Published in final edited form as:
J Immunol. 2014 August 1; 193(3): 1468–1474. doi:10.4049/jimmunol.1400551.

CXCL17 is a major chemotactic factor for lung macrophages

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Abstract

Chemokines are a superfamily of chemotactic cytokines that direct the movement of cells throughout the body under homeostatic and inflammatory conditions. The mucosal chemokine CXCL17 was the last ligand of this superfamily to be characterized. Several recent studies have provided greater insight into the basic biology of this chemokine and have implicated CXCL17 in several human diseases. We sought to better characterize CXCL17's activity *in vivo*. To this end, we analyzed its chemoattractant properties *in vivo* and characterized a *Cxcl17*$^{-/-}$ mouse. This mouse has a significantly reduced number of macrophages in their lungs compared to WT mice. Additionally, we observed a concurrent increase in a new population of macrophage-like cells that are F4/80^{+}CDllc^{mid}. These results indicate that CXCL17 is a novel macrophage chemoattractant that operates in mucosal tissues. Given the importance of macrophages in inflammation, these observations strongly suggest that CXCL17 is a major regulator of mucosal inflammatory responses.

Introduction

The immune system is a complex network of cell types that are widely disseminated throughout the body. Despite these long distances, the cells retain the ability to interact and communicate so they can mount effective immune responses. To accomplish this, cells of the immune system rely on the production of cytokines. A subset of these cytokines regulate the trafficking of immune cells in the body and are therefore called chemotactic cytokines, or chemokines.

The human chemokine superfamily includes 48 secreted ligands and 19 receptors (1-3). Chemokines have a distinctive 3-dimentional structure, which relies on four conserved cysteine residues that form disulfide bonds. The distribution of these four cysteine molecules within the chemokine structure is the criteria used to divide the superfamily into subclasses: CC, CXC, CX3C and XC (1-3). In addition to their role in the development of immune responses, some chemokines are also expressed under homeostatic conditions in specific

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tissues. This group of chemokines, known as homeostatic chemokines, are important for directing the homing of subsets of immune cells into secondary and tertiary immune tissues (1, 2).

CXCL17 was the last chemokine ligand of the human chemokine superfamily to be described (4). Consequently, there are few reports describing the biology of CXCL17. Several recent studies have documented a role for CXCL17 in tumor progression (5-9), inflammation (10) and human disease (11). These studies suggest that CXCL17 fulfills important physiological roles *in vivo* under both homeostatic and inflammatory conditions. Furthermore, these findings suggest that CXCL17 is an important player underlying the pathologies observed in these diseases. To gain a better understanding of CXCL17's role *in vivo*, we sought to obtain and analyze a CXCL17 deficient (*Cxcl17*$^{-/-}$) mouse.

Knockout mice are common research tools utilized to study chemokine biology. CXCR4 or CXCL12 deficient mice, for example, revealed that this receptor/ligand axis is critically involved in development (12-14). Similarly, mice lacking CCR7 expression pointed to a pivotal role of the CCL21/CCR7 axis in lymphocyte homing to lymph nodes (15-18). Although these chemokines/receptors have been well characterized, their respective knockout mice were critical tools that aided in the characterization and understanding of their functional roles (15, 19-21). These observations underscore the importance of a given chemokine deficient mouse in understanding its biology.

Human and mouse Cxcl17 share 71% sequence identity at the amino acid level (4). Also, a similar pattern of CXCL17 expression in mucosal tissues of both human and corresponding murine tissues has been observed (4, 5, 9-11). Therefore, we predicted that a *Cxcl17*$^{-/-}$ mouse would provide us with important information about CXCL17's *in vivo* activity. The remarkably similar CXCL17 expression patterns in both human and mouse suggests that the phenotype of a CXCL17 deficient mouse will mirror the biology of human CXCL17.

Prior to studying a *Cxcl17*$^{-/-}$ mouse, we wanted to gain further insight into CXCL17's expression profile and *in vivo* chemotactic activity. Comparison of the gene expression profiles of Cxcl17 in germ free (GF) and specific pathogen free (SPF) mice established that CXCL17 exhibits both a homeostatic and inflammatory expression profile, which classifies it as a 'dual' chemokine (1-3). In line with previous *in vitro* chemotaxis studies, we were able to demonstrate that *in vivo*, CXCL17 preferentially chemoattracts macrophages, and to a lesser extent dendritic cells (DCs).

Given our previous findings of CXCL17's robust expression in the lung and association with idiopathic pulmonary fibrosis (IPF) (11), we focused our phenotypic analysis on the lungs of the *Cxcl17*$^{-/-}$ mice. Our findings indicate that the *Cxcl17*$^{-/-}$ mice exhibit a paucity of macrophages in their lungs. Through population specific analyses, we observed a significant reduction in the numbers of alveolar (AMs) but not interstitial macrophages (IMs) in *Cxcl17*$^{-/-}$ mice compared to WTs. Interestingly, we also observed a significant increase in a population of F4/80^{+}CDllc^{mid} cells that have macrophage-like characteristics.

This study represents the first in-depth analysis of CXCL17's biology in an *in vivo* context. We have established that CXCL17 is a dual (homeostatic and inflammatory) chemokine, the

conditions under which CXCL17 is expressed the mucosal tissues and also present the first analysis of CXCL17's chemotactic activity *in vivo*. Finally, we describe the phenotype of the *Cxcl17*$^{-/-}$ mouse, which indicates that CXCL17 is a critical chemotactic factor regulating lung macrophages. Taken together, we conclude that CXCL17 is an important mucosal macrophage chemotactic factor.

Materials & Methods

Mice

Cxcl17$^{+/-}$ mice were obtained from the Knock Out Mouse Project (KOMP) at UC Davis (Genentech ID UNQ473). *Cxcl17*$^{+/-}$ mice were bred to generate *Cxcl17*$^{-/-}$ mice. Wild type (WT) mice for breeding purposes were purchased from Jackson Laboratories (http://jaxmice.jax.org/). All mice used were housed in the same specific pathogen free (SPF) facility with a 12h dark/light cycle with autoclaved bedding and irradiated food. All breeding and handling of the mice were performed under an approved IACUC protocol.

Germ Free Mice

Germ free (GF) and specific pathogen free (SPF) mice were generous gifts from Mauro Teixeira (Universidade Federal de Minas Gerais, Brazil). Tissue samples from each mouse were stored in RNALater (Qiagen, Valencia, CA) prior to total RNA extraction. RNA extraction and Q-PCR were performed as described below.

Genotyping PCR

Earpunches were collected from 3 week old mice upon weaning. DNA was extracted from the earpunches using a kit (Bioland Scientific, Paramount, CA). Primers specific to the Cxcl17 targeting construct were used in a 35-cycle conventional polymerase chain reaction (PCR) to determine the genotype of each mouse. The amplified DNA products were analyzed on a 1% agarose gel. Primers: CXCL17 forward 5′-CTCTTCCGACCACAGTATCC-3′, CXCL17 reverse 5′-CTACAGTTGCAGACATGTTGG-3′, Neo forward 5′-GCAGCGCATCGCCTTCTATC-3′, Neo reverse 5′-CTACAGTTGCAGACATGTTGG-3′.

