Cytosolic Entry Controls CD8+ T-Cell Potency during Bacterial Infection

Keith S. Bahjat,1* Weiqun Liu,1 Edward E. Lemmens,1 Stephen P. Schoenberger,2 Daniel A. Portnoy,3 Thomas W. Dubensky, Jr.,1 and Dirk G. Brockstedt1

Cerus Corporation, Concord, California; Division of Cellular Immunology, La Jolla Institute for Allergy & Immunology, San Diego, California; and Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California

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Interaction with host immunoreceptors during microbial infection directly impacts the magnitude of the ensuing innate immune response. How these signals affect the quality of the adaptive T-cell response remains poorly understood. Utilizing an engineered strain of the intracellular pathogen Listeria monocytogenes that infects cells but fails to escape from the phagosome, we demonstrate the induction of long-lived memory T cells that are capable of secondary expansion and effector function but are incapable of providing protective immunity. We demonstrate that microbial invasion of the cytosol is required for dendritic cell activation and integration of CD40 signaling, ultimately determining the ability of the elicited CD8+ T-cell pool to protect against lethal wild-type L. monocytogenes challenge. These results reveal a crucial role for phagosomal escape, not for delivery of antigen to the class I major histocompatibility complex pathway but for establishing the appropriate cellular context during CD8+ T-cell priming.

CD8+ T cells are an essential component for host defense against a variety of bacterial and viral infections. Recognition of cognate peptide-major histocompatibility complex (MHC) complexes, in combination with costimulatory molecules, leads to the activation, expansion, and acquisition of effector function by the antigen-specific CD8+ T cell. Following 7 to 9 days of expansion, the effector T-cell population undergoes a rapid contraction phase, leaving a population of long-lived memory T cells (22). It is this memory T-cell pool that provides lifelong protective immunity against subsequent infection. Understanding how host-pathogen interactions affect the potency and longevity of this memory T-cell pool is critical for the rational design of efficacious vaccines.

For decades, Listeria monocytogenes has served as a model system for studying basic aspects of innate and acquired cell-mediated immunity (41). A hallmark of the L. monocytogenes model is that infection with sublethal doses results in long-lived CD8+ T-cell-mediated immunity (23, 30), while heat-killed bacteria fail to provide protection (32, 55). This is a fundamentally different observation with implications for the development of vacuole-to-cytosolic entry mechanisms to discriminate between phagosome-confined and cytosolic pathogens. Cytokine profiles from cells infected with cytosolic L. monocytogenes are distinct from those induced by their vacuole-bound LLO-deleted counterparts (34, 40, 50). How these cytokines affect antigen presentation and the subsequent cellular immune response remains largely unknown. Thus, compartmentalization of either the antigen or the pathogen itself are possible mechanistic reasons supporting the observation that only live cytosolic L. monocytogenes elicits a protective T-cell response (6, 13).

Of central importance to the generation of adaptive immunity to intracellular pathogens is how dendritic cells (DC) acquire antigens for class I-restricted presentation. DC are absolutely required for the priming of L. monocytogenes-specific T cells (28). Through cross-presentation, DC can present L. monocytogenes-derived antigen via the MHC class I pathway without becoming directly infected (1, 9, 54). Cross-presentation involves movement of endocytosed antigen from the vacuole to the cytosol for proteasomal degradation, and then translocation into MHC class I-containing compartments for presentation to CD8+ T cells (21, 44). Priming of CD8+ T-cell responses to nonsecreted L. monocytogenes-derived antigens underscores the efficiency and relevance of this pathway (49, 56). It is therefore paradoxical that while cross-presentation is sufficient for induction of CD8+ T-cell responses to a nonsecreted antigen, vacuole-confined LLO− L. monocytogenes strains are believed not to be immunogenic (3, 7, 13).

In this report, we demonstrate that L. monocytogenes mutants that lack LLO (LLO−Lm) do in fact provoke a robust primary and secondary CD8+ T-cell response but fail to provide substantial long-lived protective immunity. These results illustrate a critical role for cytosolic entry by L. monocytogenes: not to secrete antigen into the MHC class I pathway but in-
stead to elicit innate signals that improve the potency of the adaptive T-cell response. Thus, bacterial compartmentalization does not directly limit the ability of the DC to acquire antigen but rather influences the ability to mature and elicit competent CD8+ T-cell memory.

**MATERIALS AND METHODS**

Mice, Six- to 12-week-old female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Studies were performed under animal protocols approved by the Cerus Institutional Animal Care and Use Committee.

