Defining the Helper T Cell Contribution to *Helicobacter pylori* Gastritis

by

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Abstract

Mouse models of *H. pylori* disease develop chronic gastritis due to an immune response dependent upon IFNγ and CD4+ T cells, but several reports demonstrate that IL-17A, a cytokine associated with mucosal inflammatory responses, is elevated in gastric punch biopsies of infected human patients. To investigate the role of IL-17A in *H. pylori* gastritis I used C57Bl/6 (B6), IL-17A knockout (17AKO), IFNγ knockout (IFNγKO), and CIITA transgenic (CIITA-Tg) mice in two distinct mouse models of disease. 17AKO mice developed less gastritis than B6 mice with greater bacterial colonization in simple infection experiments, but 17AKO and B6 T cells promoted equivalent gastritis and *H. pylori* colonization in adoptive transfer recipients, indicating that while IL-17A contributes to gastritis other inflammatory mechanisms may be at work in adoptive transfer. T cells from CIITA-Tg mice do not produce IL-17A but promote gastritis and cytokine expression similar to B6 T cells in recipient mice, but CIITA-Tg T cells recovered from transferred mice were hyporesponsive to *H. pylori* antigen stimulation, suggesting that gastritis progresses without antigen-specific Th1 or Th17 cells. Several innate inflammatory cytokines were induced by infection in all mice that developed disease, indicating that non-T cell effector populations contribute to disease development. To explore these cells I isolated leukocyte populations from gastric mucosa to analyze them via flow cytometry. Both the quantity of T cells and the ratio of regulatory to effector T cells in transfer experiments affected the quantities of lymphocytes, granulocytes and myeloid cells *in situ* as well as influencing gastritis

severity. Transfer of IFNγKO and 17AKO T cells significantly altered the populations of leukocytes but not disease severity, suggesting that gastritis in adoptive transfer requires T cells to develop disease without needing specific T cell cytokines. Together, these observations indicate that IL-17A and IFNγ both contribute to gastritis development in *H. pylori* infected mice and may be necessary for early promotion of gastritis, but disease may continue in an antigen-independent fashion late in infection. We have also clearly underestimated the importance of innate effector cells in mouse models of *H. pylori* gastritis, as these cells actively participate to promote disease development.

Chapter 1

Background

An introduction to *Helicobacter pylori* and disease

Helicobacter pylori is a spiral-shaped microaerophile that efficiently colonizes the gastric mucosa of the human stomach, an acidic environment with an average pH of around 5. The bacteria can even survive considerable travel through the gastric lumen, where the pH is often less than 2. It is currently estimated that *H. pylori* infects almost 75% of the world population (120), with up to 90% of the population in developing countries affected. Several studies suggest infection occurs most often vertically early in life, via parent-to-child transmission (175, 176). *H. pylori* infection appears to be life-long, barring chemotherapeutic interventions like antibiotic treatment (47). For the great majority of those infected, *H. pylori* behaves as a commensal organism (46), but it may also trigger an immune response in the gastric mucosa, that promotes a mild and mostly painless inflammation. Unusually, the immune response fails to resolve or cure the bacterial infection, giving rise to a persistent inflammatory condition known as chronic gastritis (48). Furthermore, even if the patient has a strong memory immune response to *H. pylori* after chemical eradication of the infection, this immunologic memory fails to prevent re-infection (108, 150).

For an estimated 10% of the afflicted, their chronic gastritis is severe enough to cause symptoms including pain and gastric reflux, prompting those patients to seek clinical help (120). In some patients, *H. pylori* gastritis progresses to the best-known

manifestation of infection, peptic ulcers with erosion of the mucosal layer (131). A different long-term consequence of chronic *H. pylori* infection and gastritis is the development of a variety of gastric cancers, including adenocarcinomas of the mucosal epithelium, and lymphomas that develop from the immune cells responding to the gastric infection (198).

Endoscopic examinations reveal that *H. pylori* infection and gastritis are multifocal, with lesions scattered across the antrum and corpus of the stomach. Afflicted patients are often diagnosed through a combination of bacteriological tests in combination with histopathological analysis of gastric punch biopsies (43). Inflamed gastric mucosa presents with marked infiltration of immune cells into the lamina propria and sub-mucosal layers of the tissue (for an overview, see (45)). A hallmark of *H. pylori* gastritis is the presence of numerous polymorphonuclear cells (PMNs), also known as neutrophils, responding to infection at the site of the inflammatory lesions, a quality also known as "activity". "Chronicity" reflects invasion of the gastric tissue with mononuclear cells, a broad category of cells that includes monocytes, macrophages, dendritic cells, and both B and T cell lymphocytes. Atrophy of the glandular tissue of the stomach is taken as evidence of a prolonged chronic inflammatory response. It is also possible to directly stain for *H. pylori* bacteria, which may be seen in dense films on the surface of the gastric epithelia.

Bacterial virulence

H. pylori expresses two proteins that are considered to be bacterial toxins: VacA and CagA. Secreted into the lumen of the stomach as a multimer, VacA binds to gastric epithelial cells and opens pores in cell membranes (for review, see (180)), promoting the

formation of vacuoles and occasionally cell death (33). Strains of *H. pylori* that express VacA are associated with a higher incidence of peptic ulceration (12). CagA is part of a pathogenicity associated island, or cagPAI, that encodes the toxin and a type IV secretion system. Several reports have demonstrated that CagA is injected by *H. pylori* into the cytosol of host epithelial cells through this protein "needle" (153, 193). Once inside the epithelial cells, CagA acts on the eukaryotic cytoskeleton to alter cell morphology, and some CagA polymorphisms are associated with more severe disease or an increased risk for the development of cancer (10, 15, 79). However, sequencing of several clinical isolates from gastritis patients shows that VacA-/CagA- strains are capable of causing disease (177, 218), demonstrating that these two proteins are dispensable for pathogenicity.

Numerous other factors produced by *H. pylori* are associated with disease development, but closer inspection reveals that these are primarily colonization or survival factors. Non-motile and aflagellated strains of *H. pylori* cannot penetrate the thick mucus layer that overlays and protects the glandular epithelium, and are subsequently flushed from the stomach (57, 134). BabA is a bacterial membrane protein that binds to Lewis blood antigens, polysaccharides found expressed not only on blood cells but also gastric epithelial cells, and BabA is the adhesion factor most strongly associated with gastric inflammation (169). *H. pylori* produces numerous such Lewis antigens itself, and can phase-shift through production of several different antigens within a few cell divisions (16), a mechanism believed to maximize adhesion to epithelial cells. Urease A and B raise the local pH by converting host urea into ammonia, enhancing bacterial survival and colonization in the harsh environment of the gastric lumen (52).

Mutations that delete or render non-functional any of these molecules reduce the fitness of the bacteria in the gastric environment and prevent disease development.

In lieu of actual virulence factors expressed by *H. pylori*, what then provokes disease development in infected patients? Numerous studies have examined the effect of direct co-culture of *H. pylori* with gastric epithelial cells *in vitro* in attempts to answer this question. While expression of CagA and VacA promotes cytopathic effects in cultured epithelial cells (84), direct contact with live *H. pylori* may trigger epithelial cells to produce IL-8 (36, 181), which is a strong chemoattractant for neutrophils. Some reports also demonstrate that *H. pylori* may induce apoptosis in epithelial cells (144, 163), an event that would attract monocytes to enter the tissue and remove dead cells. Whether these events occur *in vivo* is still debated, and it remains unclear if these epithelial effects are sufficient to trigger severe tissue inflammation in infected mucosa.

The immune response to *H. pylori* infection

Although what events or molecules may trigger *H. pylori* gastritis remain a mystery, the human immune response to gastric infection is well described. As mentioned above, the florid inflammatory response to *H. pylori* infection is characterized as invasion of the gastric tissue by cells of both the innate and antigen-specific arms of the immune system. The presence of invasive PMNs in the gastric mucosa (45, 51), noted above, is likely a response to the elevated production of IL-8 observed in infected gastric epithelia (63, 118, 145, 220). PMNs can kill bacteria through either degranulation or phagocytosis, although it is unclear what effect either mechanism may have upon *H. pylori* infection because neutrophils are not observed in contact with the lumen. Other phagocytes found in inflamed gastric mucosa include macrophages and dendritic cells,

both of which arise from the myeloid lineage of innate, or non-antigen specific immune cells. Studies have shown that *in vitro* co-culture of *H. pylori* with macrophage cell lines stimulates production of pro-inflammatory chemokines and cytokines like IL-1β, IL-6 and TNFα (35, 63, 64, 118). Primary cultures of bone marrow-derived dendritic cells mature after exposure to *H. pylori* and produce numerous chemoattractant molecules as well (75, 103). It has been proposed that immature dendritic cells resident in the gastric mucosa sample *H. pylori* bacteria from the contents of the gastric lumen, mature into active antigen-presenting cells, and produce the first inflammatory cytokines in response to *H. pylori* infection (133, 149).

In addition to the innate immune cells found in the mucosa of patients with *H. pylori*-mediated gastritis, diseased individuals also develop marked antigen-specific immune responses to infection. B and T lymphocytes are found in the inflamed, infected tissue (14, 190, 217), indicating that both the humoral and cell-mediated arms of the adaptive immune system are active in fighting the infection. Patients develop serum antibody titers against numerous *H. pylori* antigens (172, 217), including urease and LPS (80, 223), with IgG2 the most common antibody isotype seen. It is clear that the humoral response to *H. pylori* infection is robust, and some groups have suggested using serologic testing as a diagnostic for infection (165). Some reports also show that anti-*H. pylori* antibodies are also found in the gastric mucosa of infected patients (172, 217), but it is unclear how or if these antibodies are able to affect bacterial colonization or growth in the gastric lumen.

Of the T cell populations, both CD4+ and CD8+ T cells, or "helper" and "killer" T cells, are found in the inflammatory infiltrate in patients with *H. pylori* gastritis (122).

CD4+ T cells recovered from the peripheral blood or biopsies of gastric mucosa from *H. pylori*-infected patients can be stimulated *in vitro* to produce pro-inflammatory cytokines such as IFNγ and TNFα by co-culture of the T cells with *H. pylori*-loaded antigen presenting cells (17, 76). Killer T cells, while sometimes found in high numbers in gastritis patients, do not appear to significantly contribute to inflammation due just to *H. pylori* infection (73, 122). Several studies also demonstrate that increased numbers of *H. pylori* specfic regulatory T cells, the CD4+/CD25+/FoxP3+ population, are found in increased numbers in both the gastric mucosa and peripheral blood of infected patients (124, 125, 170). The origin of the greater numbers of *H. pylori*-specific regulatory T cells remains unclear, but it has been hypothesized that this population of cells may effectively prevent the inflammatory response from clearing the bacterial infection (125).

At the molecular level, the inflammatory response to *H. pylori* infection in the stomach is characterized by elevated expression of numerous cytokines. The inflammatory mediators found most elevated in infected gastric mucosa are IFNγ, IL-1β, IL-6, IL-8 and TNFα (35, 63, 64, 118, 220). Some reports also suggest that expression of the cytokines IL-4, IL-10, and TGFβ in gastric mucosa are increased in response to *H. pylori* infection (4, 40, 78, 81, 118, 220), but the contribution of these molecules to disease development is not well understood, especially as IL-10 and TGFb are both immunoregulatory cytokines that inhibit the immune response.

A model of the immune response to *H. pylori* infection

For most of the last 20 years, the activity of antigen-specific helper T cells has been categorized as either a "Th1" or "Th2" type of immune response, and each category is associated with a consistent pattern of cytokine expression. These two response

phenotypes also correlate with the older concepts of cell-mediated and humoral immunity (for review, see (225)). Inflammatory Th1 responses are associated with production of the effector cytokines IFNγ and TNFα, and helper T cells are polarized towards a Th1 response by exposure to IL-12, a cytokine produced by antigen-presenting cells such as macrophages and dendritic cells. At the same time, IFNγ triggers maturation of monocytes into active macrophages, and enhances phagocytosis by dendritic cells. Th2-polarized cells activated by antigen-specific stimulation characteristically produce IL-4, IL-5 and IL-10, are associated with B cell maturation, and are not normally associated with tissue inflammation.

The inflammatory response to *H. pylori* infection of the gastric mucosa implies that disease is mediated by a Th1 response to the bacteria. It is generally held that the *H. pylori*-specific helper T cells found in the gastric mucosa are driving inflammation by production of IFNγ and TNFα, both of which are found expressed at high levels in the gastric mucosa of infected patients (35, 64, 118). Also, studies have shown expression of IL-12 in the stomachs of infected patients (78, 164), and that this cytokine is critical for the production of IFNγ by the helper T cells found in the infected gastric mucosa (164). While several other pro-inflammatory cytokines are seen expressed in *H. pylori*-infected, inflamed mucosa, the T cell-derived cytokines are considered to be the most important ones for promoting disease (see Figure 1.1).

Animal models of *H. pylori* disease

Attempting to define and characterize all of the factors that contribute to disease in a genetically diverse human population is difficult at best, especially in combination with the multitude of varied *H. pylori* strains found clinically. It is unsurprising that

numerous efforts have been made to develop small animal models in which to test hypotheses about disease development in response to *H. pylori* infection. Numerous species were investigated for their suitability for experimental manipulation and their similarity to the histopathology seen in human patients, including primates, rats, gerbils, gnotobiotic piglets, and several strains of mice (for reviews, see (49, 148)). Initially mice were deemed unsuitable as an animal model because numerous groups repeatedly failed to colonize the animals with pathogenic clinical isolates. Lee et al. demonstrated that repeated passage of a pool of clinical isolates could select for a strain, known as SS1, which both efficiently colonized the mouse stomach and caused significant disease (110). Furthermore, *H. pylori* infected mice do not spontaneously resolve either gastric inflammation or cure the infection once *H. pylori* colonization is established.

Gastritis in infected mice is histologically similar to disease found in humans, as the inflammatory infiltrate includes both neutrophils and mononuclear cells, the latter population including B and T lymphocytes, macrophages and dendritic cells (for review, see (49)). The glandular architecture can be disrupted in severe inflammation, and metaplastic changes in cell morphology are also observed. The main criticism leveled at the use of mice as a model for *H. pylori* disease stems from the failure of infected mice to develop disease symptoms like those found in chronically inflamed human patients: *H. pylori*-infected mice do not develop either gastric or duodenal ulcers, and they do not develop either gastric adenocarcinomas or lymphomas due to infection. Several other *Helicobacter* species do colonize the mouse stomach and can promote the development of lymphomas and other neoplasms in chronically infected mice consequent to gastritis (109, 209), but this is not seen in *H. pylori* infected mice.

There are other animal models, notably gerbils, which reliably develop gastritis pathology that very closely resembles that of disease in humans, with florid inflammation and mucosal ulcerations in the stomach, but this animal species is not in common usage (127). Mice are the predominant small animal model for studying *H. pylori* disease, thanks to the ready availability of genetically engineered strains and immunologic reagents for studying the immune response. In my research presented in this document, I use two distinct mouse models of *H. pylori* disease, simple infection and adoptive transfer, which I describe here.

Simple infection

The simple infection model, also known as direct infection, is just that, infecting inbred strains of mice, such as C57 Bl/6 (B6) or BALB/c, with a mouse-adapted strain of *H. pylori*. In inflammation-prone strains, such as B6, mild-to-moderate gastritis develops gradually over the course of 6 to 9 months after infection with *H. pylori* SS1 (110). Other *H. pylori* strains can be used that promote a similar development of disease in both time of onset and severity in this model. Simple infection experiments have demonstrated the importance of numerous colonization factors in promoting gastritis (13, 52, 53, 159, 187), as well as the ability of *H. pylori* strains to induce disease in the absence of the toxins VacA and CagA (13, 53).

The first strength of the simple infection model lies in the ability to track the development of disease over time, which is difficult in human patients. Shi et al. found a spike in inflammatory cytokine mRNAs in the gastric mucosa of mice in the first few days following *H. pylori* infection, and the elevation of cytokine expression ebbed after two weeks (183). This suggests that presentation of novel *H. pylori* antigens is actively

taking place in the stomach tissue at this early stage of infection, although it remains unclear why this fails to trigger gastritis at this time. However, it is a common practice to collect tissue samples to track disease development at time points at a few time points shortly before and immediately following the peak development of gastritis and look for time-dependent changes (e.g. as in (184)).

The other strength of the simple infection model is the ability to use cytokine- and cytokine receptor-deficient mice to examine the contribution of those molecules to the development and progression of *H. pylori* gastritis. IFNγ knockout mice fail to develop gastric inflammation after *H. pylori* infection (186), and Panthel et al. found that *H. pylori* colonization levels were 6 to 8-fold greater in mice lacking components of the TNFα receptor (160), suggesting that the inflammatory response and pro-inflammatory mediators are important for promoting gastritis and control of bacterial colonization. Conversely, histological gastritis was found to be significantly more severe in IL-4 and IL-10 knockout mice infected with *H. pylori* (25, 26, 88, 186). Together, these cytokine findings strongly suggested that *H. pylori* gastritis is a Th1-mediated disease in mice, driven by expression of the Th1 cytokines IFNγ and TNFα, and inhibited by the Th2 and regulatory cytokines IL-4 and IL-10.

Adoptive transfer

In the second mouse model of *H. pylori* disease, we use immunodeficient mice, such as *Prkdc*^{scid} (for severe-combined immunodeficiency, or SCID) mice or recombinase-1 or -2 knockout (RAG1- or RAG2-KO) mice on a B6 background. These strains lack B and T lymphocytes and cannot develop antigen-specific responses to infection, and infection of these mice with *H. pylori* does not provoke the development of

gastritis (59), confirming the hypothesis that *H. pylori*-mediated disease is due to an antigen-specific response to infection. A typical experiment starts with infecting the immunodeficient mice with *H. pylori* and then adding populations of immune effector cells back to the infected mice. The specific order of events does not affect disease development, so long as infection and adoptive transfer both occur within the time-frame of a few weeks of each other (K. A. Eaton, personal communication).

When bulk splenocytes are transferred into infected SCID mice, significant histological gastritis is observable within 4 weeks (55, 56), much faster than the onset of disease in simple infection. Furthermore, histological inflammation is markedly more robust and florid in the gastric mucosa of infected recipient mice, and disease severity reaches a peak about 8 weeks after the adoptive transfer. Of note, infected B6 SCID mice, transferred with B6 splenocytes, may actually clear the bacterial infection after prolonged gastritis, an event that is not observed in either humans or mice directly infected with *H. pylori* (56).

The adoptive transfer disease model allows us to define the contributions of specific populations of antigen-specific immune cells to the initiation of gastritis in *H. pylori* infected recipient mice. The initial report describing this model employed bulk splenocytes isolated from a congenic, immunocompetent mouse (59), but further investigation demonstrated that CD4+ helper T cells alone are both necessary and sufficient to initiate gastritis in infected recipient mice (55). Additionally, CD4+ T cells recovered from infected, recipient mice produced IFNγ in response to stimulation with *H. pylori*-loaded antigen presenting cells (55). Together these findings strongly support the

characterization of *H. pylori*-mediated gastric inflammation as a disease driven by Th1-polarized helper T cells that produce IFNγ in response to *H. pylori* infection.

Model similarities and disparities

Although there are a number of parallels between the two disease models and human gastritis, the differences highlight how disease development may differ between these two models. In both simple infection and adoptive transfer, *H. pylori* colonization is inversely correlated to the severity of inflammation (53, 56), a phenomenon not observed in human patients where no correlation is observed between gastritis and the density of *H. pylori* colonization (198). This is taken as evidence that the inflammatory immune response is negatively affecting *H. pylori* colonization of the gastric mucosa. Qualitatively, histological gastritis is similar between both mouse models, and to human disease, as the same cell populations are observed entering the infected gastric mucosa. Patterns of cytokine expression are similar between the mouse models and biopsies from patients, with increased expression of IFNγ, TNFα, IL-1β and IL-6 seen in response to *H. pylori* infection (as mice lack an evident homolog of human IL-8, it is difficult to compare the expression of that cytokine to the various orthologs that are inconsistently assessed in the literature).

Quantitatively, there are striking differences between the two experimental approaches. Clearly, the speed with which histological gastritis develops is an issue, as adoptively transferred mice show signs of disease in a month, versus the 6 to 9 months required in simple infection (56, 110). By the same token, disease severity is also greater in the adoptive transfer model than in directly infected mice, and this more severe inflammation appears to cure *H. pylori* infection, which does not occur in simple

infection or in humans (56). The methods for assessing cytokine mRNA expression are not consistent across the literature, so it is difficult to compare the levels of inflammatory cytokines between samples from simple infection and adoptive transfer experiments, but the general trend suggests there is greater cytokine expression in directly infected mice, although mRNA levels may not directly correlate with actual protein production.

The reason for these disparities between experimental methods is unclear at this point, but observations about the density of gastric colonization early in the two mouse models suggest a hypothesis (Figure 1.1). *H. pylori* is able to colonize the gastric mucosa of immunodeficient mice more efficiently than immunocompetent mice by several orders of magnitude, even for a few weeks after adoptive transfer (56, 59), although this difference decreases as inflammation develops. The greater density of bacterial colonization would activate and mature more tissue-resident dendritic cells, enabling the antigen-presenting cells to attract more T cells to the gastric sub-mucosa. This would encourage more efficient activation and expansion of the *H. pylori*-specific CD4+ T cells in the transferred cell population, giving rise to the more precipitous and florid inflammatory response seen in adoptively transferred mice.

An observation from other adoptive transfer disease models suggests a second possible hypothesis to explain the discrepancy between our two disease models. Numerous groups have found that severe intestinal inflammation spontaneously arises after adoptive transfer of bulk splenocytes or enriched CD4+ T cells into immunodeficient mice (for a recent review, see (158)). Depending upon the model system, this colitis requires the presence of particular gut microbiota to trigger the inflammatory response (67, 195). However, other groups have observed that increasing

the proportion of regulatory T cells in the adoptive transfer inhibits the development of disease (31, 219). This finding has been interpreted as a suggestion that regulatory T cells are not well represented in the population of T cells enriched from splenocytes, or that regulatory T cells do not successfully engraft into recipient mice (for review, see (112)). Prior to this work we have not specifically explored the role, or number, of transferred regulatory T cells given to recipient mice in our adoptive transfer gastritis model (see Chapter 4). However, we found previously that transfer of primarily CD4+ $CD45RB^{lo}$ cells, a population of T cells that does include regulatory T cells, into infected recipient mice does not promote the development of disease (56). Whether the presence or absence of regulatory T cells significantly affects the progression of gastritis in our adoptive transfer model in comparison to the simple infection model remains to be determined. Overall, the numerous similarities shared by transfer colitis models and our transfer gastritis model suggest we may be seeing disease develop incidentally, and that *H. pylori* colonization simply directs the inflammatory focus towards the gastric mucosa.

Testing the Th1 hypothesis

The work of Smythies et al. showed that gastritis does not develop in *H. pylori* infected IFNγ-KO mice (186), demonstrating that production of this inflammatory cytokine is critical for disease. However, while IFNγ in mice is produced primarily by T cells, some activated macrophages (21), dendritic cells (143) and natural killer cells (65) can also produce this cytokine. If IFNγ is critical for promoting inflammation in *H. pylori*-infected gastric mucosa, which came first: the antigen-specific CD4+ T cell response, or the innate response of activated phagocytes? To answer this question, we looked at the development of gastritis in infected SCID mice transferred with CD4+ T

cells from B6 T-bet knockout (T-bet KO) mice (50). T-bet, or *T-box transcription factor 21*, is the transcription factor that specifically regulates IFNγ expression in T cells (203). We hypothesized that T cells were the key source of IFNγ production in *H. pyori*-mediated gastritis and that in the absence of T cell-produced this cytokine, inflammation would not develop in infected recipient SCID mice.

As a control, we examined gastritis in directly infected T-bet KO mice, and found no inflammation at either 12 or 24 weeks after infection. This finding was expected at these early time points, in part due to the failure of T cells to produce IFNγ, but also because there is evidence that dendritic cells are defective in activation and T cell stimulation in the absence of T-bet (123). If the antigen-specific response cannot be initiated in T-bet KO mice, then a lack of inflammation is unsurprising. However, counter to our hypothesis, *H. pylori*-infected SCID mice transferred with T-bet KO CD4+ T cells did develop moderate gastritis that was less severe but not significantly different in character from inflammation seen in infected recipients of B6 cells (50). We looked at cytokine levels as mRNA in gastric mucosa and as protein in serum, and saw that mice with T-bet KO cells had significantly, and sometimes dramatically, lower levels of IFNγ, TNFα, and IL-6 than did infected mice given B6 cells. In contrast, levels of IL-12 mRNA in gastric mucosa and IL-12 protein in serum were equivalent between infected groups of recipient mice. Together our adoptive transfer results suggest that induction of the T cell response is not significantly affected by the absence of T-bet in CD4+ T cells, and demonstrates that gastric inflammation can occur in the absence of the well characterized pro-inflammatory Th1 response.

We repeated our adoptive transfer experiments using T-bet KO deficient recipients in an effort to reconcile our simple infection and adoptive transfer results. We could not be certain that the lack of disease found in directly infected T-bet KO mice was due to a defect in antigen presentation or due to the reported T cell defect. By examining gastritis development in immunodeficient T-bet KO recipient mice, we could confirm where the functional defect occurs in the other disease model. Surprisingly, we found that even in RAG-2 x T-bet double knockout mice, T-bet KO CD4+ T cells were able to promote gastric inflammation in response to *H. pylori* infection (data not shown). These findings together confirm that *H. pylori*-mediated gastritis can occur in the absence of an antigen-specific Th1-polarized CD4+ T cell response, forcing us to consider other hypotheses about how an inflammatory response can occur in the absence of IFNγ and Th1 effector CD4+ T cells.

Dissertation overview

The body of this dissertation explores possible answers to the question of how *H. pylori*-mediated gastritis can develop in the absence of IFNγ, in addition to more carefully assessing the contribution of non-T cell immune effector cells and cytokines contribute to disease development. In Chapter 2, I present results from my exploration of the how the third, novel arm of the polarized CD4+ T cell responses, Th17, contributes to disease development. I hope to demonstrate that Th17 cells, which express the hallmark cytokine IL-17A, not only contribute to disease development in infected mice, but also that Th1- and Th17-polarized CD4+ T cells synergize to promote development of the most severe inflammation.

Chapter 3 reports the results of our collaboration with Drs. Hanief Sofi and Cheong-Hee Chang, where we employed their class II transactivator transgenic (CIITA-Tg) mouse strain to explore the contribution of the whole Th17 CD4+ cell phenotype to disease development. Their transgenic model was initially designed to explore the effect of thymocyte expression of major histocompatibility complex II on CD4+ T cell development, but they found a defect in Th17 polarization in CIITA-Tg mice. I report on our findings that demonstrate that, as I found in Chapter 2, Th17-polarized T cells contribute to development of gastritis, but we also discovered that inflammation could be maintained in the absence of a *H. pylori*-specific Th1, Th2, or Th17 cell response.

I present an improved method for analyzing the cellular immune response in *H. pylori*-infected inflamed gastric mucosa in Chapter 4. I report how I adapted a protocol for isolating lymphocytes from intestinal epithelia to work with generating single-cell suspensions of the gastric mucosa, allowing me to analyze the gastric lamina propria leukocytes through flow cytometry. Also, we explored how gastric leukocyte populations isolated from adoptively transferred mice change with input of different T cell populations, including varied numbers of T cells, populations of regulatory T cells, and use of cytokine-deficient T cells. I hope to demonstrate how these findings enhance our understanding of disease development in the absence of particular pro-inflammatory cytokines, and improve our understanding of the adoptive transfer model of *H. pylori* disease.

Figure 1.1: Initial model for development of *H. pylori* gastritis

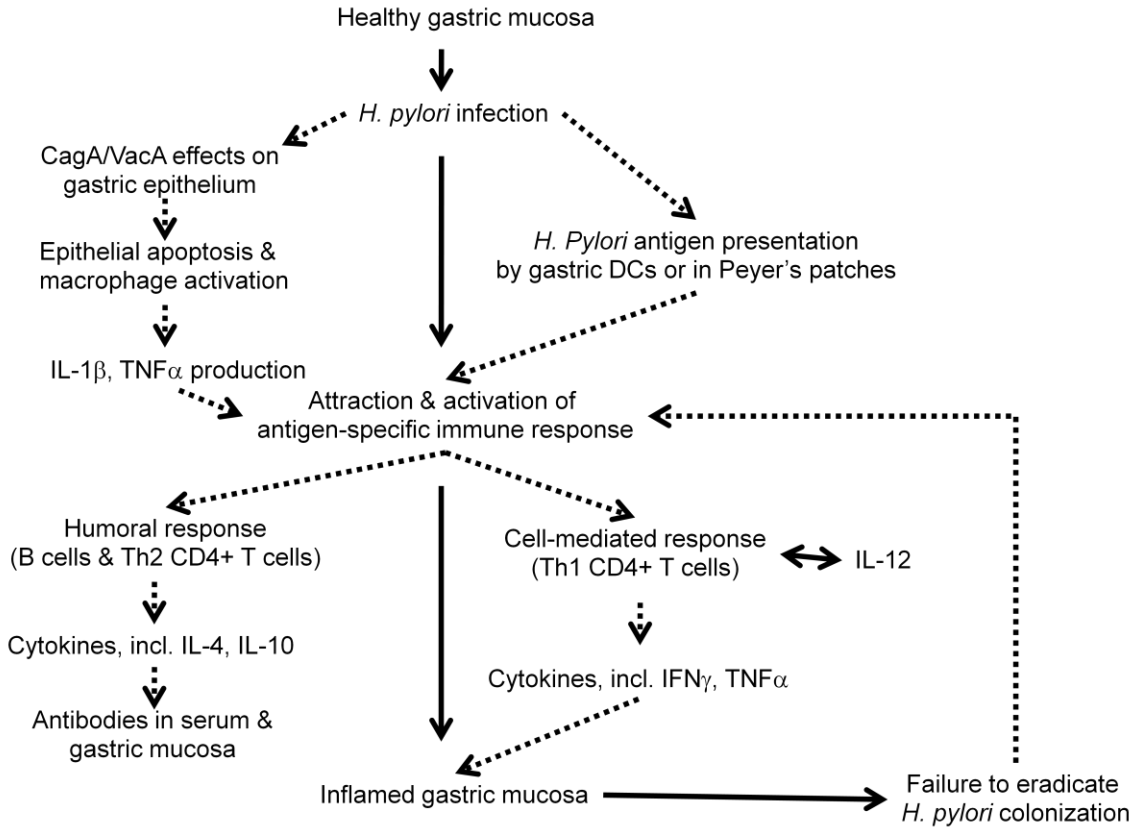


Figure 1.1: An initial model of the development of *H. pylori*-mediated gastritis in humans and mice. Solid lines denote the accepted course of events in *H. pylori* disease development. Dashed lines indicate events that are related to disease development, but have not been confirmed as necessary for disease. Note that Th1-linked events are the only CD4+ T cell response involved in actively promoting inflammation.

Chapter 2

The Role of Th17 and Th1 Polarized Helper T Cells in *H. pylori* Gastritis in Mice

ABSTRACT

Mouse models of *H. pylori* disease develop chronic gastritis in an immune response dependent upon the action of IFNγ and CD4+ T cells. Recent attention has focused on the contribution of IL-17, a cytokine associated with mucosal inflammatory responses and a molecule found elevated in gastric punch biopsies of infected human patients. I found increased IL-17A expression in the absence of IFNγ in infected RAG-2 knockout (RAG2-KO) mice adoptively transferred with T-bet knockout (T-bet KO) T cells. To further explore the contribution of IL-17A to *H. pylori* gastritis, I used IL-17 knockout (17AKO), T-bet KO, and C57Bl/6 (B6) mice in two models of disease. Adoptive transfer of 17AKO T cells to infected RAG2-KO mice produced equivalent disease with significantly lower colonization than in mice with B6 cells. In the absence of IL-17 in these mice we found IFNγ expression unchanged but several other pro-inflammatory cytokines were increased versus B6 recipients. Simple infection of 17AKO and T-bet knockout mice produced significantly reduced gastritis with greater bacterial colonization than in B6 mice. Infected 17KO mice had lower IFNγ and IL-1β expression than B6 recipients, but elevated IL-17F levels. Induction of non-T cell derived cytokines such as IL-1β and IL-6 was also significantly greater in infected 17AKO mice than in infected B6 mice. T-bet KO mice did not significantly produce any cytokines in response to *H. pylori* infection. My findings demonstrate that Th1 and Th17 polarized cells both

contribute to the development of *H. pylori* gastritis, but that cytokines of the innate immune system are more important to disease progression and infection control than previously understood.

BACKGROUND

I have described in Chapter 1 the Eaton laboratory's previous findings regarding the contribution of the T-bet transcription factor to the development of gastritis in *H. pylori* infected mice (50). Briefly, T-bet is the transcription factor that regulates T cell production of IFNγ, and T-bet knockout (T-bet KO) mice therefore lack a pro-inflammatory Th1 type response, and mice lacking CD4+ T cell expression of T-bet should not develop disease when infected with *H. pylori*. When we directly infected T-bet KO mice, we found these mice failed to develop histological gastritis. However, adoptive transfer of T-bet KO CD4+ T cells into *H. pylori*-infected SCID or RAG2 knockout (RAG2-KO) mice promoted the development of moderate to severe gastritis. We observed similar results even when we transferred RAG-2 x T-bet double knockout (DKO) mice with T-bet KO CD4+ cells and could not detect IFNγ mRNA in infected recipient mice, demonstrating that *H. pylori* gastritis can still develop in the complete absence of IFNγ.

These results disagreed with the extant model of *H. pylori* gastritis as a Th1-mediated disease, and we were forced to reconsider the roles of IFNγ and CD4+ T cells in promoting *H. pylori*-mediated gastritis in our mouse models. A suggestion that we explore the relatively new category of polarized helper T cells known as Th17 gave us a possible explanation for the observed gastritis in infected SCID mice transferred with T-bet CD4+ cells.

Th17 polarized helper T cells produce the inflammatory cytokines IL-17A, IL-22 and IL-17F in both humans and mice (99, 213), and constitute a second pro-inflammatory T cell group in addition to IFNγ-producing Th1 polarized cells (161). In humans, expression of IL-17A *in situ* is linked to elevated IL-8 production (107, 126), and this in turn leads to increased neutrophil infiltration (107), and as mentioned previously a neutrophilic infiltrate is a histologic hallmark of *H. pylori* gastritis (45). *In vitro*, development of the Th17 phenotype follows the same rules as promotion of the Th1 and Th2 phenotypes, with exclusive expression of some cytokines (161), and expression of other molecules inhibitory to the development of the other phenotypes (77). It has also been demonstrated that a number of *in vivo* inflammation models initially described as IFNγ and Th1 cell dependent are instead Th17-driven (27, 100, 224). These findings together suggested a new hypothesis for our adoptive transfer results: in the absence of IFNγ, IL-17A expression by Th17-polarized CD4+ T cells drive the development of gastric inflammation in *H. pylori*-infected, adoptively transferred mice.

In general, Th17 cells are found to strongly participate in two categories of immune responses: autoimmune conditions including encephalitis (100, 202) and uveitis (8, 224); and mucosal/epithelial immune responses including airway inflammation (116, 137, 147), psoriasis (71, 213), and colitis (82, 105, 113). Findings by Luzza, et al. promoted the idea that the Th17 response is involved in *H. pylori* gastritis as they found increased IL-17A expression in gastric punch biopsies from human patients (126), and they were the first group to find elevated IL-17A in infected human stomachs (20, 85, 96, 138). Mouse models involving other *Helicobacter* species also demonstrate the strong role played by IL-17A and Th17 cells in controlling inflammation and infection in the

lower bowel (82, 105, 113). In light of these findings, I proposed a complementary and more radical hypothesis, that Th17 polarized CD4+ T cells contribute to the development of gastritis in combination with Th1 polarized cells in *H. pylori*-infected mice.

To investigate the contribution of IL-17A and Th17 cells to *H. pylori*-mediated gastritis in mice, I re-examined samples from our T-bet KO adoptive transfer experiments and found increased IL-17A expression (see Results). Once we obtained 17AKO mice, I initiated parallel experiments in our two models of *H. pylori* gastritis to examine the contributions of IL-17A to disease progression: simple infection of immunocompetent 17AKO mice and tracking at 3 month intervals for 18 months; and infection of RAG2-KO mice then adoptively transferred with 17AKO CD4+ cells and analyzed after 8 weeks. I will discuss how my findings agree with, but go beyond, the two other reports which were published during the course of my investigations (183, 184) in the discussion.

EXPERIMENTAL DESIGN

With my initial work looking at IFNγ and IL-17A levels in our previous T-bet KO adoptive transfer experiments in hand, I planned two series of experiments with 17AKO mice. The first experiments employed our adoptive transfer methodology, where we infected immunodeficient mice lacking T and B cells and then subsequently transferred CD4+ cells enriched from the spleens of congenic mice (55, 59). Here, I infected RAG2-KO with *H. pylori* SS1, and two weeks later adoptively transferred these mice with either B6 or 17AKO CD4+ cells. These mice were sacrificed 8 weeks after transfer, and thus these mice were infected for a total of 10 weeks. I harvested stomach tissue to collect samples for histology, quantitative *H. pylori* culture and RNA analysis. These

experiments consisted of 5 mice per experimental group, with at least three independent replicates of each group.

For the second series of experiments, I compared several strains of mice in the simple infection model. 30 mice each of the B6, 17AKO and T-bet KO strains were infected with *H. pylori* SS1. At each 3 month time point after infection, 5 mice were sacrificed, until the end of the experiment at 15 months after infection. I harvested stomach tissue to collect samples for histology, quantitative *H. pylori* culture, RNA analysis. One sample every 3 months does not provide very fine resolution to determine the exact timing of critical events in disease progression, but given the slow nature of this model of *H. pylori*-mediated gastritis in mice, this sampling rate should provide us with a great deal more information than we previously possessed. Table 2.1 details the number of mice in each experimental group and at each time point, as both 17AKO and T-bet KO mice suffered from unexpected health problems secondary to their cytokine deficiencies, preventing me from having 5 mice at each time point.

Histological analysis and quantitative culture as tools for examining disease progression are *de rigeur* for the Eaton laboratory, but examination of cytokine expression is novel for the group, and a significant investment of both time and money. I selected cytokines representative of both depth and breadth in an effort to maximize a return on that investment. IFNγ is the hallmark effector molecule of Th1 polarized CD4+ helper T cells while IL-17A currently holds that distinction for Th17 cells (for review, see (99)). Another member of the IL-17A family, IL-17F has pro-inflammatory properties and may heterodimerize with IL-17A (24, 116, 216), and could complement the loss of IL-17A expression in 17AKO mice (208).

IL-1β, IL-6 and TNFα are pro-inflammatory cytokines produced by both innate immune effector cells and non-immune cells such as myocytes and fibroblasts, and elevated expression of both IL-1β and TNFα have been reported in human patients (35, 64, 87, 221). IL-12 components p40 and p35 constitute the Th1-polarizing cytokine IL-12, but IL-12p40 also combines with IL-23p19 to make IL-23, a cytokine that maintains the Th17 phenotype in polarized effector CD4+ T cells (3, 157). I decided against tracking IL-23p19 expression at the start of these experiments due to evidence suggesting that Th17 cells do not require the cytokine for induction (119, 215) and may not need IL-23 to commit to IL-17A production (196).

IL-10 and TGFβ are immunoregulatory cytokines without well-understood roles in *H. pylori*-mediated gastritis (55, 132), but I considered these molecules important in light of the reported counter-balance between Th17 and Treg CD4+ cells (for review, see (97, 210)). It is also interesting to note that *in vitro*, IL-6 and TGFβ together promote polarization of responding CD4+ T cells toward a Th17 phenotype while TGFβ alone supports development of Treg cells (97).

RESULTS

Increased IL-17A expression in the absence of T-bet

I assessed *H. pylori*-infection driven changes in IFNγ and IL-17A expression in the gastric mucosa of infected RAG2-KO mice adoptively transferred with T-bet KO CD4+ cells. Previously we demonstrated significantly lower IFNγ expression in T-bet transferred, *H. pylori*-infected SCID mice (50), but we confirmed our histological findings using RAG2-KO and RAG2 x T-bet DKO recipient mice (data not shown). There was no difference in the severity or frequency of histological gastritis between infected SCID, RAG2-KO and RAG2 x T-bet DKO mice transferred with the same type of CD4+ cell (data not shown). Our findings in infected recipient DKO mice were an important result as they clearly demonstrated that the lack of gastric inflammation in *H. pylori*-infected T-bet KO mice stemmed from the failure of the CD4+ T cells to produce IFNγ, and not due to any defect in the antigen presenting cells in T-bet KO mice (123).

IFNγ expression was lower in infected RAG2-KO mice transferred with T-betKO cells than in mice given B6 cells (Figure 2.1a), although the observed difference in this experiment was less than we found in our experiments in SCID mice (50). *H. pylori* infection induced greater expression of IL-17A in RAG2-KO mice with T-bet KO cells than observed in B6 cell recipients (Figure 2.1b). This result suggested the ability of Th17 polarized CD4+ T cells to promote inflammation when IFNγ expression is markedly reduced.

IL-17A does not contribute to gastritis in adoptive transfers

Severe and widespread gastritis with gastric epithelial metaplasia developed in both groups of infected recipient mice whether they received B6 or 17AKO T cells

(Figure 2.2). Extensive disease developed in both infected groups, with no statistical differences in the extent of gastritis between the groups (Figure 2.3). I found significantly lower *H. pylori* colonization in the stomachs of mice receiving 17AKO cells compared to infected recipients of B6 cells (Figure 2.4). These observations suggest that IL-17A does not contribute to gastritis in *H. pylori*-infected recipient mice, and may hinder efforts to control bacterial colonization, and both of these findings contradict my initial hypothesis.

Cytokine expression profiles in adoptive transfer

Using mRNA isolated from gastric tissue, I assessed changes in the expression of hallmark Th1 and Th17 cytokines in response to *H. pylori* infection in recipient mice. IFNγ expression was induced equally by infection in both groups of recipient mice (Figure 2.5). In these experiments, I could only detect IL-17A mRNA in infected recipients of B6 cells (Figure 2.6), so I was unable to calculate the fold induction of expression upon *H. pylori* infection. However, even in infected RAG2-KO mice transferred with B6 T cells, the expression of IL-17A mRNA is minimal. Induction of IL-17F was significantly greater in the infected recipients of 17AKO cells than I found in mice with B6 cells (Figure 2.7), an observation in line with the work of von Vietinghoff, who found greater expression of IL-17F in 17AKO mice (208).

H. pylori-mediated gastritis is also associated with increased expression of several primarily non-T cell derived pro-inflammatory cytokines, notably IL-1β and TNFα (118, 145, 220). I examined the mRNA expression of these two cytokines and IL-6 in order to assess the contribution of other immune effector cells to *H. pylori*-mediated gastritis. IL-1β and IL-6 mRNA were both significantly more induced in 17AKO cell recipient mice

compared to recipients of B6 cells (Figures 2.8 and 2.9). TNFα expression was induced equally in both groups of infected mice (Figure 2.10). These observed increases in non-T cell derived inflammatory mediators are consistent with previous reports, and also suggest that the immune system in *H. pylori* infected mice is compensating for the loss of the pro-inflammatory IL-17A in recipient mice.

Induction of both IL-12p40 and IL-12p35 was greater in recipients of 17AKO cells than in mice given B6 cells (Figure 2.11), but this increase was not significant for expression of IL-12p35. These findings suggest that in the absence of the Th17 cytokine IL-17A, the inflammatory response to *H. pylori* infection is promoting the polarization of Th1 phenotype helper T cells, although there is no corresponding increase in IFNγ expression in 17AKO recipient mice. Expression of the regulatory cytokines IL-10 and TGFβ was also significantly more induced in 17AKO cell recipients than in recipients of B6 cells (Figure 2.12), although it is unclear why these inhibitory cytokines are increased in the absence of IL-17A expression.

Cytokine deficiencies impede gastritis development in simple infection

Following on the results of my adoptive transfer experiments, I continued to explore disease development in the simple infection model, and I tracked the progression of gastritis in *H.pylori*-infected B6, 17AKO and T-bet KO mice. B6 and 17AKO mice developed mild-to-moderate mutifocal gastritis, characterized by mononuclear or mixed inflammatory infiltrate, while T-bet KO mice failed to develop significant disease (Figures 2.13). B6 mice developed significant gastritis by 9 months after infection, with continued inflammation until the end of the experiment at 15 months after infection, without significant variation (Figure 2.14). We found mild but significant gastritis in

17AKO mice after 6 months of infection and this degree of inflammation continued until the end of the experiment. Infected T-bet KO mice did not develop disease by 6 months after infection, in agreement with our previous findings (50), and although these mice appeared to develop mild gastritis 15 months after infection, the gastritis score was not significantly different from uninfected mice. Inflammation in 17AKO mice was significantly lower than in B6 mice at all time points once B6 mice developed disease, demonstrating that IL-17A does contribute to the development of *H. pylori* gastritis.

H. pylori colonized the strains of mice roughly in inverse proportion to inflammation (Figure 2.15). 17AKO and T-bet KO mice were both significantly more highly colonized than B6 mice after 3 months, and T-bet KO mice displayed significantly greater colonization than 17AKO mice from 9 months after infection, continuing through the rest of the experiment. This observation confirms that IL-17A, like IFNγ, also contributes to control of bacterial colonization in the stomach of *H. pylori* infected mice.

Cytokine expression profiles in simple infection

IFNγ expression in gastric mucosa was induced by *H. pylori* infection in both B6 and 17AKO mice, but not in T-bet KO mice (Figure 2.16). Coincident with the greatest inflammation at 9 months post infection, IFNγ mRNA was significantly more induced in B6 mice compared to 17AKO mice, but this was the only time I observed a statistically significant difference between these two strains. As in the adoptive transfer experiments, IL-17A was undetectable in uninfected groups or in 17AKO mice (data not shown), and while expression of this cytokine increased with time in infected B6 mice (Figure 2.17), I cannot calculate the fold induction. Induction of IL-17F was consistently higher in 17AKO mice than either B6 or T-bet KO mice at all times, although these differences

were only significant 6 months after infection (Figure 2.18). These changes in the pattern of infection-induced expression of T cell-produced inflammatory cytokines in simple infection agree with the observations in adoptively transferred mice.

IL-1β mRNA increased with the time of infection in a similar pattern in both B6 and 17AKO mice (Figure 2.19), and while induction of this cytokine was dramatic, there was no significant difference in induction between infected B6 and 17AKO mice. Induction of IL-6 also increased with time in infected B6 and 17AKO mice (Figure 2.20), but I did not observe consistently greater cytokine expression in the strains of mice with significant gastritis. For example, B6 mice expressed significantly more IL-6 at 9 months post infection, and at 15 months induction was significantly greater in 17AKO mice but induction of IL-6 expression was equivalent at 12 months post infection. There were no significant changes in IL-6 expression in infected T-bet KO mice throughout the course of infection. In general, induction of TNFα increased with time in infected B6 and 17AKO mice (Figure 2.21), but expression was significantly greater in B6 mice at nine months after infection. Again, infection of T-bet KO mice prompted no significant changes in TNFα expression throughout the course of infection. In these experiments the lack of IL-17A does not appear to influence the expression of non-T cell derived inflammatory cytokines.

In the simple infection experiments, IL-12p40 expression in infected B6 mice mirrors the progression of histological gastritis (Figure 2.22a). There is no clear pattern of cytokine induction in 17AKO or T-bet KO mice. IL-12p35 expression in mice does not significantly change over the course of infection, until 15 months after infection when IL-12p35 expression in B6 and 17AKO mice dramatically increases (Figure 2.22b). IL-

10 expression in B6 and 17AKO mice increased in parallel over the course of the experiment, rising sharply at 15 months after infection (Figure 2.23a), but T-bet KO mice did not follow this pattern with IL-10 expression peaked at 9 months after infection in these mice. I observed similar expression patterns for TGFβ in the various strains of mice throughout the course of infection (Figure 2.23b). Without a clearly defined role for these cytokines in the development of *H. pylori* gastritis in the simple infection mouse model of disease, it is unclear what these cytokines changes signify in the absence of other data, such as protein concentrations or cellular function assays.

DISCUSSION

Adoptive transfer findings and "Th" effector cytokines

In our relatively rapid adoptive transfer disease model, severe gastritis develops by 8 weeks after T cell transfer into infected mice. I observed that infected RAG2-KO mice transferred with 17AKO cells developed histologically equivalent gastritis, and the extent of inflammation was slightly reduced but not significantly lower than seen in recipients of B6 cells. Furthermore, in mice with 17AKO cells, we found significantly reduced *H. pylori* colonization. I take these findings as evidence that while my initial hypotheses, which state that Th17 cells contribute to gastritis development in the absence of Th1 cytokines, and that Th17 cells contribute to gastritis simultaneously with Th1 cells, are both partly correct. But these results also suggest an unexpected, underlying immune behavior that promotes inflammation in this model of *H. pylori* disease. It is clear that CD4+ T cells are required to initiate *H. pylori* gastritis (55, 56), but Th1 or Th17 cell-specific inflammatory cytokines may not be necessary for the continued progression of disease. We initially predicted two possibilities for the development of *H.*

pylori-mediated gastritis in the absence of IL-17A expression. In the first model, IL-17A contributes to gastritis only in the absence of an IFNγ-driven Th1 inflammatory response, as I found in infected RAG2-KO mice transferred with T-bet KO cells (Figure 2.1). Following this hypothesis, the loss of IL-17A should not affect gastritis or IFNγ expression in infected mice.

The other possibility sees IL-17A and IFNγ competing against each other to affect the severity of disease and control disease progression. In this alternate model, we would expect to see an increase in IFNγ expression in 17AKO mice. Both of these hypotheses are predicated on the model of helper T cell polarization where "Th" phenotypes are reciprocally balanced, and differentiation to either Th1 or Th17 precludes development of the other phenotype. To my surprise these findings demonstrate IL-17A contributes to disease progression independent of the expression levels of IFNγ. Furthermore, I find evidence that IL-17A expression may be required for the maximum expression of IFNγ in the simple infection model (Figure 2.16), as IFNγ expression was significantly lower in 17AKO mice than in B6 mice at 9 months post infection, when gastritis peaked in both strains.

Simple infection findings and comparison to previously published work

I show here that IL-17A a) appears to compensate for the absence of IFNγ in *H. pylori*-mediated gastritis, b) contributes to the maximum development of disease, and c) helps control gastric colonization by *H. pylori*. Looking at samples from our previous work with infected RAG2-KO mice adoptively transferred with T-bet KO cells, I found increased expression of IL-17A in the absence of CD4+ T cell-produced IFNγ, a

mechanism which could explain our previous findings of severe gastritis in these mice (50).

As a direct extension of this previous work I examined how IL-17A contributed to disease development in both mouse models of *H. pylori* disease, adoptive transfer and simple infection. Direct infection provoked mild gastritis in 17AKO mice, associated with a 100- to 1,000- fold greater colonization by *H. pylori* in 17AKO mice versus B6 mice. The two recent publications about the role of IL-17A in mouse models of *H. pylori*-mediated gastritis stopped at this point, after determining that IL-17A promotes maximum development of gastric inflammation in *H. pylori*-infected mice, and possibly contributes to controlling bacterial colonization (183, 184). Like these prior reports, my results demonstrate a role for IL-17A in disease and colonization control, but my experimental design allowed me collect markedly more data than published by any other research group, even as my schedule postponed when I could publish my findings. I have completed a more in-depth cytokine profile, over a greater length of time than previously reported, illuminating more of the events that occur in early gastritis, than either of the previous publications.

