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The Chemokine Receptor CXCR3 Attenuates the Control of Chronic Mycobacterium tuberculosis Infection in BALB/c Mice

Soumya D. Chakravarty,* Jiayong Xu,† Bao Lu,‡ Craig Gerard,‡ JoAnne Flynn,§ and John Chan2*†

The chemokine receptor CXCR3 plays a significant role in regulating the migration of Th1 cells. Given the importance of Th1 immunity in the control of tuberculous infection, the results of the present study demonstrating that CXCR3-deficient BALB/c mice are more resistant to Mycobacterium tuberculosis, compared with wild-type mice, is surprising. This enhanced resistance manifests in the chronic but not the acute phase of infection. Remarkable differences in the cellular composition of the pulmonic granuloma of the CXCR3−/− and wild-type mice were found, the most striking being the increase in the number of CD4+ T cells in the knockout strain. In the chronic phase of infection, the number of CD69-expressing CD4+ T lymphocytes in the lungs of CXCR3−/− mice was higher than in wild-type mice. Additionally, at 1 mo postinfection, the number of IFN-γ-producing CD4+ T cells in the lungs and mediastinal lymph nodes of the CXCR3-deficient strain was elevated compared with wild-type mice. Pulmonic expression of IFN-γ, IL-12, TNF-α, or NO synthase 2, the principal antimycobacterial factors, were equivalent in the two mouse strains. These results indicate that: 1) CXCR3 plays a role in modulating the cellular composition of tuberculous granuloma; 2) CXCR3 impairs antimycobacterial activity in chronic tuberculosis; and 3) in the absence of CXCR3, mice exhibit a heightened state of CD4+ T lymphocyte activation in the chronic phase of infection that is associated with enhanced CD4+ T cell priming. Therefore, CXCR3 can attenuate the host immune response to M. tuberculosis by adversely affecting T cell priming. The Journal of Immunology, 2007, 178: 1723–1735.

Tuberculosis remains a principal infectious cause of mortality worldwide, resulting in at least 1.5 million deaths annually (1). It has been estimated that one-third of the world’s population has been infected with Mycobacterium tuberculosis (2). The majority of infected individuals, however, do not manifest disease. Yet, it is generally thought that a significant number of these asymptomatic individuals harbor a latent infection capable of subsequent reactivation to cause active disease (1).

The tuberculous granuloma, a hallmark of tuberculosis, is a highly structured and dynamic entity made up of a wide variety of immune cells (3–6). Immunohistochemical studies have shown that specific subsets of immune cells infiltrating the lungs of M. tuberculosis-infected mice and humans are organized in distinct spatial relationships (3, 4, 6). For example, while pulmonic granulomatosus T lymphocytes appear to infiltrate in a diffuse pattern, B cells exist in the lungs in discrete aggregates (3, 4, 6). The granuloma is generally thought to play a significant role in host defense against the tubercle bacillus. Experimental evidence has shown that reactivation tuberculosis as a result of anti-TNF-α therapy, a form of anti-inflammatory treatment used for a wide spectrum of diseases, is associated with granuloma disorganization (7). This latter observation suggests that protection requires precise cell-cell interactions between specific leukocyte subsets. Despite this apparent importance of the integrity of the granulomatous structure in conferring protective immunity against M. tuberculosis, the precise mechanisms by which immune cells are recruited to infected tissues remain poorly characterized.

Chemokines play an important role in the recruitment, migration, and trafficking of immune cells to specific sites in inflammatory tissues. The precision of these trafficking mechanisms can be seen in the homing of specific cell types into peripheral organs. However, direct evidence that chemokines and their receptors play a role in regulating the migration of inflammatory cells to the tuberculous granuloma is just beginning to emerge (8, 9). In a murine TNF-α blockade model, neutralization of this cytokine, which is a potent regulator of the expression of chemokines and their receptors (10), causes reactivation tuberculosis that is associated with granuloma disorganization (11, 12) and altered expression of CXCL9 and CXCL10 by macrophages, the primary cell type that interacts with M. tuberculosis in the host (13). CXCL9 and CXCL10, as well as CXCL11, are the ligands for the chemokine receptor CXCR3, an important receptor expressed on Th1 cells. Indeed, CXCR3 has been implicated in various Th1-regulated physiological and pathological states (14–16). Given the significance of Th1 immunity in protection against the tubercle bacillus, we hypothesized that mice deficient in CXCR3 would exhibit enhanced susceptibility to M. tuberculosis infection. Alternatively, a hypersusceptibility phenotype may not be observed due to the presence of alternative trafficking receptors on Th1 cells such as CCR5 and CCR2. To test this hypothesis, the host response to an aerogenic challenge with the virulent Erdman strain of M. tuberculosis was evaluated.
Materials and Methods

Animals

Eight- to 10 wk-old male and female BALB/c mice, C57BL/6 mice (Charles River Laboratories), and their CXC3/−/− counterparts (17) were used for all studies. CXC3/−/− mice were bred at the Albert Einstein College of Medicine (Bronx, NY) and in specific pathogen-free facilities.

M. tuberculosis-infected mice were maintained in our biosafety level 3 (BSL-3) facility. CXC3/−/− mice were routinely genotyped by PCR using extracted tail DNA. The PCR primer sequences used were as follows: CXC3, 5′-GCTTCTCGGCTGGTGTATG-3′ (forward) and 5′-TAGCCTCAGTACAGCGACG-3′ (reverse); Neo/mycine cassette (Neo), 5′-CTTGAGGGAGGCTATTC-3′ (forward) and 5′-AGGTGAGATGACAAGAGATC-3′ (reverse); IL-2 (positive control), 5′-CTAGGGCAAGAATGAAACTTGCATGAT-3′ (forward) and 5′-CTAGGGCAAGAATGAAACTTGCATGAT-3′ (reverse) (source of Neo and IL-2 primer sequences was Jackson Laboratories Genotyping Technical Support). All primers were synthesized by Invitrogen Life Technologies. All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine.

