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Riley C. Myers, Chad W. Dunaway, Michael P. Nelson, Jennifer L. Trevor, Alison Morris and Chad Steele

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STAT4-Dependent and -Independent Th2 Responses Correlate with Protective Immunity against Lung Infection with *Pneumocystis murina*

Riley C. Myers,* Chad W. Dunaway,* Michael P. Nelson,* Jennifer L. Trevor,* Alison Morris,^{†,‡} and Chad Steele*

Although it is clear that the loss of CD4⁺ T cells is a predisposing factor for the development of *Pneumocystis* pneumonia, specific Th mechanisms mediating protection are not well understood. Th1, Th2, and Th17 responses have each been implicated in protective responses during infection. As STAT4 may promote Th1 and Th17 development, yet antagonize Th2 development, we investigated its role in *Pneumocystis murina* host defense. STAT4 was required for Th1 and, unexpectedly, Th2 responses in the lungs of C57BL/6 (BL/6) and BALB/c mice 14 d postchallenge, but only BALB/c *Stat4*^{-/-} mice demonstrated susceptibility to *P. murina* lung infection. BL/6 *Stat4*^{-/-}, but not BALB/c *Stat4*^{-/-}, mice maintained an enhanced alternatively activated (M2) macrophage signature in the lungs, which we have previously reported to be associated with enhanced *P. murina* clearance. In addition, anti-*P. murina* class-switched Abs were increased in BL/6 *Stat4*^{-/-} mice, but not BALB/c *Stat4*^{-/-} mice. Supporting our experimental observations, plasma from HIV-infected individuals colonized with *Pneumocystis jirovecii* contained significantly lower levels of the Th2 cytokines IL-4, IL-5, and IL-13 compared with HIV-infected individuals who were not colonized. Collectively, our data suggest that robust local and systemic Th2-mediated responses are critical for immunity to *Pneumocystis*. *The Journal of Immunology*, 2013, 190: 6287–6294.

Pneumocystis *jirovecii* is an opportunistic fungal pathogen that colonizes the lower airway and alveolar spaces in the lung causing *Pneumocystis* pneumonia (PCP). The development of PCP is closely associated with AIDS, and it is the leading cause of morbidity and mortality in the HIV-infected patient population (1). Although AIDS patients are highly susceptible to PCP, other individuals with suppressed immune systems are also at risk for *Pneumocystis* infection. Rheumatoid arthritis and cancer patients receiving B cell depletion therapies such as rituximab and ofatumumab (2) are susceptible to fatal PCP. *Pneumocystis* colonization is associated with chronic obstructive pulmonary disease severity (3) and is a potential contributor to mortality in infants with sudden unexpected death (4). Despite the widespread implementation of high active antiretroviral therapy and use of antibiotics against *Pneumocystis*, the mortality rate due to PCP continues to be ~10% (5), and as high as 30% if requiring intensive care (6), indicating that current treatments have reached a limit on the ability to resolve infection.

CD4⁺ T cells are required for clearance of *Pneumocystis* (7), yet the mechanism by which they specifically control the infection is not well understood. CD4⁺ T cell-mediated immunity to *Pneumocystis murina* is complicated, as mice deficient in the Th1 signature cyto-

kine IFN- γ or the Th2 signature cytokine IL-4 are not more susceptible to infection than wild-type (WT) mice (8). One week after *P. murina* infection, there is a 4:1 ratio of Th2:Th1 cell expansion, with a 2:1 ratio during the peak of infection at day 14 (9), suggesting an early role for Th2 responses. Supporting this, within the first 7 d of infection, inflammatory responses and leukocyte recruitment in response to *P. murina* challenge were defective in *Stat6*^{-/-} BALB/c mice, suggesting that Th2 responses mediate multiple aspects of anti-*P. murina* host defense. However, mice deficient in the anti-inflammatory cytokine IL-10 have accelerated lung clearance of *P. murina* and increased production of IL-12, IL-18, and IFN- γ (10), implicating enhanced Th1-associated responses in augmented protection. Regulatory T cells (Tregs) also play a role in host defense, as depletion of Tregs resulted in enhanced proinflammatory Th1 and Th2 responses during *P. murina* infection (11). Additionally, Ab-mediated neutralization of IL-17 in CD4-competent mice resulted in a significantly higher fungal burden, suggesting Th17 cells may be involved in immune responses against *P. murina* (12).

Optimal development of Th1 cells requires the transcription factor T-bet and the activation of STAT4 by IL-12 signaling (13). STAT4 is also downstream of the IL-23R, suggesting that it may play a role in Th17 development (14). Finally, STAT4-mediated CD4 T cell programming antagonizes Th2 development (15). Therefore, to further understand the contribution of STAT4 to CD4⁺ T cell responses during *P. murina* infection, we evaluated fungal host defense in C57BL/6 (BL/6) and BALB/c *Stat4*^{-/-} mice. Unexpectedly, we not only found that Th2 responses mediated protection against *Pneumocystis* lung infection, but that STAT4 was required for optimal Th2 responses in BALB/c mice.

Materials and Methods

Mice

C57BL/6, BALB/c, and BALB/c *Stat4*^{-/-} mice were obtained from The Jackson Laboratory (Bangor, ME). *Stat4*^{-/-} mice on a C57BL/6 background were provided by M. Kaplan (Indiana University). All mice used in

*Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294; [†]Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261; and [‡]Department of Immunology, University of Pittsburgh, Pittsburgh, PA 15261

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Address correspondence and reprint requests to Dr. Chad Steele, School of Medicine, University of Alabama at Birmingham, 1900 University Boulevard, Tinsley Harrison Tower 437A, Birmingham, AL 35294. E-mail address: chadsteele@uab.edu

Abbreviations used in this article: MLN, mediastinal lymph node; PCP, *Pneumocystis* pneumonia; Treg, regulatory T cell; WT, wild-type.

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experiments were 8–12 wk of age. All animals were housed in a specific pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care–certified facility and handled according to Public Health Service Office of Laboratory Animal Welfare policies after review by the University of Alabama Institutional Animal Care and Use Committee.

