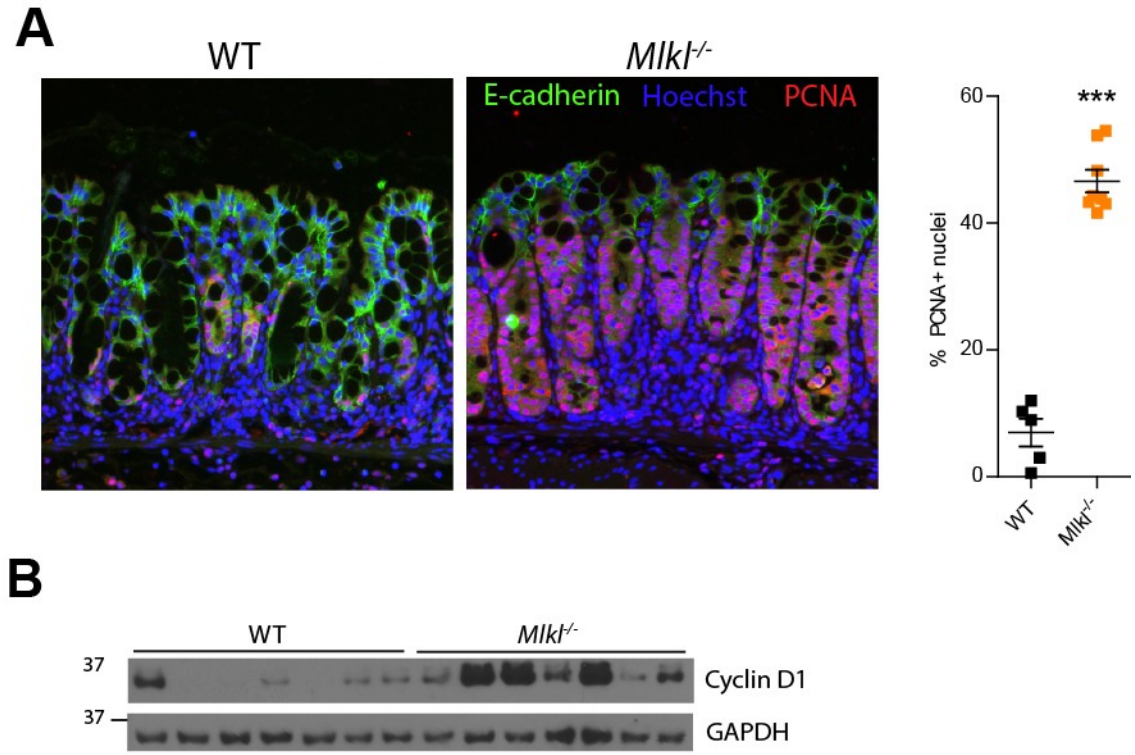

Figure 5. *Mlkl*$^{-/-}$ mice have elevated intestinal epithelial regeneration.
A) Colon sections from WT and *Mlkl*$^{-/-}$ mice on experimental Day 7 were stained for E-cadherin (Green), PCNA (RED), and Hoeschst (Blue). The number of PCNA positive nuclei within the E-cadherin positive crypt region was counted and expressed as a percentage. Each data point represents an individual mouse. B) Colon lysates from experimental Day 7 mice were immunoblotted. Data represent the mean +/- SEM. Significance was determined by student's t-test. ($^{***}p < 0.001$).

