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Pneumocystis infection alters the activation state of pulmonary macrophages

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ABSTRACT

Recent studies show a substantial incidence of *Pneumocystis jirovecii* colonization and infection in patients with chronic inflammatory lung conditions. However, little is known about the impact of *Pneumocystis* upon the regulation of pulmonary immunity. We demonstrate here that *Pneumocystis* polarizes macrophages towards an alternatively activated macrophage-like phenotype. Genetically engineered mice that lack the ability to signal through IL-4 and IL-13 were used to show that *Pneumocystis* alternative macrophage activation is dependent upon signaling through these cytokines. To determine whether *Pneumocystis*-induced macrophage polarization would impact subsequent immune responses, we infected mice with *Pneumocystis* and then challenged them with *Pseudomonas aeruginosa* 14 days later. In co-infected animals, a higher proportion of macrophages in the alveolar and interstitial spaces expressed both classical and alternatively activated markers and produced the regulatory cytokines TGFβ and IL-10, as well as higher arginase levels than in mice infected with *P. aeruginosa* alone. Our results suggest that *Pneumocystis* reprograms the overall macrophage repertoire in the lung to that of a more alternatively-activated setpoint, thereby altering subsequent immune responses. These data may help to explain the association between *Pneumocystis* infection and decline in pulmonary function.

1. Introduction

Pneumocystis jiroveci is an opportunistic fungal pathogen that often causes pneumonia in immunocompromised patients. Recently it was recognized that colonization with *Pneumocystis* is associated with a decline in pulmonary function in smokers and patients with severe forms of chronic obstructive pulmonary disease (COPD) (Morris et al., 2000, 2004b, 2008a) as well as cystic fibrosis (CF) (Calderon et al., 2010; Friaza et al., 2010; Sing et al., 2001). In addition to colonization and infection with multiple microorganisms, these patients have the hallmarks of chronic lung diseases (airway inflammation, airway remodeling, and parenchymal damage). This creates a complex microenvironment that complicates our understanding of the role of microbes during the development of pulmonary damage and repair. It is unknown whether the presence of *Pneumocystis* alters the homeostatic set point of immune activation, nor how this influences subsequent pulmonary insults.

Macrophages play a key role in the clearance of *Pneumocystis* infection (Ezekowitz et al., 1991; Limper et al., 1997; Masur and Jones, 1978; O'Riordan et al., 1995). They account for the majority of inflammatory cells recovered in bronchoalveolar lavage (BAL) samples from COPD patients and are localized to sites of alveolar destruction. The influence of specific macrophage characteristics on other immune cell types during pulmonary infection with *Pneumocystis* are poorly understood. Macrophages have been described along a continuum of gene expression patterns and functional status (Mantovani et al., 2002; Montaner et al., 1999; Nelms et al., 1999; Stout and Suttles, 2004). In contrast to the classical

Abbreviations: COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; CAM, classically activated macrophage; AAM, alternatively activated macrophage; BAL, bronchoalveolar lavage; MR, mannose receptor; CFU, colony forming unit; TSB, trypticase soy broth; EDTA, ethylenediaminetetraacetic acid; MOI, multiplicity of infection; FBS, fetal bovine serum; ANOVA, analysis of variance.

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activation of macrophages (CAM) induced by Th1 cytokines to an inflammatory effector cell that utilizes reactive oxygen species for pathogen elimination (Mantovani et al., 2002; Munder et al., 1998), alternatively-activated macrophages (AAM) not only orchestrate inflammation during Th2-type responses, but also play a role in the regulation of inflammation and the coordination of extracellular matrix protein production responsible for tissue remodeling and repair (Gordon, 2003). AAMs demonstrate marked up-regulation of mannose receptor (MR), CD23, and arginase 1, the latter of which is thought to be responsible for inhibition of CAM responses in mice (Bansal and Ochoa, 2003; El Kasmi et al., 2008; Herbert et al., 2004, 2010). The role of alternative macrophage activation during *Pneumocystis* infection and colonization is unknown and represents an opportunity to better understand the role of *Pneumocystis* colonization in patients with chronic lung diseases.

Evidence suggests that alternative macrophage activation plays a role in the pathogenesis of COPD and CF and could have significant implications in the evolution of inflammation and pulmonary remodeling in this setting (El Kasmi et al., 2008; Pesce et al., 2009a,b; Wilson and Wynn, 2009; Wynn and Barron, 2010). Our previous work found that immune responses to acute bacterial pneumonia can be altered by manipulating alternative macrophage activation. Induction of an AAM-like phenotype in a mouse model of *Pseudomonas* pulmonary infection causes a blunted neutrophil influx and decreased peribronchiolar inflammation (Feola et al., 2010). The induction of arginase 1 expression in these mice infected with *Pseudomonas*, as well as in other models of infection including *Schistosoma mansoni*, appears to be protective through the suppression of excessive inflammation (Feola et al., 2010; Pesce et al., 2009a). Although much is known of the function of arginase in the coordination of remodeling and repair, several studies have also investigated its role in the inflammatory response to pathogens that elicit both Th1- and Th2-type responses. With Th1-dominant infections, *Arg1* expression counters effective responses by suppressing NO production, as shown in mouse models of *Mycobacterium* species and *Toxoplasoma gondii* infection (El Kasmi et al., 2008). Conversely however, in the Th2-dominant response to *Schistosoma mansoni*, macrophage-specific expression of arginase-1 regulates the pathologic aspects of Th2-driven inflammation and fibrosis (Herbert et al., 2004; Pesce et al., 2009b).

