

# Modulation of mucosal effector and regulatory T cell responses by *Helicobacter pylori*

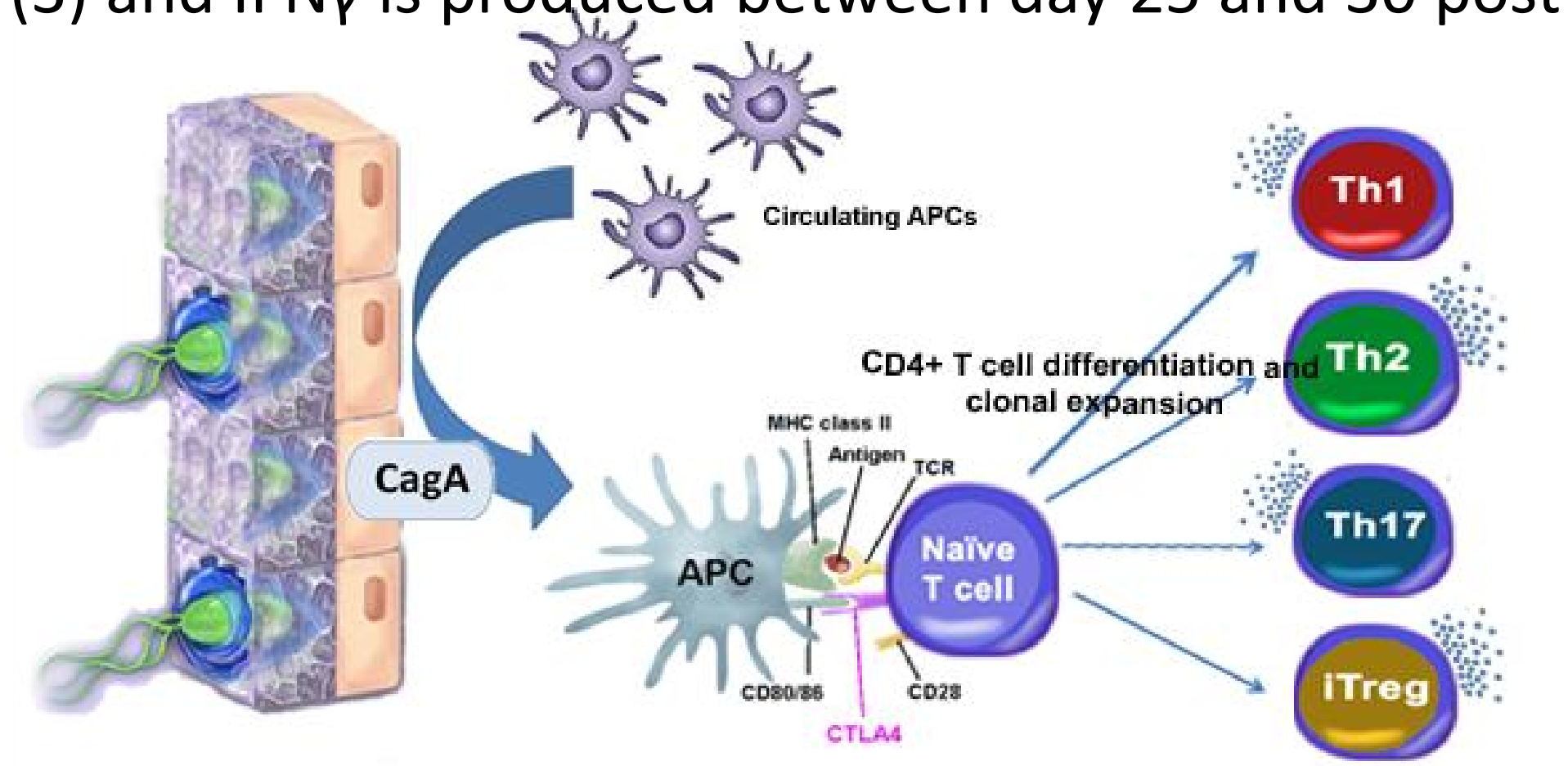
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## 1. Introduction

