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ORIGINAL ARTICLE

A MyD88-dependent IFNγR-CCR2 signaling circuit is required for mobilization of monocytes and host defense against systemic bacterial challenge

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Monocytes are mobilized to sites of infection via interaction between the chemokine MCP-1 and its receptor, CCR2, at which point they differentiate into macrophages that mediate potent antimicrobial effects. In this study, we investigated the mechanisms by which monocytes are mobilized in response to systemic challenge with the intracellular bacterium *Francisella tularensis*. We found that mice deficient in MyD88, interferon-γ (IFNγ)R or CCR2 all had defects in the expansion of splenic monocyte populations upon *F. tularensis* challenge, and in control of *F. tularensis* infection. Interestingly, MyD88-deficient mice were defective in production of IFNγ, and IFNγR-deficient mice exhibited defective production of MCP-1, the ligand for CCR2. Transplantation of IFNγR-deficient bone marrow (BM) into wild-type mice further suggested that mobilization of monocytes in response to *F. tularensis* challenge required IFNγR expression on BM-derived cells. These studies define a critical host defense circuit wherein MyD88-dependent IFNγ production signals via IFNγR expressed on BM-derived cells, resulting in MCP-1 production and activation of CCR2-dependent mobilization of monocytes in the innate immune response to systemic *F. tularensis* challenge.

***Keywords*:** bacteria; interferon; chemokine; monocyte; infection

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Introduction

The mammalian organism is confronted at all times with a vast microbial menagerie consisting of a range of bacterial species, some of which are pathogenic and must be eliminated from the host. The mammalian immune system has developed a specialized set of immune mechanisms to detect and combat these pathogenic microorganisms [1]. The initial immune response is mediated by innate immunity, which is characterized by the rapid induction of an inflammatory response designed to control infection and activate the subsequent adaptive response. The innate immune response is induced via the recognition of pathogen-associated molecular patterns by an array of pattern recognition receptors such as the Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [2, 3]. These in turn lead to the activation of an array of cytokines, chemokines, adhesion molecules and antimicrobial peptides that result in proinflammatory immune responses that promote elimination of the pathogen [2, 4].

Upon pathogen recognition, a defining feature of the innate immune response is the mobilization and trafficking of myeloid cells, including neutrophils and monocytes, to infected tissues, where they phagocytose and kill pathogenic microbes [5]. In particular, monocytes have been shown to play a critical role in the control and elimination of intracellular bacteria such as *Listeria monocytogenes*, which has been extensively used as a model pathogen for studying the innate immune response

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to intracellular bacterial infection [6]. Mice deficient in CCR2, the receptor for the monocyte chemokines MCP-1 and MCP-3, fail to mobilize monocyte populations in response to infection by intracellular bacteria and have impaired bacterial clearance [7-9]. In response to *L. monocytogenes* infection, Ly6C+ monocytes accumulate in the spleen, where they mature into TNF-α- and iNOS-producing inflammatory monocytes [10]. These cells in turn act as antimicrobial effector cells and can promote the generation of adaptive T-cell responses [10].

Activation of effective monocyte responses to intracellular bacteria such as *F. tularensis* is enhanced via production of interferon-γ (IFNγ) by activated NK and NKT cells early on, and subsequently by antigen-specific activation of CD4+ Th1 cell subsets [11-13]. IFNγ has been shown to induce antimicrobial genes such as iNOS and LRG47, as well as to promote nutrient sequestration and the expression of antigen presentation molecules in multiple cell types [14, 15]. Although the role of IFNγ in the activation of antimicrobial genes in monocytes is well characterized, a potential role of IFNγ in regulating other innate immune system functions, particularly the mobilization of myeloid cells, is not well defined.

In the present study, the mechanism by which monocytes are mobilized to sites of infection, in particular the spleen, after systemic *Francisella tularensis* challenge was investigated. *F. tularensis* is a gram-negative, intracellular bacterium that is the causative agent of tularemia, a potentially fatal zoonosis in humans if inoculation occurs via the pulmonary route [16]. Recognition of *F. tularensis* by the innate immune system is largely mediated by TLR2 and MyD88 [16, 17] and requires myeloid cells, including monocytes, as depletion of myeloid cells in mice using an anti-Gr-1 antibody (which recognizes the monocyte surface marker Ly6C as well as the granulocyte marker Ly6G) results in uncontrolled infection [18]. Further, resolution of *F. tularensis* infection requires IFNγ signaling and the activation of antimicrobial genes including TNF-α and iNOS [19-22].

Although prior work has suggested that IFNγ production plays a role in the formation of hepatic granulomas in mice upon *F. tularensis* challenge, the mechanism by which IFNγ mediates myeloid cell trafficking, and the identity and the functional importance of the mobilized cells have not been established [23]. In the present study, we demonstrate that IFNγR signaling in bone marrow (BM)-derived cells is required for the production of the monocyte chemokine MCP-1 and mobilization of monocyte populations in the spleen following bacterial challenge. Furthermore, we show the production of IFNγ and resultant MCP-1 in response to systemic *F. tularensis* challenge occurs via an MyD88-dependent mechanism. Lastly, effective control of *F. tularensis* infection requires TNF-α and NO production by the IFNγR/CCR2-mobilized monocytes. Taken together, this research identifies a new MyD88-dependent signaling mechanism proceeding via IFNγR/CCR2 that governs the mobilization and activation of monocytes in response to challenge with a systemic intracellular bacterium.

