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*J Immunol* 2009; 183:8004-8014; Prepublished online 23 November 2009;
doi: 10.4049/jimmunol.0901937
http://www.jimmunol.org/content/183/12/8004

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In a Murine Tuberculosis Model, the Absence of Homeostatic Chemokines Delays Granuloma Formation and Protective Immunity

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Mycobacterium tuberculosis infection (Mtb) results in the generation of protective cellular immunity and formation of granulomatous structures in the lung. CXCL13, CCL21, and CCL19 are constitutively expressed in the secondary lymphoid organs and play a dominant role in the homing of lymphocytes and dendritic cells. Although it is known that dendritic cell transport of Mtb from the lung to the draining lymph node is dependent on CCL19/CCL21, we show in this study that CCL19/CCL21 is also important for the accumulation of Ag-specific IFN-γ-producing T cells in the lung, development of the granuloma, and control of mycobacteria. Importantly, we also show that CXCL13 is not required for generation of IFN-γ responses, but is essential for the spatial arrangement of lymphocytes within granulomas, optimal activation of phagocytes, and subsequent control of mycobacterial growth. Furthermore, we show that these chemokines are also induced in the lung during the early immune responses following pulmonary Mtb infection. These results demonstrate that homeostatic chemokines perform distinct functions that cooperate to mediate effective expression of immunity against Mtb infection.

Immunity to tuberculosis (TB) is characterized by the induction and recruitment of IFN-γ-producing T cells to the lungs, IFN-γ-dependent activation of Mycobacterium tuberculosis (Mtb)-infected macrophages and subsequent mycobacterial control (1). The formation of an organized pulmonary granuloma containing recruited lymphocytes and mononuclear cells is essential for immunity and to limit tissue damage (2). However, the cascade of early signals that is induced following Mtb infection, which mediate cell recruitment and granuloma formation are not well understood.

Homeostatic chemokines such as the CXCL13, CCL19, and CCL21 are expressed in secondary lymphoid organs and direct the steady-state homing and localization of lymphocytes and dendritic cells (DC) within these organs (3, 4). CCL19 and CCL21 are expressed by stromal cells in the paracortical T cell zones and expressed by follicular DC as well as by stromal cells, accumulation of Ag-specific IFN-γ-producing T cells, development of the granuloma, or for control of mycobacteria in the lung. CXCL13 is expressed by follicular DC as well as by stromal cells in the B cell areas and orchestrates the homing of CXCR5 expressing lymphocytes to the follicular areas of the secondary lymphoid organs (10). It is not known whether the homeostatic chemokine CXCL13 is required for priming, initiation, or maintenance of immune responses to Mtb or whether it is essential for granuloma formation. Homeostatic chemokines are also induced in the lung during infection and inflammation and initiate the recruitment of immune cells (11, 12). The accumulation of lymphocytes and mononuclear cells in response to infection and inflammation can resemble ectopic lymphoid follicles. These structures contain well-established B and T cell areas, defined germinal centers (GCs) and HEVs and have been termed iBALT (inducible bronchus associated lymphoid tissue) (13). It has been suggested that granulomas resulting from Mtb infection contain areas that resemble ectopic lymphoid follicles both in humans (14) and mice (9, 15). However, it is not known whether homeostatic chemokines have a role to play in the generation of lymphoid structures during TB.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901937
To investigate the relationship between homeostatic chemokine expression and protective cellular responses and granuloma formation, we compared the immune response of wild-type mice with those of mice lacking the homeostatic chemokines following Mtb infection. Normal mice possess at least two independent CCL21 genes, Cca2a and Cca2b. The Cca2a gene encodes a serine at position 65 of CCL21 (CCL21-Ser) and is known to be expressed in both secondary lymphoid organs and lymphatics. However, the Cca2b gene, encodes leucine at position 65 (CCL21-Leu) and is expressed only in the lymphatic endothelium of peripheral tissues. We have used plt/plt mice (16) that do not express CCL21-Ser (Cca2a) or CCL19 protein in secondary lymphoid organs but continue to express CCL21-Leu (Cca2b) at reduced levels in lymphatic endothelium (17, 18). Furthermore, the plt/plt mice demonstrate abnormalities in DC and lymphocyte migration as well as in lymph node organization and size (19, 20). In the present study using the plt/plt mice, we show that CCL19/CCL21 is required for DC accumulation in the DLN, optimal priming and generation of activated IFN-γ-producing T cells, accumulation of IFN-γ Ag-specific cells in the lung, and granuloma formation and protection following Mtb challenge.

