Coordinated Parasitic and Microbial Mechanisms Regulate Effector CD8$^+$ T cell Heterogeneity during Helminth Infection

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TABLE OF CONTENTS

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ABSTRACT

Intestinal helminth infection is a neglected tropical disease affecting over 2 billion people worldwide. It is well-established that helminth infection elicits a potent type 2 immune response to promote parasite expulsion, yet infections are often chronic suggesting that helminths have evolved ways to become, in many cases, symbiotic with their hosts. Recent evidence suggests that helminths can persist in mammalian hosts by inducing tissue repair mechanisms to counteract their tissue-invasive nature. Evolution may have favoured persistent habitation by helminths as new data have revealed that infection can activate bystander protective mechanisms against certain viral and bacterial infections. Surprisingly, evidence suggests that anti-microbial defense during helminth infection is mediated by $CD8^+$ T cells. A prominent example of such protection occurs during *Heligmosomoides polygyrus bakeri* (*Hpb*) infection, a natural parasite of mice. *Hpb* infection induces expansion of a memory-like $CD8^+$ T cell population within the secondary lymphoid organs, referred to as "virtual memory" (TVM) cells that mediate protective immunity to bacterial and viral infection in a co-infection setting. In addition, unpublished data from our lab indicate that the gut microbiota activates a subset of intestinal IFNγ producing $CD8^+$ T cells that limit *Hpb*-induced tissue damage. However, the relationship between TVM cells and microbiota-dependent $CD8^+$ T cell activation during *Hpb* infection remains unknown. In this thesis, I hypothesized that distinct populations of $CD8^+$ T cells emerge in the gut-draining lymph nodes during *Hpb* infection and are differentially regulated by the gut microbiota. Using a combination of genetic, gnotobiotic and immunophenotyping approaches, we discovered early activation of a $CD8^+$ T cell subset, distinct from TVM cells, that express a type 1 immune signature in an IFNγ-dependent, yet microbiota-independent manner. Conversely, we found that TVM cell expansion relied on a delayed type 2 immune response and their effector phenotype was restrained by the gut

microbiota. Overall, our studies describe unexpected diversity within the activated $CD8^+$ T cell compartment during *Hpb* infection and reveal how parasites and the gut microbiota converge to impact effector $CD8^+$ T cell heterogeneity during infection.

RÉSUMÉ

L'infection par les helminthes intestinaux est une maladie tropicale négligée affectant plus de 2 milliards de personnes dans le monde. Il est bien établi que l'infection par les helminthes déclenche une puissante réponse immunitaire de type 2 pour provoquer l'expulsion du parasite, mais les infections sont souvent chroniques. Cela suggère que les helminthes ont évolué de manière à devenir symbiotiques avec leurs hôtes. Des preuves récentes suggèrent que les helminthes peuvent persister dans les hôtes mammaliens en induisant des mécanismes de réparation des tissus pour contrecarrer leur nature invasive dans les tissus. L'évolution a peut-être favorisé la persistance des helminthes, car de nouvelles données ont révélé que l'infection peut activer des mécanismes de protection contre certaines infections virales et bactériennes. Évidemment, il y a la preuve qui suggère que la défense antimicrobienne pendant l'infection par les helminthes est médiée par les cellules T CD8+. L'infection par Heligmosomoides polygyrus bakeri (Hpb), un parasite naturel de la souris, est un exemple marquant d'une telle protection. L'infection par Hpb induit l'expansion d'une population de cellules T CD8+ de type mémoire dans les organes lymphoïdes secondaires, appelées cellules "mémoire virtuelle" (TVM), qui contribuer à l'immunité protectrice contre les infections bactériennes et virales dans le cadre d'une co-infection. En outre, des données non publiées de notre laboratoire indiquent que le microbiote intestinal active un sous-ensemble de cellules T CD8+ qui produisent de l'IFNγ dans l'intestin et qui limitent les lésions tissulaires induites par l'Hpb. Cependant, la relation entre les cellules TVM et l'activation des cellules T CD8+ qui sont dépendantes du microbiote pendant l'infection par Hpb reste inconnue. Dans cette thèse, j'ai émis l'hypothèse que des populations distinctes de cellules T CD8+ émergent dans les ganglions lymphatiques qui drainent l'intestin au cours de l'infection par Hpb et sont régulées de manière différentielle par le microbiote intestinal.

En utilisant une combinaison d'approches génétiques, gnotobiotiques et d'immunophénotypage, nous avons découvert l'activation précoce d'un sous-ensemble de cellules T CD8, distinctes des cellules TVM, qui expriment une signature immunitaire de type 1 d'une manière dépendante de l'IFNγ, mais indépendante du microbiote. À l'inverse, nous avons constaté que l'expansion des cellules TVM dépendait d'une réponse immunitaire de type 2 retardée et que leur phénotype effecteur était limité par le microbiote intestinal. Dans l'ensemble, nos études décrivent une diversité inattendue au sein du compartiment des cellules T CD8+ activées pendant l'infection par Hpb et révèlent comment les parasites et le microbiote intestinal convergent pour influer sur l'hétérogénéité des cellules T CD8+ effectrices pendant l'infection.

INTRODUCTION

Intestinal helminth infection is a neglected tropical disease affecting over 2 billion people worldwide (Pullan et al., 2014; Hotez et al., 2008). It is well-established that helminth infection elicits a potent type 2 immune response characterized by the generation of interleukin (IL)-4 and -13 producing Th2 cells, mucous excretion and tissue-reparative macrophages that collectively promote parasite expulsion (Vacca et al., 2022). Although heavy parasite burden can cause significant host morbidity, infection is often chronic yet asymptomatic. This fascinating phenomenon begs two questions: how and why did these macroscopic parasites evolve to take up long-term residence in different mammalian species?

Understanding **how** chronic helminth infection can occur requires decoding the ways that this parasitic worm can evade host resistance mechanisms and initiate disease tolerance pathways. While the former has been readily explored the concept of disease tolerance in mammalian hosts is relatively novel (Medzhitov et al., 2012), especially in the context of helminth infection (King & Li, 2018). In fact, a recent study from our lab discovered that infection of mice with the parasitic roundworm *Heligmosomoides polygyrus bakeri* (*Hpb*) resulted in the IFNγ-dependent recruitment of natural killer (NK) cells to the small intestine lamina propria that limited infection-induced intestinal bleeding (Gentile et al., 2020). Additional unpublished work from our lab has unexpectedly revealed that $\alpha\beta^+$ $CD8^+$ T cells are the primary producers of IFNγ that drive NK cells to the lamina propria during early *Hpb* infection (unpublished). To the best of our knowledge, this early expansion of $CD8^+$ T cells has not been reported before. Thus, characterization of this population is needed to understand pathways that initiate the tissue-healing immune response to helminths.

Moreover, to answer **why** helminths may have evolved to require parasitism within humans for their survival, one would include the analysis of potential benefits acquired by infected humans. While helminthiasis may generally cause immunomodulation that dampens the immune response against subsequent infection (Maizels et al., 2018; Reynolds et al., 2015), emerging evidence has shown that parasitized individuals can gain protection against certain viral and bacterial infections (Lin et al., 2019; Rolot et al., 2018). As most infected individuals live in impoverished regions that increase their susceptibility to additional pathogens (Stelekati et al., 2012), the study of concurrent infections is particularly relevant to chronic diseases such as helminthiasis. In support of this concept, it has recently been reported that *Hpb*-induced IL-4 expands virtual memory $CD8^+$ T (TVM) cells for control of viral infection in mice (Lin et al., 2019). However, the unexpected expansion of anti-viral effector cells by a type 2 cytokine motivates further investigation into the mechanisms underlying development and function of TVM cells.

While results from our lab and others suggest that multiple host defense mechanisms induced upon *Hpb* infection are mediated by $CD8^+$ T cells (Gentile, 2020), the most-studied CD8 T cell subset during *Hpb* infection is the TVM cell population (Lin et al., 2019; Rolot et al., 2018; Hussain et al., 2023). However, our unpublished data revealed that the expansion of $CD8^+$ T cells in the small intestine of *Hpb*-infected mice occurs prior to the onset of a detectable type 2 immune response (Gentile, 2020). Furthermore, like TVM cells, small intestinal $CD8^+$ T cells produce IFNγ and expand in a cognate antigen-independent manner during *Hpb* infection (unpublished). These preliminary results led us to postulate that this early induction of $CD8^+$ T cells could be either an uncharacterized unique subset or a precursor of TVM cells.

The known master regulators of $CD8^+$ T cell differentiation and function are T-box transcription factors, T-box expressed in T cells (Tbet) and Eomesodermin (Eomes) (Intlekofer et

al., 2005). Tbet and Eomes can exert cooperative roles in $CD8^+$ T cells (Pearce et al., 2003), and their shared sequence homology suggests partial functional redundancy (Papioannou et al., 2014). Nevertheless, they each have distinct regulation and roles in $CD8^+$ T cells (Pearce et al., 2003). For example, Tbet and Eomes are upregulated in early effector $CD8^+$ T cells, but as these cells differentiate towards a memory phenotype, they lose expression of Tbet while increasing Eomes expression (Joshi et al., 2011). While Eomes regulation has been examined in the TVM population during *Hpb* infection, a kinetic analysis of Tbet and Eomes expression during early *Hpb*-induced $CD8^+$ T cell activation has yet to be performed.

Both type 1 and 2 immune cytokines have been shown to regulate $CD8^+$ T cell differentiation (Krummel et al., Carty et al., 2014). Krummel et al. (2018) have recently reported that $CD8^+$ T cells can autonomously skew their own differentiation towards memory through paracrine secretion of IFNγ during bacterial infection. Additionally, IL-4 has been shown to promote Eomes expression in $CD8^+$ T cells through a STAT6-dependent pathway (Carty et al., 2014). However, additional studies are needed to understand the mechanisms by which *Hpb*-induced IFNγ and IL-4 signalling affect $CD8^+$ T cell differentiation.

As *Hpb* strictly resides within the gastrointestinal tract, it is important to consider the effect of the gut microbiota. This vast ecosystem, consisting of bacteria, viruses, and fungi, has been implicated in the maintenance and development of the immune system (Wu et al., 2012). Specifically, certain commensal bacteria species have been shown to mediate $CD8^+$ T cell effector responses against *Listeria monocytogenes* infection and promote anti-tumour immunity (Tanoue et al., 2019). Furthermore, Moyat et al. (2022), have demonstrated that a complex bacterial community induces the IFNγ response and inhibits IL-4-mediated pathways during *Hpb* infection.

However, the role of the complete microbiota on type 1 and 2 immune signals and its effects on downstream $CD8^+$ T cell development during *Hpb* infection remains to be elucidated.

Given the contribution of $CD8^+$ T cells to disease tolerance against helminth infection as well as their role in anti-viral immunity in the setting of virus-helminth co-infection, this MSc thesis seeks to characterize early $CD8^+$ T cell activation during *Hpb* infection and the impact of classical type 1 and type 2 cytokine signaling as well as the gut microbiota on the expansion and differentiation of this critical immune cell compartment.

REVIEW OF RELEVANT LITERATURE

1. Immunity against helminth infection

1.1. Types of immunity activated by helminth infection

1.1.1. Type 2 immunity

The type 2 immune response is well-established to be induced during helminth infection (Vacca et al., 2022), although the exact mechanism of protection may be species-specific. Particularly, the roundworm model parasite, *Hpb,* activates a highly polarized type 2 response in mice that begins at 4 days post infection (4 dpi.) (Anthony et al., 2006), shortly after ingested larvae encyst into the muscularis mucosae of the small intestine (Sukhdeo et al., 1984). Intestinal perturbations from tissue-dwelling stages of *Hpb* cause the release of alarmins from epithelial cells (including tuft cells) as they damage the intestinal mucosa (Hewitson et al., 2015; Johnston et al., 2015). This damage signal recruits type 2 innate lymphoid cells and type 2 helper (Th2) cells that produce type 2 associated cytokines, such as interleukin (IL)-4, IL-5, and IL-13, to promote influx of innate immune cells such as macrophages, mast cells, eosinophils to the intestine (Reynolds et al., 2012; Vella et al., 1992; Patnode et al., 2014). The complex coordination between type 2 immune components and the intestinal microenvironment act to elicit parasite clearance and promote tissue repair.

Additional key anti-helminth, type 2 immune-associated mediators are the antibody and epithelial responses. Circulating IgG1 and IgE levels increase dramatically following infection (McCoy et al., 2008) and are negatively correlated with parasite burden in humans (Hayes et al., 2004). The mechanism of action of polyclonal and parasite-specific antibody responses were shown to impair larval development and limit adult fecundity (McCoy et al., 2008). Furthermore, differentiated cells of the intestinal epithelial layer have also been shown to play an important role

in anti-helminth immunity. Specifically, goblet cells expand in a T cell-dependent manner in response to *Hpb* infection (Hashimoto, 2009) and secrete defence molecules and mucins, the key components of mucus to inhibit *Hpb* and *Nippostrongylus brasiliensis* persistence (Herbert et al., 2009).

