Regulation of Chemokines, CCL3 and CCL4, by Interferon γ and Nitric Oxide Synthase 2 in Mouse Macrophages and During Salmonella enterica Serovar Typhimurium Infection

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Background. Interferon γ (IFN-γ) increases the expression of multiple genes and responses; however, the mechanisms by which IFN-γ downmodulates cellular responses is not well understood. In this study, the repression of CCL3 and CCL4 by IFN-γ and nitric oxide synthase 2 (NOS2) in macrophages and upon Salmonella typhimurium infection of mice was investigated.

Methods. Small molecule regulators and adherent peritoneal exudates cells (A-PECs) from Nos2−/− mice were used to identify the contribution of signaling molecules during IFN-γ–mediated in vitro regulation of CCL3, CCL4, and CXCL10. In addition, infection of bone marrow–derived macrophages (BMDMs) and mice (C57BL/6, Ifn-γ−/−, and Nos2−/−) with S. typhimurium were used to gain an understanding of the in vivo regulation of these chemokines.

Results. IFN-γ repressed CCL3 and CCL4 in a signal transducer and activator of transcription 1 (STAT1)–NOS2–p38 mitogen activated protein kinase (p38MAPK)–activating transcription factor 3 (ATF3) dependent pathway in A-PECs. Also, during intracellular replication of S. typhimurium in BMDMs, IFN-γ and NOS2 repressed CCL3 and CCL4 production. The physiological roles of these observations were revealed during oral infection of mice with S. typhimurium, wherein endogenous IFN-γ and NOS2 enhanced serum amounts of tumor necrosis factor α and CXCL10 but repressed CCL3 and CCL4.

Conclusions. This study sheds novel mechanistic insight on the regulation of CCL3 and CCL4 in mouse macrophages and during S. typhimurium oral infection.

Keywords. interferon γ; nitric oxide; nitric oxide synthase 2; macrophages; infection; chemokines; cytokine.

Interferon γ (IFN-γ) is produced by activated T cells, natural killer cells, and macrophages and demonstrates the following pleiotropic effects: antiviral, antibacterial, definition of T-helper subsets, induction of major histocompatibility complex molecules, cell survival, and others [1, 2]. IFN-γ is important for immune cell responses against infectious microbes, and individuals with loss of IFN-γ or its receptor show greater susceptibility to less virulent pathogens [3]. The binding of IFN-γ to its cognate receptors on target cells leads to activation of the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway, which is important in modulating gene expression [4]. Stat1−/− mice are also highly susceptible to Listeria monocytogenes and viral infections [5]. Therefore, IFN-γ and its downstream signaling molecules in the host are important in the regulation of immune responses, especially against infectious diseases.

IFN-γ–mediated regulation of cytokines and chemokines is important in modulating inflammatory responses. Upon intraperitoneal infection of mice with Salmonella enterica serovar Typhimurium (S. typhimurium), endogenous IFN-γ increases serum amounts...
of interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) [6]. Chemokines are secreted by a variety of cells and are important for several inflammatory responses. IFN-γ induces the expression of chemokine (C-C) ligand 2 (CCL2), CCL5, chemokine (CXC) ligand 9 (CXCL9), and CXCL10 in bone marrow–derived macrophages [7]. Importantly, in a model of experimental autoimmune uveitis, dysregulation of a number of chemokines in the absence of IFN-γ is observed and is correlated with altered cellular infiltration in the eyes [8]. However, the mechanisms by which IFN-γ modulates the expression of chemokines are not well understood, and is the focus of this study. Two types of mouse macrophages were used: unstimulated peritoneal exudate cells (A-PECs) and bone marrow–derived macrophages (BMDMs). An initial screen identified CCL3 to be lowered upon IFN-γ treatment in A-PECs (data not shown). Further studies demonstrated that CCL3 and its paralog, CCL4, which shares approximately 68% amino acid sequence similarity, were repressed with IFN-γ; however, CXCL10 was induced. CXCL10 is important in regulating T-cell responses and trafficking [9], whereas CCL3 and CCL4 attract a variety of leukocytes in vitro [10, 11]. Enhanced CCL3 amounts are also found in several autoimmune diseases [12–14], and it has been shown to play protective roles in a variety of infectious diseases [15–18]. Interestingly, CCL3 and CCL4, along with CCL5, share a common receptor, chemokine (C-C) receptor 5 (CCR5), which is a coreceptor for human immunodeficiency virus (HIV) on CD4+ T cells and macrophages [19, 20]. In addition, CCL3 and CCL5 also bind to CCR1 and CCR3. Notably, CCL3, CCL4, and CCL5 are secreted more in HIV-exposed individuals and are known to functionally inhibit the entry of the virus into host CD4+ T cells [19, 21]. Hence, it is important to understand the regulation of CCL3 and CCL4.