*Helicobacter pylori* is a gram-negative, microaerophilic bacterium of the Epsilonproteobacteria and the dominant member of the gastric microbiota that has colonized the stomach mucosa since early in human evolution. *H. pylori* usually does not cause illness, but colonization with strains bearing the *cag* (cytotoxin-associated gene) pathogenicity island (or also designed CagA+ strains) is associated with increased risk of noncardia gastric adenocarcinoma and peptic ulcer disease. *H. pylori* infection is associated with a marked infiltration of CD4+ T cells in the gastric mucosa and secondary lymph nodes, which promotes chronic inflammation and persistence. CD4+ T cell responses are both necessary and sufficient to clear the response in gastritis due to *H. pylori* infection in mice (1). The CD4+ T cell profile in *H. pylori* infection shows a Th1-polarized phenotype in mice, where IFN $\gamma$ , secreted predominantly by Th1 cells, is essential for *H. pylori* clearance, but at the same time may promote the formation of preneoplastic lesions (2). Consistent with the enhanced Th1 responses, T-bet is essential to induce a Th1 response (3) and IFN $\gamma$  is produced between day 25 and 30 post-infection (4).



**Figure 1. Schematic representation of the effect of *Helicobacter pylori* infection on CD4+ T cell differentiation in the gastric lamina propria.**

Even though the role of CD4+ Th17 cell responses in *H. pylori* infection has not been fully elucidated, there are several studies that demonstrate the presence of Th17 cells in the gastric mucosa, as well as spleen and mesenteric lymph nodes of infected mice (4, 5). Regulatory CD4+ T cell numbers are elevated in *H. pylori*-associated gastritis, where Tregs are positively correlated with the grade of chronic inflammation and the number of lymphoid follicles (6). Thus, CD4+ T cell differentiation plays an important role in regulating *H. pylori* infection. However, little time-course data are available. To develop a fully calibrated computational model of the *H. pylori* infection, time-course data is strongly and urgently needed. Recent studies of Shi et al. (4) show time-course data in systemic tissues, but no data are presented at the mucosa level. MIEP has generated time-course data related to the effect of *H. pylori* infection on the CD4+ T cell differentiation process to facilitate the development and calibration of a computational model of immune responses to *H. pylori*. Please refer to poster B534 for further *H. pylori* modeling studies conducted by MIEP.

