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CD25$^+$ T cells induce *H. pylori*-specific CD25$^-$ T cell anergy but are not required to maintain persistent hyporesponsiveness

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Summary

The gastric pathogen *Helicobacter pylori* (*H. pylori*) infects over half the world's population. The lifelong infection induces gastric inflammation but the host fails to generate protective immunity. To study the lack of protective *H. pylori* immunity, CD4$^+$CD25$^+$ T_{reg} cells were investigated for their ability to down-regulate *H. pylori*-specific CD4$^+$CD25$^-$ cells in a murine model. CD25$^-$ lymphocytes from infected mice were hyporesponsive to antigenic stimulation *in vitro* even in the absence of CD25$^+$ T_{reg} cells unless treated with high dose IL-2. Transfer of CD45RB^{hi} naïve CD25$^-$ cells from infected mice into *rag1*$^{-/-}$ mice challenged with *H. pylori* resulted in severe gastritis and reduced bacterial loads, whereas transfer of CD45RB^{lo} memory CD25$^-$ cells from *H. pylori*-infected mice resulted in only mild gastritis and persistent infection. CD25$^-$ cells stimulated in the absence of CD25$^+$ cells in *rag1*$^{-/-}$ mice promoted bacterial clearance, but lost this ability when subsequently transferred to wild type mice harboring CD25$^+$ cells. These results demonstrate that CD25$^+$ cells induce anergy in CD25$^-$ cells in response to *H. pylori* infection but are not required to maintain hyporesponsiveness. In addition, CD25$^+$ cells are able to suppress previously activated CD25$^-$ cells when responding to *H. pylori* challenge *in vivo*.

Keywords

Anergy; Helicobacter pylori; inflammation; regulatory T cells; mouse

Introduction

CD4$^+$CD25$^+$ regulatory T cells (T_{reg} cells) have been identified in the peripheral tissue of mice and humans where they help prevent the development of autoimmunity by down-regulation of self-reactive T cells that escape thymic education [1]. Whereas the level of regulatory CD25^{hi} T cells in human blood ranges from 2 – 4%, a distinct population of regulatory CD25$^+$ cells make up approximately 10% of the mouse peripheral T cell pool [2-4]. T_{reg} cells are further characterized by the expression of the transcription factor *Foxp3* and by their anergic response to *in vitro* stimulation [5,6]. The suppressive activity of these cells is contact-dependent and requires TCR activation [7]. It has recently been shown that T_{reg} cells are also necessary for the maintenance of tolerance to food antigens and

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commensal flora of the gut [8,9] as transfer of $CD4^+$ T cells depleted of T_{reg} cells into immunodeficient mice results in the spontaneous development of colitis and wasting disease [10,11]. T_{reg} cells were shown to prevent the development of colitis when simultaneously transferred with a $CD25^+$ T_{reg} cell-depleted population [9,12].

T_{reg} cells have been identified in the host response to specific pathogens [13-16]. Reduced and non-protective immune responses have been described for the parasites *Plasmodium yoelii* and *Leishmania major* and the fungus *Pneumocystis carinii* which contributes to the ability of these microorganisms to establish persistent infection of the host [14-16]. Recently, $CD25^+$ T_{reg} cells have also been identified in the gastric mucosa of *Helicobacter pylori* (*H. pylori)*-infected patients [17-19]. *H. pylori* is a gram-negative bacterium that infects the stomach of more than half the world's population and in many developing nations its prevalence exceeds 80% of the population [20,21]. Infection persists for the life of the host and is accompanied by active-chronic inflammation and an adaptive immune response that fails to generate protective immunity. Several early studies on *H. pylori* immunity indicated that infection might induce T cell hyporesponsiveness as both peripheral blood lymphocytes and gastric lymphocytes from *H. pylori*-positive patients were shown to respond to *in vitro* stimulation by *H. pylori* antigen with low cytokine secretion and proliferation relative to *H. pylori*-negative patients [22,23]. In mice, the *in vitro H. pylori*-specific recall response of T cells from experimentally infected animals is significantly weaker than mice immunized with *H. pylori* antigens [24,25] and limiting the contribution of $CD25^+$ cells can result in increased gastritis and reduced bacterial loads [26,27].

In the present study we investigated the extent of the regulatory activity of $CD4^+CD25^+$ T_{reg} cells during *H. pylori* infection in mice. We demonstrate here that the presence of T_{reg} cells at the time of T cell activation in the gastric mucosa results in the generation of a population of $CD25^-$ *H. pylori*-specific anergic T cells. Although several reports have suggested that these *H. pylori*-specific $CD25^-$ T cells become responsive when $CD25^+$ T_{reg} cells are removed [26-28], our *in vitro* and *in vivo* data suggest that once stimulated in the presence of T_{reg} cells, this hyporesponsive $CD25^-$ population remains anergic even in the absence of $CD25^+$ regulatory T cells. Additionally, we demonstrate that T_{reg} cells are not only capable of inducing *H. pylori*-specific anergy in naive $CD25^-$ cells, but are capable of down-regulating anti-*H. pylori* immunity in previously activated $CD25^-$ cells as well.

Results

Foxp3 expression is increased in the gastric mucosa during *H. pylori* infection

Prior to investigating the role of $CD25^+$ regulatory T cells during *H. pylori* infection, we confirmed the recruitment of these cells to the gastric mucosa in our model of *H. pylori* infection by measuring *Foxp3* mRNA levels in gastric biopsies by quantitative PCR. *Foxp3* positive T_{reg} cells have been previously reported to reside in the gastric mucosa after *H. pylori* infection as has been demonstrated in both human and murine tissue [17,26]. In our model, *Foxp3* expression was increased almost three fold compared to naïve control mice four weeks after challenge (data not shown). Although the increase was not statistically significant we observed a comparable increase in two independent experiments.

