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Early Infection Termination Affects Number of CD8\(^+\) Memory T Cells and Protective Capacities in *Listeria monocytogenes*-Infected Mice upon Rechallenge\(^1\)

Kuo-En Tseng,\(^2\)* Cheng-Yu Chung,\(^2\)† Weng Siong H’ng,* and Shih-Lien Wang\(^3\)*

Here, we reevaluate the effects of early termination of infection on primary T cell expansion, subsequent memory cell development, and protective immunity. Using a murine *Listeria monocytogenes* (LM) infection model, we found the primary expansions of both CD4\(^+\) and CD8\(^+\) T cells were affected even when ampicillin was given as late as 60 h postinfection (p.i.). Subsequent development of CD8\(^+\) memory T cells was also impaired, although to a lesser extent, and only mice that received ampicillin at 24 h p.i. revealed a significant decrease in memory CD8\(^+\) T cells. Upon rechallenge with 1 \(\times\) 10\(^5\) CFU of LM, all ampicillin-treated mice cleared LM as effectively, and they generated similar amounts of Ag-specific CD8\(^+\) T cells as with untreated mice. However, mice that received ampicillin at 24 h p.i. lost their protective abilities when rechallenged with 7.5 \(\times\) 10\(^5\) CFU of LM. Ampicillin treatment also revealed early down-regulation of B7.1 and B7.2, but not CD40, on dendritic cells 72 h p.i. Our results have several important implications: 1) they argue against the hypothesis that brief exposure of T cells to an Ag is sufficient for full-fledged primary T cell responses and subsequent memory T cell development in vivo; 2) they suggest the existence of a reservoir of memory T cells, more than the immune system can possibly expand during secondary infection; and 3) they suggest that protective capacity is correlated with the number of preexisting memory T cells and that secondary expanding T cells play a limited role, at least in murine LM infection. *The Journal of Immunology*, 2009, 182: 4590–4600.

Introduction

Induction of protective immunity is the goal of vaccine immunization, and it is implemented by memory B cells, memory T cells, and preexisting Abs from plasma cells. Longevity of these three kinds of cells is the basis for long-term protection upon reencounter with infectious agents. Indeed, it has been documented that neutralizing Abs and CTLs persisted several decades after initial infection of polio virus (1) or vaccination of vaccinia virus, respectively (2). Unlike preexisting Abs, which can take after initial infection of polio virus (1) or vaccination of vaccinia virus, respectively (2). Unlike preexisting Abs, which can take

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During the past few years, several investigators have shown that relatively brief TCR engagement is sufficient to drive CD8\(^+\) T cell proliferation for several days and to acquire effector and memory function. This phenomenon has been described as “programmed T cell development” or “Ag-independent T cell proliferation” (9–17). While some studies have stimulated CD8\(^+\) T cells in vitro (9–12), others use a murine *Listeria monocytogenes* (LM)\(^\dagger\) infection model and terminate the infection, and hence the presence of Ag, earlier in vivo by ampicillin (Amp) treatment (13–15). Although three in vivo experiments showed that primary CD8\(^+\) T cell response was unaffected even if Amp treatment was given as early as 24 h post infection (p.i.), there were conflicting results about whether primary CD4\(^+\) T cell expansion was impaired after Amp treatment (14, 15). While Williams and Bevan showed that CD4\(^+\) T cell expansion was highly dependent on the continued presence of infection (15), Corbin and Harty claimed that Amp treatment as early as 24 h p.i. only had a minimal impact on the expansion of CD4\(^+\) T cells (14). Consistent with Williams and Bevan, a recent report that controlled the presence of Ag and the duration of TCR engagement by engineering a mouse line expressing a MHC class II-restrictive epitope under the control of a tetracycline-inducible promoter showed that CD4\(^+\) T cells required the continuous presence of an Ag for full expansion (18). Additionally, a new study also suggested that, as with CD4\(^+\) T cells, primary expansion of CD8\(^+\) T cells might require a prolonged engagement of TCR and Ag (19). In an attempt to dissect the Ag engagement requirement and other signals during in vivo infection, as others had suggested that inflammation itself might influence

\(^{\dagger}\) Abbreviations used in this paper: LM, *Listeria monocytogenes*; Amp, ampicillin; DC, dendritic cells; LLO, listeriolysin O; Moxi, moxifloxacin; p.i., postinfection; SEB, staphylococcal enterotoxin B.
the expansion of CD8+ T cells (20), those authors transferred peptide-pulsed dendritic cells (DCs) expressing diphtheria toxin receptor into mice infected with LM without expressing that peptide and controlled the duration of Ag exposure by injection of diphtheria toxin at specific time points. Their results indicated that primary CD8+ T cell expansion was indeed impaired if the duration of Ag engagement was shortened, even though the infection itself progressed normally. However, this conclusion is apparently in conflict with previous studies in which primary CD8+ T cell response was normal after early termination of LM infections by Amp treatment (13–15). However, it may be argued that the nature of peptide-pulsed DCs is different from that of DCs activated by infection.

Due to the importance of this issue, and due to the conflicting data, we decided to reexamine the effects of prematurely ending infection on primary T cell responses, development of memory cells, and protective immunity. We report that early termination of LM infection by Amp treatment affected primary T cell expansion, subsequent development of memory CD8+ T cells, and the protective capacities of mice upon rechallenge.

Materials and Methods

Mice and bacteria

Normal 6- to 10-wk-old female BALB/c and male C57BL/6 mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan. CB1 mice were an F1 cross of BALB/c female and C57BL/6 male mice. Animals were housed in a specific pathogen-free facility with an individual ventilation cage system at the Laboratory Animal Center, Tzu-Chi University, Hualien, Taiwan, and they were cared for in accordance with the Institutional Animal Care and Use Committee of Tzu-Chi University. The use of animals was reviewed and approved by the Institutional Animal Care and Use Committee. LM strain 10403S was provided by Dr. Eric Paner (Memorial Sloan-Kettering Cancer Center, New York, NY). LM-OVA, a strain of LM engineered to express OVA, was a kind gift from Dr. H. Shen (University of Pennsylvania). LM 10403S and LM-OVA were grown in brain heart infusion broth (Difco) to log phase and were administered i.v. through tail veins. The LD50 of LM was ~5–6 × 104 CFU in BALB/c mice.