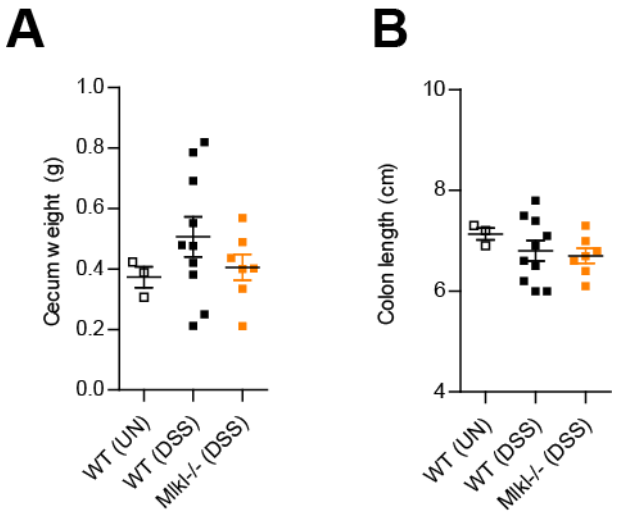

Figure S1. *Mlkl*$^{-/-}$ mice have equivalent colon length and cecum weight on Day 5 post-DSS. A-B) WT and *Mlkl*$^{-/-}$ mice were administered 5% DSS in the drinking water for 5 days before being sacrificed. Data represent the mean +/- SEM.

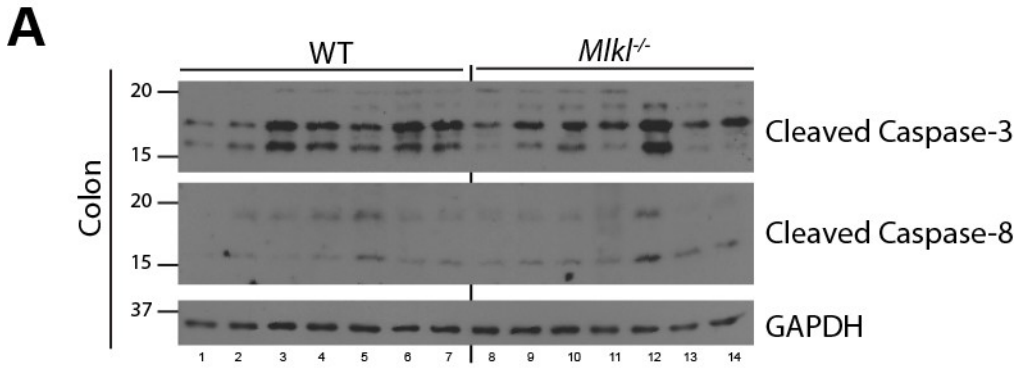

Figure S2. Colon caspase-8 and caspase-3 levels are equivalent in WT and *Mlkl*$^{-/-}$ mice after DSS.
A) Colon lysates from experimental Day 7 mice were immunoblotted for necrosome proteins.

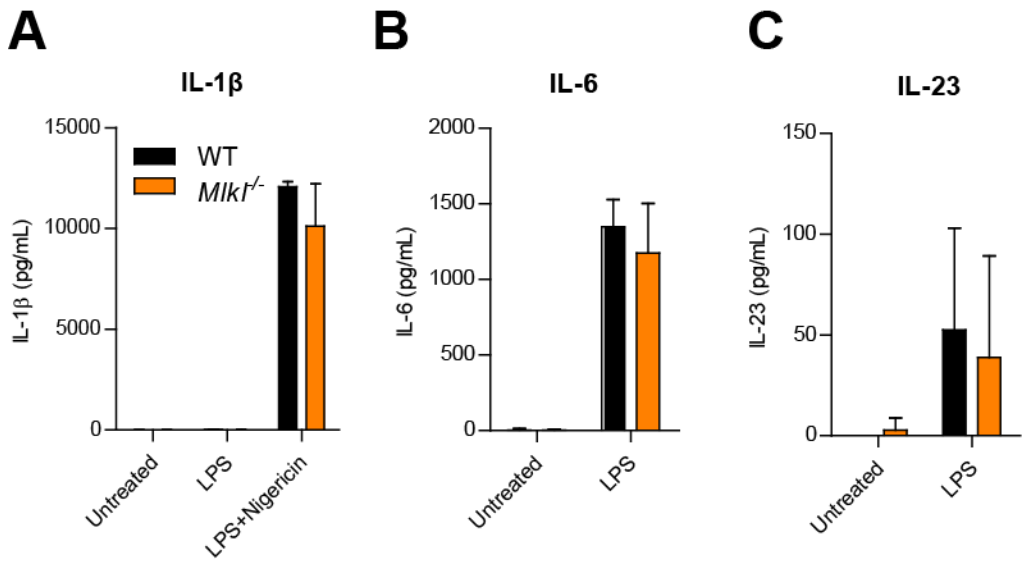

Figure S3. BMDCs from *Mlkl*$^{-/-}$ mice have equivalent pro-inflammatory cytokine production compared to control BMDCs from WT mice