In vivo analysis of CXCL17-mediated chemotaxis

100 ng of recombinant mouse Cxcl17 (rmCxcl17) (R&D Systems, Minneapolis, MN) or sterile 1X PBS vehicle were administered to three wild type (WT) mice for each condition via intraperitoneal (i.p.) injection. 48 hours post injection, both groups of mice were sacrificed and the cells of each of their peritoneal cavities were collected by washing the peritoneum with 6ml of sterile 1X PBS. Prior to staining, the peritoneal cells were counted and treated with a blocking buffer (4% FBS in 1X PBS) for 30 minutes at 4°C. After blocking, the cells were washed with 2ml of 1X PBS and stained for flow cytometry analyses with a granulocyte specific marker (Gr-1), macrophage specific marker (F4/80) and a dendritic cell (DC) specific marker (CDllc) to determine the cellular composition for the peritoneal cavities from each mouse (both antibodies from Biolegend, San Diego, CA). The AlexaFluor 488 anti-mouse Gr-1 was used at a concentration of 0.1 μg per 10^6 cells, and the PerCP anti-mouse F4/80 and APC anti-mouse CD11c antibodies were used at 0.05 μg per

10^6 cells, all in a total volume of 100 μl 1X PBS. A FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) was used to read all tubes by flow cytometry, and the data were processed and analyzed using FlowJo (Tree Star, Inc.).

Collection of Primary Cells for Chemotaxis Assays

All primary cells were collected from 3-6 week old WT female C57BL/6-B129 mice housed in a specific pathogen free facility. Splenocytes were collected using manual dissociation followed by red blood cell lysis with ACK lysing buffer. Lung intraepithelial cells were collected by manual dissociation after a 1 hour collagenase digestion at 37°C. Pleural and peritoneal cells were obtained by washing each respective cavity with sterile 1X PBS. No further processing of these cells was required prior to their use in the chemotaxis assay. All animal procedures were performed according to approved IACUC protocols.

Chemotaxis Assays

The chemotaxis assays were performed using 24 well transwell migration plates (Corning, NY), which contain an upper insert and lower chamber. 200ng/ml of recombinant chemokine (R&D Systems) in 600ul of Chemotaxis buffer (C-buffer) (incomplete RPMI, Mediatech, Manassas, VA) was added to the bottom chamber of the transwell plate. The transwell plates used in these assays had 5.0 μm sized pores. $1.0x10^6$ cells were used as the input cell number for all cell lines tested unless otherwise noted. Prior to their addition to the top insert assay plate, the cells were washed three times in C-buffer. The assay was incubated at 37°C and 5% CO_2 for 18-20 hours. Chemotaxis was periodically monitored using a microscope.

Quantification of Chemotaxis By Flow Cytometry

This protocol was adapted from Proudfoot et al. (22). Briefly, at the termination of the chemotaxis assay, the chemotaxed cells were collected from the bottom chamber of the plate, spun down in FACS tubes, and then resuspended in 200ul of 1X PBS. Standards were generated by making 10-fold dilutions of cells ranging from $1.0x10^6$ to $1.0x10^2$ cells in 200ul of 1X PBS. The cell counts for the standards and all of the chemotaxed cells were recorded as the number of events counted in 30 seconds. Since the precise number of cells was known for the standards, their cell counts were used to generate a standard curve. The trendline and equation resulting from this standard curve was used to calculate the relative number of cells that chemotaxed for each cell line or primary cell analyzed. A FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was used for these quantification experiments.

Q-PCR Analysis

Total RNA was extracted from mouse tissues using TRIzol (Invitrogen, Carlsbad, CA) and subsequently purified using Qiagen's RNEasy columns and DNase digest (Qiagen). Equal concentrations of RNA were used for each tissue sample in a reverse transcription reaction to synthesize cDNA (Qiagen). Each reaction used 50 ng/ml of each cDNA, gene specific primers, and gene specific UPL probes to quantitatively detect CXCL17 and control gene

transcripts in each tissue sample. The results were processed in Excel and analyzed using GraphPad Prism (http://www.graphpad.com/).

FACS Staining of Immune Cell Populations

Spleen, lymph nodes, bone marrow, and cells from peritoneal and pleural lavages, and lungs were collected from WT and *Cxcl17*$^{-/-}$ mice. Solid organs were passed through 40 μm strainers rinsed with 1X PBS to make single cell suspensions. 0.25 - 1x10^{6} cells, depending of total numbers obtained from the organs, were transferred to FACS tubes. FC receptors were blocked using blocking buffer (4% FBS in 1X PBS) and 0.1 μL of unlabelled anti-CD16/CD32 (anti-FC receptor). Screening of the macrophage/monocyte/DC populations was achieved by staining the cells with the following cocktail: PerCP anti-mouse F4/80, Alexa488 anti-mouse CD11c (iCyt, Champaign, IL), PE anti-mouse B220 and APC anti-mouse CD11b (iCyt). For all stainings, the antibodies were used at 1:1000 in 1X PBS and adding 100 μL of each mix per tube. Finally, all samples were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson) and the data was analyzed using FlowJo software (Tree Star, Inc) and GraphPad Prism (http://www.graphpad.com/).

Immunohistochemical Staining of Tissues

Tissue sections from formalin fixed, paraffin-embedded samples were selected from autopsy files for normal human bronchial mucosa. Two 5-mm tissue punches were taken from the relevant tissues and included in a paraffin block containing four different samples.

The tissue sections were mounted on siliconized slides and xylol was used to remove the paraffin. The tissue sections were then rehydrated in ethanol solutions at progressively lower concentrations. Epitope retrieval was achieved by placing the tissue sections in a pressure cooker for 15 minutes. The resulting slides were incubated with an anti-human CXCL17 antibody (R&D Systems) or an isotype control. The primary antibody was incubated in a polymer-based visualization kit (DAKO, Carpinteria, CA) for 10 minutes per the manufacturer's instructions. The completed reaction was revealed with diaminobenzidine and counterstained with hematoxylin. Positive staining was evaluated and recorded by a pathologist.

Results

CXCL17 is a Dual Chemokine (inflammatory and homeostatic)

We and others (4, 5, 9-11) have previously profiled CXCL17's expression in both humans and mice. These gene expression analyses have established that CXCL17 is highly expressed in mucosal tissues of the digestive and respiratory system as well as in the female reproductive tract (4, 5, 9-11).

Chemokines have been divided into three different expression groups: homeostatic, inflammatory or dual, based on their expression patterns (1-3). The dual classification means that the ligand is expressed under both homeostatic and inflammatory conditions. In the case of CXCL17, its expression classification (homeostatic versus inflammatory) has yet to be established. To determine CXCL17's expression characteristics and properly classify it, we

analyzed the expression pattern of Cxcl17 in tissues from germ free (GF), specific pathogen free (SPF) and normally housed mice. SPF and mice housed in normal conditions will be colonized by “normal” microbial flora, and therefore will have a low level of inflammation at their mucosal sites as a result of this colonization (23-26); due to their microbial sterility, the GF mice should not exhibit even basal inflammation at their mucosal surfaces. Therefore, CXCL17 expression in GF and SPF/normal mice should reflect the chemokine's homeostatic and inflammatory expression profile at mucosal sites, respectively.

The expression of Cxcl17 in the mucosal tissues of these mice was determined using quantitative RT-PCR (qPCR) (Figure 1). In agreement with our previous observation in human and murine tissues, Cxcl17 is robustly expressed in the trachea, lung and tongue with lower expression detected in intestinal tissues. Within gut tissues, Cxcl17 expression is lower in the GF and SPF samples compared to normally housed mice. This trend is also observed in the stomach, tongue and lung of the differentially housed mice. However, Cxcl17 expression in the trachea of GF and SPF mice was not significantly reduced compared to mice housed in a normal vivarium. These data indicate that in the trachea, CXCL17 is expressed under homeostatic conditions, but its expression under varying conditions in other mucosal tissues indicates that it is upregulated under inflammatory conditions. We conclude that CXCL17 is a dual (homeostatic/inflammatory) chemokine.

