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Distinct Responses of Splenic Dendritic Cell Subsets to infection with *Listeria monocytogenes*: maturation phenotype, level of infection, and T cell priming capacity *ex vivo*

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Abstract

To determine the relative contributions of DC subsets in the development of protective immunity to *Listeria monocytogenes* we examined the relationship between maturation, bacterial burden, and T cell priming capacity of four well characterized subsets of splenic DC following infection with *Lm*. CD8 α^+ , CD4 $^+$, and CD8 α^- CD4 $^-$ DC and the B220 $^+$ plasmacytoid DC (pDC) were compared for abundance and costimulatory molecule expression at 24, 48, and 72h post i.v. infection. We further determined the bacterial burden associated with each DC subset and their relative capacities to prime CD8 $^+$ T cells at 24hpi. The CD8 α^+ DC displayed the highest level of maturation, association with live bacteria, and T cell activation potential. Second, the CD4 $^+$ DC were also mature, yet were associated with fewer bacteria, and stimulated T cell proliferation, but not IFN- γ production. The CD8 α^- CD4 $^-$ DC showed a modest maturation response and were associated with a high number of bacteria, but failed to induce T cell proliferation *ex vivo*. pDC displayed a strong maturation response, but were not associated with detectable bacteria and also failed to stimulate T cell activation. Finally, we measured the cytokine responses in these subsets and determined that IL-12 was produced predominantly by the CD8 $^+$ DC, correlating with the ability of this subset DC to induce IFN- γ production in T cells. We conclude that *Listeria*-specific CD8 $^+$ T cell activation in the spleen is most effectively achieved by infection-induced maturation of the CD8 α^+ DC subset.

Keywords

dendritic cells; *Listeria*; T cell activation; costimulation; innate immunity

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Introduction

Dendritic cells play a major role in the activation of adaptive immunity by capturing, processing, and presenting antigen to naïve T cells in lymphoid organs [1]. DC are important for protective immunity to the Gram-positive, intracellular bacterial pathogen, *Listeria monocytogenes* (*Lm*) [2;3]. Infection with sub-lethal doses of wild type *Lm* induces the maturation of DC *in vivo* and primes protective T cell responses [4;5]. In contrast, infection with even high doses of an avirulent mutant *Lm* that cannot enter the host cell cytoplasm (referred to herein as vacuolar *Lm*) is not as effective at inducing DC maturation [4;5;6], and is, for the most part, ineffective in conferring protection [7;8;9;10;11;12]. In fact, new evidence suggests that recognition of *Lm* that are not able to escape the phagosome actually suppresses protective T cell responses [11].

A range of migratory and resident dendritic cell subsets have been identified in the spleen [13;14;15]. The resident, splenic conventional DC (cDC) include CD8 α ⁺, CD4⁺, and CD8 α ⁻CD4⁻ DC (DNDC). The interferon-producing, B220⁺ DC are the plasmacytoid DC (pDC) [13;16;17]. CD8 α ⁺DC play an important role in the initiation of CD8⁺ T cell responses and possess potent cross-presentation activity, reviewed in [18]. The expression of 33D1 by CD8 α ⁻ DC is associated with the co-expression of antigen processing machinery for MHC class II presentation and thus the ability to preferentially present antigen to CD4⁺ T cells [19]. Finally, pDC are best known for their production of type I interferon upon viral infection, but their role in antigen presentation is modest [20;21;22].

While CD8 α ⁺ DC have been predominantly implicated in the priming of the CD8⁺T cell responses critical to protective immunity to *Lm* [23;24;25], the role of other DC subsets in the immune response to *Lm* has not been well defined. In particular, little has been done to examine the number maturation state of individual DC subsets in the first 72 hours following infection. Intriguingly, CD8 α ⁺ DC have also been shown to be required for establishing *Lm* infection in the spleen [25]. Therefore, assessing their role in antigen presentation vs. transport of bacteria (and their antigens) into lymphoid organs has been experimentally difficult using approaches to deplete these cells. Furthermore, the relationship between direct infection and antigen presentation/T cell priming remains to be defined.