Human subjects

Persons with documented HIV infection who were 18 y of age or older and had at least one visit to the University of Pittsburgh Medical Center's HIV/AIDS clinic were recruited between July 1, 2007 and September 30, 2010. Recruitment was performed by using posted advertisements and word of mouth and by contacting patients in a research registry. All participants signed written informed consent forms, and the University of Pittsburgh Institutional Review Board approved the protocol. Participants were excluded if they had new or increasing respiratory symptoms (cough, shortness of breath, and dyspnea) or fevers within the past 4 wk. All participants also performed an oral wash by gargling with sterile saline for 1 min. For determination of *Pneumocystis jirovecii* colonization, DNA extraction was performed on sputa and oral washes using a DNeasy kit (Qiagen, Valencia, CA). *Pneumocystis* colonization was determined by nested PCR of the mitochondrial large subunit rRNA, as previously described (16). DNA extraction and PCR were carried out in separate rooms, and all PCRs were performed in a UV box. Positive and negative controls were included in each reaction mixture. A subject was considered *P. jirovecii* colonized if PCR of either induced sputum or oral wash demonstrated human *Pneumocystis* by DNA sequencing in duplicate reactions. For determination of Th cytokine levels, plasma from participants who were colonized with *P. jirovecii* ($n = 50$) or who were not colonized ($n = 53$) was analyzed using a human 41-plex cytokine and chemokine kit (catalogue HCYTMAG-60K-PX41; Millipore) and the Bio-Plex multiplex suspension cytokine array system, according to the manufacturer's instructions (Bio-Rad Laboratories). Bio-Plex analysis of plasma samples was conducted at the University of Alabama at Birmingham and approved by the University of Alabama at Birmingham Institutional Review Board.

P. murina isolation and inoculation

P. murina was prepared as previously described (17, 18). In brief, C.B-17 SCID mice previously inoculated with *P. murina* were injected with a lethal dose of ketamine/xylazine, and the lungs were aseptically removed and frozen at -80°C in 1 ml PBS. Frozen lungs were homogenized through a 70 μm filter and pelleted at $300 \times g$ for 10 min at 4°C . The pellet was resuspended in 1 ml PBS, and a 1:10 dilution was stained with modified Giemsa stain (Diff-Quik). The number of *P. murina* cysts was quantified microscopically, and the concentration was adjusted to 2×10^6 cysts/ml. For in vivo challenge, mice were anesthetized with isoflurane and administered 2×10^5 cysts in a volume of 0.1 ml via intratracheal inoculation. Some preparations were also adjusted to 2×10^6 cysts/ml, and 50 μl aliquots were placed into tubes containing 200 μl 90% FBS supplemented with 10% DMSO and stored at -80°C . Using this storage method, stable *P. murina* viability, as determined by quantitative real-time PCR, can be maintained for >1 y.

CD4^+ T cell isolation and culture

Mice were anesthetized with i.p. ketamine/xylazine and sacrificed by exsanguination 14 and 28 d postinoculation. Both lungs were collected and minced in IMDM (Sigma-Aldrich, St. Louis, MO) supplemented with 1% penicillin-streptomycin-glutamine (Mediatech, Herndon, VA), 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA), and 0.4 mg/ml polymyxin B (Thermo Fisher), followed by incubation for 60 min with 1 mg/ml tissue culture grade type IV collagenase (Sigma-Aldrich) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through sterile 70 and 40 μm nylon filters, and RBCs were lysed with RBC lysis buffer. Lymph node cells were excised, and cells were isolated by forcing tissue through a sterile 70 μm nylon filter followed by RBC lysis. CD4^+ T cells were isolated using mouse CD4 Flowcomp Dynabeads (catalogue 114-61D; Invitrogen, Carlsbad, CA), per the manufacturer's protocol, to 90–95% purity. The CD4^+ T cells were then plated at 1×10^6 cells/ml in IMDM and stimulated with 2 $\mu\text{g}/\text{ml}$ anti-CD3 (clone 145-11) and 1 $\mu\text{g}/\text{ml}$ anti-CD28 (catalogue 102112; BioLegend, San Diego, CA) for 48 h at 37°C in 5% CO_2 . Controls included cells incubated with medium alone. The supernatants were clarified by centrifugation, and the protein levels of 23 cytokines and chemokines were determined using Bio-Plex multiplex suspension cytokine array, according to the manufacturer's instructions (Bio-Rad Laboratories). The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories).

Real-time PCR analysis of *P. murina* rRNA in lung tissue

Total RNA was isolated from the right lung of mice inoculated 14 and 28 d prior with *P. murina* by a single-step method using TRIzol reagent

(Invitrogen Life Technologies), according to the manufacturer's instructions. The RNA was then transcribed to cDNA (iScript cDNA synthesis kit; Bio-Rad), and real-time PCR for *P. murina* rRNA was performed, as described previously (17, 18).

Alternative macrophage activation marker analysis

Total RNA was isolated from the right lung of mice inoculated 14 and 28 d prior with *P. murina* by a single-step method using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. The RNA was then transcribed to cDNA (iScript cDNA synthesis kit; Bio-Rad), and real-time PCR for the M2 marker *Remla* (Mm00445109_m1; Applied Biosystems) and the M1 marker *iNOS* (Mm00440485_m1; Applied Biosystems) was performed (iQ Supermix; Bio-Rad). Gene expression was normalized to GAPDH mRNA levels (primers/probe from Applied Biosystems) using the $2^{-\Delta\Delta\text{Ct}}$ method, as previously described (18). The left lung was homogenized in PBS supplemented with Complete Mini protease inhibitor tablets (Roche), clarified by centrifugation, and stored at -80°C . CCL17/TARC (catalogue MCC170) levels were determined by ELISA (R&D Systems, Minneapolis, MN).

Pneumocystis-specific Ab ELISA

Blood was collected weekly for 28 d from the tail vein, and sera were frozen at -20°C . A sonicate of *P. murina* was prepared as previously described (19, 20). Briefly, *P. murina* inoculum was sonicated and clarified by centrifugation, and then coated onto microtiter plates (Sigma-Aldrich) at 1 $\mu\text{g}/\text{well}$ overnight at 4°C . The wells were then blocked with 10% BSA (Equitech Bio, Kerrville, TX) for 1 h at 37°C . Plates were washed with PBS containing 0.05% Tween 20. Sera were serially diluted and incubated at 37°C for 1 h. Abs were detected by incubating plates with anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG2c conjugated to HRP (Southern Biotech, Birmingham, AL) for 1 h at 37°C , followed by incubation with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich). Endpoint data are expressed as the inverse \log_2 of the dilution at which the OD450 was 0.1.

Statistical analysis

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, San Diego, CA). Comparisons between groups were made with paired or unpaired two-tailed Student *t* test. For comparisons of plasma cytokines between *P. jirovecii*-colonized and noncolonized participants, a nonparametric Mann-Whitney *U* test was employed. Significance was accepted at $p \leq 0.05$.