1.1.2. Type 1 immunity

Type 2 immunity against gastrointestinal helminth infection is regulated by regulatory T cells (Tregs) (Grainger, 2010) and limited by type 1 immunity (Else, 1994). Generally, the type 1 immune response is characterized by production of type-1-associated cytokines interferon (IFN)-γ, IL-12, IL-18, TNFα, and reactive oxygen species that activate key immune players: $CD8^+$ T cells, natural killer (NK) cells, macrophages, and type 1 helper (Th1) cells that coordinate immune reactions that mediate intracellular pathogen killing (Annunziato et al., 2015). Though excessive production of IFNγ during helminth infection was associated with increased worm burden (Reynolds & Maizels, 2012), recent studies have suggested that the induction of type 1 immune response is necessary for disease tolerance mechanisms (Nusse et al., 2018; Progatzky et al., 2021; Gentile et al., 2020). Disease tolerance, only recently appreciated in mammalian immunity, is a strategy of host protection that acts to reduce negative impacts of infection while not affecting pathogen burden (Medzhitov et al., 2012).

Our lab has recently found that the recruitment of NK cells to murine small intestine during initial days of *Hpb* infection (<4 dpi) limited vascular injury in IFNγ-signalling-dependent manner (Gentile et al., 2019). Similar results were obtained by Nusse et al. indicating that IFNγ production during early *Hpb* infection also prevented tissue damage by contributing to the regeneration of the intestinal epithelium (2018). Furthermore, following *Hpb* egress from the submuscosa, a tissue-wide IFNγ transcriptional response was shown to be required for tissue repair (Progatzky et al.,

2021). These results suggest that activation of the type 1 immune response is necessary to maximize host defense against intestinal helminths. However, the pathways that initiate a type 1 immune response to helminths are still unknown.

1.1.3. Type 3 immunity

Although induction of type 3 immune response has been reported during *Hpb* infection, there is no evidence that suggests that it promotes immunity against *Hpb* (Reynolds et al., 2014). However, it has been shown to promote immunity toward infection of other helminths, such as *N. brasiliensis*, where it can activate protective and pathological type-3-immune-mediated roles during helminth challenge (Weaver et al., 2013). The key player reported during helminth-induced type 3 immunity is the type 3 helper (Th17) cell. Driven by pro-inflammatory cytokines, IL-6 and IL-17A, and maintained by IL-23, Th 17 cells are a recently discovered $CD4^+$ T cell subset that produces cytokines IL-17A, IL-21, GM-CSF, and IL-22 at mucosal barrier sites to aid in defense and tissue repair (Weaver et al., 2013). During *N. brasiliensis* infection in the lungs, early production of IL-17A by Th17 cells suppresses IFNγ signalling that subsequently promotes type 2 immunity and efficient worm expulsion mechanisms (Ajendra et al., 2020). This role of type 3 immunity is recapitulated during infection with *Trichinella spiralis* where Th17 cells were necessary to drive intestinal contractility, a necessary component of parasite expulsion (Steel et al., 2019).

1.2. Contribution of the microbiota on helminth-induced immune responses

1.2.1. Effect of helminth infection on the microbiota

The ability of enteric helminths to persist within mammalian intestines is thought to occur not only through modulation of the host immune response (Karo-Atar et al., 2022), but also via manipulation of the complex microbial communities present at these sites (Rapin and Harris,

2018). Indeed, through several different bacterial 16S rRNA sequencing techniques, *Hpb* infection has been shown to alter bacterial composition in the gastrointestinal tract (Walk et al., 2010; Rausch et al., 2013; Reynolds et al., 2014; Zaiss et al., 2015; Su et al., 2018; Ramanan et al., 2016; Rapin et al., 2020). Specifically, *Hpb* infection led to an outgrowth of *Lactobacillaceae* in the small intestine (Reynolds et al., 2014) and ileum (Walk et al., 2010) and provided resistance against *Bacteroides vulgatus* outgrowth in Nod2-deficient mice (Ramanan et al., 2016). Notably, helminth infection may contribute to microbiota modification through direct antimicrobial activity of their excretory/secretory products (Kato et al., 1996; Abner et al., 2001; Hewitson et al., 2011; Rausch et al., 2018), and by indirect alteration of host immune pathways such as those that affect mucus composition (Marillier et al., 2008; Hasnain et al., 2011; Hasnain et al., 2012; Tsubokawa et al., 2015) and intestinal nutrient absorption (Shea-Donohue et al., 2001).

1.2.2. Role of microbiota during helminth infection

On the other hand, while the role of the microbiota on helminth infection has been less studied, it has been proposed that complex host-helminth-microbiota interactions have evolved overtime such that disruption of any one of these partners may affect the other players of the ecosystem (Rapin et al., 2020). Multiple reports have shown that the microbiota can affect resistance against helminth infection. While earlier studies have shown that germ-free mice infected with *Hpb* harboured less adult worms and eggs (Chang and Wescott, 1972; Wescott et al., 1968; Weinstein et al., 1969; Rausch et al., 2018), these experiments did not account for possible anaerobic bacterial contamination in the germ-free mice from using fecal-grown *Hpb.* Thus, we (Russell et al., 2021) and others (Zaiss et al., 2015; Moyat et al., 2022; Reynolds et al., 2014) have begun to perform infection using *Hpb* reared with auxotrophic HA107 *Escherichia coli* which cannot colonise germ-free mice. Although these recent studies have shown that *Hpb* infection in

germ-free and antibiotic-treated mice exhibited increased worm and egg burden (Moyat et al., 2022), vancomycin treatment, associated with increased *Lactobacillus,* or administration of *Lactobacillus taiwanensis* were shown to promote *Hpb* susceptibility in BALB/c mice (Reynolds et al., 2014). Interestingly, these observations were accompanied by different immune responses; in particular, germ-free infection elevated systemic type 2 immune responses while decreasing Th1 responses (Moyat et al. 2022), whereas, *Lactobacillus* species abundance positively correlated with IL-17A and Treg frequencies, and had no association with type 2 cytokines nor IFNγ production (Reynolds et al., 2014)

2. CD8 T cells and their role during helminth infection

2.1. Subsets of CD8 T cells and their origins

During the early stages of conventional antigen-associated T cell activation, Tbet and Eomes promote cytotoxic effector capacities of the $CD8^+$ T cell by inducing the expression of cytolytic molecules such as perforin and granzyme B (Pearce et al., 2003). Then, in the contraction phase, a subset of effector $CD8^+$ T cells will adopt a memory phenotype, which will allow for more efficient and rapid immunity against subsequent infection of the same or similar pathogen (Pipkin et al., 2010; Banerjee et al., 2010; Josh et al., 2011). While both Tbet and Eomes promote IL-15 signalling that stabilizes memory cell populations (Intlekofer et al., 2005), knockout murine studies have shown that Tbet drives terminal effector differentiation (Joshi et al., 2007) while Eomes promotes memory formation (Kaech et al., 2002; Intlekofer et al., 2005; Banerjee et al., 2010). Overall, Tbet and Eomes are important determinators of $CD8^+$ T cell function and fate.

Memory CD8 T cells are a heterogeneous population that can be identified by their extracellular and intracellular markers. Recently, a subset of $CD8^+$ T cells found in secondary lymphoid organs, called virtual memory $CD8^+$ T (TVM) cells, has been recently discovered to be

activated independently of their cognate antigen and to respond more quickly than their naïve counterparts. Like antigen-experienced "true memory" $CD8^+$ T (TTM) cells, TVM cells exhibit a $CD44^{hi}CD122^+CXCR3^{hi}$ memory phenotype. However, the upregulation of the α4 integrin (CD49d) observed in TTM cells in response to TCR activation does not occur in TVM cells (Martinet et al., 2015). The current model of TVM cell development in C57BL/6 mice is that they arise from $Eomes^{hi}$ precursors that have higher affinity for self-peptide/MHC in the thymus (Drobek et al., 2018; Miller et al, 2020). They later acquire the differentiated $CD44^+CD49d^{lo}CXCR3^+CD62L^+$ phenotype upon exposure to IL-15 in the periphery (Sosinowski et al, 2013).

2.2. Impact of helminth infection on CD8 T cells

2.2.1. TVM cells

Type 2 immunity, in addition to induction of anti-helminth immunity, also promotes the rapid and sustained expansion of TVM cells (Rolot et al., 2018; Lin et al., 2019; Hussain et al., 2023). Unlike their TTM counterparts, TVM cells are poised to expand and produce IFNγ in response to inflammatory cytokines (IL-12, IL-15 and IL-18) in a cognate-antigen independent manner (Hamilton et al., 2006; Haluszczak et al., 2009). As a result, TVM cells can reduce bacterial burden to a higher extent than do naïve $CD8^+$ T cells (Sosinowski et al, 2013). This innate-like behaviour of TVM cells is postulated to aid in defense against difficult-to-clear pathogens (Lee et al., 2010), which may be an evolutionary advantage for parasitized hosts to induce TVM cells during infection. Specifically, this innate-like memory population can expand upon helminth-induced IL-4 signals (Rolot et al., 2018; Lin et al., 2019; Hussain et al., 2023). In support of this idea, TVM cells induced during *Hpb* and *Schistosomaisis* infections have been shown to result in protection against bacterial and viral coinfections (Rolot et al., 2018; Lin et al.,

2019). However, the mechanisms that regulate TVM cell expansion during helminth infection are still not completely understood.

2.2.2. Other CD8 T cell subsets

While the main subset of $CD8^+$ T cells studied during intestinal murine helminth infection is the TVM cell, other types of $CD8^+$ T cells have been reported to be induced during other parasite-mediated diseases (Metwali et al., 2006). $CD8^+$ T cells induced during helminth infection have been shown to exert both anti-helminthic and immunopathological effects. Whether $CD8^+$ T cells play a protective or pathological role may be correlated to the specific time of infection. In human and murine filariasis, type-1-associated cytokines (i.e., IFNγ, TNFα) were produced by $CD8^+$ T cells during initial stages of infection and were proposed to be cytotoxic against the parasite (Folkard et al., 1995; Babu et al., 2003). In contrast, filariasis-infected individuals presenting with pathology had elevated proinflammatory $CD8^+$ T cells in comparison to asymptomatic patients (Anuradha et al., 2014). In support of this finding, chronic infection of *Echinococcus multilocularis* in humans was reported to drive proliferation and oligoclonality of peripheral $CD8^+$ T cells that were associated with increased parasitic lesions in the liver (Manfras et al., 2002).

2.3. Impact of the microbiota on CD8 T cells

Interactions between the micro-organisms residing within mammalian barrier sites (e.g., gastrointestinal tract, skin) are necessary for immune development and activation (Berg et al., 1996; Thursby et al., 2017). In particular, $CD4^+$ and $CD8^+$ T cells are exposed to and can be primed by environmental and microbiota-derived antigens to differentiate into effector cells with functions ranging from regulatory to proinflammatory (Atarashi et al., 2017; Tanoue et al., 2019). Recent advances in studies of the microbial impacts on $CD8^+$ T cell development have shown that the microbiota can induce $CD8^+$ T cell differentiation and activation (Tanoue et al., 2019; Bachem et

al., 2019). IFNγ production from $CD8^+$ T cells in the intestine was induced by a specific consortium of 11 bacterial strains from healthy human donors (Tanoue et al., 2019). Additionally, *Staphylococcus epidermidis* on the skin was shown to promote skin-specific noninflammatory accumulation of IFNγ+ and IL-17+ $CD8^+$ T cells (Naik et al., 2012; Naik et al., 2015; Linehan, 2018; Chen et al., 2019). Activation of $CD8^+$ T cells was mediated by TLR2 and Dectin-1 signalling (Chen et al., 2019) and antigen presentation by major histocompatibility (MHC) class I on dendritic cells (Tanoue et al., 2019; Linehan et al., 2018). This microbial-activated immune response may be a mechanism of commensal bacteria to prevent pathogen colonization and promote tissue repair after injury. Indeed, bacteria transplantation protected against *Listeria monocytogenes*, *Leishmania major* and *Candida albicans* infection (Tanoue et al., 2019, Naik et al., 2012; Naik et al., 2015; Linehan et al., 2018). Furthermore, gut microbiota-derived products were also shown to promote $CD8^+$ T cell differentiation and function. Microbiota-derived SCFAs promoted $CD8^+$ T cell memory cell differentiation through promotion of oxidative phosphorylation (Bachem et al., 2019) and $CD8^+$ T cell cytotoxic responses by increasing glycolysis and mitochondrial respiration (Trompette et al., 2018). Therefore, the bacterial component of the microbiota and their derived products have been shown previously to be involved in the activation of $CD8^+$ T cells. Future studies into how the microbiota and parasite coordinate $CD8^+$ T cell activation and differentiation during infection will provide a better understanding of possible pathways through which tissue protection and intracellular pathogen infection can be improved.

HYPOTHESIS & AIMS

We hypothesize that differentiation of effector $CD8^+$ T cells during *Hpb* infection requires the gut microbiota and IFNγ signalling. To address this hypothesis, we pursued the following objectives:

1. To determine the kinetics and phenotype of proliferating $CD8^+$ T cells over the course of *Hpb* infection.
2. To examine the impact of the microbiota, type 1 and 2 immune responses on $CD8^+$ T effector cell heterogeneity during *Hpb* infection.

MATERIALS & METHODS

Mice, infection and treatments

Mice

All mice were handled with accordance to the McGill University Health Centre Research Institute Animal Resource Division with approved animal use permit no. 7977. Female and male mice were used at 8-10 weeks of age. Wild-type (WT), IFNγR-KO and STAT6-KO mice on a C57BL/6 (B6) background were obtained from The Jackson Laboratory and kept under specific pathogen-free (SPF) conditions. Germ-free mice (GF) were obtained from the McGill Centre for Microbiome Research and maintained under sterile conditions. All animals were bred and/or housed at the McGill University Health Centre Research Institute.

