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Distinct *in vivo* dendritic cell activation by live versus killed *Listeria monocytogenes*

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Immunization of mice with live or heat-killed *Listeria monocytogenes* (HKLM) efficiently primes pathogen-specific $CD8^+$ T cells. T lymphocytes primed by HKLM, however, undergo attenuated proliferation and do not fully differentiate. Thus, only infection with live bacteria induces long-term, $CD8^+$ T cell-mediated protective immunity. In this study we demonstrate that live and heat-killed bacteria, while both associating with Mac-3^+CD11b^{hi} cells, localize to distinct splenic areas following intravenous inoculation. While HKLM localize to the marginal zone and the splenic red pulp, live *L. monocytogenes* are carried to the T cell zone of splenic white pulp. Despite these differences, *in vivo* depletion of CD11c-expressing cells prevents priming of naive T cells by either HKLM or live *L. monocytogenes*. Analysis of CD11c^{hi} dendritic cells (DC) reveals that infection with live *L. monocytogenes* induces higher levels of CD40, CD80 and CD86 expression than immunization with HKLM. Our results suggest that $CD8^+$ T cell priming following HKLM immunization or live infection is mediated by DC and that the disparate outcomes of priming can be attributed to suboptimal conditioning of DC in the absence of live, cytosol-invasive bacteria.

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Introduction

Listeria monocytogenes is a Gram-positive intracellular bacterium that causes severe infections in immunocompromised individuals and pregnant women [1]. In immunocompetent mice, intravenous injection of a small number of *L. monocytogenes* causes an acute infection of spleen and liver that is cleared within 7 to 8 days [2, 3]. Neutrophils and monocytes are rapidly recruited to sites of bacterial infection and are required for initial control of *L. monocytogenes* infection [4, 5]. Interestingly, in the infected spleen, live *L. monocytogenes* are mostly found in T cell zones of the white pulp [6]. Complete clearance of bacteria depends upon T cells, and immunodeficient SCID mice develop chronic, ultimately lethal listeriosis [7]. $CD8^+$ T cells mediate protective immunity, since immune mice depleted of memory $CD8^+$ T cells are susceptible to re-challenge with a lethal dose of *L. monocytogenes* [8, 9].

Although *L. monocytogenes* is believed to infect macrophages in the spleen, priming of $CD8^+$ T cells appears to be mediated predominantly by dendritic cells (DC). Indeed, depletion of $CD11c^+$ cells using an engineered mouse model, where the diphteria toxin receptor is expressed under the control of CD11c promoter, has demonstrated that DC are essential for

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Abbreviations: **HKLM:** Heat-killed *Listeria monocytogenes* · **LLO:** Listeriolysin O

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priming of Listeria-specific $CD8^+$ T cells [10]. Once $CD8^+$ T cells are primed during the course of live infection, they undergo programmed expansion and differentiation into memory T cells in an antigen-independent fashion [11–13].

In a previous study, we demonstrated that memory $CD8^+$ T cell generation and protective immunity can be uncoupled [9]. Our results demonstrated that, in contrast to infection with live *L. monocytogenes*, immunization with heat-killed *L. monocytogenes* (HKLM) primed $CD8^+$ T cells that did not acquire cytolytic and IFN-γ-secreting effector functions or down-regulate CD62L and, importantly, did not confer protective immunity. Although $CD8^+$ T cells primed with live *L. monocytogenes* versus HKLM were functionally distinct, the mechanisms underlying this disparity remained undefined. Although we favored the notion that live infection induces an inflammatory milieu that is conducive to $CD8^+$ T cell proliferation and differentiation, mixed immunization with live bacteria and HKLM demonstrated that the inflammatory response induced by live infection did not promote differentiation of T cells primed by HKLM. One possible explanation for this result is that live bacteria and HKLM localize to distinct sites in the spleen. Furthermore, $CD8^+$ T cell priming is mediated by $CD11c^{hi}$-expressing DC, thus it is also conceivable that HKLM and live bacteria promote the differentiation of distinct DC populations with differing capacities to induce $CD8^+$ T cell differentiation.

In order to study these possibilities, we investigated the localization of live *L. monocytogenes* and HKLM in spleens following intravenous inoculation. As previously described, live bacteria localize to the T cell zone of the white pulp. In contrast, HKLM remain in the marginal zone and the splenic red pulp. *In vivo* depletion of $CD11c^{hi}$-expressing cells, however, demonstrated that $CD8^+$ T cell priming following live infection or HKLM immunization is mediated by DC. Analysis of DC following intravenous inoculation with live bacteria or HKLM demonstrated enhanced CD40, CD80, CD86 and MHC class II up-regulation during live infection. Thus, our results support a model of disparate DC activation following live versus HKLM immunization determining the extent of $CD8^+$ T cell proliferation and differentiation.

