Pathogen-Induced Inflammatory Environment Controls Effector and Memory CD8+ T Cell Differentiation

Joshua J. Obar, Evan R. Jellison, Brian S. Sheridan, David A. Blair, Quynh-Mai Pham, Julianne M. Zickovich and Leo Lefrançois

*J Immunol* 2011; 187:4967-4978; Prepublished online 10 October 2011; doi: 10.4049/jimmunol.1102335
http://www.jimmunol.org/content/187/10/4967

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/10/11/jimmunol.1102335.DC1

**References**

This article cites 83 articles, 40 of which you can access for free at:
http://www.jimmunol.org/content/187/10/4967.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
Pathogen-Induced Inflammatory Environment Controls Effector and Memory CD8+ T Cell Differentiation

Joshua J. Obar,* Evan R. Jellison,* Brian S. Sheridan,* David A. Blair,* Quynh-Mai Pham,* Julienne M. Zickovich,* and Leo Lefrançois*

In response to infection, CD8+ T cells integrate multiple signals and undergo an exponential increase in cell numbers. Simultaneously, a dynamic differentiation process occurs, resulting in the formation of short-lived effector cells (SLECs; CD127highKLRG1low) and memory precursor effector cells (CD127highKLRG1low) from an early effector cell that is CD127lowKLRG1high in phenotype. CD8+ T cell differentiation during vesicular stomatitis virus infection differed significantly than during Listeria monocytogenes infection with a substantial reduction in early effector cell differentiation into SLECs. SLEC generation was dependent on Ebi3 expression. Furthermore, SLEC differentiation during vesicular stomatitis virus infection was enhanced by administration of CpG-DNA, through an IL-12–dependent mechanism. Moreover, CpG-DNA treatment enhanced effector CD8+ T cell functionality and memory subset distribution, but in an IL-12–independent manner. Population dynamics were dramatically different during secondary CD8+ T cell responses, with a much greater accumulation of SLECs and the appearance of a significant number of CD127highKLRG1high memory cells, both of which were intrinsic to the memory CD8+ T cell gene. These subsets persisted for several months but were less effective in recall than memory precursor effector cells. Thus, our data shed light on how varying the context of T cell priming alters downstream effector and memory CD8+ T cell differentiation. The Journal of Immunology, 2011, 187: 4967–4978.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11 $16.00

*Center for Integrated Immunology and Vaccine Research, Department of Immunology, University of Connecticut Health Center, Farmington, CT 06030; and †Department of Immunology and Infectious Diseases, Montana State University, Bozeman, MT 59718

Received for publication August 11, 2011. Accepted for publication September 1, 2011.

This work was supported by National Institutes of Health Grants ROI-AI41576 (to L.L.), ROI-AI74657 (to L.L.), F32-AI074277 (to J.J.O.), P20-RR020185 (to J.J.O.), T32-AI07080 (to D.A.B.), and F32-AI84328 (to E.R.J.). Additionally, the Montana State University Agricultural Experiment Station and equipment grants from the M.J. Murdock Charitable Trust supported this work.

Address correspondence and reprint requests to Dr. Leo Lefrançois or Dr. Joshua J. Obar, Department of Immunology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030 (L.L.) or Department of Immunology and Infectious Diseases, Montana State University, 960 Technology Boulevard, Bozeman, MT 59718 (J.J.O.). E-mail addresses: llefranc@neuron.uchc.edu (L.L.) and joshua.obar@montana.edu (J.J.O.).

The online version of this article contains supplemental material.

Abbreviations used in this article: DPEC, double-positive effector cell; EEC, early effector CD8+ T cell; KLRG1, killer cell lectin-like receptor subfamily G, member 1; LCMV, lymphocytic choriomeningitis virus; L. monocytogenes, L. monocytogenes-OVA; MLEC, memory precursor effector cell; MPEC, memory precursor effector cell; ODN, oligodeoxynucleotide; SLEC, short-lived effector cell; T Rexa, central memory T cell; T VSE, effector memory T cell; VSV, vesicular stomatitis virus; VSV-OVA, vesicular stomatitis virus expressing OVA; WT, wild-type.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11 $16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102335

The Journal of Immunology

© 2017 by The American Association of Immunologists, Inc.
these studies, it was demonstrated that secondary memory CD8+ T cells had elevated levels of granzyme B and decreased levels of CD62L and CD27 (25, 26). Furthermore, global genetic analysis revealed drastic differences in memory T cells after primary through quaternary antigenic stimulation (27). However, the effector/memory differentiation dynamics in these situations has remained understudied. More importantly, whether all pathogens and vaccine vectors induce similar effector CD8+ T cell differentiation remains an open question. In this study we demonstrate that effector CD8+ T cell differentiation differs substantially after vesicular stomatitis virus (VSV) and Listeria monocytogenes infections. These differences were tied to the composition of the inflammatory milieu induced by each infection. Inflammation not only altered SLEC/MPEC differentiation, but it also had a striking effect on the functionality of the effector CD8+ T cell population and composition of the memory population by limiting the differentiation of CD62Llow effector memory T cell (Tem) cells. Additionally, multiple encounters with Ag dramatically altered SLEC/MPEC differentiation in a memory cell-intrinsic manner. Thus, our data shed light on the fact that effector and memory CD8+ T cell differentiation is dynamically controlled and varies depending on the context of the activation, that is, the type of priming pathogen or the number of times the cell is stimulated with the same Ag.

Materials and Methods

Mice

Female C57BL/6 and B6-ly5.2 mice between 5 and 8 wk old were purchased from the National Cancer Institute, whereas female B6.129S1-Il12a<sup>tm1Jm/J</sup> (p35<sup>−/−</sup>) and B6.129S7-Ifng<sup>tm1JjJj/J</sup> (Ifn-γ<sup>−/−</sup>) mice between 6 and 8 wk old were purchased from The Jackson Laboratory. Female B6.129<sup>−/−</sup> X Ebi3<sup>−/−</sup> (Ebi3<sup>−/−</sup>) mice were provided by Dr. David Pascual (Montana State University). Female B6.129P2.IFNg<sup>tm1K1J/J</sup> (IL-18<sup>−/−</sup>) mice were provided by Dr. Mark Jutila (Montana State University). OT-I Rag<sup>−/−</sup> mice were bred in the University of Connecticut Health Center animal facility. OT-I mice deficient for IFNAR were provided by Dr. John Harty (University of Iowa), whereas OT-I mice deficient for IL-12β or both IL-12β and IFNAR were provided by Dr. Matthew Mescher (University of Minnesota). All animal protocols were approved by either the University of Connecticut Health Center or Montana State University Institutional Animal Care and Use Committee.