Results

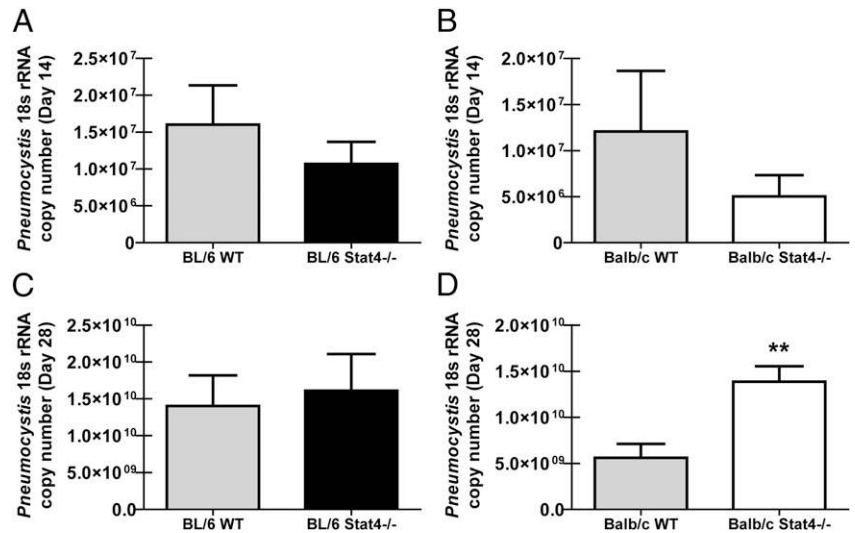
Differential susceptibility to *P. murina* lung infection between *Stat4*^{-/-} mice on BL/6 versus BALB/c backgrounds

Mice deficient in the IFN- α R (21, 22), IL-12p35 (23), or IL-23p19 (12), all of which signal through STAT4 (13, 14, 24), have delayed clearance of *P. murina*, suggesting that STAT4 contributes to anti-*P. murina* responses in the lung. As *Stat4*^{-/-} mice are available on the Th1 skewed BL/6 background and the Th2 skewed BALB/c background, BL/6 and BALB/c WT and *Stat4*^{-/-} mice were challenged with *P. murina* and fungal burden was determined 14 and 28 d thereafter. There was no difference in lung burden between BL/6 WT and BL/6 *Stat4*^{-/-} mice (Fig. 1A) or BALB/c WT and BALB/c *Stat4*^{-/-} mice (Fig. 1B) 14 d postchallenge. In contrast, whereas BL/6 *Stat4*^{-/-} mice had similar *P. murina* lung burden as BL/6 WT mice (Fig. 1C), BALB/c *Stat4*^{-/-} mice had a significantly higher burden in the lungs compared with BALB/c WT mice (Fig. 1D). Thus, despite both strains having a deficiency in STAT4, susceptibility to *P. murina* lung infection was uniquely observed in mice on the BALB/c background.

BL/6 and BALB/c *Stat4*^{-/-} mice demonstrate impaired CD4^+ Th2 responses in the lung

Due to the unexpected susceptibility difference between BL/6 *Stat4*^{-/-} mice and BALB/c *Stat4*^{-/-} mice, we questioned whether this could be explained by differences in lung CD4^+ T cell responses. On day 14 after *P. murina* inoculation, CD4^+ T cells were purified from enzymatic lung digest cell suspensions and stimulated for 48 h with anti-CD3 and anti-CD28. As assessed by Bio-

FIGURE 1. Differential susceptibility to *P. murina* lung infection between *Stat4*^{-/-} mice on BL/6 versus BALB/c backgrounds. C57BL/6 WT and *Stat4*^{-/-} mice and BALB/c WT and *Stat4*^{-/-} mice were administered 2 × 10⁵ *Pneumocystis* cysts via intratracheal inoculation. (A and B) Fourteen and (C, D) 28 d postinoculation, lungs were collected and *Pneumocystis* burden was determined by real-time PCR for *Pneumocystis* rRNA copy number. The figure illustrates representative data from one of two independent studies with an n = 5 mice per group. Data are expressed as mean *Pneumocystis* rRNA copy number. Data are expressed as mean + SEM. **p < 0.01 (unpaired two-tailed Student t test).



Plex, production of the Th1 signature cytokines IL-2 and IFN-γ by CD4⁺ T cells from the lungs of BL/6 *Stat4*^{-/-} mice (Fig. 2A) and BALB/c *Stat4*^{-/-} mice (Fig. 2B) was significantly reduced compared with their WT counterparts, as expected. However, unexpectedly, IL-4, IL-5, and IL-13 production by lung CD4⁺ T cells were also significantly reduced in BL/6 *Stat4*^{-/-} mice (Fig. 2A) and BALB/c *Stat4*^{-/-} mice (Fig. 2B). Thus, *Stat4*^{-/-} mice on both backgrounds exhibited global defects in Th1-type and Th2-type cytokine production during *P. murina* infection. As a role for STAT4 in IL-17A production by CD4⁺ T cells has been reported (14), we questioned its production by lung CD4⁺ T cells during *P. murina* infection. IL-17A production by CD4⁺ T cells isolated from the lungs of BALB/c *Stat4*^{-/-} mice, which were susceptible to *P. murina* infection, was similar to CD4⁺ T cells isolated from the lungs of BALB/c WT mice (Fig. 2B). In contrast, lung CD4⁺ T cell-mediated production of IL-17A was significantly less in BL/6 *Stat4*^{-/-} mice (Fig. 2A), which were protected from *P. murina* infection (compared with BALB/c *Stat4*^{-/-} mice). By 28 d postchallenge, IL-4, IL-5, and IL-13 production by lung CD4⁺ T cells returned in BL/6 *Stat4*^{-/-} mice, as did IL-17A, although IFN-γ remaining impaired (Fig. 2C). However, lung CD4⁺ T cells in BALB/c *Stat4*^{-/-} mice continued to display significantly impaired production of IFN-γ, IL-4, IL-5,

and IL-13 (Fig. 2D). Furthermore, IL-17A production by lung CD4⁺ T cells was now also impaired at 28 d postchallenge. Thus, resistance to *P. murina* lung infection in BL/6 *Stat4*^{-/-} mice was associated with increased lung CD4⁺ Th2 responses, whereas susceptibility to *P. murina* lung infection in BALB/c *Stat4*^{-/-} mice correlated with significantly attenuated lung CD4⁺ Th1, Th2, and Th17 responses.

