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J Immunol 2004; 173:6694-6702; doi: 10.4049/jimmunol.173.11.6694
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Shortening the Infectious Period Does Not Alter Expansion of CD8 T Cells but Diminishes Their Capacity to Differentiate into Memory Cells¹

Matthew A. Williams and Michael J. Bevan²

Following a primary immune response, a portion of effector T cells gives rise to long-lived memory cells. Although primary expansion and differentiation of effector CD8 T cells is dictated by a brief exposure to Ag, it is unclear whether full memory differentiation is also programmed within the same short window. By carefully modulating the kinetics of *Listeria monocytogenes* infection, we analyzed the requirements for the programming of effector and memory T cell development in vivo. We find that although limiting the infectious period to the first 24–48 h does not impact the size of the primary CD8 response, the ensuing memory population is significantly diminished. This effect is particularly pronounced in the development of tissue-homing memory cells and is inversely proportional to the initial infectious dose. In contrast to CD8 responses, the differentiation of primary CD4 responses was highly dependent on the continued presence of the infection. Shortening the duration of the infection greatly reduced the development of CD4 effector responses in the spleen and prevented their trafficking to peripheral sites of infection.

We propose that the stimulus received by CD8 T cells during the early stages of infection largely contribute to the differentiation of CD8 effector cells, whereas continued or distinct signals received at later stages influence their ability to differentiate into memory cells. *The Journal of Immunology, 2004, 173: 6694–6702.*

Following acute bacterial or viral infection, Ag-specific CD8 T cells undergo a remarkable phase of expansion and differentiation. Some estimates calculate that naive CTL precursors may expand as much as 10,000-fold after initial priming (1). Large populations of Ag-specific CTL are generated in mice following a variety of acute bacterial and viral infections, including *Listeria monocytogenes* (LM)³ (2), lymphocytic choriomeningitis virus (3, 4), and vaccinia virus (5). Upon resolution of the infection and the disappearance of Ag, 90–95% of these Ag-specific cells die, leaving behind a smaller population of memory cells. These cells persist long-term in the absence of further antigenic stimulation and at much higher frequencies than naive precursors. Also, they respond much more quickly to rechallenge, possessing the ability to produce effector cytokines such as IFN-γ and TNF-α after only brief stimulation, as well as rapidly reacquiring high levels of cytolytic activity (6, 7).

Recent studies have found that naive CD8 T cells require only a brief Ag-dependent instructional period before entering an Ag-independent program of proliferation and differentiation. As little as 2–2.5 h of antigenic stimulation in vitro is sufficient to induce multiple rounds of division, and 24 h of stimulation can lead to full differentiation into effector CTL (8–10). In vivo, abrupt termination of LM infection by ampicillin treatment begun as early as 24 h postinfection (p.i.) leads to unimpaired development of effector CTL populations at the peak of the immune response, as well as protective immunity (11). Further studies suggest that the onset and kinetics of contraction are also programmed during the early phases of the immune response (12). Nonetheless, it is clear that after initial stimulation, many factors continue to modulate the eventual fate of CD8 T cells, such as the persistence of Ag during chronic infection (13) or stimulation by extrinsic factors such as IL-2 (14).

During infection, CD4 responses, although not as large as CD8 responses, follow a similar pattern of expansion, differentiation, contraction, and initial establishment of memory (15, 16). Following in vitro stimulation, CD4 cells can also undergo a brief Ag-independent proliferative program (17). However, compared with CD8 T cells, optimal survival and acquisition of effector function appear to require longer Ag stimulation periods, as well as cell-extrinsic growth factors such as IL-2 (18–20). To date, little has been done in vivo to study the length of stimulation required for the optimal expansion and differentiation of pathogen-specific CD4 effector and memory populations during acute infection.