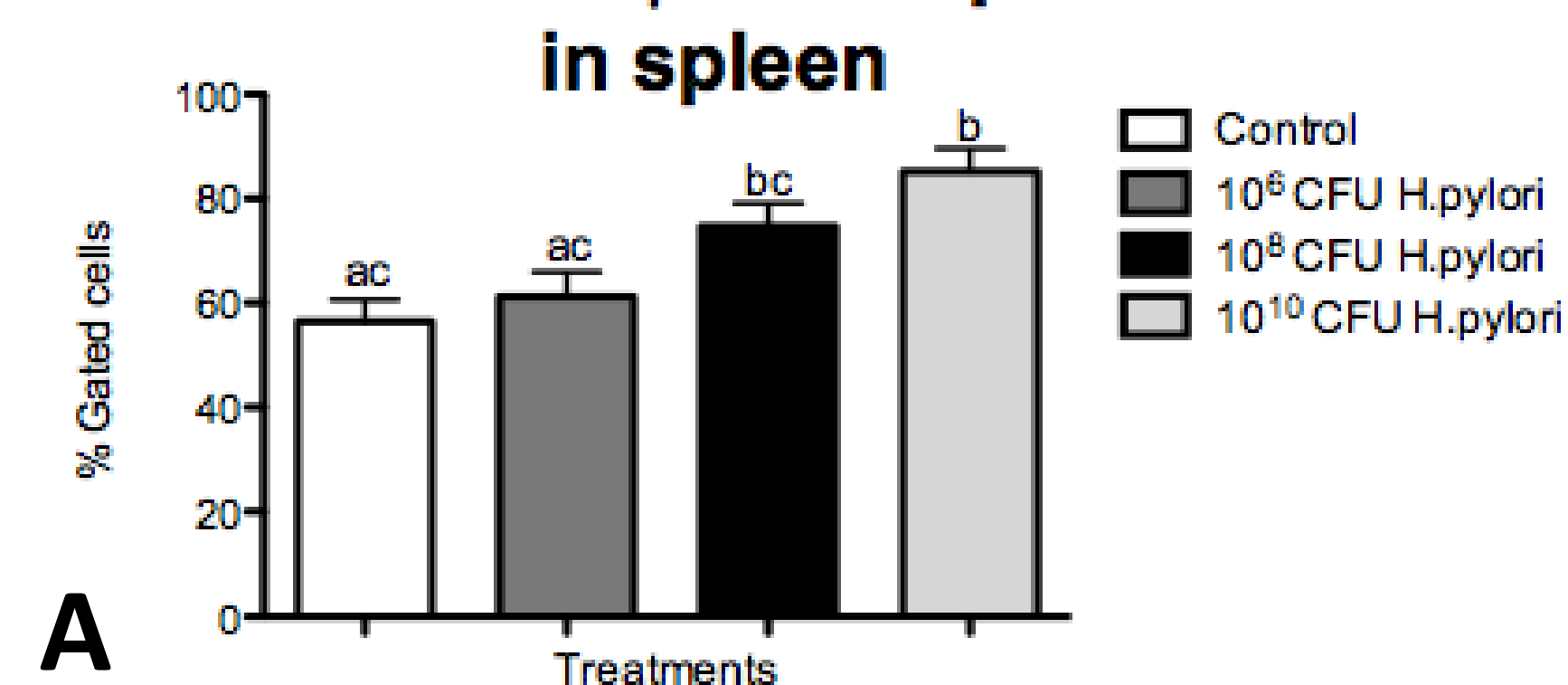
## 2. Materials and Methods

Eight-week-old C57BL/6 mice were fasted for 12 h and infected with *H. pylori* 26995 (European CagA+ strain) via orogastric gavage. To optimize the infective dose mice were challenged with 0, 10<sup>6</sup>, 10<sup>8</sup> or 10<sup>10</sup> CFU. The time-course study was run with 10<sup>10</sup> CFU of *H. pylori* 26995 and mice were euthanized on days 7, 14, 30 and 60 post-infection. Mice were housed at the animal facilities of Virginia Tech. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Virginia Tech and met or exceeded requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act. Body weights and disease activity were assessed daily. Stomach, blood, spleen, Peyer's patches and gastric and duodenal lymph nodes were collected 14 days post-infection for histology, immunological and microbiology analysis. We evaluated the functional profile of the responder CD4+ T cells by assessing the cytokine they produce following T cell stimulation. Colonization of the gastric mucosa by *H. pylori* was assessed by PCR and a 2% agarose gel using *H. pylori* strain 26695 specific primers for the *HP0046* gene. In addition, *H. pylori* was re-isolated from infected mice and quantified using a bacterial dilution method in Columbia plates with 5% sheep blood plus Dent supplement.