Results

Live and dead *L. monocytogenes* bacteria localize to distinct splenic regions

T cell priming occurs within the splenic white pulp, an area where most T cells are localized [14, 15]. Interestingly, live *L. monocytogenes* rapidly localize to T cell areas of the splenic white pulp following infection with virulent bacteria [6, 16]. Splenic localization of intravenously inoculated HKLM is less well defined. Because T cell activation differs following immunization with live or dead bacteria, we hypothesized that bacterial antigens might be delivered to antigen-presenting cells (APC) and presented to T cells distinctly in these two settings. To study this question, we performed immunohistology on spleens from mice injected with HKLM or live *L. monocytogenes*. To clearly identify splenic follicles, the marginal zone, the red and the white pulp including the central arteriole, we used phalloidin staining which binds cellular actin (red). We validated this approach (Fig. 1A) using anti-IgD and anti-MOMA-1 staining, which delineate B cell zones (green) and marginal zones (blue), respectively.

Since it is difficult to detect low numbers of live bacteria (5,000) inside organs of infected mice at early time points (not shown), we injected high numbers of bacteria to increase their frequency and to be able to detect them on tissue sections (10^9 for 1 h, 10^5 for 24 h). At 1 h after inoculation, *in vivo* bacterial replication is minimal, enabling comparison between similar doses of live and dead bacteria (10^9) (Fig. 1B, middle panels). Inoculation of higher doses of bacteria increases the number of infected follicles within spleens (data not shown) but does not effect either bacterial distribution inside infected foci (Fig. 1B, upper and lower right panels) or T cell responses [11]. At early time points following intravenous inoculation, live *L. monocytogenes* and HKLM localized to the marginal zone, defining the border between the red pulp and the white pulp area (Fig. 1B, middle panels). However, 24 h following injection, live *L. monocytogenes* were predominantly localized to the T cell zones, whereas HKLM remained within either the marginal zone or the red pulp (Fig. 1B, lower panels). In both settings, at 1 h or 24 h after inoculation, most bacteria were inside cells that were positive for Mac-3 (Fig. 2), a protein expressed by some DC as well as activated tissue macrophages [17]. After 24 h infection with live *L. monocytogenes*, bacteria localized inside the white pulp were surrounded by $CD11c^+$ DC (Fig. 2). Of note, whether mice were injected with live or dead *L. monocytogenes*, few $CD11c^+$ DC were associated with bacteria (Fig. 2). HKLM did not enter the white pulp at later time points (48 or 72 h, data not shown).

Live *L. monocytogenes* induce relocation of $Mac\text{-}3^+$ cells inside T cell zones

Localization of live bacteria to the splenic T cell zone could occur by two mechanisms. First, bacteria could be transported to the T cell zone by macrophages. Alter-

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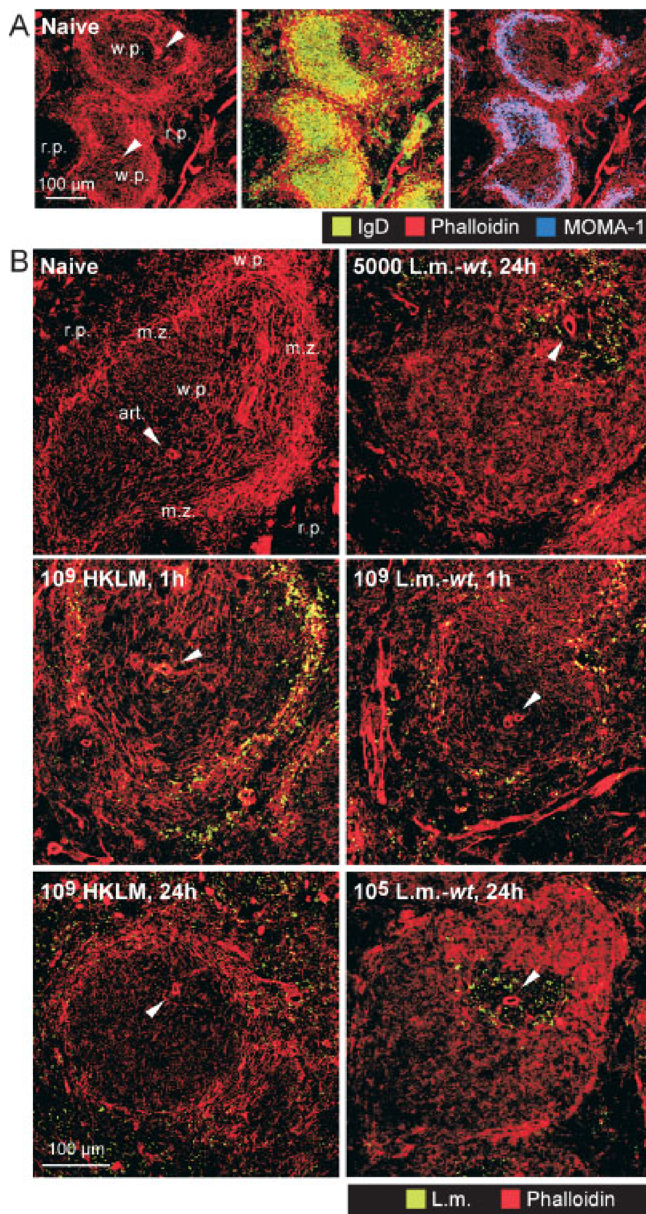

Fig. 1. Distinct compartmentalization of live and heat-killed *L. monocytogenes* in the spleen. Phalloidin was used to delineate the central arteriole (art, white arrows) of the follicle and the marginal zone (m.z., in red). (A) IgD (green) and MOMA-1 (blue) stain B cell and marginal zones, respectively. (B) BALB/c mice were infected with live *L. monocytogenes* (10^9 bacteria/mouse at 1 h or 10^5 at 24 h) or immunized with HKLM (10^9/mouse). Spleens were harvested at the indicated time points and sectioned and stained as described in the Materials and methods. Bacteria were stained with a polyclonal antiserum and are green. Photomicrographs are shown for individual mice and are representative of six to nine mice per group.

