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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-

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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 yr 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 with gargling with sterile saline for 1 min. For determination of *Pneumocystis jiroveci* 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 RNA, as previously described (16). DNA extraction and PCR were carried out in separate rooms, and all reactions were performed in a UV box. Positive and negative controls were included in each reaction mixture. A subject was considered infected if *P. jiroveci* (n = 50) or who were not colonized (n = 55) was analyzed using a human 41-plex cytokine and chemokine kit (catalogue HCYTMMAG-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 × 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 × 10⁶ cysts/ml. For in vivo challenge, mice were anesthetized with isoflurane and 200 μl containing 2 × 10⁶ cysts/ml was injected into the lung infection between *P. murina*–colonized and noncolonized participants, a nonparametric Mann–Whitney U test was employed. Significance was accepted at p ≤ 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-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 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-
Fourteen days after intratracheal infection, 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 isolated from the lungs of BALB/c Stat4−/− mice, which were susceptible to P. murina infection. By 28 d postchallenge, IL-4, IL-5, and IL-13 production by lung CD4+ T cells from BL/6 Stat4−/− mice was similar to CD4+ T cells isolated from the lungs of BALB/c WT mice (Fig. 2A, 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 (RELMTS/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 Stat4−/− mice.
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...
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-γR (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
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. m u r i n a* 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. m u r i n a* 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. m u r i n a* 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. m u r i n a* 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 required at some level for Th2 responses in BALB/c mice more than BL/6. It was of interest to note that *P. m u r i n a* 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. m u r i n a* host defense. *P. m u r i n a* host defense was not dependent on T-bet, the master
P. murina characterized, the mechanism for the apparent STAT4-dependent, unpublished observations). As the intrinsic parameters for cyto-
fection in BALB/c 28 d postchallenge. As this suggested that BALB/c production in the lungs of BL/6 and BALB/c Stat4 defense were most likely enhanced in BL/6, but not BALB/c protected, this led us to hypothesize that other mechanisms of host were susceptible to infection whereas BL/6 mice. CD4+ T cell–mediated production of IL-4 and IL-13 in the lungs is normally critical for M2 macrophage activation, but they paired, suggesting that enhanced M2 macrophage activation con-

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 BALB/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 mediates IL-23R signaling (14), and IL-17A has been shown to be STAT4 is thought to be partially involved in Th17 differentiation by making its way to the lungs of BL/6 mice. CD4+ T cell–mediated production of IL-4 and IL-13 in the lungs of BL/6 mice 14 or 28 d postinfection. How-

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 pre-

In summary, our study establishes that local and systemic TH2-mediated immunity contributes to multiple aspects of host de-
defense and correlates with resistance against Pneumocystis lung infection. An unexpected finding from this work was the im-
pairment 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 phe-
nomenon during P. murina infection as well as to identify specific STAT4-dependent mechanisms critical for TH2 development. Al-

The authors have no financial conflicts of interest.

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