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Inflammation Directs Memory Precursor and Short-Lived Effector CD8$^+$ T Cell Fates via the Graded Expression of T-bet Transcription Factor

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SUMMARY

As acute infections resolve, effector CD8$^+$ T cells differentiate into interleukin-7 receptor^{lo} (IL-7R^{lo}) short-lived effector cells (SLECs) and IL-7R^{hi} memory precursor effector cells (MPECs) capable of generating long-lived memory CD8$^+$ T cells. By using another SLEC marker, KLRG1, we found that KLRG1^{hi} effector cells began appearing early during infection and were committed to downregulating IL-7R. Unlike IL-7R^{hi} MPECs, KLRG1^{hi} IL-7R^{lo} SLECs relied on IL-15, but IL-15 could not sustain their long-term maintenance or homeostatic turnover. The decision between SLEC and MPEC fates was regulated by the amount of inflammatory cytokines (i.e., IL-12) present during T cell priming. According to the amount of inflammation, a gradient of T-bet was created in which high T-bet expression induced SLECs and low expression promoted MPECs. These results elucidate a mechanism by which the innate immune system sets the relative amounts of a lineage-determining transcription factor in activated CD8$^+$ T cells and, correspondingly, regulates their memory cell potential.

INTRODUCTION

In response to acute viral or bacterial infections, antigen-specific CD8$^+$ T cells rapidly expand and differentiate into effector cells to help clear infection. Subsequently, most effector cells die, leaving behind a few memory CD8$^+$ T cells that protect from reinfection. Conceptually, effector CD8$^+$ T cells can be divided into at least two subsets, memory precursor effector cells (MPECs) that can become long-lived memory CD8$^+$ T cells and short-lived effector cells (SLECs) that do not. Currently, it is not well known when during their differentiation the effector cells make the critical decision to become MPECs or SLECs or what factors control this process.

Historically, identification of MPECs and SLECs within the effector CD8$^+$ T cell population has proven difficult because most of the T cell attributes first studied (e.g., CD44^{hi} and CD11a^{hi}) were acquired uniformly by effector CD8$^+$ T cells (Dutton et al., 1998). However, further analyses have found subsets of CD8$^+$ T cells that differentially express the IL-7 receptor alpha-chain (IL-7R), L-selectin (CD62L), CCR7, Killer cell lectin-like receptor G1 (KLRG1), CD27 and CD28, and others, that, depending on the attribute examined, differ according to their localization, effector functions, or potential to become protective memory CD8$^+$ T cells (de Bree et al., 2005; Huster et al., 2004; Kaech et al., 2003; Sallusto et al., 1999; Schluns et al., 2000; Voehringer et al., 2001; Wherry et al., 2003). Nevertheless, a greater understanding of how these different subsets form during infection is needed.

IL-7R has been identified as a marker of MPECs after acute infections, because at the peak of effector CD8$^+$ T cell expansion, ~5%–20% of antigen-specific CD8$^+$ T cells express IL-7R (referred to as IL-7R^{hi}) and have a substantially greater potential to form memory CD8$^+$ T cells when compared to IL-7R^{lo} effector CD8$^+$ T cells (Huster et al., 2004; Kaech et al., 2003; Schluns et al., 2000). This finding raises two pertinent questions: when during infection do the activated CD8$^+$ T cells commit to becoming IL-7R^{hi} MPECs or IL-7R^{lo} SLECs and what signals regulate this decision?

An effector CD8$^+$ T cell will see many signals during infection that could affect its gene expression and longevity. Some signals that can influence this process are the strength and duration of antigenic stimulation, costimulation, CD4$^+$ T cell help, and inflammatory cytokines (e.g., IL-12, IFN-α and IFN-β, IFN-γ; Bachmann et al., 2004; Badovinac and Harty, 2006; Khanolkar et al., 2004; Kolumam et al., 2005; Lang et al., 2005; Mescher et al., 2006; Wherry et al., 2003). Because of spatial and temporal differences in antigen load and local cytokine milieus, it is likely that two individual effector CD8$^+$ T cells will be exposed to unique sets of signals and this will lead them to differentiate to varying degrees or along separate cell lineages. Similarly, in the specification of developing

CD4$^+$ T cells, differential exposure to cytokines and antigenic stimuli can instruct cell-fate decisions toward T helper 1 (Th1), Th2, Th17, or Treg lineages (Weaver et al., 2006). Because of their profound effects, these lineage-determining cytokines are now referred to as signal 3 during T cell priming (Mescher et al., 2006).

This study investigated how and when IL-7R^{hi} MPECs and IL-7R^{lo} SLECs formed during a primary immune response to an acute viral infection. We found that the NK cell inhibitory receptor KLRG1 can serve as an early indicator of effector CD8$^+$ T cells committed to adopting an SLEC fate. Moreover, a critical determinant of the SLEC or MPEC fate decision was the amount of inflammation the CD8$^+$ T cells were exposed to at the time of priming, and in particular IL-12 was an instructive signal in this process. IL-12 could modulate the expression of the transcription factor T-bet (encoded by *Tbx21*) in a dose-dependent manner and, in accordance, we identified that the relative amounts of T-bet regulated the SLEC or MPEC fate decision. High amounts of T-bet induced KLRG1^{hi} IL-7R^{lo} SLECs, but lower amounts promoted the development of KLRG1^{lo} IL-7R^{hi} MPECs. Our data presented here detail a model of how inflammatory cytokines, through a gradient of T-bet expression, regulate the formation of memory CD8$^+$ T cells.

RESULTS

KLRG1 Is a Marker of Short-Lived Effector CD8$^+$ T Cells

To understand how MPECs and SLECs formed and acquired different cell fates during acute viral infection, we transferred ~1 × 10^4 Thy1.1$^+$ naive P14 (LCMV GP$_{33-41}$ specific) T cell receptor (TCR) transgenic (tg) CD8$^+$ T cells into naive wild-type (WT) recipients to make "P14 chimeric mice" that were subsequently infected with LCMV. Next, we compared day 7 IL-7R^{hi} and IL-7R^{lo} P14 effector CD8$^+$ T cells with Affymetrix GeneChips. Of the ~400 genes that differed substantially between IL-7R^{hi} and IL-7R^{lo} effector CD8$^+$ T cells, the expression of several NK cell receptors (KLRG1, Ly49c, KLRE1, 2B4, and Ly49h) was increased in IL-7R^{lo} SLECs (Figure 1A). However, this phenotype did not extend to other NK cell receptors such as NKG2A/C, NKG2D, and CD94 (data not shown). We focused on KLRG1 because of the large differential in its mRNA and protein expression (Figures 1A and 1B). Moreover, previous reports showed that KLRG1 was a marker of "terminally differentiated" mature NK cells, CD8$^+$ and CD4$^+$ T effector memory (T$_{EM}$), and mast cells (Chtanova et al., 2005; Kaech et al., 2003; Ortega et al., 1991; Robbins et al., 2005; Voehringer et al., 2001). It is possible that KLRG1 expression may indicate a common program of terminal differentiation in these cell types.

We examined day 8 LCMV-specific endogenous and P14 CD8$^+$ T cells in the blood, spleen, liver, lung, and inguinal lymph node (LN) and found mostly KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells (Figure 1B; Figures S1 and S3 in the Supplemental Data available online; and data not shown). During "the contraction phase" (days 8–30 postinfection, pi), KLRG1^{hi} IL-7R^{lo} cells sharply declined in number (~40-fold decrease) and continued to gradually decay thereafter ($t_{1/2}$ ~65–80 days; Figure 1C; Figure S1). In contrast, the magnitude of KLRG1^{lo} IL-7R^{hi} effector cell contraction was considerably less between days 8 and 30 pi (~4-fold decrease), and after that, KLRG1^{lo} IL-7R^{hi} cell numbers remained stable in all tissues except for the lung. Consequentially, KLRG1^{lo} IL-7R^{hi} CD8$^+$ T cells predominate the memory cell population at later times (Figures 1B and 1C). Notably, KLRG1^{hi} memory cells were reduced in their ability to homeostatically turn over, which likely contributed to their decline over time (Figure 1D).

