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*J Immunol* published online 15 June 2012
http://www.jimmunol.org/content/early/2012/06/15/jimmunol.1102579

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/06/15/jimmunol.1102579_9.DC1

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Changes in Functional but Not Structural Avidity during Differentiation of CD8+ Effector Cells In Vivo after Virus Infection

Samuel Amoah, Rama D. Yammani, Jason M. Grayson, and Martha A. Alexander-Miller

By the peak of the CD8+ T cell response, the effector cell pool consists of a heterogeneous population of cells that includes both those with an increased propensity to become long-lived memory cells (memory precursor effector cells; MPEC) and those that are terminally differentiated cells (short-lived effector cells; SLEC). Numerous studies have established the critical role that functional avidity plays in determining the in vivo efficacy of CD8+ effector cells. Currently, how functional avidity differs in MPEC versus SLEC and the evolution of this property within these two populations during the expansion and contraction of the response are unknown. The data presented in this study show that at the peak of the effector response generated after poxvirus infection, SLEC were of higher functional avidity than their MPEC counterpart. Over time, however, SLEC exhibited a decrease in peptide sensitivity. This is in contrast to MPEC, which showed a modest increase in peptide sensitivity as the response reached equilibrium. The decrease in functional avidity in SLEC was independent of CD8 modulation or the amount of Ag receptor expressed by the T cell. Instead, the loss in sensitivity was correlated with decreased expression and activation of ZAP70 and Lck, critical components of TCR membrane proximal signaling. These results highlight the potential contribution of avidity in the differentiation and evolution of the T cell effector response after viral infection.

The Journal of Immunology, 2012, 189: 000–000.

A critical aspect of the anti-viral immune response is the ability to generate long-lived memory CD8+ T cells that can rapidly respond after secondary Ag encounter. Recent studies have defined markers that identify effectors with increased potential to become memory cells (1–3). These cells express the IL-7 receptor α-chain (CD127), which is upregulated after transient loss as a result of activation. Effector cells with an intrinsically low survival and proliferative potential have been termed short-lived effector cells (SLEC) and are marked by the expression of killer cell lectin-like receptor G1 (KLRG1). Effector cells that express neither have been termed early effector cells (EEC) (4).

Although these markers allow identification of cells that differ in their capacity to give rise to memory cells, the properties/signals that result in the differentiation and maintenance of cells along the memory precursor effector cells (MPEC) versus SLEC pathway are only beginning to be unraveled. Recent studies identified high expression of CD25 as a marker of cells with a propensity to become SLEC (4–6). CD25 was found to be heterogeneously expressed in a transient manner within the responding effector population. The isolation and transfer of CD25high cells at early times post-infection (i.e., prior to selective upregulation of KLRG1 or CD127) revealed that this population preferentially differentiated into SLEC (5). Although IL-2 signaling appears to promote differentiation into SLEC, the mechanistic basis for sustained high-level CD25 expression is unclear. For example, whether the capacity for high and sustained expression of CD25 is associated with intrinsic properties of those cells or whether it is the result of stochastic encounter with Ag remains to be defined.

The CD8+ T cell response generated after virus infection is an amalgam of a number of individual clones that undergo rapid expansion. This yields a population of cells that are heterogeneous with regard to their functional properties. Heterogeneity within the polyclonal response can take the form of differences in the pattern of cytokines produced as well as the cytolytic exerted in response to TCR engagement. Increased breadth in the effector functions present in responding cells is associated with increased efficacy in vivo (7). An additional attribute that is predictive of efficacy is the sensitivity to peptide Ag; that is, functional avidity (8–18). Among the polyclonal virus-specific effector population there exist cells that differ substantially in the amount of peptide required to induce lysis or secrete cytokines. In vitro cells can be identified that differ in peptide requirement by up to 5-logs (8). The difference in peptide sensitivity among these effectors is likely defined by both intrinsic properties (e.g., TCR affinity) and modulation that occurs as a result of peptide encounter (e.g., changes in CD8 level/isoform or differences in the regulation of signaling cascades).

