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Regulatory T Cells Selectively Control CD8+ T Cell Effector Pool Size via IL-2 Restriction

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Regulatory T cells (Treg) are key players in maintaining immune homeostasis but have also been shown to regulate immune responses against infectious pathogens. Therefore, Treg are a promising target for modulating immune responses to vaccines to improve their efficacy. Using a viral vector system, we found that Treg act on the developing immune response early postinfection by reducing the extent of dendritic cell costimulatory molecule expression. Due to this change and the lower IL-2 production that results, a substantial fraction of CD8+ effector T cells lose CD25 expression several days after activation. Surprisingly, such Treg-dependent limitations in IL-2 signaling by Ag-activated CD8+ T cells prevent effector differentiation without interfering with memory cell formation. In this way, Treg fine-tune the numbers of effector T cells generated while preserving the capacity for a rapid recall response upon pathogen re-exposure. This selective effect of Treg on a subpopulation of CD8+ T cells indicates that although manipulation of the Treg compartment might not be optimal for prophylactic vaccinations, it can be potentially exploited to optimize vaccine efficacy for therapeutic interventions. *The Journal of Immunology, 2011, 187: 3186–3197.

T he T cell limb of the adaptive immune system provides a crucial contribution to host defense. Ag-driven activation of specific precursors within the naive T cell pool by presentation of peptide–MHC molecule ligands in conjunction with costimulatory signals and differentiation-guiding cytokines leads to the development of acute effector cells and also the production of long-lived memory cells. The latter equip the host that survives an initial infection with the capacity to mount a more rapid and effective response upon re-exposure to the same organism should Ab fail to be protective on its own.

One key player in regulating the adaptive immune system is a population of CD4+ T cells called regulatory T cells (Treg). Foxp3 is an essential transcription factor for the development and function of Treg (1). These T cells, either produced during differentiation in the thymus (natural Treg) or induced actively among conventional T cells by a combination of Ag stimulation and cytokine exposure in peripheral sites (induced Treg), possess a variety of mechanisms that constrain effector T cell (Teff) responses. Among the many reported ways in which Treg depress effector immunity, the most well documented involve the production of immunosuppressive cytokines such as IL-10 and TGF-β and the expression of anticytotoxic molecules such as CTLA-4 (2–5). Additionally, in vitro studies established the interference of Treg with IL-2 production, primarily through limitation of cosignaling by DC but also by competition for availability of this cytokine, based on the high level of CD25 expression on this suppressive T cell subset (6, 7).

Although clearly playing a major role in maintaining tolerance to self, Treg have also been reported to affect the magnitude of T cell responses to infectious agents (8). Although a plethora of mechanisms regarding how Treg exert their function on conventional CD4+ T cells have been described in vitro, insights concerning the dominant in vivo mechanism(s) particularly with respect to CD8+ T cell responses are still lacking. Such insights are not only crucial for refining our understanding of Treg biology but are also pivotal in allowing for specific manipulation of Treg action without adversely affecting immune homeostasis.

During an acute infection, several subtypes of Ag-specific CD8+ T cells can be discriminated, based on changes involving expression of Bcl-2, cytokine receptors such as CD127 and CD25, homing molecules like CCR7 or CD62L (9), and transcription factors such as T-bet, eomesodermin, and Blimp-1 (10). A large fraction of activated cells are short-lived effector cells (SLEC; CD127lo, CD62Llo, Bcl-2lo, Bcl-6lo, T-bethi, and Blimp-1hi) that
migrate to the sites of infection, produce cytokines, kill infected cells, and then typically die themselves. A smaller number become long-lived memory cells that contribute to enhanced protection against future infection by the same organism. Both SLEC and memory T cells (Tmem) can be further divided into additional subpopulations. Effector cells can produce an array of cytokines (polyfunctional effectors) or may differentiate to a state in which they only produce a single cytokine (monofunctional effectors). Interestingly, the number of polyfunctional but not monofunctional T cells correlates with protection against Leishmania infection, whereas both populations likely contribute to immunopathology during an overt immune response (11). Tmem can be further divided into effector memory (EM) and central memory (CM) T cells. The former reside in peripheral tissues, whereas the latter are found in secondary lymphoid organs and have a high capacity for self-renewal.

The development of many of these CD8 T cell subpopulations is influenced by the cytokine IL-2. It contributes to the expansion of CD8\(^+\) T cells and plays a crucial role in programming and maintaining a functional memory CD8\(^+\) T cell response (12). Recently, it has become clear that very different levels of IL-2–dependent signaling are necessary for the development of distinct CD8\(^+\) T cell subsets. Although CM CD8\(^+\) T cells only seem to require low or transient exposure to IL-2, SLEC are critically dependent on high-level, prolonged signals from this cytokine (13, 14). These effects of such robust IL-2 signals on SLEC can be seen not just at the cellular but also at the epigenetic level (15).

Given these emerging data on a differential role of IL-2 on CD8\(^+\) T cell subsets and the central importance of IL-2 in Treg homeostasis, activation, and function, we sought to investigate whether Treg might help shape the nature of CD8\(^+\) T cell responses by exerting divergent effects on different CD8\(^+\) T cell subpopulations. To specifically address the relevance of this hypothesis in the context of T cell-directed vaccination, we used modified vaccinia virus Ankara (MVA), which, among other poxviruses, represents a mainstay viral vector system being evaluated in clinical trials of therapeutic and prophylactic vaccination (16). Therefore, results from this study could have a direct impact on future clinical studies involving live viral vector vaccines. Additionally, when manipulating the Treg compartment, the replication-deficient nature of the virus in vivo minimizes effects seen not just at the cellular but also at the epigenetic level (15).