CXCL17 preferentially chemoattracts macrophages in vivo

Previous studies have analyzed CXCL17's chemotactic activity via *in vitro* analyses (e.g. chemotaxis assays) (4, 6, 9). These assays are important tools for the initial studies into a chemokine's chemotactic activities, but they may not always reflect its chemotactic activity under normal *in vivo* conditions. We therefore sought to analyze the chemotactic activity of CXCL17 in a more physiological context. To this end, we injected wild type (WT) mice with Cxcl17 i.p. and the cellular contents of the peritoneal cavities were collected and analyzed by flow cytometry. As shown in Figure 2 and Supplementary Figure 1, the peritoneal cavities of mice injected with recombinant Cxcl17 contained significantly more in $F4/80^{+}CDllb^{+}$ macrophages by 24 h and 48h than the cavities of mice that received PBS (Figure 2). We observed no significant difference in the recruitment of dendritic cells (DCs). It has been reported that CXCL17 recruits neutrophils (9), but we did not observe changes in the recruitment of these cells to the peritoneal cavity following Cxcl17 injection (data not shown). These data indicate that CXCL17 is a potent macrophage chemotactic factor in vivo.

Phenotype of a Cxcl17 deficient mouse

To gain further insight into the biology of CXCL17, we obtained a $Cxcl17^{+/-}$ mouse strain. These animals were cryo-recovered from the Mutant Mouse Regional Resource Center (MMRRC) at UC Davis (27, 28). We obtained the animals following successful resuscitation, at which point the mice were heterozygous for the *Cxcl17* deletion. The first of four exons was targeted via homologous recombination to inactivate the *Cxcl17* gene on chromosome 7. Careful breeding and genotyping yielded $Cxcl17^{-/-}$ mice (supplemental Figure 2). Homozygous $Cxcl17^{-/-}$ mice show no defects in breeding, gross anatomy development, and gained weight normally (data not shown).

Given that this is the first report of a *Cxcl17*$^{-/-}$ mouse, we performed an in-depth immunophenotyping analysis. Analysis of the major lymphoid compartments (lymph nodes (LN), spleen, bone marrow, and peritoneal cavity) revealed no significant differences between WT and *Cxcl17*$^{-/-}$ mice (data not shown). Next we focused on sites where CXCL17 is robustly expressed; specifically, we analyzed the lungs of the *Cxcl17*$^{-/-}$ mice for possible defects or alterations in the immune cell populations.

We also compared the *in vitro* chemotaxis activity of cells isolated from lungs of WT or *Cxcl17*$^{-/-}$ mice. Lung cells from WT mice showed robust chemotaxis responses to rmCxcl17 (Figure 3). More than 90% of the lung cells that chemotax in response to rmCxcl17 were F4/80^{+}, indicating that they are macrophages (data not shown). The chemotactic activity of these cells is sensitive to pertussis toxin (PTX), indicating signaling through GαI (29-33). When we tested cells from the lungs of *Cxcl17*$^{-/-}$ mice, we observed significantly reduced chemotaxis in response to Cxcl17 (Figure 3). Based on this observation, we hypothesized that there was likely a defect in the populations of macrophages or DCs in *Cxcl17*$^{-/-}$ mouse lungs.

Like many tissues, the lung contains several subpopulations of important immune cells including DCs and macrophages, which have been shown to be the major cell types that respond to CXCL17 by chemotaxis (4, 7, 9, 10). To investigate potential changes to these populations, we used previously reported flow cytometric approaches, which allowed us to analyze DCs, alveolar (AMs), and interstitial macrophage (IMs) populations of the lung (34).

Analysis of the composition of macrophage and DCs of the lungs of *Cxcl17*$^{-/-}$ mice revealed that *Cxcl17*$^{-/-}$ mice have a significant decrease in the number of AMs compared to WT mice (Figure 4). There is also a small decrease in DCs in *Cxcl17*$^{-/-}$ mice. We observed no changes in the population of IMs between WT and *Cxcl17*$^{-/-}$mice. Interestingly, there was a significant increase in a population of F4/80^{+} CDllc^{mid} cells (Figure 4). Forward versus side scatter characteristics indicate that these are macrophage-like cells.

Given that CXCL17 is a chemokine, and therefore a chemoattractant, these data suggest that this new macrophage-like population of cells is dependent on CXCL17 expression within the lung to reach their final niche. The alterations in the AMs population could be due to a compensatory effect in response to increased numbers of F4/80^{+} CDllc^{mid} cells.

Recent studies have demonstrated that each tissue has specific macrophage populations that can identified by their unique gene expression profile, which includes cell surface markers, transcription factors, and transporters (35). We used this information to confirm that subpopulations of macrophages are altered in the lungs of *Cxcl17*$^{-/-}$ mice (Figure 4). Due to the novelty of the biomarkers specific for this population of cells there are no antibodies available to directly stain and identify them. We therefore used qPCR instead to quantitate the mRNA expression of these markers in both WT and *Cxcl17*$^{-/-}$ lung samples. As shown in Figure 5, two lung macrophage specific markers, ABCC3 and NR1D1, are significantly reduced in the lungs of *Cxcl17*$^{-/-}$ mice compared to WTs. Both ABCC3, a transporter, and NR1D1, a transcription factor, are highly expressed in macrophages (35-39) and are strongly

expressed in cells of the monocyte lineage (DCs and macrophages) in the lung (supplementary figure 2).

These data indicate that Cxcl17 is an important macrophage recruitment factor for certain populations of lung macrophages. To further explore this, we stained for CXCL17 in the human lung. As shown in Figure 3, CXCL17 is strongly expressed in bronchial epithelium, as described (9, 11) and importantly, it is also strongly expressed in endothelial cells of blood vessels of the lung. The latter observation strongly suggests that CXCL17 mediates recruitment of blood cells from the circulation to the lung.

Discussion

CXCL17 was the last chemokine ligand described (4) and therefore we are still making significant discoveries about its biology. We and others (4, 5, 9-11) have established that CXCL17 exhibits a predominant mucosal expression pattern. In the present study we aimed to further investigate the function of CXCL17 *in vivo*.

To date, most studies involving CXCL17 have investigated roles for this mucosal chemokine in cancer (5-9). CXCL17 has been shown to promote tumor growth by acting as a pro-angiogenic factor (5, 9, 10) and by increasing tumor cell proliferation (6) in several types of cancer. Conversely, Hiraoka et al. (7) have shown that CXCL17, in conjunction with ICAM2, is upregulated in the precursor lesion of pancreatic cancer where it may play a role in immune surveillance.

Two recent studies have also investigated the connection between CXCL17 and inflammation. Lee et al. (10) have reported that CXCL17 exerts anti-inflammatory effects on LPS-activated macrophages. Lachance et al. (40) observed an increase of CXCL17 expression in a mouse model of acute cutaneous hypersensitivity. Given these findings, we wanted to establish whether inflammatory conditions are required to drive CXCL17 expression, and if so, in which mucosal tissues.

By analyzing tissues from GF, SPF, and standard (non SPF) housed mice, we found that CXCL17 is expressed in some tissues (i.e. trachea) under homeostatic conditions but is also induced upon microbial colonization in various gut and lung tissues (Figure 1). The expression of CXCL17 in the trachea was similar in all three groups of mice, indicating that no inflammatory stimulus is required to induce CXCL17 expression in this tissue. Conversely, CXCL17 expression is significantly elevated in tissues of the digestive tract, tongue and lungs from mice housed under standard conditions compared to GF or SPF housed mice (Figure 1). GF mice are not colonized by normal flora, and therefore lack the basal level of inflammation that is normally found in these tissues when they are colonized. In line with this, we observed that CXCL17 expression was restored when mice were housed under normal conditions that allowed them to be colonized by normal flora (Figure 1).

