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Foxp3+ Regulatory T Cells Impede the Priming of Protective CD8+ T Cells

James M. Ertelt,*†‡ Jared H. Rowe,*†‡ Margaret A. Mysz,*†‡ Charanjeet Singh,†§ Monika Roychowdhury,†§ Marijo N. Aguilera,*†‖ and Sing Sing Way*†‡

T cell activation is controlled by incompletely defined opposing stimulation and suppression signals that together sustain the balance between optimal host defense against infection and peripheral tolerance. In this article, we explore the impacts of Foxp3+ regulatory T cell (Treg) suppression in priming Ag-specific T cell activation under conditions of noninfection and infection. We find the transient ablation of Foxp3+ Tregs unleashes the robust expansion and activation of peptide-stimulated CD8+ T cells that provide protection against Listeria monocytogenes infection in an Ag-specific fashion. By contrast, Treg ablation had nonsignificant impacts on the CD8+ T cell response primed by infection with recombinant L. monocytogenes. Similarly, nonrecombinant L. monocytogenes administered with peptide stimulated the expansion and activation of CD8+ T cells that paralleled the response primed by Treg ablation. Interestingly, these adjuvant properties of L. monocytogenes did not require CD8+ T cell stimulation by IL-12 produced in response to infection, but instead were associated with sharp reductions in Foxp3+ Treg suppressive potency. Therefore, Foxp3+ Tregs impose critical barriers that, when overcome naturally during infection or artificially with ablation, allow the priming of protective Ag-specific CD8+ T cells. The Journal of Immunology, 2011, 187: 2569–2577.

Defining the signals that control the expansion and activation of protective Ag-specific T cells is an important prerequisite for designing therapies aimed at targeting these adaptive immune components for boosting host defense against infection. Although some molecular signals capable of stimulating CD8+ T cell activation in vitro have been identified (1, 2), the essential components required for priming protective CD8+ T cells in vivo in the context of infection or immunization remain incompletely defined. For example, although cytokines such as IL-12, type I IFN, or IL-21, each together with cognate Ag and costimulation, stimulate the expansion of CD8+ T cells in vitro, these cytokines are jointly nonessential for priming the expansion of protective pathogen-specific CD8+ T cells after in vivo infection with the intracellular bacterium Listeria monocytogenes (3–6). This discordance is likely attributed to the more complex balance between immune stimulation and suppression signals that regulate T cell activation in vivo, and other differences in the relative activation of immune components during infection compared with noninfection conditions. These include the stimulation of immune cells through a growing list of pattern recognition receptors that recognize defined pathogen-associated ligands, the activation of more complex inflammatory cytokine cascades, and upregulation of costimulation and cytokine signals by APCs that are each difficult to recapitulate using in vitro models of T cell stimulation (7–11). Therefore, dissociating the parameters that permit the priming of pathogen-specific T cells during infection from noninfection conditions, using relevant in vivo models, is required.

Regulatory T cells (Tregs), identified as the Foxp3+ subset of CD4+ T cells, play pivotal roles in controlling the balance between immune stimulation and suppression during both conditions of noninfection and infection (12–14). Mice with naturally occurring or experimentally induced sustained defects in Foxp3+ develop fatal systemic autoimmunity that is associated with the activation of self-reactive T cells and APCs (15–19). By extension, the potency of many immune adjuvants coincides with their ability to either directly dampen Treg suppression or indirectly by reducing the impacts of Treg suppression on target cells (20–25). Although these associations suggest overriding Treg suppression may represent an important prerequisite for priming protective T cells in vivo, the specific limitations imposed by Foxp3+ Tregs on the expansion and activation of Ag-specific T cells have not been clearly identified.

To interrogate the impacts of Treg suppression on priming T cells in vivo, we enumerated the effects of Foxp3+ cell ablation on the expansion of Ag-specific CD8+ T cells initially after stimulation with purified peptide designed to mimic Ag exposure under noninflammatory conditions. Related experiments investigated the relative impacts of Treg suppression on the pathogen-specific T cell response primed by infection with recombinant L. monocytogenes engineered to express the same Ag, nonrecombinant L. monocytogenes administered with purified peptide, and potential shifts in Treg suppressive potency after this infection. We used Foxp3DTTR transgenic mice that express the human high-affinity diphtheria toxin (DT) receptor or Foxp3GFP reporter mice that express GFP, each with Foxp3 that allows the targeted ablation or isolation, respectively, of Tregs based on their lineage-defining marker (19, 26). These experiments demonstrate Foxp3+ Treg suppression imposes critical limitations for priming protective
CD8\(^+\) T cells after stimulation during noninflammatory conditions that are overcome by acute infection.