For Treg depletion, mice were injected with 1 μg diphtheria toxin (DTX; Calbiochem) for three consecutive days starting on day 1 postvaccination unless otherwise stated. For Treg activation/amplification, 1 μg recombinant murine IL-2 (PeproTech) was incubated for 5–10 min at room temperature with 5 μg anti-IL-2 Ab (JES5-16; BioXcell) to allow for complex formation and the resulting material injected 3 d prior to immunization unless otherwise stated. For in vivo blocking studies, 150 μg anti-CTLA-4 (UC10-4B9; BioXcell) or isotype control (eBio299Arm; eBioscience) was given i.p. on days −1 and 0 prior to immunization. For blocking of IL-10, 500 μg anti–IL-10R (1B1.3A; BioXcell) or isotype control (2A3) was given i.v. 30 min before vaccination. TGF-β signaling was inhibited by repeated i.p. injections of a TGF-β RI kinase inhibitor II (400 μg/injection) (Calbiochem).

Materials and Methods

Mice

C57BL/6, MHC class II (MHC II) knockout (KO), IL-10 KO, CD40 KO, and CD86 KO mice were obtained from The Jackson Laboratory. DEREG mice were derived from in-house breeding under specific pathogen-free conditions following institutional guidelines. C57BL/6 CD45.1 congenic and OT-I TCR transgenic RAG1-deficient mice were obtained from Taconic Laboratories through a special contract with the National Institute of Allergy and Infectious Diseases. Foxp3-DTR mice were kindly provided by Dr. Alexander Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY).

For the generation of bone marrow chimeras, C57BL/6 CD45.1-congenic mice were gamma-irradiated with two doses of 600 rad from a cesium source and subsequently reconstituted with a mixture containing 5 × 10\(^6\) each of C57BL/6 CD45.1, MHC II KO, and CD40 KO (CD45.2) bone marrow cells. All animal procedures used in this study were approved by the Animal Care and Use Committee, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Treg manipulation and Ab treatment

For Treg depletion, mice were injected with 1 μg diphtheria toxin (DTX; Calbiochem) for three consecutive days starting on day 1 postvaccination unless otherwise stated. For Treg activation/amplification, 1 μg recombinant murine IL-2 (PeproTech) was incubated for 5–10 min at room temperature with 5 μg anti-IL-2 Ab (JES5-16; BioXcell) to allow for complex formation and the resulting material injected 3 d prior to immunization unless otherwise stated. For in vivo blocking studies, 150 μg anti-CTLA-4 (UC10-4B9; BioXcell) or isotype control (eBio299Arm; eBioscience) was given i.p. on days −1 and 0 prior to immunization. For blocking of IL-10, 500 μg anti–IL-10R (1B1.3A; BioXcell) or isotype control (2A3) was given i.v. 30 min before vaccination. TGF-β signaling was inhibited by repeated i.p. injections of a TGF-β RI kinase inhibitor II (400 μg/injection) (Calbiochem).

Vaccines and vaccination

MVA (cloned isolate Ilnew) expressing the entire OVA gene was generated as described previously (20). Female mice between 8 and 12 wk of age were vaccinated with 10\(^6\) IU MVA, i.v. or i.p. in total volume of 200 or 500 μl PBS, respectively. VV-expressing OVA was kindly provided by Drs. J. Yewdell and J. Bennink (National Institutes of Health).

DC isolation, analysis, and injection

To mature DC in vivo, mice were immunized with MVA i.v. the day before DC isolation. Spleen suspensions were digested for 30 min at 37°C with collagenase II and DNase I (Sigma-Aldrich) and then were treated for 5 min with EDTA. Then cells were washed, stained, and analyzed by flow cytometry.

Quantification of Ag-specific T cell responses and Ab staining

Splenocytes from vaccinated C57BL/6 mice were stimulated with either H-2Kb-presented VV-specific peptides A3L270–278, B11S25, or OVA257-264 or with a control peptide (galactosidase96) for 5 h in the presence of 1 mg/ml brefeldin A (Sigma-Aldrich) (21). Cells were stained with ethidium monooxide bromide (Invitrogen) and blocked with anti-CD16/CD32–Fc Block (BD Biosciences). Cells were stained with Abs specific for CD8
(5110: Caltag Laboratories), CD69 (H12F3; BioLegend), CD4 (LT4), CD11c (N418), CD25 (PC61), CD45.2 (104), CD62L (MEL-14), CD70 (FR70), CD80 (16-10A1), CD86 (GL1), killer cell lectin-like receptor G1 (KLRG1) (2F1), CD127 (A7R34), CTLA-4 (UC10-4B9), GTR (DTA-1), ICAM-1 (3E2), ICOS (7E17G9), and I-Ab (M5/114,15.2), all from BD Biosciences. Intracellular cytokine staining was performed with anti–IFN-γ (XMG1.2), anti–TNF-α (MP6-XT22; both from BD Biosciences), and anti–IL-2 (JES6-5H4; eBioscience) using the Cytofix/Cytoperm kit (BD Biosciences). FACS staining was performed using anti-Foxp3 (FJK-16s) and permeabilization buffers from eBioscience. The following tetramers were obtained through the National Institutes of Health Tetramer Facility: B8R30 and OVA257. Data were acquired by FACS analysis on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Quantitative RT-PCR

Spleens from mice treated with PBS or IL-2–Ab complexes were stabilized in RNAlater until further processing. Tissues were homogenized in TRIzol (Invitrogen), and aqueous phase-containing RNA was separated by addition of 1-bromo-3-chloropropane (Molecular Research Center). Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Quantitative RT-PCR for IL-2 was performed using FAM-labeled TaqMan MGB probes (Applied Biosystems). IL-2 mRNA levels were normalized to the housekeeping gene ACTB (actin).

