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Sequential MyD88-Independent and -Dependent Activation of Innate Immune Responses to Intracellular Bacterial Infection

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Summary

Microbial infections induce chemokine and cytokine cascades that coordinate innate immune defenses. Infection with the intracellular bacterial pathogen *Listeria monocytogenes* induces CCR2-dependent monocyte recruitment and activation, an essential response for host survival. Herein we show that invasive *L. monocytogenes*, but not killed or noninvasive bacteria, induce secretion of MCP-1, the requisite chemokine for monocyte recruitment. Induction of MCP-1, but not TNF or IL-12, following *L. monocytogenes* infection is MyD88 independent. Consistent with these results, MyD88 deficiency does not impair monocyte recruitment to *L. monocytogenes* infected spleens, but prevents monocyte activation. Our results indicate that distinct microbial signals activate innate immune responses in an ordered, step-wise fashion, providing a mechanism to specify and modulate antimicrobial effector functions.

Introduction

Listeria monocytogenes is a Gram positive intracellular bacterial pathogen that causes severe infections in immunocompromised hosts (Gellin and Broome, 1989). Upon phagocytosis by macrophages, *L. monocytogenes* escapes the vacuole by secreting listeriolysin O (LLO), an essential virulence factor (Bielecki et al., 1990). In the cytosol, *L. monocytogenes* expresses ActA, which polymerizes actin, enabling bacterial mobility and cell-to-cell spread (Cossart and Bierne, 2001). While both ActA and LLO-deficient bacteria are avirulent upon inoculation of mice, ActA-deficient bacteria induce long-term, CD8 T cell-mediated protective immunity while LLO-deficient bacteria do not (Bouwer et al., 1994; Harty and Bevan, 1995). Thus, invasion of host cell cytosol provides an essential stimulus that promotes development of protective adaptive immunity.

T cell-mediated immune responses become operative 4 to 5 days following bacterial inoculation and are essential for complete clearance of *L. monocytogenes* from infected mice (Bhardwaj et al., 1998; Busch and Pamer, 1999). Prior to this, innate immune responses restrain in vivo bacterial proliferation and spread. Mice lacking interferon-γ, TNF, or their specific receptors have inadequate innate immune responses and are highly susceptible to infection with virulent *L. monocytogenes* (Buchmeier and Schreiber, 1985; Huang et al., 1993; Pasparakis et al., 1996; Pfeffer et al., 1993; Rothe et al., 1993). Several lines of investigation have implicated monocyte recruitment in early defense against infection. Antibody-mediated blockade of CD11b or deficiency of the CCR2 chemokine receptor markedly enhance susceptibility to *L. monocytogenes* infection by preventing monocyte recruitment to infected tissues (Rosen et al., 1989; Kurihara et al., 1997). Recent studies demonstrated that monocytes recruited to *L. monocytogenes* infected spleens differentiate into Tip-DCs, a dendritic cell population that produces large amounts of TNF and iNOS (Serbina et al., 2003).

Toll-like receptors (TLRs) constitute a family of pattern recognition molecules that initiate intracellular signaling cascades upon exposure to microbial molecules (Barton and Medzhitov, 2003; Takeda et al., 2003). Distinct TLRs respond to LPS, lipoteichoic acid, bacterial flagellin, peptidoglycan, CpG DNA, and bacterial lipoproteins, leading to activation of macrophages and dendritic cells, production of inflammatory cytokines, acquisition of microbicidal activities, and enhanced antigen presentation. Detection of bacterial products in cytosolic compartments is mediated, at least in part, by Nod-1 and Nod-2 proteins. These molecules induce NFκB activation by detecting muramyl di- and tri-peptides that derive from the degradation of Gram-negative bacterial cells walls (Girardin et al., 2003; Inohara and Nunez, 2003). In contrast to Nod-1- and Nod-2-mediated signaling, TLR molecules signal through MyD88, a cytosolic adaptor molecule that binds the cytosolic TIR domain of TLRs and the death domain of the IL1-receptor-associated-kinase IRAK (Takeda et al., 2003). Given its central role in innate immune signaling, it is not surprising that mice lacking MyD88 have defective innate immune responses and succumb to infection with *L. monocytogenes* (Edelson and Unanue, 2002; Seki et al., 2002).

Chemokines are among the first products of an inflam-

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matory response to infection. MCP-1 is a 76 amino acid chemokine that is induced by a range of inflammatory stimuli including microbial infection (Rollins, 1997; Yoshimura et al., 1989). The predominant, if not exclusive, receptor for MCP-1 is CCR2, which is expressed on a subset of monocytes and also on activated T cells (Luther and Cyster, 2001). Transgenic mice expressing high levels of MCP-1 have increased susceptibility to *L. monocytogenes* infection, likely reflecting abnormal monocyte recruitment in this setting (Rutledge et al., 1995). Mice lacking MCP-1 have decreased pulmonary granulomatous responses to *Schistosoma mansoni* eggs but normal resistance to pulmonary infection with *Mycobacterium tuberculosis* (Lu et al., 1998). MCP-1 is produced in vivo during the course of *L. monocytogenes* infection (Barsig et al., 1998; Flesch et al., 1998); however, the mechanisms promoting its induction in vivo remain undefined.

Because Tip-DC recruitment to the spleens of *L. monocytogenes* infected mice is CCR2 mediated, we investigated the in vivo induction of MCP-1 expression following bacterial inoculation. We find that MCP-1 is induced rapidly following *L. monocytogenes* infection, but only by bacterial strains that access the host cell cytosol. MCP-1 induction does not require MyD88, indicating that a MyD88-independent, cytosolic detection mechanism is responsible for monocyte recruitment to sites of bacterial infection. Once recruited, however, monocyte differentiation into TipDCs is MyD88 dependent, suggesting that innate immune activation is multitiered and depends on input from sequential and distinct microbial stimuli.

Results

MCP-1 Is the Principal CCR2 Ligand during In Vivo *L. monocytogenes* Infection

Mice lacking the CCR2 chemokine receptor are highly susceptible to *L. monocytogenes* infection (Kurihara et al., 1997). Recently, we described a novel dendritic cell subset, TipDCs, that is recruited to the spleen in a CCR2-dependent manner and is the major source of TNF and iNOS early during *L. monocytogenes* infection (Serbina et al., 2003). While several monocyte chemoattractant proteins (MCPs) can bind to CCR2, MCP-1 was shown to be a major ligand for CCR2 in a variety of inflammatory settings (Huang et al., 2001; Lu et al., 1998; Tesch et al., 1999). To examine the role of MCP-1 in Tip-DC recruitment, we compared susceptibilities of wild-type and mice lacking CCR2 and MCP-1 to *L. monocytogenes* infection. Figure 1A demonstrates that both CCR2- and MCP-1-deficient mice had increased numbers of *L. monocytogenes* in spleens 3 days following bacterial inoculation when compared to the wild-type mice. The susceptibility of CCR2 or MCP-1-deficient mice was even more apparent 5 days following infection (Figure 1A). In comparison to CCR2$^{-/-}$ mice, we detected greater mouse-to-mouse variability with MCP-1$^{-/-}$ mice, with occasional, albeit rare, mice demonstrating nearly wild-type levels of resistance to infection. The reason for this disparity remains obscure.

We next examined whether Tip-DCs were recruited to the spleen in the absence of MCP-1. Tip-DCs recruited to the spleen, when analyzed 48 hr after bacterial inoculation, express intermediate levels of CD11b and CD11c and high levels of Mac-3 (Serbina et al., 2003). Flow cytometric analysis revealed that the percentages of CD11b^{int}/Mac-3^{high} cells were significantly reduced in the spleens of MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice when compared to infected wild-type spleens (55% and 84% reduction, respectively) while the percentages of neutrophils (CD11b^{high}/Mac-3^{low}) were increased, most likely reflecting the increased microbial numbers in the knockout mice. Although MCP-1 appears to be the major ligand governing Tip-DCs recruitment, the small amount of CCR2-dependent, MCP-1-independent Tip-DCs recruitment that we detected suggests that other CCR2 ligands may function in place of MCP-1 (Figures 1A and 1B). However, as shown in Figure 1A, this residual cellular recruitment is insufficient to confer resistance to *L. monocytogenes* infection in MCP-1$^{-/-}$ mice.