A-C) BMDCs were cultured *in vitro* and stimulated with 5 ng/mL LPS for 4 hours followed by 20 μM Nigericin for 1 hour. Data represent the mean +/- SEM.

CHAPTER 5 – DISCUSSION

The innate immune response and cell death pathways play pivotal roles in the host response to infection. Physical barriers, mucosal surfaces, AMPs, and various immune cell subsets provide initial protection against microbes and separate the host from the environment. Cell death eliminates microbial replication niches to prevent further dissemination. Innate immunity and cell death are thus tightly interconnected and can be activated by similar stimuli and regulated by common proteins. Careful control of these processes is required to prevent inappropriate activation that can lead to the development of inflammatory disorders and autoimmune diseases. The work of this thesis examines how inflammation and cell death are engaged in metabolic disease and IBD.

5.1 The role of caspase-12 in metabolic disease and inflammation

The inflammasome and caspase-1 respond to foreign and endogenous signals to initiate an inflammatory response. Activation of this pathway has been determined to have metabolic consequences in the obese state and can lead to insulin resistance and T2D (1). In chapter 2 of this thesis, we assessed the effect of two members of the inflammatory caspase sub-family, caspase-11 and -12, in murine models of metabolic disease. As the *Casp12*$^{-/-}$ strain generated with 129 mouse embryos also lacked caspase-11, we obtained a second C57/BL6 *Casp12*$^{-/-}$ *Casp11*$^{+/+}$strain as well. Loss of caspase-12 in both strains of mice resulted in obesity, insulin resistance, and adipose tissue inflammation while *Casp11*$^{-/-}$ mice were equivalent to controls.

Interestingly, we found no striking metabolic abnormalities or changes in caloric intake of the *Casp12*$^{-/-}$ mice to explain their increased weight gain. It is possible that this may be due to subtle differences accumulating over an extended period of time that were not detected. This explanation could be tested by feeding the *Casp12*$^{-/-}$ mice and control animals an equivalent daily amount of food as opposed to *ad libitum*. If the caspase-12 deficient mice continued to become obese this would imply an increased extraction of calories from the food or a reduction in energy consumption.

The obese phenotype of the *Casp12*$^{-/-}$ mice could be rescued by ablation of *Nlrp3*. It is difficult to determine if caspase-12 is acting directly within the Nlrp3 inflammasome pathway or in a parallel pathway. Some insight into this question could be achieved through a bone marrow chimera experiment to determine in which compartment Nlrp3 is required. An interesting possibility is that both genes may affect the composition of the microbiome. Preliminary sequencing of fecal samples from *Casp12*$^{-/-}$ mice suggested differences in certain families of bacteria compared to WT mice (Data not shown). Dysbiosis has been demonstrated to be a causative factor of obesity in people and mice (539). Interestingly, deficiency in inflammasome components has been demonstrated to allow colonization by microbiota that can directly affect bodyweight of the host (330). Therefore a shift in the intestinal bacteria of *Casp12*$^{-/-}$*Nlrp3*$^{-/-}$ mice could offer an explanation for the reduction in adiposity of these mice.