To determine the role of the homeostatic chemokine CXCL13 in response to Mtb infection, we used mice that lack the expression of CXCL13 (Cxc13−/− mice) (21, 22). Cxc13−/− mice have defects in B cell trafficking (22, 23), organization of B cells in the lymphoid organs (21), generation of GCs (24), and development of lymph nodes (21). Despite the lack of peripheral lymph nodes, we show that following Mtb challenge, Cxc13−/− mice prime Ag-specific IFN-γ-producing cells in the spleen and accumulate activated T cells in the lungs similar to wild-type mice. However, our data show a novel role for CXCL13 in spatial organization of the lymphocytes within the inflammatory lesion, optimal activation of phagocytes, and control of Mtb. These data suggest that homeostatic chemokines CCL19/CCL21 and CXCL13 play distinct roles in initiation and maintenance of cellular responses that contribute to protective immunity and granuloma formation during Mtb infection.

Materials and Methods

Animals

C57BL/6 (B6) mice were purchased from The Jackson Laboratory. Cxc13−/− (22) and plt/plt (16) mice on the B6 background were obtained from J. Oyster (University of California, San Francisco, CA) and bred at the Trudeau Institute (Saranac Lake, NY). The plt/plt mice were crossed with Cxc13−/− mice to generate double knockout (DKO) mice. ESAT6 (early secreted antigenic protein 6) αβ TCR transgenic (Tg) mice, recognizing IAα/ESAT61-20 were generated by Dr. G. Winslow (Wadsworth Center, Albany, NY) and Dr. D. Woodland (Trudeau Institute, Saranac Lake, NY) (25). The ESAT6 TCR Tg mice were crossed to Rag1−/− mice. Experimental mice were age- and sex-matched and used between the ages of 8 and 10 wk. Mice were used in accordance with National Research Council and Trudeau Institute International Animal Care and Use Committee guidelines.

Experimental infection

The H37Rv strain of Mtb was grown in Proskauer Beek Medium containing 0.05% Tween 80 to mid-log phase and frozen in 1 ml of aliquots at −70°C. For aerosol infections, the mice were exposed to a 70-μm nylon tissue strainer (BD Falcon) for 45 min at 37°C, to remove B cells and macrophages. The Tg T cells were harvested and used as APC at a concentration of 1 × 105 cells/well. The resultant suspension was treated with Gey’s solution to remove any residual RBC. Cells were subsequently panned on goat anti-mouse IgG H chain +L chain-coated (Jackson Immunoresearch Laboratories) Primaria flasks (BD Falcon) for 45 min at 37°C, to remove B cells and macrophages. The Tg T cells were next enriched using a CD4 T cell-negative isolation kit (Miltenyi Biotec). A sample of the sorted cells was analyzed to confirm purity and to evaluate the surface expression of the activation markers CD69 and CD44. Purified Tg T cells were labeled with 0.5 μm CFSE (Invitrogen) for 10 min at 37°C. A total of 1 × 106 Tg T cells (200 μl) were transferred i.v. into Mtb-infected mice. Twelve hours later, mice were sacrificed, and the surface expression of CD69 and CD44 on transferred Tg T cells was determined.

Flow cytometry

Single cell suspensions were stained with fluorochrome-labeled Abs specific for CD4 (clone GK1.5), CD69 (clone HIF2S), CD44 (clone IM7), CD11c (clone HL3), MHC class II I-A<sub>d</sub> (clone AF6-120.1), GL-7 (clone GL-7), and CD95 (clone 15A7). Cells were collected on a BD FACSCalibur flow cytometer using CellQuest software or on a DakoCyAn ADP flow cytometer. Cells were gated based on their forward and side scatter characteristics and the frequency of specific cell types determined using FlowJo (Tree Star).

Real-time PCR

RNA was extracted from lung tissue as previously described (29). RNA samples were treated with DNase and reverse transcribed. cDNA was then amplified using FAM-labeled probe and PCR primers on the ABI Prism 7700 sequence detection system. Fold increase in signal over that derived from uninfected lungs was determined using the threshold cycle (ΔΔCt) calculation. The primer and probe sequence for murine gapdh, Ifng, Tnf, Facility). Endogenous biotin was neutralized with avidin followed by biotin (Sigma-Aldrich). Sections were probed with goat anti-mouse CD3ε to detect CD3 lymphocytes (clone M-20; Santa Cruz Biotechnology). F4/80 to detect macrophages (rat anti-mouse F4/80, clone CI: A3-1; Serotec, B220 to detect B cells (rat anti-mouse B220, clone RA3-6B2; BD Pharmingen) and inducible NO synthase (iNOS) to detect activated macrophages (goat anti-iNOS, clone M-19; Santa Cruz Biotechnology). Primary Abs were detected with secondary Abs conjugated to CY3 for iNOS and CD3 (Cy3 Donkey Fab anti-goat; Jackson ImmuNoResearch Laboratories), donkey anti-rat Ab conjugated to Alexa Fluor 488 for F4/80 (Molecular Probes) and streptavidin-Alexa Fluor 488 for B220 (Molecular Probes). Slow fade gold antifade with DAPI (Molecular Probes) was used to counterstain tissues and to detect nuclei. Images were obtained with a Zeiss Axio Plan 2 microscope and were recorded with a Zeiss Axio Cam digital camera. Caudal lobes from four mice per group underwent morphometric analysis in a blinded manner using the morphometric tool of Zeiss Axioplan microscope, which determines the area defined by the squared pixel value for each granuloma as previously described (27).