In this study, the mechanisms by which IFN-γ represses CCL3 and CCL4 in A-PECs were investigated and the functional roles of STAT1, nitric oxide synthase 2 (NOS2), p38 mitogen-activated protein kinases (p38MAPK), and the activating transcription factor 3 (ATF3) were elucidated. These findings were extended to an infection scenario using oral inoculation of mice with S. typhimurium. Overall, this study reveals in vitro and in vivo roles of IFN-γ and NOS2 in the regulation of CCL3 and CCL4.

METHODS

Chemicals and Other Reagents

Recombinant mouse IFN-γ was obtained from PeproTech Asia. Lipopolysaccharide from Escherichia coli serotype O55: B5 (LPS), SP600129 (SP), NG-methyl-L-arginine (LNMA), S-nitroso-N-acetyl-penicillamine (SNAP), fludarabine (F), L-arginine (L-Arg), and L-norvaline (Nor) were obtained from Sigma Aldrich. SB202190 (SB) was obtained from Merck–Millipore. Tauradoline was obtained from Enzo Life Sciences.

Isolation and Culturing of Resident A-PECs

A-PECs were isolated by injecting 0.32 M sucrose solution into the mouse peritoneal cavity; the recovered solution was centrifuged at 500 g for 10 minutes [22]. The cells were resuspended in Roswell Park Memorial Institute medium 1640 (Sigma) supplemented with 5% heat-inactivated fetal calf serum (Invitrogen), 5 μM β-mercaptoethanol (Sigma), 100 μg/mL penicillin, 250 μg/mL streptomycin, and 50 μg/mL gentamicin (Hismedia Laboratories, India) and plated at a cell density of approximately 2 × 10^7 cells per well in a 96-well plate. The cells were left to adhere for 45 minutes at 37°C in a humidified carbon dioxide incubator (Sanyo) and washed 3 times with phosphate-buffered saline (PBS; Sigma). A similar procedure was used to obtain and work with adherent PECs from mice treated with 4% Brewer’s thioglycollate (TG) medium for 4–5 days.

Cytokine and Chemokine ELISAs

The amounts of cytokines (IFN-γ and TNF-α) in the supernatants and sera were estimated using enzyme-linked immunosorbent assay (ELISA) kits from eBioscience. The amounts of chemokines (CCL3, CCL4, and CXCL10) in the supernatants and sera were quantified using ELISA kits from PeproTech. The manufacturer’s protocols were followed for quantification along with appropriate standards (approximately 31–1000 pg/mL). 3,3',5,5'-Tetramethylbenzidine was used as the substrate, and optical density readings were obtained at 450 nm using the VersaMax ELISA plate reader.

Bacterial Strain and Culture

S. typhimurium NCTC 12023 were cultured in Luria broth and streaked on Salmonella-Shigella (SS) agar plates [6]. A single colony from the plate was cultured overnight in 3 mL sterile Luria broth (LB) and used to inoculate 50 mL sterile LB at 0.2% and grown for 3–4 hours (log phase culture) or overnight (stationary phase culture) at 37°C, 160 rpm. Bacterial culture was centrifuged, washed with PBS, and resuspended in PBS for further experiments.

Mice, Infections, and Colony Forming Units (CFU) Analysis

C57BL/6, C57BL/6.Ifnγ−/−, and C57BL/6.Nos2−/− mice (aged approximately 6–8 weeks) were obtained from the Central Animal Facility, Indian Institute of Science (IISc). The mice received humane care, and experiments were performed according to approved procedures issued by the institutional animal ethics committee. Mice were orally fed approximately 10^8 CFU of S. typhimurium in a final volume of 0.5 mL PBS. On day 4 post infection, mice were sacrificed, and blood and other organs were collected. The organs were weighed and homogenized in PBS; CFU were enumerated on SS agar plates. Blood collected by cardiac puncture...
immediately upon sacrificing the mice was allowed to clot and centrifuged. Serum was separated and stored at −20°C for further analysis.

Statistical Analysis
All data were plotted using GraphPad Prism 5 software. The significance was obtained by performing a 2-tailed unpaired t test. P values <.05, .01, and .001 were represented as *, **, and ***, respectively. Also, significance was calculated and represented with respect to untreated cells (Figures 1–5) or control mice (Figures 6 and 7) at the same time point in each figure.