Mycobacteria and infection of mice

To maintain virulence, M. tuberculosis Erdman strain (Trudeau Institute, Saranac Lake, NY) bacterial stocks were generated by passaging through mice as previously described (18). Mice were infected via the aerosol route and placed in a closed air aerosolization system (In-Tox Products). Confirmation of CFU delivery was determined by harvesting the lungs of three or four infected mice one day postinfection. At the appropriate times postinfection, tissue bacillary load was quantified by plating serial dilutions of tissue homogenates onto Middlebrook 7H10 agar (Difco Laboratories) as previously described (11).

Histopathological and immunofluorescence studies

Tissue samples for histological studies were fixed in 10% normal buffered formalin followed by paraffin embedment. Six-micrometer sections stained with H&E were examined. For immunofluorescence detection of different leukocyte subsets, lung samples procured from infected mice were embedded in OCT (Tissue-Tek; Sakura), snap frozen in liquid nitrogen, and stored at −80°C until sectioning. Six-micrometer sections were cut and transferred onto positively charged slides (Fisher SuperFrost) and air dried. Sections were fixed in 3.7% paraformaldehyde (37% paraformaldehyde stock, diluted 1/10 in PBS; Electron Microscopy Sciences) for 10 min at 4°C. Sections were blocked against nonspecific binding using 5% goat serum in 0.1% BSA/PBS for 15 min. Sections were then incubated with primary mAbs specific to the following cell surface markers of interest: rat anti-mouse CD4 (CD4+ T cells), CD8 (CD8+ T cells), and CD19 (B cells) (BD Pharmingen). Sections were washed in 0.1% BSA/PBS several times before incubation with goat anti-rat secondary Ab conjugated to the following fluorophores: Alexa Fluor 555, 594, or 647 (Molecular Probes). Upon completing incubation with the secondary Ab and after several more washes in 0.1% BSA/PBS, sections were stained with 0.3 μM DAPI (Molecular Probes) and then washed in 1× PBS. Sections were immediately mounted with a ProLong antifade reagent (Molecular Probes) and then washed in 1× PBS. Sections were immediately mounted with a ProLong antifade reagent (Molecular Probes) and then washed in 1× PBS. The mounted slides were air dried.

Flow cytometric analysis of lung cells

CD45+ cells from lungs of mice infected with M. tuberculosis were obtained by preparing single cell suspensions with subsequent immunomagnetic separation using CD45+ microbeads and MACS MS separation columns (Miltenyi Biotec) as previously described (4). Three- or four-color flow cytometric analyses of these cells were completed using the following fluorochrome-conjugated Abs: anti-CD4-PE, anti-CD8-allophycocyanin, anti-CD3-PerCP, anti-CD45-PE, anti-CD45-allophycocyanin, anti-CD45-Cy5, anti-Ly6G-allophycocyanin or anti-Ly6G-FlTC, anti-CD19-PE, anti-CD11c-PE, anti-CD2L-PE, anti-CD69-PE (BD Pharmingen), and anti-F4/80-PE (Caltag Laboratories). An appropriate isotype control mAb for each fluorochrome (BD Pharmingen) was included in all analyses. CD45+ cells (1 × 106) were stained using the above Abs in FACS buffer (PBS containing 5% mouse serum, 10% FBS, and 0.01% NaCN) for 40 min at 4°C. Following additional washes in FACS buffer and PBS, the cells were fixed in 1× immediately prior to 30 min incubation with the milliQ water. This solution was added to the stained cells, which were then collected using a FACSSkalar (BD Biosciences) cytometer. Analysis was done using CellQuest software (BD Biosciences).

Intracellular cytokine staining

Single cell suspensions of the lung were prepared as described above. To obtain single cell suspensions of the mediastinal lymph node (MLN), lymph node tissues from three mice were dissected, pooled together, treated with collagenase D (10 mg/ml; Roche) in 10 ml of DMEM, and incubated at 37°C for 20 min. MLNs were then mechanically disrupted and forced through a 70-μm cell strainer (BD Falcon). The resulting cell suspension was washed in DMEM at room temperature before treatment with RBC lysis buffer (BioSource ammonium chloride potassium (ACK) lysis buffer) at 4°C. Finally, cells were washed in buffer consisting of 1× PBS with 0.5% FBS and 2 mM EDTA. Intracellular cytokine staining of total lung and MLN cells was completed using a BD Pharmingen mouse intracellular cytokine staining kit according to the manufacturer’s protocols with slight modifications. Briefly, lung or MLN cells were cultured at 0.5 × 10^6 cells/ml in 10% FBS plus RPMI 1640 medium with the presence of a leukocyte activation mixture from BD Pharmingen (consisting of PMA, ionomycin, and a BD Pharmingen GolgiPlug, which contains brefeldin A) for 4 h at 37°C. Following stimulation, cells were collected, washed in complete medium, spun down, and stained for T lymphocyte surface markers as noted previously. After further washes in FACS buffer, cells were treated with Cytofix/Cytoperm buffer (BD Biosciences), washed two times with permeabilization buffer (containing FBS and 0.1% saponin) from BD Biosciences, and stained with anti-IFN-γ-PE (20 μl prediluted; BD Pharmingen) for 15 min at 4°C. A PE-conjugated isotype control mAb and a purified blocking Ab against IFN-γ to ensure staining specificity were included for all analyses. After two subsequent washes with BD Biosciences permeabilization buffer followed by one wash with FACS buffer, cells were resuspended in 200 μl of FACS buffer and collected on a FACSCalibur flow cytometer (BD Biosciences).