A distinguishing feature of Tip-DCs is their production of iNOS during *L. monocytogenes* infection. Therefore, intracellular iNOS expression in the spleens of wild-type, MCP-1$^{-/-}$, and CCR2$^{-/-}$ mice was examined 2 days following bacterial inoculation. Consistent with the decrease in Tip-DCs numbers, the percentage of iNOS-expressing cells was dramatically reduced in the spleens of MCP-1$^{-/-}$ mice (Figure 1D), confirming that MCP-1 is the principal mediator of CCR2-dependent innate immune responses.

MCP-1 Production during *L. monocytogenes* Infection Is Contingent on Cytoplasmic Localization of Bacteria

Because MCP-1 production appears to be essential for the initiation of innate immune responses following *L. monocytogenes* infection, we decided to determine the kinetics of MCP-1 induction. MCP-1 production in the spleens was observed as early as 6–12 hr postinfection and was dramatically increased between 24 and 48 hr postinfection (Figure 2A), suggesting direct correlation between MCP-1 levels and the in vivo bacterial burden. Although it is established that induction of MCP-1 is an NF-κB-dependent process (Ueda et al., 1994, 1997), the stimuli driving MCP-1 secretion in vivo remain incompletely defined. Recently NF-κB activation by cytoplasmic, but not vacuolar, *L. monocytogenes* has been demonstrated (O'Riordan et al., 2002). We therefore investigated whether invasion of host cell cytosol was required for MCP-1 induction by *L. monocytogenes*. Interestingly, MCP-1 production was not observed in vivo when mice were inoculated with an avirulent *L. monocytogenes* strain that lacks the LLO gene and thus remains within a vacuolar cellular compartment. Similarly, inoculation of mice with a preparation of heat-killed Listeria (HKLM) also failed to induce in vivo MCP-1 production. In contrast, robust MCP-1 production was observed in response to inoculation with the avirulent *L. monocytogenes* strain lacking ActA, which accesses the host cell cytosol but cannot move from cell to cell (Figure 2B). Thus, our results indicate that, in the setting of *L. monocytogenes* infection, MCP-1 induction correlates more closely with the ability of the organism to access host cell cytosol than the ability to replicate in vivo and cause disease (i.e., virulence).

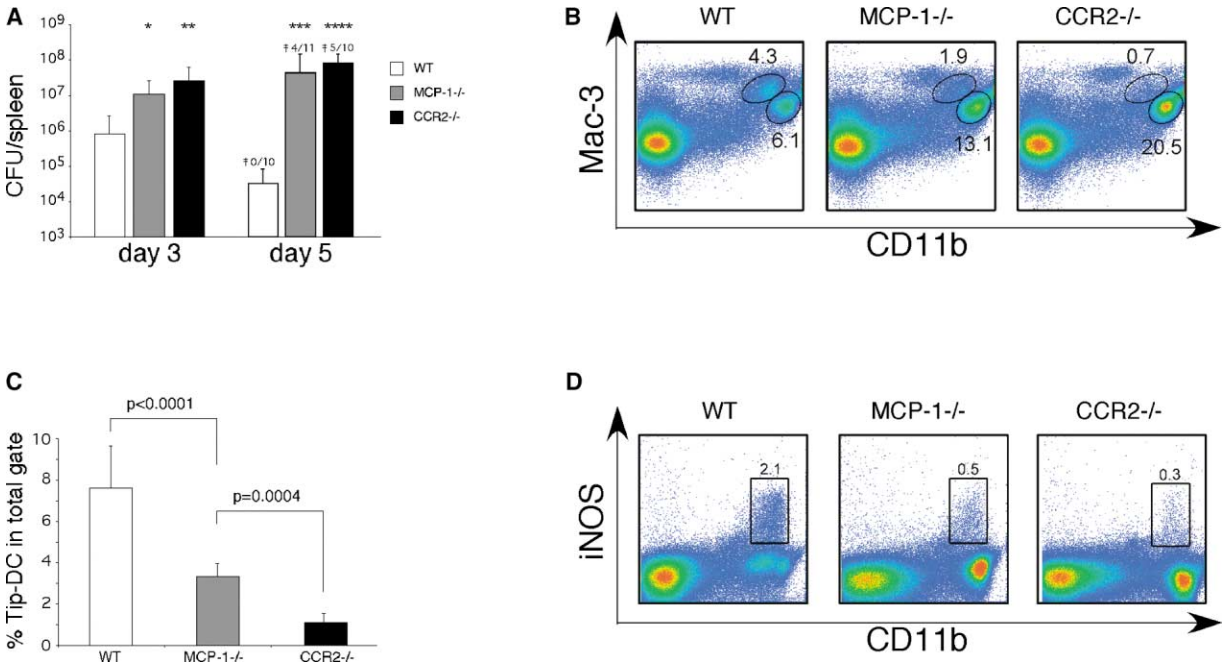

Figure 1. MCP-1-Mediated Recruitment of Tip-DCs

(A) C57BL/6, MCP-1$^{-/-}$, and CCR2$^{-/-}$ mice were infected intravenously with 3,000 live *L. monocytogenes*, spleens were removed at the indicated times, and viable bacteria were quantified. Mean numbers of colony forming units (CFU) from groups of 10–13 mice are shown. Error bars represent standard deviation. *P = 0.03, **P = 0.02, ***P = 0.01, ****P = 0.0003 as compared to wild-type controls. Double dagger, number of dead animals/total in the group.

(B) Splenocytes from C57BL/6, MCP-1$^{-/-}$, and CCR2$^{-/-}$ mice infected with *L. monocytogenes* for 24 hr were stained for CD11b and Mac-3. A large gate was drawn on the live lymphocyte/monocyte population and expression of CD11b and Mac-3 was analyzed. The percentages of CD11b^{int}/Mac-3^{high} and CD11b^{high}/Mac-3^{low} cells are indicated above each gate. Representative dot plots for three mice per group are shown. This experiment was repeated four times with similar results.

(C) Percentages of CD11b^{int}/Mac-3^{high} cells in wild-type, CCR2$^{-/-}$, and MCP$^{-/-}$ spleens 1 day following bacterial infection. Each bar represents the average of 12–14 mice from four independent experiments. Error bars represent standard deviation.

(D) Splenocytes from wild-type, MCP-1$^{-/-}$, and CCR2$^{-/-}$ mice infected with *L. monocytogenes* for 48 hr were stained for intracellular iNOS. The percentage of CD11b^{+}NOS2^{+} cells is indicated on each plot. Representative dot plots for three mice per group are shown. This experiment was repeated twice with similar results.

It is likely that cells infected with *L. monocytogenes* are the major source of MCP-1 in vivo. However, as the precise nature of these cells is not known, we sought to confirm our in vivo results with in vitro studies using bone marrow derived macrophages. Infection of macrophages with wild-type or ActA-deficient *L. monocytogenes* induced MCP-1 secretion as early as 6 hr postinfection, while infection with LLO-deficient *L. monocytogenes,* even in 10-fold excess, failed to induce chemokine secretion (Figure 3A). However, macrophages readily produced TNF in response to all bacterial strains tested (Figure 3B), suggesting that distinct mechanisms induce the secretion of MCP-1 as opposed to TNF. Because the numbers of intracellular wild-type and mutant *L. monocytogenes* are likely to differ, we examined macrophages infected for 6 hr with each of the bacterial strains. Figure 3C demonstrates that bacteria were readily detectable in macrophages infected with wild-type and ActA-deficient *L. monocytogenes*. As expected, wild-type bacterial staining often colocalized with actin staining, reflecting bacterial invasion of the cytosol. Actin staining was not seen with the ActA-deficient strain of *L. monocytogenes*, since this strain does not polymerize actin. LLO-deficient *L. monocytogenes* were also readily detectable in macrophages 6 hr after infection. In fact, the number of bacteria present in macrophages infected with the high dose (50:1) of LLO-deficient *L. monocytogenes* was much larger than in either wild-type or ActA-deficient infected macrophages. Thus, the absence of MCP-1 production by macrophages infected with LLO-deficient *L. monocytogenes* cannot be attributed to the paucity of intracellular bacteria.

