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TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection

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Summary

Dendritic cells (DCs) present microbial antigens to T cells and provide inflammatory signals that modulate T cell differentiation. While the role of DCs in adaptive immunity is well established, their involvement in innate immune defenses is less well defined. We have identified a TNF/iNOS-producing (Tip)-DC subset in spleens of *Listeria monocytogenes*-infected mice that is absent from CCR2-deficient mice. The absence of Tip-DCs results in profound TNF and iNOS deficiencies and an inability to clear primary bacterial infection. CD8 and CD4 T cell responses to *L. monocytogenes* antigens are preserved in CCR2-deficient mice, indicating that Tip-DCs are not essential for T cell priming. Tip-DCs, as the predominant source of TNF and iNOS during *L. monocytogenes* infection, orchestrate and mediate innate immune defense against this intracellular bacterial pathogen.

Introduction

Listeria monocytogenes is a Gram-positive bacterium that causes severe infections in immunocompromised individuals (Gellin and Broome, 1989). In a mouse model, intravenously inoculated bacteria are rapidly cleared from the bloodstream and predominantly infect the spleen and liver. At the cellular level, *L. monocytogenes* bacteria are phagocytosed by macrophages and escape the vacuole by secreting listeriolysin O (LLO), a membranolytic protein that is essential for bacterial virulence (Bielecki et al., 1990). *L. monocytogenes* replicates in the cytosol of infected macrophages and can induce directed actin polymerization, enabling bacterial spread to neighboring cells (Cossart and Mengaud, 1989). During typical murine *L. monocytogenes* infection, bacteria proliferate in vivo for 2 to 3 days and then, upon induction of an antigen-specific CD8 T cell response, are cleared from the spleen and liver (Busch and Pamer, 1999).

While clearance of *L. monocytogenes* infection is T cell dependent, early control of infection depends upon a functioning innate immune system (Bancroft et al., 1991; Harty and Bevan, 1996). Innate immune responses to *L. monocytogenes* have been extensively studied, and although our understanding remains incomplete, there are a few well-defined inflammatory events that, while distinct and still unconnected, provide a primitive working scaffold. It is clear, for example, that the production of reactive nitrogen intermediates by inducible nitric oxide synthase (iNOS) comprises one of the major mechanisms for killing of *L. monocytogenes* in vivo (Shiloh et al., 1999). Upstream of this, it is known that production of IFN-γ and TNF is also essential for innate immune defense against *L. monocytogenes* infection (Buchmeier and Schreiber, 1985; Pasparakis et al., 1996). Further upstream, blocking recruitment of myelomonocytic cells to the liver and spleen with monoclonal antibodies against the type 3 complement receptor markedly enhances the in vivo proliferation of *L. monocytogenes*, indicating that recruitment of CR3 (a.k.a. Mac-1 or CD11b) expressing cells to sites of infection is essential for the success of innate immune defenses (Rosen et al., 1989).

Many questions remain, however. While iNOS induction is essential for bacterial clearance, it remains unclear which cells produce NO. Are NO-producing cells recruited to sites of infection, or is NO production restricted to cells that are infected with *L. monocytogenes*? With respect to in vivo TNF and IFN-γ production during *L. monocytogenes* infection, it remains unclear which cells produce these essential cytokines, and which cells are these cytokines' targets. Finally, with respect to CR3-mediated recruitment of cells to sites of *L. monocytogenes* infection, since many cells express CR3 it remains unclear which recruited cells mediate the antimicrobial effects.

The spleen, which is a major site of *L. monocytogenes* infection, is a complex tissue that is histologically divided into red pulp, white pulp, and marginal zone. Each of these regions contains distinct macrophage and dendritic cell populations. The marginal zone contains two major populations of macrophages: marginal zone macrophages and metallophilic macrophages. Marginal zone macrophages are large, have long cell processes, are MHC class II negative, and are highly phagocytic. They interact closely with marginal zone B cells. Metallophilic macrophages, on the other hand, are not highly phagocytic but have processes that extend into the white pulp (Kraal, 1992). The white pulp contains dendritic cells, particularly within the T cell zones (Nolte et al., 2000). Histologic studies of *L. monocytogenes*-infected spleens have demonstrated that bacteria localize to T cell zones in the white pulp (Conlan, 1996). Some studies suggest that early infection with *L. monocytogenes* induces marked apoptosis of T lymphocytes (Merrick et al., 1997). How *L. monocytogenes* is cleared

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from the bloodstream, which macrophages take up bacteria, and how bacteria localize to the T cell zone of the white pulp remain undefined.

It is increasingly clear that dendritic cells contribute to T cell-independent immune responses. Dendritic cells are well equipped to detect microbial pathogens by expressing distinct Toll-like receptor (TLR) combinations, enabling recognition of microbial molecules and consequent induction of cytokines (Jarrossay et al., 2001; Kadowaki et al., 2001). In humans and mice, plasmacytoid predendritic cells secrete IFN-α when stimulated with viral products and differentiate into CD11c$^+$ DCs upon exposure to microbial products (Asselin-Paturel et al., 2001; Cella et al., 1999; O'Keeffe et al., 2002; Siegal et al., 1999). Another recently defined DC subset in human peripheral blood produces large amounts of TNF in response to LPS (Schakel et al., 2002). A role for circulating DCs in clearance of bacteria from the blood stream was suggested by the finding that CD11c$^+$CD11b$^+$ dendritic cells transport killed bacteria to the splenic marginal zone (Balazs et al., 2002). While CD11c-expressing DCs are essential for priming of naive T cells responding to *L. monocytogenes* infection (Jung et al., 2002), the role of DCs in innate immune defense against this infection is unknown.

In this report we demonstrate that *L. monocytogenes* infection induces the recruitment of a novel, TNF- and iNOS-producing (Tip) DC subset to the spleen. Recruitment of Tip-DCs is dependent upon CCR2 signaling, and the absence of this DC subset results in uncontrolled bacterial replication and host death. While Tip-DCs are the major producers of TNF and iNOS in the spleen of *L. monocytogenes* infected mice, they are not a significant reservoir of bacterial infection. These studies identify a novel DC subset that specializes in defense against bacterial infection and demonstrate that TNF and iNOS are produced by uninfected cells that are recruited to the site of infection in a CCR2-dependent fashion.

Results

Normal Development of *L. monocytogenes*-Containing Lesions in the Spleens of CCR2-Deficient Mice

To dissect innate immune mechanisms that mediate *L. monocytogenes* clearance, we characterized cellular responses to infection in wild-type mice and mice lacking the CCR2 chemokine receptor, which targets monocytes to sites of inflammation. As previously described, mice lacking CCR2 are unable to control in vivo bacterial growth and generally die within 4 to 5 days following inoculation (Kurihara et al., 1997). Interestingly, in vivo bacterial replication is similar in wild-type and CCR2$^{-/-}$ mice for the first 48 hr after inoculation, but beyond this time period the number of bacteria in the spleens of CCR2$^{-/-}$ mice continues to increase, while wild-type mice begin to clear bacteria (data not shown).

Since CCR2 is implicated in the migration of macrophages (Kuziel et al., 1997) and dendritic cells (Sato et al., 2000), we examined the distribution of these cells in spleens of wild-type and CCR2$^{-/-}$ mice. Uninfected CCR2$^{-/-}$ spleens were normal by immunohistology, with well-defined areas of white pulp containing T and B cells and normal distributions of Mac-3$^+$ (Flotte et al., 1983; Ho and Springer, 1983) and CD169$^+$ (Crocker et al., 1991) macrophages in the red and white pulp (Figure 1A). DEC-205$^+$ cells also localized similarly to the white pulp areas in wild-type and CCR2$^{-/-}$ mice (Figure 1A).

