



Combination Immunotherapy with Passive Antibody and Sulfasalazine Accelerates Fungal Clearance and Promotes the Resolution of *Pneumocystis*-Associated Immunopathogenesis

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ABSTRACT The pulmonary immune response protects healthy individuals against *Pneumocystis* pneumonia (PcP). However, the immune response also drives immunopathogenesis in patients who develop severe PcP, and it is generally accepted that optimal treatment requires combination strategies that promote fungal killing and also provide effective immunomodulation. The anti-inflammatory drug sulfasalazine programs macrophages for enhanced *Pneumocystis* phagocytosis and also suppresses PcP-related immunopathogenesis. Anti-*Pneumocystis* antibody opsonizes *Pneumocystis* organisms for greater phagocytosis and may also mask antigens that drive immunopathogenesis. Thus, we hypothesized that combining antibody and sulfasalazine would have the dual benefit of enhancing fungal clearance while dampening immunopathogenesis and allow the rescue of severe PcP. To model a clinically relevant treatment scenario in mice, therapeutic interventions were withheld until clear symptoms of pneumonia were evident. When administered individually, both passive antibody and sulfasalazine improved pulmonary function and enhanced *Pneumocystis* clearance to similar degrees. However, combination treatment with antibody and sulfasalazine produced a more rapid improvement, with recovery of body weight, a dramatic improvement in pulmonary function, reduced lung inflammation, and the rapid clearance of the *Pneumocystis* organisms. Accelerated fungal clearance in the combination treatment group was associated with a significant increase in macrophage phagocytosis of *Pneumocystis*. Both passive antibody and sulfasalazine resulted in the suppression of Th1 cytokines and a marked increase in lung macrophages displaying an alternatively activated phenotype, which were enhanced by combination treatment. Our data support the concept that passive antibody and sulfasalazine could be an effective and specific adjunctive therapy for PcP, with the potential to accelerate fungal clearance while attenuating PcP-associated immunopathogenesis.

KEYWORDS *Pneumocystis*, antifungal therapy, immunomodulation, immunopathogenesis, pulmonary infection, respiratory pathogens

Pneumocystis jirovecii is an opportunistic pathogen that causes life-threatening pneumonia (*Pneumocystis* pneumonia [PcP]) in immunocompromised patients. The morbidity and mortality secondary to PcP are significant when one considers that, according to the Centers for Disease Control and Prevention (CDC), the incidence of PcP is 9% for hospitalized patients with HIV/AIDS and 1% for solid-organ transplant patients, with an overall incidence of 40 cases per 1,000 person-years in these populations in the United States (1). With the increasing utilization of immunomodulatory agents, the pool of patients at risk of developing PcP will likely increase (2). For example, soon after the introduction of rituximab, a monoclonal antibody (MAb) that targets the CD20

Citation Hoy Z, Wright TW, Elliott M, Malone J, Bhagwat S, Wang J, Gigliotti F. 2020.

Combination immunotherapy with passive antibody and sulfasalazine accelerates fungal clearance and promotes the resolution of *Pneumocystis*-associated immunopathogenesis. *Infect Immun* 88:e00640-19. <https://doi.org/10.1128/IAI.00640-19>.

Editor Liise-anne Pirofski, Albert Einstein College of Medicine

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Received 14 August 2019

Returned for modification 30 August 2019

Accepted 10 October 2019

Accepted manuscript posted online 14 October 2019

Published 22 January 2020

antigen on B lymphocytes, reports of PcP associated with B cell depletion began to appear in the literature (3). Even though there are established treatments for PcP, mortality remains high and has changed little in the past several decades (1, 4). A major contributor to the pathogenesis of PcP that leads to respiratory failure and, ultimately, death is the immune response elicited by this fungal pathogen (5–9), and we hypothesize that effective control of immunopathogenesis is the critical factor that current treatment regimens fail to adequately address. Therapies that effectively target PcP-related immunopathogenesis are likely to be a necessary feature of treatments able to reduce the persistently high mortality rates among patients that develop severe PcP. Current treatments involve the use of high-dose corticosteroids (CS) for the purpose of suppressing immunopathogenesis. However, CS do not always provide a benefit for PcP patients, and their effectiveness remains unclear (10–14). Given the pleiotropic effects of CS, it seems likely that off-target effects may counteract some of the expected anti-inflammatory benefits during PcP and that more specific targeting of PcP-related immunopathogenesis, such as with specific antibody, would improve clinical outcomes.

