The Role of NLRs in Induction and Resolution of Intestinal Inflammation

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A thesis submitted for degree of Doctor of Philosophy,

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Abstract

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Innate immune activation is thought to play a central role in IBD pathogenesis because genetic polymorphisms in *NOD2* and *NLRP3*, cytosolic innate immune receptors belonging to the NLR family, have been associated with IBD susceptibility. However, the mechanisms through which NLR mutations predispose to IBD remain unclear. The aim of this project was to dissect the functional roles of different NLRs in intestinal inflammation. Using the well-established DSS-induced colitis model as well as experimental models of IBD based on infection with *Helicobacter hepaticus*, we found that *Nod2* expression was significantly increased at the peak of intestinal inflammation, and remained elevated throughout the resolution process. This observation suggests a possible role for Nod2 in the resolution of inflammation. Conversely, upon infection with the acute intestinal pathogen *Citrobacter rodentium*, *Nlrp3*$^{-/-}$ mice suffered from increased bacterial colonization as early as 3 days post infection, resulting in exacerbated intestinal inflammation and severe weight loss. Analysis of irradiation bone marrow chimeras showed that the protection required Nlrp3 activation in the non-haematopoietic compartment. Furthermore, this protective mechanism was independent of the inflammasome-associated cytokines IL-1β or IL-18. Therefore, this study implicates Nlrp3 activation in intestinal tissue cells as having a crucial role in controlling pathogenic bacterial colonization, providing a potential mechanism by which NLRP3 polymorphisms could lead to increased susceptibility to IBD.

This thesis is dedicated to my parents Jing Zhao and Bin Song for their unconditional support and guidance

Without Fleming, no Chain or Florey;

without Florey, no Heatley;

without Heatley, no penicillin.

- Sir Henry Harris

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Abbreviations

A/E	attaching and effacing
AIEC	adherent-invasive *E. coli*
AMP	anti-microbial peptide
APC	antigen presenting cell
CD	Crohn's disease
CLR	C-type lectin receptor
CX3CR1	CX3C chemokine receptor 1
DAMP	danger associated molecular pattern
DAP	meso-diaminopimelic acid
DC	dendritic cell
DSS	dextran sodium sulphate
EHEC	enterohemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
FACS	fluorescence activated cell sorting
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
ILC	innate lymphoid cells
i.p.	intraperitoneal
i.v.	intravenous
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
LDH	lactate dehydrogenase
LEE	locus of enterocyte effacement
LPL	lamina propria leukocyte
LPS	lipopolysaccharide
LRR	leucine-rich repeat
mAb	monoclonal antibody
MAMP	microbe associated molecular pattern
MDP	muramyl dipeptide
MLN	mesenteric lymph node
NLR	Nod-like receptors / nucleotide-binding domain and leucine-rich repeat containing protein
NLRP3	human NLR family, pyrin domain containing 3
Nlrp3	mouse NLR family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
NOD2	human nucleotide-binding oligomerization domain containing 2
Nod2	mouse nucleotide-binding oligomerization domain containing 2
PAMP	pathogen associated molecular pattern
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
RLR	retinoid acid-inducible gene-1-like receptor
ROS	reactive oxygen species
SFB	segmented filamentous bacteria
SNP	single nucleotide polymorphism
SPF	specific pathogen-free
T3SS	type III secretion system

T4SS	type IV secretion system
TLR	Toll-like receptors
TNBS	trinitrobenzene sulfonic acid
Th1	T helper 1 cell
Th2	T helper 2 cell
Th17	T helper 17 cell
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
UC	ulcerative colitis
WT	wild-type

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Chapter 1 – General introduction

1.1 The immune system

1.1.1 Mammalian immune system

One of the main functions of the immune system is to protect the host from pathogenic infections. However, the type and the magnitude of the protective immune responses vary greatly depending on the location as well as the type of infection encountered. The innate immune system has evolved a range of receptors known as pattern recognition receptors (PRRs) to detect a wide variety of highly conserved molecular patterns found predominantly on foreign microbes. Upon detection the PRRs signal to produce rapid but non-specific innate immune responses. On the other hand, the adaptive immune system has evolved an enormous repertoire of highly variable lymphocyte receptors, generated through genetic recombination, that recognise pathogen-specific antigens. Furthermore, the adaptive system exhibits immunological memory, where faster and stronger immune responses are induced upon secondary encounter with the same pathogen. Both the innate and adaptive immune systems work together, and because of the potential for immune responses to cause tissue pathology, they are tightly regulated to avoid inappropriate reactions towards self or innocuous targets.

1.1.2 Intestinal immune system

The human intestinal surface covers an area of approximately $100m^2$, and is directly exposed to an estimated 10^{14} commensal bacteria (1, 2). We derive essential nutrients from our microbiota, which includes all microorganisms in the intestine, and they can

also provide protection from pathogens, therefore, maintaining a healthy balance with our microbiota is crucial. However, the proximity of such a large number of foreign organisms and the large number of local immune cells creates a challenge for the intestinal immune system.

To achieve a peaceful coexistence, there are several barriers separating the microbiota from the underlying tissue. The outermost layer comprises a viscous mucous that impedes bacterial attachment (1) and allows retention of high concentrations of anti-microbial peptides (AMPs) and antibodies of the IgA isotype close to the epithelium (3). Under this protective layer lies a single layer of intestinal epithelial cells (IECs), which have a variety of protective functions. Beneath the epithelium, the intestinal lamina propria is populated by huge numbers of innate and adaptive leukocytes that produce a range of anti-microbial immune effectors. In addition, there are also specialized structures of organized lymphoid tissues, such as Peyer's patches (4), isolated lymphoid follicles (5), and cryptopatches (6) that are important sites of immune induction in the gut.

1.2 Intestinal immune cells

1.2.1 Intestinal epithelial cells

The first site of bacteria-host contact in the intestine is the vast intestinal epithelium, formed by a single layer of IECs that is constantly exposed to hundreds of trillions of commensals and sporadically to pathogens (7). There are several specialized subtypes of IECs, comprising absorptive enterocytes that take up water and nutrients; goblet cells that secrete mucous; Paneth cells that secrete antimicrobial peptides; enteroendocrine cells that sense the luminal environment and transmit signals to enteric neurons across the basolateral membrane; as well as microfold (M) cells that lack overlying mucous, which allows sampling of antigens from the lumen and transportation to the underlying dendritic cells inside organized lymphoid tissues, such as the Peyer's patch (8, 9).

To increase the surface area in order to allow for more efficient absorption of nutrients, the intestinal epithelium forms folds that both project into the lumen (villi) and away from the lumen (crypts). At the bottom of the crypts lie epithelial stem cells, which generate new epithelial cells that migrate to the tip and are subsequently lost by apoptosis (10). In between the IECs, paracellular tight junctions prevent fluids and particles passing across the epithelium (1).

The first barrier to the microbiota is exclusion, and this is a central function of goblet cells that secrete MUC2, forming thick layer of mucous on surface of the epithelium (11). The importance of this mucous layer in preventing excessive barrier penetration by luminal bacteria was revealed by the finding that mice lacking Muc2 develop spontaneous colitis (12).

In the small intestine, Paneth cells secrete a range of AMPs including lysozymes, defensins, cathelicidins, calprotectins and C-type lectins such as RegIIIγ (13, 14). As Paneth cells are located at the bottom of crypts, this creates a sterile environment, thus protecting stem cells from infection and preventing bacterial translocation into systemic sites (15). The importance of Paneth cell function is highlighted by the association between ileal Crohn's disease (CD) and genetic variants in *TCF4*, a transcription factor important in Paneth cell maturation and function (14). Furthermore, CD patients carrying the T300A disease-risk allele of the autophagy gene *ATG16L1*, also exhibited abnormalities in their Paneth cells, with defective secretory granule morphology, as did transgenic mice rendered hypomorphic for the Atg16l1 protein (16).

IECs also express many different PRRs (1) that can trigger secretion of IL-8 and other inflammatory factors upon sensing pathogenic invasion or damage (17). Furthermore, Paneth cells respond to infection by secreting AMPs (18-20), chemokines and cytokines including TNF-α (21). Some of the factors secreted by IECs can modulate downstream immune responses through effects on dendritic cells (DCs) and T cells (22, 23). Conversely, under steady state conditions, conditioning factors released by healthy IECs, such as thymic stromal lymphopoietin (TSLP) and IL-25, maintain hypo-responsiveness of the intestinal immune system towards commensal bacteria. These conditioning factors limit DC production of the p40 subunit of IL-12 and IL-23, and promote IL-10 secretion, which avoids priming of T helper 1 (Th1)-cell responses and stimulates T regulatory (Treg)-cell and T helper 2 (Th2)-cell responses (24).

1.2.2 Intestinal myeloid cells

Intestinal mononuclear phagocytes and antigen-presenting cells (APCs) are very heterogeneous, comprising various subsets of macrophages and DCs (25, 26). Recent data suggest that these different myeloid populations may be derived from similar precursors that have been subjected to different stimuli (27).

In the small intestine mononuclear phagocytes are important in uptake of pathogenic and commensal bacteria, as well as innocuous antigens or apoptotic IECs. IECs undergo apoptosis under normal physiological conditions and the resulting TGF-β secretion promotes a tolerogenic condition during steady state by favouring the induction of $Foxp3^+$ Treg cells (28). In addition, myeloid cell production of IL-10, such as that derived from intestinal macrophages further promotes $Foxp3^+$ Treg function (29, 30). Consistent with an anti-inflammatory phenotype, during the steady state intestinal macrophages are hypo-responsive to bacterial stimuli through Toll-like receptors (TLRs), in terms of inflammatory mediator secretion, although they retain potent phagocytic and bactericidal activities (31, 32).

Under homeostatic conditions, DCs expressing the integrin CD103 can migrate from the intestinal lamina propria to the mesenteric lymph nodes, and preferentially induce tolerogenic $Foxp3^+$ Treg cells (33-36), whilst DCs expressing the CX3C chemokine receptor 1 (CX3CR1) can sample antigens from the lumen (37, 38), but do not migrate to MLN and do not prime naïve T cells (39).

During pathogenic invasion, intestinal macrophages and DCs adopt a different phenotype, characterized by the production of pro-inflammatory cytokines and chemokines that can trigger host protective immune responses (26). However, excessive activation of intestinal

myeloid cells is associated with chronic inflammatory conditions, such as inflammatory bowel disease (IBD), indicating that their activation must be tightly controlled (40).

1.2.3 Intestinal eosinophils

Eosinophils are a granulocyte population that are typically thought to participate in anti-parasite defence (41). However, direct evidence for this role is scarce, especially for eosinophils in the intestine (42). As eosinophils in the large intestine express the surface molecules CD11b and F4/80, they may have been mistaken for macrophages, which also express these markers (43). More recently it has been shown that eosinophils co-express the cell surface markers CCR3 (44) and Siglec-F (45), which has allowed accurate identification of these cells. Using these markers, large numbers of eosinophils have been detected in the intestinal lamina propria leukocyte (LPL) compartment (43, 46). Recent reports demonstrating that the eosinophil-selective chemokine CCL11 is up-regulated in paediatric UC patients (47), and that eosinophils accumulate within inflammatory lesions in some mouse models of intestinal inflammation (47-49), suggest that eosinophils could play an important role in intestinal immunity and pathology.

Intestinal eosinophils have relatively long lifespan compared to eosinophils in circulation, surviving for >7 days (46). Whilst the cytokines IL-5, IL-3, and GM-CSF are important for eosinophil output, their recruitment to the intestine during steady state and during inflammation is largely dependent on CCL11 and CCR3 (42). CCL11 is a cytokine constitutively expressed by mononuclear cells in the lamina propria of the intestine (50, 51), and increases further during inflammation (47, 52). Furthermore, mice deficient in CCR3 also showed severely reduced eosinophil levels in the intestine both during steady state as well as during inflammation (44, 52, 53).

Studies to decipher eosinophil function *in vivo* have been hampered by the fact that most eosinophil depletion studies were performed using antibodies against markers that were not entirely eosinophil-specific (42). However, recently two eosinophil-deficient transgenic mouse strains were generated: ΔdblGATA-1 (54) and TgPHIL (55). Somewhat surprisingly, these mice do not suffer from any apparent deficiencies and do not have impairment in intestinal worm expulsion during primary infections (42).

Interestingly, a study recently described an antibacterial function of intestinal eosinophils where they rapidly released mitochondrial DNA to trap extracellular bacteria (56). Together with experiments reporting eosinophilia and eosinophil peroxidase-mediated tissue damage in dextran sodium sulphate (DSS) colitis, these data suggest that intestinal eosinophils may participate in a range of protective and inflammatory responses in the gut (42). Furthermore, eosinophils have been shown to play a role in both the regulation of colonic epithelial barrier function (57) as well as the resolution of inflammation (58). Given the above evidence, the role of eosinophils in the intestine clearly requires further investigation.

1.2.4 Intestinal lymphocytes

There are many effector cells of the adaptive immune system in the intestine including intraepithelial lymphocytes (IELs), B cells, as well as T cells.

B cells make up an abundant cell population in the gut, with a striking preponderance of IgA-secreting plasma cells (59). Whilst the induction of IgA production occurs mainly in the Peyer's patches, class switching to IgA also occurs in isolated lymphoid follicles and mesenteric lymph nodes (60). IECs also help to promote class-switching towards the

production of IgA through the production of factors such as TGF-β, BAFF and APRIL (61). Finally, IgA is transported across the epithelium and released into the mucus layer in an IEC-mediated process of transcytosis, where it acts to reduce bacterial attachment and translocation (61, 62).

IELs are a population of T cells found within the intestinal epithelium and are extremely abundant in the intestine (63). There are several subsets of IELs, including T cells characterized by CD8αα TCRαβ cell surface marker expression that release immune regulatory cytokines such as IL-10 and TGF-β (64). These cells are protective during the T cell transfer model of colitis (65, 66). Conversely, IELs characterized by TCRγδ expression recognize class-I like molecules on stressed IECs (64). TCRγδ IELs can function without TCR activation (67, 68) and have been reported to produce IL-17A in response to infection (69, 70). In addition, they have also been shown to promote resolution and repair through secretion of keratinocyte growth factor (71) and by restraining excessive TCRαβ T cell activation (72).

Many T cells expressing TCRαβ and CD4 surface markers can be found in the intestinal lamina propria (73). Upon TCR activation in the presence of distinct cytokine environments, naïve $CD4^+$ T cells can differentiate into a number of effector subsets including Th1 and Th2 (74), T helper 17 (Th17) (75), as well as Tregs (76, 77).

Th1 cells express the transcription factor Tbet (T box expressed in T cells) and secrete cytokines like IFN-γ and lymphotoxin to combat intracellular pathogens. (74, 78-80). Th2 cells express the transcription factor GATA-3 and secrete cytokines IL-4, IL-5, and IL-13 to fight against extracellular pathogens (74, 78, 80).

In addition to Th1 and Th2 populations, there is an enriched population of Th17 cells in the intestine (81, 82). These cells express IL-23R (83), polymorphisms of which have

been found to be associated with IBD in humans (84, 85). Furthermore, Th17 cells produce cytokines such as IL-17A, IL-17F, IL-21 and IL-22, all of which have been found to be upregulated in IBD patients (86-91). However, experimental studies in mouse models of IBD have reported conflicting roles for IL-17A and IL-17F in intestinal inflammation. In acute colitis induced following administration of the toxic chemical DSS, IL-17A was protective, whereas IL-17F was pathogenic (92). However, in *Helicobacter hepaticus*-induced innate immune colitis neutralization of IL-17A with a specific monoclonal antibody attenuated colitis, indicating that IL-17A was promoting colitis in this model (93). Furthermore, in the T cell transfer model of IBD, IL17A and IL-17F were reported to have redundant pro-inflammatory roles (94). Another Th17 cell-derived cytokine reported to contribute to intestinal pathology is IL-21, which may drive tissue remodelling through induction of matrix metalloproteinases (95). In contrast, IL-22 has been reported to protect against intestinal inflammation in several models, by inducing epithelial protective responses such as antimicrobial peptide production and by promoting wound healing by IECs (96). Thus, Th17 cytokines may have diverse effects on intestinal homeostasis depending on the context in which they are produced and the presence of addition factors in the local environment.

Another $CD4^+$ T cell population enriched in the intestine are Treg cells, which comprise both $FOXP3^+$ as well as $FOXP3^-$ cells (97). The transcription factor FOXP3 is important for immunoregulation as defective FOXP3 results in inflammatory disease in mice and humans (97). In particular, human patients with mutations in *FOXP3* develop immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which often manifests with intestinal inflammation (97). One group of $FOXP3^+$ Treg cells, termed natural Treg, acquire the expression of this transcription factor during development in the thymus, whilst another group of $FOXP3^+$ Treg cells are induced in the intestine from

naïve $CD4^+$ T cell precursors through TGF-β (34) $FOXP3^+$ Treg cells are abundant in the intestine and can control inappropriate responses against dietary and microbial stimuli (97). Furthermore, Treg cells have been shown to control intestinal inflammation in murine colitis models (98-100). The production of immune suppressive factors, particularly TGF-β and IL-10, plays a critical role in the control of intestinal inflammatory responses by Treg cells (101, 102). The importance of IL-10 is further highlighted by the fact that mice deficient in IL-10 develop spontaneous colitis (103) and by recent findings that mutations in *IL-10R* subunits are associated with early-onset severe IBD in humans (104).

1.2.5 Innate lymphoid cells

Recent studies have identified novel populations of tissue resident leukocytes, lacking cell lineage-specific markers that can rapidly respond to factors released during infection or stress. Due to their lack of defined lineage markers, these cells have been termed innate lymphoid cells (ILCs) (93). ILCs could be classified into several subsets, all of which share the requirement of the transcriptional repressor Id2 during development and lack rearranged antigen receptors (105). ILC subsets include cells that share characteristics of NK-like cells or lymphoid tissue-inducer cells, as well as several newly described cell types (105).

In terms of intestinal homeostasis, a population of $CD90^+$ $CD4^-$ ILCs have been identified that accumulated and drove innate intestinal inflammation in the inflamed colons of $Rag^{-/-}$ mice infected with *H. hepaticus* (93). These ILCs expressed IL-23R and the Th17 transcription factor RORγt and they secreted IL-17A, IL-22 and IFN-γ upon stimulation with IL-23. A similar population of ILCs was shown to provide early protection from

Citrobacter rodentium infection, by secreting IL-22 (106). Other ILC populations were identified in different experimental systems, including ILCs that secreted IL-4 and IL-13 in response to parasite infections (107-109). Thus, ILCs are tissue resident leukocytes that respond rapidly to stress or infection by producing cytokines normally associated with different types of effector T cell subsets. They appear to be involved in early protection against pathogens, but may also contribute to tissue damage during inflammation.

1.3 Pattern recognition receptors

The major classes of PRRs include the TLRs, the Nod-like receptors or nucleotide-binding domain and leucine-rich repeat containing proteins (NLRs), the retinoid acid-inducible gene-1-like receptors (RLRs), as well as the C-type lectin receptors (CLRs). Together they provide sensors for detection of all types of pathogens, both intra- and extra-cellular (110).

1.3.1 Toll-like receptors

The most well characterised PRR family is the TLR family. TLRs recognise a range of conserved molecules present in bacteria or viruses but absent in host cells, such as lipopolysaccharides (LPS), flagellin and ssRNA (110). In the intestine, TLR sensing is important in both IECs and haematopoietic cells and they complement each other to maintain intestinal homeostasis (17, 111, 112). An important role for TLRs in protecting the intestine against injury was highlighted by findings that mice lacking *Tlr2*, *Tlr4*, *Tlr5* or *Tlr9* or their common downstream TLR signalling adaptor *Myd88,* exhibited increased susceptibility to DSS colitis (113-118).

This suggests the TLR sensing of the intestinal microbiota plays a key role in intestinal homeostasis. Indeed, TLR signals have been shown to induce proliferative and anti-apoptotic factors in IECs, to promote IEC restitution, and to fortify intercellular tight junctions (112). In addition, Paneth cells have been shown to directly sense enteric bacteria through TLR activation and this sensing induced AMP production that limited bacterial translocation (15, 119). Furthermore, TLR2 signalling in Treg cells has been found to be important in protection against T-cell-mediated colitis in mice administered Polysaccharide A derived from the human commensal *Bacteroides fragilis* (120). Although TLR signals in IECs are tissue-protective in the gut, other studies have shown that excessive activation of TLR/MyD88 signalling in the haematopoietic cells in the intestine can drive chronic intestinal inflammation (111). Similarly, dendritic cells sensing of intestinal microbiota components through TLR/MyD88-dependent signalling was shown to be essential for T-cell proliferation and intestinal pathology (121). Together, these data suggest that TLR activation in the intestine is complex and that downstream signalling in different cellular compartments can trigger either protective or pathogenic responses.

1.3.2 Nod-like receptors

The NLRs are cytosolic pattern recognition receptors with orthologs that can be found in both animals and plants (122, 123). There are currently 22 known human NLR proteins and at least 34 mouse NLR genes (124). They are mainly expressed on immune cells such as myeloid cells and T cells, although some are also expressed by non-haematopoeitic cells, such as endothelial cells and epithelial cells (125, 126). NLR proteins are characterized by a variable N-terminal protein-protein interaction domain, a central

nucleotide-binding oligomerization domain (NOD), and a C-terminal leucine-rich repeat (LRR) domain.

NOD1 and NOD2 detect breakdown products of bacterial cell wall peptidoglycan termed meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively (127-130). These ligands may be introduced via type III (T3SS) or type IV (T4SS) secretion systems in bacteria (131), or via internalization by endocytosis (132, 133). In addition to detecting bacteria, recent reports have also shown that NOD2 can participate in anti-viral response by activation of MAVS and IRF to promote IFN-β production in response to viral ssRNA (134).

Interestingly, polymorphisms in *NOD2 (CARD15)*, and to a lesser extent *NOD1,* have been associated with susceptibility to IBD (135-137). Similarly, mice deficient in *Nod1* or *Nod2*, or bearing a CD-associated susceptibility allele of human *NOD2*, showed increased susceptibility to DSS induced colitis (138, 139). The proposed mechanisms involved through which *NOD2* mutations predispose to IBD will be discussed in detail in section 1.3.3. In addition to IBD, *NOD1* mutations are also associated with asthma (140) and *NOD2* mutations are associated with Blau syndrome (141), GvHD (142), and early-onset sarcoidosis (143), further highlighting the roles of these NLRs in regulating inflammatory responses.

NOD1 and NOD2 signal in an analogous fashion to TLR, activating RIP2 kinase, which triggers a signalling cascade that results in the activation of NF-κB and MAPK (126). However, many other NLRs respond to various stimuli by forming an inflammasome and subsequently inducing the activation of caspase-1 (Figure 1.1). Inflammasomes are multi-molecular cytosolic complexes made up of NLR, an adaptor protein ASC, and caspase-1

(144). Each inflammasome is named after the NLR in it, such as the NLRP1 inflammasome, the NLRP3 inflammasome, and the NLRC4 inflammasome (Figure 1.1).

The adaptor protein ASC has a N-terminal PYD domain and a C-terminal CARD domain, and through homotypic protein-protein interactions they are able to recruit PYD- and CARD-containing proteins such as the NLRs and caspase-1 (144). The inflammasome complex activates pro-caspase-1 through autocleavage (145), and active caspase-1 then promotes the processing of pro-IL-1β and pro-IL-18 into their mature forms, which are secreted via an unconventional secretion pathway (146). This caspase-1-dependent unconventional secretion pathway has been suggested to be involved in the secretion of a number of inflammatory, cytoprotective, or tissue repair proteins lacking a secretion signal (147).

It is thought that for optimal IL-1β secretion, cells need to receive their first signal from TLR stimulation, which results in the synthesis of pro-IL-1β (144, 148). A second danger signal is then required to trigger the formation of the inflammasome, which results in caspase-1 activation and the subsequent maturation and secretion of IL-1β (149). To date, the exact role of ASC has not yet been fully elucidated and it is not clear whether all inflammasomes require ASC. For example, in *in vitro*-reconstituted NLRP1 inflammasomes, ASC was not essential, but instead functioned to enhance caspase-1 activation (150). In other reports, ASC has been shown to oligomerize into a supramolecular structure (pyroptosome) through its PYD domain, subsequently activating caspase-1 (151). In addition to processing and secretion of IL-1β and IL-18, activated caspase-1 can also induce an inflammatory form of cell death named pyroptosis, which results in cell lysis and concomitant release of IL-1β and IL-18 (152).

MDP, a derivative of bacterial peptidoglycan, is a well-known NLR ligand that induces the activation of NOD2, NLRP1, and NLRP3 (149). It has been shown that MDP stimulation results in the association of NOD2 with NLRP1, which then leads to caspase-1-dependent IL-1β secretion (153). In addition, Muruve *et al.* have shown that internalized adenoviral DNA also induced maturation of pro-IL-1β through a NLRP3- and ASC-dependent process. Interestingly, it was shown in the same study that inflammasomes could also be activated by bacterial, viral, and mammalian DNA in the cytoplasm through an ASC-dependent (but NLRP3-independent) process (154).

Thus, much remains to be learned about how the activation of different types of inflammasomes is regulated in response to distinct stimuli.

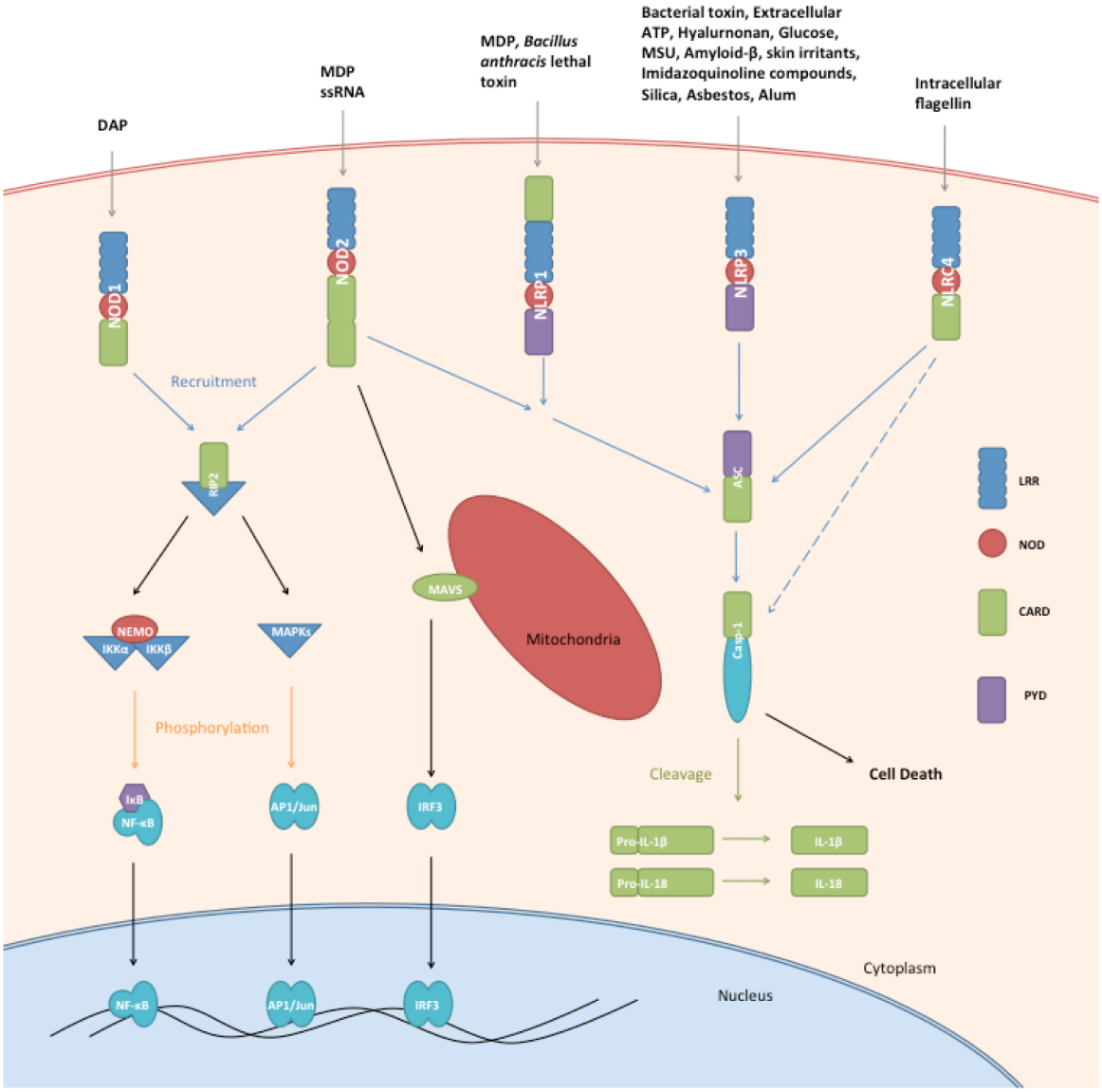

Figure 1.1 NLR Signalling Pathways (124, 126, 146, 149) NOD1 senses intracellular DAP and NOD2 senses MDP. Both NOD1 and NOD2 then recruit the adaptor protein RIP2, which subsequently binds to the IKKs resulting in the translocation of NF-κB into the nucleus. Also, recruitment RIP2 can also result in the activation of the MAPKs, resulting in the translocation of AP1/Jun into the nucleus. Upon activation by ssRNA, NOD2 can also interact with MAVS, leading to the activation of IRF3. NLRP1, NLRP3, and NLRC4 (and also NOD2) can form inflammasomes upon activation by binding the adaptor protein ASC and caspase-1. These multi-molecular complexes activate caspase-1, which mediates processing of pro-IL-1β and pro-IL-18 into their active forms for secretion. Inflammasome-mediated caspase-1 activation can also result in an inflammatory form of cell death termed pyroptosis.

1.3.3 Role of NOD2 in intestinal homeostasis

Although the aetiology of IBD is not yet known, the innate immune system has been shown to be an important player ever since three common variations in the LRR region of *CARD15*, the gene that encodes NOD2, were identified to be the susceptibility to CD (135, 136). In fact**,** individuals homozygous or compound heterozygous for the NOD2 susceptibility alleles have an odds ratio of 17.1 (155). Of the susceptibility alleles, the L1007fsinsC frameshift mutation confers the greatest risk (156) and has been shown to be defective in MDP sensing and the resulting NFκB activation (128, 130). Although other common CD susceptibility mutations such as R702W and G908R are less studied, R702W has been associated with structuring behaviour and granulomas (157), whilst G908R has been associated with ileal involvement (158).

Whilst the mechanism through which mutations in *NOD2* predispose to CD is not yet clear, it has been reported that there is an overproduction of NOD2 in the inflamed mucosa of CD patients, which subsequently contributes to increased activation of NF-κB (159). However, patients with ileal CD and patients with *NOD2* susceptibility alleles also showed reduced α-defensin expression in the intestine (160, 161), suggesting that impaired control of intestinal bacteria could contribute to IBD susceptibility (161, 162).