5.2 A role for caspase-12 in humans

The majority of the human population is fixed for a non-functional truncated caspase-12 allele, though approximately 20% of people of African descent express the full length gene (196). It has been estimated that the inactive allele arose ~100–500 thousand years ago due to positive selection as opposed to neutral genetic drift (540). Resistance to sepsis has been hypothesized to be a driving factor accounting for the evolutionary loss of caspase-12 (189). Mouse models and *in vitro* studies have implicated an anti-inflammatory role for caspase-12 in regulating the inflammasome, NF-κB activation, and NOD signalling (189, 191, 192). However, despite these observed anti-inflammatory effects, the role of caspase-12 in humans is controversial. In recent studies, the caspase-12 genotype was reported to confer no effect on clinical parameters related to rheumatoid arthritis, malaria infection, community-acquired pneumonia, candidemia, or systemic lupus erythematosus (198-200, 541, 542).

In chapter 2 of this thesis we sought to determine if the loss of Caspase-12 in humans correlated with the metabolic disease found in *Casp12*$^{-/-}$ mice. To do this we collaborated with two teams of investigators who genotyped their patient cohorts for the $T^{125}C$ SNP encoding full length caspase-12. The $T^{125}C$ SNP correlated with reduced serum and adipose tissue inflammatory markers in the Yale Pediatric Obesity cohort, a similar trend to what we observed in mice. To expand on these findings, a more in depth analysis of inflammation in the adipose tissue would

be interesting. For example, an assessment of inflammasome activation would determine if this pathway was similarly elevated in humans as in mice. The elevated numbers of macrophages observed in caspase-12 deficient humans could also be separated and studied after liposuction biopsies. While it did not appear that caspase-12 was expressed in immune cells infiltrating the adipose tissue from our bone-marrow chimera experiment, it would be important to see if this was also true in human cells. It is difficult to determine if the $T^{125}C$ genotype would have any influence on proposed therapy for these patients, however these findings suggest that they may be less suitable candidates for anti-inflammatory medication to treat metabolic disease.

In spite of this correlation with adipose tissue inflammation, the results from the Dallas Heart Study suggested that caspase-12 did not influence metabolic parameters such as serum glucose, serum triglycerides, or insulin resistance. While we cannot precisely determine the reason for this discrepancy with our mouse data, there are multiple possible explanations. In contrast to the murine homolog, human caspase-12 lacks catalytic activity and is repressed by estrogen. Interestingly, correlation of the $T^{125}C$ SNP and reduced inflammation from the Yale cohort was only observed in male obese teenagers, but not female. Caspase-12 expression was not quantified in the Yale study however, and it is unknown if reduced levels in the female patients could explain this gender difference. This inhibitory role of estrogen was determined through the generation of mice over-expressing human caspase-12 in a *Casp12*$^{-/-}$ background (191). It would be interesting to determine if these mice were protected from obesity, similarly to WT mice expressing the murine protein. Other unknown host specific differences between the proteins may also have an effect.

The mouse is a model organism used to study human disease that has many advantages. Mice have shorter life spans, reproduce quickly, are relatively cost-effective, and can be easily genetically manipulated. However, findings in mice do not always correlate with human data, especially regarding complex diseases that are dependent on multiple factors as is the case with obesity. Inbred mice lack the genetic diversity of humans and are maintained in a carefully controlled environment. They are fed a constant diet and have equal opportunities for exercise. These variables are impossible to control for in a clinical cohort and can cloud results. Therefore subtle changes that may produce an observable phenotype in mice may be unapparent in similar

human studies because of confounding factors. Due to the relatively low expression of the functional $T^{125}C$ caspase-12 allele a large cohort is required to obtain an adequate sample size of patients, especially regarding homozygous individuals. Future clinical studies are required to confirm the DHS data and confirm if caspase-12 affects obesity and metabolic disease in humans.

Overall the discrepancies between murine and clinical studies indicate that the striking immunoregulatory and metabolic effects of caspase-12 in mice and *in vitro* may not directly translate to humans or are severely dampened. Therefore there is a lack of overwhelming evidence to suggest why caspase-12 was originally lost in the human population or why it has been maintained by a small percent of people primarily in Africa. In recent years, significant progress has been made in our understanding of the other inflammatory caspases, caspase-1 and -11. Our efforts to decipher the function of caspase-12 in humans have fallen behind and warrant future studies to identify a potential physiological role.

