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Eosinophils Contribute to Early Clearance of *Pneumocystis murina* Infection

Taylor Eddens,*† Waleed Elsegeiny,*‡ Michael P. Nelson, ‡ William Horne, ‡ Brian T. Campfield, ‡ Chad Steele, ‡,1 and Jay K. Kolls ‡,1

*Pneumocystis jirovecii* is a host-specific fungal pathogen that causes a diffuse interstitial pneumonia in immunocompromised individuals (1). *Pneumocystis* remains the most common serious opportunistic infection in the HIV/AIDS population and is a frequent complication in developing countries where combination antiretroviral therapy and anti-*Pneumocystis* prophylaxis are difficult to implement (2–7). In developed countries, the incidence of *Pneumocystis* infection has continued to rise owing to the expansion of the immunosuppressed population (8, 9). One study estimates that 75% of cases of *Pneumocystis* pneumonia are in non–HIV-immunosuppressed individuals, such as those receiving immunosuppressive drug therapy for hematologic malignancy and post-transplantation rejection (9). In fact, in the non–HIV-infected immunosuppressed population, *Pneumocystis* infection tends to have increased morbidity, such as higher rates of mechanical ventilation, and increased mortality compared with the HIV-positive population (10–12).

Given the opportunistic nature of *Pneumocystis*, much can be gleaned about the host immune response required for clearance of *Pneumocystis* by examining the populations that are susceptible to infection. The HIV/AIDS population provides the strongest evidence; a clear inverse correlation exists between decreasing CD4+ T cell counts and increased susceptibility to *Pneumocystis* infection (13–16). The importance of CD4+ T cells in protecting against *Pneumocystis* pneumonia has also been demonstrated in patients with genetic immunodeficiencies, such as forms of SCID, as well as in animal models of infection (17, 18). Although CD4+ T cells have been shown to interact with various cell types, such as B cells and macrophages, throughout the course of *Pneumocystis* infection, the ability of other immune cell types to contribute to *Pneumocystis* clearance in a CD4+ T cell–dependent manner is still an area of active investigation (19–23). Identifying novel cell types that mediate immunity to *Pneumocystis* could potentially suggest unique pathways for targeted therapeutic development.

To investigate immunologic responses that may mediate clearance of *Pneumocystis*, we used RNA sequencing of whole lung at day 14 of *Pneumocystis murina* infection in CD4-depleted mice (which develop chronic progressive infection) and wild-type C57BL/6 mice (which clear by 4 wk). This analysis revealed a prominent eosinophil signature in wild-type mice compared with CD4-depleted mice. We also observed a substantial increase in recruited eosinophils in the bronchoalveolar lavage fluid of infected CD4-replete mice compared with CD4-depleted mice. With the use of hydrodynamic injection of a plasmid encoding IL-5, CD4-depleted and immunosuppressed individuals, such as those receiving immunosuppressive drug therapy for hematologic malignancy and post-transplantation rejection are also susceptible. Given the opportunistic nature of *Pneumocystis* infection, the ability of other immune cell types to contribute to *Pneumocystis* clearance in a CD4+ T cell–dependent manner is still an area of active investigation (19–23). Identifying novel cell types that mediate immunity to *Pneumocystis* could potentially suggest unique pathways for targeted therapeutic development.

To investigate immunologic responses that may mediate clearance of *Pneumocystis*, we used RNA sequencing of whole lung at day 14 of *Pneumocystis murina* infection in CD4-depleted mice (which develop chronic progressive infection) and wild-type C57BL/6 mice (which clear by 4 wk). This analysis revealed a prominent eosinophil signature in wild-type mice compared with CD4-depleted mice. We also observed a substantial increase in recruited eosinophils in the bronchoalveolar lavage fluid of infected CD4-replete mice compared with CD4-depleted mice. With the use of hydrodynamic injection of a plasmid encoding IL-5, CD4-depleted and...
Rag1−/− knockout mouse receiving plasmid expressing IL-5 (pIL5) demonstrated significant eosinophilia in the lung and decreased Pneumocystis burden 14 d post challenge. Finally, GATA1tm6Sho/J knockout mice deficient in eosinophilopoiesis had no difference in burden when treated with pIL5. With the findings taken together, this study demonstrates that one role of CD4+ T cells during Pneumocystis infection is to recruit eosinophils to the lung, which then contribute to clearance of Pneumocystis.

Materials and Methods

Mice
C57BL/6 mice, Rag1−/− knockout mice on a C57BL/6 background, BALB/c mice, and Gata1tm6Sho/J mice were all ordered from The Jackson Laboratory (24). Mice were all 6- to 8-wk-old females and were bred in the Rangos Research Building Animal Facility. All use of laboratory animals was approved and performed in accordance with the University of Pittsburgh Institutional Care and Use Committee.

