THE CONTRIBUTION OF PLAC8 TO TH1-DRIVEN INFECTION AND AUTOIMMUNITY

by

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ABSTRACT

Inflammation is critical for host protection against intracellular pathogens. Shortly after an intracellular infection, antigen-presenting cells produce IL-12 to promote $CD4^+$ T cell differentiation into T_H1 $CD4^+$ T cells that contribute to pathogen clearance by IFNγ production. However, T_H1-driven inflammation must be regulated because excessive inflammation can result in autoimmune diseases such as inflammatory bowel disease. Even though there are several drug therapies available to limit inflammation caused by T_H1-driven autoimmunity, many leave hosts vulnerable to intracellular infections by dramatically suppressing their T_H1-immune response. Before we can design novel therapeutics aimed to treat T_H1-associated autoimmunity with limited side effects, we need a better understanding of the regulatory pathways involved in this inflammatory response. In these studies, we find that the protein placenta-specific 8 (Plac8) is highly expressed within T_H1 $CD4^+$ T cells differentiated *in vitro* and may contribute to T_H1-driven inflammation. In addition to $CD4^+$ T cells, we show that Plac8 is expressed at very high basal

levels in $CD8^+$ T cells and is further induced following IL-12 stimulation. Interestingly, Plac8 suppresses IFNγ production by $CD4^+$ and $CD8^+$ T cells following IL-12 stimulation *in vitro* which suggests Plac8 could suppress T_H1-driven inflammation. Although Plac8 does not significantly regulate IFNγ production by pathogenic $CD4^+$ T cells during a T cell transfer model of colitis, we observe that Plac8 promotes optimal establishment of antigen-specific $CD8^+$ T cells following influenza infection. Furthermore, we find that Plac8 is important for limiting *Citrobacter rodentium* bacterial burdens as early as 3 days post infection. Although Plac8 ablation did not directly alter the ability of neutrophils to kill *C. rodentium in vitro*, we find that Plac8 expression may promote colonic CXCL1 production in *C. rodentium*-infected mice. Therefore, the contribution of Plac8 during *C. rodentium* infection may be to limit bacterial burdens by upregulating colonic expression of CXCL1 and increasing neutrophil recruitment to the site of infection. Although the precise function of Plac8 during these inflammatory responses remains elusive, for the first time, these data show that Plac8 has an immunoregulatory role during T_H1 immune responses and should be further explored for potential therapeutic benefit.

INDEX WORDS: inflammation, CD4 T cell, Th1 cell, inflammatory bowel disease, CD8 T cell, influenza, neutrophil, *Citrobacter rodentium*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The immune system consists of cells that serve as the host's primary defense against foreign pathogens and can be categorized as either innate or adaptive immune cells. Innate immune cells respond rapidly and non-specifically to invading organisms whereas adaptive immune cells take longer to respond but function more precisely. The innate immune system consists of epithelial cell barriers such as skin and mucosal membranes, phagocytic cells, inflammatory cells, dendritic cells (DCs), and even soluble mediators like cytokines and complement proteins. The ability of these cells and mediators to act non-specifically is important because they provide host protection until the adaptive immune response is activated which can take up to four or five days. Once lymphocytes are activated, B cells induce a humoral immune response by producing antibodies that neutralize invading pathogens, and activated T cells induce a cell-mediated immune response by secreting soluble cytokines that increase the efficiency of surrounding immune cells or by directly lysing cells containing the invading pathogen.

In general, cell-mediated immunity can be categorized as type 1 (T_H1), type 2 (T_H2), or type 3 (T_H17) (**Fig. 1.1**). Type 1 immunity consists of $CD4^+$ T_H1 cells, $CD8^+$ cytotoxic T (T_C1) cells, and group 1 innate lymphoid cells (ILC-1s)/NK cells that express the T-bet transcription factor and secrete IFNγ [1]. IFNγ is critical for the activation and enhanced phagocytosis of mononuclear phagocytes, including macrophages and microglial cells [2]. IFNγ is thought to exert many of its effects through the activation of over 200 IFNγ-responsive genes [3]. During viral infections, these IFNγ-responsive genes can prevent viral entry, viral replication, viral release and

transmission, and viral reactivation [4]. Type 2 immunity consists of CD4$^+$ T$_H$2 cells, CD8$^+$ T$_C$2 cells, and ILC-2s that express the transcription factor GATA-3 and produce IL-4, IL-5, and IL-13 which provide protection against helminth infections [1]; however, ILC-2s are primarily known for their copious production of IL-13 [5]. IL-4 is also a major contributor to allergic inflammation [6] and can induces expression of several pro-inflammatory mediators such as IL-6, GM-CSF, and VCAM-I adhesion molecule [7]. IL-5 primarily contributes to eosinophil activation [8] and IL-13 is most known for its contribution to host protection against gastrointestinal helminth infections and potent stimulation of tissue fibrosis [9]. Lastly, type 3 immunity consists of CD4$^+$ T$_H$17 cells, CD8$^+$ T$_C$17 cells, and ILC-3s that express the RORγt transcription factor and produce IL-17A and IL-22 [1]. IL-17 signaling leads to the induction of pro-inflammatory cytokines including IL-6, IL-1, TNF, and many proinflammatory chemokines ensuring recruitment of inflammatory cells to the site of infection [10, 11] Whereas IL-22 actively promotes mucosal host defense against bacterial pathogens by increasing host production of anti-microbial peptides and increasing epithelial cell proliferation [12]. Type 3 immunity is known for protection against extracellular bacteria and fungi.

These categories are helpful in organizing infection types, but immune responses elicited by some infectious diseases can be more complex. For example, *Citrobacter rodentium*, is an extracellular, mouse-specific pathogen used to study human enteropathogenic *Escherichia coli* infections, and is known to start with a strong CD4$^+$ T$_H$17 T cell response but gradually transitions toward a CD4$^+$ T$_H$1 cell response to clear the infection [13]. There are also parasites like *Schistosoma mansoni* and *S. japonicum* which induce a different CD4$^+$ T cell response depending on the stage of the pathogen's life cycle [14]. In addition, hosts with co-infections may elicit an untraditional CD4$^+$ T cell response to pathogen infections. In one study, patients infected with a

helminth infection induced a $CD4^+$ T_H2 cell response following mycobacterium tuberculosis infection, which is typically known to be a robust $CD4^+$ T_H1 cell-centric pathogen [15]. Overall, these studies demonstrate that cell-mediated immunity is determined by many variables and cannot be determined by pathogen type alone.

As $CD4^+$ T cells begin differentiating in response to inflammation, there are several regulatory mechanisms in place to promote the fidelity of differentiating $CD4^+$ T cells toward one $CD4^+$ T_H cell lineage. For example, IL-4 stimulation is critical for T_H2 differentiation but IL-4 also suppresses T_H1 differentiation through inhibition of IL-12Rβ2 expression [16] and in T_H1 cells, T-bet is shown to induce IFNγ expression while actively suppressing T_H2 differentiation by silencing IL-4 expression [17, 18]. Other regulatory mechanisms work through epigenetic modulations which allow master transcription factors easier accessibility to lineage-specific genes. There is evidence of differential placement of super-enhancers, which are large clusters of transcriptional enhancers aimed to promote gene expression [19], upstream of lineage-specific effector cytokines and cytokines receptors [20]. In addition, some studies have shown regulation of histone acetylation on lineage-specific effector cytokine loci in T_H1, T_H2, and T_H17 $CD4^+$ T cells [21, 22].

However, $CD4^+$ T cell commitment to only one lineage has been challenged, as multiple studies have suggested that differentiated $CD4^+$ T_H cells have "plasticity" and can be redirected toward other $CD4^+$ T_H subsets. For example, IFNγ producing $CD4^+$ T cells isolated from the spinal cord of mice during an experimental autoimmune encephalomyelitis (EAE) model were shown to previously expressed IL-17A, suggesting that these $IFN\gamma^+$ $CD4^+$ T_H1 cells may have originated from $CD4^+$ T_H17 cells [23]. In addition, $CD4^+$ T_H17 cells transferred into animal models of diabetes and cancer have shown to reach a more $CD4^+$ T_H1 cell-like phenotype post-transfer [24,

25]. There is also evidence that T regulatory cells can lose expression of their master transcription factor, FOXP3 and obtain the ability to secrete pro-inflammatory cytokines [26]. And lastly, LCMV-specific naïve $CD4^+$ T cells differentiated *ex vivo* under T_H2 conditions were able to co-express GATA-3 and T-bet and secrete IFNγ following *in vivo* transfer into an LCMV-infected host [27]. Although these studies have demonstrated alterations in the phenotype of $CD4^+$ T_H cells, it is not clear if these cells completely differentiate from one lineage into another which makes many of these results controversial.

Although these cell-mediated immune responses are designed to protect hosts against various pathogens, dysregulation of these inflammatory responses can result in autoimmunity. According to the NIH, autoimmune diseases affect more than 24 million people in the United States and are most prevalent in young populations during the prime of their working and reproductive years [28]. There are three major phases associated with autoimmune diseases: initiation, propagation, and resolution. Autoimmune diseases can be initiated through genetic predisposition and/or environmental factors [29-31]. For example, hosts with genetic polymorphisms in *IL23R* are shown to be more susceptible to developing Crohn's disease, psoriasis, and ulcerative colitis [32] and patients infected with Epstein-Barr virus are more susceptible to developing multiple sclerosis [33].

After the initiation phase, the disease shifts into a propagation phase where most patients will begin presenting with clinical symptoms. During the propagation phase, there is an increased accumulation of effector T cells and a decreased number of functional regulatory T cells (Tregs) in the tissues [34, 35]. The propagation phase is self-perpetuating in nature because effector T cells target self-antigens, which are oftentimes unable to be eliminated and this inflammatory response is left unregulated because there is a deficiency in Treg cell function [28]. In addition, this

inflammatory environment can lead to the recruitment and activation of other immune cells and cause excessive tissue damage that can expose effector T cells to new antigenic epitopes that can restart this vicious cycle through a process called epitope-spreading [28, 36]. During the propagation phase, many of the autoimmune diseases have an inflammatory cytokine that can be utilized as a biomarker for disease progression. For example, onset of systemic lupus erythematosus (SLE) is associated with an increase in type I interferon production [37], an increase in TNF is associated with both rheumatoid arthritis and psoriatic arthritis [38, 39], and an increase in C-reactive protein is associated with IBD [40].

Oftentimes inflammatory resolution will occur, and it is thought to be conducted primarily by Tregs. Although Tregs may have difficulty controlling the initial inflammation during the propagation phase, as effector T cells start secreting IL-2 to promote effector T cell activity and proliferation, this increase in IL-2 cytokine production will also promote Treg activity and proliferation [41]. In fact, Tregs will oftentimes outcompete effector T cells for paracrine IL-2 signaling because FOXP3 directly promotes high-affinity IL-2 receptor expression [42], and IL-2 signaling can further promote IL-2 receptor expression through the activation of Stat5 [43]. As a result, effector T cell responses are oftentimes followed by a wave of Treg-mediated suppression during autoimmune diseases. In addition, signaling through T cell inhibitory receptors have shown to be important for controlling these inflammatory responses. For example, after T cell activation, they will begin to express CTLA-4, which can function to suppress various immune responses [44]. Patients who inherit one mutant allele of CTLA-4 are more susceptible to the development of symptoms associated with autoimmune diseases and increased infiltration of CD4 T cells into various organs [45]. Unfortunately, patients who are unable to properly generate inhibitory T cell

receptors or functioning Tregs may fail to resolve inflammation which can result in a progressive, non-resolving disease.

Understanding and successfully treating autoimmunity has proven challenging, but there are currently drug therapies available for treating some these autoimmune diseases. Most of these treatments rely on neutralizing inflammatory cytokines, such as TNF, and have proven to be successful in treatment of rheumatoid arthritis and IBD [46, 47]. However, one of the limitations of current therapeutic strategies is that they rely on neutralizing and target present in the propagation phase of infection and do not fix the fundamental problems that are causing the source of inflammatory dysregulation. In addition, these therapies oftentimes require life-long treatment and will leave patients susceptible to infectious diseases. Therefore, future research aimed to understand the regulation of these inflammatory responses is critical for identifying potential therapeutic targets that can lead to longer lasting, safer therapeutic alternatives.

PLACENTA-SPECIFIC 8 (Plac8)

Plac8 is a small (~16 kDa), cysteine-rich protein, originally found in the spongiotrophoblast layer in mouse placental tissue [48] and subsequently determined to be expressed by both lymphoid and myeloid cells and intestinal and lung epithelial cells [49]. In epithelial cells, Plac8 contributes to proper cilia development and motility [50], and in neutrophils, Plac8 promotes intracellular killing of various bacterial pathogens [49]. However, the contribution of Plac8 toward the function of other cell types remains unknown. Through data mining, we determined that Plac8 is one of the most highly expressed T_H1 genes and is found to be expressed 58 times higher in T_H1 cells compared to undifferentiated $CD4^+$ T cells (T_H0) and 10 times higher in WT cells compared to STAT4-deficient cells [51]. Although Plac8 expression has been associated with T_H1 $CD4^+$ T cells *in vivo* [52] and Plac8 induction has been associated with many

T_H1 pathogens such as hepatitis B [53] and Ebola [54], the role of Plac8 during Th1-driven inflammation has not been determined.

Most studies assessing the role of Plac8 have been confined to the field of cancer biology. These studies demonstrated that Plac8 protein expression is upregulated at the sites of colon, prostate, and pancreatic cancers [55-60]. Gene silencing of Plac8 limits the spread of pancreatic cancers, although there are conflicting explanations regarding the mechanism. One report suggested that Plac8 promotes apoptosis through its physical interaction with Akt1 and Mdm2, two key elements in the c-Myc-p53 apoptotic pathway [61]. Another report demonstrated that siRNA silencing of Plac8 in pancreatic cancer cell lines inhibits autophagy, an evolutionarily conserved process in which cytoplasmic components and organelles are catabolized and recycled as an adaptation to cellular stress and is an essential process for maintaining pancreatic cancer metabolism [58]. However, a more recent study using a murine pancreatic cancer model showed that Plac8 contributes to pancreatic cancer growth through autophagy-independent mechanisms involving Kras-driven cell-cycle progression [57]. These conflicting reports suggest that the functional role of Plac8 may be context-dependent but also emphasizes the high therapeutic potential of this gene for cancer treatment.

The role of Plac8 during bacterial infections has also been investigated. One of the earliest studies demonstrated high levels of Plac8 protein expression in granules from activated neutrophils, and *Plac8*$^{-/-}$ neutrophils have impaired anti-bacterial function [49]. In this study WT and *Plac8*$^{-/-}$ mice were injected intraperitoneally (i.p.) with *Klebsiella pneumoniae* and the peritoneal cavities were lavaged at 1 and 4 hours post infection (p.i.) and assessed for neutrophil recruitment and bacterial burdens. *Plac8*$^{-/-}$ mice had increased neutrophil numbers, but it was deemed to be a result of the increased bacterial burdens present in the peritoneal cavities of *Plac8*$^{-}$

$^{/-}$ mice relative to the WT controls and not because of differences in neutrophil recruitment capacity. Because neutrophils directly kill *K. pneumoniae,* and *Plac8*$^{-/-}$ mice had higher bacterial burdens and neutrophils present at the site of infection, they hypothesized that Plac8 was important for optimal neutrophil-mediated anti-microbial function. To test this hypothesis, Ledford *et al*. co-cultured WT or *Plac8*$^{-/-}$ neutrophils with opsonized *K. pneumoniae in vitro* for 1 hour. They found that cultures given *Plac8*$^{-/-}$ neutrophils had increased bacterial numbers relative to the WT controls, indicating that Plac8 was important for optimal neutrophil killing. Furthermore, Ledford *et al*. determined that Plac8 was important for neutrophil intracellular killing of *K. pneumoniae*. This was assessed by recruiting neutrophils to the peritoneal cavity with thioglycolate or injecting *K. pneumoniae* i.p., and waiting 30 minutes before harvesting the neutrophils from the peritoneal cavity. After lysing neutrophils and plating the bacteria, it was determined that *Plac8*$^{-/-}$ neutrophils harbored a higher number of *K. pneumoniae* relative to their WT counterparts, demonstrating that Plac8 contributes to the intracellular killing of *K. pneumoniae*.

Ledford *et al*. also published a study demonstrating the importance of Plac8 in myeloid cell recruitment during a contact hypersensitivity response [62]. Using bone marrow chimera mice, this defect was determined to be myeloid-derived because WT mice that received *Plac8*$^{-/-}$ bone marrow had a significantly attenuated response to oxazolone compared to *Plac8*$^{-/-}$ mice that received WT bone marrow. Ultimately, it was determined that the site of inflammation in the *Plac8*$^{-/-}$ mice had significantly lower levels of the pro-inflammatory cytokine IL-6 and chemokine CXCL1 6 hours after antigen exposure. Because IL-6 and CXCL1 are required for proper recruitment and activation of myeloid cells, it was concluded that *Plac8*$^{-/-}$ mice had decreased cellular infiltrates during a contact hypersensitivity response as a result of decreased IL-6 and CXCL1 levels.

Plac8 has also been implicated in *Chlamydia muridarum*-specific $CD4^+$ T cell responses [52]. Johnson *et al.* isolated *Chlamydia muridarum*-specific $CD4^+$ T cells from the spleens of C57BL/6 female mice 7 days after *C. muridarum* challenge. These cells were subsequently cultured and with inactivated *C. muridarum* or *C. muridarum*-antigens and were passaged several times to develop $CD4^+$ T cell clones. Interestingly, Plac8 mRNA levels were increased in *Chlamydia muridarum*-specific $CD4^+$ T cell clones that killed the bacteria through nitric oxide-independent mechanisms. When infecting *Plac8*$^{-/-}$ mice with *Chlamydia muridarum*, it was determined that *Plac8*$^{-/-}$ mice were unable to clear the infection as quickly as WT mice and *Plac8*$^{-/-}$ mice developed more severe immunopathology following infection. Johnson *et al.* hypothesized that *Chlamydia muridarum*-specific $CD4^+$ T cells were killing bacteria either through nitric-oxide dependent mechanisms or Plac8-dependent mechanisms. To investigate their hypothesis further, *Plac8*$^{-/-}$ and WT mice were treated with medroxyprogesterone at 52 dpi (once the bacterial burden was almost cleared) to see if this drug-induced nitric oxide inactivation would inhibit bacterial clearance by *Plac8*$^{-/-}$ mice. Interestingly, bacterial replication was increased in *Plac8*$^{-/-}$ mice following medroxyprogesterone treatment, whereas bacterial replication in the WT mice was unaffected following treatment. This suggests that Plac8-expressing $CD4^+$ T cells in the WT mice were able to clear bacteria through an NO-independent mechanism.

Plac8 upregulation has been associated with several T_H1 pathogens, and Plac8 has been described as an IFN-stimulated gene [53, 63]. Wieland *et al*. observed that Plac8 was one of the highest genes induced in the livers of chimpanzees that were infected with hepatitis B virus [53], and Pankla *et al*. determined that Plac8 was one of many genes upregulated in the whole blood of patients with septicemic melioidosis caused by the T_H1 bacterium, *Burkholderia pseudomallei* [63]. In addition, Plac8 expression was significantly upregulated in PBMCs isolated from

cynomolgus macaques 4 days after Ebola virus infection compared to PMBCs isolated from uninfected hosts [54]. Altogether, these data show that Plac8 may be contributing to the host T_H1 immune response and highlights the importance of continued research of Plac8 in these settings.

Although several studies have suggested Plac8 expression is associated with T_H1 $CD4^+$ T cells, a more recent study utilizing single-cell RNA sequencing unexpectedly found that Plac8 was one of the most highly expressed genes in T_H2 $CD4^+$ T cells isolated from mice sensitized and subsequently challenged with house dust mite allergen [64]. In this study, T_H2 $CD4^+$ T cells were isolated from the mediastinal lymph node (mdLN) and bronchioalveolar lavage fluid (BAL) 15 days after the challenge period. Although data previously described from Wei *et al*. found little to no Plac8 expression in T_H2 $CD4^+$ T cells [51], it is important to note that Wei *et al*. utilized T_H2 $CD4^+$ T cells differentiated *in vitro* whereas Tibbitt *et al*. utilized T_H2 $CD4^+$ T cells differentiated *in vivo*. Altogether these data show that Plac8's function in $CD4^+$ T cells is likely complex and context dependent but highlights the potential for Plac8 to be an important contributor to cell-mediated immunity.

INTESTINAL IMMUNITY

The gastrointestinal tract consists of the small and large intestines. Starting from the proximal ends, the small intestine can be further divided into the duodenum, jejunum, and the ileum, whereas the large intestine can be divided into the cecum, colon, and rectum. The gastrointestinal tract contains numerous microorganisms, with the majority residing within the colon. Microorganisms exist on all environmentally exposed surfaces of the human body, but it has been cited that most reside in the gastrointestinal tract with more than 10^{14} microorganisms and more than 1000 species [65, 66]. Collectively, these microorganisms are referred to as the host's microbiome.

Recently the microbiome has received much attention due to the discovery that these commensal organisms significantly influence host metabolic and immunological responses. For example, lean mice have a different intestinal microbiome than obese mice. Transplantation of the intestinal microbiome from lean mice into obese mice resulted in the ability of obese mice to normalize their body weights [67]. In addition, it has been shown that the host's microbiome can skew $CD4^+$ T cell responses. One study showed that mice from Taconic Farms had a more T_H17-biased immune response that was attributed to the presence of segmented filamentous bacteria. To demonstrate this, they transferred this commensal bacteria into mice from the Jackson Laboratory, which were not T_H17 biased, and this transfer resulted in a higher production of IL-17A and IL-22 by their $CD4^+$ T cells [68].

