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IL-2 Induction of Blimp-1 Is a Key In Vivo Signal for CD8+ Short-Lived Effector T Cell Differentiation

Salix Boulet,* Jean-François Daudelin,* and Nathalie Labrecque*†‡

During infection or vaccination, only a small proportion of CD8+ T cells differentiate into memory cells. The mechanisms underlying the differentiation of CD8+ T cells into short-lived effector cells (SLECs) or memory precursor effector cells are poorly defined. It was recently shown in infectious models that the transcriptional repressor B lymphocyte–induced maturation protein 1 (Blimp-1) enhances the formation of SLECs. The factors controlling Blimp-1 expression leading to the in vivo formation of SLECs are still not known. However, it has been shown that cytokines such as IL-2 induce Blimp-1 expression in vitro. In this study, we took advantage of the low-inflammation model of dendritic cell immunization to study the role of the IL-2/Blimp-1 axis in SLEC differentiation as well as the importance of Blimp-1 expression in memory precursor effector cells for proper CD8+ memory generation. Our results show that Blimp-1 deficiency affects effector differentiation and function in the absence of inflammation. Unexpectedly, memory generation was not affected in Blimp-1-deficient OT-I cells responding to vaccination. In addition, modulation of the bioavailability of IL-2 by injection either of a blocking Ab or of the cytokine, demonstrates a link between IL-2, Blimp-1 induction, and SLEC formation in wild-type cells. Conversely, injection of IL-2 had less effect on Blimp-1–deficient CD8+ T cells, indicating that the effect of IL-2 on in vivo SLEC differentiation is mediated by Blimp-1. In conclusion, IL-2 induction of Blimp-1 expression is a key regulator of SLEC differentiation in vivo. The Journal of Immunology, 2014, 193: 1847–1854.

O nce CD8+ T cells encounter their Ag, a molecular program that allows for their differentiation into effector cells is initiated. Effector T cells may then contribute to the proper control of the pathogenic agent through cytotoxicity and cytokine secretion. This function completed, contraction of the effector T cell pool results in the survival of only a few memory cells that will provide immunological protection.

Considerable efforts have been made to disentangle the molecular events regulating the stepwise formation of effector and memory T cells (1). These studies have been facilitated by the discovery of molecular markers that can be used at the effector stage to distinguish CD8+ T cells more likely to become memory T cells from those that will fail to survive the contraction phase (1). Memory precursor effector cells (MPECs) are CD127hi and KLRG1lo and have the potential to remain as long-lived CD8+ T cells. Conversely, short-lived effector cells (SLECs) are terminally differentiated cells. They express increased levels of KLRG1 and lower levels of CD127 (CD127loKLRG1hi) and die by apoptosis following clearance of an infection (2, 3). B lymphocyte–induced maturation protein 1 (Blimp-1), encoded by the gene Prdm1, is one of the key molecules that promote the differentiation of SLECs during infection (4, 5). Indeed, during acute infections, CD8+ effectors lacking Blimp-1 generate fewer SLECs, and as a consequence, more MPECs and long-lived memory T cells are generated. Moreover, effectors lacking Blimp-1 expression produce less granzyme B (grzmB), more IL-2, and more TNF-α, a phenotype that probably reflects their differentiation into MPECs (4, 5).

Although Blimp-1 is required for SLEC formation and proper acquisition of effector functions, it is also expressed in MPECs, albeit at a lower level than in SLECs (4, 6). It is therefore intriguing that MPECs from Prdm1 conditional knockout (KO) mice are not affected in their differentiation into memory cells. In addition, more MPECs and long-lived memory CD8+ T cells are generated in the absence of Blimp-1, suggesting that its expression in CD127hi KLRG1lo cells might be deleterious to memory formation. Following dendritic cell (DC) vaccination, memory CD8+ T cell generation is accelerated, and most of the effectors acquire an MPEC phenotype at the peak of the T cell response (7). This results from a decrease in inflammatory signals normally available during an infection. Therefore, DC vaccination offers an opportunity to study the importance of Blimp-1 in the context of low inflammation and to dissect out the role of Blimp-1 expression in MPECs and in their differentiation into long-lived CD8+ memory T cells. Because DC-based vaccination is a promising approach to treat cancer patients, it is important to decipher the role of transcription factors such as Blimp-1 in effector and memory CD8+ T cell differentiation in this system.

