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Manipulating the Rate of Memory CD8+ T Cell Generation after Acute Infection

Vladimir P. Badovinac* and John T. Harty2*†

Infection with Listeria monocytogenes elicits expansion in numbers of Ag-specific CD8+ T cells, which then undergo programmed contraction. The remaining cells undergo further phenotypic and functional changes with time, eventually attaining the qualities of memory CD8+ T cells. In this study, we show that L. monocytogenes-specific CD8+ T cell populations primed in antibiotic-pretreated mice undergo brief effector phase, but rapidly develop phenotypic (CD127low, CD43high) and functional (granzyme Blow, IL-2-producing) characteristics of memory CD8+ T cells. These early memory CD8+ T cells were capable of substantial secondary expansion in response to booster challenge at day 7 postinfection, resulting in significantly elevated numbers of secondary effector and memory CD8+ T cells and enhanced protective immunity compared with control-infected mice. Although early expansion in numbers is similar after L. monocytogenes infection of antibiotic-pretreated and control mice, the absence of sustained proliferation coupled with decreased killer cell lectin-like receptor G-1 up-regulation on responding CD8+ T cells may explain the rapid effector to memory CD8+ T cell transition. In addition, antibiotic treatment 2 days post-L. monocytogenes challenge accelerated the generation of CD8+ T cells with memory phenotype and function, and this accelerated memory generation was reversed in the presence of CpG-induced inflammation. Together, these data show that the rate at which Ag-specific CD8+ T cell populations acquire memory characteristics after infection is not fixed, but rather can be manipulated by limiting inflammation that will in turn modulate the timing and extent to which CD8+ T cells proliferate and up-regulate killer cell lectin-like receptor G-1 expression. The Journal of Immunology, 2007, 179: 53–63.

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fter infection or immunization with strong adjuvant, Ag-specific CD8+ T cells undergo expansion in numbers, followed by contraction, eventually generating a memory pool of Ag-specific cells that can remain stable for life (1–5). Under these circumstances, the acquisition of memory characteristics by populations of Ag-specific CD8+ T cells, including the ability to respond vigorously to booster (secondary) immunization and generate substantially higher numbers of secondary memory T cells, takes ~40 days (6).

Interestingly, a small fraction of Ag-specific CD8+ T cells at the peak of the expansion phase possesses some of the phenotypic properties of long-term memory cells and may be the precursors to CD8+ T cell memory (1). In partial support of this notion, high-dose booster immunization at the peak of the primary response elicited secondary expansion of Ag-specific CD8+ T cells after some (7), but not all types of infections (6, 8). However, booster immunizations at the peak of the expansion phase stimulated only modest secondary expansion and a relatively small increase in secondary memory CD8+ T cell numbers compared with booster immunizations at 40 days or later postprimary infection. In the case of Listeria monocytogenes infection, it has been suggested that the proliferation of the small fraction of early memory CD8+ T cells might be diminished by the large effector CD8+ T cell pool that rapidly clears the infection (Ag) upon secondary challenge (7). Therefore, some Ag-specific CD8+ T cells may already possess memory-like characteristics at the peak of primary expansion; however, the phenotype and function of those cells are masked by the large effector CD8+ T cell pool of the same specificity. In summary, efficient boosting of primary memory responses requires a relatively long interval between immunizations, suggesting that Ag-specific CD8+ T cell populations may acquire memory characteristics at a slow and potentially fixed rate.

Immunization of mice with LPS-matured, peptide-coated bone marrow-derived dendritic cells (DC) stimulates similar expansion in numbers of Ag-specific CD8+ T cells as L. monocytogenes infection (8, 9). However, in striking contrast to infection, most peptide-DC-prime plus CD8+ T cells acquire the large effector CD8+ T cell pool of the same specificity. In this study, these early memory cells are able to respond vigorously to even modest booster immunization as early as 4 days after the initial immunization. Confection with L. monocytogenes or administration of CpG oligodeoxynucleotides (ODN) to induce inflammation prevented the early development of memory characteristics after DC immunization in wild-type, but not IFN-γRII–/– CD8+ T cells, without compromising the magnitude or kinetics of the CD8+ T cell response. These data suggested that, at least after DC immunization, the rate at which Ag-specific CD8+ T cell populations acquire memory characteristics can be accelerated, and that inflammation, acting on the responding T cells, may control the rate of memory CD8+ T cell differentiation (8).
Whether manipulating inflammation after infection or immunizations with strong adjuvant will also accelerate memory CD8⁺ T cell development remains an important question. For example, decreasing the interval between initial priming and booster immunization has the potential to enhance vaccine efficacy, particularly in situations in which time is of the essence, such as immunotherapy of cancer, in response to bioterrorism, or pandemic infection (10).

In this regard, DC immunization is currently under evaluation in the clinic; however, the need to obtain and propagate DC from each patient limits this approach.