Pneumocystis infection time course and primary infections

Twenty-five C57BL/6 female mice were CD4 depleted using weekly i.p. administration of 0.3 mg GK1.5 mAb per mouse and were subsequently challenged with 2.0 × 10^4/ml P. murina cysts, using oropharyngeal inoculation as previously described (22, 25, 26). Twenty-five age-matched C57BL/6 female mice were inoculated at the same time but were not CD4 depleted. Five mice from each group were then sacrificed at days 0, 3, 7, 10, and 14. Four BALB/c and Gata1tm6Sho/J mice were also inoculated with Pneumocystis oropharyngeally and sacrificed at day 14. Six uninfected BALB/c mice were used as naive controls.

RNA isolation and quantitative RT-PCR

Lung RNA was purified using TRIzol Reagent (Life Technologies). Briefly, lungs were homogenized, and following the addition of chloroform, RNA in the aqueous phase was collected and precipitated in isopropanol. Following centrifugation, the RNA was washed with 75% ethanol, centrifuged again, and then resuspended in nuclease-free water. Following incubation at 55˚C, RNA was quantified using a Nanodrop, and 1 μg RNA was converted to cDNA using the iScript cDNA synthesis kit per the manufacturer’s instructions (Bio-Rad). Pneumocystis burden was then quantified using SsoAdvanced quantitative RT-PCR (qRT-PCR) universal probes supersmix (Bio-Rad), with primers and a probe specific for P. murina small subunit (SSU) rRNA with a standard curve of known Pneumocystis SSU rRNA concentrations. SSU primer and probe sequences are as follows: Forward: 9′-GATCGAGTCTCTCTGTCGAGTC-3′; Reverse: 5′-TCATTCAAGGCATGTTCGCAATCCT-3′; probe: 5′-GACCTGTGGTCAAACTACGAACTCGTGGTGTAATGTCGGTAGGAGAGTCCGGATGTACAGTGGTAGTGGACACTCTCAGTG-3′. The products were then used in the following primers: Prg2, Epx, Il5, Clec3a, Muc5ac, Muc5b, and Il13 (Applied Biosystems). Prior to sequencing, the RNA was further purified using a QIAGEN RNA cleanup kit with DNase treatment.

RNA sequencing

Total RNA from mouse whole lung was used to perform RNA sequencing. Each sample was assessed using a Qubit 2.0 fluorometer and Agilent Bioanalyzer TapeStation 2200 for RNA quantity and quality. Library preparation was done using Illumina TruSeq Stranded mRNA sample prep kit. The first step in the workflow involves purifying the poly-A-containing mRNA molecules using poly-T oligo attached magnetic beads. Following purification, the mRNA is fragmented into small pieces, using divalent cations. The cleaved RNA fragments are copied into first-strand cDNA using reverse transcriptase and random primers. Strand specificity is achieved using deoxyuridine triphosphate in the Second Strand Marking Mix, followed by second-strand cDNA synthesis using DNA polymerase 1 and RNase H. These cDNA fragments then have the addition of a single “A” base and subsequent ligation of the adapter. The products are then purified and enriched with PCR to create the final cDNA library. The cDNA libraries are validated using KAPA Biosystems primer premix kit with Illumina-compatible DNA primers and the Qubit 2.0 fluorometer. Quantity is assessed using the Agilent Bioanalyzer TapeStation 2200. The cDNA libraries were pooled at a final concentration of 1.8 pM. Cluster generation and 75-bp paired read single-indexed sequencing was performed on Illumina NextSeq 500s.