At present, there is no information regarding how peptide sensitivity within the responding effector impacts the differentiation program with regard to SLEC versus MPEC generation. It is reasonable to speculate that interaction of higher-avidity cells with APC results in a quantitatively or qualitatively different signal compared with that generated in low-avidity cells. Increased signaling in responding cells could lead to increased CD25 expression, as has been reported under conditions of high-level TCR engagement (19, 20), thereby promoting SLEC commitment in these cells (5).
A number of studies support a role for tuning of TCR signaling in effector cell differentiation. For example, the tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 (SHP-1), an important negative regulator of TCR signaling, has been shown to play a role in SLEC formation (21). In the absence of SHP-1, SLEC were increased suggesting that indeed augmented or prolonged signals from the TCR can drive an effector cell toward terminal differentiation (21). Thus, an attractive model was that cells with intrinsically higher avidity would be more likely to receive strong activating signals and as such would have an increased propensity to differentiate along the SLEC pathway.

In this work, we report the results of our studies evaluating the regulation of structural and functional avidity in the MPEC and SLEC populations present after viral infection. Our results suggest that the SLEC versus MPEC differ in peptide sensitivity; however, this is independent of TCR affinity or the level of expression of CD8 and TCR. Further, we find that cells exhibiting the MPEC phenotype during the acute response exhibit a modest increase in avidity during the establishment of the memory response. In contrast, SLEC significantly decrease avidity over this same period of time. The decreased avidity observed in SLEC at later times is associated with decreased expression of both Lck and ZAP70. Together these data suggest that avidity plays a role in the fate of effector cells in vivo after viral infection and that avidity within the SLEC and MPEC populations evolves as the memory response is established.

Materials and Methods

Mice and infections

Six to ten-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. Mice received 1 x 10^6 PFU vaccinia virus (VACV)-GP33 (22) or 2 x 10^6 PFU lymphocytic choriomeningitis virus (LCMV) Armstrong by i.p. injection.

Tetramer dissociation

A total of 1 x 10^6 spleen cells from vaccinia-infected mice were stained for 1 h at room temperature with B8R tetramer (National Institutes of Health Tetramer Core Facility, Emory University, Atlanta, GA), KLRG1 (Abcam), CD127, and CD44 (BioLegend) Abs. Cells were then washed and resuspended in media containing 50 μg/ml anti-MHC class I Ab (BioLegend) to prevent tetramer rebinding. Cells were incubated at 37°C for the indicated times with additional CD127 Ab added to prevent the apparent loss of the MPEC population, which occurred over time due to dissociation of the CD127 Ab. Cells were subsequently washed and fixed prior to staining with CD8α (BD Biosciences) for 30 min on ice.

Intracellular cytokine staining and flow cytometry

A total of 1 x 10^6 spleen cells from VACV- or LCMV-infected mice were cultured for 5 h in media containing Golgi Plug (BD Biosciences) and graded concentrations of the immunodominant B8R peptide. Cells were then incubated for 30 min on ice with CD127, CD44 (BioLegend), KLRG1 (Abcam), and CD8α (BD Biosciences) and in some cases CD8β (BioLegend) Abs. After washing, cells were permeabilized using Cytofix/Cytoperm (BD Biosciences) and then stained for IFN-γ (BD Biosciences). Samples were acquired on a BD FACS Canto II, and 1 x 10^6 events were routinely acquired. Data were analyzed using FlowJo software (Tree Star).

TCR Vβ analysis

A total of 1 x 10^6 spleen cells from VACV-infected mice (day 7 postinfection) were stained with Abs against CD8α, CD44, CD127, KLRG1, and Vβ (2–14) together with B8R tetramer for 30 min on ice. Samples were then washed twice and acquired on a BD FACS Canto II. Data were analyzed using FlowJo software (Tree Star).

