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

Wolfgang Kastenmuller, Georg Gasteiger, Naeha Subramanian, Tim Sparwasser, Dirk H. Busch, Yasmine Belkaid, Ingo Drexler and Ronald N. Germain

J Immunol 2011; 187:3186-3197; Prepublished online 17 August 2011;
doi: 10.4049/jimmunol.1101649
http://www.jimmunol.org/content/187/6/3186

Supplementary Material http://www.jimmunol.org/content/suppl/2011/08/19/jimmunol.1101649.DC1

References This article cites 58 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/187/6/3186.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulatory T Cells Selectively Control CD8+ T Cell Effector Pool Size via IL-2 Restriction

Wolfgang Kastenmüller,*1 Georg Gasteiger,†‡‖‡,1.2 Naeha Subramanian,*
Tim Sparwasser,§1 Dirk H. Busch,§‡‖‡ Yasmine Belkaid,§ Ingo Drexler,†‡‖‡‖‡ and
Ronald N. Germain*

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 immnosuppressive cytokines such as IL-10 and TGF-β and the expression of anticosstimulatory 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 mechanisms(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 CDS2L (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-2hi, 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 that are difficult to control when using replicating pathogens, such as changes in viral replication and secondary changes in inflammation as well as the duration of Ag presentation. This is highly relevant because these factors have a well-established role in influencing the heterogeneity of activated T cells (17). Therefore, MVA allows for distinction of direct versus indirect effects of Treg due to changes in pathogen clearance and consequently on Ag dose and the level of inflammation. Finally, MVA has the same cellular tropism as vaccinia virus (VV), it replicates its genome, it induces the entire cascade of viral gene expression, and the infected cells undergo apoptosis in a manner similar to VV-infected targets in vivo (18, 19). There is, however, a block in the assembly of virions, preventing the production of viral progeny. Therefore, MVA can serve as a well-controlled model of synchronized nonreplicating infection to study fundamental aspects of CD8+ T cell responses to complex pathogens (20).

Using MVA and an experimental approach that allowed us to either deplete Treg or enhance their suppressive capacity in vivo, we found that Treg selectively inhibit the generation of SLEC while preserving the induction of CM cells. This differential effect of Treg on CD8+ T cell subpopulations was due to a limitation in the availability of IL-2, a key cytokine required selectively for optimal SLEC generation. Such a limitation was achieved at least partly by Treg-mediated CTLA-4–dependent downregulation of DC-mediated CD80/CD86 costimulation and a consequent reduction of IL-2 production by Ag-specific T cells. Importantly, a well-timed administration of IL-2 during the developing immune response allows Treg-mediated suppression to be overridden and increases the amount of SLEC without impairing Treg function. Our study thus reveals an unappreciated differential effect of Treg on CD8+ T cell subpopulations and suggests that transient depression of Treg function may be a promising means of enhancing the efficacy of therapeutic vaccinations that require generation of a large number of acute effector cells, as in cancer immunotherapy. However, this manipulation may be of little value for prophylactic vaccinations, given the minimal effect of Treg depletion on memory CD8+ T cell numbers.

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 × 10⁶ 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 postimmunization 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 × 10⁶ each of C57BL/6 CD45.1, MHC II KO, and CD40 KO (CD45.2) bone marrow cells. All 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.

Viruses and vaccination
MVA (cloned isolate IInew) expressing the entire OVA gene was generated as described previously (20). Female mice between 8 and 12 wk of age were vaccinated with 10⁷ 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, B8R20, or OVA257 or with a control peptide (galactosidase) for 5 h in the presence of 1 µg/ml brefeldin A (Sigma-Aldrich) (21). Cells were stained with ethidium monoazide bromide (Invitrogen) and blocked with anti-CD16/CD32–F(ab)2 (BD Biosciences). Cells were stained with Abs specific for CD8.
Treg CONTROL SLEC VIA IL-2 RESTRICTION

(5110: Caltag Laboratories), CD69 (H12F3; BioLegend), CD4 (L2T4),
CD11c (N418), CD25 (PC61), CD45.2 (104), CD62L (MEL-14), CD70
(FR70), CD80 (16-10A1), CD86 (GL1), killer cell lectin-like receptor G1
(KLRG) (2F1), CD127 (A7R34), CTLA-4 (UC10-4B9), GITR (DTA-1),
ICAM-1 (3E2), ICOS (7E.17G9), and L-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). Foxp3 staining was performed using anti-Foxp3 (FSK-16b)
and permeabilization buffers from eBioscience. The following tetramers
were obtained through the National Institutes of Health Tetramer Facility:
B8R<sub>30</sub> and OVA<sub>357</sub>. Data were acquired by FACs analysis on an LSR II
flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree
Star).