**Materials and Methods**

**Mice**

C57BL/6 (B6) and Lys.2/Cr (CD45.1\(^+\)) mice were purchased from The National Cancer Institute (Bethesda, MD), Foxp3\(^{DTR}\) and Foxp3\(^{GFP}\) were kindly provided by Dr. Alexander Rudensky (Memorial Sloan-Kettering, New York, NY) (19, 26), and each backcrossed over 15 generations to the B6 background. Wild-type (WT) OT-1 TCR transgenic mice maintained on a CD90.1 CD45.2 background and IL-12R\(^-\) deficient OT-1 transgenic mice maintained on a CD90.2 CD45.2 background have been described (27, 28).

CD19Cre and ROSAiDTR mice were purchased from The Jackson Laboratory and intercrossed to generate CD19\(^{GFP}\) ROSA\(^{DTR}\) mice (29, 30). For cell ablation, purified DT (Sigma-Aldrich) dissolved in saline was injected i.p. (50 \(\mu\)g/kg) 1 d before and on the day of peptide stimulation and/or L. monocytogenes infection. For IL-12 neutralization, purified anti-IL-12p40 (clone 17.8) or isotype control rat IgG2a Ab was inoculated i.p. (1.0 mg per mouse) 1 d prior to peptide stimulation and/or L. monocytogenes infection (31, 32). All experiments were performed under University of Minnesota Institutional Animal Care and Use Committee-approved protocols.

**Cell staining, stimulation, and adoptive cell transfer**

Fluorophore-conjugated Abs for cell surface and intracellular staining were purchased from eBioscience or BD Biosciences. CD8\(^+\) T cells harvested from OT-1 TCR transgenic mice were infected with Foxp3\(^{DTR}\) or Foxp3\(^{GFP}\) B6 control mice (10\(^5\) cells per mouse). OVA\(_{257-264}\) peptide (University Biochemical Research; 95% purity) was inoculated intravenously (200 \(\mu\)g per mouse) 1 d after CD8\(^+\) T cell transfer from OT-1 TCR transgenic mice. For tracking the response among endogenous CD8\(^+\) T cells, H-2K\(^b\) dimer X (BD Biosciences) loaded with OVA\(_{257-264}\) peptide was used for staining, as described (6, 33). For cytotoxic production, splenocytes were stimulated ex vivo with OVA\(_{257-264}\) peptide (1 \(\mu\)M) for 5 h in cultures supplemented with GolgiPlug (BD Biosciences). JAWS II cells (CLR-11904; American Type Culture Collection) were grown in media supplemented with 20% FCS, 1% Hepes, and 5 ng/ml GM-CSF (34, 35). For stimulation, JAWS II cells were either pulsed with OVA\(_{257-264}\) peptide (5 \(\mu\)M, 2 h at 37°C) or no peptide control in complete media, washed three times with serum-free media, and injected i.v. into mice (1 \(\times\) 10\(^5\) cells per mouse in 200 \(\mu\)l).

**Infections**

Recombinant L. monocytogenes-OVA (36) and nonrecombinant L. monocytogenes-10403S (37) were each grown in brain heart infusion media at 37°C, back-diluted to log-phase (OD\(_{600}\) 0.1), washed and resuspended in sterile saline, and injected i.v. (10\(^8\) CFUs per mouse; 0.2 LD\(_{50}\)). The number of CFUs in organ homogenates was enumerated 3 d postinfection, as described (6, 33).

**Suppression assays**

GFP\(^+\) Tregs were isolated from Foxp3\(^{GFP}\) reporter mice by first enriching for CD4\(^+\) T cells, using negative selection (Miltenyi Biotec), and then sorting for GFP\(^+\) cells (FACS(Aria). Responder T cells from naive CD45.1\(^+\) mice were labeled with CFSE (5 \(\mu\)M for 10 min), cocultured in 96-well round-bottom plates (5 \(\times\) 10\(^4\) cells per 100 \(\mu\)l) with purified GFP\(^+\) Tregs at a 1:1 ratio, and serial 2-fold dilutions of Treg to responder T cells. Shifts in Treg suppressive potency were enumerated by comparing the proliferation (CFSE dilution) in responder CD8\(^+\) T cells after coculture with GFP\(^+\) Tregs isolated from mice before and after L. monocytogenes infection, and stimulation with anti-mouse CD3 (1 \(\mu\)g/ml) for 3 d.