Next, wild-type and CCR2$^{-/-}$ mice were infected with *L. monocytogenes*, and splenic bacterial localization was characterized. In infected wild-type mice, *L. monocytogenes*-containing lesions are found in T cell areas of the white pulp, where bacteria are associated with Mac-3$^+$ cells (Figure 1B and data not shown). Mac-3 is an antigen expressed on some dendritic cells and on activated tissue macrophages (Flotte et al., 1983; Ho and Springer, 1983). Mac-3$^+$ cells were already present in the splenic white pulp 24 hr postinfection (data not shown) with increasing infiltration 48 hr postinfection. Localization of Mac-3$^+$ cells and *L. monocytogenes* to the white pulp occurred normally in CCR2$^{-/-}$ spleens (Figure 1B), indicating that CCR2-mediated chemotaxis is not essential for this process. Furthermore, the pattern of DEC-205$^+$ staining was similar in wild-type and CCR2$^{-/-}$ spleens, indicating that localization of DEC-205$^+$ DCs in proximity to sites of *L. monocytogenes* infection is also CCR2 independent (Figure 1B).

CD11b^{int}/CD11c^{int}/Mac-3^{high} Cell Subset Is Absent in Spleens of *L. monocytogenes*-Infected CCR2$^{-/-}$ Mice

Although histologic analysis did not reveal abnormalities in the localization of Mac-3$^+$ or DEC-205$^+$ cells in CCR2$^{-/-}$ mice, we decided to further characterize macrophage and dendritic cell subsets in wild-type and CCR2$^{-/-}$ mice following *L. monocytogenes* infection. We found that the numbers of CD11b$^+$ cells were similar in wild-type and CCR2$^{-/-}$ mice while the numbers of CD11c$^+$ cells increased in wild-type mice but remained unchanged in CCR2$^{-/-}$ mice following infection (Figure 2A). Flow cytometric analysis of dissociated spleen cells identified a population of CD11b^{int} (intermediate) cells, a proportion of which also expressed CD11c (gate R2, 3.6% of total collagenase-digested splenocytes), that were present in wild-type mice 48 hr following infection but absent in CCR2$^{-/-}$ mice (gate R2, 0.7%) (Figure 2B). CD11b^{int} cells expressed high levels of Mac-3 1 and 2 days postinfection (Figure 2C). In contrast to wild-type mice, CD11b^{int}/Mac-3^{high} cells were not present in spleens of infected CCR2$^{-/-}$ mice. Surface staining of the CD11b^{int}/CD11c^{int} cells demonstrated upregulation of B7.1, B7.2, CD40, and MHC class II, particularly 48 hr following bacterial infection (Figure 2D). The CD11b^{int}/CD11c^{int} cell population was morphologically similar to CD11c^{hi} DCs, with pleomorphic nuclei, cytoplasmic extensions, and numerous vacuoles (Figure 2E). Unlike macrophages, CD11b^{int}/CD11c^{int} cells did not adhere to plastic culture plates upon in vitro cultivation.

Normal T Cell Priming in *L. monocytogenes*-Infected CCR2$^{-/-}$ Mice

Priming of pathogen-specific T lymphocytes is predominantly mediated by DCs. Recent studies demonstrated an essential role for CD11c-expressing cells in the priming of *L. monocytogenes*-specific CD8 T cells (Jung et al., 2002). To determine whether the CD11b^{int} DC subset identified in wild-type mice could prime naive T cells, CD11b^{int}/CD11c^{low} (R1, see gates shown in Figure 2B),

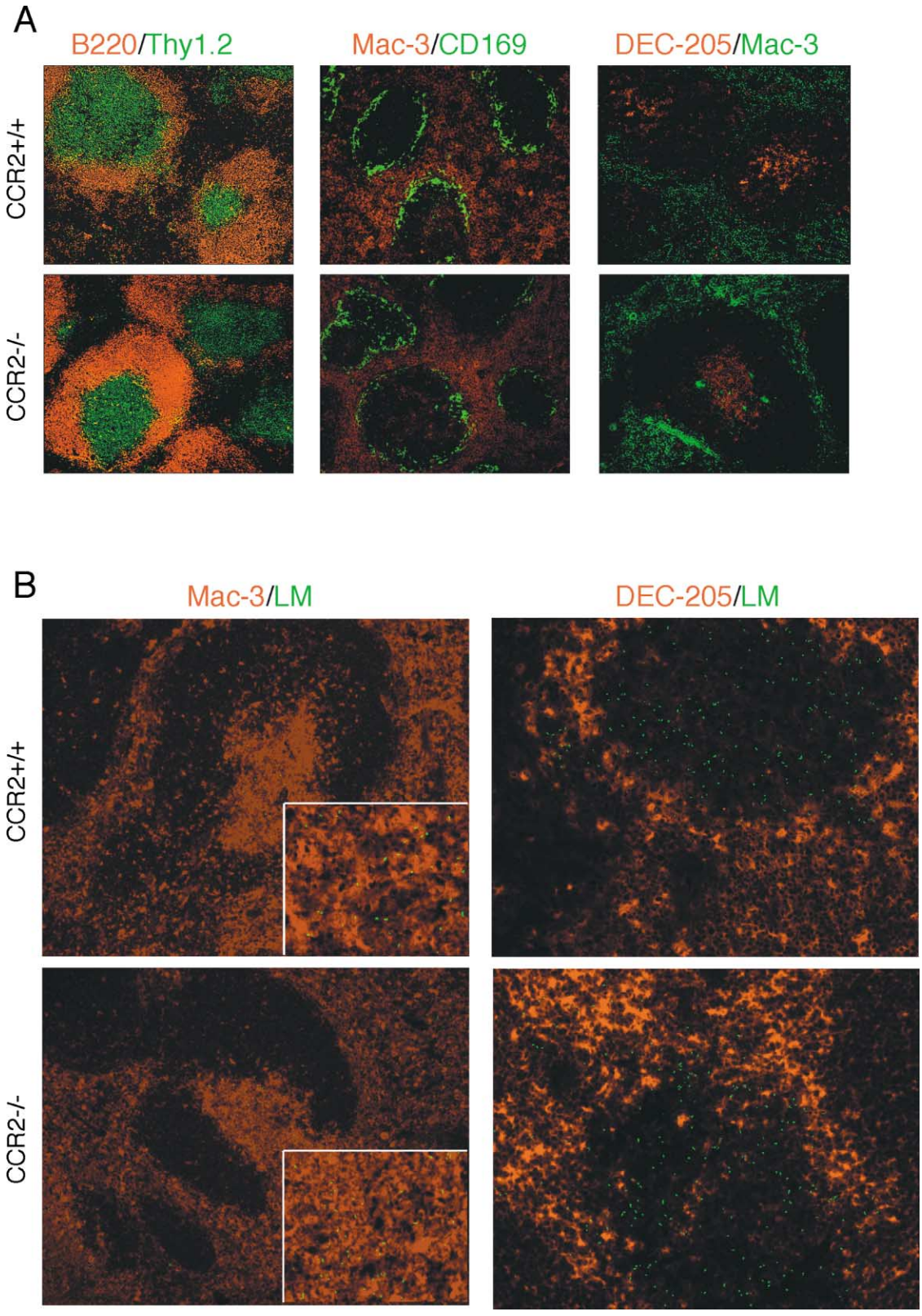

Figure 1. Normal Splenic Architecture and Lesion Formation in CCR2$^{-/-}$ Mice

(A) Expression of macrophage/dendritic cell markers in the spleens of naive wild-type and CCR2$^{-/-}$ mice. Left: expression of B220 (red) and Thy1.2 (green). Middle: expression of Mac-3 (red) and CD169 (green). Right: expression of DEC-205 (red) and Mac-3 (green).

(B) Colocalization of *L. monocytogenes* with macrophages and dendritic cells in the spleens of wild-type and CCR2$^{-/-}$ mice infected for 48 hr. Left: staining for Mac-3 (red) and *L. monocytogenes* (green). Right: staining for DEC-205 (red) and *L. monocytogenes* (green). All panels are 100× magnification, while insets are 200×.

