The role of IL-1beta in intestinal inflammation

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Abstract

Although very high levels of IL-1β are present in the intestines of patients suffering from Inflammatory Bowel Diseases (IBD), little is known about the contribution of IL-1β to intestinal pathology. In the work presented in this thesis, I used several mouse models of chronic intestinal inflammation to address the role of IL-1β in driving innate and adaptive immune pathology in the intestine. My results showed that IL-1β promotes innate immune pathology in *Helicobacter hepaticus*-triggered innate intestinal inflammation, by augmenting the recruitment of granulocytes and the accumulation and activation of a population of IFN-γ- and IL-17A-producing innate lymphoid cells (ILC). To specifically investigate the role of IL-1R signaling on pathogenic T cell responses in the intestine, I used a T cell transfer colitis model. My results demonstrated a key role for IL-1R signals in promoting the accumulation and survival of pathogenic $CD4^+$ T cells in the colon, particularly $CD4^+$ IL-$17A^+$ Th17 cells. Finally, because mutations in the *NOD2* gene have been strongly associated with susceptibility to IBD, I investigated the contribution of NOD2 in the *H.hepaticus*-induced innate immune IBD model. I found that NOD2 expression was significantly increased in the inflamed colon. Furthermore, my preliminary studies suggested that NOD2 played a pro-inflammatory role in innate immune colitis, but that NOD2 had little impact on *H.hepaticus* colonization of the mouse intestine. In summary, my results identify multiple mechanisms through which IL-1β promotes intestinal pathology and suggesting that targeting IL-1β, or innate immune receptors, may represent a useful therapeutic approach in IBD.

I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie

Nothing shocks me. I'm a scientist.

Indiana Jones

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Abbreviations

AIEC adherent/invasive *E.coli*
AMP anti-microbial peptides
APC antigen presenting cell
APC Allophycocyanin
ATG16L1 Autophagy-related protein 16-1
ATP adenosine triphospate
BCL B-cell lymphoma
BSA bovine serum albumin
bp base pair
CAPS cryopyrin-associated periodic syndromes
CARD caspase recruitment
CD Crohn's disease
CFU colony forming units
CP cryptopatch
CTLA-4 cytotoxic T lymhphocyte associated antigen 4
DAMP danger associated molecular pattern
DC dendritic cells
DNA deoxyribonucleic acid
DSS dextran sulphate sodium
EAE experimental autoimmune encephalomyelitis
EDTA ethylenediaminetetraacetic acid
ER endoplasmic reticulum
EU endotoxin unit
FACS fluorescence activated cell sorting
Fc fragment constant
FCS foetal calf serum
FITC fluorescein isothiocyanate
FOXP3 forkhead box P3
GALT gut associated lymphoid tissue
GATA-3 Trans-acting T-cell-specific transcription factor-3
GF germ free
GLUT glucose transporter
GWAS genome-wide association studies
H&E haematoxylin and eosin
Hh + αIL-10R *H.hepaticus* infection and αIL-10R mAb treatment
HPRT hypoxanthine phosphoribosyl transferase
IBD inflammatory bowel disease
IEL intra epithelial lymphocyte
IFN interferon
Ig immunoglobulin
IKK IkB kinase
IL interleukin
ILF isolated lymphoid follicle
i.p. intraperitoneal

IPEX immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF interferon regulatory factor
ITAM immune-tyrosine activatory motif
JAK janus kinase
KGF keratinocyte growth factor
KO knock out
Lin lineage
LP lamina propria
LPS lipopolysaccharide
LT lymphotoxin
LTi lymphoid tissue inducer
M microfold
mAb monoclonal antibody
MAP mitogen associated protein
MAMP microbe associated molecular pattern
MHCI/II major histocompatibility complex type I/II
min minute
MLN mesenteric lymph node
MOI multiplicity of infection
mRNA messenger ribonucleic acid
MyD88 myeloid differentiation response gene 88
OD optical density
N/A not available
ND not detectable
NACHT nucleotide-binding and oligomerization
NF-kB nuclear factor kappa-light-chain-enhancer of activated B cells
NK natural killer
NOD nucleotide-binding oligomerisation domain
NLR nucleotide-binding domain and leucine rich repeat containing receptor
PAMPS pathogen associated molecular patterns
PBS phosphate buffered saline
PCR polymerase chain reaction
PE phycoerythrin
Peg polyethylene glycol
PerCP peridinin chlorophyll protein
PP Peyer's Patch
PRR pattern recognition receptor
R receptor
RA retinoic acid
RA receptor antagonist
RAP receptor accessory protein
RAG recombinase activating gene
RIP receptor interacting protein
RNA ribonucleic acid
RPMI Roswell Park Memorial Institute medium
RIP receptor interacting protein

RLR retinoic acid-inducible gene-I-like receptors
ROR retinoic acid-related orphan nuclear hormone receptor
ROS reactive oxygen species
RT room temperature
SA streptavidin
SCID severe combined immunodeficiency
SFB segmented filamentous bacteria
SPF specific pathogen free
STAT signal transducer and activator of transcription
Tbet T box expressed in T cells
TCR T cell receptor
TGF transforming growth factor
TIR Toll/IL-1R homology
TLR toll like receptor
TNBS 2,3,6-trinitrobenzene sulfonic-acid
TNF tumour necrosis factor
Treg regulatory T cell
UC ulcerative colitis
UPR unfolded protein response
WT wild type

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Chapter 1: General introduction

1.1 Introduction

Two branches of the immune system have evolved to defend the host against agents able to cause tissue injury: the innate immune system, which responds rapidly against invading agents, following the recognition of invariant structures shared by different types of microbes, and the adaptive immune system, which produces a specific immune response against distinct antigens and provides “memory” to the system, allowing a more vigorous response to repeated exposure to the same agent.

The immune system has evolved to contrast invasion of the host by microbial species. However, mammalian hosts harbour many complex microbial communities in their body (on the skin, in the upper respiratory tract, in the urogenital and gastrointestinal tract) (1), which provide numerous beneficial functions to the host, including harvesting nutrients and preventing infection by pathogens (2). However, these microbes bear many of the same molecular signatures characterizing harmful pathogens. Thus, at mucosal sites the immune system evolved mechanisms to distinguish between harmless and pathogenic microbes. These mechanisms are particularly relevant in the intestine, which hosts one of the more dense microbial communities on the planet (3). The disruption of the fine balance between tolerance towards commensal microflora and reaction against invading pathogens in the intestine is the underlying cause of Inflammatory Bowel Disease (IBD), a chronic inflammatory condition of the gut (4). However, the molecular and cellular mechanisms underlying the development of IBD are unclear, and a better

understanding of intestinal immunity is required for the development of novel therapeutic strategies for IBD.

1.2 The intestinal immune system

Extending over 100m^2 in humans, the intestinal epithelium is the largest mucosal surface in the body (5). It is covered with a single layer of intestinal epithelial cells (IEC), which allow swift absorption of nutrients, but also represents a major entry route for invading pathogens (6). As the gut is heavily colonized by commensal bacteria, distinct anatomical features have evolved to allow the intestinal immune system to rapidly react against invading pathogens whilst avoiding inflammatory responses against beneficial commensal bacteria and food antigens.

The gut associated lymphoid tissue (GALT) comprises both effector sites, such as the intestinal epithelium and lamina propria (LP), and secondary lymphoid structures, including the mesenteric lymph nodes (MLN), the Peyer's patches (PP) of the small intestine and isolated lymphoid follicles (ILF) and cryptopatches (CP) that are distributed throughout the intestine (6, 7) (**Fig.1-1)**. Secondary lymphoid structures are essential for the induction of adaptive immune responses and the development of tolerance to food antigens (6). Moreover, MLN allow the compartmentalization of the intestinal immune response, as antigen-loaded intestinal dendritic cells usually do not penetrate further than these structures into systemic secondary lymphoid organs (8). Antigen presentation in the MLN results in the expression of gut homing receptors on lymphocytes, such as $\alpha_4\beta_7$ and CCR9 in T lymphocytes, that promote their recirculation to the intestinal mucosa (6). The intestinal LP and epithelium are rich in innate and adaptive leukocytes, which are described in the next paragraphs.

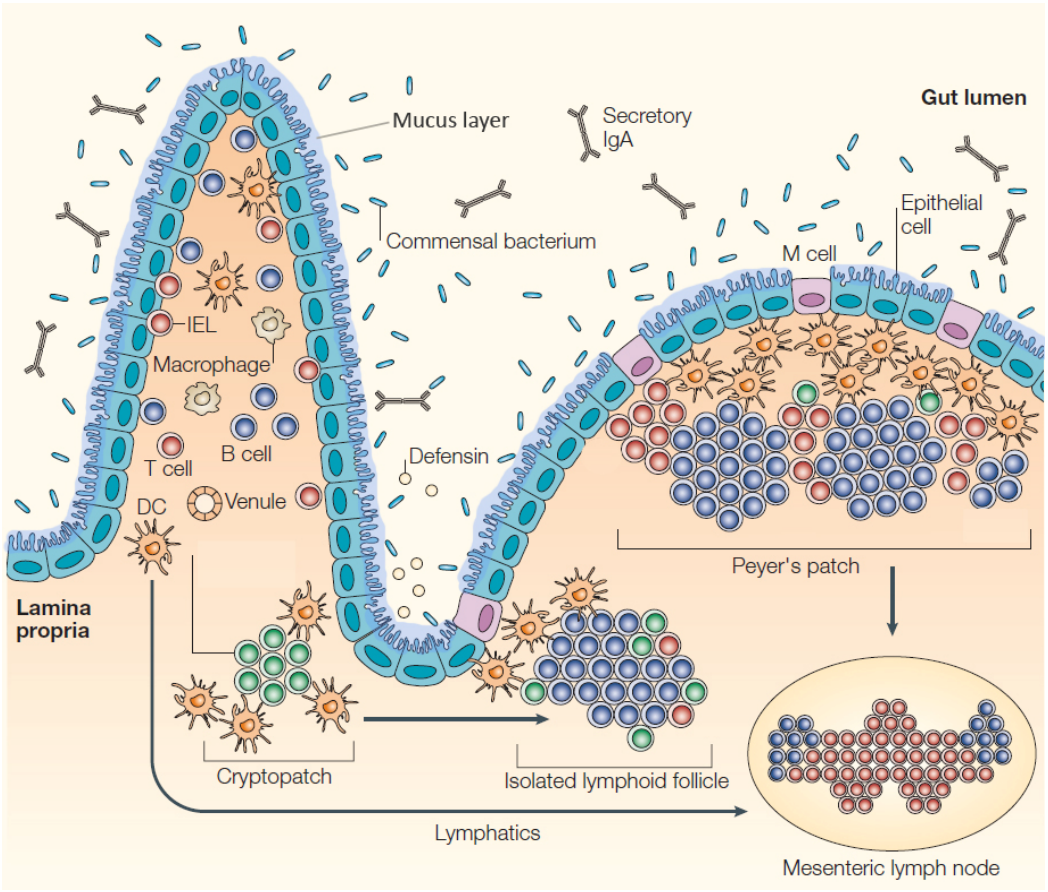


Figure 1-1. The gut associated lymphoid tissue. The intestinal epithelium separates the host connective tissue from the lumen. The intestinal epithelium is covered by a thick layer of mucous, which contributes to the exclusion of intestinal microbes, together with secretory IgA and bactericidal defensins. Intraepithelial lymphocytes (IEL) contribute to the maintenance and repair of the intestinal epithelium (9). The intestinal lamina propria (LP) is enriched in T cells, B cells and macrophages. Dendritic cells (DC) are also present in the intestinal LP and, upon capture of antigens, migrate to the mesenteric lymph nodes, where they activate T cell responses (6). Peyer's patches (PP) are formed by large B cell follicles, with intervening T-cell areas. The Peyer's patches are separated from the lumen by the follicle associated epithelium (FAE). The FAE is characterized by the presence of microfold cells (M cells), which lack the brush border and are not covered in mucous. M cells are specialized for the transport of antigens from the lumen to the sub-epithelial dome of PP, where they are sampled by DC (10). Isolated lymphoid follicles (ILF) are similar in structure to PP, but are found throughout the intestine (6). Cryptopatches are small collections of Lin-cKit^{+}IL-7Rα^{+}cells, which are important for the maturation of intestinal T cells (11) and which may differentiate into ILF (12). Figure modified from (7).

1.3 Innate intestinal immune cells

1.3.1 Intestinal epithelial cells

The host connective tissue is separated from the intestinal lumen by a thin monolayer of columnar intestinal epithelial cells (IEC). The gut epithelium comprises 5 different IEC lineages: absorptive enterocytes, goblet cells, Paneth cells, enteroendocrine cells and M cells, all of which contribute to the epithelial barrier function (13). The gut epithelium is continuously renewed; epithelial stem cells that are present in the crypts constantly generate new epithelial cells that migrate progressively to the tip to the tip of the intestinal villi and eventually undergo apoptosis and exfoliate in the lumen (14). IEC are strongly polarized, as their apical side is rich in microvilli (forming the typical "brush border") and the IEC layer is held together by paracellular tight junctions that prevent diffusion of fluids and particles present in the lumen (5). Goblet cells further enhance the epithelial barrier by secreting gel-forming mucins, such as MUC2, which form a thick layer of mucous on the surface of the epithelium, thus excluding most bacteria from the epithelial surfaces (15). Paneth cells are specialized secretory cells found at the base of the intestinal crypts in the small intestine. Paneth cells are characterized by large secretory granules, mainly containing antimicrobial peptides (AMP), such as defensins, cathelicidins and calprotectins (16). Paneth cells release the content of their granules into the intestinal lumen, preventing bacterial invasion of the crypts (17). Enteroendocrine cells are also situated at the base of the intestinal crypts, where they sense luminal signals and transmit these signals to enteric neurons by secreting different bioactive products across the basolateral membrane (18).

Microfold (M) cells play a strategic role in monitoring the luminal environment. M cells, which are found within the follicle-associated epithelium (FAE) that overlies the PP and ILF, lack a brush border and are not covered in mucous. M cells sample antigens from the lumen and transport them to the subepithelial dome, an area of the PP that is rich in dendritic cells (19), allowing transfer of luminal antigen to antigen presenting cells (APC) and representing an important portal into the intestinal immune system (6).

1.3.2 Intestinal mononuclear cells: dendritic cells and macrophages.

Intestinal mononuclear cells are a heterogenous population comprising cells with a variety of functions and surface markers (20). Classically, dendritic cells (DC) are specialized APC whose main function is to sample antigens from the environment, migrate to the peripheral lymph nodes and prime T cell activation and differentiation (21). In contrast, macrophages differentiate in peripheral tissues and are responsible for immunosurveillance and phagocytosis, although they are also able to present the antigens to T cells (22). In mice, DC are traditionally identified by high surface expression of CD11c and MHCII, whilst macrophages are conventionally defined by the expression of the CD11b and F4/80 surface markers (21, 22).

Recent advances in the field have created a more complex picture, suggesting that several myeloid APC populations are present in the gut and that they may arise from similar precursors in response to different stimuli (10). For example, it is clear that there is a population of DC expressing the integrin αE (CD103) that migrate from the intestinal LP to the MLN and promote tolerogenic T cell responses to oral antigens (23-26). In addition, most resident intestinal macrophages in the steady state are

highly phagocytic, but also unresponsive to most bacterial stimuli, such as TLR agonists (27, 28). Moreover, intestinal macrophages have been reported to produce IL-10 and support $CD4^+$ regulatory T cell function in the LP (29). Thus, under normal homeostatic conditions, most myeloid APC in the gut appear to re-enforce anti-inflammatory circuits. By contrast, high expression of the fractalkine receptor CX_3CR1 defines a population of tissue-resident "inflammatory DC"/macrophages that can uptake intestinal bacterial antigens from the lumen (30-32). In addition to these cells, other monocytic populations infiltrate the mesenteric lymph nodes and the colon during intestinal inflammation, although their precise phenotypes and functions are still unclear (10).

1.3.3 Natural killer cells

Natural Killer (NK) cells are innate cells of lymphoid origin essential in the defense against viral infections and tumors (33). NK cells can kill infected cells through the release of cytotoxic granules or ligation of death receptors (33) and can activate other immune cells by secreting pro-inflammatory cytokines, mainly IFN-γ and TNF-α (33). The activation of NK cells is determined by the integration of signals from inhibitory and activating receptors that are present on the cell surface (34). For example, cells infected by viruses or tumor cells down-regulate MHCI and other inhibitory ligands for NK cells ("missing self"), resulting in NK cell activation and lysis of the target cells (33)

NK cells have been described in the intestine, where they express the NK cell markers NKp46 and NK1.1 (35, 36). These cells are cytotoxic, can secrete IFN-γ (35) and express the regulatory cytokine IL-22 (36), although non-conventional

"NK22" cells (described below) are the main source of this cytokine in the intestine (36, 37).

1.3.4 Lymphoid tissue inducer cells (LTi) and innate lymphoid cells (ILC)

Lymphoid tissue inducer cells (LTi) are essential for the formation of lymph nodes and Peyer's patches in the fetus (38). LTi don't express lineage markers specific for T cells, B cells or myeloid cells (lin^-), but are defined by the expression of CD4, lymphotoxin-α (LT-α) and LT-β, CD127 (IL-7Rα), c-kit, RANK-L and the transcription factor RORγt (38). LTi originate from the fetal liver (39, 40) and migrate to the lymph nodes and PP anlagen in response to chemotactic signals (7). Here, they interact with stromal cells through activation of the LT-β receptor, inducing the secretion of different cytokines, which in turn recruit hematopoietic cells to the nascent lymph node (41). LTi are present also after birth (42, 43) and they contribute to the formation of ILF in the gut (44) and to tissue remodeling, as they are essential for the re-establishment of normal lymph node structure following damage induced by acute viral infection (45). LTi cells can produce IL-17A and IL-22, key players in intestinal homeostasis and immunity (38, 46), and sustain T cell-independent production of IgA by B cells (47).

Recent advances in the field showed that LTi cells are part of a broader family of innate lymphoid cells (ILC) that play a central role in modulating immune responses, especially in the gut (38). An additional type of ILC includes cells that share some surface markers with NK cells and produce IL-22, which have been designated as NK22 (37) or NCR22 (48) or ILC22 cells (38). In mice, NK22 cells are $NKp46^+$, but do not express the conventional NK marker NK1.1 (36, 49, 50). Moreover, they differ

from NK cells as they don't have cytotoxic capacity, they lack the expression of inhibitory receptors and they produce very little IFN-γ (38). The development of NK22 cells is dependent on the transcription factor RORγt and on the presence of the intestinal microflora (36, 50), but is independent of IL-15, in contrast to conventional NK cells (36). NK22 have been described in humans, where they express CD56, NKp44 and c-kit, whilst they are low or negative for NKp46 (37, 42).

Our laboratory recently described another subset of ILC, which is able to produce IL-17A, IFN-γ and IL-22 during intestinal inflammation in *Rag2*-deficient mice (51). This population, which differs from both LTI and ILC22 cells by lacking expression of c-kit and NKp46 in mice (51), plays a central role in promoting innate immune inflammation during *Helicobacer hepaticus*-induced colitis (51).

A different set of ILC was described in the context of Th2 immunity in the gut (52-55). These lin^- cells can respond to type-2 cytokines like IL-25 and IL-33 by producing IL-13, IL-4, IL-5 and IL-6 (56), promoting Th2 responses, especially in the context of helmint infection (56). Four different cell subsets were independently identified: nuocytes (52), natural helper cells (53), innate helper type 2 cells (Ih2) (54) and multipotent progenitor type 2 cells (MMPtype2) (55). MMPtype2 are probably of myeloid origin, as they can differentiate *in vitro* into mast cells, basophils and macrophages (55). In contrast, the other three cell subsets share many cell markers and functions, prompting some investigators to suggest that they might represent different effector stages of the same cell type (56). Further work is required to identify the relationship between these ILC subtypes and to clarify their functionality in intestinal homeostasis and inflammation.

1.4 Adaptive intestinal immune cells

1.4.1 Intraepithelial lymphocytes (IEL)

Intraepithelial lymphocytes (IEL) are a population of T cells associated with the gut epithelium. IEL are present at other mucosal surfaces, but are extremely abundant in the gut, particularly in the small intestine (57). Intestinal IEL include "Type a" TCR$\alpha\beta$ $CD4^+$ or CD8$\alpha\beta^+$ T cells, which are similar to conventional T cells found in other tissues (9). "Type b" IEL are far more abundant in the mucosal epithelium and include TCR$\alpha\beta$ and TCR$\gamma\delta$ T cells which express the unique co-receptor CD8$\alpha\alpha$ (mainly in the small intestine) or lack expression of a co-receptor (double negative, DN cells, mainly present in the large intestine) (9). CD8$\alpha\alpha$ TCR$\alpha\beta$ T cells are selected independently of MHCI, have a limited TCR variability and have a natural memory T cell phenotype (9). CD8$\alpha\alpha$ TCR$\alpha\beta$ T cells are mainly self-reactive (9), but their activation is not harmful to the host, as it is associated with the release of immune-regulatory cytokines such as IL-10 and TGF-β (9). Accordingly, CD8$\alpha\alpha$ $\alpha\beta$ IEL promote intestinal homeostasis, as mice lacking this mucosal T cell subset are more susceptible to acute intestinal inflammation and co-transfer of CD8$\alpha\alpha$ $\alpha\beta$ IEL was reported to be protective in the T cell transfer model of colitis (58, 59).

IEL bearing a TCR$\gamma\delta$ do not have the same MHCI restriction as conventional $\alpha\beta$ T cells, but instead recognize class-I like molecules expressed by IEC undergoing cellular stress (9). Moreover, $\gamma\delta$ T cells can probably exert many effector functions independently of TCR activation (60, 61), suggesting that $\gamma\delta$ T cells could represent a more "innate" population of T cells, with a role in the first line of host defense (62).

At mucosal surfaces, γδ IEL promote immune responses during infection by producing IL-17A (63, 64) and inducing the production of IFN-γ by NK cells (65). However, γδ IEL also play a role in the limitation of intestinal immunity and in promoting epithelial restitution and repair, for instance through the secretion of keratinocyte growth factor (KGF) (66). For example, *Tcrd*^{KO} mice (completely lacking γδ T cells) have reduced numbers of crypts in the intestine and are more susceptible to 2,4,6-trinitrobenzene sulfonic-acid (TNBS)-induced colitis (67, 68). Moreover, γδ IEL can restrain excessive TCRαβ T cell activation, as *Tcrd*^{KO} mice showed increased damage following infection with *Eimeria vermiformis* due to an uncontrolled CD4^{+} αβ T cell response (69).

1.4.2 B cells

B cells are adaptive lymphocytes generated in the bone marrow and dedicated to the production of antibodies. Antibodies have an extensive repertoire of antigen specificity, which is initially obtained through V(D)J recombination, generating immature B cells expressing IgM and IgD (70). However, in the presence of antigen, B cells further extend their receptor repetoire by somatic hypermutation (SHM) and class switch recombination (CSR), creating different antibody isotypes, including IgG, IgE and IgA (71). B cells are abundant in the GALT and mainly secrete IgA (72). Peyer's patches are the main inductive site of intestinal IgA, but class switch to IgA can also occur in ILF and MLN (73). Class switching to IgA is strongly dependent on TGF-β (72), but other classical requirements for class switching, such as T cell help through CD40-CD40L interaction, are dispensable for IgA production, as factors produced by DC, macrophages and IEC, such as BAFF (B cell activating factor) and

APRIL (A proliferation-inducing ligand), can promote IgA class switching in the absence of T cells (74). BAFF and APRIL are mainly produced in response to TLR ligation (75-77), highlighting the strong link between IgA production and the intestinal microflora.

IgA is present as a dimer in the intestine, linked through a J chain peptide that is synthesized by IgA secreting cells (78). IgA dimers are secreted into the intestinal lumen via transcytosis across IEC, that is mediated by the polymeric immunoglobulin receptor (pIgR) (79) In the lumen, IgA limits the penetration of commensal bacteria by trapping them in the mucous layer (73). IgA can also neutralize pro-inflammatory bacterial products, such as LPS and bacteral toxins (80), and protect against infection by enteric viruses (81). Moreover, IgA can modulate the expression of pro-inflammatory epitopes by intestinal bacteria (82) and promote the formation of biofilms by commensal species (83). Thus, IgA-producing B cells play a crucial role in maintaining the balance between the host and the intestinal microflora.

1.4.3 $CD4^+$ T cells

The intestinal LP is home to a population of TCR$\alpha\beta$ T cells, mainly expressing the co-receptor CD4 (84). Typically, these cells have an activated/memory phenotype (85). Different $CD4^+$ T cell subsets play different roles in intestinal homeostasis. To assess more specifically the role of $CD4^+$ T cells in the intestine, a short overview of T cell generation and activation is given below.

$CD4^+$ T cells originate in the thymus, where a productive TCR is produced following V(D)J recombination (86). Following positive and negative selection, which determines MHC-restriction and deletes auto-reactive T cell clones (86), T cells exit

into the bloodstream and recirculate through secondary lymphoid tissues (87). Recirculating T cells can be activated in secondary lymphoid organs by antigen recognition, leading to TCR activation. However, T cells are only fully activated in the presence of co-stimulatory signals on activated APC (88), such as CD80 and CD86, which interact with CD28 on T cells (88).

Activated $CD4^+$ T cell differentiate into T helper cells, which have been classified into different subsets according to their cytokine effector profile and the expression of specific transcription factors. T helper 1 (Th1) and T helper 2 (Th2) were the first subsets to be defined (89), but more recently regulatory T cells (Treg) (90) (91) and T helper 17 (Th17) (92) were also identified (**Fig.1-2**). Although the T helper subset paradigm provides a very useful framework to define T cell function and biology, it is important to note that these subsets are often heterogeneous (93, 94) and the different T helper subsets can co-operate against infection (95, 96).

1.4.3.1 Th1 cells

The Th1 subset is characterized by the production of the cytokines IFN-γ and lymphotoxin and by the expression of the transcription factor Tbet (T box expressed in T cells) (89, 97, 98). Although the strength of TCR stimulation can shape T cell responses, the cytokine milieu is the major determinant of T helper subsets differentiation (97). For Th1 cells, Tbet expression is induced by IFN-γ, IL-12 and IL-27, through the activation of STAT-1 (signal transducer and activator of transcription-1) (99-102). Tbet activation promotes Th1 differentiation by activating the *Ifng* locus (103-105) and by silencing the locus for the key Th2 transcription factor GATA-3 (Trans-acting T-cell-specific transcription factor-3) (106-109). Moreover, Tbet

activation leads to up-regulation of the β2 chain of the IL-12 receptor (IL-12Rβ2). As IL-12 promotes Th1 expansion in a STAT-4 dependant way, this process helps stabilize the commitment to the Th1 lineage (110). Both *Tbx21*^{KO} mice, which lack Tbet, and *Stat4*^{KO} mice show impaired Th1 differentiation, and are more susceptible to intracellular pathogens (111-116). Th1 cell protective activities are mainly attributed to the production of IFNγ, which activates cellular immunity, especially macrophage activation (89, 97). However, excessive Th1 responses have also been implicated in the development of inflammatory pathologies, such as type 1 diabetes (T1D) (117) and IBD (118, 119).

1.4.3.2 Th2 cells

Th2 cells are defined by the expression of GATA-3 and by the production of IL-4, IL-5 and IL-13 (89, 97). Th2 differentiation *in vitro* requires both IL-4 and IL-2, as they promote GATA-3 expression and stabilization through STAT-6 and STAT-5, respectively (97). Moreover, other transcription factors, such as nuclear factor of activated T-cells (NFAT), T cell factor-1 (TCF-1) and Maf (c-Maf), contribute to Th2 differentiation (97). Molecular mechanisms involved in Th2 differentiation repress the differentiation of Th1 cells, as GATA-3 represses IL-12Rβ2 expression (120) and TCF-1 inhibits the expression of IFN-γ (121). Th2 responses play a key part in the response against extracellular infectious agents, such as intestinal nematodes, by coordinating a complex immune response, including recruitment of eosinophils, mast cells and basophils, IgE production, increased mucus secretion by goblet cells and increased intestinal contractility (122). However, aberrant Th2 responses are implicated in the development of atopic diseases, such as asthma and allergy (123).

Traditionally, IL-9 was considered a Th2 cytokine, as it promotes mast cell function and IL-13 production, contributing to allergic inflammation (124). However, naïve $CD4^+$ T cells differentiated in the presence of TGF-β and IL-4 produce high levels of IL-9 and IL-10, but no other Th2 cytokines and do not express any defining transcription factor (125, 126). These have been termed Th9 cells and promote immune responses to intestinal helminthes and asthma (125-127). However, as yet no specific transcription factor has been associated with Th9 differentiation and it is not clear whether they truly are a separate T cell lineage.

1.4.3.3 Th17 cells

In mice, Th17 cells are characterized by the production of IL-17A, IL-17F, IL-17E, IL-21 and IL-22 (97) and they typically express the surface molecules CCR6 and IL-23R (128). In mouse and human, Th17 cells differentiate in response to TGF-β stimulation in the presence of IL-6 or IL-21 (129-132) (133-136), although TGF-β independent Th17 differentiation has been described (137). IL-1β also contributes to early Th17 differentiation (138, 139), while IL-23 plays an essential role in sustaining Th17 differentiation and promoting survival of Th17 cells (140-142). IL-17A itself, mainly produced at mucosal surfaces by γδ T cells, might promote differentiation of $CD4^+$ Th17 cells (60, 61). The main transcription factors implicated in Th17 differentiation are RORγt and RORα, members of the retinoic acid-related orphan nuclear hormone receptor family with redundant functions in Th17 differentiation (97, 128). RORγt and RORα expression is dependent on STAT3 activation by IL-6 or IL-21 (143, 144) and humans with STAT3 mutations show defective Th17 differentiation (145). However, other transcription factors can also contribute to Th17 differentiation and

expansion, such as IRF4 (146), B-cell activating transcription factor (Batf) (147), and the aryl hydrocarbon receptor (AHR) (148, 149).

Th17 are enriched at mucosal surfaces and they play a key part in the defense against bacteria and fungi, including pulmonary infection by *Klebsiella pneumonia* and *Pseudomonas aerugionosa* and mucosal infection by *Candida albicans* and *Aspergillus fumigates* (150-152). Moreover, excessive Th17 responses have been implicated in the development of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE, an experimental model of multiple sclerosis) (153, 154), psoriasis (155-157) and collagen-induced arthritis (158, 159). Th17 have also been implicated in the pathogenesis of colitis and their controversial role in this disease will be discussed later in this chapter.

1.4.3.4 Regulatory T cells (Tregs)

Although many cell types can be immuno-suppressive in specific settings, Tregs represent a specialized subset of T cells whose main function is to mediate tolerance by suppressing immune responses (160). Although many early studies showed that a population of memory $CD4^+$ T cells expressing the IL-2 receptor α chain (CD25) was able to suppress immune responses *in vitro* and *in vivo* (90, 161-166), the formal classification of regulatory T cells as a separate lineage was facilitated by the identification of the transcription factor required for their development, FOXP3 (forkhead box P3) (167-170). FOXP3 was identified in an effort to define the cause of a severe human autoimmune disorder, the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), a disease phenotype that is recapitulated by the *scurfy* mouse (167-170). Both IPEX patients and *scurfy* mice bear

loss of function mutations of FOXP3, resulting in a severe early-onset lymphoproliferative disorder affecting multiple systems (167-170).

Two main types of $CD4^+$ $FOXP3^+$ Tregs have been described, possibly sharing similar functions *in vivo*: natural Tregs (nTreg) that differentiate into fully functional $FOXP3^+$ Tregs in the thymus; and induced Tregs (iTreg) that differentiate from naïve $CD4^+$ T cells in the periphery (160). nTreg generation in the thymus is dependent on high affinity recognition of self antigens (160), although some evidence suggests that the affinity of nTreg for self antigens is well below that observed in conventional effector T cells specific for foreign antigens (171). TCR stimulation results in up-regulation of CD25 and IL-2R-mediated signals lead to the induction of FOXP3, possibly by activation of STAT-5 (172, 173). Other signals, such as stimulation by the common gamma chain (γc) cytokines IL-7 and IL-15 and co-stimulation through CD28 are important for the development of nTreg (174-178). These signals result in the activation of a complex transcriptional programme, including the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway and of other transcription factors, including NFAT and AP-1 (activator protein 1) (179). However, FOXP3 expression is crucial to maintain the Treg pool, as loss or attenuation of FOXP3 expression in Tregs results in severe autoimmune pathology (180, 181).

Whilst the majority of Treg cells in the spleen and blood originate in the thymus, the GALT is a preferential site for the generation of peripheral or induced Treg cells (iTreg) (182). The intestine is the perfect environment for the provision of the two main signals required for iTreg differentiation: suboptimal or chronic TCR stimulation

and high amounts of TGF-β (160). Both these signals promote FOXP3 expression, although the underlying mechanisms involved are not completely characterized (160). Nevertheless, there is strong evidence that intestinal APC play an important role in driving mucosal Treg differentiation. For example, $CD103^+$ DC play a key role in promoting iTreg differentiation in the MLN, by producing both TGF-β and retinoic acid (RA), which promote FOXP3 expression (25, 183). Macrophages from the small intestinal LP can also induce differentiation of iTregs, in a process dependent on IL-10, RA and TGF-β (29).

Treg cells have been shown to exert their suppressive function on a plethora of immune cell types *in vitro* and *in vivo*, including monocytes, macrophages, DC, granulocytes, T and B cells and NK cells (184). *In vivo,* Tregs are likely to suppress effector T cell activation by directly interacting with DC, rather than with T cells (185, 186). Treg can strongly down-modulate the pro-inflammatory functions of DC, by blocking co-stimulatory molecules through the expression of CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) and by promoting IL-10 production by DC (187, 188). In the intestine, Tregs also mediate immune-suppression by producing soluble factors, such as IL-10, TGF-β and IL-35 (189, 190). Moreover, Treg can dampen immune responses by depleting the microenvironment of growth factors, such as IL-2, or pro-inflammatory molecules, such as extracellular ATP, thereby limiting the activation of other immune cells (191, 192). Other regulatory T cell populations have been described, but are as yet poorly characterized. In particular, $FOXP3^-$ Tr1 cells may be potent sources of IL-10 and can play an important role in modulating immune responses against infection at mucosal surfaces (193-195).

It is striking that a limited population of Treg cells (comprising 5-10% of the peripheral $CD4^+$ T cell pool) can control immune responses elicited by different agents in extremely different environments. A possible explanation for the efficacy of Treg-mediated immune regulation is that Treg are "fine-tuned" to control polarized immune responses (128), as Tregs can acquire expression of transcription factors specific for polarized T cell lineages. For instance, expression of interferon regulatory factor 4 (IRF4) by Tregs is required for the suppression of Th2 responses (196), whilst STAT-3 deficient Tregs fail to control Th17 cells, resulting in the development of spontaneous colitis (197).

1.4.3.5 Follicular helper cells

Follicular helper T cells (Tfh) reside in secondary lymphoid organs and provide B cells with the help necessary to differentiate into high affinity plasma cells (198). Tfh cells are characterized by high expression of CXCR5, ICOS (inducible costimulator) and PD-1 (programmed death 1) (198), and produce IL-21 (198). Tfh are identified by the expression of high levels of B-cell lymphoma 6 (BCL-6) (198), although low levels of BCL-6 are found in all activated T cells (199). Tfh are found in intestinal PP, where they promote IgA production through the secretion of TGF-β and IL-21 (200).

1.4.3.6 Plasticity of T cell subsets

Recent evidence suggests that $CD4^+$ T helper effector cell phenotypes are not completely fixed, as committed Thelper cells can produce different cytokines from those determined by their differentiation status (201). For instance, Th17 cells can produce IFN-γ *in vivo* (157, 202, 203), Th1 cells can make IL-13 (204) and Th2 clones can produce IFN-γ (205). Moreover, transcription factors also reveal variable

expression and function. For instance, FOXP3 expression can be transiently expressed by activated T cells and cells that lose FOXP3 can produce pro-inflammatory cytokines (206-209), while $FOXP3^{+}Tbet^{+}$, $FOXP3^{+}ROR\gamma t^{+}$ or $FOXP3^{+}GATA3^{+}$ $CD4^{+}$ T cells have been identified *in vivo* (126, 210-212). In view of these findings, a new model has been proposed, where $CD4^{+}$ T cells are not fully committed to their helper subsets, but can fine-tune their effector functions in response to changing environmental stimuli (201).

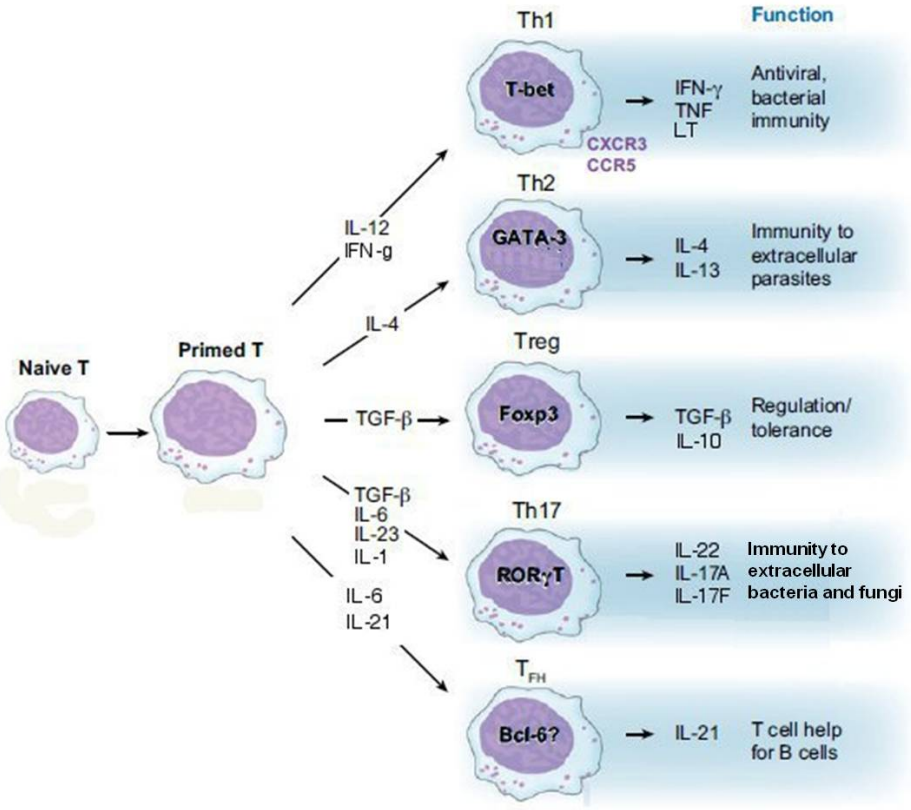


Figure 1-2. Schematic representation of T helper subsets and functions. Different $CD4^+$ T helper subsets are generated in the presence of specific polarizing cytokines. Each subset is characterized by the expression of a distinct key transcription factor and by the production of different effector cytokines that contribute to different aspects of host immunity. Modified from (213)

1.5 Intestinal microflora

The intestine harbours a vast amount of bacteria, referred to as the intestinal microflora. Recent advances in deep genomic sequencing have led to an unprecedented understanding of the complexity of this unique ecosystem (2). Despite its high density, the diversity of the system at a division level is surprisingly low, with only 8 of the 55 known bacterial divisions present in the gut (214). In humans, the dominant divisions are Cytophaga-Flavobacterium-Bacteroides and Firmicutes, with Proteobacteria and Actinobacteria also represented, although more rare, as well as viruses, fungi and protists (214-216). In contrast, at a species level, the intestinal microflora is extremely diverse and inter-individual variation is very high (214). Each person's "microbiome" is unique, supporting the hypothesis that genetic factors, "legacy effects" (which include events in early post-natal life) and environmental effects define the composition of the intestinal microflora (214, 217-221).

Our relationship with the intestinal microflora is mutalistic, as both the host and the bacteria benefit from it (222). Indeed, the intestine represents a perfect anaerobic bioreactor for the growth of intestinal bacteria (215). In exchange for this "safe harbour", the microbiota exerts a plethora of advantageous functions for the host. One of the most important functions of the intestinal microflora is to contribute to efficient nutrient acquisition, by fermenting non-digestible carbohydrates into absorbable small chain fatty acids (2), by synthesizing vitamins and by promoting the absorption of ions (223). Accordingly, the intestinal microflora plays an essential role in modulating host metabolism and changes in its composition have been associated

with obesity and malnutrition (224). Secondly, intestinal bacteria support development and maintenance of the GALT, by stimulating epithelial cell proliferation, differentiation and repair (225-227), by favouring the accumulation of plasma cells and $CD4^+$ T cells in the intestinal LP (228), and by promoting the formation of secondary lymphoid structures such as PP and ILF (44, 228). Signals from the intestinal microflora are also important for the development of the systemic immune compartment (81, 229) and alterations in the intestinal flora have been associated with the development of extra-intestinal autoimmune diseases, such as type 1 diabetes (230) and asthma (231, 232). Finally, the intestinal microbiota itself contributes to protection against infection by pathogens, as beneficial intestinal bacteria are extremely adapted to the intestinal niche, making it difficult for "newcomers" to successfully colonize the host (215). Intestinal bacteria also express antibacterial bacteriocins, which can limit the growth of competing species (233) and promote the production of protective IgA and mucins, further fortifying the intestinal barrier (82, 234-236).

The equilibrium between host immune responses and intestinal microflora is maintained through a series of adaptations. First of all, many intestinal bacteria express modified versions of lipopolysaccharide (LPS), making them less immuno-stimulatory (237, 238) and, as previously discussed, intestinal bacteria are generally excluded from the epithelium layer by the presence of a thick layer of mucus (13). As noted above, in the steady state, innate and adaptive immune responses in the intestine are skewed towards hypo-responsivess and induction of tolerance (13). Intestinal bacteria may play a key role in maintain tolerance during homeostasis in

several ways: by inducing expression of IL-10 (193, 239-241), by down-regulating NF-κB signalling (242) and by promoting the generation of Tregs (239, 243, 244). Finally, the structure of the GALT ensures that the systemic immune system is kept immunologically ignorant towards intestinal bacteria, efficiently compartmentalizing intestinal immune responses (245).

1.6 Pattern recognition receptors (PRR)

PRR are the key molecules involved in the activation of the innate immune system. These germline-encoded receptors recognize microbe-associated molecular patterns (MAMP), typically molecules essential for microbes' survival and shared by a class of microorganisms (246). PRR can also recognize danger-associated molecular patterns (DAMP), molecules of host origin released in conditions of cellular stress or tissue injury (247). Four main classes of PRR have been identified so far: Toll-like receptors (TLR), nucleotide-binding domain and leucine rich repeat containing receptors (NLR) retinoic acid-inducible gene-I (RIG-I)-like receptors (RLR) and C-type lectin receptors (CLR). Together, they ensure that coordinated protective immune responses are rapidly elicited following tissue injury or microbial infection. CLR are a heterogeneous family of transmembrane PRR characterized by the expression of a "C-type lectin-like" domain (CTLD) in the extracellular region (248). CLR mainly recognize carbohydrates on pathogens and play a key role in anti-fungal responses (248). The prototypical CLR, Dectin-1, recognizes fungal β-glucans and activates the proinflammatory NF-κB, NFAT and mitogen associated protein kinase (MAPK) pathways mainly through activation of spleen tyrosine kinase (Syk) and Caspase recruitment domain-containing protein 9 (CARD9) (248). Intracellular RIG-I-like

receptors (RLR) include RIG-I, melanoma differentiation-associated protein-5 (MDA-5) and other structurally related uncharacterized proteins (249) and play a key role in promoting anti-viral responses by inducing the expression of type 1 IFN, especially in non-myeloid cells (250).

1.6.1 Toll-like receptors (TLR)

TLR are an exceptionally conserved family of PRR, initially identified in *Drosophila*, where they play an essential role both in development and in immune responses (251). The TLR family includes 10 members in humans and 13 in mice (252) (**Table 1-1**), which are variably expressed by many leukocytes, including DC, macrophages, granulocytes, B cells and some T cell subsets (252). Moreover, TLR are also expressed by non-hematopoietic cells, including epithelial cells and fibroblasts (253). Different TLR are localized at different cellular sites: specifically, TLR1, 2, 4, 5 and 6 are expressed at the cell surface, while TLR3, 7, 8 and 9 are expressed in endosomes, so that the ligands for these latter TLR need to be internalized in order to trigger signaling (253). TLRs are type I transmembrane molecules characterized by a conserved structure that includes an extracellular domain containing a variable number of leucine-rich repeats (LRR) that have a conserved amino-acid motif and mediate ligand recognition (253). The intracellular domain of TLR is characterized by the presence of a signaling Toll/IL-1R homology (TIR) domain, shared with the receptors for IL-1 family cytokines (254).

TLR recognize a plethora of different MAMPs (**Table 1-1**). However, the same TLR can recognize many different agonists: for instance, TLR4 recognizes extremely different structures, from LPS to fibronectin (253). In part, this could be due to the use of

different partners for the recognition of specific ligands: for instance, TLR4 associates with CD14 and MD2 to bind LPS (253), whilst TLR2 can dimerize both with TLR1 and TLR6 (253). TLR signaling is triggered by the oligomerization of TLR following ligand recognition, which brings the intracellular TIR domains in close proximity, allowing the recruitment of adaptor molecules (254). Currently, four adaptor molecules have been identified: myeloid differentiating factor-88 (MyD88), MyD88 adapter-like (Mal), TIR domain-containing adapter-inducing IFN-β (TRIF), and TRIF-related adapter molecule (TRAM) (255). MyD88 is required for the signaling of all TLR except TLR3, which signals through TRIF (255). TLR4 signals both through MyD88 and by recruiting TRAM, translocating to the endosomal compartment and triggering TRIF signalling (255). The function of Mal is less clear, but it is thought to stabilize MyD88 recruitment in TLR2 and TLR4 signaling (255).

Signals through MyD88 lead to the activation of the NF-κB and MAPK pathways, triggering a strong pro-inflammatory transcriptional response (255) (**Fig.1-3**). Triggering of TRIF results in both NF-κB activation and IRF-3-mediated transcription of IFN-α and IFN-β, resulting in a coordinated type-1 interferon-mediated immune response (255) (**Fig.1-3**). Importantly, signaling through IL-1R and IL-18R is also MyD88-dependent, at least in part, and results in a similar signaling cascade as described for membrane TLR (254).

TLR	Associated molecule(s)	Ligand/agonists	Cellular localization
TLR1	TLR2	Triacyl lipopeptides	Cell surface
TLR2	TLR1, TLR6, CD14	Peptidoglycan, lipoproteins, zymosan	Cell surface
TLR3	Not known	Doube-stranded RNA Single-stranded RNA	Endosomes
TLR4	CD14, MD2	LPS, mannan, taxol, HSP60, fibronectin, viral envelope proteins	Cell surface (endosomes)
TLR5	Not known	Flagellin	Cell surface
TLR6	TLR2	Diacyl-lipopeptides, lipoteichoic acid	Cell surface
TLR7	Not known	ssRNA viruses, RNA from bacteria Imidazoquinoline compounds	Endosomes
TLR8	Not known	ssRNA viruses	Endosomes
TLR9	Not known	CpG DNA motifs dsDNA viruses	Endosomes
TLR10	Not known	Not known	Cell surface
TLR11	Not known	Profilin-like molecule	Cell surface
TLR12	Not known	Not known	Not known
TLR13	Not known	Vescicular stomatits virus	Endosomes

Table 1-1. Toll-like receptors ligands and localization. All known TLR are indicated, together with their possible partners and activating molecules/agents. For many activating agents binding to the TLR has not been demonstrated, hence they should be considered as agonists. TLR4 is likely to be endocytosed following LPS recognition at the plasma membrane (256). Note that TLR11, 12 and 13 are not found in human. Adapted from (257) and (258).

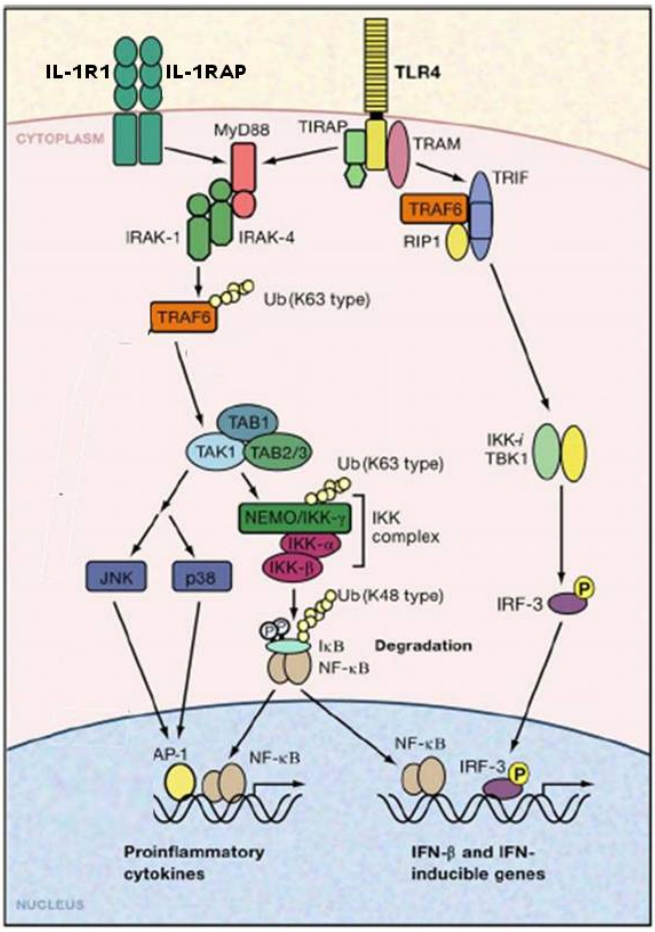


Figure 1-3. Overview of TLR signaling. Ligand recognition leads to dimerization of TLR, bridging the intracellular TIR domains and prompting the recruitment of adaptor molecules such as MyD88. MyD88 recruits IL-1R associated kinase-4 (IRAK4) and IRAK-1. IRAK-4 phosphorylates IRAK-1, which associates with TNFR-associated factor 6 (TRAF6). TRAF6 catalyzes its own ubiquitination and ubiquitinates IKK-γ, activating the IKK complex, which phosphorylates IκB, leading to its degradation. NF-κB can now migrate to the nucleus and coordinate the expression of pro-inflammatory cytokines. Ubiquitination of TRAF6 also results in recruitment of TGF-β activated kinase 1 (TAK1) and of TAK1 binding proteins TAB1, TAB2 and TAB3. TAK1 phosphorylates both IKK-β and MAP kinase kinase 6, reinforcing NF-κB activation and promoting MAP kinase signaling, which also leads to the expression of proinflammatory genes. TLR3, TLR7 and TLR9 are localized on lysosomal membranes (not shown) and are activated following internalization of microbial antigens. TLR3 signal independently of MyD88 through TRIF, which can also mediate MyD88-independent TLR4 signaling. TRIF activates RIP-1 through TRAF6, activating NF-κB. TRIF also activates TANK-binding kinase-1 (TBK1) via TRAF3, which phosphorylates IRF-3 and IRF-7, resulting in the expression of type-1 interferons. Modified from (253).

1.6.2 NLR

Nucleotide-binding domain and leucine rich repeat containing receptors (NLR) are cytoplasmic PRR characterized by the presence of a nucleotide-binding and oligomerization (NACHT) domain. Typically, NLR also express a C-terminal LRR and an N-terminal interaction domain, which can be a caspase recruitment (CARD) domain, a pyrin domain (PYD) or a baculovirus inhibitory of apoptosis domain (BIR) (259). NLR are expressed both in immune and non-immune cells, including myeloid cells, T cells, endothelial cells and epithelial cells (260). NLR recognize a vast array of MAMP and DAMP, and coordinate different innate immune responses (**Table 1-2**). Residing in the cytoplasm, these molecules are perfectly placed to sense stress and danger signals. Depending on their signaling pathway, NLR can be classified in three major classes: non-inflammasome NLR, inflammasome NLR and regulatory NLR. Regulatory NLR include novel members of the family, such as NLRX1, which localizes to the mitochondria, where it modulates ROS responses and attenuates type 1 IFN production by interacting with members of the RLR family (261).

NLR	Activating bacterial motif / agonist	Microbes recognized
NOD1	Meso-DAP g-D-Glu-DAP (iE-DAP) L-Ala-g-D-Glu-meso-DAP (TriDAP)	*S.flexneri (G-)* *H.pylori (G-)* *E.coli(G-)* *Chlamydia species (G-)* *Pseudomonas species (G-)* *L.monocytogenes (G+)*
NOD2	MurNAc-L-Ala-D-isoGln (MDP) N-glycolylated MDP ssRNA	*L.monocytogenes (G+)* *S.pneumoniae (G+)* *S.typhimurium (G-)* *S.flexneri (G-)* *M.tubercolosis (G+,a.f.)* *Trypanosoma cruzi* *Respiratory syncytial virus*
NLRP1	Lethal factor from *B.anthracis* MDP	
NLRP3	MDP Bacterial RNA Bacterial toxins (Aerolysin, Maitotoxin, Nigericin, Adenlyate cyclase toxin) "Danger" signals (Extracellular ATP, Hyalurnonan, Glucose, MSU, Amyloid-β) Skin irritants (TNP-Cl, TNCB, DNFB) Particulate agents (silica, asbestos, alum)	*S.aureus (G+)* *L.monocytogenes (G+)* *N.gonorrhoeae(G-)* *B.pertussis (G-)* *C.albicans* *S.cerevisiae* *Sendai virus* *Influenza virus* *Adenovirus* *Encephalomyocarditis virus*
IPAF (NLRC4)	Intracellular flagellin	*S.typhimurium (G-)* *L.monocytogenes (G+)* *S.flexneri (G-)* *L.pneumophila (G-)* *P.aeruginosa (G-)*

Table 1-2. NLR and their activating agonists. NLR are activated by many microbes and microbes-associated molecular motifs. Some NLR, especially NLRP3, may also act as sensors for endogenous and exogenous danger signals. Although NLR are currently described as receptors, no direct evidence exists that they interact directly with their agonist, the only exception being NLRP1, which has been shown to directly interact with MDP in a cell free system (262).

DAP:diaminopymelic acid; MDP: muramyldipeptide; (G+): Gram positive; (G-): Gram negative; (G+,a.f.): Gram-positive acid fast; ATP: adenosine triphosphate; MSU: mono-sodium urate crystals; TNP-Cl: trinitrophenylchloride; TNCB: trinitrochlorobenzene; DNFB: dinitrofluorobenzene.

Modified from (260)

1.6.2.1 Non-inflammasome NLR

NOD1 and NOD2 are the main NLR signaling in a non-inflammasome dependent fashion. Both molecules sense products derived from the breakdown of the bacterial cell wall peptidoglycan: NOD1 is activated by *meso*-diaminopimelic acid (DAP), which is generally found in Gram-negative bacteria, whereas NOD2 detects muramyl dipeptide, which is common to both Gram-negative and Gram-positive bacteria (263-266). Importantly, these cell wall components need to be transported inside the cell to activate NOD1 and NOD2. In some cases this is mediated through the action of specialized type III or type IV bacterial secretion systems (267). Alternatively, it has been proposed that MDP can be internalized by endocytosis (268, 269) and then translocated to the cytosol by an oligopeptide transporter (268).

Triggering of NOD molecules results in the activation of the NF-κB pathway (**Fig.1-4**). Activation of NOD2 leads to its translocation to the plasma membrane and recruitment of receptor interacting protein-2 (RIP2) (270). In turn, NOD2-RIP2 recruits the ubiquitin ligases cIAP1 and cIAP2 and mediates the polyubiquitination of IKK-γ, activating the IKK complex (271) and ultimately promoting the translocation of NF-κB to the nucleus, where it induces a pro-inflammatory programme. NOD2-RIP2 also recruits TAK1 and the TAK-1 binding proteins TAB1-3 (272). TAK1 activates the IKK complex and promotes the activation of the MAP kinase pathway, although this process has not been not fully characterized (273).

NOD1 and NOD2 clearly play a role in defense against bacterial infection. NOD signaling induces a coordinated anti-microbial response, including production of chemokines, pro-inflammatory cytokines, antimicrobial peptides and reactive oxygen

species (274). More recently, it has been proposed that NOD2 also contributes to anti-viral defenses by promoting IFN-β production through activation of MAVS and IRF3 (275). In humans, mutations in NOD1 are associated with asthma (276), whilst gain of function mutations in NOD2 are linked to Blau syndrome and early-onset sarcoidosis (277, 278). Moreover, mutations in both NOD1 and NOD2 are associated with IBD, as will be discussed later in this chapter (279-281).

1.6.2.2 Inflammasome NLR

Some NLR, notably NLRP3, NLRP1 and IPAF (NLRC4), signal through the formation of a multi-molecular scaffold known as the inflammasome (282) (**Fig.1-4**). Inflammasome formation is mediated by oligomerization of NLR, resulting in recruitment of the precursor form of the proinflammatory caspase, caspase-1 (pro-casp1)(282). Clustering of pro-casp1 in the inflammasome results in auto-cleavage and formation of active caspase 1 (282). In turn, activated caspase-1 mediates the processing of the precursor forms of the cytokines IL-1β and IL-18, pro-IL1β and pro-IL-18, which are cleaved into their active forms by caspase-1 and then secreted through a poorly understood unconventional secretion pathway (259). Recent evidence suggests that caspase-1 activation might lead to the secretion of other leaderless proteins involved in inflammation and tissue repair (283), suggesting that the formation of the inflammasome has a wider impact on innate immunity. Some NLR, such as NLRP3, require the presence of the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD) to form an inflammasome, whilst others, such as NLRP1 and IPAF, can form inflammasomes in an ASC-independent way, although the recruitment of ASC seems to increase the efficiency of the process

(262, 284, 285). Importantly, expression of pro-IL-1β and of many NLR, including NLRP3, is under the control of TLR signaling, strongly linking these two key arms of innate immune sensing (286, 287).

NLRP3 is an extremely promiscuous NLR, activated by a wide range of microbes, stress signals and exogenous particles (**Table 1-2**) (259). The extreme diversity of stimuli able to activate this pathway suggests that currently unknown interacting proteins might confer specificity to the NLRP3 response. Alternatively, all NLRP3 agonistic signals might converge in the activation of an intracellular inflammatory pathway, which in turn activates NLRP3 (259). Support for the latter hypothesis has grown since the discovery that many NLRP3 agonists induce the generation of reactive oxygen species (ROS) and ROS blockade inhibits inflammasome activation (288-291). However, alternative models of NLRP3 activation have been proposed. Extracellular ATP is a potent activator of the NLRP3 inflammasome (292-295), as ATP can activate the ion channel P2X7, leading to K^+ efflux and opening of the pannexin-1 membrane pore, allowing the influx of NLRP3 agonists into the cytosol (296-298). Alternatively, particulate agents such as mono-sodium urate crystals and fibrillar amyloid-β can promote NLRP3 activation by inducing phagosomal destabilization (299, 300). Further investigation is required to better characterize the mechanisms of activation of NLRP3.

In contrast to NLRP3, IPAF presents a more selective pattern of activation. IPAF senses intracellular flagellin, which is generally delivered by bacterial secretion systems (259). Accordingly, IPAF is important in the immune response against flagellated bacteria such as *Salmonella typhimurium, Legionella pneumophila* and

Pseudomonas aeruginosa (284, 285, 301). However, as *P.aeruginosa* can activate IPAF in both a flagellin-dependent and independent way (302) and non-flagellated bacteria such as *Shigella flexneri* also activate the IPAF inflammasome (303), it is likely that ligands other than flagellin can induce IPAF activation. Other NLR can also form inflammasomes: for instance the NLRP1 inflammasome is responsible for the susceptibility to *Bacillus anthracis* letal toxin in mouse (304). Finally, NOD2 has been reported to interact with NLRP1 and NLRP3, promoting inflammasome formation and IL-1β processing upon MDP stimulation (305, 306).

On top of inducing cytokine activation, the inflammasome can trigger a specific type of cell death, that has been termed "pyroptosis" (307, 308). Pyroptosis is a caspase-1 dependent form of cell death, that is independent of activation of classical pro-apoptotic caspases (308). In contrast to apoptosis, pyroptosis is characterized by the release of cytoplasmic components, including activated IL-1β and IL-18, into the extracellular space (308), and by nuclear condensation and DNA cleavage, without the DNA laddering typical of apoptosis (309, 310). Pyroptosis is strongly pro-inflammatory and it is an important defense mechanism against some bacterial infections, such as *Listeria monocytogenes, S.typhimurium* and *S.flexneri,* (303, 311-313). However, caspase-1 activation has been linked with pro-survival signals *in vitro* (314) and caspase-1 activation does not always lead to pyroptosis. Hence the overall contribution of pyroptosis to NLR-induced immunity is unclear.

Hyper-activation of the inflammasome has been implicated in many human diseases. Specifically, gain of function mutations in NLRP3 are responsible for a group of auto-inflammatory diseases collectively known as cryopyrin-associated periodic

syndromes (CAPS), characterized by high levels of systemic IL-1β (315-317). CAPS are also associated with mutations in other genes encoding molecules involved in the regulation of IL-1β activity, such as IL-1RA (318, 319). Moreover, dysfunctions in the NLRP3 inflammasome have been associated with gout (292). Finally, mutations in NLRP3 have been associated with susceptibility to IBD (320) and this will be discussed later in this chapter.

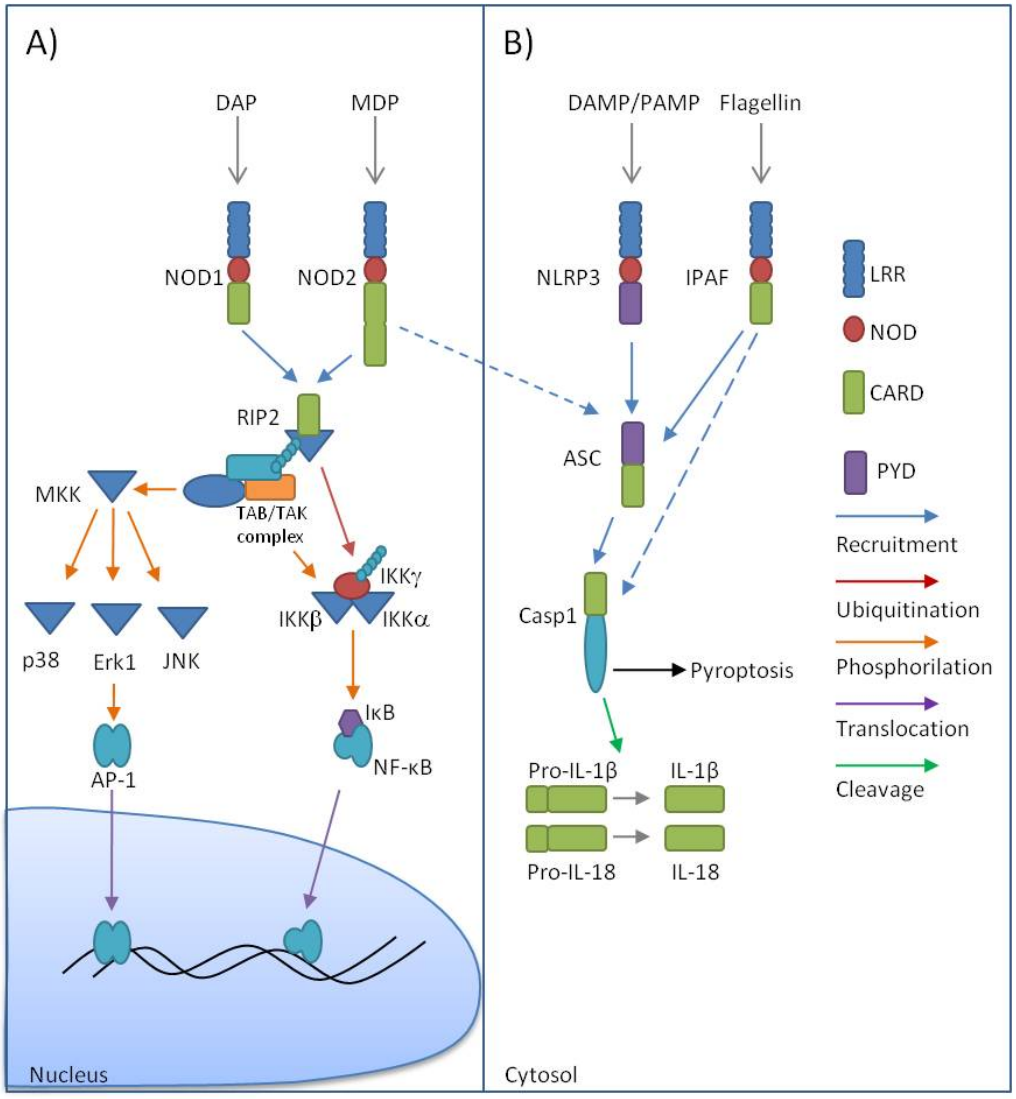


Figure 1-4. Overview of NLR signaling.

A) Non inflammasome NLR activation results in recruitment of RIP2 through CARD-CARD interactions. NOD2/RIP2 activate the NF-κB pathway through polyubiquitination of IKKγ (NEMO). NOD2 promotes polyubiquitination of RIP2, recruiting the TAK1/TAB1-3 complex, which activates the IKK complex by phosphorylating IKKβ. TAK1 also activates the MAPK pathway.. A review of non-inflammasome NLR signaling is provided in (274). B) Upon activation, inflammasome NLR self-oligomerize through NACHT domain interaction and recruit caspase-1 through CARD-CARD interactions. NLR lacking a CARD domain, such as NLRP3, recruit caspase-1 through interaction with the adaptor molecule ASC. Formation of the inflammasome promotes activation of caspase-1. Active caspase-1 cleaves proIL-1β and proIL-18 into active cytokines, which are then released into the extracellular space. Caspase-1 might also target other molecules. Activation of caspase-1 can also lead to pro-inflammatory cell death, or pyroptosis, through poorly understood mechanisms. Inflammasome NLR signaling is reviewed in (259).

1.7 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gut, resulting from a breakdown of the homeostatic mechanisms required to maintain a mutualistic relationship with the intestinal microflora (4). Clinically, inflammatory bowel disease includes two main forms of intestinal inflammation, Crohn's disease (CD) and ulcerative colitis (UC) (4), although 10-15% of IBD cases cannot be clearly classified (321). Despite the differences in clinical manifestation, genetic and environmental causes triggering CD or UC overlap significantly, suggesting that both these diseases arise from the disruption of core homeostatic mechanisms in the intestine (4).

Clinically, CD presents as a patchy, transmural inflammation, which can affect any part of the gastrointestinal tract. CD is typically relapsing and most patients soon develop complications, such as abscesses, fistulas and strictures (322). UC is also relapsing, but inflammation is generally non-transmural and limited to the colon (322). The incidence of CD in Europe ranges between 0.7-9.8 cases/100,000 person-years, whilst UC is more common, with 1.5-20.3 cases/100,000 person-years, with higher incidence in Northern European countries (323). Typically, UC and CD are initially treated with anti-inflammatory and immunosuppressant drugs, including corticosteroids (322). The understanding of the mechanisms underlying the development of IBD facilitated the development of novel biological therapies, which significantly improved the management options for these chronic conditions. In particular, αTNF-α monoclonal antibodies (mAb) are now commonly used in clinical practice in cases where traditional drugs fail to induce or maintain remission (324).

However, surgery is still a frequent outcome for many IBD patients, especially those suffering from CD (322), highlighting the need for novel therapeutic strategies.

The specific aetiology of IBD is currently unknown, but the disease arises from the combination of genetic and environmental factors (4), as highlighted by the rate of disease concordance in identical twins, which is only 30-35% in CD and 10-15% in UC (325). However, genome-wide association studies (GWAS) have underscored the contribution of genetic polymorphisms to IBD susceptibility and have identified 99 distinct genetic loci associated with IBD (217, 326-331). The increased disease risk associated with most of these indivdual loci is extremely low (NOD2 confers the highest risk in Caucasians, with an odds-ratio of 17.1 in homozygotes for risk alleles) (332), highlighting the complexity of IBD pathogenesis, and suggesting that mutations in several pathways may be needed to develop disease. However, in some cases rare mutations in key regulatory genes could also contribute to IBD pathogenesis, as shown by the presence of rare forms of familial IBD, such as that observed in patients homozygous for mutations in *Il10ra* or *Il10rb*, encoding for the subunits of the IL-10 receptor (4).

Environmental factors also play an essential role in IBD pathogenesis. However, decades of research failed to identify life-style risk factors universally associated with IBD (323). A notable exception is cigarette smoke: smokers have a decreased risk of developing UC than non-smokers (333), but smoking doubles the risk of developing CD (333). More recently, the advent of metagenomics has allowed an unprecedented analysis of the main environmental factor associated with IBD pathogenesis, the intestinal microflora.

1.7.1 Microflora-host interactions in IBD

It is now widely accepted that IBD results from an alteration of the balance between intestinal microflora and host immune system (334). The evidence for an association between IBD and altered microflora is particularly strong for CD. Indeed, antibiotics can be beneficial in the treatment of CD and UC and fecal stream diversion was able to ameliorate disease in some CD patients (335, 336). In addition, antibodies and/or T cell responses directed against enteric bacteria and fungi are present in the majority of patients suffering from CD (337-340). Similarly, most animal models of IBD are dependent on the presence of intestinal bacteria (341-343) and antibiotic or probiotic treatment ameliorated intestinal inflammation in some models (336).

Despite some association of CD with infection by *Mycobacterium avis paratubercolosis* (MAP), the causative agent of spontaneous granulomatous enterocolitis in cattle (344), no single microbe has been causatively associated with IBD. In contrast, recent evidence suggests that IBD, particularly CD, might result from changes in the overall composition of the intestinal microflora, a phenomenon known as “dysbiosis” (214, 334). Metagenomic studies reported that IBD patients have significantly less diversity in their intestinal microflora community (221, 345, 346), with decreased representation of the *Bacteroidetes* order and of the *Lachnospiraceae* family (221). In parallel, intestinal bacteria with reported anti-inflammatory effects, such as *Faecalibacterium prausnitzii* are decreased in CD patients (241). However, it is currently unclear whether the observed dysbiosis is cause or consequence of IBD. Significant alterations of the intestinal microflora are also associated with the development of intestinal inflammation in mice. Specifically, T-cell deficient mice lacking Tbet (*Tbx21*^{KO} *Rag2*^{KO} mice, or TRUC) spontaneously

developed intestinal inflammation that was partially transmissible to WT mice through cross-fostering (347, 348). Moreover, T cell clones specific for commensal bacterial antigens were able to transmit colitis upon transfer into T cell-deficient mice (349-351) and infection of immuno-deficient susceptible mouse strains with the Gram-negative bacterium *Helicobacter hepaticus* results in intestinal inflammation (352). Overall, these mouse studies clearly show that while alterations in the microflora are necessary for the development of severe colitis, they are not sufficient, as they must be associated with genetic susceptibility of the host.

In parallel with changes in the composition of the intestinal microflora, CD might also be associated with a more intimate association between the intestinal epithelium and the gut microflora. This hypothesis is supported by studies in mice, showing that mice lacking MUC2, the main component of the mucous layer, which separates the intestinal bacteria from IEC, develop spontaneous colitis (353). Moreover, several studies demonstrated an increased frequency of adherent/invasive *E.coli* (AIEC) in patients suffering from ileal Crohn's disease (354-357). AIEC adhesion to IEC in CD patients is mediated by abnormal expression of CAECAM-6 (carcinoembryonic antigen-related cell adhesion molecule-6) (358) and transgenic expression of CAECAM-6 in mice resulted in severe colitis that was associated with colonization by AIEC (359). However, it is not clear whether colonization with AIEC is specific to CD, as it has also been reported in some colon cancer patients (360).

In spite of the clinical association between CD and altered microflora, the cellular and molecular mechanisms associated with this process are still unclear. Recent studies provided significant examples on how intestinal bacteria can modulate host

immunity. Colonization of mice with segmented filamentous bacteria (SFB) was shown to promote the development of intestinal Th17 cells (361-363) and SFB colonization was protective against acute intestinal inflammation following infection with *C.rodentium* (361). Other bacterial species exert immuno-modulatory activity: for instance, infection of mice with *Bacteroides fragilis,* or with a combination of *Clostridia* species, boosted IL-10 production by $FOXP3^+$ and $FOXP3^-$ T cells in the colon, promoting resistance to intestinal inflammation (240, 364, 365).

1.7.2 Helicobacter hepaticus

Whilst the identification of a single bacterial agent associated with the development of chronic colitis in humans has been elusive, in mice, colonization with *Helicobacter* species is strongly associated with the development of chronic intestinal inflammation in susceptible strains (352). The best studied representative of the murine intestinal helicobacters is *Helicobacter hepaticus*, a Gram-negative, microaerophilic, flagellated bacteria originally identified in the colon and liver of A/JCr mice, where it causes typhlitis (caecal inflammation) and liver inflammation (352). *H.hepaticus* is widely distributed in animal colonies around the world (366) and can cause intestinal inflammation in a number of immunodeficient strains, such as mice deficient in IL-10 (367, 368), T and B cells (Rag^{KO} mice)(369, 370), NF-κB subunits (371) or SMAD3 (372). The intestinal crypts of the large intestine are the natural niche of *H.hepaticus* (373), which can also be found in the liver, gall bladder and bile canaliculi (373). Upon colonization of WT mice, *H.hepaticus* rapidly becomes a dominant member of the caecal microflora, inducing at the same time a strong decrease in the overall diversity of the bacterial community (374). However,

H.hepaticus did not induce intestinal inflammation upon mono-colonization of germ-free *Il10*^{KO} mice, suggesting that interplay between *H.hepaticus,* the host and other bacterial species is necessary for the development of colitis (343, 375). However, liver inflammation was still observed upon monocolonization of susceptible germ free strains with *H.hepaticus,* suggesting that different mechanisms are involved in the development of *H.hepaticus* induced typhlitis and hepatitis (375). Expression of flagellin is also required for *H.hepaticus* colonization of the intestine (376) and several virulence factors have been implicated in *H.hepaticus* pathogenicity, including cytolethal distending toxin (CDT) (377), hydrogenase (378), urease (379) and the flavoenzime PutA (380). Moreover, the *H.hepaticus* genome contains a putative pathogenicity island, (HHGI1), which has been proposed contain other virulence factors involved in the induction of disease (379, 381). In parallel, *H.hepaticus* evolved a series of adaptations to avoid activation of the host immune compartment. For instance, *H.hepaticus* can evade recognition by TLR5 (382) and *H.hepaticus* LPS can suppress TLR4 responses in IEC (383). Moreover, recent data propose that *H.hepaticus* has a Type VI secretion system (T6SS), which induces the repression of pro-inflammatory gene expression in IEC, limiting intestinal immune responses (384).

1.7.3 PRR in intestinal inflammation

Pattern recognition receptors (PRR) play a central role in intestinal inflammation by regulating the function of both epithelial and haematopoietic intestinal cells and polymorphisms in various PRR, such as NOD2, NOD1, TLR2, TLR4 and TLR9, have been associated with IBD susceptibility (4, 385).

1.7.3.1 TLR

Immunohistochemistry studies and expression analysis revealed that IEC express a wide range of PRR, including TLR2, TLR3, TLR4, TLR5 and TLR9 (386). However, TLR signaling is strongly down-regulated, both by modulation of TLR expression/localization and by expression of specific inhibitors of TLR signaling (386). The expression of many TLR is strongly compartimentalized in the intestine, particularly in the colon. For instance, TLR4 or TLR5 are only found at the basal membrane of colonic IEC, avoiding contact of these receptors with luminal MAMP (387-391). IEC also express molecules, such as TOLLIP (Toll-interacting protein) (392), SIGIRR (Single immunoglobulin IL-1R-related molecule, also known as TIR8) (393), PPARγ (Peroxisome proliferator-activated receptor γ) (394) and TNFAIP3 (Tumor necrosis factor, alpha-induced protein 3, or A20) (395), that actively inhibit TLR signaling by interfering with NF-κB activation (396).

TLR signaling in the epithelium is essential in promoting proliferation and repair following tissue injury, as mice lacking the common adaptor MyD88, TLR2 or TLR4 are more susceptible to DSS (dextran sodium sulfate)-induced intestinal injury (227, 397, 398). Similarly, conditional deletion of the NF-κB subunit IKKγ in IEC, leading to suppressed NF-κB signaling in IEC, resulted in spontaneous colitis, with increased apoptosis of IEC (399). TLR signaling in the epithelium is also important for the fortification of the intestinal barrier. TLR2 signals can promote tight junction formation during repair from intestinal injury (400) and TLR signals induce IgA production and expression of anti-microbial peptides (386, 401). Specifically, MyD88

signaling in Paneth cells is required for their expression of antimicrobial lectins, CRP-ductin and RELMβ (resistin-like molecule-β) (17).

While tonic TLR signals in the epithelium promote intestinal homeostasis, prolonged activation of TLR, particularly in intestinal hematopoietic cells, drives intestinal inflammation (402). Indeed, MyD88 signaling is required for the development of colitis in *Il10*^{KO} mice (403) and in mice bearing a myeloid cell-specific deletion of STAT-3, which makes myeloid cells insensitive to IL-10 (404). Experiments analyzing the role of downstream NF-κB signaling in intestinal inflammation epitomize the dual role of TLR signals in intestinal inflammation. Thus, although IEC-specific deletion of the downstream signaling molecule IKK-β results in increased susceptibility to DSS-induced intestinal injury (405), in *Il10*^{KO} mice ablation of IKK-β in IEC does not prevent the development of colitis whereas deletion of IKK-β in myeloid cells confers protection from intestinal pathology (405).

1.7.3.2 NLR

Genetic analysis has revealed links between NLR and IBD (4). Polymorphisms in *NOD2* are strongly associated with the development of CD in the Western world, as 30-40% of CD patients carry *NOD2* mutations in at least one allele (the frequency in the general population is ~10%) (406). Around 80% of CD-associated *NOD2* variants fall into three individual mutations (R702W, G908R and 1007fs), localizing to the C-terminal LRR and potentially disrupting the sensor function of NOD2 (4). This hypothesis is supported by the finding that monocytes from CD patients bearing *NOD2* mutations show decreased responses to MDP stimulation (407). A "loss of function" model is also supported by reports that Paneth cells from patients bearing

mutated *NOD2* alleles express less α- and β-defensins (408, 409), although inflammation itself could account for the observed decrease in defensin expression (410). Nevertheless, a similar decrease in the expression of specific α-defensin was observed in *Nod2*^{KO} mice (411). In parallel, a "loss of regulatory function" model has also been proposed, as NOD2 stimulation down-regulates TLR-induced activation of NF-κB signalling in DC *in vitro* (412, 413), although this effect was not observed by all investigators (414). Moreover, transfer of ovalbumin (OVA)-specific CD4^{+} T cells into *Nod2*^{KO} recipient mice followed by oral administration of OVA-expressing *E.coli* resulted in increased colitis compared to WT recipients, consistent with the hypothesis of an increased activation state of *Nod2*^{KO} APC (415). In addition, MDP administration protects mice from acute dextran sulphate sodium (DSS)-induced colitis, although this effect could be due to modulation of IEC rather than APC (416). Other investigators propose a "gain of function" model to explain how *NOD2* mutations predispose to IBD. Indeed, macrophages from mice bearing the most common CD-associated human *NOD2* variant selectively responded to MDP stimulation by producing high levels of IL-1β (417). In addition, these mice were more susceptible than WT mice to acute intestinal injury (417). More recently, it has been proposed that in humans mutated forms of *NOD2* can inhibit IL-10 expression in APC, by suppressing the IL-10 inducer heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (418). Surprisingly, *Nod2*^{KO} mice do not develop spontaneous intestinal inflammation, although they are more susceptible to mucosal infection by *Listeria monocytogenes* (411). Moreover, *Nod2*^{KO} mice show increased Th1 responses in the intestine, suggesting that this PRR could play an important role in skewing adaptive immune responses (419, 420). In summary, NOD2 clearly plays an

extremely complex role in the intestine, modulating different aspects of intestinal homeostasis, including barrier function, bacterial handling and control of both innate and adaptive immune responses.

Polymorphisms in *NOD1* have also been associated with CD (279). NOD1 expression by IEC plays a key role in the control of infection by invasive Gram-negative bacteria in the intestine (421-423) and the recognition of the non-invasive Gram-negative bacteria *Helicobacter pylori* by gastric epithelial cells is also dependent on NOD1 (267). Finally, expression of NOD1 by non-hematopoietic cells is essential for the development of ILF in the mouse intestine, and has a strong influence on the composition of the microbial flora (44). Thus, mutations in *NOD1* might significantly alter the host-bacteria equilibrium in the intestine. Little information is currently available on the role of other NLR in the pathogenesis of IBD, although a genetic association of CD with NLRP3 has been reported (320).

1.7.4 Effector mechanisms in IBD

Clinical research and work in animal models have provided crucial information on the inflammatory pathways activated in IBD. However, it is often difficult to understand the overall effect of a specific pathway, as effector mechanisms in intestinal inflammation are deeply intertwined and often the same pathway can deliver both protective and pathogenic functions. As most IBD patients present with similar symptoms, despite the strong genetic variability, researchers hoped that biological therapies targeting shared downstream effector functions could benefit all patients (322). Whilst this was partially accomplished by novel therapeutics, such as αTNF-α therapy, a more realistic perspective will probably require tailoring therapies to

different populations of patients. Moreover, combinatorial therapy, targeting different effector pathways at the same time, might be necessary to induce and maintain remission.

