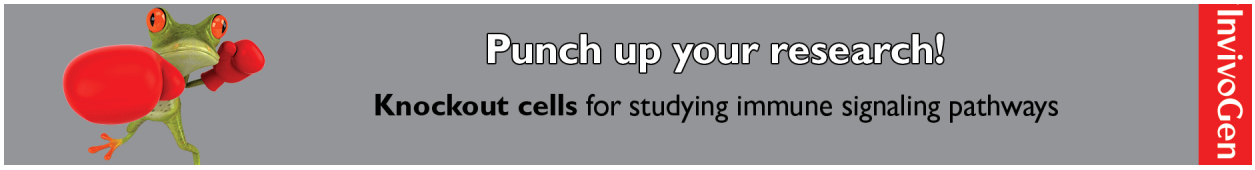

The Journal of Immunology

Role of CD4 T Cell Help and Costimulation in CD8 T Cell Responses During *Listeria monocytogenes* Infection

Devon J. Shedlock, Jason K. Whitmire, Joyce Tan, Andrew S. MacDonald, Rafi Ahmed and Hao Shen

This information is current as of May 16, 2017.

J Immunol 2003; 170:2053-2063; ;
doi: 10.4049/jimmunol.170.4.2053
http://www.jimmunol.org/content/170/4/2053

References This article **cites 77 articles**, 46 of which you can access for free at:
http://www.jimmunol.org/content/170/4/2053.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2003 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

Role of CD4 T Cell Help and Costimulation in CD8 T Cell Responses During *Listeria monocytogenes* Infection[1]

Devon J. Shedlock,[2]* Jason K. Whitmire,[2,3‡] Joyce Tan,[4‡] Andrew S. MacDonald,[†] Rafi Ahmed,[‡] and Hao Shen[5]*

CD4 T cells are known to assist the CD8 T cell response by activating APC via CD40-CD40 ligand (L) interactions. However, recent data have shown that bacterial products can directly activate APC through Toll-like receptors, resulting in up-regulation of costimulatory molecules necessary for the efficient priming of naive T cells. It remains unclear what role CD4 T cell help and various costimulation pathways play in the development of CD8 T cell responses during bacterial infection. In this study, we examined these questions using an intracellular bacterium, *Listeria monocytogenes,* as a model of infection. In CD4 T cell-depleted, CD4$^{-/-}$, and MHC class II$^{-/-}$ mice, *L. monocytogenes* infection induced CD8 T cell activation and primed epitope-specific CD8 T cells to levels commensurate with those in normal C57BL/6 mice. Furthermore, these epitope-specific CD8 T cells established long-term memory in CD4$^{-/-}$ mice that was capable of mounting a protective recall response. In vitro analysis showed that *L. monocytogenes* directly stimulated the activation and maturation of murine dendritic cells. The CD8 T cell response to *L. monocytogenes* was normal in CD40L$^{-/-}$ mice but defective in CD28$^{-/-}$ and CD137L$^{-/-}$ mice. These data show that in situations where infectious agents or immunogens can directly activate APC, CD8 T cell responses are less dependent on CD4 T cell help via the CD40-CD40L pathway but involve costimulation through CD137-CD137L and B7-CD28 interactions. ***The Journal of Immunology,*** **2003, 170: 2053–2063.**

The murine model of listeriosis has proven to be a powerful system for the investigation of immune responses to bacterial infection (1). *Listeria monocytogenes* is a Gram-positive, facultative intracellular bacterium that replicates within the cytosol of both phagocytic and nonphagocytic cells following bacteria-mediated endosomal escape (2). Once in the host cell cytosol, this bacterium employs an actin-based mechanism of locomotion to spread into neighboring cells without encountering the extracellular milieu (3, 4). Upon *L. monocytogenes* infection, activated macrophages, neutrophils (5), NK cells (6), and $\gamma\delta$ T cells (7) are critical for the initial control of bacterial growth. Adaptive immune responses take several days to develop and play no apparent role in the early control of a primary infection. Upon reinfection, Ag-specific memory T cells mount a prompt response, mediating rapid bacterial clearance and providing protective immunity against otherwise lethal challenges (8). Although it has been well established that CD8 T cells play a critical role in protective immunity to *L. monocytogenes* (9–11), the contribution of CD4 T cells to antilisterial immunity remains less well defined. Inconsistent results have been reported from studies using in vivo depletion of CD4 T cells, the adoptive transfer of purified T cell subsets, and CD4 T cell KO mice (10, 12–14). During a primary response, it is known that naive CD4 T cells directly contribute to antilisterial defense by differentiating into Th1 cells (15) and facilitating the granulomatous response (16). In addition, CD4 T cells have been shown to work synergistically with CD8 T cells in mediating protective immunity (17). Thus, it is hypothesized that CD4 T cells provide help for the efficient induction of CD8 T cell responses during *L. monocytogenes* infection.

The development of primary CD8 T cell responses is thought to be assisted directly by CD4 T cells in two ways: 1) the activation of professional APC, which increases their ability to deliver costimulation to Ag-specific naive CD8 T cells and 2) the secretion of cytokines that facilitate CTL expansion and activity (18). The latter pathway of CD4 T cell help has long been recognized and involves the differentiation of naive CD4 T cells into Th1 helpers that secrete distinctive patterns of cytokines, which augment CTL expansion and enhance the production of cytotoxic molecules (19). However, several recent studies have indicated that the first pathway is the dominant form of CD4 T cell help for the priming of naive CD8 T cells (20–23). This pathway entails the engagement of the CD40 ligand (L)[6] on CD4 T cells to the CD40 molecule on dendritic cells (DC). DC activation and maturation ensues, resulting in the up-regulation of B7-1 (CD80) and B7-2 (CD86) on DC, which subsequently interact with CD28 on CD8 T cells and provide the costimulatory signal for the efficient priming of naive Ag-specific CD8 T cells (24, 25). Additional costimulatory molecules, such as TNFR superfamily members like CD137 (4-1BB),

Departments of *Microbiology and [†]Pathobiology, University of Pennsylvania, Philadelphia, PA 19104; and [‡]Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322

Received for publication July 29, 2002. Accepted for publication December 16, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants AI-45025 and AI-46184 (to H.S.) and AI-30048 (to R.A.), and National Institutes of Health Training Grant AI-07632 (to D.J.S.).

[2] D.J.S. and J.K.W. contributed equally to this work.

[3] Current address: Department of Neuropharmacology, CVN9, The Scripps Research Institute, 10550 North Torrey Pines Road, San Diego, CA 92037.

[4] Current address: Department of Immunology, IMM26, The Scripps Research Institute, 10550 North Torrey Pines Road, San Diego, CA, 92037.

[5] Address correspondence and reprint requests to Dr. Hao Shen, Department of Microbiology, University of Pennsylvania School of Medicine, 3610 Hamilton Walk, Philadelphia, PA 19104-6076. E-mail address: hshen@mail.med.upenn.edu

[6] Abbreviations used in this paper: L, ligand; TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; rLm, recombinant *L. monocytogenes*; LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; NP, nucleoprotein; LLO, listeriolysin O; DC, dendritic cell; HKLm, heat-killed *L. monocytogenes*; MOI, multiplicity of infection.

Copyright © 2003 by The American Association of Immunologists, Inc.

0022-1767/03/$02.00

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

also play a role in T cell activation (26–29). Ligation of CD137 with its ligand (CD137L), which is expressed on NK cells, B cells, macrophages and DC, provides a CD28-independent costimulatory signal leading to CD4 and CD8 T cell expansion, cytokine production, development of CTL effector function, and promotion of cell survival (26, 27, 30–33). Although "conditioning" of APC by CD4 T cells through costimulatory interactions has been well established as a major form of help for CD8 T cell responses to model Ags, the requirements and mechanisms of CD4 T cell help in vivo are not fully understood, particularly for CD8 T cell responses to bacterial infection.

It has been shown recently that many bacterial products are recognized by Toll-like receptors (TLR) on DC and macrophages. These include recognition of peptidoglycan by the heterodimer of TLR2 and TLR6, LPS and lipoteichoic acid by TLR4, flagellin by TLR5, and bacterial DNA by TLR9 (34–37). Stimulation with these bacterial products induces DC activation and maturation, upregulates MHC class II, CD80, and CD86, and enhances their ability to activate T cells in secondary lymphoid organs (35). These recent findings suggest that bacterial infection may directly activate DC through TLR without the engagement of CD40-CD40L interaction with CD4 T cells and thus allow efficient priming of CD8 T cells in the absence of CD4 T cell help. In this context, it is not known what role various costimulatory pathways may play in the induction of the CD8 T cell response to bacterial infection.

In this study, we examined quantitatively the contribution of CD4 T cells to the induction, maintenance, and recall response of *Listeria*-specific CD8 T cells. We used recombinant *L. monocytogenes* (rLm) expressing well-defined MHC class I-restricted epitopes to characterize Ag-specific CD8 T cell responses. Our results show that rLm infection induces similar levels of CD8 T cell activation in normal C57BL/6 and CD4 T cell-deficient mice. Ag-specific CD8 T cell responses developed normally during the initial expansion phase but established a slightly lower level of immunological memory in the absence of CD4 T cell help. These memory CD8 T cells persisted in $CD4^{-/-}$ mice and were capable of mounting a protective recall response, although fewer effector cells were generated during the recall response compared with C57BL/6 mice. We further showed that incubation of murine DC with heat-killed or live *L. monocytogenes* resulted in DC activation and maturation that were accompanied by up-regulation of B7-1 and B7-2 expression. Analysis of requirements for costimulation showed that although *Listeria*-specific T cell responses were induced independently of CD40-CD40L interactions, they were dependent upon the B7-CD28 pathway. In addition, T cell activation and epitope-specific CD8 T cell expansion was moderately affected in $CD137L^{-/-}$ mice. Altogether, these data are consistent with a model whereby *L. monocytogenes* infection bypasses CD4 T cell help by directly activating APC to prime epitope-specific CD8 T cells through a CD40-independent but CD28-dependent pathway. However, optimal levels of CD8 T cell memory are established in the presence of CD4 T cell help.

Materials and Methods

Mice

Adult male or female C57BL/6, $CD4^{-/-}$ (C57BL/6-*Cd4*$^{tm1\ Mak}$), and MHC class $II^{-/-}$ (C57BL/6Tac-*Abb*^{tm1} N5) mice were purchased from the National Cancer Institute (Frederick, MD), The Jackson Laboratory (Bar Harbor, ME), and Taconic Farms (Germantown, NY), respectively. $CD40L^{-/-}$, 4-1BB$L^{-/-}$, and $CD28^{-/-}$ mice, as described previously by others (30, 38, 39), were bred in-house. Mice were cared for in accordance with Institutional Animal Care and Use Committee-approved protocols at the University of Pennsylvania School of Medicine Animal Facility (Philadelphia, PA) or at Emory University (Atlanta, GA).