BL/6 Stat4^{-/-} mice demonstrate enhanced lung M2 macrophage polarization

We have recently reported that an increase in alternatively activated (M2) alveolar macrophages correlated with an enhanced ability to clear *P. murina* (18). To determine whether M2 responses were different between BL/6 *Stat4*^{-/-} mice and BALB/c *Stat4*^{-/-} mice, we assessed markers of M2 macrophage populations in the lung. Fourteen days post-*P. murina* challenge, there were lower lung mRNA levels of the M2 macrophage marker *Retnla* (RELM-α/FIZZ-1) (Fig. 3A) in BL/6 *Stat4*^{-/-} mice, but not BALB/c *Stat4*^{-/-} mice (Fig. 3B), despite both of these strains demonstrating lower Th2 responses by lung CD4⁺ T cells (Fig. 2A, 2B). Lung levels of CCL17, a chemokine produced by M2 macrophages, were also not different between WT BL/6 mice and BL/6 *Stat4*^{-/-} mice (Fig. 3C) and WT BALB/c mice and BALB/c

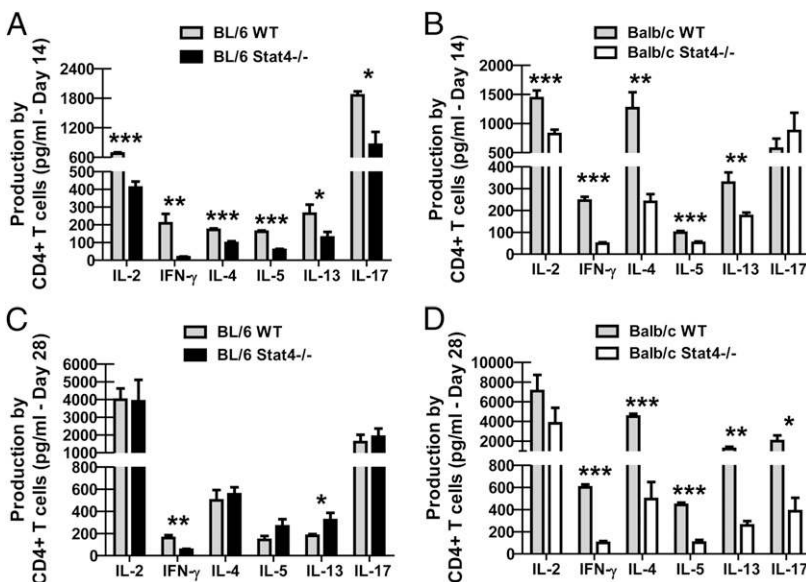
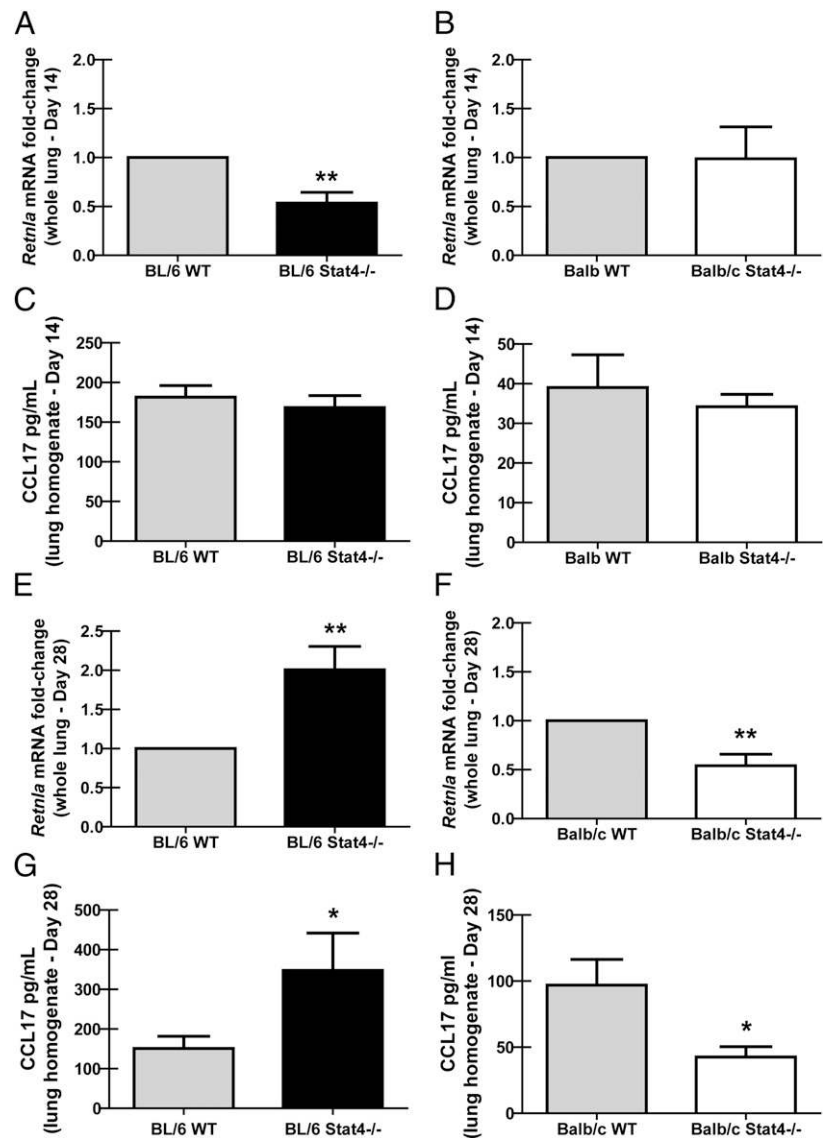


FIGURE 2. BL/6 and BALB/c *Stat4*^{-/-} mice demonstrate impaired CD4⁺ Th2 responses in the lung. C57BL/6 WT and *Stat4*^{-/-} mice and BALB/c WT and *Stat4*^{-/-} mice were administered 2 × 10⁵ *Pneumocystis* cysts via intratracheal inoculation. (A and B) Fourteen and (C, D) 28 d postinoculation, the lungs were collected and enzymatically digested, and CD4⁺ T cells were isolated via Dynabeads, followed by stimulation with 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 48 h. Th cytokine levels were quantified in clarified coculture supernatants by Bio-Plex. Cumulative data are shown from two to three independent experiments with cells cultured in duplicate or triplicate. Data are expressed as mean pg/ml + SEM. For both graphs, *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired two-tailed Student t test).

FIGURE 3. BL/6 *Stat4*^{-/-} mice demonstrate enhanced lung M2 macrophage polarization. C57BL/6 WT and *Stat4*^{-/-} mice and BALB/c WT and *Stat4*^{-/-} mice were administered 2×10^5 *Pneumocystis* cysts via intratracheal inoculation. (A–D) Fourteen and (E–H) 28 d postinoculation, the right lung was collected, total RNA was isolated and transcribed to cDNA, and quantitative real-time PCR was performed for *Retnla* (A, B, E, F). Gene expression was normalized to *Gapdh*, and fold changes between WT (set at 1) and *Stat4*^{-/-} mice were determined using the $2^{-\Delta\Delta Ct}$ method. Of note, there were no differences in the δ Ct values (Ct value of *Retnla* minus Ct value of *Gapdh*) when comparing C57BL/6 WT mice and BALB/c WT mice at 14 and 28 d postchallenge. For CCL17 analysis (C, D, G, H), mice were infected as described, and, 28 d postinoculation, the left lung was collected and homogenized in PBS supplemented with Complete Mini protease inhibitor tablets, and supernatants were clarified by centrifugation. CCL17 levels were determined in lung homogenate supernatants by ELISA. Cumulative data are shown from three independent studies with $n = 4-6$ mice/group per study. (A, B, E, F) Data are expressed as mean fold change. $**p < 0.01$ (paired two-tailed Student *t* test). (C, D, G, H) Data are expressed as mean pg/ml + SEM. $*p < 0.05$ (unpaired two-tailed Student *t* test).