Analysis of Lck and ZAP70 levels

Splenocytes were harvested at day 7 or day 30 postinfection. Cells were labeled with anti-CD8α, anti-CD44, anti-CD127, anti-KLRG1, and B8R tetramer allophycocyanin. After surface staining, cells were fixed with Lyse/Fix buffer (BD Biosciences) for 10 min at room temperature. Immediately after, cells were centrifuged and resuspended in 100 μl Cytofix/Cytoperm (BD Biosciences) for 20 min on ice. Cells were then stained with PE-conjugated anti-Lck Ab (BD Biosciences) or purified anti-ZAP70 Ab (Cell Signaling) for 20 min. In the case of ZAP70, Ab was detected with an Alexa Fluor 488-conjugated secondary Ab (Molecular Probes). For analysis of phosphorylated protein, CD8α/CD44/KLRG1 or CD127 populations were sorted on a FACS Aria instrument after enrichment of CD8α cells using a CD8α + T cell isolation kit II (Miltenyi Biotec). Isolated cells were stained on ice with B8R tetramer and washed with cold FM1. At 1 min after removal from cold conditions, fix/lyse buffer (BD Biosciences) was added and cells incubated for 10 min at 37°C. Cells were permeabilized with 90% ice-cold methanol for 30 min on ice prior to staining with Abs specific to phosphorylated ZAP70 (BD Biosciences) or phosphorylated Lck [anti-phospho-scr (Tyr 416); EMD Millipore]. In the case of anti-phospho-scr, Ab was detected by addition of an Alexa Fluor 488-conjugated secondary Ab (Molecular Probes). Cells were also analyzed at a later time (10 min). Relative differences among the populations at this time followed the same pattern, although overall levels of phosphorylated molecules had decreased.

Statistical analysis

All significance analysis was performed using a two-tailed Student t test.

Results

Differentiation of MPEC and SLEC after VACV infection

Current data suggest that CD8+ T cells possess the potential to differentiate along both the SLEC and MPEC pathways (23). That said, it is clear that within a polyclonal response, a subset of cells will commit to the SLEC pathway whereas others will become memory precursors. Whether peptide sensitivity is associated with one pathway versus another remains to be determined. Prior to addressing this issue, we first established the kinetics of differentiation in our model system of i.p. infection with VACV. C57BL/6 mice were infected with 10^6 PFU VACV. Infections were staggered to allow for concurrent analysis of the populations present at each time point. On days 7, 11, 14, and 30 postinfection, spleens were isolated, and the frequency and number of EEC, MPEC, and SLEC specific for the immunodominant B8R epitope determined by tetramer staining. On day 7 postinfection, most of the cells present, assessed by both percentage and number, were found within the EEC population (Fig. 1). By day 11, however, there was a sharp decrease in both the number and percentage of both EEC and SLEC, with a majority now displaying the MPEC phenotype (Fig. 1B). The distribution of effectors among the MPEC and SLEC populations remained relatively constant between days 11 and 30, although absolute numbers were diminishing in all populations. This suggests the three populations are undergoing cell loss throughout this time period. As expected, these data show an early SLEC population that subsequently undergoes contraction coupled with an increased MPEC population as the immune response progresses.

Initial differentiation of effector cells along the MPEC versus SLEC pathways is affected by Vβ usage

The effector pool generated as a result of infection is the result of the recruitment and expansion of distinct naïve precursors. Although a single cell can give rise to both MPEC and SLEC (23), it is not clear whether there are differences with regard to the propensity to do so in the context of a polyclonal response. We used TCR Vβ analysis to follow the differentiation of cells within the polyclonal response. We reasoned that if individual clones differed in their propensity to give rise to SLEC versus MPEC, then this
B8R-specific SLEC or MPEC was determined using a panel of V