Real-time RT-PCR

Total RNA was isolated from the lungs of M. tuberculosis-infected mice using the TRIzol (Invitrogen Life Technologies) extraction protocol with slight modifications. Briefly, isopropanol-purified RNA was washed in 75% ethanol, then 100% ethanol, and resuspended in nuclelease-free water. RNA samples were subsequently incubated at 65°C for 10 min followed by treatment with SUPERase-In RNase inhibitor (Ambion) and then subjected to RNase-free DNase (Promega) digestion for 15 min at 37°C. The reaction was neutralized with the addition of nuclelease-free water. The RNA obtained was subjected to a second round of TRizol extraction and purification as described above. After quantification by absorbance at 260 nm, RNA quality and purity were assessed by running equal concentrations on a 1.5% formaldehyde agarose gel. Reverse transcription of total lung RNA was completed with a first-strand cDNA synthesis kit for RT-PCR (avian myeloblastosis virus) (Roche Diagnostics) by oligo(dT) priming according to the manufacturer’s protocol. To procure RNA from granulomatous lesions, laser capture microdissection (LCM) of formalin-fixed, paraffin-embedded lung tissue from M. tuberculosis-infected wild-type and CXC3/−/− mice was undertaken. Briefly, 8-μm sections were cut and placed onto 76 × 26 polyethylene naphthalate film 2.0 glass slides (Leica Microsystems) and air dried. Upon staining with H&E, tissue sections were cut using a Leica AS LMD system. Dissected granulomatous tissue from individual mice were collected in 0.2-ml PCR tubes and subjected to RNA extraction and purification using the RNeasy Micro kit (Qiagen) according to the manufacturer’s protocol. LCM-procured RNA was then subjected to reverse transcription to synthesize cDNA using Sensiscript reverse transcriptase (Qiagen) and random primers (Roche) in accordance with the manufacturer’s instructions. For total lung RNA-derived cDNA, relative expression of the genes of interest in wild-type and CXC3/−/− mice was assessed by real-time PCR using molecular beads (Integrated DNA Technologies) as previously described (19); for cDNA derived from LCM-procured RNA. Power SYBR Green PCR master mix (Applied Biosystems) was used. Levels of gene expression measured were normalized to the housekeeping gene β-actin. The β-actin real-time PCR was always run in parallel with that of the other genes of interest. The PCR protocol used was as follows: 10 min at 95°C; 40 cycles of 20 s at 95°C, 20 s at 55°C, and 30 s at 72°C. The real-time PCR was completed on an ABI PRISM 7700 (for the SYBR Green method, an ABI PRISM 7900 was used) (Applied Biosystems), with data collection and analysis done using its Sequence Detection System software version 1.9.

3 Abbreviations used in this paper: MLN, mediastinal lymph node; LCM, laser capture microdissection; NOS2, NO synthase 2; Treg, regulatory T cell.
The absence of CXCR3 expression in CXCR3<sup>−/−</sup> BALB/c mice through PCR genotypic analysis of tail DNA. Recovered genomic DNA was subjected to PCR analysis using primers specific for CXCR3 and the phosphoglycerate kinase (PGK) neomycin cassette insert (lanes 1–4), with additional IL-2 specific primers used in lane 4 only. The IL-2 served as a positive control for the PCR. The products for CXCR3 (246 bp), the phosphoglycerate kinase neomycin cassette insert (280 bp), and IL-2 (342 bp) are shown. +/+ wild type; −/−, CXCR3<sup>−/−</sup>.

**Figure 1.**

Statistical analysis

Where appropriate, data points were subjected to the unpaired Student's t test to determine statistical significance using SigmaPlot 2002 (SPSS) and GraphPad Prism 4 software. Log transformation of CFU values was undertaken before statistical analysis for normalization purposes. p < 0.05 was considered statistically significant.

**Results**

Infection kinetics of M. tuberculosis-infected CXCR3<sup>−/−</sup> BALB/c mice indicate that this knockout mouse strain controls chronic tuberculosis better than wild-type animals

To confirm the correct genetic background of the mouse strains used in this study, PCR genotyping of a representative sample of CXCR3-deficient littermates in both the BALB/c and the C57BL/6 background was routinely done (Fig. 1). Before the commencement of all experimental investigations presented in this study, the genotypes of the mice used were confirmed for CXCR3 gene disruption each time.