The intestinal immune system must balance tolerance with immunity to prevent the host from responding to harmless commensal microbial antigens while still appropriately responding to invading microbial pathogens (**Fig. 1.2**). One of the major players in this immune regulation are intestinal epithelial cells (IECs). IECs are a single layer of epithelial cells organized in a crypt-villus formation and separate the intestinal lumen from the underlying lamina propria. There are many different types of IECs that are categorized as either absorptive (enterocytes) or secretory (Paneth, goblet, enteroendocrine, and tuft cells) [69]. Although the morphology and functions of IECs are different, these cells originate from the same stem cell progenitor in the crypt zone marked by the leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) [70]. The enterocytes (columnar epithelial cells) are responsible for absorbing digested nutrients as well as maintaining a physical and chemical barrier against invading pathogens. The barriers between the cells are called tight junctions and they are composed of claudins, zona occludens (ZOs), and junctional adhesion molecules (such as JAM1) [71-73]. The secretory epithelial cells have a

variety of functions. Paneth cells secrete a variety of antimicrobial proteins to prevent bacterial invasion, goblet cells secrete gel-forming mucins and these mucins are filled with neutralizing secretory IgA to create a distance between the microorganisms and epithelial cells, enteroendocrine cells secrete hormones to regulate digestion and initiate crypt epithelial cell proliferation, and tuft cells secrete IL-25 which is shown to be protective against helminth infections [69, 74]. The IECs also secrete a variety of immunoregulatory cytokines (in addition to IL-25) such as thymic stromal lymphopoietin (TSLP) and transforming growth factor-β (TGFβ) to prevent an immune response against the commensal microorganisms [75, 76].

The lamina propria contains a dense population of DCs, macrophages (MΦs), lymphocytes, and mast cells that are referred to as the gut-associated lymphoid tissue (GALT). MΦs in the lamina propria are able to sample, process, and present antigens locally or within the mesenteric lymph node (mLN) to mount an immune response [77, 78]. The literature has conflicting nomenclature between the DCs and MΦs in the gut because of their phenotypical and functional plasticity. However, the most recent distinction describes two different populations: the pre-DC-derived $CD11c^{+}CD103^{+}$ DCs and the monocyte-derived $CD11c^{lo}F4/80^{+}CX_{3}CR1^{hi}$ MΦs [79, 80]. The DCs are able to traffic to secondary lymphoid organs or remain in the LP to present antigens to naïve $CD4^{+}$ T cells [81]. These DCs also secrete TGFβ which allows naïve $CD4^{+}$ T cells to differentiate into an anti-inflammatory phenotype called forkhead box P3 ($FOXP3^{+}$) regulatory T cells [81, 82]. In addition, DCs within the GALT provide retinoic acid to T cells during antigen presentation, which results in the upregulation of the α4β7 integrin by T cells and gut tropism [83]. The MΦs have less migratory potential and are known for their transepithelial dendrites that squeeze between the epithelial cells to sample exogenous antigens within the lumen [84]. In addition, these MΦs produce high levels of IL-10 which promotes regulatory T cell activity [85].

Even though most of the IECs are found in the villus/crypt formation, there are smaller portions that reside above intestinal follicles such as the Peyer's patches (PPs) and isolated lymphoid follicles (ILFs). These epithelial cells are referred to as the follicle-associated epithelium and are overall better suited for antigen sampling than the villous/crypt regions because of the presence of microfold (M) cells. M cells are able to actively transport antigens (or whole microorganisms) from the lumen into the underlying follicles where the DCs and MΦs can more readily process and present antigens to lymphocytes [86]. To demonstrate the importance of M cells, studies have shown that mice with dysfunctional M cells have a significantly weakened immune response against pathogenic bacteria such as *Salmonella* Typhimurium [87].

INFLAMMATORY BOWEL DISEASE

As mentioned, it is important for the intestinal environment to maintain homeostasis. Unfortunately, there are some people who lack intestinal immune regulation, which results in chronic inflammation referred to as inflammatory bowel disease (IBD). In the United States and Europe, about 3.7 million people suffer from IBD [88]. Crohn's disease (CD) and ulcerative colitis (UC) are two major clinically defined forms of chronic IBD, and it is currently hypothesized that these diseases are caused by an abnormal immune response directed against commensal flora due to genetic and environmental factors [89, 90]. CD is found throughout the gastrointestinal tract and is characterized by having intermittent sites of edema and ulcers creating a "cobblestone" appearance, whereas UC is restricted to the colon and is found to have patches of ulcers [89]. Patients with CD will oftentimes experience persistent diarrhea, abdominal pain, and even blocking of the intestine due to swelling and scar tissue, while those who suffer from UC will commonly have loose, bloody stool and severe urgency for a bowel movement. Although IBD

research has intensified, more research still needs to be conducted on the immunopathology of the disease to understand which factors lead to disease progression.

Currently, there is no single mouse model that can capture the complexities of human IBD, but each model provides valuable insight that has been utilized to understand the pathogenesis of human IBD. In general, the most common ways IBD is studied in mice is through (1) genetic manipulation to induce spontaneous disease (2) chemical treatment (3) adoptive transfer of effector T cells in a host that has no Tregs [91]. Utilizing genetically modified mice to study IBD is beneficial for identifying the function of a molecule or cell type during colitis. For example, *Muc2*$^{-/-}$ mice which have a loss of epithelial barrier function which results in increased inflammation [92], *MDR1a*$^{-/-}$ mice which causes an increased intestinal permeability and translation of the bacteria into the lamina propria [93], mice that overexpress IL-7 develop colitis through enhanced survival of pathogenic CD4^{+} T cells [94], and *IL-10*$^{-/-}$ mice which develop colitis through impaired MΦ function [95].

Dextran sulfate sodium (DSS) colitis is a chemical-induced colitis model whereby DSS is administered to mice in their drinking water for a short period of time and will induce colitis through altering mucosal barrier function and injuring colonic epithelial cells [96]. Because this model induces a potent innate inflammatory response, it has been used to determine the important protective roles of TLR2, TLR4, and MyD88 signaling in epithelial cells and macrophages during colitis [97-99]. This model has also been used to determine the protective role of IL-1β signaling in repairing damaged epithelial cells and in the reconstitution of the epithelial cell barrier [100]. Although the DSS model has increased our understanding of human IBD, it is important to use the DSS model with caution because this model causes a more widespread, indiscriminate lesion formation in the colon than human IBD.

The haptenating agent 2,4,6-trinitrobenzene sulfonic acid (TNBS) is another common chemical-induced colitis model [101]. This model is used to determine the contribution of T_H1 $CD4^+$ T cells, neutrophils, and macrophages to colitis and results in elevated levels of IFNγ which closely resembles CD [102]. During TNBS induced colitis, it is known that blocking IFNγ production through anti-IL-12p40 treatment can alleviate symptoms associated with the disease [103]. These studies paved the way for humanized anti-IL-20p40 antibody treatments for patients with Crohn's disease that are unresponsive to the anti-TNF therapies [104]. Originally it was thought that anti-IL-12p40 treatments were working to directly neutralize IL-12, but more recent studies have determined that the p40 receptor subunit can also couple with p19 to form IL-23 which is important for stabilizing the T_H17-mediated immune response [105]. As a result, follow up studies were conducted to determine if IL-12 or IL-23 drives the dominate effector response in these models. Interestingly, it was found that $p19^{-/-}$ mice, which lack IL-23, actually develop worse colitis symptoms than their wild type counterparts [106]. Subsequent studies determined that the exacerbated disease pathology found in $p19^{-/-}$ mice after TNBS treatment was associated with increased levels of IL-12 [106] and that IL-17A production by T_H17 cells can suppress induction of T-bet in $CD4^+$ T cells reduce T_H 1 CD4 T cell IFNγ production [107]. Therefore, pathology driven by colitis in the TNBS model is primarily driven by T_H1 $CD4^+$ T cells, while T_H17 $CD4^+$ T cells provide more of an immunoregulatory role.

Adoptive transfer models of colitis are also utilized to study human IBD. This model works by introducing naïve, effector $CD4^+$ T cells into a $Rag^{-/-}$ host which lacks T and B cells, including the $FOXP3^+$ Tregs responsible for maintaining peripheral tolerance [108]. Five-eight weeks after the T cells are transferred, mice develop colitis due to autoreactivity against the commensal microbiota [108]. Through utilizing this model, it has been determined that Tregs can exert their

suppressive function on pathogenic $CD4^+$ T cells by secreting IL-10, TGF-β, and IL-35 [109, 110]. Although extensively studied, the role of T_H1 $CD4^+$ T cell and T_H17 $CD4^+$ T cell effector functions have been challenging to define in this model. The earliest studies determined that the adoptive transfer model of colitis are predominantly driven by T_H1 $CD4^+$ T cells because anti-IFNγ treatment of mice can alleviate colitis [111] and naïve T cells lacking T-bet fail to induce colitis in this model following adoptive transfer [112]. However, after it was determined that IL-23 can promote the differentiation of T_H17 $CD4^+$ T cells, it raised the possibility that T_H17-associated cytokines IL-17, IL-21, IL-22, and IL-23 might have a larger contribution to colitis progression than IFNγ. Subsequent studies determined that adoptively transferred *p19*$^{-/-}$ naïve $CD4^+$ T cells [113], IL-23 receptor deficient naïve $CD4^+$ T cells [114], nor RORγt deficient naïve $CD4^+$ T cells [115] resulted in colitis in *Rag*$^{-/-}$ hosts which provides convincing evidence that the T_H17 immune response is important in driving colitis in this model. Although T_H17-associated functions are important during this model, the T_H1 immune response still appears to be the main effector T cell response in this model because there is a far greater number of $IFN\gamma^+$ $CD4^+$ T cells than IL-$17A^+$ $CD4^+$ T cells present in the lamina propria [116]. However, there is increasing evidence that T_H17 $CD4^+$ T cells in this model are inherently plastic and can also give rise to $IFN\gamma^+$ $CD4^+$ T cells [117, 118] which makes the distinction between the pathology driven by T_H1 $CD4^+$ T versus T_H17 $CD4^+$ T challenging to determine.

CITROBACTER RODENTIUM

Colitis can also be studied in mice by utilizing infectious pathogens like *Citrobacter rodentium*. *C. rodentium* is a mouse-restricted pathogen that shares 67% of its genome with human enteric pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) [119]. *C. rodentium* colonizes the intestinal mucosa through the formation of attaching

and effacing (A/E) lesions which allows close association of the bacteria to the apical surface of intestinal epithelial cells and is also a defining characteristic of EPEC and EHEC [120]. Initially, *C. rodentium* colonizes the caecal patch, a major lymphoid structure in the caecum before infecting the colon and rectum [121]. As *C. rodentium* enters the lower gastrointestinal tract, it initiates virulence factors associated with the locus of enterocyte effacement (LEE) pathogenicity island, such as formation of a type III secretion system (T3SS) and other effector proteins that are critical for their survival within a host [119, 122]. After peak of bacterial load, infected epithelial cells will begin shedding into the intestinal lumen, releasing cells colonized with *C. rodentium* until complete clearance of the pathogen which can take 2-3 weeks post infection [121, 123]. Because of the similarities between *C. rodentium* and human EPEC and EHEC, utilizing *C. rodentium* in mice has become the standard *in vivo* model for determining more about the pathogenic life cycles of these organisms and which virulence mechanisms are essential for their pathogenesis.

C. rodentium infection causes colitis characterized by crypt hyperplasia, goblet cell depletion, and epithelial cell damage [124]. Damaged epithelial cells secrete IL-33 which upregulates expression of REG3γ, an anti-microbial peptide that increases spatial separation between intestinal epithelial cells and microbes [125]. Epithelial cells will also produce anti-microbial REG3β which induces IL-17A production by ILC-3s to promote neutrophil recruitment [126]. Once neutrophils are at the site of infection, they secrete IL-22 and IL-17A which further promotes the production of REG3γ and REG3β [127], enhances the epithelial cell barrier by strengthening tight junctions, and promotes epithelial cell proliferation [128]. In general, neutrophil recruitment in humans is primarily mediated through neutrophil upregulation of CXCR1 and CXCR2 which bind to CXCL8 (IL-8) [129]. However, mice do not produce CXCL8 and instead upregulate CXCR2 and CXCR4 which bind to CXCL1 (KC), CXCL2, CXCL12, and

CXCL15 [130-132]. During *C. rodentium* infection, studies have demonstrated that neutrophils are primarily recruited by CXCL1 and CXCL2 [133-135]. Neutrophil recruitment during *C. rodentium* infection is critical because neutrophils are required for preventing bacteremia, gangrenous mucosal necrosis, and infection-associated diarrhea [133-135].

Once neutrophils are recruited to the site of infection, they eliminate pathogens through a variety of different mechanisms. The first way is through degranulation, a process where neutrophils release a series of antimicrobial compounds such as MPO, defensins, lysozymes, bactericidal/permeability-increasing protein (BPI), and a number of serine proteases to kill bacteria [136]. Neutrophils also produce reactive oxygen species (ROS) through a respiratory burst that damages invading bacteria [137]. Upon stimulation, neutrophils can also undergo NETosis which is an active form of cell death that results in the release of decondensed chromatin which kills microbes by trapping them and exposing them to a high concentration of antimicrobials [138, 139]. And lastly, neutrophils can kill invading bacteria through phagocytosis. Here, bacteria are engulfed by the neutrophils and are contained within a phagosome that fuses with granules, releasing antimicrobial molecules into the phagosome and killing the bacteria [140].

In addition to neutrophils, $CX3CR1^+$ MΦs, $CCR2^+$-derived MΦs, and $CD11b^+F4/80^+Gr1^+$ inflammatory monocytes also contribute to early *C. rodentium* control [141-143]. $CX3CR1^+$ MΦs support IL-22 production by ILC3s which is important for maintaining the intestinal epithelial cell barrier and inducing the production of REG3γ and REG3β [141]. After infection, $CCR2^+$ MΦs give rise to $CDllc^+CD11b^+F4/80^+CD103^-$ intestinal monocytes which secrete IL-1β and also leads to increased IL-22 production by ILC3s [142]. And inflammatory monocytes are recruited by CCL2 and contribute to pathogen control by secreting IL-12 to promote $CD4^+$ T cell differentiation into T_H1 $CD4^+$ T cells [143].

Studies have shown that mice require both $CD4^+$ T cells and B cells for the clearance of *C. rodentium*, but $CD8^+$ T cells are dispensable [144]. It is suggested that $CD4^+$ T follicular helper (T_{FH}) cells, a subset of $CD4^+$ T cells that help B cells produce antibodies against foreign pathogens, play a critical part in the clearance of *C. rodentium*. However, it has been challenging to tease apart the impact of the effector $CD4^+$ T cells from the $CD4^+$ T_{FH} cells [145]. It is well documented that *C. rodentium* infection elicits a mixed $CD4^+$ T cell response including: T_H1 $CD4^+$ T cells, which are characterized by IFNγ and TNF production, and T_H17 $CD4^+$ T cells, which are known to secrete IL-17A, IL-17F, and IL-22 [146, 147].

T_H1-associated cytokines, IFNγ and TNF, are critical for limiting morbidity associated with *C. rodentium* infection. To demonstrate the importance of T_H1 $CD4^+$ T cell IFNγ production during *C. rodentium* clearance, Shiomi *et al*. adoptively transferred of B cells and $IFN\gamma^{-/-}$ OT-II $CD4^+$ T cells or $IFN\gamma^{+/+}$ OT-II $CD4^+$ T cells into *Rag1*$^{-/-}$ mice prior to *C. rodentium*-OVA infection [148]. *Rag1*$^{-/-}$ mice that were given $IFN\gamma^{-/-}$ OT-II cells had a significantly higher *C. rodentium* burden 14 dpi, significantly higher histopathological scores 14 dpi and 28 dpi, and significantly lower levels of OVA-specific IgG present in the serum and feces 7 dpi and 14 dpi [148]. To assess the importance of TNF production during *C. rodentium* infection, Gonçalves *et al*. infected *TNFRp55*$^{-/-}$ and *TNFRp55*$^{+/+}$ mice with *C. rodentium*. TNF signaling was not important for clearance of *C. rodentium*, but TNF signaling did contribute to significant control of bacterial burdens between 7 dpi and 14 dpi and limited mucosal thickening [149].

Although there is not a lot known about the long-term fate of activated $CD4^+$ T cells following *C. rodentium* infection, a recent study demonstrated that *C. rodentium* infection can induce a long-lived population of $CD4^+$ T_{RM} cells [150]. Bishu *et al*. designated these cells as $CD4^+$ T_{RM} cells because they were $CD4^+CD44^{hi}CD69^+$ cells that did not express lymph node

homing markers and were detached from the systemic circulation following *C. rodentium* infection. Interestingly, many of these $CD4^+$ T_{RM} cells coproduced IL-17A and IFNγ following *C. rodentium* challenge and variably expressed $CD103^+$, which is a phenotypic marker associated with classic T_{RM} cells [150, 151]. Altogether, *C. rodentium* infection elicits a robust $CD4^+$ T_H1 cell-mediated immune response which makes this an ideal pathogen to study T_H1-immunity.

INFLUENZA VIRUS

Influenza viruses are another commonly used pathogen to study T_H1 cell-mediated immune responses. Influenza viruses are a group of negative-sense, single-stranded, segmented RNA viruses that are a member of the *Orthomyxoviridae* family [152]. They can be classified as type A, B, or C viruses with type A being the most prevalent in humans, birds, and other warm blooded animals [152]. IAV can be further categorized based on the antigenic variation of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). There are currently 18 HA and 11 NA subtypes types known [153]. On average, influenza viruses infect 5-10% of adults and 20-30% of children under the age of five every year worldwide [153]. These infections are most severe in very young, elderly, and chronically ill individuals and can result in 250,000-500,000 annual deaths [153]. Influenza A virus is one of the most common respiratory diseases and is associated with high morbidity and mortality [154]. In addition, it has a significant economic burden of roughly $90 billion annually in the United States alone [155]. These compounding factors make it one of the most significant infectious diseases in the western world.

Influenza viruses are primarily spread when a healthy individual inhales virus-containing droplets from a cough or sneeze from an infected individual [154]. Once the virus enters the uninfected host, it targets the epithelial cells of the upper and lower respiratory tract [152]. In humans, the HA on the influenza virus binds to the sialic acid attached to galactose in a α-2,6

configuration [156]. Next, the HA is cleaved by proteases and the virus is endocytosed into an endosome where the acidic environment will uncoat the virion and allow the virus to begin its intracellular replication [157]. After replication, the progeny will make their way toward the cellular membrane and bind to sialic acid residues [158]. There, they are released from the cell after NA cleaves the sialic acid linkage, and the progeny look for new cells to infect [158]. Viral replication peaks around 48 hours after infection, and a person remains contagious for five to seven days after initial exposure [154].

Laboratory mice are commonly used for studying immune responses to influenza infection because of their relative availability, ease of genetic manipulation, and abundance of species-specific reagents. Unfortunately, many IAVs that are pathogenic and infectious in humans are unable to infect laboratory mice. In order to utilize IAV in a laboratory setting, several mouse-adapted viruses have been synthesized to closely resemble IAVs that infect humans, including A/Puerto Rico/8/1934 (PR8, H1N1) and A/HK-x31(x31, H3N2) (**Fig. 1.3**). Even though these two viruses are different IAV subtypes, all of their proteins, excluding the HA and NA, are identical [159]. Therefore, any cell-mediated protection established against one of these viruses will lead to cell-mediated protection against the other virus. Traditionally, these viruses can be used together to assess the protective capacity of established memory T cells where x31 is used during a primary infection because it initiates an immune response without causing severe pathology in wild type (WT) mice while PR8 is used as the challenge virus because of its higher virulence [160].

During IAV infection, lung epithelial cells play an important role initiating the innate immune response. These cells are equipped with several different pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), retinoic acid inducible gene-1 (RIG-I), and nucleotide-

binding oligomerization domain (NOD) like receptors (NLRs) that are able to sense viral antigen and recruit other immune cells to the site of infection [161]. Activation of these receptors leads to IFNα/β and IFNλ production which induces an anti-viral state in surrounding epithelial cells [162, 163]. In addition, PRR activation leads to the production of chemokines and pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF, CCL2, CCL3, and CXCL10 which recruit alveolar MΦs and monocytes that further drive the inflammatory response [164]. NK cells are also recruited and serve as cytotoxic effector cells that lyse virally infected cells in an antigen-nonspecific manner. As IAV infects epithelial cells, it leaves behind traces of IAV HA type 1 on the outside of the cell [165]. The NK cells will then use its receptors, NKp44 and NKp46, to recognize the residual HA and initiate cell lysis through the release of perforin and granzymes [165]. In addition, NK cells release ample amounts of IFNγ which contributes to early viral control and inflammation [166].

The most effective way to clear an IAV infection is through a robust T_H1 immune response. Around seven days post-infection, activated lymphocytes provide humoral and cell-mediated protection. B cells produce neutralizing antibodies to prevent the virus from invading into new epithelial cells [167] and $CD8^+$ T cells are able to protect the host through lysis of virally infected cells in an antigen-specific manner [168]. In addition, T_H1 $CD4^+$ T cells can contribute to viral clearance through direct lysis, but they are better known for IL-2 and IFNγ production [169]. As the virus is cleared, some T cells can survive in the absence of viral antigen and inflammation. These T cells are designated T memory (T_{mem}) cells, and they provide a rapid response against reinfection.

ANTI-VIRAL $CD8^+$ T CELL IMMUNITY

A protective $CD8^+$ T cell response against influenza viruses is advantageous because of the ability of $CD8^+$ T cells to recognize internal viral proteins, such as nucleoprotein (NP) and matrix

protein (M1), which are relatively conserved across different subtypes of influenza viruses [168, 170]. The initial step of activation begins when a naïve $CD8^+$ T cell recognizes a peptide sequence of about eight to ten amino acids long associated with major histocompatibility complex class-I (MHC-I) on the surface of an antigen presenting cell (APC), such as a DC [171, 172]. Only $CD8^+$ T cells with T cell receptors (TCRs) complementary to the short peptide sequence are able to begin the steps of $CD8^+$ T cell activation [173]. In addition to TCR stimulation, it is important that the T cell receives co-stimulation via CD28, CD40, CD27, and/or inducible T cell costimulator (ICOS) along with stimulation through receptors for inflammatory cytokines such as IL-12 or IFNα to ensure efficient activation [174, 175]. These activated T cells will clonally expand about 10^5-fold over the course of a week and target infected epithelial cells in an antigen-specific manner [176]. While $CD8^+$ T cells are not able to prevent initial infection, $CD8^+$ T cells can secrete perforin and granzymes to directly lyse infected cells before the viral progeny can be released to infect surrounding cells [177, 178].

The $CD8^+$ T cell response to an acute viral infection typically occurs in three phases: expansion, contraction, and memory [151]. During the expansion phase, antigen-specific $CD8^+$ T cells undergo rapid proliferation, and differentiate into effector cytotoxic lymphocytes (T_{eff}) in attempt to control the infection [151]. After the virus has been eliminated, about 90-95% of these $CD8^+$ T_{eff} will undergo apoptosis and die to maintain immune homeostasis [151]. The remaining antigen-specific $CD8^+$ T cells will develop into functional $CD8^+$ T_{mem} cells that serve to protect the host against subsequent infection by a heterologous virus [151].

