The role of inflammasomes in intestinal inflammation



Naren Srinivasan
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A thesis submitted for degree of Doctor of Philosophy,
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Abstract

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Single Nucleotide Polymorphisms (SNPs) in the intracellular pattern recognition receptor gene *NLRP3* are associated with susceptibility to Crohn's disease, a form of inflammatory bowel disease (IBD). Following cell damage or infection, NLRP3 triggers the formation of inflammasomes, a multimolecular protein complex containing NLRP3, ASC and caspase-1, which mediate secretion of IL-1β and IL-18. NLRP3 inflammasome activation in macrophages has been implicated in protection against several pathogens, but whether NLRP3 activation in tissue cells contributes to protective immunity against bacterial pathogens is unknown. We show that upon infection with the attaching/effacing (A/E) intestinal pathogen *Citrobacter rodentium*, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice displayed higher bacterial colonization, more weight loss and exacerbated intestinal inflammation. We further show that Nlrp3 inflammasome activation in intestinal epithelial cells (IECs) acts rapidly after infection to limit bacterial replication and penetration, and inhibits the development of inflammatory pathology in the gut. We also show that epithelial Nlrp3-mediated protection is independent of the classical inflammasome cytokines IL-1β and IL-18. Thus an Nlrp3-Asc circuit in IECs regulates early defense against a mucosal pathogen and limits inflammation in the intestine.

Nlrp3 inflammasome activation has also been implicated in protection in acute models of experimental colitis, but its role in chronic models of colitis is unknown. We found that Asc signaling is necessary for the development of innate chronic intestinal inflammation driven by *Helicobacter hepaticus*. Thus while deficient inflammasome signaling in tissue cells increases susceptibility towards enteric pathogens, excessive inflammasome activation can drive chronic intestinal inflammation.

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Abbreviations

AIEC	adherent/invasive E.coli
AMP	anti-microbial peptides
APC	antigen presenting cell
APC	Allophycocyanin
ATG16L1	Autophagy-related protein 16-1
ATP	adenosine triphosphate
BSA	bovine serum albumin
CARD	caspase recruitment
CD	Crohn's disease
CFU	colony forming units
DAMP	danger associated molecular pattern
DC	dendritic cells
DNA	deoxyribonucleic acid
DSS	dextran sulphate sodium
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
Fc	fragment constant
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FOXP3	forkhead box P3
GALT	gut associated lymphoid tissue
GATA-3	Trans-acting T-cell-specific transcription factor-3
GF	germ free
GWAS	genome-wide association studies
H&E	haematoxylin and eosin
Hh + αIL-10R	H.hepaticus infection and αIL-10R mAb treatment
HPRT	hypoxanthine phosphoribosyl transferase
IBD	inflammatory bowel disease
IEL	intra epithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IKK	IkB kinase
IL	interleukin
i.p.	intraperitoneal
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF	interferon regulatory factor
ITAM	immune-tyrosine activatory motif
JAK	janus kinase
KO	knock out
Lin	lineage
LP	lamina propria
LPS	lipopolysaccharide
LTi	lymphoid tissue inducer
M	microfold
mAb	monoclonal antibody
MAP	mitogen associated protein
MHCI/II	major histocompatibility complex type I/II
Min	minute
MLN	mesenteric lymph node
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid

MyD88	myeloid differentiation response gene 88
OD	optical density
N/A	not available
ND	not detectable
NACHT	nucleotide-binding and oligomerization
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NOD	nucleotide-binding oligomerization domain
NLR	nucleotide-binding domain and leucine rich repeat containing receptor
PAMPS	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PP	Peyer's Patch
PRR	pattern recognition receptor
RA	retinoic acid
RA	receptor antagonist
RAP	receptor accessory protein
RAG	recombinase activating gene
RIP	receptor interacting protein
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RLR	retinoic acid-inducible gene-I-like receptors
ROR	retinoic acid-related orphan nuclear hormone receptor
ROS	reactive oxygen species
RT	room temperature
SA	streptavidin
SCID	severe combined immunodeficiency
SFB	segmented filamentous bacteria
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
Tbet	T box expressed in T cells
TCR	T cell receptor
TGF	transforming growth factor
TIR	Toll/IL-1R homology
TLR	toll like receptor
TNBS	2,3,6-trinitrobenzene sulfonic-acid
TNF	tumour necrosis factor
Treg	regulatory T cell
UC	ulcerative colitis
WT	wild type

Chapter 1. General Introduction

1.1 Host defence and the innate immune system

1.1.1 Innate immunity

Found among multiple branches in the evolutionary tree of life, the innate immune system is one of the most ancient forms of antimicrobial defence. The innate immune system forms the first line of defence against pathogenic insults. At the frontline of this network are a range of cell types including; haematopoietic cells, such as neutrophils, macrophages, dendritic cells (DCs), basophils, eosinophils, mast cells, NK cells and other NK-like cells; and non-haematopoietic cells, such as epithelial cells and stromal cells, pericytes and myofibroblasts, which have only recently emerged as bonafide players in the innate immune response. Innate immunity is rapidly inducible and once activated triggers a cascade of host processes aimed at clearing the invading pathogen, collectively known as inflammation (Medzhitov, 2007).

Inflammatory responses can be broken down into four main components:

i. Inducers which initiate the inflammatory response e.g. pathogens
ii. Sensors that detect the inducers (e.g Pattern Recognition Receptors (PRRs)) and signal to produce,
iii. Mediators including cytokines and chemokines that act on,
iv. Target tissues to promote their adaptation or eradication of the insulting inducer.

1.1.2 Pattern Recognition Receptors

Sensors of the innate immune were first proposed over two decades ago by Charles Janeway in a seminal paper, “Approaching the asymptote, evolution and revolution in immunology” (Janeway, 1989). Inflammation, while essential for antimicrobial immunity, if inappropriately targeted can result in damage to host tissues, as evidenced by numerous autoimmune disorders, which creates the need for discrimination between *self* and *nonself*. The adaptive immune system handles this problem largely by deleting auto-reactive T cells during negative selection. Innate immunity relies on germ-line encoded receptors known as Pattern Recognition Receptors (PRRs) which detect

highly conserved, invariant motifs found mainly on microbes known as Pathogen Associated Molecular Patterns (PAMPs) (Janeway, 1989) . PAMPs are ideally suited for detection for three main reasons. First, PAMPs are often generic for an entire class of microbe e.g. lipopolysaccharide (LPS), which is detected by Toll-like receptor 4 (TLR4), is found on the outer membrane of all gram-negative bacteria (Medzhitov, 2007). This means that the host doesn't have to encode multiple receptors for distinct bacterial species. Second, PAMPs are often highly conserved structures, such as flagellin, DNA, and outer membrane components that are essential for microbial survival. Specificity for such conserved essential structures limits evasion through mutation (Medzhitov, 2007). Lastly, PAMPs are found predominantly on microbes, which is thought to allow for discrimination of *self* from *nonself* and the avoidance of autoimmunity (Medzhitov, 2007).

1.1.3 Innate immune effector functions

The initiation of an innate immune response can lead to clearance of invading pathogens via a number of effector mechanisms. These effector host processes can be divided into direct versus indirect antimicrobial mechanisms. Direct mechanisms include; the production of antimicrobial peptides (AMPs) and complement that are directly bactericidal (Porter et al., 2002); phagocytic engulfment and intracellular breakdown of the microbe (Stuart and Ezekowitz, 2005); production of toxic reactive oxygen and nitrogen intermediates via respiratory burst (Segal, 2005); and more recently characterized degradative modules such as autophagy (Kundu and Thompson, 2008). Indirectly, the innate immune response deals with pathogens through the instruction of an adaptive immune response tailored to the invading pathogen (Iwasaki and Medzhitov, 2010). This instruction involves the maturation of antigen presenting cells (APCs) such as dendritic cells (DCs) leading to increased expression of co-stimulatory molecules (eg CD80, CD83 and CD86) that are necessary for T cell activation, and also upregulation of chemokine homing receptors such as CCR7 that allow DCs to migrate from peripheral tissues to draining lymph nodes where they can prime T cells (Iwasaki and Medzhitov, 2010). Additionally, the activation of PRRs results in the skewing of unique T-helper cell responses that are optimized to help clear the inducing pathogen. DCs are able to skew naive T cells towards distinct T-helper subsets via the cytokines they produce. For instance, in response to viral infection, the engagement of certain TLRs leads to the

production of IL-12p70 which signals via the IL-12 receptor to trigger STAT4, a transcription factor that drives the production of IFN-γ and the upregulation of T-bet, a key master transcription factor responsible for Th1 lineage differentiation (Zhu et al., 2010). IFN-γ, in turn feeds back onto the infected cell via IFN- γ receptor and promotes antiviral immunity by upregulating MHC class I expression and other antiviral enzymes such as PKR (Schroder et al., 2004).

PRRs are subdivided into four major classes; Toll-like receptors (TLR), NOD-like receptors (NLRs), RIG-like receptors (RLR) and C-type lectin-like receptors (CLRs). This thesis primarily deals with TLRs and NLRs and thus these will be discussed in detail below.

1.1.4 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are the most well characterized family of PRRs and were also the first class of PRRs to be defined (Kawai and Akira, 2010, 2011; Lemaitre et al., 1996). So far 10 TLRs have been discovered in humans, while 12 functional TLRs have been found in mice, with TLR1-9 being conserved between mouse and man (Kawai and Akira, 2010, 2011). TLRs are type I transmembrane proteins made up of three domains; a leucine-rich repeat (LRR)-rich ectodomain that mediates PAMP recognition, a transmembrane domain, and a Toll-interleukin-1 receptor (TIR) domain that mediates signal transduction via homotypic interactions with TIR-containing adapter molecules including MyD88, Mal, TRIF and TRAM. Dimerization of TLRs is thought to facilitate such interactions by bringing together TIR domains forming a signalling platform where adapters can dock (O'Neill and Bowie, 2007).

TLRs can be subdivided into two main groups, extracellular TLRs present on the plasma membrane, and intracellular TLRs expressed in endosomal compartments. This compartmentalization, coupled with unique ligand specificities and adapter utilization, allows for their functional specialization (Table 1.1). Interestingly, the majority of the intracellular TLRs (TLR3, 7, 8, 9 and 13) sense nucleic acids either derived from viruses or bacteria (Blasius and Beutler, 2010). It is thought that this compartmentalization of nucleic acid sensing TLRs is an important strategy in avoiding the detection of host DNA (which is usually excluded from the endosome) and consequently autoimmunity. Interestingly, TLR9 signalling requires proteolytic cleavage of its ectodomain by endosomal compartment-resident proteases, and this is hypothesized

to prevent activation of TLR9 on the cell surface (Ewald et al., 2008; Park et al., 2008). Consistent with this idea, Mouchess and colleagues recently demonstrated that chimeric mice harbouring haematopoietic cells expressing a transmembranal mutant TLR9 that does not require ectodomain cleavage for activation, developed spontaneous systemic inflammation and anaemia (Mouchess et al., 2011). Proteolytic regulation of TLRs is a conserved mechanism for other intracellular nucleic acid sensing TLRs members including TLR3 and TLR7, suggesting that this is a conserved regulatory strategy (Ewald et al., 2008).

TLR	Location	Adapters	Ligand(s)
1	cell surface	MyD88, Mal	triacyl lipopeptides
2	cell surface	MyD88, Mal	LTA, PGN, lipoproteins
3	endosome	TRIF	dsRNA
4	cell surface	MyD88, Mal, TRIF, TRAM	LPS
5	cell surface	MyD88, TRIF?	flagellin
6	cell surface	MyD88, Mal	diacyl lipopeptides
7	endosome	MyD88	ssRNA
8	endosome	MyD88	ssRNA
9	endosome	MyD88	unmethylated CpG DNA motifs, 2' deoxyribose phosphate backbone
10	unknown	unknown	unknown
11	endosome		profilin
12	endosome	MyD88	profilin
13	endosome		23S ribosomal RNA

Table 1.1 Toll-like receptors (TLRs): Location and ligands. Adapted from (Takeda and Akira, 2007).

TLRs utilize five main adapters in signalling; MyD88, TRIF, Mal, TRAM and SARM (O'Neill and Bowie, 2007). MyD88 is utilized by all the TLRs (except TLR3) and upon recruitment, triggers the activation of the master transcription factor NFκB, mitogen-activated protein kinases (MAPK) and IRF1/5 (Kawai and Akira, 2010). TLR3, by contrast, signals via the adapter TRIF which drives the transcription of type 1 interferons via the transcription factor IRF3 as well as a late wave of NFκB activation (Kawai and Akira, 2011). Interestingly, TRIF is thought to signal from intracellular endosomal compartments rather than the plasma membrane (Kagan et al., 2008). TLR4 is unique among the TLRs in its capacity to signal via both MyD88 and TRIF (Kagan et al., 2008; Watts, 2008). The differential ability of TLRs to utilize the adapters MyD88 and TRIF is important in diversifying the responses evoked by ligand recognition. The differences in signalling adapter 'preference' have been elegantly dissected and can be explained by the discovery of sorting adapters and downstream adapter molecules. At 'steady state' MyD88 normally localises to the cytosol and requires the sorting adapter Mal for delivery to the plasma membrane-bound TIR domain-containing TLRs. Mal is found mainly in the plasma membrane where it binds to phosphoinositide-containing phosphatidyl-inositol-4, 5-bisphosphate (PIP_2). Indeed, Mal deficiency renders macrophages unresponsive to TLR2 and TLR4 agonists and this can be rescued by a mutant MyD88 expressing a PIP_2 binding domain that enables its trafficking to the plasma membrane (Barton and Kagan, 2009). Presumably, TLR7, 8 and 9 do not require Mal for signalling as MyD88 is present in the cytosol in close proximity to endosomes wherein these TLRs are located, however this still remains to be formally demonstrated. Similar to Mal being a sorting adapter for MyD88, TRAM is a sorting adapter for TRIF with affinity for plasma membrane PIP_2 (Barton and Kagan, 2009). TRAM differs from Mal in its exclusive involvement in TLR4 signalling. Finally SARM has recently been characterized as a negative regulator of TRIF (O'Neill and Bowie, 2007).

Fitting with the idea of functional specialization of TLRs, double stranded RNA viruses are sensed by TLR3 which signals via TRIF to induce the transcription of type 1 interferon, which mediates crucial roles in antiviral immunity (Alexopoulou et al., 2001; Hoebe et al., 2003). An additional mechanism of tailoring of the innate immune response revolves around co-receptor dimerization. While most TLRs form homodimers, TLR2 can form heterodimers with TLR1 and TLR6 that may

provide a further degree of specificity in pathogen detection (Ozinsky et al., 2000; Takeuchi et al., 2001; Takeuchi et al., 2002). Furthermore, certain TLRs can cooperate with non-TLRs for signalling e.g. TLR4 and TLR6 have shown to form a heterodimer with the scavenger receptor CD36 in their response to amyloid β (Stewart et al., 2010). Additionally, TLR4 co-localizes with MD2 for the detection of LPS, and requires delivery of LPS to this complex by LPS-binding protein (LBP) via the co-receptor CD14 (Akashi-Takamura and Miyake, 2008).

TLR recognition of a cognate PAMP initiates an inflammatory response that usually clears the invading pathogen. The key protective functions for TLRs in immunity against infection are highlighted by a plethora of data demonstrating heightened susceptibility of TLR deficient mice in a variety of infection models (Table 1.2). However, while insufficient protective immunity can result in mortality, excessive innate immune responses can perpetuate deleterious immunopathology that accompanies some viral infections (Tchivilev et al., 2008).

TLR	Pathogen	Phenotype
TLR4	*S. typhimurium* *N. meningitis*	↓ survival ↓ survival
TLR2	*S. aureus* *S. pneumoniae*	↓ survival
TLR1/2	Mycobacteria, meningococci	↓ cytokine production
TLR2/6	Mycoplasma	↓ cytokine production
TLR3	West Nile Virus	↑ resistance
TLR5	*S. typhimurium* *P.aeruginosa*	no phenotype
TLR7/8	Influenza LCMV	↓cytokine production ↓ clearance
TLR9	*K. pneumoniae* *L. pneumophila* MCMV	↓ survival ↓ survival ↓ clearance
TLR11	*T. gondii*	↓ survival

Table 1.2 Susceptibility phenotypes of TLR deficient mice in models of infection adapted from (Carpenter and O'Neill, 2007).

1.1.5 NOD-like receptors

Among the classes of PRRs, NLRs are second only to TLRs in terms of scrutiny. In stark contrast to TLRs, NLRs are restricted exclusively to cytosolic compartments. NLRs are found in both plant and animal kingdoms, often in greater numbers in the former, for instance *Arabidopsis* harbours roughly 160 NLRs in contrast to just 20 in most mammals (Ausubel, 2005). However they are thought to have arisen independently, via convergent evolution in nature, indeed NLR-like proteins are absent in insects (von Moltke et al., 2013).

Structural features

The prototypic features that define NLRs include the possession of three domains.

i. A N-terminal domain made up of either a caspase-recruitment domain (CARD), Pyrin domain (PYD) or Baculovirus Inhibitor of apoptosis Repeat domain (BIR). An exception to this is NLRX1, which possesses an N terminus with no apparent homology to any of these three domains. Another exception is CIITA, which harbours an acidic activation domain. NLRs are often classified into different subgroups based on the N-terminal domain that they encode, as these determine downstream signalling pathways. It is interesting to note that both CARD and PYD domains are death domain-fold containing motifs, suggesting an overarching conserved role of the NLR family in cell death. Indeed CARD: CARD domain homotypic interactions mediate cross-talk between certain NLRs and the Caspases (Davis et al., 2011; Schroder and Tschopp, 2010; von Moltke et al., 2013).

ii. A central Nucleotide-binding oligomerization domain (NOD), also termed the NACHT domain. NACHT sequences encode nucleoside triphosphatase domains (NTPase) that can bind and hydrolyze ATP, and are thought to mediate oligomerization of NLRs, a prerequisite in the initiation of downstream signalling. More recent work has put forward the idea that the NACHT domain might also serve as a site for regulation by certain secondary messengers (discussed later) (Davis et al., 2011; von Moltke et al., 2013).

iii. The C-terminal domain of NLRs, similar to TLRs, is composed of leucine rich repeat sequences (LRRs) that in some cases can directly bind to PAMPs (Kofoed and Vance, 2011; Zhao et al., 2011). However, the precise mechanism of agonist activation of NLRs

remains to be determined and other studies have hinted at an auto-inhibitory role for LRRs in some NLRs (Hu et al., 2013; Poyet et al., 2001).

As mentioned, NLRs are divided into four main families based on their N-terminal domains, with NLRX1 belonging to its own family (Table 1.3). As can be observed, the precise molecular identity of the ligands/agonists for the majority of NLRs remains uncharacterized.

NLR	N-terminal domain	Ligand/Agonist	Reference
CIITA	Acidic activation	unknown	
NAIP (1,2,5,6)	BIR	Flagellin, Type III rod proteins	(Kofoed and Vance, 2011; Zhao et al., 2011)
NOD1	CARD	iE-DAP	(Chamaillard et al., 2003)
NOD2	CARD	MDP	(Girardin et al., 2003a; Girardin et al., 2003b)
NLRC3	CARD	unknown	
NLRC4	CARD	Flagellin, TypeIII rod proteins	(Franchi et al., 2006; Miao et al., 2006; Miao et al., 2010b)
NLRC5	CARD	unknown	
NLRP1	PYRIN	Anthrax lethal toxin	(Boyden and Dietrich, 2006)
NLRP2	PYRIN	unknown	
NLRP3	PYRIN	ATP, MSU, Toxins, oxidized mitochondrial DNA, alum, silica, UV radiation, amyloid β	(Schroder and Tschopp, 2010; von Moltke et al., 2013)
NLRP4	PYRIN	unknown	
NLRP5	PYRIN	unknown	
NLRP6	PYRIN	unknown	
NLRP7	PYRIN	Microbial lipopeptides	(Khare et al., 2012)
NLRP8	PYRIN	unknown	
NLRP9	PYRIN	unknown	
NLRP10	PYRIN	unknown	
NLRP11	PYRIN	unknown	
NLRP12	PYRIN	*Yersinia pestis*	(Vladimer et al., 2012)
NLRP13	PYRIN	unknown	
NLRP14	PYRIN	unknown	
NLRX1	?	ssRNA, dsRNA, and poly(I:C)	(Hong et al., 2012)

Table 1.3 NLRs: structure and agonists

Signalling

In general, upon activation, NLRs signal via two main routes that culminate in two very distinct responses. Many NLRs, as will be discussed below, signal to coordinate transcriptional responses via master transcription factors such as NFκB and AP-1 (Davis et al., 2011). However remarkably, numerous other NLRs, rather than driving transcriptional responses, potentiate post-translational modifications that culminate in the activation of a key enzyme known as Caspase-1 (Davis et al., 2011) (Martinon et al., 2002).

Transcriptional regulation

NOD2 is the most well studied member of the NLR family and thus its signalling pathway can be used as a 'textbook' case. Upon recognition of its cognate ligand, muramyl dipeptide (MDP) (Girardin et al., 2003a; Girardin et al., 2003b), NOD2 self oligomerizes via NACHT domains and recruits the signalling adapter molecule Receptor-Interacting serine/threonine-Protein kinase 2 (RIP2) (Park et al., 2007). RIP2 and NOD2 interact via homotypic CARD: CARD interactions that result in the polyubiquitination and activation of this adapter protein. RIP2, in turn, activates the Inhibitor-kappaB kinase gamma (IκKγ also termed NFκB essential modulator (NEMO)) via K63-linked polyubiquitination. This leads to the recruitment of a kinase, TAK1 that further activates the IκKB complex triggering the subsequent phosphorylation, polyubiquitination and degradation of the inhibitor of NFκB, IκBα. The degradation of IκB releases repression of NFκB, allowing it to move into the nucleus and drive transcription of a multitude of inflammatory genes e.g. IL-6, TNF-α and CCL2 (Kanneganti et al., 2007). Interestingly, other NLR family members such as NLRC5, NLRC3 and NLRP12 and can negatively regulate NFκB (Cui et al., 2010; Davis et al., 2011; Schneider et al., 2012).

Some NLRs also affect type 1 IFN production. NLRX1 for instance negatively regulates type 1 IFN production by sequestering a signalling adapter for RIG-I, Mavs (Allen et al., 2011; Lei et al., 2012; Moore et al., 2008). Recent work on NLRP4 also uncovered a negative regulatory role on type I interferon signalling (Cui et al., 2012). NOD2 has also been implicated in driving type I IFN

production that might explain its reported protective role in certain viral infections (Sabbah et al., 2009).

Finally, NOD1/2 signalling can induce MAPKs such as p38, extracellular signal-regulated protein kinase (ERK), and c-jun N-terminal kinase (JNK) (Kanneganti et al., 2007). This pathway, similar to NFκB signalling, relies on RIP2 and TAK1 as adapter molecules. However, NLR signalling has not been through as rigorous a biochemical dissection as TLR signalling and as such, multiple gaps in our knowledge concerning its signalling remain unaccounted for.

Post-translational modifications and the Inflammasome

A number of NLRs, upon activation, trigger the formation of a multi-protein complex known as the inflammasome (Martinon et al., 2002; Martinon et al., 2009). The inflammasome is composed of at least two proteins: an NLR and the enzyme caspase-1. The primary and most well characterized function of inflammasomes is to cleave and activate caspase-1 (Martinon et al., 2002), a cysteine protease that cleaves peptides specifically after aspartic-acid sequence (Cerretti et al., 1992; Ghayur et al., 1997; Kuida et al., 1995; Thornberry et al., 1992; Yamin et al., 1996). Caspases can be divided into initiator, executioner and inflammatory caspases. The initiator caspases (caspase-2, 8, 9 and 10) are named for their ability to initiate the apoptotic cascade by cleaving and activating executioner caspases (caspase-3, 6 and 7) that in turn cleave defined cellular substrates such as PAK2 and DNA fragmentation factor (Rodrigue-Gervais and Saleh, 2013). Inflammatory caspases include caspase-1, caspase-12, murine caspase-11 and human caspase-4 and 5 and are defined by a CARD domain followed by a cysteine residue-containing catalytic domain (Rodrigue-Gervais and Saleh, 2013). Interestingly, a common feature for caspase activation revolves around the formation of multimolecular platforms such as the death-induced signalling complex (DISC) and the PIDDosome (a caspase-2 activating complex) (Rodrigue-Gervais and Saleh, 2013; Tinel and Tschopp, 2004). These aggregates promote the oligomerization of caspases enabling them to form a functional active site and catalyze downstream signalling cascades. Caspase-1 is synthesized as a 45kDa pro-enzyme composed of 2 heterodimeric complexes known as p10 and p20 (Cerretti et al., 1992; Ghayur et al., 1997; Kuida et al., 1995; Martinon et al., 2002; Thornberry et al., 1992). Upon activation this pro-enzyme undergoes auto-proteolysis via an induced-proximity mechanism, which

removes the N-terminal and an internal fragment yielding the active form made up of a p10 and p20 subunit (Salvesen and Dixit, 1999). Caspase-1 has two well-defined substrates, the precursors of the inflammatory cytokines IL-1β and IL-18 (Cerretti et al., 1992; Ghayur et al., 1997; Thornberry et al., 1992). These cytokines are made in cells as inactive, zymogens that require cleavage for activation. Thus, activated NLRs promote caspase-1 activation that in turn cleaves and activates pro-IL-1β and pro-IL-18 for secretion via a poorly described process.

Recent advances in inflammasome biology have improved our understanding of the molecular events governing the activation of this multi-protein complex. In the following section I will review the latest developments in the field. As this thesis primarily deals with Nlrp3, I will focus my discussion on this NLR.

1.1.6 The Nlrp3 inflammasome

Nlrp3, also known as cryopryin, was discovered through genetic mapping of two autosomal dominant fever syndromes; Familial Cold Autoinflammatory Syndrome (FCAS) and Muckle-Wells Syndrome (MWS) (Hoffman et al., 2001). As these syndromes were associated with excessive IL-1β, and caspase-1 had previously been linked to IL-1β activation, the link between Nlrp3, caspase-1 and IL-1β was made soon after and the inflammasome was born (Martinon et al., 2002). Nlrp3 is a pyrin domain (PYD) containing NLR protein and therefore lacks the necessary CARD domain required for direct interactions with Caspase-1. Bridging this gap is a unique PYD and CARD domain containing protein known as Pycard or Asc (apoptosis-associated speck-like protein containing a CARD domain) (Martinon et al., 2002; Masumoto et al., 1999; Srinivasula et al., 2002). Interestingly, as its name suggests early studies showed that Asc formed ‘specks’ upon activation and was associated with celldeath (McConnell and Vertino, 2000). These ‘specks’ probably represent multi-molecular protein aggregates made up of Nlrp3, Asc, Caspase-11 and Caspase-1 (Martinon et al., 2002). However, Asc specks have never been reported *in vivo* thus their existence may be an *in vitro* artefact.

Activation

Nlrp3 requires two signals for activation. Signal 1 regulates the expression of Nlrp3 and occurs both transcriptionally and post-translationally. Activated TLRs drive NF-κB-dependent signals that transcriptionally induce *Nlrp3*, pro-*Il1β* and pro-*Il18* expression (Bauernfeind et al., 2009). Furthermore, Nlrp3 protein is repressed by ubiquitination and requires additional signals (part of signal 1) to relieve repression through a deubiquitinating enzyme, BRCC3 (Juliana et al., 2012; Py et al., 2013). Signal 2 triggers Nlrp3 activation and inflammasome assembly and can be provided by an astonishing array of stimuli including microbes, and non-microbial endogenous and exogenous activators. Microbial stimuli associate with Nlrp3 activation include bacteria (Table 1.4) fungal pathogens such as *Candida albicans*, and *Aspergillus fumigatus* (Gross et al., 2009; Hise et al., 2009; Said-Sadier et al., 2010; Tomalka et al., 2011) and viruses including influenza A and vesicular stomatitis virus (Ichinohe et al., 2010; Rajan et al., 2011).

Pathogen	Bacterial components
Aeromonas hydrophila	Aerolysins
Aeromonas veronii	Aerolysin
Bacillus brevi	Gramicidin
Bordatella pertussis	Pore-forming activity of adenylate cyclase toxin (CyaA)
Chlamydophila pneumoniae	?
Clostridium difficile	TcdA and TcdB toxins
Escherichia coli	RNA
Klebsiella pneumonia	?
Listeria monocytogenes	Listeriolysin O (LLO)
Mycobacterium marinum	RD-1/ESX-1
Mycobacterium tuberculosis	ESAT-6 (early secretory antigenic target 6)
Neisseria gonorrhoeae	Lipooligosaccharide
Salmonella typhimurium	Unknown SPI-2 dependent signal
Streptococcus pneumonia	Pneumolysin
Streptococcus pyogenes	Streptolysin O
Streptomyces hygroscopicus	Nigericin
Staphylococcis aureus	Hemolysins; particulate peptidoglycan
Vibrio cholera	?; Hemolysins
Yersinia pseudotuberculosis	Unknown Type three secretion system-dependent substrate
Yersinia pestis	?

Table 1.4 Bacterial Nlrp3 activators (adapted from (von Moltke et al., 2013))

Non-microbial Nlrp3 stimuli include self-derived activators such as ATP, succinate, amyloid β, hyaluronan, monosodium urate (MSU) crystals and cholesterol crystals (reviewed in (Davis et al., 2011). Inorganic, environmental activators can also trigger the Nlrp3 inflammasome and comprise of crystalline and particulate matter such as asbestos, alum, silica, nanoparticles (TiO_2), as well as other stimuli such as UV radiation (reviewed in (Davis et al., 2011)).

Models of Nlrp3 inflammasome activation

Given the extremely wide and varied sources of signal 2 in inflammasome activation, many speculated that unlike most PRRs, Nlrp3 did not directly engage each of the aforementioned stimuli. Rather, it was believed that the stimuli must converge on a common signal upstream of Nlrp3 activation. The nature of this common signal has been under intense investigation and yielded many competing and controversial models of Nlrp3 activation that are discussed below.

The channel model

Originally, it was shown that extracellular ATP could serve as a second signal for Nlrp3 activation *in vitro*. ATP release serves a good 'danger signal' as it is stored in all cells could act as a universal marker for cell damage. ATP activates Nlrp3 in a manner that depends on the purinergic ATP-receptor, P2X7, which in turn mediates a K^+ efflux, concomitant with the opening of a channel known as pannexin 1 (Ferrari et al., 2006; Pelegrin and Surprenant, 2006). The opening of this hemichannel was initially proposed to allow bacterial products, such as MDP, to enter the cell's cytosol and directly engage Nlrp3 (Tschopp and Schroder, 2010). While this model potentially explained ATP's ability to trigger Nlrp3, it could not explain how large molecules like asbestos could fit through pannexin 1 hemichannels. Further work showed that many agonists could activate Nlrp3 independently of P2X7 bringing the general utility of this model into question (He et al., 2013).

Phagosomal rupture model

Sterile particulate and crystalline matter are thought to activate Nlrp3 via phagosomal damage. Due to their large size and inability to breakdown into small fragments, these particles induce a state of

'frustrated phagocytosis' that leads to lysosomal rupture that somehow triggers Nlrp3 activation. Supporting this model were early studies showing that Nlrp3 activation by some particulate matter depended on phagocytosis, phagosomal acidification and the lysosomal proteinase, cathepsin B (Hornung et al., 2008). While other reports confirmed cathepsin B involvement for non-particular matter such as nigericin (Hentze et al., 2003), other studies using cathepsin B deficient macrophages showed that this proteinase was dispensable for Nlrp3 activation by other particulate agonists, including MSU, hemozoin and alum (Dostert et al., 2009). Although these conflicting results may potentially reflect redundancy between cathepsins, precisely how cathpesins triggered Nlrp3 activation remains uncharacterized. Notably, a recent study showed that Nlrp3 co-localized with phagosomes containing Gram-negative bacterial cargo and regulated phagosomal function (Sokolovska et al., 2013), suggesting that Nlrp3 could monitor the phagosome for signs of damage.

ROS model

One of the most compelling hypothesis put forth to explain Nlrp3 activation was reactive oxygen species (ROS). Many agonists induce ROS production making this an attractive convergent signal for Nlrp3 activation. Consistent with this idea was the finding that inflammasome activation could be inhibited via ROS scavengers such as N-acetyl-cysteine (Zhou et al., 2010). The molecular events downstream of ROS were studied further and shown to involve a complex of an oxidoreductase, thioredoxin, and thioredoxin-interacting protein (TXNIP) (Zhou et al., 2010). In resting cells, TXNIP is complexed with thioredoxin. When intracellular ROS concentrations rise, TXNIP is released from thioredoxin and binds to the LRR domain of Nlrp3 and triggers its activation (Zhou et al., 2010). Fitting with this hypothesis, knockdown of TXNIP abrogated Nlrp3 activation by a number of agonists (Zhou et al., 2010). While ROS seems an attractive secondary signal for Nlrp3 activation, a number of studies have reported conflicting results (Bauernfeind et al., 2009; Kanneganti and Lamkanfi, 2013; Munoz-Planillo et al., 2013). One reason to account for the discrepant results may lie in the fact that ROS inhibitors have off-target effects and have been shown to inhibit the NFκB pathway that might affect signal 1 instead of signal 2 (Bauernfeind et al., 2009). Furthermore, if ROS indeed represents the secondary signal activating Nlrp3, it remains unclear how TLR agonists that also stimulate ROS production would fail to trigger Nlrp3.

Mitochondrial damage

More recent work on the mechanism of Nlrp3 activation has implicated a role for mitochondrial damage. The first study to suggest this showed that mitochondrial derived ROS was responsible for Nlrp3 activation, consistent with the ROS model (Zhou et al., 2011). In addition, Nlrp3 and Asc co-localized with mitochondria upon activation in this study. Furthermore, selectively blocking mitochondrial respiratory chain transporters uncoupled the electron transport chain and increased the mitochondrial ROS levels that triggered Nlrp3 activation (Zhou et al., 2011). Another study showed that Nlrp3 co-localized with oxidized mitochondrial DNA generated during apoptosis, and was sufficient to trigger inflammasome activation (Shimada et al., 2012). However, this report is somewhat puzzling as apoptosis is generally considered 'silent' and anti-inflammatory.

A recent study showed that Nlrp3 coupled with the adapter protein Mavs via its N-terminal domain. Mavs directly bound Nlrp3 and co-localized to mitochondria (Subramanian et al., 2013). Mavs deficient BMDMs showed impaired caspase-1 activation in response to non-crystalline Nlrp3 agonists such as ATP and nigericin. While this study supports the notion that Nlrp3 may survey the mitochondria for damage, it does not clarify how Asc and caspase-1 couple with Nlrp3-Mavs. Mavs possesses a CARD domain and thus could theoretically interact with either Asc or Caspase-1. Finally, it was recently demonstrated that upon administration of the antibiotic linezolid Nlrp3 can bind a mitochondrial lipid, cardiolipin, and interference with cardiolipin synthesis blocked Nlrp3 activation (Iyer et al., 2013).

Why might Nlrp3 survey mitochondrial homeostasis? Evolutionarily, the mitochondrion is thought to derive from symbiotic bacteria. The endosymbiotic theory proposes that organelles such as the mitochondrion and plastids evolved from free-living bacteria that were subsumed as 'endosymbionts'. Thus mitochondria represent the most 'foreign' component of 'self'. Detection of mitochondrial damage perhaps evolved as an early means of keeping symbiosis in check.

Calcium (Ca^{2+}) signalling

Three recent studies have implicated Ca^{2+} signalling in Nlrp3 activation (Lee et al., 2012; Murakami et al., 2012; Rossol et al., 2012). Interestingly LPS and Ca^{2+} were sufficient to induce Nlrp3 activation *in vitro*. This activation was dependent upon signalling via the G-coupled protein

receptor CASR (calcium-associated signalling receptor). Ca^{2+} promoted Nlrp3 activation by promoting release of intracellular Ca^{2+} stores and by relieving inhibition by cAMP (Lee et al., 2012). Intracellular Ca^{2+} promoted the assembly of inflammasome components while cAMP was found to bind to the NBD domain of Nlrp3 and directly anatagonize its oligomerization (Lee et al., 2012). Interestingly CAPS (Cryopyrin Associated Periodic Syndromes) patients harboured mutations in the NBD domain of Nlrp3 that made Nlrp3 more resistant to inhibition by cAMP (Lee et al., 2012). While novel, these studies did not identify the precise mechanisms through which Ca^{2+} potentiates Nlrp3 activation. One way in which Ca^{2+} might activate Nlrp3 may be via through inducing mitochondrial damage, however it is difficult to determine which is the more proximal event in this setting (Murakami et al., 2012). While these studies support a role for extracellular Ca^{2+} in the triggering of Nlrp3 activation, a recent study suggests that this might be attributed to an artefact. Munoz-Planillo *et al* showed that extracellular Ca^{2+} could indeed trigger Nlrp3 activation but that this activity depended on the medium in which the stimulation was performed (Munoz-Planillo et al., 2013). While Ca^{2+} triggered Nlrp3 activation in RPMI, it failed to do so in IMDM, DMEM or HBSS medium. As RPMI has a higher $[PO_4^{3-}]$, addition of Ca^{2+} leads to the formation of calcium phosphate crystals that trigger Nlrp3 activation independently of CASR (Munoz-Planillo et al., 2013). These results raise doubts over the credibility of Ca^{2+} as a convergent signal for Nlrp3 activation. Taken together these studies highlight the fact that there is still no universally accepted mechanism of how Nlrp3 is activated and that different agonists may cause different types of cellular stress which converge upon a proximal event in Nlrp3 activation.

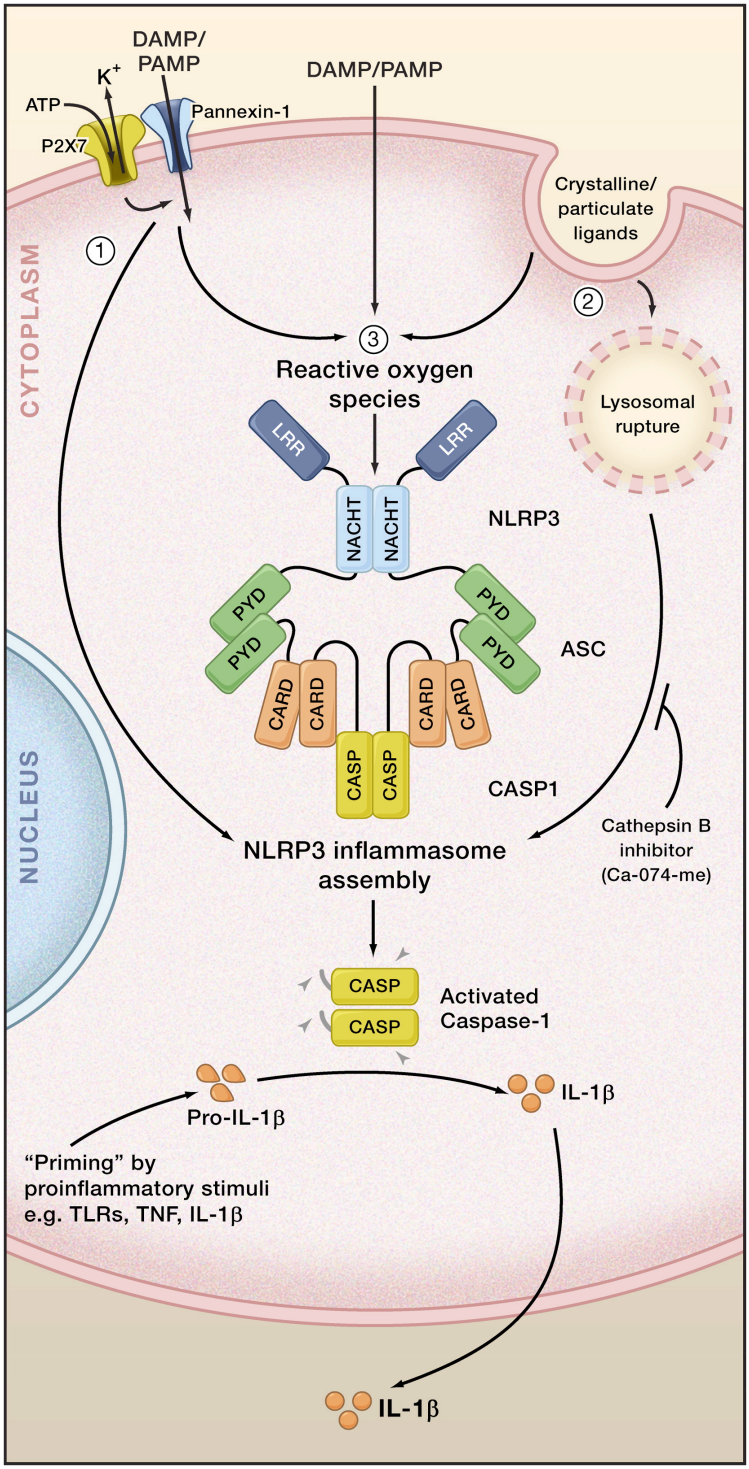


Figure 1.1 Models of Nlrp3 activation adapted from (Schroder and Tschopp, 2010). At resting state, Nlrp3 is inactive due to autoinhibition by its LRR domains. Activation requires two signals. Signal 1 primes pro-IL-1β and Nlrp3 while signal 2 is required to relieve Nlrp3 inhibition and promote its oligomerization with Asc and caspase-1. Three major models of signal 2 exist. Firstly the channel model holds that an efflux in K^+ promotes Nlrp3 inflammasome assembly (1). Recently this model has been modified to include Ca^{2+} signalling in the promotion of Nlrp3 inflammasome activation. Secondly, phagosomal rupture is thought to trigger Nlrp3 via a cathepsin-dependent mechanism (2). This mechanism may account for how large indigestible particles trigger Nlrp3 inflammasome activation. Thirdly, ROS derived from damaged mitochondria may trigger Nlrp3 inflammasome activation via the release of Thioredoxin interacting protein (TXNIP) (3).

Regulatory proteins involved in inflammasome activation

As can be seen from the discordant studies above, the molecular events governing Nlrp3 activation still require further investigation. Apart from the secondary signals that govern Nlrp3 activation, a number of regulatory proteins have been identified. A few key proteins are discussed below:

Protein kinase R (PKR)

PKR was recently shown as a general regulator of inflammasome activation (Lu et al., 2012). PKR is a serine threonine kinase that is a general sentinel for cellular stress responses, activated in response to nutrient starvation, cytokines, PAMPs and DNA damage (Kang and Tang, 2012). Upon activation, PKR affects a range of cellular pathways including the MAPK, NFκB, IRF3, apoptotic and autophagy pathways (Kang and Tang, 2012). Stretching this already impressive list of functions is the finding that PKR regulates Nlrp3, Nlrc4, Nlrp1 and Aim2 activation (Lu et al., 2012). Lu *et al* found that upon *in vitro* activation by cognate agonists, PKR physically coupled to the above PRRs and regulated caspase-1 activation and IL-1β release (Lu et al., 2012). Indeed, BMDMs deficient in PKR were unresponsive to inflammasome agonists. Precisely how PKR drives inflammasome activation remains to be determined but it is interesting to note that PKR was also required for bacterial-induced cell-death, suggesting a possible role upstream of caspase-11 (see below) (Hsu et al., 2004).

Caspase-11

Caspase-11 (or caspase-4/5 in humans) was originally characterized as an upstream regulator of caspase-1 in the earliest descriptions of the inflammasome (Martinon et al., 2002; Wang et al., 1996; Wang et al., 1998). Caspase-11 physically associated with caspase-1, but could not directly process pro-IL-1β (Wang et al., 1996). Thus it was proposed that caspase-11 modulated inflammasome activation upstream of caspase-1. Recently there has been renewed interest in this protease. A key paper showed that only a subset of Nlrp3 activators such as cholera toxin B (CTB), and certain Gram negative bacteria e.g. *Citrobacter rodentium*, triggered activation of a 'non-canonical' inflammasome dependent on caspase-11 for cell death and cytokine processing (Kayagaki et al., 2011). Other Nlrp3 triggering stimuli, such as ATP and Nigercin, and non-Nlrp3

stimuli do not engage a caspase-11-dependent inflammasome. Interestingly, the non-canonical stimuli triggered pro-IL-1β processing in an Nlrp3-Asc-Caspase-1 dependent manner, but induced cell death in a manner that was independent of these proteins but dependent on caspase-11 (Kayagaki et al., 2011). These results indicated that caspase-1 and caspase-11 have differing upstream triggers. Which PRRs are upstream of caspase-11? This question was elegantly addressed by Rathinam *et al* who showed that nearly all Gram negative bacteria triggered caspase-11 activation transcriptionally via an LPS: Tlr4-driven autocrine loop (Rathinam et al., 2012). Tlr4 signalling triggers the production of type1IFN via TRIF, which signals in an autocrine loop through Ifnar (type1IFN receptor and Stat1, resulting in transcription of caspase-11 (Rathinam et al., 2012). Further characterization of this pathway has led to the speculation that caspase-11 may be involved in responses against bacteria that escape the phagocytic vacuole (Aachoui et al., 2013). To this end, infection with *Legionella pneumophila*, a normally phagosome-contained bacterium, cannot activate caspase-11. However *L. pneumophila* mutants lacking *sdhA,* a virulence factor that promotes phagosomal integrity, escape the phagosome and trigger caspase-11 activation (Aachoui et al., 2013). In agreement with a general role for caspase-11 in cytosolic sensing, but disputing a role for Tlr4 in caspase-11 activation, caspase-11 can be activated via LPS transfection in BMDMs derived from *Tlr4*$^{-/-}$ mice (Kayagaki et al., 2013). This exciting finding hints at the existence of a novel intracellular LPS sensor that awaits discovery. The relevance of caspase-11 *in vivo* was assessed using sepsis models wherein resistance to LPS-driven septic shock in mice is observed in caspase-11 deficient mice but not caspase-1 deficient mice (Kayagaki et al., 2011). Fascinatingly, *Tlr4*$^{-/-}$ mice were equally susceptible to LPS-induced septic shock as WT mice (Kayagaki et al., 2013). However, several key questions remain to be answered in this field, including: what determines whether a stimulus engages a canonical versus a non-canonical inflammasome? How does caspase-11 potentiate caspase-1 activation? How does caspase-11 drive cell-death (pyroptosis)? Which PRR(s) are upstream of caspase-11 activation?

GBP5

GBP5 (guanylate binding protein 5) belongs to a class of IFN-γ inducible proteins known as the GBPs (guanylate binding proteins). Some of these proteins such as GBP5 contain a guanosine 5′-

triphosphatases (GTPase) domain fused to a CARD domain. Shenoy *et al* found that cells lacking GBP5 are deficient in their responses towards Nlrp3 agonists (Shenoy et al., 2012). Surprisingly, GBP5 bound to the PYD domain of Nlrp3 and promoted caspase-1 activation downstream of soluble Nlrp3 agonists e.g. Nigericin and ATP, and pathogens such as *Listeria monocytogenes,* however GBP5 was dispensable for responses against particulate and crystalline stimuli such as alum (Shenoy et al., 2012). *In vivo, Gbp5*$^{-/-}$ mice displayed slightly increased susceptibility towards oral *L. monocytogenes* infection and protection against LPS driven septic shock (Shenoy et al., 2012). However, these observations were not directly linked to impaired caspase-1 activation and thus require verification.

1.2 The gastrointestinal tract: challenges to the notion of self

Given the abundance of commensal bacteria (and accompanying PAMPs) that reside within most mammalian hosts, how do we avoid chronic inflammation? Nowhere is this more relevant than in the gastrointestinal tract where roughly 100 trillion bacteria are thought to reside, outnumbering our own cells by a factor of 10 (Backhed et al., 2005). The microbiota, a term used to describe the entire collection of microbes harboured by our bodies, provides us with a number of benefits. Indeed, we coexist in a symbiotic relationship where, in exchange for nutrients and residence, our microbiota provide us with resistance towards pathogens, increased efficiency in nutrient extraction from complex plant polysaccharides and promote development of the gut associated lymphoid tissues (GALT) (Backhed et al., 2005). We exist in a delicate balance with our microbiota, a breakdown in which is thought to precipitate chronic intestinal inflammation known as Inflammatory Bowel Disease (IBD).

1.3 Inflammatory Bowel Disease (IBD): a breakdown in intestinal homeostasis

IBD is a chronic relapsing and remitting disease that may present clinically as Crohn's Disease (CD) or Ulcerative Colitis (UC). CD is characterized by transmural inflammation that is patchy and can affect any part of the gastrointestinal tract, however the terminal ileum is most commonly

affected (Xavier and Podolsky, 2007). By contrast UC involves superficial inflammation that spreads from the rectum into the colon to differing degrees (Xavier and Podolsky, 2007). While CD is thought to be a predominantly Th1/Th17 mediated disease, UC is characterized by type 2 responses. Familial associations suggest a strong host genetic contribution to IBD wherein first-degree relatives of IBD patients have roughly a 5-fold increased risk of developing the disease (Xavier and Podolsky, 2007). Genome Wide Association Studies (GWAS) have been instrumental in uncovering the genetic contribution to IBD, identifying a plethora of risk-associated alleles (Khor et al., 2011). Such genes regulate pathways that have evolved to maintain intestinal homeostasis and are discussed below. In most cases however carrying these common alleles only increases one's risk of disease modestly, thus multiple factors, genetic and environmental, may need to collude to manifest in IBD. Supporting this is the finding that there is only a 30-35% and a 10-15% concordance rate between monozygotic twins for CD and UC, respectively (Khor et al., 2011). Among the environmental factors influencing susceptibility to IBD, the microbiota looms the largest. IBD manifests at the intersection between a genetically susceptible host and a colitogenic microbiota. Some of the main factors that maintain homeostasis, and how their breakdown results in IBD, are discussed below.

1.4 Mechanisms enforcing intestinal homeostasis

1.4.1 Intestinal epithelial cells (IEC)

Mucus and antimicrobial peptides (AMPs)

The intestine is made up of a monolayer of intestinal epithelial cells (IECs) that line the intestine. Underlying this monolayer are immune effector sites, such as the lamina propria, and more organized lymphoid structures such as the Peyer's patches, cryptopatches and isolated lymphoid follicles (Artis, 2008). Overlaying the outer epithelial surface are two thick layers of mucus, secreted by specialised goblet cells. These comprise a loose outer layer of 100μm depth and a firmer inner layer of roughly 50 μm in depth that together form a physical and chemical barrier (Johansson et al., 2008). Intriguingly while the outer mucus layer harbours some bacteria, the inner layer is completely sterile and devoid of any bacteria in the colon (Johansson et al., 2011;

Johansson et al., 2008). This suggests that at steady state, commensal bacteria require some effort to penetrate the thick layer of mucus to reach the intestinal epithelium and represents a mechanism by which excessive immune reactions maybe avoided. Fitting with this idea, certain parasites such as *Entamoeba histolytica* encode proteases that cleave the major mucus-forming protein, Muc2, enabling them to penetrate the mucus layer and reach the epithelium where they can drive disease (Lidell et al., 2006). Additionally, *Muc2*$^{-/-}$ deficient mice spontaneously develop intestinal inflammation (Van der Sluis et al., 2006). The mucus layer presents both a physical and a chemical barrier to the microbiota. The mucus is laden with antimicrobial peptides (AMPs) that are directly bactericidal and secretory IgA that can effectively neutralize LPS (Boullier et al., 2009). Indeed the sterility of the inner mucus layer in *Myd88*$^{-/-}$ mice, which lack certain AMPs, is lost in the intestine (Vaishnava et al., 2008; Vaishnava et al., 2011). Thus, AMPs fortify the epithelial barrier and prevent aberrant immune responses against commensals. Paneth cells are IECs that are specialized in producing large amounts of AMPs including lysozyme, cryptidins and defensins (Porter et al., 2002). Paneth cells are found at the bottom of intestinal crypts next to intestinal stem cells and are though to reside there to maintain the sterility of the intestinal stem cell niche (Sato et al., 2011). Additionally Paneth cells produce numerous growth factors such as EGF, Wnt3 and TGFα that maintain the intestinal stem cell niche (Sato et al., 2011).

While the physical exclusion of the microbiota is useful, some PAMPs such as LPS are soluble and thus may be able to reach the epithelium and the underlying lamina propria. To avoid this the intestinal epithelium has evolved a means of detoxifying LPS via an enzyme known as intestinal alkaline phosphatase (IAP) (Bates et al., 2007) and zebra fish lacking IAP showed higher rates of neutrophil recruitment to the gut (Bates et al., 2007). The mucus layer contributes to intestinal homeostasis in humans too as mutations in *MUC19* are associated with susceptibility to CD (Khor et al., 2011).