Bacteria and virus

The construction of the rLm strains used has been described in detail previously (40). The rLm strain XFL203 (referred to as rLm33 in this report) expresses the glycoprotein epitope gp_{33-41} (gp33) from lymphocytic choriomeningitis virus (LCMV). The gp33 epitope is secreted by rLm33 as part of a listeriolysin O (LLO)-PhoA fusion protein under the control of the hemolysin promoter (40). Similarly, the rLm strain XFL204 (referred to as rLm396) secretes the LCMV nucleoprotein (NP) epitope $NP_{396-404}$ (NP396) as part of an LLO-PhoA fusion protein. Bacterial strains were maintained as −80°C stocks in brain-heart infusion/50% glycerol, inoculated onto brain-heart agar, then grown overnight at 37°C with aeration. The rLm33 strain is slightly attenuated, with a LD_{50} of 5×10^5 CFU in C57BL/6 mice compared with 5×10^4 CFU for the wild-type 10403S strain. Mice were immunized i.v. with a sublethal dose of 5×10^4 CFU of rLm33 (~0.1 LD_{50}). At this sublethal dose (0.1 LD_{50}), bacteria were cleared by day 5 postinfection (p.i). For virus infection, mice were injected i.v. with 1×10^6 PFU of LCMV clone 13 (41). Infectious virus in serum of infected mice was measured by plaque assay on Vero cell monolayers as previously described (41).

In vivo depletion of CD4 T cells

Mice were injected i.p. with 200 μg of purified anti-mouse CD4 mAb (clone GK1.5) 2 days before (day −2) and at the time of infection (day 0) (42), resulting in >96% reduction in the number of splenic CD4 T cells at the time of rLm infection as determined by flow cytometry.

DC generation and activation

DC were generated from murine bone marrow by culture in GM-CSF (PeproTech, Rocky Hill, NJ) for 11 days as described previously (43, 44). Cells generated by this method comprised 95% DC (class II^+CD11c^+), with the remainder of the cells being predominantly granulocytes. No contaminating B cells, macrophages, CD4, or CD8 T cells were generated under these conditions, as determined by FACS using mAbs specific for B220, F4/80, CD4, and CD8-α (data not shown). For activation of DC, bone marrow-derived DC were incubated for 18 h with 100 μg/ml LPS, heat-killed *L. monocytogenes* (HKLm), or live *L. monocytogenes*. HKLm was produced by incubation at 90°C for 30 min, followed by three washes in PBS. For live *L. monocytogenes* stimulation of DC, a mixture of antibiotics including 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml tetracycline was added 4 h following the addition of bacteria.

Flow cytometry

Surface staining was performed using freshly explanted splenocytes that were stained in 1% BSA/PBS (w/v) using fluorochrome-conjugated mAbs, anti-CD8 (clone 53-6.7), anti-CD4 (clone RM4-5), anti-CD44 (clone IM7), and anti-CD62L (MEL-14), purchased from BD PharMingen (San Diego, CA). CD8 T cells specific for gp33 were quantified and phenotyped with MHC class I H-$2D^b/gp_{33-41}$ (D^b/gp33) tetramers (45). After staining, cells were fixed in 2% paraformaldehyde/PBS (w/v), and events were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

IFN-γ ELISPOT and intracellular staining assays

gp33-specific responses were measured by IFN-γ ELISPOT assay as described previously (45, 46). The capture Ab, anti-mouse IFN-γ (clone R4-6A2), and the detection Ab, biotinylated anti-mouse IFN-γ (clone XMG1.2), were purchased from BD PharMingen. gp33 peptide was used at 1 μM to stimulate CD8 T cells. Intracellular cytokine staining was performed using a Cytofix/Cytoperm kit (BD PharMingen) in accordance with the manufacturer's protocol. Briefly, splenocytes were cultured at 37°C with 5% CO_2 for 5 h in complete medium supplemented with 50 U/ml recombinant human IL-2, 1 μl/ml GolgiStop with monensin, and in either the presence or absence of 0.1 μM gp33 (KAVYNFATM) or NP396 (FQPQNGQFT) or 3.0 μM LLO190 (NEKYAQAYPNVS) peptide. Cells were then surface stained with anti-CD4, anti-CD8, and anti-CD44 or anti-CD62L mAbs, washed twice, and then incubated with Cytofix/Cytoperm to permeabilize the plasma membranes. Staining for intracellular cytokines was performed using anti-IFN-γ (clone XMG1.2) or anti-TNF-α (clone MP6-XT22) mAbs (BD PharMingen). Similar results were observed with or without IL-2 in the in vitro culture.

CTL assay

To analyze CTL responses after LCMV clone 13 challenge, splenocytes (4×10^6/well) from infected mice were used directly without in vitro culture in an ex vivo CTL assay. gp33-specific cytotoxic activity was determined in a standard 5-h ^{51}Cr release assay using peptide-coated or uncoated target cells as described previously (47).

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

T cell proliferation assay

Spleen cells from individual mice were plated in triplicate at 8×10^5 cells/well (4×10^6/ml) in 96-well plates and stimulated with HKLm (equivalent to 1×10^6 CFU) or left unstimulated. The cultures were incubated for 48 h at 37°C in 6% CO_2 and then pulsed with 50 Ci/ml [^{3}H]thymidine for 24 h. Plates were harvested using a Tomtec harvester (Tomtec, Hamden, CT), and incorporation of [^{3}H]thymidine was measured using a Microbeta Trilux scintillation counter (Wallac, Gaithersburg, MD).

Results

CD4 T cell responses to L. monocytogenes infection

To analyze a possible role of CD4 T cells in providing help to CD8 T cell responses, we first examined the CD4 T cell response to *L. monocytogenes*. C57BL/6 mice were infected with a sublethal dose of a recombinant *L. monocytogenes* strain, rLm33, which expresses the CD8 T cell epitope gp_{33-41} from LCMV. Bacteria were cleared from spleens and livers by 5 days p.i. (data not shown). To assess the activation of CD4 T cells, we analyzed surface expression of the activation marker CD44 at the peak of the T cell response at 7 days p.i. (Fig. 1*A*). In infected mice, more CD4 T cells exhibited an activated (CD44^{high}) phenotype in comparison to naive mice (Fig. 1*A*). This activation was accompanied by the expansion of the CD4 T cell population (Fig. 1*B*). The number of total CD4 T cells per spleen expanded 6-fold, from 4×10^6 in naive mice to 25×10^6 cells in infected mice. Thus, *L. monocytogenes* infection induced vigorous activation and expansion of the CD4 T cell subset in C57BL/6 mice, as seen previously (48–50).

To assess the Ag-specific CD4 T cell response, we stimulated splenocytes from rLm33-immunized C57BL/6 mice with HKLm in vitro and measured [^{3}H]thymidine incorporation, a method traditionally used to measure the presence of *Listeria*-specific CD4 T cells (Fig. 1*C*). In this assay, the proliferative response stimulated by HKLm is exclusively due to *Listeria*-specific CD4 T cells and not CD8 T cells or B cells since only in vitro depletion of CD4 T cells abolishes proliferation (51). No proliferation above background levels was detected when splenocytes from naive control mice were stimulated with HKLm, whereas splenocytes from C57BL/6 mice previously immunized with rLm33 proliferated extensively in response to HKLm stimulation (Fig. 1*C*). In addition, we analyzed the CD4 T cell response to a recently identified, MHC class II-restricted epitope ($LLO_{190-201}$) from LLO (52). LLO190-specific CD4 T cells were readily detected by ELISPOT and intracellular IFN-γ staining as they made up 3–5% of total splenic CD4 T cells and reached 1×10^6 cells/spleen on day 7 p.i. (Fig. 1*D* and data not shown). Altogether, these results demonstrate that a robust *Listeria*-specific CD4 T cell response occurs following *L. monocytogenes* infection.

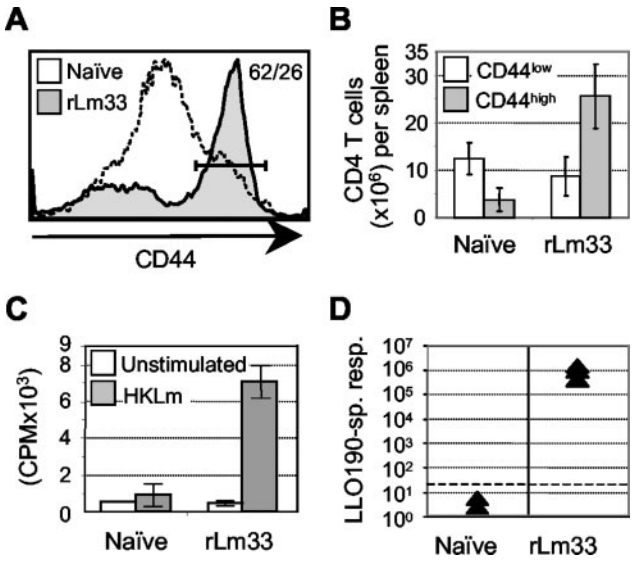

FIGURE 1. CD4 T cell responses to *L. monocytogenes* infection. C57BL/6 mice were infected with a sublethal dose of rLm33. *A*, Activation of CD4 T cells was examined 7 days p.i. by FACS analysis of CD44 expression. Numbers (62/26) indicate percentages of splenocytes that are activated (CD44^{high}) CD4 T cells in infected/naive mice. FACS histograms are from a representative mouse of three in each group. *B*, Absolute numbers of activated (CD44^{high}) and resting (CD44^{low}) CD4 T cells in the spleen of naive and rLm33-infected mice at day 7 p.i. Data represent the average and SD of three mice per group. *C*, *Listeria*-specific CD4 T cell responses were measured by an in vitro proliferative response to HKLm. Splenocytes from naive or rLm33-infected mice were cultured with or without HKLm for 48 h and then pulsed with [^{3}H]thymidine for 24 h. Data represent the average and SD of triplicate assays of three mice per group. *D*, Absolute numbers of LLO_{190}-specific cells per spleen were determined by ELISPOT assay. Each triangle represents a single mouse and the limit of detection is indicated by the dashed line.