Results

IFNγR expression is required for MCP-1 production and expansion of monocyte populations in the spleen after systemic F. tularensis challenge

IFNγR signaling is a crucial mediator of immune responses to intracellular bacterial pathogens, including *F. tularensis* [19, 24]. Thus, we asked whether IFNγR signaling participates not only in the activation of antimicrobial mechanisms but also in the mobilization of monocytes during intracellular bacterial challenge using an established systemic *F. tularensis* infection model in mice [19, 25]. Wild-type (WT) and IFNγR-deficient mice were inoculated intravenously with 2×10^4 colony-forming units (CFUs) of *F. tularensis*, and, consistent with prior published results [19, 25], IFNγR-deficient mice but not WT mice were highly susceptible to this *F. tularensis* challenge (Supplementary information, Figure S1). These data indicate that IFNγR signaling is critical for host defense against *F. tularensis*. To assess how IFNγR signaling affects monocyte populations in the spleen after infection, we harvested spleens from infected WT and IFNγR-deficient mice and from uninfected control mice 24 h after *F. tularensis* challenge (Figure 1A-1C). We observed a significant expansion of CD11b+ Ly6C^{hi} monocytes in the spleens of WT mice 24 h after infection. In contrast, there was no increase in splenic monocytes in IFNγR-deficient mice relative to uninfected controls. On the other hand, IFNαβR-deficient mice did not exhibit susceptibility to infection or defective expansion of monocyte populations after inoculation with *F. tularensis,* indicating that type I interferons are not required for host defense against *F. tularensis* (Supplementary information, Figure S1 and data not shown). These findings suggest that IFNγR signaling is crucial for the expansion of splenic monocyte populations after systemic *F. tularensis* challenge.

Previous work has described a critical role for the monocyte chemokine MCP-1 in mobilizing monocytes to the periphery in response to systemic bacterial challenge. As there was a defective expansion of monocyte populations in the spleens of IFNγR-deficient mice in response to systemic *F. tularensis* challenge (Figure 1A-1C), we hypothesized that this may be related to defective MCP-

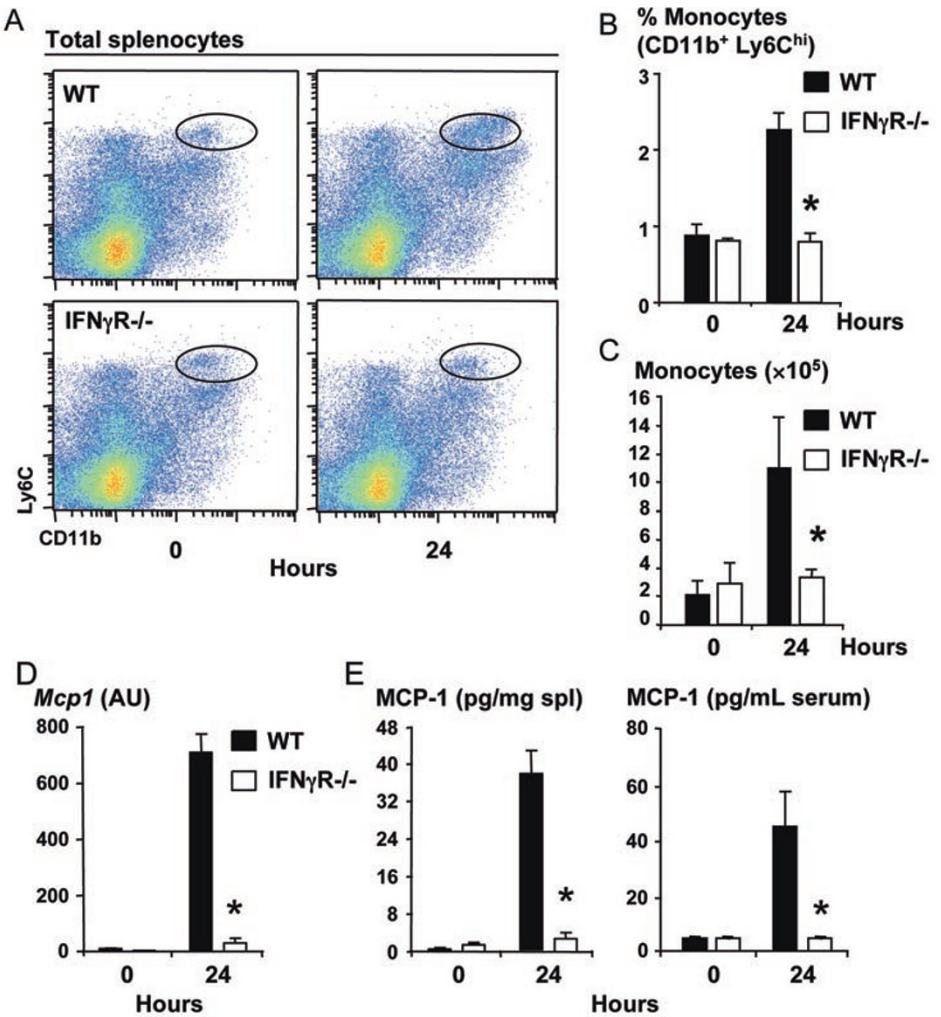

Figure 1 IFNγR expression is required for MCP-1 production and expansion of monocyte populations in the spleen after systemic *F. tularensis* challenge. WT and IFNγR−/− mice (n = 3/grp) were inoculated intravenously with 2 × 10^4 CFU *F. tularensis*. After 24 h, infected mice were euthanized along with uninfected (0 h) controls (n = 3/grp). **(A)** Representative analysis dot plot of RBC-depleted total splenocytes showing monocytes (CD11b+, Ly6C^{hi} population) in control and *F. tularensis*-infected WT and IFNγR−/− mice. Monocyte populations are shown as **(B)** mean proportion of total splenocytes ± SD obtained from dot plots described in **(A)**. **(C)** Mean absolute number of splenic monocytes ± SD. **(D)** Mean mRNA levels ± SD of MCP-1 from control and infected WT and IFNγR−/− mice. **(E)** Mean protein levels ± SD of MCP-1 from spleen homogenates and sera of WT and IFNγR-deficient mice. *p < 0.05 WT versus IFNγR−/− mice. Data are representative of at least five repeated experiments.

1 production in these mice. We therefore determined the induction of MCP-1 mRNA in the spleens of WT mice, IFNγR-deficient mice, and uninfected control mice by Q-PCR 24 h after *F. tularensis* inoculation. There was a profound impairment in MCP-1 mRNA induction in the spleens of IFNγR-deficient mice compared with WT mice after *F. tularensis* infection (Figure 1D). Similarly, MCP-1 protein production as measured by ELISA was also severely impaired in the spleens and sera of IFNγR-deficient mice compared with WT control mice after *F. tularensis* infection (Figure 1E). Collectively, these data reveal a crucial role for IFNγR signaling in the activation of MCP-1 production and the subsequent expansion of splenic monocyte populations.