To address these questions, we sought to determine how distinct DC subsets responded to either wild type or vacuolar *Lm in vivo* during the first 72hpi. We examined the response (costimulatory molecule expression, number, and level of infection) of four splenic DC subsets and their capacity to activate naïve CD8⁺ T cells following *Lm* infection. These findings offer novel insights as to how individual DC subsets contribute to the overall response to an intracellular pathogen, and indicate that optimal T cell priming is achieved by DC that are both mature and infected.

Materials and Methods

Bacteria

Lm strains 10403s (wild type), DP-L2319 (vacuole-retained), DP-L4056 (wt *Lm*OVA), and DP-L4027 (Δhly *Lm*OVA) were obtained from Dr. Daniel Portnoy (University of California, Berkeley, CA). DP-L2319, which harbors deletions in the genes encoding Listeriolysin O (LLO) and two phospholipases (Δhly $\Delta plcA$ $\Delta plcB$), herein referred to as “vacuolar,” cannot enter the cytosol of host cells. DP-L4027 has a single deletion of the *hly* gene that encodes for LLO. DP-L4056 and DP-L4027, which secrete the ovalbumin protein have been described previously [12].

Mice

C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) or Charles River Laboratories (Wilmington, MA). OT-I/Rag1^{-/-} TCR transgenic mice specific for OVA₂₅₇₋₂₆₄ presented by K^b were purchased from The Jackson Laboratory (Bar Harbor, ME). CD11c-DTR-GFP mice which have been previously described [26] were obtained from The Jackson Laboratory (Bar Harbor, ME).

Infection of mice and bacterial enumeration

Mice were infected with 5×10^4 or 5×10^5 cfu wild type *Lm* or *LmOVA* (10403s or DP-L4056, respectively), 5×10^8 cfu vacuolar *Lm* or vacuolar *Lm-OVA* (DP-L2319 or DP-L4027, respectively), or mock treated (sterile 1X PBS). For bacterial enumeration ex vivo, sorted splenic DC subsets were lysed in water and dilutions plated on BHI agar to assess bacterial counts. Bacterial counts were normalized per 10^3 DC or per 10^6 total splenocytes. GraphPad Prism software was used for statistical analysis.

Staining strategy for flow cytometric identification of splenic DC

At the times indicated post infection, spleens were harvested and digested with Collagenase IV (Worthington Chemical) for 30 min followed by red blood cell lysis, to obtain a single cell suspension. Because the majority of splenocytes are T and B cells, we used a combination of antibodies against CD3 and CD19 labeled with the same fluorophore to exclude these cells from our analysis (referred to as a “dump channel”). Anti-CD3 (145-2C11) and anti-CD19 (1D3; both in PerCP Cy5.5), were used in combination with anti-CD11c APC (HL3) to identify all DC subsets in first step gating. To pinpoint specific cDC subsets, we used: anti-CD8 α Pacific Blue (53-6.7), and anti-CD4 FITC (3/23). Anti-CD45R/B220 Pacific Blue (RA3-6B2) and anti-Ly6C FITC (AL-21) were used to define pDC. To determine the maturation status of the specific DC subsets, we used one of the following antibodies as an additional stain: anti-CD86 PE (GL1), anti-CD80 PE (16-10A1), anti-CD40 PE (3/23), or anti-I-A^b PE (AF6-120.1), all from BD Pharmingen. After excluding T and B cells, CD8 α ⁺ DC were defined as: CD11c⁺CD8 α ⁺CD4⁻; CD4⁺ DC: CD11c⁺CD8 α ⁻CD4⁺; DNDC: CD11c⁺CD8 α ⁻CD4⁻B220⁻; and pDC: CD11c⁺ B220⁺ Ly6C⁺. Cells were then fixed with 2% PFA and acquired using a BD FACSCanto II with Diva software for acquisition and analysis (BD Biosciences).

