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High Viral Burden Restricts Short-Lived Effector Cell Number at Late Times Postinfection through Increased Natural Regulatory T Cell Expansion

Samuel Amoah, Beth C. Holbrook, Rama D. Yammani, and Martha A. Alexander-Miller

Generating and maintaining a robust CD8⁺ T cell response in the face of high viral burden is vital for host survival. Further, balancing the differentiation of effectors along the memory precursor effector cell pathway versus the short-lived effector cell (SLEC) pathway may be critical in controlling the outcome of virus infection with regard to clearance and establishing protection. Although recent studies have identified several factors that have the capacity to regulate effector CD8⁺ T cell differentiation—for example, inflammatory cytokines—we are far from a complete understanding of how cells choose the memory precursor effector cell versus SLEC fate following infection. In this study, we have modulated the infectious dose of the poxvirus vaccinia virus as an approach to modulate the environment present during activation and expansion of virus-specific effector cells. Surprisingly, in the face of a high virus burden, the number of SLECs was decreased. This decrease was the result of increased natural regulatory T cells (Tregs) generated by high viral burden, as depletion of these cells restored SLECs. Our data suggest Treg modulation of differentiation occurs via competition for IL-2 during the late expansion period, as opposed to the time of T cell priming. These findings support a novel model wherein modulation of the Treg response as a result of high viral burden regulates late-stage SLEC number. *The Journal of Immunology*, 2013, 190: 000–000.

CD8⁺ T cells are a critical contributor to the clearance of viral infection. Following infection, naive Ag-specific cells will undergo marked expansion and in the process differentiate to become effector cells with the capacity to mediate viral clearance. After the peak of CD8⁺ T cell response, >90% of these cells will die during the contraction phase of the response, with the surviving cells giving rise to a stable self-renewing memory pool (1).

Recent data show that effector cells with increased potential to persist long term as self-renewing memory cells, termed memory precursor effector cells (MPECs), can be identified by the expression of the IL-7R- α (CD127) at the peak of CD8⁺ T cell expansion (2, 3). In contrast, short-lived effector cells (SLECs) are identified by the expression of KLRG1, a marker of replicative senescence (4). As suggested by their name, SLECs have limited potential to persist long term and are thought to be terminally differentiated (5, 6).

Inflammatory cytokines, shown to be important for the generation of optimal effector responses as well as memory formation (7), appear to drive these effector cells down the terminally differentiated pathway. For example, prolonged exposure to IL-2 has

been reported to promote SLEC generation (8). IL-12, another important proinflammatory cytokine, can also promote SLEC differentiation by increasing expression of the transcription factor T-bet (5, 9). Thus, in addition to providing general signals promoting naive CD8⁺ T cell activation, inflammation is a potent regulator of SLEC formation (10). Certainly, the inflammatory environment can differ across infections. Thus, it is perhaps not surprising that distinct mediators contribute to T cell differentiation when individual infectious models are interrogated. For example, IL-12 is dispensable for SLEC differentiation following infection with vaccinia virus (VV), lymphocytic choriomeningitis virus, or vesicular stomatitis virus, but not *Listeria monocytogenes* (11, 12).

Although initially associated with regulating the immune response to autoantigens by suppression of self-reactive T cells (13–15), CD4⁺CD25⁺ forkhead box protein 3 (Foxp3)-expressing cells, known as natural regulatory T cells (nTregs) (16), have also been shown to negatively regulate the immune response during viral infections. For example, depletion of nTregs by administration of anti-CD25 Ab, followed by infection with VV or HSV, resulted in elevated CD8⁺ T cell responses (17–19). As a result of their constitutive expression of the high-affinity IL-2 α -chain (CD25), nTregs possess the potential to take up significant amounts of IL-2 from the environment. In support of this, recent studies have implicated nTregs as a modulator of effector cell expansion through IL-2 uptake (20).

Although virus burden can exert significant influence over the size of the effector cell response (21, 22), it is not clear whether it regulates the differentiation of effector cells. Further, whether virus dose-dependent differences in the size of the response selectively have an impact on effectors in a differentiation-dependent fashion is unknown. In this study, we have addressed these questions by infecting mice with either a high or a low dose of VV. We observed that the virus-specific response in mice infected with a low dose of VV was skewed toward SLECs. The selective increase in SLECs in mice infected with low doses could not be explained by increased

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Abbreviations used in this article: DC, dendritic cell; EEC, early effector cell; MPEC, memory precursor effector cell; nTreg, natural regulatory T cell; SLEC, short-lived effector cell; Treg, regulatory T cell; VV, vaccinia virus.

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proliferation in this population. However, depletion of nTregs, which we found to be present in increased numbers at late times postinfection in mice infected with high doses, led to an increase in the number of B8R-specific cells and a skewing of the response toward SLECs. Treatment of mice with recombinant human IL-2 or depletion of regulatory T cells (Tregs) following the initiation of the response postinfection with the high dose resulted in increased SLECs, supporting a mechanism whereby Tregs regulate effector cells by IL-2 uptake at the postpriming stage. These data provide evidence for a role for viral burden in effector cell differentiation, with increased viral dose inhibiting the number of SLECs at the peak of the response through increasing the number of nTregs during the expansion phase.

Materials and Methods

Mice and infections

Female 6- to 10-week-old C57BL/6 mice (Frederick Cancer Research Facility, National Cancer Institute, Frederick, MD) were used throughout this study. Mice were maintained in the Wake Forest University School of Medicine animal facilities, under specific pathogen-free conditions and in accordance with approved Institutional Animal Care and Use Committee protocols. Infections were done with 1×10^6 (high dose) or $1-3 \times 10^4$ (low dose) PFU of VV-GP33 (23) delivered i.p.

Cell staining and flow cytometry

For detection of CD8⁺ T effector cells, a total of 1×10^6 spleen cells from VV-infected mice were incubated for 30 min on ice with B8R Tetramer (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA) together with anti-CD127 (SB/199; BioLegend), -CD44 (IM7; BioLegend), -KLRG1 (2F1/KLRG1; BioLegend), and -CD8 α (53-6.7; BD Biosciences) Abs. Cells were then washed twice and samples acquired on a BD FACSCanto II flow cytometer. For analysis of T-bet and eomes, cells were stained as above, followed by fixation and permeabilization using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) per the manufacturer's instructions. Cells were incubated with T-bet PE-Cy7 (eBio4B10; eBioscience) and eomes Alexa Fluor 488 (Dan11mag; eBioscience) diluted in 1 \times Permeabilization Buffer. Isotype controls were used in negative control samples. Active caspase 3 in B8R⁺ cells was assessed using anti-caspase 3, active form (C92-605; BD Biosciences), following permeabilization with BD Cytotfix/Cytoperm Kit per the manufacturer's instructions. For detecting Tregs, cells were stained with Abs specific for CD4 (GK1.5; BioLegend), CD44 (IM7; BioLegend), CD25 (PC61; BioLegend), CTLA4 (UC10-4B9; BioLegend), CD69 (H1.2F3; BioLegend), and GITR (YG1TR 765; BioLegend) Abs for 30 min on ice. After washing, cells were incubated for 20 min in the dark at room temperature in a Foxp3 Fix/Perm Buffer (BioLegend). Cells were then washed and incubated for 15 min in the dark at room temperature in Foxp3 Perm Buffer (BioLegend). Following washing, cells were stained with anti-Foxp3 (MF-14; BioLegend) in Perm Buffer for 30 min in the dark at room temperature, after which they were washed. Data were acquired using a BD FACSCanto II and analyzed using FlowJo software (TreeStar). For analysis of dendritic cells (DCs), mice received anti-CD25 (PC-61.5.3; Bio X Cell) Ab on day 1 prior to infection with the high virus dose. On day 2 postinfection, DCs in the spleen were assessed by flow cytometric analysis for the expression of CD80 and CD40. Cells with high levels of CD40 (3/32; BD Biosciences) and CD80 (16-10A1; BD Biosciences) were considered mature.

