Vol.2, No.1, 25-39 (2012)
http://dx.doi.org/10.4236/oji.2012.21004

Open Journal of Immunology

Innate-like CD4 T cells selected by thymocytes suppress adaptive immune responses against bacterial infections

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Received 21 September 2011; revised 16 December 2011; accepted 30 December 2011

ABSTRACT

We have reported a new innate-like CD4 T cell population that expresses cell surface makers of effector/memory cells and produce Th1 and Th2 cytokines immediately upon activation. Unlike conventional CD4 T cells that are selected by thymic epithelial cells, these CD4 T cells, named T-CD4 T cells, are selected by MHC class II expressing thymocytes. Previously, we showed that the presence of T-CD4 T cells protected mice from airway inflammation suggesting an immune regulatory role of T-CD4 T cells. To further understand the function of T-CD4 T cells, we investigated immune responses mediated by T-CD4 T cells during bacterial infection because the generation of antigen specific CD4 T cells contributes to clearance of infection and for the development of immune memory. The current study shows a suppressive effect of T-CD4 T cells on both CD8 and CD4 T cell-mediated immune responses during Listeria and Helicobacter infections. In the mouse model of *Listeria monocytogenes* infection, T-CD4 T cells resulted in decreasedfrequency of *Listeria*-specific CD8 T cells and the killing activity of them. Furthermore, mice with T-CD4 T cells developed poor immune memory, demonstrated by reduced expansion of antigen-specific T cells and high bacterial burden upon re-infection. Similarly, the presence of T-CD4 T cells suppressed the generation of antigen-specific CD4 T cells in *Helicobacter pylori* infected mice. Thus, our studies reveal a novel function of T-CD4 T cells in suppressing anti-bacterial immunity.

Keywords: Bacterial Infection; Innate-Like CD4 T Cells; Immune Suppression

1. INTRODUCTION

It is well established that conventional murine CD4 T cells are selected on thymic epithelial cells (TEC) that express MHC class II [1]. In humans, studies have shown that CD4 T cells can be generated by a pathway that is independent of TEC-expressed MHC class II [2-4]. The alternative cell types supporting CD4 T cell development seem to be hematopoietic cells, particularly thymocytes [5-7]. Using mouse models, we and others have demonstrated that indeed MHC class II-expressing thymocytes successfully mediate CD4 T cell selection independent of TEC-expressed MHC class II [8,9]. CD4 T cells selected by MHC class II on thymocytes are called thymocyte-selected CD4 (T-CD4) T cells, as distinguished from conventional epithelial cell-selected CD4 (E-CD4) T cells.

E-CD4 T cells are well studied and known to modulate adaptive immunity by differentiating into helper cell subsets and producing cytokines according to environmental signals. Th1 cells produce the pro-inflammatory Th1 cytokine IFN-γ and facilitate CD8 T cell-mediated cellular immunity against intracellular pathogens [10], whereas Th2 cells produce cytokines IL-4, IL-5 and IL-13 that are critical for B cell differentiation and antibody-mediated humoral immunity [10,11]. More recently, IL-17-producing Th17 cells have been discovered and studied in various contexts [12-14]. In addition to regulating on-going immune responses, E-CD4 T cells are required for the memory development of CD8 T cell and B cell immunity in various infection models [15-20].

Unlike E-CD4 T cells, T-CD4 T cells rapidly produce Th1 and Th2 cytokines upon TCR stimulation *in vitro* and *in vivo* [21], resembling invariant natural killer T (iNKT) cells in their innate-like functional characteristics [22-25]. Moreover, T-CD4 T cells maintain Th2 cytokine production under Th1-skewing conditions [21] but are poor IL-17 producers under Th17-skewing conditions *in vitro* [26]. Therefore, T-CD4 T cells seem to be potent

effector cells. Unexpectedly, however, mice possessing T-CD4 T cells are protected from allergen-induced airway inflammation [21] and development of experimental autoimmune encephalomyelitis (EAE) was reduced in the presence of T-CD4 T cells [27], which indicated that T- and E-CD4 T cells function differently in physiological contexts. However, the role of T-CD4 T cells in immune responses against bacterial infections has not been addressed.

Hosts protect themselves from infection by mounting appropriate innate and adaptive immune responses tailored toward pathogens. Intracellular pathogens such as *Listeria monocytogenes* are taken up primarily by phagocytes and can be destroyed in the phagosomes of antigen presenting cells (APC) upon infection. However, they can escape into the cytosol through listeriolysin O (LLO)-dependent mechanisms [28], and thence are processed and presented through the MHC class I pathway, inducing robust cellular immunity [29,30]. *Listeria*-specific conventional CD4 and CD8 T cells exhibit similar responding kinetics of activation, expansion and contraction [31], robustly producing the Th1 cytokine IFN-γ, which is critical for the anti-microbial activity of macrophages [32] and the up-regulation of MHC expression on APC [33]. In addition, CD8 T cells directly lyse infected cells as an important mechanism of bacterial clearance [31,34,35]. Immunological memory protects host organisms by clearing recurrent infections with enhanced rapidity and effectiveness. Studies have reported the essential role of CD4 T cells in the establishment and development of memory immunity against *L. monocytogenes* infection [16,17,31]. In the absence of CD4 T cells, although mice are able to mount a primary immune response to eliminate the bacteria with a similar efficiency to CD4-sufficient hosts, they suffer from a defective memory immune response upon re-infection and succumb from high bacterial loads [18,19,36-38].

Helicobacter pylori infection causes gastritis due to IFN-γ production by *Helicobacter*-specific Th1 cells. In *H. pylori*-infected mice, CD4 T cells are necessary and sufficient for induction of gastritis [39]. In both mice [40] and humans [41], IFN-γ is elevated in association with gastritis due to Helicobacter, and antigen-specific Th1 cells are present in inflamed mucosa [41]. IFN-γ-deficient mice [42] and T-bet knockout mice [43] fail to develop gastritis in response to *H. pylori.* Thus, the evidence strongly supports the hypothesis that in both mice and humans gastritis due to *H. pylori* is a Th1- and IFN-γ-dependent disease. In spite of this evidence, several published studies have suggested that gastritis due to *H. pylori* gastritis is not absolutely dependent on the Th1 response. We and others showed that while knockout mice deficient in either IFN-γ or T-bet fail to develop gastritis in response to *H. pylori* [42,43], immunodeficient recipients of IFN-γ or T-bet knockout CD4 T cells do develop gastritis, albeit of less severity than recipients of CD4 T cells from C57BL/6 mice [39,43]. Thus, in some situations, *H. pylori* gastritis can be Th1-independent.

In the current study, we show that T-CD4 T cells play an immunosuppressive role during infection by two different bacterial pathogens, implicating a possible regulatory function for these cells during microbial challenge.

2. MATERIALS AND METHODS

2.1. Mice

CIITA^{Tg} (Tg) and WT littermates were bred and kept under specificpathogen-free conditions in the animal facility at the University of Michigan Medical School. C57BL/6 mice (males and females) at 7 - 8 wk of age were purchased from Jackson or NCI. CD45.1^{+} B6 mice (B6.SJL-*Ptprc*^{a}/BoyAiTac) and CD45.1^{+} A$\beta^{-/-}$ mice (B6.SJL-*Ptprc*^{a}/BoyAiTac *H*2-*Ab*1^{tm1Gru}) (7 - 8 wk of age) were purchased from Taconic. Helicobacter-freespecific-pathogen-free female C57BL/6J-*Prkdc*^{scid} (severe, combined, immunodeficient, SCID) mice were obtained from Jackson laboratories. All mice used were 6 - 12 wk of age. 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*. All experimental procedures andprotocols were approved by the University Committee on Use and Care of Animals.