Hyporesponsive $CD25^-$ cells are activated by high dose IL-2 and antigen

We have previously demonstrated *H. pylori*-specific anergy in bulk spleen cells from *H. pylori*-infected mice that could be induced to produce IFNγ in the presence of *H. pylori* and high dose IL-2 (1000 U/ml) [24]. To control for the possibility that other cell types in the spleen cell population might contribute to the responsiveness of the T cells, purified $CD4^+$ cells were cultured in the presence or absence of high dose IL-2. Figure 1 shows that the

$CD4^+$ cells from infected mice displayed activity comparable to naïve mice when stimulated by antigen alone. This activity was significantly less than observed for antigen-stimulated $CD4^+$ cells from immune mice ($P < 0.001$). The addition of IL-2 resulted in IFNγ production by cells from infected mice comparable to the response of the cells from immune mice.

We then assessed the role of $CD25^+$ cells in preventing the T cells from responding to antigen exposure *in vitro*. Spleen cell populations from infected mice in which the $CD25^+$ cells had been depleted by either complement-mediated killing or by fluorescence activated cell sorting remained anergic when stimulated with *H. pylori* lysate antigen. However, activity could be recovered only when cells were simultaneously exposed to antigen and high dose IL-2 (data not shown).

The cellular response of the infected mice was further analyzed by specifically measuring the activity of the $CD4^+$ cells in the presence or absence of $CD25^+$ cells. Initially, $CD25^+$ cells were depleted from bulk spleen cells by complement-mediated killing and the $CD25^-$ cells were stimulated *in vitro* with *H. pylori* lysate antigen. Removal of the $CD25^+$ cells did not result in the ability of the $CD25^-$ cells to respond to antigen (Figure 2A). Flow cytometric assessment of cell populations depleted of $CD25^+$ cells by complement-mediated lysis showed that less than one percent of the cells continued to stain positively for CD25 compared to approximately 11% for untreated cells (data not shown). We then purified $CD4^+$ cells from spleens by positive selection and then further fractionated the cells into $CD25^+$ and $CD25^-$ populations by affinity column isolation. The direct stimulation of $CD4^+$ cells from naïve mice with *H. pylori* antigen generated low levels of activity (Figure 1). Similar observations have been made by others [29]. Therefore we stimulated the fractionated cells with antigen-pulsed macrophages in which soluble antigen was removed by washing prior to co-culture with the fractionated spleen cells. $CD4^+CD25^-$ cells failed to respond to *H. pylori* antigen but were induced to produce significantly greater quantities of IFNγ when co-stimulated with high dose IL-2 ($P = 0.008$) (Figure 2B).

$CD4^+CD25^-$ cells from infected mice promote gastritis and reduce bacterial load

Since $CD25^-$ T cells from *H. pylori*-infected mice were hyporesponsive *in vitro*, we transferred these cells into immunodeficient *rag1*$^{-/-}$ mice prior to challenge with live *H. pylori* to measure their responsiveness *in vivo* in the absence of regulatory T cells. Since mice deficient in the rag1 enzyme lack mature T cells and B cells, the $CD25^-$ cells from the donor mice would be stimulated in the absence of any recipient mouse lymphocytes. We observed significant reductions in bacterial load in *rag1*$^{-/-}$ mice reconstituted with $CD25^-$ cells from naïve donors (Figure 3). These mice had the lowest average number of bacteria and displayed the most severe inflammation, significantly greater than experimentally infected WT control mice (Figure 4, $P < 0.006$). Transfer of bulk $CD4^+$ cells from *H. pylori*-infected mice into *rag1*$^{-/-}$ recipients resulted in a significant decrease in bacterial load compared to infected WT control mice (Figure 3, $P < 0.05$) and mild gastric inflammation (Figure 4). Contrary to our expectations, we also observed decreased bacterial load in *rag1*$^{-/-}$ mice receiving $CD4^+CD25^-$ cells from *H. pylori*-infected mice that was statistically equivalent to groups transferred with $CD4^+CD25^-$ cells from naïve donors. Histologic examination of the gastric mucosa demonstrated that *rag1*$^{-/-}$ mice reconstituted with $CD4^+CD25^-$ cells from *H. pylori*-infected mice responded with mild inflammation (Figure 4).

Memory $CD25^-$ cells promote mild gastritis and persistent *H. pylori* colonization

Our data demonstrate that adoptive transfer of purified $CD25^-$ cells from *H. pylori*-infected donors into immunodeficient mice promotes inflammation that is hostile to *H. pylori* upon challenge (Figures 3 and 4). This is in contrast to the hyporesponsiveness we observed *in*

vitro. We next determined whether transfer of bulk $CD25^-$ cells from infected mice, as performed above, might include a memory and naïve population of $CD25^-$ cells capable of generating distinct responses. The $CD25^-$ population from *H. pylori*-infected mice was fractionated into $CD45RB^{hi}$ (naïve) and $CD45RB^{lo}$ (memory) populations and then separately transferred into *rag1*$^{-/-}$ recipients followed by challenge with *H. pylori*. As demonstrated in Figure 5, *H. pylori* challenge of *rag1*$^{-/-}$ mice reconstituted with either bulk $CD25^-$ cells or purified $CD4^+CD25^-CD45RB^{hi}$ naïve cells from *H. pylori*-infected mice resulted in significantly decreased levels of bacterial colonization relative to infected WT controls ($P < 0.05$). In contrast, challenge of *rag1*$^{-/-}$ mice reconstituted with purified $CD4^+CD25^-CD45RB^{lo}$ memory cells from *H. pylori*-infected mice failed to reduce bacterial colonization and had bacterial loads that were significantly greater than from transfer of unfractionated $CD25^-$ cells or purified $CD4^+CD25^-CD45RB^{hi}$ naïve cells from infected mice ($P < 0.05$). The increased levels of bacteria in these mice were comparable to *H. pylori*-infected WT control mice. These data demonstrate that after *in vivo* challenge with *H. pylori*, the *H. pylori*-specific memory $CD25^-$ cells remain unresponsive despite the absence of $CD25^+$ regulatory T cells resulting in persistent colonization.