Although CXCL17's chemotactic activity has been previously analyzed (4, 6, 7, 9, 10) this property had not yet been investigated in an *in vivo* setting under non-disease conditions. To investigate this, we injected WT mice i.p. with recombinant Cxcl17 and analyzed the cells recruited to the peritoneal cavity after 72 hours. We observed a significant increase in the

number of macrophages recruited to the peritoneal cavities of these mice (Figure 2). These results are in line with previous *in vitro* findings (4, 7, 9, 10). It has also been previously reported that CXCL17 will chemoattract DCs *in vitro* (4, 7), but we did not observe significant DC recruitment in our *in vivo* model (Figure 2). This difference may be due to differences in regional DC populations. Pisabarro et al. (4), observed that PBMC-derived DCs chemotax in response to CXCL17. Given that recent studies have documented strong diversity of regional DC populations (41-43) it is likely that the DCs found in blood are different from the DCs that would respond to i.p. injected CXCL17. Alternatively this may reflect a difference between mouse and human CXCL17 biology.

An important tool for studying CXCL17's biology *in vivo* is a *Cxcl17*$^{-/-}$ mouse. Our initial observations indicated that the *Cxcl17*$^{-/-}$ mouse is viable and fertile. Several chemokine receptor/ligand knockout mice have been generated and some of these strains were not viable (44, 45). Given the observed viability of this mouse, we conclude that Cxcl17 does not play a significant role in development. The *Cxcl17*$^{-/-}$ mice also gained weight and otherwise exhibited normal development, indicating that CXCL17 is not likely to play a role in metabolism, unlike other mucosal chemokines like CXCL14 (21, 46).

Immuno-phenotyping of the *Cxcl17*$^{-/-}$ mouse revealed insights about CXCL17's *in vivo* activity. We chose to focus on the lung, which is a site with robust CXCL17 expression (9, 11) (Figure 1) and a tissue where we have previously established a role for CXCL17 in human disease because it is highly upregulated in idiopathic pulmonary fibrosis (IPF) (11). Using both flow-cytometric and gene expression analyses, we observed a significant reduction in the number of macrophages present in the lungs of the *Cxcl17*$^{-/-}$ compared to WTs (Figures 4, 5). These findings agree with previous studies that have shown that CXCL17 chemoattracts cells from the myeloid lineage (4, 7, 9, 10) (and our unpublished data).

Given that several macrophage subpopulations exist in the lung, we investigated whether all lung macrophage populations were altered by the absence of CXCL17, or, alternatively, whether only a specific group was affected. Using cell specific biomarkers, we identified the reduced lung macrophage population as alveolar macrophages (AMs) (34) (Figure 4). We did not observe significant differences in the interstitial macrophage (IMs) population (34) (Figure 4). We also observed a significant increase in a population of cells that are F4/80^{+}Cdllc^{mid} (Figure 4) in the *Cxcl17*$^{-/-}$ mouse. The size and complexity of these cells indicate that they are macrophage-like, and distinct from the AMs and IMs populations. Their possible relation to AMs remains a topic for future studies.

We conclude that CXCL17 is an important macrophage chemoattracting factor in the lungs. The decrease in the AMs population is due to the absence of CXCL17 in the lungs. Chemokines are chemotactic molecules and are important for various populations of cells to home into tissues under homeostatic conditions (1-3). Conflicting data has been presented about the origin of AMs: some studies suggest that AMs originate as circulating blood monocytes that traffic into the lung (47-50) while other groups have generated data indicating that AMs arise from IMs (48, 51). A recent study analyzed the transcriptome and phenotypic similarities between AMs and IMs (52). These authors showed that IMs more

closely resemble the phenotype and gene expression profile of monocytes (52). Furthermore, the chemokine receptor expression profile of IMs, not AMs, more closely resembles the expression profile of monocytes (52). These studies showed that AMs and IMs differ at the transcriptional level; along with out results, these observations suggest that AMs depend on Cxcl17 to home to the lung.

We should note that the CXCL17 receptor has not yet been identified; therefore this unidentified receptor may mediate the homing of alveolar macrophages into the lung. This hypothesis is also supported by our chemotaxis studies (Figure 3) and immunohistochemical staining of human lung (Figure 3B). The fact that CXCL17 is expressed by the endothelial cells surrounding blood vessels (Figure 3B) strongly suggests that this chemokine is an important extravasation factor, a property shared by other members of the chemokine superfamily (53-56). CXCL17 is also strongly expressed in the bronchial epithelium, which is a site where alveolar macrophages are normally located (49, 57). It is therefore possible that CXCL17 may have other functions on alveolar macrophages besides allowing them (or their precursors) to home to the lung. The normally germfree environment of the airways also justifies the homeostatic expression of CXCL17 in the trachea (and likely bronchus). Taken together, our data suggests that CXCL17 mediates the recruitment of alveolar macrophages or their precursors to the lung.

These observations also support the hypothesis that CXCL17 may play a role in the pathogenesis of idiopathic pulmonary fibrosis (IPF) (11). We have reported elevated levels of CXCL17 in the BALf of IPF patients (11). IPF patients are known to have an increased numbers of macrophages in their lungs, which could be due to the elevated CXCL17 levels.

Our findings identify CXCL17 as an important macrophage recruitment factor. CCL2 is the other well characterized chemokine ligand that has been shown to robustly recruit macrophages through interactions with its cognate ligand, CCR2 (58-61). Mice genetically deficient for either the ligand or the receptor show significant defects in macrophage recruitment under inflammatory and homeostatic conditions (54, 58, 62). The $Cxcl17^{-/-}$ mice represent another chemokine knockout mouse strain that exhibits a significant macrophage phenotype. Given the mucosal expression pattern of CXCL17, we would have expected the macrophage defects to be in mucosal tissues, as is indeed the case. Our results predict that AMs express the CXCL17 receptor. We should note that we have already confirmed that CXCL17 does not bind CCR2 (unpublished observation). Taken together, our results strongly suggest that CXCL17 and its receptor represent important macrophage chemotactic factors in mucosal tissues. Given the importance of the CCL2/CCR2 axis in macrophage recruitment, these observations strongly suggest that CXCL17 and its receptor play an important role in mucosal inflammatory responses. We are currently focusing on the identification of the CXCL17 receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Mauro Teixeira from the Universidade Federal de Minas Gerais, Brazil for his generous gift of the tissue samples from germ free and specific pathogen free mice.

This project is supported by an NIH NIAID grant R01 AI093548-01A1 (to AZ).