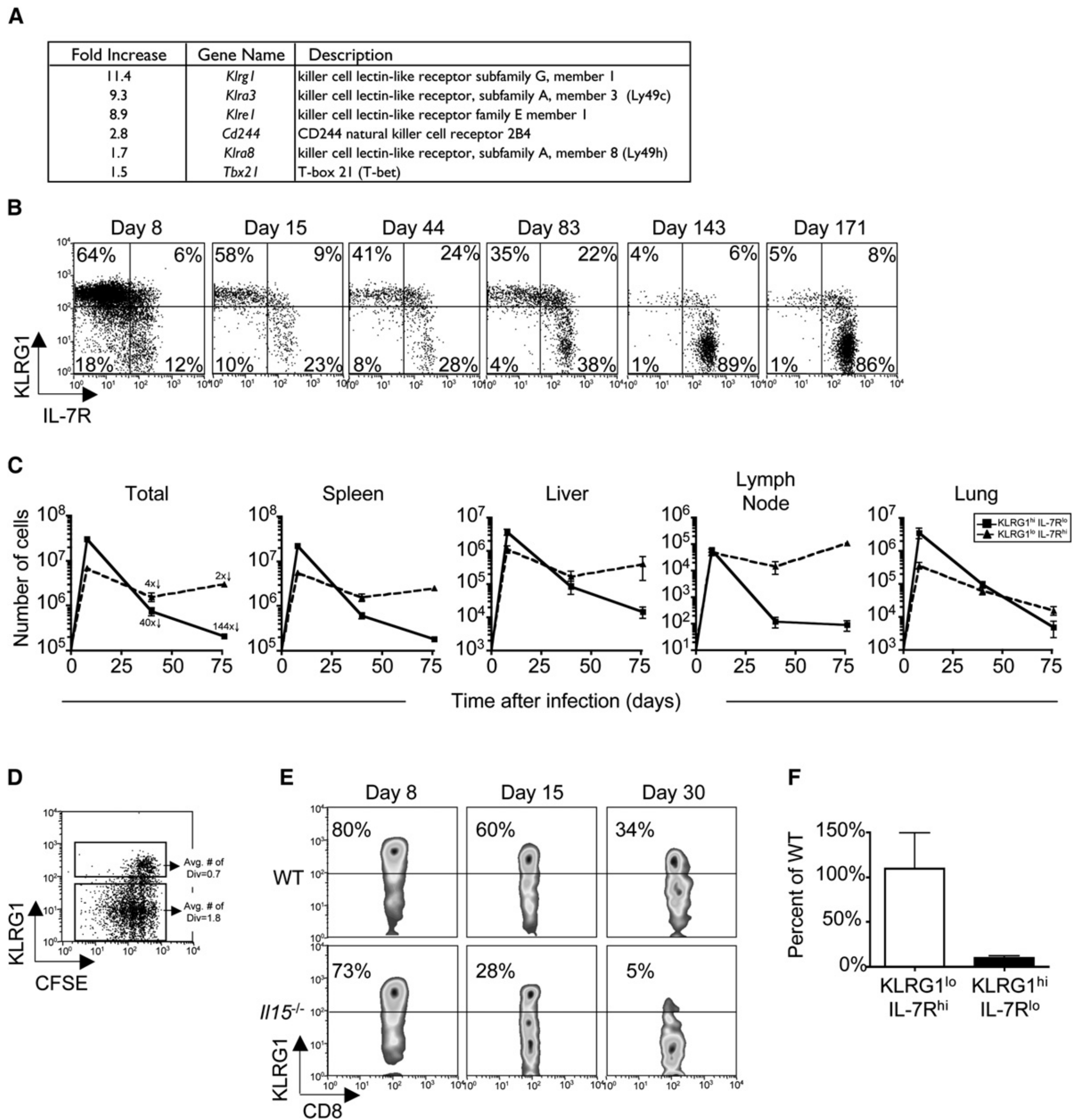
To confirm that KLRG1^{hi} IL-7R^{lo} CD8$^+$ T cell decline was due to death rather than conversion, equal numbers of FACS-sorted day 8 P14 KLRG1^{hi} IL-7R^{lo} and KLRG1^{lo} IL-7R^{hi} effector cells were transferred into day 8 infected recipients and followed for 2–3 months after transfer. There was little to no evidence of conversion between donor KLRG1^{hi} IL-7R^{lo} and KLRG1^{lo} IL-7R^{hi} cells during this time and ~8-fold more KLRG1^{lo} IL-7R^{hi} cells survived (Figure S2 and data not shown). Therefore, similar to previous results (Huster et al., 2004; Kaech et al., 2003), these data showed that the short-lived fate of KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells was relatively fixed after acute infection.

KLRG1^{hi} IL-7R^{lo} CD8$^+$ T Cells Require IL-15 for Survival

Although KLRG1^{hi} IL-7R^{lo} cells were relatively short-lived, we examined whether IL-15 sustains their survival because they cannot receive IL-7 signals and together, IL-7 and IL-15 promote memory CD8$^+$ T cell longevity and self-renewal (Hand et al., 2007; Schluns and Lefrancois, 2003). We tested the generation and survival of SLECs and MPECs after LCMV infection in WT and *IL15*$^{-/-}$ mice. KLRG1^{hi} IL-7R^{lo} and KLRG1^{lo} IL-7R^{hi} cells had formed equally in both groups of mice (day 8 pi), but subsequently the KLRG1^{hi} IL-7R^{lo} cells in the *IL15*$^{-/-}$ mice rapidly disappeared (Figure 1E and data not shown). Similar to previous work, this suggested that IL-15 was required for KLRG1^{hi} IL-7R^{lo} cell survival after infection (Yajima et al., 2006). To test this directly, equal numbers of day 8 KLRG1^{hi} IL-7R^{lo} and KLRG1^{lo} IL-7R^{hi} P14 effector CD8$^+$ T cells were sorted and transferred into WT and *IL15*$^{-/-}$ mice. Although the KLRG1^{lo} IL-7R^{hi} cells survived equally well in the *IL15*$^{-/-}$ and WT animals, in the absence of IL-15, >90% of the transferred KLRG1^{hi} IL-7R^{lo} cells rapidly died (Figure 1F). Thus, SLECs could not re-express IL-7R to survive in *IL15*$^{-/-}$ mice, and consequentially they were acutely dependent on IL-15. In light of the data in Figures 1C and 1D, this shows that SLECs can see IL-15, but it was insufficient to maintain their long-term survival or HT.

KLRG1 Marks Cells Early during Infection that Are Committed to Becoming IL-7R^{lo} SLECs

To determine when SLECs and MPECs emerged during infection, we followed KLRG1 expression on LCMV-specific endogenous and P14 effector CD8$^+$ T cells during



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Fold Increase	Gene Name	Description
11.4	*Klrg1*	killer cell lectin-like receptor subfamily G, member 1
9.3	*Klra3*	killer cell lectin-like receptor, subfamily A, member 3 (Ly49c)
8.9	*Klre1*	killer cell lectin-like receptor family E member 1
2.8	*Cd244*	CD244 natural killer cell receptor 2B4
1.7	*Klra8*	killer cell lectin-like receptor, subfamily A, member 8 (Ly49h)
1.5	*Tbx21*	T-box 21 (T-bet)

Figure 1. KLRG1^{hi} IL-7R^{lo} Effector CD8^{+} T Cells Are Short Lived and Require IL-15 for Survival

(A) The mRNA of IL-7R^{hi} and IL-7R^{lo} Thy1.1^{+} effector CD8^{+} T cells from P14 chimeric mice were compared on day 7 of LCMV infection by Affymetrix GeneChips. Table shows the increase in expression for selected NK receptors (IL-7R^{lo} > IL-7R^{hi} cells) and are the average of three independent experiments.

(B and C) Analysis of MPEC and SLEC subsets after LCMV infection of P14 chimeric mice. Data are representative of at least five independent experiments including at least four animals per time point.

(B) Plots are gated on Thy1.1^{+} P14 T cells and show expression of KLRG1 and IL-7R in blood over time.

(C) Line graphs show KLRG1^{hi} IL-7R^{lo} (squares) and KLRG1^{lo} IL-7R^{hi} (triangles) P14 CD8^{+} T cell numbers in the spleen, liver, lung, inguinal lymph node (LN), and total from all tissues. The magnitude of contraction between days 8–40 and 8–75 is indicated.

(D) CFSE-labeled P14 memory CD8^{+} T cells from day ~40 pi were transferred into naive mice and then analyzed for CFSE and KLRG1 expression 4–6 weeks later. Data are representative of five independent experiments.

(E) WT and *IL15*$^{-/-}$ mice were infected with LCMV, and D^{b} GP$_{33-41}$ MHC class I tetramer^{+} CD8^{+} T cells were analyzed for KLRG1 expression 8, 15, and 30 days pi. Similar data were observed for NP$_{396-404}$-specific CD8^{+} T cells (data not shown). Data are representative of three independent experiments.

(F) KLRG1^{hi} IL-7R^{lo} or KLRG1^{lo} IL-7R^{hi} P14 CD8^{+} T cells were sorted day 8 pi and transferred in equal numbers into WT or *IL15*$^{-/-}$ recipients for 10–15 days. Bar graph shows the average number of donor cells (mean ± SEM) recovered from *IL15*$^{-/-}$ recipients normalized to the number recovered from WT recipients for the KLRG1^{hi} IL-7R^{lo} (white bar, n = 3) and KLRG1^{lo} IL-7R^{hi} (black bar, n = 3) cell populations. Data are representative of three independent experiments.

LCMV infection. KLRG1^{hi} effector CD8$^+$ T cells first appeared 4–5 days pi and after at least 7–10 cell divisions, and these cells progressively increased in number and frequency until the peak of expansion (7–8 days pi) in all tissues examined (Figure 2A; Figure S3; data not shown; note: "KLRG1^{lo}" includes both intermediate and low expression). Next, we examined IL-7R expression on KLRG1^{hi} and KLRG1^{lo} effector CD8$^+$ T cells during infection. By days 4–5 pi, IL-7R expression was decreased compared to naive CD8$^+$ T cells but was indistinguishable between KLRG1^{hi} and KLRG1^{lo} cells (Figure 2B; note histograms in bottom row). Between days 6 and 8 pi, IL-7R expression gradually increased on the KLRG1^{lo} cells and decreased on the KLRG1^{hi} cells, albeit ~10% were IL-7R^{hi} (Figure 2B). Thus, between days 5 and 8 pi, KLRG1^{hi} and KLRG1^{lo} effector CD8$^+$ T cells appeared to differentiate and mature along two distinct cell lineages: KLRG1^{hi} IL-7R^{lo} SLECs and KLRG1^{lo} IL-7R^{hi} MPECs.