Reconstitution of germ-free mice

GF mice were colonized with an SPF microbiota (xGF) through oral administration of a fecal slurry (150 µl per mouse by gavage). This solution was made by homogenizing fresh fecal pellets from uninfected B6 WT mice in PBS to a concentration of 62.5 g/mL, followed by centrifugation at 700 × g for 5 minutes to obtain the supernatant that is diluted by ten-fold in PBS. xGF were used 3-4 weeks after microbial reconstitution.

Hpb Infection

Mice were infected by gavage with 200 L3 *Hpb* larvae diluted in sterile water and at the indicated time points, tissues were harvested for analysis. Fecal-reared infectious L3 *Hpb* larvae were generated by methods previously described (Johnston et al., 2015). For infection of GF and xGF mice, sterile L3 *Hpb* larvae were generated as described by our lab (Russell et al., 2021).

BrdU adminutesistration

1 mg of BrdU dissolved in sterile PBS was intraperitoneally injected into mice 12 hours prior to sacrifice and water with BrdU (0.8 mg/mL) was provided ad libitum 3-4 days before harvest as described previously (Rocha et al., 1990).

Cell preparation

mLN cell extraction

Mesenteric lymph nodes (mLN) were crushed with a sterile syringe plunger and filtered through a 70 μm strainer into FACS buffer (PBS, 10 μM HEPES, 10% heat-inactivated fetal bovine serum (FBS). Cells were washed and resuspended in FACS buffer prior to enumeration using a haemocytometer and trypan blue exclusion.

Small intestinal lamina propria cell extraction

The last 10 cm of the small intestine (ileum) was harvested, then fat tissue and Peyer's patches were removed. After the tissue was cut longitudinally and washed in cold wash buffer (Hanks Balanced Salt Solution (HBSS), 10 μM HEPES, and 2% FBS), the intestine underwent two incubations (37°C, 250 rpm, 20 minutes) in pre-warmed EDTA buffer (HBSS, 5 mM EDTA, 10% FBS, 15 mM HEPES), followed by filtration through a 100 μm strainer to separate the small intestinal lamina propria from the intestinal epithelium. The tissue was then washed twice in cold wash buffer before being incubated (37°C, 250 rpm, 25 minutes) in digestion buffer (RPMI 1640, 10% FBS, 15 mM HEPES, 100 U/mL of DNAse, 200 U/mL of Collagenase VIII). The digestion was stopped by adding 35 mL of cold R10 buffer (RPMI 1640, 10% FBS, 15 mM HEPES, 1% L-glutamine, 1% penicillin/streptomycin (P/S)) and then passed through a 100 μm filter and

centrifuged at 2500 rpm for 7 minutes at 4 °C. Erythrocytes were lysed in red cell lysis solution (155 mM NH_4Cl, 0.12 mM EDTA, 10 mM $KHCO_3$) before leukocytes were enumerated using trypan blue exclusion.

***Ex vivo* restimulation**

Single cells extracted from the mLN of 14-day post infected mice were stimulated using two methods after resuspension in T cell media (RPMI, 10% FBS, 1% P/S, 10 mM HEPES, 70 μM β-mercaptoethanol). Innate stimulation used IL-12 (5 ng/mL), IL-18 (10 ng/mL), IL-2 (5 ng/mL) for 16 hours at 37°C, with brefeldin A and Golgi stop (BD Biosciences) added at the last 3 hours of incubation. Unbiased restimulation was performed using phorbol 12-myristate 13-acetate (PMA, 20 ng/mL) and ionomycin (1 μg/mL), with addition of brefeldin A and Golgi stop for 4 hours at 37°C. IFNγ production was assessed by intracellular staining and flow cytometry.

Flow cytometry

Dead cells from the single cell suspensions prepared as described above were stained with Fixable Viability Dye (eBioscience) in PBS for 23 minutes at 4 °C, followed by incubation with anti-Fc receptor (clone 2.4G2. BD Biosciences) in FACS for 7 minutes at 4 °C. Cells were then incubated with fluorochrome-conjugated antibodies against surface antigens diluted in FACS for 30 minutes at 4 °C. Extracellular antibodies used were the following: CD62L (MEL-14), CD4 (GK1.5), CD44 (IM7), TCRβ chain (H57-597), CD8α (53-6.7), CD39 (Duha59), CD49d (R1-2), CD22 (Cy34.1), CXCR3 (CXCR3-173). Cells were fixed and permeabilized according to the FoxP3 Fix/Perm kit (eBiosciences) prior to intracellular staining with the following intracellular antibodies: Tbet (O4-46), Eomes (Dan11mag), Ki67 (SolA15), IFN-γ (XMG1.2), GATA3 (TWAJ) and GZMA (3G8.5.

For experiments with BrdU, staining followed instructions from the Biolegend Phase-Flow™ BrdU Cell Proliferation Kit for Flow Cytometry with use of the antibody against BrdU (3D4). Data were acquired with a FACS Canto II or LSR Fortessa (BD Biosciences) and analyzed using FlowJo software (TreeStar).

RNA extraction and qRT-PCR

The first 0.5-1 cm of duodenum was flash frozen and stored at −80 °C before RNA was extracted using TRIzol (Sigma). cDNA samples were prepared as previously described (Meli et al., 2019). Relative gene expression was measured by qRT-PCR for *Ly6a, Cxcl9*, and *Cxcl10*. Fold expression was calculated using the ΔΔCT method where samples were normalized to the gene expression of the housekeeping gene *Hprt*. The following primers were ordered from Integrated DNA Technologies. CXCL9 forward primer (cttttcctcttgggcatcat) and reverse primer (gcatcgtgcattccttatca). CXCL10 forward primer (gcaccatgaacccaagtg) and reverse primer (ttcatcgtggcaatgatctcaaca). Ly6a forward primer (ggaggcagcagttattgtgg) and reverse primer (gctacattgcagaggtcttcc). HPRT forward primer (aggacctctcgaagtgttgg) and reverse primer (aacttgcgctcatcttaggc).

Cecal DNA extraction and qPCR

Fecal DNA was extracted using the QIAamp Fast DNA Stool Minutesi Kit (QIAGEN) according to the manufacturer's instructions. qPCR for the V1-V2 hypervariable region of the 16S rRNA gene segment was performed using the following primers: aggattagataccctggta (forward), cttcacgagctgacgac (reverse).

ELISA

Total serum IgE was quantified using a Mouse IgE ELISA Ready-SET-Go! kit (eBio/Affymetrix).

Fecundity

Feces were collected from 14-day infected mice at various time points post infection, weighed and placed in 750 µL saturated NaCl solution. The samples were homogenized and incubated at room temperature for 24 hours, then at 4 °C for at least 5 days. The eggs were enumerated and normalized to the volume of the supernatant and weight of feces.

Hematoxylin and Eosin staining

5 cm of duodenal tissue was cut longitudinally and rolled. Sample was fixed in 10% formalin for 24 hours and stored in 70% ethanol prior to paraffin-embedding done by the Histology Core at the RI-MUHC. 5 µm thick sections were placed onto microscope slides (Fisherbrand). Slides were deparaffinized and rehydrated (3 changes of 100% ethanol, 1 change of 95% ethanol, 1 minute each) to distilled water, stained with Mayer's hematoxylin (Sigma) for 15 minutes, and incubated with bluing solution for 2 minutes, followed by eosin Y staining (Sigma) for 23 seconds prior to dehydration (2 changes of 90% ethanol, 1 minute each) and clearing (3 changes of xylene, 1 minute each). Tap water rinses of 1 minute were performed after incubations with hematoxylin and bluing solution. Slides were mounted using a xylene-based mounting glue (ThermoFisher). Images were taken with an Olympus BX50 Brightfield microscope.

Immunoflurescence staining and Confocal microscopy

Tissue was prepared, and slides were deparaffinized and rehydrated as done with the H&E staining protocol. Slides were steamed with 10% citrate buffer for 20 minutes, cooled for 20 minutes and placed into PBS for 10 minutes for antigen retrieval. Slides were blocked with 1% bovine serum albumin (BSA) for 2 hours at room temperature prior to overnight primary antibody staining of UEA, DCLK and Ki67 at 4°C. Finally, staining of nuclei was done with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 15 minutes, followed by mounting with Prolong Diamond Antifade Mountant (Invitrogen Images were taken using a Zeiss LSM780 laser scanning confocal microscope and analyzed using Fiji software.

Statistical analysis

Statistical evaluation was performed by t test or one-way analysis of variance (ANOVA), or two-way ANOVA as indicated. A p value < 0.05 was considered significant. Data were analyzed using Prism v9 (GraphPad, La Jolla CA).

RESULTS

CD8$^+$ T cells expand during early *Hpb* infection in the gut-draining mesenteric lymph node.

Unpublished data from our lab found that CD8$^+$ αβ$^+$ T cells, rather than NKT cells, MAIT cells, CD4$^+$ T cells, and CD8$^+$ αα$^+$ T cells, were the predominant producers of IFNγ in the small intestinal lamina propria during 2 dpi of *Hpb* infection (Gentile, 2020). Furthermore, it was also determined that proliferating Ki67$^+$ CD8$^+$ T cells were highest in the gut-draining mesenteric lymph nodes (mLN) and lamina propria at 2 and 4 dpi, respectively (unpublished). Finally, it was observed that Ovalbumin-specific CD8$^+$ T cells transferred into TCRβ-KO mice also proliferated during early (< 4dpi) *Hpb* infection in the mLN, suggesting that *Hpb* can induce a unique IFNγ-producing CD8$^+$ T cell subset in a cognate antigen-independent manner (unpublished). To more closely examine the kinetics of CD8$^+$ T cell expansion during early *Hpb* infection, we analyzed proliferation and activation using Ki67 and bromodeoxyuridine (BrdU) labeling in activated CD44$^+$ CD8$^+$ T cells in the mLN at several timepoints (0.5, 1, 2, and 7 dpi). I limited my analysis to the mLN for most of this study as another member of the lab focused on the small intestine. My flow cytometry analysis revealed that the frequency of CD44$^+$ CD8$^+$ T cells, whether CD62L$^-$ or CD62L$^+$ began to increase at 2 dpi of *Hpb* in comparison to naïve CD8$^+$ T cells (CD44$^-$ CD62L$^+$) (**Fig 1A-D**). As Ki67 can also be expressed when cells are in the G0 phase (Miller et al., 2018), we additionally used staining protocols for BrdU, a thymidine analog that gets incorporated into the DNA of replicating cells, to assess proliferation. Accordingly, increased markers of proliferation in CD44$^+$ CD8$^+$ T cells were observed at the same time point (**Fig 1E-H**). Representative plots of select timepoints from multiple pooled experiments are shown where target populations are compared against uninfected controls and staining isotypes. We corroborated this data by performing immunofluorescent imaging of Ki67$^+$ CD8$^+$ T cells within the mLN from mice

at 2 and 7 dpi (**Fig 1I**). Together, these results confirm that $CD8^+$ T cell activation begins in the mLN at 2 dpi of *Hpb*. For these initial flow cytometry experiments, we used both BrdU and Ki67 staining to robustly identify the proliferation kinetics of the $CD8^+$ T cell population. However, for the rest of the study, we chose to exclude BrdU staining for technical ease.

CD8 T cell differentiation in the mLN changes throughout the course of *Hpb* infection.

To further characterize this early proliferating and activated $CD8^+$ T cell population, we examined their expression of master transcription factors, Tbet and Eomes. Expression of Tbet is induced by IFNγ signalling (Lighvani et al., 2001) whereas Eomes expression in $CD8^+$ T cells has been shown to be activated by IL-4 signals (Renkema et al., 2016). While it has been shown that *Hpb* induces type 1 immune signalling at 2-4 dpi (Gentile et al., 2019), followed by type 2 immunity immediately afterwards, how type 1 and 2- cytokines play a role in $CD8^+$ T cell differentiation during *Hpb* challenge still requires further examination. In support of the recently reported timeframe for type 1 immune induction during *Hpb* infection (Gentile et al., 2019), we detected an increase in $Tbet^+$ $Eomes^-$ $Ki67^+$ $CD44^+$ $CD8^+$ T cells numbers at 2 dpi that is retained until 7 dpi (**Fig 2A, B**). While the number of Tbet-expressing cells are maintained, their frequency decreases gradually. This discrepancy can be explained by the observed gradual increase in frequency and number of Eomes expression in $Ki67^+$ $CD44^+$ $CD8^+$ T cells (**Fig 2A, C**). Notably, the amount of $CD8^+$ T cells expressing both Tbet and Eomes follows a similar trend as single $Eomes^+$ $CD8^+$ T cells, yet their proportions do not significantly change over time (**Fig 2A, D**). Overall, these results suggest that the polarization of proliferating activated $CD8^+$ T cells follows the induction of type 1 and 2 immune responses during *Hpb* infection.