**Bacterial strains.** DNA sequences encompassing the hly (LLO) promoter and encoding amino acids 1 to 441 of LLO were amplified from the wild-type L. monocytogenes strain DP-L4056 and cloned into pPL2 (31). Ovalbumin (OVA) sequences were amplified from pDP3616 (25) and cloned in frame with LLO in L. monocytogenes (LLO-Lm, LLO-Lm-Lm (LP-L4027 Aδ), and actA-Lm (LP-L4029 AδΔa) strains have been described previously (31). Strains carrying pPL2-LLO-OVA in the aforementioned strains are denoted as Lm-OVA, LLO-Lm-OVA (0.1 50% lethal dose [LD50] = 1 x 106 CFU) and Lm-OVA (0.1 LD50 = 1 x 104 CFU), respectively. KBMA-Lm (killed but metabolically active L. monocytogenes) (ΔactA/Δambl/Δa) and KBMA-Lm-OVA (0.1 LD50 = 3 x 104 particles) are isogenic strains without or with the OVA antigen expression cassette, respectively. KBMA-Lm and KBMA-Lm-OVA were psoralen and UVA treated to mimic a noninvasive infection (11). Photochemically treated bacteria (KBMA-Lm) were washed once with Dulbecco’s phosphate-buffered saline, resuspended in 8% dimethyl sulfoxide, and then stored at 80°C. The LD50 for each strain corresponds to a dose resulting in approximately 1 x 106 CFU per spleen at the peak of expansion. Median lethality values in C57BL/6 mice were determined as described previously (12).

**Immunizations and protective immunity.** Bacteria for immunization were prepared from stationary-phase overnight cultures in brain heart infusion broth. Bacteria were washed and resuspended in Hank’s balanced salt solution (HBSS) prior to intravenous (i.v.) injection. The anti-CD40 antibody, clone FGK-45 (46), was used at 100 µg per animal via intraperitoneal injection 24 h following L. monocytogenes immunization. Bacterial injection stocks were plated to confirm CFU. Protective immunity to lethal challenge was assessed by i.v. injection of 2 LD50 (1 x 104 CFU) of wild-type L. monocytogenes (strain DP-L4056). Seventy-two hours postinfection, spleens and livers were harvested and then homogenized in distilled water plus 0.5% NP-40, and 10-fold serial dilutions were plated onto brain heart infusion agar plates. Colonies were enumerated 48 h later.

CD8+ T cells were depleted by administering 250 µg of anti-CD8 monoclonal antibody (clone 2.43) via intraperitoneal injection on days 5, 6, and 7 after primary immunization.

**Peptides.** OVA257-264 (SIINFEKL), LLO296-304 (VAYGROVYL), LLO300-311 (NEKYQAYPWS), p60217-226 (KYGVSVQDI), HSV-gB2 (SSIEFARL), and g-galactosidase (β-Gal) (TPHPARIGL) were synthesized by SynPep Corporation (Dublin, CA).

**Reagents for flow cytometry.** CD3 fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-Cy7 (clone 145-2C11), CD4+ FITC (clone GK1.5), CD8 PE-Cy7 or allophycocyanin-Cy7 (clone 56-3.7), CD19 PE (clone 1D3), CD40L PE (clone MR1), CD44 FITC or PE-Cy5.5 (clone IM7), CD62L PE or PE-Cy7 (clone MEL-14), purified CD16/32 (clone 93), and CD69 PE (clone H1.2F3) were purchased from eBioscience (San Diego, CA). CD11b FITC (clone M1/70) was purchased from BD Biosciences. Cd11b is a marker of myeloid DCs, which are particularly abundant in spleen. CD3 is a marker of T cells, which are the primary population of interest in this study. FlowJo software (Treestar, Ashland, OR) was used for data analysis.

**RESULTS**

**LLO-deleted L. monocytogenes strains fail to elicit protective immunity.** To examine CD8+ T-cell responses in L. monocytogenes-infected mice, we constructed a series of recombinant L. monocytogenes strains that secrete a fusion protein comprised of a nonfunctional N-terminal fragment of LLO linked in frame to OVA (Fig. 1A). The expression of OVA by the challenge strain, LLO Lm-OVA-immunized mice demonstrated minimal protective immunity as determined by the reduction in CFU within the spleen. Thus, addition of a high-affinity CD8+ T-cell epitope resulted in robust immunity to OVA257-264, whereas LLO Lm-OVA-immunized mice demonstrated minimal protective immunity that was not significantly different from unimmunized controls. Mice immunized with LLO Lm-OVA or LLO Lm-OVA did not rescue the ability of LLO-deleted L. monocytogenes strains to elicit protective immunity.

**LLO-deleted L. monocytogenes elicits primary CD8+ T-cell responses in vivo.** Next, we examined whether OVA- or LLO-specific CD8+ T-cell responses were primed following immunization with LLO Lm-OVA. Due to differences in vivo growth between the strains utilized, we immunized animals at 10% of the 50% lethal dose (0.1 LD50) for each strain. This approach standardized the maximum number of bacteria present per animal for each bacterial strain, allowing a comparable antigen dose between experimental groups. Mice immunized with LLO-expressing strains, such as actA-Lm-OVA or LLO Lm-OVA and reproducible, leading to approximately 4% OVA 257-264-specific CD8+ T cells (Fig. 2A and C). Use of Kp-OVA257-264 multimers demonstrated that these were CD62L-negative effector CD8+ T cells (Fig. 2B). At least twofold lower in magnitude than the response to actA-Lm-OVA, the primary CD8+ T-cell response in LLO Lm-OVA-immunized mice was robust and reproducible, leading to approximately 4% OVA257-264-specific CD8+ T cells (Fig. 2A to C). In contrast, immunization with heat-killed actA-Lm-OVA (HK-Lm-OVA) failed to elicit LLO- or OVA-specific CD8+ T cells.
LLO-deleted *L. monocytogenes* strains expressing OVA does not elicit protective immunity. (A) Construction of *L. monocytogenes* strains expressing OVA linked to nonfunctional LLO using the pPL2 site-specific integration vector. This permits the measurement of MHC class I- and II-restricted T-cell responses without the expression of functional LLO. (B) C57BL/6 mice immunized with either HBSS, actA-*Lm*-OVA (1 × 10^7 CFU), or LLO-*Lm*-OVA (3 × 10^8 CFU) were challenged 25 days later with 1 × 10^8 CFU of either wild-type *L. monocytogenes* (wt-Lm) or wild-type Lm-OVA (wt-Lm-OVA). Spleens were harvested three days after challenge and plated for CFU. Data represent one experiment of two.