**SLEC present at day 7 are of higher avidity than MPEC**

Given that avidity may influence the number of Ag contacts that a cell receives, coupled with our understanding that repeated Ag contact can drive a cell toward a more terminally differentiated phenotype, we tested the hypothesis that functional avidity could contribute to the differentiation fate of an effector cell. To determine if functional avidity differed within MPEC and SLEC populations, splenocytes were isolated at the peak of the effector response (i.e., day 7 postinfection) and stimulated with titrated concentrations of B8R peptide. The peptide dose-response curves for MPEC and SLEC from a representative animal (Fig. 3A) as well as averaged data (Fig. 3B) are shown. We found a similar increase in the sensitivity of SLEC versus MPEC in the lymph nodes at day 7 postinfection (Supplemental Fig. 1). Notably, cells in the periphery (i.e., the lung) showed no skewing in SLEC toward higher avidity (Supplemental Fig. 1). This may reflect selection for SLEC with inherently lower avidity for either entry into or survival within this tissue. Alternatively, it may reflect regulation of these cells in the lung. This possibility is intriguing given the negative regulation of effector cells that has been reported in this tissue (24–27). Together these analyses revealed a reproducible and significant difference in functional avidity between SLEC and MPEC populations in lymphoid organs at early times postinfection, with SLEC exhibiting increased sensitivity to peptide Ag compared with their MPEC counterpart.

SLEC and MPEC diverge in functional avidity over the course of the response

We next tested the possibility that avidity was altered over time as MPEC and SLEC populations evolved and contracted (day 14 postinfection and beyond). To assess this possibility, we analyzed the functional avidity within the MPEC and SLEC populations between day 7 and day 30 postinfection (Fig. 4, representative primary data are shown in Supplemental Fig. 2). MPEC demonstrated a significant although modest increase in avidity between the peak of the MPEC response (day 11) versus postcontraction (day 30), requiring 3.3-fold less peptide for production of IFN-γ (Fig. 4A, 4C). These findings show a movement toward higher avidity (Supplemental Fig. 1). This may reflect se-

FIGURE 1. SLEC undergo rapid contraction after the peak of the CD8+ T cell response. C57BL/6 mice were infected with $1 \times 10^6$ PFU VACV and analyzed on days 7, 11, 14, and 30 postinfection. Infections were staggered to allow for analysis on the same day. Cells are pregated on CD8+CD44+ cells. (A) Representative plots showing the frequency of B8R tetramer+ cells that express either CD127 (MPEC) or KLRG1 (SLEC). KLRG1+ and CD127+ staining on the total CD8+ population is shown for comparison. (B) Frequency (left panel) and number (right panel) of B8R tetramer+ CD8+ T cells that are MPEC (CD127+), SLEC (KLRG1+), or EEC (double negative). All data are a mean and SEM of at least six mice/group from at least two independent experiments.

FIGURE 2. TCR VB usage after VACV infection. Spleens were harvested on day 7 after infection with $1 \times 10^6$ PFU VACV. TCR VB analysis of B8R-specific SLEC or MPEC was determined using a panel of VB-specific Abs. Data shown are the mean and SEM from four independent experiments each with three mice. Mean and SEM are shown. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.002$.

FIGURE 3. MPEC and SLEC differ in functional avidity on day 7 postinfection. C57BL/6 mice were infected with $1 \times 10^6$ PFU VACV, and on day 7 postinfection, avidity was assessed by stimulation with titrated concentrations of B8R peptide. IFN-γ production was determined by ICCS. (A) Data from a representative mouse are shown. (B) Average EC_{50} for MPEC versus SLEC ($n = 31$). The EC_{50} represents the amount of peptide needed to achieve the 50% maximal percentage of IFN-γ-producing cells. **$p \leq 0.01$. 