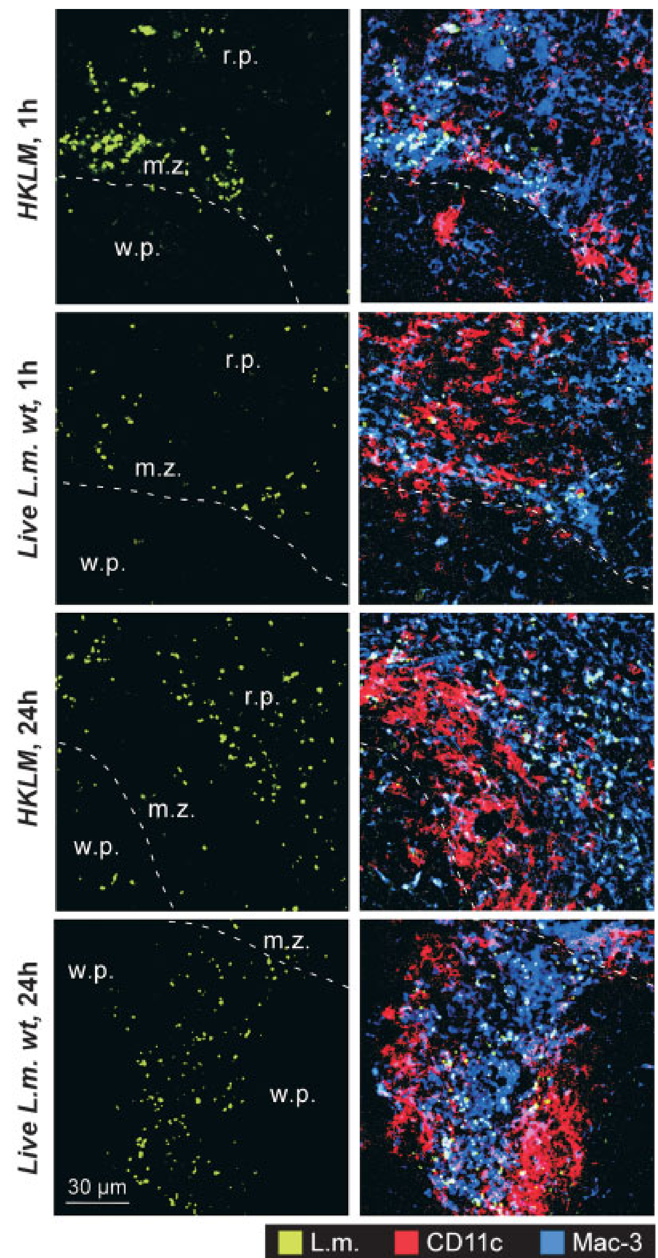

Fig. 2. Live and heat-killed *L. monocytogenes* are mostly inside $Mac3^+$ but not $CD11c^+$ cells. Macrophages/neutrophils (Mac-3-specific mAb, blue), DC (CD11c-specific antibody, red) and *L. monocytogenes* (antiserum, green) were stained. The localization of the marginal zone (m.z.) is indicated by the interrupted white line. The red pulp (r.p.) and the white pulp (w.p.) are also indicated. Most bacteria are found associated with $Mac\text{-}3^+$ cells; very few bacteria associated with $CD11c^+$ cells either 1 or 24 h after inoculation.

natively, bacteria could move from cell to cell by actin polymerization. To distinguish between these two mechanisms, we infected mice with the *L. monocytogenes* mutant *ActA*$^-$ [18], which cannot polymerize actin filaments and therefore cannot move from cell to cell. To prevent movement of *ActA*$^-$ bacteria by extracellular spread, mice were treated with high doses of the antibiotic gentamicin, an antibiotic that kills extracellular but not intracellular bacteria. *ActA*$^-$ bacteria readily localized to the splenic T cell zones (24 h) and were surrounded by $CD11c^+$ DC (Fig 3) with kinetics indistinguishable from infection with wild-type (WT) bacteria. This result suggests that $Mac3^+$ cells infected