Alveolar macrophages are the main effector cells for the removal of *Pneumocystis* from the lungs and also regulate pulmonary inflammation and lung repair (15–17). Thus, we hypothesized that targeting macrophage function would enhance fungal clearance while simultaneously removing the antigenic stimulus that drives immunopathogenesis. Studies of macrophage biology have demonstrated them to be complex cells whose function varies based on phenotype. Classically activated macrophages (CAMs), or M1 macrophages, have an inflammatory phenotype in response to exposure to lipopolysaccharide (LPS) and interferon gamma (IFN- γ). In contrast, alternatively activated macrophages (AAMs), or M2 macrophages, are proresolution and/or anti-inflammatory and can be programmed via multiple mechanisms, including exposure to interleukin-4 (IL-4) and IL-13 or antigen-antibody immune complexes (18, 19). Importantly, M2 macrophages appear to be potent effector cells for *Pneumocystis* killing (15, 20, 21) but are not absolutely necessary to eradicate *Pneumocystis* (22).

The opsonization of microorganisms facilitates the recognition and clearance of pathogens by phagocytes. Different classes of proteins act as specific or nonspecific opsonins. The role of opsonins in the clearance of fungi has not been well studied; however, there is some experimental support for their importance. For example, the fungal pathogen *Candida albicans* was shown to be more efficiently phagocytosed in the presence of mannose-binding lectin (MBL) than under conditions when the opsonin MBL was absent (23). Two opsonins shown to affect the clearance of *Pneumocystis* are complement and antibody (24, 25). Standardized assays to measure the phagocytosis of *Pneumocystis* have only recently been developed, and as a result, there is only limited experimental support for antibody acting in concert with macrophages to clear *Pneumocystis* (15, 24–26). In addition to promoting *Pneumocystis* clearance through opsonization, anti-*Pneumocystis* antibody may also provide a benefit during PcP treatment by masking or removing the *Pneumocystis* antigens that drive immunopathogenesis. Furthermore, antibody has also been shown to have nonspecific immunomodulatory effects, as exemplified by its use in diseases like idiopathic thrombocytopenic purpura or Kawasaki disease (27–32). This effect of antibody or immunoglobulin (Ig) might prove valuable in the management of PcP because of the prominent immunopathogenic component.

We have previously reported that passive antibody provides protection against PcP when used prophylactically (25, 33, 34). Similarly, sulfasalazine (SSZ) also appears to be effective for prophylaxis against *Pneumocystis* in mice and humans (15, 35–37). However, the potential utility of passive antibody or SSZ as treatment options for moderate to severe PcP has not been evaluated. Both antibody and SSZ have the potential to enhance *Pneumocystis* clearance while suppressing inflammation and PcP-related immunopathogenesis. We report here that combination treatment with passive antibody and SSZ induces a dramatic recovery of mice suffering from severe PcP, which is characterized by a shift in macrophage polarization, increased macrophage-mediated phagocytosis of *Pneumocystis*, and reduced immunopathogenesis.

RESULTS

Combination treatment with anti-*Pneumocystis* antibody and SSZ produces dramatic recovery from severe PcP. Mice displaying obvious clinical signs of PcP-related immune reconstitution inflammatory syndrome (IRIS), including a greater than 10% body weight loss and a greater than 25% increase in respiratory rate, were treated with nonspecific control antibody, passive anti-*Pneumocystis* antibody, SSZ, or a combination of anti-*Pneumocystis* antibody plus SSZ. As expected, control mice displayed characteristic symptoms of severe PcP over the course of the study, including dramatic weight loss, reduced lung compliance, and increased lung resistance (Fig. 1A to C). However, mice treated individually with *Pneumocystis*-specific passive antibody or SSZ demonstrated enhanced recovery from PcP compared to control untreated mice. The improvement was clearly demonstrated by an accelerated recovery of body weight (Fig. 1A). Furthermore, the beneficial effects of the individual treatments were markedly enhanced by combination treatment with both specific anti-*Pneumocystis* antibody and sulfasalazine. In contrast to untreated mice with PcP, mice treated with the combination therapy began to recover weight by day 6 posttreatment and had nearly completely recovered the lost body weight by day 11 posttreatment (Fig. 1A). None of the experimental groups showed a statistically significant change in pulmonary function measurements at day 6 posttreatment. However, by day 11 posttreatment, dramatic improvements in pulmonary function measurements were observed in the combination treatment group relative to the measurements for control mice with PcP. The combination therapy group had a greater than 39% higher lung compliance and 62% reduced lung resistance compared to results for untreated mice (Fig. 1B and C). In our experience, these pulmonary function measurements have proven to be reliable indicators of the severity of PcP in this model, thereby lending physiological significance to the observed results. These data demonstrate that, when used as a treatment for severe PcP, anti-*Pneumocystis* antibody plus SSZ induces a rapid recovery from the severe respiratory impairment associated with PcP.