In addition, the absence of infection and concomitant inflammation in DC vaccination allows for simple modulation of individual cytokines or chemokines provided during vaccination. This capability may serve as a tool to identify mechanisms of CD8+ T cell differentiation. The signals necessary for the in vivo induction of Blimp-1 in Ag-specific CD8+ T cells are still undefined.
It is known that IL-2 can induce Blimp-1 expression when CD8+ T cells are activated in vitro (8–10) and that prolonged IL-2 signaling is required to promote the terminal differentiation of CD8+ CD25hi effectors, which express elevated levels of Blimp-1 (11). It is therefore tempting to speculate that IL-2 signaling is directly responsible for the in vivo induction of Blimp-1 in Ag-specific CD8+ T cells to promote their differentiation into SLECs. In this article, we have evaluated whether Blimp-1 controls effector CD8+ T cell differentiation when inflammation is low and whether its expression in MPECs affects proper differentiation of functional memory CD8+ T cells. To further our understanding of the differentiation pathway controlling effector cell fate choice, we have identified an in vivo signal controlling Blimp-1 expression and SLEC differentiation.

Materials and Methods

**Mice**

B6.SJL, (C57BL/6xB6.SJL)F1 and OT-1 (12) mice were bred at the Maisonneuve-Rosemont Hospital Research Center facility (Montreal, Quebec, Canada). E8I-Cre (C57BL/6-Tg(Cd8a-cre)Itan/J) (13) and Prdm1 floxed (B6.129-Prom1tm1Clme/J) (14) mice were purchased from The Jackson Laboratory. OT-1 CD45.2+ were crossed to obtain OT-I Prdm1 floxed/floxed (Il2/Il2) mice with (KO) or without [wild-type (WT)] the E8I-Cre transgene. Prdm1 deletion was verified by PCR and quantitative PCR (see Supplemental Fig. 1). Mice were housed in a pathogen-free environment and treated in accordance with the Canadian Council on Animal Care guidelines.

**Bone marrow DC culture**

Bone marrow (BM) DCs were generated from male B6.SJL mice, as described previously (15). On day 6, 1 μg/ml LPS (Sigma-Aldrich) was added to the culture to induce maturation of DCs, and the OVA peptide (SIINFEKL; OVA257–264) (Midwest Biotech) was loaded overnight (2 μg/ml). On the seventh day of culture, DCs were purified by density centrifugation on 14.7% Nycodenz (Sigma-Aldrich) and washed three times in PBS before injection.

**Vaccination, IL-2 bioavailability modulation, and analysis of the T cell response**

At 2 d after adoptive transfer of 10^3 or 10^4 OT-I cells from female CD45.2+ mice, B6.SJL, CD45.1+ female recipient mice were immunized with 5 × 10^5 OVA-pulsed male-derived DCs. Male DCs were used to provide antigenic help (16). Effector responses (4–6 d postvaccination, according to the number of adoptively transferred cells) or memory responses (≥45 d postvaccination) were identified by flow cytometry as CD8+CD44hi even in the same mouse by surgical removal of superficial lymph nodes (LN) (17) or in the lymphoid organs of euthanized mice. The functional quality of effector and memory CD8+ T cells was evaluated as previously described (15, 18). In some experiments, the IL-2 signal was blocked by injecting i.p. 1 mg JES6-1A12 mAb (BioXcell) daily on days 0–5 postvaccination. In other experiments, 15,000 U IL-2 (Novartis) was injected i.p. twice daily from day of vaccination until day of euthanasia. As a negative control for experiments modulating IL-2 bioavailability, either PBS or an isotype control Ab (Rat IgG2a clone 2A3; BioXcell) was injected.