Histologic examination of the gastric mucosa in these mice revealed an inverse relationship between the bacterial load and the degree of gastritis (Figure 6). Whereas protected *rag1*$^{-/-}$ mice that had been reconstituted with $CD25^-$ bulk cells or $CD25^-$ naïve cells from infected donor mice displayed severe gastritis compared to infected WT control mice ($P < 0.003$), mice reconstituted with *H. pylori*-specific $CD25^-$ memory cells had a heavy bacterial load and only minimal inflammation. The gastric inflammation was equivalent to infected WT controls and statistically reduced relative to mice reconstituted with bulk or naïve $CD25^-$ cells ($P < 0.003$) These results are consistent with previous reports suggesting that increased gastritis correlates with decreased bacterial colonization [24,30,31]. These data suggest that after anergy is induced in *H. pylori*-specific $CD25^-$ cells, the $CD25^+$ regulatory T cells are no longer necessary to maintain persistent infection.

T_{reg} cells suppress previously activated *H. pylori*-specific $CD25^-$ cells *in vivo*

The data presented above indicate the presence of T_{reg} cells during activation of *H. pylori*-specific $CD25^-$ cells is sufficient to induce a permanent state of anergy in some *H. pylori*-specific cells. Because *H. pylori*-infected mice can be protected by therapeutic immunization [32,33] we next tested whether previously-activated, $CD25^-$ T cells are capable of promoting inflammation that reduces the bacterial load when introduced to wild type mice either before or after *H. pylori* infection, a system in which the recipient mice have a full complement of $CD25^+$ T cells. First, *rag1*$^{-/-}$ mice reconstituted with $CD25^-$ cells from naïve wild type mice were challenged with *H. pylori*. Challenge of these mice resulted in a population of $CD25^-$ T cells associated with a significant reduction in bacterial load relative to our infected WT control group (Figure 7, $P < 0.05$). These $CD25^-$ cells were then isolated from the spleens of the immunodeficient mice and transferred again into either naïve WT mice or *H. pylori*-infected WT recipients that contain a normal repertoire of $CD25^+$ regulatory T cells. The naïve recipients were challenged one week after transfer. Mice were harvested at four weeks post-transfer and gastric sections were removed to determine the level of bacterial colonization and gastric inflammation as described in Methods.

Transfer of the $CD25^-$ cells into naïve wild type mice that were challenged after adoptive transfer resulted in high levels of bacterial colonization, statistically greater than transfer into *rag1*$^{-/-}$ mice ($P < 0.05$) and equivalent to that of infected control mice (Figure 7). Similar results were observed when these $CD25^-$ cells were transferred into previously-infected wild type mice. Bacterial colonization levels remained high, not significantly distinct from infected WT controls. These data suggest that in addition to naïve $CD25^-$ cells,

previously-activated $CD25^-$ cells succumb to the regulatory control of $CD25^+$ T cells *in vivo*.

Gastric sections were isolated from these mice to assess the level of inflammation. As shown in Figure 8, infected WT control mice developed mild gastritis as we have observed previously. The control group of immunodeficient mice that had received $CD25^-$ cells associated with reduced bacterial loads resulted in significantly increased levels of inflammation relative to infected controls ($P < 0.03$). Transfer of these $CD25^-$ cells into either infected wild type or naïve wild type mice followed by challenge resulted in statistically equivalent levels of gastritis (1.4 ± 1.0, 1.3 ± 1.2 respectively). Further, both groups of wild type recipients of the $CD25^-$ cells displayed mild gastritis statistically similar to infected controls. These data are in accordance with other models of *H. pylori* immunity in which high gastritis correlates with decreased colonization [24,30,31].

Discussion

This study was designed to investigate the extent of $CD25^+$ T cell regulatory activity during *H. pylori* infection. T cells from *H. pylori*-infected mice remained hyporesponsive to *H. pylori* antigens *in vitro* even in the absence of $CD25^+$ T_{reg} cells. The ability of high dose IL-2 together with *H. pylori* antigen to activate these $CD25^-$ T cells is indicative of anergy. We confirmed these observations *in vivo* as transfer of *H. pylori*-specific $CD25^-$ memory cells fractionated from the T cell population of infected mice failed to promote gastritis or reduce the bacterial load when transferred into *rag1*$^{-/-}$ mice that were subsequently challenged with *H. pylori*. Therefore, using both *in vitro* and *in vivo* techniques, we demonstrate here that $CD25^+$ regulatory T cells induce a $CD25^-$ cell hyporesponse that helps give rise to the mild inflammation and persistent colonization observed during chronic *H. pylori* infection in mice.

Several reports have documented the involvement of $CD4^+CD25^+$ regulatory T cells in the host immune response of human subjects to *H. pylori* [17,26-28,34,35]. *Foxp3* expression, a surrogate marker for $CD25^+$ T_{reg} cells, has been documented in the gastric mucosa of infected humans [17,26] and the number of $CD25^+$ Treg cells is elevated in children compared to adults which is consistent with reduced levels of gastritis in the pediatric population [35].

In vivo studies by Raghavan *et al.* in the mouse model have shown that transfer of lymph node $CD25^-$ cells into immunodeficient nu/nu mouse recipients followed by *H. pylori* challenge results in severe gastritis and significantly decreased bacterial colonization relative to control nu/nu mice reconstituted with unfractionated lymph node cells [27]. More recently, Rad et al. depleted the $CD25^+$ cell population *in vivo* using CD25-specific antibodies to achieve significantly increased gastritis in *H. pylori* infected mice and a reduction in bacterial load [26]. The results obtained by these two laboratories are similar to other models of pathogenic microbial infections in which $CD25^-$ cells activated in the absence of regulatory T cells mount a protective immune response [14,15]. These studies, and others by Eaton et al. discussed below are significant in that they provide evidence that T_{reg} cells are part of the natural host response to *H. pylori* infection and that they contribute to the persistence of *H. pylori* at the gastric mucosa. However, while these studies have investigated the potential of $CD25^-$ T cells to respond to *H. pylori* infection in the absence of T_{reg} cells, they have not addressed the potential activity of $CD25^-$ T cells activated in the presence of T_{reg} cells.