To evaluate the role of CXCR3 in controlling M. tuberculosis infection, wild-type and CXCR3<sup>−/−</sup> BALB/c mice were infected via the aerosol route with a relatively low dose of bacilli (∼400 CFU) and subsequently sacrificed to determine bacterial load in the lungs and spleen of wild-type and CXCR3-deficient littermates at 30, 90, and 180 days postinfection. The bacterial burden in the lungs or spleen was not significantly different at 30 days postinfection. By 90 days postinfection, however, it was apparent from the assessment of tissue bacterial burden that the CXCR3<sup>−/−</sup> BALB/c mice exhibited enhanced capacity in controlling a tuberculous infection. Although the bacterial load in the lungs and spleen of wild-type BALB/c at 30 and 90 days postinfection was maintained at a stable level, the number of viable bacilli in these two organs in the CXCR3<sup>−/−</sup> mice decreased over this 60-day period. The difference in the pattern of disease progression resulted in a −0.5 log and −1.0 log lower bacillary load in the lungs and spleen, respectively, of the CXCR3<sup>−/−</sup> mice compared with wild-type BALB/c mice. By 180 days postinfection, the differences in tissue bacterial numbers between the two groups of mice increased further. At this chronic persistence phase of infection, the bacterial burden in the lungs (p = 0.019) and spleen (p = 0.036) of CXCR3<sup>−/−</sup> mice was ~1.5 to 2.0 logs lower than that observed in wild-type BALB/c. Together, these data strongly indicate that CXCR3<sup>−/−</sup> BALB/c mice, compared with wild-type animals, exhibit increased anti-mycobacterial capacity in the chronic phase of infection, as assessed by quantification of bacterial burden in the infected lungs and spleen. This relative resistance of CXCR3-deficient mice was also observed in tissues of CXCR3<sup>−/−</sup> BALB/c mice in the chronic phase of infection when animals were infected aerogenically with a high inoculum of M. tuberculosis Erdman strain (∼1,000 CFU) (data not shown). A 360-day survival study conducted in parallel to the above-described kinetics experiment (Fig. 2) involving the relatively low dose of infection inoculum did not reveal significant differences in the time to death between the CXCR3<sup>−/−</sup> and wild-type mice (median survival time for CXCR3<sup>−/−</sup> mice was 330 days vs 315 days for wild-type mice with a ratio of 1.05 at a 95% confidence interval of 0.8262 to 1.274; representative of three independent experiments). It is well established that immunocompetent mice infected with M. tuberculosis can maintain a stable bacterial load for a prolonged period of time before spontaneous disease progression sets in that ultimately leads to death. It is likely that the absence of CXCR3 allows the host to control chronic tuberculosis at a tissue bacterial burden set point that is lower compared to infected wild-types, yet does not enable complete clearance of the infection. Although diminished, the tissue bacterial burden of the CXCR3<sup>−/−</sup> mice did not reach a level that would allow this group to escape from the eventually spontaneous and fatal end-stage disease progression and to survive longer than the wild-type mice. Finally, it is possible...
that the mechanisms operative in the CXCR3-deficient mice are no longer effective during the end stage chronic infection, perhaps as a result of immunological exhaustion recently described in a chronic lymphocytic choriomeningitis virus infection model (20).

The ability of CXCR3−/− C57BL/6 mice to control tuberculous infection

A recent report has provided evidence that the ability of CXCR3-deficient C57BL/6 mice to control M. tuberculosis in the lungs and spleen, as assessed by tissue bacterial burden during the initial 60 days of infection, is comparable to that of wild-type mice (21). Based on the results of our study using CXCR3−/− BALB/c mice, the ability of the C57BL/6 counterpart to control an aerogenic challenge of virulent M. tuberculosis was examined in the chronic phase of infection up to 150 days postinfection. Interestingly, compared with wild-type mice, CXCR3-deficient C57BL/6 mice exhibit increased capacity to control the infection in the chronic phase, as assessed by tissue bacterial load, only in the spleen and not in the lungs (Fig. 3). This contrasts with the relative resistance phenotype of the CXCR3-deficient BALB/c mice, which is apparent in both the lungs and the spleen. In the CXCR3−/− C57BL/6 mice aerogenically challenged with a relatively low dose of the M. tuberculosis Erdman strain (~100 CFU), there were no significant differences in the pulmonic bacterial load, relative to that of the wild-type mice, at 30, 90, or 150 days postinfection. In the spleen, the bacterial numbers in the CXCR3−/− C57BL/6 mice were ~1.5 log lower than that detected in the wild-type mice at 90 days (p = 0.0029) and 150 days (p = 0.0007) postinfection. No CXCR3−/− or wild-type C57BL/6 mice succumbed to the aerosol infection for the duration of the study. A total of three C57BL/6 studies were performed (twice with an infection inoculum of ~100 CFU and once with ~500 CFU) with similar results (Fig. 3 and data not shown). These data are not inconsistent with those reported by an earlier study designed to examine the susceptibility of CXCR3-deficient C57BL/6 mice to a low-dose (~100 CFU) aerogenic M. tuberculosis H37Rv infection (21). That study reported no statistically significant differences in bacterial burden in the lungs or spleen between the wild-type C57BL/6 and CXCR3−/− groups up to 60 days postinfection, but it did not examine bacterial load at later times after the aerogenic challenge. Overall, the results of the C57BL/6 study, together with those generated from experiments using BALB/c mice, strongly suggest that CXCR3-deficiency enables the host to better control a chronic M. tuberculosis infection. Interestingly, this chronic phase-specific resistance manifests in distinct organs depending on the genetic background of the infected host.

The lung granulomatous response of M. tuberculosis-infected CXCR3-deficient BALB/c mice: histological and immunofluorescence studies

Evidence abounds that CXCR3 plays a significant role in a wide spectrum of pathophysiological conditions by virtue of its ability to regulate the migration of leukocytes, including Th1 cells, to the site of inflammation (22–24). Because Th1 immunity plays a critical role in host defense against the tubercle bacillus, potential mistrafficking of Th1 cells to the site of infection as a result of CXCR3 deficiency may further dysregulate the tuberculous granuloma. Indeed, Th1 chemokine receptors, including CCR2 and
CCR5, have been reported to affect the antituberculosis granulomatous response (8, 9). Therefore, studies were undertaken to characterize the pulmonic granulomatous tissues in *M. tuberculosis*-infected CXCR3−/− BALB/c mice. We chose to use CXCR3−/− BALB/c mice to analyze the granulomatous response because this strain, and not CXCR3-deficient C57BL/6 animals, manifests the

