Systemic Fates of Non-Classically Restricted, Commensal Specific T Cells

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Abstract

Inflammatory Bowel Diseases (IBD), encompassing both Crohn's disease and ulcerative colitis, are characterized by destruction of the gut epithelial barrier, dysbiosis and aberrant immune reactivity to the microbiota. Although multiple studies have revealed $CD8^+$ T cell signatures in the tissues and circulating PBMC of patients with IBD, the role of $CD8^+$ T cells in the initiation or perpetuation of disease has remained understudied.

Work investigating the contribution of immunity to the commensal microbiota in the skin identified a population of innate like $CD8^+$ T cells restricted by the MHC-Ib molecule H2-M3, that play critical roles in wound healing and barrier repair. However, these commensal reactive $CD8^+$ T cells have not yet been described in the gut. Thus, the aim of this DPhil project was to describe commensal specific, innate-like $CD8^+$ T cells in this tissue. To achieve this, we leveraged established H2-M3 tetramer tools to track commensal specific $CD8^+$ T cell responses during homeostasis and a model of colitis wherein colonization with the pathobiont *Helicobacter hepaticus* and treatment with αIL-10R antibody mimics genetic susceptibility and aberrant reactivity to the microbiota found in some patients with IBD.

Using a range of histological and flowcytometry measurements, we describe the architecture of the colon with and without H2-M3 restricted and MHC-Ia restricted $CD8^+$ T cells in homeostasis and colitis, illustrating that MHC-Ib restricted $CD8^+$ T cells can dominantly contribute to the $CD8^+$ T cell response during inflammation. Further, we confirm the residence of H2-M3 restricted commensal reactive $CD8^+$ T cells in the large intestine where they adopt a

primarily Tc1 phenotype, differing from their Tc17 signature in the skin. During inflammation, we also report an upregulation of *H2M3* gene expression in whole colon tissue and colon organoids as well as an expansion of commensal reactive $CD8^+$ T cells secreting pro-inflammatory cytokines. We also observed expansion of commensal reactive $CD8^+$ T cells to the colon during colitis after skin association, where they again exhibit a pro-inflammatory Tc1 phenotype. Finally, subsequent computational identification of predicted H2-M3 binding peptides produced by *Helicobacter hepaticus,* we developed novel tetramer tools which illustrate the high relative frequency of H2-M3 restricted $CD8^+$ T cells in naïve animals.

Taken together, these findings lay the groundwork for further investigation into the impact of commensal reactive $CD8^+$ T cells on the etiology or perpetuation of IBD.

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When I was at University at CSU, I passed a monument built in front of the Microbiology building almost daily. It said, “If I have seen further than others, it was by standing on the shoulders of giants.” - Isaac Newton. It comes to mind now as I write these acknowledgments because instead of just thinking it was a cheesy quote, I finally understand it.

To do science is to build, to stand atop the body of literature, and look further than past "Greats" have been allowed to see. To be a scientist is to have the privilege to peer into the unknown and experience the joy of discovering something about this world that no person on this planet has ever seen, until you look it up on PubMed and find out it was published in 1999.

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Now on to your regularly scheduled Thesis Content

Contributions

- Dr. Derron A. Alves and Kevin Bock performed histopathological analysis of murine colon tissues.
- Dr. Angelina Angelova performed the analysis of 16S-rRNA dataset.
- Dr. Juliana Perez-Chaparro performed the MIC assays and produced aliquots of *Helicobacter hepaticus.*
- The NIH tetramer core produced all peptide:MHC tetramers.

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List of Abbreviations

IBD	Inflammatory bowel diseases
CD	Crohn's disease
UC	Ulcerative colitis
IEC	Intestinal epithelial cell
Muc2	Mucin 2
SCFA	Short chain fatty acids
IEL	Intra-epithelial lymphocytes
LPL	Lamina propria lymphocytes
CD	Cluster of differentiation
IL	Interleukin
iNKT	Invariant natural killer T cell
MAIT	Mucosal associated invariant T cell
Th	T-helper cell ($CD4^+$ T cell)
DC	Dendritic cell
ILC	Innate lymphoid cell
GF	Germ free
SPF	Specific pathogen free
TCR	T cell receptor
NOD2	Nucleotide binding oligomerization domain containing 2
PRR	Pattern recognition receptor
Treg	T regulatory cell
Rag	Recombination-activating gene
SFB	Segmented filamentous bacteria
IgA	Immunoglobulin A
GALT	Gut associated lymphoid tissue
mLN	Mesenteric lymph nodes
IgA	Immunoglobulin A
MHC	Major histocompatibility complex
HLA	Human leukocyte antigen
CXCL	Chemokine (C-X-C motif) ligand
AMP	Antimicrobial peptide
fMet	*N*-formylated peptide
KGF	Keratinocyte growth factor
Tc	T-cytotoxic cell ($CD8^+$ T cell)
REGIIIγ	Regenerating islet-derived protein
MyD88	Myeloid differentiation factor 88
CTL	Cytotoxic T lymphocyte
Mta	Maternally transmitted antigen
β2m	β_2-microglobulin
TAP	Transporter associated with antigen processing
ND1	NADH dehydrogenase subunit 1

PLZF	Promyelocytic leukemia zinc finger
SLAM	Signaling lymphocyte activation molecule
SAP	SLAM associated protein
ATAC-seq	Assay for transposase-accessible chromatin with high throughput sequencing
SDI	Sociodemographic index
RNA	Ribonucleic acid
TNF	Tumor necrosis factor
NK1.1	Killer cell lectin-like receptor subfamily B, member 1
PBMC	Peripheral blood mononuclear cells
DSS	Dextran Sodium Sulfate
TNBS	2,4,6- trinitrobenzene sulfonic acid
SCID	Severe combined immunodeficiency
H&E	Hematoxylin and Eosin
NK	Natural Killer
IFNγ	Interferon-γ
Tbet	T-box transcription factor TBX21
FOXP3	Forkhead box P3
RORγT	Retinoic-acid-receptor-related orphan nuclear receptor gamma
WT	Wildtype
KO	Knockout
PCoA	Principle coordinate analysis
ASV	Amplicon sequence variants
IBA-1	Ionized calcium-binding adaptor molecule-1
MPO	Myeloperoxidase
OT-1	Ovalbumin specific $CD8^+$ TCR transgenic
H.h	*Helicobacter hepaticus*
pDC	Plasmacytoid DC
mRNA	Messenger RNA
cCM	Control culture medium
iCM	Inflammatory culture medium
PD-1	Programmed cell death protein-1
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
GATA3	GATA binding protein 3
qPCR	Quantitative polymerase chain reaction
CFU	Colony forming units
$SA1^{Tg}$	*Staphylococcus aureus* specific TCR transgenic
Nur77	Nuclear receptor NR4A1
ICI	Immune checkpoint inhibitors

1 Introduction

The “microbiota” describes the trillions of bacteria, viruses, fungi, and parasites that inhabit the surface of our tissues. Each barrier to the external world is occupied by the microbiota which serve a plethora of functions to the host. These include protection from infection[1], digestion of foodstuffs to aid in nutrient acquisition, and education to the nascent immune system[2]. To foster these vital interactions, host homeostasis hinges on adequate maintenance of barrier tissues such as the gut and skin. This “commensalism” can be contextual, with some key members of the microbiota turning pathogenic if given the opportunity[3]. Notably, it is believed that a break down in barrier function and subsequent maladaptive responses to the microbiota is a driving factor in the etiology of inflammatory bowel diseases (IBD) such as Crohn’s disease (CD) and ulcerative colitis (UC)[4]. Investigation into these diseases in particular give us a window into how barrier tissues must contain structural and immunological mechanisms to maintain the vital crosstalk between host and microbiota, while limiting their outgrowth and nefarious potential.

1.1 Establishment of the host commensal relationship: unique features of the skin and gut barriers

The gut and skin comprise the largest barrier organs in the mammalian body and host the highest density of the commensal microbiota. Although the structures of these epithelial barriers differ substantially, they both allow for the passage of molecular cues and metabolites from the microbiota that affect host physiology, while controlling aberrant inflammatory responses to the external milieu[5–7]. These two tissues vary greatly in their exposures to elements, nutrient availability, and

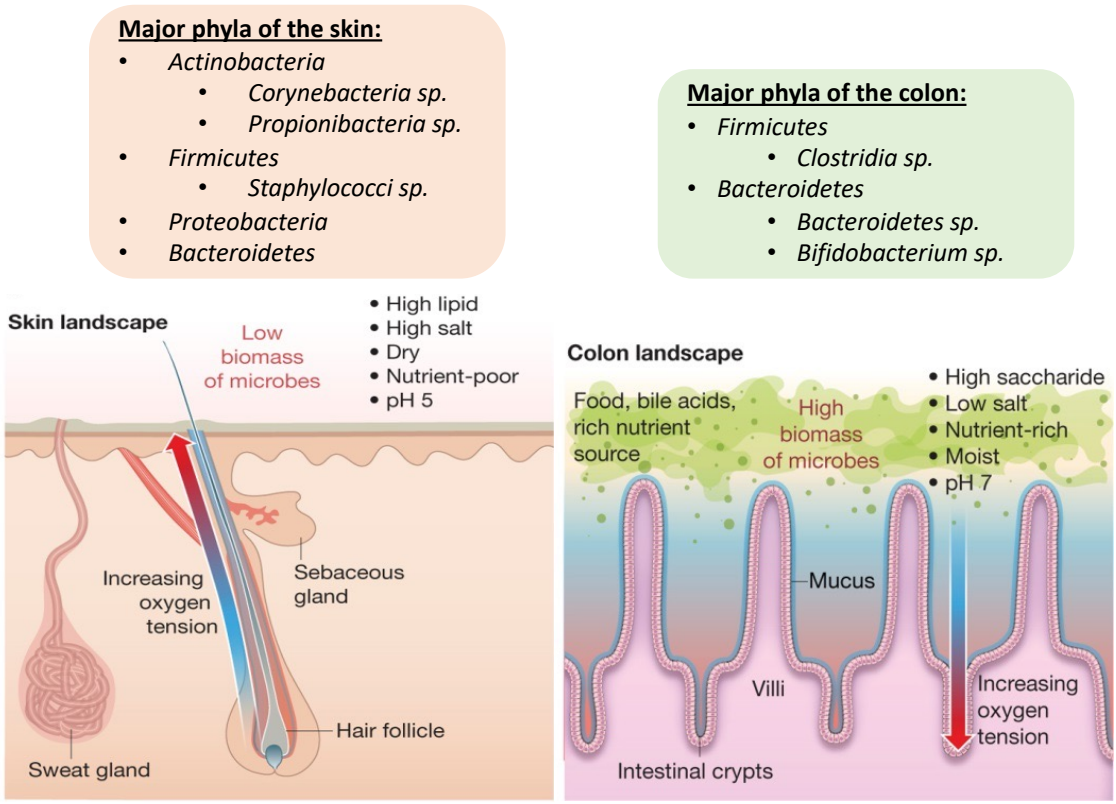


Figure 1.1: Comparison of the skin and gut barriers
Schematic of the skin and gut barriers illustrating the different structures, nutrients, oxygen availability and microbial composition. Sweat and sebaceous glands of the skin produce an environment rich in lipids and salt. Exposed to the atmosphere, the skins surface is highly oxygenated, and low in moisture, fostering the growth of a low biomass of bacterial species belonging to *Actinobacteria, Firmicutes, Proteobacteria* and *Bacteroidetes* phyla. In contrast, the colon lumen is anaerobic, nutrient dense and moist, containing a much higher biomass of bacterial species belonging largely to the *Firmicutes* and *Bacteroidetes* phyla. Mucus lining the villi of the colon maintains a physical barrier between the host and microbiota of the lumen.

Figure adapted from Y Chen *et al. Nature* (2018)

oxygen accessibility which drive variability in the microbes that inhabit them (Figure 1.1).

Serving as the outermost protective layer of the host, the skin epidermis consists of dense layers of cornified epithelial cells called keratinocytes below which resides the dermis and subcutaneous tissues. The skin is peppered with sweat glands that salinate the epithelial landscape, and sebaceous glands that create a waxy sebum containing anti-microbial peptides as well as other bioactive compounds that control outgrowth of potentially pathogenic microbes[7]. In this highly oxygenated environment, the major bacterial species that reside upon the

skin belong to the *Actinobacteria, Firmicutes, Proteobacteria* and *Bacteroidetes* phyla and are present in relatively low abundance[8].

In contrast, the gastrointestinal tract, consisting of the esophagus, stomach, small and large intestine, is home to the largest biomass of the microbiota in part due to the nutrient rich environment provided by the host's diet. The small intestine, which can be subdivided into three sections: duodenum, jejunum, and ileum, is the primary site of terminal nutrient absorption and immunological interaction with the microbiota. It is here that microbes aid in the digestion of complex food nutrients allowing for the absorption of key metabolites by the host. The large intestine, comprised of the cecum, colon, rectum, and anus serves as the major site of water reabsorption, and harbors the densest biomass of bacteria[9].

The lumen of the intestine is anaerobic and bacteria belonging to the *Firmicutes* and *Bacteroidetes* dominate[10,11]. A singular layer of intestinal epithelial cells (IEC) guard the deeper muscularis and submucosal tissues of the gut from the high microbial burden of the intestinal lumen. IECs are connected by tight junctions, and between the enterocytes reside a variety of specialized cells that create physical and enzymatic barriers to constrain encroaching microbes[12]. The small intestine contains specialized epithelial cells called Paneth cells which produce a variety of anti-microbial peptides that control pathogen and microbiota overgrowth[13]. Additionally, goblet cells produce mucin and line the intestinal epithelium of both the small and large intestine, increasing in density towards the terminal colon. It is this mucus layer that provides vital protection against invading microbes by forming a largely impenetrable matrix packed with antimicrobial

proteins and antibodies, and deficiencies in Muc2 predispose mice to intestinal inflammation and cancer development[14,15]. Chemosensory tuft cells within the epithelium are believed to be key orchestrators of immunity to helminths[16], and enteroendocrine cells respond rapidly to microbial metabolites such as short chain fatty acids (SCFA) to produce gut hormones controlling host metabolism[17]. Together, these innate mechanisms are critical mediators to establish a homeostatic balance between the host and commensal microbiota.

1.2 Immunological mediators with the microbiota in the gut

The gut supports numerous immunological cell types and structures which direct homeostatic responses to the commensal microbiota (Figure 1.2). The lymphocytes that populate both the epithelium (intra-epithelial lymphocytes-IEL) and lamina propria (lamina propria lymphocytes-LPL) mediate many interactions with the microbiota and host tissues. IELs have a peculiar predisposition for expression of the CD8α homodimer and can largely be subclassified as “induced” or “natural”[18]. $CD4^+$ T cells, $\gamma\delta^+$ T cells and $CD8^+$ T cells, as well as other TCR^- cells reside within the IEL with incompletely understood functions[18]. However, multiple subsets of IEL do, in part, depend on the microbiota for their residence. Germ free (GF) mice, devoid of a microbiota, have remarkably reduced numbers of IEL compared to specific pathogen free (SPF) mice[19] and animals deficient in NOD2, an intracellular pattern recognition receptor (PRR), also have very few IEL[20]. Notably, loss of function mutations in NOD2 also strongly associate with susceptibility to Crohn’s disease[21,22] collectively suggesting that IELs might play key roles in maintaining host homeostasis with the microbiota that has yet to be fully understood.

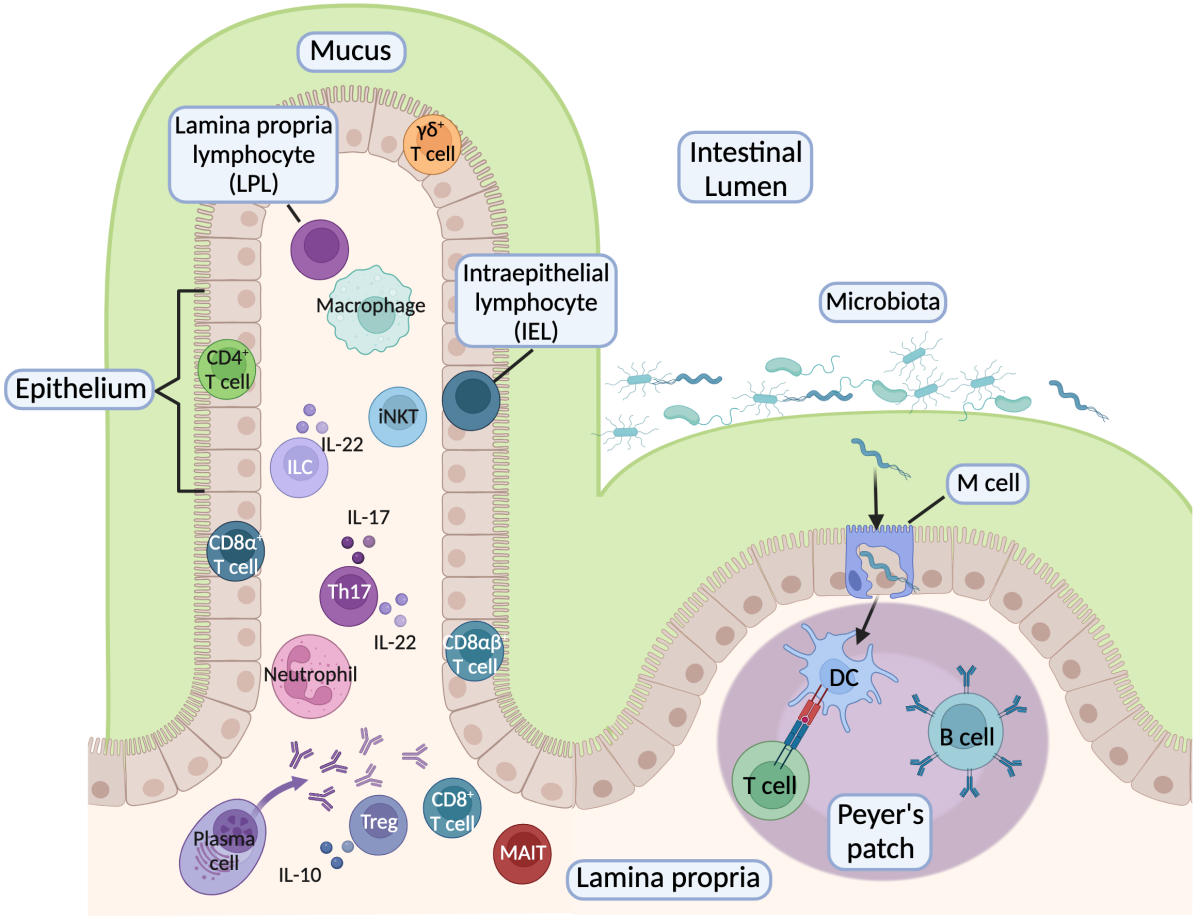


Figure 1.2: Immunological mediators of gut homeostasis with the microbiota
Within the epithelium of the gut resides IEL consisting of CD$\alpha\alpha^+$, CD8$\alpha\beta^+$, CD4$^+$, and $\gamma\delta^+$ T cells of elusive function. In the lamina propria, ILCs and T cells produce barrier maintaining cytokines such as IL-17 and IL-22 in response to the commensal microbiota. Tregs produce the tolerogenic cytokine IL-10 that constrain pathological responses to microbes. Plasma cells produce antibodies, notably, secretory IgA, which plays vital roles in constraining encroaching bacteria. CD8$^+$ T cells establish residency in the lamina propria with limited known functions. MAIT cells recognize riboflavin derivatives sourced from the microbiota presented on the MHCIb molecule, MR1, and iNKT cells respond microbially derived lipids presented on CD1d. Neutrophils and Monocytes phagocytose microbes and are potent stimulators of downstream inflammation or adaptive immune responses. M cells residing above Peyer's patches, specialized lymphoid structures of the small intestine, sample the milieu of the lumen to DCs which initiate T and B cell responses in the gut.

Abbreviations: CD, Cluster of Differentiation; IL, Interleukin; IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; iNKT, invariant natural killer T; MAIT, mucosal-associated invariant T; Th17, T helper 17, DC, dendritic cell; ILC, innate lymphoid cell.

Figure created with BioRender.com.

Beneath the epithelium resides the lamina propria, and all LPL within. Various CD4$^+$ T cell subsets play vital roles in maintaining homeostasis with the commensal microbiota. Most notably, T regulatory (Treg) CD4$^+$ T cells can tune other CD4$^+$ T response to commensals. Indeed, CD4$^+$CD25$^+$ Treg cells are sufficient to both prevent and cure colitis driven by CD4$^+$ CD45RB^{high} T cells into

Rag$^{-/-}$ mice[23]. Further, IL-10$^{-/-}$ mice develop spontaneous colitis driven by colonization with members of the microbiota such as *Helicobacter hepaticus*[24] and resulting CD4^{+} T cell activation, illustrating the delicate balance between pathogen and commensal held in check by IL-10 signaling. The microbiota can also induce a Treg response itself; production of SCFA by the microbiota induces colonic Tregs[25]. T-helper (Th)-17 CD4^{+}T cells are highly dependent on the microbiota and can be induced after colonization with bacterial species residing close to the intestinal epithelium such as Segmented Filamentous Bacteria (SFB)[26]. Th17 cells are contextually pathogenic, providing increased protection against some intestinal infections, yet have also been implicated in driving pathology in IBD[26,27]. IL-22, a member of the IL-10 family of cytokines, is produced by Type 3 Innate Lymphoid cells (ILC3), and CD4^{+} T cells, and is critical in maintaining the gut barrier as IL-22$^{-/-}$ mice suffer dissemination of intestinal bacteria and resulting systemic inflammation[28]. CD8^{+} T cells, although present in the gut lamina propria, have not been well studied as much as their IEL counterparts and have most often been investigated in the context of immunization or viral infection[29]. Although, emerging research indicates the commensal microbiota also play profound roles in modulating CD8^{+} T cell responses that can have systemic effects in the context of cancer[30–32]. Innate-like T cells, such as mucosal associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells are also present in the gut lamina propria and will be discussed in more detail in future sections[2]. Further, monocytes and neutrophils are innate sensors of the microbiota, which protect against pathogenic insults by phagocytosing encroaching bacteria and directing subsequent immune responses through antigen presentation[33,34]. Notably, plasma cells, robust producers of secretory IgA,

also reside within the gut and are vital in maintaining compartmentalization of the microbiota[35].

The gut associated lymphoid tissues (GALT) comprise all structured sites of immunological innervation. This includes the Peyer's patches of the small intestine and lymphoid follicles, which sample luminal antigens through specialized epithelial cells called microfold (M) cells, driving T cell and B cell activation[36]. Further, the GALT also comprises the mesenteric lymph nodes which drain the small and large intestine and are sites of antigen presentation, T and B cell activation and orchestrate many of the local and systemic responses to the microbiota and gut pathogens[37].

Together, the network of immune cells of the gut and GALT are impacted by the microbiota and can in turn regulate downstream responses to the microbes of the small and large intestine to maintain host homeostasis.

1.3 Innate-like T cells mediate early interactions with the microbiota

(Text adapted from Ansaldo, Farley & Belkaid, Annual Review of Immunology, 2021 of which T. K. Farley was co-first author and primary contributor to the following passage)[2]

Upon birth, the neonate must contend with a barrage of novel microbial stimuli derived from the maternal vaginal or skin microbiome and establish homeostatic set points for the lifetime of the organism. A growing body of evidence implicates innate-like or unconventional T cells, such as those restricted by non-polymorphic major histocompatibility complex class Ib (MHC-Ib) molecules, as playing key roles in the early introduction to the microbiota. These unconventional T cells recognize a set of conserved antigens such as modified peptides, metabolites, or lipids[38]. MAIT cells, iNKT cells and $\gamma\delta$ T cells express semi-invariant TCR's,

greatly limiting the repertoire of antigens compared to classically MHC restricted T cells[39]. Some $CD8^+$ T cells with more diverse TCR repertoires are restricted by other MHC-Ib molecules such as H2-M3, which presents *N*-formylated peptides derived from bacteria and mitochondria[40]. Qa-1, the murine functional homologue of HLA-E in humans, canonically presents peptides derived from MHC-I leader sequences but has been shown to also bind some pathogen derived peptides from *Salmonella typhimurium, Salmonella enterica* serovar Typhi*, Listeria monocytogenes* and *Mycobacterium tuberculosis*[39,41]*.* By recognizing a conserved set of microbially derived peptides, unconventional T cells are believed to act as a bridge between innate pattern recognition receptors (PRR) and the highly polymorphic adaptive immunity provided by classical T and B cell responses.

Typically, innate-like T cells acquire effector functions during development, prior to thymic egress, and seed tissues before conventional adaptive immune cells, making them well poised to rapidly respond to the earliest microbial encounters[42,43]. Further, MHC-Ib molecules uniquely present antigens with conserved motifs that can originate from a large constituency of the microbiota. MAIT cells recognize riboflavin derivatives derived from microbial metabolism[44] and iNKT cells are restricted by the MHC-Ib molecule CD1d which presents microbe-derived lipid antigens[45]. Thus, the assembly of innate-like MHC-Ib restricted T cells are ideal orchestrators of the early life communication between the microbiota and host immune system.

Adequate seeding of tissues with unconventional T cells can depend on exposure to specific microbes during key developmental windows. For example, iNKT cell accumulation in mucosal tissues is regulated by the microbiota in the

postnatal period. Increased CXCL16 expression in the colon and lungs of adult germ-free mice leads to aberrant accumulation of iNKT cells and greater susceptibility to asthma and colitis[46] which can only be rescued by neonatal exposure to the microbiota. Furthermore, neonatal but not adult exposure to sphingolipids produced by *Bacteroides fragilis* can prevent aberrant iNKT cell accumulation in the colon by locally inhibiting iNKT cell proliferation[47]. The development of MAIT cells is also dependent on early-life exposure to microbes that produce riboflavin derivatives during an early-life window[48], and impaired microbiota-derived antigen exposure in early life leads to irreversible defects in MAIT cells[48–50]. Microbes belonging to the order *Enterobacterales*, *Proteus mirabilis* and *Klebsiella oxytoca*, are sufficient for MAIT cell development, and perhaps uncoincidentally, members of this order are highly enriched in the neonatal gut and decrease in abundance with age[51].

Taken together, these studies argue that the microbiota establish homeostatic set points of unconventional T cell populations in early life. These fundamental interactions between unconventional T cells and early-life microbiota also support the idea that innate-like immune responses may have been sustained throughout evolution to promote early responses to the microbiota prior to the establishment of adaptive immune responses.

1.4 Innate-like T cells persist as sentinels of the microbiota throughout life

(Text adapted from Ansaldo, Farley & Belkaid, Annual Review of Immunology, 2021 of which T. K. Farley was co-first author and primary contributor to the following passage)[2]

In addition to their role in early life, unconventional and innate-like lymphocytes contribute to the long-term maintenance of homeostatic responses to the

microbiota. Indeed, as discussed above in Section 1.3. the ability of unconventional T cells to respond to canonical antigens derived from a large fraction of the microbiota makes these cells powerful regulators of tissue physiology, with an increasingly appreciated role in tissue repair (Figure 1.3). Throughout life, barrier tissues are marred by frequent inflammatory insults requiring rapid induction of repair mechanisms that are under the control of several redundant cell subsets and pathways. One of the first indications of a role for the microbiota in tissue repair was the observation that, in the gut, TLR activation by commensals is required to protect the host from acute injury[52]. Dendritic epidermal T cells (DETC) were the first subset of unconventional T cells linked to tissue repair, a property that has since been attributed to numerous other subsets of innate-like or non-classically restricted T cells[53].

Within the gut, intraepithelial $\gamma\delta^+$ T cells comprise the largest population of innate-like T cells within the epithelium and contribute to mucosal repair via the production of keratinocyte growth factor (KGF)[54]. Additionally, MAIT cells from both humans and mice constitutively express a wound healing program[48,55,56]. Within the skin, MAIT cells can make up a significant portion of lymphocytes: up to 40% of the $\alpha\beta$ T cells in mice and approximately 2% of $CD3^+$ lymphocytes in humans[48,57]. The enrichment within this tissue could be explained by the fact that a significant portion of the skin microbiota express the machinery associated with the production of MAIT cell antigens[48]. Following skin colonization with *S. epidermidis*, a commensal capable of synthesizing riboflavin derivatives, MAIT cells significantly expand and contribute to wound repair[48,56]. Similarly, *S.*

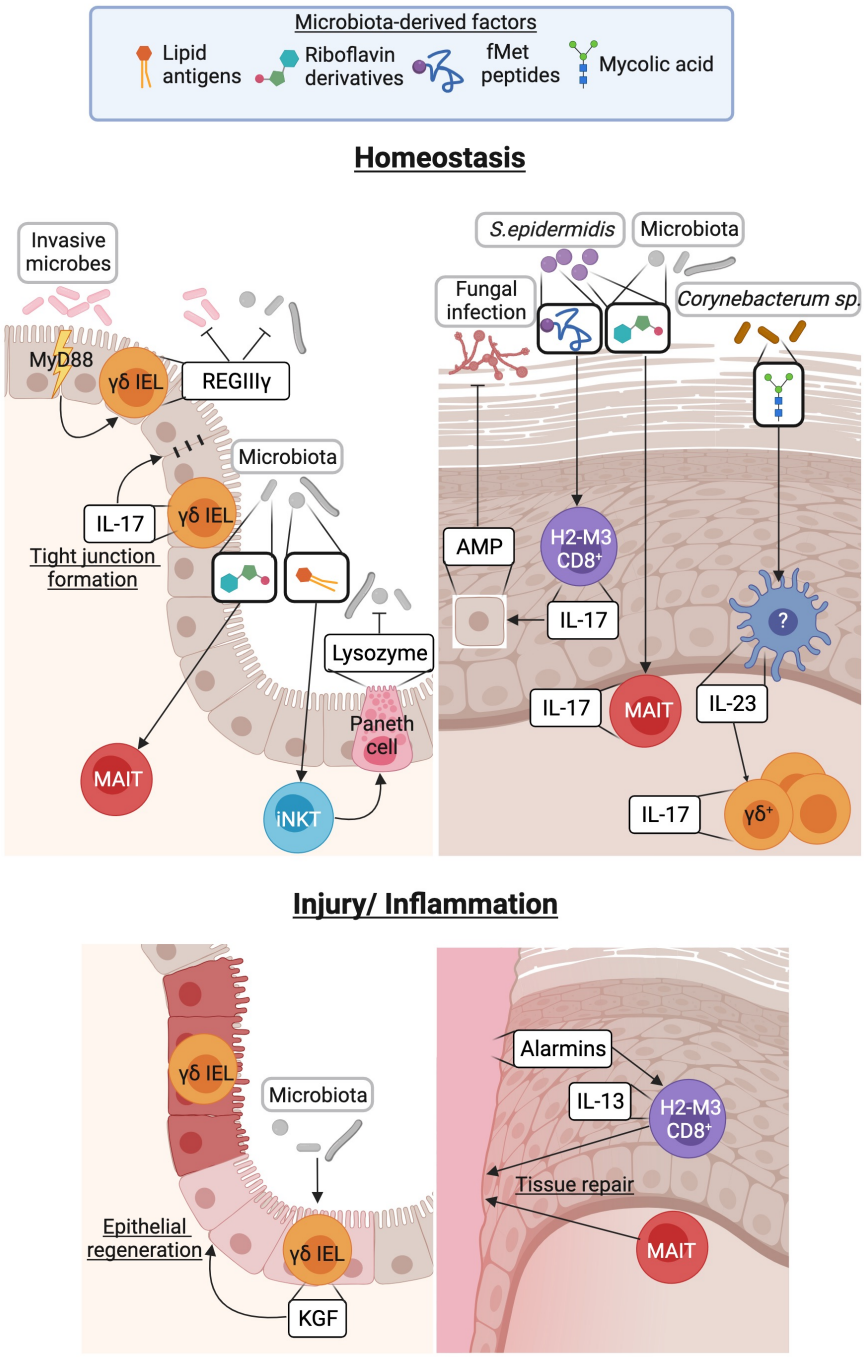


Figure 1.3: Unconventional T cells engage in a lifelong dialog with the microbiota and control tissue homeostasis
(Figure legend on next page)

epidermidis promotes the induction of nonclassical MHC-Ib-restricted CD8⁺ T cells[58–61]. Topical exposure to a particular clade of *S. epidermidis* also stimulates the induction and tissue accumulation of H2-M3-restricted CD8⁺ T cells specific for commensal-derived *N*-formyl-containing peptides[60]. Within the epidermis these commensal-specific IL-17-producing CD8⁺ T cells can promote tissue repair by

Figure 1.3: Unconventional T cells engage in a lifelong dialog with the microbiota and control tissue homeostasis

Unconventional T cells, including γδ T cells, MAIT cells, iNKT cells, and Tc17 cells, seed barrier tissues such as the skin and gut, where they control numerous physiological tissue responses. These include regulation of the microbiota composition and promotion of tissue repair. In response to epithelial MyD88 signaling, gut epithelium–resident γδ T cells produce the AMP REGIIIγ, which in turn regulates microbial composition and localization. Colonization can also be regulated indirectly in response to microbial antigens: Microbiota-derived lipids induce iNKT-dependent Paneth cell secretion of lysozyme. Within the skin, IL-17-producing H2-M3-restricted CD8+ T cells accumulate in the epidermis in response to Staphylococcus epidermidis–derived N-formylated peptides. These T cells promote the production of AMPs in an IL-17A-dependent manner, thereby enhancing protection against subsequent infection. MAIT cells and γδ T cells are also enriched within the skin and respond to microbiota-derived metabolites, microbiota-induced cytokines, or both. In the gut, γδ T cell–derived IL-17A contributes to tight-junction formation between enterocytes, and following tissue injury these cells are potent producers of the epithelial mitogen KGF. Skin-resident unconventional T cells, including MAIT cells and H2-M3-restricted $CD8^+$ T cells, also contribute to wound healing in an IL-13-dependent manner. Figure adapted from image created with BioRender.com.

Abbreviations: AMP, antimicrobial peptide; fMet, N-formylated peptide; IEL, intraepithelial lymphocyte; iNKT, invariant natural killer T; KGF, keratinocyte growth factor; MAIT, mucosal-associated invariant T; Tc17, H2-M3-restricted $CD8^+$ T.

Figure adapted from Ansaldo *et al., Annual Review of Immunology* (2021)

releasing IL-13 in response to tissue alarmins and provide heterologous protection against fungal pathogens[59,61]. Interestingly, association with *S. epidermidis* also elicited a Tc17 response in primate skin, though the restriction of these cells and antigen(s) they are responsive to, was not investigated[60]. While a human homologue of H2-M3 has not been identified[39,40], we could postulate that alternate mechanisms of antigen presentation may be in place to recognize these highly abundant microbiota-derived *N*-formylated peptides.

Unconventional T cells also regulate microbiota composition and protect against microbial invasion. For instance, $\gamma\delta^+$ T cells from the IEL compartment promote production of the antimicrobial peptide REGIIIγ at steady state and constrain the microbiota in the context of mucosal damage[62,63]. The immunomodulatory, antibacterial program expressed by $\gamma\delta^+$ T cells is controlled by the microbiota itself, and microbial sensing via epithelial MyD88 governs $\gamma\delta^+$ T cell metabolism and dynamics of epithelial surveillance[62,64]. Responses of iNKT cells to microbe-derived lipid antigens presented by CD1d[39] regulate intestinal

Paneth cell function and production of lysozyme, an enzyme that catalyzes the destruction of bacterial cell walls[65]. As such, iNKT cells can indirectly limit bacterial translocation across the intestinal barrier[65]. Further, H2-M3 restricted $CD8^+$ T cells induced by skin commensals can promote the production of antimicrobial peptides by keratinocytes, thereby promoting heterologous protection against subsequent fungal infection[59]. Similarly, within the eye, IL-17-producing $\gamma\delta^+$ T cells elicited in response to the commensal microbe *Corynebacterium mastitidis* provide heterologous protection against ocular fungal infections via local increase in antimicrobial peptides[66]. Within the skin, IL-17-producing $\gamma\delta^+$ T cells also respond to colonization with *Corynebacterium* in a mycolic acid/IL-23-dependent manner[67]. The nature of the antigens recognized by $\gamma\delta^+$ T cells remains largely unknown, but these cells are believed to also recognize lipid and phosphoantigens[68] a point of relevance for potential recognition of commensal-derived products. MAIT cells have also been proposed to contribute to antimicrobial defense, and many bacteria and yeast, including pathogens, are prolific producers of riboflavin derivatives[44,69–71].

Emerging evidence supports the idea that the fundamental role of innate-like T cells may result from their action as a network of cells with overlapping and potentially synergistic functions rather than as individual subsets. For instance, H2-M3 restricted $CD8^+$ T cells, MAIT cells, and $\gamma\delta^+$ T cells all produce type 17 cytokines in the skin, involved in barrier function and immune surveillance[48,59,60,66,67]. These cell subsets also compete for a shared physiological niche[48,50]. This phenomenon was confirmed in a patient harboring a homozygous point mutation in MR1 associated with the absence of MAIT cells and concomitant expansion of $\gamma\delta^+$ T cells[72]. This is also supported by reports of a

shared transcriptional profile between human $\gamma\delta^+$ T cells and MAIT cells[73]. Similarly, H2-M3 restricted CD8$^+$T cells and human MAIT cells share similar wound healing signatures[55,60].The redundancy of innate-like T cells and ability to compete for similar survival factors or niches may have been maintained through evolution to increase tissue resilience and long-term protection of host homeostasis.

1.5 Expanded roles for H2-M3 restricted CD8$^+$ T cells beyond infection

H2-M3 is a murine MHC-Ib molecule which selectively binds and presents *N*-formyl (fMet) containing peptides to innate-like CD8$^+$ T cells[40]. Formylation of the *N*-terminus is required for protein translation in prokaryotes and eukaryotic mitochondria, a vestige of their bacterial origin[40,74]. Formyl peptide receptors are PRR's that can recognize fMet peptides in an innate manner, stimulating chemotactic signals in phagocytes and initiating inflammatory responses[75]. Yet, the murine immune system has maintained through evolution, a subclass of innate-like CD8$^+$ T cells to respond to these microbially derived signals which are now appreciated to have pleiotropic functions during both infection and in barrier maintenance.

The hint of an MHC-Ib molecule capable of presenting fMet peptides was first reported by Fischer Lindahl et al. in 1980 where they observed unrestricted killing of target cells by CD8$^+$ cytotoxic T lymphocytes (CTL) driven by the presentation of an "unusual" maternally transmitted antigen (Mta)[76]. Follow up studies established that this MHC-Ib molecule was indeed H2-M3, associates with β_2-microglobulin (β2m), like many classical and non-classical MHC molecules, and that the Mta was a peptide derived from the N terminus of the mitochondrial

protein, NADH dehydrogenase subunit 1 (ND1)[77–79]. Through work by Wang and colleagues, it is also now understood that H2-M3 is retained intracellularly in the endoplasmic reticulum in a peptide-receptive state where upon exposure to N-formylated peptides, the complex is loaded by tapasin and stabilized by TAP then subsequently shuttled to the cell surface for antigen presentation[80,81].

H2-M3 has since become one of the best characterized MHC-Ib molecules and a large body of work has focused on the H2-M3 restricted $CD8^+$ T cell response in the context of infection with intracellular bacteria, in particular, *Listeria monocytogenes*[40]. Some of the first mentions of a non-classical response to *L. monocytogenes* occurred in the late 1980's when two subsets of $CD8^+$, or at the time, Lyt-2^+ cells, were described to contribute to the CTL response to infection[82]. They were designated as "non-MHC- restricted" yet perplexingly peptide specific, as these CTL required antigen for their function, but were able to lyse bone marrow derived macrophages devoid of classical MHC molecules *H-2K*, *D,* and *L*[82]. Following suspicion that these CTL's may be restricted by the H-2B molecule H2-M3, Kurlander and colleagues developed a strain of mice containing a non-functional H2-M3 allele which abrogated CTL killing of mutant macrophages presenting *Listeria* peptides[83]. Simultaneously, utilizing a CTL line derived from a *L. monocytogenes* primed mouse, Pamer and colleagues also confirmed that H2-M3 can present bacterial peptides during infection[84]. Subsequent studies on the kinetics of these non-classical responses compared to classically restricted CTL responses established an early innate-like role for H2-M3 restricted $CD8^+$ T cell responses to infection, with minimal contribution to the secondary CTL response (Figure 1.4 B)[85,86]. This is not because H2-M3 restricted $CD8^+$ T cells lack the ability to form memory, but rather, MHC-Ia restricted memory $CD8^+$ T cells prevent

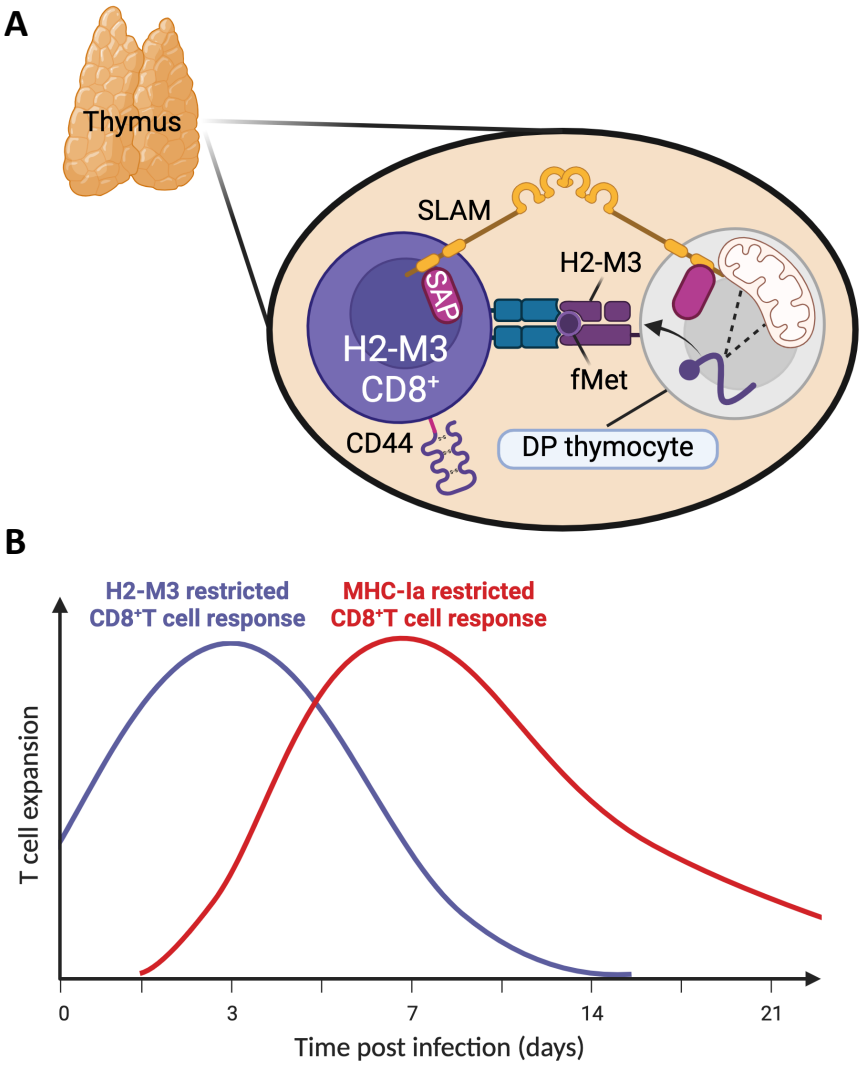


Figure 1.4: Thymic development of H2-M3 restricted CD8⁺ T cells endows innate-like phenotype
A. H2-M3 restricted CD8⁺ T cells can be selected on both epithelial cells, and hematopoietic cells of the thymus. However, like other innate-like T cells, only selection on hematopoietic cells, also known as double positive (DP) thymocytes, endows their pre-activated phenotype and expression of activation markers such as CD44. Innate-like H2-M3 restricted CD8⁺T cells require SAP signaling, and presentation of *N*-formyl containing peptides on H2-M3, potentially derived from the mitochondria, for their development. **B.** After their development in the thymus, innate-like H2-M3 restricted CD8⁺ T cells can act as early sentinels of disease, responding rapidly and robustly to infectious agents before the MHC-Ia restricted CD8⁺ T cell response can emerge.

Abbreviations: SLAM, signaling lymphocyte activation molecule; SAP, SLAM associated protein

Figure created with Biorender.com

Figure 1.4 B adapted from Anderson & Brossay, *Immunogenetics* (2016)

antigen presentation during subsequent challenge[87]. One caveat to these findings is that they have largely been carried out *ex-vivo* or *in-vitro*, with minimal knowledge about H2-M3 restricted T cell responses in tissue outside of spleen

and lymph node. This is especially interesting considering the primary site of infection for *L. monocytogenes* is the gastrointestinal epithelium. Urdahl et al., reported that MHC-Ib restricted $CD8^+$ T cells can accumulate in the lungs of mice infected with *Mycobacterium tuberculosis,* however, they did not assess the relative contribution of H2-M3 restricted compared to other MHC-Ib restricted $CD8^+$ T cells in their study[88].