As discussed above, Raghavan et al. transferred $CD25^-$ cells from naïve mice into immunodeficient recipients prior to challenge [27]. Rad et al. employed a different strategy

in that $CD25^+$ cells were depleted *in vivo* by application of CD25-specific antibodies [26]. This depletion however was accomplished before infection was established and therefore the *H. pylori*-specific $CD25^-$ cells were activated in the absence of T_{reg} cells [26]. Eaton *et al.* have developed a useful model for studying host immunity against *H. pylori* infection using adoptive transfer of wild type T cells into SCID mouse recipients that subsequently get challenged with *H. pylori* [36-38]. These mice develop severe gastritis and over time significantly reduce and in some cases eliminate the *H. pylori* load from the gastric mucosa [37]. When fractionated populations were transferred into SCID mice, naïve T cells ($CD45RB^{high}$) were capable of promoting severe gastritis that depleted the Helicobacter population while memory T cells ($CD45RB^{low}$) did not [36]. Similar to the study by Raghavan et al., the donor cells used by Eaton *et al.* were obtained from naïve mice. The present report is distinct from these prior studies in that the donor populations employed to investigate the potential activity of $CD25^-$ T cells were obtained from *H. pylori*-infected mice and therefore the *H. pylori*-specific memory cells were originally activated in the presence of T_{reg} cells.

In vitro analysis of $CD25^+$ T cell regulatory activity often is performed by depleting $CD25^+$ T cells from the lymphocyte population to assess the ability of the $CD25^-$ cell to respond to antigen in their absence [7]. Evidence that the $CD25^+$ T cells are required for ongoing suppression is obtained when the $CD25^-$ T cells proliferate or produce cytokine in recall assays. We recently demonstrated that *H. pylori*-specific $CD25^-$ cells from infected mice remain hyporesponsive even in the absence of $CD25^+$ T cells, an observation consistent with the presence of anergic cells [24]. We relied upon the adoptive transfer model in immunodeficient mice developed by Eaton et al to test these observations *in vivo* [36-38]. Initially, adoptive transfer of $CD25^-$ cells from *H. pylori*-infected wild type donor mice resulted in significant gastritis and a reduction in bacterial load. This observation was contrary to expectations given the anergic response of these cells noted *in vitro*. Our subsequent transfer in which $CD25^-$ memory cells were compared to the $CD25^-$ naïve cells from *H. pylori* infected mice demonstrated that the naïve $CD25^-$ cells were associated with bacterial clearance whereas memory $CD25^-$ cells from the same donor mice remained hyporesponsive upon infection. Although Eaton et al have described the role of $CD45RB^{high}$ cells in promoting inflammation capable of killing *H. pylori* [36], this is the first study to demonstrate that the *H. pylori*-specific $CD25^-$ cells induced during infection of wild type mice are in fact hyporesponsive and do not require ongoing suppression to remain down-regulated.

The present study also demonstrates that $CD25^-$ T cells activated in the absence of Treg cells and which reduce the bacterial load in the SCID adoptive transfer model become ineffective when transferred into wild type mice. Transfer into wild type recipients reintroduces dominant regulatory T cells into the response and results in mild inflammation and persistent infection. Using this population of $CD25^-$ cells, we were able to determine that T_{reg} cells are not only capable of influencing naïve $CD25^-$ cells, but are also capable of rendering previously-activated $CD25^-$ cells ineffective. These findings are in accordance with previous studies investigating the extent of $CD25^+$ regulatory control in a murine model of colitis [39]. Transfer of $CD4^+CD25^+$ regulatory T cells four weeks after established $CD4^+CD45RB^{hi}$-induced colitis resulted in resolution of disease as early as two weeks after transfer of regulatory T cells. Similarly, we demonstrate here that $CD25^+$ T_{reg} cells maintain the ability to influence previously-activated responsive $CD25^-$ cells during *H. pylori* infection.

The ability of resident T_{reg} cells to suppress previously activated donor $CD25^-$ T cells is in contrast with studies demonstrating that animals harboring an existing *Helicobacter* infection can be protected by therapeutic immunization [32,33,40]. Therapeutic

immunization studies indicate that activation of T cells under certain circumstances results in a population of T cells that is not influenced by the T_{reg} cells that are part of the host response to infection. Therefore either the nature or frequency of proinflammatory T cells produced here in the absence of T_{regs} must be different than the T cell response induced by immunization. Further analysis will be required to characterize these two types of responses.

Several recent reports have begun to elucidate the development of the host T cell response to *H. pylori* infection. This response consists, in part, of immunoregulatory $CD25^+$ T_{reg} cells that actively suppress other *H. pylori*-specific cells from promoting heightened inflammation [26,27,41]. In the mouse model, we have demonstrated that the presence of CTLA-4 on T_{regs} is necessary to suppress *H. pylori* associated protective gastritis [24]. The presentation of antigen by the gastric epithelium and the involvement of the co-receptor B7H1 may also be promoting the induction of suppressive $CD4^+CD25^+$ T_{reg} cells that have been demonstrated *in vitro* to decrease the proliferative activity of activated T cells [42]. Mechanistically, it appears that IL-10 production by T_{reg} cells may play a role in the suppressive activity of these cells [36,43,44] although IL-10 deficient T_{reg} cells were also capable of significantly reducing the proinflammatory effects of *H. pylori*-specific $CD25^-$ cells in an adoptive transfer model [41].

$CD25^+$ regulatory T cells are typically associated with active suppression. The present study however demonstrates an alternate mechanism of immune down-regulation by this cell type. The observation that *H. pylori*-specific $CD25^-$ T cells remain unresponsive even in the absence of $CD25^+$ T cells both *in vitro* and *in vivo* provides compelling evidence for the induction of anergy. These observations should enhance our understanding of *H. pylori* immunopathogenesis, and may be relevant to our understanding of the immunopathogenesis of other microbial infections and the maintenance of immunologic homeostasis in the gastrointestinal tract.