Compartmentalization of PRRs

While the mucus provides a robust barrier against translocation of commensals and PAMPs, additional layers of security must exist to reinforce this exclusion in instances where the mucus barrier might be weakened. One such means is the compartmentalization of PRRs. Two key

examples concern Tlr4 and Tlr5. It was observed that Tlr4 and CD14 localize at the bottom of the crypt in the colon while Tlr4 is located intracellularly in the small intestine (Hornef et al., 2002; Ortega-Cava et al., 2003). Thus, commensal-derived LPS is unlikely to be able to easily access these sites, in contrast to invasive pathogen-derived LPS Another example of such compartmentalization concerns Tlr5. Tlr5 is exclusively expressed on the basolateral side of the epithelium thereby necessitating a breach of the epithelial barrier for a bacterium to deliver agonistic flagellin (Gewirtz et al., 2001).

Autophagy

Autophagy is a catabolic process of self-digestion that evolved as a response to starvation. It works by removing old or damaged organelles by encasing them in a characteristic double membrane and then targeting them for degradation by fusion with lysosomes (Deretic and Levine, 2009). Autophagy was originally implicated in intestinal homeostasis by GWAS showing that single nucleotide polymorphisms (SNPs) in a key autophagy gene *ATG16L1* were associated with heightened risk to CD (Hampe et al., 2007). Paneth cell abnormalities were reported in both patients carrying this risk allele as well as mice rendered hypomorphic for *Atg16l1* (Cadwell et al., 2008; Cadwell et al., 2010). These abnormalities increased the susceptibility of hypomorphic mice to an acute model of intestinal inflammation, in the presence of norovirus (Cadwell et al., 2010).

NFκB

NFκB is a master transcription factor involved in the induction of a wide range of genes. Normally NFκB is sequestered in the cytoplasm, complexed with an inhibitor, IκB. In order to translocate to the nucleus and drive transcription, NFκB first needs to dissociate from IκB. For this to occur, another set of proteins, the IκK complex which is made up of catalytic α,β and regulatory γ subunits, must first be activated (Pasparakis, 2008). Upon activation by a range of stimuli, the IκK complex phosphorylates IκB, targeting it for proteasomal degradation. This relieves NFκB from inhibition and enables it to translocate into the nucleus and drive gene transcription. A crucial role for NFκB activation in the maintenance of intestinal homeostasis was demonstrated by the finding that mice deficient in IκKγ (and thus NFκB activation) specifically in IECs developed spontaneous

colonic inflammation (Nenci et al., 2007). This study postulated that NFκB activation was required for anti-apoptotic signalling in IEC, as the colitic phenotype could be rescued by crossing the mice to TNFα receptor deficient mice. Furthermore, intestinal inflammation was mediated by excessive innate immune responses as inflammation was abolished upon crossing to a *Myd88*$^{-/-}$ background (Nenci et al., 2007). Another study showed that mice lacking IκKβ in IECs showed reduced expression of a Th2-skewing cytokine, TSLP (Zaph et al., 2007). This led to impaired Th2 immunity against an enteric parasite, *Trichuris muris*, and severe Th1-driven intestinal inflammation (Zaph et al., 2007). Thus, NFκB may promote intestinal homeostasis through conditioning adaptive immune responses.

1.4.2 Intestinal macrophages

A population of resident macrophages exists in the lamina propria that possesses a unique phenotype that reinforces the tolerance of the microbiota. Intestinal macrophages are highly refractory to PAMP stimulation, producing lower levels of pro-inflammatory cytokines such as IL-6, IL-12 and TNFα upon stimulation (Smythies et al., 2005). This may be partially ascribed to the fact that this population of macrophages do not express many key stimulatory receptors including CD14, complement receptors, CR3 and CR4, Fc receptors including Fcα and Fcβ, or growth factor receptors for IL-2 or IL-3 (Smythies et al., 2005). Interestingly however, these macrophages retain antibacterial potential as they are unimpaired in phagocytosis and efficiently kill *S. typhimurium* and *E.coli* (Smythies et al., 2005). Furthermore, stromal cell derived TGFβ plays a role in inhibiting cytokine release from these macrophages (Smythies et al., 2005). STAT3 signalling in intestinal macrophages is also important in the maintenance of this tolerogenic phenotype as mice lacking STAT3 specifically in macrophages develop spontaneous intestinal inflammation (Takeda et al., 1999). This was attributed mainly to a lack of cell intrinsic IL-10R signalling (Takeda et al., 1999). A pro-inflammatory macrophage phenotype has also been associated with human IBD. In the inflamed CD gut, inflammation is perpetuated by a distinct CD14^{+} subset of macrophages that are not refractory to stimulation and produce large amounts of IL-23, IL-6 and TNFα than typical resident macrophages (Kamada et al., 2008).

1.4.3 Lamina propria dendritic cells (DCs)

DCs are key mediators between the innate and the adaptive immune systems. Instead of killing microbes like macrophages, DCs phagocytose microbes in the gut and migrate to the mesenteric lymph node (MLN) where they can prime naïve $CD4^+$ T cells (Varol et al., 2010). During this priming, T cells upregulate the CCR9 and α4β9 that enables them to migrate to the intestine (Varol et al., 2010). Intestinal myeloid cells can be differentiated by distinct expression of the markers CD11b, CD103 and CX3CR1 (Varol et al., 2010). $CD11b^+$ $CD103^+$ DCs and $CX3CR1^+$ mononuclear phagocytes are the main mononuclear phagocytes within the intestine. Antigens taken up in the lamina propria are exclusively transported to the MLN by $CD11b^+$ $CD103^+$ DCs in a CCR7 dependent manner (Varol et al., 2010). $CD11b^+$ $CD103^+$ DCs promote homeostasis by preferentially driving $Foxp3^+$ Treg differentiation in the MLN through the production of TGFβ and retinoic acid (Sun et al., 2007) (Coombes et al., 2007). Lamina propria DCs also promote tolerance against commensals through the induction of IgA^+ plasma cells in a T cell-independent manner in Peyer's patches (Macpherson and Uhr, 2004). Importantly the MLN is an anatomical adaptation that allows mucosal immune responses to be generated whilst preventing systemic inflammatory responses against the microbiota (Hooper and Macpherson, 2010; Macpherson and Uhr, 2004).

1.4.4 Innate lymphoid cells (ILCs)

ILCs are a recently discovered population of innate cells defined by a lack of expression of rearranged antigen receptors, a lack of myeloid and DC markers and a lymphoid morphology, that are found at enriched frequencies in mucosal tissues (Spits et al., 2013). These cells are divided into three groups named ILC1, 2 and 3 based on their phenotype and function (Spits et al., 2013). ILC3 cells play important roles in the containment of commensals and in protective immune responses against enteric pathogens. Depleting ILCs in $Rag^{-/-}$ mice leads to the systemic dissemination of commensals and systemic inflammation that can be prevented by administering IL-22 (Sonnenberg et al., 2012). ILC3 are the major innate source of IL-22 in the lamina propria. IL-22 signals via IL-22R expressed on IEC to drive STAT3 phosphorylation and induction of AMPs including Reg3γ and Reg3β (Pickert et al., 2009) (Sonnenberg et al., 2011; Zheng et al., 2008). Importantly, mice deficient in Reg3γ lose the 50μm sterile inner mucus layer that segregates

the microbiota from the IECs and display higher faecal IgA levels and higher frequencies of IFN-γ^+ $CD4^+$ T cells (Vaishnava et al., 2011). A recent study suggested that ILC may also be capable of inducing tolerance. A proportion of ILC3 were shown to express MHCII and to be able to present antigen, however rather than inducing T cell proliferation like APCs, ILC3s do not express co-stimulatory molecules and thus are thought to limit T cell responses (Hepworth et al., 2013). Consistent with this regulatory role, deletion of MHCII specifically on Rorγt^+ ILCs resulted in spontaneous intestinal inflammation that was dependent on $CD4^+$ T cells and bacteria (Hepworth et al., 2013). While ILC3s are clearly important in protecting against commensal dissemination in some settings, excessive ILC activity can drive intestinal inflammation in some chronic models of colitis (Buonocore et al., 2010). In these models, ILCs respond to IL-23 to produce high levels of IFN-γ and IL-17 that are pathogenic (Buonocore et al., 2010). In addition to ILCs, the intestine has a number of other small populations of innate leukocytes, including intraepithelial $CD8^+$ T cells, iNKT cells, and γδ T cells, that have been suggested to play important roles in the maintenance of intestinal homeostasis. However as they were not investigated in this thesis, they are not discussed in detail here.

1.4.5 Regulatory T cells (Tregs)

One of the most essential mechanisms that engenders tolerance against commensals is the presence of Tregs in the intestine. Tregs are a subset of $CD4^+$ $CD25^+$ T cells dependent on the lineage skewing transcription factor Foxp3 (Sakaguchi et al., 2010). The majority of Tregs acquire Foxp3 expression during their development in the thymus and are sometimes termed natural nTreg cells. However, there is a subpopulation of naïve $CD4^+$ T cells that acquire Foxp3 in a TGFβ-dependent manner in the intestine and these are termed induced iTreg cells (Izcue et al., 2009). Germ-free mice have significantly fewer $Foxp3^+$ Tregs in their colon compared to conventionalized mice and this can be reversed through colonization with a mix of *Clostridium* species (Atarashi et al., 2011). Thus while we maintain some spatial segregation between our microbiota and the intestinal immune system, we clearly sample some bacteria/bacterial products and may in fact require them to promote robust tolerance. Evidence for a non-redundant role for Tregs in intestinal tolerance was observed in humans with a loss of function mutation or deletion in *FOXP3*. These patients develop

a severe fatal inflammatory disease known as Inflammatory Polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) that is characterized by intestinal inflammation (Bennett et al., 2001). Numerous models of colitis have shown that Tregs prevent chronic intestinal inflammation (Izcue et al., 2009). Most notably, the co-transfer of $Foxp3^+$ Tregs can prevent intestinal inflammation induced by lymphopenia-induced naïve T cell proliferation (Hori et al., 2003).

A key function of Tregs is to effectively compete with effector $CD4^+$ T cells in the intestinal niche. To do so Tregs may sometimes express transcription factors and other costimulatory molecules that are classically associated with colitogenic T cell subsets, such as Th17 and Th1 cells (Littman and Rudensky, 2010). For example, selective deletion of the Th17-assocaited transcription factor STAT3 in $Foxp3^+$ Tregs leads to spontaneous intestinal inflammation driven by Th17 cells (Chaudhry et al., 2009). Similarly, Tregs lacking T-bet, the lineage skewing transcription factor for Th1 cells, are unable to control Th1 mediated inflammation when transferred into *Foxp3*$^{-/-}$ scurfy mice (Koch et al., 2009).

1.4.6 IL-10 and TGFβ

IL-10 is a dominant anti-inflammatory cytokine in the intestine. Its fundamental role was evidenced by the observation that *Il10*$^{-/-}$ mice develop spontaneous intestinal inflammation (Kuhn et al., 1993), and from recent findings that mutations in IL-10 receptor genes *IL-10RA* and *IL-10RB* are strongly linked to early onset severe IBD (Glocker et al., 2009). The colitis that emerges in *Il10*$^{-/-}$ mice is dependent on the microbiota since when these mice were reared germ-free, they did not develop disease (Sellon et al., 1998). IL-10 acts to repress excessive innate immune responses on myeloid cells as the inflammation is abrogated when *Il10*$^{-/-}$ mice are crossed to $LysM^{Myd88KO}$ mice (Hoshi et al., 2012). There are multiple sources of IL-10 in the intestine including myeloid cells, B-cells and T cells. $Foxp3^+$ Tregs are a non-redundant source of IL-10 at steady state in the colon since mice lacking IL-10 specifically from $Foxp3^+$ T cells develop spontaneous colitis (Rubtsov et al., 2008). IL-10 also promotes tolerance by signalling directly into $Foxp3^+$ T cells as Tregs lacking IL-10R were unable to suppress colitis mediated by transfer of naïve T cells and lost Foxp3 expression (Murai et al., 2009). Interestingly, intestinal macrophages served as the source of IL-10 in this setting (Murai et al., 2009). Confirming a role for IL-10 signalling in $Foxp3^+$ Tregs, deletion

of IL-10R specifically on Foxp3$^+$ Tregs led to spontaneous intestinal inflammation that was marked by an accumulation of Th17 cells (Chaudhry et al., 2011).

TGFβ is produced in great abundance in the intestine. TGFβ exists in three isoforms, TGFβ1, TGFβ2 and TGFβ3 that signal via the TGFβR complex. Akin to *Foxp3*$^{-/-}$ mice, *Tgfβ1*$^{-/-}$ mice develop severe autoimmunity and die within a few weeks of birth (Shull et al., 1992). TGFβ acts to restrain pathogenic T cell responses, as mice lacking TGFβR specifically on T cells also developed spontaneous autoimmunity and multi-organ inflammation (Li et al., 2006; Marie et al., 2006). Furthermore, naïve T cells lacking TGFβR were refractory to Treg mediated suppression in the T cell transfer model of colitis (Fahlen et al., 2005). While multiple cell types can produce TGFβ, Tregs represent a key source as *Tgfβ1*$^{-/-}$ Tregs could not suppress colitis induced by the transfer of naïve T cells (Li et al., 2006).

1.4.7 IL-23, Th1 and Th17 responses

Th1 cells are defined by a dependence on the transcription factor T-bet and by their ability to produce IFN-γ (Zhu et al., 2010). They are strongly implicated in the pathogenesis of chronic intestinal inflammation, exemplified by the finding that T-bet deficient naïve T cells were unable to drive colitis upon transfer into lymphopenic hosts (Neurath et al., 2002). Furthermore, blocking IFN-γ using monoclonal antibodies attenuated T cell transfer colitis (Powrie et al., 1994). Initial characterization of clinical samples also pointed to a predominantly Th1-driven pathology in CD, characterized by the accumulation of CD4$^+$ IFN-γ$^+$ T cells (Neurath et al., 2002; Okazawa et al., 2002). Th1 differentiation depends on IL-12R signalling and STAT4 (Zhu et al., 2010). Parallel studies using the T cell transfer model of colitis observed that while Stat4 was necessary for naïve CD4$^+$ T cells to induce disease in lymphopenic hosts, T cell-derived IFN-γ$^+$ was dispensable (Simpson et al., 1998). Furthermore, clinical trials using fontolizumab, an anti-IFN-γ monoclonal antibody, proved unsuccessful for the treatment of active Crohn's disease (Caprioli et al., 2012). Similarly, although blocking IFN-γ$^+$ with a neutralizing mAb afforded protection against experimental intestinal inflammation if given early after T cell transfer, it was ineffective against established disease (Powrie et al., 1994). Thus, it appears that Th1 cells play an important role in the induction of intestinal inflammation, but that additional mechanisms contribute to chronic

disease perpetuation. While anti-IFN-γ^+ mAb treatment prevented inflammation only if administered at the onset of disease, others had shown that anti IL-12p40 mAb could effectively treat established colitis (Neurath et al., 1995).

This enhanced potency of anti-IL-12 p40 was not fully understood until the turn of the millennium with the discovery of IL-23, a heterodimer made up of IL-23p19 complexed with the shared IL-12p40 subunit (Oppmann et al., 2000). Many of the inflammatory functions that were originally ascribed to IL-12, comprising of IL-12p40 and IL-12p35 subunits, were subsequently re-evaluated. In edifying studies, naïve T cells were transferred into cohorts of *p19*$^{-/-}$*Rag*$^{-/-}$ or *p35*$^{-/-}$*Rag*$^{-/-}$ or *p40*$^{-/-}$*Rag*$^{-/-}$ hosts to unequivocally assess the relative roles of IL-23 and IL-12 in intestinal inflammation (Hue et al., 2006; Kullberg et al., 2006). Strikingly, mice lacking IL-12 (*p35*$^{-/-}$*Rag*$^{-/-}$ hosts) developed just as severe colitis as WT mice, while mice deficient in IL-23 (*p19*$^{-/-}$*Rag*$^{-/-}$) were protected from colitis (Hue et al., 2006; Kullberg et al., 2006). These studies cemented a role for IL-23 in promoting colitis. Further work showed that IL-23R signalling directly into T cells promoted their proliferation and accumulation in the colon, as well as promoting the emergence of an IFN-γ^+ IL-17A$^+$ double positive population of T cells (Ahern et al., 2010). The precise role of these double-producers in intestinal pathogenesis remains to be demonstrated, but it is notable that IFN-γ^+ IL-17A$^+$ double positive cells have also been isolated from the inflamed colons of CD patients (Annunziato et al., 2007). IL-23 also promotes chronic intestinal inflammation by directly restraining Foxp3$^+$ Tregs as while the transfer of WT naïve T cells into *p19*$^{-/-}$*Rag*$^{-/-}$ mice did not cause colitis, transferring *Foxp3*$^{-/-}$ naïve T cells into the same host background restored disease (Izcue et al., 2008). From a human disease perspective, is it notable that several SNPs in IL-23R have been associated with both CD and UC (Cho, 2008).

Th17 cells are a relatively new subpopulation of T cells that are dependent on the transcription factor Rorγt and were initially characterized by the expression of IL-17A, IL-17F, IL-21 and IL-22 (Zhu et al., 2010). STAT3 is required for the induction of Rorγt as T cells lacking STAT3 could not differentiate into Th17 cells and showed decreased Rorγt expression (Yang et al., 2008b). Th17 cells depend on a variety of cytokines for their differentiation; early studies identified TGFβ and IL-6 as potent inducers of Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Together these cytokines induce the STAT3-dependent expression of IL-21, IL-23R and

RORγt (Ivanov et al., 2007). IL-23, while not strictly required for the initial differentiation of Th17 cells, is involved in the maintenance and continued differentiation of this cell type *in vivo* (Korn et al., 2009). Th17 cell differentiation also relies upon the transcription factor IRF4 as *Irf4*$^{-/-}$ naïve T cells display an impaired ability to differentiate into Th17 cells and *Irf4*$^{-/-}$ mice are resistant to EAE, a Th17-driven disease (Brustle et al., 2007). Recently IL-1β has also been implicated in Th17 differentiation (Chung et al., 2009; Coccia et al., 2012; Lalor et al., 2011; Mills et al., 2013; Nakae et al., 2003; Shaw et al., 2012). CD4^{+} naïve T cells lacking *Il1r1* exhibited impaired differentiation into Th17 cells and showed reduced colitogenic capacity when transferred into lymphopenic *Rag*$^{-/-}$ mice mice (Coccia et al., 2012). The role of IL-1β in driving Th17 differentiation is discussed in greater length in chapter 5 of this thesis.

Th17 cells have been implicated in IBD as their frequencies are abundant in the inflamed tissues from CD patients (Shale et al., 2013). However, studies on Th17 cells and cytokines in experimental models of colitis have yielded conflicting results. While Rorγt was required for naïve T cells to cause colitis in the T cell transfer model, IL-17A was dispensable, and IL-17A and IL-17F appeared to play redundant roles in this model (Leppkes et al., 2009). In contrast, blockade of IL-17A reduced the levels of colitis arising in Treg specific *Stat3*$^{-/-}$ mice (Chaudhry et al., 2009) and attenuated intestinal inflammation in *Helicobacter hepaticus*-infected *Rag*$^{-/-}$ mice (Buonocore et al., 2010). In acute models of colitis using DSS, IL-17A was shown to be protective while IL-17F drove disease (Yang et al., 2008a).

1.4.8 The microbiota

While other environmental factors such as smoking can influence susceptibility to IBD, the microbiota is thought to play a dominant role in influencing the course of disease. Supporting this hypothesis, a large number of spontaneous models of colitis e.g. *Il10*$^{-/-}$ mice can be rescued by re-deriving the mice germ-free or through the administration of broad-spectrum antibiotics (Sellon et al., 1998). This is also the case in humans, wherein fecal stream diversion or antibiotic administration relieves intestinal inflammation in some circumstances (Khan et al., 2011). Not all commensals have the capacity to potentiate inflammation however. Indeed certain species of bacteria exploit genetically susceptible hosts and drive disease. A well studied example is

Helicobacter hepaticus, a common member of the microbiota that coexists peacefully in wild type (WT) hosts, but drives severe colitis in B6 *Il10*$^{-/-}$ mice and 129SvEv *Rag*$^{-/-}$ hosts (Kullberg et al., 1998; Maloy et al., 2003). Another example is found in *Rag*$^{-/-}$ *Tbet*$^{-/-}$ mice that develop a spontaneous communicable colitis driven by the outgrowth of two key species *Proteus mirabilis* and *Klebsiella pneumonia* (Garrett et al., 2010). Such species that exploit genetically susceptible hosts are classically termed opportunistic pathogens although a new term that has recently been coined meaning the same is 'pathobiont' (Chow and Mazmanian, 2010).

While these studies point to a pathogenic role of the microbiota in intestinal inflammation, some members also engender tolerance and development of the GALT. Germ free mice show profound reductions in the maturation of their intestinal immune system with lower levels of CD4$^+$ T cells, smaller Peyer's patches, immature isolated lymphoid follicles, reduced IgA production, fewer IL-17 producing CD4$^+$ T cells and γδ T cells, fewer IL-10 producing Foxp3$^+$ Tregs, and reduced AMP production from IECs (Atarashi et al., 2011; Bouskra et al., 2008; Cash et al., 2006; Duan et al., 2010; Ivanov et al., 2009). In a seminal study, it was shown that the microbiota was required to promote repair of the intestinal epithelium upon damage by DSS (Rakoff-Nahoum et al., 2004). Fittingly, germ-free mice are more susceptible to DSS than SPF (specific pathogen-free) mice (Maslowski et al., 2009). Certain species seemed to have specialised to promote particular pathways within the intestine. For example, mono-colonization with segmented filamentous bacteria (SFB), but not other commensals, was able to rescue the defective Th17 induction in GF mice (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). These Th17 cells are important in defence against enteric pathogens, as WT mice lacking SFB were more susceptible to inflammation driven by the murine intestinal pathogen *C. rodentium* (Ivanov et al., 2009). Other functions, by contrast, simply involve the general detection of commensal PAMPs, for instance the susceptibility of antibiotic-treated mice to DSS–driven inflammation could be rescued by the administration of LPS or LTA (Rakoff-Nahoum et al., 2004). Overall these studies show that the microbiota regulates intestinal homeostasis but shifts in this symbiotic relationship, known as dysbiosis, can precipitate outgrowths of opportunistic pathogens that may either initiate or perpetuate intestinal inflammation.

What is the evidence for dysbiosis in human IBD? Firstly, what does a 'normal' healthy human microbiota look like? We are all born sterile, acquiring our microbiota firstly from our mothers and then later from ingested food. The adult intestinal microbiota is dominated by two main phyla, Bacteroidetes (including *Bacteroides*) and Firmicutes (which includes *Lactobacillus, Bacillus and Clostridium*) (Eckburg et al., 2005). Bacteroidetes is the major phylum making up >90% of the microbiota. Two low abundance phyla also present within the intestine are Actinobacteria and Proteobacteria. At lower branches of the taxonomic tree, the picture is less clear. Since most of the bacterial species are unculturable, metagenomic approaches are used to sequence bacteria. Using this approach across a wide range of different cohorts, it was recently proposed that the human microbiota stratifies into just three main groups or 'enterotypes' classified according to the relative levels of three genera of bacteria *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (Arumugam et al., 2011).

IBD patients display evidence of dysbiosis with specific changes in the composition of their microbiota. For example, CD patients exhibit an outgrowth in Adherent Invasive *Escherichia coli* (AIEC), which adhere close to the IECs of the ileum and are believed to exacerbate intestinal inflammation (Darfeuille-Michaud et al., 2004). Fewer lactobacillus and bifidobacteria have also been reported in the past (Honda and Littman, 2012). Importantly, others have reported reductions in the *Clostridial* clusters IV and XIVa (Frank et al., 2007), species that can promote the generation of IL-10 producing $Foxp3^+$ Tregs (Atarashi et al., 2013; Atarashi et al., 2011). Furthermore, CD patients harbour significantly less *Faecalibacterium prausnitzii*, a member of the *Clostridales* order, than non-IBD control patients (Sokol et al., 2009). This species has also been implicated in driving the production of IL-10 (Sokol et al., 2008). *Bacteroides fragilis*, a Gram-negative bacterium has also been shown to attenuate intestinal inflammation through the induction of IL-10 production from Tregs (Mazmanian et al., 2008; Round et al., 2011; Round and Mazmanian, 2010). This was attributed to a capsular polysaccharide expressed by *B. fragilis,* polysaccharide A (PSA) (Mazmanian et al., 2008). *Mycobacterium paratuberculosis* is a bacterium belonging to the Mycobacterium avium complex, that was first isolated from biopsies from CD patients two decades ago and was proposed to play a role in driving intestinal inflammation, however this link remains controversial (Chiodini et al., 1984) (Chiodini et al., 2012)). A big hurdle in linking dysbiosis to

intestinal inflammation is teasing apart causality and correlation. Under instances of intestinal inflammation, it is not surprising that the makeup of the microbiota shifts, in fact microbiota changes occur even during innocuous events such as formula feeding, fever or antibiotic consumption (Costello et al., 2012). Thus more vigorous studies must be performed in the future to assess the causal role of the microbiota in driving IBD.

1.4.9 Innate immunity and pattern recognition

Innate immunity is intricately linked with susceptibility to IBD. From a genetic perspective, many PRRs, downstream adapter proteins and cytokines have been linked with susceptibility to IBD. Experimental models of colitis have also revealed protective and pathogenic effects of innate signalling. These are discussed in greater detail below:

TLRs in intestinal inflammation

A number of TLRs have been linked to disease phenotype in CD, these include TLRs1, 2, 4, 6 and 9 (Oostenbrug et al., 2005; Pierik et al., 2006; Torok et al., 2004). Experimentally, one of the first studies to demonstrate beneficial interactions between the microbiota and the innate immune system was carried out by Rakoff-Nahoum *et al*. Using the DSS acute model of intestinal inflammation, they found that mice lacking Myd88 were more susceptible than WT controls to intestinal inflammation (Rakoff-Nahoum et al., 2004). This result, at the time, was surprising as the dogma was that aberrant immune responses against commensals drove intestinal inflammation. DSS works primarily by damaging the intestinal epithelium and thus removing the barrier between commensals and the underlying lamina propria. Depleting the microbiota in WT mice using antibiotics phenocopied the result in *Myd88*$^{-/-}$ mice. Furthermore antibiotic treated mice could be rescued through the oral administration of LPS or LTA, showing definitively that TLR-based recognition of commensal bacteria could aid repair and restitution of the intestinal epithelium (Rakoff-Nahoum et al., 2004). TLR2 also contributes to the maintenance of homeostasis by maintaining tight junction proteins between IECs (Cario et al., 2004). Myd88-dependent signalling in IECs is important in the production of AMPs such as Reg3γ that maintain the segregation between the microbiota and IECs (Vaishnava et al., 2008; Vaishnava et al., 2011). TLR5 has been

strongly linked to intestinal inflammation as some investigators have reported spontaneous intestinal inflammation and rectal prolapase in a proportion of *Tlr5*$^{-/-}$ mice, however this remains highly controversial as in many animal facilities these mice show no disease (Letran et al., 2011) (Vijay-Kumar et al., 2007). TLR9 recognition of commensal DNA can restrain Treg differentiation as *Tlr9*$^{-/-}$ mice display lower frequencies of intestinal Foxp3^{+} Tregs (Hall et al., 2008).

Although these studies have highlighted the beneficial aspects of TLR and Myd88 signalling in intestinal defence and repair processes, studies in chronic IBD models and some spontaneous models of intestinal inflammation have also highlighted pathogenic roles for this axis. Specifically, haematopoietic Myd88 signalling is required for the induction of colitis by the bacterium *Helicobacter hepaticus* (Asquith et al., 2010). Similarly, *Foxp3*$^{-/-}$ mice that develop spontaneous intestinal inflammation can be partially rescued by crossing them to a *Myd88*$^{-/-}$ background (Rivas et al., 2012). Spontaneous colitis emerging in *Il10*$^{-/-}$ mice can also be rescued by crossing these mice to a *Myd88*$^{-/-}$ background (Rakoff-Nahoum et al., 2006). However, since the intestinal inflammation in *Foxp3*$^{-/-}$ mice is microbiota independent (Chinen et al., 2010), we can also conclude that Myd88 signalling perpetuates T cell responses directed towards self-peptides in the absence of regulation. Thus ultimately it may be the cell compartment in which Myd88 signalling is active that determines whether it is protective or pathogenic.

NLRs in intestinal inflammation

NOD2

NOD2 was the first gene linked to CD and still confers the strongest susceptibility to disease (Hugot et al., 2001; Ogura et al., 2001). Three main NOD2 variant alleles exist: *R702W*, *G909R* and *1007fs* (Eckmann and Karin, 2005). Notably, these mutations all occur in or near the C-terminal LRR domain of NOD2, a region that is associated with MDP ligand recognition. Individuals harbouring one copy of a major risk allele have a 2-fold increased risk of developing CD, while carrying two risk alleles increases your risk 17-fold (Eckmann and Karin, 2005). Despite over a decade of intensive study, the precise mechanisms through which variants in NOD2 contribute to CD susceptibility remain unclear, although three main hypotheses have been proposed.

i. Defective epithelial defence

NOD2 activation can trigger the production of AMPs, in particular it is linked to the production of cryptidins and defensins (Kobayashi et al., 2005). Defective production of AMPs by IECs might confer susceptibility towards CD by impairing the mucin barrier that segregates the microbiota and IECs. Alternatively, shifts in the composition of AMPs as a result of defective NOD2 sensing could favour the outgrowth of colitogenic species that drive intestinal inflammation. To this end, CD patients have reduced expression of Paneth cell-derived α defensins including HD-5 and HD-6 (Wehkamp et al., 2004). Furthermore a recent paper showed that *Nod2*$^{-/-}$ mice harbour a transmissible colitogenic flora that increased susceptibility to DSS colitis and colorectal cancer (Couturier-Maillard et al., 2013). While defective epithelial defence is a plausible hypothesis, hard evidence causally linking it to CD remains scant. Thus far much of the work studying this hypothesis has relied upon IEC cell lines that do not accurately recapitulate the *in vivo* scenario. New advances allowing primarily IEC cultivation in organoid culture should facilitate comparison of IECs grown from CD patients carrying NOD2 variants with those from control non-IBD patients.

ii. Lack of inhibition of TLR signalling

An alternative hypothesis holds that NOD2 inhibits pro-inflammatory TLR signalling in the inflamed gut. Supporting this hypothesis, pre-incubation of PBMCs with MDP leads to diminished subsequent responses towards TLR ligands (Kullberg et al., 2008; Watanabe et al., 2008). By contrast, PBMCs isolated from patients harbouring the *1007fs* NOD2 variant, were not affected by MDP treatment leading to excessive production of pro-inflammatory cytokines (Kullberg et al., 2008). Additional studies reported that transgenic mice overexpressing Nod2 were resistant to TNBS colitis (Yang et al., 2007) and that administration of MDP to WT mice afforded protection against DSS colitis in a NOD2 dependent manner (Watanabe et al., 2008). Interestingly reconstituting *Nod2*$^{-/-}$ mice with intact Nod2 but not the CD-associated frameshift mutant *1007fs* NOD2, restored responsiveness towards MDP treatment (Watanabe et al., 2008). Other studies have shown that NOD2 potentiates TLR2-dependent anti-inflammatory signals (Netea et al., 2004). PBMCs from patients harbouring the 3020insC mutant NOD2 showed defective IL-10 production when stimulated with TLR2 but not a TLR4 agonist (Netea et al., 2004).

iii. Defective autophagy

As noted above, autophagy has also been linked to intestinal homeostasis and variants in *ATG16L1* and *IRGM* are associated with susceptibility to CD (Cho, 2008). Importantly, autophagy has recently been shown to negatively regulate IL-1β production, either directly by breaking down components of the inflammasome such as Asc, or indirectly by targeting pro-IL-1β for degradation (Harris et al., 2011; Shi et al., 2012). Macrophages from mice harbouring a mutant Nod2, akin to the *1007fs* NOD2 variant in humans, secreted greater amounts of IL-1β upon stimulation with MDP (Maeda et al., 2005). Similarly, mice lacking Atg16l1 in haematopoietic cells were more susceptible to DSS colitis and this susceptibility could be rescued by blocking IL-1β or IL-18 (Saitoh et al., 2008). A crucial link was then established between Nod2 and autophagy induction, wherein Nod2 activation by MDP triggered the recruitment of Atg16l1 to the site of bacterial entry at the plasma membrane, in a Rip2 dependent fashion (Cooney et al., 2010; Travassos et al., 2010). Importantly, DCs from CD patients harbouring either NOD2 or ATG16L1 variants were defective in autophagy induction, bacterial handling and antigen presentation (Cooney et al., 2010). Further supporting a role for Nod2 in regulating autophagy, PBMCs from individuals with ATG16L1 variants produced less IL-1β upon stimulation with Nod2 ligands (Plantinga et al., 2011). Together, these data suggest that reduced autophagy, as a consequence of loss of function mutations in NOD2, may lead to defective commensal bacterial handling and excessive IL-1β production that may drive intestinal inflammation.

Apart from these three major hypotheses, others have emerged. One holds that a gain of function mutation in NOD2 leads to suppression of IL-10 transcription that may contribute to the aetiology of CD (Noguchi et al., 2009). This is supported by evidence showing that PBMCs from patients carrying the *1007fs* NOD2 variant produced less IL-10 upon stimulation with TLR2 ligands (Netea et al., 2004). Overall, it is clear that NOD2 plays a major role in the maintenance of intestinal homeostasis. Yet, the precise molecular mechanisms by which NOD2 mediates its function in the intestine and how mutations in this gene precipitate CD still requires clarification.

Inflammasomes

Recently, single nucleotide polymorphysms (SNPs) in the regulatory region of *NLRP3*, resulting in lowered expression of NLRP3, were linked with susceptibility to CD (Cummings et al., 2010; Schoultz et al., 2009; Villani et al., 2009). One study suggested that this was due to a decreased production of IL-1β from monocytes upon stimulation with LPS, (Villani et al., 2009) suggesting that a loss of function mutation in *NLRP3* leads to susceptibility to CD. Conversely, this suggests a protective function for *NLRP3* in the intestine. Recent studies in mice support the hypothesis that inflammasome activation limits intestinal inflammation. Mice deficient in *Nlrp3*, *Asc* or *Caspase-1* exhibit more severe intestinal inflammation and weight loss than wild type (WT) mice when subjected to the dextran sodium sulfate (DSS) model of intestinal inflammation (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). Interestingly, administering recombinant IL-18 rescued these mice from inflammation, showing that IL-18 derived from inflammasome activation is crucial to the maintenance of intestinal homeostasis (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). Indeed GWAS studies have associated missense variants in the IL-18 receptor accessory protein (IL18RAP) with increased risk of CD (Rivas et al., 2011). Nlrp3 is widely expressed by both haematopoietic and parenchymal cells and while some of these papers suggested a protective role of inflammasomes in haematopoietic cells (Allen et al., 2010), others contended that inflammasome activation in non-haematopoietic cell types was required (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). Inflammasome activation may exert some protective effects indirectly, by regulating the activity of cytokines that fortify epithelial barrier defence, such as IL-22 (Huber et al., 2012). For example, a recent study showed that Nlrp3-dependent IL-18 production could positively regulate IL-22 signalling by downregulating IL-22 binding protein, an inhibitor of IL-22 (Huber et al., 2012). Inflammasomes may also contribute to risk to CD through effects on the microbiota as mice deficient in Caspase-1, Asc, or Nlrp6 were recently discovered to harbour a more 'colitogenic' intestinal microbiota, associated with an outgrowth of TM7 and Bacteroidetes (Prevotellaceae), that exacerbated DSS-triggered intestinal inflammation (Elinav et al., 2011). Furthermore, this increased susceptibility to DSS colitis could be transferred to WT mice that were co-housed with the inflammasome deficient mice (Elinav et al., 2011). Interestingly other studies have also observed that mice lacking Nlrp3 exhibit dysbiosis (Hirota et al., 2010). However,

caution must be taken when interpreting these data as it is difficult to know whether dysbiosis observed in these inflammasome deficient strains was causally linked to the defective innate immune response or whether, due to different environmental conditions in distinct animal facilities, they harboured an opportunistic pathogen. Indeed it is puzzling as to how a colitogenic species that arises as a consequence of impaired inflammasome activation can still thrive and promote disease in co-housed WT mice, in which inflammasome activation is intact? Furthermore, the fact that cross-fostering the inflammasome deficient mice onto a WT foster mother rescued the dysbiotic phenotype suggests that this phenotype may have arisen stochastically and is unrelated to impaired inflammasome activation (Elinav et al., 2011). Fitting with this, a key paper recently showed that *Myd88*$^{-/-}$ and *Tlr*$^{-/-}$ mice exhibited distinct microbiotas from each other, but that these were primarily shaped by maternal transmission rather than genotype (Ubeda et al., 2012). Thus, by crossing heterozygous *Myd88*$^{-/+}$ mice and various *Tlr*$^{-/+}$ mice, the authors showed that there was no separation of the microbiota makeup between WT and KO mice from the same litter (Ubeda et al., 2012). Furthermore, the microbiota of WT littermates of *Myd88*$^{-/-}$ mice more closely resembled *Myd88*$^{-/-}$ than the WT mice from *Tlr*$^{-/-}$ colonies (Ubeda et al., 2012).

While the studies above support an overall protective function of inflammasomes in acute intestinal inflammation, they are inconsistent with earlier evidence supporting the opposite conclusion. In fact, early studies showed that blocking either IL-18 or caspase-1, using monoclonal antibodies or pralnacasan, significantly alleviated DSS induced intestinal inflammation (Loher et al., 2004; Siegmund et al., 2001a) and it was reported that *caspase-1*$^{-/-}$ mice were more resistant to DSS colitis (Siegmund et al., 2001b). These studies have been supported by more recent work that shows that mice lacking Nlrp3 are protected from DSS-induced colitis (Elinav et al., 2011) (Bauer et al., 2012; Bauer et al., 2010). It is very difficult to reconcile these disparate findings, but one potential explanation is that deficiency in inflammasome components can have protective or pathogenic effects on DSS colitis depending on composition of the microbiota. Together, these studies highlight the need for further study of how inflammasome activation may directly or indirectly modulate intestinal homeostasis.

1.5 Experimental models of Intestinal inflammation

A number of animal models of intestinal inflammation exist that recapitulate specific elements of IBD. Broadly, one can classify these into two groups, acute and chronic, that may be driven by the innate immune system or that may require sustained activation of effector T cells. The models utilised in this thesis are discussed in the section below.

1.5.1 *Helicobacter hepaticus*-driven intestinal inflammation

a. Innate model

H. hepaticus is a Gram negative microaerophilic bacterium. It is spirally shaped with polarized flagella and persistently colonizes the caecum, colon and liver of mice. *H. hepaticus* causes chronic typhlocolitis (inflammation of the caecum and colon), as well as hepatitis and systemic inflammation, upon colonization of genetically susceptible hosts (Cahill et al., 1997; Fox et al., 1994; Kullberg et al., 1998; Ward et al., 1996). The utility of this model is that it recapitulates the aetiology of IBD: a dysbiosis coupled with a genetically susceptible host. An example of such a host is the 129SvEv *Rag2*$^{-/-}$ mouse strain that develops severe intestinal and mild systemic inflammation upon colonization with *H. hepaticus* (Figure 1.2) (Maloy et al., 2003). From a bacterial perspective precisely how *H. hepaticus* drives inflammation is not known, however many facets of the host pathological innate immune response that underlies the intestinal inflammation in this model are well studied. In brief, colonization of 129SvEv *Rag2*$^{-/-}$ mice results in Myd88 signalling within haematopoietic cells and production of IL-23 and IL-1β which signals via IL-23R on innate lymphoid cells (ILCs). ILCs produce copious amounts of IL-17, IFN-γ and IL-22 that ultimately drive intestinal inflammation by eliciting inflammatory responses from innate leukocytes in the gut (Asquith et al., 2010; Buonocore et al., 2010; Coccia et al., 2012; Hue et al., 2006). In addition to these cytokines, TNFα also perpetuates innate immune-mediated inflammation as blockade with anti-TNFα antibodies attenuates the inflammation in this model (Maloy et al., 2003).

b. Lymphocyte replete model

Although *H. hepaticus* also persistently colonizes WT lymphocyte replete mice, this is not usually accompanied by intestinal inflammation. This is because *H. hepaticus* drives the induction of $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ regulatory populations that suppress *H. hepaticus*-driven intestinal inflammation in an IL-10-dependent manner (Kullberg et al., 2002). Thus, *Il-10*$^{-/-}$ mice are susceptible to *H. hepaticus*-driven intestinal inflammation while WT mice display no signs of inflammation (Kullberg et al., 1998). To reveal pathogenic T cell responses towards *H. hepaticus* in WT mice, we used a monoclonal antibody against IL-10R to block regulatory circuits (Kullberg et al., 2006). Treatment of control uninfected WT mice with anti-IL-10R did not elicit intestinal inflammation. By contrast, the treatment of *H. hepaticus* infected WT mice with anti-IL-10R for 4 weeks led to severe intestinal inflammation that affected the caecum, proximal, mid and distal colon (Kullberg et al., 2006) (Figure 1.2). Typhocolitis was characterized by epithelial cell hyperplasia, goblet cell depletion, leukocytic infiltration, abscess formation and oedema. Similar to the *H. hepaticus* innate IBD model, intestinal inflammation in *H. hepaticus* infected WT mice treated with anti-IL-10R was dependent on Myd88 and IL-23 (Asquith et al., 2010; Kullberg et al., 2006). However, rather than ILCs, this T cell-mediated pathology was characterized by accumulation of large populations of Th1 and Th17 cells in the intestinal lamina propria, that produced high levels of IFN-γ, IL-17A and IL-17F (Asquith et al., 2010; Kullberg et al., 2006).

1.5.2 T cell transfer colitis

Transfer of naïve $CD4^+$ T cells ($CD4^+$ $CD25^-$ $CD45Rb^{hi}$) into a lymphopenic *Rag*$^{-/-}$ host leads to lymphopenia induced proliferation and differentiation into effector cells that drive intestinal inflammation, systemic inflammation and wasting disease (Powrie et al., 1993) (Figure 1.2). The histological features that characterize the intestinal inflammation include leukocytic infiltration, crypt hyperplasia and goblet depletion. Inflammation in this model is characterized by the production of pro-inflammatory cytokines, including IFN-γ, IL-17A and TNFα. IL-23 is another key player in this model as IL-23 deficient *Rag*$^{-/-}$ recipients do not develop colitis and naïve $CD4^+$ T cells deficient in IL-23R cannot drive intestinal inflammation (Ahern et al., 2010; Hue et al., 2006). T-bet deficient naïve $CD4^+$ T cells are also impaired in their ability to drive colitis, although

this seems independent of their capacity to produce IFN-γ (Neurath et al., 2002; Simpson et al., 1998). In addition, the Th17 inducing transcription factor Rorγt is important, as naïve $CD4^+$ T cells deficient in Rorγt are unable to drive colitis (Leppkes et al., 2009). Importantly, the development of T cell transfer colitis requires signals from the microbiota as germ-free (GF) *Rag*$^{-/-}$ recipients are protected from disease (Stepankova et al., 2007).

Pioneering studies by Powrie *et al* showed that the co-transfer of 'antigen-experienced' $CD4^+$ $CD45Rb^{lo}$ T cells could prevent both systemic and intestinal inflammation driven by naïve $CD4^+$ cells in lymphopenic hosts (Powrie et al., 1993). Subsequent studies characterized the suppressive activity to $CD25^+$ $Foxp3^+$ regulatory T cells, and this unique model has allowed researchers to probe the mechanisms involved in Treg cell differentiation and function *in vivo*, such as suppressive cytokines and cell-surface molecules (Izcue et al., 2009). In addition to preventing the onset of inflammation, adoptively transferred Tregs can also abrogate established colitis (Mottet et al., 2003; Uhlig et al., 2006).

1.5.3 *C. rodentium* model

C. rodentium is a Gram negative murine intestinal pathogen. It belongs to the class of attaching and effacing (A/E) pathogens, which also includes the human pathogens enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC, respectively) (Mundy et al., 2005). Upon colonization of WT mice, *C. rodentium* drives an acute intestinal inflammation characterized by oedema, epithelial damage and hyperplasia (Mundy et al., 2005) (Figure 1.2). Additionally infection with *C. rodentium* leads to the formation of characteristic A/E lesions, that are raised actin-rich pedestals above the plasma membrane that enable intimate bacterial attachment to IECs (Mundy et al., 2005). *C. rodentium* infection begins with rapid colonization of the caecal patch followed by infection of the distal colon about three days later. Bacterial burdens and disease symptoms peak around 8-14 days post infection at depending upon the strain used and infectious dose (Mundy et al., 2005). In immune competent hosts, *C. rodentium* is usually cleared from around day 14- 21 in an IgG dependent manner (Vallance et al., 2002).

The virulence factors utilized by *C. rodentium* to colonize mice and drive disease are very well characterized. *C. rodentium* harbours a pathogenicity island known as the locus of enterocyte

effacement (LEE) that encodes multiple virulence factors, including components of a type III secretion system that it uses to deliver effector proteins directly into IECs (Garmendia et al., 2005). Together these effectors work together to destroy intestinal microvilli, breakdown tight junction proteins that maintain the integrity of the IEC monolayer and inhibit the reabsorption of water. As a consequence, mice develop diarrhoea and acute intestinal inflammation (Garmendia et al., 2005; Mundy et al., 2005)).

The immune responses against *C. rodentium* have been well dissected over the last two decades. In terms of innate immunity, Myd88 and Tlr2 expression in IECs is crucial for protection against *C. rodentium* induced mortality (Gibson et al., 2008a; Gibson et al., 2008b; Lebeis et al., 2007). Tlr2 was initially proposed to contribute to protection by promoting the integrity of the epithelium via tight junction proteins (Gibson et al., 2008b), but a recent paper found that Tlr2 also limited excessive pro-inflammatory responses driven by Tlr4 (Gibson et al., 2010). Furthermore, Tlr2 also seemed to contribute to epithelial integrity through the production of IL-11, as *Tlr2*$^{-/-}$ mice could be rescued by the administration of recombinant IL-11 (Gibson et al., 2010). Myd88 signalling on haematopoietic cells is also required for protection and work showing that *Il1r1*$^{-/-}$ mice are also susceptible to *C. rodentium* driven mortality indicates that IL-1R signals contribute to the MyD88 protective axis (Lebeis et al., 2009). Nod1 and Nod2 are also important in immunity against *C. rodentium*, as they contribute to protective responses by inducing early innate IL-17 production and by recruiting monocytes to the lamina propria (Geddes et al., 2011; Kim et al., 2011). Adaptive immunity is central to clearing *C. rodentium* as *Rag*$^{-/-}$ mice are persistently colonized and eventually die from infection (Simmons et al., 2003; Vallance et al., 2002).

Mice lacking IL-17, IL-12, IFN-γ and TNFα are all hyper-susceptible to *C. rodentium* infection, however they do not suffer mortality upon infection (Goncalves et al., 2001; Ishigame et al., 2009; Simmons et al., 2002). A crucial axis of protection against *C. rodentium* is driven by IL-23, because *Il23*$^{-/-}$ mice exhibit high mortality after infection (Mangan et al., 2006). In this model, IL-23 signalling promotes the production of another protective cytokine, IL-22 by $CD4^{+}$ ILCs (Sonnenberg et al., 2011). IL-22 signals via IL-22R on IECs and promotes the production of Reg3γ, indeed *Il22*$^{-/-}$ deficient mice exhibit mortality after infection that can be rescued by administration of recombinant Reg3γ (Zheng et al., 2008). While ILCs represent a key source of

early IL-22, a second wave of T cell-derived IL-22 is important in the later stages of protection against *C. rodentium* (Basu et al., 2012). The variety of innate and adaptive immune responses that are triggered during different stages of *C. rodentium* infection make it an excellent model to assay the roles of defined factors in protection and pathology during pathogenic infection of the intestine.

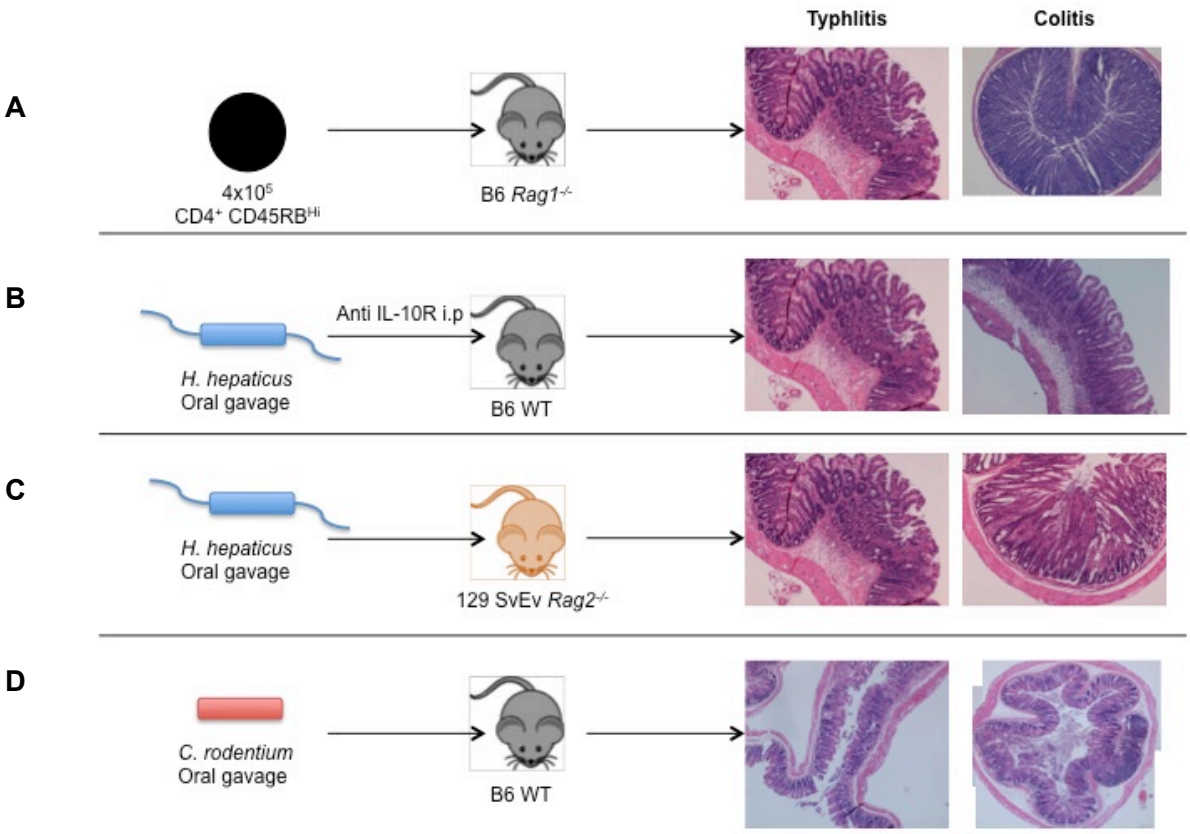


Figure 1.2 Models of IBD utilised in this thesis.

(A) T cell transfer model in B6 *Rag1*$^{-/-}$ mice

(B) *H. hepaticus* + Anti IL-10R model in lymphocyte replete B6 WT mice

(C) *H. hepaticus* innate model in 129SvEv *Rag2*$^{-/-}$ mice

(D) *C. rodentium* model in B6 WT mice

1.6 Aims of this thesis

As discussed above, there is a lot of contention over the role of inflammasomes in intestinal inflammation. Thus in this thesis I set out to use different models of intestinal inflammation to try and tease out whether inflammasome signalling played any part in maintaining intestinal homeostasis. From a less disease-focused perspective, the use of distinct models of intestinal inflammation also enabled me to uncover some surprising findings regarding how inflammasomes function in different cell types and to challenge certain established dogmas.

There is a large body of evidence suggesting that defective innate immunity can predispose one to CD through impaired bacterial handling, leading to outgrowths of AIEC for example. In light of this, I first used an infectious model of intestinal inflammation using the murine intestinal pathogen *C. rodentium*, the results are described in chapter 3. This pathogen has a tropism primarily for IECs that also enabled a dissection of inflammasome signalling in this understudied cell type in inflammasome biology.