Previously, we showed that *L. monocytogenes* infection of mice that were pretreated with antibiotics resulted in reduced expansion of Ag-specific CD8 T cells, but the generation of long-term memory CD8 T cells without contraction (11). In this study, we show that CD8⁺ T cells primed in the globally reduced inflammatory environment observed after *L. monocytogenes* infection of antibiotic-treated mice rapidly acquire memory phenotype (CD127<sup>high</sup>, CD27<sup>high</sup>, CD43<sup>low</sup>) and function (granzyme B<sup>low</sup>, IL-2 producing), including the ability to vigorously respond to booster (secondary) immunization. The magnitude of early expansion (proliferation) and naive to effector transition of CD8⁺ T cells primed in the low inflammation environment in antibiotic pretreated was similar to that observed in control-infected mice. Importantly, the sustained proliferation and up-regulation of killer cell lectin-like receptor G-1 (KLRG-1) expression on CD8⁺ T cells responding to unmanipulated infection were not observed in antibiotic-pretreated mice, suggesting that these factors might influence the rate of effector to memory CD8⁺ T cell transition. Finally, antibiotic treatment at 48 h after infection, which results in early clearance of infection and reduces the duration of inflammation, also accelerates generation of memory CD8⁺ T cells, without influencing the overall kinetics or magnitude of the CD8⁺ T cell response. Together, these data suggest that manipulations that limit inflammation, although allowing effective APC generation, can speed up the development of CD8⁺ T cells with memory phenotype and function after infection.

Materials and Methods

**Mice, bacteria, antibiotic, CpG, and BrdU treatment**

BALB/c mice and C57BL/6 mice (Thy1.2<sup>+</sup>; H-2<sup>d</sup> and H-2<sup>b</sup>, respectively) were obtained from the National Cancer Institute. OT-I TCR-transgenic (Tg) (Thy1.1<sup>+</sup>, H-2<sup>b</sup>) mice were previously described (12). Pathogen-infected mice were housed in the appropriate biosafety conditions. All mice were used at 8–12 wk of age. All animal experimental protocols followed approved Institutional Animal Care and Use Committee protocols.

The virulent *L. monocytogenes* strains 10403s (vir LM) and XFL303 (vir LM-nucleoproteins [NPs]) (13) and attenuated *L. monocytogenes* strains DP-L1942 and OVA257 expressing (Att LM-OVA) (which are responding OT-I cells. The staining procedure was according to the manufacturer’s protocol.

**Results**

**Absence of CD8⁺ T cell contraction and decreased inflammation early after *L. monocytogenes* infection of antibiotic-pretreated mice**

Previously, we showed that infection of Amp-pretreated mice with a high dose of attenuated *L. monocytogenes* resulted in development of CD8⁺ T cell memory in the absence of contraction (11). The absence of contraction correlated with decreased IFN-γ production early after infection in Amp-pretreated mice and resulted in a higher frequency of CD127⁺CD27<sup>high</sup>, CD43<sup>low</sup> (as detected by 1B11 mAb) and function (the magnitude of early expansion (proliferation) and naive to effector transition of CD8⁺ T cells primed in the low inflammation environment in antibiotic pretreated was similar to that observed in control-infected mice. Importantly, the sustained proliferation and up-regulation of killer cell lectin-like receptor G-1 (KLRG-1) expression on CD8⁺ T cells responding to unmanipulated infection were not observed in antibiotic-pretreated mice, suggesting that these factors might influence the rate of effector to memory CD8⁺ T cell transition. Finally, antibiotic treatment at 48 h after infection, which results in early clearance of infection and reduces the duration of inflammation, also accelerates generation of memory CD8⁺ T cells, without influencing the overall kinetics or magnitude of the CD8⁺ T cell response. Together, these data suggest that manipulations that limit inflammation, although allowing effective APC generation, can speed up the development of CD8⁺ T cells with memory phenotype and function after infection.

**Abs and peptides**

The following mAbs were used: anti-IFN-γ (clone XMG1.2; eBioscience), anti-CD8⁺ (clone 53-6.7; BD Pharmingen), anti-Thy-1.2 (clone 53-2.2; BD Pharmingen), anti-Thy-1.1 (clone OX-7; BD Pharmingen), anti-TNF (clone MP9-XT22; eBioscience), anti-CD127 (IL-7Ra; clone AT7K3; eBioscience), anti-CD27 (clone LG7P9; eBioscience), anti-KLRG-1 (clone 2F1; eBioscience), anti-CD43 (PE conjugated; clone 1B11; BD Pharmingen), anti-IL-2 (clone JES6-5H4; BD Pharmingen), anti-perforin (clone eBioMAK-D; eBioscience), anti-granzyme B (Caltag Laboratories), rat IgG2a and IgG2b isotype controls (clones eBR2a, keyhole limpet hemocyanin/G2b-1-2, respectively; eBioscience), and mouse IgG1 (Caltag Laboratories). Defined *L. monocytogenes* listeriolysin O (LLO)91–99 and p60217–225 H²L-restricted, lymphocytic choriomeningitis virus (LCMV) NP118–126 H²L<sup>+</sup>-restricted, and OVA257–264 epitopes were used, as previously described (13, 15, 17).