Furthermore, the adoptive transfer model employed by the Eaton laboratory demands a broader consideration of the immunologic and bacterial factors contributing to disease development than provided by just simple infection models. Just as we demonstrated previously that IFNγ is dispensable for the development of gastritis, a finding that was not without controversy, so here I have shown that IL-17A is also not required for disease, and it is clear that a great deal more work will be required to reconcile this finding with the simple infection model. By combining these two

experimental approaches, I have explored the role of IL-17A in *H. pylori*-mediated gastritis more thoroughly and completely than previously recorded.

Evidence in favor of other pro-inflammatory cytokines

In an effort to explain my findings I assessed the expression of other pro-inflammatory cytokines, including those molecules characteristic of innate inflammatory responses. Generally, while *H. pylori*-infected T-bet KO mice showed no significant changes in cytokine expression at all, 17AKO mice displayed broadly reduced expression of both T cell derived and innate inflammatory cytokines compared to infected B6 mice. This difference correlates with the very mild gastritis and increased colonization observed in infected 17AKO mice compared to B6 mice with a more robust cytokine response. Conversely, in infected RAG2-KO mice receiving 17AKO cells, with histological inflammation equivalent to mice with B6 cells but with reduced bacterial colonization, I found significant increases in the expression of IL-1β and IL-6.

It is important to note that I found significantly increased IL-17F expression in both infected 17AKO mice and infected RAG2-KO mice receiving 17AKO cells. This cytokine is an IL-17A family member secreted by Th17-polarized CD4+ T cells, and may functionally heterodimerize with IL-17A (24, 116, 216). Overexpression of IL-17F and neutrophilia have been previously reported in 17AKO mice (208), although I did not observe a difference in neutrophil infilitration in inflamed gastric mucosa (data not shown). IL-17F is reported to have other pro-inflammatory functions that overlap and are redundant with the IL-17A spectrum of activity, including neutrophil chemoattraction (116) and inducing cytokine expression in epithelial cells and monocytes (99).

There is disagreement over the pro-inflammatory or protective roles of IL-17A and F, especially in the gastrointestinal tract, with some evidence suggesting that IL-17A protects against colitis while IL-17F exacerbates inflammation (113). If IL-17F is a pro-inflammatory mediator in gastric mucosa, then over-expression of this cytokine could compensate for IL-17A in promoting histologic inflammation. However, these findings would lead me to expect increased neutrophilic infiltrate (116, 208) and a consistent increase in IL-6 expression in inflamed mucosa (71), and yet I observed neither event. It is impossible to explain the potential role of IL-17F in *H. pylori*-mediated gastritis at this time.

Inducing cytokines suggest the importance of non-T cell effectors

I consider that the observed changes and differences in IL-12p40 and IL-12p35 expression provide further evidence against the prominence of "Th" phenotype polarized CD4+ T cells in maintaining inflammation in *H. pylori*-infected mice. If IL-12 is required to promote or sustain the development of IFNγ-expressing Th1 polarized CD4+ T cells, then I would expect infection to induce expression of the IL-12 constituent molecules prior to an increase in IFNγ expression. Instead, IL-12p40 expression is only induced in B6 mice in parallel to inflammation, and IL-12p35 is not induced at all until late in the course of infection. Even in adoptive transfer recipients of 17AKO cells, I found increased p40 and p35 expression without a concordant rise in IFNγ mRNA.

In contrast, IL-23 works to maintain the Th17 phenotype in polarized CD4+ T cells, suggesting IL-12p40 expression would be induced after IL-17A expression increased. However, expression of IL-12p40 precedes that of IL-17A in infected B6 mice, and does not appear to influence the expression of IL-17F in 17AKO mice. In

comparison, in infected RAG2-KO mice with 17AKO cells, both IL-12p40 and IL-17F are significantly upregulated, but there is no evidence yet to suggest a link between IL-23 and IL-17F expression.

Resolving *H. pylori* disease and the expression of regulatory cytokines

I added IL-10 and TGFβ to my RT-PCR analysis list initially to explore the poorly understood role of imunomodulatory cytokines in *H. pylori*-mediated gastritis in mice. While some research in human gastritis patients suggests that disease victims suffer from a dearth of regulatory T cells in the gastric mucosa (173), other reports demonstrate the ability of *H. pylori* to induce the development of regulatory T cells (74, 89, 171). Disease in IL-10 deficient mice was more severe and developed with more speed than in B6 controls (132). It is unclear whether this result is due to the hyper-responsiveness of IL-10 deficient T cells to antigen stimulation, or if IL-10 otherwise modulates immune responses through influences over other non-inflammatory cytokines in addition to its reported anti-inflammatory properties.

My findings suggest that IL-10, along with TGFβ, plays a role late in disease in B6 mice, perhaps in an effort to control an active inflammatory response in the face of ineffective bacterial colonization control. In light of this, it is interesting to consider my IL-12p35 results given reports showing IL-12p35 is one component in the immunoregulatory cytokine IL-35 (30). Thus, the rise in IL-12p35 expression at 15 months of infection could be an indication of a late-term increase in the expression of a broad spectrum of regulatory cytokines in *H. pylori* infected mice. This hypothesis does not readily explain the observation of a significantly greater expression of IL-10 and TGFβ in RAG2-KO recipients of 17AKO cells than in mice given B6 cells. That

observation may reflect a difference in the development of regulatory T cells in the absence of a mature Th17 CD4+ cell effector phenotype, but this hypothesis is so far unsupported by the literature for 17AKO mice.

General conclusions

Overall, my results have come far from the experimental questions posed by my initial hypotheses. Still, I can infer several conclusions from these findings. First, IL-17A-expressing, Th17-polarized CD4+ T cells contribute to gastritis in *H. pylori*-infected mice. Next, IFNγ is probably more crucial to the development of histological gastritis in *H. pylori* infected mucosa. Third, the paradigm of helper T cells differentiating into exclusive and contradictory effector phenotypes appears to be an invalid model for the role of CD4+ T cells in *H. pylori*-mediated gastritis. Fourth, previously discounted effectors like innate inflammatory cytokines such as IL-1β and IL-6 may play a more significant role in controlling *H. pylori* colonization and promoting disease development than we have previously understood. Lastly, given the marked increases in regulatory cytokines late in infection, the immune system in chronically infected mice may recognize that the inflammatory response has failed to control *H. pylori* infection and so increases production of anti-inflammatory cytokines to dampen inflammation and reduce tissue damage.

Taken together, I find that my explorations of the contribution of IL-17A to *H. pylori*-mediated gastritis revealed more about the whole response to infection than just the participation of specialized CD4+ T cells. While we demonstrated now and previously that both Th1 and Th17 phenotype helper T cells contribute to gastric pathology in infected mice, I uncovered significant evidence suggesting pro-

inflammatory cytokines of the innate immune response also contribute meaningfully to controlling disease.

FUTURE DIRECTIONS

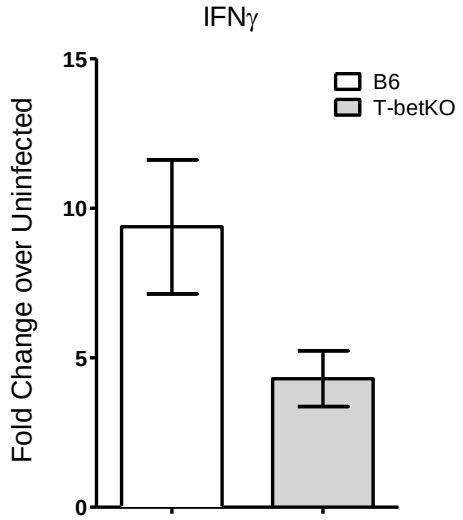
I think that while the work I present here answers many questions about the role of IL-17A in promoting the development of *H. pylori* gastritis, my findings immediately raise several other questions. Foremost, how might IL-17F contribute to *H. pylori* disease, and do the increased mRNA levels of this cytokine reflect an immune response to infection, or just disregulation in response to the loss of IL-17A (208)? IL-17F knockout mice demonstrated reduced neutrophilia in an airway-inflammation model (222), which could mean we would see reduced neutrophilic infiltrate in infected IL-17F mice and IL-17F recipients. To check the contribution of the entire Th17 response at once we could use RORγt knockout mice, which are deficient in both IL-17A and IL-17F signaling (83). However, RORγt -KO mice also lack Peyer's patches and lymph nodes (60), which appear to be crucial for *H. pylori* gastritis development (146, 149), suggesting that we do not use RORγt -KO mice in the simple infection model of disease. We would likely do better to investigate the contribution of Th17-polarized helper T cells to disease development by crossing 17AKO and 17FKO mice to generate double knockout that are functionally deficient for the hallmark Th17 cytokines.

Overall I think that we would do better to investigate the contributions of other pro-inflammatory mediators, specifically IL-1β, IL-6 and TNFα, for which we see dramatic inductions in both simple infection and adoptive transfer experiments. These cytokines are made by myeloid cells including macrophages and dendritic cells, and Kaparakis et al. recently demonstrated that deletion of macrophages reduces *H. pylori*

disease, confirming the importance of the action of innate effector cells (92). Furthermore, expression of these cytokines may influence the behavior of both innate and adaptive effector cells. The contributions of IL-1β and TNFα to *H. pylori* disease have been assessed indirectly in infected IL-1 receptor and TNF receptor 1 deficient mice (1, 205), with reduced disease found in both strains. But the adoptive transfer model allows us to identify both the source and target of these molecules. We could start with single knockout experiments: adoptive transfer of T cells from TNFα-KO, IL-1β-KO or IL-6-KO mice. I expect infected recipients of these CD4+ T cells would develop moderate gastritis, likely equivalent to greater than the degree of inflammation found in recipients of IFNγKO cells (55), as I do not think that T cells are the primary source for these pro-inflammatory mediators. However, if we used cytokine deficient mice as the recipient mice in adoptive transfer experiments or used them in simple infection experiments, I predict that we would see markedly reduced inflammation in response to *H. pylori* infection.

Figure 2.1: IFNγ and IL-17A mRNA in recipients of B6 or T-bet KO CD4+ T cells

A:



B:

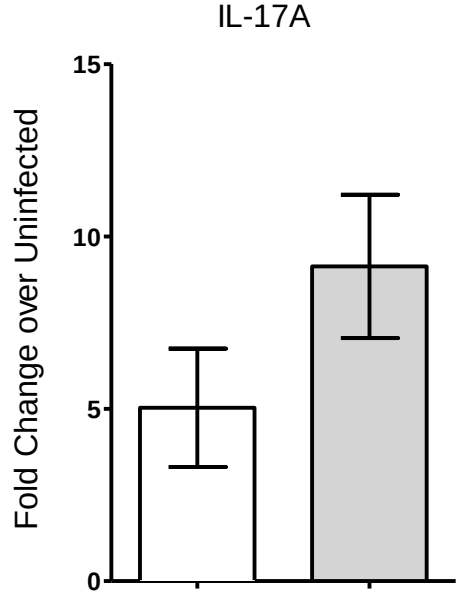


Figure 2.1: mRNA from the gastric mucosa of RAG2-KO mice adoptively transferred with either B6 (open bars) or T-bet KO (shaded bars) CD4+ T cells was analyzed for expression of (a) IFNγ and (b) IL-17A. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1 (see Methods). There were no significant differences observed between infected groups of mice.

Figure 2.2: Photomicrographs of infected RAG2-KO recipients of B6 and 17AKO cells

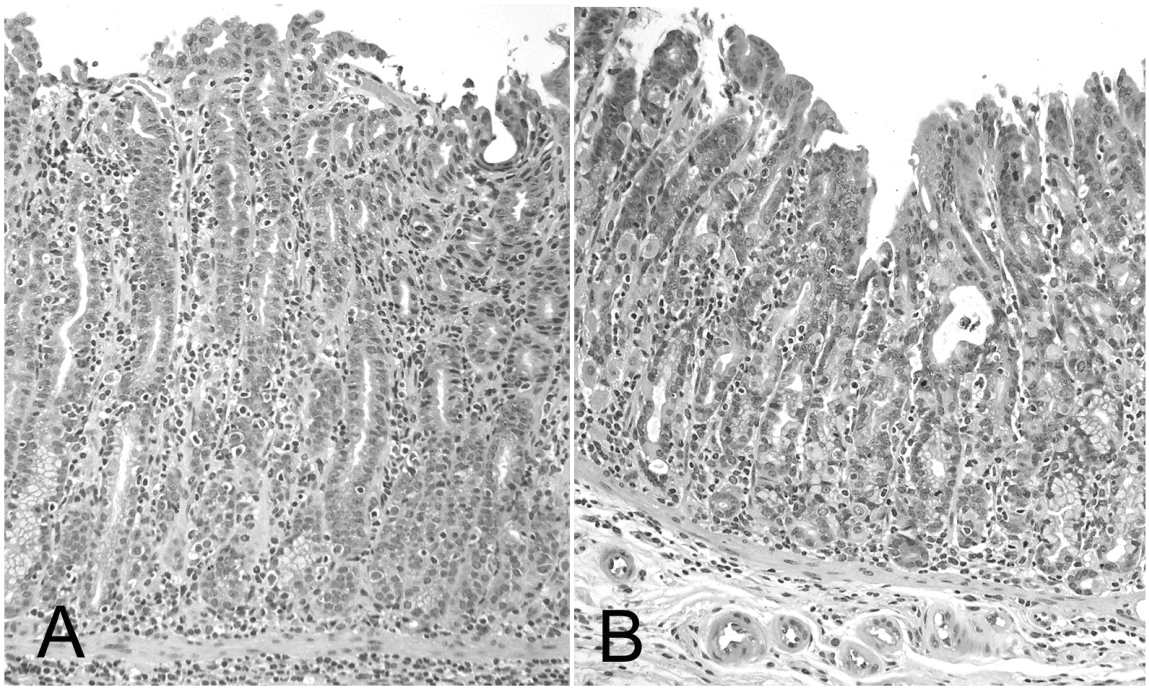


Figure 2.2: Hematoxylin and eosin stained sections of gastric fundus from *H. pylori* infected RAG2-KO mice 8 weeks after transfer with either (a) B6 or (b) 17AKO CD4+ cells. Mononuclear cells and neutrophils can be seen in the inflammatory infiltrate in both sections.

Figure 2.3: Mean gastritis score after adoptive transfer of B6 or 17AKO T cells

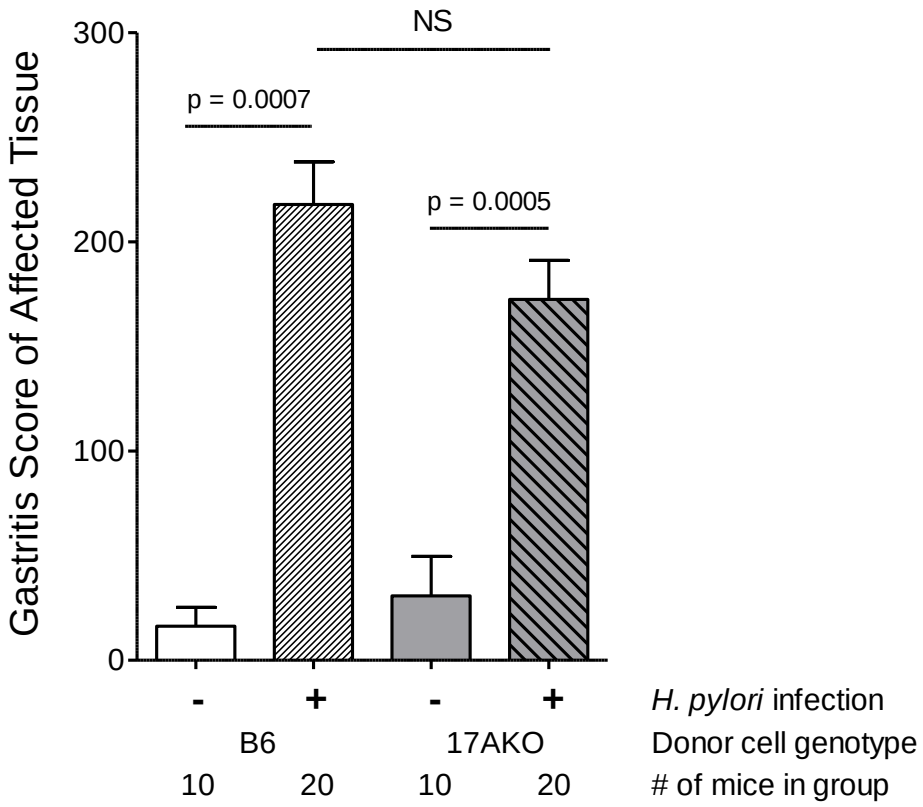


Figure 2.3: Histology score of gastric mucosa in uninfected or *H. pylori* SS1 infected RAG2-KO mice transferred with B6 or 17AKO CD4+ cells, 8 weeks after transfer. Slides were scored as described in Methods. Gastritis developed with infection but was not significantly different between infected recipient groups. The number of mice tested in each group for all adoptive transfer analyses is listed at the bottom of the graph.

Figure 2.4: *H. pylori* colonization after adoptive transfer of B6 or 17AKO T cells

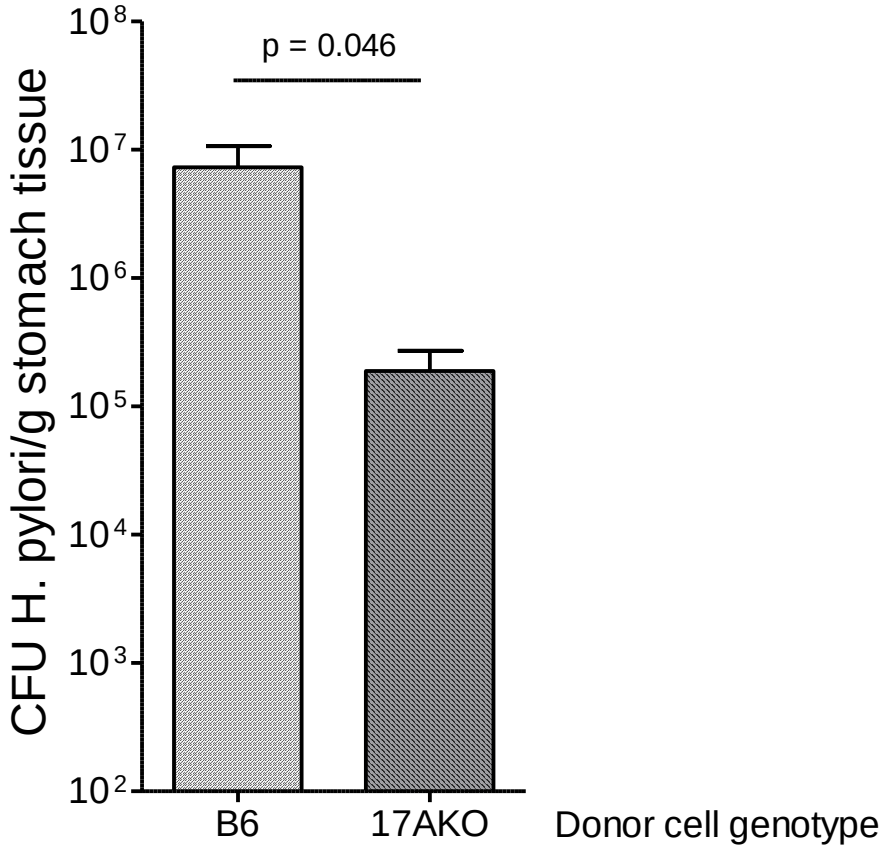


Figure 2.4: Bacterial load in *H. pylori* infected RAG2-KO mice transferred with B6 or 17AKO CD4+ cells, 8 weeks after transfer, 10 weeks after infection. Recipients of 17AKO cells had less bacterial colonization than mice with B6 cells.

Figure 2.5: Induction of IFNγ after adoptive transfer of B6 or 17AKO T cells

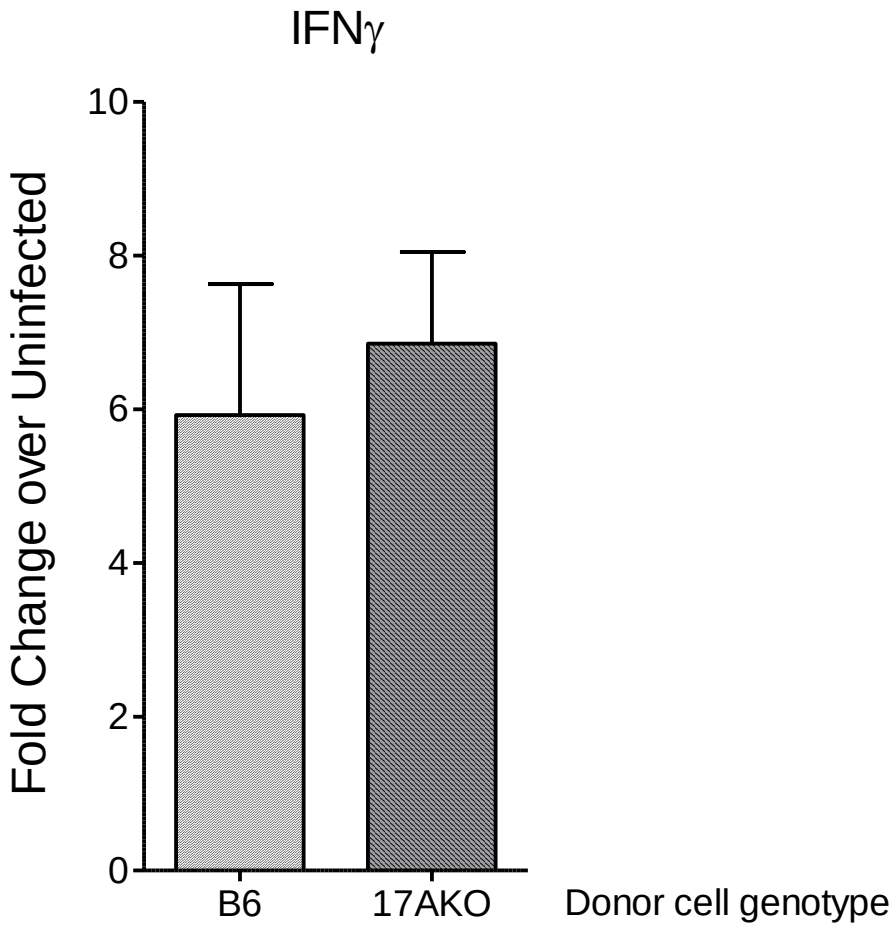


Figure 2.5: IFNγ mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. There is no statistical difference in cytokine induction between groups.

Figure 2.6: Expression of IL-17A in infected RAG2-KO mice with B6 or 17AKO T cells

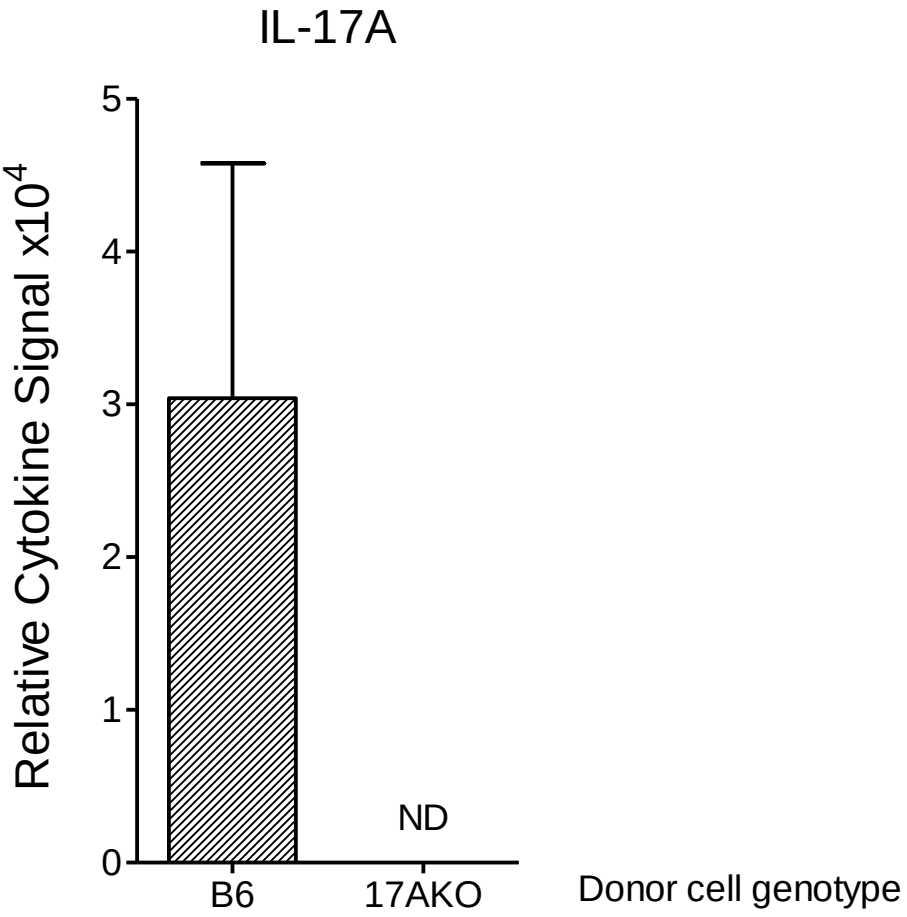


Figure 2.6: IL-17A mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are normalized to GAPDH as the signal was undetectable in uninfected mice. IL-17A expression was undetectable in mice given 17AKO T cells. ND = Not Detected

Figure 2.7: Induction of IL-17F after adoptive transfer of B6 or 17AKO T cells

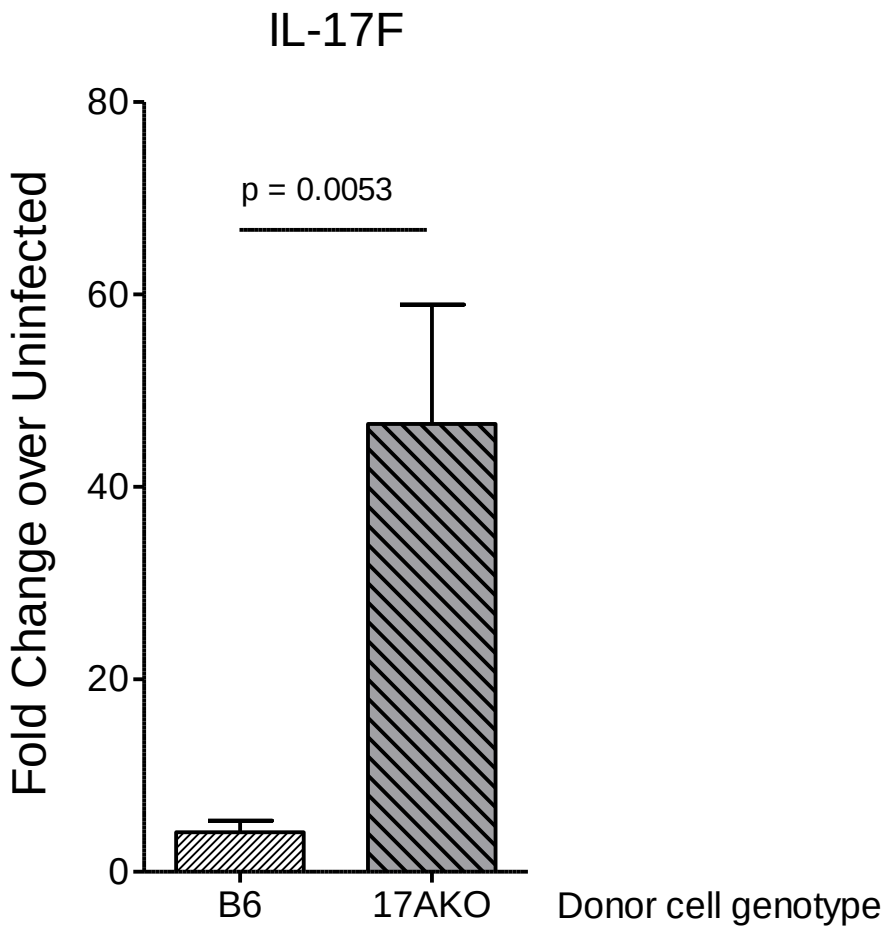


Figure 2.7: IL-17F mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells.

Figure 2.8: Induction of IL-1β after adoptive transfer of B6 or 17AKO T cells

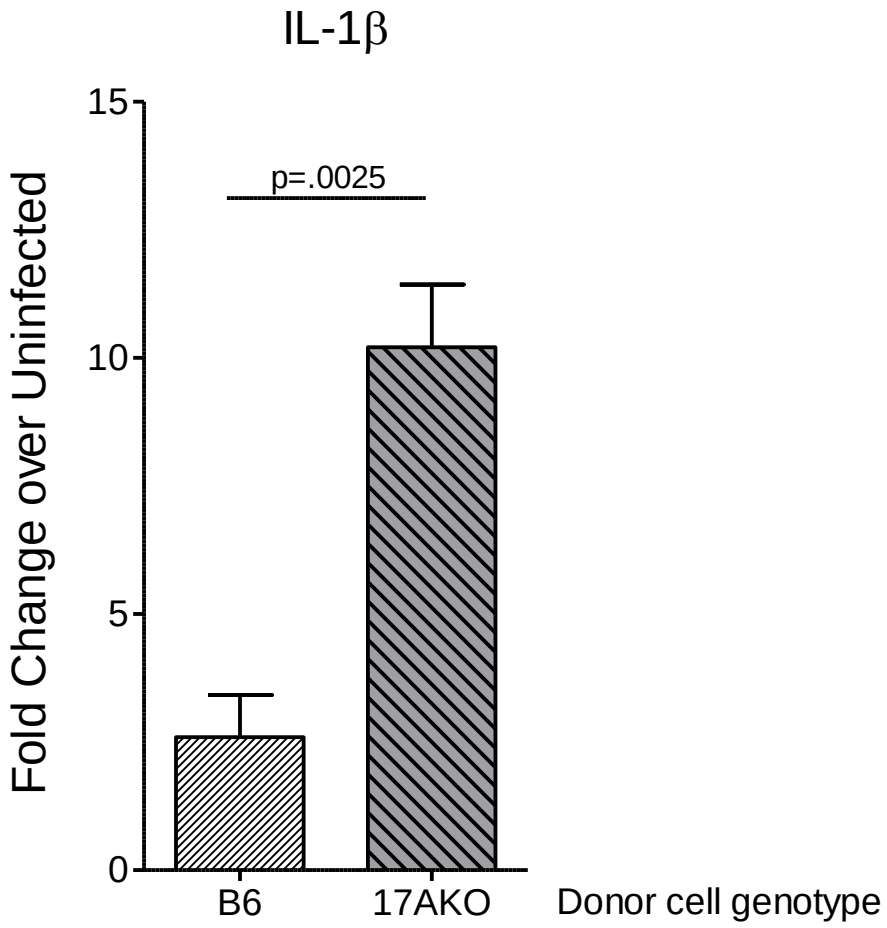


Figure 2.8: IL-1β mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. Induction of the innate inflammatory cytokine was significantly greater in recipients of 17AKO cells.

Figure 2.9: Induction of IL-6 after adoptive transfer of 17AKO T cells

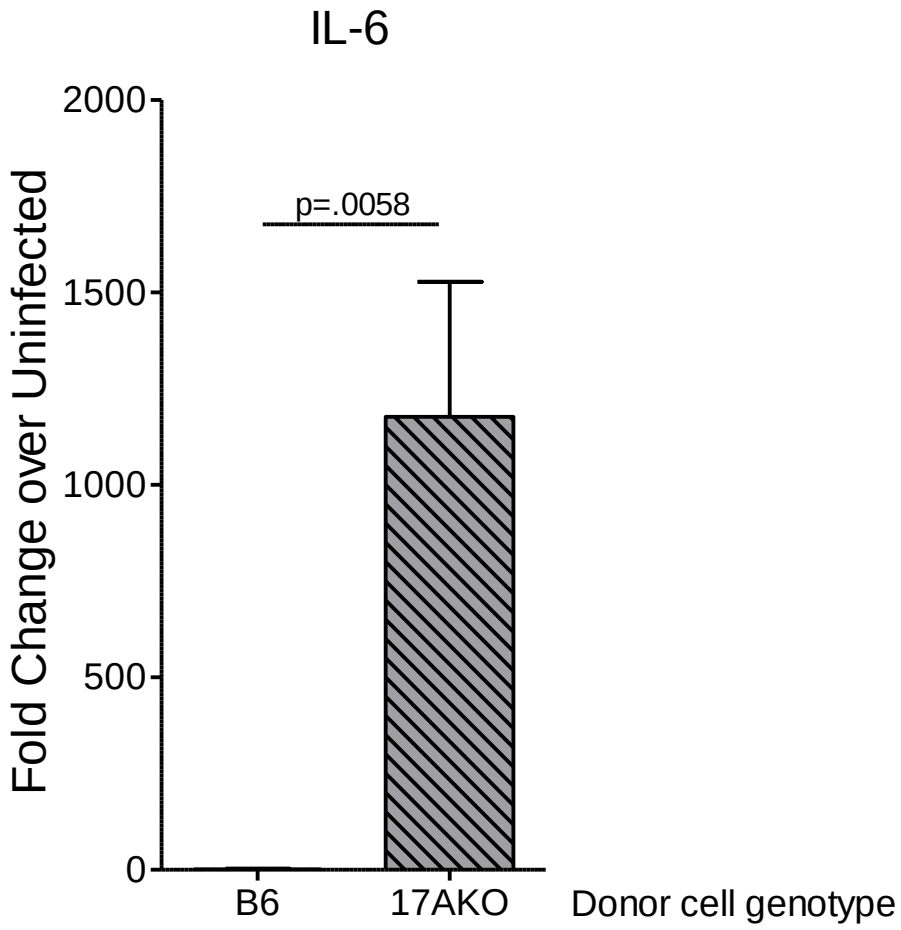


Figure 2.9: IL-6 mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. Induction of the innate inflammatory cytokine was significantly greater in recipients of 17AKO cells than in B6 cells (1.4 ± 1.4 fold induction).

Figure 2.10: Induction of TNFα after adoptive transfer of B6 or 17AKO T cells

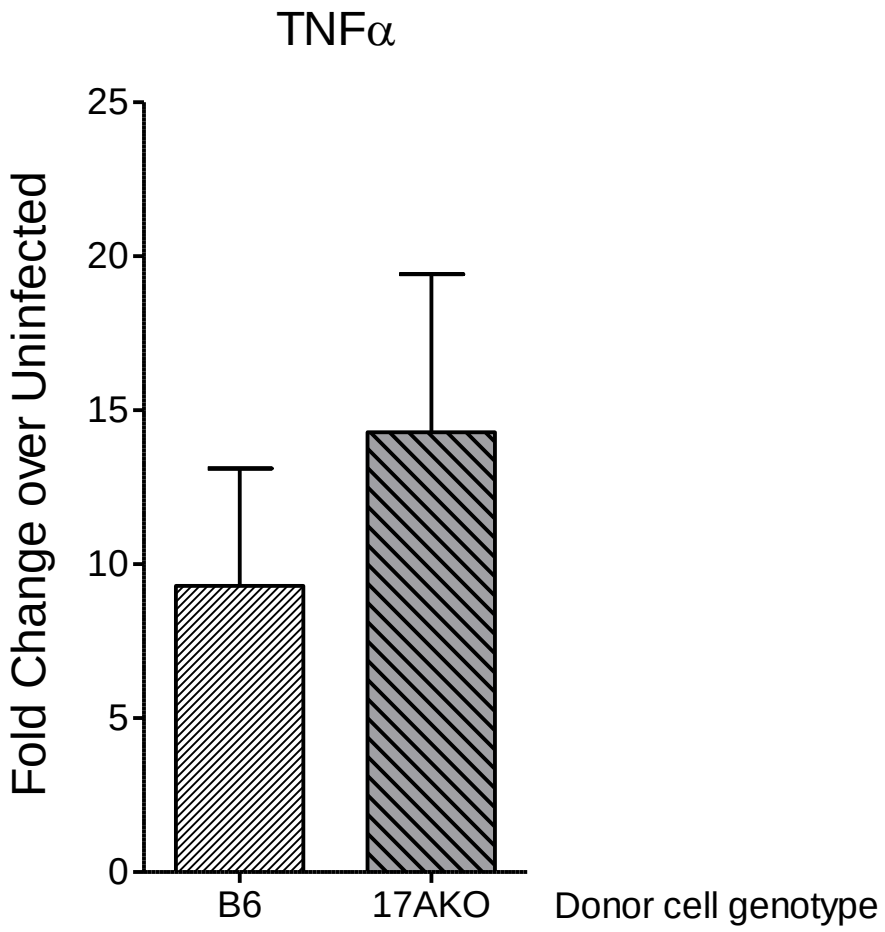
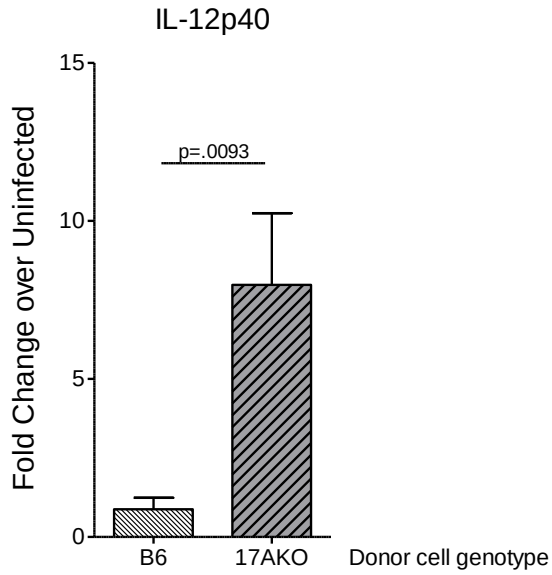


Figure 2.10: TNFα mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. There is no statistical difference in cytokine induction between groups.

Figure 2.11: Induction of IL-12 cytokines after adoptive transfer of B6 or 17AKO T cells

A.



B.

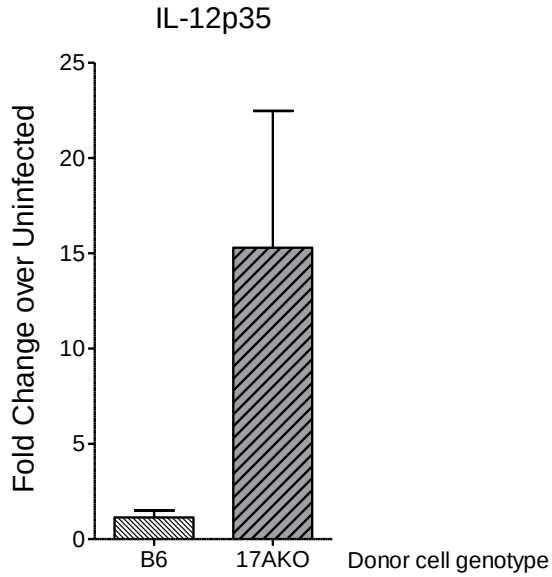
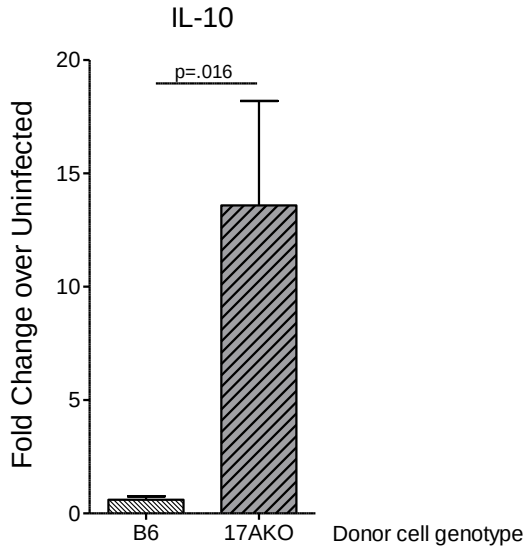


Figure 2.11: IL-12p35 and p40 mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. There is no statistical difference in induction of IL-12p35 between groups, likely due to wide individual variation between 17AKO recipients.

Figure 2.12: Induction of regulatory cytokines after adoptive transfer of B6 or 17AKO T cells

A.



B.

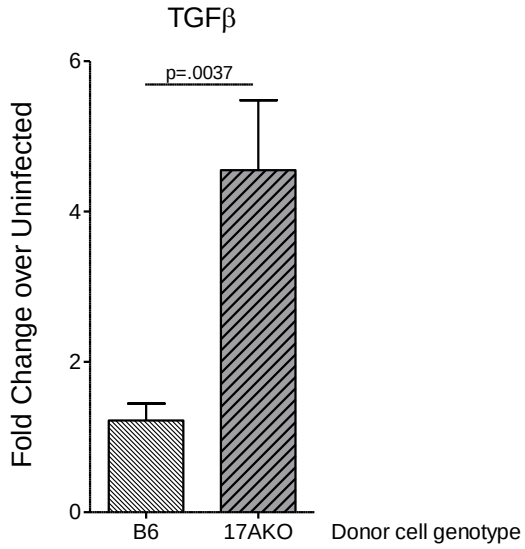


Figure 2.12: IL-10 and TGFβ mRNA from the gastric mucosa of recipient mice in adoptive transfer experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. Regulatory cytokines were significantly increased in the stomachs of 17AKO recipient mice.

Table 2.1: Summary of number of mice at each time point for each strain in simple infection experiments.

Mouse strain	Number at 3 months	Number at 6 months	Number at 9 months	Number at 12 months	Number at 15 months
B6	5	5	5	5	5
17AKO	4[a]	5	4[a]	5	5
T-betKO	5	5	3[a]	4[a]	2[a]

[a] Unexpected health difficulties secondary to the cytokine deficiencies in these mice meant that some mice died before I was able to collect samples. It is impossible to calculate any statistical significance for any samples examined from T-bet KO mice at 15 months after infection.

Figure 2.13: Photomicrographs of simple infection of B6, 17AKO and T-bet KO mice

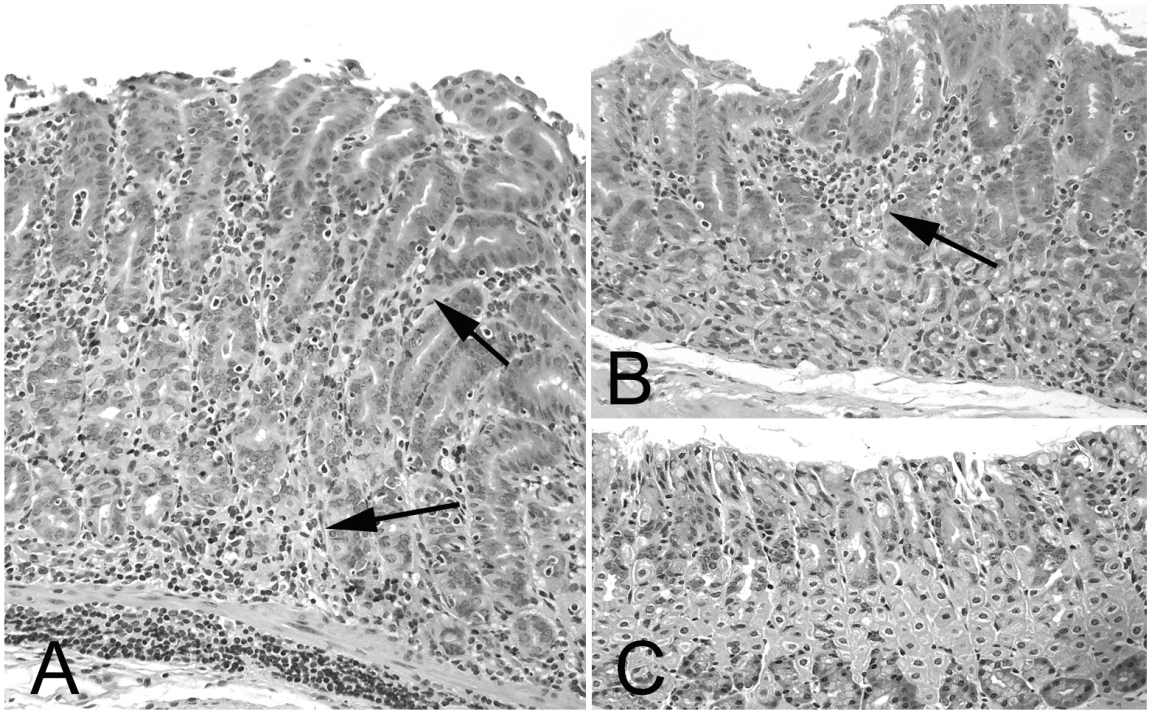


Figure 2.13: Hematoxylin and eosin stained sections of gastric fundus from *H. pylori* infected mice 9 months after infection. (a) B6 mice, (b) 17AKO mice, (c) T-betKO mice. Histological sections were scored as described in Methods. Arrows point to focal lesions with dense inflammatory infiltrate.

Figure 2.14: Mean gastritis score in simple infection in B6 and cytokine deficient mice

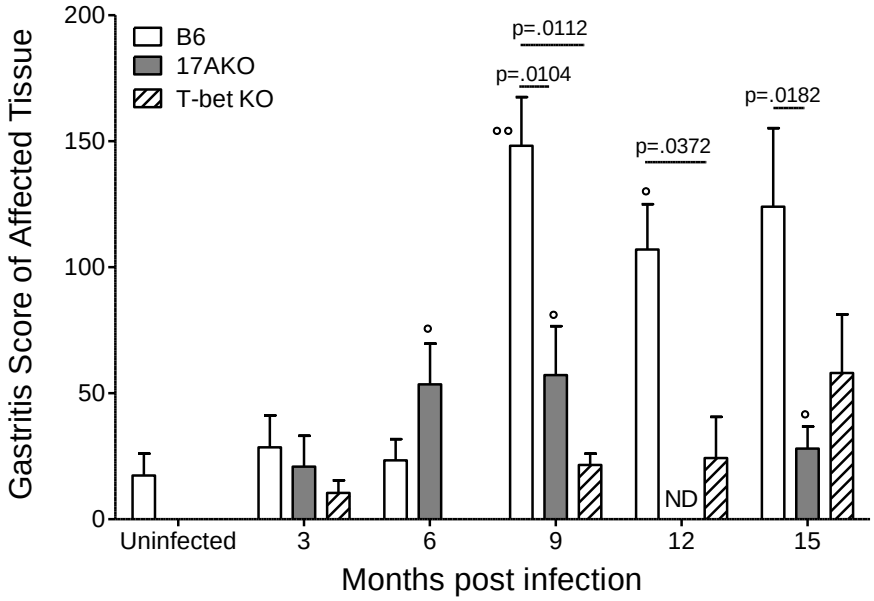


Figure 2.14: Histology scores in B6, 17AKO and T-bet-KO mice infected with *H. pylori* SS1. Slides were scored as described in Methods. ND = Not done, as I lack data for the 12 month time point in 17AKO mice due to an error in tissue fixation. ° $p<0.05$ when compared to uninfected mice of the same strain; °° $p<0.01$ when compared to uninfected mice of the same strain. When there is no marker over a column, then it is not significantly different from either uninfected mice of the same strain (circles) or from infected B6 mice (lines).

Figure 2.15: *H. pylori* colonization in simple infection of B6 and cytokine deficient mice

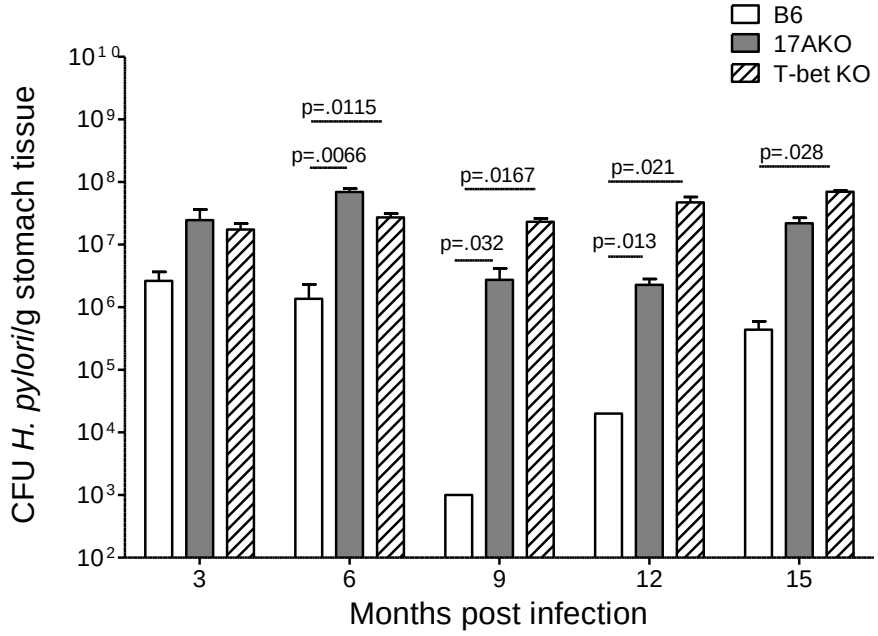


Figure 2.15: Bacterial load in B6, 17AKO and T-bet KO mice infected with *H. pylori* SS1, as colony forming units per gram of stomach tissue over the course of infection. When there is no indicator, colonization is not significantly different from *H. pylori* density in B6 mice.

Figure 2.16: Induction of IFNγ in gastric mucosa in simple infection

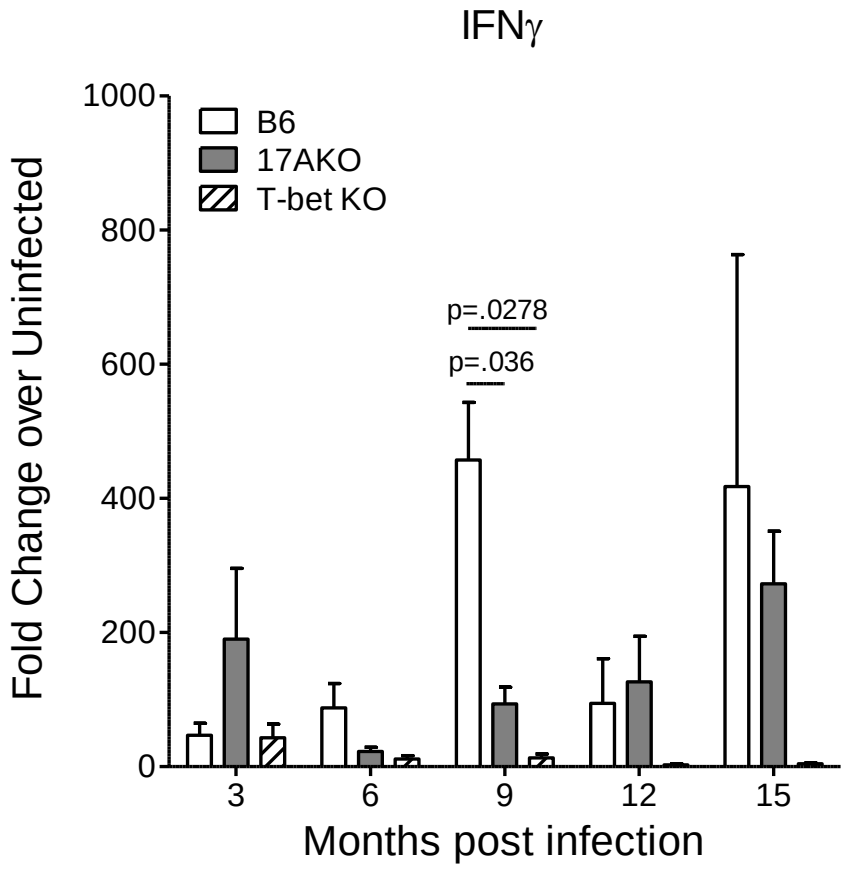


Figure 2.16: IFNγ mRNA from the gastric mucosa in simple infection experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. When there is no marker over a column, then it is not significantly different from cytokine induction in B6 mice.