Analysis of p-STAT5

Spleens were isolated and immediately placed into 5 ml PBS containing 1.6% formaldehyde. The spleen was then dissociated and placed through a 70-micron filter followed by incubation at room temperature for 15 min. Subsequently, 20 ml ice-cold methanol was added with constant vortexing. Cells were placed briefly on ice and subsequently stored at -80°C . For staining, 0.5 ml of cells were removed and washed 3 \times in staining buffer. Cells were stained with Abs to CD4, Foxp3 (clone NRRF-30; eBioscience), CD25, and p-STAT5 (clone 47; BD Biosciences), and samples were acquired on a BD FACSCanto II cytometer.

Plaque assay

Spleen or ovaries from infected mice were homogenized and centrifuged, and serial dilutions of the supernatant were added to confluent BSC-1 cells in six-well plates. Plates were incubated for 2 h at 37°C , after which the

media were aspirated and 2 ml MEM + 10% FBS was added. At 24 h later, monolayers were stained with crystal violet and plaques were enumerated.

BrdU detection and recombinant IL-2 treatments

Infected mice received 500 μl BrdU (1.6 mg/ml BrdU; Sigma-Aldrich) by i.p. injection. At 5 h later, splenocytes were isolated and stained on ice for 30 min with B8R Tetramer (National Institutes of Health Tetramer Core Facility) together with Abs to CD127, CD44 (BioLegend), KLRG1 (Abcam), and CD8 α (BD Biosciences). BrdU staining was carried out according to the supplied protocol (BD Biosciences). Recombinant human IL-2 treatment was done as previously described (24). Briefly, mice received two daily injections of 15,000 IU of recombinant human IL-2 from days 3 to 6 postinfection.

nTreg depletion

The nTregs were depleted by injecting 400 μg anti-CD25 (clone PC-61) or isotype (Bio X Cell) Ab 1 d prior to infection with VV. For late nTreg depletion, mice were injected with 30 μg anti-FR4 (BioLegend) 2.5 d following infection with virus to prevent deleterious effects of anti-CD25 Ab on activated T cells. The nTregs were also depleted by administering 30 μg anti-FR4 1 d prior to infection.

Statistical analyses

Unless otherwise stated, all statistics were done using a two-tailed Student *t* test, with $p \leq 0.05$ considered significant.

Results

CD8⁺ effector responses are skewed toward SLECs following infection with a low versus high virus dose

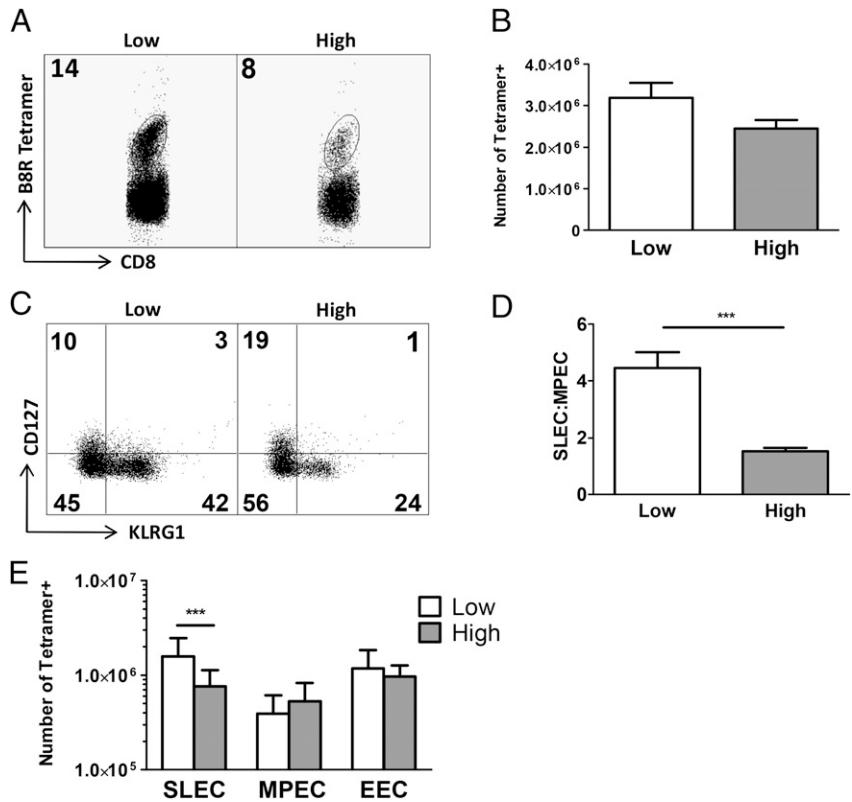
The differentiation of effector cells along the MPEC versus SLEC pathways is a highly regulated process. At present we know little about how viral burden perturbs this process. To address this question, C57BL/6 mice were i.p. infected with either a high (1×10^6 PFU) or low dose ($1-3 \times 10^4$ PFU) of VV. We then determined if the difference in virus would result in changes in the effector population generated to the immunodominant B8R₂₀₋₂₇ epitope. On day 7 postinfection, splenocytes were isolated and the number of MPECs and SLECs determined by staining with tetramer together with KLRG1 and CD127. SLECs were identified as KLRG1^{hi}CD127^{lo} and MPECs as KLRG1^{lo}CD127^{hi} (5). Early effector cells (EECs), which express neither KLRG1 nor CD127 (25), were also analyzed. On day 7 postinfection, mice infected with high-dose virus exhibited a decreased frequency of Ag-specific cells, ~ 2 -fold less when compared with mice infected with low-dose virus (Fig. 1A). When the total number of Ag-specific cells was determined, there was a trend toward more Ag-specific cells in the mice infected with low doses, although this did not reach statistical significance (Fig. 1B).

We next determined the differentiation state of the effector cells present in mice infected with disparate amounts of virus. This analysis showed a higher frequency of SLECs in animals infected with low- compared with high-dose virus (Fig. 1C), resulting in a skewing in the ratio of SLECs/MPECs within the total effector cell population (Fig. 1D). In agreement with these data, mice infected with the low dose of virus exhibited more SLECs than did those infected with high doses (Fig. 1E). No significant difference was found in the EEC or MPEC populations. The difference in the total number of B8R cells could be accounted for almost exclusively by this increase in SLECs in mice infected with low doses. Taken together, these data show that increasing the infecting dose of VV led to a selective decrease in the terminally differentiated SLEC population.

