

Rescue of CD8 T cell–mediated antimicrobial immunity with a nonspecific inflammatory stimulus

See the related Commentary beginning on page 1415.

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Reconstitution of protective immunity by adoptive transfer of pathogen-specific T cells has been successful in patients with compromised cellular immunity. The *in vivo* effectiveness of *in vitro*–expanded CD8 CTLs is variable, however. For example, adoptively transferred *Listeria monocytogenes*–specific CD8 CTLs only confer protective immunity if challenge infection occurs within 48 hours of T cell infusion. Herein we show that transferred CTLs persist in lymphoid compartments for many weeks, but that their response to bacterial challenge decreases during the first week following transfer. While T cells transferred less than 48 hours before infection proliferate, those transferred 7 days before infection die. Remarkably, treatment of mice with anti-CD40 at the time of T cell infusion reprograms transferred T cells, allowing them to proliferate and confer protective immunity upon bacterial challenge 7 days later. Our study demonstrates, for the first time to our knowledge that CD40-mediated stimuli can influence CD8 T cell activation independent of concurrent antigen exposure. The ability to modulate long-term responsiveness of CD8 T cells with a transient, nonspecific inflammatory stimulus has importation implications for adoptive immunotherapy.

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Introduction

Adoptive T cell therapy can restore pathogen-specific immunity and has effectively treated Epstein-Barr virus–associated malignancies and prevented cytomegalovirus infections in bone marrow transplant recipients (1–3). Optimizing the duration of protective immunity conferred by T cell infusions remains an important challenge. Studies in bone marrow transplant recipients and AIDS patients, as well as work in animal models, indicate that persistence of functional CD8 CTLs is diminished by the absence of CD4 T lymphocytes (4–6). Approaches to enhancing CD8 CTL survival upon transfer have included coadministration of IL-2, transfection of CTLs with chimeric GM-CSF/IL-2 receptors, and cotransfer of pathogen-specific CD4 T cells (7, 8). Recent studies have suggested that CD40 ligation can be used to enhance CD8 T cell responses to two persistent viruses, HIV and herpes simplex virus (9–11).

CD40 is a member of the TNF receptor family and is expressed on a variety of cells, including B and T lymphocytes, dendritic cells, monocytes, macrophages,

eosinophils, and endothelial cells (12). Stimulation of CD40 by its ligand, CD154, has protean immunomodulatory effects, which include upregulation of B7.1/B7.2 costimulatory molecules (13–15). The contribution of the B7/CD28 costimulatory pathway to the induction and maintenance of CD8 effector T cell responses has been studied in viral and bacterial infection models, with different results depending on the pathogen. The relative dependence of CD8 CTL responses on CD40-mediated signals likely reflects differences in the activation of innate inflammatory responses (16–18).

Listeria monocytogenes, a facultative intracellular bacterium, induces robust CD8 T cell responses that mediate long-term protective immunity in mice (19, 20). Presumably because this bacterial infection induces an exuberant inflammatory response, priming of CD8 T cells during primary infection does not depend upon the activation of the CD40/CD154 signaling pathway (18, 21). However, CD8 T cell responses are reduced in the absence of CD28, indicating that inflammatory mechanisms other than CD40 promote B7/CD28 costimulation (17).

Adoptive transfer of *L. monocytogenes*–specific CD8 T cells confers protective immunity to naive recipient mice (22, 23). Remarkably, transferred CD8 T cells rapidly lose their effectiveness, and it is a general finding that recipient mice challenged 1 week after T cell infusion have lost protective immunity (24, 25). The mechanism for this loss of protection remains undefined. In this study, we have characterized the survival, activation, and proliferation of adoptively transferred *L. monocytogenes*–specific CTLs in recipient mice. We find that transferred T cells persist but lose the ability to proliferate and to

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Nonstandard abbreviations used: T cell receptor (TCR); antigen-presenting cell (APC); carboxyfluorescein diacetate succinimidyl ester (CFSE).

confer protection during the first week following infusion. Remarkably, the responsiveness and protective capacity of transferred CD8 T cells can be dramatically improved by nonspecific activation of the CD40 signaling pathway. These studies suggest that modulation of the T cell compartment with nonspecific inflammatory stimuli can influence the antimicrobial capacity of antigen-specific T lymphocytes.

Methods

Mice and bacteria. BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Thy1.1 mice were originally obtained from Charles Surh (The Scripps Research Institute, La Jolla California, USA). *L. monocytogenes* strain 10403S (obtained from Daniel Portnoy, University of California, Berkeley, Berkeley, California, USA) and the *L. monocytogenes* LLO_{Ser92} mutant strain (mutation of the tyrosine in position 92 of listeriolysin to serine) (26) were grown in brain-heart infusion broth.

Immunization of mice with *L. monocytogenes* and generation of LLO-specific CD8 T cell lines. BALB/c Thy1.2 mice were immunized by intravenous injection of 2×10^3 *L. monocytogenes* into the lateral tail vein. Spleens were removed 7–10 days after immunization, and splenocytes were harvested by dissociation through a wire mesh and lysis of erythrocytes with ammonium chloride. Splenocytes were resuspended in RP10+, consisting of RPMI 1640 (GIBCO BRL; Life Technologies Inc. Gaithersburg, Maryland, USA) supplemented with 10% FCS, L-glutamine, HEPES (pH 7.5), β -mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μ g/ml) and gentamycin (50 μ g/ml). We incubated 40×10^6 responder splenocytes in the presence of 30×10^6 irradiated, syngeneic splenocytes that were peptide-pulsed for 1 hour at 37°C with 10^{-9} M LLO₉₁₋₉₉ peptide. Cell lines were cultured in 10 ml RP10+ medium supplemented with 0.5 ng/ml IL-2 and 4 ng/ml IL-7 (R&D Systems Inc., Minneapolis, Minnesota, USA). After 7 days, responder T cells were restimulated as described above. One to two weeks after the second in vitro restimulation, CD8 CTLs were washed with PBS and resuspended at a concentration of 6×10^6 epitope-specific CD8 CTLs per 250 μ l PBS for injection into syngeneic mice.