Materials and Methods

Reagents

Complete cell media consisted of RPMI 1640 supplemented with 10% fetal bovine serum (Gibco Life Sciences, Carlsbad, CA). MACS $CD4^+$ cell purification reagents and columns were purchased from Miltenyi Biotech (Auburn, CA) and used according to manufacturer's instructions. Low-tox M rabbit complement and Lymphocyte-M were purchased from Cedarlane Laboratories (Hornby, Ontario). Anti-CD28 antibody and IFNγ ELISA reagents were purchased from ebioscience (San Diego, Ca) and IL-2 ELISA reagents purchased from R & D (Minneapolis, MN) were used according to manufacturer's instructions. Anti-CD25, anti-CD45RB, and PE-conjugated anti-CD25 and anti-FCγRIII antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). *H. pylori* lysates were prepared by probe sonication of *H. pylori* suspensions in PBS. Sonicate was sterile filtered using 0.2 μm acrodisc filters (Pall Corporation, Ann Arbor, Mi). The *Foxp3* assay on demand (Mm00475156_m1) was purchased from ABI (Foster City, Ca)

Mice

Six to ten week old C57BL/6 female mice and lymphocyte-deficient *rag*$1^{-/-}$ male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen free conditions in microisolator units. All studies involving the use of mice were reviewed and approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Bacteria

H. pylori Sydney Strain 1 (SS1) [45] was grown on Columbia agar (Difco, Detroit, MI) supplemented with horse blood and antibiotics at 37°C for 96 h under microaerobic conditions (5% O_2, 10% CO_2). For inoculation of mice, bacteria were transferred to 10 ml Brucella broth (Difco Laboratories, Detroit) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and amphotericin B (2.5 μg/ml). Liquid cultures were established in T25 flasks and maintained at 37°C with 5% CO_2.

Infection/Immunization

Infections were performed with *H. pylori* SS1 by gavage using flexible tubing on the end of an 18 G needle. Each mouse received 500 μl of an actively growing bacterial culture of at least 0.3 OD_{450nm} on two consecutive days. Immunization was performed by intranasal administration of 100 μg *H. pylori* lysate plus 5 μg cholera toxin adjuvant in 20μl PBS on day 0, 7, 14, and 28 as previously described [25].

Complement-mediated lysis of $CD25^+$ cells

Single cell suspensions were prepared from spleens and red blood cells were removed by lysis in a hypotonic solution. Cells were resuspended in 2% FBS/PBS and incubated for 30 minutes at 4°C with anti-mouse $CD25^+$ antibody at 0.65 μg per 1×10^7 cells. Cells were then washed and resuspended in PBS with Low-tox M rabbit complement (20:1 v/v) and incubated at 37°C for 45 minutes. Viable lymphocytes were concentrated using a Lymphocyte-M gradient followed by several washes in PBS. Deletion of $CD25^+$ cells was confirmed by staining with PE-conjugated anti-CD25 antibody and assessment by flow cytometry.

Foxp3 Quantitative PCR

The ABI *Foxp3* assay was set up in accordance with the manufacturer's instructions and run against all the samples in the study using GAPDH as an endogenous control on a 384-well plate. RNA was accurately quantified using a nanodrop-1000 spectrophotometer (Nanodrop Industries). Archive cDNA was made for all samples by means of an RT reaction using ABI high-Capacity cDNA archive kit and using similar amounts of total RNA as starting material in a 100ul reaction in an ABI 9700 PCR unit. Results were generated using ABI SDS software and are presented as relative fold changes versus a designated calibrator sample. Results include 95% confidence limits.

Isolation of $CD25^+$ and $CD25^-$ cell populations by flow cytometry

$CD4^+$ splenocytes were positively selected according to manufacturer's instructions using the MACS $CD4^+$ cell purification reagents and medi-MACS columns on a magnetic support. Purified $CD4^+$ cells were incubated with FCγRIII for 15 minutes followed by incubation with PE-conjugated anti-CD25 antibody. Sorting of $CD25^+$ and $CD25^-$ cells was performed using a BD FACSAria (Franklin Lakes, NJ) at the Flow Cytometry Core Facility of the Comprehensive Cancer Center of Case Western Reserve University.

In vitro recall assay

Bulk spleen cells or $CD25^-$ spleen cells from immunized or infected mice were prepared as described above and plated in 96 well plates in 200 μl complete media and stimulated with 10 μg/ml *H. pylori* lysate. Supernatants were removed at 36 hours to determine the amount of IFNγ secretion. Designated groups were also treated with high dose (1000U/ml) IL-2 for one hour prior to stimulation with antigen. Similar assays were also performed with $CD4^+$ spleen cells prepared by positive selection using the MACS $CD4^+$ cell purification reagents and columns purchased from Miltenyi Biotech (Auburn, CA), and in some cases with $CD4^+$

cells further fractionated into $CD25^+$ and $CD25^-$ population by affinity isolation using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotech). Stimulation of $CD4^+$ cells fractionated by CD25 status was accomplished with bone marrow derived macrophages from C57BL/6 mice. Briefly, bone marrow cells isolated from hind leg femurs and tibias were grown in DMEM supplemented with 10% FBS and supplemented with 20% GM-CSF-conditioned media from Ladmac cell culture. Each well was seeded with 1×10^4 macrophages and pulsed with 10 μg/ml *H. pylori* lysate antigen for six hours. The macrophages were washed three times and then the fractionated cells were added to the wells for stimulation as described above.

Evaluation of inflammation and CFU determination

For all adoptive transfer studies, recipient mice were sacrificed 28 days post-infection by CO_2 asphyxiation and gastric biopsies from the greater curvature of the stomach was collected to assess degree of gastritis and bacterial load. To determine gastric inflammation, a biopsy strip was fixed in 10% buffered formalin. H&E staining was performed at the Willard Alan Bernaum Cystic Fibrosis Research Center core facility at the CWRU School of Medicine (Cleveland, OH). As previously described [46,47] the area of the tissue section displaying the most severe inflammation was evaluated blindly and assigned a global score from 0 – 5 based upon the following parameters: 0, no significant lesions; 1, mild infiltrate of inflammatory cells, typically along the base of the glands; 2, larger focus of inflammation extending between glands and/or in submucosa; 3, patch(es) of inflammation extending between glands toward the lumen and in the underlying submucosa. Moderate mucous cell metaplasia and mild to moderate epithelial hyperplasia may be present. 4, intense transmucosal inflammatory infiltrate extending across the field, distorting glandular architecture, marked epithelial hyperplasia and extensive mucous cell metaplasia often present; 5, extensive mucosal and submucosal inflammation with disruption of glandular architecture and ulceration. To determine bacterial colonization, a biopsy strip was placed in a pre-weighed tube of 200 μl Columbia broth, the wet weight was determined and the tissue was homogenized using a disposable pellet pestle (Kontas Glass Company, Vineland, NJ). Serial dilutions were prepared and 10 μl aliquots were plated for growth as described above. Bacterial load was determined as CFU/gram of stomach tissue.

