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STAT4-dependent and independent T helper type-2 responses correlate with protective immunity against lung infection with *Pneumocystis murina*[‡]

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Abstract

Although it is clear that the loss of CD4+ T cells is a predisposing factor for the development of *Pneumocystis* pneumonia, specific T helper 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 *P. 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 days post-challenge, 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 antibodies 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 to HIV infected individuals who were not colonized. Collectively, our data suggests that robust local and systemic Th2-mediated responses are critical for immunity to *Pneumocystis*.

Introduction

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 (RA) 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 (COPD) severity (3) and is a potential contributor to mortality in infants with sudden unexpected death (SUID) (4). Despite the wide-spread implementation of high active antiretroviral therapy (HAART) and use of antibiotics against *Pneumocystis*, the mortality

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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 cytokine IFN-γ or the Th2 signature cytokine IL-4 are not more susceptible to infection than wild-type 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 days of infection, inflammatory responses and leukocyte recruitment in response to *P. murina* challenge was 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 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, antibody-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-23 receptor, 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 Dr. Mark Kaplan, Indiana University. All mice used in experiments were 8-12 weeks 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 years of age or older and had at least 1 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 weeks. All participants also performed an oral wash by gargling with sterile saline for one minute. For determination of *Pneumocystis jirovecii* colonization, DNA extraction was performed on sputa and oral washes using a DNeasy kit (Qiagen, Valencia,

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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 T helper cytokine levels, plasma from participants who were colonized with *P. jirovecii* (n = 50) or were not colonized (n = 53) was analyzed using a human 41-plex cytokine and chemokine kit (Cat. #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 (UAB) and approved by the UAB Institutional Review Board.

Pneumocystis 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 × 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 ml aliquots were placed into tubes containing 200 μl of 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 year.

CD4+ T cell isolation and culture

Mice were anesthetized with intraperitoneal ketamine/xylazine and sacrificed by exsanguination 14 and 28 days post-inoculation. Both lungs were collected and minced in Iscove's modified Dulbecco's medium (IMDM) (Sigma, St. Louis, MO) supplemented with 1% penicillin-streptomycin-glutamine (Mediatech, Herndon, VA), 10% heat-inactivated fetal bovine serum (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, St. Louis, MO) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through sterile 70 μm and 40 μm nylon filters, and red blood cells were lysed with red blood cell lysis buffer. Lymph node cells were excised and cells isolated by forcing tissue through a sterile 70 μm nylon filter followed by red blood cell lysis. CD4+ T cells were isolated using mouse CD4 Flowcomp Dynabeads (Cat # 114-61D, Invitrogen, Carlsbad, CA) per the manufactures' protocol to 90-95% purity. The CD4+ T cells were then plated at 1×10^6 cells/ml in IMDM and stimulated with 2 μg/ml anti-CD3 (clone 145-11) and 1 μg/ml anti-CD28 (Cat # 102112, BioLegend, San Diego, CA) for 48 hours 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 days 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

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(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 days 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 *Retnla* (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 $2^{-(\Delta\Delta Ct)}$ 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 (Cat # MCC170) levels were determined by ELISA (R&D Systems, Inc., Minneapolis, MN).

Pneumocystis-specific antibody ELISA

Blood was collected weekly for 28 days from the tail vein and sera was 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, St. Louis, MO) at 1 μg/well overnight at 4°C. The wells were then blocked with 10% bovine serum albumin (Equitech Bio, Inc., Kerrville, TX) for 1 hour at 37°C. Plates were washed with PBS containing 0.05% Tween-20. Sera were serially diluted and incubated at 37°C for 1 hour. Antibodies were detected by incubating plates with anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG2c conjugated to HRP (Southern Biotech, Birmingham, AL) for 1 hour at 37°C, followed by incubation with 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma, St. Louis, MO). 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 non-colonized participants, a non-parametric Mann-Whitney test was employed. Significance was accepted at p 0.05.

Results

Differential susceptibility to *P. murina* lung infection between *Stat4-/-* mice on BL/6 vs. Balb/c backgrounds

Mice deficient in the IFN-αR (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 days thereafter. There was no difference in lung burden between BL/6 WT and BL/6 *Stat4*-/- mice (Figure 1A) or Balb/c WT and Balb/c *Stat4*-/- mice (Figure 1B) 14 days post-challenge. In contrast, whereas BL/6 *Stat4*-/- mice had similar *P. murina* lung burden as BL/6 WT mice (Figure 1C), Balb/c *Stat4*-/- mice had a significantly higher burden in the lungs compared to Balb/c WT mice (Figure 1D). Thus, despite both strains having a deficiency in