Recognition of MDP induces a conformational change in NOD2, resulting in oligomerization and exposure of the CARD domain, which allows CARD-CARD binding with a downstream signal adaptor molecule serine threonine kinase RIP2 (also known as RIPK2/RICK) (123, 163)(Figure 1.1). Ubiquitinylation of RIP2 is important in the recruitment of TAK1, an essential step for RIP2-mediated NF-κB activation as well as mitogen-activated protein kinase (MAPK) activation (164, 165). Interestingly, NOD2 can also respond to viral ssRNA, through a completely separate pathway. Upon binding viral

ssRNA, NOD2 is translocated to the mitochondria and interacts with MAVS, ultimately resulting in the activation of IRF3 and production of interferon-β (134).

NOD2 is regulated by an array of intracellular proteins including the negative regulators Erbin (166), Centaurinb1 (167), AAMP (168), and Rac1GTPase (169), as well as positive regulators such as GRIM-19 (170). Furthermore, interaction of NOD2 with distinct binding partners, such as CARD9 or Bid, could lead to activation of different pathways such as p38/c-Jun or ERK/NF-κB, respectively (171)(172).

Indeed, there is evidence that NOD2 could bind NLRP1 following activation by MDP, resulting in a complex that binds pro-caspase-1, leading to caspase-1 activation and processing and secretion of IL-1β and IL-18 (153). Furthermore, a CD-associated frameshift mutation in the LRR region of *NOD2* causes spontaneous association between NOD2 and NLRP1, thus resulting in enhanced IL-1β secretion (173). These studies highlight the role of LRR region autoinhibiting NOD2 activity as well as its interaction with inflammasomes. However, other findings have suggested that NOD2 interacts with NLRP3 instead of NLRP1 (174, 175). Thus, the precise interactions between normal and CD-associated variants of *NOD2* with inflammasome components require additional study.

Despite numerous studies pointing towards activation of pro-inflammatory signalling pathways after activation of NOD2, other experimental evidence suggests that NOD2 may negatively regulate inflammatory responses in the intestine *in vivo*. For example, administration of MDP protected mice from the development of chemically-induced colitis (176). In addition, another study reported that compared to wild type (WT) mice, Nod2-deficient mice had increased susceptibility to colitis induced by intra-rectal administration of *Escherichia coli* expressing an ovalbumin peptide to mice that had

previously received ovalbumin peptide-specific T cells (177). Furthermore, over-expression of *Nod2* in transgenic mice rendered them completely resistant to peptidoglycan-induced colitis and partially resistant to trinitrobenzene sulfonic acid (TNBS)-induced colitis (178), correlating with a decreased IL-12p70 response to peptidoglycan, but not to other TLR ligands (178). Recently, Nod2's anti-inflammatory role was again highlighted in a mouse model of necrotizing enterocolitis, which showed that MDP activation of Nod2 inhibited TLR4 signalling in IECs, thus ameliorating the condition (179). Nod2's suppressive action on TLR signalling has been shown to act through IRF4 (176) and IRAK-M (180). Alternatively, the production of cryptins (181) as well as α-defensins (182) following NOD2 activation could also serve as a mechanism by which NOD2 can prevent excessive inflammation. The above data, together with the initial characterization that *NOD2* mutants associated with the susceptibility to CD are deficient in MDP recognition (128), offer a potential mechanism by which CD patients suffer from uncontrolled intestinal inflammation.

NOD2 has also been shown to be able to modulate adaptive immune responses. For example, upon sensing bacterial MDP by NOD2, human DCs became primed to induce secretion of IL-17A, but not IFN-γ, by memory Th cells (183). MDP in this case was shown to enhance bacterial TLR agonist induction of IL-23 and IL-1, which are Th17 promoting cytokines (183). This is especially interesting since the IL-23/IL-17 axis has been shown to play an important pathogenic role in intestinal inflammation (86). Furthermore, NOD2 in T cells has also been shown to play a role in defence against pathogens such as *Toxoplasma gondii*. (184). Interestingly, studies in a model of *Borrelia burgdorferi* infection have reported that whilst early NOD2 activation during infection may be pro-inflammatory, responses at latter stages of infection are anti-inflammatory (185).

Recently, NOD2 has been linked with autophagy, a complex intracellular degradation mechanism that functions to remove damaged organelles or misfolded proteins by enveloping them in double-membrane vacuoles that are subsequently fused with lysosomes (186). More specifically, NOD2 has been shown to bind a key autophagy protein ATG16L1 (187), which is striking because polymorphisms in *ATG16L1* have also been associated with CD (156). Moreover, Travassos and colleagues showed that in DCs exposed to bacteria, NOD2 co-localizes with ATG16L1 at the site of bacterial entry, which agrees well with reports showing NOD2's association with the cell membrane being important for its activation (188). Similarly, the CD-associated NOD2 frameshift mutation leads to the loss of the cell membrane-localization (188), and when this *NOD2* frameshift mutation was expressed in DCs it resulted in a reduced autophagy response to *Shigella* (187). A separate study also confirmed NOD2's association with autophagy as NOD2 stimulation induced autophagy as well as antigen presentation in DCs (189). In fact, DCs isolated from patients expressing *NOD2* or *ATG16L1* polymorphisms associated with CD failed to express MHC II and to respond to *Salmonella* (189, 190). Furthermore, autophagy-impaired macrophages exhibited reduced bacterial killing capacity against adherent-invasive *E. coli* (AIEC) (191). Whilst these studies clearly highlight that NOD2 can interact with the autophagy pathway to control bacterial handling in DCs, the consequences of this activity for intestinal homeostasis *in vivo* remain to be determined.

Together, these data show that NOD2 activation can have diverse and complex effects on intestinal homeostasis and they illustrate why deciphering the mechanism through which NOD2 polymorphisms predispose to CD has proved so elusive.

1.3.4 Role of NLRP3 and the inflammasome in intestinal homeostasis

The most well-characterized inflammasome to date is the NLRP3 inflammasome. NLRP3 detects a great range of stimuli including endogenous and environmental danger associated molecular patterns (DAMPs), as well as microbe associated molecular patterns (MAMPs) (192, 193) (Figure 1.1), although no direct binding to NLRP3 has yet been shown (194). Many studies point towards a two-step model in NLRP3 inflammasome activation. First, NF-κB activation in response to microbial stimulation induces *NLRP3* expression, either directly through PRRs (148) or indirectly through endogenous cytokines (195). Subsequently, a second signal such as bacterial toxins (196), ATP, or particulate matter such as asbestos and silica (195), activate NLRP3 to form the inflammasome complex. However, it has also been shown that TLR stimulation can also result in an endogenously released ATP, which can also promote the secretion of IL-1β and IL-18 (197).

One way NLRP3 could recognize such diverse ligands is by sensing conserved downstream signals generated by the DAMPs and MAMPs. Three mechanisms of activation have been proposed. One mechanism posits that pore formation (for example, by bacterial toxins) would not only allow extracellular ligands to gain access to the cytoplasm but also result in K^+ efflux (198), which has been shown to be crucial for the activation of both NLRP3 as well as NLRP1 inflammasomes (199). Another hypothesis suggests that lysosomal damage could explain how crystalline structures are sensed, as a damaged lysosomes result in the release of cathepsin B, which could lead to NLRP3 activation (200, 201). Finally, ROS generation could be another common mechanism by which NLRP3 is activated (202-205). In fact, autophagy has been suggested to negatively regulate inflammasome activation via inhibition of ROS generation by dysfunctional

mitochondria (202). This concept is supported by another study where the ablation of an autophagy gene, ATG16L1, in mouse haematopoietic cells led to increased inflammasome activation and IL-1β secretion in response to LPS (206).

Recently, several studies highlighted associations between *NLRP3* polymorphisms and CD susceptibility (207-209). Since *NLRP3* is mainly expressed in granulocytes and monocytes (210), Villani *et al.* assessed peripheral blood monocytes carrying the *NLRP3* risk alleles both for their expression of *NLRP3* and for their ability to secrete IL-1β. Homozygosity for the rs4353135 risk allele was found to be associated with a lower level of *NLRP3* expression, and homozygosity in the rs6672995 allele with a lower level of IL-1β secretion when stimulated with LPS (207). However, this conclusion is in contrast to previous studies suggesting a pro-inflammatory role of the inflammasome-dependent cytokines such as IL-1β and IL-18 in experimental models of intestinal inflammation (211-214). Conversely, recent findings suggest that Nlrp3 indeed plays a protective role in the intestine, as *Nlrp3*$^{-/-}$ mice suffered from exacerbated colitis flowing administration of DSS (215, 216). The exacerbated disease observed in *Nlrp3*$^{-/-}$ mice after DSS treatment was attributed to a lack of IEC protection mediated by IL-18 (215-217). Moreover, IL-18 secretion downstream of Nlrp3 inflammasome activation was also shown to protect against colitis-associated tumour formation (216, 218). Collectively, these studies suggest a predominantly protective role for the inflammasome activation in intestinal homeostasis.

1.4 Intestinal host-microbe interactions

1.4.1 Intestinal microbiota in health and disease

The microbiota comprises complex communities of microorganisms that inhabit the body surfaces (219). In the intestine during steady state, the bacterial microbiota help the host to digest plant polysaccharides and other dietary substances (220). In the lower intestines bacteria can reach densities of $\geq 10^{12}/cm^3$ intestinal contents (219). Given that these dense microbial communities are only separated from the host lamina propria by a single layer of IECs, this poses a great challenge for the host immune system as it needs to preserve this symbiotic relationship and at the same time be ready to respond rapidly to opportunistic or pathogenic invasions.

Indeed, the microbiota has recently become a major focus in gastrointestinal research (221). This has become apparent as germ-free mice revealed the importance of microbiota on the lymphoid structure development as well as epithelial function (219). For example, sensing of the microbiota through MyD88-dependent signals induces epithelial antimicrobial proteins (222, 223) and also enhances epithelial cell proliferation (224, 225).

Furthermore, the composition of the microbiota can also influence the make up of intestinal T cell subsets. For example, mono-colonization with segmented filamentous bacteria (SFB), caused an accumulation of Th17 and Th1 cells in the intestine (226, 227). Conversely, a cocktail of *Clostridium* species was shown to induce the expansion of lamina propria and systemic $Foxp3^+$ Tregs in mice (228). Furthermore, polysaccharide A from the human commensal species, *B. fragilis,* has also been shown to induce Treg development and prevent the expansion of Th17 cells (120, 229). Moreover, recent data

have suggested that the microbiota can even have extra-intestinal effects as demonstrated by observations that colonization with distinct microbiota influenced susceptibility to mouse autoimmune models, including type I diabetes (230), arthritis (231), and multiple sclerosis (232).

However, it has also been shown that the immune system is able to influence the make up of the microbiota. For example, in immunocompromised hosts such as *Nlrp3*$^{-/-}$ and *Nlrp6*$^{-/-}$ mice, changes in the configuration of the microbiota resulted in exacerbated hepatic steatosis and inflammation (233). Therefore, such a reciprocal influence between the microbiota and immune compartments requires one to consider both compartments when seeking to understand intestinal homeostasis as well as intestinal diseases.

1.4.2 Inflammatory bowel disease

IBD is a chronic, relapsing and remitting intestinal inflammatory disorder thought to be mediated by dysregulated host-microbiota interactions. (234). Although the exact aetiology is not known, multiple factors such as the host genetics, the immune system as well as the gut environment have been shown to be important (3). Furthermore, chronic inflammation in IBD patients also predisposes them to an increased risk of developing colorectal cancer (235, 236).

The major types of IBD include CD as well as ulcerative colitis (UC). CD can involve any part of the gastrointestinal tract and is transmural, characterized by dense infiltration of lymphocytes and macrophages (237, 238). Furthermore, granulomas, fissuring ulceration, as well as submucosal fibrosis could also be observed in CD patients (238). On the other hand, UC is only limited to the mucosal layer of the colon (237), and is

characterized by infiltration of lymphocytes and granulocytes (238). Other histological features of UC patients include loss of goblet cells, ulcerations, and crypt abscesses (238).

Current treatment strategies include blockade of inflammatory cytokines such as TNF-α, however, this is not effective in many patients whilst it may predispose to secondary infections in others (239). Other immune suppressive drugs used include 5'ASA for UC and corticosteroids for CD (240). Unfortunately, despite these treatment strategies many IBD patients ultimately require surgical intervention (240). Therefore, further research into the aetiology and the subsequent development of novel therapeutics is required to improve the prognosis for patients suffering from IBD.

1.5 Mouse models of intestinal infection and inflammation

1.5.1 *Helicobacter hepaticus*-induced typhlocolitis

Helicobacter hepaticus (*H. hepaticus*), is a Gram-negative spiral rod with bipolar sheathed flagella, that is a common member of the mouse intestinal microbiota (241). Although it does not cause invasive infections, it persistently colonizes the mucous layer in the cecum and colon (242). Whilst *H. hepaticus* does not cause overt disease in most immune competent mouse strains, it has pathogenic potential in susceptible hosts, for example, it has been associated with hepatocellular carcinoma in A/JCr mice and can trigger chronic intestinal inflammation in *Il10*$^{-/-}$ mice (241). *H. hepaticus* has been described as a 'pathobiont' and possesses a pathogenicity island containing a bacterial Type VI secretion system (T6SS) (243). Furthermore, *H. hepaticus* produces a number of virulence factors including a cytolethal distending toxin that is delivered intracellularly and induces host cell death (244, 245), as well as enzymes providing resistance to oxidative stress (246, 247).

Although WT mice infected with *H. hepaticus* develop strong local and systemic adaptive immune responses towards *H. hepaticus,* a protective Treg response develops which prevents excessive inflammation (248). This regulatory response can be circumvented by administration of αIL-10R mAb throughout the course of infection, leading to the development of chronic inflammation in the caecum and colon (249). Pathology is characterized by epithelial hyperplasia, marked infiltration of mononuclear leukocytes into the lamina propria and by oedema, and can lead to development of abscesses and ulceration (249). Disease in this model is known to be dependent on IL-23, which drives mixed Th1 and Th17 responses in the intestine (249). Moreover, cytokines important in driving disease in this model, such as IL-23 and TNF-α, are also implicated in human

IBD (84, 85, 250). Since IBD is a chronic disease where a genetically susceptible host might respond inappropriately to an otherwise well tolerated intestinal bacterium, the *H. hepaticus* + αIL-10R mAb model offers a unique opportunity for us to investigate disease mechanisms in an immune competent host.

Furthermore, *H. hepaticus* can also cause chronic intestinal inflammation in susceptible mice lacking immune regulatory circuits such as 129SvEv *Rag*$^{-/-}$ mice, where *H. hepaticus* triggers innate immune-mediated typhlocolitis that can eventually progress to colon cancer (100, 251-253). This innate immune IBD-like pathology is characterized by epithelial hyperplasia and the formation of crypt abscesses that are associated with marked granulocyte accumulation in the tissue. More recently, the development of innate immune typhlocolitis was shown to be dependent on a novel population of IL-23R^{+} ILCs that secreted IL-17 and IFNγ in response to IL-23 (93). Interestingly, a similar population of ILCs has also been identified in the inflamed intestine in CD patients, and shown to produce a similar panel of inflammatory cytokines in response to IL-23 (254). This innate intestinal inflammation was also shown to be critically dependent on MyD88 signaling within the haematopoeitic compartment, indicating that signaling through TLR and/or IL-1 cytokine family receptors plays a key role in pathology. Thus, the *H. hepaticus* innate immune-mediated IBD provides an excellent model with which to selectively interrogate innate immune pathways in intestinal inflammation, which is of particular interest since mutations in many genes linked to innate immunity and bacterial handling have been linked to IBD susceptibility (255).

1.5.2 Dextran sodium sulphate-induced colitis

DSS is thought to be toxic to the gut epithelial cells (256), and DSS administration in drinking water results in destruction of epithelial cells and neutrophil infiltration (257). Histologically this is characterized by loss of crypt structure and mucosal erosion (258). IBD-relevant features such as cryptitis and crypt abscesses have also been reported, although not widely reported in this model (257). Clinically, mice suffer from acute colitis that resulting in bloody diarrhoea and weight loss (259). Upon removal of DSS in drinking water, most mice eventually recover from colitis (260).

The adaptive immune system does not seem to play a role in this model as mice lacking T and B cells also suffer from severe intestinal inflammation (261). Furthermore, several factors of the innate immune system have been found to be protective in this model such as TLRs (113) and NLRs (215, 216, 262). Therefore this model is ideal for examining the role played by innate immune responses to intestinal injury.

1.5.3 *Citrobacter rodentium*-induced typhlocolitis

The non-invasive murine pathogen *C. rodentium* belongs to a group of Gram negative attaching and effacing (A/E) pathogens that includes the human pathogens Enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (263). These bacteria share many common virulence factors encoded on a pathogenicity island called locus of enterocyte effacement (LEE) and *C. rodentium* is widely used as a model to study the interactions between host and A/E pathogens because human EPEC and EHEC strains infect mice poorly (263, 264).

C. rodentium initially colonizes the caecum, and subsequently infects the distal colon (265). Like other A/E pathogens, *C. rodentium* colonizes the IECs by effacement of brush-border microvilli and attachment of bacteria to the host plasma membrane. This is achieved using a T3SS, which injects various effector proteins into host epithelial cells, including the translocated intimin receptor (Tir) that facilitates tight binding of the bacterium (266). Other translocated effector proteins modulate host signalling and inhibit water reabsorption, leading to further effacement of microvilli and the destruction of tight junctions, causing acute intestinal inflammation of the caecum and colon, as well as diarrhoea (267). *C. rodentium*-induced intestinal pathology reproduces several features of IBD, including infiltration of lymphocytes and macrophages in the lamina propria at the base of the crypts (268).

Both innate and adaptive immune responses are required for effective protection from *C. rodentium*. TLR sensing has been shown to be important in host defence against *C. rodentium* (269-271), as MyD88-deficient mice suffered from higher bacterial outgrowth due to defective epithelial turnover and impaired recruitment of neutrophils and macrophages (271). This exacerbated disease in MyD88-deficient mice is characterized by intestinal tissue necrosis, severe inflammation, systemic bacterial dissemination, and death within two weeks (271). Similarly, though TLR2-deficient mice did not suffer from increased *C. rodentium* burden, but they also experienced exacerbated disease such as intestinal cell death, ulcerations, bleeding, as well as increased mortality (270).

More recently, both IL-17 and IL-22 have been implicated in innate immune protection against *C. rodentium* as early as 4 days post infection (106, 272, 273). IL-17, conferred protection both early following infection through innate Th17 cells (273), as well as later during infection through adaptive Th17 cells (274). IL-22 is produced in the intestine

following *C. rodentium* infection to limit damage to IECs and systemic spread of bacteria through the induction of Reg family of antimicrobial proteins in IECs (272). More recently, the IL-22 induction following *C. rodentium* infection has been found to be produced by ILCs in response to lymphotoxin (106, 275-277). Ultimately, after about 3 weeks, an IgG-dependent specific antibody response is required to clear *C. rodentium*, as mice deficient in T or B cells eventually die (278).

Indeed, *C. rodentium* is a very well characterized intestinal pathogen, and the rapidly expanding understanding of the host-pathogen interaction in this model makes it an especially interesting tool to evaluate the functional relevance of NLRs (264).

1.6 Aims

The overall aim of this thesis is to examine the role NLRs play during the induction as well as during the resolution of intestinal inflammation. In particular, the two NLRs associated with IBD, NOD2 and NLRP3, will be investigated in a variety of murine colitis models to assess their contribution to or protection from intestinal inflammation. Since the identification of NOD2 mutations and susceptibility to IBD, extensive research has been done in characterizing its potential involvement in the induction of disease (173). However, little has been done to examine its role in the resolution phase of intestinal inflammation. As IBD is a relapsing and remitting disease, this might potentially overlook valuable therapeutic approaches that could modulate the activities of the strongest IBD-associated gene to date (173). Therefore, my first aim was to develop resolving intestinal inflammation models in order to determine whether Nod2 plays a role during the resolution phase of inflammation.

Secondly, recent association of NLRP3 SNPs with CD have further highlighted the importance of NLRs in intestinal inflammation (207-209). However, not much is known about NLRP3's role in the intestine. Therefore, my second aim was to assess the role Nlrp3 plays in various mouse intestinal inflammation models. Furthermore, as Nlrp3-mediated effector responses are poorly characterized in the intestine, I aim to investigate novel mechanisms involved in the regulation of intestinal immune homeostasis by Nlrp3 using both *in vivo* as well as *ex vivo* techniques.

Chapter 2 – Material and methods

2.1 Mice

2.1.1 Mouse maintenance

C57BL/6, 129SvEv, C57BL/6.129S1/Sv *Nod2*$^{-/-}$, and C57BL/6 *Nlrp3*$^{-/-}$ mice were bred and maintained at the University of Oxford under specific pathogen-free (SPF) conditions. C57BL/6.129S1/Sv *Nod2*$^{-/-}$ mice were acquired from The Jackson Laboratory (Maine, USA). C57BL/6 *Nlrp3*$^{-/-}$ mice were generated as described before (279).

2.1.2 Irradiation bone marrow chimeras

C57BL/6 WT, or *Nlrp3*$^{-/-}$ recipient mice were γ-irradiated 2x (5.5 Gy, 550 rad) and intravenously reconstituted with 10×10^6 bone marrow cells isolated from either WT C57BL/6, or *Nlrp3*$^{-/-}$ mice. They were then left for >8 weeks for the injected bone marrow cells to reconstitute.

2.2 *Helicobacter hepaticus*

2.2.1 Culture

Culture of *H. hepaticus* NCI-Frederick isolate 1A (Hh1A; strain 51449; American Type Culture Collection) was achieved on blood agar plates containing trimethoprim (5μg/ml), vancomycin (10 μg/ml) and polymyxin B (25 IU/ml) (TVP; all from Oxoid, UK) in an anaerobic jar placed under microaerophilic conditions at 37°C for 48-72 h (280). For larger cultures, *H. hepaticus* was inoculated at OD600 of 0.05 into vented Erlenmeyer flasks (Corning) containing liquid growth media (TSB supplemented with TVP and 10% FCS (Gibco, Invitrogen)). The flask was then transferred into an anaerobic jar and placed under microaerophilic conditions and incubated at 37°C on a shaking platform (150rpm). Fluorescent Live/Dead™ assay kit (Molecular Probes) was used to assess viability according to the manufacturer's instructions.

2.2.2 Screening

Regular screening were performed for presence of *Helicobacter* species as well as presence of *H. hepaticus* in local mouse colonies. Stool samples were collected and DNA was purified using Stool DNA Isolation Kit (Qiagen, Crawley, UK). Separate PCRs were performed, targeting either pan-*Helicobacter* genus-specific (16s rRNA) gene using primers (F-GCT ATG ACG GGT ATC C; R-GAT TTT ACC CCT ACA CCA) or targeting *H. hepaticus*-specific (p25) gene using primers (F-ATG GGT AAG AAA ATA GCA AAA AGA TTG CAA; R-CTA TTT CAT ATC CAT AAG CTC TTG AGA ATC) (281). Either *H. hepaticus* genomic DNA or stool DNA from *H.* hepaticus-infected mice were used as positive controls. *H. hepaticus* genomic DNA was obtained from *H.*

hepaticus cultures and isolated using DNeasy kit (Qiagen). 1.5% agarose gel was used to separate PCR products.

Pan *Helicobacter* PCR Conditions		*H. hepaticus* PCR Conditions	
Denaturation:	94˚C 2min	Denaturation:	94˚C 2min
30 cycles of:	94˚C 30s 58˚C 45s 72˚C 1min	30 cycles of:	94˚C 30s 58˚C 45s 72˚C 1min
Extension Keep at 4˚C.	72˚C 10min	Extension Keep at 4˚C.	72˚C 10min
Expected amplicon: 422bp		Expected amplicon: 705bp	

2.2.3 Quantification

For quantification of *H. hepaticus* colonization, DNA was isolated from caecal contents using the Stool DNA Isolation Kit and colonization quantified using primers and probes designed for *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) (282) by Taqman qPCR (Chromo4 detection system, MJ Research, Essex, UK). A standard curve was generated by serial dilution of *H. hepaticus* genomic DNA. Units for colonization level were expressed as [*H. hepaticus* DNA]/20 ng total caecal DNA.

2.3 *Citrobacter rodentium*

2.3.1 Culture

Luria Broth (Sigma) supplemented with 50 μg/ml nalidixic acid (Sigma) was used to grow *C. rodentium* (ICC169) at 37°C inside a shaking incubator.

2.3.2 Quantification

At indicated time points, various tissues were isolated and weighed, before homogenization in 600μl of sterile PBS with FASTPREP-24 (MP). The homogenate was then serially diluted and plated on LB agar plates supplemented with 50 μg/ml nalidixic acid and incubated at 37 °C overnight. Final values were normalized to the mass of tissue.

2.4 Induction of intestinal inflammation

2.4.1 Induction of colitis with *H. hepaticus* and αIL-10R antibody

Infection was accomplished by gavage of 1×10^8 CFU *H. hepaticus* (NCI-Frederick isolate 1A) into each SPF C57BL/6 mice on a daily basis for three days. Disease was induced by weekly i.p. injection of 1mg αIL-10R (IB1.2 clone) mAb for 4 weeks (249).

2.4.2 DSS-induced colitis

Disease was induced by dissolving 2% DSS in the drinking water of SPF C57BL/6 mice for 7 days (283). Subsequently mice were returned back to normal drinking water.

2.4.3 *C. rodentium*-induced intestinal inflammation

For infections, bacteria were grown to saturation overnight and on the following day the optical density of the culture was measured and adjusted to 0.05. This culture was allowed to grow to log phase (after 3.5h). Estimation of bacteria concentration was performed using the following equation: CFU/mL = (500000000 * OD) – 30000000. Required amount of bacteria was then centrifuged at 7000 rpm for 10min and resuspended in PBS. 200µl bacterial suspension at ~10^9 cfu/mL was gavaged into each mouse. Mice were subsequently weighed on a daily basis.

2.5 Analysis of histopathology

2.5.1 Sample preparation

At least 5mm wide strip of caecum and 7mm long piece of various sections of the colon were fixed in 10% formalin in PBS. Sections were then embedded in paraffin section and cut at 4μm thickness. They were then stained with haematoxylin and eosin (H&E) and scored by at least two researchers in a blinded fashion.

2.5.2 Scoring for *Helicobacter hepaticus*-induced intestinal inflammation

Inflammation of *H. hepaticus*-induced colitis was assessed as previously described (284). The factors considered are summarized in Table 2.1 (final score 0-12).

2.5.3 Scoring for DSS-induced intestinal inflammation

Scoring guide was based on previously established categories (Table 2.1) and was modified based on prominent features in DSS colitis. Factors considered are summarized in Table 2.2. As no proximal colon inflammation was present (260), only mid and distal sections of the colon were analysed (final score 0-12).

Table 2.1 Summary of histological scoring for *H. hepaticus*-induced 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

Table 2.2 Summary of histological scoring for DSS-induced colitis

A	Epithelium	Hyperplasia	and/or	Goblet Cell Depletion	and/or	Loss of epithelial cells		
	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	Area affected (% of section)							
	0	None						
	1	Up to 25%						
	2	25-50%						
	3	>50%						
D	Markers of severe inflammation	Loss of crypt structure	and/or	Submucosal inflammation	and/or	Oedema	and/or	Crypt abscesses
	0	None		None		None		None
	1	Mild		Mild		Mild		Few (<5)
	2	Marked		Marked		Marked		Many
	3	Substantial		-		-		-

2.5.4 Scoring for *C. rodentium*-induced intestinal inflammation

Scoring guide was based on previously established categories (Table 2.1) and was modified based on prominent features observed in *C. rodentium*-induced colitis. Factors considered are summarized in Table 2.3. Only caecum and distal colon were scored as minimal inflammation developed in other regions (final score 0-15).

2.5.5 Immunohistochemistry

Formalin-fixed paraffin sections as prepared above were baked at 60°C for 75min and then de-paraffinized. Antigen retrieval was performed by boiling slides in citrate buffer for 40min at 96°C. Anti-Ki-67 rabbit monoclonal antibody (Abcam ab16667) was used to probe the samples. Finally, samples were visualized with Peroxidase/DAB (DAKO EnVision™ Detection Systems, K5007) and counterstained with haematoxylin.

2.5.6 *In situ* TUNEL assay

Formalin-fixed paraffin sections as prepared above were baked at 60°C for 75min and then de-paraffinized. In Situ Apoptosis Detection Kit (Trevigen 4812-30-K) was used and staining performed according to manufacturer's instructions. Slides were visualized on the Zeiss Axioplan 2e with Photometrics Coolsnap HQ Camera and acquired using Metamorph software.

Table 2.3 Summary of histological scoring for *C. rodentium*-induced colitis

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	Oedema		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 Gene expression analysis

2.6.1 Quantitative polymerase chain reaction

Tissue samples were flash-frozen in liquid nitrogen and stored at -80°C. To extract RNA, samples were first homogenized with FASTPREP-24 (MP) in 600µl lysis buffer and then processed using the Qiagen RNeasy Mini Kit (Cat.: 74104). Next, cDNA was generated using oligo-dT primers (Sigma) and Superscript III reverse transcriptase (Invitrogen). Finally, gene expression was assessed using either SYBR Green reagents or Taqman reagents. For SYBR Green qPCRs, Quantace SensiMix reagents (QT505-05) were used. For Taqman qPCRs, Eurogentec qPCR MasterMix reagents (RT-QP2X-03) were used. Primers used are summarized in Table 2.1. *Hprt1*, Nod1, *Nod2*, *Nlrp3*, and *Nlrc4* primers were obtained from Qiagen Quantitect Primer Assays. *Il22, Reg3g, Retnla*, *Ear1/2* primers and Taqman probes were obtained from Applied Biosystems. Reactions were run using a Chromo4 detection system (MJ Research) and mRNA expression levels were normalized to *Hprt1* and calculated using the following formula $1000 * 2^{(-\Delta CT)}$ (284).