Adoptive transfer studies

Transfer of CD25- cells to *rag*$1^{-/-}$ mice (Figures 3 and 4)—Bulk $CD4^+$ or $CD4^+CD25^-$ cells were resuspended in PBS and 2×10^6 cells were injected i.p. into each *rag*$1^{-/-}$ mouse on Day 0. Mice were challenged with *H. pylori* SS1 on days 1 and 2.

Transfer of naïve or memory $CD25^-$ cells into *rag*$1^{-/-}$ mice (Figures 5 and 6)—$CD4^+CD25^-$ bulk, $CD4^+CD25^-CD45RB^{hi}$ (brightest 20%) or $CD4^+CD25^-CD45RB^{lo}$ (dullest 10%) cells were resuspended in PBS and 1.5×10^5 cells were injected i.p. into each *rag*$1^{-/-}$ mouse on Day 0. On Days 1 and 2, mice were infected with *H. pylori* SS1.

Transfer of $CD25^-$ cells from reconstituted *rag*$1^{-/-}$ mice into wild type mice (Figures 7 and 8)—On Day 0, 4.5×10^6 $CD25^-$ spleen cells were injected i.p. into *rag*$1^{-/-}$ recipient mice. Mice were challenged with *H. pylori* SS1 on Days 1 and 2. On Day 29, mice were sacrificed and splenocytes were removed and prepared for a second round of adoptive transfer by i.p. injection of 1×10^7 bulk splenocytes (16% $CD4^+$ measured by flow staining) into naïve or infected wild type mice. Naïve recipients were then challenged on Days 35 and 36.

Statistics

Differences between experimental groups in each experiment was evaluated by Student's T test. Differences were considered statistically significant if *P* values were less than 0.05.

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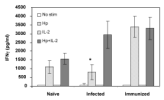

Figure 1. *H. pylori*-specific hyporesponsiveness of CD4$^+$ cells from infected mice is reversed in the presence of high dose IL-2

CD4$^+$ cells were isolated from the spleens of naïve, infected, or immunized mice and stimulated *in vitro* for 24 hours with 10 μg/ml *H. pylori* lysate (Hp), 1000 U/ml rhIL-2, or both. T cell activation was determined by production of IFNγ as quantified by ELISA. * $P <$ 0.01 compared to immunized mice similarly treated. Each value shown is the mean of each group ± the standard deviation.

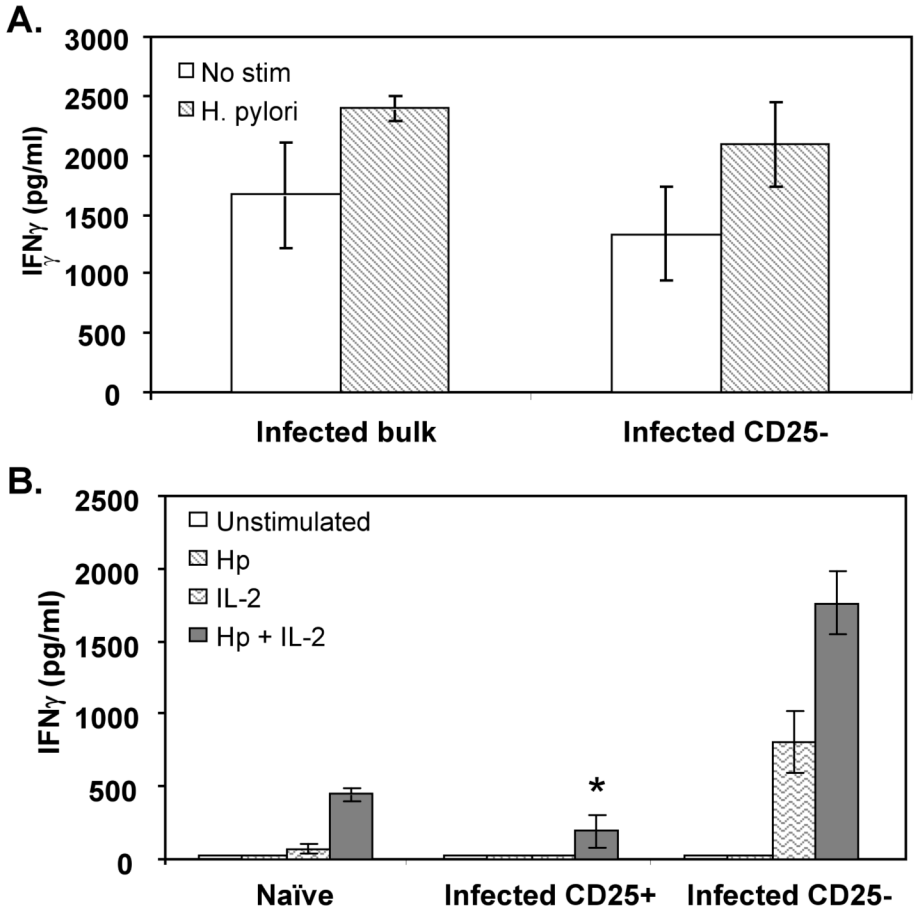

Figure 2. *H. pylori*-specific CD25$^-$ T cells remain hyporesponsive in the absence of CD25$^+$ cells but are activated by the addition of exogenous high dose IL-2

(A) Splenocytes isolated from *H. pylori*-infected mice were depleted of CD25$^+$ cells by complement-mediated killing and stimulated with 10 μg/ml *H. pylori* lysate antigen. (B) CD4$^+$ cells from infected mice fractionated into CD25$^-$ or CD25$^+$ T cell populations by flow cytometry were stimulated with antigen pulsed macrophages in the presence or absence of high dose (1000U/ml) rhIL-2. IFNγ was measured as a marker of T cell activation. CD4$^+$ cells from naïve mice were unfractionated. *$P < 0.01$ compared to CD25$^-$ cells stimulated in a comparable manner. Each value shown is the mean of each group ± the standard deviation.