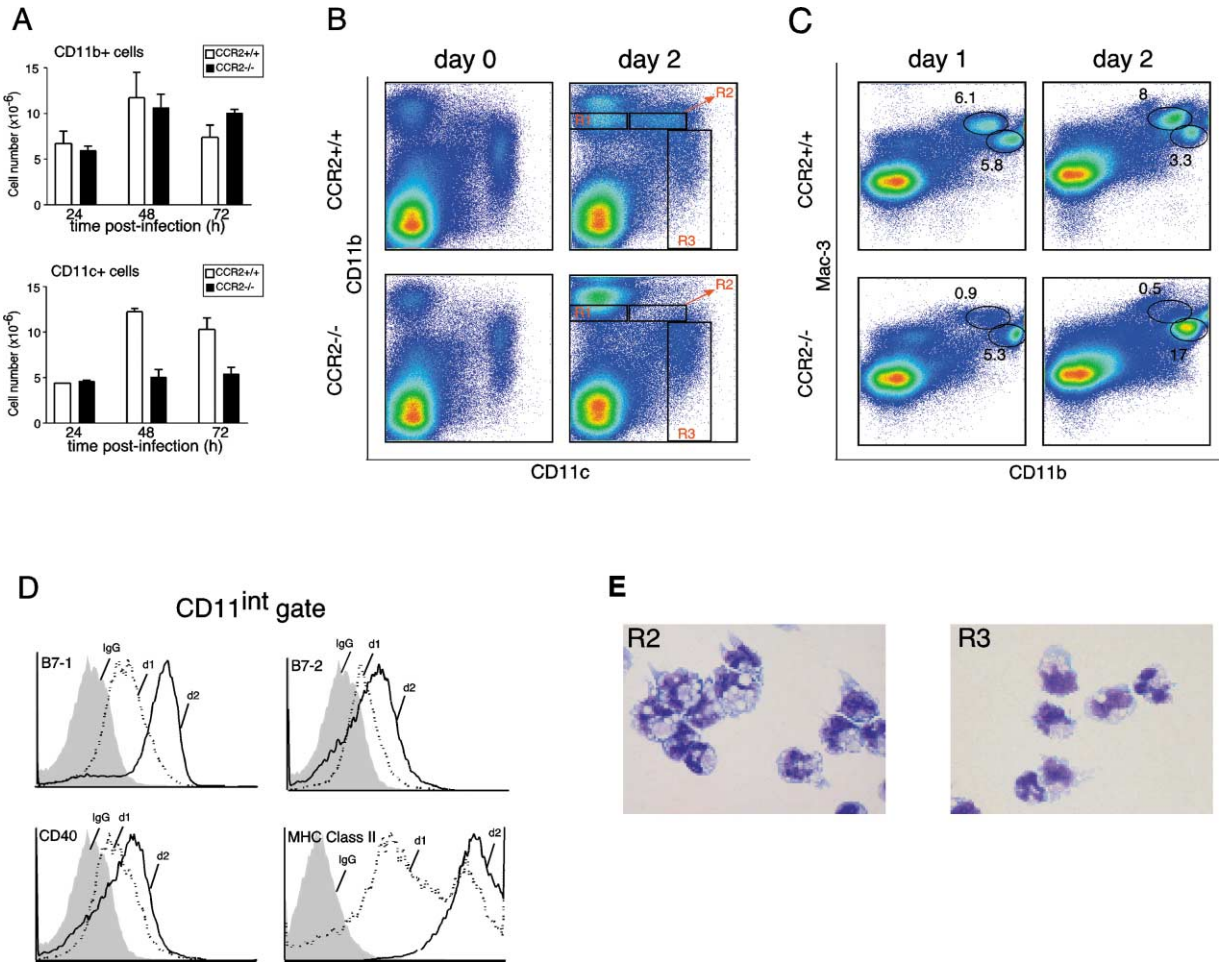

Figure 2. Characterization of CD11b$^+$/CD11c$^+$ Cell Subsets in Spleens of Wild-Type and CCR2-Deficient Animals after Bacterial Challenge

Mice were infected with 3000 *L. monocytogenes*, and spleens were harvested at the indicated times and collagenase digested.

(A) Mean numbers of MACS-purified CD11b$^+$ and CD11c$^+$ cells/10^8 total spleen cells from three to six mice per time point are shown (error bars, SD).

(B) CD11b and CD11c expression on live splenocytes. R1 (CD11b^{int}/CD11c^{low}), R2 (CD11b^{int}/CD11c^{int}), and R3 (CD11b^{low}/CD11c^{hi} cells) denote gates used for sorting of monocyte subpopulations.

(C) Expression of CD11b and intracellular Mac-3 by splenocytes.

(D) Expression of activation markers on CD11b^{int} cells (combined R1 and R2 gates depicted in [B]). In (B)–(D), representative dot plots for 6–15 mice are shown.

(E) Cytospins of cells sorted from gates R2 and R3 (as depicted in [B]) were stained with Giemsa for morphological assessment. Magnification, 600×.

CD11b^{int}/CD11c^{int} (R2), and CD11b^{low}/CD11c^{hi} cells (R3) were sorted and used as stimulators in mixed lymphocyte reactions. All three sorted subsets induced proliferation of allogeneic T cells (Figure 3A). At higher stimulator to effector ratios, proliferation induced by CD11b^{int}/CD11c^{int} cells was comparable to that induced by CD11b^{low}CD11c^{hi} cells (conventional DCs).

To determine whether the absence of CD11b^{int}/CD11c^{int} DCs in CCR2$^{-/-}$ mice limits T cell priming, we infected wild-type and CCR2$^{-/-}$ mice with an attenuated strain of *L. monocytogenes* and measured the CD8 T cell response to the immunodominant LLO_{91-99} epitope 7 days later. The percentage of LLO_{91-99}-specific CD8 T cells was larger in CCR2$^{-/-}$ mice than in wild-type control mice, indicating that the DC subset missing from CCR2$^{-/-}$ mice is not required for presentation of bacterial antigens to naive CD8 T cells (Figure 3B). To determine whether differentiation of CD8 T cells into effectors might be impaired in CCR2$^{-/-}$ mice, we measured the ability of LLO_{91-99}-specific CD8 T cells to produce TNF and IFN-γ in response to peptide stimulation. The percentage of CD8 T cells producing these cytokines in response to LLO_{91-99} stimulation in wild-type and CCR2$^{-/-}$ mice is similar (Figure 3C), indicating that CD8 T cell differentiation is not impaired by the absence of the CD11b^{int}/CD11c^{int} DC subset. Along similar lines, CD4 T cells were primed and produced TNF and IFN-γ following *L. monocytogenes* infection of CCR2$^{-/-}$ mice (Figure 3D).

CD11b^{int}CD11c^{int} Cells Are a Predominant Source of TNF-α during Infection

Since the absence of CD11b^{int}/CD11c^{int} DCs in CCR2$^{-/-}$ mice did not adversely affect T cell responses, we postulated that their absence might diminish the production