$CD8^+$ T_{eff} can be categorized by different phenotypic markers that help predict cell fate. Two surface markers used for phenotyping $CD8^+$ T_{eff} cells are killer cell lectin-like receptor G1 (KLRG1) and IL-7 receptor subunit-α (IL-7Rα or CD127) [179, 180]. KLRG1 is a surface marker

indicative of terminal differentiation but its mechanism is not clear [181]. CD127 is shown to be critical for early T cell proliferation and for long-term survival of T cells within the periphery [182, 183]. KLRG1^{hi}CD127^{lo} CD8^{+} T_{eff} are referred to as short-lived effector cells (SLECs), and these are known for their high cytotoxicity against pathogens and will undergo apoptosis during the contraction phase [179]. KLRG1^{lo}CD127^{hi} CD8^{+} T_{eff} are referred to as memory precursor effector cells (MPECs) and these cells are less active during the primary infection, but will survive the contraction phase to become CD8^{+} T_{mem} [180]. And finally KLRG1^{lo}CD127^{lo} CD8^{+} T_{eff} are called early effector cells (EECs) and these cells are able to become SLECs or MPECs over time, or undergo apoptosis [184].

In addition to phenotypic markers, there are two T-box transcription factors that are useful for identifying which CD8^{+} T_{eff} will become CD8^{+} T_{mem} versus those that will undergo apoptosis. During a T_{H}1 immune response, T-bet and eomesodermin (EOMES) play many cooperative and redundant roles for CD8^{+} T cells, most notably the controlled production of perforin and granzyme B [179, 185]. In fact, CD8^{+} T cells lacking T-bet and EOMES lose their cytotoxicity and convert into IL-17-producing CD8^{+} T cells that cause uncontrollable inflammation [186]. However, during the contraction phase, these transcription factors appear to play more unique roles. CD8^{+} T_{eff} expressing higher levels of T-bet will typically die during the contraction phase while cells expressing higher levels of EOMES will become CD8^{+} T_{mem} [187]. The mechanisms that turn on and off these transcription factors as well as how the transcription factors affect one another during the contraction phase are currently unknown, but the balance between these two transcription factors appears to be critical for CD8^{+} T cell development and function.

Two features of CD8^{+} T_{mem} are their longevity and ability to rapidly respond against secondary infection. Unlike CD8^{+} T_{eff}, CD8^{+} T_{mem} can survive for long periods of time in an

antigen- and TCR-independent manner [188, 189]. As $CD8^+$ T cells transition from their highly proliferative T_{eff} phenotype into one that is much slower during the T_{mem} phenotype, it has been shown that cytokines IL-7 and IL-15 from the common gamma chain (γc) family are crucial for stimulating basal proliferation and long-term survival of $CD8^+$ T_{mem} [190]. It has also been shown that autophagy, an evolutionarily conserved process in which cytoplasmic components and organelles are catabolized and recycled as an adaptation to cellular stress, is critical for this transition [191]. In fact, while genetic ablation of two genes critical in autophagosome formation, Atg5 and Atg7, during lymphocytic choriomeningitis virus (LCMV) or murine cytomegalovirus (MCMV) has no effect on $CD8^+$ T_{eff} function, it is detrimental to $CD8^+$ T_{mem} formation [191, 192].

The ability of $CD8^+$ T_{mem} to respond quickly to secondary infection stems from a few inherent qualities. First, $CD8^+$ T_{mem} exist at higher numbers than naïve $CD8^+$ T cells. Second, their chromatin around effector loci remain 'poised' in an open and accessible conformation allowing rapid re-expression of effector molecules, such as IFNγ [193, 194]. Third, $CD8^+$ T_{mem} are oftentimes positioned at mucosal portals which increases their chances of intercepting pathogens shortly after entry [193]. Even though most $CD8^+$ T_{mem} responses will result in pathogen control, there are several factors such as phenotype, function, and location that determine how the $CD8^+$ T_{mem} will protect the host against secondary infection [151].

Originally, $CD8^+$ T_{mem} were categorized based on the expression of two homing molecules: CD62L (L-selectin) and CCR7 [195]. Central memory T cells (T_{CM}) express both of these homing molecules, while effector memory T cells (T_{EM}) express neither [184]. CD62L and CCR7 are important for T_{mem} to enter the lymph nodes (LN); thus T_{CM} are primarily found in LNs while T_{EM} are primarily found within circulation and peripheral tissues such as the liver and lungs [151]. In addition to phenotypic differences, these memory cell subsets also have distinct functional

abilities. When exposed to a recognizable antigen, T_{CM} are known to rapidly proliferate via increased IL-2 production, whereas T_{EM} are less proliferative but will secrete high levels of effector cytokines such as IFNγ to clear the foreign antigen [196].

More recently, a third subset of $CD8^+$ T_{mem} was discovered. These cells are named tissue-resident memory T cells (T_{RM}) based on their characteristic of remaining embedded within a designated peripheral tissue, away from the circulation (Table 1) [197]. T_{RM} are commonly associated with the gastrointestinal tract, reproductive tract, respiratory tract, and skin within epithelial tissues that serve as an interface between the host and the environment [198]. This prime location allows T_{RM} to rapidly respond to a familiar antigen shortly after the host is re-exposed, thus generating a long-lasting T_{RM} response against a foreign antigen has been the goal for several mucosal vaccines. T_{RM} are classically phenotyped as $CD103^{hi}CD69^{hi}CD27^{low}$ and appear to be terminally differentiated because of their poor proliferative ability and elimination when forced into circulation [151, 199]. As research progresses, it has become clear that these surface markers are not reliable for identifying all T_{RM} [200]. Instead, techniques such as intravenous injection of fluorescent antibodies to label T cells in circulation versus those embedded within the tissue are being used to better distinguish T_{RM} from the other memory subsets [201].

SUMMARY AND STRUCTURE OF DISSERTATION

IFNγ and TNF production by T cells is one of the hallmarks of protection against many T_H1-pathogens, but excessive T_H1-cytokine production can cause autoimmunity. Therefore, it is important that T_H1-associated inflammatory responses are properly regulated. Unfortunately, T_H1-driven inflammation is a complex, pathogen-dependent process, and many of the regulatory processes are not well understood. Given Plac8's numerous associations with T_H1-driven inflammation and little insight into the contribution of Plac8 during this inflammatory response,

in these studies, we evaluate the contribution of Plac8 to immune cell function during different T_H1-associated inflammatory diseases. The primary goal of these studies is to determine if Plac8 has any regulatory functions during T_H1-driven inflammation and whether Plac8 has the potential to be utilized therapeutically. In Chapter 2 of this dissertation, we hypothesize that Plac8 is critical for optimal $CD4^+$ and $CD8^+$ T cell function during T_H1-driven inflammation. To assess the contribution of Plac8 to $CD4^+$ T cell function, we utilize *in vitro* assays in combination with the T cell transfer model of colitis and a *C. rodentium* infectious model of colitis; to determine the contribute of Plac8 to $CD8^+$ T cell function, we utilize *in vitro* assays and a mouse model of influenza infection. In Chapter 3, we hypothesize that Plac8 is important for protection against *C. rodentium* infection by promoting neutrophil-mediated killing of *C. rodentium*. To test this hypothesis, we utilize a mouse model of *C. rodentium* infection in combination with *ex vivo* assays to assess the contribution of Plac8 to neutrophil function. Chapter 4 of the dissertation will include discussions and implications of our findings, and Chapter 5 will discuss future directions.

FIGURES

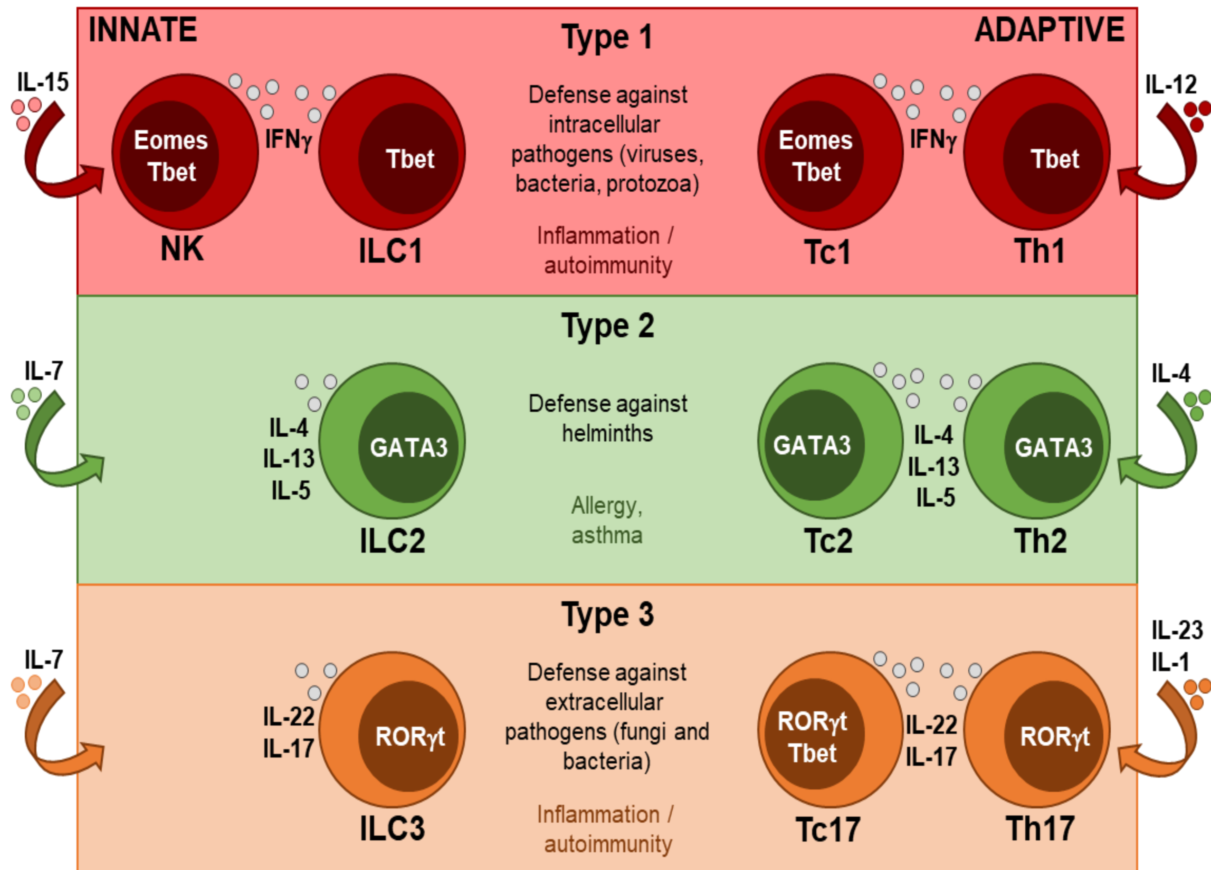


Figure 1.1: Summary of the major types of cell-mediated immune responses

Adapted from Annunziato *et al*. [1]

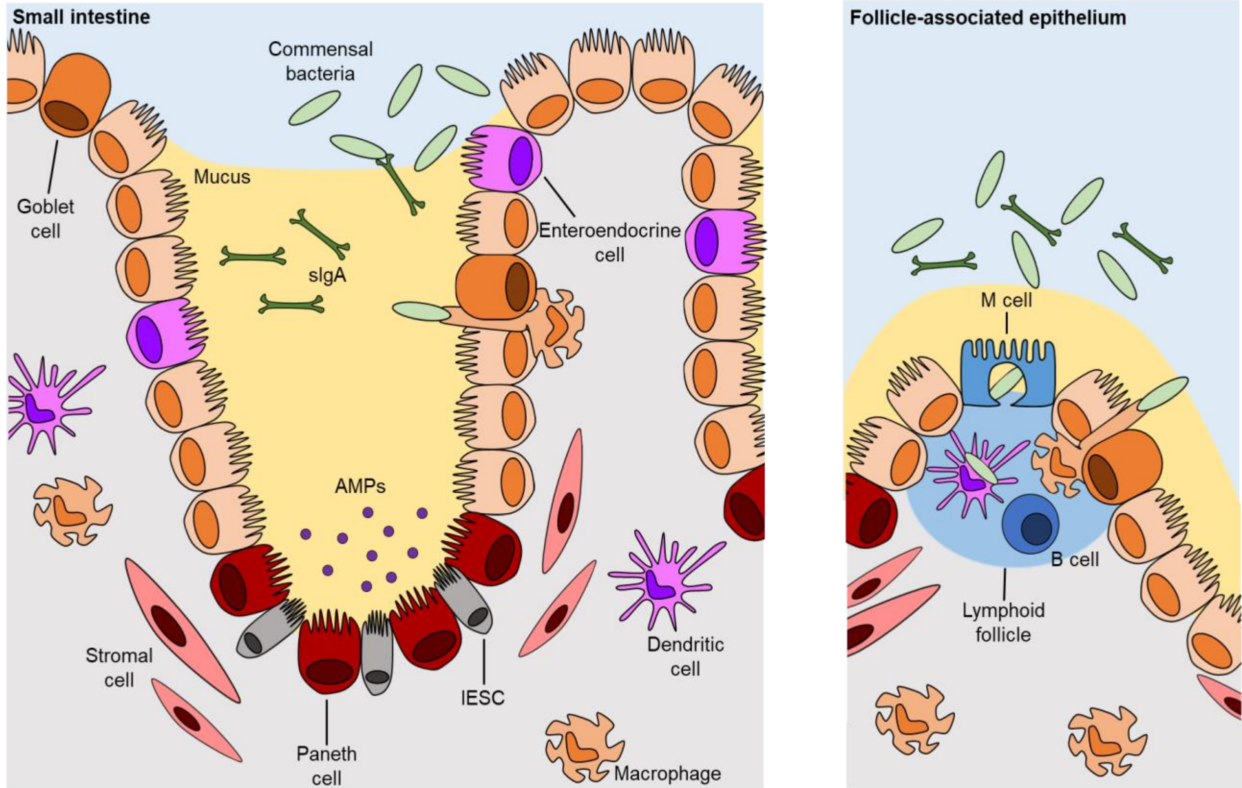


Figure 1.2: Summary of intestinal immunity

Adapted from Peterson *et al*. [202]

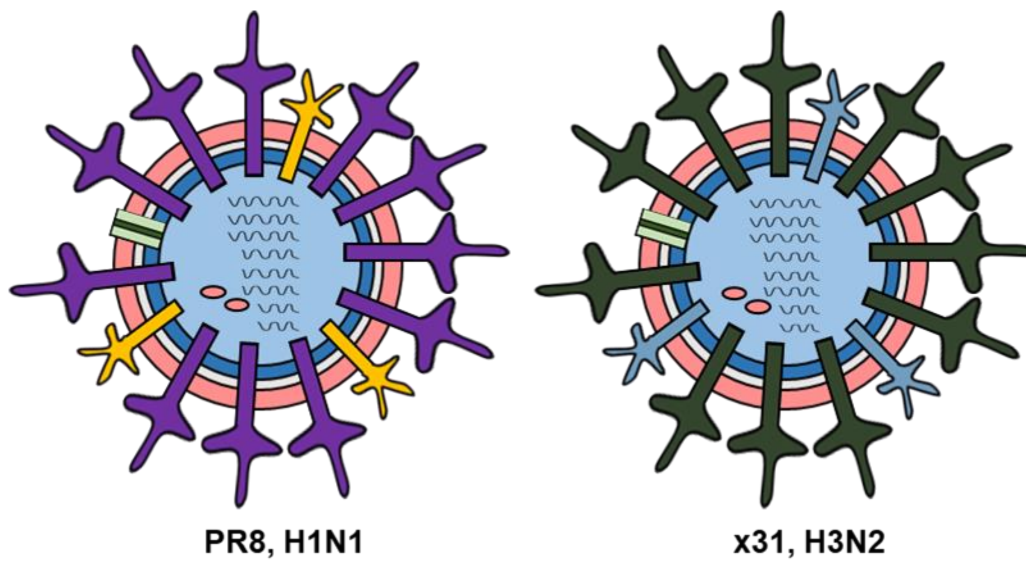


Figure 1.3: Visualization of influenza viruses: PR8 and x31

CHAPTER 2

PLACENTA-SPECIFIC 8 LIMITS IFNγ PRODUCTION BY CD4 T CELLS *IN VITRO* AND PROMOTES ESTABLISHMENT OF INFLUENZA-SPECIFIC CD8 T CELLS *IN VIVO*[1]

[1] Slade CD, Reagin KL, Lakshmanan HG, Klonowski KD, and Watford WT. Submitted to *PLOS ONE.*

ABSTRACT

During type 1 immune responses, CD4 T helper 1 (Th1) cells and CD8 T cells are activated via IL-12 and contribute to the elimination of intracellular pathogens through interferon gamma (IFNγ) production. In this study, we identified Placenta-specific 8 (Plac8) as a gene that is uniquely expressed in Th1 CD4 T cells relative to other CD4 T cell subsets and hypothesized that Plac8 may represent a novel therapeutic target in Th1 CD4 T cells. First, we determined that Plac8 mRNA in CD4 T cells was induced following IL-12 stimulation via an indirect route that required new protein synthesis. Upon evaluating the functional relevance of Plac8 expression in Th1 CD4 T cells, we discovered that Plac8 was important for suppressing IFNγ mRNA and protein production by CD4 T cells 24 hours after IL-12 stimulation, however Plac8 did not contribute to pathogenic CD4 T cell function during two models of intestinal inflammation. We also noted relatively high basal expression of Plac8 in CD8 T cells which could be further induced following IL-12 stimulation in CD8 T cells. Furthermore, Plac8 expression was important for establishing an optimal CD8 T cell response against influenza A virus via a T cell-intrinsic manner. Altogether, these results implicate Plac8 as a potential regulator of Th1 CD4 and CD8 T cell responses during Th1 T cell-driven inflammation.

INTRODUCTION

Type 1 cell-mediated immune responses consist of CD4 T helper 1 (Th1) cells and CD8 cytotoxic T cells that provide protection against intracellular pathogens primarily through secretion of interferon gamma (IFNγ) and tumor necrosis factor (TNF) following IL-12 stimulation [1, 203]. Dysregulation of type I immune responses can lead to autoimmune disorders such as rheumatoid arthritis, multiple sclerosis, or inflammatory bowel disease [204]. Although some anti-inflammatory treatments for autoimmune diseases have shown promise, most leave the patients vulnerable to infections and require life-long treatment [205]. Before safer, more efficacious therapeutics against Th1-driven inflammation can be developed, a more complete understanding of the pathways that regulate this immune response is required.

Placenta-specific 8 (Plac8) is a small, cysteine-rich protein originally identified in placental tissue [48] but subsequently determined to be expressed in a variety of epithelial tissues and immune cells [206]. Currently, Plac8 is known to regulate oncogenic cell growth in various cancerous epithelial cell lines [55-60] and promote neutrophil antimicrobial functions [206]. However, Plac8 mRNA expression in T cells [206] suggests additional T cell-specific functions are likely. For example, Plac8 mRNA and protein is highly expressed in *Chlamydia muridarum*-specific Th1 CD4 T cells [207], Th1 CD4 T cells differentiated *in vitro* using classical Th1 biasing culture conditions [51], and more recently, in Th2 CD4 T cells isolated from mice sensitized and challenged with host dust mite allergen [64]. Altogether, these studies suggest that Plac8 may have an unappreciated role in promoting CD4 T cell differentiation and/or effector functions, conferring protective cellular immunity. A better understanding Plac8's functional impact on Th1 CD4 T cells and other CD4 Th helper cell subsets is important for consideration of novel therapeutics and vaccines that may benefit from targeting this cellular pathway.

In this study, we show that Plac8 mRNA is differentially expressed by Th1 CD4 T cells compared to Th2, Th17, and iTreg T helper subsets differentiated *in vitro*. In addition, we determined that Plac8 expression is induced in CD4 T cells following Th1 stimulation with IL-12. Interestingly, Plac8 induction was via an indirect mechanism whereby IFNγ played a modest role. Parallel studies in naïve CD8 T cells revealed a surprising and, to date, an unreported high basal level of Plac8 mRNA expression relative to naïve CD4 T cells. Likewise, Plac8 expression is induced in CD8 T cells following IL-12 stimulation, suggesting a potential role for Plac8 in IL-12-driven Th1 immunity in both T cell subsets. Indeed, Plac8 suppressed IFNγ production by CD4, and to a lesser extent, CD8 T cells after IL-12 stimulation *in vitro*. Interestingly, however, *Plac8*$^{-/-}$ CD4 T cells did not induce enhanced morbidity compared to *Plac8*$^{+/+}$ CD4 T cells in a T cell transfer model of colitis in which Th1 cells are important mediators of disease. On the contrary, Plac8 was important for optimal establishment of virus-specific effector CD8 T cells in response to influenza infection. The numerical enhancement of CD8 T cells conferred by Plac8 sufficiency was T cell-intrinsic, yet unrelated to cellular proliferation and IL-2 signaling, both known to be important for effector cell differentiation. Although the precise function of Plac8 in T cells remains elusive, these data sufficiently demonstrate an immunoregulatory role for Plac8 during Th1 type immune responses that should be further explored for potential therapeutic benefit.

MATERIALS AND METHODS

Mice. Wild type (WT), C57BL/6 *IFNγ* $^{-/-}$, and C57BL/6 *Rag1*$^{-/-}$ mice were originally ordered from the Jackson Laboratory or Charles River and are bred in-house. C57BL/6 *Plac8*$^{-/-}$ mice were generated by Beverly H. Koller (University of North Carolina at Chapel Hill, Chapel Hill, NC) [206] and kindly provided by Dr. Raymond Johnson (Indiana University School of Medicine, Indianapolis, IN). C57BL/6-Tg (TcraTcrb)1100Mjb/J (OT-I) mice were originally provided by the late Dr. Leo Lefrançois (University of Connecticut, Farmington, CT), maintained on both CD45.1 or CD45.2 congenic backgrounds, and bred with *Plac8*$^{-/-}$ mice to generate *Plac8*$^{-/-}$, CD45.2^{+}, OT-I mice. To generate bone marrow chimeras, WT CD45.1/.2 heterozygote mice were irradiated with a single dose of 1,100 rads. The following day, mice were injected i.v. with $3x10^{6}$ each WT CD45.1 and *Plac8*$^{-/-}$ CD45.2 bone marrow cells and rested for 2 months for immune cell reconstitution. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Georgia (#A2018 06-013-A4). Euthanasia was performed by CO2 asphyxiation or by tribromoethanol overdose, both followed by cervical dislocation.