Despite similar IL-7R protein expression, day 5 KLRG1^{hi} effector CD8$^+$ T cells expressed ~1/3 the amount of IL-7R mRNA compared to KLRG1^{lo} cells (Figure 2C). This suggested that by day 5, KLRG1^{hi} cells were already repressing *Il7r* transcription to a greater degree and raised the possibility that they were already fully committed to becoming IL-7R^{lo} SLECs. To test this question, we transferred equal numbers of purified day 5 KLRG1^{hi} and KLRG1^{lo} effector CD8$^+$ T cells into day 5 infected animals and followed their development 4 and >60 days later. Both donor cell populations had similar engraftment and continued clonal expansion. On day 9 pi, almost all donor KLRG1^{hi} cells were IL-7R^{lo} (Figure 2D). In contrast, ~20%–30% of the donor KLRG1^{lo} cells became KLRG1^{hi} IL-7R^{lo} cells between days 5 and 9 pi, but many remaining KLRG1^{lo} became IL-7R^{hi} (Figure 2D). Approximately 60 days later, these cells gave rise to ~5-fold more memory CD8$^+$ T cells than did donor KLRG1^{hi} cells (Figure S4). Thus, as early as day 5 pi, KLRG1 upregulation marks a critical developmental decision in which effector CD8$^+$ T cells have committed to a shortened lifespan.

KLRG1^{hi} and KLRG1^{lo} Effector CD8$^+$ T Cells Have Similar Functional Properties

To determine other phenotypic and functional differences between KLRG1^{hi} and KLRG1^{lo} effector CD8$^+$ T cells, we compared their expression of several effector and memory markers, functional capabilities, and rates of division during LCMV infection. The expression of most proteins on day 5 and 8 KLRG1^{hi} and KLRG1^{lo} cells was similar, although KLRG1^{lo} cells expressed more CD27, IL-7R, and IL-2 and IL-15Rβ-chain (CD122) at day 8 (Figure 3A). Functionally, day 5 and 8 KLRG1^{hi} and KLRG1^{lo} effector CD8$^+$ T cells had comparable cytotoxic activity and ability to produce IFN-γ when restimulated (Figures 3B and 3C). However, ~2- to 4-fold more KLRG1^{lo} cells produced IL-2 at all time points examined (Figure 3C). Note that KLRG1 expression does not change during 5 hr stimulation assays (data not shown). Lastly, KLRG1^{hi} and KLRG1^{lo} effector cells had comparable rates of proliferation between days 4 and 8 pi, although we consistently observed a slightly greater fraction of KLRG1^{hi} cells in cycle at most times (Figure 3D). Therefore, KLRG1^{hi} and KLRG1^{lo} effector cells appeared functionally and phenotypically similar during LCMV infection and were distinguished only by small differences in expression of CD27 and IL-7R and their ability to produce IL-2 that evolved between days 5 and 8 pi. Given their similarities, we tested whether KLRG1 functioned in SLEC or MPEC lineage commitment by using an shRNA*i* retrovirus (RV) to "knock down" *Klrg1* in effector CD8$^+$ T cells during infection. *shKlrg1* efficiently decreased KLRG1 expression but had no other noticeable effects on SLEC formation or cytokine production, suggesting that KLRG1 does not control MPEC and SLEC fate decisions (Figure S5).

Truncating Infection Impairs KLRG1^{hi} IL-7R^{lo} SLEC Formation

Next, we studied how SLEC and MPEC fate decisions are regulated. One hypothesis was that excessive exposure of effector CD8$^+$ T cells to antigenic or inflammatory stimulation during infection diminished their memory cell developmental potential and drove their development into KLRG1^{hi} IL-7R^{lo} SLECs. We tested this by shortening the duration of *Listeria* infection with antibiotic treatment. P14 chimeric mice infected with *Listeria* expressing $GP_{33\text{-}41}$ (LM-GP33) were either left untreated (No Rx) or treated with ampicillin (Amp Rx) on day 1 pi to stop infection. Amp Rx specifically decreased the number of SLECs formed during LM-GP33 infection because similar numbers of MPECs, and subsequently memory CD8$^+$ T cells, formed in both groups of animals (Figure 4A and data not shown). Therefore, shortening the duration of infection had a greater impact on the formation of KLRG1^{hi} IL-7R^{lo} SLECs. In addition, increasing the intraclonal competition for antigenic and inflammatory signals by increasing the precursor frequency of naive P14 CD8$^+$ T cells during infection led to a corresponding decrease in the formation of KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells (Figure S6).

Inflammatory Signals Regulate Development or KLRG1^{hi} IL-7R^{lo} Effector Cells

The above experiment did not discriminate how the duration of infection regulated SLEC generation because Amp Rx reduced both antigenic and inflammatory signals. To examine the role of antigenic stimulation specifically, we used three recombinant vaccinia virus strains (referred to as rVVhp), each expressing different amounts of the LCMV epitope $GP_{33\text{-}41}$ via hairpin-mediated attenuation of protein expression (Wherry et al., 1999). In such, $GP_{33\text{-}41}$ abundance is varied whereas the kinetics of infection and viral titers are not. In accordance with previous results (Wherry et al., 1999), the frequency and number of P14 effector CD8$^+$ T cells at day 7 pi was proportional to antigen abundance (Figure 4B and data not shown). However, the frequencies of KLRG1^{hi} IL-7R^{lo} and KLRG1^{lo} IL-7R^{hi} cells formed were not greatly affected by antigen abundance (Figure 4B).

Next, we investigated how exposure to inflammation affected SLEC development by varying the amount of

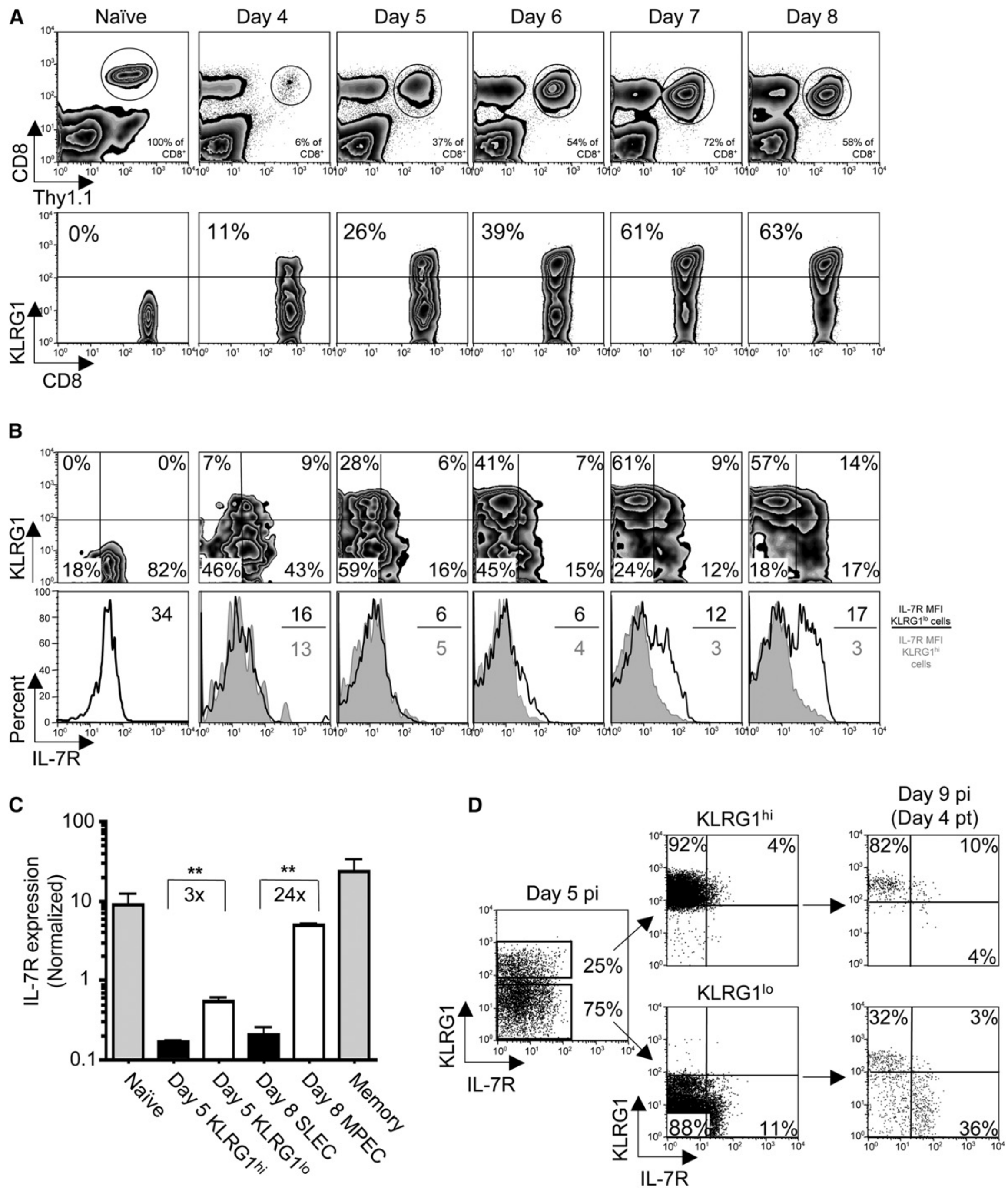

Figure 2. KLRG1 Marks Effector CD8+ T Cells Committed to an SLEC Fate

(A and B) P14 chimeric mice were infected with LCMV, and on days 4–8 pi the Thy1.1+ P14 effector CD8+ T cells were analyzed for expression of (A) KLRG1 and (B) KLRG1 and IL-7R. (B) Bottom row, histograms show IL-7R expression on KLRG1hi (filled) or KLRG1lo (open) P14 CD8+ T cells. IL-7R MFI is shown (KLRG1lo/KLRG1hi). Data are representative of at least three experiments.