It has been proposed that the selection of memory cells during contraction is a process of competition for limiting growth and survival factors following resolution of the infection (21). In this scenario, the instructional program for effector cells and memory cells would be identical, and selection of memory cells would take place after the peak of the response. Although such competition undoubtedly plays a role in the outgrowth of memory populations, recent studies have shown that memory CD8 T cell precursors are already present at the peak of the response (22, 23), suggesting that the programming of effector and memory differentiation potential early during infection are at least partially distinct processes. In the present report, we sought to assess the requirements for programming effector and memory T cell populations by modulating the kinetics and dose of LM infection. We find that the programming of effector CD8 T cell expansion and differentiation precedes the
acquisition of central memory development potential, which in turn precedes the acquisition of tissue or effector memory development potential. The pace at which memory potential is programmed is at least in part sensitive to the initial infectious dose. We further find that the stimulation of CD4 effector and memory responses is more tightly associated with the initial duration of infection, implying that the full development of Th1 responses following infection depends on repeated or extended exposure to Ag- and/or pathogen-induced stimuli.

Materials and Methods

Mice

Six-week-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions in the animal facilities at the University of Washington (Seattle, WA). OT-1 TCR transgenic mice congenic for Thy1.1 were bred and maintained in the same facilities. Mice were infected at 8–12 wk of age.

Bacterial infections and ampicillin treatments

An erythromycin-resistant recombinant strain of L. monocytogenes that expresses a secreted form of OVA (LM-OVA) was given to us by H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA). LM-OVA was grown in brain-heart infusion (BHI) broth supplemented with 5 mg/ml erythromycin. At mid-log growth phase, culture samples were measured by OD and diluted in PBS for tail vein injection into animals. Bacterial counts were further verified by plating culture dilutions on BHI agar plates and incubating overnight. For primary infections, mice were infected i.v. with 3 × 10^7 CFU LM-OVA unless otherwise indicated. For secondary infections, mice received 1 × 10^5 CFU LM-OVA and were sacrificed 3 days later. Bacterial counts in the spleen and liver were determined as described (3). Spleen single cell suspensions were obtained from perfused livers as previously described (24). Cells were resuspended in FACS staining buffer (PBS containing 0.1% sodium azide) and stained on ice for 20 min with the following cell surface Abs: CD8-FITC, CD4-FITC, CD44-PE, CD62L-PE (all from BD Pharmingen, San Diego, CA). Tetramer staining was performed following cell surface Abs: CD4-FITC or CD8-FITC, as appropriate, and permeabilized and stained for intracellular cytokine expression using reagents provided in the Cytofix/Cytoperm kit according to the manufacturer’s instructions (BD Pharmingen). The Abs used were IFN-γ, allophycocyanin and IL-2-PE (BD Pharmingen), and cells were subsequently analyzed by flow cytometry.

Flow cytometry

H-2Kb tetramers bound to the OVA-derived peptide SIINFEKL were generated as described (3). Spleen single cell suspensions were obtained by grinding the tissue and lysing RBC in a hypotonic buffer. Liver-residing lymphocytes were obtained from perfused livers as previously described (24). Cells were resuspended in FACS staining buffer (PBS containing 1% FCS, 2 mML-glutamine, 10 mM HEPES, 0.5 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 96-well plates in the presence of 1 μ/ml brefeldin A (GolgiPlug, Cytofix/Cytoperm kit; BD Pharmingen) with or without the appropriate peptide for 4–5 h at 37°C. OVA257–264 (SIINFEKL, Kβ-restricted) and listeriolysin O (LLO)/0-20 (NEKYAQAYPNVS, 1-Aβ-restricted) were added at concentrations of 0.1 and 1 μg/ml, respectively. Following the incubation, cells were stained with cell surface Abs CD4-FITC or CD8-FITC, as appropriate, and permeabilized and stained for intracellular cytokine expression using reagents provided in the Cytofix/Cytoperm kit according to the manufacturer’s instructions (BD Pharmingen). The Abs used were IFN-γ, allophycocyanin and IL-2-PE (BD Pharmingen), and cells were subsequently analyzed by flow cytometry.