The discrepancy between our results and previous publications (7) suggested that the CD8^+^ T cells we observed might lack the cytolytic activity that is required for protective immunity. To assess antigen-specific cytotoxicity, we measured epitope-specific killing using an in vivo cytotoxicity assay (Fig. 2D and E). Consistent with the T-cell quantification results, CD8^+^ T cells primed in LLO-*Lm*-OVA-immunized mice eliminated OVA257–264-loaded targets in vivo with equivalent efficiency as those mice immunized with Lm-OVA at the peak of the primary response (Fig. 2D). Cytolytic activity was not limited to OVA257–264-specific T cells, as killing of LLO296–304^+-loaded targets in LLO-*Lm*-OVA-immunized mice was also observed (data not shown). To confirm that these responses were not an anomaly of the LLO-OVA fusion antigen, we measured cytolytic activity in BALB/c mice against the endogenous secreted *L. monocytogenes* p60 antigen. LLO-*Lm* (either with or without the LLO-OVA antigen) killed p60217–225^+-loaded targets in vivo with comparable efficiency (Fig. 2E). Thus, OVA257–264^+-specific CD8^+^ T cells were functionally similar to those responding to an endogenous *L. monocytogenes* antigen.

To determine whether primary CD8^+^ T cells were capable of killing wild-type *L. monocytogenes*-infected cells in vivo, and thus capable of providing protection against lethal challenge, we challenged animals near the peak of the primary CD8^+^ T-cell response. Mice were immunized with the 0.1 LD_{90} of actA-*Lm* (1 × 10^7 CFU) or LLO-*Lm* (3 × 10^8 CFU) and were challenged 7 days postimmunization with 1 × 10^8 CFU wild-type *L. monocytogenes*. Unexpectedly, the primary response in LLO-*Lm*-immunized mice led to the rapid elimination of wild-type *L. monocytogenes* in the spleen (Fig. 2F). This was comparable to the clearance exhibited in actA-*Lm*-immunized mice, representing >100,000-fold protection. Depletion of CD8^+^ T cells prior to challenge in either the actA-*Lm* or LLO-*Lm*-immunized mice abrogated this protection. Taken together, these data show that phagosome-confined LLO-*Lm* elicited primary antigen-specific primary effector CD8^+^ T cells capable of secreting cytokines, killing peptide-pulsed targets, and protecting against lethal challenge.

**LLO-deleted *L. monocytogenes* strains elicit CD4^+^ T-cell memory.** The induction of antigen-specific CD4^+^ T cells without protective immunity (Fig. 1B) shown here is similar to the T-cell response described previously in CD4^+^ T-cell-depleted animals (27, 48, 51). To measure priming of antigen-specific CD4^+^ T cells following immunization with LLO-*Lm*-OVA, we utilized the MHC class II-restricted LLO190–201 epitope. This epitope is also present within the LLO-OVA fusion protein. Mice immunized with LLO-*Lm*-OVA mounted a primary LLO190–201^+-specific CD4^+^ T-cell response, comprising 1 to 3% of the splenic CD4^+^ T-cell population (Fig. 3A). The LLO190–201^+-specific response in mice immunized with actA-*Lm* is typically 2 to 4%. The CD4^+^ T-cell response represented a twofold reduction in magnitude compared to that elicited by actA-*Lm*-OVA, which is similar to the difference in magnitude observed for CD8^+^ T-cell frequency (Fig. 2A). Next, we assessed induction of CD4^+^ T-cell memory. Similar to the response with actA-*Lm*-OVA, LLO-*Lm*-OVA-vaccinated mice mounted a robust secondary CD4^+^ T-cell response upon challenge (Fig. 3B). The LLO190–201^+-specific CD4^+^ T cells underwent an 8- to 10-fold expansion and produced IFN-γ and CD40L upon reexposure to the LLO190–201 peptide. The CD4^+^ T-cell recall response in LLO-*Lm*-OVA-primed mice was consistently of greater magnitude than that for actA-*Lm*-OVA (Fig. 3B). Both the percentage of CD4^+^ T cells specific for LLO190–201 and the absolute number of these cells within the spleen were significantly greater in LLO-*Lm*-OVA-primed mice (Fig. 3B and C). These data suggest that the lack of protective immunity observed in LLO-*Lm*-immunized mice was not related to an inability to elicit *L. monocytogenes*-specific CD4^+^ T cells.