**Quantification of Ag-specific CD8⁺ T cell response and cytokine/chemokine determination**

The magnitude of the epitope-specific CD8⁺ T cell response was determined by intracellular cytokine (IFN-γ) staining, as described (18). The percentage of IFN-γ⁺CD8⁺ T cells in unstimulated samples from each group was subtracted from the peptide-stimulated value for determination of the percentage of Ag-specific CD8⁺ T cells. The total number of epitope-specific CD8⁺ T cells per spleen was calculated from the percentage of IFN-γ⁺CD8⁺ T cells, the percentage of CD8⁺ in each sample, and the total number of cells per spleen. The relative concentrations of various cytokines and chemokines were determined in the serum using Bio-Plex Mouse Cytokine 18-plex panel (Bio-Rad), according to the manufacturer’s recommendation.

**Materials and Methods**

**Mice, bacteria, antibiotic, CpG, and BrdU treatment**

BALB/c mice and C57BL/6 mice (Thy1.2<sup>+</sup>; H-2<sup>d</sup> and H-2<sup>b</sup>, respectively) were obtained from the National Cancer Institute. OT-I TCR-transgenic (Tg) (Thy1.1<sup>+</sup>, H-2<sup>b</sup>) mice were previously described (12). Pathogen-infected mice were housed in the appropriate biosafety conditions. All mice were used at 8–12 wk of age. All animal experimental protocols followed approved Institutional Animal Care and Use Committee protocols.

**Adoptive transfer of OT-I**

Naïve OT-I Thy1.1<sup>+</sup> cells (~3000 OT-1/recipient) were obtained from the blood of previously screened TCR-Tg donors and transferred into naïve C57BL/6 Thy1.2<sup>+</sup> mice 1 day before immunization with attenuated *L. monocytogenes* strain expressing OVA257–264 epitope (Att LM-OVA).
ability to make IL-2 after in vitro stimulation) (8). Interestingly, Ag-specific CD8\(^+\) T cells in Amp-pretreated mice at day 7 postinfection also express high levels of CD127 (11). We next asked whether \textit{L. monocytogenes} infection of Amp-pretreated mice (another low inflammatory environment) would also result in early acquisition of memory phenotype. Strikingly, the majority of LLO91–99-specific CD8\(^+\) T cells at day 7 in Amp-pretreated mice exhibited memory phenotype (CD127 high, CD27 high, CD43 low) and produced IL-2 after in vitro peptide stimulation (Fig. 2). This contrasts sharply with the LLO91–99-specific CD8\(^+\) T cells at day 7 in control-infected mice in which most of the cells exhibit a characteristic effector phenotype (CD127 low, CD27 int, CD43 high) and fail to produce IL-2 after stimulation.

However, both effector and memory CD8\(^+\) T cell populations are able to rapidly kill Ag-expressing targets in vivo despite different levels of expression of cytotoxic (granzyme B and perforin) molecules (21–24). The levels of steady-state granzyme B and perforin expression are higher on effector cells compared with long-term memory CD8\(^+\) T cells (21–24). Interestingly, LLO91–99-specific CD8\(^+\) T cells at day 7 in Amp-pretreated mice exhibited decreased levels of granzyme B and perforin expression compared with Ag-specific CD8\(^+\) T cells in control-infected mice (Fig. 3), suggesting that expression of cytotoxic molecules also correlates with their early CD8\(^+\) T cell memory phenotype. Together, these data reveal that, similar to DC immunization, the reduced inflammation observed in Amp-pretreated mice after \textit{L. monocytogenes} infection correlates with the early acquisition of memory phenotype and function by Ag-specific CD8\(^+\) T cells.

**Vigorous secondary expansion and increased memory CD8\(^+\) T cell numbers after early \textit{L. monocytogenes} boost**

An important characteristic of memory CD8\(^+\) T cells is the ability to undergo vigorous proliferation in response to secondary Ag stimulation. Consistent with this notion, early (4–6 days postimmunization) memory CD8\(^+\) T cells stimulated by DC vaccination can undergo vigorous proliferative expansion (>25-fold) in response to booster immunizations, rapidly generating large numbers of Ag-specific effector CD8\(^+\) T cells and resulting in elevated, stable memory cell numbers that are much higher than observed in \textit{L. monocytogenes}-infected mice given the same booster immunization (8). To address whether the memory phenotype CD8\(^+\) T cells at day 7 after \textit{L. monocytogenes} infection of Amp-pretreated mice acquired secondary expansion ability, we infected control- and Amp-pretreated mice, and boosted these mice at day 7 with \(10^4\) of a virulent \textit{L. monocytogenes} strain that has been engineered to express the LCMV NP118–126 epitope in addition to the endogenous \textit{L. monocytogenes} epitopes (13) (Fig. 4A). Analysis of the CD8 T cell response to the newly introduced NP118–126 epitope provides a control for any potential impact of differential clearance of the booster infection in the control- and Amp-pretreated hosts. As previously observed, LLO91–99-specific CD8\(^+\) T cells were present at high frequency (Fig. 4B) and total numbers (Fig. 4C) at day 7 in control-infected mice, and the number of these cells was \(~10\)-fold reduced in Amp-pretreated mice. Booster infection at day 7 after \textit{L. monocytogenes} infection did not increase the number of LLO91–99-specific CD8\(^+\) T cells in control-infected mice, which underwent contraction to memory
numbers representing 5% of those present at the peak of expansion. In contrast, booster infection at day 7 after the initial L. monocytogenes infection of Amp-pretreated mice caused a vigorous expansion (>40-fold) in LLO91–99-specific CD8⁺ T cells and eventually generated numbers of LLO91–99-specific memory CD8⁺ T cells that were 10-fold higher than observed in control-infected mice. Therefore, early memory phenotype Ag-specific CD8⁺ T cells primed in Amp-pretreated mice, but not the population of effector CD8⁺ T cells in control-infected mice, were able to vigorously respond to secondary (booster) immunization.