References

1. Zlotnik A, Yoshie O, Nomiyama H. The chemokine and chemokine receptor superfamilies and their molecular evolution. Genome biology. 2006; 7:243. [PubMed: 17201934]
2. Zlotnik A, Yoshie O. The chemokine superfamily revisited. Immunity. 2012; 36:705–716. [PubMed: 22633458]
3. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD, Mantovani A, Matsushima K, Murphy PM, Nibbs R, Nomiyama H, Power CA, Proudfoot AE, Rosenkilde MM, Rot A, Sozzani S, Thelen M, Yoshie O, Zlotnik A. International Union of Pharmacology. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. Pharmacological reviews. 2014; 66:1–79. [PubMed: 24218476]
4. Pisabarro MT, Leung B, Kwong M, Corpuz R, Frantz GD, Chiang N, Vandlen R, Diehl LJ, Skelton N, Kim HS, Eaton D, Schmidt KN. Cutting edge: novel human dendritic cell- and monocyte-attracting chemokine-like protein identified by fold recognition methods. J Immunol. 2006; 176:2069–2073. [PubMed: 16455961]
5. Weinstein EJ, Head R, Griggs DW, Sun D, Evans RJ, Swearingen ML, Westlin MM, Mazzarella R. VCC-1, a novel chemokine, promotes tumor growth. Biochemical and biophysical research communications. 2006; 350:74–81. [PubMed: 16989774]
6. Mu X, Chen Y, Wang S, Huang X, Pan H, Li M. Overexpression of VCC-1 gene in human hepatocellular carcinoma cells promotes cell proliferation and invasion. Acta biochimica et biophysica Sinica. 2009; 41:631–637. [PubMed: 19657564]
7. Hiraoka N, Yamazaki-Itoh R, Ino Y, Mizuguchi Y, Yamada T, Hirohashi S, Kanai Y. CXCL17 and ICAM2 are associated with a potential anti-tumor immune response in early intraepithelial stages of human pancreatic carcinogenesis. Gastroenterology. 2011; 140:310–321. [PubMed: 20955708]
8. Zhou Z, Lu X, Zhu P, Zhu W, Mu X, Qu R, Li M. VCC-1 over-expression inhibits cisplatin-induced apoptosis in HepG2 cells. Biochemical and biophysical research communications. 2012; 420:336–342. [PubMed: 22425983]
9. Matsui A, Yokoo H, Negishi Y, Endo-Takahashi Y, Chun NA, Kadouchi I, Suzuki R, Maruyama K, Aramaki Y, Semba K, Kobayashi E, Takahashi M, Murakami T. CXCL17 expression by tumor cells recruits CD11b+Gr1 high F4/80-cells and promotes tumor progression. PloS one. 2012; 7:e44080. [PubMed: 22952881]
10. Lee WY, Wang CJ, Lin TY, Hsiao CL, Luo CW. CXCL17, an orphan chemokine, acts as a novel angiogenic and anti-inflammatory factor. American journal of physiology Endocrinology and metabolism. 2013; 304:E32–40. [PubMed: 23115081]
11. Burkhardt AM, Tai KP, Flores-Guiterrez JP, Vilches-Cisneros N, Kamdar K, Barbosa-Quintana O, Valle-Rios R, Hevezi PA, Zuniga J, Selman M, Ouellette AJ, Zlotnik A. CXCL17 is a mucosal chemokine elevated in idiopathic pulmonary fibrosis that exhibits broad antimicrobial activity. J Immunol. 2012; 188:6399–6406. [PubMed: 22611239]
12. David NB, Sapede D, Saint-Etienne L, Thisse C, Thisse B, Dambly-Chaudiere C, Rosa FM, Ghysen A. Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99:16297–16302. [PubMed: 12444253]
13. Doitsidou M, Reichman-Fried M, Stebler J, Koprunner M, Dorries J, Meyer D, Esguerra CV, Leung T, Raz E. Guidance of primordial germ cell migration by the chemokine SDF-1. Cell. 2002; 111:647–659. [PubMed: 12464177]
14. Knaut H, Werz C, Geisler R, Nusslein-Volhard C. A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. Nature. 2003; 421:279–282. [PubMed: 12508118]

15. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, Lipp M. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell. 1999; 99:23–33. [PubMed: 10520991]
16. Lipp M, Burgstahler R, Muller G, Pevzner V, Kremmer E, Wolf E, Forster R. Functional organization of secondary lymphoid organs by the chemokine system. Current topics in microbiology and immunology. 2000; 251:173–179. [PubMed: 11036773]
17. Fan L, Reilly CR, Luo Y, Dorf ME, Lo D. Cutting edge: ectopic expression of the chemokine TCA4/SLC is sufficient to trigger lymphoid neogenesis. J Immunol. 2000; 164:3955–3959. [PubMed: 10754285]
18. Ohl L, Henning G, Krautwald S, Lipp M, Hardtke S, Bernhardt G, Pabst O, Forster R. Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. The Journal of experimental medicine. 2003; 197:1199–1204. [PubMed: 12732661]
19. Cook DN, Beck MA, Coffman TM, Kirby SL, Sheridan JF, Pragnell IB, Smithies O. Requirement of MIP-1 alpha for an inflammatory response to viral infection. Science. 1995; 269:1583–1585. [PubMed: 7667639]
20. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature. 1996; 382:635–638. [PubMed: 8757135]
21. Nara N, Nakayama Y, Okamoto S, Tamura H, Kiyono M, Muraoka M, Tanaka K, Taya C, Shitara H, Ishii R, Yonekawa H, Minokoshi Y, Hara T. Disruption of CXC motif chemokine ligand-14 in mice ameliorates obesity-induced insulin resistance. The Journal of biological chemistry. 2007; 282:30794–30803. [PubMed: 17724031]
22. Proudfoot AE, Power CA, Church DJ, Soler D, Mack M. Cellular assays of chemokine receptor activation. Curr Protoc Pharmacol. 2001; Chapter 12(Unit12):14.
23. Tlaskalova-Hogenova H, Sterzl J, Stepankova R, Dlabac V, Veticka V, Rossmann P, Mandel L, Rejnek J. Development of immunological capacity under germfree and conventional conditions. Annals of the New York Academy of Sciences. 1983; 409:96–113. [PubMed: 6347006]
24. Tlaskalova-Hogenova H, Cerna J, Mandel L. Peroral immunization of germfree piglets: appearance of antibody-forming cells and antibodies of different isotypes. Scandinavian journal of immunology. 1981; 13:467–472. [PubMed: 7198287]
25. Souza DG, Vieira AT, Soares AC, Pinho V, Nicoli JR, Vieira LQ, Teixeira MM. The essential role of the intestinal microbiota in facilitating acute inflammatory responses. J Immunol. 2004; 173:4137–4146. [PubMed: 15356164]
26. Tlaskalova-Hogenova H, Stepankova R, Kozakova H, Hudcovic T, Vannucci L, Tuckova L, Rossmann P, Hrncir T, Kverka M, Zakostelska Z, Klimesova K, Pribylova J, Bartova J, Sanchez D, Fundova P, Borovska D, Srutkova D, Zidek Z, Schwarzer M, Drastich P, Funda DP. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cellular & molecular immunology. 2011; 8:110–120. [PubMed: 21278760]
27. Grieder FB. Mutant Mouse Regional Resource Center Program: a resource for distribution of mouse models for biomedical research. Comparative medicine. 2002; 52:203. [PubMed: 12102564]
28. Tang T, Li L, Tang J, Li Y, Lin WY, Martin F, Grant D, Solloway M, Parker L, Ye W, Forrest W, Ghilardi N, Oravecz T, Platt KA, Rice DS, Hansen GM, Abuin A, Eberhart DE, Godowski P, Holt KH, Peterson A, Zambrowicz BP, de Sauvage FJ. A mouse knockout library for secreted and transmembrane proteins. Nature biotechnology. 2010; 28:749–755.
29. Neptune ER, Bourne HR. Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. Proceedings of the National Academy of Sciences of the United States of America. 1997; 94:14489–14494. [PubMed: 9405640]
30. Neptune ER, Iiri T, Bourne HR. Galphai is not required for chemotaxis mediated by Gi-coupled receptors. The Journal of biological chemistry. 1999; 274:2824–2828. [PubMed: 9915816]
31. Thelen M. Dancing to the tune of chemokines. Nature immunology. 2001; 2:129–134. [PubMed: 11175805]

32. Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA. The G protein-coupled receptor repertoires of human and mouse. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100:4903–4908. [PubMed: 12679517]
33. Thelen M, Peveri P, Kernen P, von Tscharner V, Walz A, Baggiolini M. Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1988; 2:2702–2706. [PubMed: 2840318]
34. Bedoret D, Wallemacq H, Marichal T, Desmet C, Quesada Calvo F, Henry E, Closset R, Dewals B, Thielen C, Gustin P, de Leval L, Van Rooijen N, Le Moine A, Vanderplasschen A, Cataldo D, Drion PV, Moser M, Lekeux P, Bureau F. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. The Journal of clinical investigation. 2009; 119:3723–3738. [PubMed: 19907079]
35. Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, Helft J, Chow A, Elpek KG, Gordonov S, Mazloom AR, Ma'ayan A, Chua WJ, Hansen TH, Turley SJ, Merad M, Randolph GJ. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nature immunology. 2012; 13:1118–1128. [PubMed: 23023392]
36. Heng TS, Painter MW. The Immunological Genome Project: networks of gene expression in immune cells. Nature immunology. 2008; 9:1091–1094. [PubMed: 18800157]
37. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, Hodge CL, Haase J, Janes J, Huss JW 3rd, Su AI. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. Genome biology. 2009; 10:R130. [PubMed: 19919682]
38. Moreau A, Le Vee M, Jouan E, Parmentier Y, Fardel O. Drug transporter expression in human macrophages. Fundamental & clinical pharmacology. 2011; 25:743–752. [PubMed: 21210849]
39. Fontaine C, Rigamonti E, Pourcet B, Duez H, Duhem C, Fruchart JC, Chinetti-Gbaguidi G, Staels B. The nuclear receptor Rev-erbalpha is a liver X receptor (LXR) target gene driving a negative feedback loop on select LXR-induced pathways in human macrophages. Mol Endocrinol. 2008; 22:1797–1811. [PubMed: 18511497]
40. Lachance PA, Hazen A, Sevick-Muraca EM. Lymphatic vascular response to acute inflammation. PloS one. 2013; 8:e76078. [PubMed: 24086691]
41. Ardavin C. Origin, precursors and differentiation of mouse dendritic cells. Nature reviews Immunology. 2003; 3:582–590.
42. Ito T, Liu YJ, Kadowaki N. Functional diversity and plasticity of human dendritic cell subsets. International journal of hematology. 2005; 81:188–196. [PubMed: 15814329]
43. Plantinga M, Hammad H, Lambrecht BN. Origin and functional specializations of DC subsets in the lung. European journal of immunology. 2010; 40:2112–2118. [PubMed: 20853496]
44. Lu M, Grove EA, Miller RJ. Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99:7090–7095. [PubMed: 11983855]
45. Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. Blood. 2000; 95:2505–2513. [PubMed: 10753828]
46. Tanegashima K, Okamoto S, Nakayama Y, Taya C, Shitara H, Ishii R, Yonekawa H, Minokoshi Y, Hara T. CXCL14 deficiency in mice attenuates obesity and inhibits feeding behavior in a novel environment. PloS one. 2010; 5:e10321. [PubMed: 20428232]
47. Landsman L, Jung S. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. J Immunol. 2007; 179:3488–3494. [PubMed: 17785782]
48. Zaslona Z, Wilhelm J, Cakarova L, Marsh LM, Seeger W, Lohmeyer J, von Wulffen W. Transcriptome profiling of primary murine monocytes, lung macrophages and lung dendritic cells reveals a distinct expression of genes involved in cell trafficking. Respiratory research. 2009; 10:2. [PubMed: 19149869]

49. Lambrecht BN. Alveolar macrophage in the driver's seat. Immunity. 2006; 24:366–368. [PubMed: 16618595]
50. Balhara J, Gounni AS. The alveolar macrophages in asthma: a double-edged sword. Mucosal immunology. 2012; 5:605–609. [PubMed: 22910216]
51. Godleski JJ, Brain JD. The origin of alveolar macrophages in mouse radiation chimeras. The Journal of experimental medicine. 1972; 136:630–643. [PubMed: 4559194]
52. Moreira AP, Hogaboam CM. Macrophages in allergic asthma: fine-tuning their pro- and anti-inflammatory actions for disease resolution. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2011; 31:485–491.
53. Lukacs NW, Strieter RM, Elner V, Evanoff HL, Burdick MD, Kunkel SL. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. Blood. 1995; 86:2767–2773. [PubMed: 7545470]
54. Kuziel WA, Morgan SJ, Dawson TC, Griffin S, Smithies O, Ley K, Maeda N. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. Proceedings of the National Academy of Sciences of the United States of America. 1997; 94:12053–12058. [PubMed: 9342361]
55. Ebnet K, Vestweber D. Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines. Histochemistry and cell biology. 1999; 112:1–23. [PubMed: 10461808]
56. Middleton J, Patterson AM, Gardner L, Schmutz C, Ashton BA. Leukocyte extravasation: chemokine transport and presentation by the endothelium. Blood. 2002; 100:3853–3860. [PubMed: 12433694]
57. Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. Nature reviews Immunology. 2014; 14:81–93.
58. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV Jr, Broxmeyer HE, Charo IF. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. The Journal of clinical investigation. 1997; 100:2552–2561. [PubMed: 9366570]
59. Yoshimura T, Robinson EA, Tanaka S, Appella E, Kuratsu J, Leonard EJ. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. The Journal of experimental medicine. 1989; 169:1449–1459. [PubMed: 2926329]
60. Valente AJ, Graves DT, Vialle-Valentin CE, Delgado R, Schwartz CJ. Purification of a monocyte chemotactic factor secreted by nonhuman primate vascular cells in culture. Biochemistry. 1988; 27:4162–4168. [PubMed: 3415979]
61. Matsushima K, Larsen CG, DuBois GC, Oppenheim JJ. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. The Journal of experimental medicine. 1989; 169:1485–1490. [PubMed: 2926331]
62. Kurihara T, Warr G, Loy J, Bravo R. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. The Journal of experimental medicine. 1997; 186:1757–1762. [PubMed: 9362535]

Abbreviations used in this paper

WT	wild type
GF	germ free
SPF	specific pathogen free
DC	dendritic cell
AMs	alveolar macrophages
IMs	interstitial macrophages
qPCR	quantitative real-time PCR

LN	lymph node
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
NR1D1	nuclear receptor subfamily 1, group D, member 1
IPF	idiopathic pulmonary fibrosis
PTX	pertussis toxin
BALf	bronchoalveolar lavage fluid

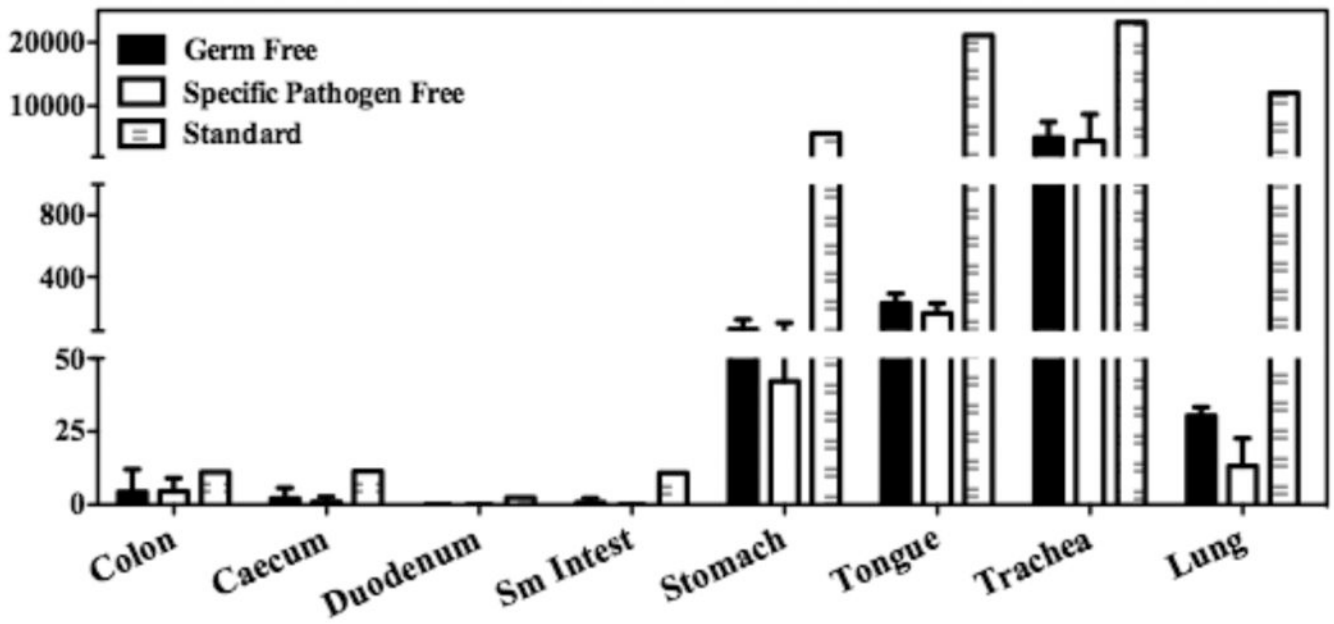

Figure 1. CXCL17's Expression Pattern In Germ Free Mouse Mucosal Tissues Demonstrates That CXCL17 is a hybrid (Both an Inflammatory and Homeostatic Chemokine)

Q-PCR was used to determine CXCL17 expression levels in tissue samples from germ free (GF) and Specific Pathogen Free (SPF) mice, which were compared to tissues from mice housed in a standard vivarium. These data suggest that CXCL17 is expressed under inflammatory conditions in many mucosal tissues, but has homeostatic expression in the colon and trachea. X-axis, tissues; y-axis, CXCL17 expression units; Sm Intest, small intestine.