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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 hours with anti-CD3 and anti-CD28. As assessed by Bio-Plex, production of the Th1 signature cytokines IL-2 and IFN-γ by CD4+ T cells from the lungs of BL/6 *Stat4*-/- mice **(Figure 2A)** and Balb/c *Stat4*-/- mice **(Figure 2B)** were significantly reduced compared to 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 **(Figure 2A)** and Balb/c *Stat4*-/- mice **(Figure 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 **(Figure 2B)**. In contrast, lung $CD4^+$ T cell-mediated production of IL-17A was significantly less in BL/6 *Stat4*-/- mice **(Figure 2A)**, which were protected from *P. murina* infection (compared to Balb/c *Stat4*-/- mice). By 28 days post-challenge, 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 (Figure 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 (Figure 2D). Furthermore, IL-17A production by lung CD4+ T cells was now also was impaired at 28 days post-challenge. 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-alpha/FIZZ-1) (Figure 3A) in BL/6 *Stat4*-/- mice, but not Balb/c *Stat4*-/- mice (Figure 3B), despite both of these strains demonstrating lower Th2 responses by lung CD4+ T cells (Figure 2A, 2B). Lung levels of CCL17, a chemokine produced by M2 macrophages, was also not different between WT BL/6 mice and BL/6 *Stat4*-/- mice (Figure 3C) and WT Balb/c mice and Balb/c *Stat4*-/- mice (Figure 3D) 14 days post-challenge. In contrast, by twenty-eight days post-*P. murina* challenge, there was significantly higher lung *Retnla* (RELM-alpha/FIZZ-1) mRNA levels (Figure 3E) and CCL17 protein levels (Figure 3G) in the lungs of BL/6 *Stat4*-/- mice, indicating that along with increased lung CD4+ Th2 responses (Figure 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-alpha/FIZZ-1) compared to Balb/c WT mice (Figure 3F) as well as a significant reduction in the concentration of CCL17 in the lungs of Balb/c *Stat4*-/- mice (Figure 3H), which correlated with attenuated lung CD4+ Th2 responses (Figure 2D). Of note, naïve 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

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marker *Nos2* between BL/6 *Stat4*-/- mice and Balb/c *Stat4*-/- mice compared to 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 antibody 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 antibody levels were assessed. BL/6 and Balb/c WT and *Stat4*-/- mice were challenged with *P. murina* and sera were collected weekly for 28 days 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 preexisting *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 antibody production compared to BL/6 WT mice. Whereas *P. murina*-specific IgG1 production by B cells in BL/6 *Stat4*-/- mice was significantly enhanced 28 days post-challenge **(Figure 4A)**, IgG2b was enhanced earlier at days 7, 14 and 21 **(Figure 4B)**. The production of *P. murina*-specific IgG2c was also significantly increased throughout the immune response in BL/6 *Stat4*-/- mice compared to BL/6 WT mice **(Figure 4C)**. In contrast, there was no difference in the levels of anti-*P. murina* antibodies of any isotype at any time point examined in Balb/c *Stat4*-/- mice compared to Balb/c WT mice **(Figures 4D, E and F)**. Thus, enhanced anti-fungal antibody 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 days post-challenge, Figure 2A). Moreover, while Balb/c *Stat4*-/- mice also exhibited defective CD4+ T cell responses in the lung (14 and 28 days post-challenge, Figures 2B and 2D), these mice had decreased M2 macrophage activation and no difference in anti-*P. murina* antibody 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 days after challenge with *P. murina* and stimulated ex vivo with anti-CD3 and anti-CD28 for 48 hours. 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 were significantly diminished compared to CD4+ T cells from the MLN of Balb/c WT mice **(Figure 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 to CD4+ T cells from the MLN of BL/6 WT mice **(Figure 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 **(Figure 5B)**. Thus, the enhanced production of anti-*P. murina* class-switched antibodies 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 antibodies in sera from Balb/c *Stat4*-/- mice, also in the presence of CD4+ T cell defects, correlated with higher lung burden.