Infection with high-dose VV is associated with an increased viral burden

Increasing the viral dose administered to the animals should lead to increased viral burden in the spleen at early times postinfection, the

FIGURE 1. Infection with a low or high dose of VV skews the response toward more SLECs in mice infected with low dose. Wild-type C57BL/6 mice were i.p. infected with either a high or a low dose of VV. At 7 d postinfection, spleens were harvested and the immunodominant B8R-specific population was assessed. **(A)** Representative dot plots showing the frequency of B8R⁺ CD8⁺ T cells in mice infected with low- or high-dose virus. Cells were prepared for analysis of the CD8⁺ CD44^{hi} population. **(B)** Averaged data showing the total number of B8R-specific CD8⁺ T cells. **(C)** Representative dot plot of B8R⁺ CD8⁺ T cells stained with anti-KLRG1 and anti-CD127 Abs. The upper left quadrant represents MPECs, the lower right quadrant SLECs, and the lower left quadrant EECs. Cells were prepared for analysis of the CD8⁺ CD44^{hi} population. **(D)** Ratio of the frequency of SLECs to MPECs averaged across multiple mice. **(E)** Averaged data showing the number of SLECs, MPECs, or EECs within the B8R⁺ CD8⁺ T cell population. Statistical analysis was performed using a Student two-tailed *t* test. Averaged data are from ≥23 mice assayed in a minimum of 7 independent experiments. ****p* < 0.001.



window for priming the CD8⁺ T cell response. In addition, we would expect the higher dose to increase systemic virus at later times (days 5–7), the point at which the effector population is undergoing continued expansion and differentiation. To assess viral load during the priming phase, spleens were isolated from mice on days 1–3 following infection. On days 1 and 2 postinfection, virus was detected in animals infected with the high virus dose, whereas virus was below the limit of detection in animals infected with the low dose (Fig. 2A). By day 3, virus began to decrease in the spleens of animals infected with the high virus dose, so that no statistically significant difference was seen between the two groups of mice.

To assess systemic virus present at later times postinfection, we measured viral load in the ovaries at days 5 and 7 postinfection. In agreement with what was observed in the spleen at early times, infection with the higher viral dose resulted in high levels of systemic virus (Fig. 2B). Thus differences in viral burden were evident throughout both the T cell activation and expansion phases. As a result, dose-associated differences in the environment could presumably regulate both early and late phases of the immune response.

The increase in SLEC/MPEC ratio is apparent at multiple times postinfection

We next determined how differences in viral burden affected the kinetics of SLEC versus MPEC generation, as it was possible that the skewing we observed at day 7 postinfection was a reflection of increased kinetics of SLEC generation or expansion in these animals. The total number of B8R-specific cells, as well as the number of B8R-specific MPECs and SLECs, was assessed on days 4–8 following infection. Irrespective of the infecting dose, the peak of the B8R-specific response occurred on day 7 (Fig. 3A). We found that the number of B8R-specific cells was significantly higher at early times in the expansion phase (days 4 and 5) following infection with the higher virus dose. However, by day 6 the difference in the number of B8R-specific cells between the two groups of animals was much less pronounced, with a trend toward higher numbers in the animals infected with the lower virus dose at days 7 and 8. Thus, infection with the high dose of virus resulted in a larger effector cell response at early times; however, the response in animals infected with the low dose of virus underwent rapid expansion between days 5 and 6 that resulted in a similar number of B8R-specific cells at this timepoint and eventually a trend toward higher numbers at later times.

We next evaluated differentiation within the effector population over the course of the response. At day 5, the ratio of SLECs/MPECs was relatively similar (Fig. 3B). The difference at day 4

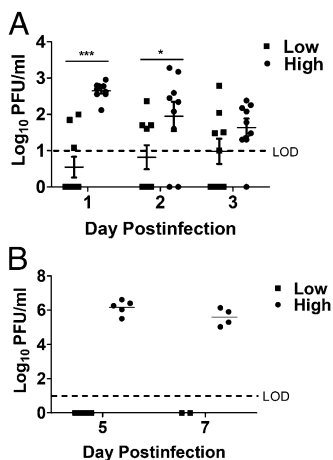


FIGURE 2. Infection with high-dose VV is associated with an increased viral burden. C57BL/6 mice were infected with a high or low dose of VV i.p. **(A)** On days 1–3, viral burden in the spleen was assessed by plaque assay. **(B)** Virus in the ovaries was measured on days 5 and 7 postinfection. Statistical analysis was performed using two-way ANOVA. **p* < 0.05, ****p* < 0.001. LOD, Limit of detection.

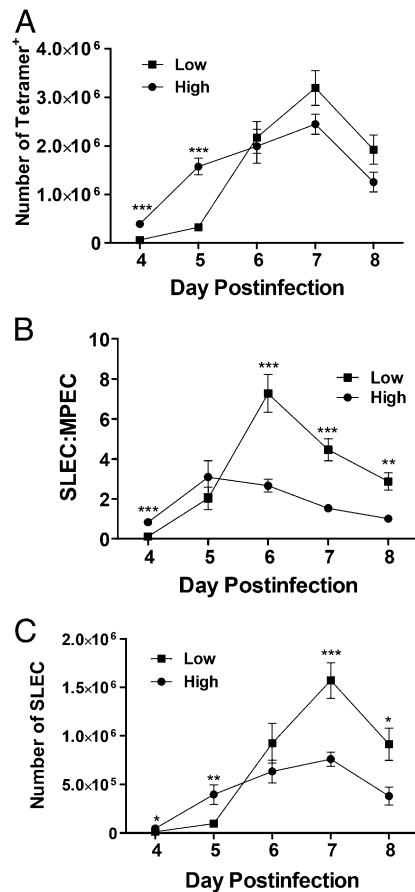


FIGURE 3. The increased SLEC/MPEC ratio is present only at late times postinfection. C57B/6 mice were infected with either a high or a low dose of VV i.p. On days 4–8 following infection, spleens were harvested and the immunodominant B8R-specific population was assessed. **(A)** Total number of B8R-specific cells, **(B)** B8R⁺ SLEC/MPEC ratio, and **(C)** B8R⁺ SLEC numbers over time. Data shown are the average of 10 mice for day 4, at least 21 mice on day 5, at least 13 mice on day 6, at least 23 mice on day 7, and 6 mice on day 8. Statistical analysis was performed using a Student two-tailed *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

was the result of the very low number of SLECs present at this time following infection with the low virus dose (Fig. 3C). By day 6 the skewing toward SLECs in mice infected with the low dose was readily observed and continued through day 8 (Fig. 3B). This skewing in ratio was associated with a robust increase in the number of SLECs present in mice infected with the low virus dose (Fig. 3C). On the basis of these data, we conclude that the increased ratio of SLECs/MPECs observed following infection with the low dose is not a transient effect related to a difference in the kinetics of B8R-specific T cell generation. Further, the increased representation of SLECs within the population was not evident until day 6 and thus was consistent with regulation at a late stage of the response.

B8R⁺ cells undergo increased proliferation on days 5–7 postinfection in mice infected with low doses

One possible explanation for the increased skewing toward SLECs in mice infected with low-dose virus was a selective increase in proliferation within this population. To test this possibility, animals received a 5-h pulse with BrdU on day 5 or 7 postinfection. Analysis of the BrdU⁺ populations showed that mice infected with the low virus dose exhibited an increase in proliferation at both timepoints, compared with mice infected with the high virus dose

(Fig. 4). This was the case for all of the populations (MPECs, SLECs, and EECs). Although SLECs appeared to be the most highly proliferating population at day 5 postinfection, MPECs exhibited higher levels of BrdU⁺ cells at day 7. Although effector cells were actively dividing in mice infected with the high virus dose at day 5 postinfection, by day 7 limited evidence of proliferation was observed. These data show that a selective increase in proliferation within the SLEC population in mice infected with the low dose of virus cannot account for the skewing in this population.