**Abs, flow cytometry, and cell sorting**

Anti–TNF-α (MP6-XT22), -BrdU (B44) Abs and 7-aminoactinomycin D (7-AAD) were purchased from BD Biosciences. Anti–CD45.2 (104), -CD44 (1IM7), -CD8 (53-6.7), -CD43 (1B11), -CD62L (MEL-14), -IL-2 (JES6-5H4), -CD25 (PC61), and Annexin V Abs were purchased from BioLegend. Anti–CD127 (7-A)R34), -KLRC1 (2F1), and -gzmB (16G6) Abs were purchased from eBioscience. Anti–IFN-γ (XMG1.2) Ab was purchased from Life Technologies. Cell surface and intracellular stainings for cytokines were performed as previously described (15). To perform 7-AAD and Annexin V stainings, cells were surface stained and washed once with PBS and once with binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2). Cells were then incubated for 15 min with 7-AAD and Annexin V diluted in binding buffer prior to acquisition on a cytometer. For intracellular staining, BrdU staining was performed as follows. Cells were fixed with the Cytofix/Cytoperm buffer (BD Biosciences) for 30 min and permeabilized with the Perm/Wash buffer (BD Biosciences) for 10 min. Cells were then refixed for 5 min prior to DNA treatment (1 h, 37˚C, 30 μg/ml 10^5 cells; Sigma-Aldrich). Finally, cells were incubated with the anti-BrdU Ab diluted in the Perm/Wash buffer for 30 min. Stainings were analyzed on the BD FACSCanto I or LSR II system. In some experiments, endogenous (CD8+CD45.2−), total OVA-specific effector cells (CD8+CD45.2+), or early effector cells (EECs) (CD8+CD45.2+CD127−KLRC1−) were sorted with a BD FACSaria III.

**Listeria monocytogenes OVA challenges**

At ≥45 d postvaccination with OVA-pulsed DCs, L. monocytogenes-OVA (2 × 10^7 CFU) challenges were performed as previously described (19).

**Quantitative real-time PCR**

Expression of Prdm1 mRNA of sorted CD8+ T cells was performed as previously described (19). Briefly, total RNA was isolated using TRIzol (Life Technologies) and reverse transcribed into cDNA using SuperScript II with oligo(dT) primers (Life Technologies). Real-time PCR was performed in triplicates using Power SYBR Green (Life Technologies) on an Applied Biosystems 7500 Real-Time PCR System. The ΔCt cycle threshold (Ct) value for each sample was determined by calculating the difference between the Ct value of the target and the Ct value of the endogenous reference gene (HPRT). Then, the ΔΔCt value for each sample was determined by subtracting the mean of the ΔCt value of the sample from the ΔCt value of a reference sample. The relative level of target gene expression was calculated using 2^−ΔΔCt.

**Statistical analysis**

Statistical analyses for differences between groups were performed using the Student t test. The Welch correction was applied for unequal variances when required. Data are presented as mean ± SEM. All tests were two sided, and p < 0.05 was considered statistically significant; *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

Blimp-1 does not affect T cell expansion and memory formation when inflammation level is low

To evaluate whether Blimp-1 expression is required for the proper differentiation of effector CD8+ T cells when the inflammation level is low, we adoptively transferred WT or Blimp-1-deficient OT-I T cells (see Materials and Methods and Supplemental Fig. 1) into naive B6.SJL recipients, followed by vaccination with DCs loaded with the OVA peptide (DC-OVA). As shown in Fig. 1A, Blimp-1 deficiency in CD8+ T cells did not alter the expansion of OVA-specific CD8+ T cells, as a similar frequency and number of OT-I T cells were recovered in the LNs, BM, and spleen at the peak of the T cell response (day 4 for the adoptive transfer of 10^6 OT-I T cells and day 6 for 10^7). These results further confirm that Blimp-1 expression is not required for CD8+ T cell expansion.

In acute infection, Blimp-1 is dispensable for the generation of memory CD8+ T cells, and its deficiency enhances memory formation (4, 5). We investigated whether Blimp-1 expression was required for memory formation in response to DC vaccination, in which the majority of effectors differentiate into MPECs. As shown in Fig. 1B, a lack of Blimp-1 expression did not preclude or enhance the formation of memory CD8+ T cells. These Blimp-1-deficient memory T cells had a similar phenotype and produced similar amounts of cytokines as WT cells following a short in vitro restimulation with their cognate Ag (Supplemental Fig. 2A). When mice were rechallenged 45 d postvaccination with a lethal dose of recombinant Listeria monocytogenes—expressing OVA (L. monocytogenes–OVA), both WT and Blimp-1-deficient OT-I T cells were recovered in the LNs, BM, and spleen at the peak of the T cell response (day 4 for the adoptive transfer of 10^6 OT-I T cells and day 6 for 10^7). These results further confirm that Blimp-1 expression is not required for CD8+ T cell expansion.
Blimp-1 influences effector T cell differentiation in a precursor-frequency–dependent manner