**FIGURE 5.** Characterization of CD4+ (A) and CD8+ (B) T cell populations among CD45+ leukocytes obtained from the lungs of CXCR3−/− and WT BALB/c mice infected with *M. tuberculosis* through flow cytometry. Absolute numbers of CD4+ and CD8+ cells at 1, 3, and 6 mo postinfection are shown. Proportions of CD45+ cells analyzed, as well as dead cells excluded by side scatter vs forward scatter properties, were comparable between wild-type (WT) and CXCR3−/− groups. For analysis, gating on the lymphocyte population based on side vs forward scatter properties was done. Data points represent the mean of data obtained from three mice per group per time point being analyzed. Error bars represent SD. C: Representative dot plots illustrating the proportions of CD4+ and CD8+ T cell subsets comprising total CD3+ T cell populations in wild-type and CXCR3−/− groups at 1, 3, and 6 mo postinfection. Data shown are representative of three independent experiments done using a low-dose aerosol infection model (inoculum size, ~200 CFU of *M. tuberculosis* Erdman strain).
observed resistance in the lungs, the organ that is the most relevant in disease pathogenesis in tuberculous infection. Histological analysis of infected tissues revealed no obvious structural differences between the granulomas in the lungs of CXCR3−/− BALB/c and wild-type mice (Fig. 4). This observation is further supported by the results of immunofluorescence studies aimed at characterizing the distribution of the various lymphocyte subsets in lung granulomatous tissues in the two groups of mice. The results (data not shown) demonstrate that by 1 mo postinfection both the CXCR3−/− and the wild-type BALB/c mice formed well-structured B cell aggregates, whereas their CD4+ and CD8+ T lymphocytes assumed a diffuse pattern of localization within the granulomatous tissues. At this 1-mo postinfection time interval, the bacterial burden in the lungs of the CXCR3−/− and wild-type BALB/c mice were comparable. Therefore, as assessed by immunofluorescence and light microscopy studies, CXCR3 deficiency per se does not alter the overall pattern of leukocyte distribution in the lungs of M. tuberculosis-infected mice.

Flow cytometric characterization of the cellular composition of the granulomatous tissues in the lungs of M. tuberculosis-infected, CXCR3-deficient BALB/c mice

To further characterize the granulomatous response of CXCR3−/− BALB/c mice, flow cytometric analysis was used to examine the cellular constituents of infected lung tissues in both the acute and chronic phase of infection. Flow cytometry was performed using single cell suspensions of immunomagnetically separated CD45+ lung cells. Results of these studies revealed that the number of CD4+ T cells was comparable between CXCR3-deficient and wild-type BALB/c mice at 30 days postinfection (Fig. 5A). However, by 90 days postinfection, the CXCR3−/− mice, compared with wild-type mice, showed a significantly higher number of CD4+ T cells in the lungs (4.2 × 10⁶ in CXCR3−/− mice and 2.8 × 10⁶ in wild-type BALB/c mice; p = 0.0008) (Fig. 5A). This difference represents a ~50% increase in this T lymphocyte subset in CXCR3-deficient BALB/c mice compared with wild-type mice. The difference in CD4+ T cells between the two mouse groups increased as the infection progressed. By 180 days postinfection, the number of CD4+ T cells in the lungs of CXCR3−/− mice (4.3 × 10⁶) was ~60% more than that in infected wild-type mice (2.6 × 10⁶) (p = 0.0275) (Fig. 5A). Flow cytometric analysis of the number of CD8+ T lymphocytes in the lungs of infected mice revealed no difference between the two groups of animals at 30 days postinfection (Fig. 5B). By 90 days postinfection, CXCR3−/− mice began to display a lower level of CD8+ T cell infiltration compared with wild-type mice, although this difference did not

**FIGURE 6.** Absolute numbers of different leukocyte subsets of CD45+ cells obtained from M. tuberculosis-infected CXCR3−/− and WT BALB/c mice through flow cytometry. At 1, 3, and 6 mo postinfection, positively enriched CD45+ cells were obtained and subjected to staining using Abs for cell surface markers of interest: CD19 (B cells), F4/80 (macrophages), CD11c (dendritic cells), and Ly6G (neutrophils). Gating on respective side vs forward scatter populations was done for each population of interest. Data points represent the mean of data obtained from three mice per group per time point being analyzed. Error bars represent SD. Data shown are representative of three independent experiments completed using a low-dose aerosol infection model (inoculum size, −200 CFU of M. tuberculosis Erdman strain).
reach statistical significance. At 180 days postinfection, as the disease progressed into a more chronic phase, there were ~57% fewer CD8\(^+\) T cells in the lungs of CXCR3-deficient mice compared with wild-type BALB/c mice \((1.2 \times 10^6\) in CXCR3\(^{-/-}\) mice and \(2.6 \times 10^6\) in wild-type BALB/c mice; \(p = 0.0087\)) (Fig. 5B). Hence, at 6 mo postinfection, the CD4\(^+\) T cell/CD8\(^+\) T cell ratio in the lungs of M. tuberculosis-infected CXCR3\(^{-/-}\) BALB/c mice was 3.6, whereas that for wild-type mice was 1.0. Of note, at 1, 3, and 6 mo postinfection, the CD3\(^+\) T cell populations studied consisted almost entirely of CD4\(^+\) and CD8\(^+\) T cell subsets in both wild-type and CXCR3\(^{-/-}\) mice (Fig. 5C).