1.7.4.1 TNF-α and IL-6

The development of αTNF-α therapy is arguably the biggest contribution of basic research to IBD therapy (324). Different αTNF-α mAbs are available for clinical use, all extremely effective in maintaining remission in patients that do not respond well to traditional therapies (322). How this is achieved is not clear, although the antibody might induce apoptosis of effector $CD4^+$ T cells in the lamina propria (324). Further confirmation of the importance of the TNF-α pathway in human disease comes from the association of CD in Asian patients with polymorphisms in *Tnfsf15*, encoding for the TNF-like cytokine TL1A (424, 425). Animal models also confirm a central role of TNF-α in driving intestinal pathology. The stability of TNF-α mRNA is controlled by AU-rich elements (ARE) at the 5'UTR (426). Mice with a deletion of these TNF-α regulatory elements ($Tnf^{\Delta ARE}$) show high levels of TNF-α secretion and develop transmural inflammation mainly in the terminal ileum, together with polyarthritis (426, 427). In spite of the success of αTNF-α therapy, it should be noted that many patients either develop resistance to this treatment or fail to respond altogether, and that αTNF-α treatment is associated with increased frequencies of infection and cancer, highlighting the need for further IBD therapies (428).

IL-6 is also elevated in the colon of CD patients and correlates with the degree of inflammation (429-431). IL-6 promotes the survival and effector functions of $CD4^+$ T cells and macrophages in the intestine (432, 433). Accordingly, treatment of patients

with active disease with humanized αIL-6R (IL-6 receptor) mAb was beneficial in a randomized controlled clinical trial (434), and strategies blocking IL-6 signaling were beneficial in both acute and chronic animal models of colitis (432, 433).

1.7.4.2 IFN-γ and Th1 cells

Early studies supported a role for Th1 cells in IBD, as mononuclear cells from CD patients showed increased IFN-γ production and T-bet expression compared to healthy controls (435) (119). In addition, treatment with αIL-12p40 ameliorated disease and reduced the frequency of Th1 cells in the lamina propria, although the beneficial effect of this treatment may also have been mediated by the modulation of the IL-23/Th17 pathway (436, 437). Furthermore, T cells lacking IFN-γ or T-bet are unable to induce colitis in adoptive transfer models (118, 119). However, the role of IFN-γ in colitis in lymphocyte-replete animals is less clear. Indeed, while treatment with αIFN-γ mAb ameliorated colitis in *Il10*^{KO} mice and in a model of *H.hepaticus*-induced intestinal inflammation (367, 438), IFN-γ deficiency did not reduce colitis in *Il10*^{KO} mice (439). Moreover, neutralization of IFN-γ was not effective in improving CD in a randomized controlled trial (440, 441). Therefore, the precise contribution of this arm of adaptive immunity to intestinal pathology is unclear, perhaps reflecting the heterogeneity of Th1 responses.

1.7.4.3 IL-23 and Th17 cells

In recent years the IL-23 pathway has emerged as a major effector pathway in intestinal inflammation (442). IL-23 shares one of its subunits, IL12p40 or IL12B, with IL-12 (443). Different investigators reported that the beneficial effects of αIL-12p40 treatment on intestinal inflammation are, at least in part, due to depletion of IL-23

(438, 444-446), as blockade of the IL-23 specific subunit (IL-23p19) also results in amelioration of colitis (447). These studies are complemented by genetic data showing a strong association between polymorphisms in the *IL23R* and in other components of the IL-23 pathway, such as *STAT3*, and susceptibility to IBD (326, 327, 448). Moreover, IL-23 is elevated in the colon of CD patients, which also harbour a characteristic population of $CD14^{+}$ macrophages expressing high levels of IL-23 in response to commensal bacteria stimulation (449-451). As IL-23 plays an important role in promoting the accumulation and maintenance of Th17 cells (442), many studies addressed the role of this T helper subset in intestinal inflammation, with contrasting results. Th17 cells are enriched in the mucosa of CD patients (450, 452, 453) and polymorphisms in both IL-17A and IL17F are associated with IBD (454), suggesting a pathogenic role for Th17 cells and cytokines of the IL-17 family in IBD. This was supported by the finding that mice lacking the IL-17R were less susceptible to acute colitis (455). Moreover, combined blockade of IL-6 and IL-17A ameliorated colitis in $Il10^{KO}$ mice (446) and blocking IL-17A ameliorated the spontaneous colitis developed by mice lacking STAT3 specifically in Tregs (197). Furthermore, transfer of bacteria-reactive Th17 cells to SCID (severe combined immunodeficiency) mice results in more severe colitis than transfer of Th1 cells (447) and transfer of $Rorc^{KO}$ or $Irf4^{KO}$ naïve T cells does not induce colitis in Rag^{KO} mice (456, 457). However, further evidence points to a more complex role of Th17 cells in intestinal inflammation. For example, deletion of IL-17A resulted in more severe disease in acute models of colitis (458) and IL-17A production by T cells was shown to be dispensable for disease in models of T cell transfer colitis, with some authors reporting exacerbated systemic disease in mice receiving $Il17a^{KO}$ T cells (456, 459, 460). Other IL-17 family cytokines,

such as IL-17F and IL-22, also appear to play a role in intestinal inflammation. For example, IL-17F is pathogenic in models of acute colitis (461) and ablation of both IL-17F and IL-17A was beneficial in T cell transfer colitis (456). In contrast, IL-22 plays a protective role in acute models of intestinal injury, by promoting epithelial restitution and repair (462). However, IL-22 is increased in the colon of CD patients and levels of IL-22 in the serum correlate with disease activity, suggesting that IL-22 could play a pathogenic role in chronic forms of intestinal inflammation (463-465). These contrasting findings underline the complexity of the role of Th17 responses and IL-17 family cytokines in intestinal inflammation and could be partly explained by the heterogeneity and plasticity of Th17 cells. For instance, recent findings from our laboratory showed that IL-23 promoted the differentiation of IL-17A$^+$IFN-γ^+ CD4$^+$ T cells in the intestine (203). Similarly, IL-17A$^+$IFN-γ^+ CD4$^+$ T cells have been recovered from the LP of CD patients (453, 466). We also reported that IL-23 could induce the production of Th17 cytokines such as IL-17A and IL-22 by subsets of innate lymphoid cells (ILC) and that blocking IL-17A was beneficial in a model of *H.hepaticus*-dependent innate immune colitis (51), adding a further layer of complexity in understanding the role of IL-17 producing cells in intestinal inflammation.

1.7.5 Immune-suppressive pathways

The presence of regulatory T cells in the gut is essential for intestinal homeostasis as individuals suffering from IPEX often present with severe intestinal inflammation (167, 169, 170) and CD4$^+$CD25$^+$FOXP3$^+$ T cells play a crucial role in preventing and limiting intestinal inflammation in animal models of disease (467). The mechanisms through which Treg exert such dominant immunosuppression in the gut are unclear,

but the immuno-modulatory cytokines IL-10 and TGF-β are of crucial importance to maintain homeostasis in the intestine (189, 468). For instance, deletion of STAT-3 in macrophages, which makes them insensitive to IL-10 stimulation, results in spontaneous colitis (197, 404). Genetic data support the hypothesis that IL-10 is a key player in intestinal homeostasis: mutations in the IL-10 receptor genes *Il10ra* and *Il10rb* lead to early onset severe IBD (469), whilst polymorphisms in *Il10* were associated with UC and CD by GWAS studies (470).

TGF-β is essential for Tregs to inhibit T cell driven colitis (369, 471-473) and T cells that cannot respond to TGF-β are refractory to Treg-mediated inhibition (474). Moreover, mice deficient in TGF-β signaling or with T cells unable to respond to TGF-β stimulation develop spontaneous intestinal inflammation (475-480). TGF-β is highly expressed in the intestine and found at even higher levels in IBD patients (481), although complex post-translational modifications of TGF-β are required for its activity, so expression data don't necessarily correlate with the presence of active TGF-β (482). However, as TGF-β promotes the differentiation of Th17 cells in a pro-inflammatory milieu (483), this cytokine may play different roles in intestinal homeostasis, depending on subtle changes in the microenvironment.

1.7.6 Emerging modules in intestinal inflammation: ER stress and autophagy

Recent evidence suggests a strong link between ER stress and IBD pathogenesis. ER stress arises from the accumulation of misfolded proteins in the ER (484) and thus typically affects highly secretory cells, such as plasma cells, Paneth cells and goblet cells (485). Cells respond to ER stress by eliciting the unfolded protein response

(UPR), which results in arrest of protein translation, up-regulation of molecular chaperones and increased protein secretion (484). However, in the presence of sustained ER stress the UPR ultimately induces cell death (484). The UPR is mediated by different signaling cascades, activated by three proximal effector molecules: eukaryotic inositol-requiring transmembrane kinase-endoribonuclease-1 (IRE1), pancreatic ER kinase (PERK) and activated transcription factor 6 (ATF6) (484). The signaling cascades downstream of IRE1 and ATF6 have both been associated with IBD pathogenesis (485). Forward genetics studies associated the UPR pathway activated by ATF6 with intestinal inflammation, as mice rendered hypomorphic for S1P, a protease that activates ATF6, were more susceptible to DSS colitis (486). More extensive evidence supports a role for the IRE1 UPR pathway in IBD. The IRE1 isoform IRE1β is specifically expressed in the intestinal epithelium and mice deficient in IRE1β are more susceptible to DSS colitis (487). Moreover, polymorphisms in *XBP-1* (X-box binding protein 1), a transcription factor downstream of IRE1 activation, are associated with CD and UC and XBP-1 activation is increased in IBD patients (488). Mice with an IEC-specific deletion of *Xbp1* developed spontaneous inflammation in the small intestine (488), which was associated with Paneth cell dysfunction, a loss of Paneth cells and goblet cells in the small intestine and exaggerated pro-inflammatory responses by IEC (488), suggesting that the UPR might control various aspects of bacterial handling in the intestine.

Other studies support the association between ER stress and intestinal inflammation. For instance, mice with point mutations in MUC2 develop spontaneous intestinal inflammation, which is associated with aberrant MUC2 folding, accumulation of

MUC2 in the ER and ER stress induction in Paneth cells (489). Moreover, rats transgenic for the MHCI allele HLA-B27 develop intestinal inflammation (490), which is linked to HLA-B27 misfolding, leading to activation of the UPR in macrophages (491). Thus, the UPR pathway seems to play a key role in maintaining the homeostasis in the intestine, particularly with regard to Paneth cell and goblet cell function.

Autophagy is a catabolic process in which cytoplasmic elements are engulfed in a double membrane "autophagosome" and then degraded through the lysosomal pathway (492). Initially identified for its role in quality control and recycling of cytoplasmic proteins and organelles, autophagy is also essential in many immunological processes, such as engulfment of intracellular bacteria, antigen presentation and lymphocyte survival (493). Polymorphisms in two autophagy genes, autophagy-related protein 16L-1 (*ATG16L1*) and immunity-related GTPase family M protein (*IRGM*), are associated with susceptibility to CD (326, 328, 494). ATG16L1 is part of a group of proteins involved in the localization of the autophagy complex (492). The mutation associated with IBD does not affect basal autophagy, but it appears to specifically impair the handling of intracellular bacteria in IEC (495). Mice that are hypomorphic for Atg16l1 show defects in Paneth cell morphology and function, which parallel the PC alterations found in IBD patients bearing mutations in *ATG16L1* (496). Autophagy is also important in regulating bacterial handling in innate immune cells: Atg16l1-deficient macrophages over-produce IL-1β in response to LPS and mice with a specific Atg16l1 defect in the hematopoietic compartment are more susceptible to acute intestinal injury, a phenotype which can be reversed by

treatment with αIL-1β and αIL-18 antibodies (497). IRGM plays a key role in autophagic control of intracellular pathogens (498-500) and the mutations associated with IBD compromise its ability to coordinate anti-bacterial autophagy by changing IRGM expression levels (501). However, the exact role of IRGM in autophagy and intestinal homeostasis is still unclear.

1.8 Animal models of intestinal inflammation

The development of animal models of intestinal inflammation contributed tremendously to the understanding of the pathogenesis of IBD (502). Broadly, animal models of colitis can be divided into acute and chronic models. Acute models, such as DSS- or TNBS-induced colitis are based on the induction of epithelial injury in the large intestine and are particularly useful to dissect epithelial repair. Infection models, such as the *Citrobacter rodentium* model of disease, also focus on acute inflammation, but add a layer of complexity by analyzing the interplay between the host and a specific pathogen (503). Chronic models of disease better represent the complex inflammatory scenario found in IBD patients, although some features of IBD, such as its relapsing/remitting nature, are not reproduced in these models. In this thesis, different models of chronic inflammation were used to analyze specific aspects of intestinal immune homeostasis.

1.8.1 T cell transfer colitis

The normal naive T cell repertoire of healthy WT mice contains clones that are reactive against the commensal flora and self antigens (504). Accordingly, transfer of naive $CD4^+$ T cells from WT mice into immunodeficient $Rag1^{KO}$ or SCID mice results in systemic and intestinal inflammation (165, 166, 505). In this model, mice develop

severe inflammation in the colon and mild inflammation in the caecum, which is accompanied by severe weight loss, splenomegaly and liver inflammation (505) (**Fig.1-5**). The intestinal inflammation is often transmural and characterized by lymphocyte infiltration, goblet cell loss and hyperplasia of the intestinal epithelium (505). Disease is mediated by the activation and accumulation of T cells in the colon, MLN and systemic tissues of recipient mice (444). Inflammation is driven by a mixed Th1/Th17 response and it is dependent on the expression of IL-23 by innate immune cells (119, 444, 459, 506). T cell-transfer colitis can be prevented by the co-transfer of $CD4^+CD25^+$ regulatory T cells (165, 166, 471), which can also cure established disease (467), in a TGF-β dependent fashion (473, 474).

The T cell transfer model provides an extremely useful setting to study intestinal inflammation, since specific genes can be deleted either in the donor naive $CD4^+$ T cell population or in the recipient $Rag1^{KO}$ mice, allowing analyses of the specific contribution of different immune compartments. However, this model also has some drawbacks. First of all, following transfer into an immunodeficient mouse, naive $CD4^+$ T cells undergo extremely high homeostatic proliferation, which might skew the overall T cell repertoire towards highly reactive cells (507). Moreover, $Rag1^{KO}$ mice lack other immune cells that might contribute to intestinal inflammation, such as NKT cells, B cells and IEL. Although this allows a "reductionist" interpretation of experimental data, the contribution of these cell populations to intestinal inflammation cannot be studied in this model. Finally, although this model is dependent on the intestinal microflora (508, 509), little is known about the host-microbe interplay underlying intestinal inflammation.

1.8.2 *Models of Helicobacter hepaticus*-induced intestinal inflammation

H.hepaticus is one of a small group of bacteria which are directly linked to the development of chronic intestinal inflammation in susceptible mouse strains (367, 369, 510), providing a great opportunity to study how the host immune system responds to bacteria-driven changes in the intestinal microenvironment. As noted above, it has become apparent that innate immune pathways, such as NLR sensing or autophagy, play an essential part in the development of IBD (4). As T and B cell deficient 129SvEv *Rag2*^{KO} mice infected with *H.hepaticus* develop severe intestinal inflammation (369, 370), this model allows the investigator to focus on innate immune pathways in the development of chronic intestinal inflammation, in the absence of any contributions of adaptive immunity (**Fig.1-5**). *H.hepaticus*-induced innate intestinal inflammation occurs in the caecum and colon, and it is accompanied by systemic inflammation in the spleen and liver (369, 370). Pathology is characterized by high levels of IL-17A, TNF-α and IFN-γ and by the accumulation of pro-inflammatory granulocytes at the site of inflammation (444). Similarly to the T cell transfer model, intestinal inflammation is dependent on IL-23 (444) and can be prevented by transfer of $CD4^+$ $CD25^+$ regulatory T cells (511). Moreover, persistent infection leads to the development of colonic cancer, providing a useful model of inflammation-induced tumorigenesis (512). Whilst useful to specifically dissect the role of innate immune pathways in chronic intestinal inflammation, the innate *H.hepaticus* model of intestinal inflammation lacks the complexity associated with the presence of a normal immune system and understates the importance of adaptive immunity in the amplification of chronic inflammation.

Infection of WT mice with *H.hepaticus* does not result in intestinal inflammation, as protective regulatory responses develop in the intestine (193). However, it is possible to reveal the pathogenic potential of *H.hepaticus* infection in WT mice by blocking IL-10 signaling, as mice receiving a blocking αIL-10R mAb throughout the course of infection develop chronic intestinal inflammation following infection with *H.hepaticus* (**Fig.1-5**) (367, 438). Intestinal inflammation in this model involves both the colon and the caecum, where it is particularly severe, with the development of abscesses and ulceration. The development of inflammation is IL-23 dependent and correlates with the appearance of a mixed Th1/Th17 response and treatment with αIFN-γ mAb partially attenuated intestinal pathology (438). In spite of its advantages, this model is still poorly characterized and results are often difficult to interpret due to the complex interplay between different immune cell populations.

In summary, the combination of T cell transfer and *H.hepaticus*-induced IBD models permit thorough analyses of the effects of manipulations of different aspects of the immune system on the development and regulation of chronic intestinal inflammation. Crucially, these models are dependent on molecules such as TNF-α and IL-23, which are strongly implicated in IBD pathogenesis in humans. Hence, these chronic models of intestinal inflammation provide excellent tools to identify and validate potential therapeutic targets for IBD therapy.

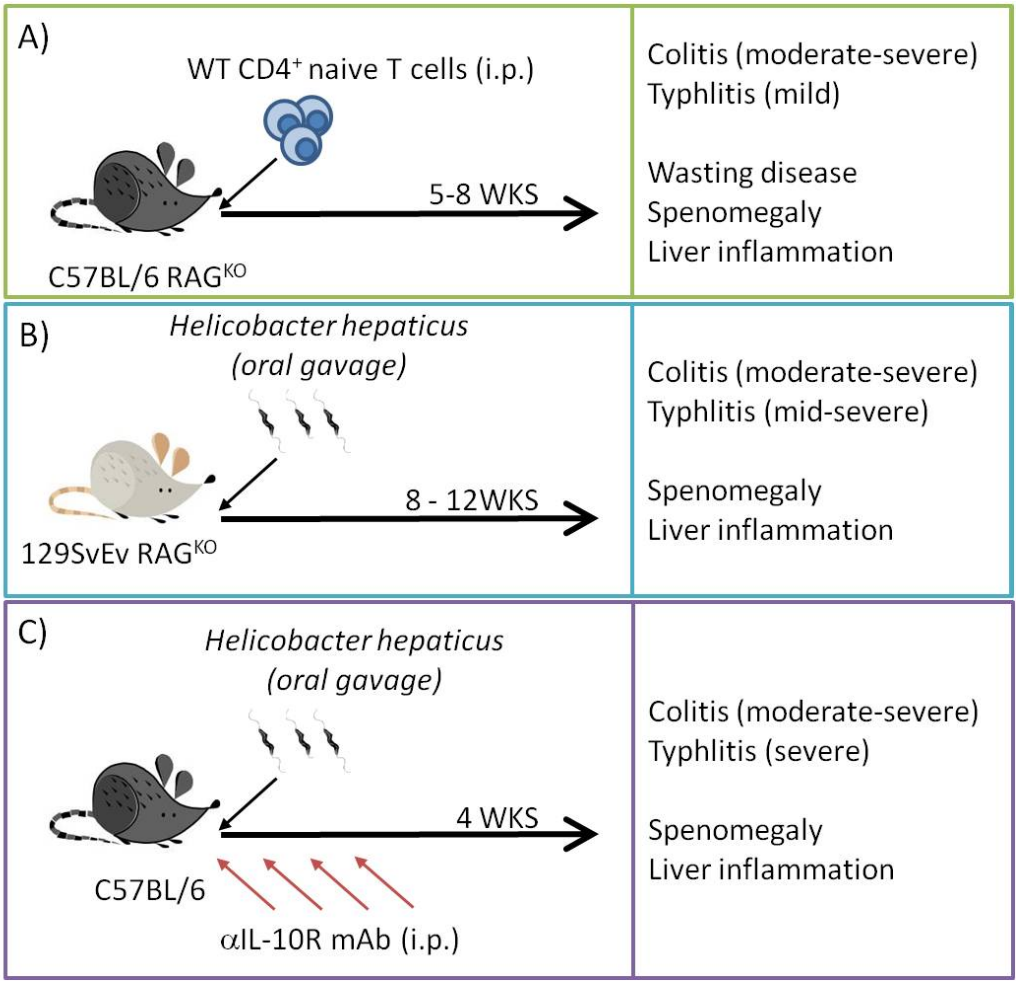


Figure 1-5. Schematic representation of mouse models of chronic intestinal inflammation employed in this thesis. A) T cell transfer colitis. B) *H.hepaticus*-induced innate immune-mediated typhlocolits. C) *H.hepaticus*-induced typhlocolitis in lymphocyte replete mice. WKS, weeks; i.p. intraperitoneally.

1.9 IL-1β and the IL-1 family

The IL-1 family is a group of 11 members mediating a vast array of inflammatory responses (**Table 1-3**). All members share a common gene structure and the genes encoding for IL-1 family cyotokines, with the exception of IL-18 and IL-33, are clustered on chromosome 2 (513). With the exception of IL-1RA (IL-1 receptor antagonist), all IL-1 cytokines lack a signal peptide for secretion and are translocated to the extracellular space by unconventional mechanisms, generally after processing by a converting enzyme (**Table 1-3**) (513). The receptors for IL-1 family members are also structurally related: they are characterized by extracellular immunoglobulin domains and intracellular Toll/IL-1 receptor (TIR) domains (514). In IL-1 family receptors, signaling is strongly homologous to TLR signaling and many responses mediated by IL-1 family receptors cannot be distinguished from TLR-mediated responses (**Fig.1-3**) (254). Most IL-1 family cytokines initially bind to a primary receptor subunit on the cell surface. However, a second subunit, or receptor accessory protein (RAP), has to be recruited for signaling, as the juxtaposition of two TIR domains is required for the recruitment of the adaptor protein MyD88 (**Fig.1-3**) (515). However, IL-1β can signal also independently of MyD88, as the activated IL-1RAP can interact with the regulatory p85 subunit of PI3K (phosphatidylinositol 3-kinase), triggering PI3K signaling, which results in activation of NF-κB and AKT (516, 517). Whilst the biological function of most IL-1 family proteins is unclear, IL-1α, IL-1β, IL-1RA, IL-18 and IL-33 have been extensively characterized (**Table 1-3**).

IL-1 family member	Receptor	Main processing enzyme
IL-1α	IL-1R1 + IL1RAP	Calpain
IL-1β	IL-1R1 + IL-1RAP	Caspase-1
IL-1RA	IL-1R1 + IL-1RAP	None
IL-18	IL-18R + IL-18RAP	Caspase-1
IL-33	IL-1RL1 (ST2) + IL-1RAP	?
IL-36RA	IL-1RL2 + IL1RAP	?
IL-36α	IL-1RL2 + IL1RAP	?
IL-36β	IL-1RL2 + IL1RAP	?
IL-36γ	IL-1RL2 + IL1RAP	?
IL-37	?	?
IL-1F10	?	?

Table 1-3. All known IL-1 family members are indicated, together with their receptors and main processing enzymes. Other enzymes might also contribute to the activation of IL-1 family members. R, receptor; RA, receptor antagonist; RAP, receptor accessory protein; RL, receptor like; ?, unknown. IL-1 family members are reviewed in (513).

IL-1α, IL-1β and IL-1RA are structurally related and all bind to IL-1R1(513). IL-1RA is an antagonist and its binding to IL-1R1 does not allow the recruitment of IL-1RAP, impairing any downstream signaling (518). As they bind to the same receptor, IL-1α and IL-1β mediate similar functions (513). However, they are differentially regulated and mediate different responses *in vivo*. IL-1α is translated into a precursor, which can be cleaved by the calcium-dependent cysteine protease calpain and secreted into the extracellular space (514). However, IL-1α processing is rare and IL-1α is seldom found extracellularly (519). Instead, active IL-1α is released by dying cells and it is involved in necrosis-induced inflammation (520, 521). Moreover, the precursor for IL-1α has a nuclear localization sequence and can act as an autocrine growth factor, particularly in endothelial cells and fibroblasts (522, 523).

IL-1β is also initially produced as a zymogen, but pro-IL-1β does not have any known functional properties (513). IL-1β transcription is tightly regulated, and it is induced by stimulation through TLR or IL-1R stimulation (considered the first signal required for IL-1β production) (524). As previously described, the IL-1β precursor is cleaved into its active form by caspase-1, following activation of the inflammasome by the NLR pathway (signal 2) (**Fig.1-6**) (525). IL-18 processing follows a similar pathway, although IL-18 release does not necessarily require a signal 1 as IL-18 is constitutively expressed in macrophages (526). Other proteases, such as proteinase-3, chymase and elastase can cleave pro-IL-1β into its active form in an NLR-independent fashion (527). The mechanisms for the secretion of IL-1β and IL-18 into the extracellular space are unclear (528), but proposed pathways include exocytosis of secretory lysosomes (529), shedding of membrane microvescicles (530) or exososomes (531), or translocation via membrane transporters, such as the ABC transporters (532).

IL-1β exerts a plethora of systemic and local effects (524). Once in the circulation, IL-1β can promote the synthesis of cyclooxygenase-2 (COX2) in the hypothalamic vascular network, inducing production of prostaglandin E2 in the brain and mediating fever (533). IL-1β also promotes the synthesis of acute phase proteins by hepatocytes, and induces macrophage differentiation and neutrophil differentiation and mobilization in the bone marrow (533-535). Moreover, IL-1β promotes recruitment of immune cells to sites of inflammation, by inducing the expression of adhesion molecules and chemoattractants by vascular endothelial cells (536-538). Locally, IL-1β modulates the function of many immune cells. IL-1 signaling on immune cells coordinates a complex pro-inflammatory programme, leading to the

up-regulation of numerous mediators of inflammation. Specifically, IL-1β promotes the expression of the inducible form of nitric oxide synthase (iNOS), COX2 and phospholipase A2 (PLA2) inducing the production of large amounts of PGE2, leukotrienes, nitric oxide (NO), platelet-activating factor and ROS (524, 539, 540). Moreover, IL-1β promotes cytokine secretion by DC and macrophages and promotes MHCII expression on APC (541-543). IL-1β also directly modulates adaptive immune responses, by promoting T cell proliferation (513), the maintenance of Th2 cells (544) and the differentiation of Th17 cells, which express high levels of the IL-1R (513, 545). Finally, IL-1β promotes B cell proliferation and T cell help, supporting class switching and T cell-dependent antibody responses (543, 546, 547).

IL-1RA is a natural antagonist for IL-1R, with no agonist activity (514). IL-1RA has a similar affinity for IL-1R as IL-1β or IL-1α, but a 100-fold molar excess of IL-1RA is required to inhibit IL-1β *in vitro* (548). The importance of IL-1RA in limiting IL-1 responses is exemplified by *Il1ra*^{KO} mice, which spontaneously develop inflammatory diseases, such as chronic inflammatory polyarthropathy (on the BALB/c background) (549) or inflammatory arterial disease (on the MFIx129 background) (550). Moreover, administration of IL-1RA is beneficial in a number of animal models of disease, including collagen-induced arthritis and streptozotocin-induced diabetes in mice (551). In addition to IL-1RA, other mechanisms are in place to limit IL-1β function, including the expression of IL-1R2, a scavenger receptor for IL-1 that lacks signaling activity (552). IL-1R2 is mainly expressed by IL-1 producing cells and limits autocrine IL-1 mediated activation (552). IL-1R2 and IL-1RAP also exist in a soluble form, which can sequester circulating IL-1 (552, 553). The importance of limiting IL-1

signaling is highlighted by the severe phenotype of patients with mutations in components of the inflammasome pathway, such as NLRP3 (554). These patients suffer from recurrent bouts of fever, rashes, urticaria, arthritis and, in the most severe forms, chronic aseptic meningitis (554). Moreover, loss-of-function mutations of IL-1RA result in severe systemic inflammation (318) (319). All these diseases can be successfully treated by administration of exogenous IL-1RA (anakinra), directly linking their pathogenesis with dysregulated IL-1β production (555).

IL-18 is expressed by dendritic cells, macrophages and epithelial cells (513). IL-18 acts together with IL-12 to induce IFN-γ expression by T cells, $CD8^+$ T cells, B cells and NK cells (556), and it was originally associated with Th1 responses. However, IL-18 can promote Th2 responses in the absence of IL-12 (557): hence IL-18 can be considered a general activator of helper T cell function. IL-18 also amplifies immune responses by promoting the expression of cytokines and chemokines by peripheral blood mononuclear cells and neutrophils (558, 559). Due to its high inflammatory capacity, mechanisms are in place to limit IL-18 activity *in vivo*, such as the expression of IL-18-binding protein (IL-18BP), a neutralizing high affinity inhibitor of IL-18 (514).

IL-33 was recently discovered as the ligand for the orphan receptor IL-1RL1 (ST2), which signals in association with IL-1RAP (560). IL-33 is produced as a precursor and, similarly to IL-1α, pro-IL-33 is biologically active as a transcription factor, particularly in high endothelial venules (561). Currently, it is not clear whether IL-33 requires cleavage to mediate its function or whether pro-IL-33 is found in the extracellular space. Similarly to IL-1RAP, a soluble form of ST2 can sequester IL-33 and inhibit its activity (562). IL-33 coordinates Th2 responses by recruiting and sustaining cytokine

production by Th2 cells (560, 563, 564) and by promoting the function and survival of eosinophils, mast cells and basophils (565-567). Accordingly, IL-33 is thought to be a key mediator of asthma (568).

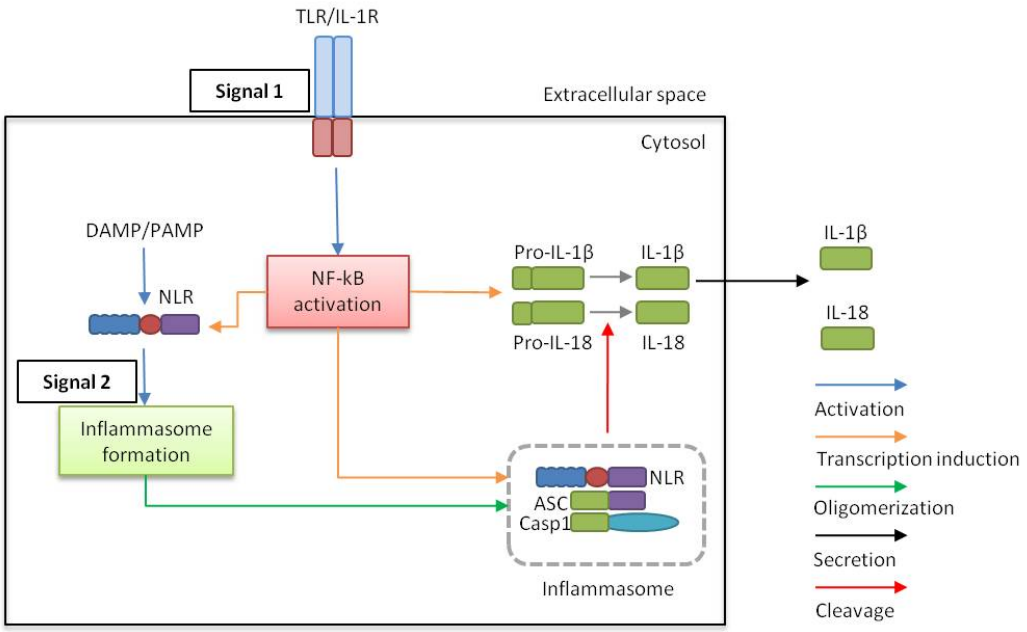


Figure 1-6. Signals required for IL-1β and IL-18 activation and secretion. The activation of IL-1β and IL-18 is dependent on two different signals. Signal 1 is delivered extracellularly by the activation of TLR or IL-1R, which triggers the activation of the NF-κB signaling pathway, resulting in up-regulation of the expression of NLR and of pro-IL-1β. Pro-IL-18 and other members of the inflammasome are constitutively expressed at high levels in macrophages. Signal 2 is delivered by the activation of intracellular NLR by PAMPs or DAMPs. Activation of NLR results in the formation of the inflammasome and activation of caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 into their active forms. The secretion of active IL-1β and IL-18 occurs by poorly characterized Golgi-independent mechanisms. The mechanisms of IL-1β activation are reviewed in (514).

1.9.1 IL-1β in intestinal inflammation

Several studies reported high levels of IL-1β production by lamina propria mononuclear cells from active colonic lesions in IBD patients (569-575). As the levels of IL-1RA are only modestly up-regulated in the colon of IBD patients, the ratio of IL-1RA to IL-1β is significantly decreased, promoting intestinal inflammation (574). Genetic data also support an association of an imbalance in the IL-1RA/IL-1β ratio in IBD. The gene for IL-1RA exists in different allelic variants, defined by the number of tandem repeats in intron 2 (576). An increase frequency of allele A2 (two tandem repeats) was found in small ethnically homogenous groups of IBD patients, such as Los Angeles-based Hispanic people or Jewish patients (577), although the association was not reported in other ethnic groups (578, 579). High levels of intestinal IL-1β are characteristic of many animal models of IBD and strategies blocking IL-1β were beneficial in models of acute intestinal inflammation in rats spontaneous colitis, rabbits immune complex colitis and in DSS-induced colitis in mice (580-582), whilst neutralization of IL-1RA exacerbated immune complex colitis in rabbits (583). Recent reports suggest that production of IL-1β might be a common effector mechanism activated by different CD-related pathways, as in animal models alterations of key pathogenic molecules such as NOD2 or ATG16L1 results in over-production of IL-1β by macrophages and increased susceptibility to acute intestinal injury (417, 497). Moreover, mice deficient in matrix metalloproteinase-7 (MMP7), which cannot produce mature cryptdins/defensins and are more susceptible to DSS-induced colitis, show higher levels of colonic IL-1β than WT mice during intestinal inflammation (584). However, recent data suggested that the association between CD and polymorphisms in *NLRP3* was linked with decreased production of IL-1β by blood

monocytes (320). Similarly, blood monocytes from patients bearing *NOD2* mutations produce less IL-1β following MDP stimulation than cells from healthy controls (407, 585, 586). These contrasting data might reflect differential functions of the IL-1β pathway in the intestine versus the systemic compartment, or differing requirements for IL-1β production between blood monocytes and tissue macrophages (587). Thus, although there is strong clinical and pre-clinical evidence that associates high levels of IL-1β with chronic intestinal inflammation, little is known about the cellular and molecular mechanisms underlying this association.

Other IL-1 family members have also been implicated in intestinal inflammation. GWAS studies identified an association of polymorphisms in the area encoding for IL18R1 and IL18RAP with susceptibility to both UC and CD, although other IL-1 family receptor such as IL-1RL1 are also present in this cluster (326, 588). IL-18 is expressed at high levels in the colons of IBD patients (589, 590), but IL-18BP is also strongly up-regulated, so the overall contribution of this cytokine to inflammation in humans is unclear (591). In mice, strategies blocking IL-18 were beneficial in the treatment of acute DSS- and TNBS-induced colonic injury (592-595) and in chronic T cell transfer colitis (596), suggesting a pathogenic role for this cytokine in intestinal inflammation. However, recent data challenge this hypothesis, as caspase 1-deficient mice developed worse colitis upon DSS administration, a phenotype which could be partially ameliorated by exogenous administration of IL-18 (597, 598). Hence, further analysis is required to fully understand the role of IL-18 in intestinal inflammation.

1.10 Aims of this thesis

The association of chronic intestinal inflammation in IBD patients with high levels of colonic IL-1β was established over 20 years ago (573). In spite of this, little research has been conducted on the relevance of IL-1β in chronic intestinal inflammation, hampering the investigation of this pathway as a potential therapeutic target. Since IL-1β has a large number of immunomodulatory effects, it is difficult to identify single cellular mechanisms underlying the overall effect of this pluripotent cytokine *in vivo*. To overcome this obstacle, I took advantage of the different models of chronic intestinal inflammation developed in our laboratory to analyze the effect of IL-1β stimulation on different branches of the intestinal immune system.

As IL-1β is key player in promoting innate immune effector functions, my first key aim was to analyze the effect of IL-1β on the intestinal innate immune system, by using a model of innate immune-mediated colitis.

Then, as growing evidence supports a role for IL-1β in effector T cell function, my next aim was to dissect the role of IL-1β and IL-1R signaling in chronic intestinal inflammation driven by T cells, using two complimentary models of chronic colitis.

Although genetic studies have strongly linked NLR polymorphims with IBD susceptibility, their role in role in experimental models of chronic intestinal inflammation has not been extensively investigated. Given its emerging importance in bacterial handling pathways, my final aim was to assess the role of NOD2 in different models of *H.hepaticus*-driven intestinal inflammation, focusing on the effects of NOD2 deficiency on host-microbe dynamics in the intestine.

Chapter 2: Materials and Methods

2.1 Mice

C57BL/6, 129SvEv, C57BL/6 *Rag1*^{KO}, 129SvEv *Rag2*^{KO}, C57BL/6 *Il1r1*^{KO}, B6.129 *Nod2*^{KO} and 129SvEv *Rag2*^{KO} *Nod2*^{KO} mice were bred in a specific-pathogen free (SPF) animal facility at the University of Oxford. C57BL/6 *Il1r1*^{KO} were a kind gift of Dr. Enzo Cerundolo and B6.129 *Nod2*^{KO} mice were from The Jackson Laboratory (Maine, USA). All mice were routinely tested for colonization by *Helicobacter* species and confirmed *Helicobacter*-free. 129SvEv *Rag2*^{KO} *Nod2*^{KO} mice were generated by back-crossing B6.129 *Nod2*^{KO} mice onto a 129SvEv *Rag2*^{KO} background using a speed congenic approach (599, 600). Thus, B6.129 *Nod2*^{KO} mice were crossed to 129SvEv *Rag2*^{KO} mice and each generation was screened using 63 polymorphic microsatellite markers to identify offspring with the highest proportion of 129SvEv congenic markers, which were used for subsequent backcrossing. At the F5 generation, when mice presented over 80% of 129SvEv congenic markers, *Rag2*^{KO} *Nod2*^{Het} mice were intercrossed, generating *Rag2*^{KO} *Nod2*^{KO} , *Rag2*^{KO} *Nod2*^{Het} and *Rag2*^{KO} *Nod2*^{WT}, which were used in *H.hepaticus* infection experiments.

2.2 Antibodies

Table 2-1 summarizes the antibodies used for *in vitro* and *in vivo* stimulation, cell purification, phenotypic analysis and *in vivo* neutralization. For *in vivo* experiments, all antibodies were certified to contain <0.1 EU endotoxin/milligram of protein by external contractors (Clinical BioManufacturing Facility, Oxford, UK, and Lonza, Basel, Switzerland).

Specificity	Clone	Fluorochrome	Concentration	Source
Cell Purification				
CD4	YTA 3.1	N/A	~ 10 µg/ml	In-house
CD4	GK1.5	N/A	~ 10 µg/ml	In-house
CD8	YTS169	N/A	~ 10 µg/ml	In-house
MHC II	TIB120	N/A	~ 10 µg/ml	In-house
Mac-1 (CD11b)	M1/70	N/A	~ 10 µg/ml	In-house
B220	RA36B2	N/A	~ 10 µg/ml	In-house
In vitro				
CD3	1-45-2C11	N/A	2.5-10 µg/ml	EBioscience
CD28	37.51	N/A	2.5-10 µg/ml	EBioscience
In vivo				
IL-10R	1B1.2	N/A	1 mg/week	In-house
IL-1β	N/A	N/A	1 mg/week	UCB Celltech
Isotype control	N/A	N/A		UCB Celltech
Surface Staining				
Fc Block (CD16/32)	93	N/A	5 µg/ml	EBioscience
CD4	RM4-5	PerCp/PE Cy5/APC	1 µg/ml	EBioscience
CD45.2	104	FITC	5 µg/ml	EBioscience
TCR-β	H57-597	PE/APC/PerCP	1 µg/ml	EBioscience
CD11b	M1/70	PerCp/APC	1 µg/ml	EBioscience
GR1	RB6-8C5	PE	0.5 µg/ml	EBioscience
CD11c	N418	APC	1 µg/ml	EBioscience
Thy1.2	30-H12	APC	1 µg/ml	Biolegend
CD25	7D4	Pe	1 µg/ml	EBioscience
CD45RB	16A	FITC	10 µg/ml	BD Biosciences
SCA-1	E13-161.7	FITC	5 µg/ml	EBioscience
SCA-1	D7	APC	2 µg/ml	EBioscience
CD40L	MR-1	Pe	2 µg/ml	BD Biosciences
OX40	OX-86	Bio	5 µg/ml	BD Biosciences
Streptavidin	N/A	Pe	0.5 µg/ml	BD Biosciences
Intracellular Staining				
IL-17A	TC11-18H10	PE/Alexa647	2 µg/ml	EBioscience
IFN-γ	XMG1.2	FITC/APC/eFluor450	2-5 µg/ml	BD Biosciences
Foxp3	FJK-16S	FITC/APC	2 µg/ml	EBioscience
Ki67	B56	FITC	N/A	BD Biosciences
Isotype Controls				
IgG1	R3-34	FITC/PE/APC	2-5 µg/ml	BD Biosciences
IgG1	P3.6.2.8.1	Alexa647	5 µg/ml	BD Biosciences
IgG1	B56	FITC	N/A	BD Biosciences
IgG2a	R35-95	FITC/PE/APC	2-5 µg/ml	BD Biosciences
IgG2a	N/A	eFluor450	2µg/ml	EBioscience
IgG2b	A95-1	PE	5 µg/ml	BD Bioscience

Table 2-1. List of antibodies used.

2.3 *Helicobacter hepaticus* culture and detection

2.3.1 *H.hepaticus* culture conditions

Helicobacter hepaticus NCI-Frederick isolate 1A (strain 51449; American Type Culture Collection) was grown on blood agar plates containing trimethoprim, vancomycin, and polymixin B (TVP, Oxoid) at 37°C in a microaerophilic environment (10%CO_2, 10% H_2, balance N_2) as previously described (369, 374). Alternatively, *H.hepaticus* was grown in microaerophilic conditions in Tryptone Soya Broth (TSB, Oxoid, Cambridge, UK), containing TVP and 10% FCS (Gibco, Invitrogen, California, USA). For liquid culture, bacteria were inoculated at 0.05 OD_{600nm} and cultured at 37°C, shaking at 150rpm.

H.hepaticus viability was confirmed using fluorescent microscopy with a bacterial live/dead kit (BacLight; Invitrogen). *H.hepaticus* was identified by morphology and by testing for urease activity, by adding 20-50μl of urease test solution (10mM KH_2PO_4, 10mM Na_2HPO_4, 14mM urea, 0.02% (w/v) yeast extract (Difco, Kansas USA), 0.002% (w/v) phenol red, pH6.8) to a cotton swab containing a sample of newly harvested bacteria. The test was considered positive for urease activity if the yellow substrate was converted into a pink product (601).

2.3.2 *Helicobacter* screening

Mice were regularly screened for colonization with *H.hepaticus* and other *Helicobacter* species. DNA was purified from stool samples using the Stool DNA Isolation Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Two separate PCR were performed, using the primers indicated in **Table 2-2**, targeting *H.hepaticus*-specific (p25) (602) or pan-*Helicobacter* genus-specific (16s rRNA) genes

(603). *H.hepaticus* genomic DNA, obtained from *H.hepaticus* cultures using DNeasy kit (Qiagen) according to the manufacturer's protocol, and stool DNA from *H.hepaticus*-infected mice were used as positive controls. PCR products were separated on a 1.5% agarose gel and mice were considered infected if a PCR amplicon of the appropriate length was detected.

Target	Primer name and sequence (5'-3')	PCR conditions	Amplicon length	Source
***H.hepaticus* p25**	p25F - ATG-GGT-AAG-AAA-ATA-GCA-AAA-AGA-TTG-CAA p-25R - CTA-TTT-CAT-ATC-CAT-AAG-CTC-TTG-AGA-ATC	94°C, 2min; [94°C, 1min, 55°C, 1min; 72°C, 1min (30 cycles)] 72°C, 10min.	705bp	(602), Sigma Aldrich
***Helicobacter* genus 16S rRNA**	C97 – GCT-ATG-ACG-GGT-ATC-C C98 – GAT-TTT-ACC-CCT-ACA-CCA	94°C, 2min; [94°C, 1min, 58°C, 90s; 72°C, 1min45s (35 cycles)] 72°C, 10min.	422bp	(603), Sigma Aldrich

Table 2-2. *Helicobacter* screening primers and PCR conditions.

2.3.3 Quantification of experimental H.hepaticus colonization.

At the termination of experiments involving infection with *H.hepaticus*, DNA was isolated from caecal contents using the Stool DNA Isolation Kit and colonization was quantified by q-PCR performed on a Chromo4 detection system (MJ Research, Essex, UK), using oligonucleotide primers and probes specific for the *H.hepaticus ctdB* gene (604) (indicated in **Table 2-5**). Colonization was determined by generating a standard curve by serial dilution of *H.hepaticus* genomic DNA and *H.hepaticus* colonization was calculated as [*H. hepaticus* DNA]/20 ng total caecal DNA. For quantification of *H.hepaticus* colonization in the stool, fresh fecal pellets were harvested in PBS and stored at -20°C. Stool DNA was isolated using Stool DNA kit (Qiagen) and samples were processed as above. For quantification of *H.hepaticus* colonization in the ileum, small pieces (~0.5cm) of terminal ileum were harvested from sacrificed mice and

snap-frozen in liquid nitrogen. DNA was extracted using the blood and tissue kit (Qiagen) and samples were processed as above.

2.4 Flow cytometry

2.4.1 Fluorescence activated cell sorting (FACS) antibodies

Monoclonal antibodies (mAb) used for FACS are described in **Table 2-1**. Stained cells were acquired on a Dako ATP Cyan (Beckton Dickinson, Oxford, UK) or on a FACS Calibur (BD Bioscience, Oxford UK) and analyzed using FlowJo (TreeStar Inc. Oregon, USA).

2.4.2 Surface staining

Single cell suspensions ($5x10^4$-$5x10^5$cells) were resuspended in 50µl of PBS (Gibco, Invitrogen) containing 0.1% BSA (Sigma Aldrich, Missouri, USA) in a 96-well plate and incubated with αCD16/32 mAb (Fc Block, Insight Biotechnology, Wembley, UK) for 15min at RT to block any non-specific binding of staining antibodies. Cells were washed by adding 200µl of PBS/0.1% BSA and centrifuging at 1350rpm (424g) for 5min. Then, cells were resuspended in PBS/0.1%BSA containing various combinations of fluorescently-labeled mAb and incubated for 20min at 4°C. After two rounds of washing in PBS/0.1% BSA, cells were resuspended in PBS/0.1% BSA for analysis or fixed in PBS/0.1% BSA containing 2% PFA (EMS, Pennsylvania, USA) or Fixation/Permeabilization buffer (eBioscience, California, USA) and maintained at 4°C protected from light for 12-48h before analysis.

For OX40 and CD40L staining, cells were restimulated in 96-well plate (round bottom) (Corning) for 3h at 37°C in 200µl RPMI 1640 (Gibco, Invitrogen) containing

10% FCS (Gibco, Invitrogen) 100 U penicillin/streptomycin (PAA, Pasching, Austria), 2mM L-glutamine (PAA), 0.05mM β-mercaptoethanol (BDH, VWR International, Pennsylvania, USA), supplemented with 0.1μg/ml PMA (Sigma Aldrich) and 1μg/ml ionomycin (Sigma Aldrich). CD40L-PE was added for the last 90min of culture and cells were then processed as described above.

2.4.3 Intracellular staining

Following surface marker staining cells were fixed in Fixation/Permeabilization Buffer (eBioscience) and stored ON at 4°C protected from light. Cells were centrifuged at 1600 rpm (596g), resuspended in 100μl of Permeabilization buffer (eBioscience) containing 2% normal rat serum (NRS) and incubated for 30-60min at 4°C protected from light. After two washes in Permeabilization buffer/2%NRS, cells were resuspended in 50μl of Permeabilization buffer containing an appropriate combination of the fluorescently-labeled anti-cytokine mAb indicated in **Table 2-1** and incubated at 4°C for 20min in the dark. Cells were washed twice in Permeabilization buffer and resuspended in PBS/0.1% BSA for acquisition.

For intracellular cytokine staining, fresh spleen, MLN or LPL cell suspensions were restimulated in 96 well plates (round bottom) (Corning) for 3h at 37°C 5% CO_2 in 200μl RPMI 1640/10% FCS containing 100U/ml penicillin/streptomycin, 2mM L-glutamine and 0.05mM β-mercaptoethanol, supplemented with 0.1μg/ml PMA, 1μg/ml ionomycin and 10μg/ml Brefeldin A (Insight Biotechnology, Wembley, UK). After 3h, cells were washed in PBS/0.1%BSA twice, cell surface markers were stained and cells were fixed as described above.

2.5 Cell isolation and sorting

2.5.1 Cell isolation from spleen and MLN

Spleen and MLN were reduced into single cell suspensions by forcing them through 70-100 µm cell strainers (Falcon, BD Biosciences). Cells were washed with ice cold PBS/0.1% BSA followed by centrifugation at 1350 rpm (424 g) for 5min. Erythrocytes were lysed by resuspending spleen cells in ACK lysis buffer (150 mM NH4CL, 10 mM, KHCO3, 0.1 mM NA2EDTA, pH 7.2-7.4) for 3min at RT, followed by washing in 15 ml PBS/0.1% BSA and filtering through a 100 µm cell strainer. Residual ACK was eliminated with a second washing step. Cells were then resuspended in ice cold PBS/0.1% BSA.

2.5.2 Isolation of colonic lamina propria leukocytes (cLPL)

Colons were cut open and any content was removed. Next, the colons were cut into small pieces (~0.5cm) and placed into a 50ml tube (Falcon, BD Biosciences) containing 10ml of ice cold PBS/0.1% BSA. Tubes were shaken to eliminate any remaining contents and supernatants were aspirated. Next, ~10ml of warm (37°C) RPMI 1640/10% FCS containing 5 mM EDTA (VWR International UK) was added to each sample and tubes were shaken (200rpm) for 15min at 37°C to remove epithelial cells. Supernatants were discarded and new RPMI/FCS/EDTA was added: this process was repeated twice more, for a total of three EDTA washes. Next, ~10ml of RPMI 10%FCS containing 15mM Hepes (PAA) was added to each sample to neutralize the EDTA activity. After aspirating the supernatants, 10ml of RPMI/10%FCS/15mM Hepes containing 0.2-0.3mg/ml type VIII collagenase (Sigma Aldrich) was added and samples incubated for 1h at 37°C with shaking (200rpm). Supernatants were

collected and filtered through a cell strainer (100 µm), topped up with cold PBS/0.1% BSA and resuspended in 10ml cold RPMI/FCS/EDTA to block collagenase activity. The collagenase digestion was repeated and the cells from the two digestions were pooled and washed with cold PBS/0.1% BSA. Cells were centrifuged and resuspended in 3ml 30% Percoll (GE, Amersham, UK) solution (P30). The cell suspension was overlaid over a discontinuous gradient comprising 3ml 75% Percoll solution (P75, bottom layer) overlaid with 4ml of 40% Percoll solution (P40, top layer), giving a P30/P45/P75 gradient. Gradients were centrifuged for 20min RT at 1800rpm, without any brake. Leukocytes were isolated from the P40/P75 interface, washed with PBS/0.1% BSA and resuspended in ice cold PBS/0.1% BSA.

2.5.3 Liver leukocytes isolation

At sacrifice, livers were perfused to eliminate circulating blood cells from the organ. Briefly, the mouse inferior vena cava was severed to create an outlet for the perfused PBS and 20-40 ml of PBS were injected through the heart. The gallbladder was removed and livers were cut into small pieces (~0.5cm) and placed in cold PBS/0.1% BSA. Supernatants were aspirated and 10ml of RPMI/10%FCS containing 0.2-0.3 mg/ml collagenase type VIII was added to each sample. Samples were then incubated at 37°C for 1h with shaking (200rpm). Cell suspensions and remaining fragments were forced through a 100 µm cell strainer, collagenase activity was blocked by adding ice cold RPMI/10%FCS/5mM EDTA and cells were washed in ice cold PBS/0.1% BSA. Cells were resuspended in 9ml of P30 and layered onto a discontinuous gradient of 9ml P75 (bottom layer) overlaid with 12ml P40 (top layer), to give a P30/P40/P75 gradient. Gradients were centrifuged for 20min at RT 1800rpm without braking and liver leukocytes were isolated at the P40/P75

interfaces. Following washing with PBS/0.1% BSA to eliminate residual Percoll, liver leukocytes were kept in ice cold PBS/0.1% BSA.

2.5.4 CD4+ T cell enrichment

Cell suspensions were prepared from spleen as described above. Cells were then incubated on ice for 20min with a panel of rat mAb against mouse surface markers αCD8, αMHCII, αCD11b, αB220 (**Table 2-1**). Cells were washed and resuspended in PBS/0.1% BSA at 10^8cells/ml. Anti-rat IgG-coated beads (Dynal, Invitrogen) were resuspended at 10^8beads/ml and combined with the cell suspension at a 1:1 ratio. The bead/cell mixture was then incubated for 20min at 4°C with constant rotation. A magnet (Dynal) was applied to deplete bead-bound cells and the remaining cell suspension was collected into a fresh tube. Cell suspensions were washed on ice cold PBS/0.1% BSA and resuspended in ice cold PBS/0.1% BSA. Typically, harvested cells were ~75% $CD4^+$ cells.

2.5.5 Purification of $CD4^+$ $CD45RB^{Hi}$ naïve T cells

$CD4^+$ T enriched spleen cells, prepared as described above, were resuspended at 10^8cells/ml and stained with fluorescently-labeled αCD4, αCD25 and αCD45RB mAb (Table 2.1) as described in section 2.4.2. Stained cells were washed in PBS/0.1% BSA and resuspended at $5x10^7$ cells/ml. After filtering, naïve T cells were purified as $CD4^+CD25^-CD45RB^{Hi}$ (471) using a MoFLo cell sorter (Dako Cyomation, Beckton Dickinson), typically to ≥98% purity.

2.5.6 Isolation of innate populations from colonic lamina propria

Lamina propria leukocytes were isolated from the colon of colitic mice or healthy controls as described in section 2.5.2 above. Cells were resuspended at 10^8cells/ml

and stained with different panels of mAb. For isolation of "conventional" innate lamina propria leukocytes, cells were stained with fluorescently-labeled mAb αCD45.2, αCD11b, αCD11c and αGr1 (**Table 2-1**) as described in section 2.4.2. Dendritic cells ($CD45^+CD11c^+CD11b^-Gr1^-$), inflammatory monocytes/macrophages ($CD45^+CD11c^+CD11b^+Gr1^-$ or $CD11c^+CD11b^+Gr1^+$) and granulocytes ($CD45^+CD11c^-CD11b^+Gr1^+$) were FACS-sorted using a MoFLo cell sorter (Dako Cyomation, Beckton Dickinson). For isolation of innate lymphoid cells (ILC), LPL were stained with fluorescently-labeled αCD45.2, lineage marker antibodies (*lin*) αSca-1 and αThy1, as described in section 2.4.2. Cells were washed, resuspended at $5x10^7$cells/ml and sorted on a MoFLo cell sorter (Dako Cyomation, Beckton Dickinson), typically to ≥98% purity. ILC were identified as $CD45^+$ *lin*$^-$Sca-1^+ and $Thy1^{Hi}$ (51).

2.6 Induction of intestinal inflammation

2.6.1 Induction of innate immune-mediated colitis by infection with *H.hepaticus*

129SvEv *Rag2*^{KO} or 129SvEv *Rag2*^{KO} *Nod2*^{KO} or 129SvEv *Rag2*^{KO} *Nod2*^{Het} mice were infected on three consecutive or alternate days with 0.5-1 x 10^8 CFU *H.hepaticus* using an oral gavage needle (22G) (Popper and Sons, Virginia, USA), as described previously (369). In all experiments, mice were infected for a minimum of 8 weeks.

At sacrifice, samples for cellular isolation (spleen, MLN, liver, LPL) were collected in ice cold PBS/0.1% BSA. Blood was collected by post-mortem cardiac puncture, placed in a BD Microtainer (BD Bioscience) for at least 30min, then centrifuged for 2min at 6500rpm (4000g), to separate the serum, which was then stored at -20°C. The caecum was cut open and the caecal contents were collected in PBS/0.1%BSA.

Colonic, caecal and hepatic samples were fixed in PBS containing 3.6% PFA for histological analysis as described in section 2.7.1. Sections of proximal, mid and distal colon, caecum, liver and spleen were snap-frozen in liquid nitrogen for RNA or protein isolation.

2.6.2 Induction of colitis in C57BL/6 mice by infection with *H.hepaticus* and αIL-10R treatment.

C57BL/6 WT or *Il1r1*^{KO} mice were infected with *H.hepaticus* as described in section 2.6.1 above. On the day of infection. and once a week throughout the course of the experiment, mice were injected i.p. with 1mg of αIL-10R mAb (**Table 2-1**), as described previously (438). After 4 weeks, mice were sacrificed and processed as described in section 2.6.1.

2.6.3 T cell transfer colitis

Naive CD4^{+} T cells were isolated as described in section 2.5.4 and injected i.p. into C57BL/6 *Rag1*^{KO} mice (4x10^{5} cells/mouse), as described previously (605). Mice were monitored weekly for weight loss and clinical signs of disease (i.e. loose feces) and animals were culled at the development of severe weight loss (10-20% of initial body weight) or when clinical symptoms of colitis became apparent (605). In some experiments, animal were culled at earlier time points, i.e. 2 weeks. At sacrifice, animal tissues were processed as described in section 2.6.1.

2.6.4 Modulation of colitis with a blocking αIL-1β antibody.

In some experiments, mice that had been subjected to one of the colitis induction protocols described above were injected i.p. with 1mf of a blocking αIL-1β mAb or

isotype control (**Table 2-1**) on day 0 of the experiment and once a week throughout the duration of the experiment.

2.6.5 Modulation of innate colitis with caspase-1 inhibitors

In some experiments, orally available caspase-1 inhibitors ((606)**Table 2-3**) (Vertex Pharmaceuticals, Abingdon, UK) were administered by oral gavage through a 22G feeding needle to *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice beginning on day 4 post-infection and continuing daily or twice a day for 8 weeks (**Table 2-3**). Caspase-1 inhibitors were prepared in indicated vehicles and vehicle-only control groups were included in the experiments.

Caspase-1 inhibitor	Vehicle	Dosage
VX-765	25% Cremophor EL	200mg/kg, once/day
VRT-761157	30% Polyethylene Glycol-400-70% 50 mM sodium citrate buffer	100mg/kg, twice/day

Table 2-3. Caspase-1 inhibitors, with their vehicles and dosage.

2.7 Assessment of intestinal and systemic inflammation

2.7.1 Assesment of intestinal inflammation

At sacrifice, sections of proximal, mid and distal colon, caecum and liver were rinsed in PBS/0.1% BSA and fixed in PBS/3.6% PFA. Sections were embedded in paraffin, cut at 4μm thickness and stained with hematoxylin and eosin. As described previously (459), intestinal inflammation was scored in a blinded fashion on a scale of 0-12 according to the parameters indicated in **Table 2-4**. Scores for colon sections were averaged together to give the colon inflammation score. Micrographs were acquired on a CoolScope Microscope (Nikon, Tokyo, Japan).

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 leucos 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

0 None

1 Submucosal inflammation OR Few crypt abscesses (<5)

2 Submucosal inflammation AND Few Crypt Absecesses (<5)

2 Many crypt abscesses (<5) OR Extensive submucosal inflamm OR Crypt branching (*Hh)*

3 Many crypt abscesses (<5) AND Extensive submucosal inflamm / Crypt branching (*Hh)*

3 Ulceration OR Extensive fibrosis

Table 2-4. Criteria used for the quantification of intestinal inflammation, based on the scoring system published in (459)

2.7.2 Assessment of liver inflammation

At sacrifice, a small piece of liver (~1cm) was harvested from the left lobe, rinsed in PBS/0.1% BSA and fixed in PBS/3.6% PFA. The tissue was embedded in paraffin, cut at 4μm in thickness and stained with H&E. Images were captured using a Coolscope Microscope (Nikon).

2.7.3 Assessment of splenic inflammation

At sacrifice, spleens were harvested in 1ml PBS/0.1% BSA and kept on ice. Spleens were weighed on an analytical balance and cell number was determined following generation of cell suspensions as described in section 2.5.1.

2.8 Analysis of gene expression by qPCR

2.8.1 RNA isolation

The RNAeasy mini kit was used to isolate total RNA from snap-frozen pieces of tissue or cell suspensions. Tissue samples were homogenized in a FastPrep™ 24 homogeniser (MP Biomedicals, Illkirch, France) in 700 µL of Buffer RLT (Qiagen) supplemented with 2-mercaptoethanol (2 rounds of homogenization at 4.0 M/s for 40s, using lysing matrix D beads (MP Biomedicals)). Following centrifugation at 13000 rpm (~16000 g) for 5 minutes, supernatants were removed and RNA isolated according to the manufacturer's instructions, including a DNAse digestion step to reduce contamination by genomic DNA. RNA concentration was determined using a NanoDrop (Thermo Scientific) and RNA was stored at -80°C. cDNA was retro-transcribed from RNA using Superscript III reverse transcriptase (Invitrogen) and Oligo dT primers (Invitrogen), according to the manufacturer's protocol.

2.8.2 Quantitative PCR (qPCR)

qPCR were performed using either SyBR Green or Taqman primers, as indicated in **Table 2-5.** For Taqman primer sets, probes were labeled with the fluorophores/quencher pair FAM/TAMRA. Where possible, primers were designed not to amplify genomic DNA and a RNA sample which was not reverse transcribed was always run as a control. For each sample, 4.0 µL of cDNA was assayed in

triplicate in a final reaction volume of 20.0 µL. For Taqman reactions, 300 nmol unlabelled primers and 100 nmol probe were used and fluorescence was detected using a Chromo4 detection system (MJ Research). For SyBR Green, primers were purchased from Qiagen and used according to manufacturer's instructions. Gene expression levels for each sample were normalized to HPRT expression. Mean relative gene expression was determined and differences in expression levels were calculated using either the $2^{-\Delta C(t)}$ or $2^{-\Delta\Delta C(t)}$ method (607).

Primer name	Sequence	Source	Method
HPRT Forward (F)	GAC-CGG-TCC-CGT-CAT-GC	(608), Sigma Aldrich	Taqman
HPRT Reverse (R)	TCA-AAC-CTG-GTT-CAT-CAT-CGC	(608), Sigma Aldrich	Taqman
HPRT Probe (P)	ACC-CGC-AGT-CCC-AGC-GTC-GTC	(608), Sigma Aldrich	Taqman
GM-CSF F	N/A	Applied Biosystems	Taqman
*cdt*B Reverse	TCG-TCC-AAA-ATG-CAC-AGG- TG	(604), Sigma Aldrich	Taqman
*cdt*B Forward	CCG-CAA-ATT-GCA-GCA-ATA-CTT	(604), Sigma Aldrich	Taqman
*cdt*B Probe	AAT-ATA-CGC-GCA-CAC-CTC-TCA-TCT-GAC-CAT	(604), Sigma Aldrich	Taqman
IL-1β	N/A	Qiagen	SYBR Green
NLRP3	N/A	Qiagen	SYBR Green
NOD1	N/A	Qiagen	SYBR Green
NOD2	N/A	Qiagen	SYBR Green
IPAF	N/A	Qiagen	SYBR Green

Table 2-5. qPCR primers

2.9 Measurement of antibody response

2.9.1 sHelAg preparation

Soluble *H.hepaticus* antigen (sHelAg) was prepared as described (367). Briefly, *H.hepaticus* was harvested from liquid culture and washed twice in PBS. The bacterial suspension was lysed by repeated (5x) cycles of freeze/thaw, followed by sonication (7x30sec, 10μm, with 1 minute resting on ice between cycles). The bacterial lysate was cleared of debris by centrifugation at ~16000g for 10min at 4°C and the supernate harvested and filtered through a 22μm filter (Qiagen). Protein concentration was determined using a Bradford assay as described in section 2.11.2 below, and sHelAg was stored at -20°C.

2.9.2 Intestinal lavages

For collection of intestinal IgA, small intestines were removed and flushed through with PBS/50mM EDTA containing EDTA-free protease inhibitor (Roche, Basel, Switzerland, 1 tablet/7ml). The resulting lavages were centrifuged at 3000rpm (~2000g) for 10min at 4°C, 3 ml of supernatant was collected and 60μl of PMSF (100mM) (Pierce, Thermoscientific, Massachusetts, USA) was added to the suspension. After further centrifugation at ~16000g for 10min at 4°C the clarified supernatants were collected and 1mM PMSF, 0.01% NaN3 and 5% FCS were added. Lavages were stored at -20°C.

2.9.3 Detection of *Helicobacter hepaticus*-specific antibodies

H. hepaticus-specific IgG and IgA antibodies were detected by ELISA. High Binding 96-well ELISA plates (Costar, Sigma Aldrich) were coated with sHelAg (10μg/ml, 50μl/well) in 0.1M carbonate buffer (0.1 M Na_2CO_3 0.1 M $NaHCO_3$, pH9.5) at 4°C

O/N. After extensive washing with PBS/0.05% Tween-20 (Sigma Aldrich), wells were blocked with PBS/5% BSA for 2h at RT and serial dilutions of serum samples were added to the wells (initial dilution 1/100 for serum, neat for intestinal lavages). After O/N incubation at 4°C and extensive washing, biotinylated goat-anti-mouse IgG (Vector Laboratories, Peterborough, UK) was added to the wells (1:500 dilution) and incubated for 1h at RT. After extensive washing, peroxidase-conjugated AvidinD (Vector Laboratories) was added to the wells (1:1000 dilution) and incubated for 1h at RT. For IgA detection in intestinal lavage samples, peroxidase-conjugated goat-anti-mouse IgA (Zymed, Invitrogen) was added directly to the wells (dilution 1:500), with no Biotin-Streptavidin amplification step. Color reactions were developed by adding 2,29-azinobis(3-ethylbenzthiazolinesulfonicacid) (ABTS) substrate (Sigma Aldrich), and OD_{405} was measured with an ELISA plate reader (Molecular Devices, California USA).

2.10 *In vitro* cell culture and cytokine secretion assays

2.10.1 *Ex vivo* cell culture

MLN, spleen and LPL cells, isolated as described in section 2.5.1, were cultured O/N at 37°C 5%CO_2 in RPMI/10% FCS/100 U/ml penicillin/streptomycin/2mM L-glutamine/0.05mM β-mercaptoethanol in flat bottom 96 well plates (Corning). After culture, supernatants were harvested for cytokine analysis and cells were lysed for RNA isolation in Buffer RLT containing 1% β-mercaptoethanol. For ILC functional assays, IL-23 was added to LPL cultures at 10ng/ml (R&D Systems, Minnesota USA).

2.10.2 *Ex vivo* organ culture

For organ culture, freshly isolated explants from the mid colon, caecum and terminal ileum of colitic mice or healthy controls were harvested and weighed. After rinsing in PBS/0.1%BSA/100U/ml penicillin/streptomycin, organ explants were cultured O/N at 37°C 5%CO_2 in RPMI/10% FCS/100 U/ml penicillin/streptomycin/2mM L-glutamine/0.05mM β-mercaptoethanol, in 48-well plates. Supernatants were harvested for cytokine analysis.

2.10.3 Infection of bone-marrow derived macrophages.

For derivation of macrophages from the bone marrow (BMDM), tibiae and femurs were isolated from 129SvEv mice and the bone marrow was flushed from them with PBS/0.1%BSA using a 26G needle. Bone marrow cells were dispersed by passing through a 19G needle and then washed in PBS/0.1% BSA. Cells were resuspended in RPMI/10% FCS/100 U/ml penicillin/streptomycin/2mM L-glutamine containing 15% L292-cell conditioned media and cultured at 37°C 5%CO_2 in 15cm bacterial plastic Petri dishes (Falcon, BD Bioscience). New medium was added on day 3 and medium was completely changed on day 6. On day 7, BMDM were detached using PBS/10mM EDTA containing 4mg/ml lidocaine (Sigma Aldrich). BMDM were then plated at 10^6cells/well in a 24-well plate and infected with *H.hepaticus* at a multiplicity of infection (MOI) of 15, and cultured O/N at 37°C 5%CO_2 in Optimem (Invitrogen). Supernatants were harvested for cytokine analysis and western blot.

2.10.4 Quantification of cytokine levels

Cytokine levels in culture supernatants were analyzed using FlowCytomix Bead Assay (EBioscience), according to manufacturer's instructions. Data were acquired on a

FACSCalibur flow cytometer and analyzed using FlowCytomix Pro Software (EBioscience). Data were normalized to cell number or to the weight of the organ explants. Levels of IL-1β secretion in organ explant supernatants were analyzed using Bio-Plex Bead Assay (Bio-Rad, California USA) and normalized by weight.

2.10.5 *In vitro* Th17 cell differentiation

For *in vitro* differentiation of Th17 cells from total $CD4^+$ T cells, $CD4^+$ T cells were enriched from the spleen as described in section 2.5.1 and cultured in 96-well plates at 37°C 5%CO_2 in RPMI/10% FCS/100 U/ml penicillin/streptomycin/2mM L-glutamine/0.05mM β-mercaptoethanol supplemented with TGF-β (2.5ng/ml) and IL-6 (50ng/ml) (both from R&D systems) plus plate bound αCD3 (2μg/ml) and αCD28 (1μg/ml) mAb (**Table 2-1**).

For differentiation of Th17 from naïve $CD4^+$ T cells, $CD4^+CD45RB^{Hi}$ T cells were FACS-sorted and cultured in 96-well plates in IMDM/10% FCS/100 U/ml penicillin/streptomycin/2mM L-glutamine/0.05mM β-mercaptoethanol in the presence of IL-1β (20ng/ml) and IL-23 (50ng/ml) plus plate bound αCD3 (2μg/ml) and αCD28 (1μg/ml). After 3 days of differentiation, cells were washed twice in PBS/0.1% BSA and analyzed as described in section 2.4.

2.10.6 Apoptosis assay

To evaluate apoptosis *in vitro*, CD4-enriched splenocytes prepared as described in section 2.5.1, were cultured in 96-well plates in RPMI/10% FCS/100 U/ml penicillin/streptomycin/2mM L-glutamine/0.05mM β-mercaptoethanol, in the presence of plate bound αCD3 mAb and αCD28 mAb (both from eBioscience) at 2.5-10μg/ml. After 3 days, cells were harvested and apoptosis was evaluated by staining

with the phosphatidyl serine binding protein Annexin V and the vital dye 7AAD (both from eBioscience) (609). Briefly, cells were washed extensively in cold PBS and resuspended in Binding Buffer (eBioscience), containing 2.5μl of Annexin V APC and 1μl of 7-AAD. The samples were gently vortexed and incubated for 15min at RT in the dark. Each sample was diluted in ~300μl of Binding buffer and promptly analyzed by FACS. Splenocytes left O/N at RT were used as a positive control for apoptosis.

2.11 Western blot

2.11.1 Preparation of colon samples

Upon termination of experiments, small sections of proximal, mid and distal colon were snap frozen in liquid nitrogen and stored at -80C. Samples were placed in tubes containing lysing matrix D beads and 500 μL of PBS containing protease inhibitors (Roche) and homogenized in a FastPrep™ 24 homogeniser (MP Biomedicals) (2x 4.0 M/s for 40sec). Following centrifugation at 13000 rpm (~16000g) to remove cellular debris, protein homogenates were aliquoted and stored at -20°C.

2.11.2 Protein quantification

Protein concentration was determined by Bradford Assay (Bio-Rad). Protein Assay solution was diluted according to manufacturer's instructions and 180μl of diluted Protein Assay solution was added to 20μl of each protein sample. A standard curve was created by 2-fold serial dilution of a 1mg/ml solution of BSA in PBS. Colour reactions were detected by reading OD_{600nm} on an ELISA reader.

2.11.3 Protein precipitation

Supernatants were concentrated with methanol/chloroform, as previously described (299). Briefly, an equal volume of methanol and 0.25 volumes of chloroform were mixed thoroughly with supernatants, followed by centrifugation at 13000rpm (16000g) at 4°C. The upper layer was discarded and the protein interface was washed by the addition of 500μl of methanol. Samples were centrifuged at 13000rpm (16000g) at 4°C for 20min, supernatants discarded and the protein pellet was air-dried. Protein samples were resuspended in Lane Marker Reducing Sample Buffer (Thermoscientific), boiled at 95°C for 5min and loaded on SDS-PAGE gels.

2.11.4 Western blot

For IL-1β detection, equal amounts of protein or supernatants were loaded on a 4-12% NuPage gel (Invitrogen) and separated by electrophoresis at 200V. For caspase-1 detection, samples were separated on 15%SDS-PAGE gels and separated by electrophoresis at 140V. Samples were transferred by wet transfer at 50V to a PVDF membrane (GE-Healthcare), that had been pre-activated in methanol. For caspase-1 blots, the membrane was then fixed by washing for 20min in PBS/0.5% PFA. Membranes were blocked with PBS/0.5% Tween-20 containing 5% milk powder (source), rinsed in PBS/0.5% Tween-20 and incubated O/N at 4°C with primary antibody: goat anti-mouse IL-1β (for supernatants, R&D 1:1000), rabbit anti-mouse IL-1β (for colon lysates, Cell Signaling 1:1000) rabbit anti-mouse caspase-1 (Santa Cruz Inc., 1:800) or rabbit anti-mouse β-actin (Cell Signaling 1:2000). After extensive washing in PBS/0.5%Tween-20, membranes were incubated for 2h at RT or O/N at 4°C with peroxidase-conjugated secondary antibodies: anti-goat IgG (R&D, 1:10000)

or anti-rabbit IgG (Dako, 1:2000). After extensive washing, blots were developed using ECL-plus (GE-Healthcare, for IL-1β blots) or Super Signal West Femto (Pierce, for caspase-1 blots). Images were captured by chemilluminescence on X-ray films (Fujifil Medical Systems, Bedford UK), which were developed using a RP X-OMAT developer (Kodak Limited, Herts, UK).

2.12 Statistics

All *in vivo* data were analyzed using Mann-Whitney test, as these variables are not normally distributed (610). All *in vitro* assays were analyzed using unpaired Student t-test (610). All analyses were performed using Prism (Graphpad Software, California USA). Differences were considered statistically significant if $p<0.05$.

Chapter 3: The role of IL-1β in *H.hepaticus*-induced innate intestinal inflammation

3.1 Introduction

IL-1β is a pro-inflammatory cytokine primarily produced by innate leukocytes that has a wide range of systemic and local effects. IL-1β was initially identified as the main inducer of fever and has a plethora of systemic effects, including the induction of acute phase protein secretion by hepatocytes (533). In the bone marrow, IL-1β promotes the differentiation of macrophages and the mobilization of neutrophils (514, 611). In peripheral tissues, IL-1β promotes leukocyte extravasation and migration by up-regulating the expression of adhesion molecules and chemokines on endothelial cells (536-538), and also activates dendritic cells, macrophages and neutrophils (524).

The potent inflammatory activity of IL-1β is reflected by the tight mechanisms in place to regulate its secretion. IL-1β is translated as an inactive 31kDa precursor (pro-IL-1β) following TLR stimulation, which is cleaved into its activated 17kDa form by caspase-1, also known as interleukin-1β converting enzyme (ICE) (612, 613). Activation of caspase-1 relies on the formation of a multimolecular scaffold known as the inflammasome, which is triggered by the activation of intracellular NOD-like receptors (NLR) by endogenous or exogenous danger signals (282, 614). Upon secretion into the extracellular space, IL-1β binds and signals via the IL-1R1, which is expressed on a wide range of cell types, including epithelial and endothelial cells, hepatocytes and innate and adaptive leukocytes (513). Regulatory mechanisms downstream of IL-1β secretion are also in place to limit the action of IL-1β, including

the production of a natural antagonist for its receptor, IL-1RA, and the expression of a decoy receptor, IL-1R2 (524).

Many clinical studies have reported high levels of IL-1β secretion by colonic lamina propria monocytes from IBD patients (569-573). High levels of IL-1β were associated with active lesions (574, 575), suggesting an important role of this cytokine in promoting localized inflammation. High levels of colonic IL-1β are also a feature of many animal models of colitis (580, 581, 615) and treatment with IL-1 blocking agents has been successful in ameliorating acute models of intestinal injury and inflammation (616-618). Furthermore, different genetic lesions associated with IBD development result in increased IL-1β in animal models. For example, macrophages from "knock-in mice" bearing the most common CD-associated variant of the *Nod2* gene produced high levels of IL-1β following stimulation with muramyl dipeptide (MDP) (417). These NOD2 mutant mice also developed exacerbated disease in response to acute dextran sulphate sodium (DSS)-induced intestinal injury, which was significantly ameliorated by the administration of recombinant IL-1RA (417). In addition, conditional deletion of the CD-linked autophagy gene *Atg16l1* in the haematopoietic system of mice resulted in increased IL-1β production following LPS stimulation and increased susceptibility to DSS-mediated intestinal injury, a phenotype reversed by co-treatment with αIL-18 and αIL-1β antibodies (497).

The role of caspase-1 in intestinal inflammation is still developing. Early work revealed a pathogenic role for caspase-1 in acute intestinal inflammation: *casp1*^{KO} mice failed to develop DSS-induced colitis (618) and caspase-1 inhibitors ameliorated DSS-colitis *in vivo* (619, 620). More recent publications reported that *casp1*^{KO} mice

were more susceptible to DSS-colitis (597, 598, 621). McAlindon and colleagues reported increased levels of expression of caspase-1 in macrophages isolated from patients suffering from IBD, suggesting that caspase-1 might be involved in disease pathogenesis (571).

To assess the role of IL-1β and caspase-1 in modulating innate leukocyte function during chronic intestinal inflammation, we used a model of *H.hepaticus*-induced colitis where pathology is entirely mediated by innate immune mechanisms.

3.2 Results

3.2.1 IL-1β plays a key role in innate intestinal inflammation.

Infection of T and B cell-deficient 129SvEv *Rag2*^{KO} mice with the Gram-negative intestinal bacterium *Helicobacter hepaticus* results in the development of chronic inflammation in the caecum and colon (369, 512). Initially, we assessed whether *H.hepaticus*-induced intestinal inflammation was associated with increased levels of IL-1β. Indeed, qPCR analysis of colonic tissue samples from *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice revealed that the induction of colitis was associated with increased levels of IL-1β mRNA (**Figure 3-1A**). However, because IL-1β is translated into an inactive precursor, which requires cleavage by activated caspase-1 for secretion (514), we measured the levels of IL-1β secretion by organ cultures from the gastrointestinal tract. Intestinal inflammation in the colon and caecum of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice was strongly associated with increased secretion of IL-1β compared to uninfected controls (**Fig.3-1B**). By contrast, no difference in IL-1β levels was observed in the ileum of *H.hepaticus* infected 129SvEv

Rag2^{KO} mice compared to uninfected controls (**Fig.3-1B**). Considering that *H.hepaticus* infection induces inflammation primarily in the colon and caecum, this result suggests that IL-1β secretion is directly linked to local intestinal inflammation. In IBD patients, innate leukocytes are mainly responsible for IL-1β secretion in the lamina propria (571, 573). Indeed we observed high levels of IL-1β secretion by purified colonic lamina propria leukocytes (cLPL) of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice (**Fig.3-1C**). To further confirm the presence of active IL-1β in the inflamed colon we performed western blot analysis of total colon homogenates, which revealed increased levels of both pro-IL-1β (31kD) and active IL-1β (17kD) in the colons of *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice (**Fig.3-1D**). Moreover, we also observed an increase in the ratio between active and pro-IL-1β in *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice (data not shown). To identify the innate sources of IL-1β in the colonic lamina propria, we used cell sorting to isolate different populations of cLPL from *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice (**Fig.3-2A**). Thus, dendritic cells ($CD11c^+CD11b^-Gr1^-$), inflammatory monocytes/macrophages ($CD11c^+CD11b^+Gr1^-$ or $CD11c^+CD11b^+Gr1^+$) and granulocytes ($CD11c^-CD11b^+Gr1^+$) were FACS-sorted from cLPL and cultured O/N. Analysis of IL-1β secretion revealed that, with the exception of $CD11c^+CD11b^-Gr1^-$ dendritic cells, all innate inflammatory populations examined contributed to IL-1β secretion in the colon of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice (**Fig.3-2B**). We were unable to isolate sufficient innate immune cells from the colon of uninfected 129SvEv *Rag2*^{KO} mice to perform a similar analysis in steady state conditions. Taken together, these data show that *H.hepaticus* induced intestinal inflammation is associated with increased IL-1β secretion by innate leukocyte populations.