Sorting of Splenic DC Subsets

Collagenase-digested splenocytes harvested at the indicated timepoints were first enriched for CD11c⁺ cells using positive selection with anti-CD11c microbeads from Miltenyi-Biotec. Resulting CD11c-enriched cells were sorted into the 4 DC populations using a BD FACS Aria and the following staining protocol: anti-CD3 (145-2C11) and anti-CD19 (1D3; both labeled with PerCP Cy5.5 to exclude contaminating T and B cells), anti-CD11c APC (HL3), anti-CD8 α Pacific Blue (53-6.7), anti-CD4 FITC (3/23), and anti-CD45R/B220 PE (RA3-6B2), all from BD Pharmingen.

Ex vivo T cell priming assays with sorted splenic DC

At 24hpi, sorted splenic DC from uninfected, wt *LmOVA*-infected or Δ hly *LmOVA*-infected mice were seeded at 2.5×10^4 cells/well in 96-well V-bottom plates (Corning). Chloramphenicol and gentamicin (each 10 μ g/ml; Sigma) were added to each well, and mock-treated splenic DC of each subset were loaded with 0.1 ng/ml of preprocessed OVA₂₅₇₋₂₆₄ peptide (SIINFEKL; from Wake Forest University School of Medicine Peptide Synthesis Facility) or 10 μ g/mL of preprocessed OVA₃₂₃₋₃₃₃ peptide (courtesy of Dr. Steven B. Mizel) for 30 min to 1h prior to the addition of T cells. Peptide was not added to DC infected with *LmOVA* or Δ hly *LmOVA*.

OVA-specific CD8⁺ T cells were isolated from the spleens of OT-I/Rag1^{-/-} TCR transgenic mice. T cells were labeled with 5 μ M CFSE (BD Pharmingen), according to the manufacturer's instructions, and added to the DC at a ratio of 2:1 (T cell:DC). T cells were harvested 72 h later and restimulated by incubation with OVA₂₅₇₋₂₆₄ peptide (2 μ g/ml) in the presence of Golgi Plug (BD Pharmingen) for 5 h. The T cells were then stained with anti-CD8 α PerCP (53-6.7; BD Pharmingen) then fixed and permeabilized for intracellular cytokine staining using the Cytofix/cytoperm kit (BD Pharmingen), according to the manufacturer's instructions. Cells were stained intracellularly with anti-IFN- γ Ab (BD Pharmingen). Following staining, cells were resuspended in 2% paraformaldehyde (PFA) and stored at 4°C until acquisition and analysis. CFSE proliferation data were analyzed, as previously described [6], using FlowJo software (TreeStar). Division index indicates the average number of divisions of the starting population of cells (accounting for both divided and undivided cells) and the proliferation index quantitates the average number of divisions of the divided cells.

Cytokine detection in DC by intracellular staining

Mice were infected i.v. with 5×10^4 Lm-OVA for 24h. Spleens were harvested, collagenase digested, and cell suspensions were incubated for 5h in the presence of Golgi Plug (BD Biosciences). Cells were then surface stained to identify DC subsets as indicated above. Cells were then fixed, permeabilized and stained with an antibody against IL-12p40 using the Cytofix/cytoperm kit according to manufacturer instructions (BD Biosciences).

Results

The number of CD8 α ⁺ and CD4⁺ splenic DC decreases after 24 hpi following infection with wild type *L. monocytogenes*

We first wanted to determine how the number and activation state of splenic DC subsets changed over the first 72 of infection with *Lm*, as this is a key time for T cell priming. To distinguish among the four previously described resident DC subsets reviewed in [27], we used a staining and gating strategy to examine only CD11c⁺ cells, then further separated the cells based on expression of CD8 α , CD4, B220, and Ly6C. This strategy defined populations of DC that were either CD8 α ⁺CD4⁻ (CD8 α ⁺ Dc), CD4⁺CD8 α ⁻ (CD4⁺ DC), CD8 α ⁻CD4⁻ (double negative, or DNDC) or B220⁺Ly6C⁺ (pDC). In supplementary Fig. 1, we have depicted the gating strategy used to include or exclude cells from the subset analysis.