To assess whether the accumulation of other leukocyte subsets in the granulomatous tissues was altered due to a deficiency in CXCR3, B cells (CD19\(^+\)), macrophages (F4/80\(^+\)), neutrophils (Ly6G\(^+\)), and dendritic cells (CD11c\(^+\)) present in the lungs of M. tuberculosis-infected CXCR3\(^{-/-}\) and wild-type BALB/c mice at various time points postinfection were analyzed by flow cytometry. The results in Fig. 6 demonstrate no significant differences in the number of B cells and macrophages throughout the course of infection. In contrast, the number of CD11c\(^+\) cells is ~54% higher in the CXCR3\(^{-/-}\) mice at 6 mo postinfection compared with that in wild-type mice \((p = 0.024)\). Finally, the lungs of M. tuberculosis-infected CXCR3-deficient mice harbor ~60% fewer neutrophils compared with those present in tuberculous wild-type animals at 6 mo postinfection (Fig. 6). The number of neutrophils in the lungs of M. tuberculosis-infected CXCR3\(^{-/-}\) mice, compared with the wild-type mice, is lower as well at 1 and 3 mo postinfection (Fig. 6), although the differences at these earlier time points did not reach statistical significance. This result is in agreement with an earlier study reporting that CXCR3-deficient C57BL/6 mice infected with the virulent H37Rv M. tuberculosis strain exhibit a defective neutrophilic response in the lungs compared with wild-type animals (21). In summary, flow cytometric analyses of the kinetics of accumulation of various leukocyte subsets in the lungs of M. tuberculosis Erdman strain-infected CXCR3\(^{-/-}\) BALB/c and wild-type mice revealed a remarkable difference in the cellular composition between the two groups.

**T cell activation during M. tuberculosis infection in CXCR3-deficient BALB/c mice**

CD4\(^+\) T cells play a critical role in engendering protective immunity against M. tuberculosis (1). This, together with the significantly increased number of CD4\(^+\) T lymphocytes during the chronic phase of tuberculous infection in the CXCR3\(^{-/-}\) mice relative to wild-type mice (Fig. 5), led us to focus on examining whether the knockout strain’s superior ability to control the tubercle bacillus (Fig. 2) was due to a more robust activation of CD4\(^+\) T cells in M. tuberculosis-infected CXCR3-deficient mice. Flow cytometric analyses of immunomagnetically selected CD45\(^+\) lung cells revealed that, at 90 days postinfection, the number of CD4\(^+\) T cells expressing the early activation marker CD69 in the lungs of CXCR3\(^{-/-}\) mice was significantly higher than that detected in wild-type mice (Fig. 7A; \(2.3 \times 10^6\) in CXCR3\(^{-/-}\) mice and \(1.5 \times 10^6\) in wild-type mice; \(p = 0.0200\)). By contrast, the number of
pulmonic CD4⁺ T cells expressing CD62L (a naive T cell marker lacking in effector T lymphocytes) was significantly higher in wild-type compared with CXCR3-deficient mice (Fig. 7A; 0.45 × 10⁶ in CXCR3⁻/⁻ mice and 0.66 × 10⁶ in wild-type BALB/c mice; \( p = 0.0236 \)). The expression of these activation status markers by the CD4⁺ T cells of tuberculous CXCR3⁻/⁻ and wild-type BALB/c mice was comparable at 2 wk and 1 mo postinfection (Fig. 7A). Thus, the postinfection time point where M. tuberculosis-infected CXCR3-deficient mice displayed a higher level of CD4⁺ T cell activation coincided with that beyond which the knockout animals exhibited a better ability to control the tubercle bacillus compared with wild-type mice (Fig. 2). These data suggest that a more robust effector CD4⁺ T cell response could account for the superior ability of CXCR3⁻/⁻ mice to control M. tuberculosis.

The events involved in the development of a CD4⁺ T cell response during the various phases of M. tuberculosis infection are just beginning to be defined (25, 26). The priming of T cells by APCs likely plays an important role in the development and subsequent maintenance of the T cell response to M. tuberculosis infection. Indeed, the results of intracellular staining studies revealed that the number of IFN-γ-producing CD4⁺ T cells in the lungs of CXCR3⁻/⁻ mice were higher than that of wild-type mice (Fig. 7B; 8 × 10⁴ (2.6% of CD4⁺ T cells) in CXCR3⁻/⁻ mice and 4 × 10⁴ (1.4% of CD4⁺ T cells) in wild-type mice). More importantly, the same was observed when CD4⁺ T cells obtained from the MLNs of infected animals were analyzed (Fig. 7B; 3.2 × 10⁴ (1% of CD4⁺ T cells) in CXCR3⁻/⁻ mice and 2 × 10⁴ (0.45% of CD4⁺ T cells) in wild-type mice). These results
suggest that enhanced T cell priming for a Th1 response is more robust in CXCR3<sup>−/−</sup> mice than wild-type mice. This may lead to the subsequent development and maintenance of an augmented CD4<sup>+</sup> T cell effector response that can confer a better ability on the knockout strain to control the tubercle bacillus in the chronic phase of infection.

**Analysis of gene expression in the lungs of M. tuberculosis-infected CXCR3<sup>−/−</sup> BALB/c mice**

The chemokine/chemokine receptor system is marked by considerable redundancy in its capacity to allow multiple ligands the ability to bind a number of receptors (27, 28). To determine whether other relevant chemokine receptors or chemokine ligands would be differentially expressed in the absence of CXCR3, we analyzed total RNA obtained from M. tuberculosis-infected CXCR3<sup>−/−</sup> and wild-type lung tissue 1 mo postinfection by real-time PCR (Fig. 8). At this time point the lung bacterial load is comparable between the two mouse groups and, therefore, any differences observed in gene expression can be reasonably ascribed to the direct or indirect effect of CXCR3. Additionally, although gene expression studies using whole infected lungs could generate potentially erroneous information due to differential expression of inflammatory molecules in granulomatous vs nongranulomatous tissues, such errors could be circumvented by using LCM-procured granulomatous tissues (19). However, we chose to use whole infected lungs for our studies, because at 1 mo postinfection the levels of the granulomatous inflammation were comparable in the two mouse strains examined based on histological examination.
secreted phosphoprotein (Spp) 1 (42–45) (Fig. 8E, MMP-9, tissue inhibitor of metalloproteinase 2 (TIMP-2), and other inflammatory mediators like matrix metalloproteinase 9 infected CXCR3-deficient and wild-type BALB/c mice, as were replication (41), was expressed to similar levels in mediating granuloma formation and subsequent tubercle bacil-