Tetrameric H2-K^d-peptide complexes. MHC-peptide tetramers for staining of epitope-specific T cells were generated as previously described (27).

Staining and flow cytometric analysis. One hundred thousand CD8 CTLs from T cell cultures or 2×10^6 splenocytes were added to wells of a 96-well plate. After incubation at 4°C for 20 minutes with unconjugated streptavidin (0.5 mg/ml; Molecular Probes Inc., Eugene, Oregon, USA) and Fc Block (BD PharMingen, San Diego, California, USA) in FACS staining buffer (SB: PBS [pH 7.45] 0.5% BSA, and 0.02% sodium azide), cells were stained with FITC-conjugated anti-CD62L (BD PharMingen), phycoerythrin-conjugated H2-K^d LLO₉₁₋₉₉ tetramers (0.25–0.5 mg/ml), peridinin chlorophyll protein-conjugated anti-Thy1.1 (BD PharMingen), and

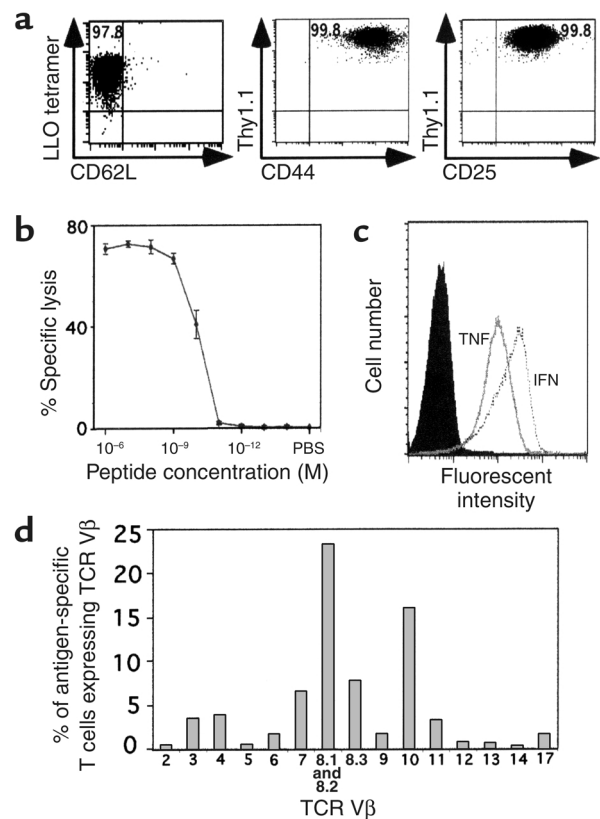


Figure 1

In vitro-expanded LLO₉₁₋₉₉-specific CD8 CTLs have an effector phenotype and retain a diverse TCR V β repertoire 7–14 days after the second in vitro stimulation with peptide-pulsed APCs. (a) CD8 CTL line stained with mAb for CD8 α , CD62L, Thy1.1, CD44, CD25, and H2-K^d tetramers complexed with LLO₉₁₋₉₉. Left panel: Dot plot gated on live CD8 and Thy1.1 T cells. Middle and right panels: Dot plots gated on live CD8 T cells. Numbers in upper quadrants represent percentages of total CD8 T cells. (b) Percentage specific lysis by the LLO₉₁₋₉₉-specific CD8 CTL line in the presence of different concentrations of LLO₉₁₋₉₉ peptide was determined by ⁵¹Cr-release assay using P815 (H2^d) target cells. (c) TNF and IFN- γ production by CD8 CTL line was assessed by standard intracellular cytokine staining following in vitro stimulation. (d) CD8 CTL line was stained for TCR V β expression with TCR V β -specific antibodies. The percentage of LLO₉₁₋₉₉-specific CD8 CTLs stained with each of the TCR antibodies is indicated. Data are representative of two independent experiments.

allophycocyanin-conjugated anti-CD8 α (BD PharMingen) in SB for 60 minutes at 4°C. Cells were also stained with either FITC- or phycoerythrin-conjugated anti-CD25 or anti-CD44 and FITC-conjugated anti-T cell receptor V β (anti-TCR V β) segments (TCR V β 2, 3, 4, 5, 6, 7, 8.1, 8.2, 8.1–8.3, 9, 10, 11, 12, 13, 14, 17). Subsequently, cells were washed three times in SB and then fixed with 1% paraformaldehyde/PBS (pH 7.4). Four-color flow cytometry was performed using a FACSCalibur or LSR flow cytometer, and data were analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems Mountain View, California, USA).

Intracellular cytokine staining. One to two weeks after the second in vitro stimulation, 5×10^6 CD8 CTLs per milliliter RPMI+ were incubated for 5 hours at 37°C and

5% CO₂ with 1 µg/ml brefeldin A (BD PharMingen) in a 24-well flat-bottom plate coated with anti-CD3 mAb (10 µg/ml) (BD PharMingen). Cells were fixed by incubation with Cytofix/Cytoperm (BD PharMingen) at 4°C for 20 minutes. Thereafter, cells were washed twice with 1× Perm/Wash buffer (BD PharMingen) and stained at 4°C for 30 minutes with FITC-conjugated anti-IFN-γ, FITC-conjugated anti-TNF, or FITC-conjugated IgG1 isotype control. Finally, cells were again washed with 1× Perm/Wash Buffer and resuspended in FACS SB for flow cytometric analysis.