**LLO-deleted *L. monocytogenes* strains elicit memory CD8^+^ T cells which undergo secondary expansion and kill peptide-loaded targets in vivo.** We next hypothesized that the lack of maximal protective immunity following LLO-*Lm* immunization might result from a failure of CD8^+^ T cells to expand and acquire effector function following a challenge with wild-type *L. monocytogenes*. To address this possibility, we tested the ability of CD8^+^ T cells to undergo secondary expansion and
kill peptide-loaded targets in vivo. Following rechallenge with a lethal dose of Lm targets, robust secondary expansion of both OVA<sub>257-264</sub> and LLO<sub>296-304</sub>-specific CD8<sup>+</sup> T cells was observed in mice immunized with LLO-Lm-OVA (Fig. 4A and B). The expansion was indistinguishable from that observed in mice immunized with actA<sup>+</sup>-Lm-OVA. Because LLO<sup>+</sup>-Lm-immunized mice demonstrated little protection against wild-type <em>L. monocytogenes</em>, we evaluated the ability of these secondary CD8<sup>+</sup> T cells to kill antigen-specific targets in vivo. Surprisingly, immunization with either LLO-Lm-OVA or actA<sup>+</sup>-Lm-OVA resulted in complete killing (>95%) of OVA<sub>257-264</sub>-loaded targets three days following a 1 × 10<sup>5</sup> CFU (2 LD<sub>50</sub>) Lm-OVA challenge, while nonimmunized mice demonstrated killing of less than 4% (Fig. 4C). Killing of LLO<sub>296-304</sub>-pulsed targets was similarly robust; however, following Lm-OVA boost, LLO-Lm-OVA-primed mice demonstrated significantly greater killing than did actA<sup>+</sup>-Lm-OVA primed mice (<i>P</i> < 0.001). In summary, LLO-deleted <em>L. monocytogenes</em> elicited robust antigen-specific CD8<sup>+</sup> T cells that produced IFN-γ and had cytolytic activity in vivo. These cells underwent secondary expansion in response to challenge, resulting in CD8<sup>+</sup> T cells that were indistinguishable from those in actA<sup>+</sup>-Lm-OVA-immunized mice with respect to cytokine production and in vivo cytotoxicity. Significantly, despite having a functional phenotype, this secondary CD8<sup>+</sup>-T-cell pool was unable to provide substantial long-term protective immunity (Fig. 1B).

**FIG. 2.** LLO-Lm elicits primary CD8<sup>+</sup> T cells that mediate protection against lethal <em>L. monocytogenes</em> challenge. (A) In C57BL/6 mice at 7 days postimmunization with actA<sup>+</sup>-Lm-OVA (1 × 10<sup>7</sup> CFU), LLO-Lm-OVA (3 × 10<sup>5</sup> CFU), or heat-killed actA<sup>+</sup>-Lm-OVA (1 × 10<sup>10</sup> particles), the percentage of LLO<sub>296-304</sub>- or OVA<sub>257-264</sub>-specific CD8<sup>+</sup> T cells was determined using intracellular cytokine staining. Numbers represent the percentages of IFN-γ-positive cells within the CD8<sup>+</sup>-T-cell population, and they are the means of three mice per group. (B) Staining of CD8<sup>+</sup> T cells with anti-CD62L and Kb-OVA<sub>257-264</sub> multimers. Data shown are gated on MHC class II-negative, CD4<sup>-</sup>CD<sup>+</sup>-positive cells within the CD8<sup>+</sup>-positive T-cell subset, and they are the means of three mice per group. (C) Absolute antigen-specific T cells per spleen at 7 days postimmunization. Antigen-specific T cells were quantified by staining for intracellular IFN-γ following restimulation with the indicated peptide (γ-axis). Each symbol represents an individual animal. Data are from a single representative experiment of at least five replicates. (D) C57BL/6 mice were immunized with HBSS (left panel), wild-type <em>L. monocytogenes</em> (5 × 10<sup>5</sup> CFU), Lm-OVA (5 × 10<sup>5</sup> CFU), or LLO-Lm-OVA (3 × 10<sup>5</sup> CFU). In vivo cytolytic activity was determined 7 days later by challenging mice with gB2 (control)- or OVA<sub>257-264</sub>-loaded targets. (E) BALB/c mice immunized with HBSS (left panel), wild-type <em>L. monocytogenes</em> (5 × 10<sup>5</sup> CFU), LLO-Lm (3 × 10<sup>5</sup> CFU), Lm-OVA (5 × 10<sup>5</sup> CFU), or LLO-Lm-OVA (3 × 10<sup>6</sup> CFU) were challenged 7 days later with β-Gal (control)- or p60<sub>17-232</sub>-loaded splenocytes. Numbers represent the percent killing of targets, and they represent the means of three mice per group. Standard deviations are indicated below. All data represent a single experiment of at least three replicates. (F) C57BL/6 mice were immunized with HBSS, actA<sup>+</sup>-Lm (1 × 10<sup>7</sup> CFU), or LLO-Lm (3 × 10<sup>5</sup> CFU). Five days later, half of the mice were depleted of CD8<sup>+</sup> T cells. On day 7 postimmunization, mice were challenged with wild-type <em>L. monocytogenes</em> (1 × 10<sup>5</sup> CFU), and CFU in the spleen were determined 3 days later. Data represent logs of protection relative to HBSS controls, graphed as the mean and standard deviations of five mice per group. One representative experiment of two is shown.
Cytosolic entry is required, but not sufficient, for protective immunity. To better characterize this CD8⁺-T-cell memory pool, we evaluated the relationship between L. monocytogenes subcellular localization and CD8⁺-T-cell potency. Unlike actA⁻ Lm, LLO⁻ Lm remains confined within the phagosome and is unable to escape into the cytosol and multiply (36). To examine the role of subcellular localization, one would need to use an appropriate experimental control for the LLO⁻ strain, one that escapes the phagosome but does not multiply within the cytosol. For this purpose, we utilized the KBMA-Lm described previously (11). Treatment of the nucleotide excision repair-deficient (ΔnuAΔIB) bacteria with psoralen and UV light leads to infrequent, randomly distributed, and irreparable DNA cross-links that prevent bacterial multiplication but allow for continued protein synthesis and metabolic activity. Consequently, KBMA-Lm escapes from the phagosome to the cytosol, where it actively secretes proteins but is unable to multiply or spread from cell to cell. In vitro tests demonstrated that metabolic activity lasts only 12 to 24 h after inactivation, thus limiting the duration of protein synthesis in vivo (W. Liu, unpublished data). First, we assessed protective immunity 60 days after immunization with L. monocytogenes strains that differed only in their ability to access the cytosol. As described earlier, immunization with LLO⁻ Lm did not elicit significant protective immunity (Fig. 5), either as a single immunization or following two immunizations separated by 14 d. In contrast, two immunizations with cytosolic KBMA-Lm generated long-lived maximal protective immunity (Fig. 5), whereas a single immunization with KBMA-Lm did not. These results demonstrated that entry of L. monocytogenes into the cytosol substantially improves CD8⁺-T-cell fitness. Only CD8⁺ T cells primed by cytosolic L. monocytogenes could incorporate a second antigen exposure into long-lived and maximal protective immunity.