(C) Bar graph shows the average IL-7R mRNA expression (normalized to the ribosomal gene *L9,* mean ± SEM) in the indicated cell populations (n = 3–5) measured by real-time PCR. **p < 0.001. Data represent the cumulative result of 3–5 independent samples.

(D) Day 5 pi KLRG1hi and KLRG1lo P14 CD8+ T cells were sorted and transferred in equal number back into day 5 LCMV infected recipients and analyzed 4 days post transfer (pt) for KLRG1 and IL-7R expression. Data are representative of at least three independent experiments.

"bystander" inflammation that P14 CD8+ T cells were exposed to during priming. P14 chimeric mice were immunized with LPS-matured dendritic cells (DCs) loaded with GP_{33-41} peptide (referred to as DC-33) with or without increasing different doses of a *Listeria* strain that does *not* express GP_{33-41} (referred to as LM). Concurrent DC-33

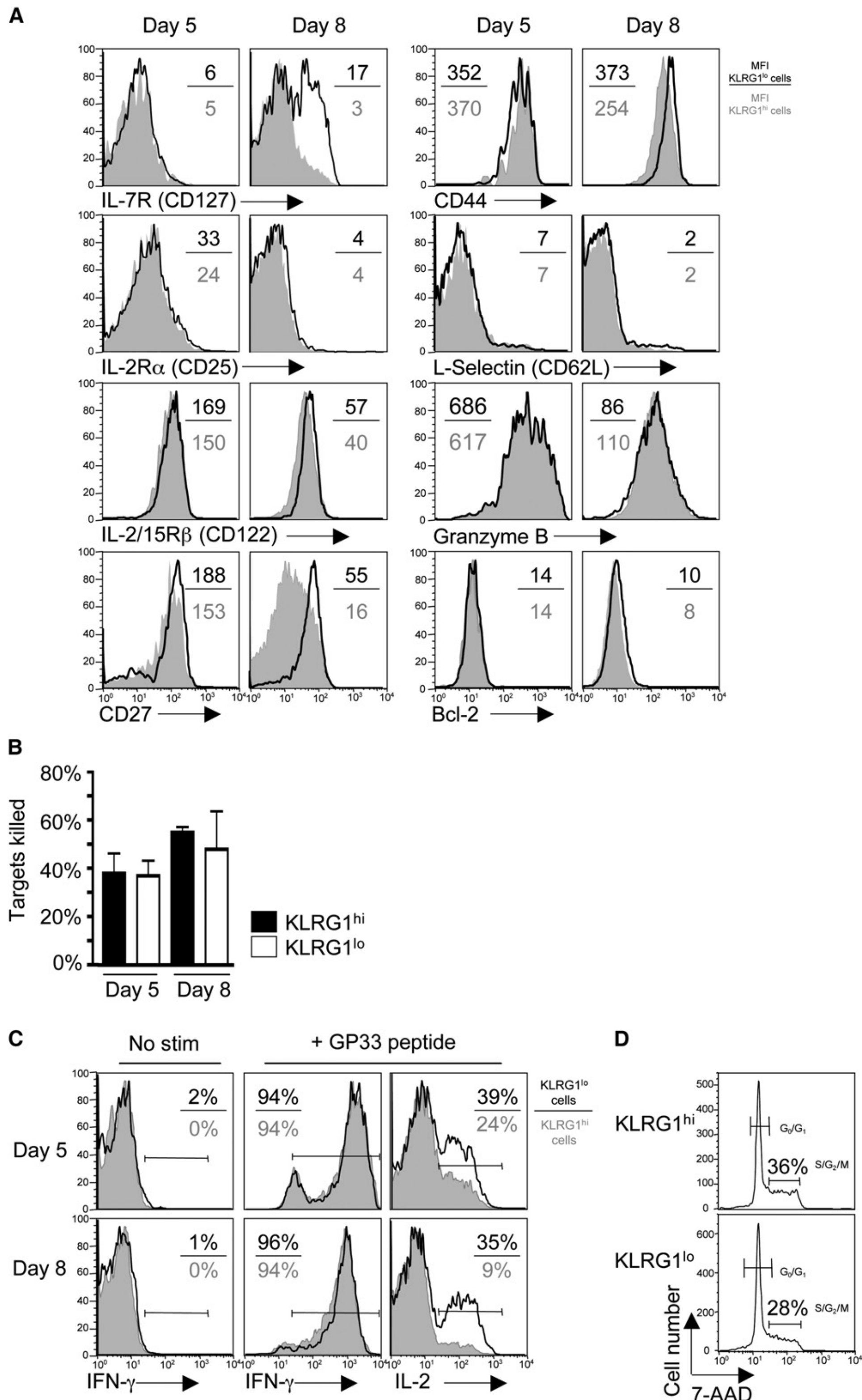

Figure 3. Phenotypic and Functional Comparisons between KLRG1^{hi} and KLRG1^{lo} Effector CD8^{+} T Cells

(A) Histograms show expression of the indicated proteins on KLRG1^{hi} (filled) and KLRG1^{lo} (open) P14 CD8^{+} T cells on days 5 and 8 pi. The MFI of KLRG1^{lo}/KLRG1^{hi} cells is shown. Data are representative of more than four independent experiments.

(B) In vivo CTL assay comparing day 5 and 8 KLRG1^{hi} (black, n = 2) and KLRG1^{lo} (white, n = 2) P14 effector CD8^{+} T cells. The average percent killing (mean ± SEM) over 4 hr was normalized to the effector:target (E:T) ratio. Data are representative of two independent experiments.

immunization with "high-dose" LM infection resulted in ~60%–75% KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells. However, as the LM dose was lowered, there was a corresponding decrease in the frequency of KLRG1^{hi} IL-7R^{lo} cells; in the absence of any overt inflammation, mostly KLRG1^{lo} IL-7R^{hi} effector CD8$^+$ T cells formed (Figure 4C). Therefore, the percentage of effector CD8$^+$ T cells that became KLRG1^{hi} IL-7R^{lo} SLECs was proportional to the amount or duration of inflammation.

LM infection produces many inflammatory cytokines, so the effect of a simpler adjuvant, CpG oligodeoxynucleotides (ODNs), was tested. Naive P14 CD8$^+$ T cells were stimulated with GP$_{33-41}$ peptide-loaded splenocytes with and without CpG ODN for 24–48 hr, transferred into naive recipients, and analyzed 5 days later. Without CpG, few KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells formed, but with CpG, ~25%–50% of the effector cells became KLRG1^{hi} and IL-7R^{lo} (Figure 4D). CpG ODN is a potent generator of both type I and II interferons and IL-12 (Krieg, 2002; and data not shown); therefore, we examined the effects of CD8$^+$ T cell priming with IL-12 and IFN-γ directly. Priming P14 CD8$^+$ T cells with IL-12+IFN-γ or IL-12, but not IFN-γ alone, generated KLRG1^{hi} IL-7R^{lo} effector cells (Figure 4D). Thus, IL-12 was a critical signal that could induce the formation of KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells.

IL-12, but Not IFN-γ, Induces T-bet Expression in a Dose-Dependent Manner

The transcription factor T-bet is critical for Th1 CD4$^+$ T cell differentiation and terminal maturation of KLRG1^{hi} NK cells (Robbins et al., 2005; Szabo et al., 2000). T-bet expression is also regulated by IFN-γ and IL-12 (Weaver et al., 2006), so we tested whether T-bet was involved in the SLEC or MPEC fate decision. First, we examined whether IL-12 or IFN-γ could induce T-bet expression in early activated CD8$^+$ T cells by using flow cytometry. IL-12, but surprisingly not IFN-γ, could induce T-bet to markedly higher levels than peptide alone, and the amount of T-bet induced directly corresponded to the concentration of IL-12 in the media (Figures 4E and 4F). These data extend from previous work (Takemoto et al., 2006) by showing that T-bet expression in activated CD8$^+$ T cells could be controlled by IL-12 in a dose-dependent manner.