Lung and DLN cell preparation

Lung tissue and DLN was prepared as described (26). Briefly, a single cell suspension was prepared from either digested lung tissue or DLN by dispersing the tissue through a 70-μm nylon tissue strainer (BD Falcon). The resultant suspension was treated with Gey’s solution to remove any residual RBC, washed twice, and counted (26).

Detection of IFN-γ-producing cells by ELISPOT assay

Ag-specific IFN-γ-producing IA<sub>α</sub>-restricted T cells from infected lungs or DLN were enumerated using peptide-driven ELISPOT as previously described (28, 29). Briefly, 96-well microtiter ELISPOT plates were coated with monoclonal anti-mouse IFN-γ and then blocked with medium containing 10% FBS. Cells from lungs and DLN were seeded at an initial concentration of 1 × 105 cells/well and subsequently diluted 2-fold. Irradiated B6 spleenocytes were used as APC at a concentration of 1 × 106 cells/well in the presence of ESAT6<sub>1-20</sub> (10 μg/ml) peptide and IL-2 (10 U/ml) (28). After 24 h, plates were washed and probed with biotinylated anti-mouse IFN-γ. Spots were visualized and enumerated using a dissection microscope. No spots were detected in cultures lacking Ag or when using cells from uninfected mice.

Naïve CD4 T cell isolation

To generate a population of Tg CD4 T cells from ESAT6 TCR Tg mice, a single cell suspension was prepared from lymph nodes and spleens by dispersing the tissues through a 70-μm nylon tissue strainer (BD Falcon). The single cell suspension was treated with Gey’s solution to remove any residual RBC. Cells were subsequently panned on goat anti-mouse IgG H chain +L chain-coated (Jackson Immunoresearch Laboratories) Primaria flasks (BD Falcon) for 45 min at 37°C, to remove B cells and macrophages. The Tg T cells were next enriched using a CD4 T cell-negative isolation kit (Miltenyi Biotec). A sample of the sorted cells was analyzed to confirm purity and to evaluate the surface expression of the activation markers CD69 and CD44. Purified Tg T cells were labeled with 0.5 μm CFSE (Invitrogen) for 10 min at 37°C. A total of 1 × 106 Tg T cells (200 μl) were transferred i.v. into Mtb-infected mice. Twelve hours later, mice were sacrificed, and the surface expression of CD69 and CD44 on transferred Tg T cells was determined.

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and inos has been published (29). The oligonucleotides used for the detection of CCL21-Leu were designed and validated by the Trudeau Institute Molecular Biology Core Facility and were forward 5'-AGACTCAGGAGCCCAAAGCA-3', reverse 5'-GTTGAAGCAGGGCAAGGGT-3', probe 5'-FAM-CCACCTCATGCTGGCCTCCGT-BHQ. Primer and probes used for the detection of Ccl19 and Cxcl13 were purchased from Applied Biosystems.

Statistical analysis

Differences between the mean of experimental groups were analyzed using the two-tailed Student t test. Differences were considered significant when \( p < 0.05 \).

Results

Homeostatic chemokines are induced in the lungs during the early immune response following Mtb infection

Mtb infection results in induction of homeostatic chemokines CCL19, CCL21, and CXCL13 in the lungs of infected mice (30). However, the kinetics of induction of these homeostatic chemokines in the lung during the early stages of infection are not known. To determine the timing of induction of homeostatic chemokines during the Mtb infection, we aerosol-infected wild-type B6 mice with a low dose of Mtb and monitored the induction of mRNA for homeostatic chemokines in the lung. Sustained induction of mRNA for the homeostatic chemokines CCL19 and CXCL13 occurred between days 18 and 21 following infection (Fig. 1A). Expression coincided with the induction of mRNA for IFN-\( \gamma \), TNF-\( \alpha \), and iNOS in the lungs of infected mice (Fig. 1B). In contrast, mRNA for CCL21 was not induced during infection. These data demonstrate that the homeostatic chemokines CCL19 and CXCL13, but not CCL21 are induced in the lung during the early phase of the immune response to Mtb infection and that this induction coincides with the expression of protective inflammatory responses in the lung.