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

Observations thus far 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 T helper cytokines in plasma from a cohort of HIV-infected individuals who were documented to be colonized with *P. jirovecii* using nested PCR compared to 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 (**Figure 6A**). In contrast, HIV-infected individuals who were colonized with *P. jiroveci* had significantly lower concentrations of the Th2 cytokines IL-4, IL-5 and IL-13 (**Figure 6A)** compared to HIV-infected individuals that 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 to HIV-infected individuals who were not colonized **(Figure 6B)**. Of note, CD4 cell numbers were not different between colonized vs. non-colonized individuals **(Figure 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 T helper response that is required for clearance is not clear. As stated previously, mice deficient in the IFN-αR (21) (22), IL-12p35 (23), or IL-23p19 (12) display delayed (significant differences in *P. murina* lung burden at 14 to 21 days post-challenge), yet ultimately intact, (no significant differences in *P. murina* lung burden at 28 days post-challenge) 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 BL/6 and Balb/c backgrounds, we demonstrate here 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 were 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 three-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 days post-challenge. This was unexpected, because in the absence of STAT4, CD4+ T cells are known to be biased towards the development of Th2 cells in other infection models (15). Th2 cytokine production did return 28 days post-infection in BL/6 *Stat4*-/- mice, but not Balb/c *Stat4*-/- mice, suggesting that STAT4 is 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 days post-challenge was three-fold lower compared to 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 days post-challenge (unpublished data). As the intrinsic parameters for cytokine signaling in determining T-helper cell fate have been well characterized, the

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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 days after infection in BL/6 *Stat4*-/- mice (but not Balb/c *Stat4*-/- mice) and 28 days after infection 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 STAT4, so it was unclear in this study whether the delayed clearance of *P. murina* was due to impaired Th17 responses or compromised STAT4-mediate host defense. The current study suggests IL-17A production by CD4+ T cells 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 fugal burdens 28 days post-challenge. As this suggested that Balb/c *Stat4*-/- mice were susceptible to infection while BL/6 *Stat4*-/- mice were protected, this led us to hypothesize that other mechanisms of host defense were 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 lab 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 are normally critical for M2 macrophage activation, but they were decreased in BL/6 *Stat4*-/- mice 14 days after infection. However, Th2 responses in the lungs returned by 28 days post-challenge 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 days post-challenge mice that may serve to initiate M2 macrophage polarization and these populations are currently being investigated. Additionally, immune complexes binding to Fc receptors on macrophages also induce alternative activation (29); consequently, the increase in anti-*P. murina* antibody production in BL/6 28 days post-challenge 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* antibodies. While it is clear that CD4+ T cells are required for *P. murina* host defense, B cells and antibodies are also important contributors to host defense (30) (31), (32) (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 if this could be a result of increased Th2 cytokine production in the MLN. Curiously,

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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 dominate CD4+ T cell response against *P. jiroveci* in humans. PBMC stimulated with the major surface glycoprotein (MSG) 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 here suggests 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.

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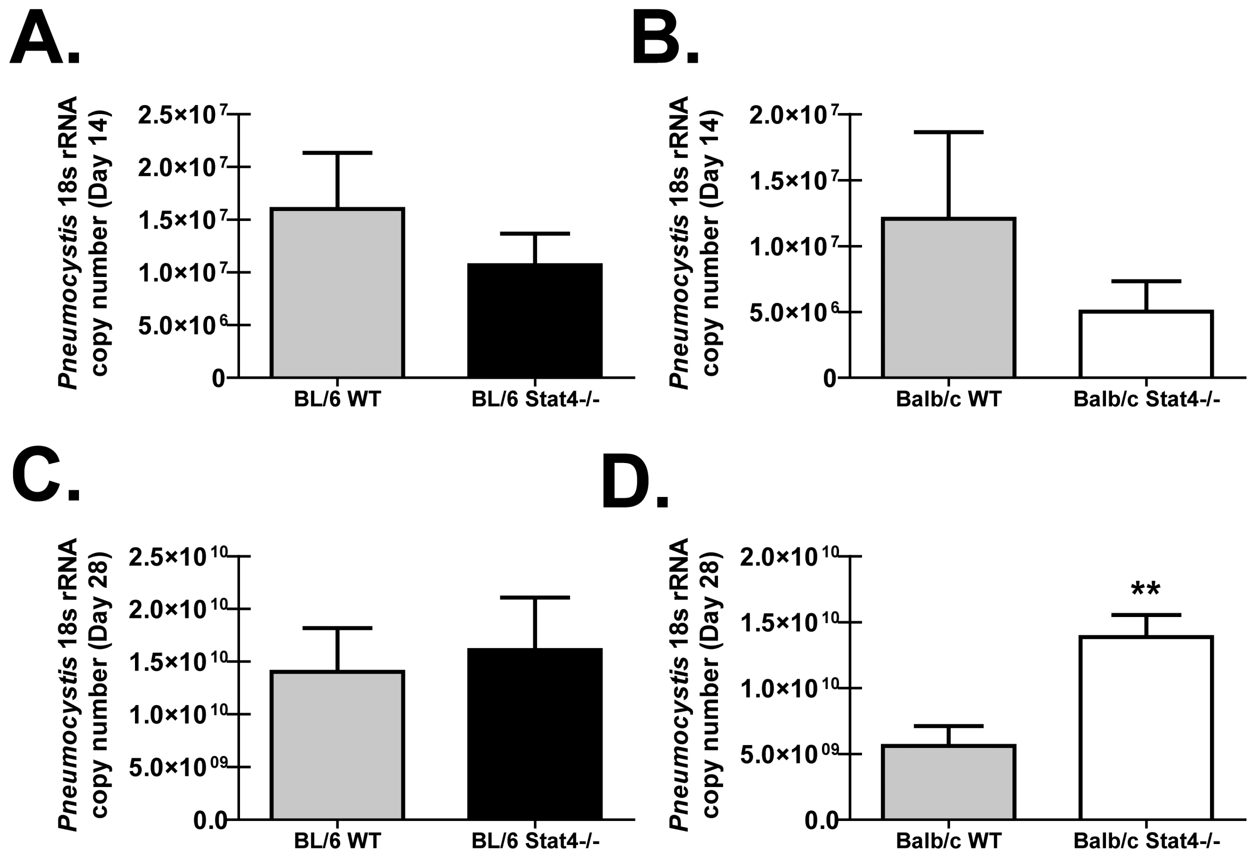