Most of the work examining the role of the inflammasome thus far has relied upon chemical models of acute intestinal inflammation. While such models are useful and high-throughput, they fail to recapitulate many aspects of inflammation observed in CD. Thus, in an attempt to address this, I used multiple chronic models of intestinal inflammation. These included bacterial driven models of chronic intestinal inflammation using *H. hepaticus* that we carried out in both lymphocyte replete and deficient settings. The results from these investigations are presented in chapter 4.

Finally, I utilized a non-pathogen driven model of intestinal inflammation using the T cell transfer model. Principles of inflammasome biology are largely derived from studies in macrophages and other myeloid cells. The T cell transfer model allowed us to study the function of inflammasomes directly in T cells. Furthermore, we also probed the involvement of inflammasome-signalling in interactions between tissues cells, APCs, and naïve T cells and Tregs, using recipients deficient in inflammasome signalling. These results are found in chapter 5.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Mice

Wild-type C57BL/6, *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were bred and maintained under specific pathogen free (SPF) conditions in animal facilities at the University of Oxford, UK. *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were gifts from Vishva Dixit, Genentech. We crossed B6 *Rag*$^{-/-}$ mice with B6 *Asc*$^{-/-}$ mice to generate B6 *Rag*$^{-/-}$ *Asc*$^{-/-}$ mice. 129SvEv *Rag*$^{-/-}$ *Asc*$^{-/-}$ mice were generated by crossing B6 *Asc*$^{-/-}$ mice with 129SvEv *Rag*$^{-/-}$ mice using a speed congenics approach (Wakeland et al., 1997). According to the protocol, B6 *Asc*$^{-/-}$ mice were crossed to 129SvEv *Rag*$^{-/-}$, and every litter was screened for 63 microsatellite markers to identify offspring with the greatest proportion of 129SvEv congenic markers. These offspring were then backcrossed to 129SvEv *Rag*$^{-/-}$ mice with repeated enrichment for 129SvEv congenic markers for 5 generations until all 63 markers were 129SvEv. Experiments were performed in accordance with the UK Scientific Procedures Act of 1986. Mice were also routinely screened for *Helicobacter spp.* and were greater than 6 weeks old when used.

2.1.2 Antibodies

Antigen	Clone	Fluorochrome	Source
Fc Block (CD16/32)	93	N/A	EBioscience
CD4	RM4-5	PerCP/PE-Cy5/APC	EBioscience
CD45.2	104	FITC	EBioscience
TCRβ	H57-597	PE/APC/PerCP	EBioscience
CD11b	M1/70	PerCP/APC	EBioscience
GR1	RB6-8C5	PE	EBioscience
CD11c	N418	APC	EBioscience
Thy1.2	30-H12	APC	Biolegend
SCA-1	E13-161.7	FITC	EBioscience
SCA-1	D7	APC	EBioscience
F4/80	BM8	APC	EBioscience
MHCII	M5/114.15	e450	EBioscience
Siglec-F	E50-2440	PE	BD Biosciences
CD25	7D4	PE	EBiosciences
CD45Rb	16A	FITC	BD biosciences
Intracellular stain			
IL-17A	TC11-18H10	PE/Alexa647	EBiosciences
IFN-γ	XMG1.2	eFluor450	BD Biosciences
Foxp3	FJK-16S	FITC/APC	BD Biosciences
Isotype controls			
IgG1	R3-34	FITC/PE/APC	BD Biosciences
IgG1	P3.6.2.8.1	Alexa647	BD Biosciences
IgG1	B56	FITC	BD Biosciences
IgG2a	R35-95	FITC/PE/APC	BD Biosciences
IgG2a	N/A	eFluor450	EBioscience
IgG2b	A95-1	PE	BD Bioscience
In vivo			
IL-10R	1B1.2	none	In house

Table 2.1 Antibodies used in this thesis.

2.2 Cell isolation and cell sorting

2.2.1 Isolation of splenocytes and cells from the MLN

Spleens and MLNs were isolated from mice, accompanying fat was removed, and organs were placed directly into PBS supplemented with 0.1% BSA (PBS/BSA) on ice, until processing. Spleens and MLNs were next mashed with the plunger-end of 2ml syringes in petri dishes and then resuspended in 5mls of PBS/BSA through a 100μm cell strainer. The cell suspensions were then centrifuged at 1400rpm for 5 min and the supernatants discarded. Cell pellets from MLNs were

resuspended in 1ml of PBS/BSA and counted. Cell pellets from spleens were resuspended in 1ml of ACK (Ammonium Chloride Potassium (150 mM NH_4Cl, 10 mM, $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2 lysis buffer for 3 min to lyse red blood cells, followed by washing in 14 ml PBS/0.1% BSA and filtering through a 100µm cell strainer. Residual ACK was removed with a second wash step. Cells were finally resuspended in PBS/0.1% BSA for counting.

2.2.2 Enrichment of $CD4^+$ T cells and cell sorting

Splenocytes were isolated as described above. Cells were next incubated with a cocktail of antibodies directed against CD8, MHCII, CD11b and B220 for 20min on ice. Thereafter cells were washed and resuspended at a concentration of 10^8 cells/ml in PBS/0.1% BSA. Anti-rat IgG coated Dynal beads (Invitrogen) were vortexed and then mixed with cells in a 1:1 ratio. This cell/bead mixture was then incubated for 20 min at 4°C on a rotator. Next, a magnet was applied to the bead mixture to separate stained cells from non-stained cells (mostly $CD4^+$ T cells). Cells were then washed in PBS/0.1% BSA before being resuspended in cold PBS/0.1% BSA. Next cells were stained with antibodies against CD4, CD25 and CD45RB for 20 min at 4^oC followed by two washes with PBS/0.1% BSA. Naive $CD4^+CD25^-CD45RB^{hi}$ T cells and $CD4^+CD25^+CD45RB^{lo}$ Tregswere sorted using a MoFLo cell sorter (Dako Cytomation, BD).

2.2.3 Isolation of colonic lamina propria leukocytes (cLPL)

Colons were cut open longitudinally and faecal contents removed before being cut into 1cm pieces and placed into a 50ml tube (Falcon, BD Biosciences) with 10ml of ice-cold PBS/0.1% BSA. After aspirating the liquid, 10ml of warm (37°C) RPMI/10% FCS containing 5 mM EDTA (VWR International UK) was added to the colon pieces and tubes were shaken (200rpm) for 15min at 37°C to remove IECs. Supernatants were discarded and this procedure was repeated twice more, for a total of three EDTA washes. Next, ~10ml of RPMI 10%FCS containing 15mM Hepes (PAA) was added to each sample to neutralize the EDTA activity. After aspirating the supernatants, 10ml of RPMI/10%FCS/15mM Hepes containing 0.25mg/ml type VIII collagenase (Sigma Aldrich) was added to samples and incubated for 1h at 37°C with shaking. Supernatants from the digest were

collected through a cell strainer (100 µm), after which 200µl of EDTA was added to block collagenase activity. Cells were then centrifuged and resuspended in 3ml 30% Percoll (GE, Amersham, UK) solution (P30). The suspension was then gently added over a gradient comprising 3ml 75% Percoll layer (P75, bottom) underlying a 4ml 40% Percoll layer (P40, top layer), making a P30/P45/P75 gradient. Gradients were centrifuged for 20min at 1800rpm, without brakes. Cells were then isolated from the P40/P75 interface and washed with PBS/0.1% BSA before resuspension in PBS/0.1% BSA.

2.2.4 Isolation and culture of bone marrow derived macrophages (BMDMs)

Tibiae and femurs from mice were isolated and the bone marrow was flushed using PBS/0.1%BSA and a 26G needle. The bone marrow was then dispersed using a 19G needle, washed in PBS/0.1%BSA and then resuspended in RPMI/10% FCS/100U/ml penicillin/streptomycin/2mM L-glutamine containing 15% L292 cell conditioned media. Cells were cultured at 37°C in a 5% CO_2 incubator in 15cm bacterial plastic petri dishes (Falcon, BD Bioscience) for three days before supplementing the medium. On day 6, the medium was completely changed before detaching BMDMs on day 7 using PBS/10mM EDTA containing 4mg/ml lidocaine (Sigma Aldrich) and plating. $2x10^5$ BMDMs/well were plated in a 24 well plate and infected with *C. rodentium* or *H. hepaticus* at various MOIs.

2.2.5 Inflammasome activation with BMDMs

BMDMs were either pre-treated with 20ng/ml LPS overnight (Alexis biochemical) or left untreated. The next day BMDMs were washed three times with PBS before infection with either *C. rodentium* or *H. hepaticus* in Optimem media (Invitrogen). For infections with *C. rodentium*, BMDMs were infected for 1.5 hours before aspirating the bacteria-containing media and adding gentamycin (100µg/ml) for another 5.5 hours. Thereafter cell supernatants were harvested and either assayed by ELISA or by immunoblotting after protein isolation and concentration. For infections with *H. hepaticus*, BMDMs were infected for 16 hours before harvesting supernatants. In experiments involving inhibitors, BMDMs were either pre-incubated with various inhibitors for 1

hour before infection (Bafilomycin A1 at 250nM, Cytochalasin D at 5μM, APDC at 500μM, YVAD at 0.3mM (all from Sigma Aldrich)) or incubated with inhibitors (KCl at 5- 130mM, NH_4Cl at 10mM and Glycine at 5mM (all from Sigma Aldrich)) throughout the course of the infection. For positive controls for inflammasome activation BMDMs were treated with either 5μM Nigericin or 5mM ATP (both from Sigma Aldrich) for 7 hours and 3 hours, respectively.

2.3 Generation of irradiation bone marrow chimeras

Mice were irradiated twice with 550 RAD from a ^{60}Co source with a gap of 3-4 hours between each irradiation. Bone marrow cells were prepared as previously described by flushing the femurs and tibiae of mice with PBS/0.1% BSA. $10x10^6$ bone marrow derived cells were then injected intravenously (i.v.) into each irradiated recipient. Mice were left to reconstitute their haematopoietic cell system for 6-8 weeks, before being infected with *C. rodentium*.

2.4 Quantitative PCR (qPCR) and microarray

2.4.1 qPCR

Caecum and colon tissues were flash-frozen in liquid nitrogen and RNA was isolated with Qiagen RNeasy Mini Kit (Cat.74104). RNA was then reverse transcribed using Superscript III reverse transcriptase and oligo dT primers (both from Invitrogen). qPCR reactions were performed in triplicate and cDNA was amplified using Quantace SensiMix reagents (QT50505) and a Chromo4 detection system (MJ Research). mRNA expression levels were analysed post normalisation to *Hprt1* (which served as a house keeping gene) expression levels using the following formula: arbitrary units = $1000 * 2^{(-\Delta CT)}$. Primers and probes were purchased from Applied Biosystems.

2.4.2 Microarray

Infected caecal tissue was placed into RNAlater and RNA was extracted using the Ambion RiboPure kit (AM1924). Subsequent steps were carried out in collaboration with Dr Dilair Baban from the Wellcome Trust Centre for Human Genetics, University of Oxford. RNA quantity was determined using a NanoDrop ND1000 while RNA quality was assessed with a BioAnalyzer 2100

(Agilent Technologies). 300ng of total RNA from each sample was used for whole-genome gene expression analysis using the Illumina Single Colour Mouse WG-6_V2_0_R0_11278593 BeadChip with direct hybridization assay (Illumina, San Diego, CA). Biotinylated cRNA was prepared using the Illumina TotalPrep-96 RNA Amplification Kit (# 4393543 Ambion, Inc., Austin, TX). After array hybridization, washing, blocking, and staining with streptavidin-Cy3, the fluorescence emissions by Cy3 were imaged with the iScan system. GenomeStudio 2011 software (Illumina, San Diego, CA) was next used to generate data that was then imported to GeneSpring GX 12 (Agilent Technologies, Inc, Santa Clara CA) and analyzed. To identify significantly differentially expressed genes, data was normalized with Shift to 75 percentile and then baseline transformed to median of all samples.

2.5 Induction and regulation of intestinal inflammation

2.5.1 *C. rodentium*

C. rodentium (ICC169) (a gift from Professor Gadi Frankel) was grown to stationary phase overnight at 37°C in Luria Broth (Sigma) supplemented with nalidixic acid (Sigma) to a concentration of 50μg/ml in a shaking incubator. The next day, the optical density (OD) of the culture was determined and then diluted to 0.05. The culture was grown to log phase for 3.5hours, harvested by ultra centrifugation and resuspended in sterile PBS. Mice were orally gavaged with 200μl of bacterial solution at a concentration of between 10^8 and 10^9 CFU per mouse. Following infection, mice were weighed every day and culled if weight loss exceeded 20% of starting weight. Mice were thereafter euthanized at various time points throughout the infection. Colon and caeca washed in PBS/0.1% BSA before fixing in a 3.6% (w/v) formaldehyde solution for assessment of intestinal inflammation.

2.5.2 T cell transfer colitis

$4x10^5$ $CD4^+CD25^-CD45RB^{hi}$ T cells (naïve T cells) were injected into B6 *Rag1*$^{-/-}$ recipients intraperitoneally (i.p.) (Powrie et al., 1993). For Treg mediated protection from colitis, $4x10^5$ naive T cells and $2x10^5$ $CD4^+CD25^+CD45RB^{lo}$ (Tregs) were mixed and injected i.p. into B6 *Rag1*$^{-/-}$ hosts (Izcue et al., 2008; Uhlig et al., 2006). Mice were weighed weekly throughout the experiment and

sacrificed when weight loss reached 20% of the original body weight before T cell transfer. After sacrifice, colon (proximal, mid and distal) and caeca were washed in PBS/0.1% BSA and then fixed in a 3.6% (w/v) formaldehyde solution for assessment of intestinal inflammation.

2.5.3 *Helicobacter hepaticus*

H. hepaticus **culture**

Helicobacter hepaticus NCI-Frederick isolate 1A was grown at 37°C on blood agar plates made with laked horse blood supplemented with the antibiotics trimethoprim (5μg/ml), vancomycin (10 μg/ml) and polymyxin B (25 IU/ml) (TVP, Oxoid) in microaerophilic conditions (10% CO_2, 10% H_2, 80% N_2) and held in vented jars (Oxoid). After 3 days of culture on blood agar plates bacteria were inoculated into liquid cultures of TSB media supplemented with 10% FCS and TVP (Gibco) in 250ml vented Erlenmeyer flasks (Corning) and were grown under microaerophilic conditions at 37°C on a shaking incubator overnight. Cultures were then centrifuged at 7500rpm for 20 min to pellet bacteria. The viability of *H. hepaticus* was determined using a fluorescent Live/Dead assay (Molecular Probes).

Quantification of *H. hepaticus* colonization

After sacrificing mice infected with *H. hepaticus*, DNA was isolated from caecal contents with the Stool DNA Isolation Kit and colonization was assessed by qPCR using primers and probes specific for the *H. hepaticus* ctdB gene (F-CCG CAA ATT GCA GCA ATA CTT; R-TCG TCC AAA ATG CAC AGG TG; Probe AAT ATA CGC GCA CAC CTC TCA TCT GAC CAT) (Ge et al., 2001). *H. hepaticus* colonization levels were calculated by generating a standard curve by serially diluting *H. hepaticus* genomic DNA and then extrapolating the [*H. hepaticus* DNA] in the sample of interest. Colonization was expressed as [*H. hepaticus* DNA]/20ng of total caecal DNA.

Lymphocyte replete (*H. hepaticus* + anti IL-10R) model

Mice were orally gavaged with $1x10^8$ cfu *Helicobacter hepaticus* with a 22G curved gavage needle for the first three days of the infection. Additionally, mice received anti-IL-10R blocking antibody

(1mg/ml) by i.p. injection once weekly for 4 weeks starting from the day of *H. hepaticus* infection. Mice were euthanized 4 weeks post infection and colon and caeca washed in PBS/0.1% BSA before fixing in a 3.6% (w/v) formaldehyde solution for assessment of intestinal inflammation.

Innate model

Mice on a 129SvEv background were orally gavaged with $1x10^8$ cfu *Helicobacter hepaticus* with a 22G curved gavage needle for the first three days of the infection. Mice were then euthanized 3 or 8 weeks post infection and colon and caeca washed in PBS/0.1% BSA before fixing in a 3.6% (w/v) formaldehyde solution for assessment of intestinal inflammation.

2.6 Assessment of intestinal inflammation

2.6.1 Histological analysis

Mice were euthanized at indicated time points during the course of infection whereupon ~0.5-1cm pieces of caecum, proximal, mid and distal colon were cut and fixed in 10% formaldehyde saline solution. 5µm sections were then cut using a microtome and stained with haematoxylin and eosin (H&E). Sections of caecum and distal colon were then scored by two researchers in a blinded fashion in accordance with the criteria listed below.

Criteria for assessment of intestinal inflammation in *H. hepaticus* driven and T cell transfer models of colitis

A	Epithelium	Hyperplasia	and/or	Goblet Cell Depletion				
	0	None		None				
	1	Mild (1.5X)		Mild (25%)				
	2	Moderate (2-3X)		Marked (25-50%)				
	3	Severe (>3X)		Substantial (>50%)				
B	Inflammation in lamina propria							
	0	None - few leucocytes						
	1	Mild - some increase in leukocytes at tips of crypts OR many lymphoid follicles						
	2	Moderate - marked infiltrate (notable broadening of crypt)						
	3	Severe - dense infiltrate throughout						
C	Area affected (% of section)							
	0	None						
	1	Up to 25%						
	2	25-50%						
	3	>50%						
D	Markers of severe inflammation	Submucosal inflammation		Crypt abscesses		Crypt branching		Ulceration OR Extensive fibrosis
	0	None		None		None		None
	1	Mild	OR	Few (<5)		None		None
	2	Mild	AND	Few (<5)		None		None
	2	Extensive	OR	Many (>5)	OR	Yes		None
	3	Extensive	AND	Many (>5)	OR	Yes		None
	3	-		-		-		Yes

Criteria for assessment of intestinal inflammation in *C. rodentium* driven intestinal inflammation

A	Epithelium	Hyperplasia	and/or	Goblet Cell Depletion	and/or	Damage
	0	None		None		None
	1	Mild (1.5X)		Mild (25%)		Mild
	2	Moderate (2-3X)		Marked (25-50%)		Marked
	3	Severe (>3X)		Substantial (>50%)		Substantial
B	Inflammation in lamina propria					
	0	None - few leucocytes				
	1	Mild - some increase in leukocytes at tips of crypts OR many lymphoid follicles				
	2	Moderate - marked infiltrate (notable broadening of crypt)				
	3	Severe - dense infiltrate throughout				
C	Submucosal	Edema		Infiltration		Follicle
	0	None	and/or	None	and/or	None
	1	Mild	and/or	Mild	and/or	Enlarged
	2	Marked	OR	Marked		-
	3	Marked	AND	Marked		-
D	Area affected (% of section)					
	0	None				
	1	Up to 25%				
	2	25-50%				
	3	>50%				
E	Markers of severe inflammation	Bleeding	and/or	Crypt abscesses	and/or	Necrosis/ulceration
	0	None		None		None
	1	Some		Few		None
	2	Marked		Many		Few/small
	3	-		-		Large/many

2.6.2 Flow cytometry

Stained cells were acquired using a Dako ADP Cyan (BD) and then analyzed using FlowJo software (TreeStar Inc. Oregon, USA).

Surface staining

Single cell suspensions (typically $1x10^6$ cells/well) were resuspended in 50µl PBS/0.1% BSA and blocked with anti-CD16/32 mAb (eBioscience) to block Fc receptors and non-specific binding of antibodies, for 15 min on ice in a round bottom 96 well plate (Corning). This was followed by two washes in PBS/0.1% BSA. Cells were then stained with a panel of antibodies for 15 min on ice in the dark before being washed at least twice in 200µl PBS/0.1% BSA, as described above. Stained samples were then fixed in 200µl PBS/2% PFA and stored in the dark at 4°C until analysis the next day.

Intracellular staining

Following surface staining described above, cells were resuspended in 100µl of permeabilization buffer (BD Biosciences or eBioscience) and incubated in the dark at 4°C overnight. The next day cells were centrifuged at 1500rpm for 5 min and resuspended in 100µl of permeabilization buffer (eBioscience) supplemented with 2% normal rat serum (NRS) and incubated for 2hours at 4°C in the dark. After this, cells were washed twice in permeabilization buffer and then resuspended in 50µl of buffer containing a panel of fluorescently conjugated antibodies and incubated for 20min at 4°C in the dark. Thereafter stained cells were washed 4-5 times in permeabilization buffer and then resuspended in PBS/0.1% BSA for acquisition.

For intracellular staining, cells were first restimulated for 3hours at 37°C in a 5% CO_2 incubator in 200µl RPMI 1640 or IMDM/ 10% FCS, 100U/ml penicillin/streptomycin, 2mM L-glutamine and 0.05mM β-mercaptoethanol containing 0.1µg/ml PMA (phorbol-12-myristate-13-acetate), 1µg/ml ionomycin and 10µg/ml Brefeldin A (Insight Biotechnology, Wembley, UK). After restimulation, cells were washed twice in PBS/0.1% BSA before surface staining and intracellular staining, as described above.

2.6.3 Colony counts of *C. rodentium*

To measure the tissue adherent *C. rodentium* levels in infected mice, tissues were first weighed and then homogenized using ceramic beads in a FASTPREP-24 (MP) in 600µl of sterile PBS. The

lysate was serially diluted in PBS, plated on nalidixic acid (50μg/ml) agar plates dilutions and then incubated at 37°C overnight before counting colonies the next day. As *C. rodentium* (ICC169) harbours a resistance plasmid against nalidixic acid, this plating method allows for the selection of *C. rodentium*. The number of colonies were normalised to the weight of the organ used for homogenization to generate CFU/g.

2.6.4 Organ culture

To measure cytokine production from tissue, tissues were first weighed and then washed in PBS supplemented with 1000U/ml penicillin, streptomycin and 0.1% BSA for 10 min to kill bacteria. Thereafter organ explants were incubated in RPMI supplemented with 1000U/ml penicillin, streptomycin, 10% FCS and 2mM L-glutamine, in 48-well plates at 5% CO_2 in a 37°C incubator for 24 hours. Supernatants were then harvested and analysed for cytokines. IL-1β and IL-18 levels were measured by sandwich ELISA (primary antibody: eBioscience 14-7012-85; secondary antibody: eBioscience 13-7112-85; Mouse IL-18 Platinum ELISA, eBioscience BMS618/2TEN). IFN-γ, IL-17A, IL-22, and TNFα were measured by Flow Cytomix Cytokine Bead Assay (Bender MedSystems).

2.7 Western blot

2.7.1 Whole tissue

Total protein extracts were made by homogenizing snap frozen caecal tissue in RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1 mM EDTA) containing a protease inhibitor cocktail (Roche). Protein levels were normalized by the Lowry assay (Biorad) and then diluted in sample buffer (60 mM Tris HCl pH 6.8, 2% SDS, 0.02% Bromophenol Blue, 5% β-Mercaptoethanol, all from Sigma Aldrich). Based on how much protein was isolated, 5-12μg was resolved by 15% SDS-PAGE gels at 140V. Samples were transferred at 50V onto a methanol-activated PVDF membrane (GE-Healthcare). The membrane was fixed with PBS/0.5% PFA for 20min and then blocked with PBS/0.5% Tween-20 (Sigma Aldrich) containing

2.5% milk powder (Tesco value) for 1 hour at room temperature. The membrane was next incubated with primary antibody (anti-caspase-1 p10 (Santa Cruz sc-514), anti-IL-18 (Abcam ab71495) or anti-Tubulin (Santa Cruz sc5286)) at 4°C overnight. The next day, the membrane was washed 4times for 5 min each with PBS/0.5% Tween-20 before incubating with secondary antibody (anti-Rabbit IgG (Santa Cruz, 1:5000) for 1-2 hours at room temperature. After 5 washes with PBS/0.5% Tween-20 for 10 min each to remove non-specifically bound secondary antibody, the membrane was visualized and imaged using ECL solution (Pierce).

2.7.2 *In vitro* BMDMs

After infection of BMDMs with either *H. hepaticus* or *C. rodentium*, cell supernatants were spun down and frozen at -20°C until further analysis. Supernatants were thawed and then concentrated with chloroform/methanol precipitation. 1 volume of methanol and 0.25 volumes of chloroform were mixed with samples into a homogenous suspension. This was then spun down for 5min at 4°C and 13000rpm. Thereafter the protein precipitates at the interface between methanol and chloroform. The top layer was gently removed and replaced with another volume of methanol before spinning again for another 20min at 4°C and 13000rpm. Thereafter the supernatant was discarded, the protein pellet air-dried and finally resuspended in1x sample buffer. Protein samples were then boiled at 95°C for 10min and placed immediately onto ice for 1minute before spinning down for 5 min at 4°C and 13000rpm. Thereafter samples were loaded as resolved on a SDS gel as described above. The methods used for inflammasome activation assays and Western blots are described in more detail in (Gross, 2012).

2.8 Fluorescence microscopy

Formaldehyde-fixed tissue sections were deparaffinized and antigen retrieval was performed in 0.01 M sodium citrate buffer in a decloaking chamber (Biocare). Slides were then blocked with normal goat serum and stained with rabbit anti-*C. rodentium* antiserum, mouse anti-E-cadherin (BD Bioscience) and/or mouse anti-Asc (Santa Cruz, sc 22514R). Slides were then stained with

AF555-conjugated goat-anti-rabbit and an AF488-conjugated goat-anti-mouse secondary antibody (Invitrogen). Finally, slides were mounted in DAPI-containing Vectashield (Vector Laboratories) and visualized using an Olympus FV1000 confocal microscope.

2.9 Statistics

Statistical significance was determined by two-way ANOVA with Bonferroni post-tests for weight curves. All other statistical significance was determined by nonparametric Mann-Whitney as the variables were not normally distributed. Differences were considered statistically significant when $p < 0.05$.

Chapter 3. The inflammasome in mucosal defence: Nlrp3/Asc activation in intestinal epithelial cells protects against *C. rodentium* infection

3.1 Introduction

NLRs have garnered much attention in recent times mainly due to the strong association of *NOD2* polymorphisms with susceptibility to Crohn's disease (CD) (Hugot et al., 2001; Ogura et al., 2001). More recently, inflammasome-forming NLRs have also been implicated in intestinal immune homeostasis. Interestingly, a number of genes associated upstream and downstream of the inflammasome pathway have been implicated in susceptibility to CD by GWAS studies, including; the *IL-18R1-IL-18RAP* locus (Barrett et al., 2008; Imielinski et al., 2009); *ATG16L1*, an autophagy gene that negatively regulates the inflammasome (Hampe et al., 2007); and *NLRP3*, an upstream inflammasome forming NLR (Villani et al., 2009). Furthermore, components of the inflammasome such as Asc and caspase-1 are highly expressed in the intestine (data not shown). Yet despite this the function of inflammasomes in the intestine remains unclear. Experimental models have thus far mainly relied on chemical models of acute intestinal inflammation e.g. DSS or TNBS. Using DSS, early studies suggested that caspase-1 and downstream cytokines such as IL-18 drove intestinal inflammation (Loher et al., 2004; Siegmund et al., 2001a; Siegmund et al., 2001b; Sivakumar et al., 2002). Few studies supported this notion, showing that *Nlrp3*$^{-/-}$ mice were protected from DSS colitis, (Bauer et al., 2012; Bauer et al., 2010; Elinav et al., 2011). DSS triggered activation of caspase-1 in an Nlrp3-dependent manner in bone marrow-derived macrophages (BMDMs) *in vitro* (Bauer et al., 2010). Yet three other studies found that mice lacking either caspase-1, Nlrp3 or Asc were susceptible to DSS and developed greater weight loss and intestinal inflammation (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). While these latter studies agreed on the general role of the Nlrp3 inflammasome during intestinal inflammation, they showed conflicting results on which cellular compartment was involved. This study is further complicated by the role of the microbiota that has been recently implicated in this model with *Asc*$^{-/-}$ mice (Elinav et al., 2011). While DSS is a useful model of intestinal inflammation, it is essentially an intestinal injury model that fails to recapitulate numerous aspects of IBD.

Regarding the role of inflammasomes in defence against intestinal pathogens, much work has utilized *Salmonella typhimurium* infection as the model of choice. Some studies indicate that caspase-1 activation is required by *S. typhimuirum* to colinize the intestine and initiate disease (Monack et al., 2000; Muller et al., 2009). However others contend that caspase-1 is protective against oral *S. typhimuirum* infections (Lara-Tejero et al., 2006; Raupach et al., 2006). Collectively, the role of inflammasomes in intestinal inflammation remains equivocal and more work is needed to clarify these issues.

To help address this, we wished to assess the role of the inflammasome in protection from pathogenic bacterial infection in the intestine. We utilized the *C. rodentium* model of acute intestinal inflammation to do so. While IBD is not an infectious disease, one factor predisposing patients, suggested by GWAS studies, may be impaired innate immunity. Thus models with enteric pathogens such as *C. rodentium* might provide us with an indirect perspective into the aetiology underlying CD in patients harbouring variants in *NLRP3*.

3.2 Results

3.2.1 *C. rodentium* activates the Nlrp3 inflammasome *in vitro*

We first asked whether the inflammasome was involved in the sensing of *C. rodentium in vitro*. To test this we infected bone-marrow derived macrophages (BMDMs) with *C. rodentium* and assayed inflammasome activation using western blotting (WB) to detect the p10 subunits that are generated through autocatalytic cleavage of activated caspase-1. We found that *C. rodentium* infection triggered caspase-1 activation in BMDMs in a dose-dependent manner (Figure 3.2). Inflammasome activation is thought to occur through a two-signal mechanism, wherein signal 1 is provided by TLR-NFκB-dependent priming of pro-caspase-1 expression (Latz et al., 2013; Martinon et al., 2009) and signal 2 is provided by an inflammasome forming NLR. Interestingly, *C. rodentium* activation of caspase-1 required priming of BMDMs with LPS, indicating that the bacterium cannot provide both signals simultaneously *in vitro* (Figure 3.2). To determine which NLR was upstream of caspase-1, we generated BMDMs from *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ mice and infected them alongside WT controls with *C. rodentium*. Caspase-1 cleavage was completely abrogated in *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs infected with *C. rodentium* (Figure 3.3), indicating that *C. rodentium* triggers an Nlrp3/Asc-dependent inflammasome in BMDMs.

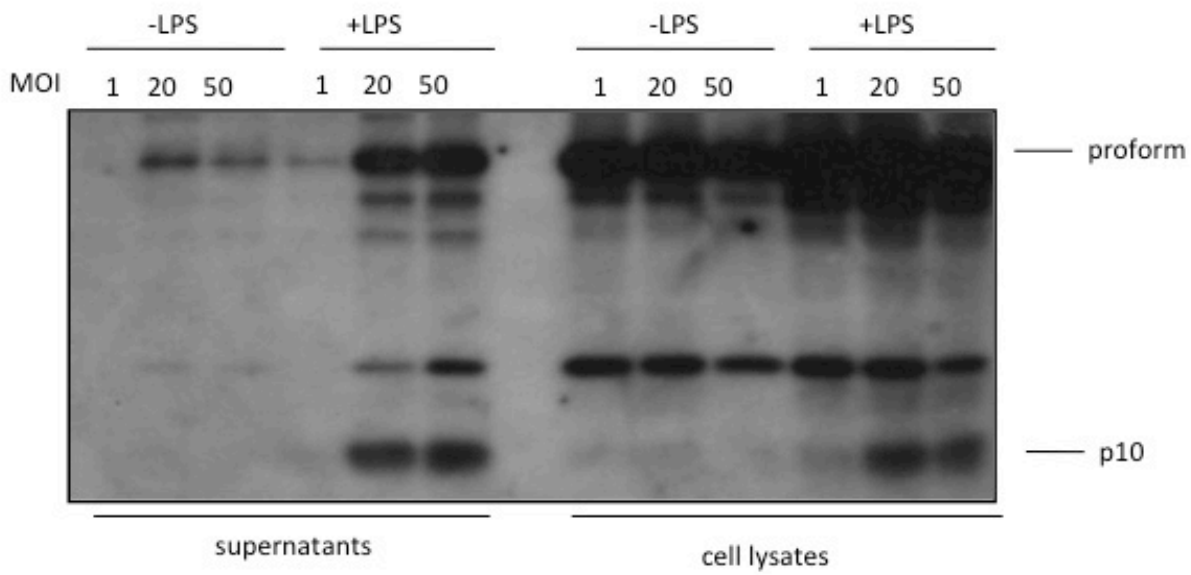


Figure 3.2. *C. rodentium* infection triggers caspase-1 activation in BMDMs *in vitro*. $2x10^5$ bone marrow derived macrophages (BMDMs) were either pre-treated with 20ng/ml lipopolysaccharide (LPS) overnight or left untreated. The next day BMDMs were infected *in vitro* with increasing multiplicities of infection (MOI) of *C. rodentium* for 1.5 hours before treating with 100μg/ml gentamycin for another 5.5 hours. Cell lysates and supernatants were then collected, protein extracted and concentrated and then immunoblotted for caspase-1 p10. Data shown represents one of two independent experiments with similar results.

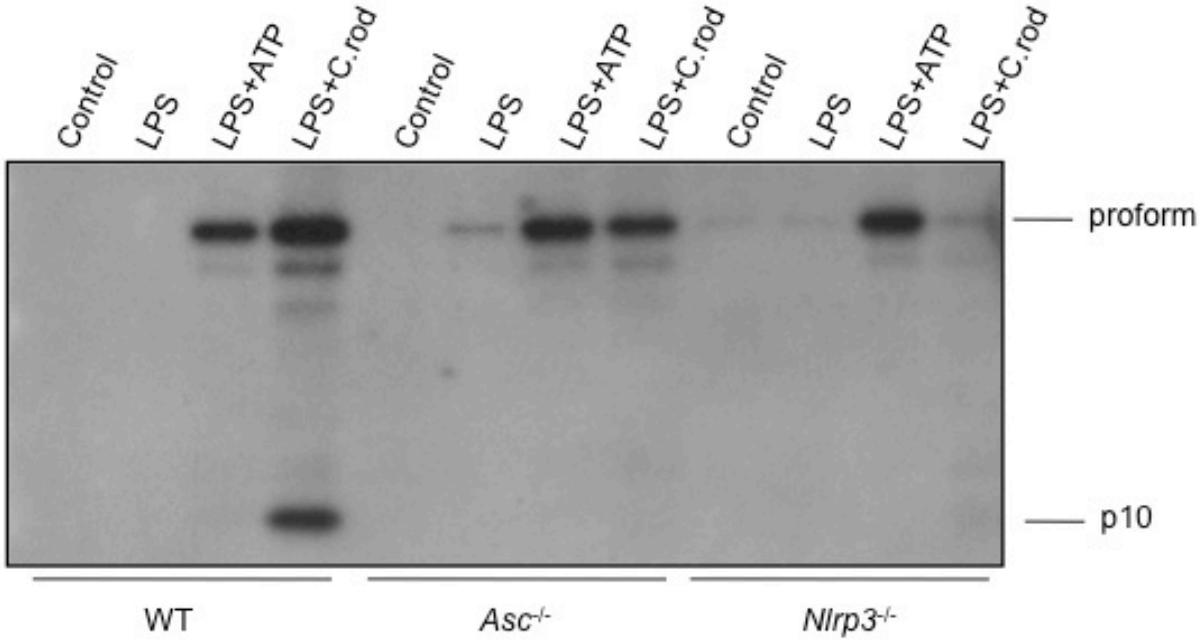


Figure 3.3. *C. rodentium* triggered caspase-1 activation in BMDMs is Nlrp3 and Asc dependent. 2x10^5 bone marrow derived macrophages (BMDMs) from wild type (WT), *Asc*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice were either pre-treated with 20ng/ml lipopolysaccharide (LPS) overnight or left untreated (control). The next day BMDMs were infected *in vitro* with *C. rodentium* (MOI 20) for 1.5 hours before treating with 100μg/ml gentamycin for another 5.5 hours. Cell supernatants were then collected, protein extracted, concentrated and then immunoblotted for caspase-1 p10. Data shown represents one of two independent experiments with similar results.

To further confirm that *C. rodentium* did indeed activate caspase-1 inflammasomes in BMDMs, we measured downstream cytokines cleaved and activated by this enzyme, IL-1β and IL-18. Consistent with the activation of caspase-1, *C. rodentium* infection triggered robust release of IL-1β and IL-18 by BMDMs in an Asc and Nlrp3-dependent manner (Figure 3.4). Again, production of these cytokines required priming with LPS, as BMDMs did not produce IL-1β and IL-18 upon infection with *C. rodentium* in the absence of prior LPS priming (data not shown).

3.2.2 *C. rodentium* induced inflammasome activation requires the phagocytosis of live bacteria and potassium efflux

Having identified that Nlrp3 sensing of *C. rodentium* was required for caspase-1 activation we next set out to ascertain the mechanisms involved. As described in the general introduction, three main models of Nlrp3 inflammasome activation have been proposed; the channel model; the lysosome rupture model; and the ROS model (Tschopp and Schroder, 2010).

To determine the mechanism through which *C. rodentium* triggered Nlrp3 activation, we utilized inhibitors to block distinct pathways associated with each model. Firstly, we observed that heat killed (HK) *C. rodentium* was unable to trigger caspase-1 activation in BMDMs, suggesting that live bacteria were required (Figure 3.5A). Furthermore, pre-treatment of BMDMs with cytochalasin, an inhibitor of actin polymerization, completely abrogated *C. rodentium* induced caspase-1 activation, indicating that phagocytosis of the bacterium was necessary (Figure 3.5A). However, blocking lysosomal acidification using either NH_4Cl or bafilomycin A, a vacuolar type H^+-ATPase inhibitor, had no effect on caspase-1 cleavage in infected BMDMs, showing that *C. rodentium* triggers caspase-1 independently of phagosomal acidification (Figure 3.5A). Furthermore glycine, a non-specific cytoprotective agent, had no effect on caspase-1 activation. As glycine prevents cell death that results from non-specific ion fluxes, we can conclude that caspase-1 activation is not due to non-specific cell death (Bergsbaken et al., 2009).

As K^+ efflux is a key feature of the channel model of Nlrp3 inflammasome activation, we tested the effect of blocking this by gradually increasing the extracellular [K^+] concentration in the medium. Interestingly, we observed a dose-dependent reduction in caspase-1 cleavage as we increased the [K^+] concentration (Figure 3.5B). Thus, *C. rodentium* induced caspase-1 activation in BMDMs

requires live bacteria that somehow induce a K^+ efflux that subsequently triggers Nlrp3 activation. To test the role of ROS, we pre-treated BMDMs with the ROS inhibitor, APDC. (4-amino-2,4-pyrrolidinedicarboxylic acid) (Zhou et al., 2011). However, treatment with APDC had no effect on caspase-1 cleavage after *C. rodentium* infection suggesting that ROS is dispensable for inflammasome activation in this setting (Figure 3.5B). Consistent with the requirement for K^+ efflux, phagocytosis and live bacteria for caspase-1 activation, inhibiting these pathways also abrogated IL-1β and IL-18 production by BMDMs (Figure 3.6). Finally, to confirm the involvement of caspase-1 we used a specific peptide inhibitor of caspase-1, YVAD (Thornberry et al., 1992). Pretreatment of BMDMs with YVAD led to a large reduction in IL-1β production and a smaller reduction in IL-18 production after stimulation with *C. rodentium* (Figure 3.6).

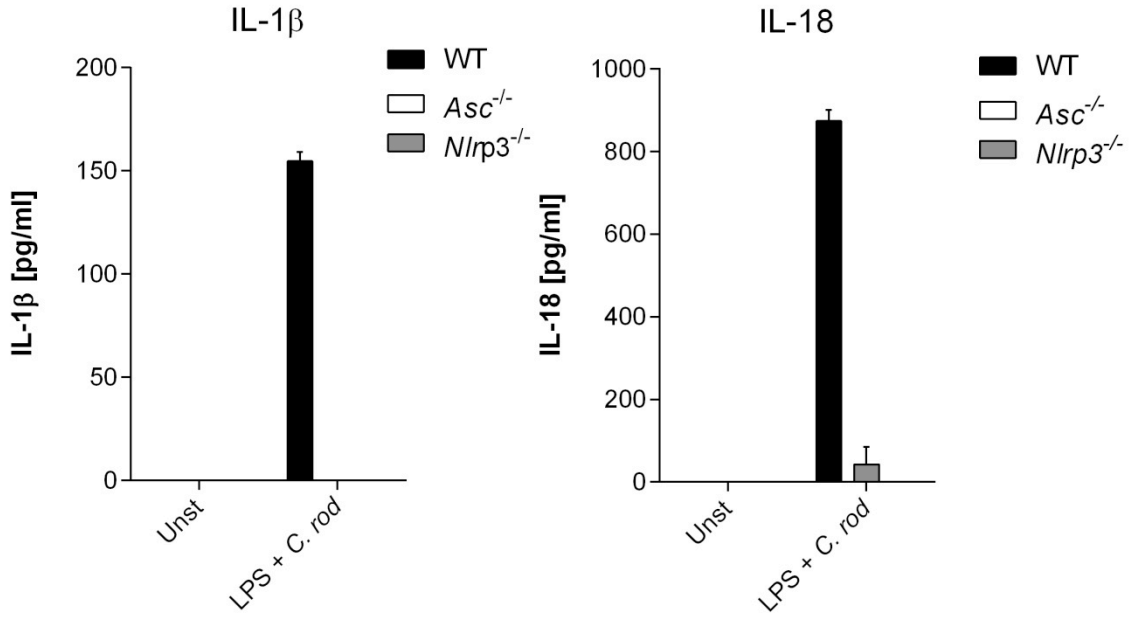


Figure 3.4. *C. rodentium* infection triggers the release of IL-1β and IL-18 by BMDMs. $2x10^5$ bone marrow derived macrophages (BMDMs) from wild type (WT), *Asc*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice were either pre-treated with 20ng/ml lipopolysaccharide (LPS) overnight or left unstimulated (unst). The next day BMDMs were infected *in vitro* with *C. rodentium* (MOI 20) for 1.5 hours before treating with 100μg/ml gentamycin for another 5.5 hours. Cell supernatants were then collected and assayed for IL-1β or IL-18 by ELISA. Bars are representative of mean±SEM and are derived from triplicate wells. Data shown represents one of two independent experiments with similar results.

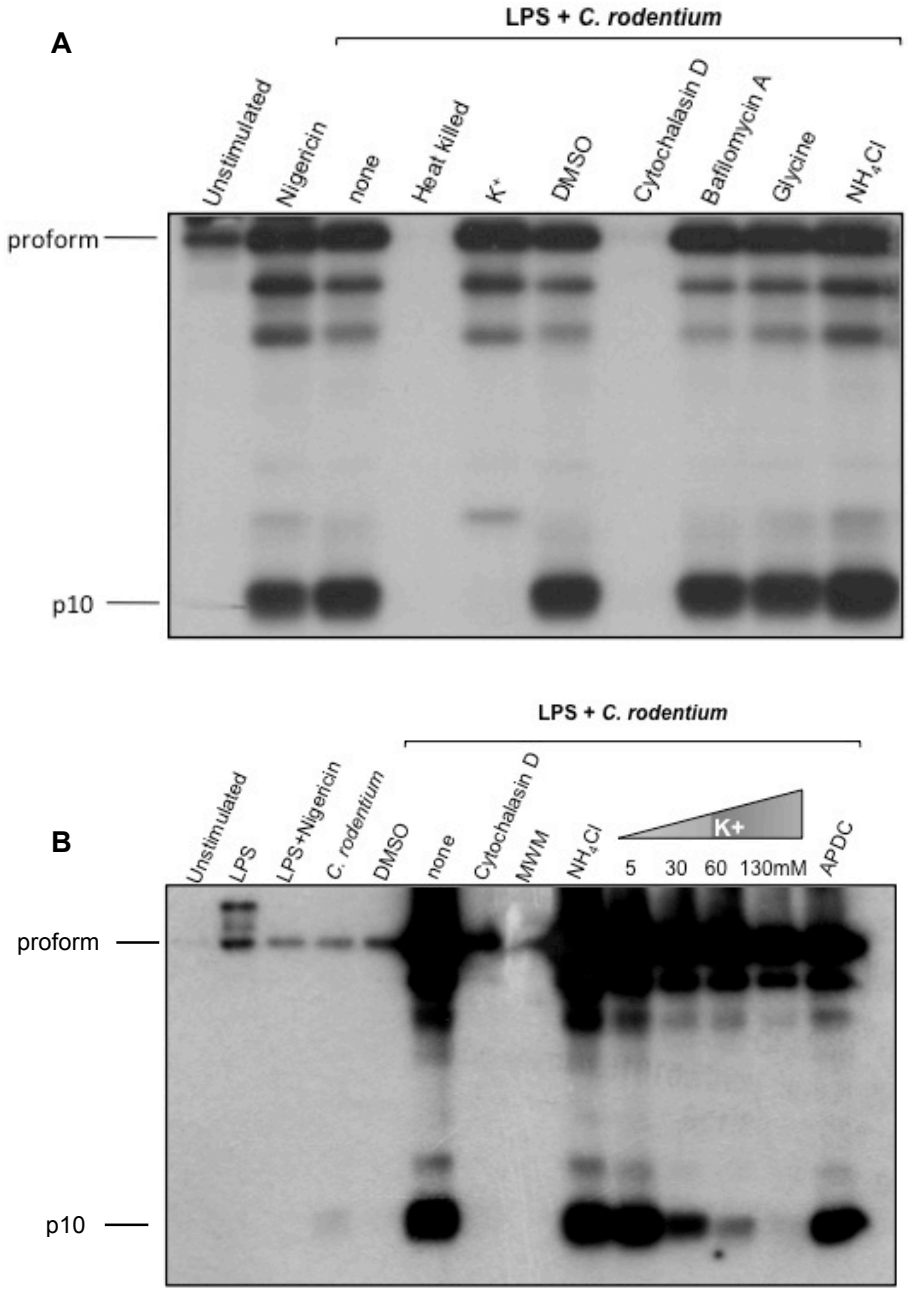


Figure 3.5. Caspase-1 activation by *C. rodentium* requires the phagocytosis of live bacteria and potassium (K^+) efflux in BMDMs. $2x10^5$ bone marrow derived macrophages (BMDMs) from wild type (WT) mice were either pre-treated with 20ng/ml lipopolysaccharide (LPS) overnight or left untreated. The next day BMDMs were pre-treated with inhibitors (concentrations in materials and methods) for 1 hour before being infected *in vitro* with *C. rodentium* (MOI 20) for 1.5 hours and then treating with 100µg/ml gentamycin for another 5.5 hours. Cell supernatants were then collected, protein extracted, concentrated and then immunobloted for caspase-1 p10. MWM is molecular weight marker. A and B represent two independent experiments in total. DMSO was used as a control for unspecific inhibitory effects.

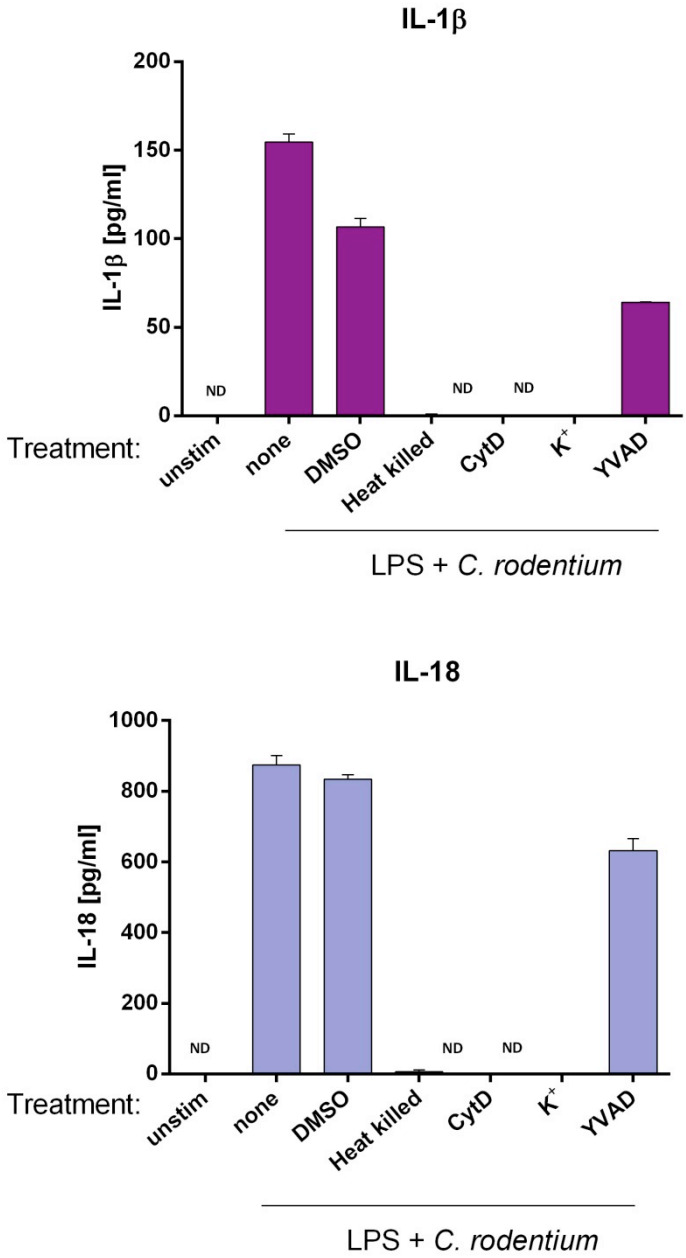


Figure 3.6. *C. rodentium* driven IL-1β and IL-18 production by BMDMs requires the phagocytosis of live bacteria and potassium efflux. $2x10^5$ bone marrow derived macrophages (BMDMs) from wild type (WT) mice were either pre-treated with 20ng/ul lipopolysaccharide (LPS) overnight or left untreated (unstim). The next day BMDMs were pre-treated with inhibitors for 1 hour before being infected *in vitro* with *C. rodentium* (MOI 20) for 1.5 hours and treating with 100µg/ml gentamycin for another 5.5 hours. Cell supernatants were then collected, protein extracted and assayed for IL-1β and IL-18 by ELISA. Data shown represents one of two independent experiments with similar results. DMSO was used as a control for unspecific inhibitory effects. Cytochalasin D (CytD). YVAD is a caspase-1 inhibitor.

3.2.3 *C. rodentium* activation of caspase-1 in BMDMs is independent of multiple virulence factors.

Next, we attempted to assess whether specific bacterial virulence factors were required for *C. rodentium* to trigger caspase-1 activation. Most pathogens that activate the Nlrp3 inflammasome use mechanisms that converge upon the production of toxins e.g. maitotoxin, or that perturb mitochondria (Latz et al., 2013) (Shimada et al., 2012). *C. rodentium* virulence is encoded primarily by the locus of enterocyte effacement (LEE) pathogenicity island (PAI) and a type three secretion system (TTSS) downstream of the LEE region (Garmendia et al., 2005). TTSS are used by numerous bacterial pathogens to directly translocate bacterial effector proteins or 'Esp' (*E*PEC *s*ecreted *p*rotein) into host cells that subsequently modulate or hijack host cell processes. We tested the role of some of the most well characterized *C. rodentium* virulence factors in caspase-1 activation by using isogenic mutants lacking defined virulence factors (Mundy et al., 2005). Thus, BMDMs were infected with mutants lacking translocated intimin receptor (Tir), EspF or EspT, proteins that mediate bacterial attachment, disruption of the mitochondrial membrane potential and formation of membrane ruffles, respectively (Bulgin et al., 2009; Garmendia et al., 2005; Raymond et al., 2011). We found that induction of IL-1β and IL-18 secretion together with caspase-1 activation in BMDMs was equivalent following infection with WT *C. rodentium* or isogenic mutants (Figure 3.7A and B). Thus, inflammasome activation by *C. rodentium* in BMDMs is independent of Tir, EspF and EspT.