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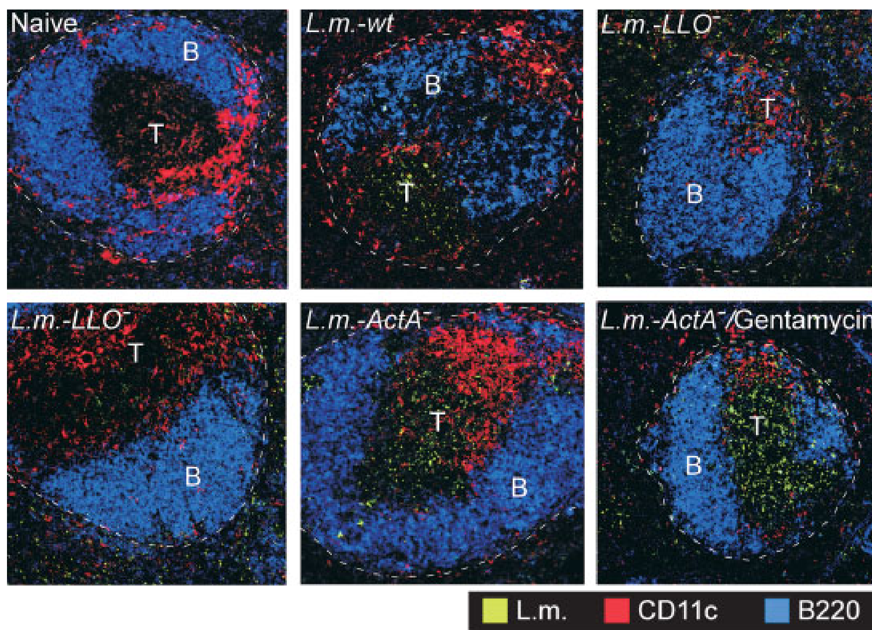

Fig. 3. Live *L. monocytogenes* relocate to splenic T cell zones. Mice were infected with WT (10^5/mouse), *LLO*$^-$ (5×10^8/mouse) or *ActA*$^-$ (10^6/mouse) *L. monocytogenes*, and spleens were harvested 24 h later and stained for bacteria (green), CD11c (red) and B220 (blue). To exclude the possibility that *ActA*$^-$ bacteria migrated to T cell zones extracellularly, mice were given 2 mg gentamicin intraperitoneally at the time of infection and 12 h later, and spleens were analyzed 24 h after infection (lower right panel). This experiment was performed twice with similar results.

with live *L. monocytogenes* may carry intracellular bacteria into the splenic T cell zone.

To determine the role of bacterial invasion of host cell cytosol in splenic localization, we infected mice with live, listeriolysin O (*LLO*)-deficient *L. monocytogenes*. *LLO*$^-$ bacteria cannot escape primary phagocytic vacuoles. Unlike *ActA*-deficient bacteria, *LLO*$^-$ bacteria did not localize to splenic T cell zones (Fig. 3). This result suggests that bacterial invasion of the cytosol is an essential stimulus that promotes the movement of infected cells into the T cell zone.

Live WT *L. monocytogenes* predominantly reside within CD11b$^+$CD11c$^-$ cells

Although histological examination suggested that live *L. monocytogenes* do not co-localize with DC, given the nature of DC and their interdigitating processes, it remained possible that bacteria directly infect DC. To address this issue, we flow-sorted splenocytes 24 h after infection with live WT *L. monocytogenes*. CD11b$^+$ and CD11c$^+$ cells were purified into distinct populations by cell sorting, lysed in water/0.1% Triton X-100 and plated onto bacterial culture plates to enumerate live bacteria. About 40–50% of live bacteria enumerated per infected mouse spleen were recovered after the purification procedure. Fig. 4A shows the sorting gates and bacterial numbers found per spleen 24 h after intravenous injection of 10^5 live WT *L. monocytogenes*. As previously suggested by tissue section analysis, very few bacteria were detected within CD11c^{hi} DC (900) as compared to CD11b$^+$ cells (18,000). This result suggests that splenic CD11c^{hi} DC are not a significant *in vivo* reservoir of *L. monocytogenes* bacteria (Fig. 4B). Of note, almost no bacteria were detected inside CD11c$^-$CD11b$^-$ cells (not shown). Only 5–10% of the initial intravenous inoculum gets to the spleen [2], suggesting that the bacteria found inside these cell subsets 24 h after infection had already undergone several rounds of divisions.

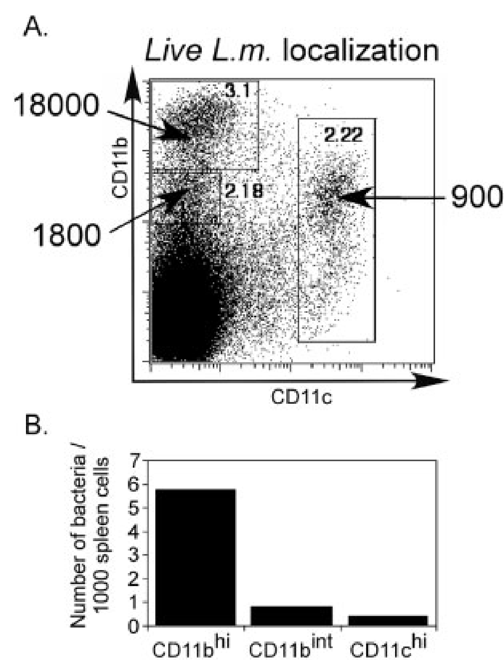

Fig. 4. Live *L. monocytogenes* are predominantly in CD11b$^+$ cells and not CD11c^{hi} DC. (A) Splenic APC were flow-sorted 24 h after immunization with live Listeria (10^5/mouse), lysed and plated onto BHI plates. The number of live bacteria per spleen is shown for each sorting gate. (B) The bar graph shows the number of bacteria per 1,000 spleen cells.