In Vivo Tip-DC Recruitment Requires Infection with Cytosol-Invasive *L. monocytogenes*

Lack of MCP-1 secretion in response to LLO-deficient *L. monocytogenes* prompted us to examine the in vivo recruitment of Tip-DCs. Mice were inoculated with ActA- or LLO-deficient *L. monocytogenes* or HKLM and Tip-DC recruitment to spleen was examined 24 hr later by flow cytometry. Consistent with the robust stimulation of MCP-1 by ActA-deficient *L. monocytogenes* infection, recruitment of CD11b^{int}/Mac-3^{high} cells to spleens was intact. In a marked contrast, recruitment of these cells to spleens of mice inoculated with LLO-deficient bacteria or HKLM was dramatically reduced (Figure 4A). Furthermore, splenocytes harvested from mice inoculated with ActA-deficient, but not LLO-deficient bacteria or HKLM produced TNF following in vitro stimulation (Figure 4B). Intracellular cytokine staining demonstrated

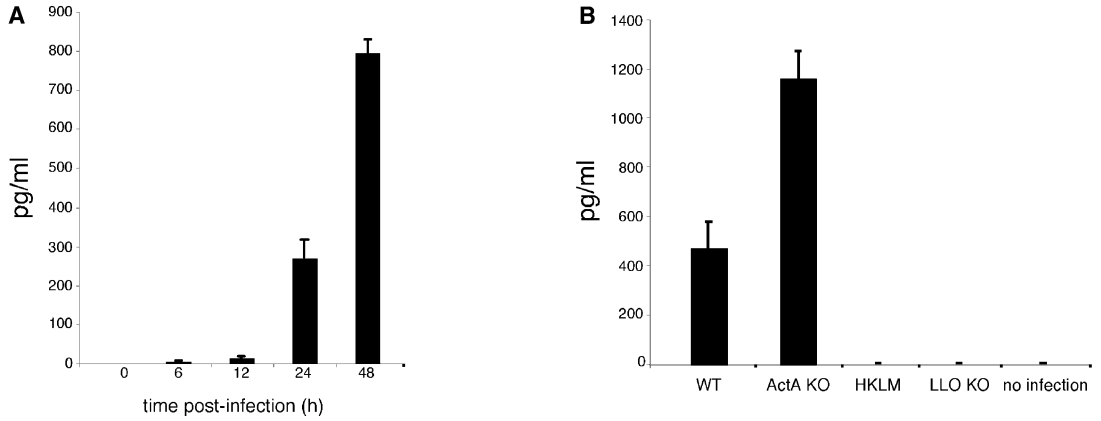

Figure 2. In Vivo MCP-1-Induction Requires Cytosol Invasion by Bacteria

(A) C57BL/6 mice were infected intravenously with 3,000 live *L. monocytogenes*, and spleens were removed at the indicated times and lysed. MCP-1 in lysates was quantified by sandwich ELISA. Each bar represents three mice and the experiment was repeated twice. Error bars show standard deviation.

(B) C57BL/6 mice were infected with 5000 wild-type, 5×10^5 ActA-deficient, 10^8 LLO-deficient *L. monocytogenes* or immunized with 10^9 HKLM. Spleens were removed 24 hr later and MCP-1 levels in spleen lysates were quantified by ELISA. Each bar represents three mice and the experiment was repeated three times. Error bars show standard deviations.

that the high level of TNF secretion in response was attributable to CD11b^{int} cell subset that was largely absent from the spleens of LLO-deficient *L. monocytogenes* and HKLM-inoculated mice (Figure 4C). Previously we demonstrated that Tip-DCs recruited to the spleen express iNOS and consequently produce large amounts of nitric oxide that likely contributes to microbial killing. Therefore, we examined iNOS expression in the spleens of mice inoculated with ActA- or LLO-deficient *L. monocytogenes* or HKLM. Although LLO-deficient, ActA-deficient, and heat-killed bacteria were readily detectable in the spleens 48 hr after inoculation, only ActA-deficient bacteria induced iNOS (Figure 4D). These findings further support the notion that invasion of host cell cytosol triggers a chain of events that results in the activation of effective innate immune defense against *L. monocytogenes* infection.

MCP-1 Is Secreted by Macrophages in the Absence of MyD88

MyD88 plays a central role in the activation of many innate immune responses including induction of the inflammatory cytokine cascades. The role of MyD88-mediated signaling in the induction of chemokines is less well defined. We thus investigated whether recruitment of Tip-DCs to *L. monocytogenes* infected spleens is a MyD88-dependent event. To this end, MCP-1, IL-12p40, and TNF production by wild-type, MyD88$^{-/-}$, and TLR2$^{-/-}$ bone marrow derived macrophages was examined. We found that MCP-1 secretion by bone marrow macrophages, while being completely dependent on cytosolic bacterial localization, was only partially dependent on MyD88-signaling (Figure 5A). In marked contrast, IL-12p40 secretion by macrophages, while independent of cytosol invasion, is completely abrogated in the absence of MyD88 (Figure 5B). Interestingly, while cytosol-invasive bacteria could trigger TNF production in the absence of MyD88, induction of TNF by noninvasive bacteria or HKLM is completely MyD88 dependent (Figure 5C). TLR-2 deficiency did not have a detectable effect on either chemokine or cytokine secretion by infected macrophages (Figures 5A, 5B, and 5C).

MCP-1 production can be induced by TNF by mechanisms that are increasingly understood at a molecular level (Boekhoudt et al., 2003; Kumar and Boss, 2000; Ping et al., 1999). To test whether MyD88-independent MCP-1 production induced by wild-type and ActA-deficient *L. monocytogenes* results from TNF secretion by macrophages, we examined secretion of MCP-1 by TNF$^{-/-}$ bone marrow derived macrophages. We found that TNF deficiency had no impact on MCP-1 production by macrophages (Figure 5D). Our results indicate that MCP-1 secretion by bone marrow derived macrophages is TNF- and MyD88-independent but can be augmented by MyD88-mediated signals.

MyD88-Independent Recruitment of Tip-DCs Is followed by MyD88-Dependent Activation

To determine whether our results with bone marrow derived macrophages reflected in vivo processes, we next examined regulation of MCP-1 production and Tip-DC activation in *L. monocytogenes* infected MyD88$^{-/-}$ mice. MCP-1 production in the spleens of MyD88$^{-/-}$ mice 24 hr following infection was comparable to that observed in the wild-type controls, indicating that in vivo MCP-1 production also does not depend on MyD88 mediated signaling (Figure 6A). Consistent with intact MCP-1 production, CD11b^{int}/Mac-3^{high} cells were present in the spleens of MyD88-deficient mice 1 and 2 days postinfection (Figure 6B; data not shown). In line with the results of our macrophage experiments, in vivo induction of MCP-1 was also TNF independent (Figure 6A). To rule out possible contributions of IL-1 and IL-18 in the induction of MCP-1, we infected mice deficient in IL-1β-converting enzyme (ICE) and examined MCP-1 secretion 24 hr later. MCP-1 levels in the spleens of ICE$^{-/-}$ mice were similar to those observed in the wild-type control mice, demonstrating complete indepen-