Quantitative RT-PCR

Splenocytes 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 addi-
tion 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 (Ap-
plied Biosystems). IL-2 mRNA levels were normalized to the house-
keeping gene ACTB (actin).

In vivo cytotoxicity assay

Splenocytes were incubated in the presence of B8R<sub>30</sub>, OVA<sub>357</sub>, 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 de-
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 signifi-
cance using two-tailed Student t tests.

Online supplemental material

Supplemental Fig. 1 shows phenotypic changes of Foxp3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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
pre-requisite 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 ad-
ministration 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, GITR, 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<sup>+</sup> 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 de-
pleted 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<sup>+</sup> 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<sup>+</sup> 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 cyto-
toxicity assay provided data consistent with these findings. We
found an increased killing capacity of Ag-specific CD8<sup>+</sup> 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 re-
call 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<sup>+</sup> 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
in the initial priming phase (Fig. 1G, 1H). These findings indicate that during a primary antiviral re-
sponse, Treg primarily control the peak number of antiviral ef-
ector CD8<sup>+</sup> 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<sup>+</sup> T cells can be divided into several sub-
populations based on their capacity for cytokine production and
their surface protein expression. Early after priming, CD8<sup>+</sup> T cells
can be classified as SLEC (CD62L<sup>+</sup>/CD127<sup>+</sup> or KLRG<sub>hi</sub>/
CD127<sup>+</sup>), EM precursors (CD62L<sup>−</sup>/CD127<sup>+</sup>), and CM

Downloaded from http://www.jimmunol.org/ by guest on July 25, 2017
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. 2B, 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.

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 treatment. Indeed,
experiments. Bars show mean values; error bars show SEM. **

OM-1 T cell numbers. Data are representative of three independent

performed control population to allow for estimation of differences in absolute
dilution was assessed 3 d later. Cell counts were normalized to cotrans-

points postimmunization (days 1, 3, or 5), CFSE-labeled OT-1 T cells and

Ab complexes or mock on day

of DEREG mice (4)


FIGURE 4. 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 multimerB6R-specific immune response on day 8 postimmunization.
Days on the x-axis represent initiation of Treg depletion (for three con-
secutive days) postpriming. Data show total numbers of B6R-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 cotrans-
ferred control population to allow for estimation of differences in absolute
OT-1 T cell numbers. Data are representative of three independent
experiments. Bars show mean values; error bars show SEM. **p < 0.01.

Treg depletion on day 5 or later had no significant effect on total
SLEC numbers on day 8 as compared with mock-treated animals.
Importantly and in line with our previous results, total numbers of
CM remained unaltered irrespective of the timing of Treg de-
pletion (data not shown).

Given the well-established capacity of Treg to inhibit T cell
division in vitro, we decided to compare the proliferation of
adaptively transferred OT-1 T cells by CFSE dilution in mock-
or IL-2 complex-treated mice to see if the effect of Treg on SLEC was
mediated by a change in the extent of cell division. We transferred
CFSE-labeled OT-1 T cells on days 1, 3, or 5 post-virus inoculation
and analyzed the transferred cells 3 d later, covering time points
within and outside the window of Treg-mediated suppression (Fig.
4B). Surprisingly, we found no differences in OT-1 CFSE dilution
with respect to number of divisions as well as total numbers be-
tween IL-2 complex- and mock-treated animals, suggesting that
Treg influence neither the recruitment into cell cycle nor the early
cell divisions of virus-activated CD8+ T cells. Because Treg
seemed to exert their suppression during days 1–3 p.i., leading to
significantly reduced T cell numbers on day 8, we concluded that
Treg manipulated the programming of CD8+ T cells early during
the initiation of the immune response. To address this presumptive
difference in CD8+ T cell programming, we decided to further
characterize the phenotype of OT-1 T cells primed in IL-2 com-
plex- versus mock-treated animals.