In vivo immunization and treatment

Six- to 10-wk-old BALB/c female or CB1 female mice were used in all experiments. For analysis of primary lysteriolysin O (LLO190-99)specific CD8+ T cell response, BALB/c mice were infected with 1500 CFU of LM, and spleens were removed for investigation on day 7 p.i. For analysis of primary CD4+ and CD8+ T cell responses, CB1 mice were infected with 1 × 106 CFU of LM-OVA, and spleens were removed on day 7 p.i. For analysis of secondary LLO190-99-specific CD8+ T cell response, infected BALB/c mice were rechallenged with 1 × 103 LM on day 35 p.i., and spleens were taken out for analysis 5 days later. For expansion of TCRVβ8+ T cells, BALB/c mice were injected with 20 μg of staphylococcal enterotoxin B (SEB; a kind gift from Dr. Y. S. Lin, National Cheng-Kung University, Taiwan) i.p., and splenocytes were analyzed 72 h later. Mice receiving Amp (Amresco) treatment were injected i.p. with 1 mg of Amp in PBS at various time points and given drinking water supplemented with 2 mg/ml Amp. Ampwater was given for 7 days during primary infection. 5 days during secondary infection, or 72 h after administration of SEB. For mexifloxacin (Moxi) treatment, mice were given three injections of 3 mg Moxi/PBS i.p. with a 12-h interval for each injection, starting at various time points after LM infection. Moxi was kindly provided by Bayer Pharmaceuticals. For analysis of in vitro CD8+ T cell proliferation, CFSE-labeled CD8+ T cells were treated with 5 μg/ml Con A (Sigma-Aldrich) with or without 50 or 100 μg/ml Amp or with either 3 or 6 μg/ml of Moxi. CFSE profile was analyzed 72 h later.

Bacteria quantification

Spleens were removed and viable bacteria counts were made by homogenizing the spleens through a wire mesh into PBS containing 0.05% Triton X-100. Subsequently, aliquots of several 10-fold serial dilutions were plated onto brain-heart infusion agar plates (Difco; BD Diagnostics) and CFUs were counted after 24 h of incubation.

Results

Early termination of infection affects primary CD8+ T cell response

To end LM infection early, infected mice were given 1 mg Amp i.p. plus 2 mg/ml Amp in drinking water, a protocol previously used by other investigators (15). We first tested whether this protocol could effectively clear LM earlier than in nontreated controls. Consistent with previous results, infected spleens were almost free from LM 24 h after application of Amp (13, 15). Thus, we adopted this Amp treatment protocol throughout our investigation.

To evaluate the effect of early termination of infection on primary CD8+ T cell response, we treated LM-infected BALB/c mice with Amp at 24, 48, or 60 h p.i. The population of LLO190-99-specific CD8+ T cells was assayed by intracellular IFN-γ staining of LLO190-99 peptide-pulsed splenocytes from day 7 p.i. LLO190-99 is a well-recognized H-2Kd-restrictive dominant epitope in BALB/c mice after LM infection (22). The first thing we noticed was that the spleens of Amp-treated mice were much smaller than those of untreated controls. Spleen weight and total number of splenocytes in Amp-treated mice were significantly less than those in untreated mice (Fig. 1, A and B). The average spleen weight and total number of splenocytes in the 24 h Amp-treated group were only 44.4% and 60.4% of the untreated group, respectively. Neither spleen weight nor total number of splenocytes increased significantly even when Amp treatment was implemented at 48 or

Flow cytometry

For intracellular staining of IFN-γ, 2 × 106 splenocytes were pulsed with 107 M LLO190-99-OVA257-64 or LLO190-201 peptides at 37°C. Peptides were synthesized by Biomer Technology. Brefeldin A (2.5 μg/ml; Sigma-Aldrich) was added to the cell suspension 1.5 h later and splenocytes were further incubated for another 3.5 h at 37°C. Next, splenocytes were incubated in FACS staining buffer (PBS/0.5% BSA/0.02% sodium azide) for 1 h on ice in the presence of anti-CD8α-PE (BioLegend; clone 35-6-7) for LLO190-99 and OVA257-64-pulsed splenocytes or anti-CD4-PE (Caltag Laboratories; clone RM4-5) for LLO190-201-pulsed splenocytes. Dead cells were stained by staining with 1% paraformaldehyde ethidium monazide bromide (Sigma-Aldrich) and exposure to light during the last 10 min of staining to cross-link DNA-bound ethidium monozide bromide (21). Cells were subsequently washed three times in FACS staining buffer, fixed in 1% paraformaldehyde/PBS for 10 min, and permeabilized with 0.1% saponin (Sigma-Aldrich) for 10 min before applying anti-IFN-γ-FITC mAb (Caltag Laboratories; clone N418), anti-TCRVβ8-PE (eBioscience; clone KJ16), and anti-CD11c-PE (BioLegend; clone N418), anti-B7-1-FITC (BioLegend; clone 16-10A1), anti-B7-2-FITC (BioLegend; clone GL-1), anti-CD40-FITC (BioLegend; clone HM40-3), anti-1-A3-E-FITC (BioLegend; clone MS5/145.15.2), and anti-H-2Kd-FITC (BioLegend; clone AF6-88.5). Flow cytometric acquisition was performed on a multicolor flow cytometer for detection of memory CD8+ cells. The collected data were further analyzed with CellQuest Pro software (BD Biosciences).

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Cell preparation

Spleens were removed from naive mice or infected mice at various time points. Splenocytes were harvested by dissociation through a wire mesh, followed by lysis of erythrocytes with ammonium chloride, and they were subsequently resuspended in RP10, which consisted of RPMI 1640, In vitro supplemented with 10% FCS, l-glutamine, HEPEs, 2-ME, penicillin, and streptomycin. Cell numbers were determined by exclusion of 0.45% trypan blue. To purify CD8+ cells, direct MACS was performed. Briefly, total splenocytes were incubated with anti-CD8 microbeads (Miltenyi Biotec) for 15 min at 4°C according to the manufacturer’s instructions. After washing with MACS buffer (0.5% BSA/PBS with 5 mM EDTA), CD8+ cells were eluted through an LS column. The purified CD8+ cells were resuspended in PBS and labeled with 5 μM CFSE (Molecular Probes) for 10 min at 37°C. After washing with cold RP10, the labeled CD8+ cells were resuspended in RP10.
Early termination of infection affects primary CD8\(^+\) T cell responses. BALB/c mice were left untreated or treated with 1 mg of Amp/ PBS i.p. plus 2 mg/ml Amp in drinking water at either 24, 48, or 60 h after i.v. administration of 1500 CFU of LM. On day 7 p.i., splenocytes were pulsed with 10\(^{-6}\) M LLO\(_{91–99}\) peptide for 5 h before staining for CD8 and intracellular IFN-\(\gamma\). Naïve splenocytes were included to verify the specificity of staining. The average spleen weights (A) and total splenocytes (B) for each group are presented. C, The representative flow plots display CD8 staining on the \(y\)-axis and IFN-\(\gamma\) staining on the \(x\)-axis. The numbers in the quadrants are the percentages of cells in each quadrant. D, The numbers of LLO\(_{91–99}\)-specific CD8\(^+\) IFN-\(\gamma\)-cells for each group. The data represent the average of four mice and one of three independent experiments. E, From three independent experiments, the relative percentages of LLO\(_{91–99}\)-specific CD8\(^+\) IFN-\(\gamma\)-cells from the Amp-treated group to untreated mice are enumerated and averaged. Error bars represent the SD. *\(, p < 0.001\) when compared with the untreated group.