Results

Shortening the infectious period results in normal expansion of CD8 effector cells but diminished memory development

Oral treatment of mice with ampicillin effectively limits LM infection in vivo (11). In our hands, a combination of oral and i.p. ampicillin treatment resulted in the clearance of an LM-OVA within 24 h from the spleen (Fig. 1A). Similar kinetics of bacterial clearance were seen in the liver (data not shown). T cell priming in the spleen was undetectable as soon as 1 day following ampicillin treatment, as CFSE-labeled OT-1 T cells injected at this time point failed to divide significantly after 3 days. In contrast, CFSE-labeled indicator cells injected into untreated animals at the same time point divided six to eight times (Fig. 1B). Therefore, by days 2–3 p.i. in ampicillin-treated mice, T cell differentiation is likely to be independent of both Ag and pathogen-driven inflammatory responses.

We sought to use this system to assess whether early signals required for the programming of effector CD8 T cell differentiation occurred with similar kinetics as those required for the programming of memory cell differentiation. Mice infected with 3 × 10^7 CFU LM-OVA received ampicillin treatment at 24 or 48 h p.i. and were analyzed at 7 or 35 days p.i. for the development of OVA257–264-specific immune responses in the spleen and liver.

CFSE assays

OT-1 splenocytes were incubated in 10 μM CFSE in RPMI 1640 (Molecular Probes, Eugene, OR). After 10 min the staining was halted by the addition of cold RPMI 1640. Cells were washed, resuspended in PBS, and 2 × 10^6 transgenic T cells were injected i.v. into recipient B6 mice. Three days later, splenocytes were surface stained with CD8-PE and Thy1.1-allophycocyanin (BD Pharmingen) and analyzed by flow cytometry.

Intracellular cytokine staining and peptides

Splenocytes and liver lymphocytes were resuspended in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 10 mM HEPES, 0.5 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 96-well plates in the presence of 1 μ/ml brefeldin A (GolgiPlug, Cytofix/Cytoperm kit; BD Pharmingen) with or without the appropriate peptide for 4–5 h at 37°C. OVA257–264 (SIINFEKL, Kβ-restricted) and listeriolysin O (LLO)/0-20 (NEKYAQAYPNVS, 1-Aβ-restricted) were added at concentrations of 0.1 and 1 μg/ml, respectively. Following the incubation, cells were stained with cell surface Abs CD4-FITC or CD8-FITC, as appropriate, and permeabilized and stained for intracellular cytokine expression using reagents provided in the Cytofix/Cytoperm kit according to the manufacturer’s instructions (BD Pharmingen). The Abs used were IFN-γ, allophycocyanin and IL-2-PE (BD Pharmingen), and cells were subsequently analyzed by flow cytometry.

FIGURE 1. Ampicillin treatment rapidly clears LM-OVA and halts Ag presentation. Mice were infected with 3 × 10^7 CFU LM-OVA and either left untreated (B6) or given ampicillin (2 mg/ml in drinking water, plus 1 mg i.p.) 24 h p.i. (B6+Amp). A. Mice were harvested from each group and bacterial clearance was measured in the spleen at the indicated time points. Error bars represent the SEM (n = 3). Results are representative of three separate experiments. B. A total of 2 × 10^6 CFSE-labeled Thy1.1^+ OT-1 C8 D T cells were injected into ampicillin treated or untreated mice at 24 or 48 h p.i. Three days later, spleens were harvested and assessed for expression of CD8, Thy 1.1, and CFSE fluorescence. Representative histograms display CFSE fluorescence of CD8^+Thy1.1^+ cells (n = 2/group).
When ampicillin treatment was initiated as early as 24 h p.i., effector CD8 responses in both the spleen and liver were comparable in size to untreated control mice (Fig. 2, A and B). Although the percentage of CD8 T cells in the spleen that were OVA-specific was consistently higher in ampicillin-treated mice (Fig. 2A), their spleen size was consistently smaller than that of untreated mice, leading to similar overall numbers (Fig. 2B). These results are in accordance with previously published data demonstrating that early clearance of infection with ampicillin did not prevent expansion and differentiation of effector CD8 T cells (11). In contrast to the lack of effect at day 7 p.i., mice treated with ampicillin at 24 h p.i. failed to develop a full cohort of memory cells, resulting in ~3- to 4-fold fewer OVA-specific CD8 in the spleen and ~7- to 10-fold fewer OVA-specific CD8 T cells in the liver by day 35 p.i., as compared with untreated control mice (Fig. 2, A and B). When ampicillin treatment was initiated 48 h p.i., CD8 memory levels in the spleen and liver were only slightly decreased (~2-fold) when compared with untreated controls. Throughout the course of four separate experiments, the contraction in the spleen of the total number of OVA-specific CD8 T cells in untreated B6 mice averaged 10- to 12-fold between day 7 p.i. (peak of the CD8 response) and 5–6 wk p.i. (establishment of stable memory), whereas the average contraction in mice receiving ampicillin treatment 24 h p.i. approached 40-fold. The differences in the liver were even more apparent. OVA-specific CD8 cells in untreated mice contracted ~30-fold, whereas in mice treated with ampicillin 24 h p.i., average contraction topped 200-fold. Throughout these and subsequent experiments, intracellular IFN-γ staining correlated with H-2Kb/OVA257–264 tetramer staining (data not shown).

