REGULATION OF INTESTINAL IMMUNE RESPONSES BY THE RETINOIC ACID INDUCIBLE TRANSCRIPTION FACTOR HIC1

by

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Abstract

The intestinal immune system must be able to respond to a wide variety of infectious organisms while maintaining tolerance to non-pathogenic microbes and food antigens. The Vitamin A metabolite all-*trans*-retinoic acid (atRA) has been implicated in the regulation of this balance, partially by regulating T cell and innate lymphoid cell (ILC) responses in the intestine. However, the molecular mechanisms of atRA-dependent intestinal immunity and homeostasis remain elusive. The work herein investigates the role for the POK/ZBTB family transcriptional repressor Hypermethylated in cancer 1 (HIC1, ZBTB29) in the regulation of immune cell responses in the intestine. Using genetically modified mice I demonstrate that *Hic1* is specifically expressed in immune cells of the intestine at steady state in an atRA dependent manner. I further show that mice with a T cell-specific deletion of *Hic1* have reduced numbers of T cells in the intestine. In addition, I demonstrate that HIC1-deficient T cells overproduce IL-17A *in vitro* and *in vivo*, and fail to induce intestinal inflammation in multiple murine models of inflammatory bowel disease, identifying a critical role for HIC1 in the regulation of T cell function in the intestinal microenvironment under both homeostatic and inflammatory conditions. In other studies, I investigated the cell-intrinsic role of HIC1 in intestinal ILC populations. I demonstrate that in the absence of HIC1, group 3 ILCs (ILC3s) are lost, resulting in increased susceptibility to infection with the murine bacterial pathogen *Citrobacter rodentium*. In addition, the loss of ILC3s leads to a local and systemic increase in IFN-γ-producing T cells that prevents the development of protective immunity against infection with the parasitic helminth *Trichuris muris*. Together the results presented here provide further insight into atRA mediated immune responses in the intestine as atRA-dependent expression of HIC1 in T cells and ILC3s regulate intestinal homeostasis, inflammation and protective immunity.

Lay summary

Inflammation of the gut is a major cause of disease and death around the world, with over 200,000 Canadians suffering from a form of inflammatory bowel disease (IBD). Local gut cells and white blood cells control the immune response in the intestine and must balance fighting infection with shutting down inflammation to prevent IBD. Vitamin A helps keep this balance, but its exact role is not fully known. This study shows that the molecule HIC1, found only in white blood cells within the gut, is controlled by vitamin A and is required for the development of IBD. Removal of HIC1 from certain cell types results in fewer white blood cells in the gut and prevents severe inflammation but can also lower response to infection. This study provides new information about the cells and molecules that affect the gut immune system, which may be useful for making new treatments for IBD.

Preface

A version of Chapter 3, at the time of writing, is in press: **Burrows K**, Antignano F, Bramhall M, Chenery A, Scheer S, Korinek V, Underhill TM, Zaph C. The transcriptional repressor HIC1 regulates intestinal immune homeostasis. *Mucosal Immunology.* 2017. doi: 10.1038/mi.2017.17. I designed and conducted 80% of experiments and wrote the manuscript. Dr. Frann Antignano conducted 10% of the experiments. Dr. Alistair Chenery conducted 10% of the experiments. Dr. Michael Bramhall provided intellectual content. Dr. Sebastian Scheer provided intellectual content. Dr. Vladimir Korinek provided *Hic1*^{Citrine} mice and intellectual content. Dr. T. Michael Underhill provided *Hic1*$^{fl/fl}$ mice and intellectual content. Dr. Colby Zaph designed experiments and edited the manuscript.

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List of abbreviations

αLP	α4β7-expressing lymphoid progenitor
A/E	Attaching and effacing
Ab	Antibody
ADH	Alcohol dehydrogenase
AICD	Activation induced cell death
ALDH	Retinal dehydrogenase
AMP	Antimicrobial peptide
APC	Antigen presenting cell
atRA	All-*trans*-retinoic acid
BCL6	B-Cell CLL/Lymphoma 6
BCR	B cell receptor
BM	Bone marrow
BTB	Broad-complex tramtrac, bric-à-brac
CCR	C-C motif chemokine receptor
CD	Cluster of Differentiation
ChILP	Common helper-like ILC progenitor
CLP	Common lymphoid progenitor
CNS	Central nervous system
CtBP	C terminal binding protein
CTL	Cytotoxic T lymphocyte
DAMP	Danger associated molecular pattern

DC	Dendritic cell
DSS	Dextran sodium sulfate
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
GC	Germinal centre
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
HIC1	Hypermethylated in cancer 1
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILC1	Group 1 ILC
ILC2	Group 2 ILC

ILC3	Group 3 ILC
iT_{reg}	Inducible regulatory T cell
Lin	Lineage
LP	Lamina propria
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer cell
M cell	Microfold cell
MALT	Mucosal associate lymphoid tissue
MHC	Major histocompatibility complex
MHCI	MHC class I
MHCII	MHC class II
mLN	Mesenteric lymph nodes
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
NKT cell	Natural killer T cell
nTreg	Natural regulatory T cell
PAMP	Pathogen associated molecular pattern
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PLZF	Promyelocytic leukemia zinc finger
POK/ZBTB	POZ and Kruppel/Zinc Finger and BTB
PP	Peyer's Patch
PRR	Pattern recognition receptor

RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
ROR-γt	RAR – related orphan receptor-gamma t
RXR	Retinoid X receptor
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
T_{FH}	T follicular helper cell
TGFβ	Transforming growth factor beta
T_H	T helper cell
ThPOK	T helper-inducing POZ/Kruppel-like factor
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
T_{reg}	regulatory T cell
T_{RM}	Tissue resident memory cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
VAD	Vitamin A-deficient
qRT-PCR	Quantitative real time polymerase chain reaction

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Chapter 1: Introduction

1.1 Overview

The intestinal immune system encompasses an immense network of cells responsible for protection from invading organisms at a site with direct contact with the external environment. Immune cells at this barrier site are under constant barrage from microorganisms and other potential antigens. Therefore, these intestinal immune cells must maintain a tightly regulated balance between activation and tolerance in order to provide rapid protection from harmful pathogens while not reacting to innocuous antigens. Within this network of cells, T cells and innate lymphoid cells (ILCs) have been shown to play important roles in maintaining this balance as both cell types contribute to promoting proper immune responses to remove pathogens as well as promoting tolerance in order to prevent inflammatory diseases. Dysregulated responses by either T cells or ILCs can lead to chronic inflammation or persistent infections [1,2]. Therefore, it is critical to further our understanding of the factors that influence proper function of these cells under homeostasis as well as during infection or inflammation. This dissertation will focus on the role of a transcription factor – Hypermethylated in cancer 1 (HIC1, ZBTB29) – in T cell and ILC function in regards to intestinal helminth and bacterial infections as well as multiple models of intestinal inflammation.

1.2 The immune system

The immune system is an organization of cells and molecules that have evolved specialized roles to defend the host from invading pathogens. Cells of the immune system each serve different purposes but act in concert to form appropriate responses to different pathogens.

These responses are carried out by two main entities: the innate and the adaptive immune systems. The innate immune system is thought to be much more evolutionary ancient but is critical as it is the first to respond to a pathological challenge [3]. In contrast, the adaptive immune system is believed to have evolved more recently and offers a more specific response with longer lasting protection through immunological memory; which allows its responses to improve upon repeated exposure to the same pathogen [3,4]. Together, these systems work to protect the host from invading virus, bacteria and parasites as well as harmful toxins.

1.2.1 Adaptive immunity

The adaptive immune system is comprised solely of T-lymphocytes and B-lymphocytes. At homeostasis these cells can be found at secondary lymphoid structures such as: the spleen, lymph nodes and mucosa associated lymphoid tissues (MALT) in distinctive T and B cell zones.

B cells are generated in the bone marrow and confer antibody (Ab) mediated humoral immunity [5]. B cells express genetically rearranged B cell receptors (BCR) on their cell surface, which are designed to detect antigens (foreign peptides). Once B cells become activated through their BCR they secrete Abs, which are designed to bind to specific epitopes on antigens and initiate a range of immune responses. Initially, activated B cells will produce immunoglobulin (Ig) M Abs [5]. IgM Abs serves multiple purposes such as activation of the complement pathway, opsonization of targets for phagocytosis, or neutralization of toxins [5]. However, extrinsic signals can cause activated B cells to undergo further somatic hyper-mutation and class-switching, which result in the production of higher affinity Abs of different isotypes (IgG, IgE or IgA) [5]. Each class of Ab has a different function based on its constant heavy chain (Fc). The Fc region can determine which anatomical locations are accessible to the Ab. IgM is restricted to blood and

lymph due to its large size as a pentamer, IgG and IgE are monomers and can freely diffuse into tissues from the blood [5]. While, IgA forms dimers, and is actively transported across the mucosal epithelium where it plays an important role in neutralizing pathogens and toxins [5].

T cells develop in the thymus and are characterized by their expression of a T cell receptor (TCR) that can also undergo genetic rearrangement during development in order to recognize an unlimited combination of peptides bound to major histocompatibility (MHC) complexes expressed on other cells. T cells can be further characterized by their function and expression of cell surface molecules. Cytotoxic T lymphocytes (CTLs) express cluster of differentiation 8 (CD8) in order to recognize peptide presented on MHC class I (MHCI) molecules and are responsible for killing infected cells through the release of cytotoxic mediators such as perforin or granzyme [6]. T helper (T_H) cells are defined by their expression of CD4, which allows them to recognize peptide loaded onto MHC class II (MHCII) molecules of professional antigen presenting cells (APCs) [7]. T_H cells function by releasing cytokines to influence the responses of other cell types and can further be broken down into subtypes based on which cytokines they produce.

1.2.1.1 T helper cell subsets

Following activation of naïve $CD4^+$ T_H cells by APCs, co-stimulatory molecules and the cytokine milieu will program T_H cells to differentiate into one of several lineages. Each T_H cell lineage has its own set of master transcription factors that control lineage fate and stability as well as production of characteristic cytokines that carry out its function (Summarized in **Figure 1.1**). The T_H1 lineage, which is important for immunity to viral and intracellular bacterial infections, is driven by interleukin 12 (IL-12) and promotes expression of signal transducer and

activator of transcription (STAT) 4 and TBET, which in turn activate expression of interferon gamma (IFN-γ) [7]. T_H2 differentiation, which is critical for antibody mediated humoral immune responses as well as immunity to helminth infections, is induced by IL-4. T_H2 cells are characterized by their expression of transcription factors STAT6 and GATA3 as well as the key cytokines IL-4, IL-5 and IL-13 [7]. T_H17 differentiation is important for defense against extracellular pathogens and barrier integrity at mucosal surfaces. The T_H17 lineage is induced by IL-6 and transforming growth factor β (TGFβ) to express STAT3 and RAR–related orphan receptor (ROR)-γt and produce key cytokines IL-17A, IL-21 and IL-22 [8]. Finally, regulatory T (T_{reg}) cells are on the opposite end of the spectrum from the other T_H cell lineages and are responsible for dampening immune responses and preventing immunopathology [9]. T_{reg} cells can further be categorized into thymically derived natural (nT_{reg}) or peripheral induced (iT_{reg}) cells that respond to IL-2 and TGFβ [10]. Both subsets of T_{reg} cells are characterized by expression of master transcription factors STAT5 and forkhead box p3 (FOXP3) and produce immunosuppressive cytokines IL-10 and TGFβ [9].

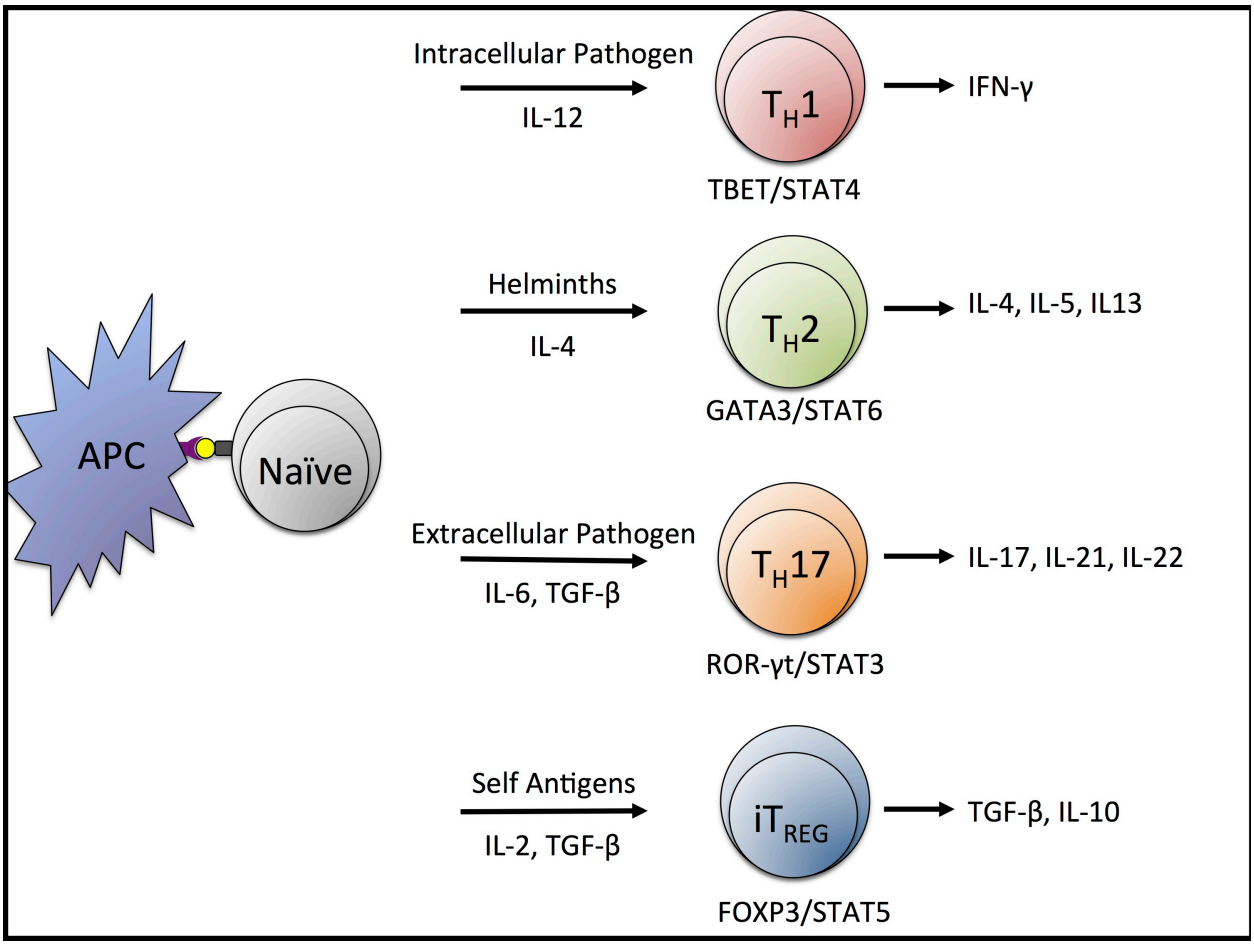

Figure 1.1 $CD4^+$ T_H cell lineages.

Upon presentation of antigen from professional antigen presenting cells, naïve $CD4^+$ T_H cells can differentiate into one of several lineages depending on the stimuli. T_H1 cells differentiate in response to intracellular pathogens (virus, bacteria) and express TBET and STAT4 and produce IFN-γ. T_H2 cells emerge in response to helminth infections and produce IL-4, IL-5 and IL-13 as well as express GATA3 and STAT6. T_H17 cell differentiate to combat extracellular pathogens (bacteria, fungi) and express STAT3, ROR-γt and produce IL-17 and IL-22. T_{reg} cells emerge to dampen immune responses and prevent immunopathology by expressing STAT5 and FOXP3 and producing IL-10 and TGFβ.

Classically, these T_H lineages were thought to be quite stable with mechanisms such as transcriptional silencing of lineage promiscuous genes and epigenetic regulation reinforcing differentiation. For example, the T_H1 cell transcription factor TBET can block transcriptional programs as well as influence epigenetic modifications (e.g. DNA methylation and histone modifications) that silence lineage promiscuous genes such as *Il4* and block the differentiation of T_H2 cells [11–13]. However, evidence is emerging that demonstrates plasticity among T_H cell lineages. T cells have now been shown to repolarize into mixed or alternative fates and exhibit adaptability to changing environments [14,15]. This phenomenon has been regularly observed in regards to the T_H17 lineage. Through lineage tracing experiments, studies have shown that during models of inflammatory bowel disease T_H17 cells can become more T_H1-like and begin to express TBET and produce IFN-γ [16–18]. However, under different conditions, it has also been demonstrated that T_H17 cells can take on a more regulatory phenotype by producing IL-10 and ceasing production of IL-17A [19,20]. Thus demonstrating that the classic T_H paradigm of linear differentiation may not fully encompass the complexity of T_H cell fates and a better understanding of the molecules and mechanisms that control T_H cell fates and functions is required.

1.2.2 Innate immunity

The innate immune system is much more primitive than the adaptive immune system and serves as the first line of defense against pathogens. Cells of the innate immune system include: epithelial cells, myeloid cells, and innate lymphoid cells (ILCs). Epithelial cells are under appreciated for the role they play in the immune system but are extremely important as they serve as the physical barrier from the external environment, excrete antimicrobial peptides and

form a close relationship with commensal flora, which conveniently outcompetes potentially pathogenic bacteria for nutrients [21]. Myeloid cells also contribute to innate immunity in a number of ways. They perform phagocytosis of pathogens, produce different proteases and enzymes to kill pathogens as well as produce cytokines and chemokines to instruct other immune cells [22]. Myeloid cells are a broad group of immune cells that consist of monocytes, macrophages, mast cells, and granulocytes (basophils, eosinophils, neutrophils) [23]. Although certain dendritic cells (DCs) also belong to the monocyte lineage, conventional DC development diverges from myeloid cell differentiation earlier in hematopoiesis to form its own lineage [24]. Similar to macrophages, DCs are also responsible for production of cytokines and phagocytosis of pathogens; however, they also perform a unique function of constantly sampling its environment through a process known as micropinocytosis [24,25].

Innate immune cells are activated through germ line pattern recognition receptors (PRRs), which include: Toll like receptors (TLRs), NOD-like receptors and C-type lectin receptors [22,26,27]. These PRRs have each evolved to recognize certain pathogen associated molecular patterns (PAMPs). Some examples of PAMPs include: fungal beta-glucan (detected by TRL2) viral double stranded RNA (detected by TLR3), bacterial lipopolysaccharide (LPS) (detected by TLR4) and bacterial flagellin (detected by TLR5) [22]. Upon activation of these PRRs, different signaling cascades are used in order to activate specific cellular functions in order to combat the specific pathogen detected.

Although the primary role of innate immune cells is to control an infection, they must also signal to activate adaptive immune cells. This function is largely carried out by macrophages and DCs, which act as a bridge between the innate and adaptive immune system. Upon uptake of antigen (extracellular proteins or pathogens), these cells will enzymatically break

down these antigens into short polypeptide chains that are then loaded onto MHCII molecules and exported to the cell surface [28]. These cells can then migrate into lymph nodes where they come in contact with $CD4^+$ T_H cells and can activate them through interactions with their TCR. Similarly, DCs can load antigen onto MHCI and activate $CD8^+$ CTLs through a process known as cross-presentation [29].

1.2.2.1 Innate lymphoid cells

Innate lymphoid cells (ILCs) are a newly characterized subset of innate immune cells that bear a striking resemblance to adaptive lymphoid cells but do not rely on the genetic rearrangement of receptors such as the TCR to function. These ILCs lack all expression of known myeloid and dendritic cell markers such as: CD11b, CD11c and Gr1, and express transcription factors and cytokines associated with T_H cells [2]. As such, ILCs have been categorized into three main groups based on their resemblance to T_H cell subsets (Summarized in **Figure 1.2**).

Group 1 ILCs (ILC1s) express surface markers CD161 (NK1.1) and CD335 (NKp46) as well as T_H1 associated genes TBET and IFN-γ and are important for defense against intracellular pathogens but have minimal cytotoxic capabilities [30]. Classic natural killer (NK) cells are also grouped into the ILC1 subset and resemble $CD8^+$ CTLs as they induce cellular killing of viral infected cells or tumor cells through the release of perforin and granzyme [30]. NK cells can be identified by their expression of NK1.1, NKp46 and the transcription factor EOMES [30].

Group 2 ILCs (ILC2s) (previously called: innate helper 2 cells, natural helper cells and nuocytes) are characterized by their similarity to T_H2 cells. They express surface receptors for IL-33 (ST2), IL-25 (IL-17RB) and TSLP (TSLPR), as well as the transcription factor GATA3

and produce cytokines IL-4, IL-5 and IL-13 in order to facilitate allergic responses and immunity to helminth infections [31].

Finally, group 3 ILCs (ILC3s), which closely resemble T_H17 cells, express ROR-γt and are critical for immunity to extracellular pathogens as well as for promoting normal epithelial barrier function. ILC3s can further be characterized into subgroups based on expression of other surface marker molecules and secretion of cytokines. The subgroups of ILC3s have both overlapping and distinct functions. Every subset has been shown to produce IL-22 but C-C motif chemokine receptor (CCR)6^+ ILC3s can also produce IL-17A to combat fungal infections [32–34]. $CCR6^+$ ILC3s can also be classified as $CD4^+$ Lymphoid tissue inducer (LTi) cells [33] that produce lymphotoxins and are critical for the generation of secondary lymphoid structures [35,36]. Conversely, $CCR6^-$ ILC3s mostly express natural cytotoxic receptor (NCR; NKp46) and TBET and produce IFN-γ and has been shown to be critical in tissue inflammation [37,38]. There is also a heterogeneous population of NCR^- ILC3s that contains an LTi-like population that is important for cryptopatch and isolated lymphoid follicle development [39–41] as well as a population that can further differentiate into NCR^+ ILC3s [42].

Developmentally, ILCs are derived from the same common lymphoid progenitor (CLP) that gives rise to T and B cells [43]. This CLP will generate an ILC specific α4β7 expressing lymphoid progenitor (αLP) that depends on nuclear factor IL-3 induced (NFIL3) and will no longer support T or B cell development [44,45]. This progenitor can then form either an EOMES expressing NK progenitor or an inhibitor of DNA binding 2 (ID2) driven common helper innate lymphoid cell progenitor (ChILP) [46,47]. Fetal liver derived LTi cells can then be generated directly from ChILPs, but the other ILC lineages must first go through a committed ILC precursor (ILCP) that is driven by promyelocytic leukemia zinc finger (PLZF) [48]. Finally, ILCPs

can differentiate into one of the ILC subsets. A committed ILC2 progenitor (ILC2p) has been identified in the bone marrow [49], however, the existence of an ILC1 or an ILC3 progenitor is still not fully understood but evidence suggest a population of ILC3p exists in human tonsils [50]. As ILCs are still relatively newly defined, the factors that control their differentiation and function are yet to be fully elucidated.

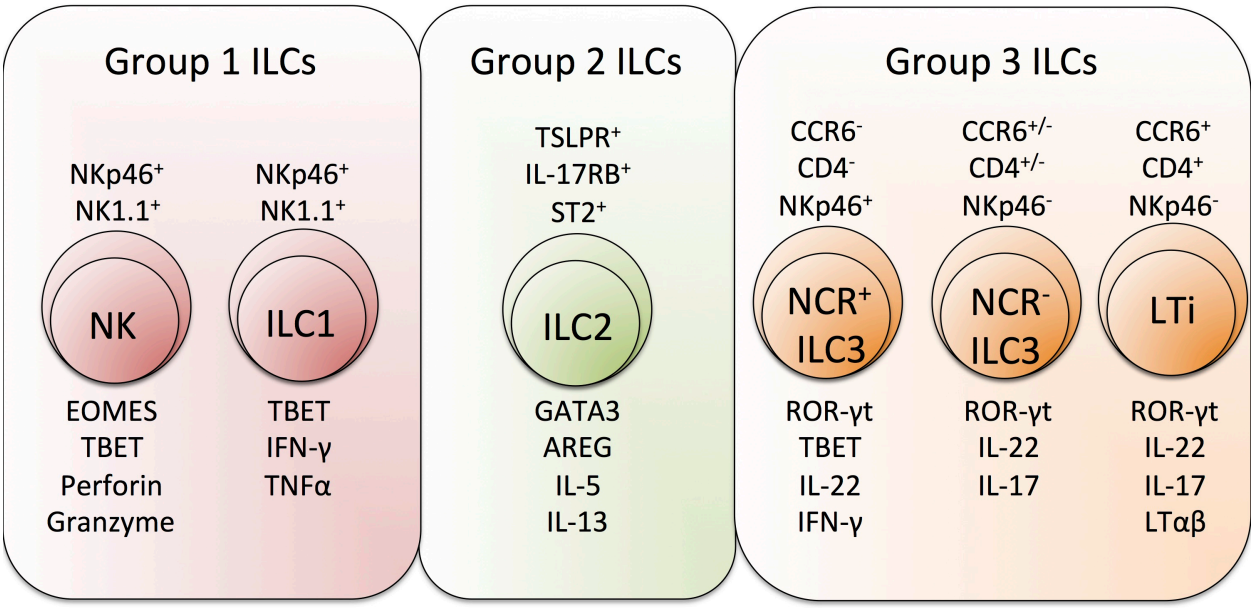

Figure 1.2 Innate lymphoid cell subsets.

Innate lymphoid cell subsets closely resemble T_H cell subsets and can be defined by similar expression of transcription factors and cytokines. Group 1 ILCs consist of NK cells and ILC1s and are characterized by the expression of TBET and IFN-γ. Group 2 ILCs produce IL-4, IL-5 and IL-13 and express GATA3. Group 3 ILCs all express ROR-γt and IL-22 but can be subdivided based on slightly specialized functions and expression of surface molecules NKp46, CD4 and CCR6.

1.3 Intestinal immune organization

The gastrointestinal tract is one of the major tissues of the mucosal immune system (others include: the nasal/oral cavities, the lungs, and the urogenital tract). The gastrointestinal tract is a barrier organ with a large surface area exposed to the external environment, ranging from 30 to 300 square metres in humans [51], and is uniquely required to interact with the trillions of commensal bacteria that dwell within the gut. Consequently, the gastrointestinal tract harbors the greatest number of immune cells in the body and these cells are required to analyze the contents of the intestinal lumen to distinguish the potential hazardous pathogens from the innocuous antigens and commensals and respond accordingly.

The first line of defense within the intestine is a single layer of epithelial cells that provide a physical barrier to separate the lumen from the cells of the intestinal lamina propria. Intestinal epithelial cells (IECs) can be characterized into numerous different cell types including: goblet cells, paneth cells, enterocytes, microfold cells, and tuft cells. Each cell type has a different function within the gut such as absorbing nutrients, secreting hormones and immune surveillance. Specifically, goblet cells are responsible for producing mucins that make up the mucous layer that protects epithelial cells from the luminal contents [21]. Paneth cells can secrete antimicrobial peptides (AMPs) such as β-defensins that protect from bacterial infections and regulate the commensal populations [21]. Tuft cells are chemosensory and are a key source of IL-25 that promotes a type 2 immune response [52]. Microfold cells (M cells) sample antigens from the lumen and deliver them to the mucosal associated lymphoid tissues (MALT) [21].

Within the epithelium there are populations of intra-epithelial lymphocytes (IELs) that are capable of rapidly responding to antigens. The IEL population is heterogeneous and is represented by both conventional and nonconventional T cell subsets. IEL can be broken down

into 2 major groups based on their expression of either the common αβ TCR or an unconventional γδ TCR. TCRγδ$^+$ IELs do not have a clearly defined TCR specificity but have been shown to have multiple functions including: cytokine production, lysis of infected cells, as well as regulation of epithelial cell integrity by producing keratinocyte growth factor [53–55]. In contrast, conventional TCRαβ$^+$ IELs are better defined and appear to be antigen experienced CD8$^+$ CTLs, as they resemble peripheral memory T cells, accumulate over time and express the tissue resident memory T cell (T_{RM}) surface markers CD69 and CD103 [56,57].

Under the epithelium in the lamina propria (LP) is a large and heterogeneous population of immune cells that include T cells, plasma cells, DCs, macrophages, neutrophils, eosinophils, mast cells and ILCs. LP $CX_3CR_1^+$ mononuclear phagocytes and LP DCs are responsible for sampling luminal contents of the intestine either by extending dendrites through epithelial cells into the lumen or through goblet cell associated passages in order to take up antigen [58,59]. Upon processing of antigen, DCs can then migrate to draining lymph nodes where they can induce tolerance or activate T and B cell responses [58,60]. The DC populations in the gut can be divided into two main groups, the CD103$^+$CD11b$^+$ DC are the dominant population that is critical in driving T_H17 responses while the minor population of CD103$^+$CD11b$^-$ DCs does not yet have a clearly defined role [61,62]. Eosinophils and mast cells are two populations that represent granulocytes within the intestinal LP and are responsible for the release of cytokines, cytotoxic mediators, and chemokines in response to parasitic helminth infections [63]. Another granulocytic population in the LP is the neutrophils, which can eliminate bacterial infections by multiple mechanisms including: phagocytosis, degranulation and release of antimicrobial proteins (e.g. lysozyme), and by releasing neutrophil extracellular traps (a mixture of DNA, enzymes and proteins that immobilizes pathogens) [64]. The plasma cells found in the LP are responsible for

producing IgA that can permeate the epithelial layer and enter the lumen of the intestine in order to neutralize toxins and pathogenic microbes [65]. The T cell population of the LP, unlike the IELs, consists of both $CD8^+$ and $CD4^+$ $TCR\alpha\beta^+$ cells with very few $TCR\gamma\delta^+$ cells. In addition, the LP T cells consist of both effector and memory populations with a large portion of IL-17A producing T_H17 cells as well as $FOXP3^+$ regulatory T cells to promote tolerance [66]. Finally, all 3 subtypes of ILCs are present in the intestinal LP. ILC3s represent the largest group of ILCs and are critical in maintaining intestinal barrier function as well as regulating commensal bacterial populations and containing bacterial infections until the adaptive immune system can respond [67–70]. ILC1s and ILC2s are also present in the LP, although at much lower numbers and are involved in inflammation and immune responses to intestinal helminth infections, respectively [71,72].