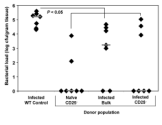

Figure 3. Adoptive transfer of bulk CD4$^+$ or CD4$^+$CD25$^-$ cells from *H. pylori*-infected donors results in decreased bacterial colonization

Bulk CD4$^+$ or purified CD4$^+$CD25$^-$ spenocytes isolated from naïve or *H. pylori*-infected mice were adoptively transferred into immunodeficient recipient mice on day zero. All mice were then challenged with live *H. pylori* on days one and two. Mice were sacrificed on day 28 and the level of bacterial colonization was compared to infected WT control mice. Grey bars represent the median for each group. N = seven mice per group. * $P < 0.05$ for all three transfer groups compared to infected WT control.

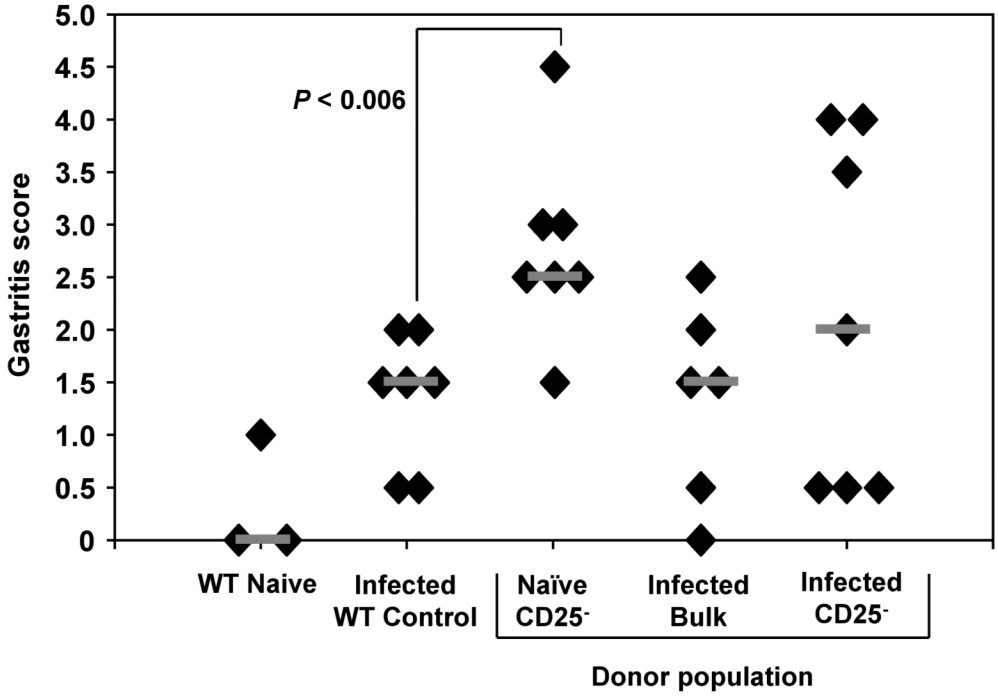

Figure 4. Adoptive transfer of bulk CD4$^+$ or CD4$^+$CD25$^-$ cells from *H. pylori*-infected donors results in mild gastric inflammation

Bulk CD4$^+$ or purified CD4$^+$CD25$^-$ splenocytes isolated from naïve or *H. pylori*-infected mice were adoptively transferred into immunodeficient recipient mice on day zero. All mice were then challenged with live *H. pylori* on days one and two. Mice were sacrificed on day 28 and the level of gastric inflammation was compared to infected WT control mice. N = seven mice per group.

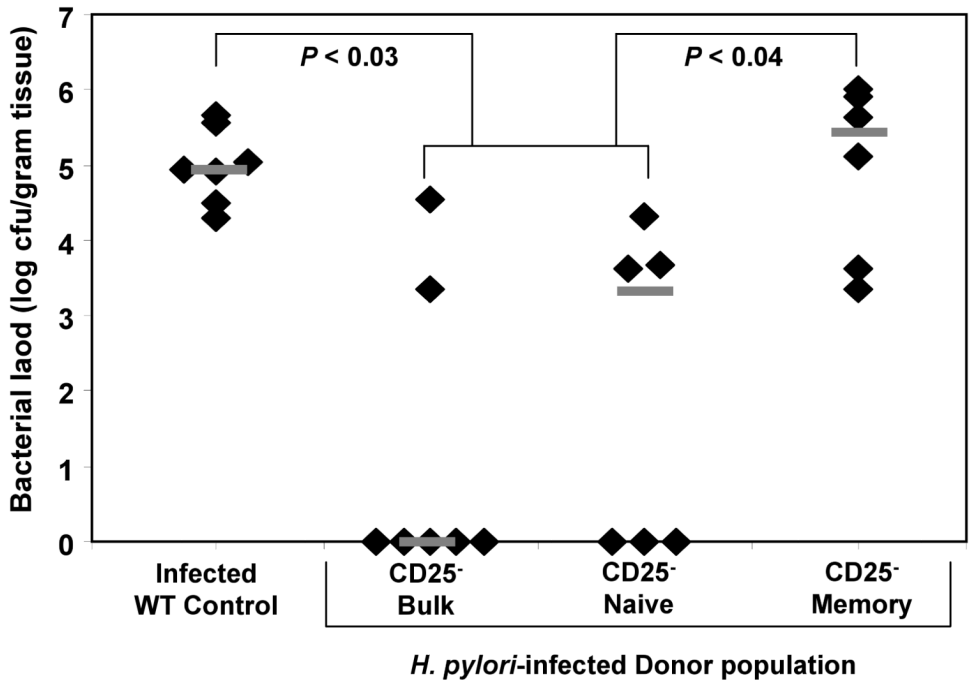

Figure 5. Memory $CD4^{+}CD25^{-}$ cells from *H. pylori*-infected mice remain hyporesponsive after *in vivo* challenge

Bulk CD25- cells, $CD25^{-}$ $CD45RB^{hi}$ (naïve) cells or $CD25^{-}$ $CD45RB^{lo}$ (memory) cells were prepared from the spleens of *H. pylori* infected mice and transferred into immunodeficient recipient mice on day zero. Mice were challenged with live *H. pylori* on days one and two and sacrificed 28 days post-challenge. Grey bars represent the median for each group. N = six mice per group.