Figure 2.17: Expression of IL-17A in gastric mucosa in simple infection

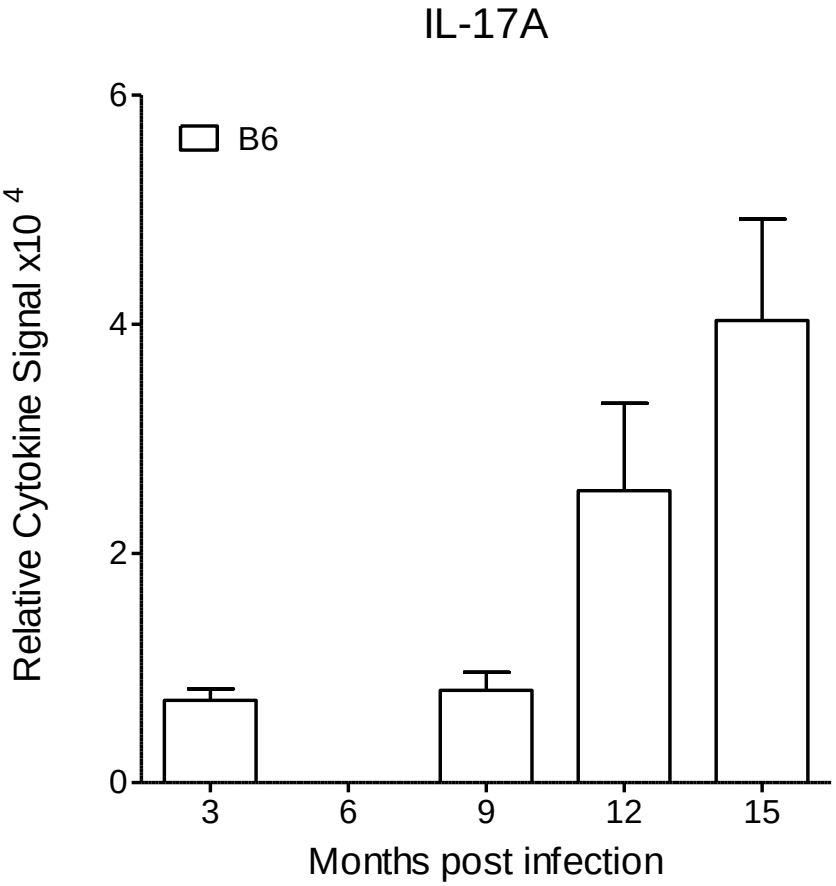


Figure 2.17: IL-17A mRNA from the gastric mucosa of *H. pylori* infected B6 mice. Results are normalized to GAPDH as the signal was undetectable in uninfected mice. IL-17A expression was not detected in either infected 17AKO or T-bet KO mice (not shown).

Figure 2.18: Induction of IL-17F in gastric mucosa in simple infection

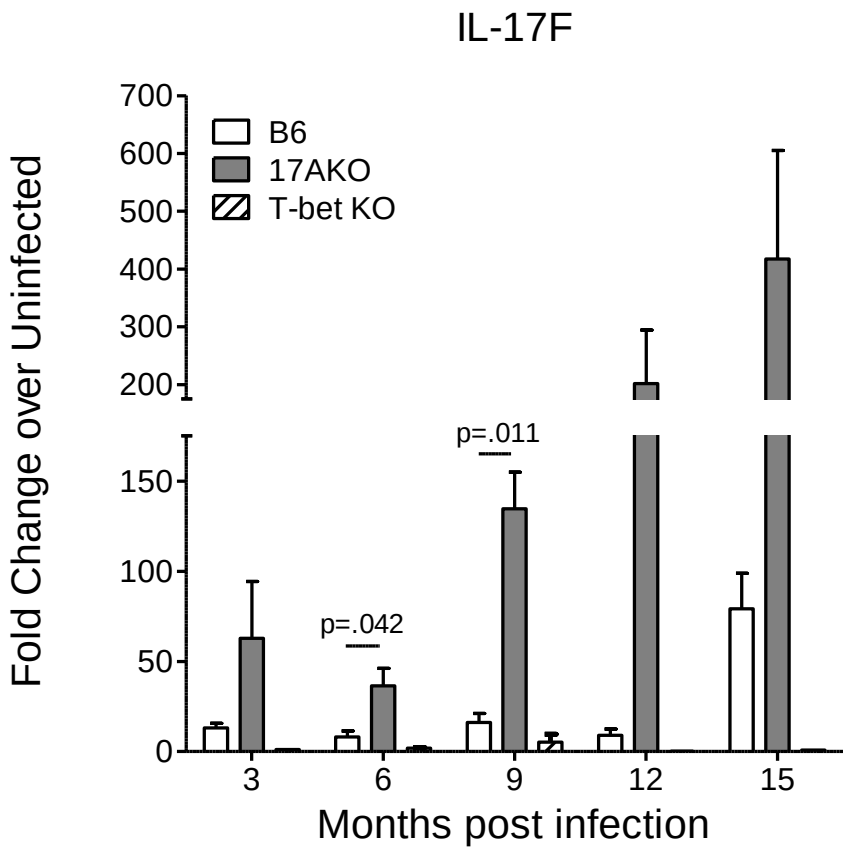


Figure 2.18: IL-17F mRNA from the gastric mucosa in simple infection experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. When there is no marker over a column, then it is not significantly different from cytokine induction in B6 mice. The lack of significance in IL-17F induction late in infection is probably due to the wide individual variation in expression in recipients of 17AKO cells.

Figure 2.19: Induction of IL-1β in gastric mucosa in simple infection

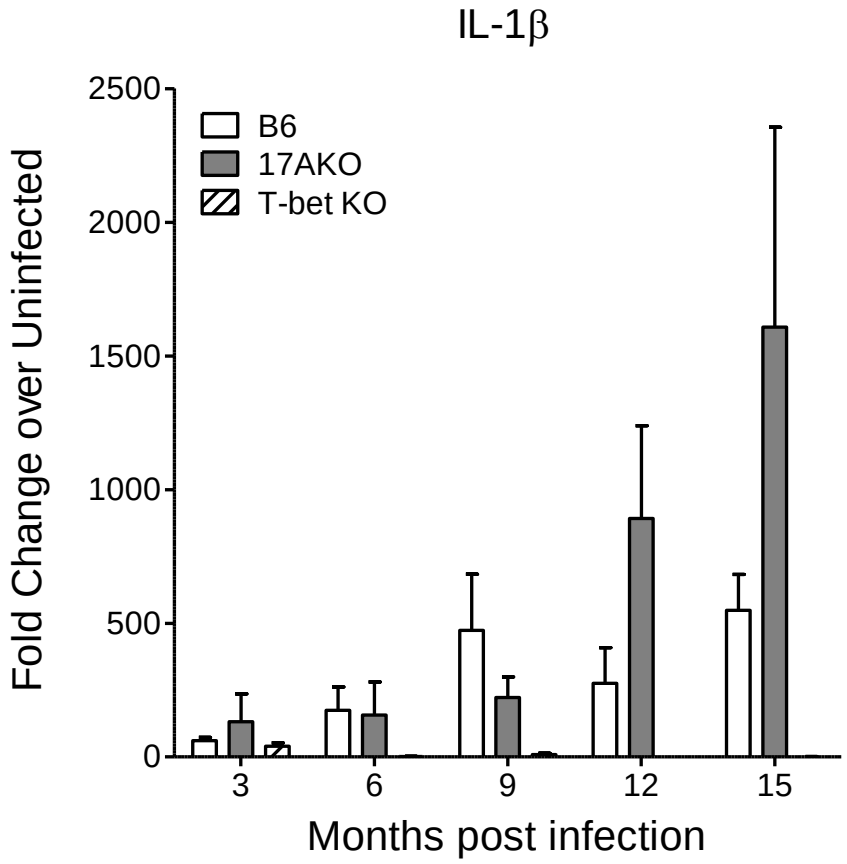


Figure 2.19: IL-1β mRNA from the gastric mucosa in simple infection experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. When there is no marker over a column, then it is not significantly different from cytokine induction in B6 mice. Thus, there are no significant differences in induction of IL-1β expression with *H. pylori* infection, but the trends are strongly suggestive.

Figure 2.20: Induction of IL-6 in gastric mucosa in simple infection

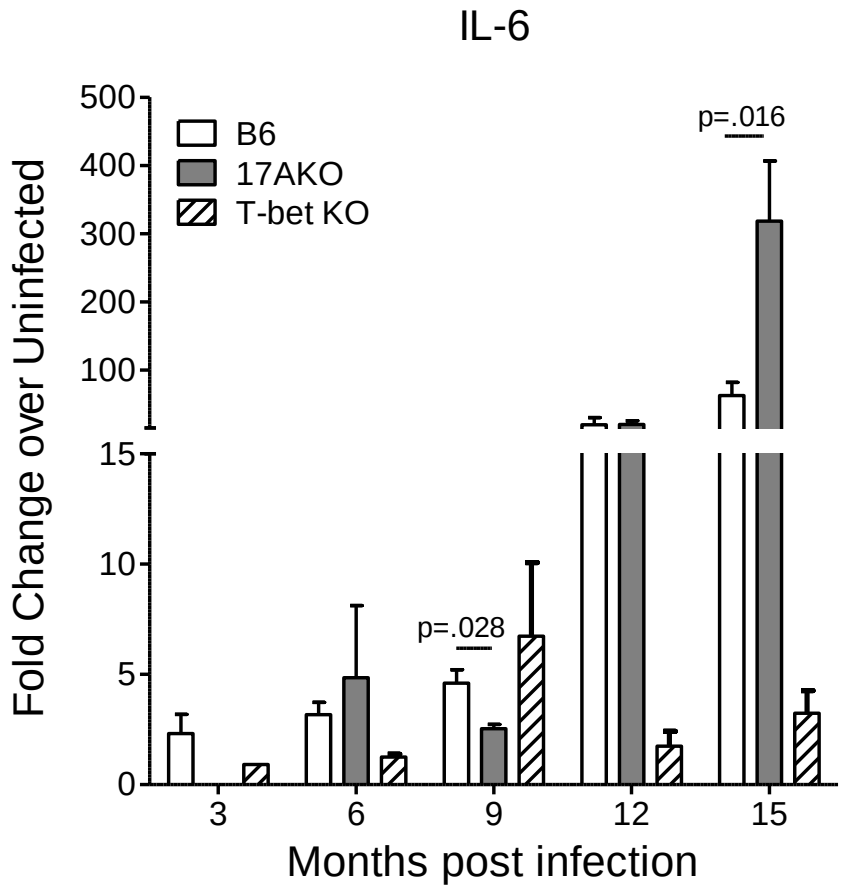


Figure 2.20: IL-6 mRNA from the gastric mucosa in simple infection experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. When there is no marker over a column, then it is not significantly different from cytokine induction in B6 mice.

Figure 2.21: Induction of TNFα in gastric mucosa in simple infection

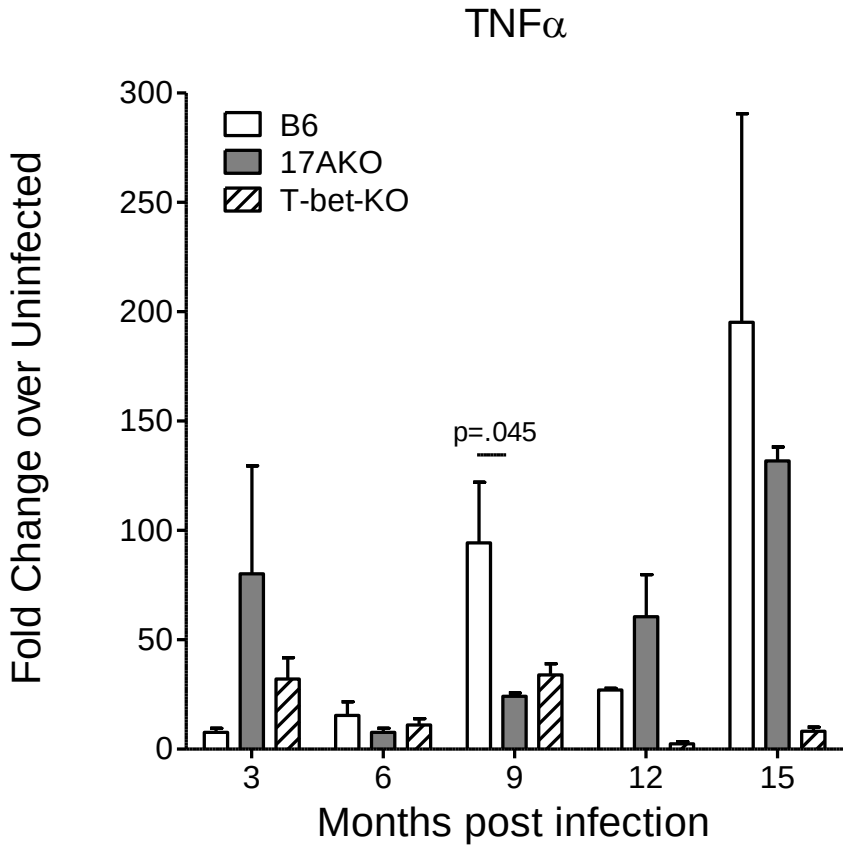
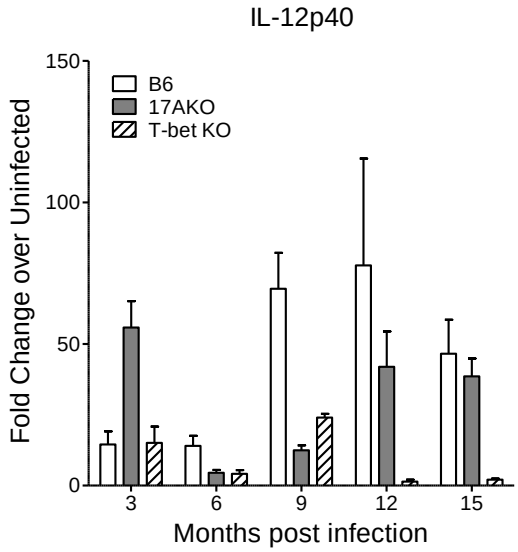


Figure 2.21: TNFα mRNA from the gastric mucosa in simple infection experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. When there is no marker over a column, then it is not significantly different from cytokine induction in B6 mice.

Figure 2.22: Induction of IL-12 cytokines in gastric mucosa in simple infection

A:



B:

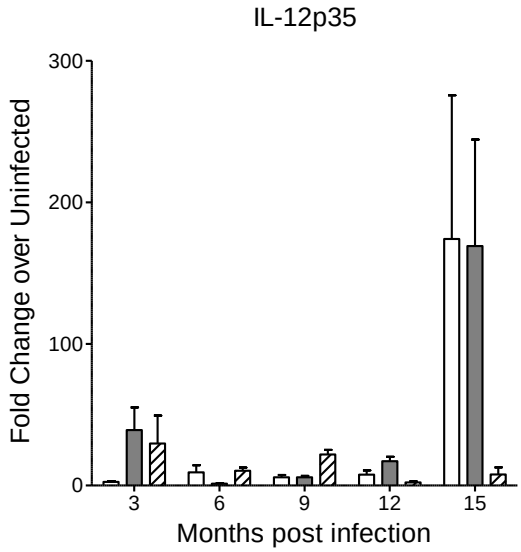
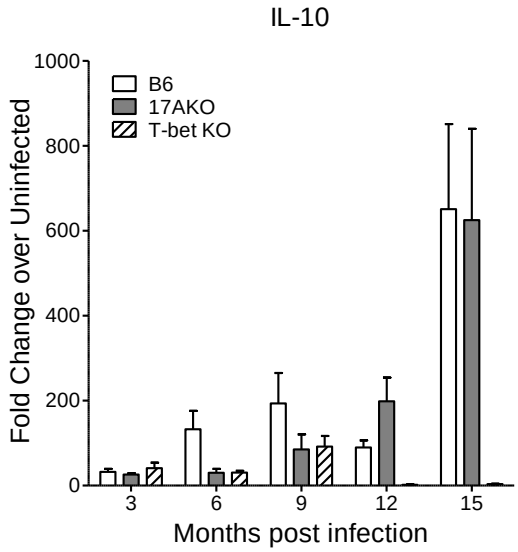


Figure 2.22: IL-12p40 and IL-12p35 mRNA from the gastric mucosa in simple infection experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. There were no significant differences in induction of either IL-12 cytokine between any of the groups of mice examined.

Figure 2.23: Induction of regulatory cytokines in gastric mucosa in simple infection

A:



B:

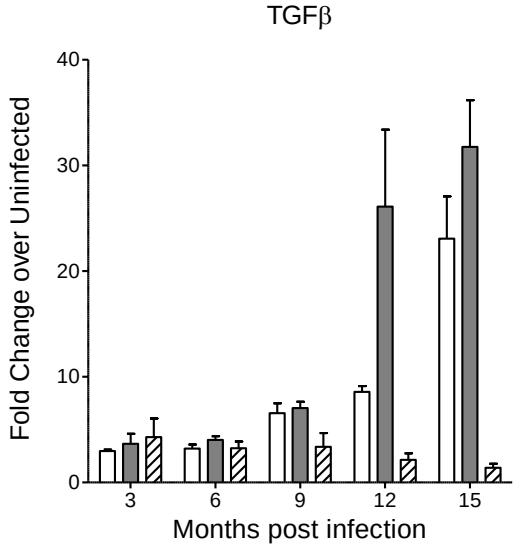


Figure 2.15: IL-10 and TGFβ mRNA from the gastric mucosa in simple infection experiments. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. There were no significant differences in the induction of either regulatory cytokine between any of the groups of mice, although expression trends upward with time in both infected B6 and 17AKO mice.

METHODS

Mice strains and housing

Helicobacter-free C57Bl/6J mice (B6) were purchased from Jackson Laboratories (Bar Harbor, ME), and congenic T-bet KO and RAG2-KO mice were bred and maintained in-house at the University of Michigan. IL-17A KO mice (17AKO) were a generous gift from Dr. Yoichiro Iwakura of the Center for Experimental Medicine in Tokyo, Japan (147). No known mouse pathogens are present in the mouse colonies as determined by routine periodic screening of sentinel mice. Mice were maintained in static microisolator cages and offered non-supplemented commercial mouse chow and water *ad libitum*. The University of Michigan Animal Care and Use Committee approved all animal experiments.

H. pylori culture and inoculation

Plate cultures of *H. pylori* SS1 were used to inoculate overnight broth cultures in 10 mL of Brucella broth with 10% fetal calf serum and 50 μl of Skirrow's antibiotic supplement. Cultures were centrifuged and washed once and then resuspended in sterile phosphate-buffered saline (PBS), counted on a hemacytometer, and diluted to a final concentration of $1x10^8$ bacteria/mL. B6, 17AKO, T-bet KO, and RAG2-KO mice were given 100 μl of sterile 0.5 M Na_2CO_3 via gastric feeding tube followed by100 μl of the bacterial suspension giving a total dose of $1x10^7$ of *H. pylori* SS1 per mouse. This procedure was repeated on the following day, for a total of two inoculations.

Adoptive transfer

Two weeks after the RAG2-KO mice were inoculated with *H. pylori*, we harvested spleens from WT or 17AKO mice. Splenocytes were dissociated and then

suspended in 450 μL of 1x PBS with 0.5% bovine serum albumin, and mixed with 50 μL of anti-CD4 L3T4-coated Miltenyi MACS Beads (Miltentyi Biotec, Auburn, CA) for 15 minutes at 4 °C. The cell-bead mixture was layered onto a pre-wetted Miltenyi MS magnetic column and washed twice in MACS buffer, before the columns were removed from the magnet and the cells were removed with an additional 500 μL of buffer. The CD4-enriched cells were washed twice in media and resuspended to a concentration of $1x10^7$ cells/mL in sterile PBS.

Groups of both uninfected and *H. pylori*-infected RAG2-KO mice received 100 μL of either WT or 17AKO cell suspension via intraperitoneal injection, for a final adoptive transfer of $1x10^6$ CD4-enriched cells per mouse.

Tissue collection, histology scoring and RNA profiling

For the simple infection model, 4-5 mice were euthanized at 3, 6, 9, 12 and 15 months after infection and stomachs were harvested for histology, assessment of bacterial load, and RNA isolation. Adoptively transferred mice were euthanized at 8 weeks after transfer, a total of 10 weeks after infection, and their stomach tissues were harvested in the same way. For histology, 1 mm-wide strips from the greater curvature were emersion-fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5μm sections, and stained with hematoxylin and eosin. Bacterial colonization was quantified by plate counts of serially diluted gastric homogenate.

For RNA isolation samples were stored for no more than three months in 300 μl of TRIzol at –80 ºC (Invitrogen, Carlsbad, CA) and total RNA was purified according to the TRIzol protocol. mRNA was then further enriched with Qiagen RNEasy columns, and stored at –80 ºC until analysis. Using SA Biotech's cDNA first strand synthesis kit

and protocol (SA Biosciences, Frederick, MD), mRNA samples were prepared for qPCR of mouse cytokine genes, which was performed on custom SuperArrays (CAPM-0752A) using SA Biotech's SYBR green master mix and protocol, on a Stratagene MX3000p thermal cycler. The arrays included probes for the following genes: IL-1β, IL-6, IL-17A, IL-17F, IFNγ, TNFα, and GAPDH, which served as housekeeping control.

Extent of gastritis was scored as previously described (51). Briefly, adjacent 200x microscopic fields were examined for the presence of gastric infiltrate severe enough to displace glands, presence of neutrophilic inflammation, and/or presence of gastric epithelial metaplasia. Two longitudinal sections of gastric fundus were scored in their entirety, and the percentage of positive fields in all three categories was added together to calculate the total score. All sections were scored blind, without prior knowledge of their source.

Data handling and statistical analyses

All graphs were made using GraphPad Prism 5.01 software. This program automatically calculated and graphed the standard error of the mean (SEM) for all column graphs. All matching datasets were tested for statistical significance through application of two-tailed Student's t-test using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Experimental groups that are not statistically significantly different are unmarked.

For qPCR analyses, SYBR Green cycle threshold was established automatically using the Stratagene MX Pro software, based on ROX fluorescence. Any well with a SYBR Green cycle threshold greater than 35 cycles was counted as a negative result. Expression of cytokine genes was normalized to GAPDH expression, and we calculated

the fold-change in mRNA levels by averaging the values of every uninfected mouse in a group, and then dividing the result from each infected mouse by this.

Chapter 3

MHC II Expression by CD4+ T Cells Suppresses *H. pylori*-Specific T Cell Responses But Not Gastritis

ABSTRACT

Helicobacter pylori is associated with chronic gastritis, peptic ulcer disease, gastric carcinoma, and gastric MALT lymphoma. Disease occurs only in a subset of infected individuals, however, most likely because of host differences in immune response and subsequent severity of gastritis. Gastritis is T cell-dependent, and associated with Th1 bias and IFNγ expression in gastric mucosa, but the specific factors that contribute to differences in host response remain poorly understood. In this chapter I report on a collaborative investigation into the contribution of thymocyte-selected CD4+ T cells (T-CD4) to the pathogenesis of immune response, gastritis, and bacterial clearance in *H. pylori*-infected mice. CIITA transgenic (CIITA-Tg) mice express MHCII on thymocytes, and generate T-CD4 as well as endothelium-selected CD4 T cells (E-CD4). We compared *H. pylori*-infected SCID recipients of CIITA T (Tg-CD4) cells with infected recipients of congenic C57BL/6 T cells (B6 CD4). Gastric IFNγ, TNFα, and IL-1β expression were elevated in infected recipient mice compared to uninfected mice regardless of donor T cell phenotype. Gastritis and bacterial colonization did not differ in the two recipient groups. T cells recovered from both groups were viable and capable of producing cytokines in response to CD3 stimulation. However, while B6 CD4 T cells recovered from infected mice produced IFNγ in response to *H. pylori* antigen, recovered

Tg-CD4 cells were profoundly hyporesponsive to *H. pylori* antigen. In another experiment we compared B6 and CIITA-Tg mice directly infected with *H. pylori* and found no differences in gastritis or bacterial colonization, although CIITA-Tg mice expressed less IFNγ in response to infection that did B6 mice. Total numbers of IFNγ+ CD4+ T cells declined with infection in both B6 and CIITA-Tg mice, but the population of *H. pylori*-specific IFNγ+ CD4+ T cells increased in both groups of mice. These results suggest that 1) MHC II transgenic T cells that produce both IFNγ and IL-4 may regulate antigen-specific T helper responses, and 2) T cell-dependent gastritis due to *H. pylori* is independent of antigen-specific Th1 and Th17 cells.

BACKGROUND

H. pylori gastritis in humans is an unusual inflammatory disease in that the immune response is not protective, and there is no association between the severity of gastritis and the level of bacterial colonization (212). This is in contrast to mouse models, in which gastritis results in suppression of bacterial colonization (55, 56), and vaccination has been at least partly effective (for review, see (2)). In most models, the extent of gastritis is inversely proportional to the bacterial colonization density, and in some models in which inflammatory response is severe, bacterial colonization is eventually eliminated, and the gastric mucosa returns to normal morphology (56, 132). In humans, although there have been scattered reports of spontaneous clearance of infection (130), most people, once infected, remain so for life, regardless of disease severity.

A possible mechanism for this difference in immune clearance between humans and mice is the difference in the early development and thymic education of CD4+ T

cells in the two species. In mice, MHC II expression is limited to thymic stromal epithelial cells, and T helper cell selection is epithelium limited (114). In humans, both thymic epithelial cells and some developing T cells express MHCII, and T cell selection can occur via contact with either epithelial cells or thymic T cells. Thus, mice have a single population of epithelium-selected T cells (E-CD4 cells), while humans have a mixed population of E-CD4 and thymocyte-selected T cells (T-CD4 cells).

Li et al. and Patel et al. described a transgenic mouse line in which the human class II transactivator was placed under control of the murine CD4+ promoter (CIITA-Tg) (114, 162). These mice generate populations of both E- and T-CD4+ T-cells allowing us to examine how this mixed population typical of human T cell development influences T cell subsets and response to infectious agents and it has been shown that T – CD4 and E-CD4 cells respond differently to mitogenic stimulation. Normal E-CD4 cells harvested from B6 mice express IFNγ in response to mitogenic stimuli *in vitro* under Th1 polarizing conditions, but under the same conditions, mixed populations of E- and T-CD4 cells from CIITA-Tg mice produce both IFNγ and IL-4, the hallmark cytokines of Th1 and Th2 responses, respectively (115). Thus these mice provide a tool to investigate the role of T-CD4 cells in susceptibility to *H. pylori* colonization, as well as to determine how the co-expression of IFNγ and IL-4 affects antigen-specific immune response to a Th-1 inducing pathogen.

EXPERIMENTAL DESIGN

Drs. Chang and Sofi approached me after I presented some of the initial findings I reported in Chapter 2. They wished to explore the functionality of their CIITA-Tg mice after determining that Tg CD4 cells produce significantly less IL-17A production (since

reported in (188)). Together we hoped that by combining the potential Th17 defect in their transgenic mice with our apparently Th17-dependent system, both groups could gain useful insights into our respective areas of investigation. We hypothesized that we would find reduced gastritis in infected mice with CIITA-Tg CD4+ T cells.

We chose to start with adoptive transfer experiments to quickly test our hypothesis, and analyzed the mice initially according to the Eaton lab's protocol: I took samples for quantitative culture, histological staining, and the recent addition of mRNA profiling. For mRNA analysis of gastric mucosa, I measured the expression levels of IFNγ, IL-4 and IL-17A to follow helper T cell cytokines that should be affected by the CIITA transgene. I also checked IL-12p35 and p40 to track induction of helper T cell phenotypes, and IL-1β, IL-6 and TNFα to follow the innate immune response, as well as IL-10 and TGFβ1 to track regulatory cytokines.

On subsequent adoptive transfer experiments we expanded our analyses to include measuring cytokine profiles T cells recovered from the recipient mice. At the time of sample collection, Dr. Sofi harvested the spleens from our recipient mice and re-isolated CD4+ cells from these animals. He stained a small aliquot of these cells for flow cytometry analysis, to confirm the presence of engrafted CD4+ cells in adoptively transferred mice (data not shown). Dr. Sofi then stimulated the remaining recovered cells in one of two ways. Using half of the cells, he non-specifically activated the T cells via plate-bound anti-CD3 and soluble anti-CD28 antibodies in order to expand the whole T cell population. The other half of the recovered cells were co-cultured with splenic antigen presenting cells pre-loaded with *H. pylori* lysate to expand the antigen-specific T cells. After five days of culture under either condition, Dr. Sofi briefly restimulated the

cells with mitogen and then collected the culture supernatants and assayed them for IFNγ, IL-4, or IL-17A levels to check Th-specific cytokine production.

At the same time as we started the second round of adoptive transfer experiments, we also began one simple infection experiment with CIITA-Tg mice. We sacrificed the mice after 12 weeks for a number of logistical reasons, including concerns over an ongoing *Helicobacter spp.* infection in the animal facilities, which we felt could confuse our results. We sacrificed these mice and I took samples for histology, culture and mRNA profiles. Due to the availability of reagents, I only analyzed the mRNA levels of IFNγ, IL-17A, TNFα, IL-12p35 and p40, and IL-10. Dr. Sofi harvested the spleens from these mice, isolated CD4+ T cells, and co-cultured with plate-bound anti-CD3 and soluble anti-CD28 or with *H. pylori*-loaded antigen presenting splenocytes to later test them for antigen-specific cytokine production via flow cytometry. He then stained for cytoplasmic cytokine expression as an indirect way to assess the cytokine profiles of the mice tested.

RESULTS

Histology and bacterial colonization in adoptive transfer

As expected, severe, widespread gastritis with gastric epithelial metaplasia was present in *H. pylori*-infected SCID recipients of B6 CD4+ T cells (Figure 3.1). Gastritis was also present in *H. pylori*-infected recipients of Tg-CD4 cells. Gastritis was extensive in both groups, and there was no statistical difference in extent of gastritis or bacterial colonization between the groups that received B6 cells and those that received Tg-CD4 cells (Table 3.1). Uninfected mice did not develop gastritis.

Gastric mucosal cytokine expression in adoptive transfer

IFNγ expression was markedly induced by *H. pylori* infection, regardless of the source of transferred CD4+ cells, but the increase in IFNγ expression (8-fold) was significantly greater in infected mice receiving B6 cells than in mice with transgenic T cells (2.6-fold) as shown in Figure 3.2. In contrast, neither Th17 nor Th2 associated cytokines were significantly elevated in any group above a 2-fold threshold. IL-17A levels did not differ significantly between any of the experimental groups, regardless of infection or CD4+ status (Figure 3.3). In comparison to the results presented in Chapter 2, I was able to detect some IL-17A expression in uninfected, recipient SCID mice, allowing me to calculate the fold induction of IL-17A expression in response to infection. IL-4 expression in all tissues was at or below the limit of detection in all groups (data not shown).

Infection led to significant but similar increases in innate pro-inflammatory cytokines in both groups of mice, with induction of IL-1β reaching 10-fold on average (Figure 3.4). IL-6 expression was the least induced of the innate cytokines, with barely a 2-fold increase in mRNA level for either group (Figure 3.5), and TNFα expression increased by nearly 4-fold on average (Figure 3.6). We also found no difference in IL-10 expression induced by *H. pylori* infection, as both groups of mice saw an average 2-fold increase in mRNA levels (Figure 3.7). All other cytokines tested demonstrated less than a 2-fold change in mRNA expression between infected and uninfected mice, suggesting their expression may not be biologically relevant in this model.

Cytokine responses of CD4+ splenocytes from recipient mice

CD4+ cells were recovered from all groups of recipient SCID mice regardless of donor or infection status. CD4+ cells were 7.2 ± 0.6% of total splenocytes from uninfected B6 cell recipients; 30.9 ± 1.9% in infected B6 CD4+ recipients; 6.5 ± 1.0 % in uninfected Tg-CD4 recipients; 28.0 ±1.4 % in infected Tg-CD4 recipients. In all cases, recovered cells were viable and produced cytokines in response to non-specific stimulation. In response to plate-bound CD3, recovered cells from all groups of mice produced between 3500-7200 pg/ml of IFNγ (Figure 3.8) and 1500-3800 pg/ml of IL-17 (Figure 3.9), and there was no difference in any of the groups, regardless of donor cell type or infection. Tg-CD4, but not B6 cells produced IL-4 in response to CD3 (2600-5900 pg/ml) (Figure 3.10), compatible with previous studies demonstrating the ability of Tg-CD4 cells to produce Th2 as well as Th1 cytokines.

Cytokine response to *H. pylori* antigen differed depending on donor cell and infection. As expected, co-culture with *H. pylori*-pulsed dendritic cells induced production of IFNγ by CD4+ T cells recovered from *H. pylori*-infected B6 cell recipients (Figure 3.11). IL-4 and IL-17A levels were either low or undetectable (Figure 3.12 and data not shown). In contrast to B6 CD4+ cells, Tg-CD4 cells recovered from infected recipient mice failed to produce any of the three T cell effector cytokines in response to *H. pylori* antigen stimulation (Figures 3.11 and 3.12). Thus, the presence of T-CD4 in these mice not only failed to induce IL-4 or IL-17, but also suppressed *H. pylori*-specific IFNγ responses in infected recipient mice.

Histology and colonization in simple infection

We expected that the shortened duration of the experiment would limit the development of inflammation, and we did not find gastritis in any group of mice that was significantly different from a value of zero (Table 3.2). While there was some evidence of inflammatory infiltrate in B6 mice, this was not different from the background of histological inflammation seen in uninfected mice. The mean gastritis score was lower in CIITA-Tg mice than in B6 mice, in both uninfected and infected groups, but these differences were not significant. *H. pylori* colonization was greater in CIITA-Tg mice compared to B6 mice, but not significantly so. It is difficult to make conclusions about the effect of E- and T-CD4 cells upon disease development in *H. pylori* infected mice based on these results.

Gastric mucosal cytokine expression in simple infection

At the molecular level, *H. pylori* infection triggered a strong increase in IFNγ expression in B6 mice (Figure 3.13), which was markedly greater than induction in infected CIITA-Tg mice, but this difference was not significant, likely due to the limited sample number. IL-17A expression increased similarly by an average of 14-fold in both groups of infected mice (Figure 3.14). TNFα expression increased moderately in infected B6 mice to a level almost 2-fold greater than in transgenic mice (Figure 3.15), but this difference was not significant. All other cytokines tested demonstrated less than a 2-fold change in mRNA expression between infected and uninfected mice, suggesting that their expression may not be biologically relevant in this model.

Intracellular cytokine staining of CD4+ splenocytes in simple infection

We analyzed CD4+ splenocytes recovered from uninfected and infected mice for cytokine expression after stimulating the cells *in vitro* for 5 days to evaluate the Th profiles of these mice, and their *H. pylori*-specific T cells in particular. After stimulating the cells non-specifically with plate-bound anti-CD3 and soluble CD28 antibodies, we found that *H. pylori* infection appeared to reduce the number of IFNγ producing cells in both groups of mice (Figure 3.16), and also reduced the proportion of IL-4 producing cells in CIITA-Tg mice (Figure 3.17). In contrast, co-culture of isolated CD4+ cells with *H. pylori*-loaded antigen presenting cells suggests that the number of *H. pylori*-responsive helper T cells increased in both Th1- and Th2-polarized populations (Figures 3.18 and 3.19). IL-17A-expressing CD4+ T cells never comprised a population greater than 0.5% of all CD4+ T cells tested (data not shown).

DISCUSSION

Adoptive transfer findings and the responses of recovered T Cells

This study resulted in several unexpected findings. Our finding that adoptive transfer of E-CD4 cells induced antigen-specific IFNγ-producing CD4+ cells in *H. pylori*-infected mice, is compatible with previous studies by us (50, 55, 56) and others (139, 140, 174) indicating that disease due to *H. pylori* is CD4-dependent and Th1-associated. Surprisingly, however, we showed that adoptive transfer of mixed E- and T-CD4 cells either fails to induce, or suppresses, antigen-specific Th1 cells. T cells recovered from *H. pylori*-infected E-CD4 recipients, as expected, produced high levels of IFNγ in response to *H. pylori*-pulsed antigen-presenting cells. In contrast, CD4+ cells recovered from infected recipients of mixed E- and T-CD4 cells failed to produce IFNγ in

response to *H. pylori*-pulsed antigen presenting cells, although they were viable and functional as indicated by their strong cytokine response to non-specific CD3 stimulation. Thus, the presence of T-CD4 cells in this population appears to interfere with *H. pylori*-specific, but not non-specific, IFNγ production by recovered CD4+ cells.

The most surprising finding from our adoptive transfer experiments is that in spite of the absence of an antigen-specific Th1 response *in vitro* and significantly lower induction of IFNγ *in vivo*, the extent of gastritis and bacterial colonization in mice with E- and T-CD4 cells were not statistically different from recipients of E-CD4 cells. Thus, the absence of antigen-specific IFNγ-producing CD4+ cells in recipient mice did not affect disease outcome. This unexpected result is not due to an IL-4 mediated suppression of IFNγ expression by Tg-CD4 cells, as we observed no significant differences in IL-4 expression between experimental groups.

Gastritis due to *H. pylori* is not absolutely dependent on a Th1 response (20, 50, 55, 138), and we have shown that gastritis in mice can occur in the absence of IFNγ-producing CD4+ cells (50, 55). However, in the current study, *H. pylori*-stimulation of CD4+ splenocytes recovered from recipients of mixed E- and T-CD4 cells not only did not induce IFNγ, but did not induce either IL-4 or IL-17, suggesting that gastritis occurred in these mice in the absence of *H. pylori*-specific Th1, Th2, and Th17-biased CD4+ cells. The presence of gastritis in the absence of antigen-specific Th1 cells raises the question of which cells and cytokines induce disease. As indicated above, gastritis due to *H. pylori* has traditionally been attributed to antigen-specific Th1 T cells that produce IFNγ resulting in activation of a florid immune and inflammatory response (38, 39).

In the adoptive transfer experiments, gastric IFNγ mRNA expression was elevated in both groups of infected recipient mice, but antigen-specific Th1 cells were only detected in recipients of E-CD4 cells. Some studies have suggested that other T helper subsets, such as Th2 and Th17, may contribute to *H. pylori* gastritis under some conditions, but these cell subsets were also non-responsive to *H. pylori* in recipients of mixed E- and T-CD4 cells. Thus, we were not able to recover *H. pylori*-specific Th1, Th2, or Th17 cells from the spleens of *H. pylori*-infected recipient mice, in spite of severe gastritis and pro-inflammatory cytokine upregulation in the stomach.

Simple infection results

In our simple infection experiment, our histology and colonization results are insufficient to allow us to conclude anything about the effect of E- and T-CD4 cells upon the development of *H. pylori* gastritis. However, we may be able to infer something about the very early development of disease in these mice from the cytokine mRNA data and responses of CD4+ splenocytes. The decreased induction of both IFNγ and TNFα seen in CIITA-Tg mice compared with B6 mice predicts that inflammation would be reduced in these mice. It appears that this finding agrees with the initial profile of Th-polarized CD4+ T splenocytes recovered from these mice, as it appears that the total populations of Th1 and Th2 cells are reduced by *H. pylori* infection. However, looking specifically at *H. pylori*-specific CD4+ T cells, we see increased populations of Th1 and Th2 cells in infected mice compared to uninfected mice. The marked increase in IL-4-producing antigen-specific T cells seen in infected CIITA-Tg mice agrees with the expected phenotype of CIITA-Tg cells.

These results also clearly disagree with the changes in T cell populations observed in the adoptive transfer experiments, but this discrepancy may be due to the shortened duration of this experiment. As disease develops more gradually in directly infected mice, it is possible that whatever unknown event crippled the antigen-specific T cell response in mice given CIITA-Tg T cells has not yet occurred in this single experiment with infected CIITA-Tg mice, especially if the loss of antigen-specific responsiveness is linked to the intensity of the T cell activity. Without repeating the experiment for a longer time, we will be unable to confidently answer these questions.

Interactions Between T Cells and Innate Immune Effectors

In the current study, the cytokines that were most prominent and consistent in both groups of infected recipient mice were those that are generally associated with innate immunity: TNFα and IL-1β, as well as IFNγ. This presents us with a conundrum. If, as is well-established (55, 140), CD4+ T cells are essential to induce gastritis in *H. pylori*-infected mice, but as suggested here, antigen-specific T cells are not essential and expression of effector T cell cytokines is variable, what is the mechanism by which gastritis develops in this model? We propose that the pathogenesis of this disease involves stimulation of innate immune cells by non-antigen specific CD4+ T cells. In this hypothesis, specific *H. pylori* molecular patterns activate pathogen response receptors on innate immune cells. These cells are capable of inducing the inflammatory cytokines characteristic of *H. pylori* infection even in the absence of T helper cytokines. However, the presence of CD4+ T cells appears to be essential to permit full expression of the florid inflammatory response associated with disease due to *H. pylori*. Thus, the presence of CD4+ T cells, regardless of antigen specificity, intensifies release of proinflammatory

cytokines IFNγ, TNFα, and IL1-β from innate immune cells resulting in gastritis and other manifestations of disease.

There is some precedent for the suggestion that T cells can activate innate immune cells in an antigen-independent manner. To our knowledge, there have been no investigations of the role of T cells in activating innate cells in response to *H. pylori* per se, but studies of T cell mediated diseases similar to *H. pylori* gastritis provide support for the role of T cells in activating innate immune cells. A commonly used model of inflammatory bowel disease utilizes the adoptive transfer of T effector cells (CD4+ $CD45RB^{hi}$ cells) to immunodeficient mice (167) (for recent reviews, see (128, 158)). This model is similar to our *H. pylori* adoptive transfer model in that disease is CD4+ T cell and bacteria-dependent, and is associated with elevated levels of IFNγ and TNFα. Disease is most commonly attributed to bacteria-specific Th1 cells, and most studies have focused on the T cell subsets and cytokines that contribute to disease.

Few studies have actually examined the role of CD4+ cells in activating innate immune cells, but a study performed by Corazza and colleagues (32) presents data that support our findings. In that study, colitis was dependent on the adoptive transfer of T cells, but only in the presence of innate immune cells that were capable of producing TNFα. Adoptive transfer into TNFα+ recipients induced colitis regardless of the TNFα expression of the transferred T cells. However, adoptive transfer into TNFα-deficient RAG-deficient recipients failed to induce colitis, even when the T cells were capable of producing TNFα. These results are similar to ours in that they indicate that CD4+ T cell stimulation of cytokine production by innate immune cells, rather than innate immune

activation of antigen-specific T cells, was the principal pro-inflammatory mechanism in this model.

Other models have produced similar results. In a study of Th1-dependent lung inflammation, Clark et al. (28) showed that the inflammatory cells were not T cells of donor origin, but were innate cells of recipient origin, and that disease was attributable to T cell mediated activation of pulmonary macrophages. Other studies have demonstrated that T cell dependent colitis in recipient mice is MHCII-independent (101, 207), and results in activation of lamina propria dendritic cells to produce IFNγ and TNFα, and to activate NKT cells (101). While preliminary, all of these studies suggest that CD4+ T cells may directly activate resident innate immune cells resulting in antigen-non-specific inflammation.

The role of innate immunity in disease due to *H. pylori* itself has only recently been investigated, and few in vivo studies are published, but recent studies have suggested that innate immune response contributes to disease. *H. pylori* expresses several pro-inflammatory molecular patterns and is recognized by both Toll-like receptors and NOD1 (68). One study showed that deletion of macrophages in a mouse model suppressed the development of *H. pylori* gastritis, indicating that these antigen non-specific effector cells are a principal mediator of disease (92). These reports together with our findings here suggest we more closely investigate the contributions of innate immune cells and their pro-inflammatory cytokines in the development of *H. pylori* gastritis.

General Conclusions

These results suggest there are two distinct stages in the development of *H. pylori*-mediated disease. The first stage depends upon T cells that may be specific for *H. pylori* antigens (146) to initiate inflammation. Now we demonstrate that inflammation can be propagated without an antigen-specific Th1- or Th17-polarized response to infection. It is possible that innate immune effector cells play the same roles in these two stages of disease, but if T cells do not behave consistently in this disease model, why would we assume that of other cell types? Also, as the CIITA-Tg model was designed to allow investigation of human-like behavior in T cell development (114), it is possible that our findings with Tg-CD4 re-isolated from spleen reflect a similar failure of the systemic T cell response in human patients with chronic gastritis.

Taken together, I find that these observations reveal more about the entire immune response to *H. pylori* infection, in much the same way as I described my work with 17AKO mice in Chapter 2. We know more know about the potential sequence of events promoting gastric inflammation than previously, and have gained further confirmation about the importance of the innate immune response to infection.

FUTURE DIRECTIONS

There are three issues that I would address to continue this work. First, I would attempt to recover Tg-CD4 cells from the stomachs of adoptively transferred mice and profile their antigen responsiveness (see Chapter 4 for a description of a method that could allow for this experiment). I expect that transgenic T cells recovered from infected gastric mucosa would display the same lack of response to *H. pylori* antigens, confirming that the splenic samples analyzed above are representative of the failure of the systemic

immune response to infection. This experiment would also prove that Tg-CD4 cells are not defective in their ability to traffic through the various compartments of the immune system.

Next, I would complete simple infection experiments with CIITA-Tg mice on an extended schedule similar to the work conducted in Chapter 2. In addition to collection all of the normal stomach samples at each 3-month time point, I would also harvest splenic CD4+ cells and profile their cytokine production after both antigen-independent and *H. pylori*-specific stimuli. If I found that CIITA-Tg mice demonstrated the same loss of antigen-responsiveness after several months of infection that would confirm the unusual behavior is due to the transgenic genotype and the mixed populations of E- and T-CD4 cells, rather than a previously unknown defect in the adoptive transfer system.

One significant question remains, however: is the loss of the Tg-CD4 antigen response to *H. pylori* a unique event due in part to either the bacterial species or the unusual gastric environment? Or do CIITA-Tg CD4+ T cells respond similarly to other chronic inflammatory stimuli? To address this, I would I would compare the behavior of transgenic mice and Tg-CD4 cells in chronic inflammatory models with two other bacterial species: *Helicobacter felis*, a normal mouse gastric pathogen, and *Citrobacter rodentium*, a colitogenic pathogen. If T cells recovered after a prolonged infection with either pathogen were also incapable of responding to bacterial antigens, then we would know that CIITA-Tg CD4+ T cells cannot sustain a prolonged response to infection. However, if Tg-CD4 cells only lost the ability to respond to *H. felis* that would suggest that the gastric milieu significantly affects T cell behavior. The third possibility is that recovered transgenic T cells could maintain their responsiveness to *H. felis* and *C.*

rodentium antigens, which would demonstrate that *H. pylori* triggers the loss of function in T cells.

ACKNOWLEDGEMENTS

I would like to acknowledge the work of Dr. Hanief Sofi in this report. We collaborated extensively on experimental design and he maintained the CIITA-Tg mouse colony, performed the CD4+ T cell isolations, and conducted all of the *in vitro* T cell stimulation and flow cytometry experiments reported here.

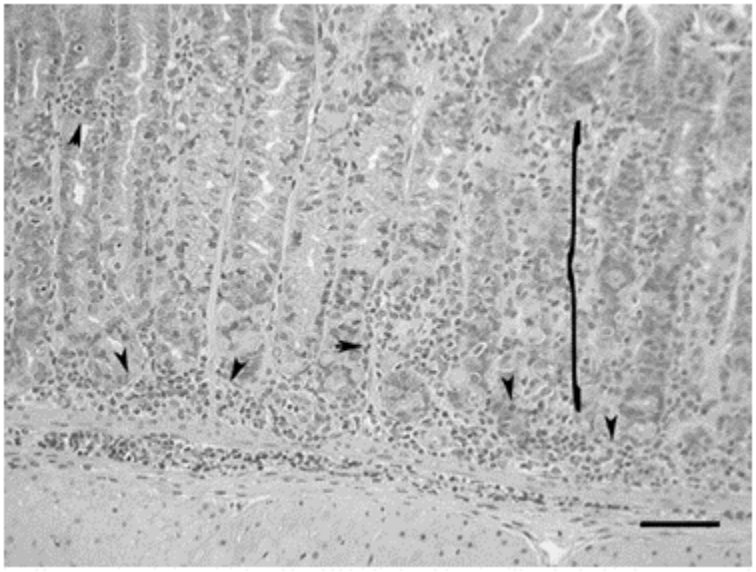
Table 3.1: Gastritis scores and bacterial colonization density in *H. pylori*-infected and uninfected SCID recipients of B6 or TG-CD4 T cells.

Donor cell type	*H. pylori* Infection	Number of mice in group[a]	Mean gastritis Score[b]	CFU *H. pylori*/g stomach tissue
B6 CD4	-	13	11.3 ± 7.9	None detected
B6 CD4	+	15	135.4 ± 28.8**	$1.9x10^7 \pm 1.0x10^7$
Tg-CD4	-	16	6.1 ± 3.1	None detected
Tg-CD4	+	18	151.6 ± 25.0**	$9.0x10^6 \pm 2.9x10^6$

[a]Total number of mice per group in 4 separate experiments
[b]Slides were scored as described in the methods. ** $p < 0.01$ when comparing the infected group to the uninfected recipients of the same cells.

Figure 3.1: Histologic inflammation in adoptive transfer

A:



B:

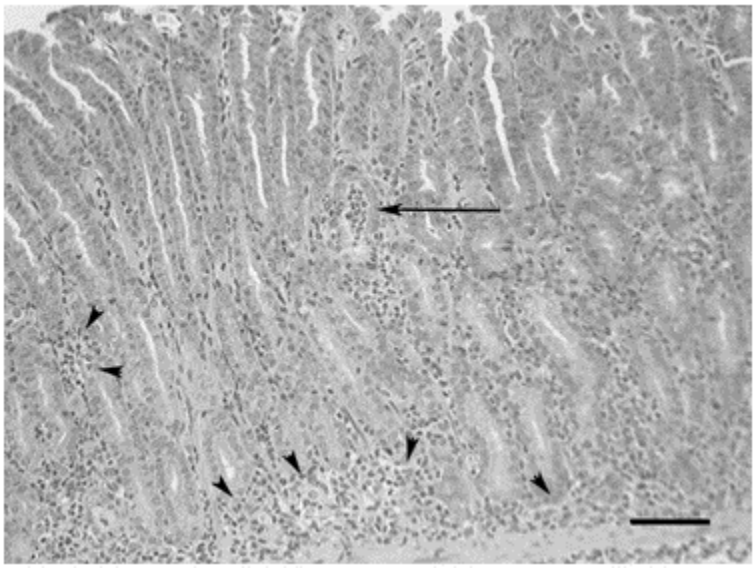


Figure 3.1: Hematoxylin and eosin-stained sections of gastric mucosa from *H. pylori*-infected SCID recipient mice. A: B6 CD4+ cells recipient. Inflammatory infiltrate (arrowheads) consists of neutrophils, lymphocytes, and macrophages. Bracket indicates metaplastic glands. B: Tg-CD4 recipient. Inflammatory infiltrate (arrowheads) is similar to B6 CD4 recipient. In this field, all glands are metaplastic as indicated by loss of parietal cells and replacement by mucus-type cells. Arrow indicates a gland abscess. Bars = 50μm.