5.3 Inhibiting the inflammasome as a therapeutic strategy for metabolic disease

Given the widespread activation of the inflammasome in numerous diseases there has been a great deal of interest in inhibiting it and its downstream effects. The results of Chapters 2 and 3 of this thesis implicate a role for inflammasome activation as a causative factor in metabolic disease. The inflammasome can be targeted at multiple checkpoints including component recruitment and complex formation, caspase-1 activation, as well as downstream targets such as IL-1β. Inhibitors have been developed to repress inflammasome activity at all three of these junctures and tested in pre-clinical and clinical studies (543).

As NLRP3 is the primary inflammasome PRR primarily responsible for obesity driven inflammation, antagonists targeting its activation may be of particular use to treat T2D and insulin resistance (1). Glyburide is a sulfonylurea compound that blocks potassium-ATP channels in pancreatic β-cells and has been prescribed to treat insulin resistance (544). Interestingly, it was also demonstrated to inhibit the Nlrp3 inflammasome, though it is unknown to what extent its anti-inflammatory abilities confer its efficacy (545). Other inhibitors such as

MCC950 and β-hydroxybutyrate have also been demonstrated to repress the Nlrp3 inflammasome in preclinical studies and may have potential as therapeutic agents (546, 547). Caspase-1 inhibitors have also been tested clinically though none have been approved for use. Pralnacasan is an inhibitor that was demonstrated to be effective in treating metabolic disease in a mouse model of obesity (4). The drug has undergone clinical trials for inflammatory diseases such as rheumatoid arthritis and osteoarthritis, but despite showing promising anti-inflammatory activity in phase 1 and 2 trials, it was withdrawn due to the development of liver toxicity in mice (548).

IL-1β release occurs downstream of inflammasome activation and has been linked to multiple inflammatory diseases (549). There are multiple strategies that have been developed to block activity of the cytokine *in vivo*. These include the use of recombinant IL-1Ra and neutralizing monoclonal antibodies (543). Anakinra is a recombinant IL-1Ra that is currently approved for the treatment of rheumatoid arthritis and has been successful in treating joint disease and CAPS patients (550). Its ability to resolve aspects of metabolic disease has also been tested. In a randomized placebo-controlled trial, anakinra administration has been demonstrated to increase insulin production and reduce levels of the inflammatory biomarkers CRP and IL-6 in T2D patients (372). The benefits of IL-1 blockade were still evident 39 weeks after treatment had concluded in a follow-up study (505). Other studies have also indicated an improvement in pancreatic β-cell function and insulin secretion (551, 552). These beneficial effects may be attributed to the blunting of IL-1β toxicity to β-cells (504).

The short half-life of anakinra necessitates a daily injection protocol, however monoclonal antibodies targeting IL-1β have extended half-lives and represent an alternate method for curtailing IL-1 signalling. IL-1β neutralizing monoclonal antibodies such as Canakinumab and Cevokinumab have shown promising results in clinical tests to increase insulin secretion in T2D patients (553, 554). A large scale study named Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) is currently underway to confirm the involvement of IL-1β in atherosclerosis and diabetes development. It will assess the effects of Canakinumab administration in a cohort of 17,200 stable post myocardial infarction patients over a 4 year period (555). A beneficial effect will be an important step in gaining approval for inflammasome pathway anti-inflammatories in the treatment of metabolic disease.

5.4. Necroptosis in colitis and disease

Necroptotic cell death has been linked to multiple diseases (245). Recent work over the past few years has linked IEC death by this pathway as a contributing factor to colitis (5-7, 9, 528). In chapter 4 we examined the role of the currently identified most downstream effector molecule of necroptosis, Mlkl, for its involvement in IBD. The DSS model supported a detrimental role for Mlkl in the development of experimental colitis. While intestinal cell death was reduced in *Mlkl*$^{-/-}$ mice, elevated levels of the repair cytokine IL-22 and IEC proliferation were likely contributing factors to their survival.