Intestinal immune cells are further organized in the peyer's patches (PP) and mesenteric lymph nodes (mLN), which make up the intestinal MALT. PPs are lymphoid aggregates found along the small intestine while the mLN are aggregates found throughout the mesentery. Both structures contain large numbers of naïve B and T cells awaiting antigenic stimulation, however delivery of antigen varies between these sites. PPs are located above epithelial M cells that will allow the passage of antigen into this lymphocyte rich site through a process called transcytosis where APCs can then process and present the antigen to lymphocytes [73]. Conversely, the mLN rely on the migration of intestinal DCs through the lymphatics to deliver antigen [58]. Given this complex and enormous system, all of the factors and mechanisms that control intestinal immune homeostasis have yet to be fully understood. (Summarized in **Figure 1.3**)

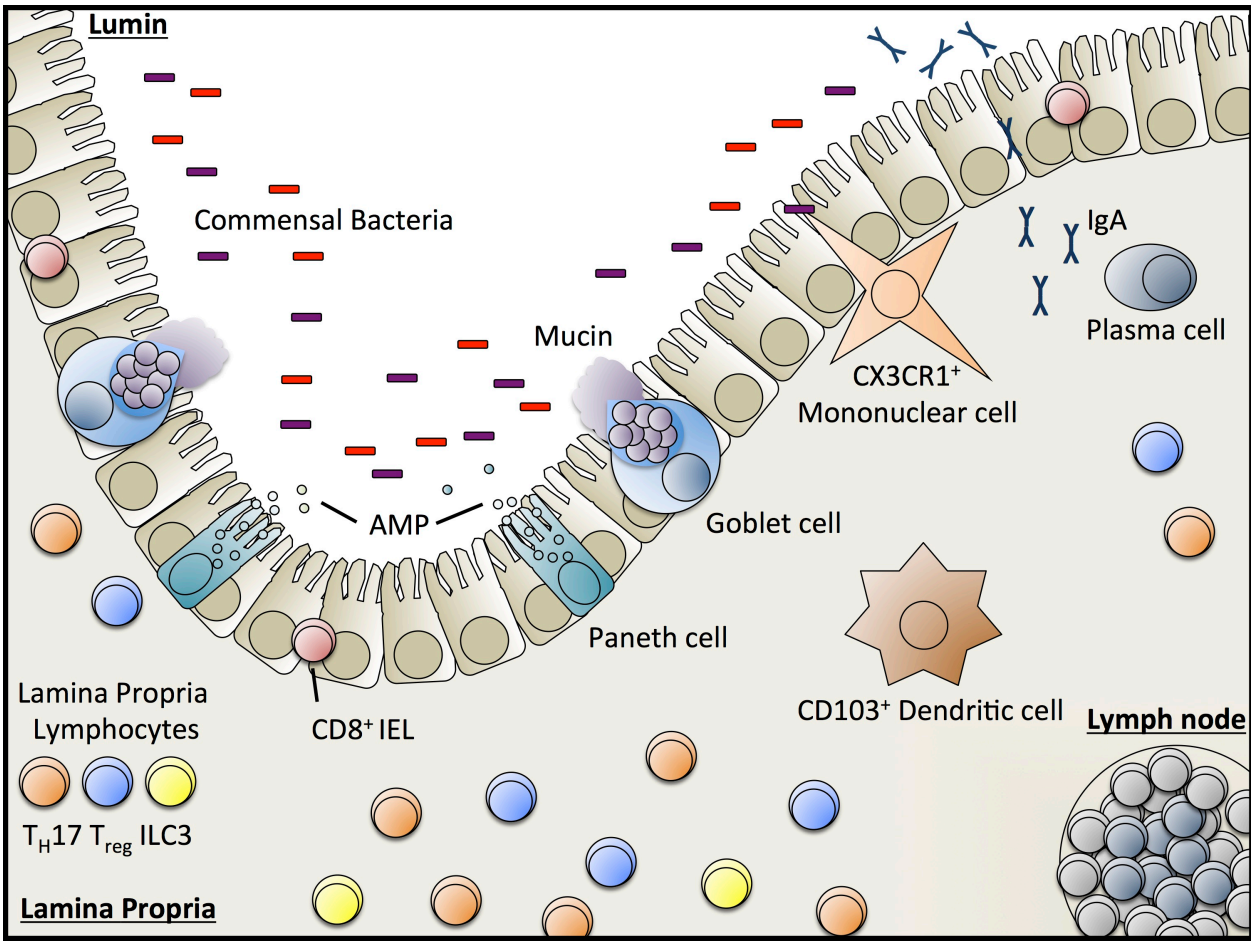

Figure 1.3 Simplified diagram of intestinal immune cell organization.

A single layer of epithelial cells separates the intestinal lumen from the lamina propria. Within the epithelial layer, goblet cells secrete mucins and paneth cells secrete antimicrobial peptides (AMP) to control the microbial populations. $CD8^+$ lymphocytes integrate within the intestinal epithelial layer. In the lamina propria, $CX3CR1^+$ mononuclear cells and dendritic cells process and present antigens. IgA producing plasma cells secrete antibodies that cross the epithelial layer. T_H17, T_{reg} and ILC3 make up the majority of the lamina propria lymphocyte population. Reservoirs of naïve T and B cells reside in nearby lymph nodes.

1.3.1 Inflammatory bowel disease

Loss of immune regulation within the gastrointestinal tract can lead to a chronic inflammatory condition known as inflammatory bowel disease (IBD), which encompasses two main clinical entities: Crohn's disease and ulcerative colitis (UC). IBD is emerging as a global disease with the incidence and prevalence increasing worldwide but with a bias towards westernized countries [74,75]. Both Crohn's disease and UC are associated with combinations of multiple pathogenic factors including environmental changes, genetics, abnormal gut microbiota and a broadly dysregulated immune response. Despite the similarities in regards to pathogenic causes, the presentation of each disease can be quite different. Crohn's disease can affect any part of the gastrointestinal tract (oral cavity, esophagus, stomach, small intestine, cecum, or colon) and is associated with segmented areas of chronic inflammation with granuloma formation, ulcers and fistulas as well as luminal narrowing and intestinal adhesions [76,77]. Patients with Crohn's disease can experience common symptoms such as: fever, abdominal pain, diarrhea, and weight loss and will experience a characteristic series of episodes of relapsing disease followed by states of remission [76,77]. UC, on the other hand, is characterized by chronic inflammation of only the colonic mucosa. Clinical features of UC include: ulceration, edema and hemorrhaging along the colon [76,78]. UC patients present with similar symptoms as Crohn's disease such as abdominal pain and diarrhea however, they will also present with anemia if colonic bleeding is severe [76,78].

As chronic inflammation is ultimately a dysregulated immune response, research has mainly focused on the immune abnormalities in IBD pathogenesis. As many of the relevant antigens in IBD are of microbial origins it is believed that, as the intestinal barrier and immune tolerance are broken down, mucosal innate and adaptive immune cells mount a pro-inflammatory

response towards commensal bacteria. Myeloid cells within the intestine are activated through PRRs and begin to expand and produce chemokines and pro-inflammatory cytokines such as IL-1β, IL-6 and IL-12 as well as tumor necrosis factor alpha (TNFα). ILCs will also become active during IBD and produce more cytokines, recruit lymphocytes, and organize inflammatory cells into isolated lymphoid follicles [79]. Upon recruitment of lymphocytes, T cells are polarized into T_H cell subsets with IFN-γ producing T_H1 cells dominating in Crohn's disease [80] (**Figure 1.4**) while IL-13 producing T_H2 cells define UC patients [81]. As there is an increase in pro-inflammatory cells, there is an associated loss of regulatory cells such as T_{reg} cells and other innate immune cells capable of producing immunoregulatory cytokines such as IL-10 and TGFβ [82–86]. Further, reports have also shown that in some cases of IBD regulatory cell numbers can appear normal, however, their effectiveness in dampening immune responses is critically impaired [83,87]. Interestingly, T_H17 cell are present in high number of the intestinal LP during both diseases compared to healthy controls [88,89], however the role of T_H17 cells during the pathogenesis of IBD remains controversial. Initial studies in mice showed T_H17 cells began to produce IFN-γ and became pathogenic [16,90,91], while other studies have outlined a protective function of IL-17A and T_H17 cells in the pathogenesis of IBD [92–94]. In contrast, one molecule that is considered central to progression of both diseases is TNFα and targeted neutralization has been a proven therapeutic, however, not all patients respond to treatment and some lose response or become intolerant over time [95–97]. Thus more investigation is needed into the factors that influence immune cell function in the onset and progression of IBD in order to find new therapeutic targets.

1.3.1.1 Experimental models of IBD

Most of our understanding of the immune response during IBD comes from studies using animal models of intestinal inflammation. These models use a variety of chemical, genetic and cell mediated techniques to break immunological tolerance in the intestine. Examples of experimental models of IBD in this dissertation are: anti-CD3ε induced inflammation, T cell transfer colitis, and dextran sodium sulfate (DSS) induced colitis.

Anti-CD3ε induced inflammation is an acute, T_H1/T_H17 driven form of small intestinal inflammation. Intraperitoneal injection of a CD3ε-specific antibody induces a 'cytokine storm' with inflammation localized mainly in the small intestine [98]. By mimicking antigen, the CD3ε-specific antibody triggers signaling through the TCR that leads to activation-induced cell death (AICD) of intestinal T cells [99]. Consequently, phagocytic engulfment of apoptotic T cells by antigen presenting macrophages and DCs, leads to systemic up-regulation of IL-6 and TGF-β [100,101]. The combination of these cytokines is important for the differentiation and recruitment of T_H17 cells to the small intestinal LP. Intriguingly, inflammation with this model is transient and will subside after five days allowing for the study of both disease initiation and resolution [102]. Lineage tracing experiments with this model have offered great insight into the plasticity of T_H17 cells with pathogenic IFN-γ^+ and IFN-γ^+IL-17A$^+$ T cells dominating initial stages of disease while IL-17A$^+$ and IL-17A$^+$IL-10$^+$ T cells dominate during disease resolution [19,102]. Interestingly, this model has also been validated as an *in vivo* model of tolerization and is now under study in human clinical trials due to the presence of strong immunoregulatory T cells at the resolution of disease [101,103].

T cell transfer colitis is a powerful model to study specific, cell-intrinsic roles in the development of IBD. In this system, naïve CD45RB^{high} CD25$^-$ CD4$^+$ T cells are isolated from

donor mice by fluorescence-activated cell sorting (FACS) and transferred into immunodeficient recipient mice [104]. The immunodeficient recipients lack T_{reg} cells, thus the transferred T cells are allowed to react to the commensal microbiota of the recipient mice unhindered and cause chronic intestinal inflammation [105]. At approximately four weeks post transfer, recipient mice will begin to exhibit weight loss and morbidity associated with the onset of colitis. Disease is driven by T_H1 cell differentiation with high production of IFN-γ and TNFα – further, inhibition of T_H1 cell differentiation is sufficient to prevent disease [106]. This model offers great insight into the role of T cells in the development of disease as genetically altered donor mice, such as gene-deficient mice, can be used to look at T cell-intrinsic roles of specific genes that could be associated with IBD. Conversely, gene-deficient T_{reg} cells can be transferred with naïve T cells in order to study the cell-intrinsic effect of different genes on T_{reg} function during intestinal inflammation [107].

The previous two models of intestinal inflammation are both T cell driven, however not all forms of colitis rely on T cells for disease. Another model of colitis that is driven by innate immune cells is the chemical insult model of DSS induced colitis. This model involves chemically induced epithelial cell damage, which leads to bacterial translocation and activation of sub-epithelial innate immune cells and therefore does not require T or B cells for development of disease [108,109]. After seven days of exposure to DSS in their drinking water, mice develop acute colitis that results in weight loss, bloody stool, diarrhea and colon shortening. Within the colon, disease is characterized by high levels of IFN-γ and TNFα, large amounts inflammatory cell infiltrate, sloughing of epithelial cells and complete loss of crypt architecture [108,109]. Together, these models are critical to our understanding of disease pathogenesis in IBD.

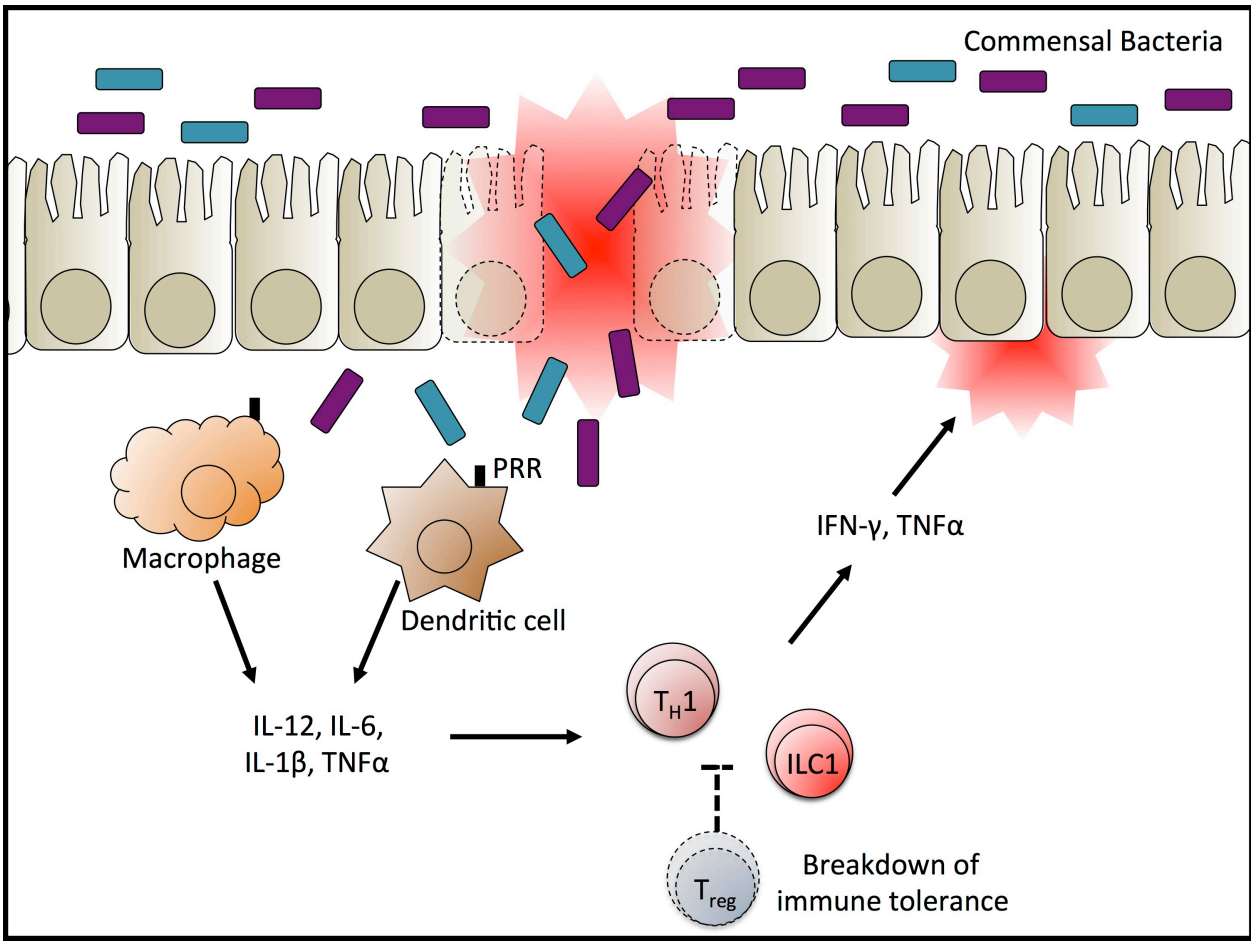

Figure 1.4 Schematic of IFN-γ driven intestinal inflammation.

IBD is characterized by an immune response targeted against commensal bacteria. Breakdown of the epithelial cell barrier or immune tolerance will lead to a commensal bacteria activation of resident innate immune cells through pattern recognition receptors (PRR). Release of inflammatory cytokines (TNFα, IL-12, IL-6, IL-1β) will activate ILC1 cells to produce IFN-γ. T_H1 cells will differentiate under these inflammatory conditions and produce more IFN-γ and TNFα initiating more damage to intestinal tissue, further progressing the cycle of chronic inflammation.

1.3.2 Intestinal infections

Enteric pathogens are often associated with diarrhea, and acute diarrheal illnesses are a major cause of morbidity and mortality worldwide, associated with an estimated 4.8 million deaths in children under the age of five [110]. Further, it is estimated that over a billion people worldwide are infected with some form of intestinal helminth, which can cause severe growth and cognitive deficiencies in infected children [111]. In addition, the Center for Disease Control and Prevention estimates that each year in the United States 48 million people become ill as a result of infections with food-borne pathogens, 128,000 are hospitalized, and 3,000 die [112,113]. Therefore it is important to continue research into these often neglected diseases to identifying the mechanisms required for optimal immunity to infection, which will promote the development and optimization of treatments against these deadly intestinal infections. In order to study these diseases many experimental models of intestinal infections have been developed. This dissertation will focus on the bacterial infection model of *Citrobacter rodentium* and the parasitic helminth infection model of *Trichuris muris.*

1.3.2.1 *Citrobacter rodentium* infection model

The murine mucosal pathogen *Citrobacter rodentium* is an attaching and effacing (A/E) bacteria that provides a laboratory model for human enteric pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC). *C. rodentium* shares 67% of its genome with both EPEC and EHEC [114] and is similarly transmitted via the fecal-oral route in order to colonize the intestinal mucosa, particularly the cecum and colon [115]. Bacterial colonization of these pathogens is characterized by the formation of A/E lesions, which are intimate bacterial attachment to the intestinal epithelium, effacement of the brush border

microvilli and the formation of pedestal-like structures underneath the adherent bacterium [116]. Although *C. rodentium* is only considered an opportunistic infection in humans, EPEC and EHEC are defined as major gastrointestinal risks to humans globally. EPEC is a major cause of infantile diarrhea, and is associated with high rates of morbidity and mortality in developing countries [110], while EHEC is prevalent in developed countries and depending on the strain can produce highly potent toxins (e.g. Shiga toxin) that cause kidney failure [117].

Immunity to *C. rodentium* relies heavily on the ability to mount a strong T_H17/ILC3 response in the intestinal mucosa to ultimately up regulate production of antimicrobial peptides and recruit phagocytes to kill the pathogenic bacteria. Once *C. rodentium* breaches the epithelial barrier, different PAMPS such as lipopolysaccharide (LPS) or peptidoglycan will activate various TLRs on innate immune cells. Epithelial cells will undergo hyperplasia and resident DCs and macrophages will produce proinflammatory cytokines IL-1β, IL-6, IL-23, and TNFα [118]. ILC3s will also become activated and produce high levels of IL-17A and IL-22 [70,119,120]. At a later time point, T_H17 cells are recruited to the site of infection and further contribute to the production of both IL-17A and IL-22 [121,122]. These two cytokines are key for resistance of *C. rodentium*. IL-22 instructs intestinal epithelial cells to secrete antimicrobial peptides such as REGIIIβ and REGIIIγ [38,41,123]. IL-17A helps in the recruitment of neutrophils that are important for the direct killing and phagocytosis of the bacteria [124] (Summarized in **Figure 1.5**). This model provides insight into the development of T_H17 responses and was key to the discovery of ILC3s [119]; moreover, recent studies have shown that this model can be used to study other diseases such as ulcerative colitis, Crohn's disease and colon tumorigenesis, further highlighting the power and breadth of this model in studying mucosal immune responses [125–127].

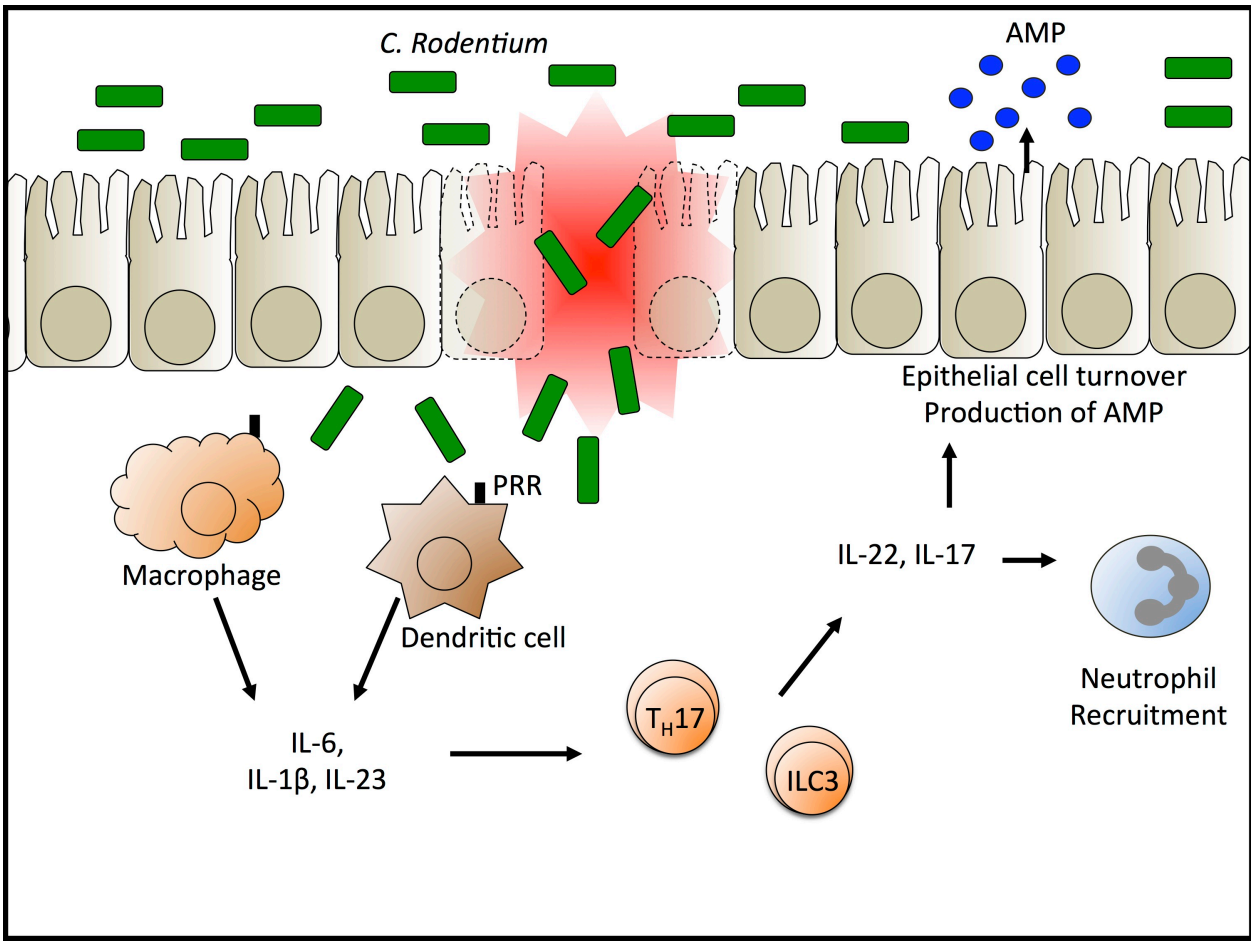

Figure 1.5 Schematic of intestinal immune response to *Citrobacter rodentium*.

Infection with *C. rodentium* induces a robust ILC3/T_H17 response in the intestine. The bacterial pathogen will activate innate immune cells to produce cytokines (IL-6, IL-23, IL-1β), which will activate resident ILC3 cells to produce IL-22 and IL-17A. T_H17 cells will differentiate under these conditions and produce more IL-22 and IL-17A. IL-17A will facilitate recruitment of neutrophils while IL-22 will instruct epithelial cells to produce anti-microbial peptides (AMP) to combat the invading bacteria.

1.3.2.2 *Trichuris muris* infection model

Soil-transmitted helminths are the most prevalent infectious microorganisms of humans, with approximately two billion people infected [111]. *Trichuris spp.* is a soil-transmitted nematode parasite that can infect humans and animals. *T. trichuria* is the human infecting whipworm that currently infects 800 million people worldwide [111]. Chronic infections are common in children and are associated with clinical symptoms ranging from impaired nutritional status and growth retardation to anemia, enteropathy, and intestinal obstruction [111]. *Trichuris spp*. has a simple life cycle consisting of larval development in the cecum of a single primary host, molting into mature worms, shedding of eggs into the feces, then embryonation of the eggs in the soil, where finally mature eggs can be ingested by a new host to start the cycle a new [128]. *T. muris* is a naturally-occurring nematode parasite in wild rodent populations that has been isolated for infections in laboratory mice as a model for human trichuriasis [128,129]. *T. muris* is an intestinally restricted parasite, which allows for its use as an experimental model to study localized mucosal immune responses.

Immunity to *T. muris* critically depends on the development of a strong T_H2 response against the helminth parasite. T_H2 cytokines will cause physiological changes in the intestinal microenvironment including rapid intestinal epithelial cell turnover, goblet cell hyperplasia and smooth muscle contractility in order to physically expel the parasite [128]. Immune cells are activated in the intestinal mucosa as the parasite burrows into the colonic epithelium, damaging cells and allowing the release of danger associated molecular patterns (DAMPs) and cytokine alarmins (e.g. IL-25, IL-33 and Thymic Stromal Lymphopoietin (TSLP)) [128]. These signals will then activate innate immune cells such as DCs, ILC2s and basophils, which in turn will produce cytokines to promote the differentiation of T_H2 cells [128]. Together, these cells will secrete large

quantities of IL-4, IL-5 and IL-13, which elicit the physiological changes in the intestine and expulsion of the worms [128] (Summarized in **Figure 1.6**). Interestingly, not all strains of laboratory mice can mount proper anti-*T.muris* T_H2 immune responses. Wild type C57Bl/6 mice are resistant to infection but due to slight genetic differences between strains many mice will mount a non-protective T_H1 response and remain chronically infected and are described as susceptible (e.g. AKR mice) [130]. Further, the use of gene-deficient mice on a C57Bl/6 background has been valuable to gain insight into cell-intrinsic factors that are critical for T_H2 immune responses in the intestine [131–134]. Interestingly, the infective dose of *T.muris* eggs can also influence the type of immune response from wild type C57Bl/6 mice, with a high dose (150 – 200) of eggs triggering a protective T_H2 response and clearance of worms, while a low dose (30) of eggs results in a T_H1 response and chronic infection [129]. Thus, the *T.muris* model of infection offers a powerful system in which to study the factors that control the differentiation of T cells into T_H1 or T_H2 fates within the intestine.