**Statistics**

The percent and total cell numbers, and log10 number of recoverable CFUs postinfection were first determined to be normally distributed. The differences in each group were then analyzed using the unpaired Student t test (Prism, GraphPad) with \(p < 0.05\) taken as statistical significance.

**Results**

**Regulatory T cells impede peptide-stimulated CD8\(^+\) T cell expansion and activation**

To identify the limitations imposed by Treg suppression for priming T cell in vivo, the impacts of Foxp3\(^+\) cell ablation on the expansion of CD8\(^+\) T cells with specificity to a defined nonself Ag were evaluated. We used adoptively transferred CD8\(^+\) T cells with specificity to the H-2K\(^b\) OVA\(_{257-264}\) peptide derived from OT-1 TCR transgenic mice that are identified among cells in recipient mice on the basis of CD90.1 expression (27). After stimulation with OVA\(_{257-264}\) peptide in Treg-sufficient mice, these cells expand only modestly (<50-fold), which is consistent with results from previous reports (3) (Fig. 1A). By contrast, DT treatment 1 d prior and on the day of peptide inoculation triggered significantly more (>100-fold, \(p < 0.001\)) expansion of OVA-specific CD90.1\(^+\) CD8\(^+\) cells in Foxp3\(^{GFP}\) compared with Foxp3\(^{WT}\) controls (Fig. 1A). The expansion of these CD8\(^+\) T cells occurred in an Ag-specific fashion because only background levels were recovered from Treg-ablated mice without peptide stimulation (Fig. 1A). In parallel with expansion, peptide-stimulated CD8\(^+\) T cells also proliferated more rapidly with accelerated kinetics of CFSE dilution in Treg-ablated mice than in Treg-sufficient mice (Fig. 1B). Furthermore, the impacts of transient Treg ablation on the proliferation and expansion of peptide-stimulated CD8\(^+\) T cells were not limited only to adoptively transferred cells because increased numbers of Ag-specific among endogenous CD8\(^+\) T cells were also identified by staining with OVA\(_{257-264}\) peptide-loaded H-2K\(^b\) dimers in Treg-ablated mice compared with Treg-sufficient controls (Supplemental Fig. 1). Of importance, OVA-specific CD8\(^+\) T cell expansion was uniquely triggered by the ablation of Foxp3\(^+\) Tregs and not due to nonspecific immune activation caused by DT-induced cell death, because only background levels were found after peptide stimulation and DT treatment in CD19\(^{-}\)ROSA\(^{DTR}\) mice, in which a >30-fold increase in apoptotic cell death occurs (29, 30) (Supplemental Fig. 2).

We next compared the impacts of Treg suppression on the activation of peptide-stimulated CD8\(^+\) T cells. The relative expression of T cell activation markers, such as CD25 and C62L and production of IFN-\(\gamma\) were found to each parallel the more robust expansion of peptide-stimulated cells in Treg-ablated mice (Fig. 1A, 1C). Compared with the few OVA-specific CD8\(^+\) T cells recovered from Treg-sufficient mice that were predominantly CD25\(^lo\) and C62L\(^lo\), and produced little to no cytokine, the expanded population of these cells primed in Treg-ablated mice upregulated CD25 and IFN-\(\gamma\) production and downregulated C62L expression (Fig. 1A, 1C). These findings indicate that Foxp3\(^+\) Tregs suppress both the expansion and the activation of peptide-stimulated CD8\(^+\) T cells in vivo. In related experiments, we extended these studies to investigate the impact of transient Treg ablation on the kinetics whereby CD8\(^+\) T cells expand and contract after peptide stimulation. Compared with the level of OVA-specific CD8\(^+\) T cells at day 5 after peptide stimulation in Treg-ablated mice, reductions in both percent and total cell numbers became apparent beginning day 10 and steadily declined through day 30 (Fig. 1D). As expected, only background levels of OVA-specific CD90.1\(^+\) CD8\(^+\) T cells were found in peptide-stimulated Treg-sufficient control mice at each of these time points. Together, these results demonstrate that Treg ablation primes the activation, expansion, and subsequent contraction of peptide-stimulated CD8\(^+\) T cells.