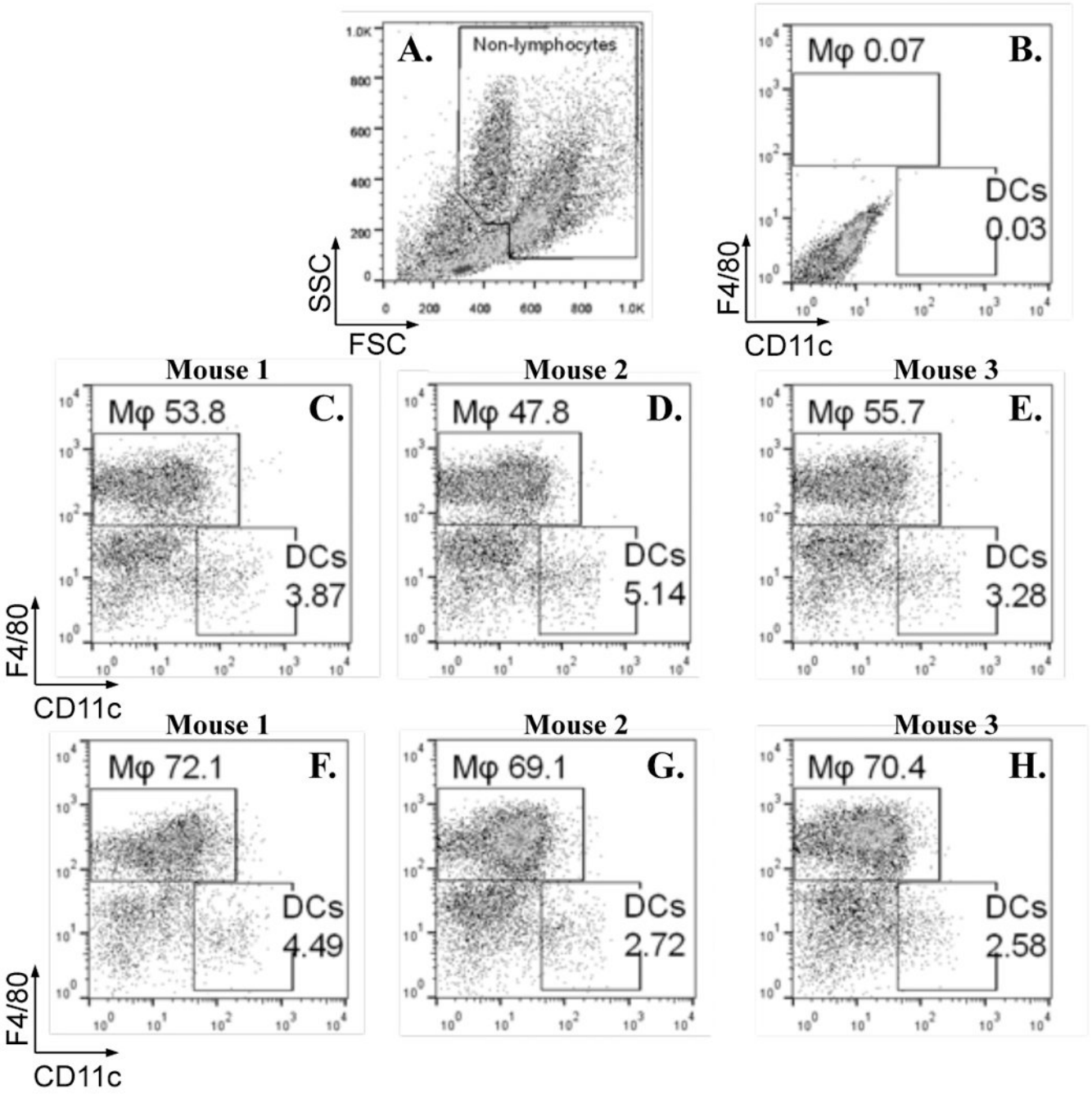

Figure 2. Intraperitoneal Injection of rmCxcl17 Increases Recruitment of Macrophages 48 Hours Post Injection

After injecting rmCxcl17 into the peritoneal cavities of three mice, a significant increase in macrophages (Mϕ), but not dendritic cells (DCs) was observed (F.-H.) compared to mice injected with PBS vehicle (C.-E.). The specific cell populations were determined by staining the cells with cell specific antibodies (F4/80, macrophages; CDllc, DCs; Gr-1, granulocytes). This experiment was performed twice with n=3 mice per group. See also Supplementary Figure 2.

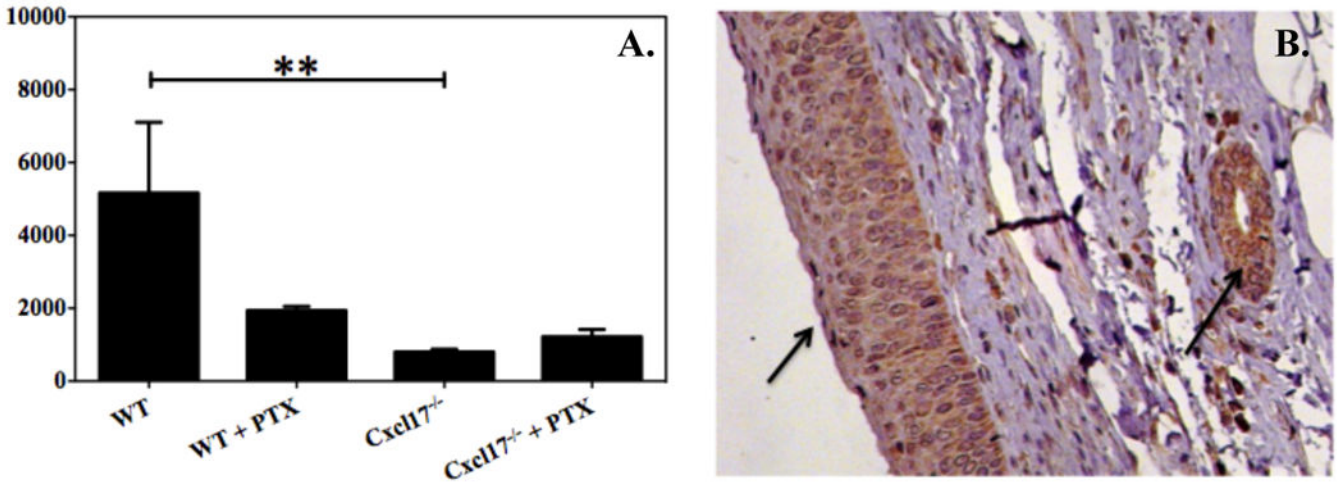

Figure 3. CXCL17 is An Important Chemoattracting Factor In The Lung

A. Single cell suspensions of lung cells from WT and *Cxcl17*$^{-/-}$ mice showed differences in their chemotactic activity in response to recombinant Cxcl17. The *Cxcl17*$^{-/-}$ mouse lung cells demonstrate a significant reduction in the number of chemotaxing cells compared to WT mouse lung cells. Treatment with *Bordatella pertussis* toxin (PTX) reduced chemotaxis of WT lung cells. This experiment was performed with n=3 mice per group. **p=0.03. B. The endothelial cells surrounding blood vessels of the lung stain positive for CXCL17. The arrows show positive CXCL17 staining at the luminal facing side of the respiratory mucosa and the endothelial cells.