60 h p.i. This suggests that Amp treatment early in infection effectively limited the expansion of immune cells in the infected spleens.

Intracellular IFN-\(\gamma\) staining revealed that Amp-treated mice had smaller percentages of LLO\(_{91–99}\)-specific CD8\(^+\) T cells in their splenocytes (Fig. 1C). The percentages of LLO\(_{91–99}\)-specific CD8\(^+\) T cells in total splenocytes were \(~56\%\), \(62\%\), and \(71\%\) of untreated mice for 24-, 48-, and 60-h Amp-treated groups, respectively. This staining was highly specific, as naïve mice showed very small amounts of CD8\(^+\) IFN-\(\gamma\)-staining. Next, we calculated the total LLO\(_{91–99}\)-specific CD8\(^+\) T cells in spleens for each group. As we expected, the difference between the untreated group and Amp-treated groups was even more substantial, as both total splenocytes and percentage of LLO\(_{91–99}\)-specific CD8\(^+\) T cells were smaller in Amp-treated groups. Indeed, in one experiment, the total LLO\(_{91–99}\)-specific CD8\(^+\) T cells for untreated, 24-, 48-, and 60-h Amp-treated groups were 6.93 \(\times\) 10\(^5\), 2.03 \(\times\) 10\(^5\), 1.83 \(\times\) 10\(^5\), and 2.22 \(\times\) 10\(^5\), respectively (Fig. 1D). The averages of relative percentages of total LLO\(_{91–99}\)-specific CD8\(^+\) T cells in Amp-treated vs untreated groups from three independent experiments were 24.9\%, 25.6\%, and 40.8\% for 24-, 48-, and 60-h Amp treatment, respectively (Fig. 1E). The differences between untreated and all Amp-treated groups were statistically significant (\(p < 0.001\)).

Thus, our results strongly indicate that early termination of LM infection by Amp treatment significantly decreased the population of primary Ag-specific CD8\(^+\) T cells. CD8\(^+\) T cell response was still dramatically affected even when Amp treatment was initiated as late as 60 h p.i. However, when Amp treatment was given to infected mice 72 h p.i., we found no statistical difference in either the total number of splenocytes or in the numbers of total Ag-specific CD8\(^+\) T cells between the untreated and Amp-treated groups (data not shown).

Early termination of infection affects both primary CD8\(^+\) and CD4\(^+\) T cell responses

We had observed that Amp treatment had a dramatic effect on primary CD8\(^+\) T cell response after LM infection. To rule out the possibility that our observation was a phenomenon specific only to a particular epitope, and to see whether CD4\(^+\) T cell response was also affected, we sought to analyze the effect of Amp treatment on several epitope-specific T cell responses, both CD8\(^+\) and CD4\(^+\), in a single experiment. To achieve this, CB1 mice (an F\(_1\) cross of BALB/c female \(\times\) C57BL/6 male) were infected with LM-OVA and Amp was administered at 24, 48, or 60 h p.i. as described above. This enabled us to simultaneously analyze LLO\(_{91–99}\), an H-2K\(d\)-restrictive epitope, OVA\(_{257–64}\), an H-2K\(b\)-restrictive epitope, and LLO\(_{190–201}\), an H-2Ab-restrictive epitope (23). As in previous experiments, we found Amp-treated CB1 mice had a smaller total number of splenocytes than did untreated mice day 7 p.i. (data not shown). Intracellular IFN-\(\gamma\) staining also showed that Amp-treated mice had smaller percentages of LLO\(_{91–99}\)-specific CD8\(^+\) T cells (data not shown), OVA\(_{257–64}\)-specific CD8\(^+\) T cells (Fig. 2A, upper panels), and LLO\(_{190–201}\)-specific CD4\(^+\) T cells (Fig. 2A, lower panels) in their spleens than did untreated mice. As expected, mice treated with Amp at 24 h p.i. had a more significant decrease in their T cell responses. Interestingly, early termination of infection seemed to have a more severe impact on CD4\(^+\) T cell response than on CD8\(^+\) T cell response. For example, in the group treated with Amp at 24 h p.i., the percentage of OVA\(_{257–64}\)-specific CD8\(^+\) T cells was \(\sim32.3\%\) of untreated group, while the percentage of LLO\(_{190–201}\)-specific CD4\(^+\) T cells was only \(11.1\%\) of untreated group. In fact, the number of CD4\(^+\) IFN-\(\gamma\)-cells detected in mice treated with Amp at 24 h p.i. was only slightly higher than the background staining controls.

Enumeration of total Ag-specific T cells in spleens clearly demonstrated that premature termination of LM infection limited the expansion of both CD8\(^+\) and CD4\(^+\) T cells. For all three epitopes analyzed, Amp treatment led to significant decreases in the number of total Ag-specific CD8\(^+\) and CD4\(^+\) T cells (Fig. 2B), although different epitopes seemed to be affected differently. CD4\(^+\) T cell expansion was more sensitive to premature termination of infection, and the number of Ag-specific CD4\(^+\) T cells decreased most
FIGURE 2. Early termination of infection affects both primary CD4+ and CD8+ T cell responses. CB1 mice (F1 BALB/c × C57BL/6) were infected with 1 × 10^4 CFU of LM-OVA and left either untreated or given Amp 24, 48, or 60 h p.i. as described in Fig. 1. On day 7 p.i., splenocytes were pulsed with 10^-6 M LLO_{91-99} or OVA_{327-334}, or 10^-6 M LLO_{190-201} before staining for CD8 (LLO_{91-99} and OVA_{327-334}) or CD4 (LLO_{190-201}) and intracellular IFN-γ. A. The representative flow plots display CD8 (upper panels) or CD4 (lower panels) staining on the y-axis and IFN-γ staining on the x-axis. The upper half was pulsed with OVA_{327-334} peptide, while the lower half was pulsed with LLO_{190-201} peptide. The numbers in the quadrants are the percentages of cells in each quadrant. B. The numbers of LLO_{91-99}-specific CD8+ IFN-γ+ cells (CD8LLO), OVA_{327-334}-specific CD8+ IFN-γ+ cells (CD8OVA), and LLO_{190-201}-specific CD4+ IFN-γ+ cells (CD4LLO) are calculated for each group. The data represent the average of four mice and one of two independent experiments. Error bars represent the SD. #, p < 0.01; *, p < 0.05 when compared with the untreated group.