Data analysis

Raw reads from an Illumina NextSeq500 in fastq format were trimmed to remove adapter/primer sequences. Trimmed reads were then aligned using BWA (version 0.5.9), settings aln -o 1 -e 10 -i 5 -k 2 -t 8 against the mouse genomic reference sequence. Additional alignment and processing using were done with the Picard tools (version 1.58), including local realignment and score recalibration (not duplicate marking) to generate a final genomic aligned set of reads. Reads mapping to the genome were characterized as exon, intron, or intergenic (outside any annotated gene), using the matched annotation for the genomic reference sequence. The remaining unmapped reads from the genomic alignment were then aligned to a splice reference created using all possible combinations of known exons (based on annotation described above) and then categorizing these as known or novel splice events. These aligned data are then used to calculate gene expression by taking the total of exon and known splice reads for each annotated gene to generate a count value per gene. For each gene a normalized expression value also is generated in two ways: 1) RPM (reads per million mapped), which is calculated by taking the count value and dividing it by the number of million mapped reads; and 2) RPKM (reads per million per kilobase), which is calculated by taking the RPM value and dividing it by the kb length of the longest transcript for each gene. The RPM values are subsequently used for comparing gene expression across samples to remove the bias of different numbers of reads mapped per sample. RPKM values are subsequently used for comparing relative expression of genes with one another to remove the bias of different numbers of mapped reads and alignment transcript lengths. In addition to gene expression measurements, nucleotide variation was also detected using the GATK (version 1.3-25, -dcov 2000 -stand_call_conf 30.0 -stand_emit_conf 10.0 -A DepthOfCoverage -A BaseCounts -A AlleleBalance), which identified single nucleotide and small insertion/deletion (indel) events using default settings. Mapped exonic reads per wild-type sample were as follows: 44,291,011; 43,821,698; and 41,308,864. Mapped exonic reads per GATA1 knock-down sample were as follows: 23,305,058; 43,821,698; 122,834,770; and 29,805,804. Data were then filtered on a quality score of 20 and probes for eosinophil-associated genes: Ear11, Ear3, Ear4, Ccl2, Ccl12, Ccr3, Prg2, Ccl11, Ccl7, Il13, Ear10, Il5ra, Ear2, Csf2rb, Ccl5, Il5, Ear1, Il4 (did not pass quality filter), and Epx (did not pass quality filter).

Data availability

The RNA sequencing data contained in this paper are publicly available through the Sequence Read Archive BioProject number: PRJNA276529. Further information can be found at http://www.ncbi.nlm.nih.gov/bioproject/. Bromchalveolar lavage

Wild-type C57BL/6 and GATA1-treated mice infected with Pneumocystis were anesthetized at day 14 post infection, and a 20 gauge Exel Safelet Catheter (Exel International) was inserted into the cricoid cartilage. The needle was then removed, and 1 ml aliquots were inserted and removed from the lung, using a 1-ml syringe (10 ml total). BAL cells were then spun at 300 × g for 10 min, resuspended in PBS, and counted using trypan blue stain. A total of 1 × 10^6 cells were then transferred to a round-bottom 96-well plate for staining, and the remainder of cells were transferred to TRIzol Reagent for RNA isolation (as described above). Naive (uninfected) mice were also examined as a control.

IL-5 and Eotaxin-1 Luminex on lung homogenate

Lung was collected in PBS containing protease inhibitors (Roche) and homogenized. We used a Bio-Plex Pro Assay (23-plex; Bio-Rad) according to the manufacturer’s recommendations. Briefly, the plate was treated with Bio-Plex assay buffer, followed by vortexing and two washes. Lung homogenates (undiluted), standards, and blanks were then added to the plate and incubated at room temperature for 1 hour, shaken at 850 rpm, covered. Following three washes, detection Abs were then diluted and added to each well. The plate was incubated as above. Following three washes, diluted streptavidin-PE was added to each well and incubated at room temperature for 20 min on shaker. The plate was then washed three times and resuspended in assay buffer, and beads were quantified using a Bio-Plex MAGPIX (Bio-Rad).

Flow cytometry

BAL cells or cells from digested lung (1 × 10^6 total) were spun at 300 × g for 3 min, resuspended in PBS, and pelleted once more. Cells were then resuspended in PBS containing 2% heat-inactivated FBS and 0.4 mg/μl anti-CD16/CD32 (eBioscience, clone: 93). Following a 15-min incubation at 4˚C, cells were stained with the following Abs: SglcF-PE (BD Pharmingen, Annexin V-PE (BD Pharmingen), and 1:25 dilution of the relevant Abs. Following washing, the stained cells were acquired and analyzed using a BD FACSCalibur on list mode using CellQuest software.

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clone: E50-2440), CD11b-APC (BioLegend, clone: M1/70), GR1-PE-Cy7 (BD Pharmingen, clone: RB6-8C5), CD11c-FITC (eBioscience, clone: N418), and F4/80-APC-e780 (eBioscience, clone: BM8). Following an hour of incubation at 4°C, cells were washed with PBS, pelleted, and fixed (BD Cytofix). Cells were then analyzed using a BD LSR II Flow Cytometer with compensation via OneComp eBeads (eBioscience).