T-bet Controls Formation of KLRG1^{hi} IL-7R^{lo} Effector CD8$^+$ T Cells

Because IL-12 induced T-bet expression and SLEC formation, we tested whether T-bet was necessary for SLEC development. Therefore, we infected WT and *Tbx21*$^{-/-}$ mice directly with LCMV (Figure 5A) or, to examine the cell-autonomous role of T-bet, we transferred small numbers of WT and *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells into WT mice that were subsequently infected with LCMV (Figure 5B). Compared to WT effector CD8$^+$ T cells, <10% of *Tbx21*$^{-/-}$ effector cells were KLRG1^{hi} and IL-7R^{lo} (Figures 5A and 5B). This likely accounted for the ~4- to 9-fold reduction in the total number of *Tbx21*$^{-/-}$ effector cells. Interestingly, similar numbers of KLRG1^{lo} IL-7R^{hi} MPECs formed in WT and *Tbx21*$^{-/-}$ mice (Figure 5C). Similar to a previous study, *Tbx21*$^{-/-}$ effector CD8$^+$ T cells expressed relatively normal amounts of Granzyme B and IFN-γ, but IL-2 production was greatly elevated (Juedes et al., 2004; data not shown). Most *Tbx21*$^{-/-}$ effector CD8$^+$ T cells were CD27^{hi} and expressed lower amounts of Ly49c, KLRE1, CD244 (2B4) mRNA, suggesting that multiple SLEC-associated attributes were dependent on T-bet (data not shown). These data suggested that T-bet plays a necessary cell-intrinsic role in SLEC formation. In support of this, IL-12 induction of KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells required T-bet (Figure 5D).

Next, we determined whether T-bet expression was sufficient for KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cell generation by transducing activated WT P14 CD8$^+$ T cells with MSCV RV expressing T-bet and GFP or GFP alone and transferring them into naive recipients. Transduction of cells with control MSCV RV did not induce KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells; however, if T-bet was overexpressed, >50% of the cells became KLRG1^{hi} IL-7R^{lo} SLECs by day 5 after transfer (Figure 5E). Strikingly, >80%–90% of the WT or *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells transduced with T-bet RV became KLRG1^{hi} and IL-7R^{lo} when transferred into LCMV-infected recipients (Figure 5F). Furthermore, most KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells formed by T-bet overexpression were short-lived and contracted by day 30+ after transfer (Figure 5E; data not shown). Together with that above, these data show that T-bet is necessary and (with overexpression) sufficient to specify a population of naturally arising short-lived KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells.

A Gradient of T-bet Expression Specifies a SLEC or MPEC Fate

In light of the T-bet RV overexpression data, we next examined whether KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells naturally express more T-bet than KLRG1^{lo} IL-7R^{hi} cells. DNA microarrays and real-time PCR showed that T-bet mRNA was increased ~1.5- to 2-fold in IL-7R^{lo} SLECs compared to IL-7R^{hi} MPECs at day 7 pi, and immunoblotting confirmed that KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cells contained ~3- to 4-fold more T-bet protein than did KLRG1^{lo} IL-7R^{hi} cells (Figures 1A and 6A; data not shown).

Although T-bet expression was lower in MPECs, we tested whether it functions in these cells. By comparing WT and *Tbx21*$^{-/-}$ "MPECs" with Affymetrix GeneChips,

(C) Splenocytes from day 5 and 8 LCMV-infected P14 chimeric were stimulated with GP$_{33-41}$ peptide and analyzed for IFN-γ and IL-2 production by intracellular cytokine staining. Histograms show IFN-γ (left and center) and IL-2 (right) production by KLRG1^{hi} (filled) and KLRG1^{lo} (open) Thy1.1$^+$ P14 CD8$^+$ T cells. IL-2 plots are gated on IFN-γ-producing cells. Similar results were found in endogenous LCMV-specific CD8$^+$ T cells stimulated with NP$_{396-404}$, GP$_{33-41}$, and GP$_{276-284}$. Data are representative of more than three independent experiments.
(D) Histograms show the percent of KLRG1^{hi} (top) and KLRG1^{lo} cells (bottom) P14 CD8$^+$ T cells in S, G2, and M phases of the cell cycle on day 5 pi with 7-AAD. Data are representative of three independent experiments.

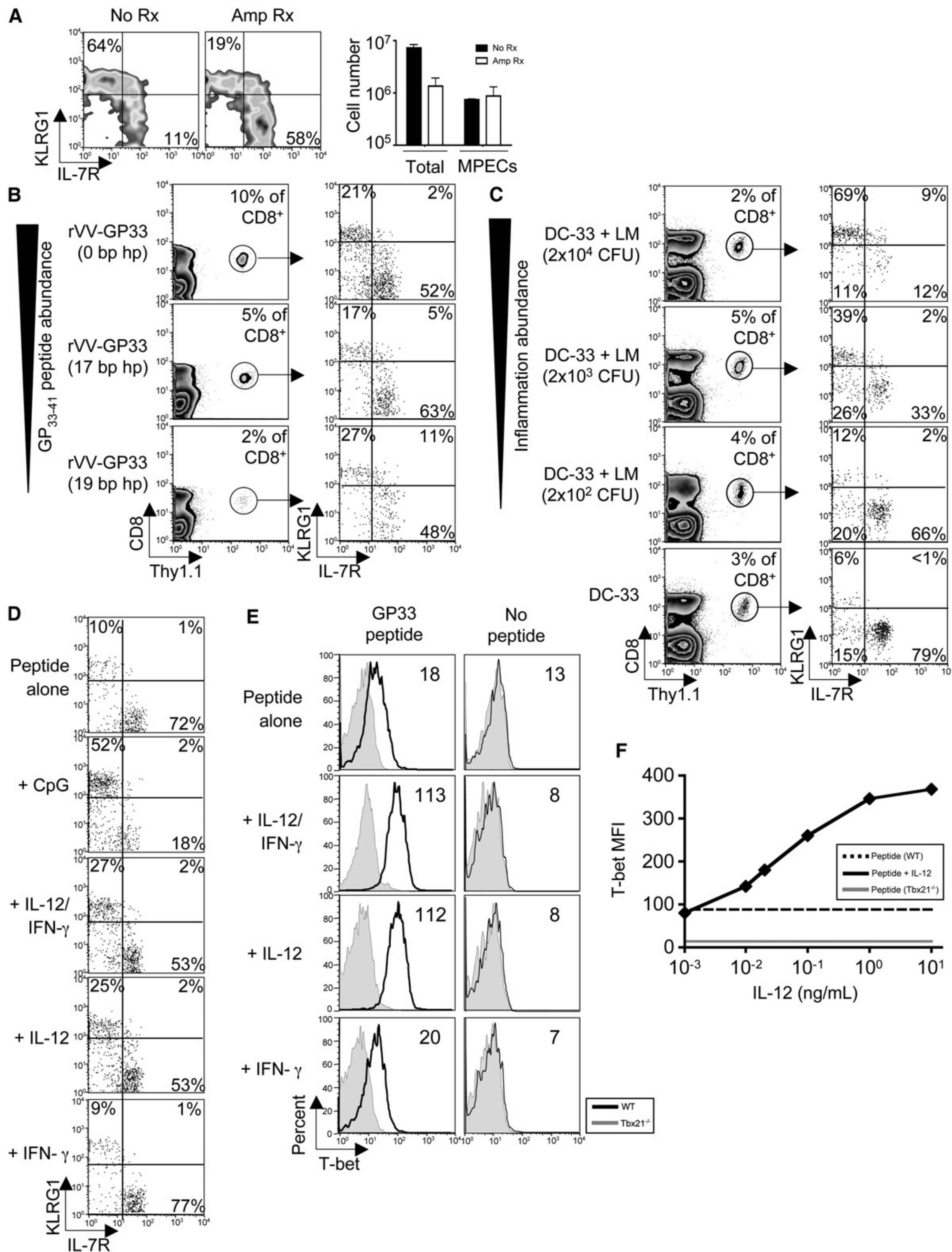

Figure 4. Inflammation Regulates KLRG1^{hi} IL-7R^{lo} SLEC Formation

(A) P14 chimeric mice were infected with LM-GP33 and 1 day pi were either left untreated (No Rx, black bars, n = 3) or treated with ampicillin (Amp Rx, white bars, n = 3). FACS plots show KLRG1 and IL-7R expression and bar graphs show the average number of total or KLRG1^{lo} IL-7R^{hi} MPECs (mean ± SEM) on day 7 pi. Data are representative of five independent experiments.

(B) P14 chimeric mice were infected with rVVhp -0, -17, or -19 (see text), and 8 days pi, Thy1.1^{+} P14 CD8^{+} T cells were analyzed for KLRG1 and IL-7R expression. Data are representative of three independent experiments.

we found that T-bet regulated ~20% of the MPEC-specific genes (data not shown). Moreover, although *Tbx21*$^{-/-}$ effector cells initially expressed relatively normal amounts of CD122 at day 5 pi, T-bet was required for sustained CD122 expression after this time point (Figure 6B). Thus, low amounts of T-bet were necessary for normal MPEC development. This finding suggested a model in which T-bet functions distinctly in SLECs and in MPECs: high T-bet amounts induced terminal SLEC differentiation, whereas low amounts promoted MPEC development.

To directly test whether the amount of T-bet expression dictates SLEC or MPEC formation, we varied the amount of T-bet in LCMV-specific effector CD8$^+$ T cells during infection by two separate methods. First, we examined the effects of *Tbx21* gene dosage by comparing *Tbx21*$^{+/+}$, *Tbx21*$^{+/-}$, and *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells for their ability to form SLECs and MPECs 7 days pi and memory cells ~50 days pi. These experiments showed that as the T-bet copy number decreased, so did the frequency and number of KLRG1^{hi} IL-7R^{lo} SLECs (Figure 6C; Figure S7). Conversely, the frequency of KLRG1^{lo} IL-7R^{hi} "MPECs" increased (*Tbx21*$^{-/-}$ > *Tbx21*$^{+/-}$ > *Tbx21*$^{+/+}$); however, similar numbers formed in all three groups of effector CD8$^+$ T cells (Figure 6C; Figure S7). These data show that a 50% decrease in T-bet expression affects the lineage commitment of SLECs considerably more than MPECs.