Figure 1. Differential susceptibility to *P. murina* lung infection between *Stat4*-/- mice on BL/6 vs. Balb/c backgrounds

C57BL/6 WT and *Stat4*-/- mice and Balb/c WT and *Stat4*-/- mice were administered 2×10^5 *Pneumocystis* cysts via intratracheal inoculation. (A/B) Fourteen and (C/D) twenty-eight days post-inoculation, 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 is expressed as mean *Pneumocystis* rRNA copy number. Data are expressed as mean + SEM. ** represents a P value of < 0.01 (Unpaired two-tailed Student's t test).

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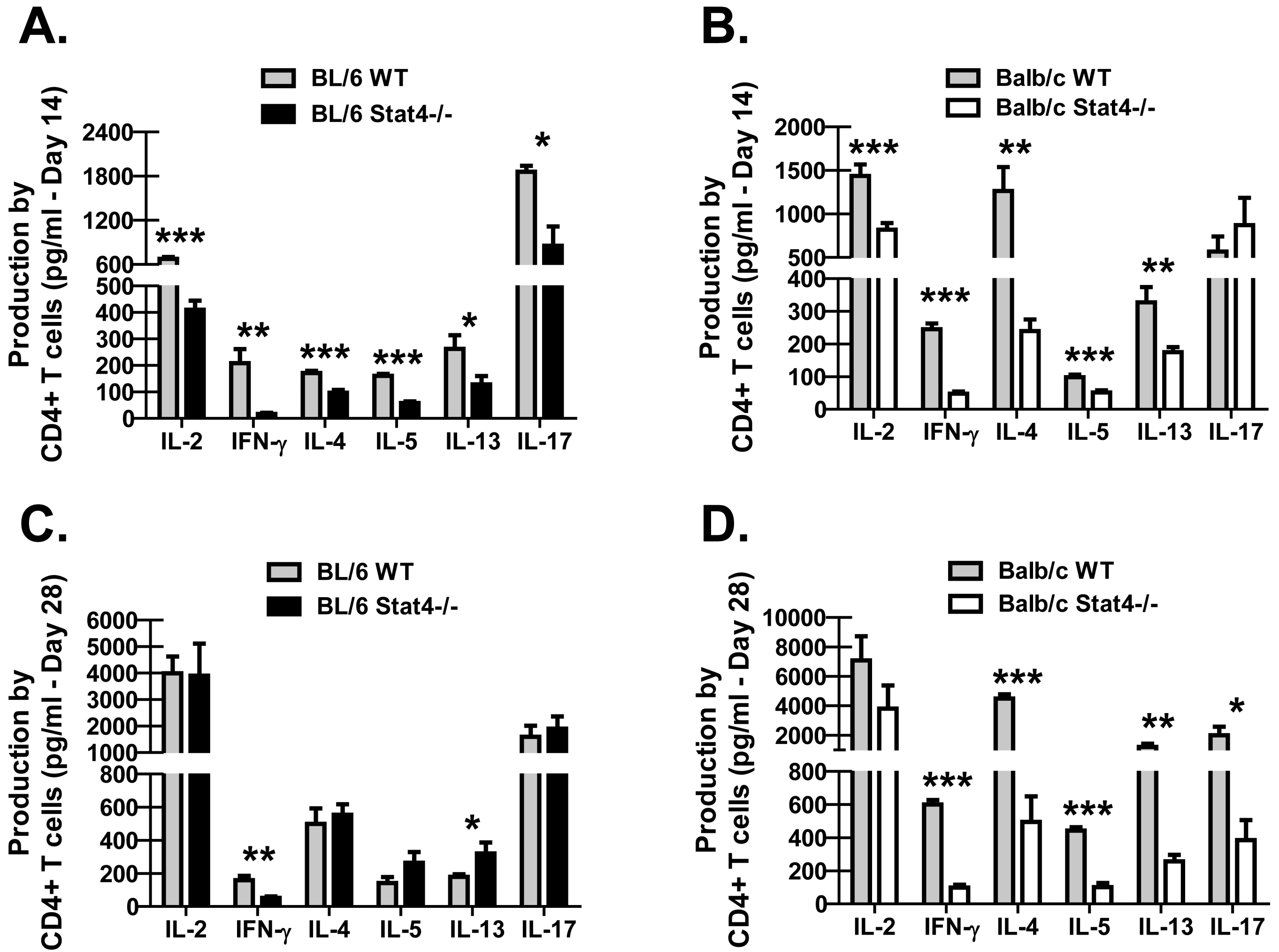