Cell purification. Pooled spleens and lymph nodes were collected from WT or *Plac8*$^{-/-}$ mice and processed by gently pressing them through a 70 µm filter. Where noted, CD4 and CD8 T cells were subsequently purified using a mouse T cell negative selection kit (Stemcell Technologies, Vancouver, Canada) according to manufacturer's instructions. T cells were stained for 15 min at 4°C in PBS + 0.1% BSA (Gemini Bio-Products) using anti-mouse antibodies purchased from eBioscience and Tonbo Biosciences (both San Diego, CA): CD16/CD32 (93), CD4 (IM7), CD8 (53-6.7), CD44 (IM7), CD62L (MGL-14), CD45RB (C363.16A), and CD25

(PC61.5). Propidium iodide (Sigma-Aldrich) was used to exclude dead cells (PI^+) from the sorted population. For T cell *in vitro* assays, live cells were designated as $CD4^+$ or $CD8^+$ T cells before sorting out naïve T cells ($CD62L^+$, $CD44^{lo}$) or memory T cells ($CD44^{hi}$). For the T cell transfer model of colitis, live $CD4^+$ T cells were sorted as ($CD25^-$, $CD45RB^{hi}$) effector cells.

T cell culture. Naïve T cells were counted and plated at $1x10^6$ cells/mL on a 96 well culture plate coated with 5 μg/mL of α-CD3 (145-2C11) and α-CD28 (37.51) (Invitrogen, Carlsbad, CA). Cells were cultured for 3 days at 37°C in 5% CO_2 and complete RPMI (RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.01 M HEPES and 50 µM 2-mercaptoethanol). Differentiation conditions for CD4 T cells were: Th1 (10 ng/mL IL-12 + 5 μg/mL αIL-4), Th2 (10 ng/mL IL-4 + 5 μg/mL αIFNγ and αIL-12), Th17 (10 ng/mL IL-6 + 5 ng/mL TGF-β + 5 μg/mL αIL-2), or iTreg (10 ng/ml TFG-β + 40 IU/mL rhIL-2) (PeproTech, Rocky Hill, NJ and BD Sciences, San Jose, CA), or no additional cytokines (Th0) for the 3 days. Prior to IL-12 or IFNγ (PeproTech) stimulation, T cells were plated with 5 μg/mL of α-CD3 and α-CD28 for 3 days. Cycloheximide (CHX) pretreatment was at 10 µg/mL for 30 min prior to assay where indicated. IFNγ protein from supernatants was measured using an IFNγ ELISA (eBioSciences).

RT-qPCR. RNA was isolated from CD4 and CD8 T cells using an EZ-RNA extraction kit (Omega Bio-Tek, Norcross, GA) and was converted to cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA). The relative expression levels of *plac8* and *ifnγ* and was determined by using predesigned TaqMan probe and primer sets relative to an actin endogenous control (Applied Biosystems, Grand Island, NY) and reference sample using the $\Delta\Delta C_T$ method.

T cell transfer model of colitis. *Rag1*$^{-/-}$ mice were injected i.p. with 3x10^5 WT or *Plac8*$^{-/-}$ naïve $CD4^+CD25^-CD45RB^{hi}$ effector T cells. Mice were weighed prior to adoptive transfer and weekly thereafter until significant weight loss was observed in the WT control group. Splenic, mesenteric lymph node (mLN)-derived and lamina propria (LPL) lymphocytes were isolated. Cells were resuspended to 1x10^6 cells/mL before *ex vivo* stimulation with 50 ng/mL PMA, 0.5 µg/mL ionomycin, and Golgi transport inhibitor according to the manufacturer's directions (BD Biosciences) for 4 h at 37°C. Cells were surface stained for CD4 (RM4-5) and TCR□ (H57-597) prior to fixation in 4% formalin (Protocol), resuspension in BD Perm/Wash (San Jose, CA), and intracellular staining with monoclonal antibodies reactive to IFNγ (XMG1.2), TNF (MP6-XT22), and IL-17A (eBio1787). Samples were run on the BD LSRII flow cytometer, and data analysis was performed using FlowJo software.

Influenza model. WT, *Plac8*$^{-/-}$, or mixed bone marrow chimera mice were intranasally infected with 10^4 pfu/mL influenza A/HK-x31 (X31, H3N2) or 10^3 pfu/mL of recombinant X31 expressing ovalbumin (X31-OVA) in 50 µL PBS. OT-I adoptive transfers of 1x10^3 WT and 1,000 *Plac8*$^{-/-}$ OT-I cells were given i.v. one day prior to X31 infection. Mice were monitored until 8 or 10 dpi to quantitate the peak effector CD8 T cell response for X31-OVA or X31, respectively. To evaluate memory CD8 T cell responses, mice were euthanized at 35 dpi. BAL, mdLN, lung, and spleen were collected at indicated time points and processed as previously described [208]. Cells were stained with a combination of CD8 (53-6.7), CD44 (IM7), CD45.1 (A20), CD45.2 (104), TCR Vα2 (B20.1), CD25 (PC61.5), and the influenza nuclear protein (NP) MHC class-I [H-2Db/ASNENMETM] tetramer conjugated to brilliant violet 421 obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA) at RT for 1 h. For proliferation studies, mice were administered 1 mg/100 µL of BrdU (Sigma) i.p. 12 hours prior to sacrifice, and single cell

suspensions were intracellularly stained with an αBrdU (BU1/75) antibody. Plaque assays were done by serially diluting lung homogenates and plating them on MDCK cells as described [209].

Statistics. *P* values were determined by unpaired or paired two-tailed Student's t-test or by One-way ANOVA with Tukey's post-hoc analysis using Prism software. Statistical significance is considered $p \leq 0.05$.

RESULTS

Plac8 mRNA is induced in T cells following IL-12 stimulation and suppresses IFNγ production by T cells in vitro

To date, Plac8 has been shown to be expressed by Th1 CD4 T cells stimulated with α-CD3 and α-CD28 in the presence of IL-12 *in vitro* [51] and by Th1 CD4 T present after *Chlamydia muridarum* infection *in vivo* [207]. However, to our surprise, a recent publication also noted high expression of Plac8 within Th2 cells [64]. To determine whether Plac8 expression is differentially regulated among CD4 T cell subset(s), we sought to globally assess Plac8 expression across a variety of *in vitro* differentiated CD4 T cell subsets. To this end, naïve CD4 T cells were sort-purified from mouse spleens and lymph nodes and plated with immobilized α-CD3 and α-CD28 supplemented with either IL-12 (Th1 conditions), IL-4 (Th2 conditions), TFG-β and IL-6 (Th17 conditions), TGF-β and IL-2 (iTreg conditions), or media alone (Th0) for three days [210] before being harvested and assessed for Plac8 mRNA expression via RT-qPCR. Differentiation of the specific CD4 T cell subsets was verified by assessing the expression of Th subset-specific signature cytokines or transcription factors by RT-PCR (**S2.1 Fig.**). When the *in vitro* differentiated CD4 T cell samples were normalized to the level of Plac8 mRNA expression in undifferentiated Th0 cells, CD4 T cells cultured under Th1 conditions expressed significantly higher levels of Plac8 mRNA relative to all other subsets assayed (**Fig. 2.1A**), consistent with the limited subset analysis by Wei et al. [51] (**Fig. 2.1B**).

Because IL-12 is critical to the differentiation of Th1 CD4 T cells, we next determined whether IL-12 directly or indirectly induces Plac8 mRNA expression in Th1 cells. TCR-activated CD4 T cells were pretreated with or without cycloheximide (CHX) prior to IL-12 stimulation. CHX prevents new protein synthesis by interfering with protein translocation, thereby ceasing any

protein intermediates produced upon IL-12 stimulation. Plac8 expression was significantly lower in CHX pre-treated CD4 T cells compared to their untreated counterparts, demonstrating that IL-12 induced Plac8 expression through a protein intermediate (**Fig. 2.1C**). Given that IFNγ is a key product of the IL-12 signaling pathway and is important for further differentiating Th1 CD4 T cells [211], we examined whether Plac8 mRNA expression by Th1 CD4 T cells was dependent on IFNγ signaling. To test this, naïve CD4 T cells were isolated from C57BL/6 wild type (WT) mice and *IFNγ* $^{-/-}$ mice and cultured under Th1 conditions for three days, followed by quantitation of Plac8 mRNA expression. Although Plac8 mRNA induction after Th1 polarization trended lower in *IFNγ* $^{-/-}$ versus WT CD4 T cells, this was not statistically significant (**Fig. 2.1D**). Likewise, although TCR-activated CD4 T cells stimulated directly with IFNγ for 6 h upregulated Plac8 mRNA expression, the level of expression observed was only a fraction of that observed in CD4 T cells stimulated with IL-12 (**Fig. 2.1E**). Altogether, these data show that while IL-12 significantly induces Plac8 expression in CD4 T cells via a protein intermediate, IFNγ produced downstream of IL-12 plays only a modest role in Plac8 mRNA induction. Therefore, other unidentified protein intermediates downstream of IL-12 signaling contribute to Plac8 induction in CD4 T cells.

Because CD8 T cells also robustly respond to IL-12 stimulation, we next characterized Plac8 mRNA expression within these cells. First, naïve (CD62L^{+}, CD44^{lo}) and memory (CD44^{hi}) phenotype CD8^{+} T cells, as well as CD4 T cells as a comparison, were isolated from spleen and lymph nodes, and the basal expression of Plac8 mRNA within these populations was quantified by RT-qPCR directly *ex vivo*. Naïve CD4 T cells expressed relatively low basal levels of Plac8 mRNA compared to other assayed cell types and thus were used as a reference point (**Fig. 2.1F**). Plac8 mRNA expression was nearly 100-fold higher in naïve CD8 T cells relative to naïve CD4 T

cells and there was about 30 times more Plac8 expression within memory CD4 T cells compared to naïve CD4 T cells (**Fig. 2.1F**). To determine if IL-12 similarly induced Plac8 mRNA expression within CD8 T cells, naïve CD8 T cells were TCR-activated for three days prior to stimulation with increasing concentrations of IL-12 for 24 h. Like CD4 T cells, Plac8 mRNA expression within CD8 T cells was significantly induced in a concentration-dependent manner (**Fig. 2.1G**). These data show that CD8 T cells express higher levels of Plac8 mRNA relative to CD4 T cells, and that Plac8 can be further induced in CD8 T cells following IL-12 stimulation, although the fold induction in the latter was significantly lower given their higher basal level of Plac8 expression.

Plac8 mRNA is preferentially expressed by Th1 versus other CD4 T cell subsets (**Fig. 2.1A**). Therefore, it is possible that Plac8 plays an important role in either driving Th1 cell differentiation or promoting Th1 CD4 T cell effector function. To determine if Plac8 contributes to the differentiation of Th1 CD4 T cells, naïve WT and *Plac8*$^{-/-}$ CD4 T cells were isolated and differentiated in Th1 conditions for three days, and polarization was subsequently assessed by measuring intracellular IFNγ staining by flow cytometry. Overall, there was no difference in either the frequency of IFNγ-producing CD4 T cells or IFNγ mean fluorescence intensity between WT and Plac8$^{-/-}$ Th1 cells (**Fig. 2.2A-B)**, suggesting that Plac8 does not contribute to Th1 differentiation. To determine if Plac8 contributes to differences in T cell IFNγ production, WT and *Plac8*$^{-/-}$ CD4 or CD8 T cells were TCR-activated with α-CD3 and α-CD28 for three days prior to IL-12 stimulation for 24 h. After IL-12 stimulation, supernatants were collected, and the mRNA isolated from cell pellets was converted into cDNA for analysis of IFNγ gene expression by RT-qPCR. *Plac8*$^{-/-}$ CD4 T cells stimulated with IL-12 had higher levels of IFNγ mRNA compared to WT CD4 T cells and the same trend was observed in CD8 T cells (**Fig. 2.2C**), suggesting that Plac8 may negatively regulate IFNγ transcription. Indeed, *Plac8*$^{-/-}$ CD4 T cells consistently

secreted higher levels of IFNγ protein as determined by ELISA (**Fig. 2.2D**). These data suggest that although Plac8 is not essential for Th1 cell differentiation, Plac8 constrains IFNγ production by CD4 T cells early after IL-12 stimulation.

Given that Plac8 inhibits IFNγ production by CD4 T cells *in vitro*, we sought to evaluate whether Plac8 functionally impairs Th1 CD4 T cells *in vivo* by utilizing a well-established T cell transfer model of colitis in which adoptively transferred $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells induce colonic inflammation in *Rag*$^{-/-}$ recipients within 8-12 weeks post-transfer [212]. Inflammation in this colitis model is driven by the recognition of gut flora by self-reactive T cells through the IL-23/IFNγ/IL-17 axis [213]. IFNγ produced by Th1 cells in this model is known to be critical for disease pathogenesis, because transfer of *IFNγ* $^{-/-}$ CD4 T effector cells fails to induce disease in *Rag1*$^{-/-}$ hosts [214]. In addition, since development of disease is completely dependent on the transferred CD4 T cell population, this model will allow us to evaluate the direct effect of Plac8 on pro-inflammatory CD4 T cell function *in vivo*. Therefore, to determine if Plac8 contributes to IFNγ suppression *in vivo*, *Rag1*$^{-/-}$ recipients received either WT or *Plac8*$^{-/-}$ $CD4^{+}CD45RB^{hi}CD25^{-}$ T cells, and morbidity was assessed via weight loss over time. There was no difference in weight loss between the two groups of animals at any time point analyzed (**Fig. 2.3A**). Ten weeks post-transfer, CD4 T cells were evaluated for TNF, IL-17A, and IFNγ cytokine production via intracellular cytokine staining; however, there was no difference in either the frequency (**Fig. 2.3B**) or total cell number (**Fig. 2.3C**) of cytokine-producing WT and *Plac8*$^{-/-}$ cells in the spleen, mesenteric lymph nodes (mLN), or colon. Because we saw an increase in CD4 T cell IFNγ production 24 h-post IL-12 stimulation *in vitro*, and the T cell transfer model of colitis induces chronic inflammation over several weeks, we hypothesized that Plac8's impact on CD4 T cell IFNγ may be limited to models of acute inflammation. To this end, we utilized an acute infectious

model of colitis in response to *Citrobacter rodentium*. This pathogen elicits a potent Th1 and Th17 response; however, IFNγ production by CD4 T cells is indispensable [148]. To specifically address the T cell-intrinsic regulation of the response, we utilized mixed bone marrow chimeras whereby irradiated CD45.2/.1 heterozygote hosts were reconstituted with 1:1 ratio of WT (CD45.1) and *Plac8*$^{-/-}$ (CD45.2) bone marrow. Because the WT and *Plac8*$^{-/-}$ T cells are exposed to the same environmental stimuli in this model, any differences noted between the two cell types are due to intrinsic differences within the T cells themselves. However, assessment of cytokine production by WT and *Plac8*$^{-/-}$ CD4 T cells 14 days post *C. rodentium* infection revealed no differences in TNF□ IL-17A, or IFNγ production (**S2.2 Fig.**). Therefore, while our *in vitro* stimulation data suggested that Plac8 may function to constrain IFNγ production, our functional analyses suggests that Plac8 does not contribute to T cell-mediated inflammation *in vivo,* at least in the specific Th1-dependent disease models tested.

Plac8$^{-/-}$ hosts establish fewer memory CD8 T cells after influenza infection

Because CD8 T cells express relatively high basal levels of Plac8 mRNA (**Fig. 2.1F**), and there were trending differences in IFNγ regulation between WT and *Plac8*$^{-/-}$ CD8 T cells stimulated with IL-12 (**Fig. 2.2C-D**), we hypothesized that Plac8 may contribute to CD8 T cell function *in vivo*. To test this, we utilized a mouse-adapted influenza A virus (IAV) infection model, because IAV induces a robust Th1 immune response in C57BL/6 WT mice [160], and CD8 T cells play a critical role in viral clearance [215]. First, we determined whether Plac8 contributed to viral clearance by assessing lung viral titers from WT or *Plac8*$^{-/-}$ mice infected with 10^4 pfu of X31 (**Fig. 2.4A**); however, there were no differences in viral titers on 3, 5, or 7 days post infection (dpi) between WT and *Plac8*$^{-/-}$ mice (**Fig. 2.4B**) indicating that Plac8 does not significantly impact early CD8 T cell viral control.

In other cell types, Plac8 is known to regulate cellular proliferation [57, 216, 217], and modified proliferation can impact CD8 T cells not only acutely during the effector phase of the anti-viral response, but also the development of subsequent memory CD8 T cells. Therefore, we next assessed whether Plac8 is important for the establishment of the immunodominant influenza nucleoprotein (NP)-specific CD8 T cells within WT and *Plac8*$^{-/-}$ mice. WT and *Plac8*$^{-/-}$ mice were infected with 10^4 X31, and NP-reactive CD8 T cells were examined in the bronchoalveolar lavage fluid (BAL) as well as in lung, spleen and lung-draining mediastinal lymph node (mdLN) tissues at the peak of the effector (10 days post infection, dpi) and memory (35 dpi) CD8 T cell response (**Fig. 2.4A&C**). At 10 dpi, all tissues assessed harbored an equivalent frequency and number of NP-specific CD8 effector T cells (**Fig. 2.4D**). However, there was a significantly lower frequency and number of NP-specific memory CD8 T cells in the *Plac8*$^{-/-}$ IAV infected mice compared to WT mice at the 35 dpi memory time point. These data suggest that Plac8 is important for the establishment of memory CD8 T cells.

Plac8 promotes effector CD8 T cell establishment through a T cell-intrinsic mechanism that is not proliferation

Plac8 protein is expressed by other immune and stromal cells, such as macrophages and lung epithelial cells [206], that participate in the immune response to influenza, and can indirectly influence the influenza-specific CD8 T cell response. Therefore, we cannot differentiate if the phenotype observed at 35 dpi (**Fig. 2.4D**) is the result of a CD8 T cell intrinsic or extrinsic mechanism. To circumvent this limitation, we utilized a mixed bone marrow chimera model whereby CD45.1/.2 WT recipient mice were lethally irradiated and reconstituted 1:1 with bone marrow from CD45.2 *Plac8*$^{-/-}$ and CD45.1 WT mice (**Fig. 2.5A**). After a two-month engraftment period, chimeric mice were infected with 10^4 pfu of X31, and BAL, spleen, mdLN, and lungs were

harvested 10 or 35 dpi. Although each recipient mouse was given a 1:1 mix of bone marrow, not all hosts engrafted equally. Therefore, to negate any engraftment variability, $CD8^+$ T cells derived from WT ($CD45.2^+$) and *Plac8*$^{-/-}$ ($CD45.1^+$) mice were identified, and the frequency of $CD44^{hi}$, NP-reactive cells from each genotype was quantitated **(Fig. 2.5B)**. At 10 dpi, *Plac8*$^{-/-}$ $CD44^{hi}$, NP-specific CD8 T cells were present at a lower frequency than their WT NP-specific counterparts in the lung, BAL and spleen, whereas memory NP-specific CD8 T cells derived from *Plac8*$^{-/-}$ mice were additionally reduced in the lung (**Fig. 2.5C-D**). These data suggest that Plac8 is regulating the CD8 T cell response through a T cell-intrinsic mechanism.

To examine whether the phenotype observed at the effector time point could be explained by differences in CD8 T cell proliferation, another cohort of chimeric mice were infected with X31, and the proliferation of NP-specific CD8 T cells were characterized 8 dpi, just prior to the peak proliferative CD8 T cell response, via 5-Bromo-2'-deoxyuridine (BrdU) incorporation. Simultaneously, surface expression of CD25, the high affinity IL-2Rα chain, was measured to evaluate potential differences in cellular responsiveness to the IL-2 growth factor. However, no differences in either CD25 surface expression or BrdU incorporation were observed at the assayed time (**Fig. 2.5E**). Altogether, these data demonstrate that Plac8 is important for the establishment of effector CD8 T cells 10 dpi, independent of modified proliferation or IL-2 signaling.

Given that WT and *Plac8*$^{-/-}$ mice could harbor different numbers of NP-specific CD8 T cell precursors, which could directly impact the magnitude of the NP-specific CD8 T cell response, we repeated the influenza studies using an OT-I T cell adoptive transfer model whereby all transgenic OT-I cells (WT or *Plac8*$^{-/-}$) are TCR $V\alpha2^+$ $CD8^+$ T cells that recognize a specific ovalbumin (OVA) peptide in the context of MHC class I. WT CD45.1/.2 recipient mice received 1,000 WT ($CD45.1^+$) and 1,000 *Plac8*$^{-/-}$ ($CD45.2^+$) OT-I cells one day prior to infection with a recombinant

X31 virus expressing the OVA antigen (X31-OVA) (**Fig. 2.6A**). BAL, spleen, mdLN, and lung were harvested at the peak of the OT-I cell response and at a memory time point (8 and 35 dpi, respectively), and OT-I cells were identified as $CD8^{+}$, $CD44^{hi}$, and TCR $V\alpha2^{+}$ cells before distinguishing a $CD45.1^{+}$ (WT) or $CD45.2^{+}$ (*Plac8*$^{-/-}$) origin (**Fig. 2.6B**). There were significantly less *Plac8*$^{-/-}$ OT-I cells in all analyzed tissues at 8 dpi, and significantly less *Plac8*$^{-/-}$ OT-I cells in the spleen at 35 dpi (**Fig. 2.6C**). Because the starting amount of adoptively transferred WT and *Plac8*$^{-/-}$ OT-I cells are equal, and there are significantly fewer *Plac8*$^{-/-}$ OT-I T cells at the peak of the T cell response, these observations provide strong evidence that Plac8 promotes effector CD8 T cell establishment through a T cell-intrinsic mechanism.

DISCUSSION

In this study, we have determined that Plac8 is highly and uniquely expressed by Th1 CD4 T cells compared to Th2, Th17, and iTreg CD4 T cells. We also found that Plac8 is indirectly induced following IL-12 stimulation in CD4 T cells and that IFNγ plays a modest role in its induction. While Plac8 significantly suppressed IFNγ production in IL-12 stimulated CD4 T cells *in vitro*, this phenotype was not captured in either a T cell transfer model of colitis or following *Citrobacter rodentium* infection. Interestingly, and unexpectedly, we found that naïve CD8 T cells express significantly higher basal levels of Plac8 compared to naïve CD4 T cells (~100x greater) and that IL-12 similarly induces Plac8 mRNA expression within CD8 T cells. Further, the functional effect of Plac8 deficiency was more relevant to CD8 T cell responses as *Plac8*$^{-/-}$ mice harbored fewer effector and, in some cases, memory antigen-specific CD8 T cells after IAV infection.