**Eosinophil culture and Pneumocystis killing assay**

Bone marrow–derived eosinophils were generated using a previously described protocol (27) and per our previous work (28). Briefly, bone marrow was isolated from naive BALB/c mice, and cells were plated at $1 \times 10^6$ cells/ml in RPMI 1640 containing 20% FBS (Irvine Scientific, Santa Ana, CA), 2 mM glutamine, 25 mM HEPES, 1× MEM nonessential amino acids, 1 mM sodium pyruvate (all from Life Technologies BRL, Rockville, MD), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 100 ng/ml stem cell factor, and 100 ng/ml FLT3-L (both from PeproTech). After 4 d, cells were replated in the above media supplemented with 10 ng/ml IL-5. After 10 d, bone marrow cells were fully differentiated into eosinophils. As previously reported (27), samples of $1 \times 10^5$ cells were taken for RNA analysis each time media were changed, for real-time PCR analysis of Epx for eosinophil development and Mpo (Applied Biosystems) for neutrophil development. In addition, cells were cytopspun onto glass slides, Giemsa stained, and analyzed for morphology and purity by a murine pathologist in the Comparative Pathology Laboratory at the University of Alabama at Birmingham. On day 10, bone marrow–derived eosinophils were enumerated and used in experiments. Bone marrow–derived eosinophils (1 × 10^5) were then cocultured with 1 × 10^5 Pneumocystis cysts in 100 μl for 18 h at 37°C and 5% CO₂ alone or in the presence of 10 ng/ml IL-4 and IL-13. Controls included P. murina cultured in the absence of eosinophils as well as in the presence or absence of IL-4 and IL-13. Total RNA was isolated from the contents of each well using TRZol LS reagent (Invitrogen, Carlsbad, CA), and Pneumocystis SSU burden was calculated as above. Percent killing was defined as previously described (20).

**pIL5 and pCMV hydrodynamic injection**

An untagged murine IL-5 expression vector (pIL5; Origene, MC208784) and an empty vector pCMV6 control (Origene, PS100001) were grown in Mix and Go E. coli (Zymo Research) in 200 ml Luria–Bertani containing kanamycin and were prepared using an EndoFree Plasmid Maxi Kit (QIAGEN) per the manufacturer’s instructions. Following quantification of vector, 10 μg vector was added to 2 ml Ringer’s solution (0.9% NaCl, 0.03% KCl, and 0.016% CaCl₂) and injected i.v. via the tail vein within 5 s, as previously described (29, 30).

**FIGURE 1.** RNA sequencing of whole lung shows a prominent CD4-dependent eosinophil signature at day 14 of Pneumocystis infection. (A) Wild-type or GK1.5-treated CD4-depleted C57BL/6 mice were infected with $2.0 \times 10^6$ cysts per milliliter of Pneumocystis and were sacrificed at day 3, 7, 10, or 14 following infection (n = 5 at each time point). Pneumocystis burden was calculated by qRT-PCR of the SSU rRNA, and a significant decrease was seen at day 14. *p < 0.01 by the Student t test. (B) RNA sequencing of whole-lung RNA at day 14 in GK1.5-treated and wild-type mice shows increase in expression in genes associated with eosinophils (n = 4 in each group). (C) Histogram of expression values from the heat map in (B). *p < 0.05 by the Student t test. Similar to the expression pattern seen by RNA sequencing, a 10-fold increase in Prg2 is seen at day 14 by qRT-PCR. p > 0.05. (D) Eosinophil culture and Pneumocystis killing assay. Bone marrow–derived eosinophils were generated using a previously described protocol (27) and per our previous work (28). Briefly, bone marrow was isolated from naive BALB/c mice, and cells were plated at $1 \times 10^6$ cells/ml in RPMI 1640 containing 20% FBS (Irvine Scientific, Santa Ana, CA), 2 mM glutamine, 25 mM HEPES, 1× MEM nonessential amino acids, 1 mM sodium pyruvate (all from Life Technologies BRL, Rockville, MD), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 100 ng/ml stem cell factor, and 100 ng/ml FLT3-L (both from PeproTech). After 4 d, cells were replated in the above media supplemented with 10 ng/ml IL-5. After 10 d, bone marrow cells were fully differentiated into eosinophils. As previously reported (27), samples of $1 \times 10^5$ cells were taken for RNA analysis each time media were changed, for real-time PCR analysis of Epx for eosinophil development and Mpo (Applied Biosystems) for neutrophil development. In addition, cells were cytopspun onto glass slides, Giemsa stained, and **analyzed for morphology and purity by a murine pathologist in the Comparative Pathology Laboratory at the University of Alabama at Birmingham. On day 10, bone marrow–derived eosinophils were enumerated and used in experiments. Bone marrow–derived eosinophils (1 × 10^5) were then cocultured with 1 × 10^5 Pneumocystis cysts in 100 μl for 18 h at 37°C and 5% CO₂ alone or in the presence of 10 ng/ml IL-4 and IL-13. Controls included P. murina cultured in the absence of eosinophils as well as in the presence or absence of IL-4 and IL-13. Total RNA was isolated from the contents of each well using TRZol LS reagent (Invitrogen, Carlsbad, CA), and Pneumocystis SSU burden was calculated as above. Percent killing was defined as previously described (20).**
IL-5 ELISA
Serum IL-5 was quantified using BioLegend ELISA MAX Mouse IL-5 ELISA kit per the manufacturer’s instructions. Briefly, a 96-well plate was coated with capture Ab and stored overnight at 4˚C. Following washes with PBS + 0.05% Tween 20, the plate was blocked with assay diluent for 1 h at room temperature. Serum samples (diluted 1:20) and IL-5 standard were diluted in assay diluent, added to the plate, and incubated overnight at 4˚C. Detection Ab and diluted Avidin-HRP were then added to the plate, with washes between additions, and the plate was developed with tetramethylbenzidine substrate in the dark. Absorbance was then measured at 450 nm.