Stat4^{-/-} mice (Fig. 3D) 14 d postchallenge. In contrast, by 28 d post-*P. murina* challenge, there were significantly higher lung *Retnla* (RELM- α /FIZZ-1) mRNA levels (Fig. 3E) and CCL17 protein levels (Fig. 3G) in the lungs of BL/6 *Stat4*^{-/-} mice, indicating that, along with increased lung CD4⁺ Th2 responses (Fig. 2C), BL/6 *Stat4*^{-/-} mice had increased M2 macrophage activation. In contrast, BALB/c *Stat4*^{-/-} mice had significantly lower lung mRNA levels of *Retnla* (RELM- α /FIZZ-1) compared with BALB/c WT mice (Fig. 3F) as well as a significant reduction in the concentration of CCL17 in the lungs of BALB/c *Stat4*^{-/-} mice (Fig. 3H), which correlated with attenuated lung CD4⁺ Th2 responses (Fig. 2D). Of note, naive BL/6 *Stat4*^{-/-} mice did not demonstrate evidence of increased CCL17 or *Retnla* mRNA levels (data not shown). There was no difference in the mRNA levels of the M1 macrophage marker *Nos2* between BL/6 *Stat4*^{-/-} mice and BALB/c *Stat4*^{-/-} mice compared with their respective WT controls (data not shown). We further did not observe any differences in the pro-M2 cytokine IL-33 in either strain of WT or *Stat4*^{-/-} mice (data not shown). Thus, BALB/c *Stat4*^{-/-} mice, which are susceptible to *P. murina* infection (relative to BL/6 *Stat4*^{-/-} mice), had diminished M2 macrophage activation, suggesting that intact/enhanced M2 macrophage activation in BL/6 *Stat4*^{-/-} mice is a possible protective mechanism.

P. murina-specific Ab levels are elevated in serum of BL/6 *Stat4*^{-/-} mice, but not BALB/c *Stat4*^{-/-} mice

To gain further insight into potential mechanisms of resistance and susceptibility between BL/6 *Stat4*^{-/-} mice and BALB/c *Stat4*^{-/-} mice, *P. murina*-specific serum Ab levels were assessed. BL/6 and BALB/c WT and *Stat4*^{-/-} mice were challenged with *P. murina* and sera were collected weekly for 28 d and analyzed by ELISA. The level of anti-*P. murina* IgM was similar between BL/6 and BALB/c *Stat4*^{-/-} mice and their respective controls, suggesting that STAT4 does not play a role in the production of pre-existing *P. murina*-specific natural IgM or in IgM produced during the immune response against *P. murina* (data not shown). However, BL/6 *Stat4*^{-/-} mice had enhanced anti-*P. murina* class-switched Ab production compared with BL/6 WT mice. Whereas *P. murina*-specific IgG1 production by B cells in BL/6 *Stat4*^{-/-} mice was significantly enhanced 28 d postchallenge (Fig. 4A), IgG2b was enhanced earlier at days 7, 14, and 21 (Fig. 4B). The production of *P. murina*-specific IgG2c was also significantly increased throughout the immune response in BL/6 *Stat4*^{-/-} mice compared with BL/6 WT mice (Fig. 4C). In contrast, there was no difference in the levels of anti-*P. murina* Abs of any isotype at any time point examined in BALB/c *Stat4*^{-/-} mice compared with

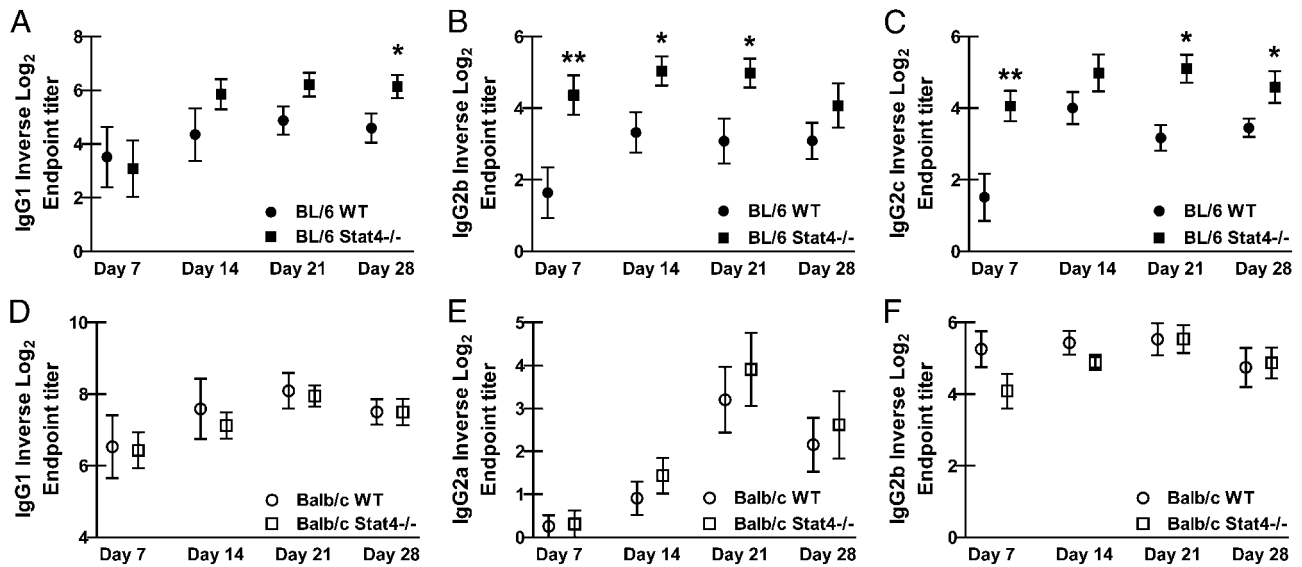


FIGURE 4. *P. murina*-specific Ab levels are elevated in serum of BL/6 *Stat4*^{-/-}, but not BALB/c *Stat4*^{-/-}, mice. (**A–C**) C57BL/6 WT and *Stat4*^{-/-} mice and (**D–F**) BALB/c WT and *Stat4*^{-/-} mice were administered 2×10^5 *Pneumocystis* cysts via intratracheal inoculation, followed by bleeding mice weekly for 28 d. *P. murina*-specific (A, D) IgG1, (B, E) IgG2b, (C) IgG2c (C57BL/6), and (F) IgG2a (BALB/c) were determined by ELISA. Cumulative data are shown from three independent studies with $n = 3$ –4 mice/group per study. Data are expressed as the mean per group of the natural log of the dilution at which the OD450 is 0.1 + SEM. * $p < 0.05$, ** $p < 0.01$, respectively (unpaired two-tailed Student *t* test).