SLECs from animals infected with high and low doses exhibit similar levels of active caspase 3 positivity

Another possible explanation for the decreased number of SLECs in effectors following infection with the high virus dose was increased apoptosis in this population. To test this possibility, we examined cells for the presence of active caspase 3 at day 6 and day 7 postinfection. As shown in Fig. 4E and F, no evidence was found of increased apoptosis in SLECs from animals infected with high doses. In fact, apoptosis was minimal at these times. Thus, the reduced number of SLECs in animals infected with high doses cannot be explained by increased apoptosis in these cells.

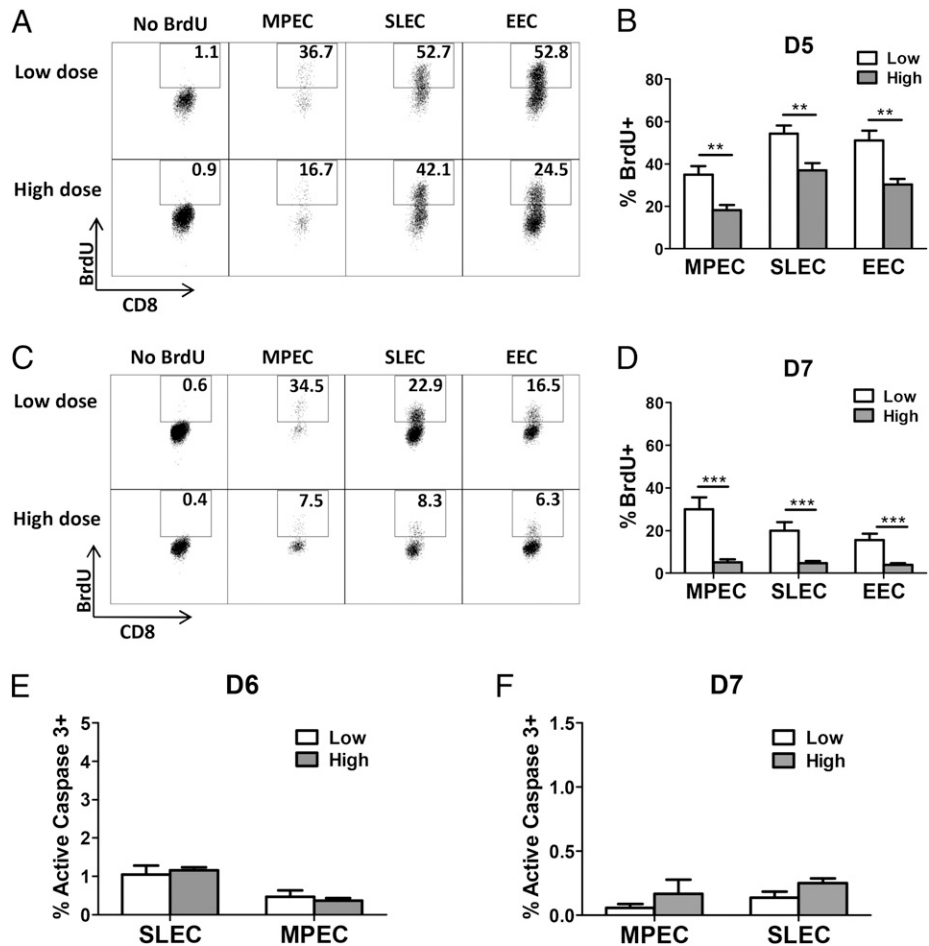
B8R⁺ cells from animals infected with low doses exhibit an increased level of T-bet at day 5 postinfection

Inflammatory signals that promote SLEC generation also increase expression of the transcription factor T-bet (5). Thus, we determined whether B8R⁺ effector cells from animals infected with low doses exhibited increased expression of T-bet. EEC, MPEC, and SLEC populations were evaluated on day 5 and day 6 postinfection. On day 5 postinfection, the expression of T-bet was significantly higher in EECs and MPECs from mice infected with the low dose of virus (Fig. 5A). T-bet expression was also increased in SLECs (although this did not reach statistical significance; *p* = 0.067). Of interest, the level of eomes was also increased in these effectors (Fig. 5B). By day 6, levels of T-bet were similar in effectors generated following infection with the high versus low dose of virus (Fig. 5C), whereas the decrease in eomes was sustained in effectors from mice infected with the high virus dose (Fig. 5D). Although the role of T-bet and eomes under these conditions is not fully elucidated from these studies, the altered expression of these molecules in effectors from mice infected with the high versus low virus dose is in agreement with the differential regulation of effector differentiation observed. Further, the reduced expression of T-bet at day 5 in the animals infected with high doses is consistent with a reduced presence of SLECs over time.

nTregs control SLEC number in mice infected with high-dose virus

Recent evidence points to the indirect contribution of nTregs to effector cell differentiation through regulation of IL-2 present in the environment (20). As a first step in evaluating the potential for nTregs to play a role in control of the SLEC pool following infection with VV, Treg number was analyzed over the course of infection. A significant increase in the number of nTregs was observed at day 5 in mice that received the high virus dose, resulting in a significantly higher number of nTregs in mice infected with the high versus low virus dose (Fig. 6A). The time-frame for nTreg increase coincided with the reduced relative expansion of B8R⁺ cells in animals infected with high doses versus those infected with low doses. Expansion of nTregs in mice infected with low doses was not observed until later in the response (day 7) (Fig. 6A).

FIGURE 4. B8R-specific effectors undergo increased proliferation in mice infected with low- compared with high-dose virus. C57BL/6 mice were infected with either a high or a low dose of VV i.p. On day 5 or 7 postinfection, mice received BrdU by i.p. injection. At 5 h later, BrdU incorporation was assessed. Representative dot plots as well as the average frequency of B8R⁺ MPECs and SLECs on day 5 (**A, B**) and day 7 (**C, D**) postinfection are shown. Data in (**B**) are the average of eight animals and in (**D**) of six animals analyzed in at least three independent experiments. On days 6 (**E**) and 7 (**F**) postinfection, MPECs and SLECs were assessed for the presence of active caspase 3. No significant differences were found between effectors isolated from animals infected with high doses versus those infected with low doses. Statistical analysis was performed using a Student two-tailed *t* test. ***p* < 0.01, ****p* < 0.001.



To determine whether the increased number of nTregs was involved in regulating SLEC number in animals infected with the higher virus dose, nTregs were depleted by administration of anti-CD25 or isotype control Ab 1 d prior to infection. Injection with anti-CD25 resulted in significant depletion of CD25⁺Foxp3⁺ cells (Fig. 6B, 6C). Consistent with previously published data that show nTreg-mediated suppression of the B8R-specific response (17), mice depleted of nTregs showed a significant increase in the number of B8R-specific cells (Fig. 6D). Concomitant with the increase in Ag-specific cells was an increase in the SLEC population (Fig. 6E) that resulted in skewing of the B8R-specific re-

sponse toward SLECs (Fig. 6F). No significant increase in the number of MPECs was observed (Fig. 6E). As CD25 is also expressed on activated T cells, administration of anti-CD25 Ab could potentially affect these cells. To ensure that the results we obtained were not due to direct effects on activated T cells, we treated mice with anti-FR4 Ab, an alternative approach to the depletion of nTregs (26). The results from these studies were similar to those obtained following anti-CD25 treatment (Supplemental Fig. 1). Together, these data show that the presence of increased nTregs following infection with high virus dose resulted in a reduced SLEC population.