H2-M3 restricted $CD8^+$ T cells express the activation marker CD44 even in naïve mice that is not dependent on the microbiota as was previously postulated[60,89]. The innate-like phenotype of H2-M3 restricted $CD8^+$ T cells is endowed by their selection process in the thymus (Figure 1.4 A). Studies utilizing H2-M3 restricted transgenic mouse lines showed that H2-M3 is required for both positive and negative selection in the thymus[90]. H2-M3 restricted $CD8^+$ T cells are selected on both thymic epithelial cells, and hematopoietic cells, however, the hallmark "innate-like" activated phenotype present on these cells, is derived from positive selection on hematopoietic cells, presumably against a mitochondrial antigen[91,92]. This selection process is shared with other non-classical T cells such as iNKT's, but they differ in their expression of the transcription factor PLZF[91]. Further, the innate-like phenotype is dependent on the signaling lymphocyte activation molecule (SLAM) associated protein (SAP) pathway, like iNKT cells[93,94].

More recently, H2-M3 restricted $CD8^+$T cells have been revealed to also play key roles in response to a non-inflammatory commensal exposure at the skin barrier. In SPF mice, the skin epithelium is sparsely populated with $CD8^+$ T cells, however, following association with a particular clade of common human skin commensal *Staphylococcus epidermidis*, Naik and colleagues first reported a

rapid expansion of IL-17A producing $CD8^+$ T cells (Tc17) within the skin epithelium[58,59]. Notably, mice that were associated with *S. epidermidis* had enhanced barrier immunity and heterologous protection against infection with a fungal pathogen[58,59]. Subsequent studies established a requirement for H2-M3 in driving these responses, and transcriptomic analysis of the Tc17 population elicited after topical association with *S. epidermidis* compared to the inflammatory Tc1 elicited by intradermal infection with the same microbe, revealed a tissue repair and wound healing signature[60]. This was confirmed *in-vivo* utilizing a model of wound repair in which *S. epidermidis* associated animals had significantly improved wound healing compared to unassociated controls, an effect that was lost in the absence of H2-M3[60]. Further, ATAC-seq of *S. epidermidis* specific H2-M3 restricted $CD8^+$ T cells showed a poised Type 2 signature, and in response to tissue alarmins released upon epithelial injury, commensal specific Tc17 were able to rapidly produce IL-13 which is key to their wound healing capabilities[61]. Taken together, these studies established a role for H2-M3 restricted $CD8^+$ T cell responses in mediating critical functions for tissue homeostasis in response to commensal exposure. Further, it illustrates the pleiotropic effects of $CD8^+$ T cells, beyond their well characterized cytotoxic role during infection. Yet, the contributions of these commensal specific, H2-M3 restricted T cells to tissue physiology at other epithelial barriers such as the gut, remains unexplored.

1.6 Inflammatory Bowel Diseases: loss of tolerance to the microbiota and barrier disruption

Although the composition of a “healthy” microbiota remains to be defined, as has been illustrated in previous sections, host homeostasis relies heavily on the adequate colonization of microbes and the development of tolerogenic responses

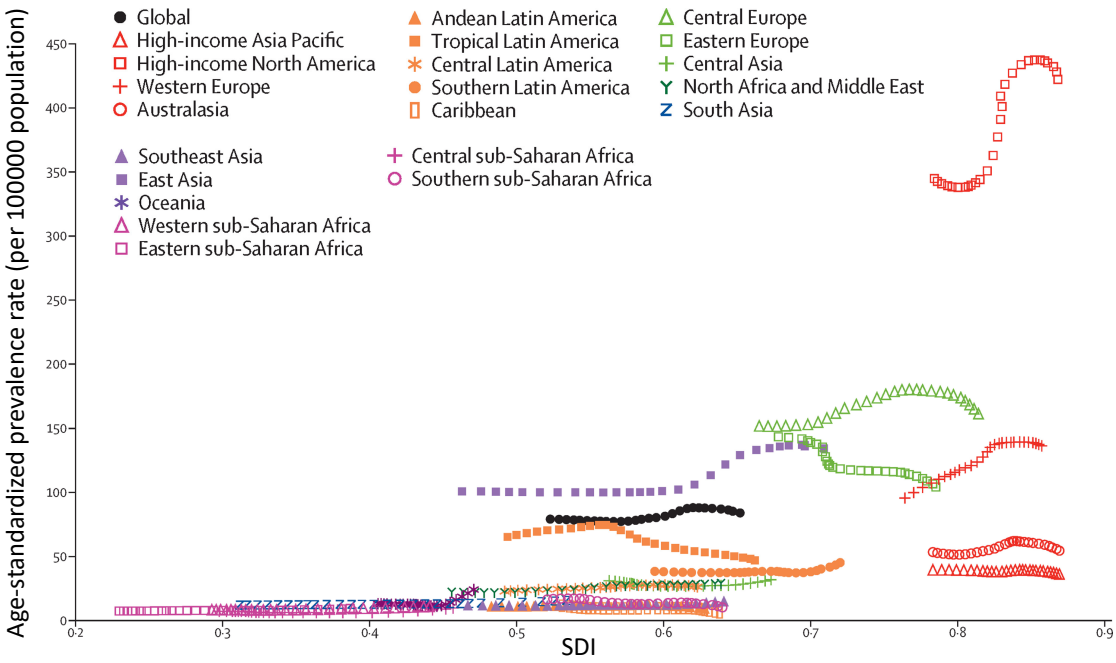


Figure 1.5: Prevalence of inflammatory bowel disease increases with sociodemographic index
For each of the 21 global burden of disease region studied, points from left to right depict the estimated age-standardized prevalence rate from each year between 1990-2017.

Abbreviations: SDI, Sociodemographic index

Figure adapted from Alatab *et al.*, *The Lancet Gastroenterology & Hepatology* (2020)

that maintain barrier integrity. Inflammatory bowel diseases (IBD), which encompass Crohn's disease and ulcerative colitis, the two most common forms, are diseases in which environmental triggers such as the gut microbiota, couple with genetic predisposition resulting in breakdown of homeostatic immunity at the gut barrier, and inflammation of the tissue [4,5]. Most recent estimates of the global burden of IBD predict that over 6.8 million people worldwide suffer from disease[95]. Although these diseases are often grouped together, due to their chronic relapsing activity of acute flares and periods of remission, they have vastly different clinical manifestations, treatments, and outcomes [4]. UC is most common and characterized by continuous inflammation isolated to the colon and rectum primarily affecting the mucosa, which at its most severe results in ulcerating lesions[96]. In contrast, CD patients present with discontinuous and transmural skip lesions that can affect any part of the gastrointestinal tract from the mouth to the

anus[97]. Regardless of UC or CD diagnosis, patients with IBD all have hallmark disease traits of loss of tolerance to the microbiota, aberrant immune activation, and epithelial barrier destruction. Thus, many treatments hinge on suppressing immune responses and inducing epithelial repair following flares[4]. Although many patients reach remission with available treatments, there are still a significant fraction of individuals that are non-responsive to therapy with no known cure.

Comprehensive analysis of the global burden of IBD indicates that disease prevalence is increasing over time and highest in countries with high sociodemographic index (SDI), a measure of socioeconomic development (Figure 1.5)[95,98]. Amongst a multitude of potential environmental triggers associated with disease induction, countries with high SDI have widespread access to sterilization techniques and antibiotics which drastically alter the microbiota of the population, drawing intrigue into a potential correlation[99–101]. Additionally, intestinal microbiome studies of patients with IBD illustrate a profound remodeling of microbial communities. Patients with IBD often present with a collapse of microbial diversity with dominance of microbes such as *Escherichia coli* belonging to the *Enterobacteriaceae* family and decrease in *Clostridium* species belonging to the phyla Firmicutes[102–106]. Longitudinal studies in CD have demonstrated that such microbiota alterations can even occur very early on in disease development, yet it is difficult to disentangle if such alterations are a result of inflammation or a driving factor of disease[104,105].

The immune system of the gut is greatly altered during IBD as well. Single cell-RNA sequencing analysis of the colon immune landscape of patients with UC reveal rewiring of interactions between immune cells of the gut during disease,

interestingly illuminating $CD8^+$ T cells as major producers of the pro-inflammatory cytokine TNFα[107]. Further, immune responses to the microbiota itself are altered during disease, microbiota reactive $CD4^+$ T cells are reduced in the circulation of patients with CD, but in the tissue have a higher propensity to produce IL-17A[108]. Interestingly, using a novel method to identify commensal MHC Class II epitopes derived from the microbiota, Pedersen and colleagues have also illustrated how commensal reactive $CD4^+$ T cells can shift from a tolerant IL-10 response to a potentially inflammatory IL-17A response during Crohn's disease[109]. It should be highlighted, however, that despite the overwhelming presence of Th17 and other IL-17A producing cells in IBD, blockade of IL-17A has not proven to be an effective clinical treatment, even exacerbating disease[110,111]. Due to its role in tight junction formation and barrier repair, it is feasible that IL-17 production during active disease is an effort to repair the inflamed tissue and is thus not a viable target in treatment of IBD[27].

Taken together, IBD is fundamentally a set of diseases with profound alterations in the native microbiota, epithelial barrier destruction and immune dysfunction. As such, the most effective therapies target key inflammatory mediators of disease including TNFα, IL-12/23, and the integrin $\alpha4\beta7$, but still, many patients do not reach remission[112]. Therefore, there is a dire need to expand upon the knowledge of the factors mediating inflammation in IBD, to identify novel targets for improved treatments.

1.7 $CD8^+$ T cells in IBD: prognostic markers or perpetuators of disease?

$CD8^+$ T cells have been largely understudied in IBD research. Some models utilizing transgenic autoreactive $CD8^+$ T cells implicate them potential initiators of

disease[113,114] however, these studies better recapitulate autoimmune disease, wherein the immune response is directed towards self. Although self-reactive $CD8^+$ T cells might be present in patients with IBD, inflammation is believed to be driven more by a maladaptive response to the commensal microbiota, and most research has focused on commensal reactive $CD4^+$ T cell subsets. Notably, in a model of epithelial barrier disruption with compounded IFNγ dysregulation, $NK1.1^+CD8^+$ T cells were implicated in driving disease in the absence of iNKT[115]. Recent studies have shown $CD8^+$ T cell signatures in circulating PBMC and tissue samples of patients with IBD with varying prognostic correlations, but a common emergence of a Tc17 signature[107,116,117]. Additionally, exhaustion of $CD8^+$ T cells has been linked to improved clinical outcomes in patients with Crohn's disease, indicating that $CD8^+$ T cell functionality might contribute to inflammation[116,118]. Further, $CD8^+$ T cell responses to the commensal microbiota have been reported in humans at steady state and are altered during inflammatory bowel disease[119–121]. However, these studies lack mechanistic insight. To our knowledge, there is no known function of commensal specific $CD8^+$ T cells during colitis. Further, no one has investigated the potential contribution of MHC-Ib restricted $CD8^+$ T cells, which might be poised and able to respond during inflammation more rapidly.

1.8 Mouse models of Inflammatory Bowel Disease

While human epidemiological and clinical studies have improved our understanding of IBD, animal models are essential to dissect the underlying mechanisms of IBD and are often used to test potential therapeutics. No one model recapitulates the full spectrum of disease, therefore multiple models are leveraged to recapitulate different aspects of IBD. These include infection-induced models, chemically induced models, immune mediated T cell transfer models and

genetic models. Table 1.1 summarizes some of the most studied mouse models to date, and their dependence on the microbiota[4,122].

Table 1.1: Commonly studied mouse models of intestinal inflammation

Mouse model	Site of inflammation	Mechanism of action	Influence of Microbiota
Dextran Sodium Sulfate (DSS)	Colon	Epithelial barrier disruption and immune activation by luminal contents and microbiota	Influences reproducibility, mixed dependence for pathology [123–126]
2,4,6-trinitrobenzene sulfonic acid (TNBS)	Colon	Hapten reagent that induces Th1 responses to modified self-proteins [127]	Required for pathology but probiotics reduce severity [128,129]
Oxazolone colitis	Colon	Th2 mediated colitis [130]	Unknown
IL-10 signaling deficiency (IL-10$^{-/-}$ or anti IL-10R blocking antibody)	Colon	CD4^{+} T cell driven, loss of tolerance to microbiota	Required for pathology, particularly *Helicobacter* spp. [24,131,132]
Adoptive T cell transfer (RAG$^{-/-}$/ SCID recipient)	Colon	Adoptive transfer of naïve CD4^{+} T cells that react to microbiota in the absence of Treg[133]	Required for pathology, CD4^{+} T cells respond to commensal antigens
TNFΔ^{ARE}	Ileum and colon	Global constitutive expression of TNFα. Expression on epithelial cells alone also is sufficient for ileitis[134]	Required for pathology[135]
Anti-CD40 in RAG1$^{-/-}$	Colon	Myeloid cell derived proinflammatory IL-	Independent of the microbiota[136]

		23, IL-1β and IL-12[136]	
***Citrobacter rodentium* infectious colitis**	Colon	Infection by *C.rodentium*, mimics human infection with enteropathic and enterohemorrhagic *E.coli*	*C.rodentium* requires commensals for colonic colonization[137]
***Salmonella enterica* infectious enterocolitis**	Small intestine	Infection of epithelial cells, release of proinflammatory cytokines and induction of innate and adaptive responses[138]	Germ Free mice are highly susceptible to *Salmonella* infection, microbiota aids in colonization resistance[139]

1.8.1 Helicobacter hepaticus αIL-10R driven colitis: a model of genetic susceptibility and loss of tolerance to the microbiota

In the early 1990's the first genetic knockout of IL-10 was created by Kuhn and colleagues[131]. Although initially created to investigate the function of IL-10 across the immune system, the most profound effect of IL-10 deficiency was the spontaneous induction of enterocolitis that involved the entire intestinal tract. The first hint that intestinal inflammation could be linked to the microbiota was when transfer of IL-10$^{-/-}$ mice into a specific pathogen free environment abrogated the small intestinal inflammation, however colitis persisted[131].

Following this hallmark study, Kullberg and colleagues established that colonization with a single commensal bacterium, *Helicobacter hepaticus* was sufficient to induce IL-12 and IFNγ driven colitis in genetically susceptible IL-10 deficient animals[24]. Since then, *H. hepaticus* is now commonly referred to as a "pathobiont" lending to its ability to colonize the cecum and colons of immunocompetent animals as a member of the microbiota while also driving

severe disease in mice deficient in IL-10 signaling. This is a pivotal model for IBD as IL-10 signaling defects are among the strongest factors associated with the development of infantile IBD[4]. It has since been utilized often as a model of colitis in which genetic predisposition can drive adherent immune responses to members of the microbiota, both hallmark features of IBD.

1.9 Research hypothesis and experimental aims

Commensal reactive T cells in the gut play pivotal roles in homeostasis and are perturbed within the inflammatory environment of IBD. Further, innate-like T cells act as sentinels of the microbiota and are key mediators between innate and adaptive immune responses and modulate tissue homeostasis. However, the contribution of commensal reactive, innate-like $CD8^+$ T cells in the gut has remained unexplored. Further, how these commensal reactive $CD8^+$ T cells may contribute to the immune response during inflammation in the gut is also unknown. Previous studies of H2-M3 restricted $CD8^+$ T cells to a skin commensal illuminated a wound healing signature after elicitation by a commensal exposure. Thus, we hypothesized that commensal reactive $CD8^+$ T cells would be present in the gut, a site of high antigenic exposure, with potential wound healing capabilities at homeostasis. In this thesis, we utilize the *Helicobacter hepaticus* αIL-10R model of colitis to characterize the innate-like commensal reactive $CD8^+$ T cell response in the colon during inflammation and measure the contribution of innate like $CD8^+$ T cells to pathology.

2 Materials and Methods

2.1 Mice

Specific Pathogen- free (SPF) Wildtype (WT) C57BL/6 mice were purchased from Taconic Biosciences. $K^bD^{b-/-}$ (B6.129P2-*H2-Kb*^{tm1} *H2-Db*^{tm1} N12 mice were kindly provided by Dr. D. Margulies (NIAID, NIH). WildR mice were kindly provided by Dr. B. Rehermann. H2-M3$^{-/-}$ mice were kindly provided by Dr. C. Wang (Northwestern University) and bred in house with WT C57BL/6. All mice were bred and maintained under SPF conditions at an American Association for the Accreditation of Laboratory Animal Care (AAALAC)- accredited animal facility at NIAID and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals. All experiments were performed at NIAID under an Animal Study Proposal (LHIM-2E or LHIM-3E) approved by the NIAID Animal Care and Use Committee. Sex- and age- matched mice between 6 and 15 weeks of age were used for each experiment when possible.

2.2 Mouse models

2.2.1 Helicobacter hepaticus and αIL-10R induced colitis

Frozen aliquots of *Helicobacter hepaticus* (MT140035/HH-2) were thawed to room temperature and then centrifuged at 21130 rcf for 5 min at 4°C and resuspended in PBS to an optical density measured at 600nm ($OD600_{nm}$) of 5. Mice were gavaged with $1x10^8$ colony forming units (CFU) of *Helicobacter hepaticus* (*H.h*) using a 24G needle on days 1 and 2. On days 1 and 7 mice were injected intra-peritoneally (i.p.) with 1mg of αIL-10R blocking antibody (Clone: 1B1.3A, BioXCell) in 0.2 mL of PBS. Mice were sacrificed and tissues collected at day 14 after disease initiation.

2.2.2 Dextran Sodium Sulfate model of colitis

Wildtype and H2-M3$^{-/-}$ mice were cohoused for 3 weeks prior to induction of colitis. Mice were provided drinking water with 2.3% Dextran Sodium Sulfate salt (DSS) (ref: J14489-22, ThermoFisher Scientific) for 7 days, then returned to normal drinking water for an additional 7 days. Fresh DSS was provided every 2 days. Body weights were monitored daily, and mice were sacrificed at day 14, or if they lost >20% of their initial body weight.

2.2.3 Staphylococcus epidermidis topical association

Mice were topically associated with *Staphylococcus epidermidis* (NIHLM087) with approximately 5mL of culture suspension (10^9 CFU/mL) across the entire skin surface using a sterile swab. Commensal association was repeated every other day for four times total. Mice were sacrificed and tissues were analyzed at day 14 post association, unless otherwise indicated.

2.2.4 S. epidermidis gut colonization

Staphylococcus epidermidis (NIHLMO87) culture was centrifuged at 2916 rcf for 5 min at 4℃ then resuspended in PBS to an $OD600_{nm}$ = 10. Mice were gavaged with 200uL of *S. epidermidis* using a 24G needle for three consecutive days.

2.2.5 Staphylococcus aureus topical association

Mice were topically associated with *Staphylococcus aureus* (42F02) with approximately 5mL of culture suspension (10^9 CFU/mL) across the entire skin surface using a sterile swab. Commensal association was repeated every other day for four total times. Colitis was induced as described above (Section 2.2.1). Mice were sacrificed and tissues were analyzed at day 21 post association.

2.2.6 Adoptive Transfer of commensal reactive T cells

SA1^{Tg} and Bowie^{Tg} mice were backcrossed to a CD45.1 *Rag1*$^{-/-}$ background to limit dual TCR expression and facilitate transferred cell identification. 0.5-1x10^{5} SA1^{Tg} CD4^{+} T cells or Bowie^{Tg} CD8^{+} T cells were transferred to CD45.2 recipient mice by intravenous injection in the tail vein one day before the first topical association with *S. epidermidis* (NIHLM087) or *S. aureus* (42F02).

2.2.7 Vancomycin treatment

Mice were treated with 0.5g/L Vancomycin (ref: 94747-5G Sigma-Aldrich) with 3g/L sweetener (Splenda) in the drinking water throughout the course of topical association with *S. epidermidis*. Water was refreshed every two days.

2.3 Microbial culture

2.3.1 Helicobacter hepaticus

Helicobacter hepaticus (MT140035/HH-2) (*H.h*) was grown on non-pre-reduced blood agar plate (TSA with 5% Horse Blood) (ref. B21372X Fischer Scientific) at 37°C for 5-7 days under microaerophilic atmosphere using a GasPak 100 (ref. 260627 BD BBL) and gas mix (85% N_2, 10% H_2, 5% CO_2). *H.h* was harvested with a sterile cotton swab and transferred to a vented Erlenmeyer flask (ref. 89098-258 VWR) containing Tryptic Soy Broth (TSB) (ref. CUS-0279 KD Medical) with 10% Fetal Bovine Serum (FBS) (ref. 89510-186 VWR) and Skirrow Campylobacter Selective Supplement (ref. OXSR0069E Fischer Scientific). The liquid culture was incubated at 37°C under microaerophilic atmospheric conditions for up to a week shaking at 146rpm, by doubling the culture volume every 2-3 days. *H.h* was then collected, and optical density was measured at 600nm ($OD600_{nm}$). The culture was centrifuged at 10,000 rpm for 25 min at 4°C and resuspended in TSB (without supplement) to an $OD600_{nm}$ =10. An equal volume

of 50% filtered sterilized glycerol was added to the bacterial suspension, and aliquots were stored in 1.5mL tubes at -80°C until use.

2.3.2 Staphylococcus epidermidis

Staphylococcus epidermidis (NIHLM087) was streaked on a Columbia blood agar (ref. R01217 Fischer Scientific) (5% Sheep Blood) at 37°C, over-night. Colonies were picked and resuspended in 3 mL of TSB (ref. CUS-0279 KD Medical) to obtain a suspension at $OD600_{nm}$ =~0.3. 1.9 mL of the bacterial suspension was transferred to a vented Erlenmeyer flask (ref. 89098-258 VWR) containing 50mL TSB and incubated at 37°C for 4-8 hr until the culture reached an $OD600_{nm}$ =~0.8.

2.3.3 Staphylococcus epidermidis- Ova

Staphylococcus epidermidis-Ova was streaked on TSA containing 10ug/mL chloramphenicol (ref. C0378 Sigma) at 37°C, over-night. Colonies were picked and culture grown as stated above for *S. epidermidis* and carried out in TSB containing 10ug/mL chloramphenicol.

2.3.4 Staphylococcus aureus

Staphyloccocus aureus (42F02) was cultured in by inoculating TSB and incubating the culture for 4 hr at 37°C with shaking at 200 rpm.

2.4 Minimum inhibitory concentration (MIC) assay

A stock concentration of vancomycin (ref: 94747-5G Sigma-Aldrich) was prepared in Mueller Hinton (MH) Broth II pH (+/- .2) (ref 90922 Sigma). 100μl per well of MH broth was added to a sterile 96 round bottom well plate, and serial dilutions of vancomycin were created ranging from 0.025-128 μg/mL by transferring 100μl of antibiotic, with MH broth alone as the negative control (Table 2.1). *S. epidermidis* (NIHLM087) was grown and prepared to a McFarland 0.5 in MH broth

(~$OD600_{nm}$=0.125). 10 μl (~$5x10^5$ CFU/mL) of bacterial solution was added to each well except for the negative control column and the plate was incubated at 37°C for 16-20 hours and results compared to CLSI/ EUCAST guidelines to determine MIC.

Table 2.1: Setup to assess MIC of *S. epidermidis* against vancomycin

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	well
Broth	Broth + *S.epidermidis*	0.025	0.5	1	2	4	8	16	32	64	128	ug/mL

2.5 16S rRNA gene profiling

2.5.1 Sample processing and sequencing

Fecal pellet and ileum sample DNA was extracted using the MagAttract PowerMicrobiome DNA/RNA kit (Qiagen). 16*S* V4 rDNA libraries were created using 100ng of DNA as a template, V4 region-targeting primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Amplicons were sequenced on a MiSeq (Illumina) using the v3 MiSeq Reagent Kit (Illumina) by the NIAID Microbiome Core facility. Analysis of the data was performed by QIIME2 DADA2 algorithm and SILVA database (v138.1) was used for taxonomic assignments of produced ASVs.

2.5.2 Quality Control

QC revealed a starting dataset with 51 samples represented by 477 ASVs (106 species). All samples and 312 ASVs (90 species) were retained after sequence depth filtering (at 55000 reads/sample), ASV presence at 0.025% relative

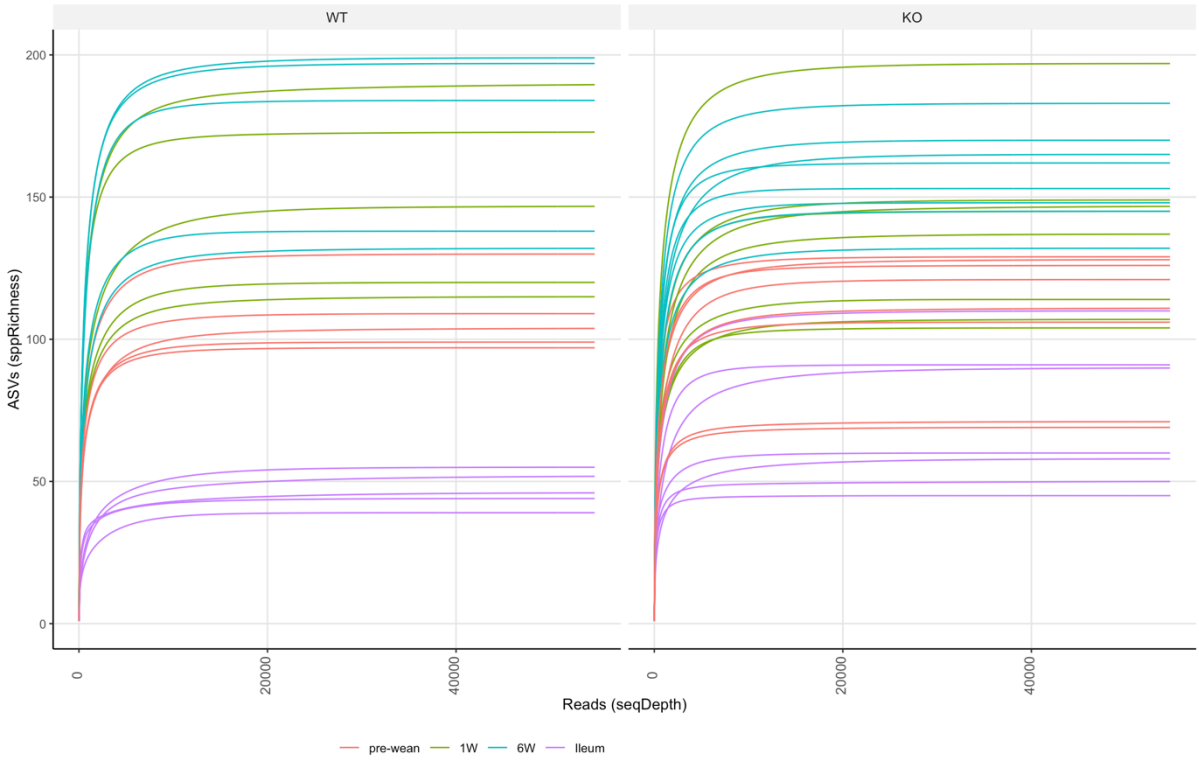


Figure 2.1: QC seqDepth of reads

abundance and ASV prevalence of 10% across all samples (5 samples). (Figure 2.1).

2.6 Tissue Processing

2.6.1 Cell isolation from colon

Colons and cecum were excised and opened longitudinally, and rigorously shaken in PBS. Tissue was cut into 2 cm sections and placed in a 50 mL beaker with complete RPMI 1640 media (2mM L-glutamine, 1mM sodium pyruvate, 1mM non-essential amino acid, 50uM β-mercaptoethanol, 20mM HEPES, 100U/ml of penicillin, 100mg/mL streptomycin) with 5 mM EDTA and 0.145 mg/ml dithiothreitol for 20min at 37°C with a stirbar and constant spinning. To remove epithelial cells, tissue was shaken in a 50mL conical tube containing complete RPMI (no FBS) supplemented with EDTA three times and strained using a tea strainer. Tissue was returned to the beaker and minced using scissors. 10 mL of

complete medium containing 500 mg/mL DNase I (Sigma-Aldrich) and 100 mg/ml Liberase TL (Roche) with continuous stirring at 37°C for 25min. Digestion was stopped with complete RPMI supplemented with 3% FBS, and tissue was strained through a 70μm filter and single cells suspension centrifuged at 1500rpm for 5min at 4°C. Cells were resuspended in 4mL of 37% Percol and spun at 1800rpm for 5min at 4°C, cell pellet was then resuspended in media and used for downstream analysis.

2.6.2 Cell isolation from skin

Ear pinnae were excised and separated into ventral and dorsal sheets then digested in complete RPMI 1640, supplemented with 0.25 mg/mL of Liberase TL purified enzyme blend (Roche). Tissue was digested for 90 min at 37°C and 5% CO_2, and then processed using the Medicon/Medimachine tissue homogenizer system (BD) and brought to a single cell suspension after filtration through a 50μm cell strainer.

2.6.3 Cell isolation from spleen and lymph nodes

Cells from peripheral lymph nodes and spleen were isolated by mashing tissue through a 70μm cell strainer to generate single cell suspensions unless otherwise noted.

2.7 In-vitro re-stimulation

For detection of basal cytokine potential, single-cell suspensions from various tissues were cultured directly *ex vivo* in a 96-well U-bottom plate in complete RPMI 1640 supplemented with 10% FBS and stimulated with 50 ng/mL of phorbol myristate acetate (PMA) (Sigma-Aldrich) and 5 mg/mL of ionomycin (Sigma-Aldrich) in the presence of brefeldin A (1:1000, GolgiPlug, BD Biosciences) for 2.5

hours at 37°C in 5% CO_2. After stimulation, cells were assessed for intracellular cytokine production as described below.

2.8 Flow cytometry

2.8.1 Tetramer staining

Single cell suspensions were incubated with titrated concentrations of tetramer in addition to anti-CD16/32, to minimize non-specific binding. Staining of spleens was carried out in 15mL conical tubes, all other tissues were stained in 96 well U bottom plates, for 1 hour at room temperature in complete RPMI 1640 media supplemented with 10% FBS. Cells were washed and then used for downstream enrichment or further surface staining.

2.8.2 Surface and intracellular staining

Single cell suspensions were plated into 96 well U bottom plates for staining, all centrifugation steps were at 1500rpm for 5min and counting beads were added to wells for cell number quantification. All surface antibody mixes (Table 2.2) were prepared in Hanks Balanced Salt Solution (HBSS) in addition to Rat gamma globulin and anti-CD16/32 to minimize non-specific binding and incubated for 25min on ice. Cells were then washed and centrifuged. If necessary, intracellular staining was carried out using the FoxP3/Transcription factor staining buffer kit (eBioscience) and intracellular staining antibody mixtures (Table 2.2) were prepared in permeabilization buffer (eBiosciences) and incubated at 4°C overnight. Otherwise, cells were fixed with BD Cytofix/Cytoperm (BD Biosciences) for 30 min on ice. All cells were washed twice after staining and resuspended in HBSS for acquisition.

Table 2.2: Antibodies

Primary antibody	Fluorochrome	Clone	Manufacturer
CD3	BUV737	17A2	BD Bioscience
CD3	BV650	17A2	Biolegend
CD4	BV510	RM4-5	Biolegend
CD4	BV570	RM4-5	Invitrogen
CD4	BV650	RM4-5	Biolegend
CD8α	APC	53-6.7	Invitrogen
CD8α	BV480	53-6.7	BD Bioscience
CD8α	PE-CF594	53-6.7	BD Bioscience
CD8β	BV650	H35-17.2	BD Bioscience
CD8β	PerCP-eF710	H35-17.2	Invitrogen
CD8β	BV605	H35-17.2	BD Bioscience
CD11b	eF450	M1/70	Invitrogen
CD11b	BUV737	M1/70	BD Bioscience
CD19	eF450	eBio1D3	Invitrogen
CD19	NovaFlourYellow 730	eBio1D3	Invitrogen
CD39	PE-Fire700	Duha59	Biolegend
CD44	AF700	IM7	Invitrogen
CD44	BUV805	IM7	BD Bioscience
CD45	APC-Cy7	30-F11	Invitrogen
CD45	BUV395	30-F11	BD Bioscience
CD45.1	BV510	A20	Biolegend
CD45.2	APC AF780	104	Invitrogen
CD49a	BV750	Ha31/8	BD Bioscience

CD62L	BUV615	MEL-14	BD Bioscience
CD62L	BUV563	MEL-14	BD Bioscience
CD69	PE-Cy5	H1.2F3	Biolegend
CD90.1	BV605	OX-7	Biolegend
CD90.2	BV605	53-2.1	Biolegend
CD90.2	BV785	30-H12	Biolegend
CD103	BUV496	M290	BD Bioscience
Eomes	eF450	Dan11mag	Invitrogen
FOXP3	eF660	FJK-16s	Invitrogen
FOXP3	AF700	FJK-16s	Invitrogen
GATA3	APC	TWAJ	Invitrogen
GATA3	Alexa488	TWAJ	Invitrogen
Granzyme B	APC Fire750	QA16A02	Biolegend
Helios	PE-eF610	22F6	Invitrogen
IFNγ	BV605	XMG1.2	Biolegend
IFNγ	FITC	XMG1.2	Invitrogen
IFNγ	APC	XMG1.2	Invitrogen
IL-10	BV711	JES5-16E3	BD Bioscience
IL-17A	BV421	TC11-18H10.1	Biolegend
IL-17A	PE-Cy7	TC11-18H10.1	Biolegend
IL-22	APC	IL22JOP	Invitrogen
Ki67	AF532	SolA15	Invitrogen
Ly6C	Alexa488	HK1.4	Invitrogen
Ly6G	PE	1A8	BD Bioscience
MHC-II	eF450	M5/114.15.1	Invitrogen
MHC-II	NovaFluor Blue 610-70S	M5/114.15.1	Invitrogen

NK1.1	BUV661	PK136	BD Bioscience
NK1.1	eF450	PK136	Invitrogen
NK1.1	Pe-TxR	PK136	BD Bioscience
NKG2A/CE	BUV615	20d5	BD Bioscience
RORγT	PE-CF594	Q31-378	BD Bioscience
RORγT	PE	B2D	Invitrogen
T-bet	PE-Cy7	4B10	Biolegend
T-bet	BV421	4B10	Biolegend
TCR β	BUV737	H57-597	BD Bioscience
TCR β	PerCP Cy5.5	H57-597	Invitrogen
TCR $\gamma\delta$	eF450	GL-3	Invitrogen
TCR $\gamma\delta$	Pe-TxR	GL-3	BD Bioscience
TCR $\gamma\delta$	SB780	GL-3	Invitrogen
TNFα	AF700	MP6-XT22	BD Bioscience

2.8.3 Acquisition and analysis

Flow cytometric acquisition was performed on a BD Fortessa X-20 utilizing or Cytek Aurora following bead based (BD Biosciences) compensation. Data was analyzed using FlowJo v10 software.

2.9 Pathology and Immunohistochemistry

Mice were euthanized at day 14 and tissues harvested. The cecum and colon from each animal were submitted to the IDPS laboratory in 10% neutral buffered formalin. A longitudinal section of the entire cecum and a cross section of the ascending colon approximately 1.0-1.5 cm from the cecum/cecal-colic junction were made and placed in tissue cassettes. Tissue cassettes were processed and embedded in paraffin according to established protocols, sectioned at 5 μm, and

stained with hematoxylin and eosin (H&E). Sections were evaluated in a blinded fashion by the same pathologist (D.A.A), and a combined score of what was seen in the cecum and ascending colon, respectively, was assigned based on the following scoring system.

- Inflammatory infiltrate: 0 (none); 1 (increased presence of inflammatory cells); 2 (infiltrate also in submucosa); 3 (transmural)
- Goblet cell loss: 0 (none); 1 (<10%); 2 (10-50%); 3 (>50%)
- Crypt density: 0 (normal); 1 (decreased by <10%); 2 (decreased by 10-50%); 3 (decreased by >50%)
- Crypt hyperplasia: 0 (none); 1 (slight increase in crypt length); 2 (2-3-fold increase in crypt length); 3 (>3-fold increase in crypt length)
- Muscle thickening: 0 (none); 1 (slight); 2 (strong); 3 (excessive)
- Submucosal inflammation: 0 (none); 1 (individual cells); 2 (infiltrate(s)); 3 (large infiltrates(s))
- Crypt abscess: 0 (absent); 3 (present)
- Ulceration: 0 (absent); 3 (present)

  NOTE This scoring table was adapted from Koellink PJ, Wildenberg ME, Stitt LW, et al. *J Chron's and Colitis* (2018) [140]

An average mucosal thickness for each section was also annotated. Using digital camera software, linear measurements were made from the base of the crypt nearest the muscularis mucosa to the mucosal surface. Three random areas from

each cecum and ascending colon were measured and the average mucosal thickness was calculated for each. Additionally, serial sections of cecum and colon were made for immunohistochemistry for T-cells (CD-3), cytotoxic/suppressor T-cells (CD8), B-cells (B220), IBA-1 (for macrophages and/or dendritic cells), myeloperoxidase/MPO (for granulocytes, specifically neutrophils), and Ki67 nuclear staining.

The rabbit anti-Ki67 polyclonal antibody (Abcam, ab15580, lot GR3452706-1) was diluted to 1:250 and applied in a standard protocol on the Leica Bond RX auto-staining platform. Epitope retrieval was performed using the citrate based ER1 reagent (Leica Biosystems, AR9961). Detection with DAB chromogen was completed using the Bond Polymer Refine Detection kit (Leica Biosystems, DS9800). Slides were finally cleared through gradient alcohol and xylene washes prior to mounting and cover slipping. Sections were examined by light microscopy using an Olympus BX51 microscope and photomicrographs were taken using an Olympus DP28 camera.

Image analysis was performed on the *H. hepaticus* infected tissues to provide quantitative analysis and show the amount of staining per area of analysis. The images were digitized on a bright-field Leica Aperio AT2 slide scanner with 40x magnification. The Images were uploaded through eslide manager which is a web-based digital pathology information management system used for digital slide viewing and image analysis. The Aperio Color Deconvolution V9 base algorithm was tuned to recognize a target chromogen (in this case, 3,3′-Diaminobenzidine, DAB). After tuning, and adjusting the parameters of the algorithm, the settings were saved as a macro, which was used for all the

slides. Following image capture, the algorithm was applied to detect Ki67 nuclear immunostaining with DAB chromogen in individual mucosal epithelial cells and quantified based on the intensity of positive nuclear immunostaining.

2.10 Tetramer based Stem Cell PE and APC positive enrichment

Tissues were prepared as described above and single cell suspensions stained for 1 hour at room temperature with tetramer (PE or APC) in complete RPMI 1640. Samples were then magnetically enriched for bead-bound cells utilizing EasySep PE or APC Positive Selection Kit II (StemCell) and an EasySep Multistand (StemCell) as described in[141].

2.11 Co-culture assay

$CD45^+$ $CD3^+$ $CD90.2^+$ $CD8\beta^+$ T cells (> 95% purity) were FACsorted from the colon lamina propria tissue or mesenteric lymph nodes of $K^bD^{b-/-}$ mice 14 days after colitis induction with *H.h* αIL-10R using a FACSAria cell sorter. For splenic DC purification, single-cell suspensions from the spleen of congenic CD45.1 wild-type mice were magnetically enriched for $CD11c^+$ cells by positive selection using CD11c MicroBeads and MACS separation columns (Miltenyi Biotec). Purified splenic dendritic cells (SpDC) and $CD8\beta^+$ T cells were co-cultured at a 15:1 ratio (~$5x10^3$ $CD8\beta^+$ T cells- if possible) in a 96-well U-bottom plate in complete medium for 16 hours at 37°C in 5% CO_2. Brefeldin A (GolgiPlug) was added for the final 4 hours of culture. fMet peptides (Genscript) were added with purified $CD8\beta^+$ T cells at a final concentration of 15 µg/ml. $CD8\beta^+$ T cell cytokine production following co-culture was assessed by flow cytometry after surface and intracellular cytokine staining.

2.12 Analysis of published datasets

Analysis of RNA-seq from [142] Heuberger et al., *Mucosal Immunol (2023)* and microarray gene expression from[143] Ilott et al., *ISME (2016*) was conducted using the MIG Transcriptome Explorer R package. Analysis of NICHE-seq dataset from[144] Gu et al., *BioRxiv* was conducted by using the CZ CELLxGENE suite hosted by the Wellcome Sanger Institute: https://cellxgene.cziscience.com/. Accessed (September 2023).

2.13 Quantification and Statistical Analysis

Statistical analysis was performed using Prism v9.1 (GraphPad Software Inc., La Jolla, CA, USA). Statistical details of experiments can be found in figure legends.

3 Assessing the roles of H2-M3 restricted and classical CD8$^+$ T cell responses in the large intestine during homeostasis and inflammation

3.1 Introduction

The intestinal lamina propria and epithelial compartments are densely populated by CD8$^+$ T cells, however, the exact functions and specificities of these cells remains largely unknown. Although multiple studies have reported CD8$^+$ T cell signatures in the tissues and circulating PBMC of patients with IBD[107,119,145], there are limited known causal roles for CD8$^+$ T cells in disease development or perpetuation. However, utilizing mouse models of autoreactive CD8$^+$ T cells, some studies suggest that CD8$^+$ T cells might be initiators of inflammation[113,114]. Further, in the absence of iNKT cells and dysregulated IFNγ production, NK1.1$^+$ CD8$^+$ T cells can mediate colitis[115] .Thus, further exploration into the exact functions of CD8$^+$ T cells in IBD is needed.

MHC-I molecules have evolved to present a multitude of different antigens to T cells, and only the most recently evolved MHC-Ia molecules are believed to present a highly diverse set of peptides to CD8$^+$ T cells. More ancient MHC-Ib molecules have been sustained throughout evolution and can present non-canonical antigens such as microbial metabolites, lipids, and modified peptides to MHC-Ib restricted cells[39]. Notably, some innate-like CD8$^+$ T cells are restricted by “middle aged” MHC-Ib molecules, such as Qa-1 and H2-M3 and can rapidly respond to antigen, bridging innate and adaptive responses[39,41,146]. There has been little investigation into the contributions or functions of innate-like MHC-Ib restricted CD8$^+$ T cells in the colon at steady state, or during models of IBD, despite the high antigenic load from the commensal microbiota. However,

Dasgupta et al., reported that a Qa-1^{b} restricted CD8^{+} T cell population residing in the colon expressing the CD8$\alpha\alpha$ homodimer is capable of conferring protection in the dextran sodium sulfate (DSS) model of colitis[147]. Further, skin epithelium resident, commensal specific H2-M3 restricted CD8^{+} T cells bear a tissue repair phenotype[58,60,61], however their residence in the colon, potential impact on tissue physiology or contribution to inflammation during colitis, has not yet been investigated.

Additionally, studies investigating the function of H2-M3 restricted CD8^{+} T cells during infection have suggested potential cross reactivity with microbiota derived antigens[148] yet the potential impact of these commensal reactive cells on the microbiota has not been assessed.

3.2 Aim

In this chapter, we describe the landscape of the large intestine in the presence or absence of H2-M3, or MHC-Ia molecules, in both homeostasis and during acute models of colitis. Further, we also investigate the impact of H2-M3 genotype on the gut microbiota composition and hypothesize potential downstream consequences for tissue physiology.