To assess the ability of memory CD8 T cells in ampicillin-treated mice to persist long-term, we analyzed the generation and maintenance of memory cells over a long time course in mice that received ampicillin 24 h p.i. Once again, treatment with ampicillin resulted in similarly sized effector populations in the spleen and liver at day 7 as compared with untreated mice, whereas 3-fold fewer OVA-specific memory cells in the spleen and 6-fold fewer memory cells in the liver were detected by 5 wk p.i. However, memory populations in both groups of mice were stable in the spleen and liver, maintaining the same 3- and 6-fold disparity, respectively, through 11 wk (Fig. 2C). In other experiments we have found the disparity to remain constant through 100 days p.i. (data not shown). Therefore, it seems likely that the events dictating the differences in memory cell formation in ampicillin-treated mice occur early during infection. Notably, shortening the infectious period did not noticeably alter the timing of the peak effector response nor the onset of contraction, as has previously been reported (12).

**Shortening the infectious period disrupts the development of CD8 effector memory cells**

Upon observing that the development of tissue CD8 memory cells in the liver was particularly impacted by ampicillin treatment 24 h p.i., we hypothesized that perhaps the development of effector
memory cells was especially deficient in these mice. OVA-specific, IFN-γ-producing CD8 T cells were analyzed at various time points p.i. for their ability to coproduce IL-2. CD8 memory cells capable of secreting both IFN-γ and IL-2 have previously been demonstrated to belong largely to the central memory subset (25). At day 35 p.i., we found that early ampicillin treatment resulted in a higher percentage of IFN-γ-producing cells that also produced IL-2, as compared with untreated control mice (Fig. 3A). This relationship held true over an 11-wk time course (Fig. 3B). We also directly stained splenocytes with K\(^{b}\)/OVA\(_{257-264}\) tetramer and analyzed them for CD62L. CD8 memory cells in ampicillin-treated mice converted more rapidly to a CD62L\(^{\text{high}}\) central memory phenotype than did memory cells from untreated animals (Fig. 3, C and D).

These results indicate that early termination of infection leads to the generation of fewer effector memory cells and are in accord with our previous observation that in comparing ampicillin-treated (24 h) and untreated mice, the disparity for liver CD8 memory cells was greater than that for spleen (~7 to 10-fold vs ~3 to 4-fold over the course of four experiments). We further found that the disparity in numbers of IFN-γ\(^+\) IL-2\(^-\) memory cells was only ~2 to 2.5-fold, whereas the disparity in numbers of IFN-γ\(^+\) IL-2\(^-\) cells was typically ~5-fold (data not shown).