2.2. *L. monocytogenes* Infection

The recombinant strain of *L. monocytogenes* expressing a secreted form of ovalbumin (rLM-OVA) waspreviously described [44] rLM-OVAwas grown in brain heart infusion broth (Difco) to mid-exponential phase followed by washing with PBS prior to injection *i.v.* into mice. 5×10^5 rLM-OVA are equivalent to 0.5 LD_{50} for infection.

2.3. T- and E-CD4 T Cell Generation

T- and E-CD4 T cells were generated by transferring BM cells from CIITA^{Tg} (Tg) or WT to lethally irradiated A$\beta^{-/-}$ (MHC class II-deficient) or WT hosts, respectively. Eight weeks after the transfer, the hosts were sacrificed and CD4 T cells were enriched from total splenocytes using MACS anti-mouse CD4 microbeads (Miltenyi Biotec).

2.4. Adoptive Transfer Model

For Listeria infection using the adoptive transfer model, the recipients were sub-lethally irradiated (500 rad). Three

days later, each of them received ~3 × 10^7 CD4 T cell-depleted splenocytes from WT mice mixed with 10^7 E-CD4 or T-CD4 T cells obtained from BM chimeric mice using anti-mouse CD4 microbeads (Miltenyi Biotec). The mice were rested overnight and then inoculated with rLM-OVA as indicated in each experiment via the *i.v.* route. For Helicobacter infections, groups of both uninfected and *H. pylori*-infected SCID mice were received 100 µl of CD4 enriched cells from WT or Tg mice via intraperitoneal injection, for a final dose of ~1 × 10^6 CD4 T cells per mouse.

2.5. Stimulation of rLM-OVA-Specific T Cell Population *ex Vivo* and Cytokine Intracellular Staining

Splenocytes from infected and naïve mice were stimulated with SIINFEKL peptide (1ug/ml, Biomatik Corporation) to detect rLM-OVA-specific CD8 T cells or with LLO (listeriolysin O) 190-201 to detect rLM-OVA-specific CD4 T cells. Splenocytes were incubated with 1 µg/ml peptide for five hours, and monensin was added before the last three hours. After five hours, cells were washed and stained with anti-CD4 and anti-CD8 antibody. Next, cells were fixed in 2% paraformaldehyde for 30 min at room temperature, permeabilized with 0.2% saponin (Sigma), and stained with anti-IFN-g (XMG1.2) for flow cytometry.

2.6. *In Vivo* Killing Assay

Splenocytes from naive mice, depleted of red blood cells, were split into two portions. One was labeled with a high concentration of CFSE (5.0 nM, 2 × 10^7 cells/ml) and pulsed with OVA 257 - 263 peptides as the target population. The control was labeled with a low concentration of CFSE (0.5 nM, 2 × 10^7 cells/ml) without peptides. Cells were washed and then the two populations were mixed at 1:1 ratio (4 - 5 × 10^6 cells each). Cells were injected into rLM-OVA infected or PBS-treated mice. Mice were euthanized at indicated time points, and single-cell suspensions of spleenswere analyzed by flow cytometry.The killing efficiency was calculated as follows: 100 – ([(% peptide pulsed in infected/% unpulsed in infected)/(%peptide pulsed in uninfected/% unpulsed in uninfected)] × 100).

2.7. Quantification of Listeria Load

Livers were removed and put into 14-ml tubes containing 10 ml PBS with 0.2% NP40. The tissues were homogenized by using a homogenizer (The Lab Depot, Inc.) at maximum speed for 30 seconds. Tissue homogenates were subjected to 10-fold serial dilutions and then plated onto Luria broth agar plates. The number of colonies formed were counted after 24 hour of incubation at 37˚C.

2.8. *H. pyloriinfection*

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 hemacytometer, and diluted to a final concentration of 1 × 10^8 bacteria/ml. SCID mice were given 100 µl of sterile 0.5 M Na_2CO_3 via gastric feeding tube followed by100 ml of the bacterial suspension giving a total dose of 1 × 10^7 of *H. pylori* SS1 per mouse. This procedure was repeated on the following day, for a total of two inoculations.

2.9. Cytokine Analyses of Helicobacter Infected Samples

T cell depleted splenocytes from uninfected C57Bl/6J mice were irradiated with 3000 rads and used as APC. Splenic CD4 T cells from adoptively transferred mice were enriched, 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 (50 mg/ml) for 72 hrs. The other set of cells were stimulated with 5 mg/ml plate-bound anti-CD3e (145-2C11), 1 mg/ml anti-CD28 (37.51), and 50 U of IL-2 (Roche, Indianapolis, IN) at a concentration of 1 × 10^6 CD4 T cells/ml for 72 hrs. The supernatants from both stimulation conditions were collected, and cytokine production was measured by ELISA.

2.10. Histology

1 mm wide strips from the greater curvature were emersion-fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 5 µm sections, and stained with hematoxylin and eosin (HE). Extent of gastritis was scored as previously described. Briefly, adjacent 200× 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.

2.11. Flow Cytometry

Antibodies specific for CD4 (GK1.5), CD8 (53-6.7), CD45.1 (A20), CD45.2 (104), TCRβ (H57-597), NK1.1 (PK136), Ly-6G (Gr-1), CD11c (HL3), CD11b (M1/70), F4/80 (6F2), I-A^b (AF6-120.1), H-2K^b (AF6-88.5) were from PharMingen, BD Bioscience (Mountain View, CA).

The antibody against Foxp3 (FJK-16s; eBioscience) was used according to the staining protocol provided by the company. Samples were analyzed using a FACS Canto flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

2.12. RNA Analyses

Total RNA of the splenocytes from infected with Listeria or Helicobacter and control mice were extracted using TRIzol (Invitrogen). The PCR reactions were performed and analyzed using the iCycleriQ™ (BioRad). Conditions: 95˚C for 5min, followed by 40 cycles of 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 40 sec. Primers: GAPDH Forward (F): ctccactcacggcaaattca, Reverse (R): cgctcctggaagatggtgat. IL-1 F: caaccaacaagtgatattctccatg, R: gatccacactctccagctgca. IL-10 F: ggttgccaagcctatcgga, R: acctgctccactgccttgct. IFN-g F: tcaagtggcatagatgtggaagaa, R: tggctctgcaggattttcatg. TNF-α F: ccccaaagggatgagaagtt, R: cacttggtggtttgctacga. To measure cytokine expression from Helicobacter infected samples, mice were euthanized and stomachs were harvested eight weeks after adoptive transfer. mRNA was isolated as above, and cDNA was synthesized and analyzed on custom SuperArrays (CAPM-0752A) using SA Biotech's SYBR green master mix. The arrays contained probes for IL-1β, IL-6, TNFα, and GAPDH. IL-4 mRNA expression levels were determined separately by performing qPCR with IL-4 specific primers of our design (F: aacgtcctcacagcaacgaa, R: tgcagctccatgagaacact).

2.13. Statistical Analysis

The statistical analysis was done using Prism software. A two-tailed *t*-test was used for statistical analysis. *p* values of ≤ 0.05 were considered significant, and *p* values > 0.05 were not indicated and were considered statistically insignificant.