Lung digestion
The right superior lobe of the lung was physically digested using scissors, followed by 1.5-h incubation in collagenase/DNase in a 37˚C shaker at 250 rpm. Single-cell suspensions were strained using a 70-μm filter, pelleted.

FIGURE 2. CD4-dependent recruitment of eosinophils to the lung at day 14 of Pneumocystis infection. (A) BAL of naive (uninfected), wild-type, and GK1.5-treated CD4-depleted mice 14 d post inoculation with Pneumocystis shows a large population of cells with high side scatter in wild-type mice (left panel). The cells were gated as shown (left panel), and a SiglecF+CD11b+ population was seen in the wild-type animals, but not the naive or GK1.5-treated animals (right panel). (B) Significant increase in percentage of SiglecF+CD11b+ cells in wild-type animals compared with naive and GK1.5-treated animals (n = 4–5). ****p < 0.0001 by one-way ANOVA with Tukey’s multiple comparisons. (C) qRT-PCR for Epx (top) and Prg2 (bottom) on RNA extracted from BAL cell pellets shows significant increase in expression in wild-type animals compared with naive and GK1.5-treated animals. *p < 0.05 by one-way ANOVA with Tukey’s multiple comparisons.

FIGURE 3. Eosinophils contribute to control of Pneumocystis infection both in vitro and in vivo. (A) BALB/c and Gata1<sup>imdbsho/J</sup> knockout mice were infected with Pneumocystis and sacrificed at day 14 post infection, and SSU burden was quantified by qRT-PCR. ***p < 0.01 by Student t test. Uninfected BALB/c mice have no detectable Pneumocystis burden. (B) qRT-PCR for Epx (left) and Prg2 (right) on RNA from whole lung shows significant increase in BALB/c mice infected with Pneumocystis compared with uninfected BALB/c and infected Gata1<sup>imdbsho/J</sup> knockout mice. *p < 0.05 by the Kruskal–Wallis test with Dunn’s multiple comparisons test. (C) Bone marrow–derived eosinophils from BALB/c mice demonstrate anti-Pneumocystis activity when cocultured in vitro for 24 h at an eosinophil to P. murina cyst ratio of 100:1. ***p < 0.0001, Student t test. (D) Bone marrow–derived eosinophils show enhanced killing activity when cocultured with Pneumocystis in the presence of 10 ng/ml of IL-4 and IL-13 compared with Pneumocystis alone. **p < 0.01 by Student t test.
and then resuspended in 10 ml PBS. Following enumeration using trypan blue, cells were stained for flow cytometry as described above.

**Histology**

The left main bronchus was clamped using forceps, and 250 µl 10% formalin was injected into the bronchus. The lung tissue was submerged in 10% formalin, paraffin embedded, and processed by the Children’s Hospital of Pittsburgh Histology Core. Sections were then stained using H&E and periodic acid–Schiff.

**Statistics**

All statistics were performed using GraphPad Prism 6. Briefly, an unpaired, two-tailed Student t test, with \( p < 0.05 \) considered significant, was used for all studies except for the BALB/c pIL5 treatment. Given the non-Gaussian distribution for the BALB/c pIL5 treatment, a Mann–Whitney nonparametric rank test was performed, with \( p < 0.05 \) considered significant. For studies with three groups, a one-way ANOVA with Tukey’s multiple comparisons was used, with \( p < 0.05 \) considered significant. A Kruskal–Wallis nonparametric test with Dunn’s multiple comparisons test was used for gene expression analysis in the BALB/c primary challenge experiment, with \( p < 0.05 \) considered significant. Linear regression was also performed using Prism, and Pearson’s correlation coefficient calculations were performed.