To continue the classification of this early activated $CD8^+$ T cell population, we compared it to the recently established *Hpb*-mediated IL-4-dependent population of virtual memory $CD8^+$ T (TVM) cells. TVM cells are a subset of $CD8^+$ T cells that expands independently of cognate antigen within the mLN and spleen (Lin et al., 2019; Rolot et al., 2018; Hussain et al., 2023). We found that the expansion of TVM cells ($CXCR3^+$ $CD49d^{lo}$ of $Ki67^+$ $CD44^+$ $CD8^+$ T cells) begins at 5 dpi in the mLN (**Fig 2E, F**). The expression patterns of $Eomes^+$ subpopulations, whether $Tbet^+$ or $Tbet^-$, followed the similar progressive expansion as TVM cells, while single positive Tbet-expressing $CD8^+$ T cells do not (**Fig 2A-F**). In concordance, we observed that TVM markers were induced in $Ki67^+$ $CD44^+$ $CD8^+$ T cells that express Eomes, rather than those that express only Tbet (**Fig 2G, H**). These results suggest that early proliferating $Tbet^+$ $CD8^+$ T cells may represent a unique population that precedes the generation of canonical TVM cells.

Type 1 and 2 immune signalling drives CD8 T cell differentiation during *Hpb* infection.

As Tbet expression in $CD8^+$ T cells correlated with the dynamics of helminth-activated type 1 signalling, we postulated that Tbet expression in $CD8^+$ T cells would require type 1 cytokine signalling. Using mice with a germline deletion of IFNγ-receptor (IFNγR-KO), we found a decreased frequency of $Tbet^+$ $Ki67^+$ $CD44^+$ $CD8^+$ T cells within the mLN of 2 and 4 days *Hpb*-infected IFNγR-KO mice compared to WT controls (**Fig 3A, B**). The number of $Tbet^+$ $Ki67^+$ $CD44^+$ $CD8^+$ T cells in IFNγR-KO mice consistently followed the same downward trend at 4 dpi (**Fig 3A, B**). Conversely, the expression of Eomes in activated $CD8^+$ T cells was largely not changed in mice lacking IFNγ signalling, except for a significant increase observed at 4 dpi during early infection (**Fig 3A, C**). We next tested the association between Eomes expression in $CD8^+$ T cells and type 2 immunity by using mice that lacked the gene that encodes signal transducer and

activator of transcription 6 (STAT6-KO), which is a master transcriptional regulator that initiates and maintains type 2 immunity (Maier et al., 2012). Expectedly, mice that lack STAT6-mediated responses had decreased amounts of $Ki67^+$ $CD44^+$ $CD8^+$ T cells that expressed Eomes in the mLN at 7 dpi (**Fig 3E, F**), while Tbet expression in activated $CD8^+$ T cells did not change at early or late time points (**Fig 3E, G**). Accordingly, activated $CD8^+$ T cells that expressed Tbet and Eomes were partially decreased in both IFNγR-KO and STAT6-KO mice (**Fig 3A, D, E, H**). These results propose that $CD8^+$ T cell differentiation is affected by type 1 and 2 signalling pathways induced during *Hpb* infection in the mLN.

To further examine the hypothesis that differentiation of $CD8^+$ T cells corresponds to signalling pathways induced during *Hpb* infection, we examined the requirement of TVM cell expansion and development on STAT6-mediated type 2 signalling from 2 to 14 dpi. As $CD44^+$ $CD49d^{lo}$ clearly denotes $CD8^+$ T cells that have been activated but have not encountered their cognate antigen, we used this staining phenotype to identify TVM cells. We observed an ablated percentage of $CD44^+$ $CD49d^{lo}$ TVM cells in the mLN of STAT6-KO mice at 5 and 14 dpi (**Fig 4A**). Furthermore, while the percentage of Eomes-expressing TVM cells was also decreased in STAT6-KO mice compared to controls at 5 and 14 dpi (**Fig 4B**), the frequency of additional TVM markers CXCR3 and CD62L was not changed at any timepoint (**Fig 4C**). The number of TVM cells and its canonical markers were also decreased at 14 dpi in the mLN of STAT6-KO mice (**Fig 4A-C**). These results suggest that TVM cell expansion and development during *Hpb* infection require IL-4 and/or IL-13 signals.

To further examine the impact of type 2 cytokine signaling on TVM cell development and differentiation, we initiated a collaboration with Dr. Benjamin Dewals at the University of Liège, an expert in TVM cell biology (Rolot et al., 2018; Yang et al., 2023). Ongoing studies from his

laboratory have demonstrated that TVMs uniquely expression CD22, CD39 and Granzyme A compared to other CD8+ effector T cell subsets (Yang et al., 2023). To test if the expression of these novel markers of TVM cells was dependent on type 2 cytokine signalling, we phenotyped TVM cells expanded after 14 dpi of *Hpb* in STAT6-KO mice. Decreased expression of CD22, CD39, and Granzyme A in TVM cells was observed in *Hpb*-infected mice that lacked type 2 signalling compared to WT controls (**Fig 4D-E**). Given that CD22 and CD39 have been described to inhibit the activation of other immune cell subsets (Smith et al., 1998; Noble et al., 2016), these results suggest that while IL-4 signalling promotes the expansion of TVM cells during *Hpb* infection it may also activate an auto-regulatory loop to prevent hyperactivation of this T cell subset with innate-like properties.

IL-4 can drive Eomes activity in $CD8^+$ T cells that leads to IFNγ production (Lee et al., 2013; Carty et al., 2013). Therefore, we predicted that IFNγ production within the TVM cell compartment would be impaired in mice that lack IL-4 signalling. Following *ex vivo* stimulation of cells from the mLN of *Hpb*-infected STAT6-KO mice at 14 dpi, our results showed that the percentage of IFNγ production in TVM cells was equivalent to that of wild-type controls (**Fig 4F**), yet the number of $IFN\gamma^+$ TVM cells were decreased. These results suggest that IL-4 signalling is necessary for IFNγ production in *Hpb*-expanded TVM cells but does not affect the equilibrium of IFNγ-expressing vs non-expressing TVM cells.

The microbiota drives intestinal Type 1 immune target gene activation but may not play a role in Tbet expression by *Hpb*-activated $CD8^+$ T cells.

Our results demonstrate that a previously undefined subset of $CD8^+$ T cells expands in the mLN during early *Hpb* infection in an IFNγ-signalling-dependent manner. To broaden our

understanding of $CD8^+$ T cell differentiation upon infection of a strictly enteric helminthic pathogen, an important factor to consider is the gut microbiota, a dynamic ecosystem of bacteria, viruses, protists, and fungi that plays non-redundant roles towards immune development and activation (Berg et al., 1996; Thursby et al., 2017). To begin to explore the role of the gut microbiota on $CD8^+$ T cell differentiation, we first established a model of GF *Hpb* infection (Russell et al., 2021). We infected germ-free (GF) mice with GF *Hpb* larvae (Hpb^{GF}) that were tested by our lab to have maintain sterility in GF mice while inducing similar infectivity and fitness as larvae generated from previously established rearing techniques (Johnson et al., 2015; Russell et al., 2021). Since GF mice have enlarged ceca due to their lack of commensal gut microbes that would otherwise metabolize complex carbohydrates (Gustafsson et al., 1970; Lindstedt et al., 1965), we observed that the cecal weights and cecal 16S rDNA levels of infected GF mice resembled those of uninfected GF mice (**Fig 5A, B**). Additionally, cecal weights and 16S rDNA levels of Hpb^{GF}- infected mice were increased and decreased, respectively, in comparison to those of GF mice reconstituted with a diverse microbiota from specific-pathogen-free mice (hereafter referred to as xGF; **Fig 5A, B**). Together, these results suggest that our GF *Hpb* infection model maintains sterility.

To assess the immunogenicity of this model of GF *Hpb* infection, we examined classical components of the helminth-induced type 2 immune response. While GF infection yielded a higher number of Th2 cells in the mLN and lamina propria (**Fig 5D**) and higher total IgE levels in the serum (**Fig 5E**), type 2-associated epithelial cells, such as tuft and goblet cells (marked by Double cortin-like kinase (DCLK) and Ulex europaeus agglutinin (UEA), respectively), were not overtly different (**Fig 5F**). However, more extensive imaging and quantification is required to confirm the epithelial cell phenotype. The increase in type 2 immune markers was not due to a change in

parasite load, as egg count, a proxy for parasite burden, was not changed (**Fig 5C**). In concordance with previous studies (Moyat et al., 2022), these results suggest that there is an elevated type 2 immune response in mice lacking a microbiota during helminth infection. While type-2-associated molecules and cell types have been associated in repair (Monticelli et al., 2011; Wynn et al., 2012; Rosenberg et al., 2013), overexuberant or chronic type 2 immune signals during helminth infection are linked to accelerated fibrosis (Mentink-Kane et al., 2011). To determine if GF infection correlates with increased fibrosis, we examined the muscularis mucosae in the small intestine of *Hpb*^{GF} -infected mice after 14 dpi. We observed an increase in inflammatory infiltrates and cell debris within regions that previously harboured developing *Hpb* larvae (**Fig 5G**), suggesting that the microbiota may play a role in regulating the type 2 immune response to ultimately result in proper wound repair.

As the gut microbiota can activate IFNγ production by CD8$^+$ T cells (Tanoue et al., 2019) and enhance type 1 immunity for defense against infection (Thiemann et al., 2017), we hypothesized that the microbiota drives IFNγ signalling during *Hpb* infection. Within the infected small intestine of GF mice compared to xGF controls, we observed a decrease in expression of IFNγ target genes: Cxcl9, Cxcl10, and Ly6a (**Fig 6A**). These data suggest that IFNγ signalling is impaired during GF *Hpb* infection. Thus, we next sought to examine the role of the gut microbiota in IFNγ-dependent CD8$^+$ T cell differentiation. Flow cytometric analysis of cells from the mLN of infected GF mice showed a significant increased frequency and non-significant increased abundance of Ki67$^+$ CD44$^+$ CD8$^+$ T cells increased at 4 dpi compared to xGF infected controls (**Fig 6B**). No changes were seen in the proportion or number of Tbet-expressing proliferating activated CD8$^+$ T cells between xGF and GF groups (**Fig 6C**). These results imply that type 1

immune genes activated by microbial signals play a larger role in regulating the local (intestinal) type 1 immune response than in the differentiation of peripheral $CD8^+$ T cells during *Hpb* infection.

Microbial signals regulate the expression of CD39 and Granzyme A in TVM cells during *Hpb* infection.

Although our preliminary data demonstrate a lack of microbial importance on Tbet expression by $CD8^+$ T cells during early *Hpb* infection, the gut microbiota may still control TVM expansion. As an increase in Th2 cells was found during GF *Hpb* infection, we hypothesized that increased IL-4 signals would enhance TVM expansion, which has previously been reported to be IL-4-dependent (Rolot et al., 2018; Lin et al., 2019). Unexpectedly, the frequency and number of total $CD44^+$, $CD49d^{lo}$ TVM cells and TVM cells that expressed additional canonical markers (CXCR3, CD62L) were not different between hosts with or without a commensal microbiota (**Fig 7A, B**). Furthermore, development of TVM cells requires Eomes expression because it regulates the expression of CD122, a receptor that is necessary for IL-15-dependent TVM maintenance (Sosinowski et al., 2013). It has been shown that the expression of Eomes in TVM cells in unprimed hosts is dependent on type 1 IFN signalling (Martinet et al., 2015). Given that GF mice have compromised type I IFN signaling (Schaupp et al., 2020; Abt et al., 2012), we postulated that the TVM cells from unprimed GF hosts would also have impaired Eomes expression. However, we found that Eomes expression was intact in TVM cells from uninfected GF mice and was further retained in TVM cells expanded upon GF *Hpb* infection (**Fig. 7C**). Our results show that any potential microbially-derived source of type 1 IFN does not affect the expression of Eomes in *Hpb*-expanded TVM cells.

To further characterize the role of the microbiota on the development of TVM cells, we analyzed its role in expression of immunoregulatory markers expressed by TVM cells. We found that the CD22 expression in $CD44^{+}$, $CD49d^{lo}$ TVM cells was not changed between xGF and GF hosts. We additionally observed that the frequency of CD39 and Granzyme A was increased in GF hosts at 14 dpi, whereas the abundances of CD39- and Granzyme A-expressing TVM cells were not changed compared to controls (**Fig 7D-E**). These results suggest that the microbiota can partially regulate the *Hpb*-induced TVM cell subset. Moreover, as type 1 immune signalling is enhanced upon microbial sensing, we predicted that TVM cells generated in the absence of the microbiota during *Hpb* infection would have an impaired ability to produce IFNγ. However, IFNγ production by *ex-vivo* stimulated TVM cells from 14 day post-infected mLNs was not affected by microbiota-derived signals (**Fig 7F-G**), suggesting canonical TVM cell effector functions activated during *Hpb* infection may be regulated independently of the microbiota.