The results of our studies revealed no significant differences in the gene expression levels for either chemokine receptors (except that for CXCR3, used as a control for the assay; \( p = 0.0006 \)) or chemokine ligands of interest (Fig. 8, A and B), including CXCL9 and CXCL10, the ligands for CXCR3. The cytokines IFN-\( \gamma \), TNF-\( \alpha \), and IL-12 have been clearly established as playing important roles in host defense against M. tuberculosis (1, 29). Controversy notwithstanding, type I IFNs (IFN-\( \alpha \) and IFN-\( \beta \)) have been implicated in affecting the ability of the host to control M. tuberculosis infection (30). In examining relative gene expression levels of these cytokines among the two groups of mice, no significant differences can be detected (Fig. 8, C and D). Expression studies on molecules involved in macrophage M. tuberculosis sensing and killing mechanisms, namely NOS2 (31–33), TLR2 (34–37) (Fig. 8C), LRG-47 (Fig. 8E), the IFN-\( \gamma \)-dependent, NOS2-independent macrophage anti-tuberculous effector mechanism (38), and P2rx7, a member of a family of ion channels gated by extracellular ATP implicated in mediating intracellular mycobacterial killing (39, 40), showed no significant differences between CXCR3\(^{-/-}\) and wild-type BALB/c (Fig. 8E). ICAM-1, shown to be of importance in mediating granuloma formation and subsequent tubercle bacillary containment as a means of controlling their dissemination and replication (41), was expressed to similar levels in M. tuberculosis-infected CXCR3-deficient and wild-type BALB/c mice, as were other inflammatory mediators like matrix metalloproteinase 9 (MMP-9), tissue inhibitor of metalloproteinase 2 (TIMP-2), and secreted phosphoprotein (Spp) 1 (42–45) (Fig. 8E). Activated leukocyte cell adhesion molecule (ALCAM; CD166), the ligand for CD6, an important modulator in T cell activation and proliferation (46), displayed a slightly higher level of expression in CXCR3\(^{-/-}\) mice compared with wild type, but this difference did not achieve statistical significance (Fig. 8E). Lastly, evidence exists that regul-

### Discussion

The chemokine superfamily represents a large number of small soluble proteins (8–10 kDa) capable of binding directly to G-protein coupled receptors (28, 51). Chemokines and their receptors have been implicated in various functions ranging from the homeostatic maintenance of the hemopoietic system to tissue repair to mediating host immune responses through their ability to modulate leukocyte migratory and trafficking patterns (52–55). More recent evidence suggests that chemokines may play a role in regulating the interactions between immune cells (56–58). The chemokine receptor CXCR3 binds the \( \alpha \)-chemokines CXCL9, CXCL10, and CXCL11 and is primarily expressed on activated T cells, B cells, NK cells, and dendritic cells. CXCR3 is preferentially expressed on CD4\(^{+}\) Th1 cells (59, 60). The role of CXCR3 in directing Th1 cell trafficking has been evinced in a wide array of experimental systems. For example, CXCR3 blockade using anti-CXCR3 mAb decreases recruitment of Th1 cells into the peritoneal cavity subsequent to adjuvant-induced peritonitis (61). In a murine model of cardiac transplantation, CXCR3\(^{-/-}\) recipients and anti-CXCR3 mAb-treated wild-type mice showed extended cardiac allograft survival coupled with a significant decline in the recruitment of CD4\(^{+}\) and CD8\(^{+}\) T cells into the transplanted organ (17). CXCR3 and its ligand CXCL10 have also been shown to...
mediate the trafficking and infiltration of T cells at the site of lung allograft rejection (62).

Given the significance of CXCR3 in directing the trafficking of Th1 type cells and the importance of this lymphocyte subset in engendering protection against *M. tuberculosis*, it is not unreasonable to predict that mice deficient in this receptor may be more susceptible to the tubercle bacillus than wild-type animals. However, the present study has provided evidence that CXCR3-deficient mice, compared with their wild-type counterparts, are better able to control tuberculous infection. The results of studies using BALB/c and C57BL/6 CXCR3-deficient mice have revealed a strain-dependent tissue specificity of this relative resistance phenotype. Thus, while CXCR3−/− BALB/c mice show superior resistance compared with wild-type mice in the lungs and spleen, the resistance phenotype of the CXCR3-deficient C57BL/6 animals is apparent only in the latter organ. This strain-dependent tissue specificity is not surprising for the following reasons: 1) it is well established that the control of *M. tuberculosis* involves multiple host factors (63–65); 2) the level of susceptibility to the tubercle bacillus spans a wide range among the numerous congenic mouse strains studied (66); and 3) different animal species display specific patterns of organ sensitivity to the tubercle bacillus (67).