Pathogens, infections, and treatments

Both the recombinant VSV expressing OVA (VSV-OVA) (28) and recombinant L. monocytogenes expressing OVA (L. monocytogenes-OVA) (29) have been previously described. For primary infections, mice were infected i.v. with either 10<sup>7</sup> PFU VSV-OVA (Indiana) or 10<sup>3</sup> CFU L. monocytogenes-OVA. To study the recall response, mice were challenged with 2 × 10<sup>6</sup> PFU VSV-OVA (New Jersey) or 5 × 10<sup>4</sup> CFU L. monocytogenes-OVA. Some VSV-infected mice were treated i.p. with 50 µg oligodeoxynucleotide (ODN) 1826 (InvivoGen) given 1 h postinfection. Furthermore, some VSV-infected mice were treated i.p. with two doses of 0.5 µg recombinant murine IL-12 (PeproTech) given 1 and 24 h post-infection.

Systemic cytokine/chemokine analysis

Three groups of mice were analyzed: 1) L. monocytogenes infected, 2) VSV infected, and 3) VSV infected and treated with 50 µg ODN 1826. Peripheral blood was collected at 6, 12, 24, 48, and 72 h. Serum was collected 3 h later by centrifugation. Serum was then frozen at −80°C until used. Three different Luminex-based multiplex bead assay plates were used for quantification: 1) IFN-α and IFN-β (Affermtrix); 2) G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IL-23, IP-10, RANTES, and TNF-α (Affermtrix); and 3) IL-6, IL-10, IL-22, IL-25, IL-28B, MCP-5, MIP-3α, and MIP-3β (Millipore).

Tissue sample preparation and flow cytometric analysis

The H-2Kb tetramer containing the OVA-derived peptide SIINFEKL was generated as previously described (30, 31). Analysis of endogenous Ag-specific CD8+ T cells throughout the entire immune response was conducted as has previously been described (32).

Intracellular cytokine staining

Cells were incubated with 1 µg/ml OVA or VSV-N peptide plus 1 µl GolgiPlug (BD Biosciences) in medium at 37°C for 4.5 h. Cells were stained for cell surface Ags on ice for 15 min. Cells were then fixed and rendered permeable using BD Cytofix/Cytoperm (BD Biosciences) before staining with Abs to IFN-γ and TNF-α.

Secondary memory adoptive transfer

To generate secondary memory mice C57BL/6 mice were initially infected with 10<sup>5</sup> CFU L. monocytogenes-OVA and then >200 d later mice were rechallenged with 5 × 10<sup>8</sup> CFU L. monocytogenes-OVA. Approximately 30 d after secondary infection mice were sacrificed and their spleens collected. CD8+ T cells were enriched using magnetic beads and the CD8+ enrichment kit and autoMACS (Miltenyi Biotec). Cells were then stained with anti-CD11a, anti-CD44, anti-KLRG1, anti-CD127, anti-class II, and anti-CD4. Memory phenotype cells (class II<sup>+</sup>CD44<sup>high</sup>CD44<sup>high</sup>) were sorted into three populations: 1) CD127<sup>high</sup>KLRG1<sup>low</sup> (MPEC), 2) CD127<sup>high</sup>KLRG1<sup>high</sup> (double-positive effector cells [DPECs]), and 3) CD127<sup>low</sup>KLRG1<sup>high</sup> (SLEC). Cells were sorted using a FACS Aria (BD Biosciences). After sorting, an aliquot of cells was further stained with anti-CD8α and OVA/K<sub>α</sub> to enumerate the number of OVA/K<sub>α</sub>-specific CD8+ T cells in each memory population. For adoptive transfer 10<sup>5</sup> OVA/K<sub>α</sub>-specific CD8+ T cells were transferred to naive CD45.1<sup>+</sup> C57BL/6 mice. One day later mice were infected with 10<sup>4</sup> CFU L. monocytogenes-OVA.

Statistical analysis

Statistical significance was determined by either a paired Student t test or one-way ANOVA using Prism 5 (Graphpad Software). Significance was set as p < 0.05.

Results

Effector CD8+ T cell subset generation differs between L. monocytogenes and VSV infection

During the CD8+ T cell response, a large number of cytotoxic effector cells are generated. Recent studies have identified subsets of effector cells generated after lymphocytic choriomeningitis virus (LCMV) infection based on CD127 and KLRG1 expression (3, 4). At the peak of the response to LCMV infection the predominant CD8+ T cell population is CD127<sup>low</sup>KLRG1<sup>high</sup> representing SLECs, while a small population of CD127<sup>high</sup>KLRG1<sub>low</sub> MPECs is also found. To examine whether the effector cell subsets present following VSV and L. monocytogenes infections were similar to those found after LCMV infection, mice were infected i.v. with 10<sup>7</sup> PFU recombinant VSV-OVA or 10<sup>3</sup> CFU recombinant L. monocytogenes-OVA. Subsequently, OVA/K<sub>α</sub>-specific CD8+ T cells were monitored longitudinally using a recently described tetramer enrichment protocol (32, 33) to examine the phenotype of early responding populations. On day 3 postinfection with either VSV or L. monocytogenes, the early effector CD8+ T cells (EEC) were primarily CD127<sup>low</sup>KLRG1<sub>low</sub> Two days later, the SLEC and MPEC populations began to emerge after either infection (Fig. 1A). Notably, approximately twice as many EECs remained after VSV infection as compared with L. monocytogenes infection. By day 8 the effect was even more dramatic, with ~75% of tetramer+ cells being SLECs, whereas only a small population of MPECs (~5%) emerged after L. monocytogenes infection. Comparatively, 8 d after VSV infection, 40–50% of tetramer+ cells remained as EECs with ~30 and ~20% of the cells being SLECs and MPECs, respectively. Over time, CD127<sup>high</sup>KLRG1<sub>high</sub> memory CD8+ T cells gradually became the dominant population after either infection, but this occurred much more rapidly following VSV infection (Fig. 1). Fifteen days after VSV infection, ~50% of the population was MPECs; whereas this occurred only at ~100 d after L. monocytogenes infection.
population of DPECs, that is, CD127hiKLRG1hi cells, was also observed after L. monocytogenes infection, which was less evident during VSV infection (Fig. 1). In fact, ∼50 d after L. monocytogenes-OVA infection, the Ag-specific pool was distributed roughly equally between MPEC, SLEC, and DPEC subsets. Quantification of the effector populations at the peak of the CD8+ T cell response revealed that the number of MPECs/EECs, rather than the overall number of OV A/Kb-specific CD8+ T cells, more closely correlated to the size of the memory population (Supplemental Fig. 1). Thus, effector CD8+ T cell populations generated by VSV and L. monocytogenes infection substantially differed in kinetics and composition.

