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Original article

Characterization of chemokine and chemokine receptor expression during *Pneumocystis* infection in healthy and immunodeficient mice

Lisa R. Bishop [a], Michail S. Lionakis [b], Monica Sassi [a], Philip M. Murphy [c], Xiaojun Hu [d], Da Wei Huang [d], Brad Sherman [d], Ju Qiu [d], Jun Yang [d], Richard A. Lempicki [d], Joseph A. Kovacs [a,*]

[a] *Critical Care Medicine Department, NIH Clinical Center, National Institutes of Health, Bethesda, MD 20892, USA*
[b] *Fungal Pathogenesis Unit, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[c] *Laboratory of Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[d] *Laboratory of Immunopathogenesis and Bioinformatics, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA*

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Abstract

We examined gene expression levels of multiple chemokines and chemokine receptors during *Pneumocystis murina* infection in wild-type and immunosuppressed mice, using microarrays and qPCR. In wild-type mice, expression of chemokines that are ligands for Ccr2, Cxcr3, Cxcr6, and Cxcr2 increased at days 32–41 post-infection, with a return to baseline by day 75–150. Concomitant increases were seen in Ccr2, Cxcr3, and Cxcr6, but not in Cxcr2 expression. Induction of these same factors also occurred in CD40-ligand and CD40 knockout mice but only at a much later time-point, during uncontrolled *Pneumocystis* pneumonia (PCP). Expression of CD4 Th1 markers was increased in wild-type mice during clearance of infection. Ccr2 and Cx3cr1 knockout mice cleared *Pneumocystis* infection with kinetics similar to wild-type mice, and all animals developed anti-*Pneumocystis* antibodies. Upregulation of Ccr2, Cxcr3, and Cxcr6 and their ligands supports an important role for T helper cells and mononuclear phagocytes in the clearance of *Pneumocystis* infection. However, based on the current and prior studies, no single chemokine receptor appears to be critical to the clearance of *Pneumocystis*.
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Keywords: *Pneumocystis*; PCP; Chemokines; Chemokine receptor; Knock-out mice

1. Introduction

Pneumocystis is an opportunistic fungal pathogen that causes severe pneumonia in immunocompromised hosts [1]. Healthy individuals may become infected with *Pneumocystis*, but infection is rapidly cleared by a robust immune response [2,3]. A better understanding of the immune response to *Pneumocystis* in immunocompetent hosts should lead to a better understanding of disease in immunocompromised hosts.

Chemokines and chemokine receptors play an important role in host responses to infections, functioning to coordinate leukocyte trafficking, as well as modulating several other cellular biological processes such as survival, proliferation, differentiation, and anti-microbial activity [4]. The potential role of chemokines in *Pneumocystis* infection has been examined in a number of *in vitro* and in vivo studies [5–16]. In vitro studies have demonstrated an increase in Cxcr2 ligands [6,17], while in vivo studies, which were primarily performed in immunosuppressed mice, have shown increased

* Corresponding author. Building 10, Room 2C145, MSC 1662 Bethesda, MD 20892-1662, USA. Tel.: +1 301 496 9907; fax: +1 301 480 2466.
E-mail address: jkovacs@mail.nih.gov (J.A. Kovacs).

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expression of Ccr2, Ccr5, and Cxcr3 ligands [9–11,14,15,18]. However, no study to date has comprehensively examined chemokine and chemokine receptor responses in immunocompetent hosts.

In a prior microarray study we identified a robust immune response to *Pneumocystis* infection in immunocompetent mice that was biphasic in nature, the second phase of which coincided with clearance of infection [19]. In contrast, CD40-ligand knockout mice (CD40L-KO), which are highly susceptible to *Pneumocystis* infection, showed a much more muted response during the same time period. Notably, a large number of chemokines and chemokine receptors were upregulated during the second phase in immunocompetent but not CD40L-KO mice.

The current study was undertaken to better understand the role of chemokines and chemokine receptors in host responses to *Pneumocystis* in both wild-type and CD40L-KO mice over time, through examination of RNA expression as determined by microarray analysis as well as by real-time quantitative polymerase chain reaction (PCR). To gain insight into cell-specific determinants of anti-*Pneumocystis* pulmonary immunity, we also used microarray analysis to examine RNA expression in purified lung CD4 cells and alveolar macrophages. We further examined the role in clearance of *Pneumocystis* infection of two chemokine receptors, Ccr2 and Cx3cr1, which are characteristically expressed on inflammatory and resident monocytes/macrophages, respectively, using Ccr2-KO and Cx3cr1-KO mice [20].