3. RESULTS

3.1. CD8 T Cell-Mediated Immune Response Was Decreased in Mice with T-CD4 T Cells

To ascertain the role of T-CD4 T cells during bacterial infection, we compared the immune responses against *L. monocytogenes* infection between two groups of mice: CIITA^{Tg} (Tg) and wild type (WT) mice. Tg mice express MHC class II on both thymocytes and TEC due to the expression of the CIITA transgene directed by the CD4 promoter [9]. Therefore, thymocytes can be selected by thymocytes and TEC generating a mixture of T- and E-CD4 T cells. An indirect measurement suggests that approximately 10% - 20% of peripheral CD4 T cells are T-CD4 T cells [45]. By contrast, WT mice possess E- but not T-CD4 T cells because they express MHC class II only on TEC in the thymus. Therefore, the differences in the immune responses between Tg and WT mice are likely attributable to the presence of T-CD4 T cells in addition to E-CD4 T cells in Tg mice.

To facilitate the detection of *Listeria*-specific Tcell populations, we used a recombinant strainof *L. monocytogenes*expressing a secreted form of chicken ovalbumin (rLM-OVA). The anti-OVA response was used as an indicator of anti-*Listerial* responses of CD8 T cells [44]. Tg and WT mice were inoculated intravenously with rLM-OVA or PBS as a control. Tissues were collected and analyze on day 7 after infection. Infected WT and Tg mice had bacterial counts that were below the limit of detection at the time of analysis suggesting efficient clearance of bacteria in both groups of mice. As shown in **Figure 1(a)**, total as well as CD4 and CD8 T cell numbers were comparable between Tg and WT groups. To measure the *Listeria*-specific T cell response, CD4 and CD8 splenocytes were stimulated separately *ex vivo* with rLM-OVA-specific peptides and T cells recognizing the cognate peptides were detected by intracellular IFN-γ staining [18]. Significantly fewer CD8 T cells from Tg mice expressed IFN-γ than in WT mice upon peptide stimulation (**Figure 1(b)**), whereas IFN-γ^+ CD4 T cells in response to rLM-OVA were slightly increased in Tg compared to WT mice (**Figure 1(c)**). Therefore, the presence of T-CD4 T cells partially suppressed immune responses of CD8 T cells during bacterial infection.

Induction of cytotoxicity is another important indicator of CD8 T cell function, in addition to IFN-γ expression [33-35]. Therefore, an *in vivo* killing assay was employed to measure rLM-OVA-specific CD8 T cell killing efficiency on day 7 [17,46]. As expected, in naïve mice, the ratio of CFSE^{hi} to CFSE^{lo} populations remained 1:1 (**Figure 1(d)**). However, the CFSE^{hi} population was decreased in both groups of infected mice indicating that it was recognized and eliminated by rLM-OVA-specific CD8 T cells (**Figure 1(d)**), and the difference in the killing efficiency between the two infected groups was not significant. Therefore, the lower number of IFN-γ CD8 T cells stimulated by the infection, compared to the wild type controls, did not compromise killing activity in Tg mice with T-CD4 T cells.

3.2. Memory Immune Responses against *L. monocytogenes* Are Impaired in the Presence of T-CD4 T Cells

It has been demonstrated that E-CD4 T cells play a critical role in the establishment of optimal immune memory of CD8 T cells against *L. monocytogenes* [18,19, 36,37]. Therefore, we asked whether T-CD4 T cells perform the same function as E-CD4 T cells do in the course of CD8 T cell memory generation. Tg and WT mice were

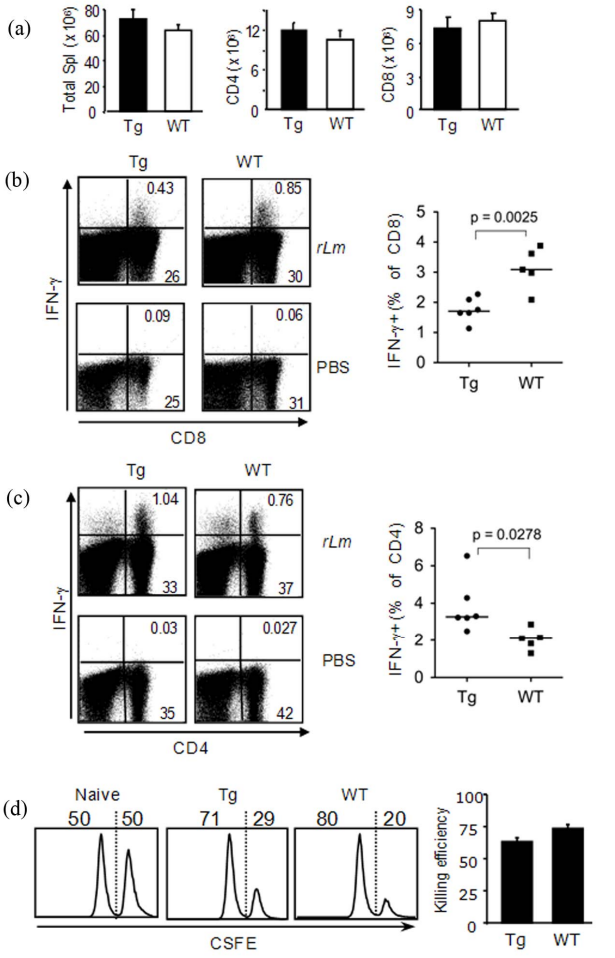


Figure 1. Reduced anti-*listerial* responses in Tg mice during primary infection. Tg and WT littermates were infected intravenously with rLM-OVA (5×10^4) or PBS. Mice were euthanized and analyzed 7 days after infection. (a) Numbers of the total splenocytes and of the indicated cell populations are shown; (b) and (c) Frequencies of IFN-γ-producing rLM-OVA-specific CD8 (b) and CD4 (c) T cells. The values in representative FACS profiles are percentages of the total splenocyte population; the graphs on right show the percentage of antigen specific CD8 (IFN-γ + CD8/total CD8) (b) and CD4 (IFN-γ + CD4/total CD4) (c) T cells. The bars indicate the median value; (d) *In vivo* killing assay. A mixture of OVA peptide-loaded target cells ($CFSE^{hi}$) and control cells ($CFSE^{lo}$) were injected into recipient mice. Mice were euthanized 3 hours later and the composition of the injected cells in the spleenswere analyzed by flow cytometry. The numbers above histograms indicate the percentages of $CFSE^{hi}$ and $CFSE^{lo}$.

infected with a low dose of rLM-OVA, rested for a month, and challenged with a high dose of rLM-OVA. Three days after the challenge, the host memory response was analyzed using the same parameters as above. The numbers of total splenocytes and CD4 T cells were equivalent in both mouse groups and CD8 T cells were slightly reduced in Tg mice although there was no statistical significance (**Figure 2(a)**). When antigen specific responses of CD8 T cells were examined, rLM-OVA-specific CD8 T cells in Tg mice were decreased 2-fold compared to WT mice (**Figure 2(b)**). Therefore, Tg mice consistently exhibited decreased CD8 T cell response against rLM-OVA during primary infection and secondary challenge. However, unlike CD4 T cell responses during the 7 days of infection, the CD4 T cell compartment during challenge showed a similar response to rLM-OVA between Tg and WT mice rLM-OVA between Tg and WT mice (**Figure 2(c)**). To compare the efficiency of bacterial clearance, *L. monocytogenes* burdenin