Figure 3.2: Induction of IFNγ after adoptive transfer of B6 or CIITA-Tg T cells

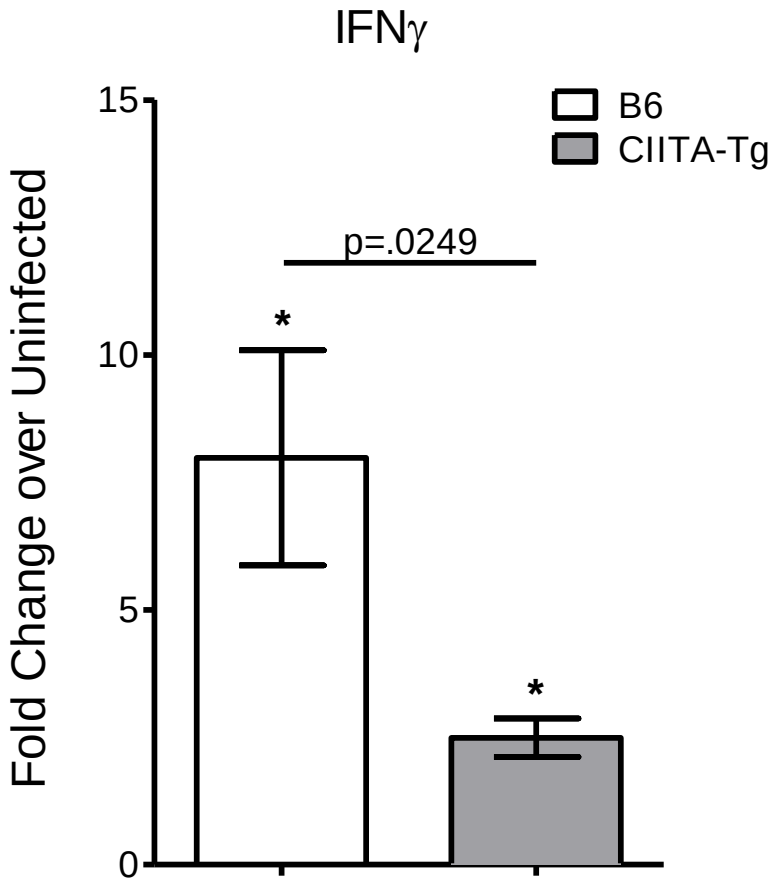


Figure 3.2: IFNγ mRNA from the gastric mucosa in *H. pylori* infected mice adoptively transferred with B6 or CIITA-Tg CD4+ T cells. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. * $p < 0.01$ when comparing relative cytokine mRNA values between uninfected controls and infected mice of the same group.

Figure 3.3: Induction of IL-17A after adoptive transfer of B6 or CIITA-Tg T cells

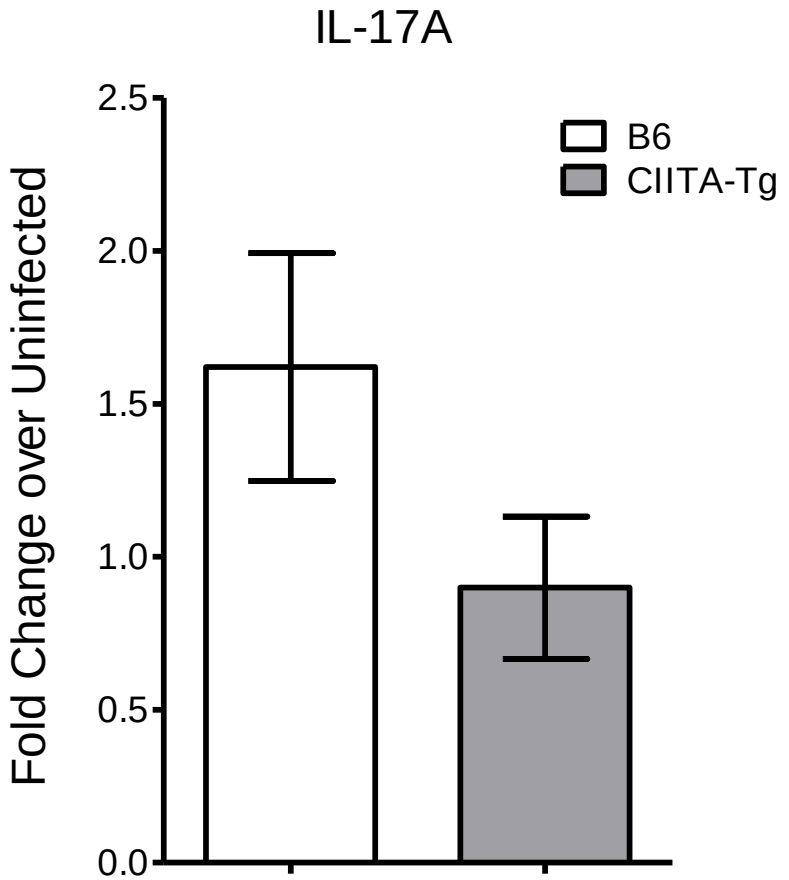


Figure 3.4: IL-17A mRNA from the gastric mucosa in *H. pylori* infected mice adoptively transferred with B6 or CIITA-Tg CD4+ T cells. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. There is no statistical difference in IL-17A induction between infected groups, and IL-17A expression in infected mice is not significantly different from that in uninfected mice.

Figure 3.4: Induction of IL-1β after adoptive transfer of B6 or CIITA-Tg T cells

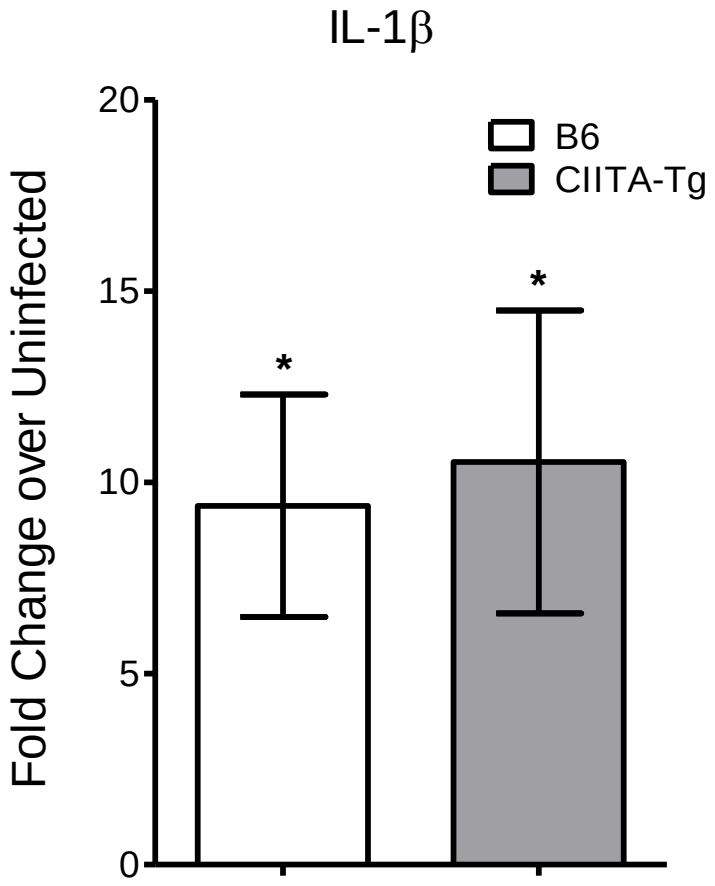


Figure 3.4: IL-1β mRNA from the gastric mucosa in *H. pylori* infected mice adoptively transferred with B6 or CIITA-Tg CD4+ T cells. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. * $p < 0.01$ when comparing relative cytokine mRNA values between uninfected controls and infected mice of the same group. There is no statistical difference in IL-1β induction between infected groups.

Figure 3.5: Induction of IL-6 after adoptive transfer of B6 or CIITA-Tg T cells

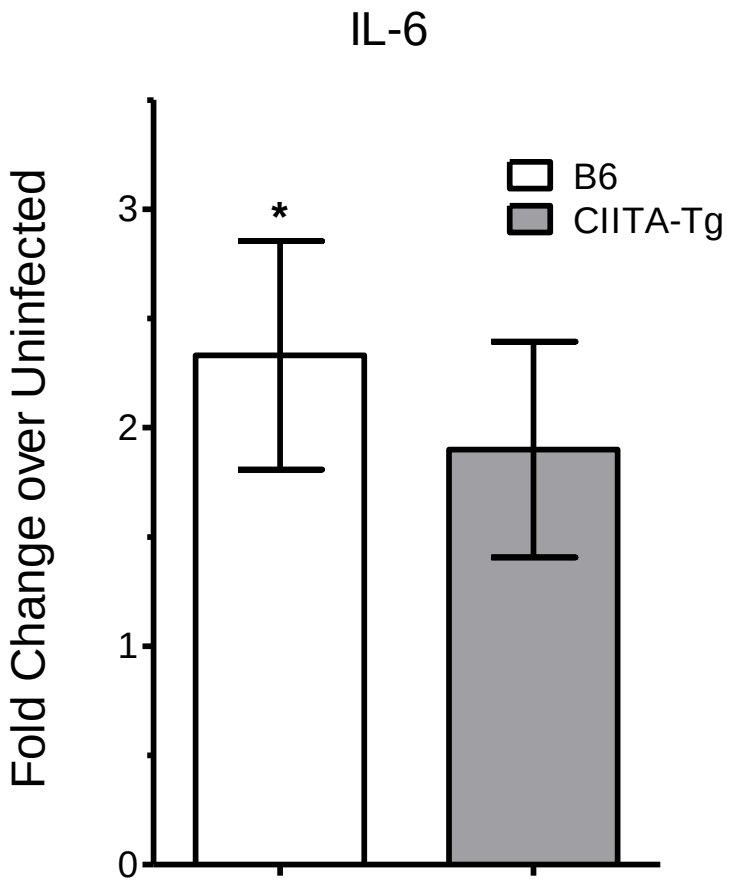


Figure 3.5: IL-6 mRNA from the gastric mucosa in *H. pylori* infected mice adoptively transferred with B6 or CIITA-Tg CD4+ T cells. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. * $p < 0.01$ when comparing relative cytokine mRNA values between uninfected controls and infected mice of the same group. There is no statistical difference in IL-6 induction between infected groups.

Figure 3.6: Induction of TNFα after adoptive transfer of B6 or CIITA-Tg T cells

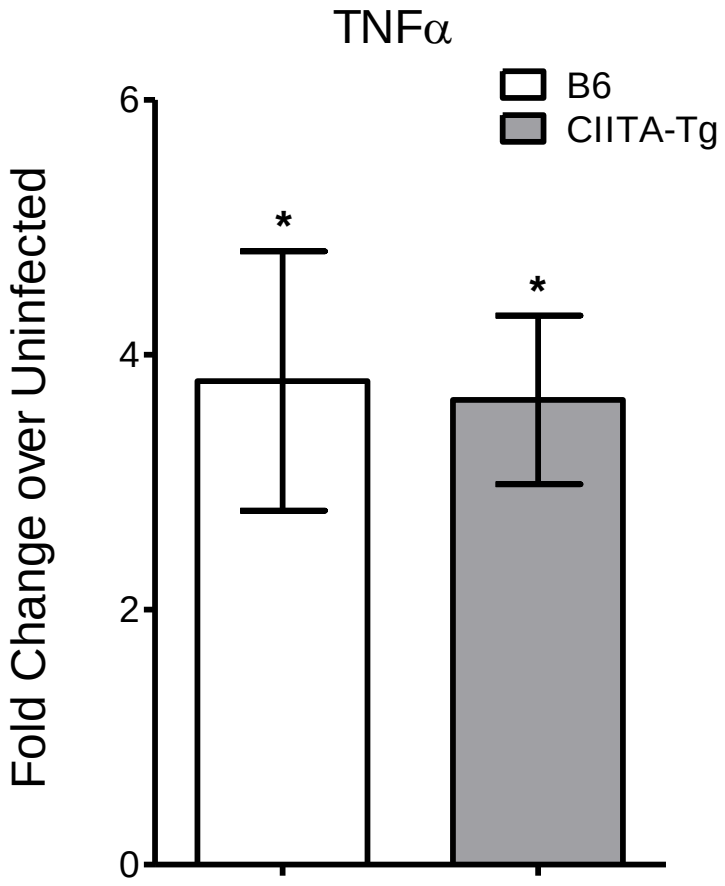


Figure 3.6: TNFα mRNA from the gastric mucosa in *H. pylori* infected mice adoptively transferred with B6 or CIITA-Tg CD4+ T cells. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. * $p < 0.01$ when comparing relative cytokine mRNA values between uninfected controls and infected mice of the same group. There is no statistical difference in TNFα induction between infected groups.

Figure 3.7: Induction of IL-10 after adoptive transfer of B6 or CIITA-Tg T cells

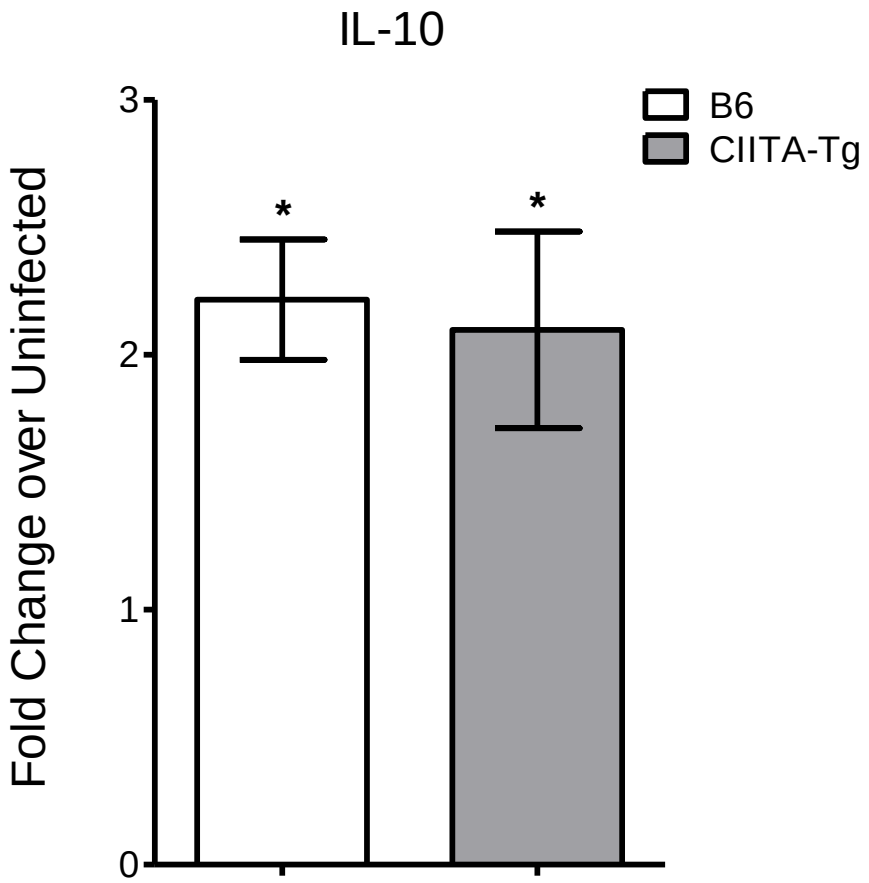


Figure 3.7: IL-10 mRNA from the gastric mucosa in *H. pylori* infected mice adoptively transferred with B6 or CIITA-Tg CD4+ T cells. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice transferred with the same genotype of cells. * $p < 0.01$ when comparing relative cytokine mRNA values between uninfected controls and infected mice of the same group. There is no statistical difference in IL-10 induction between infected groups.

Figure 3.8: IFNγ production from antibody-stimulated recovered CD4+ T cells

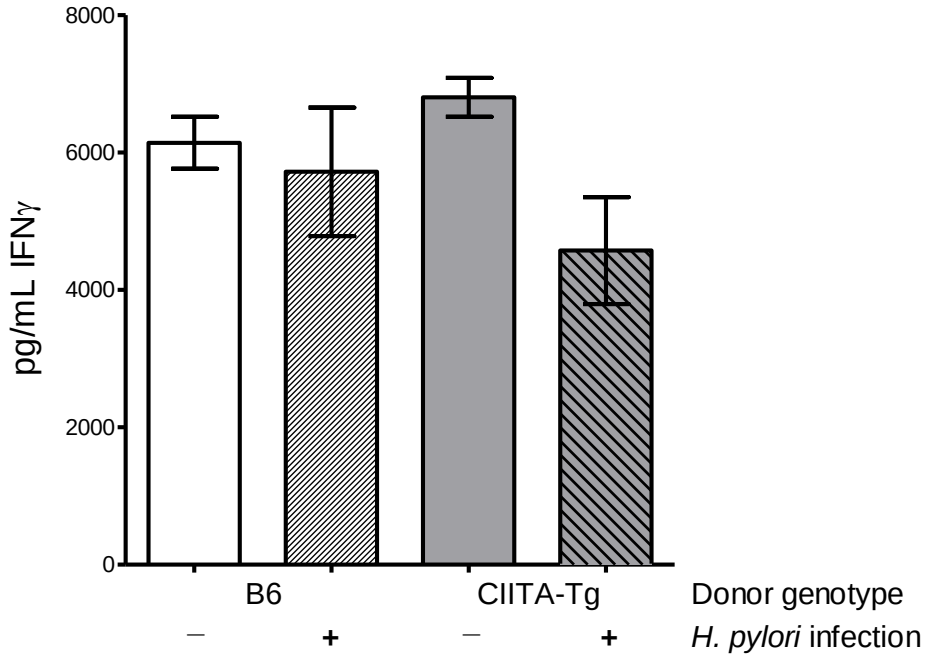


Figure 3.8: IFNγ production from CD4+ T cells recovered from adoptively transferred SCID mice, pooled and then stimulated with plate-bound anti-CD3 and soluble anti-CD28. Bars are the average of 2 (uninfected) or 3 (infected) replicates of each condition, and each replicate used CD4+ T cells pooled from 5 mice. There were no statistical differences in cytokine production between groups.

Figure 3.9: IL-17A production from antibody stimulated recovered CD4+ T cells

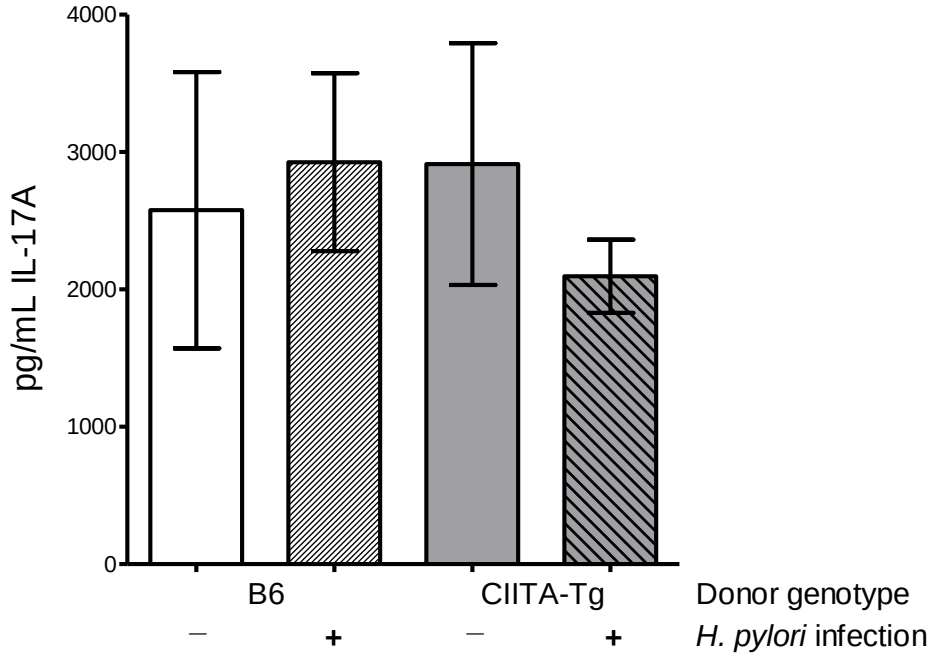


Figure 3.9: IL-17A production from CD4+ T cells recovered from adoptively transferred SCID mice, pooled and then stimulated with plate-bound anti-CD3 and soluble anti-CD28. Bars are the average of 2 (uninfected) or 3 (infected) replicates of each condition, and each replicate used CD4+ T cells pooled from 5 mice. There were no statistical differences in cytokine production between groups.

Figure 3.10: IL-4 production from antibody stimulated recovered CD4+ T cells

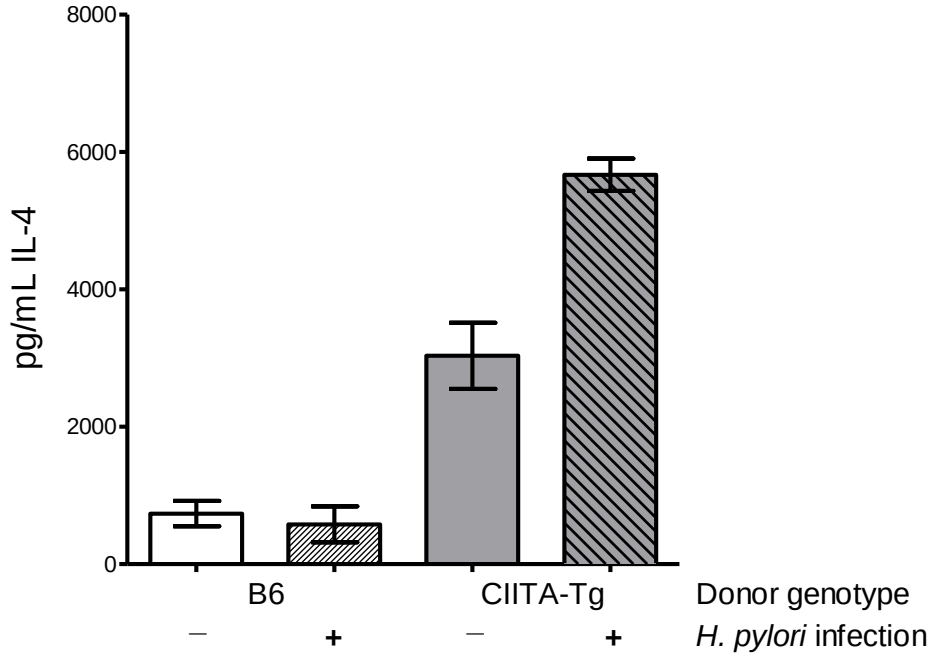


Figure 3.10: IL-4 production from CD4+ T cells recovered from adoptively transferred SCID mice, pooled and then stimulated with plate-bound anti-CD3 and soluble anti-CD28. Bars are the average of 2 replicates of each condition, and each replicate used CD4+ T cells pooled from 5 mice. I am unable to calculate the statistical significance of the groups due to the limited number of repeats.

Figure 3.11: IFNγ production from antigen stimulated recovered CD4+ T cells

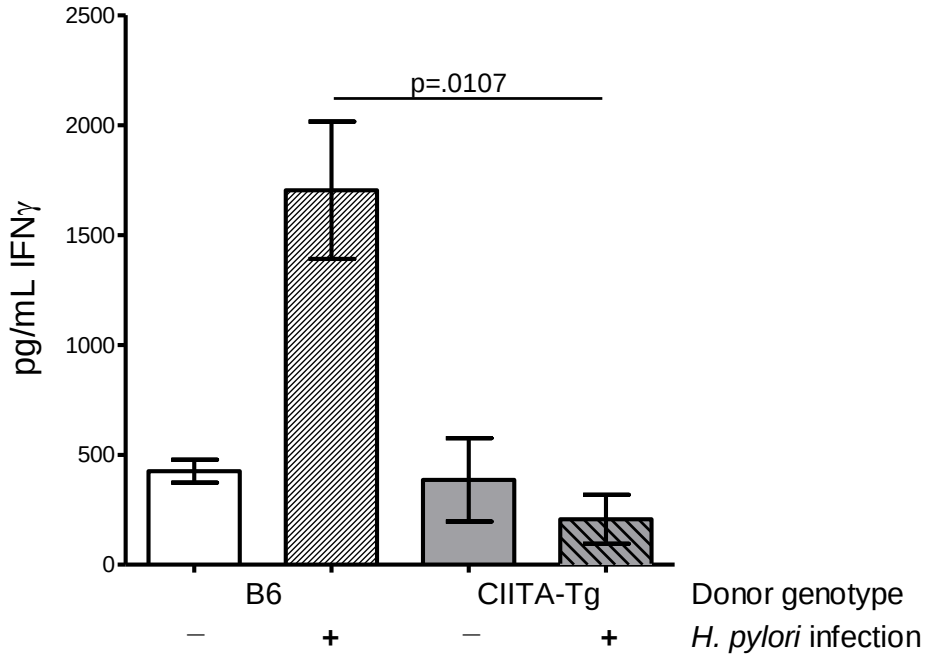


Figure 3.11: IFNγ production from CD4+ T cells recovered from adoptively transferred SCID mice, pooled and then stimulated with splenic antigen presenting cells loaded overnight with *H. pylori* lysate. Bars are the average of 3 replicates of each condition and each replicate used CD4+ T cells pooled from 5 mice. Unless otherwise indicated, there was no significant difference in cytokine production between assay groups.

Figure 3.12: IL-4 production from antigen stimulated recovered CD4+ T cells

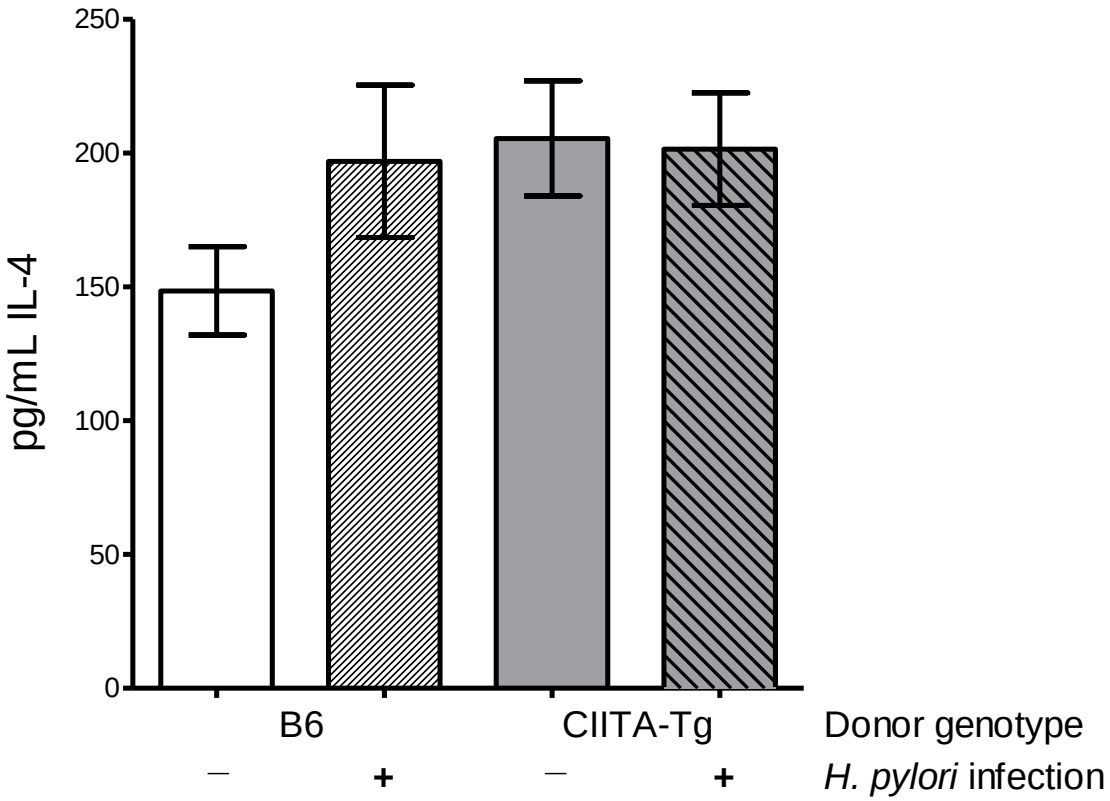


Figure 3.12: IL-4 production from CD4+ T cells recovered from adoptively transferred SCID mice, pooled and then stimulated with splenic antigen presenting cells loaded overnight with *H. pylori* lysate. Bars are the average of 3 replicates of each condition and each replicate used CD4+ T cells pooled from 5 mice. There was no significant difference in cytokine production between assay groups.

Table 3.2: Gastritis scores and bacterial colonization density in *H. pylori*-infected and uninfected B6 and CIITA-Tg mice.

Genotype	Infection status	Number of mice	Mean gastritis score[a]	*H. pylori* cfu/g stomach tissue
B6	-	4	24.3 ± 15.9	ND
B6	+	5	39.7 ± 23.3	$2.33x10^5 \pm 7.57x10^5$
CIITA-Tg	-	5	8.4 ± 7.8	ND
CIITA-Tg	+	4	7.1 ± 7.1	$5.89x10^7 \pm 2.13x10^7$

[a]Slides were scored as described in the methods.
ND = Not detected

Figure 3.13: Induction of IFNγ in gastric mucosa in simple infection of B6 and CIITA-Tg mice

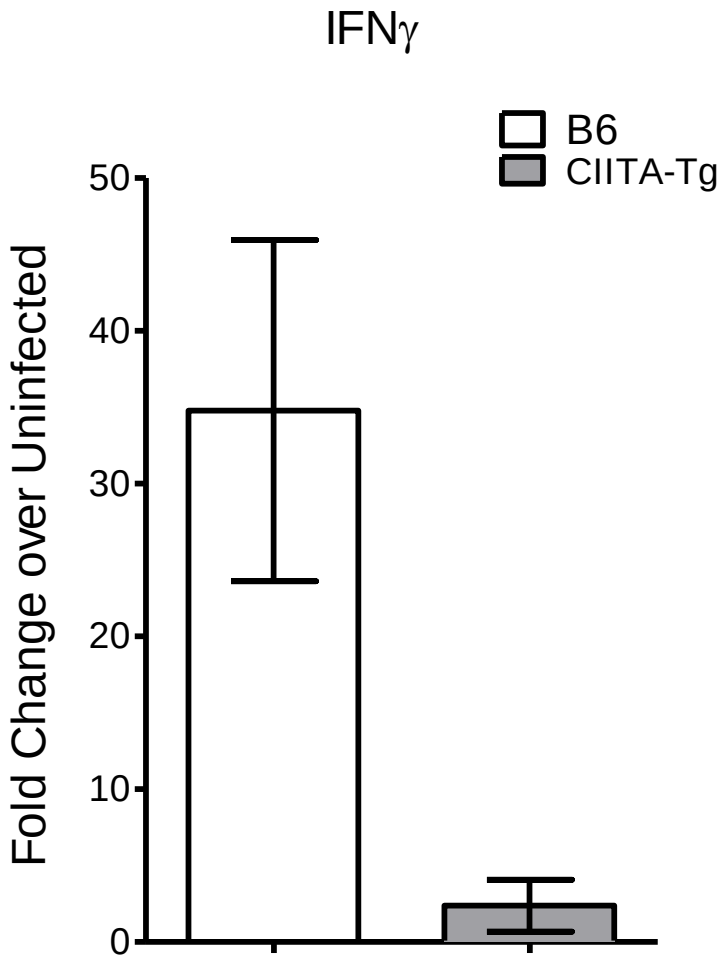


Figure 3.13: IFNγ mRNA from the gastric mucosa in a simple infection experiment. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice of the same genotype. There was no significant difference in cytokine induction between infected groups.

Figure 3.14: Induction of IL-17A in gastric mucosa in simple infection of B6 and CIITA-Tg mice

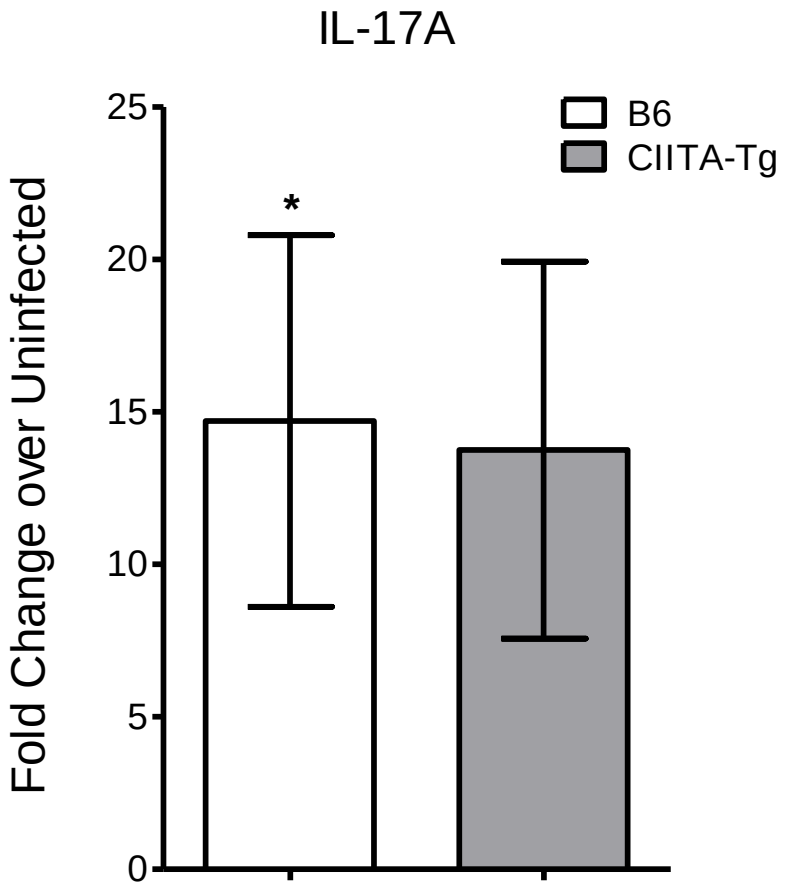


Figure 3.14: IL-17A mRNA from the gastric mucosa in a simple infection experiment. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice of the same genotype. * $p < 0.01$ when comparing relative cytokine mRNA values between uninfected controls and infected mice of the same group. There was no significant difference in cytokine induction between infected groups.

Figure 3.15: Induction of TNFα in gastric mucosa in simple infection of B6 and CIITA-Tg mice

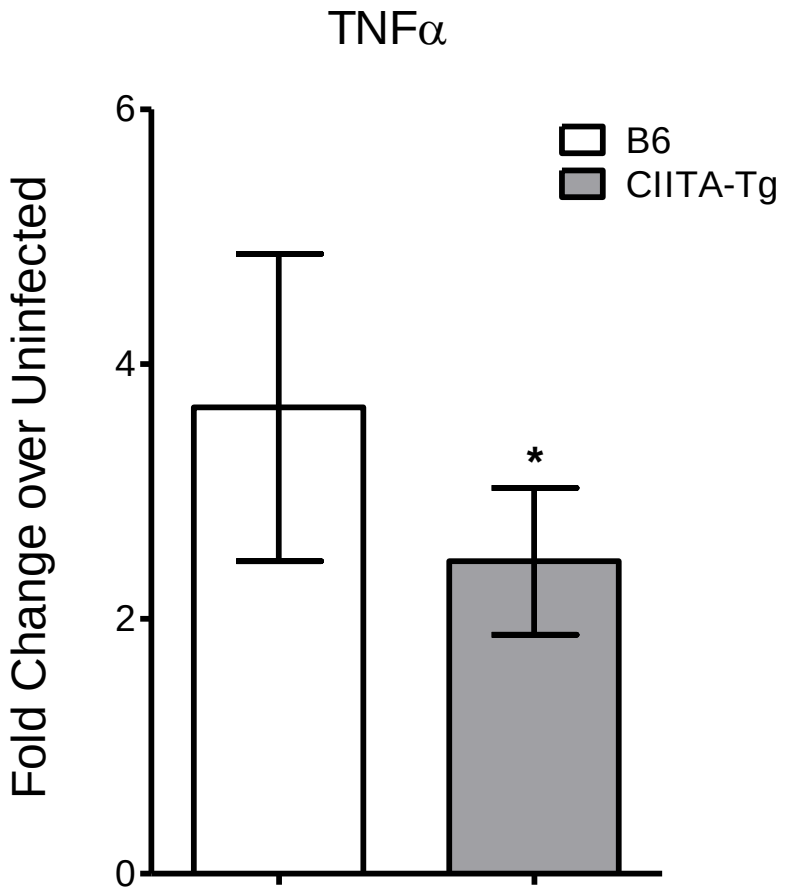


Figure 3.15: TNFα mRNA from the gastric mucosa in a simple infection experiment. Results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1, calculated by dividing the level of cytokine expression in infected mice by the average expression in uninfected mice of the same genotype. * $p < 0.01$ when comparing relative cytokine mRNA values between uninfected controls and infected mice of the same group. There was no significant difference in cytokine induction between infected groups.

Figure 3.16: IFNγ+ CD4+ splenocytes from mice after antibody stimulation

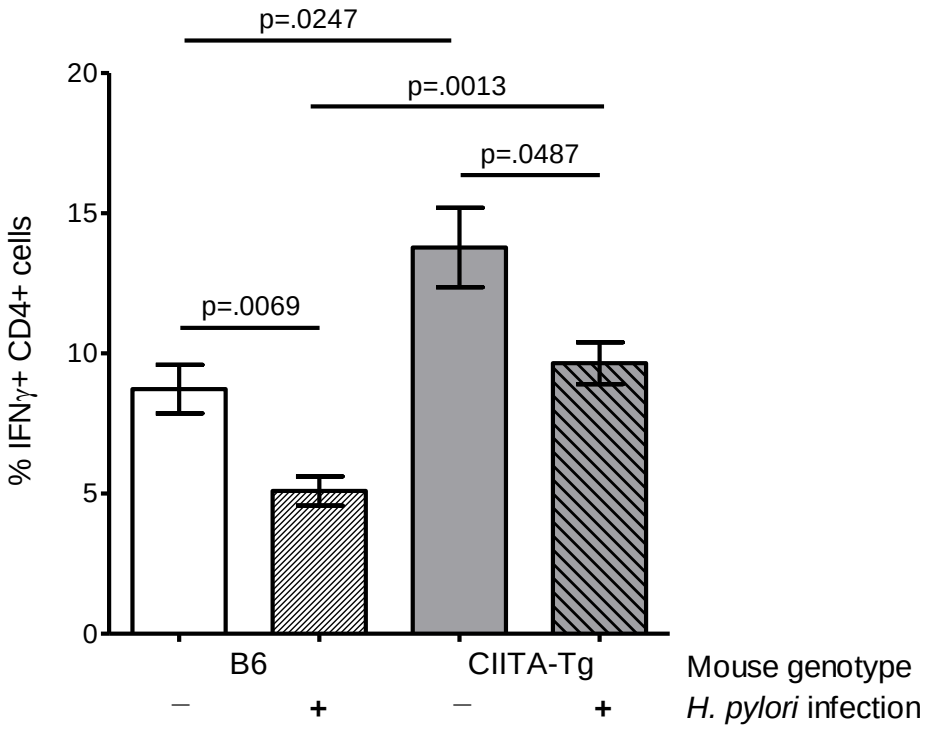


Figure 3.16: IFNγ+ CD4+ T cells isolated from uninfected and infected mice and then stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies. Cells were stained for intracellular cytokine expression after restimulation with PMA and ionomycin (see Methods). Bars are the average of 4 or 5 mice (see Table 3.2).

Figure 3.17: IL-4+ CD4+ splenocytes from mice after antibody stimulation

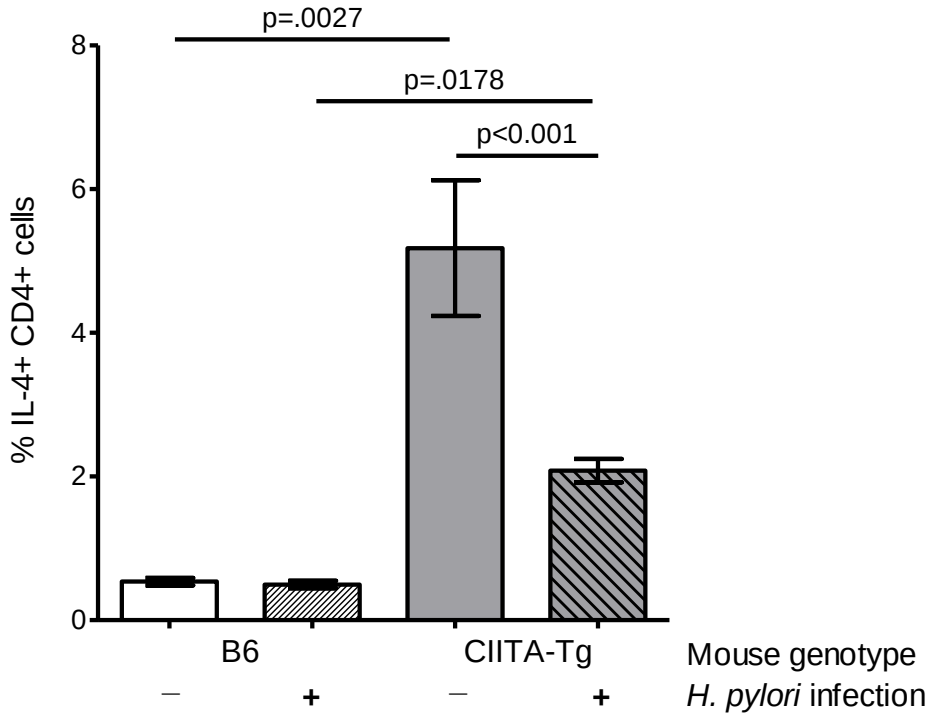


Figure 3.17: IL-4+ CD4+ T cells isolated from uninfected and infected mice and then stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies. Cells were stained for intracellular cytokine expression after restimulation with PMA and ionomycin (see Methods). Bars are the average of 4 or 5 mice (see Table 3.2). There was no significant difference in IL-4 expression in T cells between B6 mouse groups.

Figure 3.18: IFNγ+ CD4+ splenocytes from mice after antigen stimulation

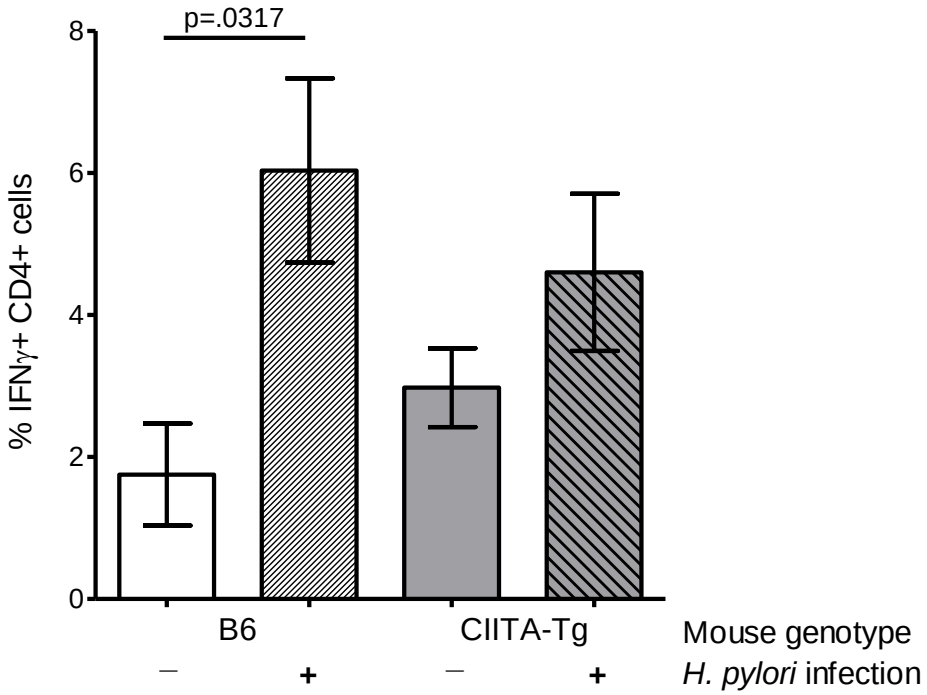


Figure 3.18: IFNγ+ CD4+ T cells isolated from uninfected and infected mice and then stimulated with splenic antigen presenting cells loaded overnight with *H. pylori* lysate. Cells were stained for intracellular cytokine expression after restimulation with PMA and ionomycin (see Methods). Bars are the average of 4 or 5 mice (see Table 3.2). There was no significant difference in IFNγ expression in T cells between CIITA-Tg mouse groups.

Figure 3.19: IL-4+ CD4+ splenocytes from mice after antigen stimulation

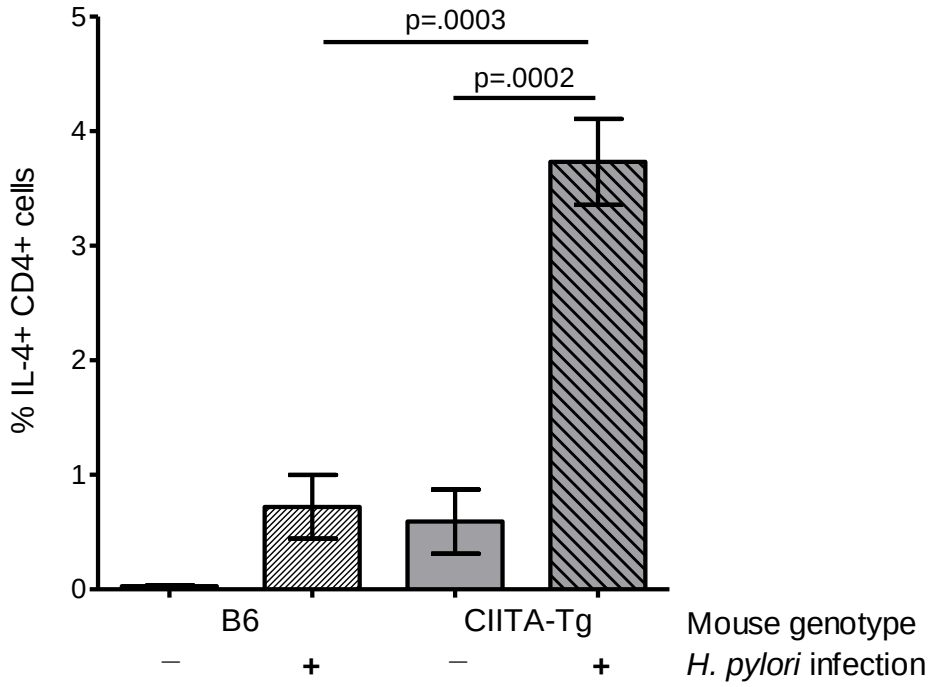


Figure 3.19: IL-4+ CD4+ T cells isolated from uninfected and infected mice and then stimulated with splenic antigen presenting cells loaded overnight with *H. pylori* lysate. Cells were stained for intracellular cytokine expression after restimulation with PMA and ionomycin (see Methods). Bars are the average of 4 or 5 mice (see Table 3.2). There was no significant difference in IL-4 expression in T cells between B6 mouse groups.

METHODS

Mice and housing

C57BL/6J mice and congenic mutant mice were used. CIITA-Tg and C57BL/6J donor mice were obtained from our breeding colony at the University of Michigan. CIITA-Tg mice have been described previously (115). No known mouse pathogens are present in the mouse colony as determined by routine periodic screening of sentinel mice. Mice were maintained in static microisolator cages and offered non-supplemented commercial mouse chow and water *ad libitum*. Helicobacter-free specific-pathogen-free C57Bl/6J-*Prkdc*^{scid} (severe, combined, immunodeficient, SCID) mice were obtained from Jackson laboratories. The number of mice in each experimental group is indicated in Table 1. All animal experiments were approved by the University of Michigan Animal Care and Use Committee.

H. pylori culture and inoculation

Overnight broth cultures of *H. pylori* strain SS1 in 10 ml of Brucella broth with 10% fetal calf serum were centrifuged, washed, resuspended in sterile phosphate-buffered saline (PBS), counted on a hemocytometer, and diluted to a final concentration of $1x10^8$ bacteria/ml. B6, CIITA-Tg, and SCID mice were given 100 μl of sterile 0.5 M Na_2CO_3 via gastric feeding tube followed by100 μl of the bacterial suspension giving a total dose of $1x10^7$ of *H. pylori* SS1 per mouse. This procedure was repeated on the following day, for a total of two inoculations.

Adoptive transfer and time course

Two weeks after the SCID mice were inoculated with *H. pylori*, splenocytes from WT or CIITA-Tg mice were dissociated, suspended in 450 μl of MACS buffer (PBS with

0.5% bovine serum albumin), and incubated with 50 μl of anti-CD4 L3T4-coated Miltenyi MACS Beads (Miltenyi Biotec, Auburn, CA) for 15 minutes at 4º C. The cell-bead mixture was layered onto a pre-wetted Miltenyi MS magnetic column and washed twice in MACS buffer, before the columns were removed from the magnet and the cells were collected with an additional 500 μl of MACS buffer. The CD4-enriched cells were washed twice in media and resuspended to a concentration of $1x10^7$ cells/ml in sterile PBS. This procedure results in a population containing >95% CD4+ cells (data not shown). Groups of both uninfected and *H. pylori*-infected SCID mice received 100 μl of either WT or CIITA-Tg cell suspension via intraperitoneal injection, for a final dose of $1x10^6$ CD4-enriched cells per mouse.

Tissue collection, histology scoring and RNA profiling

Eight weeks after adoptive transfer, mice were euthanized and stomachs were harvested for histology, assessment of bacterial colonization, and RNA isolation. Spleens and mesenteric lymph nodes were harvested for flow cytometry and functional analyses as described below. For histology, 1 mm-wide strips from the greater curvature were emersion-fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5μm sections, and stained with hematoxylin and eosin. Bacterial colonization was quantified by plate counts of serially diluted gastric homogenate.

For RNA isolation samples were stored for no more than three months in 300 μl of TRIzol at –80º C (Invitrogen, Carlsbad, CA) and total RNA was purified according to the TRIzol protocol. mRNA was then further enriched with Qiagen RNEasy columns, and stored at –80º C until analysis. Using SA Biotech's cDNA first strand synthesis kit and protocol (SA Biosciences, Frederick, MD), mRNA samples were prepared for qPCR

of mouse cytokine genes, which was performed on custom SuperArrays (CAPM-0752A) using SA Biotech's SYBR green master mix and protocol, on a Stratagene MX3000p thermal cycler. The arrays contained probes for the following genes: IL-1β, IL-6, IL-12a, IL-12b, IL-17A, IFNγ, TNFα, and GAPDH, which served as housekeeping control. IL-4 mRNA expression levels were determined separately by performing qPCR with IL-4 specific primers of our design (5' AACGTCCTCACAGCAACGAA, 3' TGCAGCTCCATGAGAACACT) and Stratagene Brilliant SYBR Green Master Mix and protocol (Stratagene, La Jolla, CA). In all cases ROX dye was used as a fluorescence control in order to set cycle threshold values.