After clearance of LCMV infection, the magnitude of *Tbx21*$^{+/+}$, *Tbx21*$^{+/-}$, and *Tbx21*$^{-/-}$ effector CD8$^+$ T cell contraction was proportionally decreased according to T-bet copy number, but similar sized memory CD8$^+$ T cell populations were yielded (as may have been predicted by MPEC numbers; Figure S7). Thus, at the population level, *Tbx21*$^{+/-}$ and *Tbx21*$^{-/-}$ effector cells appeared more efficient at generating memory CD8$^+$ T cells, but it is likely that this effect was due to their impaired SLEC formation rather than to increased MPEC formation. It may be that during LCMV infection, the number of MPECs formed is capped by other mechanisms. Importantly, the lowered expression of T-bet in *Tbx21*$^{+/-}$ P14 cells was sufficient to maintain CD122 expression as well as to partially suppress IL-2 production in the memory CD8$^+$ T cells (Figure 6D; data not shown). It is not known yet whether the *Tbx21*$^{-/-}$ memory CD8$^+$ T cells, with reduced CD122 expression, will be maintained long-term or gradually decay as in *IL15*$^{-/-}$ mice (Schluns and Lefrancois, 2003). *Tbx21*$^{-/-}$ memory CD8$^+$ T cells express higher amounts of IL-7R compared to WT cells (Figure 6C), and therefore, it is possible that IL-7 may compensate for reduced CD122 expression as described previously (Kieper et al., 2002; data not shown).

We also regulated T-bet activity (via its nuclear localization) by fusing it to the estrogen receptor α (T-bet:ER; Matsuda et al., 2007). *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells were reconstituted with T-bet:ER RV and transferred into LCMV-infected recipients that were subsequently treated with different doses of tamoxifen (Tm) during infection. After 7 days, we found that the frequency of KLRG1^{hi} IL-7R^{lo} SLECs directly correlated with the amount of tamoxifen administered, ranging from ~10% to >60% at the lowest and highest Tm doses, respectively (Figure 6D). As expected, all doses of Tm rescued *Tbx21*$^{-/-}$ MPEC CD122 expression, showing again that low amounts of T-bet were required for normal MPEC gene expression (data not shown). Together, these data suggested a model (Figure S8) in which IL-12 (and likely other cytokines) regulate T-bet expression in a dose-dependent manner during infection to influence the memory T cell potential of effector CD8 T cells.

DISCUSSION

During acute viral or bacterial infections, effector CD8$^+$ T cells undergo a complex and coordinated differentiation program, part of which involves the generation of at least two effector cell subsets that have different fates and memory cell developmental potential (Badovinac et al., 2004; Huster et al., 2004; Kaech et al., 2003). This study was designed to investigate how and when during infection these cell-fate decisions occur. We found that inflammatory signals (like IL-12) present during CD8$^+$ T cell priming can dictate a T-bet expression gradient and regulate effector CD8$^+$ T cell-fate determination. Higher amounts of T-bet induced a terminally differentiated SLEC state, associated with increased KLRG1 expression and stable IL-7R repression, and acute dependence on IL-15 for survival, but not long-term persistence or homeostatic turnover. In contrast, effector cells expressing lower amounts of T-bet remained KLRG1^{lo} and some were capable of becoming IL-7R^{hi} MPECs that responded to both IL-7 and IL-15 and developed into long-lived, self-renewing memory CD8$^+$ T cells.

To date, KLRG1 is the best-described SLEC marker during acute infection, although it is unlikely that it plays a significant role in their formation. In humans and mice, most acute infections result in mainly KLRG1^{lo} IL-7R^{hi} memory CD8$^+$ T cells, but chronic or latent viral infections

(C) P14 chimeric mice were concurrently immunized with DC-33 and varying doses of *Listeria* (*not* expressing GP$_{33\text{-}41}$). FACS plots show KLRG1 and IL-7R expression on day 7 P14 effector CD8$^+$ T cells. Data are representative of four independent experiments.

(D) Purified naive Thy1.1$^+$ P14 CD8$^+$ T cells were stimulated with GP$_{33\text{-}41}$ peptide-loaded cells ± CpG ODN or the indicated cytokines for 24–48 hr and then transferred into naive recipients. Thy1.1$^+$ P14 CD8$^+$ T cells were analyzed for KLRG1 and IL-7R expression 5–6 days pt. Data are representative of at least three independent experiments.

(E and F) Naive WT or *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells were stimulated as in (D) with IL-12 or IFN-γ or both (E) or decreasing concentrations of IL-12 (F). Data are representative of three independent experiments.

(E) Histograms show T-bet expression in WT (open) or *Tbx21*$^{-/-}$ (filled) Thy1.1$^+$ CD44^{hi} CD8$^+$ T cells, and the T-bet MFI is indicated. Data are representative of at least three independent experiments.

(F) Line graph shows the MFI of T-bet with either peptide alone (dashed), the indicated concentration of IL-12 (solid), or *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells + IL-12 (gray).

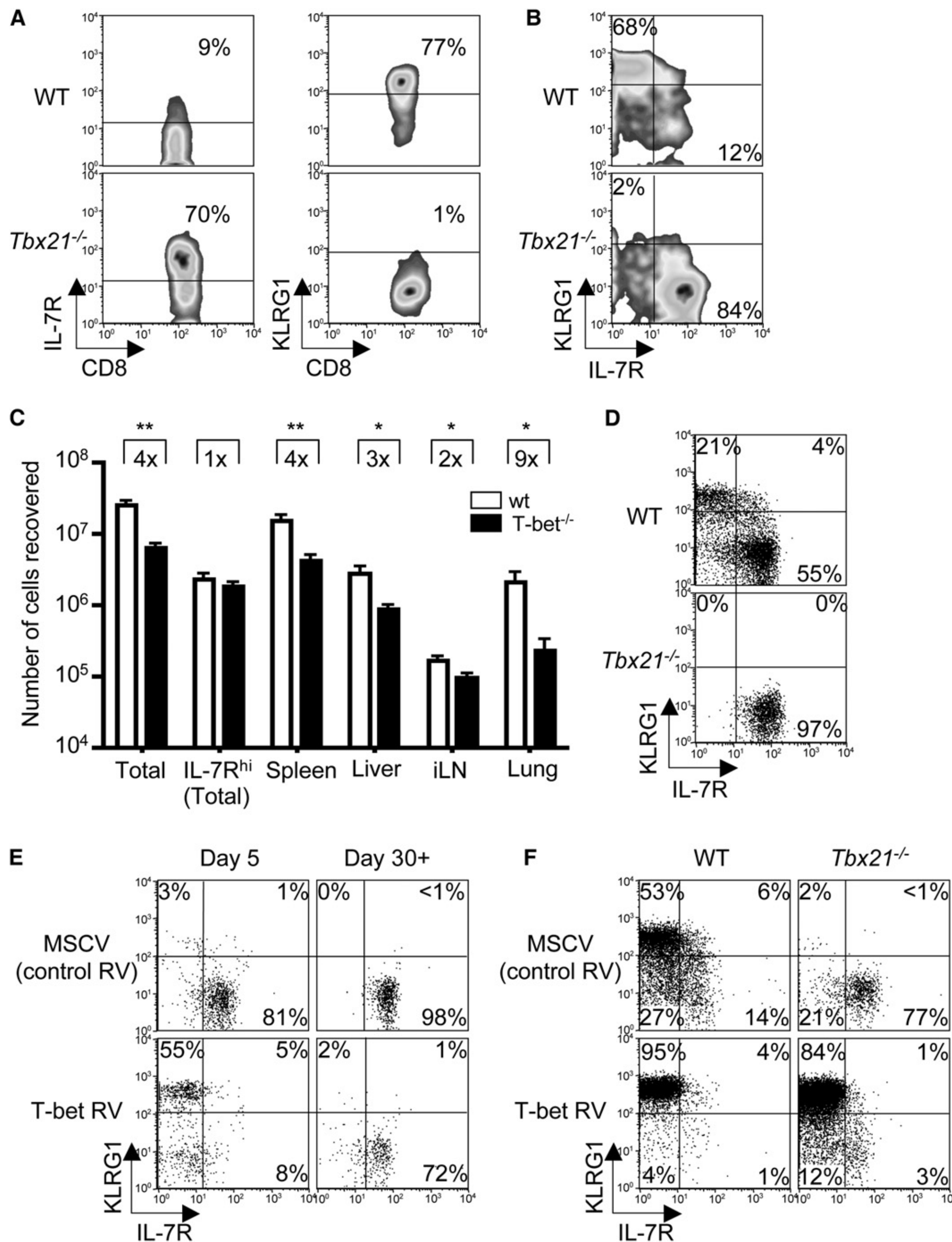

Figure 5. T-bet Expression Is Necessary and Sufficient for Development of KLRG1^{hi} SLECs

(A) WT or *Tbx21*$^{-/-}$ mice or (B) WT mice containing ~1 × 10^4 WT or *Tbx21*$^{-/-}$ P14 CD8+ T cells were infected with LCMV and analyzed 7–8 days later for IL-7R and KLRG1 expression on GP_{33-41}-specific CD8+ T cells (A) or Thy1.1+ P14 CD8+ splenocytes (B). Similar data to (A) were observed with $NP_{396-404}$-specific CD8+ T cells (data not shown). Data in (A) and (B) are representative of more than four independent experiments.