A hallmark of immunity to Mtb is cellular accumulation and development of organized granulomatous response in the lung (2). To determine whether induction of chemokines correlated temporally with cellular events in the lung, we followed the kinetics of initiation and generation of the granuloma formation in the lungs of Mtb-infected wild-type B6 mice. The earliest signs of granuloma formation were evident by day 18 and progressed to the formation of well-established granulomata from day 21 through day 30 postinfection (Fig. 2A). Granuloma formation coincided with the recruitment of CD3 \( \text{T} \) cells and B220 cells on day 18 postinfection (Fig. 2B). The increase in size of the granulomata between days 21 and 30 (Fig. 2A) coincided with the formation of distinct lymphoid-like structures in the lung. These included defined areas of B220 cells that were surrounded by CD3 lymphocytes (Fig. 2B). Furthermore, the area occupied by the B220\(^+\) cells progressively increased from day 18 to day 30 postinfection with Mtb (Fig. 2, C and D). Thus, there is a temporal correlation in the induction of homeostatic chemokines, recruitment of lymphocytes, and granuloma formation. The timing of these events also correlated with the expression of protective immune responses in the lung.

FIGURE 1. Homeostatic chemokines are induced in the lungs during the early immune response following Mtb infection. B6 mice were infected with ~75 CFU of Mtb via the aerosol route and at specific times after infection, lung tissue was harvested and processed to extract RNA. The presence of mRNA for different homeostatic chemokines CCL19, CCL21, and CXCL13 (A) and protective molecules IFN-\( \gamma \), TNF-\( \alpha \), and iNOS (B) was determined by real-time PCR, and the \( \log_{10} \)-fold increase in mRNA was determined for four infected mice vs four uninfected mice. Data points represent the mean and SD for four mice for each time point. One experiment representative of two is shown.
FIGURE 2. Early induction of homeostatic chemokines correlate with the initiation of granuloma formation in the lung following Mtb infection. B6 mice were infected with ~75 CFU of Mtb via the aerosol route and at specific times after infection, lung tissue was harvested and processed as described. Upon harvest, the caudal lobe of the lung from each of four mice per group was perfused with 10% formal saline solution, embedded in paraffin, sectioned, and stained using H&E. A, Morphometric analysis of area covered by the granuloma in H&E-stained sections of Mtb-infected B6 lungs at different time points during the early immune response was determined. Data points represent the mean and SD for four mice for each time point. B, Lung sections from infected B6 mice at different time points postinfection were stained for B220 (green) and CD3 (red) and shown at a magnification of ×40. Data are representative of four mice per group. Morphometric analysis of total area (C) and average area (D) covered by the B cell areas (B220<sup>+</sup>) in sections of infected lungs of B6 mice at different time points following infection. Data points represent the mean and SD for four mice for each time point. One experiment representative of two is shown.

The expression of homeostatic chemokines is important for initiation of granuloma formation following Mtb infection

Following low-dose aerosol infection with Mtb, the lungs of wild-type mice developed a mononuclear infiltrate by day 20 postinfection (Fig. 3, left column) that progressed toward a mix of defined lymphocytic areas intermixed with macrophage aggregates by day 50 (Fig. 3, middle column). Well-defined B220<sup>+</sup> cell areas and activated iNOS-producing macrophages were present by day 50 in wild-type B6 mice (Fig. 3, right column). In contrast to the granulomatous response in wild-type mice, the absence of either CCL19/CCL21 (plt/plt mice), CXCL13 (Cxc13<sup>−/−</sup>), or all homeostatic chemokines (DKO) resulted in poorly developed granuloma on day 20 (Fig. 3, left column). There was also a failure to develop the pronounced lymphocytic areas observed in the wild-type mice on day 50 (Fig. 3, middle column). Although iNOS-producing activated macrophages were detected in chemokine-deficient mice on day 50 postinfection, the organization of defined B220<sup>+</sup> cell areas in the lung was absent in the Cxc13<sup>−/−</sup> and DKO infected lungs, and markedly compromised in the plt/plt mice (Fig. 3, right columns). These data suggested that in the absence of homeostatic chemokines, initiation and organization of lymphoid areas and granulomas in the lungs of infected mice is markedly disrupted.