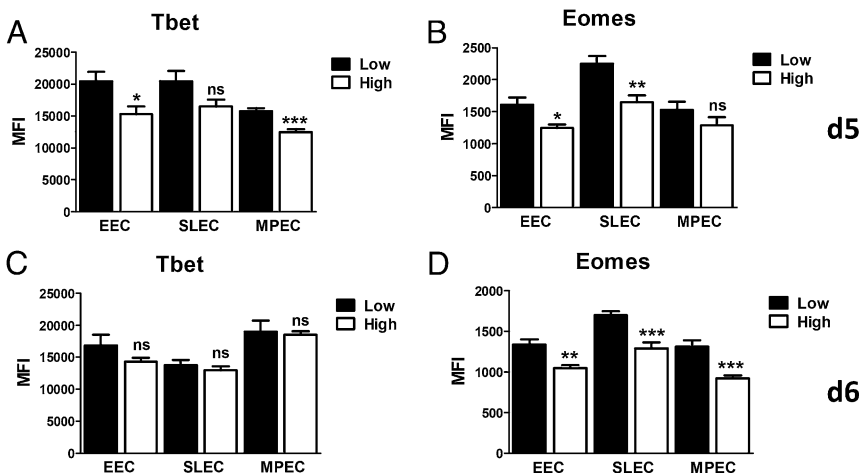


FIGURE 5. Effector cells from mice infected with the high virus dose exhibit decreased levels of T-bet at day 5 postinfection. C57BL/6 mice were infected with either a high or a low dose of VV. On day 5 (**A, B**) and day 6 (**C, D**) postinfection, splenocytes were isolated and CD8⁺ B8R⁺ SLECs, MPECs, and EECs assessed for expression of eomes and T-bet. For each virus dose, results are the average of six or seven mice analyzed in two independent experiments. Statistical analysis was performed using a Student two-tailed *t* test. Significance testing was performed across the two virus doses. **p* < 0.05, ***p* < 0.005, ****p* < 0.001.

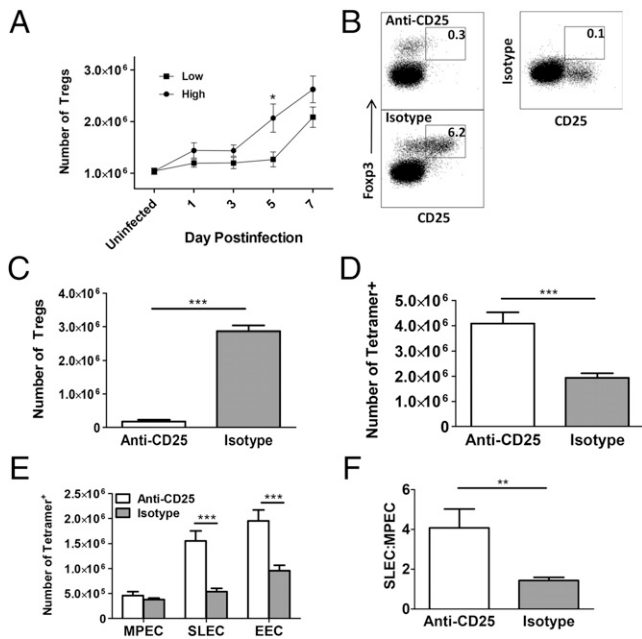


FIGURE 6. nTregs control SLEC number in mice infected with high-dose virus. **(A)** C57BL/6 mice were infected with either a high or a low dose of VV. The number of CD25⁺Foxp3⁺ Tregs present over days 1–7 following infection was determined. **(B–F)** Mice infected with the high virus dose were treated with 400 μ g of anti-CD25 or isotype control Ab 1 d prior to infection. On day 7 postinfection, nTregs were analyzed. **(B)** Representative dot plots and **(C)** average number of nTregs present in treated mice. **(D)** Total number of B8R-specific cells. **(E)** Effector cell numbers and **(F)** ratio of SLECs to MPECs following anti-CD25 or isotype Ab treatment. Data shown for **(A)** are an average of 10 mice from days 1–3, at least 6 mice from days 5–7 analyzed in a minimum of two independent experiments. For **(B)–(F)**, data are an average of at least nine mice from four independent experiments. Statistical analysis was performed using a Student two-tailed *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

nTregs generated following infection with the high virus dose exhibit increased expression of the activation markers CD25 and GITR and decreased expression of CTLA4

Given the increased number of nTregs in mice infected with the high dose of virus, we were interested to determine whether these nTregs exhibited differential expression of markers known to be involved in nTreg function or proliferation. To this end we assessed the expression of CD25, CD44, GITR, and CTLA4. The nTregs from mice infected with the high virus dose had significant increases in CD25 and GITR (Fig. 7A, 7B), whereas the level of CTLA4 expressed on these cells was decreased (Fig. 7D). No difference in CD44 was observed (Fig. 7C). IL-2 signaling and GITR engagement are known to be associated with increased survival, proliferation, and function of nTregs (27–29). In contrast, CTLA4 engagement results in decreased proliferation of these cells (30, 31). Thus, the differential expression pattern of these markers on nTregs from mice infected with high doses is consistent with their increased number and function.

Late depletion of nTregs resulted in an increased SLEC/MPEC ratio

Our analysis of the emergence of SLECs following infection with high- versus low-dose virus was consistent with a late effect of nTregs—that is, skewing did not occur until \geq day 6 postinfection (Fig. 3). On the basis of this result, we hypothesized that depletion of nTregs at a postpriming stage would still promote an increased SLEC/MPEC skewing similar to that observed in mice infected with

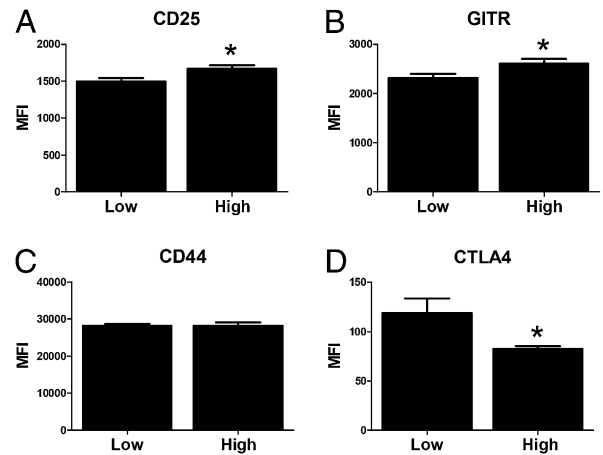


FIGURE 7. nTregs from mice infected with the high virus dose exhibit increased levels of CD25 and GITR, but decreased CTLA4. On day 6 following infection, nTregs identified by staining with CD4 and Foxp3 were assessed for the expression of CD25 **(A)**, GITR **(B)**, CD44 **(C)**, and CTLA4 **(D)**. The data are generated from seven or eight individual animals analyzed across two independent experiments. Statistical analysis was performed using a Student two-tailed *t* test. **p* < 0.05.

the high virus dose that were depleted of nTregs prior to infection. To determine if this were the case, we administered anti-FR4 Ab at day 2.5 postinfection. We chose this approach to minimize potential effects of administering anti-CD25 Ab during the activation and proliferation stages of the T cell response when activated T cells also express high levels of CD25. Treatment of mice with anti-FR4 at day 2.5 resulted in fewer Tregs at day 7 postinfection (Fig. 8A, 8B). Further, depletion of nTregs led to a significant increase in the SLEC population (Fig. 8C and data not shown) and, as a result, increased skewing within the population toward SLECs (Fig. 8D). A previous study reported that nTregs could decrease SLECs via inhibition of DC maturation (32). However, in agreement with the late-stage effect of nTregs on SLEC number in our model, nTreg depletion had no significant effect on the maturation of DCs following infection with the high virus dose (Fig. 8E).