FIGURES

Figure 1

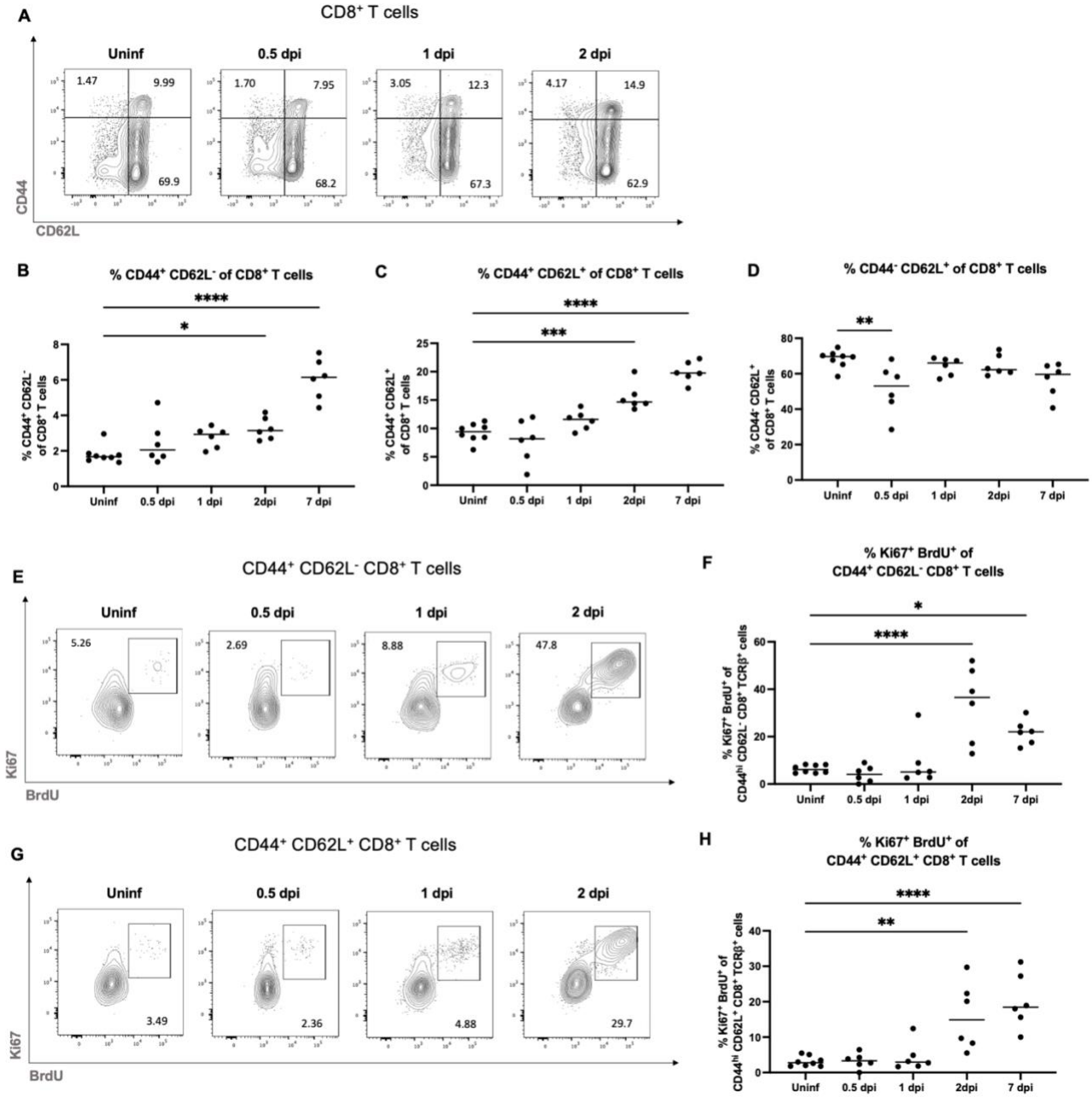

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Figure 1 continued

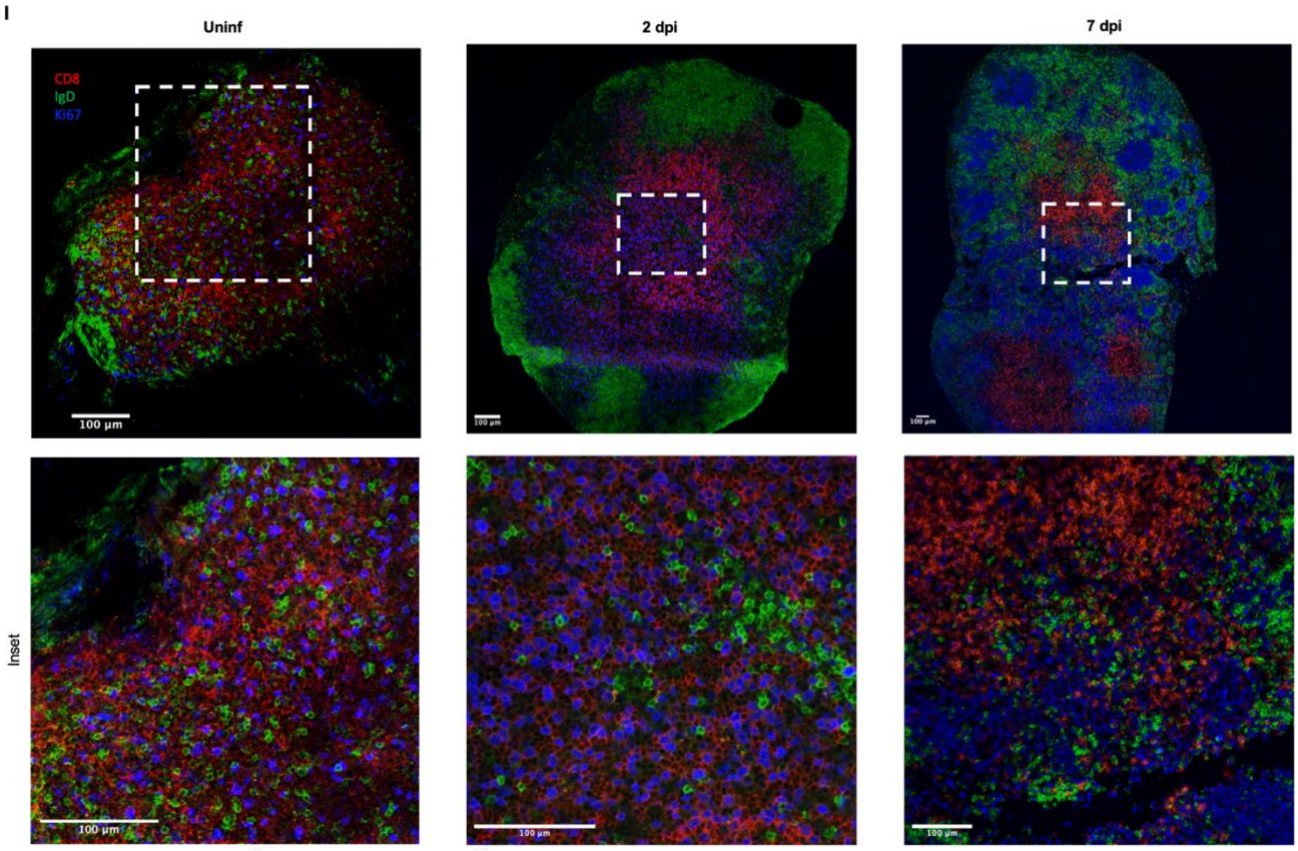

Figure 2

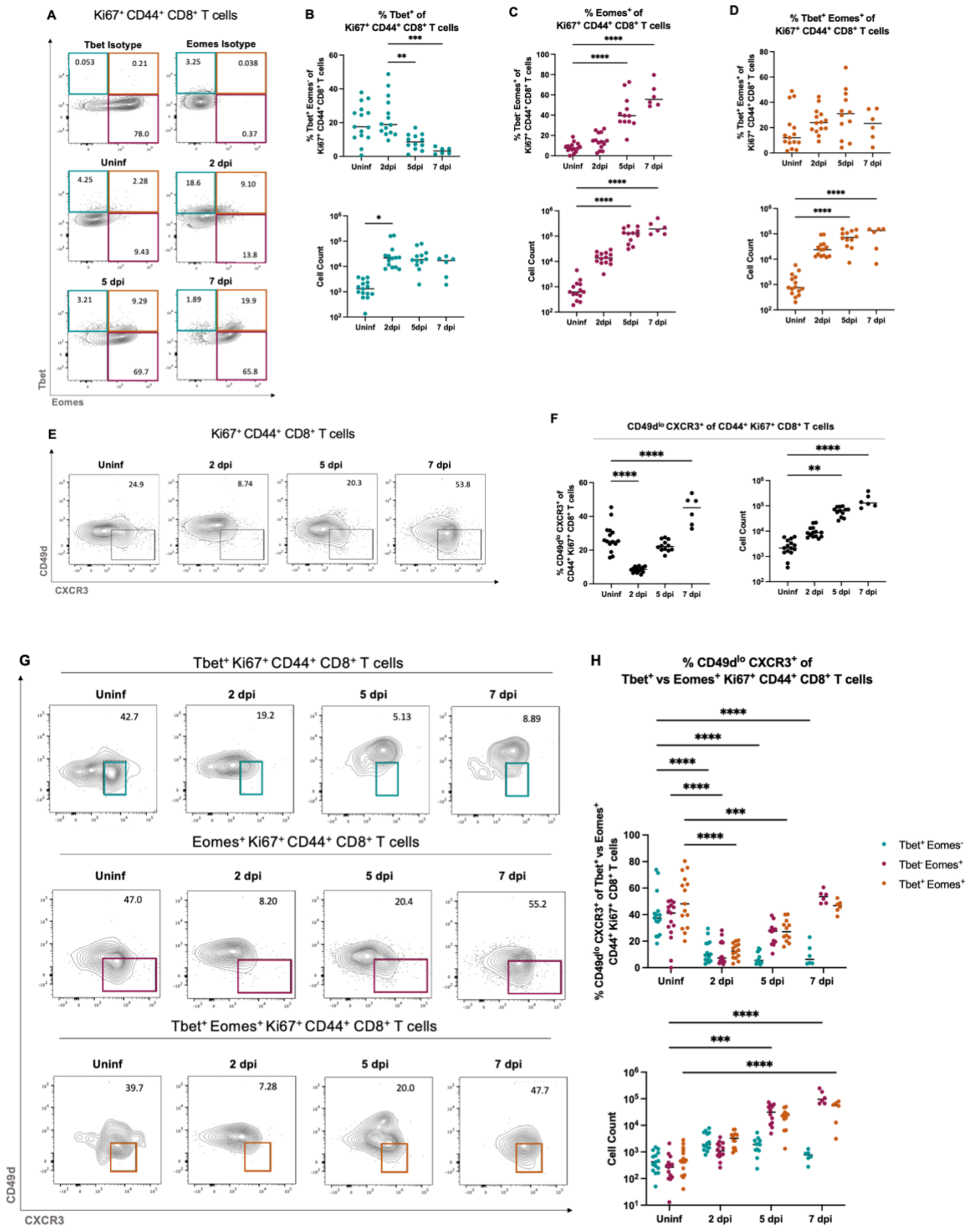
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Figure 3