Detailed descriptions of methods, namely nitrite estimation, cell viability, reverse-transcription polymerase chain reaction (RT-PCR), estimation of intracellular protein amounts, siRNA-mediated knockdown, and histological analysis, are included in the Supplementary Methods section.

RESULTS
IFN-γ Represses CCL3 and CCL4 in A-PECs
Initial experiments were designed to study the effects of IFN-γ on A-PECs. The addition of IFN-γ did not affect cell survival (Figure 1A); however, Cxcl10 was rapidly induced. Interestingly, the transcripts of Ccl3 and Ccl4 were repressed at later time points (Figure 1B) and further quantification using quantitative RT-PCR is required; however, these changes were validated at the protein level using ELISA. Although CXCL10, a well-known IFN-γ-induced chemokine [10], was induced (Figure 1C), CCL3 and CCL4 were repressed in the supernatant of cells treated with different doses of IFN-γ (Figure 1F and I). Also, CXCL10 was induced in the supernatants of A-PECs as early as 6 hours post IFN-γ treatment, and levels were maintained up to 36 hours (Figure 1D and E). On the other hand, CCL3 and CCL4 amounts were repressed later, that is, 24 hours post IFN-γ treatment and remained repressed for 36 hours (Figure 1G, H and J, K). Therefore, in response to IFN-γ in A-PECs, the kinetics of repression of the transcripts and secreted amounts of CCL3 and CCL4 were delayed as compared with CXCL10 induction.

STAT1 Activation is Critical for IFN-γ-Mediated Regulation of CCL3, CCL4, and CXCL10
Next, the role of the canonical JAK-STAT signaling pathway in response to IFN-γ was evaluated. The intracellular phospho-STAT1 (p-STAT1) amounts increased with time in A-PECs treated with IFN-γ (data not shown). This increase in intracellular p-STAT1 amounts upon IFN-γ treatment was abolished in the presence of fludarabine, a STAT1 inhibitor [23] (Supplementary Figure 1A). Fludarabine treatment inhibited CXCL10 induction as well as repression of CCL3 and CCL4 in the presence of IFN-γ (Supplementary Figure 1D–F).

NOS2 Mediates Repression of CCL3 and CCL4 by IFN-γ
As some of the responses to IFN-γ are nitric oxide (NO) dependent [24], the possible role of this molecule in regulating chemokines was evaluated. Nitrite was induced in response to IFN-γ in the supernatants of A-PECs (Figure 2A). To study the functional contribution of NO, an inhibitor of NO synthases, LNMA, was used [25]. Studies with this inhibitor clearly demonstrated that NO in macrophages does not affect CXCL10 induction but represses CCL3 and CCL4 amounts (Supplementary Figure 2A–D). Of the 3 isozymes of NOS, primary macrophages are known to express NOS2 [26]; therefore, the role of NOS2 in the repression of CCL3 and CCL4 in response to IFN-γ was investigated. IFN-γ-induced nitrite in the supernatants of A-PECs from C57BL/6 mice was absent in A-PECs from Nos2−/− mice (Figure 2A), clearly establishing NOS2 as the major source of NO in these cells. RT-PCR analysis of C57BL/6 and Nos2−/− A-PECs treated with IFN-γ revealed that induction of Ifi1 and Cxcl10 transcripts was similar in both strains of mice (Figure 2B). Although induction of CXCL10 was unaffected (Figure 2C), the repression of CCL3 and CCL4 by IFN-γ observed in C57BL/6 A-PECs was abolished in Nos2−/− A-PECs both at the transcript level (Figure 2B) and in culture supernatants (Figure 2D and E).

Activation of p38MAPK Regulates IFN-γ-Mediated CCL3 and CCL4 Repression
MAPKs are known to play crucial roles in regulating IFN-γ responses in macrophages [7]. The roles of the 2 stress-induced kinases, p38MAPK and c-Jun N-terminal kinase (JNK), were investigated. In response to IFN-γ, intracellular amounts of p-JNK were not significantly altered (data not shown); however, the amounts of intracellular p-p38MAPK increased significantly with time post IFN-γ treatment in A-PECs (Figure 3A). To evaluate the functional contribution of p38MAPK, a well-known inhibitor, SB202190 [27], was used. SB202190 did not affect the induction of CXCL10 (Figure 3D); however, the repression of CCL3 and CCL4 in the supernatants of A-PECs by IFN-γ was rescued by SB202190 (Figure 3E and F).