CTL assays. Standard Cr-release assays using ⁵¹Cr-labeled P815 target cells were performed as previously described (28). For peptide titrations, the percentage specific lysis was determined over a range of different peptide concentrations at a constant effector-to-target ratio of approximately 20:1.

Carboxyfluorescein diacetate succinimidyl ester labeling. CD8 CTLs were washed with PBS and resuspended at 5 × 10⁷ per milliliter in PBS containing 1 µM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Inc.). The cell suspension was incubated at 37°C, 5% CO₂ for 10 minutes and immediately washed with cold RPMI 1640/10% FCS before transfer into mice.

Infection of mice with *L. monocytogenes*, harvesting of spleen, and bacterial quantification. Thirty minutes, 48 hours, or 7 days after transfer of 6 × 10⁶ LLO₉₁₋₉₉-specific Thy1.1 CD8 CTLs, Thy1.2 recipient mice were infected with 5 × 10³ *L. monocytogenes* 10403S or LLO₉₁₋₉₉ via lateral tail vein injection. Seventy-two hours after infection, spleens and livers were removed and viable bacterial counts in these organs were assessed by homogenizing the tissues through a wire mesh into PBS containing 0.05% Triton X-100. Subsequently, aliquots were plated onto brain-heart infusion agar plates (Life Technologies Inc.) and CFUs were counted after 24–48 hours of incubation.

In vivo administration of mAb's. Anti-CD40 mAb was purified from the FGK-45 hybridoma, and 100 µg was injected intraperitoneally into recipient mice at the indicated time points.

Results

Characterization of in vitro-generated epitope-specific CD8 CTL line. To characterize the ability of adoptively transferred CD8 T cells to confer protective immunity, we generated LLO₉₁₋₉₉-specific CD8 T cell lines by in vitro peptide stimulation. This epitope induces an immunodominant T cell response with a complex TCR repertoire (29). Previous work indicated that the activation status could influence the capacity of CD8 T cells to confer protective immunity (29, 30). Therefore, we characterized the LLO₉₁₋₉₉-specific CD8 CTL lines by surface staining for activation marker and assessed their effector function by cytotoxic assays and intracellular cytokine staining. We also determined their TCR Vβ repertoire after in vitro stimulation. More than 97% of epitope-specific CD8 CTLs expressed low levels of CD62L and high levels of CD44 and CD25 (Figure 1a). Cytotoxicity assays with titrations of the targeting peptide demonstrated a high

degree of specific lysis, as well as peptide sensitivities that were comparable to those seen after short-term in vitro restimulation of primary and recall LLO₉₁₋₉₉-specific T cells (31) (Figure 1b). Intracellular cytokine staining demonstrated that the entire population of in vitro-expanded LLO₉₁₋₉₉-specific CD8 CTLs produced TNF and IFN (Figure 1c). After two in vitro restimulations, the Vβ repertoire of the T cell line retained a distribution similar to that seen in LLO₉₁₋₉₉-specific T cells undergoing recall responses in vivo (29) (Figure 1d). Taken together, these results characterize the in vitro-expanded CD8 CTLs as a population of epitope-specific, effector T cells with a broad TCR repertoire.

We next investigated the recovery rate of in vitro-expanded CD8 CTLs after transfer into naive, syngeneic recipient mice. The Thy1 disparity between the CTL line (Thy1.2) and the recipients (Thy1.1) allowed us to track the transferred cells. Of 6 × 10⁶ LLO₉₁₋₉₉-specific CTLs transferred 2–3 days earlier,

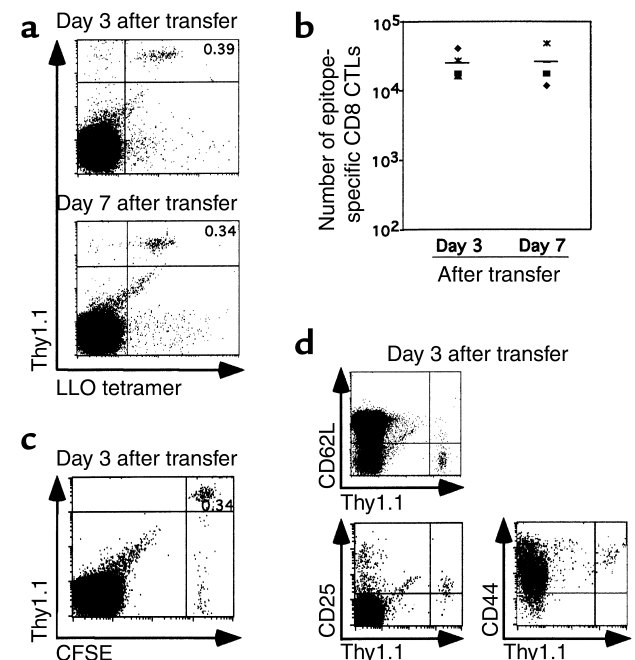
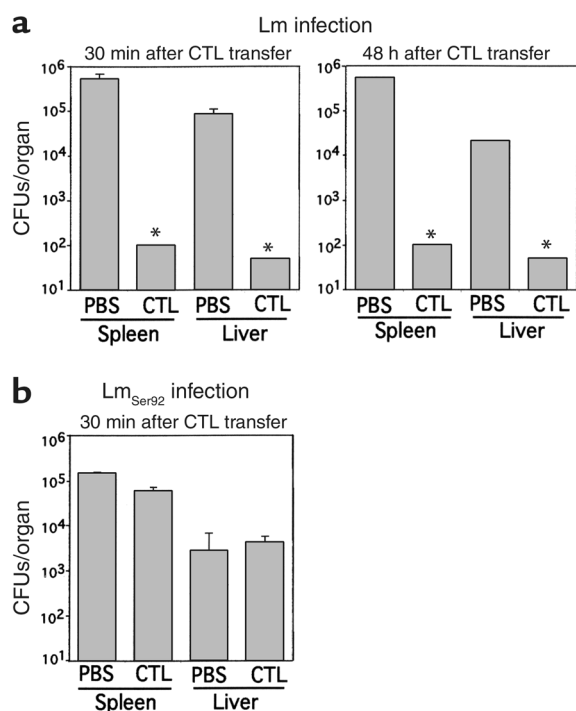