Amp-pretreated mice exhibited a slight delay in complete clearance of the booster L. monocytogenes infection compared with control-infected mice (Fig. 4D). However, this is unlikely to account for the extreme difference in CD8 T cell expansion in these groups because this modestly prolonged infection in the Amp-pretreated mice did not result in increased numbers of NP118–126-specific CD8⁺ T cells compared with control-infected mice (Fig. 4B). Thus, the slightly prolonged infection was unlikely to underlie the enhanced secondary response of LLO91–99-specific CD8⁺ T cells in the Amp-pretreated mice. Taken together, these data reveal that L. monocytogenes infection of Amp-pretreated mice generates populations of Ag-specific CD8⁺ T cells with memory phenotype and function, including the ability to respond to low-dose booster immunization within 1 wk after priming. This is in contrast to populations of Ag-specific CD8⁺ T cells in control-infected mice that exhibited a effector phenotype and substantially less proliferation in response to relatively low-dose booster immunizations.

**Increased memory CD8⁺ T cell numbers in antibiotic-pretreated prime-boosted mice provide increased protection from challenge infection**

The degree of immunological protection against L. monocytogenes challenge directly correlates with the number of memory CD8⁺ T cells (17). To assess the impact of increased memory CD8⁺ T cell numbers on immune protection, and formally validate the idea that accelerated memory CD8⁺ T cell generation followed by early booster immunization could benefit the host, all groups of mice described in Fig. 3 were challenged after 2 mo with a high dose (5 × 10⁷/mouse; ~50 LD₅₀) of virulent L. monocytogenes. Three days later, the bacterial burden was determined in the spleens and livers of those mice as well as in naive control mice. All of the naive mice succumbed to the high-dose L. monocytogenes challenge by day 3 postinfection (Fig. 5). Control mice that received either the prime-boost or booster immunization alone survived the challenge infection. These groups had similar numbers of memory cells (Fig. 4C) and also exhibited similar reduction in bacterial numbers (Fig. 5) compared with the high level of infection observed in naive mice. In contrast, the 10-fold higher numbers of LLO91–99-specific memory CD8⁺ T cells in Amp-pretreated and boosted mice (Fig. 4C) provided substantially better protection with undetectable levels of bacteria in the spleen (limit of detection was ~80 CFUs) and 100- to 500-fold less bacteria in the liver compared with nonantibiotic-treated immune mice. Thus, the early generation of memory-like CD8⁺ T cells in Amp-pretreated mice enabled the rapid increase in immunity after booster immunization.
Decreased proliferation during the late expansion phase of CD8\(^+\) T cell responses correlates with early acquisition of memory characteristics

Our previous studies suggested that inflammation and not the degree of proliferation influenced the rate of memory CD8\(^+\) T cell development after peptide-DC immunization (8). Infection of mice pretreated with antibiotics creates a situation in which both inflammation and duration of infection are minimized during the initial events of CD8\(^+\) T cell activation (Fig. 1) (11). As a consequence, the magnitude of CD8\(^+\) T cell expansion is substantially decreased compared with the expansion in infected control mice (Fig. 1C) (11). To determine whether the extent to which Ag-specific CD8\(^+\) T cells divide (proliferate) after infection influences the acquisition of memory CD8\(^+\) T cell characteristics, we adoptively transferred low numbers of Thy1.1/Tg cells (3000/mouse; OT-I (12)) into naive C57BL/6 Thy1.2 mice at the time of Amp pretreatment and before L. monocytogenes -OVA (Att LM-OVA) infection (Fig. 6A).