CD8 T cell responses to L. monocytogenes infection in CD4 T cell-depleted mice

In light of the strong CD4 T cell response we and others have observed during *L. monocytogenes* infection, we next analyzed the contribution of CD4 T cells to CD8 T cell responses by depleting CD4 T cells in vivo using anti-CD4 mAbs (GK1.5). As expected, few CD4 T cells were detected in the spleens of anti-CD4 mAb-treated mice (Fig. 2*A*). These and untreated control mice were infected with a sublethal dose of rLm33. Bacteria were cleared from spleens and livers of both CD4 T cell-depleted and untreated mice by day 5 p.i. (data not shown). Expression of CD44 was up-regulated on CD8 T cells in rLm33-infected mice compared with naive mice (Fig. 2*A*). Similar levels of CD8$^+$CD44^{high} cells were observed between CD4-depleted and untreated control mice that were infected with rLm33, with the majority of cells exhibiting an activated (CD44^{high}) phenotype. Interestingly, slightly fewer CD44^{low} cells were present in the CD4-depleted than in the undepleted mice. Although the reason for this minor difference is not known, the results nevertheless indicate that a complete pool of CD4 T cells is not required for the activation of CD8 T cells during *L. monocytogenes* infection.

To examine the generation of epitope-specific CD8 T cells, we quantified CD8 T cells specific to the gp33 epitope in rLm33-infected mice by intracellular IFN-γ staining (Fig. 2*B*). In CD4 T cell-depleted mice, ~2.1% ± 0.4 of splenic CD8 T cells from three mice produced IFN-γ after in vitro stimulation with gp33 peptide and a similar frequency (2.1% ± 1.2) was detected in mice not depleted of CD4 T cells. IFN-γ-producing cells all expressed a CD44^{high} phenotype as expected and were specific to the gp33 epitope, since neither cultures without peptide nor splenocytes from uninfected mice that were stimulated with gp33 had IFN-γ-producing cells. Thus, similar frequencies of gp33-specific IFN-γ-producing CD8 T cells were induced by infection with rLm33 in both untreated and CD4 T cell-depleted mice, suggesting that CD4 T cells are not required for the generation of epitope-specific CD8 T cells.

CD8 T cell response to L. monocytogenes infection in CD4$^{-/-}$ and MHC II$^{-/-}$ mice

Results from rLm33-infected mice depleted of CD4 T cells suggest that CD4 T cells are not required for the priming of naive CD8 T cells during *L. monocytogenes* infection. However, in vivo mAb-mediated depletion of CD4 T cells is transient and may not be 100% effective. Thus, the possibility exists that a small residual population of CD4 T cells may be sufficient to influence the CD8

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

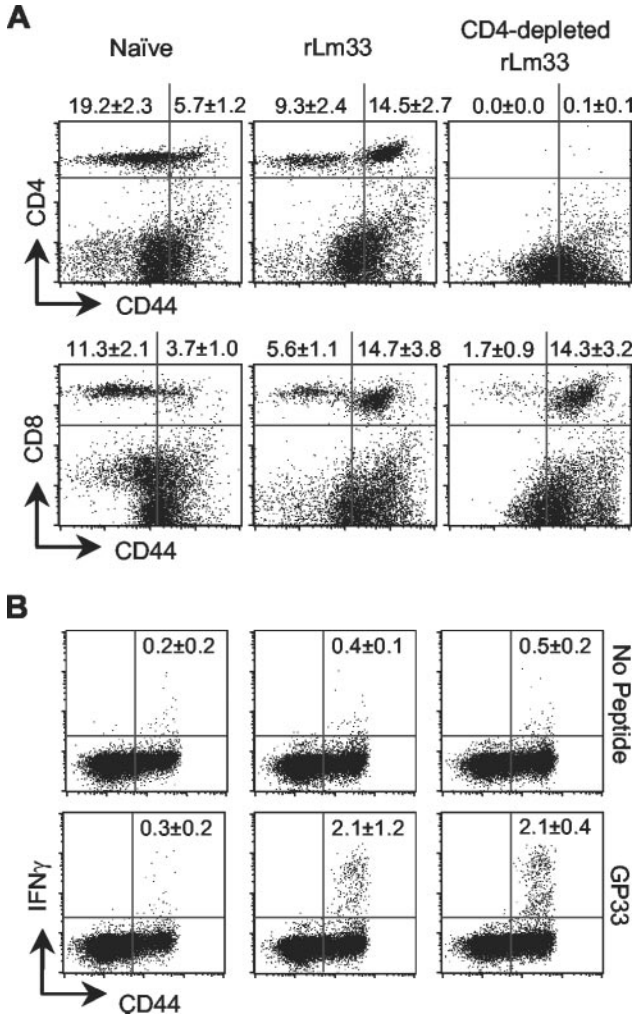

FIGURE 2. CD8 T cell responses in CD4 T cell-depleted mice following *L. monocytogenes* infection. C57BL/6 mice were depleted of CD4 T cells by in vivo administration of anti-CD4 mAb (GK1.5). CD4 T cell-depleted and control mice were infected with a sublethal dose of rLm33. *A*, Activation of CD4 and CD8 T cells was examined on day 7 p.i. by FACS analysis of CD44 expression. Numbers above gates indicate mean percentage and SD of splenocytes that are activated ($CD44^{high}$) or resting ($CD44^{low}$) CD4 and CD8 T cells. *B*, gp33-specific cells were quantified by intracellular IFN-γ staining after in vitro stimulation of splenocytes with or without gp33 peptide. FACS plots are gated on $CD8^+$ cells and numbers within gate indicate the mean percentage and SD of CD8 T cells that produce IFN-γ. Each FACS plot is a representative mouse of three from each group. Similar results were observed from two independent experiments.

T cell response to *L. monocytogenes* infection. To resolve this issue, we examined CD8 T cell responses in CD4 T cell-deficient mice following infection with rLm33.

Similar to CD4 T cell-depleted mice, $CD4^{-/-}$ mice were fully capable of controlling rLm33 infection in the spleens and livers by day 5 p.i. (data not shown). On day 7 p.i., the activated CD8 T cell population ($CD8^+CD44^{high}$) in the spleens of $CD4^{-/-}$ mice rose from 7.1% ± 1.4 in naive to 28% ± 7.7 in infected mice, compared with an increase from 4.5% ± 0.9 in naive to 25% ± 5.2 in infected C57BL/6 mice. These results show that the activation of CD8 T cells was not diminished in $CD4^{-/-}$ mice (Fig. 3*A*). The slightly higher percentage of activated CD8 T cells in $CD4^{-/-}$ mice may reflect an enrichment of the CD8 T cell population due to the lack of CD4 T cell expansion. To analyze the expansion quantitatively, we compared the absolute numbers of total splenocytes and of CD8 T cells in C57BL/6 and $CD4^{-/-}$ mice (Fig. 3*B*). On day 7 p.i., the number of total splenocytes expanded from 6×10^7 to 18×10^7 cells in C57BL/6 but only to 13×10^7 cells in $CD4^{-/-}$ mice. This reduced expansion of total splenocytes in $CD4^{-/-}$ mice was most likely due to the lack of CD4 T cells but might also reflect reduced CD8 T cell and/or B cell proliferation and macrophage infiltration that are dependent on CD4 T cell help. Although the CD8 T cell populations in both C57BL/6 and $CD4^{-/-}$ mice expanded to ~41×10^6 cells/spleen, the fold of expansion was less in $CD4^{-/-}$ mice (2.6-fold) than in C57BL/6 mice (4.3-fold). This reduced expansion may be due in part to the fact that $CD4^{-/-}$ mice started with a greater number of CD8 T cells (18×10^6/spleen in naive $CD4^{-/-}$ compared with 10×10^6/spleen in naive C57BL/6 mice, Fig. 3*B*; Ref. 53). Notably, almost all of the CD8 T cell expansion in both mouse strains was due to increases in the number of activated $CD8^+CD44^{high}$ cells, while the number of resting $CD8^+CD44^{low}$ cells declined slightly or remained relatively unchanged after rLm33 infection (Fig. 3*B*). On the other hand, $CD8^+CD44^{low}$ cells, as the percentage of total splenocytes, decreased markedly after rLm33 infection (Fig. 3*A*). This was likely attributable to expansion of activated T and B cells and infiltration of phagocytic cells, thus making $CD8^+CD44^{low}$ cells a smaller percentage of total splenocytes. Thus, *L. monocytogenes* infection induced similar activation and expansion of CD8 T cells in normal C57BL/6 and $CD4^{-/-}$ mice.

We next examined whether rLm33 infection of $CD4^{-/-}$ mice generated epitope-specific CD8 T cells, as was observed in mAb-mediated CD4 T cell-depleted mice. At day 7 p.i., gp33-specific CD8 T cells were detected in spleens of both C57BL/6 and $CD4^{-/-}$ mice, as measured by tetramer and intracellular IFN-γ staining (Fig. 3, *C* and *D*). Approximately 2.9% ± 0.9 of splenic CD8 T cells from rLm33-infected C57BL/6 mice produced IFN-γ after in vitro stimulation with the gp33 peptide, whereas 1.6% ± 0.3 of splenic CD8 T cells in rLm33-infected $CD4^{-/-}$ mice were specific to the gp33 epitope at day 7 (Fig. 3*D*). Similar levels of gp33-specific CD8 T cells at day 7 p.i. were also detected by D^b/gp33 tetramer staining in rLm33-infected normal and $CD4^{-/-}$ mice, 2.46% ± 0.8 and 1.66% ± 0.2, respectively (Fig. 3*C*). Furthermore, we infected MHC $II^{-/-}$ mice with rLm33 and rLm396, which expresses an H-$2D^b$-restricted $NP_{396-404}$ epitope of LCMV, and observed similar levels of epitope-specific T cell responses between MHC $II^{-/-}$ and normal C57BL/6 mice for both epitopes (data not shown). Together, the results from CD4 T cell-depleted, $CD4^{-/-}$ and MHC $II^{-/-}$ mice, using two different CD8 T cell epitopes, clearly demonstrate that *L. monocytogenes* infection induces normal CD8 T cell responses in the absence of CD4 T cell help.