Figure 2

CD8 CTLs maintain an effector phenotype and do not proliferate after transfer into naive, syngeneic recipients. (a–d) Three and seven days after transfer of 6 × 10⁶ Thy1.1 CD8 CTLs into Thy1.2 BALB/c recipients, splenocytes were stained for CD8α, Thy1.1, CD44, CD25, and with LLO₉₁₋₉₉ H2-K^d tetramers. (a) Dot plots are gated on live CD8 T cells, and the percentage of cells positive for Thy1.1 (y axis) and LLO₉₁₋₉₉ (x axis) is shown. (b) Absolute number of cells that stained positive for Thy1.1 and LLO₉₁₋₉₉ on days 3 and 7 after transfer; each point represents an individual mouse. (c) LLO₉₁₋₉₉-specific CD8 CTLs were labeled with CFSE prior to transfer, and the percentage of CFSE⁺ Thy1.1 CD8 T cells 3 days after transfer is indicated. (d) Activation-marker expression of LLO₉₁₋₉₉-specific T cells was determined 72 hours after transfer. Staining for Thy1.1 is shown on the x axis, and staining with LLO₉₁₋₉₉ H2-K^d tetramers and for CD62L, CD25, and CD44 is shown on the y axis. Dot plots are gated on live CD8 T cells. (a, c, and d) Dot plots are representative of three to four mice per group.



approximately 2.5×10^4 epitope-specific CTLs were recovered from the recipient spleen, where they accounted for 0.3–0.4% of the total CD8 T cell population. Seven days after transfer these numbers remained essentially unchanged (Figure 2, a and b). Interestingly, transferred CTLs did not proliferate within the naive recipient, as demonstrated by their high CFSE fluorescence 72 hours after transfer (Figure 2c). While transferred T cells remained CD62L-low and CD44-high, their level of CD25 expression did decrease in comparison with in vitro-maintained T cell lines (Figure 2d).

Figure 4

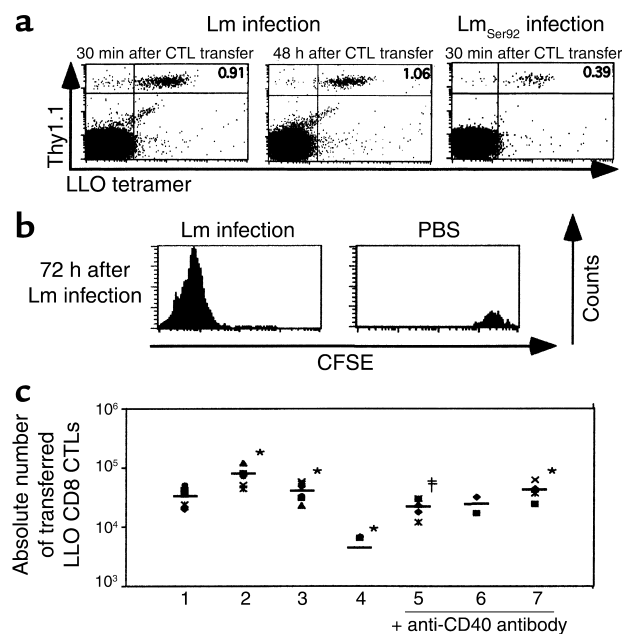
Protective immunity conferred by transferred CTLs correlates with their expansion 72 hours after infection. (a) We infused 6×10^6 T cells into recipients, followed by infection with wild-type *L. monocytogenes* or LLO_{Ser92} *L. monocytogenes* 30 minutes or 48 hours after transfer. Seventy-two hours after infection, splenocytes were stained for CD8 α , Thy1.1, and with LLO₉₁₋₉₉ H2-K^d tetramers. Dot plots are gated on live CD8 lymphocytes. Staining for Thy1.1 is shown on the y axis, tetramer staining on the x axis. Dot plots represent 4–6 animals per group. (b) CD8 CTLs were labeled with CFSE and transferred into recipient mice that were either infected or left uninfected. 72 hours after transfer CFSE fluorescence of Thy1.1⁺, LLO₉₁₋₉₉-specific T cells was determined. (c) Absolute number of transferred T cells, determined by Thy1.1 and H2-K^d tetramers, is plotted for various conditions. Lane 1, Lm_{Ser92} infection 30 minutes after CTL transfer; lane 2, Lm infection 30 minutes after CTL transfer; lane 3, Lm infection 48 hours after CTL transfer; lane 4, Lm infection 7 days after CTL transfer; lane 5, no Lm infection, day 3 after CTL transfer; lane 6, no Lm infection, day 7 after CTL transfer; lane 7, Lm infection 7 days after CTL transfer. 5, 6, and 7 received 100 μ g anti-CD40 antibody intraperitoneally 2 days and 1 day before CTL infusion and 12 hours after infection. Each point represents an individual mouse. * and † achieves statistical significance ($P < 0.05$ by Student's *t* test).

Figure 3

LLO₉₁₋₉₉-specific CD8 CTLs confer a high degree of protective immunity to wild-type *L. monocytogenes* (Lm) infection 30 minutes or 48 hours after T cell infusion (a). T cell recipients are not protected from a challenge with LLO_{Ser92} *L. monocytogenes* 30 minutes after CTL infusion, demonstrating in vivo antigen specificity (b). CFUs per organ are shown on the y axis in log scale. Time interval between CTL transfer and infection is indicated. Control animals received PBS, while experimental animals received 6×10^6 LLO₉₁₋₉₉-specific CD8 CTLs. *Lower limit of detection. Data are the mean and SD of two to three animals per group and are representative of two independent experiments.