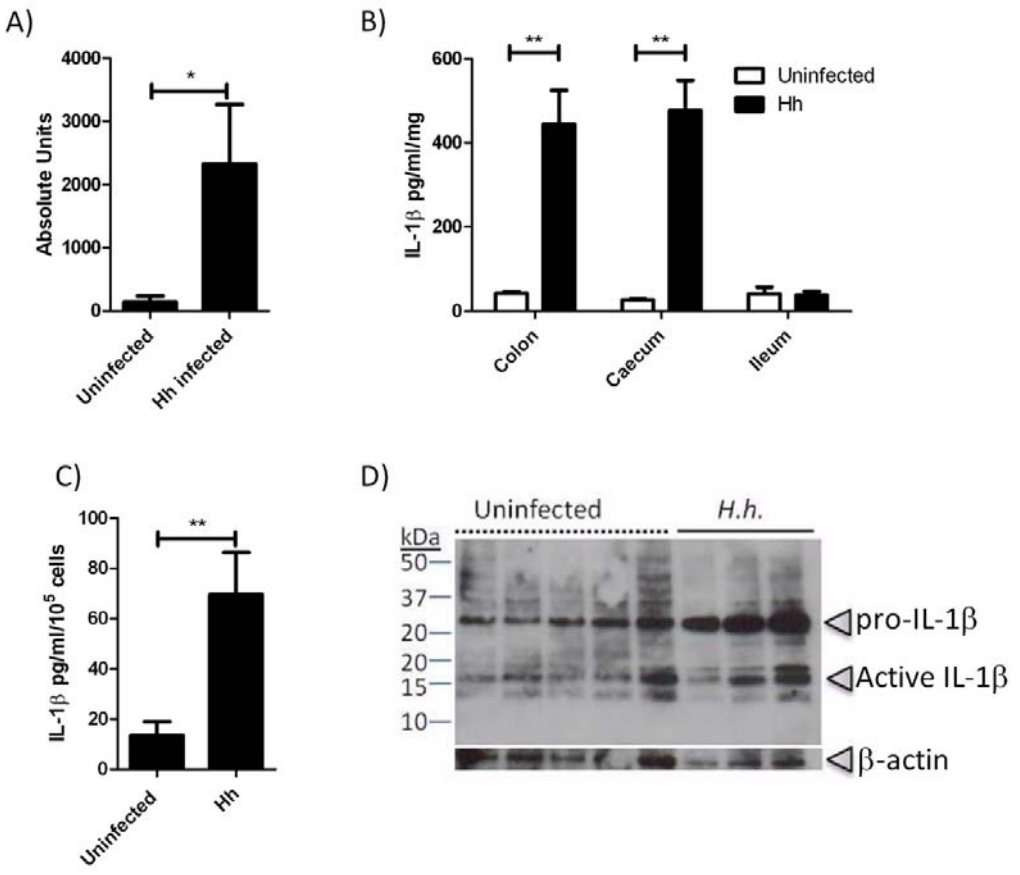


Figure 3-1. *H.hepaticus* induced innate immune driven typhlocolilts is associated with IL-1β secretion. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* and sacrificed at peak disease (>8 weeks after infection). All results are shown as mean ± SEM. A) Expression levels of IL-1β. mRNA was purified from colonic tissue samples and cytokine gene expression was evaluated by qPCR (n=3 for unifected control, 5 for *H.hepaticus*-infected mice). B) IL-1β secretion from organ explants from colon, caecum and ileum incubated O/N in complete medium (n= 3 for uninfected control, 14 for *H.hepaticus*-infected mice, pooled from 2 independent experiments). C) IL-1β levels in the supernatants of colonic lamina propria leukocytes (cLPL) cultured O/N in complete medium (n= 7 for uninfected controls, 22 for *H.hepaticus*-infected mice, pooled from 3 separate experiments). D) αIL-1β western blot on colon homogenates. Colons of *H.hepaticus* infected colitic 129 SvEv mice and uninfected controls were homogenized in RIPA buffer. Equal amounts of protein were separated on a 4-12% SDS-PAGE gel. A western blot was performed using rabbit anti-mouse IL-1β mAb (Cell signaling). To control for loading errors, the blot was stripped and re-rpobed for β-actin. Each lane represents an individual mouse, One representative experiment of two is shown.

*p<0.05, **p<0.01 (Mann-Whitney test).

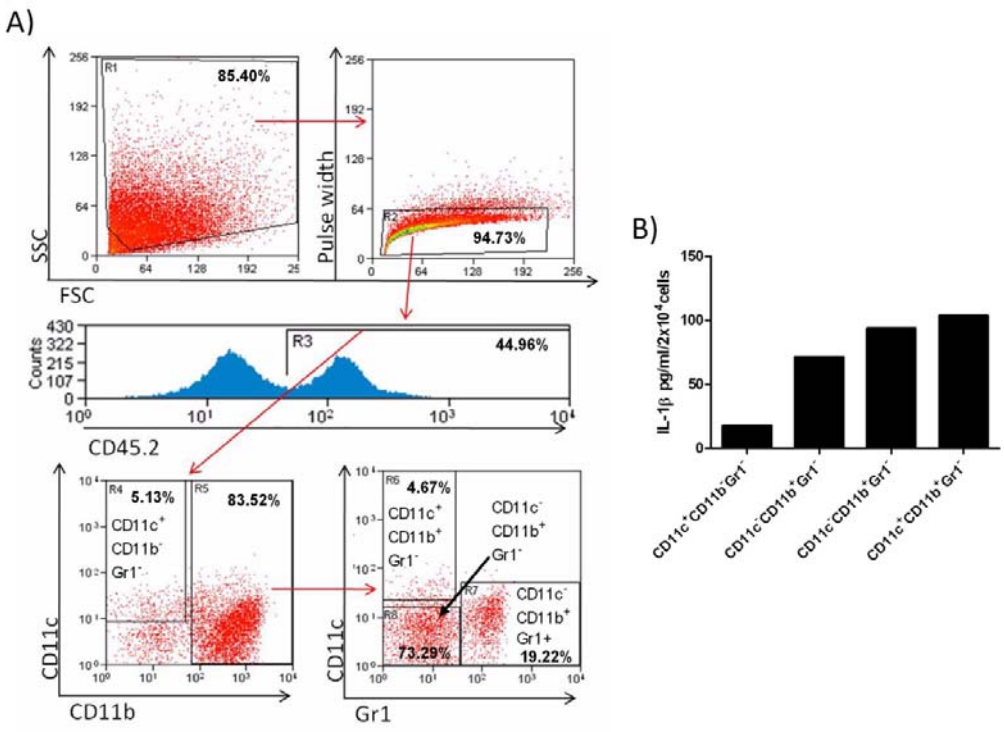


Figure 3-2. Different innate leukocytes contribute to IL-1β secretion in the colon of *H.hepaticus*-infected 129SvEv Rag2^{KO} mice. 129SvEv Rag2^{KO} mice were infected with *H.hepaticus* and culled 2 or 8 weeks post infection. At sacrifice, innate leukocytes were isolated from the colonica lamina propria and separated into the indicated populations by FACS sorting. Sorted cells were cultured O/N in complete media. One representative experiment out of 2 is shown. A) FACS sorting strategy: following a generous morphological gate, duplets were excluded and analysis was limited to CD45.2^{+}cells. Then, dendritic cells were identified as CD11c^{+}CD11b^{+}GR1^{-} cells, whilst inflammatory monocytes/macrophages were separated as CD11c^{-}CD11b^{+}Gr1^{-} and granulocytes as CD11c^{-}Cd11b^{+}Gr1^{+} cells. B) IL-1β levels in the supernatants were measured by Flowcytomix Multiplex assay (eBioscience).

To formally assess the requirement for IL-1β in *H.hepaticus*-induced innate intestinal inflammation, we blocked the activity of IL-1β with a neutralizing monoclonal antibody (αIL-1β). Treatment of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice with αIL-1β resulted in significant attenuation of colitis compared to mice treated with an isotype control antibody (**Fig.3-3A,C**). Although caecal inflammation was not significantly attenuated (**Fig.3-3B**), hepatic inflammation was also reduced by administration of αIL-1β, as illustrated by the decreased number of inflammatory foci (**Fig.3-3C**). Moreover, systemic inflammation was also reduced following IL-1β blockade, as shown by decreased splenomegaly and spleen cellularity in αIL-1β treated animals (**Fig.3-4**). Treatment with αIL-1β had no effect on *H.hepaticus* colonization levels in the gut (**Fig.3-5**). These results identify a role for IL-1β in promoting intestinal and systemic innate inflammation following *H.hepaticus* infection. To further characterize the effect of blocking IL-1β, we examined the levels of pro-inflammatory cytokines secreted by purified cLPL from the different treatment groups. Consistent with previous studies in our lab (444), we observed an increase in pro-inflammatory cytokine production by cLPL from *H.hepaticus* infected colitic mice (**Fig.3-6**). However, following administration of αIL-1β we observed significantly decreased levels of IL-6, IFN-γ, IL-17A and IL-1β when compared to isotype control treated animals (**Fig.3-6**). These results support a crucial role of IL-1β in promoting pro-inflammatory cytokine cascades in innate immune colitis.

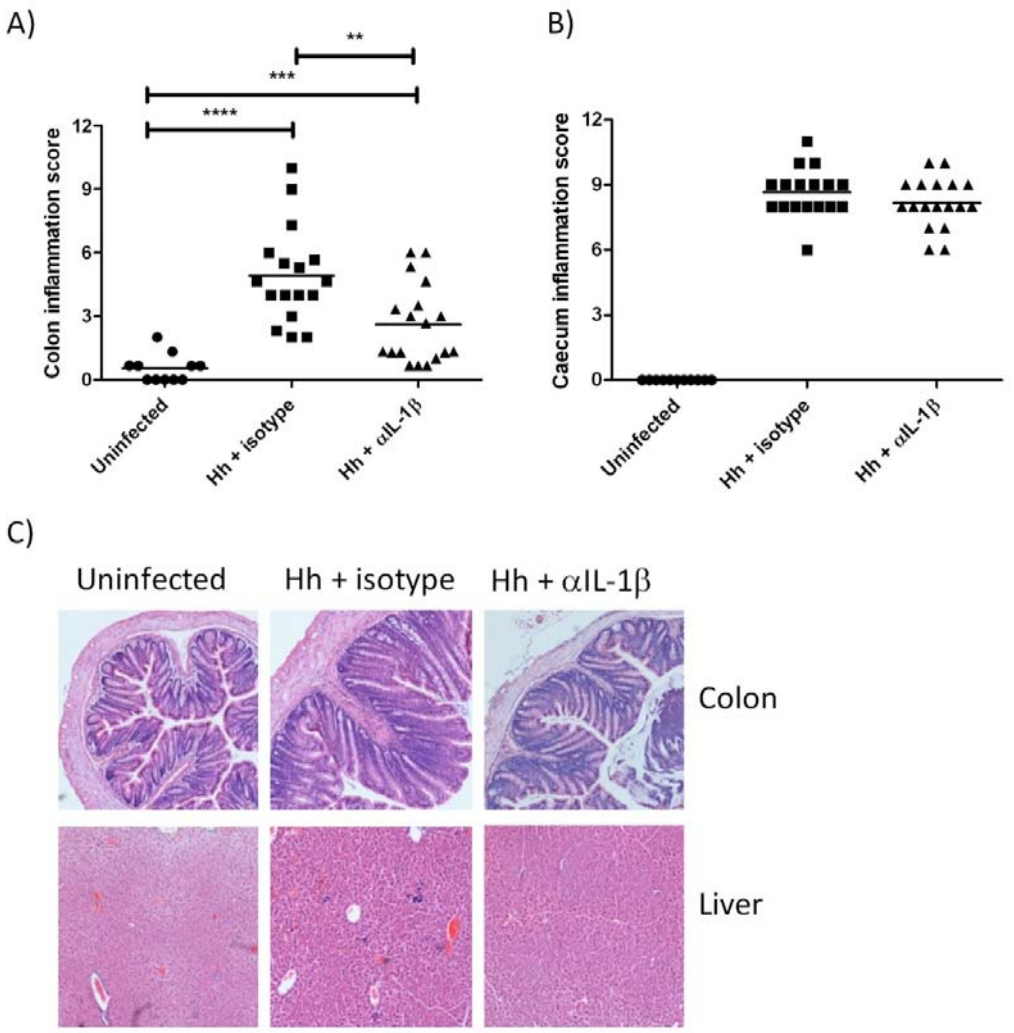


Figure 3-3. IL-1β drives innate immune colitis. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus*, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.) and sacrificed after 8 weeks of infection. Each symbol represents an individual mouse and data are pooled from four independent experiments (n=9-16). Bars represent the mean histological score for each group. A) Colon inflammation. B) Caecum inflammation. C) Representative photomicrographs of mid-colon and liver sections (original magnification 50x).

**p<0.01, ***p<0.001, ****p<0.0001 (Mann-Whitney test).

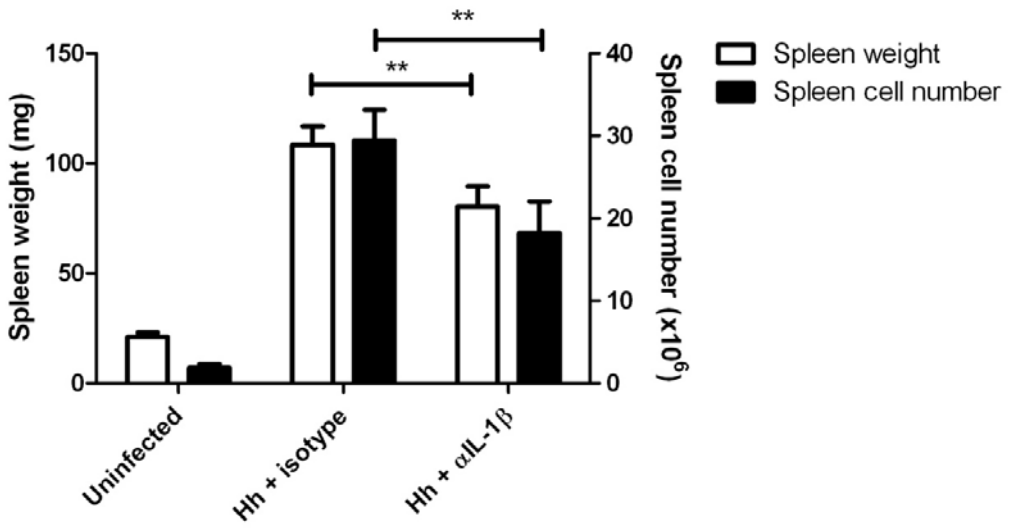


Figure 3-4. IL-1β drives systemic disease in *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus*, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.) and sacrificed after 8 weeks of infection. At sacrifice, spleen weight and spleen cellularity were determined. Data are shown as mean ± SEM from 3 pooled independent experiments (n=9-16).

**p<0.01 (Mann-Whitney test).

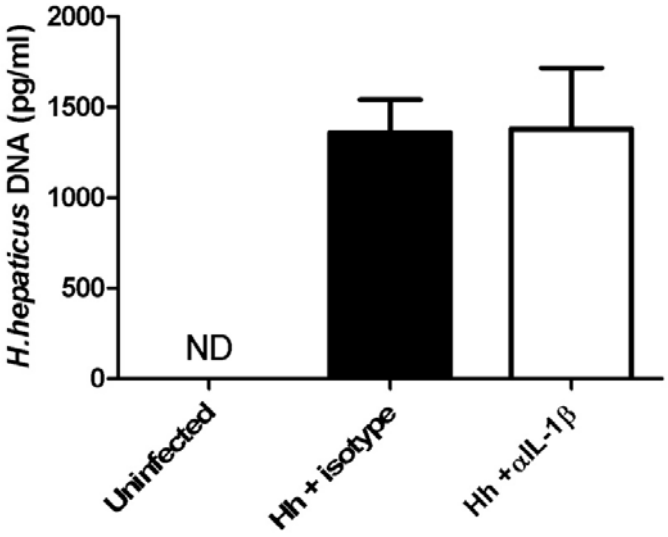


Figure 3-5. Treatment with αIL-1β does not affect *H.hepaticus* colonization. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus*, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.) and sacrificed after 8 weeks of infection. DNA was isolated from cecal contents and levels of *H.hepaticus* DNA were determined using a q-PCR method based on the *cdtB* gene (604). One representative experiment out of three is shown. Data are expressed as mean ± SEM (n=3-6). ND, not detectable.

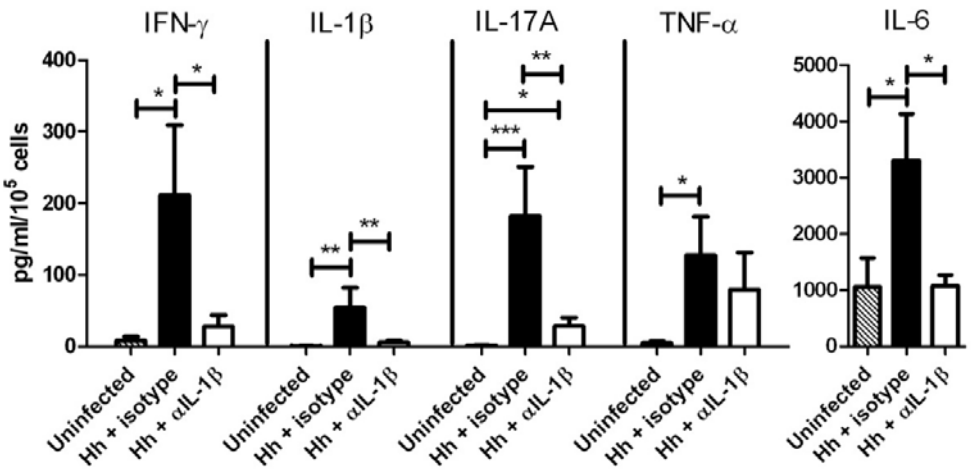


Figure 3-6. IL-1β drives the pro-inflammatory cytokine response in the colon of *H.hepaticus*-infected 129SvEv *Rag2*^{KO}. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus*, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.) and sacrificed after 8 weeks of infection. Colonic lamina propria leukocytes were isolated from the described mice groups and cytokine secretion after O/N culture in complete media was determined. Data are shown as mean ± SEM from 3 pooled independent experiments (n=9-16).

*p<0.05,**p<0.01, ***p<0.001 (Mann-Whitney test).

3.2.2 IL-1β blockade decreases granulocyte recruitment and accumulation of IL-17A producing innate lymphoid cells in the colon.

H.hepaticus induced innate colitis is associated with pronounced accumulation of $CD11b^{+}Gr1^{Hi}$ granulocytes in the colon and spleen (444). Thus, we isolated leukocytes from different tissues of *H.hepaticus*-infected αIL-1β–treated and isotype control treated 129SvEv *Rag2*^{KO} mice and analyzed the frequency and accumulation of granulocytes by flow cytometry. As expected, *H.hepaticus*-mediated inflammation was associated with significant increases in the frequency and accumulation of $CD11b^{+}Gr1^{Hi}$ cells in the colon, liver and spleen (**Fig3-7**). Blocking IL-1β in *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice resulted in a decrease in the frequency of $CD11b^{+}Gr1^{Hi}$ cells in the colon compared to mice treated with an isotype control, whilst not affecting frequencies in the spleen or liver (**Fig3-7A**). However, the accumulation of $CD11b^{+}Gr1^{Hi}$ cells was partially dependent on IL-1β both in the colon and spleen, but not in the liver, of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice (**Fig.3-7B-D**).

We recently identified a novel population of $Sca1^{+}$ $Thy1.2^{Hi}$ innate lymphoid cells (ILC) that play an important role in innate immune mediated intestinal inflammation (51). Thus, we analyzed the frequency, accumulation and function of these cells in the colon following αIL-1β treatment. As previously reported (51), we observed an expansion of ILC in the colon and liver of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice (**Fig.3-8A-D**). Treatment with αIL-1β did not affect the frequency of ILC in the colon or liver of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice (**Fig.3-8A-B**). However, we observed significantly decreased numbers of ILC in the colons of *H.hepaticus*-

infected 129SvEv *Rag2*^{KO} mice that received αIL-1β compared to those treated with an isotype control (**Fig.3-8C**). The inhibitory effect of αIL-1β on ILC accumulation was specific to the colon and was not observed in the liver (**Fig.3-8D**), where ILC also mediate pathology following *H.hepaticus* infection (51). During *H.hepaticus*-induced intestinal inflammation, ILC produce the pro-inflammatory cytokines IL-17A and IFN-γ, and these responses are enhanced following stimulation with IL-23 (51). Intracellular cytokine staining revealed that the observed decrease in colonic ILC number following αIL-1β treatment was accompanied by a significant decrease in IL-17A-producing ILC and by a reduction in the number of IFN-γ-producing ILC (**Fig.3-9**). These results indicate a role of IL-1β in promoting the accumulation and activation of ILC in the colon. As we have shown that ILC are the major IL-23 responsive population in this innate colitis model (51), we cultured cLPL and liver leukocytes from αIL-1β treated *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice in the presence of IL-23 (**Fig.3-10A-B**). Blockade of IL-1β *in vivo* resulted in decreased production of both IL-17A and IFN-γ by IL-23 stimulated cLPL *in vitro* (**Fig.3-10A**). Secretion of IL-17A and IFN-γ following IL-23 stimulation of liver leukocytes was also impaired (**Fig.3-10B**), showing that IL-1β plays a key role in promoting ILC function *in vivo.* Taken together, these data suggest that IL-1β plays multiple roles in promoting innate immune responses in the colon, by modulating recruitment, accumulation and function of key pro-inflammatory cell types.

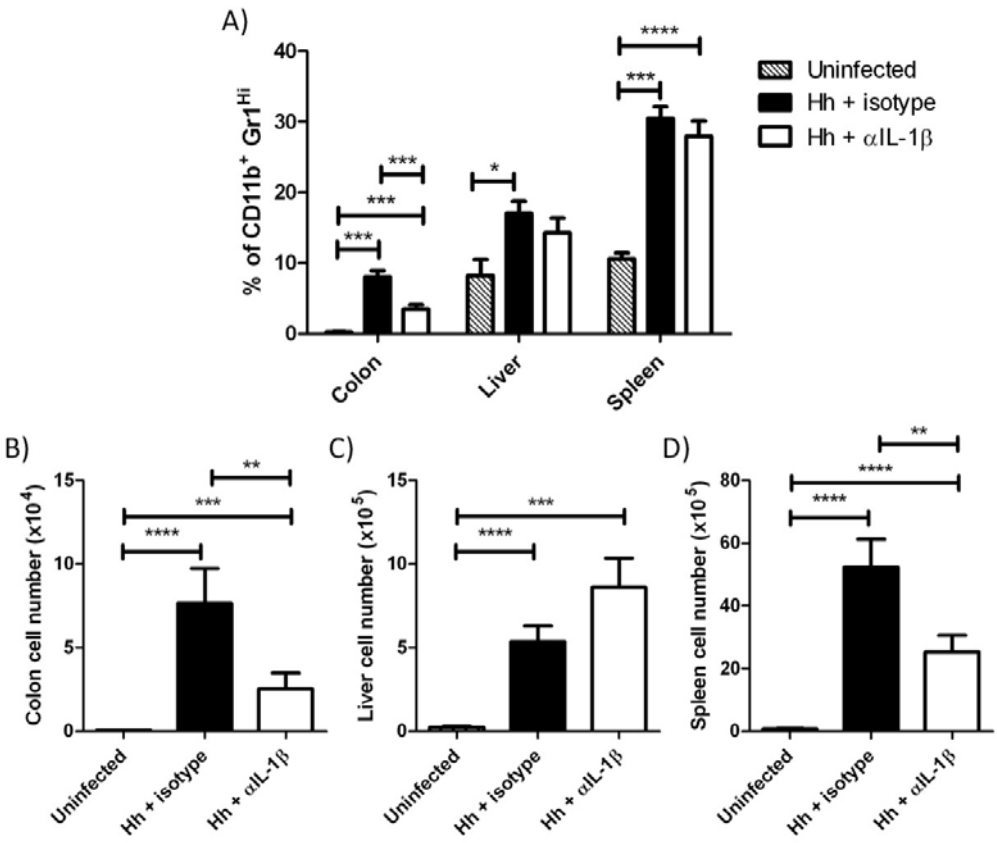


Figure 3-7. Blocking IL-1β impairs the recruitment and accumulation of proinflammatory CD11b^{+}Gr1^{Hi} granulocytes in the inflamed colon. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus*, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.) and sacrificed after 8 weeks of infection. Leukocytes were isolated from the colon, liver and spleen and analyzed by flow cytometry. All data are shown as mean ± SEM from 3 pooled independent experiments (n=9-16). A) Frequencies of CD11b^{+}Gr1^{Hi} cells in the colon, liver and spleen. B) Numbers of CD11b^{+}Gr1^{Hi} cells in the colon. C) Numbers of CD11b^{+}Gr1^{Hi} cells in the liver. D) Numbers of CD11b^{+}Gr1^{Hi} cells in the spleen.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (Mann-Whitney Test).

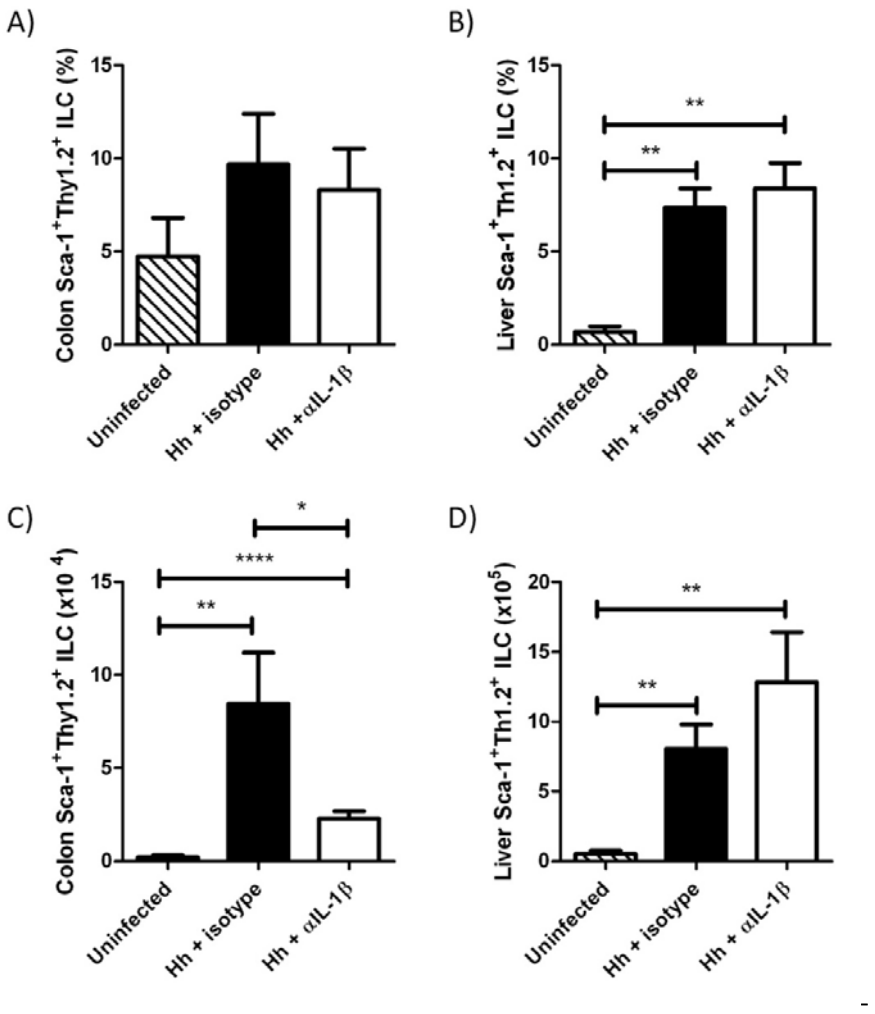


Figure 3-8. αIL-1β treatment reduces the accumulation of innate lymphoid cells (ILC) in the colon. 129SvEv *Rag2*^{KO} mice were infected with H.hepaticus, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.). After 8 weeks, mice were sacrificed and colonic lamina propria leukocytes (cLPL) were isolated and analyzed by FACS. Innate lymphoid cells were identified as CD45.2^{+}lin^{-}Sca1^{+}Thy1.2^{Hi} cells. Data are shown as mean ± SEM and are pooled from two independent experiments (n= 6-11). A) Frequency of Sca1^{+} Thy1.2^{Hi} innate lymphoid cells (ILC) in the colon. B) Frequency of Sca1^{+} Thy1.2^{Hi} innate lymphoid cells (ILC) in the liver. C) Total numbers of Sca1^{+} Thy1.2^{Hi} innate lymphoid cells (ILC) in the colon. D) Total numbers of Sca1^{+} Thy1.2^{Hi} innate lymphoid cells (ILC) in the liver.

*p<0.05, **p<0.01, ****p<0.001 (Mann-Whitney Test).

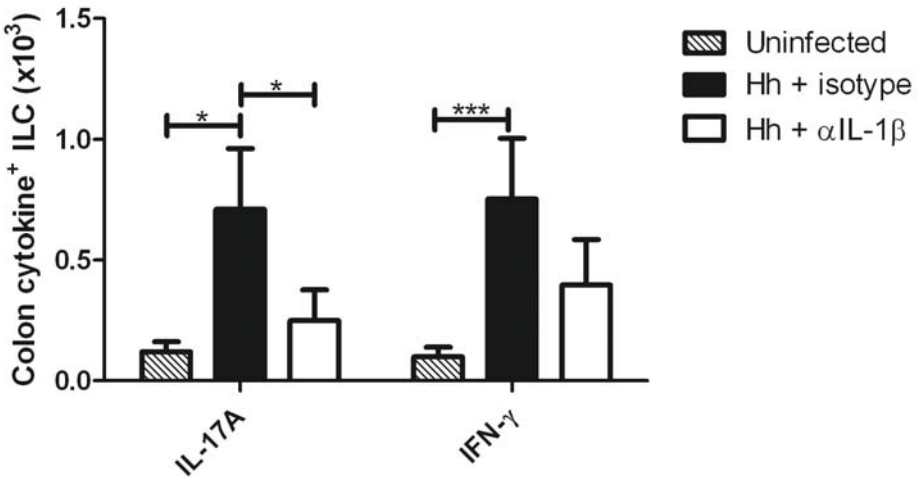


Figure 3-9. αIL-1β treatment reduces the accumulation of IL-17A producing innate lymphoid cells (ILC) in the colon. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus*, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.). After 8 weeks, mice were sacrificed and colonic lamina propria leukocytes (cLPL) were isolated. Cells were cultured overnight and cytokine secretion was inhibited by treatment with Brefeldin-A (10μg/ml) for the last 3h of culture. Numbers of IL-17A and IFN-γ producing cells were analyzed by intracellular staining and FACS analysis. Data are shown as mean ± SEM and are pooled from two independent experiments (n= 6-11).

*p<0.05, ***p<0.001 (Mann-Whitney Test).

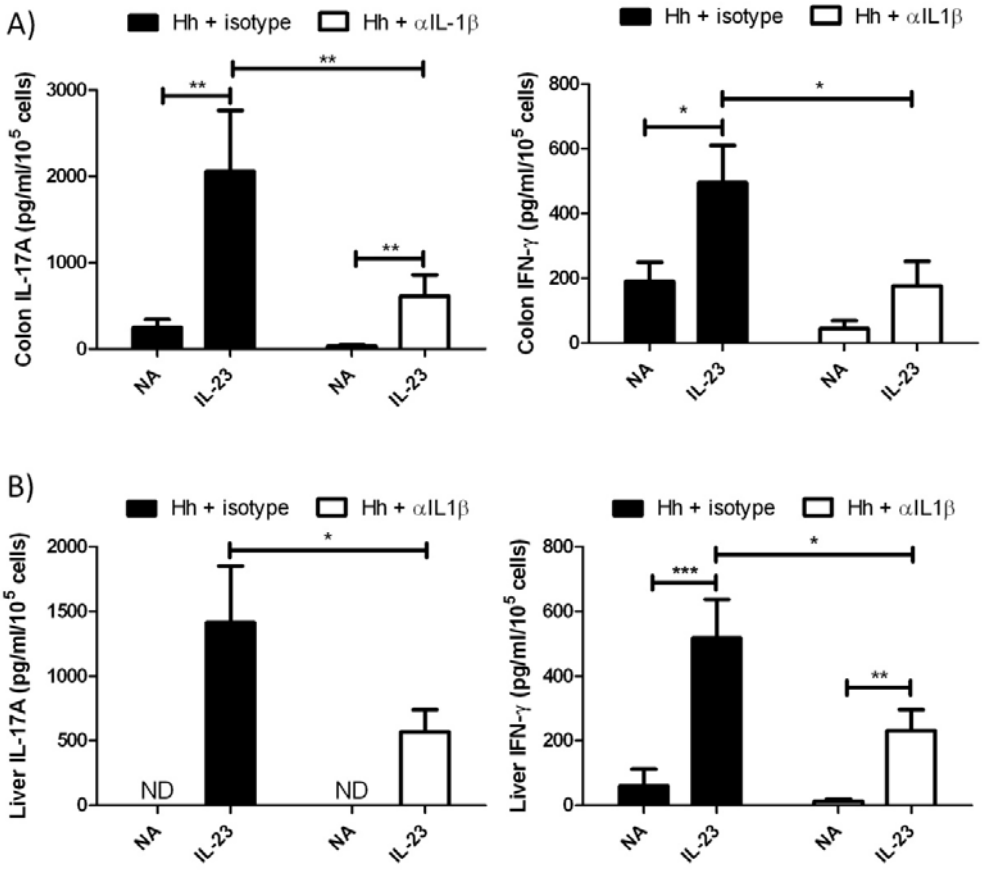


Figure 3-10. IL-1β promotes ILC function *in vivo*. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus*, treated weekly with 1mg of αIL-1β antibody or isotype control (i.p.). After 8 weeks, mice were sacrificed and colonic lamina propria leukocytes (cLPL) and liver leukocytes were isolated. Following O/N culture in the presence or absence of IL-23 (10ng/ml), cytokine secretion in the supernatants was evaluated by Flowcytomix Multiplex Assay (eBioscience). Data are represented as mean ± SEM from 2 pooled independent experiments (n=6-10). ND, not detectable.

*p<0.05, **p<0.01 (Mann-Whitney Test).

3.2.3 *In vitro* infection of macrophages with *H.hepaticus* results in caspase-1 dependent secretion of IL-1β.

To explore the molecular mechanisms underlying *H.hepaticus* induced IL-1β secretion, we turned to an *in vitro* infection system. We found that infection of bone marrow derived macrophages (BMMF) with *H.hepaticus* induced secretion of active IL-1β (**Fig.3-11A-B**). Moreover, as shown by western blot, *H.hepaticus* infection induced caspase-1 activation in BMMF (**Fig.3.11C**). To test whether *H.hepaticus* induced IL-1β secretion by BMMF was caspase-1 dependent, we used VX-765, a selective small molecule inhibitor of caspase-1 activity that is rapidly hydrolyzed into its active form, VRT-043198 (606, 622) (**Fig.3-11D**). VX-765 has been shown to specifically inhibit caspase-1 in a cell-free system and to selectively inhibit IL-1β and IL-18 secretion by *Staphylococcus aureus*- stimulated human blood monocytes (606). Treatment of BMMF with VX-765 completely blocked IL-1β secretion following *H.hepaticus* infection (**Fig.3-11E**). Taken together, these data show that *H.hepaticus* infection of macrophages results in caspase-1 dependent IL-1β secretion *in vitro*.

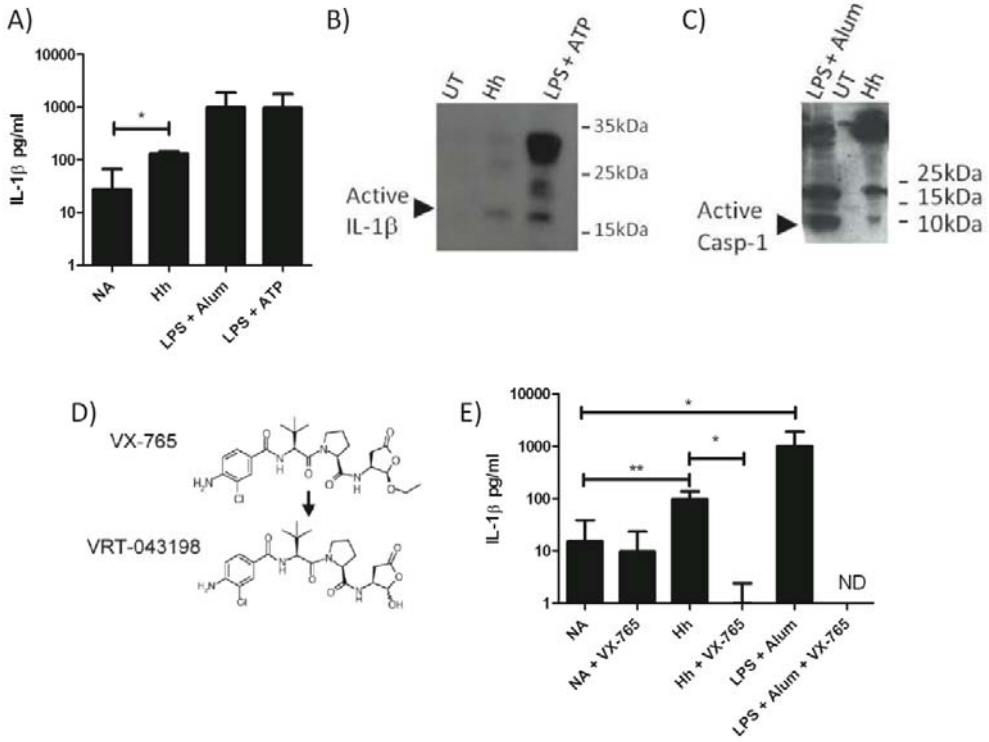


Figure 3-11. *H.hepaticus* induced IL-1β secretion from bone marrow-derived macrophages is caspase-1 dependent. Bone marrow derived macrophages were infected with *H.hepaticus* (MOI=15, 10^6cells/well) O/N. As a positive control for activation of caspase-1 inflammasomes, BMDM were treated with LPS (20ng/ml) O/N and restimulated with Alum (5mg/ml) or ATP (5mM) for 30min. IL-1β was assayed using Flowcytomix Multiplex Assay. Data are shown as mean ± SEM (n-3-6 from 3 independent experiments) ND, not detectable. A) IL-1β secretion in the culture supernatants. B) WB against IL-1β on precipitated culture supernatants. Culture supernatants were separated on a 4-20% SDS-PAGE gel and probed with goat anti-mouse IL-1β (R&D systems). Arrow indicates active IL-1β (17kD). One representative blot out of 3 is shown. C) WB against caspase-1 on precipitated culture supernatants. Culture supernatants were separated on a 15% SDS-PAGE gel and probed with rabbit anti-mouse caspase-1 (Santa Cruz, Inc.). Arrow indicates active caspase-1 (10kD). One representative blot out of 3 is shown. D) Structure of VX-765 and VRT-043198, from (606). E) IL-1β secretion in the culture supernatants. When indicated, VX-756 (6μM) was added to the cell culture, starting 1h before infection with *H.hepaticus* or treatment with alum.

*p<0.05, **p<0.01 (Student unpaired t-test).

3.2.4 *In vivo* efficacy of caspase-1 inhibitors in modulation of innate colitis is masked by effects of drug excipients.

Next, we examined whether *in vivo* inhibition of caspase-1 activity would result in amelioration of pathology in *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice. Initially, we investigated the efficacy of the VX-765 because, after oral administration, VX-765 is readily transformed into its active form and it had been shown to ameliorate pathology in models of rheumatoid arthritis and skin inflammation, when administered by oral gavage twice a day at 100mg/kg (606). Thus, we infected 129SvEv *Rag2*^{KO} mice with *H.hepaticus* and administered 200mg/kg VX-765 by oral gavage, once per day throughout the course of the experiment. As VX-765 was prepared in 25% Cremophor EL, a version of polyethoxylated castor oil, a control group received 25% Cremophor EL only. Although treatment of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice with VX-765 resulted in a decrease in colon inflammation compared to *H.hepaticus* infected untreated mice (**Fig.3-12A**), this effect appeared to be largely due to the delivery vehicle, because daily oral administration of Cremophor EL alone resulted in similar attenuation of colitis (**Fig.3-12A**). Caecal inflammation was unaffected by treatment with VX-765 or Cremophor EL (**Fig.3-12B**). Moreover, we observed a comparable reduction in systemic inflammation following treatment of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice with either VX-765 or Cremophor EL, as shown by reduced spleen weight and spleen cellularity (**Fig.3-12C**). However, FACS analysis did not reveal any changes in the frequency of $CD11b^{+}$ $Gr1^{Hi}$ granulocytes in the spleen of VX-765 or Cremophor EL treated mice compared to *H.hepaticus* infected 129SvEv *Rag2*^{KO} controls (**Fig.3-12D**). Analysis of caecal DNA showed that treatment with Cremophor EL modestly reduced *H.hepaticus*

colonization (**Fig.3-13**), potentially contributing to the observed reduction in inflammation.

Next, we evaluated the efficacy of the novel caspase-1 inhibitor "Compound 2" in modulating innate colitis. Similarly to VX-765, Compound 2 is a pro-drug that is rapidly converted by esterases *in vivo* to its active form, a small molecule reversible inhibitor of caspase-1 (Vertex Pharmaceuticals, Incorporated., unpublished observations). To overcome the vehicle effect observed in the VX-765 experiments Compound 2 was prepared in 30% Polyethylene Glycol-400-70% 50 mM sodium citrate buffer (Peg-400). Preliminary studies from Vertex Pharmaceuticals showed that Peg-400 had no modulatory effects in acute models of intestinal inflammation (Dr. P.Weber, personal communication). In addition, to increase the duration of the exposure to the drug, the frequency of administrations was increased. Hence, cohorts of 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* and administered Compound 2 (100mg/kg) in Peg-400 or Peg-400 alone, by oral gavage, twice a day for 8 weeks. At sacrifice, histological analysis of intestinal inflammation revealed that, whilst there was a trend for decreased inflammation in the colon of *H.hepaticus*-infected mice treated with Compound 2 compared to those that received Peg-400 (**Fig.3-14A**), the high variability observed in both drug- and vehicle-treated mice prevents any precise conclusion from these data. Similar variability was observed when analyzing systemic inflammation (**Fig.3-14C-D**) and caecal inflammation (**Fig.3-14B**). Moreover, in this experiment unusually low levels of colitis and systemic inflammation were observed in 129SvEv *Rag2*^{KO} mice receiving *H.hepaticus* alone (**Fig.3-14A,C,D**). Further revealed that treatment of *H.hepaticus* infected 129SvEv

Rag2^{KO} mice with Peg-400 or Compound 2 did not significantly affect the accumulation of CD11b^{+} Gr1^{Hi} cells in the spleen (**Fig.3-15A**), or colon (**Fig.3-15B**), although there was a trend towards an increased frequency of granulocytes in the colon (**Fig.3-15B**). Finally, Peg-400 or Compound 2 administration to *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice did not affect *H.hepaticus* colonization levels (**Fig.3-16**). Overall these results show that studies to assess the effect of orally available caspase-1 inhibitors in models of colitis must consider the potential high impact of commonly used drug excipients on intestinal physiology.

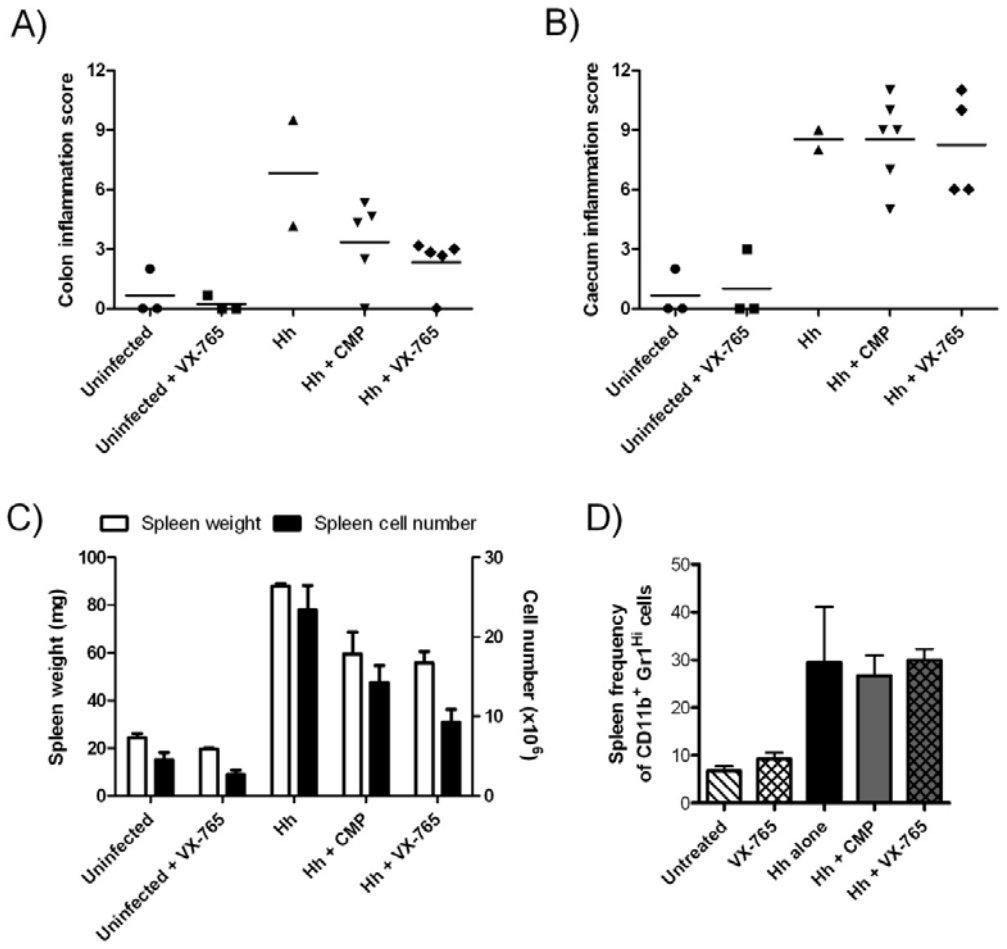


Figure 3-12. Cremophor EL attenuates innate colitis and systemic inflammation. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* and treated daily with VX-765 (200mg/kg) in 25% Cremophor EL (VX-765) or 25% Cremophor EL only (CMP) by oral gavage. Mice were sacrificed at week 8 post infection. A) Colon inflammation scores. Each symbol represents an individual mouse (n=2-7). Bars represent the mean histological score for each group. B) Caecum inflammation scores. Each symbol represents an individual mouse (n=2-7). Bars represent the mean histological score for each group. C) Spleen weights and splenocyte numbers. Data are expressed as mean ± SEM (n=2-7). D) FACS analysis of the frequency of CD11b^{+}Gr1^{Hi} in the spleen. Data are expressed as mean ± SEM (n=2-7).

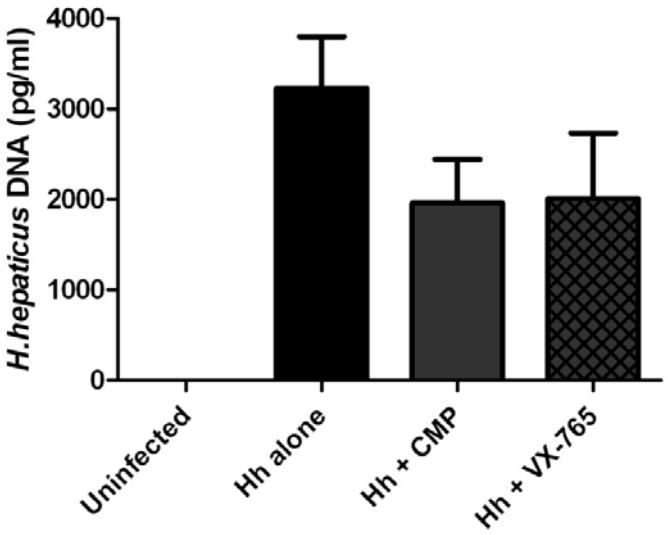


Figure 3-13. Cremophor EL reduces *H.hepaticus* colonization in 129SvEv *Rag2*^{KO} mice. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* and treated daily with VX-765 (200mg/kg) in 25% Cremophor EL (VX-765) or 25% Cremophor EL only (CMP) by oral gavage. After 8 weeks, the mice were sacrificed and DNA was isolated from cecal contents. Levels of *H.hepaticus* DNA were determined using a q-PCR method based on the *cdtB* gene (604). Data are expressed as mean ± SEM (n=2-7).

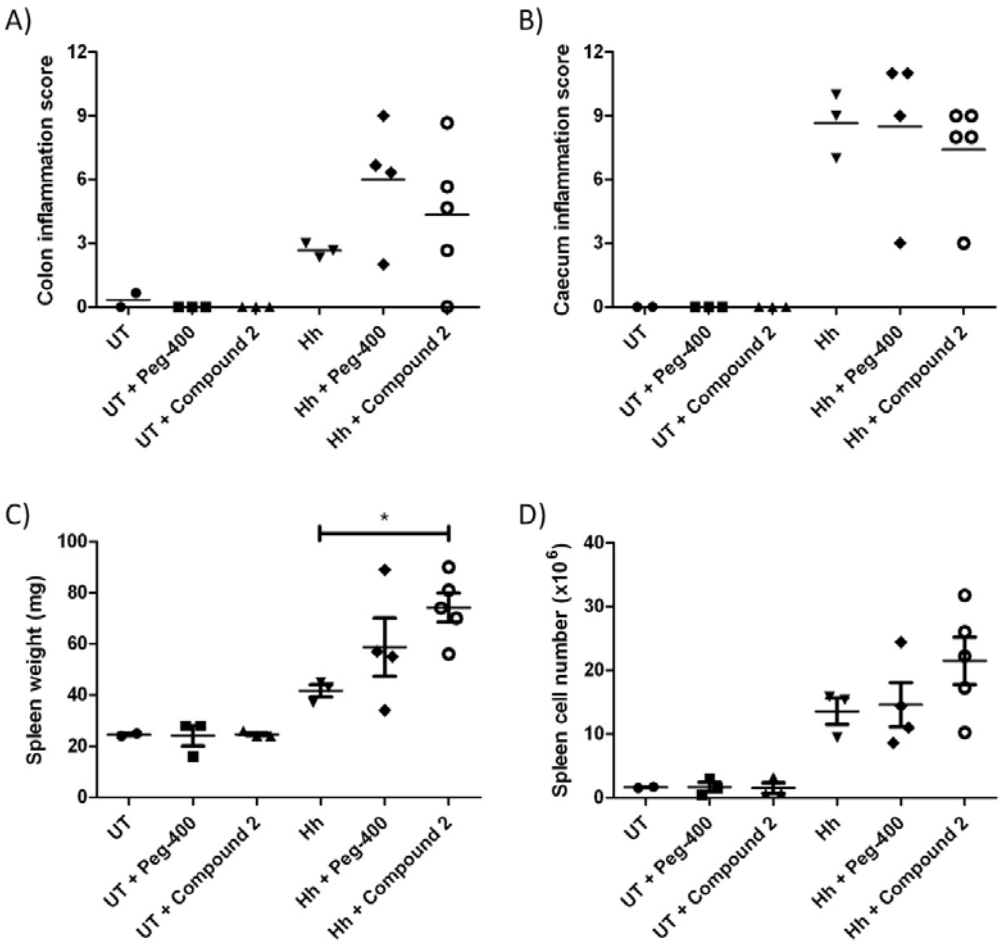


Figure 3-14. Effects of-administration of Polyethylene glycol 400 or Compound 2 in *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* and treated twice a day with Compound 2 (100mg/kg) in 30% PEG-400 or 30% PEG-400 only (Peg-400) by oral gavage. Mice were sacrificed after 8 weeks. Each symbol represents an individual mouse. Bars represent the mean for each group. A) Colon inflammation score. B) Caecum inflammation score. C) Spleen weights. D) Splenocyte numbers.

*p<0.05 (Mann-Whitney test).

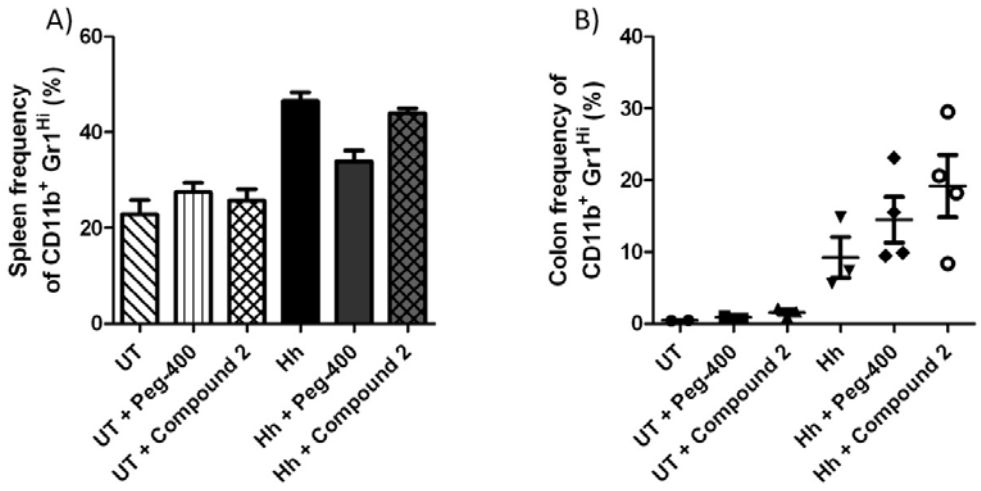


Figure 3-15. Effects of Peg-400 or Compound 2 treatment on the accumulation of CD11b^{+} Gr1^{Hi} granulocytes in *H.hepaticus*-infected 129SvEv *Rag2^{KO}* mice. 129SvEv *Rag2^{KO}* mice were infected with *H.hepaticus* and treated twice a day with **Compound 2** (100mg/kg) in 30% PEG-400 or 30% PEG-400 only (Peg-400) by oral gavage. After 8 weeks the mice were sacrificed, and leukocytes isolated from the spleen and colon were analyzed by FACS. A) Frequencies of CD11b^{+} Gr1^{Hi} cells in the colon. Data are shown as mean± SEM (n=2-5). B) Frequencies of CD11b^{+} Gr1^{Hi} cells in the spleen.

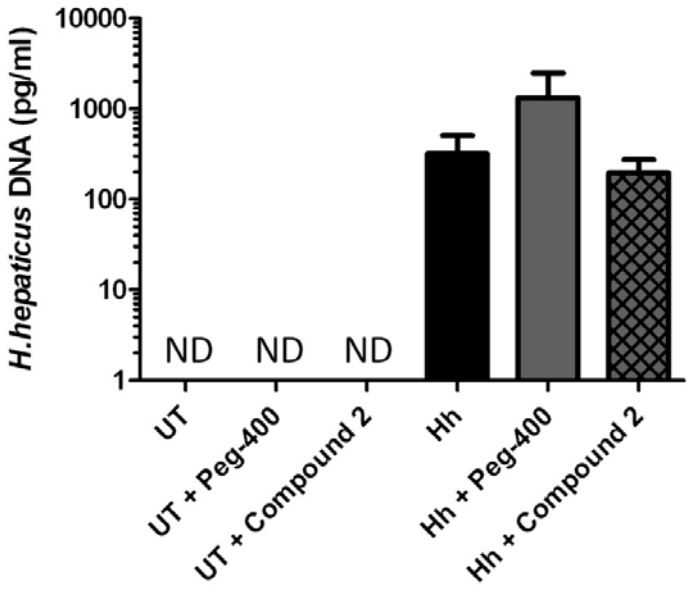


Figure 3-16. Treatment with Peg-400 or Compound 2 does not affect *H.hepaticus* colonization. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* and treated twice a day with Compound 2 (100mg/kg) in 30% PEG-400 or 30% PEG-400 only (Peg-400) by oral gavage. After 8 weeks, the mice were sacrificed and DNA was isolated from cecal contents. Levels of *H.hepaticus* DNA were determined using a q-PCR method based on the *cdtB* gene (604). Data are expressed as mean ± SEM (n=2-7). ND, not detectable. UT, untreated.

3.3 Discussion.

IL-1β is known to play a crucial role in the promotion and amplification of innate immune responses. However, its specific role in intestinal inflammation is unclear. Here, we used a T cell-independent model of intestinal inflammation to dissect the cellular mechanisms through which IL-1β contributes to innate immune-driven intestinal pathology. We found that IL-1β was essential for the development of severe innate immune pathology driven by the intestinal pathogen *H.hepaticus*. We identified an intestine-specific role for IL-1β in driving classical pathways of innate immune inflammation, by promoting the recruitment of granulocytes to the colon. Moreover, we observed a crucial role for IL-1β in supporting innate "type-17" responses in the colon, by promoting the accumulation and function of IL-17A producing innate lymphoid cells.

Using a T cell-independent model of colitis we assessed the role of IL-1β in driving innate intestinal inflammation. In accordance with the central role played by IL-1β in orchestrating innate inflammatory responses (513, 524), we observed attenuation of *H.hepaticus*-induced colitis following αIL-1β treatment. The common adaptor protein, MyD88, is responsible for signalling downstream of the IL-1R (514) and previous studies from our laboratory showed *Myd88*^{KO} mice to be completely protected from *H.hepaticus*-induced colitis (623). Interestingly, *H.hepaticus* infected *Myd88*^{KO} mice are completely protected from both typhlitis and colitis, whereas only colitis was significantly decreased in αIL-1β treated mice, implying that IL-1β contributes mainly to pro-inflammatory MyD88-dependent signals in the colon. Consistent with this hypothesis, *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice treated

with αIL-1β exhibited a selective decrease in neutrophil recruitment in the colon, which is in accordance with the prominent role of IL-1β in promoting neutrophil infiltration to sites of inflammation (538, 624). Others have reported that blocking the IL-1β pathway could ameliorate DSS-triggered intestinal inflammation (616-618). Of note, IL-1R deficiency in this model did not perturb granulocyte recruitment to the colon. However DSS colitis represent acute models of colitis, where granulocyte recruitment is secondary to the extensive epithelial damage caused by the initial bacterial or chemical injury. By contrast, *H.hepaticus*-induced intestinal pathology in 129SvEv *Rag2*^{KO} mice is primarily associated with chronic activation of innate leukocytes in the lamina propria (623). Hence the mechanisms underlying intestinal pathology and leukocyte recruitment are profoundly different between these models of colitis.

Our data show that IL-1β acts to boost IL-17A production in the intestine of *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice. Given that IL-17A production contributes to pathology in this model (51), it is possible that the decreased inflammation observed following treatment with αIL-1β is due, at least in part, to its effects on IL-17A. We further observed that IL-1β was required for the accumulation of a novel population of IL-17A producing innate lymphoid cells (ILC). Fewer numbers of Sca1^{+} Thy1.2^{Hi} ILC were present in the colon of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice receiving αIL-1β compared to those treated with an isotype control. In addition, ILC isolated from *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice that were treated with αIL-1β had reduced frequencies of IL-17A and IFN-γ secreting cells, which correlated with decreased secretion of these cytokines in response to IL-23 stimulation. Thus, IL-1β

signals promote the accumulation and effector functions of ILC in the intestine. Moreover, we observed a similar modulation of IL-23 induced IL-17A and IFN-γ secretion by ILC in the liver of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice receiving αIL-1β compared to those treated with an isotype control. However, no effect of IL-1β on ILC accumulation was observed in the liver. This would suggest that the role of IL-1β in promoting ILC function is conserved between different organs, whilst the effects on ILC accumulation are colon-specific. A role for IL-1β in promoting the *in vitro* proliferation and function of a distinct ILC type has been proposed in humans. NK-22 or RORC^{+}CD127^{+} NK-like cells are present at mucosal sites and can secrete both IL-22 and IL-17A in response to IL-23 (37, 42). Recently, it has been shown that IL-1β can synergize with other cytokines, such as IL-7, IL-15 or IL-23 to promote proliferation and effector cytokine secretion by human NK-22 cells *in vitro* (625, 626). Moreover, IL-1β stimulation has been shown to promote IL-22 secretion by NKp46^{+} Rorγt^{+} ILC from mouse small intestine (627). Thus, IL-1β may play a crucial role in modulating the functions of several types of ILC at mucosal sites. The exact mechanisms underlying these effects remain unclear, but it is possible that IL-1β modulates cytokine receptor expression or signal transduction in these cells, as IL-1β stimulation alone has only minor effects on ILC biology (37, 42). Alternatively, IL-1β could mediate its effects by mediating cell survival signals or via indirect mechanisms. IL-1β has been shown to promote the secretion of prostaglandin-E2 (PGE2) by macrophages, via the induction of cyclooxygenase-2 expression (COX-2) (628). Since PGE2 was reported to promote IL-17 production in T cells (629, 630), it is possible that IL-1β might promote IL-17 responses in ILC through a similar pathway.

Although we found that *H. hepaticus* induced secretion of active IL-1β by macrophages in a caspase-1-dependent manner, our attempts to inhibit colitis using orally administered caspase-1 inhibitors were unsuccessful, mainly because of the unexpected immuno-modulatory effects of the drug vehicles. Excipients are commonly used to improve the solubility, stability and oral availability of poorly water-soluble drugs. Cremophor EL is a non-ionic surfactant, commonly used to emulsify and stabilize hydrophobic drugs. Significant immuno-suppressant effects of daily intra-muscular (i.m) administration of Cremophor EL were reported in a model of chronic granulomatous colitis, induced in Lewis rats by intramural injection of peptidoglycan/polysaccharide into the distal colon (631). Daily i.m. administration of Cremophor EL alone resulted in a reduction of colonic MPO activity, crypt depth and mucosal permeability compared to untreated colitic rats (631). Whilst these effects might partially be attributed to a stress response to repeated i.m. injections, it has been shown that Cremophor EL exerts anti-inflammatory effects in rabbits following aortic perfusion (632). Thus, far from being an inert excipient, Cremophor EL exerts profound immuno-modulatory effects, as confirmed by our findings that daily oral administration of Cremophor EL alone in *H.hepaticus* 129SvEv *Rag2*^{KO} mice resulted in decreased intestinal and systemic inflammation.

In contrast to the protective effect of Cremophor EL on innate colitis, our studies using Compound 2 emulsified in Peg-400 did not result in any significant attenuation of intestinal inflammation. However, interpretation of this experiment was complicated by the high intra-group variability and by the uncharacteristically low levels of pathology found in the control *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice.

Peg-400 is a water-miscible polymer often used to increase the solubility and oral availability of poorly water-soluble drugs (633). Moreover, Peg-400 is used in the clinic to evaluate intestinal permeability in IBD patients (634) and no toxic or inflammatory effects of this procedure have been reported, although it has limited laxative properties (635, 636). Furthermore, administration of Peg-3350 to TNBS-treated colitic rats did not result in increased inflammation and was reported to ameliorate colitis (637, 638). Thus, these experiments were unable to provide any clear information on the role of caspase-1 activation in innate intestinal inflammation. Our results underline the importance for the development of novel vehicle formulations for oral delivery of immuno-modulatory drugs to the intestine.

In addition to caspase-1, other enzymes might contribute to IL-1β processing at sites of inflammation. Indeed, several enzymes, including neutrophil elastase, proteinase 3, cathepsin G, chymase, granzyme A and MMPs have been shown to cleave pro-IL-1β, producing mature IL-1β with biological activity (527). An important pathogenic role for serine protease-mediated processing of IL-1β has been demonstrated in models of inflammatory arthritis (527). Thus, while caspase-1 deficiency conferred only minimal protection from experimental arthritis, strategies blocking both caspase-1 and serine proteases resulted in strong amelioration of pathology (639, 640). Unfortunately, most of the mature IL-1β peptides have a similar molecular weight (17-18kDa), making it impossible to distinguish products from different enzymes on a western blot (527). However, specific inhibitors for different IL-1β processing enzymes are available, making it possible to design strategies to specifically assess the contribution of different enzymes to IL-1β processing *in vivo*.

The shared conspicuous granulocyte accumulation observed in the inflamed intestinal mucosa of UC patients and in *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice, suggests that the latter model may provide a useful system for testing the different inhibitors capable of processing IL-1β.

Taken together, the data presented in this chapter clearly define a pathogenic role for IL-1β in innate immune-driven *H.hepaticus* dependent intestinal inflammation. Moreover, we identified specific cellular mechanisms underlying the pro-inflammatory role of IL-1β in chronic colitis. Finally, we highlighted the problems associated with the oral delivery of drugs to the inflamed intestine. Further analysis of the IL-1β/caspase-1 axis may reveal novel therapeutic strategies in IBD.

Chapter 4: Role of IL-1β in *H.hepaticus*-driven T cell-dependent inflammation

4.1 Introduction

Having established a pathogenic role for IL-1β in driving *H.hepaticus*-induced innate intestinal inflammation, we next investigated whether this cytokine was required for bacterial-driven chronic colitis in the presence of both innate and adaptive immunity. In immune competent C57BL/6 (WT) mice, *H.hepaticus* persistently colonizes the large intestine, rapidly becoming the dominant species of the cecal microbiota (641). Although strong adaptive immune responses are elicited during chronic *H. hepaticus* infection, at the same time, the host develops a regulatory T cell response that prevents the development of intestinal inflammation (193). However, when the crucial anti-inflammatory cytokine IL-10 is blocked by administration of an αIL-10R antibody to *H. hepaticus*-infected WT mice (Hh + αIL-10R), regulation is circumvented, unleashing *H. hepaticus*-specific $CD4^+$ T cell effector responses, leading to the development of chronic intestinal inflammation (438). In particular, strong IL-23 dependent Th17 and Th1 responses are associated with intestinal inflammation in this model (438).

IL-1β plays an established role in driving $CD4^+$ T cell expansion and proliferation. IL-1β can promote Th1 effector function, although the IL-1 receptor (IL-1R1) is expressed only at low levels on Th1 cells (545). In addition, Th17 cells express IL-1R1 at high levels (138) and IL-1β promotes Th17 differentiation and expansion (133, 138, 139). Indeed, $Il1r1^{KO}$ $CD4^+$ T cells show impaired differentiation into Th17 cells *in vitro* and in murine models of EAE (60, 138, 139).

$CD4^+$ $FOXP3^+$ regulatory T cells also express IL-1R1 (642, 643) and IL-1 has been shown to inhibit the suppressive function of Tregs and to promote the expansion of non-regulatory T cells *in vitro* (644, 645). Moreover, it has been proposed that scavenging of IL-1 from the microenvironment might be one of the regulatory mechanisms used by Tregs to dampen the inflammatory response (643). However, it has also been proposed that IL-1β promotes the differentiation of $FOXP3^+$ Tregs into IL-$17A^+$ $CD4^+$ T cells (138).

Mice defective in IL-1R1 are insensitive to stimulation by IL-1β and IL-1α and show reduced delayed-type hypersensitivity responses (646, 647). Initially, *Il1r1*^{KO} mice were described to be more susceptible than C57BL/6 mice to systemic infection with *Listeria monocytogenes* (647), but the difference was most likely due to incomplete backcrossing of chimeric *Il1r1*^{KO} mice onto the C57BL/6 strain (646). However, subsequent studies confirmed that *Il1r1*^{KO} mice were more susceptible to infection by different bacteria, including *Staphylococcus aureus* (648, 649), *Streptococcus pneumoniae* (650)*, Bordetella pertussis* (651) and *Mycobacterium tuberculosis* (652). However, the mechanisms underlying this phenotype differ between experimental settings. For instance, increased susceptibility to *S. aureus* was due to delayed neutrophil recruitment at the site of infection in *Il1r1*^{KO} mice (648), a phenotype also noted during sterile inflammation (624). In contrast, *Il1r1*^{KO} mice were more susceptible to *B. pertussis* infection and showed increased lung pathology because of an exacerbated immune response (651).Hence, the net effect of IL-1β and IL-1R signaling on limiting infection and associated pathology may differ dramatically for different bacterial infections.

Recently, *Il1r1*^{KO} mice were reported to be more susceptible to acute intestinal inflammation induced by DSS administration (653) or by infection by the attaching/effacing bacterium *C.rodentium* (506). In contrast, administration of IL-1β blocking agents was beneficial in DSS colitis (582, 616-618), as discussed in chapter 3

In light of the contradictory results from acute models of intestinal infection and inflammation, we used the Hh + αIL-10R model to better characterize the role of IL-1β and IL-1R signaling in intestinal inflammation. Previous studies from our laboratory indicated that this model of intestinal inflammation was dependent on the common signaling adaptor MyD88 (623). Since IL-1R signals through MyD88, we hypothesized that IL-1β might play a pathogenic role in this model of colitis. We used two approaches to determine the role of IL-1β/IL-1R signaling in the Hh + αIL-10R IBD model:

a. Blocking IL-1β activity in vivo using a monoclonal antibody (αIL-1β).
b. Using *Il1r1*^{KO} mice.

4.2 Results

4.2.1 IL-1β promotes *H.hepaticus*-driven intestinal inflammation in immune-competent mice.

To assess the role of IL-1β in intestinal inflammation in a lymphocyte-replete setting, we used a model of colitis where *H.hepaticus* infection of C57BL/6 (WT) mice is accompanied by the administration of a αIL-10R antibody (Hh + αIL-10R), leading to the development of severe typhlocolitis and systemic inflammation (438). Firstly, we examined whether *H.hepaticus*-induced intestinal inflammation in WT mice was

associated with increased levels of IL-1β. To measure the concentration of active IL-1β in the inflamed intestine we analyzed the levels of IL-1β secretion by organ cultures from the gastrointestinal tract of Hh + αIL-10R WT mice (**Fig.4-1**). Intestinal inflammation in the colon and caecum of Hh + αIL-10R WT mice was associated with increased IL-1β secretion, whereas neither *H. hepaticus*-infection alone nor treatment with αIL-10R alone elicited high levels of IL-1β (**Fig.4-1**).

To evaluate the functional outcome of IL-1β secretion on intestinal inflammation, we blocked IL-1β activity *in vivo* by treating Hh + αIL-10R WT mice with a αIL-1β antibody. Administration of αIL-1β antibody resulted in a significant decrease in inflammation in the colon of Hh + αIL-10R WT mice (**Fig.4-2A,C**), with a trend towards decreased inflammation in the caecum also observed (**Fig.4-2B,C**). However, this was not associated with decreased systemic inflammation, with similar spleen weight and spleen cellularity observed in Hh + αIL-10R WT mice receiving the αIL-1β antibody or the isotype control (**Fig.4-3**). Moreover, treatment with αIL-1β did not significantly affect *H. hepaticus* colonization (**Fig.4-4**).

Next, we assessed the levels of pro-inflammatory cytokine production in the intestine of Hh + αIL-10R WT mice treated with a αIL-1β antibody by measuring cytokine production by organ cultures from the colon and caecum of treated animals. Surprisingly, we observed similar levels of many pro-inflammatory cytokines in the colon and caecum of Hh + αIL-10R WT mice treated with either αIL-1β or an isotype control (**Fig.4-5**). This suggests that blocking IL-1β does not significantly inhibit all intestinal pro-inflammatory responses in this model.

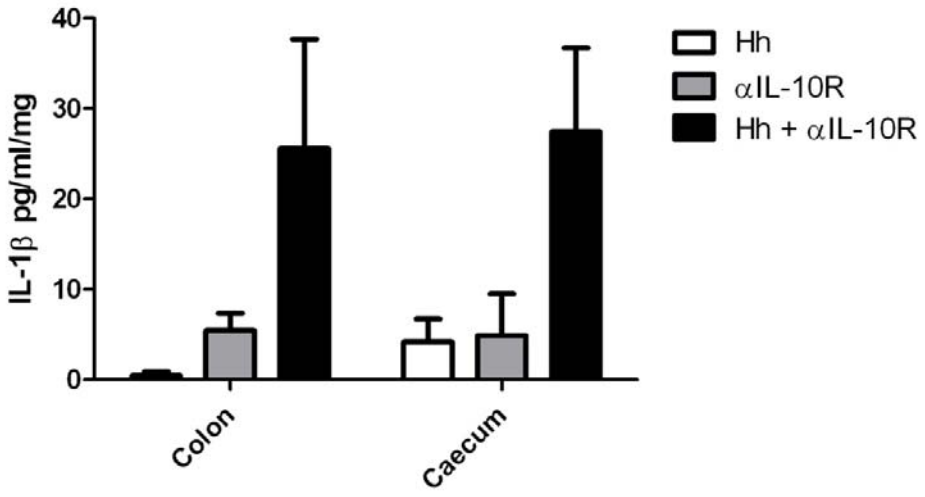


Figure 4-1. *H.hepaticus*-driven intestinal inflammation is associated with increased levels of IL-1β in the colon and caecum of colitic mice. C57BL/6 mice were infected with *H.hepaticus* and treated with αIL-10R (1mg/mouse/week) (Hh + αIL-10R). After 4 weeks the mice were culled and organ explants from colon and caecum were isolated and incubated O/N in complete medium. Results show IL-1β levels in the supernatants normalized to the weight of the explants Results are shown as mean ± SEM (n= 2 for αIL-10R mice and 10 for *H.hepaticus*-infected or *H.hepaticus* + αIL-10R treated mice).

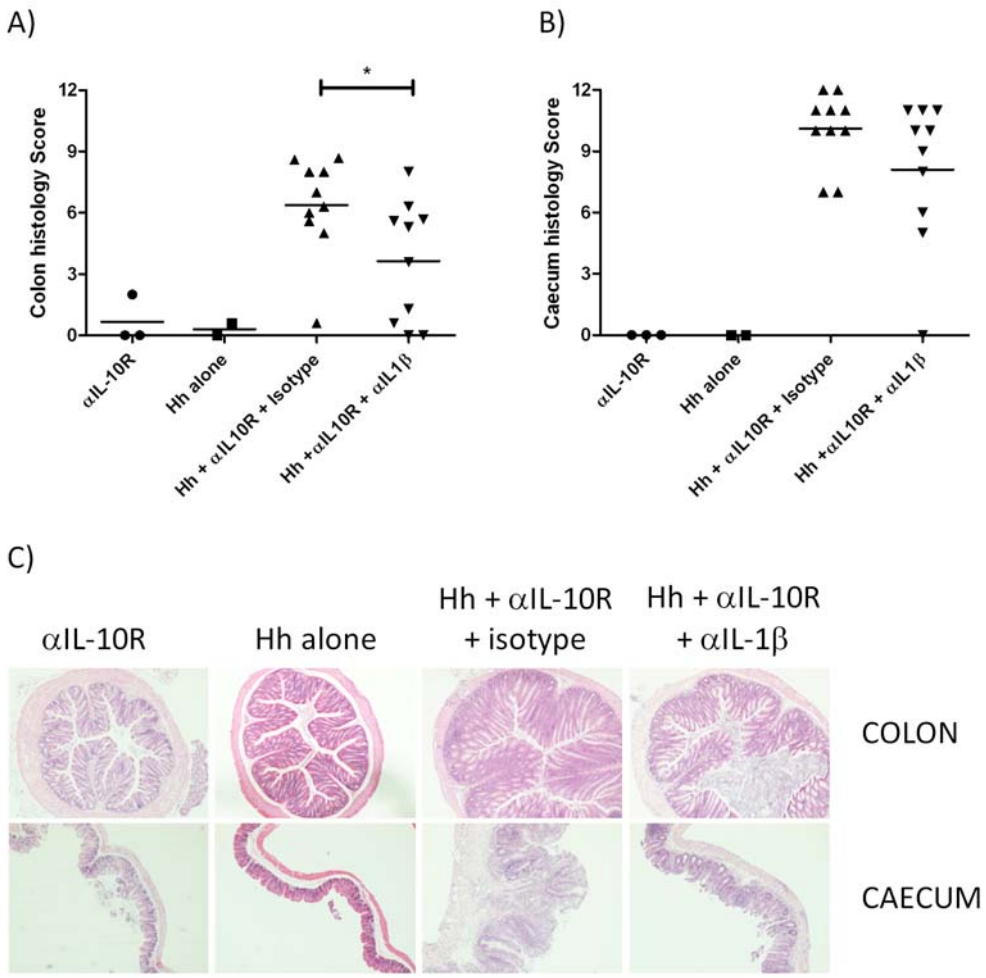


Figure 4-2. Treatment with αIL-1β results in reduced inflammation in the colon and caecum of lymphocyte-replete mice. C57BL/6 mice were infected with *H.hepaticus* and treated weekly with αIL-10R (Hh + αIL-10R) and concurrently treated with αIL-1β or an isotype control (1mg/mouse/week, i.p.). Mice were sacrificed 4 weeks after infection and pathology was assessed histologically. Histology was scored in a blinded fashion to avoid bias. Each symbol represents an individual mouse and data are pooled from 2 independent experiments (n=3 for αIL-10R alone, 2 for Hh alone, 10 for Hh +αIL-10R + isotype and 10 for Hh + αIL-10R + αIL-1β). Bars represent the mean histological score for each group. *p<0.05, calculated using the Mann-Withney test. A) Colon inflammation. B) Caecum inflammation. C) Representative micrograph pictures (original magnification=50X)

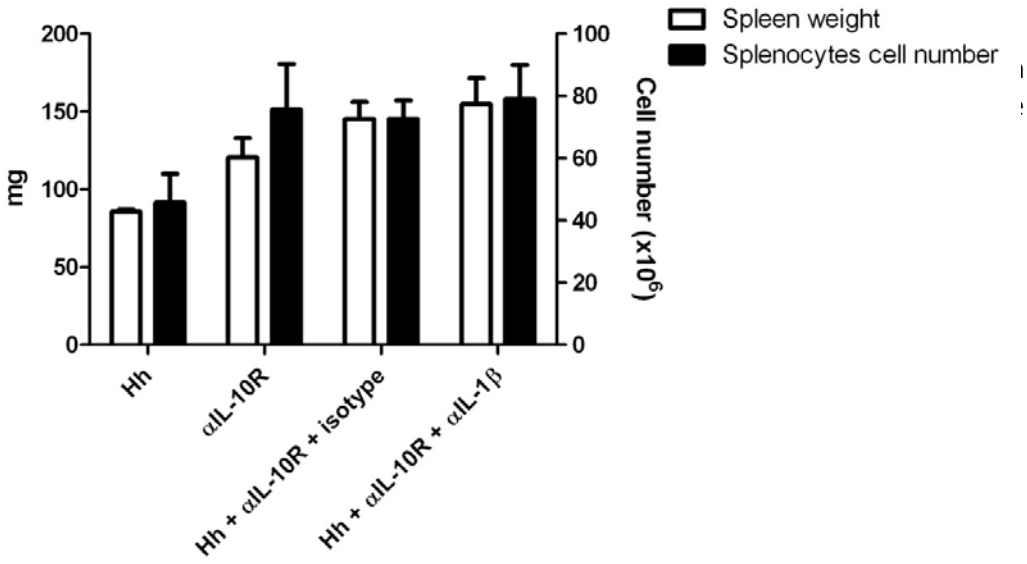


Figure 4-3. αIL-1β treatment does not affect systemic disease in *H.hepaticus*-infected αIL-10R treated WT mice. C57BL/6 mice were infected with *H.hepaticus* and treated weekly with αIL-10R (Hh + αIL-10R) and concurrently treated with αIL-1β or an isotype control (1mg/mouse/week, i.p.) for 4 weeks. At sacrifice, spleen weight and splenocytes cell numbers were evaluated. All data are shown as mean ± SEM (n=2-10 from 2 independent experiments).

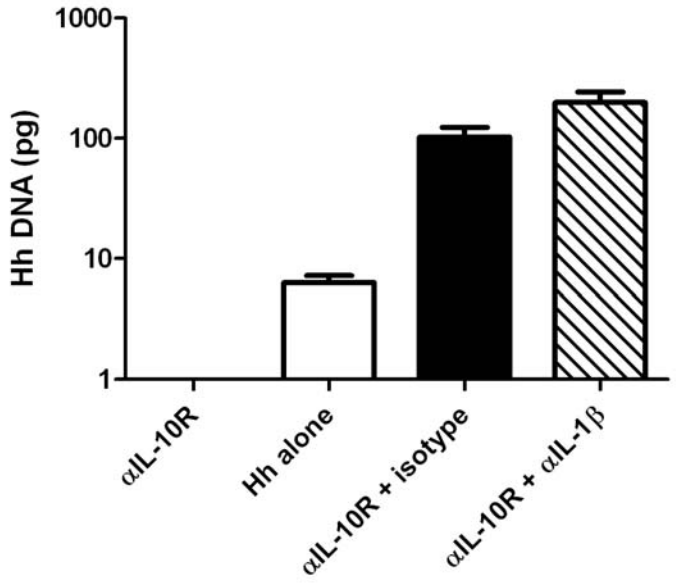


Figure 4-4. αIL-1β treatment does not affect H.hepaticus colonization in lymphocyte replete mice. C57BL/6 mice were infected with *H.hepaticus* and treated weekly with αIL-10R (Hh + αIL-10R) and concurrently treated with αIL-1β or an isotype control (1mg/mouse/week, i.p.) for 4 weeks. At sacrifice, DNA was isolated from cecal contents and levels of *H. hepaticus* DNA were determined using a q-PCR method based on the *cdtB* gene (604). Data are expressed as mean ± SEM (n=2-10 from 2 independent experiments).