Developmental potential of effector CD8+ T cell subsets

Although the population kinetics just described suggested that EECs may be progenitors to the other effector subsets, we wanted to test the developmental potential of each of the populations. To do so, a small number of OT-I cells were transferred and the mice were infected with VSV-OVA (Fig. 2). Five days later EECs, SLECs, and MPECs were purified by sorting and transferred to infection-matched mice. Two days after transfer, the purified SLECs largely remained as SLECs and did not produce any MPECs. In contrast, the sorted EEC population not only generated both MPECs and SLECs, but also additional EECs. Purified MPECs developed into MPECs, as well as EECs. Thirty-five days after transfer, SLEC-derived cells were undetectable; in contrast, sorted EECs primarily developed into CD127hiKLRG1lo memory CD8+ T cells, whereas transferred MPECs focused exclusively to CD127hiKLRG1lo memory CD8+ T cells. Thus, EECs have the greatest developmental potential with the ability to generate all effector CD8+ T cell lineages.

Universal granzyme B expression by effector CD8+ T cell subsets, but hierarchal loss of expression

Although the EEC population appears to be highly activated, we wanted to confirm whether all such cells had lytic potential. To do this, mice were infected with VSV-OVA or L. monocytogenes-OVA and subsequently granzyme B levels in the responding CD8+ T cell subsets were assessed by flow cytometry. Five days post-infection each of the Ag-specific CD8+ T cell subpopulations contained high levels of granzyme B (Supplemental Fig. 2). Interestingly, only 2 d later (day 7 postinfection) certain effector CD8+ T cell subpopulations began to lose granzyme B expression in a hierarchical manner: first CD62Lhi MPECs followed by...
CD62L<sup>low</sup> MPECs, and then the EEC population followed by the SLEC subset (Supplemental Fig. 2). Furthermore, there were notable differences in granulocyte B expression depending on the infection. In general, VSV-specific effectors expressed less granulocyte B as compared with L. monocytogenes-specific effectors, and the VSV-specific MPEC populations rapidly lost granulocyte B expression. Thus, there appeared to be a hierarchical programming of at least granulocyte B expression in the effector subpopulations.

**IL-12 and IFN-αβ differentially regulate SLEC differentiation after L. monocytogenes and VSV infection**

Optimal activation of the CD8<sup>+</sup> T cell response has been shown to be controlled by three distinct signals during priming: 1) pMHC/TCR engagement, 2) costimulation, and 3) cytokines (34). Recent work demonstrates that SLEC differentiation following LCMV and L. monocytogenes infection is controlled at the time of priming by the inflammatory milieu (3, 9, 35). Two of the cytokines shown to be intimately involved in activation, differentiation, expansion, and survival of CD8<sup>+</sup> T cells are IL-12 and IFN-αβ (36–41). To study the effects of IL-12 and IFN-αβ signaling on the differentiation of SLEC and MPEC populations, equal numbers of wild-type (WT) and either IL-12<sub>r</sub><sup>-/-</sup>, IFNAR<sup>-/-</sup>, IL-12<sub>r</sub><sup>−/-</sup>/IFNAR<sup>-/-</sup> OT-I cells were cotransferred into WT C57BL/6 mice (Fig. 3A). Effector CD8<sup>+</sup> T cell differentiation following either VSV-OVA or L. monocytogenes-OVA infection was measured in the peripheral blood. Following VSV-OVA infection there was no difference observed between WT and IL-12<sub>r</sub><sup>-/-</sup> OT-I cells in their ability to generate the effector subsets (Fig. 3B), but an ~2-fold reduction in expansion was noted (data not shown). In contrast, following L. monocytogenes-OVA infection IL-12<sub>r</sub><sup>-/-</sup> OT-I cells did not undergo differentiation to the SLEC population as efficiently as did WT OT-I cells, which resulted in a relative increase in EECs and MPECs (Fig. 3B) and an ~5-fold reduction in expansion was observed (data not shown). Moreover, following either L. monocytogenes-OVA or VSV-OVA infection IFNAR<sup>-/-</sup> and IL-12<sub>r</sub><sup>-/-</sup>/IFNAR<sup>-/-</sup> OT-I cells failed to generate the SLEC population as well as WT OT-I cells (Fig. 3C, 3D). In all cases, EEC and MPEC subsets were increased, suggesting that inflammatory signals acting at the EEC stage were driving SLEC development and that MPEC generation was the “default” pathway. Furthermore, IFNAR<sup>-/-</sup> and IL-12<sub>r</sub><sup>-/-</sup>/IFNAR<sup>-/-</sup> OT-I cells failed under as robust expansion after both VSV and L. monocytogenes infection when compared with WT OT-I cells; during VSV infection, effector CD8<sup>+</sup> T cell expansion was ~9-fold and ~100-fold reduced, respectively, whereas during L. monocytogenes infection effector CD8<sup>+</sup> T cell expansion was ~6-fold and ~400-fold reduced, respectively (data not shown). Thus, our results indicated that both IL-12 and IFN-αβ were important factors driving SLEC differentiation following L. monocytogenes infection, whereas only IFN-αβ was important following VSV infection. However, in either case some SLEC differentiation was apparent, indicating that additional factors were involved in the SLEC differentiation pathway, such as perhaps IL-2 (9, 10).