Anti-*Pneumocystis* antibody and SSZ accelerate fungal clearance. To determine whether anti-*Pneumocystis* antibody or SSZ promoted *Pneumocystis* clearance from the lungs during a clinical treatment scenario, total fungal lung burdens were determined by quantitative real-time PCR (qPCR) (38), and lung cyst burdens were determined by Gomori's methenamine silver (GMS) staining, as described previously (39). At day 6 posttreatment, the anti-*Pneumocystis* antibody, SSZ, and combination treatment groups had a trend toward fewer total organisms and cyst forms than control mice, but the differences were not statistically significant (Fig. 2A and B). However, by day 11 posttreatment the anti-*Pneumocystis* antibody- and SSZ-treated groups had a 1- to 2-log reduction in the total lung *Pneumocystis* burden relative to the control group (Fig. 2A). Furthermore, the anti-*Pneumocystis* antibody-plus-SSZ combination treatment group displayed a greater than 3-log average reduction in total lung *Pneumocystis* burden compared to that for the untreated control mice with PcP (Fig. 2A). Several mice in the combination treatment group had an undetectable *Pneumocystis* burden, while the burden in the remaining mice was very near the limit of detection, indicating the nearly complete clearance of *Pneumocystis* in the combination treatment group. A similar pattern of accelerated clearance was found when the *Pneumocystis* burden was assessed by the use of microscopic cyst counts, although the cyst counts were lower than the total *Pneumocystis* burden determined by qPCR (Fig. 2B). These results demonstrate that anti-*Pneumocystis* antibody plus SSZ promotes the rapid clearance of an established *Pneumocystis* infection.

Accelerated *Pneumocystis* clearance in mice treated with anti-*Pneumocystis* antibody plus SSZ is associated with the earlier induction of macrophage phagocytosis. Based on prior work, we hypothesized that anti-*Pneumocystis* antibody and SSZ would accelerate the kinetics of *Pneumocystis* clearance by promoting lung macrophage phagocytosis of *Pneumocystis*. To quantify *Pneumocystis* phagocytosis by macrophages, we used an imaging flow cytometer-based method that we previously described (15). Mice with PcP treated with either anti-*Pneumocystis* antibody or SSZ did