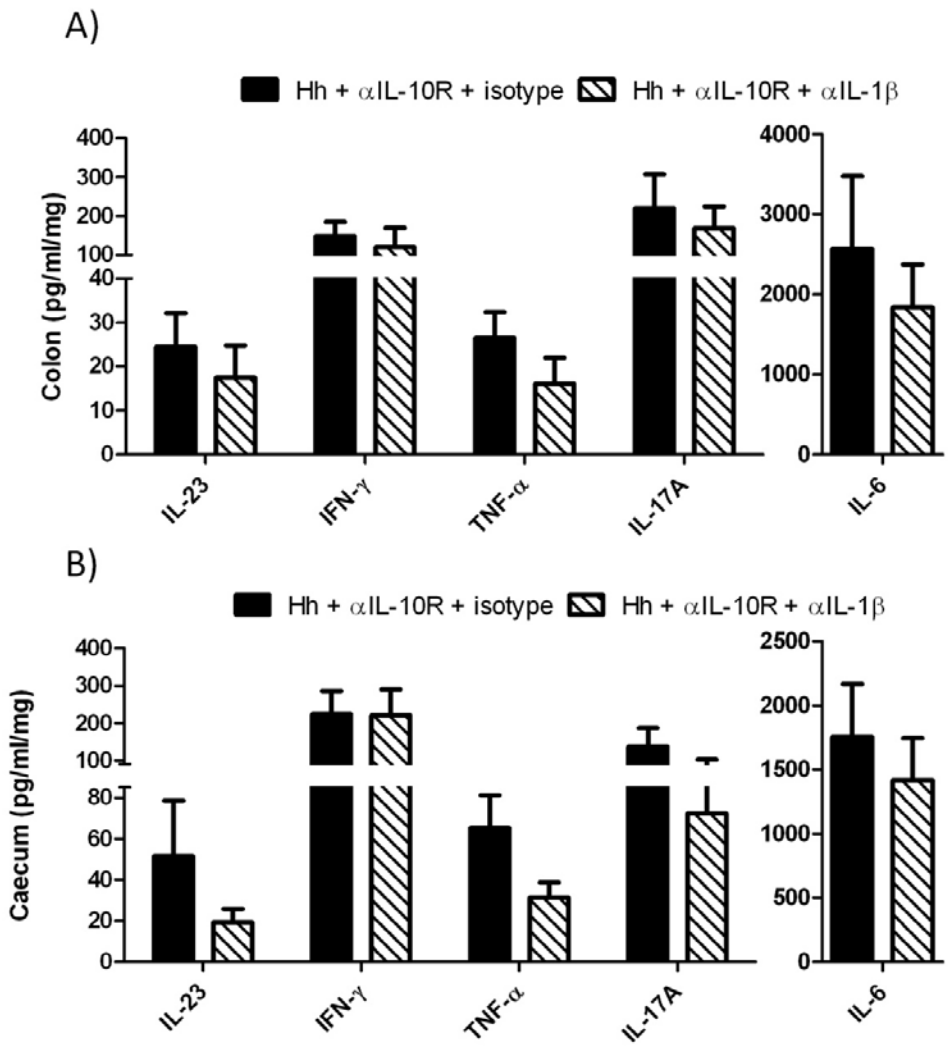


Figure 4-5. No effect of αIL-1β treatment on the cytokine production in the intestine of αIL-10R-treated *H.hepaticus*-infected mice. C57BL/6 mice were infected with *H.hepaticus* and treated weekly with αIL-10R (Hh + αIL-10R) and concurrently treated with αIL-1β or an isotype control (1mg/mouse/week, i.p.) for 4 weeks. At sacrifice, organ explants were cultured overnight in complete RPMI, and cytokine release in the supernatants was measured. Results represent mean cytokine levels (±SEM) normalized by weight of intestinal tissue (n=2-10 from 2 independent experiments).A) Cytokine secretion from colon explants. B) Cytokine secretion from caecum explants.

4.2.2 Moderate effect of IL-1β blockade on the recruitment of CD4+ T effector cell populations in the inflamed intestine.

The development of intestinal inflammation in Hh + αIL-10R WT mice is associated with the expression of both Th1 and Th17 responses in the colon (438) as well as the appearance of IL-17A$^+$IFN-γ^+ double producing CD4$^+$ T cells (203). Hence, we examined the effects of IL-1β blockade in modulating colonic CD4$^+$T cell infiltrates in this model. Thus, we isolated lymphocytes from the colonic lamina propria (cLP) and MLN of Hh + αIL-10R WT mice that received αIL-1β or isotype control and evaluated the frequencies of IL-17A$^+$IFN-γ^-, IL-17A$^-$IFN-γ^+ and IL-17A$^+$IFN-γ^+ CD4$^+$ T cells by intracellular FACS analysis. As expected, Hh + αIL-10R WT mice developed strong pro-inflammatory CD4$^+$ T cell responses in the colon, as shown by the significantly increased frequencies of IL-17A$^+$ and IFN-γ^+ CD4$^+$ T cells observed, when compared to mice receiving Hh or αIL-10R alone (**Fig.4-6A,B**). Treatment of Hh + αIL-10R WT mice with αIL-1β resulted in a minor decrease in the frequency of both IL-17A$^+$IFN-γ^- and IL-17A$^+$IFN-γ^+ CD4$^+$ T cells in the colon compared to Hh + αIL-10R WT mice that received an isotype control (**Fig.4-6A**). Thus, the proportion of total IL-17A$^+$ CD4$^+$ T cells in the colon was significantly decreased in αIL-1β treated cohorts compared to those treated with isotype control, whereas the proportion of total IFN-γ^+ CD4$^+$ T cells was not significantly different (**Fig.4-6B**). In the MLN of Hh + αIL-10R WT mice, we observed small, but significant, increases in the frequencies of IL-17A$^+$ and IFN-γ^+ CD4$^+$ T cells compared to mice receiving Hh alone (**Fig.4-6C**). However, we did not find any significant differences in CD4$^+$ T cell effector responses in MLN between Hh + αIL-10R WT mice that received αIL-1β or isotype control (**Fig.4-6C**). Overall, these

results suggest that IL-1β blockade marginally affects the differentiation/recruitment of IL-17A producing CD4+ T cells in the lamina propria.

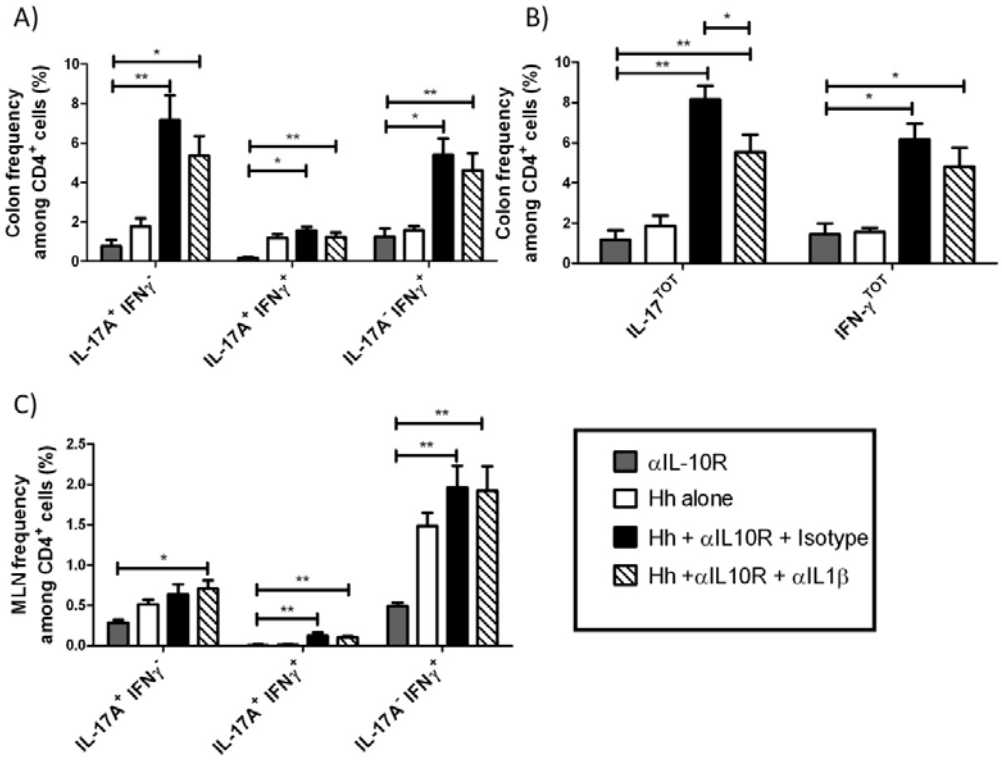


Figure 4-6. Characterization of CD4$^+$ T cell effector populations in the colon and MLN of αIL-1β treated *H.hepaticus*-infected αIL-10R treated mice. C57BL/6 mice were infected with *H.hepaticus* and treated weekly with αIL-10R (Hh + αIL-10R) and concurrently treated with αIL-1β or an isotype control (1mg/mouse/week, i.p.) for 4 weeks. At sacrifice, lymphocytes were isolated from the cLP and MLN and the frequencies of IL-17A$^+$ and IFNγ$^+$ cells were evaluated by intracellular staining and FACS analysis. All data are gated on CD4$^+$ T cells. Data are shown as average ± SEM (n=2-10 from 2 independent pooled experiments). A) Frequency of pro-inflammatory populations in the cLP. B) Frequencies of total IL-17A$^+$ and total IFN-γ$^+$ T cells in the colon. C) Frequency of pro-inflammatory populations in the MLN

*p<0.05, **p<0.005, Mann-Whitney test.

4.2.3 The differentiation of FOXP3$^+$ CD4$^+$ T cells is independent of IL-1β in the inflamed intestine.

The attenuated pathology observed in mice receiving the αIL-1β antibody could be explained by an increased frequency of CD4$^+$ regulatory T cells (Treg) in the gut, which might limit disease through IL-10-independent mechanisms. Hence, we analyzed the frequency of FOXP3$^+$ CD4$^+$ T cells in the colon lamina propria of Hh + αIL-10R WT mice receiving either αIL-1β antibody or an isotype control (**Fig.4-7**). We found that Hh + αIL-10R WT mice harboured increased frequencies of FOXP3$^+$ CD4$^+$ T cells in the colon compared to mice infected with *H. hepaticus* alone (**Fig.4-7**). However, blocking IL-1β did not alter the frequency of regulatory FOXP3$^+$ CD4$^+$ T cells in the colon of Hh + αIL-10R WT mice (**Fig.4-7**). This effect was also observed in the MLN, where the frequency of FOXP3$^+$ CD4$^+$ T cells was lower than what observed in the colon (**Fig.4-7**). Similarly, no difference in Treg frequency was observed between *H.hepaticus*-infected αIL-10R treated mice receiving αIL-1β or an isotype control (**Fig.4-7**). Interestingly, treatment with αIL-10R alone also increased the frequency of FOXP3$^+$ CD4$^+$ T cells in the colon (**Fig.4-7**), suggesting that IL-10 regulates FOXP3$^+$ CD4$^+$ T cell frequencies independently of ongoing infection or inflammation in the gut.

Recently, it has been proposed that IL-1β promotes the differentiation of FOXP3$^+$ CD4$^+$ regulatory T cells from IL-17A-producing cells (138). Intracellular staining analysis revealed a small population of FOXP3$^+$ IL-17A$^+$ CD4$^+$ T cells in the colon of Hh + αIL-10R WT mice, that was increased relative to the frequencies found in mice infected with Hh only or treated with αIL-10R alone (**Fig.4-8**). However, we did not

observe any significant difference in the frequency of $FOXP3^{+}$ $IL\text{-}17A^{+}$ $CD4^{+}$ T cells between Hh + αIL-10R WT mice receiving either αIL-1b or an isotype control (**Fig.4-8**).

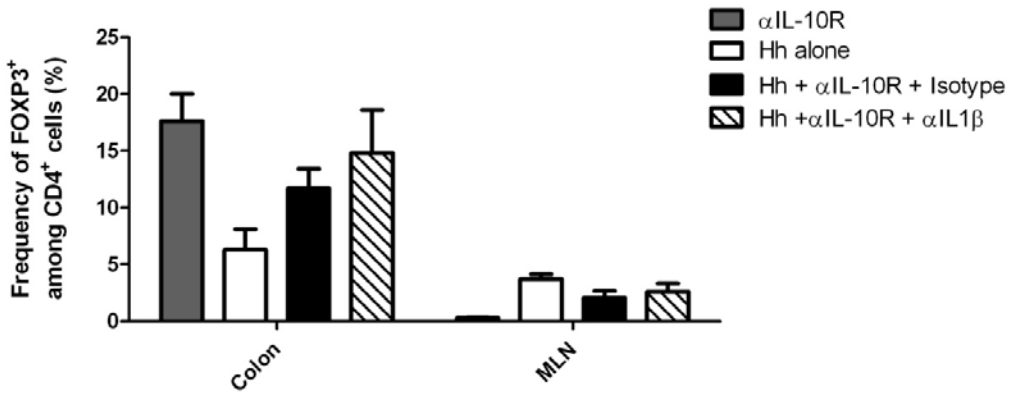


Figure 4-7. IL-1β blockade does not affect the frequency of regulatory FOXP3$^+$ T cells in the colon of *H.hepaticus*-infected αIL-10R-treated WT mice. C57BL/6 mice were infected with *H.hepaticus* and treated weekly with αIL-10R (Hh + αIL-10R) and concurrently treated with αIL-1β or an isotype control (1mg/mouse/week, i.p.) for 4 weeks. At sacrifice, lymphocytes were isolated from the cLP and MLN and the frequency of FOXP3$^+$T cells was evaluated by intracellular staining and FACS analysis. All data are gated on CD4$^+$ T cells. Data are shown as average ± SEM (n=2-10 from 2 independent pooled experiments).

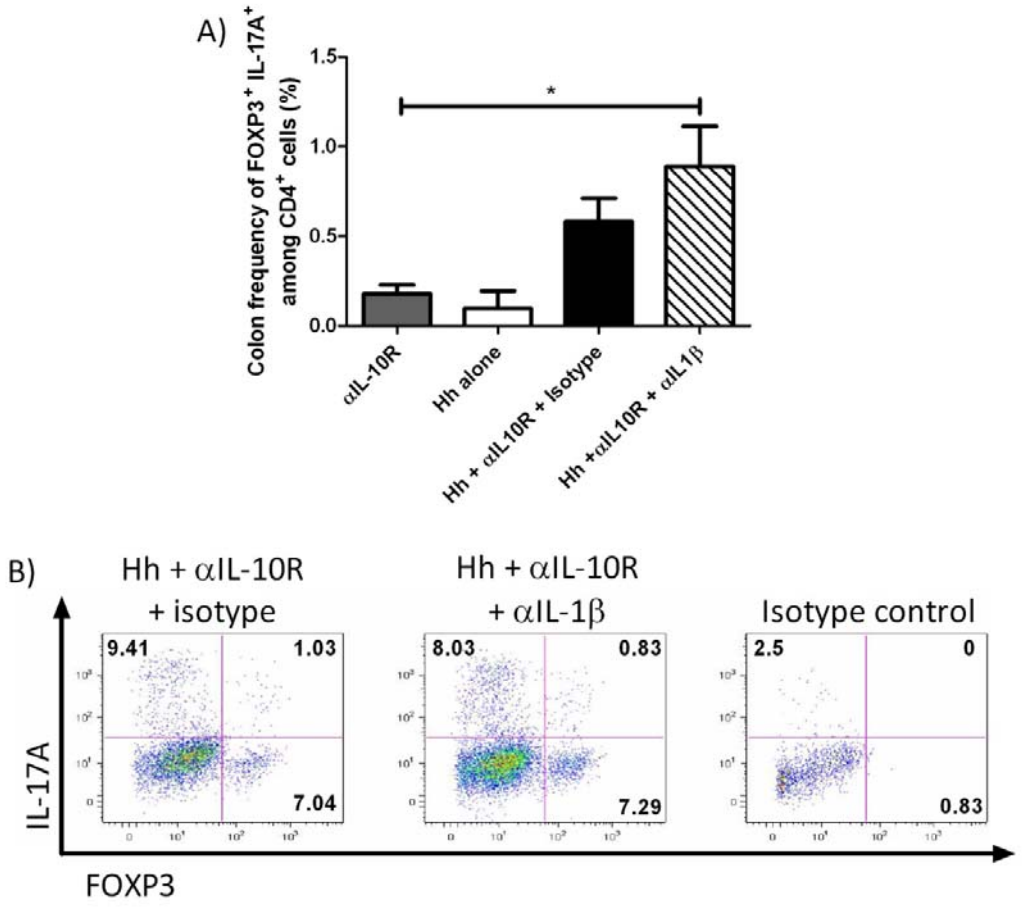


Figure 4-8. IL-1β blockade does not affect the frequency of CD4$^+$ FOXP3$^+$ IL-17A$^+$ T cells in the colon of *H.hepaticus*-infected αIL-10R-treated WT mice. C57BL/6 mice were infected with *H.hepaticus* and treated weekly with αIL-10R (Hh + αIL-10R) and concurrently treated with αIL-1β or an isotype control (1mg/mouse/week, i.p.) for 4 weeks. At sacrifice, lymphocytes were isolated from the cLP and the frequency of IL-17A$^+$ FOXP3$^+$ T cells was evaluated by intracellular staining and FACS analysis. All data are gated on CD4$^+$ T cells. A) Frequency of FOXP3$^+$ IL-17A$^+$ CD4$^+$ T cells in the colon. Data are gated on CD4$^+$T cells and shown as average ± SEM (n=2-10 from 2 independent pooled experiments). B) Representative FACS plots. Numbers represent frequencies of events in the quadrant.

*p<0.05, Mann Whitney test.

4.2.4 IL-1R1 signaling is dispensable for the development of intestinal inflammation in *H. hepaticus*-infected WT mice treated with anti-IL-10R.

Overall, the data presented so far show that IL-1β promotes *H. hepaticus*-dependent chronic intestinal inflammation in the WT host. To further investigate the role of the IL-1 pathway in intestinal inflammation, we set to analyze the role of IL-1R signaling. To this end, cohorts of *Il1r1*^{KO} and WT mice were infected with *H.hepaticus* and treated with αIL-10R antibody (Hh + αIL-10R). To our surprise, Hh + αIL-10R *Il1r1*^{KO} mice developed comparable colitis and caecal inflammation to Hh + αIL-10R WT mice (**Fig.4-9A-C**). Moreover, Hh + αIL-10R *Il1r1*^{KO} mice developed comparable systemic inflammation to Hh + αIL-10R WT mice, as shown by similar increases in spleen weight and spleen cell number, compared to controls that were infected with Hh only (**Fig.4-10**). Analysis of cytokine secretion by cLPL revealed a more complex scenario. Although no statistically significant differences were observed, cLPL isolated from Hh + αIL-10R *Il1r1*^{KO} mice showed a trend towards reduced IL-1β, TNF-α and IL-17A secretion compared to cLPL from Hh + αIL-10R WT mice (**Fig.4-11**). Overall, these results indicate that IL-1R1 signaling is dispensable for the induction of systemic and intestinal inflammation following infection with *H. hepaticus* and blockade of the IL-10R pathway. However, IL-1R1 signaling might augment the secretion of some pro-inflammatory cytokines in the colon.

Previous reports showed that *Il1r1*^{KO} mice may exhibit defects in bacterial handling and were more susceptible than WT mice to several systemic and mucosal bacterial infections (647, 649, 654). Hence, we evaluated the levels of *H. hepaticus*

colonization in WT and *Il1r1*^{KO} mice in the presence or absence of αIL-10R treatment. However, we found that that, irrespective of the presence or absence of overt inflammation, *Il1r1*^{KO} mice showed comparable levels of *H. hepaticus* colonization to WT mice (**Fig.4-12).**

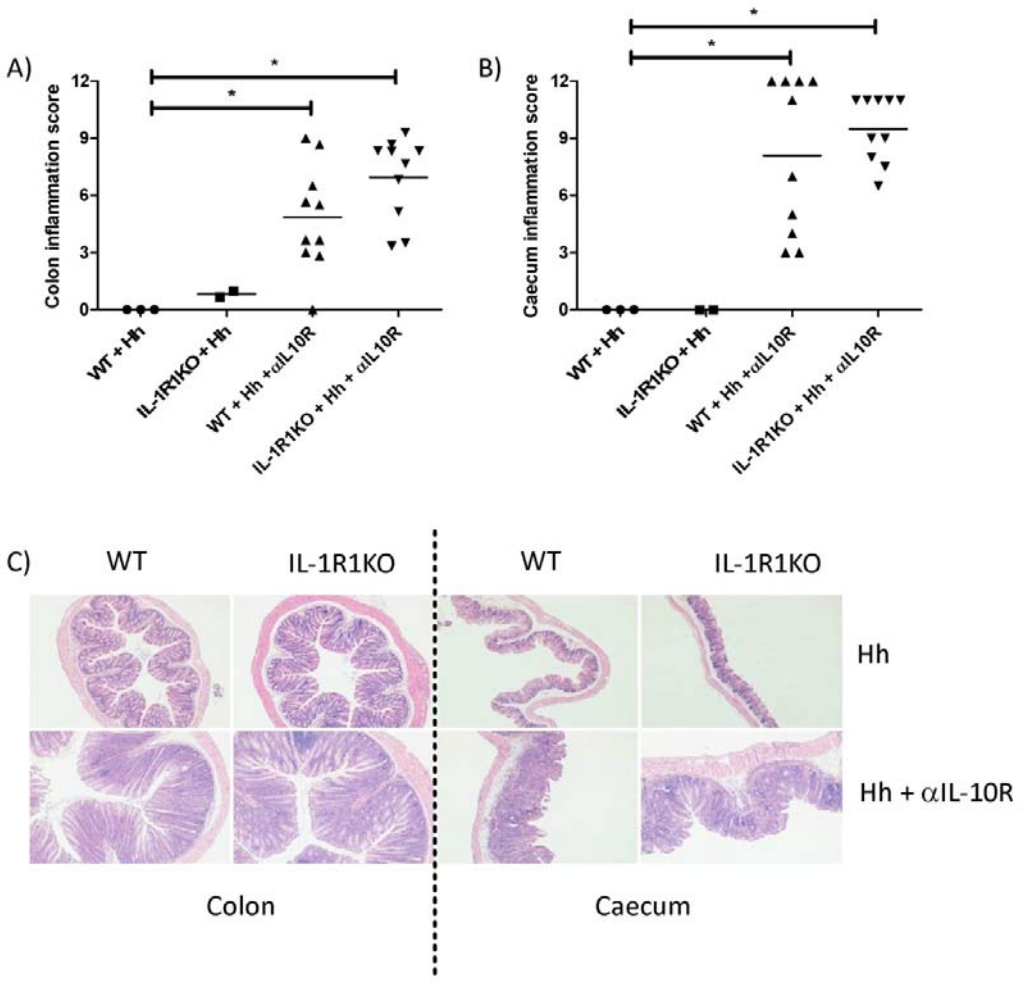


Figure 4-9. IL-1R1 deficiency does not affect the development of intestinal inflammation following infection with *H. hepaticus* and αIL-10R treatment. *Il1r1*^{KO} or C57BL/6 WT mice were infected with *H. hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.) (Hh + αIL-10R). After 4 weeks, mice were sacrificed and intestinal pathology was assessed histologically in a blinded fashion to avoid bias. Each symbol represents an individual mouse and data are pooled from 2 independent experiments. Bars represent the mean histological score for each group (n=2 for *Il1r1*^{KO} + Hh, 3 for WT + Hh, 10 for WT + Hh + αIL10R and *Il1r1*^{KO} + Hh + αIL-10R). A) Colon histological score. B) Caecum histological score. C) Representative micrographs. Original magnification =50x

*p<0.05, Mann Whitney test

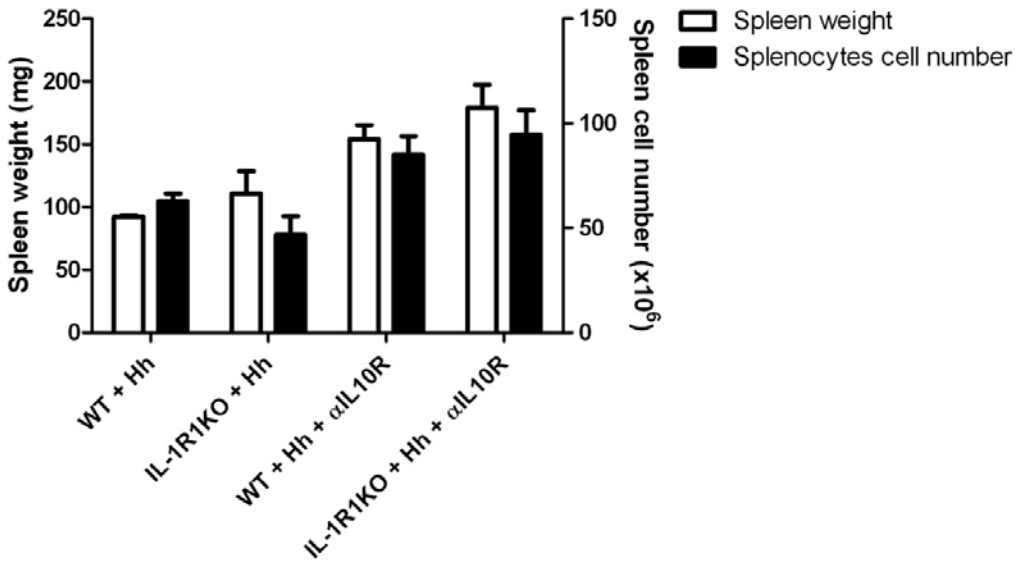


Figure 4-10. IL-1R1 deficiency does not affect systemic disease in *H. hepaticus*-infected αIL-10R-treated mice. *Il1r1*^{KO} or C57BL/6 WT mice were infected with *H. hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.) for 4 weeks (Hh + αIL-10R). At sacrifice, spleen weight and splenocyte cell numbers were evaluated. All data are shown as mean ± SEM (n=2-7 from 2 independent pooled experiments).

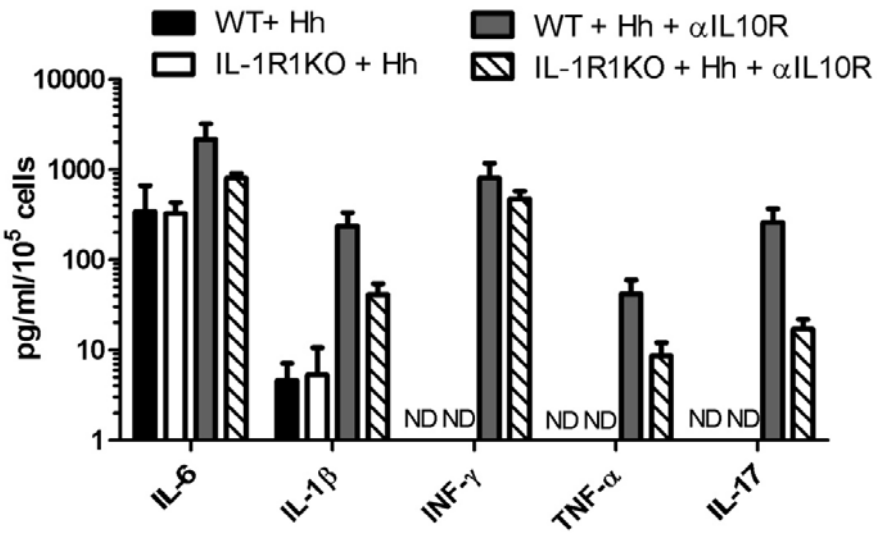


Figure 4-11. Comparable pro-inflammatory cytokine secretion by cLPL from colitic *Il1r1*^{KO} and WT mice. *Il1r1*^{KO} or C57BL/6 WT mice were infected with *H. hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.) for 4 weeks (Hh + αIL-10R). At sacrifice, cLPL were isolated from and cultured overnight in complete RPMI medium. Cytokine levels in the supernatants were assessed by Flowcytomix Bead Assay. All data are shown as mean ± SEM (n=2-7 from 2 independent pooled experiments). ND= not detectable.

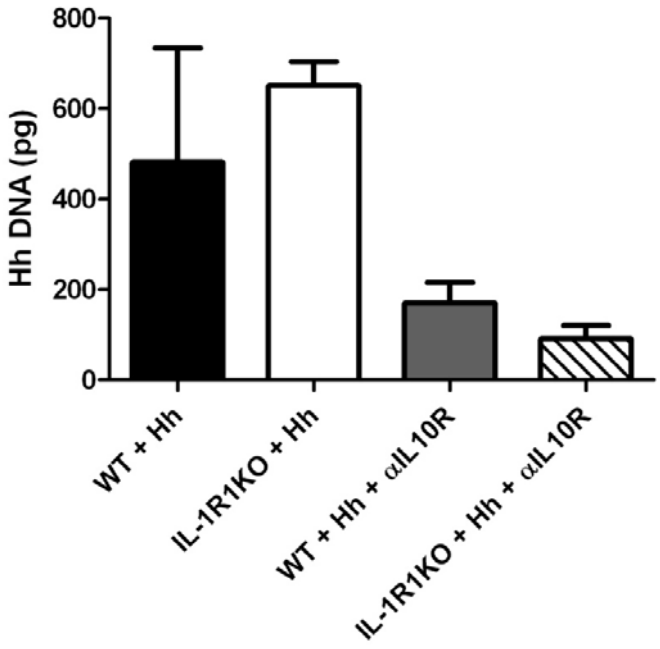


Figure 4-12. IL-1R1 deficiency does not affect *H. hepaticus* colonization. *Il1r1*^{KO} or C57BL/6 WT mice were infected with *H.hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.) for 4 weeks (Hh + αIL-10R). At sacrifice, DNA was isolated from cecal contents and levels of *H. hepaticus* DNA were determined using a q-PCR method based on the *cdtB* gene (604). Data are expressed as mean ± SEM (n=2-10 from 2 independent pooled experiments).

4.2.5 IL-1R signaling is dispensable for the accumulation of pro-inflammatory leukocyte populations during chronic intestinal inflammation.

IL-1 has an established role in inducing neutrophil recruitment through the induction of chemokines by endothelial cells (524, 538) and *Il1r1*^{KO} mice exhibited defective neutrophil recruitment in some models of infection (648, 655, 656) and sterile inflammation (624). One of the features of systemic inflammation induced by *H.hepaticus* infection and αIL-10R treatment is the accumulation of CD11b^{+}Gr1^{Hi} granulocytes in the spleen (438, 623). To test if this was dependent on IL-1R signaling we analyzed the frequency of CD11b^{+}Gr1^{Hi} cells in the spleen of WT and *Il1r1*^{KO} mice, following Hh + αIL-10R. As expected, we observed a significant increase in the frequency of granulocytes in the spleen of WT mice following Hh + αIL-10R, compared to WT mice infected with Hh only (**Fig.4-13**). However, Hh + αIL-10R *Il1r1*^{KO} mice showed frequencies of CD11b^{+}Gr1^{Hi} cells comparable to those observed in Hh + αIL-10R WT mice (Fig.4-14), suggesting that in this model IL-1R1 signaling is dispensable for neutrophil recruitment in the spleen.

Next, we examined the frequency of pro-inflammatory CD4^{+} T cell populations in the colon and MLN of Hh + αIL-10R mice. As previously mentioned, IL-1R signalling has been shown to be crucial for Th17 differentiation *in vitro* and *in vivo* (138, 139). However, our intracellular staining analysis revealed that Hh + αIL-10R *Il1r1*^{KO} mice exhibited similarly increased frequencies of IL-17A^{+}IFN-γ^{-}, IL-17A^{+}IFN-γ^{+}, IL-17A^{-}IFN-γ^{+} CD4^{+} T cells in the colon as found in Hh + αIL-10R WT mice, although a slightly reduced frequency of IL-17A^{+} IFN-γ^{-} CD4^{+}T cells was observed (**Fig.4.14A**). By contrast, in the MLN the frequencies of IL-17A^{+} IFN-γ^{+} CD4^{+} T cells were significantly

decreased and IL-17A^{+} IFN-γ^{+} CD4^{+} T cells were also reduced in Hh + αIL-10R *Il1r1*^{KO} mice compared to Hh + αIL-10R WT mice (**Fig.4.14B**). However, calculation of total cell numbers revealed comparable accumulation of pro-inflammatory CD4^{+} T cell populations in the colon and MLN of Hh + αIL-10R *Il1r1*^{KO} mice and Hh + αIL-10R WT mice (**Fig.4-15A-B**).

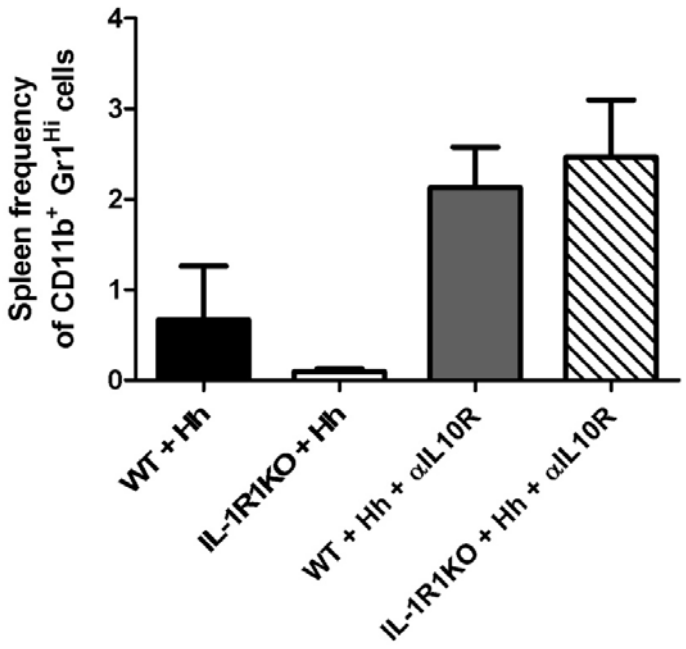


Figure 4-13. No effect of IL-1R1 deficiency on the frequency of CD11b^{+} Gr1^{Hi} granulocytes in the spleen of *H. hepaticus*-infected αIL-10R-treated mice. *Il1r1^{KO}* or C57BL/6 WT mice were infected with *H. hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.) for 4 weeks (Hh + αIL-10R). At sacrifice, lymphocytes were isolated from the spleens and the frequency of CD11b^{+}Gr1^{Hi} granulocytes was evaluated by FACS analysis. All data are shown as mean ± SEM (n=2-10 from 2 independent pooled experiments).

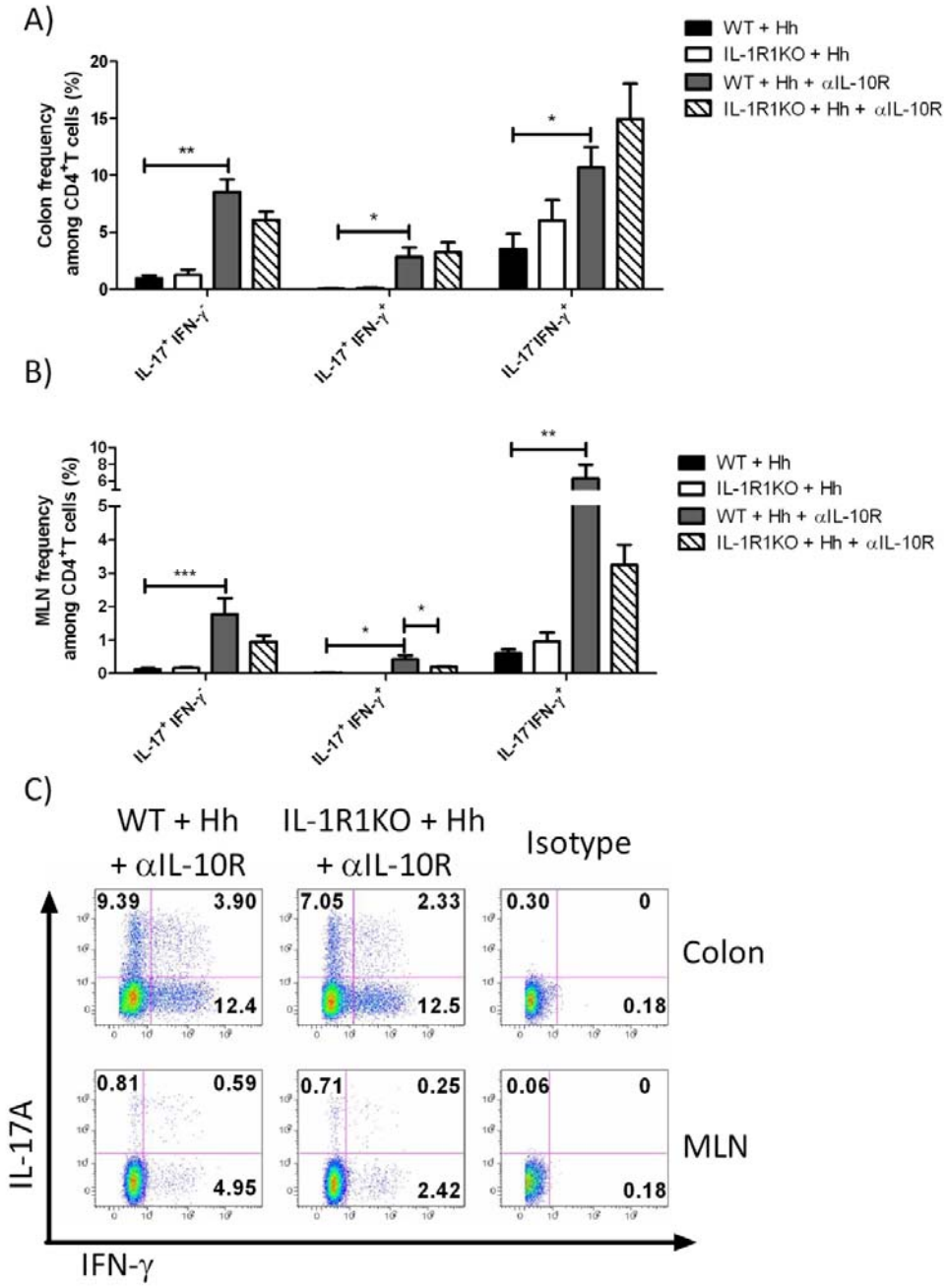


Figure 4-14. Characterization of pro-inflammatory CD4$^+$ T cell populations following *H.hepaticus* infection and αIL-10R treatment in *Il1r1^{KO}* mice. *Il1r1^{KO}* or C57BL/6 WT mice were infected with *H.hepaticus* orally (10^8CFU), treated with αIL-10R antibody (1mg/week i.p.) (Hh + αIL-10R) and culled after 4 weeks. Lymphocytes were isolated from the cLP and MLN. The frequencies of pro-inflammatory CD4$^+$ T cell populations were assessed by intracellular staining and FACS analysis. All data are shown as mean ± SEM (n=2-10 from 2 independent experiments). A) Frequency of pro-inflammatory CD4$^+$ T cell populations in the colon. B) Frequency of pro-inflammatory CD4$^+$ T cell populations in the MLN. C) Representative FACS plots. *p<0.05, **p<0.005 Mann Whitney. Numbers represent frequencies of events in the quadrant.

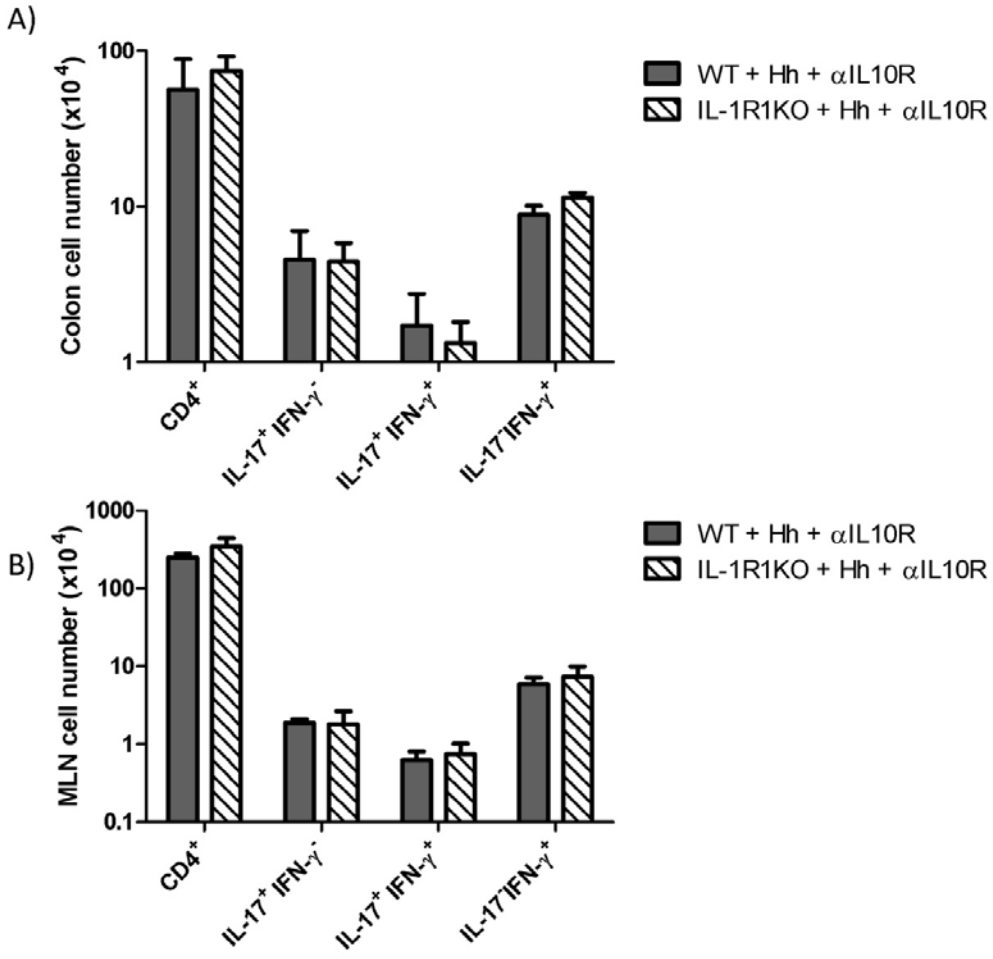


Figure 4-15. Accumulation of pro-inflammatory CD4$^+$ T cells in the colon and MLN of *H.hepaticus*-infected αIL-10R-treated C57BL/6 mice is not dependent on IL-1R signaling. *Il1r1^{KO}* or C57BL/6 WT mice were infected with *H.hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.) (Hh + αIL-10R). After 4 weeks, the mice were culled and lymphocytes were isolated from the cLP and MLN. The numbers of pro-inflammatory CD4$^+$ T cell populations were assesed by FACS analysis. All data are shown as mean ± SEM (n=2-10 from 2 independent experiments). A) Number of pro-inflammatory CD4$^+$ T cells in the colon. B) Number of pro-inflammatory CD4$^+$ T cells in the MLN

4.2.6 IL-1R expression is required for the differentiation of FOXP3$^+$IL-17A$^+$ CD4$^+$ T cells in the intestine.

Finally, we analyzed the frequency of FOXP3$^+$ Treg cells in the colon and MLN of *Il1r1*^{KO} mice following Hh + αIL-10R. We observed similar frequencies of FOXP3$^+$ T cells in the colon of Hh + αIL-10R WT mice and of Hh + αIL-10R *Il1r1*^{KO} mice (**Fig.4-16**), suggesting that IL-1 signaling is not required for Treg accumulation at this site. As previously shown, induction of colitis by Hh + αIL-10R was not associated with an expansion of the FOXP3$^+$ Treg cell compartment in the MLN (438). However, we did observe a significant decrease in the frequency of FOXP3$^+$ CD4$^+$ T cells in the MLN of Hh + αIL-10R *Il1r1*^{KO} mice compared to Hh + αIL-10R WT mice (**Fig.4-16**).

It has been proposed that IL-1β delivers a pro-inflammatory signal to Treg, promoting their differentiation into IL-17A-producing cells (138). In agreement with this finding, we observed decreased frequencies of FOXP3$^+$ IL-17A$^+$ CD4$^+$ T cells in the colon of Hh + αIL-10R *Il1r1*^{KO} mice when compared to Hh + αIL-10R WT mice (**Fig.4-17**).

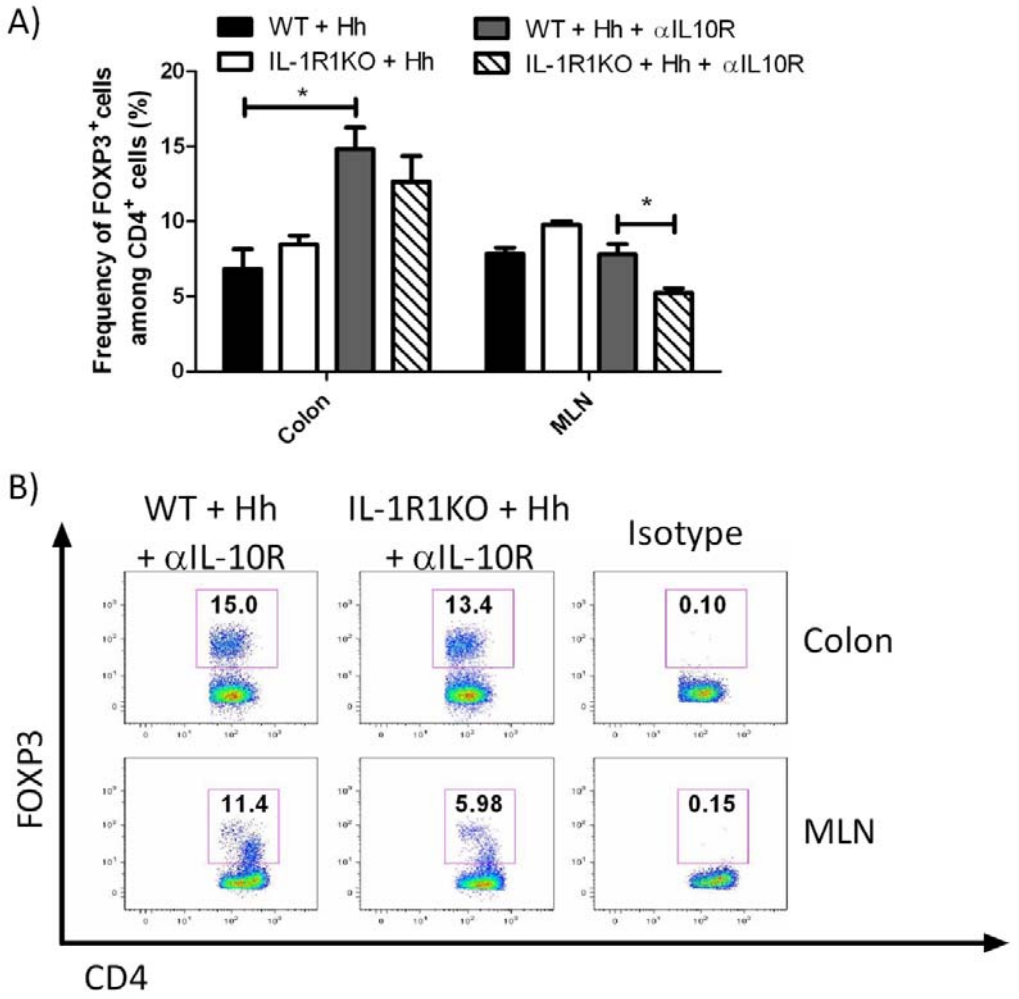


Figure 4-16. Frequencies of FOXP3$^+$ CD4$^+$ T cells are comparable in colon but reduced in MLN of colitic *Il1r1^{KO}* mice. *Il1r1^{KO}* or C57BL/6 WT mice were infected with *H.hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.) for 4 weeks (Hh + αIL-10R). At sacrifice, lymphocytes were isolated from cLP and MLN and the frequencies of CD4$^+$ FOXP3$^+$ T cell populations were assessed by intracellular staining and FACS analysis. All data are gated on CD4$^+$ T cells. A) Frequencies of FOXP3$^+$ CD4$^+$ T cells in the cLP and MLN. Data are shown as mean ± SEM (n=3-10 from 2 pooled independent experiments).B) Representative FACS plots. Numbers represent frequencies of events in gate.

*p<0.05 Mann Whitney test

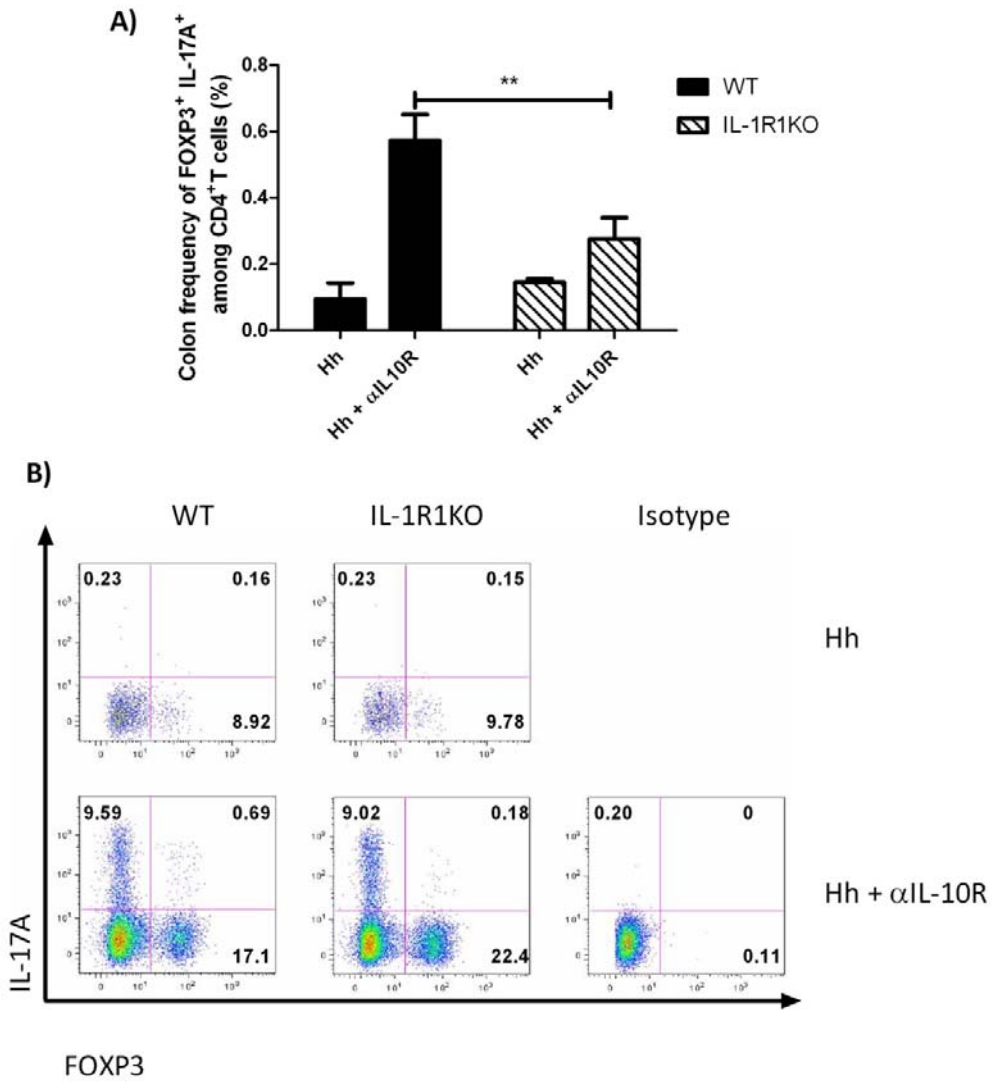


Figure 4-17. Reduced frequency of FOXP3$^+$ IL-17$^+$ CD4$^+$ T cells in the inflamed colon in *Il1r1^{KO}* mice. *Il1r1^{KO}* or C57BL/6 WT mice were infected with *H.hepaticus* orally and treated with αIL-10R antibody (1mg/week i.p.). After 4 weeks, the mice were culled and lymphocytes were isolated from the cLP. The frequencies of FOXP3$^+$IL-17A$^+$ CD4$^+$ T cell populations were assessed by intracellular staining and FACS analysis. Data are shown as mean ± SEM (n=2-10 from 2 independent experiments).A) Frequency of FOXP3$^+$IL-17A$^+$ CD4$^+$ T cells in the colon. Data are gated on CD4$^+$ T cells and are shown as mean ± SEM (n=2-53-10 from 2 pooled independent experiments). B) Representative FACS plots. Numbers represent frequencies of events in the quadrant.

4.3 Discussion

Here, we used a model of *H.hepaticus*-dependent chronic colitis in the lymphocyte replete mouse to assess the role of IL-1β and IL-1R signaling in intestinal inflammation. Our study yielded contrasting results: whilst blocking IL-1β with a monoclonal antibody resulted in amelioration of intestinal inflammation, mice deficient in IL-1R1 developed intestinal inflammation in a similar way to WT mice. Different factors might contribute to this discrepancy.

The simplest explanation for the observed difference between mice treated with αIL-1β and *Il1r1*^{KO} mice is that, when IL-1R signaling is completely missing, other pro-inflammatory pathways play a compensatory role in intestinal inflammation. Such redundancy was already reported for this colitis model when single TLR were genetically ablated (657, 658). Moreover, the fact that the absence of IL-1R signaling is compensated for in other biological processes is exemplified by the fact that *Il1r1*^{KO} mice reproduce normally, whilst IL-1β is essential for the implantation of the blastocyst in the uterus, as i.p.injections of IL-1RA inhibits blastocyst implantation in the uterus of superovulated mice (659).

It should also be considered that IL-1R1 binds both IL-1α and IL-1β and triggers intracellular signaling downstream of both of these cytokines (513). Hence, IL-1R deficiency doesn't perfectly recapitulate the pharmacological blocking of IL-1β, as signaling from both IL-1α and IL-1β is lost. Since treatment with αIL-1β resulted in amelioration of disease, whilst *Il1r1*^{KO} mice developed normal levels of inflammation, it is possible to hypothesize that IL-1α could play a protective role in intestinal inflammation. However, such anti-inflammatory functions of IL-α have not been

described and IL-1α has been implicated in promoting sterile inflammation, specifically in response to cell death (514). As most IL-1α is retained intracellularly, this cytokine is mainly released by dying cells, promoting inflammation and neutrophil recruitment (521, 660) Moreover, it has been shown that peritoneal macrophages and dendritic cells also secrete IL-1α (and IL-1β) following injection of necrotic cells (520). In addition, transgenic mice over-expressing IL-1α develop severe arthritis following abnormal secretion of IL-1α by macrophages and synoviocytes, resulting in excessive recruitment of neutrophil and macrophages to the joint (661). Hence, whilst it is possible that IL-1α exerts an anti-inflammatory effect in intestinal inflammation, the current literature on this cytokine does not support this hypothesis. However, it has been proposed that IL-1α can promote keratinocyte proliferation during wound healing, by inducing the release of mitogenic factors such as KGF (keratinocyte growth factor) from dermal fibroblasts (662, 663). Hence, it is possible that IL-1α promotes repair of the epithelium in the intestine, as intestinal epithelial cell lines express IL-1R1 (664). However, it should be noted that IL-1β can also promote the release of KGF by dermal fibroblasts (662). Moreover, a complete KO mouse for IL-1α does not exist, as the IL-1α-deficient mice currently available still retain expression of the N-terminal pro-piece, which can exert functional activities (514). Hence, a clear definition of the role of IL-1α in intestinal inflammation might prove challenging.

Secondly, IL-1R1-independent functions of IL-1β have been described, particularly in the brain. For instance, intracerebroventricular (i.c.v.) injection of IL-1β exacerbates brain damage caused by cerebral ischemia, and this effect was also observed *Il1r1*^{KO}

mice (665) (666). Moreover, IL-1β promoted survival of retinal ganglion cells following axotomy of the optical nerve through both IL-1R1-dependent and independent mechanisms (667). Intriguingly, a similar phenomenon has been shown in the intestine, following infection with the parasite *Trichuris muris* (668). In this model, WT mice infected with *T. muris* expel the parasite in about 20 days, following the induction of a strong Th2 immune response. Although this protected phenotype was recapitulated in *Il1r1*^{KO} mice, by contrast, IL-1α-deficient or IL-1β–deficient mice were unable to expel the parasite (668). Moreover, MLN cells from *T. muris*-infected *Il1r1*^{KO} mice responded to *ex vivo* stimulation with IL-1β and IL-1α (668). These data suggest that IL-1α and IL-1β promoted a protective response against *T.muris* independently of IL-1R1. Further studies are required to identify another receptor able to bind IL-1β and deliver an intracellular signal. IL-1R2 is a good candidate, as it binds IL-1β with high affinity (514). However, IL-1R2 is thought to be a scavenger receptor, as its cytoplasmatic tail lacks the TIM domain required for signal transduction (514). Nevertheless, i.c.v. administration of a blocking αIL-1R2 antibody inhibited the induction of fever following i.c.v injection of IL-1β (669). The expression pattern of IL-1R2 in the intestine is not known and further characterization will be required, including defining specific IL-1R2-expressing cell populations. Although IL-1R2-deficient mice are not currently available, monoclonal antibodies could be used to test the role of IL-1R2 in intestinal inflammation.

Another possible contributing factor could be that *Il1r1*^{KO} mice might host a significantly different intestinal microbiota than C57BL/6 WT mice. Recent evidence suggests that deficiency in a single component of the immune system drastically

alters the composition of the intestinal microbiota, resulting in differential susceptibility to intestinal inflammation (670). In support of this hypothesis, it is notable that *Il1r1*^{KO} mice are prone to obesity, especially in old age (671), although pre-obese *Il1r1*^{KO} mice already show metabolic changes (671). Since obesity is strongly correlated with profound changes in the composition of the intestinal microbiota (224), it is possible that *Il1r1*^{KO} have a different microbiota from WT mice. Hence, any anti-inflammatory effect of the absence of IL-1R1 signaling could be negated by the presence of more colitogenic intestinal bacteria. Indeed, although colonization with *H.hepaticus* is a necessary bacterial trigger for the induction of colitis, it is not sufficient to induce colitis, as germ-free *Il10*^{KO} mice do not develop disease following infection with *H.hepaticus*. It is likely that the induction of colitis in the Hh + aIL10R model follows a similar pattern, hence the composition of the microbiota might play an important role in the induction of disease. However, similar patterns of disease severity were observed in *Il1r1*^{KO} and WT mice whether the mice were co-housed, which minimizes inter-strain differences in the bacteria microflora, or whether they were housed separately during the duration of the experiment, arguing against a contribution of the intestinal microflora to intestinal pathology in *Il1r1*^{KO} mice.

Lastly, it is possible that IL-1β mediates both pro- and anti-inflammatory activities during intestinal inflammation. In this case, the incomplete blocking achieved by aIL-1β administration might hamper the pro-inflammatory functions of IL-1β, without inhibiting completely its anti-inflammatory activities.

Despite extensive analysis of Hh + αIL-10R mice receiving αIL-1β we were unable to definitively identify which cellular or molecular pathways underlies the observed decrease in pathology. $CD4^+$ T cell responses in the intestine are crucial in promoting inflammation in this model (193). However, we did not observe any significant changes in the frequency or accumulation of $CD4^+$ T cells in the colon of Hh + αIL-10R mice receiving either αIL-1β or the isotype control. Moreover, the frequency and accumulation of pro-inflammatory Th1 and Th17 cell subsets was only minimally altered following administration of αIL-1β. However, we did observe a significant reduction in the overall proportion of IL-17A producing cells, comprising both cells that produce IL-17A only and cells that produce both IL-17A and IFN-γ. Considering compelling evidence linking IL-1β and Th17 differentiation in models of EAE (Refs), we decided to further investigate the effects of IL-1R deficiency on $CD4^+$ T cell differentiation in the gut in a model of T cell transfer as described in chapter 5.

In addition to $CD4^+$ T cells, other cellular pathways might be responsible for the observed decrease in pathology following treatment with αIL-1β. As we have demonstrated in chapter 3, IL-1β plays a central role in the recruitment of pro-inflammatory granulocytes in the intestine. Unpublished observations from our laboratory show an increase in the frequency of granulocytes in the colon following induction of colitis in C57BL/6 WT mice by Hh + αIL-10R (R.Szabady). Hence, it is possible that blocking IL-1β results in decrease recruitment of granulocytes to the colon in this model.

IL-1β also plays a central role in modulating the function of γδ T cells. Specifically, IL-1β stimulation, in synergy with IL-23, induces IL-17 production by γδ T cells from the

spleen and the peritoneum (60, 61, 672, 673). Moreover, IL-1R1^{+} CD62L^{-} γδ T cells from the small intestine lamina propria rapidly produce IL-17A in response to stimuli from the intestinal microflora (672). Hence, the protective action of αIL-1β treatment could be due to modulation of the γδ T cell compartment. Indeed, γδ T cells play a pathogenic role in models of chronic colitis in the absence of TCRαβ cells (674). However, induction of colitis by Hh + αIL-10R treatment is not associated with an expansion of intestinal γδ T cells or IL-17A producing γδ T cells (Dr. Matthew Shale, unpublished observations). Moreover, we did not observe a significant decrease in IL-17A production by colonic lamina propria cells from Hh + αIL-10R WT mice treated with αIL-1β. Intriguingly, γδ T cells also play a protective role in acute model of intestinal inflammation, mainly because of their ability to secrete factors promoting repair of the epithelium, such as keratinocyte growth factor (KGL) (675). As IL-1α can induce KGL production by fibroblasts in models of wound repair (662, 663), it would interesting to assess the role of IL-1R1 expressing γδ T cells in restitution of the epithelium in intestinal inflammation, by examining the functional status of γδ T cells during intestinal inflammation in Hh + αIL10R *Il1r1*^{KO} mice. Moreover, the functional relevance of γδ T cells in this model of intestinal inflammation could be address by inducing *H.hepaticus*-driven colitis in *Tcrd*^{KO} mice.

Finally, it is possible that the pathogenic activity of IL-1β extends beyond the immune system. IL-1β is known to promote microvascular thrombosis associated with intestinal inflammation, by acting on the coagulant pathway (676). Moreover, IL-1β can directly inhibit the contractility of gut smooth muscle, leading to decreased intestinal motility, a common feature of chronic intestinal inflammation in humans

(677). Furthermore, IL-1β can alter the responsiveness of neurons in the myenteric plexus (the major nerve supply to the gut), although the consequences of this action are not fully characterized (678).

If we compare the results obtained by using αIL-1β to modulate colitis with those obtained with *Il1r1*^{KO} mice, it is striking that the only cellular signature that differs between these experimental settings is the modulation of FOXP3^{+} IL-17A^{+} CD4^{+} T cell frequency. Indeed, this small cell population was not altered by treatment of Hh + αIL-10R mice with αIL-1β. In contrast, Hh + αIL-10R *Il1r1*^{KO} mice showed significantly lower frequencies of FOXP3^{+} IL-17A^{+} CD4^{+} T cells compared to Hh + αIL-10R WT mice. Previous reports showed that FOXP3^{+} Tregs could produce IL-17A and upregulate CCR6 in the presence of IL-1β stimulation *in vitro,* particularly in the presence of other pro-inflammatory stimuli like IL-23, in a process dependent on STAT3 and RORγt (144, 207, 679, 680). Moreover, IL-1R1 deficiency prevented the differentiation of FOXP3^{+} IL-17A^{+} T cells in models of EAE (138). However, the function of these double positive cells remains unclear: some authors propose that FOXP3^{+}IL-17A^{+} CD4^{+} T cells lose their suppressive ability *in vitro* (144, 681), whilst others report that they are still able to suppress T cell proliferation (679, 680). Their functionality *in vivo* has not been addressed. Our data associate decreased frequencies of FOXP3^{+}IL-17A^{+} CD4^{+} T cells in Hh + αIL-10R *Il1r1*^{KO} mice with a trend towards deterioration of colitis. However, FOXP3^{+}IL-17A^{+} cells represent a minimal proportion of CD4^{+} T cells, so further experiments analyzing their function are required. Moreover, no differences in the frequencies of this cell subset was observed following administration of αIL-1β to Hh + αIL-10R WT mice, suggesting

that the differentiation of FOXP3^{+}IL-17A^{+} CD4^{+} T cells might be independent of IL-1β and dependent on other IL-1R1-mediated signals in the intestine.

Clearly, the pleiotropic effects of IL-1β hamper a clear interpretation of the results presented in this chapter. Our studies using αIL-1β support a pathogenic role for IL-1β in intestinal inflammation in the lymphocyte replete host, but fail to clearly identify the cellular pathways targeted by this cytokine. The observed effect on Th17 cell differentiation prompted us to further investigate the role of IL-1R signaling on T cells in a more controlled model of T cell driven intestinal inflammation, as detailed in chapter 5.

Chapter 5: Role of IL-1R signalling in T cell transfer induced colitis.

5.1 Introduction

IL-1β was initially identified as "lymphocyte activating factor" (LAF) for its ability to promote T cell proliferation in the presence of an antigen or a mitogen (682). More recently, the role of IL-1β on T cell proliferation and T helper subset differentiation has been examined in more detail. IL-1R is differentially expressed by distinct Th effector subsets, with Th2 cells expressing higher levels of IL-1R1 than Th1 cells (544). However, Th1 effector function can still be modulated by IL-1β: for instance, IL-1β can increase the production of IFN-γ by staphylococcal enterotoxin B (SEB)-activated Th1 cells *in vitro* (683).

More recently, it has been shown that IL-1β plays a key role in promoting Th17 differentiation in humans and in mice (60, 138, 139, 144). Th17 cells express high levels of the IL-1R (138, 545) and Th17 cell differentiation can be induced by IL-1β stimulation in the presence of IL-23 or TGF-β (133) (137, 138, 157, 202). IL-1β promotes Th17 cell differentiation by up-regulating the key Th17 transcription factors RORγt and IRF-4 (137, 138). Moreover, signaling via IRAK-1, downstream of the IL-1R, can augment Th17 cell differentiation by phosphorylating another important Th17 transcription factor, STAT3 (684). Consistent with a role for IL-1β in promoting Th17 cell differentiation, *Il1r1*^{KO} mice were found to be resistant to the development of experimental autoimmune encephalitis (EAE) following immunization with myelin oligodendrocyte glycoprotein (MOG), because of an attenuated Th17 response (139). Impaired induction of EAE was also observed upon

transfer of *Il1r1*^{KO} CD4^{+} T cells into C57BL/6 *Rag1*^{KO} mice that were subsequently immunized with MOG (138).

The intestine is a preferential site for Th17 cell differentiation: approximately 20% of CD4^{+}TCR$\alpha\beta^{+}$ T cells in the small intestine and around 10% in the colon express IL-17A (362, 685, 686). In contrast, the frequency of Th17 cells in extra-intestinal sites is very low (<0.1% of CD4+ T cells) in the steady state (362). Numerous studies have demonstrated that stimuli from the intestinal microflora play a central role in promoting Th17 cell differentiation in the mouse intestine (361-363, 365, 686-688). For instance, colonization by segmented filamentous bacteria (SFB) was shown to drive the differentiation of Th17 cells in the small intestine (361-363). Moreover, ATP of bacterial origin can also promote the differentiation of Th17 cells in the colon (686) and agonists for TLR-5 and TLR-9 were also reported to promote Th17 cell differentation in the gut (687, 688).

Although IL-1β has been linked to Th17 cell responses, the role of IL-17A in the development of intestinal inflammation is controversial. In acute models of intestinal injury, IL-17A plays a disease protective role, with IL-17A-deficient mice showing increased pathology and leukocyte infiltration after DSS-administration (458). However, studies in chronic inflammatory models have highlighted a more complex role for IL-17A. Studies from our laboratory demonstrated a pathogenic role for IL-17A in *H. hepaticus* driven innate immune IBD (51). Similarly, administration of an αIL-17A antibody ameliorated the spontaneous colitis that developed in mice bearing a conditional deletion of the transcription factor Stat3 in Foxp3^{+} Treg cells (197). Th17 cells are enriched in the inflamed gut both in animal IBD models and in

human IBD patients (438, 444, 450, 452) and $CD4^+$ T cells lacking the transcription factor RORγt, that directs Th17 differentiation, could not transfer colitis to C57BL/6 *Rag1*^{KO} mice (456). However, T cell derived IL-17A is not absolutely required for the development of intestinal pathology in T cell transfer models of colitis and it has been proposed that T cell derived IL-17A and IL-17F might play a redundant role in driving intestinal inflammation (456, 459, 460). These conflicting results might be explained by an as yet undiscovered additional pathogenic function of Th17 cells. Alternatively, a complex network of pro-inflammatory cells/factors may contribute to IL-17A-mediated pathology *in vivo* (128).

Here, we used the classic T cell transfer model of colitis to assess the role of IL-1R-mediated modulation of T cell function in intestinal inflammation. This approach presented two key advantages:

1. By comparing naïve $CD4^+$ T cells from WT and *Il1r1*^{KO} mice we could assess the cell intrinsic modulation of T cell activity by IL-1R, without modulating the ability of any other cells to respond to IL-1R signals.
2. Adoptive transfer of WT and *Il1r1*$^{KO}CD4^+$ T cells into identical B6 *Rag1*^{KO} recipient mice allowed us to circumvent any potential contribution of intestinal microbiota differences between WT and *Il1r1*^{KO} mice to the induction of colitis.

5.2 Results

5.2.1 IL-1R1 signaling on T cells drives intestinal and hepatic inflammation.

To address whether IL-1β could also promote deleterious adaptive immune responses within the intestine, we transferred naïve $CD4^+CD45RB^{Hi}$ T cells isolated from either WT or *Il1r1*^{KO} mice into C57BL/6 *Rag1*^{KO} recipients and assessed the development of pathology. As expected, adoptively transferred WT naïve $CD4^+$ T cells induced weight loss and severe intestinal, hepatic and systemic inflammation in C57BL/6 *Rag1*^{KO} recipients (**Fig. 5.1-A**). By contrast, *Il1r1*^{KO} naïve $CD4^+$ T cells failed to induce weight loss upon transfer to C57BL/6 *Rag1*^{KO} mice compared with recipients of WT naïve $CD4^+$ T cells (**Fig. 5.1A**). Despite attenuated wasting disease, no significant difference was observed in either splenomegaly or spleen cellularity in C57BL/6 *Rag1*^{KO} mice receiving WT or *Il1r1*^{KO} naïve $CD4^+$ T cells (**Fig. 5.1B**). C57BL/6 *Rag1*^{KO} mice transferred with *Il1r1*^{KO} naïve $CD4^+$ T cells showed significantly reduced intestinal pathology in both the caecum and the colon **(Fig.5.2A-C**). Liver pathology was also affected, with fewer inflammatory foci visible in C57BL/6 *Rag1*^{KO} mice receiving naïve *Il1r1*^{KO} $CD4^+$T cells (**Fig.5.2C**). The attenuated intestinal inflammation in the colon of C57BL/6 *Rag1*^{KO} mice receiving *Il1r1*^{KO} naïve $CD4^+$ T cells was accompanied by significant reductions in secretion of the pro-inflammatory cytokines IL-6, IFN-γ, TNF-α and IL-17A by cLPL (**Fig.5.3**), indicating a general dampening of the intestinal inflammatory response in the absence of IL-1R1 signaling on T cells.

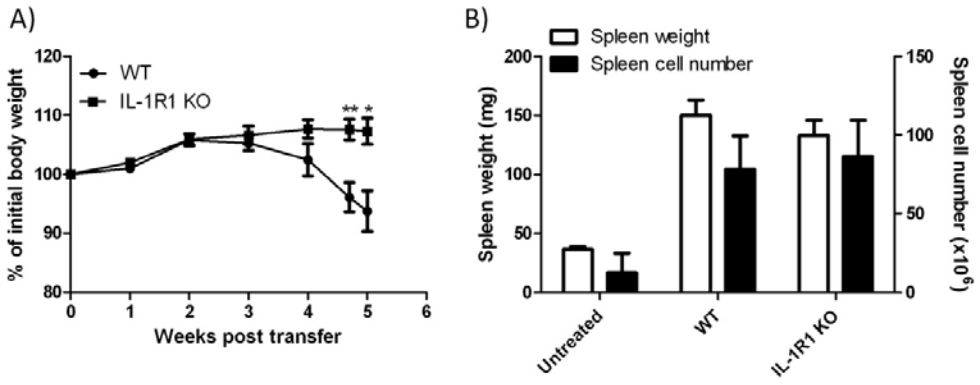


Figure 5-1.IL-1 signaling on T cells is required for the development of wasting disease after naïve CD4$^+$ T cell transfer into *Rag1^{KO}* mice. C57BL/6 *Rag1^{KO}* mice were reconstituted by i.p. injection of 4x10^5 CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from C57BL/6 WT or *Il1r1^{KO}* mice. (A) Representative weight loss curves, shown as percentage of initial weight. (B) Spleen weight and spleen cell number. Data represent mean ± SEM from one representative experiment out of 3 similar experiments (n=3 for untreated controls, n=8 for naïve CD4$^+$T cell recipients).

*p<0.05, **p<0.01 Mann Whitney test

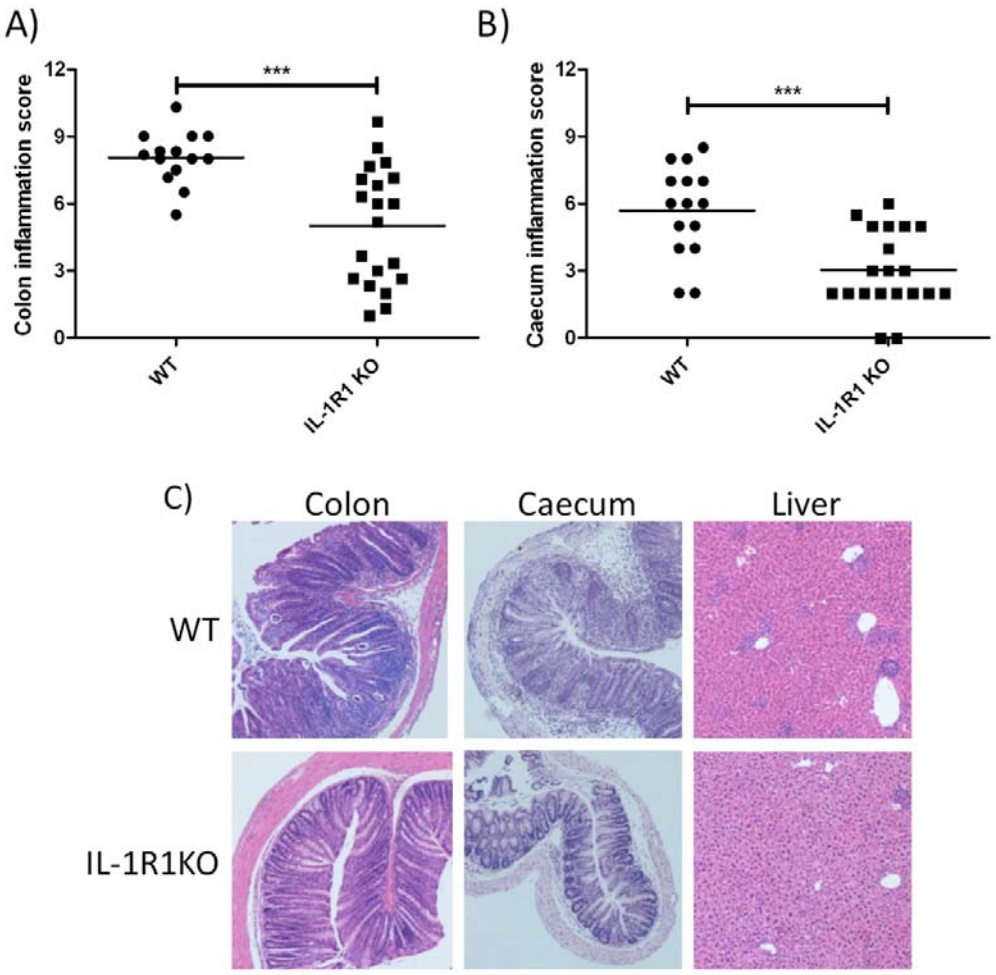


Figure 5-2. T cell driven intestinal inflammation is attenuated in the absence of IL-1R signalling on T cells. C57BL/6 *Rag1*^{KO} mice were reconstituted by i.p. injection of 4x10^5 CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from C57BL/6 WT or *Il1r1*^{KO} mice. Animals were sacrificed when recipients of WT naive CD4+ T cells developed clinical signs of inflammation (~5 weeks after transfer) and assessed for intestinal and hepatic inflammation. Data are from 3 pooled independent experiments (n=14 for recipients of WT naive CD4$^+$ T cells and n=20 for recipients of *Il1r1*^{KO} naïve CD4$^+$ T cells). Bars show the mean score. A) Colon inflammation scores. B) Caecum inflammation scores. C) Representative photomicrographs of colon, caecum and liver (original magnification 50x).

***p<0.001 Mann Whitney test

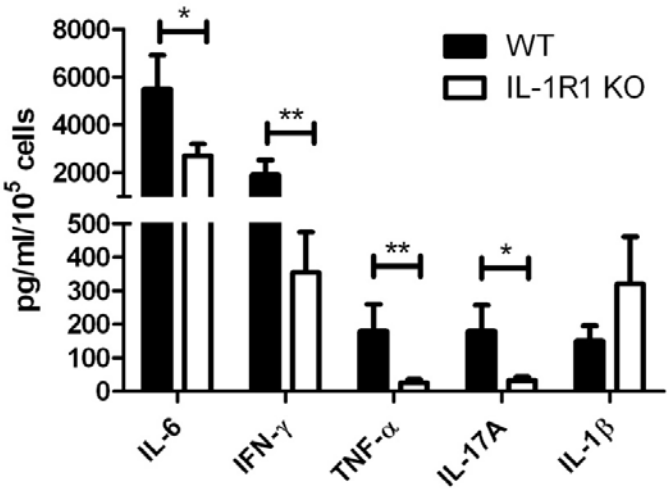


Figure 5-3. Impaired cytokine response from cLPL upon adoptive transfer of IL-1R deficient naive CD4^{+} T cells. C57BL/6 *Rag1^{KO}* mice were reconstituted by i.p. injection of 4x10^{5} CD4^{+} CD25^{-} CD45RB^{Hi} T cells isolated from C57BL/6 WT or *Il1r1^{KO}* mice. Animals were sacrificed when recipients of WT naive CD4+ T cells developed clinical signs of inflammation (~5 weeks after transfer). LP leukocytes were isolated from the colon and cultured O/N in complete medium. Cytokine secretion was evaluated by FlowCytomix Cytokine assay (eBioscience). Data are shown as mean ± SEM (n=10-14 from 2 pooled independent experiments).

*p<0.05, **p<0.01 Mann Whitney test

5.2.2 IL-1R1 signaling promotes accumulation of Th17 cells in the colon.

We next analyzed the effects of cell-intrinsic IL-1R signals on the differentiation and accumulation of $CD4^+$ T cells in the colon. When compared with recipients of WT naïve $CD4^+$ T cells, C57BL/6 *Rag1*^{KO} mice that received *Il1r1*^{KO} naïve $CD4^+$ T cells exhibited a 50% decrease in the total number of $CD4^+$ T cells present in the colon (**Fig.5-4A**), although the frequency of $CD4^+$ T cells amongst lamina propria leukocytes was unaffected (**Fig.5-4B**). This suggests that IL-1R expression by T cells is essential to drive their accumulation in the intestine. Moreover, the effect of IL-1R signaling on $CD4^+$ T cell accumulation was specific to the intestine, because similar $CD4^+$ T cell numbers were found in the MLN or spleen of C57BL/6 *Rag1*^{KO} mice receiving WT or *Il1r1*^{KO} naive $CD4^+$ T cells (**Fig.5-4A**).

The T cell transfer model of colitis is characterized by accumulation of Th1 and Th17 cells in the colon (444, 689). Recently, it has been proposed that IL-1β plays a key role in promoting the differentiation of Th17 cells (60, 138, 139). In agreement with these findings, naïve $CD4^+$ T cells from *Il1r1*^{KO} mice cultured in the presence of IL-1β and IL-23 exhibited decreased Th17 cell differentiation compared to WT naïve $CD4^+$ T cells (**Fig.5-5A-B**). Similarly, when Th17 cell differentiation was induced by culturing splenic $CD4^+$ T cells with IL-6 and TGF-β, *Il1r1*^{KO} $CD4^+$T cells exhibited decreased differentiation into Th17 cells when compared to WT $CD4^+$ T cells (**Fig.5-5C-D**). These data show that IL-1R signaling augments Th17 cell differentiation *in vitro.* To assess the role of IL-1R expression on T helper cell differentiation *in vivo*, we isolated $CD4^+$ T cells from the colon of C57BL/6 *Rag1*^{KO} mice receiving WT or *Il1r1*^{KO} naïve $CD4^+$T cells and performed intracellular staining for IL-17A and IFN-γ production. We found that IL-1R expression was dispensable for Th1 and Th17 cell differentiation in the MLN

and colon, as shown by similar frequencies of these effector cells observed in recipients of WT or *Il1r1*^{KO} naïve CD4^{+} T cells (**Fig.5-6A,B**). Furthermore, we observed similar numbers of CD4^{+} IL-17A^{-}IFN-γ^{+} Th1 cells and double-secreting CD4^{+} IL-17A^{+}IFN-γ^{+} T cells in the colon of C57BL/6 *Rag1*^{KO} mice receiving *Il1r1*^{KO} or WT naïve CD4^{+} T cells (**Fig.5-6C**). By contrast, we observed a specific defect in the accumulation of CD4^{+} Th17 cells, as shown by a 50% decrease in the number of IL-17A^{+}IFN-γ^{-} T cells found in the colon of C57BL/6 *Rag1*^{KO} mice receiving *Il1r1*^{KO} naïve CD4^{+} T cells when compared to recipients of WT naïve CD4^{+} T cells (**Fig.5-6C**). Together, these data indicate a role for T cell-intrinsic IL-1R1 signalling in promoting the accumulation of Th17 cells in the colon.