3.3 Results

3.3.1 Classically restricted CD8^{+} T cells dominate at steady state in the large intestine lamina propria and intraepithelial compartments

To understand the impact of H2-M3 restricted CD8^{+} T cells on the large intestine architecture at steady state, we first analyzed the gross histology of the proximal colon and cecum of wildtype, H2-M3$^{-/-}$ and H2K^{b}D$^{b\ -/-}$ animals which are devoid of classically restricted CD8^{+} T cells. These sections of tissue were selected because they are the primary site of infection during *Helicobacter hepaticus*

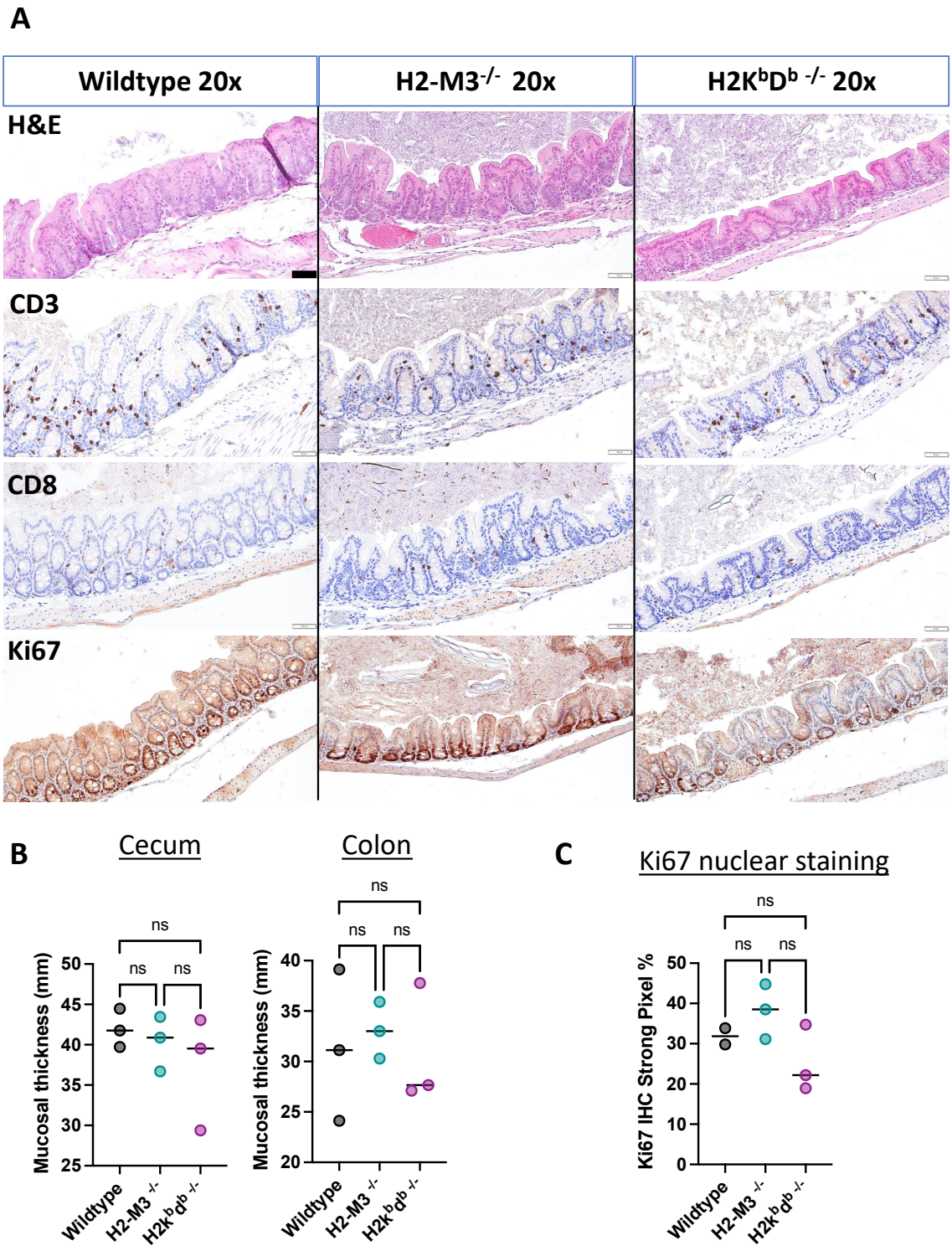


Figure 3.1: MHC-I expression does not affect colon or cecum architecture at steady state. A. Gross histology of cecum of naïve Wildtype (n=3), H2-M3$^{-/-}$ (n=3) and H2K^{b}D$^{b-/-}$ (n=3) animals **B.** Quantification of mucosal thickness of cecum and colon. **C.** Quantification of Ki67 IHC pixel strength in colon. Scale bar = 50μm. Data representative of 1 experiment.

ileocolitis[132] and thus were used as a baseline. Hematoxylin and eosin (H&E) staining showed no major histological changes between groups (Figure 3.1 A) nor significant differences in the mucosal thickness in either the cecum or proximal

colon (Figure 3.1 B). Immunohistochemistry staining of CD3 in the cecum revealed comparable densities of total T cells within the tissue for all genotypes (Figure 3.1 A). CD8 staining also showed comparable numbers of $CD8^+$ cells in wildtype and H2-$M3^{-/-}$ mice, with fewer but detectable $CD8^+$ cells in the $H2K^bD^{b\ -/-}$ animals suggesting the presence of a small population of MHC-Ib restricted $CD8^+$ T cells in the colon at steady state (Figure 3.1 A). Because H2-M3 restricted, commensal reactive $CD8^+$ T cells have a tissue regeneration and wound healing function in the skin epithelium, we also performed Ki67 nuclear staining to assess cellular proliferation in the absence of H2-M3 to assess whether these cells might have an impact on epithelial proliferation at steady state. Ki67 nuclear staining revealed no significant differences between all genotypes (Figure 3.1 A and C). Taken together, these results suggest no dominant effect of H2-M3 nor $H2K^b$ or $H2D^b$ restricted $CD8^+$ T cells on the large intestine architecture at steady state. Additionally, in the absence of MHC-Ia, there remains a detectable population of $CD8^+$ T cells in the colon during homeostasis.

Our histological analysis of the cecum and proximal colon restricted us from getting a holistic view of the entire tissue immune landscape and therefore we also assessed the cellular composition of the large intestine by flow cytometry. For these experiments, the colon and cecum were processed together and both the lamina propria and epithelial compartments were analyzed. In the lamina propria, we observed no noticeable differences in the frequency or cell number of $CD4^+$ or $CD8\beta^+$ T cells between wildtype and H2-M3 deficient animals (Figure 3.2 A). $CD4^-$ $CD8\beta^-$ T cells, which comprise, in part, a population of commensal reactive, MHC-II restricted T cells in the colon LPL were also unchanged[149]. In $H2K^bD^{b\ -/-}$ mice, there was a significant reduction in the $CD8\beta^+$ T cell population by

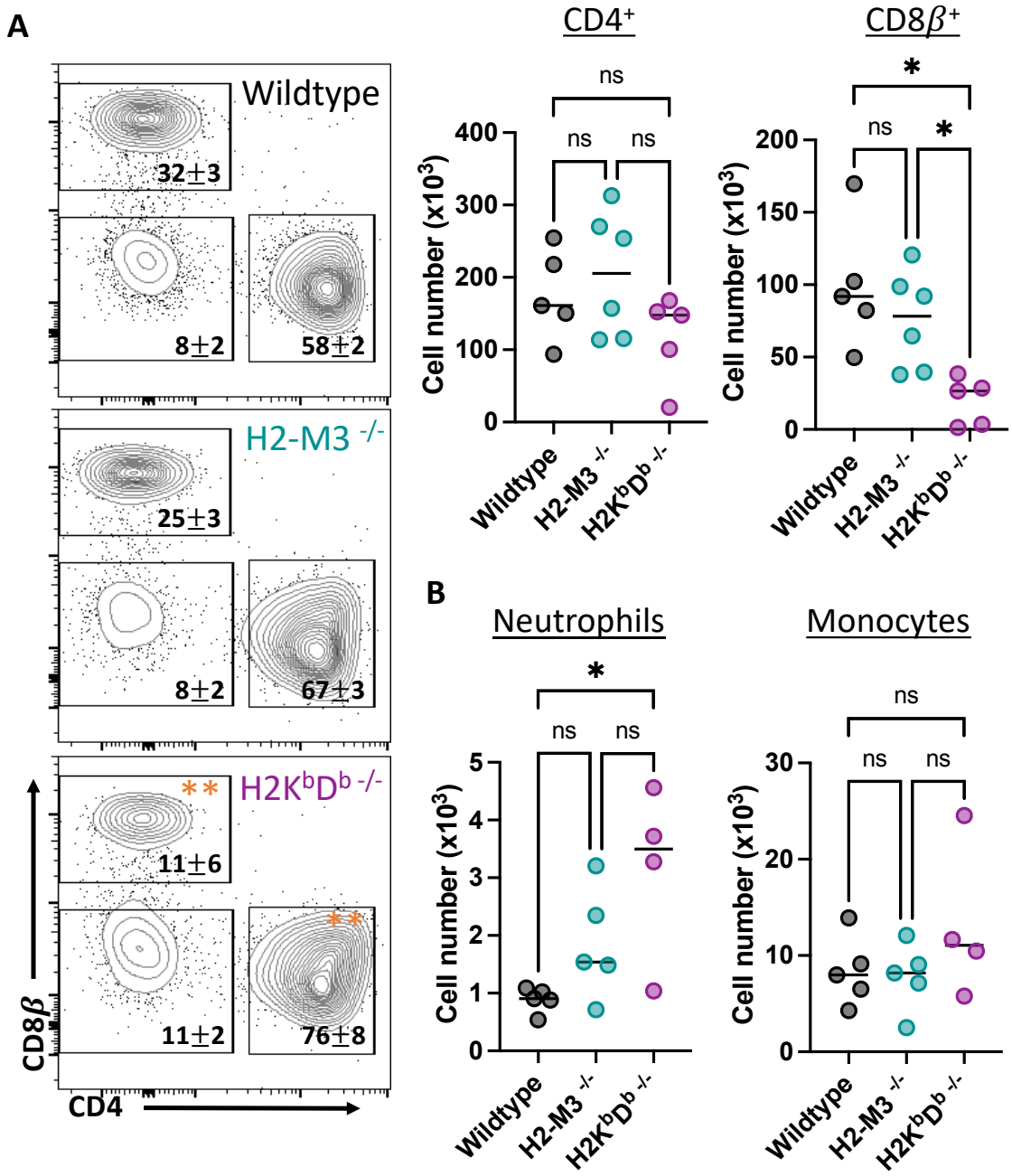


Figure 3.2: MHC-Ia restricted CD8⁺ dominate the murine large intestine at steady state. A. Frequencies and numbers of CD4⁺ and CD8β^+ T cells in large intestine (LI) lamina propria of naïve Wildtype (WT) (n=5), H2-M3$^{-/-}$(n=6), K^{b}D$^{b-/-}$(n=5) mice. Plots are gated on Live CD45⁺ Thy1.2⁺ TCRβ^+. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Stars indicate significance compared to wildtype **B.** Numbers of Neutrophils and Monocytes in LI lamina propria of indicated mice. Monocytes were identified by Live CD45⁺ CD11b⁺ Ly6C⁺ and Neutrophils further identified by LY6G⁺ expression. One Way ANOVA was used to measure significance * p<0.05, ** p<0.01. Data representative of 1 experiment.

both frequency and cell number, indicating that at steady state, the large intestine lamina propria is dominantly populated by CD8β^+ T cells restricted by MHC-Ia molecules, although there remain a few MHC-Ib restricted CD8⁺ T cells (Figure 3.2 A).

We also assessed the impact of H2-M3 or $H2K^bD^b$ deficiency on the innate populations of neutrophils and monocytes in this tissue which may indicate low-level inflammation. $H2\text{-}M3^{-/-}$ animals exhibited no significant difference in neutrophil or monocyte populations compared to wildtype, however, $H2K^bD^{b\ -/-}$ mice displayed a significant increase in neutrophils (Figure 3.2 B). Although histological analysis did not show an increase in neutrophils in $H2K^bD^{b\ -/-}$ animals, this more sensitive measurement revealed the possibility that classically restricted $CD8^+$ T cells might restrain low level inflammation in the colon LPL, or that non-classically restricted $CD8^+$ T cells promote it. Further, MHC-I is also a canonical inhibitory signal for Natural Killer (NK) cells. Thus, loss of MHC-I in the colon might unleash NK cells, which could account for this slight increase in neutrophil infiltration.

To interrogate this further, we assessed the cytokine potential and transcription factor expression of the $CD8^+$ T cell compartment in these mice. Under steady state conditions, the majority of $CD8^+$ T cells in all genotypes produced the type 1 cytokines IFNγ and TNFα, with low expression of Granzyme B indicating minimal cytotoxic potential at homeostasis (Figure 3.3 A). A large subset of commensal reactive, H2-M3 restricted $CD8^+$ T cells in the skin express IL-17A contributing to their wound healing and tissue repair phenotype in this tissue[58,60,61]. Therefore, we also assessed the IL-17A production of $CD8^+$ T cells in the absence of H2-M3 and classically restricted $CD8^+$ T cells.

In all genotypes, there was a population of IL-17A producing $CD8^+$ T cells in the large intestine lamina propria at steady state, however, the frequency was unchanged in both $H2\text{-}M3^{-/-}$ and $H2K^bD^{b-/-}$ animals which suggests a tissue

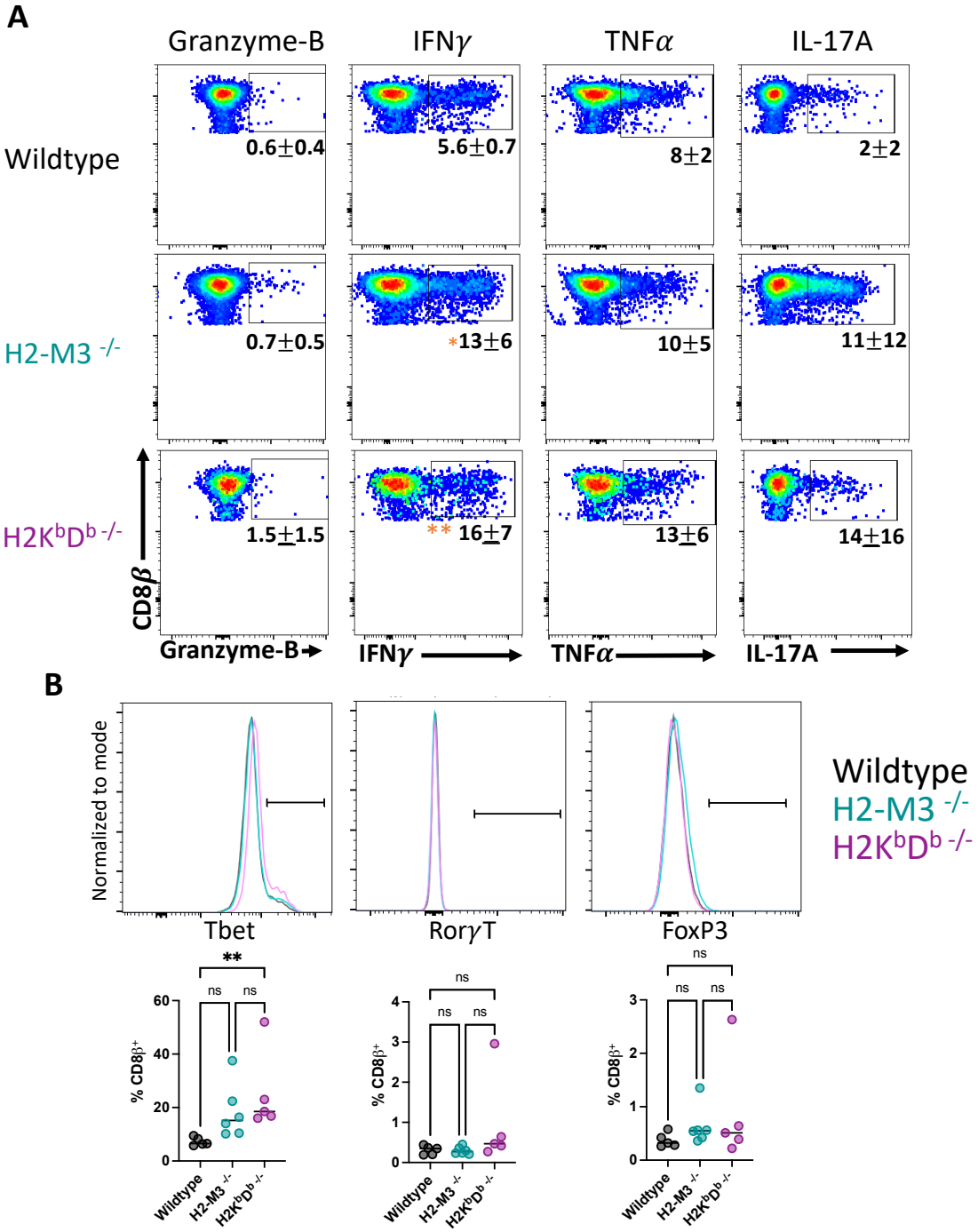


Figure 3.3: Absence of MHC-I impacts cytokine potential of CD8⁺T cells in LI LPL. A. Frequencies of cytokine production of Granzyme-B⁺, IFNγ^+, TNFα^+, or IL-17A⁺ CD8β^+T cells isolated from the LI lamina propria of naïve Wt, H2-M3$^{-/-}$, and K^{b}D$^{b-/-}$ animals. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Significance compared to Wt control populations **B.** Expression of T-bet, RorγT and FoxP3 by CD8β^+T cells in LI lamina propria. One Way ANOVA was used to measure significance * p<0.05, ** p<0.01. Data representative of 1 experiment.

specific cytokine profile, more so than an MHC restriction skewing as is seen with other innate like T cells[150] (Figure 3.3 A). Interestingly, in the absence of both H2-M3 and H2K^{b}D^b, CD8$^+$ T cells displayed significantly increased IFNγ production

compared to wildtype CD8$^+$ T cells (Figure 3.3 A). Non-classically restricted CD8$^+$ T cells are reported to be "preset"[151], therefore it is possible that in the H2K^{b}D$^{b-/-}$ animals, this is a result of the heightened activation state of the residual MHC-Ib restricted CD8$^+$ T cell population. However, H2-M3$^{-/-}$ animals are still replete with classically restricted CD8$^+$ T cells thus, it is also conceivable that expression of broad Class I molecules may play a regulatory role that is not yet understood. In line with these observations, the transcription factor that governs Type 1 cytokine production Tbet[152], was elevated in CD8$^+$ T cells from H2K^{b}D$^{b-/-}$ animals with a non-significant increase in H2-M3$^{-/-}$ mice (Figure 3.3 B).

Transcription factors FOXP3 and RORγT were not expressed in this population compared to CD4$^+$ FOXP3$^+$ T_{reg} and RORγT$^+$ Th17 T cells (data not shown). These results indicate that classically and non-classically restricted CD8$^+$ T cells exhibit a Type 1 cytokine profile in the large intestine lamina propria at steady state and express the transcription factor Tbet. Further, they suggest that in the absence of MHC-Ia restricted CD8$^+$ T cells, there is a slight inflammatory signature, potentially due to the increased production of IFNγ by non-classically restricted CD8$^+$ T cells. These results are preliminary and would need to be repeated with more mice to confirm the findings.

The intestinal epithelium is densely populated with CD8$^+$ T cells. CD8$^+$ IEL can be largely classified by the expression of either the CD8$\alpha\beta$ or CD8$\alpha\alpha$ co-receptors. Overall, H2-M3 and H2K^{b}D^b deficiency did not affect the relative frequencies of CD8$\alpha\beta$ or CD8$\alpha\alpha$ IEL, however, in the absence of H2K^{b}D^b, the numbers of CD8$^+$ IEL were significantly decreased (Figure 3.4). This was

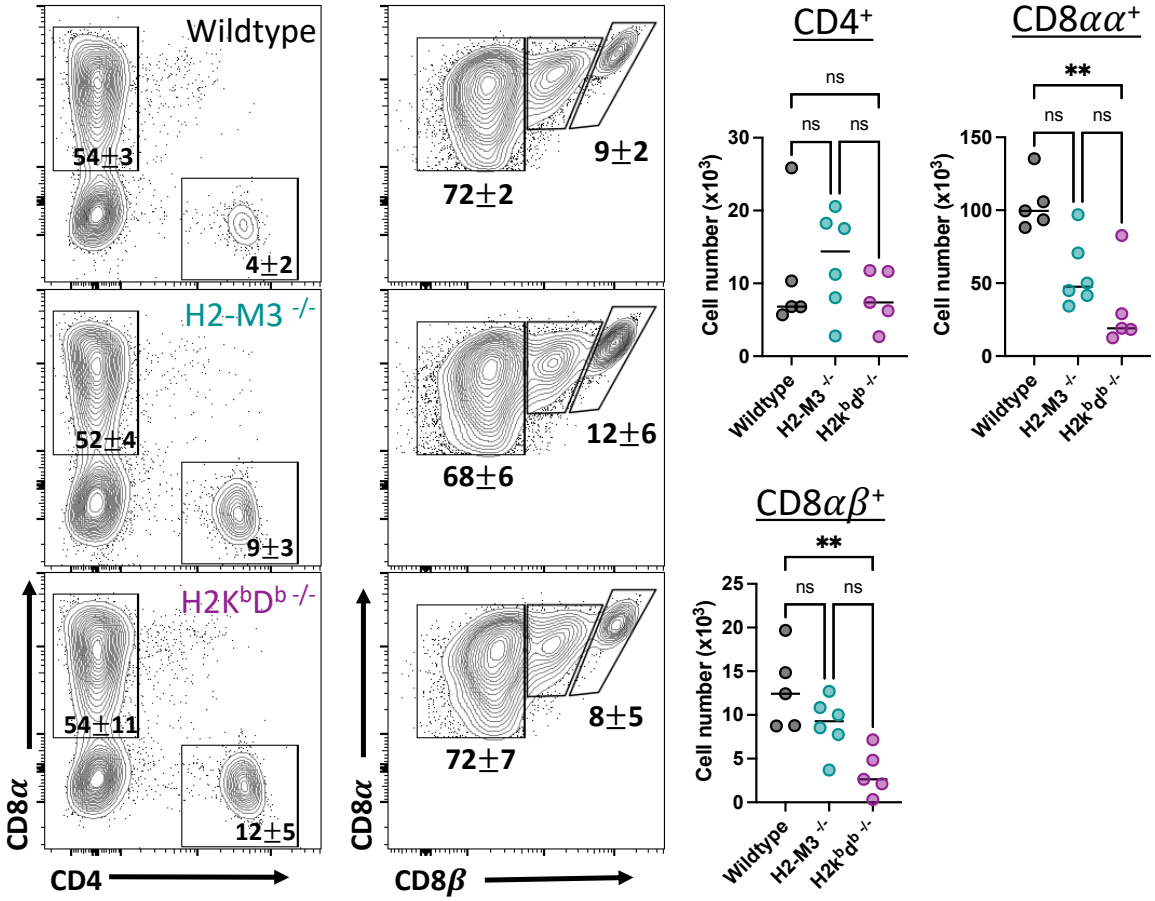


Figure 3.4: Majority of CD8 intra-epithelial lymphocytes are dependent on MHC-Ia expression at steady state. Frequencies and numbers of CD4$^+$, CD8$\alpha\alpha^+$, CD8$\alpha\beta^+$ T cells in LI epithelium of WT, H2-M3$^{-/-}$, K^{b}D$^{b-/-}$ mice. Plots are gated on Live CD45$^+$ TCRβ^+. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. One Way ANOVA was used to measure significance ** p<0.01. Data representative of 1 experiment.

distributed across both CD8$\alpha\beta^+$ or CD8$\alpha\alpha^+$ populations indicating that the majority of CD8$^+$ IEL arise from a classically restricted precursor. These results deviate from a previously published study indicating that only CD8$\alpha\beta^+$ IEL are affected by H2K^{b}D^b deficiency[153].

These results collectively illustrate that MHC-Ia restricted CD8$^+$ T cells dominantly occupy both the large intestine lamina propria and epithelium at steady state. However, in the absence of H2K^{b}D^b, non-classically restricted CD8$^+$ T cells, likely comprised of H2-M3 or Qa-1 or Qa-2 restricted cells, can still populate these tissues to a much smaller degree. The exact functions of these cells remain to be elucidated. More detailed analysis of surface activation

markers, cytokine production, or RNA sequencing would aid in functional understanding.

3.3.2 H2-M3 deficiency affects microbiome composition

H2-M3 selectively presents 5-6 amino acid long, *N*-formyl (fMet) containing, peptides that are produced by both prokaryotes and mitochondria[79,154]. Studies investigating H2-M3 restricted $CD8^+$ T cells in the context of infection indicated a role in controlling primary infection with intracellular pathogens with a dispensable role during reinfection[84,85] . Notably, Lenz and Beven reported fMet specific cytotoxic $CD8^+$ T cells in infection-naïve animals suggesting potential cross reactivity with antigens from the commensal microbiota[148]. Similarly, recent studies investigating these responses in the skin illustrated their ability to respond to a non-inflammatory exposure to a commensal[60]. However, the direct impact of H2-M3 on the commensal microbiota has not yet been elucidated.

To assess the potential impact of H2-M3 restricted $CD8^+$ T cells on the gut microbiota, we set up harem breeders with a H2-$M3^{+/-}$ male and females that were either H2-$M3^{+/+}$ or H2-$M3^{-/-}$ (Figure 3.5). Harem breeding was chosen to control for maternal microbiota and all pups used for the experiment were born within the same week. The harem was cohoused for the time of breeding and separated for birth and weaning. Pups from each dam were genotyped prior to weaning and separated into cages based on genotype, with samples from H2-$M3^{+/+}$ and H2-$M3^{-/-}$ pups assessed. Pre-wean, week 1 and week 6 post weaning fecal samples were collected as well as terminal ileum luminal contents which were then used for 16S rRNA amplicon sequencing.

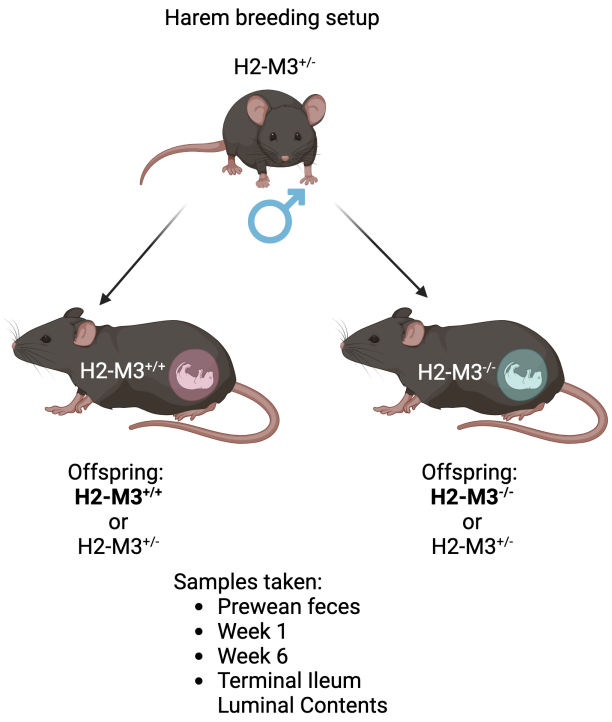


Figure 3.5: Schematic of experimental setup to assess the impact of H2-M3 on microbiota composition

We analyzed multiple alpha diversity indices including Species Richness, Chao1 index, Shannon Diversity, and Inverted Simpson Diversity, to encapsulate measurements with differing weights for rare bacterial species. All Alpha Diversity metrics measured showed no significant impact of H2-M3 genotype (Figure 3.6 A). Instead, time and sample type were significant factors driving differences; Alpha Diversity increased with age and was much higher in fecal samples compared to terminal ileum samples (Figure 3.6 A). Due to the considerable differences between the longitudinal fecal samples and the ileum samples, all future analyses focused on fecal samples to control for sample type. Additionally, fecal samples are a more accurate depiction of the potential role for H2-M3 in the large intestine, which was the focus of this thesis. We then completed linear modeling of the Alpha diversity measurements across timepoints in fecal microbiome samples which also showed no significant differences in the trends between wildtype and H2-M3$^{-/-}$ animals (Figure 3.6 B).

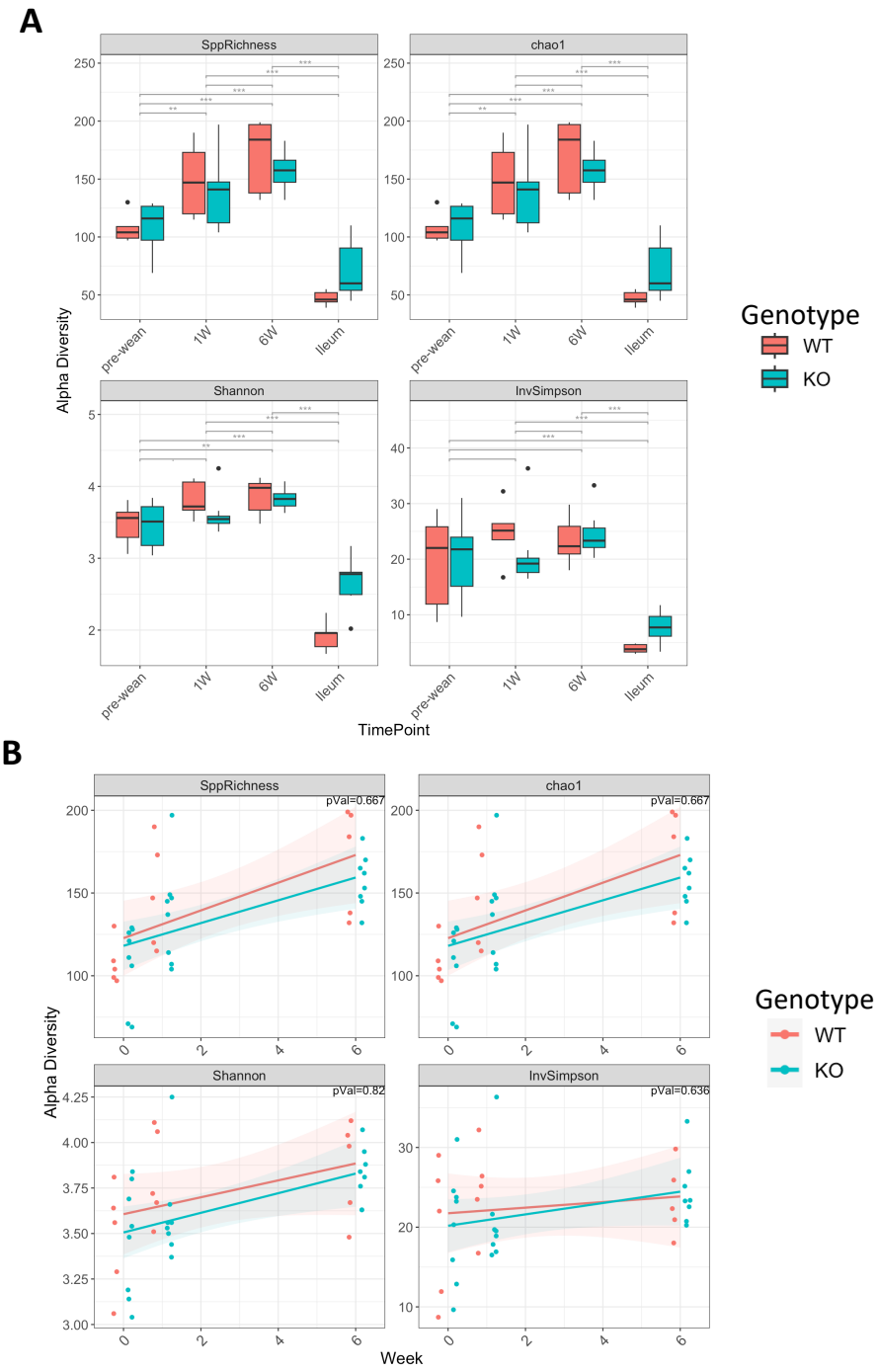


Figure 3.6: Time and sample type drive majority of differences in alpha diversity. A. Longitudinal analysis of alpha diversity measured by Species Richness (SppRichness), chao1 index, Shannon Diversity and Inverted Simpson Diversity (InvSimpson) of Wildtype [WT] (n=5) vs H2-M3$^{-/-}$ [KO](n=8) animals across timepoints (Pre-wean-6 weeks) and sample type (Feces vs Ileum) **B.** Linear model of alpha diversity metrics of fecal samples across timepoints. Data representative of 1 experiment.

Beta diversity is a more comprehensive analysis of the composition of the microbial community and can reveal taxonomic differences that are lost in alpha diversity metrics. PCoA analysis of longitudinal fecal samples revealed clustering

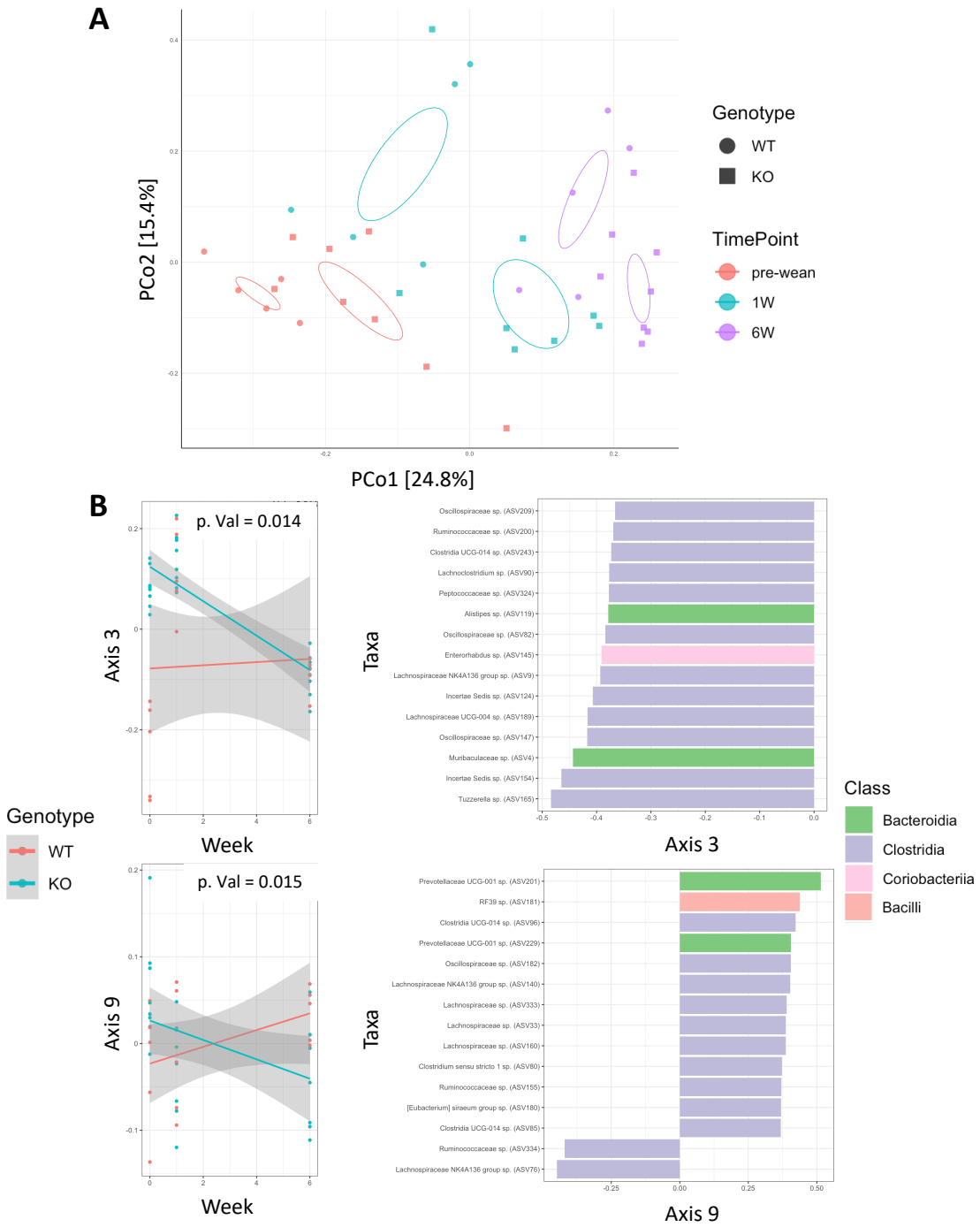


Figure 3.7: **H2-M3 deficient animals have altered beta diversity. A.** Principal coordinates analysis (PCoA) with Bray distances based on genotype for fecal samples. Each dot represents an individual mouse at indicated timepoints. Percentages represent the variance explained by each PC and ellipses indicate 80% confidence intervals **B.** Taxonomic loading and trajectory on Axis 3 and 9 driving significant differences between genotypes. Statistics determined by PERMANOVA analysis.

of samples by both genotype and timepoint (Figure 3.7 A) indicating a potential role for H2-M3 in driving differences in the fecal microbial composition. PCoA is a method which reduces high dimensional analyses to two dimensions, therefore, to

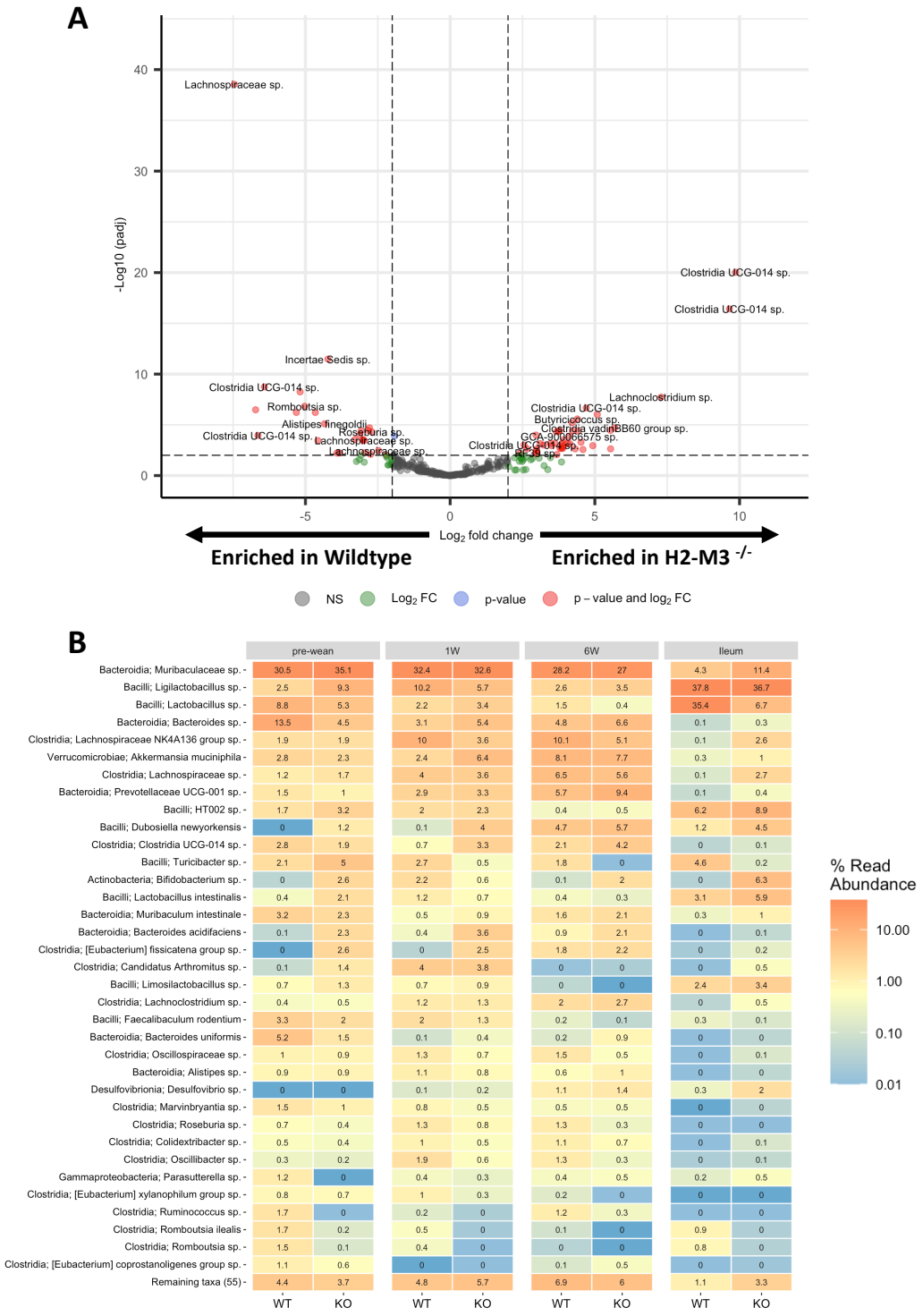


Figure 3.8: *Clostridia sp.* are enriched in H2-M3$^{-/-}$ mice. A. Volcano plot of differential abundance of bacterial taxa in fecal samples between genotypes measured by DESeq2 analysis, corrected for variance between timepoints. Thresholds used were significance < 0.01; $Log_2FC > 2$. Total taxa = 312. **B.** Read abundance of top 35 taxa at species level grouped by genotype faceted by timepoint

interrogate the taxonomic differences driving this clustering we investigated the taxonomic loading on each axis. This analysis revealed that axes 3 and 9 were

most significant in influencing clustering differences between wildtype and H2-M3$^{-/-}$ samples, driven by alterations in species belonging to the class *Clostridia* (Figure 3.7 B). Further, DEseq2 analysis assessing the differential abundance of individual amplicon sequence variants (ASV) showed a significant enrichment of multiple ASV's belonging to class *Clostridia* in H2-M3$^{-/-}$ animals compared to wildtype (Figure 3.8 A). Notably, *Clostridia* sp. were among the most highly abundant taxa in all samples analyzed (Figure 3.8 B), supporting a hypothesis that these differences might be physiologically relevant. DEseq2 analysis also revealed a significant enrichment of one *Lachnospiracea sp.* ASV in wildtype animals (Figure 3.8A). However, without knowledge of the relative abundance of this ASV in the samples, it is possible that this result is due to fluctuations in very small microbial populations. Further analysis would need to be conducted to assess the relevance of this result.

Taken together, although these results are preliminary and would need to be repeated and controlled with the maternal microbiota, they suggest a role of H2-M3 restricted CD8^{+} T cells in modulating the gut microbiota. Putative H2-M3 binding peptides can be computationally identified in multiple species of both pathogenic and commensal bacteria[60,84,148,155]. Thus, it is highly likely that members of the commensal microbiota produce peptides that can elicit an H2-M3 restricted response, which could in turn modulate the microbiota and surrounding tissue.

3.3.3 H2-M3 deficiency alters the cellular infiltration during a model of acute epithelial barrier destruction

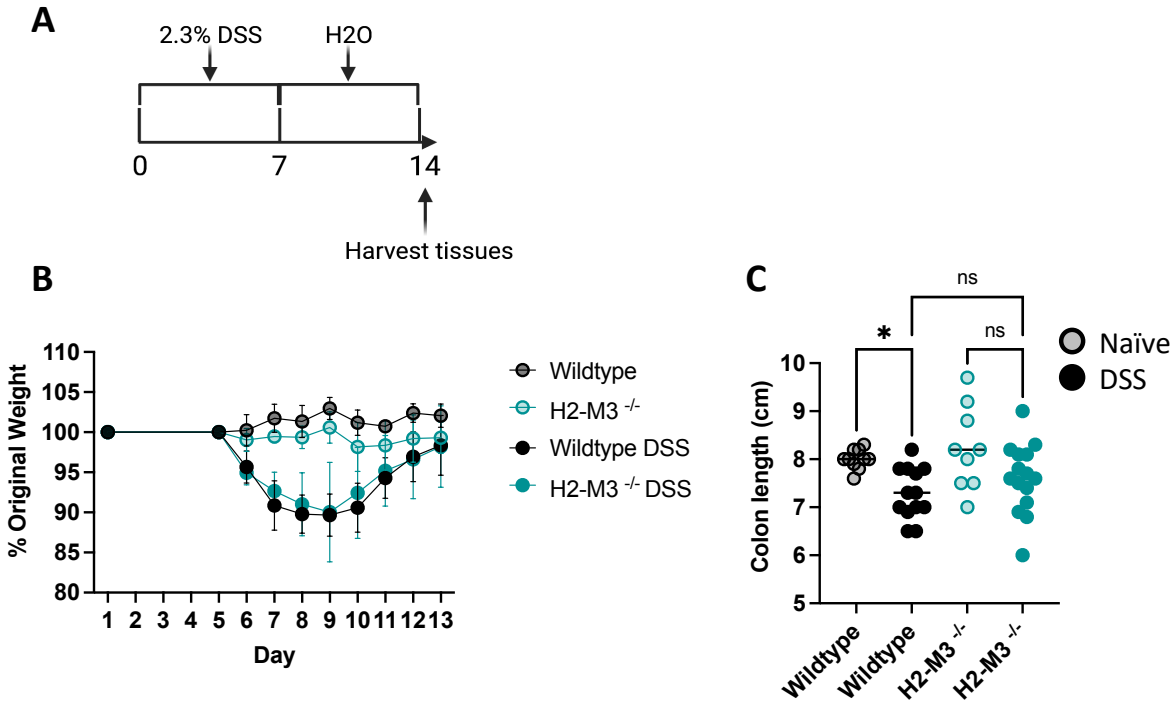


Figure 3.9: Clinical outcomes of DSS colitis are unaffected by H2-M3. A. Schematic of Dextran Sodium Sulfate (DSS) model of colitis **B.** Changes in body weight during experimental period **C.** Colon length of naïve and DSS treated Wildtype and H2-M3$^{-/-}$ mice. One way ANOVA was used to measure significance * p <0.05. Data representative (**B**) or cumulative (**C**) of 3 independent experiments.

Prior research in the Belkaid lab investigating the role of commensal specific, H2-M3 restricted CD8^{+} T cell responses in the skin epithelium illuminated a wound healing and tissue repair phenotype[60]. Thus, to understand whether H2-M3 restricted CD8^{+} T cells might play a role in maintaining the gut barrier, we employed a model of colitis wherein treatment with dextran sodium sulfate (DSS) disrupts the colonic epithelium leading to inflammation in this tissue.