May be reflected in the VB distribution among the various effector cell populations. We first tested whether VB usage correlated with the differentiation into SLEC versus MPEC. We assessed the responses on day 7 postinfection, when the effector population was at its peak and both SLEC and MPEC populations were readily detected. As above, B8R-specific SLEC and MPEC were identified by staining with KLRG1 and CD127 Abs, respectively. Although VB2 and VB8.1/2 were the dominant regions used by both MPEC and SLEC populations, comparison of the usage across the repertoire for the MPEC and SLEC populations revealed a bias in the usage of VB3, 6, 7, 8.1/2, and 9, with these regions being more highly represented within the MPEC populations (Fig. 2). These data are consistent with nonrandom selection within the effector population with regard to differentiation into SLEC versus MPEC.
avidity within the MPEC population. In contrast, SLEC exhibited a significant decrease in avidity between their peak response at day 7 and day 30 (Fig. 4B). SLEC present at day 30 required a 3.1-fold more peptide to elicit effector function compared with MPEC at days 7, 11, 14, or 30 postinfection. The evolution of avidity over time within the two populations resulted in a 3.1-fold difference in avidity between the MPEC and SLEC present at day 30, with MPEC now being the higher-avidity population.

Changes in functional avidity within MPEC or SLEC populations over time are not associated with altered TCR levels

We were interested in the mechanism responsible for the differences in avidity present at the various times postinfection. As a first step, we tested the possibility that changes in the level of TCR expression correlated with the observed differences in peptide sensitivity. Splenocytes were isolated at days 7–30 postinfection and stained with Abs specific to CD8, CD44, CD127, KLRG1, TCRβ, along with tetramer. As shown in Fig. 5, TCR levels on B8R-specific cells were similar within the SLEC populations across all time points. This was also the case for MPEC. Thus, changes in the level of TCR over time could not account for the changes in peptide sensitivity that occurred in the MPEC or SLEC population over time. There were differences in the level of TCR when comparing MPEC to SLEC populations at days 11–14, with MPEC expressing higher levels of TCR compared with SLEC. However, when comparing the avidity of the two populations over time, there was not a consistent correlation with avidity.

Changes in functional avidity are not associated with altered structural avidity

We then tested the possibility that as the response evolved, there was a selection for T cells bearing receptors with different affinity (i.e., lower in the case of SLEC or higher in the case of MPEC) or that changes occurred in the organization of the TCR within the membrane that altered the ability to interact efficiently with the APC. Either of these would change the ability of cells to bind and retain tetramer. We first determined the level of tetramer binding within the SLEC and MPEC populations at days 7, 11, 14, and 30 postinfection (Fig. 6A). No significant differences were found in the intensity of tetramer staining either over the course of the response for MPEC or SLEC or between the two cell types. Although the level of tetramer binding under saturating conditions was similar, a more sensitive approach for measuring TCR affinity/structural avidity is found in the analysis of tetramer dissociation. Splenocytes were isolated at day 7 or day 30 postinfection and stained for 1 h at room temperature with B8R tetramer together with CD127, KLRG1, and CD44 Abs. Cells were then washed and resuspended in media containing anti-MHC class I Abs to prevent rebinding of tetramer during the dissociation period. To assess loss of tetramer binding, cells were incubated at 37°C, conditions under which high-affinity TCR clones selectively maintain tetramer binding. CD127 Ab was included during the dissociation period to optimize detection of MPEC, as we had noted in initial studies that there was a time-dependent loss in MPEC during the assay period due to CD127 Ab dissociation. At the indicated times over a 4-h period, samples were fixed and subsequently stained with Ab to CD8α. No significant difference in tetramer dissociation in SLEC (Fig. 6B) or MPEC (Fig. 6C) present at day 7 versus day 30 was observed. Of note, there was also no difference when comparing dissociation in MPEC versus SLEC. Thus, changes in structural avidity could not account for the altered functional avidity.

Contribution of CD8 to time-dependent changes in functional avidity in SLEC

CD8 is expressed on the cell surface as either a heterodimer consisting of αβ-chains or as an αα homodimer. A number of studies have indicated that expression of the heterodimeric form of CD8 is associated with increased sensitivity to peptide Ag (28–30). In addition, we have previously shown that an increased ratio of CD8β/CD8α staining is associated with higher avidity (29). On the basis of the potential for changes in either the absolute level or isoform of CD8 to alter avidity, we investigated CD8 regulation as a mechanism to account for the changes in avidity observed in MPEC and SLEC over time. In SLEC, neither CD8α (Fig. 7A) nor CD8β (Fig. 7B) levels were significantly modulated over the time...
course assessed. In contrast, the level of both CD8α and CD8β increased significantly over time in the MPEC population. Evaluation of the ratio of CD8β/CD8α did not reveal significant changes, suggesting that the representation of CD8αβ versus αα was constant (Fig. 7C). However, the overall increase in CD8 may contribute to the increased peptide sensitivity observed in MPEC present at later times postinfection.