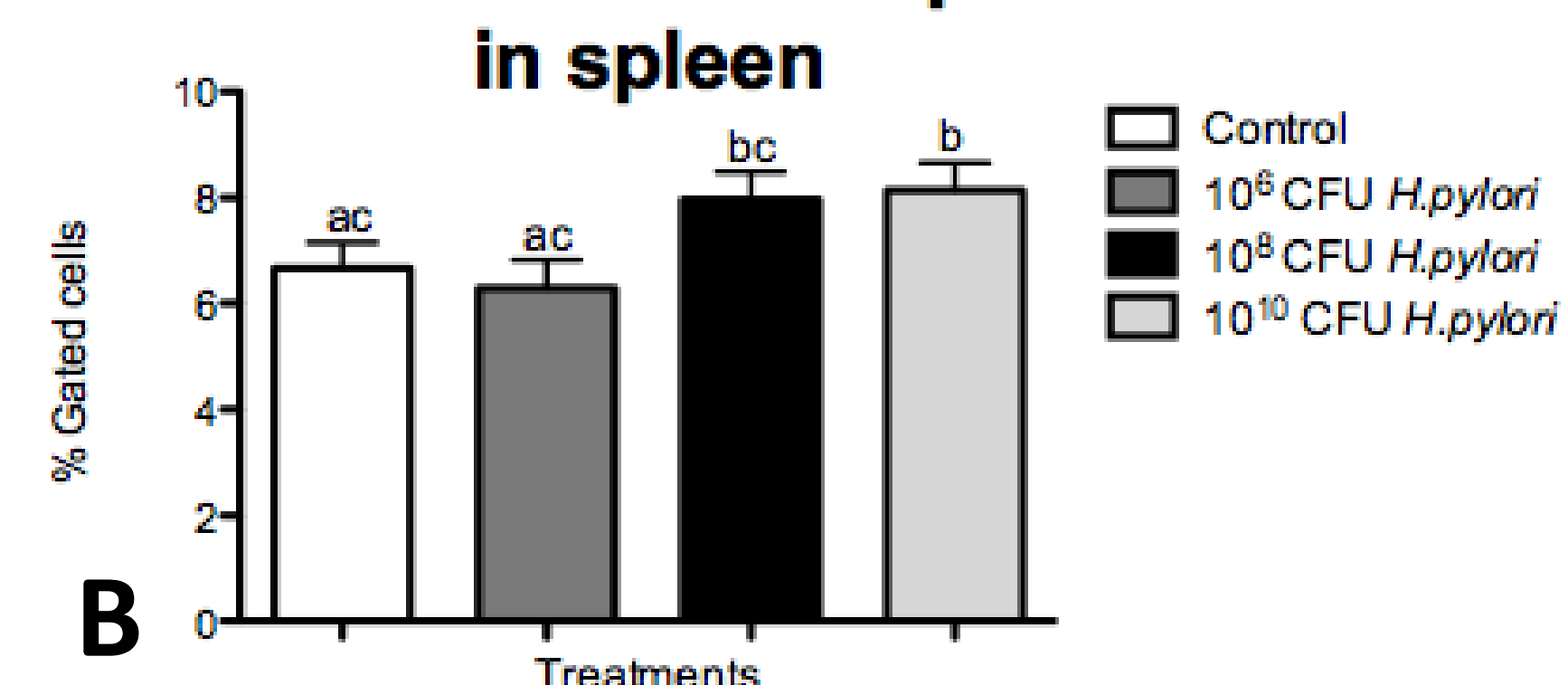
## 3. Results and Discussion

Spleen, gastric lymph nodes and Peyer's patches-derived cells were isolated in sterile conditions and single cell suspensions were used for proliferation and functional assays to determine the effect of *H. pylori* on cytokine production. Our data demonstrate that *H. pylori* infected mice at doses 10<sup>8</sup> and 10<sup>10</sup> CFU had greater percentages of CD4+ IFN $\gamma$ + Tbet+ T cells in the spleen (**Figure 2A**), indicating that *H. pylori* induced differentiation of CD4+ T cells into a T helper 1 (Th1) phenotype. Flow cytometry results also demonstrate a parallel increase of cells with immunoregulatory function with increasing doses of *H. pylori*. Specifically, the percentages of regulatory T cells (Treg), defined as CD4+ FOXP3+ T cells in the spleen increased in the group of mice inoculated with the highest dose of *H. pylori* (**Figure 2B**). However, the Th1 response predominated over the Treg response in this study since the percentages of Treg cells were lower than Th1 cells. These effector responses are in line with the gastric histopathological results which show a higher lamina propria leukocytic infiltration as the dose is increased from 10<sup>6</sup> to 10<sup>10</sup> CFU (**Figure 2C**). Moreover, gastric atrophy and epithelial hyperplasia were both showing an increase when the dose was increased up to 10<sup>10</sup> CFU (data not shown).