Optimal generation of IFN-γ-producing CD4 effector and memory cells requires a longer infectious period

To assess the kinetics with which CD4 effector and memory precursors are recruited, we analyzed CD4 responses in the spleen and liver specific for the dominant I-A\(^b\)-restricted CD4 T cell LLO\(_{190-201}\) (26). Mice were infected with LM-OVA and either left untreated or given ampicillin 24 or 48 h p.i. At various time points during the effector and memory phase of the immune response, mice were harvested and analyzed for the development of IFN-γ-producing CD4 cells following ex vivo stimulation with the LLO\(_{190-201}\) peptide. Mice treated with ampicillin 24 h p.i. generated 5- to 10-fold fewer CD4 effectors in the spleen by day 7 when compared with untreated animals (Fig. 4, A and B). Even ampicillin treatment as late as 48 h p.i. partially impaired the development of CD4 effectors in the spleen, resulting in a 2- to 3-fold decrease. Similarly to our observations of the CD8 response, differences in the liver were again greater, as ampicillin treatment 24 h p.i. virtually abolished the trafficking of CD4 effector cells to this organ. When ampicillin treatment was delayed until 48 h p.i., IFN-γ-producing CD4 T cells were detected in the liver at day 7, but at 10-fold lower levels than untreated mice. At day 35, mice treated with ampicillin at 24 h p.i. generated ~4- to 5-fold fewer memory CD4 in the spleen, whereas mice treated 48 h p.i. did not differ from untreated animals. In the liver, no CD4 memory cells were detected in mice treated 24 h p.i., whereas mice treated 48 h p.i. suffered only a mild decrease of liver-residing CD4 memory cells, as compared with control animals (Fig. 4, A and B).

We further assessed the persistence of CD4 memory cells over a long time course. Between 4 and 11 wk, although the total numbers of memory CD4 T cells decreased in both ampicillin-treated and untreated groups, the relative fold difference between the two groups stayed the same (Fig. 4C). In separate experiments, the

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**FIGURE 3.** Shortening the duration of infection results in particularly impaired development of CD8 effector memory. At various time points p.i. untreated (B6) and ampicillin-treated (24 h p.i., B6+Amp) mice were harvested and tested for the presence of OVA-specific CD8 in the spleen. A, Representative flow charts display IFN-γ and IL-2 staining in the spleen at 5 wk p.i. (gated on CD8) following ex vivo restimulation with OVA\(_{257-264}\). Number (upper right) represents the percentage of CD8 "IFN-γ" cells that are also IL-2\(^-\). B, Percentages of CD8 "IFN-γ" cells that were also IL-2\(^-\) were quantified over an 11-wk time course. Error bars represent SEM (n = 2–3/group). Results are representative of three separate experiments. C, Fresh splenocytes were stained 35 days p.i. with Abs for CD8 and CD62L, and with the K\(^{b}\)/OVA\(_{257-264}\) tetramer. Representative histograms are gated on CD8\(^+\) tetramer-positive cells and display CD62L staining. Numbers represent the percentage of CD62L\(^{\text{high}}\) positive. D, The percentages of tetramer staining cells that are CD62L\(^{\text{high}}\) at 1 or 5 wk p.i. are displayed for mice that had received no treatment (B6) or ampicillin treatment 24 h p.i. (B6+Amp). Error bars represent the SEM (n = 3).
disparity in memory CD4 T cells in untreated and ampicillin-treated mice remained constant through 100 days (data not shown). These results demonstrate a requirement for a longer stimulation in the recruitment of CD4 effector responses, as compared with CD8. However, unlike what was observed for the CD8 response, the recruitment of effector CD4 precursors did not precede that of memory CD4 precursors.

**Shortening the primary infectious period does not impact the quality of the secondary recall response**

Because ampicillin treatment led to the generation of fewer memory cells, we sought to determine whether they would respond to and protect against a lethal rechallenge. Six weeks p.i., untreated and ampicillin-treated (24 h) mice were given a high dose of LM-OVA (1 × 10⁷ CFU). Three days later, mice were harvested and checked for the expansion of CD8 and CD4 in the spleen and liver, as well as for clearance of the pathogen. Despite the disparity in size of their initial CD8 and CD4 memory populations, by day 3 post-rechallenge the difference between the groups is <2-fold (Fig. 5, A and B). In the liver, the differences are once again greater, with ~5-fold fewer activated CD8 and >10-fold fewer CD4 responding to the rechallenge in ampicillin-treated mice (Fig. 5, A and C). By day 3 post-rechallenge, both groups of mice completely cleared the pathogen from the spleen, and ampicillin-treated mice only had residual CFUs in the liver (Fig. 5D). Clearly, despite their fewer numbers, memory cells in ampicillin-treated mice are not defective and are quite capable of mounting a protective recall response.