Determining factors that regulate Plac8 expression in T cells may help to identify how it can be utilized therapeutically during T cell-driven inflammation. Data mined from Wei *et al.* showed that Plac8 mRNA expression was high in Th1 CD4 T cells relative to Th2 CD4 T cells differentiated *in vitro* [51] and Johnson *et al*. found that Plac8 mRNA and protein expression is associated with Th1 *Chlamydia muridarum*-specific CD4 T cells [207]. However, more recently, Tibbitt *et al.* identified Plac8 as one of the most highly upregulated genes in Th2 CD4 T cells isolated from mice sensitized and challenged with house dust mite (HDM) allergen *in vivo* [64]. These seemingly contradictory results might be due to variations in the differentiation conditions found *in vivo* versus *in vitro*. For example, IL-1α and TSLP, which are not typically included in *in vitro* cultures, including ours, are secreted during HDM sensitization [218] and their impact on Plac8 expression in CD4 T cells has not been determined. Altogether, these results indicate that

Plac8 induction in CD4 T cells may be complex and context dependent, and a careful evaluation of the environments that induce Plac8 expression in CD4 T cells will be important as we continue ascribing Plac8's function in these cells.

Our *in vitro* studies firmly identify IL-12 as a factor that increases Plac8 mRNA expression in CD4, and to a lesser extent, CD8 T cells. Since our data demonstrate that IL-12 induces Plac8 expression *indirectly* (**Fig. 2.1C**), and the fact that Plac8 is referred to as an IFN-stimulated gene [53, 63], we initially hypothesized that autocrine IFNγ signaling, secondary to IL-12, was responsible for the significant Plac8 expression observed in Th1 CD4 T cells. However, direct stimulation of T cells with IFNγ failed to induce Plac8 expression to a similar extent as IL-12, suggesting that IL-12 signaling induces Plac8 mRNA expression indirectly via a largely IFNγ-independent mechanism. Genomic analysis of 1kb upstream of Plac8's promoter sequence identified putative binding sites for C/EBPα, C/EBPβ, NF-κB, Sp1, and IRF8, but not Stat4 which is directly regulated by IL-12 [219]. C/EBPα is the only one of these transcription factors implicated in limiting IFNγ production by Th1 CD4 T cells through a T cell-intrinsic manner [220], consistent with the phenotype we observed in our Plac8 study. This suggests that C/EBPα could be regulating Plac8 expression in response to IL-12 stimulation; however, it remains unclear whether IL-12 can *directly* regulate C/EBPα production in CD4 T cells.

We determined that Plac8 suppresses IL-12-induced IFNγ production by CD4 T cells at the transcriptional level (**Fig. 2.2C**). While these findings were initially unexpected, a previous study demonstrated that Plac8 is important for brown fat differentiation through associating with C/EBPβ and binding to the C/EBPβ promoter to induce its transcription [221], so the possibility that Plac8 could function as a transcriptional regulator is not unprecedented. Despite Plac8's regulation of IFNγ production by CD4 T cells *in vitro*, Plac8 ablation did not apparently alter T

cell effector function in a T cell transfer model of colitis (**Fig. 2.3**). This may be because IL-12p70 is known to be dispensable for colitis, but IL-23 and IFNγ are not [113, 148], suggesting that IFNγ production by CD4 T cells may result from IL-23 stimulation rather than IL-12 stimulation in this model. It is also possible that Plac8's effect on CD4 T cell IFNγ production occurs transiently, and the magnitude of the enhancement of the IFNγ response in *Plac8*$^{-/-}$ T cells may not be large enough to manifest during a model of chronic inflammation such as this colitis model.

Surprisingly, Plac8 was important for the establishment of effector influenza-specific CD8 T cells through a T cell-intrinsic manner when utilizing a mixed bone marrow chimera model and an OT-I T cell adoptive transfer model (**Fig. 2.5C and 2.6C**). However, when comparing the NP-specific CD8 T cell response in the complete WT versus *Plac8*$^{-/-}$ influenza mouse model, Plac8 was important for the establishment of memory, but not effector, CD8 T cells (**Fig. 2.4E**). Because the impact of Plac8 on the influenza-specific CD8 T cell response is different when utilizing a model where Plac8 is globally depleted versus a model where Plac8 is specifically depleted in CD8 T cells, this data suggests that Plac8 may contribute to the function of other immune cell(s) during influenza infection. Plac8 protein is expressed by macrophages and lung epithelial cells, two key components during IAV infection [206], but there is no information regarding Plac8's function within these cells. Therefore, it will be important to determine Plac8's impact on global immune cell function and CD8 T cell-specific functions during influenza infection.

When Plac8 was depleted in CD8 T cells, we observed a decrease in *effector* CD8 T cell establishment relative to WT CD8 T cells which led us to hypothesize that Plac8 promotes proliferation within CD8 T cells. This hypothesis is supported by Plac8's documented role in promoting proliferation in multiple cancerous epithelial cell lines [57, 216, 217]. However, in our model we found that Plac8 does not regulate the proliferation of antigen-specific CD8 T cells as

measured by similar surface expression of CD25 and BrdU incorporation 8 dpi (**Fig. 2.5E**). We also compared the WT and KO effector CD8 T cells based on their expression of surface markers IL-7Rα and KLRG1 which are indicative of effector CD8 T cell fate, however there were no differences in the expression of these surface markers, suggesting that Plac8 does not impact CD8 T cell differentiation pathways during influenza infection (data not shown). An alternate hypothesis is that Plac8 may promote CD8 T cell survival after IAV infection because it has been shown to promote cancer cell survival by limiting apoptosis [61, 222]. Because apoptotic cells are rapidly scavenged *in vivo* by phagocytes, quantifying apoptosis *in vivo* remains challenging [223]. Currently, the best way to identify cell death *in vivo* relies on measuring the amounts of intracellular molecules released by dying cells [224]; however many of these markers are not unique to cell death, and their cellular sources cannot be defined [225], making this assessment at the moment equivocal.

Determining Plac8's direct impact on Th1-driven inflammation has proven to be complex; however, these studies demonstrate that Plac8 has potential to serve as a viable therapeutic target. Through our CD4 T cell experiments, we have shown that Plac8 can limit IFNγ production by CD4 T cells, suggesting that Plac8 could be utilized to limit cellular inflammation and provide a longer-lasting, more efficacious alternative to the current anti-inflammatory treatments. In addition, we determined that global Plac8 expression promotes the establishment of memory CD8 T cells following influenza infection, which may promote efficacy rates in the current influenza vaccines. Although the precise function of Plac8 during Th1-driven inflammatory responses remains elusive, for the first time, these data show that Plac8 serves an immunoregulatory role during Th1 type immune responses and should be further explored for potential therapeutic benefit.

ACKNOWLEDEMENTS

The authors would like to thank Julie Nelson in the Center for Tropical and Emerging Global Diseases Flow Cytometry Core Facility for flow sorting, Jamie Barber in the CVM Cytometry Core Facility for LSRII assistance, and UGA's Coverdell Rodent Vivarium staff members for animal care.

FIGURES

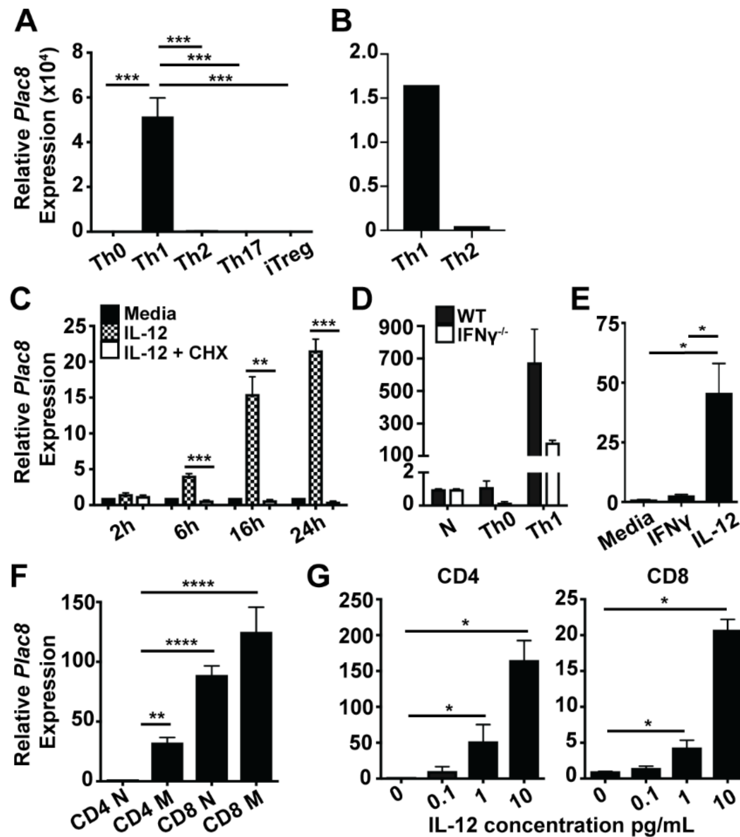


Figure 2.1. Plac8 mRNA is induced within T cells following IL-12 stimulation

(**A**) Naïve (CD44^{lo}CD62L^{+}) CD4 T cells were sort purified, differentiated over 3d into the indicated Th subsets *in vitro*, and the relative level of Plac8 mRNA expression determined. The level of Plac8 mRNA expression in undifferentiated Th0 CD4 T cells was set to 1 as the baseline expression level. (**B**) Plac8 expression in Th1 and Th2 *in vitro* differentiated CD4 T cells was determined by microarray [51]. (**C**) Naïve CD4 T cells were TCR activated with immobilized α-CD3 and α-CD28 for 3d prior to IL-12 stimulation for the indicated hours in the presence (white bars) or absence (hatched bars) of cycloheximide (CHX) pretreatment or media alone (filled bar). Plac8 expression was normalized to untreated Th0 CD4 T cells. (**D**) Naïve CD4 T cells were sort

purified from WT and *IFNγ*$^{-/-}$ mice, TCR activated in the presence or absence of IL-12 (Th1 and Th0, respectively) for 3d. Relative Plac8 expression levels were calculated using the naïve sample of each genotype as the baseline. (**E**) Naïve CD4 T cells were TCR activated and subsequently stimulated for 6h with IFNγ or IL-12 and Plac8 mRNA levels determined. (**F**) Naïve (N, $CD44^{lo}CD62L^{+}$) and memory (M, $CD44^{hi}$) $CD4^{+}$ and $CD8^{+}$ T cells were sort purified from mice based on surface marker expression. Relative expression levels of Plac8 were normalized to CD4 N. (**G**) CD4 or CD8 T cells were TCR activated for 3d prior to stimulation with increasing concentrations of IL-12 for 24h. A, D, and F are representative of 3 experiments and C, E, and G are representative of 2 experiments. P values for C, D, F, and G were determined by unpaired Student's t test. P values for A and E were calculated by one-way ANOVA and Tukey's post hoc analysis. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$)

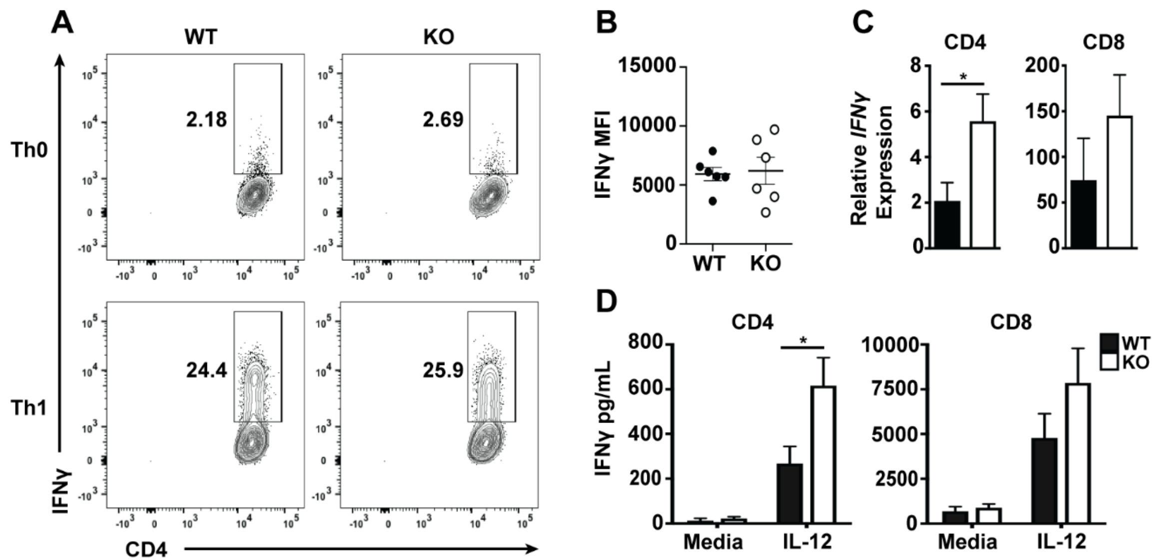


Figure 2.2. Plac8 negatively regulates IFNγ production by T cells following IL-12 stimulation

WT or *Plac8*$^{-/-}$ (KO) naïve (CD44^{lo}CD62L^{+}) CD4 T cells were sort purified and TCR stimulated with α-CD3 and α-CD28 in the presence or absence of IL-12 (Th1 or Th0, respectively) for 3d (**A,B**). Cells were subsequently harvested, stimulated with PMA and ionomycin for 4h, and the frequency (**A**) of intracellular IFNγ levels and mean fluorescence intensity (MFI) (**B**) was determined by flow cytometry. Data are representative of two-independent experiments. Other WT or KO naïve CD4 T cells were TCR stimulated with α-CD3 and α-CD28 for 3d prior to IL-12 stimulation for 24h. Relative IFNγ mRNA levels (**C**) and protein levels (**D**) were determined by RT-qPCR or ELISA. Relative IFNγ expression was calculated using T cells that were not stimulated with IL-12 as the baseline. Each group has at least 6 samples. P values were determined by unpaired Student's t test. * ($P < 0.05$)

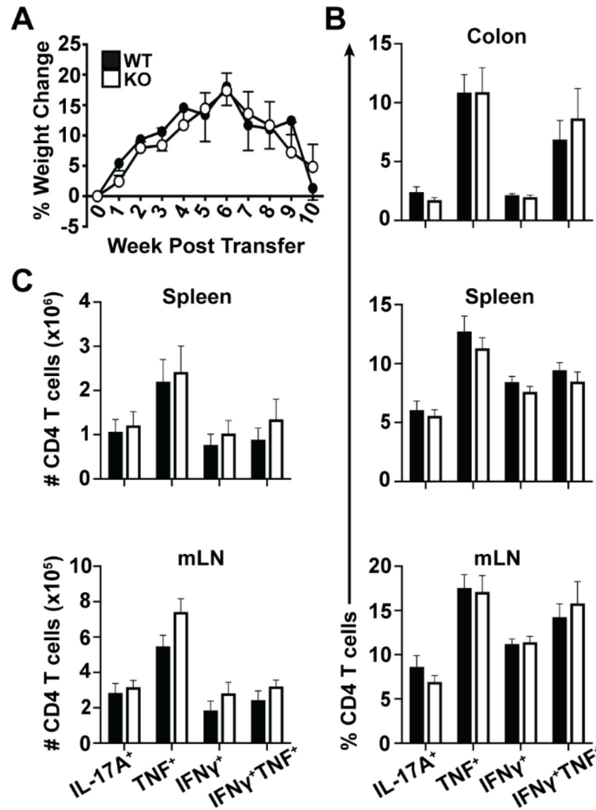


Figure 2.3. Plac8 does not suppress IFNγ production by pathogenic CD4 T cells during colitis

WT or *Plac8*$^{-/-}$ (KO) CD4^{+}CD45RB^{hi}CD25^{-} effector T cells were injected into *Rag1*$^{-/-}$ recipients. (**A**) Mice were weighed weekly and % weight change was calculated. Data shown are the daily mean % weight change for each group and representative of three independent experiments and 6 mice per group. After colitis onset, mice were sacrificed and single cells were isolated from the indicated tissues and stimulated with PMA and ionomycin for 4h. The frequency (**B**) and total cell number (**C**) of the indicated cytokine-producing CD4 T cells was determined by intracellular flow cytometry. B and C are pooled data from 3 independent cohorts totaling 17 hosts/group.

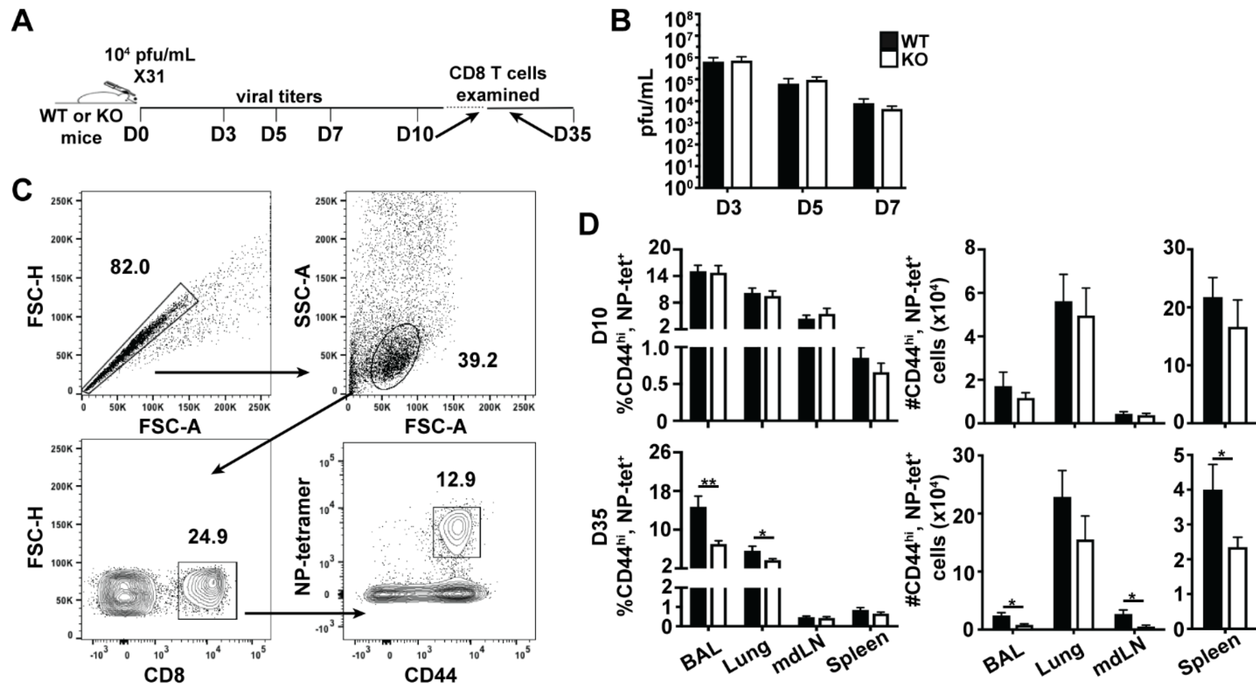


Figure 2.4. Plac8$^{-/-}$ hosts establish fewer memory CD8 T cells after influenza infection

(**A**) WT or *Plac8$^{-/-}$* (KO) mice were intranasally infected with 10^4 pfu/mL of X31. (**B**) On 3, 5, and 7 dpi, mouse lungs were harvested and viral titers determined via plaque assays. N $\geq$ 6 mice/ time point. (**C**) Gating strategy used to identify influenza NP-specific CD8 T cells by flow cytometry. (**D**) The frequency and total cell number of NP-specific CD8 T cells isolated from the indicated tissues at 10 or 35 dpi. Data represents 12 animals (3 pooled cohorts) or 17 animals (four pooled cohorts) for 10 and 35 dpi, respectively. *P* values were determined by unpaired Student's *t* test. * ($P < 0.05$), ** ($P < 0.01$)

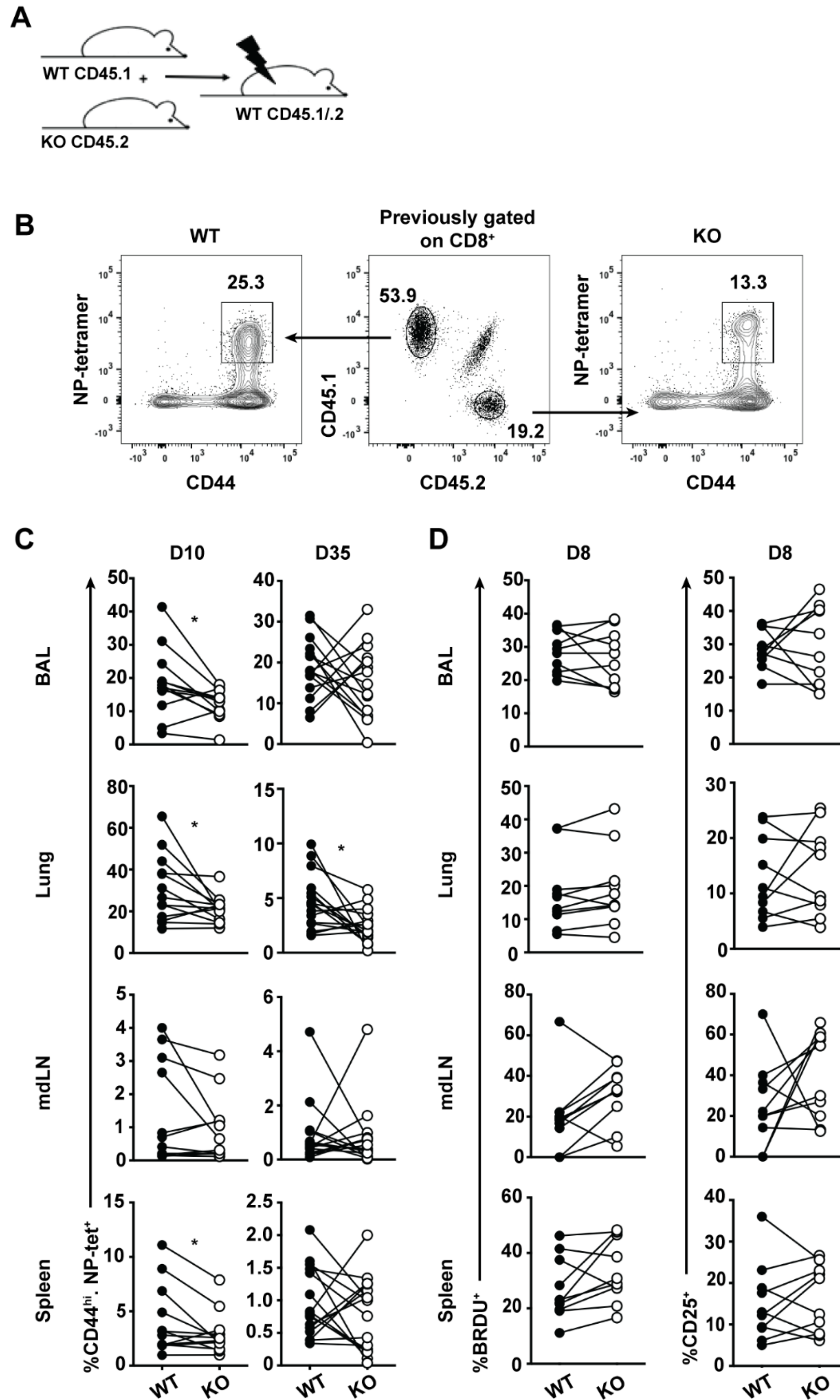


Figure 2.5. Plac8 promotes effector CD8 T cell establishment through a T cell-intrinsic mechanism, independent of proliferation