Cytosolic entry promotes dendritic cell activation and CD8⁺-T-cell priming in vivo. A possible mechanism underlying the inability of LLO⁻ Lm immunization to elicit CD8⁺ T cells with the requisite potency to provide protective immunity is that T cells did not receive the appropriate signals during priming. We hypothesized that antigen-presenting cell (APC) activation during L. monocytogenes infection depends upon critical cytosolic signals. This premise is supported by previous data from cultured DC (14) but has not been examined within the context of the intact animal. Because few DC directly take up L. monocytogenes during active infection (37), it was important to examine activation in vivo, where tertiary cells are most likely to provide maturation signals in trans, in response to bacterial stimuli. We compared DC activation in vivo following immunization with cytosolic KBMA-Lm or phagosome-confined LLO⁺ Lm to eliminate bacterial multiplication and persistence as variables. We examined the activation of splenic DC 24 h after immunization, determined in pilot studies to be the peak of DC activation (data not shown). Immunization with actA⁻ Lm and KBMA-Lm (strains that enter the cytosol) led to upregulation of the costimulatory molecules such as CD80 and CD86, which led to a 5- to 10-fold increase in surface CD80 and CD86 in both the CD8α⁺ and CD8α⁻ DC populations (Fig. 6). In contrast, upregulation of costimulatory molecules was 5- to 10-fold lower in LLO⁻ Lm-immunized mice and only differed slightly from HBSS-treated animals.

Because LLO⁺ Lm elicited almost no DC maturation in vivo, we questioned whether sufficient innate signals would be generated to promote synergy with anti-CD40 antibody. CD40 ligation promotes DC maturation and enhances T-cell stimulatory capacity (15). The ability of a DC to receive CD40 signals depends upon the presence of microbial priming signals (38, 47). Addition of anti-CD40 to an LLO⁺ Lm immunization elicited only twofold greater costimulatory molecule expression than anti-CD40 alone (Fig. 6). This result was in stark contrast to the combination of anti-CD40 with KBMA-Lm, which led to a 5- to 10-fold increase in surface CD80 and CD86 among CD8α⁺ DC (relative to anti-CD40 alone). Together, these experiments demonstrated that cytosolic entry is required for L. monocytogenes-induced DC maturation in vivo (KBMA-Lm > LLO⁺ Lm) and that bacterial multiplication enhanced this effect (actA⁻ Lm > KBMA-Lm). Although
LLO\(^{-}\) Lm interacts with cell surface and phagosomal pattern recognition receptors (24, 53), these stimuli were insufficient to deliver the microbial priming signals necessary for synergy with anti-CD40. Cytosolic entry and bacterial growth were critical for optimal activation and maturation of CD8\(^{+}\) DC in vivo in response to \textit{L. monocytogenes}.