Using this approach, we now demonstrate that the overall number of CD8 α ⁺ and CD4⁺ DC in the spleen decreased significantly after 24 hours of infection with wild type *Lm* (Fig. 1A). These decreases were on the order of 50-100-fold loss in the CD8 α ⁺ DC by 72h, and an approximate 10-fold loss in the CD4⁺ population by 48h. Yet, no significant decrease was observed in the DNDC and pDC subsets compared to mock infected animals. A significant decrease in the total number of splenocytes was also observed after 48h infection with WT *Lm*, but this 2-3-fold decrease could not completely account for the more dramatic losses in the CD8 α ⁺ and CD4⁺ DC. In contrast, the number of CD8 α ⁺ and CD4⁺ DC in vacuolar *Lm*-infected mice remained steady (Fig. 1A), as did the total number of splenocytes following infection with the vacuolar mutant *Lm* (Fig. 1B). Surprisingly, the numbers of pDC at 48h and 72h and of DNDC at 48h were significantly increased over mock in the mice infected with vacuolar *Lm*.

Splenic DC subsets mature to different extents following listerial infection

The extent of DC maturation in specific splenic DC subsets has not been well established following i.v. infection. We therefore determined how splenic DC subsets responded to *Lm*

infection *in vivo*. We also wanted to determine how the DC maturation response compared with the development of protective immunity *in vivo*, by assessing the maturation of DC following infection with either bacteria that efficiently induce protection (Wt *Lm*) or a strain that requires a much higher dose to induce protection (vacuolar *Lm*). We infected C57BL/6 mice with either 5×10^4 Wt *Lm* or 5×10^8 vacuolar *Lm* and harvested total splenocytes at 12, 24, 48, or 72 hpi. We examined the maturation state of the DC subsets at this time using the multistep gating strategy described in the methods. The expression of CD86, CD80, and CD40 were quantitated based on median fluorescent intensity compared to the level expressed by DC from mock-infected mice.

Among the three subsets of cDC (CD8 α^+ , CD4 $^+$, and DNDC) we first examined the CD8 α^+ DC as these have been reported to be critical for protective immunity to *Lm* and priming CD8 $^+$ T cell responses [3;23]. We found that these cells strongly up-regulated the expression of CD40, CD80, and CD86 upon infection with wild type *Lm* (Fig. 2A) by 24 hpi. In contrast, the expression of costimulatory molecules by CD8 α^+ DC in response to vacuolar *Lm* was barely above the mock level at all times tested except for an early and transient two-fold increase at 12h. The maturation response of the CD4 $^+$ DC elicited by wild type *Lm* was lower in magnitude (data not shown) but fold induction over basal levels was comparable or slightly greater than the CD8 α^+ DC (Fig. 2B). However the peak of maturation in this subset occurred at 48 hpi in contrast to the CD8 α^+ DC, whose maturation peaked closer to 24h. Again, there was no significant increase in costimulatory molecules on CD4 $^+$ DC in response to vacuolar *Lm*. Interestingly, DNDC did not appear to upregulate CD40 or CD80 to a great extent in response to either wild type *Lm* or vacuolar *Lm* over DC from mock-infected animals, but did modestly up-regulate CD86 at 24, 48, and 72hpi (Fig. 2C). There was also a small, but significant up-regulation of CD86 by these DNDC in response to vacuolar *Lm*. Finally, we observed a strong maturation response in the pDC population following exposure to wild type *Lm* (Fig. 2D) that peaked around 24 hpi. While the level of costimulatory molecule expression on the splenic pDC was substantially lower than the cDC on a per-cell basis (data not shown), the fold induction over background was comparable. Thus, following infection with Wt *Lm*, we observed different extents of maturation exhibited by the four DC subsets examined and distinct kinetics with which co-stimulatory molecule expression was up- or down-regulated. However, in all DC subsets, the response to vacuolar *Lm* was weak and transient or virtually non-existent.