Substantially. Also, T cell expansion was impaired most significantly in the group treated with Amp at 24 h p.i.

Thus, our data clearly showed that early termination of LM infection by Amp treatment affected the expansion of multiple epitope-specific T cells, both CD8+ and CD4+. This meant that Amp treatment had a general effect on T cell priming and successful T cell expansion after LM infection; however, we first had to rule out the possibility that Amp treatment inhibited T cell proliferation nonspecifically. In other words, Amp might inhibit T cell proliferation directly through an unknown mechanism, not through affecting the other immune components related to T cell activation or priming. To eliminate this possibility, we tested the effect of Amp treatment on SEB-induced T cell expansion. SEB is a superantigen and will interact with Vβ3, Vβ7, Vβ8.1–8.3, Vβ11, and Vβ17 of TCR on T cells to induce proliferation and expansion of T cells (24). Since Ag processing and activation of Ag-presentation cells are not required for superantigens to initiate T cell expansion, Amp treatment should not affect T cell expansion induced by them unless Amp directly impairs T cell proliferation. Therefore, BALB/c mice were given 20 μg of SEB i.p. with or without Amp treatment 24 h after SEB injection. Splenocytes were stained for CD8 and TCRVβ8 to evaluate the expansion of CD8+ TCRVβ8+ T cells 72 h after SEB treatment since preliminary experiments indicated that CD8+ TCRVβ8+ T cells peaked at 72 h after SEB treatment (data not shown). We found SEB-treated mice had twice the percentage of CD8+ TCRVβ8+ T cells compared with naive mice (Fig. 3A). Nonetheless, mice that received both SEB and Amp treatments also revealed a similar increase. As shown by the total number of CD8+ TCRVβ8+ T cells, SEB-treated mice, regardless of whether they had received Amp treatment, all had a 2.5-fold increase compared with untreated mice (Fig. 3B). The difference is statistically significant (p < 0.01). Thus, we concluded that Amp treatment did not impair the proliferation of CD8+ T cells induced by SEB administration, and that CD8+ and CD4+ T cell responses affected by Amp treatment after LM infection were not due to a nonspecific effect of Amp on T cell proliferation. We also analyzed the expansion of CD4+ TCRVβ8+ T cells after SEB treatment; however, we were unable to find significant expansion. This was probably because there was a higher number of CD4+ TCRVβ8+ T cells in naive spleens before SEB treatment (data not shown).

To further solidify our conclusion, we tested the effect of Amp on T cell proliferation directly in vitro. To this end, CD8+ T cells were enriched by MACS and induced to proliferation with Con A. Amp needed to be added to assay its effect on T cell proliferation, so first we tried to estimate the concentration of Amp in sera of Amp-treated mice 24 h after Amp treatment. To further strengthen our finding that early termination of infection affects primary T cell responses, we sought to treat the mice with other antibiotics. Unfortunately, most commonly used antibiotics,
staining on the or treated with 20/H9262 either in vitro or in vivo. A
medium in vitro (data not shown). This was probably due to their
unlike Amp, could not clear LM rapidly in vivo, even though they
were very effective in inhibiting LM growth in brain-heart infusion
medium in vitro (data not shown). This was probably due to their
failure to penetrate into cells or to their accumulation in the com-
partment other than cytosol. Finally, we turned to the antibiotics Moxi,
which has been shown to effectively kill intracellular LM both in cell and animal models (25, 26). At first, we used the same
treatment protocol as Amp to see whether Moxi could clear LM
rapidly in vivo under our experimental conditions. However, it
turned out to be ineffective. We suspected that this was because mice did not drink much Moxi/water. Thus, we modified the treat-
ment protocol as 3 mg of Moxi/PBS i.p. at 24 h p.i. plus another
two i.p. injections of 3 mg of Moxi/PBS every 12 h. This treatment
protocol effectively cleared LM. Untreated mice had 724-fold and
315-fold more bacteria than did Moxi-treated mice at 48 and 72 h
p.i., respectively (Fig. 4A). This result seemed satisfactory; how-
ever, we did notice that this outcome was less effective than the
Amp treatment protocol (data not shown). Also, we consistently
found that there were more bacteria at 72 h p.i. compared with 48 h
p.i. under this Moxi treatment protocol. We thought that this was
because Moxi was given at a 12-h interval and that it was unable
to maintain a high enough concentration to completely kill LM all
the time. Nonetheless, we decided to continue this protocol and to
Test whether under this treatment Moxi could affect CD8 T cells
response, instead of spending time to modify the treatment
protocol.

Thus, we treated LM-infected BALB/c mice with the Moxi pro-
tocol described above at 24, 48, or 60 h p.i. The population of LLO91–99-specific CD8 T cells was assayed by intracellular
IFN-γ staining of LLO91–99 peptide-pulsed splenocytes from day
7 p.i. There were significant decreases in the numbers of LLO91–99-specific CD8 T cells in mice starting Moxi treatment at
24 or 48 h p.i. compared with untreated mice (Fig. 4, B and C; p <
0.01). However, unlike Amp treatment, the number of LLO91–99-
specific CD8 T cells was not statistically significant between un-
treated mice and mice given Moxi at 60 h p.i. We thought this
result reflected the fact that Moxi treatment was not as effective as
Amp treatment in clearing LM in vivo.

Thus, our data showed that, as with Amp, Moxi treatment af-
ected primary CD8 T cell response. To rule out the possibility that Moxi inhibited CD8 T cell proliferation or activation non-
specifically, we tested the effect of Moxi on Con A-induced CD8 T cell proliferation. Using standard concentrations of Moxi, sera
from mice 2 h after receiving two or three 3 mg Moxi injections
were estimated to have between 1.5 and 3 μg/ml of Moxi. There-
fore, we tested whether 3 and 6 μg/ml of Moxi would affect Con
A-induced CD8 T cell proliferation. CD8 T cells all proliferated
vigorously with or without Moxi treatment, and we were unable
to find any significant difference among all Con A-stimulated
groups (Fig. 4D).