ATF3 Mediates Repression of CCL3 and CCL4 by IFN-γ
Analysis of the promoters of mouse Ccl3 and Ccl4 revealed the presence of a consensus-binding sequence to ATF3 (Figure 4A). Also, addition of IFN-γ led to an increase in the intracellular amounts of ATF3 in A-PECs with time (Figure 4B). To understand the functional contributions by ATF3, experiments with an inducer, taurolidine [28], were performed. Addition of taurolidine induced ATF3 amounts in A-PECs (Supplementary Figure 2E) and repressed CCL3 and CCL4, but not CXCL10, in C57BL/6 (Supplementary Figure 2F, G, and H) and Nos2−/− (Supplementary Figure 2I, J, and K) A-PECs. To confirm the role of ATF3 in IFN-γ-mediated repression of CCL3 and CCL4,
siRNA-mediated knockdown of ATF3 in A-PECs was performed (Figure 4E). Knockdown of ATF3 did not affect IFN-γ–induced nitrite and CXCL10 amounts (Figure 4F and G); however, reduced repression of CCL3 and CCL4 by IFN-γ was observed upon ATF3 knockdown (Figure 4H and I).

IFN-γ and NOS2 Repress CCL3 and CCL4 During *S. typhimurium* Infection in BMDMs

Having established the mechanism of regulation of CCL3 and CCL4 in A-PECs (Figure 2F), the roles of IFN-γ and NOS2 were studied during intracellular replication of *S. typhimurium*...
in BMDMs. Addition of IFN-γ inhibited intracellular replication of S. typhimurium (Figure 5A) and repressed the S. typhimurium infection–induced production of CCL3 and CCL4 (Figure 5C and D), but not CXCL10 (Figure 5B). Also, higher CFU were recovered upon S. typhimurium infection of BMDMs from Nos2−/− mice (Figure 5E). Notably, the absence of NOS2 led to increased amounts of CCL3 and CCL4, but not CXCL10, in the supernatants upon S. typhimurium infection (Figure 5F–H).

Figure 2. Interferon γ (IFN-γ) mediated repression of CCL3 and CCL4 is nitric oxide synthase 2 dependent. A, Nitrite amounts in the supernatants of adherent peritoneal exudates cells (A-PECs) from C57BL/6 and Nos2−/− mice after 24 hours of treatment with or without 5 U/mL of IFN-γ. B, Reverse-transcription polymerase chain reaction analysis of expression of indicated genes post 24 hours of treatment with or without IFN-γ (5 U/mL) in A-PECs from C57BL/6 and Nos2−/− mice. The amounts of CXCL10 (C), CCL3 (D), and CCL4 (E) present in the supernatants of A-PECs from C57BL/6 and Nos2−/− mice after 24 hours of treatment with or without 5 U/mL of IFN-γ. Data are represented as mean ± standard error from 3 independent experiments. Significance is represented with respect to untreated controls. F, A schematic model depicting the pathway leading to regulation of chemokines CXCL10, CCL3 and CCL4 by IFN-γ in A-PECs is shown.
IFN-γ and NOS2 Regulate CCL3 and CCL4 Amounts

IFN-γ and NOS2 Repress CCL3 and CCL4 In Vivo upon *S. typhimurium* Infection of Mice

Next, the regulation of CXCL10, CCL3, and CCL4 during oral infection of mice with *S. typhimurium* was investigated. Mice lacking IFN-γ or NOS2 had higher CFUs in different organs on day 4 post *S. typhimurium* oral infection compared with C57BL/6 (Figure 6A–D). Also, histopathological examination of livers revealed greater damage in Ifn-γ−/− and Nos2−/− mice upon infection when compared with C57BL/6-infected mice (Figure 6E). Consistent with previous studies [6, 29], both Ifn-γ−/− and Nos2−/− mice were more susceptible to *S. typhimurium* infection compared to C57BL/6 mice.

Importantly, analysis of uninfected and infected mice sera revealed that induction of IFN-γ in sera upon infection was similar between C57BL/6 and Nos2−/− mice (Figure 7A). However, Ifn-γ−/− and Nos2−/− mice had significantly less TNF-α and CXCL10 induction compared with C57BL/6 mice upon infection (Figure 7B and C). Most importantly, amounts of CCL3 and CCL4 were significantly larger in both Ifn-γ−/− and Nos2−/− mice sera upon infection compared to C57BL/6
mice (Figure 7D and E). Therefore, endogenous IFN-γ and NOS2 increase TNF-α and CXCL10 but repress CCL3 and CCL4 amounts in vivo during S. typhimurium infection of mice.