CD8 α^+ DC and DNDC had the highest bacterial burdens at 24 hours post infection

Our timecourse analysis of splenic DC numbers and maturation status revealed different patterns of response to infection among the four subsets examined. Thus, we next wanted to assess the level of direct infection of each DC subset *ex vivo* to determine if infection level correlated with maturation level. For these experiments, we infected groups of C57BL/6 mice *i.v.* with wild type or vacuolar *Lm* and measured the numbers of viable bacteria associated with each DC subset in the spleen 24h post infection, (when the peak of CD8 α^+ DC and pDC maturation occurs). Following infection, spleens were harvested and cDC and pDC selected by cell sorting. The sorted cells were then lysed and lysates plated on BHI agar to measure the number of viable bacteria associated with each subset. CFU in the liver were also enumerated to ensure that mice had similar overall bacterial burden at 24 hpi (Supp. Fig. 2).

In Fig. 3A and B, the data indicate that the CD8 α^+ DC, DNDC, and CD4 $^+$ DC were infected with low, but detectable numbers of bacteria, but we were not able to detect live bacteria associated with the pDC. To address which subsets were infected with the vacuolar *Lm*, mice were infected with 5×10^8 cfu vacuolar bacteria and the level of infection determined in splenic cDC and pDC (Fig. 3A). In a pattern similar to the wild type *Lm*, we observed a low level of infection in each of the cDC subsets but no detectable vacuolar bacteria in the pDC.

Thus, although we did observe significant maturation of pDC in response to vacuolar *Lm*, we could not detect infection of these cells. Because each DC subset constitutes a different proportion of the total splenocyte population (DNDC make up a larger proportion than CD8 α ⁺ DC or CD4⁺ DC), we also normalized the number of cfu to the number of splenocytes. When calculated based on number of bacteria in each subset per million splenocytes, we observed that there were no significant differences between the number of viable bacteria associated with CD8 α ⁺ DC vs. DNDC (Fig. 3B). Finally, the CD4⁺ DC had fewer bacteria than either CD8 α ⁺ DC or DNDC per total splenocytes. Thus, when considering the spleen as a whole, the majority of the *Lm* were associated primarily with the DNDC and CD8 α ⁺ DC, but the CD8 α ⁺ DC had a higher number of bacteria per cell.

CD8 α ⁺ DC and CD4⁺ DC prime naïve OVA specific CD8⁺ T cells *ex vivo*

To test the ability of sorted splenic DC subsets to prime naïve OVA-specific T cells, following infection with either wild type *Lm*-OVA or vacuolar *Lm*-OVA, we performed T cell priming assays *ex vivo*. Representative dot plots of proliferation and IFN γ production are shown in Figure 4A. Compiled data quantitating the relative proliferative responses are presented in figures 4B and 4C. We observed that both the CD8 α ⁺ DC and, to a lesser extent, CD4⁺ DC were able to induce OVA specific CD8⁺ T cell proliferation (Fig. 4A and B). The CD8 α ⁺ DC promoted T cells to undergo several rounds of proliferation and to produce high levels of IFN γ . In contrast, the CD4⁺ DC were also capable of inducing proliferation but to a much lesser extent than the CD8 α ⁺ DC and strikingly, CD4⁺ DC did not activate the T cells to produce substantial IFN γ . Neither the DNDC nor the B220⁺ pDC induced significant proliferation of the OVA specific CD8⁺ T cells nor their production of IFN γ . Finally, none of the four DC subsets sorted from mice infected with vacuolar *Lm* stimulated either significant T cell proliferation or IFN γ production. Intriguingly, all four DC subsets stimulated T cell proliferation and IFN γ production when loaded with exogenous OVA peptide *in vitro*. Thus, the distinct T cell activation capacities of DC subsets *ex vivo* likely stemmed from differences in the relative levels of antigen presented. We also wanted to determine which of the four splenic DC subsets could prime CD4⁺ T cell responses *ex vivo* following *Lm* infection *i.v.* Surprisingly, we were unable to detect activation of OVA-specific CD4⁺ T cells (OT-II) by any of the DC subsets tested (data not shown).