Overt inflammation mediated through IL-12 enhances SLEC differentiation during VSV infection

Our previous result indicated that the inflammatory milieu induced by L. monocytogenes and VSV infection differed significantly. Specifically, IL-12-mediated signals appeared to be lacking during VSV infection because IL-12β deficiency had minimal effect on CD8<sup>+</sup> T cell differentiation (Fig. 3B). To examine the early systemic inflammatory milieu induced during VSV and L. monocytogenes infection systemic infection serum was collected 6, 12, 24, 48, and 72 h postinfection and analyzed using a multiplex cytokine/chemokine panel (Supplemental Fig. 3). VSV infection induced high levels of IFN-α, whereas L. monocytogenes infection did not. In contrast, L. monocytogenes infection induced a low level of IFN-β, which was not evident after VSV infection. L. monocytogenes infection was a potent inducer of Th1 proinflammatory cytokines, as evidenced by IL-6, IL-12p70, and IFN-γ production; whereas VSV infection failed to elicit these cytokines, at least to levels that were detectable in the blood. Additionally, both IL-2 and IL-22 were increased following L. monocytogenes infection, but were not after

**FIGURE 3.** Differential role of IFNAR and IL-12<sub>r</sub> in effector CD8<sup>+</sup> T cell differentiation following VSV and L. monocytogenes infection. A, WT (CD45.1/2<sup>+</sup>) OT-I CD8<sup>+</sup> T cells and cytokine receptor-deficient (CD45.2<sup>+</sup>) OT-I CD8<sup>+</sup> T cells were cotransferred to CD45.1<sup>+</sup> mice. Mice were infected 18 h later i.v. with either VSV-OVA or L. monocytogenes-OVA. Seven days later the OVA/K<sup>+</sup>-specific CD8<sup>+</sup> T cells in the PBLs were monitored for differentiation of effector CD8<sup>+</sup> T cell subsets based on KLRG1 and CD127 expression. Three sets of mixed OT-I adoptive transfers were analyzed: WT/IL-12<sub>r</sub><sup>-/-</sup> (B), WT/IFNAR<sup>-/-</sup> (C), and WT/IL-12<sub>r</sub><sup>-/-</sup>/IFNAR<sup>-/-</sup> (D). Each bar represents the median ± 1 SEM. Each graph represents the mean of four to five mice per group and at least two independent experiments. Statistical significance was determined using a paired Student t test. *p < 0.05, **p < 0.01.
VSV infection. Following both L. monocytogenes and VSV infection the expression of IP-10 (CXCL10), G-CSF, and IL-16 was observed.

Considering the major differences in the cytokine milieu generated in response to the different infections, it was of interest to test whether altering the inflammatory conditions resulted in alterations in effector cell subset development. To this end, we treated VSV-infected mice with 50 μg ODN 1826, a type B CpG-containing oligonucleotide that resulted in induction of IL-12p70, IL-9, and IL-22, as well as enhanced expression of IP-10 and G-CSF (Supplemental Fig. 3, blue lines). Treatment of mice with ODN 1826 at the time of VSV infection did not alter effector CD8+ T cell expansion (Fig. 4A). However, ODN 1826 treatment did result in significantly greater SLEC differentiation as compared with control mice (Fig. 4B, 4C), with loss of approximately half the EEC and MPEC subsets. Treatment of mice with a control oligonucleotide lacking the CpG motif or ODN 1584, a type A CpG-containing oligonucleotide that does not induce IL-12p70 expression, did not alter the effector CD8+ T cell differentiation (data not shown). Because IL-12 has been shown to be important in the induction of T-bet and subsequent SLEC differentiation (3), we next tested whether IL-12 was necessary for the ODN 1826-induced SLEC differentiation, as well as whether IL-12 was sufficient to promote SLEC formation. To test this hypothesis C57BL/6 and p35−/− mice were either left untreated, treated with 50 μg ODN 1826, or treated with recombinant murine IL-12 at the time of VSV-OVA infection. Infection of C57BL/6 and p35−/− mice resulted in similar effector CD8+ T cell expansion and differentiation (Fig. 4A–C). Treatment of C57BL/6 mice with ODN 1826 significantly enhanced SLEC differentiation (Fig. 4B, 4C). However, ODN 1826 treatment of p35−/− mice did not result in augmentation of SLEC differentiation (Fig. 4B, 4C), indicating that IL-12 was necessary for the enhanced SLEC differentiation following ODN 1826 administration. Treatment of C57BL/6 mice with recombinant murine IL-12 at the time of VSV infection resulted in an enhancement of SLEC differentia-

**FIGURE 4.** ODN 1826 enhances SLEC differentiation through an IL-12- and Ebi3-dependent mechanism. A–C, C57BL/6 and p35−/− mice were infected i.v. with VSV-OVA. After viral infection, some mice were treated with either 50 μg ODN 1826 (within 2 h) or 0.5 μg rIL-12 (two doses, 1 and 24 h). Seven days later the VSV-N/Kb−specific CD8+ T cells in the spleen were identified by tetramer staining. Absolute numbers of VSV-N/Kb−specific CD8+ T cells (A) during effector CD8+ T cell differentiation were then assessed based on KLRG1 and CD127 expression by flow cytometry (B), and each effector subpopulation was quantified as a percentage of the tetramer+CD8+ T cell population (C). D and E, C57BL/6 and Ebi3−/− mice were infected i.v. with VSV-OVA. After viral infection, some mice were treated with 50 μg ODN 1826 (within 2 h). Seven days later the OVA/Kb−specific CD8+ T cells in the spleen were identified by tetramer staining. Absolute numbers of OVA/Kb−specific CD8+ T cells (D) during effector CD8+ T cell differentiation were then assessed based on KLRG1 and CD127 expression by flow cytometry (E) and each effector subpopulation was quantified as a percentage of the tetramer+CD8+ T cell population (F). For B and E, representative contour plots show the expression of KLRG1 and CD127 on the Ag-specific CD8+ T cells. In the upper right corner of each plot is the mean percentage of the population in each quadrant, which is graphed in C and F. Statistical significance was determined using a one-way ANOVA. *p < 0.05, **p < 0.01. These data are representative of four to five mice per group and two independent experiments.
differentiation was still observed in Ebi3 (Fig. 4) while decreasing the proportion of EECs and MPECs. ODN 1826 significantly enhanced SLEC differentiation (1.73-fold). ODN 1826 treatment enhances effector CD8+ T cell cytokine and furthermore appeared to act independently of IL-12.

D largely independent of Ebi3 (Fig. 4). ODN1826, or 1 g during VSV infection by treatment with ODN 1826 would enhance TEM precursors, as well as whether IL-12 was necessary and limited SLEC differentiation observed during VSV infection appears to be due to a lack of IL-12-mediated events, which can be mimicked with IL-12-inducing adjuvants.