**Peptide-stimulated CD8\(^+\) T cells primed in the absence of Tregs are protective**

To more definitively evaluate the functional properties of peptide-stimulated CD8\(^+\) T cells primed in Treg-ablated mice, we compared their ability to confer protection against virulent L. monocytogenes engineered to stably express a truncated form of OVA that contains the OVA\(_{257-264}\) peptide (L. monocytogenes-OVA) (36). Significantly reduced numbers of bacterial CFUs were recovered from mice initially stimulated with OVA\(_{257-264}\) peptide in the absence of Tregs, compared with Treg-sufficient controls day 3.
CD90.1+ CD8+ T cells 60 h (day 2.5) and 120 h (day 5) after stimulation day of peptide inoculation. B Tregs. Each group of mice received DT treatment 1 d before and on the cognate peptide in the presence (Foxp3-WT) or absence (Foxp3-DTR) of CD25 and IFN-γ (filled histogram) or unlabeled cells (gray line histogram). (Foxp3-DTR) (black line histogram), compared with unstimulated cells with cognate peptide in Treg-sufficient (Foxp3-WT) or Treg-ablated mice.

FIGURE 1. Foxp3+ Treg ablation primes the expansion and activation of peptide-stimulated T cells. A. Percent and number of OVA-specific CD90.1+ CD8+ T cells among splenocytes day 5 after stimulation with cognate peptide in the presence (Foxp3-WT) or absence (Foxp3-DTR) of Tregs. Each group of mice received DT treatment 1 d before and on the day of peptide inoculation. B. CFSE expression among OVA-specific CD90.1+ CD8+ T cells 60 h (day 2.5) and 120 h (day 5) after stimulation with cognate peptide in Treg-sufficient (Foxp3-WT) or Treg-ablated mice (Foxp3-DTR) (black line histogram), compared with unstimulated cells (filled histogram) or unlabeled cells (gray line histogram). C. Expression of CD25 and IFN-γ production by CD90.1+ (line histogram) or bulk CD8+ T cells (filled histogram) day 5 after peptide stimulation in Treg-sufficient mice without peptide stimulation (Fig. 5A). Furthermore, purified peptide with L. monocytogenes-OVA infection also stimulated the robust expansion of OVA-specific CD8+ T cells that mirrored the effects of Foxp3+ Treg ablation (Fig. 5A compared with Fig. 1A). The expansion of these cells was Ag specific and required stimulation with OVA peptide because only background levels were found in nonrecombinant L. monocytogenes-infected mice without peptide stimulation (Fig. 5A). Together, these findings indicate L. monocytogenes infection overrides suppression imposed by Foxp3+ Tregs and primes the expansion and activation of peptide-stimulated CD8+ T cells.

L. monocytogenes infection overrides the impacts of Treg suppression

The primary expansion kinetics and protective capacity of peptide-stimulated CD8+ T cells primed in the absence of Tregs bear striking resemblance to the pathogen-specific T cell response primed by L. monocytogenes infection (2, 6, 40). These parallels led us to investigate the impact of Treg suppression on the OVA257–264-specific CD8+ T cell response after L. monocytogenes-OVA infection between Treg-ablated and Treg-sufficient control mice, the robust levels of IFN-γ, IL-2, and TNF-α produced by cells isolated from mice primed initially with OVA257–264 peptide in the absence of Foxp3+ Tregs illustrate the highly activated nature of these cells (Fig. 3B). Reciprocally, reduced IFN-γ production by cells isolated from mice initially stimulated in the presence of Tregs is consistent with a functionally more tolerant phenotype previously described for CD8+ T cells stimulated in vivo with purified peptide (3, 38, 39).

In related experiments, we compared the secondary expansion kinetics of OVA-specific CD8+ T cells after L. monocytogenes-OVA infection. Consistent with their ability to confer protection against L. monocytogenes infection in an Ag-specific fashion, OVA-specific CD8+ T cells re-expanded rapidly in mice initially primed with peptide in the absence of Tregs (Fig. 3A). Although differences in L. monocytogenes-OVA pathogen burden and T cell precursor frequency at the time of infection preclude a direct comparison of the secondary OVA-specific CD8+ T cell response after L. monocytogenes-OVA infection between Treg-ablated and Treg-sufficient control mice, the robust levels of IFN-γ, IL-2, and TNF-α produced by cells isolated from mice primed initially with OVA257–264 peptide in the absence of Foxp3+ Tregs illustrate the highly activated nature of these cells (Fig. 3B). Reciprocally, reduced IFN-γ production by cells isolated from mice initially stimulated in the presence of Tregs is consistent with a functionally more tolerant phenotype previously described for CD8+ T cells stimulated in vivo with purified peptide (3, 38, 39).