**Results**

**RNA sequencing of whole lung identifies an eosinophil signature early in Pneumocystis infection**

To further understand the role of CD4+ T cells in *Pneumocystis* infection, we examined *Pneumocystis* burden in wild-type and GK1.5-treated, CD4-depleted C57/Bl6 mice. In this study, at day 14 using quantitative real-time PCR, wild-type mice begin to clear infection, as CD4-depleted mice had a higher fungal burden at this time point (Fig. 1A). As an unbiased approach to investigating potential mechanisms of fungal clearance, we used RNA sequencing of whole lung at this time point to examine the signatures of potential effector cells. Strikingly, several genes associated with eosinophil function and recruitment, such as prg2 (major basic protein), il5ra (IL-5 receptor α), ccr3, ccl11 (eotaxin-
1), and ccl24 (eotaxin-2), were all significantly upregulated at day 14 of infection in wild-type animals (Fig. 1B, 1C). Another specific eosinophil marker, eosinophil-associated ribonuclease 2 (ear2), was also significantly upregulated in wild-type animals, whereas less specific eosinophil-associated ribonucleases (ear5, ear10, ear11) also had higher expression in wild-type animals. Importantly, in addition to a robust eosinophil signature at day 14 in wild-type animals, IL-5 had significantly higher expression in wild-type animals at the transcriptional level at day 7 and day 10 post infection with Pneumocystis (Fig. 1D). Importantly, prg2, an eosinophil-associated gene, had a 10-fold increase in expression at day 14 by qRT-PCR, similar to that detected by RNA sequencing (Fig. 1D). Furthermore, protein levels of IL-5 and eotaxin-1 (CCL11) were significantly higher in wild-type animals at day 14 when compared with CD4-depleted mice (Fig. 1E).

**Eosinophils are present in BAL fluid early in infection**

To further clarify the CD4+ T cell–dependent eosinophil response to Pneumocystis infection, we sought to define the cell populations in the BAL fluid of wild-type and CD4-depleted animals at day 14. Cell populations in naive mice were also analyzed. A population of cells with high side scatter was present in animals with intact CD4+ T cell responses, but absent in naive and CD4-depleted animals infected with Pneumocystis (Fig. 2A, left panel). After gating on all cells, a population of SiglecF+CD11b+ cells was noted in the wild-type animals, but this population was substantially reduced back to naive levels in animals treated with GK1.5 (Fig. 2A, right panel). The SiglecF+CD11b+ population represented over 60% of cells in BAL fluid in wild-type mice, whereas less than 2% of cells were SiglecF+CD11b+ in mice treated with GK1.5 (Fig. 2B). In addition, the RNA from BAL cell pellets was enriched for transcripts associated with eosinophils; Epx and Prg2 expression was nearly 1000-fold higher in BAL cell pellets from wild-type mice when compared with naive and CD4-depleted mice (Fig. 2C).

**Eosinophils contribute to control of Pneumocystis infection both in vitro and in vivo**

To determine the role of eosinophils in Pneumocystis infection, we used a loss-of-function approach and infected eosinophilopoiesis-deficient Gata1tm6Sho/J mice and BALB/c controls. Gata1tm6Sho/J mice had an increased Pneumocystis burden at day 14 post infection compared with control BALB/c mice, whereas uninfected BALB/c had no detectable Pneumocystis burden (Fig. 3A). BALB/c mice had an increase in Epx expression and a modest increase in Prg2 expression compared with Gata1tm6Sho/J mice and BALB/c uninfected controls (Fig. 3B). Eosinophils cultured from BALB/c bone marrow also demonstrated anti-Pneumocystis activity in vitro (Fig. 3C). Furthermore, bone marrow–derived eosinophils displayed increased Pneumocystis killing activity when cocultured with IL-4 and IL-13 (Fig. 3D).
Hydrodynamic injection of IL-5 promotes Pneumocystis clearance in CD4-depleted C57BL/6 and Rag1−/− mice

To induce eosinophilia prior to Pneumocystis infection, we used hydrodynamic injection with either a pIL-5 or an empty plasmid control (pCMV) in C57BL/6 mice treated with GK1.5 or Rag1−/− mice 3 d prior to infection (Fig. 4A). At day 2 following infection, mice treated with pIL5 had more than a log-fold increase in serum IL-5 (Fig. 4B). At day 14 of infection, treatment with pIL5 resulted in an increased abundance of a high side scatter population and a SiglecF+CD11b+ population in both CD4-depleted C57BL/6 and Rag1−/− mice (Fig. 4C). Although these populations were present by flow cytometry in the groups treated with pCMV alone (Fig. 4C, left panel), C57BL/6 and Rag1−/− mice treated with pIL5 had significantly more SiglecF+CD11b+ cells as measured by both percentage and total cell number recovered from the lung (Fig. 4D).