BALB/c WT mice (Fig. 4D–F). Thus, enhanced anti-fungal Ab production in addition to increased M2 macrophage activation in BL/6 *Stat4*^{-/-} mice is sufficient for protection during *P. murina* infection in the absence of robust CD4⁺ T cell responses in the lung (14 d postchallenge; Fig. 2A). Moreover, whereas BALB/c *Stat4*^{-/-} mice also exhibited defective CD4⁺ T cell responses in the lung (14 and 28 d postchallenge; Fig. 2B, 2D), these mice had decreased M2 macrophage activation and no difference in anti-*P. murina* Ab production, rendering these mice susceptible to *P. murina* infection.

CD4⁺ Th2 responses in the draining lymph nodes are elevated in BL/6 *Stat4*^{-/-} mice, but significantly impaired in BALB/c *Stat4*^{-/-} mice

The observation of higher *P. murina*-specific IgG levels in sera from BL/6 *Stat4*^{-/-} prompted us to determine whether a difference in systemic CD4⁺ T cell responses between BL/6 and BALB/c WT and *Stat4*^{-/-} mice existed. CD4⁺ T cells from the mediastinal lymph nodes (MLN) of BL/6 and BALB/c WT and *Stat4*^{-/-} mice were isolated 14 d after challenge with *P. murina* and stimulated ex vivo with anti-CD3 and anti-CD28 for 48 h. Similar to CD4⁺ T cells from the lungs, the production of IL-4, IL-5, IL-13, IL-2, and IFN- γ by CD4⁺ T cells from the MLN of BALB/c *Stat4*^{-/-} mice was significantly diminished compared with CD4⁺ T cells from the MLN of BALB/c WT mice (Fig. 5A). In contrast, CD4⁺ T cells from the MLN of BL/6 *Stat4*^{-/-} mice produced significantly more IL-4, IL-5, and IL-13 compared with CD4⁺ T cells from the MLN of BL/6 WT mice (Fig. 5B). IL-17A production was significantly reduced in MLN CD4⁺ T cells from BL/6 *Stat4*^{-/-} mice, yet there was no difference in IFN- γ production (Fig. 5B). Thus, the enhanced production of anti-*P. murina* class-switched Abs observed in sera from BL/6 *Stat4*^{-/-} mice correlated with lack of susceptibility of these mice in the presence of CD4⁺ T cell defects. In contrast, no changes in anti-*P. murina* class-switched Abs in sera from BALB/c *Stat4*^{-/-} mice, also in the presence of CD4⁺ T cell defects, correlated with higher lung burden.

Lower Th2 cytokine levels in plasma correlate with *P. jirovecii* colonization in HIV-infected individuals

Observations to date suggest that Th2 immunity mediates protection from *P. murina* infection. Therefore, to determine the cytokine response in the periphery in humans during *P. jirovecii* colonization, we examined the levels of Th cytokines in plasma from a cohort of HIV-infected individuals who were documented to be colonized with *P. jirovecii* using nested PCR compared with HIV-infected individuals who were not colonized. Assessment of Th1, Th2, Th17, and Treg-associated cytokine levels in plasma from this cohort revealed no differences in the Th1 cytokine IFN- γ , the Th17 cytokine IL-17A, and the Treg cytokine IL-10 (Fig. 6A). In contrast, HIV-infected individuals who were colonized with *P. jirovecii* had significantly lower concentrations of the Th2 cytokines IL-4, IL-5, and IL-13 (Fig. 6A) compared with HIV-infected individuals who were not colonized. Dichotomizing Th2 cytokine levels as above and below the median for the cohort demonstrated that HIV-infected individuals who were colonized with *P. jirovecii* were significantly more likely to have levels of the Th2 cytokines IL-4, IL-5, and IL-13 that were below the cohort median compared with HIV-infected individuals who were not colonized (Fig. 6B). Of note, CD4 cell numbers were not different between colonized versus noncolonized individuals (Fig. 6C). Thus, these data suggest that colonization with *P. jirovecii* is less likely if Th2 responses are induced.

Discussion

Although CD4⁺ T cells are the central effector cell mediating host defense against *Pneumocystis*, the type of Th response that is required for clearance is not clear. As stated previously, mice deficient in the IFN- α R (21, 22), IL-12p35 (23), or IL-23p19 (12) display delayed (significant differences in *P. murina* lung burden at 14–21 d postchallenge), yet ultimately intact (no significant differences in *P. murina* lung burden at 28 d postchallenge) organism clearance, suggesting that some aspects of Th1 responses may provide protection against *Pneumocystis*. However, by comparing the immune response against *P. murina* in *Stat4*^{-/-} on the