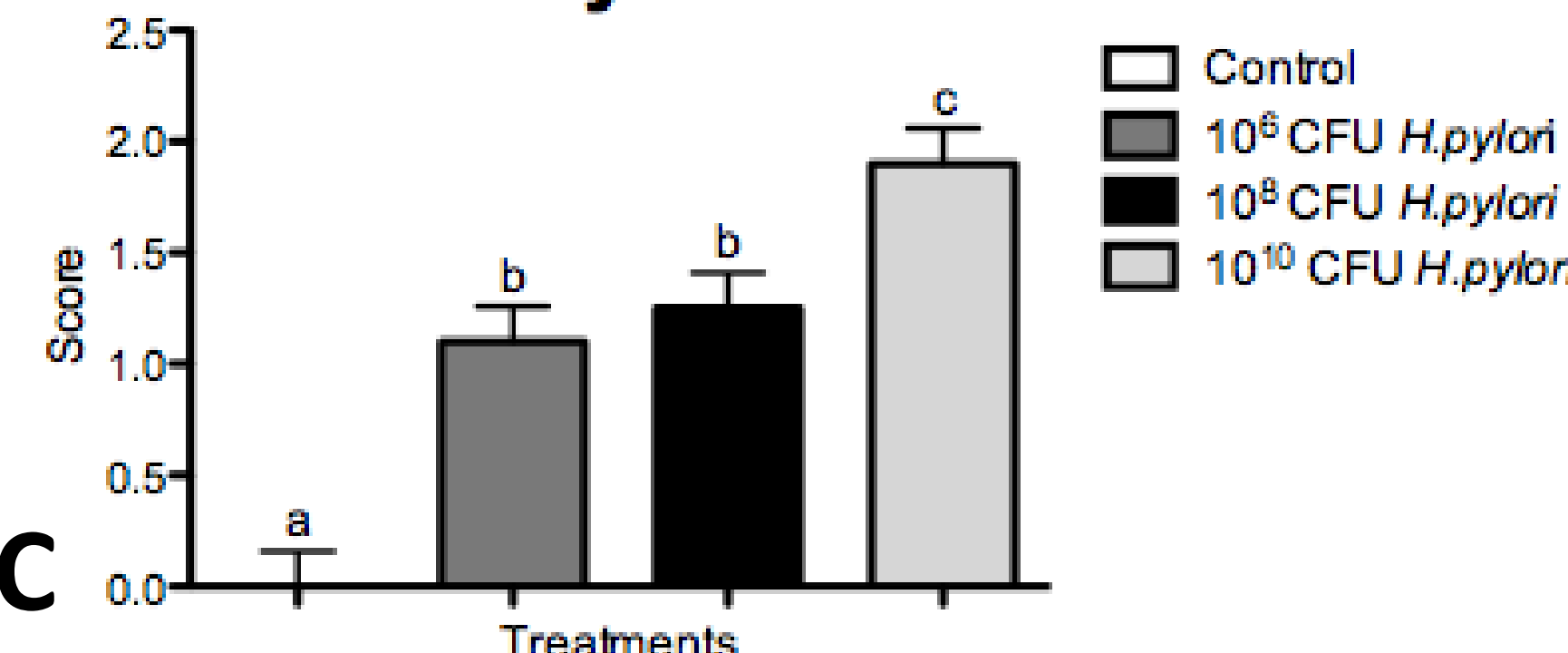
### CD4+ Tbet+ IFN $\gamma$ + T cell production in spleen



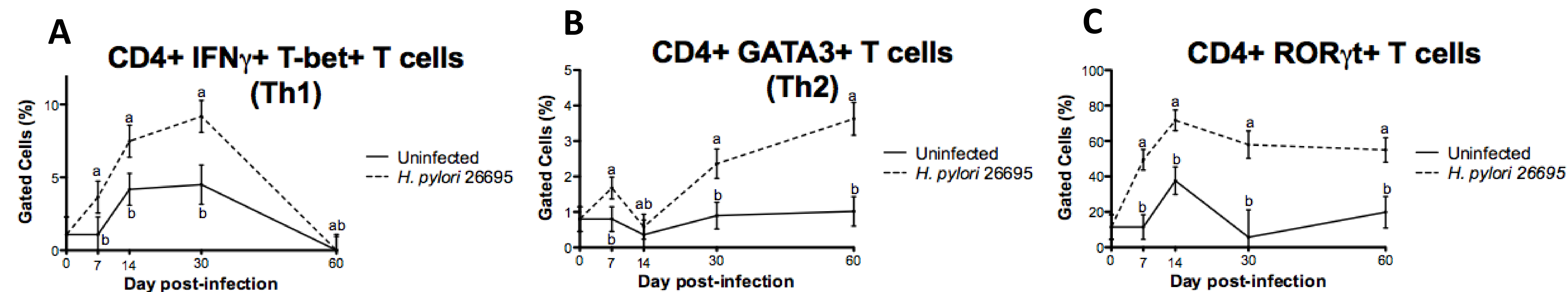
### CD4+ FOXP3+ T cell expression in spleen



### Leukocytic infiltration



**Figure 2. *Helicobacter pylori* effect on cytokine production and tissue damage with increasing concentrations of infectious dose.** Increasing infectious doses of *Helicobacter pylori* promote CD4+ T cell differentiation into a predominant Th1 phenotype characterized by IFN $\gamma$  and Tbet expression (A) as well as an increase in the percentages of FOXP3+ regulatory T (Treg) cells on day 14 post-infection (B). Splenocytes were stimulated for 6 hours with 5  $\mu$ g/mL of plate-bound anti-mouse CD3 antibody. The presence of CD4+ T cells with regulatory and effector phenotype was assessed by intracellular flow cytometry and based on the expression of FOXP3, Tbet and IFN $\gamma$ . Leukocytic infiltration in the gastric mucosa increases with increasing concentrations of *H. pylori* infectious dose from 10<sup>6</sup>, 10<sup>8</sup> to 10<sup>10</sup> CFU (C). Data are represented as means of groups of ten mice. Means without a common letter superscript are significantly ( $P < 0.05$ ) different.