Extent of gastritis was scored as previously described (51). Briefly, adjacent 200x microscopic fields were examined for the presence of gastric infiltrate severe enough to displace glands, presence of neutrophilic inflammation, and/or presence of gastric epithelial metaplasia. Two longitudinal sections of gastric fundus were scored in their entirety, and the percentage of positive fields in all three categories was added together to calculate the total score. All sections were scored blind, without prior knowledge of their source.

Flow cytometry and CD4+ T-cell functional analyses

Splenocytes from uninfected C57 Bl/6J mice were depleted of CD4+ by negative selection with magnetic beads, as described above, and CD8+ T cells were removed in the same manner, using anti-CD8 Ly-2-coated Miltenyi MACS Beads. The remaining cells were irradiated with 3000 rads and used as antigen-presenting cells (APCs). Splenic CD4+ T cells from adoptively transferred mice were enriched with anti-CD4 L3T4-coated magnetic beads as described above. These enriched CD4+ T cells were pooled and

then split into two sets. One set was co-cultured with APCs (1:2 ratio) that had been loaded overnight with *H. pylori* bacterial lysate (50μg/ml) for 72hrs. The other set of cells were stimulated with 5 μg/ml plate-bound anti-CD3ε (145-2C11), 1 μg/ml anti-CD28 (37.51), and 50 U of IL-2 (Roche, Indianapolis, IN) at a concentration of $1x10^6$ CD4+ T-cells/ml for 72 hrs. The supernatants from both stimulation conditions were collected, and cytokine production was measured by ELISA. For intracellular cytokine staining, cultured cells were re-stimulated with 50 ng/ml phorbol myristyl acetate and 1.5 μM ionomycin (Calbiochem) for 5 h. Monensin (Sigma–Aldrich) at 3 μM was added during the last 3 h of stimulation. Cells were fixed in 2–4% paraformaldehyde and permeabilized with 0.2% saponin (Sigma–Aldrich), and followed by intracellular staining with anti-IL4, anti-IL-17A and anti-IFN-γ antibodies.

Data handling and statistical analyses

All graphs were made using GraphPad Prism 5.0 software. This program automatically calculated and graphed the standard error of the mean (SEM) for all bar graphs. All matching datasets were tested for statistical significance through application of two-tailed Student's t-test using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). For all graphs, significant differences between groups ($p < 0.05$) are indicated by horizontal bars with the p-value indicated above. Experimental groups that are not statistically significantly different are unmarked.

For qPCR analyses, SYBR Green cycle threshold was established automatically using the Stratagene MX Pro software, based on ROX fluorescence. Any well with a SYBR Green cycle threshold greater than 35 cycles was counted as a negative result.

Expression of cytokine genes was normalized to GAPDH expression, and we multiplied these values by 10,000 for ease of visualization. To calculate fold-induction of cytokine expression, we divided the average mRNA value of infected mice by the average mRNA value of those uninfected mice that received the same CD4+ T-cells. A threshold value of two-fold was chosen to indicate a biologically significant increase in mRNA expression.

Chapter 4

Flow Cytometric Analysis of Single Cell Suspensions of Leukocytes Isolated from Inflamed Gastric Mucosa

ABSTRACT

H. pylori gastritis is histologically characterized by infiltration of the infected gastric mucosa by neutrophils and mononuclear cells, including lymphocytes and myeloid cells. Much attention has been paid to molecular events, such as the actions of bacterial virulence factors, and the participation of numerous pro-inflammatory cytokines. While we know that CD4+ T cells are required to initiate disease in mouse models of disease, the participation of other immune cell types in mice is poorly understood, especially in the adoptive transfer model of gastritis which develops more florid inflammation. I isolated and profiled leukocytes from the gastric lamina propria of *H. pylori* infected adoptively transferred mice using fluorescent antibody staining and flow cytometry, which method enabled me to consistently identify populations of CD4+ T cells, CD11c+ myeloid cells and GR-1+ granulocytes from the spleens and stomachs of infected SCID and RAG2-KO recipient mice. I then tested the ability of the method to discern differences in gastric leukocyte populations in recipient mice transferred with different populations of donor CD4+ cells. Transfer of different quantities of B6 CD4+ cells demonstrated that fewer T cells produced more severe disease and larger populations of T cells and granulocytes than in the stomachs of mice given more CD4+ cells. Mice transferred with enriched regulatory T cells or a 1:1 mixture of regulatory

and effector T cells developed milder gastritis with smaller populations of all cell types examined than did mice given just effector CD4+ cells. IFNγ and IL-17A deficient (IFNγKO and 17AKO, respectively) CD4+ cells promoted development of similar gastritis compared to mice that received B6 cells, but recipients of IFNγKO cells had larger populations of all cell populations compared to the other two groups of mice. These findings together demonstrate the utility and sensitivity of the technique and provide us with another means by which to examine the role of cells and cytokines in *H. pylori* gastritis.

BACKGROUND

Speaking broadly, the gastric mucosa is a difficult tissue within which to conduct bacterial pathology experiments. Many research groups limit themselves to commenting on gross pathology and simple histological analysis for multiple reasons. The small size of the mouse stomach restricts the number of possible parallel analyses that can be performed on a single sample. The severe environment of the stomach mucosa, including low pH and secretion of multiple digestive enzymes, limits molecular analysis of the immune response to infection to mRNA profiling of cytokine expression. The single, small gastric lymph node in mice, so unlike the intestine's mesenteric lymph nodes and Peyer's patches, prevents easy cellular analysis of the immune response to bacterial infection. As such, the majority of pathological studies of *H. pylori*-mediated gastritis limit their analyses to simple histochemical stains and immunohistochemistry.

Cellular analysis of the immune response to *H. pylori* infection remains an important goal, especially in light of our recent adoptive transfer findings (Chapters 2 and 3). In demonstrating that neither T cell-produced IFNγ or T cell-produced IL-17A are

required for disease progression ((50, 55) and Chapter 2) in *H. pylori*-infected mice, and also showing that an antigen-specific T cell response is not required to maintain the inflammatory response (Chapter 3), we are challenging the model of the primacy of the CD4+ T cell in *H. pylori*-mediated gastritis. Dr. Eaton's previous work established the necessity and sufficiency of CD4+ T cells for the development of *H. pylori*-mediated gastritis (55, 56), but our results strongly suggest that we investigate the contributions of non-T cell populations in the gastric mucosa. These findings underline the need for a consistent method for cellular analysis of the immune response to *H. pylori* infection.

One possible method enabling this sort of analysis would be to disassociate the gastric mucosa into a single-cell suspension, which would allow for both descriptive and functional analyses of recovered cells. There are numerous reports studying the behavior of primary gastric epithelial cells isolated from human and animal stomachs (102, 106, 135, 185, 194), and some reports investigated the response of these cells to *H. pylori* infection *ex vivo* or *in vitro* (102, 135, 155, 156, 204). Others have generated *H. pylori*-specific CD4+ T cell clones from human gastric punch biopsies (37, 190) using similar methods, which mostly involve mechanical disruption of the tissue to generate experimental populations of cells. It is strange to consider that such methods have apparently not yet been applied in a small animal model to perform a bulk analysis of the whole immune response to *H. pylori* infection of the stomach. The work I present here provides an experimental tool to describe consistently the leukocyte populations found in the whole of the inflamed gastric mucosa in *H. pylori*-infected mice.

EXPERIMENTAL DESIGN

The earliest protocols for generating single cell suspensions from gastric mucosa are over 30 years old (106, 189) and while there have been subsequent updates to incorporate new reagents and improved laboratory equipment, these methods uniformly focus on the isolation of gastric epithelial cells. I looked to the isolation of lamina propria and intraepithelial lymphocytes (LPL and IEL, respectively) from intestinal mucosa for a sample protocol, and found the work of LeFrancois and Lycke (111).

Their protocol intended to release the greatest part of the leukocyte populations from the thin layers of the small and large intestines through numerous, serial wash steps, before finally subjecting the tissue to harsh proteolytic digestion with collagenase. LeFrancois and Lycke sought to culture their harvested LPL and IEL, allowing the isolated cells to recover from the digestion process, before profiling or otherwise manipulating their cell populations. While still part of the gastrointestinal tract, gastric mucosa is notably thicker than intestinal tissue, and I worried that the lengthy protocol and rough treatment with collagenase would hinder my ability to profile the isolated cells in the short term with fluorescent antibody staining and flow cytometry. As detailed in the first section of the Results, I improved the isolation protocol for gastric lamina propria lymphocytes (GLPL) by removing wash steps and selecting a protease blend optimized for digestion of extracellular matrix.

We chose to focus on *H. pylori*-infected, adoptively transferred SCID and RAG2-KO mice, to maximize our chances to harvest measureable quantities of gastric leukocytes, as adoptively transferred mice reliably generate severe histological gastritis, with more cellular infiltrate than immunocompetent mice directly infected with *H. pylori*

(55, 59). Also, in addition to guaranteeing a steady supply of experimental animals, this model allows us to examine the specific contributions of innate immune effector cells without resorting to either antibody-mediated depletion of cell populations or transgenic mouse strains deficient for specific cell lineages, as both approaches come with caveats. Antibody depletion does not guarantee complete removal of any single cell population, and many mouse strains deficient in an entire innate immune cell lineage also suffer from severe health problems (i.e. *op/op* mice, which lack all phagocyte cell types, including osteoclasts critical for bone-remodeling, suffer from pronounced growth defects (211)).

To confirm the robustness and accuracy of the GLPL model, we assessed the leukocyte profiles from the stomachs of *H. pylori*-infected RAG2-KO mice adoptively transferred with different quantities of B6 CD4+ cells. Our *ex vivo* yield of CD4+ cells should reflect the input quantity, in addition to further defining the limits of the adoptive transfer model. Additionally, we varied the quality of our adoptive transfer input in some experiments by enriching our donor splenocytes for CD4+/CD25+ lymphocytes, a population consisting mostly of regulatory helper T cells. We then transferred these enriched regulatory T cells alone or mixed 1:1 with a 'normal' population of CD4+ splenocytes. We hypothesized that mice receiving large numbers of regulatory T cells would not develop marked histological disease, which should be reflected in the GLPL profiles from these mice.

Our eventual goal was to profile gastric leukocytes in SCID mice transferred with CD4+ T cells harvested from various cytokine knockout mice. Based on our cytokine mRNA profiles in mice adoptively transferred with T-bet KO or 17AKO CD4+ cells, we predicted that we should find differences in the populations of cells responding to *H.*

pylori infection. To test this, we isolated gastric leukocytes from SCID mice adoptively transferred with IFNγKO or 17AKO CD4+ cells.

RESULTS

Protocol optimization

I adapted my protocol from the work of LeFrancois and Lycke, who sought to isolate IELs and LPLs from the various tissues of the mouse intestine (111). Unlike the results they propose in their protocol description (see Table 4.1), which includes multiple repeated washes to gently flush lymphocytes from the loose layers of the intestinal epithelium, my preliminary work demonstrated that I would be unable to release many cells from stomach tissue without protease treatment (data not shown). I kept several of the initial wash steps in order to dilute and remove the viscous and proteolytic gastric mucus, and to neutralize the gastric pH, and limiting the number of washes also markedly reduced the length of the protocol. To improve my total cell yield, I also explored another protease treatment biased towards digestion of extra-cellular matrix proteins than cell-surface proteins, and based upon those results I established our isolation protocol as described in Materials and Methods. The total cell yields and viabilities of the various protocols are detailed in Table 4.1.

The need for a protease digestion to isolate tissue leukocytes suggested that I might be unable to detect and identify any single-marker leukocyte population through flow cytometry due to proteolytic destruction of the epitopes required for antibody staining. I harvested the spleens from the mice and used each animal's bulk splenocytes as untreated control cells stained in parallel with the freshly isolated gastric leukocytes from the same mouse, which were all *H. pylori*-infected SCID mice adoptively

transferred with B6 CD4+ cells. To set flow cytometer parameters and establish population gates I analyzed the forward and side scatter plot of the bulk splenocytes to establish a single pan-leukocyte gate (Figure 4.1). Looking only at events that fell within the leukocyte gate, I examined each single-stained population of cells in order to establish sub-gates in each dye color and establish color compensation. These sub-gates allowed me to assess both the strength of staining, or mean fluorescence intensity (MFI) of each population, as well as to measure the number of stain-positive cells as a percentage of the whole leukocyte gate. After setting the flow cytometer parameters using harvested bulk splenocytes (Figure 4.1), I then analyzed the splenocytes from each mouse in multiply-stained samples, followed by analyzing each tube of multiply-stained gastric cells (Figure 4.2). For FoxP3 staining of regulatory T cells, I employed a similar process (Figure 4.3)

As shown in Figure 4.4, I did find significant differences in the expression levels of CD4 and CD11c on those cell populations, with reduced CD4 expression on gastric leukocytes compared to splenocytes, without a significant difference in the quantity of CD4+ cells expressed as a % of leukocytes in either spleen or stomach (Figure 4.5). I found higher expression of CD11c on gastric cells than on splenocytes, again without a significant difference in the % of CD11c+ cells between spleen and stomach. These findings demonstrated that after two rounds of protease digestion, I would be still able to specifically detect and identify single-marker populations of freshly isolated gastric leukocytes.

Leukocyte profiles in infected recipient mice

I then compared the relative abundance of cell populations in the stomach and spleen in infected recipient SCID mice with B6 CD4+ cells, in order to understand how

in situ leukocyte profiles varied from those found in central lymphoid tissues. Table 4.2 lists the specific cell markers for which I stained and the populations I initially examined. On occasion I observed small numbers of CD8+ or CD79a+ cells, which were likely transferred in with the bulk of CD4+ cells in the initial adoptive transfer, but these populations averaged 2.5% or less of the total leukocyte gate in the stomach (CD8a+: 1.48 ± 0.35% across three separate GLPL isolation experiments, CD79a+: 2.4 ± 0.60%), so for the rest of this work I focused on helper T cells, granulocytes and monocytes/macrophages.

I did not find significant differences in the relative abundance of CD4+ or CD11c+ cells between stomachs and spleen in SCID mice with B6 cells (Figure 4.5), but there was a significantly larger population of GR-1+ cells in the spleens than in the stomachs. Values for cell populations varied with each experimental group of transferred mice, but these numbers were generally consistent within a single GLPL isolation group (data not shown), allowing us to assess the individual yields from each animal and determine if the isolation and transfer succeeded in any single instance. I also looked for correlation between the relative abundance of each of the three populations (CD4+, CD11c+, and GR-1+) we followed, and any of the histological characteristics (chronicity, activity and metaplasia) and the total inflammation score, but there was no consistent association between any cell type and any histological value (data not shown).

Gastric leukocyte profiles reflect adoptive transfer input

We performed GLPL analysis on infected recipient RAG2-KO mice transferred with our 'standard' number of $1x10^6$ B6 CD4+ cells, a 'half-dose' of $5x10^5$ cells, or a '10x' dose of $1x10^7$ cells (see Table 4.3), in order to test whether cell populations isolated

through our GLPL method would reflect differences in the material input. Histological analysis of the mouse groups showed that the 10x group developed significantly less inflammation than the standard- and half-dose groups (Table 4.3). Antibody staining of bulk splenocytes demonstrated that the 10x group on average had a higher percentage of CD4+ cells than the other groups, indicating that the adoptive transfer had successfully engrafted in these mice (Figure 4.6). The GLPL profiles from these mice stands in contrast to the splenic profiles, as there were significantly greater percentages of CD4+ cells in the stomachs of half-dose mice than in either of the other groups (Figure 4.7). Looking at CD11c+ population, we found the stomachs of the 10x group held significantly lower percentages of these cells than we saw in the standard group (Figure 4.8a). The gastric tissue of half-dose mice also had a higher percentage of GR-1+ cells than any other group (Figure 4.8b). When we looked at regulatory T cells as a percentage of the whole CD4+ GLPL population, we found that Treg numbers followed the number of CD4+ T cells transferred, with the most Tregs found in the 10x group (Figure 4.9).

To understand the limits of the isolation method and its ability to detect potentially rare populations of cells, we also examined the GLPL profiles of *H. pylori*-infected RAG2-KO mice transferred with a 'normal' population of CD4-enriched B6 splenocytes, a 'Treg' population of B6 splenocytes enriched for regulatory cells through CD4 and CD25 selection (see Methods), or a 'mixed' transfer with a 1:1 ratio of normal and Treg enriched cells (see Table 4.4). As expected, mice receiving Treg or mixed transfers developed very mild gastritis (Table 4.4), with significantly lower histological inflammation values in both groups compared to those mice receiving the normal population of CD4+ cells. To confirm that the transferred cells successfully engrafted,

we assessed the CD4+ populations in recipient mice and found similar percentages of CD4+ cells in mice receiving normal and Treg cells, with significantly greater numbers of CD4+ cells in mice receiving the mixed population (Figure 4.10).

As we found the greatest inflammation in those mice transferred with the normal population of CD4+ cells, we expected to find the largest populations of immune effector cells in that group of mice. Indeed, we found that mice with the unfractionated CD4+ adoptive transfer had the largest populations of CD4+, CD11c+ and GR-1+ cells in their stomachs (Figures 4.11 and 4.12). We also saw that the percentage of regulatory T cells as part of the CD4+ GLPL population was highest in mice transferred with just Treg cells, and lowest in mice receiving a normal population of CD4+ cells (Figure 4.13).

Gastric leukocyte populations in recipients of cytokine-deficient CD4+ cells

We analyzed the GLPL populations of *H. pylori*-infected recipient SCID mice transferred with B6, IFNγKO or 17AKO CD4+ cells to determine whether we could find differences between those groups of mice which displayed no significant differences in the semi-quantitative measures of histological scoring (Table 4.5). The various transfers engrafted equally well, as measured by splenic CD4+ population (Figure 4.14), but we found significantly different numbers of CD4+ cells in the stomachs of these mice (Figure 4.15). Mice receiving IFNγKO cells had the greatest percentage of CD4+ cells in their GLPL, followed by mice with 17AKO cells, and mice transferred with B6 cells had the smallest population of CD4+ cells of all groups. This pattern was repeated when we examined the CD11c+ populations (Figure 4.16a). When we examined GR-1+ cells however, recipients of B6 and 17AKO cells had equivalent populations of granulocytes, but just as with the other two cell groups, IFNγKO recipients had the highest percentage

of GR-1+ cells (Figure 4.16b). We did stain these GLPL isolates for regulatory T cells, and observed that mice transferred with B6 cells have a relatively larger fraction of Tregs in their inflamed gastric mucosa than mice receiving either IFNγKO or 17AKO cells (Figure 4.17), although we did not examine the transfer input to assess the population of regulatory T cells as a subset of the transferred CD4+ splenocytes.

DISCUSSION

Utility of the GLPL isolation method for cellular analysis

In this work I adapted an extant protocol for the cellular analysis of intestinal epithelial and lamina propria lymphocytes (111) into a tool for the analysis of gastric lamina propria lymphocytes. I started this work in an effort to provide a new way to analyze the immune response to *H. pylori* infection in the mouse stomach, given the limitations inherent in working in such a small and environmentally unfriendly tissue. After optimizing the isolation protocol for maximum cell yield and viability, I demonstrated that the resulting single-cell suspensions could be successfully analyzed through fluorescent antibody staining and flow cytometry, and confirmed there are differences in leukocyte populations found in the spleen and the stomach. Additionally, this isolation method delivers consistent GLPL populations within single experimental groups.

Furthermore, this technique is sensitive to relatively minor changes in the adoptive transfer experimental protocol, as demonstrated by a clear distinction between GLPL populations in mice transferred with $5x10^5$ or $1x10^6$ CD4+ cells. We also demonstrated that through flow cytometric analysis we can distinguish between histologically different disease states: as we saw severe inflammation in mice transferred

with a normal population of CD4+ splenocytes we found significant populations of immune effector cells, when compared to non-inflamed RAG2-KO mice that received Treg or mixed CD4+ populations. Lastly, we present initial findings suggesting that GLPL profiles can reveal previously undetected differences in gastric inflammation in recipient mice transferred with a variety of cytokine deficient CD4+ cells.

It is also useful to note the similarities and contrasts between the gastric lamina propria and splenic populations of leukocytes. Under standard conditions of our adoptive transfer protocol, we find that helper T cells and monocytes/macrophages are found in equivalent numbers in both tissues. But we see lower CD4 expression and higher CD11c expression on gastric cells than on splenocytes, and both of these behaviors signify activation of those two cell types. These observations suggest those immune effector populations are activated in the *H. pylori*-infected stomach. In comparison to the first two cellular populations, we find markedly reduced numbers of GR-1+ cells in the stomach, compared to splenocytes, but the mouse spleen is also a hematopoietic organ, so we would expect to see larger numbers of granulocytes and their precursors in the bulk splenocytes.

We must note that counter to our initial expectations, GLPL profiles do not statistically correlate with the histopathological findings from the same mouse or experimental group of mice. Our findings suggest that the percentage of CD4+ cells in the gastric tissue trends towards positive correlation with the histological inflammation score, especially in the experiments where we changed the number of transferred cells and mixed normal and regulatory T cells, but this is only suggestive. This discrepancy between GLPL flow cytometry and histopathological scoring probably arises from two

complicating factors. First, both our current histological scoring method, and the GLPL flow cytometry as presented here, are semi-quantiative methods, making it difficult to argue that correlations of cell populations to observed histopathology are meaningful. Secondly, the multifocal nature of *H. pylori* gastric lesions dictates that these two analysis methods will rarely agree, as histological techniques reveal the distribution of inflammatory cells in tissue sections, but the GLPL method samples the whole of the stomach without necessarily providing specific contextual information.

Adoptive transfer input affects disease development

Due in part to our previous work with the adoptive transfer model of disease ((55), Chapters 2 and 3), we started to question the role of the CD4+ T cell in disease development in *H. pylori*-infected recipient mice. As one way to address this issue, we asked whether smaller or larger numbers of transferred CD4+ cells required would affect the extent of disease development. The initial quantity of $1x10^6$ CD4+ splenocytes was determined by Dr. Eaton when she initially developed the model, with a million T cells achieving two aims as both a convenient number of cells with which to work, and a sufficient quantity to consistently promote disease development (55). We had expected the half-dose to produce limited disease in infected recipient mice, and that we would be unable to distinguish between the standard and 10x doses. As shown above, we observed directly the opposite result, with the greatest histological inflammation and most abundant populations of CD4+ and GR-1+ immune effector cells found in those mice receiving the half-dose of CD4+ cells.

Relying only on the splenic CD4+ profiles in the recipient mice, which directly reflected the quantity of input cells, we would be hard-pressed to immediately explain our

histological findings. With GLPL profiles of the three experimental groups we found that half-dose mice had significantly greater numbers of CD4+ cells in the gastric mucosa than in recipients of the standard or 10x dose of cells. This finding then strongly suggests that homeostatic proliferation, the process by which transferred CD4+ T cells can repopulate an immunodeficient host in an MHC II-dependent but antigen independent fashion (for review, see (18)), drives preferential expansion of the CD4+ populations in peripheral tissues versus the central lymphoid organs. It may be that antigen non-specific population expansion somehow sensitizes or partially activates the CD4+ T cell population, enabling the rapid development of severe gastritis in response to *H. pylori* infection seen in adoptively transferred mice. I hypothesize that if we followed disease development in infected RAG2-KO mice transferred with $1x10^7$ CD4+ T cells, we would find similar disease kinetics as found in the simple model of infection, with these mice only developing a moderate gastritis that does not resolve with time (see Chapter 2).

The idea that the adoptive transfer-triggered expansion in the CD4+ T cell effector population is further supported by our findings in RAG2-KO mice transferred with populations of regulatory T cells, or evenly mixed numbers of regulatory T cells and otherwise un-enriched CD4+ cells. As demonstrated by Shen et al., when regulatory T cells comprise a large fraction of an adoptively transferred population of CD4+ cells, homeostatic proliferation of T cells is curtailed in recipient mice (182). Our GLPL profile of CD4+ cells in the gastric mucosa of infected recipient mice is in direct agreement with Shen's findings. The GLPL profiles contrast with the number of CD4+ cells found in recipient spleens, suggesting adoptively transferred regulatory T cells may preferentially traffic to the central lymphoid organs, and not travel to the peripheral tissues.

Unfortunately, we have not consistently collected data about the Treg population in the spleens of these adoptively transferred animals, and doing so in the future would allows to better characterize the behavior of regulatory T cells in adoptive transfer. At this point, we do not have enough information to confidently state whether Tregs exhibit different traffic patterns from normal CD4+ T cells, or if there is another explanation for our observations.

Cytokine KO T cells promote equivalent disease with different GLPL profiles

I developed this method specifically to explore differences in the immune effector cell populations in *H. pylori*-infected gastric mucosa after I found that adoptive transfer of 17AKO T cells into recipient SCID mice produced histological inflammation equivalent to recipient mice transferred with B6 T cells (Chapter 2), which followed upon our similar findings with the adoptive transfer of T-bet KO T cells (50). While histopathological scoring showed no qualitative or quantitative difference in disease between experimental groups, changes in cytokine mRNA levels demonstrated the loss of IL-17A had a marked effect (Chapter 2). GLPL analyses presented here prove that infected SCID mice transferred with CD4+ cells lacking pro-inflammatory cytokines specific to either Th1- or Th17-polarized helper T cells give rise to significant changes in the populations of immune effector cells in the gastric mucosa.

The difference in splenic and gastric CD4+ populations was an unexpected finding, with significantly greater percentages of IFNγKO and 17AKO T cells in the stomachs of recipient mice compared to mice with B6 cells, a difference not reflected in the splenic profiles. In concert with our transfer dose experiments, the finding of increased numbers of cytokine knockout T cells in the stomach while the splenic profiles

remain the same, regardless of the genotype of transferred cells, suggests that cytokine knockout T cells are proliferating more in the peripheral tissue than in the spleen. As our transfer dose experiments suggested, increased T cell proliferation in the stomach appears to lead to greater disease in infected mice. In support of this hypothesis, we also found fewer regulatory T cells in the stomachs of mice transferred with IFNγKO or 17AKO T cells, which also agrees with our transfer dose findings, although it is important to consider that regulatory T cells from a cytokine knockout background may not behave normally.

Increased T cell proliferation leading to greater numbers of gastric T cells could explain how CD4+ cells deficient for necessary pro-inflammatory cytokines (50, 55) can promote histologically equivalent disease. A greater number of T cells present in the stomach, even with reduced inflammatory potential because they lack a particular pro-inflammatory molecule, can promote similarly severe disease. We found greater numbers of CD11c+ and GR-1+ cells in mice transferred with cytokine deficient T cells compared to recipients of B6 cells, suggesting that the presence or action of a greater number of T cells attracts larger populations of other effector cells, although we had expected to find a larger population of granulocytes in the stomachs of 17AKO recipients than in mice with B6 cells, as IL-17A deficient mice are reported to have neutrophilia (208).

General conclusions

As presented here, the GLPL isolation method coupled with flow cytometry gives us a powerful new tool for analysis of the inflamed gastric mucosa in *H. pylori*-infected immunodeficient mice adoptively transferred with various populations of CD4+ T cells.

The ability to determine how various T cell-specific factors contribute to the development of *H. pylori* gastritis has been the great strength of the adoptive transfer model. As demonstrated by our previous work with T-bet KO CD4+ T cells (50) and my work here with 17AKO and CIITA-Tg CD4+ T cells (Chapters 2 and 3), we have discovered limits to the utility of the adoptive transfer model as it was initially developed. GLPL profiles re-invigorate this model of disease development by showing that we can glean still more information about disease mechanisms.

The work shown here provides us with several significant insights into the behavior of the immune system in response to *H. pylori* infection of the gastric mucosa. While these observations do not lend support to any particular hypothesis about the role of CD4+ T cells arising from either Chapter 2 or Chapter 3, the suggestion that antigen non-specific proliferation of T cells may trigger an antigen-specific disease response is novel. Also, significant differences in non-T cell immune effector populations, seen both in the experiments with varied CD4+ T cell dosages and with the adoptive transfer of cytokine deficient T cell experiments, confirm the importance of innate immune effector populations in the development of *H. pylori*-mediated gastritis.

FUTURE DIRECTIONS

There were several experiments in progress at the time I wrote this report. We were completing two more engrafting dose experiments and two more IFNγKO adoptive transfers, and those results need to be incorporated into the full version of this report.

We should complete the profile of GLPL populations by staining for CD16 to look for natural killer cells, as it has been proposed that these cells have a role in promoting inflammation in infected human patients (76, 152), and we can then determine

if those cells constitute a measureable population of host-derived cells in infected gastric mucosa. Once we have profiled the leukocyte populations most likely to contribute to disease development, we can narrow our focus to explore the functional state of the various cell types. For T cells alone, we can track T cell activation via CD134, which I had used in my initial experiments, and also follow the traffic of memory T cells ($CD62L^{lo}/CD44^{hi}$) through the inflamed gastric mucosa. This latter population is of greater interest after our findings that I reported in Chapter 3, with the loss of *H. pylori*-antigen specificity in CIITA-Tg cells in adoptive transfer.

We can also employ a number of advanced cell staining techniques to explore the functional states of at least the CD4+ T cells. Intracellular staining of T cells for cytokine production would be a good way to let us directly examine protein expression in the harsh environment of the stomach, although it could be technically difficult without treating the isolated leukocytes with mitogen to promote strong cytokine production (see Chapter 3-Methods). Changes in mRNA levels serve as a good proxy for expression of inflammatory mediators, but it is difficult to determine how issues of translational regulation affect cytokine production given the small size of our gastric samples, and cytoplasmic protein staining most closely approaches the issue of in situ cytokine expression. If we could examine the cytokine production by T cells directly from the site of gastric mucosa, I think we would gain a much greater insight into the role of the T cell response to *H. pylori* infection.

ACKNOWLEDGEMENTS

I would like to acknowledge the able assistance of David Loeffler and Clint Fontaine in this work. David assisted me early in the optimization of the isolation

protocol and flow cytometry techniques, and refining experimental design. Together the two gentlemen performed several of the isolations and analyses in the transfer dose experiments as well as the experiments with transfer of cytokine-deficient T cells.

Table 4.1: Comparison of cell yields and protocols for isolating gastric lamina propria leukocytes

	Initial protocol		Final protocol	
Solution/media	Number of repeats	Duration	Number of repeats	Duration
CMF/HEPES	6x	5’ each	1x	5’ each
CMF/FCS/EDTA	4x	15’ each	2x	10’ each
RPMI-10	2x	5’ each	1x	5’ each
RPMI-10 + Protease	3x w/Collagenase	60’ each	2x w/Liberase-TM	45’ each
CMF/HEPES	2x	5’ @ 850x g	1x	5’ @ 850x g
Final viable cell yield	$7.58x10^5 \pm 9.16x10^4$ [a]		$2.51x10^6 \pm 4.92x10^5$ [b]	

[a]Average cell yield from 2 separate experiments with 6 mice total.
[b]Average cell yield from 3 separate experiments with 11 mice total.

Figure 4.1: Flow cytometry plots of splenocytes from adoptively transferred mice

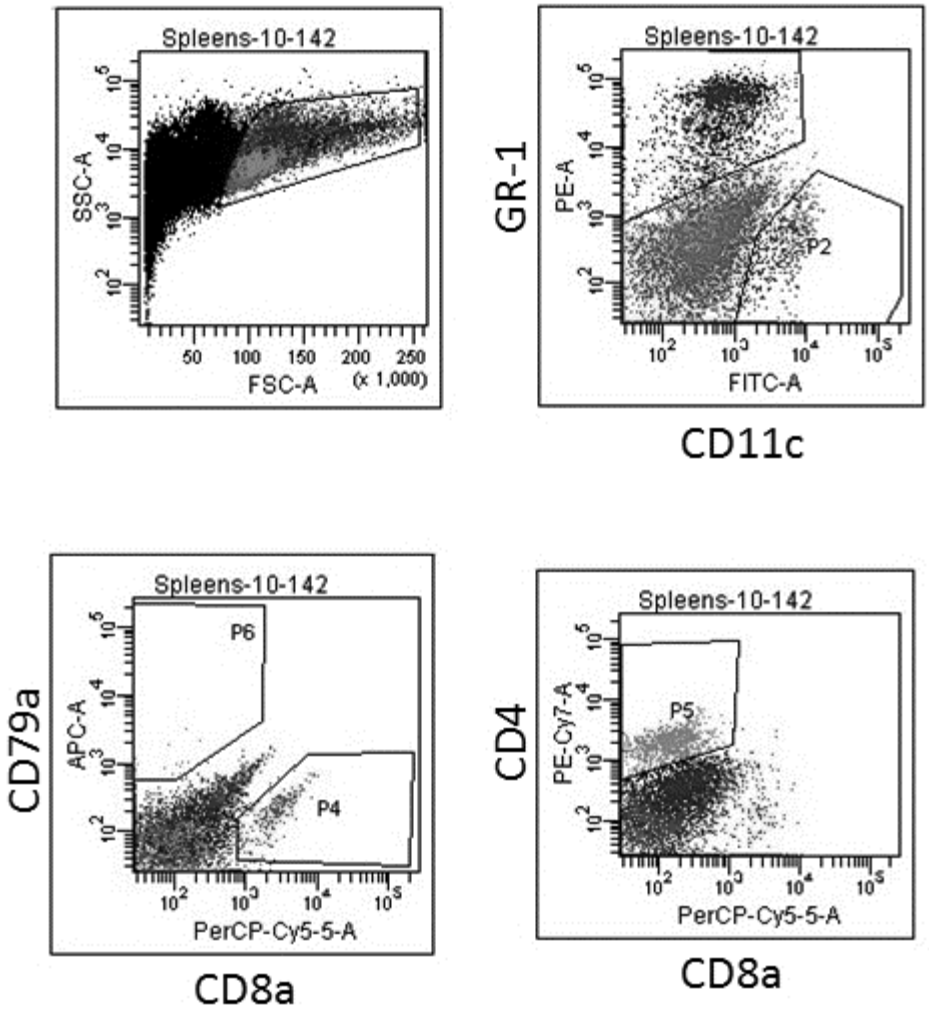


Figure 4.1: Flow cytometry dot plots of splenocytes from *H. pylori* infected SCID mice adoptively transferred with B6 CD4+ cells. Splenocytes were isolated from mice 8 weeks after transfer and 10 weeks after infection. Leukocytes were gated on size and granularity (upper left) and all gates are subsidiary to that first gate. Populations were then examined on 2-color dot plots to assess single positive populations for stain brightness (Mean Fluorescence Intensity (MFI)) and number of events (see Methods).

Figure 4.2: Flow cytometry plots of gastric leukocytes from adoptively transferred mice

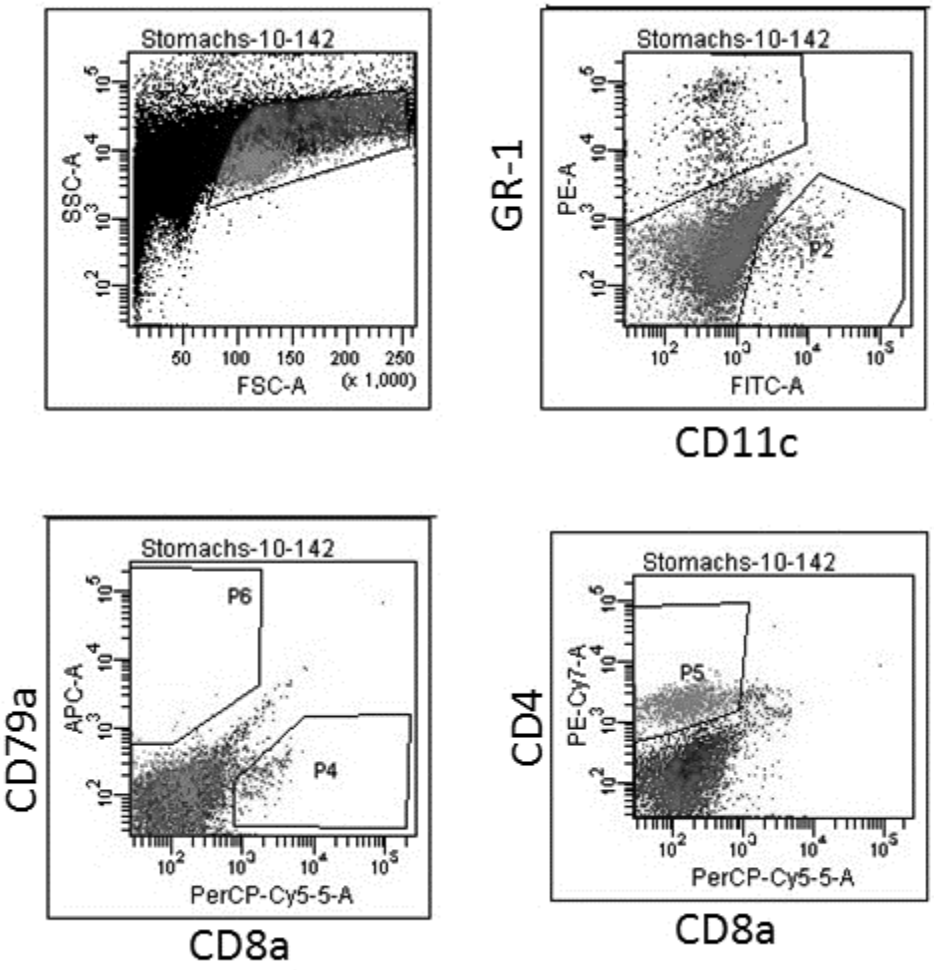


Figure 4.2: Flow cytometry dot plots of gastric cells from *H. pylori* infected SCID mice adoptively transferred with B6 CD4+ cells. Gastric cells were isolated from mice 8 weeks after transfer and 10 weeks after infection (see Methods). Splenocytes were used to gate leukocytes on size and granularity (upper left) and all gates are subsidiary to that first gate. All gastric leukocyte gates are copies of splenocytes gates (see Figure 4.1). Populations were then examined on 2-color dot plots to assess single positive populations for stain brightness (Mean Fluorescence Intensity (MFI)) and number of events (see Methods).

Figure 4.3: Flow cytometry plots of regulatory CD4+ cells from the stomachs of adoptively transferred mice

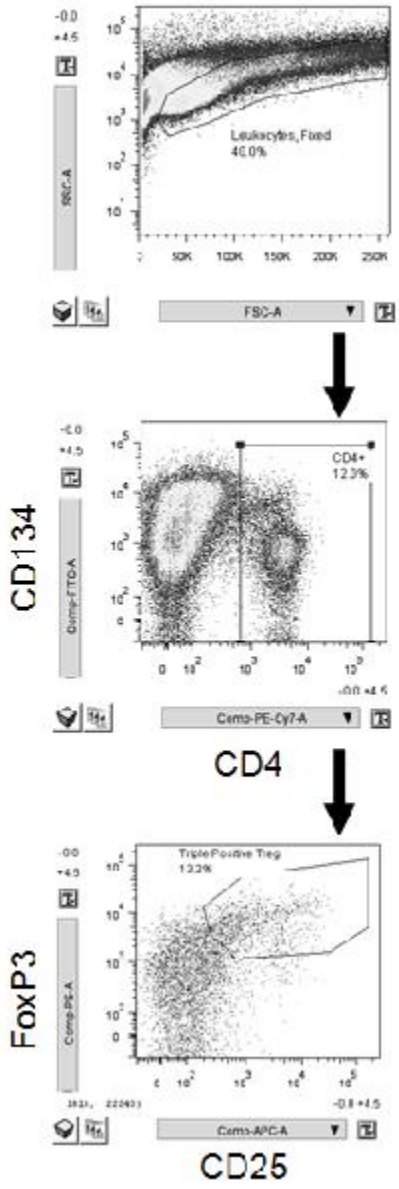


Figure 4.3: Flow cytometry dot plots of fixed and permeabilized gastric leukocytes from *H. pylori* infected SCID mice adoptively transferred with B6 CD4+ cells. Gastric cells were isolated from mice 8 weeks after transfer and 10 weeks after infection (see Methods). Leukocytes were gated on size and granularity (top) and then sub-gated on CD4 expression. CD4/CD25/FoxP3 triple positive cells were then gated to count the number of events as a subset of CD4+ cells (see Methods).

Figure 4.4: Mean fluorescent intensity of cell surface markers in spleen and stomach

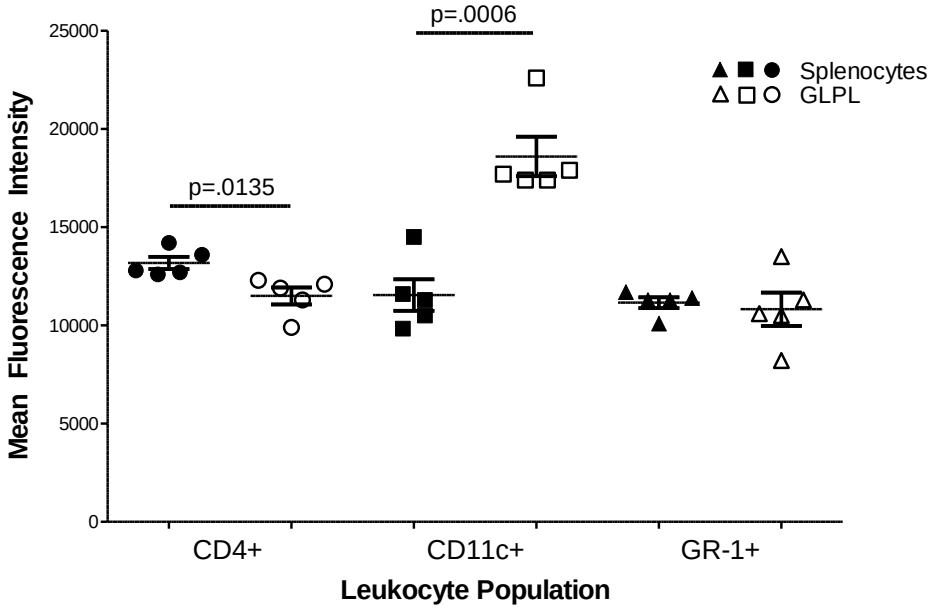


Figure 4.4: Mean fluorescence intensity (MFI) of antibody-staining on leukocytes isolated from the spleens and stomachs of *H. pylori*-infected B6 cell recipient SCID mice. The entire leukocyte fraction was first identified in splenic populations and that gate was then used to examine gastric leukocytes (see Figure 4.1). The MFI of all events in the specific gate was determined by the flow cytometer (see Methods).

Figure 4.5: Leukocyte populations in spleen and stomach of adoptively transferred mice

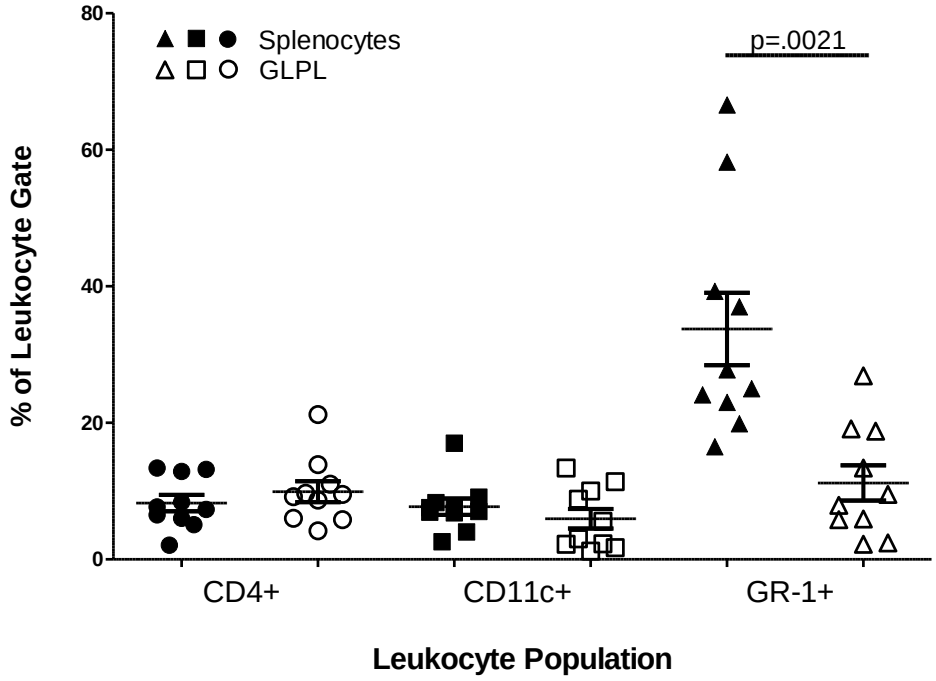


Figure 4.5: Relative abundance of single-stained populations of leukocytes isolated from the spleens and stomachs of *H. pylori*-infected B6 cell recipient SCID mice. The entire leukocyte fraction was first identified in splenic populations and that gate was then used to examine gastric leukocytes (see Figure 4.1). Abundance of events as a percentage of total events in the leukocyte gate was calculated by the flow cytometer (see Methods).

Table 4.2: Cell surface markers selected for initial profiling of gastric lamina propria leukocytes and splenocytes harvested from *H. pylori*-infected SCID and RAG2-KO mice transferred with CD4+ T cells.

Marker	Cell population	Tissues stained
CD4	Helper T cells	Stomach, spleen
CD8a	Killer T cells	Stomach, spleen
CD11c	Monocytes, macrophages, dendritic cells	Stomach, spleen
CD25	Regulatory T cells, activated T cells	Stomach
CD79a	B cells	Stomach, spleen
CD134	Activated T cells	Stomach
FoxP3	Regulatory T cells	Stomach, spleen
GR-1 (Ly-6G)	Neutrophils, eosinophils, dendritic cells	Stomach, spleen

Table 4.3: Gastritis scores in *H. pylori*-infected RAG2-KO mice adoptively transferred with different numbers of B6 CD4+ cells.

Group	Transfer of B6 CD4+ Cells	Number of mice[a]	Mean gastritis score[b]
Normal	$1x10^6$	9	264.8 ± 16.4
Half-dose	$5x10^5$	10	270.1 ± 20.2
10x dose	$1x10^7$	8	98.3 ± 45.3*

[a]Total number of mice per group in 2 separate experiments.
[b]Slides were scored as described in the methods.
* $p < 0.01$ when compared to the normal dose group.

Figure 4.6: $CD4^+$ splenocytes in mice transferred with different numbers of T cells

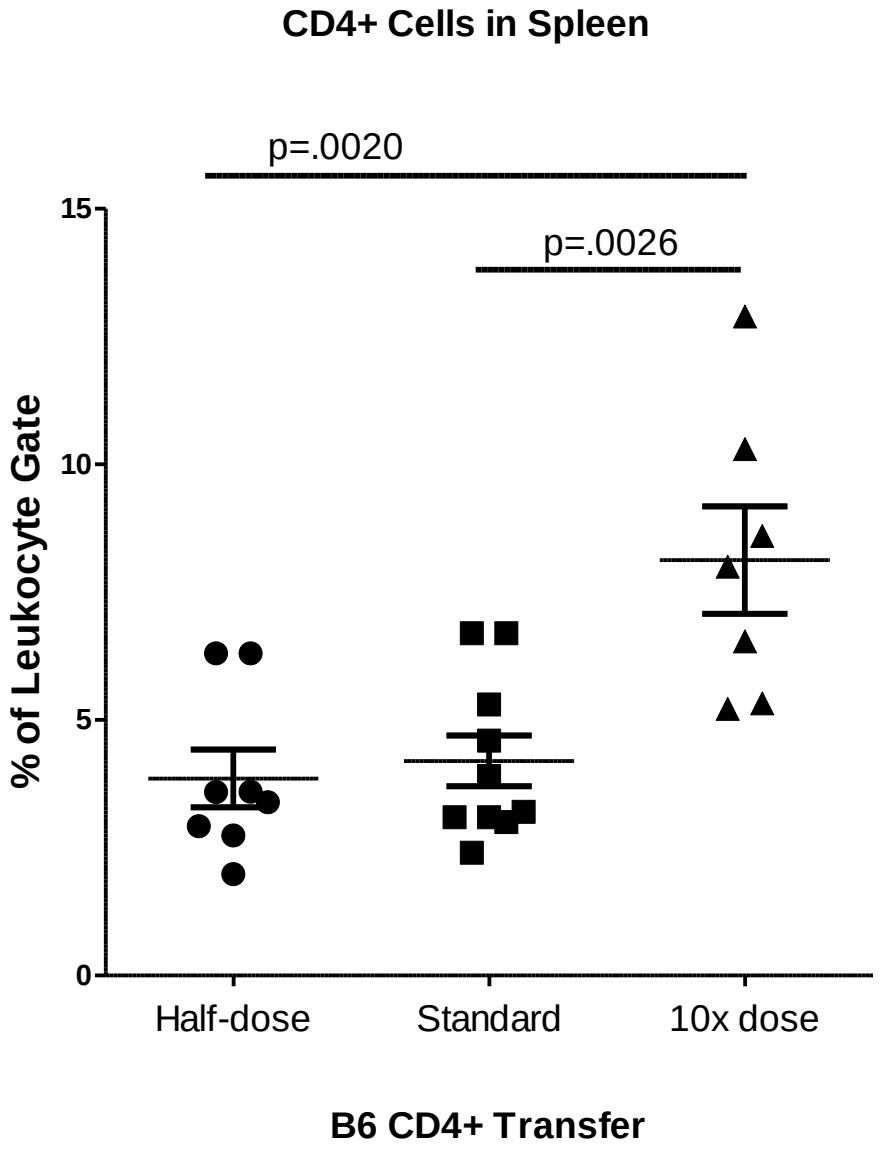


Figure 4.6: $CD4^+$ cells as a percentage of the total leukocyte gate of splenocytes from *H. pylori* infected RAG2-KO mice adoptively transferred with different quantities of B6 CD4+ cells. Gates were assigned as described in Figure 4.1, and cells were isolated and stained as described in Methods. There was no statistical difference in the number of $CD4^+$ cells seen in the spleens of mice that received either $5x10^5$ or $1x10^6$ B6 CD4+ cells.