In response to influenza and lymphocytic choriomeningitis virus infections, Blimp-1 is required for the proper differentiation of effectors into SLECs (4, 5). We therefore evaluated whether Blimp-1 deficiency in CD8+ T cells affects the acquisition of effector functions and phenotype in a context of low inflammation. WT and Blimp-1–deficient OT-I effectors expressed normal levels of the activation markers CD44 and 1B11 (Supplemental Fig. 2C), and they produced similar amounts of IFN-γ (Fig. 2A). However, with a low precursor frequency, Blimp-1–deficient OT-I CD8+ T cells produced less grzmB (Fig. 2A, 2B) and more IL-2 and TNF-α (Fig. 2A). This led to an increase in the frequency of cells making both IFN-γ and IL-2 or TNF-α (Fig. 2B). Functional differences between WT and KO effector cells were lost if 10^6 OT-I cells were transferred prior to vaccination (Fig. 2).

We also evaluated the impact of Blimp-1 deficiency on the SLEC/MPEC differentiation choice. As shown in Fig. 3, at the peak of the response, when a high precursor frequency of OT-I T cells was used, most effectors differentiated into MPECs even when Blimp-1 was present. However, when a low frequency of OT-I naive T cell precursor was used, a significant fraction of the effectors acquired an SLEC phenotype only when Prdm1 was not deleted (Fig. 3A, 3B). This observation was particularly obvious when looking at SLEC cell numbers (Fig. 3C). Moreover, when both WT and Prdm1^−/− OT-I T cells with low precursor frequency were cotransferred in the same host, SLECs were predominantly observed in the WT OT-I effector population (Supplemental Fig. 2D, 2E). This finding suggests that Blimp-1 is intrinsically required for efficient SLEC differentiation upon DC vaccination and that MPEC/SLEC differentiation may be controlled by similar molecular events in the presence or absence of inflammation.
The level of Blimp-1 expression induced in effectors is influenced by precursor frequency

The fact that precursor frequency modulated MPEC/SLEC differentiation is intriguing, because, in both instances, naive T cells should have received similar DC signals and responded to the same low inflammatory milieu. To further understand why almost no SLECs were generated when a high frequency of OT-I naive T cell precursor was used, we evaluated whether Blimp-1 expression was differentially induced as a function of the number of naive CD8+ T cells transferred prior to vaccination. To accomplish this, Prdm1 mRNA expression was measured in effectors at the peak of the response. As shown, in Fig. 3D, a high level of Prdm1 mRNA expression was induced only in effectors that were obtained following DC vaccination of mice adoptively transferred with a low precursor frequency of naive OT-I T cells. This result suggests that high precursor frequency of naive T cells affects the signals that are perceived during Ag presentation or the expansion phase, which then interferes with the differentiation program of the effectors.

These results offer a unique opportunity to identify the signals that regulate Prdm1 expression in vivo. It is well documented that IL-2 increases Blimp-1 expression in effector cells from mice injected with IL-2 (8–10). Furthermore, it was shown that prolonged IL-2 signals promote the generation of SLECs (11, 20). Because no SLECs are generated when a high precursor frequency of OT-I T cells is used, we hypothesized that this occurs because of an increased competition for IL-2 by Ag-specific CD8+ T cells. To support this idea, we compared CD25 expression on effectors from adoptive transfers with 10^5 and 10^6 OT-I cells. In the course of an immune response, CD25 levels on Ag-specific CD8+ T cells have been shown to reflect bioavailable IL-2 levels (11). As shown in Fig. 3E, both at day 4 and at day 6 postvaccination, CD25 expression was increased on effectors when 10^5 OT-I cells, compared with 10^6 cells, were transferred. Thus, augmenting the number of CD8+ T cell precursors resulted in decreased bioavailable IL-2. Of interest, although Blimp-1 targets CD25, the levels of the IL2Rα-chain on WT and KO cells were similar in these early stages of the response (data not shown), which is consistent with a recent publication reporting that Blimp-1 represses CD25 expression during the late stages of a CD8+ T cell response (21).