To address the possibility that the level of T cell function induced by the different DC subsets correlated with the relative levels of IL-12 produced by these cells, we measured the frequency of IL-12 producing CD8⁺ and CD4⁺ DC following 24h *Lm* infection *in vivo* by intracellular cytokine staining. As in the previous experiments, we first excluded T and B cells (CD3⁺ or CD19⁺) then examined only the CD11c⁺ cells among the remaining population. The expression of IL-12 vs. CD8 α by the total CD11c⁺ DC is illustrated in figure 5A. We observed that in *Lm*-infected mice, the majority of IL-12 production was detected in the CD8⁺ population both in terms of frequency of IL-12-expressing cells as well as in the amount of cytokine expressed per cell. We also consistently observed a low frequency of IL-12 producing cells in this DC subset even in the absence of infection (mock, figure 5A). However, when we specifically compared IL-12 production by CD8⁺ vs. CD4⁺ DC, we found that while both subsets responded to infection by up-regulating IL-12, a much lower frequency of CD4⁺ DC produced IL-12 in response to *Lm* infection than CD8⁺ DC (Figure 5B). Thus, these data indicate that the relative capacity of DC subsets to prime IFN- γ production by T cells (Figure 4) correlates with the frequency and level of IL-12 produced by these cells.

Discussion

To our knowledge, this is the first study to evaluate simultaneously the maturation profile, infection level, and T cell priming potency of splenic DC subsets upon systemic infection with *Listeria monocytogenes*. Previous studies have explored specific aspects of this process, such as how DC numbers and maturation profile change at later times post infection [28], or have measured the T cell priming capacity of specific DC subsets [23], yet, none has comprehensively examined these responses with regard to extent of infection in each subset during the peak of T cell priming activity. Taken together, our data demonstrate that upon infection with *Listeria*, the priming capacity of individual DC subsets depends on both the maturation state of the cells, their association with bacteria, and correlates with the level of IL-12 produced.

Our study demonstrates that the CD8 α^+ DC are major players in the initiation of protective T cell responses to *Lm* because 1) they were the most heavily infected (on a per-cell basis) of the four DC subsets examined, 2) displayed a strong maturation response to infection, and 3) were the most potent at stimulating T cell proliferation and function *ex vivo*. We also observed that the kinetics of the CD8 α^+ DC maturation was more transient than the CD4 $^+$ DC (Fig 2). This may be due to the high infection level in this population, resulting in death of these cells after 24h. Consistent with this notion, our data in Fig. 1 demonstrate that the greatest loss in number of DC was observed in this population at 48 and 72hpi. To explore the possibility that these DC were dying by apoptosis or by pyroptosis, we examined caspase-3 and caspase-1 activation in these cells at 24h post infection. However, we observed no significant difference in the levels of these markers of cell death in DC from infected vs. uninfected mice (data not shown). Thus, the mechanism through which these cells are depleted remains to be determined.

CD8 α^+ DC have been identified in several studies as being key to the development of protective immunity to a number of infectious organisms [23;25;29;30]. Several studies have employed the CD11c-DTR model for depleting DC to assess the role of DC, (including CD8 α^+ DC) in establishing protective immunity to *Lm* [31;32]. However, these studies revealed that in the *Listeria* model, CD8 α^+ DC are also required for seeding of the spleen with bacteria [25]. Thus, distinguishing between the role of DC in T cell activation vs. transport of bacteria has been difficult.

Intriguingly, we also observed a strong maturation response of the CD4 $^+$ DC upon infection. These DC were also infected, although at a lower level than the CD8 α^+ DC. One potential explanation for the failure of CD4 $^+$ DC to induce IFN γ production by CTL is that they don't express sufficient IL-12 (Fig 5) or other Th1 polarizing cytokines [33]. Data in support of this idea have come from several studies of DC responses in which the majority of IL-12 producing DC are found within the CD8 α^+ subset [34;35;36;37;38]. An alternative explanation for these findings is that CD4 $^+$ DC are not thought to be optimal for priming CTL responses because their antigen processing machinery favors presentation of antigen via MHC class II [39]. However, we were unable to detect any stimulation of CD4 $^+$ T cells in our priming assays *ex vivo*, likely due to a low level of antigen presented at the doses used for infection. Thus, the role of CD4 $^+$ DC in the priming of protective T cell responses to *Listeria* remains to be determined.