Figure 4.7: $CD4^+$ cells in the stomachs of mice transferred with different numbers of T cells

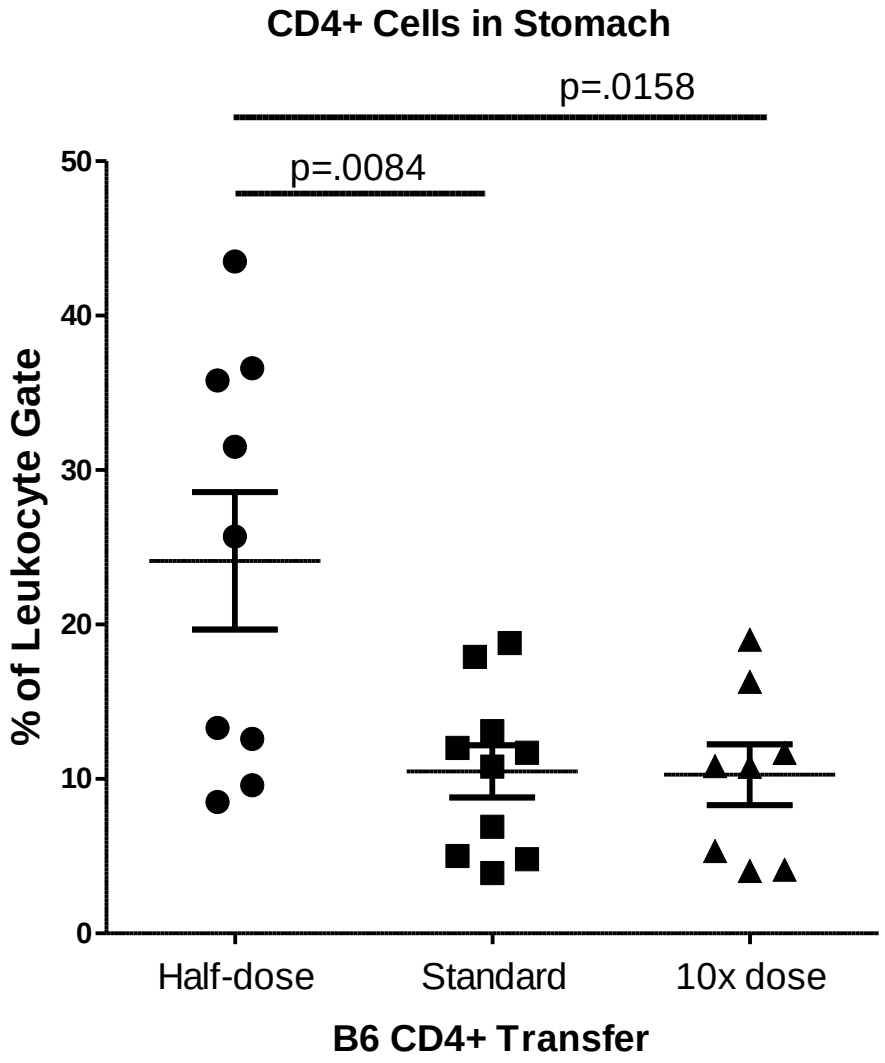
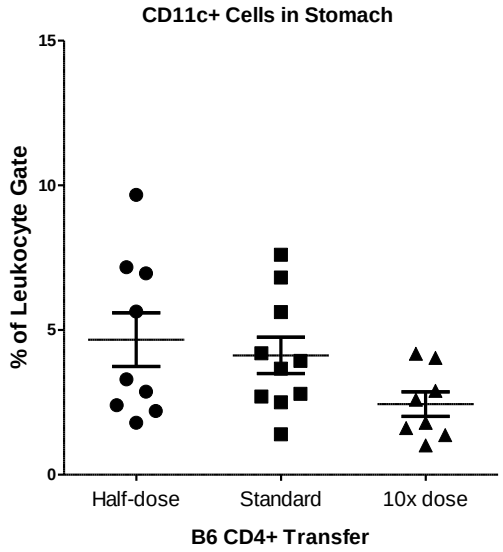


Figure 4.7: $CD4^+$ cells as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with different quantities of B6 CD4+ cells. Gates were assigned as described in Figure 4.2, and cells were isolated and stained as described in Methods. There was no statistical difference in the CD4+ population of leukocytes in mice that received either $1x10^6$ or $1x10^7$ B6 CD4+ cells.

Figure 4.8: Leukocyte populations in the stomachs of mice transferred with different numbers of T cells

A:



B:

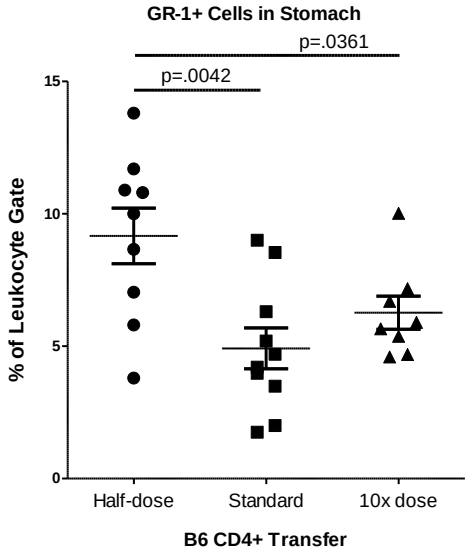


Figure 4.8: Myeloid cells (a) and granulocytes (b) as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with different quantities of B6 CD4+ cells. Gates were assigned as described in Figure 4.2, and cells were isolated and stained as described in Methods. There was no statistical difference in the fraction of CD11c+ cells between any group of mice, and no difference in the fraction of GR-1+ cells between mice that received either $1x10^6$ or $1x10^7$ B6 CD4+ cells.

Figure 4.9: Regulatory T cells in the stomachs of mice transferred with different numbers of T cells

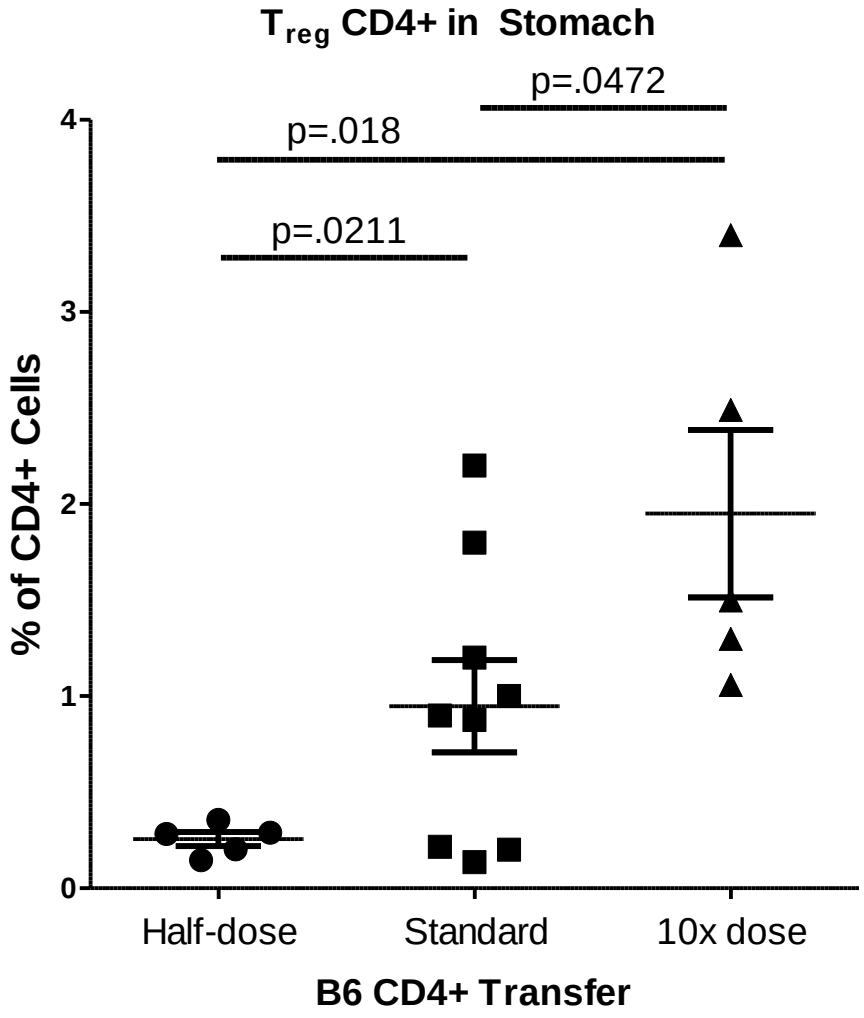


Figure 4.9: $CD4^+/CD25^+/FoxP3^+$ cells as a percentage of the CD4+ population of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with different quantities of B6 CD4+ cells. Gates were assigned as described in Figure 4.3, using fixed and permeabilized cells stained with CD4, CD25 and FoxP3. Cells were isolated and stained as described in Methods.

Table 4.4: Gastritis scores in *H. pylori*-infected RAG2-KO mice adoptively transferred with different populations of B6 CD4+ cells.

Group	Transfer of B6 Cells	Number of mice[a]	Mean gastritis score[b]
Unfractionated	$5x10^5$ CD4+ cells[c]	9	270.1 ± 20.2
T_{reg}	$5x10^5$ CD4+/CD25+/FoxP3+ cells[d]	9	152.5 ± 29.7**
Mixed	$5x10^5$ CD4+ cells and $5x10^5$ CD4+/CD25+/FoxP3+ cells	4	97.0 ± 26.9**

[a]Total number of mice per group in 2 separate experiments.
[b]Slides were scored as described in the methods.
[c]Transferred population consists of 85-95% CD4+ splenocytes (data not shown).
[d]Transferred population consists of 85-95% CD4+ splenocytes, of which 65-80% are CD4/CD25/FoxP3 triple positive (data not shown).
** $p < 0.01$ when compared to the group transferred with normal CD4+ T cells.

Figure 4.10: $CD4^+$ splenocytes in mice transferred with different populations of T cells

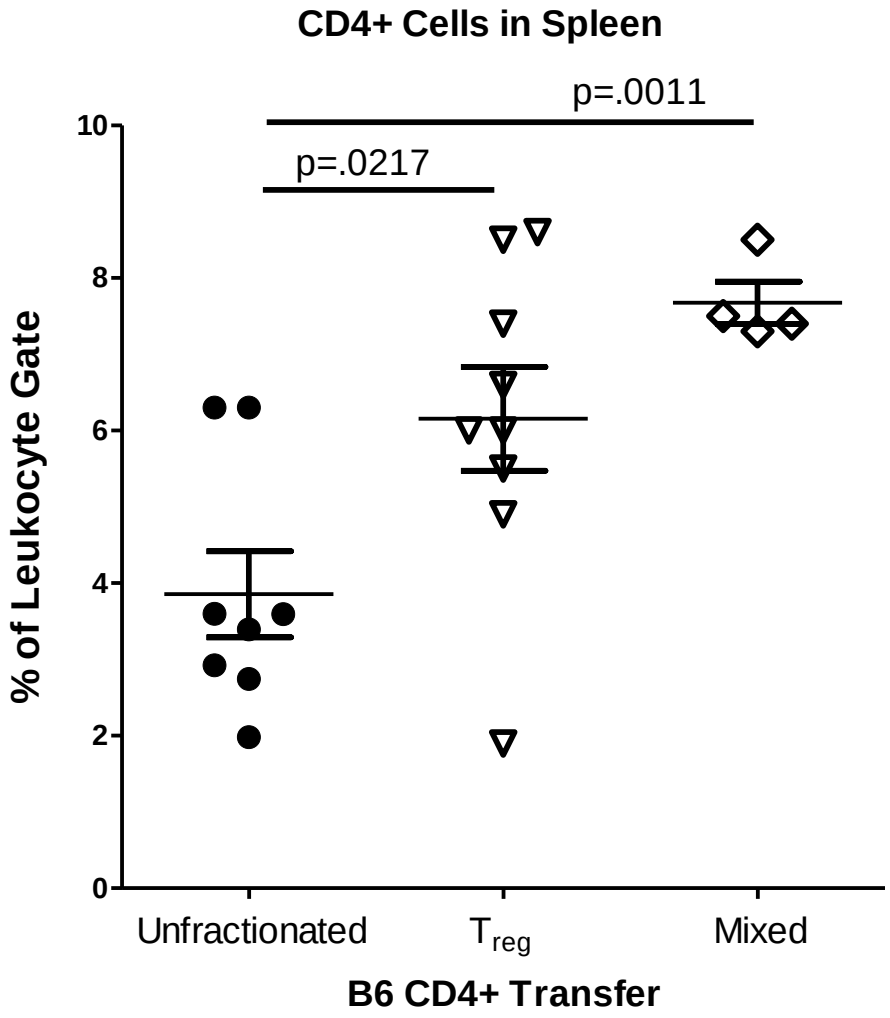


Figure 4.10: $CD4^+$ cells as a percentage of the total leukocyte gate of splenocytes from *H. pylori* infected RAG2-KO mice adoptively transferred with different populations of B6 CD4+ cells. Gates were assigned as described in Figure 4.1, and cells were isolated and stained as described in Methods. There was no statistical difference in the number of $CD4^+$ cells seen in the spleens of mice that received either T_{reg} or 1:1 mixed T_{reg} and unfractionated B6 CD4+ cells.

Figure 4.11: $CD4^+$ cells in the stomachs of mice transferred with different populations of T cells

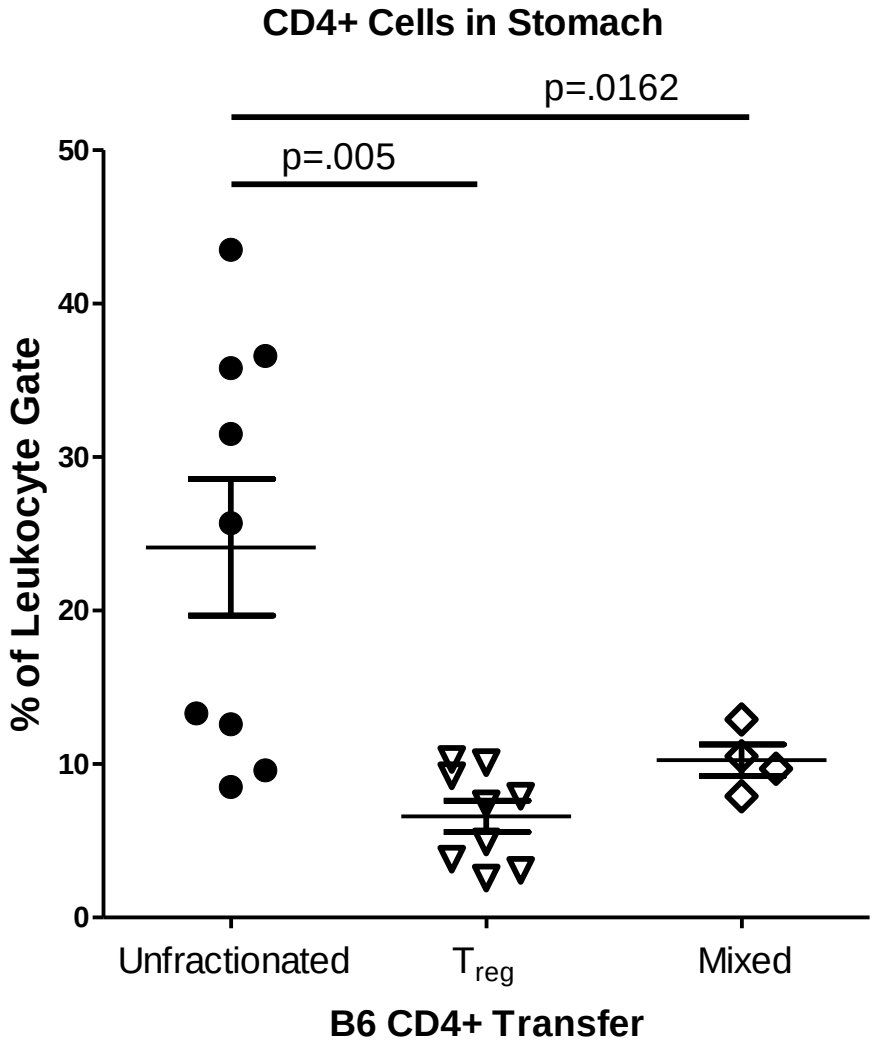
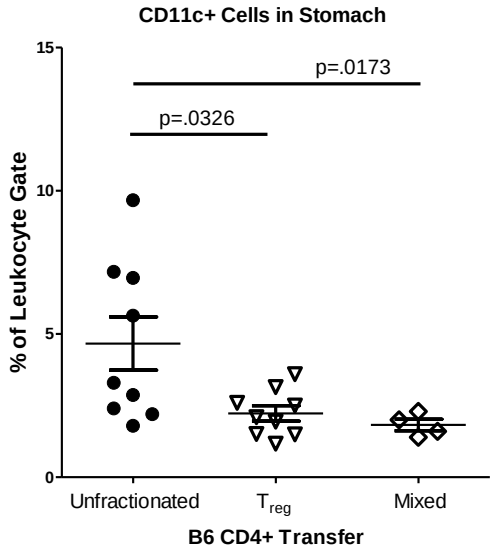


Figure 4.11: $CD4^+$ cells as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with different populations of B6 CD4+ cells. Gates were assigned as described in Figure 4.2, and cells were isolated and stained as described in Methods. There was no statistical difference in the CD4+ population of leukocytes in mice that received either T_{reg} or 1:1 mixed T_{reg} and normal effector B6 CD4+ cells.

Figure 4.12: Leukocyte populations in the stomachs of mice transferred with different populations of T cells

A:



B:

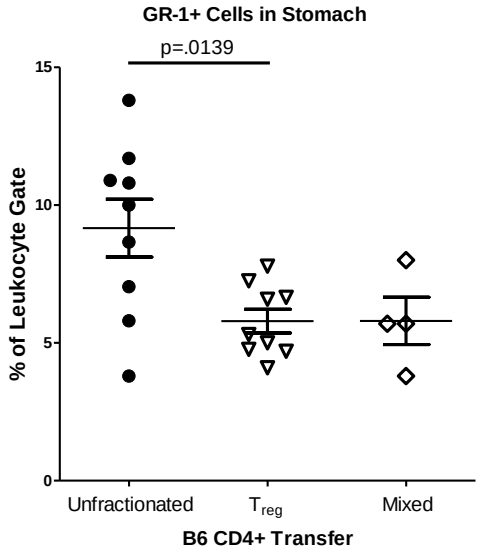


Figure 4.12: Myeloid cells (a) and granulocytes (b) as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with different quantities of B6 CD4+ cells. Gates were assigned as described in Figure 4.2, and cells were isolated and stained as described in Methods. There was no statistical differences between mice that received either T_{reg}s or 1:1 mixed T_{reg} and normal effector B6 CD4+ cells.

Figure 4.13: Regulatory T cells in the stomachs of mice transferred with different populations of T cells

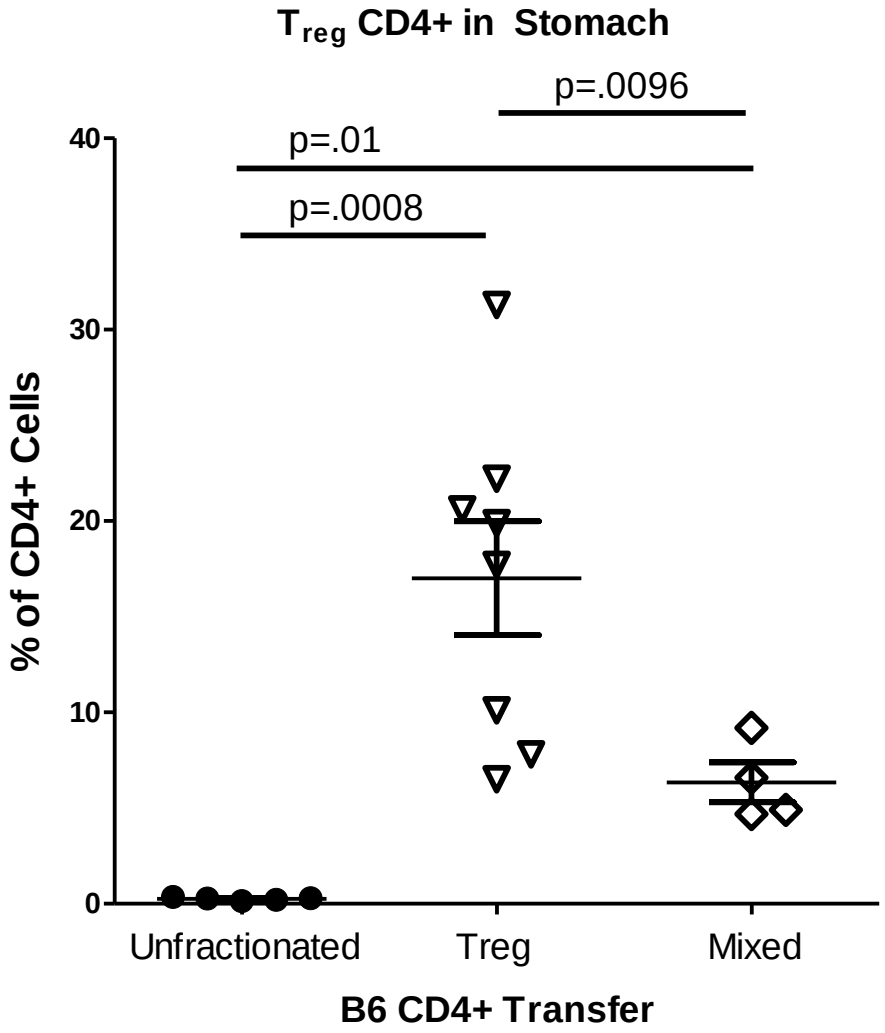


Figure 4.13: $CD4^+/CD25^+/FoxP3^+$ cells as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with different populations of B6 CD4+ cells. Gates were assigned as described in Figure 4.3, using fixed and permeabilized cells stained with CD4, CD25 and FoxP3. Cells were isolated and stained as described in Methods.

Table 4.5: Gastritis scores in *H. pylori*-infected SCID mice adoptively transferred with CD4+ T cells from B6, IFNγKO, or 17AKO mice.

Donor cell type	Number of mice	Mean gastritis score[b]
B6	19	176.2 ± 19.0
IFNγKO	4	130.8 ± 36.9
17AKO	14	149.3 ± 24.2

[a]Total number of mice per group in multiple experiments.
[b]Slides were scored as described in the methods.

Figure 4.14: $CD4^+$ splenocytes in mice transferred with cytokine-deficient T cells

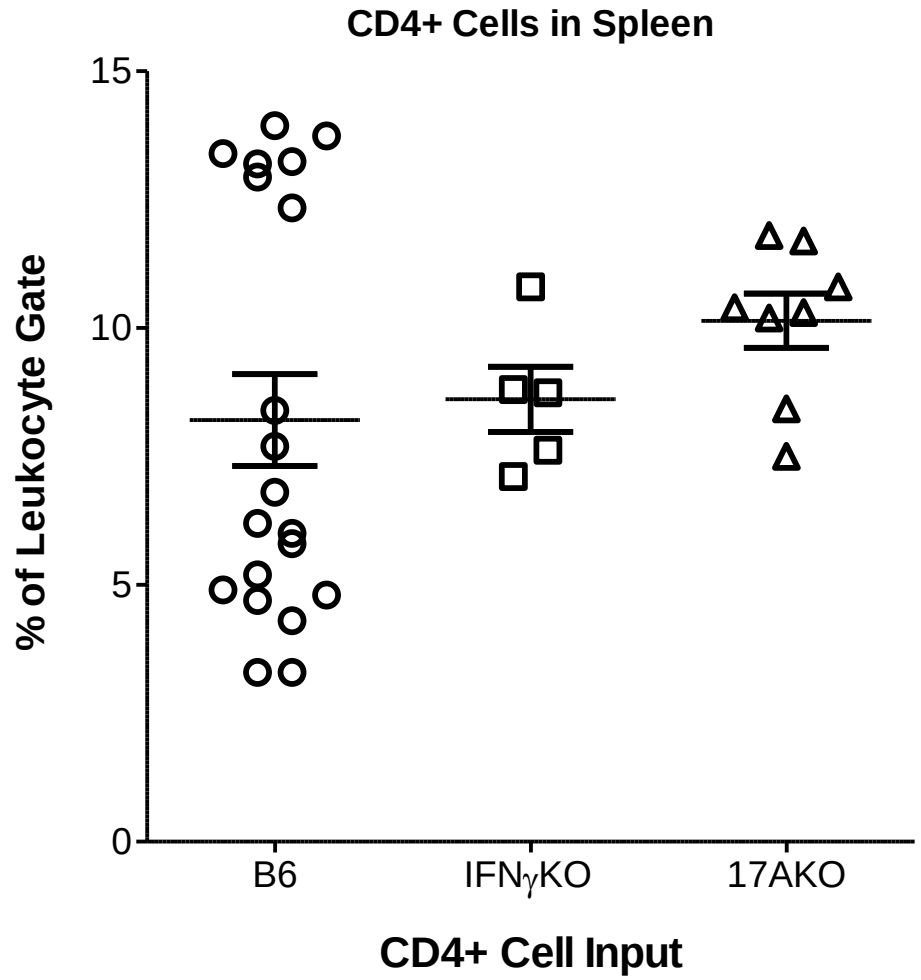


Figure 4.14: $CD4^+$ cells as a percentage of the total leukocyte gate of splenocytes from *H. pylori* infected RAG2-KO mice adoptively transferred with cytokine-deficient CD4+ cells. Gates were assigned as described in Figure 4.1, and cells were isolated and stained as described in Methods. There was no statistical difference in the fraction of $CD4^+$ cells found in any group.

Figure 4.15: $CD4^+$ cells in the stomachs of mice transferred with cytokine-deficient T cells

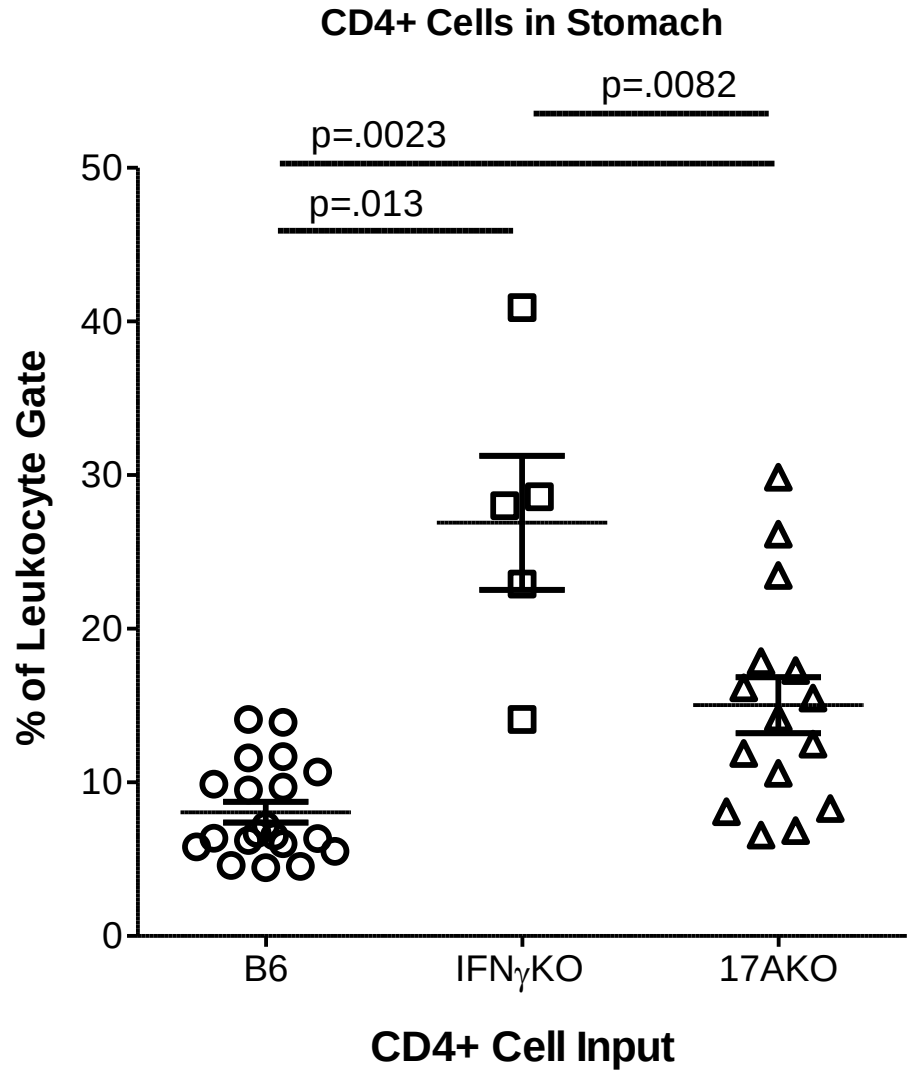
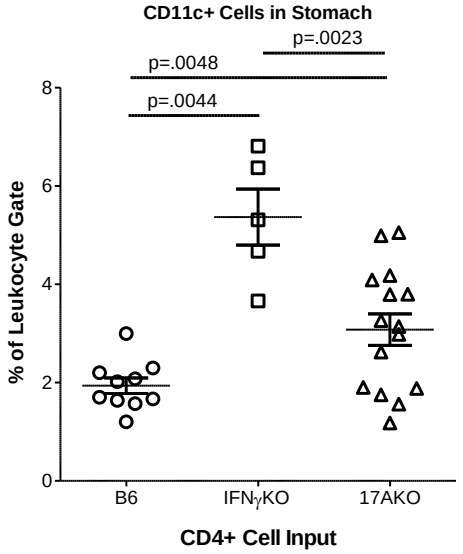


Figure 4.15: $CD4^+$ cells as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with cytokine-deficient CD4+ cells. Gates were assigned as described in Figure 4.2, and cells were isolated and stained as described in Methods.

Figure 4.16: Leukocyte populations in the stomachs of mice transferred with cytokine-deficient T cells

A:



B:

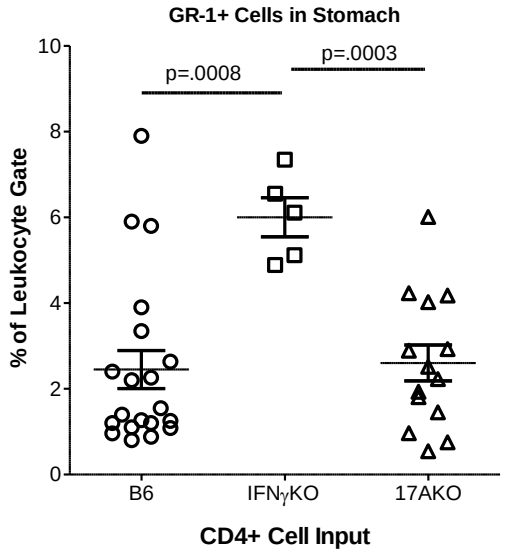


Figure 4.16: Myeloid cells (a) and granulocytes (b) as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with cytokine deficient CD4+ cells. Gates were assigned as described in Figure 4.2, and cells were isolated and stained as described in Methods.

Figure 4.17: Regulatory T cells in the stomachs of mice transferred with cytokine-deficient T cells

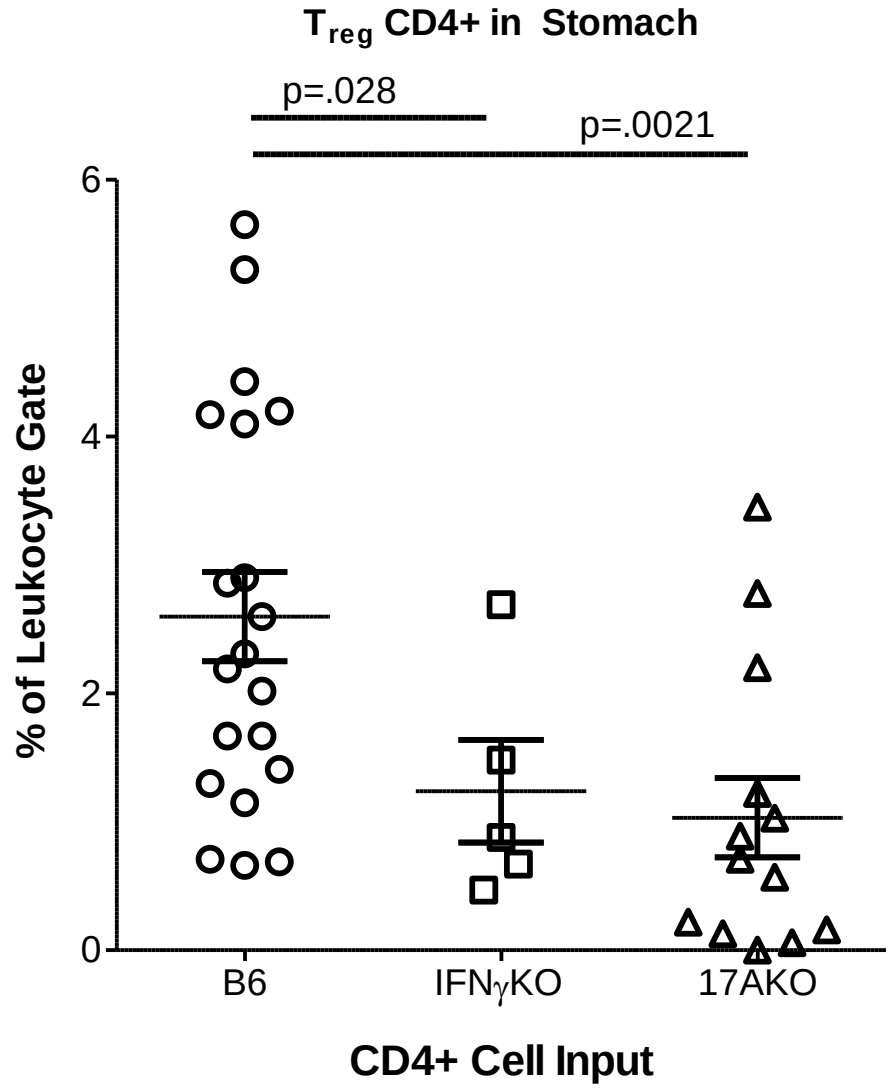


Figure 4.17: $CD4^+/CD25^+/FoxP3^+$ cells as a percentage of the total leukocyte gate of gastric lamina propria leukocytes isolated from infected RAG2-KO mice adoptively transferred with cytokine-deficient CD4+ cells. Gates were assigned as described in Figure 4.3, using fixed and permeabilized cells stained with CD4, CD25 and FoxP3. Cells were isolated and stained as described in Methods. There was no statistical difference in the fraction of T_{reg} seen between mice that received either IFNγKO or 17AKO CD4+ cells.

METHODS

Mice strains and housing

Helicobacter-free C57Bl/6 (B6), C57B6.129S7-*Ifng*^{tm1Ts}/J (IFNγ KO), C57B6.CB17-*Prkdc*^{Scid}/SzJ (SCID) and B6(Cg)-*Rag2*$^{tm1.1Cgn}$/J (RAG2-KO) mice were purchased from Jackson Laboratories and maintained in house. C57Bl/6 IL-17A knockout (17AKO) mice were bred and maintained in house. No known mouse pathogens are present in the mouse colonies as determined by routine periodic screening of sentinel mice. Mice were maintained in static microisolator cages and offered non-supplemented commercial mouse chow and water *ad libitum*. The University of Michigan Animal Care and Use Committee approved all animal experiments.

H. pylori culture and inoculation

Plate cultures of *H. pylori* SS1 were used to inoculate overnight broth cultures in 10 mL of Brucella broth with 10% fetal calf serum and 50 µl of Skirrow's antibiotic supplement. Cultures were centrifuged and washed once and then resuspended in sterile phosphate-buffered saline (PBS), counted on a hemacytometer, and diluted to a final concentration of $1x10^8$ bacteria/mL. SCID and RAG mice were given 100 µl of the bacterial suspension giving a total dose of $1x10^7$ of *H. pylori* SS1 per mouse. This procedure was repeated on the following day, for a total of two inoculations.

Adoptive transfer

Two weeks after the mice were inoculated with *H. pylori*, we harvested spleens from B6, IFNγKO or 17AKO mice. Splenocytes were dissociated and then suspended in 450 µL of 1x PBS with 0.5% bovine serum albumin, and mixed with 50 µL of anti-CD4 L3T4-coated Miltenyi MACS Beads (Miltentyi Biotec, Auburn, CA) for 15 minutes at 4

°C. The cell-bead mixture was layered onto a pre-wetted Miltenyi MS magnetic column and washed twice in MACS buffer, before the columns were removed from the magnet and the cells were removed with an additional 500 µL of buffer. The CD4-enriched cells were washed twice in media and resuspended to a concentration of $1x10^7$ cells/mL in sterile PBS. Groups of *H. pylori*-infected SCID mice received 100 µL of either B6, IFNγKO or 17AKO cell suspension via intraperitoneal injection, for a final adoptive transfer of $1x10^6$ CD4-enriched cells per mouse (see Table 4.5). For engrafting dose experiments, *H. pylori*-infected RAG2KO mice received 100 µL of B6 CD4+ cells at concentrations of $5x10^6$ cells/mL, $1x10^7$ cells/mL, or $1x10^8$ cells/mL (see Table 4.3).

For regulatory T cell engrafting experiments, B6 splenocytes were enriched for CD4+/CD25+ cells via the Miltenyi MACS Regulatory T Cell Isolation kit. Briefly, CD4+ cells were enriched by mixing splenocytes with a mixture of biotinylated antibodies against CD8, CD11b, CD45R, CD49b, and Ter-119, followed by mixing the cells with anti-biotin magnetic beads. The cell-bead mixture was layered onto a pre-wetted Miltenyi LD column and washed twice in MACS buffer, and the flowthrough containing CD4+ cells was collected. Then the CD4+ cells were mixed with anti-CD25 antibody-coated magnetic beads. The cell-bead mixture was then layered onto a pre-wetted Miltenyi MS magnetic column and washed twice in MACS buffer, before the columns were removed from the magnet and the cells were removed with an additional 500 µL of buffer. The CD4/CD25-enriched cells were washed twice in media and resuspended to a concentration of $1x10^7$ regulatory T cells/mL in sterile PBS. *H. pylori*-infected RAG2-KO mice were injected with either 50 µL of normal CD4-enriched cells, 50 µL of CD4/CD25-enriched cells, or 50 µL of each cell suspension (see Table 4.4).

Tissue collection, leukocyte isolation, and histology scoring

Adoptively transferred mice were euthanized at 8 weeks after transfer, a total of 10 weeks after infection, and their spleens and stomach tissues were harvested for histological analysis and leukocyte profiling. The forestomach was removed before opening the remaining tissue along the inferior curvature. Spleens were mashed so that the tissue was dissociated into single-cell suspensions. For histology, 1 mm-wide strips from the greater curvature were emersion-fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5µm sections, and stained with hematoxylin and eosin. The remaining tissue was placed in ice-cold 1x HBSS until processing.

Leukocytes were isolated from gastric tissue in a protocol adapted from the intestinal lymphocyte protocol of Lefrancois and Lycke (111). Briefly, stomach tissue was minced with a scalpel into pieces approximately 1 mm on a side. The pieces were rinsed twice in room temperature CMF/HEPES (1x Hank's buffered saline w/15 mM HEPES, pH 7.2) on an orbital rocker for 5 minutes each. Next, the pieces were washed twice in CMF/FCS/EDTA (CMF/HEPES w/10% fetal calf serum and 5 mM EDTA) on a shaking platform at 37 °C for 15 minutes, and then rinsed once in RPMI-10 (RPMI-1640 w/15 mM HEPES and 10% fetal calf serum, pH 7.2) for 5 minutes at room temperature to remove any traces of EDTA. Lastly, the tissue pieces were digested twice in RPMI-10 containing 15 U/mL Liberase-TM (Roche) for 45 minutes each at 37 °C. The supernatants from each wash and digestion were poured through a 40 µm nylon strainer and collected in a single silanized Erlenmeyer flask per stomach. Cells collected in the Erlenmeyer flask were spun down and resuspended in RPMI-10, stained with trypan blue and dye-excluding cells were counted to assess viable cell yield.

Flow cytometry

Single cell suspensions of splenocytes or gastric cells were split into two aliquots, one stained for CD4, CD8, CD11c, CD79a and Ly-6G (GR-1). The other set of cells was fixed, permeabilized and stained using the eBiosciences Regulatory T Cell Staining Kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Stained cells were analyzed with single-color controls on a flow cytometer. For the live cells, single-positive populations were gated and counted, while CD4+/CD25+/FoxP3+ cells were only counted from the fixed cells. Analysis of the GLPL profiles for the cytokine knockout adoptive transfer experiments were conducted after sample analysis using the FlowJo program (Tree Star Inc, Ashland, Oregon, USA, www.flowjo.com).

Data handling and statistical analyses

All graphs were made using GraphPad Prism 5.01 version 5.01 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). This program automatically calculated and graphed the standard error of the mean (SEM) for all column graphs. All matching datasets were tested for statistical significance through application of two-tailed Student's t-test using the in-built statistical analysis package. Experimental groups that are not statistically significantly different are unmarked.

Chapter 5

Overall Conclusions and Future Directions

The interaction of Th1 and Th17 phenotypes in *H. pylori* gastritis

Recognition of Th17 as a second pro-inflammatory helper T cell phenotype challenged numerous long-held assumptions about inflammatory immune responses, especially in response to mucosal infections (for review see (99)). When the Eaton lab observed *H. pylori* gastritis in infected SCID mice given T-bet KO T cells, thus disabling the Th1 response (50), we needed to look for alternatives to the Th1 response in order to explain our findings. By pairing the Th17 response with the concept of counter-balanced T cell phenotypes, I offered what I considered an attractive hypothesis, where *H. pylori* disease develops in the absence of IFNγ thanks to increased IL-17A production coming from a larger population of Th17-polarized cells arising in the absence of Th1 cells. I found initial evidence to support this idea in my mRNA analysis of tissue from mice transferred with T-betKO T cells (Chapter 2).

As per my initial hypothesis, which stated that IL-17A contributed to *H. pylori* gastritis only in the absence of IFNγ, the lack of IL-17A did not significantly affect disease in our adoptive transfer disease model (Chapter 2) even though we found significantly increased IL-17F expression in 17AKO recipients, and the expression of this homologous inflammatory mediator might explain the inflammation we found. To confirm this, collaborated with the Chang laboratory to use their CIITA-Tg CD4+ T mice, which express significantly less RORγt than B6 CD4+ T cells and so have a

marked defect in production of Th17 cytokines, including IL-17A and IL-17F (83). However, in infected mice transferred with CIITA-Tg CD4+ T cells, we found that disease developed in a fashion similar to mice receiving 17AKO cells, that disease development was not significantly affected by use of the transgenic T cells (Chapter 3). These findings suggested that as long as IFNγ expression was unaffected, IL-17A did not significantly contribute to disease development, a result that agreed with both my hypothesis and our previous observations with T-betKO T cells (50).

In the simple infection model of *H. pylori* gastritis, it is clear that the loss of IL-17A significantly affects disease development, and that the contribution of IL-17A occurs even in the presence of a fully functional Th1 response to infection. This finding disagrees with my initial hypothesis, and so I formulated a second hypothesis, positing that Th1- and Th17-polarized T cells were in competition to promote inflammation, and this suggested I should find increased IFNγ expression in the absence of IL-17A. While *H. pylori* infection promoted IFNγ expression in both B6 and 17AKO mice, infected 17AKO mice expressed less IFNγ mRNA than controls. I found a similar result in my adoptive transfer experiments, where IFNγ expression was slightly but not significantly decreased in RAG2-KO mice transferred with 17AKO T cells. Furthermore, we found significantly lower expression of both IL-17A and IFNγ expression in infected mice transferred with CIITA-Tg cells compared to B6 recipients. Together these findings discounted my second hypothesis.

The relative decrease in IFNγ expression in infected mice lacking either IL-17A or a functional Th17 response suggested a third hypothesis. Could Th1- and Th17-polarized T cells actively cooperate in order to promote maximum inflammation in *H.*

pylori infected mice? This idea, if I allow that IFNγ is more important for inflammation in disease than IL-17A, explains both our simple infection and adoptive transfer results. In the simple infection model, IL-17A expression is insufficient to induce significant inflammation without T cell-produced IFNγ. Without IL-17A, IFNγ expression promotes marked inflammation that is still significantly less than seen in B6 mice. The greatest problem with this hypothesis is its disagreement with the current immunological dogma about the balance of T cell effector phenotypes, although there are a growing number of examples that suggest this sort of cooperative behavior (93, 117, 151). This third hypothesis also explains much of our adoptive transfer results, where we see reduced inflammation, although not always a significant decrease, in the absence of particular cytokines.

To test this new hypothesis, we would need to disable both the Th1 and Th17 responses together. The simplest approach would be to use mice lacking both T-bet and RORγt, the transcription factors regulating IFNγ and IL-17A/F in CD4+ T cells respectively, and to test these T cells in the adoptive transfer model. I predict that we would see an additive effect upon disease development with significantly lower histological inflammation in mice given T-bet x RORγt double-knockout T cells than in infected mice transferred with either T-betKO or RORγt-KO T cells. I would still expect to see some inflammation in infected mice with double-knockout T cells, as suggested by the mRNA levels of innate inflammatory mediators induced by *H. pylori* infection. I do not expect these double-knockout mice to be suitable for testing the Th1/Th17 cooperative hypothesis in the simple infection model. As it is clear that disease fails to develop in a timely fashion in T-betKO mice in simple infection (Chapter 2), and RORγt-

KO mice lack Peyer's patches and lymph nodes (60) which retards or prevents the development of *H. pylori* gastritis (98, 146), I do not think that direct infection of the double-knockout mice would promote any disease development at all. Similarly, we would be unable to use IFNγ x IL-17A double-knockout mice in the simple infection model, as disease fails to develop in IFNγKO mice (186), making it difficult to sort out the contributions of both Th effector phenotypes to disease development.

The importance of the 'innate' inflammatory response to *H. pylori*

It is important to note that a cooperative model for Th1/Th17 action in *H. pylori* disease probably cannot account for all of the inflammation observed in our cytokine knockout and transgenic experiments. This issue is most apparent in mice transferred with CIITA-Tg T cells, where we find that *H. pylori* infection barely induces a 2-fold increase in IFNγ expression (Chapter 3) without a significant difference in histological inflammation. This is only a small change in cytokine expression, and ten times less than the 25-fold average increase in IFNγ expression seen in infected mice receiving 17AKO T cells (Chapter 2). In a similar way, the simple infection of 17AKO mice promotes an increase in IFNγ expression that is approximately four-fold less than that seen in infected B6 mice, but histological disease is barely reduced by half (Chapter 2). In these examples we found significant disease development occurred even when both IFNγ and IL-17A expression was comparatively reduced, suggesting that Th1 and Th17 cytokines cannot account for the whole of gastric inflammation.

We know from several reports that Th2 cytokines do not contribute to *H. pylori* gastritis (25, 88, 186), suggesting that we look to inflammatory mediators other than those produced by CD4+ T cells. There is already a significant body of literature

investigating the contribution of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα to disease in human patients, although the great majority of this work focuses on the link between these cytokines and gastric cancer (for review, see (200)). In mice these cytokines are produced by activated macrophages and dendritic cells, while TNFα may additionally be secreted by Th1-polarized CD4+ T cells. There are only a few reports that indirectly investigate the contributions of these molecules to *H. pylori* gastritis. Abiko et al. found significantly fewer granulocytes in the gastric mucosa of *H. pylori* infected IL-1 receptor type I-deficient mice compared to infected controls (1). Panthel et al. discovered that *H. pylori* colonization levels were 6 to 8-fold higher in mice lacking either TNF receptor chain p55 or both TNFR p55 and p75, suggesting a hobbled immune response to infection (160).

In all of the experiments described here, *H. pylori* infection induces significant, and sometimes dramatic, increases in the mRNA of these cytokines. On average, *H. pylori* infection increased IL-1β expression by 500-fold in B6 mice, an induction comparable to the observed rise in IFNγ mRNA levels. In comparison, infection of 17AKO mice triggers an eventual 1500-fold increase in expression in the gastric mucosa, dwarfing the induction of IFNγ by a factor of five. Induction of both IL-1β and IL-6 is greater than IFNγ expression in mice transferred with 17AKO cells, although moderately so in comparison to the rise in simple infection. We observed the same response, with greater induction of IL-1β and IL-6, in mice given CIITA-Tg CD4+ cells. TNFα expression is also induced 100-fold during simple infection in both B6 and 17AKO mice, and the increase in TNFα mRNA is roughly twice that of IFNγ in recipient mice with either 17AKO or CIITA-Tg T cells. Taken together these findings strongly suggest that

non-T cell-derived cytokines contribute significantly to *H. pylori* disease, and that possibly one or more of these innate inflammatory cytokines may rival IFNγ for importance in the development of gastritis.

In Chapter 2 I discussed exploring the contributions of 'innate' inflammatory cytokines to *H. pylori* gastritis through use of single-cytokine knockout mice, where I hypothesized that the host-derived innate effector cells are responsible for production of the non-Th cytokines. Specifically I described using IL-1β, IL-6, and TNFα deficient mice as recipient hosts in adoptive transfer experiments in an effort to define the contribution of the host innate immune system to disease development. But, are T cells directing the action of innate effector cells, or are the innate cells dictating T cell responses? Receptor-deficient strains of mice are available for IL-1β, IL-6, and TNFα, and we can use these mice as T cell donors to see how the innate inflammatory cytokines influence T cell behavior. Alternatively, we could use those receptor knockouts as recipient mice and explore how that impedes the innate response to *H. pylori* infection. Following my hypothesis, altering the genotype of transferred CD4+ T cells should not significantly affect disease development, but altering the host ability to produce or respond to the innate inflammatory mediators would significantly inhibit histological gastritis.

However, I think that we can refine all of these proposed experiments into a more elegant system that allows us to explore the interaction between host innate effector cells and transferred CD4+ cells to determine how non-T cell inflammatory cytokines affect both populations of cells. For IL-6 and TNFα, we can mix and match cytokine deficiency and cytokine-receptor deficiency, that is we could use the receptor-deficient

mice as T cell donors and the cytokine-deficient mice as recipients. This would let us clearly define the contribution of host-derived innate inflammatory mediators upon T cell behavior in the adoptive transfer model of *H. pylori* disease, as we will limit the direction of cytokine effect to go from potential T cell expression to potential host response. My hypothesis predicts that this limitation in cytokine effect should not lead to a further reduction in gastritis than simply using the receptor-deficient mice as hosts. Conversely, if we used receptor-deficient mice as our recipients and cytokine-deficient mice for T cells, we direct the cytokine effect to go from host-derived cytokine production to T cell response, neatly exploring how the production of innate inflammatory cytokines affect the CD4+ T cell response to *H. pylori* infection.

Questioning the role of CD4+ T cells in *H. pylori* gastritis

The narrow focus of the adoptive transfer model of *H. pylori* gastritis is both its greatest strength and its largest liability. Use of this model allowed us to recognize the importance of CD4+ T cells in disease development, but it also encouraged assumptions about the start and progression of gastric inflammation. These assumptions include that the immune response to *H. pylori* infection is driven almost completely by the action of antigen-specific CD4+ T cells. While these T cells may have been activated by dendritic cells presenting antigenic fragments from *H. pylori* sampled in the gastric lumen (91) or through Peyer's patches (98, 146), the prominent influence of IFNγ has led many to downplay or ignore the contribution of other cell types as discussed above.

Our findings in infected mice transferred with CIITA-Tg CD4+ cells calls into question the assumption that antigen-specific CD4+ T cell responses are necessary for the continued progression of disease. We saw similar levels of histological inflammation in

the stomachs of infected mice with CIITA-Tg cells, but CD4+ T cells recovered from these mice failed to respond to the presentation of *H. pylori* antigen. It could be argued that CIITA-Tg T cells recovered from the spleens of recipient mice do not accurately reflect the population of transgenic T cells resident in the stomach responding to *H. pylori* infection, or that the CIITA-Tg T cells cannot adequately form memory responses to stimulation. Neither of these possibilities is a likely explanation, given the extent of the Chang lab's work characterizing the CIITA-Tg model. We are left with the observation that severe, *H. pylori*-mediated gastritis can continue in the absence of an antigen-specific CD4+ Th1 or Th17 cell response.

This finding suggests two important questions. First, given that *H. pylori*-specific T cells are not required for disease progression, are antigen-specific T cells required to start disease? Much has been made of the failure of the human immune system to clear *H. pylori* infection, even though marked antigen-specific T and B cell responses are found in human patients. If it is possible that an antigen-specific response is required for neither disease initiation nor progression, then we should consider *H. pylori* an irritant instead of a pathogen whose pathology somehow invokes a T cell response. Secondly, while we have previously shown that T cells are necessary to start the inflammatory response to *H. pylori* infection, are CD4+ T cells required at all to maintain disease once inflammation is robust? The possibility that *H. pylori* gastritis is refractory to the action of the adaptive immune system would also suffice to explain the failure to clear infection in humans.