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CD11c^{hi} DC prime naive *L. monocytogenes*-specific CD8^{+} T cells following live or dead bacteria injection

Previous studies indicated that priming of Listeria-specific CD8^{+} T cells following live bacterial infection requires CD11c^{hi}-expressing DC [10]. To characterize the *in vivo* role of CD11c^{hi} DC in priming of Listeria-specific CD8^{+} T cells after HKLM immunization, we used the DTR transgenic mouse model. As shown in Fig. 5, when CD11c^{hi}-expressing cells were deleted in DTR transgenic mice by diphteria toxin treatment, adoptively transferred naive L9.6 CD8^{+} T cells remained undivided (CFSE^{hi}), regardless of whether mice were injected with live bacteria or HKLM. In contrast, in DTR mice that were not injected with the toxin, L9.6 T cells underwent activation and proliferation. These results further support the *in vivo* requirement of CD11c^{hi} DC for the priming of Listeria-specific CD8^{+} T cells following HKLM immunization.

Suboptimal activation of CD11c^{hi} DC after HKLM immunization

Although CD11c^{hi}-expressing DC are involved in priming of CD8^{+} T cells following either live infection or immunization with HKLM, the disparate CD8^{+} T cell phenotypes following these two forms of immunization remain unexplained. To determine whether DC activation is distinct following live infection or HKLM immunization, we analyzed cell surface expression of costimulatory molecules (CD80, CD86, CD40) and MHC class II molecules on CD11c^{hi} splenic DC as well as their ability to secrete the proinflammatory cytokine IFN-γ. Fig. 6A shows that the extent and the duration of CD80 and CD86 up-regulation is distinct following live infection versus HKLM immunization. Live *L. monocytogenes* infection induced 5-fold greater costimulatory molecule expression than HKLM immunization. Although up-regulation occurred more rapidly after HKLM injection (already at 7 h, grey versus black histograms), down-regulation also occurred at earlier

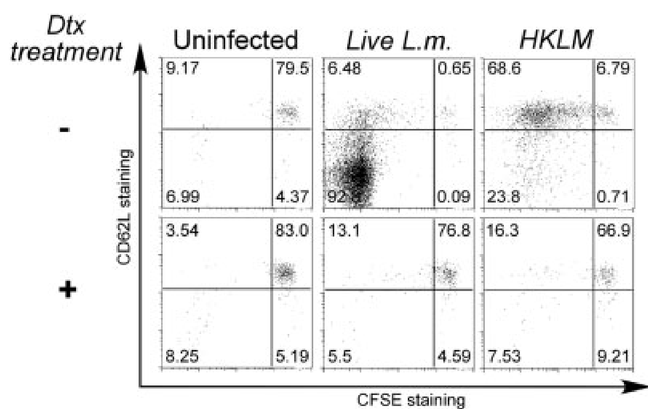

Fig. 5. CD11c^{hi} DC prime naive *L. monocytogenes*-specific CD8^{+} T cells after live infection or HKLM immunization *in vivo*. CFSE-labeled L9.6 Thy1.1^{+} T cells were transferred into BALB/c DTR transgenic mice and then either left untreated or treated with diphteria toxin. Mice were challenged with 5,000 live or 10^9 HKLM, as described in the Materials and methods, and L9.6 T cell proliferation was analyzed 4.5 days later by FACS. This experiment was done three times with two mice/condition and gave similar results.