IL-27/Ebi3 contributes to SLEC differentiation during VSV infection

Previous studies have shown that SLEC differentiation is highly dependent on T-bet (3). Infection of Tbx21−/− mice with VSV resulted in severely impaired SLEC differentiation (data not shown), but SLEC differentiation during VSV infection occurs independently of IL-12 (Fig. 4B, 4C), which is the prototypic inducer of T-bet expression (42). Thus, it was of interest to test whether other cytokines that induced T-bet expression could regulate effector cell subset development. Other cytokines known to regulate T-bet expression include IL-18 (43), IL-27 (44), and IFN-γ (45, 46). To this end, we infected C57BL/6, IL-18−/−, IFN-γ−/−, and Ebi3−/− mice with VSV-ova. IL-18 had minimal effect on the differentiation of effector CD8+ T cells during VSV infection with or without ODN 1826 treatment (Supplemental Fig. 4A). Also, IFN-γ deficiency had only a slight impact on SLEC differentiation after ODN 1826 treatment (Supplemental Fig. 4B). More interestingly, although effector CD8+ T cell expansion was largely independent of Ebi3 (Fig. 4D), differentiation of SLECs was markedly impaired in Ebi3−/− mice, with nearly a 2-fold reduction in SLEC differentiation (Fig. 4E, 4F). Simultaneously, there was an increase in the proportion of MPECs found, whereas EECs remained largely unchanged (Fig. 4E, 4F). Next, we examined whether Ebi3 expression was necessary for the ODN 1826-mediated enhancement of SLEC differentiation after VSV infection. To this end, C57BL/6 and Ebi3−/− mice were infected with VSV-ova and 1 d later treated with 50 μg ODN 1826. Similar to our previous results, treatment of C57BL/6 mice with ODN 1826 significantly enhanced SLEC differentiation (1.73-fold increase) while decreasing the proportion of EECs and MPECs (Fig. 4E, 4F). Similarly, ODN 1826 treatment enhanced SLEC differentiation was still observed in Ebi3−/− mice as compared with controls (1.79-fold increase), but the overall proportion of SLECs still significantly decreased compared with ODN 1826 treated C57BL/6 mice (Fig. 4E, 4F). Thus, Ebi3, and therefore likely IL-27, played a prominent role in the generation of SLECs and furthermore appeared to act independently of IL-12.

ODN 1826 treatment enhances effector CD8+ T cell cytokine production during VSV infection

Generating polyfunctional effector and memory CD8+ T cells provides optimal protective capabilities (47). Previous results show that after L. monocytogenes infection the effector CD8+ T cell population is largely polyfunctional, producing both IFN-γ and TNF-α and producing high levels of IFN-γ on a per cell basis (48). In contrast, after VSV infection the effector CD8+ T cell population appeared to be of lower quality (data not shown). Therefore, we tested whether enhancing the inflammatory milieu during VSV infection by treatment with ODN 1826 would enhance the functionality of the responding CD8+ T cells. To test this we treated C57BL/6 or p35−/− mice with PBS, 50 μg ODN1826, or 1 μg rIL-12. Seven days later we analyzed cytokine production from the effector CD8+ T cells found in the spleen by intracellular cytokine staining after a 4.5-h restimulation with peptide. VSV infection of C57BL/6 or p35−/− mice induced a substantial population of IFN-γ producers but only ∼25% of these produced TNF-α (Fig. 5). When C57BL/6 mice were treated with either ODN 1826 or rIL-12 the quality of the effector CD8+ T cells improved as judged by production of higher levels of IFN-γ on a per cell basis (Fig. 5A) and ~60% of the IFN-γ+ cells producing TNF-α (Fig. 5B). Surprisingly, the functionality of the effector CD8+ T cells was similarly enhanced in ODN 1826-treated p35−/− mice. Thus, while IL-12 was partially sufficient for increasing the functionality of the effector CD8+ T cells induced by VSV infection, IL-12 was not necessary for the enhancement of effector CD8+ T cells function following ODN 1826.

ODN 1826 treatment limits formation of CD62Llow effector memory precursors during VSV infection

Memory CD8+ T cells are a heterogeneous population. Based on CD62L and CCR7 expression at least two populations of memory cells have been described (49, 50). These populations are central memory cells that express both CD62L and CCR7, whereas effector memory cells lack expression of these proteins (50). Our previous results show that following VSV infection many more effector memory CD8+ T cell precursors are generated as compared with L. monocytogenes infection (51). In this study we examined whether treatment of VSV-infected mice with ODN 1826 altered the distribution of central memory T cell (TCM) and TEM precursors, as well as whether IL-12 was necessary and sufficient for such alterations. To test this we treated C57BL/6 or p35−/− mice with PBS, 50 μg ODN 1826, or 1 μg rIL-12. Seven days later we analyzed the proportion of Ag-specific CD62Lhigh MPECs within the lymph nodes (Fig. 6A, 6B), but similar effects were also observed in the spleen (data not shown). The percentage of CD62Lhigh MPECs was significantly enhanced after CpG treatment and this effect was partially IL-12 independent (Fig. 6B). IL-12 injection did not enhance CD62Lhigh MPEC development. However, the absolute number of CD62Lhigh MPECs was not significantly changed irrespective of the treatment (Fig. 6C), whereas the absolute number of CD62Llow MPECs after VSV infection was markedly lower in C57BL/6 mice treated with ODN-1826, but not rIL-12 (Fig. 6D). The effect of CpG treatment...

FIGURE 5. ODN 1826 enhances effector CD8+ T cell cytokine functional through an IL-12-independent mechanism. C57BL/6 and p35−/− mice were infected i.v. with VSV-OVA. After viral infection, some mice were treated with either 50 μg ODN 1826 (within 2 h) or 0.5 μg rIL-12 (two doses, 1 and 24 h). Seven days postinfection the potential of CD8+ T cells to produce IFN-γ and TNF-α was assessed by intracellular cytokine staining. Either IFN-γ mean fluorescent intensity (A) or the percentage of IFN-γ+TNF-α+ cells (B) was examined. For each panel a representative FACs plot showing the typical cytokine profile and gating is shown. Graphs show the median for four to five mice per group and are representative of at least three independent experiments. Statistical significance was determined using a one-way ANOVA and a Newman–Keuls posttest.
was again largely IL-12 independent. The differences in total numbers of cells compared with percentages can be explained by our findings showing that CpG treatment reduced the overall MPEC population (Fig. 4). Nevertheless, within the MPEC compartment, CpG treatment enhanced CD62L expression in an IL-12-independent manner, similar to the enhancement of cytokine production by CpG treatment (Fig. 5).