Here, we demonstrate that *Pneumocystis* infection induces characteristics of alternative macrophage activation both *in vitro* and *in vivo*. We also show that the *Pneumocystis*-induced AAM phenotype persists and alters the immune response to a secondary bacterial challenge. Additional studies in IL-4Rα$^{-/-}$ mice suggest that *Pneumocystis*-mediated macrophage polarization is, to an extent, dependent upon IL-4 and/or IL-13 signaling, although this absence does not alter the kinetics of *Pneumocystis* clearance in these mice. These studies provide evidence for immune modulation as a result of colonization or infection with *Pneumocystis* that may influence the progression of pathology associated with chronic lung diseases.

2. Materials and methods

2.1. Mice

Five- to 8-wk-old C57BL/6, BALB/c, and BALB/c-Il4ra^{tCAMSz}/J (IL-4Rα$^{-/-}$) mice were obtained from Jackson Laboratories, bred, and maintained at the AALAC accredited University of Kentucky Animal Facility. The IL-4Rα$^{-/-}$ mouse is homozygous for the *Il4ra* ^{tCAMSz} targeted mutation. IL-4Rα is a common chain that is present in IL-4 and IL-13 receptors and is required for transmitting signals for both cytokines (Kondo et al., 1993; Russell et al., 1993). These mice are unable to respond to IL-4/IL-13 to induce alternative macrophage activation. Mice were housed in pathogen-free isolation, given food and water *ad libitum*, and transferred to a biosafety housing unit after infection. *Pneumocystis* was maintained in a colony of Rag1$^{-/-}$ mice as a source for all infections. All experimental animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Enumeration of inoculated Pneumocystis organisms

To prepare *Pneumocystis* for inoculation, lungs removed from infected Rag1$^{-/-}$ mice were pushed through a stainless steel mesh and collected in HBSS. Debris was removed by centrifugation, and aliquots of lung homogenates were spun onto glass slides, fixed with methanol, stained using DiffQuik (Siemens, Germany) reagents, and the number of *Pneumocystis* nuclei were counted. To infect mice, animals were anesthetized lightly with isoflurane gas, and 10^5–10^7 *Pneumocystis* nuclei, depending upon the experiment, were injected intratracheally (i.t.) in 100 μl of PBS. For detection of *Pneumocystis* burden, the right lung lobes of each animal were excised, minced, and digested in RPMI 1640 supplemented with 2% fetal calf serum, 1 mg/ml collagenase A, and 50 U/ml DNase. Digested fractions were pushed through mesh screens, and aliquots were spun onto glass slides and stained with Diff-Quik for microscopic enumeration. Lung burden is expressed as $\log_{10}$ *Pneumocystis* nuclei per animal with a lower limit of detection of $\log_{10}$ 3.23.

2.3. Infection with P. aeruginosa

The clinical mucoid strain *P. aeruginosa* M57-15, a gift from Anna Van Heeckeren, PhD, Case Western University, was used in these experiments. The bacteria were grown in trypticase soy broth (TSB) to late log phase or early stationary phase. The method for incorporation of the bacteria into agarose beads, essential to induce prolonged infection in mice, was adapted from previously described methods (Nacucchio et al., 1984; van Heeckeren and Schluchter, 2002). *P. aeruginosa*-laden agarose beads were diluted to achieve an inoculum of 1 to 2×10^5 CFU per 100 μl, which is approximately the lethal dose 10 percent (LD_{10}). Fourteen days after *Pneumocystis* inoculation as described above, *P. aeruginosa*-laden beads were instilled intratracheally using a blunted 24-gauge curved inoculation needle while the animals were under isofluorane anesthesia. To confirm the actual inocula given, an aliquot of the bead preparation was homogenized and plated on *Pseudomonas* selection agar immediately after infection and counted after overnight incubation.

Mice were humanely euthanized on day 0 prior to infection with *P. aeruginosa* and on post-infection days 3 and 10. Bronchial alveolar lavage and lung digest samples were obtained as described above. An aliquot was plated to assess bacterial burden by manual CFU counting on *Pseudomonas* selection agar. This agar was used to avoid contamination from upper airway flora. We have verified that the lavage procedure does not significantly affect bacterial counts of homogenized lung tissue.

2.4. Isolation of macrophages from alveolar space and lung interstitium

Mice were humanely euthanized by exsanguination under deep anesthesia. The lungs were lavaged with 1 ml aliquots of HBSS containing 3 mM EDTA. Lungs were then removed and digested in medium as discussed above. After removing an aliquot for enumeration of *Pneumocystis* organisms, lung fragments were then filtered and red blood cells lysed in a hypotonic solution and analyzed as