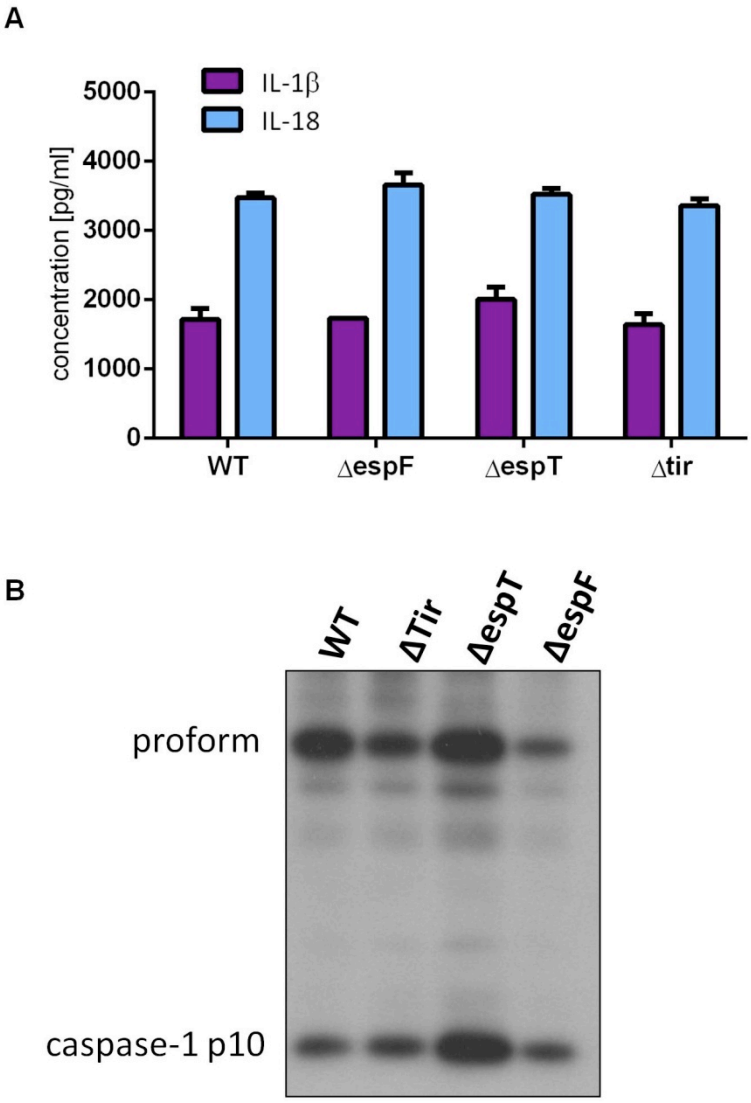


Figure 3.7 *C. rodentium* induces inflammasome activation in BMDMs independently of Tir, espT and espF. 2x10^5 bone marrow derived macrophages (BMDMs) from wild type mice were pre-treated with 20ng/ml lipopolysaccharide (LPS) overnight. The next day BMDMs were infected *in vitro* with *C. rodentium* or isogenic mutants lacking defined virulence factors (ΔespF, ΔespT or Δtir) (MOI 20) for 1.5 hours before treating with 100μg/ml gentamycin for another 5.5 hours. Cell supernatants were then collected and assayed for IL-1β or IL-18 by ELISA (A) or proteins isolated, concentrated and immunoblotted for caspase-1 p10 (B). Bars are representative of mean±SEM and are derived from triplicate wells. Data shown represents one of two independent experiments with similar results.

3.2.4 Nlrp3 and Asc protect against *C. rodentium* induced intestinal inflammation

Having ascertained that *C. rodentium* infection triggers caspase-1 activation via Nlrp3/Asc *in vitro*, we wished to determine the role of the inflammasome during *C. rodentium* infection *in vivo*. *C. rodentium* infection is a well characterized model of acute intestinal infection and inflammation.

We therefore infected cohorts of wild type (WT), *Nlrp3*$^{-/-}$and *Asc*$^{-/-}$ mice with *C. rodentium* and monitored various disease parameters. We first observed that whereas WT mice only displayed a minor and transient drop in body weight at 10 days post infection (p.i.), both *Nlrp3*$^{-/-}$mice and *Asc*$^{-/-}$ mice lost significant amounts of body weight during the first two weeks of *C. rodentium* infection before rapidly regaining their original weight (Figure 3.8). This was most pronounced in the *Asc*$^{-/-}$ mice, which lost around 15% of their starting weight (Figure 3.8). Overall, this suggests that signalling via Nlrp3 and Asc limits bacterial-driven weight loss. Furthermore, the differential weight loss between *Nlrp3*$^{-/-}$and *Asc*$^{-/-}$ mice suggests that other NLRs that interact with Asc may be involved in protective immunity against *C. rodentium*.

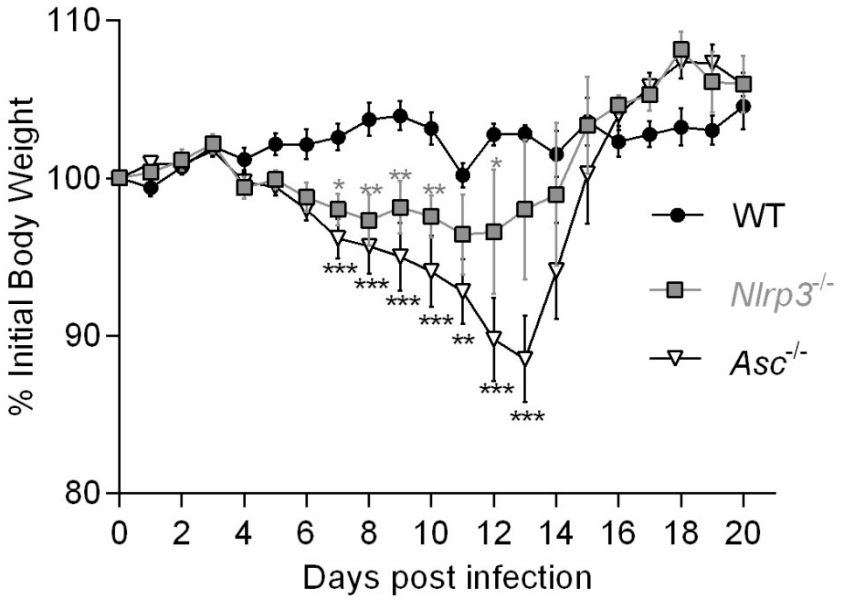


Figure 3.8 *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice show exacerbated weight loss upon *C. rodentium* infection. WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and then weighed daily for three weeks until sacrifice. Symbols denote mean body weights (±SEM) as a percentage of the initial body weight (n =16). Statistical significance was determined by the two-way ANOVA test followed by Bonferroni correction. Significance was compared to WT mice, * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Data shown represents one of three independent experiments with similar results.

We next determined the impact of Nlrp3 and Asc on *C. rodentium*-induced intestinal inflammation. Like WT mice, uninfected control *Nlrp3*$^{-/-}$and *Asc*$^{-/-}$ mice showed no histological abnormalities or any evidence of spontaneous intestinal inflammation (Figure 3.9). Consistent with other reports, WT mice developed only mild intestinal inflammation after infection with *C. rodentium* that was most evident in the distal colon after 8 and 14 days p.i. (Figure 3.9) (Mundy et al., 2005). In contrast, mice lacking Nlrp3 or Asc developed severe intestinal inflammation in their caeca (typhlitis) and distal colons (Figure 3.9 A, B&C). This was characterized by increased crypt hyperplasia, epithelial damage, oedema and submucosal infiltration (Figure 3.9B). The exacerbated pathology observed in *Nlrp3*$^{-/-}$and *Asc*$^{-/-}$ mice was evident after 8 and 14 days p.i. in the caecum and after 14 days p.i. in the distal colon (Figure 3.9). Notably, *Asc*$^{-/-}$ mice displayed a trend towards more severe typhlitis compared to *Nlrp3*$^{-/-}$ mice at 8 days p.i. (Figure 3.9A). Finally, *Nlrp3*$^{-/-}$and *Asc*$^{-/-}$ mice returned to homeostasis after 21 days p.i. and displayed no signs of persistent intestinal inflammation (data not shown). Thus, Nlrp3 and Asc signalling serve to limit *C. rodentium* driven intestinal inflammation.

As the inflammasome is a key facet of innate immune defence we asked whether the susceptibility to *C. rodentium* was a consequence of impaired anti-bacterial defence. We measured *C. rodentium* in the faecal contents during the first five days of infection and found that *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice showed a strong trend towards harbouring more *C. rodentium* compared to WT mice, which was significant in *Asc*$^{-/-}$ mice on day 5 p.i. (Figure 3.10). Faecal colonization levels reflect luminal bacterial contents, which, while instructive, do not accurately reflect the adherent *C. rodentium* that efface the epithelium and potentiate disease. Thus we next measured adherent bacterial burdens in the caecum and distal colons of *C. rodentium* infected mice throughout the disease period.

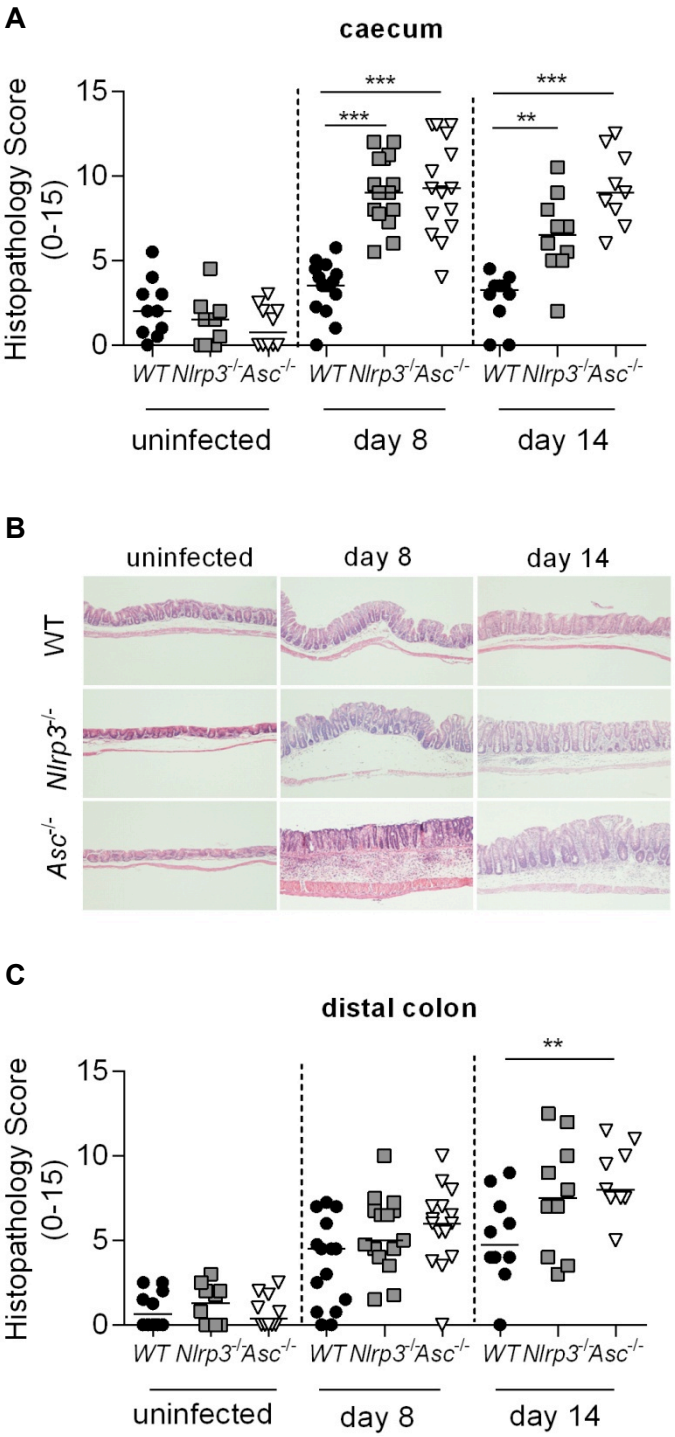


Figure 3.9 *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice develop severe intestinal inflammation after *C. rodentium* infection.

WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and then sacrificed after either 8 or 14 days post infection (p.i.). Inflammation scores in the caecum (A) and representative photomicrographs depicting H&E staining of *C. rodentium* infected caeca (magnification x50) (B). Inflammation scores in the distal colon (C). Results were pooled from two to three independent experiments (n = 9-15 per group). Horizontal bars represent the median and each symbol represents an individual mouse. Statistical significance was determined by the Mann-Whitney test. (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

We found that *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice harboured significantly higher *C. rodentium* burdens at both 8 and 14 days p.i. in the caecum and distal colon, relative to WT mice (Figure 3.11 A & B). This result indicates that activation of the inflammasome limits *C. rodentium* tissue burdens. Next, we sought to determine the localization of *C. rodentium* in the intestine. Using immunofluorescence we observed that in WT mice, *C. rodentium* localized mainly to the luminal surface of the intestinal epithelium lining the caecum (Figure 3.11C). By contrast, *C. rodentium* were present deep within the base of the cecal crypts in *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice (Figure 3.11C). Thus signalling via Nlrp3 and Asc restricts penetration of *C. rodentium* in the cecal crypts. Indeed, consistent with this increased penetration, we found that while the spleens of *C. rodentium* infected WT mice were completely sterile, 80% of *Asc*$^{-/-}$ mice and 50% of *Nlrp3*$^{-/-}$mice harboured low but detectable levels of *C. rodentium* (Figure 3.12A). Accompanying this systemic translocation, *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice displayed significant splenomegaly at 8 and 14 days p.i (Figure 3.12B), which was associated with an increased accumulation of neutrophils (CD11b^{+} Gr1^{hi}) (Figure 3.13A, B). Overall, these data suggest that signalling via Asc and Nlrp3 limits translocation of *C.rodentium* into systemic sites and in doing so prevents excessive systemic inflammatory responses.

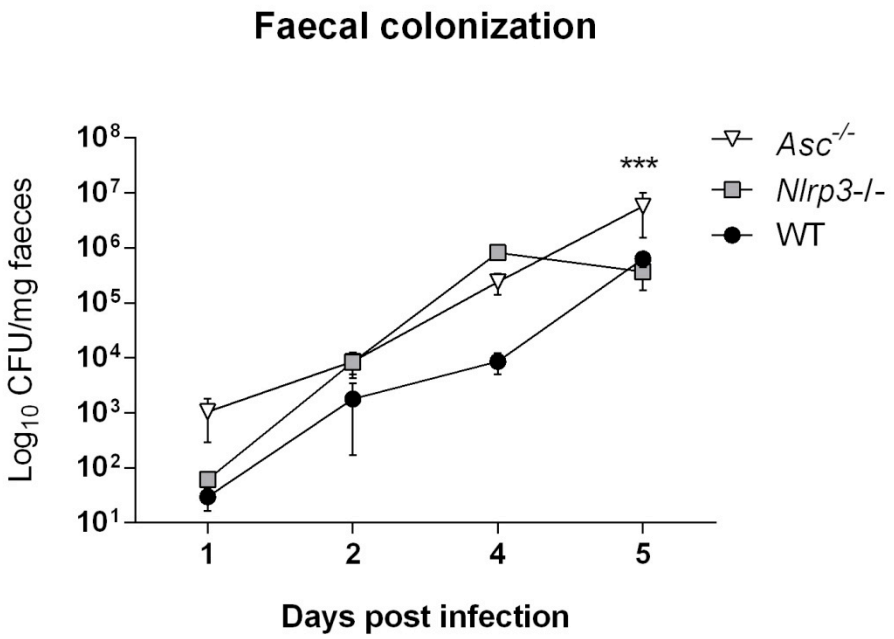


Figure 3.10 *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice display higher faecal *C. rodentium* colonization. WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium*. Faecal pellets from mice were harvested for the first five days of infection and plated out on *C. rodentium* selective plates. *C. rodentium* CFU were normalized per mg of faeces. Data show mean CFU±SEM (*n* = 3-5 per group) from one of two independent experiments with similar results. Statistical significance was determined by the Mann-Whitney test. (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

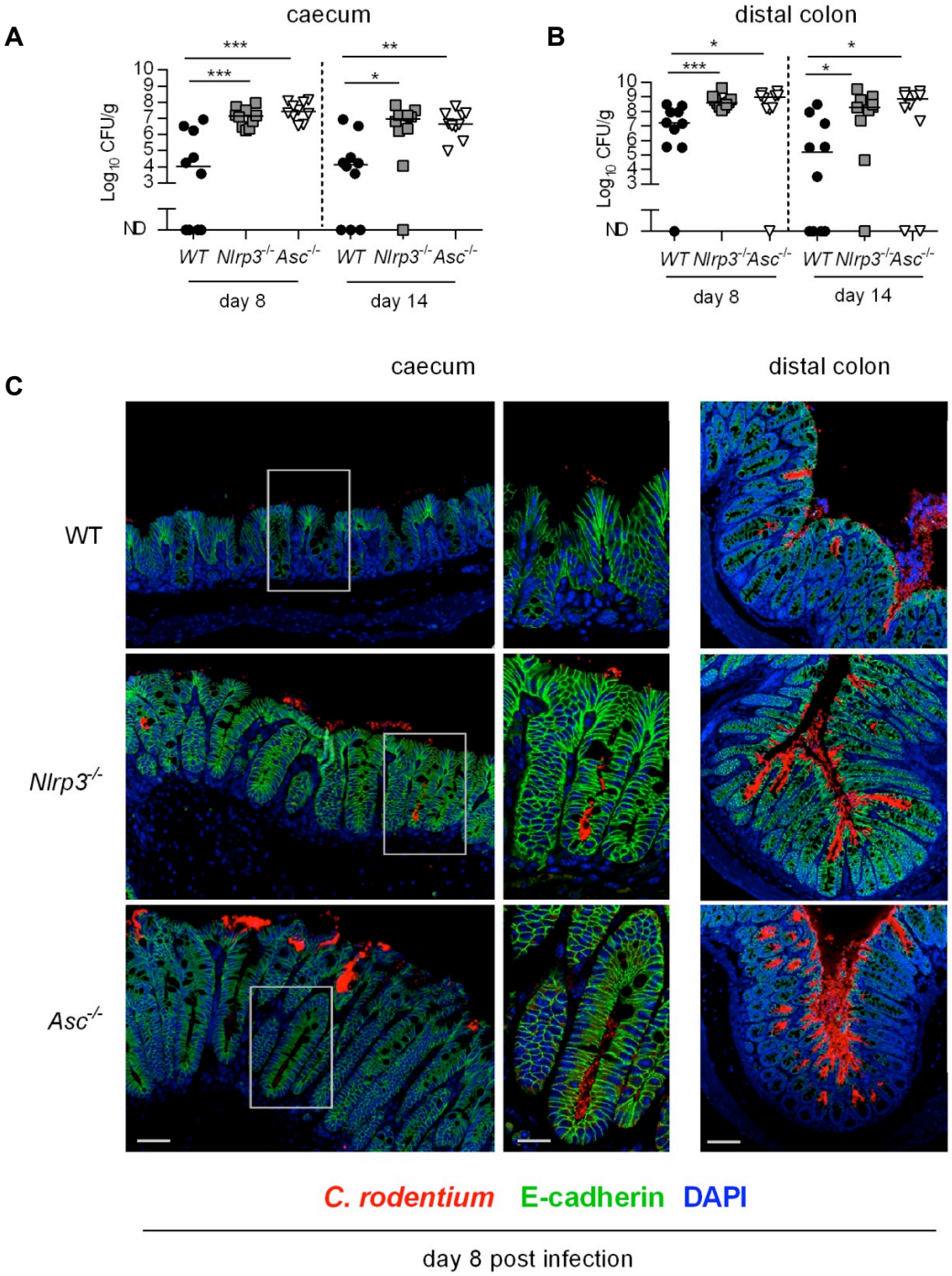


Figure 3.11 *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice harbour higher adherent *C. rodentium* burdens. WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and sacrificed after either 8 or 14 days p.i. (A,B) *C. rodentium* burdens were measured in the caecum (A) and distal colon (B) (ND, no detectable bacteria). (C) Caeca isolated from WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice at 8 days p.i. were stained for *C. rodentium* (red), E-cadherin (green) and DAPI (blue). Scale bars = left panel 30µm, mid panel 15µm, right panel 70µm. Data represent pooled results from at least two independent

experiments. Horizontal bars represent the medians and each symbol represents an individual mouse (n = 10 per group). Statistical significance was determined by the Mann-Whitney test. (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$)

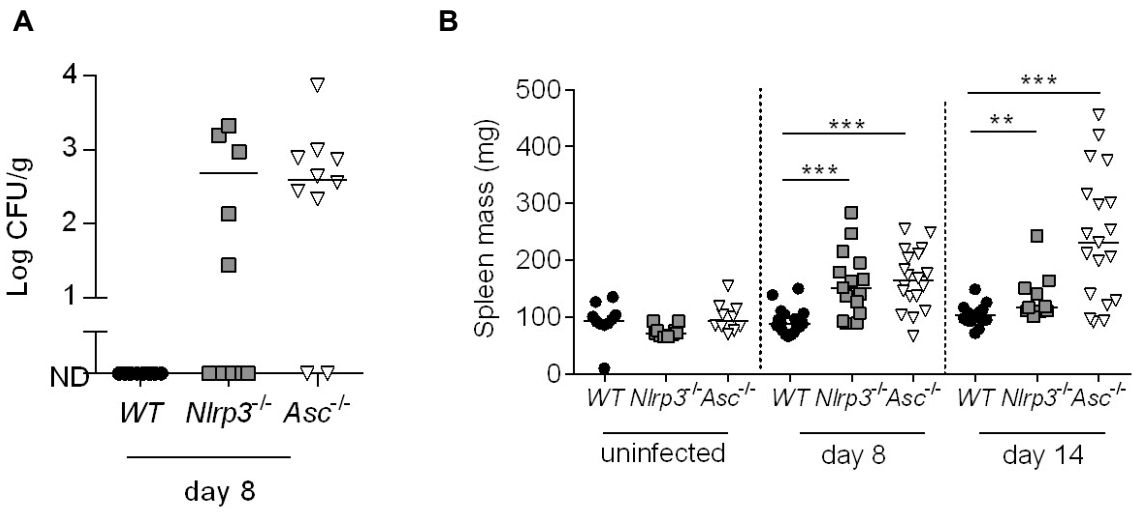


Figure 3.12 Signalling via Nlrp3 and Asc limits systemic translocation of *C. rodentium*. WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and then sacrificed after either 8 or 14 days p.i. (A) *C. rodentium* burdens in the spleen at day 8 p.i. normalized to spleen weight. (B) Spleen weights during the course of infection. Each symbol represents a single animal and horizontal bars indicate group medians. Data represent pooled results from at least two independent experiments (n = 9-20 per group). Statistical significance was determined by the Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$)

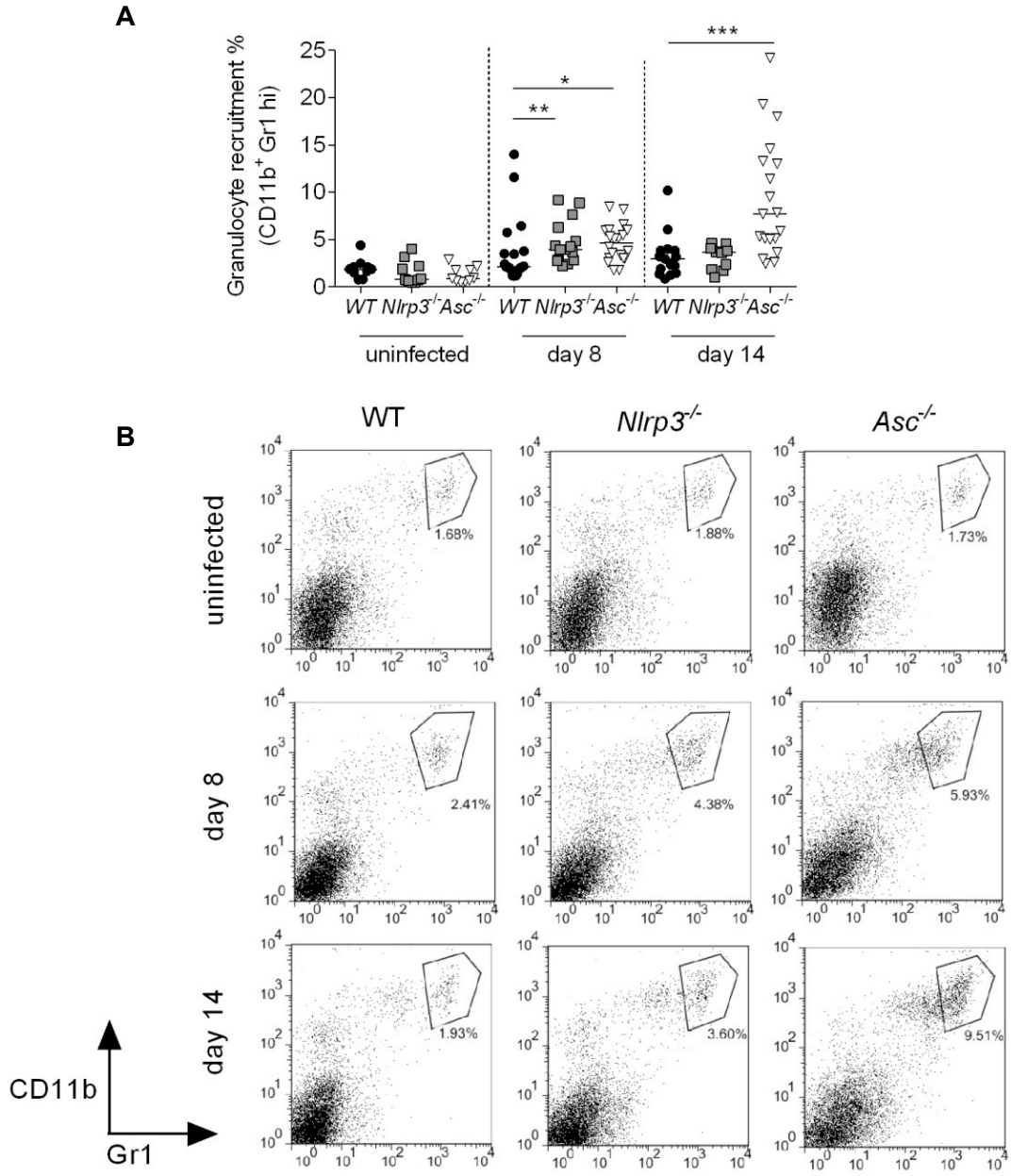


Figure 3.13 Signalling via Nlrp3 and Asc limits granulocyte accumulation in the spleen during *C. rodentium* infection. WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and then sacrificed after either 8 or 14 days p.i. (A) Frequency of granulocytes in the spleen (CD11b$^+$ Gr1^{hi}). (B) Representative FACS plots showing gating used to identify splenic granulocytes (CD11b$^+$ Gr1^{hi}). Each symbol represents a single animal and horizontal bars indicate group medians. Data represent pooled results from at least two independent experiments (n = 9-20 per group). Statistical significance was determined by the Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$)

3.2.5 Nlrp3-dependent protection against *C. rodentium* is independent of caspase-1 cleaved cytokines

As we observed very similar phenotypes between *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ we assumed that this phenotype was due to impaired caspase-1 inflammasome activation. Furthermore, given that *Il1r*$^{-/-}$ mice are known to be extremely susceptible to *C. rodentium* (Lebeis et al., 2009), we reasoned that a lack of caspase-1 cleaved cytokines such as IL-1β might underlie the susceptibility of *Nlrp3*$^{-/-}$ and Asc$^{-/-}$ mice. To test this hypothesis this we measured the levels of IL-1β, IL-1α and IL-18 in caecal organ culture explants isolated from *C. rodentium* infected WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice. We observed no differences in the levels of IL-1β or IL-1α between the genotypes at steady state and 8 days p.i., demonstrating that these cytokines were unimpaired in the absence of either *Nlrp3* or *Asc* (Figures 3.14A, B). Furthermore, while *Asc*$^{-/-}$ mice were completely abrogated in their cecal IL-18 production both at steady state and 8 days p.i., *Nlrp3*$^{-/-}$ mice showed equivalent levels of IL-18 compared to WT mice (Figure 3.14C). As ELISAs may not distinguish between pro-and active forms of these caspase-1 cleaved cytokines, we wanted a more accurate measurement of the caspase-1 activation status in *C. rodentium* infected mice. Thus we assayed caspase-1 activation in cecal tissue homogenates isolated from WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice at 8 days p.i. Consistent with their comparable levels of IL-18 production, caspase-1 cleavage to the active p10 subunit was equivalent in WT and *Nlrp3*$^{-/-}$ mice (Figure 3.14D). In contrast, caspase-1 activation was almost completely abrogated in *Asc*$^{-/-}$mice at 8 days p.i. (Figure 3.14D). Taken together, these data collectively suggest that the major susceptibility phenotype observed in *Nlrp3*$^{-/-}$mice is independent of activation of caspase-1 and secretion of associated cytokines.

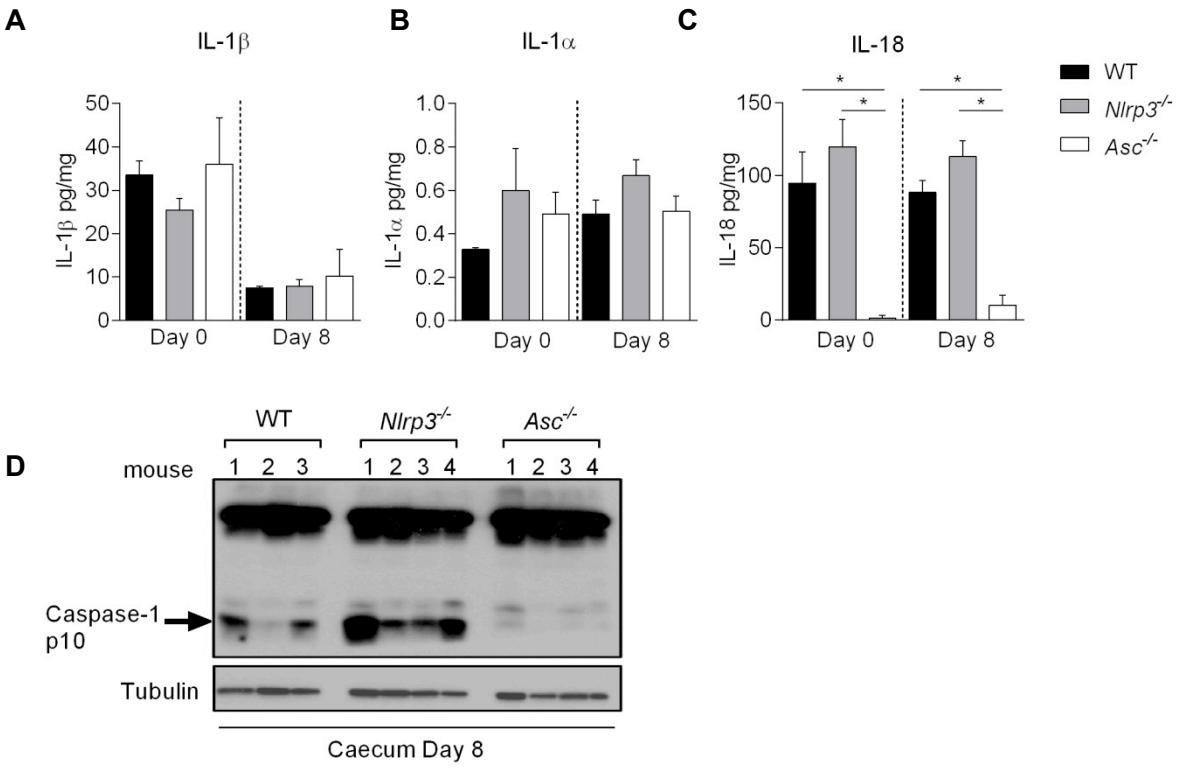


Figure 3.14 Nlrp3-dependent protection against *C. rodentium* is independent of caspase-1 activation. Cohorts of WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed at 8 days p.i. Cecal tissue explants were cultured overnight and levels of IL-1β (A), IL-1α (B), and IL-18 (C), were assayed in the supernatants by ELISA. Data represent mean cytokine levels ±SEM (*n* = 4 per group). (D) Immunoblot analysis of caecal tissue homogenates probed with antibody to caspase-1 p10 and tubulin. Each lane represents an individual mouse (*n* = 3-4 per group). Statistical significance was determined by the Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$). Data shown represents one of two independent experiments with similar results.

Our results also suggested that the difference in caspase-1 activation and IL-18 production between *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$mice might underlie the slight difference in disease severity between the two genotypes. To test this and determine the role of IL-18 in *C. rodentium* infection we infected *Il18r*$^{-/-}$ mice. We observed that *Il18r*$^{-/-}$mice showed a trend towards increased typhlitis after 8 days p.i. compared to WT mice, although this did not reach statistical significance (Figure 3.15A). However, *Il18r*$^{-/-}$mice harboured significantly higher levels of adherent *C. rodentium* in their caeca at 8 days p.i. and around 80% had detectable *C. rodentium* in their spleen (Figure 3.15B). Thus, IL-18 production plays a role in protection against *C. rodentium* colonization and its absence in *Asc*$^{-/-}$ mice may explain the increased disease severity relative to *Nlrp3*$^{-/-}$mice.

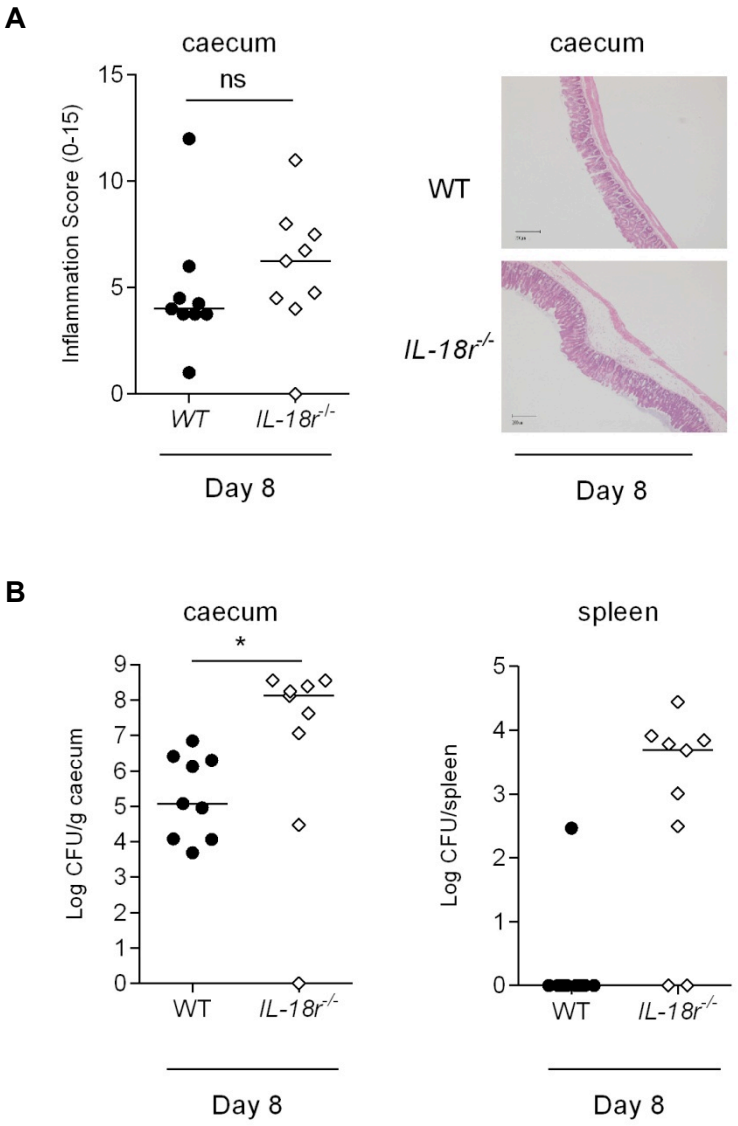


Figure 3.15 IL-18 signalling limits *C. rodentium* tissue burdens and systemic translocation. Cohorts of WT and *Il18r*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 8 days p.i. Inflammation scores in the caecum and representative photomicrographs depicting H&E staining of *C. rodentium* infected caeca (magnification x50) (A). *C. rodentium* burdens in the caecal tissue and spleen were also determined (B). Each symbol represents a single animal and horizontal bars indicate group medians. Results depicted are pooled from two independent experiments (n=9 per group). Statistical significance was determined by the Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

3.2.6 Nlrp3 and Asc activation in intestinal epithelial cells protects against *C. rodentium* driven disease

We next wished to determine which cell compartment Asc and Nlrp3 signalling provided protection against *C. rodentium*. To do so we generated irradiation bone marrow (BM) chimeric mice by transferring isolated bone marrow from either *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ mice into irradiated WT hosts and vice versa. After haematopoietic cell reconstitution was complete, we infected the BM chimeras with *C. rodentium*. Consistent with our previous observations we found that BM chimeras lacking Nlrp3 or Asc in all cell compartments (*Nlrp3*$^{-/-}$→*Nlrp3*$^{-/-}$) or (*Asc*$^{-/-}$ → *Asc*$^{-/-}$) were significantly more susceptible to *C. rodentium* induced weight loss compared to WT→WT controls (Figure 3.16A, B). We observed that BM chimeras with selective restoration of Nlrp3 in haematopoietic cells (WT→*Nlrp3*$^{-/-}$) were not protected from weight loss (Figure 3.16A). In contrast, BM chimeras with selective expression of Nlrp3 in non-haematopoietic cells (*Nlrp3*$^{-/-}$ →WT) completely protected against weight loss (Figure 3.16A), indicating that non-haematopoietic cell expression of Nlrp3 conferred dominant protection from weight loss. Similarly, BM chimeras selectively expressing Asc in their non-haematopoietic cells (*Asc*$^{-/-}$→WT) were completely protected from weight loss during *C. rodentium* infection (Figure 3.16B). Notably, BM chimeras with selective restoration of Asc in haematopoietic cells (WT→ *Asc*$^{-/-}$) also exhibited partial protection against weight loss, however this was much less than when Asc was expressed in non-haematopoietic cells (Figure 3.16B).

Consistent with a dominant role for non-haematopoietic cell Nlrp3 and Asc expression in affording protection from weight loss, we observed that intestinal inflammation levels in the BM chimeras clustered according to the non-haematopoietic genotype of the mice (Figure 3.17A, B). Thus, BM chimeric mice selectively lacking Nlrp3 in non-haematopoietic cells (WT→*Nlrp3*$^{-/-}$) developed severe typhilitis comparable to that seen in *Nlrp3*$^{-/-}$→*Nlrp3*$^{-/-}$ controls (Figure 3.17A). Conversely, BM chimeras that expressed Nlrp3 only in their non-haematopoietic cells (*Nlrp3*$^{-/-}$→WT) were protected from *C. rodentium* induced typhlitis (Figure 3.17A). Likewise, BM chimeric mice selectively lacking Asc in their non-haematopoietic cells (WT→ *Asc*$^{-/-}$) exhibited severe typhlitis similar to that found in *Asc*$^{-/-}$→ *Asc*$^{-/-}$ controls (Figure 3.17B). Once again however, haematopoietic Asc expression played a minor role in protection as *Asc*$^{-/-}$→WT BM chimeras were slightly, but

significantly, more susceptible to typhlitis relative to WT→WT controls at 14 days p.i. (Figure 3.17B). Thus, these data collectively indicate that non-haematopoietic Nlrp3 and Asc expression affords dominant protection against *C. rodentium* induced weight loss and intestinal inflammation.

Finally we determined whether, fitting with our earlier hypothesis, the susceptibility to weight loss and intestinal inflammation was accompanied by impaired control of *C. rodentium* burdens in the gut. Indeed, we found that BM chimeric mice selectively lacking Nlrp3 (WT→*Nlrp3*$^{-/-}$) or Asc (WT→ *Asc*$^{-/-}$) in their non-haematopoietic cells harboured similar high levels of adherent *C. rodentium* in their caeca and distal colons as *Nlrp3*$^{-/-}$→*Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$→ *Asc*$^{-/-}$controls (Figure 3.18A, B). Consistent with a minor role for Asc expression in haematopoietic cells in protection from *C. rodentium*-induced disease, *Asc*$^{-/-}$→WT BM chimeras had significantly higher cecal bacterial burdens relative to WT→WT controls, although these were still significantly lower than the *C. rodentium* levels observed in *Asc*$^{-/-}$→ *Asc*$^{-/-}$controls (Figure 3.18B). Similarly, BM chimeric mice lacking Nlrp3 in their haematopoietic cells (*Nlrp3*$^{-/-}$→WT) showed a trend towards higher *C. rodentium* burdens in their distal colons relative to WT→WT controls (Figure 3.18A), suggesting a minor role for haematopoietic cell Nlrp3 expression. Taken together, these data show that Nlrp3 and Asc expression in non-haematopoietic cells plays a dominant role in limiting *C. rodentium* driven disease.

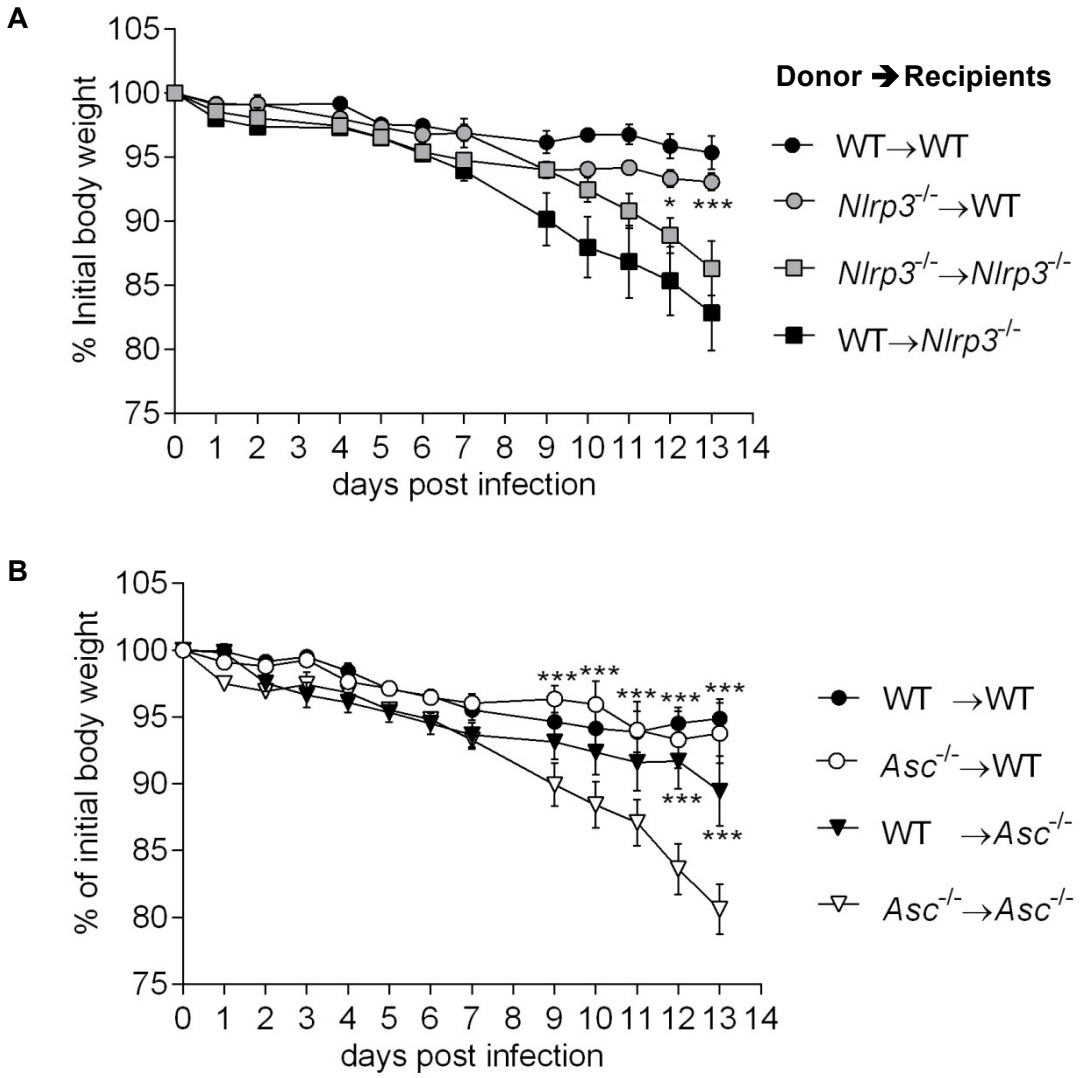


Figure 3.16 Nlrp3 and Asc expression in non-haematopoietic cells protects against *C. rodentium* driven weight loss. Irradiation bone marrow chimeras were generated and orally infected with ~10^9 *C. rodentium*. Body weights of *Nlrp3*$^{-/-}$ (A) and *Asc*$^{-/-}$ (B) bone marrow chimeras. Symbols denote mean weights (±SEM) as a percentage of the initial body weight. Data represent pooled results from two to three independent experiments (*n* = 6-11 per group). Statistical significance was determined between chimera groups and controls i.e. *Asc*$^{-/-}$ → *Asc*$^{-/-}$ or *Nlrp3*$^{-/-}$ → *Nlrp3*$^{-/-}$ respectively, by two-way ANOVA (* = P < 0.05; ** = P < 0.01; *** = P < 0.001).

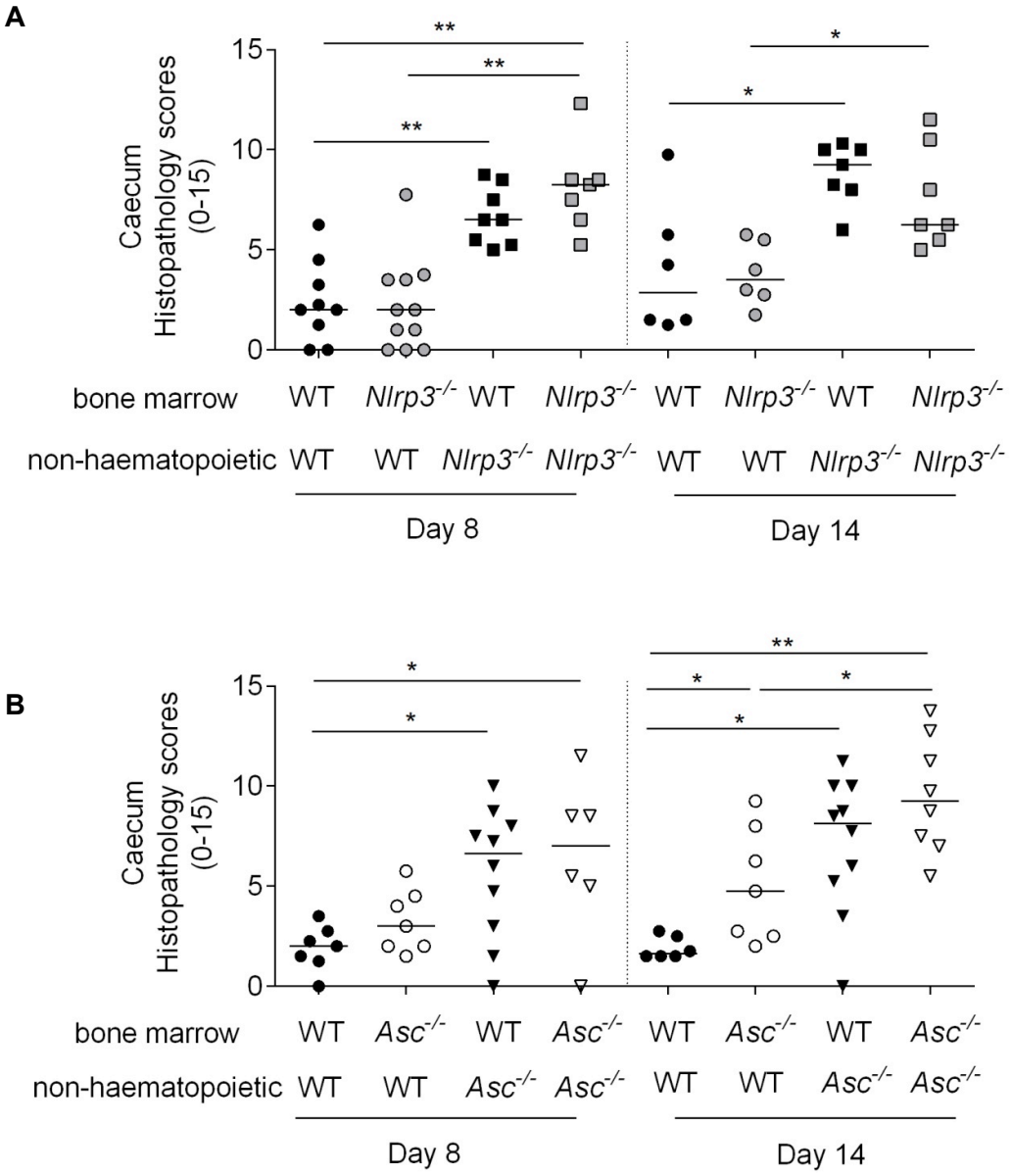


Figure 3.17 Signalling via Nlrp3 and Asc in non-haematopoietic cells protects against *C. rodentium* driven intestinal inflammation. Irradiation bone marrow chimeras were generated and orally infected with ~10^9 *C. rodentium* and sacrificed 8 or 14 days p.i.. Inflammation scores in the caecum of *Nlrp3*$^{-/-}$ (A) and *Asc*$^{-/-}$ (B) bone marrow chimeras were assessed. Data represent pooled results from two to three independent experiments, each symbol represents a single mouse and horizontal bars represent medians (n = 6-11 per group). Statistical significance was determined by the Mann-Whitney test. (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

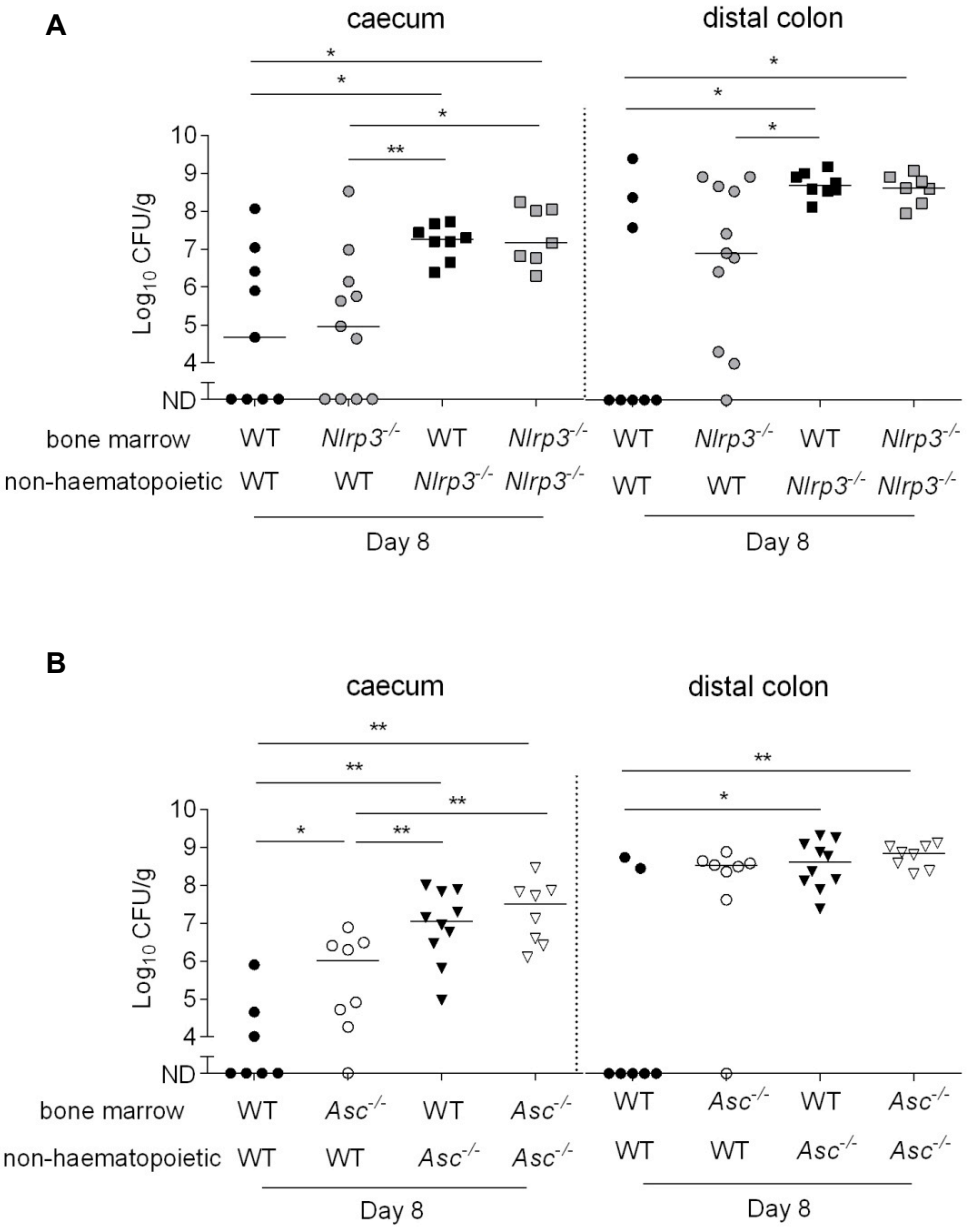


Figure 3.18 Signalling via Nlrp3 and Asc in non-haematopoietic cells protects against *C. rodentium*. Irradiation bone marrow chimeras were generated and orally infected with ~10^9 *C. rodentium* and sacrificed 8 days p.i. Adherent *C. rodentium* burdens in the caecum and distal colons of *Nlrp3*$^{-/-}$ (A) and *Asc*$^{-/-}$ (B) bone marrow chimeras were enumerated. Each symbol represents a single mouse and horizontal bars represent the medians. Data represent pooled results from two to three independent experiments (*n*= 7-11 per group). Statistical significance was determined by the Mann-Whitney test. (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

To investigate whether Nlrp3 or Asc-mediated protection correlated with caspase-1 activation we assessed caspase-1 cleavage in cecal tissue homogenates isolated from the BM chimeric mice at day 8 p.i. with *C. rodentium*. We found that caspase-1 activation (p10) was completely absent in the caeca of BM chimeric mice selectively lacking Asc in non-haematopoietic cells (WT→*Asc*$^{-/-}$), whereas caspase-1 p10 was readily detectable in BM chimeric mice selectively lacking Asc in haematopoietic cells (*Asc*$^{-/-}$→WT) (Figure 3.19A). Thus, most caspase-1 activation occurs in non-haematopoietic cells in the gut through a pathway that is Asc-dependent. Consistent with this conclusion, we also discovered that IL-18 processing in the cecum was dependent on Asc expression in the non-haematopoietic cell compartment (Figure 3.19C). By contrast, caspase-1 activation was intact in all the Nlrp3 BM chimera groups (Figure 3.19B), confirming our earlier conclusion that the *Nlrp3*$^{-/-}$ susceptibility phenotype is independent of caspase-1 activation.