Establishment, maintenance, and functionality of CD8 T cell memory following L. monocytogenes infection of $CD4^{-/-}$ mice

Our results have clearly demonstrated that CD4 T cells are not required for inducing epitope-specific CD8 T cell responses to rLm infection. We next examined the role of CD4 T cells in the establishment and maintenance of memory CD8 T cells by enumerating gp33-specific CD8 T cells at different days p.i. with rLm33. At days 7 and 15, the frequencies and total numbers of gp33-specific CD8 T cells in C57BL/6 and $CD4^{-/-}$ mice were not significantly different (Fig. 4). By day 21, $CD4^{-/-}$ mice had ~5-fold fewer gp33-specific cells than C57BL/6 mice, indicating a slightly less efficient establishment of memory CD8 T cells in the absence of CD4 T cell help. However, from days 21 to 150, the frequencies and total numbers of gp33-specific CD8 T cells did not signifi-

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

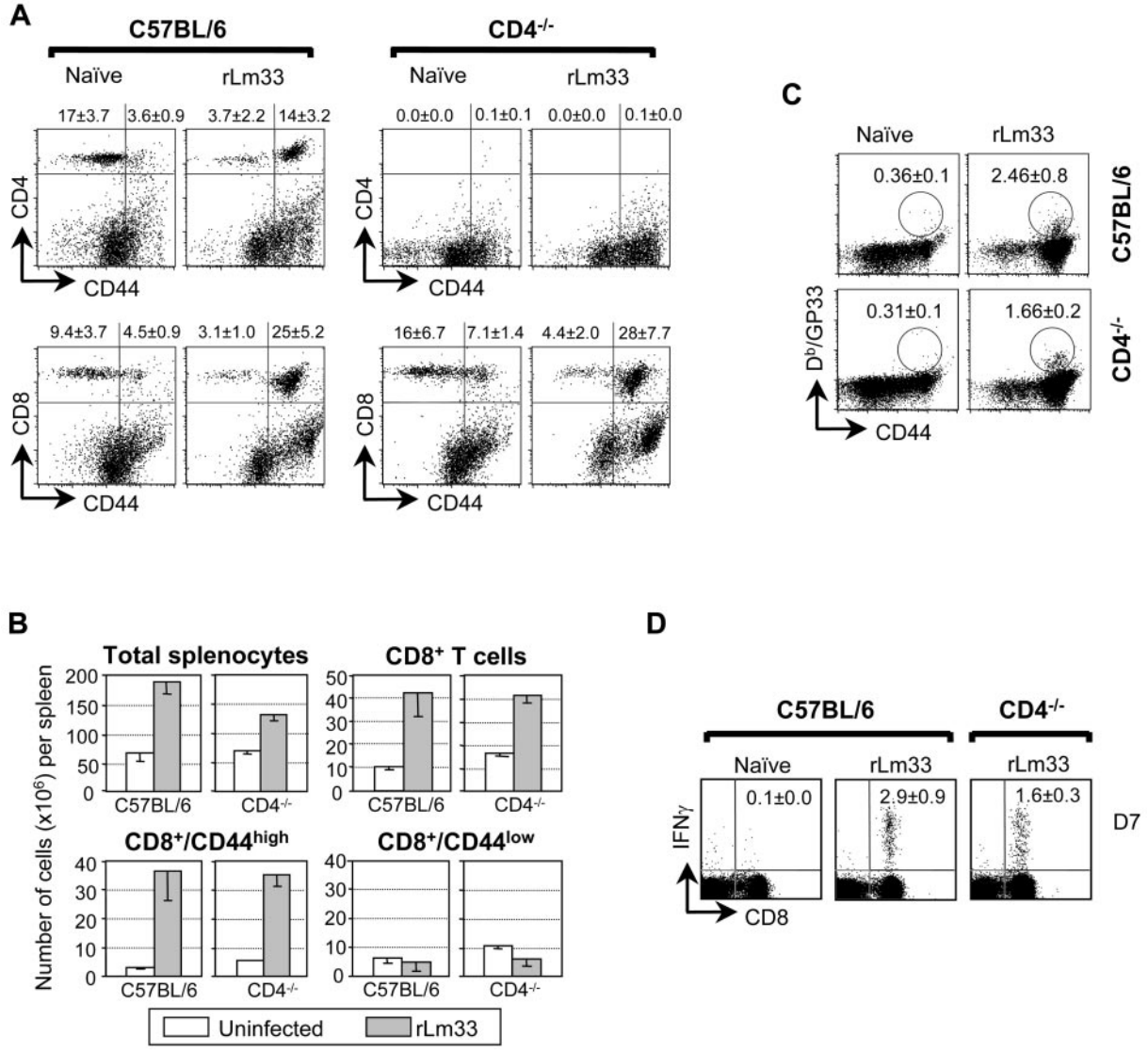

FIGURE 3. CD8 T cell responses to *L. monocytogenes* infection in CD4$^{-/-}$ mice. C57BL/6 and CD4$^{-/-}$ mice were infected with a sublethal dose of rLm33 and CD8 T cell activation and expansion were analyzed on day 7 p.i. *A*, FACS analysis of CD44 expression on CD4 and CD8 T cells. Numbers indicate mean percentage and SD of splenocytes that were activated (CD44^{high}) or resting (CD44^{low}) CD4 and CD8 T cells. *B*, Absolute numbers of total splenocytes, CD8 T cells, activated (CD8$^+$CD44^{high}) and resting (CD8$^+$CD44^{low}) CD8 T cells in the spleen of uninfected and rLm33-infected mice. *C*, Tetramer D^b/gp33$^+$ CD8 T cells in rLm33-infected C57BL/6 and CD4$^{-/-}$ mice. Numbers above gates represent mean percentage and SD of tetramer-positive CD8 T cells in the spleen. *D*, Induction of gp33-specific CD8 T cells in CD4$^{-/-}$ mice following rLm33 infection. gp33-specific CD8 T cells were determined by staining for intracellular IFN-γ of splenocytes after in vitro stimulation with gp33 peptide. Shown are FACS plots with numbers within gate indicating the mean percentage and SD of CD8 T cells that were specific to the gp33 epitope. Each FACS dot plot in *A*, *C*, and *D* is from a mouse representative of three per group. Data in *B* represent the averages of three mice per group, with lower error bars indicating SD. Similar results were observed from three independent experiments.

cantly change in the spleens of either C57BL/6 or CD4$^{-/-}$ mice. Thus, epitope-specific CD8 T cells are maintained up to at least 150 days in CD4$^{-/-}$ mice, indicating that CD4 T cells are not required for the long-term persistence of memory CD8 T cells.

We next investigated whether gp33-specific memory CD8 T cells were capable of conferring protection in the absence of CD4 T cell help. We challenged rLm33-immunized C57BL/6 and CD4$^{-/-}$ mice with clone 13, a virulent strain of LCMV that causes immunosuppression and a prolonged infection in naive mice. C57BL/6 and CD4$^{-/-}$ mice previously immunized with rLm33 exhibited massive activation of CD8 T cells in response to challenge with clone 13, with a majority of CD8 T cells expressing an activated (CD44^{high}) phenotype at 7 days postchallenge (Fig. 5*A*). This is in contrast to nonimmunized mice which had relatively low levels of CD8 T cell activation following LCMV clone 13 infection. The enhanced activation of CD8 T cells in rLm33-immunized mice correlated with a strong recall response mounted by the gp33-specific memory CD8 T cells in both C57BL/6 and CD4$^{-/-}$ mice (Fig. 5*B*). On day 7 after LCMV challenge, C57BL/6 mice previously immunized with rLm33 had 10-fold more gp33-specific IFN-γ-producing cells (~3.1 × 10^5/spleen) than nonimmunized mice (~3.5 × 10^4/spleen). Similarly, CD4$^{-/-}$ mice previously immunized with rLm33 mounted a stronger gp33-sepcific response than nonimmunized mice (~1.5 × 10^5 cells in rLm immunized compared with ~2.2 × 10^4 cells in nonimmunized). A 3-fold greater number of IFN-γ-producing cells was detected in rLm-immunized C57BL/6 mice than in rLm-immunized CD4$^{-/-}$ mice. These data suggest that although CD4 T cells are not required

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

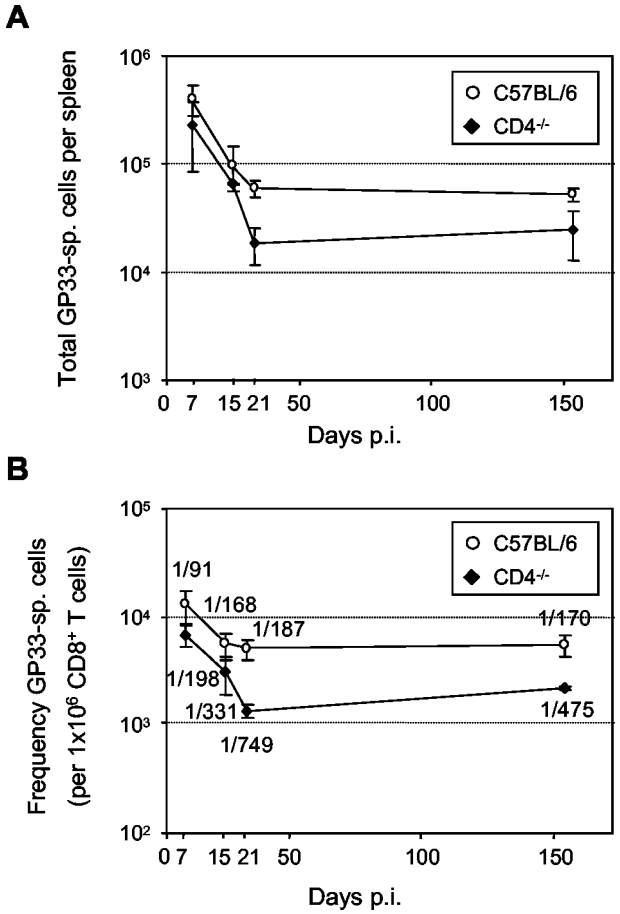

FIGURE 4. gp33-specific CD8 T memory cells are maintained in CD4$^{-/-}$ mice following rLm33 infection. gp33-specific CD8 T cells in the spleens of C57BL/6 and CD4$^{-/-}$ mice were quantified by ELISPOT on days 7, 15, 21, and 150 following rLm33 infection. *A*, Total number of gp33-specific cells per spleen. *B*, Frequency of gp33-specific cells per 1×10^6 CD8 T cells. Numbers within the graph represent the frequency of Ag-specific cells within the CD8 T cell population at each time point. Data represent the averages of at least three mice per group, with error bars indicating SD.

during the recall response of memory CD8 T cells they may augment gp33-specific CD8 T cell expansion.