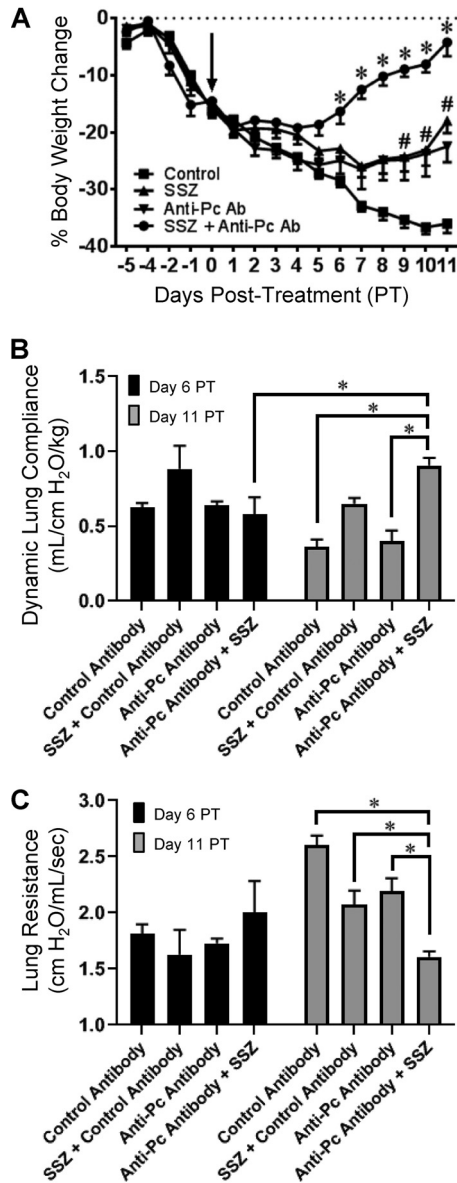


FIG 1 Accelerated recovery from PcP in mice treated with anti-*Pneumocystis* (anti-Pc) antibody, SSZ, or a combination of both. *Pneumocystis*-infected SCID mice were immune reconstituted with wild-type splenocytes. (A) Treatments were started at 8 days postreconstitution (arrow), when the mice displayed obvious signs of PCP (a >10% body weight loss and a >25% increase in respiratory rate). Body weight loss was used to noninvasively monitor the progression of disease following treatment. For body weight loss, values are the mean \pm 1 standard error measurement (SEM). *, $P < 0.05$ for mice in the SSZ plus anti-*Pneumocystis* antibody (Ab) combination treatment treatment group compared to the results for mice in the control treatment group at the same time; #, $P < 0.05$ for mice in the SSZ or anti-*Pneumocystis* antibody treatment group compared to the results for mice in the control treatment group at the same time. For time points up to and including day 6 posttreatment (PT), data are for ≥ 11 mice per group. For time points later than day 6 posttreatment, data are for ≥ 6 mice per group. (B and C) Pulmonary function, as assessed by dynamic lung compliance (B) and lung resistance (C), was measured at 6 and 11 days posttreatment. Values are the mean \pm 1 standard error measurement. *, $P < 0.05$ between groups, as indicated. At the day 6 time point, data are for 4 mice per group. At the day 11 time point, data are for 5 to 7 mice per group.

not show a significant increase in macrophage phagocytosis of *Pneumocystis* at day 6 posttreatment compared to results for control mice (Fig. 2C). However, mice with PcP treated with the combination of anti-*Pneumocystis* antibody plus SSZ showed a 3-fold increase in the number of lung macrophages with ingested *Pneumocystis* organisms at this time (Fig. 2C). There was no difference between the groups at day 11 posttreat-

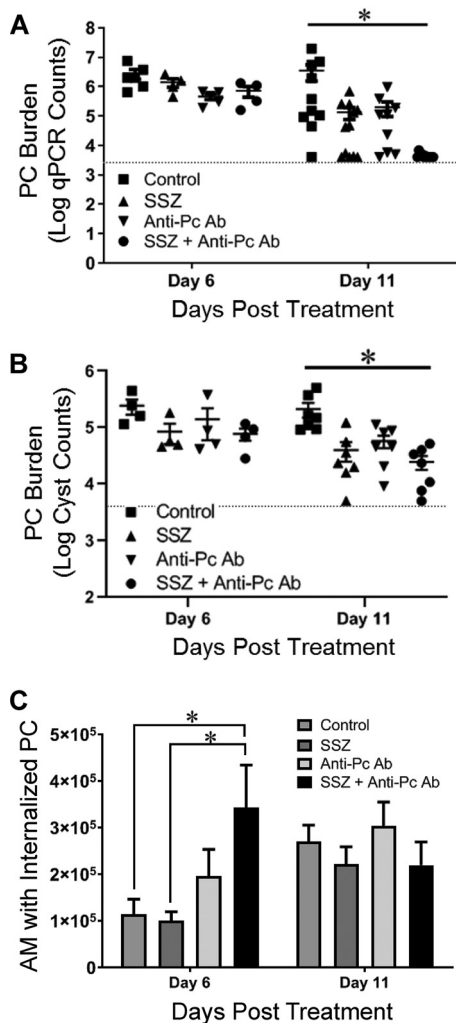
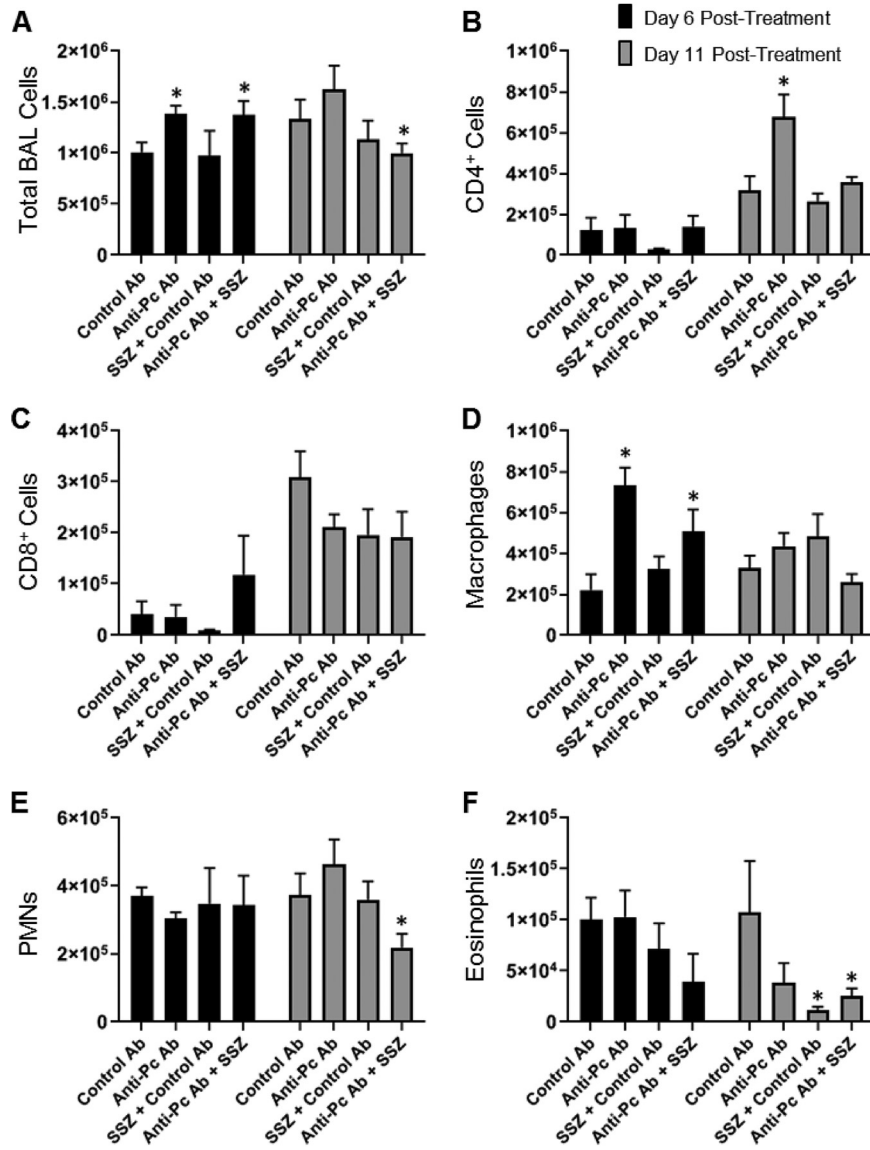