Multiple studies have shown that the DSS model of colitis is highly dependent on the commensal microbiota[156,157] thus, to control for microbiota differences, H2-M3$^{-/-}$ and wildtype mice were cohoused for 3 weeks prior to the induction of colitis and for the duration of the model. Mice were treated with 2.3%

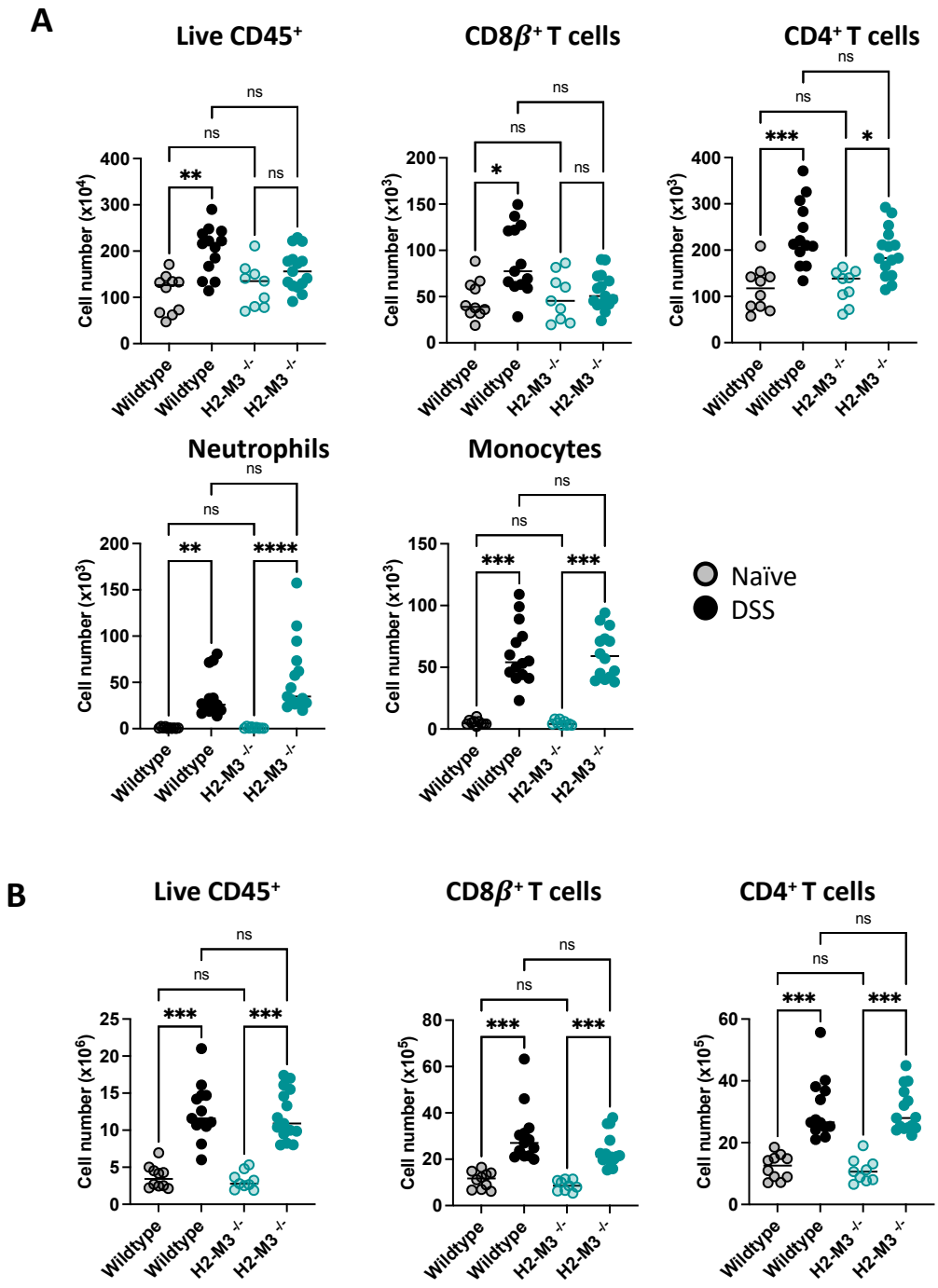


Figure 3.10: H2-M3$^{-/-}$ have reduced cellular infiltrate in colon LPL during colitis. Numbers of Live CD45$^+$ cells, CD8β^+, CD4$^+$ T cells (gated on Live CD45$^+$ Thy1.2$^+$ TCRβ^+), Monocytes and Neutrophils (gated on Live CD45$^+$ CD11b$^+$ Monocyte identified as Ly6C$^+$ and Neutrophils further identified by LY6G$^+$ expression) from **A.** LI lamina propria or **B.** pooled mesenteric lymph nodes of naïve or DSS treated, wildtype or H2-M3 $^{-/-}$ animals. One way ANOVA was used to measure significance * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Data cumulative of 3 independent experiments.

DSS in their drinking water for 7 days which was then replaced by normal drinking water until sacrifice at day 14 (Figure 3.9 A). Disease progression was measured by weight loss throughout the course of the model (Figure 3.9 B) and colon length

at sacrifice (Figure 3.9 C) neither of which were significantly different between genotypes.

Interestingly, when we assessed the total leukocyte infiltration at day 14 after initiation of colitis, there was a significant increase in total $CD45^+$ cells in the large intestine lamina propria of wildtype animals during DSS colitis, but a non-significant increase in the H2-$M3^{-/-}$ animals (Figure 3.10 A). Monocyte and neutrophil infiltration were comparable between genotypes, however, the total $CD8\beta^+$ infiltration in the H2-$M3^{-/-}$ mice was not significantly elevated during colitis, suggesting that H2-M3 restricted $CD8^+$ T cells could play a non-redundant role in the inflammatory response to DSS colitis (Figure 3.10 A). However, it should be noted that there were not significant differences between the H2-$M3^{-/-}$ and wildtype $CD8^+$ T cell infiltration during colitis (Figure 3.10 A). These differences were not seen in the gut draining, mesenteric lymph nodes, suggesting a tissue specific role for H2-M3 in driving these responses (Figure 3.10 B).

Future studies will investigate the long-term impact of H2-M3 deficiency on epithelial barrier healing in this model.

3.3.4 MHC-Ib restricted $CD8^+$ T cells contribute to the $CD8^+$ T cell response during colitis

Inflammatory bowel diseases are inflammatory disorders with complex etiology, likely arising from underlying genetic abnormalities exacerbated by environmental signals such as the commensal microbiota. No model system fully recapitulates disease, thus, to better understand the impact of H2-M3 restricted $CD8^+$ T cells on acute colitis, we also utilized the *Helicobacter hepaticus* model wherein colonization with this pathobiont in the absence of IL-10 signaling induces a $CD4^+$ T cell driven ileocolitis[24,132] .

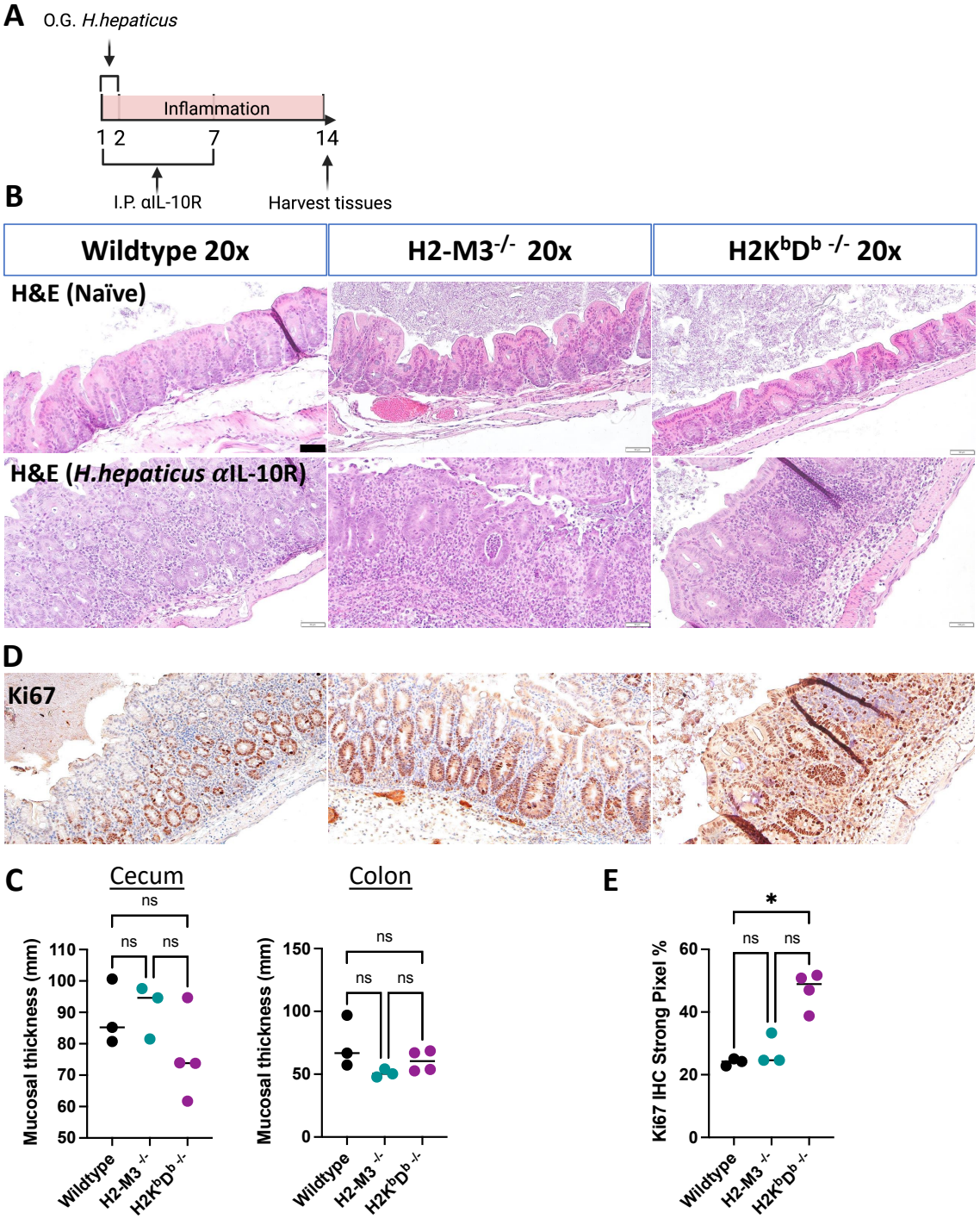


Figure 3.11: H2K^{b}D^{b} $^{-/-}$ mice have higher Ki67 nuclear staining during colitis. A. Schematic of *Helicobacter hepaticus* induced model of colitis. **B.** Representative images of gross histology (H&E) of cecum of Naïve or *H.hepaticus* and αIL-10R treated Wildtype (n=3), H2-M3$^{-/-}$ (n=3) and H2K^{b}D$^{b-/-}$ (n= 3 or 4) animals. Scale bar = 50μm **C.** Quantification of mucosal thickness of cecum and colon in *H.h* and αIL-10R treated animals . **D.** Representative images of nuclear Ki67 IHC staining in cecum of *H.h* and αIL-10R treated animals. **E.** Quantification of nuclear Ki67 IHC staining in cecum. One way ANOVA was used to measure significance * p<0.05. Data representative of 1 experiment.

To compare the contribution of H2-M3 restricted CD8^{+} T cells and MHC-Ia restricted CD8^{+} T cells in this model, we colonized wildtype, H2-M3$^{-/-}$ and

Genotype	Inflam. Infiltrate	Goblet cell loss	Crypt Density	Crypt hyperplasia	Muscle Thickening	Submucosal Inflam.	Crypt Abscess	Ulceration	Avg. Score
Wildtype	2±0	2±0	1±0	2±0	0±0	2±0	2±1.7	0±0	11±1.7
H2-M3$^{-/-}$	2±0	3±0	1±0	2±0	0±0	2±0	3±0	0±0	13±0
H2K^{b}D$^{b\,-/-}$	2±0	3±0	0.75±0.5	2±0	0±0	2±0	3±0	0±0	12±2

Table 3.1: H2-M3$^{-/-}$ and H2K^{b}D$^{b\,-/-}$ mice have similar histological scores during colitis.
Cecum of Wildtype, H2-M3$^{-/-}$, and H2K^{b}D$^{b\,-/-}$ mice were scored for the degree of inflammatory (inflam.) infiltrate, goblet cell loss, crypt density, crypt hyperplasia, muscle thickening, submucosal inflammation, crypt abscess and ulceration. Scores are reported ± SD. Details of scoring system in Section 2.9 (Methods).

H2K^{b}D$^{b-/-}$ animals with *Helicobacter hepaticus* while blocking IL-10 signaling using an αIL-10R blocking antibody (Figure 3.11 A). Gross histology of the cecum and colon revealed an increase in crypt length in all genotypes consistent with this model (Figure 3.11 B) and no significant differences in overall histological scoring (Table 3.1). Further, mice exhibited an increase in mucosal thickness that was similar between groups (Figure 3.11 C). Interestingly, Ki67 nuclear staining was significantly higher in H2K^{b}D$^{b-/-}$ animals which was more pronounced in cells infiltrating the tissue compared to the crypts of wildtype and H2-M3$^{-/-}$ animals (Figure 3.11 D and E) indicating a high proliferative capacity of infiltrating cells, or the inflamed tissue itself, in the absence of MHC-Ia restricted CD8^{+} T cells.

To better understand the cellular composition of the immune infiltrate during colitis, we also conducted immunohistochemical staining of the colon and cecum for markers of T cells (CD3/ CD8), macrophages (IBA-1), B cells (B220) and Neutrophils (MPO). Overall, we observed more pronounced immune infiltration in the cecum of challenged mice compared to the colon in this model (Figure 3.12 B). Interestingly, despite having lower CD8^{+} T cell in the tissue of H2K^{b}D$^{b-/-}$ animals at steady state, during inflammation, these differences were abrogated, and we observed considerable infiltration of CD8^{+} T cells in H2K^{b}D$^{b-/-}$ animals (Figure 3.12 A and B). Further, there was a notable emergence of

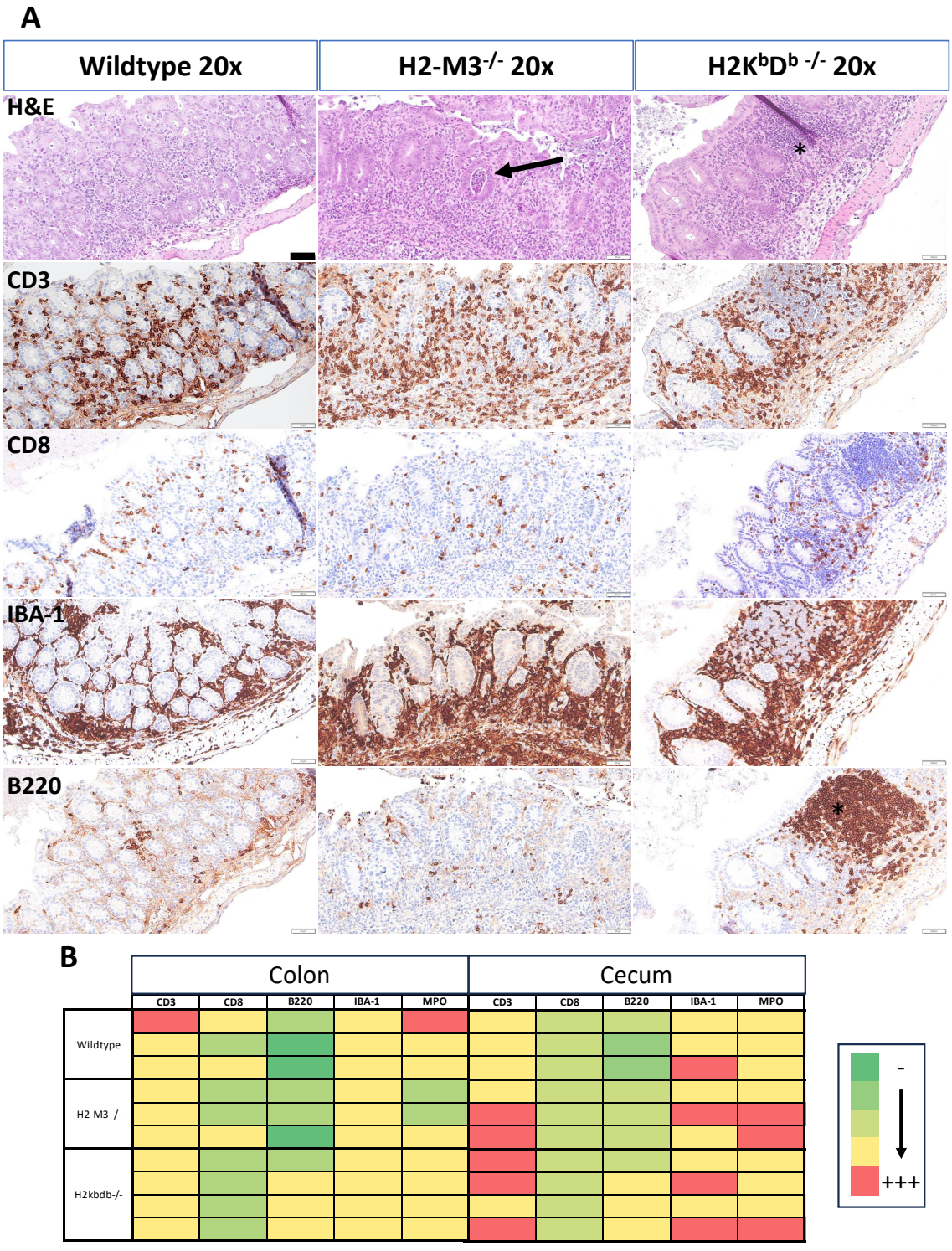


Figure 3.12: H2-M3$^{-/-}$ and H2K^{b}D$^{b\ -/-}$ mice have altered colon pathology during colitis. A. Representative images of gross histology (H&E) and IHC markers (CD3= T cells, B220= B cells, IBA-1= macrophages/DC's, MPO= Neutrophils) of cecum of *H.hepaticus* and αIL-10R treated Wildtype (n=3), H2-M3$^{-/-}$ (n=3) and H2K^{b}D$^{b-/-}$ (n=4) animals. * = mononuclear cell infiltration. Arrow indicates crypt abscess. Scale bar = 50μm **B.** Heatmap of IHC marker scores for Colon and Cecum. Data representative of 1 experiment.

mucosal lymphoid nodules comprised of B220 expressing B cells (denoted by *) more often in H2K^{b}D$^{b-/-}$ animals (Figure 3.12 A), and overall, slightly higher cellular

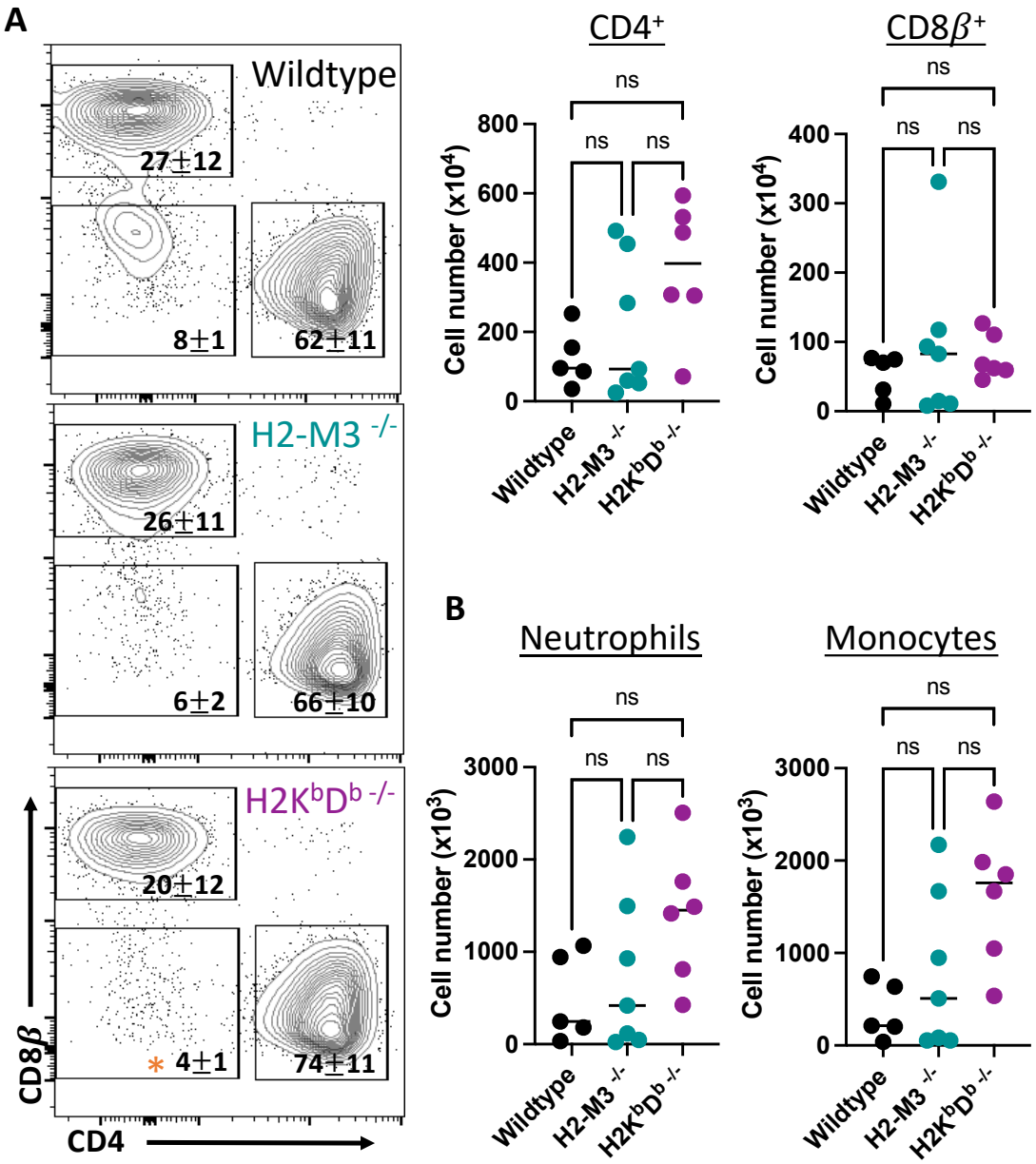


Figure 3.13: Non-classically restricted CD8⁺T cells infiltrate the colon LPL during colitis A. Frequencies and numbers of CD4⁺ and CD8β⁺ T cells in large intestine (LI) lamina propria of *H.h* and αIL-10R treated Wildtype (WT) (n=5), H2-M3$^{-/-}$(n=7), K^{b}D$^{b-/-}$(n=6) mice. Plots are gated on Live CD45⁺ Thy1.2⁺ TCRβ⁺. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Stars indicate significance compared to wildtype **B.** Numbers of Neutrophils and Monocytes in LI lamina propria of indicated mice. Monocytes were identified by Live CD45⁺ CD11b⁺ Ly6C⁺ and Neutrophils further identified by LY6G⁺ expression. One Way ANOVA was used to measure significance. Data representative of 1-3 experiments.

infiltration of macrophages and neutrophils in H2-M3$^{-/-}$ and H2K^{b}D$^{b-/-}$ mice (Figure 3.12 B). Details of quantification are described in methods. These results suggest that the absence of MHC-Ia or H2-M3 might alter the pathology of colitis. Whether this is due to local expression of MHC-I, or the modulation of the frequencies of MHC-Ib or H2-M3 restricted CD8⁺ T cells remains to be addressed.

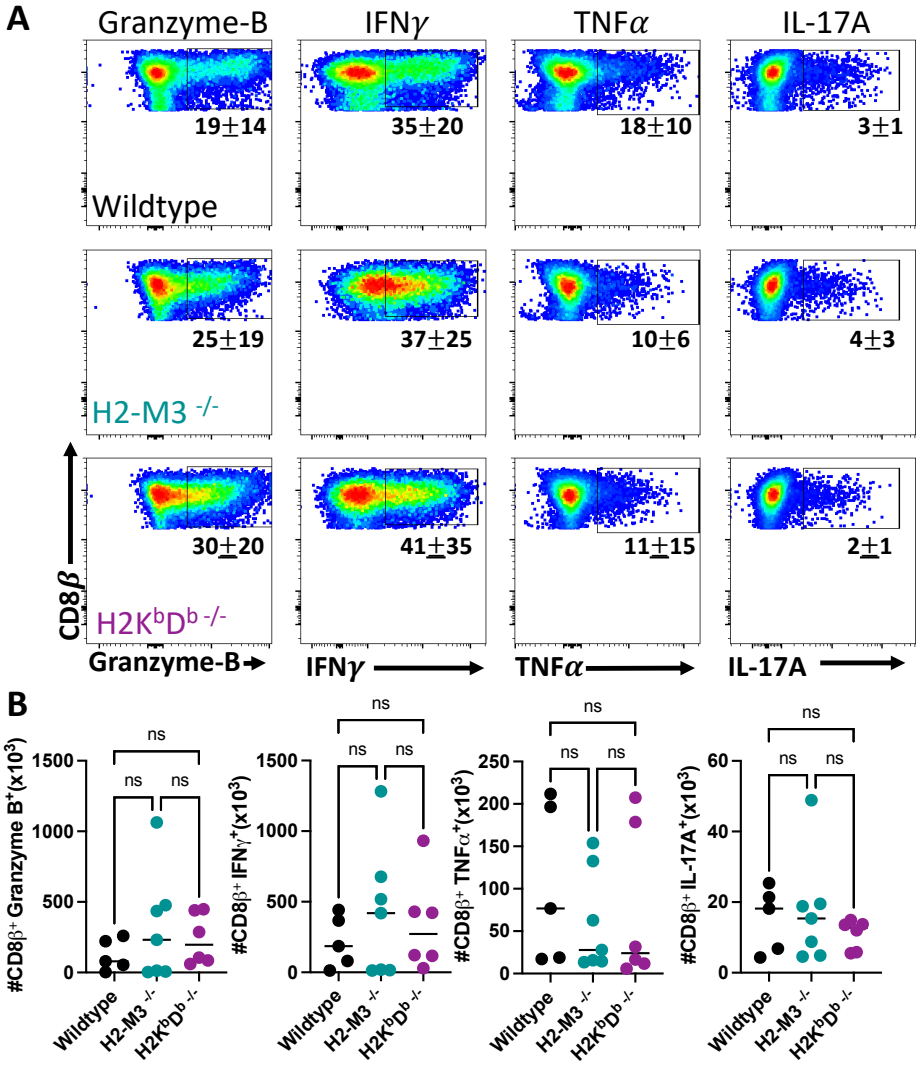


Figure 3.14: **MHC-Ia and MHC-Ib restricted CD8⁺T cells exhibit similar phenotypes during colitis. A.** Frequencies of cytokine production of Granzyme-B⁺, IFNγ^+, TNFα^+, or IL-17A⁺ CD8β^+T cells isolated from the LI lamina propria of *H.h* and αIL-10R treated Wt, H2-M3$^{-/-}$, and K^{b}D$^{b-/-}$ animals. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. **B.** Quantification. One Way ANOVA was used to measure significance. Data representative of 1 experiment.

We wished to elaborate upon these findings with flow cytometry and further characterize the MHC-Ib restricted CD8⁺ T cell infiltrate during colitis in the H2K^{b}D$^{b-/-}$ mice. In depth analysis of the T cell, monocyte, and neutrophil populations of the large intestine lamina propria revealed equal frequencies and numbers of CD4⁺ and CD8β^+ T cells during inflammation, confirming that MHC-Ib restricted CD8⁺ T cells can dominantly respond during colitis in the absence of MHC-Ia (Figure 3.13 A). Further, neutrophil and monocyte populations were

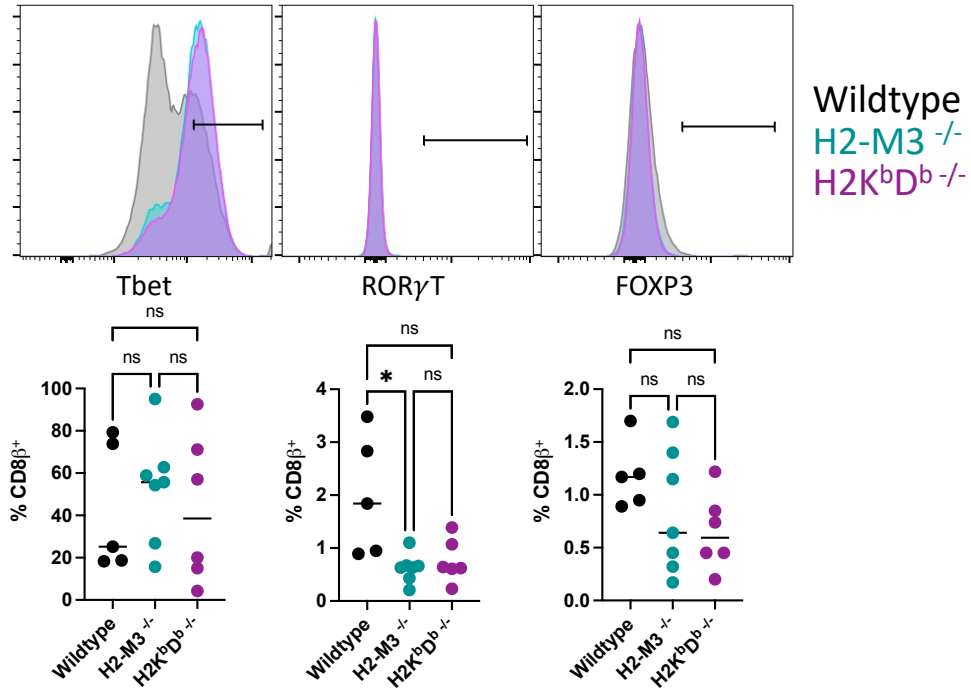


Figure 3.15: **MHC-Ia and MHC-Ib restricted CD8⁺T cells express Tbet.**
Expression of T-bet, RorγT and FoxP3 by CD8β^+T cells in LI lamina propria. One Way ANOVA was used to measure significance. Data representative of 1 experiment.

unchanged between groups, indicating a similar overall inflammatory response to *Helicobacter hepaticus* in the setting of IL-10 signaling deficiency (Figure 3.13 B).

Finally, we assessed the cytokine potential of CD8⁺ T cells in wildtype, H2-M3$^{-/-}$ and H2K^{b}D$^{b-/-}$ animals to understand if there are functional differences between MHC-Ia and MHC-Ib restricted CD8⁺ T cells in this model. During inflammation there was robust production of Type 1 (IFNγ and TNFα) as well as the cytotoxic granule, Granzyme B by the infiltrating CD8⁺ T cells (Figure 3.14 A and B). In line with this observation, the majority of infiltrating CD8⁺ T cells expressed the transcription factor Tbet, with minimal expression of RORγT and FOXP3 (Figure 3.15).

Taken together, these results support the hypothesis that there is a CD8⁺ T cell "niche" during colitis, that can be filled by MHC-Ib restricted CD8⁺ T cells during inflammation. Further, under these acute conditions, there is no observable

impact of H2-M3 on *Helicobacter hepaticus* αIL-10R induced colitis, and CD8$^+$ T cells that infiltrate the lamina propria during inflammation adopt a cytotoxic Tc1 phenotype regardless of their restriction.

3.4 Discussion

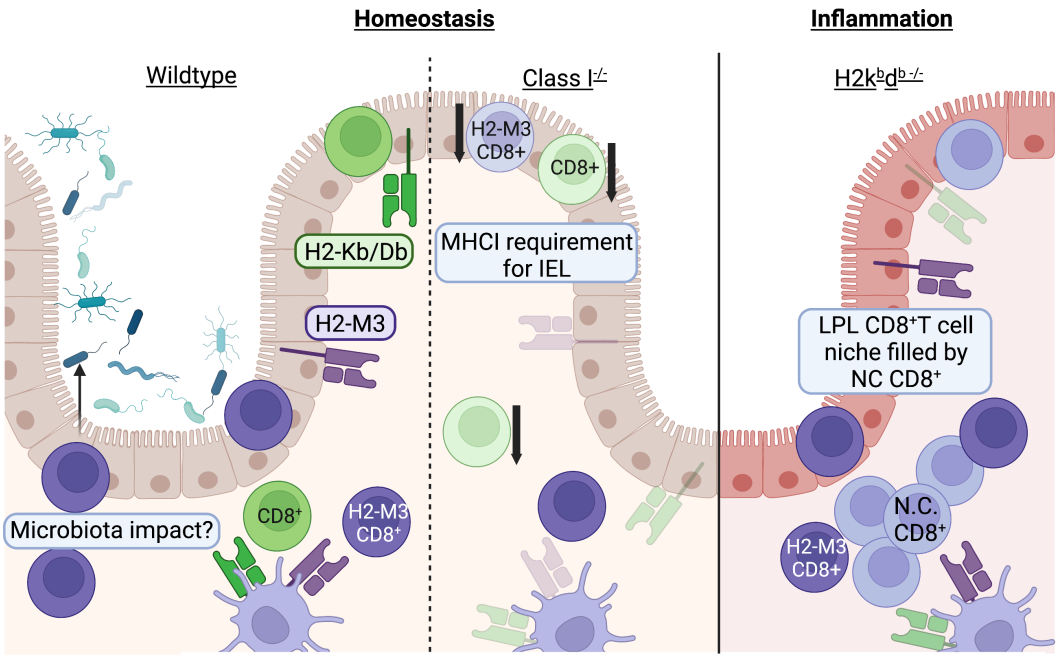


Figure 3.16: Summary of findings

In this chapter we describe the landscape of the large intestine in the absence of H2-M3 or MHC-Ia during homeostasis and in two distinct models of colitis and investigate the impact of H2-M3 and MHC-Ia restricted CD8$^+$ T cells in these different conditions. We found that during steady state, both the large intestine epithelium and lamina propria are dominantly populated by MHC-Ia restricted CD8$^+$ T cells, however during inflammation, MHC-Ib restricted CD8$^+$ T cells can fill the “niche” of the colon LP and adopt a cytotoxic Type 1 phenotype while expressing the transcription factor Tbet. We observed no noticeable impact of H2-M3 on the clinical readouts of colitis in either model used, however it should be noted that in the absence of H2K^b and H2D^b, Qa-1 and Qa-2 restricted CD8$^+$ T

cells remain which we did not track in this study. Although it is likely that H2-M3 restricted $CD8^+$ T cells are occupying the “niche” during inflammation, it is feasible that Qa-1 and Qa-2 restricted $CD8^+$ T cells do as well which has interesting clinical implications when considering these molecules are the known functional murine homologue of HLA-E[39].

There are limited studies investigating the impact of MHC-Ib restricted $CD8^+$ T cells on the development or perpetuation of colitis. Using a model of T cell transfer colitis, Das et al. reported that MHC-Ib restricted $CD8^+$T cells can induce colitis whereas MHC-Ia restricted, OT-1 cells do not[158]. This study did not describe the relative contributions of various MHC-Ib restricted $CD8^+$ T cells. In contrast, Dasgupta et al., reported that Qa-1^b restricted $CD8^+$ T cells in the colon IEL conferred protection in the DSS model of colitis[147]. These conflicting results illustrate the need to use more specialized tools to assess the relative contributions of different populations of MHC-Ib restricted $CD8^+$ T cells to colitis, and multiple models to fully assess the functional consequences. Our work suggests that, at the timepoints we assessed, MHC-Ib restricted $CD8^+$ T cells act similarly to MHC-Ia restricted $CD8^+$ T cells during colitis, although their exact roles have yet to be elucidated.

Additionally, we interrogated the role of H2-M3 restricted T cells in modulating the gut microbiota illuminating a *Clostridia* signature. Notably, *Clostridia* sp. are robust producers of short chain fatty acids such as butyrate, which has profound immunomodulatory and metabolic effects[159,160]. The impact of this altered microbiome on tissue physiology remains to be addressed.

3.5 Conclusion

Taken together, this work is the first to assess the potential contribution of H2-M3 restricted $CD8^+$ T cells to murine gut immunity during homeostasis and colitis, as well as the potential impact of commensal reactive $CD8^+$ T cells on the gut microbiota.

3.6 Future outlook and study limitations

Prior reports of commensal specific H2-M3 restricted $CD8^+$ T cells indicate they possess a wound healing and tissue repair signature in the skin driven by their Tc17 and poised Type 2 phenotype [60,61]. However, in the gut, it appears that most $CD8^+$ T cells, regardless of their restriction, adopt a Tc1 phenotype in both homeostasis and inflammation. It should be noted, that all timepoints used in these experiments were the peak of disease, perhaps masking any potential wound healing signature of H2-M3 restricted $CD8^+$ T cells. Future studies should investigate the long-term consequences of H2-M3 deficiency on recovery from colitis, particularly in the DSS model which induces epithelial barrier disruption. To further test the wound healing potential in a less disruptive model of epithelial injury, we could employ colonic tissue biopsy[161]. It is feasible that at these acute timepoints, the overwhelming inflammatory response makes it difficult to elucidate a more refined role of these cells in tissue repair, therefore testing the impact of H2-M3 genotype on the re-epithelialization of the colon after biopsy might be more physiological. It should also be noted that the tissue itself might inform the function of these cells, as suggested by the Type 1 phenotype seen in most $CD8^+$ T cells in the $H2K^bD^{b\,-/-}$ mice (Figure 3.3). Thus, it would also be interesting to see what tissue specific cues orchestrate these commensal specific $CD8^+$ T cell responses, which might be viable targets for therapies in IBD.

The *Clostridia* sp. signature seen in the absence of H2-M3 is also intriguing as these members of the microbiota are prolific producers of SCFA which are immunomodulatory metabolites[25,31,159,162]. It is interesting to hypothesize that in the absence of H2-M3 restricted $CD8^+$ T cells, there could be an outgrowth of *Clostridia* sp. which could potentially improve barrier function due to SCFA production[25,31,159]. One way to test this hypothesis would be to measure fecal SCFA production, which we would do in future studies. A limitation to this study, however, is that we did not collect the maternal microbiota. Although we attempted to control for this by cohousing the mothers during breeding, it is feasible that this did not fully equilibrate the microbiota and therefore the differences we see between genotypes is confounded by a maternal effect, and not a genotype effect. An improvement to this study would be to co-house the mothers for at least 3 weeks prior to breeding and take fecal samples to ensure full microbiota equilibration.

4 Tracking commensal reactive CD8$^+$ T cell responses in the colon during homeostasis and inflammation

4.1 Introduction

In the previous chapter, we described the infiltration of non-classically restricted CD8$^+$ T cells in the colon during inflammation as well as altered pathology in the absence of MHC-Ia restricted CD8$^+$ T cells. However, we were unable to distinguish the impact of H2-M3 restricted CD8$^+$ T cells compared to CD8$^+$ T cells restricted by other MHC-Ib molecules that might still be present in H2K^{b}D$^{b-/-}$ mice.

Early northern blot analyses of murine tissues indicated broad tissue distribution of H2-M3 in the thymus, liver, kidney, spleen, and testes, like other MHC-Ia molecules[78]. A following study utilizing a novel antibody against H2-M3 measured the surface expression of the molecule in multiple cell lines and primary cells isolated from the spleen, lymph nodes and Peyer's patch[81]. However, the detailed cell types capable of presenting H2-M3 in the gut, remains undescribed. Further, although IFNγ is a known inducer of H2-M3 expression[163], the impact of colitis on expression and potential resulting recruitment of H2-M3 restricted CD8$^+$ T cells has not yet been defined.

There are currently no known markers to discriminate H2-M3 restricted CD8$^+$ T cells from other activated CD8$^+$ T cells[164] therefore, the only way to track a *bona fide* H2-M3 response is through the utilization of tetramer tools. Historical research on H2-M3 restricted CD8$^+$ T cell responses focused on their roles during infection with the pathogen *Listeria monocytogenes* indicating cytotoxic function during primary infection but a dispensable role during re-encounter[85,89,164]. However, CD8$^+$ T cell functions may be altered depending on the context and

location of antigen exposure. Indeed, work in the Belkaid lab leading to the development of H2-M3 restricted tetramers specific to the skin commensal *Staphylococcus epidermidis,* elucidated a role for H2-M3 restricted, IL-17A producing $CD8^+$ T cells in maintaining the skin barrier[60]. Yet, these commensal specific, H2-M3 restricted responses have not been characterized in the gut.

Further, $CD8^+$ T cell responses to the commensal microbiota have been reported in humans at steady state and are altered during inflammatory bowel disease[119–121]. However, to our knowledge, no one has tracked how inflammation impacts the native $CD8^+$T cell responses to the commensal microbiota during colitis using tetramer tools.

4.2 Aim

In this chapter, we aimed to assess the expression of H2-M3 compared to other MHC-Ib molecules during homeostasis and inflammation, utilizing publicly available datasets developed by the Powrie laboratory. Further, we develop a model system to study H2-M3 restricted $CD8^+$ T cell responses to commensal exposure in the colon, utilizing established tetramer tools. Finally, we wished to assess how colitis, induced by *Helicobacter hepaticus* and αIL-10R treatment, alters the H2-M3 restricted, commensal specific $CD8^+$ T cell response.

4.3 Results

4.3.1 H2-M3 mRNA is expressed within niche's supporting the homeostatic immune response to Helicobacter hepaticus

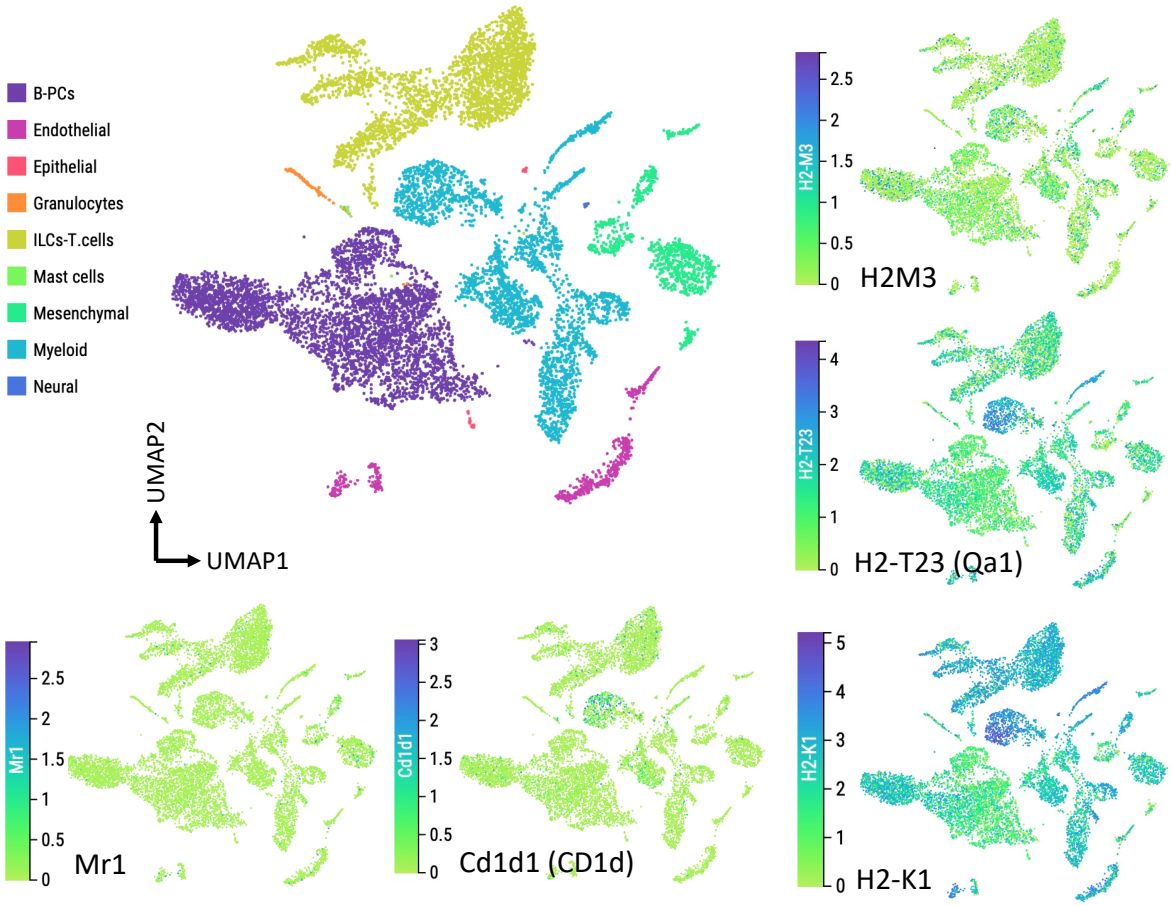


Figure 4.1: MHC-Ib gene expression is variable among cell types. UMAP visualization of total cell subtypes in publicly available Niche-seq dataset (left) and overlap on the global UMAP plot of the expression of specific genes.

The bacterium *Helicobacter hepaticus (H.h)* is a contextual pathogen, able to stably colonize immunocompetent mice, while in the absence of IL-10 signaling, induces colitis[24,132]. Colitis is held at bay by the induction of *H. hepaticus* specific Treg cells which constrain the pathogenic Th17 response to the same microbe[165]. The Powrie lab recently developed a NICHE-seq dataset to identify the network of cells that support the development of *H.h* specific Treg cells in the colon lamina propria, mesenteric lymph node, cecal patch, and small lymphoid aggregates[144].