Work by Nagai et al. answers the first question and sheds light on the second. They found that inflammation failed to develop when the T cell repertoire was restricted

to a single T cell receptor, in either simple infection or adoptive transfer experiments using OT-II x RAG2-KO mice (T cells from these mice only recognize an ovalbumin peptide) (146). In another experiment they demonstrated that gastric inflammation lessened, but was not eliminated, after two weeks of antibody-mediated depletion of CD4+ T cells from *H. pylori* infected, wild-type mice at 2 months post infection. A report from Stuller et al. also addresses the second question, as they found that when *H. pylori*-infected B6 mice were used as donors for adoptive transfer experiments, transfer of memory ($CD45RB^{lo}/CD25^{-}$) CD4+ T cells induced only a very mild gastritis in recipient RAG1-KO mice (197). That observation suggests that active responses by antigen-specific CD4+ T cells are not required to maintain gastric inflammation late in disease.

Combining these findings with our work we can state that *H. pylori*-mediated gastritis in mice arises from an antigen-specific CD4+ T cell response to infection, but once inflammation is established in infected stomachs, disease continues in an antigen-independent fashion. The continued presence of CD4+ T cells in the inflamed gastric mucosa may help maintain inflammation at its most florid, but the exact mechanism by which helper T cells contribute to a non-antigen driven inflammatory response remains to be elucidated. An adaptation of our adoptive transfer method, such as intracellular cytokine staining, could allow us to confirm this hypothesis by examining the role of other cytokines in addition to IFNγ and IL-17A. Gastric inflammation is established 4 weeks after adoptive transfer of CD4+ T cells into infected recipient mice, although disease severity is greatest after 8 weeks (59). We could delete the transferred T cell population after the 4 week time point, and then give the recipient mice T cells from a T

cell receptor-transgenic strain that are thus unable to respond to *H. pylori* antigens. If the hypothesis of antigen-independent maintenance of *H. pylori* disease is correct, then we should find no difference in disease development between groups of recipient mice reconstituted with either transgenic T cells or T cells from normal B6 mice.

Reconciling the Simple Infection and Adoptive Transfer models of disease

The adoptive transfer model has been instrumental in defining several of the limiting parameters of *H. pylori* disease development in mice, although the model leads to experimental results that appear dramatically different from the infection of immunocompetent mice with *H. pylori*. We report several instances here and in prior work (50) where adoptive transfer of knockout or transgenic T cells promotes severe inflammation in recipient mice while simple infection of the same strain of mice leads to significantly lower inflammation compared to infected B6 mice. We have been hard-pressed to explain these disparate results, but I believe that development of the gastric lamina propria leukocyte isolation method and flow cytometry profiling for the first time grants us some insight into a fundamental difference between the two disease models.

In several murine models of inflammatory bowel disease, adoptive transfer of CD4+ T cells into otherwise healthy immunodeficient recipient mice (including SCID and RAG2-KO mouse strains) can induce severe colitis (for a recent review, see (158)). Rapid expansion of transferred T cells in a recipient mouse is driven initially by the presentation of self-antigen on appropriate MHC molecules in a process known as homeostatic proliferation (201). However, several reports demonstrate the presence of potentially pathogenic gut bacteria can transform the homeostatic proliferation of transferred helper T cells into inflammatory disease (105, 195), as presentation of

bacterial antigens by dendritic cells resident in Peyer's patches and other GALT generates the colitogenic response in adoptively transferred mice (66, 67). Colitis is also driven mostly by Th1-polarized CD4+ T cells (41, 168), although some reports show that Th17 cells may also contribute in some models (61, 154). Co-transfer of increasing number of regulatory T cells abrogates disease development in most models of transfer colitis.

How do these colitis disease models relate to our findings in two different models of *H. pylori* gastritis in mice? From our work presented in Chapter 4, we know that adoptive transfer of smaller numbers of CD4+ T cells produces more severe disease than in mice given greater numbers of cells. We found less gastric inflammation in mice receiving 10^7 cells than in mice given 10^6 or $5x10^5$ cells, with significantly more CD4+ cells in the stomachs of mice with $5x10^5$ cells. T cell transfers constituted with 50% or more of regulatory T cells also prevent or suppress disease development (Chapter 4). These findings suggest the homeostatic proliferation of transferred cells in recipient mice may drive disease development. We also know that IFNγ production by T cells does contribute to disease severity (50), as does IL-17A albeit to a lesser degree (Chapters 2 and 4, (183, 184)). Lastly, reports by Nagai et al. and Kiriya et al. strongly suggest that Peyer's patches along the small intestine are major, if not the primary, sites for *H. pylori* antigen presentation by dendritic cells prior to induction of gastritis (98, 146) although this is still actively debated. Their observations are in line with recent work by Feng et al., who in two reports demonstrated that homeostatic proliferation requires innate immune activation by microbiota antigens in the mouse intestine (67) and that

spontaneous colitis arising from adoptive transfer requires T cell responses to microbiota gut antigens (66).

In light of these reports and our own findings, I suggest that we consider the adoptive transfer model of *H. pylori* gastritis as a variant of transfer colitis. This is not to argue that the adoptive transfer model is without merit as a means to study *H. pylori* disease when compared to the results from the simple infection model. The speed of disease development combined with the ability to mix-and-match genotypes between recipient and donor, or the ability to rapidly assess the pathogenicity of bacterial mutants, recommends adoptive transfer as an investigative tool. However, the severity of inflammation and the confounding influence of pathogenic T cell proliferation blunt the efficacy of the method in comparison to the simple infection model when working to identify the contributions of single cytokines or to separate the roles of the adaptive and innate immune responses to infection. So long as we recognize these caveats, then the adoptive transfer method remains a useful tool in studying *H. pylori* disease development.

Proposing a new model for the immune response to *H. pylori* infection in mice

I believe that the work I have reported here demonstrates that many of our original assumptions about the development of *H. pylori* gastritis are incorrect, and also that these observations grant us significant insight into the broader and significantly more complex story of the immune response to *H. pylori* infection in mice.

A summary of what we know about the initiation and progression of *H. pylori* gastritis in mice:

1) Antigen-specific CD4+ T cells are required to initiate *H. pylori* gastritis (146).
2) T cell expression of IFNγ is not required for disease development in adoptively transferred infected mice although it contributes to the severity of inflammation (50).
3) IL-17A is not required for disease development in either the adoptive transfer or simple infection models of disease, although it contributes to the severity of inflammation in both models (Chapter 2).
4) Significantly increased numbers of CD11c+ and GR-1+ cells in the gastric mucosa are associated with more severe disease (Chapter 4).
5) IL-1β and TNFα expression significantly and markedly increase in both models of infection, regardless of the genotype of the infected mice or of the donor T cells (Chapters 2 and 3).
6) Loss of either the IL-1R (1) or TNFR1 (160) reduces disease in infected mice.

And a summary of what we know about the maintenance of inflammation in *H. pylori* infected mice:

7) H. pylori colonization of the mouse gastric mucosa continues even after 15 months of severe gastritis (Chapter 2)

8) IFNγ and IL-17A from antigen-specific CD4+ T cells are not required to maintain florid gastritis in infected recipient mice (Chapter 3).
9) CD4+ T cells are not required to maintain gastric inflammation in infected mice, although they contribute to the severity of disease (146).
10) Memory CD4+ T cells from infected mice are hyporesponsive to further stimulation with *H. pylori* antigen (197).
11) Expression of 'innate' inflammatory mediators increases significantly after the peak of Th cytokine expression around 6-9 months after infection (Chapter 2).
12) Expression of immunoregulatory cytokines increases significantly a year or more after the initial *H. pylori* infection (Chapter 2).

Together, these data suggest a new model describing immune response to *H. pylori* infection in mice and humans, and are summarized in Figure 5.1. Following initial colonization of the gastric mucosa by *H. pylori*, chronic partial activation of the antigen presenting cells by live bacteria (76, 90) eventually leads to antigen-specific CD4+ T cell activation and expression of IFNγ. In turn, this promotes the full inflammatory response, attracting monocytes, neutrophils, and more T cells, and the mucosal milieu prompts IL-17A production in parallel with Th1 cytokines. However, the gastric environment precludes actual clearance of infection through the usual effector cell mechanisms of phagocytosis and degranulation. Chronic stimulation of the antigen-specific T cell response with a continued influx of *H. pylori* promotes development of anergic/ hyporesponsive memory T cells (197) and regulatory T cells (125, 171). While the regulatory T cells start damping the T cell response to *H. pylori* infection after several

months (Chapter 2), the inflammatory response in the gastric mucosa has weakened the epithelial barrier sufficiently that peptic secretions irritate the tissue. This damage, in combination with chronic activation of phagocytic cells by ongoing *H. pylori* infection, attracts more innate immune effectors, which propagate an antigen-independent immunopathological response that fails to clear the initial infection.

With this model I suggest a probable chronology of specific events in the development of *H. pylori* gastritis, and outlines specific roles for each of the cell populations observed so far in inflamed gastric mucosa. Much of the work needed to confirm this model involves tracking *H. pylori* disease development in the absence of innate effector cells, or following disease progression after a population of innate effector cells such as macrophages or neutrophils have been deleted. If this model is correct, then in the absence of a cell population such as neutrophils we should find that gastritis severity should peak shortly after infection and then histological inflammation should abate to a mild gastritis. Alternatively, if we deleted an innate effector population after establishment of robust inflammation, we should observe a significant and marked reduction in gastritis. This hypothetical structure incorporates both the work I report here and numerous other findings, and allows for several new experimental targets for further investigation.

Figure 5.1: Updated model for development of *H. pylori* gastritis

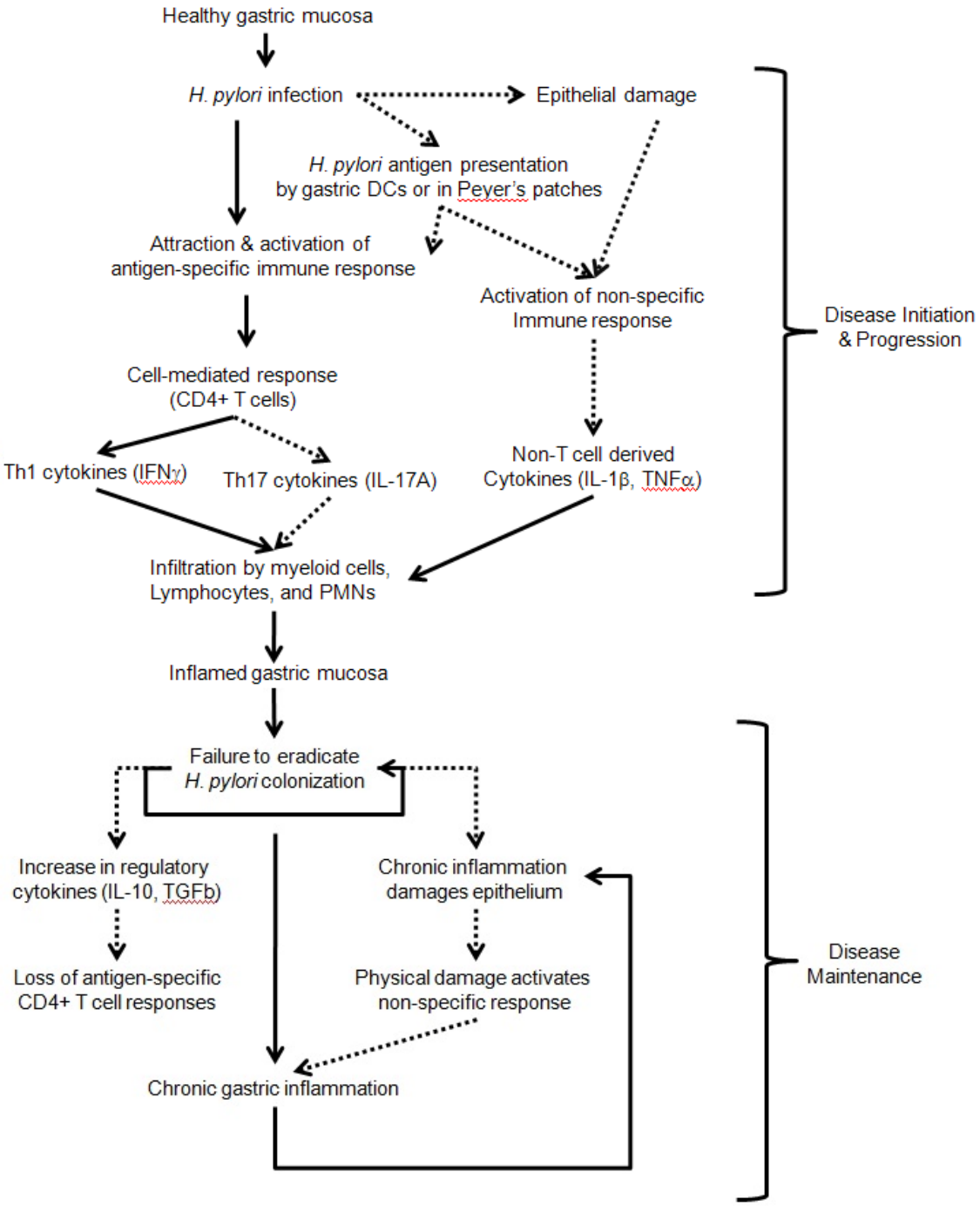


Figure 5.1: An updated model of the development of *H. pylori*-mediated gastritis in humans and mice incorporating my findings. Solid lines denote events confirmed as critical for *H. pylori* disease development. Dashed lines indicate observed and hypothetical events that likely contribute to the development of gastritis

Appendices

Appendix 1

The Contribution of IL-12p35 and p40 to *H. pylori* Gastritis

BACKGROUND

The current model of disease development in *H. pylori* infected humans and mice posits gastritis as the result of IFNγ production by antigen-specific, Th1-polarized CD4+ T cells, which is supported by several reports demonstrating that IFNγ is crucial for inflammation in infected gastric mucosa (50, 55, 178, 186). Recent recognition of the Th17 effector T cell phenotype prompted some to ask whether this second pro-inflammatory response associated with mucosal inflammatory responses, might contribute to *H. pylori*-mediated gastritis. In addition to reports of elevated levels of IL-17A found in the gastric mucosa from infected human patients (20, 96, 126, 138), two groups reported on the contribution of IL-17A to gastritis in a mouse model of *H. pylori* disease (183, 184), in addition to my own work reported in Chapter 2.

The early events in the initiation of *H. pylori* disease are poorly understood and hardly studied. Algood et al. reported profiling cytokine expression in B6 mice for several days immediately after infection with *H. pylori*, and reported significant changes in the expression of several of both T cell-produced and innate inflammatory cytokines (7). Unfortunately, they did not extend their profiles beyond three weeks after infection, when several groups have demonstrated that induction of gastric inflammation in B6 mice can take several months, often 6 or more, before achieving moderate gastritis (58,

110). That report also failed to examine other cytokines that may influence or otherwise shape the immune response, such as IL-12, the cytokine responsible for promoting Th1 polarization in activated CD4+ T cells.

IL-12 is composed of two separate subunits, IL-12p35 (or IL-12a) and IL-12p40 (or IL-12b). Of note, both proteins function in other cytokines: IL-12p35 combines with IL-27B to produce an immunomodulatory cytokine, IL-35 (30). IL-12p40 combines with IL-23p19 to make IL-23, the pro-Th17 cytokine which aids in lineage commitment. Both IL-12 and IL-23 are made by dendritic cells co-cultured with *H. pylori* (75, 76, 94), suggesting that antigen presenting cells can promote either of the pro-inflammatory Th1 or Th17 antigen-specific responses, or possibly both, in response to gastric infection with *H. pylori*. If *H. pylori*-mediated gastritis is both Th1 and Th17 dependent as suggested by numerous reports, then disease development should depend upon the expression of both IL-12p35 and IL-12p40. By studying *H. pylori* gastritis in mice lacking either IL-12p35 or IL-12p40, I should be able to explore some of the early events in the development and progression of gastritis in a mouse model of disease.

EXPERIMENTAL DESIGN

These experiments were originally planned in parallel with the simple infection experiments reported in Chapter 2. The only differences between these experiments and those reported in Chapter 2 are that I did not perform any adoptive transfer experiments with mice lacking IL-12p35 or IL-12p40 (see Future Directions), and as I did not reserve any knockout mice for uninfected controls, I was unable to calculate the fold-induction upon *H. pylori* infection in these mice. In every other respect, these experiments are equivalent to those presented in Chapter 2.

RESULTS

Cytokine deficiencies impede gastritis development in infected mice

We tracked gastritis progression in *H. pylori*-infected B6, 12aKO and 12bKO mice. All strains of mice developed mild-to-moderate multifocal gastritis, characterized by mononuclear or mixed inflammatory infiltrate (Figure A1.1). B6 mice developed significant gastritis by 9 months after infection with continued inflammation until the end of the experiment at 15 months after infection without significant variation. 12aKO mice developed mild gastritis at 3 months, and inflammation continued without significant variation through the end of the experiment. 12bKO mice developed mild gastritis late in the course of infection. Gastritis in B6 mice was the most severe at 9 months post infection, and at this time disease was greater in B6 mice than in either 12aKO or 12bKO mice.

H. pylori colonized the strains of mice roughly in inverse proportion to inflammation (Figure A1.2), as B6 mice were the least colonized strain at 9 months after infection. Bacterial load in 12aKO mice was always less than in 12bKO mice, but this difference was only significant for the first 9 months of infection.

Pro-inflammatory effector cytokine profiles

I assessed changes in the expression of Th1 and Th17 cytokines using mRNA isolated from the gastric tissue of *H. pylori*-infected mice. In infected B6 and 12aKO mice, IFNγ expression (Figure A1.3a) increased in parallel with histological inflammation. We observed the greatest inflammation in B6 mice at 9 and 15 months after infection, when levels of IFNγ were greatest, and cytokine expression was also significantly greater than in either 12aKO or 12bKO mice at these time points. IFNγ

expression peaked in 12aKO mice at 9 months, and mRNA levels for IFNγ did not significantly change over time in 12bKO mice.

IL-17A mRNA was limited in all strains, without significant differences in expression between strains until at 15 months after infection (Figure A1.3b). The pattern of IL-17F expression was similar in all mice to that of IL-17A expression, without significant differences in cytokine levels until late in infection (Figure A1.3c). While 12bKO mice expressed significantly more IL-17F than B6 mice at 12 months post infection, IL-17F levels in B6 mice were greater at 15 months after infection than in either 12aKO or 12bKO mice.

Numerous reports demonstrate an association between *H. pylori* gastritis and expression of a number of non-T cell derived pro-inflammatory cytokines, most commonly IL-1β, IL-6 and TNFα (35, 63, 64, 118, 145, 220). I measured the mRNA levels of these three cytokines to assess the contribution of innate immune effector cells in the absence of the IL-12 and IL-23 cytokines that drive and support inflammatory helper T cell polarization. IL-1β levels in B6 mice increased with the length of infection (Figure A1.4a) while cytokine expression in 12aKO mice peaked at 9 months and did not significantly change in 12bKO mice until late in the experiment. Expression of IL-1β in 12bKO mice was consistently less than in either infected B6 or 12aKO mice except at 12 months post infection.

IL-6 expression in all infected strains of mice followed a very similar pattern, without any marked change in mRNA levels until 12 and 15 months post infection (Figure A1.4b). Expression of this cytokine increased in all mice at 12 months, but decreased significantly in 12aKO and 12bKO mice compared to B6 mice. mRNA for

TNFα in 12aKO mice was significantly greater than in B6 mice at 6 months, but expression decreased after 9 months (Figuure A1.4c). In B6 mice, TNFα expression changed in parallel with tissue inflammation, and cytokine expression in 12bKO mice did not significantly increase or decrease over the course of the experiment.

Inducing and regulatory cytokine profiles

To see if IL-12p35 and IL-12p40 contributed to the development of *H. pylori* gastritis outside of their contributions to IL-12 and IL-23, I tracked the expression of the mRNA for these two cytokines in infected mice. IL-12p35 expression did not vary through most of the course of infection in either B6 or 12bKO mice, until mRNA levels for this cytokine increased in both strains of mice after 12 or 15 months of infection (Figure A1.5a). Expression levels for IL-12p40 followed a similar pattern in both B6 and 12aKO mice, with cytokine expression peaking in B6 mice at the 12 month time point and in 12aKO mice at 9 months post infection (Figure A1.5b). There was no significant difference in IL12p40 mRNA between these strains of mice until at 15 months post infection.

mRNA levels for the regulatory cytokines IL-10 and TGFb increased with the length of infection in all strains of mice (Figure A1.6), until expression of the regulatory cytokines decreased in 12aKO and 12bKO mice at 15 months post infection when both strains of mice expressed significantly less IL-10 and TGFb than I found in B6 mice. It is interesting to note that TGFb expression was significantly lower in 12aKO mice than in B6 mice at 3 months post infection, when I observed that gastritis was greater in 12aKO mice than in B6 mice.

DISCUSSION

Lack of "inducing" cytokines hinders disease development

In this appendix I show that IL-12p35 and IL-12p40 both contribute to the development of *H. pylori* gastritis in infected mice. 12aKO mice developed mild gastritis by the earliest time point I examined, which may reflect an increased inflammatory response in the absence of the regulatory cytokine IL-35, which includes IL-12p35 (30). It is important to note that when B6 mice developed significant, moderately-severe gastritis after 9 months of infection, this inflammation was significantly greater than disease found in infected 12aKO mice. 12bKO mice developed gastritis only late in infection, with significantly less inflammation in comparison to B6 mice at 9 months. Loss of the 12 month histology data prevents me from firmly establishing the start of disease in these mice, although the very mild gastritis evident at 9 months post infection suggests that inflammation had started by that time.

As we have reported previously, *H. pylori* colonization of the gastric mucosa is inversely correlated with the degree of inflammation (56, 58, 110), and we observe this pattern in B6 mice over the course of the experiment. Bacterial load in the stomachs of infected 12aKO mice changes insignificantly with time, suggesting that the basal level of inflammation observed in these mice exerts a constant effect on colonization density. Of note, although mean gastritis scores are statistically equivalent after 15 months of infection in all three strains of mice, colonization densities are significantly greater in 12bKO mice than in B6.

Taken together, these findings demonstrate that IL-12 and possibly IL-23 significantly contribute to the development of gastric inflammation in *H. pylori* infected

mice. At the same time, IL-23, more than IL-12, may be more important for the control of bacterial colonization in the stomach. It is most likely that IL-12p35 and IL-12p40 are influencing disease development and bacterial load by the action of their compound cytokines, and instead the observed effects are due to the cytokines' effects upon T cells.

Potential sample degradation at 15 months

It is important to note that for my 15 month time point RNA samples, my cytokine expression levels may be incorrect for 12aKO and 12bKO mice. The consistent and marked decrease in mRNA levels for all cytokines tested at that time point, when compared to the evident histological inflammation at that time, suggests that my samples were contaminated with RNAse or else my commercial PCR plates were sub-standard. Analysis of the GAPDH signal for each of these samples demonstrates that RNAse contamination is not an issue (data not shown), and suggests that most likely the observed drop in cytokine expression for those samples stems from a manufacturing fault.

T cell activity in the absence of IL-12 and IL-23

In the absence of either IL-12 component, IFNγ expression is severely curtailed in infected gastric mucosa, and by extension, the action of Th1-polarized effector cells is also limited in 12aKO and 12bKO mice. The hypothesis that *H. pylori*-mediated gastritis is primarily driven by Th1-polarized helper T cells arose from several groups reporting the absence of disease in infected IFNγ-KO mice (55, 186), and is supported by the findings of Guiney et al., who demonstrated *in vitro* that exposure to *H. pylori* promotes production of IL-12 in dendritic cells (75). The findings I report here supports the original Th1-centric hypothesis for disease development, and also strengthens Guiney's

results suggesting an early course of events in the immune response to *H. pylori* infection.

In contrast, the loss of IL-23 in 12bKO mice seems to barely affect the expression of either IL-17A or IL-17F. Again, this result is not surprising given the current model whereby production of IL-23 maintains but does not induce expression of Th17 cytokines, and that IL-23 also aids in lineage commitment in Th17-polarized cells (196). The contribution of Th17 effector T cells to controlling gastric colonization by *H. pylori* late in infection may be hindered by the loss of IL-23, but that is not certain.

Innate inflammatory activity without IL-12 and IL-23

Expression of the innate inflammatory cytokines IL-1β and TNFα is unaffected by the loss of either IL-12p35 or IL-12p40 early in the course of infection, and expression of the innate cytokines follows a similar path in infected B6 and 12aKO mice. In 12aKO mice especially, an early increase in the expression of TNFα may explain the mild gastritis that developed in these prior to the development of inflammation (and TNFα expression) seen in B6 mice. These observations suggest that once the initial inflammatory response to infection has begun, the initial participation of innate effector cells and cytokines in developing *H. pylori* disease is uncoupled from the action or direction of pro-inflammatory T cells.

By comparison in 12bKO mice I found relatively little change in the expression of innate inflammatory cytokines until late in infection. Levels of IL-1β, IL-6 and TNFα did not significantly change in infected 12bKO mice until 12 months post infection, when expression of all three innate cytokines increased. This observation suggests that IL-12-

driven activation of pro-inflammatory Th1 CD4+ T cells may be amongst the earliest of events contributing to the development of *H. pylori* disease.

Inducing cytokines in the absence of their partner

Changes in the expression of the IL-12 component cytokines in the absence of their IL-12 partner may illuminate some of the other possible roles of these functional subunits. IL-12p35 expression, in the absence of IL-12p40 in 12bKO mice, increases late in infection, a pattern that both parallels the apparent development of inflammation in 12bKO mice, but also recalls the change in cytokine mRNA levels for the regulatory cytokines IL-10 and TGFb. Conversely, IL-12p40 expression in both 12aKO and B6 mice follows a distinctly different pattern from that of IFNγ mRNA. It is possible but unlikely that IL-12p40 expression coordinates with the expression of IL-23p19, as the mRNA levels of IL-12p40 are not synchronous with the expression of either IL-17A or IL-17F, suggesting that IL-12p40 is not significantly contributing to the maintenance of Th17 polarized cells at that stage of disease in infected mice.

Regulatory cytokine expression

Regulatory cytokine expression, as followed in the simple infection experiments reported here and in Chapter 2, is a conundrum. In all strains examined where gastritis develops, mRNA for IL-10 and TGFb increases markedly with time, but this increase does not appear to inhibit the progression of gastritis in any infected mouse strain. It is tempting to speculate that regulatory cytokines are acting to dampen the immune response to *H. pylori* infection, but are ineffective in the face of high expression of IL-1β and IFNγ observed in infected mice. Alternatively, as regulatory cytokine expression appears to be trending upwards, it is possible that histological gastritis at 18 months or

longer post infection could be reduced by the effect of these immunomodulatory cytokines after the end of the time course I established.

General conclusions

In summary, the results I report here support the hypothesis that IFNγ-producing, Th1-polarized helper CD4+ T cells promote the development of *H. pylori* gastritis due, in part, to the expression of IL-12 by antigen presenting cells stimulated with *H. pylori*. In contrast, it is also apparent that the contribution of Th17-polarized helper T cells to *H. pylori*-mediated gastritis in infected mice is unaffected by the loss of IL-23, a cytokine that supports Th17 polarization but does not induce it. I also present more evidence suggesting that the innate inflammatory response to *H. pylori* infection is either partially uncoupled or partially independent of the antigen-dependent T cell response to infection.

FUTURE DIRECTIONS

To expand upon these studies and to possibly complete the story, it would be interesting to specifically examine the development of *H. pylori* gastritis in our adoptive transfer model of disease, using either IL-12p35 or IL-12p40 deficient SCID or RAG2-KO hosts. As we have demonstrated previously with T-betKO mice (50) and in Chapter 2 with 17AKO mice, disease can develop in the absence of the hallmark pro-inflammatory cytokine of either Th1 or Th17 T cells. This however does not rule out that Th1 or Th17 cells may be producing other cytokines in response to polarizing signals from IL-12 or IL-23. By performing adoptive transfer experiments in which the recipient mice are unable to polarize or activate the effector phenotype of adoptively transferred T cells, we may be further able to distinguish between the contributions of the innate and adaptive immune responses to *H. pylori* infection.

Figure A1.1: Mean gastritis scores in infected 12aKO and 12bKO mice

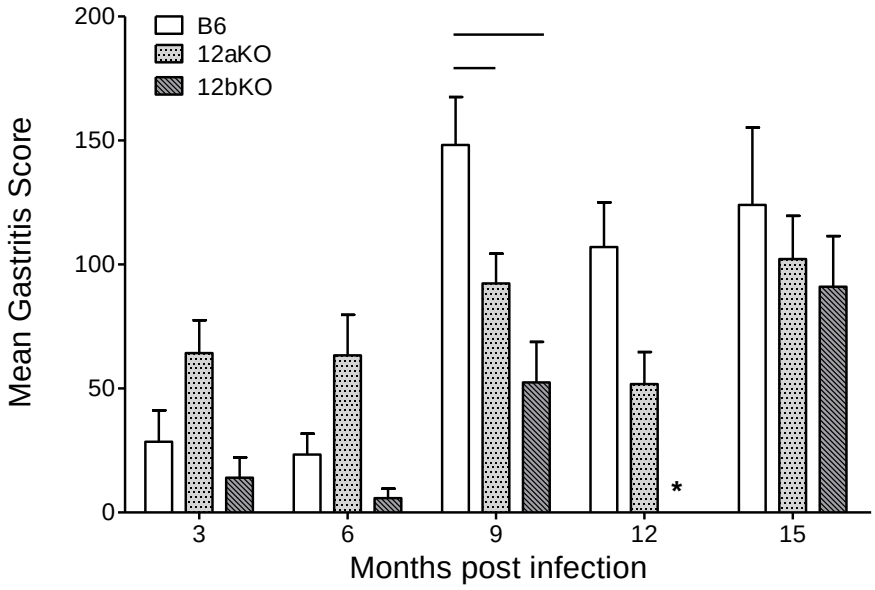


Figure A1.1: Histology scores in B6, IL-12p35-KO and IL-12p40-KO mice infected with *H. pylori* SS1. Slides were scored as described in Methods. * I lack data for the 12 month time point in IL-12p40-KO mice due to an error in tissue fixation. Bars indicate significant differences between two populations of at least $p<0.05$.

Figure A1.2: *H. pylori* colonization in 12aKO and 12bKO mice

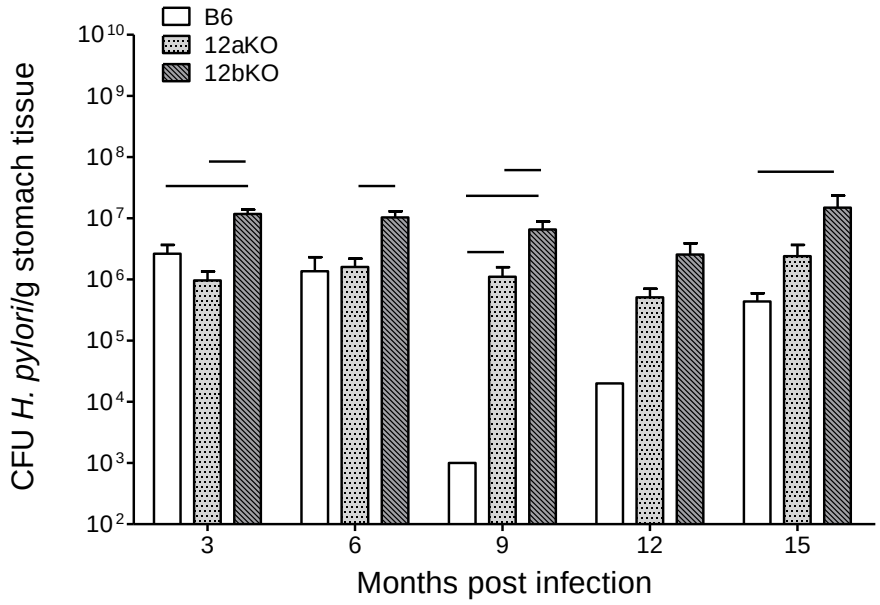


Figure A1.2: Bacterial load in B6, IL-12p35-KO and IL-12p40-KO mice infected with *H. pylori* SS1, as colony forming units per gram of stomach tissue over the course of infection Bars indicate significant differences between two populations of at least $p<0.05$.

Figure A1.3: Expression of "Th" effector cytokines in gastric mucosa

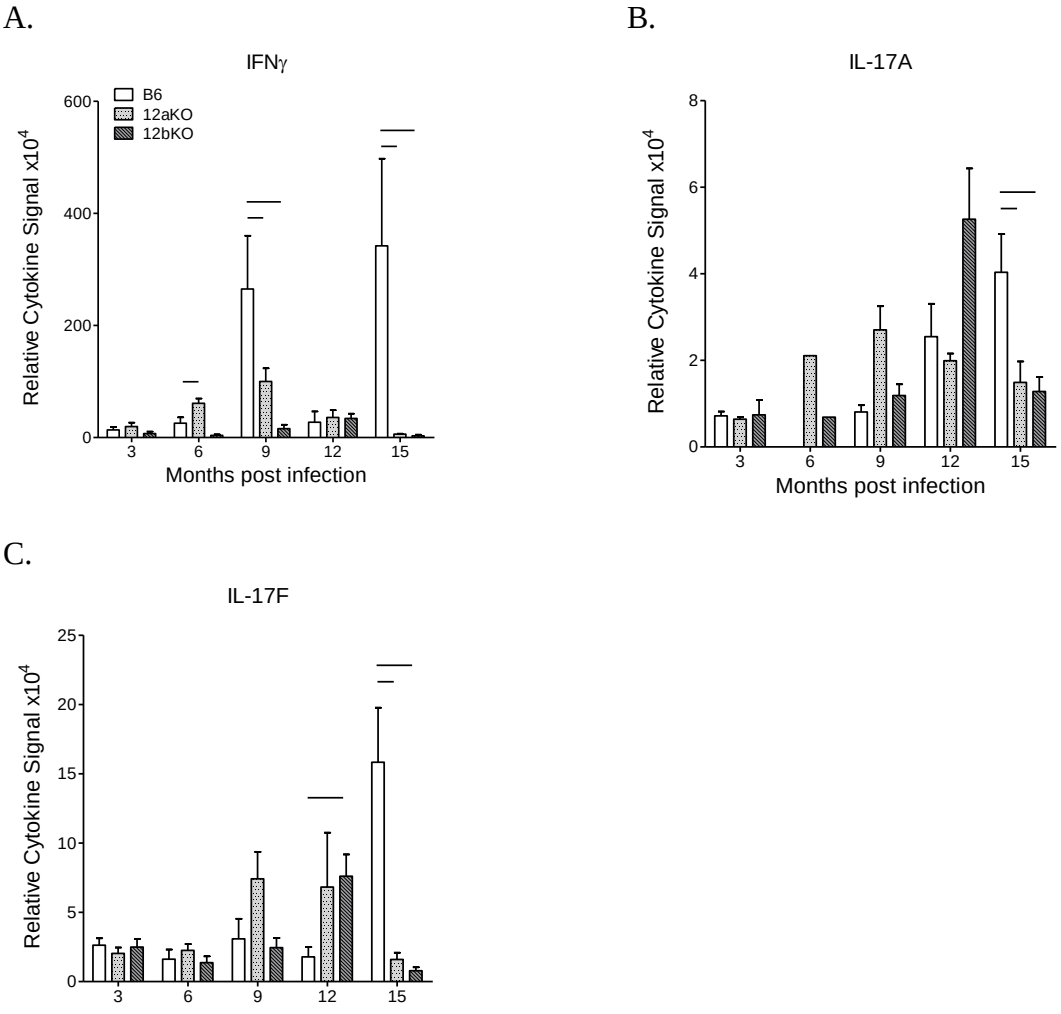


Figure A1.3: mRNA from gastric mucosa was analyzed for expression of T cell inflammatory cytokines and results are expressed as mRNA signal normalized to GAPDH expression. IFNγ (a), IL-17A (b) and IL-17F (c). Bars indicate significant differences between two populations of at least $p<0.05$.

Figure A1.4: Expression of innate pro-inflammatory cytokines in gastric mucosa

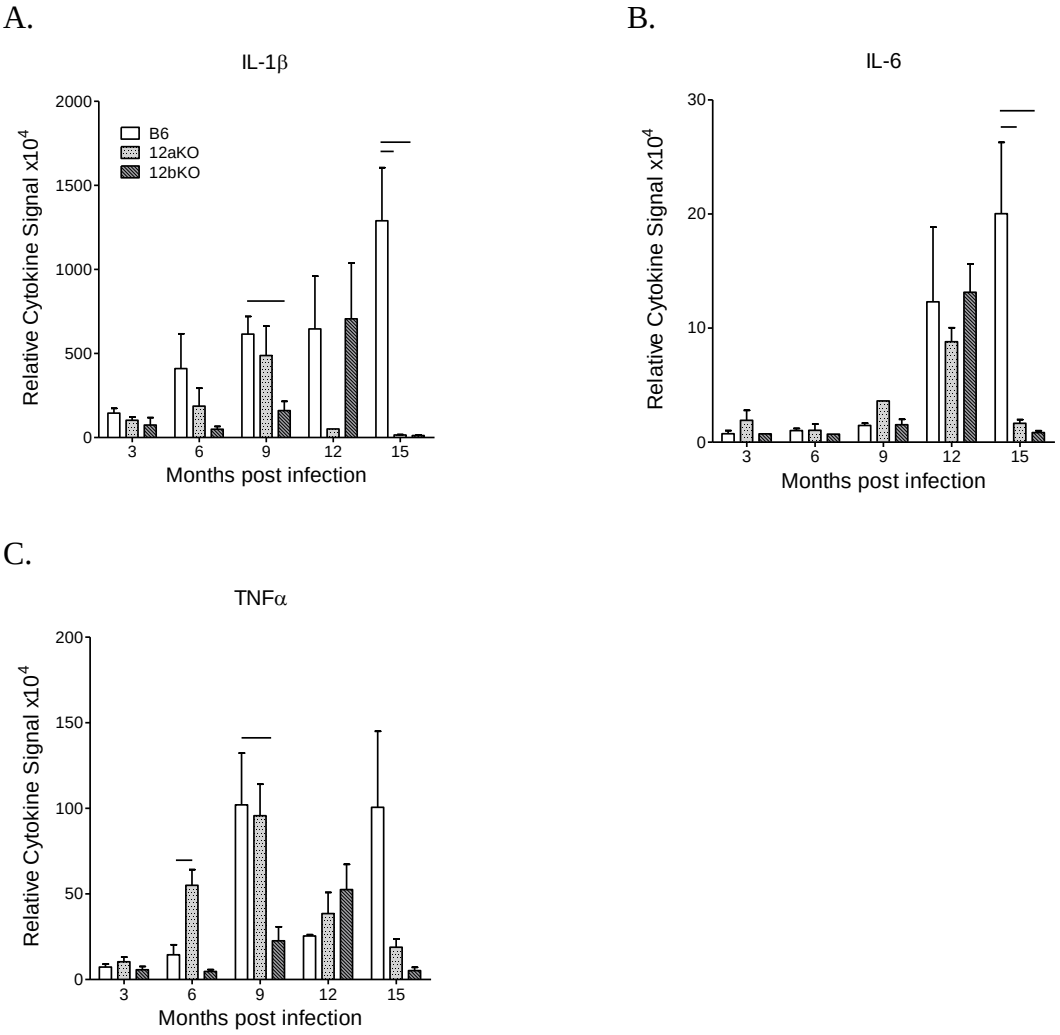
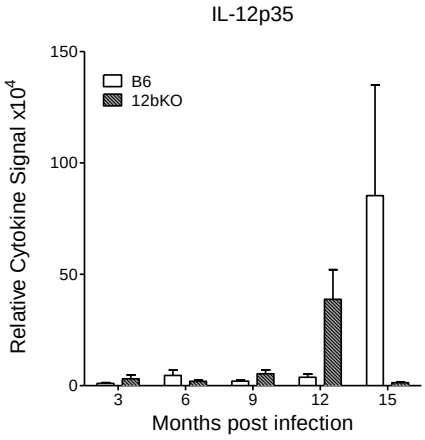


Figure A1.4: mRNA from gastric mucosa was analyzed for expression of T cell inflammatory cytokines and results are expressed as mRNA signal normalized to GAPDH expression. IL-1β (a), IL-6 (b) and TNFα (c). Bars indicate significant differences between two populations of at least $p<0.05$.

Figure A1.5: Expression of "Th" inducing cytokine in gastric mucosa
A.



B.

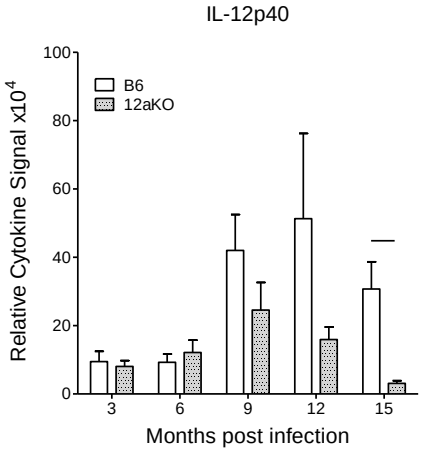
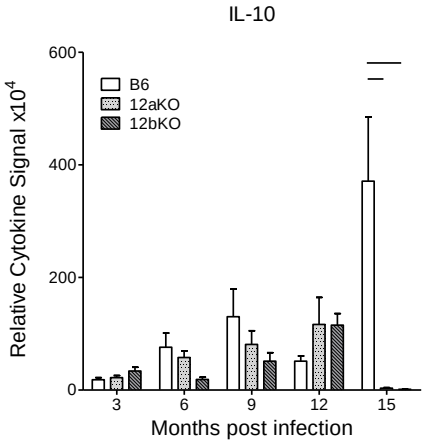


Figure A1.5: mRNA from gastric mucosa was analyzed for expression of "Th" polarizing cytokines, and results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. IL-12p35 (a), and IL-12p40 (b). Bars indicate significant differences between two populations of at least $p<0.05$.

Figure A1.6: Expression of regulatory cytokines in gastric mucosa
A.



B.

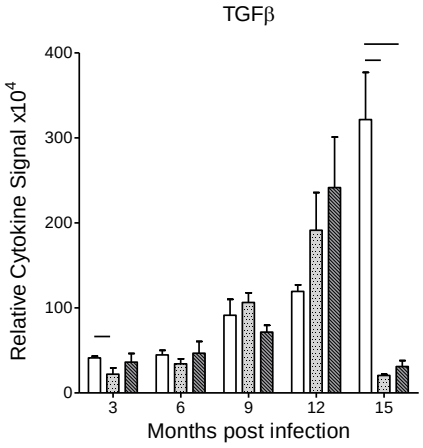


Figure A1.6: mRNA from gastric mucosa was analyzed for expression of regulatory cytokines, and results are expressed as the fold change in mRNA levels upon infection with *H. pylori* SS1. IL-10 (a), and TGFβ (b). Bars indicate significant differences between two populations of at least $p<0.05$.

METHODS

Mice strains and housing

Helicobacter-free C57Bl/6J mice (B6), B6.129S1-*Il12a*^{tm1Jm}*/J* (12aKO) and B6.129S1-*Il12b*^{tm1Jm}*/J* (12bKO) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in-house at the University of Michigan. No known mouse pathogens are present in the mouse colonies as determined by routine periodic screening of sentinel mice. Mice were maintained in static microisolator cages and offered non-supplemented commercial mouse chow and water *ad libitum*. The University of Michigan Animal Care and Use Committee approved all animal experiments.

H. pylori culture and inoculation

Plate cultures of *H. pylori* SS1 were used to inoculate overnight broth cultures in 10 mL of Brucella broth with 10% fetal calf serum and 50 μl of Skirrow's antibiotic supplement. Cultures were centrifuged and washed once and then resuspended in sterile phosphate-buffered saline (PBS), counted on a hemacytometer, and diluted to a final concentration of $1x10^8$ bacteria/mL. B6, 17AKO, T-bet KO, and RAG2-KO mice were given 100 μl of sterile 0.5 M Na_2CO_3 via gastric feeding tube followed by100 μl of the bacterial suspension giving a total dose of $1x10^7$ of *H. pylori* SS1 per mouse. This procedure was repeated on the following day, for a total of two inoculations.

Tissue collection, histology scoring and RNA profiling

For the simple infection model, 4-5 mice were euthanized at 3, 6, 9, 12 and 15 months after infection and stomachs were harvested for histology, assessment of bacterial load, and RNA isolation. Adoptively transferred mice were euthanized at 8 weeks after transfer, a total of 10 weeks after infection, and their stomach tissues were harvested in

the same way. For histology, 1 mm-wide strips from the greater curvature were emersion-fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5μm sections, and stained with hematoxylin and eosin. Bacterial colonization was quantified by plate counts of serially diluted gastric homogenate.

For RNA isolation samples were stored for no more than three months in 300 μl of TRIzol at –80 ºC (Invitrogen, Carlsbad, CA) and total RNA was purified according to the TRIzol protocol. mRNA was then further enriched with Qiagen RNEasy columns, and stored at –80 ºC until analysis. Using SA Biotech's cDNA first strand synthesis kit and protocol (SA Biosciences, Frederick, MD), mRNA samples were prepared for qPCR of mouse cytokine genes, which was performed on custom SuperArrays (CAPM-0752A) using SA Biotech's SYBR green master mix and protocol, on a Stratagene MX3000p thermal cycler. The arrays included probes for the following genes: IL-1β, IL-6, IL-17A, IL-17F, IFNγ, TNFα, and GAPDH, which served as housekeeping control.

Extent of gastritis was scored as previously described (51). Briefly, adjacent 200x microscopic fields were examined for the presence of gastric infiltrate severe enough to displace glands, presence of neutrophilic inflammation, and/or presence of gastric epithelial metaplasia. Two longitudinal sections of gastric fundus were scored in their entirety, and the percentage of positive fields in all three categories was added together to calculate the total score. All sections were scored blind, without prior knowledge of their source.

Data handling and statistical analyses

All graphs were made using GraphPad Prism 5.01 software. This program automatically calculated and graphed the standard error of the mean (SEM) for all

column graphs. All matching datasets were tested for statistical significance through application of two-tailed Student's t-test using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Experimental groups that are not statistically significantly different are unmarked. For qPCR analyses, SYBR Green cycle threshold was established automatically using the Stratagene MX Pro software, based on ROX fluorescence. Any well with a SYBR Green cycle threshold greater than 35 cycles was counted as a negative result. Expression of cytokine genes was normalized to GAPDH expression and then multiplied by 10,000 for ease of visualization.

Appendix 2

Profile of Behavioral Defects in *H. pylori* SS1 Lipopolysaccharide Mutant SS1:0826

BACKGROUND

H. pylori is a spiral bacteria, nominally placing it amongst Gram-negative organisms, and as such, the species does produce a lipopolysaccharide (LPS) membrane component analogous to LPS found in well-characterized Gram-negative pathogens like *E. coli* and *Salmonella* (for a review, see (142)). Unlike other LPS molecules however, *H. pylori* LPS is more appropriately described as a lipo-oligo-saccharide with fewer sugar moieties and a structure characterized by a highly branched structure (142). Terminal sugars on *H. pylori* LPS include a slew of Lewis antigen molecules, ranging from Lewis(a) (Le^a) to Le^x, Le^y and H-1 (141), and several groups initially hypothesized these sugars provided a type of mimicry-based immunological camouflage to evade the immune response (9, 214). More recent reports have demonstrated that several of these Lewis antigens are substrates for lectins expressed on host epithelial cells (70), which promotes the hypothesis that *H. pylori* LPS functions as an adhesion factor, and does not work a non-specific activator of inflammatory immune responses.

When Logan et al. initially characterized *H. pylori* SS1::0826kan (SS1:0826) as a deletion mutant with altered LPS structure, they were operating on the hypothesis of *H. pylori* LPS as an immunological mediator, whether as a promoter of inflammation or evasive mimic (121). They reported that in the absence of surface Le^x and Le^y produced by the *hp0826*gene product, SS1:0826 infection of CD1 mice failed to promote disease.

This work was confirmed by Eaton et al. who demonstrated that even in the rapid and severe adoptive transfer model of disease, infection of recipient mice with SS1:0826 failed to induce disease (54). It is important to note that while both groups observed significantly lower gastric colonization in mice infected with SS1:0826 of 20-100 fold difference when compared to infection with the parental SS1 strain, this was not necessarily considered an important factor in disease formation.

HP0826 encodes for a beta-1,4-galactosyltransferase, and is best characterized for its contributions to the branched structure of *H. pylori* LPS (121), but the protein may be involved in more than just construction of surface polysaccharides. Glycoproteins are adhesion factors for many eukaryotic cells, and even before the most recent reports on the role of *H. pylori* LPS in bacteria-host cell interactions, I hypothesized that the failure of SS1:0826 to induce gastric disease in infected mice did not stem from a failure to activate the immune system. Instead, loss of HP0826 activity could affect the structure and function of bacterial glycoproteins and possibly interfere with adhesion to host epithelial cells in the gastric mucosa.

EXPERIMENTAL DESIGN

The Eaton laboratory initially described the behavior of SS1:0826 in the adoptive transfer model of *H. pylori*-mediated gastritis as a failure to induce an inflammatory immune response due to loss much of the structure of *H. pylori* LPS (54). The lab was actively investigating the *in vitro* response of bone marrow-derived dendritic cells (BMDCs) when I joined them, and I expanded the repertoire of experimental methods as they addressed this question. I tested the response of components of the immune system to provocation with *H. pylori* SS1 and SS1:0826. Specifically I mixed the bacteria with

mouse bone marrow-derived dendritic cells (BMDCs) in overnight experiments and assessed the cellular response through flow cytometry methods. I stained the BMDCs for co-stimulatory and antigen presentation-related activation markers: CD11c, CD40, CD80, CD86, and MHC II. Results are presented as the mean fluorescent intensity of the marker-bright populations as a proxy for the density of those markers on the cell surface.

However, mindful of the multitude of possible contributions that a sugar-modifying enzyme might make to other bacterial functions, I proposed that we test the ability of SS1:0826 to perform in other assays. First, we needed to confirm that our mutant strain had not reverted to wild-type, and so I built a quick capture ELISA to test for expression of Lewis antigens in bacterial lysates. I decided to build an ELISA as there is no anti-HP0826 antibody yet commercially available, so investigating protein function rather than directly looking for the presence or absence of the protein would be easier. Once I knew that our strains were behaving as expected (see Results), I also assessed the short-term ability of BMDCs to interact with bacteria through either bacteria-cell adhesion or phagocytosis of the bacteria in a flow cytometry-based heterotypic adhesion assay.

I adapted an existing assay methodology (44), but the method can be summarized as staining two distinct populations of cells with two different permanent membrane-binding, fluorescent dyes, and then looking for single-color bright and two-color bright populations of events on the flow cytometer. In the assay I report here, I instead only used BacLight Red to stain the bacteria, which are too small to be consistently detected by the flow cytometer, and then measured the population of BacLight Red-bright dendritic cells. I then also employed this methodology to assess the adhesion of SS1 and

SS1:0826 to the human gastric epithelial cell line AGS, which was selected for ease of culture as well as its reported ability to produce mucus *in vitro*.