Table 2.4 Primer Sequences

Gene Name	Sequence
Il17a Forward	GCTCCAGAAGGCCCTCAG
Il17a Reverse	CTTTCCCTCCGCATTGACA
Il17a Probe	ACCTCAACCGTTCCACGTCACCCTG
Il17c Forward	CCTCTAGCTGGAACACAGTGC
Il17c Reverse	GCGGTTCTCATCTGTGTCG
Il17f Forward	GAGGATAACACTGTGAGAGTTGAC
Il17f Reverse	GAGTTCATGGTGCTGTCTTCC
Il23 Forward	AGCGGGACATATGAATCTACTAAGAGA
Il23 Reverse	GTCCTAGTAGGGAGGTGTGAAGTTG
Il23 Probe	CCAGTTCTGCTTGCAAAGGATCCGC
Ifng Forward	GGATGCATTCATGAGTATTGC
Ifng Reverse	GCTTCCTGAGGCTGGATTC
Ifng Probe	TTTGAGGTCAACAACCCACAGGTCCA
Mertk Forward	GCCAAGGCCGCATTGCCAAA
Mertk Reverse	ACGCCAAAAGCCCACACGTCA
Arg2 Forward	CCTTGCGTCCTGACGAGATCCG
Arg2 Reverse	TTTAGGTGGCATCCCAACCTGG

2.6.2 Microarray

Caecal tissue samples were harvested into RNAlater and RNA was extracted using the Ambion RiboPure kit. Subsequent steps were performed in collaboration with Dr Dilair Baban from the Wellcome Trust Centre for Human Genetics, University of Oxford. NanoDrop ND1000 was used to measure RNA quantity and BioAnalyzer 2100 (Agilent Technologies) was used to measure RNA quality. 300ng of total RNA was used from each sample 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). The Illumina TotalPrep-96 RNA Amplification Kit (# 4393543 Ambion, Inc., Austin, TX) was used to prepare biotinylated cRNA. Following array hybridization, washing, blocking, and streptavidin-Cy3 staining, iScan system was used to image the fluorescence emissions by Cy3. GenomeStudio 2011 software (Illumina, San Diego, CA) was then used to generate data that was imported to GeneSpring GX 12 (Agilent Technologies, Inc, Santa Clara CA) for further analysis. To identify significantly differentially expressed genes, data was normalized with Shift to 75 percentile and baseline transformed to median of all samples.

2.7 Flowcytometry

2.7.1 Antibodies

Antibodies used in flowcytometric analyses are summarized in Table 2.5 below.

Table 2.5 FACS 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
TCR-b	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
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

2.7.2 Isolation of lamina propria leukocytes

Leukocytes from lamina propria of colon and caecum were isolated as described previously (285). Briefly, tissue samples were cut into 0.5 cm pieces and incubated in complete RPMI 1640 containing 5mM EDTA (VWR International UK) at 37°C for 15min to remove the epithelium. Remaining tissue samples were then incubated at 37°C for 1h in complete RPMI 1640 containing 15 mM Hepes (PAA) and 0.2-0.3 mg/ml type VIII collagenase (Sigma Aldrich) to release the leukocytes. The digest was repeated and the pooled supernatants were resuspended in 30% percoll in a 15 ml tube (Falcon, BD Biosciences). This was then overlayed onto the 40% percoll layer of percoll solution in a 15 ml tube (Falcon, BD Biosciences) with 75% percoll at the bottom. Following centrifugation at 1800rpm with the brakes off for 20min, the leukocyte fraction was isolated from the interface between 40% and 75% percoll.

2.7.3 Staining for FACS analysis

Following incubation with anti-Fc receptor (anti CD16/32 antibody) and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) for 15min at 4°C. Cells were washed and incubated for another 15min at 4°C with antibodies against surface markers. Finally cells were fixed in 2% formaldehyde overnight before acquisition using FACSCalibur, Cyan (Dako). Data was exported to FlowJo (Tree Star) software for further analysis.

2.8 Organ culture

2.8.1 Overnight tissue organ culture

In order to measure secreted cytokines from intestinal tissue, colon and caecum samples were weighed and washed in PBS + BSA and penicillin/streptomycin for 10min to prevent bacterial overgrowth. Subsequently tissue samples were transferred into RPMI supplemented with 10% FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin and 5×10^{-5} M β-mercaptoethanol and incubated overnight at 37°C in an atmosphere containing 5% CO_2. Supernatants were then harvested by centrifugation and frozen at -20°C before further analysis. Levels of IL-1β (primary antibody: eBioscience 14-7012-85; secondary antibody: eBioscience 13-7112-85) and IL-18 (Mouse IL-18 Platinum ELISA, eBioscience BMS618/2TEN) were measured by ELISA, whilst IFN-γ, IL-17A, IL-22, and TNFα were measured using Flow Cytomix Cytokine Bead Assay (Bender MedSystems).

2.8.2 Polarized organ culture

Protocol was adapted from (286). Briefly, caeca were isolated and washed in HBSS supplemented with nalidixic acid to prevent bacterial overgrowth during incubation. With the luminal side facing upwards, a cloning cylinder (Sigma C1059) was attached with surgical glue (Vetbond, 3M 1469SB). This was then transferred onto a metal grid sitting inside a centre well dish (Falcon 353037). Media (DMEM with Glu and Pyruvate + 15% FBS + 1X NEAA + 200ng/mL EGF (Sigma E4127) + 1X ITES (Lonza 17-839Z) + 50μg/mL nalidixic acid) was added to the compartment below the tissue as well as inside

the cylinder. After 2h incubation in a standard incubator, supernatants from the cloning cylinder was harvested and cell death was measured by LDH assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega G1782).

2.9 Statistics

Statistical significance between two treatment groups in this thesis was calculated using the nonparametric Mann-Whitney test. For weight loss curves specifically, statistical significance on each day was determined by two-way ANOVA with Bonferroni post-tests. In all cases * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Chapter 3 – Role of NLRs during the induction and resolution of intestinal inflammation

3.1 Introduction

The identification of variants in genes encoding for PRRs such as *NOD2* and *NLRP3* being associated with CD (85, 135, 136, 207-209) has created an increased focus on the involvement of innate immune activation in the maintenance of intestinal homeostasis.

NOD2 and NLRP3 belong to the recently characterized family of intracellular innate immune receptors called the NLRs (287). NOD2 is known to be activated in response to ssRNA (134) and MDP, a derivative of bacterial peptidoglycan (130, 288). By contrast, NLRP3 is activated in response to a diverse array of stimuli including MAMPs, DAMPs, and environmental irritants (124, 126). Furthermore, whilst NLRP3 stimulation results in the activation of the inflammasome, leading to caspase-1 activation and IL-1β and IL-18 secretion, NOD2 stimulation signals primarily through activation of NF-κB as well as MAP kinases, leading to distinct patterns of inflammatory mediators (124).

Although the mechanisms through which *NOD2* mutant alleles predispose to CD remain unclear, it is known that the NOD2 mutations associated with CD susceptibility interferes with MDP recognition (128). For instance, the NOD2 L1007insC mutation is located LRR region and upon MDP recognition fails to bind the downstream RIP2 molecule, ultimately resulting in decreased NF-κB activation (289). Interestingly, stimulation with MDP protected mice from development of chemically induced colitis, providing evidence for an anti-inflammatory function for Nod2 in the intestine (176). Similarly, MDP activation of Nod2 was shown to ameliorate disease in a mouse model of necrotizing enterocolitis, and this was attributed to inhibited TLR4 signalling in IEC (179). The

findings suggest a protective function of NOD2 in intestinal homeostasis and that CD-associated *NOD2* mutations result in a loss of function. Specifically, pro-inflammatory signalling through TLR2 and TLR4 is thought to be dampened by NOD2 (176, 180). Furthermore, NOD2 is also been reported to play a critical role in fortifying the intestinal barrier by driving production of AMPs in human and mouse (161, 181). However, evidence also exists that suggest a gain of function mutation for the CD-associated *NOD2* variants. For example, the L1007fsinsC mutation in *NOD2* has been shown to suppress IL-10 transcription in PBMCs (290). Furthermore, MDP was shown to enhance bacterial agonist induction of IL-23 and IL-1β (183), which is of particular interest because IL-23 is a key mediator of chronic intestinal inflammation (86). However, these conflicting functions of NOD2 is further complicated by our poor understanding of the mechanisms driving inflammation in the intestine. For instance, NOD2 activation by MDP has also been shown to prime DCs to induce IL-17A-producing memory T cells in mouse and human (183). Though these Th17 cells have been shown to promote intestinal inflammation in some models, others have shown a protective function (3). Therefore, the Th17-promoting function of NOD2 may be either protective or inflammatory, depending on the context of inflammation.

More recently, single nucleotide polymorphisms (SNPs) in the *Nlrp3* (which codes for cryopyrin) region have also been associated with CD susceptibility (207). *Nlrp3* is mainly expressed in granulocytes and monocytes (210), and peripheral blood monocytes expressing *NLRP3* risk alleles were found to express lower levels of *NLRP3* and to secrete less IL-1β when stimulated with LPS (207). This study suggests that impaired NLRP3 inflammasome function might also contribute to CD susceptibility.

Since IBD is a remitting and relapsing disease, understanding the balance between maintenance and resolution of inflammation has significant clinical relevance in terms of treatment. The fact that inflammatory responses are usually accompanied by resolution of inflammation and tissue repair suggests that part of the inflammatory program functions to restore tissue homeostasis. Indeed, existing data indicate that low levels of LPS stimulation through TLRs can selectively induce suppression of tissue-damaging genes (291). This is further supported by studies implicating TLR signalling with induction of cytoprotective responses (113, 292). Since it is evident that NLRs are important in intestinal homeostasis, and that in the case of NOD2 can also protect against intestinal inflammation (176, 179), this demands a better understanding of the role they may play during the resolution phase of intestinal inflammation. However, studies in resolution of intestinal inflammation are limited largely by the lack of appropriate mouse IBD models. Therefore, the primary aim of this chapter is to develop and assess models of resolution of acute and chronic intestinal inflammation. The second aim is to profile the expression of NLRs during different phases of intestinal inflammation.

3.2 Results

3.2.1 Resolution of typhlocolitis following cessation of αIL-10R antibody administration in *Helicobacter hepaticus* infected WT mice.

Our lab has previously established a chronic IBD model in C57BL/6 WT mice where typhlocolitis (inflammation of the caecum and colon) is induced by oral infection with *H. hepaticus*, followed by i.p. administration of αIL-10R monoclonal antibody (mAb) on a weekly basis for 4 weeks (249). Blockade of IL-10 leads to the loss of dominant suppression and reveals potent Th1 and Th17 responses against *H. hepaticus*, which drive chronic typhlocolitis (249). Previous studies have demonstrated that Treg cells are the major sources of immune suppressive IL-10 in the gut and that they can cure established intestinal inflammation (99, 293). Therefore, we hypothesized that cessation of αIL-10R mAb administration would allow chronic intestinal inflammation to resolve, as the mAb was cleared from the circulation and IL-10 (Treg) activity was restored. To test this hypothesis, we sacrificed cohorts of mice at various time points after the disease induction protocol described above. As previously described, after four weeks of disease induction, mice had severe typhlocolitis characterised by dense leukocytic infiltrates, oedema, epithelial hyperplasia, and crypt abscesses (Figure 3.1). At 8 weeks (4 weeks after cessation of αIL-10R mAb administration), some resolution of inflammation was apparent in both colon and caecum (Figure 3.1 b-d). Finally, at 12 weeks (8 weeks after cessation of αIL-10R mAb), inflammation was almost completely resolved in both the colon and caecum, with a largely normal intestinal architecture (Figure 3.1 b-d). These observations indicate that the *H. hepaticus* + αIL-10R mAb IBD model can also serve as a good model to study the "natural" resolution of chronic intestinal inflammation.

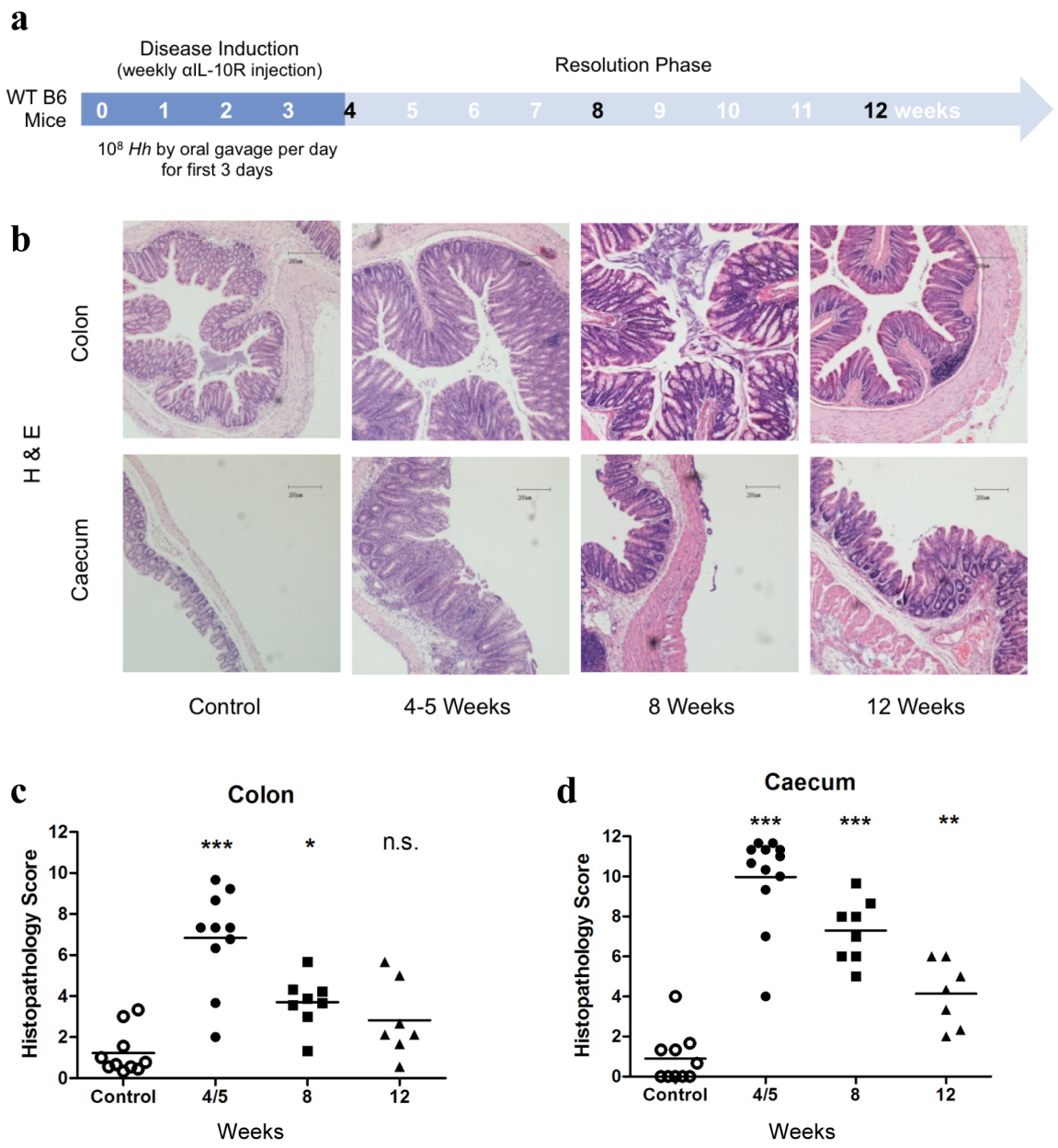

Figure 3.1 Resolution of intestinal inflammation following cessation of αIL-10R antibody administration in *H. hepaticus* infected WT mice. a, C57BL/6 mice were infected with 1 × 10^8 CFU *H. hepaticus* by daily gavage for three days, followed by weekly i.p. injection of 1mg αIL-10R antibody for 4 weeks. At indicated time points (black numbers), mice were sacrificed for analysis of intestinal inflammation. **b,** Representative mid colon and caecum histology sections stained with H&E. (scale bars = 200μm) **c,** Histopathology scores of proximal, mid, and distal sections of colon stained with H&E. **d,** Histopathology scores of caecal sections stained with H&E. Data pooled from two separate experiments. Control samples obtained from uninfected or antibody-treated uninfected mice. Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

3.2.2 *Nod2* expression increases at peak disease and remains elevated throughout resolution of chronic typhlocolitis

To assess whether NLR expression was altered during the induction or resolution of intestinal inflammation, colon and caecum tissue samples were isolated at peak disease (week 4/5), at the early resolution time point (week 8), as well as at the long-term resolution time point (week 12). For each sample, RNA was extracted, and *Nod1*, *Nod2*, *Nlrp3*, and *Nlrc4* expression evaluated using qPCR (Figure 3.2). Significant *Nod2* upregulation was observed in the colon at peak disease (week 4/5), and remained significantly elevated throughout the resolution of disease at both week 8 and week 12 (Figure 3.2). By contrast, no significant *Nod2* upregulation was observed in the caecum at any time point during induction or resolution of IBD (Figure 3.2). Expression of *Nod1* was significantly increased in the caecum at peak disease (week 4) and was also significantly elevated in the colon and caecum at the late resolution time point (week 12), although the magnitude of these increases appeared to be lower than those observed with *Nod2* (Figure 3.2). In contrast, expression of other NLRs, such as *Nlrp3* and *Nlrc4*, remained unchanged in both the caecum and colon throughout the induction and resolution of chronic intestinal inflammation.

The increase in *Nod2* expression could have been due to upregulation of the gene in the healing tissue and/or due to an influx of *Nod2*-expressing haematopoietic cells, such as monocytes, that have been shown to accumulate during colitis (294). Therefore, FACS analysis was performed on isolated colonic lamina propria leukocytes (LPLs) at various time points after disease induction. Although there was a trend towards increased $CD11b^+$ cell accumulation in the colon during the induction and resolution of intestinal inflammation, there were no significant changes in the proportions of $CD11b^+$ cells and

$CD4^+$ T cells at any time point (Figure 3.3). Although preliminary, these results suggest that local modulation of *Nod2* expression in tissue resident cells in the gut may account for the increased *Nod2* expression observed during the resolution phase of chronic colitis. Taken together, these data suggest that upregulation of *Nod2* may be important during the resolution phase of this chronic model of intestinal inflammation.

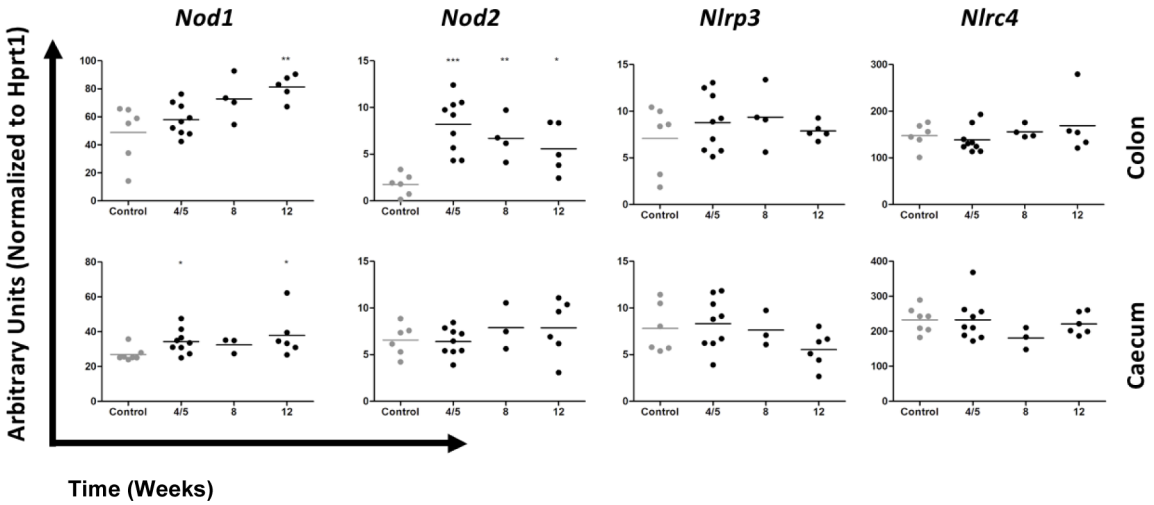

Figure 3.2 *Nod2* mRNA expression level increases at peak disease and remains elevated throughout resolution of chronic typhlocolitis. C57BL/6 mice were infected with 1×10^8 CFU *H. hepaticus* by daily gavage for three days, followed by weekly i.p. injection of 1mg αIL-10R antibody. At the indicated time points, cohorts of mice were sacrificed and pieces of intestinal tissue snap frozen. Following RNA isolation from tissue samples, mRNA expression levels in various tissues were analysed by quantitative PCR and values were normalized to *Hprt1*. Data from a single experiment (n = 3-9 mice/group). Control samples obtained from uninfected or antibody-treated uninfected mice. Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

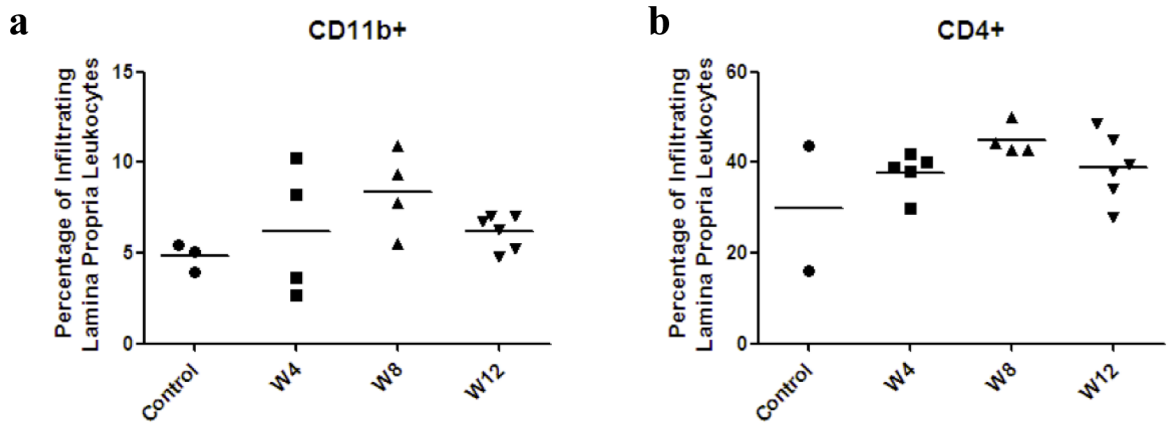

Figure 3.3 Comparable levels of CD11b$^+$ and CD4$^+$ cells throughout resolution of inflammation in *H. hepaticus* and αIL-10R mAb induced thyphlocolitis. C57BL/6 mice were infected with 1 × 10^8 CFU *H. hepaticus* by daily gavage for three days, followed by weekly i.p. injection of 1mg αIL-10R antibody. At the indicated time points, lamina propria leukocytes from colons were isolated and analysed by FACS. **a,** frequency of CD11b$^+$ cells **b,** frequency of CD4$^+$ cells. .Data from a single experiment (n = 2-6 mice/group). Control samples obtained from uninfected mice.

3.2.3 Resolution of acute DSS colitis

To complement the chronic model of intestinal inflammation, the well-characterized DSS colitis model was also assessed to confirm our findings during the resolution of intestinal inflammation. DSS is toxic to gut epithelial cells, causes increased exposure to luminal bacteria resulting in diarrhoea and intestinal inflammation (283). Disease induction in C57BL/6 mice was achieved by administration of 2% DSS in the drinking water for 7 days, after which mice were provided with regular drinking water. To assess the kinetics of disease induction and resolution, cohorts of mice were sacrificed at regular intervals (Figure 3.4a). Histological analysis showed that significant intestinal pathology was present by day 7 and peaked around day 10 (Figure 3.4 b & c). Histopathological features of DSS colitis include epithelial hyperplasia, crypt structure loss, and leukocyte infiltration (Figure 3.4 b). After day 10 the inflammation began to resolve although significant pathology was still evident on day 19-21, but by day 33 intestinal architecture had returned to normal (Figure 3.4 b & c). The weight loss that accompanies this colitis demonstrated a similar pattern of kinetics of disease induction, peaking around day 10, however the recovery was more rapid as weight had returned to normal levels by around day 21 (Figure 3.4 d). Analysis of colonic epithelial cell proliferation using Ki-67 staining showed high levels of proliferation of colonic crypt epithelial cells during peak disease (day 10) and also during the resolution phase (day 19), although this had also returned to normal by day 33 (Figure 3.5). Taken together, these data confirmed that DSS-induced colitis could serve as an acute model of resolution of acute intestinal inflammation.

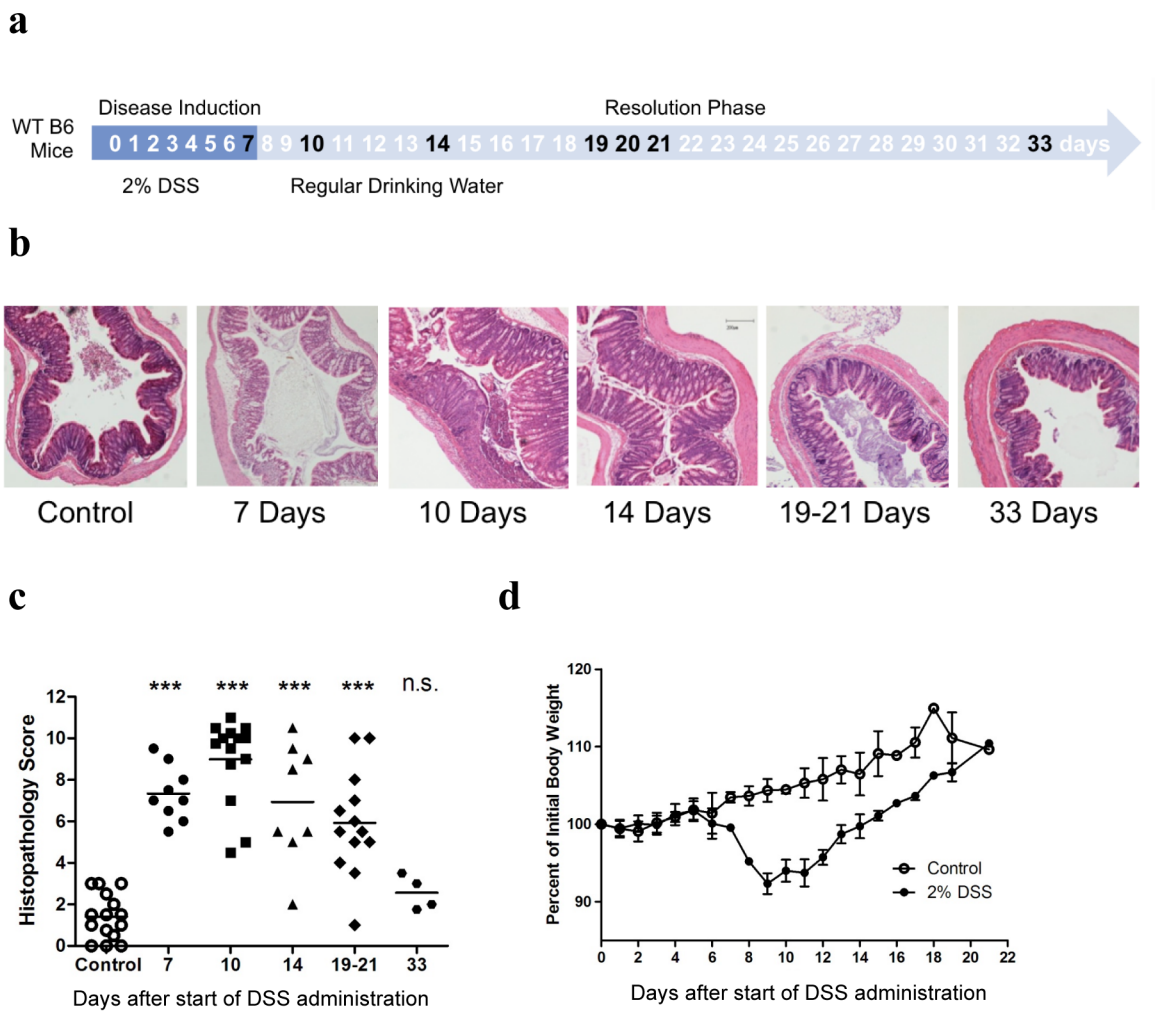

Figure 3.4 Induction and resolution of DSS colitis a, C57BL/6 mice were administered 2% DSS in drinking water for 7 days, then switched back to normal drinking water, and at the indicated time points (black numbers) they were sacrificed for analysis of intestinal inflammation. **b,** Representative sections of distal colon stained with hematoxylin and eosin (H&E). **c,** Combined histopathology scores of mid and distal sections of colon. Data pooled from three separate experiments (n = 4-15 mice/group). Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. *** = $P < 0.001$. **d,** Pooled body weight curve from three separate experiments. Control samples obtained from mice not treated with DSS.

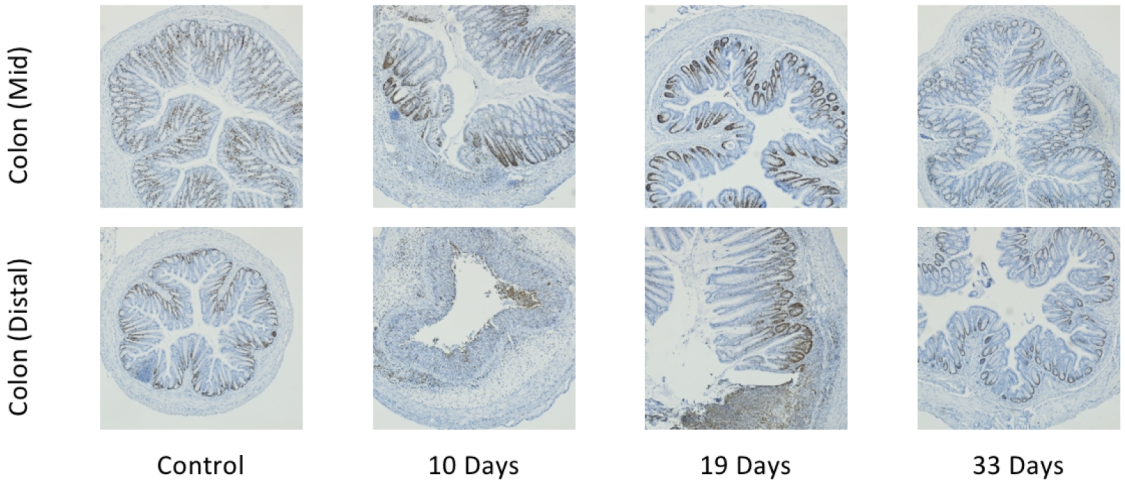

Figure 3.5 Hyperproliferation of intestinal epithelium during the induction and resolution of DSS colitis. C57BL/6 mice were administered 2% DSS in drinking water for 7 days, then switched back to normal drinking water, and at the indicated time they were sacrificed for analysis of intestinal inflammation. Different segments of the colon were formalin-fixed, paraffin embedded, and stained for Ki-67, using immunohistochemistry and counterstained with hematoxylin. Control samples obtained from untreated mice.