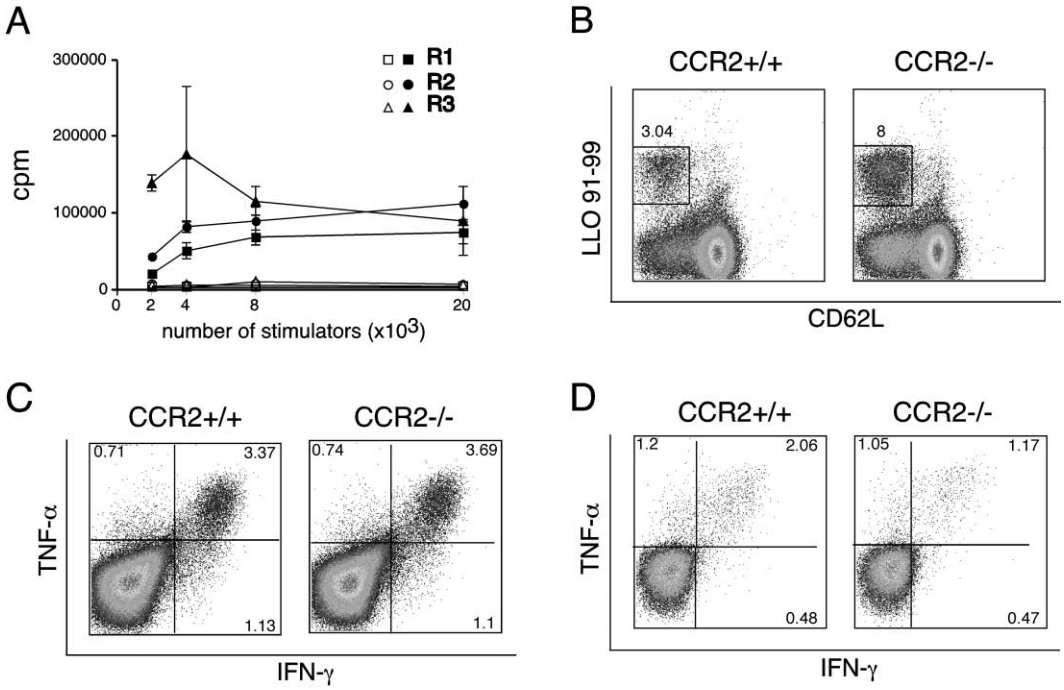

Figure 3. Intact T Cell Priming in CCR2$^{-/-}$ Mice

(A) CD11b^{int}/CD11c^{low} (R1), CD11b^{int}/CD11c^{int} (R2), and CD11b^{low}/CD11c^{hi} (R3) cells were sorted from gates depicted in Figure 2B and used as stimulators in allogeneic MLR. Proliferation of allogeneic, BALB/c T cells in response to sorted C57BL/6 CD11b^{+}/CD11c^{+} subsets is shown. Closed symbols represent BALB/c responders while open symbols represent control C57BL/6 responders. Results are mean counts per minute (error bars, SD). This experiment was repeated twice.

(B) Splenocytes from wild-type and CCR2$^{-/-}$ mice were stained 7 days following attenuated *L. monocytogenes* infection for CD62L expression and with H2-K^{d}/LLO$_{91-99}$ tetramers. Plots show live, CD8-gated T cells, and the percentage of tetramer-positive T cells is indicated.

(C) Splenocytes from *L. monocytogenes*-infected wild-type and CCR2$^{-/-}$ mice were stimulated in vitro with LLO$_{91-99}$ and stained for intracellular TNF-α and IFN-γ. Plots are gated on CD62L^{-}CD8α^{+}, live lymphocytes.

(D) Immune splenocytes were stimulated in vitro with HKLM, and CD4 T cells were analyzed by intracellular TNF-α and IFN-γ staining. Plots are gated on live, CD62L^{lo}, CD4^{+} T lymphocytes. The percentage of cytokine-positive cells is indicated, and representative dot plots for three mice per group are shown. Each of these experiments was repeated twice.

of other inflammatory mediators. IFN-γ, a cytokine that is essential for defense against *L. monocytogenes* infection (Buchmeier and Schreiber, 1985), was present in decreased amounts in spleens derived from CCR2$^{-/-}$ mice (Figure 4A). Intracellular cytokine staining of CD11b^{int} cells did not demonstrate significant IFN-γ production, and NK cells were present in equal numbers in wild-type and CCR2$^{-/-}$ mice (data not shown). Thus, the cellular source of IFN-γ during the first day of bacterial infection remains unclear. Secretion of two other monocyte-derived cytokines, IL-12 and IL-18, appeared to be near normal in CCR2-deficient spleens (Figures 4B and 4C). Interestingly, IFN-α, a cytokine that is produced in very high quantities by plasmacytoid pre-DCs in response to viral infection (Cella et al., 1999; Siegal et al., 1999), was expressed in greater quantities in CCR2$^{-/-}$ mice than in wild-type mice (Figure 4D), suggesting that CD11b^{int}/CD11c^{int} DCs are distinct from the plasmacytoid pre-DC.

Mice lacking TNF are highly susceptible to *L. monocytogenes* (Pasparakis et al., 1996), but the in vivo cellular source of TNF has not been well defined. Furthermore, the downstream antimicrobial mechanisms that depend on TNF production are also incompletely defined. We therefore next characterized the production of TNF by splenocytes from *L. monocytogenes*-infected mice. In vitro measurements of cytokine production by spleen cells 24 hr following *L. monocytogenes* infection showed that CD11b^{+} splenocytes from CCR2$^{-/-}$ mice produced markedly less TNF upon stimulation with HKLM than CD11b^{+} cells from wild-type mice (Figure 4E and data not shown). CD11b^{-} cells from either mouse strain did not produce detectable amounts of TNF in this assay. Naive splenocytes stimulated with HKLM produced minimal amounts of TNF.

We next examined the cellular source of TNF-α in wild-type animals. Intracellular cytokine staining revealed that the CD11b^{int} DC population in wild-type mice produced prodigious amounts of TNF with and without in vitro stimulation with HKLM. In contrast, CCR2$^{-/-}$ mice lacked the cells that produced large amounts of TNF (Figure 4F). Gating on the CD11b^{int} cells in wild-type spleens revealed that CD11c expression correlates with TNF production (data not shown).

Impaired iNOS Expression in CCR2$^{-/-}$ Spleens

The role of TNF in antimicrobial defense is complex but most likely indirect. Many cell types express TNF receptors, and ligation of receptors can induce apoptosis in some circumstances and activation in others. TNF has been implicated in DC differentiation (Caux et al., 1992), lymphoid organogenesis (Pasparakis et al., 1996),