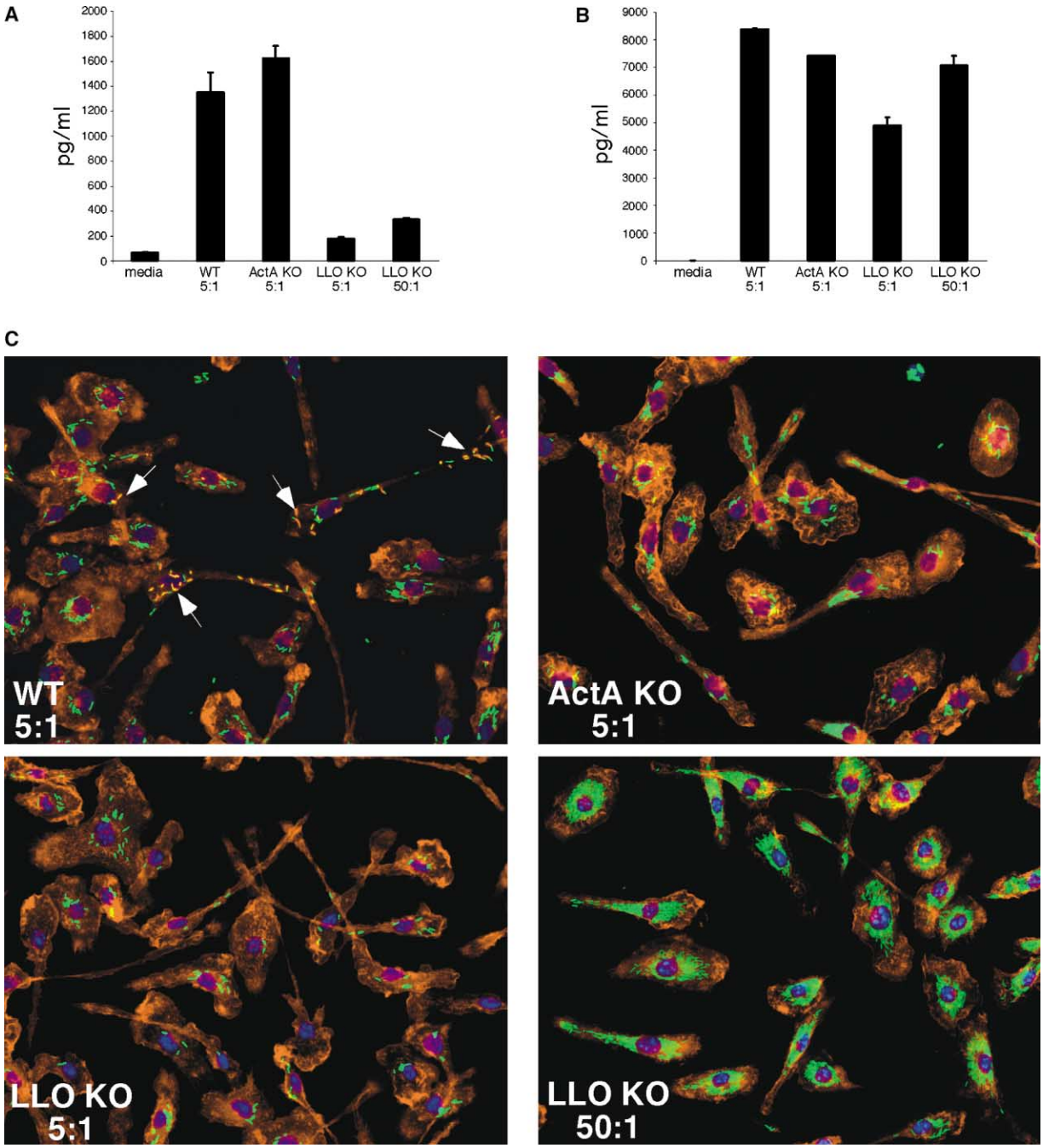

Figure 3. Distinct Regulation of MCP-1 and TNF Production by Macrophages

Macrophages were grown from bone marrow precursors of C57BL/6 mice and infected with wild-type or ActA-deficient *L. monocytogenes* strains at 5:1 bacteria:macrophage ratio, LLO-deficient *L. monocytogenes* at 5:1 or 50:1 ratio, or HKLM at 50:1 ratio. MCP-1 (A) and TNF (B) in culture supernatants were quantified 6 hr later by ELISA. Each bar represents the average of three wells and this experiment was repeated twice. Error bars show standard deviations. (C) Macrophages were stained for *L. monocytogenes* (green) and F-actin (red) 6 hr postinfection. Arrows indicated cytoplasmic bacteria. Experiment was repeated twice.

dence of MCP-1 production from signaling through IL-1 and IL-18 (Figure 6A).

Activated Tip-DCs produce TNF and iNOS and express high levels of MHC class II and costimulatory molecules (Serbina et al., 2003). We decided, therefore, to measure TNF production by MyD88$^{-/-}$ splenocytes by intracellular cytokine staining. Wild-type spleen cells obtained from mice 1 day following infection secrete TNF and can be induced to produce larger amounts of TNF by in vitro stimulation with HKLM. Splenocytes from CCR2$^{-/-}$ mice lack Tip-DCs and, therefore, do not produce significant amounts of TNF with or without HKLM stimulation. TNF secretion by MyD88-deficient splenocytes was markedly diminished, suggesting that TipDC

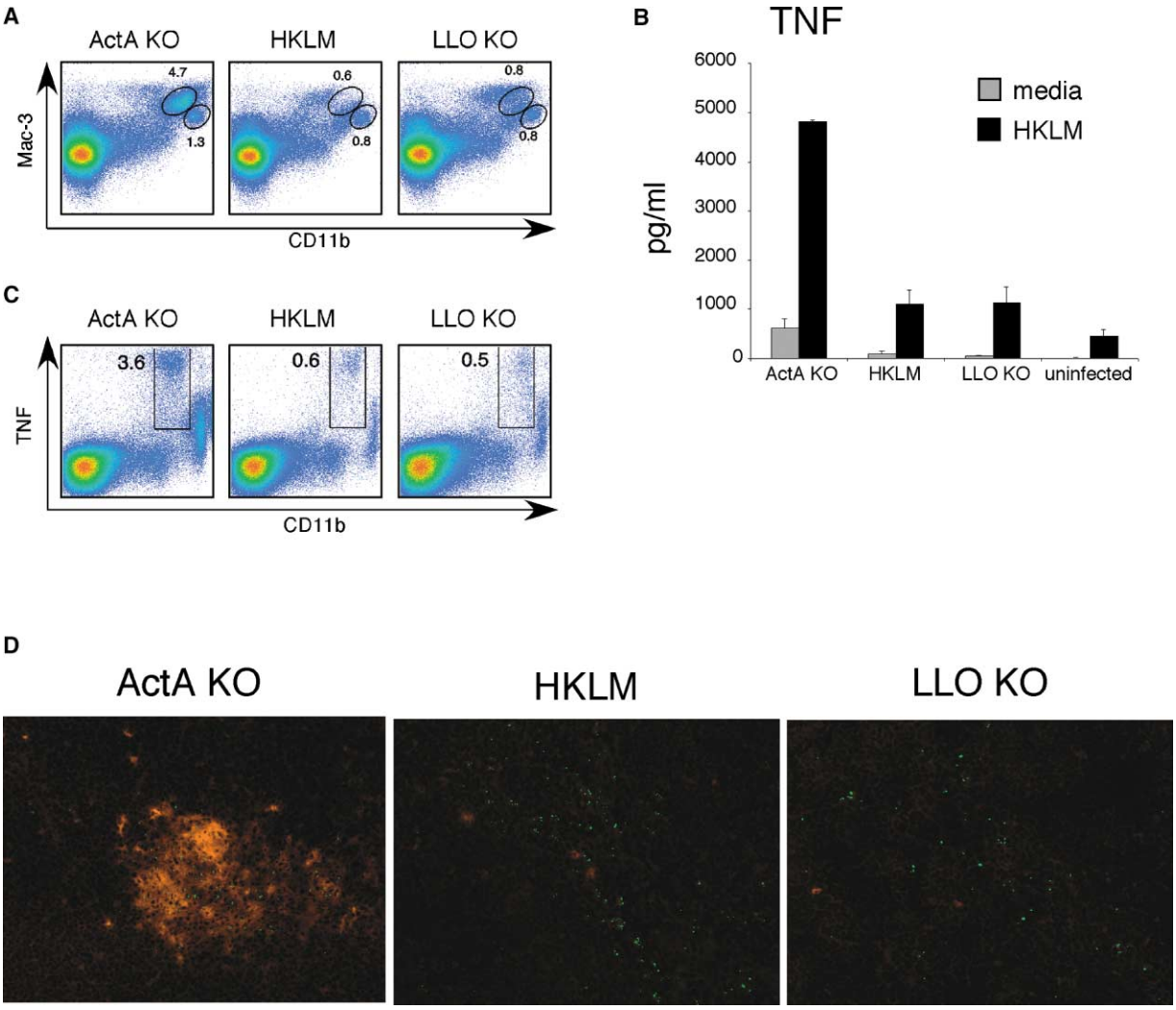

Figure 4. Cytosol Invasion by *L. monocytogenes* Is Required for Tip-DCs Recruitment to Spleen

C57BL/6 mice were inoculated with 5×10^5 ActA-deficient or 10^8 LLO-deficient *L. monocytogenes* or immunized with 10^9 HKLM.