IFNγR expression in the BM compartment is necessary for MCP-1 production and expansion of monocyte populations in the spleen after systemic F. tularensis challenge

IFNγR is expressed in a wide range of tissues, including BM-derived and non-BM-derived cell types. Therefore, we wanted to determine which compartment

utilized IFNγR signaling to regulate expansion of splenic monocyte populations in response to systemic *F. tularensis* infection. WT recipient mice were lethally irradiated and reconstituted with either WT or IFNγR-deficient BM, generating WT BM→WT and IFNγR-deficient BM→WT chimeric mice. Eight weeks after reconstitution, these mice were inoculated intravenously with *F. tularensis*. WT BM→WT mice had increased proportions of monocytes in their spleens 24 h after infection; in contrast, IFNγR-deficient BM→WT mice had virtually no expansion of splenic monocytes (Figure 2A).

Further, a similar defect in splenic MCP-1 levels in *F. tularensis*-infected IFNγR-deficient BM→WT chimeric mice was observed compared with WT BM→WT control mice (Figure 2B). These data suggest that IFNγR expression in BM-derived cells is required for MCP-1 production and the mobilization of monocyte populations in response to *F. tularensis* challenge. Similar to IFNγR-deficient mice, IFNγR-deficient BM→WT chimeric mice had a substantially increased bacterial burden in the spleen, suggesting that BM expression of IFNγR is also required for host defense against *F. tularensis* (Figure 2C).

IFNγR signaling induces MCP-1 production in BM-derived cells and acts synergistically with innate sensing mechanisms to induce MCP-1 in vitro

Our data suggest that BM expression of IFNγR is necessary for the production of MCP-1 in response to systemic *F. tularensis* challenge. MCP-1 can be produced by monocytes/macrophages, which arise from the BM [26-28]. Thus, we evaluated the ability of IFNγ to induce MCP-1 production, using primary BM-derived macrophages (BMMs) as a model. Treatment of BMMs with IFNγ resulted in significantly increased MCP-1 mRNA expression as determined by QPCR (Figure 3A). Furthermore, the effect of IFNγ on MCP-1 protein production was dose-dependent, as determined by ELISA 24 h after stimulation (Figure 3B).

During systemic infection, monocytes and macrophages in the spleen likely encounter both *F. tularensis* and IFNγ simultaneously. As pattern recognition receptors such as the TLRs also activate MCP-1 expression, we assessed whether IFNγR and TLRs interact in the induction of MCP-1 expression. As *F. tularensis* can be recognized via TLR2 [16], BMMs were stimulated with the TLR2 ligand Pam3CysK in the presence or absence of IFNγ. Interestingly, a synergistic induction of MCP-1 mRNA was observed in BMMs treated with both Pam3CysK and IFNγ (Figure 3C). Furthermore, to more closely simulate our *in vivo* bacterial challenge model, BMMs were cultured with *F. tularensis* in the presence or absence of IFNγ and culture supernatants were harvested at 24 h for analysis of MCP-1 levels by ELISA. *F. tularensis* infection alone induced a modest amount of MCP-1 protein. However, addition of IFNγ to the *F. tularensis*-stimulated BMM cultures induced a synergistic increase in MCP-1 production, which was similar to our results with the

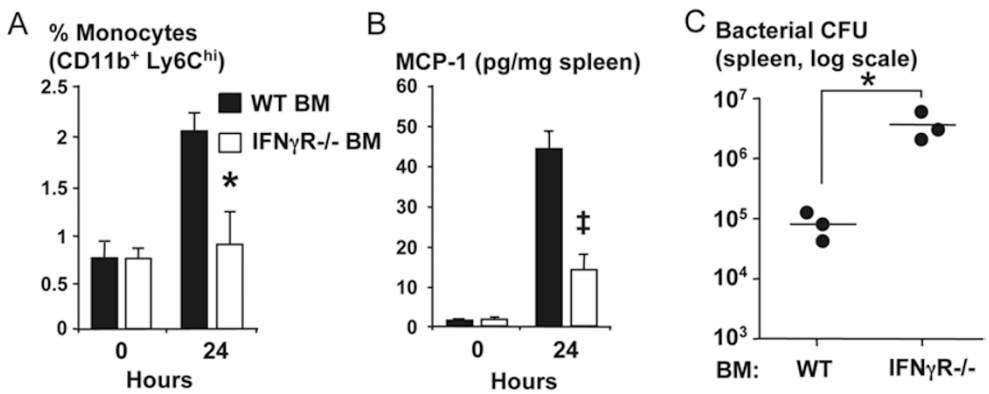

Figure 2 IFNγR expression in the bone marrow compartment is necessary for MCP-1 production and expansion of monocyte populations in the spleen after systemic *F. tularensis* challenge. WT BM→WT and IFNγR−/− BM→WT mice (n = 3/grp) were inoculated intravenously with 2 × 10^4 CFU *F. tularensis*. After 24 h, infected mice were euthanized along with uninfected controls (n = 3/grp). **(A)** Mean proportion of splenic monocytes (CD11b+, Ly6C^{hi} population) ± SD was determined as in Figure 1A in control and *F. tularensis*-infected WT BM→WT and IFNγR−/− BM→WT mice. **(B)** Mean protein levels ± SD of MCP-1 from spleen homogenates and sera of WT and IFNγR-deficient mice. **(C)** *F. tularensis* CFU recovered from spleens of WT BM→WT and IFNγR−/− BM→WT mice (n = 3/grp) three days after inoculation with 2 × 10^4 CFU *F. tularensis*. *p < 0.05 WT BM→WT versus IFNγR−/− BM→WT mice, ‡p < 0.01 WT BM→WT versus IFNγR−/− BM→WT mice. Data are representative of at least three repeated experiments.

TLR2 ligand (Figure 3D). Taken together, these data suggest that IFNγ is sufficient to activate MCP-1 production in BMMs. Furthermore, the IFNγ-dependent activation of MCP-1 can be amplified by TLR2 or *F. tularensis* stimulation.