In addition to secreting cytokines, another effector function of CD8 T cells critical for the control of LCMV infection is cytotoxicity. We thus measured CTL activities by performing direct ex vivo CTL assays using gp33 peptide-coated targets (Fig. 5*C*). There was little to no killing by splenocytes from naive mice challenged with clone 13. Splenocytes from both C57BL/6 and CD4$^{-/-}$ mice previously immunized with rLm33 and then challenged with clone 13 were able to mediate target cell lysis, although higher levels of cytolysis was observed in C57BL/6 than in CD4$^{-/-}$ mice. The higher levels of killing by cells from C57BL/6 mice likely reflect greater numbers of gp33-specific T cells in these mice, as shown in Fig. 5*B*. These results indicate that memory CD8 T cells from rLm-immunized CD4$^{-/-}$ mice were functional effectors capable of IFN-γ secretion and the killing of target cells.

The enhanced recall response by memory gp33-specific CD8 T cells provided protection against challenge with LCMV clone 13 in both C57BL/6 and CD4$^{-/-}$ mice (Fig. 5*D*). At day 21 postchallenge, nonimmunized C57BL/6 and CD4$^{-/-}$ mice had high levels of virus in the blood ($\sim 6 \times 10^4$ PFU/ml of serum). In contrast, rLm33-immunized C57BL/6 mice had no detectable virus while immunized CD4$^{-/-}$ mice exhibited a 100- to 1000-fold decrease in viral titers compared with naive mice ($<10^2$ PFU/ml of serum). The slight differences in protection between immunized C57BL/6 and CD4$^{-/-}$ mice correlate with fewer gp33-specific IFN-γ-producing cells and lower gp33-specific cytotoxicity observed in CD4$^{-/-}$ mice (Fig. 5, *B* and *C*). Nevertheless, these results show that rLm33 immunization of CD4$^{-/-}$ mice induces memory CD8 T cells that are capable of mounting a recall response that provides protective immunity against challenge with the LCMV clone 13.

Direct activation of DC by L. monocytogenes

Our results have clearly demonstrated that CD8 T cell responses can be generated in the absence of CD4 T cells following *L. monocytogenes* infection. Since CD4 T cells are known to help CD8 T cell responses by activating APC such as DC, we next examined the possibility of an alternative pathway of DC activation. Recent data have shown that bacterial products can directly activate macrophages and DC through TLR; thus, we analyzed the effect of *L. monocytogenes* on DC activation and maturation. We cultured bone marrow-derived immature DC with HKLm or live *L. monocytogenes* in vitro and measured the surface expression of MHC class II, B7-1, B7-2, and CD40, markers known to be up-regulated upon DC activation (Fig. 6). HKLm at a multiplicity of infection (MOI) of 5 and 500 stimulated up-regulation of these molecules in a dose-dependent manner and to levels comparable to those induced by LPS stimulation. Incubation of DC with live *L. monocytogenes* also stimulated DC activation. Although HKLm at a MOI of 500 stimulated DC activation similar to that of LPS, live *L. monocytogenes* at a MOI of only 0.1 exhibited this same effect. Furthermore, incubation with live *L. monocytogenes* at a 1 log greater dosage (MOI = 1.0) stimulated DC activation marker up-regulation that surpassed that of 100 μg/ml LPS, with the greatest effect on the expression of B7-2. These data indicate that *L. monocytogenes* can directly activate DC and induce surface expression of costimulatory molecules necessary for efficient priming of naive T cells.

Role of CD40-CD40L, CD137-CD137L, and CD28-B7 interactions for the induction of CD8 T cell responses to L. monocytogenes

Activation of APC by CD4 T cells depends on CD40-CD40L interaction and results in up-regulation of B7-1/B7-2 molecules, which in turn interact with CD28 and provide a costimulatory signal for efficient priming of naive CD8 T cells. The CD137-CD137L interaction is known to provide a CD28-independent costimulatory signal to CD8 T cells. We examined whether blockade of these costimulatory interactions would reduce CD8 T cell responses to *L. monocytogenes* infection. Mice deficient in CD40L, CD137L, or CD28 were infected with rLm33, which was cleared in the spleens and livers of CD40L$^{-/-}$, CD137L$^{-/-}$, and C57BL/6 mice by day 7 p.i. (data not shown). In contrast, CD28$^{-/-}$ mice retained a very low bacterial load ($\sim 2.8 \times 10^3$ CFU/spleen on day 7) but cleared the infection by day 13, indicating an increased susceptibility as seen previously by Mittrücker et al. (54).

Activation of CD4 and CD8 T cells was measured by staining for CD44 surface expression on day 7 after rLm33 infection of C57BL/6, CD40L$^{-/-}$, CD137L$^{-/-}$, and CD28$^{-/-}$ mice (Fig. 7*A*). In CD40L$^{-/-}$ mice, both CD4 and CD8 T cells were activated to levels comparable to those observed in C57BL/6 mice, with the majority of CD4 and CD8 T cells exhibiting the activated (CD44^{high}) phenotype. In contrast, activation of CD4 and CD8 T cells was greatly reduced in CD28$^{-/-}$ mice, and the percentages of

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

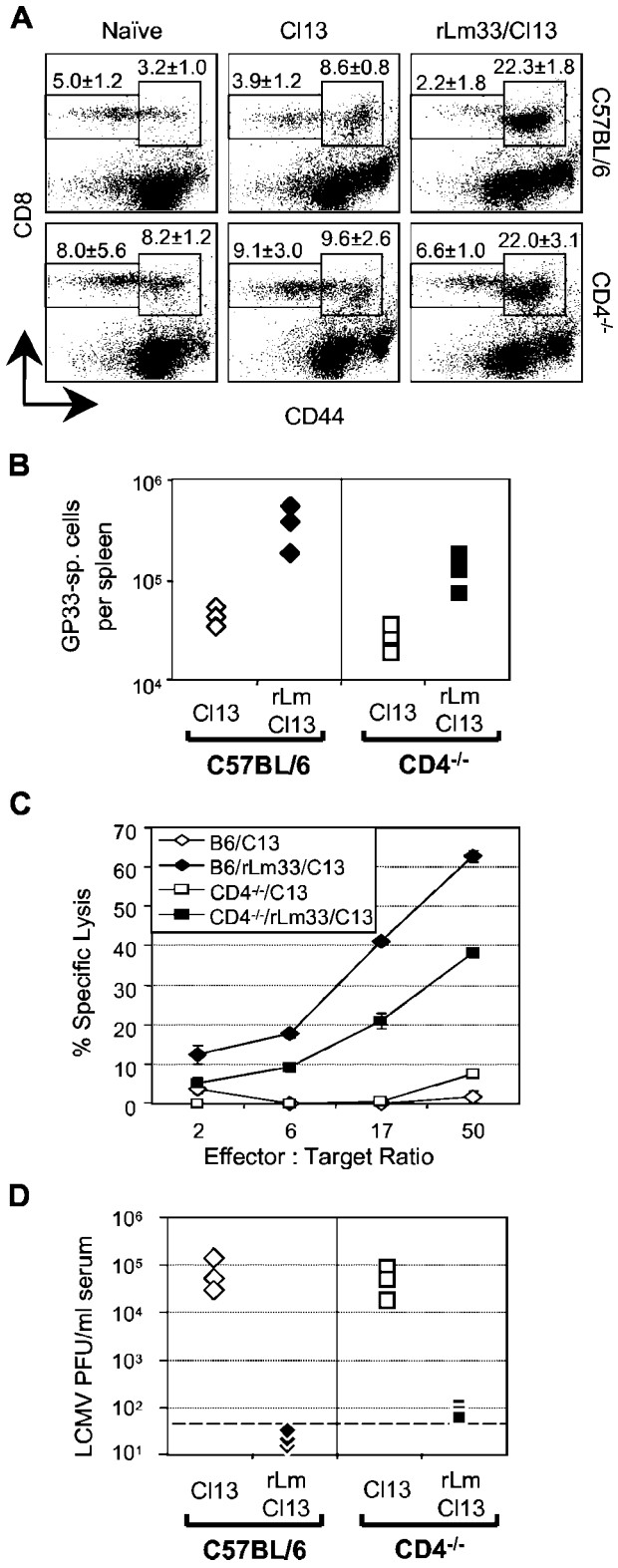

FIGURE 5. Recall response of memory CD8 T cells in the absence of CD4 T cell help. C57BL/6 and CD4$^{-/-}$ mice were immunized with rLm33. These immunized and nonimmunized control mice were then challenged with LCMV clone 13. On day 7 after LCMV challenge, spleens were harvested from naive, clone 13-infected (Cl13), and rLm33-immunized then clone 13-infected (rLm33/Cl13) mice for analysis of CD8 T cell responses. *A,* Activation of CD8 T cells was assessed by FACS analysis of CD44 expression. Numbers represent mean percentage and SD of splenocytes that are activated (CD44^{high}) or resting (CD44^{low}) CD8 T cells. *B,* Numbers of gp33-specific cells per spleen were determined by ELISPOT assay. *C,* gp33-specific cytotoxicity was measured by ex vivo CTL assay and the percentages of specific lysis represent the averages from three mice per group, with error bars indicating SD. *D,* LCMV titers in the serum were quantified by plaque assay at day 20 after LCMV challenge. A dot plot from a representative mouse of three per group is shown in *A* and each symbol in *B* and *D* represents an individual mouse.

CD4^{+}CD44^{high} and CD8^{+}CD44^{high} cells were at the background levels of uninfected mice. Interestingly, activation of CD4 T cells was relatively normal while CD8 T cell activation was diminished in CD137L$^{-/-}$ mice. Consistent with the activation data, rLm33-infected C57BL/6 and CD40L$^{-/-}$ mice had similar numbers of epitope-specific T cells (~4.5×10^5/spleen at 7 days p.i., Fig. 7*B*) while fewer gp33-specific cells were observed in infected CD28$^{-/-}$ mice ($6.1 \pm 3.9 \times 10^4$/spleen). In mice lacking CD137L, total numbers of gp33-specific CD8 T cells were slightly lower than in C57BL/6 and CD40L$^{-/-}$ mice but higher than in CD28$^{-/-}$ mice. These data indicate that although the CD40-CD40L pathway is not required, signaling through the CD137-CD137L and CD28-B7 pathways is important for naive CD8 T cell activation and proliferation during *L. monocytogenes* infection.