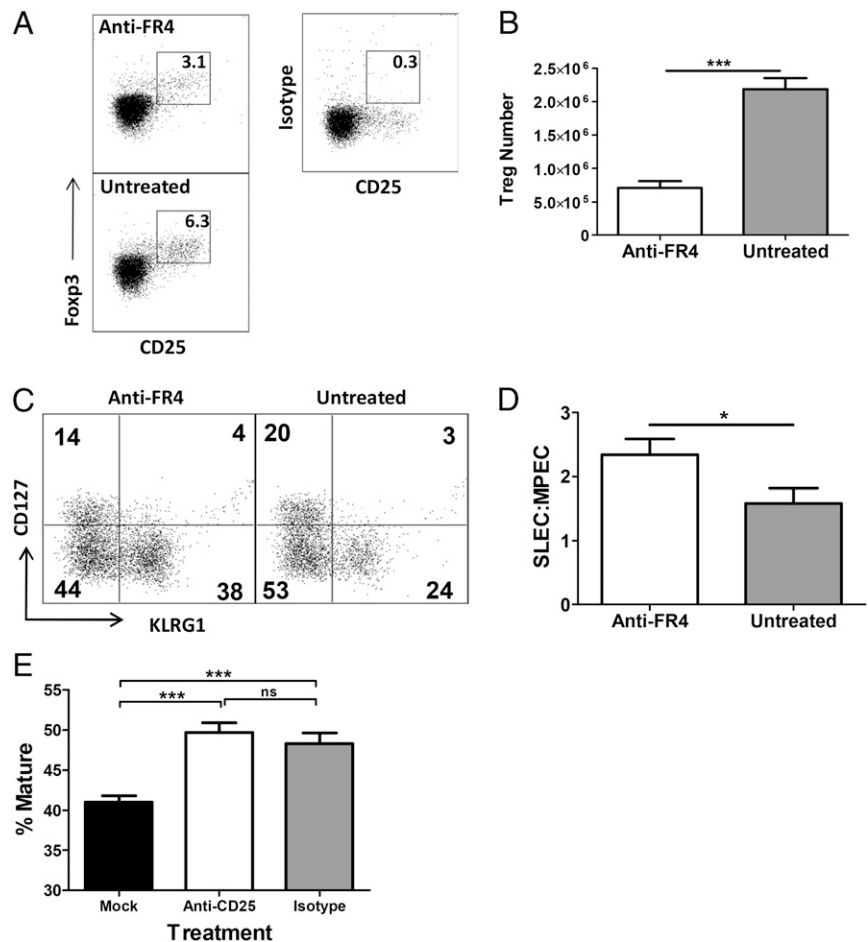
IL-2 administration at late times following infection with the high virus dose increased SLEC expansion and resulted in an SLEC/MPEC ratio similar to that observed in mice infected with the low virus dose

The nTregs constitute a major consumer of IL-2 within the immune responders. If the generation of high numbers of nTregs as a result of high viral burden were limiting late-stage SLECs by decreasing available IL-2, administration of this cytokine should restore SLECs. To determine if this were the case, mice infected with high doses received 15,000 IU of recombinant human IL-2 twice daily on days 3–6 postinfection. The data in Fig. 9A and 9B show that this treatment resulted in an increased frequency and number of B8R-specific cells on day 7 postinfection. Further, mice that received IL-2 had an increased frequency of SLECs (Fig. 9C) that resulted in an SLEC/MPEC ratio similar to the one observed in mice infected with the low virus dose (Fig. 9D; compare with Fig. 1D). Taken together, these data suggest that increased VV burden resulted in increased nTregs, which in turn decreased SLEC number through consumption of IL-2.

nTregs present at day 5 in mice infected with high virus dose exhibit increased p-STAT5 compared with those in mice infected with low virus dose

The above data are consistent with the model that IL-2 utilization by nTregs limits cytokine availability for use by CD8⁺ effectors.

FIGURE 8. nTregs regulate SLEC number at a step subsequent to priming of the CD8⁺ T cell response. C57BL/6 mice were infected with a high dose of VV i.p. On day 2.5 postinfection, mice were treated with 30 μ g of anti-FR4 Ab. Responses were analyzed on day 7 postinfection. **(A)** Representative dot plot of CD25 and Foxp3 staining following pre-gating on CD4⁺ T cells. **(B)** Number of nTregs in mice treated with anti-FR4 Ab versus untreated mice. **(C)** Representative dot plot of B8R⁺CD8⁺ T cells in untreated mice or in mice that received anti-FR4 Ab following staining with KLRG1 and CD127 Abs. **(D)** Ratio of SLECs/MPECs within the B8R⁺CD8⁺ T cell population in treated versus untreated animals. Data shown are the average of six animals analyzed in two independent experiments. **(E)** Mice received PC61 Ab on day 1 prior to infection with the high virus dose. On day 2 postinfection, the time at which DCs demonstrate maximal maturation in our preliminary studies, DCs in the spleen were assessed for the expression of CD80 and CD40 as indicators of maturation. The percentage of mature CD11c⁺ cells is shown. Data shown are the average of at least eight animals analyzed in three independent experiments. Statistical analysis was performed using a Student two-tailed *t* test, except in (C), in which a one-tailed *t* test was used. **p* < 0.05, ****p* \leq 0.0005.



Thus, we reasoned that nTregs present in animals infected with the high virus dose should exhibit evidence of increased IL-2R engagement. To test this possibility, phosphorylation of STAT5 was measured following fixation immediately following spleen isolation, as previously described (33). We assessed two timepoints, day 5 and day 6 postinfection. At day 5 following infection, a significantly higher percentage of nTregs from animals infected with high doses were positive for p-STAT5 staining, compared with Tregs from the low-dose counterpart (Fig. 10). Of note, by day 6, nTregs from animals infected with both high and low doses showed similar levels of p-STAT5. The increased phosphorylation of STAT5 in nTregs present at day 5 following infection with the high dose of virus—a timepoint at which B8R⁺ effectors from animals infected with low, but not high, doses underwent robust expansion—supports the model of increased consumption of IL-2 by these cells compared with nTregs present in animals infected with the low virus dose.

Discussion

The expansion of effector cells following virus infection is a critical determinant of effective clearance. In this article, we report the finding that viral burden dictates the nature of the CD8⁺ effector T cell response with regard to both number and differentiation state. Paradoxically, high viral load following VV infection resulted in fewer SLECs within the responding effector cell pool. This constraint on the effector cell response was the result of nTreg-mediated restriction of the SLEC population. The regulation of the effector cell response was selective for SLECs, as MPEC numbers were similar regardless of viral burden. Modulation of this population resulted in a skewing of the response

toward SLECs in animals infected with the high virus dose. The skewing was a late event, as it was not apparent until day 6 postinfection.

We reasoned that the increase in SLEC number following low-dose infection was potentially due to selective expansion of SLECs in mice infected with low doses. However, although we found that a greater percentage of SLECs were undergoing proliferation in mice infected with the low versus high virus dose, MPEC and EEC populations exhibited a similar increase in proliferating cells. Thus, selective proliferation of SLECs could not account for the increase in cell number.