FIG 2 Accelerated alveolar macrophage (AM)-mediated fungal clearance in anti-*Pneumocystis* antibody- and SSZ-treated mice. (A and B) The lung *Pneumocystis* (PC) burden in experimental mice was measured at 6 and 11 days posttreatment by two distinct methods: quantitative real-time PCR (qPCR) of the single-copy *Pneumocystis kexin* gene in lung homogenates (A) and direct Gomori’s methenamine silver staining of cysts in lung homogenate preparations (B). The limit of detection for each assay is indicated by a dotted line. Values are the mean ± 1 SEM. *, $P < 0.05$ between the control group and the SSZ-plus-anti-*Pneumocystis* antibody-treated group. At the day 6 time point, data are for 4 mice per group. At the day 11 time point, data are for ≥10 mice per group for qPCR analyses and 7 mice per group for cyst counts. (C) Macrophage phagocytosis of *Pneumocystis* was quantified by multispectral imaging flow cytometry, as we have described previously (15). Values are the mean ± 1 SEM. *, $P < 0.05$ between the indicated groups. At the day 6 time point, data are for 4 mice per group. At the day 11 time point, data are for 5 to 7 per group.

ment. All control mice with PcP and mice treated with either anti-*Pneumocystis* antibody or SSZ showed an increase in the number of macrophages with ingested *Pneumocystis* between day 6 and day 11 posttreatment. In contrast, mice with PcP treated with the combination of anti-*Pneumocystis* antibody plus SSZ showed a decrease in the number of macrophages with ingested *Pneumocystis* over this time (Fig. 2C). This was likely due to the high rate of *Pneumocystis* clearance in this group, which left fewer *Pneumocystis* organisms in the lungs by day 11. These data indicate that an early enhancement of macrophage phagocytosis of *Pneumocystis* leads to the accelerated fungal clearance observed in the anti-*Pneumocystis* antibody-plus-SSZ-treated group.