**Effector cell population dynamics differ between primary and secondary CD8+ T cell responses**

One strategy that has commonly been used to improve vaccine efficacy is a prime-boost regimen, in which the same Ag is given repeatedly over time typically in different vaccine vectors (21–24). Only recently have the functional outcomes of this prime-boost protocol been explored; repeated encounters with Ag resulted in memory populations that were more cytotoxic and less able to proliferate to subsequent Ag encounter (25–27). The reasons for these differences have not been determined. To address how repeated encounters with the same Ag impact effector and memory differentiation, we compared the CD8+ T cell response in mice infected with 10^3 CFU L. monocytogenes (1° L. monocytogenes) or 10^6 PFU VSV-OVA (1° VSV) to that of memory mice (>90 d after initial infection) challenged with 5 × 10^5 CFU L. monocytogenes-OVA (2° L. monocytogenes-L. monocytogenes-OVA or 2° VSV-L. monocytogenes) or 2 × 10^5 PFU VSV-OVA (2° VSV-OVA-VSV or 2° VSV-VSV). Postinfection, mice were serially bled and the OVA/Kb-specific CD8+ T cell response was analyzed. Surprisingly, the composition of the secondary responding populations was drastically different from what was observed during the primary response. This was especially evident during secondary VSV infection (Fig. 7A). In all cases, the KLRG1high population predominated for substantially longer in the secondary response (Fig. 7A). This resulted in a much more protracted appearance of MPECs in the secondary response. As compared with the primary response, the recall response was comprised of a much greater population of CD127highKLRG1high (DPEC) cells. Even at ~150 d after secondary infection, ~60–70% of the memory cells expressed KLRG1low and ~40% of those cells coexpressed CD127 (data not shown). Thus, in the secondary response CD127 expression and the absence of KLRG1low did not correlate with survival.

We next tested whether the enhanced SLEC differentiation observed in the secondary response was cell intrinsic or the result of possible changes in the inflammatory milieu during the secondary infection. For these experiments we used the VSV model, as the difference between the primary and secondary responses was most dramatic (Fig. 7A). Experimentally, 10^5 memory OT-I cells (CD45.1/2+) were cotransferred with 10^6 naive OT-I cells (CD45.1+) to naive C57BL/6 mice that were infected 1 d later with VSV-OVA. Analysis of both the naive and memory OT-I cells revealed that a much greater proportion of the secondary CD8+ T cells expressed KLRG1 than did the cells responding for the first time (Fig. 7B). Additionally, many more primary cells expressed CD62L than did reactivated memory cells (Fig. 7B). These data implied that memory CD8+ T cells had undergone genetic modifications that predisposed the cells to differentiate toward a SLEC phenotype upon secondary encounter with Ag, whereas the primary responders were largely influenced by environmental factors.

**Recall and differentiation potential of the individual secondary memory CD8+ T cell subsets**

Relatively little is known about the role of the effector/memory subsets in recall responses, especially with respect to the CD127highKLRG1high (DPEC) population. To test the capabilities of these three populations of secondary memory cells, memory phenotype CD8+ T cells (CD11ahighCD44high) from CD45.2+ C57BL/6 mice previously infected and challenged with L. monocytogenes were separated into CD127highKLRG1low, CD127highKLRG1high, and CD127lowKLRG1high subsets. After sorting, 10^4 OVA/Kb-specific CD8+ T cells of each population were transferred individually into naive CD45.1+ C57BL/6 mice (Fig. 8A). One day later mice were infected with 10^4 CFU L. monocytogenes-OVA and were subsequently bled at the indicated times to monitor expansion and effector cell differentiation. Each memory subset generated a readily detectible effector CD8+ T cell population in the blood, indicating that each had undergone substantial expansion. CD127highKLRG1low memory cells mounted a robust tertiary expansion, whereas CD127lowKLRG1high memory cells had a substantially diminished ability to undergo expansion (Fig. 8B). Interestingly, CD127lowKLRG1high memory cells exhibited an intermediate ability to undergo expansion upon re-encounter with Ag (Fig. 8B).

More intriguing was the subsequent effector cell differentiation from each purified memory subset. Recall of the CD127lowKLRG1high population resulted in expansion of effector CD8+...
T cells that exclusively retained the same phenotype (CD127low KLRG1high) even into memory (Fig. 8C). When the CD127high KLRG1high memory population was recalled, effector CD8+ T cells were largely CD127low KLRG1high in phenotype; with time, a CD127high KLRG1low memory population emerged, but CD127high KLRG1low cells never emerged (Fig. 8C). Strikingly, only the CD127high KLRG1low memory population appeared to contain true “memory stem cells,” which were capable of regenerating all effector/memory cell subsets following rechallenge. The effector CD8+ T cell response generated by the sorted CD127high KLRG1low population was largely dominated by cells of CD127low KLRG1high phenotype, but small numbers of the other three effector CD8+ T cell populations could be found. With time, CD127high KLRG1high and CD127high KLRG1low phenotype cells increased in frequency (Fig. 8C), similar to what was observed during the secondary immune response in intact mice (Fig. 7A). Nonetheless, even by ~200 d after challenge only a small fraction of the cells were of the IL-7Rhigh KLRG1low phenotype (~10%). Thus, we have demonstrated the importance of generating large numbers of CD127high KLRG1low CD8+ T cells during prime-boost vaccination protocols to generate a memory pool that has “stem cell”-like properties.