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Because inflammatory cytokines such as IL-12 produced in response to *L. monocytogenes* infection can directly stimulate the activation of protective CD8+ T cells (3, 41), related experiments explored whether these adjuvant effects of *L. monocytogenes* on the expansion and activation of peptide-stimulated CD8+ T cells required IL-12. We found that administration of neutralizing Abs against IL-12 p40, compared with isotype control Abs, 1 d prior to OVA-peptide stimulation and *L. monocytogenes* infection had no significant impact on the expansion or activation of OVA-specific CD8+ T cells (Fig. 5C). To more specifically investigate the direct impacts of IL-12 stimulation on CD8+ T cells—as well as address the possibility that in vivo neutralization with anti–IL-12 Ab was incomplete or nonspecific because IL-23 also shares the same IL-12 p40 subunit—complementary experiments compared the expansion and activation of CD8+ T cells from IL-12R–deficient OT-1 and IL-12R–sufficient (WT) OT-1 TCR transgenic mice each on the CD45.2 background after adoptive transfer into CD45.1 recipients. As with the results after anti–IL-12 neutralization, no significant differences in expansion or IFN-γ production were found for IL-12R–deficient compared with WT OT-1 cells after stimulation with OVA peptide and *L. monocytogenes* infection (Fig. 5C). Therefore, although IL-12 can stimulate T cell activation and is produced in response to *L. monocytogenes* infection (3, 32, 42), the adjuvant effects of *L. monocytogenes* on the expansion and activation of peptide-stimulated CD8+ T cells do not require IL-12 stimulation.

**FIGURE 2.** Peptide-stimulated CD8+ T cells primed in the absence of Tregs protect against *L. monocytogenes* infection. Bacterial CFUs in the spleen (top) or liver (bottom) day 3 postinfection with either *L. monocytogenes*-OVA or *L. monocytogenes*-10403s for the indicated mice stimulated with OVA257–264 peptide (and treated with DT 1 d prior to and on the day of peptide inoculation) 30 d prior to infection. These data are representative of three experiments containing 8–12 mice per group.

**FIGURE 3.** Secondary expansion and cytokine production for peptide-stimulated CD8+ T cells primed initially in Treg-ablated or Treg-sufficient mice. A. Percent and number of CD90.1+ CD8+ cells among splenocytes prior to (D30), or days 3 (D30 + 3) and 5 (D30 + 5) after *L. monocytogenes*-OVA infection in mice treated with peptide 30 d prior. B. Representative FACS plots demonstrating percent of IFN-γ, IL-2, and TNF-α producing CD90.1+ CD8+ T cells day 5 after secondary *L. monocytogenes*-OVA infection (D30 + 5 after peptide stimulation) directly ex vivo (No stim) or after peptide stimulation. These data are representative of three experiments containing 8–12 mice per group. Bar, 1 SE.

**FIGURE 4.** Foxp3+ Treg ablation does not impact the expansion and activation of Ag-specific CD8+ T cells primed by recombinant *L. monocytogenes*-OVA infection. A. Percent and number of OVA-specific CD90.1+ CD8+ T cells among splenocytes day 5 after *L. monocytogenes*-OVA infection in Foxp3-WT or Foxp3-DTR mice, each treated with DT 1 d prior and on the day of infection. B. Expression of CD25 and IFN-γ production by CD90.1+ (line histogram) or bulk CD8+ T cells (filled histogram) day 5 after *L. monocytogenes*-OVA infection in Treg-ablated (Foxp3-DTR) or Treg-sufficient (Foxp3-WT) controls. These data are representative of three experiments containing 10–12 mice per group.

CDS8+ T cells (Fig. 5C). To more specifically investigate the direct impacts of IL-12 stimulation on CD8+ T cells—as well as address the possibility that in vivo neutralization with anti–IL-12 Ab was incomplete or nonspecific because IL-23 also shares the same IL-12 p40 subunit—complementary experiments compared the expansion and activation of CD8+ T cells from IL-12R–deficient OT-1 and IL-12R–sufficient (WT) OT-1 TCR transgenic mice each on the CD45.2 background after adoptive transfer into CD45.1 recipients. As with the results after anti–IL-12 neutralization, no significant differences in expansion or IFN-γ production were found for IL-12R–deficient compared with WT OT-1 cells after stimulation with OVA peptide and *L. monocytogenes* infection (Fig. 5C). Therefore, although IL-12 can stimulate T cell activation and is produced in response to *L. monocytogenes* infection (3, 32, 42), the adjuvant effects of *L. monocytogenes* on the expansion and activation of peptide-stimulated CD8+ T cells do not require IL-12 stimulation.