Protective immunity conferred by CTL line specific for a single dominant epitope. We next assessed the ability of in vitro-generated CD8 CTLs to confer protection to naive syngeneic recipients. For these studies we infused 6×10^6 LLO₉₁₋₉₉-specific CTLs intravenously and challenged recipients with *L. monocytogenes* 10403S 30 minutes or 48 hours later. Bacterial counts in liver and spleen, obtained 72 hours after infection, demonstrated a high degree of protective immunity in animals that received CTLs (Figure 3a). This level of protection was comparable to that seen in immune animals responding to rechallenge infection (results not shown). To confirm the specificity of CD8 CTL-mediated immunity, we challenged recipient mice with LLO_{Ser92} *L. monocytogenes*. This mutant strain retains virulence, but LLO₉₁₋₉₉-specific T cells cannot recognize infection because of a mutation in an essential anchor residue of LLO₉₁₋₉₉. As expected, recipients of 6×10^6 LLO₉₁₋₉₉-specific CTLs were fully susceptible to infection with 5,000 LLO_{Ser92} *L. monocytogenes* (Figure 3b).

Protective immunity conferred by CD8 CTLs correlated with their rapid expansion. Ex vivo tetramer staining for LLO₉₁₋₉₉-specific CTLs clearly demonstrated expansion of transferred CD8 CTLs already 3 days after infection, at which time the population increased in size by a



factor of roughly to 2.7, to approximately 1% of the total CD8 T cell population (Figure 4a). Given the low recovery of transferred CTLs in the spleen, we wanted to know whether the observed expansion was due to recruitment of T cells into the spleen or to in situ proliferation. Therefore, the CTL line was labeled with CFSE, and recipients were infected 30 minutes after infusion of T cells. Three days after infection, all transferred CTLs were CFSE-low, suggesting that the observed expansion resulted from T cell proliferation (Figure 4b). In terms of absolute numbers of antigen-specific T cells, whereas infection 30 minutes after transfer led to the greatest expansion of epitope-specific T cells, infection 48 hours after CTL infusion resulted in significantly lower expansion (Figure 4c). Expansion of the host's endogenous CD8 T cell population was not seen at this early time point during infection (27). Furthermore, CD8 CTL expansion was not seen in animals infected with LLO_{Ser92} *L. monocytogenes*, confirming once again the in vivo specificity of the transferred CTLs (Figure 4, a and c).

Previous studies have shown that protective immunity to *L. monocytogenes* conferred by adoptively transferred T cells is lost when challenge infection is delayed beyond 24–48 hours (24, 25). To confirm this with our system, we delayed challenge infection for 7 days after transfer of LLO₉₁₋₉₉-specific CTLs. As expected, recipients were not protected, as assessed by bacterial counts in liver and spleen 72 hours after infection (Figure 5a). Loss of protection, however, could not be explained by the loss of transferred T cells, since the absolute number of transferred cells recovered from recipients 7 days after transfer was comparable to that 2 and 3 days after transfer (Figures 2a and 4c). The lack of protective immunity was accompanied by a dramatic decline in the number of epitope-specific CTLs in response to infection. Ex vivo LLO₉₁₋₉₉ tetramer staining did not detect any CD8 CTLs in the recipients' spleens 72 hours after infection (Figure 6b). These results suggested that transferred CTLs developed a functional defect in vivo during the 7 days following transfer.

CD4 memory T cells do not maintain CD8 CTL-mediated protective immunity. Previous work from our laboratory indicated that CD4 T cell help is not required for CD8 T cell responses and memory following *L. monocytogenes* infection (32). It is possible, however, that in vitro-cultured LLO₉₁₋₉₉-specific CTLs might require *L. monocytogenes*-specific CD4 T cells for responsiveness. To address this possibility, we infused 5×10^3 LLO₉₁₋₉₉-specific T cells into recipient mice. Seven days later, splenocytes from a “memory” mouse that had been immunized with LLO_{Ser92} *L. monocytogenes* were transferred into the recipients, followed 30 minutes later by an infection with 5×10^3 *L. monocytogenes* 10403S.

Spleen cells from the “memory” mouse contained *L. monocytogenes*-specific CD4 T cells, but no LLO₉₁₋₉₉-specific T cells, circumventing the possibility of competition between epitope-specific CD8 T cells for antigen-presenting cells (APCs). Figure 6a demonstrates that transfer of immune splenocytes did not restore protection conferred by the CD8 CTL line. To determine whether an existing *L. monocytogenes*-specific immune compartment could rescue CTL expansion, LLO₉₁₋₉₉-specific T cells were transferred into mice previously immunized with LLO_{Ser92} *L. monocytogenes*. Infection of these mice 7 days later did not result in the expansion of the transferred CD8 CTLs (Figure 6b). These experiments indicate that CD4 T cell help does not provide a sufficient stimulus to maintain responsiveness over time.