3.2.4 Nod2 expression increases at peak disease and remains elevated throughout colitis resolution following administration of DSS

At each time point, segments of colon and caecum, and mesenteric lymph nodes (MLNs) were isolated, RNA was extracted and *Nod1*, *Nod2*, *Nlrp3*, and *Nlrc4* expression evaluated using qPCR (Figure 3.6). No changes were observed in the level of *Nod1* expression at any time point in colon or caecum. In the colon, although *Nod2* levels were unchanged at the onset of inflammation (day 7), *Nod2* showed significant upregulation at peak disease (day 10), and its expression level remained elevated throughout the resolution process (day 14-21), although by day 33 this was not significant (Figure 3.6). In contrast, no significant changes in *Nod2* expression were observed in the caecum or MLN at any time point (Figure 3.6). Although *Nlrp3* expression in the colon was elevated at peak disease (day 10), it returned to control levels at the beginning of the resolution phase (day 14). In the caecum, *Nlrp3* was observed to be significantly downregulated in caecum on day 7 and 21. Furthermore, *Nlrc4* was also significantly down regulated in the colon on day 21, and had no change at any time point in the caecum. Interestingly, for all of the NLRs measured, no significant changes was observed at any time point in the MLN. This suggests that most changes are restricted to intestinal tissue. Taken together, these observations again confirm that *Nod2* is upregulated during resolution of intestinal inflammation.

To determine whether *Nod2* expression changes were associated with changes in leukocyte populations during induction and resolution of DSS colitis, lamina propria leukocytes were isolated and analysed by FACS. $CD11b^+$ cells in the colon were observed to be significantly increased at day 7 and day 10. As *Nod2* increase starts on day 10, this could suggest that the infiltrating monocytes on day 7 may have upregulated

Nod2 expression in the tissue. Futhermore, $CD4^+$ T cells accumulated at later time points during resolution (Figure 3.7). Whilst this may indicate increased involvement of the adaptive immune response, this observation could also be a reflection of the decreased $CD11b^+$ cells during the resolution phase. These data, whilst showing clear changes in the make up of LPL population during inflammation, does not show any correlation with the observed pattern of *Nod2* expression.

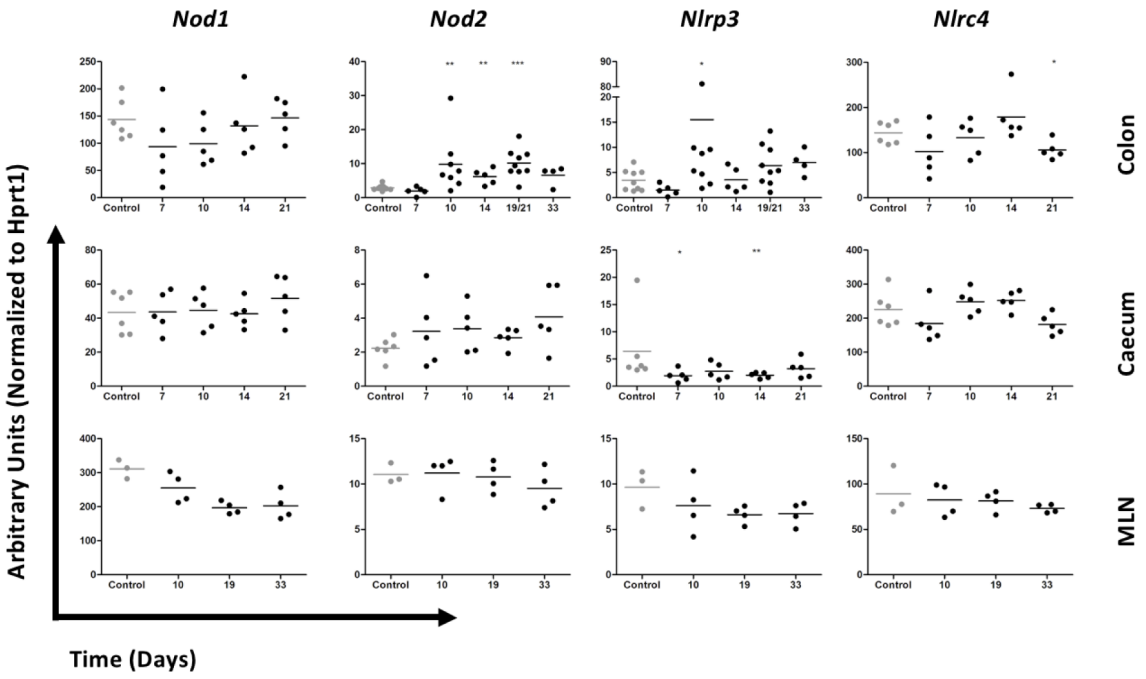

Figure 3.6 NLR expression levels during the induction and resolution of DSS colitis. C57BL/6 mice were administered 2% DSS in drinking water for 7 days, then switched back to normal drinking water. mRNA expression levels in various tissues were analysed by quantitative PCR and values were normalized to *Hprt1* (n = 3-9 mice/group). Control samples obtained from mice not treated with DSS. Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. *p<0.05, **p<0.01, ***p<0.001.

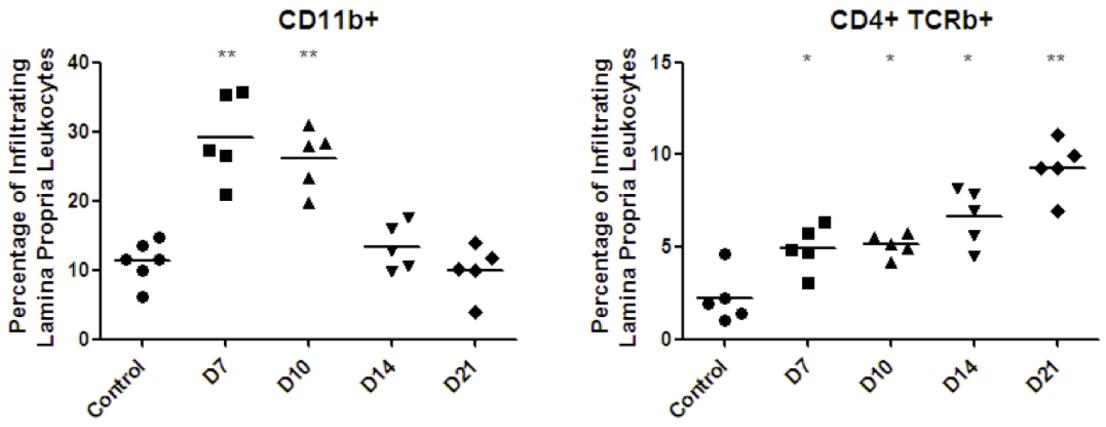

Figure 3.7 Distinct trends in the frequency of CD11b$^+$ and CD4$^+$ TCRb$^+$ cells throughout the resolution phase of DSS induced colitis. C57BL/6 mice were administered 2% DSS in drinking water for 7 days, then switched back to normal drinking water. At indicated time points, mice were sacrificed and LPLs were isolated and analysed by FACS. **a,** frequency of CD11b$^+$ cells **b,** frequency of CD4$^+$ TCRβ$^+$ cells Data from a single experiment (n = 5-6 mice/group). Control samples obtained from mice not treated with DSS. Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. * = $P < 0.05$; ** = $P < 0.01$.

3.2.5 MDP administration does not appear to protect against DSS colitis

Local delivery of MDP, which is detected by Nod2, has been reported by Watanabe and colleagues to be protective against DSS colitis if administered in a prophylactic manner (176). This has been shown by *in vitro* studies where Nod2 pre-stimulation in DC resulted in reduced pro-inflammatory cytokines induced by multiple TLR agonists (176). These data suggest that Nod2 may in fact prevent or attenuate disease induction by DSS. Alternatively, this could suggest that Nod2 is involved in resolution of DSS colitis. Therefore we decided to compare early versus late MDP administration, to determine whether Nod2 activation works prophylactically or therapeutically. However, a pilot study in which (100μg) MDP was injected i.p. on the first three days of DSS colitis experiment did not fully protect against weight loss (Figure 3.8 a), and only reduced colitis minimally on day 10 (Figure 3.8 b).

Similarly, therapeutic MDP injections at the time of peak DSS-induced disease (days 10, 11, 12) did not alter the kinetics of recovery of body weight (Figure 3.8 a), and had no significant impact on intestinal pathology during the resolution phase (Figure 3.8 b). Taken together, this pilot study did not reveal any beneficial effect of MDP when administered either prophylactically or therapeutically to mice subjected to DSS-induced colitis.

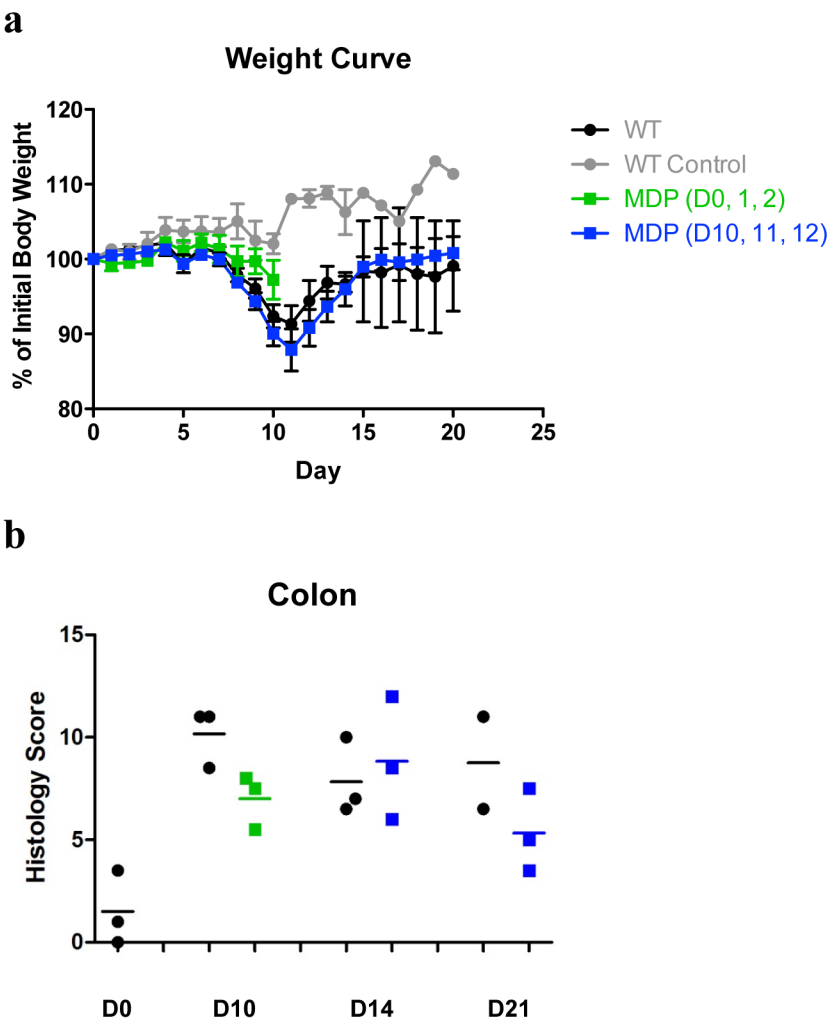

Figure 3.8 Evaluation of MDP administration in DSS-induced colitis. C57BL/6 mice were administered 2% DSS in drinking water for 7 days, then switched back to normal drinking water. MDP (100 µg/mouse/day) was administered i.p. to indicated groups for three consecutive days either at the beginning of the experiment (D0) or after peak disease (D10). **a,** Body weight curve **b,** Average histopathology scores of mid and distal sections of colon. Data from one pilot experiment (n = 2-3 mice/group). Control samples obtained from untreated mice.

3.2.6 Preliminary DSS colitis experiment using *Nod2*$^{-/-}$ mice on a mixed strain background

To further characterize the functional role of Nod2 during the resolution of intestinal inflammation, studies with *Nod2*$^{-/-}$ mice need to be performed. However, the *Nod2*$^{-/-}$ mice in our facility were obtained on a mixed 129SvEv and C57BL/6 background. Whilst the mice were being backcrossed onto the C57BL/6 background, small cohorts of *Nod2*$^{-/-}$ and *Nod2*$^{+/-}$ littermates were obtained and used in a preliminary DSS colitis experiment. Following treatment with 2% DSS administered in the drinking water, only *Nod2*$^{-/-}$ and *Nod2*$^{+/-}$ littermates exhibited a similar degree of weight loss (Figure 3.9 a), and also exhibited similar levels of intestinal pathology at peak disease (day10) and during the resolution phase (day 20) (Figure 3.9 b).

However, it should be noted that only low levels of intestinal pathology were observed in this experiment and the number of mice used was limited. This could be due to strain-dependent susceptibility to DSS, which has been documented in other studies (260). Therefore experiment needs to be repeated again after full backcrossing of these animals onto the C57BL/6 background.

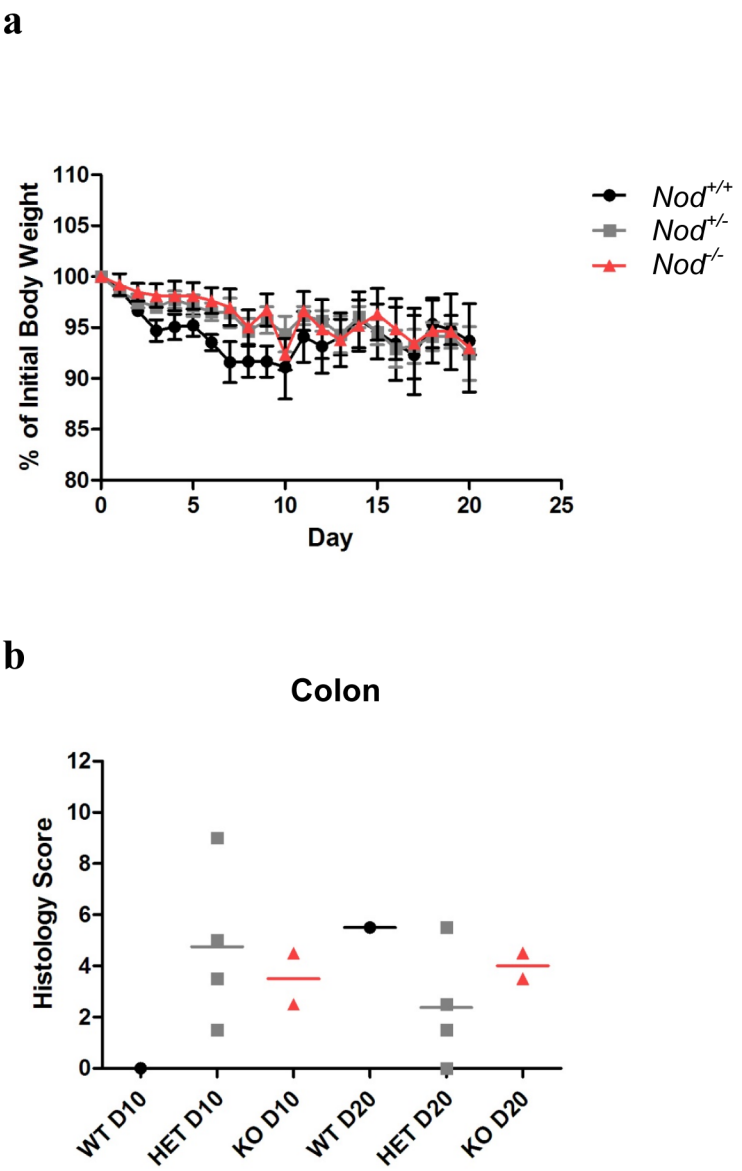

Figure 3.9 Evaluation of Nod2-deficient mice in DSS-induced colitis. *Nod2*$^{+/+}$, *Nod2*$^{+/-}$, *and Nod2*$^{-/-}$ mice on a mixed C57BL/6 x 129SvEv mixed background were administered 2% DSS in drinking water for 7 days, then switched back to normal drinking water. **a,** Body weight curve **b,** Average histopathology scores of mid and distal sections of colon. Data from one pilot experiment. (n = 1-4 mice/group)

3.3 Discussion

Despite being established as one of the major disease risk alleles for CD (135, 136), *NOD2*, like other IBD disease-susceptibility genes, has a low disease penetrance amongst people who carry associated risk alleles (3, 295). Therefore it is widely thought that IBD is caused by a genetic defect in combination with other factors such as an environmental trigger (3, 295).

The results described in this chapter show that of the NLRs characterized (including *Nod1, Nod2, Nlrp3, and Nlrc4*), *Nod2* has an expression profile that suggest an involvement during the resolution phase of intestinal inflammation. Both acute (DSS) and chronic (*H. hepaticus* αIL-10R) IBD models revealed that *Nod2* expression levels were significantly increased at the peak of intestinal inflammation, but also remained elevated throughout the resolution process. Whilst the induction of *Nod2* during inflammation is expected, since it has been shown to be upregulated following TLR and cytokine stimulation (296, 297), it is perhaps more surprising to find that the upregulation of *Nod2* continues during resolution. If Nod2 is part of a natural feedback circuit induced in the inflamed intestine to initiate/facilitate resolution, then a defective Nod2 may predispose IBD patients to chronic intestinal pathology due to defective resolution of inflammation. In support of this hypothesis mice engineered to carry a CD-associated variant of *Nod2* do not develop spontaneous intestinal inflammation (139). Hence, it is possible that the disease-associated mutations in *NOD2* may in fact be insufficient to trigger intestinal inflammation per se, but instead results in a failure to control excessive inflammation.

During resolution, there are many mechanisms that NOD2 could act through. NOD2 could inhibit of TLR responses directly, which has been reported for TLR2 (176, 178) and TLR4 (179). This anti-inflammatory effect could be a natural regulatory mechanism

where Nod2 could act to reduce inflammatory responses in myeloid cells. Conversely, Nod2 could synergize with TLR responses to induce IL-23 and IL-1, which ultimately promotes an antibacterial Th17-mediated immunity (183). By removing invading bacteria from stimulating further inflammation, Nod2 would thereby reduce inflammation. Although this mechanism may be operating early during inflammation, but it cannot explain the sustained *Nod2* upregulation observed at later time points.

Alternatively, Nod2 could also be facilitating resolution through an indirect mechanism by limiting host exposure to luminal bacteria. Upon sensing of its bacterial ligand MDP, Nod2 has been suggested to play a host-defensive role through the elaboration of anti-microbial molecules (cryptdins) (181). Deficiency in Nod2 resulted in a susceptibility to *Listeria monocytogenes* in the gastrointestinal tract (181). In addition, another study also reported that Nod2 is crucial for Paneth cells to secrete antimicrobial proteins (182). Together, these studies illustrate a mechanism that would decrease exposure to inflammatory stimuli derived from the microbiota, thus reducing inflammation and facilitating resolution.

More recent studies have established NOD2 to be important in inducing autophagy via recruitment of ATG16L1, another strong genetic risk factor for CD, to the plasma membrane. (187, 189). Cooney *et al.* showed that *NOD2* disease risk variant-expressing DCs have a defect in autophagy induction, bacterial killing, bacterial trafficking, and antigen presentation. Indeed, their work complements parallel findings that NOD2 activation could trigger potent antigen-specific adaptive immunity (183, 298). An appropriate adaptive response could enhance clearance of the inflammatory stimulus and allow restoration of intestinal homeostasis. Furthermore, recent studies have shown that autophagy can dampen inflammatory responses. For example, autophagy can remove

intracellular components such as damaged mitochondria that induce inflammation (202, 299) or cell death (300). In addition, autophagy has recently been shown to dampen inflammation directly by removing active inflammasomes in macrophages (301). Thus, the link between NOD2 and autophagy has revealed a potential mechanism by which NOD2 could contribute to resolution of inflammation.

In the intestine, a key factor that could impact on how NOD2 functions during inflammatory responses is the cell type in which NOD2 is activated. As noted above, NOD2 in antigen presenting cells contributes to proper bacterial handling, which will direct suitable immune response to eliminate a specific pathogen (189, 302). Since *NOD2* is expressed in many cell types, so the observed upregulation of *Nod2* levels in colonic tissue could come from many different sources. This phenomenon could be a reflection of infiltration by leukocytes expressing *Nod2* at a high level, as observed in histology sections at peak disease. However, during resolution *Nod2* levels remained high despite a drastic reduction in infiltrating leucocytes, pointing to the possibility of increased *Nod2* expression in non-immune cells. This would make sense as non-immune cells like the IECs make up a large percentage of the intestinal tissue, and therefore any changes in their gene expression might be detected in the whole tissue samples used in this study. In addition, as shown by the Ki-67 staining, the IECs undergo active repair and proliferation during the resolution process, and upregulated genes during this time may be important in promoting resolution or repair. It is known that IECs also express *NOD2* (296), and therefore the observed upregulation of *Nod2* in tissue during resolution could be directing key repair processes in the epithelium. Indeed Nod2 has been found to be important in bacterial killing in the epithelium (181, 182).

Future studies will also focus on the assessment of cellular compartments Nod2 may be acting in during resolution of inflammation. Attempts have been made to isolate IECs either by biochemical means using EDTA and sorbitol gradient (303), or by laser capture microdissection. However, both methods require extensive sample processing and therefore need to be optimized further in order to preserve sample quality. Another approach to assess the compartment responsible for the increased *Nod2* expression during resolution is by generating bone marrow chimeras using *Nod2*$^{-/}$ and WT mice.

Furthermore, more quantitative measures of resolution also need to be established to allow for better comparison between treatment groups. An attempt has been made to mathematically model DSS weight loss curves, through which factors such as the rate of recovery may be quantified. However, this approach was unsuccessful mainly due to variations between animals within each experimental group as well as fluctuations in daily weight. Therefore, more biochemical assays will be employed such as ELISA measurement of the inflammatory marker serum amyloid A in the serum as well as hemoccult tests for blood in faecal pellets.

If Nod2 does play a role in the resolution of inflammation, it would be extremely interesting to explore the therapeutic potential of this molecule. By activating Nod2's anti-inflammatory or pro-resolving functions using MDP, one could prime the intestine to better handle the insult of microbes upon breach of the epithelium caused by DSS administration (176). As this treatment did not work in our pilot study, differences in mouse strains, living conditions, the MDP dosage, as well as the DSS dosage will need to be optimized to reveal the effect of Nod2 during the resolution phase of intestinal inflammation. Ultimately, once the *Nod2*$^{-/-}$ mice are backcrossed onto the C57BL/6 strain,

both DSS as well as *H. hepaticus* αIL-10R resolving colitis models will be used to investigate Nod2's functional involvement in the resolution of inflammation.

Chapter 4 – Nlrp3 in non-haematopoietic cells confers protection against *Citrobacter rodentium* colonization and intestinal pathology

4.1 Introduction

NOD2 is not the only NLR that has been associated with IBD. Indeed, SNPs of *NLRP3* were shown to be genetically associated with susceptibility to CD in specific populations (207-209). One of the SNPs correlated with a decreased expression of *NLRP3* by peripheral blood monocytes and another SNP showed a reduction in IL-1β secretion upon stimulation with LPS (207). In addition, several recent studies suggested that the inflammasome plays a protective role in maintaining epithelial barrier integrity, as *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ *and Casp1*$^{-/-}$ mice were all more susceptible to DSS colitis than WT mice (215-217). This inflammasome-mediated protection for DSS colitis appeared to be dependent on IL-18 (215). However the findings are in contrast to previous studies using blocking antibodies against IL-1β and IL-18, which suggested a pathogenic role for these cytokines in DSS colitis (211, 212, 214, 304, 305). Similarly, another recent study reported that *Nlrp3*$^{-/-}$ mice were less susceptible to DSS colitis and that pharmacological inhibition of Caspase-1 ameliorated DSS-colitis (306). Thus there remains some confusion over the protective and pathogenic roles of inflammasomes in acute DSS colitis.

To investigate whether Nlrp3 was involved in the induction or regulation of bacteria-triggered colitis, we employed two complementary models. As a model of chronic colitis we used the *H. hepaticus* + αIL-10R mAb model in WT mice (249). Previous studies have identified MyD88 as a key innate immune signalling pathway through which *H. hepaticus* induces inflammation; however this induction was independent of TLR2, 4, and 5 (111). Since the inflammasome is upstream of IL-1β and IL-18, and the receptors for

these cytokines signal through MyD88-dependent pathways, we hypothesized that Nlrp3 inflammasomes might contribute to the induction of inflammation by *H. hepaticus*.

In addition, we also employed the *C. rodentium*-induced model of intestinal inflammation to evaluate the role of Nlrp3 inflammasome in bacteria-triggered acute intestinal inflammation. Various modules of the adaptive and the innate immune system have been implicated in defence against *C. rodentium*. Mice deficient in MyD88, a signalling adaptor for most TLRs as well as for IL-1R and IL-18R, are hypersusceptible to *C. rodentium* infection, exhibiting very high mortality rates and severe colitis (269). This phenotype was partially TLR2-dependent and partly IL-1R-dependent, suggesting a role for IL-1β in protective immunity against *C. rodentium* (269). However, the nature of the inflammasome responsible for this protective response remained unclear. We therefore hypothesized that Nlrp3 might play a protective role during *C. rodentium* infection.

The experiments described in this chapter aimed to determine the role of Nlrp3 activation in intestinal inflammation. We therefore assessed mice lacking the Nlrp3 protein (279) in two different models of intestinal inflammation. By utilizing chronic and acute models of colitis described above, we aimed to gain a better understanding of how Nlrp3 contributes to the protection from or development of intestinal inflammation in an infectious setting. Furthermore, as *Nlrp3* is expressed in many different cell types, we also aimed to determine if distinct roles, such as protective versus pathogenic, of Nlrp3 activation in IECs versus haematopoietic cells exist like for TLRs (111).

4.2 Results

4.2.1 Nlrp3 deficiency exacerbates *H. helicobacter*-induced chronic intestinal inflammation.

As shown previously (249), infection of C57BL/6 WT mice with *H. hepaticus* together with the concomitant administration of αIL-10R monoclonal antibody (mAb) triggers severe inflammation in the caecum and colon (typhlocolitis) (Figure 4.1). However, compared to WT mice, *Nlrp3*$^{-/-}$ mice developed significantly more severe inflammation in the caecum (typhlitis) (Figure 4.1 a) and comparable inflammation in the colon (colitis) (Figure 4.1 b). In contrast, *H. hepaticus* + αIL-10R systemic disease was completely Nlrp3-independent, as comparable spleen mass as well as granulocytic infiltration was observed between WT and *Nlrp3*$^{-/-}$ mice (Figure 4.2), although there was a trend towards an increased splenic infiltration of granulocytes in *Nlrp3*$^{-/-}$ mice. The comparable disease in WT and *Nlrp3*$^{-/-}$ mice was accompanied by similar *H. hepaticus* colonization levels (Figure 4.3) as well as similar levels of inflammatory cytokines in isolated LPLs (Figure 4.4).

Taken together, these results suggest that although Nlrp3 does not play a major role in *H. hepaticus*-mediated chronic intestinal inflammation, the significant increase in caecal pathology as well as the trend towards increased levels of granulocyte infiltration in the spleen, imply a minor protective effect of Nlrp3 in this model.

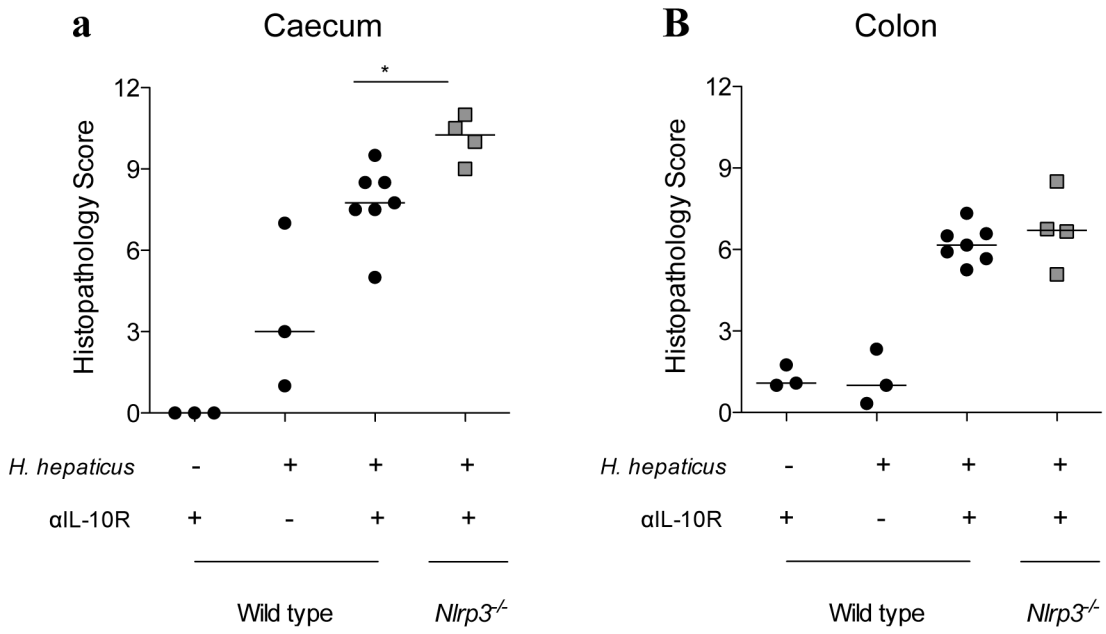

Figure 4.1 Exacerbated typhlitis in *Nlrp3*$^{-/-}$ mice following *H. hepaticus* infection and αIL-10R mAb treatment. WT and *Nlrp3*$^{-/-}$ mice were infected orally with 10^8 CFU *H. hepaticus* by daily gavage for three days, followed by weekly i.p. injection of 1mg αIL-10R antibody. Mice were sacrificed for analysis 4 weeks following initial infection and intestinal tissue samples harvested and stained with H&E. **a,** Histopathology scores of caecal sections and **b,** mean histopathology scores of proximal, mid, and distal sections of colon stained with H&E. Datafrom a single pilot experiment (n = 3-7 mice/group). Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. * = $P < 0.05$.