To understand the impact of CD40 signaling on the adaptive immune response, we enumerated OVA\(_{257-264}\)-specific CD8\(^{+}\) T cells undergoing secondary expansion. (A) C57BL/6 mice immunized with act\(A^{-}\) Lm-OVA (1 \times 10^7 CFU) or LLO\(^{-}\) Lm-OVA (3 \times 10^8 CFU) were boosted with 1 \times 10^5 CFU Lm-OVA 14 days later. Five days postboost, spleens were harvested and the frequency of antigen-specific CD8\(^{+}\) T cells was determined by IFN-\(\gamma\) intracellular cytokine staining. The peptide used for restimulation is indicated on the far left. The number in each plot represents the percentage of the CD8\(^{-}\)-T-cell population, followed by the standard deviation. The percentage is the mean of five to six mice per group from a single representative experiment of four. (B) Absolute numbers of LLO\(_{296-304}\)-specific (left) and OVA\(_{257-264}\)-specific (right) CD8\(^{+}\) T cells 5 days after challenge with Lm-OVA. (C) The in vivo cytolytic function of the recalled CD8\(^{+}\)-T-cell population was assessed using an in vivo cytotoxicity assay. Three days following boost immunization with Lm-OVA, mice received an equivalent mixture of differentially CFSE-labeled targets loaded with the indicated peptides. Upper panels show percent killing of the indicated target population relative to the HSV-gB2 control. The numbers in the upper left corners indicate the percentages of LLO\(_{296-304}\) targets killed, while the numbers in the upper right corners indicate the percentages of OVA\(_{257-264}\) targets killed. Lower panels depict the frequency of epitope-specific CD8\(^{+}\) T cells determined by intracellular cytokine staining the day of target cell transfer. Percentages represent the means of three mice per group from a single representative experiment of two replicates.
CD8+ T cells at 7 days postimmunization. Surprisingly, the addition of anti-CD40 had no effect on the frequency of antigen-specific CD8+ T cells following immunization with LLO-Lm-OVA (Fig. 6B). In contrast, the combination of KBMA-Lm-OVA with anti-CD40 elicited four- to six-fold more OVA257-264-specific CD8+ T cells than KBMA-Lm-OVA alone. These results suggest that during L. monocytogenes infection, escape from the vacuole is a prerequisite for the integration of CD40 signaling by the APC.

Anti-CD40 synergizes with cytosolic entry to maintain protective immunity. CD8+ T-cell-mediated protection following immunization with cytosolic L. monocytogenes (KBMA-Lm) was measurably different from that elicited by phagosome-confined bacteria (LLO-Lm). We observed this difference as a 10,000-fold improvement in protective immunity following two immunizations with cytosolic KBMA-Lm (Fig. 5). Thus, while CD8+ T cells appeared to be phenotypically similar (primary and secondary expansion and IFN-γ production; Fig. 2 to 4), they differed fundamentally in their ability to mediate protective immunity. Assessment of T-cell avidity by peptide titration showed that actA-Lm, LLO-Lm, and KBMA-Lm all elicited CD8+ T cells with equivalent avidity (data not shown). This led us to test whether CD8+ T cells elicited by either LLO-Lm or KBMA-Lm would be receptive to surrogate CD4+ T-cell help (27), provided by the anti-CD40 antibody described above (46). As expected, mice immunized with LLO-Lm elicited a minimal reduction in spleen CFU either 15 or 60 days postimmunization (Fig. 7A). In contrast, KBMA-Lm induced a 1,000-fold reduction in splenic CFU at 15 days postvaccination but did not maintain this protection at day 60. Consistent with the in vivo activation phenotype of DC, the effect of anti-CD40 following immunization was dependent on the subcellular location of the bacterium. Following immunization with cytosolic KBMA-Lm, anti-CD40 significantly improved the potency and longevity of protection against wild-type L. monocytogenes (1,000-fold increase at 60 days). In distinct contrast, noncytotoxic LLO-Lm was unable to elicit protection, regardless of whether we administered the anti-CD40 antibody. These results support the hypothesis that movement of the bacterium from the phagosome to the cytosol is a required step for the priming of protective CD8+ T cells.

Secondary expansion and cytolytic activity are independent of protective immunity. Neither LLO-Lm nor KBMA-Lm elicited protective immunity 60 days after a single immunization, suggesting a decrease in either T-cell function or numbers over time. To test this hypothesis, we tracked the frequency of OVA257-264-specific CD8+ T cells following immunization that led to complete protection (actA-Lm and KBMA-Lm plus anti-CD40), waning protection (single immunization with KBMA-Lm), or no protection (LLO-Lm). We observed a similar pattern of primary expansion and contraction irrespective of protective immunity. All groups maintained detectable numbers of antigen-specific CD8+ T cells over 48 days (Fig. 7B). The size of the interleukin-7 receptor-α+ (IL-7Rα+)
CD8⁺-T-cell memory pool was greatest in the groups that demonstrated protective immunity (actA⁻⁻⁻⁻ Lm and KBMA-Lm plus anti-CD40), although the difference in the populations was less than 10-fold (Fig. 7C). Interestingly, KBMA-Lm and LLO⁻⁻⁻⁻ Lm elicited similar numbers of antigen-specific CD8⁺ T cells at day 14. This result contrasts their ability to mediate clearance of wild-type L. monocytogenes after 2 weeks, where KBMA-Lm-immunized mice had 10- to 100-fold fewer CFU 3 days after challenge (Fig. 7A). On day 60, we challenged animals with vaccinia virus expressing ovalbumin and tracked secondary expansion over the next 7 days. All groups demonstrated rapid and sustained secondary expansion of antigen-specific CD8⁺ T cells regardless of the size of the CD8⁺-T-cell memory pool. This result was consistent with our observations after L. monocytogenes challenge (Fig. 3) and suggests that the speed and magnitude of secondary expansion in vivo alone is not predictive of protective immunity.