(**A**) Mixed bone marrow chimeras were generated by injecting WT CD45.1 and KO CD45.2 bone marrow 1:1 into irradiated CD45.1/.2 recipients and resting for 2 months for reconstitution. (**B**) Gating strategy to identify the genotype of influenza NP-specific CD44^{hi} CD8 T cells responding to infection based on CD45 status. (**C**) Chimeras were infected with influenza X31 and the frequency of NP-specific CD8 T cells of derived from WT or KO bone marrow was determined 10 (n=13) and 35 (n=16) dpi using the gating strategy in (**B**). Lines connect the frequency of WT and KO representation in a single host and the data are pooled from 3 independent experiments. (**D**) Mice were infected as in (**C**) and subsequently injected with BrdU i.p. 12h before a harvesting the tissues 8 dpi. Data depict BrdU incorporation within CD44^{hi}, NP-tetramer^{+} CD8 T cells over the 12h labelling period and CD25 expression by these cells (**E**) in 2 pooled independent cohorts of mice (n=11). P values were determined using paired Student's t test. * ($P < 0.05$)

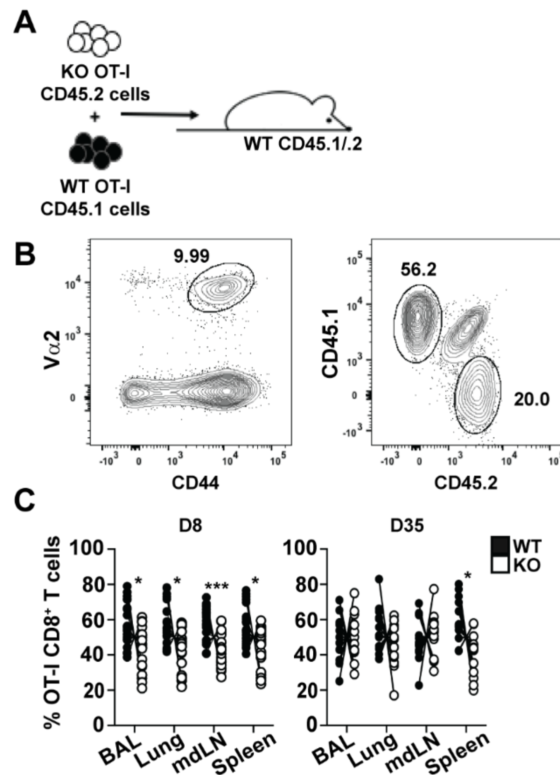
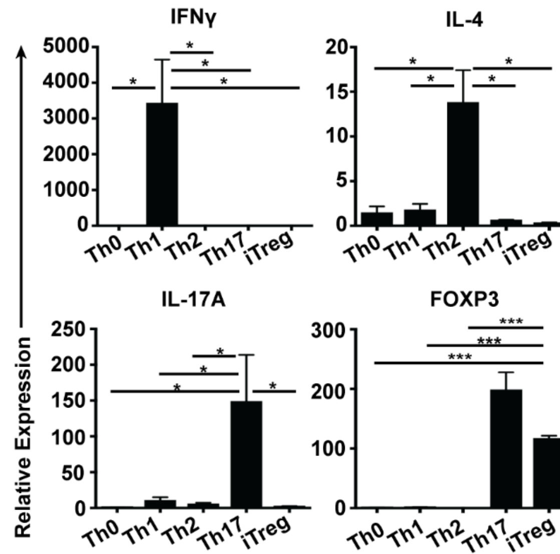


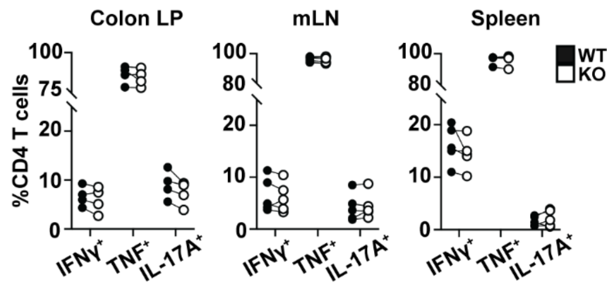
Figure 2.6. Plac8's impact on effector CD8 T cell establishment is unrelated to differences in NP-specific precursors

(**A**) Mice received a 1:1 mix of WT (CD45.1) and *Plac8*$^{-/-}$ (CD45.2) OT-I cells one day prior to infection with 10^3 X31-OVA. (**B**) OT-I cells were identified as CD44^{hi} and TCR Va2$^+$ CD8 T cells and genotyped based on CD45 status. (**C**) BAL, lung, mdLN, and spleen were harvested 8 dpi and 35 dpi and the frequency of WT vs Plac8$^{-/-}$ OT-I cells participating in the response to infection was determined. Lines connect the frequency of the two donor OT-I populations within a single recipient. *P* values were determined by paired Student's *t* test. * ($P < 0.05$), *** ($P < 0.001$)



S2.1. Efficiency of CD4 Th cell differentiation in vitro

Naïve CD4 T cells were TCR-activated by α-CD3 and α-CD3 and differentiated in the presence of IL-12 (Th1 conditions), IL-4 (Th2 conditions), TFG-β and IL-6 (Th17 conditions), TGF-β and IL-2 (iTreg conditions) or media alone (Th0) for 3d. Relative expression of the indicated Th subset signature cytokine or transcription factor were determined by RT-qPCR using Th0 cells as the baseline expression level. Data are representative of three independent cohorts. *P* values were determined by one-way ANOVA * ($P < 0.05$), *** ($P < 0.001$).



S2.2. Plac8 does not affect inflammatory cytokine production by CD4 T cells after Citrobacter rodentium infection

C57BL/6 CD45.2/.1 heterozygote hosts were irradiated with a single dose of 1,100 rad and reconstituted with 3 million WT (CD45.1) and 3 million *Plac8*$^{-/-}$ (CD45.2) bone marrow cells. Two months after immune cell reconstitution, hosts were infected with 1 x 10^9 to 3 x 10^9 CFU of a luminescent strain of *Citrobacter rodentium* ICC180 (kindly provided by Gad Frankel at Imperial College, London, United Kingdom), via gastric gavage in a total volume of 200µl. The dose was confirmed through retrospective plating on LB agar plates. Three days post gastric gavage, mice were anesthetized using isoflurane and imaged for 30 seconds using an IVIS Lumina imager (PerkinElmer) to confirm infection status. 14 dpi, lymphocytes were isolated from the lamina propria, spleen, mesenteric lymph nodes and resuspended to 1x10^6 cells/mL before *ex vivo* stimulation with 50 ng/mL PMA, 0.5 µg/mL ionomycin, and Golgi transport inhibitor according to the manufacturer's directions (BD Biosciences) for 4 h at 37°C. Cells were then surface stained for CD45.1 (A20), CD45.2 (104), CD4 (RM4-5), and TCRβ (H57-597) at 4°C for 20 min before being intracellularly stained for IFNγ (XMG1.2), TNF (MP6-XT22), and IL-17A (eBio1787) as described in materials and methods. Lines between black (WT) and white (KO) circles represent a single host. This experiment contains 5 mice and is a representative of 3 independent experiments.

CHAPTER 3

PLACENTA-SPECIFIC 8 LIMITS BACTERIAL BURDENS AND PROMOTES CXCL1 EXPRESSION DURING CITROBACTER RODENTIUM INFECTION[2]

[2]Slade CD, Rada B, Klonowski KD, and Watford WT. To be submitted to *Journal of Leukocyte Biology.*

ABSTRACT

Citrobacter rodentium is an extracellular, mouse-specific bacterial pathogen that colonizes the gastrointestinal tract through the formation of attaching and effacing lesions. After infection, neutrophils contribute to *C. rodentium* control primarily by phagocytosing bacteria and by secreting IL-22 to strengthen the epithelial cell barrier. Previous reports have determined that the cysteine-rich protein, Plac8, promotes neutrophil-mediated killing of bacteria; however, the contribution of Plac8 to the control of *C. rodentium* infection has yet to be determined. Because neutrophils are essential for early *C. rodentium* control, we hypothesize that Plac8 ablation will cause increased *C. rodentium* burdens in mice through a reduction in neutrophil–mediated killing. In these studies, we determined that *Plac8*$^{-/-}$ mice have significantly higher *C. rodentium* burdens throughout the course of infection with increased gut permeability observed at 3 days post infection compared to their wild type counterparts. Although Plac8 has previously been shown to promote neutrophil-mediated killing of bacteria, here we found that Plac8 deficiency did not affect neutrophil-mediated killing of *C. rodentium ex vivo*. Instead, Plac8 ablation resulted in a reduction of colonic CXCL1 expression 4 days after *C. rodentium* infection. Because CXCL1 is one of the primary chemokines required for neutrophil recruitment during *C. rodentium* infection, and neutrophils are essential for limiting *C. rodentium* burdens, we conclude that Plac8 likely controls *C. rodentium* burdens by promoting neutrophil recruitment to the site of infection. Overall, these results highlight the importance of Plac8 in protecting hosts against enteropathogenic infection and suggests that Plac8 has potential to be utilized therapeutically to regulate neutrophil recruitment during human enteropathogenic infections.

INTRODUCTION

Citrobacter rodentium is a mouse-specific extracellular bacterial pathogen that adheres to the apical surface of intestinal epithelial cells through the formation of attaching and effacing (A/E) lesions [120]. Because the A/E lesions formed by *C. rodentium* are indistinguishable from those formed by the human pathogens enteropathogenic E. coli (EPEC) and enterohaemorrhagic *E. coli* (EHEC), *C. rodentium* is commonly utilized as a model organism to study the pathogenesis of these human bacterial pathogens [119]. Soon after *C. rodentium* infection, epithelial cells produce antimicrobial peptides such as Reg3γ which is important for maintaining intestinal homeostasis by promoting spatial separation between the epithelial cells and bacteria [226] and Reg3β which triggers the secretion of IL-17A by type 3 innate lymphoid cells to begin neutrophil recruitment [126]. In addition, innate microbial sensing pathways lead to the activation of the NLRP3 inflammasome in macrophages, which results in the production of IL-1β [227]. IL-1R signaling induces CXCL1 production by stromal cells, which promotes the recruitment of neutrophils to the site of infection where they contribute to early *C. rodentium* control via phagocytosis and secretion of IL-22 which strengthens the epithelial cell barrier [128, 228-231]. Therefore, it is the cooperation of intestinal epithelial cells and neutrophils which limits *C. rodentium* replication and dissemination; however, many of the underlying mechanisms that regulate the protective functions of these cell types during *C. rodentium* infection remain poorly understood.

Placenta-specific 8 (Plac8) is a cysteine-rich protein originally identified in placental tissue [48] but has since been associated with a variety of cell types important for controlling *C. rodentium* infection, including neutrophils and intestinal epithelial cells [49]. Plac8 is highly expressed in neutrophil granules and is known to promote intracellular killing of some bacterial species including *Klebsiella pneumoniae* and *E. coli* [49]. It is also highly expressed on the apical

surface of intestinal epithelial cells [232], however its potential immunologic role in epithelial cells remains unexplored. Plac8-deficiency during a contact hypersensitivity response has been shown to limit myeloid cell recruitment and to be associated with lower levels of the inflammatory cytokine IL-6 and chemokine CXCL1 [62]. Currently, the impact of Plac8 on *C. rodentium* infection remains unknown. However, given the association of Plac8 with neutrophil-mediating killing in other bacterial models, we hypothesize that Plac8 is important for limiting *C. rodentium* burden by promoting bacterial killing within neutrophils.

In this study, we determined that Plac8 is required to significantly reduce *C. rodentium* burdens throughout the course of infection and that Plac8 ablation causes a significant increase in gut permeability 3 days post infection. However, we observed no difference in neutrophil control and killing of *C. rodentium ex vivo* between *Plac8*$^{-/-}$ and *Plac8*$^{+/+}$ neutrophils. Instead, *Plac8*$^{-/-}$ mice had decreased levels of CXCL1 mRNA present in their colons 4 days post *C. rodentium* infection relative to WT mice. These results suggest that *Plac8*$^{-/-}$ mice may develop increased *C. rodentium* burdens because of impaired neutrophil *recruitment* and not because of impaired neutrophil *function*. Overall, these results demonstrate that Plac8 is required for optimal protection against *C. rodentium* infection and, for the first time, suggest that Plac8 could have an immunoregulatory role in chemokine production within epithelial cells during an infectious disease model.

MATERIALS AND METHODS

Mice. Wild type (WT) C57BL/6 mice were originally purchased from the Jackson Laboratory or Charles River and are now bred in-house. C57BL/6 *Plac8*$^{-/-}$ mice were generated by Beverly H. Koller (University of North Carolina at Chapel Hill, Chapel Hill, NC) [206] and kindly provided by Dr. Raymond Johnson (Indiana University School of Medicine, Indianapolis, IN). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Georgia (#A2018 06-013-A4). Euthanasia was performed by CO_2 asphyxiation followed by cervical dislocation.

Citrobacter rodentium ICC180. Gad Frankel at Imperial College (London, United Kingdom) generously provided the luminescent *C. rodentium* strain (ICC180) [123]. Mice were infected with 2 x 10^9 to 5 x 10^9 *C. rodentium* in total volume of 200 µL PBS via a gastric gavage and doses were confirmed through retrospective plating on LB agar plates. In addition, mice were anesthetized with isoflurane, given an abdominal shave, and imaged for 30 secs 3 dpi using an IVIS imager (PerkinElmer, Waltham, MA) to verify infection status. Mice without luminescent signal 3 dpi were excluded from these studies. To quantify CFU of *C. rodentium* per gram of feces, samples were diluted in 100 µL of PBS per 0.01 g of feces before being serially diluted and plated on LB agar plates in triplicate. Plates were imaged the next day using the IVIS imager, and CFUs were calculated using the luminescent bacterial colonies.

Neutrophil Assays. Bacterial control and killing assays were conducted utilizing 1,000,000 neutrophils/mL media (HBSS containing 10 mM HEPES, 5 mM glucose, and 1% mouse serum) with the indicated MOI of *C. rodentium* (ICC180). Neutrophils were isolated from the bone

marrow from the femur and tibia and enriched using the neutrophil isolation kit (Stemcell Technologies, Vancouver, Canada). *C. rodentium* was opsonized with 100 µL of 100% murine serum at 37°C for 30 min prior to co-culturing with neutrophils. To calculate neutrophil bacterial control, *C. rodentium* and neutrophils were plated in 100 µL in a white, 96-well plate and luminescence was measured by a Varioskan Flash luminometer (Thermo Fisher Scientific) every min for 3 h. To calculate neutrophil killing of *C. rodentium*, 500 µL of neutrophils and bacteria (MOI 1) were incubated at 37°C for 30 min or 60 min and gently shaken every 10 min to prevent the neutrophils from settling. Once harvested, samples were treated with 0.1% of Triton X-100 for 5 min to lyse neutrophils before doing a cell spin and being serially diluted and plated in triplicate on LB agar plates.

FITC-dextran. Naïve mice or mice 3 dpi with *C. rodentium* were fasted 4 h before and after being gavaged with 150 µL of the permeability tracer 4-kDa FITC-dextran (Sigma-Aldrich) resuspended in PBS at a concentration of 80 mg/mL. 4 h post treatment, blood was collected via the trail vein and FITC-dextran was quantified using the Varioskan Flash luminometer at excitation wavelength of 494nm and emission wavelength of 517nm for 0.1 secs.

RT-qPCR. Mice were infected with C. rodentium (ICC180) for 4 days prior to sacrifice. Snips of the most distal colon were harvested into mRNA lysis buffer and homogenized. RNA was isolated from the tissue using an EZ-RNA extraction kit (Omega Bio-Tek, Norcross, GA) and was converted to cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA). The relative expression levels of *il23a* (Q9EQ14), *il17a* (Q62386), *il22* (Q9JJY9), *il6* (P08505), *cxcl1* (P12850), *reg3β* (P35230), and *reg3γ* (O09049) (Thermo Fisher Scientific) were calculated based on $2^{\Delta CT}$ values, whereby ΔC_T was calculated as C_T of an actin endogenous control (Applied Biosystems, Grand Island, NY) - C_T of gene of interest.

Statistics. *P* values were determined by unpaired two-tailed Student's t-test analysis or by One-way ANOVA with Tukey's post-hoc analysis using Prism software. Statistical significance is considered $p \leq 0.05$.

RESULTS

Plac8 limits *Citrobacter rodentium* burdens

C. rodentium colonizes the mouse intestinal mucosa through the formation of A/E lesions that anchors the bacteria to the apical surface of intestinal epithelial cells [120]. Shortly after infection, neutrophils are recruited to the site by chemokines like CXCL1 [134], where they can phagocytose bacteria and secrete IL-22 to increase the integrity of the gut epithelium and prevent dissemination of *C. rodentium* [128]. We hypothesize that the cysteine-rich protein, Placenta-specific 8 (Plac8) may serve as a regulator of the innate immune response against *C. rodentium* infection because it is known to promote neutrophil intracellular killing of other bacterial species such as *Klebsiella pneumoniae* and *E. coli* [49], it promotes the production of CXCL1 [62], and it is highly expressed on the apical surface of intestinal epithelial cells where *C. rodentium* initiates infection [232]. To evaluate the impact of Plac8 on *C. rodentium* control, WT and *Plac8*$^{-/-}$ mice were infected with 2-5 x 10^9 of luminescent *C. rodentium* ICC180. On 3, 5, 7, 10, 12, and 14 dpi, mice were imaged using an IVIS imager and bacterial burdens were compared based on the radiance of intensity (ROI) values in WT versus *Plac8*$^{-/-}$ mice (**Fig. 3.1A**). Here, it was determined that Plac8 significantly limits *C. rodentium* burden over the course of infection, but it has little to no effect on *C. rodentium* burdens in the fecal pellets (**Fig. 3.1B&C**). Because *C. rodentium* present in the fecal pellets is a representation of the bacterial burden present in the intestinal lumen and we only observed significant differences between the ROIs of the WT and *Plac8*$^{-/-}$ mice, we hypothesized that the *Plac8*$^{-/-}$ mice could have increased intestinal permeability and bacterial dissemination compared to WT mice.

Plac8-deficiency leads to increased disruption of the intestinal epithelial cell barrier during *C. rodentium* infection

As *C. rodentium* infection progresses, bacteria damage the intestinal epithelial cell barrier as they penetrate into the lamina propria [233], ultimately contributing to increased gut permeability. Because we observed an increased bacterial burden in *Plac8*$^{-/-}$ mice relative to WT mice based on ROI that was not detectable in fecal pellets, we investigated whether there was evidence of disruption of the intestinal epithelial cell barrier in the *Plac8*$^{-/-}$ mice. WT and *Plac8*$^{-/-}$ mice were gavaged with 4-kDa FITC-dextran before infection and 3 dpi. In this assay, FITC-dextran traverses the intestinal epithelial barrier and can be quantified in the sera of the mice as a measure of disruption to intestinal barrier function. There were no differences in FITC-dextran levels in the sera of naïve WT and *Plac8*$^{-/-}$ mice, which suggests that Plac8 does not contribute to the basal integrity of the intestinal barrier (**Fig. 3.2**). However, 3 days following *C. rodentium* infection, there were significantly higher concentrations of FITC-dextran circulating in the sera of *Plac8*$^{-/-}$ mice relative to the WT mice (**Fig. 3.2**). To assess differences in bacterial dissemination, spleens and livers were harvested 8 and 11 dpi, and bacterial burdens were assessed by plating as described by Acuff *et al*. [234]. However, neither WT nor *Plac8*$^{-/-}$ mice had any detectable *C. rodentium* in these tissues at these time points (data not shown). Overall, these data show that Plac8-deficiency causes increased permeability of the intestinal epithelial cell barrier after *C. rodentium* infection, but it is insufficient to result in dissemination of *C. rodentium* to distal organs.

Plac8-deficency does not affect neutrophil-mediated killing or control of *C. rodentium ex vivo*

In an attempt to determine why there were increased bacterial burdens in *Plac8*$^{-/-}$ mice, we evaluated the impact of Plac8 deficiency on neutrophil-mediated killing and control of *C.*

rodentium because (1) Plac8 is known to contribute to neutrophil-mediated killing of other bacterial species [49] and (2) neutrophil killing is essential for protection against *C. rodentium* [134]. To test this, neutrophils were isolated from the bone marrow of WT and *Plac8*$^{-/-}$ mice and co-cultured with *C. rodentium* for 30 and 60 min. After incubating the WT or *Plac8*$^{-/-}$ neutrophils with *C. rodentium*, neutrophils were lysed before plating the remaining bacteria and calculating the CFUs of *C. rodentium* for each condition. As expected, the presence of WT neutrophils significantly reduced the CFUs of *C. rodentium* relative to cultures with bacteria alone; however, no differences were seen in cultures that were incubated with either WT or *Plac8*$^{-/-}$ neutrophils, suggesting that Plac8 does not contribute to neutrophil-mediated killing of *C. rodentium* (**Fig. 3.3A&B**). Although neutrophils are primarily known for their ability to directly kill bacteria, they also produce bacteriostatic agents such as neutrophil gelatinase-associated lipocalin which can limit bacterial growth [235]. To determine if Plac8 contributed to neutrophil bacteriostatic effects during *C. rodentium* infection, we co-cultured WT or *Plac8*$^{-/-}$ neutrophils with *C. rodentium* and measured the overall luminescence of the sample for 3 h. Again, we observed that WT neutrophils limited the growth of bacteria relative to bacteria alone, but there were no differences in *C. rodentium* growth in conditions containing either WT or *Plac8*$^{-/-}$ neutrophils (**Fig. 3.3C**). These unexpected results suggest that the increased *C. rodentium* burdens are caused by a defect other than neutrophil-mediated control and killing of the bacteria.