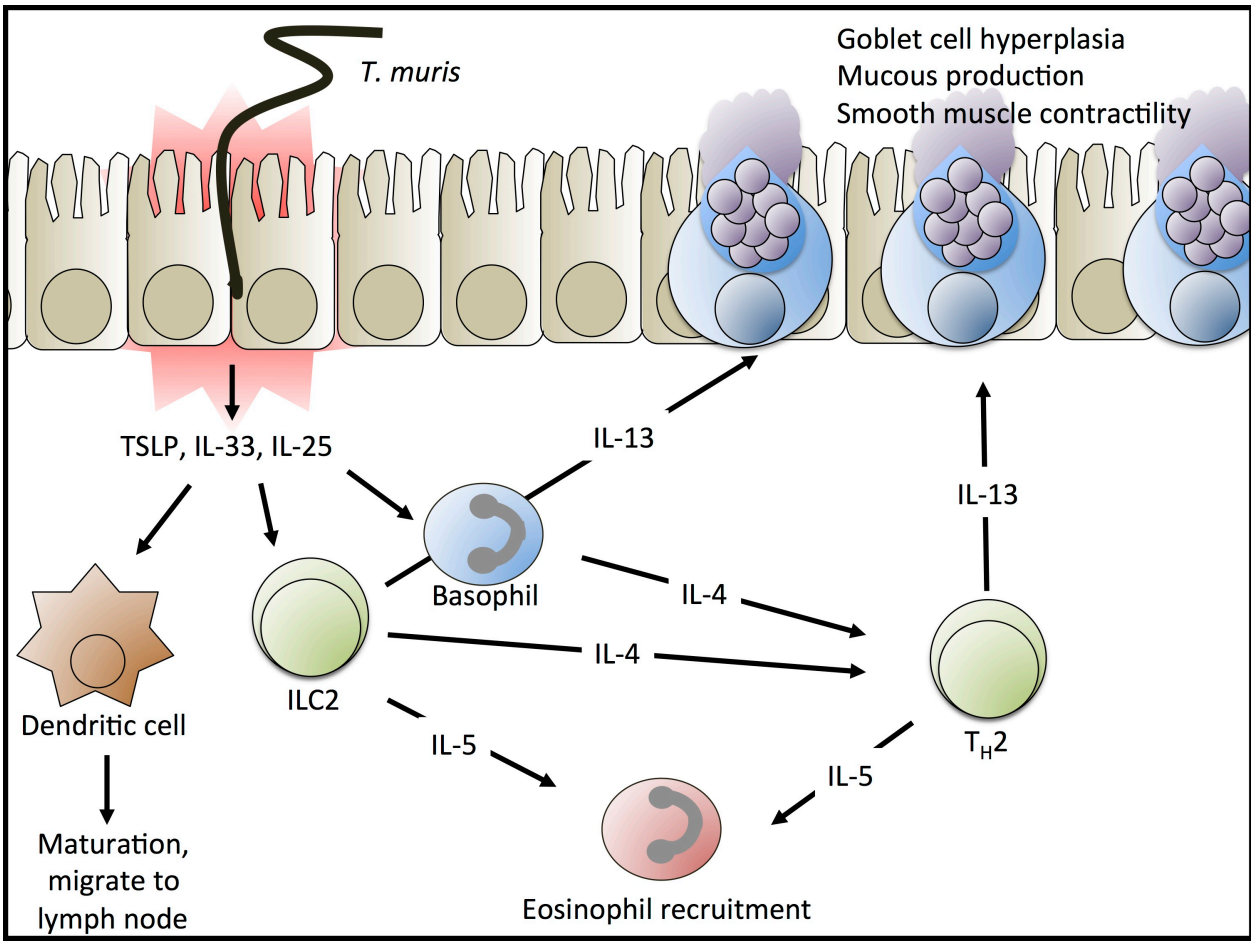

Figure 1.6 Schematic of intestinal immune response to *Trichuris muris*.

Clearance of a *T. muris* infection relies heavily on a robust T_H2 response in the intestine. The parasite will cause the release of alarmins (IL-25, IL-33 and TSLP) from intestinal epithelial cells. These cytokines will then activate local DCs to migrate to lymph nodes where they can then facilitate the differentiation of T_H2 cells. Local basophils will produce IL-4 and help promote Th2 differentiation. Alarmins will also activate local ILC2s to produce cytokines (IL-4, IL-5, IL-13). Upon activation, Th2 cells will further contribute to the production of cytokines (IL-4, IL-5, IL-13). IL-5 will recruit eosinophils while IL-13 will induce goblet cell hyperplasia, mucous production and smooth muscle contractility in order to expel the worms.

1.3.3 All-*trans*-retinoic acid and intestinal immunity

Micronutrient deficiency, such as vitamin A deficiency (VAD), is being recognized as an important factor contributing towards the global burden of infectious diseases. VAD has been closely linked with infection related morbidity and mortality in children [135–137]. Specifically, VAD has been associated with the inability of vaccines to illicit protective immunity [138,139] as well as dysregulated intestinal immune responses, which increase the risk of gastroenteritis in affected children [137]. The vitamin A metabolite, all-*trans*-retinoic acid (atRA), plays an important role in shaping intestinal homeostasis and immunity by regulating both the innate and adaptive immune systems. Intestinal epithelial barrier integrity and permeability, induction of oral tolerance and secretion of IgA by intestinal plasma cells all require atRA for proper function [140,141]. These findings, along with the discovery that in only some cases vitamin A supplements can reverse the associated morbidity in these children [135], highlight a critical need for focusing research into the downstream mechanisms of atRA signaling in order to combat intestinal infection related mortality.

Humans and mice are unable to synthesize vitamin A and are thus dependent on dietary replenishment through ingestion of foods containing retinoids or carotenoids [142]. After intake, vitamin A is stored in the form of retinol esters with approximately 80% of the body's vitamin A stored in hepatic stellate cells [143]. Once retinol esters are released into circulation, they are quickly hydrolyzed into retinol, which can then be taken up by cells and converted into retinal by alcohol dehydrogenases [144]. Finally, if the cell expresses the required retinal dehydrogenase, retinal can be converted into retinoic acid (RA) [144]. atRA is the most physiological abundant form of RA compared to other isoforms such as 9-cis- or 13-cis-RA. Within the intestine, intestinal epithelial cells and a subset of CD103-expressing intestinal LP DCs are important

sources of atRA, through their expression of alcohol dehydrogenase isoforms [145–147]. Once synthesized, atRA can be sensed by cells in the intestinal LP through the expression of nuclear RA receptors (RARs) and retinoid X receptors (RXRs) [148]. Each subset of RA receptors has three isoforms: RAR-α, β, γ and RXR-α, β, γ. RA can bind to and activate an RAR that forms a heterodimer with the corresponding RXR, which then leads to transcriptional regulation of gene expression by binding to retinoic acid response elements (RARE) [149]. RAR-α is the dominant RAR expressed by various intestinal immune cells such as T cells, B cells, DCs and ILCs and is important in their function [150–154].

Functionally, atRA is critical for imprinting gut tropism on lymphocytes through the up regulation of intestinal homing molecules: CCR9 and the integrin α4β7 [147,155]. Through the use of mice raised on vitamin A deficient diets or genetically altered mice expressing a dominant negative RAR-α, it was shown that intestinal $CD4^+$ T cells, $CD8^+$ T cells, plasma cells, ILC1s and ILC3s all rely on atRA signaling for migration into the intestinal mucosa [147,156–158]. However, atRA functions beyond lymphocyte trafficking. The differentiation of both iT_{reg} and T_H17 cells are influenced by atRA. TGF-β can synergize with atRA to boost the differentiation of iT_{reg} cells at the expense of T_H17 differentiation [159–161] and limit T cell plasticity through a mechanism involving miR-10 [162]. Interestingly, it has also been shown that atRA signaling occurs early in T cells during an inflammatory response indicating that it is important in both effector and regulatory responses [151]. Further, intestinal ILC2s migrate independently of atRA but are expanded in the absence of atRA signaling, providing enhanced type 2 immune responses [156,163]. Finally, atRA can also influence the differentiation and expansion of intestinal DC subsets through activation of a transcriptional profile in a subset of pre-mucosal DCs [164]. Thus atRA plays a complex role in mucosal immunity and is critical for intestinal immune cell homeostasis

and function, however the exact molecular mechanism for how atRA influences different immune cell functions are not fully defined.

1.4 POK/ZBTB transcription factors

The pox virus and zinc finger krüppel-type (POK) proteins, which are also known as zinc finger and broad-complex, tramtrac, bric-à-brac (ZBTB) proteins are a large and diverse family of transcription factors with 49 known members that play critical roles in a variety of biological processes such as gastrulation, limb formation, cell cycle progression, and gamete formation [165]. They are characterized by varying numbers (from 3 to ~13) of C_2H_2 krüppel-like zinc fingers on their carboxyl termini that are responsible for DNA binding with a broad-complex, tramtrac, bric-à-brac (BTB) domain near the amino terminus of each protein [166] (**Figure 1.7**). The BTB domain is used as a protein–protein interaction interface, and is important for homo- and hetero-dimerization as well as recruitment of other transcriptional co-regulators such as histone deacetylases, nuclear corepressors (N-CoR1 and -2), C-terminal binding protein (CtBP), as well as the E3 ubiquitin ligase Cullin 3 (CUL3), which are particularly important to mediate rapid epigenetic changes in the chromatin structure of target genes through histone methylation or acetylation [167,168].

In the immune system, POK/ZBTB proteins are key regulators in cellular differentiation and function [169] and have emerged as a family of transcription factors that are indispensable for different aspects of immune system development (**Table 1.1**). For example, B-Cell CLL/Lymphoma 6 (BCL6, ZBTB27) is critical for the development of germinal centres (GCs) following immunization or infection. BCL6 is expressed at high levels in GC B cells [170] and T follicular helper (T_{FH}) cells [171] that together control the germinal centre response. Promyelocytic

leukemia zinc finger (PLZF, ZBTB16) is required for NKT cell development [172] and expression of PLZF has recently been shown to identify a multipotent progenitor of innate lymphoid cells [48]. T helper-inducing POZ/Kruppel-like factor (ThPOK, ZBTB7B) is a master regulator of T_H cell development in the thymus [173,174]. Thus studies have highlighted the critical functions mediated by POK/ZBTB transcription factors within the immune system and emphasize how the deregulation of these transcription factors can lead to immune deficiencies, however, not all family members have been studied in the context of immunity and therefore warrant further investigation.

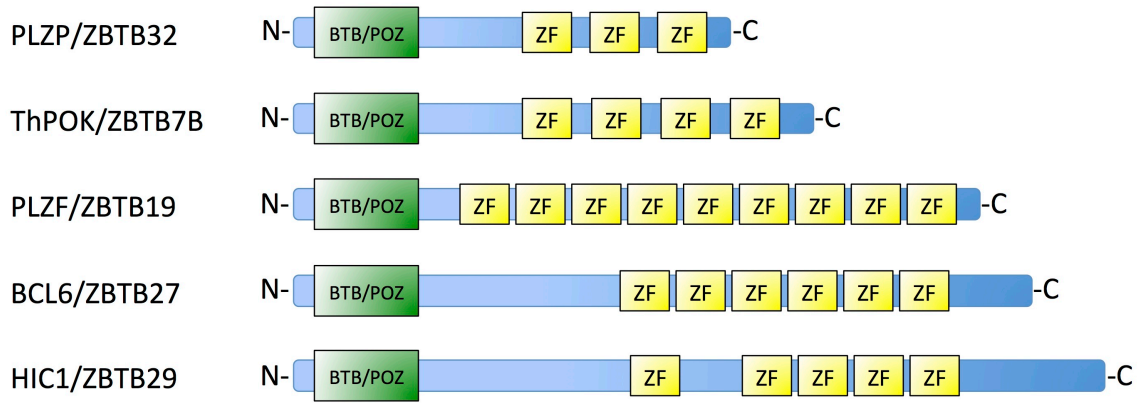

Figure 1.7 Structure of POK/ZBTB family transcription factors.

Five examples of the POK/ZBTB family of transcription factors characterized by varying numbers of C_2H_2 krüppel-like zinc fingers on their carboxyl termini with a BTB domain near the amino terminus of each protein.

POK/ZBTB family member	Immune cell function	Reference
ZBTB7A, LRF	T and B cell development	175
ZBTB7B, ThPOK	T cell lineage commitment	174,176
ZBTB16, PLZF	Development of NK T cells, γδ T cells and ILCs	48,172,177–179
ZBTB17, MIZ	Early B cell commitment	180
ZBTB27, BCL6	Germinal centre B cell and follicular T_H cell development	171,181–183
ZBTB28, BAZF	CD8 T cell memory formation	184
ZBTB32, PLZP	NK cell function	185

Table 1.1 Roles of POK/ZBTB family transcription factors in regulating the immune system.

1.4.1 Hypermethylated in cancer 1

Hypermethylated in cancer 1 (HIC1, ZBTB29) is a member of the POK/ZBTB family and has been shown to regulate fundamental cellular processes such as cell growth and survival [186,187]. HIC1 was originally described as a tumor suppressor gene that is epigenetically silenced through DNA methylation in various human cancers [188,189] and it has been proposed that HIC1-dependent repression of SIRT1, a NAD^+-dependent class III histone deacetylase (HDAC) that deacetylates and inactivates P53, is critical for modulating P53-dependent DNA damage responses [190]. Further, HIC1 has been described as a quiescent marker for cells that have exited the cell cycle, with HIC1 bound to the promoters of genes that promote progression through the cell cycle such as *Ccnd1* and *Cdkn1c* [191]. HIC1 mediates repression through the recruitment of co-repressors such as C terminal binding protein (CtBP), nucleosome remodeling and histone deacetylase (NuRD) complex, and polycomb repressive complex 2 (PRC2). Interestingly, HIC1 can also interfere with several essential signaling pathways by recruiting transcriptional activators away from their target sites. For example, HIC1 is capable for sequestering transcription factor 4 (TCF4), a key transcription factor in Wnt/β-catenin signaling [192]. HIC1 directly binds to TCF4 and forms nuclear substructures called "HIC1 nuclear bodies" in order to divert TCF4 from its transcriptional targets thus dampening the Wnt/β-catenin signaling pathway [192]. Similarly, HIC1 associates with STAT3 and inhibits STAT3 DNA binding activity via its C-terminal domain, which thus suppresses STAT3 target gene expression (e.g. *Vegf* and *Myc*) and in turn antagonize STAT3-mediated cell growth [193,194].

HIC1 has never been studied in the context of immunology; however, two unrelated genome-wide expression screens for genes in T_H cells regulated by atRA signaling have identified *Hic1* as an RA/RARα-dependent gene, although no functional analyses were carried

out [195,196]. Further, an unrelated *in silico* study identified that the *Hic1* gene contains multiple RAREs [197]. Given the relationship between atRA signaling and HIC1 expression in T_H cells it is likely that HIC1 will play a role in atRA dependent mucosal immunity.

1.5 Rationale, hypothesis and specific aims

Dysregulated T cell responses at mucosal sites are characteristic of several inflammatory diseases of the gastrointestinal tract. A better understanding of the factors that underlie the development, migration and function of $CD4^+$ T_H cells at mucosal sites would aid in the development of novel or improved treatment options. atRA is an important immune modulator in the intestine with clear role in both regulatory and effector T cell responses [151,159–161]. Pathogenesis of IBD has been linked to genetic or environmental factors that impact atRA levels in the intestine [198–200], yet the factors that control atRA-mediated signaling within intestinal immune cells during homeostasis and disease are not well-defined.

ILCs have been identified in large numbers at barrier sites such as the intestine where they are critical in promoting protective immunity to pathogens [2]. A better understanding of the mechanisms that control and maintain ILC populations in the intestine would help in the production of novel therapeutics for chronic infections. Interestingly, atRA has been identified as an important factor that regulates ILC differentiation and function, and similarly to T_H cells, Vitamin A status can significantly alter ILC responses within the intestine [156,163]. However, much is still yet to be elucidated about the molecular mechanism of how atRA controls ILC development and function.

The differentiation of all hematopoietic cells is a tightly regulated process that is controlled primarily by the expression of lineage-specific sets of transcription factors, with T_H

cell and ILC subsets sharing many of the same sets of transcription factors. The POK/ZBTB family of transcriptional repressors has been shown to play critical roles in T_H cell differentiation and function as well as ILC development [48,171,173–175]. The POK/ZBTB transcription factor HIC1 has been identified as a potential atRA responsive gene in T_H cells [195–197]; however, no functional studies have been carried out to determine a role for HIC1 in any hematopoietic cell differentiation pathway or immune cell function in the intestine where atRA signaling is critical. Therefore, there is a need to better characterize the role of HIC1 in atRA mediated intestinal immune responses.

Based on this rationale, ***I hypothesize that HIC1 will play an essential role in atRA mediated mucosal immune responses during intestinal infections and inflammatory disease.***

I have devised three specific aims to test this central hypothesis:

1. **To determine the expression pattern of *Hic1* in leukocytes of various tissues**. The expression of *Hic1* will be characterized in various immune cell populations within different tissues and the role of atRA in *Hic1* expression will be assessed.

2. **To investigate how HIC1 regulates intestinal immune homeostasis.** Genetically modified mice with HIC1-deficiency in various hematopoietic cells will be analyzed for alterations in distinct immune cell populations in the intestine at steady state.

3. **To assess how HIC1 regulates immune responses during intestinal infections and inflammation.** The *T. muris and C. rodentium* models of intestinal infection will be used to determine the effect of hematopoietic HIC1 deficiency on immunity to infections. Further the T cell transfer, DSS induced and anti-CD3e antibody induced models of inflammation will be used to determine the role for HIC1 in immune cells during the development of intestinal inflammation.

Chapter 2: Methods

2.1 Ethics statement

Experiments were approved by the University of British Columbia Animal Care Committee (Protocol numbers: A13-0010, A15-0196) and were in accordance with the Canadian Guidelines for Animal Research.

2.2 Mice

The generation of *Hic1*^{Citrine} mice has been described [201] and the generation of *Hic1*$^{fl/fl}$ mice will be described elsewhere (manuscript in preparation). *Cd4*-Cre mice were obtained from Taconic, *Vav*-Cre mice were obtained from T. Graf (Centre for Genomic Regulation, Barcelona, Spain) and *CD11c*-Cre (B6.Cg-Tg (Itgax-cre)1-1Reiz/J) and RORc-Cre (B6.FVB-Tg (RORc-cre)1Litt/J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained in a specific pathogen-free environment at the UBC Biomedical Research Centre animal facility.

2.3 Diet studies

Vitamin A-deficient (TD.09838) diet was purchased from Harlan Teklad Diets (Madison, WI). At day 14.5 of gestation, pregnant females were administered the vitamin A-deficient diet and maintained on diet until weaning of litter. Upon weaning, females were returned to standard chow, whereas weanlings were maintained on special diet until use.

2.4 Antibodies and flow cytometry

Absolute numbers of cells were determined via hemocytometer or with latex beads for LP samples. Intracellular cytokine (IC) staining was performed by stimulating cells with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 750 μg/ml ionomycin, and 10 μg/ml Brefeldin-A (Sigma, St. Louis, MO) for 4 hours and fixing/permeabilizing cells using the eBioscience IC buffer kit. All antibody dilutions and cell staining were done with phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS), 1 mM Ethylenediaminetetraacetic acid (EDTA), and 0.05% sodium azide. Fixable Viability Dye eFluor 506 was purchased from eBioscience (San Diego, CA) to exclude dead cells from analyses. Prior to staining, samples were Fc-blocked with buffer containing anti-CD16/32 (93, eBioscience) and 1% rat serum to prevent non-specific antibody binding. Cells were stained with fluorescent conjugated anti-CD11b (M1/70) anti-CD11c (N418), anti-CD19 (ID3), anti-CD5 (53-7.3), anti-CD8 (53.67), anti-CD3 (KT3)(2C11), anti-NK1.1 (PK136), anti-B220 (RA3-6B2), anti-Ter119 (Ter119), anti-Gr1 (RB6-8C5) produced in house, anti-CD4 (GK1.5), anti-TCRβ (H57-597), anti-MHCII (I-A/I-E) (M5/114.15.2), anti-F4/80 (BM8), anti-IL17a (17B7), anti-FOXP3 (FJK-16s), anti-TCRγδ (eBioGL3), anti-CD45 (30-F11), anti-CD45RB (C363.16A) anti-α4β7 (DATK32), anti-CCR9 (eBioCW-1.2), anti-IFN-γ (XMG1.2), anti-CD25 (PC61.5), anti-IL-13 (eBio13A), anti-CD90.2 (53-2.1), anti-GATA3 (TWAJ), anti-ROR-γt (B2D), anti-TBET (eBio4B10), anti-FLT3 (A2F10), anti-CKIT (ACK2), anti-IL-22 (IL22JOP), anti-Ki67 (SolA15) purchased from eBioscience, anti-CD103 (M290), anti-CD69 (H1.2F3), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD64 (X54.5/7.1.1), anti-CD127 (5B/199), anti-pSTAT3 (pY705) purchased from BD Biosciences (San Jose, CA). Data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

2.5 RNA isolation and quantitative real-time PCR

Tissues were mechanically homogenized and RNA was extracted using the TRIzol method according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA). cDNA was generated using High Capacity cDNA reverse transcription kits (ThermoFisher Scientific). Quantitative PCR was performed using SYBR FAST (Kapa Biosystems, Wilmington, MA) and SYBR green-optimized primer sets run on an ABI 7900 real-time PCR machine. Cycle threshold (C_T) values were normalized relative to beta-actin (*Actb*) gene expression. The primers used were synthesized de novo:

Hic1:

forward 5'-AACCTGCTAAACCTGGACCAT-3'

reverse 5'-CCACGAGGTCAGGGATCTG-3'

Il17a:

forward 5'-AGCAGCGATCATCCCTCAAAG-3'

reverse 5'-TCACAGAGGGATATCTATCAGGGTC-3'

Il22:

forward 5'-ATGAGTTTTTCCCTTATGGGGAC-3'

reverse 5'-GCTGGAAGTTGGACACCTCAA-3'

Ifng:

forward 5'- GGATGCATTCATGAGTATTGCC-3'

reverse 5'-CCTTTTCCGCTTCCTGAGG-3'

Il4:

forward 5'- TCGGCATTTTGAACGAGGTC -3'

reverse 5’- CAAGCATGGAGTTTTCCCATG-3’

Il13:

forward 5’-CCTGGCTCTTGCTTGCCTT-3’

reverse 5’- GGTCTTGTGTGATGTTGCTCA-3’

Foxp3:

forward 5’- CCCAGGAAAGACAGCAACCTT-3’

reverse 5’- TTCTCACAACCAGGCCACTTG-3’

Rorc:

forward 5’- TCCACTACGGGGTTATCACCT-3’

reverse 5’- AGTAGGCCACATTACACTGCT-3’

Socs3:

forward 5’- TGCAAGGGGAATCTTCAAAC-3’

reverse 5’- TGGTTATTTCTTTTGCCAGC-3’

Irf4:

forward 5’- CCGACAGTGGTTGATCGACC-3’

reverse 5’- CCTCACGATTGTAGTCCTGCTT-3’

Ahr:

forward 5’- GCCCTTCCCGCAAGATGTTAT-3’

reverse 5’- GCTGACGCTGAGCCTAAGAAC-3’

Il23r:

forward 5’- AACAACAGCTCGGATTTGGTAT-3’

reverse 5’- ATGACCAGGACATTCAGCAGT-3’

Reg3g:

forward 5'- CCGTGCCTATGGCTCCTATTG -3'

reverse 5'- GCACAGACACAAGATGTCCTG -3'

Actb:

forward 5'-GGCTGTATTCCCCTCCATCG-3'

reverse 5'-CCAGTTGGTAACAATGCCATGT-3'.

2.6 Cell culture

$CD4^+$ T cells were isolated from spleen and LNs by negative selection using an EasySep Mouse $CD4^+$ T cell isolation kit (StemCell Technologies, Vancouver, BC). 5 x 10^5 $CD4^+$ cells were cultured for up to 5 days in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 5 x 10^{-5} 2-ME with 1 μg/ml plate bound anti-CD3 (145-2C11) and anti-CD28 (37.51). $CD4^+$ T cells were polarized under T_H17 cell- (20 ng/ml IL-6, 10 ng/ml IL-23, TNF-α, IL-1β, 1 ng/ml TGF-β1, 10 μg/ml anti-IFN-γ and anti-IL-4), T_H1 cell- (10 ng/ml IL-2, IL-12 and 5 μg/ml anti-IL-4), T_H2 cell- (10 ng/ml IL-2, IL-4 and 5 μg/ml anti-IFN-γ) or iT_{reg} cell- (10 ng/ml IL-2 and TGFβ1) promoting conditions. In some cases, cells were plated as above in the presence of 10 nM retinoic acid (Sigma).

2.7 ELISA

IL-17A production was analyzed from supernatants taken on day 4 of $CD4^+$ T cell *in vitro* culture using commercially available antibody pairs (eBioscience).

2.8 Anti-CD3ε -induced intestinal inflammation

Mice were administered 30 μg of anti-CD3ε antibody (145-2C11) in 400μl PBS by intraperitoneal injection. Animals were monitored daily after injection and euthanized at 48 hours post injection. At endpoint, sections of the distal ileum were fixed in 10% buffered formalin, embedded in paraffin and stained with H&E. Histological inflammation were blindly scored on a scale of 0 to 4, where 0 represented a normal ileum and 4 represented severe inflammation. Specific aspects such as infiltrating immune cells, crypt length, epithelial erosion, and muscle thickness were taken into account.

2.9 T cell transfer colitis

$CD4^+$ cells were enriched from spleens and peripheral LNs (pLNs) of *Hic1*$^{fl/fl}$ or *Hic1*$^{\Delta T}$ mice with an EasySep Mouse $CD4^+$ T cell isolation kit (StemCell Technologies) and stained with anti-CD4, anti-CD25 and anti-CD45RB. Naive $CD4^+CD25^-CD45RB^{hi}$ cells were purified by cell sorting. $CD4^+CD25^-CD45RB^{hi}$ naive T cells (4×10^5) were injected intraperitoneally into age- and sex-matched *Rag1*$^{-/-}$ mice, which were monitored for weight loss and sacrificed 6 weeks after initiation of the experiment. At endpoint, proximal colon was fixed, embedded, and stained with H&E. Histological inflammation was scored as above.

2.10 Dextran sodium sulfate induced colitis

Mice were exposed to 3.5% DSS in their drinking water for 7 days before returning to regular drinking water for one final day. Mice were monitored for weight loss and sacrificed on day 8. At endpoint, proximal colon was fixed, embedded, and stained with H&E. Histological inflammation was scored as above.

2.11 Isolation of intraepithelial and lamina propria leukocytes

Peyer's patches were removed from the small intestine, which was cut open longitudinally, briefly washed with ice-cold PBS and cut into 1.5 cm pieces. Tissue was incubated in 2mM EDTA PBS for 15 minutes at 37°C and extensively vortexed. Supernatants were collected and pelleted, then re-suspended in 30% Percoll solution and centrifuged for 10 minutes at 1200 rpm. The pellet was collected and used as intraepithelial leukocytes. Remaining tissue was digested with Collagenase/Dispase (Roche) (0.5 mg/mL) on a shaker at 250 rpm, 37°C, for 60 minutes, extensively vortexed and filtered through a 70μm cell strainer. The flow-through cell suspension was centrifuged at 1500rpm for 5 min. The cell pellet was re-suspend in 30% Percoll solution and centrifuged for 10 minutes at 1200 rpm. The pellet was collected and used as lamina propria leukocytes.

2.12 Cell lysis, immunoprecipitation and immunoblotting

Cells were lysed and immunoprecipitation carried out using antibodies against STAT3 (C-20; Santa Cruz, Dallas, TX) and FLAG (M2; Sigma). Immunoblotting was carried out using antibodies against STAT3, HIC1 (H-123; Santa Cruz) and GAPDH (GA1R; in house).

2.13 Chromatin immunoprecipitiation

Naive $CD4^+$ T cells were activated and T_H17 polarized for 3 days, followed by crosslinking for 8 minutes with 1% (vol/vol) formaldehyde. Cells were collected, lysed and sonicated. After being precleared with protein A agarose beads (EMD Millipore, Billerica, MA), cell lysates were immunoprecipitated overnight at 4 °C with anti-STAT3 (C-20, Santa Cruz) or

normal rabbit IgG (Cell Signaling, Danvers, MA). After washing and elution, crosslinks were reversed for 4 h at 65°C. Eluted DNA was purified and samples were analyzed by quantitative real time PCR on a 7900 Real-Time PCR system (Applied Biosystems). Primer sets used for analysis are: *Il17a* promoter forward 5'- CACCTCACACGAGGCACAAG -3' and reverse 5'-ATGTTTGCGCGTCCTGATC -3'; *Il5* promoter forward 5'-AAGTCTAGCTACCGCCAATA-3' and reverse 5'- AGCAAAGGTGAGTTCAATCT-3'. Each Ct value was normalized to the corresponding input value.

2.14 Retroviral transduction

Platinum E cells were transiently transfected using the calcium phosphate method with MigR1 expression plasmids encoding GFP alone or FLAG-HIC1. Viral supernatants were collected after 48h, supplemented with 8 μg/ml polybrene (EMD Millipore) and added to T cells that had been activated under T_H17 cell-polarizing conditions for 48 h. T cells (2×10^6) were incubated in 24 well plates with 1 ml viral supernatants. After 24 hours, viral supernatant was replaced with conditioned culture medium and cells were cultured under T_H17 cell-polarizing conditions for an additional 3 days.

2.15 *Citrobacter rodentium* infection

Mice were infected by oral gavage with 0.1 ml of an overnight culture of Luria-Bertani (LB) broth grown at 37°C with shaking (200 rpm) containing 2.5×10^8 colony forming units (CFU) of *C. rodentium* (strain DBS100) (provided by B. Vallance, University of British Columbia, Vancouver, BC). Mice were monitored and weighed daily throughout the experiment and sacrificed at various time points. For enumeration of *C. rodentium,* fecal pellets or livers

were collected in pre-weighed 2.0 ml microtubes containing 1.0 ml of PBS and a 5.0 mm steel bead (Qiagen, Hilden, Germany). Tubes containing pellets or livers were weighed, and then homogenized in a TissueLyser (Retche/Qiagen) for a total of 6 mins at 20 Hz at room temperature. Homogenates were serially diluted in PBS and plated onto LB agar plates containing 100 mg/ml streptomycin, incubated overnight at 37°C, and bacterial colonies were enumerated the following day, normalizing them to the tissue or fecal pellet weight (per gram).