Anti-*Pneumocystis* antibody and SSZ alter the lung cytokine environment. To determine whether the combination of anti-*Pneumocystis* antibody and SSZ improves



PcP by reducing the number of immune cells in the lungs, cells were recovered from the bronchoalveolar lavage (BAL) fluid of experimental mice. At day 6 posttreatment, few significant differences in the number of BAL fluid cells recovered were noted between the treatment groups (Fig. 3). However, there was a significant increase in the total number of BAL fluid cells recovered from the anti-*Pneumocystis* antibody and anti-*Pneumocystis* antibody plus SSZ groups at this time (Fig. 3A). The increase in the total number of cells in the anti-*Pneumocystis* antibody-treated groups was almost entirely attributed to an increased number of macrophages in these groups (Fig. 3D). No differences in the number of BAL fluid total lymphocytes, polymorphonuclear leukocytes (PMNs), CD4⁺ T cells, or CD8⁺ T cells were observed at this time (Fig. 3). In contrast, by day 11 posttreatment, the mice treated with anti-*Pneumocystis* antibody plus SSZ had fewer total BAL fluid cells than control mice (Fig. 3A). The reduced number of total BAL fluid cells recovered was almost entirely accounted for by fewer PMNs in

the BAL fluid of anti-*Pneumocystis* antibody-plus-SSZ-treated mice than in the BAL fluid of the controls (Fig. 3E). Thus, with the exception of PMNs, we found few differences in the cellular composition of the alveoli in treated and untreated mice that would explain the beneficial effects of anti-*Pneumocystis* antibody plus SSZ. We have previously reported that PMNs do not contribute significantly to PcP-related immunopathogenesis but are faithful markers of the severity of PcP (40). Thus, the reduced numbers of PMNs in the anti-*Pneumocystis* antibody-plus-SSZ-treated group support our findings of improved lung function and less severe disease in the combination treatment group.

It is likely that the lack of change in immune cell numbers over the short course of treatment is related to the fact that we withheld the initiation of treatment until after the animals were already experiencing the immunopathological consequences of PcP. Therefore, we hypothesized that the beneficial effects of anti-*Pneumocystis* antibody plus SSZ were related to immune modulation, rather than a reduction in immune cell numbers in the lung. Several cytokines and chemokines known to be involved in the host immune response to PcP were measured in the BAL fluid of experimental mice. Tumor necrosis factor alpha (TNF- α) is an important proinflammatory cytokine with a direct role in promoting PcP-related immunopathogenesis (38, 41). By day 11 post-treatment, anti-*Pneumocystis* antibody-plus-SSZ-treated mice demonstrated a 50% reduction in the amount of TNF- α in the lung compared to that in untreated mice, which coincided with the better lung function in this group (Fig. 4A). Elevated lung levels of the Th1 cytokine IFN- γ are also associated with PcP-related immunopathogenesis (8, 15). IFN- γ levels were significantly reduced at both day 6 and day 11 posttreatment in the anti-*Pneumocystis* antibody-plus-SSZ-treated group compared to that in the untreated control group with PcP (Fig. 4B). IL-4 and IL-5 levels in the BAL fluid of experimental mice were also measured, but no differences were found (data not shown). CCL2 is a chemokine that regulates monocyte/macrophage recruitment and polarization. CCL2 is produced by lung epithelial cells during *Pneumocystis* infection (42–44) and is associated with PcP-related immunopathogenesis (15, 42). CCL2 levels were significantly reduced in all animals treated with anti-*Pneumocystis* antibody and/or SSZ at 11 days posttreatment (Fig. 4C).

Anti-*Pneumocystis* antibody and SSZ promote an M2 macrophage phenotype.

The finding that the Th1 cytokines IFN- γ and TNF- α were downregulated by anti-*Pneumocystis* antibody plus SSZ treatment indicated that suppression of the Th1 response may be beneficial for recovery from PcP. Polarization status regulates the antifungal effects of macrophages, and prior studies have indicated that M2 macrophages are effectors for *Pneumocystis* clearance and may also dampen lung inflammation. To test the effect of anti-*Pneumocystis* antibody and SSZ on the macrophage phenotype in the environment of the *Pneumocystis*-infected lung, we administered these agents or control nonspecific antibody to mice with obvious signs of PcP-related IRIS and harvested alveolar macrophages 3 days later. Administration of sulfasalazine resulted in a 16-fold increase in the M2/M1 macrophage ratio in the alveolar space compared to that in control mice with PcP, while administration of anti-*Pneumocystis* antibody resulted in an approximately 33-fold increase in the M2/M1 ratio (Table 1). Combination treatment with anti-*Pneumocystis* antibody and SSZ resulted in an even more pronounced shift in the macrophage phenotype, demonstrating a 47-fold increase in the M2/M1 ratio present in the lung compared to that in control mice with PcP. The beneficial effects of anti-*Pneumocystis* antibody and SSZ on PcP in a treatment scenario are associated with a reduction in lung Th1 cytokine levels and an increase in M2 macrophage polarization.