Therefore, our data clearly showed that both Amp and Moxi
treatment affected primary CD8 T cell response after LM infec-
tion, and that early termination of infection did have a severe im-
port on T cell expansion.

Amp treatment does not affect the secondary CD8 T cell
responses during either primary or secondary infection

Next, we wanted to see whether Amp treatment would impair
memory CD8 T cell response in the same way it impaired pri-
mary response. Therefore, we rechallenged immune BALB/c mice
with 105 CFU of LM 35 days after primary infection, and spleno-
cytes were examined for LLO91–99-specific CD8 T cells by in-
tracellular IFN-γ staining 5 days postrechallenge as described
above. Amp treatments were given 24 h after primary infection
or 24 h after rechallenge. To our surprise, mice that received Amp
treatment during either primary or secondary infection, as
well as untreated mice, all showed similar percentages of

![FIGURE 3. Amp treatment does not affect CD8 T cell proliferation either in vitro or in vivo. A and B, BALB/c mice were left untreated (naive) or treated with 20 μg of SEB i.p., and half of the SEB-treated mice were give Amp (SEB/Amp) 24 h later as described. Splenocytes were prepared and stained for CD8 and TCRVß 72 h after SEB treatment. A. The representative flow plots display CD8 staining on the y-axis and TCRVß staining on the x-axis. The numbers in the quadrants are the percentages of cells in each quadrant. B. The numbers of total CD8 T cell Vß T cells are calculated for each group. The data represent the average of four mice and one of two independent experiments. Error bars represent the SD. *, p < 0.01 when compared with the untreated group. There is no statistical signif-
icance between SEB-treated and SEB/Amp-treated groups. C. CD8 T cells were isolated from splenocytes by MACS and labeled with CFSE. CFSE-labeled CD8 T cells (2 × 106) were treated with 5 μg/ml Con A with or without 50 or 100 μg/ml Amp. Cells without treatment of Con A were included as a nonproliferative control. The plots show the representative CFSE profiles in triplicate 72 h later. The numbers in the plots are the average of percentages of cells in the region. There is no statistical signif-
icance between Amp-treated and untreated groups.](http://www.jimmunol.org/)

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Our results indicated that, unlike the primary response, Amp treatment during secondary response did not influence the expansion of Ag-specific CD8⁺ T cells. This discrepancy deserves further investigation. Additionally, the fact that secondary CD8⁺ T cell response was normal in mice that received Amp treatment 24 h after primary infection implied that memory CD8⁺ T cells existed and functioned normally in those mice, even though their primary CD8⁺ T cell expansion had been impaired significantly.

**Protection capacity upon rechallenge of LM correlates with the population of primary memory CD8⁺ T cells**

Did a normal secondary CD8⁺ T cell response in mice that received Amp treatment in primary infection mean those mice had a normal development of memory T cells? These questions were addressed by direct detection of memory CD8⁺ T cells by intracellular IFN-γ staining on day 35 p.i. The results showed that the percentage of LLO01–99-specific CD8⁺ T cells had significantly decreased compared with the percentage on day 7 p.i. (Figs. 1 and 6A). In fact, the numbers of CD8⁺ T cells in mice treated with Amp at 24 h p.i. were only slightly greater than background staining. However, unlike day 7 p.i., untreated mice and mice treated with Amp at 48 h p.i. had very similar percentages of Ag-specific CD8⁺ T cells. Indeed, the number of total LLO01–99-specific cells was only significantly different between the untreated group and the group treated with Amp at 24 h p.i. (Fig. 6B; p < 0.05).

Thus, our data showed that the development of memory CD8⁺ T cells was affected by Amp treatment in primary infection, although to a less extent than effector CD8⁺ T cells. Only mice treated with Amp at 24 h p.i. revealed a significant reduction in memory CD8⁺ T cells. Surprisingly, even though untreated mice and mice treated with Amp at 24 h p.i. had a 3-fold difference in...
numbers of LLO91–99-specific memory CD8⁺ T cells, they gave rise to similar amount of LLO91–99-specific CD8⁺ T cells 5 days after rechallenge.

Next, we were curious as to whether Amp-treated mice had the same protective immunity as untreated mice. When we

FIGURE 5. Amp treatment in either primary or secondary infection does not affect the secondary CD8 T cells responses. BALB/c mice were infected with 1500 CFU of LM and left untreated or given Amp 24 h p.i. All mice were rechallenged with 10⁵ CFU of LM 35 days after primary infection. Half of the mice that did not receive Amp in primary infection were given Amp as described 24 h after rechallenge, and the rest of the mice were left untreated during secondary infection. Thus, there are three groups of mice: mice that did not receive Amp in either primary or secondary infection (No Amp/No Amp), mice that received Amp only in primary infection (24 h Amp/No Amp), and mice that received Amp only in secondary infection (No Amp/24 h Amp). Splenocytes were prepared 5 days after secondary infection and pulsed with 10⁻⁶ M LLO₉₁₋₉₉ peptide before staining for CD8 and intracellular IFN-γ. A, The representative flow plots display CD8 staining on the y-axis and IFN-γ staining on the x-axis. The naive splenocytes are included to verify the specificity of staining. The numbers in the quadrants are the percentages of cells in each quadrant. B, The numbers of LLO₉₁₋₉₉-specific CD8⁺ IFN-γ⁺ cells are enumerated for each group. The data represent the average of four mice and one of two independent experiments. There is no statistical significance in the numbers of LLO₉₁₋₉₉-specific CD8⁺ IFN-γ⁺ cells among these three groups.