2.16 *Trichuris muris* infection

Propagation of *Trichuris muris* eggs and infections were performed as previously described [202]. Mice were infected with approximately 150 – 200 embryonated *T. muris* eggs by oral gavage and monitored over a period of 21 days. Sacrificed mice were assessed for worm burdens by manually counting worms in the ceca using a dissecting microscope. Cecal tissues were fixed overnight in 10% buffered formalin and paraffin-embedded. A total of 5-μm-thick tissue sections were stained with periodic acid–Schiff (PAS) for histological analysis. The mLN were excised and passed through a 70 μm cell strainer to generate a single-cell suspension. mLN cells (4×10^6/mL) were cultured for 72 h in media containing 1 μg/mL each of antibodies against CD3 (145-2C11) and CD28 (37.51; eBioscience, San Diego, CA). Cytokine production from cell-free supernatant was quantified by ELISA using commercially available antibodies (eBioscience).

2.17 *In vivo* neutralization of IFN-γ

Mice were infected with *T. muris* as described above. On day 4 post infection, mice were injected i.p. with 500 μg of either control IgG or anti-IFN-γ (XMG1.2) (produced in-house by

AbLabBiologics, Vancouver, BC), constituted in sterile PBS. Mice were repeatedly injected thereafter on days 8, 12, and 16 prior to sacrifice on day 21.

2.18 Serum ELISA

Serum was collected from mice 21 days post-infection with *T. muris*. Immulon plates (Thermo Fischer Scientific) were coated with 5 μg/mL of dialyzed *T. muris* antigen overnight at 4°C. Wash buffer was PBS containing 0.05% Tween 20. Plates were blocked and serum samples were diluted in 3% bovine serum albumin in PBS/0.05% Tween 20. Serum samples were incubated on plates for 1 hour at room temperature. Plates were then incubated with rat anti-mouse IgG1 or IgG2a conjugated to horseradish peroxidase (BD Pharmingen) for 1 hour at room temperature. Plates were developed using 3,3', 5,5'-tetramethylbenzidine (TMB) substrate (Mandel Scientific, ON) and stopped with 1N HCl. Plates were read at 450 nm on a Spectramax 384 (Molecular Devices, CA).

2.19 Antibiotic treatment

Mice received antibiotics (0.5 g/l of each ampicillin, gentamicin, neomycin and metronidazole, 0.25g/l vancomycin, with 4 g/l Splenda for taste) in their drinking water from weaning until euthanization (~10 week of age).

2.20 Statistics

Data are presented as mean ± standard error of the mean (S.E.M). A two-tailed Student's t-test using GraphPad Prism 5 software determined statistical significance. Results were considered statistically significant with $P < 0.05$.

Chapter 3: The transcriptional repressor HIC1 regulates intestinal T cell immune homeostasis

3.1 Overview

Among intestinal lymphocytes, $CD4^+$ T_H cells represent a major population that are important in mediating diverse host protective and homeostatic responses. However, accumulation of $CD4^+$ T_H cells in the intestine is also a key feature of IBD [203]. Through production of cytokines that sustain and amplify inflammation, $CD4^+$ T_H cells have been identified as potential drivers of disease in IBD and therefore represent an attractive therapeutic target [203]. atRA is an important mediator of $CD4^+$ T_H cell function in the intestine through multiple mechanisms including promoting intestinal T cell homing as well as playing a central role in both effector T cell activation and the differentiation of iT_{reg} cells [147,155,159–161]. However, despite the dependence on atRA for proper intestinal T cell function, the molecular mechanisms downstream of atRA signalling that control T cell homeostasis remain unknown. In this chapter, we identify HIC1 as an atRA inducible regulator of intestinal immune responses under homeostatic and inflammatory conditions. Using *Hic1* reporter mice, we demonstrate that *Hic1* is expressed in immune cells specifically in the intestinal lamina propria and intraepithelial compartment but not in other lymphoid or non-lymphoid tissues in the steady state. Further, we demonstrate that HIC1 expression is reliant on atRA signaling as mice raised on a vitamin A deficient diet lose *Hic1* expression in intestinal T cells. Moreover, *in vitro* stimulated T cells upregulated HIC1 expression in response to exogenous atRA in culture. To assess the function of HIC1 in intestinal T cells, we generated mice with a conditional deletion of *Hic1* specifically in

T cells. In the absence of HIC1 in T cells, we observe a significant reduction in the frequency of T cells in the lamina propria and intraepithelial compartment of the intestine, coincident with increased T_H17 cell responses. Further, loss of HIC1 specifically in $CD4^+$ T_H cells renders mice resistant to the development of intestinal inflammation, suggesting that HIC1 is required for the pathogenicity of $CD4^+$ T_H cells *in vivo*. Together these results demonstrate that HIC1 plays a central role in intestinal immune homeostasis and inflammation.

3.2 Results

3.2.1 *Hic1* expression in immune cells is restricted to the intestine

To begin to address the role of HIC1 in the immune system, we examined the expression of *Hic1* in $CD45^+$ leukocytes in various tissues of mice with a fluorescent reporter gene inserted in the *Hic1* locus (*Hic1*^{Citrine} mice) [201]. Citrine expression in $CD45^+$ cells was restricted to the intestine, with the only detectable Citrine-positive cells in the lamina propria and intraepithelial space (**Figure 3.1A**). Further characterization of the leukocytes in the LP revealed that a majority of T cell receptor β (TCRβ) chain-expressing $CD4^+$ and $CD8^+$ T cells and TCRγδ$^+$ $CD8^+$ T cells in the LP expressed *Hic1* (**Figure 3.1B**) and that TCRβ$^+$ $CD8^+$ and TCRγδ$^+$ $CD8^+$ intraepithelial lymphocytes (IELs) also expressed *Hic1* (**Figure 3.1C**). We also found that most $MHCII^+$ $CD11c^+$ $CD64^-$ dendritic cells (DCs) and $MHCII^+$ $CD64^+$ $F4/80^+$ macrophages expressed *Hic1* (**Figure 3.1B**). However, *Hic1* expression was not generalized for all lymphocytes in the LP, as $B220^+$ B cells did not express *Hic1* (**Figure 3.1B**). Thus, *Hic1* expression in immune cells specifically identifies intestinal resident populations.

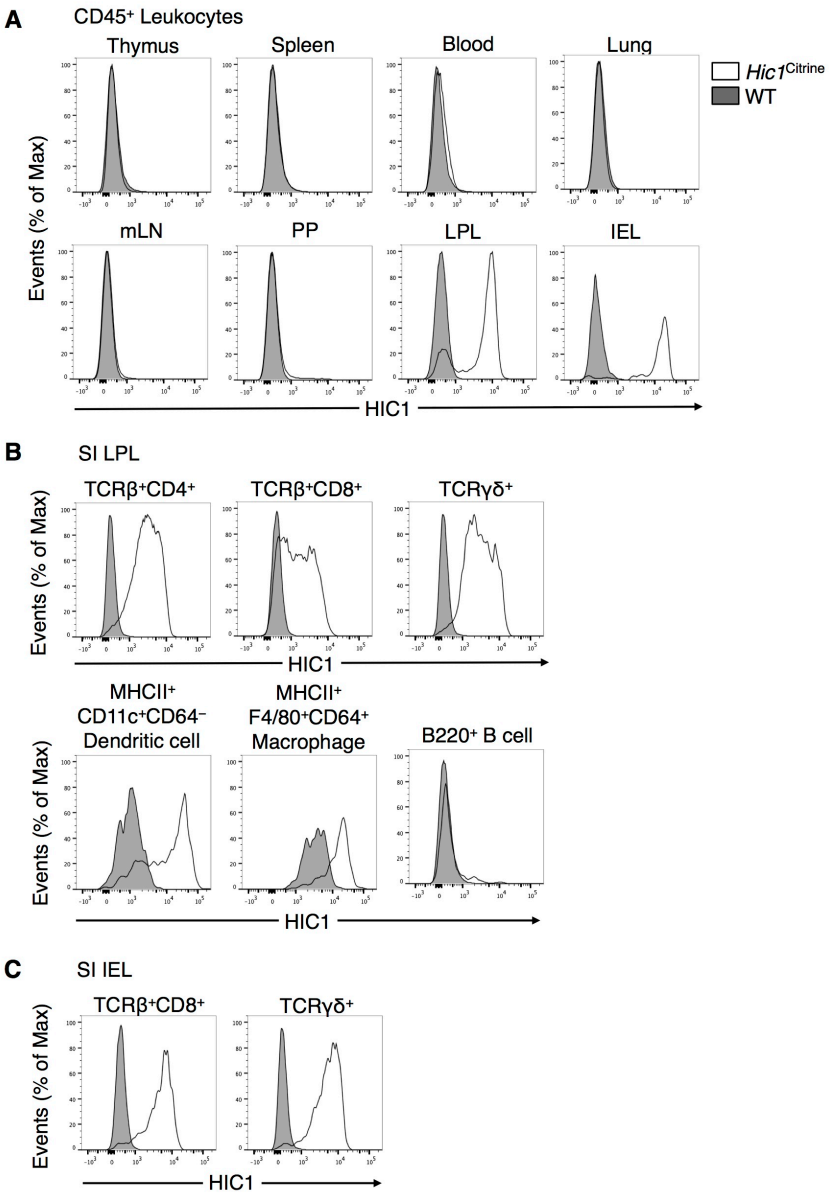

Figure 3.1 *Hic1* expression in immune cells is restricted to the intestine.

(A) Steady state thymus, spleen, blood, lung, mesenteric lymph node (mLN), peyer's patch (PP) and intestinal lamina propria and intraepithelial leukocytes (LPL and IEL) were analyzed by flow cytometry for *Hic1*^{Citrine} reporter expression in total CD45$^+$ leukocytes. (B) TCRβ$^+$CD4$^+$ T cells, TCRβ$^+$CD8$^+$ T cells, TCRγδ$^+$ T cells, MHCII$^+$CD11C$^+$CD64$^-$ Dendritic cells, MHCII$^+$F4/80$^+$CD64$^+$ Macrophages or B220$^+$ B cells were analyzed by flow cytometry for *Hic1*^{Citrine} reporter expression from the intestinal lamina propria or (C) intraepithelial compartment. (A-C) Data are representative of 3 independent experiments.

3.2.2 T cell-intrinsic HIC1 regulates intestinal immune homeostasis

As *Hic1* was expressed in multiple immune cell populations in the LP, we next sought to determine the cell-intrinsic functions of HIC1 by focusing on its role specifically in T cells. To do this, we crossed mice with *loxP* sites flanking the *Hic1* gene (*Hic1*$^{fl/fl}$ mice) with mice that express the Cre recombinase under control of the *Cd4* enhancer and promoter (*Cd4*-Cre mice) to generate mice with a T cell-intrinsic deletion of *Hic1* (*Hic1*$^{\Delta T}$ mice). *Hic1*$^{\Delta T}$ mice displayed normal thymic development (**Figure 3.2A and B**) and had normal frequencies of CD4$^+$ and CD8$^+$ T cells in the spleen (**Figure 3.2C**) or mesenteric lymph nodes (mLN) (**Figure 3.2D**). HIC1 also had no effect on the activation state of splenic CD4$^+$ T cells, as we observed equivalent expression of CD62L and CD44 between *Hic1*$^{f/f}$ and *Hic1*$^{\Delta T}$ mice (**Figure 3.2E**). Finally, HIC1 was not required for the development of FOXP3$^+$ CD25$^+$ CD4$^+$ T cells in the spleen or mLN (**Figure 3.2F**). Thus, HIC1 is dispensable for peripheral T cell homeostasis. However, we found that the frequency and number of CD4$^+$ and CD8$^+$ TCRβ$^+$ cells in the LP (**Figure 3.3A and B**) and CD8$^+$ T cells in the intraepithelial compartment (**Figure 3.3A and B**) was significantly reduced in the absence of HIC1, demonstrating that T cell-intrinsic expression of HIC1 is required for intestinal T cell homeostasis.

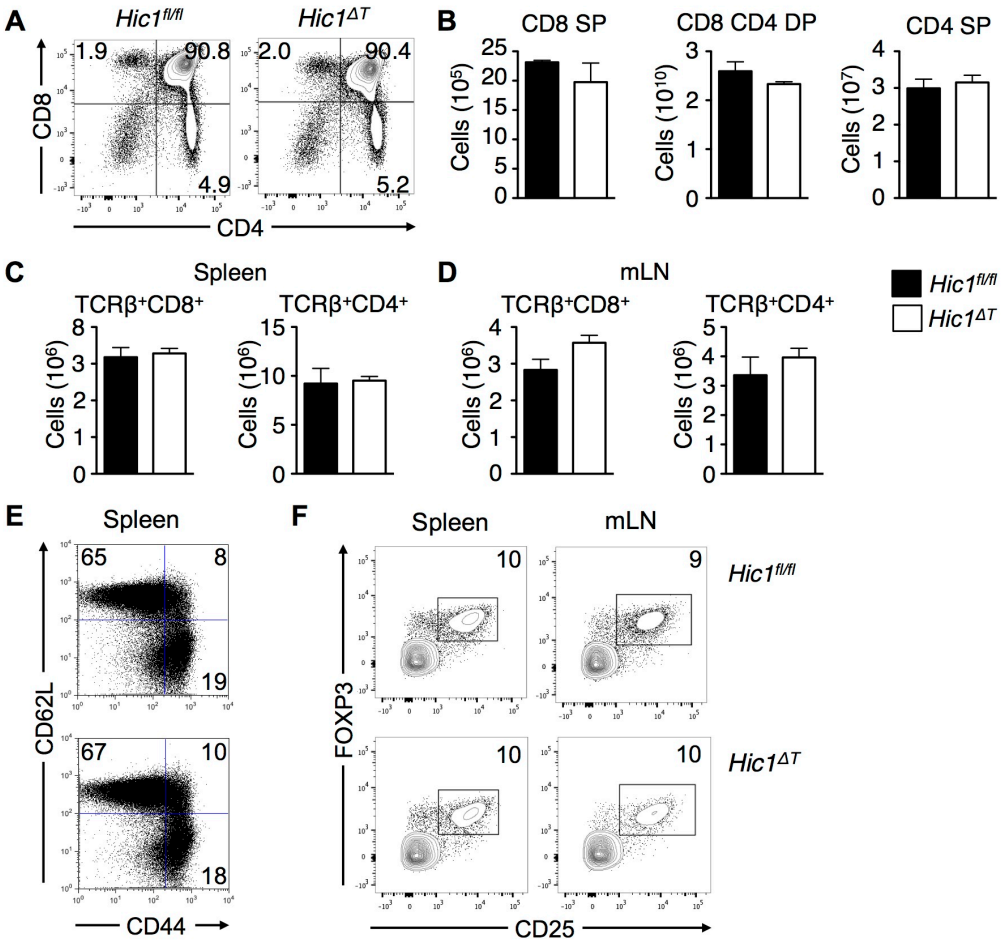

Figure 3.2 Normal central and peripheral T cell responses in the absence of HIC1.

(A-D) Thymus, spleen and mesenteric lymph nodes (mLN) from *Hic1*$^{fl/fl}$ and *Hic1*$^{\Delta T}$ mice were analyzed for CD4$^+$ and CD8$^+$ T cell frequencies and numbers by flow cytometry. (SP=single positive, DP=double positive). (E) CD62L$^+$ and CD44$^+$ frequencies of splenic CD4$^+$ T cells were analyzed by flow cytometry. (F) CD25$^+$FOXP3$^+$ T$_{reg}$ frequencies were analyzed from the spleen and mLN by flow cytometry. (B-D) Data are pooled from 3 independent experiments (n=4-6 per group). * $P < 0.05$. Error bars indicate SEM.

Lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes (IELs) display an activated, memory phenotype that include the markers CD69 and CD103, cell surface molecules that are important for retention in the intestinal microenvironment. CD69 has been shown to negatively regulate expression of sphingosine-1-phosphate receptor (S1PR1), which must be down regulated to establish tissue-residency [204,205], while CD103 binds to the epithelial cell-expressed E-cadherin and is required for maintenance of intestinal T cells [206]. We found that HIC1-deficient $CD4^+$ and $CD8^+$ T cells in the LP and intraepithelial compartments expressed significantly reduced levels of CD69 and CD103 (**Figure 3.3C and D**). Strikingly, there was an almost complete loss of $CD103^+$ $CD69^+$ $CD4^+$ T cells in the LP (**Figure 3.3C and D**). Thus, the reduced frequency and number of $CD4^+$ and $CD8^+$ LPLs and IELs in *Hic1*$^{\Delta T}$ mice is associated with reduced expression of CD69 and CD103.

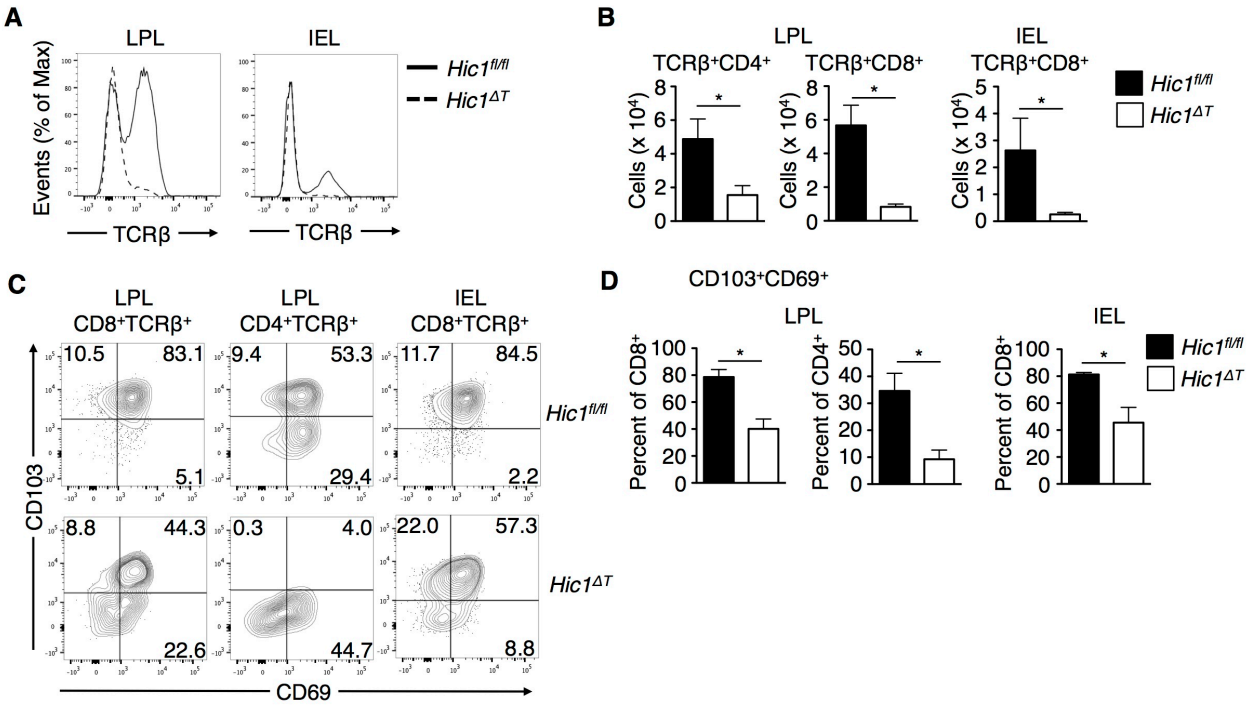

Figure 3.3 T cell-intrinsic HIC1 regulates intestinal T cell homeostasis.

Intestinal lamina propria (LPL) and intraepithelial (IEL) lymphocytes were isolated from *Hic1*$^{fl/fl}$ or *Hic1*$^{\Delta T}$ mice. (A) Frequency of TCRβ$^+$ T cells and (B, C) total number of TCRβ$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ T cells were analyzed by flow cytometry. (D) Flow cytometry analysis and (E) quantification of CD103 and CD69 surface marker expression from IEL and LPL TCRβ$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ T cells. (A, D) Data are representative of 3 independent experiments. (B, C, E) Data are pooled from 3 independent experiments (n=6-7 per group). * *P* < 0.05. Error bars indicate SEM.

3.2.3 HIC1 is a negative regulator of IL-17A production by CD4$^+$ T cells

In the intestinal LP, CD4$^+$ T_H17 and T_{reg} cells are found at higher frequencies than other T_H cell subsets [66]. Analysis of the CD4$^+$ LPLs showed that although frequency of FOXP3$^+$ T_{reg} cells were equivalent between *Hic1$^{fl/fl}$* and *Hic1$^{\Delta T}$* mice, the frequency of ROR-γt$^+$ IL-17A-expressing T_H17 cells in *Hic1$^{\Delta T}$* mice was significantly increased (**Figure 3.4A and B**). Further, splenic T cells in *Hic1$^{\Delta T}$* mice displayed a small but significant increase in the frequency of IL-17A expression T_H17 cells (**Figure 3.4C**). These results suggest that in addition to regulating T cell numbers in the intestine, HIC1 deficiency in T cells had a specific effect on T_H17 cell differentiation. To directly examine the role of HIC1 in T_H cell differentiation, we stimulated T_H cells that were purified from the spleen and peripheral lymph nodes (pLNs) of naïve *Hic1$^{fl/fl}$* and *Hic1$^{\Delta T}$* mice (**Figure 3.4D**) under diverse polarizing conditions. HIC1 deficiency had no effect on the development of T_H1, T_H2 or TGF-β-induced T_{reg} (iT_{reg}) cells (**Figure 3.5A and B**). Consistent with our *in vivo* results, we observed a significant increase in the production of IL-17A by HIC1-deficient T_H cells activated under T_H17 cell-promoting conditions, with heightened frequency and mean fluorescence intensity of IL-17A$^+$ cells (**Figure 3.4E**), resulting in a significant increase in the levels of secreted IL-17A (**Figure 3.4G**), which was also associated with increased *Il17a* expression in HIC1-deficient T_H17 cells (**Figure 3.4F**). Retroviral transduction of HIC1 into differentiating T_H17 cells resulted in a complete inhibition of IL-17A production (**Figure 3.4H and I**). Taken together, these results suggest that HIC1 is a cell-intrinsic negative regulator of IL-17A production during T_H17 cell differentiation.

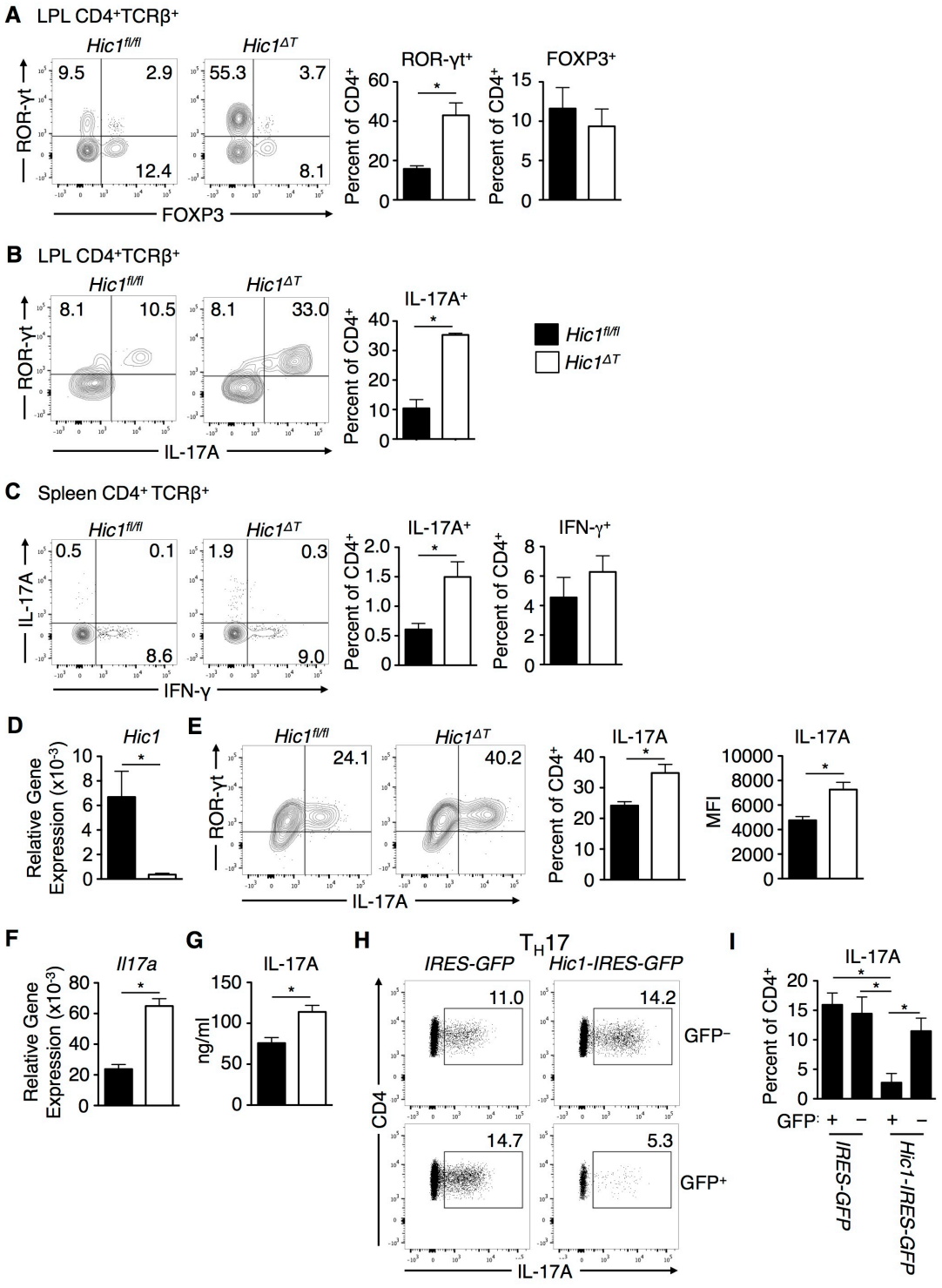
A LPL CD4+TCRβ+
Hic1fl/fl
Hic1ΔT
9.5
2.9
12.4
55.3
3.7
8.1
ROR-γt
FOXP3
ROR-γt+
FOXP3+
Percent of CD4+
B LPL CD4+TCRβ+
8.1
10.5
8.1
33.0
IL-17A
IL-17A+
Hic1fl/fl
Hic1ΔT
C Spleen CD4+ TCRβ+
0.5
0.1
8.6
1.9
0.3
9.0
IFN-γ
IL-17A+
IFN-γ+
D
Hic1
Relative Gene Expression (x10-3)
E
24.1
40.2
IL-17A
MFI
F
Il17a
G
ng/ml
H
TH17
IRES-GFP
Hic1-IRES-GFP
11.0
14.2
14.7
5.3
GFP−
GFP+
CD4
I
GFP:
IRES-GFP
Hic1-IRES-GFP

Figure 3.4 HIC1 is a negative regulator of IL-17A production by CD4$^+$ T cells.

(A, B) Steady-state intestinal lamina propria (LPL) or (C) Spleen CD4$^+$ T cells were isolated from *Hic1$^{fl/fl}$* or *Hic1$^{\Delta T}$* mice and analyzed by flow cytometry for intracellular expression of ROR-γt, FOXP3 and IL-17A. Data are pooled from 3 independent experiments (n=5-6 per group). *Hic1$^{fl/fl}$* or *Hic1$^{\Delta T}$* splenic CD4$^+$ T cells were activated under T$_H$17 cell-polarizing conditions and analyzed for: (D) *Hic1* mRNA expression by quantitative RT-PCR, (E) intracellular ROR-γt and IL-17A frequency and mean fluorescence intensity (MFI) by flow cytometry, (F) IL-17A protein production by ELISA, and (G) *Il17a* mRNA expression by qRT-PCR. Data are pooled from 4 independent experiments (n=4-8 per group). (H-I) Analysis of IL-17A expression from T$_H$17 cell-polarized cells that were retrovirally transduced with MigR1-IRES GFP (empty vector) or MigR1-*Hic1*-IRES GFP expression vectors. GFP$^+$ indicated successful retroviral infection. (I) Data are pooled from 3 independent experiments (n=3 per group). * *P* < 0.05. Error bars indicate SEM.

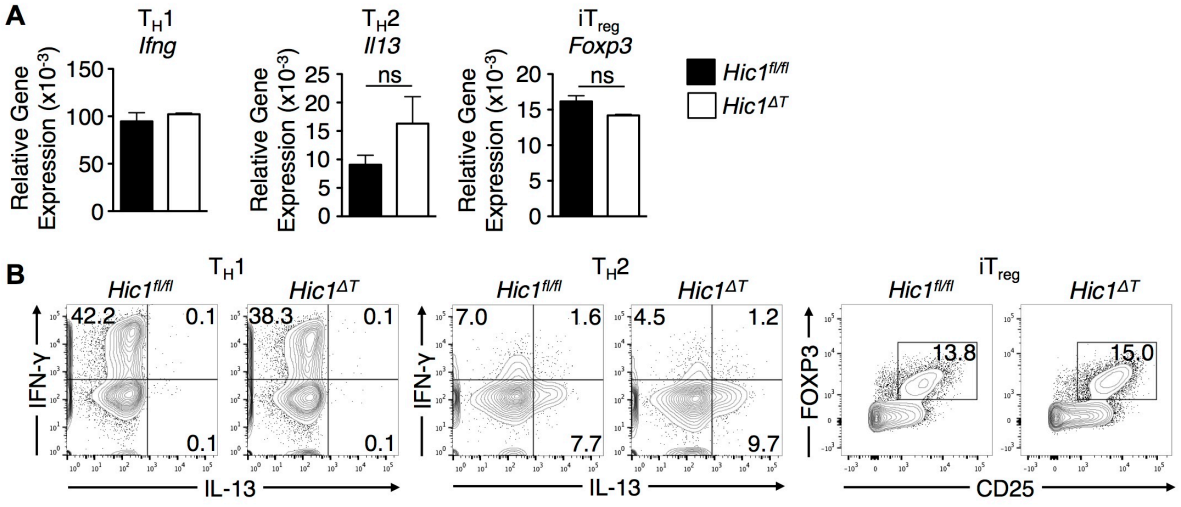

Figure 3.5 HIC1 is dispensable for T$_H$1, T$_H$2 and iT$_{reg}$ cell differentiation.