The DNDC represent a more heterogeneous subset of cells that expresses CD11c, as well as CD11b (data not shown) but none of the other subset markers examined here. These are also the most numerous of the DC examined. While these cells were associated with a high proportion of the total bacteria, on a per-cell basis they had fewer bacteria than the CD8 α^+ DC and thus, were not preferentially infected. Additionally, these DC displayed only a

modest maturation response, in the form of CD86 upregulation alone. This may be due to the differentiation state of these cells. DC with this phenotype have been identified in the spleen and are thought to be the precursors of several cDC subsets (pre cDC) [17;40;41]. Thus, their ability to mature fully in response to bacterial infection may not be completely developed. DC lacking CD8 and CD4 have also been associated with suppressor activity in cancer as well as infectious model systems [42;43;44;45]. While it is not clear if the DNDC in our studies had inhibitory activity, it is likely that the failure of this population to prime CTL activation likely stems from their poor maturation level, in spite of their association with bacteria.

While pDC did strongly upregulate costimulatory molecule expression in response to wild type *Lm*, their association with viable bacteria was below the level of detection. These findings indicate that either pDC are capable of “bystander” maturation without direct infection, or perhaps pDC are associated with very low numbers of *Lm*. Because we were unable to detect T cell activation by these cells following *Lm* infection, we favor the hypothesis that these cells were not directly associated with bacteria, and did not present bacterial antigens. This is not surprising given that pDC are not highly phagocytic and likely unable to take up intact bacteria [20]. However, cross-presentation capacity has recently been reported in pDC [20;21]. As a control, we determined that when splenic pDC were given pre-processed peptide antigen they were indeed capable of priming naïve, antigen specific CD8⁺ T cells (Fig. 4).

Perhaps as a consequence of their inability to induce DC maturation, vacuolar *Listeria* are poor inducers of protective T cell responses *in vivo* [4-12]. This failure to induce maturation may also affect the localization of the bacteria within the lymphoid organs. One study that addressed this issue [4] demonstrated that while wild type bacteria localized to the T cell zones of the spleen following infection, both killed *Lm* and Δhly *Lm* were absent from the T cell zones and instead localized to the red pulp and marginal zones. This finding has been confirmed and extended in three more recent publications examining the formation of innate immune cell clusters in the T cell zones of the spleen following i.v. *Lm* infection [32;46;47]. They observed that *Lm* were transported by DC into the T cell zones of the spleen, but this transport occurred only upon infection with WT *Lm*, not vacuolar *Lm*. Thus, the level of infection and T cell priming capacity of a given DC subset likely depends on its access to bacteria as well as its ability to gain access to the T cell zone.

In summary, our findings provide a clear illustration that individual DC subsets have distinct roles in initiating immunity against *Lm*. Our study indicates that activation of T cells following the first 24h of infection depends on the DC having both infection and a strong maturation response. This study further provides important insights for targeting DC subsets in the design of vaccines against intracellular pathogens and invites further investigation into the impact of individual DC subsets on protective immune responses to other infectious organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Splenic DC subset numbers decrease 24 hours after *Listeria* infection

Splenocytes were harvested from mice infected with 5×10^4 wild type *Lm*, 5×10^8 cfu vacuolar *Lm*, or mock treated with sterile PBS for 12-72h. Cells then stained for DC subsets as described in materials and methods. The frequency of gated splenic DC subtypes was determined using FACSDiva analysis software. The total number of DC subsets in the spleen for each timepoint was then back calculated from this frequency using counts of total splenocytes harvested following collagenase digestion using the following equation: Number of DC subset = % DC subset $\times$ [% CD11c⁺ $\times$ (% live $\times$ total # splenocytes)] Statistical analysis was performed by One Way ANOVA followed by a Tukey's post test. Data shown are compiled from four independent experiments, 5 total mice per group per timepoint.