MyD88-dependent signaling is required for IFNγ-driven MCP-1 production and expansion of splenic monocyte populations in response to systemic F. tularensis challenge

Previously published work has demonstrated that the adaptor protein MyD88, which is utilized by IL-1R, IL-18R, and most TLRs to initiate signaling, is a critical mediator of host defense against *F. tularensis* [29, 30]. Consistent with these findings, we observed that MyD88-deficient mice are highly susceptible to systemic *F. tularensis* challenge and cannot effectively clear bacteria (Supplementary information, Figure S2). However, we specifically wanted to ascertain whether MyD88 was required for the IFNγR-dependent MCP-1 production and expansion of splenic monocytes. After systemic *F. tularensis* challenge, MyD88-deficient mice had the same defect in expansion of splenic monocyte populations (Figure 4A) as we previously observed in IFNγR-deficient mice (Figure 1A and 1C). Furthermore, we found that MCP-1 protein levels in the spleens of infected MyD88-deficient mice were substantially decreased compared with WT mice (Figure 4B). Given these findings, we hypothesized that MyD88 may either be required for production of IFNγ or that it may be necessary for production of MCP-1 via a different mechanism that does not impact IFNγ production. Therefore, IFNγ levels in the sera of WT and MyD88-deficient mice were measured after systemic challenge with *F. tularensis*. Consistent with prior

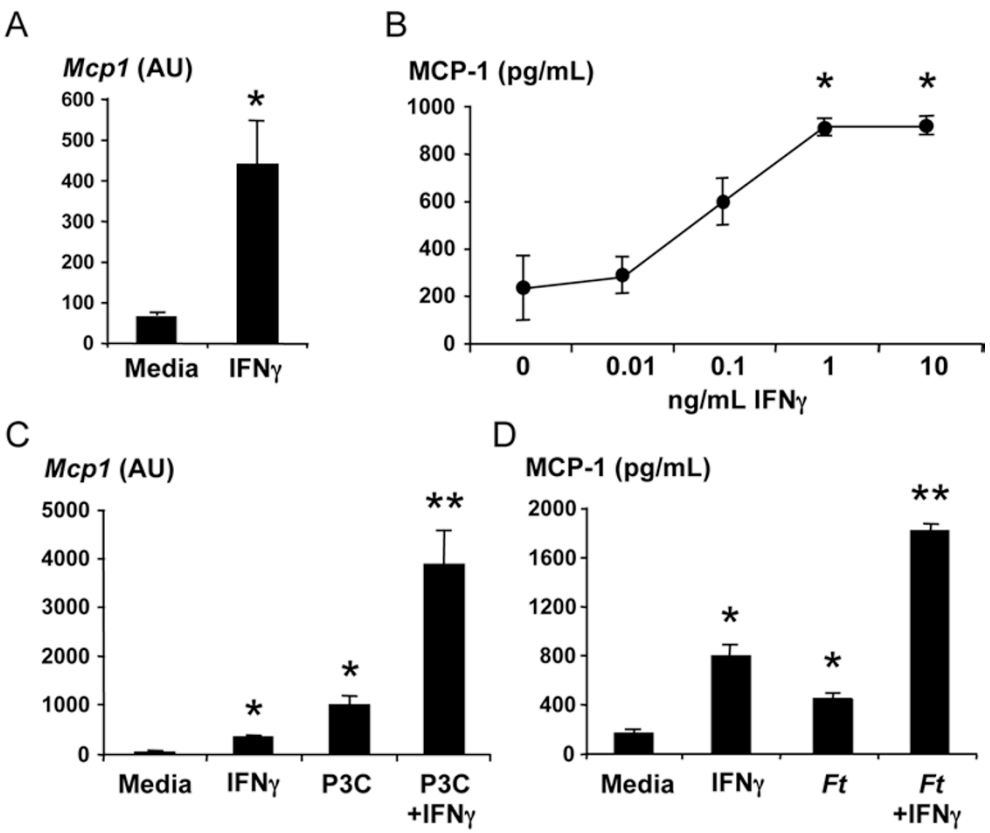

Figure 3 IFNγR signaling induces MCP-1 production in bone marrow-derived cells and acts synergistically with innate sensing mechanisms to induce increased levels of MCP-1 *in vitro*. **(A)** Mean MCP-1 mRNA levels ± SD from WT control BMMs and WT BMMs stimulated with 0.1 ng/ml IFNγ for 6 h prior to harvest. **(B)** Mean MCP-1 protein levels ± SD from supernatants of WT control BMMs and WT BMMs stimulated with the indicated concentrations of IFNγ for 24 h prior to harvest. **(C)** Mean MCP-1 mRNA levels from control BMMs and BMMs stimulated with 1 ng/ml IFNγ, 0.01 ng/ml Pam3CysK, or both for 6 h prior to harvest. **(D)** Mean MCP-1 protein levels from supernatants of control BMMs and BMMs stimulated with 1 ng/ml IFNγ, MOI 1 *F. tularensis*, or both for 24 h prior to harvest. *$p < 0.05$ versus media control, **$p < 0.05$ versus media control and other data points. Data are representative of at least three repeated experiments.

published results [31], there was no detectable IFNγ in the sera of infected MyD88-deficient mice compared to robust IFNγ production in WT mice (Figure 4C). These findings suggest that the production of IFNγ is dependent upon MyD88-mediated signals.