**The kinetics with which effector and memory T cell responses are programmed is sensitive to the initial infectious dose**

To assess the role of increased antigenic stimulation early during infection in driving the development of CD8 memory, we challenged naive mice with a high dose (3 × 10⁷ CFU) and a normal priming dose (3 × 10⁶ CFU) of LM-OVA. Mice were either treated with ampicillin at 24 h p.i. (interrupting T cell stimulation) or at 72 h p.i. (ensuring clearance of the pathogen in the recipients of the high dose). Following high dose infection ampicillin treatment effectively cleared the bacteria within 24 h (data not shown). We harvested mice 1 and 5 wk p.i. and analyzed spleens and livers for the development of CD8 and CD4 T cell responses. Effector CD8 responses in the spleen and liver were similar for both infectious doses at day 7 p.i., regardless of when ampicillin treatment was initiated. By day 35, ampicillin-treated mice generated fewer CD8 memory cells in the spleen and liver than untreated control mice. However, although the disparity in mice receiving the low dose of LM-OVA was 4.7-fold in the spleen, the disparity in mice receiving the high dose was <2-fold. Similarly, although a 9-fold...
difference in liver-residing memory CD8 T cells was observed in groups receiving the low dose, only a 3-fold difference was seen in the livers of mice receiving the high dose (Fig. 6).

CD4 responses in ampicillin treated mice were infectious dose-dependent at the effector phase. As previously observed, ampicillin treatment 24 h p.i. resulted in the generation of fewer CD4 effector cells in the spleen by day 7. However, although the disparity at the low dose was 7-fold, the disparity at the high dose was only 2-fold. At day 35, we observed the same disparity in the number of CD4 memory cells in the spleen. Ampicillin treatment in mice receiving the low dose resulted in the development of 5-fold fewer memory cells, whereas ampicillin treatment in mice receiving the high dose led to only a 2-fold decrease. Despite the dose sensitivity of CD4 T cell responses in the spleen, both the high dose and low dose groups failed to generate significant effector or memory CD4 populations in the liver, indicating that the recruitment of tissue homing CD4 T cells may require further differentiation or ongoing infection at the tissue site to traffic appropriately (Fig. 6). These results suggest that the level of antigenic stimulation may play a role in determining the ability of CD8 T cells to progress from effector cells to memory cells. They further demonstrate that increasing antigenic stimulation can drive the formation of a full cohort of CD4 effector T cells.

**Discussion**

In this report, we offer a detailed analysis of the in vivo kinetics of CD8 and CD4 effector and memory cell programming in mice in which the infectious period is abruptly shortened by treatment with ampicillin. Although effector CD8 responses are similar in ampicillin-treated and untreated mice, we demonstrate that the development of a full cohort of memory cells in the spleen and liver is impaired by antibiotic treatment. A previous study also showed that strong CD8 effector responses could be induced despite an ampicillin-shortened infectious period, but did not observe any differences in the development of CD8 memory (11). In that study, memory was measured as the ability to mount a protective recall response. Interestingly, we also find that shortening the infectious period does not result in deficient protection from rechallenge. However, careful analysis of memory populations in our study reveals differences in the size and characteristics of prechallenge memory populations. These findings provide evidence that the processes leading to the programming of CD8 effector and memory cells are at least partially distinct. Other recent evidence indicates that CD8 memory cells precursors reside within and evolve from the effector CD8 population present at the peak of the response following acute infection (22, 23). Our results also support the idea that memory development potential is at least
in part a cell-intrinsic property imprinted during the early phases of an immune response, and cannot be wholly explained by competition for cytokines or growth factors necessary for survival during the contraction phase (21). What, then, are the signals early during infection that dictate the development and survival of CD8 memory cells? One possible interpretation is that the difference between programming an effector cell precursor and a memory cell precursor is quantitative. In this scenario, the overall level of Ag-specific stimulation would dictate differentiation from a CD8 effector precursor to a CD8 memory precursor. The ability of a cell to survive long-term would depend on the level of initial stimulation, as has been recently proposed (27, 28). Included in the overall stimulation are many factors, such as the Ag density on the surface of the APC, overall length of contact with the APC, input from costimulatory molecules, inflammatory cytokines such as IFNs, and growth factors such as IL-2, IL-7, and IL-15.