FIGURE 6. Protective capacity upon rechallenge of LM is correlated with the population of preexisting memory CD8⁺ T cells. A and B, BALB/c mice were infected with 1500 CFU of LM and left untreated or given Amp 24 or 48 h p.i. as described. On day 35 p.i., splenocytes were prepared and pulsed with 10⁻⁶ M LLO₉₁₋₉₉ peptide before staining for CD8 and intracellular IFN-γ. A, The representative flow plots display CD8 staining on the y-axis and IFN-γ staining on the x-axis. The naive splenocytes are included to verify the specificity of staining. The numbers in the quadrants are the percentages of cells in each quadrant. B, The numbers of LLO₉₁₋₉₉-specific CD8⁺ IFN-γ⁺ memory cells are calculated for each group. Error bars represent the SD. *, p < 0.05 when compared with untreated group. The data represent the average of four mice and one of two independent experiments. C, BALB/c mice were infected with 1500 CFU of LM and left untreated or given Amp 24 or 48 h p.i. as described. On day 35 p.i., all infected mice as well as four naive mice were infected with 7.5 × 10⁵ CFU of LM. Numbers of bacteria in spleens were calculated 48 h after secondary infection. Error bars represent the SD. *, p < 0.05 when compared with untreated group. The data represent the average of four mice and one of two independent experiments.
rechallenged infected mice with $1 \times 10^5$ CFU of LM (a lethal dose commonly used for rechallenge) at 35 days p.i., all Amp-treated groups cleared LM as effectively as did untreated mice, consistent with previous research (14, 15). No obvious difference in the number of LM in spleens 48 h after rechallenge was found (data not shown). Indeed, as shown in Fig. 4, 24-h Amp-treated mice survived the rechallenge of $1 \times 10^5$ CFU of LM and successfully gave rise to a secondary CD8$^+$ T cell response. However, our results clearly indicated mice treated with Amp at 24 h p.i. had fewer Ag-specific memory CD8$^+$ T cells. Thus, we reasoned that we might be able to demonstrate the difference in protective immunity if we rechallenged the mice with a higher dose. So, infected mice were rechallenged with $7.5 \times 10^5$ CFU of LM and the numbers of bacteria in spleens were counted 48 h after rechallenge.

The logs of total number of bacteria in spleens of naive, untreated, 24-h, and 48-h Amp-treated mice were 8.61, 3.81, 7.54, and 5.68, respectively (Fig. 6C). Obviously, untreated mice cleared LM much more effectively than did naive mice. To know whether untreated mice and Amp-treated mice had the same protective capacity, a t test was performed. Statistical significance was only obtained between untreated mice and 24-h Amp-treated mice ($p < 0.01$). This meant that 24-h Amp-treated mice had a decreased capacity to clear LM compared with untreated mice. Although mice treated with Amp at 48 h p.i. also had higher average numbers of bacteria than did untreated mice, there was a larger variation among mice, and this led to statistical insignificance when compared with untreated mice. Overall, the protective capacity of mice seemed to correlate with their numbers of total Ag-specific memory CD8$^+$ cells before rechallenge.

Amp treatment results in early down-regulation of B7.1, B7.2, and MHC class II, but not CD40 and MHC class I, on DCs

Next, we tried to explore the mechanism of limited T cell expansions after Amp treatment in primary LM infection. Since Amp treatment resulted in earlier clearing of LM, we wondered whether the expression of costimulatory molecules was also affected by early termination of infection. Therefore, mice were left untreated or treated with Amp 24 h p.i., and then expression of B7.1, B7.2, and CD40 on CD11c$^+$ DCs was analyzed by flow cytometry 48 and 72 h p.i. (Fig. 7). There was significant up-regulation of all three molecules on DCs at 48 h p.i. compared with naive mice. Nonetheless, there were no significant differences in the expression of any of the three costimulatory molecules between untreated and Amp-treated mice at 48 h p.i. By 72 h p.i., the expressions of B7.1, B7.2, and CD40 on CD11c$^+$ DCs started to decrease. However, the expression of B7.1 and B7.2 seemed to decline faster in Amp-treated mice, and these differences were statistically significant ($p < 0.05$ for both B7.1 and B7.2). Unlike B7.1 or B7.2, the expression of CD40 on CD11c$^+$ DCs was subject to the same degree of down-regulation in both untreated and Amp-treated mice at 72 h p.i. Thus, our results revealed an early down-regulation of B7.1 and B7.2 on DCs after Amp treatment in infected mice. This early down-regulation might be part of the reason for insufficient T cell expansion after Amp treatment.

We also examined the expression of MHC class I and II on splenic DCs of mice treated or left untreated with Amp (Fig. 7, D and E). We found relative high percentages of naive DCs expressing MHC class II and MHC class I (85.4% and 98.7%, respectively), and LM infection did not induce more CD11c$^+$ cells to express either MHC class I or MHC class II. This result probably indicated that splenic DCs were no longer, at least in terms of MHC expression, in their immature status after migrating into spleen. On the contrary, the percentage of MHC class II-expressing DCs significantly decreased by 72 h p.i. in untreated mice. Surprisingly, DCs from 24-h Amp-treated mice seemed to have less of a decrease in the percentage of MHC class II expression at 72 h p.i. compared with untreated mice ($p = 0.03$). No such down-regulation of MHC class I expression was found after LM infection. We also analyzed the mean fluorescent intensity on MHC class II- or class I-expressing DCs (data not shown), but we could not find any significant difference on MHC class II expression in all experimental groups. For MHC class I expression, three were significant increases in mean fluorescent intensity after LM infection, but again no significant difference was found between untreated and Amp-treated mice.

FIGURE 7. Analyses of the expression of costimulatory molecules and MHC in Amp-treated and untreated mice after LM infection. BALB/c mice were infected with 1500 CFU of LM or left uninfected (naive). Half of the infected mice were given Amp 24 h p.i. as described. Splenocytes were prepared on day 2 (No Amp D2, 24h Amp D2) or day 3 (No Amp D3, 24h Amp D3) p.i. from both Amp-treated or untreated mice and were stained for CD11c and B7.1 (A), CD11c and B7.2 (B), CD11c and CD40 (C), CD11c and MHC class II (D), and CD11c and MHC class I (E). The data presented are percentages of CD11c$^+$B7.1$^+$ (A), CD11c$^+$B7.2$^+$ (B), CD11c$^+$CD40$^+$ (C), CD11c$^+$MHC class II$^+$ (D), and CD11c$^+$MHC class I$^+$ (E) cells out of total CD11c$^+$ DCs. The data represent the average of four mice and one of two independent experiments. Error bars represent the SD. *, $p < 0.05$. 
Discussion
In this study, we reexamine the effects of shortening infection on primary T cell response, secondary CD8⁺ T cell response, and protective immunity upon rechallenge in a murine LM infection model. We found Amp treatment early in the infection effectively terminated the infection and had a significant impact on primary T cell expansion, as well as on subsequent CD8⁺ memory T cell development. More importantly, by increasing the dose of LM, the protective capacities of infected mice upon rechallenge were correlated with the number of memory CD8⁺ T cells.