Delayed accumulation of IFN-γ-producing T cells in the absence of CCL19/CCL21 but not CXCL13

Because the homeostatic chemokines are required for the development of the granulomatous response and organization of lymphoid areas in the lung, we next addressed whether there was a role for homeostatic chemokines in the acquired T cell response to infection. Analysis of intracellular IFN-γ expression by CD4 lymphocytes from the lung demonstrated that the absence of CCL19/CCL21 (Fig. 4A), but not CXCL13 (Fig. 4B), ablated the accumulation of IFN-γ-producing cells in the infected lungs on day 20 postinfection. When we determined the kinetics of the Ag-specific IFN-γ response by ELISPOT, we found that this response was delayed in the absence of CCL19/CCL21 but not in the absence of CXCL13 (Fig. 4C). The delay in recruitment also correlated with a decreased frequency of activated CD4<sup>4<sup>high</sup></sup> CD4 T cells in the lungs of plt/plt mice relative to B6 and Cxc13<sup>−/−</sup> mice at day 20 postinfection (data not shown). These data demonstrate that CCL19/CCL21, but not CXCL13, is required for early accumulation of Ag-specific IFN-γ-producing cells in the lungs of Mtb-infected mice.

To investigate the impact of the homeostatic chemokine deficiency on the accumulation of activated Ag-specific cells within the infected lung, we adoptively transferred purified CFSE-labeled ESAT6 TCR Tg CD4 T cells into infected wild-type and chemokine-deficient mice at different time points postinfection and compared the frequency of activated Tg cells accumulating in the lung. Twelve hours following transfer, the activation status of transferred ESAT6 TCR Tg T cells demonstrated that despite the absence of CXCL13, activated CD4 Ag-specific T cells accumulated in the lungs of day 20-infected mice (Fig. 4D). In contrast, the absence of CCL19/CCL21 abrogated accumulation of activated CD4 Ag-specific T cells in the lungs of day 20 infected mice.
These data suggest that CCL19/CCL21 but not CXCL13 are critical for accumulation of activated T cells during the early immune response in the lungs following Mtb infection.

To determine whether the delayed accumulation of IFN-γ-producing cells in the infected lungs of plt/plt mice would impact expression of immunity, we evaluated the expression of protective molecules in the lung by real-time PCR. mRNA transcripts for IFN-γ and iNOS were induced in the lungs of infected B6 mice by day 20, and the transcript expression was maintained over time. However, the early induction of both IFN-γ and iNOS mRNA was delayed in the absence of CCL19/CCL21 (Fig. 5A). This delay also correlated with the absence of activated iNOS-producing F4/80-expressing macrophages in the plt/plt mice at day 20 compared with the B6 controls (Fig. 5B). This deficiency was overcome by day 50 at both mRNA and protein levels for iNOS (Fig. 5, A and B). These data suggest that the delay in recruitment of activated IFN-γ-producing T cells to the lungs of plt/plt mice results in a delay in macrophage activation. To determine whether this delay in generation of acquired cellular responses affects bacterial control, B6 and plt/plt mice were infected with Mtb and the bacterial burden in the lungs determined (Fig. 5C). The wild-type B6 and plt/plt mice had similar bacterial burden in the lung on day 20, suggesting that initial infection and replication of the bacteria in the lungs of these mice was comparable. However, plt/plt mice were less able to limit bacterial growth after day 20 compared with the wild-type mice, indicating that the delay in the generation of acquired immunity resulted in the establishment of higher bacterial numbers.

The generation of IFN-γ-producing T cells is compromised in the absence of CCL19/CCL21

The plt/plt mice have defective lymph nodes and compromised DC migration (7). Furthermore, transport of Mtb to the lung DLN via DC is likely to be dependent on CCL19/CCL21 (8), suggesting that poor accumulation of IFN-γ-producing cells in the infected lung could reflect poor induction of Ag-specific, IFN-γ-producing T cell responses in lymph nodes. To address this issue, we compared the frequency and number of cells within the DLN of wild-type and plt/plt mutant mice. As expected, although the number of white blood cells (WBCs) in the lungs of B6 and plt/plt mice were similar on day 20 following infection, the number of WBCs in the DLN of plt/plt mice was significantly lower (Fig. 6A). Also as reported previously (8), the lymph nodes of plt/plt Mtb-infected mice had markedly reduced the frequency and number of CD11c+ cells (Fig. 6, B and C). To assess the impact of the reduced number of CD11c+ cells on initiation of IFN-γ T cell responses in the DLN, we next evaluated the frequency of Ag-specific IFN-γ-producing cells in the DLN using ELISPOT. Despite the low number of WBCs in the DLN, and the decreased CD11c+ cells in the lymph nodes, the frequency of Ag-specific IFN-γ-producing cells in the infected plt/plt mice was similar to that detected in infected wild-type mice (Fig. 6D). However, despite this similar frequency, the reduced number of cells found in the plt/plt lymph nodes resulted in significantly fewer IFN-γ-producing cells in the lymph nodes of infected plt/plt mice (Fig. 6E). These data suggest that reduced DC accumulation in the DLN in the plt/plt.
mice, results in induction of reduced magnitude of IFN-γ responses in the lymphoid organs and delayed accumulation of IFN-γ-producing population in the lungs. Furthermore, we also found a reduced number of Ag-specific IFN-γ-producing cells in the spleens of day 20-infected plt/plt mice when compared with B6-infected mice (Fig. 6F).