In vivo IL-2 availability controls Blimp-1 expression and SLEC differentiation in a Blimp-1–dependent manner

To test the importance of IL-2 for the in vivo differentiation of SLECs, we evaluated whether decreasing IL-2 availability affects SLEC differentiation when the Ag-specific T cell precursor frequency is low. Compared with PBS (Fig. 4) or isotype control (Supplemental Fig. 3), injections, treating mice with a blocking anti–IL-2 mAb during the priming and expansion phase of the T cell response severely reduced the number of SLECs that were generated (Fig. 4A–C) and led to a reduction of Prdm1 expression (Fig. 4D). It is therefore likely that Ag-specific T cells received fewer IL-2 signals in vivo when their precursor frequency is high, which leads to lower expression of Blimp-1 and reduced SLEC generation.

We then determined whether increasing IL-2 availability enhanced the formation of SLECs when the Ag-specific T cell precursor frequency is high. Recipient mice were therefore adoptively transferred with 10^5 naive OT-I CD8+ T cells, immunized with DC-OVA, and injected twice daily with rIL-2 (15,000 U per injection). In response to IL-2, the peak of the response was delayed, and so mice were euthanized at day 6 postvaccination for analysis. As shown in Fig. 5A–C, IL-2 injection significantly increased the generation of SLECs. Effector cells from IL-2–injected mice had more Prdm1 mRNA (Fig. 5D), consistent with the idea that IL-2 signaling in Ag-specific T cells increases Blimp-1 expression, which then promotes the generation of SLECs in vivo. This idea is further supported by the fact that IL-2 injection directly increased Prdm1 mRNA in CD127+ KL.RG1– EECs (Fig. 5E), which contain SLEC precursors (22, 23). Therefore, the increase in Prdm1 mRNA observed in sorted effector cells from mice injected with IL-2 (Fig. 5D) is not simply a reflection of the increased percentage of SLECs, which express more Blimp-1 than MPECs; but, rather, indicates that IL-2 increases Blimp-1 expression in effector cells. The role of the in vivo IL-2/Blimp-1 axis in SLEC differentiation was then tested using Blimp-1–deficient T cells. As shown in Fig. 5A–C, IL-2–induced SLEC differentiation was impaired when Blimp-1–deficient OT-I T cells were used, and this was particularly striking when total numbers were compared (Fig. 5C). This observation...
demonstrates that IL-2 induction of SLEC differentiation in vivo depends on Blimp-1.

As noted before, when $10^6$ OT-I cells were transferred and mice were injected i.p. with PBS or left untreated, CD25 levels on effectors were low (see Fig. 3E). In addition, Prdm1 mRNA relative expression, both at the peak of the CD8$^+$ T cell response (day 4; see data from Fig. 3D, $10^6$ group) and during the early stage of the contraction phase (day 6; see data from Fig. 5D, PBS group), remained low in experiments with high precursor frequency (compare with Fig. 3D, $10^5$ group). Taken together, these data clearly suggest that, at high precursor frequency, the IL-2/Blimp-1 axis is less efficient. Intriguingly, when $10^6$ OT-I cells were transferred prior to DC-OVA vaccination (Fig. 5A), SLECs were generated at day 6 postvaccination (compare with Fig. 3A, in which cells were characterized at the peak of the response, day 4). This increase in the number of SLECs during the early stage of the contraction of the response was Blimp-1 independent, as transferred OT-I Blimp-1 KO cells were able to generate this effector population in similar numbers (Fig. 5C, right panel). This is further illustrated by the fact that expansion of SLEC populations between day 4 and day 6 postvaccination was similar between Blimp-1 WT (5.7-fold expansion) and KO (7.5-fold expansion) cells if $10^6$ OT-I cells were adoptively transferred (Fig. 6A). However, in response to IL-2 injection, which delays the peak of the CD8$^+$ T cell response, the expansion of MPECs from day 4 to day 6 was similar between WT and KO cells (4.5$\times$ and 4.4$\times$, respectively), whereas the number of SLECs expanded 316$\times$ from day 4 to day 6 in WT cells and 159$\times$ in KO OT-I cells (Fig. 6B). The total number of effector cells generated following DC-OVA vaccination with IL-2 treatment was similar whether $10^6$ Blimp-1 WT or KO cells were transferred (Fig. 6C), confirming that the total expansion of effectors was unaffected by Prdm1 deletion, but that the IL-2/Blimp-1 axis particularly affected SLEC generation. This axis may be more important during the expansion phase of the CD8$^+$ T cell response.