Our finding that Amp treatment affected both primary expansions of CD4⁺ and CD8⁺ T cells probably adds even more confusion to the existing data regarding the effect of early termination of infection on T cell responses. As noted earlier, many investigations used the same Amp treatment model in LM infection and claimed that there was no effect on primary CD8⁺ T cell response (13–15). Our results, however, get some support from an early investigation by North and colleagues that showed Amp treatment in LM-infected mice significantly impaired the primary T cell response, which was revealed by loss of protective ability when the splenocytes from Amp-treated mice were transferred into naive mice (27). Although those investigators did not differentiate which T cells, CD4⁺ or CD8⁺, were affected, it is reasonable to infer from their data that CD8⁺ T cell response was severely compromised after Amp treatment, since it is well documented that transferring Listeria-specific CD8⁺ T cells into naive hosts effectively conveys protection upon LM infection (28–30). Thus, their results suggest that CD8⁺ T cell expansion during primary LM infection is severely impaired by Amp treatment, in agreement with our results. In fact, we think that their data are most consistent with our results in general, suggesting that both CD4⁺ and CD8⁺ T cell responses are affected. In terms of CD4⁺ T cell response, our results are consistent with Williams and Bevan (15) but contrary to those of Corbin and Harvy (Fig. 2 of Ref. 14). A more recent study controlling the expression of cognate Ag in transgenic mice indicated that Ag persistence is required throughout the expansion phase of CD4⁺ T cell response (18). This is consistent with our result, although Amp treatment may not just affect Ag persistence in an LM model.

One finding that strongly suggests that both CD8 and CD4 T cell responses are impaired in primary LM infection after Amp treatment is that both spleen weight and total number of splenocytes were significantly decreased in Amp-treated mice (Fig. 1, A and B). This phenomenon has been mentioned in another report (15) and indicates a failure of full immune response and expansion of cells, most likely CD4⁺ and CD8⁺ T cells.

In general, our data show that the earlier the Amp treatment was given, the more severely impaired were the primary T cell responses. However, even Amp treatment initiated 60 h p.i. revealed substantial decreases in spleen weight, number of total splenocytes, and number of Ag-specific CD8⁺ and CD4⁺ T cells. It surprised us that the numbers of Ag-specific T cells in 60-h Amp-treated mice were not much different from 48-h Amp-treated mice or even 24-h Amp-treated mice. This suggests that full-fledged T cell responses need to meet a minimal stimulation requirement and that T cell responses are not linearly related to time after infection. Indeed, when mice were given Amp treatment 72 h p.i., we were unable to discern any significant difference in any parameters when compared with untreated mice, including spleen weight, number of splenocytes, or number of Ag-specific CD8⁺ T cells. From these results, we infer that the minimal time required to ensure a full-fledged T cell response is around 72 h. Interestingly, North and colleagues claimed that they could still discern the impact of Amp treatment on T cell responses even if Amp was given 5 days after LM infection (27).

Besides Amp treatment, Moxi treatment also resulted in impaired primary CD8⁺ T cells. Thus, this strengthens our conclusion that early termination of infection impairs primary T cell responses, and our finding is not nonspecific with regard to the effects of particular antibiotics. Under our Moxi treatment protocol, its impact on primary CD8⁺ T cell expansion is not as grave as Amp treatment, and, unlike Amp treatment, Moxi treatment starting from 60 h p.i. no longer affects CD8⁺ T cell response. Interestingly, this result correlates well with less efficiency of Moxi treatment in clearing LM in vivo. Nonetheless, collectively, the data seem to emphasize the importance of minimal stimulation requirement for a full-blown T cell response. In our opinion, this requirement is around the first 72 h of infection.

Contrary to primary response, Amp treatment during secondary response did not affect the expansion of Ag-specific CD8⁺ T cells (Fig. 5). This result implies that, unlike in primary infection, Amp treatment in secondary infection does not affect Ag presentation or related machinery required for T cell priming and activation. Alternatively, this result may mean that activation and proliferation of memory CD8⁺ T cells can still progress normally even though immune components related to T cell priming and activation are affected by Amp treatment as they are in primary response. In other words, there are differential requirements for activation and proliferation of naive and memory T cells. While there is some evidence that supports the differential activation of naive and memory T cells (31, 32), the former possibility cannot be excluded. During secondary LM infection, immune mice clear LM very rapidly, usually within 48–72 h (33), even when they are rechallenged with a high dose of LM (usually 10⁸ CFU). Therefore, there is probably not much difference between the kinetics of bacterial clearance in untreated mice and mice treated with Amp at 24 h p.i. Thus, the different kinetics of bacterial clearance may at least partly account for the different effect of Amp treatment on primary and secondary T cell responses.

Although the numbers of Ag-specific CD8⁺ T cells in mice treated with Amp at 24 h were only ~25% of those of untreated mice at day 7 p.i. (Fig. 1E), both groups gave rise to a similar population of Ag-specific CD8⁺ T cells after rechallenge (Fig. 5). Analyzing the memory CD8⁺ T cells at day 35 after primary infection revealed that untreated mice had 3-fold more Ag-specific memory CD8⁺ T cells than did mice treated with Amp at 24 h p.i. This result surprised us, and it implies that the immune system might harbor a reservoir of memory T cells, more cells than the system can possibly expand during a secondary infection. This might also suggest that under the rechallenge dose of 10⁷ CFU of LM, the machinery activated for T cell activation and expansion can only sustain the full-fledged responses of a fraction of the memory cell reservoir. Interestingly, we did not detect a significant increase in the number of CD8⁺ T cells even when the untreated mice were rechallenged with 7.5 × 10⁶ CFU of LM (data not shown). Additionally, the fate of the “unexpanded memory CD8⁺ T cells” after secondary response is also a mystery. Are they maintained in their inactivated state or purged from the system due to insufficient activation? This phenomenon deserves further investigation.