CCR2-deficient mice exhibit defective expansion of splenic monocyte populations and host defense in response to F. tularensis challenge

To directly test whether IFNγR-dependent MCP-1 production plays an important role in the mobilization of monocytes to the spleen in the context of systemic *F. tularensis* challenge *in vivo*, we examined the expansion of CD11b+ Ly6C^{hi} monocyte populations in the spleens of CCR2-deficient mice. These mice lack the receptor for MCP-1 and are thus unable to mobilize monocytes to the periphery upon bacterial challenge [7, 31, 32]. Similar to IFNγR-deficient mice, we observed defective expansion of splenic monocytes in CCR2-deficient mice after *F. tularensis* challenge (Figure 5A). These findings directly link MCP-1 to the IFNγ-dependent splenic monocyte expansion observed *in vivo*. We then hypothesized that if the monocyte populations mobilized to the spleen are critical to host defense against *F. tularensis*, then infected CCR2-deficient mice should have increased bacterial CFU in their spleens. We therefore challenged WT, IFNγR-deficient, and CCR2-deficient mice with *F. tularensis*, and subsequently assessed bacterial CFU in spleens from these mice. Notably, there were approximately tenfold more *F. tularensis* CFU in the spleens of CCR2-deficient mice compared with WT control mice three days after inoculation, indicating that CCR2-driven mobilization of monocytes to the spleen is required for control of bacterial numbers (Figure 5B). Interestingly, we observed tenfold more bacterial CFU in the spleens of IFNγR-deficient mice than in CCR2-deficient mice, suggesting that IFNγR is required for additional host defense functions aside from driving the CCR2-dependent expansion of splenic monocytes (Figure 5B). Taken together, these observations define a host defense circuit in which IFNγR signaling in BM-derived cells activates production of MCP-1, which is in turn required for the mobilization of monocytes to the spleen and effective control of *F. tularensis* numbers there.

IFNγR-deficient and CCR2-deficient mice have severe defects in bacterial clearance and in production of TNF-α and nitric oxide following systemic F. tularensis challenge

Prior work has suggested that upon reaching areas of infection, monocytes produce TNF-α and NO, critical effector mechanisms for clearing intracellular bacterial pathogens such as *F. tularensis* [10]. Thus, we examined whether CCR2-dependent mobilization of monocytes to the spleen was required for TNF-α and NO production in response to *F. tularensis*. Thus, we harvested spleens from WT, IFNγR-deficient and CCR2-deficient mice 24 h after *F. tularensis* challenge to assess production of TNF-α and NO. We found that intracellular TNF-α production in CD11b+ splenocytes harvested from *F. tularensis*-infected IFNγR-deficient and CCR2-deficient

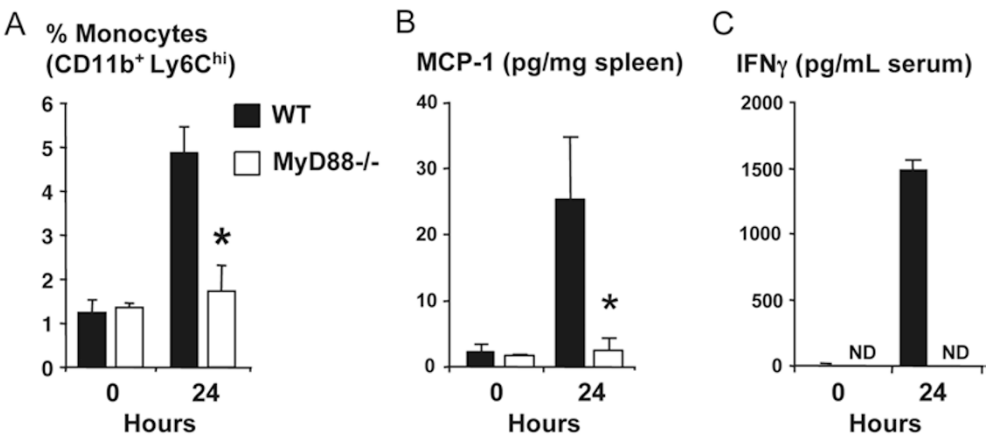

Figure 4 IFNγ-driven MCP-1 expression and expansion of splenic monocyte populations in response to systemic *F. tularensis* challenge occurs via MyD88-dependent signaling. WT and MyD88−/− mice (n = 3/grp) were inoculated intravenously with 2 × 10^4 CFU *F. tularensis*. After 24 h, infected mice were euthanized along with uninfected (0 h) controls (n = 3/grp). **(A)** Mean proportion of splenic monocytes (CD11b+, Ly6C^{hi} population) ± SD was determined as in Figure 1A in control and *F. tularensis*-infected WT and MyD88−/− mice. **(C)** Mean mRNA levels ± SD of MCP-1 from control and infected WT and MyD88−/− mice. **(D)** Mean protein levels ± SD of IFNγ in sera from control and infected WT and MyD88−/− mice. ND, not detected. *p < 0.05 WT versus MyD88−/− mice (Student's t-test). Data are representative of at least three repeated experiments.

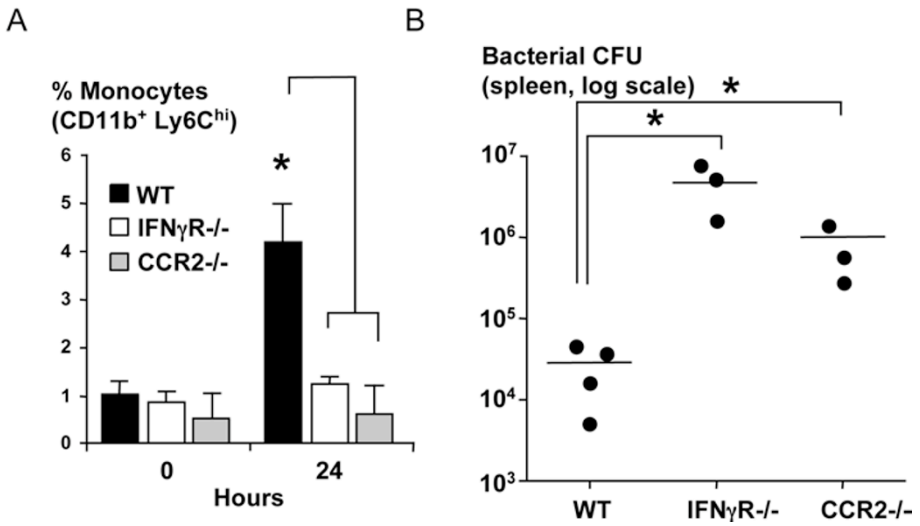

Figure 5 CCR2-deficient mice exhibit defective expansion of splenic monocyte populations and host defense in response to systemic *F. tularensis* challenge. WT mice, IFNγR−/− mice, and CCR2−/− mice (n = 3/grp) were inoculated intravenously with 2 × 10^4 CFU *F. tularensis*. After 24 h, infected mice were euthanized along with uninfected controls (n = 3/grp). **(A)** Mean proportion of splenic monocytes (CD11b+, Ly6C^{hi} population) ± SD was determined as in Figure 1A in control and *F. tularensis*-infected WT mice, IFNγR−/− mice, and CCR2−/− mice. **(B)** *F. tularensis* CFU recovered from the spleens of WT, IFNγR−/− mice, and CCR2−/− mice three days after intravenous inoculation with 2 × 10^4 CFU *F. tularensis*. *p < 0.05 WT versus IFNγR−/− or CCR2−/− mice. Data are representative of at least three repeated experiments.