In vivo cytotoxicity assay

Splenocytes were incubated in the presence of B8R30, OVA257, or control peptide for 45 min at 37°C and washed extensively. These splenocytes were labeled with CFSE (Invitrogen) at different concentrations, mixed at similar numbers, and adoptively transferred into immunized or naive hosts. At different times posttransfer, splenocytes were isolated and analyzed by FACS. Ag-specific killing was calculated based on the relative numbers the different labeled, peptide-pulsed splenocytes recovered from immunized animals in comparison with those recovered from naive hosts.

Cell labeling

Splenocytes or isolated OT-1 T cells were labeled with 1 μM Cell Tracker Green or 100 μM of Cell Tracker Blue (Invitrogen) as previously described (22).

Statistical analysis

All statistical analyses were performed using GraphPad Prism4 (GraphPad) or Excel software (Microsoft). Results are expressed as means ± SEs of the means. Differences between groups were analyzed for statistical significance using two-tailed Student t tests.

Online supplemental material

Supplemental Fig. 1 shows phenotypic changes of Foxp3+ cells after IL-2–Ab complexes treatment. Supplemental Fig. 2 shows in vivo cytotoxicity assay in the acute and memory phase after mock or DTX treatment in DEREG mice and also shows memory subpopulations in the acute phase combining CD62L/CD127 or KLRG1/CD127 staining. Supplemental Fig. 3 shows functional and phenotypical analysis of effector CD8+ T cells after mock or DTX treatment in DEREG mice using replication-competent VV-expressing OVA. Supplemental Fig. 4 shows CD25 expression on T cells and CD80/CD86 expression on DC in DEREG mice after mock or DTX treatment.

Results

Treg regulate acute but not memory or recall CD8+ T cell responses

Treg have previously been reported to suppress antiviral T cell responses, but neither the mechanisms by which they do so nor the impact on specific aspects of the cell-mediated response to viruses have been examined in detail. To study these issues during infection with virus, we used two strategies that allowed us to either amplify or to ablate regulatory T cells. To study T cell responses in the absence of Treg, we used a mouse model (DEREG) that allows for DTX-based selective depletion of this cell subpopulation (23). Treg depletion in this DTR model is transient (6–8 d) and therefore does not cause fatal autoimmunity in adult mice. This is a prerequisite for long-term analysis of the effects of Treg-mediated suppression on the adaptive immune response to vaccination. Enhancement of Treg suppression was achieved through the administration of IL–2–Ab complexes. As recently described (24), this treatment leads to specific proliferation and activation of Treg, including the upregulation of CD25, ICOS, GTR, CTLA-4, and ICAM-1 (Supplemental Fig. 1), without detectable activation of DC or conventional T cells in the absence of Ag administration. On day 8 after inoculation with a nonreplicating VV (MVA) encoding the model Ag OVA, we detected a robust CD8+ T cell response against the immunodominant viral epitope B8R, the subdominant epitope A3L, and OVA, as measured by intracellular IFN-γ staining after a brief in vitro stimulation with the respective immunogenic determinants (peptides) (Fig. 1A). When we depleted Treg through administration of DTX, we found a 2- to 3-fold higher response against B8R and OVA, but not against A3L. This suggested that Treg control the magnitude of the CD8+ T cell response to the B8R and OVA determinants. In agreement with this notion, amplification and activation of Treg by treatment with IL-2 complexes 3 d prior to immunization led to the opposite effect, a 2-fold decrease in the CD8+ T cell response against B8R and OVA as compared with PBS-treated control mice (Fig. 1B). The latter changes again occurred without an effect on the A3L response.

We next examined how the antiviral T cell response evolved over time in the presence or absence of Treg by performing a kinetic analysis of B8R-specific T cell numbers using multimer staining. Interestingly, we found that the differences seen at the peak of the immune response (day 8) in Treg-depleted mice as compared with control-infected animals diminished over time (Fig. 1C). Indeed, in the memory phase (day 60) we found no difference in the spleen with respect to the frequency of B8R-specific IFN-γ-producing or tetramer-binding T cells between mice that were depleted of Treg during the priming or mock-treated (Fig. 1E). An in vivo cytotoxicity assay provided data consistent with these findings. We found an increased killing capacity of Ag-specific CD8+ T cells at the peak of the acute response shortly after Treg manipulation but not in the memory phase (Supplemental Fig. 2A–C). Consistent with these data, recall responses at the day 60 time point were similar, irrespective of whether the mice were depleted of Treg or mock-treated in the initial priming phase (Fig. 1G). Notably, Ab titers in Treg-depleted or mock-treated animals were identical, arguing against different levels of virus neutralization during recall responses (data not shown). The kinetic analysis of B8R-specific T cell responses in mice that were treated with IL-2 complexes or mock-treated similarly revealed the transient effect of Treg-mediated suppression on CD8+ T cell responses (Fig. 1D). However, in contrast to Treg depletion (Fig. 1E) IL-2 complex-mediated Treg activation before priming did have a small but detectable effect on day 60 memory responses in the spleen (Fig. 1F). Importantly, recall responses at the day 60 time point were similar irrespective of whether the mice were depleted of Treg or treated with IL-2 complexes in the initial priming phase (Fig. 1G, 1H). These findings indicate that during a primary antiviral response, Treg primarily control the peak number of antiviral effector CD8+ T cells, with only a minor effect on the number of Tmem produced or the intrinsic capacity of those cells to mount a recall response.

Treg control the expansion of SLEC

Ag-activated CD8+ T cells can be divided into several subpopulations based on their capacity for cytokine production and their surface protein expression. Early after priming, CD8+ T cells can be classified as SLEC (CD62L+/CD127+ or KLRG1hi/CD127+), EM precursors (CD62Lhi/CD127hi), and CM
precursors (CD62L+/CD127+) (25, 26). In immunized mice following depletion of Treg, we found an increase in SLEC (CD62L- CD127- as compared with immunized mock-treated animals (Fig. 2A, Supplemental Fig. 2D). Conversely, IL-2 complex treatment before immunization led to fewer SLEC as compared with mock-treated animals (Fig. 2C). CD8+ T cell responses were analyzed using B8R- and OVA-specific multimers or ex vivo restimulation with B8R, OVA, A3L, and control peptides followed by intracellular IFN-γ staining. C and D show kinetic analysis of B8R-specific multimer-binding CD8+ T cells in the blood of immunized DEREG (C) or C57BL/6 (D) mice. Data are representative of three independent experiments. Bars show mean values; error bars show SEM. *p < 0.05, **p < 0.01.