IL-2 treatment could either enhance proliferation or survival of SLECs or increase the conversion of effectors into CD127$^-$ KLRG1$^+$ cells. However, IL-2 did not reduce apoptosis of Ag-specific CD8$^+$ T cells (Supplemental Fig. 4A), and although it increased proliferation of effectors, it did so similarly in Blimp-1 WT and KO cells, in both MPECs and SLECs (Supplemental Fig. 4B). Taken together, these data indicate that IL-2, in a Blimp-1–dependent manner, is likely to increase the conversion rate of effectors into SLECs rather than favoring their proliferation or their survival.

**Discussion**

Using a DC vaccination protocol, we were able to study the role of Blimp-1 in the MPEC–to–CD8$^+$ T cell memory transition and the importance of IL-2 in Blimp-1–mediated SLEC differentiation. In a context in which the majority of effector cells at the peak of the response are MPECs, Blimp-1 deficiency does not affect the generation of memory T cells. This finding contrasts with other reports showing an enhanced memory formation in the absence of Blimp-1 (4), a difference that may relate to the low inflammation (CD8$^-$CD45.2$^+$) were sorted at the peak of the response following adoptive transfer of $10^6$ or $10^5$ OT-I cells. (E) Expression of CD25 on effectors isolated from the blood of mice transferred with $10^6$ or $10^5$ OT-I cells prior to DC-OVA vaccination. Results are expressed as a percentage of CD8$^+$ cells and were analyzed at day 4 and day 6 postvaccination. For all panels, mean ± SEM is shown for at least two independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 