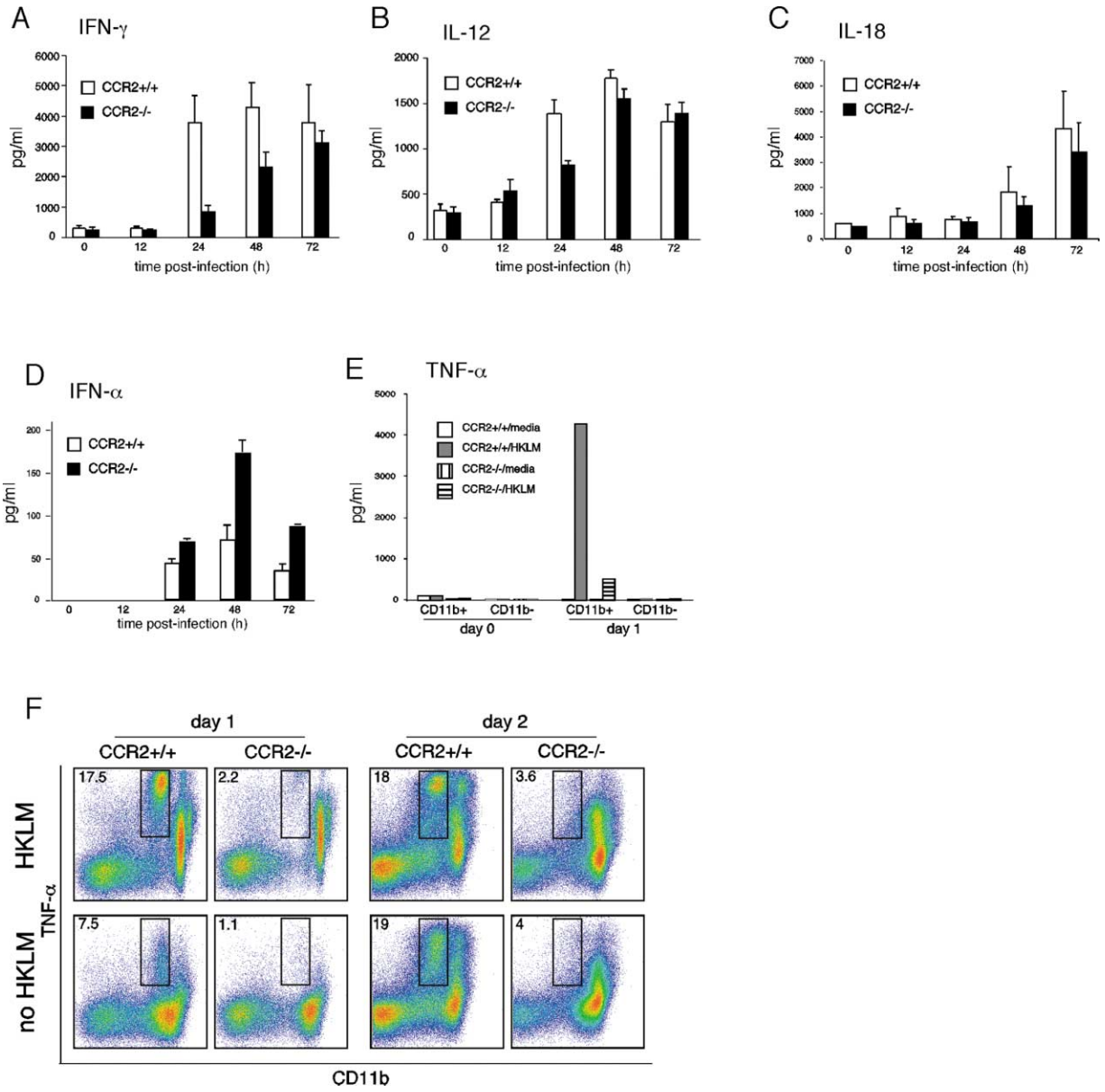

Figure 4. CD11b^{int} Cells Are Major TNF Producers in Response to Bacterial Challenge

(A–D) (A) IFN-γ, (B) IL-12, (C) IL-18, and (D) IFN-α levels were determined in spleen lysates by ELISA. Each bar represents the mean value from 4–11 mice (error bars, SD).

(E) CD11b-positive and CD11b-negative cell fractions were MACS purified from uninfected or 1 day-infected spleens and cultured for 48 hr with or without HKLM stimulation. TNF secretion in supernatants was measured by ELISA. Mean values for triplicate wells from three mice are shown. This experiment was repeated three times.

(F) Splenocytes from mice infected for 1 or 2 days were cultured with or without HKLM stimulation for 4 hr in the presence of BFA and stained for intracellular TNF and surface CD11b and CD3. Upper panels show cells that were stimulated in vitro with HKLM, while lower panels show cells that were stained without in vitro stimulation. Plots show cells gated on CD3-negative lymphocytes/monocytes. The numbers in the left upper quadrant represent the percentage of TNF^{+} cells in the indicated gate. Representative dot plots for three mice per group are shown, and the experiment was repeated three times.

and in the initiation of inflammatory cascades that mediate autoimmunity (Tracey and Cerami, 1994) or immune defense against pathogens (Flynn et al., 1995; Pasparakis et al., 1996). Thus, it is likely that TNF initiates a series of processes that lead to in vivo killing of bacterial pathogens. One of the major mechanisms for bacterial killing is the production of nitric oxide (NO) by inducible nitric oxide synthase (MacMicking et al., 1997). Immunohistochemical staining of spleens from *L. monocytogenes*-infected mice demonstrated a near absence of iNOS in CCR2$^{-/-}$ mice, while iNOS production was readily detectable in wild-type mice (Figure 5A). Consistent with this finding, NO production by splenocytes derived from *L. monocytogenes*-infected CCR2$^{-/-}$ mice was markedly reduced in comparison to splenocytes derived from infected wild-type mice (Figure 5B), sug-

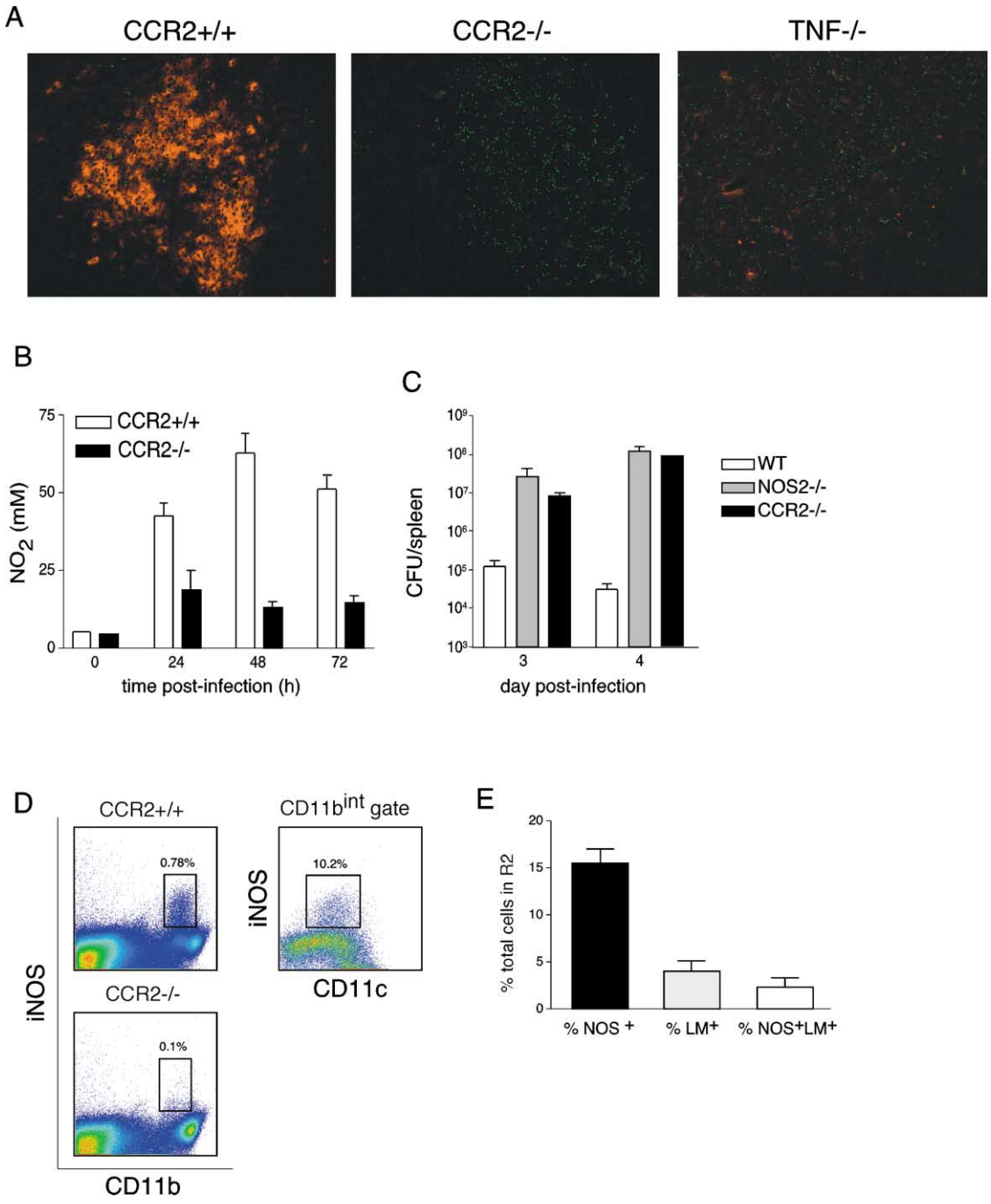

Figure 5. iNOS Expression by CD11b^{int}CD11c^{int} Cells during Infection

(A) Frozen sections from spleens of wild-type, CCR2$^{-/-}$, and TNF$^{-/-}$ mice infected for 48 hr were stained for iNOS (red) and for *L. monocytogenes* (green).

(B) NO production by splenocytes from wild-type and CCR2$^{-/-}$ mice infected with *L. monocytogenes*. Each bar represents three mice, and the experiment was repeated six times (error bars, SD).

(C) The number of viable bacteria in the spleens of C57BL/6, CCR2$^{-/-}$, and INOS$^{-/-}$ mice was determined 3 and 4 days following intravenous infection with 3000 virluent *L. monocytogenes* 10403s. Plotted values represent mean numbers of bacterial colony-forming units (CFU) from groups of three to four mice per time point (error bars, SD).