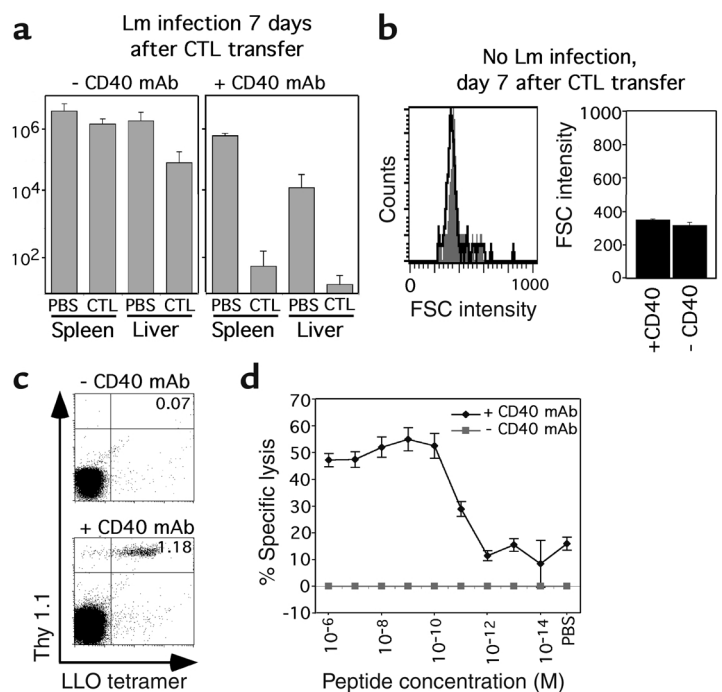


Figure 5

Protective immunity and expansion of transferred CTLs is lost 7 days after infusion. Loss of protection is prevented by anti-CD40 antibody (FGK45) treatment. (a) Recipients received 6×10^6 LLO₉₁₋₉₉-specific CTLs and were infected with wild-type *L. monocytogenes* 7 days later. Seventy-two hours after infection bacteria were counted in liver and spleen. Left panel: no FGK45; right panel 100 μ g FGK45 intraperitoneally 2 days and 1 day before CTL transfer and 12 hours after infection. Control animals received PBS. (b) Left panel: Forward scatter (FSC) for LLO₉₁₋₉₉-specific CTLs 7 days after transfer in the presence (gray histogram) or absence (black line) of FGK45. Right panel: mean FSC intensity for two to three mice per group. (c) Seventy-two hours after infection, splenocytes were stained for CD8 α , Thy1.1, and with LLO₉₁₋₉₉ H2-K^d tetramers. Right upper quadrants show percentages of total CD8 lymphocytes. (d) Two weeks after transfer of CTLs and administration of FGK45, as described in a but without infection of the recipients, splenocytes were restimulated in vitro with LLO₉₁₋₉₉. Percentage of specific lysis in the presence of different concentrations of LLO₉₁₋₉₉ peptide was determined by standard ⁵¹Cr-release assay, using P815 (H2^d) target cells. Diamonds, plus in vivo FGK45; squares, without in vivo FGK45. Data in a are the mean and SD of two to three animals per group and are representative of two independent experiments. Dot plots in c are representative of five to six animals. Data in d are the mean and SD of two animals.

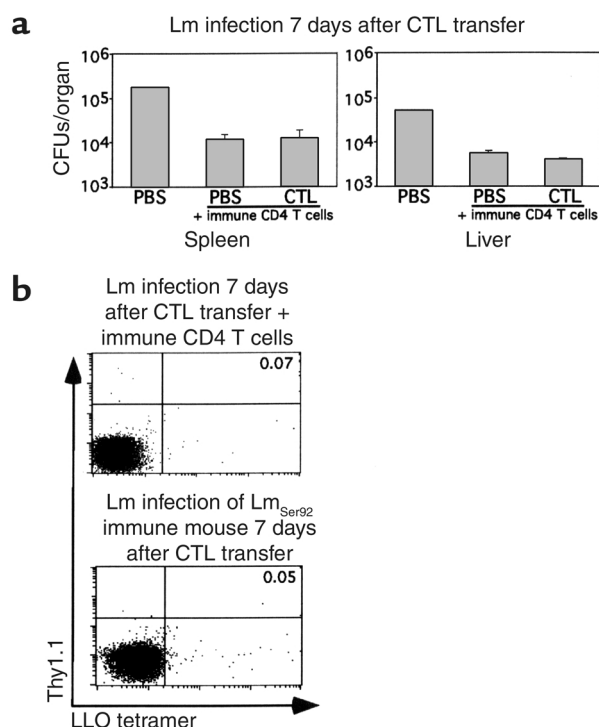


Figure 6 CD4 T cell memory does not restore the ability of infused LLO₉₁₋₉₉-specific CD8 CTLs to expand or to confer protective immunity 7 days after transfer. **(a)** We transferred 6×10^6 LLO₉₁₋₉₉-specific CTLs into recipient mice and infected the mice with wild-type *L. monocytogenes* 7 days later. Thirty minutes prior to infection, 50×10^6 splenocytes from a BALB/c mouse immunized more than 28 days earlier with LLO_{Ser92} *L. monocytogenes* were infused into the CTL recipients. Seventy-two hours after infection, spleens and livers were cultured; bacterial counts are plotted on the y axis. **(b)** We transferred 6×10^6 LLO₉₁₋₉₉-specific CTLs into recipient mice that had been infected with LLO_{Ser92} *L. monocytogenes* more than 28 days earlier. Seven days after CTL infusion, recipients were infected with wild-type *L. monocytogenes*, and 72 hours later, splenocytes were stained for CD8 α and Thy1.1 and with LLO₉₁₋₉₉ H2-K^d tetramers. Dot plots are gated on live CD8 T cells. Staining for Thy1.1 is shown on the y axis, and tetramer staining is shown on the x axis. Numbers in the upper right quadrants represent percentages of total CD8 T cells. Data are representative of two animals per group.

CD40 activation can restore protective immunity. A number of recent studies demonstrate that CD40-CD154 ligation can enhance effector T cell responses to pathogens (9–11). We reasoned that an inflammatory stimulus induced by CD40 activation might enhance the responsiveness of transferred CTLs to delayed infection.