Figure 2. BL/6 and Balb/c *Stat4*-/- mice demonstrate impaired CD4+ Th2 responses in the lung
C57BL/6 WT and *Stat4*-/- mice and Balb/c WT and *Stat4*-/- mice were administered 2×10^5 *Pneumocystis* cysts via intratracheal inoculation. (A/B) Fourteen and (C/D) twenty-eight days post-inoculation, the lungs were collected, 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. T helper cytokine levels were quantified in clarified co-culture supernatants by Bio-Plex. Cumulative data are shown from two to three independent experiments with cells cultured in duplicate or triplicate. Data is expressed as mean pg/ml + SEM. For both graphs, *, ** and *** represent P values of < 0.05, < 0.01 and < 0.001, respectively (Unpaired two-tailed Student's t test).

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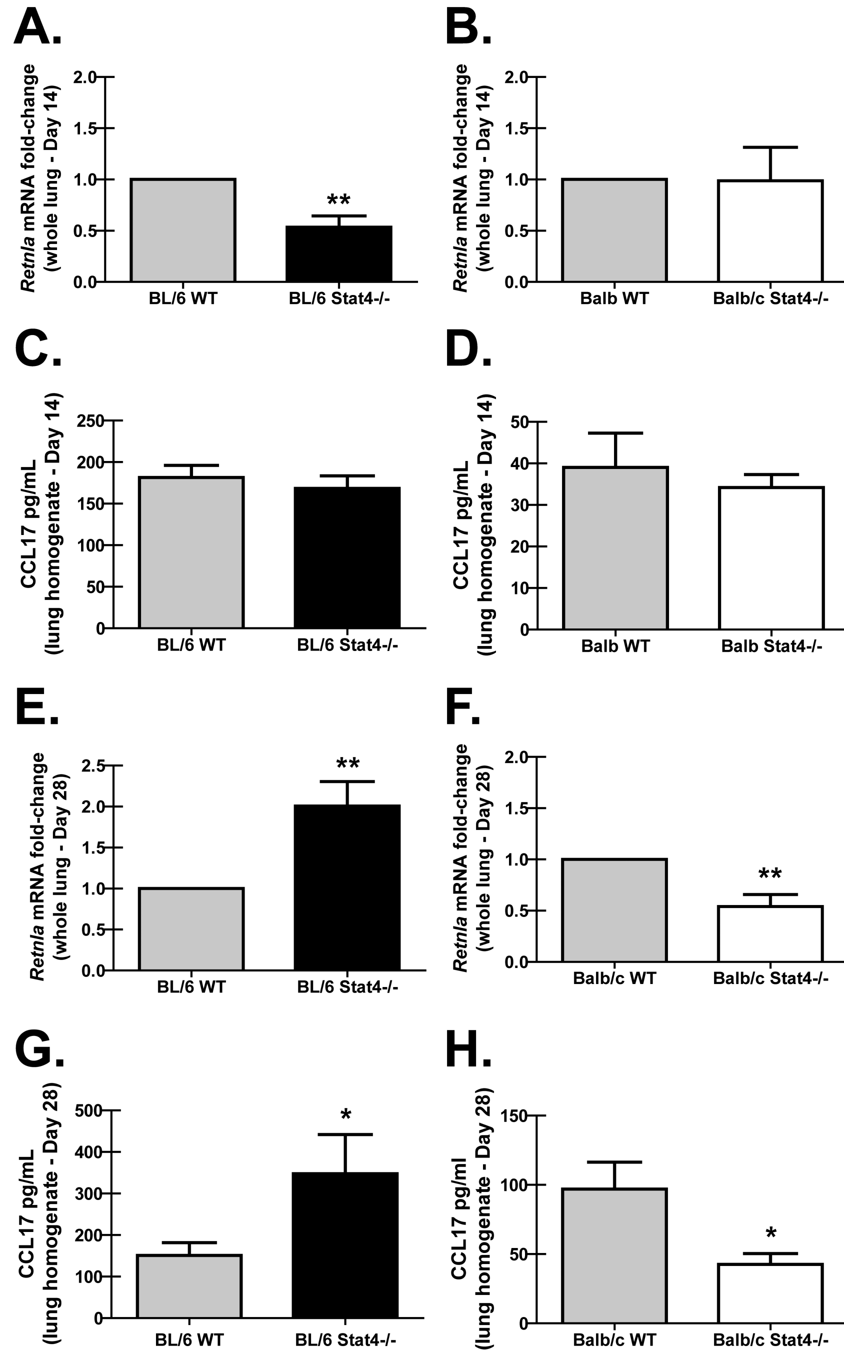