(D) Intracellular iNOS staining of splenocytes from C57BL/6 and CCR2$^{-/-}$ mice infected with *L. monocytogenes* for 24 hr. Left: expression of CD11b and iNOS in the lymphocyte/monocyte gate. The percentage of CD11b^{int}iNOS^{+} cells is indicated above each gate. Right: expression of iNOS and CD11c in CD11b^{int} gate. Shown are representative dot plots for two mice per group, and the experiment was repeated twice.

(E) Percentage of iNOS^{+} and *L. monocytogenes*$^{+}$ cells in the cytospins of cells sorted from R2 gate (as depicted in Figure 2B).

Table 1. Effect of $CCR2^{+/+}$ PBMCs Transfer on *L. monocytogenes* Growth in Spleens of $CCR2^{-/-}$ Mice

	Mean CFU (10^{-5})	% CFU Reduction Relative to No Transfer Control	P Value
Experiment 1			
No transfer	332.5 ± 105	80.8	0.036
10^6 $CCR2^{+/+}$ PBMC	63.5 ± 11		
Experiment 2			
No transfer	1.6 ± 0.4	58.3	0.05
1.5×10^6 $CCR2^{+/+}$ PBMC	0.6 ± 0.1		
Experiment 3			
No transfer	13.3 ± 1.1	70.4	0.0034
0.5×10^6 $CCR2^{+/+}$ PBMC	3.9 ± 1.4		
Experiment 4			
No transfer	10.3 ± 3.6	−16.5	0.74
2×10^6 $CCR2^{-/-}$ PBMC	12.0 ± 5.1		

Ficoll-purified blood mononuclear cells from naive (Experiment 3) or infected (Experiments 1 and 2) wild-type or naive $CCR2^{-/-}$ mice (Experiment 4) were adoptively transferred into $CCR2^{-/-}$ mice, and recipients were infected with *L. monocytogenes*. Bacterial counts in the spleens of recipient and control mice 72 hr postinfection are shown. Mean colony-forming units ±SD in groups of two to three mice are shown.

gesting that decreased in vivo bacterial killing in $CCR2^{-/-}$ mice results from diminished NO production. Interestingly, $TNF^{-/-}$ mice also produced markedly decreased amounts of iNOS upon *L. monocytogenes* infection, supporting the notion that deficient TNF production in $CCR2^{-/-}$ mice results in decreased iNOS synthesis (Figure 5A).

iNOS Is Expressed by $CD11b^{int}/CD11c^{int}$ Cells

Mice with genetic deletions of the iNOS gene have increased susceptibility to *L. monocytogenes* infection (MacMicking et al., 1997). The severity of infection in $CCR2^{-/-}$ and $iNOS^{-/-}$ mice 72 and 96 hr following bacterial inoculation is similar (Figure 5C), consistent with a common defect in bacterial killing in these mouse strains. To determine which cells produce iNOS, we stained splenocytes from infected mice for intracellular iNOS. In wild-type mice the greatest amount of iNOS is detected in the $CD11b^{int}$ cells that are lacking in $CCR2^{-/-}$ mice (Figure 5D). iNOS expression also correlates with the expression of intermediate amounts of CD11c (Figure 5D, right). In contrast to TNF, which is produced by the majority of $CD11b^{int}/CD11c^{int}$ cells (Figure 4F), iNOS is produced by a smaller fraction of $CD11b^{int}/CD11c^{int}$ DC population. To determine whether iNOS expression is limited to *L. monocytogenes*-infected cells, sorted $CD11b^{int}/CD11c^{int}$ DCs were double stained for iNOS and *L. monocytogenes*. While 15% of $CD11b^{int}/CD11c^{int}$ DCs expressed iNOS, less than 5% were infected with *L. monocytogenes* (Figure 5E). While most, but not all, *L. monocytogenes*-infected $CD11b^{int}/CD11c^{int}$ DCs expressed iNOS, the majority of iNOS-producing cells were uninfected.

To determine whether the $CD11b^{int}/CD11c^{int}$ DC population in wild-type mice is recruited from the blood stream during *L. monocytogenes* infection, we purified PBMCs from wild-type or $CCR2^{-/-}$ mice and transferred them into $CCR2^{-/-}$ mice at the time of bacterial inoculation. Table 1 demonstrates that $CCR2^{-/-}$ recipients of wild-type PBMCs had significantly fewer bacteria in the spleen 72 hr following bacterial inoculation than control $CCR2^{-/-}$ mice in three independent experiments. In contrast, transfer of $CCR2^{-/-}$ PBMC had no effect on bacterial growth in the spleens of recipient mice. These results strongly suggest that CCR2 mediates recruitment of peripheral blood monocytes to the infected spleen, rather than in situ activation of splenic macrophages.

Discussion

We have identified a novel DC subset that is the major producer of TNF and iNOS during the first 48 hr of bacterial infection. The establishment of successful innate immune defenses following intracellular bacterial infection depends critically on the presence of this TNF and iNOS-producing-DC subset. Although various macrophage and dendritic cell populations are present in the spleen during *L. monocytogenes* infection, Tip-DCs play a unique and essential role in the orchestration and implementation of antimicrobial defenses. The recruitment of Tip-DCs to infected spleens requires CCR2-mediated signaling. Remarkably, the majority of Tip-DCs are not directly infected with *L. monocytogenes,* suggesting that they are activated indirectly. Although we refer to CCR2-recruited, TNF-, and iNOS-producing cells as DCs, we recognize that differentiating between dendritic cells and activated macrophages is a tricky and perhaps imprecise business. Nevertheless, we believe the following findings, not individually but in aggregate, justify labeling Tip-DCs as dendritic cells. (1) Tip-DCs do not express CD14 or F4/80 (data not shown), but many, by 48 hr, express CD11c. (2) Tip-DCs express high levels of costimulatory molecules and MHC class II. (3) Tip-DCs prime naive, alloreactive T cells in a mixed lymphocyte reaction. (4) Tip-DCs have the morphologic appearance of dendritic cells. (5) Tip-DCs are not adherent to plastic. (6) Tip-DCs are not present in an uninfected spleen but rather, without being directly infected, are recruited into the spleen following systemic infection.

In addition to their role in T cell priming, DCs are increasingly implicated in early innate responses to microbial infection. Consistent with their role as sentinels detecting microbial invasion, DC subsets express a range of pattern recognition receptors that respond to distinct microbial products (Jarrossay et al., 2001; Kadowaki et al., 2001). In mice, DCs that resemble plasmacy-

toid cells produce massive amounts of IFN-α in response to infection with murine cytomegalovirus (Dalod et al., 2002), while in humans plasmacytoid pre-DCs produce IFN-α and undergo maturation in response to influenza virus (Cella et al., 1999). In contrast to the TNF/iNOS-producing DCs we describe, the murine IFN-α producing DCs are CD11b$^-$, B220$^+$, and CD8α$^+$. Interestingly, however, both Tip-DCs and IFN-α-producing DCs stain with RB6-8C5 mAb, which binds Ly6C/G, but do not stain with DEC-205, which stains conventional CD11c^{hi} DCs (data not shown and O'Keeffe et al., 2002). Interestingly, a GR-1$^+$ monocyte subset that migrates to the sites of inflammation has been identified in the blood stream of uninfected mice (Geissmann et al., 2003 [this issue of *Immunity*]). This monocyte subset expresses CCR2 and thus may represent a precursor of Tip-DCs.

There are examples of DCs mediating in vivo innate inflammatory responses to microbial products. CD8α$^+$, DEC-205$^+$ DCs produce IL-12 upon stimulation with some microbial products (Sousa et al., 1997), and injection of mice with Toxoplasma extract induces CCR5-dependent recruitment of CD8α DCs to the periarteriolar sheath and production of IL-12 (Aliberti et al., 2000). In this setting, IL-12 production by DCs is tightly controlled by lipoxin A_4-mediated downregulation of CCR5 (Aliberti et al., 2002). A different role for leukotrienes in DC differentiation was previously demonstrated in studies of monocyte trafficking to lymph nodes (Robbiani et al., 2000). In that system, emigration of DCs from skin to lymph nodes depends on lipid transporter multidrug resistance protein 1 (MDR-1)-mediated transport of cysteinyl leukotriene C_4, which enhances DC responsiveness to CCL19, enabling entry into lymphatic vessels. Our finding that CD11b$^+$ cells are recruited to the infected spleen and differentiate within 48 hr into DCs is reminiscent of Randolph's finding that inflammatory stimuli drive monocyte trafficking to lymph nodes and their differentiation into DCs (Randolph et al., 1999). Whether similar eicosanoid-mediated mechanisms control recruitment and activation of Tip-DCs will require further investigation. Interestingly, CCR2 signaling has been implicated in the induction of TNF transcription and thus may drive the expression of TNF by Tip-DCs (Sodhi and Biswas, 2002).