***Hic1$^{fl/fl}$* or *Hic1$^{\Delta T}$* splenic CD4$^+$ T cells were activated under T$_H$1, T$_H$2 or iT$_{reg}$ cell-polarizing conditions and analyzed for: (A) *Ifng*, *Il13* or *Foxp3* mRNA expression by quantitative RT-PCR, and (B) intracellular IFN-γ, IL-13 and FOXP3 by flow cytometry. Data are pooled from 2 independent experiments (n=6 per group). * *P* < 0.05. Error bars indicate SEM.**

3.2.4 Retinoic acid regulates expression of *Hic1* in T cells *in vitro* and *in vivo*

The vitamin A metabolite atRA plays a critical role in intestinal immune homeostasis [207]. Previous studies have shown that mice raised on a diet lacking vitamin A (VAD diet) have defects in T_H cell activation and intestinal migration, resulting in an overall impairment in T cell-driven immune responses in the intestine [147,151,155]. To test whether atRA influenced *Hic1* expression in intestinal T_H cells, we raised *Hic1*^{Citrine} mice on a VAD diet. We found that LP T_H cells from VAD mice failed to express *Hic1* (**Figure 3.6A**), suggesting that *Hic1* expression in T_H cells in the LP is dependent upon the presence of atRA. Consistent with this, addition of atRA to T_H cells isolated from spleen or lymph nodes of mice activated *in vitro* with antibodies against CD3 and CD28 resulted in an upregulation of *Hic1 mRNA and* HIC1 protein levels (**Figure 3.6B and C**). Analysis of *Hic1* expression using *Hic1*^{Citrine} mice further demonstrated that treatment of activated T_H with atRA led to increased *Hic1* expression (**Figure 3.6D**). Expression of *Hic1* was dependent upon T_H cell activation, as addition of atRA to T_H cells in the absence of T cell receptor stimulation had no effect on the expression of *Hic1* mRNA (**Figure 3.6E**). Thus, these results identify *Hic1* as an atRA-responsive gene in activated T_H cells.

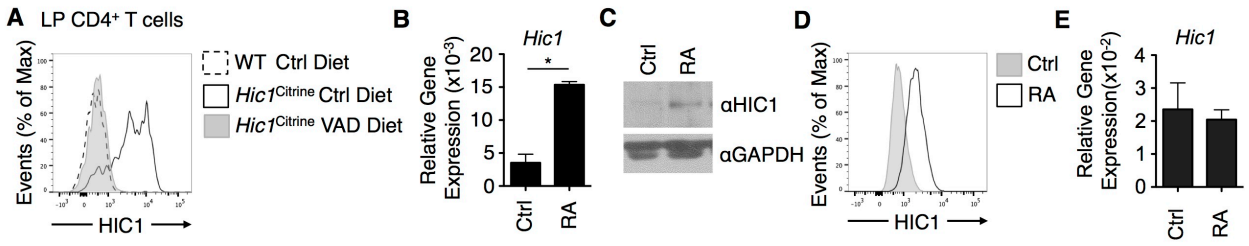

Figure 3.6 Retinoic acid regulates expression of *Hic1* in CD4$^+$ T cells.

(A) *Hic1* reporter expression in intestinal lamina propria (LP) CD4$^+$ T cells from *Hic1*^{Citrine} mice fed a control diet, *Hic1*^{Citrine} mice fed a vitamin A deficient (VAD) diet, and controls fed a control diet was analyzed by flow cytometry. Data are representative of 3 independent experiments. (B-D) Splenic T_H cells were activated with antibodies against CD3 and CD28 in the presence or absence of 10 nM RA and HIC1 levels were measured by (B) quantitative RT-PCR (C) western blot and (D) *Hic1* reporter expression. (B) Data are pooled from 2 independent experiments (n=4 per group). (C and D) Data are representative of 2 independent experiments. (E) Expression of *Hic1* in naïve splenic CD4$^+$ T cells that were treated with 10 nM RA for 16 hours was analyzed by qRT-PCR. Data are pooled from 2 independent experiments (n=4 per group). * $P < 0.05$. Error bars indicate SEM.

3.2.5 HIC1 is dispensable for expression of intestinal homing receptors

atRA is critical for migration of immune cells to the intestine [147] through the upregulation of intestinal homing molecules including α4β7 integrin and CCR9 [155]. Our results showing that *Hic1*$^{\Delta T}$ mice had reduced numbers of intestinal T cells could be due to reduced expression of intestinal homing molecules, resulting in impaired intestinal migration. However, we failed to observe any reduction in expression of α4β7 and CCR9 on T_H cells in the presence or absence of HIC1 following activation in the presence of atRA (**Figure 3.7A**). Further, we found that T_H cells isolated from the Peyer's patches or mLN of *Hic1*$^{\Delta T}$ mice were not deficient in expression of CCR9 (**Figure 3.7B**). These results demonstrate that other HIC1-dependent mechanisms such as reduced expression of CD69 and CD103 were responsible for the paucity of T_H cells in the LP and that HIC1 is dispensable for RA-dependent induction of intestinal homing molecules.

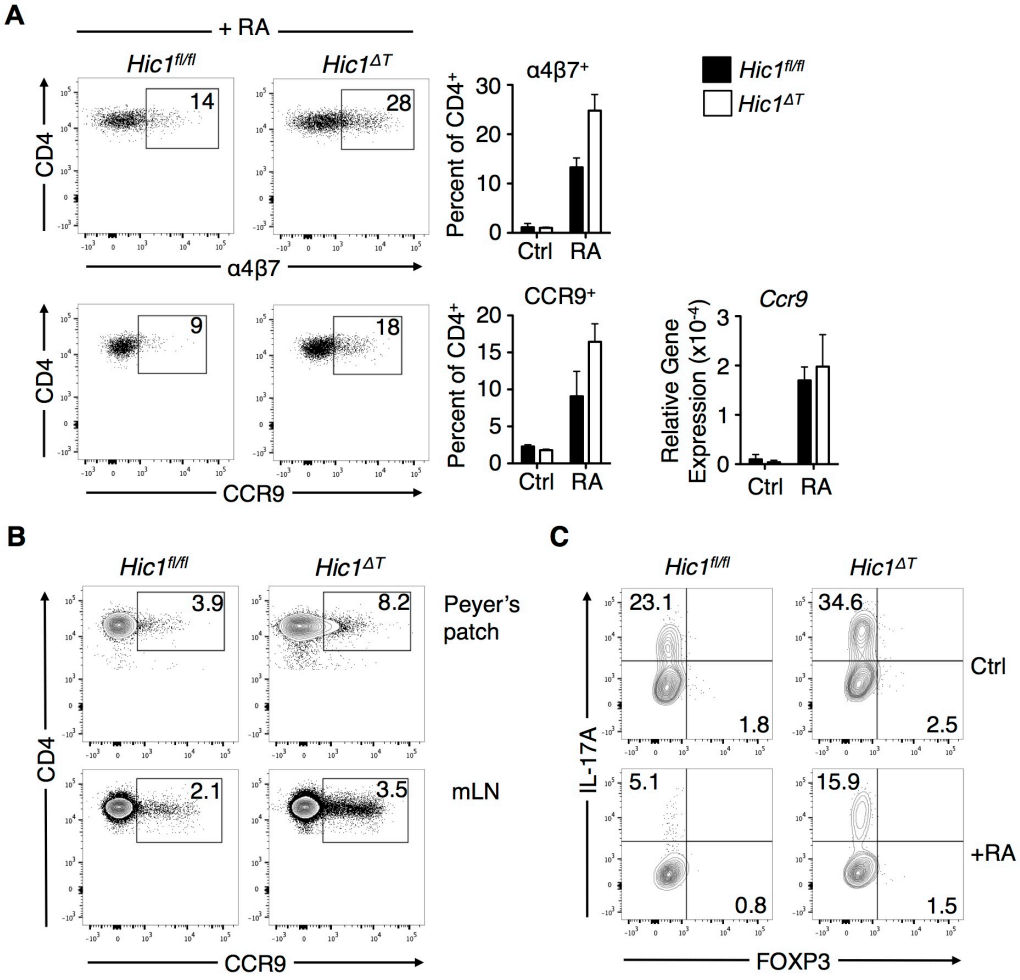

Figure 3.7 HIC1 is not required for all RA-mediated effects on CD4$^+$ T cells.

(A) Splenic T_H cells were activated with antibodies against CD3 and CD28 in the presence of 10 nM RA; α4β7$^+$ and CCR9 levels were measured by flow cytometry and quantitative RT-PCR. Data are pooled from 2 independent experiments (n=4 per group). (B) CD4$^+$ T cells were isolated from the mesenteric lymph nodes (mLN) and peyer's patches (PP) and analyzed for CCR9 expression by flow cytometry. Data are representative of 2 independent experiments. (C) Splenic CD4$^+$ T cells were activated under T_H17 cell-polarizing conditions in the presence or absence of 10 nM RA and analyzed for intracellular IL-17A and FOXP3 by flow cytometry. Data are representative of 3 independent experiments. * $P < 0.05$. Error bars indicate SEM.

3.2.6 HIC1 is not required for inhibitory effects of atRA on T_H17 cell differentiation

Several studies have demonstrated that RA can negatively affect the differentiation of T_H17 cells [159,208–210] although the precise mechanisms remain unclear. Based on our data showing heightened IL-17A production by HIC1-deficient T_H17 cells *in vitro* and *in vivo*, we hypothesized that expression of HIC1 would be required for the RA-dependent reduction in T_H17 cell differentiation. In contrast to our expectations, addition of RA to either HIC1-sufficient or -deficient T_H cells led to a significant reduction in the expression of IL-17A (**Figure 3.7C**). Thus, although HIC1 is up regulated by atRA in T_H cells and HIC1-deficient T_H17 cells express increased levels of IL-17A *in vitro* and *in vivo*, HIC1 is dispensable for atRA-dependent regulation of T_H17 cell responses *in vitro*.

3.2.7 HIC1 regulates T cell-mediated inflammation in the intestine

We next examined whether dysregulated T_H cell responses observed in naive *Hic1*$^{\Delta T}$ mice had any effect on the development of intestinal inflammation. Despite the reduced numbers of T_H cells in the LP of *Hic1*$^{\Delta T}$ mice and the dysregulated production of IL-17A by HIC1-deficient T_H17 cells, we failed to observe any significant differences in intestinal architecture between naïve *Hic1*$^{fl/fl}$ or *Hic1*$^{\Delta T}$ mice (**Figure 3.8A**, left panels). After induction of intestinal inflammation with intraperitoneal injection of a monoclonal antibody against CD3 [98,102,211], *Hic1*$^{\Delta T}$ mice displayed less intestinal inflammation compared to control *Hic1*$^{fl/fl}$ mice (**Figure 3.8A and B**). Although we observed fewer $CD4^+$ T cells in the intestine of treated *Hic1*$^{\Delta T}$ mice (**Figure 3.8C**), we did observe an increase in the number of $CD4^+$ T cells in the LP of treated *Hic1*$^{\Delta T}$ mice compared to naïve *Hic1*$^{\Delta T}$ mice (**Figure 3.3A and B**), further demonstrating that intestinal migration is not completely impaired in the absence of HIC1. Analysis of cytokine

production by T_H cells from the LP and mLN of anti-CD3 treated mice identified an increased frequency of IL-17A-producing T_H cells without any changes in the frequency of IFN-γ-producing cells (**Figure 3.8A and B**), similar to our results under steady-state conditions. Thus, T cell-intrinsic expression of HIC1 modulates inflammation in the intestine, potentially by negatively regulating IL-17A production.

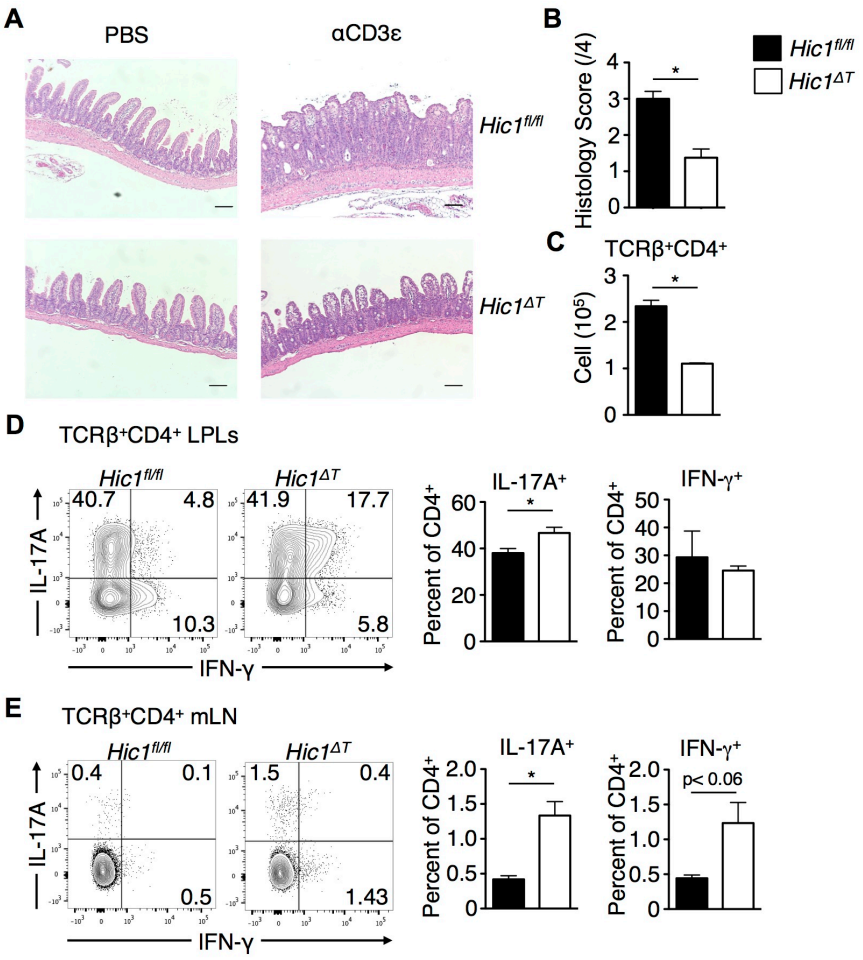

Figure 3.8 HIC1 is required for the development of intestinal inflammation.

Mice received anti-CD3ε antibody by intraperitoneal injection. (A) At 48 hours post-injection, mice were sacrificed and analyzed for intestinal tissue pathology and inflammatory infiltrate by H&E staining. Scale bar represents 100μm. (B) Histological scores. (C) Total numbers of intestinal lamina propria (LPL) TCRβ$^+$CD4$^+$ T cells were quantified by flow cytometry. (D, E) Intracellular expression of IL-17A and IFN-γ from (D) LPL and (E) mLN CD4$^+$ T cells were analyzed by flow cytometry. (A-E) Data are from 3 independent experiments (n=8-9 per group). * $P < 0.05$. Error bars indicate SEM.

To directly assess the cell-intrinsic role of HIC1 in intestinal inflammation, we employed a T cell transfer model of intestinal inflammation [104]. We adoptively transferred naïve $CD4^+$ $CD25^-$ $CD45RB^{high}$ T cells isolated from either $Hic1^{fl/fl}$ or $Hic1^{\Delta T}$ mice into $Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice that received control T cells from $Hic1^{fl/fl}$ mice began losing weight around 4 weeks post-transfer (**Figure 3.9A**) and showed significant intestinal inflammation by 6 weeks post-transfer (**Figure 3.9B**). Strikingly, we found that $Rag1^{-/-}$ mice that received T cells from $Hic1^{\Delta T}$ mice continued to gain weight and did not develop severe intestinal inflammation (**Figure 3.9A – C**). Associated with the reduced disease, there were significantly fewer $CD4^+$ T cells in the intestinal LP (**Figure 3.9D**). Furthermore, consistent with our results under homeostatic conditions as well as following α-CD3 treatment, we found that the absence of HIC1 in T cells resulted in heightened production of IL-17A with no significant effect on the frequency of IFN-γ-positive cells (**Figure 3.9E and F**). Thus, HIC1 expression in T cells is critically required for the development of intestinal inflammation, possibly by limiting expression of IL-17A.

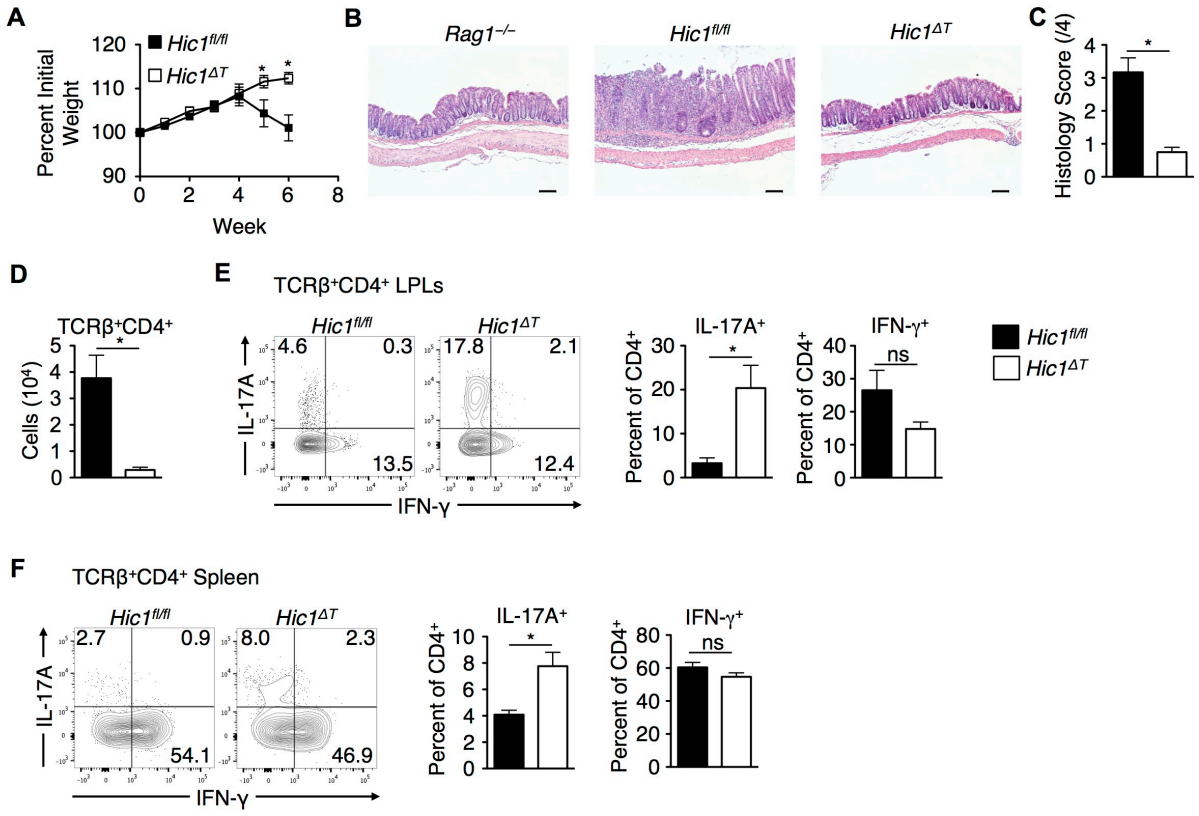

Figure 3.9 HIC1-deficient T cells fail to promote intestinal inflammation following adoptive transfer into *Rag1*$^{-/-}$ mice.

CD4^{+}CD25^{-}CD45RB^{hi} naive T cells (4 × 10^{5}) from *Hic1*$^{fl/fl}$ or *Hic1*$^{\Delta T}$ mice were transferred into *Rag1*$^{-/-}$ mice and monitored for colitis. (A) Weight loss (percentage of initial weight) was calculated for each mouse over 6 weeks. (B) At 6 weeks post-transfer, mice were sacrificed and analyzed for intestinal tissue pathology and inflammatory infiltrate by H&E staining. Scale bar represents 100μm. (C) Histological scores. (D) Total number of TCRβ^{+}CD4^{+} T cells isolated from intestinal lamina propria (LPL). (E, F) Intracellular expression of IL-17A and IFN-γ from (E) LPL and (F) Spleen CD4^{+} T cells were analyzed by flow cytometry. (A-F) Data are pooled 2 of 4 independent experiments (*n* = 4–8 per experiment). Statistics compare *Rag1*$^{-/-}$ mice that received *Hic1*$^{fl/fl}$ T cells to those that received *Hic1*$^{\Delta T}$ T cells. * *P* < 0.05. Error bars indicate SEM.

To resolve whether HIC1-deficient T cells were unable to initiate intestinal inflammation or if they offer a protective role during development of intestinal inflammation; we performed a T-cell 'co-transfer' experiment. We adoptively transfer either $CD4^+$ $CD25^-$ $CD45RB^{high}$ T cells isolated solely from *Hic1*$^{fl/fl}$ mice or T cells isolated from both *Hic1*$^{fl/fl}$ and *Hic1*$^{\Delta T}$ mice that were mixed at a 1:1 ratio into *Rag1*$^{-/-}$ mice. All *Rag1*$^{-/-}$ mice received the same number of *Hic1*$^{fl/fl}$ T cells in order to drive disease, with half of the mice receiving the additional T cells from *Hic1*$^{\Delta T}$ mice. We hypothesized that if HIC1-deficient T cells played a protective role, they would limit disease progression. Strikingly, we found that *Rag1*$^{-/-}$ mice that received a mixture of HIC1-sufficient and HIC1-deficient T cells displayed a significant difference in weight loss by 4 weeks post transfer and an overall delayed development of disease (**Figure 3.10A**). Associated with the reduced weight loss, histological analysis of the intestine revealed significant reduction in intestinal inflammation by 6 weeks post-transfer (**Figure 3.10B and C**). Thus, HIC1-deficient T cells offer protection in the development of T cell transfer driven intestinal inflammation.

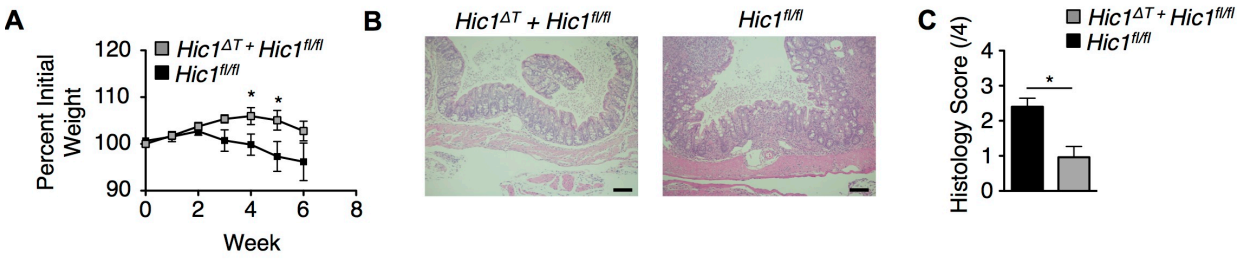

Figure 3.10 HIC1-deficient T cells prevent intestinal inflammation following adoptive transfer with wild type T cells into *Rag1*$^{-/-}$ mice.

CD4^{+}CD25^{-}CD45RB^{hi} naive T cells (4 × 10^{5}) from *Hic1*$^{fl/fl}$ or a 1:1 mixture of naïve T cells from *Hic1*$^{\Delta T}$ and from *Hic1*$^{fl/}$ mice (4 × 10^{5} from each group; 8 × 10^{5} total) were transferred into *Rag1*$^{-/-}$ mice and monitored for colitis. (A) Weight loss (percentage of initial weight) was calculated for each mouse over 6 weeks. (B) At 6 weeks post-transfer, mice were sacrificed and analyzed for intestinal tissue pathology and inflammatory infiltrate by H&E staining. Scale bar represents 100µm. (C) Histological scores. Data are pooled 2 independent experiments (*n* = 8 per group). * *P* < 0.05. Error bars indicate SEM.

Finally, to determine if HIC1-deficient T cells play a protective role in a non-T cell driven model of intestinal inflammation, we employed dextran sodium sulfate (DSS) induced colitis. This model involves chemically induced epithelial cell damage, which leads to bacterial translocation and activation of sub-epithelial innate immune cells and therefore does not require T or B cells for development of disease [108,109]. *Hic1*$^{fl/fl}$ mice and *Hic1*$^{\Delta T}$ mice were exposed to 3.5% DSS in their drinking water for 7 days before returning to regular drinking water for one final day. DSS-treated *Hic1*$^{\Delta T}$ mice exhibited attenuated weight loss in comparison to treated *Hic1*$^{fl/fl}$ mice (**Figure 3.11A**). At day 8 after DSS treatment, sections from the colon of DSS-treated *Hic1*$^{\Delta T}$ mice revealed negligible damage to the epithelium and a minimal presence of inflammatory cells. In contrast, tissue sections from DSS-treated *Hic1*$^{fl/fl}$ mice exhibited severe mucosal inflammatory cell infiltrate, sloughing of epithelial cells and complete loss of crypt architecture (**Figure 3.11B and C**). Therefore, HIC1-deficient T cells offer a protective function in the development of innate immune cell driven intestinal inflammation.

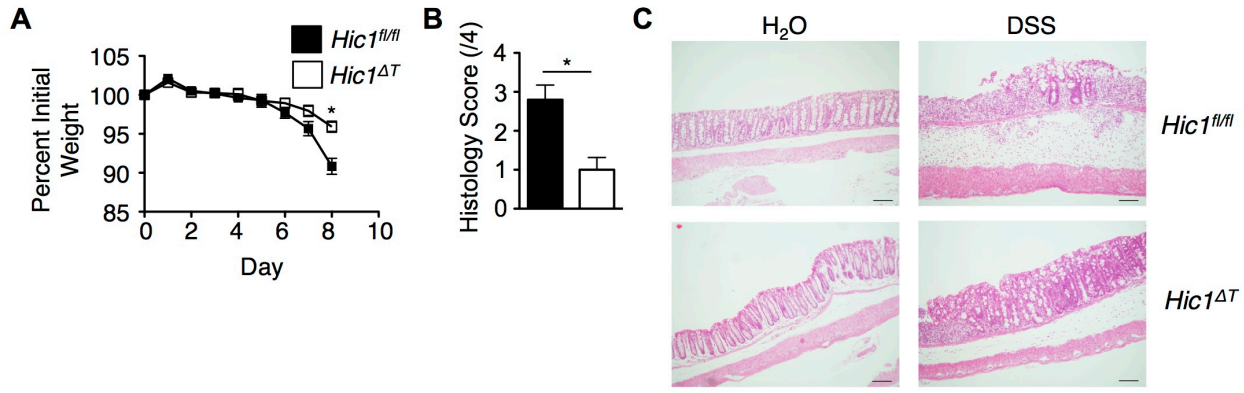

Figure 3.11 HIC1-deficient T cells limit inflammation during DSS induced colitis.

***Hic1*$^{fl/fl}$ mice and *Hic1*$^{\Delta T}$ mice were exposed to 3.5% DSS in their drinking water for 7 days and monitored for colitis. (A) Weight loss (percentage of initial weight) was calculated for each mouse over 8 days. (B, C) At 8 days post-DSS administration, mice were sacrificed and analyzed for intestinal tissue pathology and inflammatory infiltrate by H&E staining. (B) Histological scores. (C) Representative images. Scale bar represents 100µm. Data are pooled from 3 independent experiments (n=13 per group). * $P < 0.05$. Error bars indicate SEM.**

3.2.8 HIC1 is required to limit STAT3 signaling in T_H17 cells

In addition to directly repressing target genes, HIC1 has been shown to negatively regulate gene expression by several mechanisms including the recruitment of co-repressors such as the CtBP, NuRD and PRC2 complexes to target genes to mediate gene repression [191,212]. Further, HIC1 has also been shown to indirectly repress transcription by binding to transcriptional activators and preventing binding to target genes. HIC1 has been shown to interact with and inhibit DNA binding of transcription factors such as TCF4 and STAT3 [192–194]. As HIC1-deficient T_H17 cells produce heightened levels of the STAT3 target gene IL-17A [213], we hypothesized that increased STAT3 activity was associated with HIC1 deficiency. We first examined the levels of IL-6-induced active phosphorylated STAT3 (pSTAT3) in HIC1-sufficient and -deficient T_H17 cells by flow cytometry. We found that loss of HIC1 had no effect on the frequency of pSTAT3-positive T_H cells (**Figure 3.12A**), suggesting that HIC1 did not affect upstream STAT3 activation. However, co-immunoprecipitation studies in T_H17 cells of either native HIC1 (**Figure 3.12B**) or retrovirally-transduced FLAG-tagged HIC1 (**Figure 3.12C**) demonstrated that HIC1 and STAT3 interacted in T_H17 cells. Thus, our results suggest that HIC1 limits T_H17 cell differentiation by binding to STAT3, inhibiting its DNA binding and transcriptional activation. Consistent with this hypothesis, we found increased STAT3 binding to the *Il17a* promoter in the absence of HIC1 (**Figure 3.12D**), whereas there was no difference in binding at an irrelevant site (*Il5* promoter). Examination of mRNA expression for other known STAT3 target genes associated with T_H17 cell differentiation [213] revealed slight but insignificant increases in gene expression of *Rorc, Socs3, Irf4, Ahr or Il23r* (**Figure 3.12E**). Thus, HIC1 does not appear to directly regulate the molecular machinery that controls T_H17 cell differentiation.