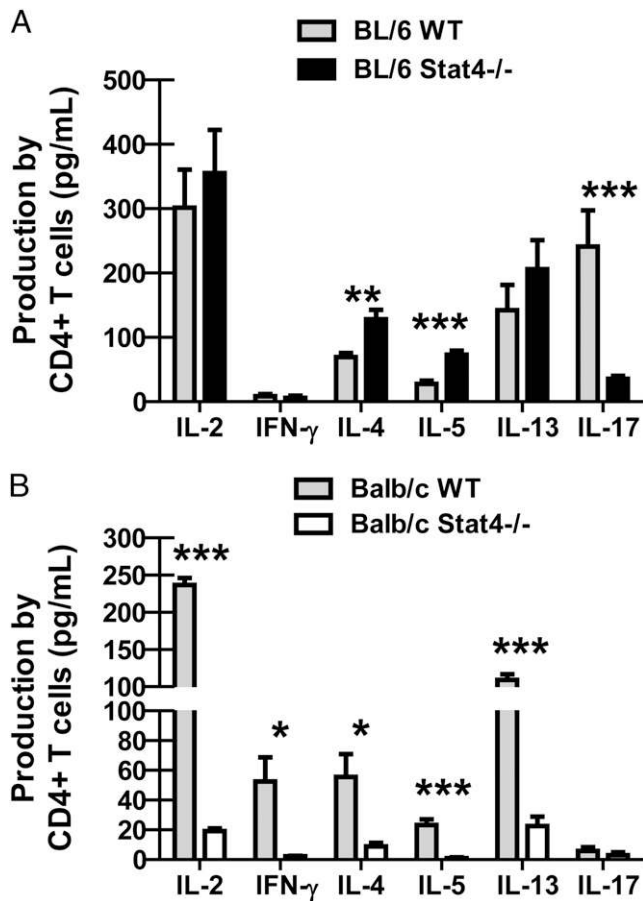


FIGURE 5. CD4⁺ Th2 responses in the draining lymph nodes are elevated in BL/6 *Stat4*^{-/-} mice, but significantly impaired in BALB/c *Stat4*^{-/-} mice. (A) C57BL/6 WT and *Stat4*^{-/-} mice and (B) BALB/c WT and *Stat4*^{-/-} mice were administered 2×10^5 *Pneumocystis* cysts via intratracheal inoculation. Fourteen days postinoculation, the mediastinal lymph nodes were collected and manually digested, and CD4⁺ T cells were isolated via Dynabeads, followed by stimulation with 2 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 for 48 h. Th cytokine levels were quantified in clarified coculture supernatants by Bio-Plex. Cumulative data are shown from two to three independent experiments with cells cultured in duplicate or triplicate. Data are expressed as mean pg/ml + SEM. For both graphs, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired two-tailed Student *t* test).

BL/6 and BALB/c backgrounds, we demonstrate in this study that STAT4 paradoxically contributes to Th2-mediated responses, which significantly contribute to multiple aspects of *P. murina* host defense.

The production of signature cytokines from the Th1 and Th2 lineages of CD4⁺ T cells in the lungs was all affected by *Stat4* deficiency in both strains of mice during *P. murina* infection. STAT4 phosphorylation is critical in IL-12-induced IFN- γ production (13), and, in the absence of STAT4, IFN- γ production by CD4⁺ T cells from the lungs was significantly decreased during *P. murina* infection. IL-2 production by CD4⁺ T cells was also reduced 3-fold on both genetic backgrounds, indicating the expected impaired Th1 development during *P. murina* infection in the lungs of *Stat4*^{-/-} mice. However, a striking finding was that lung CD4⁺ T cell-mediated production of Th2-type cytokines was also significantly reduced in BL/6 and BALB/c *Stat4*^{-/-} mice 14 d postchallenge. This was unexpected, because, in the absence of STAT4, CD4⁺ T cells are known to be biased toward the development of Th2 cells in other infection models (15). Th2 cytokine production did return 28 d postinfection in BL/6 *Stat4*^{-/-} mice, but not BALB/c *Stat4*^{-/-} mice, suggesting that STAT4 is

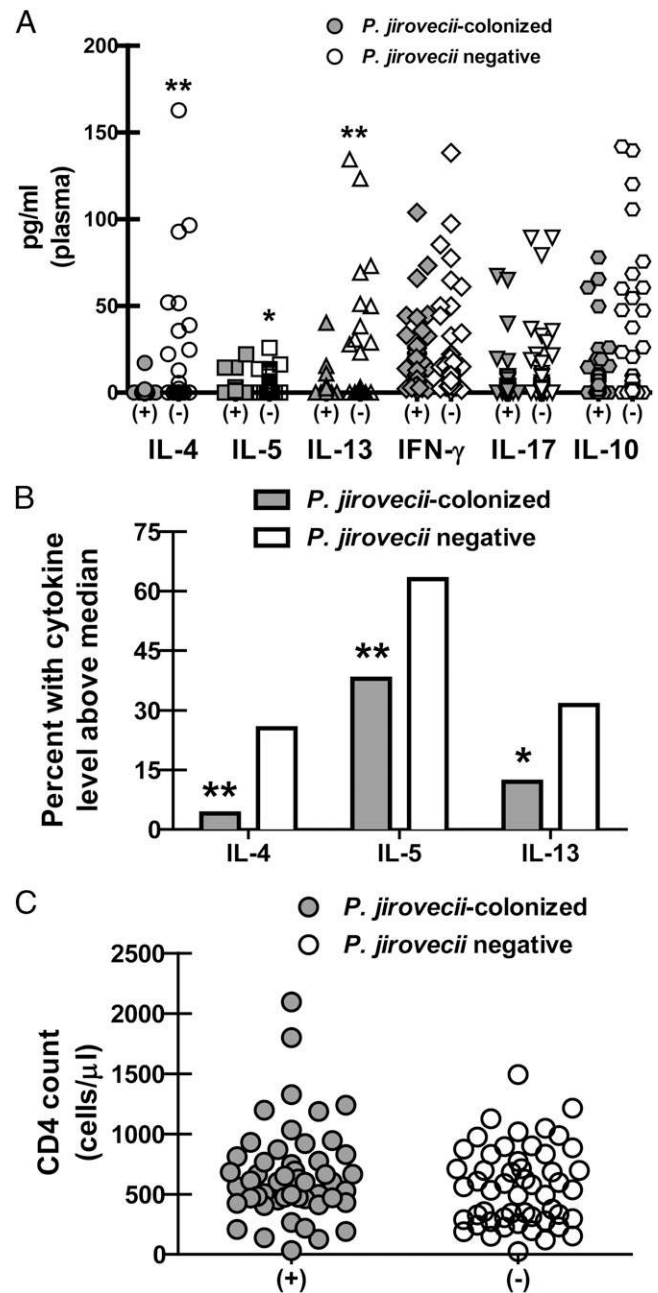


FIGURE 6. Lower Th2 cytokine levels in plasma correlate with *P. jirovecii* colonization in HIV-infected individuals. (A) Plasma was collected from a cohort of HIV-infected individuals who were subsequently confirmed to be colonized with *P. jirovecii* via nested PCR ($n = 50$). Controls included HIV-infected individuals who were negative for *P. jirovecii* by nested PCR ($n = 53$). Th cytokine levels were quantified in clarified coculture supernatants by Bio-Plex. Data are expressed as pg/ml (each symbol represents a single individual). * $p < 0.05$, ** $p < 0.01$ (nonparametric two-tailed Mann-Whitney *U* test). (B) Percentage of *P. jirovecii*-colonized versus noncolonized individuals with detectable Th2 cytokines above the median for each. * $p < 0.05$, ** $p < 0.01$ (χ^2 test). (C) CD4 cell numbers in peripheral blood of *P. jirovecii*-colonized versus noncolonized HIV-infected individuals.

required at some level for Th2 responses in BALB/c mice more than BL/6. It was of interest to note that *P. murina* lung burden in BALB/c WT mice at 28 d postchallenge was 3-fold lower compared with that in BL/6 WT mice, lending additional support for Th2 responses being important in *P. murina* host defense. *P. murina* host defense was not dependent on T-bet, the master

regulator of the Th1 cell lineage, as BALB/c *Tbx21*^{-/-} mice had no defect in *P. murina* clearance 28 d postchallenge (C. Steele, unpublished observations). As the intrinsic parameters for cytokine signaling in determining Th cell fate have been well characterized, the mechanism for the apparent STAT4-dependent, T-bet-independent effect on Th2 immunity in the lung during *P. murina* infection may be due to extrinsic factors that have yet to be examined.