**Day 30 SLEC exhibit reduced levels of both total and activated Lck and ZAP70 protein**

We next tested the hypothesis that differences in the expression of signal transduction molecules could account for the reduced responsiveness of late-stage SLEC. Splenocytes were isolated on day 7 or day 30 postinfection and the presence of MPEC and SLEC within the B8R-specific population identified by Ab staining. The level of Lck was modestly higher (1.2-fold) in MPEC versus SLEC at day 7 postinfection. In contrast to Lck, levels of ZAP70 did not differ between these early populations.

The decreased levels of protein suggested that the level of activated Lck and ZAP70 would be reduced in these cells after TCR engagement. To test this possibility directly, KLRG1+ and CD127+ populations present on day 7 and day 30 were sorted and incubated with tetramer. Sorting was performed as our preliminary studies showed that KLRG1 and CD127 staining were lost during the permeabilization procedure, prohibiting identification of SLEC within the bulk population after permeabilization. The use of tetramer allowed both detection of Ag-specific cells and stimulation through the TCR. We analyzed these responses by comparing the level of phosphorylated molecules in day 30 B8R-specific SLEC or MPEC populations to those at day 7. Changes in the positive direction represent increased levels of phosphorylated molecules in the population present at day 30, whereas a negative value represents a decreased level of phosphorylated protein. The data in Fig. 8C and 8D show that B8R-specific SLEC present at day 30 have reduced levels of phosphorylated Lck and ZAP70 compared with the cells at day 7, in agreement with changes in the total protein level. In contrast, MPEC exhibited an increase in the levels of phospho-Lck and phospho-ZAP70. This was intriguing given that we saw no significant increase in total protein and suggests augmented signaling efficiency in these cells. Together
these data are consistent with a model wherein the decreased sensitivity in SLEC present at later times is a result of decreased signaling in the cells.

Avidity within the MPEC and SLEC is regulated in an epitope-dependent fashion

The preceding data establish the differential regulation of avidity within the VACV B8R-specific effector population during the acute response and at later times as memory is established. We sought to determine whether the patterns observed for the B8R-specific response reflected general patterns with regard to effector cells or whether there were epitope-specific or virus-specific factors that regulated this process. To address this question, we evaluated the response to two epitopes (NP396–404 and GP276–286) at day 8 and day 30 after i.p. infection with the Armstrong strain of LCMV. Day 8 was chosen as this is the time point of the peak response. Fig. 9 shows SLEC specific for the GP276 epitope present at day 8 postinfection were of significantly higher avidity compared with MPEC, similar to what was observed for the B8R response. There was a similar trend with the NP396-specific response, although this did not reach statistical significance. Notably, the changes observed during the period of contraction and memory generation were variable compared within the LCMV response as well as across infections (i.e., the VACV-specific response). Whereas on average GP276-specific SLEC present at day 30 required more peptide compared with their day 7 counterpart, day 30 NP396-specific SLEC trended toward increased peptide sensitivity. We also did not detect significant decreases in the peptide requirement of MPEC present at day 30 versus day 7 for either epitope (Fig. 9). Thus, although SLEC appear generally to be skewed toward high avidity during the acute response, the regulation of avidity within the effector population over time is dependent on the epitope assessed.