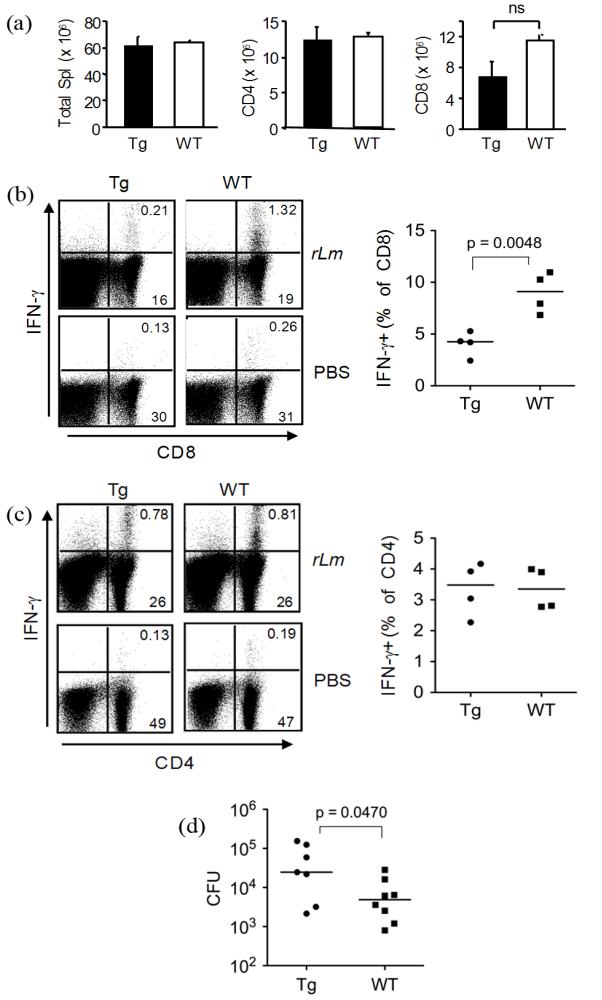


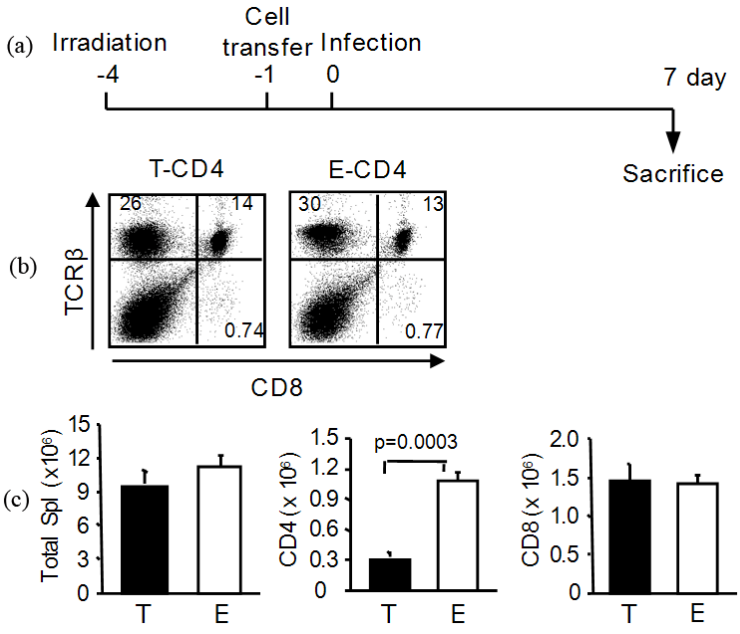
Figure 2. Poor memory response against listeria in Tg mice. Tg and WT littermates were inoculated intravenously with 4×10^3 rLM-OVA and rested for one month before challenge with 5×10^5 rLM-OVA or PBS as a control. All the mice were euthanized and analyzed 3 days after the secondary infection. (a) Numbers represent the total splenocytes and the indicated cell populations; (b) and (c) Frequencies of IFN-γ producing rLM-OVA-specific CD8 (b) and CD4 (c) T cells. Experiments and data analyses were done as described in **Figure 1**; (d) Numbers of viable bacteria from liver homogenates are depicted. The bars indicate median values. The colony forming units from mice treated with PBS were below the detection limit of 100 CFU/mouse liver.

the liver was measured using the same group of mice. We observed that the bacteria loads in Tg mice were almost ten fold of that in WT mice (**Figure 2(d)**). These data demonstrate that Tg mice with both E- and T-CD4 T cells exhibit impaired immunity to *L. monocytogenes* infection.

3.3. T-CD4 T Cells Were Responsible for the Reduced Anti-Listerial Response

The reduced efficiency of the anti-*Listerial* response in Tg mice suggested that T-CD4 T cells might negatively regulate immune function. However, it was not yet clear whether the difference was directly due to T-CD4 T cell function or it was secondary to other unknown differences between Tg and WT mice. Recently, we reported that IL-4 produced by T-CD4 T cells induces the generation of CD8 T cells with the effector/memory phenotype by expressing IFN-γ and Eomes [47]. Although the function of these innate CD8 T cells during Listeria infection is not known, the difference in immune responses could be due to innate CD8 T cells not directly by T-CD4 T cells. To determine whether CD8 T cells themselves influence the outcome of infections, we established an adoptive transfer mouse model in which different groups only differed in CD4 T cell populations. To obtain T- and E-CD4 T cells exclusively, we constructed bone marrow (BM) chimeric mice (Materials and Methods). In $A\beta^{-/-}$ micethat received Tg BM cells, all CD4 T cells become T-CD4 T cells because they have to be selected by donor MHC class II expressing thymocytes. E-CD4 T cells were generated by transferring WT BM to WT mice. Total T- or E-CD4 T cells were isolated from spleens eight weeks after BM transplantation and were mixed with CD4 T cell-depleted splenocytes from naïve WT mice for adoptive transfer (**Figures 3(a)** and **(b)**). C57BL/6 recipients were sub-lethally irradiated three days before adoptive transfer to facilitate the reconstitution of the incoming cells. The irradiation abolishes the immune responsiveness of the remaining host T cells and thus the T cell immune responses are primarily attributable to the transferred cells [48,49]. To distinguish transferred cells from recipient cells, the congenic markers CD45.1 and CD45.2 were used.

The recipients were analyzed 7 days after infection (**Figure 3(a)**). The number of CD4 T cells in T-CD4 T cell recipients was lower than that in E-CD4 T cell recipients, although transferred CD8 T cells were similar in number between the two groups (**Figure 3(c)**). To address whether the decreased T-CD4 T cell number was caused by infection, we compared the repopulation of adoptively transferred T- and E-CD4 T cells in naïve hosts (**Figure 3(d)**). In our infection experiments shown in **Figure 3(a)**, the infection was done one day after cell transfer. Therefore, we examined mice at days 1 and 8 after transfer that correspond to the time points of infection and to 7 days after infection at which time we analyzed the immune responses. In addition, we examined cells at day 3 (equivalent to day 2 of infection) when innate immunity peaks to assess the cell numbers.The numbers of transferred T-CD4 T cells declined initially but remained fairly constant afterward. At day 8, the difference in cell numbers of T-CD4 T cells compared to that of E-CD4 T cells was similar to what was observed on day 7 in infected hosts. Therefore, the decline of the T-CD4 T cell population in recipients was unlikely due to the infection or immune responses.