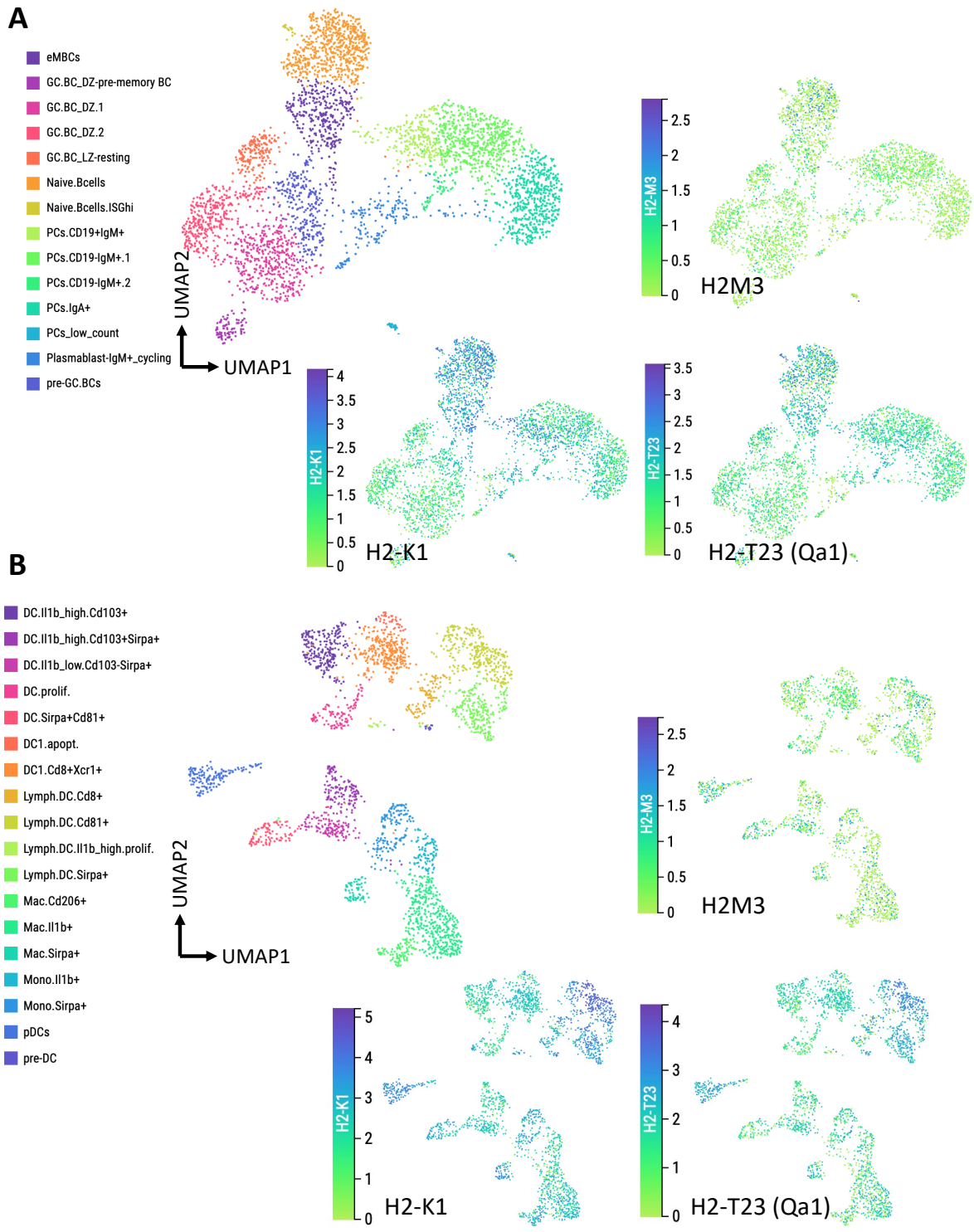


Figure 4.2: H2-M3 gene expression parallels Qa1 and MHC-Ia. A. UMAP visualization of total B cell subsets in publicly available Niche-seq dataset (left) and overlap on UMAP plot of the expression of specific genes. **B.** UMAP visualization of total Myeloid cell subsets in publicly available Niche-seq dataset (left) and overlap on UMAP plot of the expression of specific genes.

Thus, to better understand which cells might be presenting H2-M3 restricted antigens in response to *H. hepaticus,* or colonization with other potential

commensals, we analyzed this dataset for gene expression of H2-M3 and other MHC-Ib/a molecules.

Analysis of the total populations within the dataset illuminated low expression level of *H2-M3* compared to the closely related MHC-Ib molecule, *Qa-1* as well as the MHC-Ia molecule *H2-K1* (Figure 4.1). This is consistent with previous reports suggesting H2-M3 gene expression is at least 20 times lower than total class I mRNA[78]. Expression of *H2-M3*, *Qa-1* and *H2-K1* were highest in myeloid and B cell clusters which is where we focused subsequent analyses. Non-classically restricted T cells can often compensate for each other in tissues, competing for similar survival factors and producing comparable cytokines [2]. Thus, we also assessed the transcripts of other MHC-Ib molecules within this dataset to compare to H2-M3. Interestingly, *MR1* transcripts were not detectable in this dataset at all, and *CD1d* was only expressed in the myeloid cluster, indicating differential expression of MHC-Ib transcripts, and more similar expression of *H2-M3* to *Qa-1* (Figure 4.1). It should be noted that this dataset is limited by the cells that exist within the niche supporting Treg development in response to *H. hepaticus* and thus might not be comprehensive.

Deeper analysis of the B cell cluster revealed that *H2-M3* expression was almost exclusively in naïve B cells, with minimal expression in the rest of the dataset (Figure 4.2 A). *Qa-1* and *H2-K* expression was greater but still following a similar expression profile of *H2-M3* with highest expression in the naïve B cell cluster. However, these similarities diverged in early Memory B cells (eMBCs) and multiple plasma cell (PC) subsets. (Figure 4.2 A). *H2-M3* expression in myeloid clusters was more sparce, with highest expression in plasmacytoid dendritic cells

(pDCs) and CD81$^+$ lymph node resident DC's (Lymph.DC.CD81$^+$) (Figure 4.2 B). Interestingly, pDC's are potent inducers of Type 1 immune responses, notably anti-viral immunity, which might provoke a preferential skewing of H2-M3 restricted CD8$^+$ T cells to a cytotoxic or Type 1 phenotype in this tissue. *Qa-1* and *H2-K1* expression was higher in all myeloid subsets with a similar concentration in pDC's and Lymph.DC.CD81$^+$ subsets (Figure 4.2 B). Together, these results indicate that *H2-M3* gene expression is highest in naïve B cells, pDC's and CD81$^+$ DCs at steady state and the expression profile is similar to *Qa-1* and classical MHC-Ia. These results will help direct the crossing of newly developed H2-M3$^{fl/fl}$ mice to better identify which of these cell types are required for antigen presentation in the colon and gut associated lymphoid organs.

4.3.2 Oral gavage with commensal induces a local and systemic H2-M3 restricted CD8$^+$ T cell response.

Prior research in the Belkaid lab investigating commensal specific H2-M3 restricted CD8$^+$ T cells utilized the skin commensal *S. epidermidis* (*S. epi*) isolated from a human subject[8]. Topical association with *S. epi* is sufficient to induce both a local and systemic H2-M3 restricted response, which is trackable utilizing the (f)MIIINA:H2-M3 tetramer[60]. Despite primarily being considered a skin microbe, *Staphylococcus epidermidis* is frequently isolated from the stool of healthy humans[166], and recently has been shown to colonize the murine gut during colitis[167]. Therefore, we sought to utilize the tools already available in the Belkaid lab to assess the H2-M3 restricted CD8$^+$ T cell response to a commensal in the gut by colonizing mice with *S. epi* and tracking the response utilizing the (f)MIIINA:H2-M3 tetramer.

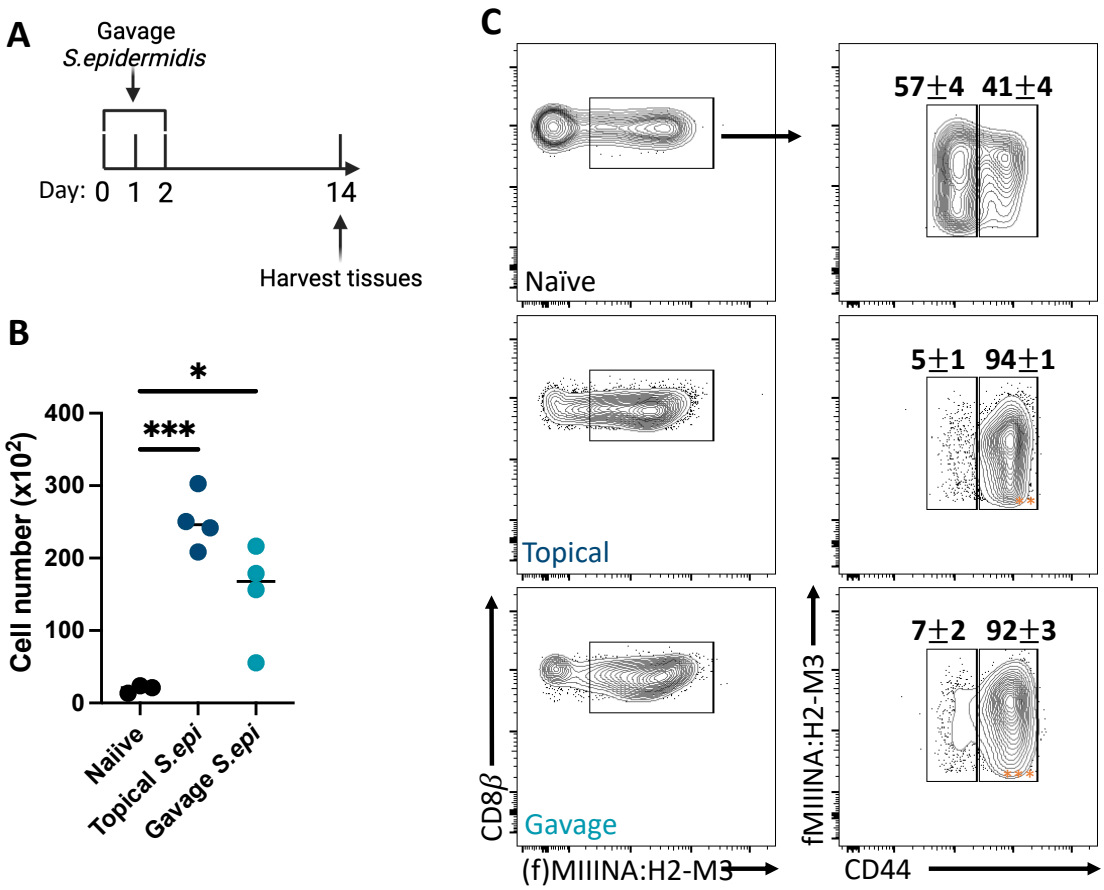


Figure 4.3: Oral *S.epidermidis* elicits a systemic commensal specific CD8 T cell response. A. Schematic of model to study commensal specific H2-M3 restricted CD8⁺T cell responses in the gut following oral gavage with *S.epidermidis* **B.** Cell numbers of (f)MIIIINA:H2-M3⁺ CD8⁺T cells in spleen following topical association and oral gavage with *S.epidermidis* **C.** Representative FACS plots of enriched spleens from mice topically associated or orally gavaged with *S.epidermidis.* Plots are gated on CD45⁺ lineage⁻ (MHCII, CD11b, γδTCR, NK1.1) CD90.2⁺ CD3ε⁺ CD8β⁺. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Stars in FACS plots indicate significance compared to wildtype. One way ANOVA was used to measure significance. * p<0.05, ** p <0.01, *** p <0.001. Data representative of 3+ independent experiments.

Mice were gavaged with *S. epi* on three consecutive days and sacrificed at day 14, which was the timepoint that has been utilized previously to assess (f)MIIINA:H2-M3⁺ T cell responses[60] (Figure 4.3 A). Mice exposed to *S. epi* at both the skin and gut exhibited increased (f)MIIINA:H2-M3⁺ cells in the spleen (Figure 4.3 B), with 92-94% expressing the activation marker CD44 (Figure 4.3 C). Mice gavaged with *S. epi* had fewer (f)MIIINA:H2-M3⁺ T cells in their spleen than mice topically associated, potentially indicating a difference in antigen production or colonization between the two sites (Figure 4.3 B). In these experiments, we were unable to confirm long term colonization by *S. epidermidis*

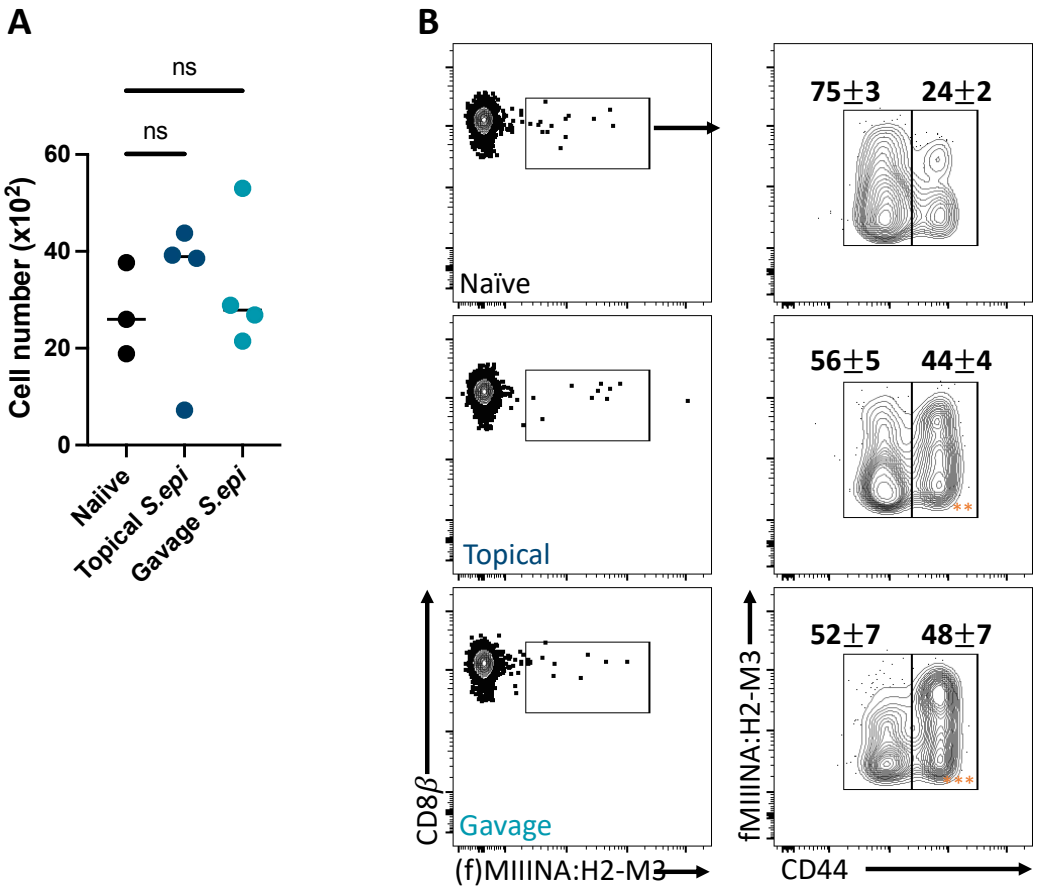


Figure 4.4: Commensal specific CD8 T cells exhibit an activated phenotype in gut draining lymph node regardless of site of exposure. A. Cell numbers of (f)MIIIINA:H2-M3$^+$ CD8$^+$T cells in mesenteric lymph node (mLN) following topical association and oral gavage with *S.epidermidis* **B.** Representative FACS plots of mLN from mice topically associated or orally gavaged with *S.epidermidis.* Plots are gated on CD45$^+$ lineage$^-$ CD90.2$^+$ CD3ε$^+$ CD8β$^+$. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Stars in FACS plots indicate significance compared to wildtype. One way ANOVA was used to measure significance.** p <0.01, *** p <0.001. Data representative of 3+ independent experiments.

in the gut because this strain has not been manipulated to express a resistance cassette for culture from fecal samples. qPCR measurements could be used to assess colonization of *S. epidermidis* in the gut, however the presence of genetic material does not confirm live microbe.

Interestingly, the mesenteric lymph nodes contained comparable numbers of (f)MIIIINA:H2-M3$^+$ cells between mice that were naïve, topically associated, and gavaged with *S. epi* (Figure 4.4 A). However, in the mice that were exposed to *S. epi*, (f)MIIIINA:H2-M3$^+$ cells expressed higher levels of CD44, indicating a more activated phenotype (Figure 4.4 B). These results imply that oral gavage with a

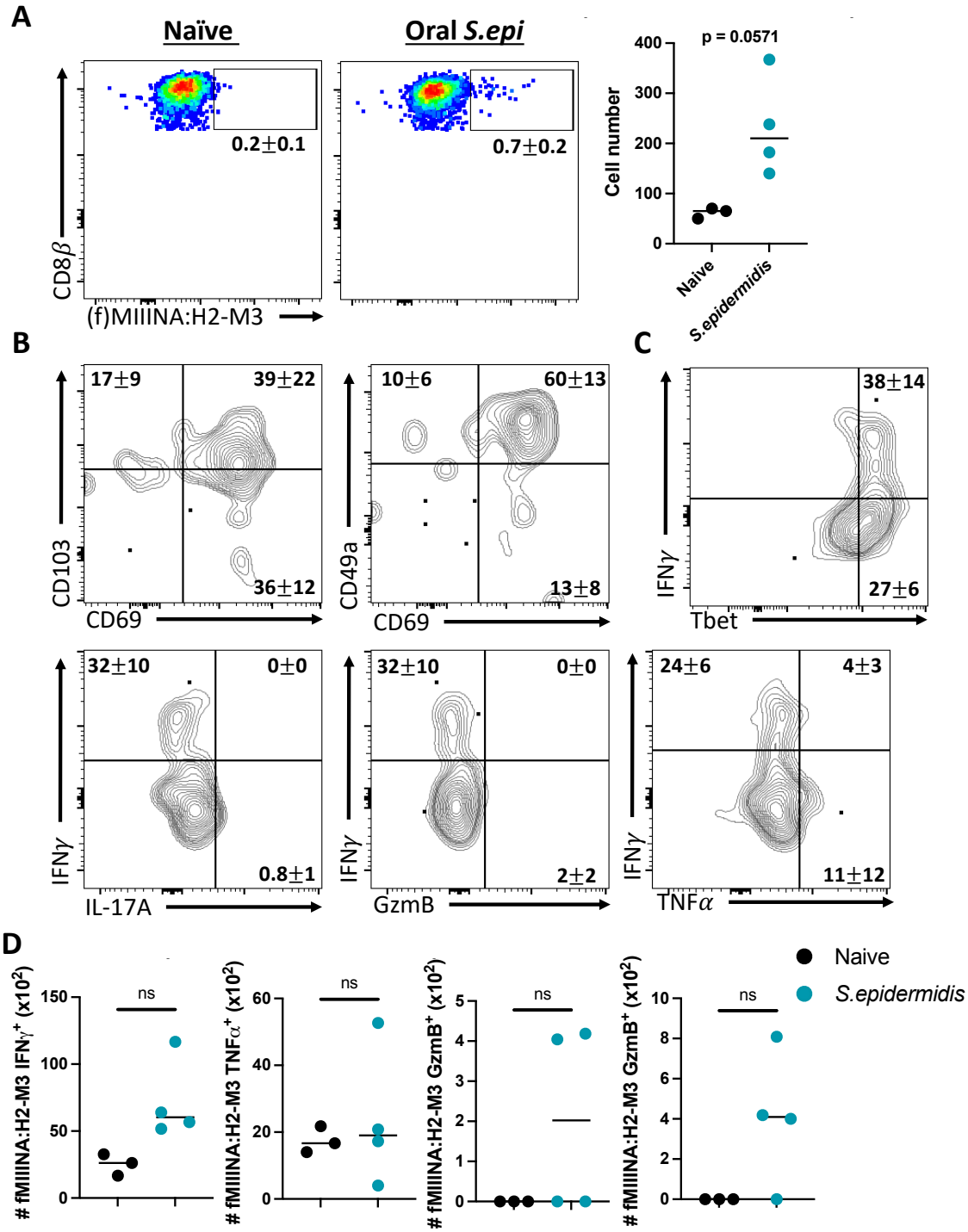


Figure 4.5: Commensal specific CD8 T cells populate the colon LPL after oral exposure. A. Representative FACS plots with frequencies and corresponding cell numbers of (f)MIIIINA:H2-M3$^+$ CD8$^+$T cells from colon lamina propria (LPL) of naïve mice or mice or orally gavaged with *S.epidermidis.* Plots are gated on CD45$^+$ lineage$^-$ CD90.2$^+$ CD3ε$^+$ CD8β$^+$. **B.** Frequencies of CD69$^+$, CD103$^+$, CD49a$^+$, IFN-γ^+, IL-17A$^+$ and TNFα^+ (f)MIIINA:H2-M3$^+$T cells extracted from colon LPL of *S.epidermidis* gavaged mice. **C.** Frequency of Tbet+ and IFNγ^+Tetramer$^+$ cells in colon LPL. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. **D.** Quantification of cytokine producing cells. Mann-Whitney was used to measure significance. Data representative of 1-2 independent experiments.

commensal microbe is sufficient to induce a systemic H2-M3 restricted T cell response. Additionally, regardless of the site of exposure, commensal reactive,

H2-M3 restricted CD8$^+$T cells can disseminate to distal lymph nodes bearing an activated phenotype.

Further, (f)MIIINA:H2-M3$^+$ cells were isolated from the colon lamina propria following oral gavage with *S. epi* (Figure 4.5 A) as well as the small intestine (data not shown). (f)MIIINA:H2-M3$^+$ cells expressed tissue residency markers such as CD103, CD69, and CD49a, and produced Type 1 cytokines IFNγ and TNFα at steady state (Figure 4.5 B and D). This differs from their previously described Tc17 signature in the skin[58,60,61], indicating potential tissue specific cues that drive the cytokine potential of these cells in response to antigen. In line with these observations, the majority of (f)MIIINA:H2-M3$^+$ cells isolated from the colon LPL express the transcription factor Tbet (Figure 4.5 C).

Taken together these results collectively demonstrate the successful creation of a model system to track H2-M3 restricted, commensal reactive CD8$^+$ T cell responses in the colon. Using this model system, we also show that H2-M3 restricted CD8$^+$ T cells express residency markers within this tissue where they produce largely type 1 cytokines at steady state. Future experiments using parabiosis could assess the long-term residency, or migratory capacity of these commensal specific CD8$^+$ T cells after elicitation in the gut.

4.3.3 H2-M3 gene expression increases during Helicobacter hepaticus αIL-10R induced colitis in colon tissue and cultured organoids

In Chapter 3 we describe the infiltration of MHC-Ib restricted CD8$^+$ T cells during *H. hepaticus* αIL-10R induced colitis, however the impact of this model on class I expression, and specifically H2-M3, is unknown. Further, the expression profile of H2-M3 compared to other MHC-Ib molecules during colitis also remains to be described. Therefore, to assess the impact of colitis on H2-M3 expression, we

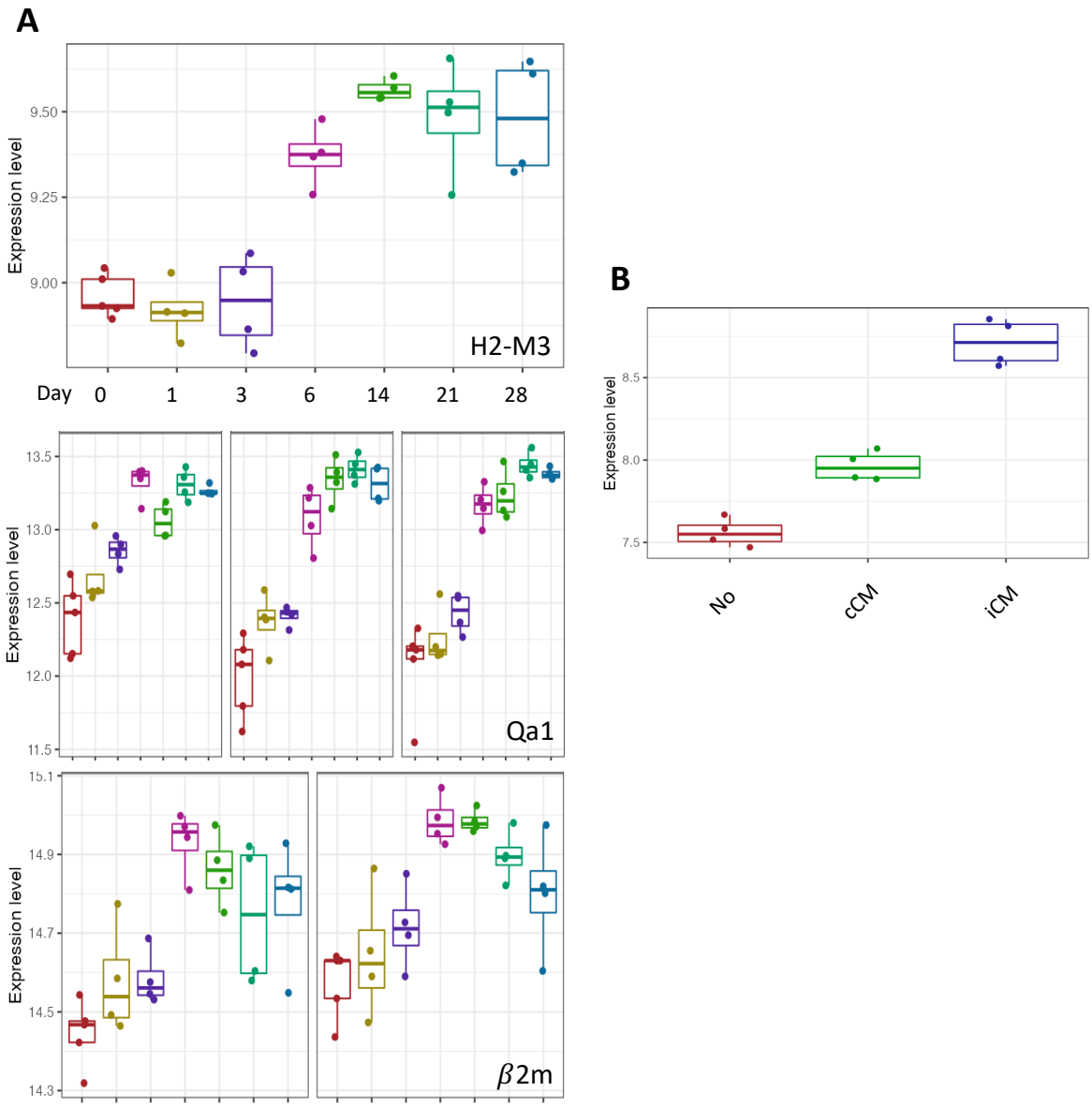


Figure 4.6: Colitis induces MHC Class I gene expression. A. Microarray dataset of whole colon tissue analyzed for H2-M3, Qa1 and β2m expression throughout the course of *Helicobacter hepaticus* αIL-10R colitis. **B.** Bulk RNA sequencing of murine colon organoids co-cultured with control Culture Medium (cCM) from cultured lamina propria lymphocytes (LPL) of naïve mice, or inflammatory Culture Medium (iCM) from LPL of *Helicobacter hepaticus* αIL-10R treated mice.

utilized a publicly available microarray dataset wherein whole colon tissue was processed for mRNA expression at multiple timepoints during *H. hepaticus* αIL-10R induced colitis[143].

We observed that *H2-M3* expression steadily increased during colitis, with sustained expression even after the peak of inflammation at day 14 (Figure 4.6 A). *Qa-1* expression also increased during inflammation and was sustained, similarly

to *H2-M3*. Both *H2-M3* and *Qa-1*, as well as all classical MHC molecules require β_2 microglobulin (β_2m) for their complete assembly and surface expression[168], therefore we also assessed the impact of colitis on $\beta_2 m$ gene expression to better understand the impact of inflammation on broad class I expression. $\beta_2 m$ gene expression also increased with colitis, but decreased after day 14, potentially indicating that protein expression of H2-M3, Qa-1 and other Class I molecules that associate with β_2 microglobulin might decrease after the peak of inflammation has passed (Figure 4.6 A). These results illustrate that *H2-M3* gene expression parallels other MHC-Ib molecules during colitis and is upregulated early in response to inflammation in whole colon tissue.

In their 1999 study, Chiu, and colleagues, reported no protein expression of H2-M3 on intestinal epithelial cells (IEC) with and without stimulation with fMet peptides, unlike many other cell types they analyzed[81]. However, IEC are highly sensitive to processing methods and thus it is feasible that there were insufficient live cells to draw accurate conclusions. Culture of $Lgr5^+$ intestinal stem cells allow for the creation of stable colon IEC organoids which develop many of the traditional cell types that reside within the colonic epithelium including Paneth cells, goblet cells, enteroendocrine cells and enterocytes[169]. Thus, to understand whether H2-M3 is expressed in the colon epithelium and how this expression might be affected by *H. hepaticus* αIL-10R colitis, we analyzed a publicly available RNA sequencing dataset produced by the Powrie lab investigating the impact of *H. hepaticus* αIL-10R colitis on colon IEC organoids[142]. For these experiments, lamina propria lymphocytes from the colon of naïve and colitic animals were first cultured overnight to produce either control culture medium (cCM) or inflammatory

culture medium (iCM). cCM and iCM was then subsequently used to stimulate colon IEC organoids which were collected for RNA sequencing[142].

Analysis of this dataset revealed an increase in *H2-M3* expression following culture with cCM above baseline, suggesting that there are factors native to the lymphocytes of the lamina propria that can induce expression in IEC (Figure 4.3 B). Further, *H2-M3* gene expression increased considerably in organoids exposed to iCM indicating that factors produced during colitis further elevate expression in IEC (Figure 4.6 B). Taken together, these results indicate that H2-M3 RNA expression increases in both whole colon tissue, and specifically in the epithelium during colitis and thus might aid in the recruitment or retention of H2-M3 restricted $CD8^+$ T cells within this tissue during inflammation.

4.3.4 Commensal reactive $CD8^+$ T cells expand in the colon during H.hepaticus colitis

After establishing that we could identify commensal reactive, H2-M3 restricted $CD8^+$ T cells in the colon, we then sought to characterize these responses during colitis. We gavaged mice with *S. epidermidis* as before, and induced colitis with *Helicobacter hepaticus* colonization and αIL-10R treatment (Figure 4.7 A).

(f)MIIINA:H2-$M3^+$ cells could be identified in the colon LPL of both the *S. epi* gavaged and *H.h* αIL-10R + S*. epi* groups, indicating adequate antigen exposure and initiation of a local immune response (Figure 4.7 B and C). Additionally, there was a significant expansion of (f)MIIINA:H2-$M3^+$ cells in the

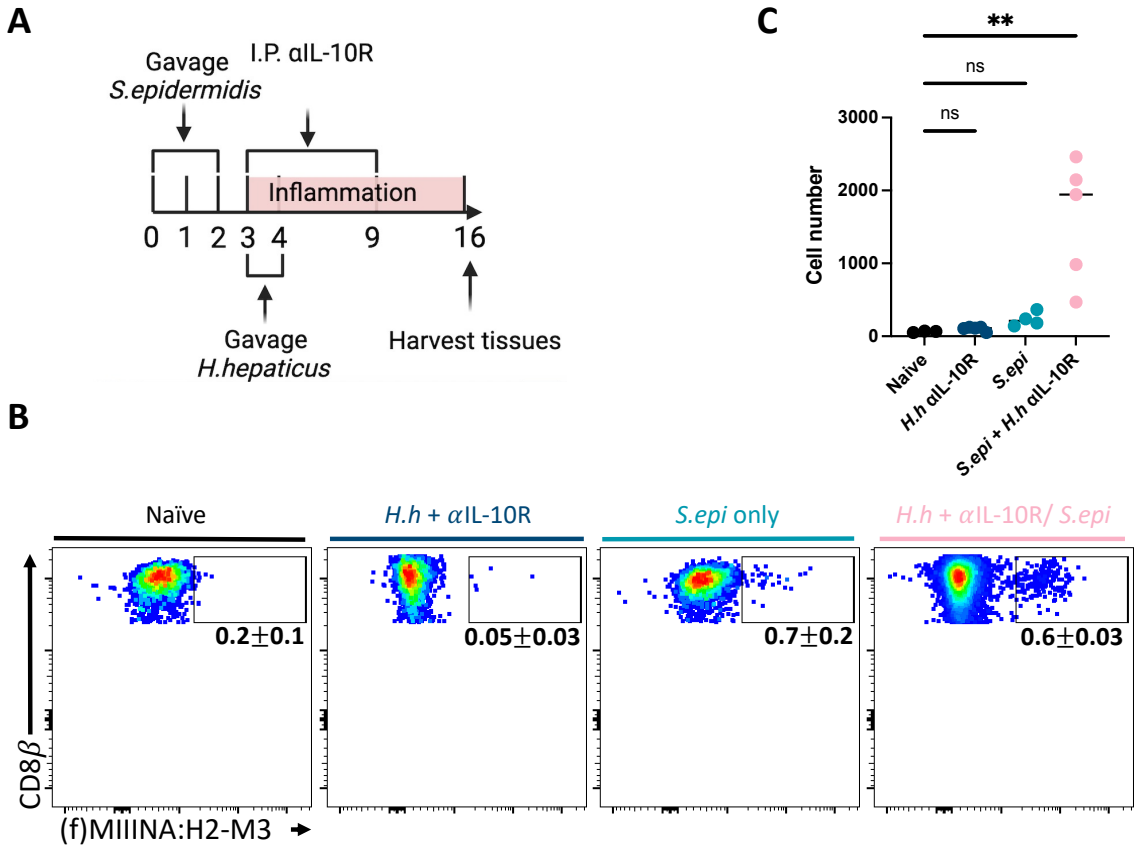


Figure 4.7: Commensal specific CD8⁺ T cells expand in colon during colitis. A. Schematic of model to study H2-M3 restricted CD8⁺ T cells during colitis **B.** Representative FACS plots with frequencies and **C** corresponding cell numbers of (f)MIIIINA:H2-M3⁺ CD8⁺T cells from colon LPL of naïve, *H.hepaticus* +αIL-10R treated, and *S.epidermidis* gavaged mice with and without *H.hepaticus* +αIL-10R. Plots are gated on CD45⁺ lineage⁻ CD90.2⁺ CD3ε⁺ CD8β⁺. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Data representative of 2 independent experiments.

colon LPL of colitic mice (Figure 4.7 B and C). Notably, *H.h* αIL-10R was not sufficient to induce a (f)MIIINA:H2-M3⁺ response, indicating that the expansion we observed was a *S. epi* reactive response and not due to inflammation alone, and suggests that within our colony of SPF mice, the native microbiota is unable to produce the (f)MIIINA peptide to immunogenic levels in homeostasis or colitis (Figure 4.7 B and C). We also observed that oral gavage with *S. epidermidis* in the absence of IL-10R signaling does not induce colitis (data not shown). (f)MIIINA:H2-M3⁺ cells also express high levels NKG2ACE, and CD39 and lower CD69 expression which are hallmarks of activation (Figure 4.8 A). Further, we observed relatively low cytokine production during inflammation, with an increased

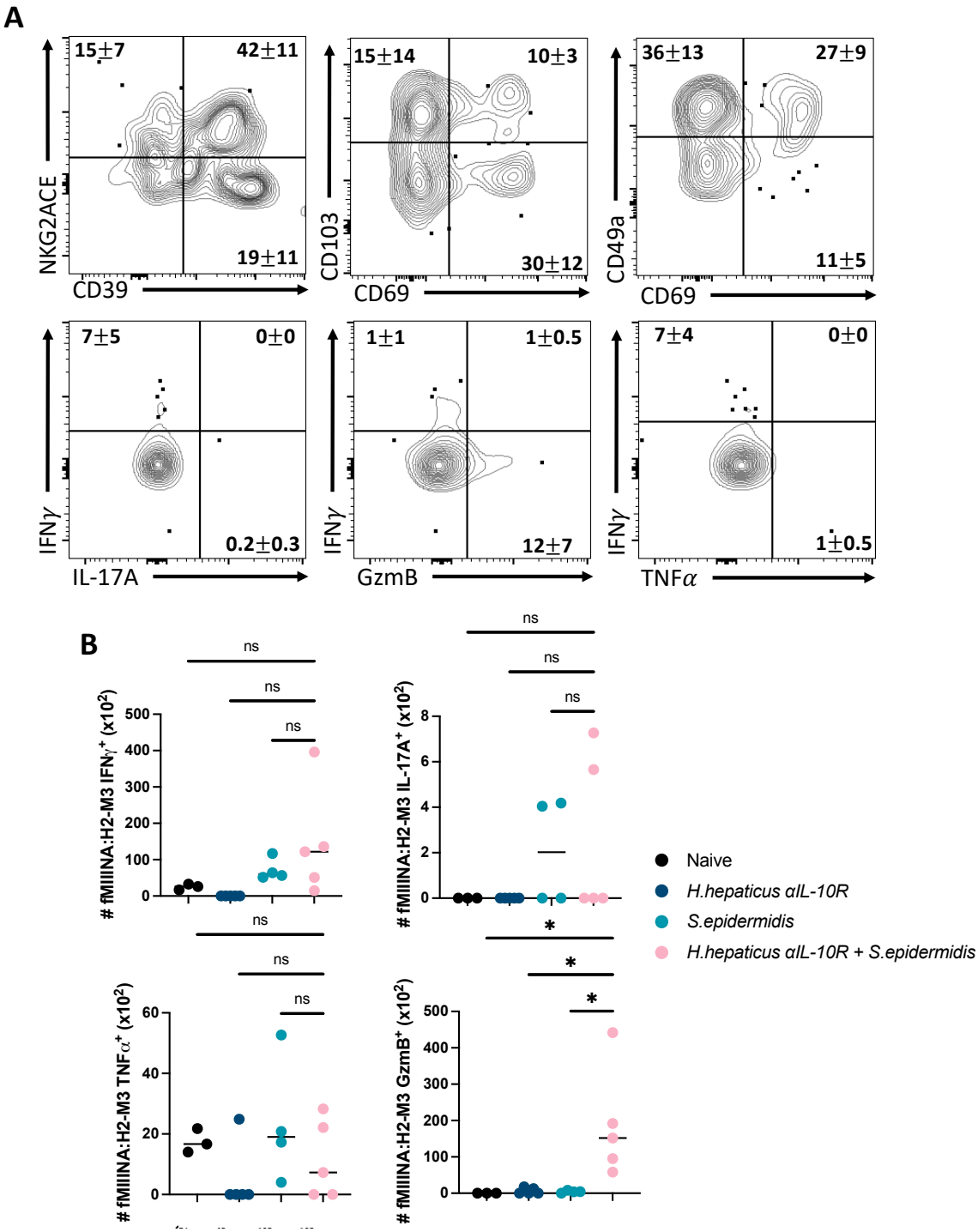


Figure 4.8: Commensal specific CD8⁺ T cells express Granzyme B and markers of activation during colitis. **A.** Frequencies of NKG2ACE⁺, CD39⁺, CD69⁺, CD103⁺, CD49a⁺, IFN-γ^+, IL-17A⁺ Granzyme B⁺ and TNFα^+, (f)MIIINA:H2-M3⁺ T cells extracted from colon LPL of *S.epidermidis* + *H.h* αIL-10R treated mice. Plots are gated on CD45⁺ lineage⁻ CD90.2⁺ CD3ε⁺ CD8β⁺ (f)MIIINA:H2-M3⁺ cells. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. **B.** Cell numbers of IFN-γ^+, IL-17A⁺ Granzyme B⁺ and TNFα^+, (f)MIIINA:H2-M3⁺ T cells. Data representative of 1 experiment.

expression of the cytotoxic granule, granzyme B during colitis (Figure 4.8 A and B) compared to their non-cytotoxic, Tc1 phenotype during homeostasis (Figure

4.5 B).

Taken together, these results indicate that commensal reactive H2-M3 restricted CD8⁺ T cells expand in the colon during colitis. Additionally, commensal reactive H2-M3 restricted CD8⁺ T cells exhibit a potential exhaustion phenotype due to the expression of CD39 and low cytokine potential during inflammation, however this would need to be confirmed by measuring the expression of other exhaustion markers such as PD1 and CTLA4[170,171] .

4.4 Discussion

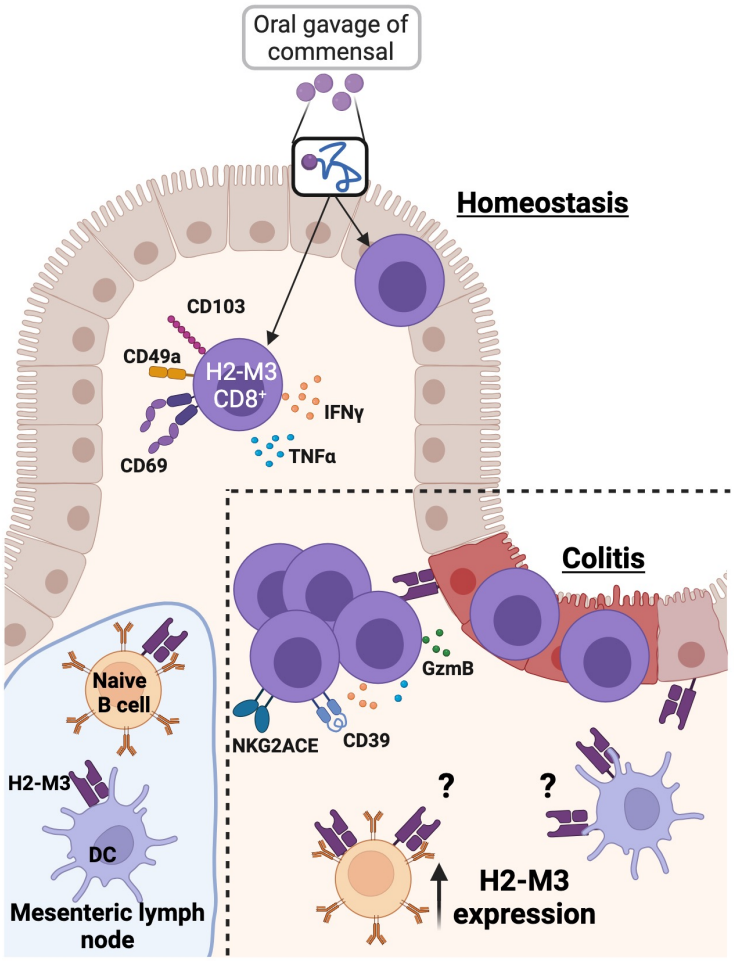


Figure 4.9: Summary of findings

In this chapter, we show that following commensal exposure in the gut, H2-M3 restricted CD8⁺ T cells can reside within this tissue where they express tissue

residency markers and produce type 1 cytokines such as IFNγ and TNFα. During the peak of colitis, these commensal reactive $CD8^+$ T cells expand in numbers yet maintain a limited capacity for cytokine production. This could support a hypothesis that the primary function of these cells is during homeostasis, and within the inflammatory environment they become exhausted. The impact of chronic inflammatory stimulus on the H2-M3 restricted, commensal reactive response has not yet been described.

In this chapter we also investigate the cell types capable of presenting H2-M3 in the colon during homeostasis and the *Helicobacter hepaticus* αIL-10R model of colitis. We confirm previous reports of low mRNA expression of *H2-M3* compared to MHC-Ia and contrast the expression profile of H2-M3 with other MHC-Ib molecules such as MR1, CD1d and Qa-1[78]. We show that the cells expressing the highest transcripts of *H2-M3* were naïve B cells, pDC's and $CD81^+$ lymph node resident DC's, and that IEC are responsive to inflammatory mediators during colitis driving an increase in *H2-M3* expression as well. These results will help inform the Cre lines we will utilize with H2-$M3^{fl/fl}$ mice that were developed for this project. It will be interesting to dissect whether antigen presentation is necessary for tissue residence, expansion during colitis, or cytokine production.

Interestingly, *H2-M3* expression more similarly mimics *Qa-1* expression in the datasets analyzed which may be due in part by their shared designation of "middle aged" MHC-Ib molecules[39]. Qa-1 is considered the murine functional homologue of HLA-E in humans, and it is proposed that due to selective pressures, these molecules arose in the two species by convergent evolution[172,173]. Thus, it is intriguing to consider a potential functional overlap

between H2-M3, Qa-1, and HLA-E restricted $CD8^+$ T cells. No human homologue of H2-M3 has yet been described, however, HLA-E has been characterized as presenting non-canonical peptides derived from intracellular pathogens such as *Mycobacterium tuberculosis* and *Salmonella enterica* Serovar Typhi[174–176]. Further work would be needed to investigate whether HLA-E, or another non-classical MHC I molecule, is capable of presenting fMet peptides in humans.

4.5 Conclusion

This work is the first to establish that commensal reactive, H2-M3 restricted $CD8^+$ T cells can reside within the colon and that they are responsive during inflammation. Further, it recapitulates key findings from human studies[118], providing a valuable platform for future exploration and functional investigation into the role of $CD8^+$ T cells in IBD. Finally, we show that transcripts for MHC-I molecules, and their necessary cofactors, are increased during colitis.

4.6 Future outlook and limitations

The potential of commensal reactive $CD8^+$T cells to become exhausted during colitis is intriguing and is supported by studies of chronic infection on $CD8^+$ T cells[170,171]. To test this, future studies could investigate the surface expression of other exhaustion markers such as PD1 and CTLA-4, and more fully illustrate the impact of inflammation on the function of these cells. This is of interest as recent studies have linked exhausted $CD39^+$ $CD8^+$ T cells to clinical outcomes in IBD[116,118]. Further studies would need to investigate whether these cells lose function during inflammation and truly become exhausted, or if the more activated and potent cytokine producing cells are lost during the cell extraction and ex-vivo stimulation process.