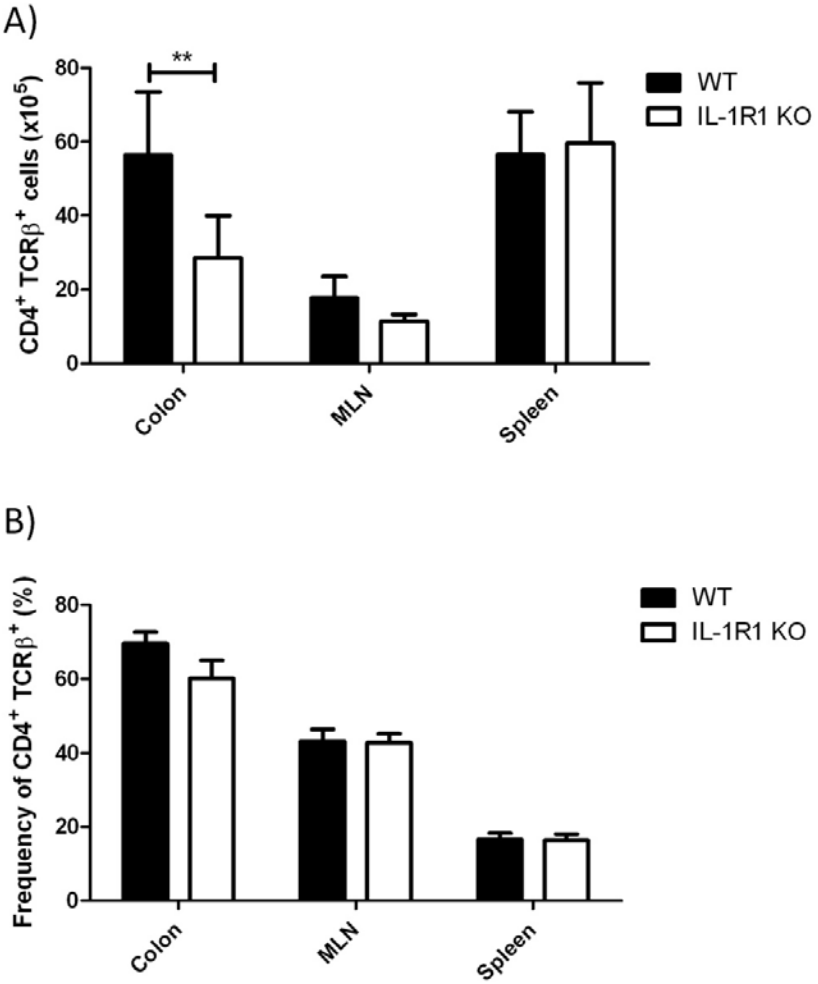


Figure 5-4. Reduced accumulation of $CD4^+$ T cells in the gut in the absence of IL-1R1 signalling. C57BL/6 *Rag1*^{KO} mice were reconstituted by i.p. injection of $4x10^5$ $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ T cells isolated from C57BL/6 WT or *Il1r1*^{KO} mice. Animals were sacrificed when recipients of WT naive CD4+ T cells developed clinical signs of inflammation (~5 weeks after transfer). LP leukocytes were isolated from the colon and analyzed by FACS analysis. A) Total numbers of $CD4^+$ T cells in the colon, MLN and spleen. B) Frequencies of $CD4^+$ T cells in the colon, MLN and spleen.

All data are shown as mean ± SEM (n = 20-21 from 3 pooled independent experiments).

**p<0.01 Mann Whitney test

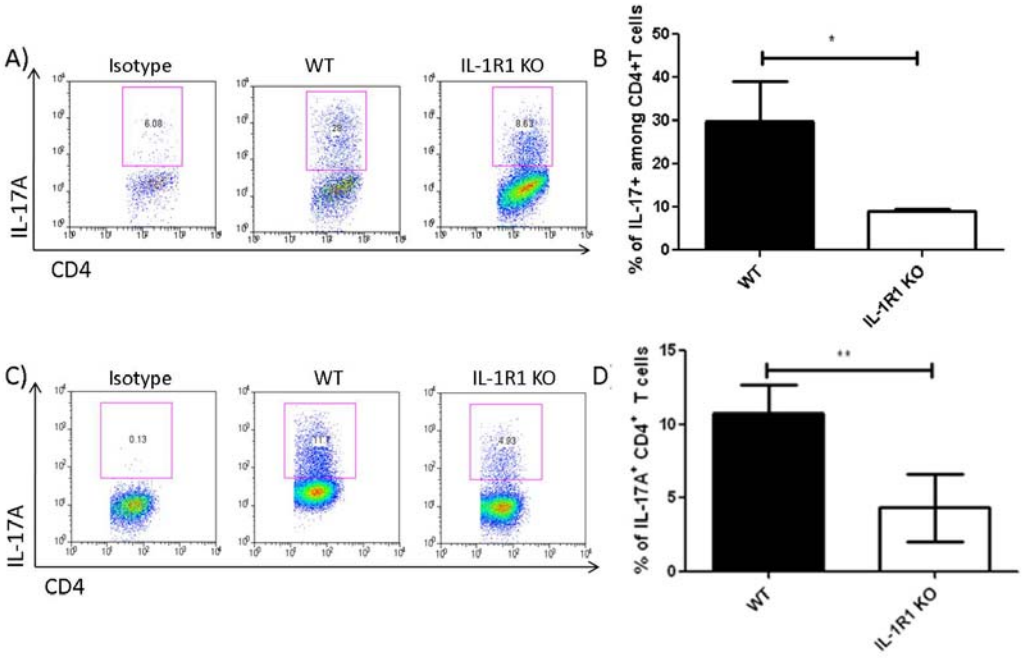


Figure 5-5. IL-1R expression on T cells augments Th17 differentiation *in vitro*. (A-B) Naïve $CD4^{+}CD45RB^{Hi}$ T cells were FACS-sorted from the spleen of WT or $Il1r1^{KO}$ mice and cultured in the presence of IL-1β (20ng/ml) and IL-23 (50ng/ml) plus plate bound αCD3 (2ug/ml) and αCD28 (1ug/ml). After 3 days, the frequency of IL-17A-producing cells was analyzed by intracellular FACS staining. Data are shown as representative plots (A) and mean ± SD (B) (n=3). (C-D) $CD4^{+}$ T cells were isolated from the spleen of WT or $Il1r1^{KO}$ mice and cultured in the presence of TGF-β (2.5ng/ml) and IL-6 (50ng/ml) plus plate bound αCD3 (2μg/ml) and αCD28 (1μg/ml). After 3 days, the frequency of IL-17A-producing cells was analyzed by intracellular FACS staining. Culture in the absence of cytokines did not result in the differentiation IL-17A producing cells. Data are shown as representative plots (C) and mean ± SD (D) from two independent experiments (n=4).

*p<0.05,**p<0.01 Mann Whitney

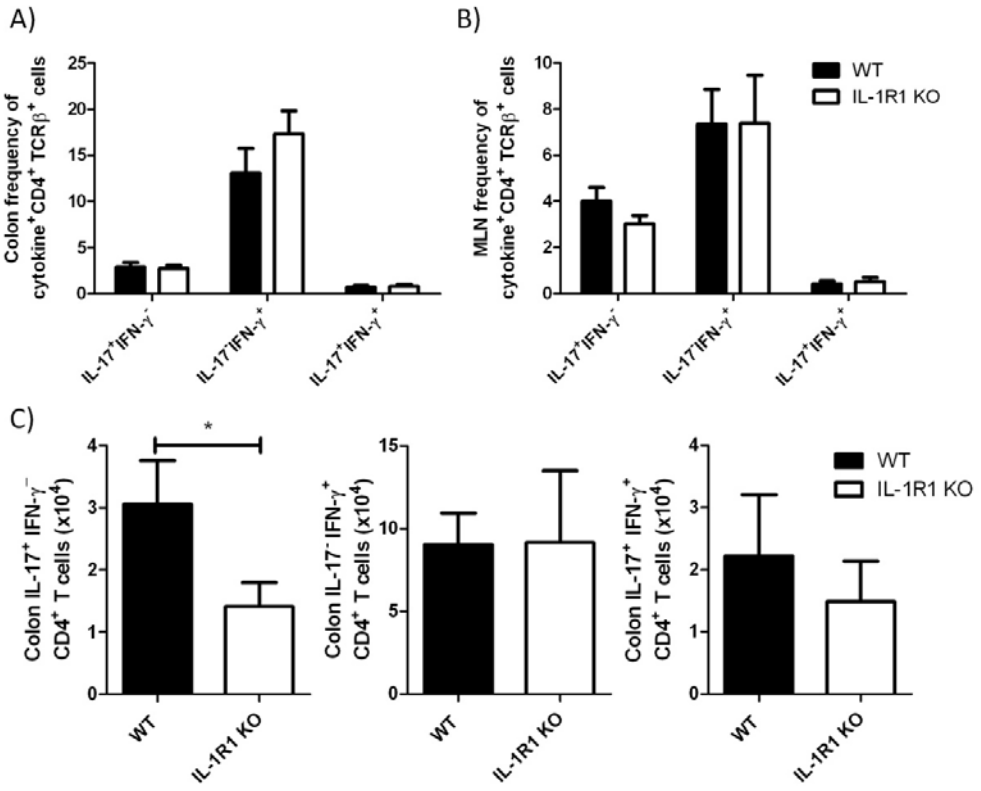


Figure 5-6. Impaired Th17 accumulation in the gut in the absence of IL-1R signalling. C57BL/6 *Rag1*^{KO} mice were reconstituted by i.p. injection of 4x10^5 CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from C57BL/6 WT or *Il1r1*^{KO} mice. Animals were sacrificed when recipients of WT naive CD4+ T cells developed clinical signs of inflammation (~5 weeks after transfer). LP leukocytes were isolated from the colon and analyzed by FACS analysis. A) Frequencies of indicated T helper subsets among CD4$^+$ T cells in the colon. B) Frequencies of indicated T helper subsets among CD4$^+$ T cells in the MLN. C) Total numbers of indicated CD4$^+$ Th cell populations in the colon. All data are shown as mean ± SEM (n = 20-21 from 3 pooled independent experiments).

*p<0.05,**p<0.01 Mann Whitney

5.2.3 IL-1R deficiency does not affect CD4+ T cell proliferation or early migration to the colon *in vivo*.

Next, we examined whether the defective accumulation of Th17 cells in the absence of cell-intrinsic IL-1R expression could be due to defective proliferation of the *Il1r1*^{KO} T cells in the colon. However, CD4^{+} T cells and Th17 cells isolated from the colon of C57BL/6 *Rag1*^{KO} mice that received *Il1r1*^{KO} or WT naïve CD4^{+} T cells expressed the proliferation marker Ki67 to a similar extent (**Fig.5-7**). Alternatively, we hypothesized that the absence of IL-1R signals could compromise the ability of CD4^{+} T cells to migrate to the colon. To test this hypothesis, we transferred *Il1r1*^{KO} or WT CD4^{+}CD45RB^{Hi} T cells into C57BL/6 *Rag1*^{KO} mice and sacrificed them two weeks after transfer, prior to the onset of colitis. At this early time point, no difference in the total number of CD4^{+} T cells or Th17 cells present in the colon was observed between C57BL/6 *Rag1*^{KO} mice that received *Il1r1*^{KO} or WT naïve CD4^{+} T cells (**Fig.5-8A**). Moreover, Ki67 expression by total CD4^{+} T cells or by Th17 cells was again comparable in WT or *Il1r1*^{KO} CD4^{+} T cells (**Fig.5-8B**). Together, these results excluded an effect of IL-1R expression in preventing T cell migration to the colon or in impairing early T cell proliferation.

Recently, it has been reported that IL-1β promotes the production of GM-CSF by Th17 cells, promoting their pathogenicity in models of EAE (690, 691). Hence, we tested whether the levels of GM-CSF were up-regulated in the colon of C57BL/6 *Rag1*^{KO} mice that received *Il1r1*^{KO} or WT naive CD4^{+} T cells (**Fig.5-9**). Our analysis showed that GM-CSF was expressed at similar levels in the colon of recipients of either WT or *Il1r1*^{KO} naive CD4^{+} T cells (**Fig.5-9**), arguing against a role for IL-1β in promoting GM-CSF in this model. However, as this experiment only analyzes the

overall levels of GM-CSF expression in the inflamed colon, further investigation on the ability of *Il1r1*^{KO} CD4^{+}T cells to produce GM-CSF in the gut will be required.

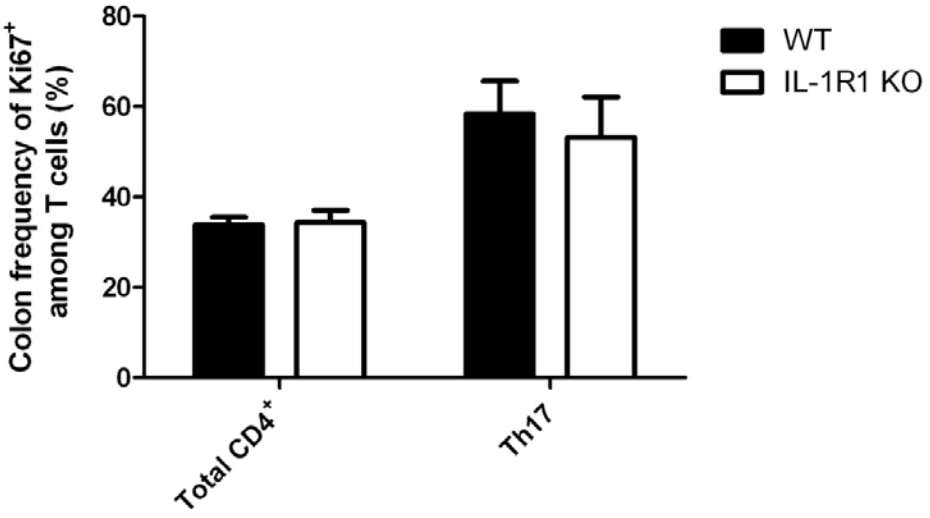


Figure 5-7. IL-1R expression has no effect on T cell proliferation in the colon following adoptive transfer. C57BL/6 *Rag1*^{KO} mice were reconstituted by i.p. injection of 4x10^5 CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from C57BL/6 WT or *Il1r1*^{KO} mice. Animals were sacrificed when recipients of WT naive CD4+ T cells developed clinical signs of inflammation (~5 weeks after transfer). Colonic lamina propria lymphocytes (cLPL) were isolated and cytokine expression and Ki67 expression by cLPL CD4$^+$ T cells was assessed by intracellular FACS staining. Th17 cells were identified as CD4$^+$TCRβ^+IL-17A$^+$IFN-γ^- cells. Data represent mean ± SEM (n = 20-21 from 3 pooled independent experiments).

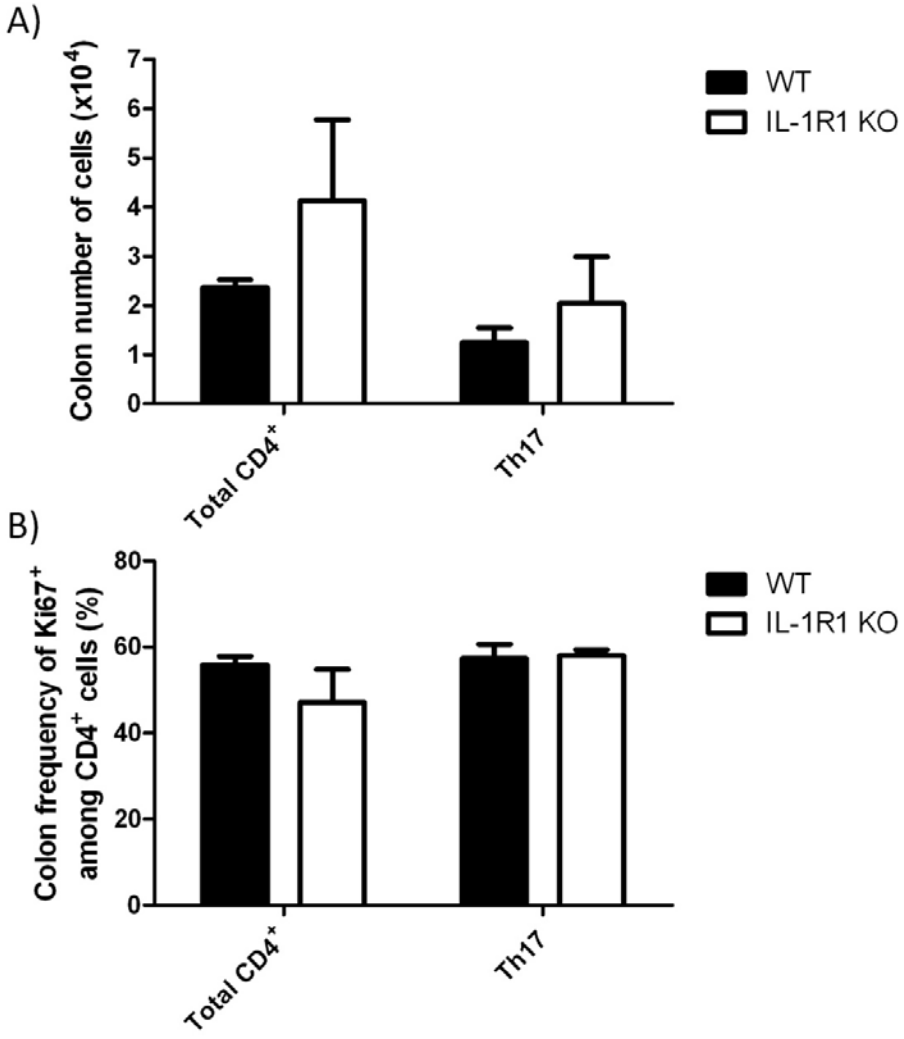


Figure 5-8. IL-1R expression has no effect on early T cell migration or proliferation in the gut. C57BL/6 *Rag1*^{KO} mice were reconstituted by i.p. injection of 2x10^6 CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from C57BL/6 WT or *Il1r1*^{KO} mice. Two weeks later, mice were sacrificed and cytokine expression and Ki67 expression by cLPL CD4$^+$ T cells was assessed by intracellular FACS staining. Th17 cells were identified as CD4$^+$TCRβ^+IL-17A$^+$IFN-γ^- cells. Data represent mean ± SEM (n=5 mice/group). A) Total number of CD4$^+$ T cells and Th17 cells in the colon. B) Ki67 expression by isolated cLPL CD4$^+$ T cells.

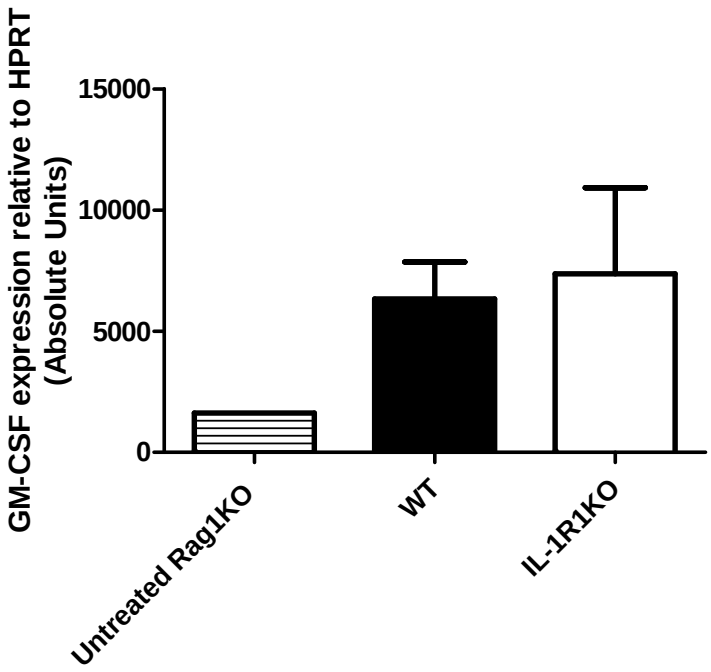


Figure 5-9. No effect of T cell IL-1R1 deficiency on the expression of GM-CSF in the colon following T cell transfer in C57BL/6 *Rag1*^{KO} mice. . C57BL/6 *Rag1*^{KO} mice were reconstituted by i.p. injection of 4x10^5 CD4$^+$ CD25$^-$ CD45RB^{Hi} T cells isolated from C57BL/6 WT or *Il1r1*^{KO} mice. Animals were sacrificed when recipients of WT naive CD4+ T cells developed clinical signs of inflammation (~5 weeks after transfer). RNA was extracted from sections of proximal, mid and distal colon and GM-CSF expression was assessed by RT q-PCR. Data are shown as mean ± SEM (n=1 for untreated *Rag1*^{KO} mice, n= 6 for recipients of WT naive CD4$^+$ T cells and 4 for *Il1r1*^{KO} recipients).

5.2.4 IL-1R1-deficient CD4+ T cells are more susceptible to apoptosis *in vitro*.

Finally, we hypothesized that the defective accumulation of *Il1r1*^{KO} CD4^{+} T cells could be due to increased apoptosis. To assess this point, we tried to quantify apoptotic T cells *in vivo* by staining colon sections from colitic mice using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), combined with αCD3 immunofluorescence staining (692). However, the frequency of CD4^{+}apoptotic cells in this assay was too low to allow quantification of apoptotic events, probably reflecting the rapid clearance of apoptotic cells *in vivo* (data not shown). Therefore, we assessed the susceptibility to apoptosis of WT and *Il1r1*^{KO} CD4^{+} T cells following stimulation with αCD3 and αCD28 monoclonal antibodies *in vitro* (693). We found that *Il1r1*^{KO} CD4^{+} T cells consistently exhibited significantly higher levels of apoptosis than WT CD4^{+} T cells under these conditions (**Fig. 5-10**), supporting the hypothesis that IL-1R signaling promotes T cell survival during inflammation, by preventing activation-induced apoptosis.

Costimulatory molecules play a central role in promoting T cell survival during immune reactions OX40 is a member of the TNF/TNFR family of costimulatory molecules expressed on activated T cells (694). The OX40/OX40L pathway promotes the clonal expansion of naive T cells and delivers anti-apoptotic signals, favouring the survival of effector T cells (695). The OX40/OX40L pathway has been associated with intestinal inflammation, as OX40^{+} cells are increased in frequency in the colon of IBD patients (696) and blockade of OX40-OX40L interactions was shown to be beneficial in different animal models of colitis (697, 698). Moreover, OX40 expression on T cells is required for the accumulation of CD4^{+} IL-17A^{+} T cells in T cell transfer colitis (699).

Since IL-1β promotes the expression of OX40 on activated T cells (543), we tested whether $CD4^+$ T cells from *Il1r1*^{KO} mice were defective in OX40 expression. However, we observed that following *in vitro* activation splenic $CD4^+$ T cells from *Il1r1*^{KO} mice expressed OX40 at a similar level and frequency as WT $CD4^+$ T cells (**Fig.5-11A,B**).

The expression of CD40L, another member of the TNF/TNFR family of costimulatory molecules, is also increased following IL-1β stimulation of T cells (543). CD40L activation is essential for *in vivo* priming of T cell responses (700). Moreover, the ligand for CD40L, CD40, is expressed on APC and CD40L/CD40 interactions deliver a crucial pro-inflammatory signal to DC, sustaining T cell responses (701). $CD40L^+$ $CD4^+$ T cells are over-represented in the colon of CD patients (702) and different strategies blocking CD40L were shown to ameliorate pathology in the T cell transfer model of colitis (349, 703, 704). However, our analysis showed that splenic $CD4^+$ T cells from *Il1r1*^{KO} and WT mice expressed comparable levels of CD40L following activation *in vitro* (**Fig.5-11C**) and that the frequency of $CD40L^+$ $CD4^+$ T cells was also comparable between *Il1r1*^{KO} and WT $CD4^+$ T cells (**Fig.5-11 D**). Overall, these data show that the expression of costimulatory molecules upon *in vitro* T cell activation is independent of IL-1R signaling.

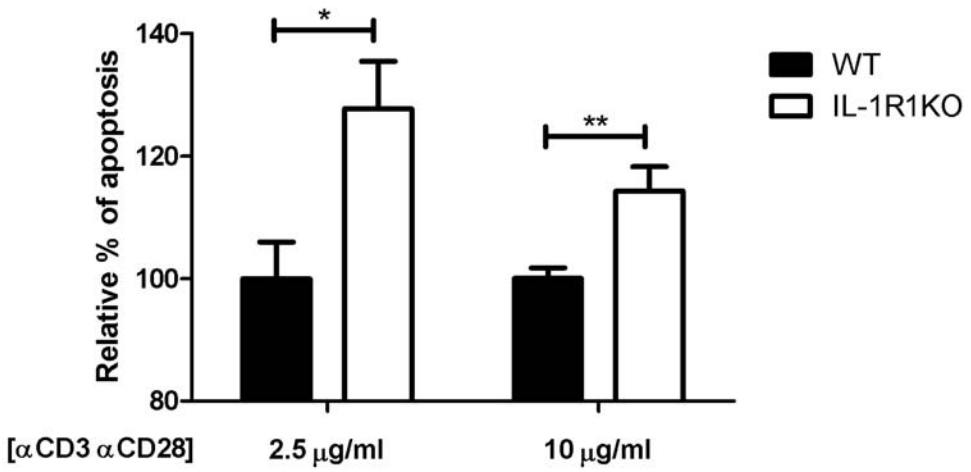


Figure 5-10. IL-1R signalling modulates CD4$^+$ T cell apoptosis *in vitro*. Purified CD4$^+$ T cells from WT or *Il1r1*^{KO} mice were cultured for three days in the presence of plate bound αCD3 and αCD28 at the indicated concentrations. Apoptosis was assessed using FACS analysis, following staining with the phosphatidyl serine binding protein Annexin V and the vital dye 7AAD (693). Annexin V$^+$ 7AAD$^-$ cells were identified as early apoptotic events. The average frequency of Annexin V$^+$ 7AAD$^-$ cells in WT cells was set as a baseline (100%) and data were expressed as relative % apoptosis compared to the baseline (n=10 mice per group from 4 pooled independent experiments).

*p<0,05, **p<0,01 Student t-test

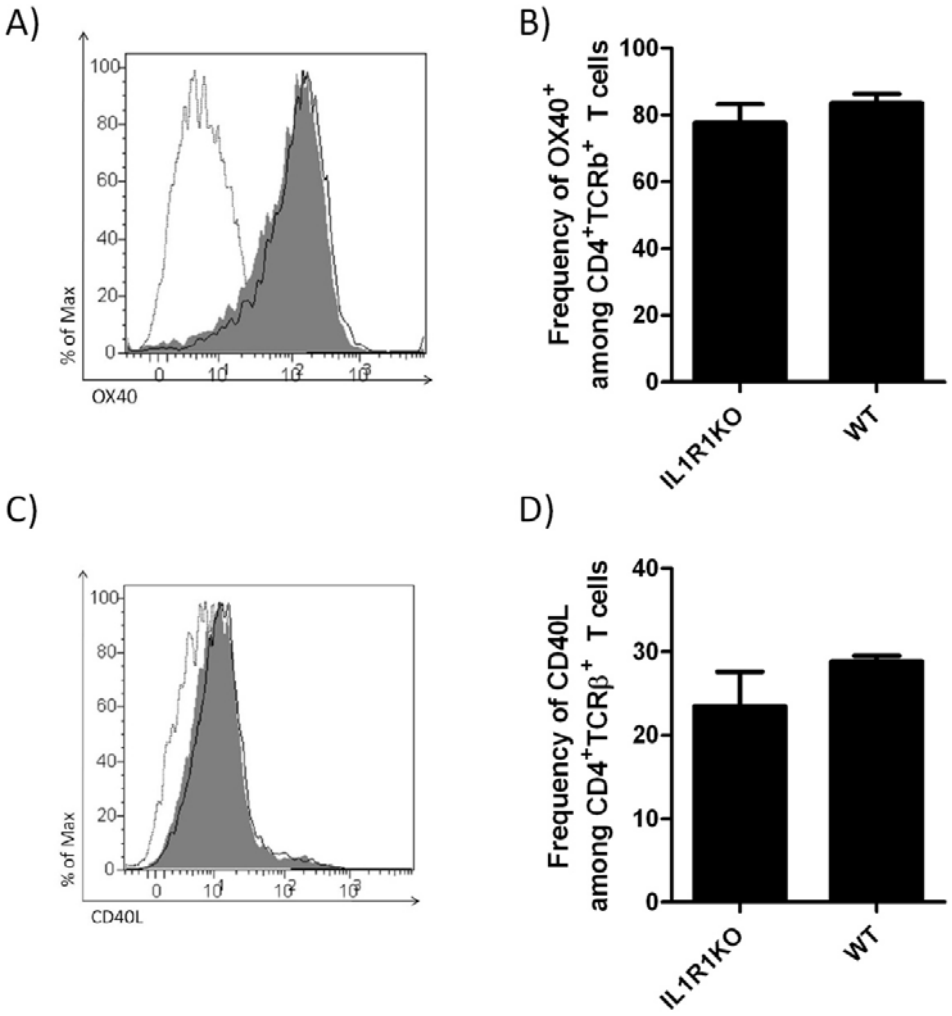


Figure 5-11. IL-1R deficiency does not inhibit CD40L or OX40 expression by activated CD4$^+$ T cells. CD4$^+$ T cells were isolated from the spleen of WT or *Il1r1*^{KO} mice and stimulated *in vitro* with PMA (0.1 µg/ml) and ionomycin (1 µg/ml). After 4h, expression of co-stimulatory molecules was analyzed by FACS. A) CD40L expression. Representative histograms are shown. Dotted line = isotype control, black line = WT CD4$^+$ T cells, filled histogram = *Il1r1*^{KO} CD4$^+$ T cells. Data are gated on CD4$^+$ TCRβ^+ cells (n=3 experimental replicates per group). B) Frequency of CD40L expressing cells. Data are shown as mean ± SEM (n=3 experimental replicates per group). C) OX40 expression. Representative histograms are shown. Dotted line = isotype control, black line = WT CD4$^+$ T cells, filled histogram = *Il1r1*^{KO} CD4$^+$ T cells. Data are gated on CD4$^+$ TCRβ^+ cells (n=3 experimental replicates). D) Frequency of CD40L expressing cells. Data are shown as mean ± SEM (n=3).

5.2.5 The decreased pathogenicity of IL-1R-deficient CD4+ naïve T cells is not due to increased accumulation of FOXP3+ Treg cells.

$CD4^+$ $FOXP3^+$ Treg cells play an essential role in limiting intestinal inflammation (467). Previous studies from our laboratory have illustrated that pro-inflammatory cytokine signalling, such as IL-23R signalling, can hamper Treg responses *in vivo* (203, 459). Hence, we assessed whether the reduction in intestinal inflammation observed in C57BL/6 *Rag1*^{KO} mice receiving *Il1r1*^{KO} $CD4^+$ naive T cells was due to increased Treg cell responses. We found that C57BL/6 *Rag1*^{KO} mice receiving *Il1r1*^{KO} $CD4^+$ naive T cells, $CD4^+$ $FOXP3^+$ T cells represented a significantly higher proportion of the $CD4^+$ T cell pool present in the colon, when compared to recipients of WT naive $CD4^+$ T cells (**Fig.5-12A**). However, analysis of total cell numbers showed that the total number of $CD4^+$ $FOXP3^+$ T cells was significantly reduced in the colonic lamina propria of C57BL/6 *Rag1*^{KO} mice transferred with *Il1r1*^{KO} $CD4^+$ naive T cells compared to recipients of WT naive $CD4^+$ T cells (**Fig.5-12B**). To further investigate regulatory responses in the colon, the levels of IL-10 in the tissue were analyzed by qPCR. Similar levels of IL-10 expression were found in total colon tissue homogenates isolated from C57BL/6 *Rag1*^{KO} mice receiving either *Il1r1*^{KO} or WT naive $CD4^+$ T cells (**Fig.5-12C**). However, cLPL isolated from C57BL/6 *Rag1*^{KO} recipients of *Il1r1*^{KO} naive $CD4^+$ T cells secreted significantly lower levels of IL-10 compared to cLPL from C57BL/6 *Rag1*^{KO} mice transferred with WT naive $CD4^+$ Tcells (**Fig.5-12D**). Taken together, the decreased accumulation of $CD4^+$ $FOXP3^+$ Tregs in the colon and decreased IL-10 expression by colonic lamina propria lymphocytes in C57BL/6 *Rag1*^{KO} mice receiving *Il1r1*^{KO} $CD4^+$ naive T cells suggests that the increased frequencies of $CD4^+$ $FOXP3^+$ Tregs are unlikely to be responsible for the decreased intestinal

inflammation observed in these mice. Recent publications suggest a role for IL-1β in inducing IL-17A production by $CD4^+$ $FOXP3^+$ Tregs (138, 144). However, $CD4^+$ $FOXP3^+$IL-$17A^+$ T cells were undetectable in the colonic lamina propria of C57BL/6 $Rag1^{KO}$ mice receiving either WT or $Il1r1^{KO}$ $CD4^+$ T cells (data not shown) suggesting that this T cell population does not play a role in this model of intestinal inflammation.

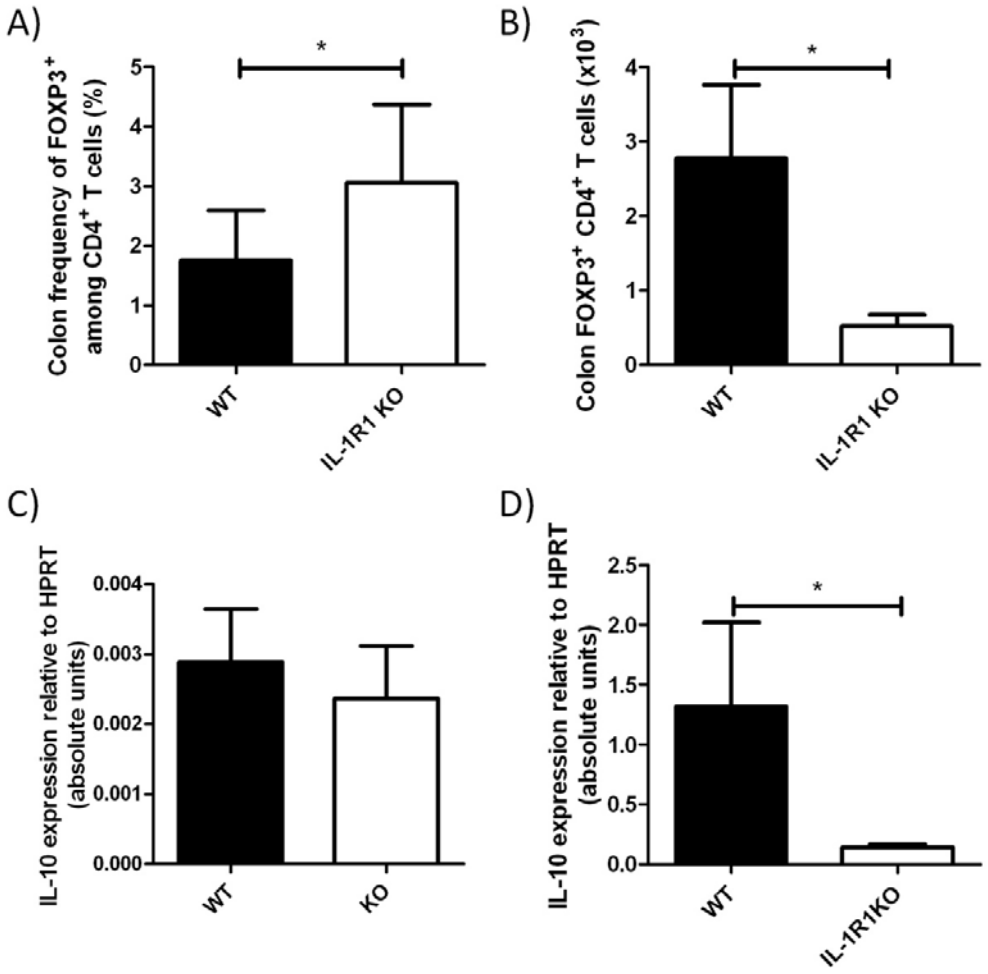


Figure 5-12. Increased frequency, but decreased accumulation of $CD4^+$ $FOXP3^+$ T cells and IL-10 production in mice receiving *Il1r1*^{KO} naïve $CD4^+$ T cells. C57BL/6 *Rag1*^{KO} mice were reconstituted by i.p. injection of $4x10^5$ $CD4^+$ $CD25^-$ $CD45RB^{Hi}$ T cells isolated from C57BL/6 WT or *Il1r1*^{KO} mice. Animals were sacrificed when recipients of WT naive CD4+ T cells developed clinical signs of inflammation (~5 weeks after transfer). Frequencies (A) and total numbers (B) of $CD4^+$ $FOXP3^+$ T cells in the colon as evaluated by intracellular FACS analysis. (n= 20-21 from 3 pooled independent experiments). C) IL-10 expression in total colon tissue homogenates (n=10-13 from 2 independent pooled experiments), and by isolated cLPL (D, n=8 mice per group) as evaluated by qPCR.

All data are shown as mean ± SEM. *p<0.05 Mann Whitney test

5.3 Discussion

IL-1β plays a well established role in promoting $CD4^+$ T cell responses. Traditionally, IL-1β is seen as a costimulator of T cell activity (514). More recently, it has been proposed that IL-1β promotes Th17 differentiation *in vitro* and *in vivo* (513). Here, we used a model of $CD4^+$ T cell driven colitis to analyze the role of T cell intrinsic IL-1R signaling in promoting T cell responses in the intestine. We found that IL-1 signaling on T cells was required for the accumulation, but not differentiation, of Th17 cells in the intestine and for the development of severe intestinal inflammation in the T cell transfer model of colitis. The reduced ability of *Il1r1*^{KO} naïve $CD4^+$ T cells to induce severe colitis upon transfer to C57BL/6 *Rag1*^{KO} recipients was associated with reduced accumulation of $CD4^+$ T cells in the colon. However, the similar numbers of $CD4^+$ T cells found in the spleen or liver in C57BL/6 *Rag1*^{KO} recipients of WT or *Il1r1*^{KO} $CD4^+$ T cells suggests that while not required for the reconstitution of lymphopenic mice, T cell-intrinsic IL-1R signaling is a prerequisite for T cell accumulation in the intestine. Mechanistic analysis revealed that this effect was not due to defective proliferation or migration of *Il1r1*^{KO} $CD4^+$ T cells, suggesting a role of IL-1β in promoting T cell survival. This was further supported by our data showing that *Il1r1*^{KO} $CD4^+$ T cells exhibited increased apoptosis following *in vitro* αCD3 and αCD28 stimulation *in vitro*. Previous work showed that IL-1β stimulation of T cells results in AKT phosphorylation (139, 517, 705), an event strongly associated with pro-survival signals (706). Moreover, IL-1β can promote the expansion of naïve and memory $CD4^+$ T cells following *in vivo* immunization, an effect which is only partially due to increased proliferation, further suggesting that IL-1β promotes T cell survival *in vivo* (705). These results are consistent with previous findings that naïve *MyD88*^{KO}

$CD4^+$T cells also exhibited impaired ability to accumulate in the intestine and mediate colitis after transfer into C57BL/6 *Rag1*^{KO} mice (623, 707, 708). Furthermore, $CD4^+$ T cells re-isolated from C57BL76 *Rag1*^{KO} mice transferred with *MyD88*^{KO} naïve $CD4^+$T cells showed reduced expression of the anti-apoptotic proteins Bcl2 and Bcl-xL, suggesting a role for MyD88 signals in promoting T cell survival(708).

We observed that defective $CD4^+$ T cell accumulation in the intestine following transfer of naïve *Il1r1*^{KO} $CD4^+$ T cells was primarily due to decreased numbers of Th17 cells. Previous studies in humans and mice have identified a role for IL-1β, in conjunction with the pro-inflammatory cytokines IL-6 and IL-23, in promoting Th17 differentiation (60, 133, 137-139) and auto-inflammatory diseases characterized by high levels of IL-1β are associated with a marked Th17 signature at the effector site (316-319, 549, 709, 710). Furthermore, T cell intrinsic IL-1R expression was necessary for the induction of experimental autoimmune encephalomyelitis (EAE) *in vivo,* as adoptive transfer of *Il1r1*^{KO} $CD4^+$ T cells resulted in decreased frequencies of Th17 cells in the central nervous system (138, 139). Our *in vitro* studies confirmed the requirement for IL-1R expression in Th17 differentiation. However, comparable frequencies of IL-17 producing $CD4^+$ T cells were found in the colon of C57BL/6 *Rag1*^{KO} mice receiving WT or *Il1r1*^{KO} naïve $CD4^+$ T cells, which may reflect the highly polarizing microenvironment and the propensity for Th17 lineage commitment in the colon to be driven by other factors (361, 685, 686). Although dispensable for Th17 differentiation in the gut, IL-1R signaling was necessary to promote their accumulation, as shown by decreased numbers of Th17 cells in the colon of C57BL/6 *Rag1*^{KO} receiving *Il1r1*^{KO} naïve $CD4^+$ T cells. This effect was specific to Th17 (IL-17A$^+$

IFNγ$^-$) cells and was not observed for Th1 cells (IL-17A$^-$IFNγ$^+$) or double-positive (IL-17A$^+$IFNγ$^+$) CD4$^+$ T cells, showing that *Il1r1*^{KO} T cells are not generally impaired in their ability to differentiate into Th effector cells. This selective defect in Th17 responses could be explained by high levels of expression of the IL-1R1 by Th17 cells, which may render them more sensitive to, or dependent on, IL-1R stimulation (138, 545). Since proliferation and migration to the gut of *Il1r1*^{KO} naïve CD4$^+$ T cells was unimpaired, it is possible that the main function for IL-1R signals in modulating Th17 biology in the gut is to promote their survival during inflammation. This hypothesis is consistent with other studies showing that IL-1β can promote Th17 expansion and cytokine production *in vitro* in the absence of TCR stimulation (139), suggesting a role of IL-1β in homeostatic maintenance of Th17 cells. Recently, Atarashi et al. proposed that, in the colon, ATP of bacterial origin promotes Th17 differentiation through the activation of a specific subset of CD70^{Hi} CD11cl^{ow} mononuclear cells (686). Moreover, administration of non-hydrolysable ATP in a T cell transfer model of colitis resulted in exacerbated intestinal inflammation, accompanied by increased accumulation of IL-17A-producing CD4$^+$ T cells (686). Since ATP is known to activate the inflammasome, it is possible that bacterial ATP plays a role in IL-1β secretion in the intestine.

Our finding of increased frequencies of CD4$^+$ FOXP3$^+$ T cells in the colon of C57BL/6 *Rag1*^{KO} mice receiving *Il1r1*^{KO} CD4$^+$ naïve T cells suggest that IL-1R signaling prevents the differentiation of CD4$^+$ FOXP3$^+$ T cells in the intestine. Indeed, it has been shown that IL-1β preferentially promotes the expansion of effector T cells over Treg cells (644, 645). Moreover, mice deficient in IRAK-1, a signaling molecule downstream of IL-1R1, have increased frequencies of CD4$^+$ CD25$^+$ FOXP3$^+$ T cells in the steady state

(684). Recent studies demonstrated that IL-1β could down-regulate FOXP3 expression by Tregs and promote their expression of the Th17 transcription factor RORγt *in vitro* (138, 144). However, we did not observe any differentiation of $CD4^+$ $FOXP3^+IL\text{-}17A^+$ T cells in the colon of C57BL/6 $Rag1^{KO}$ mice transferred either with WT or $Il1r1^{KO}$ naïve $CD4^+$ T cells. Moreover, in spite of the increased frequency of $CD4^+$ $FOXP3^+$ T cells, we found decreased total numbers of $CD4^+$ $FOXP3^+$ T cells in the colon of C57BL/6 $Rag1^{KO}$ mice receiving $Il1r1^{KO}$ $CD4^+$ naïve T cells, compared with recipients of WT naïve $CD4^+$ T cells. In parallel, we observed a reduction in the levels of IL-10 expression by cLPL from C57BL/6 $Rag1^{KO}$ mice transferred with $Il1r1^{KO}$ $CD4^+$ naïve T cells when compared to recipients of WT naïve $CD4^+$ T cells, although IL-10 expression in colon tissue homogenates were similar. Thus, a lack of T cell intrinsic IL-1R signaling does not appear to promote the preferential accumulation or regulatory function of $CD4^+$ $FOXP3^+$ T cells in the colon, making it unlikely that the reduced pathology observed upon transfer of $Il1r1^{KO}$ $CD4^+$ naïve T cells is due to increased $FOXP3^+$ Treg activity. However, it is intriguing that IL-1R deficiency on T cells resulted in decreased accumulation of both $CD4^+$ $IL\text{-}17A^+IFN\text{-}\gamma^-$ Th17 cells and $CD4^+$ $FOXP3^+$ Treg cells, as the differentiation of these different $CD4^+$ T cell subsets is strongly related. In fact, dual-expressing $RORgt^+$ $FOXP3^+$ $CD4^+$ T cells have been identified *in vitro* and *in vivo* and plasticity between these subsets has been reported . The prevailing dogma is that inflammatory cytokines, such as IL-1β, should promote the differentiation of Th17 cells at the expense of $CD4^+$ $FOXP3^+$ Treg cells (459, 644, 645). However, our data suggest a different hypothesis, where IL-1R signals are required for the maintenance of both Th17 cells and $FOXP3^+$ Treg cells, again

suggesting that IL-1β primarily promotes T cell survival in the intestine, rather than promoting T cell differentiation.

Overall, the data presented in this chapter show that IL-1R signaling on T cells is required for the development of intestinal and hepatic inflammation in a model of T cell transfer induced colitis. Moreover, we showed that T cell-intrinsic IL-1R signals play a key role in promoting the accumulation of pathogenic $CD4^+$ T cells in the intestine. This effect was not due to defective proliferation or migration to the colon, supporting the hypothesis that IL-1R signaling promotes T cell survival in the inflamed intestine. Moreover, we observed a specific effect of IL-1R signaling in promoting the accumulation of Th17 cells in the inflamed gut. These data suggest that the main role for IL-1R in intestinal inflammation is to promote the survival of pathogenic effector T cell populations. Interestingly, the therapeutic effect of αTNF-α administration in IBD patients is at least in part mediated by the induction of apoptosis in $CD4^+$ T cells (324). By extension, blockade of IL-1R signals might prove beneficial in IBD through similar mechanisms.

Chapter 6: Preliminary analysis of the role of NOD2 in *H.hepaticus*-driven intestinal inflammation

6.1 Introduction

NOD2 is the prototypical member of the family of intracellular pattern recognition receptors termed NOD-like receptors (NLR). Like most NLR, NOD2 comprises an N-terminal caspase-recruitment domain (CARD), a central oligomerization domain (NACHT) and a C-terminal series of leucine-rich repeats (LRR) (614). The exact function of NOD2 is unclear, but it has been shown that NOD2 can recognize *N*-acetyl muramyl dipeptide (MDP), a component of bacterial peptidoglycan (264) and *N*-glycolyl MDP from *Mycobacteria spp* (711). Following activation, NOD2 forms a complex with receptor interacting protein 2 (RIP-2), leading to the activation of the NF-κB pathway and the MAPK cascade, promoting adaptive and innate immune responses(250). Moreover, it has been proposed that NOD2 might participate in the formation of inflammasomes by interacting with other NLR, such as NLRP3 and NLRP1, to induce IL-1β secretion (305, 306).

In the intestine, NOD2 is expressed in intestinal epithelial cells (712), particularly Paneth cells (713), intestinal myofibroblasts (714), endothelial cells (715) and leukocytes such as macrophages and dendritic cells (281). Genome-wide association studies (GWAS) identified a strong association between polymorphisms in *NOD2* and Crohn's disease (CD) in people of European descent (326), confirming previous findings by linkage studies (280). The vast majority of CD-associated mutations localize to the C-terminal LRR, suggesting a possible disruption of the sensor function of NOD2. However, the exact role of NOD2 mutations in the pathogenesis of IBD

remains unclear and several hypotheses have been proposed to explain how they might predispose to the development of IBD:

1. Loss-of-function mutations could lead to decreased responses against intestinal bacteria, disrupting the fine balance between micro-flora and host in the human intestine. This hypothesis is supported by transfection studies suggesting that *NOD2* mutations are associated with decreased responses to MDP (716, 717). In addition, NOD2 plays a key role in promoting the production of anti-microbial peptides (AMP) by Paneth cells in the small intestine and some patients with mutations in *NOD2* show decreased α-defensin responses (410, 718). Furthermore, NOD2 can synergize with TLR signalling *in vitro* and *in vivo,* promoting the production of pro-inflammatory cytokines by dendritic cells and macrophages (414, 719, 720). Hence, mutations in *NOD2* would impair the ability of the host to control the intestinal microflora, leading to increased bacterial translocation and/or the emergence of colitogenic flora, promoting intestinal inflammation.
2. Alternatively, loss-of-function mutations in *NOD2* could abrogate its ability to down-regulate TLR mediated responses to bacterial constituents, leading to excessive pro-inflammatory responses. Indeed, it has been shown that priming of dendritic cells with MDP downregulates TLR2 responses to peptidoglycan (412, 415). Although this effect was not observed by all investigators (414), studies in mice corroborated this idea, as administration of MDP protected mice from DSS colitis through NOD2-mediated activation of IRF4, a negative regulator of TLR signalling (416).

3. Conversely, some CD-associated *NOD2* mutations might be gain-of-function. Indeed, macrophages from 'knock-in' mice bearing the most common human CD-associated *NOD2* allele secreted higher levels of IL-1β following MDP stimulation (417). However, this hypothesis is inconsistent with clinical evidence, as peripheral blood monocytes from IBD patients bearing *NOD2* mutations secrete less IL-1β than healthy controls following MDP stimulation (407, 585).
4. Finally, in human peripheral blood monocytes, it has been proposed that mutated versions of *NOD2* can inhibit IL-10 transcription by suppressing the DNA-binding activity of the crucial IL-10 inducer heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (418). Hence, mutations in *NOD2* would disrupt a crucial immunoregulatory pathway required for the control of intestinal inflammation, leading to the development of IBD.

The development of animal models has not clarified the role of NOD2 in intestinal inflammation. Neither $Nod2^{KO}$ mice nor 'knock-in' mice bearing the most common CD-associated human *NOD2* allele developed spontaneous colitis (411, 417), although $Nod2^{KO}$ mice were found to be more susceptible to mucosal bacterial infections (411, 721). $Nod2^{KO}$ mice were also reported to be more susceptible to 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (419), but not to DSS-induced intestinal inflammation (411). Additional studies have reported increased Th1-like pro-inflammatory responses in the ileum of $Nod2^{KO}$ mice in the steady state and following *H.hepaticus* infection (419, 722), as well as increased numbers of Payer's patch in the small intestine (419).

Thus far, most studies analyzing the function of NOD2 in intestinal inflammation in mice have focused on ileal inflammation, in accordance with the data showing an effect of NOD2 deficiency on Paneth cell function and development of Payer's patches (408, 409, 419) and strong associations between *NOD2* mutations and ileal disease in CD patients (723). However, *NOD2* mutations are also associated with colonic disease (406, 724) and *NOD2* mutated alleles are still more frequent in IBD patients suffering from colonic disease only compared to the general population (4). Thus, there is a need to better assess the role of NOD2 in models of chronic colitis.

Here, we present preliminary data addressing the role of NOD2 in chronic models of inflammation of the large intestine. Moreover, we also assess the impact of NOD2 on *H. hepaticus* colonization in the healthy and inflamed intestine.

6.2 Results

6.2.1 NOD2 is up-regulated during *H.hepaticus*-induced innate immune colitis.

Since the expression of NOD2 in the intestine is mainly restricted to innate immune cells and Paneth cells (713, 725), we first evaluated the role of NOD2 in the innate model of chronic typhlocolitis triggered by *H.hepaticus* infection in 129SvEv *Rag2*^{KO} mice as described in chapter 3. Initially, we assessed whether the induction of colitis resulted in changes in the expression of NOD2 and other NLR in the organs affected by *H.hepaticus*-induced inflammation. Our analysis revealed that NOD2 was significantly up-regulated in the intestinal tract and in the liver of *H.hepaticus* infected 129SvEv *Rag2*^{KO} mice (**Fig.6-1**). In the liver and caecum, IPAF (NLRC4) was also significantly up-regulated (**Fig.6-1**). By contrast, in the spleen NOD2 and IPAF expression were unchanged following induction of colitis, whereas NOD1 expression was markedly increased (**Fig.6-1**). Hence, innate immune colitis was associated with a gut-specific up-regulation of NOD2 and IPAF.

Next, we evaluated the relative contributions of epithelial and cells of hematopoietic origin to the observed NOD2 up-regulation. In normal, healthy colon, expression of NOD2 is confined to resident macrophages (713). However, during inflammation, NOD2 expression in metaplastic colonic Paneth cells has been reported (713, 725). Therefore, we used the pan-hematopoietic marker CD45 to distinguish cells of hematopoietic and non-hematopoietic origin in colon digests. CD45^{+} cells were further selected using the myeloid marker CD11b, which is expressed by approximately 70% of CD45^{+} leukocytes in the colon of *H.hepaticus*-infected 129SvEv

Rag2^{KO} mice (data not shown). Our analysis of FACS sorted CD45^{-}, CD45^{+}CD11b^{-} and CD45^{+}CD11b^{+} populations revealed that although *H.hepaticus* infection did not result in NOD2 up-regulation in the non-hematopoietic (CD45^{-}) compartment, a two-fold increase in NOD2 expression was observed in CD45^{+} CD11b^{+} cells (**Fig.6-2**).

To confirm that NOD2 expression by hematopoietic cells could be directly up-regulated by *H. hepaticus* infection we turned to an *in vitro* system. Peritoneal elicited macrophages were infected *in vitro* with *H.hepaticus* and NOD2 expression was evaluated by qPCR. At 4h post-infection, a significant up-regulation of NOD2 was observed, supporting the hypothesis that *H.hepaticus* directly induces NOD2 expression in monocytic cells (**Fig.6-3**).

Taken together, these data demonstrate that *H.hepaticus*-induced innate immune colitis is associated with increased NOD2 expression in myeloid cells in the intestine.

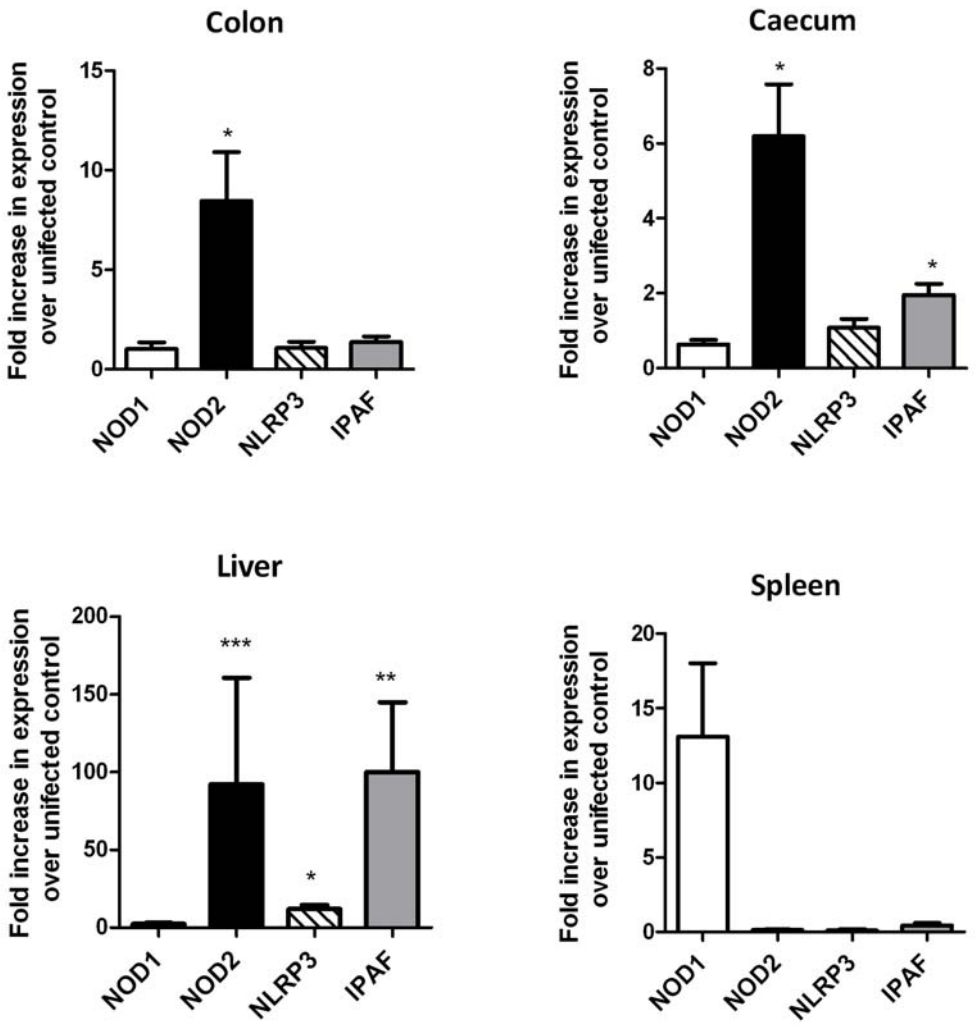


Figure 6-1. *H.hepaticus*-triggered innate immune inflammation is associated with organ specific up-regulation of NOD-like receptors. 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* (10^8 CFU, orally) and sacrificed >8 weeks after infection. mRNA was extracted from indicated organs and expression of different NLR was quantified by qPCR. Expression levels were determined relative to HPRT. Data shown represents fold increase relative to uninfected controls (mean ± SEM). Statistical significance was calculated by comparing the relative NLR expression in *H.hepaticus* infected mice vs. uninfected controls. (n=16 for colon and caecum of infected animals, n=5 for liver and spleen; n=9 for colon and caecum of uninfected controls and n=3 for liver and spleen). All data are shown as mean ± SEM. *p<0,05, **p<0.01, ***p<0.001 Mann Whitney test.

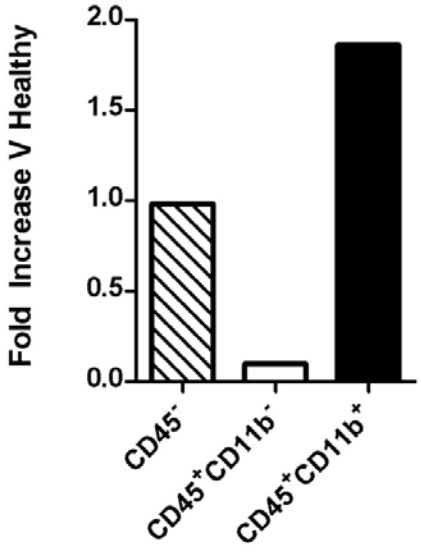


Figure 6-2. NOD2 is preferentially up-regulated in colonic myeloid cells in *H. hepaticus*-infected colitic mice. (A-B). 129SvEv *Rag2*^{KO} mice were infected with *H.hepaticus* (10^8 CFU, orally) and sacrificed >8 weeks after infection. After colon tissue digestion, $CD45^+CD11b^+$, $CD45^+CD11b^-$ and $CD45^-$ cells were FACS sorted, mRNA was purified and NOD2 expression was evaluated by qPCR. All data were normalized to HPRT expression. One representative experiment of two is shown.

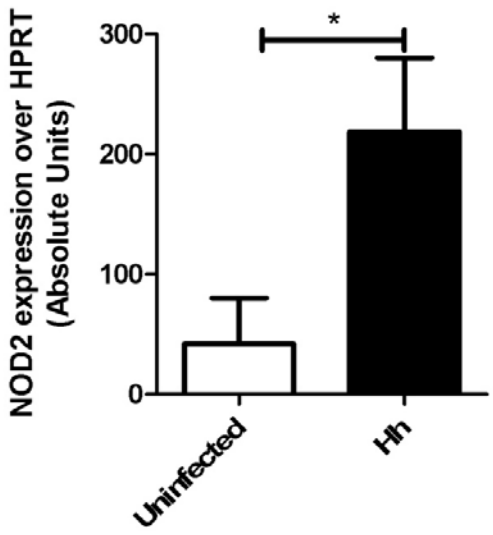


Figure 6-3. NOD2 expression is up-regulated following *H.hepaticus* infection of peritoneal elicited macrophages *in vitro*. Peritoneal macrophages (PEM) were elicited from 129SvEv by i.p. injection of 2% Biogel. PEM were harvested 3-4 days post-injection and $5x10^5$-10^6 cells were infected with *H.hepaticus* (MOI =15) for 4h. mRNA was purified and NOD2 expression was evaluated by qPCR. All data were normalized to HPRT expression. Data are expressed as mean ± SD (n=3 from 3 independent experiments).

*p<0,05 Mann Whitney test.

6.2.2 NOD2 deficiency attenuates *H.hepaticus*-induced innate immune colitis.

Next, we wanted to evaluate the functional role of NOD2 in the induction of innate immune colitis. Therefore, *Nod2*^{KO} mice that were generated on a mixed B6.129S1 background (411) were backcrossed to 129SvEv *Rag2*^{KO} using a speed congenic approach based on micro-satellite marker typing (599, 600). Unfortunately, this time consuming backcrossing process was not completed at the time this thesis was compiled. However, as a preliminary experiment, we utilized a cohort of *Nod2*^{WT}, *Nod2*^{Het} and *NOD2*^{KO} 129SvEv *Rag2*^{KO} littermates, that expressed >82% of 129SvEv micro-satellite markers. We found that, following infection with *H.hepaticus*, both 129SvEv*Rag2*^{KO} *Nod2*^{KO} mice and 129SvEv *Rag2*^{KO} *Nod2*^{Het} mice developed reduced colitis when compared to the 129SvEv*Rag2*^{KO} *Nod2*^{WT} littermate controls (**Fig.6-4A**). By contrast, in the caecum, pathology was minimally affected by the NOD2 genotype (**Fig.6-4B**). A similar phenotype was observed in the spleen, as *H.hepaticus*-infected 129SvEv *Rag2*^{KO} *Nod2*^{KO} and 129SvEv *Rag2*^{KO} *Nod2*^{Het} mice exhibited decreased splenomegaly and reduced numbers of splenocytes compared to 129SvEv *Rag2*^{KO} *Nod2*^{WT} littermate controls (**Fig.6.5**). Analysis of *H.hepaticus* colonization in the caecum revealed similar levels of *H.hepaticus* were present, irrespective of the NOD2 genotype (**Fig.6-6**). Although preliminary, these data suggest that NOD2 might play a role in promoting *H. hepaticus* induced intestinal inflammation without affecting bacterial colonization levels.

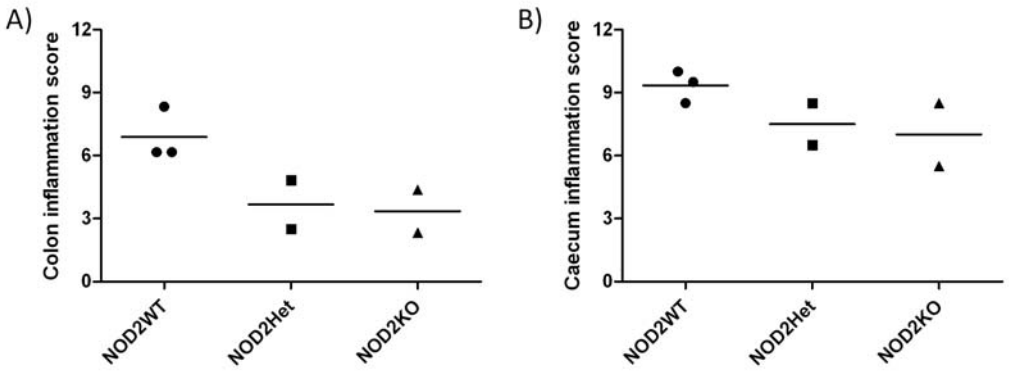


Figure 6-4. NOD2 deficiency attenuates *H.hepaticus*-induced innate immune colitis. 129SvEv *Rag2*^{KO} *Nod2*^{WT}**,** 129SveEv *Rag2*^{KO} *Nod2*^{Het} and 129SvEv *Rag2*^{KO} *Nod2*^{KO} littermates were infected with *H.hepaticus* (10^8 CFU, orally) and sacrificed 8 weeks later. Each symbol represents one mouse and bars show the average score for each group. A) Colon inflammation score. B) Caecum inflammation score.

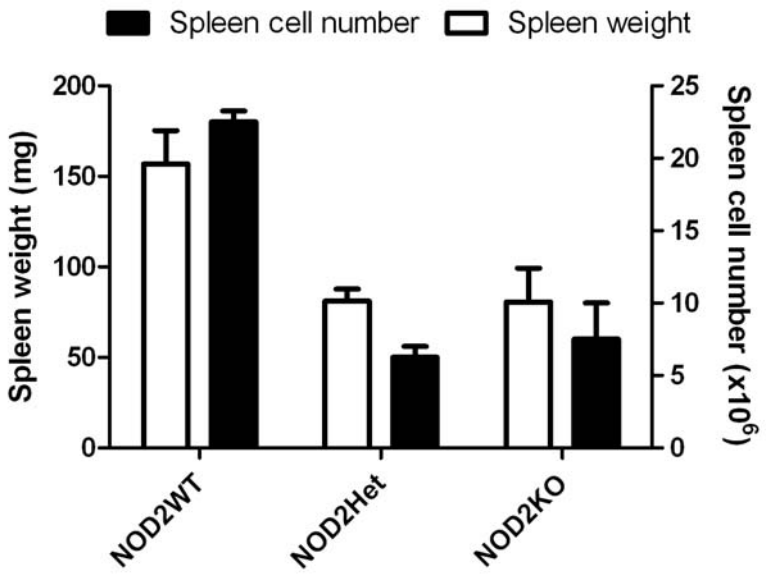


Figure 6-5. NOD2 deficiency attenuates the development of systemic pathology in *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice. 129SvEv *Rag2*^{KO} *Nod2*^{WT}, 129SveEv *Rag2*^{KO} *Nod2*^{Het} and 129SvEv *Rag2*^{KO} *Nod2*^{KO} littermates were infected with *H.hepaticus* (10^8 CFU, orally). After 8 weeks, mice were sacrificed and spleen weight and spleen cellularity evaluated. Data are shown as mean ± SEM (n=3 for *Nod2*^{WT} and 2 for *Nod2*^{Het} and *Nod2*^{KO} mice).

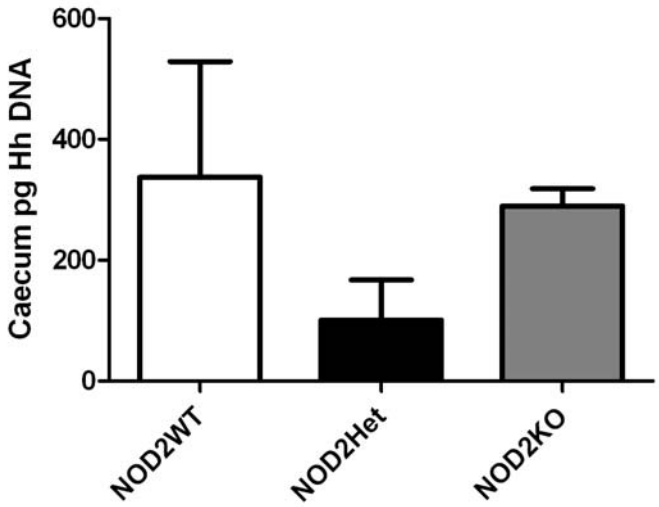


Figure 6-6. NOD2 deficiency does not influence *H.hepaticus* colonization levels in 129SvEv Rag2^{KO} mice. 129SvEv *Rag2*^{KO} *Nod2*^{WT}, 129SveEv *Rag2*^{KO} *Nod2*^{Het} and 129SvEv *Rag2*^{KO} *Nod2*^{KO} littermates were infected with *H. hepaticus* and sacrificed 8 weeks after infection. DNA was isolated from cecal contents and levels of *H.hepaticus* DNA were determined using a q-PCR method based on the *cdtB* gene (604). Data are shown as mean ± SEM (n=3 for 129SvEv *Rag2*^{KO} *Nod2*^{WT}, n= 2 for 129SveEv *Rag2*^{KO} *Nod2*^{Het} and 129SvEv *Rag2*^{KO} *Nod2*^{KO}).

6.2.3 NOD2 expression does not influence persistent *H.hepaticus* colonization of immune competent mice.

In contrast to numerous reports on the persistent nature of *H.hepaticus* infection (352, 366, 370, 373, 726), it was recently reported that immunocompetent C57BL/6 (WT) mice were able to clear *H.hepaticus*, whereas $Nod2^{KO}$ mice were unable to control or clear the infection (727). To address this issue, we analyzed the kinetics of *H.hepaticus* infection in lymphocyte replete $Nod2^{WT}$ and $Nod2^{KO}$ mice. However, as commercially available B6.129S1 $Nod2^{KO}$ mice were on a mixed background (backcrossed 7 times to C57BL/6 mice), we used both 129SvEv and C57BL/6 mice as $Nod2^{WT}$ controls. *H.hepaticus* colonization was monitored by measuring *H. hepaticus* DNA in the faeces of infected mice (727). We found that *H.hepaticus* DNA peaked in the faeces after 2 weeks of infection (**Fig.6-7A**), consistent with reports that *H. hepaticus* establishes its niche in the intestine in approximately 14 days (641). Furthermore, *H.hepaticus* DNA was present in the faeces of all mouse strains up to 63 days post-infection and no differences in *H.hepaticus* DNA levels were observed between B6.129S1 $Nod2^{KO}$ mice and $Nod2^{WT}$ controls at any time point during the infection (**Fig.6-7A**). At sacrifice, *H.hepaticus* colonization in the caecum was assessed and again, B6.129S1 $Nod2^{KO}$ mice and $Nod2^{WT}$ sufficient controls exhibited similar levels of *H.hepaticus* DNA (**Fig.6-7B**). Although the large intestine is the primary site of *H.hepaticus* colonization, *H.hepaticus* DNA has been reported to be detectable in the terminal ileum of infected mice (727). However, we observed extremely low levels of *H.hepaticus* DNA in the terminal ileum in C57BL/6 and 129SvEv $Nod2^{WT}$ control mice, close to the limit of detection of our assay (**Fig.6-7C**). Moreover, B6.129S1 $Nod2^{KO}$ mice showed comparably low levels of *H.hepaticus* DNA

in the terminal ileum as found in *Nod2*^{WT} control mice (**Fig.6-7C**). It was recently reported that *H.hepaticus* infection of B6.129S1 *Nod2*^{KO} mice resulted in the development of granulomatous ileitis (722). However, we were unable to detect granulomas or any other histological signs of inflammation in the small or large intestines of *H. hepaticus*-infected B6.129S1 *Nod2*^{KO} mice (data not shown). Taken together, these results confirm that *H.hepaticus* establishes a persistent infection in immune competent C57BL/6 and 129SvEv mice, and indicate that NOD2 does not regulate *H.hepaticus* colonization in the intestine.

Next, we evaluated whether *H.hepaticus* infection of B6.129S1 *Nod2*^{KO} mice resulted in increased antibody responses, as NOD2-mediated sensing of bacterial product promotes the activation of adaptive immunity and the production of antibodies to T cell dependent antigens (411, 728). We analyzed the levels of *H.hepaticus*-specific mucosal and systemic antibody responses using ELISAs with a preparation of soluble *H.hepaticus* antigen (sHelAg) (367). We found that both 129SvEv and C57BL/6 *Nod*^{WT} control strains mounted readily detectable *H.hepaticus*-specific IgG responses, with 129SvEv mice having significantly greater levels of serum *H.hepaticus*-specific IgG than C57BL/6 mice (**Fig.6-8A**). However, B6.129S1 *Nod2*^{KO} mice developed intermediate levels of *H. hepaticus*-specific IgG, with titers not significantly different from those found in either 129SvEv or C57BL/6 *Nod*^{WT} control strains (**Fig.6-8A**). Next, we analyzed specific mucosal IgA responses against *H.hepaticus* in the small intestine. Once again, we found that 129SvEv mice developed significantly stronger specific IgA responses against *H.hepaticus* than C57BL/6 mice (**Fig.6-8B**). Furthermore, B6.129S1 *Nod2*^{KO} mice again showed an intermediate phenotype,

developing H. hepaticus-specific IgA responses that were significantly higher than those observed in C57BL/6 NOD2^{WT} controls, but significantly lower than those found in 129SvEv NOD2^{WT} controls (**Fig.6-8B**). Taken together, these data illustrate the dominant effects of genetic strain background in regulating mucosal and systemic *H.hepaticus*-specific antibody responses, which make it difficult to decipher any effects of NOD2 deficiency using *Nod2*^{KO} mice of a mixed strain background.

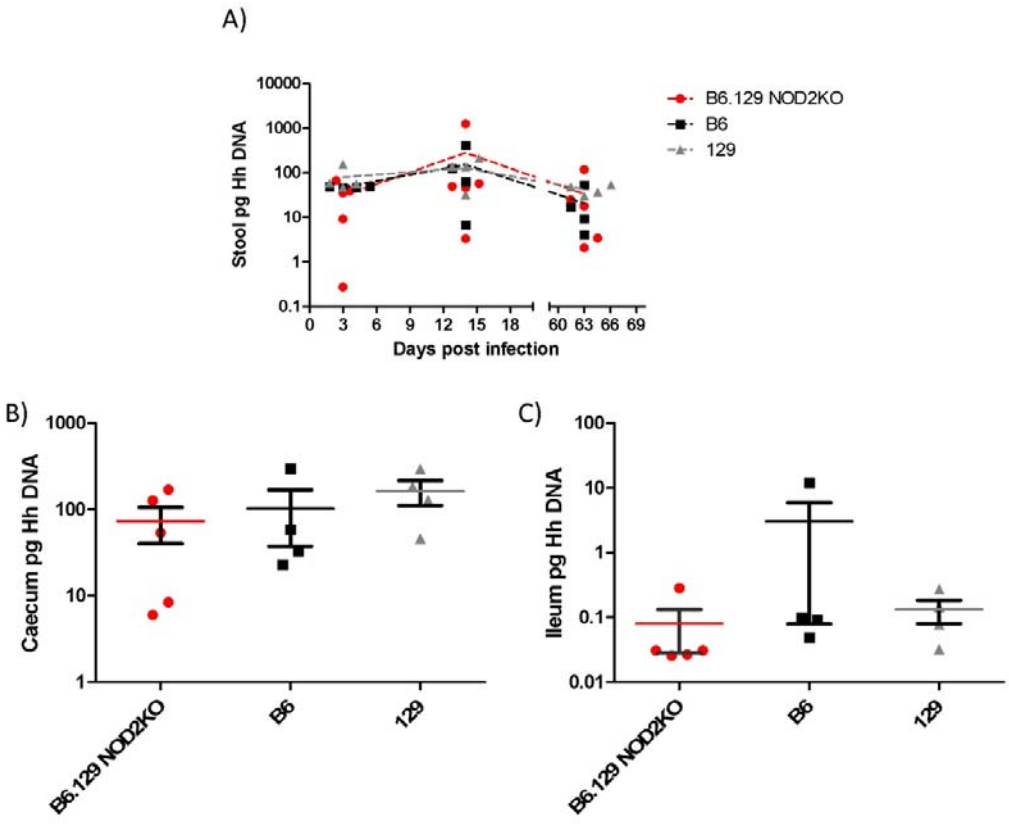


Figure 6-7. NOD2 expression does not influence *H.hepaticus* colonization in the intestine. Cohorts of B6.129S1 *Nod2*^{KO}, C57BL/6 and 129SvEv mice were infected with *H.hepaticus* (5x10^{8}CFU, orally) and stool samples were collected at day 3, 14 and 63 post-infection. After 9 weeks of infection mice were sacrificed and sections of terminal ileum and cecal contents were isolated. DNA was extracted and levels of *H.hepaticus* colonization were determined using a q-PCR method based on the *cdtb* gene (604). Each symbol represents an individual mouse and bars show the mean ± SEM. A) Quantification of *H.hepaticus* colonization in stool samples. B) *H.hepaticus* colonization in the caecum. C) *H.hepaticus* colonization in the ileum.

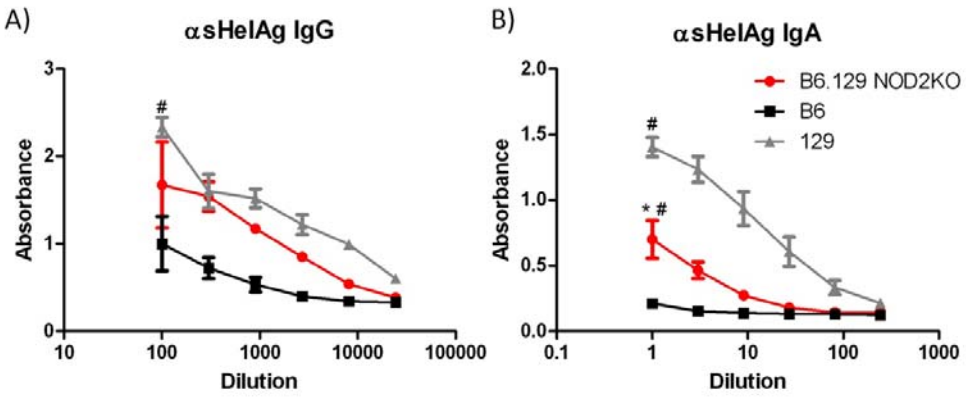


Figure 6-8. 129SvEv mice develop higher specific antibody responses against *H.hepaticus*. Cohorts of B6.129S1 *Nod2*^{KO} mice, C57BL/6 and 129SvEv mice were infected with *H.hepaticus* and sacrificed after 9 weeks of infection. At sacrifice, serum samples and small intestinal lavage samples were collected and levels of antibody response against soluble *H. hepaticus* antigen (sHelAg) evaluated by ELISA . Data are shown as mean absorbance (OD 405nm) ± SEM (n=5 for B6.129S1 *NOD2*^{KO} mice and n=4 for 129SvEv and C57BL/6 mice). A) *H.hepaticus*-specific IgG levels in serum. B) *H.hepaticus*-specific IgA levels in small intestinal lavages.

*p<0.05 when compared to 129SvEv mice, #p<0.05 when compared to C57BL/6 mice Mann Whitney test

6.2.4 Increased colonization in B6.129S1 *NOD2^{KO}* mice during *H.hepaticus*-induced colitis.

Next, we wanted to analyze the role of NOD2 in the induction of *H.hepaticus*-triggered colitis in a lymphocyte replete setting. For this purpose, we used the model described in chapter 4, where lymphocyte-replete mice develop severe typhlocolitis following infection with *H.hepaticus* and weekly treatment with 1mg αIL-10R antibody (Hh + αIL-10R) (438). To take into account the mixed genetic background of the B6.129S1 *Nod2^{KO}* mice, we used both 129SvEv and C57BL/6 mice as *Nod2^{WT}* controls. As expected (438), C57BL/6 developed severe colitis and typhlitis following Hh + αIL-10R (**Fig.6.9A-B**). However, 129SvEv mice developed only mild colitis and also had less severe caecal inflammation following Hh + αIL-10R when compared to C57BL/6 mice (**Fig.6.9A-B**). Furthermore, B6.129S1 *Nod2^{KO}* mice exhibited a wide range of severities of intestinal following Hh + αIL-10R (**Fig.6.9A-B)**. Thus, again the mixed background of the B6.129S1 *Nod2^{KO}* mice used in this experiment did not allow us to derive any specific conclusions regarding the role of NOD2 in intestinal inflammation in this setting. In contrast, no significant differences between strains were observed when analyzing the systemic response following Hh + αIL-10R, with comparable spleen weights and cell numbers observed in B6.129S1 *Nod2^{KO}* and in both *Nod2^{WT}* control strains (**Fig.6-10**).

Next, we analyzed the levels of *H.hepaticus* colonization in B6.129S1 *Nod2^{KO}*, C57BL/6 and 129SvEv mice following Hh + αIL-10R. We found that C57BL/6 had decreased levels of *H.hepaticus* colonization when compared to 129SvEv mice, although the difference was not significant due to the small group sizes (**Fig.6-11**).

This data correlates well with the observed difference in intestinal inflammation between these two mice strains (**Fig.6-9**), as high levels of inflammation often correlate with lower levels of *H.hepaticus* colonization (unpublished observations). However, following Hh+αIL-10R, B6.129S1 *Nod2*^{KO} mice showed significantly increased levels of *H.hepaticus* colonization when compared to both 129SvEv and C57BL.6 *Nod*^{WT} controls (**Fig.6-11**). Hence, even in the presence of confounding genetic background effects, NOD2 might limit *H.hepaticus* colonization in these experimental settings.