Immunization with cytosolic KBMA-Lm elicited long-term protective immunity in two ways: coadministration with anti-CD40 or boost immunization at day 14. We questioned whether these additional stimuli sustained protective immunity by increasing CD8⁺-T-cell cytolytic activity. To test this possibility, mice were challenged with 1 LD₅₀ (5 × 10⁶ CFU) of Lm-OVA at 60 days postimmunization. CD8⁺-T-cell number and function were assessed by intracellular cytokine staining (data not shown) and in vivo cytotoxicity assay three days later (Fig. 7D).
We observed that immunizations that elicited a substantial reduction in tissue CFU (actA\(^-\) Lm-OVA, KBMA-Lm-OVA plus anti-CD40, and KBMA-Lm-OVA plus day 14 boost) correlated with higher baseline levels of OVA\(_{257-264}\)-specific cytotoxicity at day 63 (Fig. 7D, top row, right peak of each histogram). This cytolytic activity also correlated with OVA\(_{257-264}\)-specific CD8\(^+\)-T-cell frequency (data not shown). However, three days after Lm-OVA challenge, all groups demonstrated similar cytotoxicity toward OVA\(_{257-264}\) and LLO\(_{296-304}\)-loaded targets, regardless of whether the immunization regimen established protective immunity (Fig. 7D, bottom row). Thus, the ability to maintain a high level of immediate cytotoxicity (i.e., killing of peptide-loaded targets in the absence of infection or inflammation) correlated with resistance to lethal challenge. These results suggest that the ability of memory CD8\(^+\) T cells to recognize and kill L. monocytogenes-infected cells prior to extensive bacterial growth is more critical than secondary expansion.

**DISCUSSION**

Memory CD8\(^+\) T cells capable of rapidly identifying and killing infected cells are essential for host defense against diverse intracellular bacterial and viral pathogens. In this study, we report that known measures for defining T-cell memory, including secondary expansion and acquisition of lytic function, are not predictive of protective immunity. Infection with L. monocytogenes mutant strains unable to access the host cytosol failed to elicit protective immunity but surprisingly led to durable primary and secondary T-cell responses. On the other hand, a strain that entered the cytosol but could not propagate within this compartment promoted DC activation and early protective immunity that waned over time. Another strain that entered and multiplied within the cytosol led to long-lived CD8\(^+\)-T-cell-mediated protective immunity. These studies reveal a critical link between innate immune signaling within the host cell cytosol and CD8\(^+\)-T-cell potency. Our findings demonstrate that cytosolic access by L. monocytogenes is critical not for the delivery of antigen to the class I MHC pathway but for interaction with host cell cytosolic immunoreceptors that enhance DC activation and T-cell potency, leading to long-lived protective immunity.

We demonstrate that entry into the cytosol is necessary, but not sufficient, for induction of significant CD8\(^+\)-T-cell-mediated protective immunity following immunization with attenuated L. monocytogenes. Cytosolic L. monocytogenes strains elicit an innate host-cell transcriptional profile, including the induction of IFN-β, distinct from that of phagosome-bound LLO\(^-\) Lm (34, 40). DC serve an essential role in translating these innate signals into an appropriate adaptive response. IFN-α/β directly influences DC potency by upregulating costimulatory molecules such as CD80 and CD86 (18, 35, 45) and promoting cross-presentation of pathogen-derived antigen (33). In primary cultures of bone marrow-derived DC, in which bacteria come in direct contact with the DC, LLO\(^-\) L. monocytogenes strains are poor inducers of maturation (14). However, few CD11c\(^+\) DC are directly infected following in vivo infection with L. monocytogenes (37), so it is likely that other cells produce cytokines necessary for DC maturation. Interestingly, even though immunization with LLO\(^-\) Lm induces an almost undetectable change in DC activation, it led to a robust induction of primary and central-memory CD8\(^+\) T cells. In this case, it is possible that the lack of type I IFN, a suppressor of IL-12p70 production (2, 16), decreased the requirement for APC maturation. Consistent with this hypothesis, we have observed increased numbers of L. monocytogenes-specific CD8\(^+\) T cells in the absence of type I IFN signaling (unpublished data).