Instead, our results identify an important role for HIC1 in limiting expression of *Il17a* in T_H17 cells by inhibition of STAT3 DNA binding.

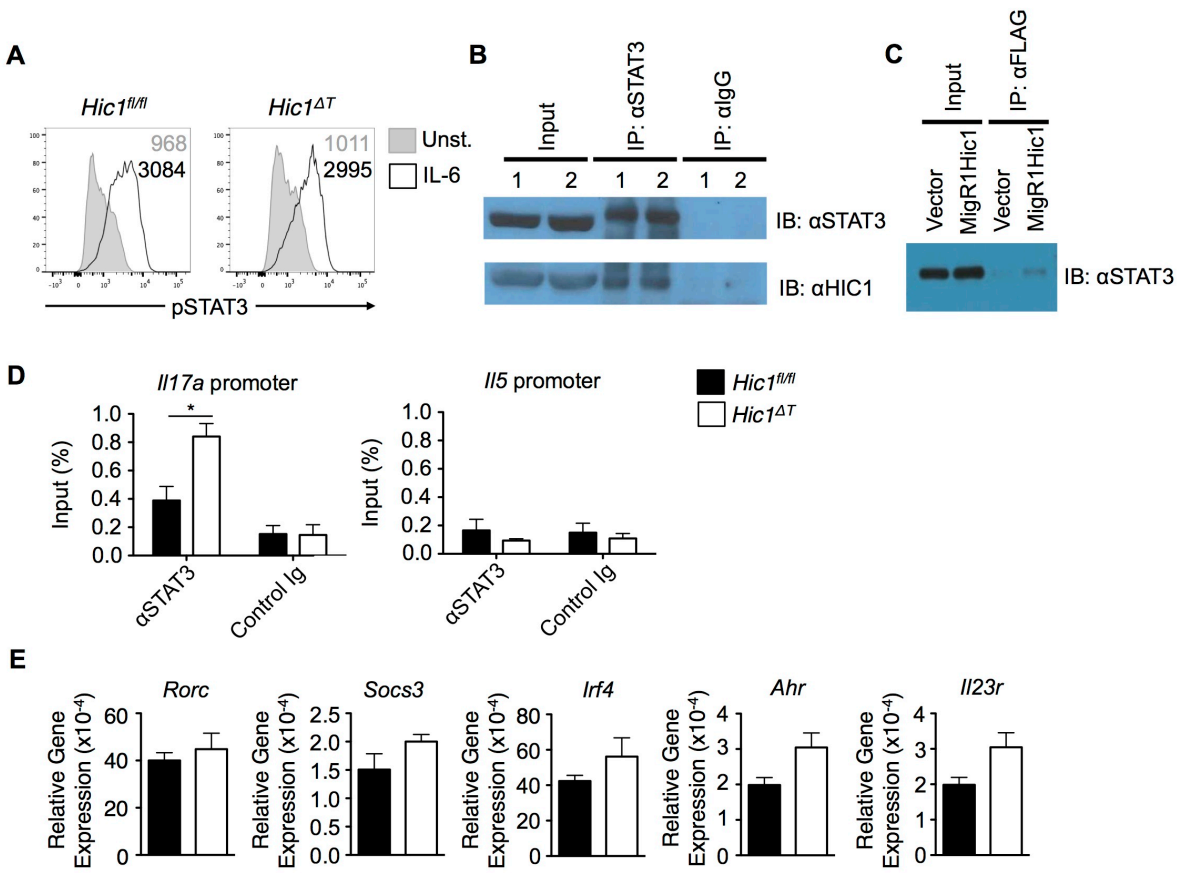

Figure 3.12 HIC1 regulates STAT3 signaling in T_H17 cells.

$CD4^+$ T cells were activated under T_H17 cell-polarizing conditions. (A) Flow cytometric analysis of STAT3 phosphorylation from T_H17 cells restimulated with or without IL-6 for 15 minutes. Numbers represent mean fluorescence intensity. Data are representative of 2 independent experiments. (B) Inputs, anti-STAT3 immunoprecipitates (IP), and normal rat serum IP from T_H17 cells were immunoblotted with anti-STAT3 and anti-HIC1 antibodies. 1 and 2 represent duplicate T_H17 cultures. Data are representative of 2 independent experiments. (C) Inputs and anti-FLAG IP from T_H17 cells retrovirally transduced with MigR1 (empty) or MigR1 FLAG-HIC1 vectors were immunoblotted with an anti-STAT3 antibody. Data are representative of 2 independent experiments. (D) Chromatin Immunoprecipitation (ChIP) analysis of STAT3 binding to the *Il17a* and *Il5* promoters in T_H17 cells. Data are from 3 independent experiments (n=6 per group). (E) Analysis of *Rorc, Socs3, Irf4, Ahr, Il23r* mRNA expression by quantitative RT-PCR. Data are from 2 independent experiments (n=6 per group). * $P < 0.05$. Error bars indicate SEM.

3.3 Discussion

We have identified the transcription factor HIC1 as a novel regulator of intestinal immune homeostasis. In the steady state, *Hic1* is specifically expressed in immune cells of the intestinal LP and intraepithelial niches in an atRA-dependent manner, and mice with a T cell-intrinsic deletion of *Hic1* have a dramatic decrease in the number of T cells in intestinal tissues. HIC1 deficiency leads to increased expression of IL-17A, possibly through the loss of STAT3 inhibition. Under inflammatory conditions, *Hic1* expression in T cells is required for intestinal pathology, identifying HIC1 as a potential therapeutic target to treat intestinal inflammation.

Our results suggest that the micronutrient atRA regulates *Hic1* expression in immune cells in the intestine. In support of this, two unrelated genome-wide expression screens for genes in T_H cells regulated by atRA signaling have identified HIC1 as an RA/RARα-dependent gene, although no functional analyses were carried out [195,196]. These results are consistent with an *in silico* study showing that the *Hic1* gene contains multiple RA receptor response elements (RAREs) [197]. However, HIC1 is dispensable for RA-mediated expression of intestinal homing molecules and atRA-dependent suppression of IL-17A production *in vitro*, suggesting that the atRA-HIC1 axis plays a specific role in regulating intestinal T cell homeostasis that remains to be defined.

T cell-intrinsic deletion of *Hic1* resulted in a significant reduction in the frequency and number of T cells in the intestinal microenvironment. Interestingly, mice raised on a VAD diet also display reduced numbers of intestinal T cells [147]. As atRA has been shown to regulate expression of intestinal homing molecules such as CCR9 and α4β7 integrin, we first suspected that defective trafficking to the intestine was responsible for the paucity of T cells in the intestines of $Hic1^{\Delta T}$ mice; however, we did not observe any defects in the ability of HIC1-

deficient T cells to express CCR9 or α4β7. In addition, we observed a significant influx of T cells into intestinal tissues upon induction of inflammation, further suggesting that migration to the intestine is not regulated by expression of HIC1. However, we did observe that the HIC1-deficient T cells present in the intestine at steady state expressed significantly lower levels of the surface molecules CD69 and CD103. These molecules are expressed by tissue-resident cells and are required for retention in tissues. Indeed, similar to our results, CD103-deficient mice have a severe reduction in the number of T cells in the intestine [206]. Thus, HIC1 appears to be required for the optimal expression of CD69 and CD103 and retention of T cells in the intestinal microenvironment.

In addition, CD69 and CD103 are characteristic markers of a subset of memory T cells known as tissue resident memory T (T_{RM}) cells. T_{RM} cells are long-lived quiescent cells that are thought to have derived from effector T cells that have migrated into non-lymphoid tissues. Interestingly, HIC1 has been shown to control fundamental cellular processes such as cell growth and survival [186,187]. HIC1 has been shown to interact with and regulate key transcription factors involved in cell cycle progression and cellular metabolism [191,214]. For example, HIC1 is involved in a regulatory feed back loop with the deacteylase SIRT1 [190], which is a key regulator of fatty acid oxidation [215]. Further, it has been demonstrated that fatty acid metabolism is key to memory T cell development and survival [216,217]. Thus, it is possible that in addition to directly regulating T cell retention in tissues, HIC1 may also be required to promote quiescence through metabolic pathways in T cells. Future studies will aim to characterize the role for HIC1 in T cell quiescence and memory development.

Another observation was the increase in the frequency of IL-17A-producing T_H17 cells in the intestine of *Hic1*$^{\Delta T}$ mice. T_H17 cells have been shown to play an important role in host

defense to extracellular bacteria and fungi [218]. However, they have also been described as pathogenic in multiple inflammatory diseases such as psoriasis and rheumatoid arthritis, and targeting IL-17A has proven to be an effective therapy [219–221]. However, the role of IL-17A in inflammatory bowel disease is controversial. Initial studies using the T cell transfer model of colitis provided the first evidence that IL-23-driven T_H17 cells were pathogenic [90]. Further, STAT3 was shown to be required to drive both colitis and systemic inflammation not only by modulating T_H17 cell differentiation but also by promoting T cell activation and survival [213]. However, targeted therapies against IL-17A in Crohn's disease have been proven ineffective [222,223] and recent studies have shown that IL-17A is important for maintaining intestinal barrier integrity and has a protective role in regard to the development of colitis [92,93]. Further, it is becoming more apparent that the T_H17 cell lineage has a degree of heterogeneity with regards to pathogenicity. For example, T_H17 cells differentiated in the presence of TGFβ are less pathogenic and produce higher levels of IL-10 while cells differentiated in the presence of IL-23 are more pathogenic and can produce IFN-γ [224,225]. In addition, T_H17 cells have a degree of plasticity and fate-mapping studies have shown that 'ex-T_H17' cells lose their ability to produce IL-17A altogether and exclusively produce IFN-γ under certain conditions [226]. As our studies show increased production of IL-17A from T_H17 cells and a lack of pathogenicity in multiple mouse models of intestinal inflammation, we hypothesize that in the absence of HIC1, T_H17 cells are skewed to a more protective lineage and do not transition into pathogenic cells. Further, it has been shown that STAT3 can inhibit transcription of key genes from other T_H cell lineages [227] and therefore may provide T_H17 lineage stability in the absence of HIC1. However, the precise molecular mechanism of how HIC1 regulates the pathogenicity of T cells remains to be fully elucidated.

In summary, we have identified the transcription factor HIC1 as an atRA-responsive cell-intrinsic regulator of T cell function in the intestine. Through its interaction with STAT3, HIC1 limits IL-17A production by T_H17 cells and is required for the development of intestinal inflammation. Together, these results suggest that HIC1 is an attractive therapeutic target for the treatment of inflammatory diseases of the intestine such as Crohn's disease and potentially other diseases associated with dysregulated T_H17 cell responses.

Chapter 4: HIC1 links retinoic acid signalling to group 3 innate lymphoid cell-dependent regulation of intestinal immunity and homeostasis

4.1 Overview

Chapter 3 focused on the role of HIC1 in intestinal T cell responses; however, in that study we also identified other intestinal immune cells (such as DCs and macrophages) that also expressed *Hic1*. Although T cells are present in vast numbers and deliver potent adaptive immune responses against antigens, the intestinal immune system also relies on innate immune cells and their rapid effector functions to maintain intestinal homeostasis [228,229]. DCs, and monocytes/macrophages are not only responsible for phagocytosis of pathogens and presentation of antigens but are also responsible for shaping the immune response through co-stimulatory molecules and cytokine secretion [228]. Further, the recently identified groups of ILCs are key orchestrators of immune defenses at mucosal surfaces [229]. Therefore, in this chapter, we sought to further characterized atRA dependent expression of HIC1 in other intestinal immune cell populations. In order to further characterize HIC1 function in other immune cells, we generated mice with either a deletion of *Hic1* in all hematopoietic cells or specifically in ROR-γt-expressing cells. At steady state, we show that HIC1 is critical for intestinal immune homeostasis by regulating ILC3s as deletion of *Hic1* results in a significant reduction in the number of ILC3s in the intestine. Further, we identify an ILC3-intrinsic role for HIC1 in regulating intestinal T_H cell responses to commensal bacteria as well as providing protective immunity to infections with the intestinal pathogens *Citrobacter rodentium* and *Trichuris muris*. These results identify a

central role for atRA-dependent expression of *Hic1* in ILC3s in the regulation of intestinal immune responses.

4.2 Results

4.2.1 *Hic1* is expressed by intestinal ILCs and is critical for intestinal immune homeostasis

We have previously shown that *Hic1* was highly expressed by T cells, DCs and macrophages within the intestinal lamina propria (LP) and intraepithelial compartments (Chapter 3). Using mice with a fluorescent reporter gene inserted in the *Hic1* locus (*Hic1*^{Citrine} mice) [201] we were able to determine that in addition to the previously identified populations, lineage-negative ($CD3^-CD5^-CD8^-CD11c^-CD11b^-CD19^-NK1.1^-Gr1^-Ter119^-B220^-$) ($lin^{neg}$) $CD90.2^+$ $CD127^+$ ILCs isolated from the intestinal lamina propria express *Hic1* (**Figure 4.1A**). Similar to our previous results, *Hic1* expression in ILCs was dependent on the availability of atRA, as *Hic1*^{Citrine} mice reared on a VAD diet did not express *Hic1* in ILCs within the LP (**Figure 4.1B**). To determine the role of HIC1 in ILCs, we first crossed mice with *loxP* sites flanking the *Hic1* gene (*Hic1*$^{fl/fl}$ mice) with mice that express the Cre recombinase under control of the *Vav* promoter (*Vav*-Cre mice) to generate mice with a hematopoietic specific deletion of *Hic1* (*Hic1*^{Vav} mice). Upon hematopoietic cell-specific deletion of *Hic1* we observed a total reduction in the number of $CD45^+$ leukocytes in the intestinal LP at steady state (**Figure 4.1C and D**). Similar to results reported in Chapter 3 using a T cell specific deletion of *Hic1*, we saw a reduction in the total number of $TCR\beta^+$ T cells (**Figure 4.1E and F**). Analysis of $CD11c^+MHCII^+$ antigen presenting dendritic cells demonstrated a large shift in DC populations.

There was a significant decrease in number of the $CD103^{+}CD11b^{+}$ DCs accompanied with a significant increase in $CD103^{+}CD11b^{-}$ DCs (**Figure 4.1G and H**). Further, we observed a significant change in ILC populations in the LP. There were significantly fewer ROR-γt^{+} ILCs (ILC3s) in the LP of *Hic1*^{Vav} mice, with a significant reduction in the number of ROR-γt^{+} $TBET^{+}$ ILC3s (**Figure 4.1I and J**). We also detected a small but significant increase in the number of $CD4^{+}$ ILC3s (also known as lymphoid tissue inducer (LTi) cells) but no change in numbers of the canonical $GATA3^{+}$ ILC (ILC2) population (**Figure 4.1I and J**). Thus hematopoietic cell-intrinsic HIC1 is critical for regulation of T cell, DC, and ILC populations in the intestinal LP.

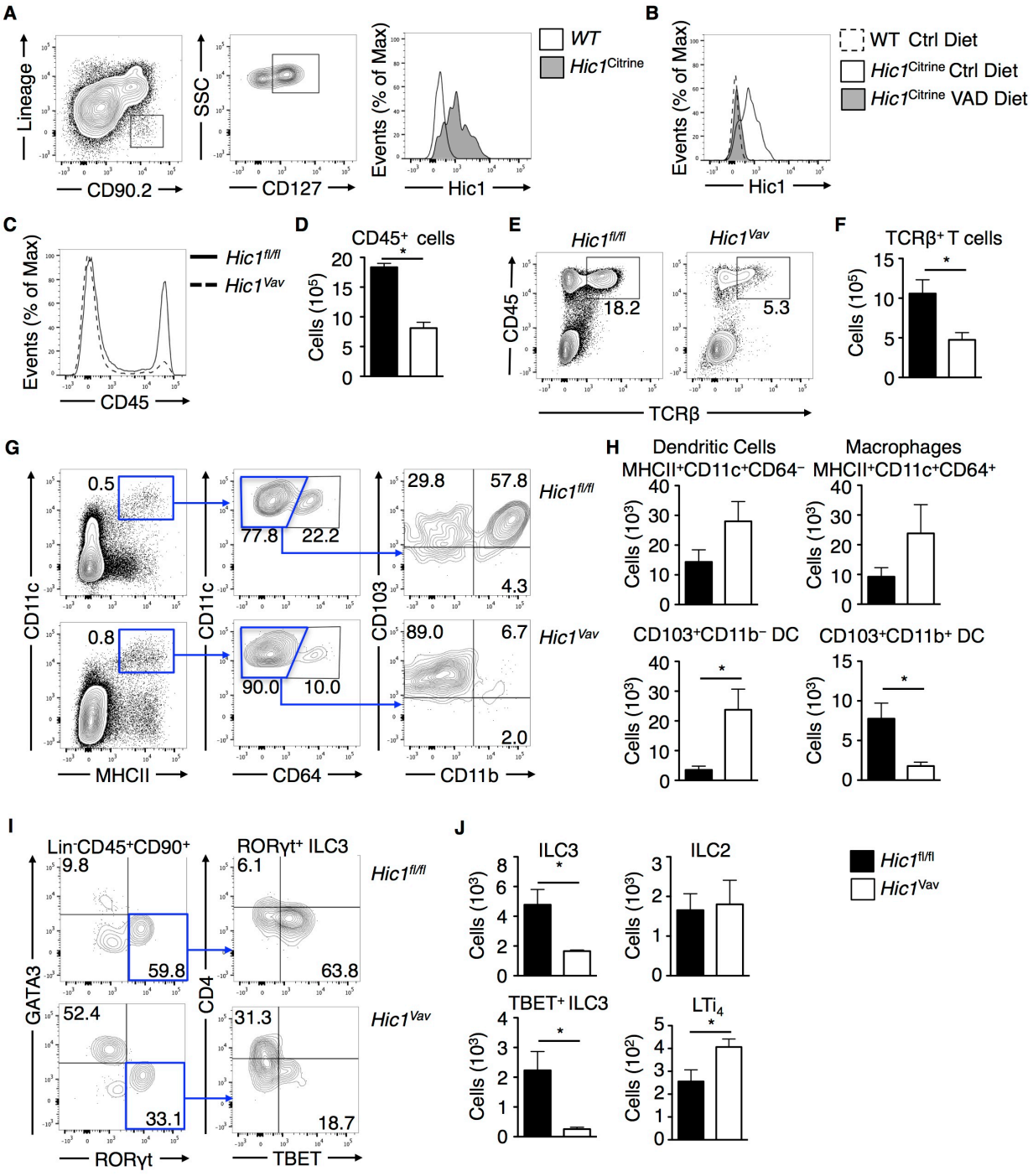
A
Lineage
CD90.2
SSC
CD127
Events (% of Max)
Hic1
WT
Hic1Citrine
B
WT Ctrl Diet
Hic1Citrine Ctrl Diet
Hic1Citrine VAD Diet
C
Hic1fl/fl
Hic1Vav
CD45
D
CD45+ cells
Cells (10^5)
E
Hic1fl/fl
Hic1Vav
18.2
5.3
TCRβ
F
TCRβ+ T cells
G
0.5
77.8
22.2
29.8
57.8
4.3
0.8
90.0
10.0
89.0
6.7
2.0
CD11c
MHCII
CD64
CD103
CD11b
H
Dendritic Cells
MHCII+CD11c+CD64−
Macrophages
MHCII+CD11c+CD64+
CD103+CD11b− DC
CD103+CD11b+ DC
Cells (10^3)
I
Lin-CD45+CD90+
9.8
59.8
RORγt+ ILC3
6.1
63.8
52.4
33.1
31.3
18.7
GATA3
CD4
RORγt
TBET
J
ILC3
ILC2
TBET+ ILC3
LTi4
Cells (10^2)

Figure 4.1 *Hic1* is expressed by intestinal ILCs and is required for intestinal immune homeostasis.

(A) ILCs (lin^{neg} $CD90^+$ $CD127^+$ cells) were analyzed by flow cytometry for $Hic1^{Citrine}$ reporter expression from the intestinal lamina propria (LP). Data representative of 2 independent experiments (B) *Hic1* reporter expression in intestinal LP ILCs from $Hic1^{Citrine}$ mice fed a control diet, $Hic1^{Citrine}$ mice fed a vitamin A deficient (VAD) diet, and controls fed a control diet was analyzed by flow cytometry. Data are representative of 2 independent experiments. (C - J) Intestinal LP cells from $Hic1^{fl/fl}$ and $Hic1^{Vav}$ mice at steady state were analyzed by flow cytometry to enumerate populations of: (C, D) $CD45^+$ leukocytes, (E, F) $TCR\beta^+$ T cells, (G, H) $CD11c^+$ $MHCII^+$ $CD64^+$ macrophages, $CD11c^+$ $MHCII^+$ $CD64^-$DCs, (I, J) ROR-γt^+ ILC3s, $GATA3^+$ ILC2s, $CD4^+$ ILC3s (LTis) and $TBET^+$ ROR-γt^+ ILC3s. Data pooled from 2 independent experiments (n=4 per group). *, $P < 0.05$; Student's *t* test. Errors bars indicate SEM.

4.2.2 HIC1 regulates intestinal ILC3 populations in a cell-intrinsic manner

To determine whether HIC1 functioned in a cell-autonomous manner in regulating the homeostasis of intestinal ILCs, we reconstituted lethally irradiated $CD45.1^+$ mice with mixed bone marrow (BM) harvested from congenic wild type ($CD45.1/2^+$) mice and $CD45.2^+$ $Hic1^{fl/fl}$ or $Hic1^{Vav}$ mice. Although a 50:50 mix of donor BM (wild type/$Hic1^{fl/fl}$) yielded chimeric recipient mice with an ~40:60 ratio of donor-derived ILC3s, a 50:50 ratio of donor BM (wild type/$Hic1^{Vav}$) only yielded a ~70:30 ratio of donor-derived ILC3s (**Figure 4.2A and B**), indicating there is a cell-intrinsic role for HIC1 as the wild type BM significantly outcompeted the $Hic1^{Vav}$ BM in developing intestinal ILC3s.

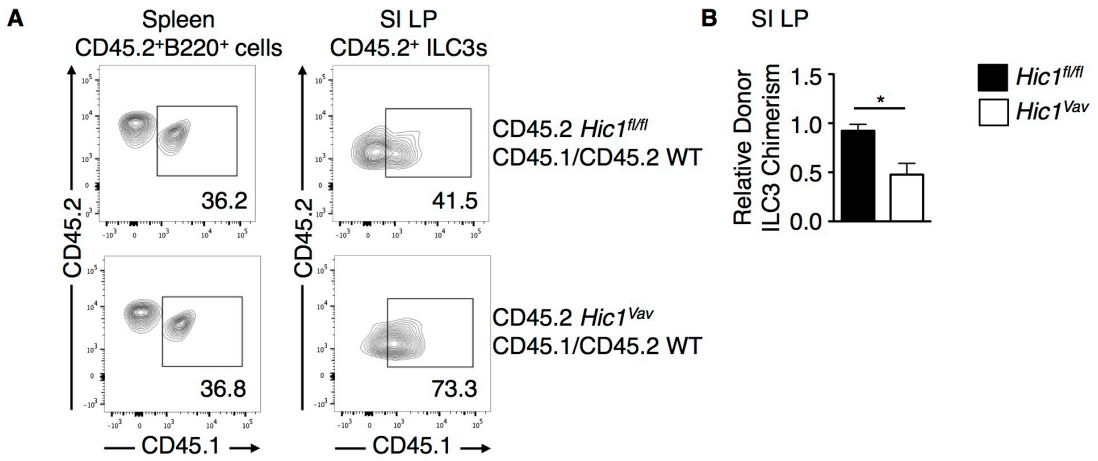

Figure 4.2 ILC-intrinsic HIC1 regulates ILC3 populations within the intestine.

Reconstitution of mixed bone marrow (BM) chimeras of *Hic1*^{Vav} BM and control *Hic1*$^{fl/fl}$ BM competed with CD45.1/2 BM and together injected into lethally irradiated CD45.1 mice was examined 12 weeks after transplant. (A) Representative plots of splenic B cells (gated on CD45.2^{+} B220^{+}) and small intestinal lamina propia (SILP) ILC3 (gated on CD45.2^{+} lin^{neg} CD90.2^{+} ROR-γt^{+}) (b) Relative ILC3 chimerism normalized to splenic B cells. Data are from two independent experiments (*n* = 6–8 mice per group).

4.2.3 HIC1 does not regulate ILC precursors in the bone marrow

As we observed a significant reduction of ILC3s in the LP in the absence of HIC1, we next tested directly whether the lack of HIC1 affected the upstream development of ILC precursors in the bone marrow. ILCs develop in the bone marrow through a lineage pathway that begins with a common lymphoid progenitor (CLP) and progresses through an α4β7-expressing lymphoid progenitor (αLP), a common progenitor to all helper-like ILCs (ChILP) and, in the case of ILC2s, an ILC2 precursor (ILC2p) [230]. Analysis of surface marker expression on lineage-negative, $CD45^+$ bone marrow cells showed that HIC1 was not required for the development of CLP, αLP, ChILP or ILC2p populations (**Figure 4.3A and B**). Thus, the reduced number of ILC3s in the LP is not due to a reduced frequency of ILC precursors and suggests that HIC1 is required for ILC3 homeostasis in the periphery.

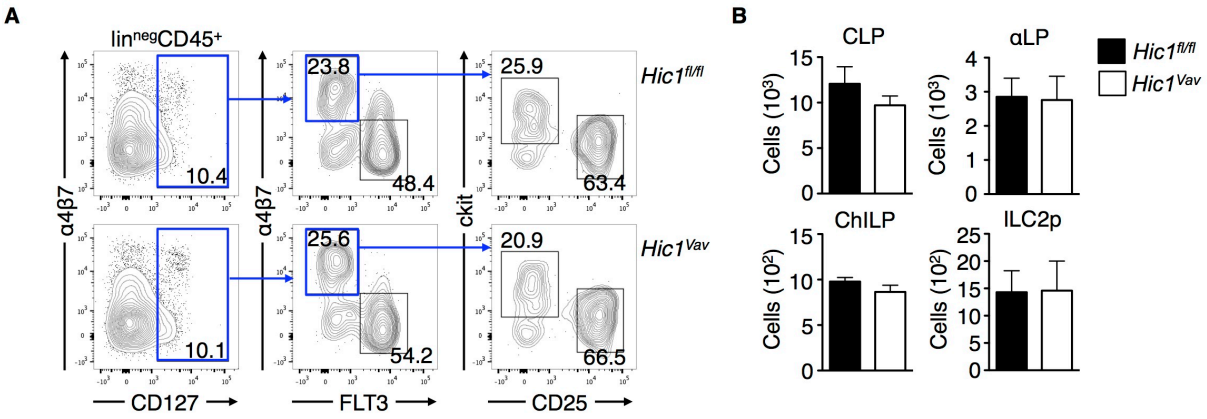

Figure 4.3 HIC1 does not regulate ILC precursors in the bone marrow.

(A) Gating strategy and (B) cell numbers of CLPs ($CD45^+$ lin^{neg} $CD127^+$ $Flt3^+$ $\alpha4\beta7^-$), $\alpha4\beta7^+$ lymphoid progenitors (αLP; $CD45^+$ lin^{neg} $CD127^+$ $Flt3^-$ $\alpha4\beta7^+$), ChILPs ($CD45^+$ lin^{neg} $CD127^+$ $Flt3^-$ $\alpha4\beta7^+$ $CD25^-$ $c\text{-}Kit^+$) and ILC2 progenitors (ILC2p; ($CD45^+$ lin^{neg} $CD127^+$ $Flt3^-$ $\alpha4\beta7^+$ $CD25^+$ $c\text{-}Kit^-$) from bone marrow of *Hic1*^{Vav} and *Hic1*$^{fl/fl}$ mice. Data are from two independent experiments (n = 4 per group). ns, not significant. *, P < 0.05; Student's t test. Errors bars indicate SEM.