Figure 3. BL/6 *Stat4*-/- mice demonstrate enhanced lung M2 macrophage polarization

C57BL/6 WT and *Stat4*-/- mice and Balb/c WT and *Stat4*-/- mice were administered 2×10^5 *Pneumocystis* cysts via intratracheal inoculation. (A/B/C/D) Fourteen and (E/F/G/H) twenty-eight days post-inoculation, the right lung was collected and total RNA isolated, 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 delta 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 days post-challenge. For CCL17 analysis (C/D/G/H), mice were infected as described and twenty-eight days post-inoculation,

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the left lung was collected and homogenized in PBS supplemented with Complete Mini protease inhibitor tablets and supernatants 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 is expressed as mean fold-change. ** represents a P value of < 0.01 (Paired two-tailed Student's t test). (C/D/G/H) Data is expressed as mean pg/ml + SEM. * represents a P value of < 0.05 (Unpaired two-tailed Student's t test).

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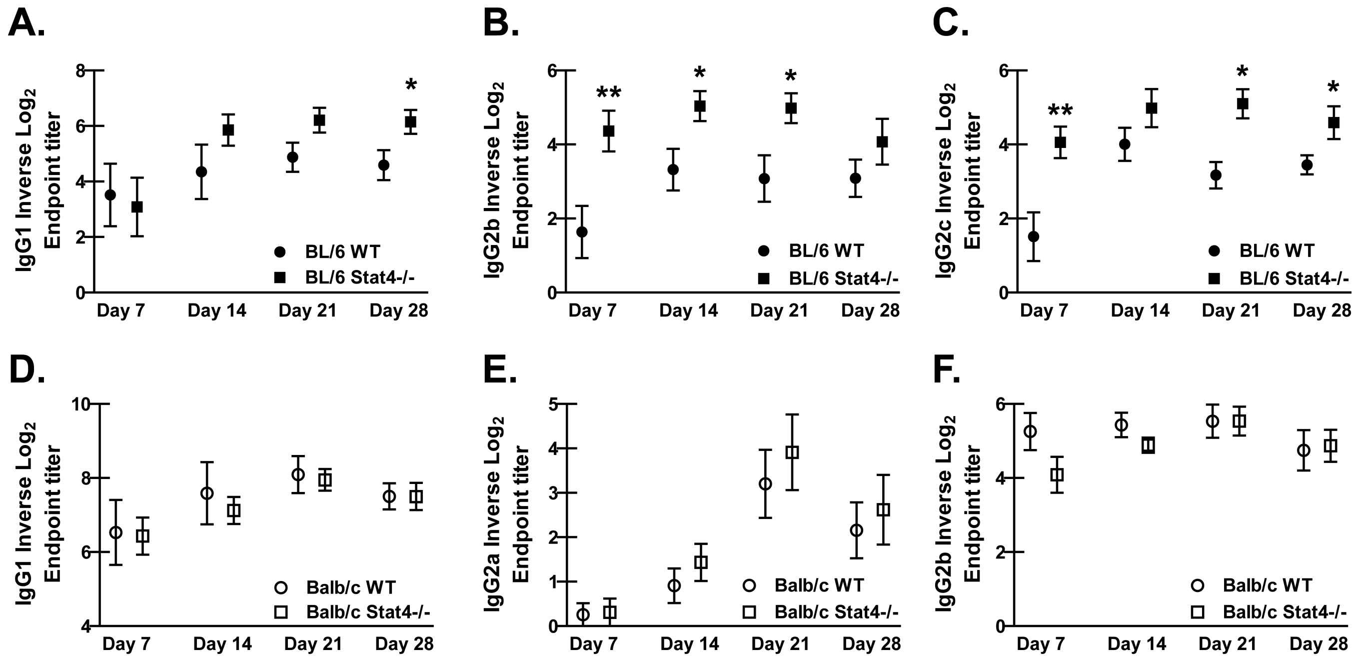