(C) Bar graph compares the average total combined number of endogenous GP_{33-41} and $NP_{396-404}$-specific CD8+ T cells (mean ± SEM) between WT (black, n = 3) or *Tbx21*$^{-/-}$ (white, n = 5) animals on day 8 of LCMV infection. Data represent the cumulative results of three independent experiments. Note similar numbers of IL-7R^{hi} effector cells in WT and *Tbx21*$^{-/-}$ animals. **$p < 0.001$, *$p < 0.01$.

(D) As in Figure 4D, WT or *Tbx21*$^{-/-}$ Thy1.1+ P14 CD8+ T cells were stimulated with IL-12, transferred into naive recipients, and analyzed for KLRG1 and IL-7R expression 5–6 days later. Data are representative of at least three independent experiments.

(E) WT P14 CD8+ T cells were transduced with control (MSCV) or T-bet-expressing RVs, transferred into naive recipients, and analyzed 5 or 30+ days later for KLRG1 and IL-7R expression on Thy1.1+ GFP+ CD8+ T cells. Data are representative of four independent experiments.

(F) WT or *Tbx21*$^{-/-}$ P14 CD8+ T cells were transduced with MSCV or T-bet RVs and transferred into recipients that were subsequently infected with LCMV. Seven days later, Thy1.1+ GFP+ splenocytes were analyzed for IL-7R and KLRG1 expression. Data are representative of more than five independent experiments.

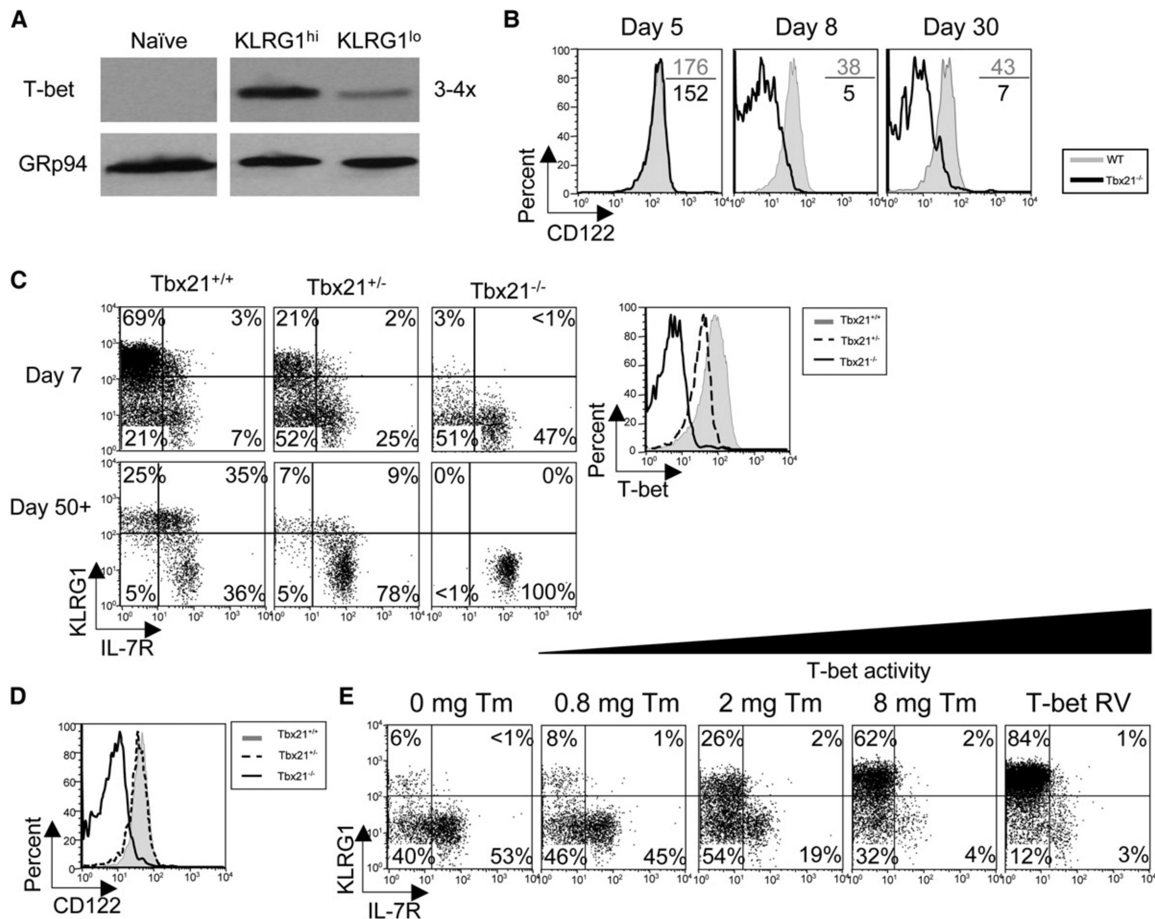

Figure 6. T-bet Functions in Both MPECs and SLECs According to an Expression Gradient

(A) Naive and day 8 KLRG1^{hi} IL-7R^{lo} SLECs or KLRG1^{lo} IL-7R^{hi} MPECs were sorted and the amount of T-bet and GRp94 protein was examined by immunoblotting. Data are representative of three independent experiments.

(B) WT and *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells were analyzed 5, 8, and 30 days pi for CD122 expression. MFI for CD122 is indicated on plots. Data are representative of more than five independent experiments.

(C) *Tbx21*$^{+/+}$, *Tbx21*$^{+/-}$, or *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells were analyzed 7 and 50+ days pi for KLRG1 and IL-7R expression and T-bet expression (bottom histogram plot). Data are representative of three independent experiments.

(D) *Tbx21*$^{+/+}$, *Tbx21*$^{+/-}$, or *Tbx21*$^{-/-}$ P14 memory CD8$^+$ T cells were analyzed 50+ days pi for CD122 expression. Data are representative of three independent experiments.

(E) *Tbx21*$^{-/-}$ P14 CD8$^+$ T cells were transduced with T-bet RV or one expressing T-bet fused to the estrogen receptor (T-bet:ER) and transferred into mice subsequently infected with LCMV. Mice were treated with 0–8 mg of tamoxifen (Tm) during infection, and on day 7 pi, GFP$^+$ donor splenocytes were analyzed for expression of KLRG1 and IL-7R. Data are representative of four independent experiments.

can produce greater frequencies of KLRG1^{hi} IL-7R^{lo} virus-specific CD8$^+$ T cells that are likely to be continually generated because of repeated exposure to viral antigens and inflammatory cytokines (Ibegbu et al., 2005; Lang et al., 2005; Sierro et al., 2005; van Leeuwen et al., 2006; Wherry et al., 2004). Furthermore, consecutive waves of infection generate mostly KLRG1^{hi} T_{EM} CD8$^+$ T cells, some of which express IL-7R and are long lived (Jabbari and Harty, 2006; Masopust et al., 2006; Voehringer et al., 2001). Therefore, it is important to emphasize that the strong correlation between KLRG1 expression and reduced CD8$^+$ T cell longevity may not hold true for CD8$^+$ T cells that have encountered antigen with inflammation repeatedly or persistently, especially if they coexpress IL-7R, as seen in a small number of KLRG1^{hi} IL-7R^{hi} primary effector CD8$^+$ T cells that appear long-lived. Moreover, it is important to emphasize that KLRG1^{hi} memory CD8$^+$ T cells have a reduced proliferative capacity and this calls into question their contribution to long-term immunologic protection (Voehringer et al., 2001).

The "decreasing potential model" for the development of memory CD8$^+$ T cells suggests that "memory T cells can only arise under conditions in which the antigenic load is limited and the stimulation of precursors ceases before a point of no return" (Ahmed and Gray, 1996). In support of this model, we and others found that altering the overall amount of stimulation per CD8$^+$ T cell (either by modulating TCR tg CD8$^+$ T cell precursor frequency

or by shortening or lengthening the duration of infection that T cells are exposed to) can profoundly affect the types of effector T cells produced or their longevity (Bachmann et al., 2006; Badovinac et al., 2004, 2005, 2007; D'Souza and Hedrick, 2006; Jelley-Gibbs et al., 2005; Lang et al., 2005). We further explored this model by separating antigenic and inflammatory signals. Although these findings do not rule out the possibility that excessive or persistent antigenic stimulation might affect SLEC or MPEC fate, they show that inflammatory signals (e.g., IL-12), through regulation of T-bet expression, play a dominant role in regulating memory cell potential. The findings presented here provide the decreasing potential model with both an early molecular signature (KLRG1) as well as a mechanism (IL-12 → T-bet → reduced longevity) for identifying and generating SLECs during primary immune responses to infection. In support of this, effector CD8$^+$ T cells from *Il12*$^{-/-}$ mice have reduced amounts of T-bet and generate more memory CD8$^+$ T cells (Pearce and Shen, 2007; Takemoto et al., 2006; W.C. and S.M.K., unpublished data). Moreover, it is worth noting that during infection, IL-12 is primarily produced by activated mature CD8α^+ dendritic cells (DCs), but over time their production of IL-12 declines (Langenkamp et al., 2000). Because a larger number of "newly" activated DCs are present early during infection, perhaps, the timing of when a naive CD8$^+$ T cell encounters a DC is critical in determining IL-12 exposure and SLEC or MPEC fate.