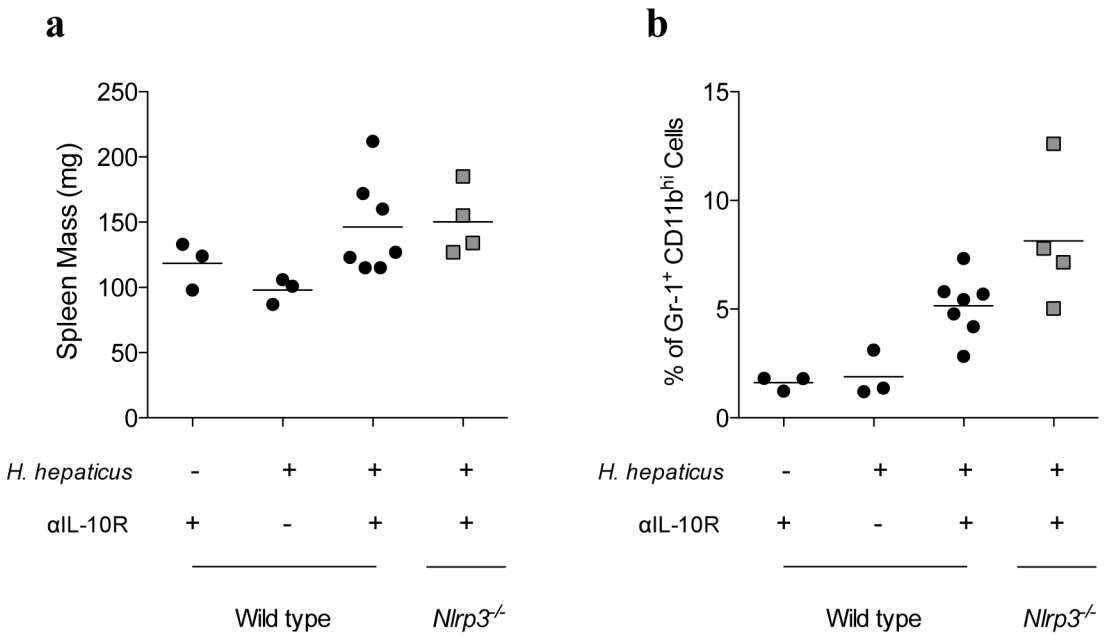

Figure 4.2 Comparable systemic inflammation in WT and *Nlrp3*$^{-/-}$ mice following *H. hepaticus* and αIL-10R antibody treatment. WT and *Nlrp3*$^{-/-}$ mice were infected orally with 10^8 CFU *H. hepaticus* by daily gavage for three days, followed by weekly i.p. injection of 1mg αIL-10R antibody. Mice were sacrificed for analysis 4 weeks following initial infection. **a,** Spleen mass, **b,** Percentage of Gr-1$^+$ CD11b^{hi} cells in spleen. Data from a single pilot experiment (n = 3-7 mice/group).

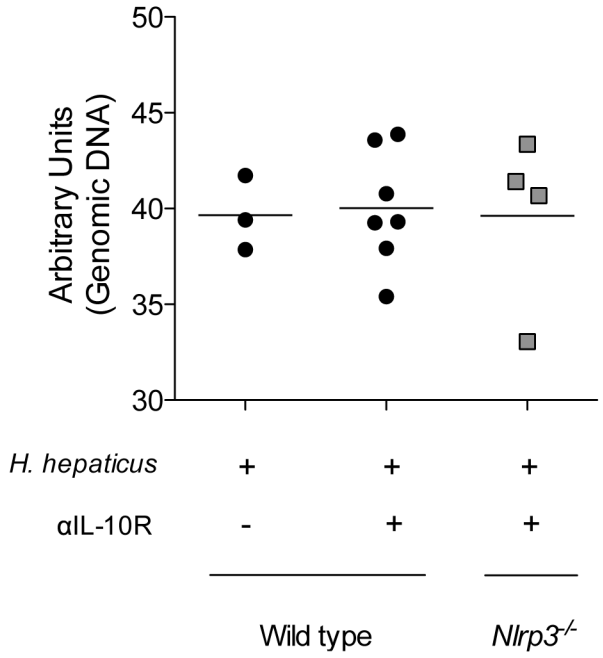

Figure 4.3 Comparable *H. hepaticus* colonization levels in WT and *Nlrp3*$^{-/-}$ mice. WT and *Nlrp3*$^{-/-}$ mice were infected orally with 10^8 CFU *H. hepaticus* by daily gavage for three days, followed by weekly i.p. injection of 1mg αIL-10R antibody. Mice were sacrificed for analysis 4 weeks following initial infection. DNA was isolated from caecal contents and *H. hepaticus* DNA was quantified by qPCR based on the *cdtB* gene. Data from a single pilot experiment (n = 3-7 mice/group).

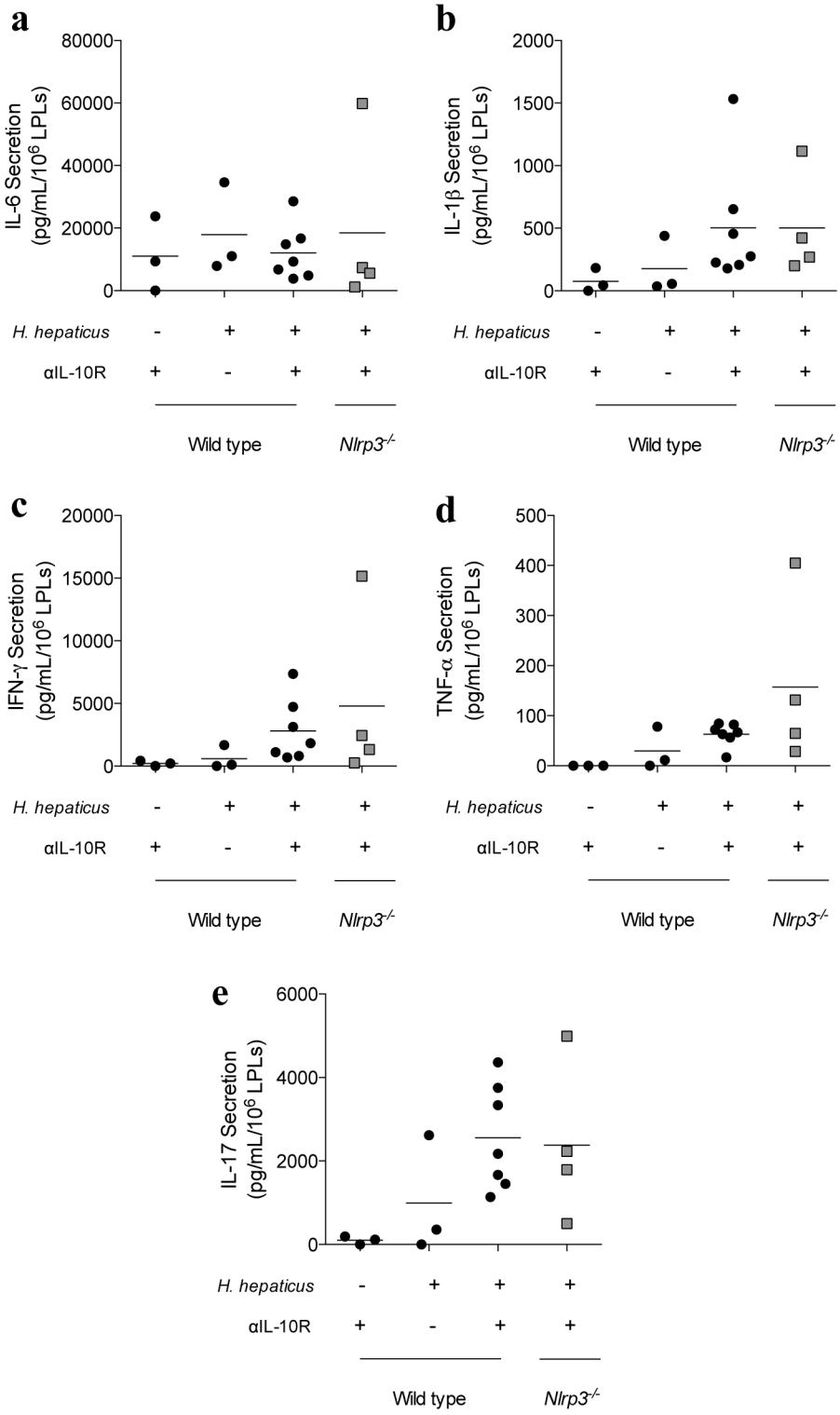

Figure 4.4 Comparable levels of inflammatory cytokines in the intestines of WT and *Nlrp3*$^{-/-}$ mice. WT and *Nlrp3*$^{-/-}$ mice were infected orally with 10^8 CFU *H. hepaticus* by daily gavage for three days, followed by weekly i.p. injection of 1mg αIL-10R antibody. Mice were sacrificed for analysis 4 weeks following initial infection. LPLs were isolated and incubated overnight. Supernatants were isolated and cytokine levels were measured using a bead cytometrix assay. **a,** IL-6; **b,** IL-1β; **c,** IFN-γ; **d,** TNF-α; **e,** IL-17. **a-e,** Data from a single pilot experiment (n = 3-7 mice/group).

4.2.2 Nlrp3 activation protects against *C. rodentium*-induced wasting disease and intestinal inflammation

To assess the role of Nlrp3 in an acute inflammatory setting in the intestine, we infected WT and *Nlrp3*$^{-/-}$ mice with *C. rodentium*. We then monitored their weight on a daily basis for three weeks. Whilst WT mice maintained their original body weight throughout the course of infection, *Nlrp3*$^{-/-}$ mice lost significant amounts of body weight over the first 14 days post-infection, before regaining their original weight by day 21 post-infection (Figure 4.5).

C. rodentium infection of WT mice leads to acute intestinal inflammation marked by mild colitis, colonic hyperplasia, goblet cell depletion and infiltration of mononuclear cells (268, 307). We confirmed that WT mice suffered only an acute, transient mild typhlitis, which had resolved by day 14 post-infection (Figure 4.6). In contrast, *Nlrp3*$^{-/-}$ mice developed severe typhlitis after infection with *C. rodentium*, characterized by crypt hyperplasia, leukocyte infiltration, submucosal inflammation, and oedema, (Figure 4.6 a). To quantify the level of inflammation, H&E stained sections of caecum and distal colon were analysed as previous studies indicated that *C. rodentium* primarily infects these two segments of the intestine (265).

Consistent with the representative photomicrographs (Figure 4.6 a), the inflammation in the caecum (typhlitis) was significantly more severe in *Nlrp3*$^{-/-}$ mice on day 8 and day 14 as compared to WT mice (Figure 4.6 b). Whilst the typhlitis subsided slightly on day 14, the inflammation was still significantly worse than in WT animals (Figure 4.6 b). In contrast, although the colitis in *Nlrp3*$^{-/-}$ mice worsened until day 14, this difference was never statistically significant when compared to WT animals (Figure 4.6 c). These

observations suggest that Nlrp3 plays an important protective role during *C. rodentium* infection, particularly in the caecum.

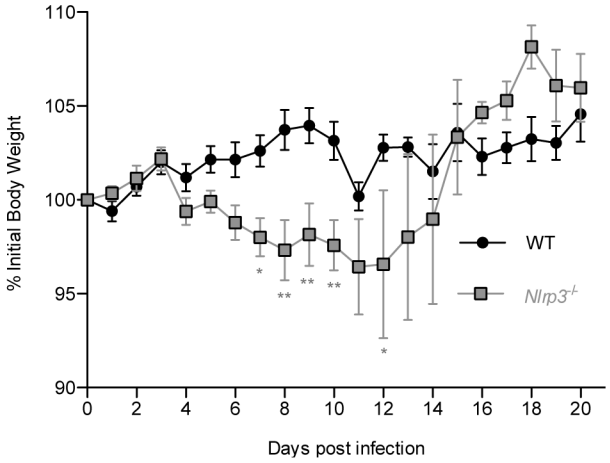

Figure 4.5 The Nlrp3 inflammasome protects against *C. rodentium*-induced wasting disease. WT and *Nlrp3*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and sacrificed 8 or 14 days post-infection. Mean body weights (±s.e.m.), throughout infection. Data represent one of three independent experiments (n = 4-16 mice/group). Statistical significance was determined by two-way ANOVA with Bonferroni post-tests. * = $P < 0.05$; ** = $P < 0.01$

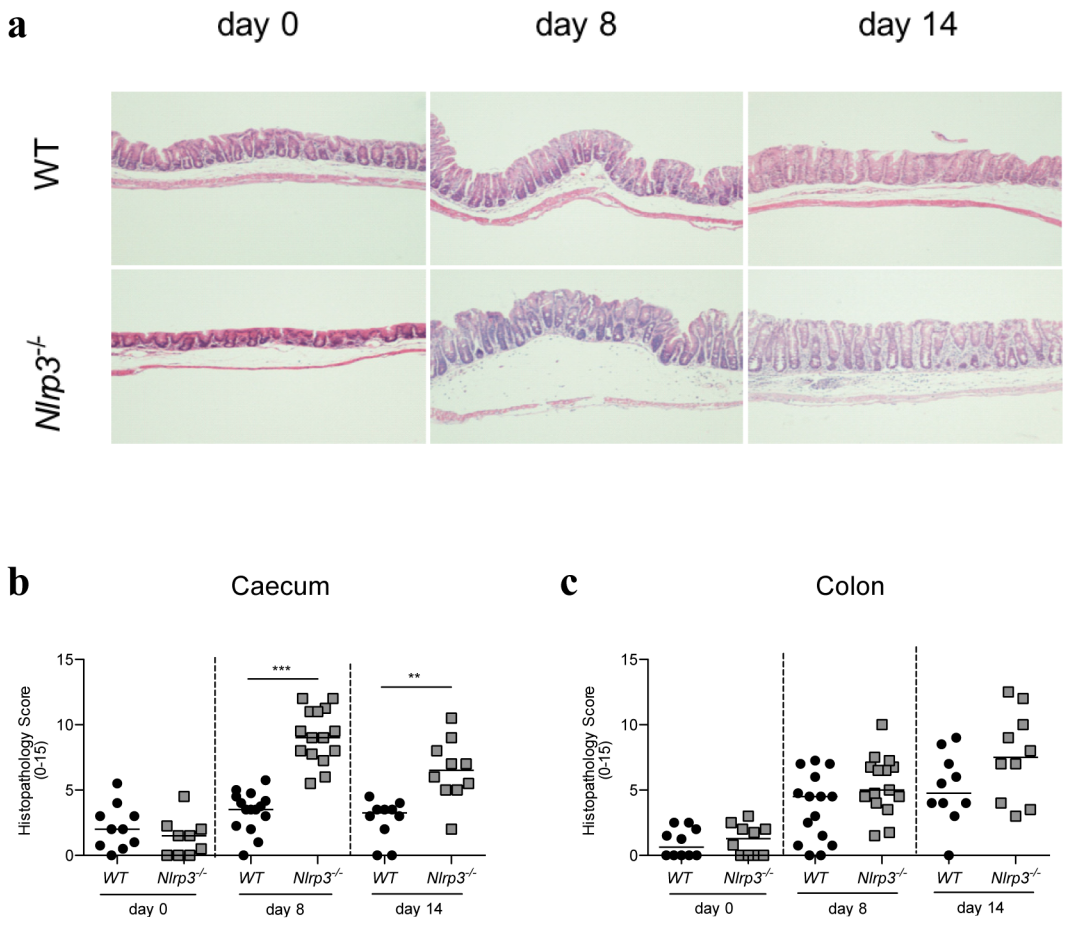

Figure 4.6 The Nlrp3 inflammasome protects against *C. rodentium*-induced intestinal inflammation. WT and *Nlrp3*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and sacrificed 8 or 14 days post-infection. Intestinal tissue samples were fixed in formol saline and paraffin-embedded sections were analysed by staining with H&E. **a,** Representative photomicrographs of H&E staining of *C. rodentium* infected caeca (×50) **b, c,** Inflammation scores in the caecum (**b**) and distal colon (**c**). Data represent pooled results from two-three independent experiments (n = 9-15 mice/group). Horizontal bars represent the medians and each symbol represents an individual mouse. Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. ** = $P < 0.01$; *** = $P < 0.001$

4.2.3 *Nlrp3*$^{-/-}$ mice have an impaired ability to control *C. rodentium* colonization

To elucidate the protective function of Nlrp3 in *C. rodentium* infection, we assessed the tissue-associated bacterial numbers in the caecum and distal colon. Consistent with exacerbated intestinal inflammation, we observed significantly higher bacterial burdens in the caeca and distal colons of *Nlrp3*$^{-/-}$ mice as compared to WT mice, both on day 8 and 14 post-infection (Figure 4.7 a & b). The magnitude of the differences in bacterial loads of the distal colon between WT and *Nlrp3*$^{-/-}$ mice was less than in the caecum on day 8 (Figure 4.7 b), consistent with the corresponding histopathology scores (Figure 4.6 b & c). *C. rodentium* is thought to cause diarrhoea primarily by weakening the tight junctions between IECs (308). One potential consequence of impaired barrier function is translocation of bacteria past the intestinal barrier into systemic sites. To assess the extent of this translocation we examined the *C. rodentium* loads in the spleen. Strikingly, we observed no translocation of *C. rodentium* in WT spleens 8 days post-infection (Figure 4.7 c). By contrast, around 50% of *Nlrp3*$^{-/-}$ mice harboured detectable numbers of *C. rodentium* the spleen. (Figure 4.7 c). These results indicate that Nlrp3 plays a key role in limiting bacterial colonization in the intestine and also prevents systemic translocation of *C. rodentium*.

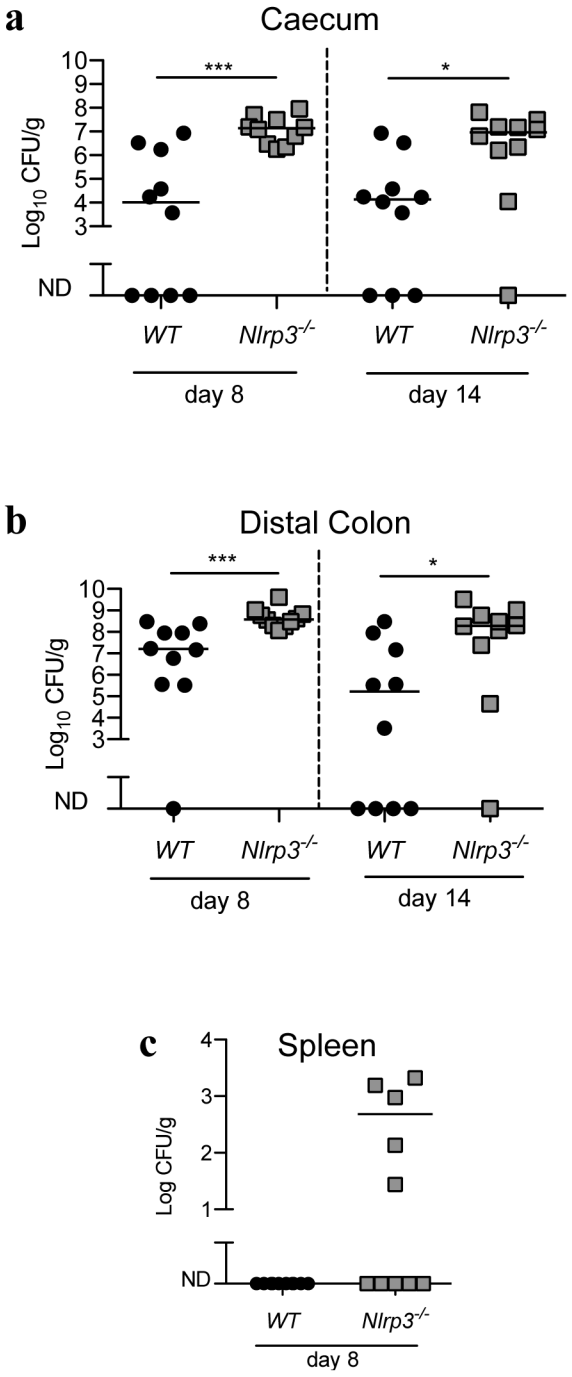

Figure 4.7 The Nlrp3 inflammasome protects against *C. rodentium* colonization. WT and *Nlrp3*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and sacrificed 8 or 14 days post-infection. Intestinal tissue samples were isolated and tissue adherent bacteria enumerated following homogenisation of samples and plating on LB plates supplemented with nalidixic acid. **a-c,** *C. rodentium* burdens in the caecum **(a)**, distal colon **(b)**, and spleen **(c)** (ND, not detectable). Data represent pooled results from two-three independent experiments (n = 9-15 mice/group). Horizontal bars represent the medians and each symbol represents an individual mouse. Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. * = $P < 0.05$; *** = $P < 0.001$

4.2.4 Similar levels of inflammatory cytokines in the caecum of WT and *Nlrp3*$^{-/-}$ mice following *C. rodentium* infection

To assess the local inflammatory environment in the intestine we isolated and incubated sections of caecum in culture media overnight to collect secreted cytokines. IL-1β and IL-18, two cytokines that are secreted following inflammasome activation, were measured by ELISA. Surprisingly, the levels in *Nlrp3*$^{-/-}$ mice are not significantly different from WT mice, indicating that this is not the protective mechanism missing the *Nlrp3*$^{-/-}$ mice (Figure 4.8 a-b).

Furthermore, we used a bead cytometrix assay for detection of cytokines previously found to be important in protection against *C. rodentium* (272, 274, 309, 310). Despite normalization of this data to the tissue mass, the results obtained varied greatly (Figure 4.8 c-f). Whilst none of the cytokines profiled gave significant differences, IL-22 levels showed a trend towards lower expression in *Nlrp3*$^{-/-}$ mice on day 8 post-infection (Figure 4.8 e). These data suggest that the exacerbated pathology in *Nlrp3*$^{-/-}$ mice is most likely not due to immunopathology, but rather due to the inability to control *C. rodentium* colonization levels.

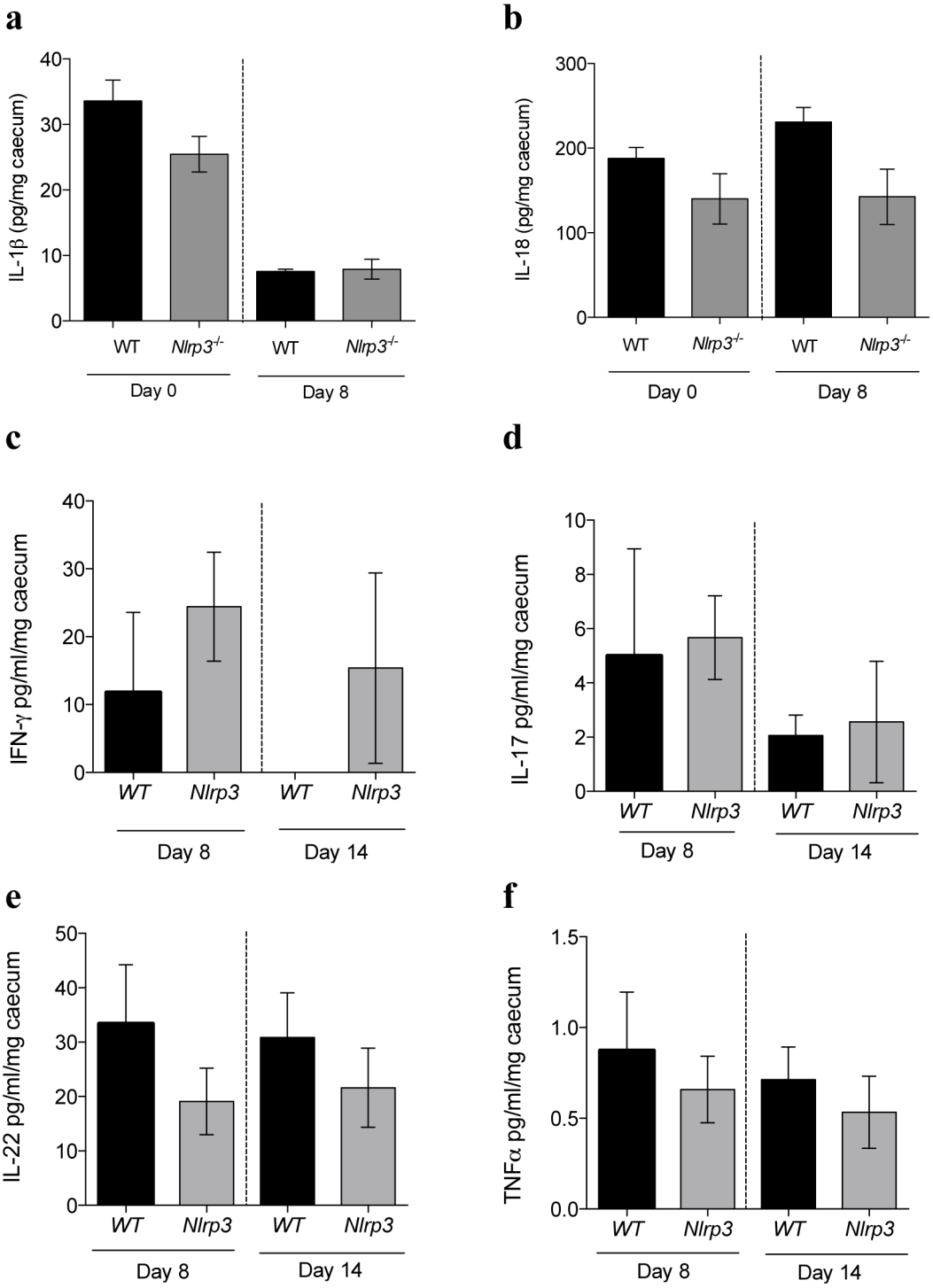

Figure 4.8 Intestinal cytokine responses following infection with *C. rodentium*. WT and *Nlrp3*$^{-/-}$ mice were infected by orally with ~10^9 cfu of *C. rodentium* and sacrificed 8 or 14 days post-infection. Intestinal samples were isolated and incubated O/N in RPMI supplemented with 10% FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin and 5 × 10^{-5} M β-mercaptoethanol. Supernatants were harvested and cytokine levels assayed by ELISA **(a-b)** using Flow Cytomix Cytokine Bead Assay **(c-f)**. Mean levels of IL-1β **(a)**, IL-18 **(b)**, IFN-γ (**c**), IL-17 (**d**), IL-22 (**e**), and TNFα (**f**) in the caecum. Data represent one or two independent experiments (n = 4-6 mice/group). Error bars represent ±s.e.m. of mean.

4.2.5 Heightened systemic immune activation in *Nlrp3*$^{-/-}$ mice

In accordance with exacerbated wasting disease, *C. rodentium* infection also induced significant splenomegaly in *Nlrp3*$^{-/-}$, but not WT mice, a day 8 and day 14 post-infection (Figure 4.9 a). This is also consistent with our observation of increased frequency of bacterial translocation into the spleen in *Nlrp3*$^{-/-}$ mice (Figure 4.7 c). The increase in spleen size correlated with a greater accumulation of granulocytes ($CD11b^{+}GR1^{high}$) in *Nlrp3*$^{-/-}$ spleens compared to WT spleens on day 8 post-infection (Figure 4.9 b).

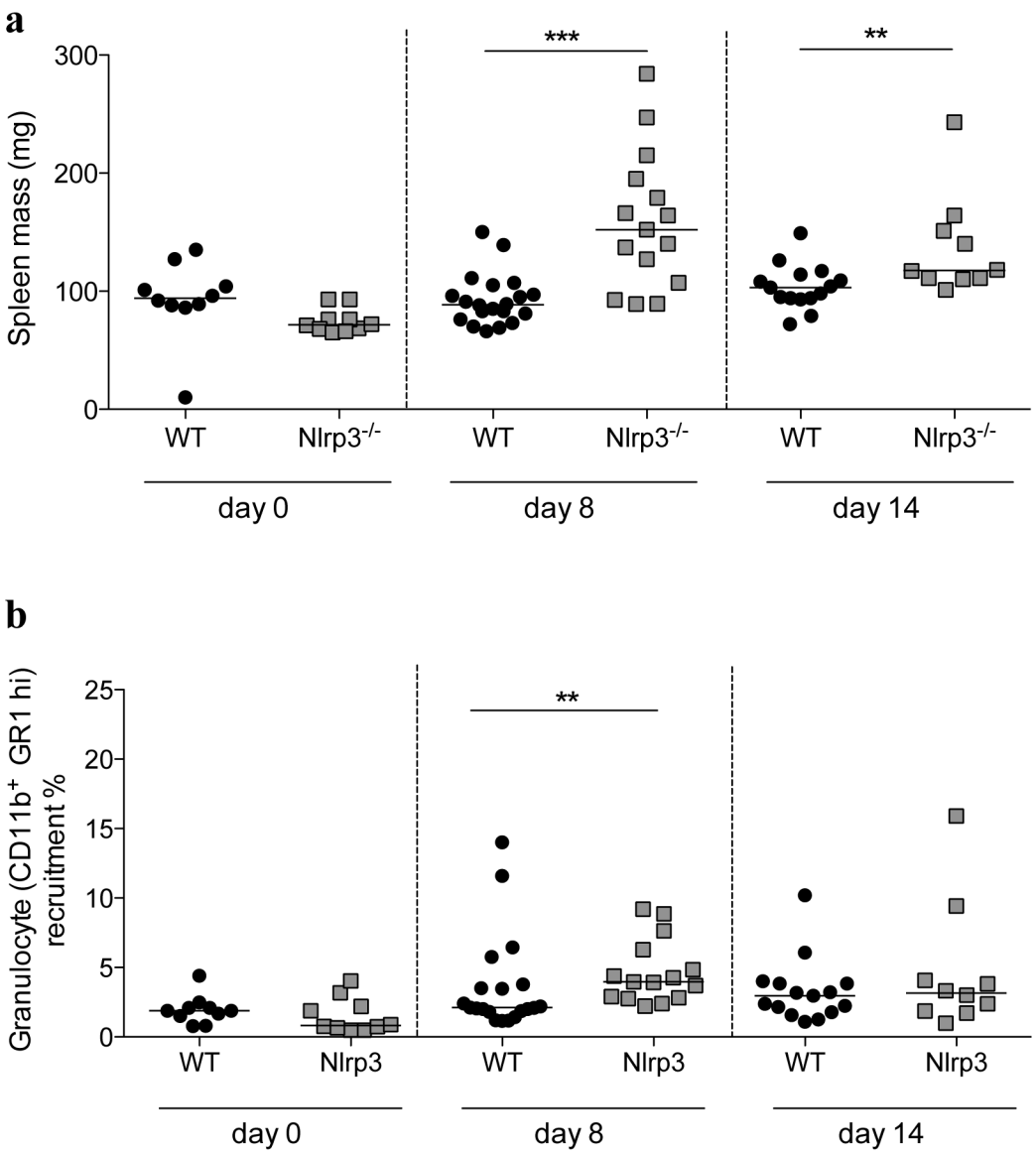

Figure 4.9 *C. rodentium* infected *Nlrp3*$^{-/-}$ mice exhibit splenomegaly with increased granulocyte infiltration. WT and *Nlrp3*$^{-/-}$ mice were orally infected with ~10^9 *C. rodentium* and sacrificed 8 or 14 days post-infection. **a**, Spleen weights **b**, Granulocyte ($CD11b^+$ $Gr1^{hi}$) frequencies as assessed by FACS analysis in the spleen**.** Each symbol represents a single animal and data represent pooled results from at least two independent experiments (n = 9-20 mice/group). Horizontal bars represent group medians. Statistical significance was calculated using the Mann-Whitney test comparing each time point versus the control. ** = $P < 0.01$; *** = $P < 0.001$

4.2.6 Control of *C. rodentium* pathology and colonization is dependent primarily on Nlrp3 expression by non-haematopoietic cells

Having found a protective role for Nlrp3 in immunity against *C. rodentium*, we attempted to identify the cell types that were responsible for mediating this protection. To address this issue, we made use of reciprocal bone marrow chimeras. Thus, to determine the role of Nlrp3 in haematopoietic cells, bone marrow cells from *Nlrp3*$^{-/-}$ mice were transferred into irradiated WT mice, creating *Nlrp3*$^{-/-}$→WT chimeric mice respectively. Conversely, to determine the contribution of Nlrp3 in non-haematopoietic cells, we created WT→*Nlrp3*$^{-/-}$ chimeric mice by injection of WT bone marrow cells into irradiated Nlrp3$^{-/-}$ mice. In addition, WT→WT and *Nlrp3*$^{-/-}$→*Nlrp3*$^{-/-}$ bone marrow reconstituted mice were generated as controls.