Discussion
Immunological memory is the foundation of vaccination. Understanding the factors governing and regulating the differentiation of the memory pool are important for understanding vaccine efficacy or failure. In this study we demonstrate that early post-infection, naive Ag-specific CD8+ T cells become activated and form an EEC pool that is CD127 low KLRG1 low in phenotype. These EECs then differentiate into MPECs (CD127 high KLRG1 low) or SLECs (CD127 high KLRG1 high), and MPECs (CD127 low KLRG1 high) to >98% purity and 10^6 cells of each phenotype were adoptively transferred to naive CD45.1+ mice. One day later those mice were challenged with 10^6 CFU L monocytogenes-OVA (LM-ova) (A). The recall kinetics (B) and subsequent tertiary effector CD8+ T cell differentiation (C) of the OVA/Kb-specific memory CD8+ T cell subpopulations was tracked in the PBLs using CD45.2 expression to identify the adoptively transferred cells and KLRG1 and CD127 expression to monitor effector cell differentiation. In the upper right corner of each plot is the mean percentage of the population in each quadrant. These data are representative of two independent experiments, each containing four to five mice per group.
The origin of the memory CD8+ T cell population has long been debated. It is thought that memory cells must go through an effector cell stage (52–54). This concept is further supported by single cell adaptive transfer (5) and cellular bar-coding (6) experiments, which demonstrate that each naive CD8+ T cell has the potential to generate all the effector and memory CD8+ T cell lineages described. Our data further support that memory CD8+ T cells pass through an obligatory effector state, as all effector CD8+ T cell subsets expressed granzyme B on day 5 postinfection. However, with time, effector CD8+ T cells lost granzyme B expression in a hierarchical manner (TCM → TEM → MPECs → EECs → SLECs), which supports the progressive differentiation model of CD8+ T cell differentiation (55–57). The hierarchical loss of granzyme B expression is likely tied to the responsivity of those populations to IL-2. IL-2 has been shown to stabilize granzyme B and perforin expression in effector CD8+ T cells (11); furthermore, we have shown that CD25/IL-2Rα is expressed at higher levels on EECs and SLECs when compared with MPECs (9). Additionally, granzyme B levels were substantially lower after VSV infection when compared with L. monocytogenes infection.

The lack of robust Th1 inflammation during VSV infection might explain why granzyme B levels were lower, because IL-6 and IL-12 enhance granzyme B expression in CD8+ T cells (11, 58). Decreased expression of granzyme B could be one of the reasons why VSV is able to persist for up to 60 d in the host (59).

The decision to become either a SLEC or MPEC teeters on the transcription factor profile of the responding CD8+ T cells, with T-bet and Blimp-1 enhancing SLEC differentiation, whereas eomesodermin (Eomes) and Bcl6 are important in MPEC differentiation (60–62). Recent work with Th1 CD4+ T cells demonstrated that T-bet repressed Tcf7 (TCF-1) expression (63); furthermore, Tcf7 is an important transcription factor for memory CD8+ T cell development (64, 65). Recent work has also begun to identify the factors regulating these critical transcription factors. We have demonstrated that IL-2 signaling is important for SLEC differentiation (9); furthermore, IL-2 enhances Blimp-1 expression while repressing Bcl6 expression (11). IL-12 has been shown to induce the expression of Thx21 (T-bet), while repressing Eomes in CD8+ T cells, resulting in the accumulation of effector cells and limiting memory development (61). Other studies have shown that IL-12 is necessary for the accumulation of KLRG1high CD8+ T cells after Toxoplasma gondii infection (66) and vaccination with peptide-pulsed dendritic cells (3, 8), but not in other systems (67). Our data support the role of IL-12 in SLEC differentiation during strong Th1-like inflammation, that is, L. monocytogenes infection or ODN 1826 treatment. However, during VSV infection there was no role for IL-12 in mediating SLEC differentiation. This resulted in a large proportion of the effector CD8+ T cells retaining a CD127+KLRG1low phenotype, reminiscent of the EEC population. This was not unique to the VSV model, as intranasal infection with influenza A virus or infection with vaccinia virus by skin scarification resulted in a similar retention of early effector CD8+ T cells with the CD127+KLRG1low phenotype (data not shown). These data fit well with a previous report demonstrating that the CD8+ T cell response during L. monocytogenes infection was highly dependent on IL-12Rβ signal, whereas responses to viral infections were not (41). Nevertheless, after VSV infection ~40% of the effector CD8+ T cell population still expressed KLRG1. In this study we showed that Ebi3, a component of IL-27 together with p28 (68), regulated SLEC differentiation during VSV infection. IL-27 is an IL-12 cytokine family member, which is an important inducer of T-bet and IL-12Rβ in both CD4+ and CD8+ T cells via a STAT1-dependent mechanism (16, 44). Interestingly, IFN-α can also induce T-bet and IL-12Rβ expression in naive T cells through a STAT1-dependent mechanism (16), which fits with our observation that IFNAR−/− CD8+ T cells had a reduced differentiation of SLECs. Our results might explain why after dendritic cell vaccination CpG DNA induced a more pronounced SLEC differentiation than did IL-12 or IL-12/IFN-γ treatment (3, 8), because this reductionist system lacked IL-27 and/or type I IFNs. In our system, we demonstrated that IFN-γ played only a minimal role, which contradicts previous work that suggests IFN-γ enhances SLEC differentiation (8, 69, 70). This difference is likely due to the magnitude of IFN-γ expression in those systems. In our hands use of ODN 1826 as an adjuvant induced minimal IFN-γ expression, whereas the other groups used live L. monocytogenes as an inflammatory adjuvant, which induces robust levels of IFN-γ.

Thus, our data together with published results support a model in which high levels of inflammatory cytokines drive maximal SLEC differentiation while limiting MPEC generation. However, depending on the pathogen or vaccination protocol only a subset of

**FIGURE 9.** Model of SLEC/MPEC differentiation under different inflammatory conditions. A, The proportion of effector CD8+ T cells that differentiate into SLECs is highly dependent on the inflammatory milieu induced by infection or vaccination. Under conditions of high inflammation, such as after L. monocytogenes (LM) infection, SLEC differentiation is significantly enhanced by the expression of inflammatory cytokines, which include type I IFNs (IFN-α/β), Ebi3 (and therefore likely IL-27), IL-12, and IFN-γ (top). In contrast, when only low levels of inflammation are induced, such as following DC vaccination (70), there is limited SLEC differentiation due to limited expression of inflammatory cytokines (bottom left). Mild inflammation results in fewer SLECs being generated, due to the limited expression of inflammatory cytokines. For example, during VSV infection type I IFNs (IFN-α/β) and Ebi3 are expressed, but IL-12 and IFN-γ are not expressed early. This inflammatory cytokine profile will result in significantly fewer SLECs being generated (middle). B, ODN 1826 treatment had significant effects on effector CD8+ T cell differentiation, which were either IL-12 dependent or independent. Enhancement of SLEC differentiation was highly dependent on IL-12, which is due to the ability of IL-12 to enhance T-bet expression (3). In contrast, enhancement of effector cytokine expression by ODN 1826 treatment occurred through an IL-12-independent mechanism. Also, ODN 1826 shifted the TCM/TEM ratio in favor of TCM generation through an IL-12-independent mechanism.
these inflammatory cytokines may be expressed, thus dampening SLEC differentiation and enhancing the number of MPECs generated (Fig. 9A). Specifically, early IL-27 and IFN-α expression enhances T-bet and IL-12Rβ expression in responding CD8+ T cells, beginning the SLEC differentiation process. Activated CD8+ T cells then respond to IL-12, if present. IL-12 signaling further augments T-bet expression in the responding CD8+ T cells enhancing SLEC differentiation. IL-12 signaling also amplifies CD25 expression on the responding CD8+ T cells (11), which promotes the expansion and differentiation of more SLECs through IL-2 signaling. Furthermore, IL-12 enhances IFN-γ production, leading to a positive feedback loop of IFN-γ on T-bet expression (71); thus, IFN-γ can enhance SLEC differentiation (8) and/or expansion (72) in CD8+ T cells if IFN-γ is highly expressed during the immune response. Subsequent survival of the SLEC population is highly sensitive to the balance of IL-15 and TGF-β, which promote its survival and death, respectively (73). A shift of this balance toward TGF-β might explain why CD8+ T cells induced by VSV infection equilibrate to a memory phenotype more quickly.