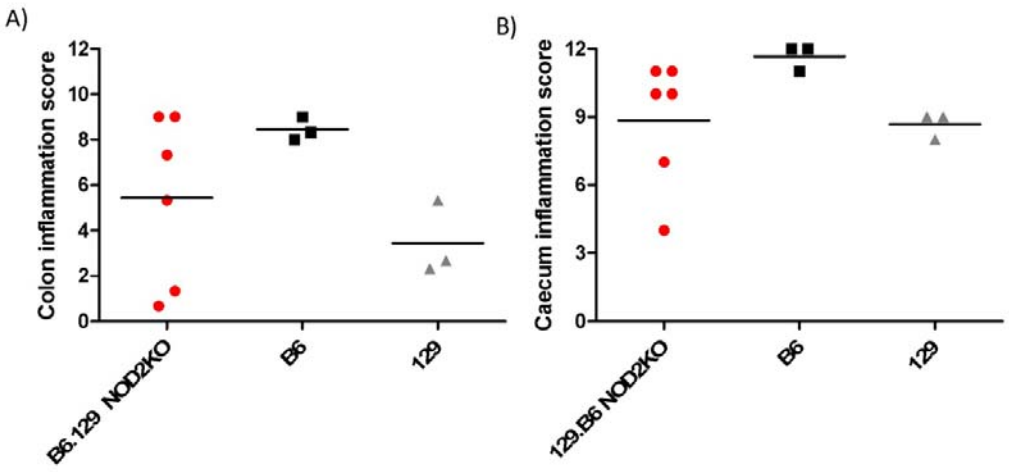


Figure 6-9. Effects of mouse strain background on *H.hepaticus* induced intestinal inflammation in lymphocyte replete mice. Cohorts of B6.129S1 *Nod2*^{KO} mice, C57BL/6 and 129SvEv mice were infected with *H.hepaticus* (10^8 CFU, orally) and treated with αIL-10R antibody (1mg/mouse, weekly). After 4 weeks, the animals were sacrificed and intestinal inflammation was evaluated. Each symbol represents an individual mouse and bars show the mean score. A) Colon inflammation score. B) Caecum inflammation score.

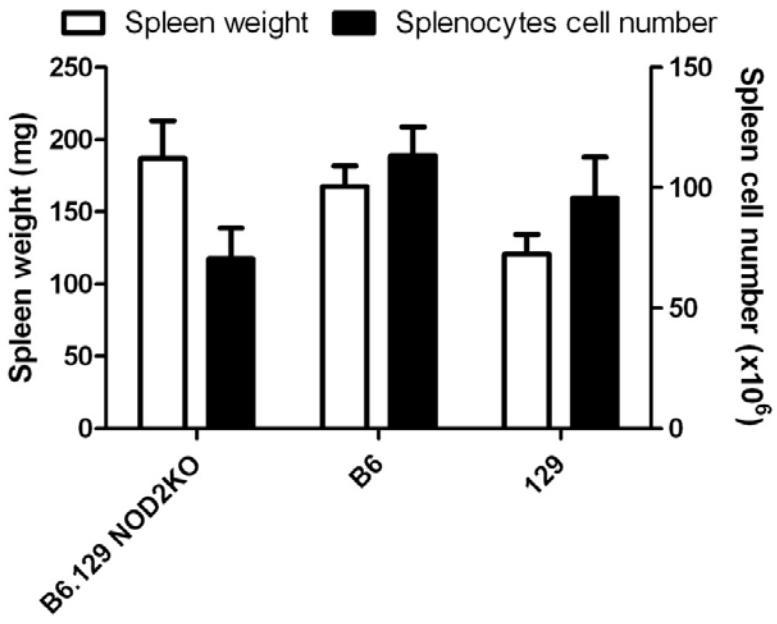


Figure 6-10. Comparable systemic inflammation following *H.hepaticus* infection and αIL-10R treatment of B6.129S1 *Nod2*^{KO} mice and *Nod2*^{WT} control mice. Cohorts of B6.129S1 *Nod2*^{KO}, C57BL/6 and 129SvEv mice were infected with *H. hepaticus* (10^8 CFU, orally) and treated with αIL-10R antibody (1mg/mouse, weekly). After 4 weeks, the animals were sacrificed and spleen weight and cellularity were evaluated. Data are shown as mean ± SEM (n= 6 for B6.129S1 *Nod2*^{KO} mice and n=3 for C57BL/6 and 129SvEv *Nod2*^{WT} control mice).

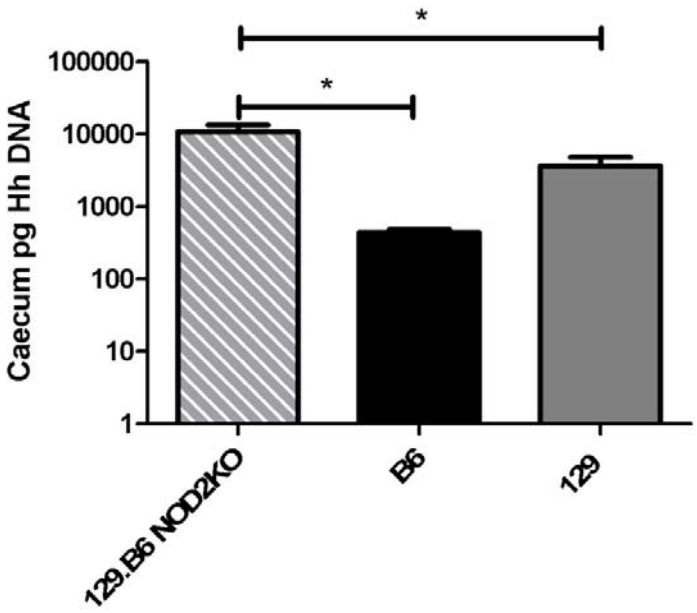


Figure 6-11. Increased *H.hepaticus* colonization in the absence of NOD2. Cohorts of B6.129S1 *Nod2*^{KO}, C57BL/6 and 129SvEv mice were infected with *H.hepaticus* and treated with aIL-10R antibody (1mg/mouse, weekly). After 4 weeks, the animals were sacrificed and DNA was isolated from cecal contents and levels of *H.hepaticus* DNA were determined using a q-PCR method based on the *cdtB* gene (604). Data are shown as mean ± SEM (n= 6 for B6.129S1 *Nod2*^{KO} mice and n=3 for C57BL/6 and 129SvEv *Nod2*^{WT} control mice).

6.3 Discussion

NOD2 was the first gene identified by genetic studies to be associated with CD (4). However, the exact contribution of *NOD2* mutations to the pathogenesis of CD is unclear. Current hypotheses focus on the role of NOD2 in bacterial handling at mucosal surfaces. However, this role has been address almost exclusively in acute models of colitis, which might not reflect the complexity of chronic disease. Here, we used two models of *H.hepaticus*-induced colitis to address the role of NOD2 in chronic intestinal inflammation. Although preliminary, these data point towards a pathogenic role of NOD2 in chronic colitis. Moreover, we assessed whether NOD2 deficiency alters the equilibrium between the host and *H.hepaticus*, a normal member of the mouse flora which can induce disease in susceptible strains. We found no contribution for NOD2 in limiting *H.hepaticus* colonization during steady state or during innate immune colitis in lymphopenic $Rag2^{KO}$ mice. However, we observed increased *H.hepaticus* colonization during colitis in lymphocyte replete $Nod2^{KO}$ mice, suggesting a potential role for NOD2-mediated modulation of adaptive immunity in limiting *H.hepaticus* colonization during inflammation. NOD2 can promote adaptive immunity both indirectly, through the activation of APC (412, 729, 730), and directly, as NOD2 deficient T cells show defective Th1 differentiation and fail to induce colitis in a T cell transfer model (420). Further investigation in the role of NOD2 in modulating intestinal adaptive immune responses will be required to understand the dynamics of the immune system/bacteria interaction in the intestine.

Initially, we focused on analyzing the regulation of NOD2 expression during *H.hepaticus*-induced innate intestinal inflammation. NOD2 is expressed at very low levels in intestinal macrophages in steady state conditions (713, 731), but is up-regulated in the inflamed colon in CD patients (731). Accordingly, we found increased levels of NOD2 expression in the inflamed colon, caecum and liver of *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice. Considering that these organs are the primary sites of *H.hepaticus* colonization (373, 444), it is tempting to speculate that NOD2 up-regulation might be directly linked to *H.hepaticus* colonization. Indeed, NOD2 was not up-regulated in the spleen, which is inflamed in this model, but is not colonized by *H.hepaticus* ((369, 370) and data not shown). Moreover, our *in vitro* data demonstrated NOD2 up-regulation in macrophages following infection with *H.hepaticus*. In addition, it has been recently reported that NOD2 expression in the terminal ileum is dependent on the microbial flora, as germ-free mice showed significantly decreased levels of NOD2 expression (727). However, NOD2 expression is also regulated by inflammatory stimuli, such as LPS or TNF-α (732), therefore it is possible that the observed NOD2 up-regulation was a result of the inflamed environment in the colon. Regardless of whether NOD2 up-regulation was induced by inflammation or by colonization with *H.hepaticus*, it was striking that NOD2 was the only NLR analyzed consistently up-regulated across sites interested by *H.hepaticus*-induced inflammation, suggesting an important role of this NLR in this model of colitis. Further analysis of sorted cell populations showed that NOD2 expression in CD45$^+$ CD11b$^+$ monocytes was increased during colitis, whilst no change was observed in CD45$^-$ cells (which likely included intestinal epithelial and stromal cells). However, NLR expression analysis of sorted populations of colonic

$CD11b^+$ cells revealed relatively low levels of NOD2 up-regulation (~2 fold) when compared to the increased NOD2 expression observed in whole tissue samples (~10 fold). This could reflect a loss of other NOD2 expressing populations, including epithelial cells, stromal cells and mast cells, during the colon digestion procedure (733) (734). Alternatively, the high levels of NOD2 up-regulation observed in the whole colon tissue may reflect the recruitment of high numbers of NOD2-expressing leukocytes to the inflamed intestine. Indeed, NOD2 expression is upregulated following maturation of blood monocytes into effector cells (732).

Other NLR were also up-regulated following *H.hepaticus* infection of 129SvEv *Rag2*^{KO} mice. Notably, the inflammasome component IPAF (NLRC4) was upregulated in the caecum and liver of colitic mice. IPAF was initially identified as an intracellular receptor for flagellin, although non-flagellated bacteria can activate the IPAF inflammasome (259). Interestingly, IPAF could mediate *H.hepaticus* recognition, as *H.hepaticus* flagellin contains an IPAF activatory motif ((735) and unpublished observations), but bears mutations impairing the activation of the other classical flagellin immune receptor, TLR5 (382). IPAF up-regulation was observed in a model of acute colitis only when mice were also infected with a pathogenic *E.coli* strain, suggesting an essential role for bacterial PAMPs in the up-regulation of its expression (736). Moreover, there is evidence that IPAF may limit intestinal inflammation in some settings, as IPAF-deficient mice were found to be more susceptible to inflammation-induced colon cancer induced by repeated cycles of DSS and azoxymethane (737). Therefore, further analysis of IPAF function in models of intestinal inflammation will be required to clarify the specific role of this NLR in

intestinal homeostasis. Interestingly, the inflamed spleen had a completely different pattern of NLR expression compared to organs directly infected by *H.hepaticus*, with a strong upregulation of NOD1. NOD1 plays a key role in limiting *Helicobacter pylori* infection in mice (267) and patients suffering from *H.pylori*-induced gastritis have higher NOD1 expression in the stomach (738). Hence, NOD1 could play a role in host defense against *Helicobacter spp.,* although the mechanisms involved could vary from species to species, as *H.hepaticus* induced NOD1 expression primarily in non-mucosal organs.

Next, we analyzed the functional role of NOD2 in *H.hepaticus*-induced innate immune colitis. Our preliminary data suggest that NOD2 might play a pathogenic role in this setting, as 129SvEv Rag^{KO} $Nod2^{KO}$ mice were protected from the development of severe colonic inflammation. A similar effect was observed in 129SvEv $Rag2^{KO}$ $Nod2^{Het}$ mice, suggesting that the presence of a single copy of NOD2 was insufficient to induced severe intestinal inflammation. These data contrast previous observations in acute models of colitis, where NOD2 was reported to play a protective role, as administration of MDP, or over-expression of NOD2, could protect mice from the development of intestinal inflammation (416, 739). These differences may reflect the different challenges and factors involved in acute and chronic inflammatory settings in the gut. Indeed, the protective effect of NOD2 in acute colitis is at least in part mediated by activation of NOD2 in intestinal epithelial cells, promoting epithelial barrier function (740). In contrast, *H. hepaticus* infection does not directly induce epithelial damage, which is a consequence of downstream inflammatory processes driven by leukocytes (623). We hypothesize that NOD2 may play a key role in

promoting pathogenic innate immune responses in *H.hepaticus*-induced colitis, as activation of the NOD2 pathway leads to the secretion of a plethora of pro-inflammatory cytokines, including IL-1β (274). Moreover, NOD2 signalling in human dendritic cells promotes IL-23 production and activation of Th17 cells (586). As we have shown that IL-23R^{+} IL-17-producing ILC are crucial effector cells in this innate model of colitis (51), it is possible that NOD2 deficiency leads to a blunted innate "type-17" response in the colon of *H.hepaticus*-infected 129SvEv *Rag2*^{KO} mice. Moreover, NOD2 deficiency could lead to decreased recruitment of pro-inflammatory innate leukocytes to the intestine, as NOD2 expression in stromal cells was recently shown to be essential for the recruitment of pro-inflammatory innate cells during *C.rodentium*-induced acute colitis, by promoting chemokine secretion (733). Our data, albeit preliminary, add a layer of complexity to the debate on how mutations in *NOD2* result in increased risk of developing Crohn's disease. In our model, a complete loss of function of NOD2 protected the mice against the development of *H.hepaticus*-induced innate immune colitis, challenging the idea that mutations in *NOD2* in human promote the development of CD primarily by abrogating the its anti-microbial function. However, complete NOD2 deletion in mice might not accurately recapitulate the function of the mutated NOD2 protein in humans.

Recently, it has been proposed that NOD2 is essential in controlling *H.hepaticus* colonization in the intestine, due to defective bactericidal activity by Paneth cells in the small intestine (727). Since our data in the 129SvEv *Rag2*^{KO} *Nod2*^{KO} mice did not support this hypothesis, we extended our analysis to lymphocyte replete mice.

Although limited by the mixed background of B6.129S1 *Nod2*^{KO} mice, our data show that NOD2 does not control *H.hepaticus* colonization in the caecum or in the ileum of lymphocyte replete mice. *H.hepaticus* colonization of the ileum in particular was barely detectable both in WT and *NOD2*^{KO} mice. Analysis of stool samples revealed comparable levels of *H.hepaticus* shedding between NOD2 sufficient and NOD2 deficient mice. In striking contrast to the data reported by Petnicki-Ocwieja and colleagues, which showed no *H.hepaticus* DNA in the stools of C57BL/6 mice after two weeks of infection (727), we could detect *H.hepaticus* DNA in the faeces of C57BL/6 mice up to 8 weeks post-infection, consistent with the general consensus of stable colonization of the murine host by this bacterium (352). Whilst technical differences might partially account for the discrepancy of our results from those presented by Petnicki-Ocwieja and colleagues, we believe that our analysis better represents the actual relationship between *H.hepaticus* and its host by analyzing the natural niche for *H.hepaticus* colonization, the large intestine. Moreover, the minimal levels of *H.hepaticus* DNA detected in the ileum argue against a significant role for *H.hepaticus* colonization of this site in driving intestinal inflammation. Our analysis of antibody responses to *H.hepaticus* and susceptibility of the B6.129S1 *Nod2*^{KO} mice to *H.hepaticus*-induced αIL-10R-triggered colitis were hampered by the profound effect of genetic background on these immune responses. Indeed, we found that 129SvEv and C57BL/6 were profoundly different in their humoral response to *H.hepaticus.* In contrast to most commensal species (234), *H.hepaticus* infection elicits potent systemic IgG responses in WT non-inflamed mice (367). 129SvEv mice showed significantly higher IgG responses against *H.hepaticus* than C67BL/6 mice. In parallel, the mucosal IgA responses were also increased in 129SvEv

mice compared to C57BL/6, although this was not associated with increased colonization. In contrast, 129SvEv mice were less susceptible than C57BL/6 mice to intestinal inflammation induced by *H.hepaticus* infection and treatment with αIL-10R. This was somewhat surprising, as 129SvEv IL-10^{KO} mice have been reported to develop worse colitis than C57BL/6 IL-10^{KO} mice (741) and 129SvEv *Rag2*^{KO} mice were more susceptible than C57BL/6 *Rag1*^{KO} mice to *H.hepaticus*-induced innate colitis (370). Although other genetic factors might contribute to the increase resistance of 129SvEv mice to *H.hepaticus*-induced αIL-10R-triggered colitis, our finding that 129SvEv mice show a stronger humoral response against *H.hepaticus* both systemically and at mucosal sites at the steady state suggests that 129SvEv mice might have a stronger "barrier" response against *H.hepaticus* in the absence of inflammation. This response might then prevent the development of severe colitis once regulatory mechanisms are disrupted. As TGF-β can promote IgA production in the intestine (742), it is tempting to speculate that 129SvEv mice could be more dependent on this regulatory cytokine to maintain intestinal homeostasis. Hence, when the IL-10 pathway is disrupted by administration of αIL-10R mAb, TGF-β dependent mechanisms might still be able to limit *H.hepaticus* induced inflammation in this mouse strain.

In conclusion, the data presented in this chapter show that NOD2 is significantly up-regulated following induction of bacteria-triggered innate colitis and it is required for the development of severe colitis in this model. Moreover, we present clear evidence to disprove the hypothesis that NOD2 is required for the control of *H.hepaticus* infection *in vivo*. We also highlighted the importance of genetic

background in defining the susceptibility to *H.hepaticus*-induced αIL-10R-triggered colitis.

Chapter 7: General discussion

7.1 Summary

In this thesis, I identified a role for IL-1β in promoting intestinal inflammation in different animal models of colitis. Specifically, I showed that blocking IL-1β was beneficial in *H.hepaticus*-driven innate colitis and in *H.hepaticus*-driven colitis in lymphocyte replete mice. Moreover, I identified a clear requirement for IL-1R signalling on T cells for the development of intestinal inflammation in a T cell transfer model of colitis, as IL-1R signalling on T cells was required for the accumulation of $CD4^+$ T cells in the inflamed colon. Th17 were particularly affected, probably reflecting the high level of expression of IL-1R1 in this population (545). I also identified a novel role for IL-1β in promoting the accumulation and pro-inflammatory function of a novel population of ILC, which also produce IL-17A during inflammation (51). Nevertheless, IL-1β clearly plays a complex role in intestinal inflammation, as *Il1r1*^{KO}mice developed disease normally upon infection with *H.hepaticus* and treatment with αIL-10R. Taken together, my data show that IL-1β may be an important conditioning factor in promoting intestinal inflammation.

In the latter part of this thesis, I presented preliminary data on the role of the NLR NOD2 in chronic intestinal inflammation. I found that, consistent with observations in human IBD patients (713, 731), the development of intestinal inflammation was accompanied by up-regulation of NOD2 expression in the colon, although NOD2 expression did not impact colonization of the large intestine by *H.hepaticus*. However, my preliminary findings suggest that the development of *H.hepaticus*-driven innate intestinal inflammation was attenuated in the absence of NOD2, even

though these data need further confirmation. In this section, I will put these findings in a broader context and propose experiments to further dissect and expand the issues presented in this thesis.

7.2 IL-1β promotes the accumulation of pro-inflammatory adaptive immune cells: a role for metabolic maintenance?

Recently, it has become apparent that IL-1β drives the expansion and effector function of IL-17 producing T cells, such as systemic $CD4^+$ T cells and γδ T cells (60, 138, 139, 673). According to my data, this also occurs in the inflamed intestine, although the effect of IL-1β at this site was mainly on maintenance/survival of $CD4^+$ T cells and Th17 cells, possibly due to the highly polarizing environment of the gut, which is enriched in a number of factors that can promote Th17 responses (361-363, 442, 686). Recently, it has been proposed that IL-1β mediates its effects also in the absence of TCR stimulation. This hypothesis, initially demonstrated for γδ T cells (60, 139), was confirmed for $CD4^+$ T cells, as IL-1β stimulation of Th17 cells sustained their phenotype even in the absence of TCR engagement (138). Upon antigen presentation in secondary lymphoid organs, T cells migrate to the inflamed tissues, where they exert their effector function (6). The stimuli required for cytokine production by effector T cells are currently unclear. Although it has been proposed that TCR stimulation by DC is required for T cell effector function in non-lymphoid organs (743), in chronic intestinal inflammation, where the antigen load is high and regulatory mechanisms are impaired, it is possible that cytokines play a crucial role in sustaining effector T cell responses, and IL-1β might represent a key factor. Moreover, recent data show that during *C.rodentium*-induced intestinal

inflammation, early "innate-like" Th17 responses are crucial to limit bacterial expansion (728), suggesting that TCR-independent mechanism of T cell activation are essential to drive early immune responses in the intestine. Intriguingly, this "innate" Th17 response was dependent on IL-6, a crucial target of IL-1β (728). As IL-1β does not promote any specific effect on T cells in the absence of other pro-inflammatory cytokines, such as IL-23 (138, 139), IL-1β might promote T cell effector functions by up-regulating the expression of other cytokine receptors, such IL-23R, by $CD4^+$ T cells, an effect that has been demonstrated *in vitro* (138, 744). Since IL-1R signaling also promotes IRF4 expression in Th17 cells (138), which is required for IL-23R up-regulation (745), IL-1β may reinforce the differentiation status of effector T cells, and particularly Th17 cells, by stabilizing their transcription programme.

My data suggesting that IL-1β promotes $CD4^+$ T cell survival is in accordance with previous reports and with the key role of MyD88 and NF-κB signals in preventing $CD4^+$ T cell death (746). Specifically, MyD88 signals have been shown to promote the expression of anti-apoptotic proteins in T cells (708, 747). Hence, MyD88-mediated IL-1β signals might promote T cell survival in a similar way.

In addition to modulation of cytokine responses and differentiation, I would like to speculate that IL-1β helps maintain pro-inflammatory T cell responses by acting on less conventional mechanisms (**Fig7-1**). T cell activation is an extremely demanding process in terms of energy requirements, as cells proliferate enormously, increase their cell mass (blastogenesis) and initiate a complex transcriptional programme requiring increased protein synthesis and changes in protein expression, localization and secretion (748). To meet these increased energy demands, activated T cells

change their metabolic profile, mainly by switching from oxidative respiration to glycolysis (748). Hence, T cells are extremely dependent on extracellular glucose for their survival and effector functions (748). Accordingly, T cell activation is linked to the expression of glucose transporters: TCR stimulation up-regulates the expression of glucose transporter 1 (GLUT1) and co-stimulatory signals induce the trafficking of GLUT1 to the cell surface (749). However, activated T cells are dependent on the presence of extrinsic factors such as cytokines to keep high levels of GLUT1 at the cell surface (750). When these factors are withdrawn, T cells rapidly undergo atrophy and apoptosis (750). My *in vitro* studies, although preliminary, suggest that IL-1β promotes T cell survival in pro-inflammatory conditions. IL-1β has been shown to regulate the expression of hexose transporters on different cell types (751) and to induce GLUT1 expression on peritoneal macrophages (752) and glial cells (753). However, its role in the modulation of GLUT1 expression on T cells has not yet been investigated. As other cytokines, such as IL-7, promote GLUT1 expression and membrane translocation (750), it would be interesting to analyze the role of IL-1β in changing the metabolic profile of activated T cells, especially because IL-1R signaling activates the PI3K/AKT pathway, which is central in promoting glucose uptake and glycolysis in activated T cells (748). Since IL-1β has a key role in regulating glucose homeostasis systemically by modulating glucoregulatory mechanisms in the brain (751, 754), and has been shown to promote glucose uptake by lymphoid organs (755), it is possible that this cytokine could promote glucose consumption by leukocytes.

The modulation of T cell metabolic function, especially at the sites of inflammation, might also condition the nature of the immune response. For instance, different helper T cell subsets might require different stimuli for survival/effector function, especially considering that they express different levels of cytokine receptors (97, 138, 545, 756). It is becoming increasingly clear that metabolites play an important role in skewing immune responses (224). For instance, withdrawal of amino acids such as arginine or tryptophan inhibits T cell responses through direct and indirect mechanisms (757, 758). Moreover, it was recently shown that withdrawal of vitamin A, the precursor of retinoic acid, from the diet resulted in decreased Th17 differentiation in the intestine, altered mucin secretion and profound changes in the intestinal micro-biota diversity and composition (759). Considering that the micro-biota can synthesize different metabolites and nutrients, it is likely that the complex communication between the host and the intestinal bacteria involves the modulation of the metabolic status of the intestinal microenvironment (224). This hypothesis is supported by recent findings showing that colonocytes use small fatty acids of bacterial origin as their main source of energy, and colonocytes from GF animals are energy-deprived and use autophagy to maintain energy homeostasis (760).

From this brief discussion, it is clear that many of the mechanisms involved in regulating immune cell metabolism are deeply intertwined with CD-related innate immune mechanisms. High levels of extracellular ATP or mitochondria alterations activate the inflammasome (293, 761). ROS production, a key activator of the NLRP3 inflammasome (259), is linked to mitochondria dysfunction and loss of ATP synthesis

(762). Moreover, autophagy is primarily a metabolic response to energy deprivation (763). Accordingly, T cells deficient in key proteins for the induction of autophagy fail to expand in response to antigenic stimuli (764). Hence, factors such as autophagy and NLR activation, which have been linked to CD susceptibility by GWAS studies (326, 331), are profoundly influenced by metabolic alterations. This field is currently focused on diseases with a clear metabolic component, such as type 2 diabetes (765). However, further investigation on the role of metabolic alterations in chronic intestinal inflammation might help elucidate key inflammatory mechanisms and identify new therapeutic interventions specifically tailored towards nutritional modulation.

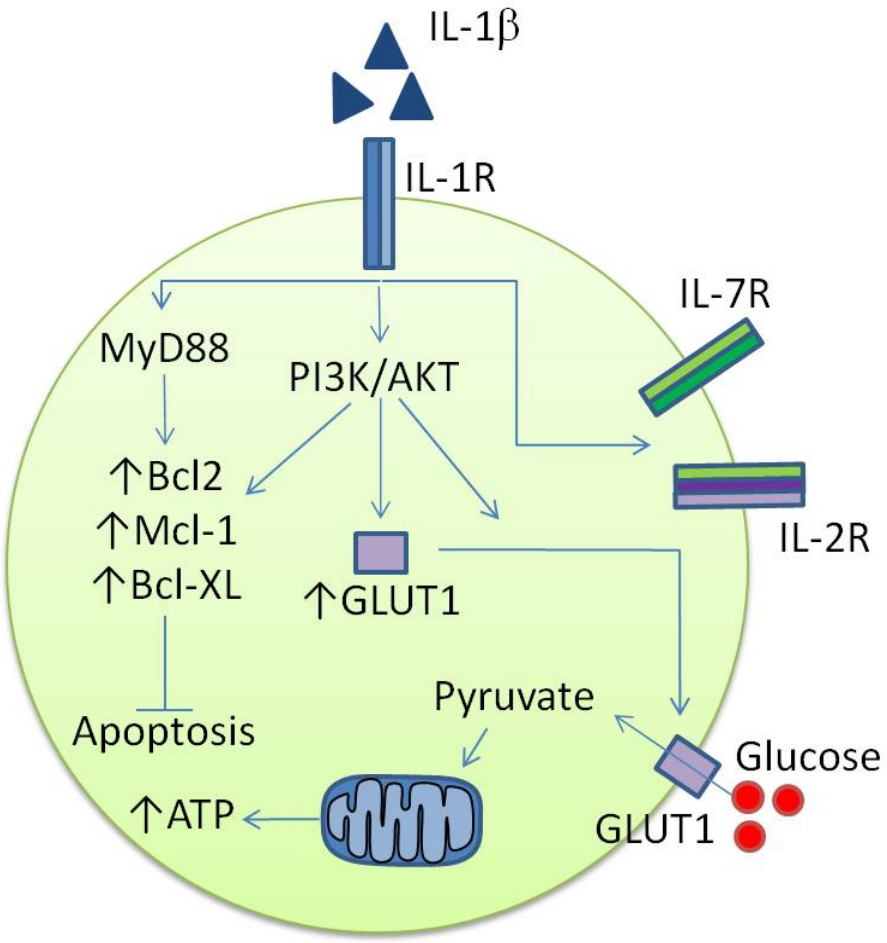


Figure 7-1. Potential influence of IL-1β on survival pathways. IL-1β might affect T cell survival by modulating different pathways. MyD88-mediated IL-1R signals might promote the expression of anti-apoptotic proteins such as Bcl2 or Mcl-1. Moreover, IL-1R signals might promote increased expression or membrane localization of receptors for extrinsic factors, such as the cytokines IL-7 and IL-2, which promote T cell survival. Finally, IL-1R signals may promote the expression and membrane trafficking of GLUT1, an essential glucose transporter for activated T cells, sustaining T cell metabolism during activation.

7.3 IL-1β modulation of ILC function: an open field.

As ILC-mediated immune responses have been less well-characterized than T cell responses, it is difficult to judge the extent to which the above considerations could be extended to this novel innate leukocyte population. However, my data showed that IL-1β was also required for the accumulation of ILC in the inflamed intestine, especially for IL-17A-producing ILC. Further work is required to address the levels of IL-1R1 expressed by these cells and the effects of *ex vivo* stimulation with IL-1β, either alone or in combination with other cytokines. One way to analyze the effects of IL-1β stimulation on ILC pathogenicity would be to investigate their phenotype and function in *H.hepaticus*-infected 129SvEv *Rag2*^{KO} *Il1r1*^{KO} mice. . However, these mice are not currently available and their generation is a lengthy process. In the meantime, other systems are available to study the role of IL-1β in ILC accumulation and function. Administration of αCD40 mAb to C57BL/6 *Rag1*^{KO} mice results in the development of intestinal and systemic inflammation (445) that is also dependent on the presence of ILC (51), the generation of C57BL/6 *Rag1*^{KO} *Il1r1*^{KO} mice would allow the investigation of the role of IL-1β in ILC function. However, ILC do not produce IL-17A during αCD40-induced colitis (51) and the induction of intestinal pathology in this model is independent of the intestinal microbiota (445), hence this system might not recapitulate the results observed in *H.hepaticus* induced colitis, as the bacterial flora might be required for IL-1R expression on ILC, as was recently reported for γδ T cells (672).

Some information regarding the role of IL-1β in modulating ILC biology can be extrapolated by data regarding a different class of intestinal ILC, NK-22 cells. As

previously mentioned these IL-22 producing cells reside in the mucosa of the small intestine in mouse and humans and play a key role in promoting epithelium restitution and repair following intestinal infection (38). Recent data show that IL-1β plays an essential role in promoting IL-22 production by NK-22 cells in humans and in mice (625-627), and IL-1β, together with IL-7 or IL-2, promoted the *in vitro* expansion of human NK-22 cells (625, 626). These findings support the hypothesis that IL-1β promotes the accumulation and effector function of colonic ILC *in vivo*. Moreover, culture with IL-1β and IL-7 promoted IL-17 production by a subset of NK-22 cells (626), suggesting that IL-1β could contribute to ILC plasticity *in vivo*. Once again, it is striking that IL-1β only has minimal effects on ILC biology *in vitro* in the absence of other cytokines, suggesting that IL-1β might have different effects on ILC biology depending on which other extrinsic factors are present in the microenvironment.

7.4 Contrasting effects of caspase-1 in intestinal inflammation.

My data support a pro-inflammatory role for IL-1β in chronic intestinal inflammation. Hence, mechanisms upstream of active IL-1β production should also play a pathogenic role in this model. Currently, there is no data available describing the role of IL-1β/IL-18 converting enzymes in chronic intestinal inflammation. However, different authors analyzed the role of IL-1b/IL18 converting enzymes in models of acute inflammation (593, 594, 597, 598, 618, 619, 766-772). Caspase-1 was the first enzyme described to cleave IL-1β and IL-18 (612, 613, 773-777), however neutrophil-derived serine proteases, such as neutrophil elastase, proteinase 3 and cathepsin G, and mast cell–derived serine protease, such as chymase and granzyme-A, can also process the IL-1β and IL-18 precursors into a secreted active form (778-782). These

enzymes might play a prominent role in IL-1β and IL-18 processing when the inflammation is characterized by dense infilitration of neutrophils or mast cells. Intererstingly, acute intestinal infiltration is characterized by increased levels of both neutrophils and mast cells and *in vivo* inhibition of chymase and proteinase 3/elastase is beneficial in models of TNBS- and DSS-induced colitis (766-769). In contrast, the role of the main IL-1β processing enzyme, caspase-1, in acute intestinal inflammation is more controversial. Initial studies reported that caspase-1 deficiency or pharmalogical inhibition resulted in protection from DSS-colitis (618, 619, 770). Early studies also reported that the main cytokines processed by caspase-1, IL-1β and IL-18, had pathogenic effects in acute colitis, as neutralization of these cytokine was beneficial in models of DSS-induced colitis (593, 594, 618, 771, 772). However, more recent studies have challenged these conclusions as two independent studies showed that caspase-1-deficient mice had increased intestinal inflammation following DSS administration (597, 598). Furthermore, the exacerbated DSS disease phenotype was attributed to a lack of caspase-1 expression by a non-haematopoietic cell compartment and was ascribed to lack of IL-18 signals from the intestinal epithelium, compromising epithelial restitution and repair (597, 598). In an attempt to reconcile these disparate findings, it has been proposed that, similarly to NF-κB activation and MyD88 signaling, IL-18 signaling has a protective effect on the intestinal epithelium, where it promotes proliferation and repair responses, but that it can also exacerbate pro-inflammatory innate immune responses in the lamina propria (783). As DSS colitis arises as a consequence of extensive epithelial injury, caspase-1 activation and subsequent IL-18 release would have a protective effect, although in some animal facilities the translocation of intestinal bacteria into the

lamina propria may elicit pathological inflammatory circuits, some of which may be driven by IL-18. (**Fig.7-2**).

It is not clear whether a similar situation exists for IL-1β, although different studies again proposed either protective or pathogenic effects of IL-1β in DSS colitis (582, 618, 653). Whilst IL-1 (α and β) promotes wound healing in the skin (662, 663), there are no reports of a similar effect in the intestine. In contrast, IL-1β was reported to increase intestinal barrier permeability *in vitro* (784, 785). Furthermore, IL-1β in the human intestine is mainly produced my monocytes/macrophages (571), whilst IL-18 is mainly expressed by epithelial cells (590, 771). Further investigation, including the generation of BM chimeras or conditional KO mice, will be required to clarify the overall role of IL-1β in acute intestinal inflammation

Further characterization of the cellular sources of IL-1β and IL-18 in the intestine is also required, as the identification of specific cell compartments producing either or both these cytokines would allow for more specific targeting. Alternatively, inflammasome formation could be studied: recently, *in vitro* data showed that it is possible to visualize inflammasome formation by immuno-fluorescence combining the use of fluorescent probes for caspase-1 and antibodies directed against IL-1β, IL-18 and members of the NLR family (786), although the feasibility of this approach *in vivo* has not yet been demonstrated.

The fact that caspase-1 dependent cytokines might have multiple effects on intestinal inflammation has important implications for the pharmacological targeting of this pathway. If caspase-1 activation in the epithelium has a protective effect on intestinal inflammation, then oral administration of caspase-1 inhibitors might not

be beneficial, as epithelial cells might be be the primary target. In contrast, systemic administration or specific targeting of immune cells could increase the likelihood of inhibition of caspase-1 activation in LP leukocytes. Alternatively, targeted delivery of caspase-1 inhibitors to specific cell compartments might be achieved through the association with lectins (for the delivery to the intestinal epithelium) or microparticles/liposomes (for the delivery to phagocytic cells). (787, 788). Considering that different enzymes, such as caspase-1, neutrophil elastase, proteinase 3, cathepsin G, chymase and granzyme-A, are able to cleave IL-1β and IL-18 (527), it would be interesting to understand whether *in vivo* these enzymes cleave both cytokines with equal activity or if, in different inflammatory settings, there is some degree of specificity for a specific cytokine, either by biochemical mechanisms or by coordinated expression of the enzyme and the cytokine precursor. If such specificity exists, one could envisage a situation where specific enzymes are inhibited to decrease the levels of IL-1β or IL-18, depending on the inflammatory condition.

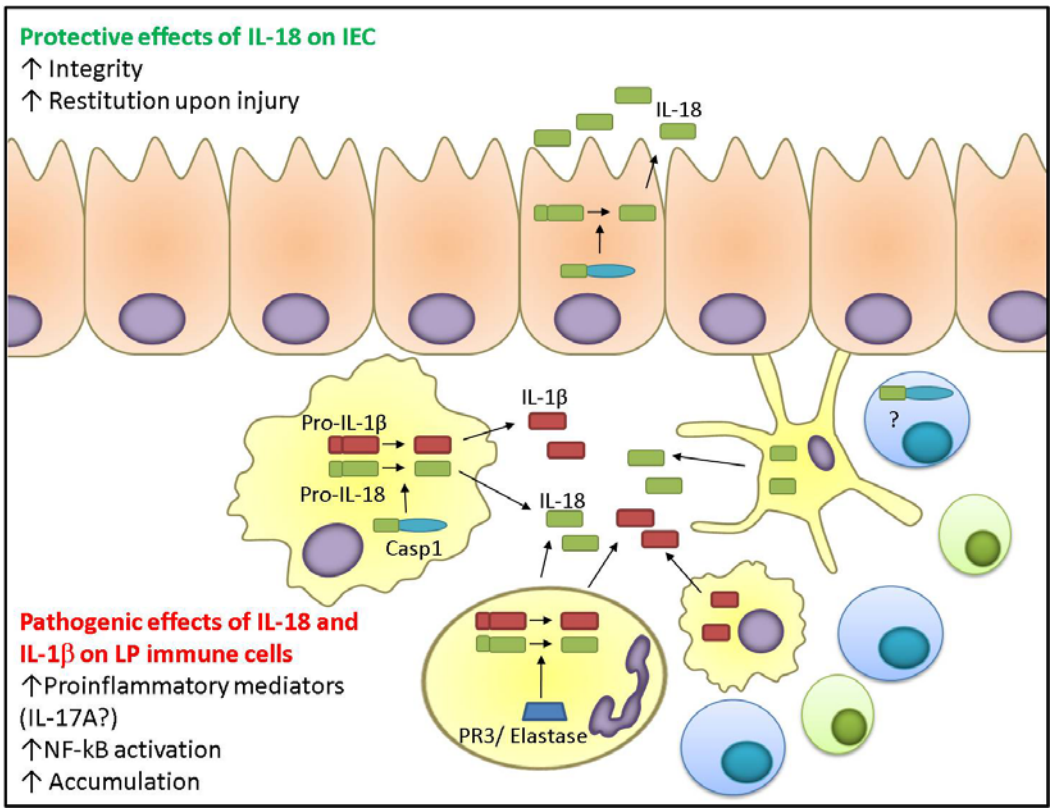


Figure 7-2. Multiple activities of IL-1β and IL-18 in the intestine. Caspase-1 activation in different intestinal compartments might result in different outcomes. As pro-IL-18 is mainly expressed in epithelial cells, activation of caspase-1 in the epithelium results in release of IL-18, which exerts a protective role on the epithelial barrier, reinforcing it and promoting restitution and repair following acute injury. In the lamina propria, innate leukocytes express both pro-IL-18 and pro-IL-1β, thus, following activation of caspase-1, or of PR3/elastase in neutrophils, these cytokines are processed and released. Both IL-18 and IL-1β can exert pro-inflammatory effects on adaptive and innate immune cells and could thus contribute to intestinal pathology. The relative activation of caspase-1 within the epithelium and haematopoietic cells may depend on the specific type of intestinal inflammation (acute, chronic, UC, CD) and might determine the pathogenic or protective role of caspase-1 dependent cytokines in the intestine.

7.5 Novel NOD2-mediated responses might contribute to intestinal inflammation.

In spite of a huge effort in characterizing the effects of NOD2 mutations and NOD2 deficiency in IBD patients and in animal models of disease, the function of this NLR in intestinal inflammation is still elusive. A lot of progress has been made in understanding NOD2 biology (789), but it is still unclear how its disruption contributes to IBD development. NOD2 seems to affect several cellular compartments, from epithelial cells to immune cells to mesenchymal cells (415, 713, 733). It is clear that NOD2 plays an essential role in the biology of the small intestine. Here, it controls the development and bactericidal function of Paneth cells (419, 713, 718), limiting bacterial burden (722, 727) and avoiding the development of deleterious Th1 responses (740). However, Paneth cells are not present in the colon (although Paneth cell metaplasia has been described in IBD patients (281), hence the function of NOD2 in this cellular compartment is limited in this organ. The role of NOD2 in colonic inflammation is less clear and here NOD2 might mainly modulate bacterial handling by innate immune cells, an idea supported by the fact that we did not observe increased levels of NOD2 expression by $CD45^-$ cells in innate colitis. Moreover, NOD2 expression by colitogenic $CD4^+$ T cells was required for their differentiation into Th1 cells and for the development of colitis in a T cell transfer model of disease (420), showing that NOD2 can also modulate adaptive immune cell function in the colon. More recently, NOD2 expression was shown to be essential for the resolution of colonic pathology following infection with *C.rodentium* (733). In this model, expression of NOD2 by colonic stromal cells was essential to induce clearance of the bacterial pathogen, by promoting CCL2 production and recruitment of

inflammatory monocytes (733). As stromal cells, particularly myofibroblasts also contribute to chronic inflammation (790), it would be worth investigating the role of NOD2 in modulating their function in chronic settings. However, the stromal compartment contains different cell types and their identification in mouse models can be difficult (791). Moreover, myfibroblasts are replenished by both the epithelial and hematopoietic cells during intestinal inflammation (792, 793), though the relative contribution of these two compartments is not known, and it is also unclear whether the phenotype of "bone marrow-derived" myofibroblasts differs from that of "epithelial-derived" cells. Hence, further work on the functional and phenotypical characterization of these cells is required before we will be able to fully understand the role of NOD2 in modulating their function. Overall, it is clear that tissue specific, and cell-specific, analysis of NOD2 is required for the understanding of its function in IBD, through the generation of conditional KO mice.

A second layer of complexity in understanding the function of NOD2 is highlighted by reports that NOD2 interacts with numerous other pathways implicated in the pathogenesis of IBD. As mentioned earlier, NOD2 can alter TLR signaling and mutated versions of NOD2 can down-regulate IL-10 synthesis in human cells (789). More recently, NOD2 signaling has been linked with other key pathways in IBD pathogenesis. Specifically, recent data suggest that NOD2 can promote autophagy in innate immune cells, by recruiting autophagosome components to the bacterial entry site, favouring bacterial degradation and antigen presentation to T cells (729, 794, 795). Strikingly, dendritic cells from IBD patients bearing NOD2 mutations had defective autophagy in response to NOD2 agonists and deficiency in bacterial

handling (729, 794). The molecular mechanisms required for this function are unclear, as RIP2 signaling was required for NOD2-mediated autophagy in dendritic cells (729), but not in epithelial cell lines (794). Current data support a model where MDP stimulation of NOD2 induces autophagy (729, 794, 795). However, emerging evidence suggests that NOD2 can also be activated by other stimuli, including recognition of viral ssRNA (275). A recent study suggested that infection with an enteric virus might be an important environmental factor for the development of intestinal inflammation in a genetically predisposed host (496, 796). Indeed, hypo-expression of the CD-associated autophagy protein ATG16L1 in mice resulted in Paneth cell abnormalities and increased susceptibility to DSS-induced colitis only when the mice were infected with a persistent murine norovirus (a ssRNA virus) (496, 796). Hence, it is possible that NOD2 recognition of enteric viruses allows a coordinated defensive response including induction of autophagy and regulation of Paneth cell function, which in turn maintains intestinal homeostasis. NOD2-mediated viral recognition might prevent intestinal inflammation by other mechanisms. Recognition of viruses by NOD2 results in IRF3 activation and type 1 interferon synthesis (275). As type 1 interferons have a beneficial effect in models of acute colitis (797), it is possible that NOD2 mutations lead to loss of type 1 interferon synthesis in the colon, promoting intestinal inflammation or limiting resolution. Hence, novel mechanisms of NOD2 activation might be involved in IBD pathogenesis (**Fig.7-3**).

Moreover, NOD2 could also promote the production of IL-1β and IL-18. Traditionally, NOD2 is considered a “non-inflammasome” NLR, as it signals mainly through the

activation of the kinase RIP-2 (250). However, different reports suggest that NOD2 can form an inflammasome in response to MDP stimulation, by interacting with NLRP1 or NLRP3 (305, 306) and *in vitro* studies in HEK-293T (human embryonic kidney) showed that co-transfection of NOD2 and pro-caspase-1 result in NOD2-caspase-1 interaction and IL-1β secretion (798). Moreover, macrophages from "knock-in" mice bearing the most common NOD2 mutation found in IBD patients secrete high levels of IL-1β following MDP stimulation (417), whilst peripheral blood mononuclear cells from IBD patients bearing NOD2 mutations show defective MDP-induced IL-1β secretion (407). Although controversial, both these pieces of evidence suggest a role for NOD2 signaling in promoting IL-1β processing and secretion. However, since NLRP3 can directly recognize MDP and trigger inflammasome formation (799), it is possible that the role of NOD2 is limited to promote expression of pro-IL-1β or secretion, rather than processing, of active IL-1β (800). Further studies will be required to address this issue and to analyze the relevance of NOD2 signaling for IL-1β processing during *in vivo* inflammation.

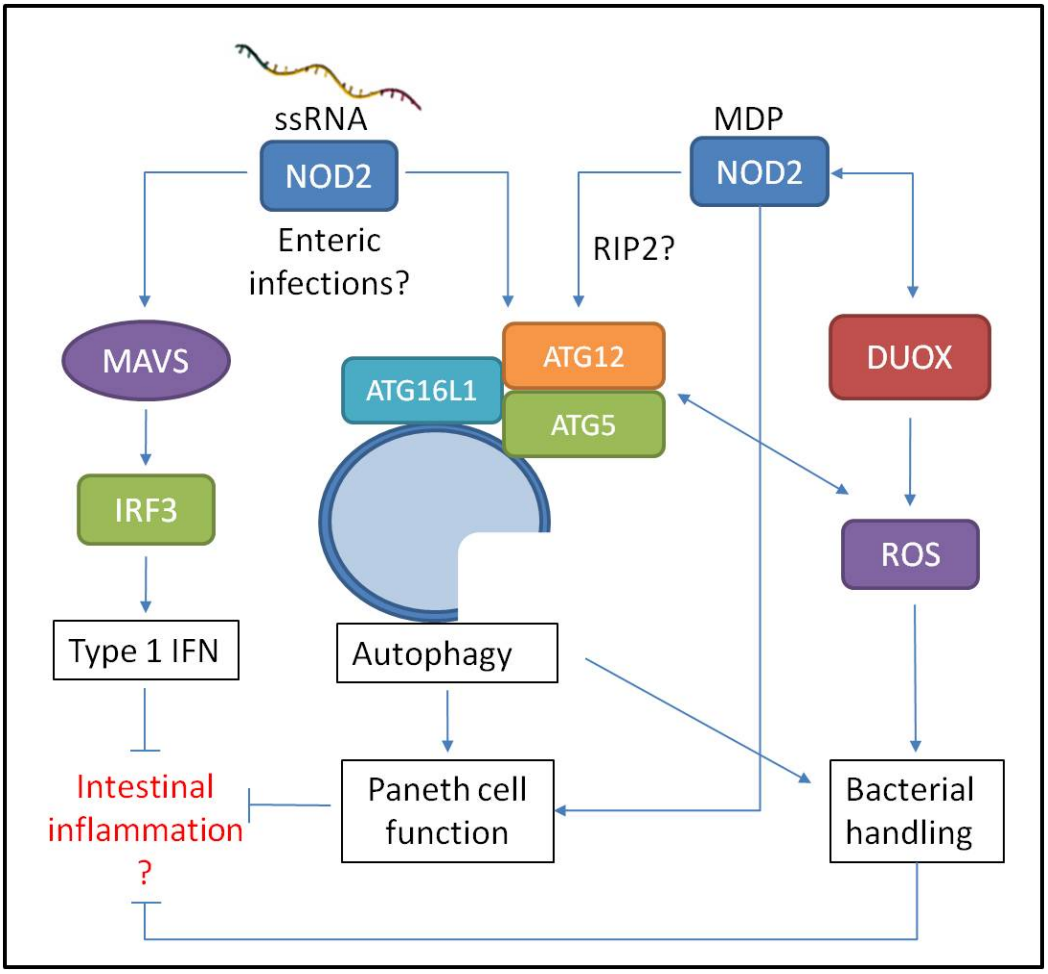


Figure 7-3. Novel mechanisms for NOD2 function in intestinal inflammation. As NOD2 can recognize ssRNA, this NLR might might play a role in enteric infections, either by promoting the production of protective type 1 interferons or by sustaining Paneth cell function by promoting autophagy, since MDP stimulation of NOD2 is able to induce formation of the autophagosome. *In vitro* data suggest that NOD2 might interact with oxidases, inducing ROS synthesis, a crucial pathway in regulating autophagy and limiting bacterial invasion. The role of these pathway during intestinal inflammation has not been investigated.

7. 6 ROS generation at the crossroad of IBD pathogenesis.

As previously mentioned, ROS synthesis is emerging as a key pathway for the activation of NLR, specifically NLRP3, responses, as most NLRP3 activators induce ROS production and inhibition of ROS synthesis prevents NLRP3 activation (259) (288-290). Moreover, recent studies report that mithocondrial damage activates the NLRP3 inflammasome through ROS generation (761, 801), suggesting that ROS represent a key factor in promoting IL-1β synthesis. In turn, IL-1β can promote the production of ROS in different cell types, including epithelial cells, monocytes and lymphocytes (540). Interestingly, the presence of elevated oxidative stress and increased production of ROS by epithelial and haematopoietic cells is a common feature of IBD intestinal lesions (802-805). Moreover, polymorphisms in *NCF4* (p40 phox), a cytosolic protein required for the activation of NADPH oxidases, are associated with IBD (329), suggesting that the generation of ROS is a key pathway in IBD pathogenesis.

Experiments in *Drosophila melanogaster* and zebrafish clearly show that ROS generation is essential for bacterial defense at mucosal surfaces, as ROS production by dual oxidase (Duox) was essential to limit intestinal bacterial proliferation and ensure host survival following enteric infection (806, 807). In particular, *Drosophila* appears to have a complex regulation of ROS synthesis, where low levels of ROS maintain a healthy balance between host and intestinal bacteria, and maximal levels of ROS are produced only following bacterial invasion of the gut epithelium (808). Similar data were obtained using colonic epithelial cell lines (809), but very little evidence is available regarding the role of ROS during intestinal inflammation in vivo.

Recently, it has been shown that the spontaneous colitis developed by mice deficient in Tak1 (TGF-β-activated kinase 1) is dependent on the generation of ROS by the intestinal epithelium and is ameliorated by the administration of an anti-oxidant (810). Further work is required to analyze the role of ROS generation in chronic colitis, in particular by characterizing the relative contribution of epithelial and hematopoietic cells to ROS generation, as leukocytes were found to be an important source of ROS in IBD patients (802, 804).

Intriguingly, ROS synthesis seems to be essential for the modulation and activation of different immune pathways associated with IBD pathogenesis. As previously mentioned, ROS production is a major stimulus for the activation of NLR signaling (259). Moreover, recent *in vitro* data suggest that NOD2 can promote bacterial killing by interacting with Duox2, a dual oxidase highly expressed in the large intestine (811, 812). Similarly, NOD2 deficiency in zebrafish was associated with uncontrolled bacterial proliferation after *S.enterica* infection and with severe defects in Duox expression (813). Moreover, the activation of NLR-homologues in plants, the pathogen-resistance (R) genes, results in ROS production (814). Hence, it is possible that one of the functions of NOD2 is to activate ROS synthesis and limit bacterial invasion *in vivo*. Autophagy is also linked to ROS synthesis as ROS generation induces autophagy (815) and, in turn, autophagy limits ROS-induced damage by scavenging oxidized proteins (816) and by eliminating damaged mitochondria (mitophagy) (817). Moreover, production of ROS following bacterial engulfment is crucial for the activation of anti-bacterial autophagy, coupling oxidative and non-oxidative bactericidal activities (818).

Overall, many lines of evidence point to an involvement of the generation of ROS in IBD pathogenesis. Deletion of oxidases in animal models often results in growth defects (819) or severe phenotypes associated with recurrent bacterial infections, similar to human Chronic Granulomatous Disease (CGD) (820), making it difficult to specifically assess the contribution of this pathway to intestinal inflammation. The generation of conditional KO would be essential to pursue this line of investigation. However, phenotypic characterization of oxidase expression in health and disease is possible, and ROS generation can be contrasted through pharmacological and nutritional approaches, allowing investigators to start to analyze this pathway *in vivo*.

7.7 Concluding remarks

In this thesis, I showed that IL-1β plays a key role in promoting adaptive and innate immune responses in intestinal inflammation, making this cytokine an attractive target for therapy. Strategies targeting IL-1β are in clinical trials for a number of acute and chronic inflammatory disorders and show promising results in terms of efficacy and safety (555, 821). Currently, strategies neutralizing IL-1β by the administration of recombinant IL-1RA (Anakinra) are approved by the U.S. Food and Drug Administration (FDA) for the treatment of moderate to severe rheumatoid arthritis in adults (821). In parallel, IL-1 trap (Rilonacept), a fusion protein containing IL-1R1- and IL-1RacP-binding motifs fused to human Fc IgG, and canakinumab (Ilaris) an αIL-1β mAb, were recently approved for the treatment of cryopyrin-associated periodic syndromes (CAPS) (821). Moroever, different IL-1β–blocking strategies were beneficial in other conditions, including type 2 diabetes and acute gout (821, 822).

No published data are available regarding the use of IL-1β blocking agents in IBD, although our data suggest that such an approach could be beneficial. Moreover, our studies clearly link IL-1β neutralization with decrease type 17 responses and granulocyte recruitment, providing useful biomarkers to assess the efficacy of IL-1β neutralization in humans. Importantly, since IL-1β blocking strategies show a similar safety profile to αTNF-α therapy and they are already in clinical practice for chronic inflammatory diseases such as rheumatoid arthritis (555, 821), it is unlikely that aIL-1b treatment in IBD patients would be associated with severe side effects. Furthermore, new αIL-1β mAb show an improved pharmacokinetic profile compared to Anakinra, with higher half lives (~20 days versus 4-6h for Anakinra) and IC50 in the range of 2-40pM (70pM for Anakinra) (555, 710), allowing for better patient compliance. Overall, we hope that our analysis will constitute the foundation for further investigation of the IL-1β pathway in IBD pathogenesis.

Strategies blocking caspase-1 activation are further away from clinical practice, although the caspase-1 inhibitor VX-765 is currently in clinical trials for the treatment of epilepsy (823). Our studies underline the importance of pharmaceutical formulation for the modulation of intestinal inflammation by caspase-1 inhibitors. Given the complex role of IL-1β and IL-18 conversion in the intestine (783), further characterization of the caspase-1 pathway in the intestine will be required for the design of appropriate caspase-1 blocking strategies for the treatment of IBD.

References

1. Dethlefsen, L., M. McFall-Ngai, and D.A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* 449:811-818.
2. Turnbaugh, P.J., R.E. Ley, M. Hamady, C.M. Fraser-Liggett, R. Knight, and J.I. Gordon. 2007. The human microbiome project. *Nature* 449:804-810.
3. Gill, S.R., M. Pop, R.T. Deboy, P.B. Eckburg, P.J. Turnbaugh, B.S. Samuel, J.I. Gordon, D.A. Relman, C.M. Fraser-Liggett, and K.E. Nelson. 2006. Metagenomic analysis of the human distal gut microbiome. *Science* 312:1355-1359.
4. Kaser, A., S. Zeissig, and R.S. Blumberg. 2010. Inflammatory bowel disease. *Annu Rev Immunol* 28:573-621.
5. Artis, D. 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* 8:411-420.
6. Mowat, A.M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 3:331-341.
7. Eberl, G. 2005. Inducible lymphoid tissues in the adult gut: recapitulation of a fetal developmental pathway? *Nat Rev Immunol* 5:413-420.
8. Macpherson, A.J., and T. Uhr. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303:1662-1665.
9. Kunisawa, J., I. Takahashi, and H. Kiyono. 2007. Intraepithelial lymphocytes: their shared and divergent immunological behaviors in the small and large intestine. *Immunol Rev* 215:136-153.
10. Persson, E.K., E. Jaensson, and W.W. Agace. 2010. The diverse ontogeny and function of murine small intestinal dendritic cell/macrophage subsets. *Immunobiology* 215:692-697.
11. Eberl, G., and S. Sawa. 2010. Opening the crypt: current facts and hypotheses on the function of cryptopatches. *Trends Immunol* 31:50-55.
12. Eberl, G., and D.R. Littman. 2004. Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. *Science* 305:248-251.
13. Garrett, W.S., J.I. Gordon, and L.H. Glimcher. 2010. Homeostasis and inflammation in the intestine. *Cell* 140:859-870.
14. Sato, T., R.G. Vries, H.J. Snippert, M. van de Wetering, N. Barker, D.E. Stange, J.H. van Es, A. Abo, P. Kujala, P.J. Peters, and H. Clevers. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459:262-265.
15. Johansson, M.E., M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G.C. Hansson. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* 105:15064-15069.
16. Ouellette, A.J. 2010. Paneth cells and innate mucosal immunity. *Curr Opin Gastroenterol* 26:547-553.
17. Vaishnava, S., C.L. Behrendt, A.S. Ismail, L. Eckmann, and L.V. Hooper. 2008. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A* 105:20858-20863.

18. Blackshaw, L.A., S.J. Brookes, D. Grundy, and M. Schemann. 2007. Sensory transmission in the gastrointestinal tract. *Neurogastroenterol Motil* 19:1-19.
19. Kyd, J.M., and A.W. Cripps. 2008. Functional differences between M cells and enterocytes in sampling luminal antigens. *Vaccine* 26:6221-6224.
20. Coombes, J.L., and F. Powrie. 2008. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* 8:435-446.
21. Steinman, R.M. 2007. Lasker Basic Medical Research Award. Dendritic cells: versatile controllers of the immune system. *Nat Med* 13:1155-1159.
22. Gordon, S. 2007. The macrophage: past, present and future. *Eur J Immunol* 37 Suppl 1:S9-17.
23. Annacker, O., J.L. Coombes, V. Malmstrom, H.H. Uhlig, T. Bourne, B. Johansson-Lindbom, W.W. Agace, C.M. Parker, and F. Powrie. 2005. Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med* 202:1051-1061.
24. Coombes, J.L., K.R. Siddiqui, C.V. Arancibia-Carcamo, J. Hall, C.M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 204:1757-1764.
25. Sun, C.M., J.A. Hall, R.B. Blank, N. Bouladoux, M. Oukka, J.R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204:1775-1785.
26. Jaensson, E., H. Uronen-Hansson, O. Pabst, B. Eksteen, J. Tian, J.L. Coombes, P.L. Berg, T. Davidsson, F. Powrie, B. Johansson-Lindbom, and W.W. Agace. 2008. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med* 205:2139-2149.
27. Smith, P.D., L.E. Smythies, M. Mosteller-Barnum, D.A. Sibley, M.W. Russell, M. Merger, M.T. Sellers, J.M. Orenstein, T. Shimada, M.F. Graham, and H. Kubagawa. 2001. Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities. *J Immunol* 167:2651-2656.
28. Smythies, L.E., M. Sellers, R.H. Clements, M. Mosteller-Barnum, G. Meng, W.H. Benjamin, J.M. Orenstein, and P.D. Smith. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 115:66-75.
29. Denning, T.L., Y.C. Wang, S.R. Patel, I.R. Williams, and B. Pulendran. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol* 8:1086-1094.
30. Niess, J.H., S. Brand, X. Gu, L. Landsman, S. Jung, B.A. McCormick, J.M. Vyas, M. Boes, H.L. Ploegh, J.G. Fox, D.R. Littman, and H.C. Reinecker. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307:254-258.
31. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J.P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2:361-367.

32. Bogunovic, M., F. Ginhoux, J. Helft, L. Shang, D. Hashimoto, M. Greter, K. Liu, C. Jakubzick, M.A. Ingersoll, M. Leboeuf, E.R. Stanley, M. Nussenzweig, S.A. Lira, G.J. Randolph, and M. Merad. 2009. Origin of the lamina propria dendritic cell network. *Immunity* 31:513-525.
33. Lanier, L.L. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 9:495-502.
34. Bryceson, Y.T., and E.O. Long. 2008. Line of attack: NK cell specificity and integration of signals. *Curr Opin Immunol* 20:344-352.
35. Chinen, H., K. Matsuoka, T. Sato, N. Kamada, S. Okamoto, T. Hisamatsu, T. Kobayashi, H. Hasegawa, A. Sugita, F. Kinjo, J. Fujita, and T. Hibi. 2007. Lamina propria c-kit+ immune precursors reside in human adult intestine and differentiate into natural killer cells. *Gastroenterology* 133:559-573.
36. Satoh-Takayama, N., C.A. Vosshenrich, S. Lesjean-Pottier, S. Sawa, M. Lochner, F. Rattis, J.J. Mention, K. Thiam, N. Cerf-Bensussan, O. Mandelboim, G. Eberl, and J.P. Di Santo. 2008. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* 29:958-970.
37. Cella, M., A. Fuchs, W. Vermi, F. Facchetti, K. Otero, J.K. Lennerz, J.M. Doherty, J.C. Mills, and M. Colonna. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457:722-725.
38. Spits, H., and J.P. Di Santo. 2011. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol* 12:21-27.
39. Mebius, R.E., T. Miyamoto, J. Christensen, J. Domen, T. Cupedo, I.L. Weissman, and K. Akashi. 2001. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. *J Immunol* 166:6593-6601.
40. Yoshida, H., H. Kawamoto, S.M. Santee, H. Hashi, K. Honda, S. Nishikawa, C.F. Ware, Y. Katsura, and S.I. Nishikawa. 2001. Expression of alpha(4)beta(7) integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J Immunol* 167:2511-2521.
41. Mebius, R.E. 2003. Organogenesis of lymphoid tissues. *Nat Rev Immunol* 3:292-303.
42. Cupedo, T., N.K. Crellin, N. Papazian, E.J. Rombouts, K. Weijer, J.L. Grogan, W.E. Fibbe, J.J. Cornelissen, and H. Spits. 2009. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol* 10:66-74.
43. Schmutz, S., N. Bosco, S. Chappaz, O. Boyman, H. Acha-Orbea, R. Ceredig, A.G. Rolink, and D. Finke. 2009. Cutting edge: IL-7 regulates the peripheral pool of adult ROR gamma+ lymphoid tissue inducer cells. *J Immunol* 183:2217-2221.
44. Bouskra, D., C. Brezillon, M. Berard, C. Werts, R. Varona, I.G. Boneca, and G. Eberl. 2008. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* 456:507-510.

45. Scandella, E., B. Bolinger, E. Lattmann, S. Miller, S. Favre, D.R. Littman, D. Finke, S.A. Luther, T. Junt, and B. Ludewig. 2008. Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nat Immunol* 9:667-675.
46. Sonnenberg, G.F., L.A. Monticelli, M.M. Elloso, L.A. Fouser, and D. Artis. 2010. CD4(+) lymphoid tissue-inducer cells promote innate immunity in the gut. *Immunity* 34:122-134.
47. Tsuji, M., K. Suzuki, H. Kitamura, M. Maruya, K. Kinoshita, Ivanov, II, K. Itoh, D.R. Littman, and S. Fagarasan. 2008. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. *Immunity* 29:261-271.
48. Satoh-Takayama, N., S. Lesjean-Pottier, P. Vieira, S. Sawa, G. Eberl, C.A. Vosshenrich, and J.P. Di Santo. 2010. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. *J Exp Med* 207:273-280.
49. Luci, C., A. Reynders, Ivanov, II, C. Cognet, L. Chiche, L. Chasson, J. Hardwigsen, E. Anguiano, J. Banchereau, D. Chaussabel, M. Dalod, D.R. Littman, E. Vivier, and E. Tomasello. 2009. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat Immunol* 10:75-82.
50. Sanos, S.L., V.L. Bui, A. Mortha, K. Oberle, C. Heners, C. Johner, and A. Diefenbach. 2009. RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat Immunol* 10:83-91.
51. Buonocore, S., P.P. Ahern, H.H. Uhlig, Ivanov, II, D.R. Littman, K.J. Maloy, and F. Powrie. 2010. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* 464:1371-1375.
52. Neill, D.R., S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, H.E. Jolin, and A.N. McKenzie. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367-1370.
53. Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.
54. Price, A.E., H.E. Liang, B.M. Sullivan, R.L. Reinhardt, C.J. Eisley, D.J. Erle, and R.M. Locksley. 2010. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* 107:11489-11494.
55. Saenz, S.A., M.C. Siracusa, J.G. Perrigoue, S.P. Spencer, J.F. Urban, Jr., J.E. Tocker, A.L. Budelsky, M.A. Kleinschek, R.A. Kastelein, T. Kambayashi, A. Bhandoola, and D. Artis. 2010. IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses. *Nature* 464:1362-1366.
56. Neill, D.R., and A.N. McKenzie. 2011. Nuocytes and beyond: new insights into helminth expulsion. *Trends Parasitol* 27:214-221.
57. Ferguson, A. 1977. Intraepithelial lymphocytes of the small intestine. *Gut* 18:921-937.

58. Poussier, P., T. Ning, J. Chen, D. Banerjee, and M. Julius. 2000. Intestinal inflammation observed in IL-2R/IL-2 mutant mice is associated with impaired intestinal T lymphopoiesis. *Gastroenterology* 118:880-891.
59. Poussier, P., T. Ning, D. Banerjee, and M. Julius. 2002. A unique subset of self-specific intraintestinal T cells maintains gut integrity. *J Exp Med* 195:1491-1497.
60. Sutton, C.E., S.J. Lalor, C.M. Sweeney, C.F. Brereton, E.C. Lavelle, and K.H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31:331-341.
61. Martin, B., K. Hirota, D.J. Cua, B. Stockinger, and M. Veldhoen. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* 31:321-330.
62. Kapsenberg, M.L. 2009. Gammadelta T cell receptors without a job. *Immunity* 31:181-183.
63. Hamada, S., M. Umemura, T. Shiono, K. Tanaka, A. Yahagi, M.D. Begum, K. Oshiro, Y. Okamoto, H. Watanabe, K. Kawakami, C. Roark, W.K. Born, R. O'Brien, K. Ikuta, H. Ishikawa, S. Nakae, Y. Iwakura, T. Ohta, and G. Matsuzaki. 2008. IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver. *J Immunol* 181:3456-3463.
64. Umemura, M., A. Yahagi, S. Hamada, M.D. Begum, H. Watanabe, K. Kawakami, T. Suda, K. Sudo, S. Nakae, Y. Iwakura, and G. Matsuzaki. 2007. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. *J Immunol* 178:3786-3796.
65. Ladel, C.H., C. Blum, and S.H. Kaufmann. 1996. Control of natural killer cell-mediated innate resistance against the intracellular pathogen Listeria monocytogenes by gamma/delta T lymphocytes. *Infect Immun* 64:1744-1749.
66. Boismenu, R., and W.L. Havran. 1994. Modulation of epithelial cell growth by intraepithelial gamma delta T cells. *Science* 266:1253-1255.
67. Komano, H., Y. Fujiura, M. Kawaguchi, S. Matsumoto, Y. Hashimoto, S. Obana, P. Mombaerts, S. Tonegawa, H. Yamamoto, S. Itohara, and et al. 1995. Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* 92:6147-6151.
68. Inagaki-Ohara, K., T. Chinen, G. Matsuzaki, A. Sasaki, Y. Sakamoto, K. Hiromatsu, F. Nakamura-Uchiyama, Y. Nawa, and A. Yoshimura. 2004. Mucosal T cells bearing TCRgammadelta play a protective role in intestinal inflammation. *J Immunol* 173:1390-1398.
69. Roberts, S.J., A.L. Smith, A.B. West, L. Wen, R.C. Findly, M.J. Owen, and A.C. Hayday. 1996. T-cell alpha beta + and gamma delta + deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. *Proc Natl Acad Sci U S A* 93:11774-11779.
70. Stavnezer, J., J.E. Guikema, and C.E. Schrader. 2008. Mechanism and regulation of class switch recombination. *Annu Rev Immunol* 26:261-292.
71. Cerutti, A. 2008. The regulation of IgA class switching. *Nat Rev Immunol* 8:421-434.

72. Suzuki, K., and S. Fagarasan. 2009. Diverse regulatory pathways for IgA synthesis in the gut. *Mucosal Immunol* 2:468-471.
73. Macpherson, A.J., K.D. McCoy, F.E. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. *Mucosal Immunol* 1:11-22.
74. Ng, L.G., C.R. Mackay, and F. Mackay. 2005. The BAFF/APRIL system: life beyond B lymphocytes. *Mol Immunol* 42:763-772.
75. He, B., W. Xu, P.A. Santini, A.D. Polydorides, A. Chiu, J. Estrella, M. Shan, A. Chadburn, V. Villanacci, A. Plebani, D.M. Knowles, M. Rescigno, and A. Cerutti. 2007. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* 26:812-826.
76. Hardenberg, G., L. Planelles, C.M. Schwarte, L. van Bostelen, T. Le Huong, M. Hahne, and J.P. Medema. 2007. Specific TLR ligands regulate APRIL secretion by dendritic cells in a PKR-dependent manner. *Eur J Immunol* 37:2900-2911.
77. Katsenelson, N., S. Kanswal, M. Puig, H. Mostowski, D. Verthelyi, and M. Akkoyunlu. 2007. Synthetic CpG oligodeoxynucleotides augment BAFF- and APRIL-mediated immunoglobulin secretion. *Eur J Immunol* 37:1785-1795.
78. Mestecky, J., J. Zikan, and W.T. Butler. 1971. Immunoglobulin M and secretory immunoglobulin A: presence of a common polypeptide chain different from light chains. *Science* 171:1163-1165.
79. Mostov, K.E., and D.L. Deitcher. 1986. Polymeric immunoglobulin receptor expressed in MDCK cells transcytoses IgA. *Cell* 46:613-621.
80. Fernandez, M.I., T. Pedron, R. Tournebize, J.C. Olivo-Marin, P.J. Sansonetti, and A. Phalipon. 2003. Anti-inflammatory role for intracellular dimeric immunoglobulin a by neutralization of lipopolysaccharide in epithelial cells. *Immunity* 18:739-749.
81. Macpherson, A.J., L. Hunziker, K. McCoy, and A. Lamarre. 2001. IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes Infect* 3:1021-1035.
82. Peterson, D.A., N.P. McNulty, J.L. Guruge, and J.I. Gordon. 2007. IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell Host Microbe* 2:328-339.
83. Bollinger, R.R., M.L. Everett, S.D. Wahl, Y.H. Lee, P.E. Orndorff, and W. Parker. 2006. Secretory IgA and mucin-mediated biofilm formation by environmental strains of Escherichia coli: role of type 1 pili. *Mol Immunol* 43:378-387.
84. Ullrich, R., H.L. Schieferdecker, K. Ziegler, E.O. Riecken, and M. Zeitz. 1990. gamma delta T cells in the human intestine express surface markers of activation and are preferentially located in the epithelium. *Cell Immunol* 128:619-627.
85. Schieferdecker, H.L., R. Ullrich, H. Hirseland, and M. Zeitz. 1992. T cell differentiation antigens on lymphocytes in the human intestinal lamina propria. *J Immunol* 149:2816-2822.
86. Robey, E., and B.J. Fowlkes. 1994. Selective events in T cell development. *Annu Rev Immunol* 12:675-705.
87. Gowans, J.L. 1959. The recirculation of lymphocytes from blood to lymph in the rat. *J Physiol* 146:54-69.
88. Sharpe, A.H. 2009. Mechanisms of costimulation. *Immunol Rev* 229:5-11.

89. Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145-173.
90. Sakaguchi, S., T. Takahashi, and Y. Nishizuka. 1982. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J Exp Med* 156:1577-1586.
91. Fowell, D., and D. Mason. 1993. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J Exp Med* 177:627-636.
92. Aggarwal, S., N. Ghilardi, M.H. Xie, F.J. de Sauvage, and A.L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 278:1910-1914.
93. Bucy, R.P., A. Panoskaltsis-Mortari, G.Q. Huang, J. Li, L. Karr, M. Ross, J.H. Russell, K.M. Murphy, and C.T. Weaver. 1994. Heterogeneity of single cell cytokine gene expression in clonal T cell populations. *J Exp Med* 180:1251-1262.
94. Bucy, R.P., L. Karr, G.Q. Huang, J. Li, D. Carter, K. Honjo, J.A. Lemons, K.M. Murphy, and C.T. Weaver. 1995. Single cell analysis of cytokine gene coexpression during CD4+ T-cell phenotype development. *Proc Natl Acad Sci U S A* 92:7565-7569.
95. Khader, S.A., G.K. Bell, J.E. Pearl, J.J. Fountain, J. Rangel-Moreno, G.E. Cilley, F. Shen, S.M. Eaton, S.L. Gaffen, S.L. Swain, R.M. Locksley, L. Haynes, T.D. Randall, and A.M. Cooper. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat Immunol* 8:369-377.
96. Zhou, L., M.M. Chong, and D.R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30:646-655.
97. Zhu, J., H. Yamane, and W.E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28:445-489.
98. Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655-669.
99. Lighvani, A.A., D.M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B.D. Hissong, B.V. Nguyen, M. Gadina, A. Sher, W.E. Paul, and J.J. O'Shea. 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A* 98:15137-15142.
100. Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260:547-549.
101. Takeda, A., S. Hamano, A. Yamanaka, T. Hanada, T. Ishibashi, T.W. Mak, A. Yoshimura, and H. Yoshida. 2003. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol* 170:4886-4890.
102. Yoshida, H., S. Hamano, G. Senaldi, T. Covey, R. Faggioni, S. Mu, M. Xia, A.C. Wakeham, H. Nishina, J. Potter, C.J. Saris, and T.W. Mak. 2001. WSX-1 is

required for the initiation of Th1 responses and resistance to L. major infection. *Immunity* 15:569-578.
103. Mullen, A.C., A.S. Hutchins, F.A. High, H.W. Lee, K.J. Sykes, L.A. Chodosh, and S.L. Reiner. 2002. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nat Immunol* 3:652-658.
104. Mullen, A.C., F.A. High, A.S. Hutchins, H.W. Lee, A.V. Villarino, D.M. Livingston, A.L. Kung, N. Cereb, T.P. Yao, S.Y. Yang, and S.L. Reiner. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 292:1907-1910.
105. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 9:765-775.
106. Grogan, J.L., Z.E. Wang, S. Stanley, B. Harmon, G.G. Loots, E.M. Rubin, and R.M. Locksley. 2003. Basal chromatin modification at the IL-4 gene in helper T cells. *J Immunol* 171:6672-6679.
107. Hwang, E.S., S.J. Szabo, P.L. Schwartzberg, and L.H. Glimcher. 2005. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* 307:430-433.
108. Koyanagi, M., A. Baguet, J. Martens, R. Margueron, T. Jenuwein, and M. Bix. 2005. EZH2 and histone 3 trimethyl lysine 27 associated with Il4 and Il13 gene silencing in Th1 cells. *J Biol Chem* 280:31470-31477.
109. Djuretic, I.M., D. Levanon, V. Negreanu, Y. Groner, A. Rao, and K.M. Ansel. 2007. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat Immunol* 8:145-153.
110. Afkarian, M., J.R. Sedy, J. Yang, N.G. Jacobson, N. Cereb, S.Y. Yang, T.L. Murphy, and K.M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 3:549-557.
111. Szabo, S.J., B.M. Sullivan, C. Stemmann, A.R. Satoskar, B.P. Sleckman, and L.H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 295:338-342.
112. Thierfelder, W.E., J.M. van Deursen, K. Yamamoto, R.A. Tripp, S.R. Sarawar, R.T. Carson, M.Y. Sangster, D.A. Vignali, P.C. Doherty, G.C. Grosveld, and J.N. Ihle. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382:171-174.
113. Kaplan, M.H., Y.L. Sun, T. Hoey, and M.J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174-177.
114. Scott, P., E. Pearce, A.W. Cheever, R.L. Coffman, and A. Sher. 1989. Role of cytokines and CD4+ T-cell subsets in the regulation of parasite immunity and disease. *Immunol Rev* 112:161-182.
115. Suzuki, Y., and J.S. Remington. 1990. The effect of anti-IFN-gamma antibody on the protective effect of Lyt-2+ immune T cells against toxoplasmosis in mice. *J Immunol* 144:1954-1956.
116. Suzuki, Y., F.K. Conley, and J.S. Remington. 1989. Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice. *J Immunol* 143:2045-2050.

117. Trembleau, S., G. Penna, E. Bosi, A. Mortara, M.K. Gately, and L. Adorini. 1995. Interleukin 12 administration induces T helper type 1 cells and accelerates autoimmune diabetes in NOD mice. *J Exp Med* 181:817-821.
118. Powrie, F., M.W. Leach, S. Mauze, S. Menon, L.B. Caddle, and R.L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* 1:553-562.
119. Neurath, M.F., B. Weigmann, S. Finotto, J. Glickman, E. Nieuwenhuis, H. Iijima, A. Mizoguchi, E. Mizoguchi, J. Mudter, P.R. Galle, A. Bhan, F. Autschbach, B.M. Sullivan, S.J. Szabo, L.H. Glimcher, and R.S. Blumberg. 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* 195:1129-1143.
120. Ouyang, W., S.H. Ranganath, K. Weindel, D. Bhattacharya, T.L. Murphy, W.C. Sha, and K.M. Murphy. 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* 9:745-755.
121. Yu, Q., A. Sharma, S.Y. Oh, H.G. Moon, M.Z. Hossain, T.M. Salay, K.E. Leeds, H. Du, B. Wu, M.L. Waterman, Z. Zhu, and J.M. Sen. 2009. T cell factor 1 initiates the T helper type 2 fate by inducing the transcription factor GATA-3 and repressing interferon-gamma. *Nat Immunol* 10:992-999.
122. Anthony, R.M., L.I. Rutitzky, J.F. Urban, Jr., M.J. Stadecker, and W.C. Gause. 2007. Protective immune mechanisms in helminth infection. *Nat Rev Immunol* 7:975-987.
123. Umetsu, D.T., J.J. McIntire, O. Akbari, C. Macaubas, and R.H. DeKruyff. 2002. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 3:715-720.
124. Noelle, R.J., and E.C. Nowak. 2010. Cellular sources and immune functions of interleukin-9. *Nat Rev Immunol* 10:683-687.
125. Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9:1341-1346.
126. Dardalhon, V., A. Awasthi, H. Kwon, G. Galileos, W. Gao, R.A. Sobel, M. Mitsdoerffer, T.B. Strom, W. Elyaman, I.C. Ho, S. Khoury, M. Oukka, and V.K. Kuchroo. 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* 9:1347-1355.
127. Staudt, V., E. Bothur, M. Klein, K. Lingnau, S. Reuter, N. Grebe, B. Gerlitzki, M. Hoffmann, A. Ulges, C. Taube, N. Dehzad, M. Becker, M. Stassen, A. Steinborn, M. Lohoff, H. Schild, E. Schmitt, and T. Bopp. 2010. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* 33:192-202.
128. Littman, D.R., and A.Y. Rudensky. 2010. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 140:845-858.
129. Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.
130. Veldhoen, M., R.J. Hocking, R.A. Flavell, and B. Stockinger. 2006. Signals mediated by transforming growth factor-beta initiate autoimmune

encephalomyelitis, but chronic inflammation is needed to sustain disease. *Nat Immunol* 7:1151-1156.

131. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.
132. Mangan, P.R., L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, R.D. Hatton, S.M. Wahl, T.R. Schoeb, and C.T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441:231-234.
133. Acosta-Rodriguez, E.V., G. Napolitani, A. Lanzavecchia, and F. Sallusto. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8:942-949.
134. Manel, N., D. Unutmaz, and D.R. Littman. 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* 9:641-649.
135. Volpe, E., N. Servant, R. Zollinger, S.I. Bogiatzi, P. Hupe, E. Barillot, and V. Soumelis. 2008. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol* 9:650-657.
136. Yang, L., D.E. Anderson, C. Baecher-Allan, W.D. Hastings, E. Bettelli, M. Oukka, V.K. Kuchroo, and D.A. Hafler. 2008. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* 454:350-352.
137. Ghoreschi, K., A. Laurence, X.P. Yang, C.M. Tato, M.J. McGeachy, J.E. Konkel, H.L. Ramos, L. Wei, T.S. Davidson, N. Bouladoux, J.R. Grainger, Q. Chen, Y. Kanno, W.T. Watford, H.W. Sun, G. Eberl, E.M. Shevach, Y. Belkaid, D.J. Cua, W. Chen, and J.J. O'Shea. 2010. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature* 467:967-971.
138. Chung, Y., S.H. Chang, G.J. Martinez, X.O. Yang, R. Nurieva, H.S. Kang, L. Ma, S.S. Watowich, A.M. Jetten, Q. Tian, and C. Dong. 2009. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 30:576-587.
139. Sutton, C., C. Brereton, B. Keogh, K.H. Mills, and E.C. Lavelle. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* 203:1685-1691.
140. Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T.B. Strom, M. Oukka, and V.K. Kuchroo. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448:484-487.
141. Nurieva, R., X.O. Yang, G. Martinez, Y. Zhang, A.D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S.S. Watowich, A.M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448:480-483.
142. Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, T. Egawa, D.E. Levy, W.J. Leonard, and D.R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8:967-974.