How does T-bet regulate the SLEC or MPEC fate decision? Similar to Th1 CD4$^+$ T cells (Szabo et al., 2000), T-bet may serve as the "master regulator" of CD8$^+$ T cell SLEC lineage commitment. However, unlike its asymmetric role in Th1 or Th2 specification, we believe that a gradient of T-bet is critical with low levels promoting MPECs and high levels inducing SLECs. A similar model has been described for PU.1 regulation of macrophage versus neutrophil development (Dahl et al., 2003). Accordingly, T-bet-influenced expression of ~50% of the SLEC-specific genes compared to ~20% of the MPEC-specific genes (data not shown). These data showed a heightened dependence on T-bet for lineage-specific gene expression in SLECs. Thus, we propose that the increased abundance of T-bet in SLECs is necessary for proper expression of these SLEC-specific genes and this explains why SLEC development is more overtly affected by T-bet deficiency. Additionally, T-bet regulates IL-12Rβ2 expression, and this positive-feedback loop can make T cells more receptive to IL-12 signals; however, it is also possible that IL-12 and T-bet act in parallel to provide separate signals to control SLEC and MPEC development.

Signals from IL-12 not only enhance T-bet expression, but might also repress Eomesodermin (Eomes), another T-box family member whose function can overlap with T-bet in CD8$^+$ T cells, leading to the speculation that T-bet and Eomes might direct SLEC and MPEC development, respectively (Takemoto et al., 2006). However, it is certainly more complicated than that because KLRG1^{hi} and KLRG1^{lo} effector cells expressed similar amounts of *eomes mRNA*, and T-bet was required for normal MPEC gene expression (data not shown). Previous work showed that in naive mice, T-bet deficiency did not affect CD122 expression in polyclonal memory CD8$^+$ T cells (Intlekofer et al., 2005). However, as shown here for antigen-specific memory CD8$^+$ T cells and as described previously for NKT cells, T-bet acts in a nonredundant manner to sustain CD122 expression (Matsuda et al., 2007; Townsend et al., 2004).

Murine cytomegalovirus (MCMV) and *Listeria* infection produce copious amounts of IL-12p70, whereas LCMV produces very little (Dalod et al., 2002), yet similar frequencies of IL-7R^{lo} SLECs form during these infections (Huster et al., 2004; Kaech et al., 2003; Sierro et al., 2005). Therefore, we predict that other inflammatory cytokines besides IL-12, which induce T-bet (Agnello et al., 2003), will also influence memory cell developmental potential. IFN-γ alone was insufficient to induce T-bet or KLRG1^{hi} IL-7R^{lo} cells, and this was surprising because IFN-γ induces T-bet in CD4$^+$ T cells and recent work has suggested that IFN-γ programs effector T cell contraction and represses IL-7R expression (Badovinac et al., 2000, 2004). Still, it is possible that IFN-γ is necessary, but not sufficient, because fewer KLRG1^{hi} IL-7R^{lo} SLECs formed in *Ifng*$^{-/-}$ mice during LCMV infection (data not shown). In support of this, in vivo CpG mediated IL-12p70 production and SLEC formation requires IFN-γ (data not shown). Accordingly, IL-12 treatment of *Ifng*$^{-/-}$ mice was sufficient to rescue CpG-mediated KLRG1^{hi} IL-7R^{lo} effector CD8$^+$ T cell induction, indicating that IFN-γ is necessary for optimal IL-12 production during immunization and infection (W.C. and S.M.K., unpublished data). Moreover, our data suggest an alternative explanation for the proposed programming of effector CD8$^+$ T cell contraction by inflammation: instead of inflammation directly controlling effector CD8$^+$ T cell contraction per se, we suggest that inflammation induces the formation of, what would naturally be, a short-lived subset of effector cells.

Although exposure to inflammatory cytokines may reduce effector CD8$^+$ T cell longevity, signals from IL-12 and type I and II IFNs also play a critical role in enhancing effector CD8$^+$ T cell expansion and function (Mescher et al., 2006). Thus, we are not proposing that better memory CD8$^+$ T cells will develop in the absence of inflammation, but rather that low exposure to inflammatory signals may promote development of functional effector cells that have greater memory potential. In contrast, excessive or prolonged exposure to inflammatory signals during infection or immunization may generate potent effector cells, but as a trade-off, their memory cell potential is decreased. This model offers a reliable mechanism to maintain T cell homeostasis because it allows the T cell response to be made in proportion to the amount and/or duration of infection, while simultaneously, it ensures restoration of normal T cell numbers once infection and inflammation ceases. Perhaps this is why KLRG1^{hi} effector cells become dependent on IL-15, a cytokine modulated by type 1 inflammatory cytokines (Doherty et al., 1996). In the future, it may become possible to tailor larger and more durable memory T cell populations by modulating the types and levels of

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inflammatory signals that they receive at the time of T cell priming, but this remains to be investigated.

EXPERIMENTAL PROCEDURES

Mice, Infections, and Treatments

Thy1.1+ P14 TCR tg mice have been described previously (Kaech et al., 2003). To make "P14 chimeric mice," ~1 × 10^4 Thy1.1+ WT, *Tbx21*$^{+/-}$, or *Tbx21*$^{-/-}$ CD8+ T cells were transferred into naive Thy1.2+ C57BL/6 (B6) mice (National Cancer Institute, Frederick, MD, and Jackson Laboratories, Bar Harbor, ME). *IL15*$^{-/-}$ mice were obtained from M. Caligiuri (Ohio State University, Columbus, OH). *Tbx21*$^{-/-}$ mice were obtained from L. Glimcher (Harvard School of Medicine, Cambridge, MA) and crossed to P14 tg mice. All animal experiments were done with approved Institutional Animal Care and Use Committee protocols. Details of infections and treatments are found in Supplemental Experimental Procedures.

CFSE, 7-AAD Labeling, Surface and Intracellular Staining, and Antibodies

Lymphocyte isolation, CFSE labeling (Invitrogen, Eugene, OR), LCMV peptide stimulations, production of MHC class I tetramers, and surface and intracellular staining was performed as described (Kaech et al., 2003). 7-AAD staining was performed according to manufacturer's directions (BD Biosciences, San Diego, CA). All antibodies were purchased from E-biosciences (San Diego, CA) except anti-Granzyme B-PE (Caltag, Burlingame, CA), Bcl-2-FITC (BD), and T-bet 647 (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-KLRG1 (2F1) hybridoma was a generous gift from D. Raulet (University of California, Berkley, CA) and was conjugated to Alexa 647 (Invitrogen, Eugene, OR). For T-bet staining, cells were permeabilized with 0.01% Triton X-100 in PBS + 0.5% FBS followed by intracellular staining. All flow cytometry was analyzed on a FACSCalibur (BD) with FloJo software (Treestar, San Carlos, CA).

Cell Isolations, Separations, and Adoptive Transfer

Details of these procedures are found in Supplemental Experimental Procedures.

In Vitro T Cell Stimulations for In Vivo Transfer and DC Immunization

DCs and naive WT B6 splenocytes were loaded with $GP_{33\text{-}41}$ peptide and either ~1 × 10^6 peptide-loaded splenocytes were cultured with ~5 × 10^4 MACS purified naive P14 CD8+ T cells for 48 hr ± 12.5 μg/mL CpG oligodeoxynucleotide 1826 (Badovinac et al., 2005), 10 ng/mL IL-12 (R&D Systems Inc., Minneapolis, MN), and/or 100 ng/mL IFN-γ (R&D Systems). For in vivo transfers, ~4 × 10^5 activated P14 CD8+ T cells from cultures stimulated for 24–48 hr with 100 ng/mL peptide-loaded splenocytes ± cytokines were i.v. transferred into naive recipients and analyzed 5–6 days later. Details of DC preparation and P14 T cell stimulations are found in Supplemental Experimental Procedures.

Retroviral Constructs and Transduction

T-bet and GFP-MSCV vectors were obtained from L. Glimcher (Szabo et al., 2000). *shKlrg1*-pSM2c RNAi vector (Open Biosystems, Huntsville, AL) was subcloned into LMP vector via XhoI and EcoRI. Generation of the T-bet:ER MSCV vector has been described previously (Matsuda et al., 2007). Details of the retroviral transduction are found in Supplemental Experimental Procedures.

In Vivo CTL and Homeostatic Turnover Assay

Details of these procedures are found in Supplemental Experimental Procedures.

Gene-Expression, Real-Time PCR, and Immunoblotting Analyses

Gene-expression analysis on IL-7R^{hi} and IL-7R^{lo} P14 effector CD8+ T cells was performed with Affymetrix Mouse 430 2.0 Array chips (Affymetrix, Santa Clara, CA). Details for the cell isolation, RNA preparation, gene-expression analysis, and immunoblotting are found in Supplemental Experimental Procedures.

Statistical Analyses

Where indicated, p values were determined by a two-tailed unpaired Student's t test. p values < 0.05 were considered significant. All graphs show averages of the mean ± SEM.

Supplemental Data

Eight figures and Experimental Procedures are available at http://www.immunity.com/cgi/content/full/27/2/281/DC1/.

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Accession Numbers

The gene-expression data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE8678.