Reduction Treg suppressive potency after *L. monocytogenes* infection

Given the potential for pathogen-associated ligands to control Treg suppression through cell-intrinsic TLR stimulation, or indirectly by activating APCs (20–24, 43–45), we investigated whether overriding the effects of Treg suppression after *L. monocytogenes* infection may reflect infection-induced shifts in Foxp3+ cell po-
The potential mechanisms whereby Foxp3\(^+\) Tregs mediate immune suppression are broadly divided into those that directly suppress effector T cell activation and others that suppress the activation of dendritic cells and other APCs (46, 47). However, dissociating the relative importance of each is complicated by the activation of both effector T cells and APCs after Foxp3\(^+\) cell ablation (19). To test this hypothesis, we compared the suppressive potency of Tregs isolated before and after L. monocytogenes infection in Foxp3\(^+\)GFP mice, where Foxp3\(^+\) cells can be purified by FACS based on GFP expression (26). We found infection did not interfere with the isolation of Tregs because GFP\(^+\) Foxp3\(^+\) cells were purified to the same extent (98–99% purity) before and at each time point postinfection (Fig. 6A). To identify potential shifts in suppression, the relative potency whereby purified GFP\(^+\) Tregs inhibit the proliferation of CFSE-labeled responder cells in coculture after stimulation with anti-CD3 Ab was enumerated. GFP\(^+\) Tregs isolated from mice within the first 3 d after L. monocytogenes infection, compared with Tregs from naive mice, were less potent at suppressing the proliferation of responder cells (Fig. 6B, 6C). In turn, CFSE dilution among responder cells progressively increased after coculture with GFP\(^+\) Tregs from mice within the first 3 d after L. monocytogenes infection, compared with cells from naive mice (Fig. 6B, 6C). By titrating the ratio of Treg to responder cells in coculture, which allows a semiquantitative assessment for these shifts in suppressive potency, we find ~4-fold reductions in suppressive potency for Tregs isolated from mice on day 3 after L. monocytogenes infection, compared with cells from naive mice (Fig. 6B). Thus, although TLR stimulation through pathogen-associated ligands and inflammatory cytokines can trigger either increased or diminished Treg suppression potency after stimulation in vitro (20–24, 43–45), the cumulative effects of in vivo infection with intact L. monocytogenes and the ensuing immune response cause reductions in Foxp3\(^+\) Treg suppressive potency.

Ag-pulsed dendritic cells do not override the impacts of Treg suppression

The potential mechanisms whereby Foxp3\(^+\) Tregs mediate immune suppression are broadly divided into those that directly suppress effector T cell activation and others that suppress the activation of dendritic cells and other APCs (46, 47). However, dissociating the relative importance of each is complicated by the activation of both effector T cells and APCs after Foxp3\(^+\) cell ablation in vivo (19). To test this potential importance whereby Treg suppression would be triggered by dendritic cells controls T cell priming, we enumerated the impacts of Foxp3 cell ablation on the expansion and activation of OVA-specific CD8\(^+\) T cells after stimulation with peptide-pulsed JAWS II dendritic cells (34, 35). These cells are a representative model for activated dendritic cells because JAWS II cells maintained in vitro constitutively upregulate the expression of molecules required for CD8\(^+\) T cell stimulation such as H-2K\(^b\) (MHC class I) and CD80 (costimulation), to a similar or even greater extent, compared with CD11c\(^+\) cells isolated ex vivo from Treg-ablated or L. monocytogenes-infected mice (Fig. 7A). Comparatively, each of these activation parameters on JAWS II cells or CD11c\(^+\) cells isolated directly ex vivo after L. monocytogenes infection or Treg ablation is significantly elevated compared with those parameters on CD11c\(^+\) cells from Treg-sufficient uninfected control mice (Fig. 7A). Interestingly, the in vivo impacts of Treg suppression were sustained even when OVA\(257–264\) peptide-pulsed JAWS II dendritic cells were used for stimulation because significantly more OVA-specific CD8\(^+\)CD45.1\(^+\) mice, and on day 5 after stimulation with OVA peptide and L. monocytogenes-10403s (bottom). Percent of IFN-\(\gamma\) production for each group of OT-1 cells (line histogram) or bulk CD8\(^+\) T cells (filled histogram) after peptide stimulation. These data are representative of three experiments containing 8–12 mice per group.