To further address the role of CCL19/CCL21 in initiation of Mtb-specific T cell responses, we analyzed the response of adoptively transferred CFSE-labeled ESAT6 TCR Tg cells. On day 20 postinfection, ESAT6 TCR Tg CD4 T cells were transferred in wild-type and plt/plt mice and 12 h later, the surface expression of CD69 on transferred cells within the DLN was compared. We found that the frequency of Tg cells expressing CD69 in the lymph nodes of B6 and plt/plt mice was comparable (Fig. 6G). These data suggest that despite the fact that plt/plt mice have several known defects in homing of T cells and DC to the secondary lymphoid organs (7), activation and polarization of IFN-γ T cells can occur in the absence of CCL19/CCL21. However, the total number of cells in the lymph nodes of plt/plt mice was much lower and this likely impacts the kinetics of the accumulation of IFN-γ-producing cells in the lungs of plt/plt mice.

CXCL13 is required for spatial organization of lymphocytes within the granulomas

We expected that the comparable frequency and number of Ag-specific IFN-γ-producing cells in the lungs of wild-type and Cxcl13−/− mice (Fig. 4, B and C) would result in comparable induction of acquired immune responses following infection. To determine whether this was the case, we performed real-time PCR analysis of infected lung tissue from wild-type and Cxcl13−/− mice and found that the early expression of IFN-γ, iNOS (Fig. 7A), and TNF-α (data not shown) were delayed in the lungs of Cxcl13−/−-infected mice. To investigate this furthermore, we compared the numbers and activation of CD11b+ and CD11c+ myeloid cells within the lungs of infected wild-type and Cxcl13−/− mice (Fig. 7, A and B). Despite the presence of an equivalent number of activated T cells (data not shown) and IFN-γ-producing T cells (Fig. 4, B and C), the induction of MHC class II expression on CD11b+ and CD11c+ cells on day 20 postinfection was significantly lower in infected Cxcl13−/− lungs, when compared with wild-type mice (Fig. 7B). This deficiency of expression of MHC class II on CD11c cells in Cxcl13−/− mice was overcome by day 50 postinfection. However, even at day 50 postinfection, CD11b+ in lungs of infected Cxcl13−/− mice expressed significantly lower levels of MHC class II expression when compared with B6 mice. Furthermore, mRNA for Fizz1 (found in inflammatory zone), a marker for alternatively activated macrophages (31, 32), was induced in the lungs of both B6 and Cxcl13−/− mice at day 20 postinfection. The levels of Fizz1 mRNA transcripts were found to decrease between days 30 and 50 postinfection in the B6-infected lungs. However, although the levels of Fizz1 mRNA transcripts decreased in the lungs of Cxcl13−/− mice, they were significantly higher compared with B6-infected lungs at day 50 postinfection (Fig. 7D). The higher levels of Fizz1 mRNA found in the lungs of Cxcl13−/− lungs at
day 50 postinfection, suggests that macrophage activation may be skewed toward the alternative phenotype in the absence of CXCL13.

These data suggest that although IFN-γ-producing T cells are recruited to the lungs of Cxcl13<sup>−/−</sup>-infected mice, they are unable to effectively activate myeloid cells in the lung and to upregulate the expression of protective molecules during the early immune response. One possible explanation for the lack of CD11b and CD11c cell activation in the presence of activated CD4 T cells is that although protective cells are recruited to the lung, they are not spatially oriented within the granulomas for optimal activation of myeloid cells. Using immunofluorescence, we therefore determined the spatial location of CD3 T cells in the early granulomas in wild-type, plt/plt, Cxcl13<sup>−/−</sup>, and DKOs. The CD3 lymphocytes were dispersed throughout the granulomata in the wild-type and plt/plt mice (Fig. 7E). However, in the absence of CXCL13, the CD3 T cells accumulated in the lungs as distinct perivascular cuffs and were not detected within granulomata (Fig. 7E). The area occupied by the perivascular cuff was significantly higher in Cxcl13<sup>−/−</sup> and DKO mice compared with B6 and plt/plt mice (Fig. 7F). These data demonstrate that although CXCL13 was not required for generation and accumulation of activated IFN-γ-producing cells to the lung, the recruited T cells required signals from CXCL13 to migrate into the granuloma and activate myeloid cells efficiently.