Splenocytes were harvested from mice infected with 5×10^4 wild type *Lm*, 5×10^8 cfu vacuolar *Lm*, or mock treated with sterile PBS for 12-72h. Cells then stained for DC subsets and surface costimulatory molecule expression. Costimulatory molecule expression of (A) CD8 α^+ DC, (B) CD4 $^+$ DC, (C) DNDC, and (D) pDC at 12-72 hours post infection with 5×10^8 vacuolar *Lm* (white bars) or 5×10^4 wild type *Lm* (black bars). Statistical analysis performed comparing to mock treated animals using a Two Way ANOVA with a Bonferroni post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data shown are compiled from four independent experiments, 5 total mice per group per timepoint.

Figure 3. Splenic CD8α⁺ and DNDC both contain live *Lm* following wild-type infection
 Splenocytes were harvested from mice infected with 5×10^5 wild type *Lm*, 5×10^8 cfu vacuolar *Lm* for 24h. Cells were then positively selected for CD11c using CD11c microbeads and Miltenyi-Biotec columns. CD11c⁺ cells were then sorted into subsets. Sorted DC subsets were then lysed in water, serially diluted, and plated on BHI plates for 48 h at 37°C. (A) Colony forming units per 1000 DC (of each subset) from vacuolar *Lm* (white bars) or wild type *Lm* (black bars) -infected mice (B) colony forming units in each DC subset normalized based on their frequency among total splenocytes. Statistical analysis performed comparing wild type *Lm* values to vacuolar *Lm* infected using a Two Way ANOVA with a Bonferroni post test. * p<0.05, ** p<0.01, *** p<0.001. Data shown are compiled from two independent experiments, 5 total mice per group.

Figure 4. Splenic CD8 α^+ DC prime naïve, antigen-specific CD8 $^+$ T cell proliferation and IFN- γ production *ex vivo* and is dependent upon cytosolic entry by *Lm*

Splenic DC subsets were sorted from mice that were infected with 5×10^5 cfu wild type *Lm*-OVA, 5×10^8 cfu vacuolar *Lm*-OVA, or mock treated with PBS for 24h, then plated in V bottom plates at a ratio of 2:1 (T cell: DC) with CFSE labeled CD8 α^+ OT-I T cells, specific for OVA₂₅₇₋₂₆₄ for 72 h. An ICS was then performed for intracellular IFN- γ production and the T cells then assessed for proliferation (dilution of CFSE) by flow cytometry. (A) Dot plot of CD8 $^+$ T cell primed by DC subsets infected with CFSE-labeled *Lm*-OVA, mock treated with PBS, or stimulated with exogenous OVA₂₅₇₋₂₆₄ peptide. (B) Division Index and (C) Proliferation Index of T cells primed by DC subsets infected with CFSE-labeled *Lm*-OVA, mock treated with PBS, or stimulated with exogenous OVA₂₅₇₋₂₆₄ peptide. Data shown are compiled from two independent experiments with 5 mice per group.

Figure 5. IL-12 production by CD8⁺ and CD4⁺ DC at 24h post infection

Mice were infected with 5×10^4 WT Lm for 24h. Spleens were harvested and incubated with Golgi plug (BD biosciences) for 5h to inhibit cytokine secretion. Cells were then stained with antibodies to DC subset markers, fixed, permeabilized and stained with antibodies to IL-12 to label intracellular stores of the cytokine. (A) Dot plots of DC depicting CD8 expression vs. IL-12. Cells shown were pre-gated to exclude T and B cells and to include only CD11c⁺ cells. Images are representative of three independent experiments. (B) IL-12 response of CD8⁺ and CD4⁺ DC prior to and following infection with WT Lm. Results are compiled from one representative experiment with 4 mice per group. * $p < 0.05$, ** $p < 0.01$.