A second possibility is that the differences in stimulating end-stage effector cells and those that go on to further differentiate into memory cells are qualitative. This model proposes that the types of activation signals required for the programming of CD8 effector cells fundamentally differ from those required for the programming of CD8 effector cells with memory potential. Distinct signals could be present in the form of specialized costimulatory molecules, cytokine growth and maturation factors, and even specialized subsets of DC. It is already known that ampicillin treatment disrupts ongoing inflammatory responses in the spleen, including production of IFN-γ and TNF-α (11). Furthermore, several costimulatory molecules play a pivotal role in the stimulation of effector and memory cells in different scenarios (29–31), and their activity likely decreases the threshold of Ag-driven stimulation for the differentiation of effector cells (20). One possible scenario is that specialized costimulatory molecules or growth factors might increase the potential of effector cells to survive through expansion and contraction and become memory cells. Recent work has demonstrated that the makeup of the dendritic cell populations in secondary lymphoid tissues is altered greatly during the early stages of immunization, resulting in the influx of subsets with distinct stimulatory capacities and functions (32–34). Under these conditions, it is likely that the characteristics of the stimulatory signals delivered to Ag-specific CD8 T cells fluctuate greatly during the first couple of days p.i.

Previous evidence indicates that at least in part, the magnitude of the signal a CD8 T cell receives can dictate its fate. As little as 2 h of interaction with Ag and APC in vitro can set the responding T cell on a pathway of programmed proliferation (8, 10). However, to acquire differentiation capacity and the potential for long-term survival, a much longer interaction is required (8, 9, 35). It is also clear that in vitro stimulation for as little as 24 h can lead to the in vivo expansion of CD8 effector cells, contraction, and long-term memory (9). Under these activation conditions, Ag presentation is likely to be fairly uniform to all responding T cells. Our own results appear to support the idea that increasing the initial Ag dose before blunting the infection can enhance the development of memory cells. It is interesting to note, however, that increasing the Ag dose did not enhance memory development in untreated mice.

Several recent studies have found a critical role for CD4 T cells in the development of fully functional and responsive CD8 memory cells (36–39). In these studies, CD4-deficient mice generate Ag-specific CD8 effector and memory cells, but upon rechallenge the memory cells respond lethargically. Thus, CD4-deficient mice rapidly control a primary LM infection but fail to control a subsequent lethal-dose rechallenge. Both the protective capacity and overall numbers of memory cells in CD4-deficient mice progressively decline over time (37). However, a role for CD4 cells during priming in dictating the generation of fully functional CD8 memory cells has also been described (38). One possible interpretation of our results is that shortening the infectious period may prevent the optimal priming of CD4 helper cells, thus interrupting the “help” needed for the priming of fully functional CD8 memory cells. However, upon rechallenge CD8 and CD4 memory cells in ampicillin-treated mice, though initially fewer in number, were found to be fully capable of generating a protective recall response. Even in the liver, with almost a 10-fold disparity in the number of