![FIGURE 5](https://example.com/figure5.png)
Tregs IMPEDE THE PRIMING OF PROTECTIVE CD8+ T CELLS

Dendritic cell does not override the in vivo impacts of Treg suppression on the expansion and activation of Ag-specific CD8+ T cells. Taken together, these results indicate Foxp3+ Treg suppression of dendritic cells, compared with effector T cells, plays a less significant role in impeding Ag-specific CD8+ T cell priming.

Discussion

Although the Foxp3+ subset of regulatory CD4+ T cells was initially identified on the basis of the essential role of these cells in maintaining peripheral tolerance and suppressing potentially detrimental self-reactive immune responses (15–17), accumulating evidence indicates that the importance of Foxp3+ Tregs readily extends to controlling the immune response against nonself pathogen-associated Ags. In this regard, although the relative impacts of Treg ablation on infection susceptibility have been characterized in numerous models of experimental infection or immunization (12, 50–54), the specific limitations imposed by Foxp3-expressing cells on the priming and expansion of Ag-specific T cells after stimulation with purified Ags have not been clearly identified. In this article, we enumerated the impacts of Treg ablation on the expansion and activation of CD8+ T cells with specificity for a defined nonself Ag after stimulation with cognate peptide. We find that transient ablation of Foxp3+ Tregs during stimulation with purified peptide without adjuvant was sufficient to prime the robust expansion and activation of Ag-specific CD8+ T cells (Fig. 1). Furthermore, CD8+ T cells stimulated with peptide in the absence of Tregs contract from peak expansion levels, are sustained through day 30 after stimulation, and provide protection in an Ag-specific fashion against L. monocytogenes infection (Fig. 2). Of interest, although stimulation with OVA257–264 peptide in Treg-ablated mice confers protection against subsequent L. monocytogenes-OVA infection, the similar number of bacterial CFUs after L. monocytogenes-OVA infection, compared with L. monocytogenes 10403s infection, in peptide-stimulated Treg-sufficient mice suggests that the modest expansion of OVA-specific CD8+ T cells primed by peptide in the presence of Tregs does not confer significant protective effects. These results are consistent with the functionally tolerant phenotype of CD8+ T cells stimulated in vivo with purified peptide without adjuvant (3, 38). Similarly, the delayed re-expansion of OVA-specific T cells after L. monocytogenes-OVA infection in these mice is also consistent with previously reported reductions in CD8+ T cell expansion after secondary stimulation with purified peptide plus LPS (3). However, the ability of these cells to re-expand at all indicates that stimulation with purified peptide under these experimental conditions was insufficient to prime a completely anergic response (55). Although our studies were not designed to address the degree of tolerance in Treg-sufficient mice that most likely reflects differences in the amount of peptide Ag used for stimulation (39), the more robust expansion and activation of CD8+ T cells in Foxp3+ cell-ablated mice clearly demonstrate that Tregs actively suppress and impose critical barriers for priming protective Ag-specific T cells under these noninflammatory, noninfection stimulation conditions.

We show that the impacts triggered by transient ablation of Foxp3+ expressing cells on the activation of peptide-stimulated T cells are in agreement with the enhanced vaccine-induced immunogenicity associated with anti-CD25 Ab coadministration (54), as well as reductions in Treg suppression triggered by many immune adjuvants and purified pathogen-associated ligands that stimulate immune cells through Toll-like and other pattern recognition receptors (20–25). In this regard, although immune adjuvants have been characterized primarily for their ability to stimulate proinflammatory cytokines and/or upregulate co-stimulation signals on APCs (7–11), our finding that transient Treg
ablation during stimulation with purified peptide alone is sufficient to prime the expansion and activation of protective CD8+ T cells illustrates the critical barriers imposed by Foxp3+ cells. Therefore, in addition to direct stimulation of APCs, overriding immune suppression dictated by Tregs represents an important consideration in designing adjuvants for stimulating T cells in vivo.