Recent studies have demonstrated that killed, polysaccharide-encapsulated Gram-positive bacteria are taken up by DCs circulating in the bloodstream and delivered to the splenic marginal zone for presentation to marginal zone B cells (Balazs et al., 2002). It is possible that intravenously administered *L. monocytogenes* bacteria are also taken up by circulating cells and delivered to the splenic marginal zone. Interestingly, in vivo depletion of CD11b-expressing cells renders mice highly susceptible to *L. monocytogenes* infection (Rosen et al., 1989). However, since bacterial targeting to spleen is similar in wild-type and CCR2$^{-/-}$ mice, we believe it is unlikely that CCR2-expressing, CD11b$^+$ cells are directly involved in clearance of inoculated bacteria from the blood stream or targeting to the splenic marginal zone. Rather, our results suggest that CCR2-mediated signaling is involved in the recruitment and/or activation of cells that are not directly involved in bacterial transport to the spleen. The finding that Tip-DCs are not bacterially infected supports this conclusion. Thus, our working model (see Figure 6) is that splenic macrophages in the marginal zone clear *L. monocytogenes* from the blood stream and, through mechanisms that remain obscure, move into the T cell zones of the white pulp. These activated, bacterially infected macrophages secrete MCP-1, which recruites CCR2-expressing monocytes from the blood stream. Monocytes, upon arrival at the site of infection, differentiate into Tip-DCs and produce TNF and iNOS, a step that is indispensable for clearance of live bacteria. IFN-γ, a cytokine crucial for innate immune responses, is also significantly reduced in the spleens of CCR2-deficient mice early during infection despite normal NK cell levels. Recently, dendritic cells were shown to induce IFN-γ secretion by NK cells (Ferlazzo et al., 2002; Gerosa et al., 2002). Thus, although Tip-DCs do not appear to secrete IFN-γ, they might provide signals augmenting cytokine secretion by other cells.

Since NO is a water- and lipid-soluble gas, it is conceivable that it exerts antimicrobial effects by diffusing into infected cells in the vicinity of Tip-DCs. Exogenous NO might also induce iNOS expression in infected cells (Sheffler et al., 1995). Since phagocytosis of live *L. monocytogenes* ultimately results in the destruction of host cells, it may be beneficial to recruit the cells capable of sensing infection and mediating microbicidal effector functions without being directly infected. Interestingly, CD8 T cell responses in the spleens of CCR2-deficient mice are larger than that observed in the wild-type spleens. Nitric oxide has been reported to be suppressive for T cell proliferation (Albina et al., 1991; Lu et al., 1996). Thus, it is possible that massive production of NO by Tip-DCs downmodulates the magnitude of T cell responses.

TNF plays an essential role in early control of infections by intracellular bacteria such as *Mycobacterium tuberculosis* (Flynn et al., 1995), *Salmonella typhimurium* (Everest et al., 1998), and *Listeria monocytogenes* (Pasparakis et al., 1996). While TNF is essential for immune defense, it is also a mediator of septic shock (Tracey and Cerami, 1994). Restricting TNF production to a specialized cell population such as Tip-DCs in a chemokine-dependent fashion ensures localized production of TNF while limiting its systemic release. Along similar lines, production of NO in response to infection is also a two-edged sword, killing invasive microbes on the one hand but causing significant tissue damage on the other hand. While dendritic cells play central roles in adaptive T cell and B cell responses, and orchestrate innate immune defenses, their direct role in microbial killing is largely undefined. iNOS production by CCR2-activated Tip-DCs indicates a direct role for these cells in microbial killing. Thus, in the setting of intracellular bacterial infection, Tip-DCs play an essential role orchestrating and, we propose, mediating innate immune defense.

Experimental Procedures

Mice and Infections

C57BL/6, CCR2$^{-/-}$, C57BL/6-H2-K^d, TNFα$^{-/-}$, and CCR2$^{-/-}$-H2-K^d mice were bred at Memorial Sloan-Kettering Research Animal Resource Center. Generation of CCR2$^{-/-}$ mice was previously de-

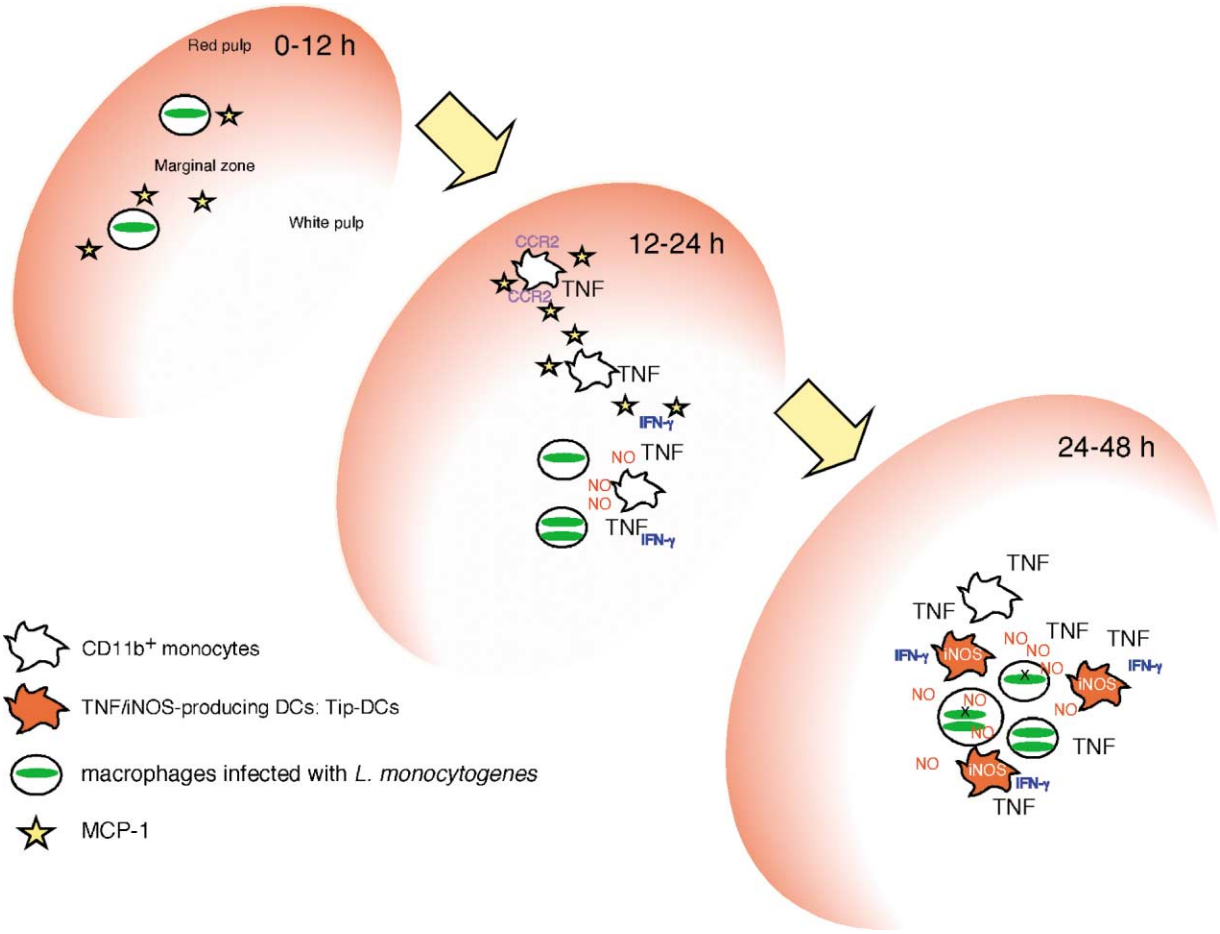

Figure 6. Tip-DCs Orchestrate Innate Antimicrobial Responses

This model describes the recruitment of CCR2$^+$ monocytes to the spleen following *L. monocytogenes* infection and their differentiation into Tip-DCs. Zero to twelve hours: Capture of *L. monocytogenes* by macrophages in the marginal zone induces MCP-1 production. Twelve to twenty-four hours: *L. monocytogenes*-infected macrophages migrate into the white pulp where CD11b$^+$ monocytes are recruited via CCR2, secrete TNF, and undergo differentiation into Tip-DCs. Twenty-four to forty-eight hours: Activated Tip-DCs, having infiltrated the lesion, produce large amounts of TNF and NO.