**DISCUSSION**

In this study, the critical roles of IFN-γ and NOS2 in repressing the production of chemokines CCL3 and CCL4 are shown, both in vitro and in vivo. Importantly, the repression of CCL3 and CCL4 was specific to IFN-γ, but not LPS (data not shown). Also, repression of CCL3 and CCL4 amounts affected by IFN-γ was observed in primary unstimulated macrophages such as A-PECs (Figure 1) and BMDMs (Figure 5), but not activated macrophages, for example, TG-elicted PECs (data not shown). Thus, the repression of CCL3 and CCL4 is ligand and cell specific. Also, LPS is known to stimulate CCL3 production by elicited macrophages [30], which are distinct from unstimulated A-PECs [31]. Studies involving unstimulated A-PECs may be useful in gaining an understanding of the macrophage responses that occur during early stages of immune challenge. This aspect of cell specificity is important because the initial phase of the immune response, which
involves unstimulated macrophages, may be geared towards generating enhanced T-cell responses, as shown with higher levels of CXCL10 and lower levels of CCL3 and CCL4. However, subsequent responses by activated macrophages may be more inflammatory due to a lack of repression of CCL3 and CCL4.

IFN-γ is known to repress CCL3 and CCL4 in the macrophage cell line ANA-1 [32]; however, the underlying molecular
mechanisms are not understood. Although CXCL10 is known to be induced in a STAT1-dependent manner in macrophages [33], STAT1-dependent repression of CCL3 and CCL4 in response to IFN-γ is shown. Also, IFN-γ-induced NO is known to modulate a variety of responses [24], and there are conflicting reports on the regulation of CCL3 by NO in different cells [34, 35]. Importantly, the repression of CCL3 and CCL4 in response to IFN-γ treatment was not observed upon inhibition of NOS activity by LNMA (Supplementary Figure 2A–D) and in cells lacking NOS2 (Figure 2). A
possibility existed that the inhibition of NOS2 led to arginine accumulation or effects of its catabolism due to arginase. However, addition of excess arginine or inhibition of arginase using the inhibitor norvaline increased amounts of IFN-γ-induced nitrite [36] but did not affect the lowering of CCL3 or CCL4 (Supplementary Figure 1G–M). Thus, NO production by NOS2 in response to IFN-γ plays a functional role in the repression of CCL3 and CCL4. It is important to note that NO alone, as provided by the NO donor SNAP, did not affect the basal production of CCL3, CCL4, and CXCL10 (data not shown). These results demonstrate that a combination of IFN-γ-specific signals, including the production of NO, leads to repression of CCL3 and CCL4.

IFN-γ-induced MAPKs are known to regulate chemokines as well as other IFN-γ-inducible genes in BMDMs [7]. Also, p38MAPK is known to stabilize CCL3 in response to LPS in human peripheral blood mononuclear cells [27]. Therefore, the possible role of MAPKs in regulating CCL3, CCL4, and CXCL10 in A-PECs was studied. Inhibitor studies revealed that activated p38MAPK, but not JNK, was important in
IFN-γ--mediated repression of CCL3 and CCL4, but not CXCL10 induction (Figure 3 and data not shown). The transcription factor ATF3 is known to be induced in response to IFN-γ in keratinocytes [37]. Also, ATF3 is known to be a transcriptional repressor of CCL4 in mouse macrophages [38] and it is inducible by NO and p38MAPK [39, 40]. The functional roles of ATF3 were addressed using two approaches: addition of taurodilone, a known ATF3 inducer that exhibits anti-inflammatory and antitumorigenic activities [28] (Supplementary Figure 2E–K) and lowering of ATF3 using specific siRNAs (Figure 4). These data are consistent with a role of IFN-γ--induced ATF3 acts as a transcriptional repressor of CCL3 and CCL4.