Non-haematopoietic cells include many radioresistant cell types including stromal cells, pericytes, fibroblasts and epithelial cells. To more precisely identify the cell type in which Asc conferred protection we stained for Asc by immunofluorescence. We found that Asc staining strongly localized to E-cadherin^{+} intestinal epithelial cells (Figure 3.20). By contrast, the underlying stromal cells and lamina propria contained very few cells with detectable Asc (Figure 3.20). These results suggest that signalling via Asc and, presumably Nlrp3, in intestinal epithelial cells protects against *C. rodentium*.

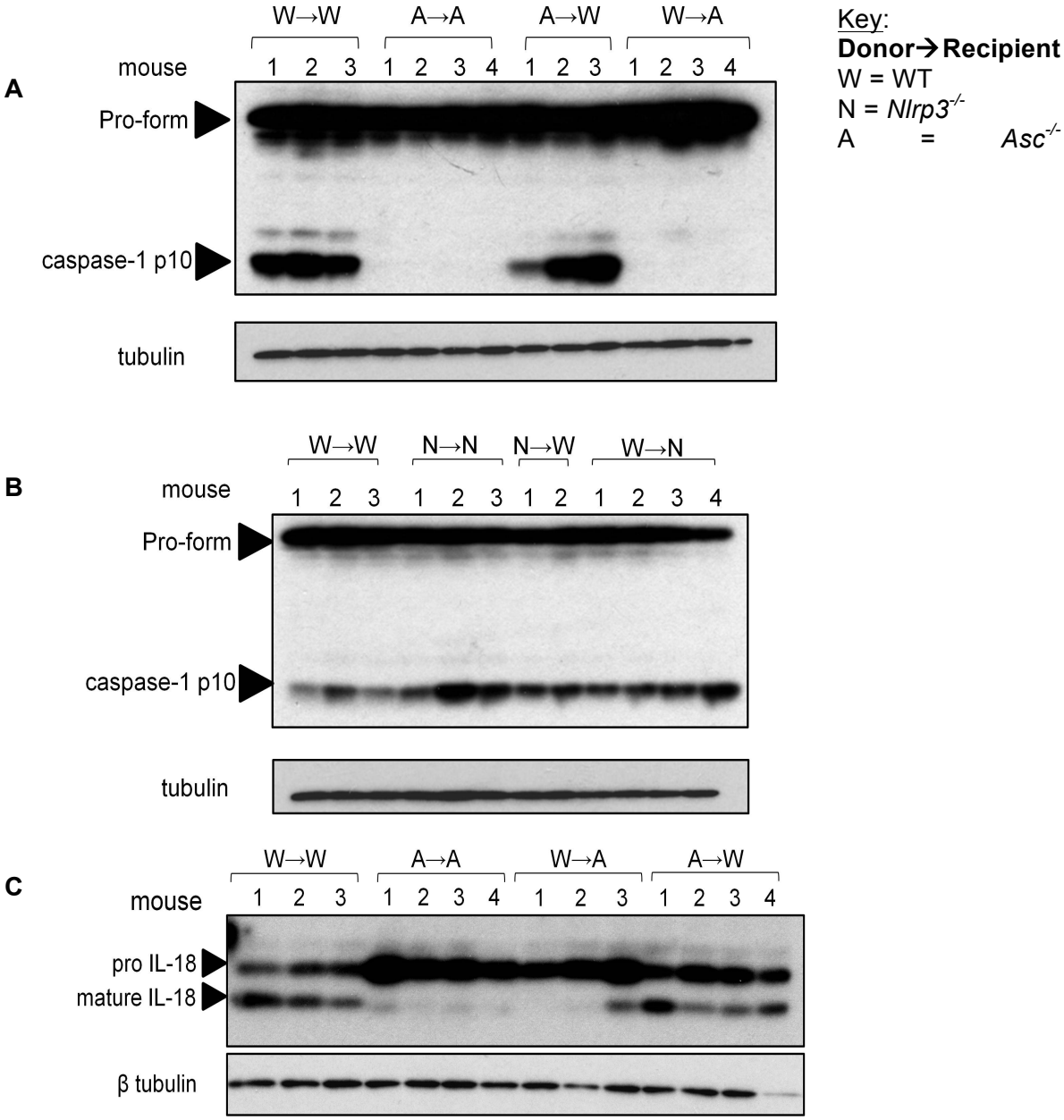


Figure 3.19 Nlrp3-dependent protection in non-haematopoietic cells is independent of caspase-1. Irradiation bone marrow chimeras were generated and orally infected with ~10^9 *C. rodentium* and sacrificed 8 days p.i. Immunoblot of caecal protein extracts from *Asc*$^{-/-}$ BM chimeric mice (A), and *Nlrp3*$^{-/-}$ BM chimeric mice (B) probed with antibody to caspase-1 and tubulin. Immunoblot analysis of caecal protein extracts from *Asc*$^{-/-}$ BM chimeric mice, probed with antibody to IL-18 and tubulin (C). Results shown are from one of two independent experiments with similar results. W = WT, N = *Nlrp3*$^{-/-}$, A = *Asc*$^{-/-}$. W→W represents WT donor bone marrow (transferred into)→ an irradiated WT recipient.

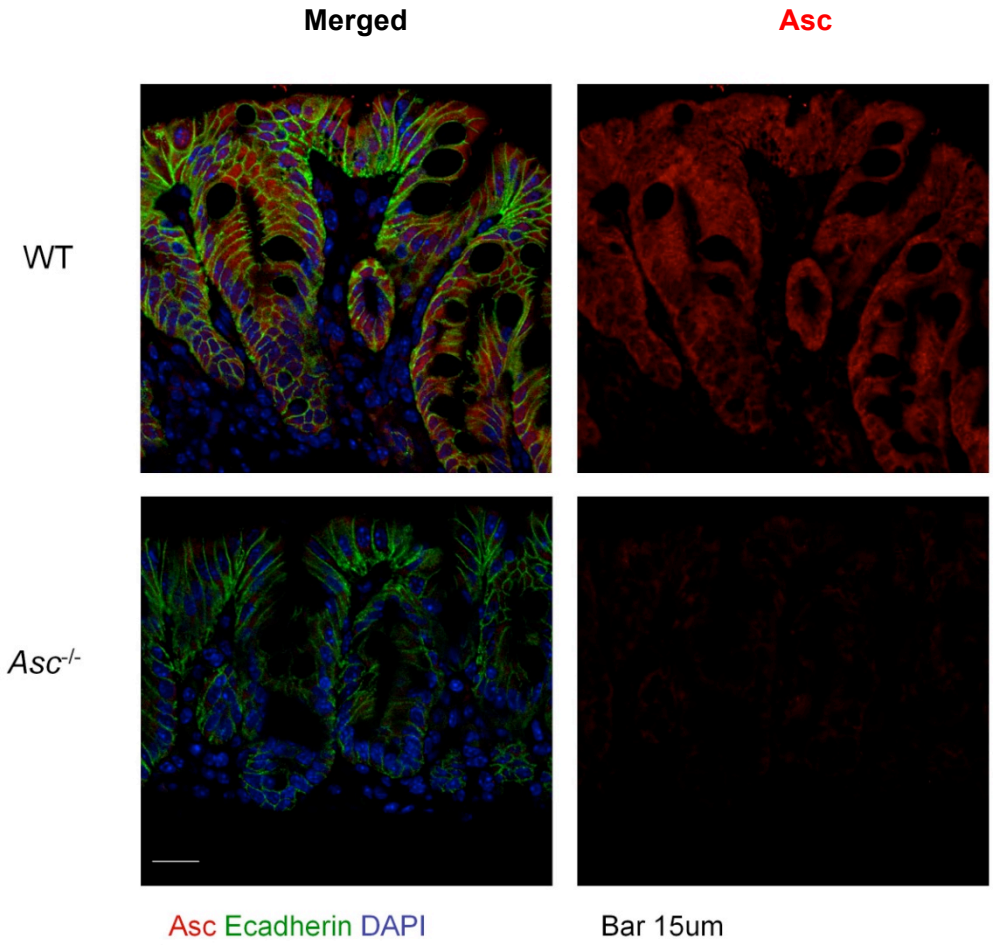


Figure 3.20 Asc is ubiquitously expressed in E-cadherin^{+} intestinal epithelial cells. Formalin fixed, paraffin embedded sections of caecal tissue samples from uninfected WT and *Asc*$^{-/-}$ mice were stained for Asc (red), E-cadherin (green) and DAPI (blue). Bar = 15µm. The results depicted above are representative of two independent experiments with a total of 4-6 sections.

3.2.7 Nlrp3 and Asc limit early *C. rodentium* colonization

We next wanted to understand whether the higher bacterial burdens were the cause of the greater intestinal inflammation or whether they were merely a consequence of it. To do so we compared the kinetics of *C. rodentium* colonization with intestinal inflammation. Through a series of time-course experiments we discovered that after just 3 days p.i., a time point before the onset of intestinal inflammation, *Nlrp3*$^{-/-}$and *Asc*$^{-/-}$mice harboured over 1000-fold more tissue-adherent *C. rodentium* in their caeca compared to WT mice (Figure 3.21A-C). This was also accompanied by clear evidence of *C. rodentium* penetration into the crypts in *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice (Figure 3.21D). Thus Nlrp3 and Asc signalling within the intestinal epithelium limit early bacterial colonization and in doing so may dampen subsequent intestinal inflammation.

Mechanistically, our results pointed to an early axis of protection that was largely independent of caspase-1. Innate lymphoid cells (ILCs) are an innate subset of cells that have been previously shown to confer early protective immunity against *C. rodentium* through the production of IL-22, which acts on epithelial cells to drive secretion of protective antimicrobial peptides, such as Reg3γ (Sonnenberg et al., 2011; Zheng et al., 2008). Since this mechanism was operational at roughly the same time we postulated the Nlrp3-Asc axis to be active and decided to investigate the ILC-IL-22 axis in *C. rodentium*-infected *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice. However, at 3 days p.i. we found equivalent frequencies of ILCs in the caeca of *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice relative to WT mice (Figure 3.22A). Furthermore, we observed that the expression of IL-22, IL-17 and Reg3γ were unimpaired in the caeca of *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice relative to WT mice after 3 days p.i. (Figure 3.22 B-D). In fact, the levels of expression of these genes were often higher in *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$mice relative to WT mice, probably reflecting the higher bacterial burdens.

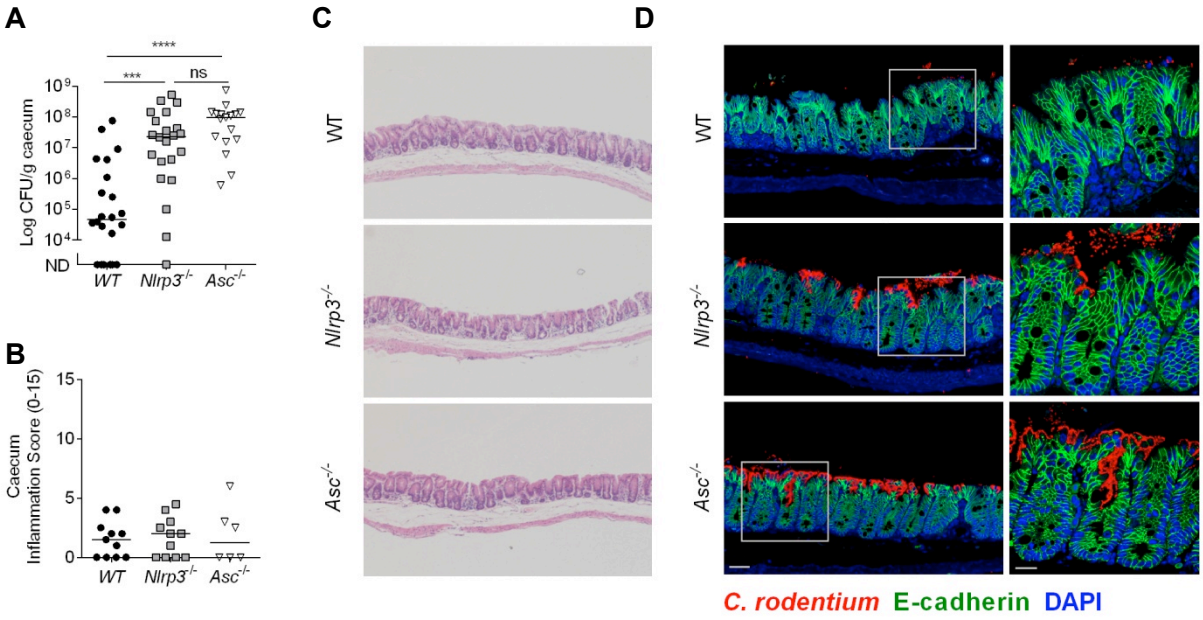


Figure 3.21 Nlrp3 and Asc activation limits early *C. rodentium* colonization. Cohorts of WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were orally infected with ~10^9 *C. rodentium* and sacrificed 3 days p.i. Thereafter *C. rodentium* burdens in the caecum (n= 17-20) were enumerated (A). Histopathology scores in the caecum (n= 6-11) (B). Representative photomicrographs of H&E stained caecum sections (magnification x50) are shown in (C). Immunofluorescence showing staining for *C. rodentium* (red), E-cadherin (green), DAPI (blue). Bar= left panel 20µm, right panel 10 µm. Horizontal bars represent medians. Data shown was pooled from two independent experiments. Statistical significance was determined by the Mann-Whitney test (* = P < 0.05; ** = P < 0.01; *** = P < 0.001).

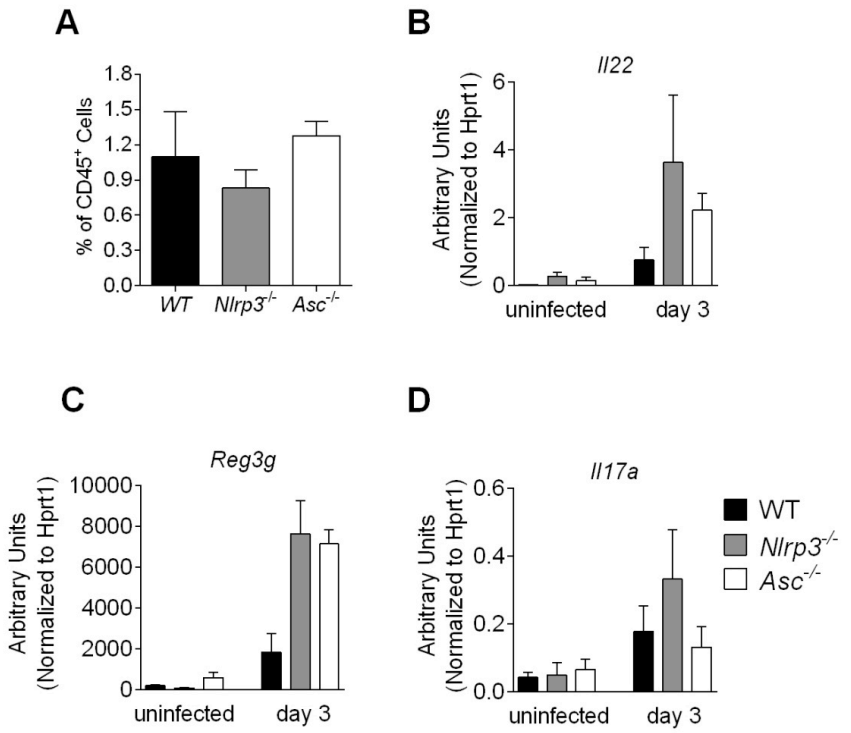


Figure 3.22 Nlrp3 and Asc dependent protection against early *C. rodentium* colonization is independent of the ILC/IL-22 axis. Cohorts of WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were either left uninfected or orally infected with ~10^9 *C. rodentium* and sacrificed at 3 days p.i. Ceca were dissected, small tissue samples removed for isolation of mRNA, and lamina propria leukocytes (LPL) isolated. Frequencies of Lin$^-$ Thy1$^+$ Sca1$^+$ innate lymphoid cells (ILC) as a percentage of CD45$^+$ LPL in the caecum were determined three days p.i. (n = 4) (A). mRNA expression of *Il22, Reg3g* and *Il17a* relative to *Hprt* was also determined (n = 3) (B-D). Data shown was pooled from two independent experiments. Statistical significance was determined by the Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

Recent work has shown that *C. rodentium* is able to induce a form of cell death known as pyroptosis in BMDMs (Kayagaki et al., 2011; Rathinam et al., 2012). In another study, Miao *et al* elegantly demonstrated that upon sensing intracellular flagellin derived from *Salmonella typhimurium*, Nlrc4 activation triggered pyroptosis in macrophages, which exposed the bacterium to neutrophil-mediated killing (Miao et al., 2010a). We hypothesized that akin to this mechanism, Nlrp3/Asc mediated pyroptosis in *C. rodentium*-infected IEC could limit bacterial burdens early during infection. As others had recently shown that *C. rodentium* induced pyroptosis was caspase-11 mediated (Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012), we compared mice naturally lacking caspase-11, 129SvEv mice, to WT C57Bl/6 mice. We found that at 3 days p.i. 129SvEv mice harboured around 100-fold more tissue adherent *C. rodentium* compared to WT C57Bl/6 mice (Figure 3.23A). Furthermore, this increased colonization in 129SvEv mice was independent of IL-1β or IL-18 (Figure 3.23B). Both these results showed that, in terms of hyper-susceptibility to increased early colonization, mice lacking caspase-11 could phenocopy mice lacking Nlrp3 or Asc. However, a major caveat to this result is that 129SvEv mice have many additional genetic differences compared to C57BL/6 mice. As we did not have access to caspase-11 deficient mice on a C57BL/6 background, we sought an alternative approach to further test our hypothesis. *C. rodentium* utilizes the virulence factor espF to induce cell death in epithelial cells *in vitro* and *in vivo* (Torchinsky et al., 2009). We reasoned that if Nlrp3/Asc-mediated cell death or pyroptosis was the mechanism that limited *C. rodentium* burdens, then infection with a ΔespF mutant *C. rodentium* that was unable to induce cell death, should result in equivalent bacterial burdens in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and WT mice. However, we found that levels of tissue-adherent ΔespF *C. rodentium* were still around 100-fold higher in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ mice compared to WT mice after 3 days p.i (Figure 3.23C). Thus, the potential role of pyroptosis in early protection from *C. rodentium* remains to be resolved.

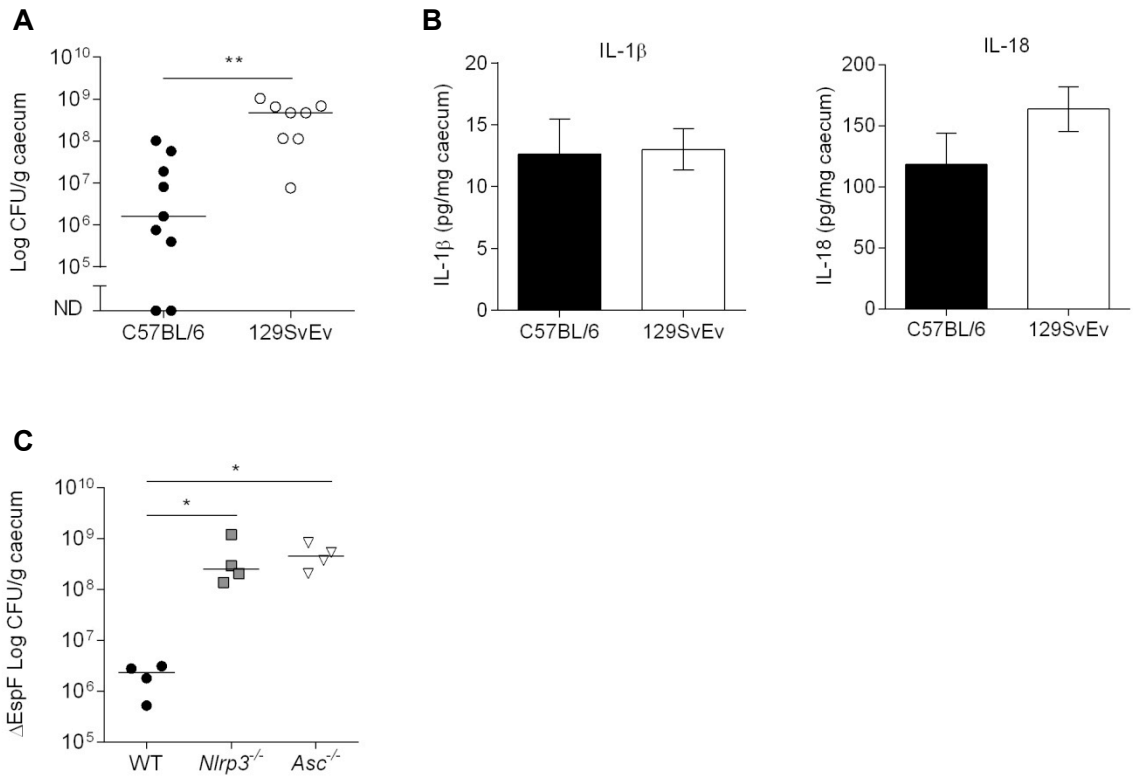


Figure 3.23 Assessing the role of pyroptosis in limiting *C. rodentium* colonization. Cohorts of C57BL/6 and 129SvEv WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were orally infected with ~10^9 of either WT (A,B) or ΔespF (C) *C. rodentium* and sacrificed at 3 days p.i. Levels of WT *C. rodentium* in the caeca of C57BL/6 and 129SvEv mice 3 days p.i. were determined (*n*=8-9) are are shown in (A). IL-1β and IL-18 levels in caecal organ explants from C57BL/6 and 129SvEv mice 3 days p.i. (*n*=4) are shown in (B). ΔespF *C. rodentium* burdens in the caeca of WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice 3 days p.i.(*n*=4) are depicted in (C). Horizontal bars represent medians. Data shown was pooled from two independent experiments. Statistical significance was determined by the Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

Finally, to get an unbiased view on the differential gene expression between *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and WT mice and identify novel pathways that might be involved, we utilized a microarray approach. We compared transcripts within caecal tissue at steady state and after 3 days p.i. Susceptibility to *C. rodentium* in *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice could theoretically arise from:

i. defective induction of a protective gene upon infection e.g. an antimicrobial peptide
ii. defective expression of a protective gene at steady state e.g. a tight junction protein
iii. excessive induction of a 'pathogenic' gene e.g. *Ifnγ*
iv. defective repression of a 'pathogenic' gene

Since *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice already harbour greater *C. rodentium* burdens at 3 days p.i. relative to WT mice, they are likely to respond proportionally by expressing more inflammatory, 'pathogenic' genes thus making it difficult to interpret categories iii. and iv. The most simple and interpretable result would fall into categories i and ii. Thus to identify protective elements that fit into these categories we first compared the genes upregulated or induced upon infection in each strain, i.e. we generated lists of genes upregulated in infected WT versus uninfected WT; infected *Nlrp3*$^{-/-}$ versus uninfected *Nlrp3*$^{-/-}$; and infected *Asc*$^{-/-}$ versus uninfected *Asc*$^{-/-}$. We found that, at 3 days p.i., WT mice only upregulated 26 genes in cecal tissue, while *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice upregulated >180 genes (Figure 3.24A). This was most likely due to the greater bacterial burdens in the latter 2 genotypes observed at this time-point. Of the 26 genes induced in infected WT mice, only 5 were exclusively upregulated in WT mice after infection, i.e. they were not induced in infected *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice (Figure 3.24A). From these five genes, two could be potentially linked to immunity against *C. rodentium* infection, namely *Gja5* and *Ccl5*. *Gja5* (or *Cx40*) encodes a connexin protein which is an integral protein that can oligomerize to form gap junctions that mediate intercellular communication between cells (Goodenough et al., 1996). The role of connexins in enteric infections is best characterized for *Shigella* species in which infection triggered opening of connexin channels has been shown to propagate the invasion and dissemination of *Shigella* in epithelial cells (Tran Van Nhieu et al., 2003). We used qPCR to validate the differential induction of *Gja5* and found a trend towards increased *Gja5* expression upon infection in WT mice but not in *Asc*$^{-/-}$ mice, although this difference did not reach statistical significance (Figure 3.24B). The other target that piqued our interest was the chemokine gene *Ccl5*, as it has been implicated in the

recruitment of T cells, basophils and eosinophils (D'Ambrosio et al., 2003). However as we were unable to validate the induction of this gene in WT mice using qPCR, we did not pursue this further (data not shown).

As we were unable to find strong candidate genes for category i., we turned our efforts to looking for genes that might not be upregulated in response to infection, but may nonetheless be defective in *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice at steady state. i.e. category ii.. As expected, we observed that *pycard* (*Asc*) expression was reduced in *Asc*$^{-/-}$ mice both at steady state and after infection (Figure 3.25). Surprisingly however, *Nlrp3* was not identified via this analysis. We found that a strong signature emerged in a group of eosinophil associated RNAses (*Ear1, 2, 4* & *10*) which were reduced in both *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice at steady state and at 3 days p.i. (Figure 3.25). We validated this change by qPCR and observed similar decreases in *Ear1/2* expression in both *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice (Figure 3.26A). While eosinophils are not well characterized in antibacterial immunity, they are abundant in the intestine (Blanchard and Rothenberg, 2009). As Nlrp3 expression in non-haematopoietic cells was required for protection against *C. rodentium*, we asked whether *Ear1/2* expression correlated with protective genotypes in the irradiation BM chimeras. However, we found that high *Ear1/2* expression correlated with haematopoietic Nlrp3 expression in the BM chimeras, but was not associated with protection from disease (Figure 3.26B). This data suggests that the main source of *Ear1/2* expression in the gut is derived from a haematopoietic cell and may be regulated by Nlrp3. However, defective *Ear1/2* expression is unlikely to explain the susceptibility of *Nlrp3*$^{-/-}$ mice to *C. rodentium*. Indeed further characterization of intestinal eosinophils at day 3p.i. with *C. rodentium* revealed equivalent frequencies of eosinophils between *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and WT mice (data not shown). Thus, the mechanism of precisely how deficiency in Nlrp3 and Asc leads to susceptibility towards *C. rodentium* remains to be discovered.

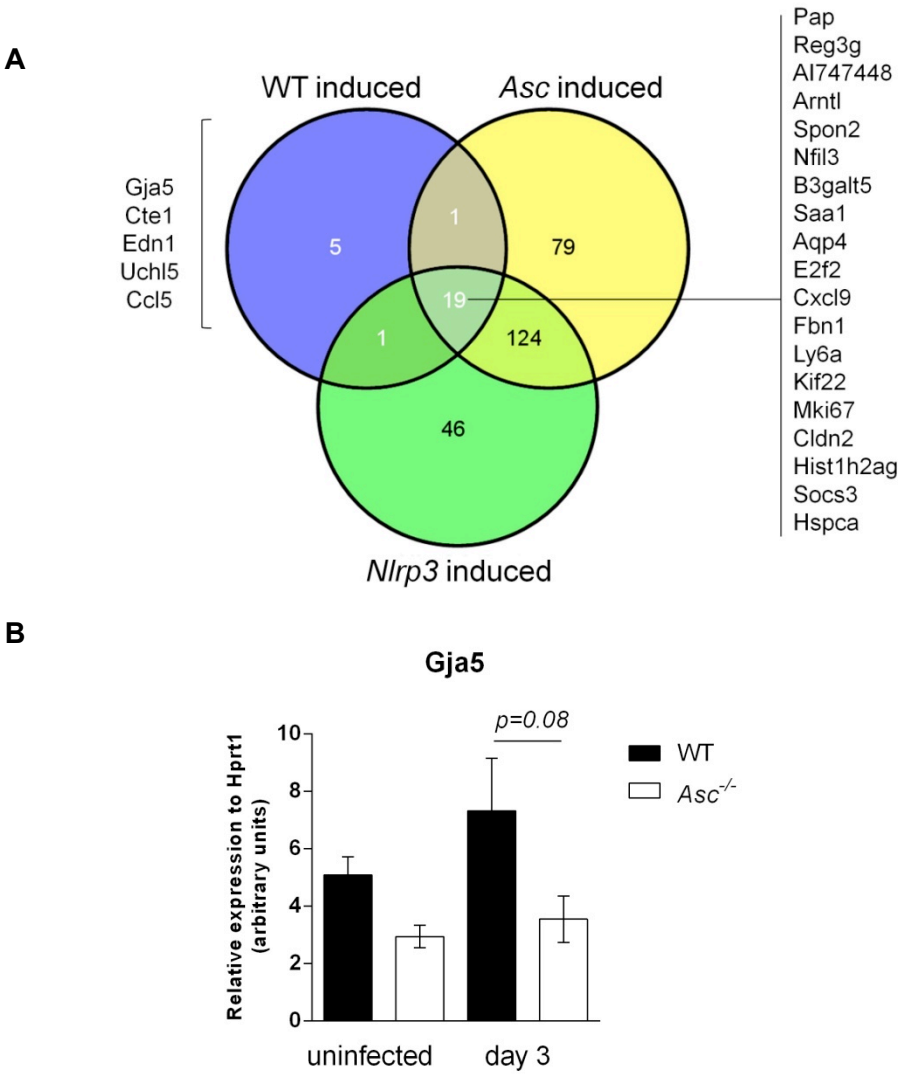


Figure 3.24 Genes upregulated WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice following infection with *C. rodentium*. Cohorts of WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were either left uninfected or orally infected with ~10^9 *C. rodentium* and sacrificed 3 days p.i. (n = 6 per group). Thereafter caecal tissue RNA was isolated and subjected to genome wide transcriptional profiling.

Above is a venn diagram showing genes upregulated (>1.5-fold) between infected and uninfected mice. Intersections denote genes upregulated in multiple genotypes during infection (A) qPCR validation of *Gja5* relative to *Hprt* (n = 5-6 per group) is shown in (B).

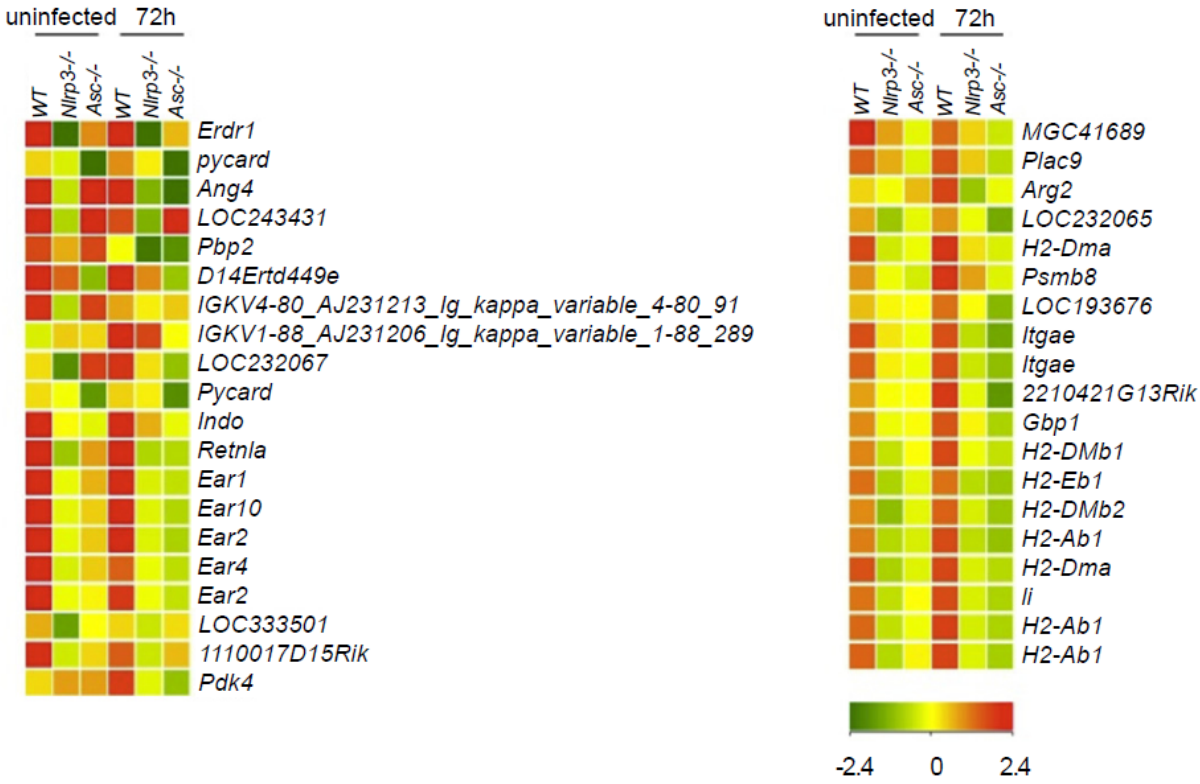


Figure 3.25 Differentially expressed genes between WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice. Cohorts of WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were either left uninfected or orally infected with ~10^9 *C. rodentium* and sacrificed at 3 days p.i (72h). (*n* = 6 per group)Thereafter caecal tissue RNA was isolated and subjected to genome wide transcriptional profiling.

Depicted is a heat map representing 33 unique genes significantly expressed at a lower level (at least 2-fold) in either *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ relative to WT mice, during steady state (uninfected) and/or 3 days p.i. (72h). Colour range (green to red) represent log transformed normalized intensity values.

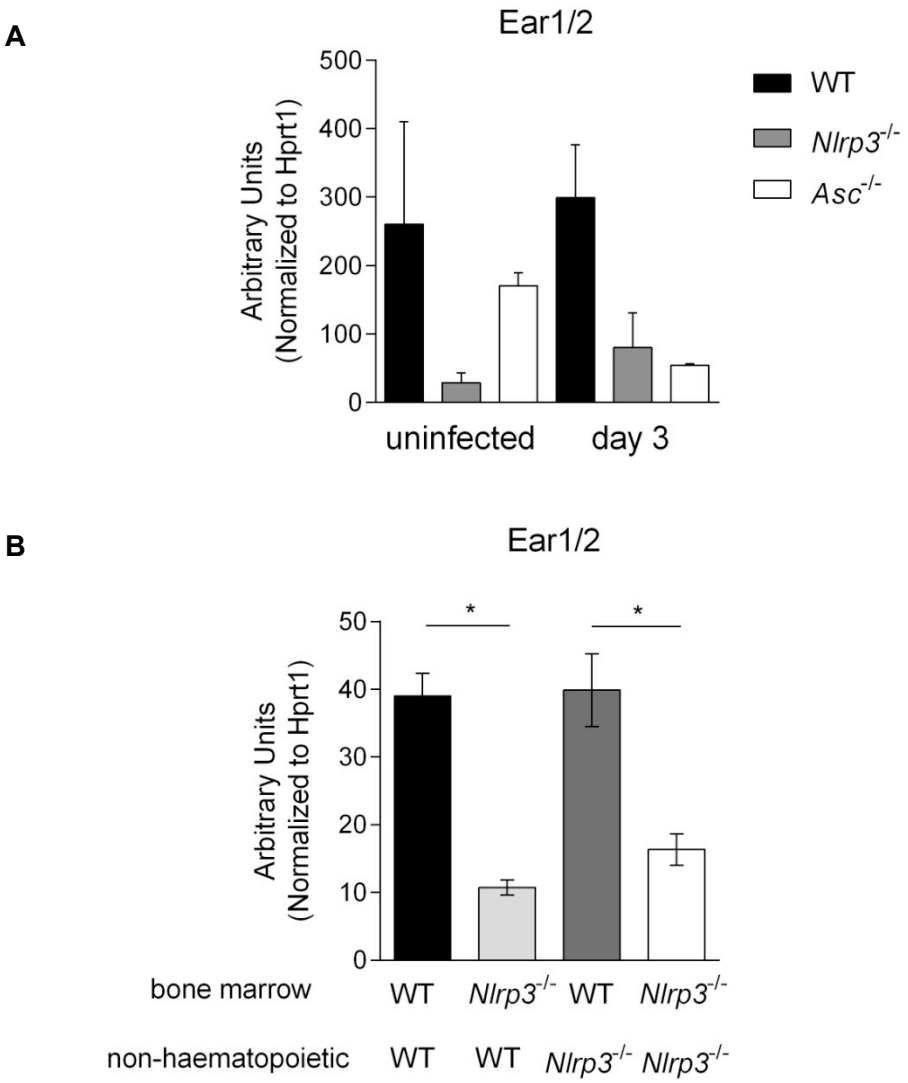


Figure 3.26 Relative gene expression of Ear 1/2 between WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice. Cohorts of WT, *Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were either left uninfected or orally infected with ~10^9 *C. rodentium* and sacrificed at 3 days p.i. Thereafter caecal tissue RNA was isolated and subjected to qPCR analyses.

(A) mRNA expression levels of *Ear1/2* relative to *Hprt* (*n* =3 per group)

(B) mRNA expression levels of *Ear 1/2* from *Nlrp3*$^{-/-}$ irradiation chimeras at 3 days p.i., relative to *Hprt* (*n* = 4 per group)

Bars represent means±SEM. Statistical significance was determined by the Mann-Whitney test (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

3.3 Discussion

Our interest in the role of innate immune responses in the maintenance of intestinal homeostasis was spurred on by a number of GWAS studies that had identified multiple biological processes associated with innate immunity, barrier function, antibacterial defence and epithelial restitution, in susceptibility to IBD (Khor et al., 2011). GWAS studies have implicated genes associated with multiple steps of the inflammasome pathway in susceptibility to CD (Villani et al., 2009). We therefore wished to understand the role of the inflammasome in intestinal homeostasis and in mucosal defence. To address the latter question in this chapter we utilized the attaching and effacing murine pathogen, *C. rodentium*.

Initially, we observed that *C. rodentium* triggered caspase-1 activation in BMDMs *in vitro*. This was dependent on Nlrp3 and Asc as BMDMs lacking these proteins were unable to activate caspase-1 upon infection. Using multiple inhibitors we also discovered that caspase-1 activation required the phagocytic uptake of live *C.rodentium*, which induced a K^+ efflux out of the cell. Our finding that live, but not heat killed, *C. rodentium* triggered caspase-1 activation is consistent with other recent findings that prokaryotic mRNA from phagocytosed live bacteria enter the cytosol to trigger Nlrp3 activation (Sander et al., 2011). However, whether live *C. rodentium* activates caspase-1 via this mechanism remains to be determined.

Recently others have demonstrated that *C. rodentium*-driven caspase-1 activation requires another member of the caspase family to license it, caspase-11 (Kayagaki et al., 2011). Caspase-11, acting upstream of caspase-1, is regulated via TRIF signalling that drives the production of IFNβ which, acting in a autocrine manner, signals via type 1 interferon receptors leading to STAT1 and IRF9-dependent caspase-11 transcription (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012). We confirmed the requirement for caspase-11 for activation of caspse-1 by *C. rodentium* in BMDMs *in vitro* (data not shown). It is tempting to speculate that the TRIF-dependency of caspase-1 activation by *C. rodentium* partially stems from the sensing of prokaryotic mRNA from live bacteria. However TLR4 deficient BMDMs are unable to upregulate caspase-11 upon *C. rodentium* infection, suggesting that LPS is probably the PAMP that induces TRIF-dependent caspase-11 induction (Rathinam et al., 2012).

Nlrp3 is a cytosolic sensor, and the activation of cytosolic proteins by phagosome-bound pathogens such as *C. rodentium* remains a mystery. Other cytosolic receptors such as Nod2 require phagosome-bound peptide transporters such as PEPT2 that 'pump' out PAMPs such as MDP (Charriere et al., 2010). While no such transporters have been discovered for Nlrp3, a recent study observed that caspase-1 could localize to bacteria containing phagosomes *in vitro* and control phagosomal pH (Sokolovska et al., 2013). Interestingly, gradually increasing the K^+ concentration inhibited *C. rodentium*-driven caspase-1 activation suggesting that infection with this bacterium somehow induces an efflux of K^+. As *C. rodentium* is a non-invasive pathogen, its effects within phagocytes are poorly characterized as at low MOIs the bacterium is efficiently killed (data not shown). Thus, how *C. rodentium* virulence activity or presence in the phagosome couples to Nlrp3 activation in the cytosol requires further characterization.

Intrigued by how *C. rodentium* infection triggered Nlrp3 activation we focussed on the virulence factors expressed by this bacterium. *C. rodentium* virulence, similar to EPEC, is controlled primarily by a chromosomally encoded pathogenicity island (PAI) called the locus of enterocyte effacement (LEE) . The LEE expresses numerous virulence genes including the type three secretion system (TTSS) and a number of effector proteins (Esp) (Holmes et al., 2010). Mitochondrial damage and the release of oxidized mitochondrial DNA have recently been described as key cellular events upstream of Nlrp3 activation, and Nlrp3 has been shown to associate with damaged mitochondria (Shimada et al., 2012; Zhou et al., 2011). Thus we focussed on virulence effector proteins that had been previously described to interfere with mitochondrial homeostasis. These included espF, an effector protein which localizes to the mitochondria in host cells and disrupts the mitochondrial membrane potential, leading to the release of cytochrome c and cell death (Holmes et al., 2010; Nagai et al., 2005). Secondly we investigated the virulence factor map (data not shown), which also colocalizes with the mitochondria and is thought to disrupt membrane potential (Luperchio and Schauer, 2001; Ma et al., 2006). We also examined the role of espT, a guanine nucleotide exchange factor which activates the small Rho GTPases, Rac1 and Cdc42 (Bulgin et al., 2009), as previous work has postulated the manipulation of small Rho GTPases as a triggering event for caspase-1 activation (Muller et al., 2010). Finally, we utilized *C. rodentium* mutants lacking Tir, the translocated receptor for the bacterial adhesin intimin that allows intimate

attachment to the epithelium, actin accumulation and pedestal formation (Deng et al., 2003). Surprisingly, isogenic mutants lacking the above virulence factors were able to activate caspase-1 in BMDMs just as efficiently as WT *C. rodentium*. We hypothesize that dispensability for these virulence factors is due to the phagocytic ability of BMDMs, which overcomes the requirement for *C. rodentium* to 'attach and efface'. That is to say attaching and effacing pathogens such as *C. rodentium* have evolved a tropism for intestinal epithelial cells, which are non-phagocytic. Thus, future studies examining the virulence determinants that confer *C. rodentium* with the ability to activate caspase-1 should be conducted with intestinal epithelial cells rather than BMDMs. The major hurdle to overcome here is that *C. rodentium* does not attach to most epithelial cell lines and so alternative sources such as *ex vivo* derived primary IEC monolayers should be investigated.

Having found that *C. rodentium* activated an Nlrp3-Asc dependent inflammasome *in vitro*, we were curious about the role of the inflammasome *in vivo*. We found that Nlrp3 and Asc signalling in IECs limits early *C. rodentium* colonization and limits subsequent intestinal inflammation. Historically, PRRs have been well characterized in haematopoietic cells such as macrophages, neutrophils and DCs. However, there is a growing body of recent literature which points towards PRRs within non-haematopoietic cells as playing key functions in protective immunity. IECs are the most numerous cell type in the GI tract and form the first line of defence against enteric pathogens. Thus they are arguably best poised to combat early invasion or, failing that, recruit other cells for support. Indeed, recent studies have shown that Tlr2, MyD88 and Nod2 signalling in non-haematopoietic cells is crucial to protection against *C. rodentium* infection (Gibson et al., 2008a; Gibson et al., 2008b; Kim et al., 2011; Lebeis et al., 2007). Our findings reinforce the notion that innate immune detection at epithelial surfaces play important roles in antibacterial immunity, and extend it to inflammasome activation in IECs.

Mechanistically, we found that the levels of caspase-1 dependent cytokines such as IL-1β, IL-1α and IL-18 were equivalent between WT and *Nlrp3*$^{-/-}$ mice, whereas IL-18 production was completely abrogated in *Asc*$^{-/-}$mice. Fitting with this, caspase-1 activation was intact in *Nlrp3*$^{-/-}$ mice but absent in *Asc*$^{-/-}$ mice after 8 days p.i.. These results suggest that Nlrp3 signalling protects against *C. rodentium* independently of caspase-1. The fact that IL-1β and IL-18 secretion and caspase-1 cleavage was independent of Nlrp3 was surprising given our *in vitro* observations, as

well the fact that *Asc*$^{-/-}$ showed only a slight trend towards increased susceptibility compared to *Nlrp3*$^{-/-}$ mice. We can surmise that protection against

C. rodentium takes two forms, both of which may utilize Asc:

i. A major Nlrp3-dependent, caspase-1 independent mechanism

ii. A minor caspase-1 dependent mechanism that may involve alternative NLR

We hypothesized that the latter mechanism was mediated by IL-18, as *Asc*$^{-/-}$ mice but not *Nlrp3*$^{-/-}$ mice lacked IL-18 production. Indeed we found that *Il18r*$^{-/-}$ mice showed a trend towards greater intestinal inflammation and harboured significantly higher bacterial burdens compared to WT mice at 8 days p.i. The major source of this IL-18 was non-haematopoietic cells, as BM chimeras lacking Asc in their non-haematopoietic compartment (WT→*Asc*$^{-/-}$) did not produce IL-18.

The major Nlrp3 dependent, caspase-1 independent mechanism of protection has not been described in the literature. However, very recent reports implicate another caspase, caspase-8, in forming 'non-canonical' inflammasomes with Asc, independently of caspase-1 (Gringhuis et al., 2012; Pierini et al., 2012; Sagulenko et al., 2013). Caspase-8 was initially characterized for its function as an initiator caspase in the cell-extrinsic TNF and Fas ligand (FasL)-driven pathway of apoptosis (Li et al., 1998). However, two recent studies showed that Aim2 and Nlrp3, whilst also driving caspase-1 activation, could promote caspase-8 activation that led to apoptosis (Pierini et al., 2012; Sagulenko et al., 2013). However, another study showed that TLR3/4 signalling via TRIF could potentiate the cleavage of pro-IL-1β via caspase-8 and that recombinant caspase-8 cleaved pro-IL-1β at a similar site to caspase-1 (Maelfait et al., 2008). In addition, FasL was recently described to trigger maturation of pro-IL-1β and pro-IL-18 in a caspase-8 dependent manner that was independent of caspase-1 (Bossaller et al., 2012). Although the previous studies described caspase-8 activities in haematopoietic cells, a recent study generated mice in which caspase-8 was selectively ablated in IECs and found that they developed spontaneous ileitis, suggesting that caspase-8 is important for IEC homeostasis (Gunther et al., 2011). Finally, the caspase-8 homolog in drosophila, Dredd, promotes antimicrobial peptide responses against Gram-negative bacteria (Leulier et al., 2000), suggesting an additional potential mechanism of protection. Therefore, it would be interesting to follow up the potential role of caspase-8 in protection against *C. rodentium*

infection. In preliminary experiments we found that mice deficient in Asc expressed lower levels of cleaved caspase-8 compared to WT mice after infection with *C. rodentium* (data not shown).

Caspase-11 was also recently implicated in responses towards *C. rodentium in vitro* (Kayagaki et al., 2011; Rathinam et al., 2012), and is upstream of caspase-1 in responses to a wide range of Gram-negative bacterial pathogens (Rathinam et al., 2012). However, while caspase-11 controls caspase-1 dependent cytokine maturation, it also independently drives a form of cell death known as pyroptosis (Kayagaki et al., 2011). Pyroptosis is an inflammatory form of cell death that differs from apoptosis in a few ways; it involves cell swelling, cell lysis and pore formation (Labbé and Saleh, 2011). Pyroptosis also shares certain features with apoptosis, namely nuclear condensation and DNA fragmentation (staining Tunel^{+}). Recently, pyroptosis has been described as a novel effector mechanism for antibacterial immunity against intracellular bacteria. For example, Nlrc4 driven pyroptosis in macrophages exposes *S. typhimurium* for clearance by neutrophils (Miao et al., 2010a). We hypothesised that a similar form of 'altruistic' cell death downstream of Nlrp3 activation could limit *C. rodentium* attachment to IECs. One prediction of this hypothesis is that there would be fewer Tunel^{+} IEC in *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice compared to WT mice after *C. rodentium* infection. However, we found very few Tunel^{+} cells in all genotypes, making such a comparison impossible (data not shown). Observing dying IEC *in vivo* is a technically difficult task due to resident macrophages that rapidly clear dying cells. In addition, although we found that naturally caspase-11 deficient 129SvEv mice harboured more tissue-adherent *C. rodentium* relative to C57BL/6 WT mice, this was most probably due to other genetic differences between these strains, as a recent paper reported that *caspase-11*$^{-/-}$ mice on a C57BL/6 background were equally susceptible to *C. rodentium* as WT C57BL/6 mice (Gurung et al., 2012). We also found that caspase-11 was dispensable for the production of IL-1β and IL-18 *in vivo*, in contrast to its fundamental requirement in these responses to *C. rodentium* in BMDMs *in vitro* (Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012). Furthermore, based on our observation that caspase-1 activation and IL-18 production occurs mainly in IECs, we predict that caspase-1 circuits are differently wired in tissue cells versus myeloid cells.

To further characterize novel Nlrp3-dependent pathways of protection, we conducted genome wide transcriptional profiling. We discovered that very few genes were induced upon infection with *C.*

rodentium exclusively in WT mice. By contrast many of the differentially expressed genes between WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice were already apparent at baseline suggesting that basal Nlrp3 and Asc signalling may contribute to early protective immunity towards *C. rodentium*. Overall, the microarray approach did not highlight many robust candidates for future characterization. This is probably because Nlrp3 and Asc functions such as caspase-1 cleavage are primarily post-translational modifications that would not necessarily be detected by transcriptional profiling. In order to identify the most proximal event upon Nlrp3 signalling that confers protection against *C. rodentium*, future studies should use proteomic approaches or 2D gel electrophoresis coupled with IEC monolayer infections. In this way differentially expressed or processed proteins that result from *C. rodentium* infection between WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ IECs may be identified by mass spectrometry.