In complementary experiments designed to enumerate the relative importance of Treg suppression in priming Ag-specific CD8+ T cells under infection conditions, the relative impacts of Treg ablation on the expansion and activation of CD8+ T cells primed by L. monocytogenes infection were enumerated. In sharp contrast to stimulation with purified peptide whereby Treg ablation results in >100-fold increased expansion of OVA-specific CD8+ T cells, Treg ablation caused no significant effects on the expansion of these same cells postinfection with recombinant L. monocytogenes that stably expresses a truncated form of OVA (Fig. 4) (36). Thus, the priming of Ag-specific T cells after L. monocytogenes infection, unlike peptide stimulation, is not subject to active suppression by Foxp3+ Tregs. Related experiments tracking Ag-specific CD8+ T cells after stimulation with purified peptide and infection with nonrecombinant L. monocytogenes revealed that these T cell stimulatory effects are not limited only to cells with specificity to L. monocytogenes-expressed Ag but also extend to those stimulated with peptide Ag during acute L. monocytogenes infection (Fig. 5). Thus, L. monocytogenes infection overrides suppression imposed by Foxp3+ Tregs that restricts the expansion and activation of T cells after stimulation under noninflammatory conditions.

The reduction in Treg potency after L. monocytogenes infection to stimulate IL-12 production, and the potency whereby this cytokine stimulates the activation of protective T cells (3, 32, 41, 42), the contribution of IL-12 to the adjuvant effects of L. monocytogenes infection was also evaluated. Complementary approaches using either neutralizing Ab or adoptively transferred T cells with targeted defects in the activation of protective T cells after stimulation with purified peptide and infection with nonrecombinant L. monocytogenes revealed that these T cell stimulatory effects are not limited only to cells with specificity to L. monocytogenes-expressed Ag but also extend to those stimulated with peptide Ag during acute L. monocytogenes infection (Fig. 5). Thus, L. monocytogenes infection overrides suppression imposed by Foxp3+ Tregs that restricts the expansion and activation of T cells after stimulation under noninflammatory conditions.

Figure 7, Ag-pulsed dendritic cells do not override the impacts of Treg suppression. A, Relative expression of MHC class I (H-2Kb) or CD80 on CD11c+ cells isolated directly ex vivo from naive Treg-sufficient mice (gray filled histogram and symbols), or mice day 5 after L. monocytogenes infection (red line histogram and symbols) or ablation of Foxp3+ Tregs (blue line histogram and symbols), compared with JAWS II cells maintained in vitro (black line histogram and symbols). B, Percent and number of OVA-specific CD90.1+ CD8+ T cells among splenocytes day 5 after stimulation with peptide pulsed or unpulsed JAWS II dendritic cells in the presence (Foxp3-WT) or absence (Foxp3-DTR) of Tregs. C, Expression of CD25 and IFN-γ production by CD90.1+ (line histogram) or bulk CD8+ T cells (filled histogram) day 5 after peptide stimulation in Treg-sufficient (Foxp3-WT) or mice ablated of Tregs (Foxp3-DTR) 1 d before and on the day of peptide inoculation. These data are representative of three experiments containing 8–12 mice per group. Bar, 1 SE.
pathogens that primarily cause persistent infection (53, 57–59). Therefore, whereas Treg suppression plays important roles in shaping the immune response that controls the severity of infection (12, 13), these findings also illustrate the potential whereby infection triggers changes in Treg suppression that control effector T cell activation. In the case of acute L. monocytogenes infection, reduced Treg suppressive potency likely permits the expansion of pathogen-specific T cells that eradicates infection. Moving forward, identifying the molecular basis whereby Tregs suppress T cell activation under noninfection conditions and discovering the specific signals triggered by infection that override Treg suppression are important areas for additional investigation and are required for designing therapies aimed at manipulating the fluid and shifting balance between immune stimulation and suppression that is controlled by Tregs.

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Disclosures

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References


Supplementary Figure 1. Foxp3+ Treg-ablation primes the expansion of endogenous peptide stimulated T cells. A. Percent and number of OVA-specific CD8+ T cells among splenocytes identified by staining with OVA257-264 H-2Kb dimer day 7 after stimulation with cognate peptide in the presence (Foxp3-WT) or absence of Tregs (Foxp3-DTR). Each group of mice received DT treatment one day before and on the day of peptide inoculation. These data are representative of three experiments containing 7-8 mice per group.
**Supplementary Figure 2.** DT induced cell death does not prime the expansion of peptide stimulated CD8⁺ T cells. Percent OVA-specific CD90.1⁺ CD8⁺ T cells day 5 after stimulation with cognate peptide with or without the DT induced ablation of CD19⁺ cells in CD19CreROSAIDTR mice (left). Percent CD19⁺ B cells among splenocytes with or without DT treatment in CD19CreROSAIDTR mice (right).