Further, H2-M3 restricted responses during *L. monocyotgenes* infection have been best characterized as aiding in the clearing of infected cells during primary infection but fail to contribute to the memory immune response to subsequent infection[85]. This is thought to be at least in part due to competition for antigen presenting cells by MHC-Ia restricted $CD8^+$ T cells during later infection[87]. However, it is interesting to consider how chronic inflammation tunes the H2-M3 restricted $CD8^+$ T cell immunity to a commensal, compared to the responses and roles during homeostasis, and what the long-term impacts are after the resolution of disease. To study this, we would need to assess (f)MIIINA:H2-$M3^+$ T cells after the peak of inflammation, likely through RNA sequencing or ATAC seq.

Finally, it should be noted that the transcriptomic data used in this chapter to identify H2-M3 expressing cells are not comprehensive of the entire cellular network of the colon, and only represent the cells involved in supporting the Treg response to *H.h* or within the colon organoid system. Therefore, future studies would need to be done to confirm H2-M3 expression in this tissue in a less biased way, such as RNAscope or spatial transcriptomics[177]. Moreover, RNA expression does not equate to protein expression and results should be interpreted with this in mind. For this project we endeavored to utilize the commercially available αH2-M3 antibody without success. Regardless, future work using H2-$M3^{fl/fl}$ mice will illuminate the importance of H2-M3 expression on various cell types for driving commensal specific responses during both homeostasis and inflammation.

5 Trafficking of commensal reactive CD8$^+$ T cells to the colon during colitis

5.1 Introduction

In Chapter 4 we describe a H2-M3 restricted commensal reactive CD8$^+$ T cell response in the gut and its expansion during colitis utilizing the (f)MIIINA:H2-M3 tetramer[60]. We also observed a dissemination of CD44^{high} commensal reactive CD8$^+$ T cells into the gut draining lymph node following topical association with *S. epidermidis* (Figure 4.4 B) which led us to ask whether these cells could contribute to the CD8$^+$ T cell response to colitis despite colonization occurring at the skin.

CD8$^+$ T cells have been largely understudied in inflammatory bowel disease research, although some models utilizing transgenic autoreactive CD8$^+$T cells implicate them as sufficient to initiate disease[113,114]. However, these studies better recapitulate autoimmune disease, wherein the immune response is directed towards self. Although self-reactive CD8$^+$ T cells might indeed be present, in IBD, inflammation is currently believed to be driven more by a maladaptive response to the commensal microbiota. Recent studies have shown CD8$^+$ T cell signatures in circulating PBMC and tissue samples of patients with IBD with varying prognostic correlations[107,116,117]. Of note, Boland and colleagues report clonal similarities between CD8$^+$ T cells in the colon and circulating PBMC in patients with ulcerative colitis, indicating trafficking between compartments[117]. Whether this is due to bystander inflammation independent of antigen or antigen-cross reactivity remains to be resolved.

5.2 Aim

In this chapter we investigate the potential contribution of commensal reactive $CD8^+$ T cell responses elicited by skin colonization to the $CD8^+$ T cell infiltration during colitis.

5.3 Results

5.3.1 Commensal reactive T cells elicited in the skin traffic to gut during colitis

To study the impact of commensal reactive $CD8^+$ T cell responses elicited in the skin on the infiltration of $CD8^+$ T cells during colitis, we first established a model wherein mice were topically associated with *Staphylococcus epidermidis* to induce a commensal reactive response we could track using the (f)MIIINA:H2-M3 tetramer. Next, we induced colitis using the *Helicobacter hepaticus* αIL-10R model (Figure 5.1 A). Topical association with *S. epi* induced an (f)MIIINA:H2-M3 Tc1 and Tc17 response in the skin as previously described[58–61] which was slightly increased in the mice treated with *H.h* αIL-10R (Figure 5.1 B and C).

IL-10 production by skin $GATA3^+$ Tregs is essential to constrain and direct the immune response to the commensal microbiota[61]. Thus, to investigate whether this increase in (f)MIIINA:H2-$M3^+$ infiltration in the ear in our model is due to the synchronous association of *S. epi* and αIL-10R treatment, we also tested the impact of IL-10R blockade on the local immune response during topical association with *S. epi.* Mice were associated with *S. epidermidis* at two-day intervals for a week and treated with αIL-10R on day 0 and day 7 (Figure 5.2 A). Ear thickness was significantly higher (Figure 5.2 B) and cell numbers of $CD4^+$, $FoxP3^+$ Treg, $CD8\beta^+$ and (f)MIIINA:H2-$M3^+$ cells all increased in the ears of mice treated with αIL-10R treatment (Figure 5.2 B). These results indicate that the

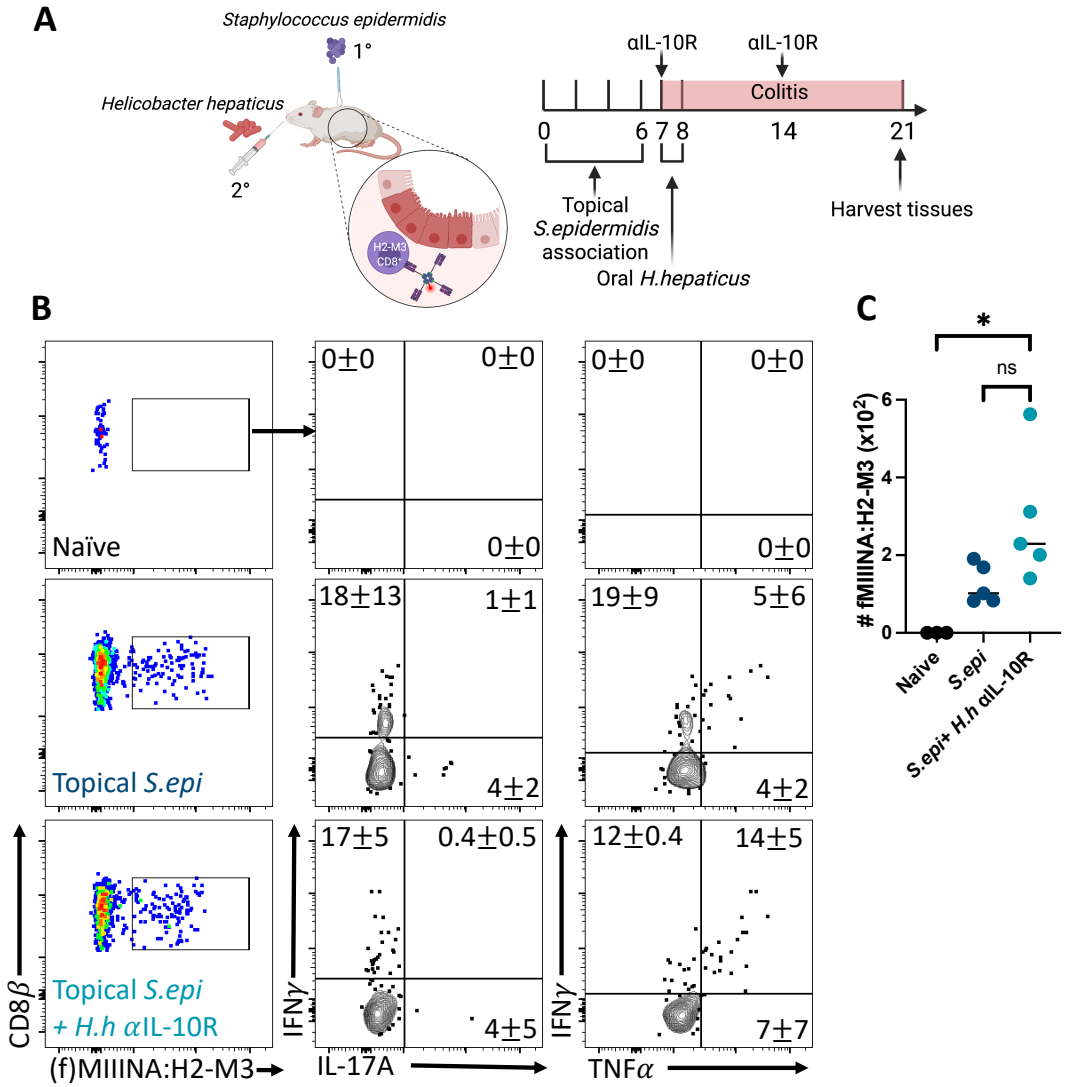


Figure 5.1: Commensal specific H2-M3 restricted CD8⁺T cells are elicited in the skin after topical association. A. Schematic of model system to track commensal specific CD8⁺T cells across tissues during inflammation **B.** Representative FACS plots of tetramer staining of lymphocytes extracted from ears of mice associated with *S.epidermidis* with or without *Helicobacter* induced colitis. Cells are gated on CD45⁺ lineage⁻ (MHCII, CD11b, γδTCR, NK1.1) CD90.2⁺ CD3ε⁺ CD8β. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. **C.** Cell numbers of (f)MIIINA:H2-M3⁺ CD8⁺ T cells. One way ANOVA was used to measure significance.** p <0.01, *** p <0.001. Data representative of 2+ independent experiments.

blockade of IL-10R signaling in our model does impact the local immune response to a skin commensal as previously described[61].

We then assessed whether commensal reactive CD8⁺ T cells that were elicited after topical association with *S. epi* can infiltrate the colon during *H.h* αIL-10R induced colitis. As observed previously, topical association with *S. epidermidis* alone was not sufficient for (f)MIIINA:H2-M3⁺ T cells to populate the

colon[60]. However,

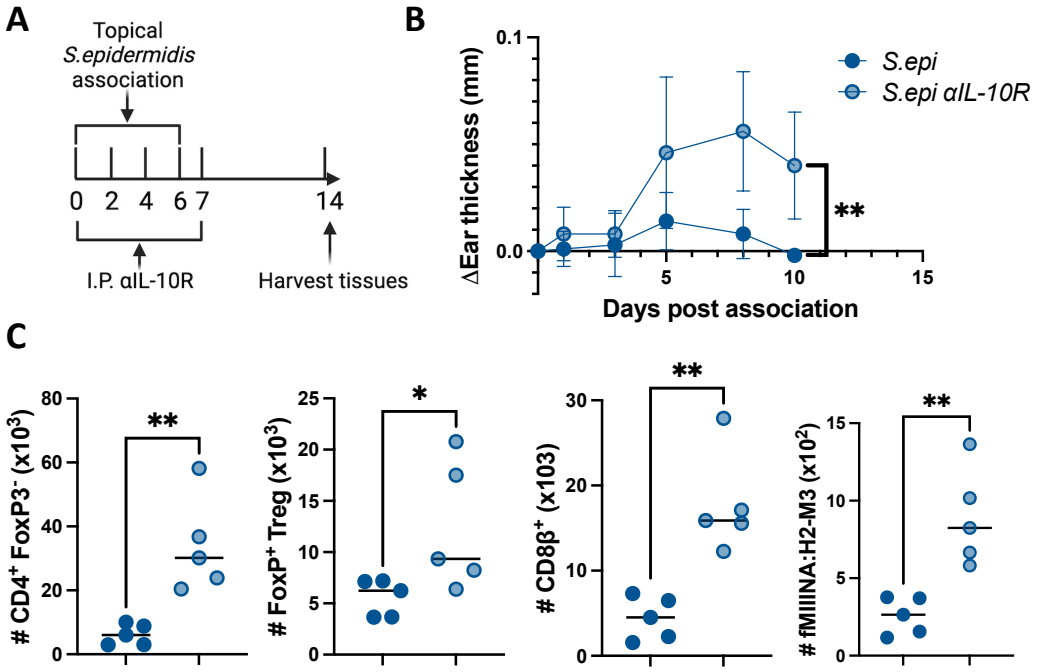


Figure 5.2: IL-10 signaling constrains low level inflammation to novel skin commensal. A. Schematic of topical *Staphylococcus epidermidis* association with αIL-10R **B.** Change in ear thickness **C.** Cell numbers of total CD8β^+, CD4$^+$FoxP3$^-$, Treg, and (f)MIIINA:H2-M3$^+$ CD8$^+$ T cells from ear of *S.epi* associated and *S.epi* + αIL-10R treated animals. Cells are gated on CD45$^+$ CD90.2$^+$ CD3ε^+. Mann-Whitney was used to measure significance.* p =0.05, ** p <0.01. Data representative of 2 independent experiments.

there was significant infiltration of (f)MIIINA:H2-M3$^+$ in both the lamina propria and epithelial compartments in mice treated with *H.h* αIL-10R (Figure 5.3 A and B). Infiltrating cells produced largely Type 1 cytokines IFNγ and TNFα and minimal production of IL-17A (Figure 5.3 A).

To identify whether this was a phenomenon unique to (f)MIIINA reactive CD8$^+$ T cells we utilized Bowie^{Tg} T cells cross to a Rag1$^{-/-}$ background, which are H2-M3 restricted and *S. epidermidis* reactive, but to a different, undescribed peptide[61]. We adoptively transferred ~2x10^5 naïve Bowie^{Tg} one day prior to topical association with *S. epi* and proceeded with the model as before (Figure 5.1 A). We observed similar infiltration of (f)MIIINA:H2-M3$^+$ and Bowie^{Tg} T cells in the colon LPL and mesenteric lymph nodes during colitis, with minimal expansion within other intestinal tissues including the duodenum, jejunum, and ileum (Figure

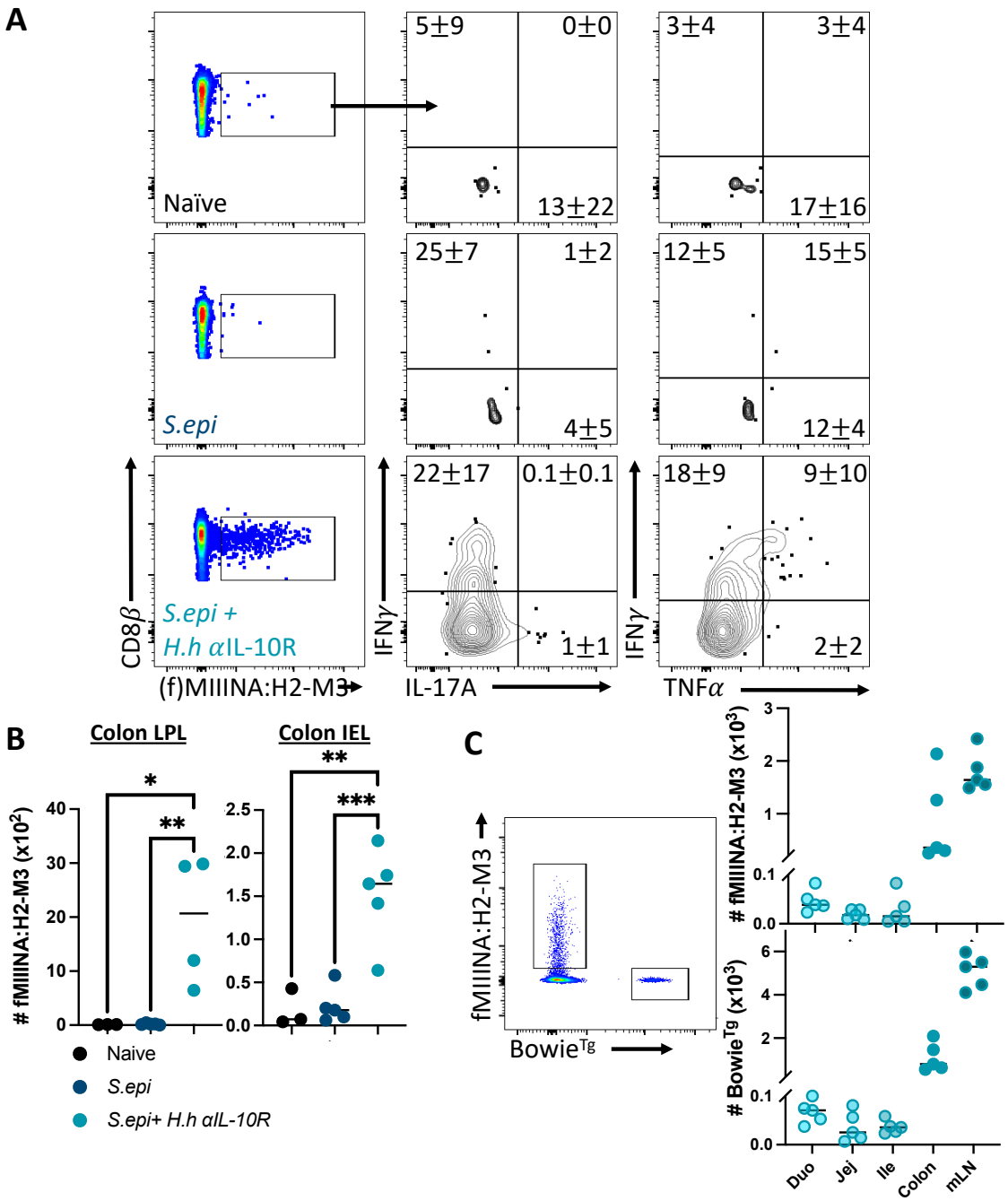


Figure 5.3: Commensal specific CD8⁺ T cells elicited by skin colonization populate colon LPL and IEL during colitis. A. Representative FACS plots of tetramer staining and cytokine production of lymphocytes extracted from colon LPL of mice associated with *S.epidermidis* with or without *Helicobacter* induced colitis. Cells are gated on CD45⁺ lineage⁻ (MHCII, CD11b, γδTCR, NK1.1) CD90.2⁺ CD3ε⁺ CD8β⁺. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD **B.** Cell numbers of (f)MIIINA:H2-M3⁺ T cells from colon LPL and IEL of naïve *S.epidermidis* associated and *H.hepaticus* +αIL-10R and *S.epidermidis* associated mice. **C.** Mice were injected with Bowie^{Tg} T cells one day prior to topical association with *S.epi*. Representative FACS plot of tetramer and Bowie^{Tg} staining in colon LPL, and quantification of cell numbers in the LPL of gut tissues and mLN. One way ANOVA was used to measure significance.* $p < 0.05$** $p < 0.01$, *** $p < 0.001$. Data representative of 1-2 independent experiments.

5.3 C). Collectively, these results illustrate that a commensal reactive H2-M3 restricted CD8⁺ T cell response to two different antigens, elicited in the skin, can

contribute to the $CD8^+$ T cell response in the colon during colitis where they produce largely Type 1 cytokines.

5.3.2 Trafficking to colon is independent of oral ingestion of S. epidermidis during association

Following topical association, mice profusely groom raising the possibility that this phenotype could be driven by potential gut colonization of *S. epidermidis* and antigen exposure within that tissue. Thus, we adopted an antibiotic treatment regimen to block colonization of *S. epidermidis* during the high microbial loads during topical association. Vancomycin was chosen for these experiments due to its selective action against $gram^+$ bacteria and low systemic absorption, thus will only affect oral ingestion and not restrict skin colonization[178].

The strain of *Staphylococcus epidermidis* used for these studies was first isolated from the skin of humans[8] which can contain species with Vancomycin resistance[179]. Therefore, we first confirmed that our strain was susceptible to vancomycin with a minimum inhibitory concentration of < 4ug/mL and that this concentration of vancomycin used was sufficient to inhibit growth in-vitro (Figure 5.4 B). Mice were given 0.5g/L of vancomycin with sweetener or sweetener alone in their drinking water one day before and throughout the course of topical association with *S. epi* to prevent colonization of the colon during this period, then taken off antibiotics for *H.h* αIL-10R treatment (Figure 5.4 A). $CD4^+$, $CD8\beta^+$ and (f)MIIINA:H2-$M3^+$ T cells were similar in the ears of all mice associated with *S. epi,* indicating that treatment with vancomycin did not impact skin colonization and the subsequent induction of a commensal reactive T cell response (Figure 5.4 C). Treatment with vancomycin also did not affect the total $CD8^+$ T cell infiltrate during colitis compared to *S. epi* association alone or with sweetener, nor did it

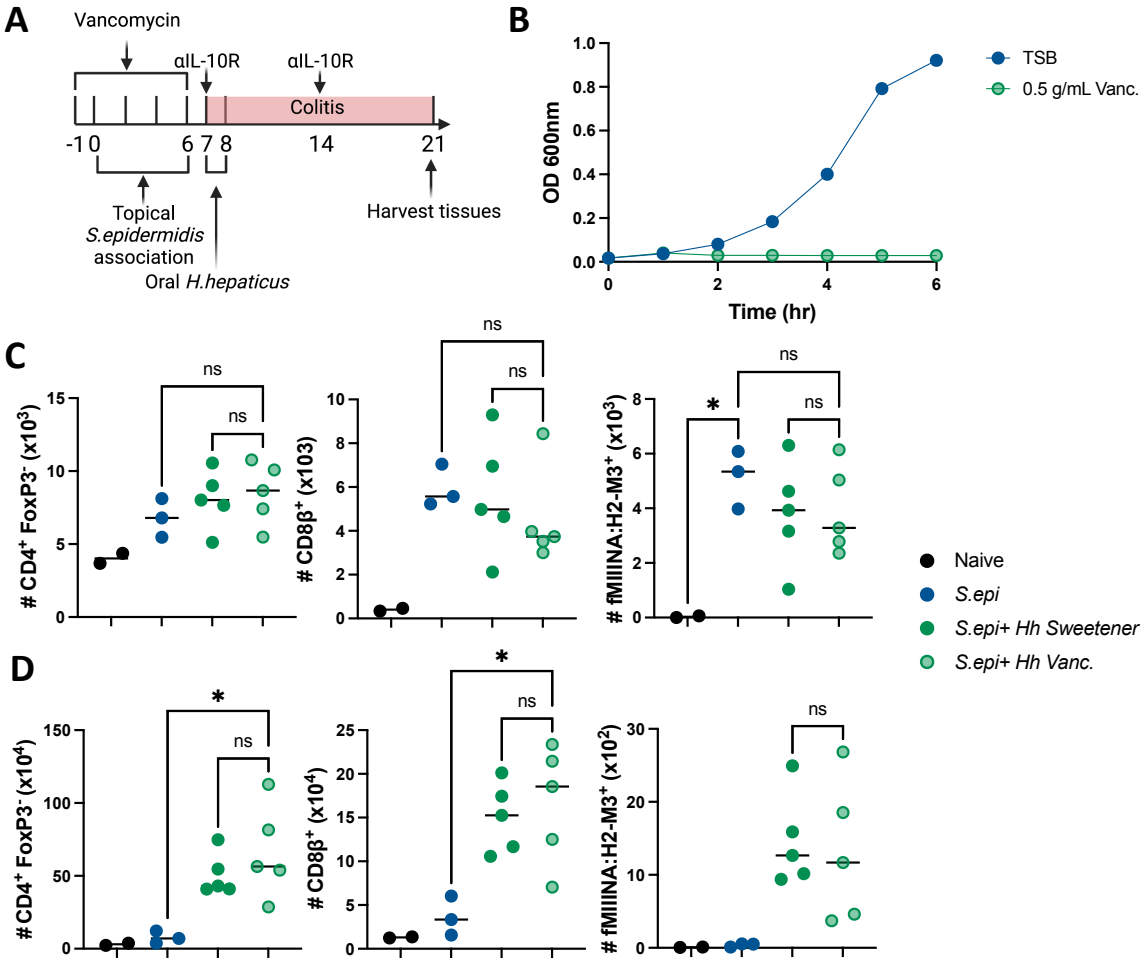


Figure 5.4: Trafficking to colon is independent of gut colonization during association. A. Schematic of experimental design **B.** Growth curve of *S.epi* with or without Vancomycin **C.** Cell numbers of total CD4$^+$Foxp3$^-$ CD8β^+ and (f)MIIINA:H2-M3$^+$ CD8$^+$ T cells from ear of Naïve, *S.epi* associated, S.epi associated and *Hh* colitis with control water, *S.epi* associated and *Hh* colitis with Vancomycin treated water. **D.** Cell numbers of CD4$^+$ FoxP3-, CD8β^+, (f)MIIINA:H2-M3$^+$ CD8$^+$ T cells from colon LPL. One way ANOVA was used to measure significance. Data representative of 1 experiment

affect the CD4$^+$ T cell response during colitis, indicating that modulation of the gut microbiota due to vancomycin did not affect the disease course of *H. hepaticus* colitis, confirming previous studies[180] (Figure 5.4 C).

Importantly, treatment with vancomycin did not affect the (f)MIIINA:H2-M3$^+$ response to colitis (Figure 5.4 D). These results suggest that oral ingestion of *S. epi* during topical association is not responsible for the emergence of commensal reactive CD8$^+$ T cells to this tissue during colitis.

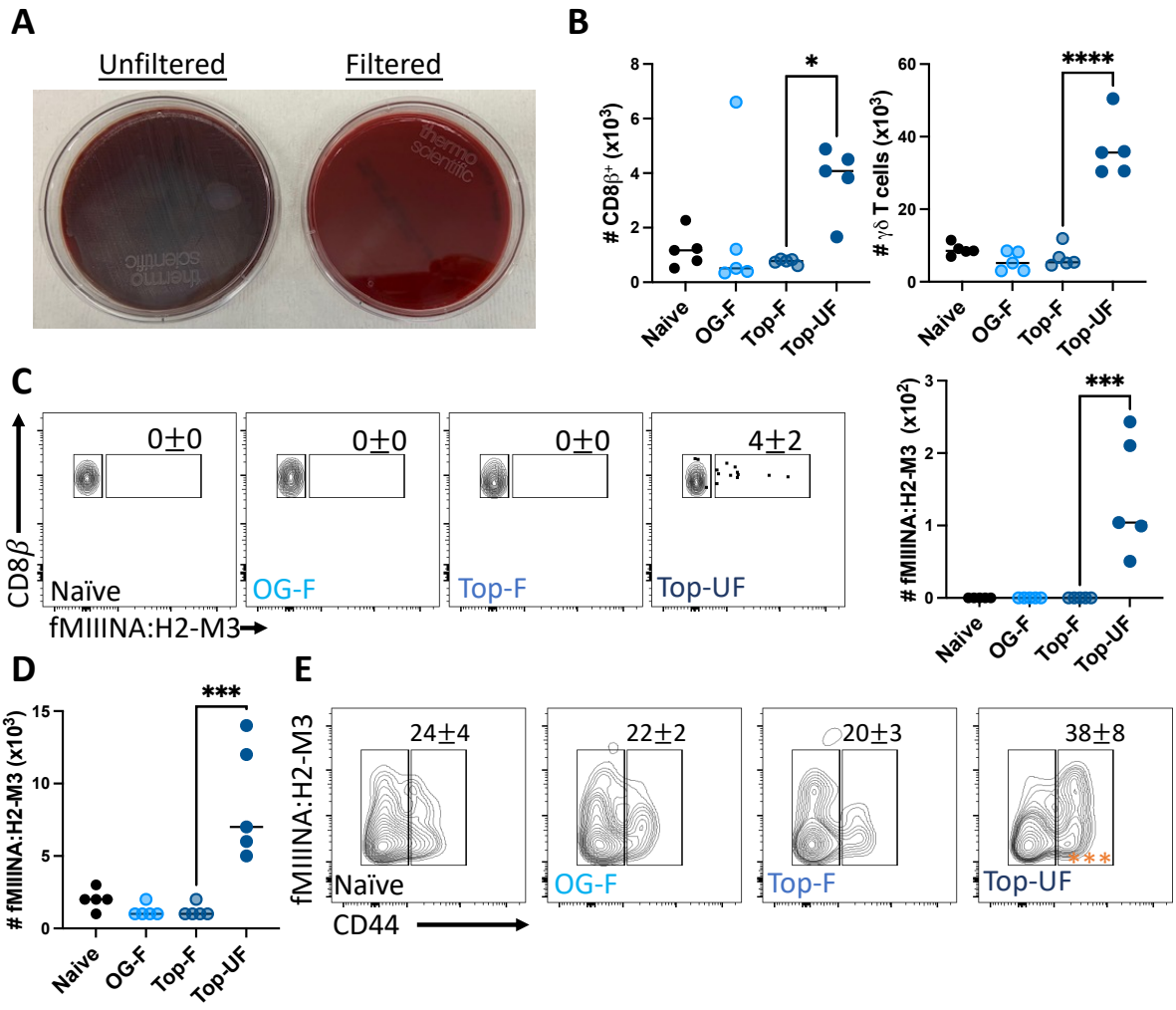


Figure 5.5: Induction of commensal specific response is dependent on live colonization. A. Blood agar plates streaked with Unfiltered (UF) and Filtered (F) TSB broth from *S.epidermidis* culture **B.** Cell numbers of $\gamma\delta$ T cells and CD8β^+ T cells from ears of mice that were naïve, gavaged with filtered broth (OG-F), topically associated with filtered broth (Top-F), or topically associated with unfiltered broth (Top-UF) **C.** Representative FACS plots with frequencies of (f)MIIINA:H2-M3$^+$ cells from the ears of Naïve, OG-F, Top-F, and Top-UF and quantification. Cells are gated on Live CD45$^+$ CD90.2$^+$ CD3ε$^+$ CD8β **D.** Quantification of (f)MIIINA:H2-M3$^+$ cells enriched from spleen of indicated groups **E.** Representative FACS plot of (f)MIIINA:H2-M3$^+$ T cells in mLN of indicated groups. Cells are gated on Live CD45$^+$ CD90.1$^+$ CD3ε$^+$ CD8β$^+$ (f)MIIINA:H2-M3$^+$. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Stars indicate significance compared to naïve. One way ANOVA was used to measure significance.* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Data representative of 1 experiment.

As bacteria grow in culture, they release antigens that could potentially induce an immune response regardless of live colonization on the skin. Further, prior studies had indicated that the (f)MIIINA antigen produced by *S. epi* was secreted, and therefore could be present in growth medium[60]. Therefore, it is feasible that despite blocking live colonization within the gut during topical association, there could be antigens in the broth, that when ingested, are

sufficient to induce a systemic commensal reactive response. To test this, we took media from *S. epi* culture and passed it through a 0.2μm filter for sterilization while allowing any antigens present in the broth to remain. Sterility of the broth was confirmed by overnight culture of the contents and no CFU's were present (Figure 5.5 A). Topical association with filtered *S. epi* media was not sufficient to induce a $CD8^+$ T cell response in the skin, indicating that the local $CD8^+$ T cell response to a commensal microbe is dependent on live colonization on the skin (Figure 5.5 B). (f)MIIINA:H2-$M3^+$ T cells were also only found in the ears of mice associated with live microbe (Figure 5.5 C). $\gamma\delta^+$ T cells also expand after colonization with *S. epi*[58], but the antigens or factors responsible have not yet been described. It is believed that $\gamma\delta^+$ T cells instead respond to cytokines or innate signaling molecules which could still be present in the filtered broth[181]. The cell numbers of $\gamma\delta^+$ T cells were unchanged in mice that were associated with filtered broth, indicating that live colonization is required for all aspects of the immune response to *S. epi* in the skin (Figure 5.5 B). Mice that were gavaged or topically associated with filtered media did not have a systemic induction of *S. epi* reactive $CD8^+$T cells (Figure 5.5 D), nor was there an expansion of $CD44^{high}$ (f)MIIINA:H2-$M3^+$ cells in the mLN suggesting that there are not antigens in the culture broth that could be responsible for this phenotype alone (Figure 5.5 E).

Taken together, these results collectively indicate that live microbial colonization with a commensal microbe on the skin is required for the systemic induction of a commensal reactive response. Whether secreted antigens alone are sufficient to drive $CD8^+$ T cells into the colon after topical association and the priming of a commensal reactive response, however, remains to be tested.

5.3.3 Commensal reactive CD4⁺ T cells do not infiltrate the colon during colitis

A recent study by Merana and colleagues described the CD4⁺ T cell response to *S. epidermidis* during DSS colitis, illustrating that commensal reactive CD4⁺ T cells are also able to traffic between the skin and gut during colitis, and that gut inflammation augments the antigen specific response to a skin commensal[167]. In their model system they first colonize neonatal mice with *S. epi* which preferentially induces a regulatory T cell response[182], as opposed to our model system in which we colonize adult mice which induces a Tc17 and Tc1 response[58]. Further, bystander CD4⁺ T cells have also been reported in infection, autoimmunity, and cancer[183], however the majority of CD4⁺ T cell responses in IBD are so far believed to be antigen specific. We were then curious whether the gut "homing" phenotype of commensal reactive CD8⁺ T cells during colitis was shared with other commensal reactive CD4⁺ T cells.

Topical association with *Staphylococcus aureus* induces a non-inflammatory, commensal reactive CD4⁺ T cell response that can be tracked using transgenic $SA1^{Tg}$ T cells which have been crossed to a $Rag^{-/-}$ background[184]. We adoptively transferred ~$2x10^5$ $SA1^{Tg}$ to mice topically associated with *S. aureus*, and then induced colitis one week later using the *H.h* αIL-10R model of colitis (Figure 5.6 A). The frequency and cell numbers of $SA1^{Tg}$ T cells were unchanged in the ears of mice with and without colitis, indicating that treatment with αIL-10R did not influence the immune response to *S. aureus* (Figure 5.5 B and C). Despite an induction of a local response in the skin, $SA1^{Tg}$ did not infiltrate the colon nor expand during colitis indicating that the homing of commensal reactive CD4⁺ T cells to the colon during colitis is not universal and may depend on the microbe (Figure 5.6 B and C). These results suggest that commensal reactive CD4⁺ T cells

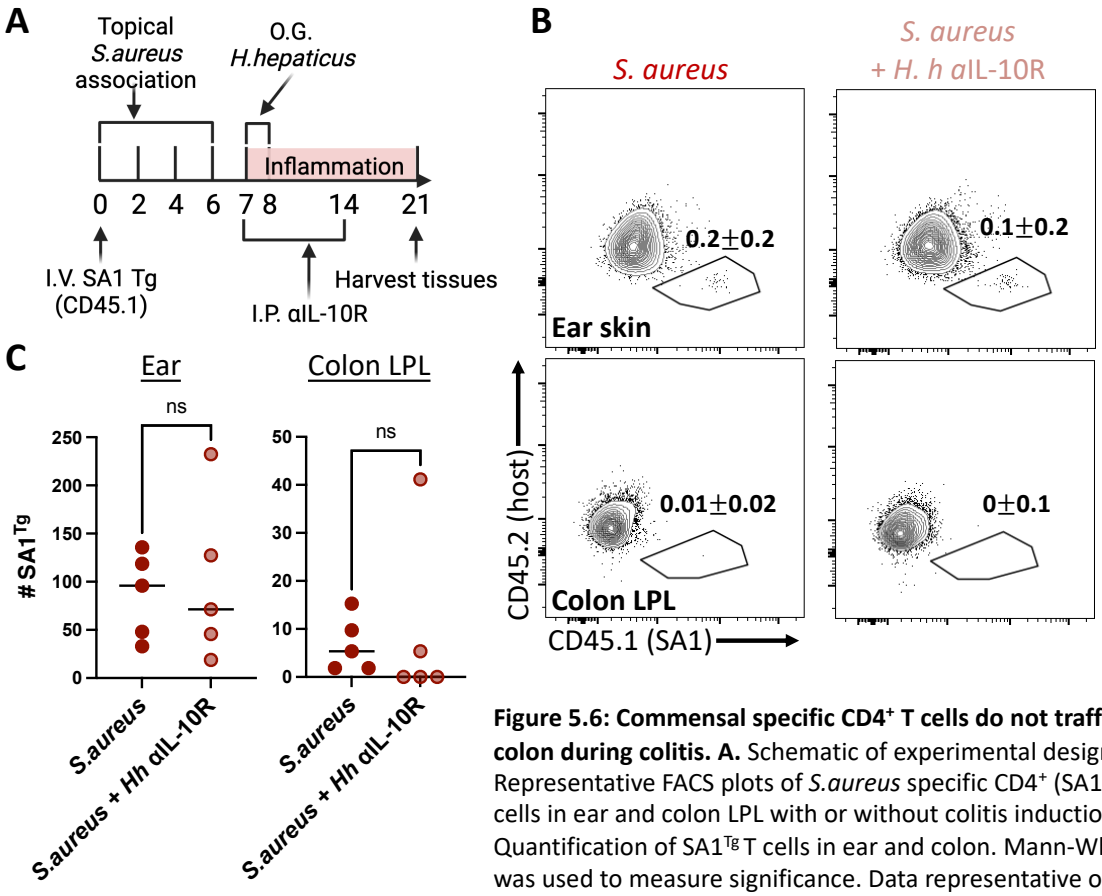


Figure 5.6: Commensal specific CD4⁺ T cells do not traffic to colon during colitis. A. Schematic of experimental design **B.** Representative FACS plots of *S.aureus* specific CD4⁺ ($SA1^{Tg}$) cells in ear and colon LPL with or without colitis induction **C.** Quantification of $SA1^{Tg}$ T cells in ear and colon. Mann-Whitney was used to measure significance. Data representative of 1 experiment.

do not infiltrate the colon during colitis, potentially indicating a lack of colonization of *S. aureus* as was observed with *S. epidermidis*[167]. Additionally, it may indicate that this phenomenon may be specific to commensal reactive CD8⁺ T cells which might maintain a capacity to respond to inflammation independent of antigen, which remains to be tested.

5.3.4 Classical and non-classical CD8⁺T cells infiltrate colon during colitis

H2-M3 restricted CD8⁺ T have been described as playing key roles in the early response to infection but are replaced by classically restricted CD8⁺ T cells at later timepoints[85,164]. We therefore wanted to compare the commensal reactive H2-M3 restricted CD8⁺ T cell response to a classically restricted CD8⁺ T cell response. Chen and colleagues engineered a strain of *S. epidermidis* NIHLM087 that expresses the OT-I peptide derived from ovalbumin[185] allowing us to

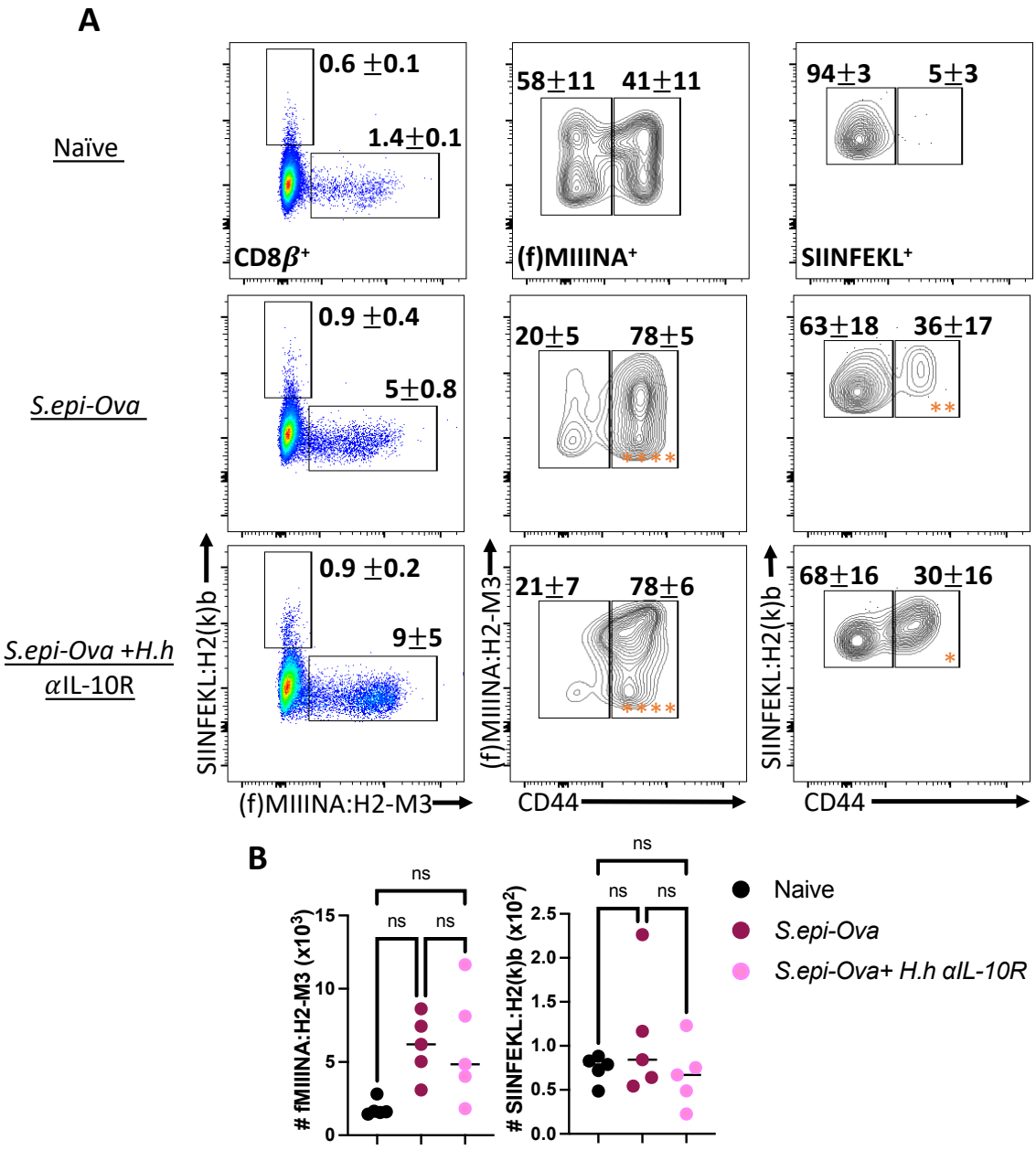


Figure 5.7: *S.epi*-Ova induces a MHC-Ia and MHC-Ib restricted systemic response that can be tracked using tetramers. A. Representative FACS plots of tetramer staining of lymphocytes enriched from the spleen mice associated with *S.epidermidis*-Ova with or without *Helicobacter hepaticus* αIL-10R induced colitis. Cells are gated on Live CD45$^+$ CD90.2$^+$ CD3ε$^+$ CD8β. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. **B.** Cell numbers of (f)MIIINA:H2-M3$^+$ and SIINFEKL:H2(k)b$^+$ CD8$^+$ T cells. One way ANOVA was used to measure significance.* $p < 0.05$, **** $p < 0.0001$. Data representative of 3 experiments.

simultaneously track (f)MIIINA:H2-M3$^+$ *S. epi* responses, as well as K^b restricted *S. epi* reactive CD8$^+$ T cell responses using the SIINFEKL:H2(k)b tetramer. Mice were associated every other day for 7 days with *S. epi*-Ova and colitis induced using the *H.h* αIL-10R model as previously described (Figure 5.1 A). Topical association with *S. epi*-Ova was sufficient to induce a systemic CD44^{high}

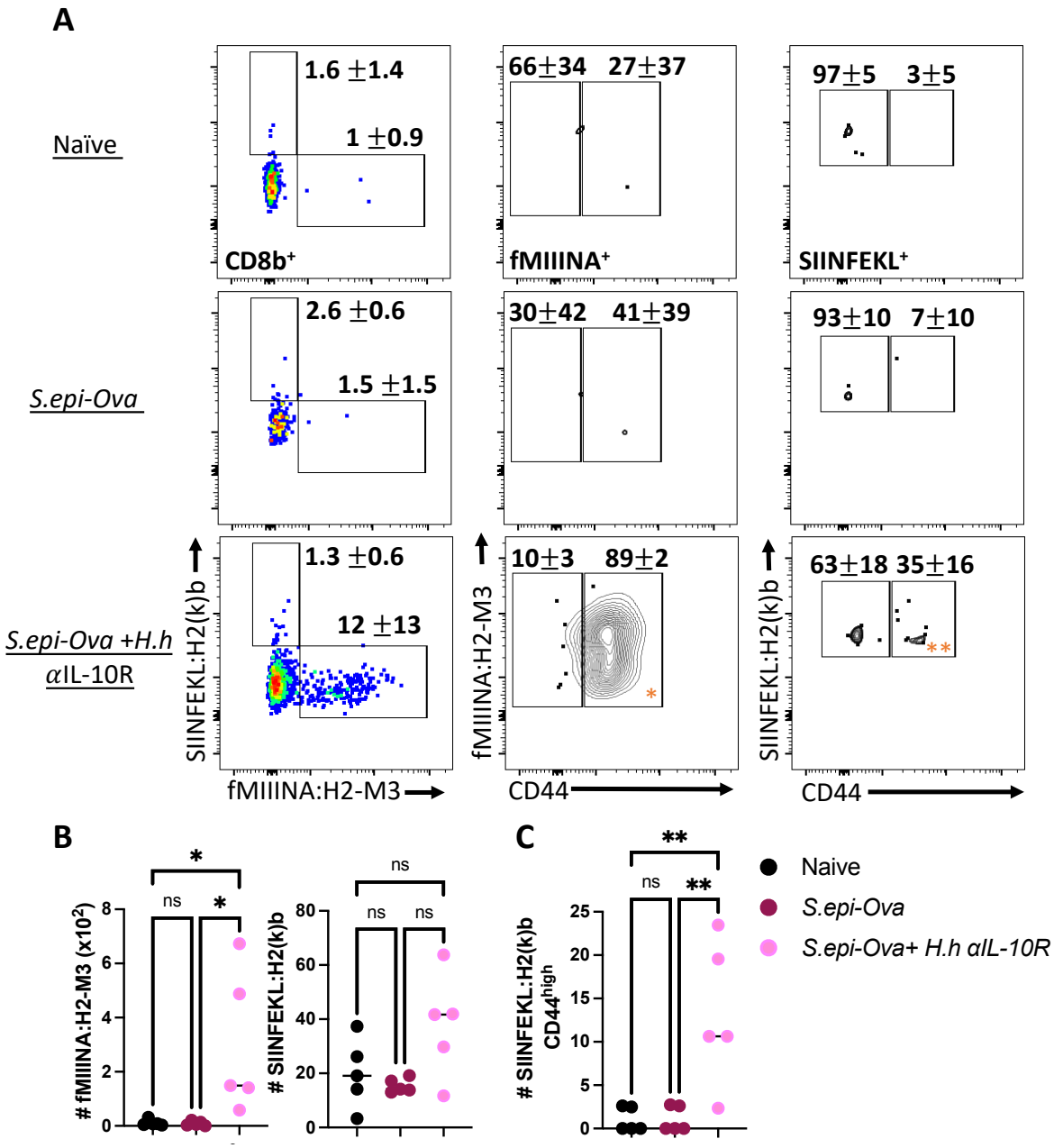


Figure 5.8: Commensal specific MHC-Ia and MHC-Ib restricted CD8⁺ T cells traffic to colon during colitis. **A.** Representative FACS plots of tetramer staining of lymphocytes enriched from the colon LPL of mice associated with *S.epidermidis*-Ova with or without *Helicobacter hepaticus* αIL-10R induced colitis. Cells are gated on Live CD45⁺ CD90.2⁺ CD3ε⁺ CD8β. Numbers in flow cytometry plots correspond to the frequency of gated populations ±SD. Stars indicate significance compared to naïve. **B.** Cell numbers of (f)MIIINA:H2-M3⁺ and SIINFEKL:H2(k)b⁺ CD8⁺ T cells. **C.** Cell numbers of CD44^{high} SIINFEKL:H2(k)b⁺ cells. One way ANOVA was used to measure significance.* $p < 0.05$, **** $p < 0.0001$. Data representative of 1 experiment.