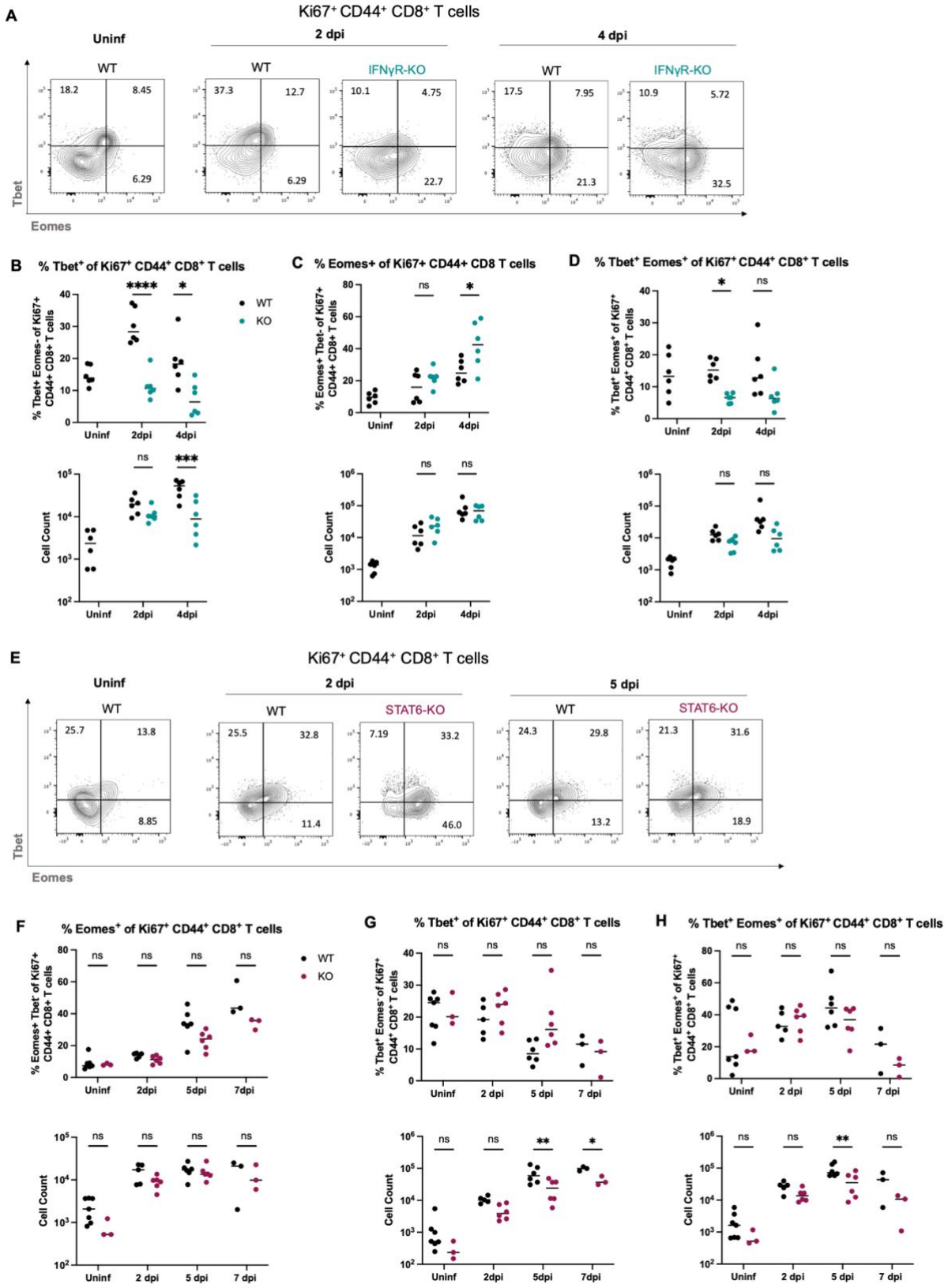
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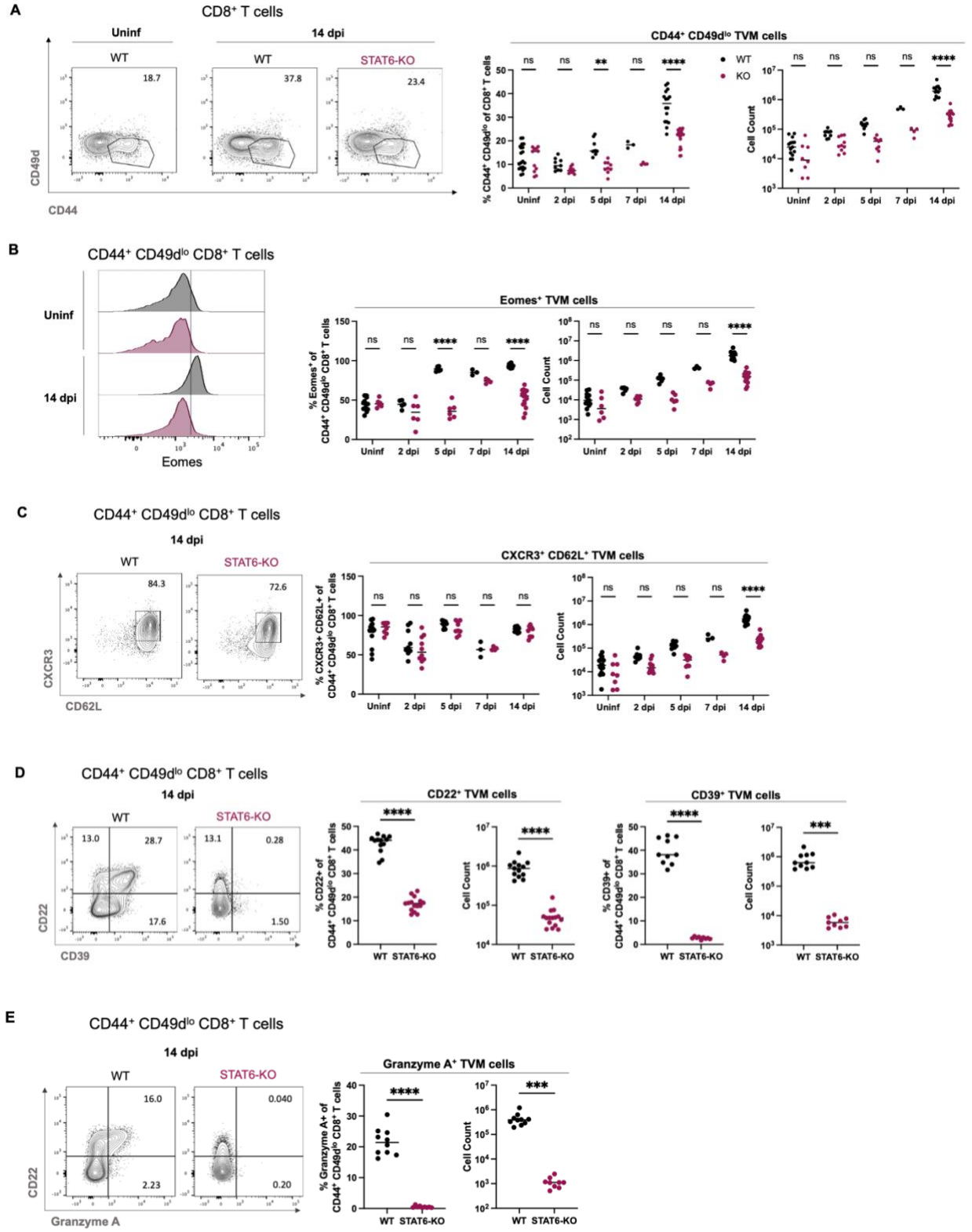
Figure 4



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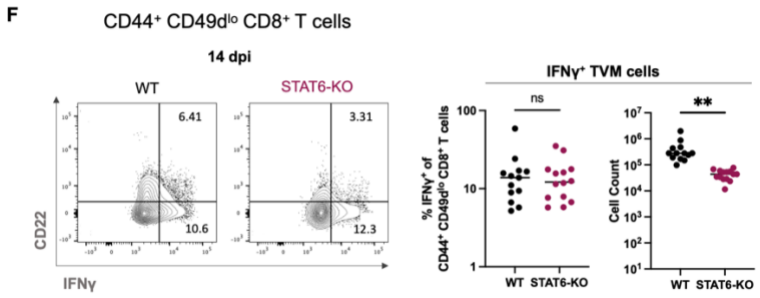
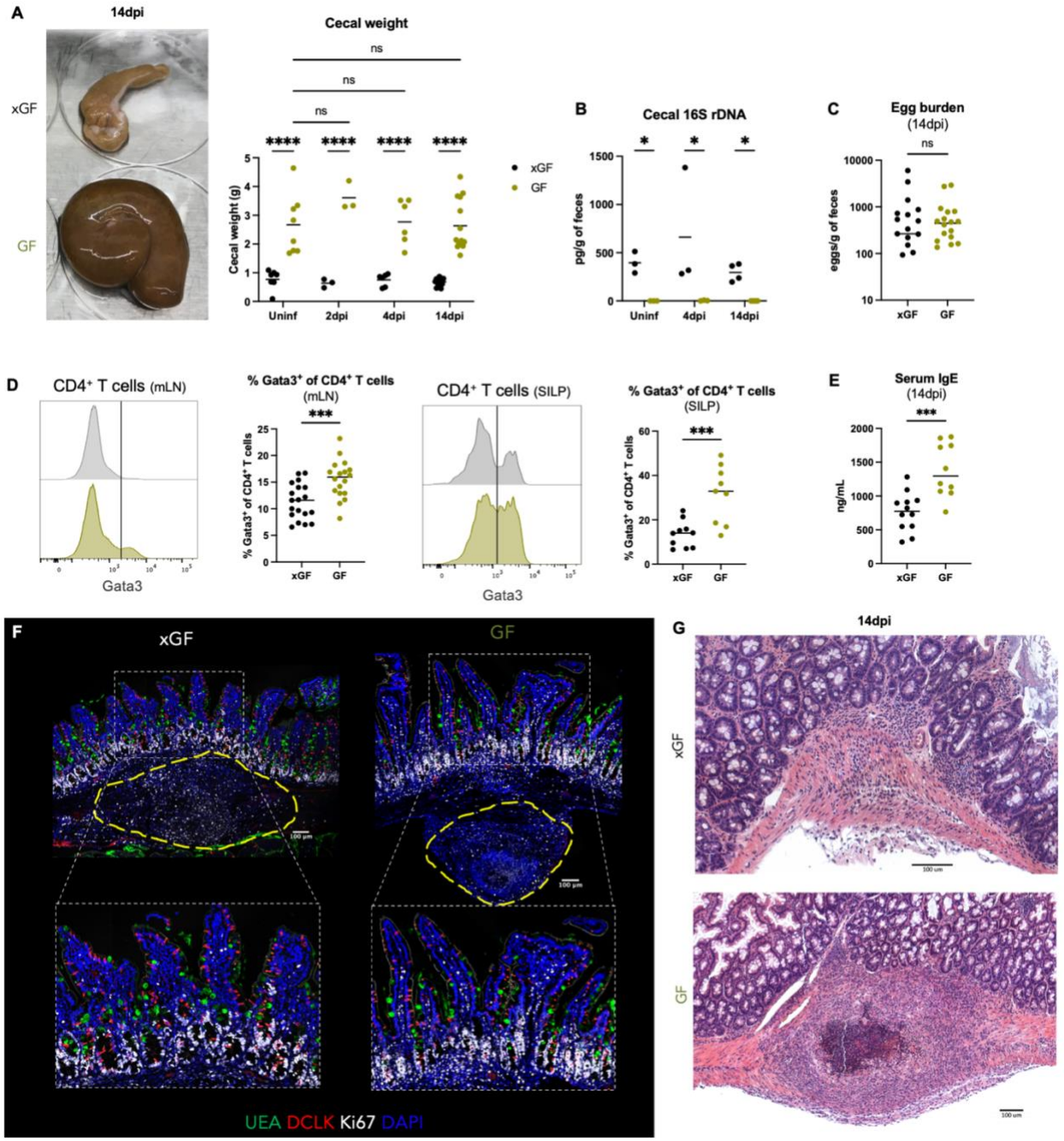


Figure 5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Cecal weight (g)
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(14dpi)
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xGF
GF
F
xGF
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UEA DCLK Ki67 DAPI
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14dpi
xGF
GF

Figure 6

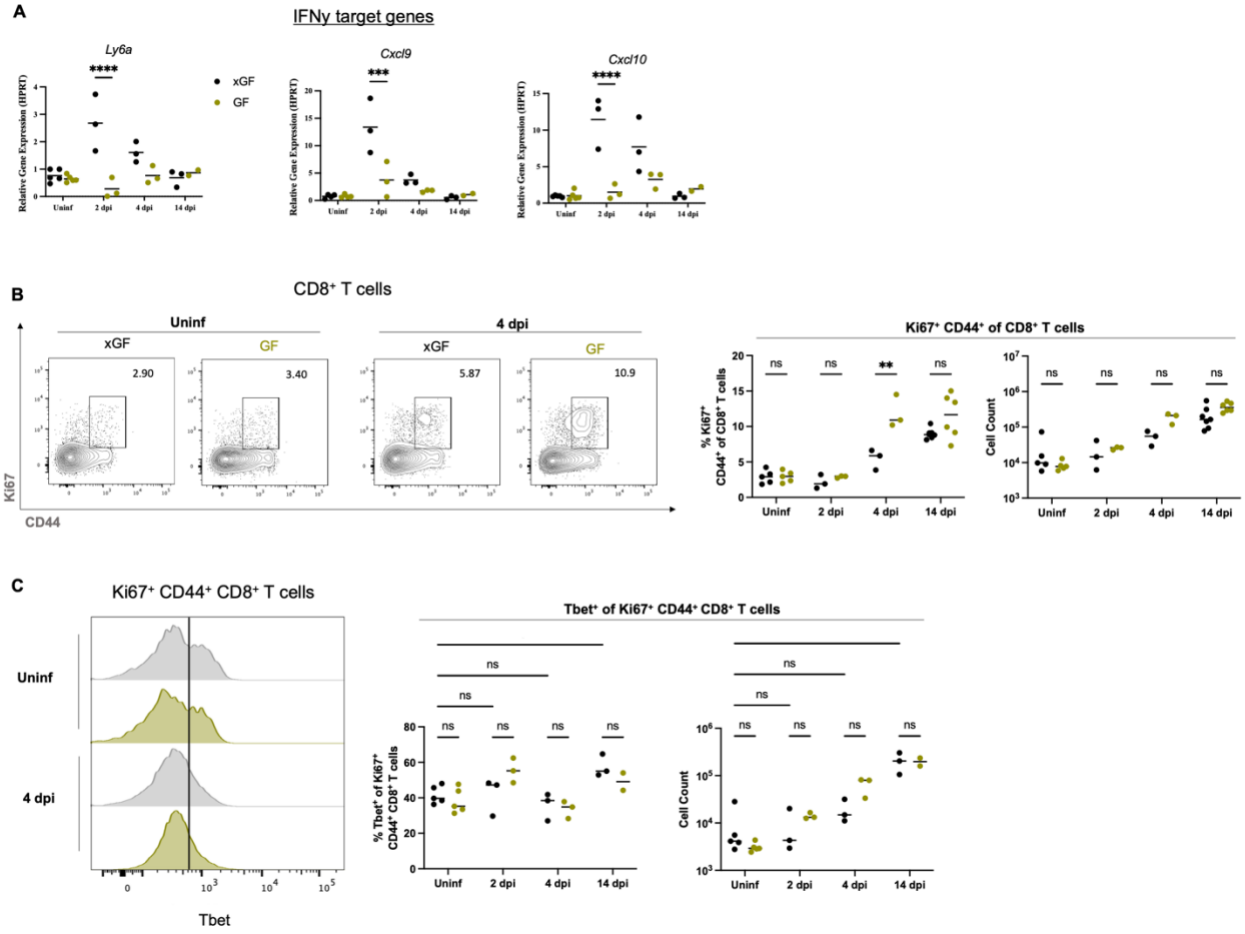
A
IFNγ target genes
Ly6a
Cxcl9
Cxcl10
xGF
GF
Relative Gene Expression (HPRT)
Uninf
2 dpi
4 dpi
14 dpi
B
CD8⁺ T cells
Uninf
4 dpi
xGF
GF
2.90
3.40
5.87
10.9
Ki67
CD44
Ki67⁺ CD44⁺ of CD8⁺ T cells
% Ki67⁺ CD44⁺ of CD8⁺ T cells
Cell Count
C
Ki67⁺ CD44⁺ CD8⁺ T cells
Uninf
4 dpi
Tbet
Tbet⁺ of Ki67⁺ CD44⁺ CD8⁺ T cells
% Tbet⁺ of Ki67⁺ CD44⁺ CD8⁺ T cells
Cell Count