Discussion

Help provided by CD4 T cells to the development of CD8 T cell responses has been studied in the context of many infections. Effective control of *Mycobacterium tuberculosis* (55), *Toxoplasma gondii* (56), and *Plasmodium* spp. (19) requires CD4 T cell help for the generation of effective CD8 T cell responses. In contrast, CD8 T cell responses to other infections such as influenza virus (57, 58) and LCMV (42) occur in the absence of CD4 T cells. This differential requirement for CD4 T cell help likely reflects the presence of many variables in the context of different infections, including the type and tropism of the pathogen, the level of costimulation delivered by DC (23), the affinity and level of presentation of CD8 T cell epitopes (59), the cytokine milieu, and the frequency of naive CD8 T cell precursors (60). It is important to note that the lack of identified epitopes in most bacterial and parasitic systems precludes quantitative measurement and kinetic analysis of Ag-specific CD8 T cell responses at the single cell level. Thus, most studies have only examined the requirement for CD4 T cells in the initial induction of a primary CD8 T cell response using bulk CTL and ELISA after in vitro restimulation and expansion. In this study, we used rLm expressing a well-defined epitope (gp33) from LCMV that allowed us to quantify the epitope-specific CD8 T cell response. By following epitope-specific T cells over time, we examined the role of CD4 T cells in the induction and contraction of primary CD8 T cell responses, in the establishment and maintenance of CD8 T cell memory, and in the recall response of memory CD8 T cells to viral challenge.

Our results show that the initial induction of a primary CD8 T cell response following systemic *L. monocytogenes* infection was normal in CD4 T cell-depleted, CD4$^{-/-}$, and MHC II$^{-/-}$ mice, consistent with previous findings in mice lacking the *class II transactivator* gene (CIITA$^{-/-}$) and orally infected MHC II$^{-/-}$ mice (61, 62). Our kinetic analysis showed that CD4$^{-/-}$ mice had slightly reduced numbers of epitope-specific CD8 T cells at later time points (>day 7 p.i.) compared with normal mice. Recent studies have shown that a single brief period of antigenic stimulation is sufficient to induce naive CD8 T cells to undergo a developmental program resulting in many cycles of cell division, acquisition of effector functions, and differentiation into memory cells (63, 64). Although extensive proliferation is induced by a single exposure to Ag, the magnitude of the response can be improved by additional factors such as IL-2 (63) and other interactions. The

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

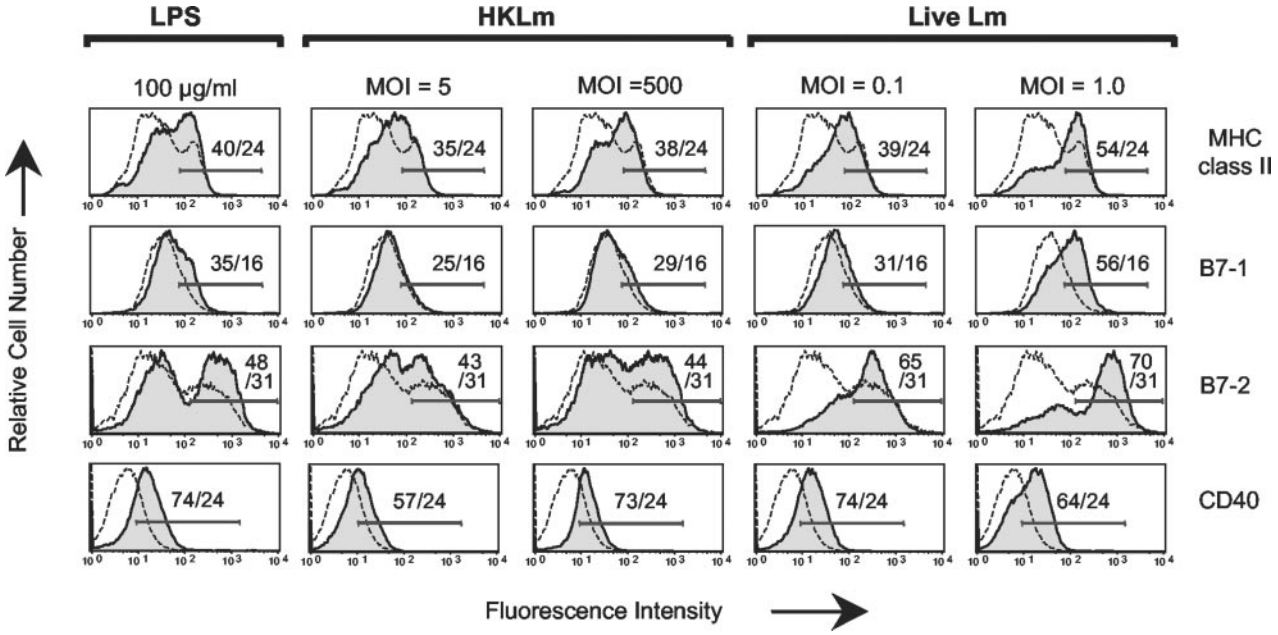

FIGURE 6. Activation of DC following in vitro culture with HKLm or live *L. monocytogenes*. A primary culture of immature murine DC was incubated in vitro with LPS, HKLm, or live *L. monocytogenes* (Lm). Antibiotics were added to cultures after 4 h to stop bacterial growth. At 18 h, cells were harvested and stained for expression of MHC class II, the costimulatory molecules B7-1 and B7-2, and CD40. Numbers indicate percentage of cells with up-regulated expression of these molecules in treated/untreated DC. The dotted lines represent unstimulated immature DC, whereas the shaded regions represent DC stimulated with either 100 μg/ml LPS, HKLm at a MOI of 5 (1.5 μg/ml), HKLm at a MOI of 500 (3.0 μg/ml), live *L. monocytogenes* at a MOI of 0.1 (0.03 μg/ml), or live *L. monocytogenes* at a MOI of 1.0 (0.30 μg/ml).

slightly weakened CD8 T cell response at the later time points in $CD4^{-/-}$ mice may reflect the absence of these extrinsic factors that serve to enhance the response to normal levels. Furthermore, a recent study of CD8 T cell responses to malaria has shown that CD8 T cells exhibit normal differentiation and proliferation during the first few days of infection while IL-4 produced by CD4 T cells enhances the continued development of the CD8 T cell response at later time points (19). It is therefore possible that CD4 T cells, through production of IL-4, also play a role in enhancing expansion and/or in curtailing contraction of CD8 T cells during murine listeriosis. This may account for the establishment of slightly lower numbers of gp33-specific memory CD8 T cells in $CD4^{-/-}$ mice. Once memory was established, however, our results showed that CD8 T memory levels remained relatively stable over a long period of time, even in the absence of CD4 T cells.

Memory CD8 T cells in rLm33-immunized $CD4^{-/-}$ mice mounted a recall response to LCMV challenge, proliferating and differentiating into effectors that provided protective antiviral immunity. However, the magnitude of the secondary response was diminished in $CD4^{-/-}$ mice compared with C57BL/6 mice. This difference was not solely due to the slightly lower number of epitope-specific memory CD8 T cells in $CD4^{-/-}$ mice than in normal C57BL/6 mice following rLm immunization. The expansion of the memory CD8 T cell population was also reduced in $CD4^{-/-}$ mice; by day 7 after LCMV challenge, the total number of gp33-specific CD8 T cells increased 5-fold in C57BL/6 mice but only 2.7-fold in $CD4^{-/-}$ mice. A diminished protective recall response has also been observed by Riberdy et al. (58) using the A/PR8/34 influenza virus to prime and the HKx31 influenza virus to recall an epitope-specific CD8 T cell response. Together, these data support the notion that CD4 T cell help functions to augment the recall response of memory CD8 T cells upon reinfection.

Although CD4 T cells may play a role in enhancing the proliferation of activated and memory CD8 T cells, they are clearly not required for the priming of CD8 T cells during *L. monocytogenes* and several other infections (42, 57, 58). This is in contrast to the critical role that CD4 T cells play in the induction of CD8 T cell responses in other experimental systems, such as immunization with protein Ags, tumor-specific CTL responses, cross-priming, and certain viral infections (65). Why is CD4 T cell help required for the induction of CD8 T cell response in some cases but not others? Interaction of CD4 T cells with DC up-regulates costimulatory molecules on the DC necessary for efficient priming of naive CD8 T cells, while cytokine production by CD4 T cells enhances the proliferation of primed CD8 T cells. In cases where CD4 T cell help is not required for priming a CD8 T cell response, it is likely that an alternative pathway of APC activation exists. One possible mechanism by which this activation may occur is direct infection of DC, resulting in the up-regulation of costimulatory molecules, as seen in influenza virus infection (57). *L. monocytogenes* infection of human DC in vitro has been reported (66), but it remains to be investigated whether direct bacterial infection activates DC to become competent APC, or in fact result in dysfunction of the host cellular machinery due to overwhelming growth of intracellular bacteria. It is also known that dsRNA intermediates produced during viral replication are recognized by TLR3 (67), leading to the activation of DC and macrophages and an IFN response (68). However, infection of DC is not a prerequisite for Ag presentation since DC are known to become activated through TLR recognition of bacterial products, such as flagellin, peptidoglycan, and lipoteichoic acid from *L. monocytogenes* (69, 70). Indeed, our data show that exposure to this bacterium was sufficient to activate DC to express costimulatory molecules B7-1/7-2. It is thus conceivable that TLR interaction with *L. monocytogenes* products may serve as an alternative pathway of DC activation that alleviates the need for CD4 T cell help to "condition" APC for efficient priming of naive CD8 T cells. The importance of DC activation through TLR during priming of CD8 T cells in the presence and absence of CD4 T cell help is currently under investigation.