The Journal of Immunology 1851

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milieu that occurs following DC vaccination. Lack or presence of inflammation may differentially affect expression of other transcription factors. It is also possible that the level of expression of Blimp-1 is much lower in MPECs generated following DC vaccination compared with infection. In that case, these levels may not be sufficient to counteract the expression of its targets, such as Bcl-6 (24, 25), a promemory transcription factor (26). Of interest, not only do Blimp-1–deficient CD8+ T cells generate a similar number of memory cells, but also these are as functional, as judged by their ability to control a challenge with a lethal dose of Listeria. Kallies et al. (5) have shown that Blimp-1–deficient memory T cells are not able to control a secondary influenza infection. However, the control of an influenza infection critically
depends on cytotoxic functions (27, 28), whereas the control of Listeria necessitates the production of IFN-γ and TNF-α by CD8⁺ effectors (29). In fact, Blimp-1–deficient primary and secondary effectors produce less grzmB, whereas their production of IFN-γ and TNF-α is normal, if not increased. Therefore, Blimp-1 is not necessary for the proper differentiation of long-lived memory T cells but is important for the acquisition of proper cytolytic potential. As in infectious models, Blimp-1 expression was required for the generation of SLECs following DC vaccination. This was particularly true when fewer precursors were adoptively transferred prior to vaccination. The molecular pathways used for the generation of terminally differentiated cells may therefore be partially independent of the level of inflammation and sensitive to competition between CD8⁺ T cells. Observations have been made for an accelerated transition to CD8⁺ memory phenotype both in conditions of low inflammation and when high numbers of precursors were transferred (7, 30). Our results show that the extent of SLEC differentiation at the peak of the response in these conditions is Blimp-1 dependent. Increased Blimp-1 expression occurred when we used a low precursor frequency of OT-I T cells in comparison with the high precursor frequency. One of the possible differences between these two conditions is that Ag-specific T cells compete more for resource, such as cytokines. We find that modulation of IL-2 availability directly affected Blimp-1 levels and SLEC differentiation in WT cells. Blimp-1–deficient cells, however, insufficiently responded to IL-2 treatment, indicating that competition for this cytokine and Blimp-1 induction are critical factors for terminal CD8⁺ T cell differentiation. We cannot exclude that cells compete for other factors or, given that the number of DCs was not increased along with the number of precursors, that they compete for Ag presentation opportunity. As the number of precursors increased, interaction time with the peptide-loaded DCs and opportunity to receive stimulatory and costimulatory signals likely decreased. Given that CD25 expression is also regulated by TCR signal strength (22, 31), sensitivity to IL-2 and, therefore, SLEC differentiation, may be decreased as a result. However, the fact that blocking IL-2 action diminished Prdm1 expression and SLEC generation when we used a reduced number of T cell precursors further supports a key role of IL-2 bioavailability in Blimp-1–mediated terminal differentiation. It is unclear why some CD127loKLRG1hi cells are formed in Blimp-1–deficient OT-I cells in response to daily IL-2 injections, but they may result from alternative IL-2–mediated pathways of terminal differentiation. The existence of a Blimp-1–independent pathway of SLEC has been previously observed (21) and is further supported by the observation that WT and KO effectors generated SLECs in similar numbers during the early stage of the contraction phase of the response if 10⁶ OT-I was transferred prior to vaccination. In addition, the fact that Blimp-1 KO SLECs are increased upon IL-2 injection suggests that a Blimp-1–independent differentiation pathway of terminal differentiation may nevertheless be dependent on IL-2. One mechanism by which IL-2 may function in a Blimp-1–independent manner is via its ability to inhibit Foxo1 (32), which may then promote effector differentiation (33–35). Alternatively, it would have been reasonable to propose that, in the absence of Blimp-1, the level of expression of several of its targets, including Eomes, Cd27, Sell, Id3, Bcl6, and Myc, should be maintained and perhaps confer a survival advantage of the few SLECs generated in Blimp-1–deficient cells (21). However, we did not measure a difference in apoptosis between Blimp-1 WT and KO cells for the time points at which SLEC differentiation was evaluated in this study. This observation is reminiscent of the fact that increased CD25 expression and IL-2 sensitivity promote differentiation and proliferation without affecting apoptosis in the expansion phase of the immune response (36). However, later expression of CD25 on Ag-specific Blimp-1–deficient CD8⁺ T cells increases their survival (21). Given that Blimp-1 is required for proper SLEC formation at the peak of the response and that CD25 expression levels are similar between WT and KO cells during expansion (not shown), the role for Blimp-1 in terminal differentiation in the early phases of the response is likely independent of its capacity to suppress cell surface expression of the IL-2 high-affinity receptor. As with IL-2 signaling, Blimp-1 function and its relative importance in the process of terminal differentiation may well be time dependent. This idea could provide an explanation for the observation of Blimp-1–dependent and –independent SLEC formation observed in our model. Our results nevertheless highlight an in vivo IL-2/Blimp-1 axis for the generation of SLECs in conditions of low inflammation. This observation is in agreement with reports showing that IL-2 can induce Blimp-1 expression in vitro (8–10, 37). It must be noted, however, that other cytokines, such as IL-4, IL-12, IL-21, and IL-27 (8, 10, 38), have been shown to induce Blimp-1 and that these may provide alternative differentiation signals, particularly in infection (8, 24). It is counterintuitive to observe that less IL-2 is available to induce Blimp-1 expression when a high frequency of precursor of naïve CD8⁺ T cells is used. Perhaps optimal per-cell dose production of IL-2 by CD8⁺ T cells occurs too late in the response to properly affect Blimp-1 expression in low inflammatory conditions when a greater number of OT-I cells are adoptively transferred. For example, in vitro studies have shown that IL-2 can induce T-bet in a dose-dependent manner, which, in turn, is a Blimp-1 inducer (39). Given that T-bet is produced in low inflammatory conditions (2), its early induction by IL-2 signal may be critical for CD8⁺
T cell terminal differentiation. However, at day 6 postvaccination, T-bet levels were not increased in mice receiving IL-2 injections (data not shown). Alternatively, others have shown that CD4 T cell help is important for the differentiation of SLECs during an immune response and that IL-2 is the critical molecule provided by CD4 help (20). It may be that at high precursor frequency of naive OT-I T cells, less IL-2 signal is available because of competition for CD4 help. Finally, it is possible that IL-2 provided by DCs during priming is a determinant for optimal CD25 induction and confers a differentiation potential (40).

In summary, our results confirm IL-2 as a key in vivo signal for the induction of Blimp-1 expression, which then is critical to generate SLECs. Furthermore, Blimp-1 expression is not necessary for the generation of functional memory CD8+ T cells. Understanding the molecular pathway controlling the generation of SLECs and memory CD8+ T cells is pivotal in the design of a better vaccination strategy using DCs, for which improvement is urgently needed to reach therapeutic efficacy in cancer patients.

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Disclosures
The authors have no financial conflicts of interest.