Colonic CXCL1 levels are downregulated in the absence of Plac8 during *C. rodentium* infection

In attempt to determine if the enhanced disruption of the intestinal epithelial cell barrier found in *Plac8*$^{-/-}$ mice relative to WT mice was caused by a defect in innate immune cell function, colon samples were harvested from WT and *Plac8*$^{-/-}$ mice 4 dpi for RT-qPCR. Samples were

evaluated for different cytokines, chemokines, and anti-microbial compounds that are known to mediate protection against early *C. rodentium* infection. Reg3β or Reg3γ are critical anti-microbial compounds produced by intestinal epithelial cells. However, there were no differences in the expression of Reg3β or Reg3γ in the colons of WT and *Plac8*$^{-/-}$ mice, suggesting that Plac8 does not contribute to the production of these anti-microbial products (**Fig. 3.4**). Colons from mice were also evaluated for levels of IL-22, a cytokine that is critical for maintaining the integrity of intestinal epithelial cells during *C. rodentium* infection [127]. Likewise, no differences in IL-22 mRNA expression between the WT and *Plac8*$^{-/-}$ mice were observed (**Fig. 3.4**). On the contrary, colons from *Plac8*$^{-/-}$ mice displayed a significant reduction in mRNA for CXCL1, a chemokine essential for neutrophil recruitment during *C. rodentium* infection [134] (**Fig. 3.4**). Therefore, it is likely that Plac8 limits *C. rodentium* burden by promoting CXCL1 secretion from intestinal epithelial cells to increase neutrophil recruitment to the site of infection.

DISCUSSION

In this study, we determined that Plac8 limits *C. rodentium* bacterial burden and maintains optimal integrity of the intestinal epithelial cells during *C. rodentium* infection. Plac8-deficency does not alter neutrophil-mediated killing or control of *C. rodentium*, but *Plac8*$^{-/-}$ mice infected with *C. rodentium* did have lower levels of colonic CXCL1 expression suggesting that neutrophil recruitment is impaired during *C. rodentium* infection in these mice.

Initially it was surprising that the absence of Plac8 did not affect neutrophil-mediated killing of *C. rodentium*, especially given that Plac8 had previously been demonstrated to promote neutrophil killing of numerous other bacterial species including: *Klebsiella pneuomiae*, *Escherichia coli*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa* [49]. However, it is important to note the neutrophil killing assays conducted by Ledford *et al.* were primarily done *in vivo*, where neutrophils were recruited into the peritoneal cavity in response to thioglycolate or other inflammatory stimuli, whereas the neutrophils utilized in our *in vitro* studies were directly isolated from the bone marrow. Although thioglycolate is the gold standard assay for assessing neutrophil recruitment to the peritoneal cavity, neutrophils only represent 60-90% of the peritoneal lavage after treatment and these neutrophils exhibit an enhanced activated phenotype [236, 237]. Because Plac8 is highly concentrated in neutrophil granules [49] and neutrophil degranulation increases upon activation [238], it is possible that Plac8 could have a differential impact on neutrophils depending on their activation level. However, because neutrophils quickly reach high activation status after being co-cultured with bacteria from the *Enterobacteriaceae* family [239], it seems unlikely that initial differences in neutrophil activation would account for the different results noted from our experiments versus those conducted by Ledford *et al*.

It is likely that the increased *C. rodentium* bacterial burden present in *Plac8*$^{-/-}$ mice is the result of impaired neutrophil recruitment caused by decreased production of the chemokine CXCL1 in the *Plac8*$^{-/-}$ mice. Previous studies have determined that Plac8 is important for regulating myeloid cell recruitment during a contact hypersensitivity response by promoting IL-6 and CXCL1 6 h post oxazolone challenge [62]. In addition, Plac8 has previously been associated with promoting IL-1β production by monocytes isolated from patients with adult Still's disease [240], and monocyte production of IL-1 is critical for driving CXCL1 production and neutrophil recruitment during *C. rodentium* infection [230]. Known sources of CXCL1 during *C. rodentium* infection are monocytes, macrophages, and intestinal epithelial cells [241, 242], and Plac8 is known to be expressed by all of these cell types [49, 243]; however, the impact of Plac8 on the function of these immune cells has not been determined. Therefore, further experimentation will be required to identify the source of CXCL1 dysregulation by Plac8 in this model. Given the very high levels of Plac8 expression within intestinal epithelial cells that are known to secrete CXCL1, our current hypothesis is that Plac8 functions within epithelial cells to promote CXCL1 secretion, and ultimately, neutrophil recruitment to the gastrointestinal tract.

In addition to CXCL1 secretion, intestinal epithelial play a crucial role in limiting early *C. rodentium* burdens in part by producing anti-microbial molecules Reg3β and Reg3γ [126, 226]. Although there was increased disruption of the intestinal epithelial barrier following *C. rodentium* infection, there were no differences in the expression of Reg3β or Reg3γ in *Plac8*$^{-/-}$ mice relative to WT mice, which suggests that Plac8 does not regulate these protective anti-microbial functions in intestinal epithelial cells. However, there are other protective functions conducted by intestinal epithelial cells that are equally important for limiting *C. rodentium* burdens to which Plac8 could contribute. Plac8 expression in intestinal epithelial cells is localized to the apical surface [232]

where *C. rodentium* comes into close contact with the cell during formation of attaching/effacing (A/E) lesions; therefore, it is possible that Plac8-deficiency could leave the intestinal epithelial cells more vulnerable to *C. rodentium* colonization. However, when comparing differences in the rate of *C. rodentium* colonization between WT and *Plac8*$^{-/-}$ mice, we have found that *Plac8*$^{-/-}$ mice are equally susceptible to *C. rodentium* colonization as their WT counterparts (data not shown), which suggests that Plac8's contributions to *C. rodentium* control occurs after successful colonization.

In addition, intestinal epithelial cells are known for their high rates of self-renewal [202] which is important for limiting *C. rodentium* burdens by shedding off epithelial cells to which *C. rodentium* has attached [233]. Silencing Plac8 in colonic epithelial tissue has been associated with decreased cellular proliferation [59], therefore, it is possible that part of the increased *C. rodentium* burdens present in *Plac8*$^{-/-}$ mice could be a result of impaired epithelial cell proliferation and renewal. It may also be possible that the increased gut permeability observed in the *Plac8*$^{-/-}$ mice could be caused an impaired ability of intestinal epithelial cells to proliferate and replace ones that have shed into the lumen. To test differences in the proliferation of colonic epithelial cells *in vivo*, one should infect WT and *Plac8*$^{-/-}$ mice with *C. rodentium*, isolate the intestinal epithelial cells as outlined by Gracz *et al* [244] and stain the epithelial cells for a proliferation marker such as Ki67 or BrdU. It would also be beneficial to take colonic histology sections to determine differences in the thickness and the integrity of infected colons from WT and *Plac8*$^{-/-}$ mice.

Neutrophils can promote integrity of gut epithelial cells during *C. rodentium* infection by secreting IL-22 [128], so we initially hypothesized that a decrease in neutrophil-derived IL-22 could contribute to the increased gut permeability in the *Plac8*$^{-/-}$ mice. However, we saw no difference in colonic accumulation of IL-22 between infected WT and *Plac8*$^{-/-}$ mice, and many

studies have shown that type 3 innate lymphoid cells (ILC3s) are the predominant source of IL-22 during the innate stages of *C. rodentium* infection [245, 246]. Therefore, it seems likely that any deficit in neutrophil-derived IL-22 in the *Plac8*$^{-/-}$ mice would be compensated for by other innate cell types, like ILC3s. Instead, we believe that having fewer neutrophils at the site to phagocytose bacteria is causing the increased bacterial burdens in the *Plac8*$^{-/-}$ mice. Neutrophils are known to play a critical role in controlling early *C. rodentium* burdens through phagocytosis [134, 231], thus the increased gut permeability observed in *Plac8*$^{-/-}$ mice may be the result of increased bacterial damage to the intestinal epithelial cells and not by the lack of effector cytokines necessary for maintaining the gut epithelial cell barrier.

Neutrophil-mediated phagocytosis is critical for early *C. rodentium* control, but neutrophils are not the only phagocytes required for host protection against enteropathogenic infections that are associated with Plac8 expression. CX3CR1^{+} macrophages [141], macrophages derived from CCR2^{+} monocytes [142], and CD11b^{+}F4/80^{+}Gr1^{+} inflammatory monocytes [143] also contribute to early *C. rodentium* control, and high Plac8 expression has been documented in both macrophages and monocytes [49, 243]. Therefore, it is possible that the increased *C. rodentium* burdens observed in *Plac8*$^{-/-}$ mice could also be compounded by functional deficiencies in other innate cell types.

Although *Plac8*$^{-/-}$ mice had increased gut permeability after *C. rodentium* infection, there was no evidence of bacterial dissemination to livers or spleens of WT and *Plac8*$^{-/-}$ mice at 8 or 11 dpi. It is important to note that gut permeability assays were performed using FITC-dextran that was 4-kDa in size, which is much smaller than a bacterium. Therefore, it is possible that the breaches in the intestinal epithelial wall were not large enough to allow *C. rodentium* to disseminate into other tissues. Because WT and *Plac8*$^{-/-}$ mice have differences in *C. rodentium*

burdens without any evidence of increased burdens present in the lumen (fecal pellets) or other tissues, we hypothesize that the increased *C. rodentium* burdens present in the *Plac8*$^{-/-}$ mice measured by luminescence may represent an increased number of bacteria that have penetrated the epithelial barrier and traversed deeper into the lamina propria. This hypothesis is further supported by the observation that the localization of the luminescence present in the infected WT and *Plac8*$^{-/-}$ mice seems to be contained within the abdominal cavity, although this needs to be confirmed experimentally.

Altogether, our studies show that Plac8 is important for host protection against *C. rodentium* infection. Plac8-deficiency caused a decrease in the colonic accumulation of the neutrophil-specific chemokine, CXCL1 during *C. rodentium* infection which may cause impaired neutrophil recruitment in the *Plac8*$^{-/-}$ mice. However, it will be important to assess the total numbers of neutrophils present in the infected colons of WT and *Plac8*$^{-/-}$ mice before this can be verified. Considering that *C. rodentium* shares many similarities to the human pathogens EPEC and EHEC, it is possible that the role of Plac8 in driving early protection against *C. rodentium* could closely resemble human responses to infection against intestinal extracellular pathogens. Therefore, delineating the specific mechanism responsible for Plac8 induction during *C. rodentium* infection could illuminate a novel therapeutic pathway that can be utilized to quickly combat these pathogenic bacteria in humans.

ACKNOWLEDEMENTS

The authors would like to thank Rick Tarleton for allowing us to use the IVIS imager, Angel Padilla for training us on the IVIS imager, and UGA’s Coverdell Rodent Vivarium staff members for the animal care.

FIGURES

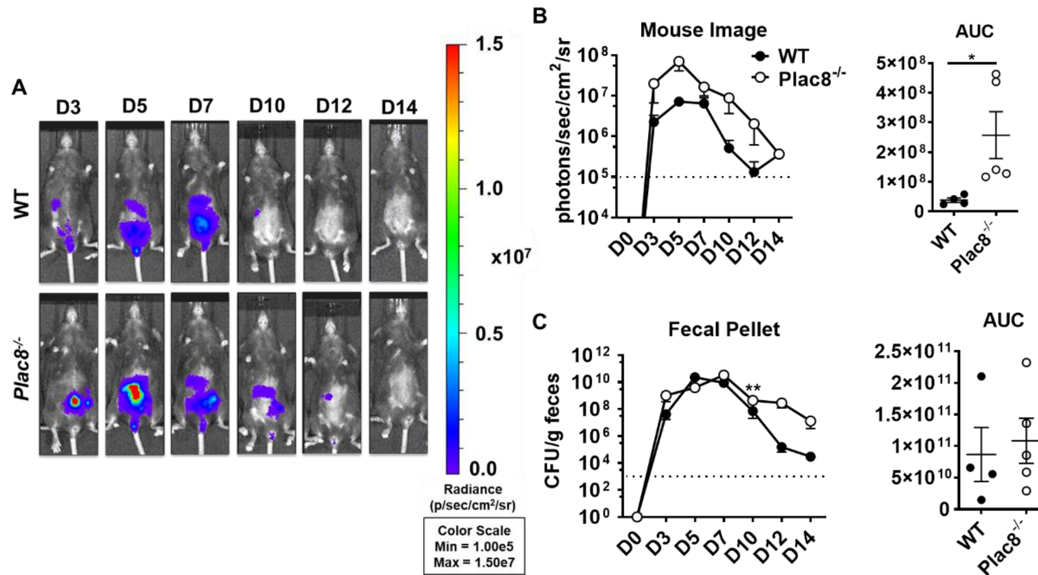


Figure 3.1. Plac8 limits Citrobacter rodentium burden.

WT and *Plac8*$^{-/-}$ mice were orally gavaged with 2 x 10^9 to 5 x 10^9 of luminescent *C. rodentium* ICC180. **A)** Mice were imaged in an IVIS imager 3, 5, 7, 10, 12, and 14 dpi. A representative mouse from the WT and *Plac8*$^{-/-}$ group are shown here. **B)** The average radiance of intensity was calculated for WT and *Plac8*$^{-/-}$ mice each day of IVIS imaging, to compare the burden of *C. rodentium* between the WT and *Plac8*$^{-/-}$ mice. **C)** Fecal pellets were collected at the time of imaging and were serially diluted and plated in triplicate on an LB agar plate. 12-16 h later, plates were imaged on the IVIS and luminescent colonies were counted to calculate the CFU of *C. rodentium* present in the fecal pellet. This experimental cohort contains 4 WT mice and 5 *Plac8*$^{-/-}$ mice and is representative of two independent cohorts. *P* values were determined by unpaired Student's *t* test. * ($P < 0.05$), ** ($P < 0.01$).

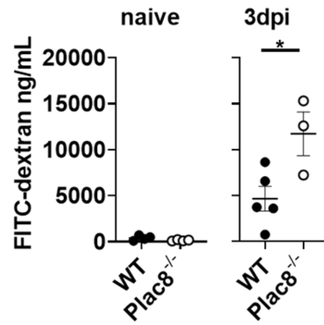


Figure 3.2. Gut integrity is significantly disrupted in the absence of Plac8 during C. rodentium infection.

A) WT and *Plac8*$^{-/-}$ mice were orally gavaged with FITC-dextran before (naïve) or 3dpi with *C. rodentium*. This experiment was conducted on one cohort. Naïve WT n = 4 and naïve *Plac8*$^{-/-}$ n = 4. 3dpi WT n = 5 and 3dpi *Plac8*$^{-/-}$ n = 3. *P* values were determined by unpaired Student's *t* test. * ($P < 0.05$). Grubbs' test was performed to omit one WT outlier.

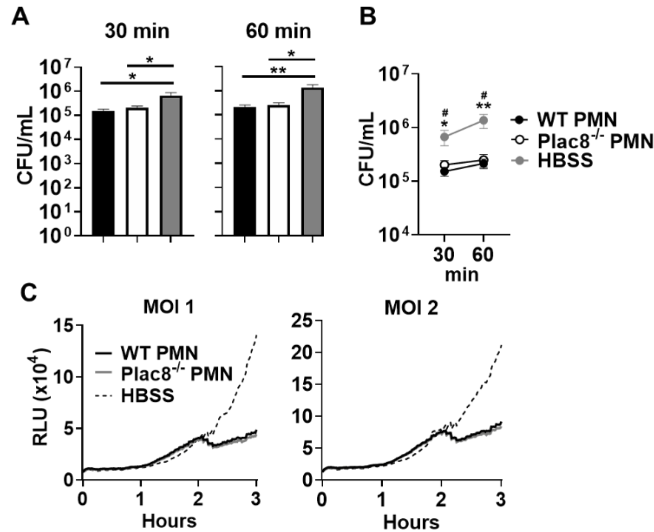


Figure 3.3. Plac8-deficiency does not affect neutrophil killing or control of C. rodentium.

Bone marrow cells were harvested from WT or *Plac8*$^{-/-}$ mice and enriched for neutrophils. **A)** 1 MOI of *C. rodentium* ICC180 was co-cultured with WT neutrophils (black bar), *Plac8*$^{-/-}$ neutrophils (white bar), or media alone (HBSS, gray bar) for 30 min or 60 min. Neutrophils were lysed before samples were serially diluted and plated on LB agar plates. 12-16 h later, colonies were counted to calculate the CFU. **B)** Kinetic representation from the experiment conducted in A. (*) represents significance relative to samples with WT neutrophils and (#) represents significance relative to samples with *Plac8*$^{-/-}$ neutrophils. **C)** 1 MOI or 2 MOIs of *C. rodentium* ICC180 was co-cultured with WT neutrophils, *Plac8*$^{-/-}$ neutrophils, or media alone (HBSS) for 3 h on a Varioskan Flash luminometer. Each minute, a value was calculated to determine the relative luminescent unit (RLU) present within each experimental condition. A and B are results from 2 independent experiments pooled together (n=6) and C is a representative cohort from an experiment conducted on 4 independent cohorts. *P* values were calculated by one-way ANOVA and Tukey's post hoc analysis. * or # ($P < 0.05$), ** ($P < 0.01$).

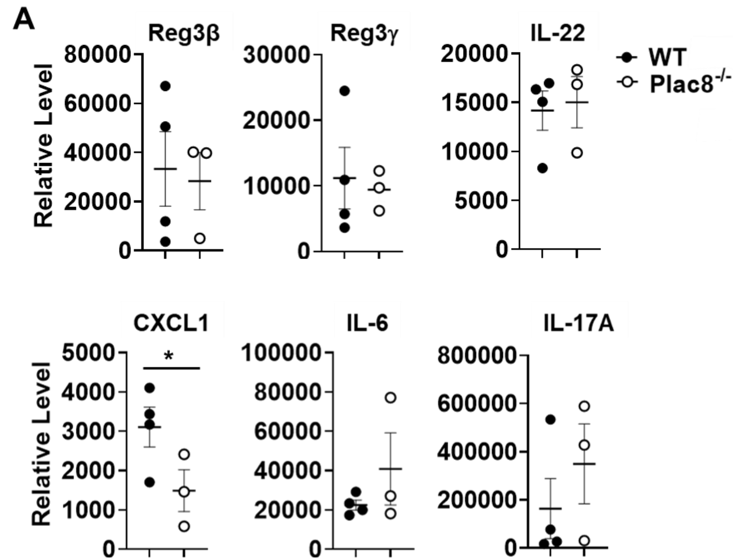


Figure 3.4. CXCL1 production is decreased in the absence of Plac8 during C. rodentium infection.

A) WT and *Plac8*$^{-/-}$ mice were infected with *C. rodentium* and 4 dpi, snips of the most distal colon were collected in mRNA lysis buffer for RT-qPCR. Samples were examined for the presence of Reg3β, Reg3γ, IL-22, CXCL1, IL-6, and IL-17A. ΔC_T values for WT and *Plac8*$^{-/-}$ colon samples were determined using an actin endogenous control. Relative expression values are graphed as $2^{-\Delta CT}$. This data was conducted with one cohort. WT, n = 4 and *Plac8*$^{-/-}$, n = 3. *P* values were determined by a one-tailed, unpaired Student's *t* test. The null hypothesis was that Plac8 ablation would decrease expression of innate immune effector molecules during *C. rodentium* infection. Grubbs' test was performed to omit one WT outlier.

CHAPTER 4

CONCLUSIONS

Plac8 expression has been associated with Th1 CD4 T cells [51, 52] and has been shown to be expressed during Th1 immune responses to viruses like HBV and Ebola [53, 54]; however, the specific role of Plac8 in Th1-driven inflammation has not been determined. Through our experiments, we found that Plac8 regulates Th1 immune cell effector functions and impacts disease outcome in a context-dependent manner. First, Plac8 significantly suppresses CD4 T cell IFNγ production following IL-12 stimulation *in vitro* (**Fig.2.2**). Second, Plac8 promoted establishment of influenza-specific effector CD8 T cells through a T cell-intrinsic manner (**Fig.2.5&2.6**). And lastly, Plac8 ablation led to increased bacterial burdens during *C. rodentium* infection, which coincided with decreased colonic CXCL1 levels (**Fig.3.1&3.4**). The proposed mechanism for each of these results are presented in model figures (**Fig.4.1-4.3**) and are discussed in detail below.

Impact of Plac8 on Th1 CD4 T cells

IFNs play a major role in the pathogenesis of many autoimmune diseases [247]. In fact, many of the genes induced by type I and type II IFNs are redundant, and it has thus proven difficult to determine which IFNs are the major contributors to disease progression [248-251]. However, it is known that IFNγ signaling specifically can lead to increased inflammation by promoting type I IFN signaling [252], promoting NF-κB activation [253], and blocking anti-inflammatory feedback mechanisms like IL-10 production [254]. In addition, IFNγ signaling can inhibit cell growth [255], promote cell death [256], and potentiate stress in a variety of different tissues [257-

259]. Therefore, determining how to modulate IFNγ expression could have a tremendous therapeutic impact in a variety of disease settings.

Through the studies presented in this dissertation, we demonstrate that Plac8 expression is highly associated with Th1 CD4 T cells (**Fig.2.1**) and that Plac8 significantly suppresses IFNγ production by CD4 T cells 24h following IL-12 stimulation *in vitro* (**Fig.2.2**); however, the contribution of Plac8 toward IFNγ suppression was not observed by pathogenic CD4 T cells during a T cell transfer model of colitis (**Fig.2.3**) or during *C. rodentium* infection (**Fig.S2.2**). There are a couple of potential explanations for this. It is important to note that the T cell transfer model of colitis and *C. rodentium* mouse models are known to induce a mixed Th1/Th17 cell immune response, and there is increasing evidence that the Th17-associated cytokine, IL-23, is more important in driving inflammation in these models than IL-12 (as reviewed by Tang *et al*. [260]). Therefore, alterations in IL-12-dependent responses may not be prominent in this model. Furthermore, IL-23 has suppressive effects on IL-12 and IL-12-dependent IFNγ production in these models of colitis [261-263]. Therefore, it is possible that the immunosuppressive effect of Plac8 on CD4 T cell IFNγ production observed *in vitro* could be minimized by the IL-23-rich environment of this model.