(f)MIIINA:H2-M3 and SIINFEKL:H2(k)b CD8⁺ T cell response (Figure 5.7 A). However, there were notably fewer SIINFEKL:H2(k)b⁺ than (f)MIIINA:H2-M3⁺ T cells, signifying immunodominance against the (f)MIIINA peptide compared to SIINFEKL (Figure 5.7 B). Further, there was no significant difference between the

total numbers of tetramer$^+$ cells in the *S. epi* + *H.h* αIL-10R treated group and the *S. epi* group, indicating that induction of colitis with this model did not affect the amplitude of a commensal reactive response (Figure 5.7 B).

In the colon lamina propria, (f)MIIINA:H2-M3$^+$ cells infiltrated during colitis and were a robust population as seen before (Figure 5.8 A and B). In contrast, SIINFEKL:H2(k)b$^+$ CD8$^+$ T cells were approximately 10-fold fewer, and there was no significant difference in the total cell numbers between colitic and non-colitic animals (Figure 5.8 B). However, the few SIINFEKL:H2(k)b$^+$ present had a significant upregulation of CD44 in colitic animals compared to *S. epi* associated alone (Figure 5.8 A and C). These results therefore collectively suggest that classical and non-classically restricted, commensal reactive CD8$^+$ T cells can infiltrate the colon LPL during colitis.

5.4 Discussion

In this chapter we describe the expansion of commensal reactive CD8$^+$ T cells in the colon during inflammation after elicitation from topical association. This potential trafficking is independent of antigen exposure in the gut by live microbial colonization during association, and antigens present in the filtered culture medium alone are insufficient to induce a commensal reactive response. We did not observe a similar expansion of commensal reactive CD4$^+$ T cells in the gut in our model, contrasting recent work investigating a similar phenomenon[167]. It should be noted, however, that in their study, Merana et al. reported colonization of the gut with *S. epi* during colitis, which could be driving the commensal reactive CD4$^+$ T cells into this tissue. Although we did not assess the colonization with *S. aureus* in the colon of the mice in our model, it is feasible that it is unable to

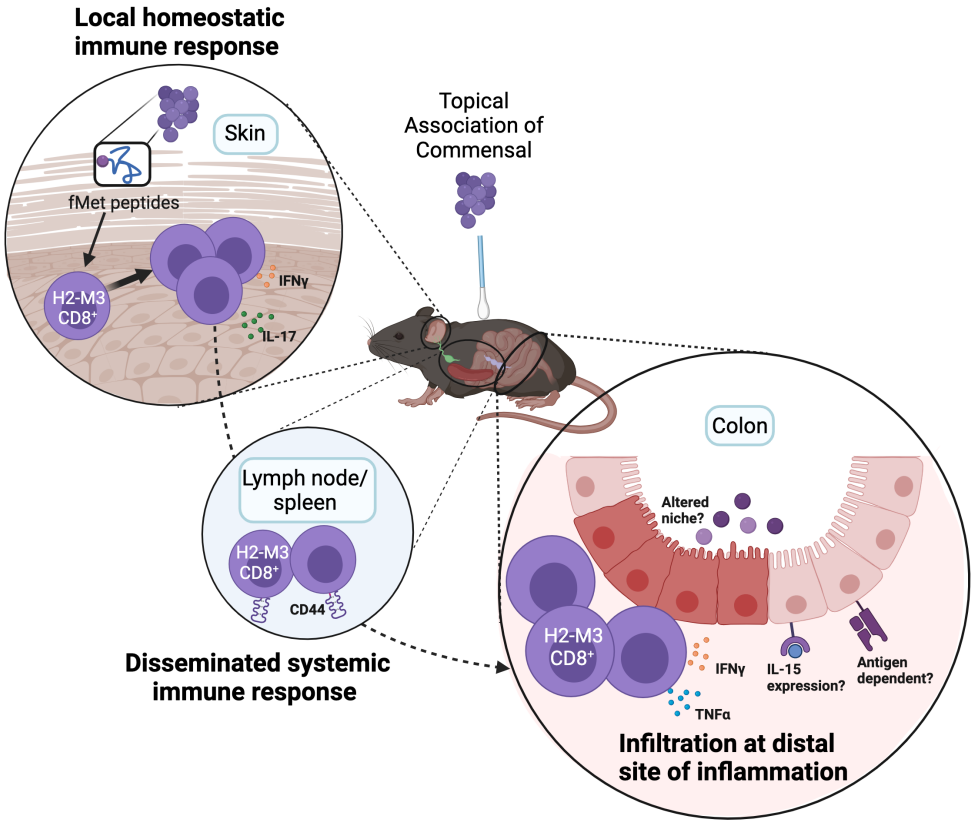


Figure 5.9 **Summary of findings**

colonize in the same way as *S. epi.* Finally, we show that both classical and non-classically restricted, commensal reactive CD8⁺ T cells can infiltrate the colon during colitis, to varying degrees.

Although from these data we cannot exclude the potential for antigen exposure in the gut by *S. epi* colonization or antigen cross-reactivity, it is feasible that this phenomenon is “bystander” activation of commensal reactive CD8⁺ T cells, likely in a similar manner to CD8⁺ T cells during infection. Although there have been multiple reports of CD8⁺ T cell signatures in circulating PBMC and biopsy samples from patients with IBD, it is unknown whether these cells are responding to commensal or reactivated viral antigens, or if it is a byproduct of inflammation[107,116,117]. Thus, our work could support the notion that these CD8⁺ T cell signatures might be due to inflammation recruiting and promoting the proliferation of memory CD8⁺ T cells that could be commensal reactive. Notably,

commensal reactive T cell responses can be quite robust, the (f)MIIINA:H2-M3$^+$ T cell response dominates SIINFEKL:H2(k)b$^+$ T cells even in the absence of overt inflammation (Figure 5.7). Thus, investigation into the collective impact of the homeostatic commensal exposures at barrier sites, resulting CD8$^+$ T cell response and the potential contribution during inflammation would be quite intriguing. It should be noted however, that further studies would need to confirm if these commensal reactive CD8$^+$ T cells are acting independently of antigen, whether inflammation itself drives an altered niche allowing for *S. epi* colonization, or if there is potential antigen cross-reactivity with other microbiota members.

Bystander activation occurs in memory CD8$^+$ T cells in the absence of cognate antigen through stimulation with cytokines such as Type I Interferons, IL-18, and IL-15[186] and was originally discovered by studying the immune response to viral infection[187]. Since then, further studies have implicated bystander CD8$^+$ T cell responses as protective in the context of acute viral infection, while also contributing to tissue injury as is observed in chronic Hepatitis D infection[186,188]. Bystander activated CD8$^+$ T cells also comprise a distinct population of tumor infiltrating lymphocytes, although their function within this context has not been elucidated[189]. The potential contribution of non-classically restricted CD8$^+$ T cells which harbor an innate-like phenotype, compared to classically restricted CD8$^+$ T cells, to bystander inflammation has not yet been addressed. Further, although commensal reactive CD8$^+$ T cells are believed to contribute to the population of bystander CD8$^+$ T cells, the limitations of tools to track them have so far restricted the ability to study them specifically.

The cytokine IL-15 produced by infected hepatocytes has been implicated in driving bystander responses during viral infection of the liver, which can contribute to tissue damage and increased pathology[188,190,191]. IL-15 has also been implicated in gastrointestinal diseases including celiac disease[192,193], and has reported to be upregulated in colonic tissue of patients with IBD[194–196]. Mice that constitutively overexpress human IL-15 in the epithelium spontaneously develop small intestinal inflammation driven by the activity of infiltrating CD8$\alpha\beta^+$ and CD8$\alpha\alpha^+$ T cells[197]. Further, IL-15$^{-/-}$ mice are resistant to DSS colitis, and depletion of CD8$^+$ T cells reduces severity of disease[198]. However, the link between IL-15 production during inflammation, and the potential contribution of bystander activated CD8$^+$ T cells to colitis has not yet been bridged. It should be noted that H2-M3 restricted CD8$^+$ T cell have been reported to require IL-15 for their development, but act independently after activation, diverging from MHC-Ia restricted CD8$^+$ T cells[199]. Therefore, it will be interesting to investigate the potential differential contributions of classical and non-classically restricted, commensal reactive CD8$^+$ T cells in colitis.

5.5 Conclusion

This work is the first to characterize the potential cross tissue involvement of commensal reactive CD8$^+$ T cells in colitis. We show that topical association with a commensal is sufficient to induce a robust systemic CD8$^+$ T cell response that can then contribute the CD8$^+$ T cell response during colitis, independent of colonization during association.

5.6 Future outlook and limitations

A significant limitation to this study is the absence of qPCR or CFU measurements to confirm the absence of *S. epi* in the gut that might be driving these responses during colitis. The *S. epidermidis* NIHLM087 isolate that we employed for these experiments does not contain an antibiotic resistance cassette, so CFU plating is not feasible, and qPCR does not confirm colonization of live microbe, only the presence of genetic material. To account for this, future studies can also utilize vancomycin throughout the course of *H. hepaticus* colitis as the modulation of the gut microbiota does not affect the disease model[180](Figure 5.4 C).

Further, to investigate whether this phenomenon is bystander activation and not driven by potential antigen availability and cross reactivity by the commensal microbiota during inflammation, future studies can also employ Nur77-GFP mice, wherein cells that have undergone TCR stimulation are labeled with GFP[200]. Bystander activated $CD8^+$ T cells should not express Nur77[201]. Further, to identify whether cells from the skin are able to traffic to the gut, or if these cells are undergoing more local recruitment and expansion, we can utilize *CAG::KikGR*[33] mice which express a photoconvertible protein[202]. This system will allow us to photoconvert cells in the ear after topical association with *S. epidermidis* and investigate their dissemination across tissues during colitis. Future studies should also investigate the impact of IL-15 production in the *H. hepaticus* αIL-10R model of colitis and whether that drives $CD8^+$ T cell infiltration in an antigen independent manner as is seen in other models of bystander activation. Experiments utilizing αIL-15 antibody treatment throughout

colitis after the initiation of the systemic commensal reactive, $CD8^+$ T cell response would aid in this investigation.

By understanding the contribution of antigen specific vs bystander activated $CD8^+$ T cells to colitis, it unveils a new potential avenue for treatment of IBD. $CD8^+$ T cells are prolific producers of TNFα which is one of most targeted cytokines in the treatment of IBD and other autoimmune disorders[203]. However, many patients develop primary or secondary resistance to αTNF treatment[204], and thus an understanding of the upstream mediators of inflammation during colitis could illuminate novel treatment options for patients with IBD. Studies investigating the potential for bystander $CD8^+$ T cells in IBD can also be conducted in humans by tracking and characterizing $tetramer^+$ cells in the tissue or PBMC of patients. For example, in their 2018 study, Kim and colleagues deployed tetramers against common human viruses such as Epstein Barr Virus and Cytomegalovirus[191], identifying NKG2D as a surface marker of a *bonafide* bystander response.

Notably, we observe a difference in trafficking of commensal reactive $CD4^+$ T cells to previously published results[167]. Although this could be due to differential colonization of *S. epi* and *S. aureus* in the gut during inflammation, which we did not assess in our model, it is also feasible that this trafficking is a product of the transgenic T cell itself. Our prior data was collected using the (f)MIIINA:H2-M3 tetramer to identify commensal reactive $CD8^+$ T cells, and Merana et al. similarly utilized a tetramer to identify commensal reactive $CD4^+$ T cells[167]. Tetramers can bind cells with diverse TCR clonotypes, allowing for the tracking of cells with a much broader repertoire and potential functions[205]. In contrast, the Tg mice used

for our experiments are crossed to a Rag1$^{-/-}$ background, eliminating the potential for TCR recombination endowing them with a fixed TCR which allows us only to track a very specific T cell response to a commensal antigen. It is therefore possible that certain TCRs allow for the dissemination of commensal specific T cells during inflammation, whereas others do not.

Finally, to better understand whether this phenomenon is shared by MHC-Ia restricted CD8^{+} T cells using *S. epidermidis*-Ova, we would need to equalize both the antigen burden and precursor frequency of the H2-M3 and H2-K^{b} restricted CD8^{+} T cells. Manipulation of *S. epidermidis*-Ova to produce both the (f)MIIINA and SIINFEKL peptide at a 1:1 ratio is logistically challenging, and would require deeper insight into the function of the protein from which the (f)MIIINA peptide is derived. However, future studies utilizing equal transfer of Bowie^{Tg} and OT-I^{Tg} T cells could circumvent discrepancies in the naïve endogenous repertoire against the two antigens.

6. Development and validation of novel H2-M3 tetramer tools

6.1 Introduction

In Chapter 3, 4 and 5 we describe the H2-M3 restricted $CD8^+$ T cell response during *H. hepaticus* αIL-10R induced colitis. In Chapter 3 we show that during colitis, MHC-Ib restricted $CD8^+$ T cells infiltrate the colon of $H2k^bd^{b-/-}$ mice. In Chapters 4 and 5, we utilize the *Staphylococcus epidermidis* reactive (f)MIIINA:H2-M3 tetramer to illustrate more specifically an infiltration of H2-M3 restricted $CD8^+$ T cells during colitis, indicating that H2-M3 restricted T cells do contribute to the MHC-Ib restricted $CD8^+$ T cell response observed in $H2k^bd^{b-/-}$ mice. To our knowledge, there are currently only 5 published H2-M3 restricted tetramers available, raising the need for additional tools to characterize these responses[60,85]. Although H2-M3 restricted $CD8^+$ T cells were originally characterized by their action during infection with the intracellular pathogen *Listeria monocytogenes, S. epidermidis* is believed to be an extracellular bacterium and the (f)MIIINA peptide that elicits an H2-M3 restricted response is a secreted antigen[60]. Presentation on H2-M3 therefore can be carried out by phagocytosis of exogenous antigens and cross presentation[206]. *Helicobacter hepaticus* is a pathobiont, and able to colonize the colon under steady state conditions but perpetuate inflammation in the absence of IL-10 signaling, we therefore hypothesized that there might be a *Helicobacter* reactive, H2-M3 restricted $CD8^+$ T cell response that we could track in homeostasis and inflammation.

6.2 Aim

In this chapter we endeavored to develop novel H2-M3 tetramer tools against *Helicobacter hepaticus* to add to the tools available to track H2-M3 restricted $CD8^+$ T cell responses to a commensal microbe.

6.3: Results

6.3.1 Identification of predicted H2-M3 binding peptides from the Helicobacter hepaticus genome

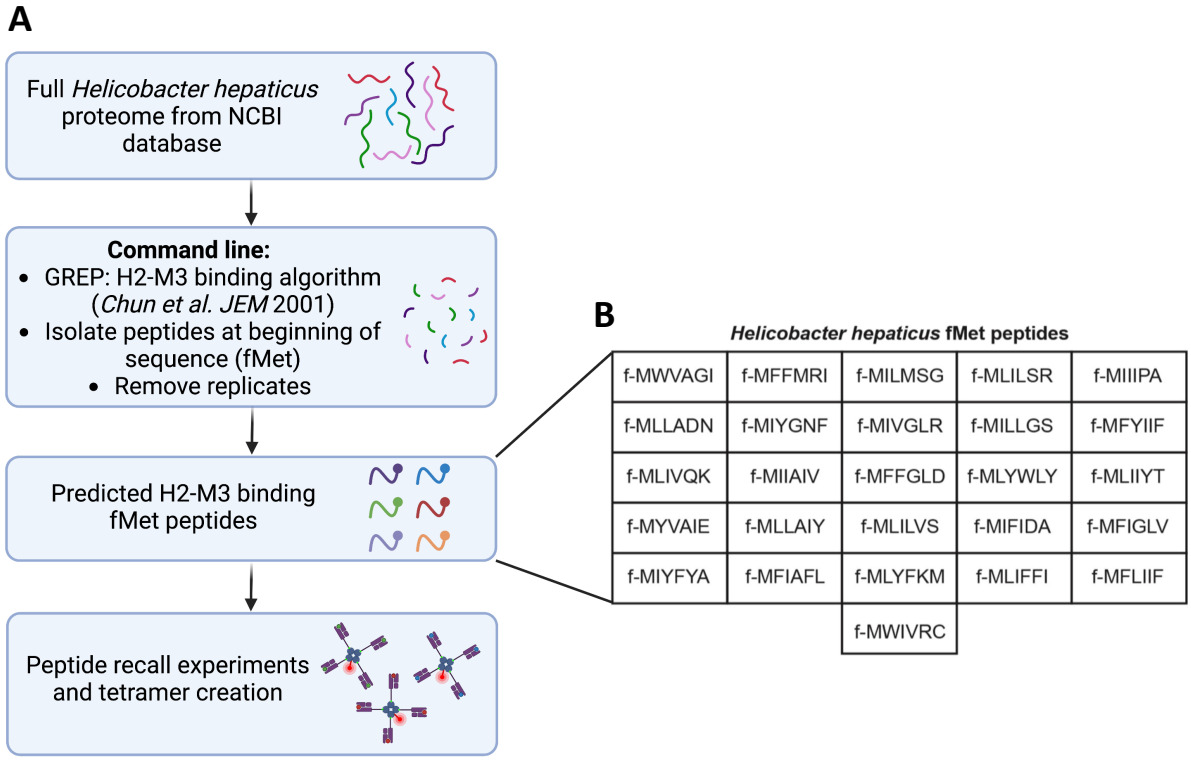


Figure 6.1: Identification of predicted H2-M3 binding peptides from *Helicobacter hepaticus* proteome. A. Computational method to identify predicted *Helicobacter hepaticus* derived peptides to produce H2-M3 tetramers **B.** 26 predicted peptides from computational analysis

H2-M3 selectively binds and presents 5-6 amino acid long *N*-formyl (fMet) containing peptides, which is required for the initiation of protein translation for prokaryotes and mitochondria[40]. This is a dynamic process which can also target non-nascent proteins for degradation[207] and is regulated by the enzyme deformylase[208]. Thus, although we cannot predict which proteins might be

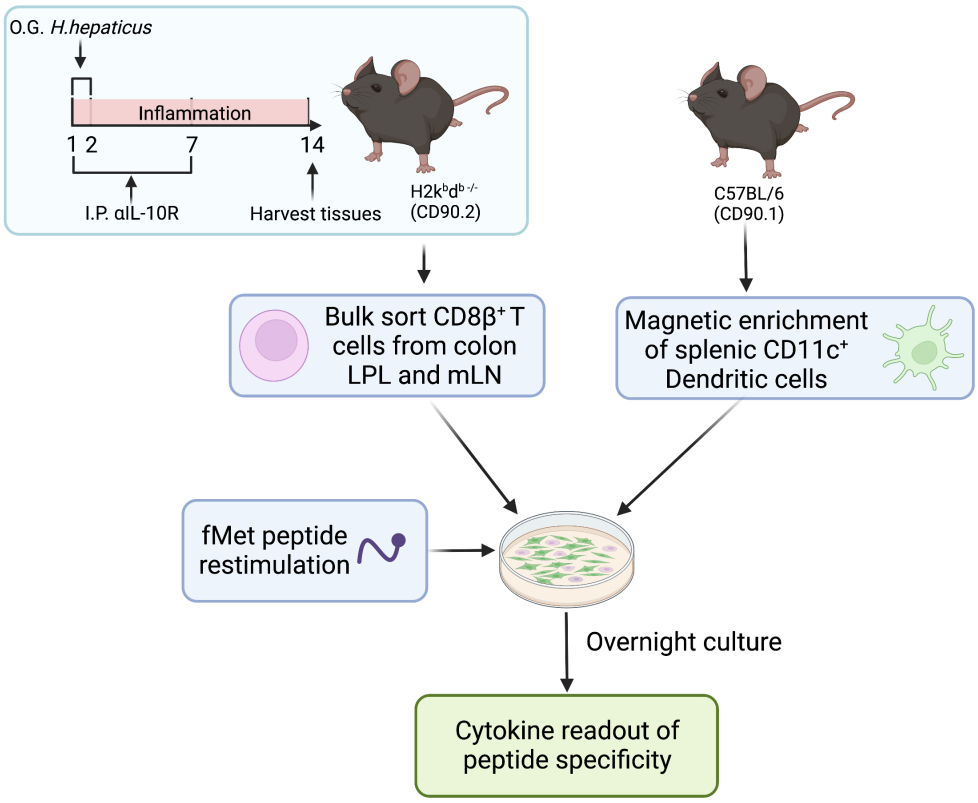


Figure 6.2: Schematic for peptide recall experiments to determine immunorelevant, H2-M3 binding fMet peptides

fMet pool 1	fMet pool 2	fMet pool 3
(f)MWVAGI	(f)MILLGS	(f)MLLAIY
(f)MFFMRI	(f)MFYIIF	(f)MLILVS
(f)MILMSG	(f)MLIVQK	(f)MIFIDA
(f)MLILSR	(f)MIIAIV	(f)MFIGLV
(f)MIIIPA	(f)MFFGLD	(f)MIYFYA
(f)MLLADN	(f)MLIIYT	(f)MLYFKM
(f)MIYGNF	(f)MLYVAIE	(f)MLIFFI
		(f)MFLIIF
		(f)MWIVRC

Table 6.1: Panel of pools of fMet peptides for *in-vitro* recall experiments

formylated and hence presented on H2-M3, theoretically, every protein in the *Helicobacter hepaticus* proteome is formylated at least in its nascent stage.

Utilizing a published H2-M3 binding motif [155], we first interrogated the *H. hepaticus*

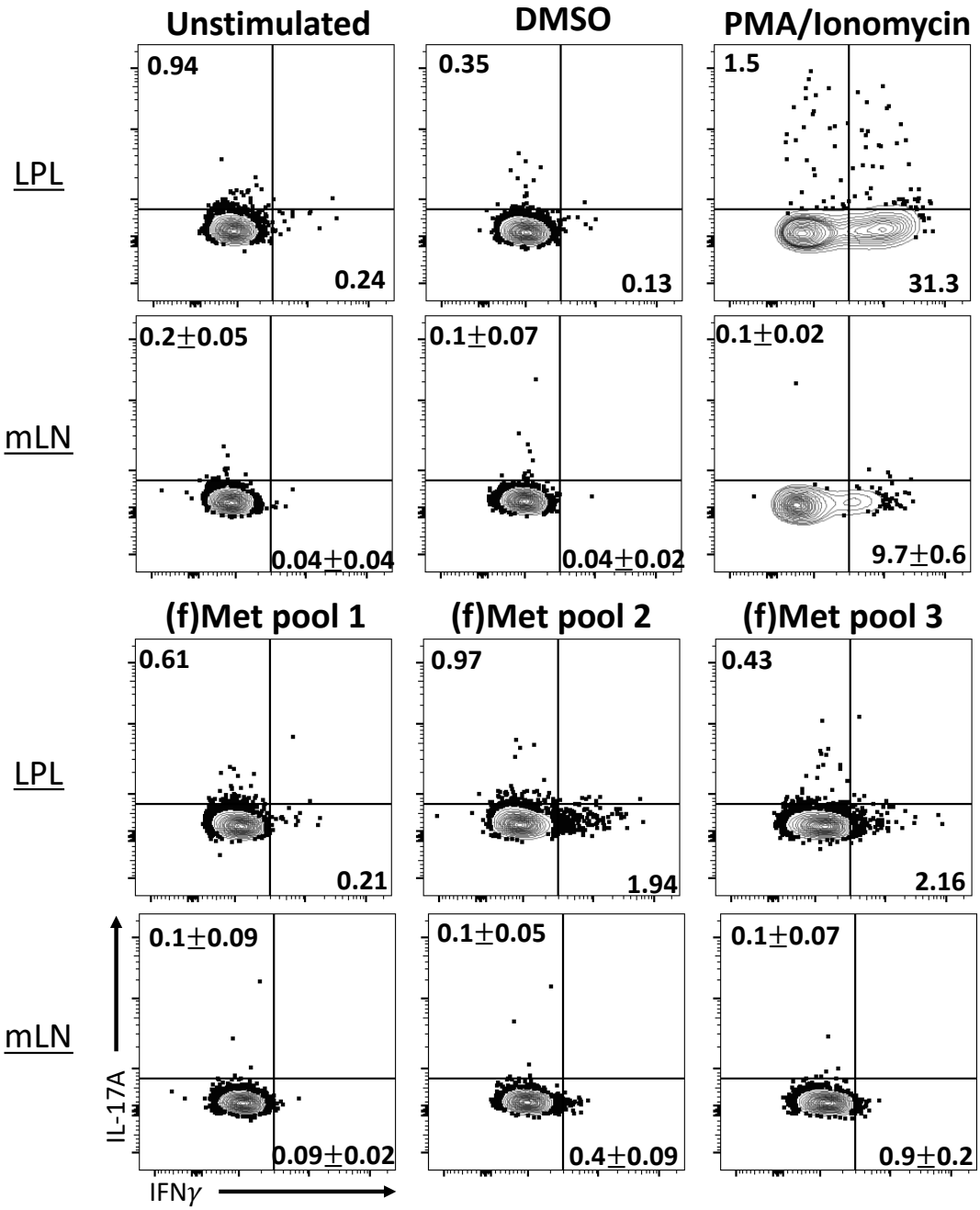


Figure 6.3: fMet pools 2 and 3 elicit cytokine response from CD8 T cells extracted from *H.hepaticus* colitis animals. *In vitro* recall of *Helicobacter hepaticus* induced $CD8^+$ T cells from FACsorted colon lamina propria lymphocytes (LPL) or mesenteric lymph nodes (mLN) from $H2k^bd^{b\,-/-}$ animals, with formyl peptide pool loaded splenic DCs. Frequencies of IL-$17A^+$ or IFN-γ^+ $CD8\beta^+$ T cells after overnight culture. Plots are gated on live $CD45^+$ $TCR\beta^+$ $Thy1.2^+$ $CD8\beta^+$ T cells. Splenic $CD11c^+$ dendritic cells were enriched from wild-type mice. Numbers in flow cytometry plots correspond to the frequencies of gated populations

genome for peptides predicted to bind H2-M3 uncovering 26 peptides (Figure 6.1 A and B).

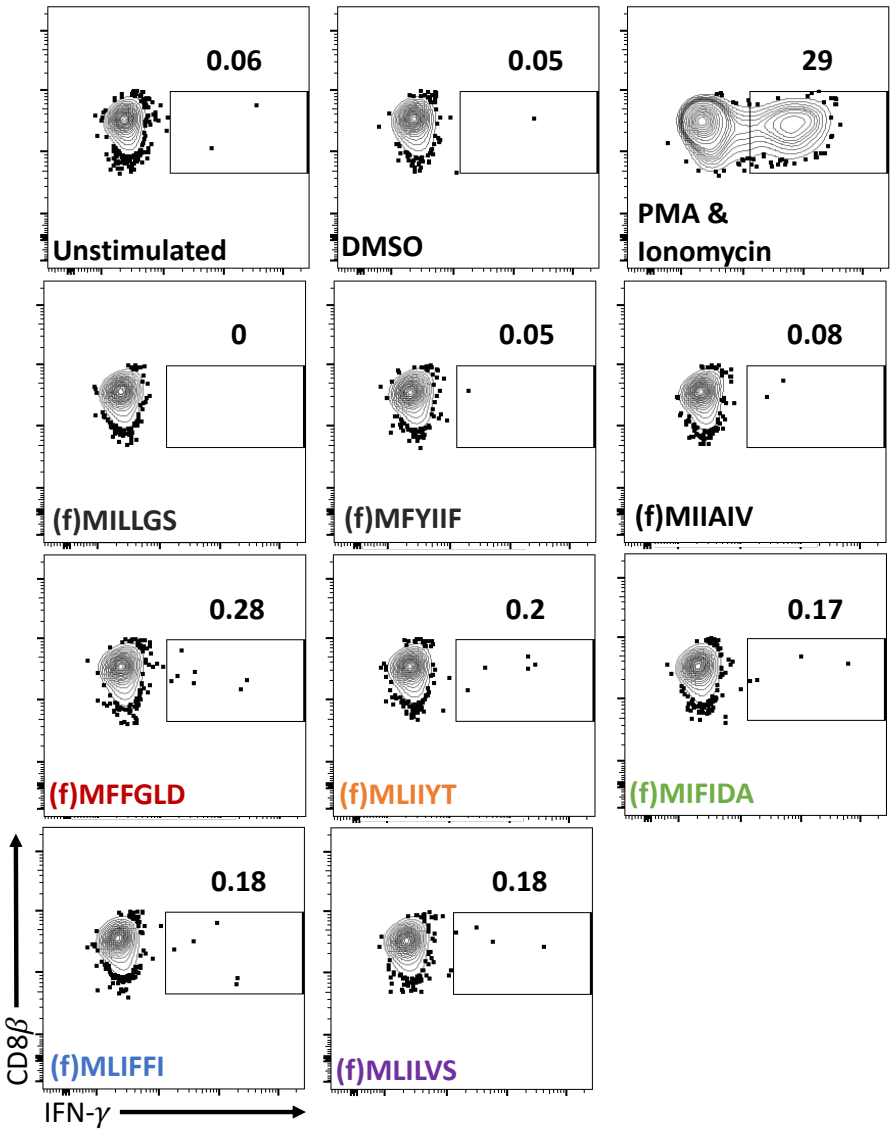


Figure 6.4: Identification of 5 immunorelevant peptides. *In vitro* recall of *Helicobacter hepaticus* induced CD8β^+ T cells from FACsorted colon lamina propria from H2k^{b}d$^{b\,-/-}$ animals, with formyl peptide loaded splenic DCs. Frequencies of IFN-γ$^+$ CD8β$^+$ T cells after overnight culture. Plots are gated on live CD45$^+$ TCRβ$^+$ Thy1.2$^+$ CD8β$^+$ T cells. Splenic CD11c$^+$ dendritic cells were enriched from wild-type mice. Numbers in flow cytometry plots correspond to the frequencies of gated populations.

Under steady state conditions, the colon lamina propria is dominantly populated by classically restricted CD8$^+$ T cells, but during colitis non-classically restricted CD8$^+$ T cells can fill the niche (Figure 3.4 and 3.13). Therefore, to enrich for H2-M3 restricted CD8$^+$ T cells that might be reactive to *Helicobacter hepaticus* fMet peptides, we sorted CD8$^+$ T cells from the colon LPL and mesenteric lymph nodes of H2k^{b}d$^{b-/-}$ mice with *H. hepaticus* αIL-10R colitis for recall experiments (Figure 6.2). Peptide pools 1 and 2 (Table 6.1) elicited IFNγ production by

Peptide	Derived protein from *Helicobacter hepaticus*	Protein function and location
(f)MFFGLD	Cytochrome d ubiquinol oxidase subunit II [Helicobacter hepaticus]	Energy metabolism/ Oxidative phosphorylation: Membrane bound
(f)MLIIYT	MULTISPECIES: divalent-cation tolerance protein CutA [Helicobacter]	Resistance to heavy metals: Cytoplasmic
(f)MIFIDA	Uroporphyrinogen decarboxylase [Helicobacter hepaticus]	Porphyrin biosynthesis: Cytoplasmic
(f)MLIFFI	Alpha/beta hydrolase [Helicobacter hepaticus]	Hydrolytic enzyme with common alpha beta fold: Unknown
(f)MLILVS	Conserved hypothetical protein [Helicobacter hepaticus ATCC 51449]	Unknown

Table 6.2: Predicted peptide function and location. Potential immunorelevant peptides with protein blasted from *Helicobacter hepaticus* proteome and known functions and locations from UniProt BLAST database

recalled $CD8^+$T cells from both colon LPL and mLN and were thus used for individual peptide recall experiments (Figure 6.3).

Individual peptide recall experiments revealed 5 fMet peptides ((f)MFFGLD, (f)MLIIYT, (f)MIFIDA, (f)MLIFFI, and (f)MLILVS)) that could stimulate IFNγ production over baseline by $CD8^+$ T cells from $CD8^+$ T cells sorted from the lamina propria of $H2k^bd^{b-/-}$ mice with *H. hepaticus* αIL-10R colitis (Figure 6.4). We therefore created fMet:H2-M3 tetramer reagents with these 5 peptides, each derived from unique proteins within the *Helicobacter hepaticus* genome (Table 6.2).

6.3.2 Validation of novel H2-M3 tetramers

Owing to their "innate-like" characteristics, H2-M3 restricted T cells are present in lymphoid organs such as the spleen of naïve mice with an activated $CD44^{high}$ phenotype even in the absence of antigen exposure[60]. Therefore, we enriched spleens of naïve mice for each tetramer. We identified a population for each

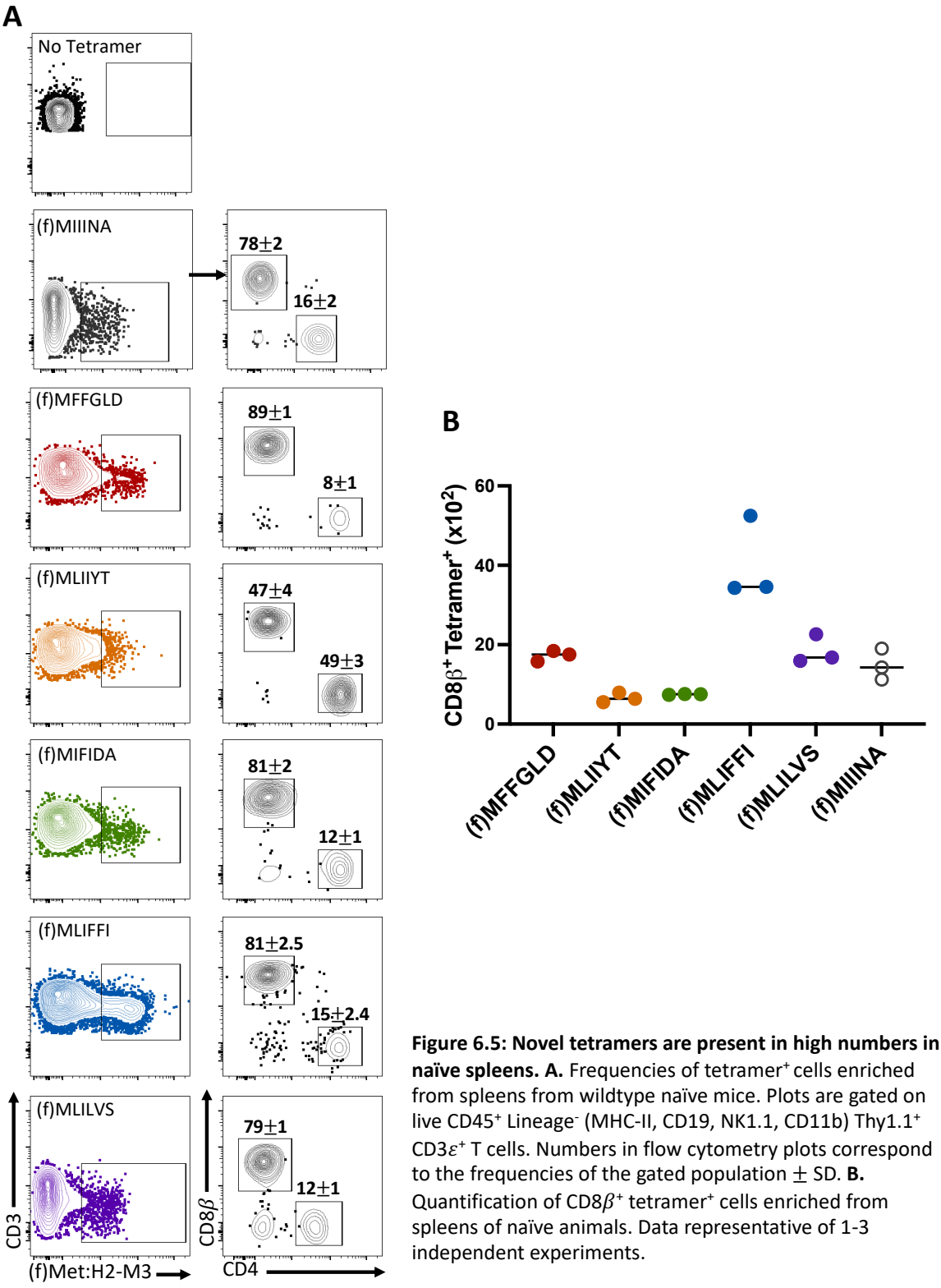


Figure 6.5: Novel tetramers are present in high numbers in naïve spleens. A. Frequencies of tetramer$^+$ cells enriched from spleens from wildtype naïve mice. Plots are gated on live CD45$^+$ Lineage$^-$ (MHC-II, CD19, NK1.1, CD11b) Thy1.1$^+$ CD3ε^+ T cells. Numbers in flow cytometry plots correspond to the frequencies of the gated population ± SD. **B.** Quantification of CD8β^+ tetramer$^+$ cells enriched from spleens of naïve animals. Data representative of 1-3 independent experiments.

tetramer, however, only 4 of the 5 stained a dominant population of CD8$^+$ T cells, with similar frequencies to (f)MIIINA:H2-M3$^+$ T cells (Figure 6.5 A). Notably, the population of (f)MLIIYT:H2-M3$^+$ was small and contained approximately 50%

CD4$^+$ T cells. MAIT cells, restricted by the MHC-Ib molecule MR1, and iNKT cells restricted by CD1d can express CD4, CD8, or neither co-receptor depending on their residence in tissue or circulation[209–211]. However, a dominant population of CD4$^+$ H2-M3 restricted T cells have not yet been described, making it more likely that this is non-specific staining. Future investigation would be warranted to see if this is a true population of CD4$^+$ H2-M3 restricted T cells.

Interestingly, we observed substantial variability in the naïve repertoire between enriched tetramer$^+$ cells. Consistent with previous work[60], (f)MIIINA:H2-M3 tetramers enriched ~2000 cells from naïve spleen, whereas (f)MLIFFI:H2-M3 tetramers enriched ~3500 cells (Figure 6.5 B). The precursor frequencies of naïve antigen specific MHC-Ia restricted CD8$^+$ T cell responses has been reported to vary between 100-1200 cells[212,213] indicating that H2-M3 restricted CD8$^+$ T cells might comprise a large proportion of CD8$^+$ T cells in the spleen of naïve animals compared to MHC-Ia restricted CD8$^+$ T cells.

H2-M3 restricted CD8$^+$ T cells exhibit a pre-activated phenotype, with up to 50% expressing CD44 on the surface even in antigen inexperienced animals[60,89]. Further, H2-M3 restricted T cells have been reported to have “promiscuous” T cell receptors, with some degree of innate cross-reactivity[214]. Thus, we stained naïve spleens with the novel H2-M3 tetramers against the well described (f)MIIINA:H2-M3 tetramer to determine any potential cross reactivity. Each of the novel tetramers stained a distinct population and exhibited minimal cross reactivity with the (f)MIIINA:H2-M3 tetramer (Figure 6.6). Further, the novel tetramer$^+$ cells all contained ~25% CD44^{high} population, in line with previous reports of H2-M3 restricted CD8$^+$ T cells[60] (Figure 6.6).

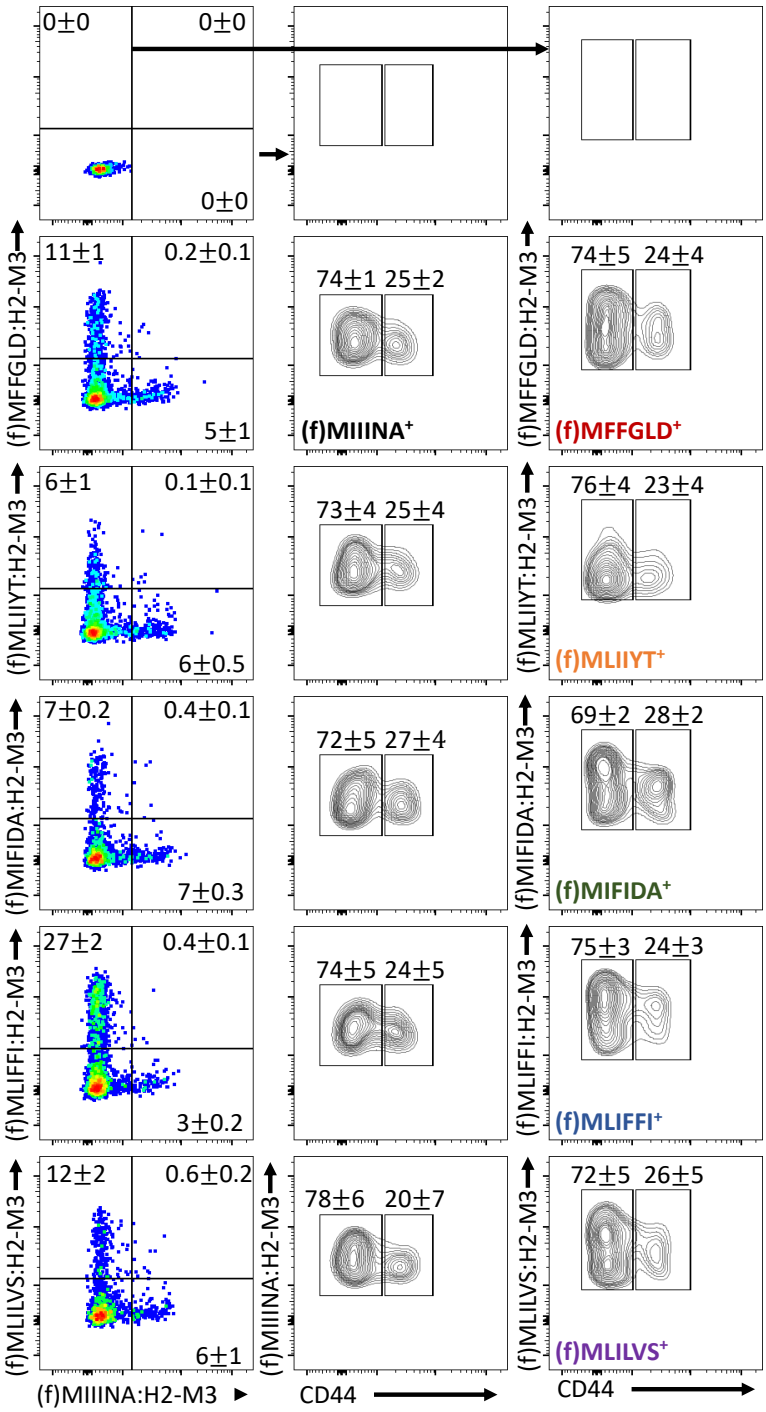


Figure 6.6: Tetramers exhibit minimal cross reactivity with other H2-M3 tetramers. Frequencies of tetramer$^+$ cells enriched from spleens from wildtype naïve mice. Plots are gated on live CD45$^+$ Lineage$^-$ (MHC-II, CD19, NK1.1, CD11b) Thy1.1$^+$ CD3ε^+ CD8β^+ T cells. Numbers in flow cytometry plots correspond to the frequencies of the gated population ± SD. Data representative of 1 experiment with 3 mice per group

Taken together, these results collectively indicate the successful development of novel H2-M3 tetramers. A final test of specificity will be to stain with both PE and APC conjugated tetramers to ensure dual staining, which we were unable to complete due to time constraints[213]. Notably, these results suggest

that H2-M3 restricted $CD8^+$ T cells might comprise a large proportion of $CD8^+$ T cells in the spleen of naïve animals compared to MHC-Ia restricted $CD8^+$ T cells that has not previously been appreciated. This could be in part due to compensation of other $CD8^+$ T cells filling a vacant niche in H2-$M3^{-/-}$ animals, illustrating the value in measuring endogenous T cell responses in immune-replete animals.