The CD8α\(^+\) DC population, previously identified as the predominant presenter of L. monocytogenes-derived peptides in vivo (4, 5), is exceptionally responsive to immunization with cytotoxic L. monocytogenes. Of particular interest, nonmultiplying KBMA-Lm elicited 2- to 10-fold higher levels of costimulatory molecule expression than phagosome-bound LLO\(^-\) Lm (Fig. 6). In addition, the actA\(^-\) Lm strain, which does multiply within the cytosol, elicited almost twofold higher levels of costimulatory molecule expression than nonmultiplying KBMA-Lm within the same compartment. This result demonstrates that recognition of bacteria within the host cell cytosol, not growth or persistence, is the minimal requirement to initiate potent DC maturation. The ability of the bacterium to grow and persist within the cell increases DC activation, likely through either an increased concentration of stimulatory ligands or increased duration of stimulus. The addition of anti-CD40 improved the magnitude of protective immunity but with the requirement that the immunizing bacteria could access the cytosol. CD40 signaling could improve T-cell priming in several ways, including (i) through enhanced expression of costimulatory molecules on DC, CD40 signaling could overcome a suboptimal antigen concentration (i.e., number of peptide-MHC complexes per DC) relative to replicating bacteria; (ii) by upregulating anti-apoptotic molecules within the DC, CD40 signaling may overcome the lack of bacterial persistence by prolonging antigen presentation (10); or (iii) by enhancing DC migration into the T-cell zone of the secondary lymphatics, based on reports that LLO\(^-\) Lm immunization leads to abnormal DC homing within the spleen (37). Thus, the inability of phagosome-confined LLO\(^-\) Lm to induce DC maturation correlates with poor CD8\(^+\)-T-cell potency and suboptimal protective immunity.

The strength of the stimulus during priming correlates directly with T-cell fitness, impacting the ability of the T cell to both respond to homeostatic signals and maintain expression of anti-apoptotic molecules (20). Our data using cytotoxic KBMA-Lm demonstrated a substantial reduction in tissue CFU upon wild-type L. monocytogenes challenge at 2 weeks postimmunization, about 1,000-fold better than with LLO\(^-\) Lm. Interestingly, this protection declined over time, while the capacity for secondary expansion remained, suggesting either that a subset of protective T cells was lost or that the potency of individual T cells waned over time. What leads to this loss of protective immunity? CD4\(^+\)-T-cell help has proven essential for the maintenance, but not the priming, of L. monocytogenes-specific protective immunity (52). The nature of the help provided by CD4\(^+\) T cells during this maintenance phase is, however, unclear. We detected IFN-γ and CD40L-producing L. monocytogenes-specific CD4\(^+\) T cells after immunization with both LLO\(^-\) and KBMA bacteria. Interestingly, the addition of anti-CD40 during immunization with KBMA-Lm did not increase protective immunity at day 14 but did lead to long-term maintenance of this response. This observation raises the possibility that CD40 ligation acts to enhance the function of the CD4\(^+\)-T-cell population. Because priming of
CD4+ T cells requires extended interaction between the T cell and DC (20, 39), the accelerated clearance of KBMA-Lm might limit the interaction of CD4+ T cells with cognate MHC II-peptide complexes. Thus, by improving DC potency and survival, anti-CD40 may facilitate improved CD4+ T-cell function.

In contrast to immunization with heat-killed L. monocytogenes, LLO-Lm elicited a robust population of primary and memory CD8+ T cells (Fig. 2) but only a minimal reduction of tissue CFU following lethal challenge with wild-type L. monocytogenes (Fig. 1B). Lauvau and colleagues elegantly demonstrated that immunization with heat-killed L. monocytogenes elicits poor expansion of antigen-specific CD8+ T cells and establishes a dysfunctional population of central-memory T cells (32). A key difference between vaccinations with heat-killed, LLO-, and replication-competent cytosolic L. monocytogenes strains is antigen load and persistence. While wild-type or actA-deleted L. monocytogenes strains multiply and secrete antigen in vivo for 4 to 9 days, L. monocytogenes CD8+ T cells lack a still-as-yet-undefined function that is required for full protective immunity. Only with a combination of metabolic activity and cytotoxic access is full protective immunity elicited (Fig. 5).

The finding that the fitness of CD8+ T cells can vary even in the presence of long-lived memory T cells is relevant for the design of vaccines for both infectious disease and cancer. In the case of therapeutic vaccines targeting malignancies or chronic infections, the presence of large numbers of effector-memory CD8+ T cells may be of lesser importance. In this setting, a delay of several hours while central-memory CD8+ T cells expand and disseminate may be inconsequential, as the desired response to these vaccines is a large effector CD8+ T-cell response. Conversely, prophylactic vaccines designed to elicit long-lived cellular immunity against a rapidly multiplying pathogen must reduce microbial burden immediately after infection. Any delay in the dissemination of cytolytic CD8+ T cells to peripheral tissues results directly in increased microbial burden. In this case, selection of vaccine vectors and regimens that maximize the maintenance of effector-memory CD8+ T cells should be a primary goal.

The induction of primary and secondary CD8+ T-cell responses is a necessary but insufficient step in the development of protective immunity to an intracellular pathogen. Antigen from phagosomal-confined LLO-deleted L. monocytogenes is cross-presented, priming primary and memory CD8+ T cells. However, without bacterial entry into the cytosol, DC in vivo express very low levels of costimulatory molecules. The result of T-cell priming by these poorly activated DC is inferior protection against lethal challenge. Our findings emphasize that a clear difference between CD8+ T-cell memory and protective immunity does exist, and while the term “memory” is frequently used to imply protective immunity, we demonstrate that this relationship is not absolute.

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