Decreased proliferation during the late expansion phase of CD8\(^+\) T cell responses correlates with early acquisition of memory characteristics

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Due to the low numbers of TCR-Tg cells transferred, the initial seeding and early proliferation of OT-I T cells were not detectable until day 4 after infection. Interestingly, similar expansion in numbers of OT-I T cells was observed at day 4 in
both control- and Amp-pretreated groups (ratio without Amp: with Amp/11011/H11011 1), suggesting that antibiotic pretreatment did not affect early expansion of Ag-specific CD8+/H11001 T cells (Fig. 6, B and C). By day 5 postinfection, OT-I T cells from both groups of mice had further expanded in numbers, although the total number of OT-I T cells in the spleen of control-infected mice was 2-fold higher than in the Amp-pretreated group (Fig. 6, B and C). Importantly, OT-I T cells primed in the Amp-pretreated mice did not further increase in numbers from day 5 to day 7, whereas substantial expansion in numbers was observed in OT-I T cells from representative mice. Numbers represent the percentage of cells positive for the granzyme B expression. B. Percentage of granzyme B-positive OT-I cells. Data are presented as mean ± SD of three mice per group.

FIGURE 6. The expansion of OT-I TCR-Tg CD8+ T cells in antibiotic-pretreated mice after L. monocytogenes infection. A. Experimental design. Amp-pretreated (W/Amp) and nontreated (W/o Amp) C57BL/6 (Thy1.2) mice that received purified naive OT-I (-3 × 10^3, Thy1.1) TCR-Tg CD8+ T cells were infected on day 0 with 5 × 10^6 of Att LM-expressing OVA257–264 (Att LM-OVA). B. Frequencies of Thy1.1+ cells in the spleens from representative mice at the indicated days postimmunization. C. Total number (mean ± SD of three mice per group) of Thy1.1+/CD8+ T cells in the spleen after Att LM-OVA challenge. D. Fold increase in numbers of Thy1.1+/CD8+ T cells is calculated by dividing total numbers of cells detected on day 5 and/or day 7 with numbers observed at day 4 postinfection.

FIGURE 7. Decreased proliferation of OT-I CD8+ T during the expansion phase in antibiotic-pretreated mice after L. monocytogenes infection. A. Experimental design. Amp-pretreated (W/Amp) and nontreated (W/o Amp) C57BL/6 (Thy1.2) mice that received purified naive OT-I (-3 × 10^3, Thy1.1) TCR-Tg CD8+ T cells were infected on day 0 with 5 × 10^6 of Att LM-OVA and treated with BrdU (2 mg/mouse i.p.) on days 5 and 6 postchallenge. B. BrdU staining of Thy1.1+/CD8+ T cells in the spleen at day 7 postinfection. Shaded histogram represents the isotype control staining; thick black line represents staining with anti-BrdU mAb of gated Thy1.1+/CD8+ T cells from three mice. Numbers represent the percentage of cells positive for the indicated molecules. C. Numbers (mean ± SD for three mice) of OT-I T cells presented as total numbers of Thy1.1+/CD8+ T cells detected in the spleen (total) or as total numbers of BrdU+ OT-I cells (BrdU pos) at day 7 postchallenge.

FIGURE 8. Amp pretreatment accelerates the transition of OT-I T cells from an effector to early memory phenotype. A. Granzyme B staining of Thy1.1+/CD8+ OT-I T cells at indicated days post-Att LM-OVA infection. Shaded histogram represents the isotype control staining; thick black line represents staining with anti-granzyme B mAb of gated OT-I T cells from representative mice. Numbers represent the percentage of cells positive for the granzyme B expression. B. Percentage of granzyme B-positive OT-I cells. Data are presented as mean ± SD of three mice per group.
rapid acquisition of CD8\(^+\) T cell memory phenotype and function.

The decreased accumulation of OT-I T cells from day 5 to day 7 observed in Amp pretreated might be due to decreased proliferation or due to increased death of Ag-specific CD8\(^+\) T cells. To distinguish between these possibilities, we repeated the OT-I T cell transfer experiment, but pulsed the mice with BrdU (2 mg/mouse i.p.) on days 5 and 6 postinfection and determined BrdU incorporation at day 7 (Fig. 7A). In this study, the reduced expansion of OT-I T cells observed in Amp-pretreated mice correlated with substantially less BrdU incorporation than observed in OT-I T cells in control mice (Fig. 7, B and C). Taken together, these results suggest that the duration of sustained proliferation during the expansion phase of the Ag-specific CD8\(^+\) T cell responses to infection might be an important variable in controlling the rate of memory CD8\(^+\) T cell generation.

Rapid effector to memory transition of Ag-specific CD8\(^+\) T cells primed in low inflammation environment

*L. monocytogenes* infection of Amp-pretreated mice could either directly generate cells with memory CD8\(^+\) T cell phenotype and function or accelerate the transition from effector to memory cells. To address these possibilities, low numbers (~3000/mouse) of naive OT-I T cells were transferred into naive Thy1.2, and these cells were analyzed for granzyme B expression at various days (starting at day 4) after infection with Att LM-OVA. Naive T cells do not express granzyme B (data not shown); however, granzyme B expression is substantially up-regulated in effector CD8\(^+\) T cells, although the levels of expression are substantially reduced in long-term

### FIGURE 9. Decreased expression of KLRG-1 on OT-I T cells in Amp-pretreated mice after *L. monocytogenes* infection. A. All of the mice (Thy1.2) received ~3 \(\times\) 10\(^3\) naive OT-I (Thy1.1), and one-half of the mice were treated with Amp (W/Amp group) 48 h before Att LM-OVA infection. Percentage of OT-I T cells from representative mice that were positive for KLRG-1 at indicated days postinfection. B. Percentage of KLRG-1-positive OT-I cells. Data are presented as mean \(\pm\) SD of three mice per group.