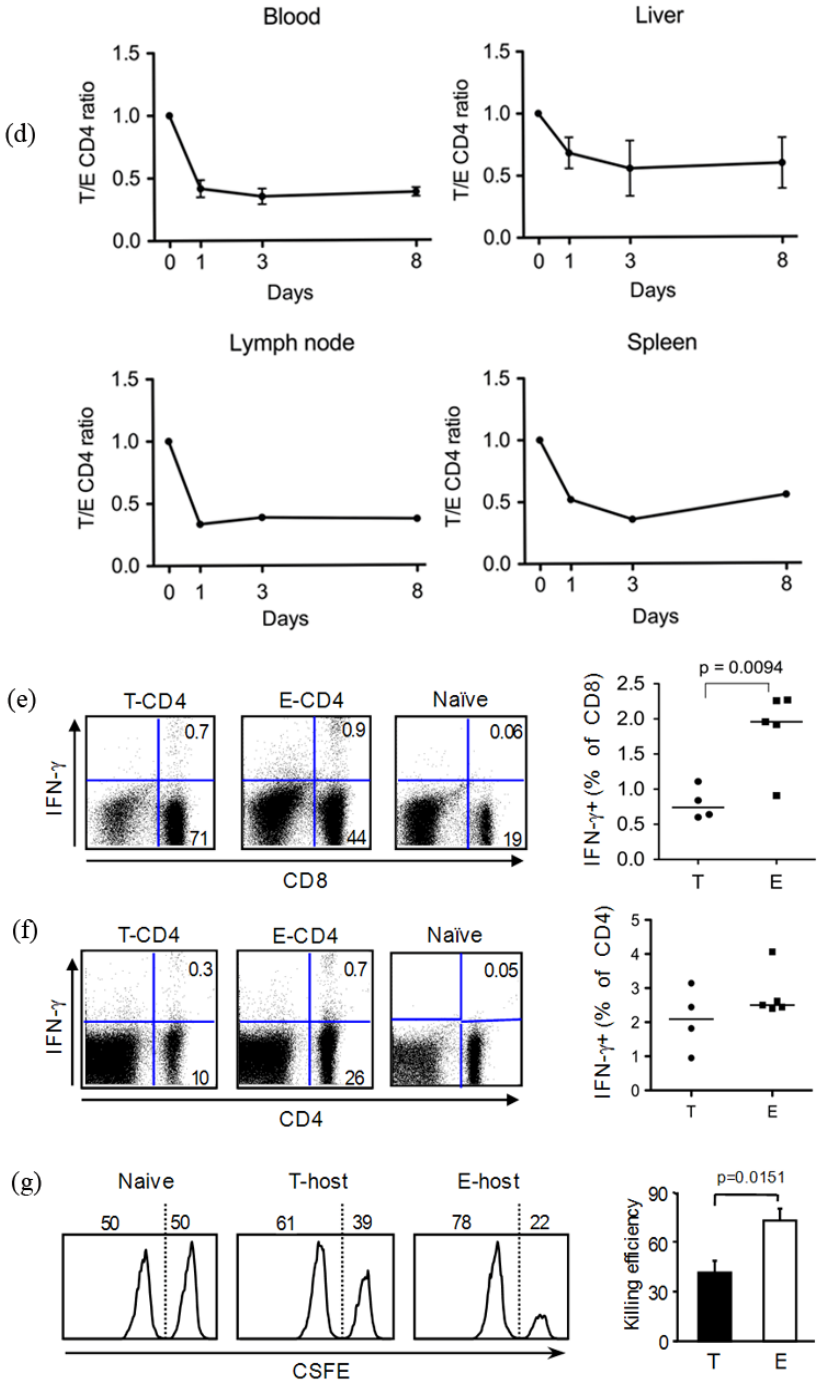


Figure 3. Effector CD8 T cell generation is compromised in the presence of T-CD4 cells. (a) A scheme of the experimental protocol. The infection dose was 2×10^4 rLM-OVA; (b) The composition of the CD45.1$^+$ input cell population prior to the transfer; (c) Numbers of the indicated cells originating from the donor on day 7; (d) T- and E-CD4 ratio after co-transfer into naïve mice. Cell populations were tracked using congenic markers CD45.1 and CD45.2 on the indicated days and the T/E-CD4 ratios were normalized to the input ratio on day 0. N = 3. (e) and (f) Frequencies of IFN-γ-producing rLM-OVA-specific CD8 (e) and CD4 (f) T cells. The FACS data shown were gated on donor populations. The values in representative FACS profiles are percentages of total donor splenocytes; the graphs on right represent percentages of total donor CD8 (e) or CD4 (f) T cells that produced IFN-γ. The bars indicate the median value; (g) *In vivo* killing assay. Experiments were performed as described in **Figure 1(d)**, except that mice were sacrificed 24 hours after cell transfer.

When antigen specific responses were examined, rLM-OVA-specific CD8 T cells were significantly reduced in T-CD4 T cell recipients (**Figure 3(e)**). In contrast, the percentage of rLM-OVA-specific CD4 T cells was comparable between the two groups despite the low T-CD4 Tcell number (**Figure 3(f)**). We next tested the killing efficiency as in **Figure 1(d)** except that we sacrificed mice after overnight instead of 3 hours after cell transfer, because both rLM-OVA-specific CD4 and CD8 T cell numbers were much lower in the adoptive transfer model. Consistent with the reduction of IFN-γ expressing CD8 T cells in T-CD4 T cell recipients, T-CD4 recipients showed significantly decreased cytotoxicity (**Figure 3(g)**). Therefore, unlike E-CD4 T cells, T-CD4 T cells do not support efficient development of rLM-OVA-specific CD8 T cells upon infection, and the decreased anti-*Listerial* immune responses can be directly attributed to the T-CD4 T cells themselves

3.4. T-CD4 T Cells Do Not Support Anti-Listerial Memory Immunity

After observing the effect of T-CD4 T cells on CD8 T cells during primary immune responses, we then tested whether the mice with transferred T-CD4 T cells were also defective in memory immunity. The adoptive transfer was performed as described in **Figure 3(a)** with modifications (**Figure 4(a)**), and a group of recipients receiving donor cells that did not contain CD4 T cells was included as an additional control. Three groups of mice receiving different populations of cells (**Figure 4(b)**) were infected and rested for a month before the challenge. These mice were analyzed three days after challenge with a high dose ofrLM-OVA. The number of total splenocytes in T-CD4 T cell recipients was slightly lower than in E-CD4 recipients although the difference was not significant (**Figure 4(c)**). Although total CD8 T cell numbers were comparable between T- and E-CD4 recipients, rLM-OVA-specific CD8 populations decreased in percentages and thus in cell numbers in T-CD4 recipients (**Figure 4(d)**), consistent with the observation made in Tg and WT hosts. In fact, CD8 T cell responses in T-CD4 recipients were similar to the mice that did not receive CD4 T cells suggesting lack of help by T-CD4 T cells. The transferred CD4 T cells, by comparison, decreased in both total and rLM-OVA-specific populations in T-CD4 recipients (**Figures 4(b)** and **(e)**). In agreement with poor CD8 T cell responses, bacterial burdens were increased in T-CD4 recipients comparable to those of the mice that did not receive CD4 T cells although the differences did not reach statistical significance (**Figure 4(f)**). Therefore, the results suggest that, during long-term immune responses, antigen specific T-CD4 T cells do not provide a sufficient help to generate a robust memory CD8 T cell response.