143. Yang, X.O., A.D. Panopoulos, R. Nurieva, S.H. Chang, D. Wang, S.S. Watowich, and C. Dong. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* 282:9358-9363.
144. Yang, X.O., R. Nurieva, G.J. Martinez, H.S. Kang, Y. Chung, B.P. Pappu, B. Shah, S.H. Chang, K.S. Schluns, S.S. Watowich, X.H. Feng, A.M. Jetten, and C. Dong. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29:44-56.
145. Milner, J.D., J.M. Brenchley, A. Laurence, A.F. Freeman, B.J. Hill, K.M. Elias, Y. Kanno, C. Spalding, H.Z. Elloumi, M.L. Paulson, J. Davis, A. Hsu, A.I. Asher, J. O'Shea, S.M. Holland, W.E. Paul, and D.C. Douek. 2008. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452:773-776.
146. Brustle, A., S. Heink, M. Huber, C. Rosenplanter, C. Stadelmann, P. Yu, E. Arpaia, T.W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* 8:958-966.
147. Schraml, B.U., K. Hildner, W. Ise, W.L. Lee, W.A. Smith, B. Solomon, G. Sahota, J. Sim, R. Mukasa, S. Cemerski, R.D. Hatton, G.D. Stormo, C.T. Weaver, J.H. Russell, T.L. Murphy, and K.M. Murphy. 2009. The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature* 460:405-409.
148. Quintana, F.J., A.S. Basso, A.H. Iglesias, T. Korn, M.F. Farez, E. Bettelli, M. Caccamo, M. Oukka, and H.L. Weiner. 2008. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453:65-71.
149. Veldhoen, M., K. Hirota, A.M. Westendorf, J. Buer, L. Dumoutier, J.C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:106-109.
150. Dubin, P.J., and J.K. Kolls. 2008. Th17 cytokines and mucosal immunity. *Immunol Rev* 226:160-171.
151. Weaver, C.T., R.D. Hatton, P.R. Mangan, and L.E. Harrington. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25:821-852.
152. Ouyang, W., J.K. Kolls, and Y. Zheng. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28:454-467.
153. Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein, and J.D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.
154. Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177:566-573.
155. Teunissen, M.B., C.W. Koomen, R. de Waal Malefyt, E.A. Wierenga, and J.D. Bos. 1998. Interleukin-17 and interferon-gamma synergize in the enhancement of proinflammatory cytokine production by human keratinocytes. *J Invest Dermatol* 111:645-649.
156. Liang, S.C., X.Y. Tan, D.P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L.A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by

Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 203:2271-2279.

157. Wilson, N.J., K. Boniface, J.R. Chan, B.S. McKenzie, W.M. Blumenschein, J.D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, J.C. Lecron, R.A. Kastelein, D.J. Cua, T.K. McClanahan, E.P. Bowman, and R. de Waal Malefyt. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 8:950-957.
158. Bush, K.A., K.M. Farmer, J.S. Walker, and B.W. Kirkham. 2002. Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein. *Arthritis Rheum* 46:802-805.
159. Lubberts, E., M.I. Koenders, B. Oppers-Walgreen, L. van den Bersselaar, C.J. Coenen-de Roo, L.A. Joosten, and W.B. van den Berg. 2004. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheum* 50:650-659.
160. Josefowicz, S.Z., and A. Rudensky. 2009. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 30:616-625.
161. Nishizuka, Y., and T. Sakakura. 1969. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* 166:753-755.
162. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184:387-396.
163. Bonomo, A., P.J. Kehn, E. Payer, L. Rizzo, A.W. Cheever, and E.M. Shevach. 1995. Pathogenesis of post-thymectomy autoimmunity. Role of syngeneic MLR-reactive T cells. *J Immunol* 154:6602-6611.
164. Powrie, F., and D. Mason. 1990. Subsets of rat CD4+ T cells defined by their differential expression of variants of the CD45 antigen: developmental relationships and in vitro and in vivo functions. *Curr Top Microbiol Immunol* 159:79-96.
165. Morrissey, P.J., K. Charrier, S. Braddy, D. Liggitt, and J.D. Watson. 1993. CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. *J Exp Med* 178:237-244.
166. Powrie, F., M.W. Leach, S. Mauze, L.B. Caddle, and R.L. Coffman. 1993. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int Immunol* 5:1461-1471.
167. Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.
168. Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27:68-73.

169. Wildin, R.S., F. Ramsdell, J. Peake, F. Faravelli, J.L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, F.D. Bricarelli, G. Byrne, M. McEuen, S. Proll, M. Appleby, and M.E. Brunkow. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27:18-20.
170. Chatila, T.A., F. Blaeser, N. Ho, H.M. Lederman, C. Voulgaropoulos, C. Helms, and A.M. Bowcock. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest* 106:R75-81.
171. Hsieh, C.S., Y. Liang, A.J. Tyznik, S.G. Self, D. Liggitt, and A.Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* 21:267-277.
172. Lio, C.W., and C.S. Hsieh. 2008. A two-step process for thymic regulatory T cell development. *Immunity* 28:100-111.
173. Burchill, M.A., J. Yang, C. Vogtenhuber, B.R. Blazar, and M.A. Farrar. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* 178:280-290.
174. Fontenot, J.D., J.P. Rasmussen, M.A. Gavin, and A.Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6:1142-1151.
175. Burchill, M.A., J. Yang, K.B. Vang, and M.A. Farrar. 2007. Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. *Immunol Lett* 114:1-8.
176. Vang, K.B., J. Yang, S.A. Mahmud, M.A. Burchill, A.L. Vegoe, and M.A. Farrar. 2008. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. *J Immunol* 181:3285-3290.
177. Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431-440.
178. Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* 6:152-162.
179. Feuerer, M., J.A. Hill, D. Mathis, and C. Benoist. 2009. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* 10:689-695.
180. Wan, Y.Y., and R.A. Flavell. 2007. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* 445:766-770.
181. Williams, L.M., and A.Y. Rudensky. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 8:277-284.
182. Murai, M., P. Krause, H. Cheroutre, and M. Kronenberg. 2011. Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. *Mucosal Immunol* 3:443-449.
183. Coombes, J.L., and K.J. Maloy. 2007. Control of intestinal homeostasis by regulatory T cells and dendritic cells. *Semin Immunol* 19:116-126.

184. Shevach, E.M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-645.
185. Tadokoro, C.E., G. Shakhar, S. Shen, Y. Ding, A.C. Lino, A. Maraver, J.J. Lafaille, and M.L. Dustin. 2006. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med* 203:505-511.
186. Tang, Q., J.Y. Adams, A.J. Tooley, M. Bi, B.T. Fife, P. Serra, P. Santamaria, R.M. Locksley, M.F. Krummel, and J.A. Bluestone. 2006. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 7:83-92.
187. Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322:271-275.
188. Yu, X., K. Harden, L.C. Gonzalez, M. Francesco, E. Chiang, B. Irving, I. Tom, S. Ivelja, C.J. Refino, H. Clark, D. Eaton, and J.L. Grogan. 2009. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* 10:48-57.
189. Rubtsov, Y.P., J.P. Rasmussen, E.Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W.R. Henderson, Jr., W. Muller, and A.Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28:546-558.
190. Collison, L.W., C.J. Workman, T.T. Kuo, K. Boyd, Y. Wang, K.M. Vignali, R. Cross, D. Sehy, R.S. Blumberg, and D.A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450:566-569.
191. Deaglio, S., K.M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J.F. Chen, K. Enjyoji, J. Linden, M. Oukka, V.K. Kuchroo, T.B. Strom, and S.C. Robson. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204:1257-1265.
192. Pandiyan, P., L. Zheng, S. Ishihara, J. Reed, and M.J. Lenardo. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* 8:1353-1362.
193. Kullberg, M.C., D. Jankovic, P.L. Gorelick, P. Caspar, J.J. Letterio, A.W. Cheever, and A. Sher. 2002. Bacteria-triggered CD4(+) T regulatory cells suppress Helicobacter hepaticus-induced colitis. *J Exp Med* 196:505-515.
194. Jankovic, D., M.C. Kullberg, C.G. Feng, R.S. Goldszmid, C.M. Collazo, M. Wilson, T.A. Wynn, M. Kamanaka, R.A. Flavell, and A. Sher. 2007. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med* 204:273-283.
195. Maynard, C.L., L.E. Harrington, K.M. Janowski, J.R. Oliver, C.L. Zindl, A.Y. Rudensky, and C.T. Weaver. 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol* 8:931-941.
196. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J.M. Kim, T.T. Chu, L. Corcoran, P. Treuting, U. Klein, and A.Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 458:351-356.

197. Chaudhry, A., D. Rudra, P. Treuting, R.M. Samstein, Y. Liang, A. Kas, and A.Y. Rudensky. 2009. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* 326:986-991.
198. Yu, D., and C.G. Vinuesa. 2010. The elusive identity of T follicular helper cells. *Trends Immunol* 31:377-383.
199. Crotty, S., R.J. Johnston, and S.P. Schoenberger. 2010. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 11:114-120.
200. Dullaers, M., D. Li, Y. Xue, L. Ni, I. Gayet, R. Morita, H. Ueno, K.A. Palucka, J. Banchereau, and S. Oh. 2009. A T cell-dependent mechanism for the induction of human mucosal homing immunoglobulin A-secreting plasmablasts. *Immunity* 30:120-129.
201. O'Shea, J.J., and W.E. Paul. 2010. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* 327:1098-1102.
202. Chen, Z., C.M. Tato, L. Muul, A. Laurence, and J.J. O'Shea. 2007. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum* 56:2936-2946.
203. Ahern, P.P., C. Schiering, S. Buonocore, M.J. McGeachy, D.J. Cua, K.J. Maloy, and F. Powrie. 2010. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* 33:279-288.
204. Hayashi, N., T. Yoshimoto, K. Izuhara, K. Matsui, T. Tanaka, and K. Nakanishi. 2007. T helper 1 cells stimulated with ovalbumin and IL-18 induce airway hyperresponsiveness and lung fibrosis by IFN-gamma and IL-13 production. *Proc Natl Acad Sci U S A* 104:14765-14770.
205. Lohning, M., A.N. Hegazy, D.D. Pinschewer, D. Busse, K.S. Lang, T. Hofer, A. Radbruch, R.M. Zinkernagel, and H. Hengartner. 2008. Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J Exp Med* 205:53-61.
206. Zhou, X., S.L. Bailey-Bucktrout, L.T. Jeker, C. Penaranda, M. Martinez-Llordella, M. Ashby, M. Nakayama, W. Rosenthal, and J.A. Bluestone. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* 10:1000-1007.
207. Xu, L., A. Kitani, I. Fuss, and W. Strober. 2007. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol* 178:6725-6729.
208. Wei, G., L. Wei, J. Zhu, C. Zang, J. Hu-Li, Z. Yao, K. Cui, Y. Kanno, T.Y. Roh, W.T. Watford, D.E. Schones, W. Peng, H.W. Sun, W.E. Paul, J.J. O'Shea, and K. Zhao. 2009. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30:155-167.
209. Komatsu, N., M.E. Mariotti-Ferrandiz, Y. Wang, B. Malissen, H. Waldmann, and S. Hori. 2009. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A* 106:1903-1908.
210. Koch, M.A., G. Tucker-Heard, N.R. Perdue, J.R. Killebrew, K.B. Urdahl, and D.J. Campbell. 2009. The transcription factor T-bet controls regulatory T cell

homeostasis and function during type 1 inflammation. *Nat Immunol* 10:595-602.

211. Zhou, L., J.E. Lopes, M.M. Chong, Ivanov, II, R. Min, G.D. Victora, Y. Shen, J. Du, Y.P. Rubtsov, A.Y. Rudensky, S.F. Ziegler, and D.R. Littman. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453:236-240.
212. Zhang, F., G. Meng, and W. Strober. 2008. Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat Immunol* 9:1297-1306.
213. King, C., S.G. Tangye, and C.R. Mackay. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* 26:741-766.
214. Peterson, D.A., D.N. Frank, N.R. Pace, and J.I. Gordon. 2008. Metagenomic approaches for defining the pathogenesis of inflammatory bowel diseases. *Cell Host Microbe* 3:417-427.
215. Backhed, F., R.E. Ley, J.L. Sonnenburg, D.A. Peterson, and J.I. Gordon. 2005. Host-bacterial mutualism in the human intestine. *Science* 307:1915-1920.
216. Scupham, A.J., L.L. Presley, B. Wei, E. Bent, N. Griffith, M. McPherson, F. Zhu, O. Oluwadara, N. Rao, J. Braun, and J. Borneman. 2006. Abundant and diverse fungal microbiota in the murine intestine. *Appl Environ Microbiol* 72:793-801.
217. Anderson, C.A., G. Boucher, C.W. Lees, A. Franke, M. D'Amato, K.D. Taylor, J.C. Lee, P. Goyette, M. Imielinski, A. Latiano, C. Lagace, R. Scott, L. Amininejad, S. Bumpstead, L. Baidoo, R.N. Baldassano, M. Barclay, T.M. Bayless, S. Brand, C. Buning, J.F. Colombel, L.A. Denson, M. De Vos, M. Dubinsky, C. Edwards, D. Ellinghaus, R.S. Fehrmann, J.A. Floyd, T. Florin, D. Franchimont, L. Franke, M. Georges, J. Glas, N.L. Glazer, S.L. Guthery, T. Haritunians, N.K. Hayward, J.P. Hugot, G. Jobin, D. Laukens, I. Lawrance, M. Lemann, A. Levine, C. Libioulle, E. Louis, D.P. McGovern, M. Milla, G.W. Montgomery, K.I. Morley, C. Mowat, A. Ng, W. Newman, R.A. Ophoff, L. Papi, O. Palmieri, L. Peyrin-Biroulet, J. Panes, A. Phillips, N.J. Prescott, D.D. Proctor, R. Roberts, R. Russell, P. Rutgeerts, J. Sanderson, M. Sans, P. Schumm, F. Seibold, Y. Sharma, L.A. Simms, M. Seielstad, A.H. Steinhart, S.R. Targan, L.H. van den Berg, M. Vatn, H. Verspaget, T. Walters, C. Wijmenga, D.C. Wilson, H.J. Westra, R.J. Xavier, Z.Z. Zhao, C.Y. Ponsioen, V. Andersen, L. Torkvist, M. Gazouli, N.P. Anagnou, T.H. Karlsen, L. Kupcinskas, J. Sventoraityte, J.C. Mansfield, S. Kugathasan, M.S. Silverberg, J. Halfvarson, J.I. Rotter, C.G. Mathew, A.M. Griffiths, R. Gearry, T. Ahmad, S.R. Brant, M. Chamaillard, J. Satsangi, J.H. Cho, S. Schreiber, M.J. Daly, J.C. Barrett, M. Parkes, V. Annese, H. Hakonarson, G. Radford-Smith, R.H. Duerr, S. Vermeire, R.K. Weersma, and J.D. Rioux. 2011. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet* 43:246-252.
218. Ley, R.E., P.J. Turnbaugh, S. Klein, and J.I. Gordon. 2006. Microbial ecology: human gut microbes associated with obesity. *Nature* 444:1022-1023.
219. Rawls, J.F., M.A. Mahowald, R.E. Ley, and J.I. Gordon. 2006. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* 127:423-433.

220. Ley, R.E., D.A. Peterson, and J.I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837-848.
221. Frank, D.N., A.L. St Amand, R.A. Feldman, E.C. Boedeker, N. Harpaz, and N.R. Pace. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* 104:13780-13785.
222. Hooper, L.V., and J.I. Gordon. 2001. Commensal host-bacterial relationships in the gut. *Science* 292:1115-1118.
223. Guarner, F., and J.R. Malagelada. 2003. Gut flora in health and disease. *Lancet* 361:512-519.
224. Kau, A.L., P.P. Ahern, N.W. Griffin, A.L. Goodman, and J.I. Gordon. 2011. Human nutrition, the gut microbiome and the immune system. *Nature* 474:327-336.
225. Alam, M., T. Midtvedt, and A. Uribe. 1994. Differential cell kinetics in the ileum and colon of germfree rats. *Scand J Gastroenterol* 29:445-451.
226. Pull, S.L., J.M. Doherty, J.C. Mills, J.I. Gordon, and T.S. Stappenbeck. 2005. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc Natl Acad Sci U S A* 102:99-104.
227. Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118:229-241.
228. Macpherson, A.J., and N.L. Harris. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 4:478-485.
229. Bauer, H., R.E. Horowitz, S.M. Levenson, and H. Popper. 1963. The response of the lymphatic tissue to the microbial flora. Studies on germfree mice. *Am J Pathol* 42:471-483.
230. Wen, L., R.E. Ley, P.Y. Volchkov, P.B. Stranges, L. Avanesyan, A.C. Stonebraker, C. Hu, F.S. Wong, G.L. Szot, J.A. Bluestone, J.I. Gordon, and A.V. Chervonsky. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455:1109-1113.
231. Braun-Fahrlander, C., J. Riedler, U. Herz, W. Eder, M. Waser, L. Grize, S. Maisch, D. Carr, F. Gerlach, A. Bufe, R.P. Lauener, R. Schierl, H. Renz, D. Nowak, and E. von Mutius. 2002. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 347:869-877.
232. Kozyrskyj, A.L., P. Ernst, and A.B. Becker. 2007. Increased risk of childhood asthma from antibiotic use in early life. *Chest* 131:1753-1759.
233. Lievin, V., I. Peiffer, S. Hudault, F. Rochat, D. Brassart, J.R. Neeser, and A.L. Servin. 2000. Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* 47:646-652.
234. Macpherson, A.J., D. Gatto, E. Sainsbury, G.R. Harriman, H. Hengartner, and R.M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288:2222-2226.
235. Suzuki, K., B. Meek, Y. Doi, M. Muramatsu, T. Chiba, T. Honjo, and S. Fagarasan. 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A* 101:1981-1986.

236. Burger-van Paassen, N., A. Vincent, P.J. Puiman, M. van der Sluis, J. Bouma, G. Boehm, J.B. van Goudoever, I. van Seuningen, and I.B. Renes. 2009. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. *Biochem J* 420:211-219.
237. Hofstad, T., N. Skaug, and K. Sveen. 1993. Stimulation of B lymphocytes by lipopolysaccharides from anaerobic bacteria. *Clin Infect Dis* 16 Suppl 4:S200-202.
238. Mandell, L., A.P. Moran, A. Cocchiarella, J. Houghton, N. Taylor, J.G. Fox, T.C. Wang, and E.A. Kurt-Jones. 2004. Intact gram-negative Helicobacter pylori, Helicobacter felis, and Helicobacter hepaticus bacteria activate innate immunity via toll-like receptor 2 but not toll-like receptor 4. *Infect Immun* 72:6446-6454.
239. Di Giacinto, C., M. Marinaro, M. Sanchez, W. Strober, and M. Boirivant. 2005. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. *J Immunol* 174:3237-3246.
240. Mazmanian, S.K., J.L. Round, and D.L. Kasper. 2008. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453:620-625.
241. Sokol, H., B. Pigneur, L. Watterlot, O. Lakhdari, L.G. Bermudez-Humaran, J.J. Gratadoux, S. Blugeon, C. Bridonneau, J.P. Furet, G. Corthier, C. Grangette, N. Vasquez, P. Pochart, G. Trugnan, G. Thomas, H.M. Blottiere, J. Dore, P. Marteau, P. Seksik, and P. Langella. 2008. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* 105:16731-16736.
242. Kelly, D., J.I. Campbell, T.P. King, G. Grant, E.A. Jansson, A.G. Coutts, S. Pettersson, and S. Conway. 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat Immunol* 5:104-112.
243. O'Mahony, C., P. Scully, D. O'Mahony, S. Murphy, F. O'Brien, A. Lyons, G. Sherlock, J. MacSharry, B. Kiely, F. Shanahan, and L. O'Mahony. 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog* 4:e1000112.
244. Geuking, M.B., J. Cahenzli, M.A. Lawson, D.C. Ng, E. Slack, S. Hapfelmeier, K.D. McCoy, and A.J. Macpherson. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* 34:794-806.
245. Macpherson, A.J., E. Slack, M.B. Geuking, and K.D. McCoy. 2009. The mucosal firewalls against commensal intestinal microbes. *Semin Immunopathol* 31:145-149.
246. Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol* 20:197-216.
247. Gallucci, S., and P. Matzinger. 2001. Danger signals: SOS to the immune system. *Curr Opin Immunol* 13:114-119.
248. Drummond, R.A., S. Saijo, Y. Iwakura, and G.D. Brown. 2011. The role of Syk/CARD9 coupled C-type lectins in antifungal immunity. *Eur J Immunol* 41:276-281.
249. Loo, Y.M., and M. Gale, Jr. 2011. Immune signaling by RIG-I-like receptors. *Immunity* 34:680-692.

250. Ting, J.P., J.A. Duncan, and Y. Lei. 2010. How the noninflammasome NLRs function in the innate immune system. *Science* 327:286-290.
251. Lemaitre, B., E. Nicolas, L. Michaut, J.M. Reichhart, and J.A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86:973-983.
252. Lavelle, E.C., C. Murphy, L.A. O'Neill, and E.M. Creagh. 2010. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. *Mucosal Immunol* 3:17-28.
253. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783-801.
254. O'Neill, L.A. 2008. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol Rev* 226:10-18.
255. O'Neill, L.A., and A.G. Bowie. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 7:353-364.
256. Kagan, J.C., T. Su, T. Horng, A. Chow, S. Akira, and R. Medzhitov. 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* 9:361-368.
257. Takeda, K., and S. Akira. 2007. Toll-like receptors. *Curr Protoc Immunol* Chapter 14:Unit 14 12.
258. Kumar, H., T. Kawai, and S. Akira. 2011. Pathogen recognition by the innate immune system. *Int Rev Immunol* 30:16-34.
259. Schroder, K., and J. Tschopp. 2010. The inflammasomes. *Cell* 140:821-832.
260. Fritz, J.H., R.L. Ferrero, D.J. Philpott, and S.E. Girardin. 2006. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* 7:1250-1257.
261. Meylan, E., and J. Tschopp. 2008. NLRX1: friend or foe? *EMBO Rep* 9:243-245.
262. Faustin, B., L. Lartigue, J.M. Bruey, F. Luciano, E. Sergienko, B. Bailly-Maitre, N. Volkmann, D. Hanein, I. Rouiller, and J.C. Reed. 2007. Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell* 25:713-724.
263. Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, M.A. Valvano, S.J. Foster, T.W. Mak, G. Nunez, and N. Inohara. 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 4:702-707.
264. Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, S.J. Foster, A.P. Moran, J.L. Fernandez-Luna, and G. Nunez. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 278:5509-5512.
265. Girardin, S.E., I.G. Boneca, L.A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M.K. Taha, A. Labigne, U. Zahringer, A.J. Coyle, P.S. DiStefano, J. Bertin, P.J. Sansonetti, and D.J. Philpott. 2003. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300:1584-1587.
266. Girardin, S.E., I.G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D.J. Philpott, and P.J. Sansonetti. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 278:8869-8872.

267. Viala, J., C. Chaput, I.G. Boneca, A. Cardona, S.E. Girardin, A.P. Moran, R. Athman, S. Memet, M.R. Huerre, A.J. Coyle, P.S. DiStefano, P.J. Sansonetti, A. Labigne, J. Bertin, D.J. Philpott, and R.L. Ferrero. 2004. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* 5:1166-1174.
268. Lee, J., I. Tattoli, K.A. Wojtal, S.R. Vavricka, D.J. Philpott, and S.E. Girardin. 2009. pH-dependent internalization of muramyl peptides from early endosomes enables Nod1 and Nod2 signaling. *J Biol Chem* 284:23818-23829.
269. Marina-Garcia, N., L. Franchi, Y.G. Kim, Y. Hu, D.E. Smith, G.J. Boons, and G. Nunez. 2009. Clathrin- and dynamin-dependent endocytic pathway regulates muramyl dipeptide internalization and NOD2 activation. *J Immunol* 182:4321-4327.
270. Lecine, P., S. Esmiol, J.Y. Metais, C. Nicoletti, C. Nourry, C. McDonald, G. Nunez, J.P. Hugot, J.P. Borg, and V. Ollendorff. 2007. The NOD2-RICK complex signals from the plasma membrane. *J Biol Chem* 282:15197-15207.
271. Bertrand, M.J., K. Doiron, K. Labbe, R.G. Korneluk, P.A. Barker, and M. Saleh. 2009. Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. *Immunity* 30:789-801.
272. Abbott, D.W., Y. Yang, J.E. Hutti, S. Madhavarapu, M.A. Kelliher, and L.C. Cantley. 2007. Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. *Mol Cell Biol* 27:6012-6025.
273. Franchi, L., N. Warner, K. Viani, and G. Nunez. 2009. Function of Nod-like receptors in microbial recognition and host defense. *Immunol Rev* 227:106-128.
274. Strober, W., P.J. Murray, A. Kitani, and T. Watanabe. 2006. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* 6:9-20.
275. Sabbah, A., T.H. Chang, R. Harnack, V. Frohlich, K. Tominaga, P.H. Dube, Y. Xiang, and S. Bose. 2009. Activation of innate immune antiviral responses by Nod2. *Nat Immunol* 10:1073-1080.
276. Hysi, P., M. Kabesch, M.F. Moffatt, M. Schedel, D. Carr, Y. Zhang, B. Boardman, E. von Mutius, S.K. Weiland, W. Leupold, C. Fritzsch, N. Klopp, A.W. Musk, A. James, G. Nunez, N. Inohara, and W.O. Cookson. 2005. NOD1 variation, immunoglobulin E and asthma. *Hum Mol Genet* 14:935-941.
277. Miceli-Richard, C., S. Lesage, M. Rybojad, A.M. Prieur, S. Manouvrier-Hanu, R. Hafner, M. Chamaillard, H. Zouali, G. Thomas, and J.P. Hugot. 2001. CARD15 mutations in Blau syndrome. *Nat Genet* 29:19-20.
278. Kanazawa, N., I. Okafuji, N. Kambe, R. Nishikomori, M. Nakata-Hizume, S. Nagai, A. Fuji, T. Yuasa, A. Manki, Y. Sakurai, M. Nakajima, H. Kobayashi, I. Fujiwara, H. Tsutsumi, A. Utani, C. Nishigori, T. Heike, T. Nakahata, and Y. Miyachi. 2005. Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome. *Blood* 105:1195-1197.
279. McGovern, D.P., P. Hysi, T. Ahmad, D.A. van Heel, M.F. Moffatt, A. Carey, W.O. Cookson, and D.P. Jewell. 2005. Association between a complex

insertion/deletion polymorphism in NOD1 (CARD4) and susceptibility to inflammatory bowel disease. *Hum Mol Genet* 14:1245-1250.

280. Hugot, J.P., M. Chamaillard, H. Zouali, S. Lesage, J.P. Cezard, J. Belaiche, S. Almer, C. Tysk, C.A. O'Morain, M. Gassull, V. Binder, Y. Finkel, A. Cortot, R. Modigliani, P. Laurent-Puig, C. Gower-Rousseau, J. Macry, J.F. Colombel, M. Sahbatou, and G. Thomas. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411:599-603.
281. Ogura, Y., D.K. Bonen, N. Inohara, D.L. Nicolae, F.F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R.H. Duerr, J.P. Achkar, S.R. Brant, T.M. Bayless, B.S. Kirschner, S.B. Hanauer, G. Nunez, and J.H. Cho. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411:603-606.
282. Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10:417-426.
283. Keller, M., A. Ruegg, S. Werner, and H.D. Beer. 2008. Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 132:818-831.
284. Franchi, L., J. Stoolman, T.D. Kanneganti, A. Verma, R. Ramphal, and G. Nunez. 2007. Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. *Eur J Immunol* 37:3030-3039.
285. Mariathasan, S., K. Newton, D.M. Monack, D. Vucic, D.M. French, W.P. Lee, M. Roose-Girma, S. Erickson, and V.M. Dixit. 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213-218.
286. Hiscott, J., J. Marois, J. Garoufalis, M. D'Addario, A. Roulston, I. Kwan, N. Pepin, J. Lacoste, H. Nguyen, G. Bensi, and et al. 1993. Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Mol Cell Biol* 13:6231-6240.
287. Bauernfeind, F.G., G. Horvath, A. Stutz, E.S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B.G. Monks, K.A. Fitzgerald, V. Hornung, and E. Latz. 2009. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183:787-791.
288. Cassel, S.L., S.C. Eisenbarth, S.S. Iyer, J.J. Sadler, O.R. Colegio, L.A. Tephly, A.B. Carter, P.B. Rothman, R.A. Flavell, and F.S. Sutterwala. 2008. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* 105:9035-9040.
289. Cruz, C.M., A. Rinna, H.J. Forman, A.L. Ventura, P.M. Persechini, and D.M. Ojcius. 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282:2871-2879.
290. Dostert, C., V. Petrilli, R. Van Bruggen, C. Steele, B.T. Mossman, and J. Tschopp. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320:674-677.
291. Petrilli, V., S. Papin, C. Dostert, A. Mayor, F. Martinon, and J. Tschopp. 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14:1583-1589.

292. Martinon, F., V. Petrilli, A. Mayor, A. Tardivel, and J. Tschopp. 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440:237-241.
293. Mariathasan, S., D.S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W.P. Lee, Y. Weinrauch, D.M. Monack, and V.M. Dixit. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.
294. Sutterwala, F.S., Y. Ogura, M. Szczepanik, M. Lara-Tejero, G.S. Lichtenberger, E.P. Grant, J. Bertin, A.J. Coyle, J.E. Galan, P.W. Askenase, and R.A. Flavell. 2006. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 24:317-327.
295. Kanneganti, T.D., N. Ozoren, M. Body-Malapel, A. Amer, J.H. Park, L. Franchi, J. Whitfield, W. Barchet, M. Colonna, P. Vandenabeele, J. Bertin, A. Coyle, E.P. Grant, S. Akira, and G. Nunez. 2006. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440:233-236.
296. Kanneganti, T.D., M. Lamkanfi, Y.G. Kim, G. Chen, J.H. Park, L. Franchi, P. Vandenabeele, and G. Nunez. 2007. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 26:433-443.
297. Kanneganti, T.D., M. Lamkanfi, and G. Nunez. 2007. Intracellular NOD-like receptors in host defense and disease. *Immunity* 27:549-559.
298. Piccini, A., S. Carta, S. Tassi, D. Lasiglie, G. Fossati, and A. Rubartelli. 2008. ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. *Proc Natl Acad Sci U S A* 105:8067-8072.
299. Halle, A., V. Hornung, G.C. Petzold, C.R. Stewart, B.G. Monks, T. Reinheckel, K.A. Fitzgerald, E. Latz, K.J. Moore, and D.T. Golenbock. 2008. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9:857-865.
300. Hornung, V., F. Bauernfeind, A. Halle, E.O. Samstad, H. Kono, K.L. Rock, K.A. Fitzgerald, and E. Latz. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9:847-856.
301. Amer, A., L. Franchi, T.D. Kanneganti, M. Body-Malapel, N. Ozoren, G. Brady, S. Meshinchi, R. Jagirdar, A. Gewirtz, S. Akira, and G. Nunez. 2006. Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. *J Biol Chem* 281:35217-35223.
302. Sutterwala, F.S., L.A. Mijares, L. Li, Y. Ogura, B.I. Kazmierczak, and R.A. Flavell. 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. *J Exp Med* 204:3235-3245.
303. Suzuki, T., L. Franchi, C. Toma, H. Ashida, M. Ogawa, Y. Yoshikawa, H. Mimuro, N. Inohara, C. Sasakawa, and G. Nunez. 2007. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. *PLoS Pathog* 3:e111.
304. Boyden, E.D., and W.F. Dietrich. 2006. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 38:240-244.

305. Pan, Q., J. Mathison, C. Fearns, V.V. Kravchenko, J. Da Silva Correia, H.M. Hoffman, K.S. Kobayashi, J. Bertin, E.P. Grant, A.J. Coyle, F.S. Sutterwala, Y. Ogura, R.A. Flavell, and R.J. Ulevitch. 2007. MDP-induced interleukin-1beta processing requires Nod2 and CIAS1/NALP3. *J Leukoc Biol* 82:177-183.
306. Hsu, L.C., S.R. Ali, S. McGillivray, P.H. Tseng, S. Mariathasan, E.W. Humke, L. Eckmann, J.J. Powell, V. Nizet, V.M. Dixit, and M. Karin. 2008. A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide. *Proc Natl Acad Sci U S A* 105:7803-7808.
307. Cookson, B.T., and M.A. Brennan. 2001. Pro-inflammatory programmed cell death. *Trends Microbiol* 9:113-114.
308. Bergsbaken, T., S.L. Fink, and B.T. Cookson. 2009. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* 7:99-109.
309. Fink, S.L., and B.T. Cookson. 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell Microbiol* 8:1812-1825.
310. Molofsky, A.B., B.G. Byrne, N.N. Whitfield, C.A. Madigan, E.T. Fuse, K. Tateda, and M.S. Swanson. 2006. Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection. *J Exp Med* 203:1093-1104.
311. Cervantes, J., T. Nagata, M. Uchijima, K. Shibata, and Y. Koide. 2008. Intracytosolic Listeria monocytogenes induces cell death through caspase-1 activation in murine macrophages. *Cell Microbiol* 10:41-52.
312. Fink, S.L., and B.T. Cookson. 2007. Pyroptosis and host cell death responses during Salmonella infection. *Cell Microbiol* 9:2562-2570.
313. Miao, E.A., I.A. Leaf, P.M. Treuting, D.P. Mao, M. Dors, A. Sarkar, S.E. Warren, M.D. Wewers, and A. Aderem. 2010. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol* 11:1136-1142.
314. Gurcel, L., L. Abrami, S. Girardin, J. Tschopp, and F.G. van der Goot. 2006. Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival. *Cell* 126:1135-1145.
315. Agostini, L., F. Martinon, K. Burns, M.F. McDermott, P.N. Hawkins, and J. Tschopp. 2004. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20:319-325.
316. Brydges, S.D., J.L. Mueller, M.D. McGeough, C.A. Pena, A. Misaghi, C. Gandhi, C.D. Putnam, D.L. Boyle, G.S. Firestein, A.A. Horner, P. Soroosh, W.T. Watford, J.J. O'Shea, D.L. Kastner, and H.M. Hoffman. 2009. Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. *Immunity* 30:875-887.
317. Meng, G., F. Zhang, I. Fuss, A. Kitani, and W. Strober. 2009. A mutation in the Nlrp3 gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses. *Immunity* 30:860-874.
318. Aksentijevich, I., S.L. Masters, P.J. Ferguson, P. Dancey, J. Frenkel, A. van Royen-Kerkhoff, R. Laxer, U. Tedgard, E.W. Cowen, T.H. Pham, M. Booty, J.D. Estes, N.G. Sandler, N. Plass, D.L. Stone, M.L. Turner, S. Hill, J.A. Butman, R.

Schneider, P. Babyn, H.I. El-Shanti, E. Pope, K. Barron, X. Bing, A. Laurence, C.C. Lee, D. Chapelle, G.I. Clarke, K. Ohson, M. Nicholson, M. Gadina, B. Yang, B.D. Korman, P.K. Gregersen, P.M. van Hagen, A.E. Hak, M. Huizing, P. Rahman, D.C. Douek, E.F. Remmers, D.L. Kastner, and R. Goldbach-Mansky. 2009. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. *N Engl J Med* 360:2426-2437.

319. Reddy, S., S. Jia, R. Geoffrey, R. Lorier, M. Suchi, U. Broeckel, M.J. Hessner, and J. Verbsky. 2009. An autoinflammatory disease due to homozygous deletion of the IL1RN locus. *N Engl J Med* 360:2438-2444.
320. Villani, A.-C., M. Lemire, G. Fortin, E. Louis, M.S. Silverberg, C. Collette, N. Baba, C. Libioulle, J. Belaiche, A. Bitton, D. Gaudet, A. Cohen, D. Langelier, P.R. Fortin, J.E. Wither, M. Sarfati, P. Rutgeerts, J.D. Rioux, S. Vermeire, T.J. Hudson, and D. Franchimont. 2009. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nat Genet* 41:71-76.
321. Geboes, K., J.F. Colombel, A. Greenstein, D.P. Jewell, W.J. Sandborn, M.H. Vatn, B. Warren, and R.H. Riddell. 2008. Indeterminate colitis: a review of the concept--what's in a name? *Inflamm Bowel Dis* 14:850-857.
322. Baumgart, D.C., and W.J. Sandborn. 2007. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* 369:1641-1657.
323. Loftus, E.V., Jr. 2004. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* 126:1504-1517.
324. Atreya, R., and M.F. Neurath. 2008. New therapeutic strategies for treatment of inflammatory bowel disease. *Mucosal Immunol* 1:175-182.
325. Spehlmann, M.E., A.Z. Begun, J. Burghardt, P. Lepage, A. Raedler, and S. Schreiber. 2008. Epidemiology of inflammatory bowel disease in a German twin cohort: results of a nationwide study. *Inflamm Bowel Dis* 14:968-976.
326. 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447:661-678.
327. Duerr, R.H., K.D. Taylor, S.R. Brant, J.D. Rioux, M.S. Silverberg, M.J. Daly, A.H. Steinhart, C. Abraham, M. Regueiro, A. Griffiths, T. Dassopoulos, A. Bitton, H. Yang, S. Targan, L.W. Datta, E.O. Kistner, L.P. Schumm, A.T. Lee, P.K. Gregersen, M.M. Barmada, J.I. Rotter, D.L. Nicolae, and J.H. Cho. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314:1461-1463.
328. Hampe, J., A. Franke, P. Rosenstiel, A. Till, M. Teuber, K. Huse, M. Albrecht, G. Mayr, F.M. De La Vega, J. Briggs, S. Gunther, N.J. Prescott, C.M. Onnie, R. Hasler, B. Sipos, U.R. Folsch, T. Lengauer, M. Platzer, C.G. Mathew, M. Krawczak, and S. Schreiber. 2007. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 39:207-211.
329. Rioux, J.D., R.J. Xavier, K.D. Taylor, M.S. Silverberg, P. Goyette, A. Huett, T. Green, P. Kuballa, M.M. Barmada, L.W. Datta, Y.Y. Shugart, A.M. Griffiths, S.R. Targan, A.F. Ippoliti, E.J. Bernard, L. Mei, D.L. Nicolae, M. Regueiro, L.P. Schumm, A.H. Steinhart, J.I. Rotter, R.H. Duerr, J.H. Cho, M.J. Daly, and S.R. Brant. 2007. Genome-wide association study identifies new susceptibility loci

for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 39:596-604.

330. Franke, A., T. Balschun, C. Sina, D. Ellinghaus, R. Hasler, G. Mayr, M. Albrecht, M. Wittig, E. Buchert, S. Nikolaus, C. Gieger, H.E. Wichmann, J. Sventoraityte, L. Kupcinskas, C.M. Onnie, M. Gazouli, N.P. Anagnou, D. Strachan, W.L. McArdle, C.G. Mathew, P. Rutgeerts, S. Vermeire, M.H. Vatn, M. Krawczak, P. Rosenstiel, T.H. Karlsen, and S. Schreiber. 2010. Genome-wide association study for ulcerative colitis identifies risk loci at 7q22 and 22q13 (IL17REL). *Nat Genet* 42:292-294.
331. Franke, A., D.P. McGovern, J.C. Barrett, K. Wang, G.L. Radford-Smith, T. Ahmad, C.W. Lees, T. Balschun, J. Lee, R. Roberts, C.A. Anderson, J.C. Bis, S. Bumpstead, D. Ellinghaus, E.M. Festen, M. Georges, T. Green, T. Haritunians, L. Jostins, A. Latiano, C.G. Mathew, G.W. Montgomery, N.J. Prescott, S. Raychaudhuri, J.I. Rotter, P. Schumm, Y. Sharma, L.A. Simms, K.D. Taylor, D. Whiteman, C. Wijmenga, R.N. Baldassano, M. Barclay, T.M. Bayless, S. Brand, C. Buning, A. Cohen, J.F. Colombel, M. Cottone, L. Stronati, T. Denson, M. De Vos, R. D'Inca, M. Dubinsky, C. Edwards, T. Florin, D. Franchimont, R. Gearry, J. Glas, A. Van Gossum, S.L. Guthery, J. Halfvarson, H.W. Verspaget, J.P. Hugot, A. Karban, D. Laukens, I. Lawrance, M. Lemann, A. Levine, C. Libioulle, E. Louis, C. Mowat, W. Newman, J. Panes, A. Phillips, D.D. Proctor, M. Regueiro, R. Russell, P. Rutgeerts, J. Sanderson, M. Sans, F. Seibold, A.H. Steinhart, P.C. Stokkers, L. Torkvist, G. Kullak-Ublick, D. Wilson, T. Walters, S.R. Targan, S.R. Brant, J.D. Rioux, M. D'Amato, R.K. Weersma, S. Kugathasan, A.M. Griffiths, J.C. Mansfield, S. Vermeire, R.H. Duerr, M.S. Silverberg, J. Satsangi, S. Schreiber, J.H. Cho, V. Annese, H. Hakonarson, M.J. Daly, and M. Parkes. 2010. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 42:1118-1125.
332. Barrett, J.C., S. Hansoul, D.L. Nicolae, J.H. Cho, R.H. Duerr, J.D. Rioux, S.R. Brant, M.S. Silverberg, K.D. Taylor, M.M. Barmada, A. Bitton, T. Dassopoulos, L.W. Datta, T. Green, A.M. Griffiths, E.O. Kistner, M.T. Murtha, M.D. Regueiro, J.I. Rotter, L.P. Schumm, A.H. Steinhart, S.R. Targan, R.J. Xavier, C. Libioulle, C. Sandor, M. Lathrop, J. Belaiche, O. Dewit, I. Gut, S. Heath, D. Laukens, M. Mni, P. Rutgeerts, A. Van Gossum, D. Zelenika, D. Franchimont, J.P. Hugot, M. de Vos, S. Vermeire, E. Louis, L.R. Cardon, C.A. Anderson, H. Drummond, E. Nimmo, T. Ahmad, N.J. Prescott, C.M. Onnie, S.A. Fisher, J. Marchini, J. Ghori, S. Bumpstead, R. Gwilliam, M. Tremelling, P. Deloukas, J. Mansfield, D. Jewell, J. Satsangi, C.G. Mathew, M. Parkes, M. Georges, and M.J. Daly. 2008. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 40:955-962.
333. Calkins, B.M. 1989. A meta-analysis of the role of smoking in inflammatory bowel disease. *Dig Dis Sci* 34:1841-1854.
334. Sartor, R.B. 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 134:577-594.
335. D'Haens, G.R., K. Geboes, M. Peeters, F. Baert, F. Penninckx, and P. Rutgeerts. 1998. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. *Gastroenterology* 114:262-267.

336. Sartor, R.B. 2004. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* 126:1620-1633.
337. Mow, W.S., C.J. Landers, A.H. Steinhart, B.G. Feagan, K. Croitoru, E. Seidman, G.R. Greenberg, and S.R. Targan. 2004. High-level serum antibodies to bacterial antigens are associated with antibiotic-induced clinical remission in Crohn's disease: a pilot study. *Dig Dis Sci* 49:1280-1286.
338. Duchmann, R., I. Kaiser, E. Hermann, W. Mayet, K. Ewe, and K.H. Meyer zum Buschenfelde. 1995. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol* 102:448-455.
339. Targan, S.R., C.J. Landers, H. Yang, M.J. Lodes, Y. Cong, K.A. Papadakis, E. Vasiliauskas, C.O. Elson, and R.M. Hershberg. 2005. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology* 128:2020-2028.
340. Dubinsky, M.C., Y.C. Lin, D. Dutridge, Y. Picornell, C.J. Landers, S. Farrior, I. Wrobel, A. Quiros, E.A. Vasiliauskas, B. Grill, D. Israel, R. Bahar, D. Christie, G. Wahbeh, G. Silber, S. Dallazadeh, P. Shah, D. Thomas, D. Kelts, R.M. Hershberg, C.O. Elson, S.R. Targan, K.D. Taylor, J.I. Rotter, and H. Yang. 2006. Serum immune responses predict rapid disease progression among children with Crohn's disease: immune responses predict disease progression. *Am J Gastroenterol* 101:360-367.
341. Rath, H.C., H.H. Herfarth, J.S. Ikeda, W.B. Grenther, T.E. Hamm, Jr., E. Balish, J.D. Taurog, R.E. Hammer, K.H. Wilson, and R.B. Sartor. 1996. Normal luminal bacteria, especially Bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. *J Clin Invest* 98:945-953.
342. Sellon, R.K., S. Tonkonogy, M. Schultz, L.A. Dieleman, W. Grenther, E. Balish, D.M. Rennick, and R.B. Sartor. 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 66:5224-5231.
343. Whary, M.T., N.S. Taylor, Y. Feng, Z. Ge, S. Muthupalani, J. Versalovic, and J.G. Fox. 2011. Lactobacillus reuteri promotes Helicobacter hepaticus-associated typhlocolitis in gnotobiotic B6.129P2-IL-10(tm1Cgn) (IL-10(-/-)) mice. *Immunology* 133:165-178.
344. Sartor, R.B. 2005. Does Mycobacterium avium subspecies paratuberculosis cause Crohn's disease? *Gut* 54:896-898.
345. Manichanh, C., L. Rigottier-Gois, E. Bonnaud, K. Gloux, E. Pelletier, L. Frangeul, R. Nalin, C. Jarrin, P. Chardon, P. Marteau, J. Roca, and J. Dore. 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 55:205-211.
346. Seksik, P., L. Rigottier-Gois, G. Gramet, M. Sutren, P. Pochart, P. Marteau, R. Jian, and J. Dore. 2003. Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. *Gut* 52:237-242.
347. Garrett, W.S., G.M. Lord, S. Punit, G. Lugo-Villarino, S.K. Mazmanian, S. Ito, J.N. Glickman, and L.H. Glimcher. 2007. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell* 131:33-45.

348. Garrett, W.S., C.A. Gallini, T. Yatsunenko, M. Michaud, A. DuBois, M.L. Delaney, S. Punit, M. Karlsson, L. Bry, J.N. Glickman, J.I. Gordon, A.B. Onderdonk, and L.H. Glimcher. 2010. Enterobacteriaceae act in concert with the gut microbiota to induce spontaneous and maternally transmitted colitis. *Cell Host Microbe* 8:292-300.
349. Cong, Y., C.T. Weaver, A. Lazenby, and C.O. Elson. 2000. Colitis induced by enteric bacterial antigen-specific CD4+ T cells requires CD40-CD40 ligand interactions for a sustained increase in mucosal IL-12. *J Immunol* 165:2173-2182.
350. Lodes, M.J., Y. Cong, C.O. Elson, R. Mohamath, C.J. Landers, S.R. Targan, M. Fort, and R.M. Hershberg. 2004. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 113:1296-1306.
351. Kullberg, M.C., J.F. Andersen, P.L. Gorelick, P. Caspar, S. Suerbaum, J.G. Fox, A.W. Cheever, D. Jankovic, and A. Sher. 2003. Induction of colitis by a CD4+ T cell clone specific for a bacterial epitope. *Proc Natl Acad Sci U S A* 100:15830-15835.
352. Fox, J.G., Z. Ge, M.T. Whary, S.E. Erdman, and B.H. Horwitz. 2010. Helicobacter hepaticus infection in mice: models for understanding lower bowel inflammation and cancer. *Mucosal Immunol* 4:22-30.
353. Van der Sluis, M., B.A. De Koning, A.C. De Bruijn, A. Velcich, J.P. Meijerink, J.B. Van Goudoever, H.A. Buller, J. Dekker, I. Van Seuningen, I.B. Renes, and A.W. Einerhand. 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* 131:117-129.
354. Barnich, N., and A. Darfeuille-Michaud. 2007. Adherent-invasive Escherichia coli and Crohn's disease. *Curr Opin Gastroenterol* 23:16-20.
355. Darfeuille-Michaud, A., J. Boudeau, P. Bulois, C. Neut, A.L. Glasser, N. Barnich, M.A. Bringer, A. Swidsinski, L. Beaugerie, and J.F. Colombel. 2004. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* 127:412-421.
356. Baumgart, M., B. Dogan, M. Rishniw, G. Weitzman, B. Bosworth, R. Yantiss, R.H. Orsi, M. Wiedmann, P. McDonough, S.G. Kim, D. Berg, Y. Schukken, E. Scherl, and K.W. Simpson. 2007. Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. *ISME J* 1:403-418.
357. Neut, C., P. Bulois, P. Desreumaux, J.M. Membre, E. Lederman, L. Gambiez, A. Cortot, P. Quandalle, H. van Kruiningen, and J.F. Colombel. 2002. Changes in the bacterial flora of the neoterminal ileum after ileocolonic resection for Crohn's disease. *Am J Gastroenterol* 97:939-946.
358. Barnich, N., F.A. Carvalho, A.L. Glasser, C. Darcha, P. Jantscheff, M. Allez, H. Peeters, G. Bommelaer, P. Desreumaux, J.F. Colombel, and A. Darfeuille-Michaud. 2007. CEACAM6 acts as a receptor for adherent-invasive E. coli, supporting ileal mucosa colonization in Crohn disease. *J Clin Invest* 117:1566-1574.
359. Carvalho, F.A., N. Barnich, A. Sivignon, C. Darcha, C.H. Chan, C.P. Stanners, and A. Darfeuille-Michaud. 2009. Crohn's disease adherent-invasive

Escherichia coli colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. *J Exp Med* 206:2179-2189.

360. Martin, H.M., B.J. Campbell, C.A. Hart, C. Mpofu, M. Nayar, R. Singh, H. Englyst, H.F. Williams, and J.M. Rhodes. 2004. Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer. *Gastroenterology* 127:80-93.
361. Ivanov, II, K. Atarashi, N. Manel, E.L. Brodie, T. Shima, U. Karaoz, D. Wei, K.C. Goldfarb, C.A. Santee, S.V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda, and D.R. Littman. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485-498.
362. Ivanov, II, L. Frutos Rde, N. Manel, K. Yoshinaga, D.B. Rifkin, R.B. Sartor, B.B. Finlay, and D.R. Littman. 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4:337-349.
363. Gaboriau-Routhiau, V., S. Rakotobe, E. Lecuyer, I. Mulder, A. Lan, C. Bridonneau, V. Rochet, A. Pisi, M. De Paepe, G. Brandi, G. Eberl, J. Snel, D. Kelly, and N. Cerf-Bensussan. 2009. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 31:677-689.
364. Round, J.L., and S.K. Mazmanian. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A* 107:12204-12209.
365. Atarashi, K., T. Tanoue, T. Shima, A. Imaoka, T. Kuwahara, Y. Momose, G. Cheng, S. Yamasaki, T. Saito, Y. Ohba, T. Taniguchi, K. Takeda, S. Hori, Ivanov, II, Y. Umesaki, K. Itoh, and K. Honda. 2010. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 331:337-341.
366. Shames, B., J.G. Fox, F. Dewhirst, L. Yan, Z. Shen, and N.S. Taylor. 1995. Identification of widespread Helicobacter hepaticus infection in feces in commercial mouse colonies by culture and PCR assay. *J Clin Microbiol* 33:2968-2972.
367. Kullberg, M.C., J.M. Ward, P.L. Gorelick, P. Caspar, S. Hieny, A. Cheever, D. Jankovic, and A. Sher. 1998. Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. *Infect Immun* 66:5157-5166.
368. Burich, A., R. Hershberg, K. Waggie, W. Zeng, T. Brabb, G. Westrich, J.L. Viney, and L. Maggio-Price. 2001. Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 281:G764-778.
369. Maloy, K.J., L. Salaun, R. Cahill, G. Dougan, N.J. Saunders, and F. Powrie. 2003. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 197:111-119.
370. Erdman, S.E., T. Pouthadis, A.B. Rogers, M. Tomszaki, B.H. Horwitz, and J.G. Fox. 2003. Susceptibility of three strains of Rag2-deficient mice to *Helicobacter hepaticus* induced colitis and colorectal cancer. *Gastroenterology* 124 (Suppl.1):A340.
371. Erdman, S., J.G. Fox, C.A. Dangler, D. Feldman, and B.H. Horwitz. 2001. Typhlocolitis in NF-kappa B-deficient mice. *J Immunol* 166:1443-1447.

372. Maggio-Price, L., P. Treuting, W. Zeng, M. Tsang, H. Bielefeldt-Ohmann, and B.M. Iritani. 2006. Helicobacter infection is required for inflammation and colon cancer in SMAD3-deficient mice. *Cancer Res* 66:828-838.
373. Fox, J.G., F.E. Dewhirst, J.G. Tully, B.J. Paster, L. Yan, N.S. Taylor, M.J. Collins, Jr., P.L. Gorelick, and J.M. Ward. 1994. Helicobacter hepaticus sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J Clin Microbiol* 32:1238-1245.
374. Young, V.B., K.A. Knox, J.S. Pratt, J.S. Cortez, L.S. Mansfield, A.B. Rogers, J.G. Fox, and D.B. Schauer. 2004. In vitro and in vivo characterization of Helicobacter hepaticus cytolethal distending toxin mutants. *Infect Immun* 72:2521-2527.
375. Fox, J.G., L. Yan, B. Shames, J. Campbell, J.C. Murphy, and X. Li. 1996. Persistent hepatitis and enterocolitis in germfree mice infected with Helicobacter hepaticus. *Infect Immun* 64:3673-3681.
376. Sterzenbach, T., L. Bartonickova, W. Behrens, B. Brenneke, J. Schulze, F. Kops, E.Y. Chin, E. Katzowitsch, D.B. Schauer, J.G. Fox, S. Suerbaum, and C. Josenhans. 2008. Role of the Helicobacter hepaticus flagellar sigma factor FliA in gene regulation and murine colonization. *J Bacteriol* 190:6398-6408.
377. Pratt, J.S., K.L. Sachen, H.D. Wood, K.A. Eaton, and V.B. Young. 2006. Modulation of host immune responses by the cytolethal distending toxin of Helicobacter hepaticus. *Infect Immun* 74:4496-4504.
378. Mehta, N.S., S. Benoit, J.V. Mysore, R.S. Sousa, and R.J. Maier. 2005. Helicobacter hepaticus hydrogenase mutants are deficient in hydrogen-supported amino acid uptake and in causing liver lesions in A/J mice. *Infect Immun* 73:5311-5318.
379. Ge, Z., A. Lee, M.T. Whary, A.B. Rogers, K.J. Maurer, N.S. Taylor, D.B. Schauer, and J.G. Fox. 2008. Helicobacter hepaticus urease is not required for intestinal colonization but promotes hepatic inflammation in male A/JCr mice. *Microb Pathog* 45:18-24.
380. Krishnan, N., A.R. Doster, G.E. Duhamel, and D.F. Becker. 2008. Characterization of a Helicobacter hepaticus putA mutant strain in host colonization and oxidative stress. *Infect Immun* 76:3037-3044.
381. Suerbaum, S., C. Josenhans, T. Sterzenbach, B. Drescher, P. Brandt, M. Bell, M. Droge, B. Fartmann, H.P. Fischer, Z. Ge, A. Horster, R. Holland, K. Klein, J. Konig, L. Macko, G.L. Mendz, G. Nyakatura, D.B. Schauer, Z. Shen, J. Weber, M. Frosch, and J.G. Fox. 2003. The complete genome sequence of the carcinogenic bacterium Helicobacter hepaticus. *Proc Natl Acad Sci U S A* 100:7901-7906.
382. Andersen-Nissen, E., K.D. Smith, K.L. Strobe, S.L. Barrett, B.T. Cookson, S.M. Logan, and A. Aderem. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci U S A* 102:9247-9252.
383. Sterzenbach, T., S.K. Lee, B. Brenneke, F. von Goetz, D.B. Schauer, J.G. Fox, S. Suerbaum, and C. Josenhans. 2007. Inhibitory effect of enterohepatic Helicobacter hepaticus on innate immune responses of mouse intestinal epithelial cells. *Infect Immun* 75:2717-2728.
384. Chow, J., and S.K. Mazmanian. 2010. A pathobiont of the microbiota balances host colonization and intestinal inflammation. *Cell Host Microbe* 7:265-276.

385. Fukata, M., and M.T. Abreu. 2009. Pathogen recognition receptors, cancer and inflammation in the gut. *Curr Opin Pharmacol* 9:680-687.
386. Abreu, M.T. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* 10:131-144.
387. Cario, E., and D.K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* 68:7010-7017.
388. Fusunyan, R.D., N.N. Nanthakumar, M.E. Baldeon, and W.A. Walker. 2001. Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes. *Pediatr Res* 49:589-593.
389. Gewirtz, A.T., T.A. Navas, S. Lyons, P.J. Godowski, and J.L. Madara. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 167:1882-1885.
390. Rhee, S.H., E. Im, M. Riegler, E. Kokkotou, M. O'Brien, and C. Pothoulakis. 2005. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc Natl Acad Sci U S A* 102:13610-13615.
391. Bambou, J.C., A. Giraud, S. Menard, B. Begue, S. Rakotobe, M. Heyman, F. Taddei, N. Cerf-Bensussan, and V. Gaboriau-Routhiau. 2004. In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal Escherichia coli strain. *J Biol Chem* 279:42984-42992.
392. Melmed, G., L.S. Thomas, N. Lee, S.Y. Tesfay, K. Lukasek, K.S. Michelsen, Y. Zhou, B. Hu, M. Arditi, and M.T. Abreu. 2003. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J Immunol* 170:1406-1415.
393. Garlanda, C., F. Riva, N. Polentarutti, C. Buracchi, M. Sironi, M. De Bortoli, M. Muzio, R. Bergottini, E. Scanziani, A. Vecchi, E. Hirsch, and A. Mantovani. 2004. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. *Proc Natl Acad Sci U S A* 101:3522-3526.
394. Dubuquoy, L., E.A. Jansson, S. Deeb, S. Rakotobe, M. Karoui, J.F. Colombel, J. Auwerx, S. Pettersson, and P. Desreumaux. 2003. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. *Gastroenterology* 124:1265-1276.
395. Turer, E.E., R.M. Tavares, E. Mortier, O. Hitotsumatsu, R. Advincula, B. Lee, N. Shifrin, B.A. Malynn, and A. Ma. 2008. Homeostatic MyD88-dependent signals cause lethal inflamMation in the absence of A20. *J Exp Med* 205:451-464.
396. O'Neill, L.A. 2008. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity* 29:12-20.
397. Fukata, M., K.S. Michelsen, R. Eri, L.S. Thomas, B. Hu, K. Lukasek, C.C. Nast, J. Lechago, R. Xu, Y. Naiki, A. Soliman, M. Arditi, and M.T. Abreu. 2005. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am J Physiol Gastrointest Liver Physiol* 288:G1055-1065.

398. Araki, A., T. Kanai, T. Ishikura, S. Makita, K. Uraushihara, R. Iiyama, T. Totsuka, K. Takeda, S. Akira, and M. Watanabe. 2005. MyD88-deficient mice develop severe intestinal inflammation in dextran sodium sulfate colitis. *J Gastroenterol* 40:16-23.
399. Nenci, A., C. Becker, A. Wullaert, R. Gareus, G. van Loo, S. Danese, M. Huth, A. Nikolaev, C. Neufert, B. Madison, D. Gumucio, M.F. Neurath, and M. Pasparakis. 2007. Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 446:557-561.
400. Gibson, D.L., C. Ma, C.M. Rosenberger, K.S. Bergstrom, Y. Valdez, J.T. Huang, M.A. Khan, and B.A. Vallance. 2008. Toll-like receptor 2 plays a critical role in maintaining mucosal integrity during Citrobacter rodentium-induced colitis. *Cell Microbiol* 10:388-403.
401. Ayabe, T., D.P. Satchell, C.L. Wilson, W.C. Parks, M.E. Selsted, and A.J. Ouellette. 2000. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. *Nat Immunol* 1:113-118.
402. Maloy, K.J., and F. Powrie. 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 474:298-306.
403. Rakoff-Nahoum, S., and R. Medzhitov. 2006. Role of the innate immune system and host-commensal mutualism. *Curr Top Microbiol Immunol* 308:1-18.
404. Kobayashi, M., M.N. Kweon, H. Kuwata, R.D. Schreiber, H. Kiyono, K. Takeda, and S. Akira. 2003. Toll-like receptor-dependent production of IL-12p40 causes chronic enterocolitis in myeloid cell-specific Stat3-deficient mice. *J Clin Invest* 111:1297-1308.
405. Eckmann, L., T. Nebelsiek, A.A. Fingerle, S.M. Dann, J. Mages, R. Lang, S. Robine, M.F. Kagnoff, R.M. Schmid, M. Karin, M.C. Arkan, and F.R. Greten. 2008. Opposing functions of IKKbeta during acute and chronic intestinal inflammation. *Proc Natl Acad Sci U S A* 105:15058-15063.
406. Economou, M., T.A. Trikalinos, K.T. Loizou, E.V. Tsianos, and J.P. Ioannidis. 2004. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. *Am J Gastroenterol* 99:2393-2404.
407. van Heel, D.A., S. Ghosh, M. Butler, K.A. Hunt, A.M. Lundberg, T. Ahmad, D.P. McGovern, C. Onnie, K. Negoro, S. Goldthorpe, B.M. Foxwell, C.G. Mathew, A. Forbes, D.P. Jewell, and R.J. Playford. 2005. Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease. *Lancet* 365:1794-1796.
408. Voss, E., J. Wehkamp, K. Wehkamp, E.F. Stange, J.M. Schroder, and J. Harder. 2006. NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. *J Biol Chem* 281:2005-2011.
409. Wehkamp, J., N.H. Salzman, E. Porter, S. Nuding, M. Weichenthal, R.E. Petras, B. Shen, E. Schaeffeler, M. Schwab, R. Linzmeier, R.W. Feathers, H. Chu, H. Lima, Jr., K. Fellermann, T. Ganz, E.F. Stange, and C.L. Bevins. 2005. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A* 102:18129-18134.
410. Simms, L.A., J.D. Doecke, M.D. Walsh, N. Huang, E.V. Fowler, and G.L. Radford-Smith. 2008. Reduced alpha-defensin expression is associated with

inflammation and not NOD2 mutation status in ileal Crohn's disease. *Gut* 57:903-910.

411. Kobayashi, K.S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R.A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307:731-734.
412. Watanabe, T., A. Kitani, P.J. Murray, and W. Strober. 2004. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* 5:800-808.
413. Hedl, M., J. Li, J.H. Cho, and C. Abraham. 2007. Chronic stimulation of Nod2 mediates tolerance to bacterial products. *Proc Natl Acad Sci U S A* 104:19440-19445.
414. Kim, Y.G., J.H. Park, M.H. Shaw, L. Franchi, N. Inohara, and G. Nunez. 2008. The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. *Immunity* 28:246-257.
415. Watanabe, T., A. Kitani, P.J. Murray, Y. Wakatsuki, I.J. Fuss, and W. Strober. 2006. Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis. *Immunity* 25:473-485.
416. Watanabe, T., N. Asano, P.J. Murray, K. Ozato, P. Tailor, I.J. Fuss, A. Kitani, and W. Strober. 2008. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. *J Clin Invest* 118:545-559.
417. Maeda, S., L.C. Hsu, H. Liu, L.A. Bankston, M. Iimura, M.F. Kagnoff, L. Eckmann, and M. Karin. 2005. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 307:734-738.
418. Noguchi, E., Y. Homma, X. Kang, M.G. Netea, and X. Ma. 2009. A Crohn's disease-associated NOD2 mutation suppresses transcription of human IL10 by inhibiting activity of the nuclear ribonucleoprotein hnRNP-A1. *Nat Immunol* 10:471-479.
419. Barreau, F., U. Meinzer, F. Chareyre, D. Berrebi, M. Niwa-Kawakita, M. Dussaillant, B. Foligne, V. Ollendorff, M. Heyman, S. Bonacorsi, T. Lesuffleur, G. Sterkers, M. Giovannini, and J.P. Hugot. 2007. CARD15/NOD2 is required for Peyer's patches homeostasis in mice. *PLoS ONE* 2:e523.
420. Shaw, M.H., T. Reimer, C. Sanchez-Valdepenas, N. Warner, Y.G. Kim, M. Fresno, and G. Nunez. 2009. T cell-intrinsic role of Nod2 in promoting type 1 immunity to Toxoplasma gondii. *Nat Immunol* 10:1267-1274.
421. Girardin, S.E., R. Tournebize, M. Mavris, A.L. Page, X. Li, G.R. Stark, J. Bertin, P.S. DiStefano, M. Yaniv, P.J. Sansonetti, and D.J. Philpott. 2001. CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO Rep* 2:736-742.
422. Kim, J.G., S.J. Lee, and M.F. Kagnoff. 2004. Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by toll-like receptors. *Infect Immun* 72:1487-1495.
423. Zilbauer, M., N. Dorrell, A. Elmi, K.J. Lindley, S. Schuller, H.E. Jones, N.J. Klein, G. Nunez, B.W. Wren, and M. Bajaj-Elliott. 2007. A major role for intestinal epithelial nucleotide oligomerization domain 1 (NOD1) in eliciting host

bactericidal immune responses to Campylobacter jejuni. *Cell Microbiol* 9:2404-2416.

424. Yamazaki, K., D. McGovern, J. Ragoussis, M. Paolucci, H. Butler, D. Jewell, L. Cardon, M. Takazoe, T. Tanaka, T. Ichimori, S. Saito, A. Sekine, A. Iida, A. Takahashi, T. Tsunoda, M. Lathrop, and Y. Nakamura. 2005. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. *Hum Mol Genet* 14:3499-3506.
425. Yang, S.K., J. Lim, H.S. Chang, I. Lee, Y. Li, J. Liu, and K. Song. 2008. Association of TNFSF15 with Crohn's disease in Koreans. *Am J Gastroenterol* 103:1437-1442.
426. Kontoyiannis, D., M. Pasparakis, T.T. Pizarro, F. Cominelli, and G. Kollias. 1999. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 10:387-398.
427. Armaka, M., M. Apostolaki, P. Jacques, D.L. Kontoyiannis, D. Elewaut, and G. Kollias. 2008. Mesenchymal cell targeting by TNF as a common pathogenic principle in chronic inflammatory joint and intestinal diseases. *J Exp Med* 205:331-337.
428. Melmed, G.Y., and S.R. Targan. 2010. Future biologic targets for IBD: potentials and pitfalls. *Nat Rev Gastroenterol Hepatol* 7:110-117.
429. Gross, V., T. Andus, I. Caesar, M. Roth, and J. Scholmerich. 1992. Evidence for continuous stimulation of interleukin-6 production in Crohn's disease. *Gastroenterology* 102:514-519.
430. Holub, M.C., E. Mako, T. Devay, M. Dank, C. Szalai, A. Fenyvesi, and A. Falus. 1998. Increased interleukin-6 levels, interleukin-6 receptor and gp130 expression in peripheral lymphocytes of patients with inflammatory bowel disease. *Scand J Gastroenterol Suppl* 228:47-50.
431. Hyams, J.S., J.E. Fitzgerald, W.R. Treem, N. Wyzga, and D.L. Kreutzer. 1993. Relationship of functional and antigenic interleukin 6 to disease activity in inflammatory bowel disease. *Gastroenterology* 104:1285-1292.
432. Atreya, R., J. Mudter, S. Finotto, J. Mullberg, T. Jostock, S. Wirtz, M. Schutz, B. Bartsch, M. Holtmann, C. Becker, D. Strand, J. Czaja, J.F. Schlaak, H.A. Lehr, F. Autschbach, G. Schurmann, N. Nishimoto, K. Yoshizaki, H. Ito, T. Kishimoto, P.R. Galle, S. Rose-John, and M.F. Neurath. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med* 6:583-588.
433. Yamamoto, M., K. Yoshizaki, T. Kishimoto, and H. Ito. 2000. IL-6 is required for the development of Th1 cell-mediated murine colitis. *J Immunol* 164:4878-4882.
434. Ito, H., M. Takazoe, Y. Fukuda, T. Hibi, K. Kusugami, A. Andoh, T. Matsumoto, T. Yamamura, J. Azuma, N. Nishimoto, K. Yoshizaki, T. Shimoyama, and T. Kishimoto. 2004. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology* 126:989-996; discussion 947.
435. Fuss, I.J., M. Neurath, M. Boirivant, J.S. Klein, C. de la Motte, S.A. Strong, C. Fiocchi, and W. Strober. 1996. Disparate CD4+ lamina propria (LP)

lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 157:1261-1270.

436. Mannon, P.J., I.J. Fuss, L. Mayer, C.O. Elson, W.J. Sandborn, D. Present, B. Dolin, N. Goodman, C. Groden, R.L. Hornung, M. Quezado, Z. Yang, M.F. Neurath, J. Salfeld, G.M. Veldman, U. Schwertschlag, and W. Strober. 2004. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 351:2069-2079.
437. Sandborn, W.J., B.G. Feagan, R.N. Fedorak, E. Scherl, M.R. Fleisher, S. Katz, J. Johanns, M. Blank, and P. Rutgeerts. 2008. A randomized trial of Ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe Crohn's disease. *Gastroenterology* 135:1130-1141.
438. Kullberg, M.C., D. Jankovic, C.G. Feng, S. Hue, P.L. Gorelick, B.S. McKenzie, D.J. Cua, F. Powrie, A.W. Cheever, K.J. Maloy, and A. Sher. 2006. IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. *J Exp Med* 203:2485-2494.
439. Kullberg, M.C., A.G. Rothfuchs, D. Jankovic, P. Caspar, T.A. Wynn, P.L. Gorelick, A.W. Cheever, and A. Sher. 2001. Helicobacter hepaticus-induced colitis in interleukin-10-deficient mice: cytokine requirements for the induction and maintenance of intestinal inflammation. *Infect Immun* 69:4232-4241.
440. Hommes, D.W., T.L. Mikhajlova, S. Stoinov, D. Stimac, B. Vucelic, J. Lonovics, M. Zakuciova, G. D'Haens, G. Van Assche, S. Ba, S. Lee, and T. Pearce. 2006. Fontolizumab, a humanised anti-interferon gamma antibody, demonstrates safety and clinical activity in patients with moderate to severe Crohn's disease. *Gut* 55:1131-1137.
441. Reinisch, W., W. de Villiers, L. Bene, L. Simon, I. Racz, S. Katz, I. Altorjay, B. Feagan, D. Riff, C.N. Bernstein, D. Hommes, P. Rutgeerts, A. Cortot, M. Gaspari, M. Cheng, T. Pearce, and B.E. Sands. 2010. Fontolizumab in moderate to severe Crohn's disease: a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study. *Inflamm Bowel Dis* 16:233-242.
442. Ahern, P.P., A. Izcue, K.J. Maloy, and F. Powrie. 2008. The interleukin-23 axis in intestinal inflammation. *Immunol Rev* 226:147-159.
443. Oppmann, B., R. Lesley, B. Blom, J.C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J.S. Abrams, K.W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J.F. Bazan, and R.A. Kastelein. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13:715-725.
444. Hue, S., P. Ahern, S. Buonocore, M.C. Kullberg, D.J. Cua, B.S. McKenzie, F. Powrie, and K.J. Maloy. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 203:2473-2483.
445. Uhlig, H.H., B.S. McKenzie, S. Hue, C. Thompson, B. Joyce-Shaikh, R. Stepankova, N. Robinson, S. Buonocore, H. Tlaskalova-Hogenova, D.J. Cua, and F. Powrie. 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 25:309-318.

446. Yen, D., J. Cheung, H. Scheerens, F. Poulet, T. McClanahan, B. McKenzie, M.A. Kleinschek, A. Owyang, J. Mattson, W. Blumenschein, E. Murphy, M. Sathe, D.J. Cua, R.A. Kastelein, and D. Rennick. 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 116:1310-1316.
447. Elson, C.O., Y. Cong, C.T. Weaver, T.R. Schoeb, T.K. McClanahan, R.B. Fick, and R.A. Kastelein. 2007. Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology* 132:2359-2370.
448. McGovern, D., and F. Powrie. 2007. The IL23 axis plays a key role in the pathogenesis of IBD. *Gut* 56:1333-1336.
449. Fuss, I.J., C. Becker, Z. Yang, C. Groden, R.L. Hornung, F. Heller, M.F. Neurath, W. Strober, and P.J. Mannon. 2006. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis* 12:9-15.
450. Nielsen, O.H., I. Kirman, N. Rudiger, J. Hendel, and B. Vainer. 2003. Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. *Scand J Gastroenterol* 38:180-185.
451. Kamada, N., T. Hisamatsu, S. Okamoto, H. Chinen, T. Kobayashi, T. Sato, A. Sakuraba, M.T. Kitazume, A. Sugita, K. Koganei, K.S. Akagawa, and T. Hibi. 2008. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* 118:2269-2280.
452. Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52:65-70.
453. Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Fili, S. Ferri, F. Frosali, F. Giudici, P. Romagnani, P. Parronchi, F. Tonelli, E. Maggi, and S. Romagnani. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med* 204:1849-1861.
454. Arisawa, T., T. Tahara, T. Shibata, M. Nagasaka, M. Nakamura, Y. Kamiya, H. Fujita, D. Yoshioka, Y. Arima, M. Okubo, I. Hirata, and H. Nakano. 2008. The influence of polymorphisms of interleukin-17A and interleukin-17F genes on the susceptibility to ulcerative colitis. *J Clin Immunol* 28:44-49.
455. Zhang, Z., M. Zheng, J. Bindas, P. Schwarzenberger, and J.K. Kolls. 2006. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm Bowel Dis* 12:382-388.
456. Leppkes, M., C. Becker, Ivanov, II, S. Hirth, S. Wirtz, C. Neufert, S. Pouly, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, B. Becher, D.R. Littman, and M.F. Neurath. 2009. RORgamma-expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* 136:257-267.
457. Mudter, J., L. Amoussina, M. Schenk, J. Yu, A. Brustle, B. Weigmann, R. Atreya, S. Wirtz, C. Becker, A. Hoffman, I. Atreya, S. Biesterfeld, P.R. Galle, H.A. Lehr, S. Rose-John, C. Mueller, M. Lohoff, and M.F. Neurath. 2008. The transcription factor IFN regulatory factor-4 controls experimental colitis in mice via T cell-derived IL-6. *J Clin Invest* 118:2415-2426.

458. Ogawa, A., A. Andoh, Y. Araki, T. Bamba, and Y. Fujiyama. 2004. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin Immunol* 110:55-62.
459. Izcue, A., S. Hue, S. Buonocore, C.V. Arancibia-Carcamo, P.P. Ahern, Y. Iwakura, K.J. Maloy, and F. Powrie. 2008. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* 28:559-570.
460. O'Connor, W., Jr., M. Kamanaka, C.J. Booth, T. Town, S. Nakae, Y. Iwakura, J.K. Kolls, and R.A. Flavell. 2009. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat Immunol* 10:603-609.
461. Yang, X.O., S.H. Chang, H. Park, R. Nurieva, B. Shah, L. Acero, Y.H. Wang, K.S. Schluns, R.R. Broaddus, Z. Zhu, and C. Dong. 2008. Regulation of inflammatory responses by IL-17F. *J Exp Med* 205:1063-1075.
462. Pickert, G., C. Neufert, M. Leppkes, Y. Zheng, N. Wittkopf, M. Warntjen, H.A. Lehr, S. Hirth, B. Weigmann, S. Wirtz, W. Ouyang, M.F. Neurath, and C. Becker. 2009. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med* 206:1465-1472.
463. Brand, S., F. Beigel, T. Olszak, K. Zitzmann, S.T. Eichhorst, J.M. Otte, H. Diepolder, A. Marquardt, W. Jagla, A. Popp, S. Leclair, K. Herrmann, J. Seiderer, T. Ochsenkuhn, B. Goke, C.J. Auernhammer, and J. Dambacher. 2006. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 290:G827-838.
464. Andoh, A., Z. Zhang, O. Inatomi, S. Fujino, Y. Deguchi, Y. Araki, T. Tsujikawa, K. Kitoh, S. Kim-Mitsuyama, A. Takayanagi, N. Shimizu, and Y. Fujiyama. 2005. Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. *Gastroenterology* 129:969-984.
465. Wolk, K., E. Witte, U. Hoffmann, W.D. Doecke, S. Endesfelder, K. Asadullah, W. Sterry, H.D. Volk, B.M. Wittig, and R. Sabat. 2007. IL-22 induces lipopolysaccharide-binding protein in hepatocytes: a potential systemic role of IL-22 in Crohn's disease. *J Immunol* 178:5973-5981.
466. Cosmi, L., R. De Palma, V. Santarlasci, L. Maggi, M. Capone, F. Frosali, G. Rodolico, V. Querci, G. Abbate, R. Angeli, L. Berrino, M. Fambrini, M. Caproni, F. Tonelli, E. Lazzeri, P. Parronchi, F. Liotta, E. Maggi, S. Romagnani, and F. Annunziato. 2008. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J Exp Med* 205:1903-1916.
467. Izcue, A., J.L. Coombes, and F. Powrie. 2009. Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol* 27:313-338.
468. Roers, A., L. Siewe, E. Strittmatter, M. Deckert, D. Schluter, W. Stenzel, A.D. Gruber, T. Krieg, K. Rajewsky, and W. Muller. 2004. T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *J Exp Med* 200:1289-1297.
469. Glocker, E.O., D. Kotlarz, K. Boztug, E.M. Gertz, A.A. Schaffer, F. Noyan, M. Perro, J. Diestelhorst, A. Allroth, D. Murugan, N. Hatscher, D. Pfeifer, K.W. Sykora, M. Sauer, H. Kreipe, M. Lacher, R. Nustede, C. Woellner, U. Baumann, U. Salzer, S. Koletzko, N. Shah, A.W. Segal, A. Sauerbrey, S. Buderus, S.B.

Snapper, B. Grimbacher, and C. Klein. 2009. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 361:2033-2045.
470. Franke, A., T. Balschun, T.H. Karlsen, J. Sventoraityte, S. Nikolaus, G. Mayr, F.S. Domingues, M. Albrecht, M. Nothnagel, D. Ellinghaus, C. Sina, C.M. Onnie, R.K. Weersma, P.C. Stokkers, C. Wijmenga, M. Gazouli, D. Strachan, W.L. McArdle, S. Vermeire, P. Rutgeerts, P. Rosenstiel, M. Krawczak, M.H. Vatn, C.G. Mathew, and S. Schreiber. 2008. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet* 40:1319-1323.
471. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 192:295-302.
472. Powrie, F., J. Carlino, M.W. Leach, S. Mauze, and R.L. Coffman. 1996. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* 183:2669-2674.
473. Li, M.O., Y.Y. Wan, and R.A. Flavell. 2007. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 26:579-591.
474. Fahlen, L., S. Read, L. Gorelik, S.D. Hurst, R.L. Coffman, R.A. Flavell, and F. Powrie. 2005. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 201:737-746.
475. Zhu, Y., J.A. Richardson, L.F. Parada, and J.M. Graff. 1998. Smad3 mutant mice develop metastatic colorectal cancer. *Cell* 94:703-714.
476. Yang, X., J.J. Letterio, R.J. Lechleider, L. Chen, R. Hayman, H. Gu, A.B. Roberts, and C. Deng. 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* 18:1280-1291.
477. Gorelik, L., and R.A. Flavell. 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12:171-181.
478. Li, M.O., S. Sanjabi, and R.A. Flavell. 2006. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 25:455-471.
479. Lucas, P.J., S.J. Kim, S.J. Melby, and R.E. Gress. 2000. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 191:1187-1196.
480. Marie, J.C., D. Liggitt, and A.Y. Rudensky. 2006. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity* 25:441-454.
481. Monteleone, G., M. Boirivant, F. Pallone, and T.T. MacDonald. 2008. TGF-beta1 and Smad7 in the regulation of IBD. *Mucosal Immunol* 1 Suppl 1:S50-53.
482. Khalil, N. 1999. TGF-beta: from latent to active. *Microbes Infect* 1:1255-1263.
483. Torchinsky, M.B., J. Garaude, A.P. Martin, and J.M. Blander. 2009. Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature* 458:78-82.