### FIGURE 10. Accelerated memory CD8\(^+\) T cell generation in mice treated with Amp after *L. monocytogenes* infection. A. BALB/c mice were infected on day 0 with 1 \(\times\) 10\(^3\) (0.1 LD\(_{50}\)) of virulent *L. monocytogenes* (vir LM), and some mice received Amp 48 h postinfection (W/Amp group). All groups were rechallenged on day 14 with 1 \(\times\) 10\(^4\) (1.0 LD\(_{50}\)) of vir LM. B. Phenotypic (CD127 and CD27\(^{high}\)) and functional (TNF and IL-2) status of IFN-\(\gamma\)/CD8\(^+\) LLO91–99-specific CD8\(^+\) T cells at days 7 and 14 postprimary infection. Numbers represent the percentage of cells positive for the indicated molecules. Data are presented as mean \(\pm\) SD of three mice per group. C. Frequencies of LLO91–99-specific CD8\(^+\) T cells from representative mice at the indicated days postinfection. Numbers represent the percentage of IFN-\(\gamma\)/CD8\(^+\) T cells in the presence (top) or absence (bottom) of peptide stimulation. D. Total number (mean \(\pm\) SD of three mice per group) of LLO91–99-specific CD8\(^+\) T cells in the spleen after primary and secondary *L. monocytogenes* infections.
memory CD8^+ T cells (24). As observed previously for the endogenous CD8^+ T cell responses (Fig. 3), the majority of OT-I T cells primed in the Amp-pretreated group did not express detectable levels of granzyme B at day 7 postinfection (Fig. 8). In contrast, OT-I T cells in both control- and Amp-pretreated groups showed an effector phenotype characterized with elevated granzyme B expression at day 4 post- *L. monocytogenes* infection (Fig. 8). Therefore, Amp pretreatment did not directly generate memory phenotype CD8^+ T cells, but instead substantially accelerated the transition from effector cells into cells with memory phenotype and function.

**KLRG-1 is not expressed on Ag-specific CD8^+ T cells primed in Amp-pretreated mice**

Recently, it has been suggested that effector CD8^+ T cells express high levels of inhibitory KLRG-1, and low levels of IL-7Ra (CD127) represent apoptosis-susceptible and terminally differentiated or senescent effector cells (19). Although the frequency of KLRG-1^- Ag-specific CD8^+ T cells (25) and the levels of KLRG-1 expression (6) decrease after the completion of the contraction phase, it has been shown that both effector and memory KLRG-1-positive TCR-Tg cells could efficiently lyse Ag-expressing targets and secrete cytokines, but were severely impaired in their ability to proliferate after Ag restimulation (26). Because Ag-specific CD8^+ T cells primed in the Amp-pretreated mice proliferate less and are able to vigorously respond to secondary challenge early after primary immunization, we next asked whether priming in low inflammation environment modulates KLRG-1 expression on responding CD8^+ T cells after *L. monocytogenes* infection. OT-I T cells do not up-regulate KLRG-1 expression by day 4 postinfection in either control- or Amp-pretreated group (Fig. 9A). KLRG-1 up-regulation was observed on responding OT-I T cells between day 4 and 7 postinfection of control mice, during the sustained proliferative response (Figs. 6C and 9, A and B). In contrast, Ag-specific CD8^+ T cells primed in Amp-pretreated mice remained negative for KLRG-1 expression during expansion (Fig. 9). Similar results were obtained for endogenous LLO91–99-specific CD8^+ T cell responses in control- and Amp-pretreated mice (data not shown). Thus, CD8^+ T cells primed in an environment of globally decreased inflammation proliferated less and failed to up-regulate KLRG-1 expression, and that, in turn, might facilitate their ability to acquire phenotypic and functional characteristics of memory CD8^+ T cells.
Accelerated memory CD8⁺ T cell generation in mice treated with antibiotic after L. monocytogenes infection