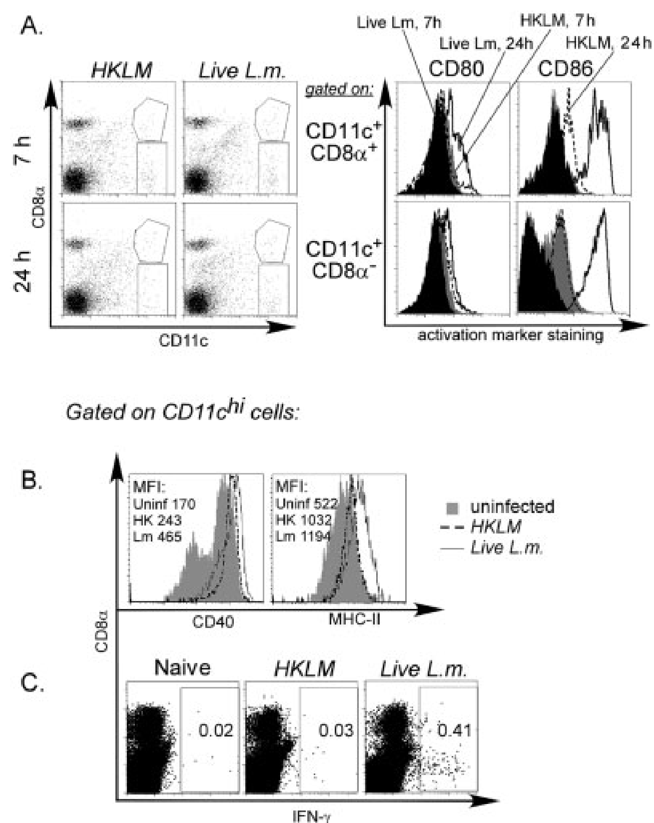

Fig. 6. CD11c^{hi} DC express lower levels of costimulatory molecules after HKLM immunization as compared to live infection. (A) Spleens from HKLM-immunized (5×10^9 bacteria) or live *L. monocytogenes*-infected mice (10^5 bacteria) harvested after 7 or 24 h were digested with collagenase and analyzed by FACS following staining with mAb specific for CD11c and CD8α (left panels, dot plots). After gating on CD11c^{+}CD8α^{+} or CD11c^{+}CD8α^{-} DC populations, the extent of their activation marker (CD80 and CD86) up-regulation was monitored (right panels, histograms). Filled (grey/black) histograms show DC from mice infected for 7 h, and open histograms with plain/dotted lines depict DC from mice infected for 24 h. Grey histograms/doted lines represent HKLM-immunized mice and black/plain lines live *L. monocytogenes*-infected mice. (B–C) Spleens from mice injected with live or heat-killed bacteria were harvested after 24 h and (B) stained for CD40 or MHC class II cell surface expression on CD11c^{hi} cells or (C) incubated in complete medium for 4–5 h with brefeldin A (2 μg/ml) and further monitored for CD11c cell surface marker expression and intracellular IFN-γ using standard intracellular cytokine staining procedure. Three mice/condition were analyzed and gave similar data in three independent experiments.

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time points (not shown). Similarly, the extent of CD40 and MHC class II cell surface expression was approximately 2- and 1.2-fold higher, respectively, 24 h after live infection as compared to HKLM immunization (Fig. 6B). Moreover, only CD11c^{hi} cells from mice injected with live *L. monocytogenes* produced IFN-γ 24 h after immunization without any *in vitro* restimulation (Fig. 6C), which correlates with costimulatory molecule up-regulation. Although few DC produced this cytokine, this result was reproducible in independent experiments and seemed to reflect their activation status. No significant IL-12 secretion by DC was detected under similar conditions (data not shown).

Discussion

While both heat-killed and live *L. monocytogenes* efficiently prime CD8$^+$ T cells, only those activated by live bacterial infection fully differentiate into effectors and provide long-term protective immunity [9]. In this report, we show that in contrast to live *L. monocytogenes*, which localize to the T cell zone, HKLM localize to the splenic marginal zone and red pulp. We further demonstrate that CD8$^+$ T cell priming following either live infection or HKLM immunization is dependent upon CD11c^{hi}-expressing cells. Our analysis of CD11c^{hi} DC showed that infection with live *L. monocytogenes* induces higher levels of cell surface CD80, CD86, CD40 and MHC class II expression than immunization with HKLM. Moreover, we detected IFN-γ-secreting CD11c^{hi} DC only in mice immunized with live *L. monocytogenes*. Thus, our results suggest that the distinct outcome of priming with live versus killed bacteria is attributable to disparate conditioning of CD11c^{hi} DC.

To dissect which factors are important for the outcome of immune responses, we analyzed the early events occurring before T cell proliferation. While live and dead bacteria are mostly found inside Mac-3$^+$CD11b$^+$ cells, only bacterial strains that generate protective immunity induce trafficking of *L. monocytogenes*-containing cells to the T cell zones; Neither HKLM nor the avirulent *LLO*$^-$ strain of *L. monocytogenes* induce protective immunity [19], and neither localize to splenic T cell zones. Thus, since T cell priming occurs during the first 24–48 h of *L. monocytogenes* infection, we speculated that local influx of Listeria-infected cells in this area might be a prerequisite for the induction of protective immunity.

Although CD11c^{hi} DC prime *L. monocytogenes*-specific naive CD8$^+$ T cells (Fig. 5), we found few live bacteria within these APC. It is likely that CD11c^{hi} DC efficiently take up Listeria-derived antigens following either live infection or immunization with HKLM, since the proportion of activated T cells is similar in both circumstances [9]. It is possible that upon recruitment to the T cell zones in the setting of live infection, infected Mac3$^+$ cells provide a stimulus for optimal T cell priming. On the other hand, the absence of inflammatory cells in the T cell zone following HKLM immunization may result in suboptimal priming.

How do *L. monocytogenes*-infected cells migrate to the T cell zone? Activated B cells have been shown to move to the T cell zones in secondary lymphoid organs by up-regulating CCR7 expression, enabling chemotaxis towards CCL19 and CCL21 [20]. Along similar lines, DC also up-regulate CCR7 upon exposure to inflammatory stimuli, which enables trafficking to splenic and lymph node T cell zones [21, 22]. Therefore, Listeria-infected cells might express CCR7 upon cytosolic invasion by *L. monocytogenes* and thus become responsive to splenic CCL19 and/or CCL21 chemokines gradients.