We intraperitoneally injected 100 μ g agonistic anti-CD40 antibody 2 days and 1 day prior to CTL transfer. Mice were infected with *L. monocytogenes* 7 days after T cell infusion, at which time they received another dose of anti-CD40. Remarkably, transferred LLO₉₁₋₉₉-specific T cells expanded in response to infection (Figures 4c and 5c). Of note, anti-CD40 did not alter the absolute number of CTLs prior to infection (Figure 4c). Moreover, CTLs extracted from mice that received anti-CD40 antibody did not differ in their forward scatter

profile from CTLs isolated from untreated mice, indicating that CD40 signaling did not result in T cell blasting in the absence of specific antigen (Figure 5b). Most importantly, protective immunity conferred by the CTL line was reestablished to a level seen in animals undergoing challenge infection up to 48 hours after transfer of the CTL line (Figure 5a).

To determine whether anti-CD40 treatment enhanced responsiveness of transferred T cells in the absence of infection, we transferred CTLs and administered anti-CD40 antibody as described above. Fourteen days later, we isolated CTLs and performed cytolytic assays after short-term in vitro restimulation with antigenic peptide. Specific lysis was only seen when animals had received anti-CD40 antibody, demonstrating yet again the capacity of in vivo CD40 activation to maintain responsiveness of transferred CD8 T cells (Figure 5d).

We subsequently determined whether the timing of anti-CD40 antibody administration influences the expansion and protective capacity of transferred CD8 CTLs. When the anti-CD40 antibody was administered 2 days and 1 day prior to CTL transfer and a booster was given 12 hours after a delayed infection, protection was fully restored (Figure 7). On the other hand, anti-CD40 antibody administration only 2 days and 1 day prior to infection or only 12 hours after delayed infection did not restore the capacity of transferred CTLs to convey protective immunity (Figure 7). These results suggest that CD40 activation initiated at the time of T cell infusion maintains transferred CD8 CTLs in a functional state.

In vivo administration of agonistic CD40 antibody enables the transferred CTL line to form a long-term memory population. CD40 stimulation promotes memory T cell generation from infused CTLs. Work in various model systems suggested that CD40-mediated stimulation is not necessary to maintain memory T cells, or to generate secondary effector T cells, but instead might function during the death phase of expanding CD8 CTLs and influence the subsequent size of the memory population (33–35). We reasoned, therefore, that in vitro-expanded CD8 CTLs responding to an in vivo infection in the presence of enhanced CD40 stimulation might give rise to a larger memory T cell population. To test this hypothesis, mice were injected with 100 μ g anti-CD40 antibody intraperitoneally 2 days and 1 day prior to the transfer of 6×10^6 CTLs. Seven days later, the animals were infected with *L. monocytogenes*, at which time they received another dose of anti-CD40 antibody. Twenty-one days after the initial infection, the mice underwent a repeated bacterial challenge with *L. monocytogenes*. Surprisingly, tetramer staining for LLO₉₁₋₉₉-specific CD8 T cells demonstrated in vivo reexpansion of LLO₉₁₋₉₉-specific CTLs derived from the T cell infusion (Figure 8a). Protective immunity conferred by the transferred CTL line could not be readily assessed, since these mice developed an endogenous immune response to the first *L. monocytogenes* infection.

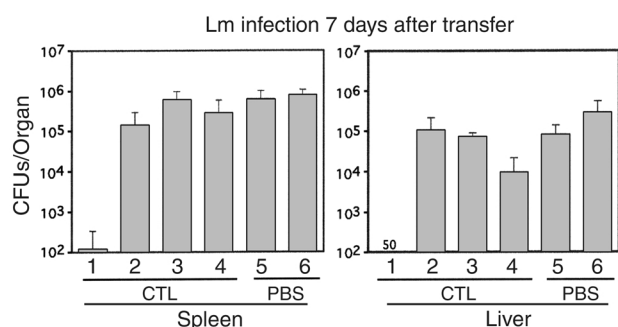


Figure 7

The timing of anti-CD40 antibody administration determines outcome of CTL response to in vivo infection. CTLs were infused into recipient mice and challenged with *L. monocytogenes* 7 days later. Bacterial counts in liver and spleen were determined 72 hours after infection. Animals received anti-CD40 antibody or an isotype control at the indicated time points. Experimental animals received 6×10^6 CTLs 7 days before infection (lanes 1–4). Control animals received PBS intraperitoneally (lanes 5 and 6). Data are representative of three animals per group. Lane 1, CD40 mAb 1 day and 2 days before CTL transfer and 12 hours after infection; lane 2, CD40 mAb 1 day and 2 days before CTL transfer; lane 3, CD40 mAb 12 hours after infection; lane 4, isotype control 1 day and 2 days before CTL transfer and 12 hours after infection; lane 5, isotype control 1 day and 2 days before CTL transfer and 12 hours after infection; lane 6, CD40 mAb 1 day and 2 days before CTL transfer and 12 hours after infection. Lower limit of detection, 50.

Transferred CD8 T cells did not reexpand to a secondary challenge when recipients did not receive anti-CD40 antibody at the time of T cell infusion (Figure 8b). These results indicate that in vivo CD40 activation enables differentiated effector CTLs to develop into memory populations following infection.

Discussion

In this report we demonstrate that adoptively transferred *L. monocytogenes*-specific CD8 CTLs persist in the recipient but lose the ability to proliferate and to confer protective immunity. Thus, the previously described finding that adoptively transferred T cells only transiently confer protection against *L. monocytogenes* is not attributable to deletion of antigen-specific T cells, but rather to a loss of the adoptively transferred T cells' ability to effectively respond to infection. We demonstrate that anti-CD40 antibody administration restores the ability of transferred T cells to mediate protection, promoting their proliferation upon repeat bacterial challenge and enhancing the development of immunologic memory.