Figure 4. *P. murina*-specific antibody levels are elevated in serum of BL/6 *Stat4*-/-, but not Balb/c *Stat4*-/-, mice

(A/B/C) C57BL/6 WT and *Stat4*-/- mice and **(D/E/F)** Balb/c WT and *Stat4*-/- mice were administered 2×10^5 *Pneumocystis* cysts via intratracheal inoculation followed by bleeding mice weekly for 28 days. *P. murina*-specific (A/D) IgG1, (B/E) IgG2b, (C) IgG2c (C57BL/6) and (F) IgG2a (Balb/c) and were determined by ELISA. Cumulative data are shown from three independent studies with n = 3-4 mice/group per study. Data is expressed as the mean per group of the natural log of the dilution at which the OD450 is 0.1 + SEM. * and ** represent P values of < 0.05 and < 0.01, respectively (Unpaired two-tailed Student's t test).

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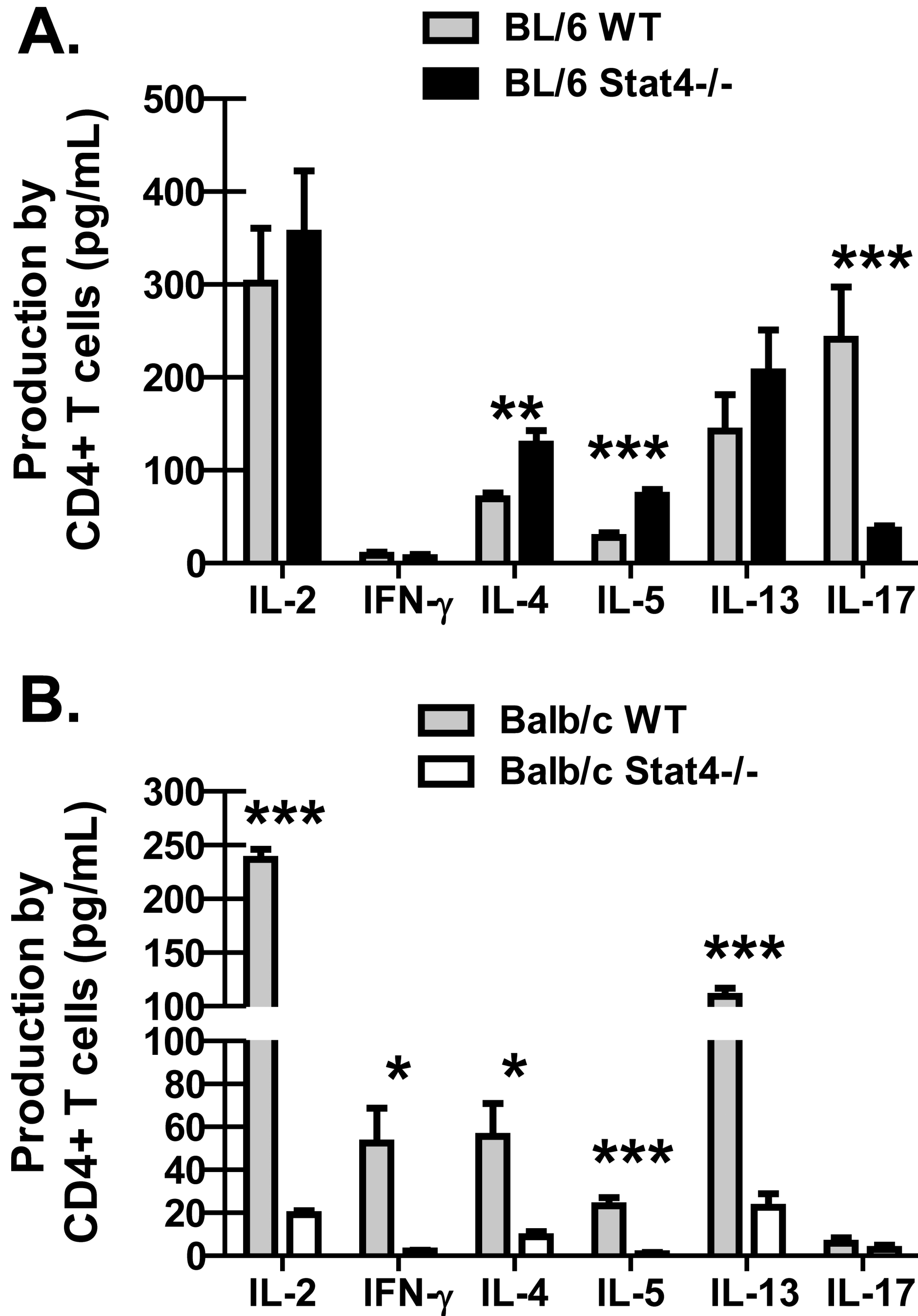