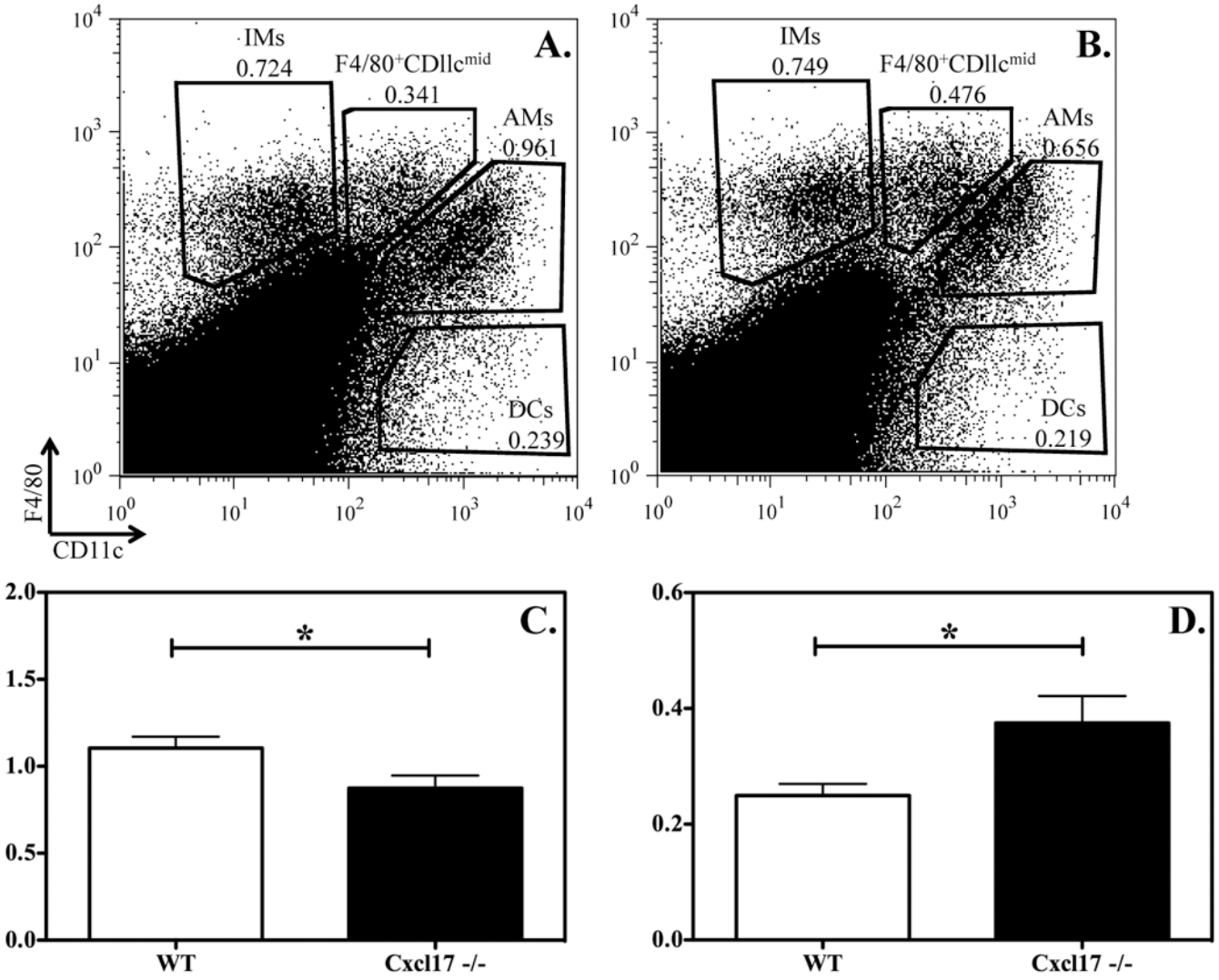

Figure 4. Altered Lung Macrophage Populations Observed In the *Cxcl17*$^{-/-}$ Mice Compared to WT Mice

Cells collected from lungs of WT (A.) and *Cxcl17*$^{-/-}$ mice (B.) were stained with fluorophore conjugated antibodies for analysis by flow cytometry. When we analyzed the macrophage populations (A-C.) we observed a significant decrease in the percentage of alveolar macrophages in *Cxcl17*$^{-/-}$ lungs compared to WT mouse lungs (C.) *p=0.05. We also observed a significant increase in a previously unidentified population of macrophage-like cells that are F4/80^{+}CDllc^{mid} (A-B., D.) *p=0.04. A-B are representative FACS plots from two separate experiments with n=at least 4 mice per group. IMs, interstitial macrophages; AMs, alveolar macrophages; DCs, dendritic cells.

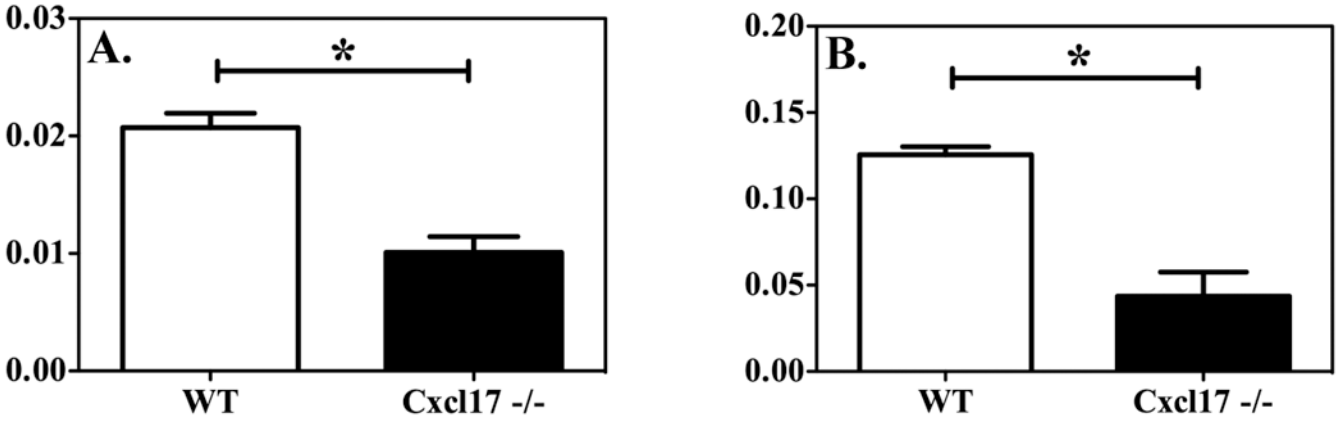

Figure 5. The Expression of Two Lung Specific Macrophage Markers Is Significantly Reduced in *Cxcl17*$^{-/-}$ Mice

The expression of lung macrophage specific markers (36), (37), (35) were measured using qPCR in single cell suspensions from WT and Cxcl17$^{-/-}$ lungs. The expression of two markers, ABCC3 (A.) and NR1D1 (B.) was significantly reduced in the cells isolated from *Cxcl17*$^{-/-}$ mouse lungs. ABCC3 *p=0.02; NR1D1 *p=0.03. Each experiment was performed twice with n=2 mice per group. Y axis, relative expression units.