mice was severely impaired compared with the TNF-α production in WT control mice (Figure 6A). Furthermore, to determine whether these splenocytes were capable of producing NO, cells from *F. tularensis*-challenged WT, IFNγR-deficient, and CCR2-deficient mice were cultured *ex vivo* with heat-killed *F. tularensis* (HKFT), and culture supernatants were evaluated for production of nitrite, a NO breakdown product. Similar to TNF-α levels, there were significantly lower levels of nitrite in the supernatants of HKFT-stimulated splenocytes from IFNγR-deficient and CCR2-deficient mice relative to WT mice (Figure 6B). Taken together, these data suggest that IFNγR-dependent CCR2 signaling is required for the mobilization of monocytes to the spleen, where they act as critical producers of TNF-α and NO and mediate clearance of *F. tularensis* infection.

Discussion

In the present study, we describe a host defense circuit activated by *F. tularensis* whereby MyD88 mediates production of IFNγ, which in turn signals via its receptor in BM-derived cells, resulting in the production of MCP-1 and subsequent CCR2-dependent mobilization of monocyte cells to the spleen. There, mobilized monocytes subsequently produce TNF-α and NO and mediate clearance of *F. tularensis*.

The role of IFNγR signaling in the activation of innate immune responses is critical to host defense in a wide range of *in vivo* models of intracellular bacterial infection, including systemic *Mycobacterium tuberculosis*, and *L. monocytogenes* infections [6, 33, 34]. Likewise, IFNγR signaling is absolutely required for control of *F. tularensis* infection, and mice lacking IFNγ are rendered moribund 5-7 days after challenge, consistent with our observations in this study [19, 35]. IFNγ activates a wide range of antimicrobial mechanisms, including induction of iNOS and subsequent NO production, which is critical for the destruction of pathogenic organisms [36]. However, the question of how IFNγ may fit into the wider signaling network that regulates the trafficking of immune cells, particularly myeloid cells, during the innate immune response to infection has not been well characterized. Thus, in the present study, we have uncovered a novel function of IFNγR signaling as a critical regulator of monocyte trafficking in response to systemic *F. tularensis* infection.

Our data show that IFNγR-deficient mice have decreased systemic levels of MCP-1, which suggests that induction of MCP-1 is a mechanism by which IFNγ orchestrates the mobilization of monocytes. We confirmed this mechanism by showing that mice deficient in CCR2, the MCP-1 receptor, also fail to mobilize monocytes to the spleen in response to *F. tularensis* challenge. Analy-

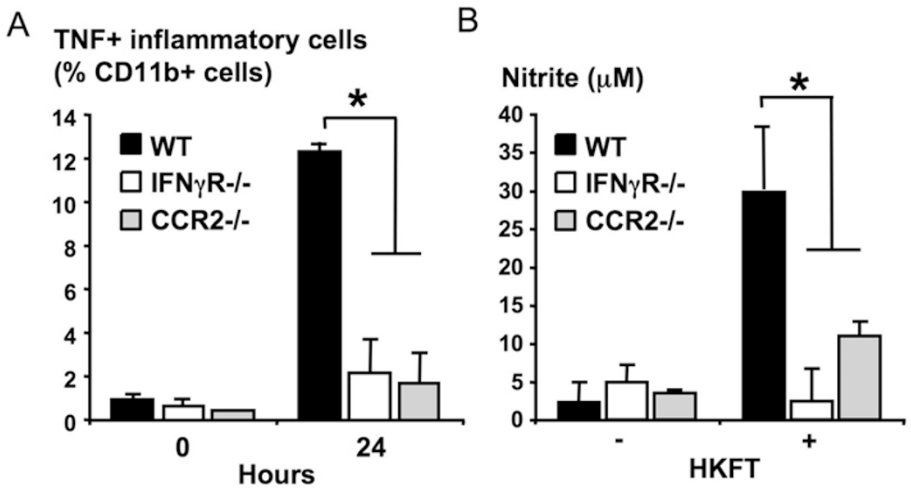

Figure 6 Mice deficient in CCR2 have similar defects as IFNγR-deficient mice in production of TNF-α and nitric oxide by inflammatory monocytes following systemic *F. tularensis* challenge. WT, IFNγR−/− mice, and CCR2−/− mice (n = 3/grp) were inoculated intravenously with 2 × 10^4 CFU *F. tularensis*. **(A)** Mean proportion ± SD of CD11b+ splenocytes producing TNF-α by intracellular staining from WT mice, IFNγR−/− mice, and CCR2−/− mice. **(B)** Mean nitrite production by splenocytes harvested from WT mice, IFNγR−/− mice, and CCR2−/− mice inoculated for 24 h with *F. tularensis* and restimulated with heat-killed *F. tularensis* in culture. *p < 0.05 WT versus IFNγR−/− or CCR2−/− mice. Data are representative of at least two repeated experiments.

sis of the MCP-1 promoter and enhancer has uncovered the presence of NF-κB and STAT-1 binding sites. *In vitro* studies have shown that MCP-1 is induced by a number of NF-κB-activating inflammatory signals, including TNF-α and TLR signaling in a wide range of cell types, including myeloid cells [26, 37]. *F. tularensis*, like many other bacteria, contains a complex set of molecular motifs that can induce NF-κB activation via TLRs [16, 38, 39]. We were therefore surprised that signaling via TLRs and other inflammatory cytokines activated by *F. tularensis* infection was not itself sufficient to drive MCP-1 production in IFNγR-deficient mice. However, our *in vitro* experiments suggest that IFNγ synergistically enhances otherwise modest *F. tularensis*-induced MCP-1 production, suggesting that *F. tularensis* alone may not efficiently trigger an immune response, and thus IFNγ functions as an amplifying loop, resulting in increased MCP-1 production and efficient mobilization of monocytes. Interestingly, *F. tularensis* LPS has been shown to be a weak activator of TLR signaling *in vitro* and *in vivo* [40-43]. Furthermore, *F. tularensis* has been shown to impair the production of inflammatory cytokines in infected cells [44]. Thus, IFNγR signaling may serve to counteract these unique properties of *F. tularensis*, which may serve as possible immune evasion mechanisms, allowing the activation of a robust host defense response to *F. tularensis*. Further studies should directly compare how efficiently different intracellular bacteria, including *F. tularensis*, or their components activate immune responses in monocytes and macrophages.