Our finding that the kinetics of CD11c^{hi} DC cell surface CD80 and CD86 up-regulation and the frequencies of IFN-γ-secreting DC differ following live infection (0.41%) versus HKLM immunization (0.03%) (Fig. 6) supports the hypothesis that killed bacteria do not fully activate DC. It has been shown *in vitro* that live bacteria are far more potent in activating bone marrow-derived DC than are HKLM [23]. This favors the idea that HKLM and live bacteria differentially activate DC *in vivo*. Several recent reports also suggest that the activation states of DC can be manipulated by pathogens, with significant implications for T cell priming. For instance, bacterial virulence factors from *Bacillus anthracis* impair *in vivo* DC function by disrupting intracellular signaling networks, thereby disarming adaptive immune responses [24]. On another level, TLR triggering on DC can block the suppressive effects of regulatory T cells by releasing specific cytokines [25]. It is possible that distinct TLR are triggered on DC by live and heat-killed bacteria. Alternatively, intracellular pattern recognition receptors such as nucleotide-binding oligomerization domain proteins (NOD) [26] might be required to fully activate DC. This trigger might be selectively activated by live, cytosol-invasive bacteria [27].

While the distinct activation of T cells by immature DC and mature DC is well characterized, it is less clear how effectively incompletely or partially activated DC stimulate naive CD8$^+$ T cells. Proliferation of naive *L. monocytogenes*-specific CD8$^+$ T cells primed *in vivo* by DC in mice immunized with heat-killed bacteria, in contrast to those in animals infected with live Listeria, undergo attenuated proliferation and do not become cytolytic and IFN-γ-secreting effectors (Fig. 5 and [9]). CD28, which recognizes CD80 and CD86 on DC, is constitutively expressed on naive T cells and is required for complete T cell priming. While CD28-deficient mice develop diminished immunity to *L. monocytogenes* infection [28], the degree of protection elicited by live

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infection of these mice exceeds that elicited by HKLM immunization of WT mice. Therefore, deficient CD80 and CD86 expression on DC does not fully explain incomplete $CD8^+$ T cell activation following HKLM immunization.

There is a long list of membrane-bound molecules on T cells that can influence T cell activation, differentiation and survival. Among them, members of the TNF receptor superfamily (such as CD40, OX40 (CD134) or 4–1BB (CD137)) have been implicated in T cell activation. Several reports have demonstrated dramatic reductions in the frequencies of antigen-specific $CD4^+$ T cells in response to lymphocytic choriomeningitis (LCMV) and vesicular stomatitis (VSV) viruses in OX40- and OX40L-deficient mice [29, 30]. In contrast to OX40/OX40-L, 4–1BB signaling largely impacts $CD8^+$ T cell responses. The frequencies of both influenza virus- and LCMV-specific $CD8^+$ T cells are decreased 2- to 10-fold in 4–1-BBL-deficient mice as compared to WT mice following primary challenge [31]. Memory T cells are also dramatically diminished in these mice several weeks after priming. Furthermore, *in vivo* stimulation of CD137 using a strong agonistic mAb enhances and broadens virus-specific $CD8^+$ T cell responses [32]. Normal $CD8^+$ T cell responses could also be restored in CD28-deficient mice upon treatment with this mAb. It might therefore be possible that HKLM immunization does not induce sufficient 4–1BBL up-regulation on $CD11c^{hi}$ DC to fully activate $CD8^+$ T cells. In support of this, a recent study using 4–1BBL-deficient mice suggested impaired $CD8^+$ T cell activation following *L. monocytogenes* infection [23].

Although the specific mechanism by which infection with live *L. monocytogenes* induces long-term protective immunity remains elusive, our studies indicate that factors induced by bacterial invasion of the host cell cytosol play a critical role in the adaptive immune response to this pathogen. Identifying the receptors and signaling pathways that are involved in this process will have important implications for vaccine development.

Materials and methods

Mice and peptides

BALB/cJ mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Females (6–8 weeks old) were used in all experiments and maintained under specific pathogen-free conditions. L9.6 TCR-transgenic mice on the BALB/cJ RAG-$1^{-/-}$ background were generated as described [9] and bear a TCR specific for the *L. monocytogenes*-derived epitope $p60_{217–225}$ presented in the context of $H2\text{-}K^d$. DTR mice express the diphteria toxin receptor (DTR) under the control of the CD11c promoter and were on the BALB/c background [10]. Mice were bred at the Memorial Sloan-Kettering Research Animal Resource Center and later at the animal facility of the Institute of Molecular and Cellular Pharmacology in Nice-Sophia Antipolis. The synthetic peptide $p60_{217–225}$ used for *in vitro* priming assays was obtained from Research Genetics (Huntsville, Alabama).

Bacterial strains

Bacteria were cultured in brain heart infusion (BHI) broth. *L. monocytogenes* strains 10403s, *ActA*$^-$ (DP-L1942) and *LLO*$^-$ (DP-L2161) were originally provided by Daniel Portnoy (University of California, Berkeley, CA). HKLM was prepared by growing *L. monocytogenes* strain 10403s in 500 ml BHI medium for 72 h at 37°C. Bacteria were washed twice in 100 ml PBS and resuspended at 5×10^{10} bacteria per ml in PBS and incubated 3 h at 70°C. Aliquots of this suspension were stored at –80°C. The complete inactivation of *L. monocytogenes* bacteria was determined by overnight culture in BHI broth.