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 im-
munization. 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 ex-
pression 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 expres-
sion of CD69 at all time points analyzed. However, at 48 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 pub-
llications, 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 mainte-
nance 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 pro-
duction 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/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 fluorescent 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 postpriming. Data are representative of three independent experiments. Bars show mean values; error bars show SEM. *p < 0.05, **p < 0.01.
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 after administration of CD86 blocking Abs. On day 8 after priming, we found significantly reduced CD8+ T cell responses against B8R and OVA as measured by IFN-γ production in mice in which we blocked CD86 as compared with isotype-treated control mice, resembling IL-2/IL-2–Ab complexes or mock-treated for 3 d prior to immunization. Anti–CD86 blocking Ab or isotype control was injected 1 d 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 1 d 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.

**TABLE 1.** Summary of Treg effects on IL-2 production in Treg cell-overexpressed mice.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Mouse Line</th>
<th>IL-2 Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased IL-2 production</td>
<td>Treg cell-overexpressed</td>
<td>High</td>
</tr>
<tr>
<td>Reduced IL-2 production</td>
<td>WT mice</td>
<td>Low</td>
</tr>
<tr>
<td>Increased IL-2 production</td>
<td>Treg cell-overexpressed</td>
<td>High</td>
</tr>
<tr>
<td>Reduced IL-2 production</td>
<td>WT mice</td>
<td>Low</td>
</tr>
</tbody>
</table>

**Discussion**

Tregs 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+ effector T cell 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 T eff, 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 T eff 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 T eff and that IL-2 seems to be dominantly acting in a paracrine fashion, demonstrating that IL-2 derived from Treg 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 T eff 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 T eff for IL-2 complexes or Isotype IgG/mouse. After 7 d, Treg cells were transferred into mice and treated with anti-CD86 or isotype control Abs 3 h prior to infection with MVA OVA, harvested the spleens, and assayed for intracellular cytokine production as above (Fig. 5C). We found a significant reduction of IL-2 and IFN-γ production by OT-1 T cells when mice were treated with anti-CD86 (Fig. 7C). Altogether, these data suggest a model in which Treg control expression of CD80 and particularly CD86 on DC in part in a CTLA-4-dependent manner. This in turn can lead to decreased cytokine production by Ag-reactive T cells interacting with these presenting cells. Diminished IL-2 levels yield a reduced effector cell pool, but are sufficient for memory precursors to develop. These findings indicate that through this mechanism, Treg regulate the size of the T eff pool with little if any impact on the generation of Tmem.
FIGURE 7. CD86 signaling is required for optimal IL-2 production in Ag-specific CD8+ T cells. Groups of mice (n = 5) received anti-CD86 or isotype Abs 3 h before i.p immunization with MVA OVA and CD8+ T cell immune response were analyzed in the spleen on day 8. Graphs show relative numbers of IFN-γ–producing CD8+ T cells after restimulation with the respective peptides (A) or total numbers of multimer (B8R)-binding CD8+ T cell subsets (B). C shows a representative bar graph of mean fluorescent 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 analysis of CD8+ T cell immune responses in wt or CD86 KO animals. Graphs show relative numbers of IFN-γ–producing CD8+ T cells after restimulation with the respective peptides (D) or total numbers of multimer (B8R)-binding CD8+ T cell subsets (E). Data are representative of two (CD86 KO) or three (anti-CD86) independent experiments. Bars show mean values; error bars show SEM. *p < 0.05, **p < 0.01.

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 functional inhibition 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 MHCI–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. 5a) has a marked reduction of IL-2 mRNA transcripts in CD4+ and CD8+ T cells (Fig. 5E) (53). In vitro, Thornton et al. (6) found a marked reduction of IL-2 mRNA transcripts in CD4+CD25− when cocultured in the presence of Treg. The suppressive activity of Treg could be enhanced by blocking CD86 and was reversed after costimulation via CD28. Apart from IL-2, blocking of CD86 during MVA infection also resulted in diminished IFN-γ production by OT-1 T cells (Fig. 5C), which has been shown to contribute to the size of the Teff pool (54). Other costimulatory molecules like CD70 seemed resistant to Treg suppression.

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 autoreactivity 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 CTLA-4 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 autoreactivity. 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 DREG mouse model (G. Punksod, 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 Teff responses (57).

As a consequence of Treg primarily regulating Teff 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 polyfunctional 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.