The decreased number of SLECs in animals infected with the high dose was also not the result of increased death, as measured by the presence of active caspase 3. Thus, one possible explanation of the increased number of SLECs is increased differentiation of effectors along this pathway in mice infected with the low virus dose; that is, a greater percentage of the dividing cells differentiate into SLECs. The increased level of T-bet in the effectors present at day 5 following infection with the low virus dose is consistent with this model. That said, these effectors also showed increased levels of eomes. It is becoming increasingly clear that the regulation of eomes and T-bet as it relates to KLRG1^{hi} versus KLRG1^{lo} expression is complex. Recently, elegant work has demonstrated the presence of further differentiation states within KLRG1^{hi} versus KLRG1^{lo} populations that diverge in the relative expression of T-bet and eomes (34). In addition, the expression of T-bet and eomes within KLRG1^{hi} versus KLRG1^{lo} lung effectors does not follow the expected pattern (35). The role of differential T-bet and eomes expression in the SLEC skewing observed in our system will require further study.

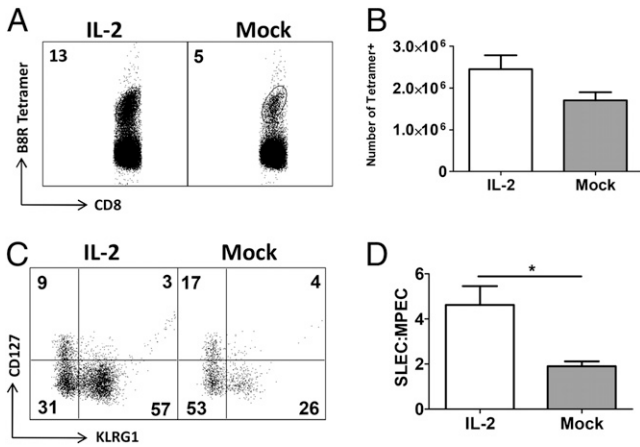


FIGURE 9. Late treatment with recombinant IL-2 results in SLEC skewing of the virus-specific CD8⁺ T cell response. C57BL/6 mice were infected with a high dose of VV i.p. On days 3–6 postinfection, mice received a twice-daily injection of recombinant IL-2. On day 7 postinfection, the B8R-specific population present in the spleen was assessed. **(A)** Representative dot plots showing the frequency of B8R⁺ CD8⁺ T cells in mice treated with IL-2 versus mock-treated animals. **(B)** Averaged data showing the total number of B8R-specific CD8⁺ T cells. **(C)** Representative dot plot of B8R⁺CD8⁺ T cells stained with anti-KLRG1 and anti-CD127 Abs following IL-2 treatment. **(D)** The SLEC/MPEC ratio within the B8R-specific population. Data shown are the average of at least 12 animals analyzed in 5 independent experiments. Statistical analysis was performed using a Student two-tailed *t* test. **p* < 0.05.

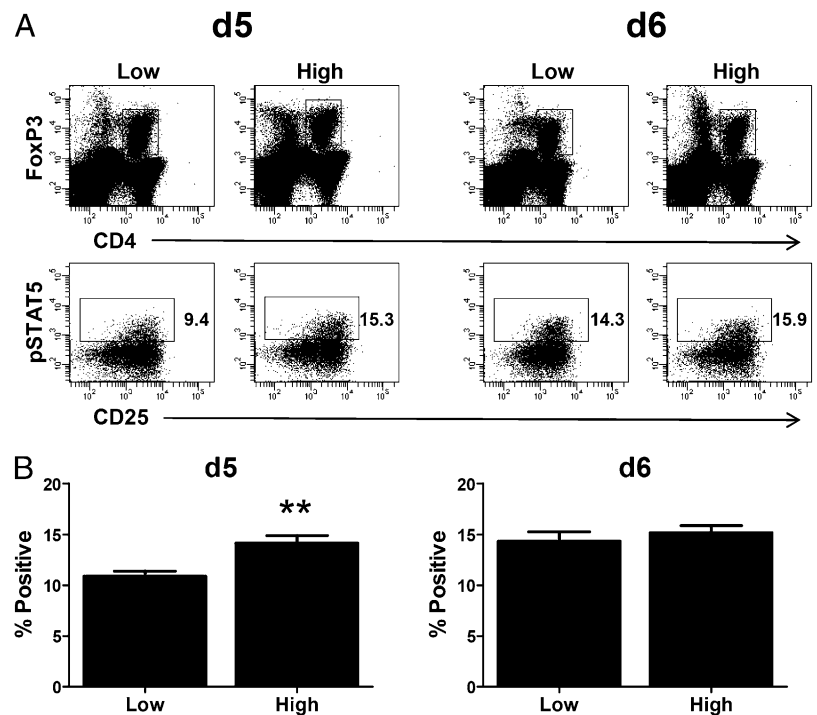
The increased proliferation observed in EEC and MPEC populations, even though the number of these cells was not increased in mice infected with the low versus high virus dose, is consistent with EECs, and perhaps MPECs, serving as sources of SLECs that are generated at later times postinfection. Although EECs are known to be efficient progenitors of SLECs (36), MPECs can also give rise to all three populations (36). One possibility is that a subpopulation of MPECs has the capacity to be shunted into the SLEC

pathway directly or via an EEC intermediate. As MPECs contain two subsets that are thought to give rise to effector memory T cells versus central memory T cells (37), it is tempting to speculate that these two subsets differ with regard to the potential to undergo further differentiation during the acute response. One might speculate that the effector memory T cell-generating subset can give rise to SLECs in the acute response, as increased IL-2 signaling results in increased effector memory T cell generation, similar to its effect on SLEC generation (8, 38). This idea fits with our model, suggesting increased IL-2 availability in the animals infected with low virus dose.

The nTregs are well established as important players in suppressing potentially damaging self-reactive T cell response (for review see Ref. 39). However, an increasing body of literature now supports their role in suppressing the immune response following viral infections with, for example, VV (17), HIV (40, 41), HSV (18, 19, 40), and HCV (40, 42). Viral load appears to be an important regulator of Treg number, as, for example, antiviral treatment during acute hepatitis B infection resulted in decreased nTregs that correlated with decreased virus load (43, 44). This result occurs potentially through increased production of IL-2, which promotes nTreg expansion and suppressive function (33). On the basis of this finding, we propose that the increased nTreg generation observed in our study in mice infected with the high compared with low VV dose is the result of increased virus burden.

The regulated expansion of nTregs in our model plays a critical role in determining effector cell fate, as their presence at high numbers was associated with a decreased SLEC population. We found that depletion of nTregs resulted in an increase in the number of SLECs comparable to that observed in mice infected with low-dose virus. This increase appeared to occur through modulation of IL-2 availability, as administration of IL-2 resulted in a larger SLEC population. The consumption of IL-2 by nTregs in mice infected with the high virus dose is supported by our finding that a larger proportion of nTregs from these mice were positive for p-STAT5 compared with the nTregs from mice infected with the low virus dose. The nTregs possess a unique ability to “starve” expanding T cells of IL-2 owing to constitutive high-level expression of CD25

FIGURE 10. nTregs from mice infected with the high virus dose exhibit increased levels of p-STAT5. On day 5 or 6 postinfection, spleens from infected animals were isolated and immediately fixed in 1.6% formaldehyde followed by methanol permeabilization. p-STAT5 levels were assessed in CD4⁺Foxp3⁺ cells by staining with Ab specific to p-STAT5. Representative data are shown in **(A)**. Averaged data are shown in **(B)**. Data for each timepoint and condition are generated from 11 individual animals analyzed across 4 experiments. Statistical analysis was performed using a Student two-tailed *t* test. ***p* = 0.002.



(20), thereby preventing IL-2 signaling that is known to be critical for shunting effectors to the SLEC pathway (8, 45), presumably through its ability to sustain Blimp-1 expression (45).