FIGURE 1. Treg regulate primary but not memory or recall responses. Groups of mice (n = 4) were immunized with MVA OVA i.p. and analyzed for Ag-specific CD8+ T cell responses in the spleen on day 8 (A, B), day 60 (E, F), day 6 postrecall (G, H), or over time in the blood (C, D). DEREG mice were treated with DTX or mock-treated (A, C, E, G), and C57BL/6 mice were treated with IL-2/IL-2-Ab complexes or mock-treated on day -3 (B, D, F, H). CD8+ T cell responses were analyzed using B8R- and OVA-specific multimers or ex vivo restimulation with B8R, OVA, A3L, and control peptides followed by intracellular IFN-γ staining. C and D show kinetic analysis of B8R-specific multimer-binding CD8+ T cells in the blood of immunized DEREG (C) or C57BL/6 (D) mice. Data are representative of three independent experiments. Bars show mean values; error bars show SEM. *p < 0.05, **p < 0.01.

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In immunized mice following depletion of Treg, we found an increase in SLEC (CD62L+/CD127+) as compared with immunized mock-treated animals (Fig. 2A, Supplemental Fig. 2D). Conversely, IL-2 complex treatment before immunization led to fewer SLEC as compared with mock-treated animals (Fig. 2C). Importantly, when calculating total numbers of activated CD8+ T cells, the CM compartment remained unaltered irrespective of depletion or amplification of Treg during priming. This is in clear contrast to absolute numbers of SLEC and EM, which were strongly affected by Treg manipulation.

Similarly, when analyzing the cytokine profile of Ag-specific CD8+ T cells on day 8, we found a relative loss of polyfunctional (IFN-γ, TNF-α, and IL-2) CD8+ T cells and an increase in monofunctional (IFN-γ only) CD8+ T cells after Treg depletion as compared with mock-treated mice (Fig. 2B). In absolute numbers, CD8+ T cells that produced IL-2 in addition to IFN-γ remained largely unaltered, whereas IFN-γ only-producing cells were strongly increased. In contrast, IL-2 complex treatment led to a shift toward more polyfunctionality of virus-specific CD8+ T cells (Fig. 2D). These cytokine data fit well with the changes in numbers of SLEC and EM in the various treatment groups.

Thus, in line with the transient effect of Treg on adaptive antiviral immunity as shown above, we found that Treg primarily regulate the number of fully differentiated, monofunctional, short-lived Teff. In contrast, CM precursor and polyfunctional IL-2 producing antiviral CD8+ T cells are largely resistant to Treg-mediated control when this regulatory compartment is manipulated acutely just prior to vaccination.
As a control for both the transient depletion of Treg and potential effects of the IL-2 complexes beyond Treg stimulation, we used another Foxp3 DTR mouse model (27). In this model, DTX-mediated Treg depletion is complete, resulting in fatal autoimmunity in adult mice. Importantly, DTX-mediated depletion in this system showed similar results on day 8 after immunization as the DEREG model we applied previously (data not shown). Additionally, pretreatment with IL-2 complexes followed by Treg depletion showed a similar enhancement of SLEC as Treg depletion alone (data not shown). This argues against a significant effect of IL-2 complexes on SLEC beyond the Treg compartment. Finally, we observed a similar differential impact of Treg depletion on SLEC over CM when immunizing with replication-competent VV, suggesting that the observed effects of Treg manipulation on effector CD8+ T cell responses may apply more broadly than to the nonreplicating vaccine vector model described above (Supplemental Fig. 3).

FIGURE 2. Treg control the size of the monofunctional, short-lived Teff pool. Groups of mice (n = 4) were immunized with MVA OVA i.p. and analyzed on day 8. DEREG mice were treated with DTX or mock-treated on days 1–3 (A, B), and C57BL/6 mice were treated with IL-2/IL-2–Ab complexes or mock-treated on day –3 (C, D). A and C show representative plots and graphs with relative and absolute numbers of CD62L and/or CD127 expression of T cells binding to B8R-loaded multimers. B and D show representative plots and graphs with relative and absolute numbers of IL-2– and/or TNF-α–producing, IFN-γ+ cells after stimulation with B8R peptide. Data are representative of five independent experiments of each type. Bars show mean values; error bars show SEM. *p < 0.05, **p < 0.01.
IL-10 and TGF-β activity do not account for Treg-mediated suppression of antiviral immunity

IL-10 has been reported to be an important mediator of Treg suppression, especially in certain models of autoimmunity. Additionally, previous studies using a bacterial infection model revealed a direct role of IL-10 on the generation of Ag-specific CD8+ T cells. The kinetics of IL-10R expression on CD8+ T cells argues for a role early during priming (28). To address a possible role of IL-10 in the present viral model system, we investigated the effect of IL-2 complex treatment in IL-10 KO mice. We found that administration of IL-2 complexes induced activated Treg that effectively inhibited CD8+ T cell responses in IL-10 KO mice (Fig. 3A). In mice without manipulation of Treg number or activity, blockade of IL-10 signaling using IL-10R blocking Abs led to a significant increase of B8R-specific CD8+ T cells, especially among SLEC and EM cells (Fig. 3B). This is compatible with the well-established role of IL-10 in limiting both innate and adaptive immune responses (29). Nevertheless, when we increased Treg function by IL-2 complex treatment, we found a similar extent of suppression of B8R-specific T cell responses in the presence or absence of IL-10R blocking Abs, arguing against a dominant role for IL-10 in the regulation described in the preceding sections.