In addition to enhancing SLEC differentiation, CpG-induced inflammation limited TEM differentiation and enhanced CD8+ T cell polyfunctionality. In contrast to the enhanced SLEC differentiation, both of these inflammation-induced alterations to the CD8+ T cell population occurred through an IL-12–independent pathway (Fig. 9B). Alterations in TEM/TEM differentiation could be the result of changes in the levels of IL-15 and IL-2, which we have previously shown to be important in TEM and TEM differentiation, respectively (51). CpG treatment has been shown to enhance IL-15 expression (74), which could maintain TEM MPEC numbers while the CD62Llow cells are driven toward terminal effectors. In addition to altering inflammatory cytokine production, CpG treatment alters expression of costimulatory molecules (75). Specifically, costimulation through OX40/OX40L interactions during vaccinia virus infection resulted in CD8+ T cells with greater functionality (76). Thus, it is likely that multiple cytokines and costimulatory pathways simultaneously play a crucial role in regulating effector CD8+ T cell differentiation.

Importantly, our studies demonstrated that multiple encounters with the same Ag had dramatically different outcomes on the CD8+ T cell response even when the same priming vector was used. This observation has important implications for the development of effective prime-boost vaccination protocols. Because of the ease of genetic manipulation and ability to induce strong cellular immune responses, both L. monocytogenes and VSV have been postulated to be effective vaccine vectors for the induction of strong T cell responses (77). Our data demonstrated that during secondary and tertiary infection more SLEC phenotype cells are generated and that these KLRG1high cells survive for prolonged periods of time. This was most dramatic following VSV infection, in which during primary infection ∼30% of the effector CD8+ T cells were of SLEC phenotype, whereas during secondary VSV infection ∼80% of the effector CD8+ T cells were of SLEC phenotype. Indeed, it might be worth considering modifying the current nomenclature since in the secondary response a substantial number of long-lived memory cells express KLRG1 either with or without CD127 expression. Previous studies have demonstrated that secondary and tertiary memory populations appear more terminally differentiated, as measured by high expression of KLRG1 and low expression of CD27 and CD62L (25–27, 78). Furthermore, we demonstrated that this enhanced SLEC formation was intrinsic to the memory CD8+ T cell, as coadaptive transfer of naive and memory CD8+ T cells into the same recipient resulted in the same phenomenon. Supporting this is the observation that in human memory CD8+ T cells the KLRG1 promoter region is in an open configuration (79). An alternative explanation for our observation is that memory cells express a different panel of cytokine/chemokine receptors that enables them to sense different cues and could result in enhancement of this terminally differentiated phenotype. Using a transcriptome approach, Wirth et al. (27) found that numerous cytokine and chemokine receptors are differentially regulated in naive and memory CD8+ T cells, such as IL-12p2, IL-2α, CXCR3, and CCR7, which are known to play important roles in regulating SLEC/MPEC differentiation (3, 9, 10, 80). Thus, naive and memory CD8+ T cells may express different cytokine receptors that could alter their ability to sense the inflammatory environment; for example, memory CD8+ T cells are known to be more sensitive to IL-18 (81). Consequently, it is crucial that we understand the factors regulating effector and memory CD8+ T cell differentiation under a range of differentiation conditions.

The heterogeneous nature of the memory populations begs the question as to which of the subpopulations are involved in responding to secondary and tertiary challenges and at what level. Because multiple encounters with the same Ags result in KLRG1high cells with prolonged life spans, we examined how both the KLRG1highCD127low and KLRG1highCD127high phenotype memory CD8+ T cells may contribute to subsequent immune responses. Interestingly, the KLRG1lowCD127high, classical memory cells had the greatest recall potential and had the ability to generate all the effector CD8+ T cell populations, including more memory precursors. In contrast, both KLRG1high memory cell populations were unable to regenerate KLRG1lowCD127high memory precursors, but they were able to contribute to the recall response to varying degrees. KLRG1highCD127high phenotype memory cells underwent robust expansion, only ∼2- to 4-fold less than the KLRG1lowCD127high memory population; in contrast, KLRG1highCD127low phenotype memory cells had relatively poor recall potential, ∼20-fold less than the KLRG1lowCD127high memory population. It has been reported that Bmi-1 is necessary for optimal CD8+ T cell expansion and TCR-mediated Bmi-1 expression is reduced in KLRG1highCD8+ T cells (82), which might explain why both KLRG1high memory CD8+ T cell populations underwent less expansion than did CD127highKLRG1low memory CD8+ T cells. Additionally, KLRG1 may actively suppress CD8+ T cell proliferation, because mAb blockade of KLRG1/E-cadherin interactions led to enhanced phosphorylated Akt, which in turn resulted in increased cyclin D, increased cyclin E, and decreased p27, resulting in more proliferation (83). Why the CD127highKLRG1high population is more competent for expansion than the CD127loKLRG1high population remains unknown. One possibility is that IL-7–mediated signaling might partially overcome the inhibitory signals mediated by KLRG1. These findings have important implications in the development of prime-boost vaccination regimens, where the timing and formulation of boosters may be a critical factor in the generation of long-term protective immunity.

Acknowledgments
We thank Drs. Matthew Mescher and John Harty for providing cytokine receptor knockout OT-I cells and Drs. Mark Jutila and David Pascual for providing IL-18 and Ebi3 knockout mice, respectively. We thank Diane Gran for expert assistance in conducting cell sorting.

Disclosures
The authors have no financial conflicts of interest.
References
Bcl-6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells. 