2. Methods

2.1. Animals

Healthy C57 black (C57BL/6) mice were obtained from the National Cancer Institute, CD40 ligand knock-out (CD40L-KO, strain B6, 129S-Tnfsf5^{tm1Imx}) and CD40 knock-out (CD40-KO, strain B6, 129P2-Cd40^{tm1Kik}/J) mice were obtained from Jackson Laboratory. Ccr2 knock-out (B6.129S4-Ccr2^{tmIIfc}/J) mice [21] were kindly provided by Dr. Joshua Farber and Cx3cr1 (B6.129-Cx3cr1^{tm1Zm}) knock-out mice were obtained from Taconic. All mouse strains were subsequently bred at the NIH. Mice were housed in microisolator cages and kept in ventilated racks. All animal work was performed under a Clinical Center Animal Care and Use Committee-approved protocol.

2.2. P. murina *infection*

Wild-type C57BL/6 and CD40L-KO mice were exposed to *Pneumocystis murina* infected seeder mice for 2 weeks, 5 weeks or 6 weeks as described in a previous study; results of immunostaining and limited Western blot analysis of lung homogenates of these wild-type animals have been previously reported [19]. CD40L-KO and CD40-KO mice, and, as controls, CD40L +/−, which were littermates of the CD40L-KO mice, were exposed to *P. murina* infected seeder mice for 5 months. Lung tissue was collected and placed in RNAlater (Qiagen) for RNA extraction and in PBS for DNA extraction. *P. murina* infection was confirmed by quantitative real-time PCR as described below.

Ccr2-KO mice were exposed to *P. murina* infected seeder mice and were sacrificed after 5, 6, 9 and 11 weeks of exposure. Uninfected Ccr2-KO mice were used as controls. Cx3cr1-KO mice and C57BL/6 wild-type mice (as controls in each cage) were exposed to *P. murina* infected seeder mice in 2 experimental cages. Mice were sacrificed after 5, 9 (Cx3cr1-KO only), and 15 weeks of exposure. Lungs were collected for quantitation of *P. murina* organisms by quantitative real-time PCR, blood was collected to obtain serum for antibody analysis and spleens were collected at weeks 9 and 11 to measure spleen cell proliferation responses following *in vitro* stimulation with *Pneumocystis* antigens [22].

2.3. Microarray studies

Microarray analysis of control and CD40L-KO mice through day 75 after *Pneumocystis* infection have been previously reported [19]. Additional microarray analysis of control (CD40L +/−), CD40L-KO, and CD40-KO mice at ~5 months following exposure, at a point when they were heavily infected with *Pneumocystis*, were performed as previously reported [19] using the Mouse Genome 430 2.0 Array (Affymetrix). To examine responses in lung CD4 cells and alveolar macrophages, microarray analysis using the Mouse Gene 2.0 ST Array (Affymetrix) was performed using bead purified CD4 cells (Miltenyi) and alveolar macrophages that were purified by adherence to plastic for ~4 h. Cells were obtained from whole lung preparations (CD4) or bronchoalveolar lavage (macrophage) from C57BL/6 mice (expressing either CD45.1 or CD45.2) at 35 days following exposure to a seeder mouse, as well as from unexposed mice. Due to the small number of cells recovered, cells from 5 animals were combined in 4 separate pools and each pool was analyzed separately. Because the RNA yield from macrophages was low even after combining cells, RNA was amplified by the Ovation Pico WTA System V2 (NuGen) prior to processing for microarray analysis. The new microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information (NCBI; U.S. National Library of Medicine, Bethesda, MD, USA) Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series Accession Number GSM1654761-92.

2.4. Real-time quantitative PCR of gene expression

RNA was extracted using RNeasy Mini Kit (Qiagen), and quantified using a NanoDrop spectrophotometer. cDNA was synthesized from 2 μg RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on an ABI Prism 7900 sequence detection system using Power Sybr Green Master Mix (Applied Biosystems) or iTaq Universal SYBR Green Supermix (Bio-Rad) to measure relative levels of gene expression of the following chemokines and receptors: Cxcl9 (monokine