Acknowledgments
We thank Alexander Rudensky for helpful discussions and the kind provision of Foxp3-DTR mice and Leon Stross and Theresa Asen for expert technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References


Fig. S1
Fig. S2

A

B

C

D

CD8+ Gate

Tet

CD8

B8R+ Gate

KLRG-1

CD127

B8R+ Gate

CD62L

CD127

mock

DTX

0 10 2 10 3 10 4 10 5

0 10 2 10 3 10 4 10 5

0 10 2 10 3 10 4 10 5

mock naive
d8
d90

CD45.1

CFSE

OVA

B8R con

CD8

CD127

KLRG-1

CD127

CD62L

mock DTX

CD8+ Gate

B8R+ Gate

B8R+ Gate

<FL5-A>: CD62L

<FL1-A>: KLRG1

<FL8-A>: CD127

<FL2-A>: B8R
Fig. S3

A

\[ \text{Bar graph showing the % Multimer+ of CD8 in B8R and SIIN.} \]

\[ \text{Mock} \quad \text{DTX} \]

\[ \text{B8R+ Gate} \quad \text{B8R} \quad \text{SIIN} \]

CD8+ Gate

<table>
<thead>
<tr>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD127</td>
</tr>
<tr>
<td>KLRG1</td>
</tr>
</tbody>
</table>

| Tet | CD8 |
|-----|
| KLRG-1 |
| CD127 |

| CD62L | CD127 |
|-------|
| CD8+ |


B

\[ \text{Bar graph showing the IFN+ cells in TNFα/IL-2.} \]

\[ \text{Mock} \quad \text{DTX} \]

\[ \text{ns} \]

\[ \text{ns} \]

\[ \text{ns} \]

\[ \text{ns} \]
Fig. S4

A

Paraortic LN  Mesenteric LN

CD25

B

CD80  CD86

CD80  CD86

MFI

**
Supplemental Material:

Figure S1

IL-2/IL-2 antibody complexes induce proliferation and activation of Treg in vivo. Foxp3-reporter mice (n=2) were treated with IL-2/IL-2 antibody complexes or mock for three consecutive days. One day later splenocytes were isolated and Treg were analyzed using several surface markers.

Figure S2

Enhanced in vivo killing after priming in the absence of Treg is observed in the acute but not in the memory phase.

Groups of DEREG mice (n=3) were immunized with MVA OVA i.p. and treated with DTX or mock on d1-d3. Congenic splenocytes were stained with different concentrations of CFSE and pulsed with B8R (bright), OVA$_{257}$ (intermediate) or control peptide (dim) before transfer (A). As control and for assessment of relative killing rates, stained and pulsed splenocytes were transferred into naïve and immunized previously depleted of Treg or mock-treated mice (B). On day 8 or d60 post prime, mice were analyzed for their capacity to lyse peptide pulsed splenocytes in vivo (C). Data are representative of three independent experiments. Bars show mean values, error bars show SEM, * p< 0.05.

(D) Treg control the size of the monofunctional, short-lived effector T cell pool: Groups of DEREG mice (n=4) were immunized with MVA OVA i.p., treated with DTX or mock-treated and analyzed on d8. Representative plots show relative distribution of KLRG-1, CD62L, and/or CD127 expression of T cells binding to B8R loaded multimers.
Figure S3

Treg control the size of the monofunctional, short-lived effector T cell pool during infection with replicating Vaccinia Virus.

Groups of DEREG mice (n=4) were immunized with 2x10^6 pfu VV OVA i.p., treated with DTX or mock-treated on d1- d3 and analyzed on d8. A shows representative plots and graphs with relative and absolute numbers of KLRG-1, CD62L and/or CD127 expression of multimer binding CD8 T cells. B shows absolute numbers of IL-2 and/or TNFα producing, IFNγ+ cells after stimulation with B8R peptide. Data are presented as mean ±SD and are representative of 3 independent experiments. ns= not significant, *=p<0.05, **= p<0.01, ***=p<0.001

Figure S4

Treg control CD25 expression on T cells and CD80/CD86 expression on DC and CD86 KO mice recapitulate Treg mediated suppression.

Groups of DEREG mice or littermates were immunized with MVA OVA i.p. treated with DTX on d1- d3 and analyzed on d3.5. OT-1 T cells were transferred on d-1 (4x 10^5) and analyzed on d3.5 in para-aortic or mesenteric LN. (A) shows representative histograms of CD25 expression on OT-1 T cells. B shows representative histograms and bar graphs of CD80/CD86 expression on DC harvested from mesenteric LN on d3.5.