It has recently been shown that GC B cells are found in the lymphoid areas of the lung during chronic pulmonary TB (15). Therefore we also addressed whether GC B cells accumulated in the lungs of infected mice during the early immune response and whether this response was dependent on CXCL13 expression. GC B cells (as identified by expression of GL-7 and CD95 (FAS)) did accumulate in the lungs at day 20 postinfection following Mtb infection in wild-type B6 mice (Fig. 8A). This accumulation of GC B cells in the lungs was dependent on CXCL13 because the frequency of GC B cells was significantly reduced in the lungs of infected Cxcl13<sup>−/−</sup> mice. This suggests that CXCL13 plays a critical role in rapid accumulation of B and T cells during TB.

To determine whether the deficiency in cellular accumulation affected bacterial control, B6, Cxcl13<sup>−/−</sup>, and DKO mice were infected with Mtb and the bacterial burden in the lungs determined (Fig. 8B). B6, Cxcl13<sup>−/−</sup>, and DKO mice all had similar levels of mycobacteria in the lung at day 20. However, unlike the wild-type mice, which controlled bacterial growth after day 20, neither the Cxcl13<sup>−/−</sup> nor the DKO mice were able to exhibit the same early control and had higher bacterial numbers in the lungs at subsequent time points. Furthermore, the DKO mice were more susceptible than the Cxcl13<sup>−/−</sup> mice suggesting that the homeostatic chemokines CCL19/CCL21 and CXCL13 cooperate to initiate, generate, and maintain protective immune responses during Mtb infection.

Discussion

The role of homeostatic chemokines in generation of the granuloma and in control of Mtb has not been definitively addressed. Our data show that homeostatic chemokines CCL19 and CXCL13 are induced in the lung during the early immune response following infection with Mtb. The induction of these homeostatic chemokines coincides with the induction of other protective molecules such as IFN-γ, iNOS, and TNF-α as well as initiation of the granulomatous response and control of mycobacteria in the lungs of infected mice. Our data conclusively show that the organization of the lymphoid structures and induction of protective immune responses during Mtb infection are dependent on homeostatic chemokine expression. Importantly, we show critical and yet distinct roles for individual homeostatic chemokines in priming, recruitment, and spatial arrangement of lymphocytes within the lymphoid...
areas during TB. Specifically, CCL19/CCL21 are required for optimal priming, activation, and accumulation of T cells in the lung, likely through their role in DC migration and priming of Ag-specific T cell responses in lymphoid organs. In contrast, we show that CXCL13 is not required for priming IFN-γ/H9253-producing Ag-specific cells, but is critical for the spatial orientation of these cells within the granuloma to activate myeloid cells and control bacteria.

The delayed accumulation of IFN-γ-producing cells in the lungs of plt/plt mice is likely caused by poor expansion of effector T cells in the lymph node rather than the failure to initiate T cell priming. For example, we find similar frequencies of Ag-specific IFN-γ-producing cells in the lymph nodes of Mtb-infected B6 and plt/plt mice. We also show that similar frequencies of adoptively transferred, naive ESAT6-specific T cells become activated in the DLN of Mtb-infected B6 and plt/plt mice, again suggesting that initial steps of T cell priming are intact. However, the lymph nodes of plt/plt mice are very small (19, 20), so the total number of T cells that expand in the lymph nodes is limited. Previously published adoptive transfer experiments show that the activation and proliferation of Mtb Ag85-specific CD4 T cells in the lymph nodes is delayed in plt/plt mice following Mtb infection (33), but these authors did not look at whether this delay impacted accumulation of effector cells in the lungs. These authors also show that DC migration and the transport of Mtb to the DLN is reduced in plt/plt mice (8) and suggest that this reduction is the limiting step in T cell priming (33). We also find a reduced number of DC recruited to the DLN of plt/plt mice. However, in both models, at least some level of T cell activation takes place in the lymph nodes of plt/plt mice (33).