FIGURE 6. Infection with a higher dose of LM-OVA reduces the effects of shortening the infectious period. Mice were infected with either 3 \times 10^3 or 3 \times 10^4 CFU LM-OVA. At each dose, one group of mice received ampicillin treatment at 24 h p.i. (B6 + Amp) whereas the control group (B6) was treated with ampicillin at 72 h p.i. to ensure bacterial clearance. Splenocytes and liver lymphocytes were harvested at 7 and 35 days p.i., restimulated with peptide ex vivo, and assessed for the presence of OVA257–264-specific CD8 and LLO190–201-specific CD8 by intracellular IFN-γ staining. Graphs display the absolute numbers of IFN-γ+ cells stimulated by the CD8 or CD4 peptide in the spleen or liver for each dose, as indicated. Numbers above each bar represent the ratio of control B6 (treated with ampicillin 72 h p.i.) to ampicillin-treated B6 (treated with ampicillin 24 h p.i.) at the indicated time point. Error bars represent the SEM (n = 3). Results are representative of two experiments.

210x475 to 551x742
CD8 memory cells and undetectable CD4 memory cells, we observed nearly complete control of the lethal rechallenge by day 3. Furthermore, following rechallenge these memory cells expanded at a pace that met or exceeded expansion in untreated mice. These results correlate with previous studies demonstrating that shortening the duration of infection did not significantly impair the establishment of protective immunity (11).

In our experiments the accumulation of effector CD8 T cells in the liver is clearly unimpared by ampicillin treatment, even though the pathogen has long since been cleared. The presence of long-term memory cells in the liver, in contrast, is greatly diminished compared with untreated controls. Currently, it is unclear whether this deficiency is due to inefficient programming of tissue homing memory cells in the spleen or the lack of chemotactic, instructional and/or survival signals in the noninflamed liver. The role of the tissue environment in dictating the development of effector memory cells with tissue homing properties remains a crucial question. We further show that blunting the infection with ampicillin treatment results in a more rapid disappearance of cells with an effector memory phenotype. One recent report has shown that effector memory cells can convert to a central memory phenotype over time, as defined by conversion from CD62Llow to CD62Lhigh. Furthermore, decreasing the initial infectious dose speeds this conversion (25). One possible interpretation of our results is that the reduced Ag load in ampicillin-treated mice resulted in a more rapid conversion to the central memory phenotype. However, we cannot rule out the possibility that the instruction of central memory and effector memory precursors are two separable processes, giving rise to distinct subpopulations.

The differentiation process for CD4 responses is more complex than for CD8 responses. Although a single exposure to Ag is sufficient to initiate several rounds of proliferation (17), full differentiation requires extended exposure to Ag (18, 19), and acquisition of effector function does not always correspond to the initiation of a proliferative program (40). Several in vitro studies have defined the relationship between level of stimulation and T cell fate, concluding that as stimulation increases, CD4 T cells are increasingly likely to proliferate, polarize to a Th1 or Th2 phenotype, and acquire full effector function, whereas increasing stimulation too much results in Ag-induced cell death (27). The overall eventual differentiation of Th1 effector cells is also likely to be dependent on both extended Ag contact and cell-extrinsic signals from cytokines.

In our hands, the development of effector CD4 responses required a longer stimulatory period than that of CD8 responses. These results support a model of progressive differentiation in which CD4 T cells require extended or repeated stimulation through multiple rounds of division before full expansion and differentiation. It has been previously reported that IFN-γ-producing Th1 effectors can develop in the spleen without developing in peripheral tissues (41). It is unclear if effector CD4 T cells with liver-homing capabilities represent a higher stage of differentiation, or if ongoing inflammatory responses in the liver are required to effectively recruit circulating CD4 cells. Another interesting finding is that unlike the phenotype we observed for CD8 T cells, the development of CD4 effector and memory precursors are temporally overlapping processes, once again suggesting that the development of antibacterial CD4 responses are much more sensitive to the continued presence of the infection than are CD8 responses.

These results have bearing on the design of vaccines targeting both CD8 and CD4 pathways. The development of CD8 effector and memory cells can be achieved following a relatively short exposure to stimulus, such as with a recombinant protein or chemically inactivated virus. Reliable generation of CD4 memory cells, however, may require strategies to extend the window of Ag presentation and inflammation, with live-pathogen vaccines likely generating the most efficient immunity.

Acknowledgments
We acknowledge B. Dere for technical assistance in the generation of MHC tetramers.

References