scribed (Kuziel et al., 1997). For most studies, CCR2$^{-/-}$ mice were backcrossed eight generations onto the C57BL/6 background, while for T cell studies, mice were crossed an additional two generations onto C57BL/6-H2-K^d mice (Kerksiek et al., 2003). iNOS$^{-/-}$ mice on the C57BL/6 background were provided by Dr. Carl Nathan (Cornell University, New York). Mice were infected intravenously with 3000 *L. monocytogenes* strain 10403S (provided by Dr. Daniel Portnoy, University of California, Berkeley). For T cell studies, mice were immunized with 1.5×10^5 attenuated ActA-deficient *L. monocytogenes* strain DP-L1942 (provided by Dr. Daniel Portnoy). At indicated times postinfection, spleens were harvested, dissociated, and digested with 0.3% collagenase type 4 (Worthington, NJ). Bacteria CFUs were determined by plating on brain-heart infusion agar plates.

Isolation of Splenic Cell Subsets

For CD11b$^+$ and CD11c$^+$ cell purification, splenocytes were collagenase digested, treated with red blood cell lysis buffer, and incubated with MACS anti-CD11b and anti-CD11c beads (Miltenyi Biotech, Auburn, CA) followed by magnetic separation according to the manufacturer's protocol. To purify CD11b^{int}CD11c^{int} and CD11c^{high} cells, spleen suspensions were incubated with monoclonal anti-CD11c-PE and anti-CD11b-APC antibodies. DAPI was used to exclude dead cells. Cell subsets were obtained by fluorescence-activated sorting with the purity of >98%. Cells were spun onto glass slides using a cytocentrifuge (Shandon, Astmoor, United Kingdom) and stained using Diff-Quik Satin Set (Dade Behring, Newark, DE) for morphological examination. Where indicated, cytospins were fixed with formaldehyde and stained for iNOS and *L. monocytogenes* as described in the histology section.

Flow Cytometry

The following antibodies were purchased from BD Pharmingen (San Diego, CA): anti-CD11b-PerCP (M1/70), anti-CD11c-PE (HL3), anti-Mac-3-FITC (M3/84), anti-B7-1-FITC (16-10A1), anti-B7-2-FITC (GL1), anti-CD40-FITC (3/23), anti-I-A/I-E-PE (M5/114.15.2), anti-CD4-PerCP (RM4-5), anti-CD8a-PerCP (53-6.7), anti-CD62L-FITC (MEL-14). Goat anti-iNOS antibody (M-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and FITC-anti-goat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Generation of PE-conjugated streptavidin H2-K^d tetramer coplexed with *L. monocytogenes*-derived $LLO_{91\text{-}99}$ peptide for detection of antigen-specific CD8 T cells was previously described (Busch et al., 1998). For analysis of CD11b$^+$/CD11c$^+$ populations, a large gate was drawn to include lymphocyte/monocyte populations, and cells were further gated on CD11b where indicated. For T cell analysis, a gate was drawn to include lymphocyte population, and cells were further gated on CD62L, CD4, and CD8α. Intracellular staining was performed by staining cells for cell surface markers, fixing in 2% paraformaldehyde, permeabilizing with Perm/Wash

Buffer (BD Pharmingen), and incubating with Mac-3-FITC or goat anti-INOS antibody followed by FITC-anti-goat IgG.

Cytokine Assays

Spleens were dissociated in ice-cold PBS containing 0.01% Triton X-100 and centrifuged at 10,000 × g. Murine IFN-γ in the supernatants was quantified by sandwich ELISA using OptEIA kit from Pharmingen, murine IFN-α was quantified using an ELISA kit from PBL Biomedical Laboratories (Piscataway, NJ), and murine TNF-α was quantified using an ELISA kit from Endogen (Woburn, MA). For TNF-α determination in culture supernatants, splenocytes from mice infected for 24 hr were cultured in vitro for 24 hr with or without 10^8/ml HKLM prepared as previously described (Lauvau et al., 2001), and TNF-α was quantified using an ELISA kit (Endogen). For intracellular cytokine staining, spleen cells were stimulated in vitro with PBS, 10^{-6} M LLO_{91-99}, or 10^8/ml HKLM in the presence of brefeldin A (BFA) for 4 hr followed by staining for intracellular TNF-α and IFN-γ according to the manufacturer's protocol (Cytofix/Cytoperm, Pharmingen). Cell cultures were grown in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin, gentamycine).

Mixed Lymphocyte Reaction

Splenocytes from BALB/c and C57BL/6 mice were enriched for T cells by magnetic separation after incubation with monoclonal anti-CD4 and anti-CD8 beads (Miltenyi Biotech). T cells were plated at 2×10^5/well in triplicate wells of 96-well, round-bottom plates in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum (FCS), L-glutamine, HEPES, 2-mercaptoethanol, and antibiotics (penicillin, streptomycin, gentamycine). C57BL/6 mice were infected with *L. monocytogenes* for 48 hr, and stimulator cell subsets were obtained from spleens by fluorescence-activated sorting on the gates depicted in Figure 2B after incubation with anti-CD11c-PE and anti-CD11b-APC monoclonal antibodies. Varying numbers of γ-irradiated stimulators were added to T cells, and cultures were incubated for 5 days. [^{3}H]thymidine was added at 1 μCi/well for the last 18 hr of culture. [^{3}H]thymidine incorporation was determined in liquid scintillation counter.

Adoptive Transfer

Blood mononuclear cells were collected from naive C57BL/6 mice or C57BL/6 mice infected with *L. monocytogenes* for 48 hr or naive $CCR2^{-/-}$ mice and purified on Ficoll gradient (Accurate Chemical and Scientific Corporation, Westbury, NY). Cells were washed with PBS and 1–2 × 106 cells were transferred intravenously into $CCR2^{-/-}$ mice.

Nitrite Assay

Splenocytes were harvested at various times postinfection and cultured for 48 hr in vitro at 2×10^5 cells/ml in 96-well flat-bottom plates. Nitrite in supernatants was measured using the Griess Reagent Kit (Molecular Probes, Eugene, OR) with $NaNO_2$ as the standard.

Histology

Spleens were harvested from uninfected mice and mice infected for 2 days with *L. monocytogenes*, and frozen 5 μm sections were prepared. Sections were acetone fixed and incubated with anti-CD45R/B220 (BD Pharmingen), rat anti-Thy1.2 (BD Pharmingen), rat anti-Mac-3 (BD Pharmingen), rat anti-CD169 (Serotec, United Kingdom), Difco Listeria O polyserum (Fisher), and goat anti-iNOS antibody (Santa Cruz). Staining for *L. monocytogenes,* B220, CD169, and Mac-3 was developed with anti-IgG-FITC and staining for iNOS, Mac-3, and DEC-205 was developed with anti-goat-IgG-biotin followed by ABC-AP Kit (Vector Laboratories, Burlingame, CA). Imaging of tissue sections was performed on a Zeiss Axioplan 2 microscope using Openlab software (Improvision, Lexington, MA).

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