484. Todd, D.J., A.H. Lee, and L.H. Glimcher. 2008. The endoplasmic reticulum stress response in immunity and autoimmunity. *Nat Rev Immunol* 8:663-674.
485. Kaser, A., and R.S. Blumberg. 2010. Endoplasmic reticulum stress and intestinal inflammation. *Mucosal Immunol* 3:11-16.
486. Brandl, K., S. Rutschmann, X. Li, X. Du, N. Xiao, B. Schnabl, D.A. Brenner, and B. Beutler. 2009. Enhanced sensitivity to DSS colitis caused by a hypomorphic Mbtps1 mutation disrupting the ATF6-driven unfolded protein response. *Proc Natl Acad Sci U S A* 106:3300-3305.
487. Bertolotti, A., X. Wang, I. Novoa, R. Jungreis, K. Schlessinger, J.H. Cho, A.B. West, and D. Ron. 2001. Increased sensitivity to dextran sodium sulfate colitis in IRE1beta-deficient mice. *J Clin Invest* 107:585-593.
488. Kaser, A., A.H. Lee, A. Franke, J.N. Glickman, S. Zeissig, H. Tilg, E.E. Nieuwenhuis, D.E. Higgins, S. Schreiber, L.H. Glimcher, and R.S. Blumberg. 2008. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell* 134:743-756.
489. Heazlewood, C.K., M.C. Cook, R. Eri, G.R. Price, S.B. Tauro, D. Taupin, D.J. Thornton, C.W. Png, T.L. Crockford, R.J. Cornall, R. Adams, M. Kato, K.A. Nelms, N.A. Hong, T.H. Florin, C.C. Goodnow, and M.A. McGuckin. 2008. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* 5:e54.
490. Taurog, J.D., J.A. Richardson, J.T. Croft, W.A. Simmons, M. Zhou, J.L. Fernandez-Sueiro, E. Balish, and R.E. Hammer. 1994. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med* 180:2359-2364.
491. Turner, M.J., D.P. Sowders, M.L. DeLay, R. Mohapatra, S. Bai, J.A. Smith, J.R. Brandewie, J.D. Taurog, and R.A. Colbert. 2005. HLA-B27 misfolding in transgenic rats is associated with activation of the unfolded protein response. *J Immunol* 175:2438-2448.
492. Levine, B., and G. Kroemer. 2008. Autophagy in the pathogenesis of disease. *Cell* 132:27-42.
493. Munz, C. 2009. Enhancing immunity through autophagy. *Annu Rev Immunol* 27:423-449.
494. Parkes, M., J.C. Barrett, N.J. Prescott, M. Tremelling, C.A. Anderson, S.A. Fisher, R.G. Roberts, E.R. Nimmo, F.R. Cummings, D. Soars, H. Drummond, C.W. Lees, S.A. Khawaja, R. Bagnall, D.A. Burke, C.E. Todhunter, T. Ahmad, C.M. Onnie, W. McArdle, D. Strachan, G. Bethel, C. Bryan, C.M. Lewis, P. Deloukas, A. Forbes, J. Sanderson, D.P. Jewell, J. Satsangi, J.C. Mansfield, L. Cardon, and C.G. Mathew. 2007. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet* 39:830-832.
495. Kuballa, P., A. Huett, J.D. Rioux, M.J. Daly, and R.J. Xavier. 2008. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS ONE* 3:e3391.
496. Cadwell, K., J.Y. Liu, S.L. Brown, H. Miyoshi, J. Loh, J.K. Lennerz, C. Kishi, W. Kc, J.A. Carrero, S. Hunt, C.D. Stone, E.M. Brunt, R.J. Xavier, B.P. Sleckman, E. Li, N. Mizushima, T.S. Stappenbeck, and H.W.t. Virgin. 2008. A key role for

autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature* 456:259-263.

497. Saitoh, T., N. Fujita, M.H. Jang, S. Uematsu, B.G. Yang, T. Satoh, H. Omori, T. Noda, N. Yamamoto, M. Komatsu, K. Tanaka, T. Kawai, T. Tsujimura, O. Takeuchi, T. Yoshimori, and S. Akira. 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 456:264-268.
498. Singh, S.B., A.S. Davis, G.A. Taylor, and V. Deretic. 2006. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* 313:1438-1441.
499. Collazo, C.M., G.S. Yap, G.D. Sempowski, K.C. Lusby, L. Tessarollo, G.F. Woude, A. Sher, and G.A. Taylor. 2001. Inactivation of LRG-47 and IRG-47 reveals a family of interferon gamma-inducible genes with essential, pathogen-specific roles in resistance to infection. *J Exp Med* 194:181-188.
500. Henry, S.C., X. Daniell, M. Indaram, J.F. Whitesides, G.D. Sempowski, D. Howell, T. Oliver, and G.A. Taylor. 2007. Impaired macrophage function underscores susceptibility to Salmonella in mice lacking Irgm1 (LRG-47). *J Immunol* 179:6963-6972.
501. McCarroll, S.A., A. Huett, P. Kuballa, S.D. Chilewski, A. Landry, P. Goyette, M.C. Zody, J.L. Hall, S.R. Brant, J.H. Cho, R.H. Duerr, M.S. Silverberg, K.D. Taylor, J.D. Rioux, D. Altshuler, M.J. Daly, and R.J. Xavier. 2008. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. *Nat Genet* 40:1107-1112.
502. Uhlig, H.H., and F. Powrie. 2009. Mouse models of intestinal inflammation as tools to understand the pathogenesis of inflammatory bowel disease. *Eur J Immunol* 39:2021-2026.
503. Luperchio, S.A., and D.B. Schauer. 2001. Molecular pathogenesis of Citrobacter rodentium and transmissible murine colonic hyperplasia. *Microbes Infect* 3:333-340.
504. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133:775-787.
505. Leach, M.W., A.G. Bean, S. Mauze, R.L. Coffman, and F. Powrie. 1996. Inflammatory bowel disease in C.B-17 scid mice reconstituted with the CD45RBhigh subset of CD4+ T cells. *Am J Pathol* 148:1503-1515.
506. Lebeis, S.L., K.R. Powell, D. Merlin, M.A. Sherman, and D. Kalman. 2009. Interleukin-1 receptor signaling protects mice from lethal intestinal damage caused by the attaching and effacing pathogen Citrobacter rodentium. *Infect Immun* 77:604-614.
507. Fry, T.J., and C.L. Mackall. 2001. Interleukin-7: master regulator of peripheral T-cell homeostasis? *Trends Immunol* 22:564-571.
508. Powrie, F., S. Mauze, and R.L. Coffman. 1997. CD4+ T-cells in the regulation of inflammatory responses in the intestine. *Res Immunol* 148:576-581.
509. Aranda, R., B.C. Sydora, P.L. McAllister, S.W. Binder, H.Y. Yang, S.R. Targan, and M. Kronenberg. 1997. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. *J Immunol* 158:3464-3473.

510. Cahill, R.J., C.J. Foltz, J.G. Fox, C.A. Dangler, F. Powrie, and D.B. Schauer. 1997. Inflammatory bowel disease: an immunity-mediated condition triggered by bacterial infection with Helicobacter hepaticus. *Infect Immun* 65:3126-3131.
511. Maloy, K.J., L.R. Antonelli, M. Lefevre, and F. Powrie. 2005. Cure of innate intestinal immune pathology by CD4+CD25+ regulatory T cells. *Immunol Lett* 97:189-192.
512. Erdman, S.E., T. Poutahidis, M. Tomczak, A.B. Rogers, K. Cormier, B. Plank, B.H. Horwitz, and J.G. Fox. 2003. CD4+ CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice. *Am J Pathol* 162:691-702.
513. Sims, J.E., and D.E. Smith. 2010. The IL-1 family: regulators of immunity. *Nat Rev Immunol* 10:89-102.
514. Dinarello, C.A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27:519-550.
515. Sims, J.E. 2010. Accessory to inflammation. *Nat Immunol* 11:883-885.
516. Sizemore, N., S. Leung, and G.R. Stark. 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. *Mol Cell Biol* 19:4798-4805.
517. Davis, C.N., E. Mann, M.M. Behrens, S. Gaidarova, M. Rebek, J. Rebek, Jr., and T. Bartfai. 2006. MyD88-dependent and -independent signaling by IL-1 in neurons probed by bifunctional Toll/IL-1 receptor domain/BB-loop mimetics. *Proc Natl Acad Sci U S A* 103:2953-2958.
518. Wang, D., S. Zhang, L. Li, X. Liu, K. Mei, and X. Wang. 2010. Structural insights into the assembly and activation of IL-1beta with its receptors. *Nat Immunol* 11:905-911.
519. Kavita, U., and S.B. Mizel. 1995. Differential sensitivity of interleukin-1 alpha and -beta precursor proteins to cleavage by calpain, a calcium-dependent protease. *J Biol Chem* 270:27758-27765.
520. Kono, H., D. Karmarkar, Y. Iwakura, and K.L. Rock. 2010. Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. *J Immunol* 184:4470-4478.
521. Chen, C.J., H. Kono, D. Golenbock, G. Reed, S. Akira, and K.L. Rock. 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* 13:851-856.
522. Maier, J.A., M. Statuto, and G. Ragnotti. 1994. Endogenous interleukin 1 alpha must be transported to the nucleus to exert its activity in human endothelial cells. *Mol Cell Biol* 14:1845-1851.
523. Kawaguchi, Y., E. Nishimagi, A. Tochimoto, M. Kawamoto, Y. Katsumata, M. Soejima, T. Kanno, N. Kamatani, and M. Hara. 2006. Intracellular IL-1alpha-binding proteins contribute to biological functions of endogenous IL-1alpha in systemic sclerosis fibroblasts. *Proc Natl Acad Sci U S A* 103:14501-14506.
524. Dinarello, C.A. 1996. Biologic basis for interleukin-1 in disease. *Blood* 87:2095-2147.
525. Mariathasan, S., and D.M. Monack. 2007. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat Rev Immunol* 7:31-40.

526. Gu, Y., K. Kuida, H. Tsutsui, G. Ku, K. Hsiao, M.A. Fleming, N. Hayashi, K. Higashino, H. Okamura, K. Nakanishi, M. Kurimoto, T. Tanimoto, R.A. Flavell, V. Sato, M.W. Harding, D.J. Livingston, and M.S. Su. 1997. Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. *Science* 275:206-209.
527. Stehlik, C. 2009. Multiple interleukin-1beta-converting enzymes contribute to inflammatory arthritis. *Arthritis Rheum* 60:3524-3530.
528. Eder, C. 2009. Mechanisms of interleukin-1beta release. *Immunobiology* 214:543-553.
529. Rubartelli, A., F. Cozzolino, M. Talio, and R. Sitia. 1990. A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J* 9:1503-1510.
530. MacKenzie, A., H.L. Wilson, E. Kiss-Toth, S.K. Dower, R.A. North, and A. Surprenant. 2001. Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 15:825-835.
531. Qu, Y., L. Franchi, G. Nunez, and G.R. Dubyak. 2007. Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J Immunol* 179:1913-1925.
532. Hamon, Y., M.F. Luciani, F. Becq, B. Verrier, A. Rubartelli, and G. Chimini. 1997. Interleukin-1beta secretion is impaired by inhibitors of the Atp binding cassette transporter, ABC1. *Blood* 90:2911-2915.
533. Dinarello, C.A. 2005. Blocking IL-1 in systemic inflammation. *J Exp Med* 201:1355-1359.
534. Stanley, E.R., A. Bartocci, D. Patinkin, M. Rosendaal, and T.R. Bradley. 1986. Regulation of very primitive, multipotent, hemopoietic cells by hemopoietin-1. *Cell* 45:667-674.
535. Ueda, Y., D.W. Cain, M. Kuraoka, M. Kondo, and G. Kelsoe. 2009. IL-1R type I-dependent hemopoietic stem cell proliferation is necessary for inflammatory granulopoiesis and reactive neutrophilia. *J Immunol* 182:6477-6484.
536. Collins, T., M.A. Read, A.S. Neish, M.Z. Whitley, D. Thanos, and T. Maniatis. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* 9:899-909.
537. Martin, T., P.M. Cardarelli, G.C. Parry, K.A. Felts, and R.R. Cobb. 1997. Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-kappa B and AP-1. *Eur J Immunol* 27:1091-1097.
538. Chou, R.C., N.D. Kim, C.D. Sadik, E. Seung, Y. Lan, M.H. Byrne, B. Haribabu, Y. Iwakura, and A.D. Luster. 2010. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. *Immunity* 33:266-278.
539. Bonizzi, G., E. Dejardin, B. Piret, J. Piette, M.P. Merville, and V. Bours. 1996. Interleukin-1 beta induces nuclear factor kappa B in epithelial cells independently of the production of reactive oxygen intermediates. *Eur J Biochem* 242:544-549.
540. Bonizzi, G., J. Piette, S. Schoonbroodt, R. Greimers, L. Havard, M.P. Merville, and V. Bours. 1999. Reactive oxygen intermediate-dependent NF-kappaB

activation by interleukin-1beta requires 5-lipoxygenase or NADPH oxidase activity. *Mol Cell Biol* 19:1950-1960.

541. Luft, T., M. Jefford, P. Luetjens, H. Hochrein, K.A. Masterman, C. Maliszewski, K. Shortman, J. Cebon, and E. Maraskovsky. 2002. IL-1 beta enhances CD40 ligand-mediated cytokine secretion by human dendritic cells (DC): a mechanism for T cell-independent DC activation. *J Immunol* 168:713-722.
542. Wesa, A., and A. Galy. 2002. Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand. *BMC Immunol* 3:14.
543. Nakae, S., M. Asano, R. Horai, N. Sakaguchi, and Y. Iwakura. 2001. IL-1 enhances T cell-dependent antibody production through induction of CD40 ligand and OX40 on T cells. *J Immunol* 167:90-97.
544. Lichtman, A.H., J. Chin, J.A. Schmidt, and A.K. Abbas. 1988. Role of interleukin 1 in the activation of T lymphocytes. *Proc Natl Acad Sci U S A* 85:9699-9703.
545. Guo, L., G. Wei, J. Zhu, W. Liao, W.J. Leonard, K. Zhao, and W. Paul. 2009. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc Natl Acad Sci U S A* 106:13463-13468.
546. Nakae, S., M. Asano, R. Horai, and Y. Iwakura. 2001. Interleukin-1 beta, but not interleukin-1 alpha, is required for T-cell-dependent antibody production. *Immunology* 104:402-409.
547. Schmitz, N., M. Kurrer, and M. Kopf. 2003. The IL-1 receptor 1 is critical for Th2 cell type airway immune responses in a mild but not in a more severe asthma model. *Eur J Immunol* 33:991-1000.
548. Arend, W.P., H.G. Welgus, R.C. Thompson, and S.P. Eisenberg. 1990. Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *J Clin Invest* 85:1694-1697.
549. Horai, R., S. Saijo, H. Tanioka, S. Nakae, K. Sudo, A. Okahara, T. Ikuse, M. Asano, and Y. Iwakura. 2000. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. *J Exp Med* 191:313-320.
550. Nicklin, M.J., D.E. Hughes, J.L. Barton, J.M. Ure, and G.W. Duff. 2000. Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. *J Exp Med* 191:303-312.
551. Sandberg, J.O., A. Andersson, D.L. Eizirik, and S. Sandler. 1994. Interleukin-1 receptor antagonist prevents low dose streptozotocin induced diabetes in mice. *Biochem Biophys Res Commun* 202:543-548.
552. Colotta, F., F. Re, M. Muzio, R. Bertini, N. Polentarutti, M. Sironi, J.G. Giri, S.K. Dower, J.E. Sims, and A. Mantovani. 1993. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 261:472-475.
553. Smith, D.E., R. Hanna, F. Della, H. Moore, H. Chen, A.M. Farese, T.J. MacVittie, G.D. Virca, and J.E. Sims. 2003. The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. *Immunity* 18:87-96.
554. Masters, S.L., A. Simon, I. Aksentijevich, and D.L. Kastner. 2009. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (*). *Annu Rev Immunol* 27:621-668.

555. Dinarello, C.A. 2010. Blocking interleukin-1beta in acute and chronic autoinflammatory diseases. *J Intern Med* 269:16-28.
556. Arend, W.P., G. Palmer, and C. Gabay. 2008. IL-1, IL-18, and IL-33 families of cytokines. *Immunol Rev* 223:20-38.
557. Hata, H., T. Yoshimoto, N. Hayashi, T. Hada, and K. Nakanishi. 2004. IL-18 together with anti-CD3 antibody induces human Th1 cells to produce Th1- and Th2-cytokines and IL-8. *Int Immunol* 16:1733-1739.
558. Puren, A.J., P. Razeghi, G. Fantuzzi, and C.A. Dinarello. 1998. Interleukin-18 enhances lipopolysaccharide-induced interferon-gamma production in human whole blood cultures. *J Infect Dis* 178:1830-1834.
559. Leung, B.P., S. Culshaw, J.A. Gracie, D. Hunter, C.A. Canetti, C. Campbell, F. Cunha, F.Y. Liew, and I.B. McInnes. 2001. A role for IL-18 in neutrophil activation. *J Immunol* 167:2879-2886.
560. Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D.M. Gorman, J.F. Bazan, and R.A. Kastelein. 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23:479-490.
561. Baekkevold, E.S., M. Roussigne, T. Yamanaka, F.E. Johansen, F.L. Jahnsen, F. Amalric, P. Brandtzaeg, M. Erard, G. Haraldsen, and J.P. Girard. 2003. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol* 163:69-79.
562. Hayakawa, H., M. Hayakawa, A. Kume, and S. Tominaga. 2007. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J Biol Chem* 282:26369-26380.
563. Chackerian, A.A., E.R. Oldham, E.E. Murphy, J. Schmitz, S. Pflanz, and R.A. Kastelein. 2007. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J Immunol* 179:2551-2555.
564. Komai-Koma, M., D. Xu, Y. Li, A.N. McKenzie, I.B. McInnes, and F.Y. Liew. 2007. IL-33 is a chemoattractant for human Th2 cells. *Eur J Immunol* 37:2779-2786.
565. Suzukawa, M., R. Koketsu, M. Iikura, S. Nakae, K. Matsumoto, H. Nagase, H. Saito, K. Matsushima, K. Ohta, K. Yamamoto, and M. Yamaguchi. 2008. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Lab Invest* 88:1245-1253.
566. Allakhverdi, Z., D.E. Smith, M.R. Comeau, and G. Delespesse. 2007. Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J Immunol* 179:2051-2054.
567. Wynn, T.A. 2009. Basophils trump dendritic cells as APCs for T(H)2 responses. *Nat Immunol* 10:679-681.
568. Smith, D.E. 2009. IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clin Exp Allergy* 40:200-208.
569. Ligumsky, M., P.L. Simon, F. Karmeli, and D. Rachmilewitz. 1990. Role of interleukin 1 in inflammatory bowel disease--enhanced production during active disease. *Gut* 31:686-689.
570. Reinecker, H.C., M. Steffen, T. Witthoeft, I. Pflueger, S. Schreiber, R.P. MacDermott, and A. Raedler. 1993. Enhanced secretion of tumour necrosis

factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol* 94:174-181.

571. McAlindon, M.E., C.J. Hawkey, and Y.R. Mahida. 1998. Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease. *Gut* 42:214-219.
572. Satsangi, J., R.A. Wolstencroft, J. Cason, C.C. Ainley, D.C. Dumonde, and R.P. Thompson. 1987. Interleukin 1 in Crohn's disease. *Clin Exp Immunol* 67:594-605.
573. Mahida, Y.R., K. Wu, and D.P. Jewell. 1989. Enhanced production of interleukin 1-beta by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. *Gut* 30:835-838.
574. Casini-Raggi, V., L. Kam, Y.J. Chong, C. Fiocchi, T.T. Pizarro, and F. Cominelli. 1995. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. *J Immunol* 154:2434-2440.
575. Ludwiczek, O., E. Vannier, I. Borggraefe, A. Kaser, B. Siegmund, C.A. Dinarello, and H. Tilg. 2004. Imbalance between interleukin-1 agonists and antagonists: relationship to severity of inflammatory bowel disease. *Clin Exp Immunol* 138:323-329.
576. Arend, W.P., M. Malyak, C.J. Guthridge, and C. Gabay. 1998. Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol* 16:27-55.
577. Tountas, N.A., V. Casini-Raggi, H. Yang, F.S. Di Giovine, M. Vecchi, L. Kam, L. Melani, T.T. Pizarro, J.I. Rotter, and F. Cominelli. 1999. Functional and ethnic association of allele 2 of the interleukin-1 receptor antagonist gene in ulcerative colitis. *Gastroenterology* 117:806-813.
578. Bioque, G., J.B. Crusius, I. Koutroubakis, G. Bouma, P.J. Kostense, S.G. Meuwissen, and A.S. Pena. 1995. Allelic polymorphism in IL-1 beta and IL-1 receptor antagonist (IL-1Ra) genes in inflammatory bowel disease. *Clin Exp Immunol* 102:379-383.
579. Hacker, U.T., M. Gomolka, E. Keller, A. Eigler, C. Folwaczny, H. Fricke, E. Albert, K. Loeschke, and S. Endres. 1997. Lack of association between an interleukin-1 receptor antagonist gene polymorphism and ulcerative colitis. *Gut* 40:623-627.
580. McCall, R.D., S. Haskill, E.M. Zimmermann, P.K. Lund, R.C. Thompson, and R.B. Sartor. 1994. Tissue interleukin 1 and interleukin-1 receptor antagonist expression in enterocolitis in resistant and susceptible rats. *Gastroenterology* 106:960-972.
581. Cominelli, F., C.C. Nast, B.D. Clark, R. Schindler, R. Lierena, V.E. Eysselein, R.C. Thompson, and C.A. Dinarello. 1990. Interleukin 1 (IL-1) gene expression, synthesis, and effect of specific IL-1 receptor blockade in rabbit immune complex colitis. *J Clin Invest* 86:972-980.
582. Arai, Y., H. Takanashi, H. Kitagawa, and I. Okayasu. 1998. Involvement of interleukin-1 in the development of ulcerative colitis induced by dextran sulfate sodium in mice. *Cytokine* 10:890-896.
583. Ferretti, M., V. Casini-Raggi, T.T. Pizarro, S.P. Eisenberg, C.C. Nast, and F. Cominelli. 1994. Neutralization of endogenous IL-1 receptor antagonist

exacerbates and prolongs inflammation in rabbit immune colitis. *J Clin Invest* 94:449-453.

584. Shi, J., S. Aono, W. Lu, A.J. Ouellette, X. Hu, Y. Ji, L. Wang, S. Lenz, F.W. van Ginkel, M. Liles, C. Dykstra, E.E. Morrison, and C.O. Elson. 2007. A novel role for defensins in intestinal homeostasis: regulation of IL-1beta secretion. *J Immunol* 179:1245-1253.
585. Li, J., T. Moran, E. Swanson, C. Julian, J. Harris, D.K. Bonen, M. Hedl, D.L. Nicolae, C. Abraham, and J.H. Cho. 2004. Regulation of IL-8 and IL-1beta expression in Crohn's disease associated NOD2/CARD15 mutations. *Hum Mol Genet* 13:1715-1725.
586. van Beelen, A.J., Z. Zelinkova, E.W. Taanman-Kueter, F.J. Muller, D.W. Hommes, S.A. Zaat, M.L. Kapsenberg, and E.C. de Jong. 2007. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* 27:660-669.
587. Netea, M.G., C.A. Nold-Petry, M.F. Nold, L.A. Joosten, B. Opitz, J.H. van der Meer, F.L. van de Veerdonk, G. Ferwerda, B. Heinhuis, I. Devesa, C.J. Funk, R.J. Mason, B.J. Kullberg, A. Rubartelli, J.W. van der Meer, and C.A. Dinarello. 2009. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* 113:2324-2335.
588. Zhernakova, A., E.M. Festen, L. Franke, G. Trynka, C.C. van Diemen, A.J. Monsuur, M. Bevova, R.M. Nijmeijer, R. van 't Slot, R. Heijmans, H.M. Boezen, D.A. van Heel, A.A. van Bodegraven, P.C. Stokkers, C. Wijmenga, J.B. Crusius, and R.K. Weersma. 2008. Genetic analysis of innate immunity in Crohn's disease and ulcerative colitis identifies two susceptibility loci harboring CARD9 and IL18RAP. *Am J Hum Genet* 82:1202-1210.
589. Monteleone, G., F. Trapasso, T. Parrello, L. Biancone, A. Stella, R. Iuliano, F. Luzza, A. Fusco, and F. Pallone. 1999. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol* 163:143-147.
590. Pizarro, T.T., M.H. Michie, M. Bentz, J. Woraratanadharm, M.F. Smith, Jr., E. Foley, C.A. Moskaluk, S.J. Bickston, and F. Cominelli. 1999. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J Immunol* 162:6829-6835.
591. Corbaz, A., T. ten Hove, S. Herren, P. Graber, B. Schwartsburd, I. Belzer, J. Harrison, T. Plitz, M.H. Kosco-Vilbois, S.H. Kim, C.A. Dinarello, D. Novick, S. van Deventer, and Y. Chvatchko. 2002. IL-18-binding protein expression by endothelial cells and macrophages is up-regulated during active Crohn's disease. *J Immunol* 168:3608-3616.
592. Siegmund, B., G. Fantuzzi, F. Rieder, F. Gamboni-Robertson, H.A. Lehr, G. Hartmann, C.A. Dinarello, S. Endres, and A. Eigler. 2001. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am J Physiol Regul Integr Comp Physiol* 281:R1264-1273.
593. Sivakumar, P.V., G.M. Westrich, S. Kanaly, K. Garka, T.L. Born, J.M. Derry, and J.L. Viney. 2002. Interleukin 18 is a primary mediator of the inflammation associated with dextran sulphate sodium induced colitis: blocking interleukin 18 attenuates intestinal damage. *Gut* 50:812-820.

594. Kanai, T., M. Watanabe, A. Okazawa, T. Sato, M. Yamazaki, S. Okamoto, H. Ishii, T. Totsuka, R. Iiyama, R. Okamoto, M. Ikeda, M. Kurimoto, K. Takeda, S. Akira, and T. Hibi. 2001. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology* 121:875-888.
595. Ten Hove, T., A. Corbaz, H. Amitai, S. Aloni, I. Belzer, P. Graber, P. Drillenburg, S.J. van Deventer, Y. Chvatchko, and A.A. Te Velde. 2001. Blockade of endogenous IL-18 ameliorates TNBS-induced colitis by decreasing local TNF-alpha production in mice. *Gastroenterology* 121:1372-1379.
596. Wirtz, S., C. Becker, R. Blumberg, P.R. Galle, and M.F. Neurath. 2002. Treatment of T cell-dependent experimental colitis in SCID mice by local administration of an adenovirus expressing IL-18 antisense mRNA. *J Immunol* 168:411-420.
597. Zaki, M.H., K.L. Boyd, P. Vogel, M.B. Kastan, M. Lamkanfi, and T.D. Kanneganti. 2010. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* 32:379-391.
598. Dupaul-Chicoine, J., G. Yeretssian, K. Doiron, K.S. Bergstrom, C.R. McIntire, P.M. LeBlanc, C. Meunier, C. Turbide, P. Gros, N. Beauchemin, B.A. Vallance, and M. Saleh. 2010. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity* 32:367-378.
599. Markel, P., P. Shu, C. Ebeling, G.A. Carlson, D.L. Nagle, J.S. Smutko, and K.J. Moore. 1997. Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat Genet* 17:280-284.
600. Wakeland, E., L. Morel, K. Achey, M. Yui, and J. Longmate. 1997. Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol Today* 18:472-477.
601. Beckwith, C.S., D.J. McGee, H.L. Mobley, and L.K. Riley. 2001. Cloning, expression, and catalytic activity of Helicobacter hepaticus urease. *Infect Immun* 69:5914-5920.
602. Feng, S., K. Ku, E. Hodzic, E. Lorenzana, K. Freet, and S.W. Barthold. 2005. Differential detection of five mouse-infecting helicobacter species by multiplex PCR. *Clin Diagn Lab Immunol* 12:531-536.
603. Fox, J.G., F.E. Dewhirst, Z. Shen, Y. Feng, N.S. Taylor, B.J. Paster, R.L. Ericson, C.N. Lau, P. Correa, J.C. Araya, and I. Roa. 1998. Hepatic Helicobacter species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* 114:755-763.
604. Ge, Z., D.A. White, M.T. Whary, and J.G. Fox. 2001. Fluorogenic PCR-based quantitative detection of a murine pathogen, Helicobacter hepaticus. *J Clin Microbiol* 39:2598-2602.
605. Powrie, F., R. Correa-Oliveira, S. Mauze, and R.L. Coffman. 1994. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* 179:589-600.
606. Wannamaker, W., R. Davies, M. Namchuk, J. Pollard, P. Ford, G. Ku, C. Decker, P. Charifson, P. Weber, U.A. Germann, K. Kuida, and J.C. Randle. 2007. (S)-1-

((S)-2-{[1-(4-amino-3-chloro-phenyl)-methanoyl]-amino}-3,3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2R,3S)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide (VX-765), an orally available selective interleukin (IL)-converting enzyme/caspase-1 inhibitor, exhibits potent anti-inflammatory activities by inhibiting the release of IL-1beta and IL-18. *J Pharmacol Exp Ther* 321:509-516.

607. Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.
608. Obst, R., H.M. van Santen, D. Mathis, and C. Benoist. 2005. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. *J Exp Med* 201:1555-1565.
609. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 184:39-51.
610. Olsen, C.H. 2003. Review of the use of statistics in infection and immunity. *Infect Immun* 71:6689-6692.
611. Smith, P.D., C. Ochsenbauer-Jambor, and L.E. Smythies. 2005. Intestinal macrophages: unique effector cells of the innate immune system. *Immunol Rev* 206:149-159.
612. Black, R.A., S.R. Kronheim, M. Cantrell, M.C. Deeley, C.J. March, K.S. Prickett, J. Wignall, P.J. Conlon, D. Cosman, T.P. Hopp, and et al. 1988. Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *J Biol Chem* 263:9437-9442.
613. Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins, and et al. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356:768-774.
614. Martinon, F., A. Mayor, and J. Tschopp. 2009. The inflammasomes: guardians of the body. *Annu Rev Immunol* 27:229-265.
615. Okayasu, I., S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, and R. Nakaya. 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98:694-702.
616. Cominelli, F., C.C. Nast, A. Duchini, and M. Lee. 1992. Recombinant interleukin-1 receptor antagonist blocks the proinflammatory activity of endogenous interleukin-1 in rabbit immune colitis. *Gastroenterology* 103:65-71.
617. Thomas, T.K., P.C. Will, A. Srivastava, C.L. Wilson, M. Harbison, J. Little, R.S. Chesonis, M. Pignatello, D. Schmolze, J. Symington, and et al. 1991. Evaluation of an interleukin-1 receptor antagonist in the rat acetic acid-induced colitis model. *Agents Actions* 34:187-190.
618. Siegmund, B., H.A. Lehr, G. Fantuzzi, and C.A. Dinarello. 2001. IL-1 beta -converting enzyme (caspase-1) in intestinal inflammation. *Proc Natl Acad Sci U S A* 98:13249-13254.
619. Bauer, C., P. Duewell, C. Mayer, H.A. Lehr, K.A. Fitzgerald, M. Dauer, J. Tschopp, S. Endres, E. Latz, and M. Schnurr. 2010. Colitis induced in mice with

dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut* 59:1192-1199.

620. Loher, F., C. Bauer, N. Landauer, K. Schmall, B. Siegmund, H.A. Lehr, M. Dauer, M. Schoenharting, S. Endres, and A. Eigler. 2004. The interleukin-1 beta-converting enzyme inhibitor pralnacasan reduces dextran sulfate sodium-induced murine colitis and T helper 1 T-cell activation. *J Pharmacol Exp Ther* 308:583-590.
621. Allen, I.C., E.M. TeKippe, R.M. Woodford, J.M. Uronis, E.K. Holl, A.B. Rogers, H.H. Herfarth, C. Jobin, and J.P. Ting. 2010. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med* 207:1045-1056.
622. Stack, J.H., K. Beaumont, P.D. Larsen, K.S. Straley, G.W. Henkel, J.C. Randle, and H.M. Hoffman. 2005. IL-converting enzyme/caspase-1 inhibitor VX-765 blocks the hypersensitive response to an inflammatory stimulus in monocytes from familial cold autoinflammatory syndrome patients. *J Immunol* 175:2630-2634.
623. Asquith, M.J., O. Boulard, F. Powrie, and K.J. Maloy. 2010. Pathogenic and protective roles of MyD88 in leukocytes and epithelial cells in mouse models of inflammatory bowel disease. *Gastroenterology* 139:519-529, 529 e511-512.
624. Chen, C.J., Y. Shi, A. Hearn, K. Fitzgerald, D. Golenbock, G. Reed, S. Akira, and K.L. Rock. 2006. MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. *J Clin Invest* 116:2262-2271.
625. Hughes, T., B. Becknell, A.G. Freud, S. McClory, E. Briercheck, J. Yu, C. Mao, C. Giovenzana, G. Nuovo, L. Wei, X. Zhang, M.A. Gavrilin, M.D. Wewers, and M.A. Caligiuri. 2010. Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. *Immunity* 32:803-814.
626. Cella, M., K. Otero, and M. Colonna. 2010. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proc Natl Acad Sci U S A* 107:10961-10966.
627. Reynders, A., N. Yessaad, T.P. Vu Manh, M. Dalod, A. Fenis, C. Aubry, G. Nikitas, B. Escaliere, J.C. Renauld, O. Dussurget, P. Cossart, M. Lecuit, E. Vivier, and E. Tomasello. 2011. Identity, regulation and in vivo function of gut NKp46(+)RORgammat(+) and NKp46(+)RORgammat(-) lymphoid cells. *EMBO J*
628. Ribardo, D.A., S.E. Crowe, K.R. Kuhl, J.W. Peterson, and A.K. Chopra. 2001. Prostaglandin levels in stimulated macrophages are controlled by phospholipase A2-activating protein and by activation of phospholipase C and D. *J Biol Chem* 276:5467-5475.
629. Boniface, K., K.S. Bak-Jensen, Y. Li, W.M. Blumenschein, M.J. McGeachy, T.K. McClanahan, B.S. McKenzie, R.A. Kastelein, D.J. Cua, and R. de Waal Malefyt. 2009. Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med* 206:535-548.
630. Yao, C., D. Sakata, Y. Esaki, Y. Li, T. Matsuoka, K. Kuroiwa, Y. Sugimoto, and S. Narumiya. 2009. Prostaglandin E2-EP4 signaling promotes immune

inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med* 15:633-640.

631. Aiko, S., E.M. Conner, J.A. Fuseler, and M.B. Grisham. 1997. Effects of cyclosporine or FK506 in chronic colitis. *J Pharmacol Exp Ther* 280:1075-1084.
632. Brunkwall, J., and D. Bergqvist. 1993. The effect of cyclosporine A dissolved in chremofore or in ethanol and of cortisone on the arterial release of prostacyclin. *J Surg Res* 55:622-627.
633. Basit, A.W., J.M. Newton, M.D. Short, W.A. Waddington, P.J. Ell, and L.F. Lacey. 2001. The effect of polyethylene glycol 400 on gastrointestinal transit: implications for the formulation of poorly-water soluble drugs. *Pharm Res* 18:1146-1150.
634. Jenkins, R.T., R.L. Goodacre, P.J. Rooney, J. Bienenstock, T. Sivakumaran, and W.H. Walker. 1986. Studies of intestinal permeability in inflammatory diseases using polyethylene glycol 400. *Clin Biochem* 19:298-302.
635. Tache, S., G. Parnaud, E. Van Beek, and D.E. Corpet. 2006. Polyethylene glycol, unique among laxatives, suppresses aberrant crypt foci, by elimination of cells. *Scand J Gastroenterol* 41:730-736.
636. Hermansky, S.J., D.A. Neptun, K.A. Loughran, and H.W. Leung. 1995. Effects of polyethylene glycol 400 (PEG 400) following 13 weeks of gavage treatment in Fischer-344 rats. *Food Chem Toxicol* 33:139-149.
637. Erdogan, B., S. Isiksoy, E. Dundar, O. Pasaoglu, and C. Bal. 2003. The effects of sodium phosphate and polyethylene glycol-electrolyte bowel preparation solutions on 2,4,6-trinitrobenzenesulfonic acid-induced colitis in the rat. *Exp Toxicol Pathol* 55:213-220.
638. Videla, S., A. Lugea, J. Vilaseca, F. Guarner, F. Treserra, A. Salas, E. Crespo, C. Medina, and J.R. Malagelada. 2007. Polyethylene glycol enhances colonic barrier function and ameliorates experimental colitis in rats. *Int J Colorectal Dis* 22:571-580.
639. Joosten, L.A., M.G. Netea, G. Fantuzzi, M.I. Koenders, M.M. Helsen, H. Sparrer, C.T. Pham, J.W. van der Meer, C.A. Dinarello, and W.B. van den Berg. 2009. Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. *Arthritis Rheum* 60:3651-3662.
640. Guma, M., L. Ronacher, R. Liu-Bryan, S. Takai, M. Karin, and M. Corr. 2009. Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. *Arthritis Rheum* 60:3642-3650.
641. Kuehl, C.J., H.D. Wood, T.L. Marsh, T.M. Schmidt, and V.B. Young. 2005. Colonization of the cecal mucosa by Helicobacter hepaticus impacts the diversity of the indigenous microbiota. *Infect Immun* 73:6952-6961.
642. Tran, D.Q., J. Andersson, D. Hardwick, L. Bebris, G.G. Illei, and E.M. Shevach. 2009. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. *Blood* 113:5125-5133.
643. Mercer, F., L. Kozhaya, and D. Unutmaz. 2010. Expression and Function of TNF and IL-1 Receptors on Human Regulatory T Cells. *PLoS ONE* 5:e8639.

644. Brinster, C., and E.M. Shevach. 2008. Costimulatory effects of IL-1 on the expansion/differentiation of CD4+CD25+Foxp3+ and CD4+CD25+Foxp3- T cells. *J Leukoc Biol* 84:480-487.
645. O'Sullivan, B.J., H.E. Thomas, S. Pai, P. Santamaria, Y. Iwakura, R.J. Steptoe, T.W. Kay, and R. Thomas. 2006. IL-1 beta breaks tolerance through expansion of CD25+ effector T cells. *J Immunol* 176:7278-7287.
646. Glaccum, M.B., K.L. Stocking, K. Charrier, J.L. Smith, C.R. Willis, C. Maliszewski, D.J. Livingston, J.J. Peschon, and P.J. Morrissey. 1997. Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. *J Immunol* 159:3364-3371.
647. Labow, M., D. Shuster, M. Zetterstrom, P. Nunes, R. Terry, E.B. Cullinan, T. Bartfai, C. Solorzano, L.L. Moldawer, R. Chizzonite, and K.W. McIntyre. 1997. Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. *J Immunol* 159:2452-2461.
648. Miller, L.S., R.M. O'Connell, M.A. Gutierrez, E.M. Pietras, A. Shahangian, C.E. Gross, A. Thirumala, A.L. Cheung, G. Cheng, and R.L. Modlin. 2006. MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against Staphylococcus aureus. *Immunity* 24:79-91.
649. Hultgren, O.H., L. Svensson, and A. Tarkowski. 2002. Critical role of signaling through IL-1 receptor for development of arthritis and sepsis during Staphylococcus aureus infection. *J Immunol* 168:5207-5212.
650. Zwijnenburg, P.J., T. van der Poll, S. Florquin, J.J. Roord, and A.M. Van Furth. 2003. IL-1 receptor type 1 gene-deficient mice demonstrate an impaired host defense against pneumococcal meningitis. *J Immunol* 170:4724-4730.
651. Zhang, X., S.E. Hester, M.J. Kennett, A.T. Karanikas, L. Bendor, D.E. Place, and E.T. Harvill. 2011. Interleukin-1 receptor signaling is required to overcome the effects of pertussis toxin and for efficient infection- or vaccination-induced immunity against Bordetella pertussis. *Infect Immun* 79:527-541.
652. Juffermans, N.P., S. Florquin, L. Camoglio, A. Verbon, A.H. Kolk, P. Speelman, S.J. van Deventer, and T. van Der Poll. 2000. Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. *J Infect Dis* 182:902-908.
653. Gonzalez-Navajas, J.M., J. Law, K.P. Nguyen, M. Bhargava, M.P. Corr, N. Varki, L. Eckmann, H.M. Hoffman, J. Lee, and E. Raz. 2010. Interleukin 1 receptor signaling regulates DUBA expression and facilitates Toll-like receptor 9-driven antiinflammatory cytokine production. *J Exp Med* 207:2799-2807.
654. Chen, C.P., M. Hertzberg, Y. Jiang, and D.T. Graves. 1999. Interleukin-1 and tumor necrosis factor receptor signaling is not required for bacteria-induced osteoclastogenesis and bone loss but is essential for protecting the host from a mixed anaerobic infection. *Am J Pathol* 155:2145-2152.
655. Schmitz, N., M. Kurrer, M.F. Bachmann, and M. Kopf. 2005. Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. *J Virol* 79:6441-6448.
656. Muller, A.J., C. Hoffmann, M. Galle, A. Van Den Broeke, M. Heikenwalder, L. Falter, B. Misselwitz, M. Kremer, R. Beyaert, and W.D. Hardt. 2009. The S. Typhimurium effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. *Cell Host Microbe* 6:125-136.

657. Boulard, O., M.J. Asquith, F. Powrie, and K.J. Maloy. 2010. TLR2-independent induction and regulation of chronic intestinal inflammation. *Eur J Immunol* 40:516-524.
658. Asquith, M. 2009. Toll-like receptors in the induction and regulation of intestinal inflammation. In Sir William Dunn School of Pathology. University of Oxford, Oxford. 229.
659. Abbondanzo, S.J., E.B. Cullinan, K. McIntyre, M.A. Labow, and C.L. Stewart. 1996. Reproduction in mice lacking a functional type 1 IL-1 receptor. *Endocrinology* 137:3598-3601.
660. Cohen, I., P. Rider, Y. Carmi, A. Braiman, S. Dotan, M.R. White, E. Voronov, M.U. Martin, C.A. Dinarello, and R.N. Apte. 2010. Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. *Proc Natl Acad Sci U S A* 107:2574-2579.
661. Niki, Y., H. Yamada, S. Seki, T. Kikuchi, H. Takaishi, Y. Toyama, K. Fujikawa, and N. Tada. 2001. Macrophage- and neutrophil-dominant arthritis in human IL-1 alpha transgenic mice. *J Clin Invest* 107:1127-1135.
662. Werner, S., and H. Smola. 2001. Paracrine regulation of keratinocyte proliferation and differentiation. *Trends Cell Biol* 11:143-146.
663. Lee, P., D.J. Lee, C. Chan, S.W. Chen, I. Ch'en, and C. Jamora. 2009. Dynamic expression of epidermal caspase 8 simulates a wound healing response. *Nature* 458:519-523.
664. Al-Ashy, R., I. Chakroun, M.E. El-Sabban, and F.R. Homaidan. 2006. The role of NF-kappaB in mediating the anti-inflammatory effects of IL-10 in intestinal epithelial cells. *Cytokine* 36:1-8.
665. Touzani, O., H. Boutin, R. LeFeuvre, L. Parker, A. Miller, G. Luheshi, and N. Rothwell. 2002. Interleukin-1 influences ischemic brain damage in the mouse independently of the interleukin-1 type I receptor. *J Neurosci* 22:38-43.
666. Boutin, H., I. Kimber, N.J. Rothwell, and E. Pinteaux. 2003. The expanding interleukin-1 family and its receptors: do alternative IL-1 receptor/signaling pathways exist in the brain? *Mol Neurobiol* 27:239-248.
667. Diem, R., M. Hobom, P. Grotsch, B. Kramer, and M. Bahr. 2003. Interleukin-1 beta protects neurons via the interleukin-1 (IL-1) receptor-mediated Akt pathway and by IL-1 receptor-independent decrease of transmembrane currents in vivo. *Mol Cell Neurosci* 22:487-500.
668. Humphreys, N.E., and R.K. Grencis. 2009. IL-1-dependent, IL-1R1-independent resistance to gastrointestinal nematodes. *Eur J Immunol* 39:1036-1045.
669. Luheshi, G., S.J. Hopkins, R.A. Lefeuvre, M.J. Dascombe, P. Ghiara, and N.J. Rothwell. 1993. Importance of brain IL-1 type II receptors in fever and thermogenesis in the rat. *Am J Physiol* 265:E585-591.
670. Elinav, E., T. Strowig, A.L. Kau, J. Henao-Mejia, C.A. Thaiss, C.J. Booth, D.R. Peaper, J. Bertin, S.C. Eisenbarth, J.I. Gordon, and R.A. Flavell. 2011. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 145:745-757.
671. Garcia, M.C., I. Wernstedt, A. Berndtsson, M. Enge, M. Bell, O. Hultgren, M. Horn, B. Ahren, S. Enerback, C. Ohlsson, V. Wallenius, and J.O. Jansson. 2006.

Mature-onset obesity in interleukin-1 receptor I knockout mice. *Diabetes* 55:1205-1213.

672. Duan, J., H. Chung, E. Troy, and D.L. Kasper. 2010. Microbial colonization drives expansion of IL-1 receptor 1-expressing and IL-17-producing gamma/delta T cells. *Cell Host Microbe* 7:140-150.
673. Lalor, S.J., L.S. Dungan, C.E. Sutton, S.A. Basdeo, J.M. Fletcher, and K.H. Mills. 2011. Caspase-1-processed cytokines IL-1beta and IL-18 promote IL-17 production by gammadelta and CD4 T cells that mediate autoimmunity. *J Immunol* 186:5738-5748.
674. Nanno, M., Y. Kanari, T. Naito, N. Inoue, T. Hisamatsu, H. Chinen, K. Sugimoto, Y. Shimomura, H. Yamagishi, T. Shiohara, S. Ueha, K. Matsushima, M. Suematsu, A. Mizoguchi, T. Hibi, A.K. Bhan, and H. Ishikawa. 2008. Exacerbating role of gammadelta T cells in chronic colitis of T-cell receptor alpha mutant mice. *Gastroenterology* 134:481-490.
675. Chen, Y., K. Chou, E. Fuchs, W.L. Havran, and R. Boismenu. 2002. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* 99:14338-14343.
676. Yoshida, H., J. Russell, E.Y. Senchenkova, L.D. Almeida Paula, and D.N. Granger. 2010. Interleukin-1beta mediates the extra-intestinal thrombosis associated with experimental colitis. *Am J Pathol* 177:2774-2781.
677. Hu, W., S. Mahavadi, F. Li, and K.S. Murthy. 2007. Upregulation of RGS4 and downregulation of CPI-17 mediate inhibition of colonic muscle contraction by interleukin-1beta. *Am J Physiol Cell Physiol* 293:C1991-2000.
678. Kindt, S., P. Vanden Berghe, W. Boesmans, L. Roosen, and J. Tack. 2010. Prolonged IL-1beta exposure alters neurotransmitter and electrically induced Ca(2+) responses in the myenteric plexus. *Neurogastroenterol Motil* 22:321-e385.
679. Voo, K.S., Y.H. Wang, F.R. Santori, C. Boggiano, K. Arima, L. Bover, S. Hanabuchi, J. Khalili, E. Marinova, B. Zheng, D.R. Littman, and Y.J. Liu. 2009. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* 106:4793-4798.
680. Valmori, D., C. Raffin, I. Raimbaud, and M. Ayyoub. 2010. Human RORgammat+ TH17 cells preferentially differentiate from naive FOXP3+Treg in the presence of lineage-specific polarizing factors. *Proc Natl Acad Sci U S A* 107:19402-19407.
681. Koenen, H.J., R.L. Smeets, P.M. Vink, E. van Rijssen, A.M. Boots, and I. Joosten. 2008. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood* 112:2340-2352.
682. Gery, I., and B.H. Waksman. 1972. Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). *J Exp Med* 136:143-155.
683. Schmitz, J., M. Assenmacher, and A. Radbruch. 1993. Regulation of T helper cell cytokine expression: functional dichotomy of antigen-presenting cells. *Eur J Immunol* 23:191-199.
684. Maitra, U., S. Davis, C.M. Reilly, and L. Li. 2009. Differential regulation of Foxp3 and IL-17 expression in CD4 T helper cells by IRAK-1. *J Immunol* 182:5763-5769.

685. Ivanov, II, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121-1133.
686. Atarashi, K., J. Nishimura, T. Shima, Y. Umesaki, M. Yamamoto, M. Onoue, H. Yagita, N. Ishii, R. Evans, K. Honda, and K. Takeda. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455:808-812.
687. Uematsu, S., K. Fujimoto, M.H. Jang, B.G. Yang, Y.J. Jung, M. Nishiyama, S. Sato, T. Tsujimura, M. Yamamoto, Y. Yokota, H. Kiyono, M. Miyasaka, K.J. Ishii, and S. Akira. 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol* 9:769-776.
688. Hall, J.A., N. Bouladoux, C.M. Sun, E.A. Wohlfert, R.B. Blank, Q. Zhu, M.E. Grigg, J.A. Berzofsky, and Y. Belkaid. 2008. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity* 29:637-649.
689. Izcue, A., J.L. Coombes, and F. Powrie. 2006. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* 212:256-271.
690. El-Behi, M., B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G.X. Zhang, B.N. Dittel, and A. Rostami. 2011. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* 12:568-575.
691. Codarri, L., G. Gyulveszi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter, and B. Becher. 2011. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 12:560-567.
692. ten Hove, T., C. van Montfrans, M.P. Peppelenbosch, and S.J. van Deventer. 2002. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut* 50:206-211.
693. Durant, L., W.T. Watford, H.L. Ramos, A. Laurence, G. Vahedi, L. Wei, H. Takahashi, H.W. Sun, Y. Kanno, F. Powrie, and J.J. O'Shea. 2010. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* 32:605-615.
694. Watts, T.H. 2005. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* 23:23-68.
695. Croft, M., T. So, W. Duan, and P. Soroosh. 2009. The significance of OX40 and OX40L to T-cell biology and immune disease. *Immunol Rev* 229:173-191.
696. Souza, H.S., C.C. Elia, J. Spencer, and T.T. MacDonald. 1999. Expression of lymphocyte-endothelial receptor-ligand pairs, alpha4beta7/MAdCAM-1 and OX40/OX40 ligand in the colon and jejunum of patients with inflammatory bowel disease. *Gut* 45:856-863.
697. Malmstrom, V., D. Shipton, B. Singh, A. Al-Shamkhani, M.J. Puklavec, A.N. Barclay, and F. Powrie. 2001. CD134L expression on dendritic cells in the mesenteric lymph nodes drives colitis in T cell-restored SCID mice. *J Immunol* 166:6972-6981.

698. Takeda, I., S. Ine, N. Killeen, L.C. Ndhlovu, K. Murata, S. Satomi, K. Sugamura, and N. Ishii. 2004. Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells. *J Immunol* 172:3580-3589.
699. Griseri, T., M. Asquith, C. Thompson, and F. Powrie. 2010. OX40 is required for regulatory T cell-mediated control of colitis. *J Exp Med* 207:699-709.
700. Grewal, I.S., and R.A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* 16:111-135.
701. Elgueta, R., M.J. Benson, V.C. de Vries, A. Wasiuk, Y. Guo, and R.J. Noelle. 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* 229:152-172.
702. Battaglia, E., L. Biancone, A. Resegotti, G. Emanuelli, G.R. Fronda, and G. Camussi. 1999. Expression of CD40 and its ligand, CD40L, in intestinal lesions of Crohn's disease. *Am J Gastroenterol* 94:3279-3284.
703. De Jong, Y.P., M. Comiskey, S.L. Kalled, E. Mizoguchi, R.A. Flavell, A.K. Bhan, and C. Terhorst. 2000. Chronic murine colitis is dependent on the CD154/CD40 pathway and can be attenuated by anti-CD154 administration. *Gastroenterology* 119:715-723.
704. Liu, Z., K. Geboes, S. Colpaert, L. Overbergh, C. Mathieu, H. Heremans, M. de Boer, L. Boon, G. D'Haens, P. Rutgeerts, and J.L. Ceuppens. 2000. Prevention of experimental colitis in SCID mice reconstituted with CD45RBhigh CD4+ T cells by blocking the CD40-CD154 interactions. *J Immunol* 164:6005-6014.
705. Ben-Sasson, S.Z., J. Hu-Li, J. Quiel, S. Cauchetaux, M. Ratner, I. Shapira, C.A. Dinarello, and W.E. Paul. 2009. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A* 106:7119-7124.
706. Kane, L.P., and A. Weiss. 2003. The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. *Immunol Rev* 192:7-20.
707. Fukata, M., K. Breglio, A. Chen, A.S. Vamadevan, T. Goo, D. Hsu, D. Conduah, R. Xu, and M.T. Abreu. 2008. The myeloid differentiation factor 88 (MyD88) is required for CD4+ T cell effector function in a murine model of inflammatory bowel disease. *J Immunol* 180:1886-1894.
708. Tomita, T., T. Kanai, T. Fujii, Y. Nemoto, R. Okamoto, K. Tsuchiya, T. Totsuka, N. Sakamoto, S. Akira, and M. Watanabe. 2008. MyD88-dependent pathway in T cells directly modulates the expansion of colitogenic CD4+ T cells in chronic colitis. *J Immunol* 180:5291-5299.
709. Lamacchia, C., G. Palmer, C.A. Seemayer, D. Talabot-Ayer, and C. Gabay. 2010. Enhanced Th1 and Th17 responses and arthritis severity in mice with a deficiency of myeloid cell-specific interleukin-1 receptor antagonist. *Arthritis Rheum* 62:452-462.
710. Gabay, C., C. Lamacchia, and G. Palmer. 2010. IL-1 pathways in inflammation and human diseases. *Nat Rev Rheumatol* 6:232-241.
711. Coulombe, F., M. Divangahi, F. Veyrier, L. de Leseleuc, J.L. Gleason, Y. Yang, M.A. Kelliher, A.K. Pandey, C.M. Sassetti, M.B. Reed, and M.A. Behr. 2009. Increased NOD2-mediated recognition of N-glycolyl muramyl dipeptide. *J Exp Med* 206:1709-1716.
712. Rosenstiel, P., M. Fantini, K. Brautigam, T. Kuhbacher, G.H. Waetzig, D. Seegert, and S. Schreiber. 2003. TNF-alpha and IFN-gamma regulate the

expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* 124:1001-1009.

713. Ogura, Y., S. Lala, W. Xin, E. Smith, T.A. Dowds, F.F. Chen, E. Zimmermann, M. Tretiakova, J.H. Cho, J. Hart, J.K. Greenson, S. Keshav, and G. Nunez. 2003. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 52:1591-1597.
714. Otte, J.M., I.M. Rosenberg, and D.K. Podolsky. 2003. Intestinal myofibroblasts in innate immune responses of the intestine. *Gastroenterology* 124:1866-1878.
715. Davey, M.P., T.M. Martin, S.R. Planck, J. Lee, D. Zamora, and J.T. Rosenbaum. 2006. Human endothelial cells express NOD2/CARD15 and increase IL-6 secretion in response to muramyl dipeptide. *Microvasc Res* 71:103-107.
716. Chamaillard, M., D. Philpott, S.E. Girardin, H. Zouali, S. Lesage, F. Chareyre, T.H. Bui, M. Giovannini, U. Zaehringer, V. Penard-Lacronique, P.J. Sansonetti, J.P. Hugot, and G. Thomas. 2003. Gene-environment interaction modulated by allelic heterogeneity in inflammatory diseases. *Proc Natl Acad Sci U S A* 100:3455-3460.
717. Inohara, N., Y. Ogura, F.F. Chen, A. Muto, and G. Nunez. 2001. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J Biol Chem* 276:2551-2554.
718. Wehkamp, J., J. Harder, M. Weichenthal, M. Schwab, E. Schaffeler, M. Schlee, K.R. Herrlinger, A. Stallmach, F. Noack, P. Fritz, J.M. Schroder, C.L. Bevins, K. Fellermann, and E.F. Stange. 2004. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 53:1658-1664.
719. Fritz, J.H., S.E. Girardin, C. Fitting, C. Werts, D. Mengin-Lecreulx, M. Caroff, J.M. Cavaillon, D.J. Philpott, and M. Adib-Conquy. 2005. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol* 35:2459-2470.
720. Park, J.H., Y.G. Kim, C. McDonald, T.D. Kanneganti, M. Hasegawa, M. Body-Malapel, N. Inohara, and G. Nunez. 2007. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J Immunol* 178:2380-2386.
721. Deshmukh, H.S., J.B. Hamburger, S.H. Ahn, D.G. McCafferty, S.R. Yang, and V.G. Fowler, Jr. 2009. Critical role of NOD2 in regulating the immune response to Staphylococcus aureus. *Infect Immun* 77:1376-1382.
722. Biswas, A., Y.J. Liu, L. Hao, A. Mizoguchi, N.H. Salzman, C.L. Bevins, and K.S. Kobayashi. 2010. Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum. *Proc Natl Acad Sci U S A* 107:14739-14744.
723. Lesage, S., H. Zouali, J.P. Cezard, J.F. Colombel, J. Belaiche, S. Almer, C. Tysk, C. O'Morain, M. Gassull, V. Binder, Y. Finkel, R. Modigliani, C. Gower-Rousseau, J. Macry, F. Merlin, M. Chamaillard, A.S. Jannot, G. Thomas, and J.P. Hugot. 2002. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet* 70:845-857.

724. Hampe, J., J. Grebe, S. Nikolaus, C. Solberg, P.J. Croucher, S. Mascheretti, J. Jahnsen, B. Moum, B. Klump, M. Krawczak, M.M. Mirza, U.R. Foelsch, M. Vatn, and S. Schreiber. 2002. Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet* 359:1661-1665.
725. Ogura, Y., N. Inohara, A. Benito, F.F. Chen, S. Yamaoka, and G. Nunez. 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 276:4812-4818.
726. Foltz, C.J., J.G. Fox, R. Cahill, J.C. Murphy, L. Yan, B. Shames, and D.B. Schauer. 1998. Spontaneous inflammatory bowel disease in multiple mutant mouse lines: association with colonization by Helicobacter hepaticus. *Helicobacter* 3:69-78.
727. Petnicki-Ocwieja, T., T. Hrncir, Y.J. Liu, A. Biswas, T. Hudcovic, H. Tlaskalova-Hogenova, and K.S. Kobayashi. 2009. Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci U S A* 106:15813-15818.
728. Geddes, K., S.J. Rubino, J.G. Magalhaes, C. Streutker, L. Le Bourhis, J.H. Cho, S.J. Robertson, C.J. Kim, R. Kaul, D.J. Philpott, and S.E. Girardin. 2011. Identification of an innate T helper type 17 response to intestinal bacterial pathogens. *Nat Med* 17:837-844.
729. Cooney, R., J. Baker, O. Brain, B. Danis, T. Pichulik, P. Allan, D.J. Ferguson, B.J. Campbell, D. Jewell, and A. Simmons. 2010. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* 16:90-97.
730. Magalhaes, J.G., S.J. Rubino, L.H. Travassos, L. Le Bourhis, W. Duan, G. Sellge, K. Geddes, C. Reardon, M. Lechmann, L.A. Carneiro, T. Selvanantham, J.H. Fritz, B.C. Taylor, D. Artis, T.W. Mak, M.R. Comeau, M. Croft, S.E. Girardin, and D.J. Philpott. 2011. Nucleotide oligomerization domain-containing proteins instruct T cell helper type 2 immunity through stromal activation. *Proc Natl Acad Sci U S A* 108:14896-14901.
731. Berrebi, D., R. Maudinas, J.P. Hugot, M. Chamaillard, F. Chareyre, P. De Lagausie, C. Yang, P. Desreumaux, M. Giovannini, J.P. Cezard, H. Zouali, D. Emilie, and M. Peuchmaur. 2003. Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. *Gut* 52:840-846.
732. Gutierrez, O., C. Pipaon, N. Inohara, A. Fontalba, Y. Ogura, F. Prosper, G. Nunez, and J.L. Fernandez-Luna. 2002. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J Biol Chem* 277:41701-41705.
733. Kim, Y.G., N. Kamada, M.H. Shaw, N. Warner, G.Y. Chen, L. Franchi, and G. Nunez. 2011. The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes. *Immunity* 34:769-780.
734. Okumura, S., K. Yuki, R. Kobayashi, S. Okamura, K. Ohmori, H. Saito, C. Ra, and Y. Okayama. 2009. Hyperexpression of NOD2 in intestinal mast cells of Crohn's disease patients: preferential expression of inflammatory cell-recruiting molecules via NOD2 in mast cells. *Clin Immunol* 130:175-185.

735. Lightfield, K.L., J. Persson, S.W. Brubaker, C.E. Witte, J. von Moltke, E.A. Dunipace, T. Henry, Y.H. Sun, D. Cado, W.F. Dietrich, D.M. Monack, R.M. Tsolis, and R.E. Vance. 2008. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. *Nat Immunol* 9:1171-1178.
736. Carvalho, F.A., N. Barnich, P. Sauvanet, C. Darcha, A. Gelot, and A. Darfeuille-Michaud. 2008. Crohn's disease-associated Escherichia coli LF82 aggravates colitis in injured mouse colon via signaling by flagellin. *Inflamm Bowel Dis* 14:1051-1060.
737. Hu, B., E. Elinav, S. Huber, C.J. Booth, T. Strowig, C. Jin, S.C. Eisenbarth, and R.A. Flavell. 2010. Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4. *Proc Natl Acad Sci U S A* 107:21635-21640.
738. Rosenstiel, P., S. Hellmig, J. Hampe, S. Ott, A. Till, W. Fischbach, H. Sahly, R. Lucius, U.R. Folsch, D. Philpott, and S. Schreiber. 2006. Influence of polymorphisms in the NOD1/CARD4 and NOD2/CARD15 genes on the clinical outcome of Helicobacter pylori infection. *Cell Microbiol* 8:1188-1198.
739. Yang, Z., I.J. Fuss, T. Watanabe, N. Asano, M.P. Davey, J.T. Rosenbaum, W. Strober, and A. Kitani. 2007. NOD2 transgenic mice exhibit enhanced MDP-mediated down-regulation of TLR2 responses and resistance to colitis induction. *Gastroenterology* 133:1510-1521.
740. Barreau, F., C. Madre, U. Meinzer, D. Berrebi, M. Dussaillant, F. Merlin, L. Eckmann, M. Karin, G. Sterkers, S. Bonacorsi, T. Lesuffleur, and J.P. Hugot. 2010. Nod2 regulates the host response towards microflora by modulating T cell function and epithelial permeability in mouse Peyer's patches. *Gut* 59:207-217.
741. Berg, D.J., N. Davidson, R. Kuhn, W. Muller, S. Menon, G. Holland, L. Thompson-Snipes, M.W. Leach, and D. Rennick. 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* 98:1010-1020.
742. Cong, Y., T. Feng, K. Fujihashi, T.R. Schoeb, and C.O. Elson. 2009. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci U S A* 106:19256-19261.
743. McLachlan, J.B., D.M. Catron, J.J. Moon, and M.K. Jenkins. 2009. Dendritic cell antigen presentation drives simultaneous cytokine production by effector and regulatory T cells in inflamed skin. *Immunity* 30:277-288.
744. Kryczek, I., S. Wei, L. Vatan, J. Escara-Wilke, W. Szeliga, E.T. Keller, and W. Zou. 2007. Cutting edge: opposite effects of IL-1 and IL-2 on the regulation of IL-17+ T cell pool IL-1 subverts IL-2-mediated suppression. *J Immunol* 179:1423-1426.
745. Huber, M., A. Brustle, K. Reinhard, A. Guralnik, G. Walter, A. Mahiny, E. von Low, and M. Lohoff. 2008. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc Natl Acad Sci U S A* 105:20846-20851.
746. Vallabhapurapu, S., and M. Karin. 2009. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* 27:693-733.

747. Gelman, A.E., D.F. LaRosa, J. Zhang, P.T. Walsh, Y. Choi, J.O. Sunyer, and L.A. Turka. 2006. The adaptor molecule MyD88 activates PI-3 kinase signaling in CD4+ T cells and enables CpG oligodeoxynucleotide-mediated costimulation. *Immunity* 25:783-793.
748. Michalek, R.D., and J.C. Rathmell. 2010. The metabolic life and times of a T-cell. *Immunol Rev* 236:190-202.
749. Jacobs, S.R., C.E. Herman, N.J. Maciver, J.A. Wofford, H.L. Wieman, J.J. Hammen, and J.C. Rathmell. 2008. Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. *J Immunol* 180:4476-4486.
750. Wofford, J.A., H.L. Wieman, S.R. Jacobs, Y. Zhao, and J.C. Rathmell. 2008. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival. *Blood* 111:2101-2111.
751. Besedovsky, H.O., and A. Del Rey. 2010. Interleukin-1 resets glucose homeostasis at central and peripheral levels: relevance for immunoregulation. *Neuroimmunomodulation* 17:139-141.
752. Fukuzumi, M., H. Shinomiya, Y. Shimizu, K. Ohishi, and S. Utsumi. 1996. Endotoxin-induced enhancement of glucose influx into murine peritoneal macrophages via GLUT1. *Infect Immun* 64:108-112.
753. Vega, C., L. Pellerin, R. Dantzer, and P.J. Magistretti. 2002. Long-term modulation of glucose utilization by IL-1 alpha and TNF-alpha in astrocytes: Na+ pump activity as a potential target via distinct signaling mechanisms. *Glia* 39:10-18.
754. Del Rey, A., E. Roggero, A. Randolf, C. Mahuad, S. McCann, V. Rettori, and H.O. Besedovsky. 2006. IL-1 resets glucose homeostasis at central levels. *Proc Natl Acad Sci U S A* 103:16039-16044.
755. Metzger, S., S. Nusair, D. Planer, V. Barash, O. Pappo, J. Shilyansky, and T. Chajek-Shaul. 2004. Inhibition of hepatic gluconeogenesis and enhanced glucose uptake contribute to the development of hypoglycemia in mice bearing interleukin-1beta- secreting tumor. *Endocrinology* 145:5150-5156.
756. Szabo, S.J., A.S. Dighe, U. Gubler, and K.M. Murphy. 1997. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 185:817-824.
757. Mellor, A.L., and D.H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 4:762-774.
758. Bronte, V., and P. Zanovello. 2005. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* 5:641-654.
759. Cha, H.R., S.Y. Chang, J.H. Chang, J.O. Kim, J.Y. Yang, C.H. Kim, and M.N. Kweon. 2010. Downregulation of Th17 cells in the small intestine by disruption of gut flora in the absence of retinoic acid. *J Immunol* 184:6799-6806.
760. Donohoe, D.R., N. Garge, X. Zhang, W. Sun, T.M. O'Connell, M.K. Bunger, and S.J. Bultman. 2011. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. *Cell Metab* 13:517-526.
761. Zhou, R., A.S. Yazdi, P. Menu, and J. Tschopp. 2011. A role for mitochondria in NLRP3 inflammasome activation. *Nature* 469:221-225.

762. Johnson, K.M., X. Chen, A. Boitano, L. Swenson, A.W. Opipari, Jr., and G.D. Glick. 2005. Identification and validation of the mitochondrial F1F0-ATPase as the molecular target of the immunomodulatory benzodiazepine Bz-423. *Chem Biol* 12:485-496.
763. Brain, O., R. Cooney, A. Simmons, and D. Jewell. 2011. Functional consequences of mutations in the autophagy genes in the pathogenesis of Crohn's disease. *Inflamm Bowel Dis*
764. Pua, H.H., I. Dzhagalov, M. Chuck, N. Mizushima, and Y.W. He. 2007. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J Exp Med* 204:25-31.
765. Schroder, K., R. Zhou, and J. Tschopp. 2010. The NLRP3 inflammasome: a sensor for metabolic danger? *Science* 327:296-300.
766. Motta, J.P., L. Magne, D. Descamps, C. Rolland, C. Squarzoni-Dale, P. Rousset, L. Martin, N. Cenac, V. Balloy, M. Huerre, L.F. Frohlich, D. Jenne, J. Wartelle, A. Belaaouaj, E. Mas, J.P. Vinel, L. Alric, M. Chignard, N. Vergnolle, and J.M. Sallenave. 2011. Modifying the protease, antiprotease pattern by elafin overexpression protects mice from colitis. *Gastroenterology* 140:1272-1282.
767. Cho, E.Y., S.C. Choi, S.H. Lee, J.Y. Ahn, L.R. Im, J.H. Kim, M. Xin, S.U. Kwon, D.K. Kim, and Y.M. Lee. 2011. Nafamostat mesilate attenuates colonic inflammation and mast cell infiltration in the experimental colitis. *Int Immunopharmacol* 11:412-417.
768. Ishida, K., S. Takai, M. Murano, T. Nishikawa, T. Inoue, N. Murano, N. Inoue, D. Jin, E. Umegaki, K. Higuchi, and M. Miyazaki. 2008. Role of chymase-dependent matrix metalloproteinase-9 activation in mice with dextran sodium sulfate-induced colitis. *J Pharmacol Exp Ther* 324:422-426.
769. Isozaki, Y., N. Yoshida, M. Kuroda, O. Handa, T. Takagi, S. Kokura, H. Ichikawa, Y. Naito, T. Okanoue, and T. Yoshikawa. 2006. Anti-tryptase treatment using nafamostat mesilate has a therapeutic effect on experimental colitis. *Scand J Gastroenterol* 41:944-953.
770. Bauer, C., F. Loher, M. Dauer, C. Mayer, H.A. Lehr, M. Schonharting, R. Hallwachs, S. Endres, and A. Eigler. 2007. The ICE inhibitor pralnacasan prevents DSS-induced colitis in C57BL/6 mice and suppresses IP-10 mRNA but not TNF-alpha mRNA expression. *Dig Dis Sci* 52:1642-1652.
771. Siegmund, B., G. Fantuzzi, F. Rieder, F. Gamboni-Robertson, A. Eigler, S. Endres, and C.A. Dinarello. 2001. Neutralization of IL-18 exerts anti-inflammatory activity in experimental colitis in mice. *Gastroenterology* 120:A315-A315.
772. Ishikura, T., T. Kanai, K. Uraushihara, R. Iiyama, S. Makita, T. Totsuka, M. Yamazaki, T. Sawada, T. Nakamura, T. Miyata, T. Kitahora, T. Hibi, T. Hoshino, and M. Watanabe. 2003. Interleukin-18 overproduction exacerbates the development of colitis with markedly infiltrated macrophages in interleukin-18 transgenic mice. *J Gastroenterol Hepatol* 18:960-969.
773. Kostura, M.J., M.J. Tocci, G. Limjuco, J. Chin, P. Cameron, A.G. Hillman, N.A. Chartrain, and J.A. Schmidt. 1989. Identification of a monocyte specific pre-interleukin 1 beta convertase activity. *Proc Natl Acad Sci U S A* 86:5227-5231.

774. Black, R.A., S.R. Kronheim, J.E. Merriam, C.J. March, and T.P. Hopp. 1989. A pre-aspartate-specific protease from human leukocytes that cleaves pro-interleukin-1 beta. *J Biol Chem* 264:5323-5326.
775. Black, R.A., S.R. Kronheim, and P.R. Sleath. 1989. Activation of interleukin-1 beta by a co-induced protease. *FEBS Lett* 247:386-390.
776. Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, K. Van Ness, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Cannizzaro, and et al. 1992. Molecular cloning of the interleukin-1 beta converting enzyme. *Science* 256:97-100.
777. Nett, M.A., D.P. Cerretti, D.R. Berson, J. Seavitt, D.J. Gilbert, N.A. Jenkins, N.G. Copeland, R.A. Black, and D.D. Chaplin. 1992. Molecular cloning of the murine IL-1 beta converting enzyme cDNA. *J Immunol* 149:3254-3259.
778. Mizutani, H., N. Schechter, G. Lazarus, R.A. Black, and T.S. Kupper. 1991. Rapid and specific conversion of precursor interleukin 1 beta (IL-1 beta) to an active IL-1 species by human mast cell chymase. *J Exp Med* 174:821-825.
779. Irmler, M., S. Hertig, H.R. MacDonald, R. Sadoul, J.D. Becherer, A. Proudfoot, R. Solari, and J. Tschopp. 1995. Granzyme A is an interleukin 1 beta-converting enzyme. *J Exp Med* 181:1917-1922.
780. Coeshott, C., C. Ohnemus, A. Pilyavskaya, S. Ross, M. Wieczorek, H. Kroona, A.H. Leimer, and J. Cheronis. 1999. Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci U S A* 96:6261-6266.
781. Sugawara, S., A. Uehara, T. Nochi, T. Yamaguchi, H. Ueda, A. Sugiyama, K. Hanzawa, K. Kumagai, H. Okamura, and H. Takada. 2001. Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. *J Immunol* 167:6568-6575.
782. Omoto, Y., K. Tokime, K. Yamanaka, K. Habe, T. Morioka, I. Kurokawa, H. Tsutsui, K. Yamanishi, K. Nakanishi, and H. Mizutani. 2006. Human mast cell chymase cleaves pro-IL-18 and generates a novel and biologically active IL-18 fragment. *J Immunol* 177:8315-8319.
783. Siegmund, B. 2010. Interleukin-18 in intestinal inflammation: friend and foe? *Immunity* 32:300-302.
784. Al-Sadi, R., D. Ye, H.M. Said, and T.Y. Ma. 2010. IL-1beta-induced increase in intestinal epithelial tight junction permeability is mediated by MEKK-1 activation of canonical NF-kappaB pathway. *Am J Pathol* 177:2310-2322.
785. Al-Sadi, R.M., and T.Y. Ma. 2007. IL-1beta causes an increase in intestinal epithelial tight junction permeability. *J Immunol* 178:4641-4649.
786. Broz, P., K. Newton, M. Lamkanfi, S. Mariathasan, V.M. Dixit, and D.M. Monack. 2010. Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J Exp Med* 207:1745-1755.
787. O'Hagan, D.T., H. Jeffery, K.J. Maloy, A.M. Mowat, D. Rahman, and S.J. Challacombe. 1995. Biodegradable microparticles as oral vaccines. *Adv Exp Med Biol* 371B:1463-1467.
788. Lavelle, E.C. 2006. Lectins and microparticles for enhanced oral vaccination. *Methods* 38:84-89.

789. Strober, W., and T. Watanabe. 2011. NOD2, an intracellular innate immune sensor involved in host defense and Crohn's disease. *Mucosal Immunol* 4:484-495.
790. Andoh, A., S. Bamba, M. Brittan, Y. Fujiyama, and N.A. Wright. 2007. Role of intestinal subepithelial myofibroblasts in inflammation and regenerative response in the gut. *Pharmacol Ther* 114:94-106.
791. Pinchuk, I.V., R.C. Mifflin, J.I. Saada, and D.W. Powell. 2010. Intestinal mesenchymal cells. *Curr Gastroenterol Rep* 12:310-318.
792. Bamba, S., C.Y. Lee, M. Brittan, S.L. Preston, N.C. Direkze, R. Poulsom, M.R. Alison, N.A. Wright, and W.R. Otto. 2006. Bone marrow transplantation ameliorates pathology in interleukin-10 knockout colitic mice. *J Pathol* 209:265-273.
793. Brittan, M., V. Chance, G. Elia, R. Poulsom, M.R. Alison, T.T. MacDonald, and N.A. Wright. 2005. A regenerative role for bone marrow following experimental colitis: contribution to neovasculogenesis and myofibroblasts. *Gastroenterology* 128:1984-1995.
794. Travassos, L.H., L.A. Carneiro, M. Ramjeet, S. Hussey, Y.G. Kim, J.G. Magalhaes, L. Yuan, F. Soares, E. Chea, L. Le Bourhis, I.G. Boneca, A. Allaoui, N.L. Jones, G. Nunez, S.E. Girardin, and D.J. Philpott. 2010. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* 11:55-62.
795. Homer, C.R., A.L. Richmond, N.A. Rebert, J.P. Achkar, and C. McDonald. 2010. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology* 139:1630-1641, 1641 e1631-1632.
796. Cadwell, K., K.K. Patel, N.S. Maloney, T.C. Liu, A.C. Ng, C.E. Storer, R.D. Head, R. Xavier, T.S. Stappenbeck, and H.W. Virgin. 2010. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell* 141:1135-1145.
797. Katakura, K., J. Lee, D. Rachmilewitz, G. Li, L. Eckmann, and E. Raz. 2005. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. *J Clin Invest* 115:695-702.
798. Damiano, J.S., V. Oliveira, K. Welsh, and J.C. Reed. 2004. Heterotypic interactions among NACHT domains: implications for regulation of innate immune responses. *Biochem J* 381:213-219.
799. Marina-Garcia, N., L. Franchi, Y.G. Kim, D. Miller, C. McDonald, G.J. Boons, and G. Nunez. 2008. Pannexin-1-mediated intracellular delivery of muramyl dipeptide induces caspase-1 activation via cryopyrin/NLRP3 independently of Nod2. *J Immunol* 180:4050-4057.
800. Ferwerda, G., M. Kramer, D. de Jong, A. Piccini, L.A. Joosten, I. Devesaginer, S.E. Girardin, G.J. Adema, J.W. van der Meer, B.J. Kullberg, A. Rubartelli, and M.G. Netea. 2008. Engagement of NOD2 has a dual effect on proIL-1beta mRNA transcription and secretion of bioactive IL-1beta. *Eur J Immunol* 38:184-191.
801. Kepp, O., L. Galluzzi, and G. Kroemer. 2011. Mitochondrial control of the NLRP3 inflammasome. *Nat Immunol* 12:199-200.

802. Mahida, Y.R., K.C. Wu, and D.P. Jewell. 1989. Respiratory burst activity of intestinal macrophages in normal and inflammatory bowel disease. *Gut* 30:1362-1370.
803. Simmonds, N.J., R.E. Allen, T.R. Stevens, R.N. Van Someren, D.R. Blake, and D.S. Rampton. 1992. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* 103:186-196.
804. Williams, J.G., L.E. Hughes, and M.B. Hallett. 1990. Toxic oxygen metabolite production by circulating phagocytic cells in inflammatory bowel disease. *Gut* 31:187-193.
805. McKenzie, S.J., M.S. Baker, G.D. Buffinton, and W.F. Doe. 1996. Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J Clin Invest* 98:136-141.
806. Ha, E.M., C.T. Oh, Y.S. Bae, and W.J. Lee. 2005. A direct role for dual oxidase in Drosophila gut immunity. *Science* 310:847-850.
807. Flores, M.V., K.C. Crawford, L.M. Pullin, C.J. Hall, K.E. Crosier, and P.S. Crosier. 2010. Dual oxidase in the intestinal epithelium of zebrafish larvae has anti-bacterial properties. *Biochem Biophys Res Commun* 400:164-168.
808. Ha, E.M., K.A. Lee, Y.Y. Seo, S.H. Kim, J.H. Lim, B.H. Oh, J. Kim, and W.J. Lee. 2009. Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. *Nat Immunol* 10:949-957.
809. Rokutan, K., T. Kawahara, Y. Kuwano, K. Tominaga, K. Nishida, and S. Teshima-Kondo. 2008. Nox enzymes and oxidative stress in the immunopathology of the gastrointestinal tract. *Semin Immunopathol* 30:315-327.
810. Kajino-Sakamoto, R., E. Omori, P.K. Nighot, A.T. Blikslager, K. Matsumoto, and J. Ninomiya-Tsuji. 2010. TGF-beta-activated kinase 1 signaling maintains intestinal integrity by preventing accumulation of reactive oxygen species in the intestinal epithelium. *J Immunol* 185:4729-4737.
811. Lipinski, S., A. Till, C. Sina, A. Arlt, H. Grasberger, S. Schreiber, and P. Rosenstiel. 2009. DUOX2-derived reactive oxygen species are effectors of NOD2-mediated antibacterial responses. *J Cell Sci* 122:3522-3530.
812. El Hassani, R.A., N. Benfares, B. Caillou, M. Talbot, J.C. Sabourin, V. Belotte, S. Morand, S. Gnidehou, D. Agnandji, R. Ohayon, J. Kaniewski, M.S. Noel-Hudson, J.M. Bidart, M. Schlumberger, A. Virion, and C. Dupuy. 2005. Dual oxidase2 is expressed all along the digestive tract. *Am J Physiol Gastrointest Liver Physiol* 288:G933-942.
813. Oehlers, S.H., M.V. Flores, C.J. Hall, S. Swift, K.E. Crosier, and P.S. Crosier. 2011. The inflammatory bowel disease (IBD) susceptibility genes NOD1 and NOD2 have conserved anti-bacterial roles in zebrafish. *Dis Model Mech*
814. Hulbert, S.H., C.A. Webb, S.M. Smith, and Q. Sun. 2001. Resistance gene complexes: evolution and utilization. *Annu Rev Phytopathol* 39:285-312.
815. Scherz-Shouval, R., and Z. Elazar. 2011. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem Sci* 36:30-38.
816. Kaushik, S., and A.M. Cuervo. 2006. Autophagy as a cell-repair mechanism: activation of chaperone-mediated autophagy during oxidative stress. *Mol Aspects Med* 27:444-454.

817. Kim, I., S. Rodriguez-Enriquez, and J.J. Lemasters. 2007. Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys* 462:245-253.
818. Huang, J., V. Canadien, G.Y. Lam, B.E. Steinberg, M.C. Dinauer, M.A. Magalhaes, M. Glogauer, S. Grinstein, and J.H. Brumell. 2009. Activation of antibacterial autophagy by NADPH oxidases. *Proc Natl Acad Sci U S A* 106:6226-6231.
819. Johnson, K.R., C.C. Marden, P. Ward-Bailey, L.H. Gagnon, R.T. Bronson, and L.R. Donahue. 2007. Congenital hypothyroidism, dwarfism, and hearing impairment caused by a missense mutation in the mouse dual oxidase 2 gene, Duox2. *Mol Endocrinol* 21:1593-1602.
820. Harbord, M., M. Novelli, B. Canas, D. Power, C. Davis, J. Godovac-Zimmermann, J. Roes, and A.W. Segal. 2002. Ym1 is a neutrophil granule protein that crystallizes in p47phox-deficient mice. *J Biol Chem* 277:5468-5475.
821. Dinarello, C.A. 2011. A clinical perspective of IL-1beta as the gatekeeper of inflammation. *Eur J Immunol* 41:1203-1217.
822. Larsen, C.M., M. Faulenbach, A. Vaag, A. Volund, J.A. Ehses, B. Seifert, T. Mandrup-Poulsen, and M.Y. Donath. 2007. Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med* 356:1517-1526.
823. Vertex Pharmaceuticals, I. 2011.