Our data demonstrate a role for MyD88-dependent signaling in the induction of IFNγ and subsequent MCP-1 production. These findings are consistent with previous work showing defective IFNγ production in *F. tularensis*-infected MyD88-deficient mice [29]. MyD88 functions downstream of three major host defense signaling mechanisms, TLRs, IL-1R, and IL-18R [30]. Prior studies have suggested a role for TLR2 in the recognition of *F. tularensis* lipoproteins, and while we have not observed decreased IFNγ production in TLR2-deficient mice (our unpublished observation), it is unclear whether TLR2 is the only TLR to recognize *F. tularensis* [16, 45]. Interestingly, *F. tularensis*-infected IL-18R-deficient mice produce less IFNγ (our unpublished observation), suggesting that signaling downstream of IL-18R may be part of the MyD88-dependent host defense mechanism described in our study. IL-18, in conjunction with IL-12, activates IFNγ production by NK cells and T lymphocytes [46]. Both NK and T cell populations produce IFNγ in response to *F. tularensis*, suggesting that NK cells and T cells may be critical sources of IFNγ during the host response against *F. tularensis* infection [23, 35, 47]. Interestingly, work using Rag1-deficient mice suggests that early IFNγ production can occur independently of T lymphocytes, as the proportion of IFNγ-producing cells is not decreased in these mice [48]. Conversely,

mice depleted of NK cells have lower proportions of IFNγ-producing cells three days after *F. tularensis* inoculation [23, 47]. Notably, one study demonstrated that the efficient formation of myeloid granulomas in the liver after *F. tularensis* infection required both IFNγ and NK cells, linking NK cell-mediated IFNγ production to myeloid cell trafficking [23]. While these findings suggest that NK cells in particular may be largely responsible for early IFNγ production, depletion of NK cells in mice prior to infection did not increase their susceptibility to *F. tularensis* challenge, suggesting that other cell types may produce enough IFNγ to sufficiently activate host defense [47, 49]. Interestingly, IFNγ expression in response to *F. tularensis* infection has been detected in dendritic cells and neutrophils, as well as hybrid NKDC populations, suggesting that IFNγ production by these non-lymphoid cell types may contribute to early activation of host defense against *F. tularensis* in the absence of T or NK cells [48]. Based on these studies, we speculate that MyD88-dependent IL-18R signaling, predominantly in NK and T cells, is a likely mechanism by which IFNγ production is activated in response to *F. tularensis*. Furthermore, the observation that myeloid populations can produce IFNγ in response to *F. tularensis* raises the interesting possibility that IL-18R/MyD88-dependent IFNγ production by these cells could contribute to the induction of MCP-1 expression.

We found that CCR2-deficient mice failed to mobilize monocytes to the spleen after *F. tularensis* challenge, and have significantly elevated bacterial numbers in the spleen compared to WT mice. These observations serve as direct evidence that monocytes are critical for efficient control of *F. tularensis* infection. CCR2-deficient mice serve as a compelling and widely used model for studying the specific contribution of monocytes to host defense, as these mice fail to mobilize monocytes to the periphery, including the spleen, upon infection or inflammation [7, 31, 32]. Importantly, the number and function of other cell types, including lymphocytes, neutrophils, tissue macrophages, and monocytes themselves, are not greatly perturbed by loss of CCR2 signaling at the steady state, making use of this model particularly advantageous [7, 31, 32]. The defects in host defense we have observed using CCR2-deficient mice to study the role of monocytes in response to *F. tularensis* infection are consistent with published studies using CCR2-deficient mice, as well as mice deficient in MCP-1 and other individual CCR2 ligands, to probe the role of monocytes in response to *L. monocytogenes* infection [5, 7, 50]. Collectively, these findings suggest that monocytes are critical mediators of the innate immune response against intracellular bacterial pathogens in general.

Monocytes are thought to control infection by intracellular bacteria in part via production of TNF-α, which activates immune responses via autocrine and paracrine mechanisms; they also activate direct antimicrobial effector mechanisms such as production of NO [5, 10]. Both mechanisms have been shown to be critical for clearance of *F. tularensis* [51, 52]. We have shown that production of TNF-α and NO is defective in IFNγR- and CCR2-deficient mice, which at least in part explains the elevated *F. tularensis* CFU we found in the spleens of these mice. Notably, we found that IFNγR-deficient mice had nearly a log more *F. tularensis* in their spleens than CCR2-deficient mice, suggesting that IFNγ activates a wider set of antimicrobial mechanisms, in which monocytes play a critical part.

Collectively, our findings describe a novel role for MyD88-dependent IFNγR signaling in the activation of MCP-1 production, leading to mobilization and activation of monocytes, which in turn mediate efficient host defense in part via TNF-α and NO production. Notably, recent work has uncovered a possible role for IFNγ in the activation of hematopoietic stem and progenitor cell proliferation *in vivo* and *in vitro* [53]. These findings suggest that IFNγ may trigger the *de novo* generation of myeloid cells to replenish the immune system in addition to inducing MCP-1-dependent mobilization of extant myeloid populations from the BM to the periphery. Furthermore, CCR2 has been shown to regulate the migration of hematopoietic progenitor cells from the BM to the periphery in response to inflammation [54]. Combining these findings with our results, the IFNγR/CCR2 signaling axis is a critical mechanism for the activation and remodeling of both immature and mature BM and peripheral hematopoietic compartments in response to inflammation and intracellular pathogen infection.