DISCUSSION

PcP is characterized by severe hypoxia and respiratory failure, and it is now clear that immune-mediated inflammatory lung injury is a major component of the pathogenesis of PcP (5, 6, 9, 15, 45). Despite the availability of effective antibiotics, the mortality rates among patients developing severe PcP remain high, likely due to the inability to adequately control immunopathogenesis during treatment. Corticosteroids are typi-

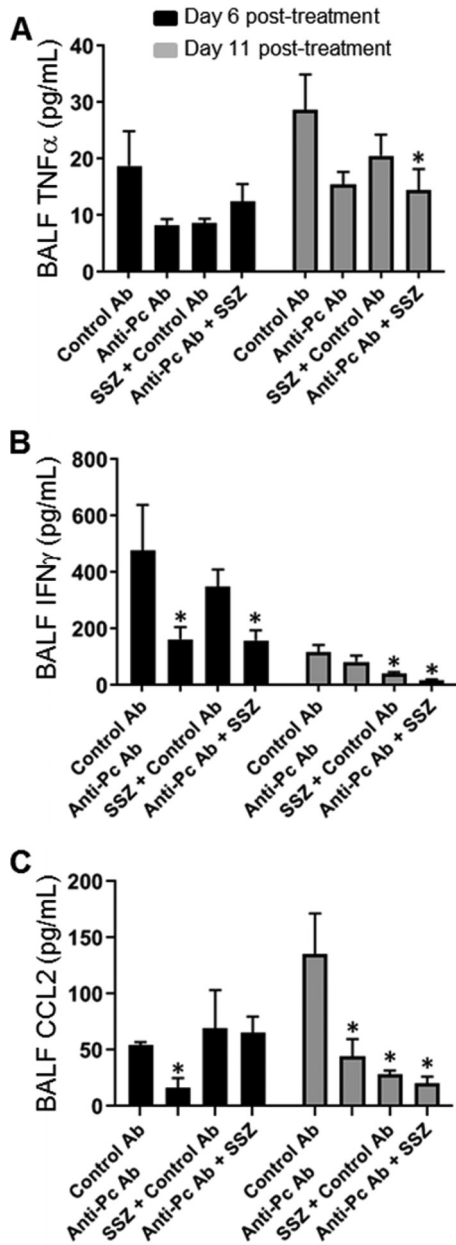


FIG 4 Effect of anti-*Pneumocystis* antibody and SSZ treatment on lung Th1 cytokine levels during PcP. Bronchoalveolar lavage (BAL) was performed on experimental mice at 6 and 11 days posttreatment. TNF- α , IFN- γ , and CCL2 levels were measured in BAL fluid (BALF) by ELISA. Values are the mean \pm 1 SEM. *, $P < 0.05$ between the indicated group and the control group at the same time point. At the day 6 time point, data are for 4 mice per group. At the day 11 time point, data are for 6 to 10 mice per group.

cally used to suppress inflammation, but recent studies suggest that the broadly acting agents do not provide a significant benefit to patients (10–14). Therefore, alternative strategies to suppress immunopathogenesis while eradicating the infection are plainly needed. Our prior studies found that the immunomodulatory drug sulfasalazine dramatically reduces the severity of PcP if it is administered before the onset of the pathogenic pulmonary immune response (15). Unexpectedly, SSZ accelerated the clearance of *Pneumocystis* through enhancement of the macrophage-mediated phagocytosis of *Pneumocystis*. We have also reported that passively administered anti-*Pneumocystis* antibody protects immunodeficient mice against *Pneumocystis* infection (24). Although these agents have been used prophylactically to prevent the onset of

TABLE 1 Effect of administration of anti-*Pneumocystis* antibody or sulfasalazine on the ratio of alveolar macrophages of the M2 to the M1 phenotype in the lung

Treatment	Phenotype (no. of macrophages ^a)		Ratio	
	M1	M2	M2/M1	Fold increase ^b
Control antibody	290	10	0.03	1
SSZ	200	100	0.5	16.6
Anti- <i>Pneumocystis</i> antibody	149	151	0.99	33.0
SSZ + anti- <i>Pneumocystis</i> antibody	125	175	1.4	46.6

^aNumber of macrophages per 300 macrophages counted.