Immunization with *L. monocytogenes*

Mice were immunized by intravenous injection of various doses (as specified in the figure legends) of live *ActA*$^-$ or *LLO*$^-$ bacteria or HKLM 10403s into the lateral tail vein.

Histology

Tissue sections were fixed for 6 h at 4°C in 2% paraformaldehyde pH 7.0, washed in PBS, incubated overnight at 4°C in a solution of 30% sucrose under agitation, and washed again in PBS. Sections were further frozen in isopentane at –80°C, and 10 μm cryostat sections were prepared. Tissues sections were incubated in 100 mM Tris HCl pH 8.0 to eliminate OCT and in a solution containing 100 mM Tris HCl pH 8.0 with saturating concentrations of 2.4.G2 mAb, 10% normal goat serum and 10% normal mouse serum. Avidin/biotin sites were saturated using the avidin/biotin blocking kit (Vector). Tissues sections were stained with FITC-coupled anti-B220, allophycocyanin-coupled anti-CD11c and anti-*L. monocytogenes* serum (Difco Listeria O rabbit polyserum, Fisher) revealed with an Alexa Fluor 568-coupled goat anti-rabbit secondary mAb. Mac-3 was revealed using an Alexa Fluor 488-coupled goat anti-rat antibody. Staining was performed in a solution containing 100 mM Tris HCl pH 8.0/Tween 0.05% and mounted in DACO medium (DAKO corporation, CA). Visualization of labeled cells on tissue sections was performed under a Leica laser scanning confocal microscope (TCS-SP) equipped with a DM-IRBE inverted microscope and an argon-krypton laser.

Antibodies and flow cytometric analysis

For flow cytometric analysis, approximately 5×10^6 cells were aliquoted per staining well of a 96-well plate. After incubation at 4°C for 20 min with Fc-block (PharMingen, San Diego, CA) in FACS staining buffer (SB; PBS pH 7.45/0.5% BSA/0.02% sodium azide), cells were stained with fluorochrome-coupled mAb in SB for 30 min at 4°C. Subsequently, cells were washed three times in SB. Flow cytometric analysis was performed using a BD LSR or a FACSCalibur flow cytometer, and data

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were further analyzed with FlowJo software (TriStar Inc, Ashland, OR).

The following mAb were purchased from BD PharMingen: anti-CD8α (53–6.7)-fluorescein isothiocyanate (FITC) or -allophycocyanin, anti-CD62L-allophycocyanin (MEL-14), anti-CD90.1-phycoerythrin (PE)(H1S-51), anti-CD11c-PE (HL3), anti-CD40- (3/23), anti-MHC class II-A/I-E^d (2G9), anti-IgD (11–26c.2a), anti-Mac-3-FITC (M3/84), anti-CD80-PE (16–10A1) and anti-CD86-PE (GL1). Phalloidin Alexa Fluor 488 and 647, Alexa Fluor 568-coupled goat anti-rabbit and Alexa Fluor 488-coupled goat anti-rat secondary mAb were purchased from Molecular Probes (Eugene, OR). Anti-MOMA-1 was obtained from BMA biomedicals AG.

Isolation of splenic cell subsets and flow cell sorting

Spleens were recovered, collagenase-digested and treated to lyse red blood cells. For DC cell sorting experiments, cell suspensions were incubated on ice in FACS SB (PBS pH 7.45/0.5% BSA) at 5×10^7 cells/ml with Fc-block 30 min and then stained 45 min with anti-CD11c-PE, anti-CD8α-allophycocyanin and DAPI for dead cell exclusion. After staining, cells were subsequently washed three times and purified using a MOFLO cell sorter at the Memorial Sloan-Kettering cell sorting facility. Cells were collected into cold RPMI 1640 containing 20% FCS. For plating experiments, cells were pelleted and lysed in water containing 0.1% Triton X-100.

CFSE labeling

Cells were washed with PBS and resuspended at 5×10^7 cells/ml in PBS containing in 10 μM 5(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes). After incubating at 37°C for 10 min in the dark, cells were immediately washed with cold RPMI/10% FCS and resuspended in PBS for intravenous injection into mice.

4.8 *Depletion of CD11c$^+$ cells in DTR transgenic mice and* in vivo *priming assay*

Thy1.2$^{+/+}$ DTR$^{+/-}$ BALB/c mice were injected with 1–2×10^6 L9.6 RAG$^{-/-}$ Thy1.1$^{+/+}$ splenocytes and treated with diphteria toxin (Sigma) 1 day later, as previously described [10]. Briefly, mice were injected intraperitoneally with 4 ng toxin/g body weight and infected or immunized 8–10 h later with 2,000 live *L. monocytogenes* or 10^9 HKLM, respectively. T cell proliferation and CD62L expression were monitored 4.5 days later by FACS analysis.

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