Strikingly, both the CD4-depleted C57BL/6 and Rag1−/− mice receiving pIL5 had a statistically significant reduction in Pneumocystis burden by day 14 of infection when compared with mice treated with pCMV (Fig. 4E). Although the average difference in burden was approximately a half-log in both cohorts, some individual mice had a greater than a log reduction in Pneumocystis burden with pIL5 treatment (Fig. 4E). A strong negative correlation existed between total number of eosinophils recruited to the lung and Pneumocystis burden in the C57BL/6-treated animals (Supplemental Fig. 1A, p = 0.0003). Recruitment of eosinophils to the lung could also be observed by H&E staining in mice receiving pIL5 (Fig. 4F). The pIL5-treated mice also had a 10-fold increase in expression of Epx and Prg2 in whole-lung RNA when compared with pCMV-treated mice (Fig. 4G). Furthermore, although pIL5 treatment increased eosinophilic lung inflammation, this was not associated with type 2 immune inflammation as measured by goblet cell hyperplasia or mucin expression (as measured by qRT-PCR of Il13, Clec3, MucSac and by PAS staining) in the pIL5-treated C57BL/6 mice (Supplemental Fig. 2).

IL-5–mediated decrease in Pneumocystis burden is abrogated in eosinophil-deficient Gata1tm6Sho/J mice

To verify the effect of pIL5-required eosinophilopoiesis, we used a genetic approach. CD4-depleted BALB/c mice were treated with pIL5 as described above, and similar levels of IL-5 were induced in the serum (Fig. 5A). Similarly, increased eosinophils were noted in the lung digest of pIL5-treated BALB/c mice (Fig. 5B). The pIL5-treated BALB/c mice had a statistically significant increase in total number and a trend toward higher percentage of SiglecF+CD11b+ cells compared with pCMV-treated BALB/c mice (Fig. 5C). Furthermore, upon sacrifice, BALB/c mice treated with pIL5 had nearly a 50% reduction in Pneumocystis burden when compared with pCMV-treated animals (Fig. 5D, p = 0.04). Burden in this case was normalized to the pCMV group as the inocula over three independent experiments varied; however, a similar reduction in burden was noted with C57BL/6 and Rag1−/− mice (Fig. 4E). Also noteworthy, four pIL5-treated mice had no induction of serum IL-5 and lacked eosinophils in the lung at day 14 by flow cytometry, likely owing to technical variation. If only

FIGURE 6. pIL5 treatment cannot rescue eosinophil-deficient Gata1tm6Sho/J knockout mice. (A) Serum IL-5 ELISA at day 2 post infection shows nearly a log increase in Gata1tm6Sho/J pIL5-treated animals (n = 4; dotted line represents limit of detection). (B) pIL5 treatment does not reduce Pneumocystis burden at day 14 post infection in Gata1tm6Sho/J mice, as measured by qRT-PCR of SSU rRNA. (C) Digested whole lung shows no difference in high side scatter populations (left panel) or SiglecF+CD11b+ cells (right panel) independent of pIL5 treatment status. (D) pIL5-treated Gata1tm6Sho/J mice showed no difference in percentage (top) or total number (bottom) of SiglecF+CD11b+ cells. (E) qRT-PCR for Epx (left) and Prg2 (right) on RNA extracted from whole lung shows no increase in eosinophil-associated genes in Gata1tm6Sho/J mice.
mice that responded to pIL5 treatment were included in the analysis, the mean percentage of Pneumocystis remaining in pIL5-treated mice would be 26% compared with 100% of pCMV-treated mice ($p < 0.0002$). The increased Pneumocystis killing in mice that recruited eosinophils to the lungs is also evident in the strong negative correlation that exists between Pneumocystis burden and total SiglecF<sup>+</sup>CD11b<sup>+</sup> recruited cells (Supplemental Fig. 1B, $p = 0.0001$). The pIL5 treatment was also associated with significant increases in eosinophil-associated genes such as Epx and Prg2 (Fig. 5E).

In contrast, Gata1<sup>tm6Sho/J</sup> mice (on a BALB/c background), mice deficient in eosinophilopoesis, were CD4 depleted and treated with pIL5 or pCMV as described above failed to show an effect of pIL5 on fungal burden, despite similar levels of IL-5 compared with previous mouse strains (Fig. 6A, 6B). Consistent with no reduction in fungal burden, no observable eosinophil recruitment was detected in the lungs of Gata1<sup>tm6Sho/J</sup> mice, as neither a high side scatter population nor a SiglecF<sup>+</sup>CD11b<sup>+</sup> was noted on flow cytometry (Fig. 6B, 6C). Furthermore, there was no induction of Epx or Prg2 in the Gata1<sup>tm6Sho/J</sup> mice treated with pIL5 (Fig. 6E).