The benefits of IL-22 induced healing in murine colitis is well known (479, 483), however it remains to be seen if the cytokine may offer therapeutic benefit. Similarly to mouse models of colitis, IL-22 is elevated in CD and UC patients (556). Initial reports suggested that it is produced by CD4+ T cells and signals subepithelial myofibroblasts to promote the release of pro-inflammatory cytokines. Elevated numbers of IL-22^{+} immune cells correlate with reduced mucosal inflammation in CD (557). A potential therapeutic role for IL-22 was demonstrated in a case-study involving a UC patient who infected himself with the helminth *Trichuris trichiura* (558). Helminth infection typically invokes a T_H2 response (559) and has been previously used in clinical trials to treat IBD (560). In this study, the helminth induced the appearance of IL-22+ helper T cells and remission of UC (558). Further insight into the function of IL-22 in human disease is required to determine if this pathway is suitable for IBD treatment. The results of

chapter 4 of this thesis suggest that IL-22$^+$ cells may be susceptible to necroptotic cell death and a combinational therapy involving necroptosis inhibitors and IL-22 inducers might thus be beneficial.

5.5 Common factors affecting both IBD and metabolic disease

The prevalence of obesity driven metabolic disease and IBD have steadily increased over the last half century and are associated with a Western lifestyle. The pathogenesis of both types of disorders requires chronic inflammation and deregulation of the immune system. Though IBD and insulin resistance share some common susceptibility loci, there is a lack of studies that indicate a strong genetic correlation between the developments of the two disorders (561). However, there have been reports suggesting an association between CD and insulin resistance or obesity (562, 563). These complex diseases share multiple contributing factors such as the intestinal microbiome, diet, and hormone signaling.

There has been an increasing focus on examining how the microbiota affects both metabolic disease and IBD. Intestinal permeability and elevated serum levels of microbial motifs, a consequence of obesity or colitis, have been linked to insulin resistance (324). In general obesity and IBD both lead to a reduction in the diversity of the GI flora (360, 564, 565). There is also preclinical evidence linking dysbiosis as a causative factor to simultaneously affect both obesity and colitis. Inflammasome deficient mice were reported to develop microbiota dependent susceptibility to both experimental colitis and obesity, possibly due to increased levels of *Prevotellaceae* bacteria (128, 330). These phenotypes could be transferred to control mice by acquisition of the pathogenic microbiota.

Diet also can influence the microbiome and affect both IBD and obesity. Dietary emulsifiers are common in Western diets and are added to many processed foods and allow lipids and water to form a homogeneous mixture. However, this effect had been hypothesized to increase intestinal permeability to the microflora (566). Treatment of mice with the emulsifiers carboxymethylcellulose or polysorbate-80 confers susceptibility to both colitis and obesity (567). The metabolic effect could be transmitted through the transfer of the gut microbiota. The dual role of the microbiome in affecting multiple pathways leading to these two diseases suggests that

a greater understanding of its functions may provide exciting opportunities for therapeutic intervention.

The work in this thesis implicated inflammatory and cell death pathways in either metabolic disease or IBD. Interestingly these pathways have been demonstrated by our lab and others to play roles in the other disease as well. In chapter 2, loss of caspase-12 was implicated as a contributing factor to obesity and insulin resistance. Caspase-12 has also been demonstrated to influence susceptibility to DSS experimental colitis and *Casp12*$^{-/-}$ mice display enhanced repair after acute treatment (408). Interestingly, the colons of KO animals have elevated NF-κB activation that impairs survival on a regiment of sustained DSS treatment. These elevated inflammatory and repair pathways also conferred susceptibility to a model of colorectal cancer. Potentially changes to the microbiota of *Casp12*$^{-/-}$ mice could also explain these phenotypes in both diseases, however further examination is required to determine this.