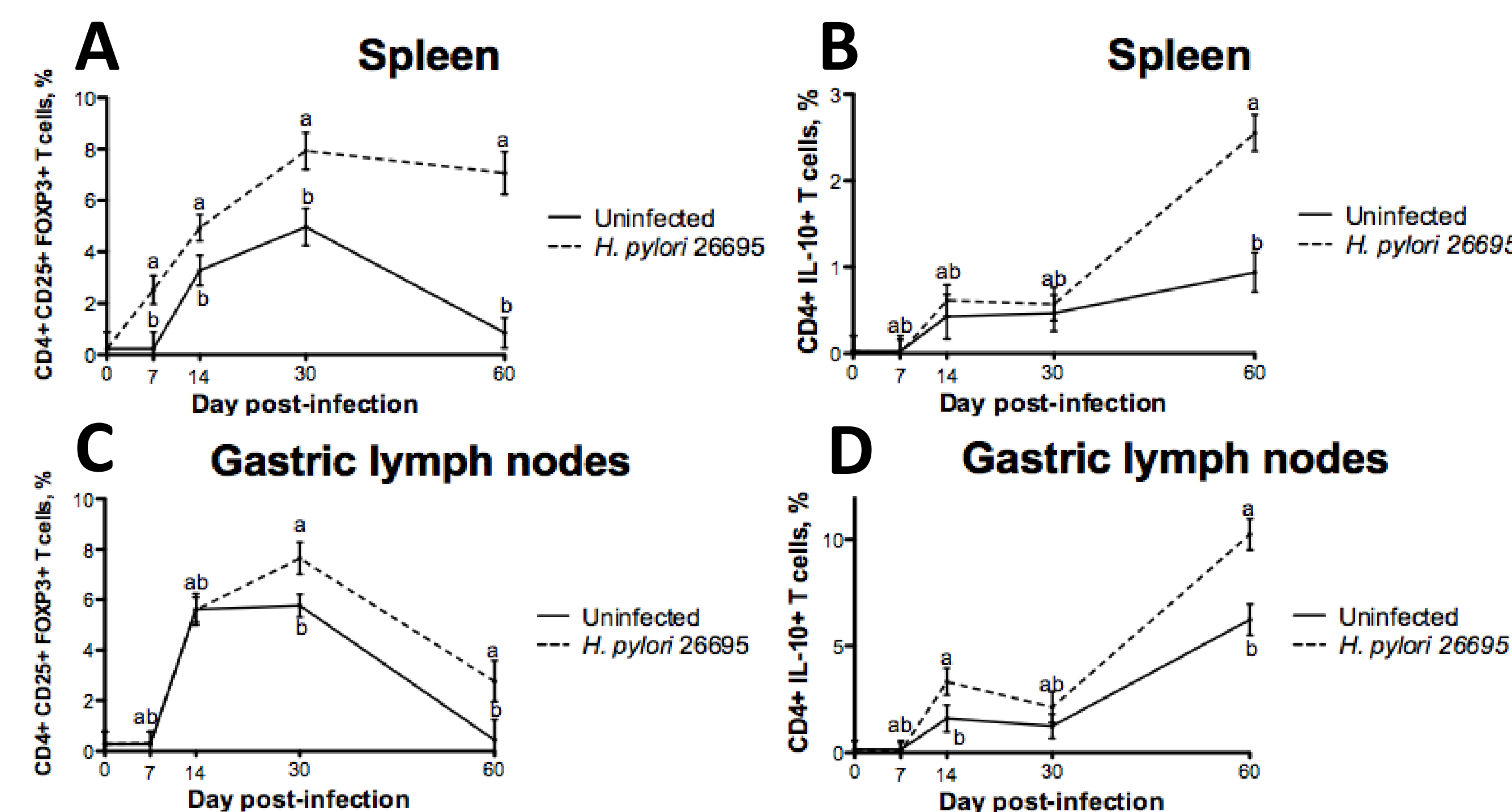


**Figure 3. Time course analyses of the effect of *Helicobacter pylori* 26695 infection on spleen T helper (Th) 1, Th2 and Th17 responses.** Splenocytes were stimulated with 5  $\mu$ g/mL of plate-bound anti-mouse CD3 and flow cytometric analyses was performed at 6 hours following stimulation to assess percentages of CD4+IFN $\gamma$ + Tbet+ T cells (A), CD4+ GATA3+ T cells (B) and CD4+ ROR $\gamma$ t+ T cells (C). Data are presented as means  $\pm$  standard error (n=10). Means without a common letter superscript are significantly ( $P < 0.05$ ) different.