In addition to potential effects upon bacterial adhesion, I wondered if deletion of the HP0826 galactosyltransferase would adversely affect other characteristics of bacterial behavior and colonization. I was most interested in the development of *H. pylori* biofilm, which had recently been reported *in vivo* in human stomachs (19, 22) and could explain the failure to colonize the gastric mucosa in infected mice seen with SS1:0826. Crystal violet staining of biomass is an acceptable proxy for measuring biofilm formation without access to a scanning electron microscope, and I tested this in cultures grown overnight in a 6-well plate with wells filled with sterile glass frit, or rough-surfaced glass beads that provide a substrate for deposition of biomass. An incidental microscope observation of the different behaviors in stationary phase *H. pylori* SS1 and SS1:0826 broth cultures led me to explore both the auto-aggregation phenotype and the dynamics of bacterial growth in broth culture.

RESULTS

O826 deficiency and surface Lewis antigen epitopes

To confirm that our 0826 mutant strains had not reverted their phenotype, I checked bacterial lysates for Le^x and Le^y antigens by ELISA, as both molecules should be absent in 0826 mutant strains (54, 121). Using 26695 as a wild-type control for Lewis antigen production, I found that both SS1:0826 and M6:0826 both produced significantly less Lewis antigens than their parent strains (Figure A2.1). Although the M6 parental strain produced more Le^x than SS1, the two 0826 mutants produced a similar amount of

Le^x. All wild-type strains produced similar amounts of Le^y, and the two 0826 mutants produced similar, lower quantities of Le^y.

SS1:0826 immunogenicity *in vitro*

I compared the ability of SS1 and SS1:0826 to activate antigen-presenting cells *in vitro* by analyzing cell surface markers of activation after overnight co-culture of BMDCs and bacteria. The two SS1 strains prompted similar changes of expression in CD11c, CD40, CD86 and MHC II (Figure A2.2). Cell surface expression of CD80 was unchanged. I also measured the ability of SS1 and SS1:0826 to adhere to, or be phagocytosed by, dendritic cells in a short time span by a flow cytometry assay. I found that BMDCs interacted with SS1 and SS1:0826 bacteria at a similar rate, with no significant differences in the amount of bacteria complexed with dendritic cells (Figure A2.3).

Bacterial adhesion to epithelial cells

Adhesion to the gastric epithelium through molecules such as BabA, a sugar-binding protein, has been demonstrated to play an important role in promoting *H. pylori* pathogenesis (). It has also been demonstrated that bacterial expression of Lewis antigens enables bacterial adhesion to epithelial cells that express galectin-3 (70), so I tested the ability of SS1 and SS1:0826 to adhere to a mucus-producing, human gastric epithelial cell line, AGS. I found that SS1:0826 bacteria adhered to AGS cells significantly less than did SS1 bacteria in a half-hour long assay (Figure A2.4). Due to the difficulties inherent in counting the number of viable bacteria before such a short term assay, I am unable to directly compare equal ratios of bacteria to cells between the SS1 and SS1:0826 strains, but the slopes of the lines are significantly different ($p<0.0001$).

0826 deficiency adversely affects bacterial behavior

To assess whether the loss of HP0826 enzymatic activity negatively impacted other functions critical for bacterial growth or communal behavior patterns, I performed a growth curve to assess whether SS1:0826 suffered from a growth defect. I observed that cultures of SS1 reached stationary phase at approximately 14 hours after the start of culture (Figure A2.5). SS1:0826 grew less efficiently than SS1, apparently going through a prolonged lag phase, until entering logarithmic growth phase at 19-20 hours after the start of culture. After 21 hours, SS1:0826 bacteria divided rapidly and total cell number was slightly greater than observed in the SS1 stationary phase culture at that time.

I also measured the ability of SS1:0826 to behave in two other assays of communal behavior. Formation of *H. pylori* biofilm has been demonstrated both *in vitro* (29, 192) and *in vivo* (19, 22), and in light of the decrease in both adhesive ability and proliferation, I assessed the ability of SS1 and SS1:0826 to form biomass that can be detected by a crystal violet staining assay after overnight growth in media. While I observed that neither SS1 nor SS1:0826 produced much biomass under normal growth conditions (Figure A2.6), SS1 bacteria produced increasing quantities of biomass as I decreased the nutritive value of the media. SS1:0826 bacteria biomass did not significantly increase under starvation conditions, and was significantly less than detectable biomass produced by SS1 bacteria in serum free Brucella broth and in complete media diluted to 10% in water.

Auto-aggregation is the phenomenon whereby bacteria in stationary phase in broth culture start to clump together and precipitate out of suspension due to decreased motility and increased mass. This behavior may be a pre-cursor to biofilm formation, or

influence the ability of bacteria to adhere to epithelial cells. I followed the auto-aggregation of SS1 and SS1:0826 in complete media over time, and observed that over the course of two hours, a stationary phase SS1 broth culture lost 20% of its OD600 value, presumably via settling of bacterial clumps to the bottom of the cuvette (Figure A2.7). In contrast, SS1:0826 bacteria in stationary phase did not significantly settle within two hours, and the slopes of lines drawn through the data points are significantly different ($p=0.0461$), suggesting that SS1:0826 suffers from an auto-aggregation defect.

DISCUSSION

SS1:0826 is as immunogenic as SS1 parent strain

In this report I demonstrate that the failure of SS1:0826 to induce an immune response in infected mice does not stem from a defect in the ability of antigen-presenting cells to recognize or respond to the bacterial strain. BMDCs co-cultured overnight with *H. pylori* expressed similar levels of cell surface markers in response to culture with SS1 or SS1:0826 strains. This suggests that the loss of Lewis antigens on the surface of SS1:0826 bacteria does not affect the immunogenicity of *H. pylori* SS1:0826 when taken up by dendritic cells. I also demonstrated that BMDCs form cell:bacteria complexes equally well with either SS1 or SS1:0826 bacteria. This interaction may be simple bacterial adhesion to the surface of the dendritic cells, or more likely it reflects phagoctosis of dyed bacteria by the dendritic cells. In hindsight, I would have predicted that BMDCs and other antigen-presenting cells would more aggressively bind to bacteria with fewer surface polysaccharides, as those bacterial molecules can inhibit phagocytosis. Together these findings clearly demonstrate that SS1 and SS1:0826 are possessed of a

similar immunogenicity, and the failure of SS1:0826 to induce an immune response to infected mice is not due to loss of the HP0826 galactosyltransferase.

SS1:0826 failure to colonize may stem from inability to adhere to epithelium

When SS1:0826 was first characterized, mention was made of its colonization defect, with a 20-100 fold reduction in bacterial load in infected stomachs in mice (54, 121). I hypothesized that this defect might stem from an inability to attach to epithelial cells, and I found that SS1:0826 in a single-bacterial-cell suspension adheres less effectively to mucus-producing gastric epithelial cells than does SS1. This finding supports the finding by Fowler et al. who demonstrated that several *H. pylori* Lewis antigens function to enhance bacterial binding by host-epithelia expressed galectin-3 (70). As 0826 mutants lack the enzymatic activity to generate most of the Lewis antigens found in *H. pylori* LPS (121) it is not surprising at this remove that they do not adhere well to host epithelium. When we also consider that the gastric lumen is an environmental surface in constant flux, with mucus production and peristaltic manipulation of the organ contents, defective adhesion to the epithelial layer is a significant hindrance to the *H. pylori* lifestyle. The failure of the immune system to respond to *H. pylori* SS1:0826 may simply stem from the inability of the bacterial strain to reside in the stomach long enough to be sampled by antigen presenting cells.

0826 deficiency affects multiple culture behaviors

There are many possible aspects of bacterial adhesion to host surfaces beyond just the ability of individual bacterial cells to adhere to epithelial cells. Formation of bacterial aggregates and deposition of biomass prior to construction of biofilm structures magnify the adhesive properties of bacteria. SS1:0826 suffers from significant defects in both of

these behaviors, compared to the parental SS1 strain. In the assays performed, it is unclear whether this loss of function is specifically due to defects in the sugar structure of *H. pylori* LPS or if deletion of *hp0826* alters protein glycosylation in the SS1:0826 strains, or possibly that quorum sensing molecules are malformed. Any of these defects could significantly affect intercellular adhesions, but as this area is little studied in *H. pylori* literature, it is unclear as to the full nature of the defect.

A more interesting finding is that SS1:0826 apparently suffers from a growth defect in comparison to the parental SS1 strain. This observation suggests that HP0826 activity is crucial to more fundamental bacterial activities than just the branched structure of *H. pylori* LPS. It is possible that surface proteins, such as ion pores or metabolite transporters, are glycosylated by HP0826 as part of their normal protein structure, and mis-fold without addition of the proper sugar groups, or are defective due to other possible biochemical defects. In combination with the other defects in bacterial behavior described above, this finding strongly suggests that HP0826 is a critical factor contributing to the function of numerous other molecules involved in *H. pylori* growth and survival in its preferred gastric environment.

General conclusions

Overall, I demonstrate here that the lack of an immune response in mice infected with *H. pylori* SS1:0826 most likely does not stem from a defect in the ability of the truncated lipopolysaccharide structure to activate antigen-presenting phagocytes like dendritic cells. Instead, it is more likely that the reduced ability of SS1:0826 to attach to gastric epithelial cells, which explains the reported defect in gastric colonization observed in mice, prevents the strain from accumulating enough bacteria *in situ* to allow for

effective sampling by host antigen-presenting cells. The other defects in bacterial culture behavior that I report here may also reflect upon the ability of *H. pylori* to colonize the gastric mucosa through intercellular interactions to promote aggregation and biofilm formation.

FUTURE DIRECTIONS

There is a great deal of work that needs to be complete before this work can be solidly presented for publication. First, all of these experiments need to be repeated to at least an n of 3, as all of the data presented is representative of 2 experiments. After that, however, I propose that we should investigate the contribution of HP0826 to protein glycosylation of membrane and extracellular proteins in *H. pylori*. This may affect as many different phenotypes as nutrient import, metabolite export, quorum sensing and biofilm scaffolding proteins, and each of these can be tested, but I would start with analysing culture lysates and various protein "cuts" by simple protein gels and Coomassie blue staining and silver stains to identify potentially affected protein species. From that point we can attempt protein sequencing on differentially-stained bands to identify affected molecules, but we can continue to assess bacterial function through other assays.

Nutrient import can be tested by profiling growth upon various nutrient-limited media, which will be difficult as *H. pylori* can be fastidious. Metabolite export can be measured via HPLC analysis of culture broth after overnight growth and comparing wild-type and mutant cultures to look for changes in the profile of aqueous or hydrophobic metabolic byproducts. Recent reports demonstrate that *H. pylori* produces auto-inducer 2 and carries a LuxR/LuxS system (69, 86), and so function of these systems can be assessed biochemically and behaviorally. Biofilm formation is usually examined via

scanning electron microscopy and structural analysis of the biomaterial deposited by bacterial cultures. I expect that deletion of *hp0826* has multiple effects upon bacterial growth and we could find defects in many or all of these phenotypes.

Figure A2.1: Lewis antigen production by *H. pylori* strains

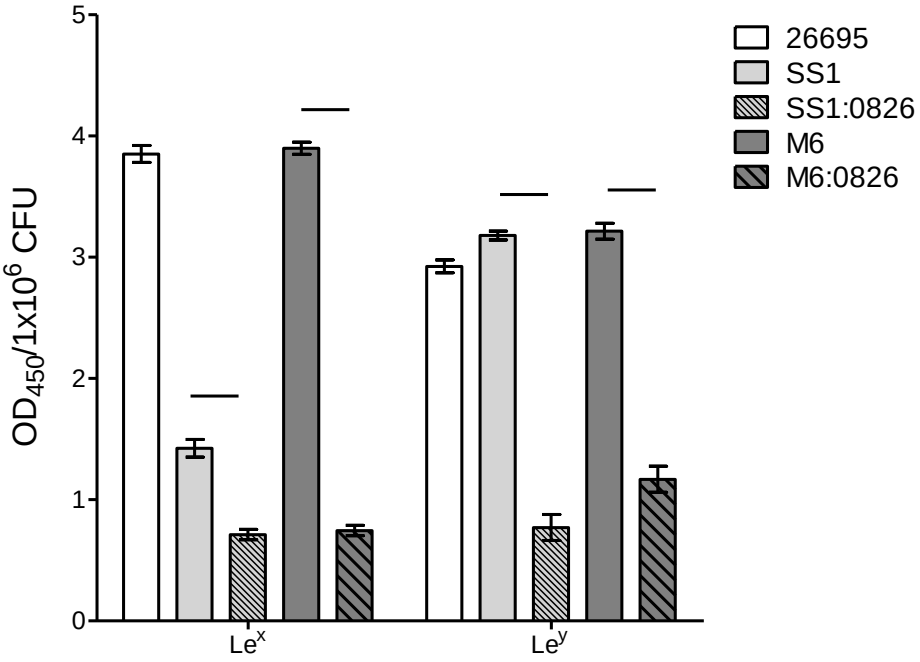


Figure A2.1: ELISA for Le^x and Le^y antigens produced by *H. pylori* strains. Bars represent a statistically significant difference between two populations of at least $p<0.05$.

Figure A2.2: Dendritic cell surface markers for activation

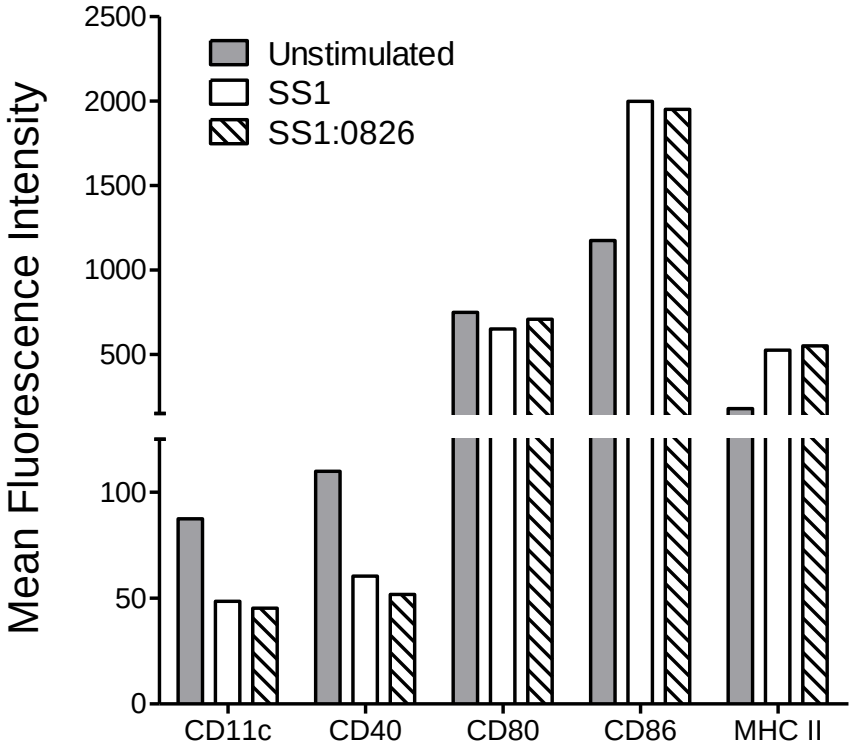


Figure A2.2: Changes in cell surface marker expression on BMDCS co-cultured overnight with *H. pylori* strains, as measured by mean fluorescent intensity of stained cells analyzed through flow cytometry.

Figure A2.3: *H. pylori*:dendritic cell adhesion

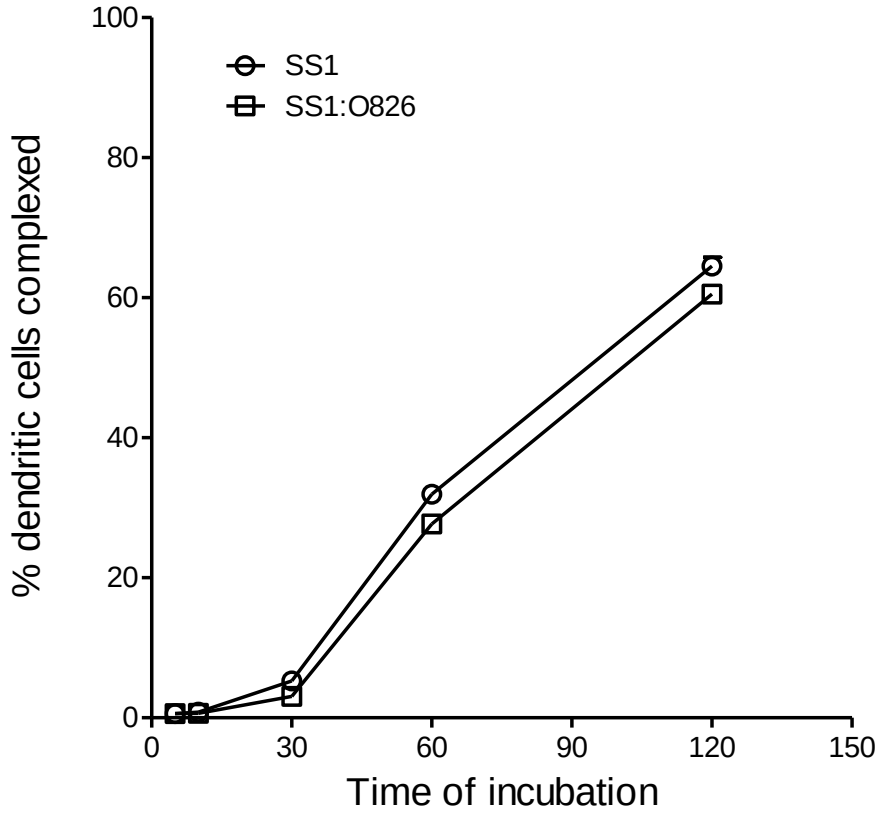


Figure A2.3: Bacterial adhesion to, or phagocytosis by, BMDCs, as measured by flow cytometry. Fluorescently dyed bacteria adhered to, or engulfed by, BMDCs, which is measured by looking for dye-bright BMDCs (see Methods).

Figure A2.4: *H. pylori*:gastric epithelial cell adhesion

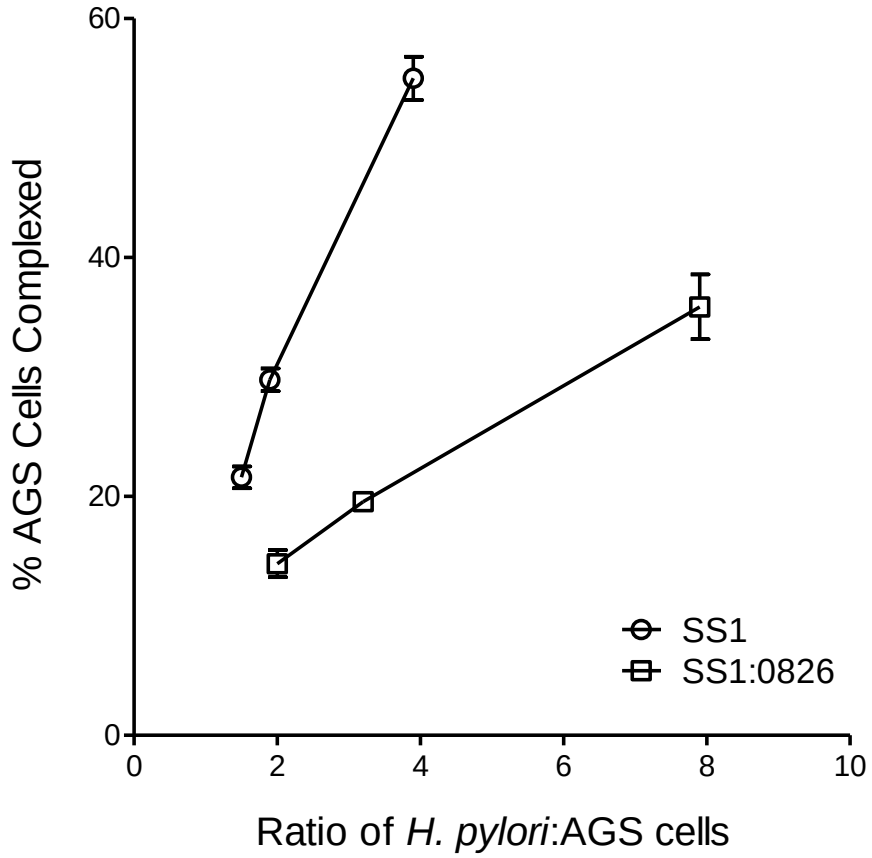


Figure A2.4: Bacterial adhesion to cells of human gastric epithelial cell line AGS. Fluorescently dyed bacteria adhered to AGS cells, which is measured by looking for dye-bright epithelial cells (see Methods).

Figure A2.5: Proliferation of *H. pylori* strains

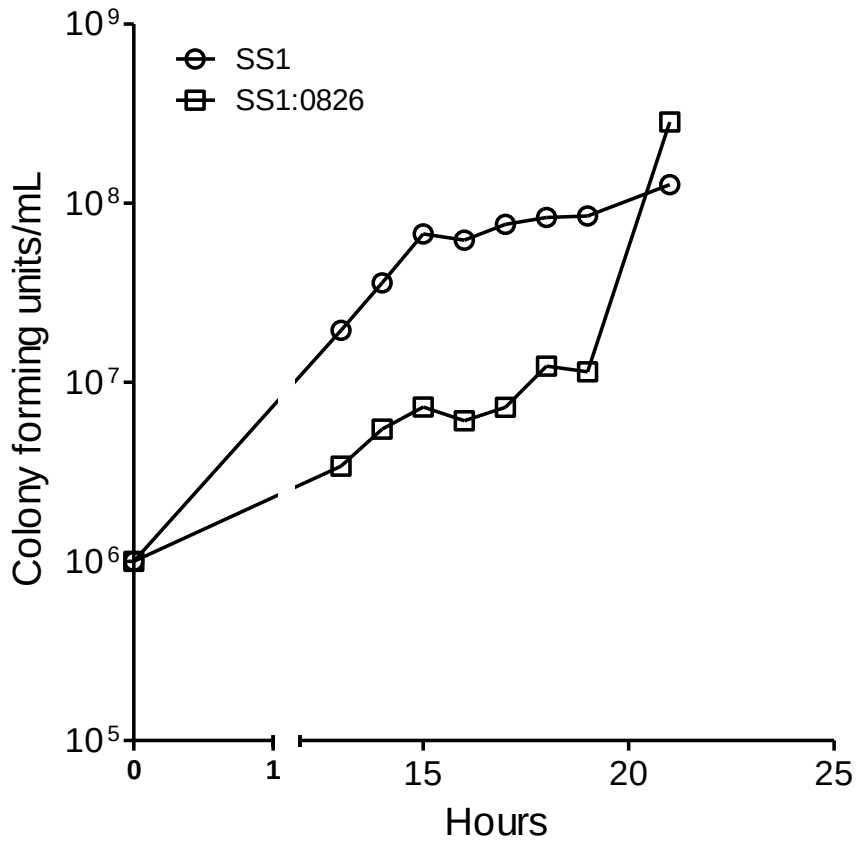


Figure A2.5: *H. pylori* bacteria in a stationary phase broth culture were seeded into a new broth culture at a concentration of $1x10^6$ cfu/mL.

Figure A2.6: Deposition of crystal-violet detectable biomass by *H. pylori* strains

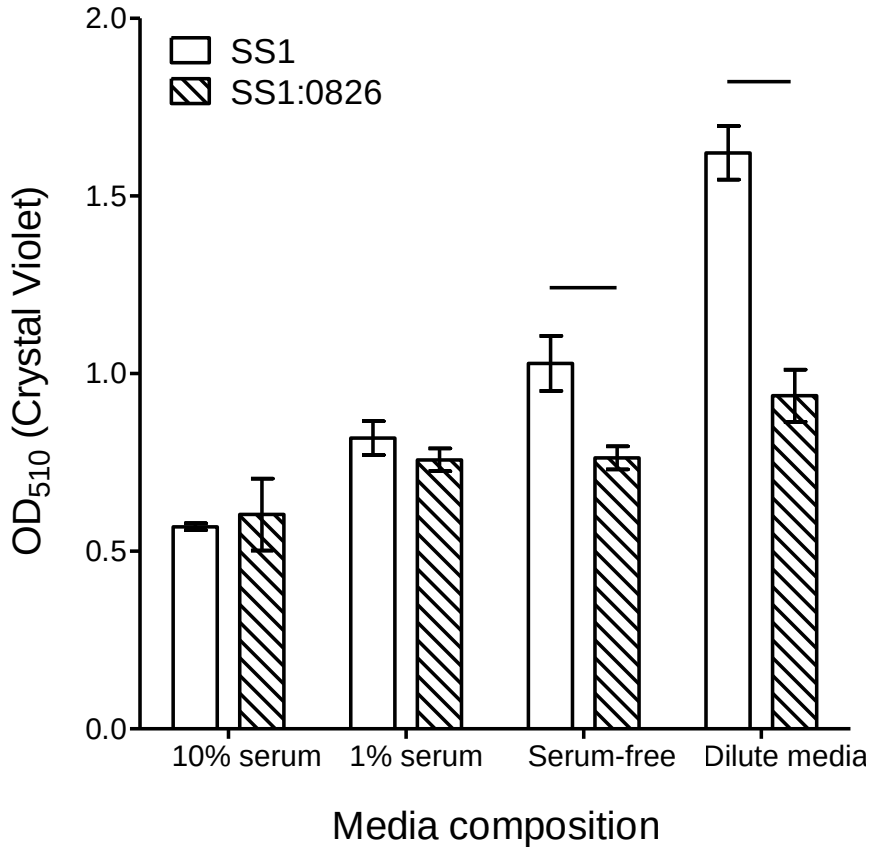


Figure A2.6: *H. pylori* SS1 and SS1:0826 were grown overnight in 6-well plates filled with glass frit in the listed culture media (see Methods). Bars represent a statistically significant difference between two populations of at least $p<0.05$.

Figure A2.7: Auto-aggregation behavior of *H. pylori* strains

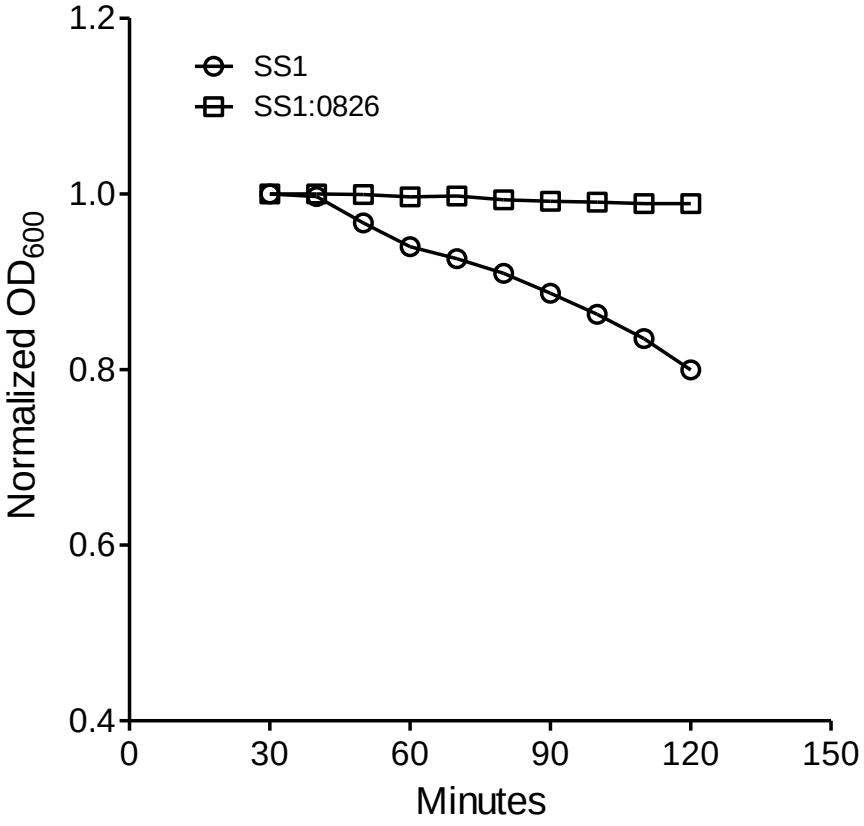


Figure A2.7: Auto-aggregation and precipitation of *H. pylori* SS1 and SS1:0826 from broth culture.

METHODS

H. pylori broth cultures

All *H. pylori* strains were grown overnight in Brucella broth with 10% fetal bovine serum under microaerophilic conditions (10% CO_2, 5% O_2) with gentle agitation. 0826 strains were cultured with the addition of 10 μg/mL kanamycin.

Proliferation of bacterial strains was assessed by allowing an overnight *H. pylori* culture to reach stationary phase, pelleting bacteria and resuspending in 1x PBS at an approximate concentration of $1x10^7$ CFU/mL measured by count on a hemocytometer. 100 μL was used to inoculate a new broth culture, and OD_{600} readings were taken every hour starting at 13 hours after inoculation, until 21 hours after the start of culture.

Auto-aggregation of bacterial cultures was assessed by pelleting the bacteria from a stationary phase overnight broth culture and resuspending in 1x PBS at an approximate OD_{600} of 1.0. Cuvettes were filled with this bacterial suspension and incubated under microaerophilic conditions at 37 °C without agitation. OD_{600} readings were taken every 10 minutes, starting 30 minutes after the start of the experiment.

BMDC isolation, and BMDC and AGS tissue cultures

Briefly, bone marrow-derived dendritic cells were isolated by harvesting bone marrow from the cavities of the leg bones of C57Bl/6J mice, breaking the marrow into a single cell suspension, and then culturing the cells for 1 week in DMEM with 10% fetal bovine serum, 10 mg/mL penicillin and 10U/mL streptomycin, and 10 U/mL each of IL-4 and GM-CSF. AGS cells were cultured in DMEM with 10% fetal bovine serum, 10 mg/mL penicillin and 10 U/mL streptomycin. Cultures were passed by trypsinization.

To co-culture BMDCs with *H. pylori* for dendritic cell activation, bacteria were centrifuged and resuspended in culture media at a concentration of $1x10^9$ CFU/mL as determined by counting bacteria on a hemocytometer. 1 mL of bacterial suspension was added to each flask of dendritic cells to effect an MOI of 100. After overnight culture, BMDCs were scraped from the flasks, aliquoted into flow cytometry tubes and then stained for the indicated markers.

ELISA for Lewis antigens

Bacterial cultures were spun down to pellet the bacteria, rinsed once in 1x PBS, and then resuspended in 1x PBS. Cultures were diluted to a final OD_{600} of 0.3, or approximately $1x10^6$ CFU/mL, and then added directly to MaxiSorp high-protein binding plates (Nunc, Nalge Nunc International, Rochester, NY) and incubated overnight at 4 °C. Plates were washed in 1x PBS, and then anti-Lewis antigen antibodies were added, BG-7 for Le^x and BG-8 for Le^y, at a dilution of 1:1000 in 1x PBS with 10% fetal bovine serum. Plates were washed again and HRP-conjugated goat anti-mouse IgG antibody was added at a dilution of 1:2000 in 1x PBS with 10% fetal bovine serum. After rinsing, plates were developed with TMB substrate and color development was stopped with 1 M H_3PO_4 stop solution. Wells were read at OD_{450}.

Flow cytometry assay for bacteria:cell adhesion

H. pylori cultures were spun down to pellet bacteria, rinsed in 1x PBS, and then resuspended in 1x PBS. Cultures were counted on a hemocytometer and diluted to an approximate final concentration of $1x10^6$ CFU/mL, and aliquots were taken for quantitative plate counts. Bacterial cultures were stained with BacLight Red (Invitrogen, Carlsbad, CA) at a concentration of 1 μM for 30 minutes in 1x PBS at 37 °C.

BMDCs were lifted by scraping the tissue culture flasks, rinsed in fresh tissue culture media and then resuspended to a final concentration of $1x10^5$ cells/mL and aliquoted into flow cytometry tubes. 300 μL of dyed bacteria was added to each tube of BMDCs to achieve an MOI of 3, and incubated at 37 °C at 5% CO_2 for the indicated time. The incubation was stopped by vortexing the tubes at maximum speed for 30 seconds and then 100 μL of 1% formalin was added to each tube to fix the bacteria:cell complexes.

AGS cultures were trypsinized, rinsed in fresh culture medium and resuspended to a final concentration of $1x10^5$ cells/mL in flow cytometry tubes. Either 100, 300 or 600 μL of bacteria was added to each tube of AGS cells to achieve different ratios of bacteria:cells. Cell/bacteria mixtures were incubated for 30 minutes at 37 °C at 5% CO_2. The incubation was stopped by vortexing the tubes at maximum speed for 30 seconds and then 100 μL of 1% formalin was added to each tube to fix the bacteria:cell complexes.

Crystal violet assay for deposition of biomass

Deposition of bacterial biomass was assessed via crystal violet staining of material left on glass beads. Briefly, 3 mL *H. pylori* cultures were inoculated in the specified media (normal Brucella broth with 10% fetal bovine serum; Brucella broth with 1% serum; Brucella broth without serum; or Brucella broth with 10% serum, diluted 1:10 in sterile water) with 100 μL approximately $1x10^6$ CFU/mL and grown in 6 well plates filled with a single layer of sterilized 5 mm diameter glass beads (glass frit). After overnight growth without agitation, cultures were aspirated and the beads were soaked in an aqueous solution of crystal violet (1% w/v) for 20 minutes at room temperature. The beads were then washed three times with deionized water to removed excess color and

air-dried. Biomass was quantitated by eluting crystal violet with an 80:20 v/v mixture of ethanol and acetone, and then measuring the OD_{570} of the eluted stain.

Statistical analysis

All graphs were made using GraphPad Prism 5.01 software. This program automatically calculated and graphed the standard error of the mean (SEM) for all column graphs. All matching datasets were tested for statistical significance through application of two-tailed Student's t-test using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Experimental groups that are not statistically significantly different are unmarked.

Appendix 3

Expression of *H. pylori* Pathogenicity Factor CagA is Unaffected by Serial Mouse Passage

BACKGROUND

Notably, *H. pylori* strains can produce two cytotoxins, VacA and CagA (11), and each of these proteins is believed to be injected into host epithelial cells via a bacterially-coded type IV secretion system (153, 193). Once inside, cellular kinases phosphorylate and activate these toxins, leading to alterations in the cytoskeleton and vacuole formation (193). CagA is responsible for a number of cytopathic effects, including a 'hummingbird' phenotype in afflicted epithelial cells (179) and apoptosis of macrophages has been attributed to CagA expression (72, 136).

The mouse-adapted *H. pylori* isolate SS1 was initially welcomed as a promising research tool, allowing development of small animal models of infectious gastritis (110). Use of SS1 has allowed us to explore many immunological events critical in the early development of *H. pylori*-mediated gastritis, but these models have not been without some controversy. It is now generally accepted that pathogenic *H. pylori* isolates carry a horizontally acquired, cytotoxicity-associated gene pathogenicity associated island (cagPAI) (23). However, reports disagree about the genetic structure of this island in SS1 and other mouse-adapted isolates, with evidence demonstrating the island is fragmented and non-functional (34). For these reasons and others, it has been argued that use of *H.*

pylori SS1 in mice does not constitute a suitable model for investigating the pathogenicity of this bacterial species.

Several reports also demonstrate *H. pylori* possesses a high degree of genetic plasticity *in vivo* and *in vitro* (6, 95, 191), raising another concern about the continuous utility of a single 'strain' in research. In one example, toxin-positive and toxin-negative isolates were recovered simultaneously from a single infected animal (191), suggesting genetic elements may pass fluidly through a community of *H. pylori in vivo*. Phase-shifting of surface Lewis polysaccharide antigens while absent the pressure of an active immune response also suggests an intrinsically high mutation rate (62, 199). The effect of mouse-adaptation upon human clinical isolates has also been shown to induce significant changes in genetic constitution and expression (5, 62, 104), in addition to extended serial passages *in vitro* leading to decreased *H. pylori* activity in several bioassays of pathogenicity (95). Together, these varied findings are evidence that *H. pylori* SS1 may experience significant shifts in pathogenic ability. Taking advantage of our significant historical collection of *H. pylori* strains and animal-passed isolates, we set out to examine the behavior of one toxin gene, *cagA*, through several different isolates of both 26695 and SS1.

EXPERIMENTAL DESIGN

The Eaton laboratory has an extensive collection of animal-passed isolates of *H. pylori* 26695, M6 and SS1 isolates, collected over more than 15 years of work exploring the development of animal models of *H. pylori* disease. Historically we have tested all three strains in mice, but we currently only employ the SS1 strain, as it reliably produces the most gastric inflammation in mice (53, 206), and we have a library of SS1 isolates

which have been serially passed in a documented chain of infection and re-isolation, granting us a unique opportunity to explore these questions. Also, SS1 is employed by a majority of groups investigating *H. pylori* gastritis in mouse models, and so we chose to focus our efforts on profiling the behavior of SS1 strains.

We elected to profile our strains completely in *in vitro* broth cultures solely to address the question of loss of CagA expression at a fundamental level. To do this, we sought to test the DNA and RNA from cultures via PCR for *cagA* sequences, and to assess the expression of CagA protein in both bacterial lysate and culture supernatant. We did not expect to find much toxin protein secreted into the broth, as export of CagA is regulated by expression of the cagPAI (23, 129). There is no published genome sequence for the SS1 strain, and so we relied upon the published sequence for the 26695 strain to design our primers, while we were able to obtain several different anti-CagA antibodies to test when building our ELISA.

To validate our assays, we included several lab-derived but animal-passed 26695 isolates and the 26695 Δcag strain, which lacks the entire cagPAI, as our negative control (Table A3.1). For our mouse studies we exclusively employ SS1, and we chose isolates that comprise a single chain of serial passages through mice, in addition to several other well characterized, previously published isolates (Table A3.1).

RESULTS

Expression of *cagA*

All 26695 and SS1 isolates tested were positive for *cagA* mRNA expression, except for 26695Δcag (Figure A3.1). We observed the greatest *cagA* levels in our 26695/Lab Passed and 26695ΔVacA isolates, even after the greater number of *in vitro*

passages experienced by these isolates. *cagA* expression in pig-passed isolate 26695/88-3887 dropped slightly upon adaptation through passage through a mouse. In general, *cagA*+ 26695 isolates expressed higher levels of *cagA* mRNA than we found in SS1 isolates, with averaged 26695 expression 119-fold greater than averaged SS1 levels. *cagA* mRNA levels in SS1-Logan were the highest of all SS1 isolates, even though this isolate had the greatest number of *in vitro* passages of our SS1 isolates. In this regard, the SS1-Logan behavior was like 26695/Lab Passed and 26695ΔVacA. In our serially mouse-passed isolates, we observed a drop in *cagA* expression from SS1/07-317 to SS1/07-564, but expression increased from SS1/07-564 to SS1/09-202.

Production of CagA

We found CagA protein in the lysate of all *cagA*+ samples (Figure A3.2). In contrast to our findings with *cagA* mRNA levels, we observed the greatest production of CagA protein in 26695/88-3887 and 26695/88-3887 Mouse, the two isolates with the lowest *cagA* expression. In SS1 isolates, we found the most CagA in the lysates of our trio of serially mouse-passed isolates. The change in expression patterns we observed between SS1/07-317, /07-564 and /09-202 was evident in protein production, as we found a significant decrease in CagA protein levels in /07-564, but this passage was followed by a significant increase in /09-202.

DISCUSSION

We investigated the potential changes in *cagA* expression and CagA production in *H. pylori* strains over repeated serial *in vitro* and *in vivo* passages to address possible shifts in pathogenic behavior due to genetic plasticity in laboratory-maintained isolates. In short, we found no consistent change in *cagA* or CagA levels across any of our

samples, no matter how long they had been maintained *in vitro* or how many passages experienced *in vivo*. We did observe a slight decrease in both mRNA and protein levels when 26695/88-3887 was passed through a mouse, but this was an insignificant change in a strain not known for its strong colonization of mice. More dramatic shifts were found in repeated passages of our mouse-adapted isolates of SS1, as we found a significant drop in CagA after 8 weeks *in vivo*, but this was subsequently followed by a significant rise in protein levels after 9 months. Our findings demonstrate that CagA production is maintained in both a mouse-adapted 26695 isolate and SS1 isolates over repeated *in vivo* serial passes, and even after a limited number of *in vitro* serial cultures.

We conducted these experiments to address the issue of *H. pylori* genetic plasticity and the stability of *cagA* expression in mouse-adapted human clinical isolate of *H. pylori*, SS1. It has been reported elsewhere that clinical *H. pylori* isolates may lose pathogenic capabilities as they undergo the process of adapting to a new host species (110, 206), especially if that adaptation grants them a prolific colonization ability in that new host (206). The suitability of *H. pylori* SS1 as a mouse model for infectious gastritis has been discussed frequently (34, 42, 166), including our own contributions to the arguments. Indeed, we have previously shown that SS1 lacks the ability to trigger IL-8 production from AGS cells (53), suggesting that mouse-adaptation cost this strain a fundamental pro-inflammatory ability. In combination with reports showing the translocation of CagA to epithelial cells from attached *H. pylori* (153), it was suggested that SS1 had either lost *cagA* expression or that the strain's cagPAI was wholly non-functional (34). While we acknowledge there are likely other meaningful mutations in

the SS1 genetic structure that interfere with various bioactivity assays, a loss or decrease in CagA production is not one of them.

Kim et al. noted that extended serial laboratory passage of SS1 on culture media prompted significant reductions in *in vitro* pathogenic behavior (95). However, even after 6 months and 64 passes *in vitro*, they found no significant differences in histological inflammation between groups of hamsters inoculated with either the parent isolate or the 64^{th}-pass culture. In our laboratory, we do not culture any single *H. pylori* isolate for more than 10 passes *in vitro* before using employing it in any subsequent experiments, but we have maintained histological gastritis and *H. pylori* SS1 infection in mice for more than 22 months (data not shown). Nor have we noted any consistent trend in loss or gain of pathogenicity, as measured by histological inflammation or cytokine expression in infected mice, over the many years we have used SS1 isolates through repeated mouse passages (data not shown). We would argue that reasonable and consistent culture, inoculation and re-isolation practices provide for a stable population of *H. pylori* SS1.

Our findings do not resolve the arguments whether mouse infection with SS1 is a suitable model in which to study bacterial pathogenicity factors as part of the process of infectious gastritis. For example, we do not address the possibility that the whole SS1 cagPAI may be non-functional, nor do we examine potential sequence changes in the *cagA* gene that may diminish the biological activity of the protein. However, we would consider that our findings help lay to rest the issue of genetic instability in the SS1 strain affecting pathogenic behaviors.

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Table A3.1: List of identities, history and characteristics of isolates tested.

Strain	Isolate	Parent Isolate	Host Species	Length of Infection	# of *in vitro* Passages	cagPAI Status?
26695	Lab Pass	26695	Human		10+	+
	88-3887		Pig		2	+
	88-3887 Mouse	88-3887	Mouse		2	+
	Δcag		Mouse		3	-
	ΔVacA		Mouse		6	+
SS1	Logan	SS1 (121)	Mouse		5	
	07-317	SS1-Eaton (54)	Mouse	8 weeks	2	
	07-564	07-317	Mouse	8 weeks	2	
	09-202	07-564	Mouse	9 months	3	

Figure A3.1: *cagA* mRNA levels in *H. pylori* broth cultures

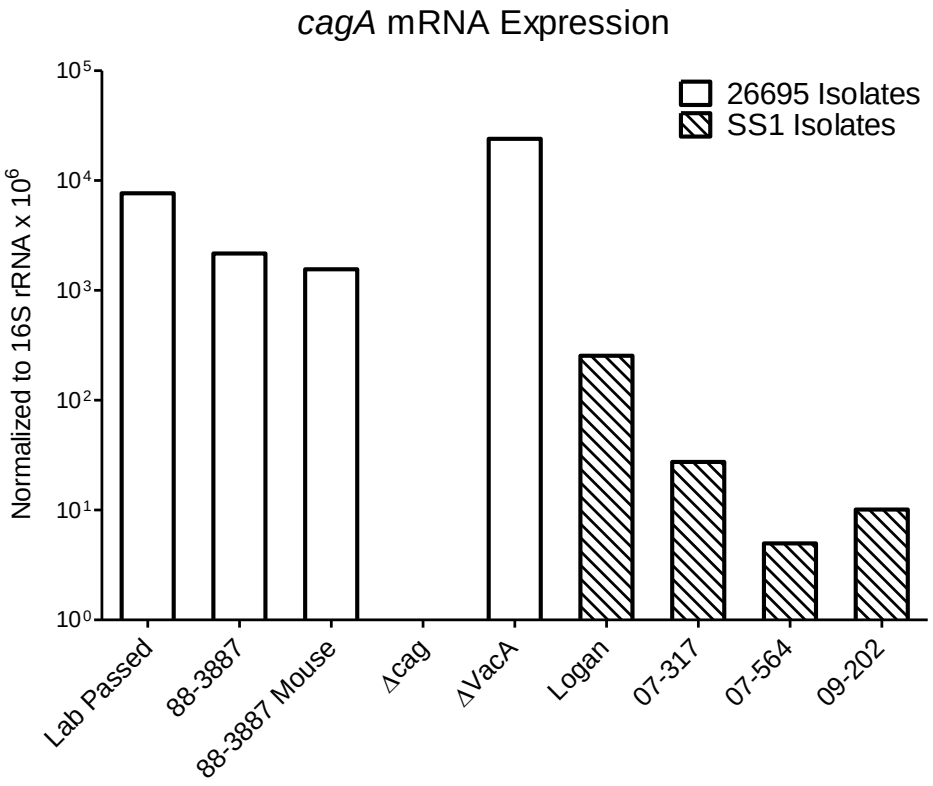

Figure A3.1: RT-PCR analysis of *cagA* mRNA expression. *cagA* primers were designed for the 5' end of the 26695 sequence. Threshold values were normalized to 16S rRNA levels, and multiplied by 106 for ease of visualization.

Figure A3.2: CagA protein in *H. pylori* broth cultures

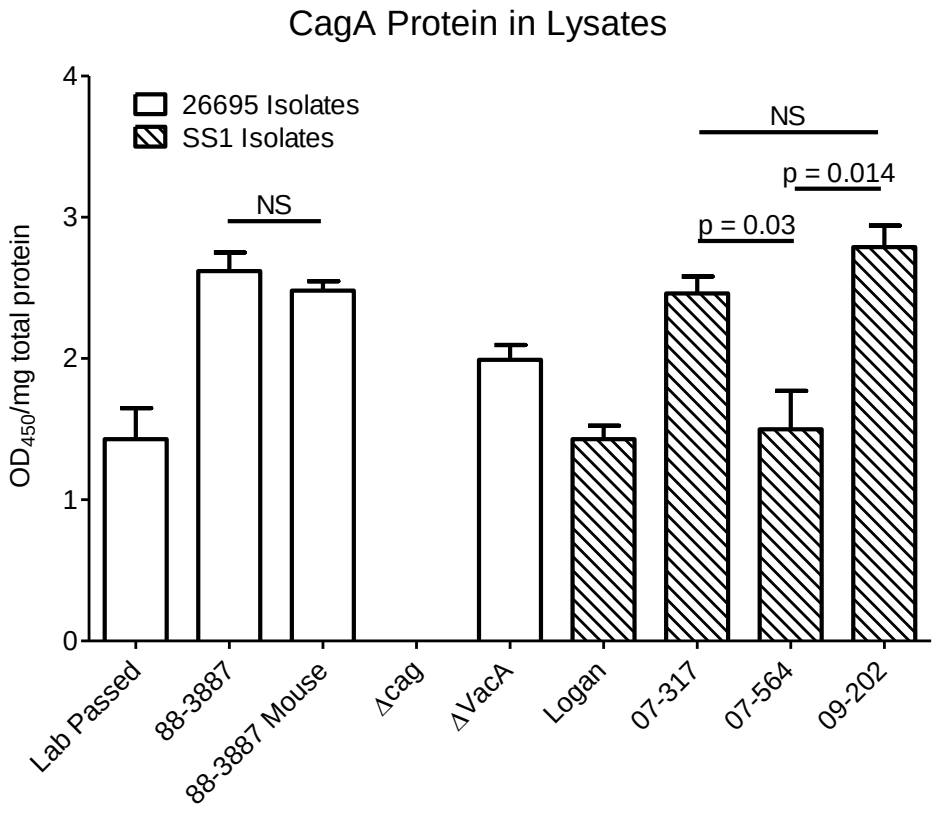

Figure A3.2: ELISA for CagA protein production in lysates of broth-cultured isolates.

METHODS

Bacterial Culture, Inoculation and Re-isolation

All cultures were stored at -80 °C in 200 μL Brucella broth with 10% fetal calf serum and 10% or 20% glycerol until they were thawed. One tube of each isolate was thawed at room temperature and we used 75 μL each to inoculate two 10 ml broth cultures (Brucella broth, 10% fetal calf serum, and 50 μL of Skirrow's antibiotic supplement). Broth cultures were incubated overnight at 37 °C in microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) on a rotary shaker at approximately 60 rpm.

Broth cultures were harvested via centrifugation at 3500 rpm (approximately 500x g) for 10 minutes, and this was sufficient to pellet all bacteria. The supernatant was removed and 500 μL were stored at -80 °C for later analysis by ELISA. The pelleted bacteria were resuspended in 1 mL of sterile 1x phosphate buffered saline and transferred into two microcentrifuge tubes. The bacteria were centrifuged again at approximately 13,000x g for 5 minutes at 4 °C. The supernatant was decanted. One pellet was resuspended and lysed in 100 μL of TRIzol reagent (Invitrogen, Carlsbad, CA), while the other was stored dry. Both pellets were stored at -80 °C for later analysis.

RNA Collection and RT-PCR

Bacterial RNA was isolated through completion of the TRIzol RNA isolation protocol once those samples were thawed to room temperature. After completion of the protocol, mRNA and rRNA were purified via the Qiagen RNEasy kit with genomic DNA digestion. Gene expression was analyzed via a SYBR-Green. *CagA* primers were designed for the 5' end of the gene (HP0547: 5': GGGCCTACTGGTGGGGATTG, 3'GCGGTAAGCCTTGTATGTCG). For a housekeeping control, primers were designed

for *H. pylori* 16S rRNA. All samples were run in duplicate. *cagA* mRNA values were normalized to 16S rRNA to account for variations in bacterial density. The normalized values were multiplied by 10^6 for ease of visualization.

Protein Collection and ELISA

Frozen culture supernatants were thawed to room temperature and then diluted 1:1 with 1x PBS with protease inhibitors (cOmplete, Mini, EDTA-free tablets, Roche Applied Science, Indianapolis, IN). Bacterial pellets were thawed to room temperature and the pellets were resuspended in 500 μL of 1x PBS with protease inhibitors. The lysates were then run through a 25 ga needle 10 times in order to shred genomic DNA. Protein concentration for both lysates and supernatants were measured via a micro-BCA test.

CagA protein production was measured via a sandwich ELISA of our own construction. Briefly, we coated Nunc MaxiSorp plates with mouse anti-CagA antibody (B237H from Abcam, Cambridge, MA) in carbonate buffer. After blocking the plate, we diluted our samples 1:50 in 1x PBS and added our dilute sample to the wells. We blocked again and then detected CagA with a rabbit anti-CagA antibody (B237H, from Santa Cruz Biotech, Santa Cruz, CA), and used an HRP-conjugated goat anti-rabbit antibody and chromogen. After stopping ELISA development with stop solution, samples were read at OD 450 All samples were tested in quadruplicate, and the OD values were normalized to the total protein concentration of either the lysate or supernatant as appropriate.

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