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

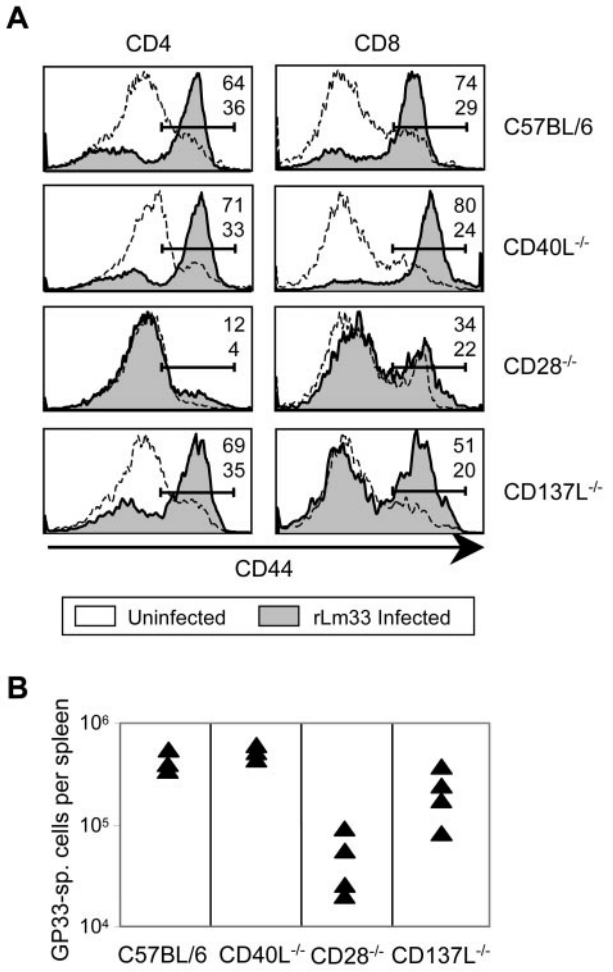

FIGURE 7. CD4 and CD8 T cell responses in CD40L$^{-/-}$, CD137L$^{-/-}$, and CD28$^{-/-}$ mice following *L. monocytogenes* infection. CD40L$^{-/-}$, CD137L$^{-/-}$, CD28$^{-/-}$, and normal C57BL/6 mice were infected with a sublethal dose of rLm33 (0.1 LD_{50}). *A*, Activation of CD4 and CD8 T cells were examined on day 7 p.i. by FACS analysis of CD44 expression. Dotted lines represent uninfected mice and the shaded regions with solid lines represent rLm33-infected mice. The numbers indicate percentage of CD4 or CD8 T cells that are activated (CD44^{high}), with the top and bottom numbers representing rLm33-infected mice and naive mice, respectively. *B*, Number of gp33-specific CD8 T cells in spleens was determined by ELISPOT assays on day 7 p.i. Each triangle represents an individual mouse.

APC conditioning by CD4 T cells involves costimulatory signaling by CD40-CD40L and B7-CD28 interactions. The engagement of CD40L on CD4 T cells by CD40 on DC results in DC activation and up-regulation of B7 molecules that in turn bind to CD28 and provide a costimulatory signal to CD8 T cells (24). In this model of tripartite cell interaction, signaling through CD40-CD40L lies upstream of DC activation. Thus, in cases where the CD8 T cell response occurs through an alternative CD4-independent pathway of DC activation, it is predicted that only the B7/CD28 costimulation, downstream of DC activation, will be essential for the induction of naive CD8 T cells. In support of this model, our results showed that the CD8 T cell response during *L. monocytogenes* infection was relatively normal in CD40L$^{-/-}$ mice but defective in CD28$^{-/-}$ mice. This is further supported by previous findings of normal *Listeria*-specific CD8 T cell responses in CD40$^{-/-}$ mice (62) and by the work of Hamilton et al. (24) who demonstrated that *L. monocytogenes* infection can overcome the requirement for CD40-CD40L interaction in CD8 T cell cross-priming by exogenous Ags. Consistent with our results, Mittrücker et al. (54) have also demonstrated the importance of CD28 in the generation and expansion of *Listeria*-specific CTL. Although previous studies have been conducted with different Ags and/or in different mouse backgrounds (24, 54, 62), our study analyzed the CD8 T cell response in CD40L$^{-/-}$ and CD28$^{-/-}$ mice in parallel, thus providing a direct comparison of how deficiencies in CD40-CD40L and B7-CD28 costimulations impact the CD8 T cell response. Furthermore, our results showed that activation of CD4 T cells, like that of CD8 T cells, was largely unaffected in CD40L$^{-/-}$ but severely reduced in CD28$^{-/-}$ mice. The relative contribution of various costimulations to the induction of epitope-specific CD4 T responses during *L. monocytogenes* infection remains to be determined.

In addition to CD40-CD40L and B7-CD28, the APC-T cell interaction involves several other ligand/receptor pairs of costimulatory molecules, including CD137/CD137L. It is less clear what role these costimulatory molecules play in CD4 and CD8 T cell responses and in the tripartite CD4 T cell-APC-CD8 T cell interaction. Our results show that the activation of CD4 T cells was normal but the activation of CD8 T cells and the induction of epitope-specific CD8 T cells were slightly reduced following *L. monocytogenes* infection of CD137L$^{-/-}$ mice. Thus, the CD137-CD137L costimulation may play a more important role in the CD8 T cell response than the CD4 T cell response. This difference has also been observed in other systems (33, 71–73), although stimulation with an agonistic mAb to CD137 can enhance both CD4 and CD8 T cell responses in vitro and in vivo (26, 74). Signaling through CD137-CD137L has been shown to induce B7 expression on DC, directly enhancing their ability to stimulate T cell proliferation (75). It has also been suggested that CD28 may provide the primary survival signal which can then be sustained by inducible costimulatory pathways such as those of CD137 and OX40 (76, 77). Consistent with this possibility, our results showed that the CD8 T cell response during *L. monocytogenes* infection was reduced in both CD28$^{-/-}$ and CD137L$^{-/-}$ mice, with a more pronounced defect in CD28$^{-/-}$ than in CD137L$^{-/-}$ mice.

In summary, this study systematically examined the role of CD4 T cells in various phases of the CD8 T cell response during *L. monocytogenes* infection. Our results show that CD4 T cells are not required for the induction of a primary CD8 T cell response nor for the establishment, maintenance, and recall response of memory CD8 T cells. However, CD4 T cells play a role in establishing optimal levels of CD8 T cell memory and in enhancing the proliferation of memory CD8 T cells during a recall response. Our results further show that *L. monocytogenes* can directly activate DC and induce a CD8 T cell response that does not require CD40-CD40L signaling but depends on the B7-CD28 and, to a lesser extent, the CD137-CD137L interaction. These results are consistent with a model in which direct activation of DC by infectious agents may overcome the requirement of conditioning APC by CD4 T cells via the CD40-CD40L interaction. This alternative pathway of DC activation may provide an explanation for the differential requirement of CD4 T cell help for CD8 T cell responses to various stimuli.

Acknowledgments

We thank members of the Shen laboratory for discussion and critical reading of this manuscript.

References

1. Unanue, E. R. 1997. Why listeriosis? A perspective on cellular immunity to infection. *Immunol. Rev. 158:5.*
2. Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs. 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes. J. Exp. Med. 167:1459.*