Infection of Amp-pretreated mice creates an environment in which inflammation is minimized throughout the initial events of T cell activation. We were also interested in whether truncating the course of infection or inflammation could accelerate the acquisition of memory characteristics in Ag-specific CD8⁺ T cells generated when the early events of infection are highly inflammatory. To address this, mice were infected with 0.1 LD₅₀ of virulent L. monocytogenes, and some mice received Amp treatment to curtail infection beginning 48 h postinfection (Fig. 10A). Importantly, this protocol results in CD8⁺ T cell responses in control- and Amp-treated mice that exhibit similar magnitudes at the peak of the response as well as minimally altered onset of contraction and memory set point (16, 17). LLO91–99-specific CD8⁺ T cells at day 7 in control and 48-h Amp-treated mice exhibited primarily an effector phenotype (Fig. 10B) (8) and failed to proliferate in response to booster immunization (8, 27). However, by day 14 after infection, LLO91–99-specific CD8⁺ T cells in 48-h Amp-treated mice displayed increased CD127 and CD27 expression as well as an increased fraction of Ag-specific cells that produce IL-2 after in vitro restimulation compared with the virtually identical frequency and total number of LLO91–99-specific CD8⁺ T cells (Fig. 10, C and D) in the control group. Consistent with this memory-like phenotype, the LLO91–99-specific CD8⁺ T cells at day 14 in 48-h Amp-treated mice underwent more vigorous secondary expansion (10-fold) after booster infection compared with the same starting number of Ag-specific CD8⁺ T cells in control-infected mice, which underwent a 3-fold expansion in total numbers in response to the same booster challenge (Fig. 10, C and D). These data demonstrate that truncating the duration of an existing L. monocytogenes infection with antibiotic treatments also accelerates the acquisition of memory characteristics and the ability to respond to booster immunization by Ag-specific CD8⁺ T cells.

Accelerated memory CD8⁺ T cell generation after antibiotic treatment of L. monocytogenes-infected mice is controlled by duration of inflammation

Previous results with DC immunization suggested that inflammation, and not the duration of Ag presentation, controlled the rate of CD8⁺ T cell memory development (8). Treatment of mice with antibiotics at 48 h after L. monocytogenes infection truncates both the infection and the associated inflammation compared with control mice (data not shown). To determine which of these parameters regulates the acquisition of memory characteristics by CD8⁺ T cells after L. monocytogenes infection, we challenged three groups of mice with actA-deficient L. monocytogenes and treated two of these groups with Amp at 48 h postinfection. One day later, one Amp-treated group received a single injection of CpG ODN to induce inflammation (Fig. 11A) (8, 11, 28, 29). At day 14 after infection, LLO91–99-specific CD8⁺ T cells in 48-h Amp-treated mice had increased expression of memory markers (CD127, CD27, and IL-2) compared with CD8⁺ T cells in control-infected mice (Fig. 11, B and C). However, CpG ODN injection prevented the early acquisition of memory phenotype in the Amp-treated group (Fig. 6, B and C) and, importantly, prevented the ability of LLO91–99-specific CD8⁺ T cells to respond vigorously to booster immunization at day 14 (Fig. 11, D and E). Similar results were obtained for CD8⁺ T cells specific for an additional L. monocytogenes-derived p60217–225 epitope (Fig. 11D and data not shown). These data suggest that the duration of inflammation, not infection, controls the rate at which CD8⁺ T cells acquire phenotypic and functional characteristics of memory cells after pathogen challenge.

Discussion

Due to their functional characteristics, memory CD8⁺ T cells provide protection and are capable of substantial expansion in numbers, leading to higher numbers of secondary memory cells, in response to booster immunization or secondary challenge. Because the level of immunological protection correlates with the number of memory CD8⁺ T cells at the time of rechallenge, increasing the quantity and quality of those cells should represent the goals of memory CD8⁺ T cell manipulation (1, 30–34). Recently, it has been shown that brief contact with Ag (24–48 h postinfection) is sufficient for naive CD8⁺ T cell to enter the developmental program that will ultimately generate CD8⁺ T cells with memory characteristics (17, 35–38). These data suggest that, at least with respect to Ag, the CD8⁺ T cell response is programmed by early events after infection. This concept allows the dissociation of early activation events (Ag dependent) and later proliferation and differentiation to memory events (Ag independent). However, it is also becoming clear that CD8 T cells must integrate a variety of signals that determine their eventual fate. In this regard, recent studies with DC immunization suggest that the rate at which CD8⁺ T cells acquire memory characteristics (including ability to vigorously proliferate in response to low-dose booster challenge) is not fixed, but depends on inflammatory signals received by the responding T cells (8). Although DC immunization may be somewhat unique, these results suggested the possibility that manipulating inflammatory responses after infection could also affect the development of T cell memory. Consistent with this notion, we show that antibiotic treatment can be used at various times pre- and post-L. monocytogenes infection to limit inflammation and accelerate the generation of populations of Ag-specific CD8⁺ T cells with memory characteristics.