Our data demonstrate that *H. pylori* 26695 infection triggered a Th1 response on day 7 post-infection followed by a slight increase in Th17 effector response on day 14 postinfection. The effector Th1 phenotype, corresponding to CD4+ IFN $\gamma$ + Tbet+ T cells is increased from day 7 to 30 post-infection and returns to baseline levels by day 60 post-infection (**Figure 3A**). The Th1 phenotype is believed to contribute to the inflammatory pathogenesis of *H. pylori* in the gastric mucosa. Additional challenge studies will be needed to characterize the switch from effector to regulatory phenotypes from day 30 to day 60. Our data also supports the presence of CD4+ Th2 cells on day 30 post-infection, which can remain active by the presence of the transcription factor GATA3 up to day 60 post-infection (**Figure 3B**). In line with the increase in GATA3 (a Th2-related transcription factor) IL-4 levels peak on day 60 post-infection (data not shown). Th17 T cells have a small but significant peak on day 14 and the expression of CD4+ ROR $\gamma$ t+ cells remain sustained until day 60 (**Figure 3C**), coexisting in lymph nodes and secondary organs with regulatory responses.

The effector response is generally sustained until day 30 when it reaches its peak and regulatory responses guided by CD4+ Treg cells (**Figure 4A, 4C**) and IL10-producing CD4+ T cells (**Figure 4B, 4D**) predominate on day 60 post-infection in the draining lymph nodes (**Figure 4A, 4B**) and the gastric mucosa (**Figure 4C, 4D**).

**Figure 4. Time course analyses of the effect of *Helicobacter pylori* 26695 infection on spleen and gastric lymph node (GLN) regulatory T cell (Treg) responses.** GLN and spleen-derived cells were stimulated with 5  $\mu$ g/mL of plate-bound anti-mouse CD3 and flow cytometric analyses were performed at 6 hours following stimulation to assess percentages of CD4+CD25+FOXP3+ Treg cells (A,C) and CD4+ IL-10+ T cells (B,D) in spleen (A, B) or GLN (C,D). Data are represented as means  $\pm$  standard error (n=10). Means without a common letter superscript are significantly ( $P < 0.05$ ) different.



To examine the influence of *H. pylori* infection on lymphoproliferative recall responses, Peyer patches and spleen-derived cells from infected and uninfected mice were stimulated *ex vivo* with inactivated *H. pylori* antigens (i.e., whole cell and whole cell sonicated) and proliferation assays were performed. On days 14 and 30 post-infection, results show greater antigen-specific proliferative responses to *H. pylori* 26695 antigens than those recovered from uninfected mice (**Figure 5A**). By day 60 post-infection lymphoproliferative responses returned to baseline levels. Interestingly, day 60 coincided with the period of increased production of IL-10 and expansion of the Treg compartment. Also, our data show how CD4+ T cells also respond specifically to *H. pylori* (**Figure 5B**).

**Figure 5. Antigen-specific lymphoproliferative recall responses of Peyer's patches-derived cells to *Helicobacter pylori*.** Cells collected on days 0, 7, 14, 30 and 60 post-infection were stimulated *ex vivo* with *H. pylori* antigens, incubated for 4 days and subjected to a lymphocyte blastogenesis test. Data are presented as mean  $\pm$  standard error (n=10). Means within time point with different letter superscripts are significantly different ( $P < 0.05$ ).

## 4. Conclusions

- ✓ *Helicobacter pylori* infection promotes CD4+ T cell infiltration in the gastric mucosa and gastric lymph nodes.
- ✓ The effector CD4+ T cell response remains active until day 30, and the immunoregulatory response by Treg and IL-10 secreting CD4+ T cells predominates on day 60 post-infection

### 5. References

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