Although histological analysis of infected lung tissues did not reveal any obvious differences among *M. tuberculosis*-infected CXCR3−/− and wild-type BALB/c mice at 1, 3, and 6 mo postinfection, results of the flow cytometric studies have shown a marked difference in the cellular composition in the pulmonic leukocyte infiltrates among the two groups. Most striking is the marked discrepancy in the numbers of CD4+ and CD8+ T cells during the chronic phase of infection (Fig. 5) that translates into a remarkable difference in the ratio of these lymphocyte subsets present in the lungs of *M. tuberculosis*-infected CXCR3−/− and wild-type BALB/c mice. At 3 mo postinfection, the CD4+/H11001 and CD8+/H11002 T cell ratio of CXCR3−/− BALB/c mice was 2.3, whereas that of wild-type mice was 1.1. At 6 mo after aerosol challenge, the ratios were 3.6 and 1.0 for the CXCR3 knockout and wild-type BALB/c, respectively. It has been observed in human tuberculosis that the CD4+/CD8+ T cell ratio derived from results of immunophenotyping experiments of bronchoalveolar lavage fluid cells correlates well with the rate of disease regression (68). However, the cause-effect relationship between the discrepancy in the ability of the two mouse groups to control *M. tuberculosis* in terms of tissue bacterial burden and the remarkable differences in the number of lung CD4+ and CD8+ T cells is unclear. The increase in pulmonic CD11c+ cells observed in *M. tuberculosis*-infected, CXCR3-deficient mice compared with wild-type mice most likely represents that of the dendritic cell population, as the numbers of macrophages (identified as F4/80+), a leukocyte subset that can also be CD11c+, are comparable in the lungs of the two mouse groups (Fig. 6). Whether the increased CD11c+ cells contribute to the higher number of CD4+ T lymphocytes in the lungs of the CXCR3−/− mice through direct or indirect interactions that lead to the expansion of T cells remains to be examined. The number of lung neutrophils in *M. tuberculosis*-infected CXCR3−/− BALB/c mice is lower than that in the wild type at all of the time points analyzed. This finding is in agreement with that of a previous study reporting that neutrophil migration in response to *M. tuberculosis* infection in CXCR3−/− C57BL/6 mice is deficient compared with that of the wild-type parental strain (21). A further determination as to whether the altered lung cell composition in the *M. tuberculosis*-infected CXCR3−/− mice results in the enhanced capacity of the knockout animals to control *M. tuberculosis* infection would be required. Equally unclear is the role of CXCR3 in regulating the cellular makeup of the tuberculous granulomatous tissues. Given the well-established role of CXCR3 in directing the trafficking of immune cells in various inflammatory states, it is reasonable to assume that the altered cellular composition observed in the lungs of *M. tuberculosis*-infected knockouts is, at least partially, due to aberrant cell migration because of CXCR3 deficiency. However, the ability of CXCR3 to regulate the migration of Th1 cells is unlikely to singularly and directly explain the phenotype observed in *M. tuberculosis*-infected, CXCR3-deficient mice.

Worthy of note, the relative superiority of CXCR3-deficient mice in containing *M. tuberculosis*, as assessed by tissue bacterial burden, manifests itself only in the chronic phase of infection, which begins between 1 and 3 mo after the initial aerosol challenge and lasts until at least 6 mo after the challenge. Thus, the relative resistance phenotype observed in the lungs of *M. tuberculosis*-infected BALB/c mice was not apparent during the initial month of infection when the bacilli replicated at a rapid rate in the host. By 3 mo after infection, the resistance phenotype became obvious. This resistance phenotype was maintained for at least up to six months after the initial *M. tuberculosis* aerosol challenge. These data suggest that the resistance phenotype observed in the CXCR3-deficient BALB/c mice could be due to the development of an antituberculous adaptive immune response more effective in controlling the disease than that of the wild-type animals. This phenotype could be the result of the more efficient T cell priming that occurs during the innate phase of the immune response in the absence of CXCR3. The robustness of T cell priming in the initial phase of tuberculous infection could have significant quantitative and qualitative effects on the development and subsequent maintenance of the antituberculous adaptive immune response, including the establishment of T cell memory, an immunological compartment that plays an important role in the control of chronic infection (69–71).

Emerging evidence suggests that the function of chemokines and chemokine receptors go beyond regulating cell trafficking (58). For instance, directly relevant to the present study, it has been reported that the interaction between CXCR3 and its ligand CXCL10 can adversely affect the priming of T cells by APCs through disruption of the immunological synapse via attenuation of the MHC-Ag/TCR stop signal (56). If this mechanism is operative during tuberculous infection in the mouse, then the absence of CXCR3 may well result in enhanced T cell priming. Indeed, our observation that there are more IFN-γ-producing CD4+ T cells in the lungs and MLNs of *M. tuberculosis*-infected CXCR3−/− mice than in those of wild-type mice at 1 mo postinfection (Fig. 7) suggests that, in a tuberculous host, this chemokine receptor can impair priming. This could translate into the development of a T cell response with a higher state of activation as indicated by an increase in CD69-expressing and a decrease in CD62L-expressing CD4+ T cells in the lungs of tuberculous CXCR3−/− mice (Fig. 7) that results in better control of the tubercle bacillus.

The roles of chemokines and chemokine receptors in regulating the host granulomatous response to *M. tuberculosis* are just beginning to be addressed. In various murine experimental tuberculosis models, disruption of the genes encoding for the chemokine receptors CCR2 and CCR5 have resulted in the aberrant trafficking of immune cells to the lungs and an altered pulmonic inflammatory response during tuberculous infection (8, 9). The CCR2 studies revealed that this receptor is dispensable for the recruitment of T cells into *M. tuberculosis*-infected lungs (72) and, although high inoculum infection results in a hypersusceptibility phenotype in CCR2−/− C57BL/6 mice compared with wild-type mice, a low-dose challenge does not alter the ability of the receptor-deficient host to control the tubercle bacilli (9). Similarly, CCR5−/− C57BL/6 mice exhibit no defect in the control of a low-dose...
11. Mohan, V. P., C. A. Scanga, K. Yu, H. M. Scott, K. E. Tanaka, E. Tsang, M. M. Mokine network can modulate the host immune response through priming in a tuberculous host is another example of how the chemokine promoter that results in increased production of MCP-1 are more susceptible to pulmonary tuberculosis (74). This increased susceptibility obviously related to the traffic-directing attributes of the chemokine receptor CXCR3-signaling chemokines. Eur. J. Immunol. 33: 2676–2686.