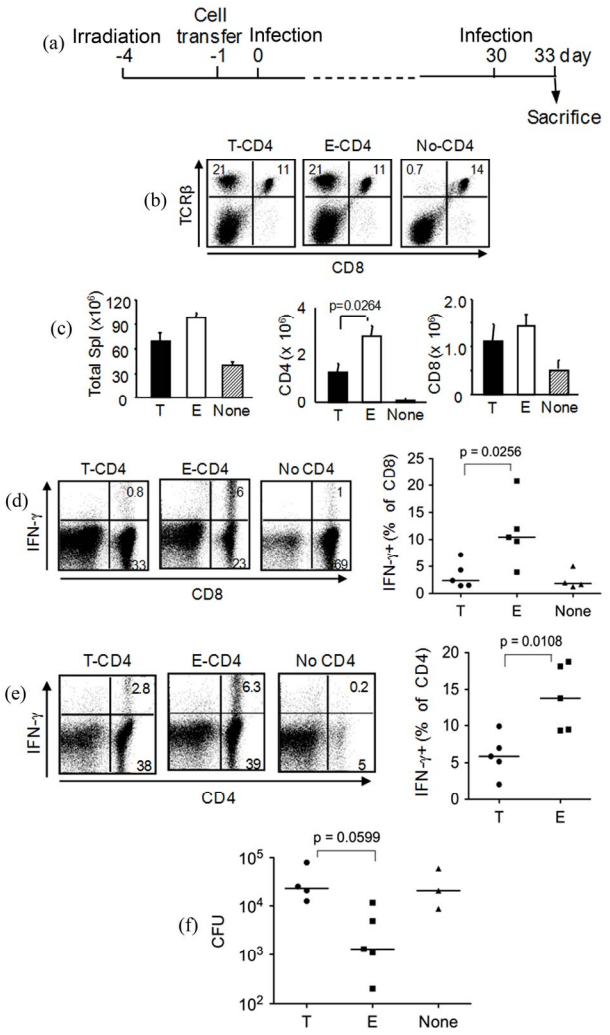


Figure 4. T-CD4 cells inhibit development of memory CD8 effector cells. (a) A scheme of the experimental protocol. Mice were infected with 2×10^3 and 5×10^5 rLM-OVA for primary and secondary infection, respectively, and then sacrificed three days after the second infection; (b) The composition of the CD45.1$^+$ input cell populations prior to the transfer; (c) The numbers represent total splenocytes and the indicated cells originating from the donor; (d) and (e) Frequencies of IFN-γ-producing rLM-OVA-specific CD8 (d) and CD4 (e) T cells (N = 5). Experimental design and data analysis were performed as described in **Figures 3(e)** and **(f)**; (f) Numbers represent viable bacteria isolated from liver homogenates. The bars indicate median values. The CFU counts of PBS-treated mice were below the detection limit.

3.5. The Presence of T-CD4 T Cells Neither Alters Innate Immunity nor the Treg Population

Having observed the suppressive effect exerted by T-CD4 T cells during bacterial infection, we asked whether T-CD4 T cells would change innate immunity because of their innate-like phenotype. However, Tg and WT groups showed increased splenic Gr1$^+$ F4/80$^+$ popu-

lations to a comparable level after infection (**Figure 5(a)**) and mRNA expression of IFN-γ, TNF-α and IL-1β from the spleens were comparable between the two groups (**Figure 5(b)**). Therefore, innate immune responses were not directly regulated by T-CD4 T cells. Next, we tested whether immune suppression was due to a change in Treg populations by the presence of T-CD4 T cells. We previously showed that natural Treg (nTreg) development can be supported by MHC class II+ thymocytes and they mediated comparable suppression [9]. To test whether the generation of nTreg would be different in BM transplanted mice, we compared Foxp3$^+$ cell populations in BM chimeras. As shown in **Figure 5(c)**, the percentages of Foxp3$^+$ cells of T-CD4 T cells were similar to

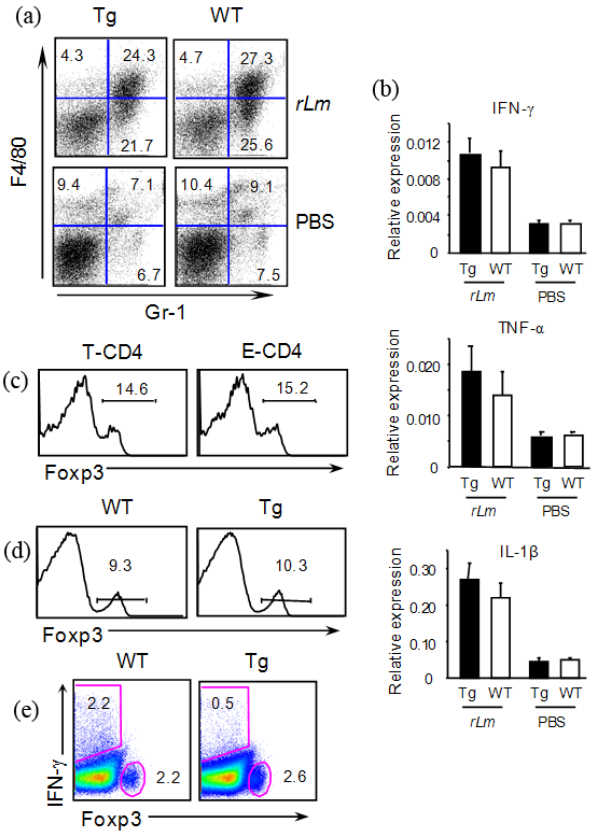


Figure 5. T-CD4 T cells does not change Treg populations ((a) and (b)) Mice were infected intravenously with 100,000 rLM-OVA and sacrificed on day 3 to assess cell populations (a) and to measure cytokine expression (b) from the spleen of Tg and WT mice. The RNA expression of the indicated cytokine genes were measured by real time PCR after reverse transcription. Relative expression of each cytokine was normalized to GAPDH. The data are representative of 4 mice in each group. (c) Representative profiles of Foxp3 expression in E- and T-CD4 T cells from [Tg→A$\beta^{-/-}$] and [WT→WT] chimeric mice. (d) and (e) WT and Tg mice were infected as in **Figure 1**. Seven days after infection, freshly isolated CD4 T cells were stained for Foxp3 expression (d) or stimulated in the presence of rLM-OVA peptides for 5 hours followed by staining of Foxp3 and IFN-γ (e).

that of E-CD4 T cells. We then asked if T-CD4 T cells could induce the generation of Foxp3$^+$ E-CD4 T cells upon infection, which would in turn suppress effector E-CD4 T cells. To test this, we infected WT and Tg mice with rLM-OVA and examined Foxp3$^+$ cells together with CD8 effector T cell generation by IFN-γ staining after primary infection as in **Figure 1**. Freshly isolated splenocytes from infected mice showed comparable percentages of Foxp3$^+$ cells (**Figure 5(d)**). When CD4 T cells from infected mice were stimulated with rLM-OVA antigens *in vitro*, IFN-γ expressing CD4 T cells were lower in the culture of CD4 T cells from Tg mice but Foxp3$^+$ cells were at a similar level between the two groups (**Figure 5(e)**). Therefore, suppression mediated by T-CD4 T cells is unlikely to be due to alteration of Treg populations.

3.6. Lack of Antigen-Specific CD4 T Cell Responses upon Helicobacter Infection in the Presence of T-CD4 T Cells

To determine if the suppressive activity of T-CD4 T cells is specific on CD8 T cells, we examined immune responses mediated by CD4 T cells using an established model of infection by *Helicobacter pylori*. We have shown that adoptive transfer of CD4 T cells to *H. pylori*-infected immunodeficient (SCID) mice is necessary and sufficient to induce severe gastritis [40]. This model allows us to measure the effect of T-CD4 T cells on E-CD4 T cells. To evaluate the role of T-CD4 cells in infection by *H. pylori*, we transferred either E-CD4 or a mixture of E- and T-CD4 T cells from WT and Tg mice, respectively, to SCID mice that were either uninfected or infected with *H. pylori* (**Figure 6(A)**). Eight weeks after transfer, we examined CD4 T cells in the spleen. The number of CD4 T cells from infected mice was similar in mice given Tg CD4 T cells and those given WT CD4 T cells, and CD4 T cells were expanded to the similar degree in the two recipient groups (**Figure 6(B)**). CD4 T cells were then stimulated by plate-bound anti-CD3 together with soluble anti-CD28 or with antigen loaded APC that were pulsed with *H. pylori* antigen. WT and Tg CD4 T cells produced equivalent amounts of IFN-γ upon anti-CD3 stimulation (**Figure 6(C)**, top left panel). In contrast, cytokine responses to *H. pylori* antigen differed depending on donor cells. As expected, CD4 T cells recovered from *H. pylori*-infected WT CD4 recipients produced IFN-γ in response to *H. pylori* antigen-pulsed antigen presenting cells as expected, whereas Tg CD4 cells recovered from infected recipient mice failed to produce IFN-γ in response to *H. pylori* antigen stimulation (**Figure 6(C)**, top right panel). Thus, the presence of T-CD4 in these mice not only failed to induce cytokine expression by the T-CD4 T cells themselves, but also suppressed *H. pylori*- specific IFN-γ responses of E-CD4 T cells. Also as expected, CD4 cells from Tg recipients