In our proposed model (**Fig.4.1**), we hypothesize that Plac8 is indirectly induced following IL-12 signaling by C/EBPα as there is a putative binding site for C/EBPα within 1kb of Plac8's promoter sequence, and C/EBPα activation is known to cause immunosuppression [264]. Specifically, there is evidence that C/EBPα activation can lead to suppression of IFNγ production by CD4 T cells through a T cell-intrinsic manner [220]. We believe that Plac8 is induced by C/EBPα after which it transcriptionally suppresses IFNγ production perhaps in cooperation with C/EBPα. This hypothesis is supported by our observation that *Plac8*$^{-/-}$ CD4 T cells express

significantly less IFNγ mRNA following IL-12 stimulation relative to WT CD4 T cells (**Fig.2.2**), and Plac8 has previously been documented as a transcription factor in adipocytes [221].

Impact of Plac8 on CD8 T cells during influenza virus infection

In contrast to its effect in CD4 T cells, Plac8 did not have a significant impact on IFNγ production by CD8 T cells (**Fig.2.2**). Instead, Plac8 promoted the establishment of influenza-specific effector CD8 T cells through a T cell-intrinsic manner (**Fig.2.5&2.6**). During influenza infection, effector CD8 T cells play a critical role in viral clearance [265]; however, effector CD8 T cells can also elicit the highest levels of immunopathology during influenza infection [266]. Therefore, it is important that this response is tightly regulated to avoid excessive host damage. Given that there is a significant reduction in effector CD8 T cell programing when Plac8 is specifically ablated from this cell population, our results suggest that it may be possible to regulate the effector CD8 T cell response through manipulating a Plac8-associated pathway within CD8 T cells. However, before we can consider utilizing this pathway to regulate the effector CD8 T cell response, it will be important to determine how Plac8 contributes to increased effector CD8 T cell programing. We determined that Plac8 did not impact effector CD8 T cell proliferation after observing no differences in NP-specific BrdU incorporation or CD25 expression between WT and *Plac8*$^{-/-}$ CD8 T cells (**Fig.2.5**). Instead, we hypothesize that Plac8 could be contributing to increased effector CD8 T cell numbers by promoting CD8 T cell survival. In this regard, Plac8 is known to promote the survival of other cell types by limiting apoptosis [61, 222].

For our effector CD8 T cell model (**Fig.4.2**), we propose that Plac8 is induced in CD8 T cells during influenza infection via IL-12 signaling because we observed that Plac8 upregulation in CD8 T cells following IL-12 signaling *in vitro* (**Fig.2.1**) and it is known that IL-12 is upregulated during influenza infection [267]. It is possible that IL-12 signaling may indirectly induce Plac8 in

CD8 T cells like what we observed in CD4 T cells (**Fig.2.1**), but this remains to be determined. After *Plac8*$^{+/+}$ CD8 T cells have an increased effector CD8 T cell programing relative to their *Plac8*$^{-/-}$ counterparts. We have determined that Plac8 is not regulating effector CD8 T cell numbers through proliferation, and instead propose that Plac8 could be important for promoting cell survival by inhibiting apoptosis.

It is well appreciated that memory CD8 T cells provide protection against subsequent heterologous influenza viral infections [268]. We observed that *Plac8*$^{-/-}$ mice have a significant reduction in memory CD8 T cell numbers 35dpi (**Fig.2.4**) relative to their WT counterparts. These results suggest that manipulation of Plac8 may also be used to modify the establishment and/or survival of influenza-specific memory CD8 T cells, which might enhance CD8 T cell-mediated protection. However, like the effector CD8 T cell data, it will be important to understand the mechanism by which Plac8 regulates memory CD8 T cell numbers in this model before it can be seriously considered as a therapeutic candidate. Unfortunately, interpreting the memory CD8 T cell data from the complete WT vs. *Plac8*$^{-/-}$ influenza mouse experiments is challenging because we first need to understand how Plac8 contributes to the function of other immune cells involved in combating influenza virus infection. Plac8 is expressed by lung epithelial cells and macrophages which play a critical role in protection against influenza infection by initiating inflammation and combating the virus before the adaptive immune response is activated [49]. However, the contribution of Plac8 to the function of these cell types during influenza infection has not been determined. Although more research needs to be conducted to understand the role of Plac8 during the influenza-specific CD8 T cell response, for the first time, these data suggest that Plac8 may be associated with a pathway that promotes the CD8 T cell response after influenza infection.

Impact of Plac8 on *Citrobacter rodentium* infection

During the course of our studies, we also determined that Plac8 significantly reduces *C. rodentium* bacterial burden as early as 3 dpi (**Fig.3.1**), prior to the initiation of an adaptive T cell response. Furthermore, Plac8 ablation was associated with lower levels of the neutrophil chemokine CXCL1 levels in the colon 4 dpi (**Fig.3.4**). Because Plac8 ablation had no apparent effect on neutrophil-mediated killing or control against *C. rodentium in vitro* (**Fig.3.3**), it is likely that the increased bacterial burdens present in *Plac8*$^{-/-}$ mice result from decreased neutrophil recruitment rather than impaired function *per se* (as depicted in **Fig.4.3**). In addition, we propose that the increased gut permeability observed in the *Plac8*$^{-/-}$ mice following *C. rodentium* infection (**Fig.3.2**) is the result of increased bacterial-damage in these mice. Altogether, this is the first time Plac8 has been associated with a pathway that may regulate CXCL1 levels and thus neutrophil recruitment during an infectious disease model.

Neutrophils are key in mediating the killing of intracellular bacteria, like *C. rodentium*, but also have a significant role in regulating the inflammatory response to various infections [269, 270]. For example, during *C. rodentium* neutrophils play a critical role in limiting epithelial cell damage by secreting IL-22 [128], neutrophils have been shown to secrete IL-12 and IL-10 during *Staphylococcus aureus* infection [271], neutrophils can promote longevity of macrophages that lead to increased helminth control [272], and neutrophils are have even been shown to directly inhibit T cell responses [273]. Therefore, understanding more about Plac8 and the pathway regulating CXCL1 production could provide more details to undercover the complexities involved for early protection against enteropathogenic infections.

We hypothesize that Plac8 could be regulating CXCL1 production in multiple ways. First, Plac8 has been characterized as a transcription factor in adipocytes [221], and it is possible that

Plac8 could be directly regulating CXCL1 production on a transcriptional level. Additionally, Plac8 has been shown to localize to neutrophilic granules [49] and by yeast two hybrid screening to interact with phospholipid scramblase 1 (PLSCR1) [274], an enzyme implicated in transmembrane bilayer migration of lipids, a process which is integral to vesicular trafficking [49, 58]. Therefore, we suspect that Plac8 may also regulate the packaging and export of CXCL1 protein from these cell types. While less evidence exists to support the latter hypothesis, it would be beneficial to determine if (1) there are differences in CXCL1 *protein* levels in the colons of WT and *Plac8*$^{-/-}$ mice, (2) if WT and *Plac8*$^{-/-}$ epithelial cells and monocytes/macrophages secrete similar levels of CXCL1 following IL-1 stimulation and (3) if there is a difference in CXCL1 intracellular accumulation in WT versus *Plac8*$^{-/-}$ epithelial cells and monocytes/macrophages following IL-1 stimulation.

In summary, while the experiments within this dissertation have provided a foundation for the implications of Plac8 in regulating inflammatory responses, additional studies need to be conducted before we can consider utilizing Plac8 therapeutically. Chapter 5 provides more information about which future experiments should be prioritized and why they are important in increasing our knowledge of Plac8 in these disease models.

FIGURES

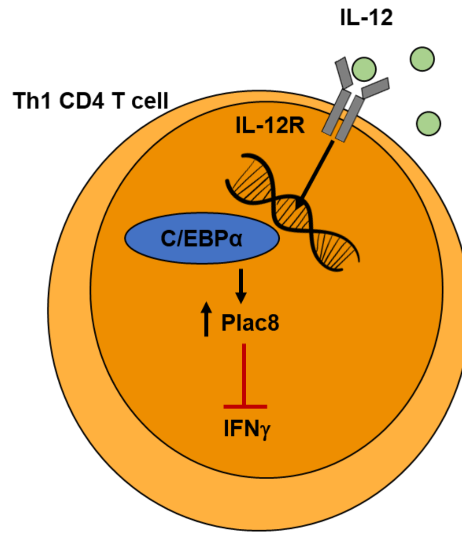


Figure 4.1: Proposed mechanism of Plac8 in Th1 CD4 T cells

Plac8 is *indirectly* induced following IL-12 stimulation in CD4 T cells (**Fig.2.1**). In this model, we propose that transcription factor C/EBPα could be the intermediate protein that induces Plac8 expression, because there is a putative binding site for C/EBPα within 1kb of Plac8's promoter sequence, and C/EBPα has a known role in limiting IFNγ production by Th1 CD4 T cells [220]. After Plac8 is induced, it suppresses IFNγ production by Th1 CD4 T cells by limiting IFNγ transcription (**Fig.2.2**). Because Plac8 induction negatively impacts IFNγ transcript levels and Plac8 is a suggested transcriptional regulator in adipocytes [221], we hypothesize that Plac8 could be operating as a transcriptional regulator in Th1 CD4 T cells to suppress Th1 CD4 T cell IFNγ production.

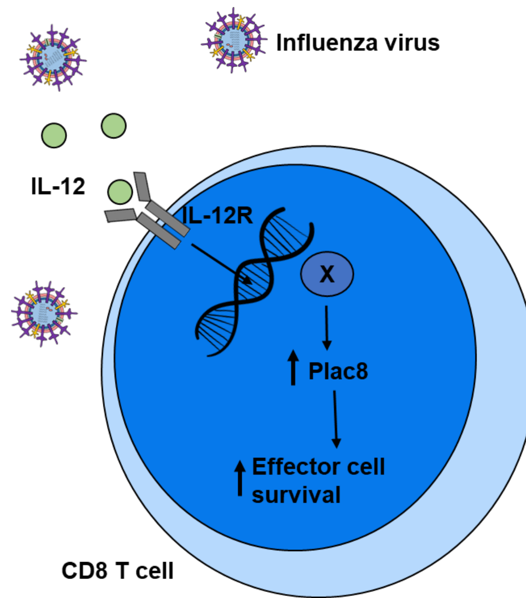


Figure 4.2: Proposed mechanism of Plac8 in CD8 T cells during influenza virus infection

Plac8 is induced following IL-12 stimulation in CD8 T cells *in vitro* (**Fig.2.1**). Because IL-12 is upregulated during influenza virus infection [267] and IL-12 signaling in CD8 T cells is critical for promoting anti-viral activity [275], we propose Plac8 could be induced by IL-12 signaling during influenza virus infection. It has not been determined if Plac8 is induced directly or indirectly by IL-12 signaling in CD8 T cells, but given that Plac8 was *indirectly* induced by IL-12 signaling in Th1 CD4 T cells (**Fig2.1**) and Th1 CD4 T cells have similar functional characteristics to CD8 T cells [1], we hypothesize that Plac8 is also induced indirectly in CD8 T cells, perhaps by C/EBPα. We observed that Plac8-ablation from influenza-specific CD8 T cells negatively impacted effector CD8 T cell programing in both a mixed chimera model and mixed OT-I adoptive transfer model of influenza (**Fig.2.5&2.6**). We determined that the differences in the effector CD8 T cell programing was not the result of an impaired ability of *Plac8*$^{-/-}$ CD8 T cells to proliferate (**Fig.2.5**). Instead, we hypothesize that Plac8 may promote effector cell survival through inhibiting apoptosis because Plac8 is known to limit apoptotic induction in other cell types [61, 222].

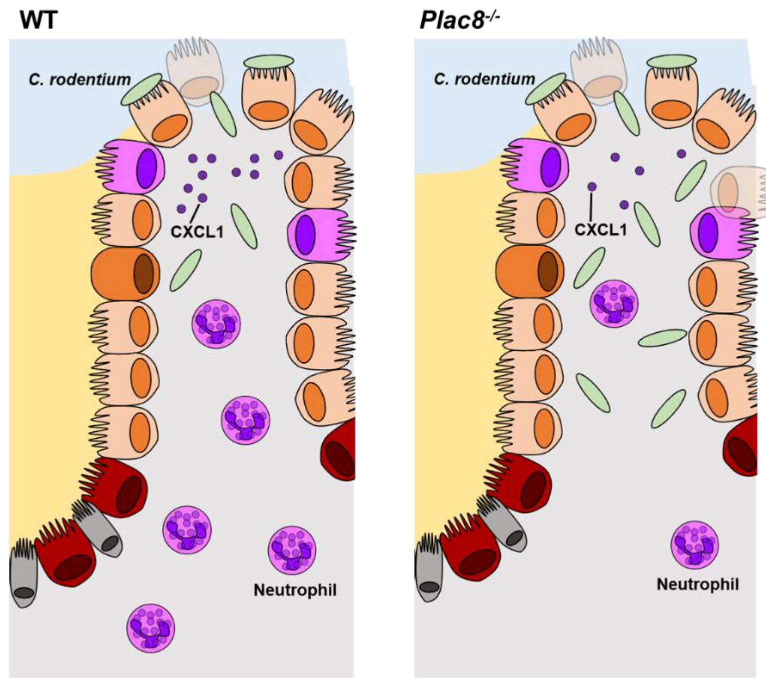


Figure 4.3: Proposed impact of Plac8 ablation during Citrobacter rodentium infection.

After *C. rodentium* infection, *Plac8*$^{-/-}$ mice have increased bacterial burdens and increased gut permeability 3dpi relative to their WT counterparts (**Fig.3.1&3.2**). Because Plac8 is known to promote neutrophil-mediated killing of other bacterial species [49], and neutrophils are critical in controlling early *C. rodentium* infection [134, 231], we initially hypothesized that the increased *C. rodentium* burdens present in the *Plac8*$^{-/-}$ mice could be the result of impaired neutrophil function. However, Plac8 ablation had no apparent impact on neutrophil-mediated killing of *C. rodentium ex vivo* (**Fig.3.3**). Instead, we observed that there were decreased levels of the neutrophil chemokine, CXCL1, present in the colons of *Plac8*$^{-/-}$ mice 4dpi relative to WT mice (**Fig.3.4**). Interestingly, Plac8 is highly expressed within intestinal epithelial cells [49, 232] which are primary sources of CXCL1 production during *C. rodentium* infection [230]. Therefore, we propose that the increased bacterial burdens present in the *Plac8*$^{-/-}$ mice is the result of decreased CXCL1 production by epithelial cells and thus impaired neutrophil recruitment to the site of infection.

CHAPTER 5

FUTURE DIRECTIONS

For this chapter, we will first discuss what we have learned about the contribution of Plac8 in regulating inflammation. Then, we will provide some of the limitations given the lack of Plac8-specific reagents. And finally, we will propose which experiments should be conducted next to expand upon the observations that were made in the research chapters.

In Chapter 2, we determined that Plac8 was important for suppressing IFNγ production by CD4 T cells following IL-12 stimulation (**Fig.2.2**) and we hypothesize that this pathway is likely regulated by transcription factor C/EBPα because C/EBPα is known to limit IFNγ production by CD4 T cells [220] and there is a putative binding site for C/EBPα ~1kb upstream of Plac8's promoter sequence. Determining the transcription factor responsible for inducing Plac8 expression will be a key part in determining how Plac8 is functioning in different cell populations and inflammatory environments. To date, the contribution of C/EBPα to the anti-viral effector CD8 T cell response has not been determined, but C/EBPα is known to promote epithelial cell regeneration [276]. Considering that *Plac8*$^{-/-}$ mice have increased gut permeability during *C. rodentium* infection (**Fig.3.2**) and epithelial cell regeneration is critical for replenishing infected intestinal epithelial cells and maintaining gut integrity during *C. rodentium* infection [233], it is possible that Plac8 could be activated downstream of C/EBPα in epithelial cells as well. There is evidence that C/EBPα plays an immunosuppressive role during inflammatory responses [264], but the specific contribution of C/EBPα to these responses still needs to be determined. To determine if C/EBPα is one of the factors that indirectly regulates Plac8 expression, it would be advantageous

to compare relative levels of Plac8 mRNA expression between WT Th1 CD4 T cells and *C/EBPα*$^{-/-}$ Th1 CD4 T cells, since Th1 cells are where the highest levels of Plac8 mRNA were observed.

Although *Plac8*$^{-/-}$ CD4 T cells had a significant increase in IFNγ mRNA and protein following IL-12 stimulation *in vitro* (**Fig.2.2**), it is important to note that the overall difference in IFNγ mRNA and IFNγ protein production was relatively modest. IL-12 stimulated *Plac8*$^{-/-}$ CD4 T cells showed a 2-3-fold increase in IFNγ mRNA and IFNγ protein production relative to WT CD4 T cells. This was surprising considering that relative Plac8 mRNA expression in Th1 CD4 T cells was several thousand-fold higher in Th1 CD4 T cells compared to the other CD4 T cell subsets. When interpreting this data, it is important to note that mRNA expression levels do not always translate into active protein expression [277, 278]. Therefore, it is possible that not all of the Plac8 mRNA observed in the Th1 CD4 T cells is actively translated into Plac8 protein. The extremely high Plac8 mRNA expression observed within Th1 cells may have significantly over-estimated the potential significance of Plac8 within these cells. In addition to the modest differences observed in IFNγ expression upon Plac8 ablation in these cells, this point is further supported by the very similar transcriptomes observed between WT and *Plac8*$^{-/-}$ Th1 CD4 T cells through RNA-sequencing (unpublished results).

One of the major obstacles in studying Plac8 functions has been the lack of a murine reactive Plac8 antibody. A Plac8 antibody would permit Plac8 protein quantitation by Western blot in different CD4 T cell subsets to determine if Plac8 protein is also highly expressed within the Th1 lineage and to quantitate how efficiently Plac8 mRNA is being translated into protein in these cells. Furthermore, a quality murine-specific Plac8 antibody would also be beneficial for 1) determining the subcellular localization of Plac8 and 2) identifying Plac8 interacting proteins through co-immunoprecipitation experiments. Both approaches could both provide new clues

about the enigmatic molecular function(s) of Plac8. For example, if we can identify Plac8 in the nucleus after stimulating T cells with IL-12, this would be strong evidence for our hypothesis that Plac8 may be acting as a transcriptional regulator for IFNγ. In addition, we could utilize the murine-reactive Plac8 antibody to perform chromatin immunoprecipitation to look for the IFNγ promoter or to determine if DNA in general is co-precipitated. We could also determine if PLSCR1 is one of Plac8's binding partners in epithelial cells like Li *et al*. demonstrated in yeast using a yeast two hybrid screen [274], and this could provide evidence that Plac8 may also regulate CXCL1 production through vesicular trafficking. Furthermore, a mouse reactive Plac8 antibody would also allow us to determine the kinetics of Plac8 protein accumulation in various cell types *in vivo* and would allow us to better understand which signals are important for Plac8 induction.

In Chapter 3, we hypothesize that the decrease in CXCL1 expression in *Plac8*$^{-/-}$ mice negatively impacts neutrophil recruitment and leaves the host more vulnerable to increased *C. rodentium* colonization. Therefore, it will be important to determine how many neutrophils are in the colons of WT vs. *Plac8*$^{-/-}$ mice 4 days post infection. It will also be important to determine the source of CXCL1 dysregulation during *C. rodentium*, given myeloid and epithelial cells are known to secrete CXCL1 [241, 242]. To separate the contribution of Plac8 to myeloid cell-derived CXCL1 versus epithelial cell-derived CXCL1, it would be advantageous to utilize a bone marrow chimera model whereby WT hosts are given *Plac8*$^{-/-}$ bone marrow and *Plac8*$^{-/-}$ hosts are given WT bone marrow. After generating these chimeric mice, mice should be infected with *C. rodentium,* and relative mRNA and protein levels of CXCL1 should be determined in the colon 4 days post infection. This data should also be correlated with bacterial burdens and neutrophil recruitment in the same mice to determine whether the source of CXCL1 dysregulation and impaired neutrophil recruitment are hematopoietic or non-hematopoietic in nature.

It is also possible that Plac8 could contribute to other functions in epithelial cells during *C. rodentium* infection outside of CXCL1 regulation. It is known that Plac8 is highly expressed with intestinal epithelial cells [49, 232], and silencing Plac8 in cancerous colonic epithelium results in decreased cellular proliferation [59]. However, the impact of Plac8 on healthy colonic epithelial cells has yet to be determined. During *C. rodentium* infection, proliferation rates of intestinal epithelial cells have a tremendous impact on disease outcome. To determine the contribution of Plac8 on the proliferation of colonic epithelial cells, one should infect WT and *Plac8*$^{-/-}$ mice with *C. rodentium*, isolate the intestinal epithelial cells for flow cytometry as conducted by Gracz *et al* [244], and utilize markers like Ki67 or BrdU to identify any differences in relative proliferation rates.

In summary, we have gathered evidence that IL-12 indirectly induces Plac8 mRNA expression in Th1 T cells and CD8 T cells, potentially via the actions of the transcription factor C/EBPα. This is supported by the identification of a putative binding site for C/EBPα within 1kb of Plac8's transcriptional start site and the observation that *Plac8*$^{-/-}$ Th1 CD4 T cells have increased IFNγ production (**Fig.2.2**), a phenotype shared with *C/EBPα*$^{-/-}$ Th1 CD4 T cells [220]. In addition, we hypothesize that Plac8 could be functioning as a transcription factor in T cells and epithelial cells. This is supported by the previous identification of Plac8 as a transcriptional regulator in adipocytes [221] and our finding that Plac8 ablation increased IFNγ mRNA levels in Th1 CD4 T cells and CD8 T cells (**Fig.2.2**) but decreased CXCL1 mRNA levels in the colons of C. *rodentium* infected mice (**Fig.3.4**). Ultimately, our studies emphasized the importance of developing a quality mouse-specific Plac8 antibody. Undertaking additional biochemical approaches, as noted above, to learn more about the subcellular localization and binding partners of Plac8 will be critical in discovering its enigmatic function(s). Overall, for the first time, these experiments provide

evidence that Plac8 may have a regulatory role during type 1 inflammatory responses, which upon further investigation, may allow us to harness this information to advance immunotherapies.

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