Since STAT1 inhibition reduced IFN-γ--mediated NO induction (Supplementary Figure 1B), NOs2 was downstream to STAT1. Inhibition of p38MAPK did not affect IFN-γ--induced NO (Figure 3C); however, activation and sustenance of p38MAPK was reduced in Nos2−/− A-PECs compared with C57BL/6 upon IFN-γ treatment (Figure 3A and B). There are conflicting reports on the regulation of NOs2 by p38MAPK in macrophages [7,41]; however, the observation that IFN-γ--regulated NOs2 increases and sustains p38MAPK activation is novel. Also, induction of ATF3 is dependent on STAT1, NO, p38MAPK, and NOs2 (Figure 4B–D). Thus, induction of ATF3 is downstream and represses CCL3 and CCL4. Overall, our study highlights the fact that IFN-γ and its signaling components, including NOs2, are important in the regulation of CCL3 and CCL4 (Figure 2F).

Next, we extended our study from A-PECs to an infection model using the intracellular pathogen, *S. typhimurium*. This aspect is an important area of study as chemokines and their receptors modulate infection and inflammatory processes. Chemokine amounts are known to be regulated upon infection of macrophages with *Borrelia burgdorferi*, *Mycobacterium tuberculosis*, and others [42, 43]. CCL3 is important in resisting infections with virulent *Cryptoccus neoformans* [15] and *Klebsiella pneumoniae* [16]. Interestingly, leukocytic infiltration and cytokine amounts are not affected during infection with *K. pneumoniae*; however, the phagocytic activity of Ccl3−/− macrophages is lower [16]. Also, inheritance of a truncated allele of Ccr5 abrogates cell surface expression and confers protection against HIV infection [19]. Studies with mice lacking Ccr5 have shown its role in modulating cytokine production, lowering T-cell responses, and enhancing resistance to *Listeria* [44]. CCR5 also lowers the infiltration of lymphocytes and dendritic cells in lungs and draining lymph nodes upon infection with *M. tuberculosis* [45]. Also, IFN-γ and NOs2 are known to be extremely important for defense against a plethora of intracellular pathogens [26, 46–48]; however, their role in modulation of chemokines is unclear. HIV infection induces NO, and inhibition of this process leads to lower CCL3 amounts [35], whereas *Trypanosoma cruzi*–infected cardiac myocytes from Nos2−/− mice produce higher CCL2, CCL4, and CCL5 amounts [49].

Addition of IFN-γ decreased the CFU and amounts of CCL3 and CCL4 upon *S. typhimurium* infection of C57BL/6 BMDMs (Figure 5A–D). Also, infection of Nos2−/− BMDMs with *S. typhimurium* resulted in higher CFU and CCL3 and CCL4 amounts (Figure 5E–H). These observations prompted us to investigate the contributions of IFN-γ and NOs2 in the regulation of CCL3 and CCL4 in vivo upon *S. typhimurium* infection in mice. Mice lacking IFN-γ and NOs2 displayed more CFU (Figure 6), which is consistent with previous studies [6, 29]. This study reveals differential roles of IFN-γ and NOs2 in modulating cytokines and chemokines during in vivo *S. typhimurium* infection: increasing TNF-α and CXCL10 but lowering CCL3 and CCL4. The observation that Ifnγ−/− and Nos2−/− mice infected with *S. typhimurium* had significantly higher amounts of CCL3 and CCL4 in sera compared with C57BL/6 mice (Figure 7D and E) is in agreement with the greater infiltration of leukocytes in tissues, for example, liver, in these mice (Figure 6). Although both IFN-γ and NOs2 are known to restrict *S. typhimurium* infection, the functional role of CCL3 and CCL4 during *S. typhimurium* infection needs to be studied. It is possible that the absence of IFN-γ or NOs2 leads to greater recruitment of leukocytes, including CCR5+ cells, as a result of higher amounts of CCL3 and CCL4. It is known that blockade of CCR1 and CCR5 using an antagonist, N-terminal methionylated RANTES, lowers cellular infiltration and bone loss during periodontal infections [50]. Therefore, it is possible that greater leukocytic infiltration into tissues may lead to more host cell damage, as observed in the livers of *S. typhimurium*–infected Ifnγ−/− and Nos2−/− mice (Figure 6). Thus, tight regulation of CCL3 and CCL4 by IFN-γ and NOs2 may be important for the host response during infections with an intracellular pathogen such as *S. typhimurium*.

Overall, our study with unstimulated A-PECs, BMDMs, and in vivo infection of mice with *S. typhimurium* identifies IFN-γ and NOs2 as key regulators of the chemokines CCL3 and CCL4. A better understanding of the regulation of these molecules may provide insights into modulation of the host immune response to effectively ameliorate several diseases in which CCL3 and CCL4 contribute significantly, including autoimmune disorders such as diabetes and infections caused by *C. neoformans*, *K. pneumoniae*, HIV, and others.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.
Notes

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References


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