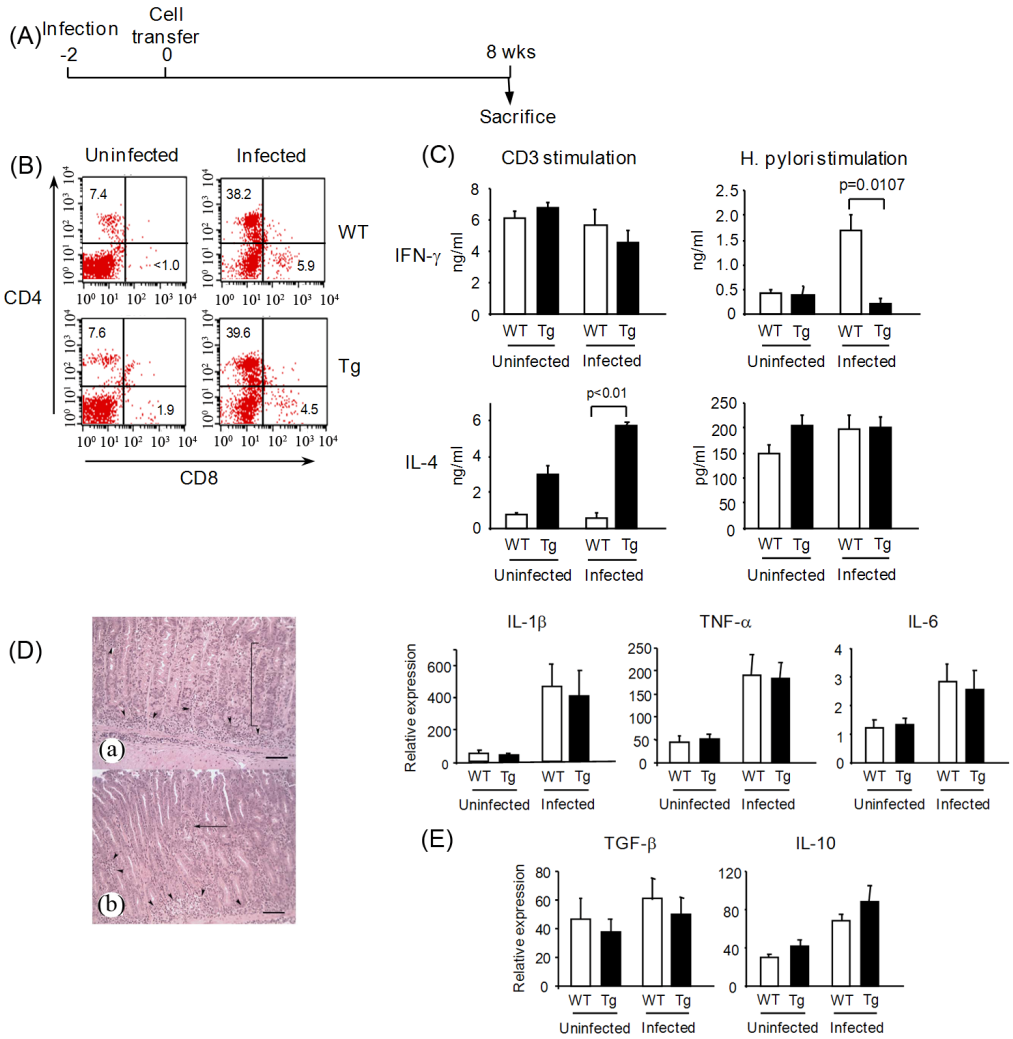


Figure 6. The presence of T-CD4 T cells inhibits the generation of Helicobacter-specific effector CD4 T cells. (A) A scheme of the experimental protocol; (B) Recovery of T cells from Helicobacter infected mice. Flow cytometric analysis of cells recovered from the spleens of SCID recipients of adoptive transfers of CD4 T cells; (C) CD4 T cells were isolated from mice and then stimulated with anti-CD3/CD28 or with *H. pylori* lysate. Supernatants were used forELISA. Bars are the average of 3 replicates of each condition, and each replicate used CD4 T cells pooled from 5 mice; (D) Hematoxylin and eosin-stained sections of gastric mucosa from H. pylori-infected SCID recipient mice. (a) WT CD4 recipient. Inflammatory infiltrate (arrowheads) consists of neutrophils, lymphocytes, and macrophages. Bracket indicates metaplastic glands. (b) Tg CD4 recipient. Arrow indicates a gland abscess. Bars = 50 μm; (E) Relative mRNA levels of cytokine genes from the gastric mucosa of uninfected and infected SCID mice that were adoptively transferred with either WT or Tg CD4 cells. Expression of cytokine genes normalized to GAPDH expression, and multiplied by 10,000 for ease of visualization.

but not from WT recipients produced IL-4 in response to anti-CD3 stimulation (**Figure 6(C)**, bottom left panel) compatible with previous studies demonstrating the ability of T-CD4 cells to produce Th2 as well as Th1 cytokines after pan-TCR stimulation [21]. But IL-4 levels upon *H. pylori* antigen stimulation showed little difference between uninfected and infected WT and Tg mice indicating the lack of IL-4 producing CD4 T cells upon infection (**Figure 6(C)**, bottom right panel).

Interestingly, although *H. pylori*-specific CD4 T cell IFN-γ production was different between recipients of Tg and WT CD4 cells, gastritis was extensive in both groups without statistically significant differences (**Figure 6(D)**). In addition, expression of proinflammatory cytokine mRNA in the gastric mucosa was elevated in both recipient groups in response to infection by *H. pylori*. The gastric mRNA levels of IL-1β, TNF-α, and IL-6 were significantly elevated in infected mice compared to uninfected mice regardless of the CD4 T cell donor (**Figure 6(E)**). Expression of anti-inflammatory cytokines TGF-β

and IL-10 did not differ between groups regardless of infection or donor cell type. These data suggest that T-CD4 suppress E-CD4 antigen-specific IFN-γ responses, and indicate that in this model, gastritis and proinflammatory cytokine production seems to be independent of IFN-γ production by antigen-specific CD4 T cells.

4. DISCUSSION

In the present study, we provide evidence for a novel suppressive function of T-CD4 T cells during adaptive immune responses against two bacterial pathogens, *Listeria monocytogenes* and *Helicobacter pylori*. The suppressive effect mediated by T-CD4 T cells appears to be broad because the presence of T-CD4 T cells inhibited the generation of both CD4 and CD8 effector T cells in response to bacterial infections. These findings are consistent with our previous work that airway inflammation was diminished in mice that have both E- and T-CD4 T cells [21] and EAE development was suppressed by having T-CD4 T cells together with E-CD4 T cells [50]. Taken together, our data support a suppressive role for T-CD4 T cells in regulating immunity in many different immune contexts.