What might this candidate effector be? Our results suggest that Nlrp3/Asc signalling in IECs limits early *C. rodentium* colonization. Protection from IECs can be cell-intrinsic, e.g. through production of mucin or antimicrobial peptides (AMPs) that directly limit *C. rodentium*. Alternatively, protection derived from IECs could be cell-extrinsic, e.g. IECs produce CCL2 in a Nod2-dependent manner that induces the recruitment of inflammatory monocytes that promote bacterial clearance (Kim et al., 2011). Based on the fact that Nlrp3/Asc-dependent protection occurred within 3 days p.i. and that we observed no impairment in the early lamina propria infiltrate (data not shown), we hypothesize that the protective mechanism operates via a cell-intrinsic manner in IEC. While we found no impairment in AMPs or Muc2 expression in *Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ mice at a transcript level (data not shown), these targets need to be scrutinized using additional methods because there may be Nlrp3/Asc-depedent post-translation modifications that regulated these proteins which would not be revealed by qPCR. An approach that one may take for instance is an IEC crypt killing assay in which we test the relative abilities of IECs from WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice to kill *C. rodentium*.

Together our data highlighting roles for Nlrp3 and Asc in IECs are consistent with other reports suggesting similar protective roles in a model of chemically induced intestinal inflammation using dextran sodium sulphate (DSS) (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Elinav et al., 2011; Zaki et al., 2010a). However while the protective mechanism in these studies was ascribed to

IL-18, we found that this only accounted for a minor part of the mechanism in protection from *C. rodentium*. Furthermore, Liu *et al* have recently confirmed our findings, observing that mice lacking caspase-1, Asc and Nlrp3 were more susceptible to *C. rodentium* infection, although they did not investigate the cell types in which the protective axis operated (Liu et al., 2012). However, in stark contrast to our findings, Liu *et al* found that IL-18 production was reduced in *Nlrp3*$^{-/-}$ mice following infection with *C. rodentium* (Liu et al., 2012). We suspect that the difference between the two findings may be due to distinct techniques used to assess IL-18 levels in the gut. While we measured IL-18 in the supernatants from organ explant cultures overnight by ELISA, Liu *et al* homogenized the whole intestinal tissue before measurement by ELISA. Our preference for using supernatants of organ cultures is that tissue homogenization is likely to release both inactive pro-IL-18 and active cleaved IL-18, which cannot be distinguished by ELISA. In contrast, as IL-18 cleavage and release is an active process, we reasoned that measuring IL-18 in the supernatant gives a more accurate representation of the levels of active IL-18. Consistent with this hypothesis, we observed the levels of cleaved caspase-1 correlated with the levels of IL-18 as measured by ELISA. Other reasons underlying these differing results might lie in the different strains of *C. rodentium* used in between our groups and the different time points at which the cytokine levels were assessed. For instance while we focused on early protection, Liu *et al* examined late time points.

As one of the leading causes of infant mortality, EPEC has a devastating impact, particularly in developing nations (Ochoa and Contreras, 2011). Mortality is thought to be primarily due to dehydration that arises from excessive diarrhoea (Ochoa and Contreras, 2011). EPEC and *C. rodentium* manipulate ion exchange in the intestine to promote diarrhoea (Hodges et al., 2008; Viswanathan et al., 2009). Sodium (Na^+), one of the primary ions taken up into IECs, is coupled to the uptake of glucose via a Na^+/glucose symport known as SGLT-1 (Lodish, 2004). In the second stage, glucose is exported into the blood via uniport proteins (Lodish, 2004). In order for the transcellular transport for glucose to operate in this way, the intracellular [Na^+] must be maintained at a low concentration so as to favour its movement down a concentration gradient from the lumen and couple this to the energetically unfavourable movement of glucose. To ensure this, Na^+ is pumped across the basolateral membrane in exchange for potassium (K^+) via a Na^+/ K^+ ATPase

pump (Lodish, 2004). Thus at steady state, a high [K^+] and a low [Na^+] is maintained in the IEC cytosol, while blood has a relatively high [Na^+] and a low [K^+] concentration. Collectively these two transporters promote the transport of salt, glucose and amino acids across the intestinal epithelium that generates an osmotic gradient drawing water from the lumen.

Interestingly, EPEC blocks the uptake of Na^+ in two ways: by blocking SGLT-1 and by inhibiting another transporter, the Na^+/ H^+ exchanger (NHE), NHE3 (Dean et al., 2006; Hodges et al., 2008). This reduction in Na^+ uptake together with the blockade of luminal chloride absorption may lead to diarrhoea as salt in the lumen draws water out of the ECM on the basolateral side of the epithelium (Viswanathan et al., 2009). Importantly as [Na^+] is reduced in the IEC cytosol, less can be pumped into the blood in exchange for K^+, which may lead to a reduction in [K^+] in the IEC cytosol. Given our data that *C. rodentium* activates an Nlrp3-dependent inflammasome in BMDMs in a mechanism involving K^+ efflux, it is tempting to speculate that Nlrp3 might sense perturbations in the delicate osmotic balances that occur upon *C. rodentium* infection. Indeed, a recent study has shown that K^+ efflux is the only cellular event that is both sufficient and necessary for Nlrp3 activation (Munoz-Planillo et al., 2013). Testing whether Nlrp3 activation can be similarly triggered by other diarrheagenic pathogens such as *V. cholerae* and rotaviral infections thus merits future investigation.

Chapter 4. The inflammasome in *H. hepaticus* driven chronic intestinal inflammation

4.1 Introduction

In the last chapter we investigated the role of Nlrp3 and Asc in an acute model of intestinal inflammation using the murine intestinal pathogen, *C. rodentium*. While such models are useful, they often do not accurately recapitulate all aspects of human IBD. In fact many of the factors that are crucial in defence against *C. rodentium* such as IL-23, mediate chronic intestinal inflammation in other models and in human disease settings (Duerr et al., 2006; Hue et al., 2006; Kullberg et al., 2006). Thus we turned to chronic models of intestinal inflammation, based on infection with the enteric murine pathogen *Helicobacter hepaticus.*

4.1.1 Innate immunity in chronic intestinal inflammation

Whilst innate immunity is key in controlling epithelial barrier function, repair and restitution after pathogenic insult or damage from chemicals and xenobiotics, excessive PRR signalling towards the microbiota is detrimental. Indeed, in contrast to findings in *C. rodentium* and DSS models of acute intestinal inflammation wherein innate immune signalling is protective (Gibson et al., 2008a; Lebeis et al., 2007; Rakoff-Nahoum et al., 2004), in chronic models of intestinal inflammation the general view is that sustained PRR signalling promotes and perpetuates pathogenic inflammatory responses (Maloy and Powrie, 2011).

Innate immune signalling is central in mediating aberrant responses against the microbiota to induce pathogenic Th1/Th17 responses that dominate intestinal inflammation. In line with this, spontaneous colitis in *Il10*$^{-/-}$ mice can be rescued by crossing these mice to *Myd88*$^{-/-}$ mice (Hoshi et al., 2012; Rakoff-Nahoum et al., 2006). Similarly, spontaneous intestinal inflammation that develops in transgenic mice selectively lacking NFκB signalling capacity in IEC can be rescued by ablating Myd88 (Nenci et al., 2007). Furthermore, mice lacking a negative regulator of MyD88 signalling, A20, spontaneously develop inflammation that can be rescued either by broad-spectrum

antibiotic administration or by knocking out Myd88 (Turer et al., 2008). Thus homeostatic microbiota driven MyD88 signalling can turn pathogenic in the absence of sufficient regulation.

We wished to ask whether excessive inflammasome activation could contribute to chronic intestinal inflammation. Supporting this idea, levels of IL-1β and IL-18 are significantly elevated in mucosal biopsies from CD patients (Casini-Raggi et al., 1995; Pizarro et al., 1999). Furthermore, mice lacking an autophagy protein in haematopoietic cells that negatively regulates the inflammasome, Atg16l1, are more susceptible to DSS colitis, which can be alleviated by anti-IL-1β, and anti-IL-18 antibody therapy (Saitoh et al., 2008).

To investigate the role of inflammasome activation in chronic intestinal inflammation we utilized a lymphocyte replete and an innate model of *H. hepaticus* (*Hh*) driven intestinal inflammation. The former model involves the infection of WT C57BL/6 mice with *Hh* and concomitant treatment with anti IL-10 receptor antibody (Kullberg et al., 2006). By contrast, the innate immune IBD model involves the chronic infection of susceptible 129SvEv *Rag*$^{-/-}$ mice with *Hh*. Interestingly, previous work from our lab showed that intestinal inflammation in both models was dependent upon MyD88 signalling in haematopoietic cells (Asquith et al., 2010). Curiously however, while *in vitro* data had implicated Tlr2 in the detection of *Hh*, it was found that Tlr2, Tlr4 and Tlr5 were dispensable for the induction of intestinal inflammation *in vivo* ((Boulard et al., 2010), M.Asquith DPhil thesis). This led us to speculate that other receptors using MyD88 as an adapter, such as the receptors for IL-1β and IL-18, might be involved. Fitting with this notion, our lab previously demonstrated that blockade of IL-1β alleviates intestinal inflammation in the innate model of colitis (Coccia et al., 2012). Here we wished to extend these studies and address the source of IL-1β in these models. Given that inflammasome activation is upstream of IL-1β and IL-18, we speculated that it might be involved in the induction of chronic intestinal inflammation.

4.2 Results

4.2.1 *Helicobacter hepaticus* activates Asc-dependent caspase-1 cleavage *in vitro*

We first wanted to ascertain whether *Hh* could activate the inflammasome *in vitro*. We assessed this by infecting BMDMs from either WT or *Asc*$^{-/-}$ mice with *Hh* overnight. We found that *Hh* infection triggered the activation of caspase-1 p10 in BMDMs in a dose-dependent manner that was dependent on Asc, since mice deficient in Asc were unable to process caspase-1 upon infection (Figure 4.1A) To test the functional relevance of this finding, we also blotted for active IL-1β. Consistent with caspase-1 activation, we found that *Hh* infection triggered the release of active IL-1β in an Asc-dependent and dose-dependent manner (Figure 4.1B). Notably, in contrast to *C. rodentium*, *Hh*-induced caspase-1 activation did not require LPS priming beforehand.

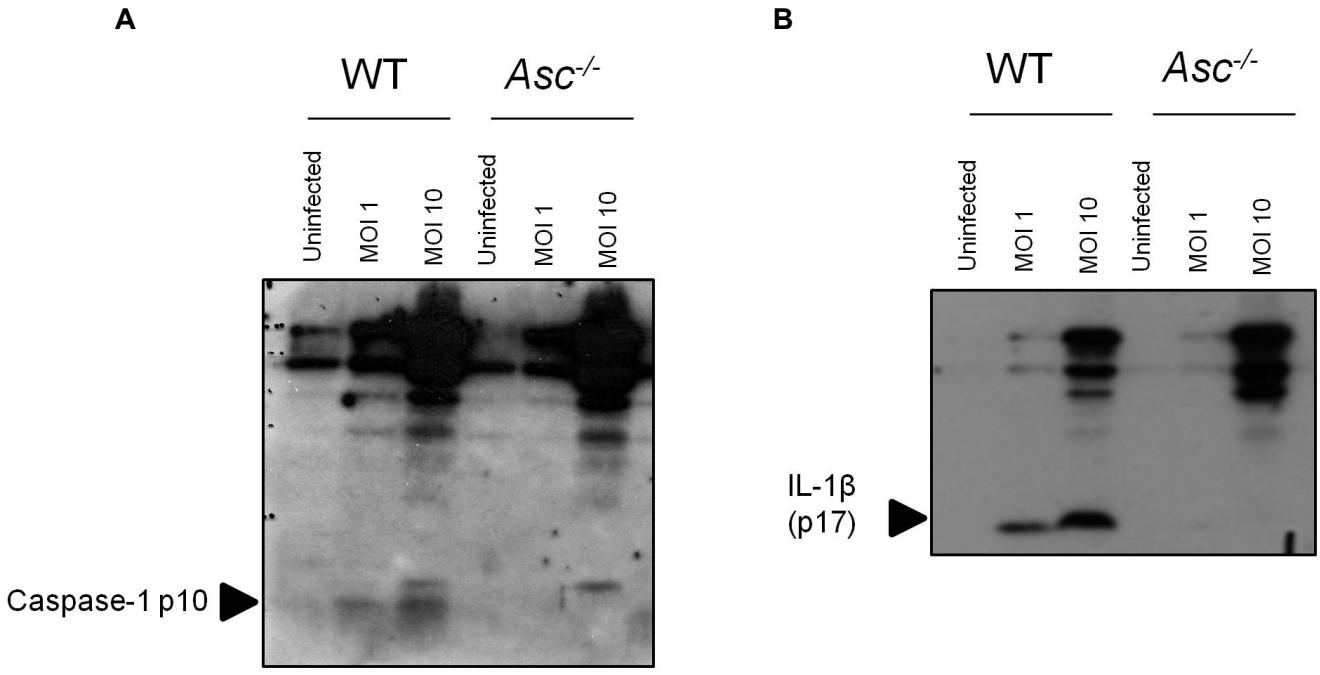


Figure 4.1 *H. hepaticus* infection triggers the activation of caspase-1 *in vitro*.

$2x10^5$ bone marrow derived macrophages (BMDMs) derived from either WT or *Asc*$^{-/-}$ mice were infected with increasing multiplicity of infections (MOIs) of *Hh* overnight or left uninfected. Following infection, supernatants were harvested, proteins isolated and concentrated and then immunoblotted for either caspase-1 p10 (A) or IL-1β p17 (B). Data shown is representative of two independent experiments with similar results.

4.2.2 Asc is dispensable for the induction of intestinal inflammation in lymphocyte replete mice

In light of our finding that *Hh* activated the inflammasome *in vitro* and other work showing that *Hh* infection induces IL-1β production *in vivo* (Coccia et al., 2012), we asked whether intestinal inflammation was dependent on Asc. To test this hypothesis we utilized a model of intestinal inflammation in B6 WT mice driven by infection with *Hh* and concomitant administration of anti-IL-10R antibody for four weeks (Hh+α-IL-10R) (Kullberg et al., 2006). In this model, the single administration of either *Hh* or anti IL-10R antibody elicits minimal inflammation while co-administration induces severe typhlocolitis. We observed that the levels of caecal inflammation were equivalent between B6 WT and B6 *Asc*$^{-/-}$ mice after Hh+α-IL-10R treatment (Figure 4.2). While not significant, the B6 *Asc*$^{-/-}$ mice showed a marked trend towards reduced inflammation in the colon compared to B6 WT mice (Figure 4.2). Systemic inflammation, as assessed by splenomegaly and splenocyte counts, was equivalent between B6 WT and B6 *Asc*$^{-/-}$ mice following Hh+a-IL-10R treatment, suggesting that Asc signalling was dispensable (Figure 4.3A,B). Finally, assessment of bacterial colonization levels in the mice revealed equivalent *Hh* colonization between the two strains (Figure 4.4).

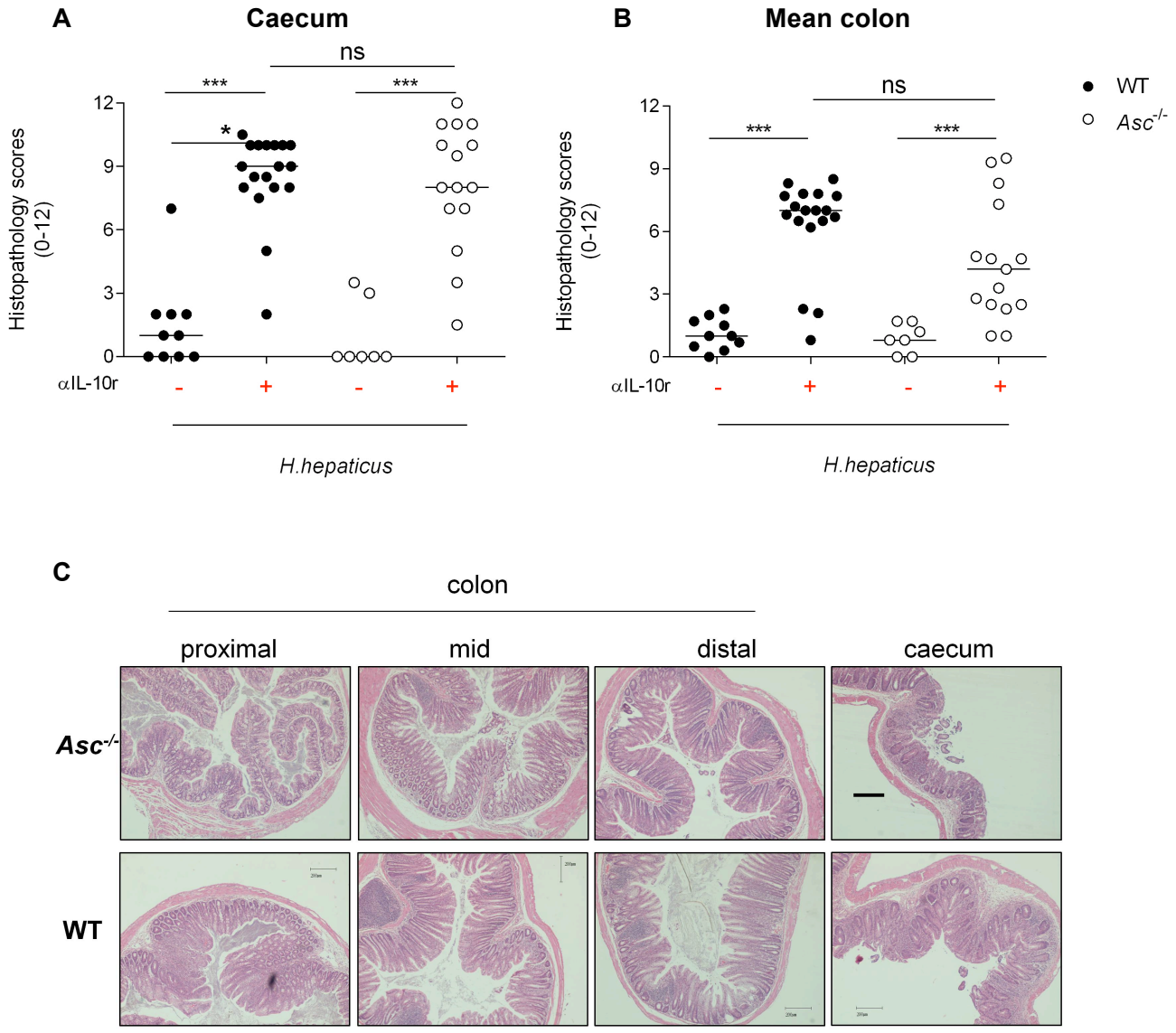


Figure 4.2 Asc-independent typhlocolitis in *H. hepaticus* and anti IL-10R treated mice.

B6 *Asc*$^{-/-}$ and WT mice were orally infected with *H. hepaticus (H.h)* and then treated with anti IL-10R antibody (1mg/wk i.p.) weekly for 4 weeks. On day 28 post-infection, mice were euthanized and assessed for caecal inflammation (A) and colonic inflammation (B). Representative histology sections from *H.h* and anti IL-10R treated animals (scale bar= 200µm) (C). Each symbol represents a single animal and data shown was pooled from 3 independent experiments (*n* = 7-18 per group). Horizontal bars represent medians. Statistical significance was determined using the Mann Whitney test (***p<0.001).

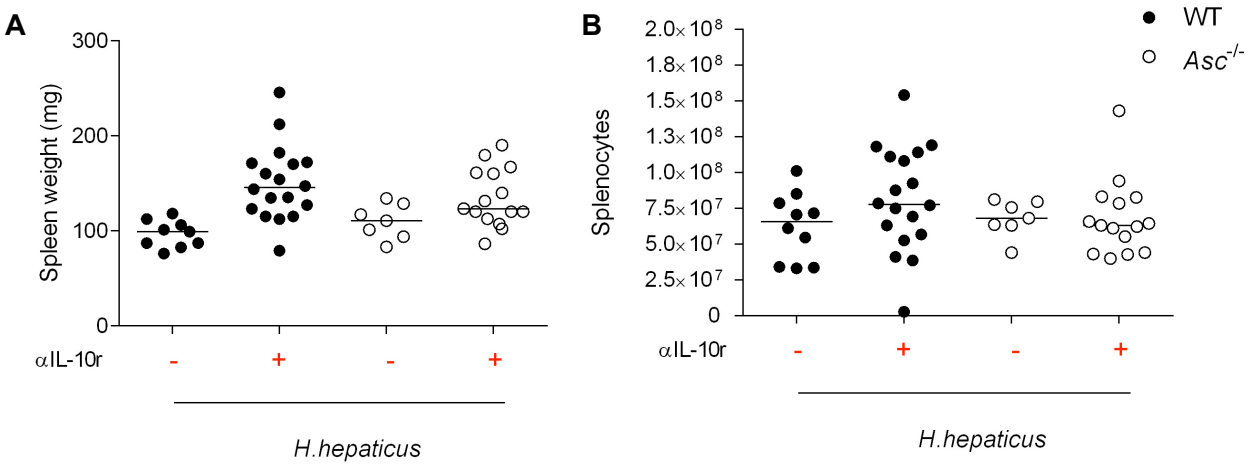


Figure 4.3 Systemic inflammation in *H. hepaticus* and anti IL-10R treated mice is independent of Asc.

B6 *Asc*$^{-/-}$ and WT mice were orally infected with *H. hepaticus (H.h)* and then treated with anti IL-10R antibody (1mg/wk i.p.) weekly for 4 weeks. On day 28 post-infection, mice were euthanized and assessed for spleen weight (A), splenocyte number (B). Each symbol represents a single animal and data shown was pooled from at least 2 independent experiments (n = 7-18 per group). Horizontal bars represent medians.

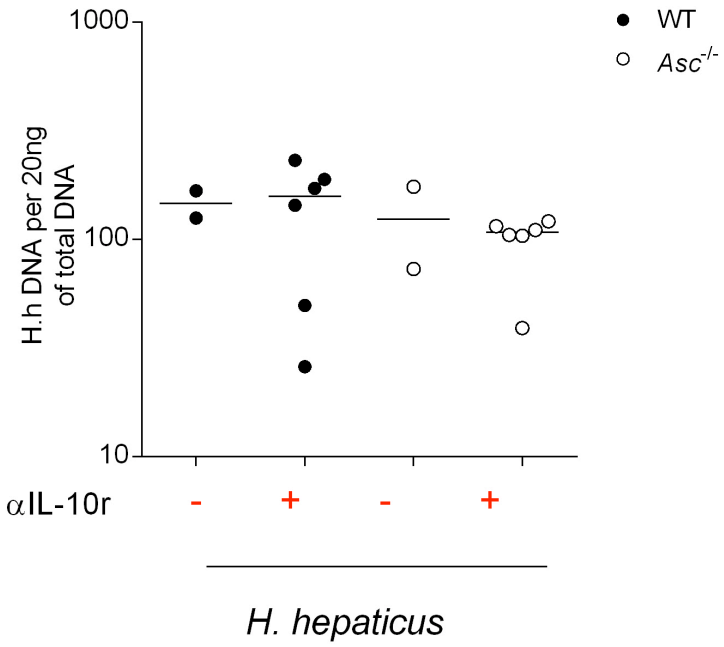


Figure 4.4 *H. hepaticus* colonization is equivalent in B6 WT and B6 *Asc*$^{-/-}$ mice.

B6 *Asc*$^{-/-}$ and WT mice were orally infected with *H. hepaticus (H.h)* and then treated with anti IL-10R antibody (1mg/wk i.p.) weekly for 4 weeks. On day 28 post-infection, mice were euthanized, caecal contents were collected and the DNA was extracted. *Hh* DNA was quantitated via real time PCR using primers directed against the *cdtB* gene. Each symbol represents a single animal and data is representative of three independent experiments (n = 2-6 mice per group).

Intestinal inflammation in the *Hh* plus anti IL-10R mAb IBD model is driven by pathogenic Th1/Th17 subsets (Ahern et al., 2010; Kullberg et al., 2006; Kullberg et al., 1998; Morrison et al., 2013). Accordingly, the inflammation is associated with the production of IFN-γ, IL-17 and TNFα. As previous reports have suggested a role for caspase-1 cleaved cytokines in the regulation of Th1 and Th17 subsets (Chung et al., 2009; Coccia et al., 2012; Shaw et al., 2012), we wanted to investigate whether the absence of Asc altered the frequencies of these cells. Consistent with the comparable intestinal inflammation levels, we saw no significant difference between the total LPL and total MLN cell numbers between B6 WT and B6 *Asc*$^{-/-}$ mice after Hh+α-IL-10R treatment (Figure 4.5), although there was a slight trend towards lower LPL numbers in the B6 *Asc*$^{-/-}$ mice. Next we determined the frequencies of Th1, Th17 and Th1/17 cells in the lamina propria of these mice. The frequencies of Th17 (IL-17^{+} IFN-γ^{-}) and Th1 (IFN-γ^{+} IL-17^{-}) cells in the LPL were equivalent between B6 WT and B6 *Asc*$^{-/-}$ mice after Hh+α-IL-10R treatment (Figure 4.6A,B). Although B6 *Asc*$^{-/-}$ mice displayed trends towards lower frequencies of double positive (IL-17^{+} IFN-γ^{+}) cells in LPL, these trends were not statistically significant (Figure 4.6C). Collectively, these data show that Asc plays a minor role in the induction of intestinal inflammation in the *Hh* anti IL-10R model.

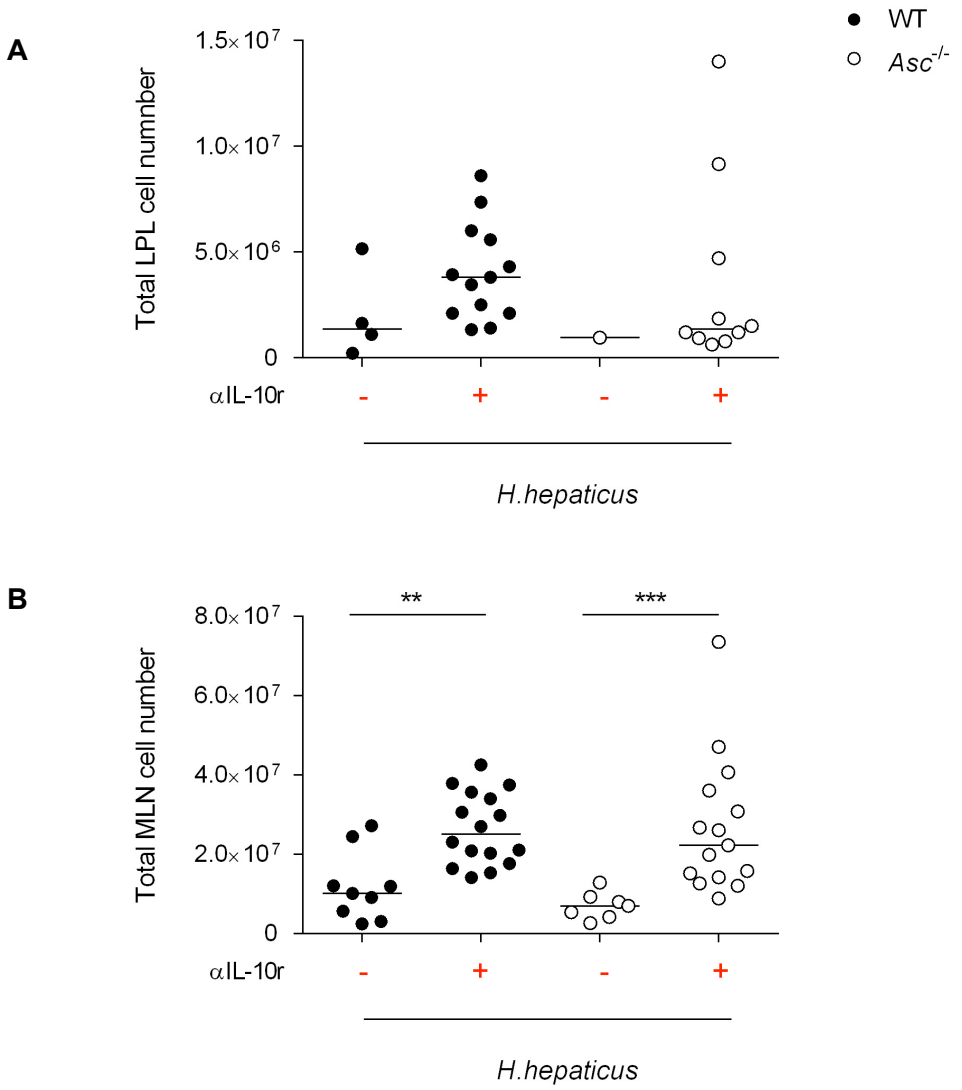


Figure 4.5 Comparable cellularity of the lamina propria and mesenteric lymph nodes in *H. hepaticus* and anti IL-10R treated WT and *Asc*$^{-/-}$ mice.

B6 *Asc*$^{-/-}$ and WT mice were orally infected with *H. hepaticus (H.h)* and then treated with anti IL-10R antibody (1mg/wk i.p.) weekly for 4 weeks. On day 28 post-infection, mice were euthanized, and pooled caecum and distal colon lamina propria leukocyte (A) and mesenteric lymph node cell numbers (B) were determined. Each symbol represents a single animal and data shown were pooled from at least two independent experiments (*n*=10-16 mice per infected group, n= 7-9 per uninfected group). Horizonal lines represent medians. Statistical significance was determined using the Mann Whitney test (* $p < 0.05$; ** $p < 0.01$; *** $p< 0.001$).

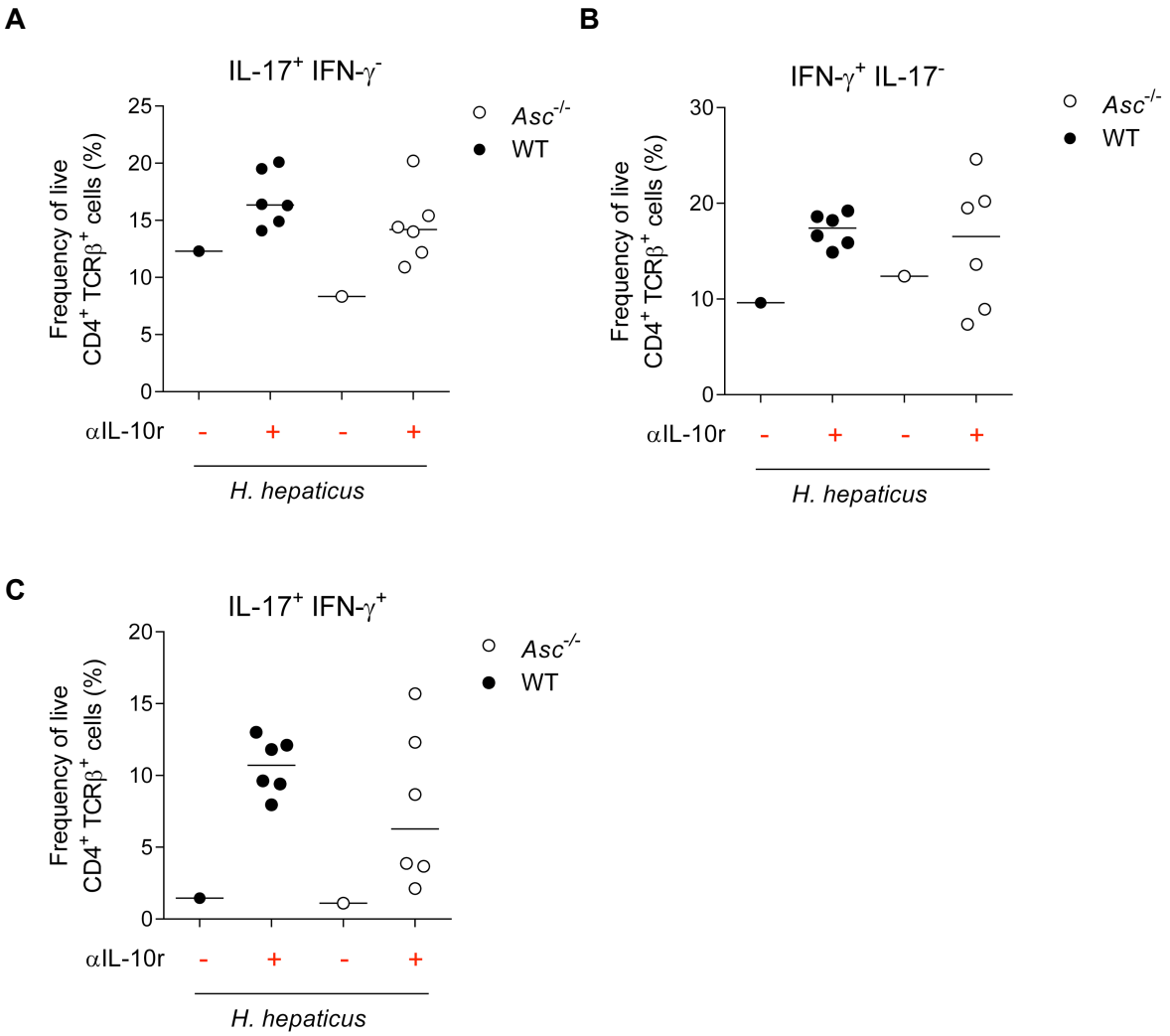


Figure 4.6 Similar Th1/Th17 frequencies in the lamina propria of WT and *Asc*$^{-/-}$ mice after *H. hepaticus* and anti IL-10R treatment.

B6 *Asc*$^{-/-}$ and WT mice were orally infected with *H. hepaticus (H.h)* and then treated with anti IL-10R antibody (1mg/wk i.p.) weekly for 4 weeks. On day 28 post-infection, mice were sacrificed and lymphocytes were isolated from the caecum and distal colon (LPL). Frequencies of IL-17^{+} IFN-γ^{-} (A), IFN-γ^{+} IL-17^{-} (B), and IL-17^{+} IFN-γ^{+} (C), amongst CD4^{+} TCRβ^{+} T cells were then assessed by intracellular staining and FACS analysis. Each symbol represents a single animal, horizontal bars represent medians and data are representative of two independent experiments with similar results (n= 6 mice per infected group).

4.2.3 Asc is required for the induction of intestinal inflammation in an innate model of experimental colitis

The lymphocyte replete model of *Hh* induced intestinal inflammation is useful because it allows one to assess the contribution of multiple cell types to intestinal inflammation. However due to the complexity of the model and the nature of the interactions, it is sometimes difficult to dissect relative roles of different immune pathways. Thus we turned to the innate model of IBD wherein *Hh* colonization of mice on a susceptible 129SvEv *Rag*$^{-/-}$ background leads to chronic intestinal inflammation (Maloy et al., 2003). The utility in this model is that it is entirely innate immune mediated, which could overcome confounding adaptive immune factors that may mask a phenotype in a lymphocyte replete animal. We first backcrossed the B6 *Asc*$^{-/-}$ mice onto a 129SvEv *Rag*$^{-/-}$ background using 'speed congenics' or 'marker assisted congenics' which yielded a fully 129SvEv *Rag*$^{-/-}$ *Asc*$^{-/-}$ strain in 5 generations (Wakeland et al., 1997). Following this, mice were colonized with *Hh* and sacrificed after 3 or 8 weeks. We found that while 129 *Rag*$^{-/-}$ mice exhibited moderate colitis after 3 or 8 weeks of infection with *Hh*, 129 *Rag*$^{-/-}$ *Asc*$^{-/-}$ mice displayed significantly reduced colitis (Figure 4.7A-C). This suggested that signalling via Asc exacerbated innate immune-mediated colitis driven by *Hh*. By contrast, the role of Asc was subtler in the caecum, although 129 *Rag*$^{-/-}$ *Asc*$^{-/-}$ mice showed a trend towards reduced typhlitis relative to 129 *Rag*$^{-/-}$ mice at both 3 and 8 weeks post-infection with *Hh* (Figure 4.7C). Asc signalling was also required for the induction of systemic inflammation as 129 *Rag*$^{-/-}$ *Asc*$^{-/-}$ mice displayed significantly reduced splenomegaly and had lower splenocyte numbers compared to 129 *Rag*$^{-/-}$ mice following infection with *Hh* (Figure 4.8). Thus, signalling via Asc contributes to the induction of innate immune mediated intestinal and systemic inflammation.

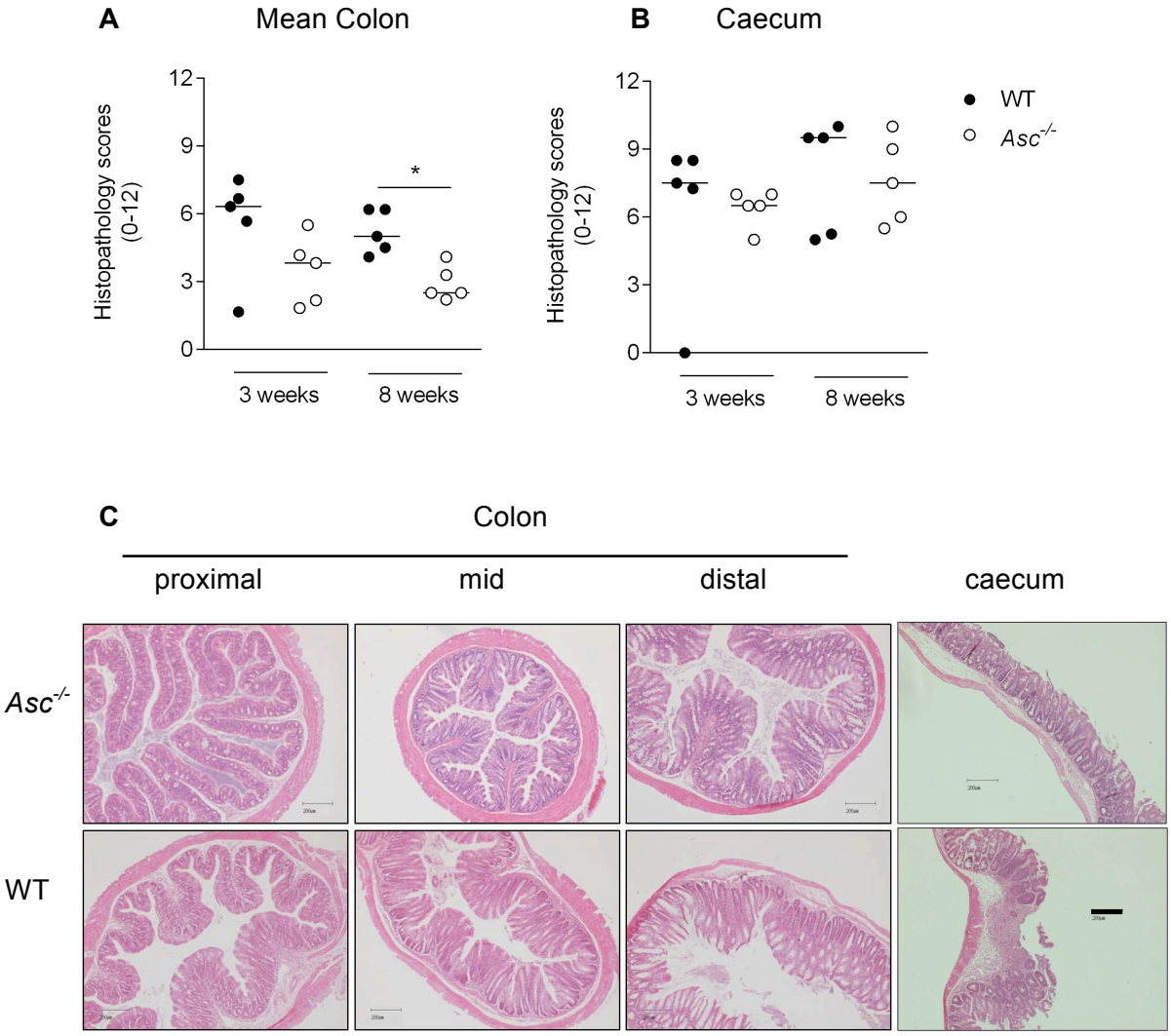


Figure 4.7 Innate immune mediated intestinal inflammation is partially dependent on Asc

129 *Rag*$^{-/-}$ mice (WT) and 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ (*Asc*$^{-/-}$) mice were infected orally with *H. hepaticus* and sacrificed after either 3 or 8 weeks post infection. They were then assessed for colonic (A) and caecal inflammation (B). Representative histology sections from the colons and caeca of 8-week infected mice (scale bar= 200µm) (C). Each symbol represents a single animal and data shown is representative of two independent experiments (*n*=5 per group). Horizontal bars represent the median and statistical significance was determined using the Mann Whitney test. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

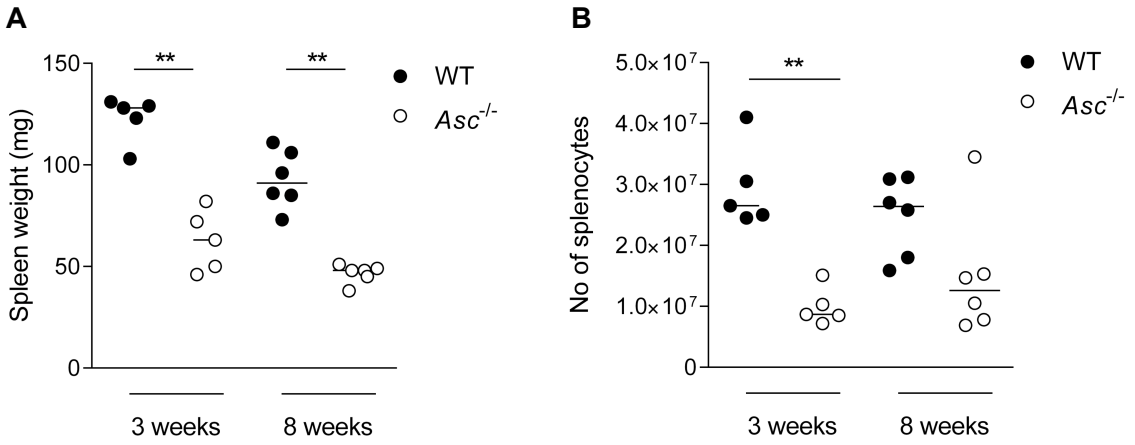


Figure 4.8 Systemic inflammation driven by *H. hepaticus* in 129SvEv *Rag*$^{-/-}$ mice is Asc dependent.

129 *Rag*$^{-/-}$ mice (WT) and 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ (*Asc*$^{-/-}$) mice were infected orally with *H. hepaticus* and then sacrificed after either 3 or 8 weeks post infection. Following infection, spleens from these mice were isolated, weighed (A) and then homogenized to assess cell numbers (B). Each symbol indicates a single animal and data shown is representative of two independent experiments (n=5-6 per group). Horizontal bars represent the median and statistical significance was determined using the Mann Whitney test. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

We next wished to determine how Asc signalling promoted colitis in this model of infection. It is well known that Myd88 signalling and IL-23 are required for the onset of *Hh* driven intestinal inflammation (Asquith et al., 2010; Hue et al., 2006). A novel IL-23-responsive subset of innate leukocytes, known as innate lymphoid cells (ILCs) was implicated in mediating intestinal inflammation in this model (Buonocore et al., 2010). Furthermore, IL-1β was recently shown to promote intestinal inflammation in this model (Coccia et al., 2012). To identify which factors downstream of Asc contributed to intestinal inflammation in this model, we first measured the levels of some of the cytokines that have previously been implicated, by culturing isolated LPLs from *Hh*-infected mice. We found that LPLs from *Hh*-infected 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice produced lower levels of IL-1β, and displayed a trend towards lower IL-23 levels, than those isolated from129 *Rag*$^{-/-}$ mice, though these differences were not statistically significant (Figure 4.9). Somewhat counter intuitively, despite displaying lower levels of intestinal inflammation, LPL from *Hh*-infected 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice produced higher levels of IFN-γ (Figure 4.9). Previously, work in our lab showed that IL-1β induced the activation and accumulation of a pathogenic subset of ILCs in the gut after *Hh* infection of 129 *Rag*$^{-/-}$ mice (Coccia et al., 2012). Thus we next wished to ascertain whether inflammasome activation was required for ILC accumulation. ILCs were identified as CD45.2^{+} Lin^{-} Thy1^{+} NKp46^{-} IL-7Rα^{+} (Spits et al., 2013)(Figure 4.10A). Fitting with reduced intestinal inflammation, we found that *Hh*-infected 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice harboured lower numbers of lamina propria leukocytes (LPLs) than *Hh*-infected 129 *Rag*$^{-/-}$ mice (Figure 4.10B). However, *Hh*-infected 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice had equivalent frequencies of ILCs in their large intestine as *Hh*-infected 129 *Rag*$^{-/-}$ mice, but showed a trend towards lower total numbers of ILCs (Figure 4.10C,D). Previously, IL-1β had been shown to induce IL-17A production from ILCs thus we investigated the frequencies of IL-17A^{+} ILCs after infection (Coccia et al., 2012). However, we found that 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice harboured equivalent numbers and frequencies of IL-17A^{+} ILCs compared to 129 *Rag*$^{-/-}$ mice after infection with *Hh* (Figure 4.11). IL-1β has been shown to promote the accumulation of neutrophils in various models of infection and sterile inflammation (Miller et al., 2007; Patton et al., 1995). Furthermore Coccia *et al* showed that blockade of IL-1β significantly reduced neutrophil infiltration in the colons of *Hh*-infected 129 *Rag*$^{-/-}$ mice (Coccia et al., 2012). Consistent with this we found significantly lower frequencies and fewer total numbers of

neutrophils ($CD11b^{+}Gr1^{hi}$) in the LPL of 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice compared to 129 *Rag*$^{-/-}$ mice after *Hh* infection (Figure 4.12A and B). Thus signalling via Asc contributes to the induction of intestinal inflammation by promoting neutrophilia. Finally, although 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice displayed milder intestinal inflammation, they displayed a marked trend towards higher levels of *Hh* compared to 129 *Rag*$^{-/-}$ mice (Figure 4.13).

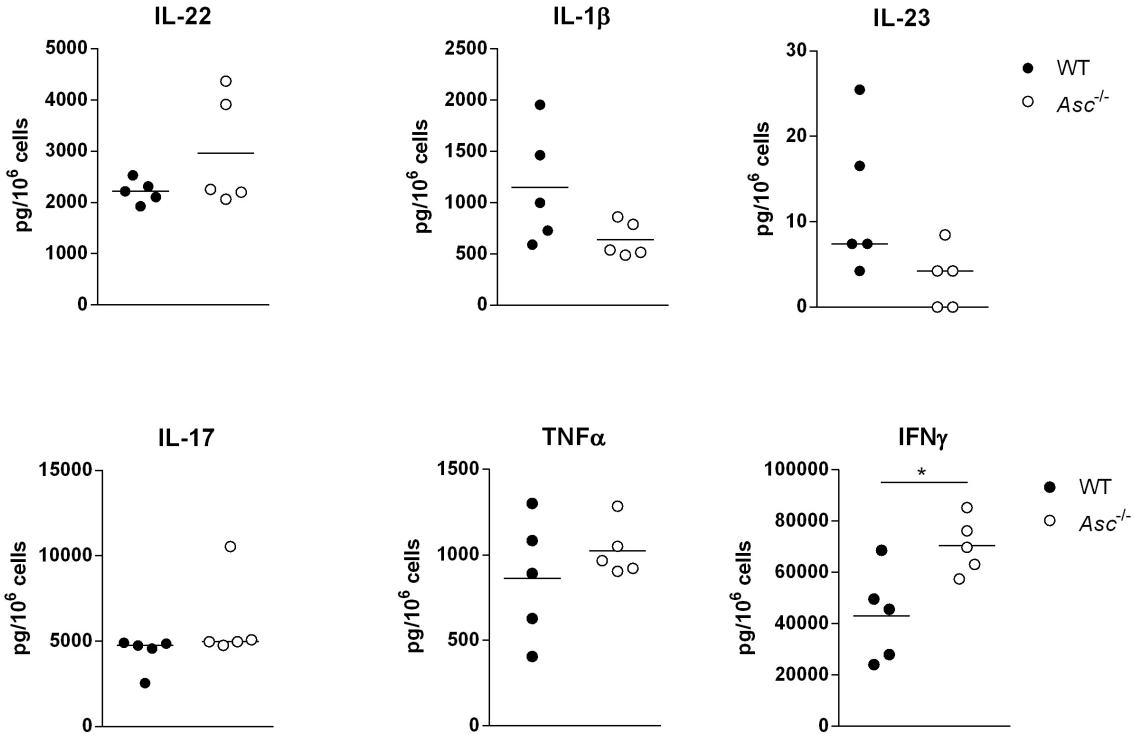


Figure 4.9 Cytokine production from lamina propria leukocytes after infection with *H. hepaticus*.

129 *Rag*$^{-/-}$ mice (WT) and 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ (*Asc*$^{-/-}$) mice were infected orally with *H. hepaticus* and then sacrificed after 3 weeks post infection. Lamina propria leukocytes (LPL) were isolated from the caecum and colons and plated out at a density of 2.5-5x10^5 cells/well and cultured overnight. Culture supernatants from LPLs were then assessed for cytokines (IL-22, IL-1β, IL-23, IL-17, TNFα and IFN-γ) using a cytometric bead assay. Cytokine levels were normalized to LPL cell numbers. Each symbol represents a single animal (n=5 mice per group) from a single pilot experiment. Horizontal bars represent the median. Statistical significance was determined using the Mann Whitney test. (* $p < 0.05$).

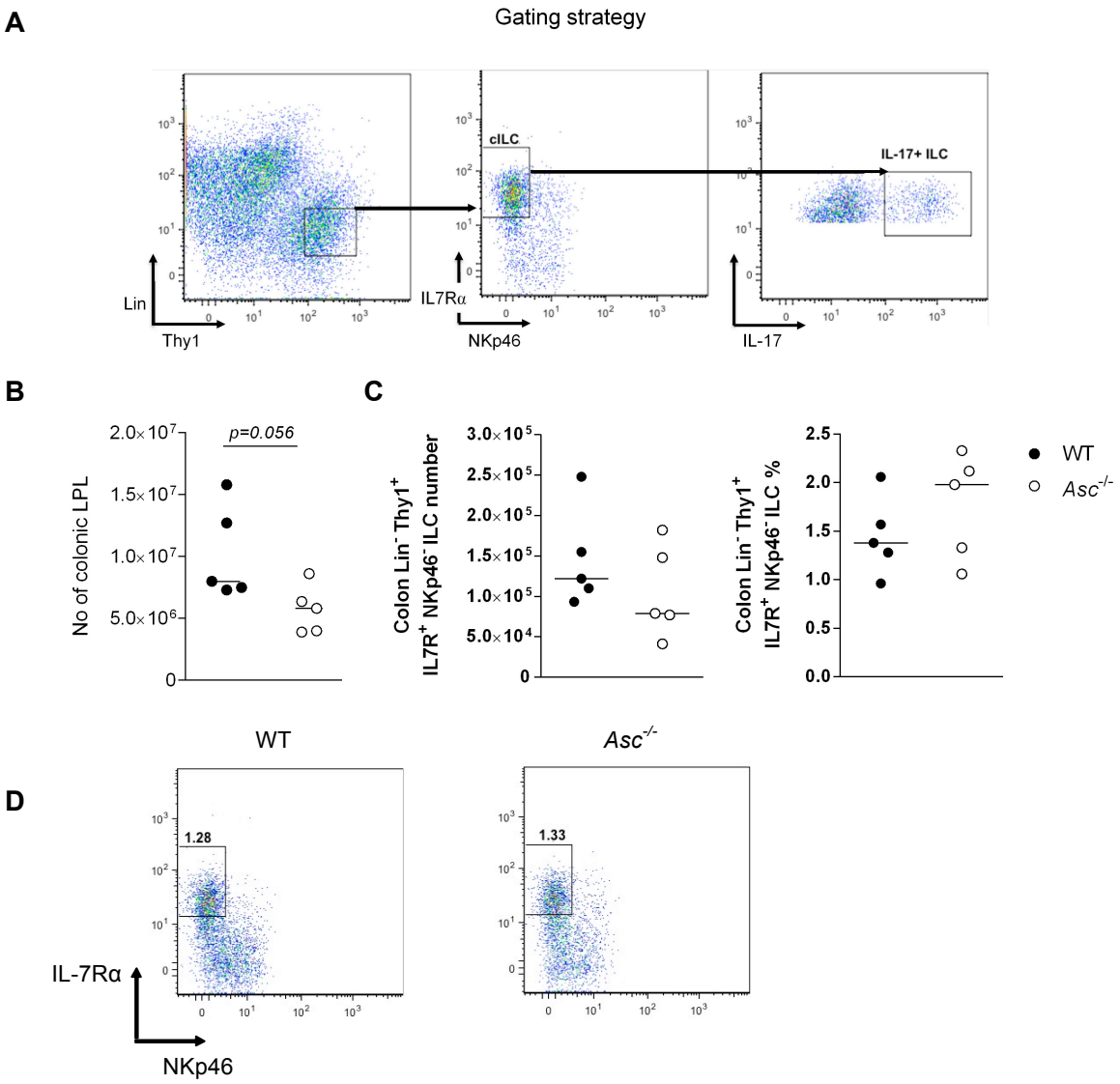


Figure 4.10 Comparable innate lymphoid cell (ILCs) accumulation in the lamina propria of *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Asc*$^{-/-}$mice after infection with *H. hepaticus*.