Materials and Methods

F. tularensis preparation

The *F. tularensis* live vaccine strain (LVS) was generously provided by Dr Karen Elkins. *F. tularensis* from frozen aliquots was streaked onto rabbit cysteine blood agar plates (Remel, Lenexa, KS) and individual colonies of *F. tularensis* were grown overnight in Mueller-Hinton Broth (MHB) supplemented with 1% Isovitalex (BD Biosciences), 0.1% glucose and ferric pyrophosphate (Sigma-Aldrich). Bacteria were harvested while in mid-log phase as determined by optical density at 600 nm wavelength in a spectrophotometer (Beckman-Coulter), washed twice with sterile PBS and diluted with sterile PBS for infection. Bacterial CFUs were verified by plating dilutions onto rabbit cysteine blood agar.

Mice

All procedures described herein were approved by and conducted in compliance with the UCLA Animal Research Committee.

6-8 week-old female C57BL/6J (WT), IFNγR-deficient, IFNαβR-deficient, and CCR2-deficient mice were obtained from the Jackson Laboratories. MyD88-deficient mice and control littermates were obtained from Dr Shizuo Akira. Mice were housed and bred in the UCLA vivarium in specific pathogen-free conditions.

Intravenous F. tularensis systemic infection model

For *in vivo* infections, mice were inoculated via the lateral tail vein with 2×10^4 CFU *F. tularensis* grown to log-phase and prepared as described above in a 200-µl bolus using a 28-gauge syringe (Becton-Dickenson), and euthanized 24 h after challenge. Groups of three mice were used in each experiment. For quantification of *F. tularensis* CFUs, spleens were homogenized to single-cell suspension and serially plated on rabbit cysteine blood agar plates for colony counting.

Generation of BM chimeras

BM reconstitution experiments were performed as previously described [55]. Whole BM was flushed from the femurs and tibiae of donor mice and depleted of RBCs using ACK lysis buffer (0.15 M ammonium chloride (NH_4Cl), 1 mM potassium bicarbonate ($KHCO_3$), and 0.1 mM EDTA (pH 7.3)). BM cells were subsequently washed twice in PBS and counted on a hemocytometer. WT recipient mice were lethally irradiated with 1100 Rad and reconstituted 24 h later by injection of 1×10^7 WT or IFNγR-deficient donor BM cells via the lateral tail vein. Mice were maintained in autoclaved cages, given irradiated feed and were maintained on sulfamethoxazole and trimethoprim oral suspension (TMS; 48 mg/ml in drinking water) for three weeks after irradiation. Mice were used for experimentation 8 weeks after BM reconstitution.

Flow cytometry

Spleens were homogenized into a single-cell suspension and depleted of RBC using ACK lysis buffer. Splenocytes were resuspended in staining media consisting of PBS containing 2% FBS and stained with anti-CD11b-APC or FITC, and anti-Ly6C-FITC or anti-Ly6C-biotin on ice for 30 min. All antibodies and SA-APC fluorochrome were obtained from BD Pharmingen (San Jose, CA). Cells were then washed and fixed with 2% PFA prior to analysis on a FACScalibur flow cytometer. For intracellular stains, 2×10^5 splenocytes were resuspended in DMEM containing 10% FBS containing 1:1 000 BD GolgiPlug and 2×10^6 heat-killed *F. tularensis* for 4 h in a 37 °C humidified incubator. Cells were subsequently stained for CD11b, fixed and permeabilized using BD Cytofix/Cytoperm, and stained for intracellular TNF-α using an anti-TNF-α-PE antibody for 30 min on ice. Cells were washed with BD PermWash and analyzed on a BD FACSCalibur. All flow cytometry data were analyzed using FlowJo (TreeStar, Inc., Palo Alto, CA, USA).

In vitro F. tularensis infection

WT BMMs were generated from whole mouse BM as previously described [56]. For *in vitro* infections, bacteria were diluted in DMEM and added at an MOI of 1 to 1×10^6 BM macrophages (for Q-PCR analysis) or 2×10^4 BM macrophages (for ELISA analysis) cultured in antibiotic-free media containing 5% heat-inactivated FBS. Bacteria were spun onto cells at 2 000 rpm for 10 min in a Beckman centrifuge at room temperature. Media was washed off 1 h later and replaced with media containing 2 µg/ml gentamicin (Sigma-Aldrich). Pam3Cys (Sigma-Aldrich) was added at 0.01 ng/ml; recombinant murine IFNγ (R&D Systems) was added at concentrations ranging from 0.01 to 10 ng/ml. For *MCP1* mRNA expression analysis experiments, cells were washed with PBS 6 h later and harvested in 1 ml of Trizol (Invitrogen). For MCP-1 ELISA, supernatants were recovered 24 h later.

Cytokine expression analysis by quantitative RT-PCR

Total RNA was harvested from BMMs or washed splenocytes using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was generated from RNA using iScript (Bio-Rad). Quantitative RT-PCR analysis of MCP-1 mRNA levels was carried out on an iCycler (Bio-Rad) using Sybr Green 2× Master Mix (Applied Biosystems). Primer sequences used are *MCP1* Fwd 5′-GCTGACCCCAAGAAGGAATG-3′, Rev 5′-GAAGACCTTAGGGCAGATGCA-3′. Data were normalized to L32 mRNA levels.

ELISAs

ELISA development kits for MCP-1 (eBioscience, San Diego, CA, USA) were used according to the manufacturer's instructions and analyzed using a 96-well plate spectrophotometer (ThermoFisher, Waltham, MA, USA). Spleens to be analyzed for cytokine levels by ELISA were weighed and homogenized in 0.5 ml PBS containing 0.05% Triton X-100 and centrifuged to remove debris. Supernatants were collected from 96-well plates and centrifuged to remove debris. All samples were plated in duplicate. Cytokine concentrations obtained from spleen homogenates were normalized to spleen mass.

Statistical analysis

The unpaired Student's *t*-test was used to compare groups; *P*-values of 0.05 or less were considered to be statistically significant.

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