Strong inflammatory responses after L. monocytogenes infection are beneficial in clearing the pathogen and, at the same time, promote the development of strong effector CD8⁺ T cell responses. Those effector Ag-specific CD8⁺ T cells, in turn, contribute to pathogen elimination (39), and after contraction undergo a relatively slow differentiation (on the population level) to acquire memory phenotype and function. The precursors to memory cells are most likely present at the peak of the expansion phase (7). In addition, high-dose booster immunization delivered at the peak of primary T cell expansion can cause these cells to undergo secondary proliferation, albeit modestly, in some, but not all models of infection (6, 7). However, this early booster immunization strategy does not uniformly lead to increased numbers of secondary memory CD8 T cells as seen when the same booster immunizations are delivered at late time points (>40 days) after priming (6–8). In contrast, Amp pretreatment before L. monocytogenes infection (shown in this study) and peptide-DC immunization (8) involve priming of naive CD8⁺ T cells in an environment of decreased inflammation, and the majority of these T cells rapidly acquire memory phenotype and functional characteristics. In the DC immunization model (8), treatments to induce inflammation, particularly IFN-γ, prevented the rapid generation of memory phenotype CD8 T cells. These data suggest that, in the context of a strong inflammatory response, the host extends the effector phase of the CD8⁺ T cell response to ensure complete clearance of infection. Thus, the rate of naive to effector to memory CD8⁺ T cell progression might be linked to the degree and duration of inflammation induced by infection or vaccination.

However, it should be noted that substantial differences are evident in the CD8⁺ T cell response kinetics between DC immunization and L. monocytogenes infection of Amp-pretreated mice.
Specifically, the CD8⁺ T cell response to DC immunization undergoes normal expansion and contraction in numbers that are unaffected by induced inflammation, suggesting that Ag display is not a variable in this system and that the in vitro TLR signals used to mature the DC are sufficient for these cells to produce the signals necessary for CD8⁺ T cell contraction (8). In contrast, Amp pretreatment causes both reduced inflammation and truncated Ag display. In this case, the CD8⁺ T cell response exhibits reduced expansion in numbers compared with control-infected mice and essentially no contraction unless inflammation is induced (11).

In the current study, we have extended our mechanistic evaluation of the impact of Amp pretreatment on the L. monocytogenes-specific CD8⁺ T cell response to C57BL/6 mice spiked with low numbers of TCR-Tg OT-I cells. These experiments showed clearly that the early CD8⁺ response (up to day 4) is numerically similar in control- and Amp-pretreated mice, and that both populations up-regulate the effector cell marker granzyme B. However, the OT-I (and endogenous) CD8⁺ T cells in Amp-pretreated mice fail to undergo the sustained late proliferation (days 5–7), never up-regulate the late effector marker KLRG-1, and rapidly lose granzyme B expression compared with OT-I T cells responding in control-infected mice. These data suggest that, in addition to inflammation, the number of divisions undertaken by the responding T cell populations may also contribute to the rate at which these cells acquire memory characteristics. Unfortunately, the low numbers of TCR-Tg cells used in our studies preclude direct analysis of cell division by CFSE dilution. However, these low numbers are essential to allow the TCR-Tg T cells to preserve the characteristics of the endogenous response (40–42).

Inflammatory cytokines such as IL-12, type I IFN, and IFN-γ have recently been recognized as important signal 3, required for effector CD8⁺ T cell survival after infection (43–46). These data suggest the possibility that the reduced expansion in CD8⁺ T cell numbers we observe after L. monocytogenes infection of Amp-pretreated mice may be due to the lack of signal 3. However, and in contrast to this notion, CpG treatment to induce inflammation in Amp-pretreated mice does not increase the magnitude of expansion, but does induce contraction of the responding CD8⁺ T cells (11). Thus, the lack of inflammation or signal 3 in the Amp-pretreated mice is not solely the reason that expansion in CD8⁺ numbers is reduced. Together, these data suggest that whereas both proliferation and inflammation may regulate the rate at which cells acquire memory characteristics, inflammation may play a dominant role in regulation of contraction, perhaps by controlling the signals delivered to the responding CD8⁺ T cells by DC during the priming interaction.

Finally, in direct support of the notion that the rate of naive to memory CD8⁺ T cell progression might be linked to the degree and duration of inflammation induced by infection, Amp treatment 2 days postinfection did not change the magnitude of Ag-specific CD8⁺ T cell expansion, contraction, or early memory cell numbers compared with nontreated mice, but, importantly, Ag-specific CD8⁺ T cells in Amp-treated mice showed accelerated acquisition of memory characteristics. Again, the duration of inflammation present early after infection seems to dictate the rate of effector to memory CD8 T cell transition because CpG treatment to induce inflammation in Amp-treated mice prevented rapid development of memory CD8⁺ T cells.

Together, these results demonstrate that the accelerated acquisition of memory characteristics by Ag-specific CD8⁺ T cells is not limited to DC immunization, but also occurred after L. monocytogenes challenge, when the course of infection and the degree of inflammation were modified by antibiotic treatment. How the DC receive maturation signals in this context remains to be determined; however, because the CD8⁺ T cell response to L. monocytogenes is absolutely dependent on DCs (47), the expansion of Ag-specific CD8⁺ T cells is a clear indication that mature, Ag-expressing DC are generated in Amp-pretreated mice. Thus, it appears that the duration of inflammation may ultimately regulate the progression of Ag-specific CD8⁺ T cells from effector to memory. Finally, these experimental models may allow the identification of the relevant inflammatory molecules that regulate the acquisition of memory characteristics, perhaps permitting judicious intervention to improve vaccine efficacy.

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Disclosures
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References