Another well-established mediator of Treg suppression is TGF-β, which has been shown to directly act on Ag-specific CD8+ T cells, causing apoptosis that especially affects SLEC. In contrast to IL-10, TGF-β seems to act rather late during priming (30). Application of a potent TGF-β kinase inhibitor did not have a significant impact on antiviral CD8+ T cells (Fig. 3C), and treatment with IL-2 complexes led to a similar suppression of virus-specific CD8+ T cells in the presence or absence of a TGF-β-specific inhibitor (Fig. 3C). In conclusion, neither IL-10 nor TGF-β seem to be central mediators of Treg suppression of adaptive antiviral immunity in the model systems studied in this paper.

Treg act early during priming

Because the obvious candidates (IL-10/TGF-β) for mediating Treg suppression did not account for the observed effects on SLEC generation, we decided to examine when during the evolving T cell response Treg execute their function to better understand how Treg act to affect SLEC numbers. To this end, we used the DEREG mouse model and depleted Treg by DTX at different times after priming, then analyzed the immune response as above (Figs. 1, 2). We found that the strongest increase of total multimer (B8R) binding CD8+ T cells and particularly of the SLEC subpopulation (CD62L+CD127- ) as analyzed on day 8 could be achieved by starting the depletion on day 1 postinfection (p.i.) (Fig. 4A). The positive effect of Treg depletion on SLEC generation diminished the later we began the toxin treatment. Indeed,
Treg regulate early during the immune response but do not inhibit initial proliferation of Ag-specific CD8+ T cells in vivo. A. Groups of DEREG mice (n = 4) were immunized with MVA OVA i.p. and analyzed for multimer+ B8R-specific immune response on day 8 postimmunization. Days on the x-axis represent initiation of Treg depletion (for three consecutive days) postpriming. Data show total numbers of B8R-specific T cells and numbers of SLEC (CD62L+/CD127-) B. Groups of B6 mice (n = 3) were immunized with MVA OVA i.p. and treated with IL-2/IL-2-Ab complexes or mock on day –3 before priming. After different time points postimmunization (days 1, 3, or 5), CFSE-labeled OT-1 T cells and celltracker blue-labeled control splenocytes were transferred, and CFSE dilution was assessed 3 d later. Cell counts were normalized to cotransferred control population to allow for estimation of differences in absolute cell numbers on day 8 as compared with mock-treated animals. Conversely, OT-1 T cells that were transferred into Treg-depleted animals showed enhanced CD25 expression (Supplemental Fig. 4A). In line with previous publications, we found a bimodal expression of CD25 on OT-1 cells 3 d postpriming. The fraction of OT-1 T cells maintaining high CD25 surface expression at this time was ∼2-fold less in IL-2-complex-treated mice as compared with PBS-treated animals, suggesting that expansion and activation of Treg was associated with a decreased availability of IL-2 that interfered with maintenance of high levels of CD25 on a substantial fraction of the previously activated cells.

**Treg limit the availability of IL-2**

In steady-state conditions, Treg are the only lymphocyte population expressing significant surface levels of CD25, accounting for the specificity of the IL-2 complex treatment when used prior to immunization. Although IL-2 signals seem to be dispensable for Treg generation, they are pivotal for survival, maintenance, and activity of the Treg compartment (31). In contrast, conventional T cells do not express CD25 during the steady state but quickly upregulate that receptor upon Ag encounter. Recently, several reports have highlighted the importance of IL-2 signaling for SLEC generation (32). In particular, it has been shown that prolonged IL-2 signaling after 3 to 4 d of Ag-driven differentiation specifically promotes SLEC formation. Treg have been shown to limit IL-2 production and have been also suggested to consume IL-2 (6, 7). Because CD8+ T cells require high levels of IL-2 for SLEC generation, we speculated that limited IL-2 availability might be the basis for the observed effects on SLEC generation (Fig. 2). The expression of the IL-2R CD25 is initially regulated by TCR signaling and then maintained and further increased by IL-2 signaling itself in a positive-feedback loop (33). Therefore, the level of CD25 expression is also an indicator of IL-2 signaling and reflects IL-2 availability. To analyze CD25 expression on Ag-specific T cells, we transferred OT-1 T cells into mice that had been pretreated with IL-2 complexes or PBS and followed their CD25 and CD69 expression over time (Fig. 5A). We found similar levels of CD25 on OT-1 T cells 8 and 24 h postpriming and comparable expression of CD69 at all time points analyzed. However, at 48 h and particularly 72 h p.i., CD25 expression was significantly reduced on a subpopulation of OT-1 T cells that had been transferred into mice pretreated with IL-2 complexes, as opposed to the OT-1 cells given to mock-treated animals. Conversely, OT-1 T cells that were transferred into Treg-depleted animals showed enhanced CD25 expression (Supplemental Fig. 4A). In line with previous publications, we found a bimodal expression of CD25 on OT-1 cells 3 d postpriming. The fraction of OT-1 T cells maintaining high CD25 surface expression at this time was ∼2-fold less in IL-2-complex-treated mice as compared with PBS-treated animals, suggesting that expansion and activation of Treg was associated with a decreased availability of IL-2 that interfered with maintenance of high levels of CD25 on a substantial fraction of the previously activated cells.