The adjuvant effects of CD40 stimulation have been demonstrated in the setting of immunization with different soluble and cell-associated antigens (13–15, 36). In these studies, in vivo antigen presentation and CD40 stimulation occurred simultaneously, supporting the widely accepted model that CD40-mediated signals upregulate costimulatory molecules on cells presenting antigen to T lymphocytes. In our study, however, in vivo CD40

stimulation and its impact on transferred T cells occurred in the absence of in vivo antigen presentation. Although it is possible that a small amount of antigen was transferred into recipient mice at the time of CTL infusion, this seems unlikely for a number of reasons. First, the concentration of LLO₉₁₋₉₉ used for stimulation was only 10^{-9} M, and excess peptide was washed from cells at the time of stimulation. Second, APCs used for CTL stimulation were irradiated and destroyed during stimulation. Third, CTL cultures were incubated for a minimum of 7 days prior to infusion into recipients, providing ample time for serum and cellular proteases to destroy residual LLO₉₁₋₉₉. Thus, in vivo CD40 stimulation stimulates *L. monocytogenes*-specific CD8 T cells in the absence of cognate interaction with the specific MHC-epitope complex. This result suggests that effector CD8 T cells interact with surrounding cells in an antigen-independent fashion, receiving stimuli that alter their differentiation and potential for memory function. The generation of immunologic synapses between T lymphocytes and dendritic cells in the absence of cognate antigen is consistent with

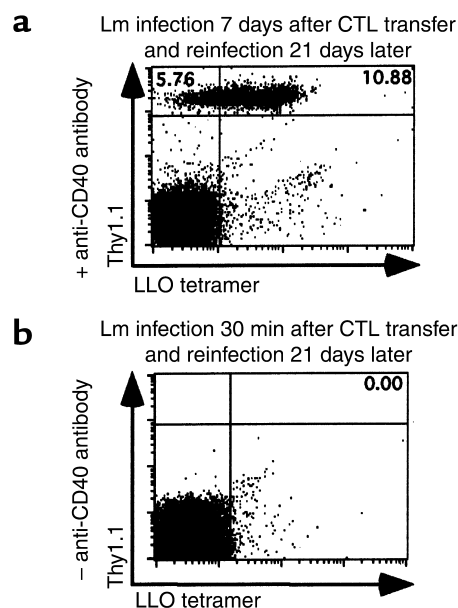


Figure 8

In vivo CD40 stimulation promotes differentiation of memory T cells from infused effector T cells. (a) CTLs were infused into recipient mice that received anti-CD40 antibody 1 day and 2 days earlier. Recipients were infected 7 days later and also received another dose of anti-CD40 antibody 12 hours after the infection. Three weeks later, mice were rechallenged with 100,000 wild-type *L. monocytogenes*, and 72 hours later, splenocytes were stained for CD8 α , Thy1.1, and CD62L, and with LLO₉₁₋₉₉ H2-K^d tetramers. Dot plots are gated on live CD8 T cells. Thy1.1 is shown on the y axis, and tetramer staining is shown on the x axis. Numbers in the upper quadrants represent percentage of total CD8 T cells. (b) Recipient mice were infected 30 minutes after CTL infusion without anti-CD40 antibody administration. These recipients were challenged 3 weeks later and analyzed as described in a. These plots are representative of three animals per group.

the notion that antigen-independent, intercellular interactions might provide meaningful stimuli that modify T cell function (37, 38).

Since CD40 stimulation did not increase the number of transferred *L. monocytogenes*-specific CD8 T cells in naive recipients, it is unlikely that increased in vivo survival or proliferation of infused CTLs accounted for their enhanced ability to provide protection. It is more likely that CTLs were reprogrammed upon infusion, optimizing their ability to proliferate and exert effector functions upon bacterial challenge. Several recent studies suggested that CD8 T cells contain internal programs that regulate their proliferation and differentiation (39–42). It is possible that anti-CD40 antibody directly ligates CD40 on transferred CD8 T cells. In fact, low-level CD40 receptor expression is detectable on the in vitro-expanded CTLs and did not change after transfer into naive or anti-CD40 antibody-treated mice (data not shown). The role of CD40 expressed on CD8 T cells was recently investigated in wild-type and CD40-deficient mice. In wild-type mice, agonistic CD40 antibody administration resulted in full activation of naive transgenic CD8 T cells responding to soluble ovalbumin. However, this effect was indirect in that CD40 receptor^{+/+} CD8 T cells transferred into CD40 receptor^{-/-} mice did not respond to ovalbumin immunization and CD40 stimulation (43). Thus, it is more likely that CD40 ligation remodels the immune compartment, providing a more suitable milieu for CTL expansion by upregulating, for example, MHC, costimulatory, and/or adhesion molecules (44, 45).

Although direct intercellular contacts may mediate the effect of CD40 stimulation in our system, cytokines such as IL-2, IL-7, and IL-15 may also contribute to the maintenance of CD8 CTL responsiveness. Ostrowski et al. investigated the effect of CD40 ligation on T cell responses of HIV-1-infected individuals in a peptide-pulsed, dendritic cell-based coculture system. In the absence of CD4 T cells, CD40 activation induced IL-15, but not IL-2 (9). Thus, CD40 activation might shift the balance toward cytokines that promote CTL survival. A murine model of T cell responses to staphylococcal enterotoxin A demonstrated an antiapoptotic effect of CD40 activation, resulting in enhanced CD4 and CD8 T cell expansion (46). Finally, it is possible that CTL expansion reflects enhanced trafficking of T cells into the spleen due to CD40-enhanced chemokine-chemokine receptor interactions (47). Differentiating between these different mechanisms will require further investigation.

Our findings support the therapeutic potential of CD40 activation (48–51) and extend its potential application to the rescue of adoptively transferred pathogen-specific CTLs. Especially in states of profound CD4 T cell deficiency, such as HIV disease, and in bone marrow transplantation patients cellular immune reconstitution with antigen-specific CD8 T cells remains a formidable challenge. In vivo CD40 ligation might represent an additional approach to improving the in vivo efficacy of adoptively transferred pathogen- and tumor-specific T cells.

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