6.3.3 Interrogating Helicobacter hepaticus specificity of novel tetramers

As these tetramers were designed against *Helicobacter hepaticus* and screened during colitis, we first tested all tetramer-stained cells for expansion and activation following *H. h* αIL-10R induced colitis to control for antigens that might be produced by *Helicobacter hepaticus* during inflammation. Many of the tetramer-stained cells had increased CD44 expression in the spleen (Figure 6.7 A). (f)MLILVS:H2-$M3^+$ cells significantly increased CD44 expression during colitis, with (f)MFFGLD:H2-$M3^+$ trending higher as well. However, treatment with *H. hepaticus* and αIL-10R alone also marginally increased the frequency of $CD44^{high}$ (f)MIIINA:H2-$M3^+$ $CD8^+$ T cells in the spleen independent of the presence of antigen (Figure 6.7 B). Therefore, we hypothesized that some of the increase in CD44 expression maybe be due to inflammation alone, and not *Helicobacter hepaticus* derived antigen recognition. To fully understand whether this response is due to antigen recognition we will utilize Nur77-GFP mice to identify whether these $CD44^{high}$ cells have undergone recent TCR stimulation.

Due to the trend in increased CD44 expression for (f)MFFGLD:H2-$M3^+$ cells, we first endeavored to more fully characterize whether this tetramer was

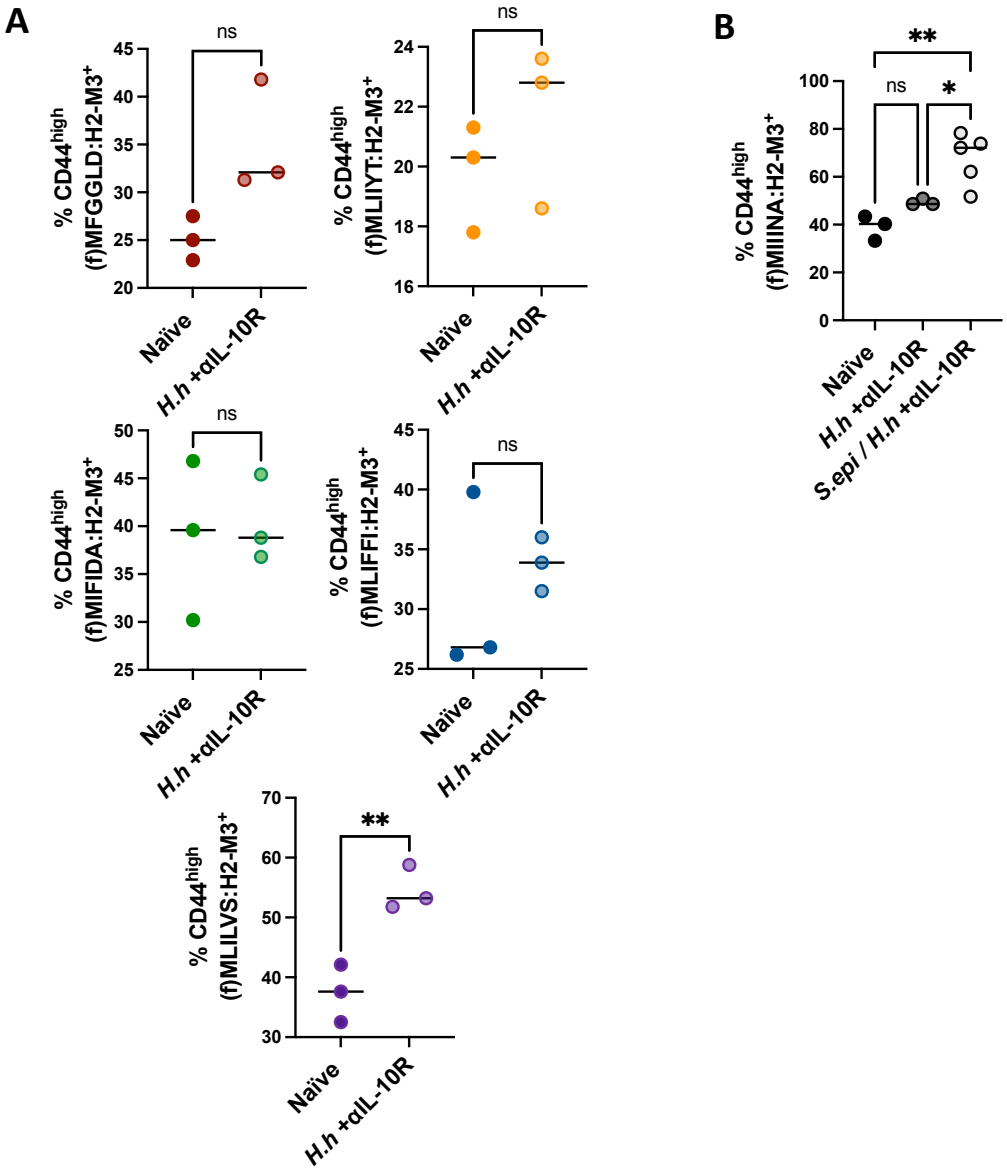


Figure 6.7: Colitis induces CD44 expression on tetramer+ cells. A. Frequency of CD44 expression of novel H2-M3 tetramer$^+$ CD8β^+ enriched from spleens of naïve and *H.hepaticus* +aIL-10R treated animals. Data representative of 1 experiment. Unpaired t test was used to measure significance ** = $p < 0.01$. **B.** Frequency of CD44 expression on (f)MIIINA:H2-M3$^+$ T cells enriched from spleens of naïve, *H.hepaticus* gavaged +aIL-10R treated, or *S.epidermidis* gavaged and *H.hepaticus* gavaged +aIL-10R treated animals. Data representative of 2 independent experiments. One way ANOVA was used to measure significance * = $p < 0.5$, ** = $p < 0.01$

specific to *Helicobacter hepaticus,* or if these results were due to inflammation induced activation.

In the spleen, total enriched (f)MFFGLD:H2-M3$^+$ cells were unchanged between naïve, αIL-10R, *Helicobacter hepaticus* gavaged and *H. h* αIL-10R treated animals (Figure 6.8 A). CD44 expression was increased in the mice

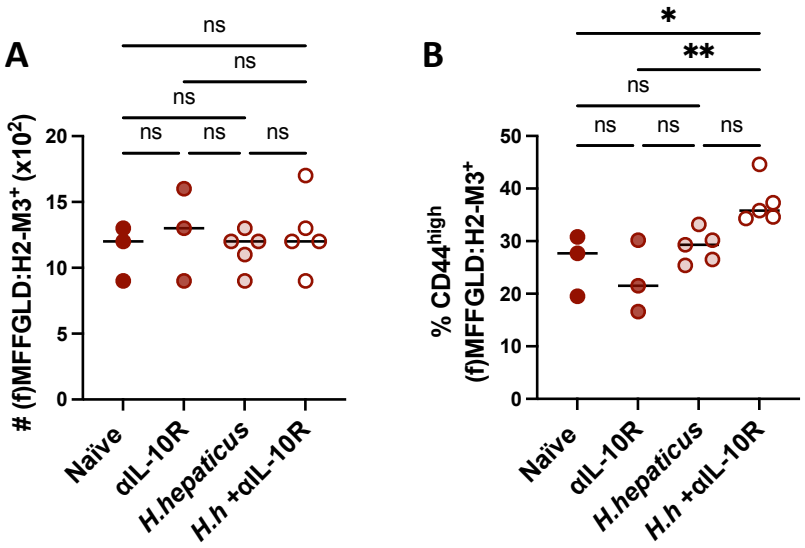


Figure 6.8: Colitis induces CD44 expression on (f)MFFGLD:H2-M3⁺ T cells in spleen. A. Quantification of total (f)MFFGLD:H2-M3⁺ CD8β^+ T cells enriched from the spleens naïve, aIL-10R treated, *H.hepaticus* gavaged, or *H.hepaticus* and aIL-10R treated mice. Data representative of 1 experiment. **B.** Frequency of CD44^{high} (f)MFFGLD⁺ CD8β^+ T cells enriched from spleens of indicated groups. Data representative of 1 experiment * = $p < 0.5$, ** = $p < 0.01$. One way ANOVA.

treated with *H.h* αIL-10R, indicating that the systemic activation status of (f)MFFGLD:H2-M3⁺ cells is dependent on colitis, and not *H. hepaticus* colonization alone (Figure 6.8 B). This could be in part due to differential protein expression and antigen availability during homeostasis or an artifact of inflammation, which would require more in-depth analysis of the contextual proteome of *Helicobacter hepaticus* during homeostasis vs inflammation. Similar to the (f)MIIINA:H2-M3 response to *S. epidermidis* in the mLN (Figure 4.5 B), total numbers of (f)MFFGLD:H2-M3⁺ were unchanged between naïve and *H. hepaticus* colonized animals (Figure 6.9 A). However, CD44 expression was significantly increased in tetramer⁺ cells colonized with *H. hepaticus* with and without αIL-10R treatment (Figure 6.9 B), suggesting that these cells might have specificity to *H. hepaticus,* or that colonization with *H. hepaticus* drives a low-level inflammation that stimulates CD44 expression. Total number of (f)MFFGLD:H2-M3⁺ cells were increased in the mLN of colitic animals, indicating that inflammation drives their

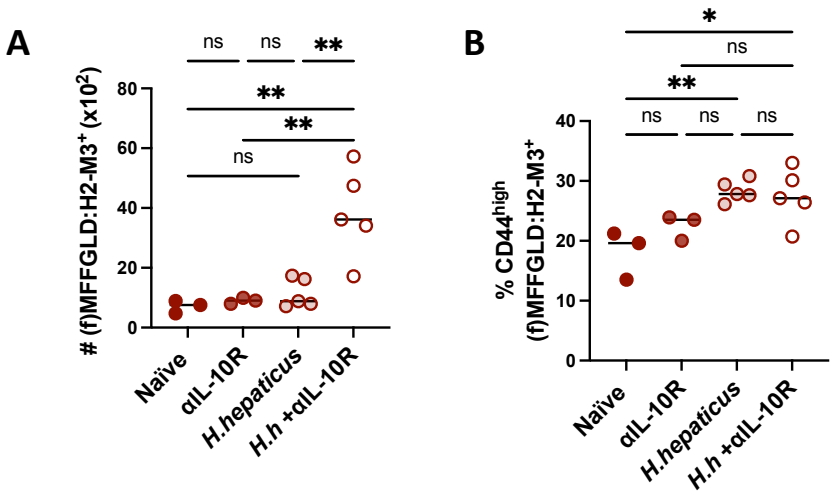


Figure 6.9: Helicobacter colonization induces CD44 expression on tetramer⁺ cells in mLN. A. Quantification of total (f)MFFGLD:H2-M3⁺ T cells in mLN of of naïve, *H.hepaticus* gavaged, or *H.hepaticus* and αIL-10R treated mice. **B.** Frequency of CD44^{high} (f)MFFGLD⁺ CD8β^+ T cells from mLN of indicated groups. One way ANOVA was used to determine significance. * = p < 0.5, ** = p < 0.01. . Data representative of 1 experiment

expansion (Figure 6.9 A). Whether this expansion is dependent on antigen remains to be determined.

In the colon LPL, we observed a slight increase of (f)MFFGLD:H2-M3⁺ following colonization with *H. hepaticus* (Figure 6.10 A & B). Further, approximately 50% of tetramer⁺ cells stained CD4⁺ T cells in the tissue, and ~10% were negative for both CD4 and CD8β indicating the possibility for non-specific staining (Figure 6.10 A). We observed a significant increase in the total number of (f)MFFGLD:H2-M3⁺ cells during colitis, however, this only amounted to approximately 150 cells (Figure 6.10 C). Comparatively, (f)MIIINA:H2-M3⁺ cells, established by colonization with *S. epidermidis,* expand to approximately 2000 during *H. hepaticus* colitis (Figure 4.7 B). It should be noted that, there could still be differential expression of antigen between (f)MIIINA and (f)MFFGLD and resulting immunodominance of the (f)MIIINA:H2-M3⁺ T cells, which was not investigated in this project.

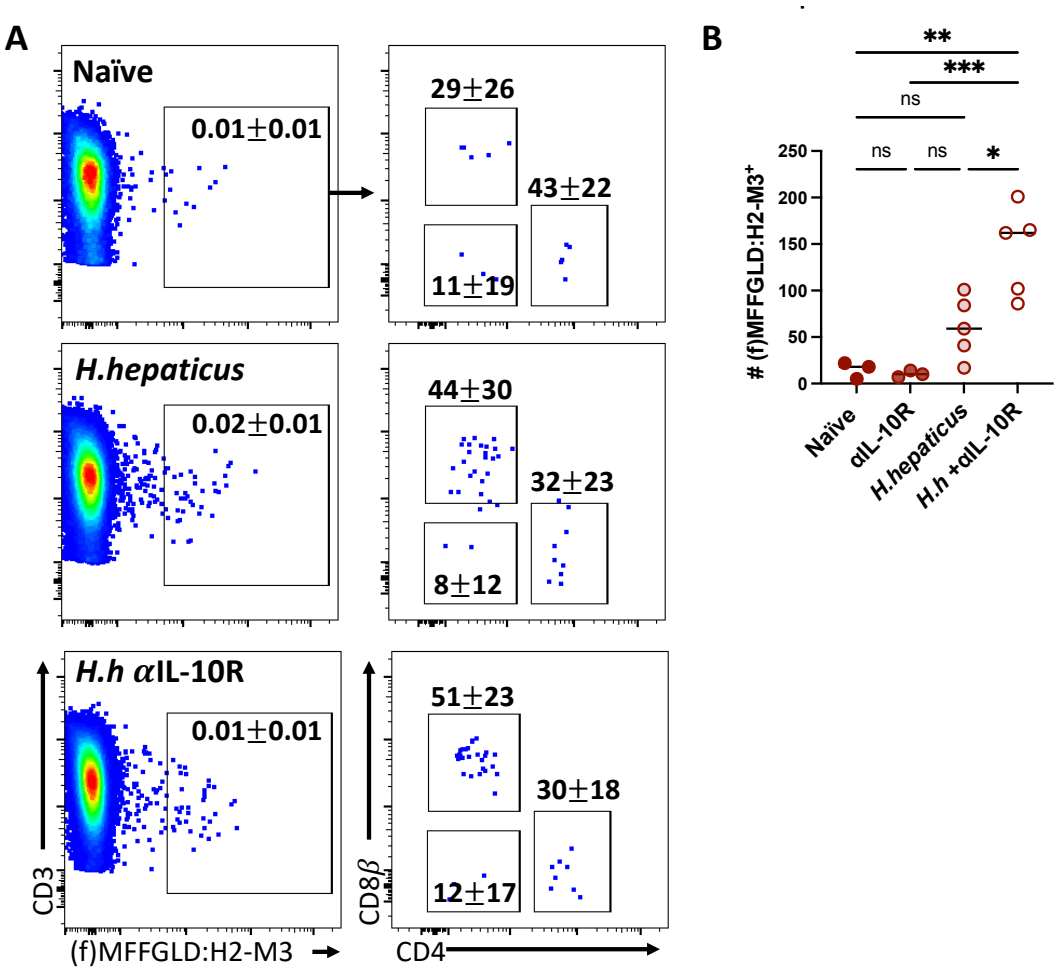


Figure 6.10: Helicobacter colonization elicits slight expansion of tetramer$^+$ cells in colon LPL. A. Frequency of CD4$^+$ and CD8β^+ tetramer$^+$ T cells from colon LPL of naïve, *H.hepaticus* gavaged, or *H.hepaticus* and aIL-10R treated mice. Plots are gated on live CD45$^+$ Lineage$^-$ (MHC-II, CD19, NK1.1, CD11b) Thy1.1$^+$ CD3ε^+ T cells. Numbers in flow cytometry plots correspond to frequency of the gated populations ± SD. **B.** Number of (f)MFFGLD$^+$ CD8β^+ T cells from colon LPL of indicated groups. Data representative of 1 experiment. One way ANOVA was used to measure significance * = $p < 0.5$, ** = $p < 0.01$, *** = $p < 0.001$

Taken together, these results suggest that although we have successfully created a novel H2-M3 tetramer that can enrich a naïve population in the spleen, it is unlikely that (f)MFFGLD:H2-M3 is specific to *H. hepaticus*. Owing to time constraints, we have not been able to complete these studies for the other 4 tetramers, and future research should repeat using these methods. Further work will investigate other tetramers created for this project for reactivity, such as (f)MLILVS:H2-M3, which also showed significant upregulation of CD44 during *H. hepaticus* colitis in preliminary experiments.

6.3.4 Investigation of potential microbiota reactive H2-M3 tetramer

In prior chapters we describe the H2-M3 restricted $CD8^+$ T cell response to colitis and show that commensal reactive H2-M3 restricted $CD8^+$ T cells can contribute to the immune response during inflammation. fMet peptides are theoretically present in all bacterial species, and many of the proteins from which these peptides are derived are conserved in multiple bacterial species present in the microbiota of SPF mice (Table 6.2). Thus, it is feasible that although we originally designed these tetramers against *H. hepaticus,* other members of the microbiota could express proteins with these peptide sequences and $CD8^+$ T cells specific to them could contribute to disease.

$CD44^{high}$ H2-M3 restricted $CD8^+$ T cells are present in germ free animals [60]. To further confirm that the novel tetramers we created stained cells with similar phenotypes, we tested three to assess their relative frequencies in GF vs SPF mice. (f)MFFGLD:H2-M3 was chosen due to its intriguing potential as a *Helicobacter hepaticus* specific tetramer, (f)MLIFFI:H2-M3 due to its relatively high frequency in SPF animals, and (f)MLIIYT:H2-M3 as a negative control. All three tetramers enriched a population of cells in the spleens of both SPF and GF animals that were approximately 17-30% $CD44^{high}$, consistent with prior reports of H2-M3 restricted $CD8^+$ T cells[60](Figure 6.11 A). Interestingly, only the (f)MLIFFI:H2-M3 tetramer enriched a greater number of cells in SPF mice, which also had significantly higher expression of CD44 (Figure 6.11 A & B). These results suggested that (f)MLIFFI:H2-M3 might stain a population of microbiota reactive, H2-M3 restricted $CD8^+$ T cells.

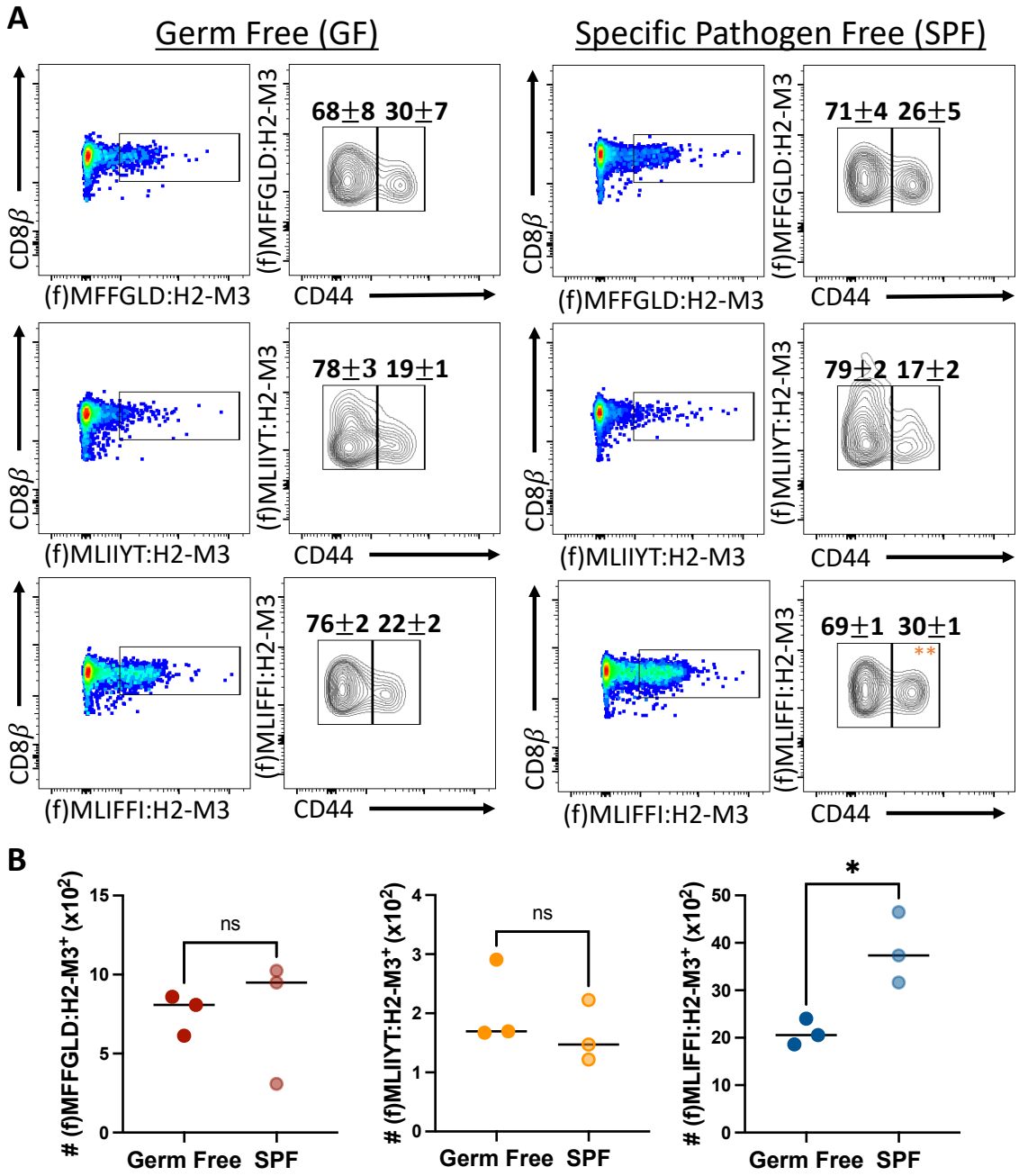


Figure 6.11: Novel tetramers are present in germ free mice. A. Frequencies of tetramer$^+$ cells enriched from spleens from wildtype SPF or GF mice. Plots are gated on live CD45$^+$ Lineage$^-$ (MHC-II, CD19, NK1.1, CD11b) Thy1.1$^+$ CD3ε^+ CD8β^+ T cells. Numbers in flow cytometry plots correspond to the frequencies of the gated population ± SD. Statistics compared to GF populations **B.** Quantification of H2-M3 restricted CD8β^+ tetramer$^+$ cells enriched from spleens of SPF or GF animals. Data representative of 1 experiment. Unpaired T test used to measure significance * = $p < 0.05$, ** = $p < 0.01$

Although SPF mice do harbor a microbiota, it is far removed from the native complexity of the microbiota with which mice have evolved. Thus, we endeavored to assess whether the (f)MLIFFI:H2-M3$^+$ T cells could be further enriched in mice with a more complex microbiota than SPF mice. “WildR” mice

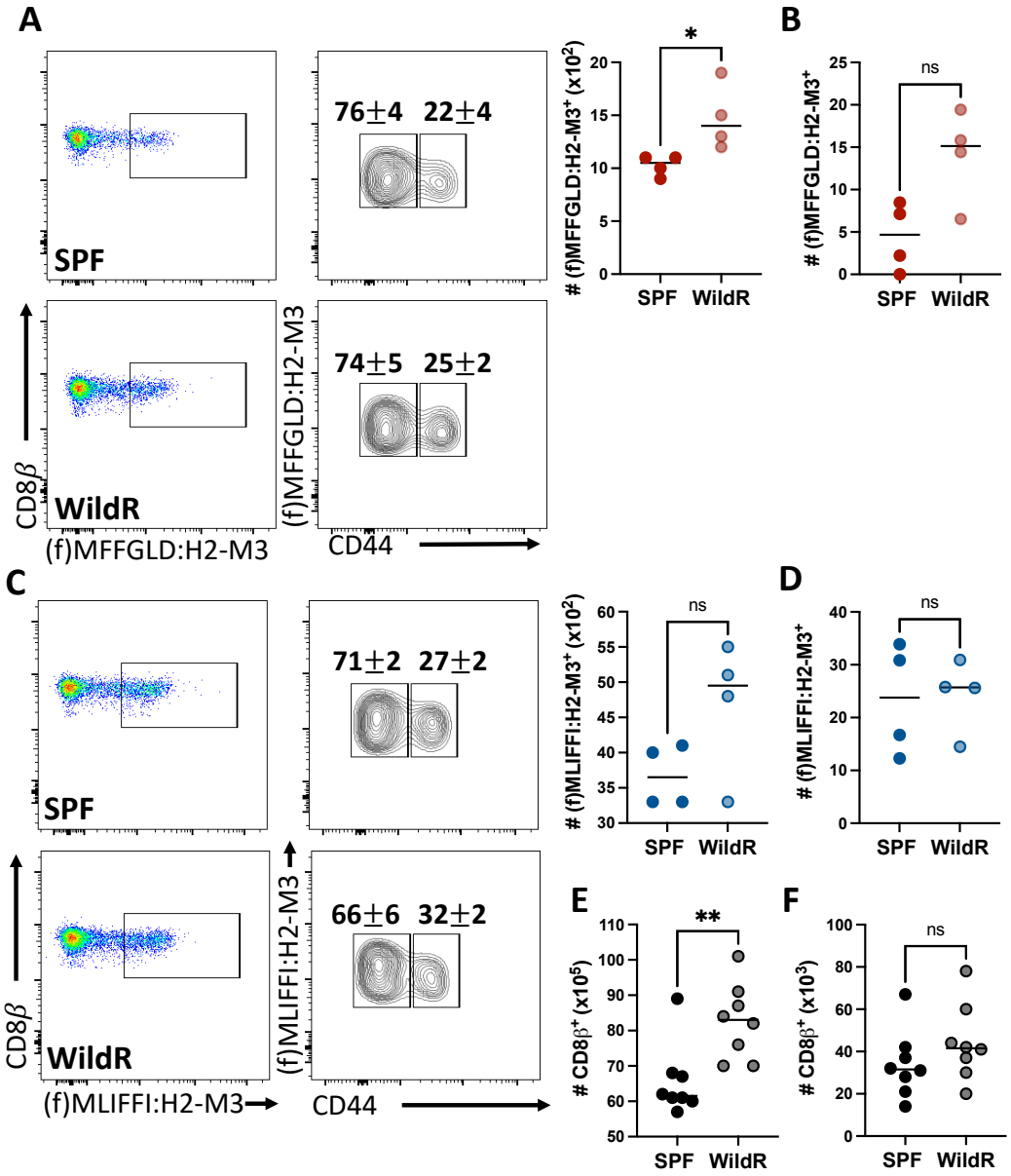


Figure 6.12: WildR microbiota induces a systemic CD8 T cell response but does not harbor novel tetramer antigens. A. Frequencies of fMFFGLD:H2-M3$^+$ cells enriched from spleens from SPF or WildR mice and cell numbers. Plots are gated on live CD45$^+$ Lineage$^-$ (MHC-II, CD19, NK1.1, CD11b) Thy1.1$^+$ CD3ε^+ CD8β^+ T cells. **B.** Quantification of H2-M3 restricted CD8β^+ tetramer$^+$ cells in colon LPL of SPF or WildR mice. **C.** Frequencies of fMLIFFI:H2-M3$^+$ cells enriched from spleens from SPF or WildR mice and cell numbers. **D.** Quantification of H2-M3 restricted CD8β^+ tetramer$^+$ cells in colon LPL of SPF or WildR mice. **E.** Quantification of CD8β^+ T cells in spleens of SPF and WildR mice. **F.** Quantification of CD8β^+ T cells in colon LPL of SPF and WildR mice. Numbers in flow cytometry plots correspond to the frequencies of the gated population ± SD. Data representative of 1 experiment. Mann-Whitney used to measure significance * = $p < 0.05$, ** = $p < 0.01$

are a laboratory mouse model in which GF C57BL/6 are reconstituted with the microbiota of indigenous wild mice[215]. This system allowed us to assess the impact of a more complex microbiota on the (f)MLIFFI:H2-M3$^+$ T cell population, and the (f)MFFGLD:H2-M3 tetramer was chosen to compare.

The total number of (f)MFFGLD:H2-M3$^+$ cells was higher in the WildR mice compared to SPF, whereas there was only a trend in (f)MLIFFI:H2-M3$^+$ cells (Figure 6.12 A & C). CD44 expression was not significantly different between groups for either tetramer, and there were no significant differences in the number of cells within the colon LPL (Figure 6.12 B & D). Total CD8$^+$ T cells were increased in WildR spleens, indicating that WildR microbiota drives an expansion of CD8$^+$ T cells universally (Figure 6.12 E). Interestingly, these differences are not seen in the colon lamina propria (Figure 6.12 F).

Taken together, these results suggest that the (f)MLIFFI:H2-M3 tetramer might stain a population of microbiota reactive H2-M3 restricted T cells, however, these are not enhanced in WildR mice.

6.4 Discussion

In this chapter we describe the creation and validation of 5 novel H2-M3 tetramers originally designed to identify *Helicobacter hepaticus* specific, H2-M3 restricted CD8$^+$ T cells. Due to time constraints, we have been unable to fully assess the specificity of each tetramer, however, 4 enrich a population of cells from naïve spleens with similar profiles as other validated H2-M3 tetramers[60]. Interestingly, the naïve repertoire of these cells varies greatly, with (f)MLIFFI:H2-M3 enriching ~3500 cells from the spleen of naïve mice. Compared to other MHC-Ia restricted CD8$^+$ T cells with naïve repertoires between 100-1200 cells[212,213], this suggests that H2-M3 restricted CD8$^+$ T cells might comprise a much larger population of CD8$^+$ T cells in the murine immune system than has been previously appreciated. Because H2-M3 restricted CD8$^+$ T cells do not have any known distinguishing cell surface characteristics and utilize diverse Vβ chains[40,60]it is difficult to establish

whether a CD8$^+$T cell is restricted by H2-M3 verses other MHC molecules without utilizing a tetramer. Further, many of the studies to investigate the function of these cells employ genetic knockouts which might create “niche” space that can be filled by other cell types, skewing homeostatic ratios. Therefore, the creation of these tools widens our understanding of the contribution of the H2-M3 restricted CD8$^+$ T cell repertoire and will help us probe their function in immune replete animals.

6.5 Conclusion

This work builds upon previous knowledge of H2-M3 restricted CD8$^+$T cell responses, confirming their high frequency even in the absence of a commensal microbiota. It also suggests that H2-M3 restricted CD8$^+$T cells might comprise a much larger subset of CD8$^+$T cells in the mouse than has been previously appreciated.

6.6 Future outlook and limitations

Although the novel tetramers created for this project exhibit similar and reproducible characteristics to other validated H2-M3 tetramers, a further test of specificity will be to stain with both PE and APC conjugated tetramers to ensure dual staining[213]. To confirm that these cells are reactive to the tetramer bound peptides, we can also immunize mice with each individual peptide, and then test for cytokine production *ex-vivo.*

Further, to better elucidate whether the (f)MLIFFI:H2-M3 tetramer stains a population of commensal reactive CD8$^+$T cells, we will utilize GF mice reconstituted with SPF microbiota to compare more directly the impact of the microbiota on this cell population.

7 General Discussion

Immune recognition of the commensal microbiota is essential in establishing homeostasis at barrier tissues[5,216]. Inflammatory bowel diseases emerge from a breakdown in the normal communication between host and microbe, leading to relapsing and progressive damage to the barrier of the gut, and incidence is increasing worldwide[95]. Particularly in regions experiencing rapid acceleration in sociodemographic index and potential alterations in the native microbiota[95,98–101]. Concomitantly, the immune system undergoes profound alterations, with notable effects on the commensal reactive T cell responses and emergent $CD8^+$ T cell signatures[107,108,116,117]. We are only just beginning to understand the complex mechanisms and cell types that are key mediators in these interactions, including the potential impact of commensal reactive $CD8^+$ T cells themselves. Thus, a better understanding of the systems underlying homeostatic recognition of the microbiota, and how those systems are altered during disease, can serve as novel interrogation points for future therapies in IBD.

7.1 Summary

$CD8^+$ T cell signatures in both tissue and PBMC have been reported in patients with IBD with limited known functional relevance[107,116,117]. In this thesis, we describe an innate-like, H2-M3 restricted, commensal reactive $CD8^+$ T cell population in the colon that has not yet been investigated, in both homeostasis and inflammation. We propose that commensal reactive $CD8^+$ T cells in the colon might be mediators of the inflammatory response during colitis, owing to their Tc1 phenotype and expression of cytotoxic mediators such as granzyme B during inflammation. We further hypothesize that commensal reactive $CD8^+$ T cells might be uniquely skilled to traffic to tissues during inflammation either due to altered

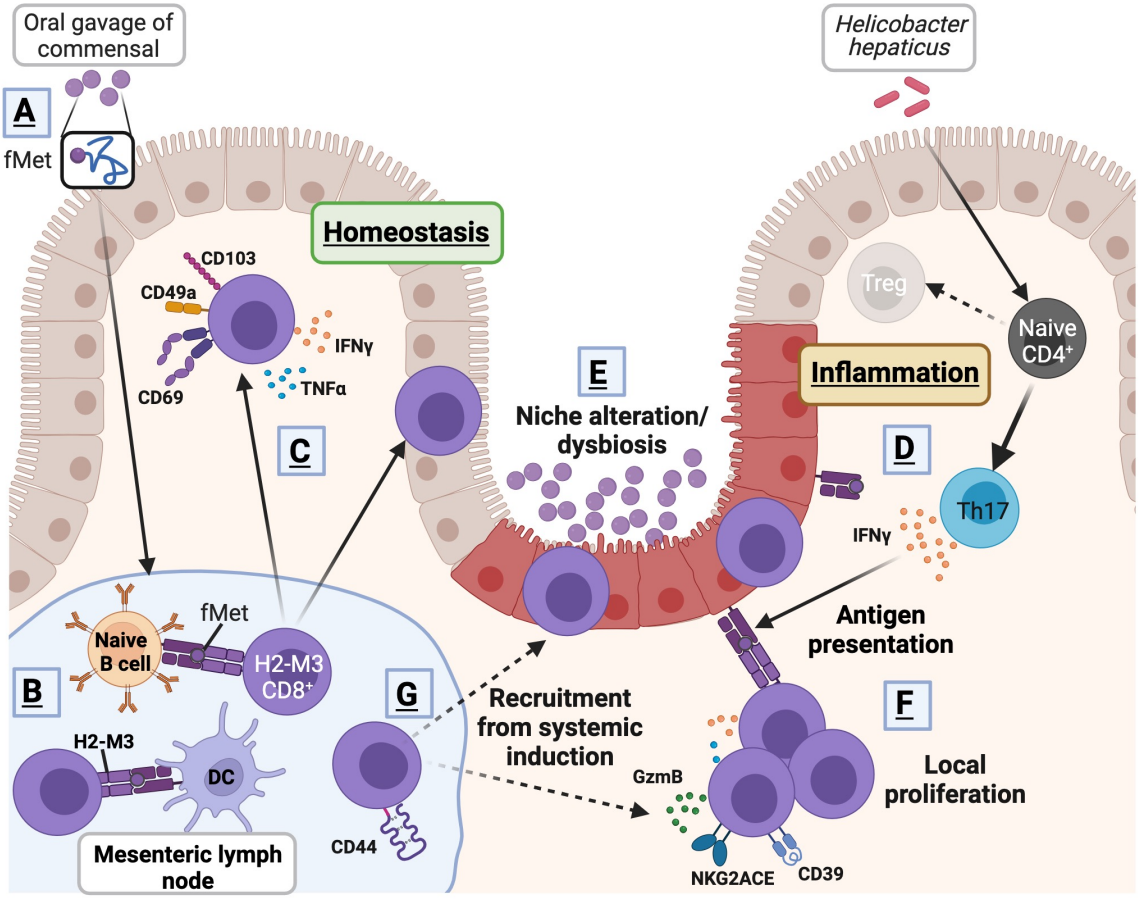


Figure 7.1 Overview of proposed model for commensal reactive CD8⁺T cells during homeostasis and inflammation

We propose that **A.** oral gavage of a commensal that produces putative H2-M3 binding peptides, in our model, *Staphylococcus epidermidis,* is sufficient to induce a systemic and local response by presentation on **B.** naïve B cells and dendritic cell subsets of the mesenteric lymph node, which are then able to **C.** seed both the lamina propria and epithelium of the gut where they establish residency and adopt a Tc1 phenotype. In our model of colitis, **D.** inflammation induced by immune dysregulation and aberrant reactivity to *Helicobacter hepaticus* can drive increased H2-M3 presentation on intestinal epithelial cells and other cells of the colon and further, **E.** alter in the niche of the gut, potentially allowing for the outgrowth of *S.epidermidis.* **F.** Increased antigen presentation or antigen availability might drive local proliferation of resident commensal reactive CD8⁺T cells, and potentially in concert with tissue derived signals **G.** aid in recruitment of commensal reactive CD8⁺T cells that have been systemically induced by exposure at other sites such as the skin.

Abbreviations: fMet, N-formylated peptide; CD, Cluster of Differentiation; IFNγ, Interferon-γ; TNFα, Tumor necrosis factor- α; Th17, T helper 17; GzmB, Granzyme B

Figure crated with Biorender.com

antigen presentation during colitis, or inflammatory cytokines that can instruct an innate-like capacity. Additionally, our data suggest that the naïve repertoire of H2-M3 restricted CD8⁺ T cells might be more robust than has been previously appreciated, instructing deeper investigation into the potential cumulative cost of microbiota reactive, innate-like CD8⁺ T cells in inflammatory diseases (Figure 6.1).

Dissecting the functional relevance of these cells during homeostasis and inflammation, and the mechanisms driving their occupation of this tissue under differing circumstances is an ongoing thread of investigation.

7.2 Divergent cytokine profiles of commensal reactive CD8$^+$ T cells depending on tissue residence

Microbiota reactive T cells play keystone roles in regulating homeostasis at barrier tissues and multiple studies have illuminated alterations in microbiota reactive CD4$^+$ and CD8$^+$ T cells during inflammatory bowel diseases[2,5,108,121]. In this study, we aimed to elucidate the role of innate-like commensal reactive CD8$^+$ T cell response in the colon during homeostasis and inflammation. We leveraged H2-M3 tetramer tools originally created to study the innate-like CD8$^+$ T cell response to *Staphylococcus epidermidis*, and a model of colitis mimicking genetic susceptibility and aberrant reactivity to the commensal microbiota. This allowed us to interrogate the impact of inflammation on the innate-like commensal-reactive CD8$^+$ T cell response in the colon amidst classically restricted CD8$^+$ T cells, a distinction that has not been achieved by previous studies.

Our data illustrates that commensal reactive, H2-M3 restricted CD8$^+$ T cells do indeed reside within the gut epithelium and lamina propria of the colon and adopt a primarily Tc1 phenotype, differing from their distinctive Tc17 signature in the skin (Figure 4.5), an indicator of their wound healing capabilities[58,60,61]. This is the first description of H2-M3 restricted CD8$^+$ T cells in the gut, adding to the known network of innate-like immune cells poised to respond to commensal antigens within this tissue[2]. These findings also raise important inquiries into the tissue specific cues that mediate the cytokine profiles and function of commensal reactive CD8$^+$ T cells elicited against the same antigen. An understanding of the

environmental signals, and transcription factors that govern the fates of commensal reactive CD8$^+$ T cells in the gut, could be exciting targets for future therapies to initiate wound healing by these cells after an inflammatory insult. However, careful modulation of these programs would need to be employed as unchecked wound healing might evolve into tumor growth and potential malignancy.

7.3 CD8$^+$T cell exhaustion: the potential impact of unleashing commensal reactive CD8$^+$T cells with immunotherapy treatments

Our data also show that commensal reactive CD8$^+$ T cells in the gut express CD39 during inflammation, recapitulating what is seen in circulating CD8$^+$ T cells from IBD patients (Figure 4.8) [118]. CD39, in collaboration with CD73, is an ectoenzyme that degrades extracellular ATP to adenosine, and is believed to identify suppressor CD8$^+$ T cells which can attenuate immune responses[217]. Thus, it is interesting to consider whether H2-M3 restricted, commensal reactive CD8$^+$ T cells might serve suppressive roles during inflammation.

Further, CD39 is also present on terminally exhausted cells, and we report a reduction of TNFα and IFNγ cytokine production by commensal reactive CD8$^+$ T cells during inflammation, a hallmark of exhaustion[170,218]. We did not measure additional markers such as PD-1 or CTLA-4, and future investigation will better elucidate whether these cells are truly exhausted. Regardless, CD8$^+$ T cell exhaustion is believed to inform the clinical outcomes of patients with IBD[116,118]. Thus, our model allows deeper investigation into the impact of exhaustion on commensal reactive CD8$^+$ T cells during colitis.

Notably, immune checkpoint inhibitors (ICI) targeting CTLA-4 and PD-1 present on exhausted cells, have proven to be remarkable therapies for patients with cancer, yet also collaterally induce colitis in some patients[219–221]. Indeed, multiple studies have illustrated that tissue resident $CD8^+$ T cells can be unleashed during ICI treatment, differentiating into cytotoxic effector cells, suggesting that exhaustion of resident $CD8^+$ T cells in the gut might be a vital mechanism in maintaining homeostasis[219,221]. Thus, our results add to a growing body of evidence that $CD8^+$ T cells, with potential reactivity to the commensal microbiota, can be key players in colitis, warranting their further investigation for the treatment of IBD and IBD like pathologies. It is also intriguing to consider that innate-like T cells, with a pre-activated phenotype, might be among the earliest inducers of inflammation during IBD.

7.4 Commensal reactive $CD8^+$ T cells as agents of inflammation across tissues

This thesis also describes the expansion of commensal reactive $CD8^+$ T cells in the colon during inflammation after topical association with *S. epidermidis* (Figure 5.3). Although we illustrate that gut colonization during topical association does not drive this response (Figure 5.4), *S. epidermidis* has been reported to occupy the colon during DSS colitis[167], therefore it is feasible that this also occurs during *Helicobacter hepaticus* αIL-10R driven colitis. Indeed, access to host tissue derived oxygen during inflammation can fuel blooms of *Enterobacteriaceae* in models of infectious colitis, further supporting this possibility[222]. Further, we show that H2-M3 gene expression is increased during colitis, potentially boosting antigen presentation during inflammation. Therefore, it is intriguing to consider

how altered niche's during colitis can drive and tune the commensal reactive $CD8^+$ T cells response to antigens now seen in the context of inflammation.

Alternatively, or perhaps additionally, these responses might be influenced by the inflammation itself and potential cytokine mediators such as IL-15. IL-15 has recently been highlighted as a key cytokine driving bystander activation of $CD8^+$ T cells that contribute to liver injury during acute Hepatitis A infection[191]. IL-15 is also elevated in circulating PBMC and colonic tissue of patients with IBD, and IL-$15^{-/-}$ mice are resistant to DSS colitis[194–196,198]. Thus, further investigation into the potential contribution of bystander activated commensal reactive $CD8^+$ T cells, which are likely present in high abundance in intestinal tissue, to colitis is warranted.

7.5 Potential comparable contributions of innate-like $CD8^+$ T cells to colitis in humans

This work highlights the intriguing role of innate-like H2-M3 restricted $CD8^+$ T cells to colitis. To date, a human homologue of H2-M3 has not been discovered and only one group has reported *N*-formyl reactive T cells in humans, albeit restricted by MHC-II [39,223]. It is feasible that *N*-formyl peptide recognition is entirely absent in humans outside of the innate formyl peptide receptors[75]. However, further, reproducible research would need to be conducted to deny this altogether.

H2-M3 restricted $CD8^+$ T cells were originally described in the context of infection and have only just recently been described as having divergent functions in response to the commensal microbiota[40,60]. Other innate-like $CD8^+$ T cells such as Qa-1 restricted $CD8^+$ T cells, have also been described as responding to pathogen derived antigens and can contribute to the inflammatory response

during infection[41,224]. Notably, Qa-1 is the functional homologue for HLA-E in humans, which has also been illustrated to present pathogen derived peptides to $CD8^+$ T cells and can play critical roles in protection from infection[175,225–227]. Notably, these HLA-E restricted $CD8^+$ T cells can have varying functions, including suppressor activity, and type 2 cytokine production, not unlike H2-M3 restricted $CD8^+$ T cells[61,174,175,227]. Thus, it would be intriguing to investigate whether commensal derived peptides could be presented on HLA-E in humans, initiating an innate-like commensal reactive $CD8^+$ T cell response to the microbiota. Translation of these findings into humans would be an exciting bridge to cross.

7.6 Conclusion

In closing, investigation into the $CD8^+$ T cell response during inflammatory bowel diseases, as well as other inflammatory diseases, is only just beginning. The microbiota is a substantial force of antigenic stimulation, likely driving a plethora of commensal reactive $CD8^+$ T cell responses with both local and systemic impacts. By understanding all cell types involved in these inflammatory disorders, we can develop more targeted therapies with reduced side effects, to bring remission to individuals in need.

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