(A) Splenocytes were stained for CD11b and Mac-3 as described in Figure 1B 1 day following inoculation. Representative dot plots for two mice per group are shown. This experiment was repeated four times.

(B) Splenocytes from 24 hr infected or uninfected mice were cultured with or without 10^7/ml HKLM for 24 hr and TNF in supernatants was assayed by ELISA. Each bar represents the average of three mice and the experiment was repeated three times.

(C) Splenocytes isolated from mice 24 hr following infection were cultured with 10^7/ml HKLM in the presence of BFA for 4 hr and intracellular TNF staining was performed. Cells were gated on lymphocytes/monocytes by size and expression of CD11b and TNF-α within the gate was analyzed. The percentage of $CD11b^{int}TNF^{+}$ cells is indicated to the left of each gate. Representative dot plots for three mice per group are shown and the experiment was repeated twice.

(D) Spleens were removed 48 hr postinfection and frozen 5 μm sections were prepared. Sections were stained for iNOS (red) and *L. monocytogenes* (green).

activation requires signaling through MyD88. Along similar lines, iNOS induction in infected, $MyD88^{-/-}$ spleens was also markedly diminished in $MyD88^{-/-}$ mice despite an abundance of bacteria (Figure 6D). MyD88 is required for signaling mediated by IL-1 and IL-18 (Adachi et al., 1998) and, thus, could contribute to Tip-DC activation in a TLR-independent fashion. To distinguish between these possibilities, we examined TNF and iNOS production by Tip-DCs in $ICE^{-/-}$ mice. Recruitment of $CD11b^{int}$/$Mac\text{-}3^{high}$ cells occurred normally in the absence of IL-1 and IL-18 (data not shown). Tip-DCs from $ICE^{-/-}$ mice responded robustly to HKLM stimulation by secreting TNF (Figure 6E) and expressed normal levels of iNOS (Figure 6F). These results indicate that MyD88-dependent activation of Tip-DCs does not involve signaling through IL-1 and IL-18.

Discussion

In this report we demonstrate that cytosol invasive *L. monocytogenes* induce MCP-1 production in vivo that is essential for Tip-DC recruitment to the spleen and the production of both TNF and iNOS. While cytokine production is MyD88 dependent and presumably mediated by one or more TLR proteins, induction of MCP-1 is MyD88 independent. The dependence of MCP-1 in-

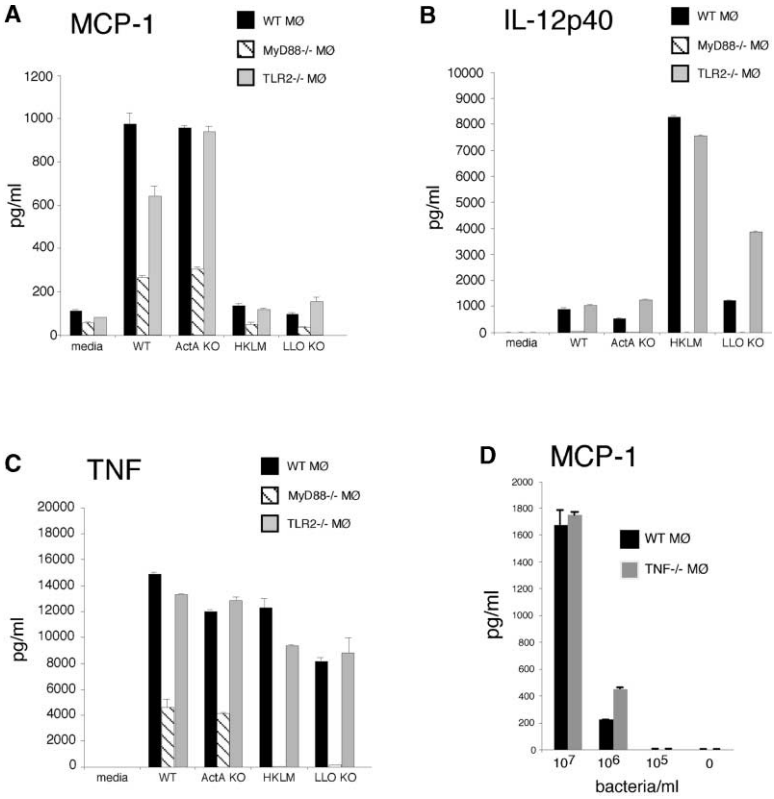

Figure 5. MyD88-Independent MCP-1 Secretion by Macrophages

Bone marrow-derived macrophages from C57BL/6, MyD88$^{-/-}$, and TLR2$^{-/-}$ mice were infected with wild-type, ActA-deficient, or LLO-deficient *L. monocytogenes* strains at 5:1 ratio. MCP-1 (A), IL-12 (B), and TNF (C) in culture supernatants were quantified 18 hr later by ELISA. Each bar represents the average of three wells and this experiment was repeated twice. Error bars show standard deviations. (C) Bone marrow-derived macrophages from C57BL/6 and TNF$^{-/-}$ mice were infected with wild-type *L. monocytogenes* and MCP-1 levels in culture supernatants were quantified 18 hr later. Each bar represents the average of three wells and this experiment was repeated twice. Error bars show standard deviations.

duction on cytosolic localization of *L. monocytogenes*, and the lack of MCP-1 induction by killed or noninvasive bacteria, strongly suggests that, in this setting, a cytosolic innate immune sensing mechanism is at play.

Microbial invasion of host cell cytosol is a calamitous event and, as might be expected, induces a commensurate cellular response. While macrophages transiently activate NF-κB upon exposure to extracellular bacteria, cytosolic invasion by *L. monocytogenes* induces much more prolonged NF-κB activation (Hauf et al., 1997). While both extracellular and cytosolic bacteria induce IL-1, IL-6, and TNF secretion by bone marrow-derived macrophages, regulation of cytokine receptor expression was only detected with cytosol invasive *L. monocytogenes* (Demuth et al., 1996). Interestingly, in HUVECs and macrophage cell lines, gene activation by cytosol-invasive *L. monocytogenes* required secretion of bacterial phospholipases (Hauf et al., 1997; Sibelius et al., 1996). A recent study confirmed that cytosol invasive *L. monocytogenes* potently induce NF-κB activation and extended this finding by showing that IFN-β is dramatically upregulated in bone marrow-derived macrophages when bacteria access the cytosol. IFN-β was similarly induced by cytosolic gram-positive and gram-negative bacteria, and was not induced by purified LPS or LTA and required p38 MAP kinase but not ERK-1 or ERK-2 activation (O'Riordan et al., 2002). Recent studies using Shigella, a gram-negative cytosol-invasive bacterium, demonstrated that Nod-1 plays an essential role in innate immune recognition by recognizing muramyl tripeptide. Although neither Nod-1 nor Nod-2 appear to play a role in cytosolic recognition of *L. monocytogenes* by epithelial cells (Girardin et al., 2003), we postulate that a similar mechanism is responsible for the recognition of gram-positive bacteria in macrophage cytosol.

MCP-1 induction has been extensively investigated. Interestingly, many studies have focused on TNF- mediated MCP-1 induction and have demonstrated that NF-κB (Ping et al., 1999; Ueda et al., 1997), STAT-1 (Zhou et al., 1998), Sp1 (Ping et al., 2000), and other yet to be defined transcription factors (Kumar and Boss, 2000) bind to two regulatory regions in the MCP-1 promoter. Recent studies indicate that TNF induces chromatin remodeling that enables transcription factors to access the MCP-1 promoter (Boekhoudt et al., 2003). While TNF undoubtedly is an important inducer of MCP-1, induction by *L. monocytoges* appears to be TNF independent (see Figure 5). Thus, our studies suggest that innate immune recognition of cytosolic bacteria activates a signaling pathway for MCP-1 induction that is TNF independent. In contrast, infection with extracellular pathogens and persistent intracellular pathogens with exclusive phagosomal localization might require secretion of exogenous cytokines such as TNF for MCP-1 induction. Since MCP-1 can be secreted by a variety of cell types, such TNF-facilitated MCP-1 secretion might not require the chemokine-secreting cell to be directly infected.