**Treg suppress IL-2 production by Ag-specific CD8+ T cells**

Next, we tested whether Treg cause such a limitation in IL-2 availability by consuming this cytokine in competition with the CD8+ T cells and/or by inhibiting IL-2 production. To this end, we performed quantitative PCR analysis of RNA from spleen, using the same experimental setting as in Fig. 5A. Interestingly, we found 5-fold fewer IL-2 transcripts in the spleen 8 and 24 h p.i. in IL-2-complex-treated mice as compared with samples from spleens of PBS-treated mice (Fig. 5B). At 48 h, there was still a 2-fold reduction in cytokine messages, whereas after 3 d, transcript levels were low and similar in both groups. To examine IL-2 production specifically within responding CD8+ T cells and on a protein level, we transferred OT-1 into mock or IL-2-complex-treated mice, immunized, and then harvested the spleens 12 h later. To assess direct ex vivo cytokine production of T cells, spleens were digested and the dissociated cells incubated in vitro without any additional stimulation for 5 h in the presence of brefeldin A. Intracellular staining for accumulated cytokines showed a significant reduction of IL-2, IFN-γ, and TNF-α production in OT-1 T cells primed in IL-2-complex-treated mice as opposed to mock-treated animals (Fig. 5C). Interestingly, cytokine...
production by OT-1 T cells inversely correlated with the frequency of Foxp3+ T cells found in mice (data not shown). From these data, we concluded that Treg reduce IL-2 production by CD8+ T cells, which becomes limiting around days 2 to 3, at a time when IL-2 seems to be crucial for SLEC generation (13).

To further examine the possible key role of Treg effects on IL-2 in determining the size of the antiviral CD8+ T cell immune response, we immunized mice and treated them with IL-2/Ab complexes or mock-treated for three consecutive days prior to immunization. OT-1 T cells were transferred on day −1 (2 × 10^6 for 8 and 24 h, 4 × 10^7 for 48 and 72 h) and analyzed in the spleen at different time points postpriming. A shows representative histograms of CD25 (upper panel) or CD69 (lower panel) expression on OT-1 T cells over time. Bar graph shows percent of CD25+ OT-1 T cells after 48 and 72 h p.i. B shows quantitative PCR of IL-2 mRNA from total spleen lysates from mice using the same experimental setup as in A. C shows a representative plot and bar graphs of mean florescent intensities of cytokines produced by OT-1 cells 12 h p.i. after 5 h ex vivo culture in the presence of brefeldin A without further stimulation. D and E show total numbers of IFN-γ–producing cells upon restimulation with the respective peptides or total numbers of multimer (B8R)-binding CD8+ T cell subsets at day 8. Graphs compare mock-treated mice or animals, which received IL-2/IL-2–Ab complexes 48 and 72 h post-priming. Data are representative of three independent experiments. Bars show mean values; error bars show SEM. *p < 0.05, **p < 0.01.

**FIGURE 5.** Treg depress CD25 expression and IL-2 production in Ag-specific CD8+ T cells. Groups of mice (n = 4) were immunized with MVA OVA i.v. and treated with IL-2/IL-2–Ab complexes or mock-treated for three consecutive days prior to immunization. OT-1 T cells were transferred on day −1 (2 × 10^6 for 8 and 24 h, 4 × 10^7 for 48 and 72 h) and analyzed in the spleen at different time points postpriming. A shows representative histograms of CD25 (upper panel) or CD69 (lower panel) expression on OT-1 T cells over time. Bar graph shows percent of CD25+ OT-1 T cells after 48 and 72 h p.i. B shows quantitative PCR of IL-2 mRNA from total spleen lysates from mice using the same experimental setup as in A. C shows a representative plot and bar graphs of mean florescent intensities of cytokines produced by OT-1 cells 12 h p.i. after 5 h ex vivo culture in the presence of brefeldin A without further stimulation. D and E show total numbers of IFN-γ–producing cells upon restimulation with the respective peptides or total numbers of multimer (B8R)-binding CD8+ T cell subsets at day 8. Graphs compare mock-treated mice or animals, which received IL-2/IL-2–Ab complexes 48 and 72 h post-priming. Data are representative of three independent experiments. Bars show mean values; error bars show SEM. *p < 0.05, **p < 0.01.

**Treg decrease the expression of CD80 and CD86 on dendritic cells in vivo**

It has been previously shown that Treg can decrease the expression of costimulatory molecules on dendritic cells (DC) in vitro. Therefore, we speculated that Treg might be able to regulate DC maturation even under highly inflammatory conditions such as viral infection in vivo and that these changes could impact the priming of CD8+ T cells and their cytokine production. We therefore immunized PBS- or IL-2 complex-treated animals with MV A i.v. and assessed the phenotype of splenic DC 24 h later (Fig. 6). MV A infection leads to a strong increase in CD86 expression and a moderate increase of CD80 and CD70 expression on DC (Fig. 6A). Importantly, CD80 and especially CD86 expression were diminished in expression on DC in IL-2 complex-treated animals. This effect of Treg could be partly reversed by Ab-mediated blocking of CTLA-4 in vivo. Of note, Treg depletion resulted in significantly higher levels of CD80 and CD70 expression on DC (Fig. 6B). Critically,
expression of both CD80 and CD86 was reduced on wt, CD40 KO, and MHC II KO DC in mice pretreated with IL-2 complexes to increase the number of activated Treg. These data indicate that Treg control the expression levels of CD80 and CD86 on DC in part via CTLA-4. Reduction of costimulatory molecule expression on DC by Treg did not require TCR–MHC II interactions in cis on the affected DC, though a requirement for Treg activation via TCR stimulation by wt DC to exert regulation on MHC II KO DC in trans cannot be ruled out.

**Reduced CD86 signaling leads to reduced IL-2 production and SLEC generation**

To look for a possible connection between the effect of Treg cells on DC maturation and the regulation of acute antiviral CD8+ T cell immunity, we analyzed the immune response elicited by MVA wt or PBS i.v. and treated with IL-2/IL-2–Ab complexes or mock-treated for 3 d prior to immunization. Anti-CTLA-4 blocking Ab or isotype control was injected 1d before and at the time of immunization. Twenty-four hours later, splenic DC were analyzed. Data show representative histograms of Foxp3 in CD4+ T cells and CD86, CD80 MHC II, and CD70 on CD11c+ cells, respectively. B, Triple bone marrow chimeras (n = 3) (CD40 KO, MHC II KO, and wt) were immunized with MVA wt i.v. and treated with IL-2/IL-2–Ab complexes or mock for three consecutive days prior to immunization. Twenty-four hours later, splenic DC were analyzed. Histograms show representative analysis of CD86 and CD80 expression by CD11c+ cells from three independent experiments.