129 *Rag*$^{-/-}$ mice (WT) and 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ (*Asc*$^{-/-}$) mice were infected orally with *H. hepaticus* and then sacrificed after 3 weeks post infection. Lamina propria leukocytes (LPL) were isolated from the caeca and colons of infected mice and then analysed by FACS. ILCs were identified as CD45.2^{+} Lin^{-} Thy1^{+} NKp46^{-} IL-7Rα^{+} (A). Total LPL cell numbers (B). Number and frequency of ILCs (C). Representative staining of ILCs in the LPL gated on live CD45^{+} lymphocytes (D). Each symbol indicates a single animal (n=5 mice per group) from a single pilot experiment. Horizontal bars represent medians. Statistical significance was determined using the Mann Whitney test.

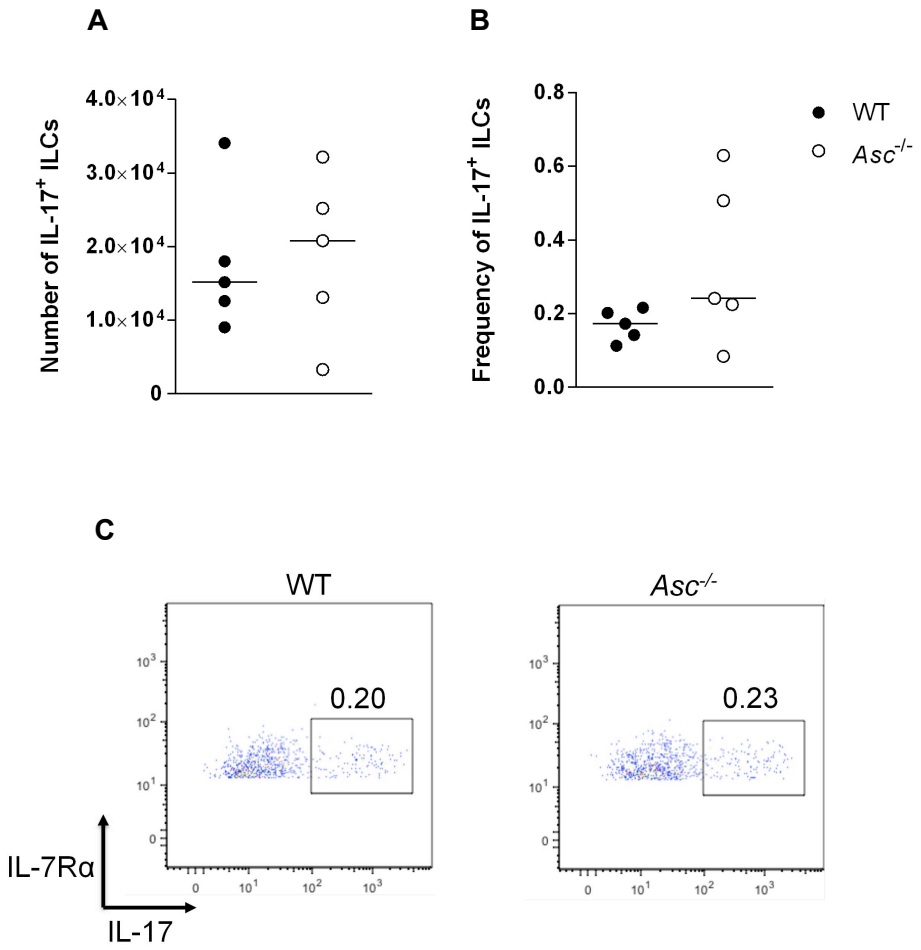


Figure 4.11 Asc is dispensable for IL-17 production by ILCs during *H. hepaticus* infection.

129 *Rag*$^{-/-}$ mice (WT) and 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ (*Asc*$^{-/-}$) mice were orally infected with *H. hepaticus* and then sacrificed after 3 weeks post infection. Lamina propria leukocytes (LPL) were isolated from the caeca and colons of infected mice and restimulated with PMA and ionomycin in the presence of brefeldin for 3h. Numbers (A) and frequencies (B) of IL-17A expressing ILCs were determined by intracellular staining and FACS analysis. Representative FACS analysis is depicted in (C). ILCs were identified as CD45.2^{+} Lin^{-} Thy1^{+} NKp46^{-} IL-7Rα^{+}. Each symbol indicates a single animal (*n*=5 mice per group) and data are derived from a single pilot experiment. Horizontal bars represent medians.

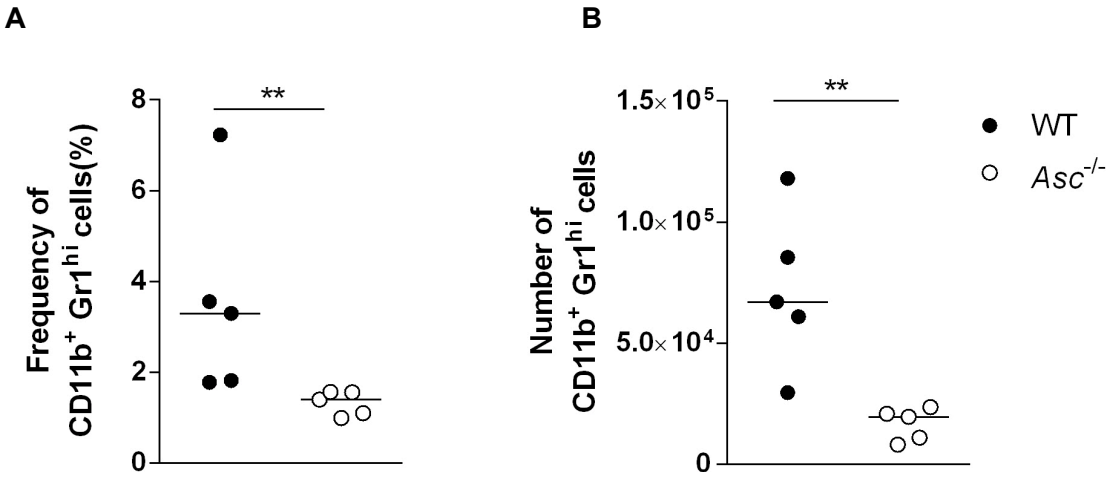


Figure 4.12 Asc signalling is required for the accumulation of neutrophils in the colon during *H. hepaticus* infection.

129 *Rag*$^{-/-}$ mice (WT) and 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ (*Asc*$^{-/-}$) mice were orally infected with *H. hepaticus* and then sacrificed after 3 weeks post infection. Lamina propria leukocytes (LPL) were isolated from the caeca and colons of infected mice. Frequencies (A) and total numbers (B) of LPL neutrophils (CD11b^{+}Gr1^{hi}) were enumerated by FACS. Each symbol represents an individual mouse (*n*= 5 mice per group) from a single pilot experiment. Horizontal lines represent medians. Statistical significance was determined using the Mann Whitney test (* $p < 0.05$; ** $p < 0.01$).

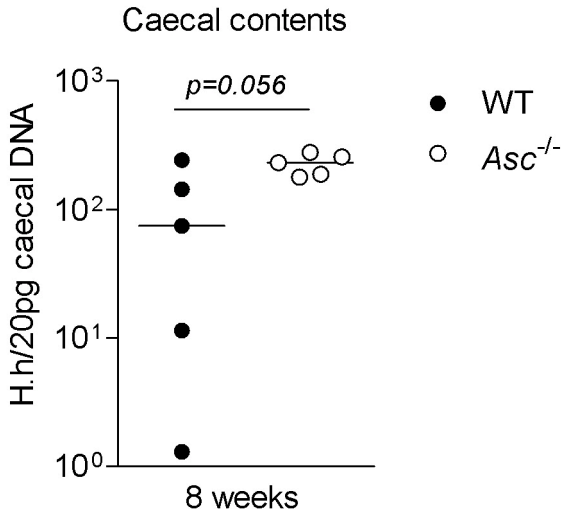


Figure 4.13 *H. hepaticus* colonization levels in 129 SvEv *Rag*$^{-/-}$ and 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice.

129 *Rag*$^{-/-}$*Asc*$^{-/-}$ (*Asc*$^{-/-}$) and 129 *Rag*$^{-/-}$ (WT) mice were orally infected with *H. hepaticus (H.h)* and euthanized 8 weeks later. Caecal contents were collected and the DNA was extracted. *Hh* DNA was quantitated via real time PCR using primers directed against the *cdtB* gene and is shown as pg per 20pg of total caecal DNA. Each symbol represents a single animal (*n*= 5 mice per group) and data shown is from a single experiment. Horizontal lines depict group medians. Statistical significance was determined by the Mann Whitney test.

4.3 Discussion

While much work has focussed on the role of the inflammasome in the context of acute intestinal inflammation (Allen et al., 2010; Bauer et al., 2010; Hirota et al., 2010; Liu et al., 2012; Zaki et al., 2010a; Zaki et al., 2010b), there have been few studies on its function in chronic intestinal inflammation. To study the function of inflammasome activation during chronic intestinal inflammation, we utilized two IBD models triggered by the microaerophilic gram-negative bacterium, *Hh*. This bacterium is often thought of as a commensal in WT hosts. However, rather than being ignored by the immune system, Hh is actively tolerated in an IL-10-dependent manner (Kullberg et al., 2002). Indeed the colonization of WT mice with Hh induces the activation of both $Foxp3^+$ Treg cells and $Foxp3^-$ Tr1-like cells that can prevent intestinal inflammation in an IL-10-dependent manner (Michael Barnes, DPhil thesis and (Kullberg et al., 2002; Kullberg et al., 1998)).

4.3.1 *H. hepaticus* and anti IL-10R antibody mediated intestinal inflammation

In the lymphocyte replete model of intestinal inflammation, tolerance towards *Hh* is broken through the blockade of IL-10R signalling. In this context, IL-23 mediates intestinal inflammation by promoting the emergence of an IFN-γ^+ IL-$17A^+$ population of T cells and by restraining Treg activity (Ahern et al., 2010; Izcue et al., 2008; Kullberg et al., 2006). From an innate perspective, work from our lab has demonstrated that MyD88 signalling is necessary for the induction of Hh driven colitis (Asquith et al., 2010). Upstream of MyD88 however, Tlr2, Tlr4 and Tlr5 are dispensable for the induction of inflammation (Mark Asquith DPhil thesis). This led us to speculate that other receptors utilizing MyD88 might be involved in the induction of Hh driven intestinal inflammation. The IL-1 receptor (IL-1R1) and IL-18 receptor (IL-18R1) both utilize MyD88 in their downstream signalling pathways (Adachi et al., 1998; Wesche et al., 1997). Thus we hypothesized that signalling via these receptors might mediate Hh driven intestinal inflammation. Additionally since IL-1β has been shown to promote Th17 differentiation (Chung et al., 2009; Shaw et al., 2012), and given the abundance of this cell type in this model of disease, we hypothesized that inflammasome-derived IL-1β might play a role.

Our results showed that signalling via Asc was dispensable for the induction of typhlitis and systemic inflammation by Hh infection plus anti IL-10R blockade (Hh+α-IL-10R). However, we observed a marked trend towards reduced colitis in *Asc*$^{-/-}$ mice compared to WT mice after Hh+α-IL-10R treatment. This was also associated with a trend towards reduced total LPL numbers and frequencies of pathogenic IFN-γ^{+} IL-17A^{+} cells in the colons of *Asc*$^{-/-}$ mice. Our data are consistent with previous data showing that *Il1r1*$^{-/-}$ mice displayed a mild attenuation of colitis but not typhlitis or systemic inflammation in this model (Margherita Coccia, DPhil thesis). Furthermore, work from our lab has also recently shown that *Il18r1*$^{-/-}$ mice develop comparable typhlocolitis to WT mice in the Hh+α-IL-10R IBD model (Oliver Harrison, personal communication), suggesting that IL-18 does not play any role. The likely explanation for our findings is either redundant sources of IL-1β and IL-18 in the absence of Asc, or in light of the phenotypes in *Il1r1*$^{-/-}$ and *Il18r*$^{-/-}$ mice, a lack of dependence on these two cytokines. Thus while IL-1β has been implicated in the differentiation of Th17 subsets at steady state (Shaw et al., 2012), and in some models of EAE and colitis (Chung et al., 2009; Coccia et al., 2012), it likely plays a partially redundant role in this disease setting. We used mice lacking Asc as a surrogate for inflammasome activation, but we cannot rule out the possibility that other NLRs that activate caspase-1 independently of Asc have a role.

4.3.2 *H. hepaticus* driven innate intestinal inflammation

Hh colonization in 129SvEv *Rag*$^{-/-}$ mice results in chronic intestinal inflammation that is primarily driven by a subset of innate lymphoid cells (ILCs) that express IL-17, IFN-γ and IL-22 (Buonocore et al., 2010). IL-23 is required for inflammation in this model as blockade of IL-23p19 leads to a complete abrogation of innate intestinal inflammation induced by Hh (Hue et al., 2006). In addition, MyD88 signalling in haematopoietic cells is required for inflammation in this setting (Asquith et al., 2010). We were prompted to investigate the role of inflammasome activation in this model as previous work from our lab had implicated IL-1β in promoting intestinal inflammation (Coccia et al., 2012). Consistent with this, we found that Hh infection of BMDMs triggered Asc-dependent caspase-1 activation and IL-1β release *in vitro*. Following this up *in vivo*, we discovered that both colitis and systemic inflammation were significantly reduced in Hh-infected 129 *Rag*$^{-/-}$

Asc$^{-/-}$ mice compared to 129 *Rag*$^{-/-}$ mice. This correlated with reduced IL-1β secretion from 129 *Rag*$^{-/-}$ *Asc*$^{-/-}$ LPLs, however we also observed a trend towards decreased IL-23 production. IL-1β has previously been shown to promote IL-17A production by ILCs by inducing the expression of IL-23 receptor directly on ILCs (Coccia et al., 2012). IL-23 signalling in turn drives IFN-γ and IL-17A production by ILCs (Buonocore et al., 2010). To see if Asc acted upstream of this pathway, we determined the levels of ILCs and their capacity to produce IL-17A after infection. However, we found no difference in the frequency of ILCs or IL-17A^{+} ILCs in the large intestines of 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ and 129 *Rag*$^{-/-}$ mice infected with Hh. Nevertheless, we observed a trend towards lower total numbers of ILCs in 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice after Hh infection, similar to that observed upon blockade of IL-1β (Coccia et al., 2012). The difference between 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice and the IL-1β blockade study was in cytokine production by ILCs, wherein we observed no reduction in this function in the absence of Asc, whereas IL-1β blockade led to a significant reduction in IL-17A and IFN-γ production by ILCs (Coccia et al., 2012). The discrepancy in the two results may stem from residual IL-1β in 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice, which we observe in supernatants from cultured LPLs. This also suggests that Asc/caspase-1 independent mechanisms of IL-1β cleavage may be operational in this model, and recent studies have provided evidence for such pathways (Bossaller et al., 2012; Gringhuis et al., 2012; Maelfait et al., 2008; van de Veerdonk et al., 2011). Apart from the effects of IL-1β on ILCs, Asc signalling is also upstream of IL-18 cleavage and activation and thus could have an effect via this cytokine. While ILCs express high levels of *Il18r1*, blockade of IL-18 using an anti-IL-18 mAb did not affect intestinal inflammation in Hh-infected 129 *Rag*$^{-/-}$ mice (Oliver Harrison, personal communication). Thus a lack of IL-18 is unlikely to underlie the protective phenotype observed in 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice.

Apart from its effects on ILCs, IL-1β has a well established function in neutrophil recruitment (Miller et al., 2007; Patton et al., 1995). Thus, blocking IL-1β in the 129 *Rag*$^{-/-}$ Hh model results in reduced neutrophil influx (Coccia et al., 2012). In line with this finding, we found significantly fewer neutrophils in the intestines and spleens of 129 *Rag*$^{-/-}$*Asc*$^{-/-}$ mice after infection with Hh. Neutrophils are thought to contribute to immunopathology by producing nitric oxide, indeed blocking this pathway slightly attenuates colitis in Hh infected 129 *Rag*$^{-/-}$ mice (Erdman et al., 2009). Thus, Asc-signalling may promote colitis through the recruitment of neutrophils. While IL-

1β production downstream of Asc signalling may potentiate these effects on neutrophils, Asc has been described to alter MAPK phosphorylation and the production of certain chemokines independent of caspase-1 and IL-1β (Hasegawa et al., 2005; Taxman et al., 2011).

Work presented in this chapter raises several questions that merit further investigation.

First, what is the cellular source of IL-1β in this model? While some work has identified intestinal macrophages as a potential source, many of the phenotypic markers used to identify macrophages e.g. CD11b and F4/80 overlap with other cell types such as eosinophils (Franchi, 2012; Shaw et al., 2012).

Second, in which cell type is Asc expression pathogenic i.e. in which cell type is signalling via Asc necessary for Hh driven intestinal inflammation? To answer this we plan to use irradiation bone-marrow chimeras and to employ recently described transgenic mice that have 'floxed' *Asc* alleles, enabling cell-type specific deletion by crossing with different Cre-expressor strains (Drexler et al., 2012).

Third, which PRR mediates the detection of Hh, upstream of Asc? Recent studies in our lab have found that 129 *Rag*$^{-/-}$*Nlrc4*$^{-/-}$ mice develop comparable Hh-triggered innate immune IBD as 129 *Rag*$^{-/-}$ mice (Sofia Nordlander, personal communication), indicating that Nlrc4 is unlikely to be involved. Preliminary work *in vitro* showed that Nlrp3 deficient BMDMs were unable to process IL-1β after Hh infection, which suggests that Nlrp3 might be upstream of Asc in this model (data not shown). This however needs to be formally assessed *in vivo* either using 129 *Rag*$^{-/-}$*Nlrp3*$^{-/-}$ mice or through Nlrp3 inhibitors such as glyburide (Lamkanfi et al., 2009).

Fourth, how does Hh trigger activation of caspase-1? That is, what are the bacterial determinants or virulence factors that mediate caspase-1 activation? Our preliminary characterization of the virulence factors involved has revealed a partial role for cytolethal distending factor (CDT) in caspase-1 activation *in vitro* (data not shown). CDT is one of few virulence factors described for Hh. It is a bacterial toxin widely expressed by enteric pathogens such as *Campylobacter jejuni*, *Salmonella typhi* and *Shigella dysenteriae* (reviewed in(Oswald et al., 2005)). CDT was the first toxin characterized that could block cell cycle at the G_2/M transition (Whitehouse et al., 1998) and it acts similar to a deoxyribonuclease that induces chromatin damage and consequently cell death (Lara-Tejero and Galan, 2000). Interestingly, CDT deficient Hh mutants drive significantly reduced

levels of typhlocolitis in *Il10*$^{-/-}$ mice *in vivo* (Young et al., 2004). It would be interesting to know how this relates to CDT-deficient Hh's ability to induce IL-1β *in vivo*.

Collectively these data demonstrate a pathogenic role for Asc signalling in a chronic model of intestinal inflammation. This is in contrast to multiple studies that have demonstrated protective functions of caspase-1, Asc and Nlrp3 in acute models of intestinal inflammation using DSS and *C. rodentium* (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Liu et al., 2012; Zaki et al., 2010a). The reasons for these contrasting roles may be due to the different functions played by inflammasomes in haematopoietic versus non-haematopoietic cells. Recent work has highlighted that IECs are major sources of IL-18 in the intestine but express little IL-1β, while mononuclear phagocytes are the main producers of IL-1β in the intestine (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Franchi, 2012; Shaw et al., 2012; Zaki et al., 2010a). Models of acute intestinal inflammation operate primarily via damage to the intestinal epithelium e.g. DSS. By contrast, chronic models of intestinal inflammation such as *Hh* driven inflammation require haematopoietic Myd88 signalling and IL-23 production and damage to the epithelium is likely a secondary event (Asquith et al., 2010; Hue et al., 2006). In a reductionist view, haematopoietic cell inflammasome activation leads primarily to IL-1β production while non-haematopoietic cell inflammasome activation drives IL-18 production. Excessive IL-1β production by APC can drive pathogenic T cell responses while IEC-produced IL-18 may mainly act by repairing the epithelium. We hypothesize then that *Hh* driven intestinal inflammation may require Asc expression in intestinal mononuclear phagocytes. If this is true then it raises the question of how non-invasive, crypt-dwelling bacteria like *Hh* trigger inflammasome activation in intestinal mononuclear phagocytes in the underlying lamina propria? This could be directly via virulence factors such as CDT, as earlier discussed, or indirectly by the sensing of damaged IECs by resident macrophages.

The distinct roles of Asc signalling in acute and chronic intestinal inflammation may also be due to the fact that each model probably tests a distinct facet of intestinal physiology and thus what we readout as 'intestinal inflammation' may not fully represent or capture the nuances of human IBD. Indeed human IBD is heterogeneous and may involve multiple aspects that operate discretely in each of the models. This could also explain why a single therapeutic such as the corticosteroid prednisone is not effective in treating all CD patients (Michetti et al., 2007). The reasons

responsible for the opposing role of Asc signalling in acute versus chronic models of intestinal inflammation will be discussed in further detail in the general discussion (Chapter 6).

Chapter 5. The role of Asc signalling in T cell dependent colitis

5.1 Introduction

Activation of the inflammasome principally results in the cleavage and secretion of IL-1β and IL-18 (Schroder and Tschopp, 2010). IBD is associated with the overproduction of IL-1β and IL-18 from gut explants suggesting that these cytokines play a pathogenic role when dysregulated (Casini-Raggi et al., 1995; Pizarro et al., 1999). While the previous chapter highlighted a potential pathogenic function for inflammasome-derived IL-1β inducing ILC activation in the gut, IL-1β and IL-18 also have pleiotropic functions on T- helper cells that may potentiate intestinal inflammation. A balance between T-helper subsets is a pre-requisite for intestinal homeostasis. Dysregulated effector $CD4^+$ T cell responses against the microbiota, particularly Th1 and Th17 responses, are believed to play key roles in driving Crohn's disease (Shale et al., 2013). In line with this paradigm, IL-17A secreting Th17 cells and IFN-γ producing Th1 cells are abundant in patients with Crohn's disease (Kanai et al., 2012). These effector subsets are kept in check by Treg cells and humans with mutations in FOXP3 develop spontaneous immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a syndrome that is marked by intestinal inflammation (Bennett et al., 2001). These studies are reinforced by experimental colitis models in mice that show that blocking pro-inflammatory cytokines such as IFN-γ and IL-17A or bolstering anti-inflammatory cytokines, such as IL-10, are therapeutically beneficial (Powrie et al., 1994; Yen et al., 2006). Thus understanding how innate factors influence T cell polarization and enable adaptation to the intestinal microenvironment is important.

5.1.1 The effects of IL-1β on $CD4^+$ T cells

Work in other models and systems have previously implicated inflammasome-derived cytokines in influencing T-helper differentiation. In particular IL-1β has been shown in numerous studies to promote Th17 cell differentiation. Th17 cells have garnered much interest of late mainly because of their abundance in the context of multiple autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, as well as other human inflammatory disorders such as CD (Korn et al., 2009).

Th17 differentiation requires a combination of signals including TCR engagement, TGFβ1, IL-1β and a STAT-3 triggering cytokine such as IL-6 or IL-21 (Korn et al., 2009). These orchestrate a number of transcription factors including IRF4 and BATF that, in conjunction with STAT3 and the lineage-imprinting factor RORγt, establish a transcriptional programme that imprints the Th17 signature (Korn et al., 2009; Veldhoen et al., 2006). IL-1β was first demonstrated to enhance IL-6 + TGFβ1-mediated induction of Th17 cells *in vitro* (Veldhoen et al., 2006). These data were confirmed *in vivo* when it was found that *Il1r1*$^{-/-}$ mice were impaired in the induction of Th17 cells after immunization with the model antigen keyhole limpet hemocyanin (KLH) (Sutton et al., 2006). Furthermore, *Il1r1*$^{-/-}$ mice were resistant to experimental autoimmune encephalomyelitis (EAE), a phenotype that could be reversed upon transfer of autoantigen-specific Th17 cells from WT mice (Sutton et al., 2006). Mechanistically, IL1R1 signalling was linked to Th17 differentiation through the upregulation of the transcription factors IRF4 and RORγt (Chung et al., 2009). Excessive IL1R1 signalling can precipitate spontaneous autoimmune disorders, for instance mice lacking the IL-1R antagonist (IL-1RA) develop spontaneous arthritis that is mediated by excessive IL-17 production (Nakae et al., 2003).

In the gut, the priming of Th17 cells is defective in *Il1r1*$^{-/-}$ mice upon *C. rodentium* infection (Hu et al., 2011). Furthermore, at steady state, the microbiota induces the differentiation of Th17 cells in the lamina propria in an IL-1β-dependent manner (Franchi et al., 2012). Recent work from our lab has also demonstrated a role for IL1R1 signalling on inflammatory effector T cells by showing that naïve T cells lacking IL1R1 had impaired colitogenic potential after transfer into *Rag*$^{-/-}$ recipient mice compared to WT naïve T cells (Coccia et al., 2012). This was partially ascribed to a specific defect in the accumulation of Th17 cells in the colon (Coccia et al., 2012).

5.1.2 The effects of IL-18 on CD4$^+$ T cells

Whereas IL-1β has a well-studied role in Th17 differentiation, IL-18 is recognized primarily for its effects on Th1 cells. Originally identified as interferon-gamma inducing factor (IGIF), IL-18 is known to synergize with IL-12 to drive IFN-γ production from CD4$^+$, CD8$^+$ T cells and NK cells (Nakanishi et al., 2001; Okamura et al., 1995). This activity is regulated through signalling via IL-18R1, which, like IL1R1, harbours a TIR-domain and signals via MyD88 driving AP1 and NFκB

activation (Adachi et al., 1998; Kanakaraj et al., 1999; Matsumoto et al., 1997; Nakanishi et al., 2001). IL-18 and IL-12 control the expression of the IL-12Rβ2 and IL-18R1 respectively (Chang et al., 2000; Yoshimoto et al., 1998) and synergize to drive IFN-γ production. While the role of IL-18 in IFN-γ induction is well appreciated, its function in Th17 responses is less well studied. However, Gutcher *et al* showed that mice lacking *Il18r1* were protected from EAE while those lacking IL-18 were as susceptible as WT mice (Gutcher et al., 2006). IL-18R1 signalling promoted pathology in this model through the induction of IL-17 production from T-helper cells (Gutcher et al., 2006), suggesting that IL-18 independent engagement of the IL-18R1 may promote Th17 skewing. Another study found that inhibiting caspase-1 ameliorated symptoms of EAE, a phenotype that could be partially reversed by administering recombinant IL-18 (Lalor et al., 2011). Interestingly in this study, IL-18 could synergize with IL-23 and drive IL-17 production from $CD4^+$ T cells in a partially IL1R1 dependent manner (Lalor et al., 2011). While these studies suggest a pro-inflammatory function for IL-18 in Th1 and potentially Th17 induction, the role of this cytokine in the gut is less understood.

Early work using the DSS model of chemically driven acute intestinal inflammation pointed to a pathogenic role for IL-18. Indeed treating animals with anti-IL-18 significantly reduced diarrhoea, weight loss and intestinal inflammation in DSS driven colitis (Siegmund et al., 2001a). It was thought that this abrogation in inflammation stemmed from a blockade in IL-18's ability to induce IFN-γ (Siegmund et al., 2001a). Similarly treatment with IL-18 binding protein (IL-18bp), an antagonist of IL-18, abrogated intestinal inflammation and weight loss induced by DSS (Sivakumar et al., 2002). Consistently other studies showed that mice lacking caspase-1 were protected against DSS driven colitis (Siegmund et al., 2001b). These findings aligned with another model of acute intestinal inflammation using the chemical Trinitrobenzenesulfonic acid (TNBS), which showed that administering recombinant human IL-18bp significantly reduced colitis and weight loss (Maerten et al., 2004). Recent work however has overturned this dogma showing that IL-18 signalling is protective in DSS intestinal inflammation (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Elinav et al., 2011; Zaki et al., 2010a). Nonetheless, the cellular targets and pathways through which IL-18 mediates such functions are poorly understood. Furthermore, the precise reasons for the discrepancies between the early and more recent studies remain unknown but may

have to do with differences in the microbiota composition in *Il18*$^{-/-}$ and WT mice that may not be accurately recapitulated using blocking antibodies or IL-18bp (Elinav et al., 2011). Few studies have looked at the role of IL-18 in chronic intestinal inflammation. One study found that adenoviral vector-mediated knockdown of IL-18 led to a slight reduction in colitis after the adoptive transfer of memory T cells into SCID recipients (Wirtz et al., 2002). By contrast, another study observed no effect of IL-18bp treatment on CD4^{+} CD45RB^{hi} reconstituted SCID mice (Maerten et al., 2004). Overall these conflicting studies highlight that the role of IL-18 in intestinal inflammation is still unclear and debated and thus warrants further investigation.

It is clear that inflammasome-derived cytokines have direct effects on CD4^{+} T cell subsets. We wanted to investigate whether these effects were apparent in the intestine and operational during intestinal inflammation. To do so, we utilized the T cell transfer model of intestinal inflammation wherein the transfer of naïve CD4^{+} CD45RB^{hi} CD25^{-} T cells into a lymphocyte deficient *Rag*$^{-/-}$ host results in lymphopenia driven proliferation and the development of systemic and chronic intestinal inflammation (Powrie et al., 1993). Co-transferring CD4^{+} CD45RB^{low} CD25^{+} Tregs can prevent the onset of inflammation (Powrie et al., 1993). The utility of this model is that it allows one to interrogate the intrinsic role of genes in naïve T cells or Tregs. In this chapter we addressed three main questions:

i. Is there a cell-intrinsic role for Asc signals for the development of pathogenic effector CD4^{+} T cells from naïve T cells?
ii. Do inflammasome/Asc-derived cytokines influence the generation of colitogenic effector CD4^{+} T cells?
iii. Do inflammasome/ Asc derived cytokines affect Treg mediated inhibition of colitis?

5.2 Results

5.2.1 Intrinsic Asc signalling is dispensable for effector T cell function

A number of studies have hinted at an inflammasome-independent role for Asc. For example, two independent studies have reported that Asc deficient $CD4^+$ T cells exhibited decreased expansion and/or survival in the periphery compared to WT $CD4^+$ T cells in mixed bone marrow chimeras (Ippagunta et al., 2011; Shaw et al., 2010). Furthermore, Asc deficient splenic T cells displayed reduced proliferation compared to WT T cells upon anti-CD3/CD28 stimulation (Narayan et al., 2011). These studies prompted us to investigate the role of Asc in effector T cell function. To do this we compared the ability of naïve T cells ($CD4^+$ $CD45RB^{hi}$ $CD25^-$) isolated from WT or *Asc*$^{-/-}$ mice to drive intestinal inflammation in lymphopenic *Rag*$^{-/-}$ recipients. We found that Asc was dispensable for the induction of colitis and typhlitis, as *Rag*$^{-/-}$ recipients of *Asc*$^{-/-}$ naïve $CD4^+$ T cells developed comparable levels of disease to *Rag*$^{-/-}$ recipients of WT naïve $CD4^+$ T cells (Figure 5.1). Similarly, Asc deficient naïve $CD4^+$ T cells induced equivalent systemic inflammation compared to WT controls as determined by splenomegaly and MLN cellularity (Figure 5.2). Thus collectively these data show that intrinsic Asc signalling in T cells is dispensable for their pathogenic function *in vivo*.

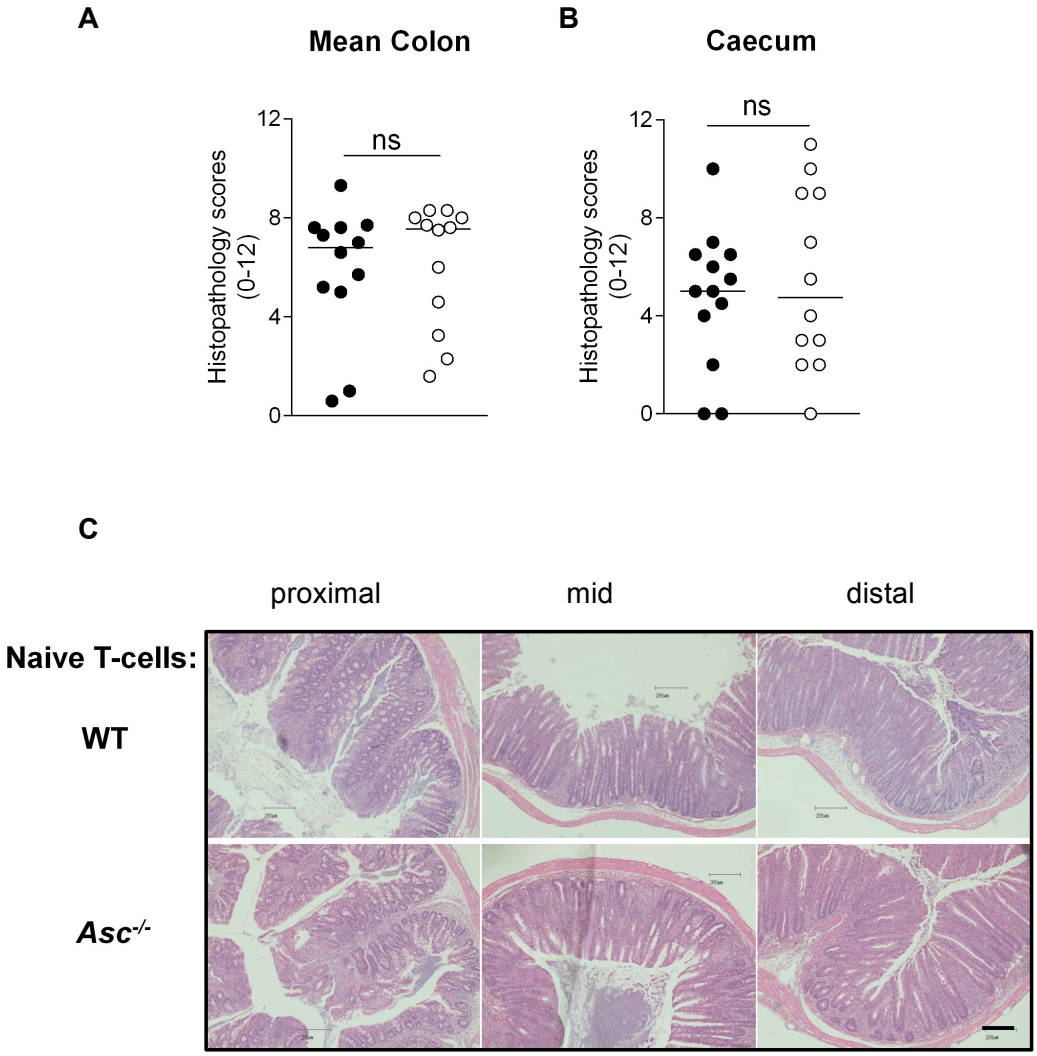


Figure 5.1 Asc expression in naive CD4$^+$ T cells is dispensable for the induction of T cell transfer colitis

Cohorts of B6 *Rag1*$^{-/-}$ mice were reconstituted with 4 x 10^5 of either WT or *Asc*$^{-/-}$ CD4$^+$CD45RB^{hi} T cells by i.p. injection. Mice were sacrificed 5-7 wks post transfer and colitis (A), typhlitis (B) were assessed histologically (n = 12 per group for A and B) Representative photomicrographs representing the proximal, mid and distal colon (scale bar= 200µm) (C) Each symbol represents a single animal and the data represents two pooled independent experiments. Horizontal lines represent group medians. Statistical analysis was conducted using the Mann Whitney test.

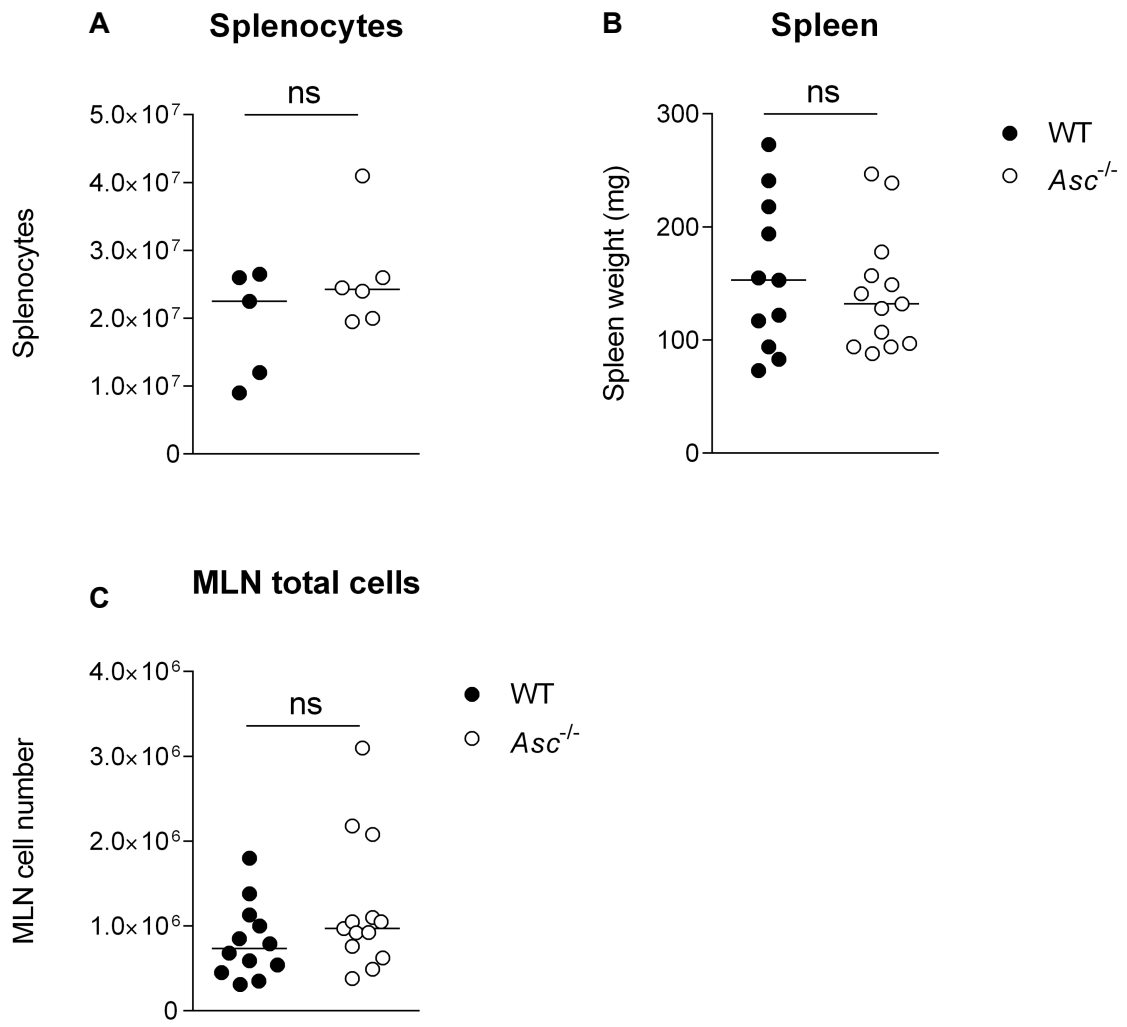


Figure 5.2 Intrinsic Asc signalling is dispensable for effector CD4$^+$ T cell expansion and systemic inflammation during T cell transfer colitis

Cohorts of B6 *Rag1*$^{-/-}$ mice were reconstituted with 4 x 10^5 of either WT or *Asc*$^{-/-}$ CD4$^+$CD45RB^{hi} T cells by i.p. injection. Mice were sacrificed 5-7 wks post transfer after which splenocyte number (A), spleen weights (B) and total MLN cell number (C) were determined. (n = 6 per group for A, and n = 12-13 per group for B and C). Data shown represents two pooled independent experiments (B, C) or a single experiment (A). Each symbol represents a single animal and horizontal lines depict medians. Statistical analysis was conducted using the Mann Whitney test.

5.2.2 Asc signalling on host APC and tissue cells is dispensable for the induction of T cell driven colitis

Next we examined the role of Asc signalling on host APC and tissue cells in the development of pathogenic T cells. Thus, we crossed *Asc*$^{-/-}$ mice to a B6 *Rag1*$^{-/-}$ background to generate *Rag*$^{-/-}$*Asc*$^{-/-}$ mice that we subsequently used as recipients in T cell transfer colitis. We found that adoptive transfer of WT naïve CD4^{+} CD45RB^{hi} T cells into *Rag*$^{-/-}$*Asc*$^{-/-}$ or *Rag*$^{-/-}$ hosts resulted in equivalent levels of colitis and typhlitis, suggesting that Asc signalling was dispensable for the induction of intestinal inflammation (Figure 5.3). The adoptive transfer of naïve T cells into a lymphopenic host also leads to the development of systemic inflammation. However, *Rag*$^{-/-}$*Asc*$^{-/-}$ or *Rag*$^{-/-}$ hosts exhibited equivalent splenomegaly after adoptive transfer of naïve CD4^{+} CD45RB^{hi} T cells (Figure 5.4), indicating that Asc was dispensable for the induction of systemic inflammation. Consistent with comparable levels of intestinal inflammation, we found similar numbers of cells in the colons and in the MLNs in *Rag*$^{-/-}$ and *Rag*$^{-/-}$*Asc*$^{-/-}$ recipients of naïve CD4^{+} CD45RB^{hi} T cells (Figure 5.5). Intestinal inflammation in the T cell transfer model of colitis is thought to be driven by a subset of IFN-γ and IL-17A producing subset of CD4^{+} T cells that is dependent on IL-23 (Ahern et al., 2010). Adoptively transferred naive CD4^{+} T cells differentiated into IL-17^{+} IFN-γ^{-} (Th17), IL-17^{-} IFN-γ^{+} (Th1) and IL-17^{+} IFN-γ^{+} (double producers) at equivalent frequencies in *Rag*$^{-/-}$ *Asc*$^{-/-}$ and *Rag*$^{-/-}$ recipients (Figure 5.6). Collectively, these data show that Asc signalling on host APC or tissue cells is dispensable for the induction of pathogenic effector T cell responses in the gut.

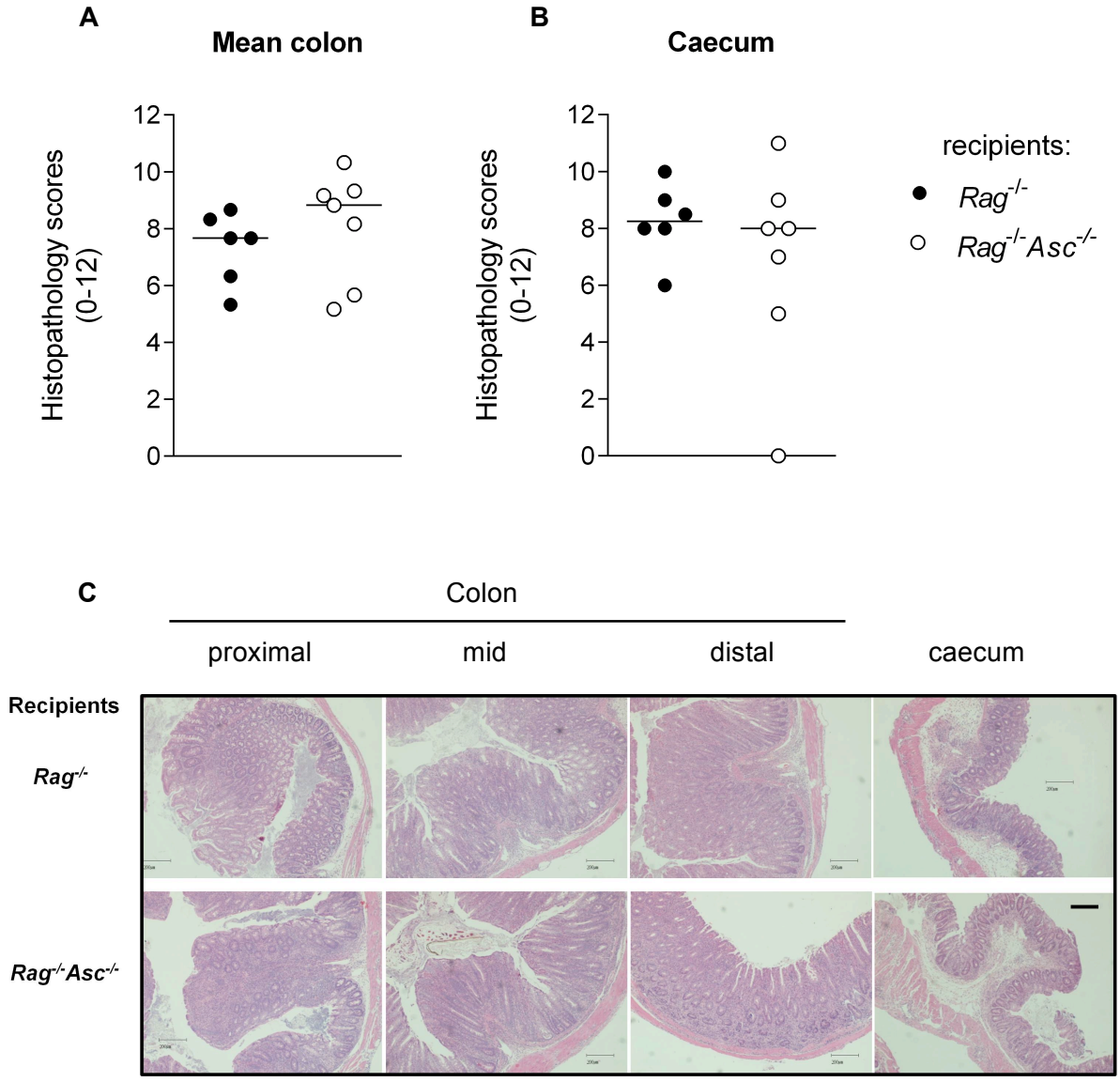


Figure 5.3 Asc expression on host APC and tissue cells is dispensable for the development of T cell driven colitis

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 WT CD4$^+$CD45RB^{hi} T cells by i.p. injection. Mice were sacrificed 5-7 wks post transfer and histopathology scores in the colon (A) and caecum (B) were assessed. Representative photomicrographs are depicted in (C) (scale bar= 200µm). Each symbol represents an individual animal. Data shown is representative of two independent experiments (n = 6-7 mice per group). Horizontal lines depict medians.

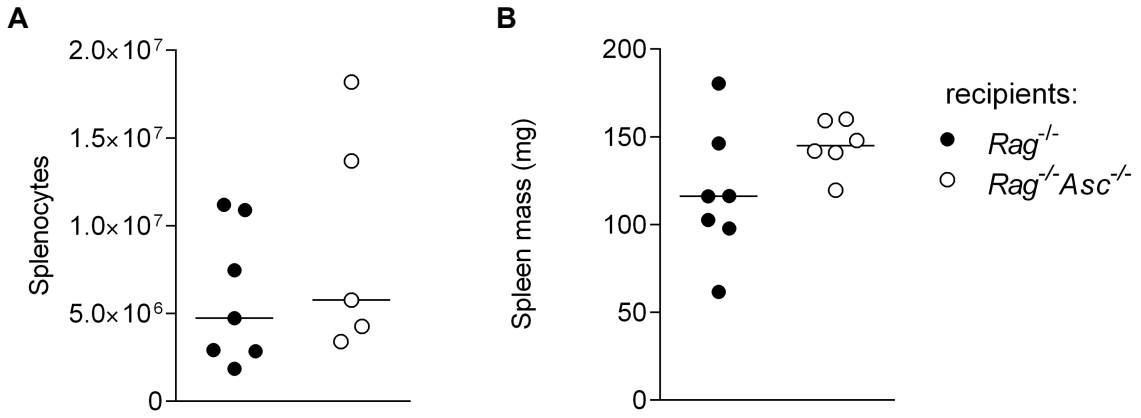


Figure 5.4 Asc expression on host cells is dispensable for the development of systemic inflammation during T cell transfer colitis.

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 WT CD4$^+$CD45RB^{hi} T cells by i.p. injection. Mice were sacrificed 5-7 wks post transfer after which splenocyte number (A) and spleen weights (B) were determined. Each symbol represents an individual animal. Data shown depict one representative of two independent experiments (*n* = 5-7 mice). Horizontal lines represent medians.

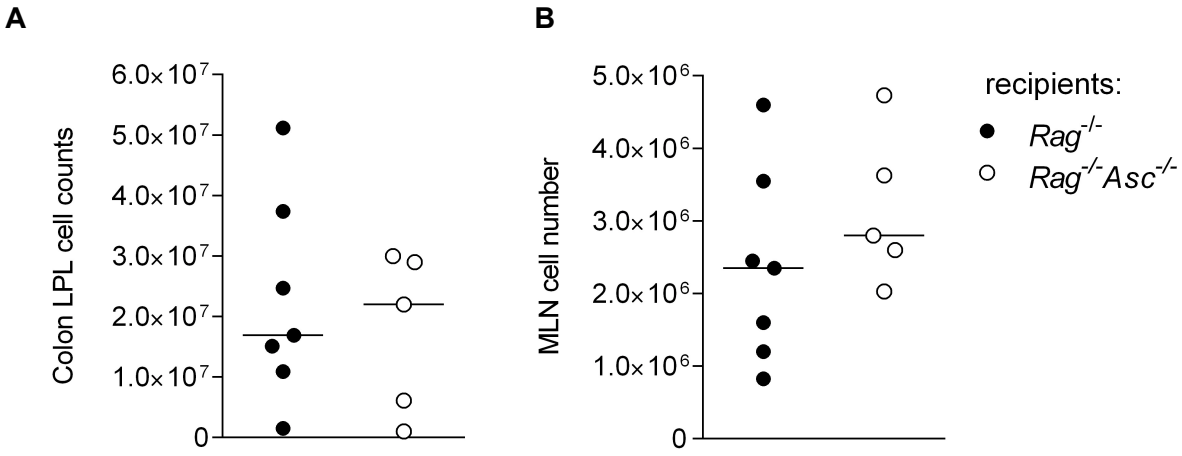


Figure 5.5 Cellularity of the lamina propria and mesenteric lymph nodes is equivalent in *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ hosts after adoptive transfer of naive CD4^{+} T cells.

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 WT CD4^{+}CD45RB^{hi} T cells by i.p. injection. Mice were sacrificed 5-7 wks post transfer after which colonic lamina propria leukocytes (LPL) were isolated and enumerated (A) and mesenteric lymph node cell numbers (MLN) were determined (B). Each symbol represents an individual animal. Data shown depict one representative of two independent experiments (*n* = 5-7 mice). Horizontal lines represent medians.