MCP-1 induction has been demonstrated to be TLR-2 and TLR-4 dependent in renal tubular epithelial cells (Tsuboi et al., 2002). In this cell type, LPS and bacterial lipoproteins but not CpG DNA induce MCP-1 transcription. Sensitivity to these microbial patterns correlates with the expression of the cognate TLR receptor. Using a variety of selective signaling inhibitors, inhibition of JNK, ERK, or p38 MAPK did not impair the induction of MCP-1 in renal tubular epithelial cells. In contrast to

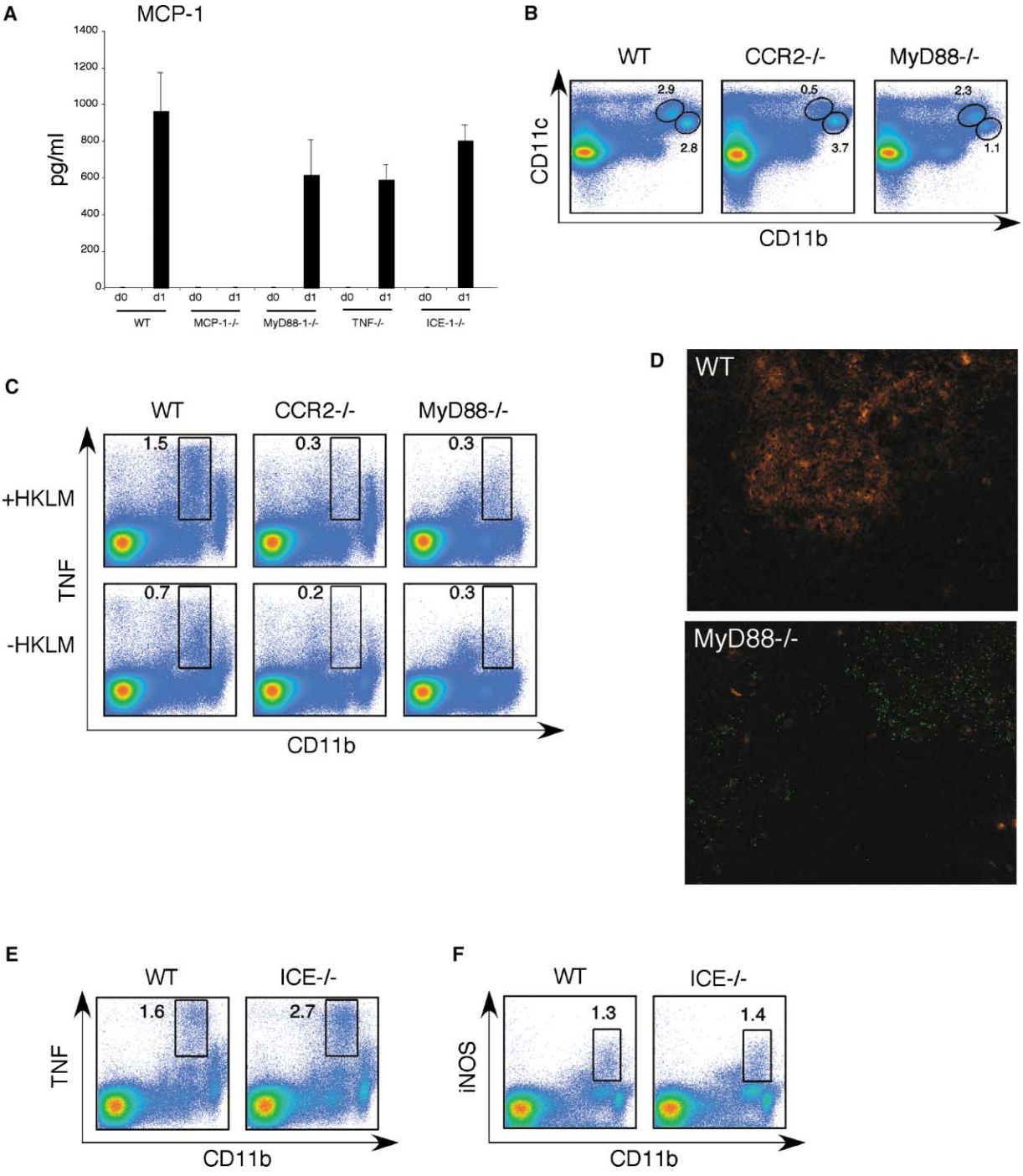

Figure 6. MCP-1 Induction during In Vivo Infection Is MyD88 Independent

(A) C57BL/6, MyD88$^{-/-}$, MCP-1$^{-/-}$, TNF$^{-/-}$, and ICE$^{-/-}$ mice were infected with 3,000 wild-type *L. monocytogenes*, spleens were removed 1 day later, and MCP-1 levels were quantified. Each bar represents the average of two to three mice and the experiment was repeated twice. Error bars show standard deviations.

(B) C57BL/6, MyD88$^{-/-}$, and CCR2$^{-/-}$ mice were infected with 3000 wild-type *L. monocytogenes* and splenocytes were stained 1 day later for CD11b and Mac-3. Representative dot plots for three mice per group are shown. This experiment was repeated twice.

(C) Splenocytes were obtained from C57BL/6, MyD88$^{-/-}$, and CCR2$^{-/-}$ mice 1 day after infection and were cultured with or without 10^7/ml HKLM for 4 hr in the presence of BFA followed by intracellular TNF staining. Representative dot plots for three mice per group are shown. This experiment was repeated twice.

(D) C57BL/6 and MyD88$^{-/-}$ mice were infected with 3,000 wild-type *L. monocytogenes*, spleens were removed 48 hr later, and frozen 5 μm sections were prepared. Sections were stained for iNOS (red) and *L. monocytogenes* (green).

(E and F) BALB/c and ICE$^{-/-}$ mice were infected with 2,000 wild-type *L. monocytogenes* and spleens were removed 48 hr later. (E) TNF secretion by splenocytes was assessed as in (C). Representative dot plots for four mice per group are shown. (F) Intracellular iNOS expression by wild-type and ICE$^{-/-}$ splenocytes. The percentage of CD11b$^+$NOS2$^+$ cells is indicated on each plot. Representative dot plots for four mice per group are shown.

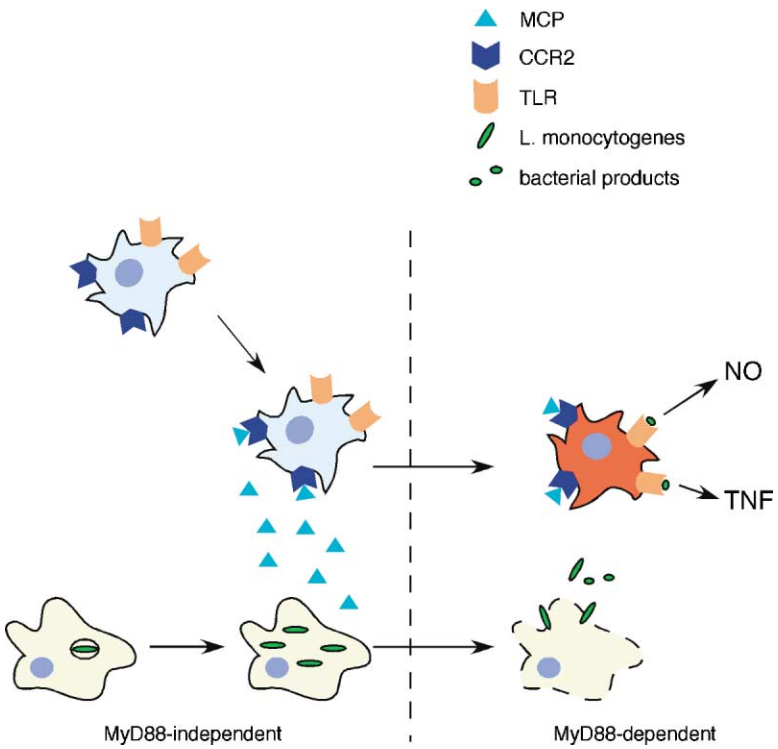

Figure 7. Two-Step Assembly of Innate Immune Defenses: MyD88-Independent Recruitment Is followed by MyD88-Dependent Activation

Step 1: presence of *L. monocytogenes* in the cytoplasm of infected macrophages induces secretion of MCP-1 in a MyD88-independent manner. MCP-1 recruits CCR2-bearing monocytes to the site of infection. Step 2: Recognition of bacterial products through TLRs induces monocyte activation and differentiation into TipDCs. MyD88-dependent secretion of TNF and iNOS by activated TipDCs ultimately leads to bacterial killing.

epithelial cells, induction of MCP-1 in RAW264.7 macrophage-like cells did not correlate with the expression of TLRs (Tsuboi et al., 2002). RAW264.7 cells express TLR-2 and TLR-4, but did not upregulate MCP-1 in response to either LPS or bacterial lipoprotein. In this cell type, however, CpG DNA potently induced MCP-1 message. The results of our studies, in combination with those of Tsuboi and colleagues, indicate that the detection of microbial products and the ensuing response differs substantially between cell types.