Discussion

After activation, T cells undergo a process of differentiation during which a percentage of effectors acquire the potential to become long-lived memory cells. Recently, our understanding of the markers associated with this fate decision has increased substantially. For example, the earliest marker of cells that will become memory appears to be the reduced expression of CD25 (5). Subsequently, these cells will re-express CD127, which promotes long-term survival and proliferation via IL-7 signaling. These capabilities are dictated, at least in part, by the expression of the transcription factor Eomes (31–33). In contrast, cells with reduced memory potential express T-bet and Blimp-1 (1, 33–35). Although these studies highlight the processes by which cells undergo differentiation along the SLEC or MPEC pathways, the mechanism through which cells initially choose one fate versus the other and the factors that promote retention in the repertoire as well as function after this process remain relatively unknown.

In the current study, we evaluated the functional and structural avidity of effectors present after poxvirus infection. We observed a statistically significant increase in the amount of peptide required by MPEC versus SLEC present at day 7 postinfection, demonstrating that cells that had differentiated into SLEC possessed higher functional avidity. Two models could account for the increased avidity observed in SLEC. First, an increase in the quality or quantity of signal that occurs, for example as a result of a higher avidity interaction with a target cell, may induce differentiation into...
SLEC. There is evidence to suggest that the nature of the signal that results from TCR engagement can impact cell fate decisions, with prolonged or sustained signaling driving SLEC generation (21, 36). For example, Ag-specific cells lacking SHP-1, a key negative regulator of TCR signaling, have more SLEC present at the peak of the T cell response compared with their wild-type counterparts (21). Further, increasing the level of presented peptide, which likely increases signaling in a quantitative manner, can promote SLEC generation in some (36) but not all cases (1). Finally, stronger and/or increased Ag contact drives CD25 expression (19, 20), a known contributor to SLEC differentiation. Differences in TCR usage within the two populations would be consistent with the selection of a subpopulation of cells that possess intrinsic differences in Ag recognition, thereby selecting them for differentiation along the SLEC pathway. That said, the selection of these cells appears independent of structural avidity and CD8 levels, consistent with a model wherein differences in avidity are dictated by variations in signaling capabilities among the individual clones.

An alternative model to explain the difference in avidity is that the program associated with SLEC differentiation results in increased avidity. Although signal transduction in SLEC versus MPEC has not been directly compared, expression analysis did reveal the presence of a limited number of signaling molecules that exhibited increased expression in SLEC compared with MPEC (37). Among these were potential regulators of NF-κB (MALT1) and ZAP70 (Tyrobp) activation. In addition, it is clear that the organization of molecules within the membrane can have profound effects on T cell signaling and activation (e.g., Refs. 38–41). Thus, an attractive possibility for increased sensitivity is the reorganization of molecules (both receptor and membrane associated cytoplasmic proteins) within lipid rafts resulting in optimal signal transduction.

In addition to these early differences in avidity that were observed as cells differentiated into SLEC versus MPEC after VACV infection, we also detected changes in peptide sensitivity within each population over the course of the response. Whereas MPEC increased their functional avidity over time, SLEC exhibited decreased avidity. This is intriguing given that SLEC were initially the higher-avidity cell type. At the population level, effector T cells have been shown to undergo marked changes in functional avidity over the course of the response, albeit the large majority of the previously reported changes occurred prior to the peak of the response (42–45). Altered avidity at the population level can result from either selective survival of a subset of effector cells or from a global change in all effectors. At present, our data do not allow us to discriminate between these two possibilities. Because the number of cells present at the later times is decreased in both SLEC and MPEC, it is possible that the effector population present at the late times postinfection reflects a subset of cells with increased survival potential. Although TCR analysis at day 30 could shed light on this process, the decreased number of Ag-specific SLEC made assessment of Vβ usage at this time point infeasible. For MPEC, the increased phosphorylation of ZAP70 and Lck observed in day 30 versus day 7 MPEC suggests that if there is selective survival of a subset of cells, those with increased signaling capabilities are at an advantage.