**FIGURE 6.** Treg depress CD80 and CD86 expression on DC in a manner dependent on CTLA-4 and independent of MHC II. A, Groups of mice (n = 3) were immunized with MVA wt or PBS i.v. and treated with IL-2/IL-2–Ab complexes or mock-treated for 3 d prior to immunization. Anti-CTLA-4 blocking Ab or isotype control was injected 1d before and at the time of immunization. Twenty-four hours later, splenic DC were analyzed. Data show representative histograms of Foxp3 in CD4+ T cells and CD86, CD80 MHC II, and CD70 on CD11c+ cells, respectively. B, Triple bone marrow chimeras (n = 3) (CD40 KO, MHC II KO, and wt) were immunized with MVA wt i.v. and treated with IL-2/IL-2–Ab complexes or mock for three consecutive days prior to immunization. Twenty-four hours later, splenic DC were analyzed. Histograms show representative analysis of CD86 and CD80 expression by CD11c+ cells from three independent experiments.

**Discussion**

Treg have been reported to play diverse roles in regulation of host defense against infection, the control of autoimmunity, and anti-tumor responses. How they affect immunity to pathogens and regulate a vigorous effector response in vivo remains undefined. In this study, we provide evidence that Treg do not globally suppress adaptive immunity in response to a virus, but have a predominant and, indeed, near-exclusive effect on SLEC in physiological conditions of Treg activation during an emerging immune response. Only in artificial conditions of preactivation of Treg do we observe a modest impact on memory cell formation.

These observations of a very selective effect of Treg on one aspect of the evoked adaptive T cell response to infection raised the question of how such selectivity is achieved. Several factors have been shown to contribute to CD8+ Teff generation including IFN-γ, IFN-α/β, and IL-12 (17). The latter two have been established to be crucial signal three elements with varying relative importance depending on the system analyzed. Absence of IFN-α/β receptors or IL-12R on T cells can lead to complete loss of functional CD8+ T cell priming (34). So although these signals are important for generation of Teff, they also seem to be crucial for the generation of a robust memory response. A more compelling case can be made for a role of IL-2 availability in the effects we observe. Thus, in contrast to IL-12 signals that are required early after T cell priming (days 1–3), IL-2 signals seem to be pivotal for optimal T eff generation, particularly later during the response (after day 3). Indeed, absence of IL-2 sensing due to the absence of CD25 leads to a dramatic reduction of SLEC during primary Ag encounter, but also impacts Tmem responses (12, 14). The important conclusion that can be drawn from these latter studies is that low-level IL-2 signaling early during the response is sufficient to drive full memory differentiation, whereas prolonged availability of IL-2 is required for Teff generation. Therefore, late changes in IL-2 availability are expected to impact on T eff generation while sparing the formation of immunological memory.

In agreement with previous research, we found a striking inhibitory influence of Treg on the production of IL-2 by Ag-activated CD8+ T cells and a capacity for late administration of long-lived IL-2 complexes to rescue the SLEC response in the presence of Treg. Therefore, we conclude that Treg limit IL-2 availability and thus specifically inhibit the size of the Teff pool while leaving just enough IL-2 to allow for Tmem generation. In vivo experiments have shown that Treg are activated via IL-2 derived from primed Teff and that IL-2 seems to be dominantly derived from CD25 and acting in a paracrine fashion, demonstrating that IL-2 derived from Teff is in principle available to Treg (35, 36). This argues that besides restricting the production of IL-2, Treg might additionally restrict IL-2 availability by competing with Teff for this critical cytokine. In vitro experiments and mathematical models derived from those experiments strongly argue that Treg via their high CD25 expression can effectively outcompete developing Teff for limited IL-2 in their environment (7, 37, 38). As stated above, IL-2 is not the only contributor to Teff generation (17), and therefore, it is likely that Treg act on Teff in additional ways, particularly those that affect Teff survival. Although TGF-β did not seem to play a role in our viral infection model, Flavell and colleagues (30) reported opposing roles of TGF-β and IL-15 in regulating survival of Teff in a bacterial infection model; the source of TGF-β was not analyzed, but could have been from cell pool, but are sufficient for memory precursors to develop. These findings indicate that through this mechanism, Treg regulate the size of the Teff pool with little if any impact on the generation of Tmem.
Treg, Finally, Treg-derived IL-10 that seems to directly act on CD8+ T cells (Fig. 3) (28) has a potential role in Treg-mediated suppression. The relative contribution of these apparently auxiliary mechanisms may vary depending on the analyzed model. Yet, the common ground is that the primary target of Treg seem to be Teff rather than Tmem cells.

Using anti-CD25 Abs to inhibit Treg function, previous studies came to the conclusion that Treg control the magnitude of both the primary and memory CD8+ T cell response (39–42). This is in contrast to our finding showing no change in memory responses after depletion of Treg using DTX-based depletion of Foxp3-positive cells (Fig. 1E). There are several differences between our model and previous studies. First, Ab-mediated Treg depletion is not fully effective because not all Treg express CD25. Second, effects of anti-CD25 Ab treatment seem to be rather long lasting compared with the transient depletion using DTX-based mouse models. This latter point is of importance because it has been shown that anti-CD25 Ab treatment influences T cell contraction and memory CD8+ T cell homeostasis (40, 43). Third, anti-CD25 Ab treatment does not actually cause depletion of Treg but rather affects Treg functionality by blocking IL-2 signaling (44). Most importantly, the IL-2R α-chain (CD25) is not specific for Treg and is also found on activated CD8+ T cells, CD4+ T cells, and B cells. Given the central role of IL-2 for CD8+ T cell differentiation, anti-CD25 Ab treatment most likely has significant direct effects on CD8+ T cells beyond blocking Treg function. In a very recent study also using anti-CD25 treatment, the authors concluded that availability for IL-2 plays a central role in regulating the size of the CD8+ T cell response, yet a possible differential effect of Treg on subpopulations of CD8+ T cells was not addressed (45). That study found that anti-CD25 treatment increased the frequency of polyfunctional CD8 T cells and the memory response on day 21. Careful elucidation of relative versus absolute numbers of CD8+ T cell subpopulations as well as analysis of immune responses at later time points (day 60) was not investigated in that study.