Toll-like receptors are positioned as guardians of innate immunity, rapidly detecting pathogen-associated motifs and inducing and amplifying inflammatory cascades. The effector mechanisms that are induced, while potentially effective at eliminating microbes, can also cause extensive tissue damage. Therefore, precise control of innate immune responses is imperative. It is also important to limit inflammatory reactions to innocuous, commensal organisms that express many of the molecular patterns recognized by TLRs. Since TLRs are constitutively expressed by cells of innate immunity prior to their interactions with microbes, limits on their activation can be imposed at the step of their recruitment. As shown in Figure 7, in the case of *L. monocytogenes* infection, our studies indicate that a MyD88-independent pathway results in MCP-1 induction and monocyte recruitment to the site of bacterial infection. Access to the cytosol appears to play a major role in this process, suggesting that a cytosolic detection mechanism, similar to the one postulated by Kuhn, Portnoy, and their colleagues, activates a signaling pathway that induces MCP-1 production. Thus, induction of TNF and iNOS by virulent *L. monocytogenes* infection can be seen as a two-step process. First, monocytes are recruited to sites of infection by MyD88-independent secretion of MCP-1 signals. Second, monocytes are activated and differentiate into Tip-DCs upon exposure to microbial products in a MyD88-dependent process.

Experimental Procedures

Mice and Infections

All mice used in this study were bred at Memorial Sloan-Kettering Research Animal Resource Center. Generation of CCR2$^{-/-}$, MCP-1$^{-/-}$, ICE$^{-/-}$, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice was previously described (Adachi et al., 1998; Kuida et al., 1995; Kuziel et al., 1997; Lauvau et al., 2001; Lu et al., 1998; Takeuchi et al., 1999). For most studies, CCR2$^{-/-}$ mice were backcrossed at least 8 generations onto the C57BL/6 background, MCP$^{-/-}$ mice were backcrossed at least 10 generations onto the C57BL/6 background, and MyD88$^{-/-}$ and TLR2$^{-/-}$ mice were backcrossed 3 generations onto the C57BL/6 background. Mice were infected intravenously with 2000-5000 *L. monocytogenes* strain 10403S, 2×10^5 attenuated ActA-deficient *L. monocytogenes* strain DP-L1942, 10^8 attenuated LLO-deficient *L. monocytogenes* strain DP-L2161 (provided by Dr. Daniel Portnoy), and 10^9 HKLM prepared as described previously (Lauvau et al., 2001). Bacteria CFUs were determined by plating on brain-heart infusion agar plates.

Flow Cytometry

Spleens were harvested 24 or 48 hr postinfection, dissociated, and digested with 0.3% Collagenase type 4 (Worthington, NJ). Erythrocytes were lysed with NH_4Cl-Tris solution and cells were stained for cell surface and intracellular markers. 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), and anti-TNF-FITC (MP6-XT22). 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). For FACS analysis, large gate was drawn to include lymphocyte/monocyte populations. Intracellular staining for Mac-3 and iNOS was performed by staining cells for cell surface markers, fixing in 2% paraformaldehyde, permeabilizing with Perm/Wash Buffer (BD Pharmingen), and incubating with anti-Mac-3-FITC or goat anti-iNOS antibody followed by FITC-anti-goat

IgG (Jackson Immunoresearch Laboratories). For intracellular TNF staining, splenocytes were stimulated in vitro with 10^8/ml HKLM in the presence of brefeldin A (BFA) or left unstimulated for 4 hr and processed according to manufacturer's protocol (Cytofix/Cytoperm, Pharmingen).

ELISAs

Murine MCP-1 was quantified using an ELISA kit from BioSource International (Camarillo, CA), and murine IL-12p40 and TNF-α were quantified using OptEIA kit from Pharmingen. To obtain lysates for cytokine assays, spleens were harvested at indicated times postinfection, mascerated in ice-cold PBS containing 0.01% Triton X-100, and centrifuged at 10,000 $\times$ g.

Cell Cultures and Immunofluorescent Staining

Macrophages were grown from bone marrow precursors in antibiotic-free DMEM medium supplemented with 33% supernatant from L-cell fibroblasts and 20% fetal bovine serum. On day 5, cells were harvested in ice-cold PBS, plated at 2–4 $\times$ 10^5/well in 96-well flat bottom plates, and various bacterial strains or HKLM were added to the cells. For immunofluorescent staining, cells were plated at 4 $\times$ 10^5/well in 8-well chamber slides (Nalge Nunc International Corp.). Bacteria were grown to log phase (A_{600} of $\sim$0.1), spun down at 10,000 rpm for 10 min, and resuspended in PBS prior to addition to cells. Unless otherwise indicated, all bacteria were added to the cells at 5:1 bacteria:cell ratio, and HKLM was added at 50:1 ratio. Total volume in the wells during infection was 100 μl for 96-well plates and 150–200 μl for 8-well chamber slides. Infection was allowed to occur for 30 min, extracellular bacteria were removed, and gentamicin-containing media was added to each well to prevent extracellular bacterial growth. Supernatants were collected 6 and 18 hr postinfection to assay for cytokines. For immunofluorescent staining, cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and stained with Texas Red-X phalloidin (Molecular Probes) and rabbit Difco Listeria O polyserum (Fisher) followed by donkey anti-rabbit IgG-FITC (Jackson Immunoresearch Laboratories). To determine TNF secretion by splenocytes, collagenase-digested spleens from mice infected for 24 hr were cultured in vitro for 24 hr with or without 10^8/ml HKLM and TNF-α in supernatants was quantified.

Histology

Spleens were harvested from mice infected for 48 hr with indicated bacterial strains and frozen 5μm sections were prepared. Acetone-fixed sections were stained using rabbit Difco Listeria O polyserum diluted 1/4000 (Fisher) and goat anti-iNOS antibody (Santa Cruz) followed by donkey anti-rabbit IgG-FITC and donkey anti-goat-IgG-biotin (Jackson Immunoresearch Laboratories). iNOS Ab reactivity was visualized using 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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References

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998). Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity *9*, 143–150.

Barsig, J., Flesch, I.E., and Kaufmann, S.H. (1998). Macrophages and hepatocytic cells as chemokine producers in murine listeriosis. Immunobiology *199*, 87–104.

Barton, G.M., and Medzhitov, R. (2003). Toll-like receptor signaling pathways. Science *300*, 1524–1525.

Bhardwaj, V., Kanagawa, O., Swanson, P.E., and Unanue, E.R. (1998). Chronic Listeria infection in SCID mice: requirements for the carrier state and the dual role of T cells in transferring protection or suppression. J. Immunol. *160*, 376–384.

Bielecki, J., Youngman, P., Connelly, P., and Portnoy, D.A. (1990). Bacillus subtilis expressing a haemolysin gene from Listeria monocytogenes can grow in mammalian cells. Nature *345*, 175–176.

Boekhoudt, G.H., Guo, Z., Beresford, G.W., and Boss, J.M. (2003). Communication between NF-kappaB and sp1 controls histone acetylation within the proximal promoter of the monocyte chemoattractant protein 1 gene. J. Immunol. *170*, 4139–4147.

Bouwer, H.G., Gibbins, B.L., Jones, S., and Hinrichs, D.J. (1994). Antilisterial immunity includes specificity to listeriolysin O (LLO) and non-LLO-derived determinants. Infect. Immun. *62*, 1039–1045.