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

3. Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart. 1992. *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. *Cell 68:521.*
4. Smith, G. A., J. A. Theriot, and D. A. Portnoy. 1996. The tandem repeat domain in the *Listeria monocytogenes* ActA protein controls the rate of actin-based motility, the percentage of moving bacteria, and the localization of vasodilator-stimulated phosphoprotein and profilin. *J. Cell Biol. 135:647.*
5. Conlan, J. W., and R. J. North. 1994. Neutrophils are essential for early anti-*Listeria* defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. *J. Exp. Med. 179:259.*
6. Dunn, P. L., and R. J. North. 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. *Infect. Immun. 59: 2892.*
7. Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. Kaufmann. 1993. Different roles of $\alpha\beta$ and $\gamma\delta$ T cells in immunity against an intracellular bacterial pathogen. *Nature 365:53.*
8. Mielke, M., S. Ehlers, and H. Hahn. 1988. The role of T cell subpopulations in cell mediated immunity to facultative intracellular bacteria. *Infection 16(Suppl. 2):S123.*
9. Naher, H., U. Sperling, and H. Hahn. 1985. H-2K-restricted granuloma formation by Ly-2^+ T cells in antibacterial protection to facultative intracellular bacteria. *J. Immunol. 134:569.*
10. Bishop, D. K., and D. J. Hinrichs. 1987. Adoptive transfer of immunity to *Listeria monocytogenes*: the influence of in vitro stimulation on lymphocyte subset requirements. *J. Immunol. 139:2005.*
11. Lukacs, K., and R. J. Kurlander. 1989. MHC-unrestricted transfer of antilisterial immunity by freshly isolated immune CD8 spleen cells. *J. Immunol. 143:3731.*
12. Czuprynski, C. J., and J. F. Brown. 1990. Effects of purified anti-Lyt-2 mAb treatment on murine listeriosis: comparative roles of Lyt-2^+ and L3T4^+ cells in resistance to primary and secondary infection, delayed-type hypersensitivity and adoptive transfer of resistance. *Immunology 71:107.*
13. Dunn, P. L., and R. J. North. 1991. Resolution of primary murine listeriosis and acquired resistance to lethal secondary infection can be mediated predominantly by Thy-1^+CD4^-CD8^- cells. *J. Infect. Dis. 164:869.*
14. Ladel, C. H., I. E. Flesch, J. Arnoldi, and S. H. Kaufmann. 1994. Studies with MHC-deficient knockout mice reveal impact of both MHC I- and MHC II-dependent T cell responses on *Listeria monocytogenes* infection. *J. Immunol. 153: 3116.*
15. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of TH1 CD4^+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science 260:547.*
16. Mielke, M. E., H. Rosen, S. Brocke, C. Peters, and H. Hahn. 1992. Protective immunity and granuloma formation are mediated by two distinct tumor necrosis factor α- and γ interferon-dependent T cell-phagocyte interactions in murine listeriosis: dissociation on the basis of phagocyte adhesion mechanisms. *Infect. Immun. 60:1875.*
17. Kaufmann, S. H. 1988. *Listeria monocytogenes* specific T-cell lines and clones. *Infection 16:S128.*
18. Wodarz, D., and V. A. Jansen. 2001. The role of T cell help for anti-viral CTL responses. *J. Theor. Biol. 211:419.*
19. Carvalho, L. H., G. Sano Gi, J. C. Hafalla, A. Morrot, M. A. de Lafaille, and F. Zavala. 2002. IL-4-secreting CD4^+ T cells are crucial to the development of CD8^+ T-cell responses against malaria liver stages. *Nat. Med. 8:166.*
20. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature 393:480.*
21. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature 393:478.*
22. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4^+ T-helper and a T-killer cell. *Nature 393: 474.*
23. Zhan, Y., A. J. Corbett, J. L. Brady, R. M. Sutherland, and A. M. Lew. 2000. CD4 help-independent induction of cytotoxic CD8 cells to allogeneic P815 tumor cells is absolutely dependent on costimulation. *J. Immunol. 165:3612.*
24. Hamilton, S. E., A. R. Tvinnereim, and J. T. Harty. 2001. *Listeria monocytogenes* infection overcomes the requirement for CD40 ligand in exogenous antigen presentation to CD8^+ T cells. *J. Immunol. 167:5603.*
25. Rolph, M. S., and S. H. Kaufmann. 2001. CD40 signaling converts a minimally immunogenic antigen into a potent vaccine against the intracellular pathogen *Listeria monocytogenes. J. Immunol. 166:5115.*
26. Cannons, J. L., P. Lau, B. Ghumman, M. A. DeBenedette, H. Yagita, K. Okumura, and T. H. Watts. 2001. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. *J. Immunol. 167:1313.*
27. Vinay, D. S., and B. S. Kwon. 1998. Role of 4-1BB in immune responses. *Semin. Immunol. 10:481.*
28. Tan, J., T. Town, M. Saxe, D. Paris, Y. Wu, and M. Mullan. 1999. Ligation of microglial CD40 results in p44/42 mitogen-activated protein kinase-dependent TNF-α production that is opposed by TGF-β1 and IL-10. *J. Immunol. 163:6614.*
29. Tan, J. T., J. K. Whitmire, K. Murali-Krishna, R. Ahmed, J. D. Altman, R. S. Mittler, A. Sette, T. C. Pearson, and C. P. Larsen. 2000. 4-1BB costimulation is required for protective antiviral immunity after peptide vaccination. *J. Immunol. 164:2320.*
30. DeBenedette, M. A., T. Wen, M. F. Bachmann, P. S. Ohashi, B. H. Barber, K. L. Stocking, J. J. Peschon, and T. H. Watts. 1999. Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J. Immunol. 163:4833.*
31. Chu, N. R., M. A. DeBenedette, B. J. Stiernholm, B. H. Barber, and T. H. Watts. 1997. Role of IL-12 and 4-1BB ligand in cytokine production by CD28^+ and CD28^- T cells. *J. Immunol. 158:3081.*
32. Hurtado, J. C., Y. J. Kim, and B. S. Kwon. 1997. Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. *J. Immunol. 158:2600.*
33. Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Siadak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, et al. 1997. 4-1BB costimulatory signals preferentially induce CD8^+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J. Exp. Med. 186:47.*
34. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity 11:443.*
35. Hertz, C. J., S. M. Kiertscher, P. J. Godowski, D. A. Bouis, M. V. Norgard, M. D. Roth, and R. L. Modlin. 2001. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J. Immunol. 166:2444.*
36. Thoma-Uszynski, S., S. M. Kiertscher, M. T. Ochoa, D. A. Bouis, M. V. Norgard, K. Miyake, P. J. Godowski, M. D. Roth, and R. L. Modlin. 2000. Activation of Toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. *J. Immunol. 165:3804.*
37. Michelsen, K. S., A. Aicher, M. Mohaupt, T. Hartung, S. Dimmeler, C. J. Kirschning, and R. R. Schumann. 2001. The role of Toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCs): peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. *J. Biol. Chem. 276:25680.*
38. Xu, J., T. M. Foy, J. D. Laman, E. A. Elliott, J. J. Dunn, T. J. Waldschmidt, J. Elsemore, R. J. Noelle, and R. A. Flavell. 1994. Mice deficient for the CD40 ligand. *Immunity 1:423.*
39. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science 261:609.*
40. Shen, H., M. K. Slifka, M. Matloubian, E. R. Jensen, R. Ahmed, and J. F. Miller. 1995. Recombinant *Listeria monocytogenes* as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity. *Proc. Natl. Acad. Sci. USA 92:3987.*
41. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice: role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med. 160:521.*
42. Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. CD4^+ T cells are required to sustain CD8^+ cytotoxic T-cell responses during chronic viral infection. *J. Virol. 68:8056.*
43. MacDonald, A. S., A. D. Straw, B. Bauman, and E. J. Pearce. 2001. CD8^- dendritic cell activation status plays an integral role in influencing Th2 response development. *J. Immunol. 167:1982.*
44. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods 223: 77.*
45. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity 8:177.*
46. Whitmire, J. K., M. S. Asano, K. Murali-Krishna, M. Suresh, and R. Ahmed. 1998. Long-term CD4 Th1 and Th2 memory following acute lymphocytic choriomeningitis virus infection. *J. Virol. 72:8281.*
47. van der Most, R. G., K. Murali-Krishna, J. L. Whitton, C. Oseroff, J. Alexander, S. Southwood, J. Sidney, R. W. Chesnut, A. Sette, and R. Ahmed. 1998. Identification of D^b- and K^b-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice. *Virology 240:158.*
48. Kursar, M., K. Bonhagen, A. Kohler, T. Kamradt, S. H. Kaufmann, and H. W. Mittrücker. 2002. Organ-specific CD4^+ T cell response during *Listeria monocytogenes* infection. *J. Immunol. 168:6382.*
49. Marzo, A. L., V. Vezys, K. Williams, D. F. Tough, and L. Lefrancois. 2002. Tissue-level regulation of Th1 and Th2 primary and memory CD4 T cells in response to *Listeria* infection. *J. Immunol. 168:4504.*
50. Mittrücker, H. W., A. Kohler, and S. H. Kaufmann. 2000. Substantial in vivo proliferation of CD4^+ and CD8^+ T lymphocytes during secondary *Listeria monocytogenes* infection. *Eur. J. Immunol. 30:1053.*
51. Roth, R., T. Nakamura, and M. J. Mamula. 1996. B7 costimulation and autoantigen specificity enable B cells to activate autoreactive T cells. *J. Immunol. 157: 2924.*
52. Geginat, G., S. Schenk, M. Skoberne, W. Goebel, and H. Hof. 2001. A novel approach of direct ex vivo epitope mapping identifies dominant and subdominant CD4 and CD8 T cell epitopes from *Listeria monocytogenes. J. Immunol. 166: 1877.*
53. Rahemtulla, A., W. P. Fung-Leung, M. W. Schilham, T. M. Kundig, S. R. Sambhara, A. Narendran, A. Arabian, A. Wakeham, C. J. Paige, R. M. Zinkernagel, et al. 1991. Normal development and function of CD8^+ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature 353:180.*
54. Mittrücker, H. W., M. Kursar, A. Kohler, R. Hurwitz, and S. H. Kaufmann. 2001. Role of CD28 for the generation and expansion of antigen-specific CD8^+ T lymphocytes during infection with *Listeria monocytogenes. J. Immunol. 167: 5620.*

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017

55. Serbina, N. V., V. Lazarevic, and J. L. Flynn. 2001. $CD4^+$ T cells are required for the development of cytotoxic $CD8^+$ T cells during *Mycobacterium tuberculosis* infection. *J. Immunol. 167:6991.*
56. Casciotti, L., K. H. Ely, M. E. Williams, and I. A. Khan. 2002. $CD8^+$-T-cell immunity against *Toxoplasma gondii* can be induced but not maintained in mice lacking conventional $CD4^+$ T cells. *Infect. Immun. 70:434.*
57. Wu, Y., and Y. Liu. 1994. Viral induction of co-stimulatory activity on antigen-presenting cells bypasses the need for $CD4^+$ T-cell help in $CD8^+$ T-cell responses. *Curr. Biol. 4:499.*
58. Riberdy, J. M., J. P. Christensen, K. Branum, and P. C. Doherty. 2000. Diminished primary and secondary influenza virus-specific $CD8^+$ T-cell responses in CD4-depleted $Ig^{-/-}$ mice. *J. Virol. 74:9762.*
59. Franco, A., D. A. Tilly, I. Gramaglia, M. Croft, L. Cipolla, M. Meldal, and H. M. Grey. 2000. Epitope affinity for MHC class I determines helper requirement for CTL priming. *Nat. Immunol. 1:145.*
60. Mintern, J. D., G. M. Davey, G. T. Belz, F. R. Carbone, and W. R. Heath. 2002. Cutting edge: precursor frequency affects the helper dependence of cytotoxic T cells. *J. Immunol. 168:977.*
61. Lauvau, G., S. Vijh, P. Kong, T. Horng, K. Kerksiek, N. Serbina, R. A. Tuma, and E. G. Pamer. 2001. Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. *Science 294:1735.*
62. Pope, C., S. K. Kim, A. Marzo, D. Masopust, K. Williams, J. Jiang, H. Shen, and L. Lefrancois. 2001. Organ-specific regulation of the CD8 T cell response to *Listeria monocytogenes* infection. *J. Immunol. 166:3402.*
63. Kaech, S. M., and R. Ahmed. 2001. Memory $CD8^+$ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol. 2:415.*
64. van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger. 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol. 2:423.*
65. Whitmire, J. K., and R. Ahmed. 2000. Costimulation in antiviral immunity: differential requirements for $CD4^+$ and $CD8^+$ T cell responses. *Curr. Opin. Immunol. 12:448.*
66. Paschen, A., K. E. Dittmar, R. Grenningloh, M. Rohde, D. Schadendorf, E. Domann, T. Chakraborty, and S. Weiss. 2000. Human dendritic cells infected by *Listeria monocytogenes*: induction of maturation, requirements for phagolysosomal escape and antigen presentation capacity. *Eur. J. Immunol. 30:3447.*
67. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature 413:732.*
68. An, H., Y. Yu, M. Zhang, H. Xu, R. Qi, X. Yan, S. Liu, W. Wang, Z. Guo, J. Guo, Z. Qin, and X. Cao. 2002. Involvement of ERK, p38 and NF-κB signal transduction in regulation of *TLR2*, *TLR4* and *TLR9* gene expression induced by lipopolysaccharide in mouse dendritic cells. *Immunology 106:38.*
69. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*
70. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature 410:1099.*
71. Takahashi, C., R. S. Mittler, and A. T. Vella. 1999. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. *J. Immunol. 162:5037.*
72. Tan, J. T., J. K. Whitmire, R. Ahmed, T. C. Pearson, and C. P. Larsen. 1999. 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. *J. Immunol. 163:4859.*
73. Wen, T., J. Bukczynski, and T. H. Watts. 2002. 4-1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production, and the development of cytolytic effector function. *J. Immunol. 168:4897.*
74. Halstead, E. S., Y. M. Mueller, J. D. Altman, and P. D. Katsikis. 2002. In vivo stimulation of CD137 broadens primary antiviral $CD8^+$ T cell responses. *Nat. Immunol. 3:536.*
75. Wilcox, R. A., A. I. Chapoval, K. S. Gorski, M. Otsuji, T. Shin, D. B. Flies, K. Tamada, R. S. Mittler, H. Tsuchiya, D. M. Pardoll, and L. Chen. 2002. Cutting edge: expression of functional CD137 receptor by dendritic cells. *J. Immunol. 168:4262.*
76. Bertram, E. M., P. Lau, and T. H. Watts. 2002. Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. *J. Immunol. 168:3777.*
77. Cooper, D., P. Bansal-Pakala, and M. Croft. 2002. 4-1BB (CD137) controls the clonal expansion and survival of CD8 T cells in vivo but does not contribute to the development of cytotoxicity. *Eur. J. Immunol. 32:521.*

Downloaded from http://www.jimmunol.org/ by guest on May 16, 2017