In contrast to selective survival of cells with intrinsic differences, an alternative model to account for changes in avidity over time is active regulation of avidity within individual cells. Such a scenario is consistent with our previous in vitro data demonstrating the ability of individual cells actively to modulate avidity after multiple Ag encounters (46). In further support of active modulation as a means to control avidity, a study by Slifka and Whitton (42) reported changes in functional avidity independent of changes in structural avidity or TCR affinity. One mechanism that appears to account for modulation is a change in the isoform or level of CD8 (29, 30, 46, 47). We did observe a significant increase in the expression of both CD8α and CD8β in MPEC over time that was associated with increased avidity. Overall higher expression of CD8 (reflected by increased expression of both CD8α and CD8β) may be an attribute of MPEC that have a survival advantage. Alternatively, cells may actively upregulate CD8 as they differentiate toward bona fide memory cells. In our studies, increased CD8 in MPEC was associated with augmented levels of both phosphorylated Lck and ZAP70. Thus, increased CD8 levels appear to promote improved efficiency with regard to the initiation of TCR signaling.

Whereas CD8 modulation may contribute to the regulation of avidity in MPEC, it cannot account for the changes observed in SLEC over time. This lower-avidity population did not exhibit decreases in CD8, nor were there changes in TCR levels or tetramer dissociation compared with their day 7 counterpart. Instead, the change in functional avidity in these cells was associated with decreased levels of two critical components of the membrane proximal signaling machinery, ZAP70 and Lck. The decreased protein levels were coupled with decreased amounts of phosphorylated protein. Increases in Lck have been previously linked to increases in avidity (42). However, this is the first report, to our knowledge, that suggests active modulation in ZAP70 by effectors as a mechanism to control the sensitivity to peptide Ag in vivo. Although the induction of decreased peptide sensitivity or nonresponsiveness in CD8+ T cells in vivo has been reported, this does not appear to be regulated by modulation of Lck and ZAP70 levels (48, 49). Thus, this mechanism may be selectively used by SLEC to downregulate Ag sensitivity.

In summary, our results are consistent with a model wherein TCR usage and/or avidity are potential contributors to the initial fate decision in CD8+ T cell effectors. In support of this, we found biased TCR usage and higher avidity in SLEC present at early times after virus infection compared with their MPEC counterpart. The peptide sensitivity of an effector plays a critical role in determining the efficiency of viral clearance. The lower avidity observed in MPEC at early times may be a mechanism to promote survival of these cells into the memory pool. Differences in avidity may be only one weapon in the arsenal of these effectors that reduces the likelihood that MPEC continue to engage Ag. This may contribute to the increased survival of these cells long-term, allowing them to populate a memory pool that can respond efficiently upon Ag reencounter. Avidity was not static in MPEC and SLEC populations over time. B8R-specific SLEC generated after poxvirus infection showed progressively decreased avidity whereas MPEC increased avidity. These changes may reflect continued Ag encounter by SLEC, which drives them toward decreased peptide sensitivity or death, effectively eliminating them from the secondary response. Deleting these highly sensitive SLEC may serve as a way for the immune system to protect against damage as the host recovers from infection. In contrast, increased sensitivity of MPEC may result in a memory population that has increased efficacy upon secondary virus encounter. Extension of our findings to another viral model, LCMV, suggested regulation of avidity over time within SLEC and MPEC populations occurs in an epitope-dependent fashion. This result points out the importance of understanding how this process is regulated. The flexibility with regard to the regulation of avidity within the effector population opens the door to interventions that would allow modulation of avidity within the population as is desired in a given context (e.g., the decrease in avidity in the context of autoreactive cells).
Acknowledgments
We thank Dr. Karen Haas for helpful comments regarding the manuscript. We thank the National Institutes of Health Tetramer Core Facility for provision of tetramer.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. SLEC in the LNs, but not the lung, are of higher avidity than MPEC on d7 p.i. Avidity was assessed by stimulation with titrated concentrations of B8R peptide. IFNy production was determined by ICCS.
Supplemental Figure 2. Representative data for analysis of avidity in MPEC and SLEC populations on data d7 and d30 postinfection with VACV. Avidity was assessed by stimulation with titrated concentrations of B8R peptide. IFNγ production was determined by ICCS. Cells were pre-gated on the CD8⁺CD44hi population.