We also find that T cells are effectively primed in Cxcl13/H11002−/− mice, which completely lack DLN, but prime T cells effectively in the spleen. Thus, failure to expand in the lymph nodes cannot be the only explanation for reduced accumulation of IFN-γ-producing T cells in the lungs of plt/plt-infected mice. In fact, other studies have demonstrated that homeostatic chemokines, such as CCL21, are important for attracting effector T cells to peripheral organs like the lungs (11). Moreover, we have found that CCL21-ser and CCL19 are important for T cell responses to influenza in the lungs, independently of all secondary lymphoid organs (12). Thus, we conclude that, although disrupted lymph node architecture and poor DC accumulation in the lymph nodes of plt/plt mice likely contribute to poor T cell expansion and reduced early accumulation of IFN-γ-producing T cells in the lungs, there are additional problems with effector cell recruitment to the lungs that also contribute to the reduced pulmonary IFN-γ T cell responses.

Consistent with this idea, even when the T cell response eventually catches up in the plt/plt mice, the granulomatous response is severely compromised and nascent lymphoid areas fail to develop in plt/plt mice as they do in wild-type infected lungs. CCL19 but not CCL21 is induced locally in the lung in response to Mtb infection and we propose that CCL19 is one of the...
primary homeostatic chemokines that is required for the initiation of the granulomatous response following Mtb infection. However we cannot formally rule out a role for CCL21 in granuloma formation. Although the T cell responses by day 30 are comparable between the plt/plt and the wild-type mice, the increased bacterial growth in the plt/plt mice suggest that the failure to initiate granuloma formation limits the efficacy of the protective responses.

In contrast, absence of CXCL13 does not impact initial priming, activation, or accumulation of activated IFN-γ-producing T cells but compromises the ability of these cells to orient correctly within the granuloma and activate myeloid cells. Thus the equivalent number of activated T cell numbers is generated and accumulate in the lungs of infected B6 (●) or Cxcl13−/− (○) mice was determined by immunofluorescence on day 20, and the area occupied by CD3 T lymphocytes in the perivascular cuffs was determined morphometrically (E).

CXCL13 is known to recruit CXCR5-expressing lymphocytes into the lymphoid organs (23) and expression of CXCL13 has been strongly associated with the generation of ectopic lymphoid follicles (34, 35). Our results demonstrate that CXCL13 is induced in the lung following Mtb infection, and we propose that similar to the events occurring in the secondary lymphoid organs, activated...
lymphocytes that accumulate in the lung up-regulate the expression of CXCR5 and respond to the CXCL13 produced in the inflamed Mtb-infected lungs. This ligand-receptor interaction then allows the T and B lymphocytes to orient themselves spatially within the lymphoid follicles. This event also appears critical for localization of T lymphocytes and macrophages within the granuloma for effective macrophage activation and bacterial control. Despite the accumulation of a comparable number of IFN-γ-producing cells in the lungs of Cxcl13−/− and B6 mice, defects in migration of activated T cells to macrophage areas in the Cxcl13−/− mice likely result in suboptimal or dysregulated activation of macrophages and higher bacterial burden. However diffusion of IFN-γ from activated T cells located in the perivascular space may contribute to the limited control of bacteria seen in the lungs of Cxcl13−/− mice.

Recent evidence suggests that B cells form lymphoid follicles and may contribute to local immune responses in the lungs during chronic Mtb infection (15). We show that GC B cells accumulate in the lung during the early immune response following Mtb infection. Furthermore, this accumulation of GC B cells is dependent on CXCL13 expression and impacts lymphoid neogenesis during Mtb infection. In this low-dose aerosol model, it is likely that the defect in the spatial orientation of T lymphocytes rather than a defect in the accumulation of GC B cells within lymphoid areas of Mtb-infected Cxcl13−/− mice is responsible for the increased susceptibility. In support of this likelihood, B cell-deficient mice
clearly lack GCs, and yet they are not more susceptible than wild-type B6 mice to low-dose aerosol Mtb infection (15). These data for the first time show that induction of CXCL13 in the lung following Mtb infection is critical for recruitment of both B and T lymphocytes to the lung and for spatial orientation of lymphocytes within granulomas.

Our data also shows that DKO mice are more susceptible than Cxcl13−/− or pltplt mice and have severe defects in lymphoid follicle organization, myeloid cell activation, and recruitment of IFN-γ-producing cells to the lung. These data suggest that the homeostatic chemokines CXCL13 and CCL19/CCL21 cooperate to promote the generation of lymphoid follicles and induction of protective immune responses during Mtb infection.

Together our data define the sequential functions performed by homeostatic chemokines in the generation and expression of protective local immune responses against Mtb infection. Specifically, CCL19/CCL21 are required for optimal priming and generation of activated IFN-γ-producing T cells, whereas CXCL13 is critical for spatial organization of the lymphocytes within the inflammatory lesion. These homeostatic chemokines therefore play different roles but cooperate to generate an effective immune response for mycobacterial control.

Disclosures

The authors have no financial conflict of interest.

References