References
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<td>Forward 1</td>
<td>5’ CAATGCTTGTCTAGTGTC 3’</td>
</tr>
<tr>
<td>Forward 2</td>
<td>5’ GATACAGGCAATTCGAACAGGGCTCAT 3’</td>
</tr>
<tr>
<td>Reverse 1</td>
<td>5’ AGTAGGTGAATGGGAGC 3’</td>
</tr>
</tbody>
</table>

B

Supplementary Figure 1: Prdm1 deletion in CD8+ T cells in E8I-Cre (C57BL/6-Tg(Cd8a-cre)1Itan/J) mice crossed with Prdm1 floxed (B6.129-Prdm1tm1Clme/J) mice. A. Set of primers used to detect deletion of Prdm1. B. Amplicon from primers Forward 1 and Reverse 1 (approx. 550bp) is only apparent in CD8+ T cells when deletion occurs, bringing annealing sites in close proximity. Amplicon from primers Forward 2 and Reverse 1 (floxed allele=270bp, WT allele=200bp) is apparent in undeleted cells. PCR was performed on sorted CD4+ or CD8+ cells from either a Prdm1 floxed mouse (WT:flox) or a Prdm1 floxed/E8I-Cre mouse (E8I-Cre:flox). 1Kb+ ladder from Life Technologies is shown. C. Prdm1 mRNA expression was measured in WT and KO effector cells sorted (CD8+CD45.2+) at the peak of the response following adoptive transfer of 10^4 OT-I cells. Naive (T_N) CD8+ T cells were sorted from recipient mice as CD45.2-CD8+CD44- cells. D. Phenotype of naive OT-I cells from Prdm1 WT and KO mice.
Supplementary Figure 2: Profile of memory and effector cells in Prdm1 WT and KO OT-I cells. A. Phenotype and function (following in vitro OVA restimulation) of memory cells (at least 45 days post-vaccination) as a function of the number of OT-I cells adoptively transferred (10⁴ or 10⁶). Overlayed are the endogenous CD8⁺ T cells (CD45.2⁻; gray), the WT OT-I cells (light full line) and the KO OT-I cells (dark full line). B. MPEC and SLEC profiles of secondary effector cells 3 days post Lm-OVA infections in Prdm1 WT vs. KO OT-I cells. Data shown from an adoptive transfer of 10⁶ precursors. C. Phenotypic histograms for CD44 and 1B11 in primary effectors. Overlay is as in A. D. 5x10⁵ Prdm1 WT (CD45.1⁺) and KO (CD45.2⁺) OT-I cells were co-transferred into recipient (CD45.1⁺CD45.2⁺) prior to DC-OVA vaccination. Mice were euthanized 6 days post-vaccination. Left, gating of the WT, KO and endogenous CD8⁺ populations in the spleen. Right, MPEC and SLEC profiles for WT and KO cells are shown. E. Statistical compilation of the percentage of SLECs and MPECs as in D.
Supplementary Figure 3: Daily PBS or isotype control injections are appropriate negative controls for αIL-2 injections. Mice adoptively transferred with 10⁴ CD45.2⁺ OT-I cells and vaccinated with OVA-loaded DCs were injected daily with PBS, a rat IgG2a isotype control antibody (clone 2A3) or the IL-2 blocking antibody JES6-1A12 and were euthanized 6 days post-vaccination. The percent of SLECs (CD127⁻KLRG1⁺) amongst CD45.2⁺ cell was then evaluated by flow cytometry.
Supplementary Figure 4: Effect of IL-2 supplementation on survival and proliferation of CD8⁺ effectors. A. CD45.1⁺ recipient mice were adoptively transferred with 10⁶ CD45.2⁺ Blimp-1 WT or KO OT-I cells two days prior to DC-OVA vaccination. Mice were then injected i.p. twice daily with PBS or IL-2, as stated in the methods section. On day 6 post-vaccination, mice were euthanized and the viability of CD45.2⁺ cells was measured by Annexin V (AnnV) staining. B. Mice were treated as in panel A, but on day 5 post-vaccination, 1mg BrdU was injected i.p. Mice were euthanized on day 6 and the proliferation of CD45.2⁺ cells was measured by staining for BrdU incorporation. Similar results were obtained if analyses were performed on day 4 post-vaccination.