In agreement with our previous results, *Nlrp3*$^{-/-}$→*Nlrp3*$^{-/-}$ (wherein Nlrp3 was deficient in both haematopoietic and non-haematopoietic compartments) displayed enhanced weight loss compared to WT→WT mice (Figure 4.10). Interestingly, restoring Nlrp3 expression in the haematopoietic compartment (WT→*Nlrp3*$^{-/-}$) conferred no protection from wasting disease, whereas selective restoration of *Nlrp3* expression in the non-haematopoietic compartment provided robust protection (*Nlrp3*$^{-/-}$→WT) (Figure 4.10). Thus, Nlrp3 expression in non-haematopoietic cells provides dominant protection against *C. rodentium* induced weight loss.

Intestinal inflammation correlated highly with weight loss trends in that an absence of Nlrp3 in the non-haematopoietic cell compartment resulted in greater levels of intestinal pathology following *C. rodentium* infection on day 8 and day 14 post-infection (Figure 4.11). Indeed the levels of typhlitis in mice with Nlrp3 deficiency in both compartments

(*Nlrp3*$^{-/-}$→*Nlrp3*$^{-/-}$) strongly clustered with the levels in chimeric mice selectively lacking Nlrp3 in the non-haematopoietic cells i.e. WT→*Nlrp3*$^{-/-}$ mice (Figure 4.11).

Earlier, we hypothesized that enhanced weight loss and intestinal inflammation in inflammasome deficient mice was a result of an impaired ability to control bacterial burdens. Bone marrow chimeric mice allowed us to identify the cell type in which Nlrp3 inflammasome activation controlled bacterial levels. Consistent with our hypothesis, when Nlrp3 was absent in both compartments (*Nlrp3*$^{-/-}$→*Nlrp3*$^{-/-}$) *C. rodentium* levels in the caecum were high (Figure 4.12). Bacterial colonization levels were equally high in chimeric mice expressing Nlrp3 only in haematopoietic cells (WT→*Nlrp3*$^{-/-}$) (Figure 4.12). In contrast, selective Nlrp3 expression in non-haematopoietic cells (*Nlrp3*$^{-/-}$→WT) was associated with significantly lower *C. rodentium* burdens in the caecum similar to those observed in WT→WT controls (Figure 4.12).

Taken together, the bone marrow chimera studies indicated that the contribution of *Nlrp3* to protection from excessive *C. rodentium* colonization and pathology is largely dependent on Nlrp3 expression within the non-haematopoietic compartment.

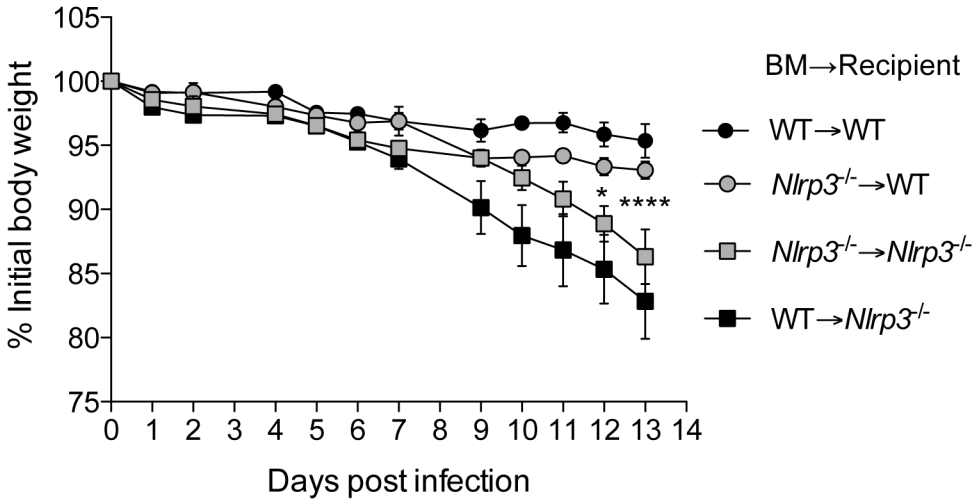

Figure 4.10 Nlrp3 expression in non-haematopoietic cells provides protection from *C. rodentium* induced weight loss. Irradiation bone marrow chimeras were infected with ~10^9 *C. rodentium* and sacrificed 8 or 14 days post-infection. Mean body weights (±s.e.m.) for each group are plotted. Statistical significances were determined between chimera groups and the complete knock-out groups (*Nlrp3*$^{-/-}$ →*Nlrp3*$^{-/-}$). (n = 6-25 mice/group) Statistical significance was determined by two-way ANOVA with Bonferroni post-tests. * = $P < 0.05$; **** = $P < 0.0001$

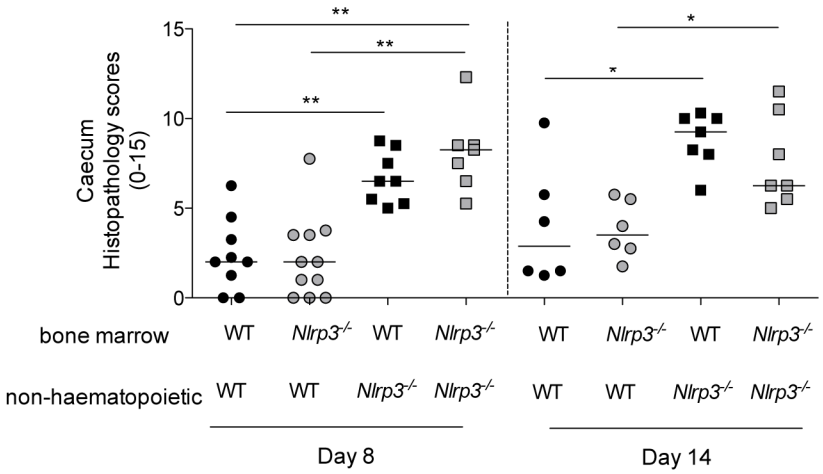

Figure 4.11 Nlrp3 expression in non-haematopoietic cells confers protection from *C. rodentium* induced intestinal inflammation. Irradiation bone marrow chimeras were infected with ~10^9 *C. rodentium* and sacrificed 8 or 14 days post-infection. Inflammation scores in the caecum were assessed as described in materials and methods Data represent pooled results from two to three independent experiments, each symbol represents a single mouse (n = 6-11 mice/group). Statistical significance was calculated using the Mann-Whitney test comparing indicated groups. * = $P < 0.05$; ** = $P < 0.01$.

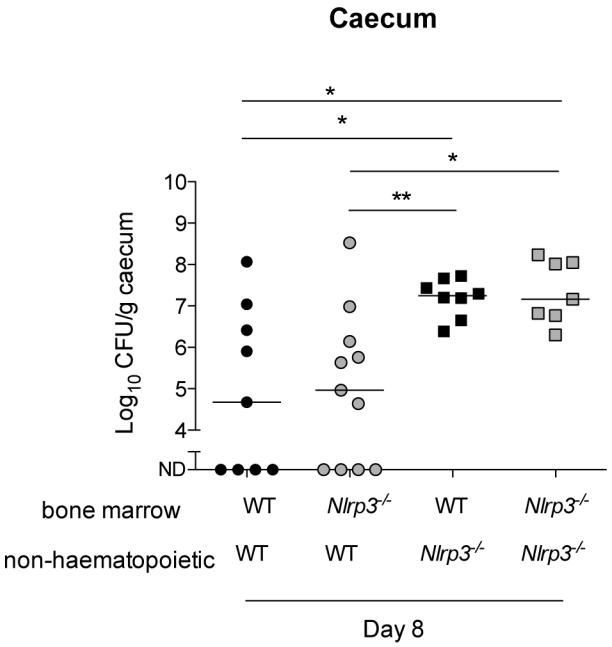

Figure 4.12 Nlrp3 expression in non-haematopoietic cells limits *C. rodentium* colonization. Irradiation bone marrow chimeras were infected with ~10^9 *C. rodentium* and sacrificed after 8. *C. rodentium* loads in the caecum on day 8 post-infection. Data represent pooled results from two to three independent experiments, each symbol represents a single mouse (n = 7-11; ND, not detectable). Statistical significance was calculated using the Mann-Whitney test comparing indicated groups. * = $P < 0.05$; ** = $P < 0.01$.

4.3 Discussion

In this study we have uncovered a crucial protective role for Nlrp3 activation in non-haematopoietic cells in response to *C. rodentium* infection. Mice deficient in *Nlrp3*$^{-/-}$ displayed higher levels of intestinal inflammation compared to WT mice after infection with *C. rodentium*. This effect has been confirmed to be mediated through the Nlrp3 inflammasome complex, as *Asc*$^{-/-}$ mice were also found to suffer from a similar pattern of exacerbated disease following infection with *C. rodentium* (Naren Srinivasan, unpublished observations).

During our study, another lab independently confirmed that both Nlrp3 as well as Nlrc4 inflammasomes were important in protecting against *C. rodentium* infection (311). They showed that the defect in inflammasomes were due to IL-1β and IL-18 (311), which contrasted with our findings. In our model the *Nlrp3*$^{-/-}$ mice produced comparable levels of the two cytokines to WT. This discrepancy may be due to the fact that we measured the secreted cytokines in organ culture supernatants, whilst Liu *et al.* measured these cytokines in tissue homogenates, which contain both pro-IL-1β and pro-IL-18 that could have been released upon cell lysis. In related publications, whilst the role of IL-1 has been confirmed to be important in *C. rodentium* infection (268, 312), and another study suggested that IL-18 was dispensible (312). Despite the differences, evidence provided by our study as well as Liu *et al* confirm that Nlrp3 plays a key role during acute bacterial infections in the intestine (311), supporting the idea that defect in protective immunity triggers IBD. Also, since one of the associated SNPs of NLRP3 resulted in decreased expression of the gene, consistent with the *Nlrp3*$^{-/-}$ phenotype of exacerbated intestinal inflammation (207).

This is in contrast to a previous report on the role of Nlrp3 in the non-haematopoietic cells in the intestine, which highlighted the protective role of IL-18 in DSS-induced intestinal inflammation (215). In support of our finding, other groups have also reported Nlrp3 inflammasome having a protective role in the intestine during inflammation induced by DSS. This is achieved through the actions of IL-18 downstream of Nlrp3 inflammasome activation. However, it remains to be seen if this protective mechanism in a chemically induced intestinal injury model holds for a pathogen-induced intestinal infection.

A recent study conducted in the UK was unable to replicate the original association between Nlrp3 SNP with CD (313). Given the fact that two other papers confirmed Nlrp3's association with CD (208, 209), it is more likely that SNPs of NLRP3 are associated with CD. However, these contradictory results are perhaps a reflection of the low penetrance of susceptibility genes for IBD in general. Furthermore, these data could also suggest a strong influence by environmental factors in triggering disease in susceptible people. Indeed, AIECs have been found frequently associated with the intestinal mucosa patients with CD (314). Although at the moment it is unclear if this association is the cause or consequence of intestinal inflammation. However, given our observation that a defective Nlrp3 in mice contribute to an increased susceptibility to *C. rodentium*, it raises the possibility that patients with disease-associated Nlrp3 variants may require an infection like *E. coli* in order to initiate disease.

Our study identifies a novel role in non-haematopoietic cells. PRR expression on IECs has been shown to regulate the production of AMPs, angiogenins, epithelial cell turnover, and barrier restitution (15, 113, 315). These innate immune mechanisms allows for the detection of pathogenic insult and are important in limiting the number of bacteria

translocating from the lumen into the mucosa. The fact that Nlrp3 offers protection against *C. rodentium* in non-haematopoietic cells such as IECs is consistent with the early protective defect observed in *Nlrp3*$^{-/-}$ mice. Furthermore, *C. rodentium* is known to target IECs by injecting a variety of virulence factors delivered through its T3SS (316). These virulence factors allow the bacteria to efficiently attach to the host cells in order to proliferate (316). Therefore, it makes sense that Nlrp3 is activated in target cell upon the detection of an infection by *C. rodentium*. In fact, Nlrp3's role in the epithelium has already been highlighted in DSS-induced intestinal injury model (215, 216). This makes sense as DSS is toxic to the intestinal epithelial cells, and Nlrp3 does respond to many different types of toxins and cellular stress (317). Together with our study, these data suggest that Nlrp3 in the intestinal epithelium is a key sensor of stress, both from chemicals as well as from pathogens.

This acute role played by Nlrp3 in non-haematopoietic cells may in fact explain the little protection provided by Nlrp3 inflammasome in mice chronically infected with *H. hepaticus* and treated with αIL-10R. First of all, any acute protection in this model is likely obscured by the involvement of dysfunctional adaptive immune response caused by αIL-10R mAb administration. Secondly, *H. hepaticus* does not directly infect IECs, which further diminishes the ability of Nlrp3 inflammasome to sense the infection. Again, in this study, the need for a specific pathogen to induce exacerbated disease in a genetically compromised host again supports the multihit model of IBD (3).

In conclusion, the data presented in this chapter clearly show that Nlrp3 plays a protective role in non-haematopoietic cells against an intestinal pathogen in an IL-1β and IL-18-independent manner. Not only is this observation interesting as it confirms the genetic association studies that Nlrp3 is important for intestinal health, but this study also

suggests that there may be novel functions of Nlrp3 independent of its classical cytokine secretion.

Chapter 5 – Novel protective mechanisms of Nlrp3 in the intestine

5.1 Introduction

Having established a protective role for Nlrp3 inflammasome in the non-haematopoietic compartment during *C. rodentium* infection, we next sought to determine the mechanism through which Nlrp3 was able to mediate this effect. One major issue to resolve is to determine if the increased bacterial colonization was driving increased intestinal pathology or vice versa. For example, limiting bacterial outgrowth through antimicrobial peptides can reduce the magnitude of inflammation elicited in response to intestinal infection (272, 318). Conversely, certain pathogens appear adapted to thrive in an inflammatory environment, such as *Salmonella*, which can utilise tetrathionate generated in an inflammatory environment as a new electron acceptor, giving this pathogen a selective advantage to outgrow the microbiota in this environment (319).

Since the bone-marrow chimera experiments indicated a major protective role of Nlrp3 activation in the non-haematopoietic compartment, and because *C. rodentium* initially infects IECs, we hypothesized that the exacerbated disease observed in *Nlrp3*$^{-/-}$ mice could be due to an impaired early innate immune response in the epithelium. Indeed, recent research has shown that immunity against *C. rodentium* involves innate immune responses over the first 4 days post-infection that control bacterial infection until adaptive immune responses can be mounted to clear the pathogen (106, 320, 321). The exacerbated intestinal inflammation phenotype observed in *C. rodentium* infected *Nlrp3*$^{-/-}$ mice could be explained by an impaired ability to control bacterial colonization and/or a defective or delayed induction of an adaptive immune response. Since *Nlrp3*$^{-/-}$ mice eventually cleared the infection, we hypothesized that an early impaired control of bacterial colonization was

responsible for the exacerbated phenotype. Indeed studies on the early phase of *C. rodentium* infection showed that IL-23-dependent production of IL-22 and IL-17A, mainly derived from ROR-γ^+ ILCs, was crucial in affording protection (106, 322). The protective effects of IL-17A and IL-22 correlated with induction of AMPs, such as RegIIIγ, from IECs (106, 322). However a link between NLRP3 inflammasome activation and innate type 17 responses in the gut remains to be established.

In addition to driving the maturation and secretion of IL-1β and IL-18, another downstream effect of Nlrp3 inflammasome activation is pyroptosis, an inflammatory form of programmed cell death driven by activation of caspase-1, that is accompanied by release of pro-inflammatory cytokines (323, 324). In fact, a recent study delineated a non-canonical pathway of caspase-11-dependent inflammasome activation that was required for the induction of pyroptosis in macrophages in response to *C. rodentium* (324). However, Nlrp3 was not reported to be involved in caspase-11-induced pyroptosis in macrophages (324), and whether this pathway functions in non-haematopoietic cells remains to be determined. Nevertheless, IEC death is a feature of *C. rodentium* infection and this is mediated through the actions of T3SS-injected virulence factors, such as EspF and Map, on mitochondria (325, 326). Given the recent papers showing that Nlrp3 senses mitochondrial dysfunction (202, 327), it is tempting to speculate that Nlrp3 might be involved in the cell death resulting from these mitochondria-linked *C. rodentium* effector proteins. Moreover, it raises the question whether Nlrp3-induced IEC death may in fact be a protective mechanism to shed infected epithelial cells, thus limiting the rate of colonization during the early stages of infection. In fact, data exists that highlight caspase-1-dependent epithelial cell extrusion upon infection with *Salmonella*, although this was reported to aid the spread of the bacteria (328). Lastly, there may be novel uncharacterised mechanisms through which NLRP3 inflammasome activation serves to

protect the intestinal epithelium against mucosal pathogens. Therefore, unbiased screening approaches such as microarrays may constitute a useful approach to identify these novel protective mechanisms.

In this chapter, by investigating an early time point after infection, we aimed to test the hypothesis that the exacerbated disease in *Nlrp3*$^{-/-}$ mice is driven by uncontrolled bacterial outgrowth. Secondly, we wanted to determine whether previously reported protective mechanisms in the *C. rodentium* model were linked to activation of the Nlrp3 inflammasome. Finally, we aimed to comprehensively characterize the early innate immune response induced by Nlrp3 activation in the intestine by performing microarray analyses of WT and *Nlrp3*$^{-/-}$ mice following infection with *C. rodentium.*

5.2 Results

5.2.1 The Nlrp3 inflammasome mediates early control of *C. rodentium* colonization

To test the hypothesis that an increased bacterial outgrowth in *Nlrp3*$^{-/-}$ mice caused the exacerbated pathology, we performed an acute infection wherein mice were infected with *C. rodentium* and then euthanized 3 days post-infection. Strikingly, we found that even as early as 3 days post-infection, *Nlrp3*$^{-/-}$ mice already harboured significantly higher levels (100-1000 fold) of tissue-adherent *C. rodentium* in their caeca compared to WT mice (Figure 5.1 a). In contrast, histological analyses of intestinal samples revealed that no overt intestinal pathology was discernable in either WT or *Nlrp3*$^{-/-}$ mice at 3 days post-infection (Figure 5.1 b). Thus, it appears that activation of the Nlrp3 inflammasome occurs early in response to *C. rodentium* infection and serves to limit bacterial colonization.

To explore the mechanism of protection conferred by the inflammasome we first measured the levels of IL-1β and IL-18, two cytokines that are processed for secretion by caspase-1 following Nlrp3 inflammasome activation (329, 330). However, after 3 days of infection, no differences in the level of IL-1β or IL-18 were observed in ceacal explants from WT and *Nlrp3*$^{-/-}$ mice (Figure 5.2). Next, we assayed caecal expression of several cytokines previously shown to be important for protection against *C. rodentium*, such as *Il17a*, *Il17f*, *Ifng*, and *Il23a*, using qPCR (274, 309, 331). We found that baseline expression of these cytokines was similar in WT and *Nlrp3*$^{-/-}$ mice. Furthermore, at 3 days post-infection, *Nlrp3*$^{-/-}$ mice expressed similar levels of *Il23a* and *Ifng* and had a trend towards increased expression of *Il17a* and *Il17f* (Figure 5.3). Thus, there was no obvious defect in these protective cytokine responses in *Nlrp3*$^{-/-}$ mice following infection with *C. rodentium.* Taken together, these results clearly show that Nlrp3-mediated protection act

at a very early time point through an IL-1β and IL-18 independent mechanism, to control *C. rodentium* colonization in the intestine.

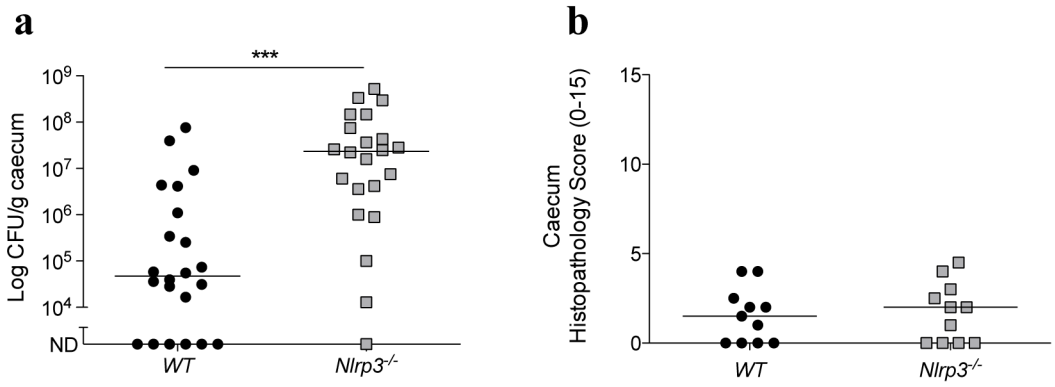

Figure 5.1 The Nlrp3 inflammasome mediates early immunity against *C. rodentium*. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 3 days post-infection. **a**, tissue adherent bacteria enumerated following homogenisation of caeca samples and plating on LB plates supplemented with nalidixic acid. (n = 17-20 mice/group). **b**, caecal samples were fixed in formol saline and paraffin-embedded sections were analysed by staining in H&E. Inflammation scores in the caecum. Bars represent medians and each symbol represents an individual mouse. Data represent pooled data from two to four independent experiments (n = 6-11 mice/group). Statistical significance was calculated using the Mann-Whitney test between WT and *Nlrp3*$^{-/-}$ mice. *** = $P < 0.001$.

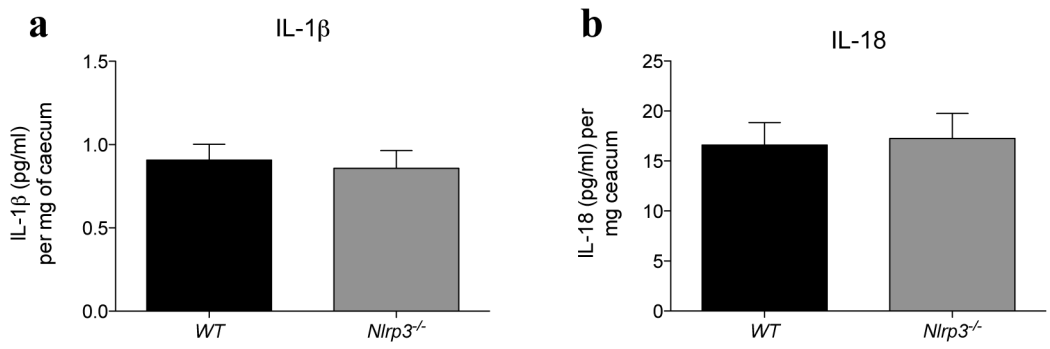

Figure 5.2 Comparable caecal IL-1β and IL-18 levels in WT and *Nlrp3*$^{-/-}$ mice at 3 days post-infection with *C. rodentium.* WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 3 days post-infection. Caecal samples were isolated and incubated overnight in media, and the supernatants harvested and cytokine levels were measured by ELISA. **a,** IL-1β. **b,** IL-18. Bars represent mean of pooled data from one of two independent experiments (n = 5 mice/group). Error bars represent ±s.e.m. of mean.

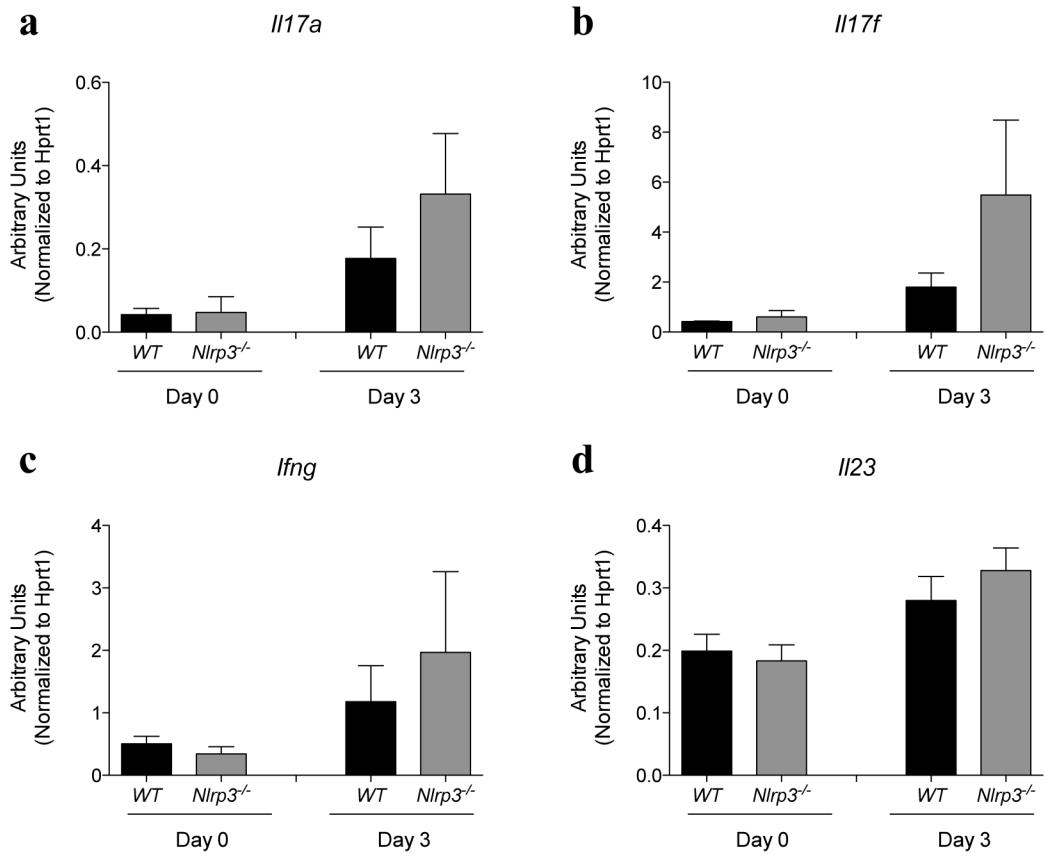

Figure 5.3 Caecal inflammatory cytokine expression in WT and *Nlrp3*$^{-/-}$ mice on day 3 post-infection with *C. rodentium*. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 3 days post-infection. At the indicated time points pieces of caecal tissue were snap frozen in liquid nitrogen. Total RNA was then isolated and mRNA expression levels were measured by qPCR and normalized to *Hprt1*. Mean mRNA levels of **a,** *Il17a*, **b,** *Il17f*, **c,** *Ifng* and **d,** *Il23a*. Error bars represent ±s.e.m. of mean.

5.2.2 Early protective effect through Nlrp3 activation is independent of ILC-IL-22 axis

Recent research has highlighted an early ILC-IL22-dependent protective mechanism against *C. rodentium* infection (106, 272). To assess the accumulation of ILCs in the intestines of WT and *Nlrp3*$^{-/-}$ mice, we performed FACS staining in infected mice 3 days post infection. However, the results showed similar levels of CD45^{+} Lin^{-} Thy1^{+} Sca1^{+} ILC in the caecum (Figure 5.4), when in both WT and *Nlrp3*$^{-/-}$ animals. In addition, qPCR assessment of mRNA expression of *Il22* and *Reg3g* (Figure 5.4 a, b) indicated that the *Nlrp3*$^{-/-}$ mice did not have a defect in the induction of these genes, as they had a trend towards increased expression compared to WT mice at day 3 post-infection.

More recently, epithelial IL-17C signalling has been found to synergize with IL-22 signalling in inducing protective antimicrobial peptides in *C. rodentium*-infected mice (332). However, we found similar levels of expression of *Il17c* in *Nlrp3*$^{-/-}$ and WT mice by qPCR at steady state or 3 days post infection (Figure 5.5 c). These results suggest that the lack of early protection in *Nlrp3*$^{-/-}$ mice is not due to defective induction of the ILC-IL-22 axis.

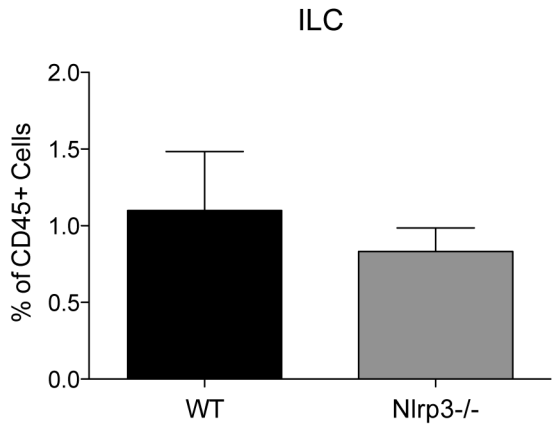

Figure 5.4 Similar intestinal ILC frequencies in WT and *Nlrp3*$^{-/-}$ mice.
WT and *Nlrp3*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and sacrificed 3 days post infection. LPLs from caecum were isolated and analysed by flow cytometry. ILCs were defined as $CD45^+$ Lin^- $Thy1^+$ $Sca1^+$ cells. (n = 4 mice/group). Data are from a single experiment. Error bars represent ±s.e.m. of mean.