Figure 7

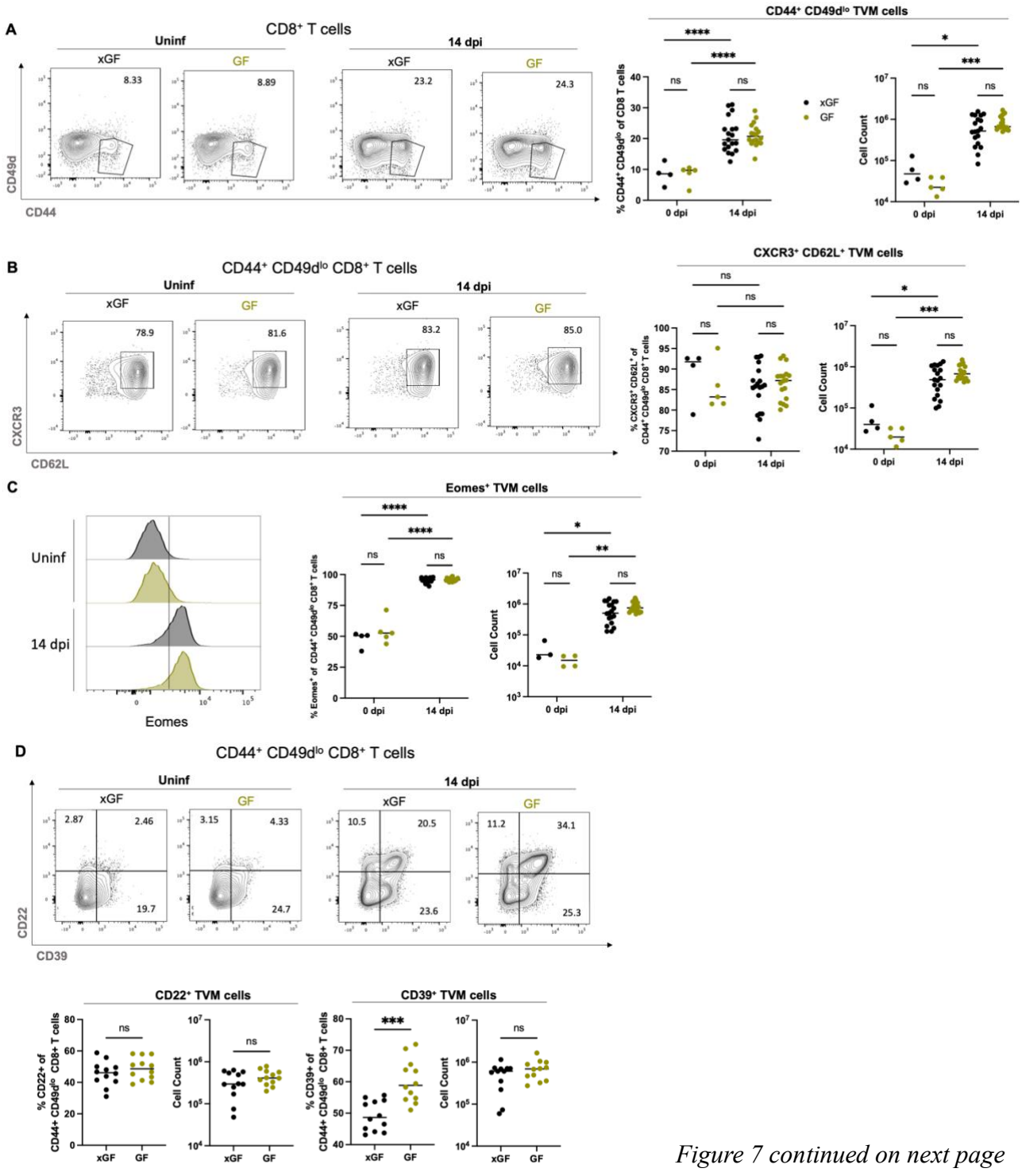

Figure 7 continued on next page

Figure 7 continued

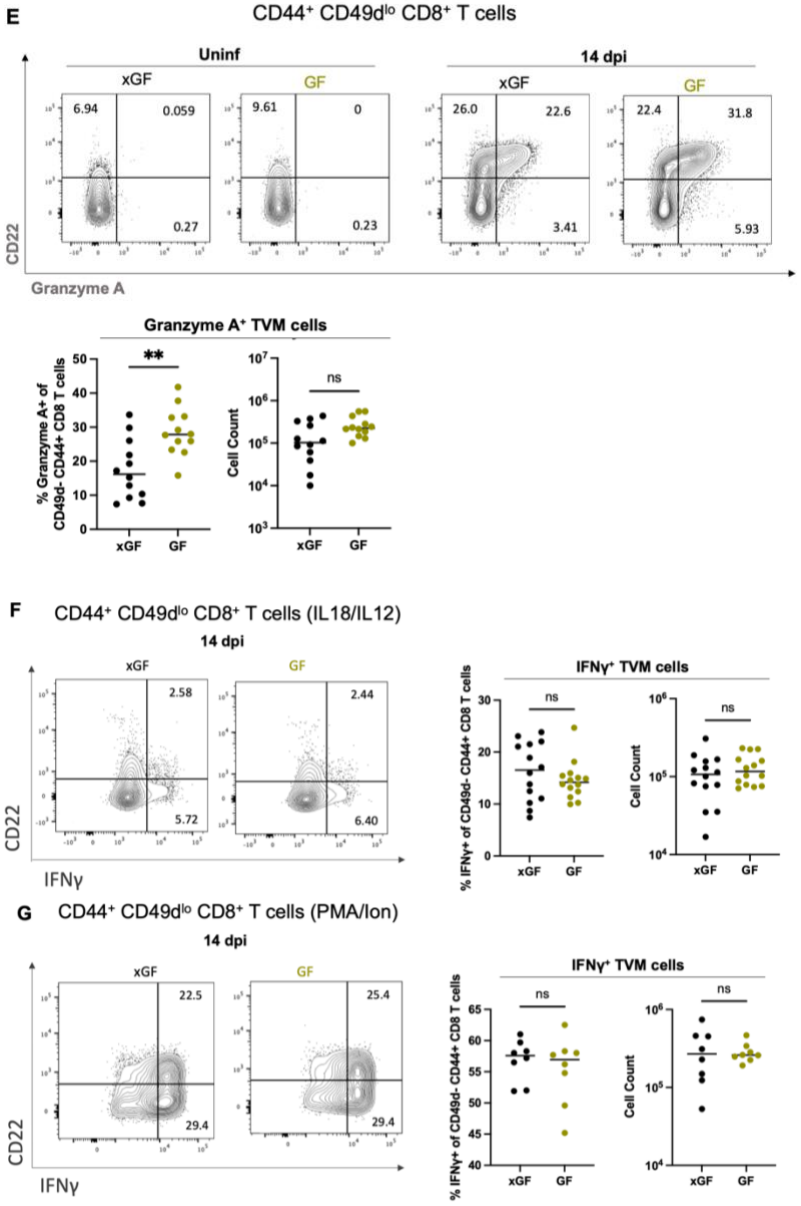

FIGURE LEGENDS

Figure 1. $CD44^+CD8^+$ T cells begin to proliferate at 2 dpi in the mLN of *Hpb*-infected mice. (**A-I**) 8-10-week-old specific-pathogen-free C57BL/6 mice were infected with 200 fecal-reared *Hpb* L3 larvae and sacrificed at the indicated days post infection (dpi). (**A-H**) Cells from the mesenteric lymph node (mLN) were analyzed by flow cytometry. (**A-D**) Representative plots (**A**) and percentages of $CD44^+CD62L^-$(**B**), $CD44^+CD62L^+$(**C**), and $CD44^-CD62L^-$ (**D**) of the total $CD8^+$ T cell population (**D**) are shown. (E-H) Representative plots and percentages of $Ki67^+BrdU^+$ cells from $CD44^+CD62L^-$ (**E, F**) and $CD44^+CD62L^+CD8^+$ T cells (**G, H**) are shown. (**I**) Immunofluorescent staining of green IgD^+ lymphocytes, blue $Ki67^+$ proliferating cells, and red $CD8^+$ T cells in the mLN of *Hpb*-infected mice at the indicated time points. (**B-D, F, H**) Each dot represents one test subject. (**B-D, F, H**) Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple-comparison test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ns = non-significant. Scale bars denote 100 μm.

Figure 2. *Hpb* infection induces an early transient population of activated $Tbet^+CD8^+$ T cells do not express markers of memory. (**A-H**) 8-10-week-old specific-pathogen-free C57BL/6 mice were infected with 200 fecal-reared *Hpb* L3 larvae and sacrificed at the indicated day post infection (dpi). (**A-H**) Cells from the mesenteric lymph node (mLN) were analyzed by flow cytometry. (**A-D**) Representative plots, percentages, and numbers of $Tbet^+Eomes^-$ (teal; **A, B**), $Tbet^-Eomes^+$ (magenta; **A, C**), and $Tbet^+Eomes^+$ (orange; **A, D**) of the total $Ki67^+CD44^+$ $CD8^+$ T cell population are shown. (**E, F**) Representative plots, percentages, and numbers of $CD49d^{lo}CXCR3^+$ cells from the total $Ki67^+CD44^+$ $CD8^+$ T cell subset are shown. (**G-H**) Representative plots (**G**), percentages (**H**), and numbers (**H**) of $CD49d^{lo}CXCR3^+$ cells from $Ki67^+CD44^+$ $CD8^+$ T cells that are

$Tbet^+Eomes^-$ (teal) or $Tbet^-Eomes^+$ (magenta) or $Tbet^+Eomes^+$ (orange) are shown. (**B-D, F, H**) Each dot represents one test subject. (**B-D, F, H**) Statistical analysis was performed by one-way (**B-D, F**) or two-way ANOVA followed by Tukey's multiple-comparison test (**H**). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ns = non-significant.

Figure 3. Differentiation of activated $CD8^+$ T cells during *Hpb* infection requires IFNγ and STAT6 signalling. (**A-H**) 8-10-week-old specific-pathogen-free C57BL/6 IFNγR-KO (**A-D**) or STAT6-KO (**E-H**) mice were infected with 200 fecal-reared *Hpb* L3 larvae and sacrificed at the indicated days post infection (dpi) and compared to wild-type (WT) controls. (**A-H**) Cells from the mesenteric lymph node (mLN) were analyzed by flow cytometry. (**A-D**) Representative plots, percentages, and numbers of $Tbet^+Eomes^-$ (**A, B**), $Tbet^-Eomes^+$ (**A, C**), and $Tbet^+Eomes^+$ (**A, D**) of the total $Ki67^+CD44^+$ $CD8^+$ T cell population from WT (black) and IFNγR-KO (teal) are shown. (**E-H**) Representative plots, percentages, and numbers of $Tbet^+Eomes^-$ (**E, F**), $Tbet^-Eomes^+$ (**E, G**), and $Tbet^+Eomes^+$ (**E, H**) of the total $Ki67^+CD44^+$ $CD8^+$ T cell population from WT (black) and STAT6-KO (magenta) are shown. (**B-D, F-H**) Each dot represents one test subject. (**B-D, F-H**) Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple-comparison test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ns = non-significant.

Figure 4. STAT6 signalling drives a specific program in TVM cells. (**A-F**) 8-10-week-old specific-pathogen-free C57BL/6 wild-type (WT) or STAT6-KO mice were infected with 200 fecal-reared *Hpb* L3 larvae and sacrificed at the indicated days post infection (dpi). (**A-F**) Cells from the mesenteric lymph node (mLN) were analyzed by flow cytometry. (**A**) Representative plots, percentages, and numbers of $CD44^+CD49d^{lo}$ cells from the total $CD8^+$ T cell population are shown.

(**B**) Representative histograms, percentages, and numbers of Eomes expression in $CD44^+CD49d^{lo}$ $CD8^+$ T cells are shown. (**C-D**) Representative plots, percentages, and numbers of $CXCR3^+CD62L^+$ (**C**), $CD22^+$ (**D**), $CD39^+$ (**D**), and Granzyme A^+ (**E**) of $CD44^+CD49d^{lo}CD8^+$ T cells are shown. (F) Representative plots, percentages, and numbers of $IFN\gamma^+CD44^+CD49d^{lo}CD8^+$ T cells after IL-12/IL-18 stimulation for 16 hrs are shown. (**A-F**) Each dot represents one test subject. (**A-F**) Statistical analysis was performed by t-test (**D, E, F**) or two-way ANOVA followed by Tukey's multiple-comparison test (**A-C**). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ns = non-significant.