Figure 5. CD4+ Th2 responses in the draining lymph nodes are elevated in BL/6 *Stat4*-/- mice but significantly impaired in Balb/c *Stat4*-/- mice

(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 post-inoculation, the mediastinal lymph nodes were collected, manually 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. T helper cytokine levels were quantified in clarified co-culture supernatants by Bio-Plex. Cumulative data are shown from two to three independent experiments with cells cultured in duplicate or triplicate. Data is expressed as mean pg/ml + SEM. For both graphs,

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*, ** and *** represent P values of < 0.05, < 0.01 and < 0.001, respectively (Unpaired two-tailed Student's t test).

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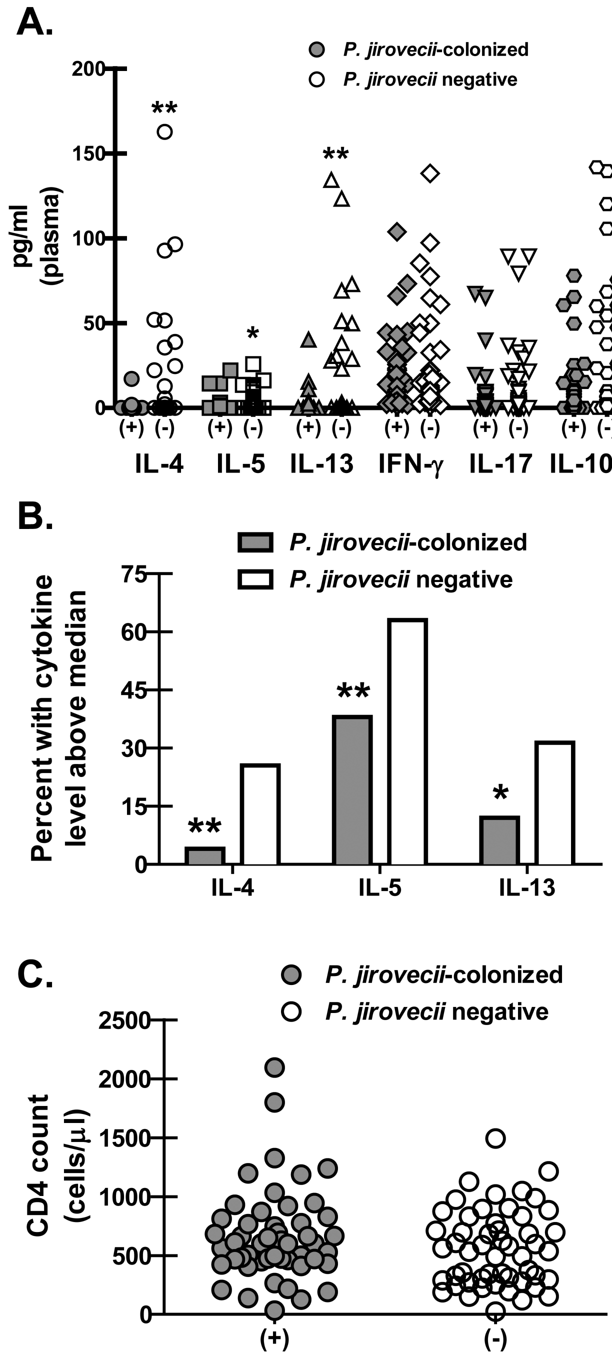

Figure 6. Lower Th2 cytokine levels in plasma correlate with *Pneumocystis jirovecii* colonization in HIV-infected individuals

(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). T helper cytokine levels were quantified in clarified co-culture supernatants by Bio-Plex. Data is expressed as pg/ml (each symbol represents a single individual. * and ** represent P values of < 0.05 and < 0.01, respectively (Non-parametric two-tailed Mann-Whitney test). **(B)** Percentage of *P. jirovecii* colonized vs. non-colonized individuals with detectable Th2 cytokines above the median for each. * and ** represent P values of < 0.05 and < 0.01,

respectively (Chi square test). **(C)** CD4 cell numbers in peripheral blood of *P. jirovecii* colonized vs. non-colonized HIV-infected individuals.

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