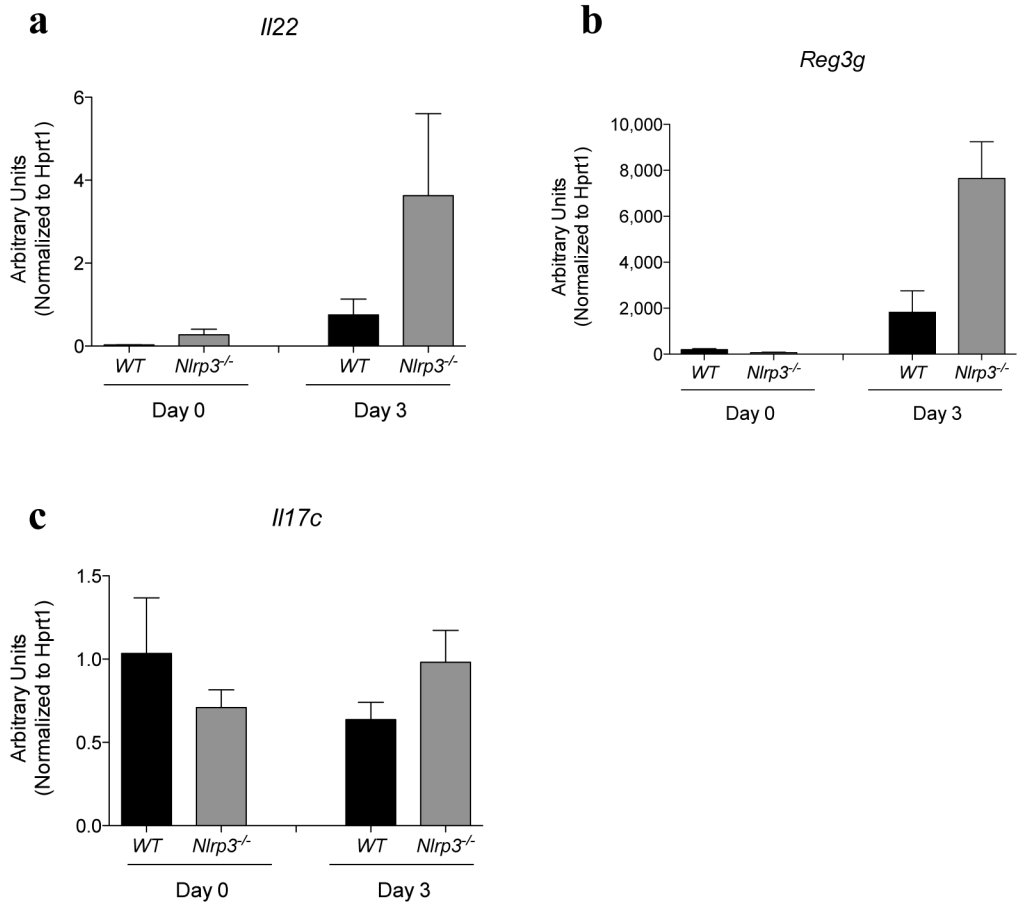

Figure 5.5 *Il22*, *Reg3g* and *IL17c* levels in WT and *Nlrp3*$^{-/-}$ mice after *C. rodentium* infection. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 3 days post-infection. At the indicated time points pieces of caecal tissue were snap frozen in liquid nitrogen. Total RNA was then isolated and mRNA expression levels were measured by qPCR and normalized to *Hprt1*. Mean mRNA levels of **a,** *Il22*. **b,** *Reg3g.* **c,** *Il17c.* (**a-c)** (n = 3 mice/group). Error bars represent ±s.e.m. of mean.

5.2.3 Assessment of IEC death as a potential early protective response to *C. rodentium*

To assess if death of infected IEC could help to limit infection and also alert the immune system, we decided to first visualize cells undergoing pyroptosis in intestinal sections, using TUNEL staining (333). However, the frequency of TUNEL-positive cells was extremely low in the intestinal sections from both WT and *Nlrp3*$^{-/-}$ mice (Figure 5.6). Whilst representative sections from day 14 post-infection are shown in Figure 5.6, sections from day 3 post-infection as well as day 8 post-infection have also been analysed and the same observations of very low levels of TUNEL^{+} cells in both WT and *Nlrp3*$^{-/-}$ mice were made (data not shown).

Since caspase-11 has been shown to be involved in *C. rodentium*-induced pyroptosis in macrophages (324), we next sought to determine if caspase-11 might be involved in clearance of *C. rodentium in vivo*. We infected both WT C56BL/6 as well as caspase-11-deficient 129SvEv mice with *C. rodentium.* On day 3 post-infection, the caeca were harvested and higher levels of *C. rodentium* were present in 129SvEv mice (Figure 5.7). Although this result could suggest that caspase-11 may be required for this early protective response, however, many other genetic differences between the two strains of mice could also have contributed to this difference.

Furthermore, to investigate if Nlrp3 detects perturbation of mitochondria function by T3SS delivery of effector proteins such as EspF, which has been shown to be important in *C. rodentium*-induced cell death (325), we tested whether a *C. rodentium* mutant lacking EspF (ΔEspF) would remove the difference in disease between WT and *Nlrp3*$^{-/-}$. However, we found that *Nlrp3*$^{-/-}$ mice again displayed about 100-1000 fold higher bacterial burdens than WT mice on day 3 post-infection with ΔEspF *C. rodentium*,

suggesting that EspF was not involved in early inflammasome activation by *C. rodentium* (Figure 5.8). This observation was not surprising as other effector proteins like Map is also known to be involved in affecting the mitochondria and therefore be involved in *C. rodentium*-induced cell death (326).

To further explore the role of programmed IEC death in our model, we decided to measure the lactate dehydrogenase (LDH) levels in the lumen of the caecum following infection with *C. rodentium*, as a marker of host cell death (324). We observed equivalent LDH activity in the caecal lumen contents from WT and *Nlrp3*$^{-/-}$ mice on day 8 post-infection with *C. rodentium* (Figure 5.9 b). Preliminary data obtained on day 3 post-infection also followed this trend of equivalent LDH activity in WT and *Nlrp3*$^{-/-}$ mice (data not shown). Therefore, we decided to look even earlier for differences in cell death, before differences in bacterial burden between *Nlrp3*$^{-/-}$ and WT mice became apparent. We found that bacterial burdens were similar in WT and *Nlrp3*$^{-/-}$ mice 1 day post-infection with *C. rodentium* (Figure 5.9 c), and again, the normalized LDH levels were similar in both groups (Figure 5.9 d).

As an alternative assay for measuring cell death, we decided to pursue an *ex vivo* polarized organ culture approach (286). However, despite many attempts to optimize this technique, preliminary data showed massive cell death and LDH release, which approached the level of positive controls that were lysed with detergent (Figure 5.10).

Taken together, these results illustrate that there is the need to develop more sensitive and robust *ex vivo* techniques that would allow the measurement of cells undergoing pyroptosis, a potentially important mechanism downstream of Nlrp3 activation.

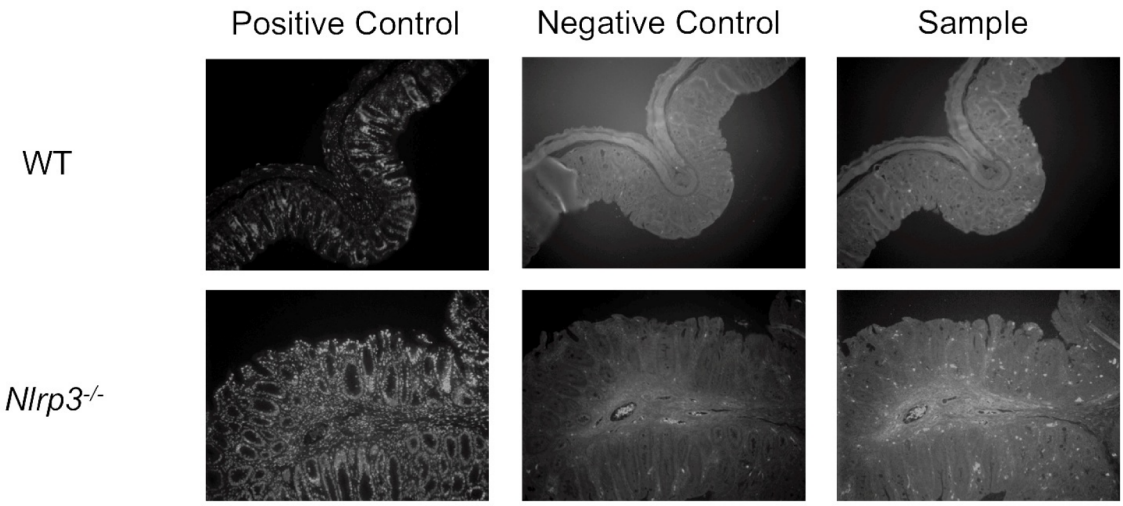

Figure 5.6 No defect in TUNEL positive cells observed in the caecum of *Nlrp3*$^{-/-}$ mice as compared to WT. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 14 days post-infection. Caecal samples were fixed in formol saline and paraffin-embedded sections were using a *in situ* TUNEL kit. Representative sample are shown above. Positive controls are generated using sections treated with TACS Nuclease to generate DNA breaks. Negative controls are generated using sections that are unlabelled.

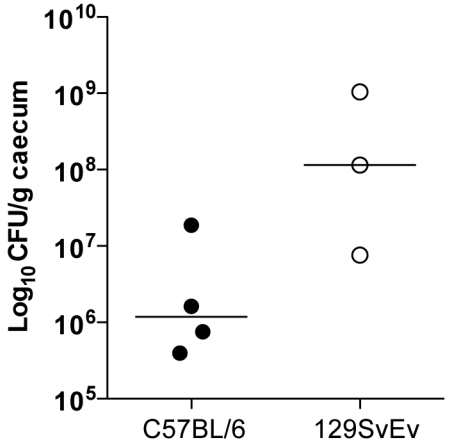

Figure 5.7 *C. rodentium* colonization in 129SvEv mice compared to C57BL/6 mice. Mice were infected with ~10^9 *C. rodentium* and sacrificed 3 days post-infection. Caecal tissue adherent bacteria enumerated following homogenisation of caeca samples and plating on LB plates supplemented with nalidixic acid. Horizontal bars represent the medians and each symbol represents an individual mouse (*n* = 3-4 mice/group). Data from a single experiment.

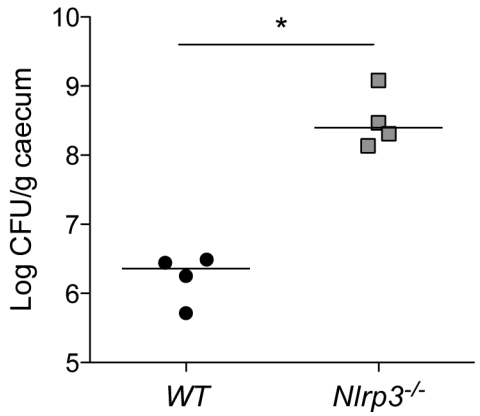

Figure 5.8 Increased level of colonization in *Nlrp3*$^{-/-}$ mice after infection with ΔEspF *C. rodentium*. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 ΔEspF *C. rodentium* and sacrificed 3 days post-infection. Tissue adherent bacteria were enumerated following homogenisation of caeca samples and plating on LB plates supplemented with nalidixic acid. Data from a single experiment (n = 4 mice/group). Statistical significance was calculated using the Mann-Whitney test between WT and *Nlrp3*$^{-/-}$ mice. * = $P < 0.05$.

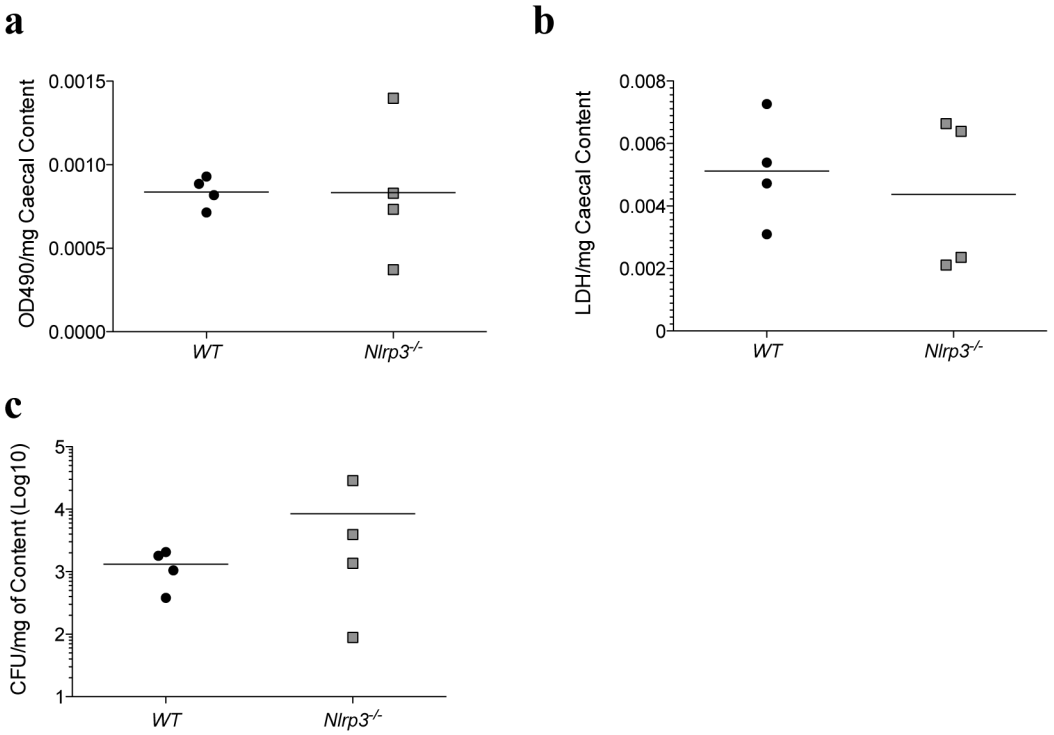

Figure 5.9 No differences in caecal supernantant LDH activity in WT and *Nlrp3*$^{-/-}$ mice. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed at various time points. Caecal contents were isolated in PBS and supernatants were analysed for LDH activity, which was normalised by weight of the caecal contents. **a,** 8 days post-infection. **b,** 3 days post-infection. **c,** CFU at day 1 post-infection. Bars represent mean of pooled data from one experiment (n = 4 mice/group).

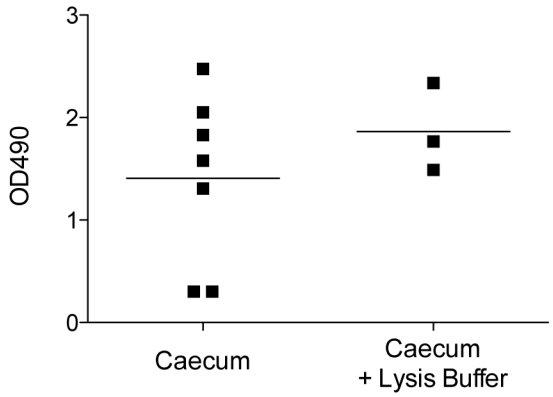

Figure 5.10 High levels of LDH release in polarized organ cultures. Caeca were isolated from *Nlrp3*$^{-/-}$ mice and set up in a polarized organ culture for 2h before supernatants were isolated for LDH assay. Bars represent mean of pooled data. Each point represents an individual experiment (n = 3-7 organ cultures).

5.2.4 Expression profiling of caecal tissue for WT and *Nlrp3*$^{-/-}$ mice 3 days post-infection with *C. rodentium*

To identify potential factors through which the Nlrp3 inflammasome mediates early protection against *C. rodentium* infection, we performed genome-wide transcriptional profiling of caecal tissue. During both steady state, as well as on day 3 post-infection with *C. rodentium*, very few genes were expressed at a lower level in the *Nlrp3*$^{-/-}$ mice than WT (Tables 5.1 and 5.2). Surprisingly, more than 150 genes were induced at significantly higher levels in *Nlrp3*$^{-/-}$ mice relative to WT mice 3 days post-infection (Figure 5.11), although this may be due to the higher bacterial levels present in the *Nlrp3*$^{-/-}$ mice. Of note, *Reg3g*, an antimicrobial peptide previously shown to be important in protection against *C. rodentium* infection, was significantly upregulated in both *Nlrp3*$^{-/-}$ as well as WT mice (Figure 5.11). This agrees well with our previous observations that many protective mechanisms, such as *Reg3g* induction, are intact in the *Nlrp3*$^{-/-}$ mice.

Interestingly, the biggest difference in gene expression between *Nlrp3*$^{-/-}$ and WT mice was the erythroid differentiation regulator 1 (*Erdr1*), which was downregulated in *Nlrp3*$^{-/-}$ mice at steady state and 3 days post-infection (Tables 5.1 and 5.2). To date, only few studies have investigated the function of this gene, which have identified *Erdr1* as an interleukin 18-regulated gene that acts as a proapoptotic factor (334, 335). In addition, the expression of *Ang4* (angiogenin 4), an intestine-specific AMP (336), was also strongly down-regulated in *Nlrp3*$^{-/-}$ mice compared to WT mice at steady state and to a lesser degree on day 3 post-infection (Figure 5.12 a), suggesting that Ang4 might contribute to the early control of *C. rodentium* colonization. However, this trend was not preserved in bone-marrow chimera experiments, therefore it might not be a key factor in protection against *C. rodentium* (Figure 5.12 b). Furthermore, 7 genes were exclusively induced

upon infection in WT mice but not in *Nlrp3*$^{-/-}$ mice (Figure 5.11). These included genes encoding a gap junction protein (*Gja5*) and the chemokine (*Ccl5*). All of these genes, although not well characterized, are interesting targets for future studies.

Fold Change		Gene
5.923463	up	*LOC386091*
2.8927803	up	*Wdfy1*
2.6390104	up	*Igh-V7183*
2.4284787	up	*Trpv6*
2.213158	up	*9626958_317*
2.2015004	up	*9626100_224*
2.0852547	up	*Cyp4b1*
2.0723717	down	*LOC333501*
2.1271422	down	*H2-DMa*
2.1976376	down	*1110017D15Rik*
2.2075124	down	*Indo*
2.376288	down	*IGKV4-80_AJ231213_Ig_kappa_variable_4-80_91*
2.4801543	down	*Ear4*
2.728001	down	*Ear2*
3.0225618	down	*Ear10*
3.0317245	down	*LOC243431*
3.1324825	down	*Retnla*
3.322534	down	*Ear1*
3.3306968	down	*Ear2*
4.0799747	down	*Ang4*
16.995226	down	*Erdr1*

Table 5.1 Genes Significantly (P < 0.05) Differentially expressed at least 2-folds between uninfected *Nlrp3*$^{-/-}$ vs. uninfected WT mice. Uninfected WT and *Nlrp3*$^{-/-}$ mice were sacrificed (n = 3). RNA was isolated from caeca and gene expression profiled using microarray.

Fold Change		Gene
8.697943	up	*Atp1b2*
8.013789	up	*LOC386091*
5.2546215	up	*Fosb*
4.9006944	up	*Krt1-23*
3.5663567	up	*Acta1*
3.0598383	up	*Wdfy1*
3.0116017	up	*Uck2*
2.9329631	up	*9626100_224*
2.890252	up	*Uck2*
2.783564	up	*Mmp3*
2.7695267	up	*Tnfaip3*
2.690243	up	*9626100_15*
2.6304424	up	*C920004C08Rik*
2.6234145	up	*9626958_317*
2.5883603	up	*Uck2*
2.4761643	up	*Mscp*
2.473403	up	*Camk2b*
2.414328	up	*Mscp*
2.3914492	up	*Cebpb*
2.3379736	up	*Trpv6*
2.2966743	up	*Mela*
2.2648134	up	*Gp38*
2.2190664	up	*Akr1c18*
2.1319032	up	*Mmp3*
2.0867195	up	*Gstm5*
2.0712802	up	*LOC380804*
2.0430684	up	*1700018O18Rik*
2.018406	up	*1300007C21Rik*
2.0145612	down	*Pbp2*
2.0565367	down	*H2-Ab1*
2.3935397	down	*Arg2*
2.9014754	down	*Retnla*
15.422449	down	*Erdr1*

Table 5.2 Genes Significantly (P < 0.05) differentially expressed at least 2 folds between *C. rodentium*-infected *Nlrp3*$^{-/-}$ vs. infected WT mice. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 3 days post infection (n = 3). RNA was isolated from caeca and gene expression profiled using microarray.

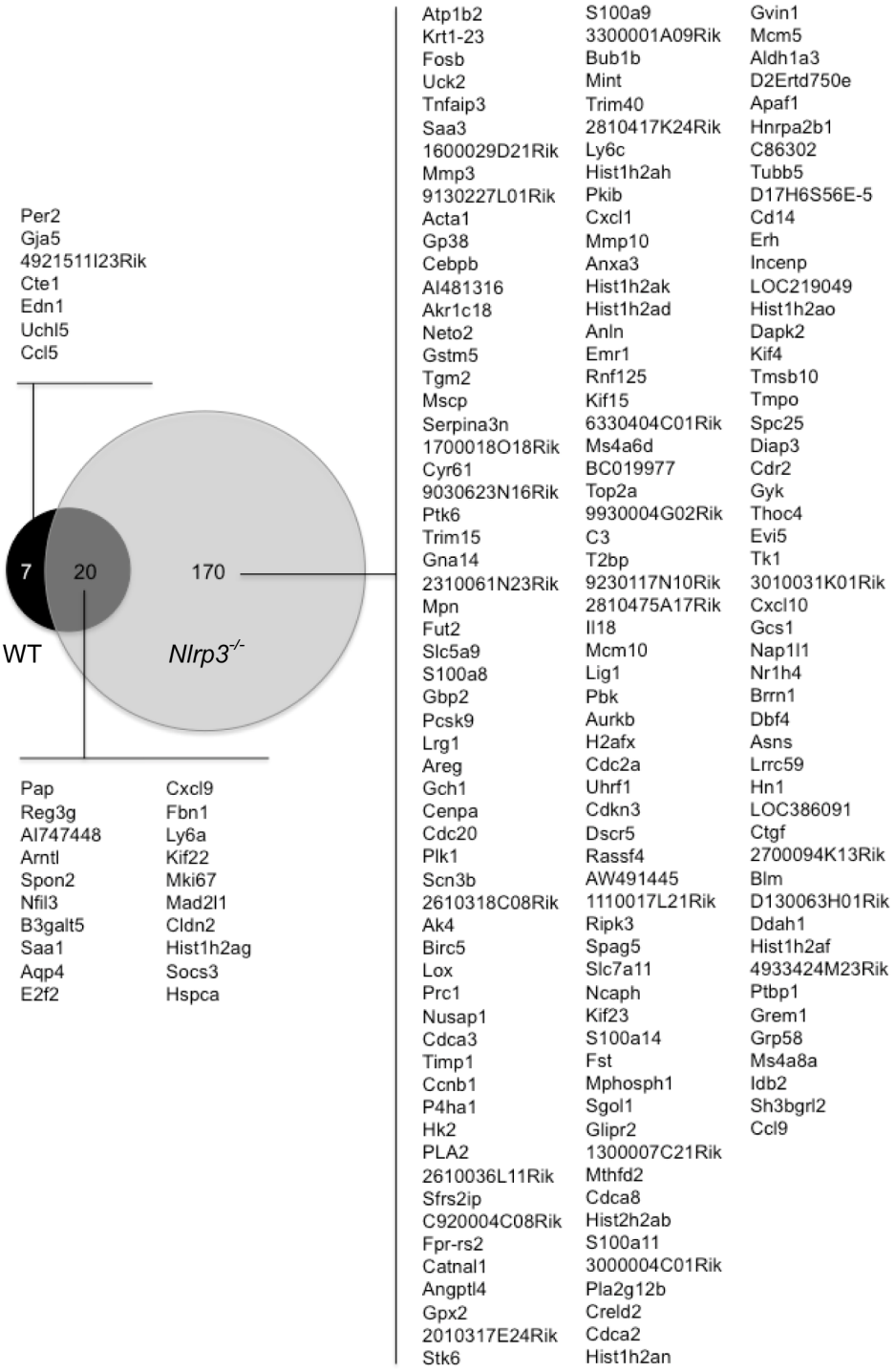

Figure 5.11 Upregulated genes upon *C. rodentium*-infection in WT or *Nlrp3*$^{-/-}$ mice. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 3 days post infection (n = 3). RNA was isolated from caeca and gene expression profiled using microarray. All of the induced genes (≥ 1.5-fold) in WT and *Nlrp3*$^{-/-}$ mice were compared and listed in the Venn diagram, which shows these genes upregulated in WT only (7); genes upregulated in both WT and *Nlrp3*$^{-/-}$ (20) and genes upregulated in *Nlrp3*$^{-/-}$ only (170)

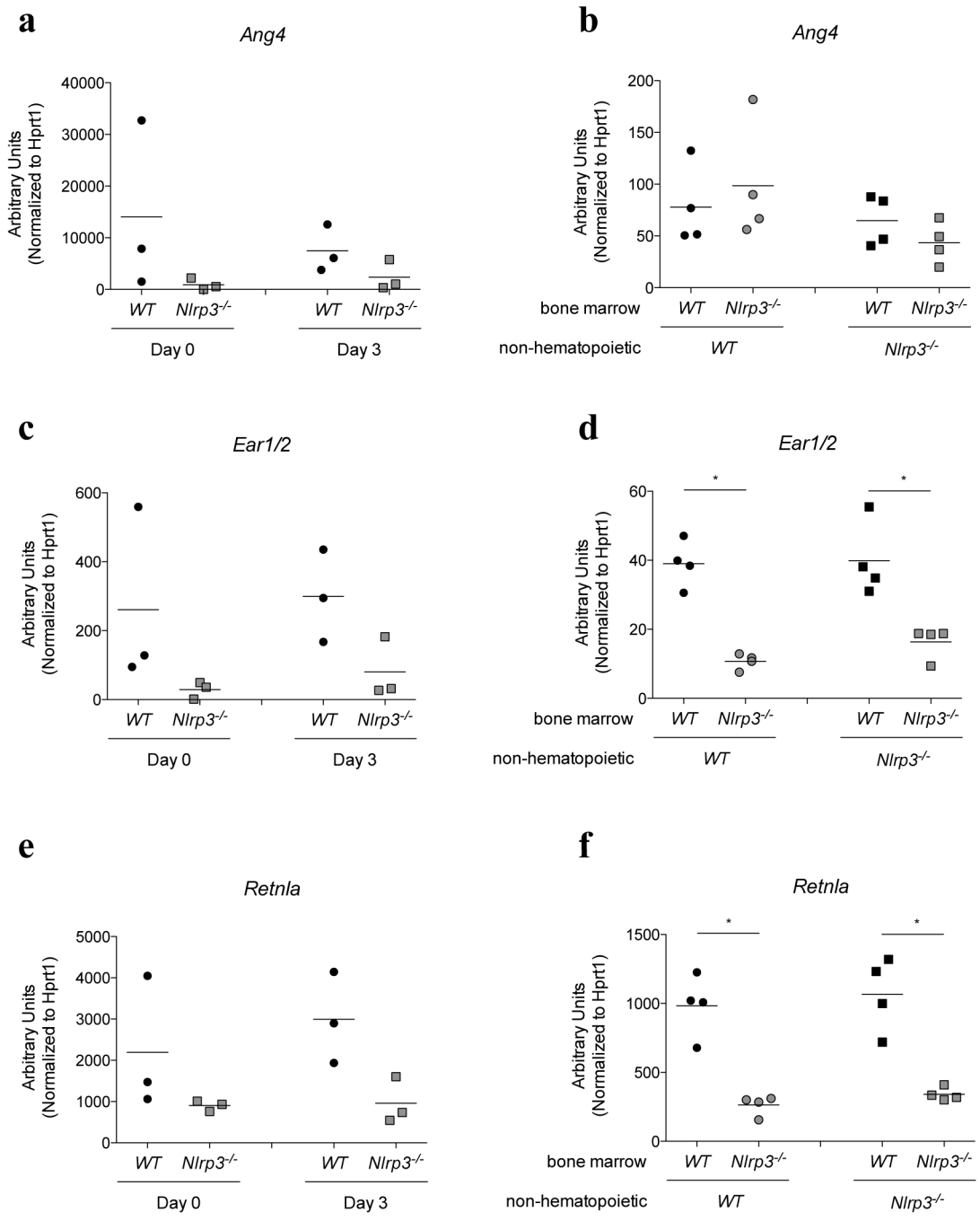

Figure 5.12 qPCR validation of selected genes that were differentially expressed in the microarray assays. WT, *Nlrp3*$^{-/-}$ or bone marrow chimera mice were infected with ~10^9 *C. rodentium* and sacrificed day 3 post infection. RNA was isolated from caeca and gene expression profiled by qPCR. **a** and **b,** *Ang4*. **c** and **d,** *Ear1/2*. **e** and **f,** *Retnla*. (**a-f**) Data obtained from one experiment (n = 3-4 mice/group). Statistical significance was calculated using the Mann-Whitney test between indicated groups. * = $P < 0.05$.

5.2.5 Expressions of eosinophil-related genes are reduced in the caeca of *Nlrp3*$^{-/-}$ mice

Of the few genes observed at a lower level in *Nlrp3*$^{-/-}$ mice during steady state, several were associated with eosinophil function or recruitment, such as Eosinophil-associated, ribonuclease A family members *Ear1*, *Ear2*, *Ear4*, *Ear10* (337) and resistin like alpha (*Retnla*) (338, 339) (Table 5.1; Figure 5.12 c and e). Although the *Ear1/2* were not more than 2-fold lower on day 3 post-infection with *C. rodentium*, they remained down regulated as shown by qPCR validation (Figure 5.12 c).

These observations prompted us to investigate the role of eosinophils during *C. rodentium* infection. However, qPCR measurement of both *Ear1/2* as well as *Retnla* expression levels in the caecum of bone marrow chimeras showed a clear association between low levels of *Ear1/2* expression and the *Nlrp3*$^{-/-}$ haematopoietic compartment (Figure 5.12 d and f). This is inconsistent with the observation that the exacerbated disease in these bone marrow chimeras correlated only with the non-haematopoietic genotype (Figure 4.10-12).

Finally, to evaluate if there was any defect in eosinophil accumulation in the intestine, LPLs were isolated from caeca of *Nlrp3*$^{-/-}$ or WT mice and stained for eosinophils (Figure 5.13). However, we did not detect any difference in the accumulation of eosinophils in the caecum of *Nlrp3*$^{-/-}$ or WT mice on day 3 post-infection with *C. rodentium* (Figure 5.14). In fact, elevated percentages of eosinophils (compared to total CD45^{+} cells) were observed in the *Nlrp3*$^{-/-}$ mice at steady state and at day 3 post-infection (Figure 5.14).

Therefore, whilst it is interesting that a Nlrp3 deficiency in the haematopoietic system leads to lower levels genes linked with eosinophils, but to a higher frequency of intestinal

eosinophils, this does not seem to be the early protective mechanism during *C. rodentium* infection.