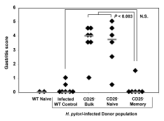

Figure 6. Memory CD4^{+}CD25^{-} cells induce mild gastritis in response to *H. pylori* infection

Bulk CD25- cells, CD25^{-} CD45RB^{hi} (naïve) cells or CD25^{-} CD45RB^{lo} (memory) cells were prepared from the spleens of *H. pylori*-infected mice and transferred into immunodeficient recipient mice on day zero. Mice were challenged with live *H. pylori* on days one and two and sacrificed 28 days post-challenge. Gastric sections were removed and the degree of inflammation based on amount and degree of cellular infiltrate and changes in tissue architecture was measured. Transfer of splenocytes from infected WT mice served as a control. N = six-seven mice per group.

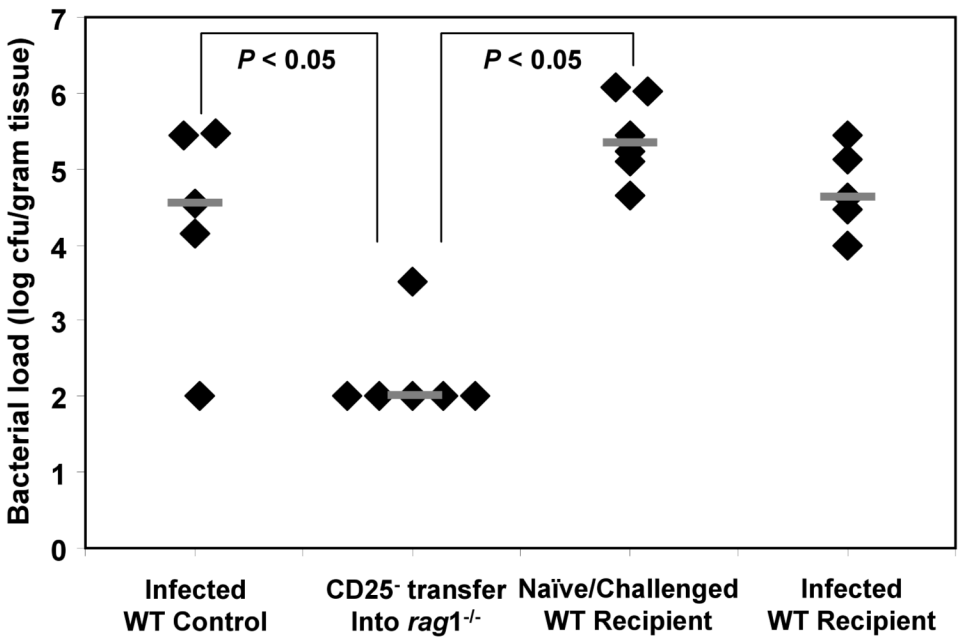

Figure 7. Previously-activated CD25$^-$ cells are not capable of reducing bacterial load in WT recipients

CD25$^-$ splenocytes isolated from naïve WT donor mice were transferred into *rag1*$^{-/-}$recipient mice on day zero followed by challenge with live *H. pylori* on days one and two. Bulk splenocytes isolated from these recipient mice were isolated on day 29 and transferred into either naïve or *H. pylori*-infected WT mice. Naïve mice were challenged with live *H. pylori* on days 35 and 36. Mice were sacrificed on day 64 and the level of bacterial colonization was compared to infected WT control mice. Grey bars represent the median for each group. N = five-seven mice per group.

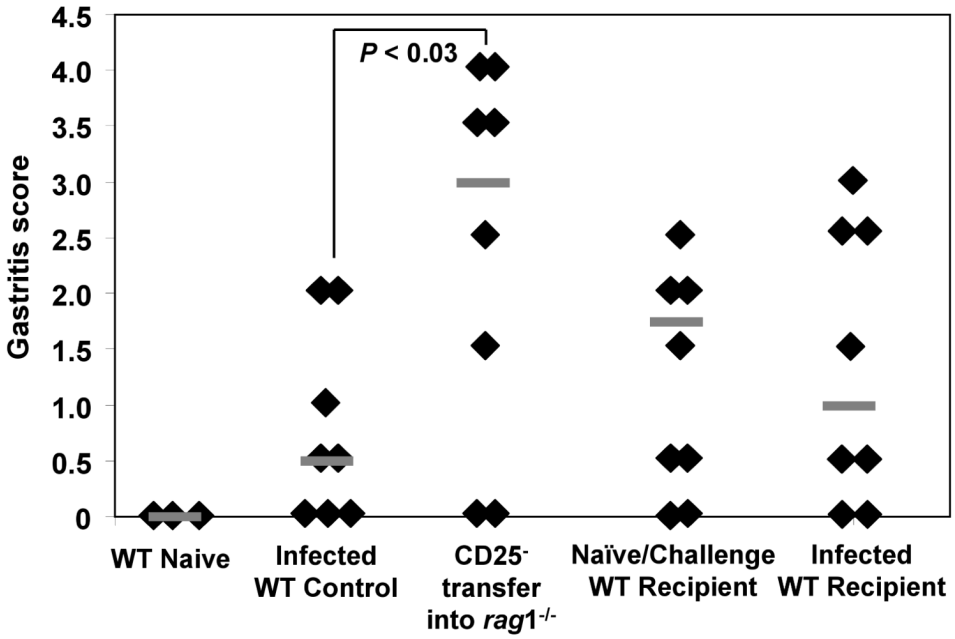

Figure 8. Transfer of previously-activated CD25$^-$ cells into WT recipients results in mild gastric inflammation

CD25$^-$ splenocytes isolated from naïve WT donor mice were transferred into *rag1*$^{-/-}$ recipients on day zero followed by challenge with live *H. pylori* on days one and two. Bulk splenocytes isolated from these recipients were isolated on day 29 and transferred into either naïve or *H. pylori*-infected WT mice. Naïve mice were challenged with live *H. pylori* on days 35 and 36. Mice were sacrificed on day 64 and gastric inflammation was measured as described in Methods and compared to infected WT control mice. N = eight mice per group.