**Discussion**

The current study used an unbiased RNA sequencing–based approach toward evaluating the role of CD4<sup>+</sup> T cells in Pneumocystis infection and suggested that CD4<sup>+</sup> T cells can recruit eosinophils to the lung by day 14 of infection by RNA sequencing. Eosinophils were also shown to have a role in immunity against Pneumocystis in a primary challenge model of eosinophil-deficient mice and in an eosinophil-based Pneumocystis in vitro killing assay. Furthermore, induction of eosinophilia via hydrodynamic injection of pIL5 was capable of reducing Pneumocystis burden in vivo in both CD4-depleted C57BL/6 and Rag1<sup>−/−</sup> mice. Finally, the same technique was able to reduce Pneumocystis burden in CD4-depleted BALB/c mice but failed to provide any therapeutic benefit in CD4-depleted Gata1<sup>tm6Sho/J</sup> mice, further implicating eosinophils as a novel cell population responsible for in vivo antifungal activity.

CD4<sup>+</sup> T cells have been established as crucial mediators to Pneumocystis owing to the high incidence of Pneumocystis in HIV/AIDS patients with low CD4<sup>+</sup> T cell counts (1, 5). One clear role of CD4<sup>+</sup> T cells in response to Pneumocystis is to stimulate Ab responses by providing costimulatory signals to B cells (19, 23). Abs against Pneumocystis are protective; however, at day 14 of infection, prior to Ab production, Pneumocystis burden has already plateaued in wild-type animals, suggesting an Ab-independent function for CD4 cells (23). As such, CD4<sup>+</sup> T cells appear to recruit eosinophils early in infection, whereas B cells are undergoing maturation into Ab-producing plasma cells and provide preliminary control of Pneumocystis burden. Importantly, IL-5 transcription and eosinophil recruitment appears to be dependent on CD4<sup>+</sup> T cells in this model, as such markers do not appear until after activation of the adaptive immune response at day 7 post infection.

Eosinophils, classically recognized as mediators of immunity against helminths, have recently been implicated in host defense against a variety of pathogens, including bacterial and viral infections (31). Recently, we have demonstrated that eosinophils contribute to host defense against the fungal pathogen Aspergillus fumigatus through a secretory factor, as eosinophils could mediate fungal killing when separated using Transwells (28). The current study extends these findings by demonstrating that eosinophil-deficient mice are more susceptible to Pneumocystis infection and that eosinophils display antifungal activity in vitro. In addition, treatment of bone marrow–derived eosinophils with IL-4 and IL-13 greatly enhanced killing of Pneumocystis, suggesting that Th2 cytokines in the lung may augment eosinophil-dependent antifungal activity.

Several lines of evidence connect Pneumocystis-related pathologic conditions and eosinophils in both human disease and mouse models. First, eosinophilia in the BAL fluid of HIV-positive patients with Pneumocystis pneumonia has been well documented; however, whether a correlation exists between high eosinophil counts and lower Pneumocystis burden in these patients is unknown (32, 33). Second, patients with a history of Pneumocystis or bacterial pneumonia had a significantly higher rate of physician-diagnosed asthma, a disease that has a well-established eosinophilic component in regard to pathogenesis (34). Murine models of Pneumocystis have also shown that STAT6, a transcription factor required for Th2 responses, is necessary for the development of airway hyperresponsiveness early in the course of infection (23). Although eosinophilia was also documented early in this murine model, the study further links a Th2 response, and potentially eosinophilia, with pathologic changes in the context of Pneumocystis infection (23). In addition, studies have shown that CD8<sup>+</sup> T cells may moderate the interactions between CD4<sup>+</sup> T cells and eosinophils, although all three cell types may contribute to Pneumocystis-driven pulmonary pathologic conditions (35).

However, the current study suggests that eosinophils are more than just the byproduct of a misguided immune response that drives airway hyperresponsiveness and disease. The current study demonstrates that eosinophils have antifungal effects against Pneumocystis infection in vitro and in vivo and appear to be recruited to the lung by CD4<sup>+</sup> T cells early in infection. In addition, the role of eosinophils as a potential therapeutic in the setting of HIV/AIDS may warrant further exploration, as IL-5–mediated eosinophilia can provide reduced Pneumocystis burden even in the setting of complete loss of T cells and B cells. Furthermore, such robust eosinophilia actually appeared to mitigate airway pathologic features (e.g., mucus production), suggesting that Pneumocystis burden may play an equally important role in driving airway hyperresponsiveness. These findings provide evidence that the specific pathways responsible for protective and pathologic effects in Pneumocystis pneumonia may be independent and may allow for the targeted use of eosinophil-based treatments for Pneumocystis while avoiding concurrent pathologic effects.

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**Disclosures**

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