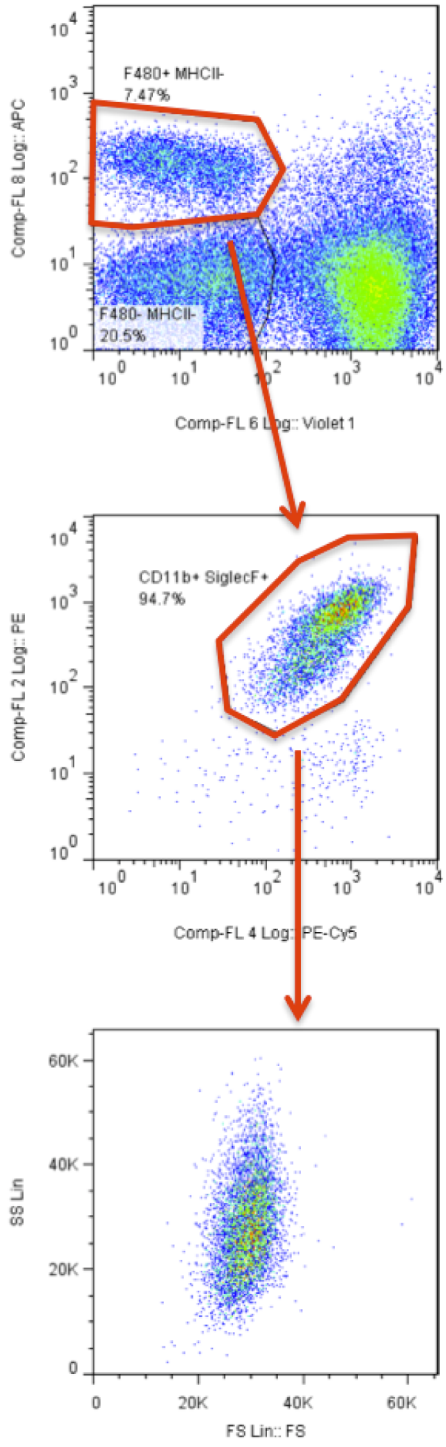

Figure 5.13 Eosinophil gating strategy. WT and *Nlrp3*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and sacrificed 3 days post infection. LPLs from caecum were isolated and analysed by flow cytometry. Eosinophils are defined as CD45$^+$ F4/80$^+$ MHCII$^-$ CD11b$^+$ Siglec-F$^+$ cells.

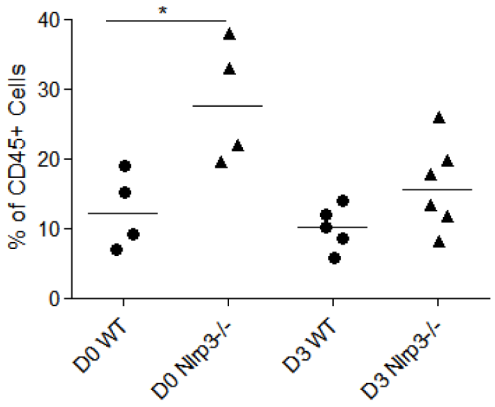

Figure 5.14 Elevated eosinophil frequencies in the caecum of *Nlrp3*$^{-/-}$ mice. WT and *Nlrp3*$^{-/-}$ mice were infected orally with ~10^9 *C. rodentium* and sacrificed 3 days post-infection. LPLs from caeca were isolated and analysed by flow cytometry. Eosinophils were defined as CD45$^+$ F4/80$^+$ MHCII$^-$ CD11b$^+$ Siglec-F$^+$ cells. Horizontal bars represent the mean and each symbol represents an individual mouse (n = 4-6 mice/group). Statistical significance was calculated using the Mann-Whitney test between WT and *Nlrp3*$^{-/-}$ mice. * = $P < 0.05$.

5.2.6 Signatures of alternatively activated macrophages are expressed at a lower level in *Nlrp3*$^{-/-}$ mice compared to WT mice

In addition to *Retnla*, which is also a marker of alternatively-activated macrophage (340), *Arg2*, which has been reported to be induced in murine macrophages upon exposure to apoptotic cell derived factors (341), was also significantly down regulated in *Nlrp3*$^{-/-}$ mice on day 3 post-infection with *C. rodentium* (Table 5.2). In fact, *Arg2* was also significantly down regulated in *Nlrp3*$^{-/-}$ mice at 14 days post-infection (Figure 5.15 c). However, *Arg2* has also been shown to be an anti-inflammatory gene (342, 343), therefore, its lower expression level observed in our study may in fact be a reflection of the higher bacterial burden in *Nlrp3*$^{-/-}$ mice. To further explore markers related to phagocytosis of apoptotic cells, we determined the expression of *Mertk*, a gene associated with the ability of macrophage to take up apoptotic cells (344). However, upon measurement of *Mertk* by qPCR on day 8 and day 14, we only observed a slight trend towards decreased expression in *Nlrp3*$^{-/-}$ mice, which was not statistically significant (Figure 5.15 a and b).

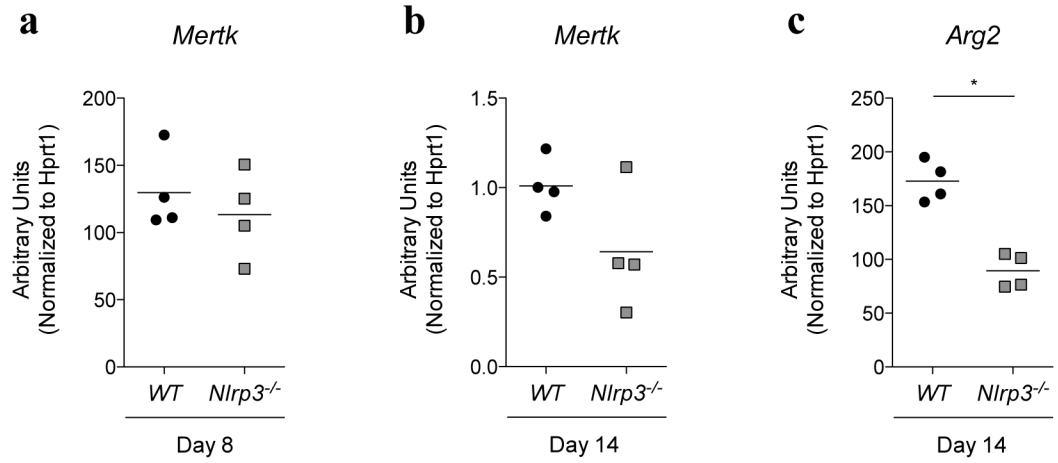

Figure 5.15 qPCR quantification of alternatively activated macrophage markers *Mertk* and *Arg2*. WT and *Nlrp3*$^{-/-}$ mice were infected with ~10^9 *C. rodentium* and sacrificed 8 days or 14 days post-infection. At the indicated time points pieces of caecal tissue were snap frozen in liquid nitrogen. Total RNA was then isolated and mRNA expression levels were measured by qPCR and normalized to *Hprt1*. Mean mRNA levels of **a** and **b,** *Mertk*. **c,** *Arg2*. Data obtained from one experiment (n = 4 mice/group). Statistical significance was calculated using the Mann-Whitney test between WT and *Nlrp3*$^{-/-}$ mice. * = $P < 0.05$.

5.3 Discussion

The intestine is constantly exposed to a large number of bacteria, and it needs to respond to potential pathogens rapidly upon challenge. Nlrp3 has been shown to be important in responding to this sudden challenge, either through chemical destruction of the epithelium (215), or through an infectious agent (311). Our results suggest that Nlrp3 responds very early to *C. rodentium* infection, which makes sense as bacteria can proliferate rapidly. Lack of Nlrp3 results in the overgrowth of the bacterium, resulting in exacerbated disease. We further showed that this Nlrp3 protective effect was independent of reported protective mechanisms in the *C. rodentium* model thus far. These data suggest that Nlrp3 operates through a novel mechanism that acts very early on to control bacterial burden in the intestine.

Recent studies have focussed on the IL-22 producing ILCs in the early phase of immunity against *C. rodentium* (106). These results point to RegIIIγ and other antimicrobial factors being key in the innate response to *C. rodentium* infection coordinated by tissue-resident innate leukocytes. In contrast, our results clearly show that whilst the Nlrp3 mediated protective response was evident as early as 3 days post-infection, *Nlrp3*$^{-/-}$ mice did not have defective expression of *Il22* or *Reg3g*. In addition, our data indicate that Nlrp3 activation in the non-haematopoietic compartment is key in controlling the bacterial burden. Since *C. rodentium* infects intestinal epithelial cells, it is possible that the activation and recognition of infection by IECs induce an early IEC-intrinsic protective response, before the involvement of immune cells such as ILCs.

Furthermore, since the secretion of IL-1β, or IL-18 was not impaired in *Nlrp3*$^{-/-}$ mice early after *C. rodentium* infection, we decided to investigate the remaining major consequence of inflammasome activation: programmed cell death called pyroptosis. In

fact, this is one of the least studied aspects of Nlrp3 inflammasome activation. Recently, a study revealed that in response to *C. rodentium*, caspase-11-dependent inflammasome activation was required for the induction of pyroptosis in macrophages (324). In order to assess if this was also true in the caecum at an early time point, we infected 129SvEv mice, which have a defective caspase-11. We observed significant increases in bacterial burden (100 fold) 3 days post-infection in 129SvEv mice compared to C57BL/6 mice, a level similar that found in *Nlrp3*$^{-/-}$ mice. It is tempting to postulate that the activation of caspase-11 in non-haematopoietic cells results in non-classical inflammasome effectors that mediate the rapid protective response towards intestinal pathogens. However, as there are many other differences between C57BL/6 mice and 129SvEv besides caspase-11, this observation highlights the need to further investigate the role of caspase-11 in response to an intestinal pathogen. In addition, Kayagaki *et al* propose that *C. rodentium* stimulates Nlrp3 in a non-canonical fashion that results in the secretion of IL-1β and IL-18 from macrophages. Whilst this is in contrast to our observations, this difference could be due to different responses observed in macrophages and IECs. The canonical activation of Nlrp3 by ATP could result in IL-1β/IL-18 secretion as well as pyroptosis, IL-1α, and HMGB1 secretion. Indeed, a recent paper has found caspase-11 to be induced by Type I IFN through a TRIF-dependent pathway following infection with Gram-negative bacteria infection of macrophages (345). It is interesting to note that only Gram-negative bacteria such as *C. rodentium* have secretion systems that allow effector translocation into the target cell (346). Following autoactivation of caspase-11, it synergizes with the Nlrp3 inflammasome and can lead to caspase-1-independent cell death (345). Although these experiments were also performed in bone marrow derived macrophages, they highlight cell death as possible outcome of Nlrp3 activation in response to infection. Therefore, it would be interesting to determine if the Nlrp3 circuitry driving pyroptosis works

differently in IECs. Together with the microarray data showing that *Erdr1*, an interleukin 18-regulated gene that acts as a proapoptotic factor (334, 335), was the most down regulated gene in *Nlrp3*$^{-/-}$ mice both during steady state as well as during infection, it is tempting to speculate that the programmed cell death pathway may be impaired in IEC in *Nlrp3*$^{-/-}$ mice.

In fact, *C. rodentium* has an intimate relationship with the IEC and many effector proteins of A/E pathogens, such as *C. rodentium* and EPEC, influence host cell death pathways (346). Whilst T3SS effector proteins EspF and Map are known to promote cell death (325, 326), other effectors such as EspZ and NleH are pro-survival factors (347, 348). It seems that these factors take control of the host cell since EPEC-infected cells do not undergo apoptosis until later during infection (349). This makes sense as the bacteria need to attach themselves to the IECs first in order to take advantage of this niche. Later on during infection, however, it is possible that cell death could benefit the pathogen to allow the bacteria to release themselves from host cells (346). This controlled attach and release mechanism illustrate how bacteria take advantage of host cells. Therefore, pathogen-mediated inhibition of early cell death could suggest the cell death may be a protective host mechanism.

Since many of the effectors injected via the T3SS of A/E pathogens can affect mitochondria, it makes sense for the host cell to closely monitor if the mitochondria stress or damage. Indeed, Nlrp3 has been shown to monitor the mitochondria (202), by sensing oxidized mitochondrial DNA (327). This mechanism potentially explains how Nlrp3 is able to sense so many different ligands, as the production of reactive oxygen species (ROS) and damage to the mitochondria have both been linked to Nlrp3 activation (193). Sensing mitochondrial dysfunction may act as an early detection system for infection.

EspF does not only lower the mitochondrial membrane potential, but also inhibits phagocytosis of the bacteria and disrupts host membrane transporters (346). Speedy detection and elimination of an infected cell could be a potential mechanism to control bacterial proliferation.

To date, most pyroptosis studies have been conducted in macrophages. Since our observations indicate a protective response in non-haematopoietic cells, it is imperative to set up a system that would allow the evaluation of epithelial cell death. However, given the difficulty in isolating and maintaining primary epithelial cells, new *ex vivo* assays need to be developed. Our experiments suggested that a lumenal LDH assay may not be sensitive enough. Conventional crypt isolation and stimulation with bacteria is non-physiological as the basolateral side is exposed to bacteria. Therefore we attempted to set up a polarized organ culture system developed for human biopsies (286). However, the residual LDH activity on isolated caeca or *ex vivo* cell death of isolated caecal tissue explants were too high to evaluate any additional cell death that may be induced. Therefore, the search for a suitable technique to measure epithelial cell death continues.

As a complementary approach to identify the potential novel mechanism involved, we chose to take an unbiased transcriptomics approach to screen the downstream effects of ablation of Nlrp3 during an intestinal infection. In fact, until now, no study has looked at the intestinal gene expression in mice lacking Nlrp3. It could be argued that the proteolytic nature of inflammasome activation might be better assessed by proteomics, however the lack of clear databases for targets (350) prompted us to perform transcriptomics as an initial screen. Upon analysis of the list of differentially regulated genes, the most noticeable defect in *Nlrp3*$^{-/-}$ mice was the down-regulation of a group of eosinophil-associated ribonucleases during steady state (Table 5.1). In fact, these genes

are also expressed at a lower level compared to WT mice during infection, although the differences were less than 2-fold. All of these genes belong to the ribonuclease A family, which comprises a large group of structurally similar proteins found in a wide range of mammals (351). Interestingly, like the major histocompatibility complex, immunoglobulins and T cells recepors (352-355), the ribonuclease A superfamily is one of the most rapidly evolving families in mammals (356). This gene duplication may be due to evolutionary pressure on the host to defend against infectious challenges (337). In fact, the eosinophil-assocated ribonucleases differentially expressed in the *Nlrp3*$^{-/-}$ have close sequence similarity to two of the major secretory proteins of human eosinophils, EDN and ECP (337). Furthermore, rat EAR-1 and EAR-2 have been shown to inhibit the growth of *E. coli* and *S. aureus* (357). In addition to the observation that *Retnla* is expressed at a lower level in *Nlrp3*$^{-/-}$ both during steady state as well as during infection, *Retnla*$^{-/-}$ mice have been reported to display substantially decreased eosinophil accumulation following DSS treatment (338).

Given all of these data, we decided to investigate the role of eosinophils in the intestine. Indeed, the intestine contains the largest population of eosinophils in our body (46). They are increasingly being appreciated by intestinal immunologists as new cell isolation procedures allow the isolation of more cells (43). In fact, eosinophils constitute more than one third of the F4/80^{+} cells historically identified as intestinal macrophages (43). More interestingly, in addition to their traditional role in type-2 immunity, eosinophils have recently been reported to exhibit antibacterial activities (56). Although Nlrp3 has been reported to be expressed at only very low levels in eosinophils (358), factors produced downstream of Nlrp3 activation in non-haematopoietic cells may be important in the induction of a protective eosinophil response. Unfortunately, despite their potential protective role in the intestine, the *Ear1/2* expression levels did not correspond to the

protective genotype of non-haematopoietic cells in the bone marrow chimera experiments. Instead, the qPCR analyses of bone marrow chimeras intestinal tissues suggested that it is may be a requirement for the haematopoietic cell compartment to express Nlrp3 in order to express normal levels of Ear proteins. In addition, the *Nlrp3*$^{-/-}$ mice also did not have a defect in accumulation of eosinophils in the intestine. Therefore, these data suggest that whilst Nlrp3 may not be expressed at a high level in eosinophils (358), the Nlrp3 inflammasome may indirectly influence eosinophil functions.

Furthermore, in addition to its links with eosinophils, *Retnla* is a well-known macrophage activation marker associated with alternatively activated macrophages (340). The fact that this gene was down regulated both during steady state and during *C. rodentium* infection in *Nlrp3*$^{-/-}$ mice suggests a defect that might involve macrophages. Indeed, phagocytosis of infected IECs undergoing programmed cell death is an important step in directing a proper response to the infection (28). In fact, a gene that has been reported to be induced in murine macrophages upon exposure to apoptotic cell derived factors (341), *Arg2*, was also significantly down regulated in *Nlrp3*$^{-/-}$ mice. Together, these data suggest that macrophage functions may also be affected in *Nlrp3*$^{-/-}$ mice. However, it is not clear whether these macrophages would be involved in anti-bacterial protection or (more likely) regulation of inflammation in host tissues.

In conclusion, whilst the current study has not definitively identified the early protective mechanism of Nlrp3 activation during intestinal infection, many interesting potential players have been uncovered. More research needs to be done to evaluate the potential Nlrp3 inflammasome involvement in IEC pyroptosis, in regulating eosinophils, as well as macrophages during intestinal infection. Revealing such mechanisms could uncover how

Nlrp3 inflammasome helps to maintain intestinal homeostasis and could allow the development of novel therapeutic strategies to treat CD patients with SNPs in *NLRP3*.

Chapter 6 – General Discussion

6.1 Summary

Data presented in this thesis have investigated the roles of two innate NLRs, encoded by by the CD-associated genes *NOD2* and *NLRP3*, in several mouse models of intestinal inflammation. In order to experimentally investigate the involvement of NOD2 in a relapsing and remitting disease such as IBD, we first characterized its expression in two models of resolving intestinal inflammation, namely the DSS-induced acute colitis model as well as in a *H. hepaticus*-induced T cell-mediated colitis model. Strikingly, in both of these models we found that *Nod2* mRNA expression was significantly increased at the peak of inflammation, and remained elevated during the resolution of inflammation. Furthermore, to explore the potential role of NLRP3 in intestinal inflammation, we infected mice lacking the inflammasome-forming Nlrp3, with the acute intestinal pathogen *C. rodentium*. *Nlrp3*$^{-/-}$ mice suffered from increased bacterial burden, resulting in exacerbated disease that was not due to a defective secretion of the inflammasome-associated cytokines IL-1β or IL-18. Interestingly, irradiation bone marrow chimera experiments demonstrated that Nlrp3 inflammasome activation in non-haematopoietic cells was important for this protective response. The aim of this chapter is to put these observations in context of emerging themes in intestinal immune homeostasis, as well as to identify future avenues of investigation to extend these studies.

6.2 Resolution as a part of the inflammatory program

The immune system has evolved many mechanisms to detect and combat pathogens that facilitate their neutralization, destruction, or explusion. Whilst these are designed to prevent pathogen-mediated damage brought on by the infection, the process of host defence can also inadvertently cause collateral damage (359). Indeed, immunopathology is the driver in many diseases initiated by an invading pathogen. For example, it is the potent host inflammatory response and consequent immune-mediated tissue damage that underlies multi-organ failure in sepsis (360). Similarly, it is the excessive host prostaglandin production during flu infection that causes the symptoms associated with flu (359). In fact, it is the cytokine storm mediated by host leukocytes that causes the life-threatening lung inflammation linked to highly virulent influenza (361). Likewise, IBD represents chronic aberrant expression of host protective inflammatory cytokines and patients are currently treated for their symptoms rather than the defective immune pathway. However, despite the availability of a plethora of anti-inflammatory agents for treatment of IBD, long-term administration is associated with side effects and many IBD patients ultimately require surgery (240). This suggests more effective treatments are needed that target the dysfunctional pathways involved to improve prognosis.

Following the discovery that polymorphisms of *NOD2* were associated with susceptibility to CD, one initial hypothesis suggested that disease-associated alleles could encode gain-of-function mutants leading to constitutive NOD2 activation and elevated synthesis of pro-inflammatory cytokines and/or chemokines (362). However, conflicting experimental evidence to support an anti-inflammatory function of NOD2, linked to inhibition of inflammatory responses mediated by triggering of TLR2 (176, 178) and TLR4 (179). These results suggested that IBD could be precipitated by loss-of-function mutations in

NOD2, leading to excessive TLR-mediated inflammatory responses. Whilst these opposing pro- and anti-inflammatory functions of NOD2 may seem puzzling if they acted at the same time, these functions could also complement each other if they were to act in a sequential fashion. Other PRR circuits can also exhibit dose and time-dependent regulatory effects to limit host pathology. For example, low levels of LPS have been previously shown to protect against a second exposure to lethal doses of LPS (291). This suggests that an important function of innate immune system may be to initiate negative regulation following activation. A lack of, or defect in, negative regulation could lead to chronic inflammation following activation and might underlie the remitting and relapsing pathology observed in IBD.

This idea is further supported by data presented in this thesis demonstrating that *Nod2*, the single most important genetic risk factor for CD (173), was upregulated in the colon during peak disease and remained elevated throughout the resolution of inflammation. This is consistent with the hypothesis that NOD2 may contribute both to the onset of inflammation, as well as to the resolution of inflammation. This trend of elevated *Nod2* expression during disease resolution was observed in two distinct models of colitis, including one acute model induced by DSS and one chronic model induced by *H. hepaticus* infection plus αIL-10R mAb administration. These observations agree well with previously reported anti-inflammatory functions of Nod2 in a colitis model (176), as well as in a GvHD model (363). Furthermore, it has recently been reported that human macrophages with CD-associated polymorphisms of NOD2 lost the ability to cross-tolerize to TLR2 and TLR4 stimulation that intact NOD2 possessed (180). Together, these data highlight a regulatory function of NOD2 that may be compromised in CD patients carrying a disease-susceptibility allele in *NOD2*.

Furthermore, NOD2 activation has been shown to induce autophagy via ATG16L1, which is also encoded by a CD-associated gene (187, 189). Interestingly, autophagy has recently been described as a mechanism that could remove active inflammasomes, thereby dampening ongoing inflammatory responses (301). In addition, autophagy has also been shown to remove cellular organelles, such as damaged, ROS-generating mitochondria, inflicted by infection/inflammation, thereby removing potential immunostimulatory factors such as ROS (202, 299). Thus, NOD2-driven autophagy could potentially contribute to intestinal homeostasis by reducing inflammasome-dependent IL-1β levels, which have been shown to contribute to intestinal pathology (364).

Taken together, resolution of inflammation through negative regulation is an important mechanism that plays a key role in the restoration of tissue homeostasis following an inflammatory response. Given the regulatory role of Nod2 and its expression during resolution, it is possible that activation of Nod2 may in fact help to initiate/facilitate this resolution process. One way to experimentally address this potential function of Nod2, would be to evaluate the kinetics of resolution of intestinal inflammation in mice lacking *Nod2* using the same IBD models as described above. Characterization of the type of cells expressing Nod2 during resolution as well as the downstream factors that are important in this process could contribute to a better understanding of resolution pathways involved in remitting and relapsing diseases such as IBD. Ultimately, development of novel agents that stimulated this natural resolution pathway could offer novel therapeutic treatments for IBD patients and those suffering from other chronic inflammatory disorders.

6.3 The Intestinal epithelium actively contributes to host defence

The first point of contact between the microbiota and the host is the intestinal epithelium. This endows these cells a unique advantage to monitor any changes in the lumen. Traditionally they have been regarded as simply a physical barrier, however, emerging evidence is supporting the role for the IECs as active members of intestinal host defence (365).

Indeed, it is known that the intestinal epithelium is important in secreting factors that prevent infection during steady state, such as mucus and AMPs (13, 14). Furthermore, IECs have been shown to express many PRRs and can actively respond to infection and participate in host defence (1, 17-21). For example, Nod1 activation in IECs has been found to be important in inducing IL-8 and human β-defensin 2 against *Campylobacter jejuni* infection (366). Furthermore, Nod2 has also been shown to be required for efficient antimicrobial defensin secretion by intestinal Paneth cells upon infection with *Listeria monocytogenes* (181). Indeed, Paneth cell dysfunction has been proposed to be an important predisposing factor for IBD development, as polymorphisms in TCF4, a transcription factor important in Paneth cell maturation and function, are associated with ileal CD (14). In addition, patients with a CD-associated risk allele of the autophagy gene *ATG16L1* showed abnormalities in Paneth cell granule size and morphology, as did mice that were rendered hypomorphic for ATG16L1 protein (16).

Moreover, recent data suggest that in addition to responding directly to a challenge, the epithelium can also produce a variety of factors that regulate the immune response. For example, epithelial cells release CX3CL1 following MyD88-dependent signalling, which promotes DC activation and dendrite extension into the lumen (37). In addition, NOD1 activation in the epithelium results in the release of MCP-1, a Th2 immunity promoting

chemokine (367). Furthermore, other epithelium-secreted factors are also involved in DC and T cell conditioning (22). For example, IL-25 and TSLP help maintain hypo-responsiveness towards commensal intestinal bacteria by limiting priming towards Th1-cell responses and by simultaneously promoting Treg-cell responses (368) and Th2 responses (24). IL-25 is a factor that is important in the response against the intestinal nematode *Trichuris muris* (369), and it acts in an autocrine manner to promote epithelial TSLP expression (370). Furthermore, epithelial cell-specific deletion of IKKβ led to defective production of TSLP, thus resulting in a failure to induce the necessary Th2 response required for parasite expulsion after *Trichuris muris* infection, which in turn led to chronic inflammatory pathology in the gut (371). In these mice, instead of a protective type-2 immune response, the severe intestinal inflammation observed was associated with increased IL-12, IL-23, IFN-γ, and IL-17A (371).

Data presented in this thesis adds to the growing list of immune protective responses that are dependent on the intestinal epithelium, rather than the intestinal leukocytes. Here we show that Nlrp3, another NLR that is encoded by a CD-susceptibility gene, plays an early protective role in non-haematopoietic cells against *C. rodentium*-induced intestinal inflammation. This makes sense as *C. rodentium* has a known tropism for IECs and its colonization involves delivery of many effector proteins into the host cell through T3SS (316). Furthermore, this agrees with previous data also highlighting a protective role of Nlrp3 in the intestinal epithelium against inflammation induced by DSS (215, 216). However, although our study has revealed an IL-1β and IL-18-independent protection mediated by Nlrp3, IL-18 has been reported to be important in Nlrp3-mediated protection from DSS-induced intestinal inflammation (215, 216), therefore the mechanisms involved appear to be distinct.

Overall, these studies further highlight the fact that the intestinal epithelium actively contributes towards a protective host response against pathogenic infection or chemical insult. However, irradiation bone marrow chimeras cannot pinpoint the exact cell type in the epithelium important for this protective effect. Potential contributions could come from epithelial cells, stromal cells, as well as long-lived tissue macrophages that may remain after irradiation. Whilst the best approach would be to engineer cell type-specific Nlrp3 knock-outs by employing the cre-lox system, this technique is difficult and time consuming. However, in the short-term it would be interesting to simply isolate separate cell populations, *ex vivo* stimulate each with ligands that activate Nlrp3, and measure caspase-1 activation as a proxy for inflammasome activation.

6.4 Nlrp3 activation coordinates a rapid response to mucosal pathogens

Nlrp3 appears to be a widely expressed sensor of cellular stress and infection because it can be activated by a myriad of structurally dissimilar stimuli (372). In addition, mutations in *NLRP3* have also been implicated in many immune-mediated diseases: the cryopyrin-associated periodic syndromes (373), CD (207-209), gout (374), type 2 diabetes (375), as well as obesity-induced insulin resistance (376). This large number of agonists as well as the variety of diseases it is involved in suggests that NLRP3 has a fundamental role in the inflammatory responses. Having such a fundamental function agrees well with the fact that NLRs are actually structurally and functionally similar to host defence proteins found in plants (377). Interestingly, these plant disease-resistance (R) proteins have nucleotide binding domains as well as leucine rich repeats, they sense pathogenic molecules and coordinate protective cellular responses, and their activation can also result in cell death (377). Furthermore, as there is little evidence for direct interaction with their agonists, these R proteins are considered indirect sensors that require additional cellular proteins (377). These features are not just similar to reports regarding mammalian NLRs, but they may even offer some hints as to how NLRs such as Nlrp3 may be operating. Indeed, Nlrp3 could also be an indirect sensor that is monitoring changes in various other cellular proteins that respond to distinct forms of cellular stress or damage. Indeed, the cytosolic localization gives the NLRs a unique advantage to sense cellular stress or damage, and this may be the reason NLRs are much more widely expressed than TLRs, that is, they may have originally evolved to monitor homeostatic perturbations. One target of Nlrp3-mediated monitoring could be the mitochondria, as mitochondrial dysfunction or damage could lead to generation of ROS or programmed cell death (202). Indeed, upon activation NLRP3 localizes to the mitochondrial membrane and defective mitophagy has been shown to induce NLRP3 activation (202).

Interestingly, mitochondria are affected by *C. rodentium*-translocated effector proteins such as EspF and Map, which cause increased cytochrome *c* release and decreased inner mitochondrial membrane potential, respectively (325, 326). In fact, decreased inner mitochondrial membrane potential that may result from interaction with such *C. rodentium* encoded effector proteins have been suggested to activate the Nlrp3 inflammasome (327). Furthermore, mitochondria-derived ROS generation has also been linked to NLRP3 inflammasome activation as ROS dissociates thioredoxin-interacting protein (TXNIP) from thioredoxin to allow TXNIP to bind to NLRP3 (378). Together, these data highlight how the cell tightly monitors the mitochondria and its ROS generation to prevent from unwanted cellular damage from ROS. It would be interesting to assess whether *C. rodentium*-induced changes in mitochondria results in the activation of Nlrp3 inflammasome, using the ΔMap mutants.

In *Salmonella*-infected macrophages, Nlrc4 inflammasome activation leads to a form of pro-inflammatory cell death known as pyroptosis, which serves to remove intracellular niches for replication (379). Given that the protective effect required Nlrp3 activation in non-haematopoietic cells in our model, and that *C. rodentium* infects IECs, whether a similar strategy is also used downstream of Nlrp3 activation in IECs upon *C. rodentium* infection remains to be investigated. In order to study such effects in the epithelium, *in vitro* culture assays need to be set up. One way to overcome the difficulty in maintaining primary IECs *in vitro* is by providing them with an extracellular matrix and growth factors (10). This would allow the stimulation of IECs directly with pathogens such as *C. rodentium.*

6.5 Conclusion

This thesis further confirmed the involvement of *NOD2 and NLRP3* in various aspects of intestinal inflammation. Whilst Nod2 may be important during the resolution phase of the intestinal inflammation, Nlrp3 activation is crucial for early protection against *C. rodentium*, suggesting that different NLR play important roles in the induction and resolution of intestinal inflammation. IBD is a complex disease that could have different aetiology in different patients and the particular functions of distinct NLRs may be vital only under defined conditions of stress or infection in the intestine, which could explain the low disease penetrance in people carrying disease-associated alleles of *NLRP3* or *NOD*2.

Furthermore, the protective host response following infection with A/E pathogen depends on Nlrp3 activation in non-haematopoietic cells, further highlighting the important role epithelial cells play in host defence in the intestine. This calls for future studies to examine this compartment as an integral part of intestinal host defence. Moreover, these data provide a potential functional link between polymorphisms in Nlrp3 and susceptibility to CD. As our data show that Nlrp3 confers protection independently of classical effector mechanisms i.e. IL-1β and IL-18, further research into this may reveal novel Nlrp3-dependent pathways that might underlie the susceptibility to CD.

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