In addition to Th1 and Th2 cytokines, IL-17A production by CD4⁺ T cells from the lungs was also negatively affected by the absence of STAT4, albeit with different kinetics: 14 d postinfection in BL/6 *Stat4*^{-/-} mice (but not BALB/c *Stat4*^{-/-} mice) and 28 d postinfection in BALB/c *Stat4*^{-/-} mice (but not BL/6 *Stat4*^{-/-} mice). STAT4 is thought to be partially involved in Th17 differentiation by mediating IL-23R signaling (14), and IL-17A has been shown to be STAT4 dependent on both the BL/6 (14) and BALB/c backgrounds (25). A role for IL-17A has been implicated in the clearance of *P. murina* (12). Neutralization of IL-17A with a mAb resulted in a higher fungal burden, and mice deficient in IL-23p19 (12), which plays a role in expanding and maintaining the Th17 fate (26), have delayed clearance of *P. murina*. IL-23p19 is an activator of STAT4, so it was unclear in this study whether the delayed clearance of *P. murina* was due to impaired Th17 responses or compromised STAT4-mediated host defense. The current study suggests IL-17A production by CD4⁺ T cells most likely did not play a critical role in *P. murina* host defense in BL/6 *Stat4*^{-/-} mice; however, we cannot exclude a role for IL-17A in protective responses in BALB/c *Stat4*^{-/-} mice.

Despite a global defect in CD4⁺ T cell-mediated cytokine production in the lungs of BL/6 and BALB/c *Stat4*^{-/-} mice, only BALB/c *Stat4*^{-/-} mice had significantly higher fungal burdens 28 d postchallenge. As this suggested that BALB/c *Stat4*^{-/-} mice were susceptible to infection whereas BL/6 *Stat4*^{-/-} mice were protected, this led us to hypothesize that other mechanisms of host defense were most likely enhanced in BL/6, but not BALB/c *Stat4*^{-/-} mice. Phagocytosis by alveolar macrophages is the predominant mechanism for clearance of *P. murina* from the lungs (27). Previous work from our laboratory has shown that increased M2 macrophage polarization correlated with enhanced clearance of *P. murina* (18). Indeed, BL/6 *Stat4*^{-/-} mice had increased M2 macrophage activation late during infection, whereas M2 macrophage activation in BALB/c *Stat4*^{-/-} mice was significantly impaired, suggesting that enhanced M2 macrophage activation contributed to protection from *P. murina* infection in BL/6 *Stat4*^{-/-} mice. CD4⁺ T cell-mediated production of IL-4 and IL-13 in the lungs is normally critical for M2 macrophage activation, but they were decreased in BL/6 *Stat4*^{-/-} mice 14 d postinfection. However, Th2 responses in the lungs returned by 28 d postchallenge and only in BL/6 *Stat4*^{-/-} mice. There may also be alternative cellular sources of IL-4 or IL-13, such as basophils or type-2 innate helper cells (28), in the lungs of BL/6 mice 14 or 28 d postchallenge mice that may serve to initiate M2 macrophage polarization, and these populations are currently being investigated. Additionally, immune complexes binding to FcRs on macrophages also induce alternative activation (29); consequently, the increase in anti-*P. murina* Ab production in BL/6 28 d postchallenge mice may have also contributed to the enhanced M2 activation.

In addition to increased M2 macrophage activation, BL/6 *Stat4*^{-/-} mice, but not BALB/c *Stat4*^{-/-} mice, had increased production of anti-*P. murina* Abs. Whereas it is clear that CD4⁺ T cells are required for *P. murina* host defense, B cells and Abs are also important contributors to host defense (30-33). *P. murina*-specific IgG2b was significantly increased in BL/6 *Stat4*^{-/-} mice early in

the immune response, whereas *P. murina*-specific IgG1 was significantly increased late. IgG1 is associated with Th2-type immunity (34) and the enhanced production was consistent with increased Th2 cytokine production in the MLN. Isotype switching to IgG2b has been associated with either Th1-type (35) or Th2-type cytokines (36) in various experimental models. Although increased IgG2b production was observed in BL/6 *Stat4*^{-/-} mice during *P. murina* infection, it is not clear whether this could be a result of increased Th2 cytokine production in the MLN. Curiously, *P. murina*-specific IgG2c was also enhanced in BL/6 *Stat4*^{-/-} mice. IgG2c is associated with Th1 immunity (37), so it was unexpected that the production of this isotype was also elevated over BL/6 WT mice.

Despite the importance of PCP as an opportunistic infection associated with HIV, there is little evidence to suggest which is the dominant CD4⁺ T cell response against *P. jiroveci* in humans. PBMC stimulated with the major surface glycoprotein of *P. jiroveci* from HIV-positive individuals with a previous history of PCP produced significantly higher concentrations of IL-4 compared with HIV-positive individuals with no history of PCP, whereas the level of IFN- γ was similar between these two groups (38). This suggests that in patients able to clear *P. jiroveci*, memory CD4⁺ T cell responses are predominantly Th2 driven. In agreement with this study and confirming our observations in mice, lower IL-4, IL-5, and IL-13 levels in plasma from HIV-positive individuals correlated with *Pneumocystis* colonization, suggesting that Th2 responses are associated with enhanced fungal host defense.

In summary, our study establishes that local and systemic Th2-mediated immunity contributes to multiple aspects of host defense and correlates with resistance against *Pneumocystis* lung infection. An unexpected finding from this work was the impairment of local and systemic Th2 responses in the absence of *Stat4*, primarily in BALB/c mice and to a lesser extent in BL/6 mice. Future studies are required to probe this observation more thoroughly to determine whether this is a strain-specific phenomenon during *P. murina* infection as well as to identify specific *Stat4*-dependent mechanisms critical for Th2 development. Although the mechanisms of how local and systemic Th2 and type-2 responses are generated and maintained during experimental *Pneumocystis* lung infection are not currently known, data presented in this work suggest that Th2 responses in humans may be protective, thus understanding the development of the Th2 response may lead to better immunotherapeutics to target *Pneumocystis* lung infection.

Disclosures

The authors have no financial conflicts of interest.

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