4.2.4 Hematopoietic specific deletion of *Hic1* results in susceptibility to intestinal bacterial infection.

ILC3s have been shown to play a significant role in resistance to infection with the attaching and effacing bacterial pathogen *Citrobacter rodentium* [38,70]. Following infection with *C. rodentium*, $Hic1^{Vav}$ mice exhibited enhanced weight loss and significantly higher bacterial burdens in the feces compared to $Hic1^{fl/fl}$ controls (**Figure 4.4A and B**). Furthermore, infected $Hic1^{Vav}$ mice but not $Hic1^{fl/fl}$ mice had dissemination of bacteria to the liver (**Figure 4.4C**), demonstrating a significant impairment in the intestinal barrier following infection. Associated with impaired bacterial containment and clearance were reduced levels of transcripts for the cytokines *Il17a* and *Il22*, as well as the intestinal antimicrobial peptide *Reg3g* (**Figure 4.4D**). Thus, HIC1 within hematopoietic cells is critical to mount a proper immune response against *C. rodentium.*

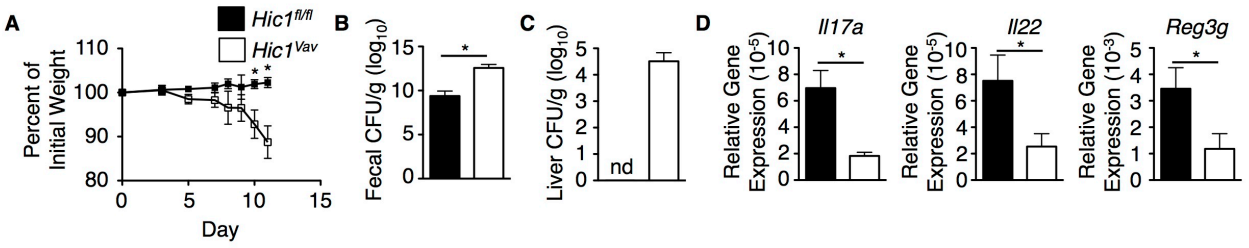

Figure 4.4 Hematopoietic deficiency of HIC1 results in susceptibility to *Citrobacter rodentium* infection. ***Hic1*^{Vav} and *Hic1*$^{fl/fl}$ mice were orally inoculated with *C. rodentium*. (A) Weight loss (percentage of initial weight) was calculated for each mouse over course of infection. (B, C) Bacterial loads (CFU/g) from fecal pellets (B) and liver (C) were measured at 11 days post inoculation. (D) Quantitative RT-PCR was performed to determine expression of *Il17a, Il22* and *Reg3g* from distal colon tissue 11 days post inoculation. Data are pooled from 2 independent experiments (n = 8-9 per group). *, P < 0.05; Student's *t* test. Errors bars indicate SEM. nd, none detected.**

4.2.5 ILC3-intrinsic *Hic1* expression is critical for defence against intestinal bacterial infection.

As T cells, $CD103^+$ $CD11b^+$ DCs and ILC3s are all important in initiating and propagating ILC3/T_H17 responses in the intestine [2,61,231,232] and these population are perturbed in $Hic1^{Vav}$ mice, we next sought to determine the effect of HIC1 deficiency in these specific cell populations during infection *C. rodentium*. We crossed $Hic1^{fl/fl}$ mice with mice expressing Cre under the control of either the *Cd4* promoter or *Itgax* promoter to generate T cell-specific ($Hic1^{CD4}$ mice) and dendritic cell-specific ($Hic1^{CD11c}$ mice) HIC1-deficient mice. Both $Hic1^{CD4}$ mice (**Figure 4.5A – C**) and $Hic1^{CD11c}$ mice (**Figure 4.5D – F**) were as resistant to infection with *C. rodentium* as control $Hic1^{fl/fl}$ mice, with equivalent weight loss, fecal bacterial burdens and expression of cytokines and antimicrobial peptide mRNA in the intestine. Thus, these results demonstrate that expression of HIC1 in T cells or CD11c-expressing cells is not required for immunity to bacterial infection and suggests loss of HIC1 in another cell population is responsible for the phenotype observed in $Hic1^{Vav}$ mice.

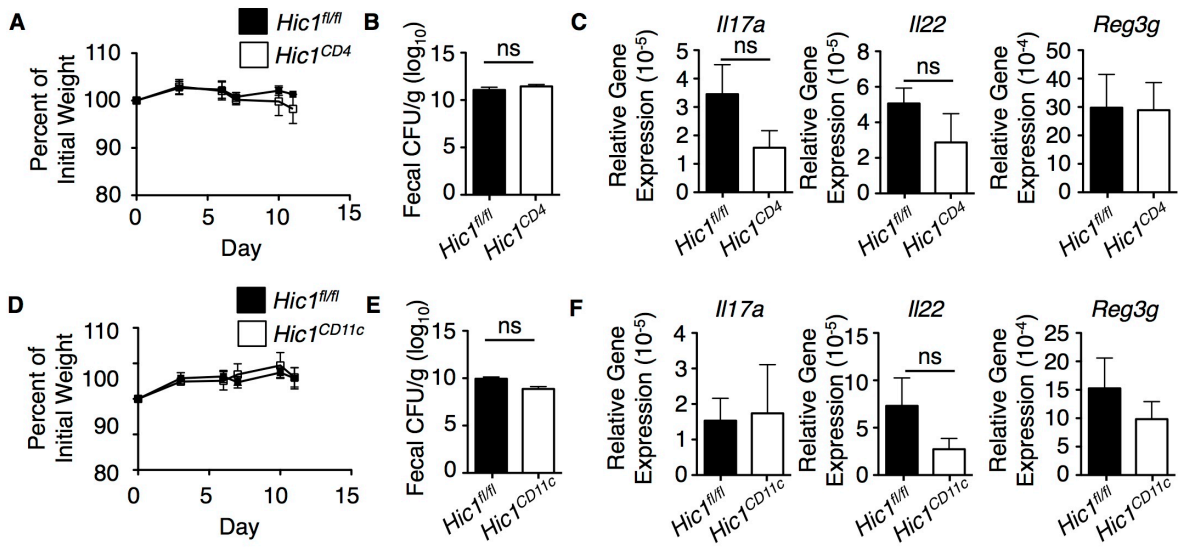

Figure 4.5 *Hic1* expression in T cells and dendritic cells in not required for immunity to *Citrobacter rodentium* infection.

(A–C) *Hic1*^{CD4} and *Hic1*$^{fl/fl}$ mice or (D–F) *Hic1*^{CD11c} and *Hic1*$^{fl/fl}$ mice were orally inoculated with *C. rodentium*. (A, D) Weight loss (percentage of initial weight) was calculated for each mouse over course of infection. (B, E) Bacterial loads (CFU/g) from fecal pellets were measured at 11 days post inoculation. (C, F) Quantitative RT-PCR was performed to determine expression of *Il17a, Il22* and *Reg3g* from distal colon tissue 11 days post inoculation. Data are pooled from 2 independent experiments (n = 6 per group, (A–C)) or (n = 5-6 per group, (D–F)). *, P < 0.05; Student's *t* test. Errors bars indicate SEM. ns, not significant.

To determine the role of HIC1 in ILC3s during infection with *C. rodentium*, we crossed *Hic1*$^{fl/fl}$ mice with mice expressing Cre recombinase under the control of the *Rorc* promoter (*Hic1*^{Rorc} mice). Following infection with *C. rodentium*, and similar to what we observed in the *Hic1*^{Vav} mice, *Hic1*^{Rorc} mice displayed increased weight loss, higher fecal bacterial burdens and increased bacterial dissemination than control *Hic1*$^{fl/fl}$ mice **(Figure 4.6A – C**). Associated with increased susceptibility was reduced expression of *Il17a*, *Il22* and *Reg3g* in intestinal tissues (**Figure 4.6D**). Based on our results, we hypothesized that HIC1 is an important regulator of ILC3 function during *C. rodentium* infection. To better examine the effect of HIC1 deletion on ILC3s, we examined the intestinal LP of *Hic1*$^{fl/fl}$ mice and *Hic1*^{Rorc} mice at day 4 post *C. rodentium* infection. Enhanced susceptibility to infection with *C. rodentium* that we observed in *Hic1*^{Rorc} mice correlated with the loss of ILC3s and reduced numbers IL-22-producing ILC3s (**Figure 4.6E and F**). Taken together, these results suggest that expression of HIC1 in ROR-γt^{+} ILC3s is critical for resistance to intestinal bacterial infection.

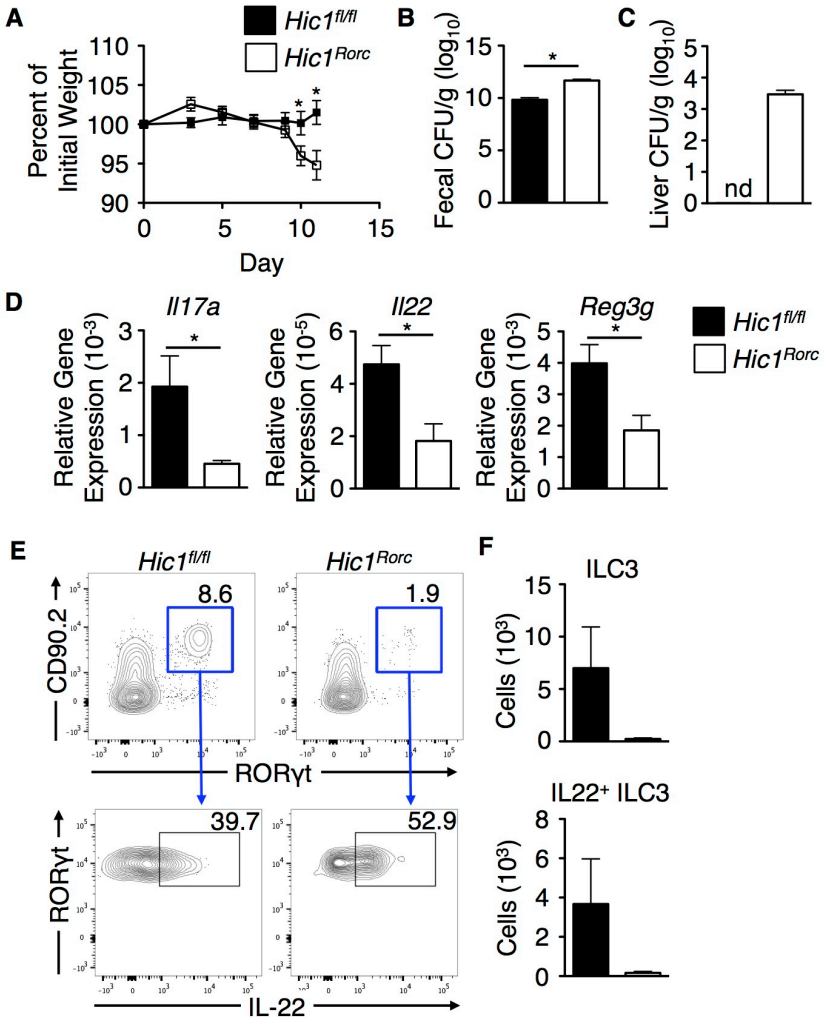

Figure 4.6 ILC3-intrinsic HIC1 is required for immunity to *Citrobacter rodentium* infection.

***Hic1*^{Rorc} and *Hic1*$^{fl/fl}$ mice were orally inoculated with *C. rodentium*. (A) Weight loss (percentage of initial weight) was calculated for each mouse over course of infection. (B, C) Bacterial loads (CFU/g) from fecal pellets (B) and liver (C) were measured at 11 days post inoculation. (D) Quantitative RT-PCR was performed to determine expression of *Il17a, Il22* and *Reg3g* from distal colon tissue 11 days post inoculation. (E, F) Total ILC3s and IL-22 producing ILC3s from intestinal lamina propria were analyzed by flow cytometry 4 days post inoculation. Data are pooled from two independent experiments (n = 7-8 per group). *, P < 0.05; Student's *t* test. Errors bars indicate SEM. nd, none detected.**

4.2.6 Hematopoietic deficiency of HIC1 results in susceptibility to intestinal helminth infection.

Our results show that HIC1 is an atRA-responsive factor that is critical for regulation of ILC3 homeostasis and function. As a recent study demonstrated that blockade of atRA signalling results in dysfunctional ILC3 responses along with a compensatory increase in ILC2 responses and enhanced immunity to infection with the intestinal helminth parasite *Trichuris muris* [163], we hypothesized that in addition to the defective ILC3 response observed in both *Hic1*^{Vav} mice and *Hic1*^{Rorc} mice, we would find enhanced ILC2 responses in the absence of HIC1. To test this, we infected *Hic1*$^{fl/fl}$ and *Hic1*^{Vav} mice with *T. muris*. In contrast to our expectations, we did not observe a heightened protective ILC2/T_H2 cell response, but found that *Hic1*^{Vav} mice were susceptible to infection. *Hic1*^{Vav} mice maintained a significant worm burden and enlarged mesenteric lymph nodes 21 days after infection (**Figure 4.7A and B**). Histological analysis revealed that *T. muris*-infected *Hic1*^{Vav} mice displayed a reduced frequency of goblet cells, with parasites embedded within the caecal epithelium (**Figure 4.7C and D**). Associated with the increased susceptibility, *Hic1*^{Vav} mice mounted a non-protective type 1 response following infection, as restimulation of the draining mesenteric lymph nodes (mLN) revealed a significant increase in secreted IFN-γ (**Figure 4.7E**) and expression of the *Ifng* in the intestinal tissues (**Figure 4.7F**), concomitant with a reduced type 2 response. We also observed a switch in the *T. muris*-specific antibody response from IL-4-dependent IgG1 to IFN-γ-dependent IgG2a in the serum of infected *Hic1*^{Vav} mice (**Figure 4.7G**). Thus, loss of HIC1 in hematopoietic cells results in increased susceptibility to infection with *T. muris*.

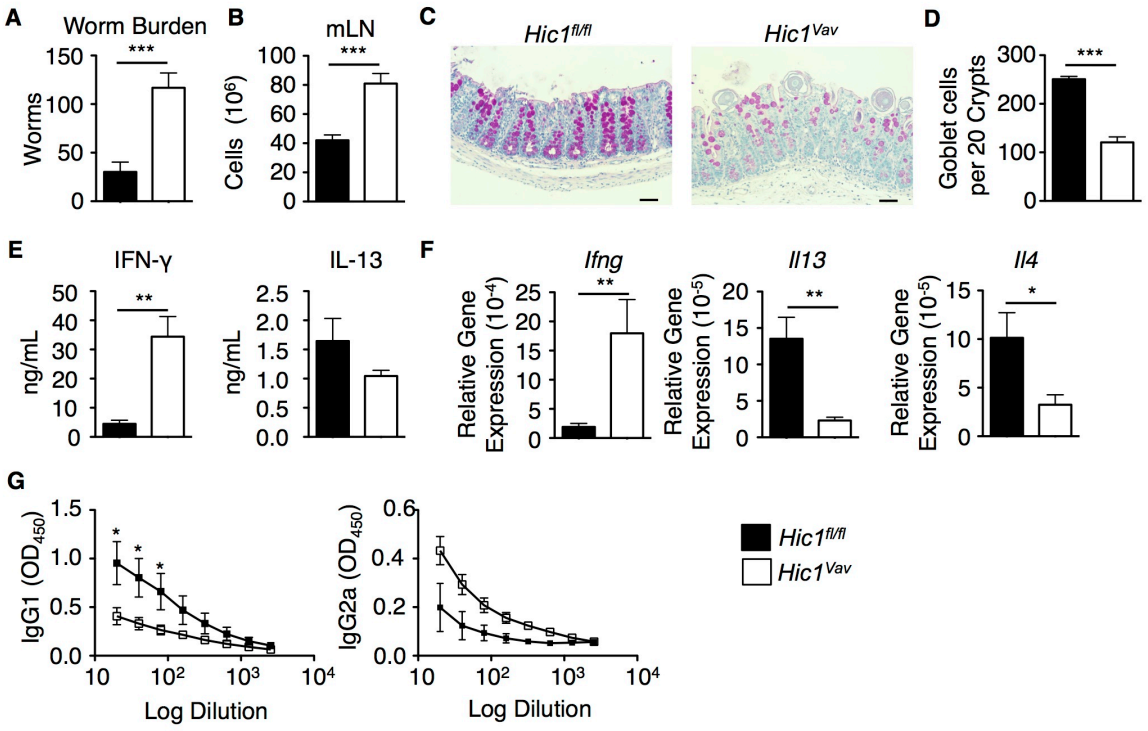

Figure 4.7 Hematopoietic HIC1 deficiency results in susceptibility to *Trichuris muris* infection.

***Hic1*^{Vav} and *Hic1*$^{fl/fl}$ mice were infected with 200 *T. muris* eggs. (A) Worm burdens were determined microscopically from cecal contents 21 days post infection. (B) Total cells of mesenteric lymph nodes (mLN) were counted. (C) PAS-stained cecal sections, scale bar = 50 µm. (D) Goblet cells were quantified from PAS-stained cecal sections. (E) Supernatants of αCD3/CD28 restimulated mLN cells were evaluated for secretion of IFN-γ and IL-13 by ELISA. (F) Quantitative RT-PCR was performed to determine expression of *Il4, Il13*, and *Ifng* from proximal colon tissue. (G) Serum *Trichuris*-specific IgG1 and IgG2a levels were quantified by ELISA. (A–F) Data are pooled from three independent experiments (n=10-12 per group). (G) Data are representative of three independent experiments (n=4 per group). *, P < 0.05; **, P < 0.01; ***, P < 0.001; Student's *t* test. Errors bars indicate SEM.**

4.2.7 HIC1 in T cells and dendritic cells is dispensable for immunity to *Trichuris muris*.

To determine which cell type required HIC1 expression to promote immunity to *T. muris*, we infected both $Hic1^{CD4}$ mice and $Hic1^{CD11c}$ mice. Strikingly, loss of HIC1 in T cells or CD11c-expressing cells had no effect on the development of protective immunity against *T. muris*. We observed equivalent expulsion of worms and expression of *Ifng* and *Il13* in the intestinal tissues between control $Hic1^{fl/fl}$ mice, $Hic1^{CD4}$ mice (**Figure 4.8A and B**) and $Hic1^{CD11c}$ mice (**Figure 4.8C and D**). Thus, we conclude that expression of HIC1 in T cells or CD11c-expressing cells is not required for immunity to *T. muris*.

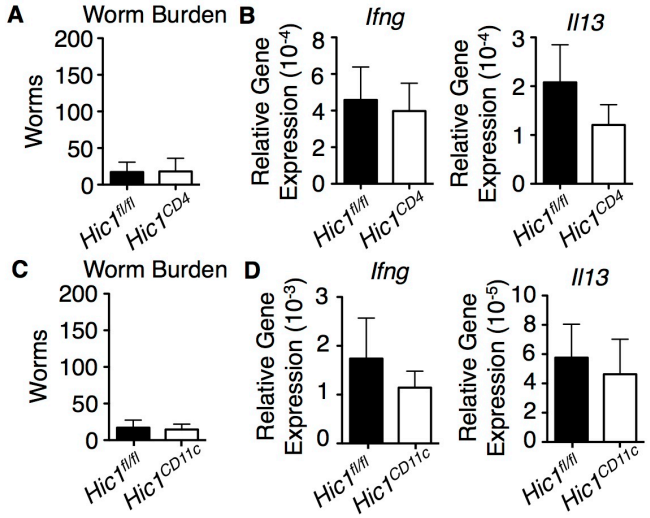

Figure 4.8 HIC1 in T cells and dendritic cells is dispensable for immunity to *Trichuris muris.*

(A, B) *Hic1*^{CD4} and *Hic1*$^{fl/fl}$ mice or (C, D) *Hic1*^{CD11c} and *Hic1*$^{fl/fl}$ mice were infected with 200 *T. muris* eggs. (A, C) Worm burdens were determined microscopically from cecal contents 21 days post infection. (B, D) Quantitative RT-PCR was performed to determine expression of *Il4, Il13*, and *Ifng* from proximal colon tissue. Data are pooled from 2 independent experiments (n = 8 per group). *, P < 0.05; Student's *t* test. Errors bars indicate SEM. ns, not significant.

4.2.8 ILC3-intrinsic expression of *Hic1* is required for immunity to *T. muris*.

As there was no observable role for HIC1 in T cell or DC function during *T. muris* infection, we next sought to determine if there was a defect in HIC1 deficient ILC3s that prevent clearance of the parasite. Surprisingly, $Hic1^{Rorc}$ mice infected with *T. muris* were susceptible to infection, maintaining a significant parasite burden at day 21 post-infection (**Figure 4.9A**). Similar to $Hic1^{Vav}$ mice, we observed enlarged mesenteric lymph nodes (**Figure 4.9B**), as well as increased inflammatory cell infiltration, reduced number of goblet cells, submucosal edema, and parasites embedded within the caecal epithelium (**Figure 4.9C and D**). Consistent with the lack of protective immunity, we found that $Hic1^{Rorc}$ mice displayed high levels of secreted IFN-γ from restimulated mLN cells as well as increased expression of *Ifng* in intestinal tissues (**Figure 4.9E and F**), which is associated with increased levels of IFN-γ-dependent IgG2a antibodies in the serum (**Figure 4.9G**). However, we failed to observe reduced expression of type 2 cytokines in the mLN or intestine (**Figure 4.9E and F**), suggesting that the heightened levels of IFN-γ was promoting susceptibility in the context of a protective type 2 immune response. Thus, ILC3-intrinsic expression of HIC1 is critically required for the development of protective type 2 immune responses against *T. muris*.

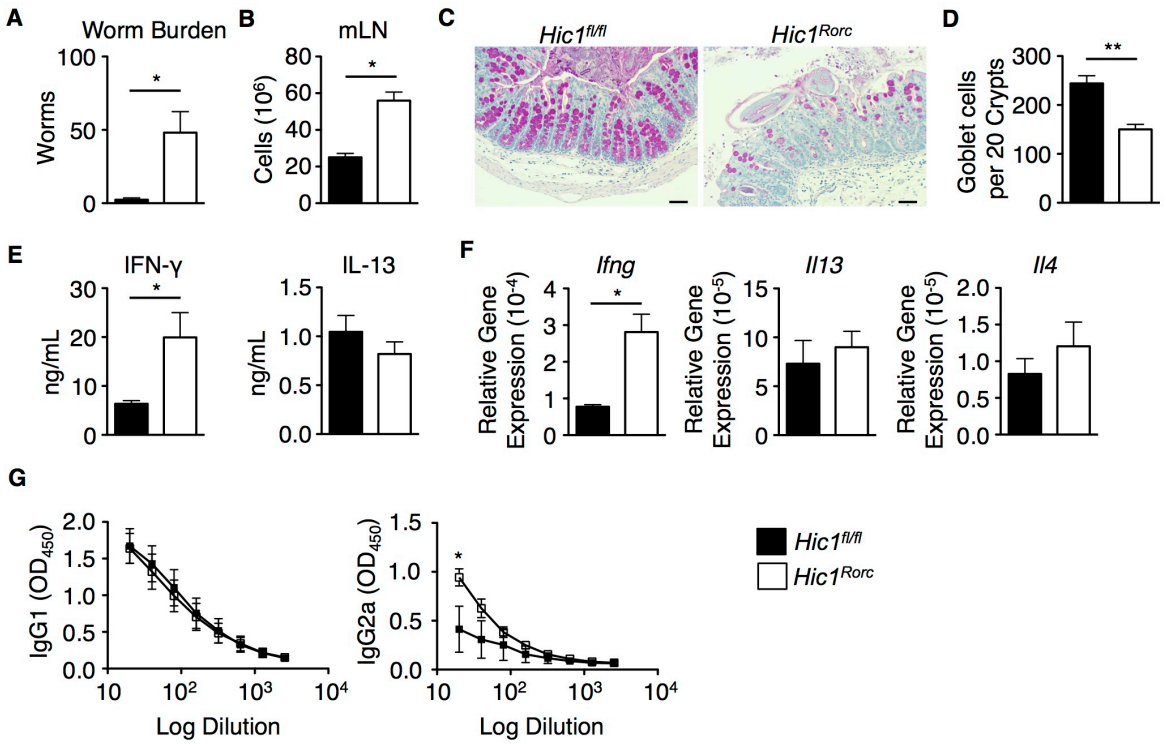

Figure 4.9 ILC3 specific deletion of *Hic1* renders mice susceptible to *Trichuris muris* infection.

***Hic1*^{Rorc} and *Hic1*$^{fl/fl}$ mice were infected with 200 *T. muris* eggs. (A) Worm burdens were determined microscopically from cecal contents 21 days post infection. (B) Total cells of mesenteric lymph nodes (mLN) were counted. (C) PAS-stained cecal sections, scale bar = 50 µm. (D) Goblet cells were quantified from PAS-stained cecal sections. (E) Supernatants of αCD3/CD28 restimulated mLN cells were evaluated for secretion of IFN-γ and IL-13 by ELISA. (F) Quantitative RT-PCR was performed to determine expression of *Il4, Il13*, and *Ifng* from proximal colon tissue. (G) Serum *Trichuris*-specific IgG1 and IgG2a levels were quantified by ELISA. (A–F) Data are pooled from two independent experiments (n=8 per group). (G) Data are representative of two independent experiments (n=4 per group). *, P < 0.05; **, P < 0.01; ***, P < 0.001; Student's *t* test. Errors bars indicate SEM.**

4.2.9 Neutralization of IFN-γ in *T. muris*-infected *Hic1*^{Rorc} mice promotes resistance

Based on our results showing heightened levels of IFN-γ, we next asked whether antibody blockade of IFN-γ would render *Hic1*^{Rorc} mice resistant to infection. We found that treatment of *T. muris*-infected *Hic1*^{Rorc} mice with α-IFN-γ antibody promoted resistance, with a complete clearance of parasites in antibody-treated mice by day 21 post infection (**Figure 4.10A**). Increased resistance was associated with increased numbers of goblet cells (**Figure 4.10B and C**) along with reduced levels of IFN-γ and heightened levels of IL-4 and IL-13 (**Figure 4.10D and E**). Antibody treatment also resulted in reduced levels of *T. muris*-specific IgG2a and increased levels of IgG1 in the serum (**Figure 4.10F**). Thus, these results suggest that ILC3-specific expression of *Hic1* is not required for resistance to *T. muris* infection when IFN-γ responses are neutralized.

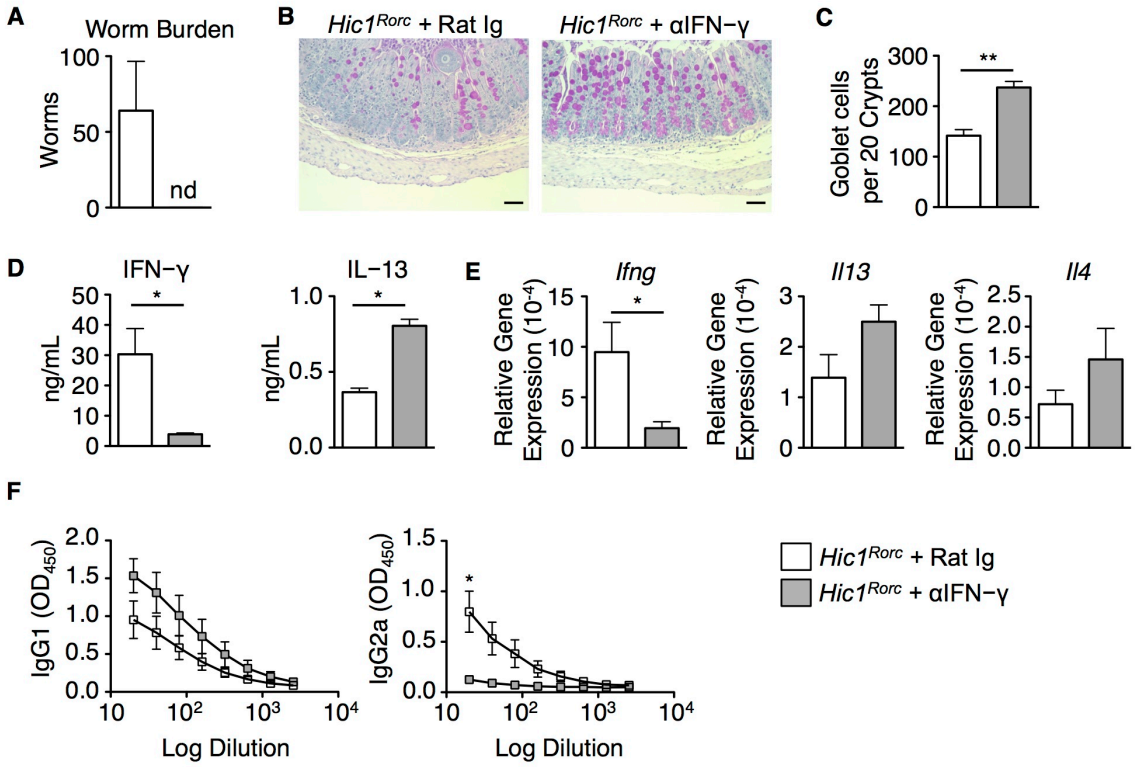

Figure 4.10 Neutralization of IFN-γ in *Trichuris muris* –infected *Hic1*^{Rorc} mice facilitates immunity to infection.