A role for nTregs in the regulation of SLECs has been recently reported (32). The basis for this effect following infection with replication-incompetent modified vaccinia Ankara was identified as a Treg-mediated decrease in DC maturation. Thus, nTregs altered effector cell generation at the priming stage. Our studies reveal an alternative strategy used by nTregs to alter SLEC number in the presence of high viral load. In this situation, regulation occurred at a kinetically distinct phase of the immune response, that is, late expansion. The late nature of this effect was demonstrated by increased SLEC number as a result of delivery of IL-2 at days 3–6 postinfection, as well as depletion of nTregs following administration of anti-FR4 at day 2.5 postinfection, a strategy that should delete Tregs at \geq day 3 postinfection. The late effect on T cells in our model is also supported by the observed similarity in DC maturation in the presence or absence of Tregs. Thus, nTregs can impact CD8⁺ T cell effector fate via multiple mechanisms, depending on the viral load.

In agreement with an effect at a postpriming stage, analysis of CD25 expression at day 4 postinfection revealed significantly increased CD25 expression on SLECs in mice infected with the high versus low dose of virus (data not shown). As high CD25 expression at this juncture is likely the result of positive feedback from sustained IL-2 signaling, nTregs seemed not to have limited T effector cell access to IL-2 at this timepoint. By day 5, the increased CD25 expression on SLECs from mice infected with the high virus dose was no longer apparent, having fallen below that on SLECs from mice infected with the low virus dose (data not shown). The loss of CD25 expression at day 5 coincided with a much higher number of nTregs. In addition, a higher percentage of these nTregs were positive for p-STAT5, an indicator of IL-2 signaling, compared with their low-dose counterpart (for review see Ref. 46). We believe that the mechanism of Treg action is primarily through IL-2, as we found no difference in the level of TGF- β or IL-10 in mice infected with the high versus low dose of virus (data not shown).

How, then, does low virus dose limit Treg expansion to promote maximal SLEC formation? Infection with the low virus dose resulted in a more limited T cell response through day 5 postinfection. Given this, we would predict that IL-2 levels are relatively low. In a previous report by Benson et al. (47), limited IL-2 levels were shown to have a more profound effect on Treg than effector T cell generation. Thus, limiting IL-2 at early times postinfection as a result of low virus burden may allow preferential use of this cytokine in local cell environments by virus-specific T cells, thereby limiting Treg expansion that would subsequently suppress the SLEC population.

In summary, our data show that the viral burden present following poxvirus infection has a significant effect on the regulation of CD8⁺ effector T cell number and fate. In contrast to what might be expected, high VV load resulted in reduced SLECs, a cell type known to be enhanced by the presence of inflammatory cytokines. Together these data reveal a novel mechanism by which viral load affects SLEC number as a result of increased nTregs that appear to limit SLEC access to IL-2 at late stages of the immune response.

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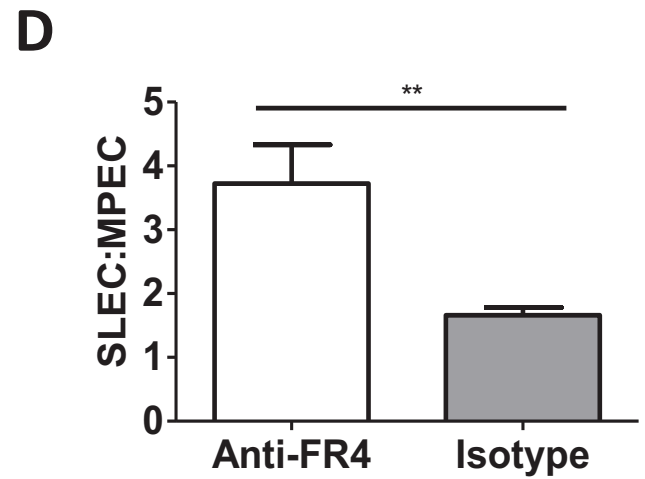
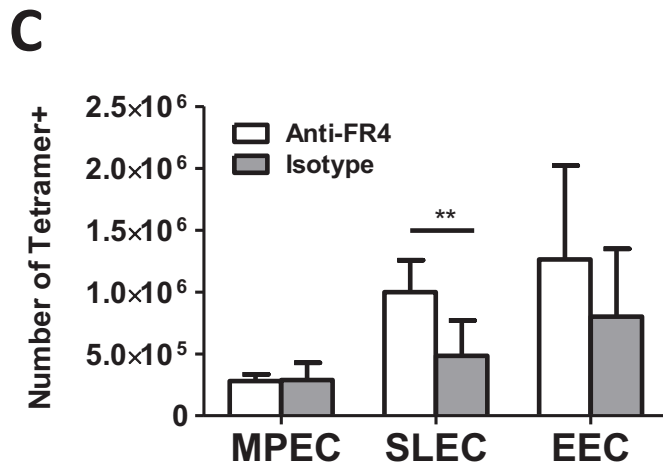
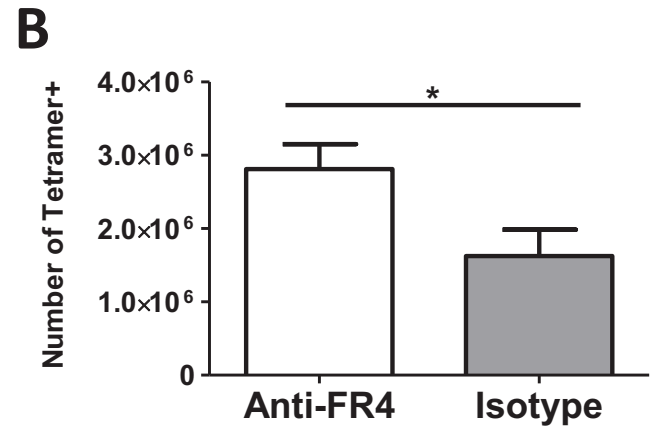
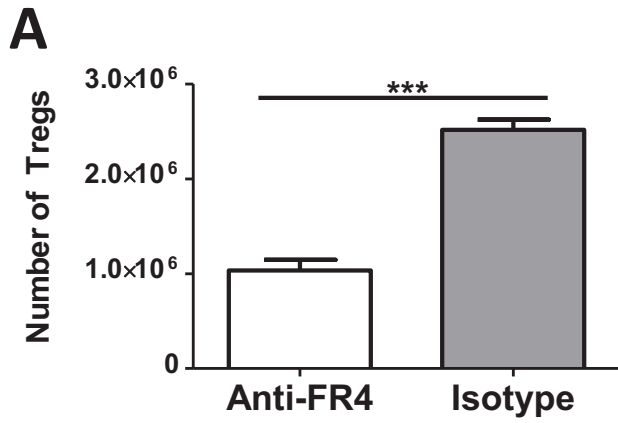
Disclosures

The authors have no financial conflicts of interests.

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Supplemental Figure 1. FR4 treated mice infected with the high virus dose have increased SLEC. Mice were treated with 30 μ g of anti-FR4 one day before infection with a high dose of VV. On d7 p.i. nTreg and effector cells were analyzed. (A) Average number of nTreg present in treated mice. (B) Total number of B8R-specific cells. (C) Effector cell numbers and (D) ratio of SLEC to MPEC following anti-FR4 treatment prior to infection with high dose VV. Data shown are the average of at least 5 animals analyzed in 2 independent experiments. Statistical analysis was performed using a Student's two tailed t-test. (*=p<0.05, **=p<0.01, ***=p<0.001).