Necroptosis was attributed to be detrimental to experimental colitis in chapter 4, however its role in obesity and related disorders has not been fully explored. A complication of excess caloric intake is the development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) that is characterized by hepatic lipid deposition, fibrosis, and hepatocyte cell death. While this cell death was originally thought to be apoptotic, recent evidence suggests that necroptosis is also involved. Mice deficient in caspase-8 in liver parenchymal cells develop NASH when fed a methionine and choline-deficient (MCD) diet (568). Deletion of the necrosome component Ripk3 ameliorated disease in this model. This study suggests that necroptosis may be a contributing factor to metabolic diseases. Adipocyte cell death is an initiating factor of adipose tissue inflammation though it is unknown if necroptosis is involved. Future studies using the DIO model in mice deficient in necrosome components will aid in determining a role.

IL-22 is a cytokine with the potential to promote intestinal repair to provide a protective role during IBD (479, 483). Interestingly, multiple studies have found IL-22 to also play a regulatory role in metabolic disease, though these results are somewhat controversial. It was determined that mice deficient in IL-22 receptor (IL-22R1), but not IL-22 cytokine, develop obesity and

insulin resistance (569). This discrepancy may be due to the ability of IL-22R1 to interact with IL-20R2 to form a receptor for IL-20 and IL-24. However, administration of exogeneous IL-22 was also capable of reducing weight gain and metabolic disease in mice. These beneficial effects of IL-22 appear to be stimulated through multiple mechanisms. Interestingly, the cytokine's effects on the GI tract may be partially responsible as intestinal permeability and serum LPS were reduced after treatment, correlating with reduced adipose tissue inflammation. The composition of the microbiota was unaffected and was not a contributing factor. IL-22 appears to also directly affect host metabolism. STAT-3 is induced by IL-22 in primary adipocytes leading to the upregulation of genes involved in fatty-acid β-oxidation and triglyceride lipolysis. Hepatocytes can also be stimulated to inhibit lipogenesis genes to reduce liver steatosis (570). IL-22 can also play a protective role in the pancreas to reduce oxidation and ER stress to promote insulin secretion (571). Despite these encouraging results, a more recent study was not able to reproduce these findings (572). While IL-22 was observed to inhibit gluconeogenesis within the liver, injection of the cytokine did not improve metabolic health. Instead, the authors hypothesized that the higher doses used in previous studies had induced cachexia and subsequently body weight loss.

Overall, these examples illustrate the interconnected pathways that can affect both metabolic disease and IBD. Activation of the immune system can affect multiple tissues and organs to alter homeostasis and promote or protect from disease. The development of therapies targeting one of these disorders may therefore also inadvertently be applicable to the other.

5.6 Final Conclusions

The rise of metabolic diseases resulting from the global obesity pandemic and the increasing number of IBD cases has placed a growing importance on unraveling their pathogenesis. The immune system plays a key underlying role in the development of both of these disorders. The body of work in this thesis provides new insight into the inflammatory caspases and necroptosis, implicating them as potential targets for therapeutic intervention through the use of murine models of disease. The work of chapter 2 detailed the ability of caspase-12 to prevent obesity and adipose tissue inflammation in mice due to expression within non-hematopoietic cells. Chapter 3 further explored the role of the inflammasome in metabolic cell types and indicated

activation in adipocytes can contribute to glucose intolerance. In chapter 4 the role of the necroptosis effector Mlkl was linked to the development of colitis. Overall these studies have provided substantial preclinical evidence for the involvement of these pathways in disease. A better understanding of these processes and related pathways may provide key discoveries that will lead to improved treatment of patients stricken by these disorders.

Original Contributions to Knowledge

1. Loss of caspase-12 in mice leads to obesity, insulin resistance and adipose tissue inflammation
2. Loss of caspase-11 in mice does not affect obesity or insulin resistance
3. Caspase-12 regulation of obesity is dependent on its role in the radioresistant compartment and is antagonized by the loss of Nlrp3
4. Expression of a hyperactive Nlrp3 allele exclusively within adipocytes contributes to glucose intolerance
5. Mlkl deficiency in mice confers protection from experimental colitis
6. The colons of DSS treated *Mlkl*$^{-/-}$ mice are characterized by increased IEC proliferation and IL-22 secretion

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