Buchmeier, N.A., and Schreiber, R.D. (1985). Requirement of endogenous interferon-gamma production for resolution of Listeria monocytogenes infection. Proc. Natl. Acad. Sci. USA *82*, 7404–7408.

Busch, D.H., and Pamer, E.G. (1999). T lymphocyte dynamics during Listeria monocytogenes infection. Immunol. Lett. *65*, 93–98.

Cossart, P., and Bierne, H. (2001). The use of host cell machinery in the pathogenesis of Listeria monocytogenes. Curr. Opin. Immunol. *13*, 96–103.

Demuth, A., Goebel, W., Beuscher, H.U., and Kuhn, M. (1996). Differential regulation of cytokine and cytokine receptor mRNA expression upon infection of bone marrow-derived macrophages with Listeria monocytogenes. Infect. Immun. *64*, 3475–3483.

Edelson, B.T., and Unanue, E.R. (2002). MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity. J. Immunol. *169*, 3869–3875.

Flesch, I.E., Barsig, J., and Kaufmann, S.H. (1998). Differential chemokine response of murine macrophages stimulated with cytokines and infected with Listeria monocytogenes. Int. Immunol. *10*, 757–765.

Gellin, B.G., and Broome, C.V. (1989). Listeriosis. JAMA *261*, 1313–1320.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zathringer, U., et al. (2003). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science *300*, 1584–1587.

Harty, J.T., and Bevan, M.J. (1995). Specific immunity to Listeria monocytogenes in the absence of IFN gamma. Immunity *3*, 109–117.

Hauf, N., Goebel, W., Fiedler, F., Sokolovic, Z., and Kuhn, M. (1997). Listeria monocytogenes infection of P388D1 macrophages results in a biphasic NF-kappaB (RelA/p50) activation induced by lipoteichoic acid and bacterial phospholipases and mediated by IkappaBalpha and IkappaBbeta degradation. Proc. Natl. Acad. Sci. USA *94*, 9394–9399.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M., and Aguet, M. (1993). Immune response in mice that lack the interferon-gamma receptor. Science *259*, 1742–1745.

Huang, D.R., Wang, J., Kivisakk, P., Rollins, B.J., and Ransohoff, R.M. (2001). Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. J. Exp. Med. *193*, 713–726.

Inohara, N., and Nunez, G. (2003). NODs: intracellular proteins involved in inflammation and apoptosis. Nat. Rev. Immunol. *3*, 371–382.

Kuida, K., Lippke, J.A., Ku, G., Harding, M.W., Livingston, D.J., Su, M.S., and Flavell, R.A. (1995). Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science *267*, 2000–2003.

Kumar, S.N., and Boss, J.M. (2000). Site A of the MCP-1 distal regulatory region functions as a transcriptional modulator through the transcription factor NF1. Mol. Immunol. *37*, 623–632.

Kurihara, T., Warr, G., Loy, J., and Bravo, R. (1997). Defects in macro-

phage recruitment and host defense in mice lacking the CCR2 chemokine receptor. J. Exp. Med. *186*, 1757–1762.

Kuziel, W.A., Morgan, S.J., Dawson, T.C., Griffin, S., Smithies, O., Ley, K., and Maeda, N. (1997). Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. Proc. Natl. Acad. Sci. USA *94*, 12053–12058.

Lauvau, G., Vijh, S., Kong, P., Horng, T., Kerksiek, K., Serbina, N., Tuma, R.A., and Pamer, E.G. (2001). Priming of memory but not effector CD8 T cells by a killed bacterial vaccine. Science *294*, 1735–1739.

Lu, B., Rutledge, B.J., Gu, L., Fiorillo, J., Lukacs, N.W., Kunkel, S.L., North, R., Gerard, C., and Rollins, B.J. (1998). Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. J. Exp. Med. *187*, 601–608.

Luther, S.A., and Cyster, J.G. (2001). Chemokines as regulators of T cell differentiation. Nat. Immunol. *2*, 102–107.

O'Riordan, M., Yi, C.H., Gonzales, R., Lee, K.D., and Portnoy, D.A. (2002). Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. Proc. Natl. Acad. Sci. USA *99*, 13861–13866.

Pasparakis, M., Alexopoulou, L., Episkopou, V., and Kollias, G. (1996). Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J. Exp. Med. *184*, 1397–1411.

Pfeffer, K., Matsuyama, T., Kundig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Kronke, M., and Mak, T.W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457–467.

Ping, D., Boekhoudt, G.H., Rogers, E.M., and Boss, J.M. (1999). Nuclear factor-kappa B p65 mediates the assembly and activation of the TNF-responsive element of the murine monocyte chemoattractant-1 gene. J. Immunol. *162*, 727–734.

Ping, D., Boekhoudt, G., Zhang, F., Morris, A., Philipsen, S., Warren, S.T., and Boss, J.M. (2000). Sp1 binding is critical for promoter assembly and activation of the MCP-1 gene by tumor necrosis factor. J. Biol. Chem. *275*, 1708–1714.

Rollins, B.J. (1997). Chemokines. Blood *90*, 909–928.

Rosen, H., Gordon, S., and North, R.J. (1989). Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. Absence of monocytes at infective foci allows Listeria to multiply in nonphagocytic cells. J. Exp. Med. *170*, 27–37.

Rothe, J., Lesslauer, W., Lotscher, H., Lang, Y., Koebel, P., Kontgen, F., Althage, A., Zinkernagel, R., Steinmetz, M., and Bluethmann, H. (1993). Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature *364*, 798–802.

Rutledge, B.J., Rayburn, H., Rosenberg, R., North, R.J., Gladue, R.P., Corless, C.L., and Rollins, B.J. (1995). High level monocyte chemoattractant protein-1 expression in transgenic mice increases their susceptibility to intracellular pathogens. J. Immunol. *155*, 4838–4843.

Seki, E., Tsutsui, H., Tsuji, N.M., Hayashi, N., Adachi, K., Nakano, H., Futatsugi-Yumikura, S., Takeuchi, O., Hoshino, K., Akira, S., et al. (2002). Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice. J. Immunol. *169*, 3863–3868.

Serbina, N., Salazar-Mather, T.P., Biron, C., Kuziel, W.A., and Pamer, E.G. (2003). TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity *19*, 59–70.

Sibelius, U., Chakraborty, T., Krogel, B., Wolf, J., Rose, F., Schmidt, R., Wehland, J., Seeger, W., and Grimminger, F. (1996). The listerial exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C synergize to elicit endothelial cell phosphoinositide metabolism. J. Immunol. *157*, 4055–4060.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. Annu. Rev. Immunol. *21*, 335–376.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443–451.

Tesch, G.H., Maifert, S., Schwarting, A., Rollins, B.J., and Kelley, V.R. (1999). Monocyte chemoattractant protein 1-dependent leukocytic infiltrates are responsible for autoimmune disease in MRL-Fas(lpr) mice. J. Exp. Med. *190*, 1813–1824.

Tsuboi, N., Yoshikai, Y., Matsuo, S., Kikuchi, T., Iwami, K., Nagai, Y., Takeuchi, O., Akira, S., and Matsuguchi, T. (2002). Roles of toll-like receptors in C–C chemokine production by renal tubular epithelial cells. J. Immunol. *169*, 2026–2033.

Ueda, A., Ishigatsubo, Y., Okubo, T., and Yoshimura, T. (1997). Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity. J. Biol. Chem. *272*, 31092–31099.

Ueda, A., Okuda, K., Ohno, S., Shirai, A., Igarashi, T., Matsunaga, K., Fukushima, J., Kawamoto, S., Ishigatsubo, Y., and Okubo, T. (1994). NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. J. Immunol. *153*, 2052–2063.

Yoshimura, T., Robinson, E.A., Tanaka, S., Appella, E., Kuratsu, J., and Leonard, E.J. (1989). Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. J. Exp. Med. *169*, 1449–1459.

Zhou, Z.H., Chaturvedi, P., Han, Y.L., Aras, S., Li, Y.S., Kolattukudy, P.E., Ping, D., Boss, J.M., and Ransohoff, R.M. (1998). IFN-gamma induction of the human monocyte chemoattractant protein (hMCP)-1 gene in astrocytoma cells: functional interaction between an IFN-gamma-activated site and a GC-rich element. J. Immunol. *160*, 3908–3916.