Figure 5. Preliminary characterization of the germ-free *Hpb* infection model. (**A-G**) 8-10-week-old germ-free (GF) C57BL/6 mice or GF mice reconstituted with a specific-pathogen-free microbiome (xGF) were infected with 200 sterile *Hpb* L3 larvae (Hpb^{GF}) and sacrificed at the indicated days post infection (dpi). (**A**) Photographs and weights of ceca from infected mice are shown. (**B**) DNA extracted from cecal samples and 16S rDNA (V1-V2) levels were quantified by qPCR. (**D**) Representative histograms and percentages of GATA3 expression in $CD4^+$ T cells in the mesenteric lymph node (mLN) and small intestinal lamina propria from 14-day-infected mice. (**C, E**) At 14 days post infection (dpi), egg burden (**C**) and total serum IgE (**E**) were enumerated. (**F, G**) Immunofluorescent staining (**F**) of green UEA^+ goblet cells, red $DCLK^+$ tuft cells, white $Ki67^+$ proliferating stem cells, and blue $DAPI^+$ nuclei and hematoxylin and eosin staining (**G**) in the duodenum of 14-day infected mice. (**A-E**) Each dot represents one test subject. (**A-E**) Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple-comparison test (**A, B**) or t-test (**C-E**). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ns = non-significant. Scale bars denote 100 μm.

Figure 6. Regulation of the microbiota on the *Hpb*-induced type-1-immune-associated response is location-dependent. (**A-C**) 8-10-week-old germ-free (GF) C57BL/6 mice or GF mice reconstituted with a specific-pathogen-free microbiome (xGF) were infected with 200 sterile *Hpb* L3 larvae (Hpb^{GF}) and sacrificed at the indicated days post infection (dpi). (**A**) Quantitative PCR was performed on IFNγ target genes, *Ly6a, Cxcl9,* and *Cxcl10* using cDNA converted from RNA extracted from the duodenum of mice. (**B**) Representative plots, percentages, and numbers of $CD44^{+}Ki67^{+}$ of the total $CD8^{+}$ T cell population (**D**) are shown. (**C**) Representative histograms, percentages, and numbers of Tbet expression in $CD44^{+}Ki67^{+}CD8^{+}$ T cells are shown. (**A-C**) Each dot represents one test subject. (**A-C**) Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple-comparison test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ns = non-significant.

Figure 7. The microbiota represses CD39 and granzyme A expression in TVM cells that expand during *Hpb* infection. (**A-G**) 8-10-week-old germ-free (GF) C57BL/6 mice or GF mice reconstituted with a specific-pathogen-free microbiome (xGF) were infected with 200 sterile *Hpb* L3 larvae (Hpb^{GF}) and sacrificed at the indicated days post infection (dpi). (**A-G**) Cells from the mesenteric lymph node (mLN) were analyzed by flow cytometry. (**A**) Representative plots, percentages, and numbers of $CD44^{+}CD49d^{lo}$ cells from the total $CD8^{+}$ T cell population are shown. (**C**) Representative histograms, percentages, and numbers of Eomes expression in $CD44^{+}CD49d^{lo}$ $CD8^{+}$ T cells are shown. (**B, D-E**) Representative plots, percentages, and numbers of $CXCR3^{+}CD62L^{+}$ (**B**), $CD22^{+}$ (**D**), $CD39^{+}$ (**D**), and Granzyme A^{+} (**E**) of $CD44^{+}CD49d^{lo}CD8^{+}$ T cells are shown. (**F, G**) Representative plots, percentages, and numbers of

$IFN\gamma^{+}CD44^{+}CD49d^{lo}CD8^{+}$ T cells after stimulations with IL-12/IL-18 for 16 hrs (**F**) and PMA/Ionomycin for 4 hrs are shown. (**A-H**) Each dot represents one test subject. (**A-H**) Statistical analysis was performed by t-test (**D-G**) or two-way ANOVA followed by Tukey's multiple-comparison test (**A-C**). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, ns = non-significant.

DISCUSSION

Our preliminary studies examined the CD8 T cell subsets induced during *Hpb* infection and their expansion kinetics, activation requirements and phenotypic markers. We have found a unique subset of IFNγ-dependent, microbiota-independent Tbet$^+$ CD8$^+$ T cells induced during early *Hpb* infection that is different from the previously identified TVM cell subset. Furthermore, we demonstrate the IL-4 and microbial sensitivity of inhibitory receptors, CD22, CD39, and effector molecule, granzyme A, in TVM cells developed during *Hpb* infection. Additionally, we performed a preliminary characterization of the germ-free *Hpb* model, with assessment of the type 1 and 2 immune responses.

Expression of Tbet is induced by IFNγ signalling (Lighvani et al., 2001), whereas Eomes expression in CD8$^+$ T cells has been shown to be activated by IL-4 (Renkema et al., 2016). Data from our lab suggests that αβ$^+$ CD8$^+$ T cells are the dominant producers of IFNγ during *Hpb* infection (Gentile, 2020), while previous studies postulate that the source of IL-4 is from invariant NK T cells (Lee et al., 2013; Weinreich et al., 2010). However, the mechanism by which *Hpb*-induced type 1 and 2-associated cytokines affect CD8$^+$ T cell differentiation has yet to be explored. Our data demonstrates that STAT6-dependent Eomes$^+$ CD8$^+$ T cells express TVM cell markers and have similar expansion kinetics as TVM cells during *Hpb* infection, which is similar to previously published data reporting IL-4-dependent expansion of TVM cells upon helminth infection (Rolot et al., 2018; Lin et al. 2019). In comparison, we report a novel subset of IFNγ-dependent Tbet$^+$ CD8 T cells that expand during early *Hpb* infection in the mLN that neither follow TVM cell expansion kinetics nor express TVM markers. While this does not preclude Tbet$^+$ Eomes$^-$ CD8$^+$ T cells from possibly being precursors for TVM cells, further studies that track these cells over time will be needed.

Our study found that the IFNγ response mediated by *Hpb* during early infection (Nusse et al., 2018; Progatsky et al., 2020; Gentile et al., 2020) contributes to $CD8^+$ T cell polarization towards a type-1-associated phenotype of Tbet expression. Whether T-bet expression is necessary for IFNγ production by transiently activated $CD8^+$ T cells should be studied to uncover mechanisms of type 1-mediated tissue protection and to further understand the regulation of IFNγ in distinct transcriptional programs within the T cell lineage (Szabo et al., 2002). Interestingly, we also observed that IFNγ signalling inhibited Eomes expression in activated $CD8^+$ T cells at 4 dpi, in support of previous data that showed how IFNγ could inhibit type 2-dependent responses during *Hpb* infection (Else et al., 1994). Furthermore, increase of IFNγ signalling at baseline in STAT6-KO mice (Mahalingam et al., 2001) could explain how the proportion of Tbet expressing cells in the activated CD8 T cell population of naïve mice was similar to frequencies seen in WT mice infected with *Hpb* at 2 dpi. These data suggest a balance between type 1 and 2 immune responses induced upon *Hpb* infection may also regulate CD8 T cell differentiation.

Hpb infection has been well-established to promote a highly polarized type 2 immune response that induces TVM cell expansion. Here we confirm that IL-4-mediated signals induce TVM cell expansion as early as 5 dpi (Lin et al., 2019) and that IL-4 can drive a specific phenotypic program in TVM cells during *Hpb* infection (Rolot et al., 2018). Though it has been shown that IL-4 can drive Eomes expression in TVM cells in the presence (Rolot et al., 2018) and absence (Renkema et al., 2016, Kurzweil et al., 2014) of helminth infection, we have specifically shown that IL-4 dependency of Eomes in TVM cells expanded from C57BL/6 mice infected with *Hpb*. Furthermore, we confirm the IL-4 dependency of CD22, CD39, and granzyme A, molecules that are part of the specific IL-4 induced transcriptional program which was demonstrated using single cell RNA sequencing by the lead principal investigator from Rolot et al. with whom we are in

collaboration (Yang et al., 2023). As helminth- and IL-4-driven $CD22^{+}$ TVM cells are enriched for clones containing self-reactive T cell receptors (Yang et al., 2023), we postulate that IL-4 can activate a heterogeneous population of TVM cells that need to be counter-regulated by CD22 or CD39 expression to prevent autoimmunity. This theory is further supported by the observed increases in expression of IFNγ, granzyme A and CD39 in $CD22^{-/-}$ mice (Yang et al., 2023). Overall, these findings reveal an important role of IL-4 in the development of TVM cells upon helminth challenge. Exploring the role of IL-15 in this specific program in TVM cells would be insightful as IL-15 has also been shown to expand TVM cells during *Hpb* infection (Hussain et al., 2022) and signals through the common gamma chain (γc) as does IL-4 (Jameson et al., 2015).

To examine the role of the microbiota on CD8 T cell dynamics during *Hpb* infection, we standardized the protocol for generation of germ-free *Hpb* larvae to be used for gnotobiotic infection (Russell et al., 2021). We reconstituted C57BL/6 germ-free mice prior to *Hpb* infection to be used as microbiota-replete controls to factor in the impaired immunological development of germ-free mice (Round & Mazmanian, 2009) and genetic background differences between control and test groups. These are important factors to consider when comparing the immune response between different germ-free helminth models and may explain discrepancies between findings of worm fitness. Since we observed no changes in egg burden, our preliminary results suggest that neither *Hpb* fecundity nor host resistance is affected by the presence of the host microbiota. On the contrary, previous groups have found reduced worm burdens in germ-free *Hpb* infection (Chang and Wescott, 1972; Wescott et al., 1968; Rausch et al., 2018). However, these groups did not account for anaerobic contamination in germ-free conditions, hence the use of sterile *Hpb* recently began to be used in studies of germ-free *Hpb* infection (Moyat et al., 2022; Zaiss et al., 2015; Moyat et al., 2022; Reynolds et al., 2014). Moyat et al. (2022) demonstrated that microbial

regulation of peristalsis and intestinal muscle contractions was required for *Hpb* resistance in C57BL/6 mice. While worm fitness was incongruent from these reports, all methods of gnotobiotic *Hpb* infection were associated with similar or slightly elevated type 2 immune responses, suggesting that other mechanisms that affect parasite resistance may be at play. Interestingly, alteration of the microbiota composition using vancomycin treatment of BALB/c mice increased *Hpb* susceptibility and this response was not associated with changes in type 2 immunity, but rather to T regulatory cell function and IL-17 production (Reynolds et al., 2014).

Preliminary results revealed that the microbiota can activate the IFNγ signature, while inhibiting type 2 immunity during *Hpb* infection (Moyat et al., 2022). With the use of axenic *Hpb* infection, we examined the role of the microbiota in host defense against *Hpb* infection and its impact on helminth-induced CD8 T cell differentiation. Our results of decreased IFNγ target gene expression in the intestine during germ-free *Hpb* infection correlates with the reduction in IFNγ-producing Th1 cells in the mLN of SPF mice that received a broad antibiotic cocktail prior to infection (Moyat et al., 2022). In addition, McFarlane et al. (2017) showed that *Hpb*-induced type 1 interferon signalling was decreased during germ-free infection. This decreased IFNγ response has been postulated to occur due to the absence or decrease in bacteria or other micro-organisms that would translocate from the lumen into the submucosa upon *Hpb*-induced epithelial damage (Moyat et al., 2022, Mcfarlane et al., 2017). This idea of bacteria translocation-activated type 1 immunity was supported by observations of increased intestinal permeability during *Hpb* egress (Moyat et al., 2022). Overall, these results suggest that the microbiota is required for the host to mount an effective intestinal *Hpb*-activated IFNγ signature. Interestingly, we observed that the increased peak in early Tbet+ CD8+ CD8 T cells was delayed in animals raised in the absence of commensal microbes, regardless of whether they received a fecal microbiota transfer prior to

infection. Thus, these results suggest that the type 1 immune response requires developmental priming from microbial signals to respond to future type 1 immune activation cues.

Moreover, even though TVM cells are present in GF mice, the role of the microbiome in its maturation has not been examined. Our findings show that another role of the microbiota during helminth infection is its inhibition of CD39 and granzyme A in TVM cells. While this downregulation of granzyme A by the presence of the microbiota supports how commensal signals control immune-mediated inflammatory responses (Wang et al., 2014; Liu et al., 2022), the role of the microbiome on granzyme A has not been reported previously. Likewise, commensal-mediated signals have been shown to attenuate inflammation through regulation of CD39 expression (Liu et al., 2022). Furthermore, we have preliminary evidence that although germ-free infection leads to a skewed type 2 immune response, this does not correlate with changes in TVM cell frequency, suggesting that other signalling pathways that can regulate TVM cell development, such as type 1 interferon or IL-15 (Martinet et al., 2015; Hussain et al., 2023), may be counteracting enhanced IL-4 signalling. In addition, TVM cells are known to be largely monofunctional, predominantly producing IFNγ after stimulation (Quinn et al., 2018). In this preliminary study, we have shown that the capacity of TVM cells to produce IFNγ is independent of type 2 signalling and the microbiota. Though type 1 IFN and IL-4 signals have both been shown to induce IFNγ production in CD8 T cells (Martinet et al., 2015; Lee et al., 2013), TVM cells from naïve mice showed a similar capacity to helminth-expanded TVM cells to control infection of murid herpesvirus 4 *in vivo* (Rolot et al., 2018). Thus, the IFNγ-producing effector function of TVM cells may be due to other compensatory mechanisms.

Overall, this thesis explores CD8 T cell dynamics during *Hpb* infection and the regulatory mechanisms of type 1 and 2 immunity in the presence or absence of the microbiota. These findings

are important towards understanding pathways involved in tissue repair and anti-viral/bacterial responses, which can pave the way for the development of therapeutics for people with helminthiasis and other diseases involving an imbalance of immune homeostasis.

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