It is very likely that the findings and underlying mechanism we describe in this study for CD8+ T cells similarly apply to CD4+ T cells. Indeed, Treg-mediated inhibition of IL-2 production in CD4+ T cells in vivo has been noted recently (46). In our experiments, we saw a similar impact on the size of Ag-specific CD4+ T cell responses on day 8 after priming, again with a dominant effect on the magnitude of the CD4+ Teff response, when manipulating the Treg compartment (data not shown). Notably, recent evidence indicates opposing roles of IL-2 on the differentiation of various Th cell subsets. IL-2 seems important for Th-1 cells, as in our study, yet inhibits generation of Th-17 cells (47, 48).

Although these studies underline the general importance of IL-2 for Th1 responses, our study demonstrates how Treg-controlled IL-2 signals regulate effector differentiation within such a response. The key platform upon which regulation takes place is the population of DC. They are the central interface linking innate and adaptive immunity, and they also contribute to the control of autoimmunity. They integrate Ag presentation and inflammatory cues and transfer that information to CD4+ and CD8+ T cells via MHC–TCR interaction and costimulatory receptors. Therefore, it seemed likely that Treg would regulate an immune response by manipulating the DC. In vitro experiments by Sakaguchi et al. (49) showed that Treg are able to change CD80 and CD86 surface expression on DC and that this depended on CTLA-4 expression on Treg. It was further shown that CTLA-4 expression on Treg and not Teff is crucial to prevent fatal autoimmunity (5, 50). In line with this, we found that in vivo, even under highly inflammatory conditions, Treg decrease costimulatory molecule expression by DCs, in particular CD86. This effect could be partially counteracted by blocking CTLA-4 (Fig. 6A) but did not seem to require MHC II–TCR interactions between the affected DC and Treg (Fig. 6B). Possibly, high-level expression of LFA-1 on Treg might be sufficient to engage DC to execute that function (Supplemental Fig. 1) (49). In an elegant model, it was recently demonstrated that Treg also regulate the extent of CD80 and
CD8+ T cells (Fig. 5) has an effect on the CD8+ T cell response in that model (57). G. Punkosdy, personal communication), nor CTLA-4 blockade the risk of subsequent autoimmunity. It should be noted that compartment and manipulate cell-mediated immunity for host benefit. Apies against infectious diseases and cancer designed to target the Treg availability of IL-2, which is required for the generation of SLEC. These results have important implications for vaccination and therapies against infectious diseases and cancer designed to target the Treg compartment and manipulate cell-mediated immunity for host benefit.

Our data thus point to two central components of Treg-mediated suppression: CTLA-4 that interferes with DC costimulation via reduced CD80/CD86 expression and CD25 to allow for Treg survival, activation, and effective competition for limited IL-2 during infection. Interestingly, studies concerning autoimmunity also identified these two pathways as being crucial components of Treg function (besides the central role of the transcription factor Foxp3). Based on these observations, it has been proposed that IL-2 and CD25-are core mediators of Treg suppression, whereas other mechanisms might represent auxiliary means of Treg-mediated regulation or have varying importance depending on the conditions/infections or tissues being analyzed (55). For example, Treg-derived IL-10 is important to control inflammation at mucosal surfaces (2), and the inflammatory pathology associated with IL-10 deficiency is largely restricted to the intestines and is eliminated in gnotobiotic mice lacking intestinal microbiota (29). Importantly, our work provides evidence that provision of IL-2 immune complexes can override Treg-mediated suppression (Fig. 5D, 5E). In a clinical setting, administration of such complexes could be a more feasible approach to boost CD8+ T cell immunity than trying to deplete the Treg compartment as a whole with the risk of subsequent autoimmunity. It should be noted that IL-2 treatment was detrimental when applied during acute lymphocytic choriomeningitis virus (LCMV) infection (56). However, neither Treg depletion, using the DEREG mouse model (G. Punksody, personal communication), nor CTLA-4 blockade has an effect on the CD8+ T cell response in that model (57). Because LCMV induces a massive expansion of T cells and high-level IL-2 production during the course of infection, one may conclude that LCMV possibly exceeds the limits of Treg-mediated suppression and that excess IL-2 further aggravates this condition. Consequently, the immune system would rely on other mechanisms to control immediate T effector responses (57).

As a consequence of Treg primarily regulating T cell over Tnem, manipulation of the Treg compartment might be highly beneficial in therapeutic settings aiming at the efficient induction of Teff, such as cancer therapy (58), but of little value for prophylactic vaccination, because numbers of multifunctional T cells (which correlate with protective immunity) remained unaltered, and Ab titers were not affected after Treg depletion (data not shown) (11).

In summary, we have used a viral vaccine model to explore the role of Treg during antiviral responses. Rather than broadly blunting the immune response, we find that Treg selectively limit the number of Teff generated while preserving the memory response. They do so by changing the amount of CD80 and CD86 displayed on DC and the availability of IL-2, which is required for the generation of SLEC. These results have important implications for vaccination and therapies against infectious diseases and cancer designed to target the Treg compartment and manipulate cell-mediated immunity for host benefit.

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References