Another interesting finding is that although the development of primary effector CD8⁺ T cells was severely compromised in all groups of mice receiving Amp treatment, only mice treated with Amp at 24 h p.i. had a significant decrease in the number of memory CD8⁺ T cells (Figs. 1, 2, and 6). This suggests that the effector CD8⁺ T cells of the mice treated with Amp at 48 and 60 h p.i. had a better ability to develop into memory T cells. A recent study
showed that effector CD8+ T cells that expressed high levels of IL-7R α-chain preferentially developed into memory CD8+ T cells (34). Those IL-7Rαhigh cells seemed to have a survival advantage. It will be interesting to see whether there is differential expression of IL-7Rα between effector CD8+ T cells of mice treated with Amp at 24 h p.i. and mice treated with Amp at 48 and 60 h p.i. We also can infer from our results that progenitor CD8+ T cells might receive signals that are important for memory T cell development from 24 h to probably around 48–60 h p.i. (regardless of whether they are related to up-regulation of IL-7Rα expression), and that these signals are significantly diminished by Amp treatment starting at 24 h p.i. Alternatively, it might not be the quality of signal but the duration of sufficient stimulation that leads to differential memory CD8+ T cell development. In the later case, a minimum of 48 h of sufficient stimulation is probably needed for full development of memory CD8+ T cells.

Does our result that early termination of LM infection by Amp treatment impaired primary CD8+ T cell expansion contradict the theory of “Ag-independent proliferation” or “programmed CD8+ T cell development”? Ag-independent proliferation of CD8+ T cells in vitro has been clearly and consistently demonstrated by many authors (9–11), and we have no evidence to oppose it. For in vivo evaluation of this theory, as we described earlier, our result was not completely consistent with some previous studies (13–15). Nonetheless, this does not mean we have evidence to indicate that Ag-independent proliferation or programmed CD8+ T cell development does not exist. In fact, although our data showed that the numbers of Ag-specific CD8+ effector T cells in mice treated with Amp at 24 h p.i. were only 25% of those of untreated mice, very few numbers of Ag-specific progenitor CD8+ T cells must go through a significant expansion and proliferation to reach those populations, even when the infection has been terminated 4–5 days earlier by Amp treatment. Considering this, our data, in a sense, agree with the theory of Ag-independent proliferation or programmed CD8+ T cell development. Therefore, our opinion is that Ag-independent proliferation or programmed CD8+ T cell development does exist, but that for a full-fledged primary CD8+ T cell response and subsequent CD8+ memory T cell development in vivo, a more prolonged Ag engagement or stimulation is required, just as in the responses of CD4+ T cells.

As was found in other investigations, we found that Amp treatment in primary infection did not affect the protective immunity of mice upon rechallenge with 10^6 CFU of LM. However, analysis of memory CD8+ T cells clearly indicated that there was a significant decrease in the numbers of memory CD8+ T cells in mice treated with Amp at 24 h p.i. Thus, we reasoned that if we rechallenged the infected mice with a higher dose of LM, the “extra” memory CD8+ T cells in untreated mice might contribute to the protection, and we might be able to discern a differential response in protective immunity between untreated and Amp-treated mice. Indeed, mice treated with Amp at 24 h p.i., unlike untreated mice and mice treated with Amp at 48 h p.i., were unable to effectively clear LM when rechallenged with 7.5 × 10^5 CFU of LM (Fig. 6C). Our results, therefore, demonstrated a correlation between the numbers of memory CD8+ T cells and protective capacities of immune mice. Interestingly, while the numbers of memory CD8+ T cells did not correlate with the size of expanded CD8+ T cells after rechallenge, they were good indicators of protective capacities. It seems to us that original memory CD8+ T cells are the major contributors to clearance of LM, that expanding memory T cells only play a limited role in protection, and that secondary expansion of memory T cells is only the consequence of successful protection. Further investigation is needed to clarify the relative importance of all contributors in protection. Also, it is important to know whether other infectious models show the same phenomenon. In this regard, it was recently documented that to protect against vaccinia virus, a high number of “effector-like” CD8+ T cells are required, and recall proliferation competence of memory T cells plays only a limited role (35). Indeed, recently, memory T cells have been proposed to contain two functional different subsets: central memory T cells with expression of CD62L and CCR7, and effector memory T cells with no or low expression of CD62L and CCR7 (36, 37). It is thought that effector memory T cells have the capacity to exert their function immediately, while central memory T cells may home to lymph organs for restimulation. Therefore, it will be interesting to know whether Amp treatment leads to differentially development of these two subsets of memory cells, not just a decrease in the total number of memory T cells. In other words, could impaired protective capacity in Amp-treated mice actually correlate with a decrease in the number of effector memory T cells? Furthermore, could normal responses of secondary expansion mean that both Amp-treated and untreated mice have similar numbers of central memory T cells? These important questions deserve further investigation, probably with the help of MHC tetramers.

Why does the premature termination of LM infection by Amp treatment affect primary T cell responses and subsequent memory T cell development (at least for CD8+ T cells)? In other words, what are the requirements for a full-fledged T cell responses and development of memory T cells? Our data suggest a minimal 72 h of continuous stimulation is required for a full-blown CD8+ T cell response. This stimulation might include the interaction of TCR and its cognate Ag in the context of the MHC/peptide complex, interaction between CD28 and B7.1 or B7.2, and, as some recent studies suggested, third signals from cytokines (38, 39). Both CD4+ and CD8+ T cells have been shown to need an Ag throughout their expansions, as previously described (18, 19). Thus, we explored the role of costimulatory molecules and MHC class II and class I. We found mice treated with Amp at 24 h p.i. had an early down-regulation of B7.1 and B7.2 on their CD11c+ DCs 72 h p.i. (Fig. 7). This suggests that insufficient stimulation from costimulatory molecules might be part of the reason for impaired T cell expansion after Amp treatment. However, we acknowledge that the degree and timing of the decrease in expression levels of B7.1 and B7.2 after Amp treatment are not comparable with the affected T cell responses. Thus, more sophisticated investigations are needed to elucidate the role of costimulatory molecules and whether they are required throughout the phase of T cell expansion.

Analysis of MHC class II and class I expression also does not give us an indication of what leads to impairment of T cell responses after Amp treatment. We found that there was a down-regulation of MHC class II on DCs by 72 h p.i., and that this down-regulation was less prominent on DCs of Amp-treated mice. Thus, there were more MHC class II+ DCs in Amp-treated mice than in untreated mice at 72 h p.i. However, we interpret down-regulation of MHC class II as a consequence of activation or TCR-MHC engagement. Thus, less turnover on MHC class II expression in 24 h Amp-treated mice may imply an early deactivation status, consistent with our findings in B7.1 and B7.2 expression. Nonetheless, whether this phenomenon has anything to do with impaired T cell response in Amp-treated mice requires further investigation.

Finally, our results might also have important implications in medical practice. Could early intervention of medicine during infection lead to an insufficient immune response and subsequently jeopardize the development of protective immunity?
Disclosures
The authors have no financial conflicts of interest.

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