Suppression by T-CD4 T cells is not likely due to an alteration in Treg populations, because T-CD4 T cells used in the assay did not contain more Treg than E-CD4 T cells based on Foxp3 staining. In addition, Treg from T-CD4 T cell recipients are functionally equivalent to those from E-CD4 T cell recipients when their suppression was tested *in vitro* [9]. Despite having the suppressive function, T-CD4 T cells are distinctive from Treg since T-CD4 T cells do not express Foxp3 (**Figure 5**). Treg cells neither have pre-made IL-4 mRNAs nor do they release Th1 and Th2 cytokines immediately after TCR stimulation as T-CD4 T cells do. Treg cells, in contrast, produce large amounts of suppressive cytokines TGF-β and IL-10 after differentiation, cytokines that are not produced in significant quantities by either T-CD4 or E-CD4 T cells (Chang and Chang, unpublished data). It is yet unclear how T-CD4 T cells exert a negative regulatory effect on other T cells. As reported, IL-4 produced by either T-CD4 or iNKT cells induce the generation of innate effector CD8 T cells [49,51,52]. Therefore, it is possible that these innate CD8 T cells exert a negative effect during Listeria infection. However, the suppressive effect on Helicobacter-specific CD4 T cells does not fully support this mechanism. Perhaps, similar to Treg, T-CD4 T cells contact the target cells directly and cause suppression, or T-CD4 T cells may act indirectly by influencing the local environment, e.g., augmenting the activity of Treg *in vivo*, which may lead to enhanced immune suppression. Further investigations into molecular mechanisms for T-CD4 T cell-mediated immune suppression is warranted.

T-CD4 T cells differ from E-CD4 T cells in several ways. T-CD4 T cells appear to be born as effector cells, expressing pre-formed mRNAs of effector T cell cytokines prior to activation [21]. This allows them to produce Th1 and Th2 cytokines shortly after TCR stimulation [21]. However, as we have shown in the current study, T-CD4 T cells suppress CD8 and CD4 T cell functions instead of providing help as E-CD4 T cells do. E-CD4 T cells are required for mounting an effective secondary immune response against bacterial infection. In the presence of E-CD4 T cells, antigen-specific CD8 T cells expand during the primary immune response against Listeria and a fraction of them become memory cells. As a result, the hosts are able to clear Listeria efficiently upon a secondary challenge. In contrast, T-CD4 T cells adversely affected primary and memory immunity against Listeria. They appear to be suppressive, as evidenced by the low number of effector CD8 T cells in the presence of T-CD4 T cells. In fact, the CD8 response in the presence of T-CD4 T cells was similar to mice that did not receive CD4 T cells further supporting that T-CD4 T cells do not function as helper cells although they do produce effector cytokines.

T-CD4 T cells resulted in suppression of memory as well as primary immune responses against Listeria infection. Perhaps memory T-CD4 T cells are generated and they actively suppressed the response of CD8 memory cells. It is equally possible that the memory CD8 T cells were generated in the presence of T-CD4 T cells but they are functionally defective. The latter seems to be the case because the host already demonstrated reduced rLM-OVA-specific CD8 T cell population during the primary response. Testing two possibilities requires a model of secondary adoptive transfer to separate the function of T-CD4 T cells in primary response from that in memory immune response. Nevertheless, the current data clearly showed that, under physiological conditions, the presence of T-CD4 T cells resulted in deficient CD8 T cell responses at both primary and memory phases.

Like in infection by *L. monocytogenes,* T-CD4 cells suppressed antigen-specific responses of E-CD4 T cells in mice infected with *H. pylori*. Thus, the presence of T-CD4 cells appeared to interfere with *H. pylori*-specific IFN-γ production by E-CD4 cells. Surprisingly, however, in spite of the absence of an antigen-specific Th1 response, the extent of gastritis in mice with E- and T-CD4 cells were not different from recipients of E-CD4 cells (**Figure 6**). Although there is strong published evidence that gastritis due to *H. pylori* is associated with IFN-γ producing Th1 T cells, gastritis in mice can occur in the absence of IFN-γ producing CD4 cells [39,43] and others have also suggested that gastritis due to *H. pylori* is not absolutely dependent on a Th1 response [53,54]. In the current study, the presence of T-CD4 cells did affect host

H. pylori-specific T cell response, but did not appear to alter the severity of gastric inflammation. This finding could provide a clue to an important difference between murine and human disease due to *H. pylori*. In mice, the extent of gastritis is inversely proportional to 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 [55,56]. In humans, in contrast, although there have been scattered reports of spontaneous clearance of infection [57], most people remain infected for life, regardless of disease severity and there is no association between the severity of gastritis and the level of bacterial colonization [58]. It is possible that the failure of humans with gastritis to clear infection is attributable to suppressive T-CD4 cells. These cells may sufficiently downregulate the immune response to result in failure of eradication but not sufficiently to affect the level of gastric inflammation and disease.

We have observed that T-CD4 T cells had lower cell recovery than E-CD4 T cells after Listeria infection when equal numbers were adoptively transferred. This was also observed in naïve recipients, and the ratio between co-transferred T- and E-CD4 T cells became stable after the initial reduction (**Figure 3**). Therefore, the decrease in total T-CD4 T cell number seems to be due to the reduced reconstitution efficiency rather than loss of T-CD4 T cells by the infection. Although underlying mechanisms for a deficit in T-CD4 T cell reconstitution are not yet understood, our data suggest that the reduced antigen-specific responses against both bacteria were not likely the consequence of a low T-CD4 T cell number in adoptively transferred mice. During a primary immune response when CD8 T cells responded equally well with or without E-CD4 T cells [18,19,36,37], the presence of T-CD4 T cells resulted in decreased host response. In addition, Tg mice that possess sufficient numbers of E-CD4 T cells still showed compromised CD8 T cell response during infection. Finally, E-CD4 T cells alone were able to mount Th1 responses against Helicobacter infection but this response was lost when T-CD4 T cells were present. Together, the data support that poor immune responses during bacterial infection is a result of suppression mediated by T-CD4 T cells.

The role of T-CD4 T cells in humans is unknown. Human T-CD4 T cells appear during gestation and decrease after birth [6], which coincides with MHC class II expression in thymocytes [59]. Moreover, fetal stem cells possess greater potential to differentiate to Treg than adult stem cells [60]. Therefore, a large number of CD4 T cells in infants seem to have the suppressor function. Interestingly, infants are known to be highly susceptible to infection [61], which we propose may be due in part to the presence of these suppressor cells at a high level. Currently, little is known the role of T-CD4 T cells in infants during the course of an immune response. But it is tempting to speculate that having a T cell compartment comprised of cells with negative function dampens adaptive immunity in infants upon infection or vaccination. Patients who have received BM transplantations can also develop T-CD4 T cells [62-65]. Studies with DiGeorge patients strongly support the development of T-CD4 T cells with suppressive function in the patients [65]. Further investigations to understand the potential role of T-CD4 T cells during the human immune response will be important to improve our treatment of infectious and autoimmune diseases.

5. ACKNOWLEDGEMENTS

We are thankful for the help of Dr. Jihoon Chang and Tim Voorhees with the comparison of cytokine levels between T- and E-CD4 culture supernatant and Drs. Phil King and Derek Sant'Angelo for critical reading of the manuscript. We are also grateful for Dr. Hao Shen who generously provided us with the recombinant Listeria strain used in our study. This study was supported by the NIH grant AI064540 to M. O'Riordan, AI043643 to K. Eaton, and AI073677 to C.-H. Chang.

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