***Hic1*^{Rorc} mice were infected with 200 *T. muris* eggs and treated i.p. with antibodies against IFN-γ or an isotype control (Rat Ig). (A) Worm burdens were determined microscopically from cecal contents 21 days post infection. (B) PAS-stained cecal sections, scale bar = 50 μm. (C) Goblet cells were quantified from PAS-stained cecal sections. (D) Supernatants of αCD3/CD28 restimulated mLN cells were evaluated for secretion of IFN-γ and IL-13 by ELISA. (E) Quantitative RT-PCR was performed to determine expression of *Il4, Il13*, and *Ifng* from proximal colon tissue. (F) Serum Trichuris-specific IgG1 and IgG2a levels were quantified by ELISA. Data are from one experiment (n=4 per group). *, P < 0.05; **, P < 0.01; Student's *t* test. Errors bars indicate SEM. nd, none detected.**

4.2.10 ILC3-intrinsic HIC1 is required to limit commensal bacteria specific T_H cell responses.

ILC3s play a central role in intestinal immune homeostasis by limiting T cell responses against commensal bacteria [233,234]. A subset of intestinal ILC3s can present commensal bacterial antigen to $CD4^+$ T cells through MHCII but lack any co-stimulatory molecules and thus induce anergy in commensal-specific T cells [234]. As we observed reduced numbers of ILC3s as well as enlarged mLN and heightened levels of IFN-γ production from restimulated T cells from the mLN of naïve and *T. muris*-infected $Hic1^{Rorc}$ mice, we hypothesized that the increased IFN-γ production was due to dysregulated T cell responses to bacteria. Consistent with this, we observe a significant reduction in the number of regulatory $MHCII^+$ ILC3s in the intestinal LP of $Hic1^{Rorc}$ mice at steady state (**Figure 4.11A and B**). Interestingly, analysis of $CD4^+$ T cells from the mLN of $Hic1^{Rorc}$ mice at steady state reveals that in comparison to control mice, $Hic1^{Rorc}$ mice exhibited significantly increased frequencies of proliferating $Ki67^+$ $CD4^+$ T cells (**Figure 4.11D**), effector/effector memory $CD44^{high}$ $CD62L^{low}$ $CD4^+$ T cells (**Figure 4.11E**) as well as IFN-γ^+ $CD4^+$ T cells (**Figure 4.11F**), indicative of disrupted immune cell homeostasis. Consistent with these responses being driven by commensal bacteria, oral administration of a cocktail of antibiotics to $Hic1^{Rorc}$ mice was associated with significantly reduced peripheral IFN-γ^+ $CD4^+$ T cells and $CD44^{high}$ $CD62L^{low}$ $CD4^+$ T cells and mLN size (**Figure 4.11C – F**). Taken together, these results suggest that ILC3-intrinsic HIC1 is required to limit commensal specific T_H cell responses in the steady state.

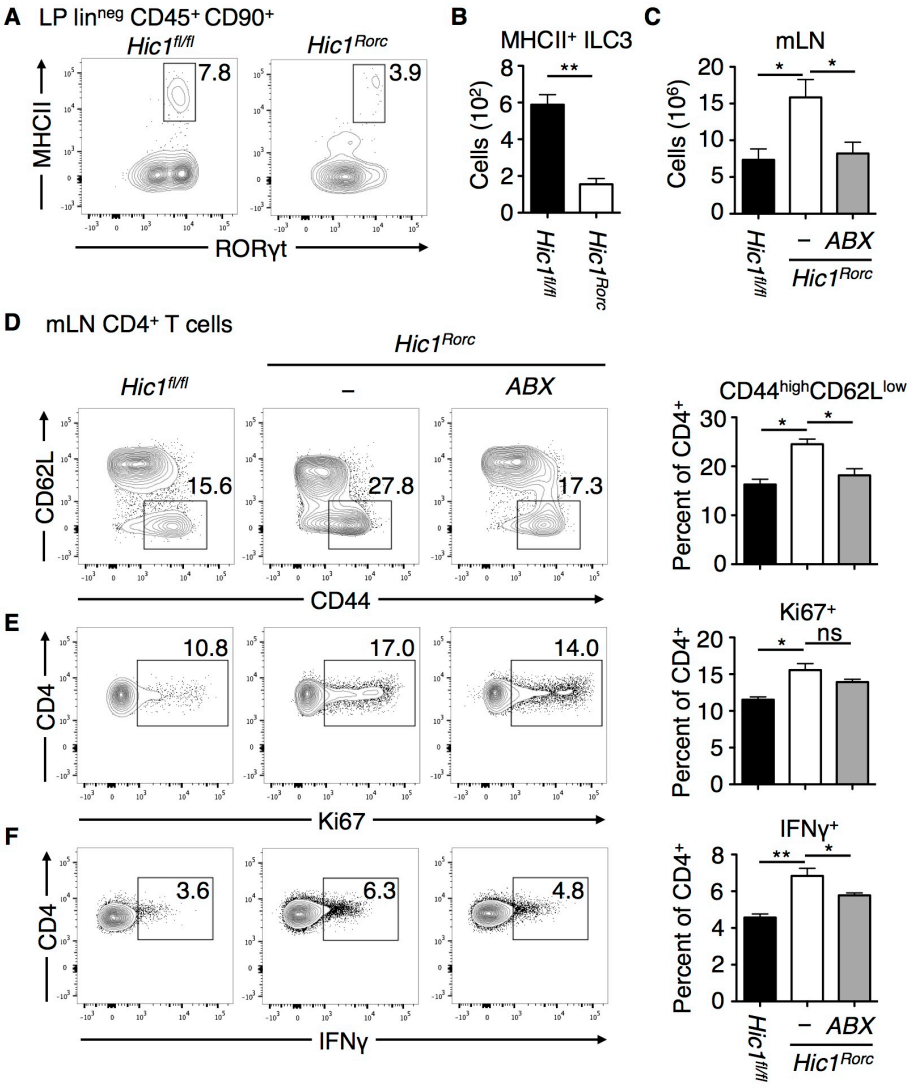

Figure 4.11 ILC3-intrinsic HIC1 is required to limit CD4$^+$ T$_H$ cell responses to commensal bacteria.

(A, B) MHCII$^+$ ROR-γt$^+$ ILC3s from intestinal lamina propria of *Hic1^{Rorc}* and *Hic1$^{fl/fl}$* mice were analyzed by flow cytometry (C–F) Mesenteric lymph nodes (mLN) from *Hic1$^{fl/fl}$ and Hic1^{Rorc}* mice treated with or without antibiotics (Abx) in their drinking water were analyzed for total cell numbers (C), and analyzed by flow cytometry for frequency of Ki67$^+$ CD4$^+$ T cells (D), frequency of CD44^{high} CD62L^{low} CD4$^+$ T cells (E), and frequency of IFN-γ-producing CD4$^+$ T cells (F). (A, B) Data are pooled from two independent experiments (n=4-5 per group). (C–F) Data are pooled from two independent experiments (n=5-7 per group). *, P < 0.05; **, P < 0.01; Student's *t* test. Errors bars indicate SEM. ns, not significant.

4.3 Discussion

Our results demonstrate that in the steady state, *Hic1* is expressed by intestinal ILCs in a Vitamin A-dependent manner. In the absence of HIC1, we observed a dramatic decrease in intestinal ILC3 numbers, which was associated with a failure to clear *C. rodentium* infection. In addition, the reduction of regulatory MHCII$^+$ ILC3s resulted in an increased frequency IFN-γ-producing T cells locally and systemically. The heightened levels of IFN-γ in the absence of HIC1 inhibited the ability of *Hic1*^{Vav} mice and *Hic1*^{Rorc} mice to mount a protective T_H2 cell-associated immune response against *T. muris* infection. Together, these results highlight an important role for HIC1 not only in regulating intestinal immune homeostasis but also in mounting proper immune responses to diverse intestinal infections.

In the absence of HIC1, we found a significant reduction in the number of ILC3s with no effect on ILC2s in the intestine. This is consistent with studies examining the role of atRA on intestinal ILC3 development and function. atRA-dependant expression of CCR9 is critical for intestinal migration of ILC3s, while intestinal ILC2 migration is independent of atRA [156]. Similarly, it has been reported that development of intestinal group 3 ILCs and postnatally formed lymphoid tissue within the intestine are dependent on atRA[235]. Specifically, our results show a reduced numbers of ROR-γt$^+$ TBET$^+$ ILC3s and an increase in the number of CD4$^+$ LTi cells. This is in agreement with studies that have demonstrated that these two lineages have distinct developmental pathways; LTi cells would develop in the fetus while TBET$^+$ ILC3s develop postnatally and rely on environmental signals including atRA [37,163,236]. Interestingly, it has been shown that atRA signalling is also important for generation of LTi cells in the fetus [153]. However, our results suggest that HIC1 is not involved in fetal LTi formation, as we find no differences in LTi numbers or lymphoid structures in the absence of HIC1. Further, the

development of ILC progenitor cells in the bone marrow is not perturbed by loss of HIC1, suggesting that the primary role of atRA dependent expression of *Hic1* is to regulate the development and function of adult cells in the periphery.

Resistance to intestinal infection with *C. rodentium* is mediated by IL-22, and ILC3s are the predominant IL-22-producing cell population during the first week of infection [69,70] with atRA signaling promoting IL-22 production by intestinal ILC3 [237]. There are contradictory studies on which ILC3 populations are key for resistance to *C. rodentium* with both $CD4^+$ LTi cells and natural cytotoxicity receptor $(NCR)^+$ ILC3s each being described as either individually critical or redundant [70,121,238]. Another study looking at $TBET^+$ ILC3s (which include NCR^+ ILC3s) demonstrated that TBET expression in a subset of ILC3s is critical for resistance to *C. rodentium* infection [239]. Our results are consistent with a role for NCR^+ or $TBET^+$ ILC3s in immunity to *C. rodentium* as $Hic1^{CD4}$ mice (deficient for HIC1 in T cells and LTi cells) are resistant to infection while $Hic1^{Rorc}$ mice (deficient for HIC1 in T cells and all ILC3s) are susceptible. Thus, *Hic1* expression in ILC3s is critical for immunity to *C. rodentium*.

In addition to a reduction in ILC3s, a recent study identified that mice raised on a VAD diet displayed increased ILC2 numbers and heightened type 2 immunity to helminth infection in the intestine [163]. However, in the absence of HIC1, we did not observe an increase in ILC2 numbers nor increased resistance to infection with *T. muris*. Instead, we detected increased production of IFN-γ and an inability to mount a protective T_H2 cell response to *T. muris* infection. Further, treatment of *T. muris*-infected $Hic1^{Rorc}$ mice with a neutralizing antibody against IFN-γ rendered the mice resistant to infection, demonstrating that HIC1-dependent responses are dispensable in the absence of IFN-γ and that the effects of atRA on ILC2s are likely independent of HIC1.

Similarly to *T. muris* infection, there was also an increased frequency of IFN-γ-producing T cells present in the mLN of *Hic1*^{Rorc} mice at steady state. Activation of these T cells relied on commensal bacteria, as administration of antibiotics that ablated the microbiota reversed the phenotype. Commensal bacteria are essential for promoting normal intestinal physiology but can trigger inflammation if not properly contained [240,241]. IL-22 has been described as having a role in preventing intestinal inflammation by influencing commensal bacterial populations [242]. In addition, regulatory MHCII$^+$ ILC3s have been shown to prevent inflammation through induction of anergy in commensal specific T_H cells [233,234]. As both IL-22 producing and MHCII$^+$ ILC3s are diminished in the absence of ILC3-intrinsic HIC1, lose of either population could contribute to the commensal bacteria dependent increase in IFN-γ producing T cells. However, previous studies have shown that IL-22-deficient mice did not develop spontaneous colitis [243]. In addition, transient blockade of IL-22, IL-17A, IL-23 or IL-17RA failed to exacerbate adaptive immune cell responses to commensal bacteria [234], suggesting that ILCs influence adaptive immune cell responses to commensals independent of cytokine production. In fact, only when ILCs are depleted with an anti-CD90 antibody or MHCII is specifically deleted in ILCs do commensal bacteria promote systemic inflammation [233,234,241]. Therefore, the anti-commensal driven increase in IFN-γ at steady state that leads to susceptibility to *T. muris* in mice with *Hic1*-deficient ILC3s is likely not due to a reduced number of IL-22 producing ILCs but rather due to the lose of regulatory MHCII$^+$ ILC3s.

Taken together, these results establish a role for the transcriptional repressor HIC1 as an atRA-responsive cell-intrinsic regulator of ILC3 cell function in the intestine, and identify a potential regulatory pathway that could be targeted to modulate ILC3 responses in the intestine.

Chapter 5: General discussion and conclusion

5.1 Summary

Summarized below are the conclusions reached from this dissertation and the overall research significance to the central hypothesis: ***HIC1 will play an essential role in atRA mediated mucosal immune responses during intestinal infections and inflammatory disease.***

Chapter 3: The transcriptional repressor HIC1 regulates intestinal T cell immune homeostasis.

1. *Hic1* is expressed by intestinal T cells in an atRA dependent manner.
2. HIC1 promotes intestinal T cell homeostasis.
3. HIC1 limits IL-17A production by T_H17 cells through interaction with STAT3.
4. T cell-intrinsic expression of *Hic1* is required for the development of multiple models of intestinal inflammation.

Studies in Chapter 3 investigated the expression pattern and role for HIC1 in intestinal T cells during homeostasis and disease. Broadly, our studies identified a novel role for HIC1 in regulating intestinal T cell populations during steady state and in promoting inflammation in the intestine. This work further contributes to our understanding of the effects of atRA on the intestinal immune system through regulation of expression of *Hic1* in intestinal immune cells. At steady state, in the absence of HIC1, we observe a significant reduction in T cell numbers; however, we discovered that there was no defect in atRA driven expression of gut trafficking molecules CCR9 and α4β7. Conversely, we observe a specific decrease in the number of resident

$CD69^+CD103^+$ T cells within the intestine. This is the first indication that atRA, through regulation of HIC1, could play a larger role than T cell trafficking to the gut and could be critical in the retention or development of intestinal T_{RM} cells. The association of Vitamin A deficiency with poor response to vaccines is well known [138,139], however, there are only a handful of studies that directly link atRA to memory T cell formation [244,245]. Our work suggests that HIC1 could be a molecular link between atRA and memory T cell development in the intestine. Further, we show that HIC1 provides a molecular mechanism for atRA inhibition of T_H cell production of IL-17A through interaction with STAT3 *in vitro* and *in vivo*. Interestingly, others have shown that atRA inhibits up regulation of *Il6r* and *Il23r* [161] – both of which are key receptors for T_H17 cell differentiation and are STAT3 target genes [213] – without altering the levels of pSTAT3 [161]. A mechanism similar to what we propose for atRA driven HIC1 suppression of IL-17A, indicating HIC1 could be responsible for atRA regulation of other STAT3 target genes. Although our *in vitro* data suggests HIC1 is not completely required for the atRA-mediated inhibition of T_H17 differentiation, indicating that there is still a yet identified mechanism involved. In regards to intestinal inflammation, our results provide further evidence to the literature that IL-17A producing T_H17 cells offer protection from disease rather than drive pathology. Previous clinical trials that have focused on the blocking IL-17A have failed; [222,223] a better treatment strategy may be to target factors that prevent T_H cell from becoming pathogenic. A recent study using an inhibitor against ROR-γt has proven effective in preventing mouse models of inflammation [246]. However, this treatment depletes T_H17 cells from the intestine and potentially leaves the animals susceptible to opportunistic infections, therefore, an appealing alternative would be to develop an inhibitor against HIC1 as it could potentially leave the T_H17 cells intact and fully capable of

mounting an anti-bacterial response (as seen in Chapter 4) but prevent the cells from becoming pathogenic during inflammatory bowel disease.

Chapter 4: HIC1 links retinoic acid signaling to group 3 innate lymphoid cell-dependent regulation of intestinal immunity and homeostasis.

1. *Hic1* is expressed by intestinal ILCs in an atRA dependent manner.
2. Hematopoietic cell-intrinsic HIC1 is required for T cell, DC and ILC homeostasis in the intestine.
3. ILC3-intrinsic HIC1 is required for resistance to *Citrobacter rodentium* infection.
4. ILC3-intrinsic HIC1 is required to limit commensal bacteria specific T_H cell responses.
5. Increased levels of commensal driven IFN-γ interfere with resistance to *Trichuris muris* infection in mice with a specific deletion of *Hic1* in ILC3s.

Studies in Chapter 4 investigated the role of HIC1 in DC, T cell, and ILC populations of the intestine during multiple infection models. With respect to the growing field of studies looking at ILCs, we identified a new transcription factor critical in maintaining proper intestinal ILC3 homeostasis as well as proper ILC3 function during challenges by the intestinal bacterial infection *Citrobacter rodentium* and the intestinal helminth parasite *Trichuris muris*. Our data is in agreement with previous reports outlining the importance of ILC3s in the production of IL-22 in order to combat *C. rodentium* infection. However, our study further identifies the importance of a $TBET^+$ ILC3 population within the intestine in defense against bacterial infections, as this population is the key population diminished upon ILC3-intrinsic deletion of *Hic1*. Interestingly,

we identified a previously unknown role for ILC3 in combating intestinal helminth infections. Although ILC3 do not contribute directly to the immune response to clear the parasites, they are critically required to limit commensal bacteria specific immune responses that would otherwise interfere with the ability to mount a proper T_H2 response to *T. muris.* Interestingly, a similar phenotype was observed in aryl hydrocarbon receptor (AHR)-deficient mice infected with *Toxoplasma Gondii* [247]. AHR-deficient mice also have a significant reduction in the number of ILC3 in the intestine and after infection with *T. Gondii,* mice displayed increased intestinal pathology associated with heightened T cell responses to commensal antigens. Together with our data, this demonstrates the critical role of ILC3s in the intestine in curtailing immune responses to commensal bacteria in order to not only mount the correct immune response but also the proper strength of response in order to limit immune pathology during infection. Although our findings do not determine a molecular role for HIC1 in maintaining ILC3 populations in the intestine, we do show that bone marrow ILC progenitors are unaffected in the absence of HIC1 suggesting HIC1 plays a role in the maintenance of peripheral adult ILC3s. Recent studies have described 'memory' like ILCs that persist after an immunological challenge in the lung and it is widely accepted that ILC3s in the intestine are long lived and relatively radioresistant due to their slow turnover [248,249]. As HIC1 is a well-known quiescent marker in cells, and these cells are highly responsive to environment cues such as atRA [37,163,236], it is probable that HIC1 is required to maintain a long lived quiescent pool of this adult ILC3 population in the intestine.

5.2 Study limitations

Many of the goals of this dissertation have been met, however, there are certain limitations and caveats that need to be addressed in these studies regarding mouse strains, experimental models and data interpretation.

To begin, the mouse models of intestinal inflammation used in Chapter 3 all focus on a T_H1 mechanism for driving disease, which roughly mimics the immune response seen in human Crohn's disease. However, the translatability of these models for ulcerative colitis is extremely limited. To further our understanding of HIC1 in all forms of IBD, a better option would have been to include the model of chronic Trinitrobenzene Sulfonic Acid (TNBS) colitis in the BALB/c strain of mice that produces a T_H2 based intestinal inflammation, which better mimics ulcerative colitis [250]. Similarly, if we could extend the models we did use onto other strains of mice it would strengthen our results and show that our results did not depend on a specific genetic background, however, we were restricted to using C57Bl/6 mice as our genetic tools – Cre/LoxP system – are limited to this strain of mice. Further, our interpretations of the data from our colitis models were clouded by multiple phenotypes within the intestine. Specifically, in regards to the anti-CD3ε antibody driven inflammation model, we could not separate the contributing effects of initiating disease with fewer T cells in the intestine from the altered cytokine production of the T cells. Further, multiple reports have described the importance of memory T cell formation in the development of T cell transfer colitis [251,252]. Therefore, if there was a defect in forming memory T cells in the absence of HIC1 as indicated by the lack of intestinal T_{RM} at steady state then both that phenotype as well as the altered cytokine production could contribute to the overall phenotype that we observed. The inclusion of T cell 'co-transfer' and DSS induced colitis experiments allowed us to determine that the altered cytokine profile of

the HIC1-deficient T cells does indeed contribute to the phenotype; however, we were never able to directly address whether or not the altered T cell numbers or effector/memory state of the T cells at homeostasis contributed to disease initiation or progression in our models.

Another major deficiency of this dissertation is the lack of molecular mechanism for HIC1 in ILCs from Chapter 4. As intestinal ILC3 are such a rare population, it proved difficult to isolate enough cells for downstream analysis; this problem was compounded when the phenotype of the mice with ILC3-specific deletion *Hic1* results in even fewer cells. FACS coupled with high throughput sequencing could help better characterize these cells, however, the population of ILCs that remain in the gene-deficient animals may simply be ILCs that have escaped genetic deletion of *Hic1*. Data analysis of Chapter 4 also provided challenges, as the *Rorc* promoter driven Cre recombinase used to generate ILC3 specific HIC1 deficient animals was not absolutely specific to ILC3s. ROR-γt is expressed early in T cell development in order to promote thymocyte survival [253] and therefore this Cre recombinase would render T cells as well as ILCs deficient for HIC1. To distinguish the effects of HIC1-deficient ILC3s from HIC1-deficient T cells, we had to use a strain of mice with a CD4 promoter driven Cre recombinase as a control group. However, recent reports have highlighted redundant functions for ILCs and T cells in the defense against *C. rodentium* infections [121]. Therefore, based on our results we cannot conclude that our *C. rodentium* infection phenotype is not due to an additive effect from a defect in both T cells and ILC3s rather than an effect in ILC3s alone.

The overall limitation of this dissertation is the ability to translate this data into clinical relevance in human disease. The mouse and human immune systems have developed under distinct evolutionary pressures and therefore display unique features. For example, human ILC3s express TLRs and can recognize certain pathogens directly through these receptors, while mouse

ILC3s rely on NCRs and signals from the milieu for activation [254,255]. Therefore, the different mechanisms utilized for innate cell activation between species complicate the translatability of our findings. Further, the inflammation models used in this dissertation rely on artificial methods to induce diseases, such as immune-deficient mice or systemic administration of a T cell activating antibody, which would not be seen in clinical presentations of human IBD. Therefore our studies should be validated in non-murine systems before being further considered for clinical relevance.

5.3 Future directions

This dissertation provides the first description of the role of HIC1 in regulating intestinal immune responses. However, many key questions remain that need to be addressed in future studies. Outlined below are a few interesting questions with potential experimental designs that could further our knowledge of the role of HIC1 in intestinal immunity.

Does HIC1 regulate T_{RM} cells or CD103 and CD69 expression in T cells from other tissues?

From Chapter 3 we found that *Hic1* is expressed in an atRA dependent manner in intestinal T cells and, in the absence of HIC1, the numbers of $CD103^+CD69^+$ T_{RM} cells are diminished in the intestine. T_{RM} cells have been characterized in multiple tissues such as the skin, lung and liver [256]. Although our broad examination of total $CD45^+$ hematopoietic cells did not indicate there was expression of *Hic1* in other tissues, reports have claimed that perfusion is not sufficient to remove circulating lymphocytes from some tissues and thus blood contamination could be masking a potential phenotype [257]. It is also interesting to note that the liver contains the

majority of stored Vitamin A with the body and a subset of skin and lung DCs can express the gene *Aldh1a2* which encodes the atRA synthesizing enzyme [258], therefore, it would be fascinating to see if atRA, and by extension HIC1, played a role in the maintenance of specific T_{RM} populations in sites distal to the intestine.

Does HIC1 play a role in immune cell tissue retention through expression of CD69 or CD103 or T cell memory formation through metabolism?

The loss of intestinal $CD103^+$ $CD69^+$ T_{RM} cells described in Chapter 3 could be attributed to two possible mechanisms. Either T cells are unable to up-regulate surface expression of CD69 or CD103, which facilitate tissue retention, or cells are unable to transition into a quiescent state that will allow them to survive long term. We have shown a direct link between atRA and HIC1 in these cells and studies have identified a correlation between human T cells treated with atRA and activation status, including CD69 expression [259] as well as CD103 expression in monocyte derived DCs treated with atRA [260] but an exact molecular mechanism has not been described. Conversely, HIC1 has been described as a cellular quiescent marker and a direct regulator of cell cycle genes as well as the metabolic regulator SIRT1 [190]. Further, the HIC1 interacting protein STAT3 has recently been identified as a critical regulator in mitochondrial function [261,262], with STAT3 playing a central role in metabolic reprogramming [263]. Recent reports have demonstrated the importance of regulating cellular metabolism in memory T cell development and survival [216,217]. Therefore, loss of HIC1 could disrupt mechanism involved in energy metabolism, such as SIRT1 levels or STAT3 function, which would influence the development of memory T cells. Analysis of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) with a

Seahorse metabolic analyzer of cultured HIC1-deficient T cells could demonstrate an altered metabolic state in these cells. Infection of mice with Lymphocytic choriomeningitis virus (LCMV) results in a strong T cell memory response including LCMV-specific T_{RM} cells that reside in the intestine, skin and liver. This infection model would allow tracking of antigen specific intestinal memory T cell formation throughout all stages of infection by using MHCI and MHCII tetramers (GP_{33-41} and $NP_{396-404}$) and intracellular cytokine staining with flow cytometry. These studies would aim to characterize a role for HIC1 in T cell quiescence and memory T cell development.

Does HIC1 offer a therapeutic or prophylactic target in treating intestinal inflammation?

The effect of deletion of *Hic1* in T cells is an increased production of IL-17A and an overall protective phenotype of T cells in regards to intestinal inflammation (Chapter 3). It would be interesting to determine if HIC1 could be used as a therapeutic as well as a prophylactic target for treatment of intestinal inflammation. Specifically, could inhibition of HIC1 turn pathogenic T cells into anti-inflammatory T cells? The generation of an inducible HIC1-deficient mouse strain by crossing *Hic1*$^{fl/fl}$ mice with mice that express a tamoxifen-inducible form of Cre driven by the *Cd4* promoter/enhancer (*Cd4*-Cre^{ERT2} mice) [264] could illuminate the timing in which HIC1 needs to be blocked in order to promote protection from disease. *Hic1* deletion could be induced in mice that have established intestinal inflammation (approximately 4-5 weeks post T cell transfer) and examine if pathogenic intestinal T cells are also lost or somehow altered in the absence of HIC1. These results would identify HIC1 as a potential therapeutic target to treat intestinal

inflammation and suggest that chemical inhibition of HIC1 may provide a novel drug for inflammation in the intestine.

Is HIC1 expressed in intestinal T cells from humans with IBD?

Our mouse model data suggests that HIC1 is an attractive target to modulate intestinal inflammation (Chapter 3). However, whether *HIC1* is expressed in intestinal T cells from humans is unknown. Collaboration with a gastroenterologist with access to human intestinal biopsies would allow us to analyze whether HIC1 is a valid target in human patients. $CD4^+$ and $CD8^+$ T cells could be isolated from intestinal biopsies from both inflamed and non-inflamed tissues of IBD patients. *HIC1* expression levels in T cells could then be compared between ulcerative colitis and Crohn's disease patients as well as healthy controls. These experiments could provide the rationale to follow up with HIC1 as a potential target in human IBD.

Does HIC1 regulate the differentiation of intestinal dendritic cell populations?

Although DCs are major producers of atRA in the intestine, recent studies have identified a cell-intrinsic role for atRA in activating a transcriptional profile in pre-DCs that allow for the differentiation of intestinal conventional DC subsets [164]. Interestingly, experiments in Chapter 3 demonstrate that intestinal DCs express *Hic1* and upon total hematopoietic specific deletion of *Hic1* in Chapter 4, intestinal DCs subsets are significantly altered. Therefore, experiments focusing on the function of HIC1-deficient DCs with a more specific *Hic1* deletion could be performed to test the role of HIC1 in intestinal DC homeostasis.

Does the absence of T cell or ILC3 –intrinsic expression of Hic1 alter the microbial populations within the intestine and how does that impact disease?

The intestinal microbiota and intestinal immune cells are co-dependent with both populations directly and in-directly influencing the other. The microbiota can directly activate ILCs through NCR as well as influence their development through metabolizing short chain fatty acids or ligands for receptors such as AHR [67,265]. Similarly, T cells and ILCs can produce IL-22, which directly influences the production of anti-microbial peptides that will alter the bacterial populations within the intestinal microbiota [242]. Further, dysbiosis of the intestinal microbiota has been linked to IBD [266] and reports indicating the loss of certain bacteria can increase the risk for developing IBD [267]. Therefore, deep-sequencing studies examining the bacterial populations within mice with T cell or ILC -intrinsic deficiencies for HIC1 at both steady state and during disease could help identify important bacterial populations that will influence the development or resolution of IBD. Further, the use of different antibiotics can be used to influence disease progression in both strains of genetically altered mice.

Does HIC1 play a role in the pathogenesis of other T_H17 driven diseases (i.e. multiple sclerosis and rheumatoid arthritis)?

Although IL-17A is becoming more associated with a protective role in IBD, other T_H17 associated diseases such as multiple sclerosis and rheumatoid arthritis are still driven by pathogenesis associated with the action of this cytokine [221,268]. Interestingly, recent reports have

highlighted a central link between inflammation in the central nervous system (CNS) and immune cells of the gut with evidence showing trafficking of antigen specific T cells between sites [19]. Therefore it would be interesting to determine if the increased IL-17A in our HIC1-deficient T cells exacerbates other models such as experimental autoimmune encephalomyelitis (EAE) or if these T cells adopt an overall anti-inflammatory phenotype that prevent development of disease in other non-intestinal inflammation models.

5.4 Concluding remarks

The findings presented in this dissertation provide the first evidence that the transcription factor HIC1 is an atRA responsive gene that is critical in the regulation of intestinal immune cells at homeostasis and during disease. Specifically, this analysis proposes that HIC1 is a major component of the molecular mechanism that drives pathogenic T cells during IBD and protective ILC responses during intestinal infections.

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