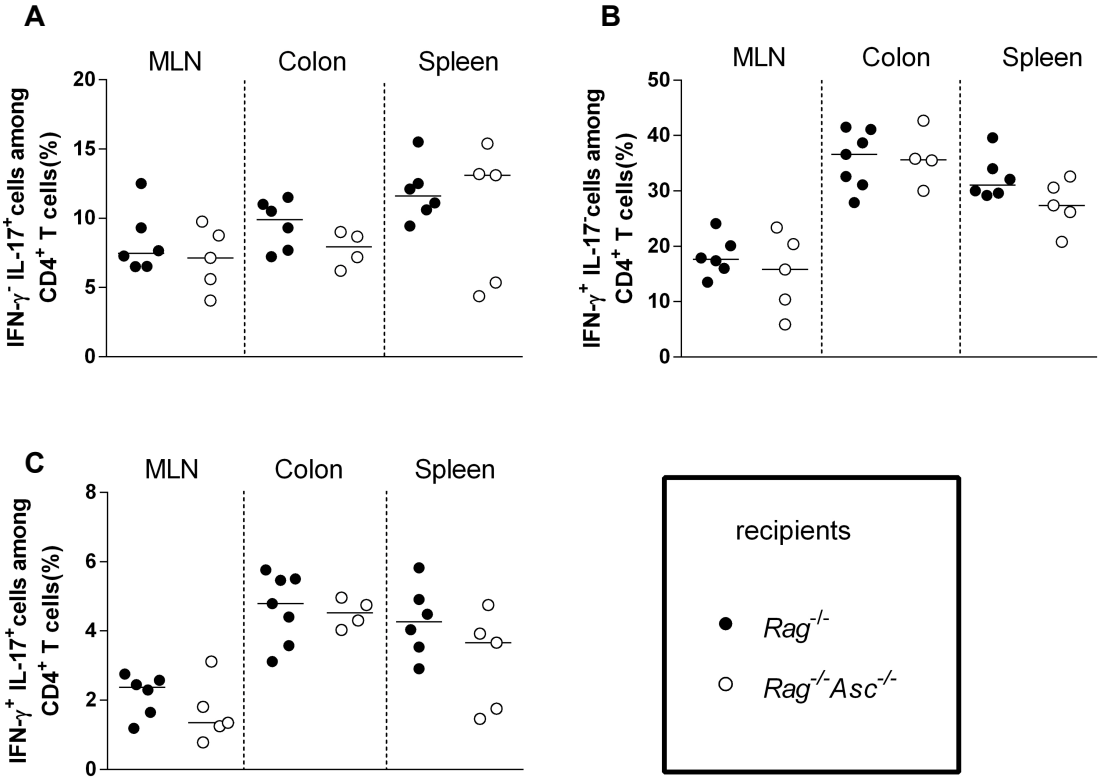


Figure 5.6 Asc-derived host signals are dispensable for the differentiation of naïve T cells into Th1, Th17 and Th1/17 cells in lymphopenic hosts

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 WT CD4$^+$CD45RB^{hi} T cells by i.p. injection. Mice were sacrificed 5-7 wks post transfer after which colonic lamina propria leukocytes (LPL) were isolated. Frequencies of Th17 (IFN-γ^- IL-17$^+$) (A), Th1 (IFN-γ^+ IL-17$^-$) (B) and Th1/17 (IFN-γ^+ IL-17$^+$) (C) amongst CD4$^+$ TCRβ^+ T cells were assessed by FACS. Each symbol represents an individual animal. Data shown depict one representative of two independent experiments (n = 4-7 mice). Horizontal lines represent medians.

5.2.3 Asc signalling on host APC or tissue cells is not required for Treg function

Co-transferring $CD4^+$ $CD25^+$ $CD45RB^{low}$ Tregs together with naïve $CD45RB^{hi}$ T cells effectively prevents the development of colitis and systemic inflammation (Powrie et al., 1993). Recent unpublished work from our lab has highlighted a crucial intrinsic role for IL-18R1 signalling in the suppressive function of Tregs in this co-transfer model (Oliver Harrison, personal communication). This strongly suggested that IL-18 might regulate the function of Tregs in the intestine. As we previously observed that levels of IL-18 were abrogated in colon explants from *Asc*$^{-/-}$ mice at steady state, we speculated that Asc-derived IL-18 might be required for Treg suppression. We tested this idea by co-transferring $CD25^+$ $CD45RB^{low}$ Tregs and naïve $CD45RB^{hi}$ T cells into either *Rag*$^{-/-}$ or *Rag*$^{-/-}$ *Asc*$^{-/-}$ hosts and monitoring the onset of disease (Figure 5.7). Both *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Asc*$^{-/-}$ recipients receiving Tregs were protected from $CD45RB^{hi}$ T cell driven wasting disease (Figure 5.8). Furthermore, although the small group sizes did not allow the differences to reach statistical significance, both *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Asc*$^{-/-}$ recipients receiving Tregs showed a comparable trend towards protection from T cell transfer colitis and typhlitis (Figure 5.9). These results collectively suggested that Asc expression in either host APC or tissue cells was dispensable for Treg mediated protection against systemic and intestinal inflammation in a lymphopenic host. Consistent with the reduced intestinal pathology, Tregs prevented splenomegaly and lymphopenia-driven expansion in the intestines equally in both *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Asc*$^{-/-}$ recipients (Figure 5.10). Thus, Asc signalling in host cells is dispensable for the pathogenic function of effector T cells and for the protective function of Tregs in the T cells transfer model of IBD.

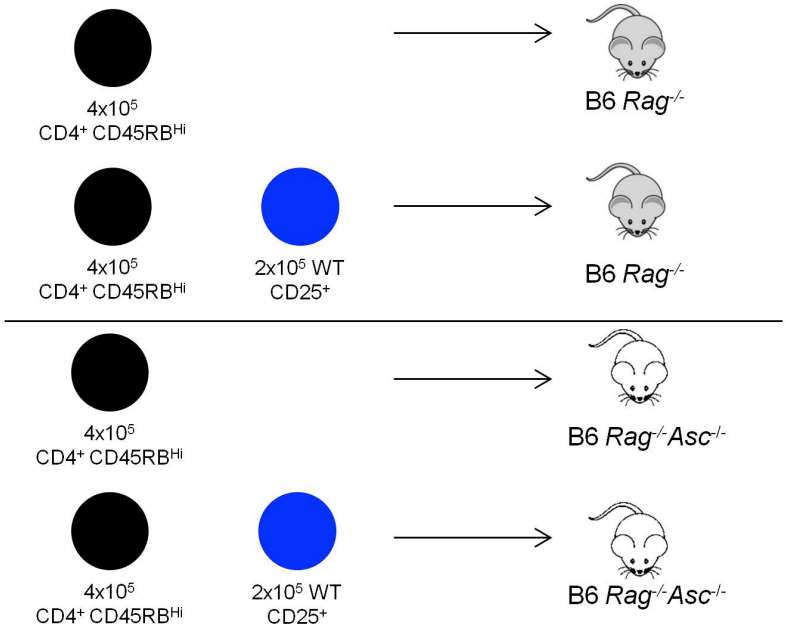


Figure 5.7 Schematic representation of T cell transfer and Treg protection

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 of WT CD4$^+$CD45RB^{hi} T cells (Black circles) by i.p. injection. Two groups of mice were also co-transferred with 2 x 10^5 CD4$^+$CD45RB^{low} CD25$^+$ regulatory T cells (Tregs, Blue circles). Mice were left to reconstitute for 6 weeks before they were euthanized and assessed for intestinal inflammation.

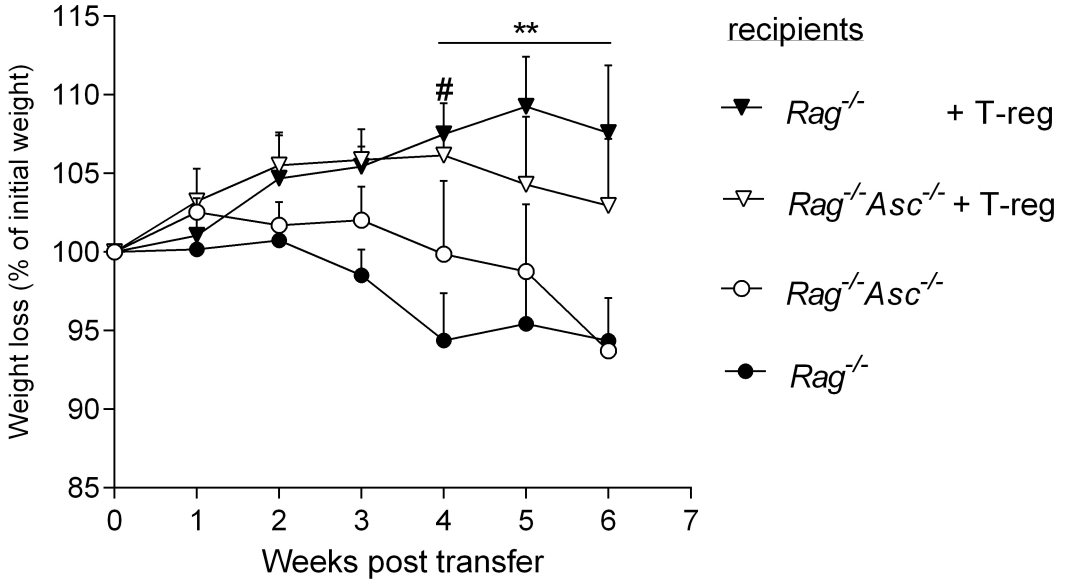


Figure 5.8 Treg protection against wasting disease is independent of Asc expression in host cells

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 WT CD4$^+$CD45RB^{hi} T cells by i.p. injection. Two groups of mice were also co-transferred with 2 x 10^5 CD4$^+$CD45RB^{low} CD25$^+$ T regulatory cells (Treg) by i.p injection. Mice were weighed weekly for six weeks before they were euthanized. Symbols denote mean weights (±SEM) as a percentage of the initial body weight (*n* = 3-7 mice per group). Data shown represent results from a single pilot experiment. Statistical significance was determined between *Rag*$^{-/-}$ and *Rag*$^{-/-}$ + Treg recipients by the two-way ANOVA analysis. (*p<0.05; **p<0.01) or between *Rag*$^{-/-}$ vs *Rag*$^{-/-}$*Asc*$^{-/-}$ + Treg recipients (#p<0.01).

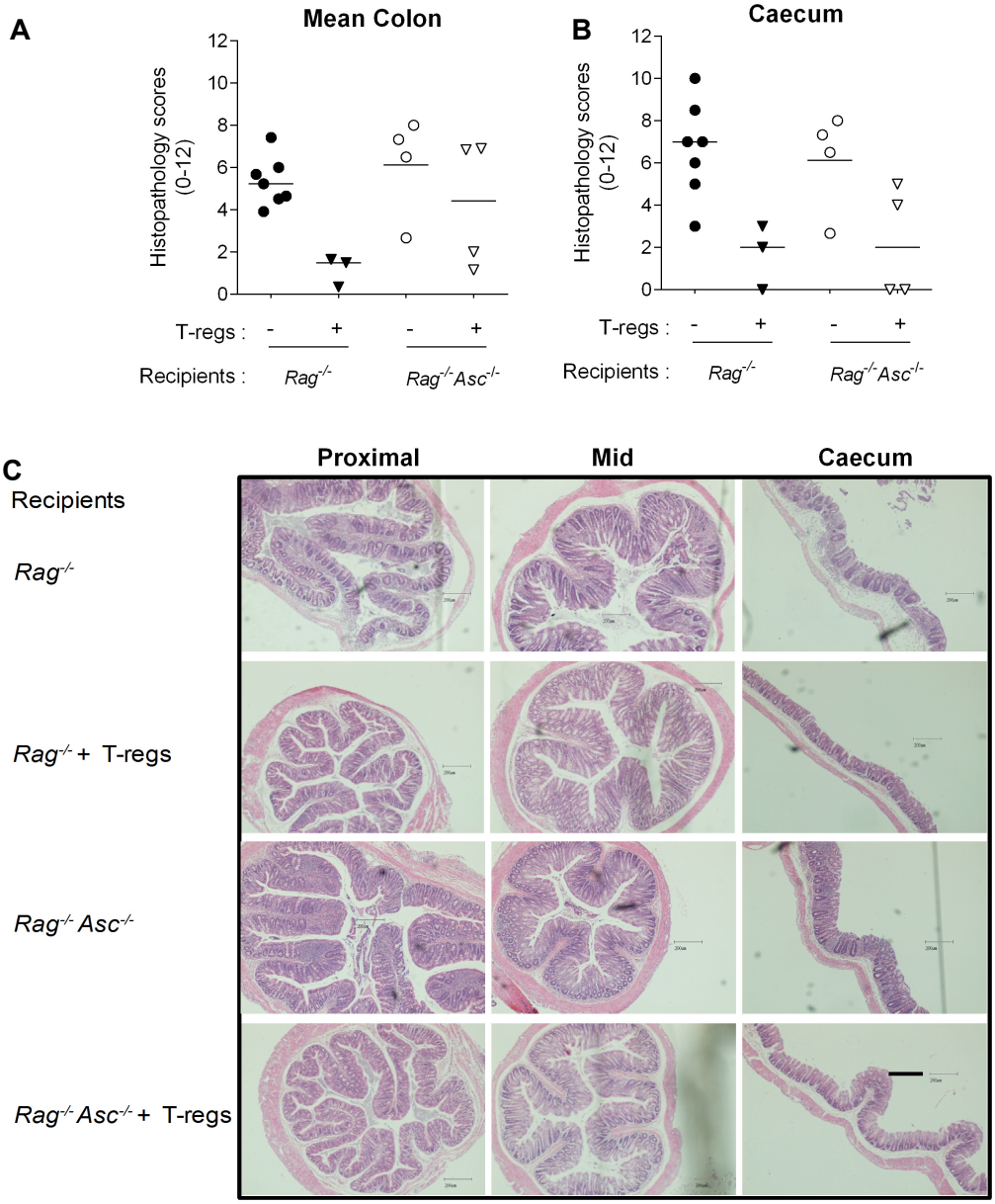


Figure 5.9 Treg mediated protection against T cell driven intestinal inflammation is independent of Asc expression in host cells.

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 WT CD4$^+$CD45RB^{hi} T cells by i.p. injection. Two groups of mice were also co-transferred with 2 x 10^5 CD4$^+$CD45RB^{low} CD25$^+$ Tregs by i.p injection. Mice were left to reconstitute for 6 weeks before they were euthanized and histopathology scores in the colon (A) and caecum (B) were assessed. Representative photomicrographs of the proximal and mid colon, and caecum are depicted in (C) (scale bar= 200µm). Each symbol represents an individual animal. Data shown is from a single pilot experiment (n = 3-7 mice). Horizontal lines depict medians.

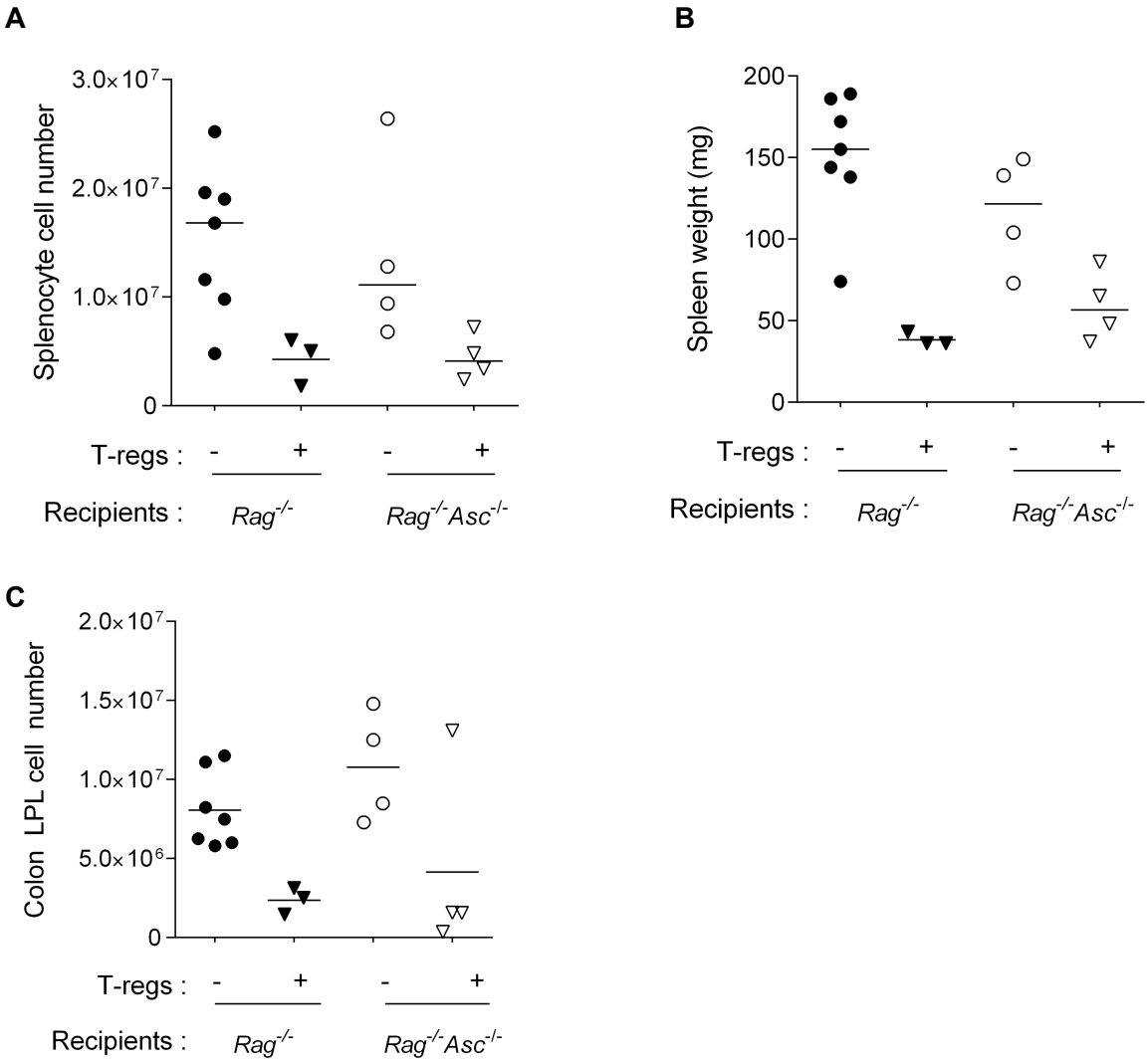


Figure 5.10 Tregs inhibit the expansion of adoptively transferred naïve T cells locally and systemically in an Asc-independent manner.

Cohorts of *Rag*$^{-/-}$ or *Rag*$^{-/-}$*Asc*$^{-/-}$ mice were reconstituted with 4 x 10^5 WT CD4$^+$CD45RB^{hi} T cells by i.p. injection. Two groups of mice were also co-transferred with 2 x 10^5 CD4$^+$CD45RB^{low} CD25$^+$ Tregs by i.p injection. Mice were left to reconstitute for 6 weeks before they were euthanized. Thereafter, splenocyte numbers (A), spleen weights (B) and colonic lamina propria leukocyte cells (LPL) numbers were determined (C). Each symbol represents an individual animal. Data shown is from a single pilot experiment (n = 3-7 mice). Horizontal lines depict medians.

5.3 Discussion

IBD manifests as a consequence of dysregulated aberrant immune responses directed against the microbiota. Work in mice and humans over the last few decades have tried to understand the identity of the microbial species and the cellular subtypes mediating inflammatory responses in IBD. Regarding the latter, $CD4^+$ T cells were identified early in the course of these studies as playing a key role in the propagation of intestinal inflammation. Work from our lab previously found that IL-1R signalling into T cells was required for the induction of severe colitis, and operated by inducing the expression of IL-23R on T cells and by promoting cell survival via the induction of anti-apoptotic factors (Coccia et al., 2012). This data complemented previous work that showed that cell intrinsic Myd88 signals also promoted naïve $CD4^+$ T cells to potentiate colitis (Fukata et al., 2008; Tomita et al., 2008). Indeed naïve $CD4^+$ T cells deficient in Myd88, drove wasting disease and colitis at a significantly slower rate than WT naïve $CD4^+$ T cells (Tomita et al., 2008) (and Mark Asquith DPhil thesis).

Interestingly these studies also indicated that cell intrinsic Myd88 signalling was required for IL-17 production from T cells. This idea was confirmed by crossing a Rorγt-GFP reporter strain to an *Il1r1*$^{-/-}$ background and demonstrated a key for microbiota-derived IL-1β in the development of small intestinal Rorγt$^+$ Th17 cells (Shaw et al., 2012). Given these findings and others that pointed to an Asc-dependent source of IL-1β from myeloid cells in the intestine (Franchi, 2012), we postulated that naïve $CD4^+$ T cells would be impaired in their ability to drive colitis in *Rag*$^{-/-}$ *Asc*$^{-/-}$ recipients. Surprisingly, we found that Asc-derived signals in host cells were dispensable for the development of colitis, as *Rag*$^{-/-}$ *Asc*$^{-/-}$ mice transferred with naïve $CD4^+$ T cells developed as severe colitis as *Rag*$^{-/-}$ recipients. This could be for a number of reasons. Firstly, while it is known that IL-1β is required for Th17 development at steady state *in vivo*, its role during lymphopenic driven expansion of naïve T cells into Th17 cells is unknown. This could be tested experimentally by using *Rag*$^{-/-}$ *Il1β*$^{-/-}$ recipients in the T cell transfer colitis model. Second, pro-IL-1β may be processed independently of Asc signalling in this model. This could be in a caspase-1 dependent manner using receptors harbouring CARD domains that can directly couple to Caspase-1 e.g. Nlrc4 (Broz et al., 2010). Alternatively, pro-IL-1β might be processed in a caspase-1 independent manner by serine proteases such as proteinase-3, elastase and cathepsin G (Netea et al., 2010). The factor

determining which enzyme cleaves IL-1β may ultimately depend on the cell type involved. Neutrophils for instance are known to cleave IL-1β independently of caspase-1, via proteinase-3 (Greten et al., 2007). Given that neutrophilia accompanies the colitis driven by naïve $CD4^+$ T cells, IL-1β may be processed by proteinase-3 thereby circumventing the requirement for Asc and caspase-1. This could be tested experimentally by using *Rag*$^{-/-}$ *proteinase-3*$^{-/-}$ recipients in T cell transfer or using inhibitors of proteinase-3.

The co-transfer of Tregs with naïve $CD4^+$ T cells can prevent the onset of systemic and intestinal inflammation (Powrie et al., 1993). Transfer of Tregs at later stages can also cure established colitis in mice, making this a potential therapy for IBD (Maloy et al., 2005; Uhlig et al., 2006). Understanding the signalling requirements for Tregs to suppress colitis *in vivo* is important to be able to boost these functions. To this end, it is known that IL-10 receptor signalling on Tregs is required for efficient suppression of naïve $CD4^+$ T cells in the T cell transfer model. Indeed while naïve T $CD4^+$ cells can induce colitis in *Rag*$^{-/-}$ *Il10*$^{-/-}$ recipients, co-transferred Tregs are unable to suppress this colitis (Murai et al., 2009). Furthermore IL-10R deficient Tregs were unable to suppress T cell transfer colitis (Murai et al., 2009). IL-10 signalling activates STAT3, and the key role of this suppressive circuit was further emphasized by a recent study showing that transgenic mice in which STAT3 was selectively ablated in Tregs spontaneously developed colitis (Chaudhry et al., 2009). We became interested in understanding the role of inflammasome signalling in influencing Treg function by recent studies that showed that cell intrinsic MyD88 signals were required for Treg-mediated suppression in T cell transfer colitis (Fukata et al., 2008). Furthermore, recent work in our lab showed that IL-18R1 signalling on Tregs was required for suppression of T cell transfer colitis *in vivo* (Oliver Harrison, personal communication). Together, these data strongly suggested that IL-18-IL-18R1 signalling via MyD88 promoted Treg mediated effector functions. As pro-IL-18 is classically cleaved by caspase-1, we wished to determine if the inflammasome regulated Treg function via IL-18. Surprisingly, we found that Tregs suppressed T cells driven colitis and systemic inflammation equally well in *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Asc*$^{-/-}$ hosts. This could be explained by a ligand distinct from IL-18 promoting Treg function in this setting. To address this possibility, *Rag*$^{-/-}$ *Il18*$^{-/-}$ mice have recently been generated in our lab (Oliver Harrison personal communication). Interestingly, there is precedence for IL-18-independent IL-18R1

signalling; Gutcher *et al* recently found that *Il18*$^{-/-}$ mice were susceptible to EAE while *Il18r1*$^{-/-}$ mice were almost completely protected (Gutcher et al., 2006). In this context, IL-18R1 signalling on non-lymphocytic haematopoietic cells was required for the induction of pathogenic Th17 cells (Gutcher et al., 2006). A second possibility to account for the lack of dependence on Asc is that caspase-1 independent mechanisms of pro-IL-18 cleavage could be operational, as discussed earlier. To test this idea we will measure the levels of IL-18 in *Rag*$^{-/-}$ *Asc*$^{-/-}$ recipient mice during T cell transfer. Interestingly, a recent study found that meprin metalliproteinases, which are proteases highly expressed in the intestine, are capable of cleaving and activating IL-18 (Banerjee and Bond, 2008). This supports the idea that non-caspase-1 modes of pro IL-18 cleavage exist.

Finally, we also tested a cell-intrinsic role for Asc signalling in effector T cell function. This was prompted by reports that Asc had caspase-1 independent effects in models of arthritis and EAE (Kolly et al., 2009; Shaw et al., 2010). This was further dissected recently and was ascribed to Asc's ability to promote the mRNA stability and expression of a guanine nucleotide exchange factor, Dock2 (Ippagunta et al., 2011). Interestingly Asc regulated lymphocyte mobility via Dock2-activation of a GTPase, Rac, which controls actin polymerization (Ippagunta et al., 2011). However, some of the striking phenotypes observed in *Asc* deficient mice, such as defective antigen presentation and defective lymphocyte migration, were called into question by a subsequent report showing that Dock2 was deficient only in a subset of *Asc*$^{-/-}$ strains (Ippagunta et al., 2012). Given these phenotypes, we hypothesized that Asc might regulate effector T cell function in a cell intrinsic manner. We tested this by transferring Asc deficient T cells into *Rag*$^{-/-}$ recipients. We observed no difference in the ability of *Asc*$^{-/-}$ T cells to driven either systemic or intestinal inflammation. Thus, intrinsic Asc expression is dispensable for effector T cell function *in vivo*. Collectively, our data suggest that Asc signalling is dispensable for the induction or regulation of T cell driven inflammation in the intestine.

Chapter 6. General discussion

6.1 Innate immune tolerance in the intestine

The question of how the immune system discriminates self from non-self is one of the most intriguing questions in immunology. In a seminal paper, Charles Janeway proposed that discrimination was achieved by germ-line encoded receptors, PRRs, that sensed and signalled in response to highly conserved pathogen-derived molecular patterns, PAMPs (Janeway, 1989; Medzhitov and Janeway, 2000). Since PAMPs were exclusive to microbes and absent in mammalian hosts, there was no danger of recognizing self and mounting an autoimmune response. Janeway's theory, elegant and prescient, rekindled interest in the role of innate immunity in immune induction. However, the theory also left many questions wanting, such as how we still responded to adjuvants like alum, or sterile inflammatory agents such as monosodium urate crystals, which lack PAMPs. Another major paradox that emerges from this theory is how mammals coexist with a vast number of foreign microbes, the microbiota, that number in the hundreds of trillions without eliciting chronic inflammation? (Srinivasan, 2010). Moreover how do we discriminate innocuous commensals from pathogens? Using the PRR framework of Janeway, this question seems problematic to answer since commensals and pathogens both express similar PAMPs such as LPS, LTA and flagellin. Indeed it is puzzling how such a massive PAMP-ridden bulk, most notably in our intestines, can reside peacefully in the absence of chronic inflammation while residing directly next to the largest collection of leukocytes in the body. A number of studies have shown that the architecture of the intestinal barrier and the compartmentalized expression of PRRs in the gut can partially account for this paradox without the need to reject Janeway's model (reviewed in (Hooper and Macpherson, 2010; Srinivasan, 2010)). For instance, TLR5 is expressed on the basolateral surface of intestinal epithelial cells (IECs), meaning that TLR5 activation will only be triggered following the translocation of bacteria or flagellin across the epithelial barrier, a consequence of pathogenic invasion (Gewirtz et al., 2001). Another important feature is a 150μm thick mucus layer in the colon that separates the microbiota from the epithelium (Johansson et al., 2011; Johansson et al., 2008). One can envisage that delivery of PAMPs to IEC-expressing PRRs

would first require the breach of this mucus layer, again a hallmark of pathogens but not commensals. While such features provide some basis for pathogen/commensal discrimination, other studies have found that at steady state commensal bacteria are constantly sampled by gut DCs, which subsequently traffic to the MLN (Macpherson and Uhr, 2004). Indeed while the majority of the area of the gut is covered in mucus, M-cells are special inductive sites which lack mucus and are thus portals of entry to both commensals and pathogens. Thus, the commensal microbiota is not entirely ignored by the innate immune system and additional factors must contribute to pathogen sensing in the gut.

6.2 The inflammasome: a discriminator between commensals and pathogens?

Janeway's model is useful to explain how we discriminate, in his words, "infectious-nonself" from "noninfectious-self". However as illustrated above, it is insufficient to explain the discrimination between pathogens and commensals. To address this, it is useful to consider the Danger model proposed by Polly Matzinger wherein the sensing of danger or alarm signals, rather than foreignness, determines whether an APC is activated or not (Matzinger, 1994, 1998, 2002a, b). While originally lacking experimental evidence, the danger model gained credence with the discovery of the inflammasome (Martinon et al., 2002). Unlike traditional PRR:PAMP activation, inflammasome activation operates via a two-signal mechanism. Signal 1 can be provided by PRR recognition of a cognate PAMP that elicits the NFκB-dependent transcriptional priming of pro-IL-1β and NLRP3. Signal 2 varies depending on the upstream PRR, but includes type III secretion system machinery (Miao et al., 2010a), cytosolic flagellin (Mariathasan and Monack, 2007), cytosolic LPS (Kayagaki et al., 2013), oxidized mitochondrial DNA (Shimada et al., 2012), mitochondrial derived ROS (Zhou et al., 2010; Zhou et al., 2011), K^+ efflux (Munoz-Planillo et al., 2013; Walev et al., 1995) and cytosolic dsDNA (Burckstummer et al., 2009; Hornung et al., 2009; Roberts et al., 2009). What is common to most of these triggers? They necessitate breach of a cell membrane to enter the cytosol, a hallmark of a pathogenic response. In addition, many of the above triggers also require microbial virulence effectors to facilitate their delivery into the cell. Pathogens may express several types of virulence factors such as adhesins, toxins, invasins, and 'injection' apparatuses known as secretion systems. Together, these virulence factors promote pathogen

growth by either hijacking host cells for use as reservoirs (e.g. viruses co-opt cells as self-perpetuating factories), by dampening immune responses directed against them, or in the most extreme cases by killing cells. The inflammasome could serve as a discriminant between pathogens and commensals through its reliance on virulence factors for activation. That is since pathogens but not commensals could theoretically provide signal 2, the ensuing inflammatory responses would be restricted against pathogens (Vance et al., 2009).

Interestingly, a similar system of detection exists in plants. In a series of influential papers, Dangl and Jones proposed that a subset of plant PRRs known as R proteins, which are homologous to mammalian NLRs, are activated in response to pathogen-mediated modifications of host cell proteins (Dangl and Jones, 2001; Jones and Dangl, 2006). Known as the Guard hypothesis, Dangl and Jones posited that in one scenario, R proteins (guards) constitutively bind a group of host cell proteins (guardees) that inhibit R protein activation but maintain them in a signalling competent state (Dangl and Jones, 2001; Jones and Dangl, 2006). Upon translocation of a microbial effector into the cell that modified the guardee, a conformational change could then release the R protein from inhibition and in doing so promote activation of effector-triggered immunity (ETI). Interestingly, some guardee proteins in plants, such as SGT1 and Hsp90, were found bound to mammalian Nlrp3 at steady state (Mayor et al., 2007), suggesting strong conservation across kingdoms.

The distinction between ETI, and what Dangl and Jones term, PAMP-triggered immunity (PTI) is instructive for understanding commensal/pathogen discrimination in mammals. In their view, the plant immune response can be characterized in four phases that proceed sequentially as follows (Jones and Dangl, 2006):

i. Pathogens induce PAMP-triggered immunity (PTI) which limits further colonization
ii. Successful pathogens deploy effectors/virulence factors which counteract PTI
iii. Virulence factors are detected by plant NLR homologs driving effector-triggered immunity (ETI) to thwart further pathogenesis. ETI is an amplified and accelerated form of PTI which can culminate in cell death at the infection site

iv. Under the pressure of natural selection, pathogens avoid ETI either by evolving means of directly suppressing ETI or by mutating or divesting off the recognized virulence factor. This leads to a continual arms race between host and pathogen.

Superimposing the above framework on the mammalian innate immune system, we see many parallels. Whilst commensals and pathogens can trigger PTI, akin to TLR signalling, only virulent pathogens can induce ETI. While still in its infancy there is accumulating evidence for the existence of ETI in mammals (Stuart et al., 2013). In support of this idea, Nlrc4-triggering pathogens, such as *Salmonella typhimurium*, lose their ability to do so when they a lack type III secretion system or flagellin (Franchi, 2012; Franchi et al., 2006; Miao et al., 2006; Miao et al., 2010b). A recent paper showed that Nod1 activation in response to *S. typhimurium* relies on sensing the activation status of small Rho GTPases, which are modified by virulence factors upon infection (Keestra et al., 2013). Furthermore, the very same Rho GTPase modulating virulence factor was required for caspase-1 activation *in vivo* (Muller et al., 2009; Muller et al., 2010). Another example of ETI in mammals is found in the context of *Yersinia Pseudotuberculosis* infections. *Y. psuedotuberculosis*, expresses a virulence factor known as YopJ which acetylates a key signalling adapter of Nod2, Rip2, favouring the dissociation of Nod2 and its subsequent interaction with caspase-1 that leads to inflammasome activation (Meinzer et al., 2012). YopJ also possesses deubiquitinase activity and can inhibit MAPK kinases and IKKβ pathways (Zhou et al., 2005) (Zheng et al., 2011). The inhibition of these pathways also triggers Nlrp3-dependent caspase-1 activation (Zheng et al., 2011). *Yersinia* species have evolved means of suppressing inflammasome activation via a virulence protein, YopK that interferes with detection of *Yersinia's* type three secretion system (T3SS), suggesting an evolutionary arm's race (see iv above). One major output of ETI in mammals may be inflammasome activation, a qualitatively distinct response from PTI, most notably exemplified through the secretion of active IL-1β and IL-18. Interestingly activation of Nlrc4 can also result in an inflammatory form of cell death, pyroptosis, which has similarities to ETI in plants (see iii above).

Collectively these lines of evidence suggest that inflammasome activation could discriminate between commensals and pathogens. However a major caveat to this work is that the majority of these mechanistic studies were performed using BMDMs *in vitro*. While these are undoubtedly

useful, they require verification *in vivo* in a suite of different cell types. Indeed the requirements for inflammasome activation can change according to the cell involved, which is probably an adaptation to its particular environment. For instance, peripheral blood monocytes (PBMCs) express a constitutively active caspase-1 and only require signal 1 i.e. a PAMP, for IL-1β secretion (Netea et al., 2009). This differential requirement makes sense in this particular context because if a microbe translocates systemically, it poses a huge threat to the host regardless of whether it is a commensal or a pathogen. Thus monocytes have relatively 'lax' requirements for IL-1β secretion, as they need to be able to respond rapidly to eradicate the translocated microbe.

The intestine is a unique environment where excessive inflammasome reactivity towards commensals could be detrimental, and thus discrimination could be beneficial. Recently one study showed that intestinal macrophages express Nlrc4 and cleave pre-loaded pro-IL-1β only in response to pathogens such as *S. typhimurium* but not commensals (Franchi, 2012). While this data suggested that Nlrc4 activation might discriminate between pathogens and commensals, this activation pathway was only observed in mice on a BALB/c background but not on the more commonly used C57BL/6 background, calling into question the general applicability of this mechanism. Furthermore, since *S. typhimurium* has a tropism for macrophages, it is difficult to tease apart whether the few commensal species used failed to trigger Nlrc4 because they couldn't survive in macrophages, or whether they lacked the secretion systems or flagellin agonists that trigger Nlrc4.

During our studies we found that active caspase-1 and mature IL-18 were present at steady state in the intestine, before the onset of infection or inflammation. IL-18 production and caspase-1 activation at steady state was Asc-dependent but did not require Nlrp3. Furthermore the bulk of caspase-1 expression occurred in non-haematopoietic cells, and based on immunofluorescence staining of IL-18 and Asc, intestinal epithelial cells were the most likely source. Thus collectively, these data suggest that caspase-1 is active at steady state in the intestinal epithelium i.e. in the absence of pathogenic infection. This surprising result could reflect a differential requirement for caspase-1 activation in epithelial cells, an understudied cell type in inflammasome biology. A key question that we are currently pursuing is whether IEC inflammasome activation is constitutive or whether it requires microbiota-derived signals. If the latter is true, then it follows that commensals

can trigger the inflammasome and that a 'virulent' signal 2 is dispensable for inflammasome activation, at least in IEC. Parallel work from our lab has shown that IL-18 production from the intestinal epithelium plays an important role in limiting intestinal Th17 cell differentiation as mice lacking IL-18 display higher frequencies of colonic Th17 cells (Oliver Harrison, personal communication). Thus, if commensals are indeed responsible for steady state caspase-1 activation and IL-18 production, this may represent and indirect mechanisms through which they regulate local Th17 cells. Distinct members of the commensal microflora can exert differential effects on the intestinal immune system. Segmented Filamentous Bacterium (SFB), a commensal bacterium that resides in the small intestine of mice, drives intestinal Th17 cell accumulation (Ivanov et al., 2009). It is tempting to speculate that certain bacteria can limit Th17 cell differentiation, by driving inflammasome-dependent IL-18 production. To test this idea we are currently orally administering combinations of antibiotics to deplete different classes of bacteria, before assessing IL-18 levels and caspase-1 activation in the intestine. Once we narrow down the class of antibiotic resistant bacteria we will undertake 16S bacterial sequencing to accurately identify the bacteria.

6.3 Dissecting inflammasome circuits in IECs

Almost all of the work in the field of inflammasome biology has centred on myeloid cells, the bone-marrow derived macrophage being the particular cell of choice. However, inflammasome activation within IEC may differ for multiple reasons, a key one being that macrophages are phagocytic while IEC are not. Thus understanding precisely how enteric pathogens such as *C. rodentium* trigger inflammasome activation in IEC will likely reveal novel insights into the molecular basis of inflammasome sensing of pathogens.

Thus far, efforts to study inflammasome function in IEC have been greatly hindered by technical barriers in culturing IEC *ex vivo*. Recently however, a breakthrough in this area has been made by the group of H. Clevers (Sato et al., 2009), who isolated whole crypts harbouring primary intestinal epithelial stem cells that could be cultured in a cocktail of growth factors to generate villus-like epithelial domains, termed organoids, which harbour multiple differentiated IEC types (Sato et al., 2009). We have recently successfully established this method in our lab and used it to confirm prominent Asc expression in IEC (data not shown). In the future, crypt isolation and culture from

Asc$^{-/-}$ and *Nlrp3*$^{-/-}$ mice will be used to generate inflammasome deficient organoids. Organoids will be challenged with live bacteria, such as *C. rodentium,* or with different types of inflammasome activators. To study this *in vivo* we plan to use a recently created transgenic Asc mouse flanked by two loxP recombination sites (Asc$^{Flox/Flox}$) by crossing it to a Villin-Cre mouse. Since Villin is expressed by all IECs, this should enable us to selectively ablate Asc signalling in IECs.

To identify the pathogen-derived molecules that drive inflammasome activation in IEC we will challenge the organoid cultures with a variety of *C. rodentium* mutants that lack defined bacterial virulence factors. These include mutants lacking the T3SS required for establishing A/E lesions and others that lack T3SS effector proteins that target host mitochondria to regulate host cell death. Indeed studies using other bacteria have shown that effector proteins delivered by T3SS or structural proteins of the T3SS itself e.g. the inner rod protein are the primary triggers for Nlrp3 and Nlrc4 inflammasome activation (Miao et al., 2010b). Together, these experiments should reveal whether the tenets of inflammasome biology are preserved in different cell types and identify novel IEC-pathogen interactions that drive host protective inflammasome responses.

6.4 Novel inflammasome effector mechanisms

Our data using the enteric pathogen, *C. rodentium*, revealed that Nlrp3 activation in the IEC compartment protected against intestinal inflammation in a caspase-1 independent manner. This suggests that Nlrp3 signalling in IECs may promote novel effector mechanisms. As activated Nlrp3 acts post-translationally to cleave cytosolic protein substrates, comparative gene expression profiling using microarrays are unlikely to reveal novel mechanisms of inflammasome function in IEC. Therefore, in order to identify such mechanisms we plan to use a quantitative proteomic approach. Future work will use the organoid culture system to identify specific proteins secreted by WT or inflammasome-deficient IEC during steady state or in response to *C. rodentium* infection. The mass-spectrometry-based iTRAQ technique will be used as it allows a quantitative comparison of protein abundance amongst different samples by comparing the relative intensities of different labels (Proteomics; Wiese et al., 2007) (Figure 6.1).

With this technique, we aim to identify proteins that are secreted from *ex vivo* derived organoid cultures after infection with *C. rodentium*. By comparing *Asc*$^{-/-}$, *Nlrp3*$^{-/-}$ and WT organoids we will

be able to restrict our analysis to proteins secreted in an Asc/Nlrp3-dependent manner (Figure 6.1). This technique has already been successfully applied in fibroblasts, to identify a novel unconventional secretion pathway mediated by caspase-1 (Keller et al., 2008). Ultimately, the identification of inflammasome regulated IEC-secreted proteins will allow us to answer how inflammasome activation in this cell compartment provides immunity against mucosal pathogens and may uncover novel pathways that might underlie the aetiology of CD in patients with *NLRP3* polymorphisms.

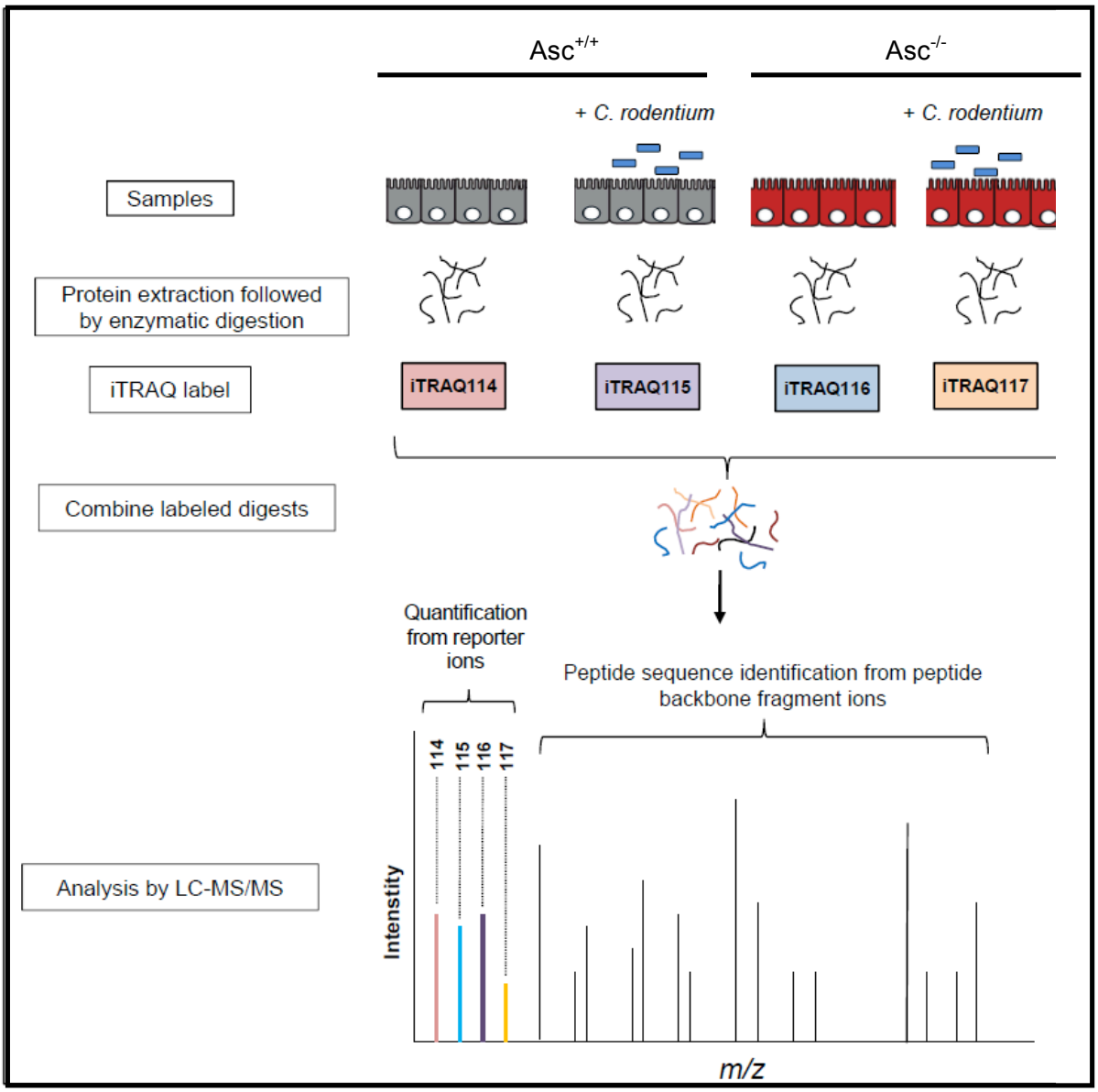


Figure 6.1 Quantitative proteomic workflow (adapted from (Proteomics)) Organoids sufficient or deficient in Asc (or Nlrp3, not shown) are either infected with *C. rodentium* or left untreated. After equalizing the protein concentrations, proteins are digested enzymatically. The peptide digest is then labelled with distinct iTRAQ tags before being mixed into a single sample. The resultant mixture is then analysed by LC-MS/MS (liquid chromatography-tandem mass spectrometry). This yields both the identity of the protein as well as the relative quantity.

6.5 Opposing roles for inflammasome signalling in chronic versus acute models of intestinal inflammation

Innate immune signalling in the intestine has both pathogenic and protective roles depending on the model used. Interestingly, in models of acute intestinal inflammation such as the DSS or the *C. rodentium* models, innate immune signalling is broadly protective. For instance, mice lacking Tlr2 or Nod2 or MyD88 are hyper-susceptible to *C. rodentium* infection (Gibson et al., 2008a; Gibson et al., 2008b; Kim et al., 2011; Lebeis et al., 2007). By contrast, studies using chronic or spontaneous models of intestinal inflammation show that innate immune signalling is mostly pathogenic. For instance, the spontaneous colitis that developed in *Il10*$^{-/-}$ mice was prevented by crossing them onto a *Myd88*$^{-/-}$ background (Rakoff-Nahoum et al., 2006). Work from our lab reinforces this dichotomy, showing protective roles for Nlrp3, Asc and Myd88 in *C. rodentium* infection, but pathogenic roles for these molecules in *Hh*-driven chronic models of colitis (Asquith et al., 2010).

The differential roles for Myd88 and Asc in acute compared to chronic colitis models stem from two main reasons. First, intestinal inflammation that manifests in *Hh*-triggered chronic models of colitis is primarily due to host immunopathology that is mediated by excessive immune responses towards *Hh*. Fittingly, blockade or ablation of pro-inflammatory cytokines, such as TNFα or IL-23 or IL-17A or IFN-γ, results in various degrees of attenuation of *Hh*-triggered typhlocolitis (Buonocore et al., 2010; Hue et al., 2006; Kullberg et al., 2006; Maloy et al., 2003). By contrast, pathological intestinal inflammation during *C. rodentium* infection is likely to be a combination of bacterial-driven epithelial damage and host immunopathology. Consistent with this, blockade of IL-17A or IFN-γ or TNFα increases susceptibility to *C. rodentium* infection and intestinal inflammation (Goncalves et al., 2001; Ishigame et al., 2009; Simmons et al., 2002). Thus while susceptibility towards *C. rodentium* is a consequence of insufficient protective immunity, susceptibility towards *Hh* may derive from over exuberant inflammatory responses stemming from defective tolerance or regulation. Since the innate immune system is at the interface of initiating protective immune responses against pathogens, innate deficiencies manifest as susceptibility against infection. By contrast, knocking out key nodes of the innate immune systems such as Myd88 and Asc in chronic models of intestinal inflammation effectively cuts the source of 'fuel' for priming and perpetuating deleterious inflammatory responses.

A second potential reason explaining the opposing roles of Asc and Myd88 signalling in acute versus chronic models of colitis may lie in the cell types primarily affected. With models such as *C. rodentium* and DSS, models that instigate colitis in an 'outside-in' manner, the primary pathophysiological mechanism may involve damage to the intestinal epithelium. By contrast, in chronic models of intestinal inflammation such as the T cells transfer model and *Hh*-driven intestinal inflammation, epithelial damage is likely to be a secondary event downstream of chronic inflammatory circuits activated in lamina propria leukocytes (Asquith and Powrie, 2010). In the acute models, epithelial repair functions of Asc, Nlrp3 and Myd88 play important roles in restoring the epithelial barrier and in doing so promoting homeostasis (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Rakoff-Nahoum et al., 2004; Zaki et al., 2010a). Myd88 signalling also contributes to intestinal homeostasis by reinforcing the spatial segregation between the intestinal microbiota and IECs, through the production of AMPs such as Reg3γ (Cash et al., 2006; Vaishnava et al., 2008; Vaishnava et al., 2011). By contrast in chronic models, the pathophysiological mechanisms that drive intestinal inflammation are primarily due a failure to regulate pro-inflammatory responses, which are propagated by myeloid APCs. Consistent with the need for tight regulation of myeloid cell activation in the lamina propria, myeloid specific STAT3 knockout mice ($LysM^{Stat3KO}$) develop spontaneous colitis due to IL-10 hypo-responsiveness that can be prevented by crossing them to a $Tlr4^{-/-}$ background (Kobayashi et al., 2003). Similarly, spontaneous colitis in $Il10^{-/-}$ mice can be prevented by crossing these mice to a myeloid-specific LysM or $CD11c^{Myd88KO}$, but not to an epithelial-specific $Villin^{Myd88KO}$ (Hoshi et al., 2012).

6.6 Concluding remarks

As discussed above, the pathophysiological models that operate in each model of intestinal inflammation differ. Inflammatory Bowel Disease (IBD) is a multifaceted, heterogeneous disease that probably encompasses aspects reflected in each model. For instance, the acute models primarily affect the intestinal epithelium, a fact that is reflected in CD GWAS studies highlighting 'epithelial' genes such as E-cadherin (*CDH1*), the ER stress protein (*XBP1*), solute transporters (*SLC22A4*) and the mucin (*MUC19*) (Khor et al., 2011). Chronic models, by contrast, are a result of a failure to regulate pathogenic innate and adaptive inflammatory responses. This is reflected in

SNPs in *IL-10*, *IL-23R, IL-2RA, TNSF15* and the inducible T cells co-stimulator, *ICOSLG* (Khor et al., 2011).

Our study was initially spurred on by reports of SNPs in *NLRP3* increasing susceptibility to CD (Villani et al., 2009). We found that Nlrp3 and Asc activation in the intestinal epithelium contributes to early mucosal defence against an enteric pathogen. Our data reinforce the mounting evidence that the intestinal epithelium plays a crucial sentinel role in preventing pathogen colonization and in so doing, limits intestinal inflammation in acute settings. Adherent invasive *E.coli* (AIEC) species are frequently associated in close apposition to the epithelium of CD patients, suggesting that these bacteria may either initiate or perpetuate intestinal inflammation in CD (Darfeuille-Michaud et al., 2004). It is conceivable that, in a similar and complementary role to *NOD2*, a major CD susceptibility gene, *NLRP3* and *ASC* limit the outgrowth and colonization of colitogenic species and thereby promote intestinal homeostasis. Fitting with this idea, one group has shown that $Asc^{-/-}$ and $Il18^{-/-}$ mice display a dysbiotic microbiota with an outgrowth in two colitogenic bacterial species, TM7 and *Prevotellaceae* (Elinav et al., 2011). While insufficient Nlrp3 and Asc may predispose mammals to enteric infections, excessive activation is known to drive autoinflammatory diseases such as the Cryopyrin- Associated Periodic Syndromes or CAPS (Aksentijevich et al., 2002; Masters et al., 2009). Our results showing pathogenic roles for Asc in chronic intestinal inflammation support this idea and advise caution in targeting these pathways in IBD therapy. Apart from IBD therapy however, boosting intestinal Nlrp3/Asc activation in IEC as a therapy against EPEC or EHEC may prove interesting in the future.

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