^bFold increase in the M2/M1 ratio compared to that achieved with the control antibody.

complications of PcP, they have not been tested in treatment scenarios. In the current study, we found that when used as a treatment for active PcP, the combination of anti-*Pneumocystis* antibody and SSZ reduced the severity of PcP-related respiratory impairment, accelerated *Pneumocystis* clearance, and promoted recovery. These findings indicate that appropriate immunomodulation has the potential to improve outcomes in patients with PcP.

In our prior studies of PcP-related immunopathogenesis, we found that depleting specific T cell subsets or blocking T cell recruitment before the onset of the *Pneumocystis*-driven immune response was highly effective for preventing immunopathogenesis. However, the suppression of immunopathogenesis in mice treated with anti-*Pneumocystis* antibody plus SSZ after the onset of PcP was not associated with a drastic reduction in T cell numbers in the lung (Fig. 3). The likely explanation for this finding is that we initiated treatment after the pathogenic immune response was already ongoing. Thus, we suspect that passive antibody and SSZ did not act by reducing the magnitude of the T cell population participating in the pulmonary immune response but did so by modulating it in a manner that promoted fungal clearance while suppressing lung injury. In support of this conclusion, we found significantly lower levels of the Th1 cytokines TNF- α and IFN- γ in the lungs of anti-*Pneumocystis* antibody-plus-SSZ-treated mice with PcP than in those of the control mice. The reduced levels of Th1 cytokines coincided with an increase in the proportion of alternatively activated M2 macrophages in the lungs of mice treated with anti-*Pneumocystis* antibody and/or SSZ. It is now recognized that macrophages can vary in their biological characteristics based on their mode of activation. CAMs, or M1 macrophages, are proinflammatory and are polarized by exposure to LPS, TNF- α , and IFN- γ . AAMs, or M2 macrophages, exhibit anti-inflammatory and proresolution activity and can be polarized by exposure to IL-4/IL-13 or antigen-antibody complexes (18, 19). In addition, recent studies have indicated that M2 macrophages promote the clearance of *Pneumocystis* from the lungs (15, 16, 20). Thus, treatments that bring about a shift in the macrophage phenotype to an M2 or anti-inflammatory phenotype would be expected to benefit PcP treatment by suppressing inflammation and immunopathogenesis while enhancing fungal clearance, as we have now reported for combination treatment with anti-*Pneumocystis* antibody plus SSZ treatment.

The precise role for antibody in the immune response to fungal infections is incompletely understood. We hypothesize that the beneficial effects of passive antibody for the treatment of PcP are likely multifaceted. Antibody likely masks immunopathogenic epitopes and clears the *Pneumocystis* antigens that drive PcP immunopathogenesis, while also opsonizing *Pneumocystis* for enhanced fungal clearance. Furthermore, the presentation of antigen complexed with antibody results in the activation of macrophage M2 polarization pathways, which may add to the beneficial effects of the antibody by increasing phagocytic potential and initiating anti-inflammatory programs. We have previously shown that SSZ enhances antibody-independent macrophage phagocytosis of *Pneumocystis* (15). We suspect that the addition of specific antibody further enhances *Pneumocystis* phagocytosis by SSZ-

stimulated lung macrophages, explaining the accelerated clearance in combination-treated mice. Indeed, we found that the combination of anti-*Pneumocystis* antibody plus SSZ enhances macrophage phagocytosis of *Pneumocystis* in the lung (Fig. 2). Specific antifungal antibodies have been shown to increase phagocytosis in a model of *Candida* fungal infection (46). It seems likely that specific anti-*Pneumocystis* antibodies help opsonize *Pneumocystis* in a similar manner and that this effect is enhanced with the addition of sulfasalazine. In our model, we waited for the *Pneumocystis* infection to create significant illness prior to starting treatment. For cryptococcal fungal infections, there has been demonstration of a modified outcome with passive administration of specific fungal antibodies to capsular glucuronoxylomannan prior to infection (47, 48). In that experiment, monoclonal antibodies were given up to 24 h prior to infection, and mice that received antibody had a lower fungal burden. Another study from the same group evaluated more specific groups of anticryptococcal antibodies and found that passive administration prior to infection decreased the fungal burden, except for a few antibodies that led to worse infection with increased mortality (49). In the same study, C3 complement-deficient mice had improved protection with specific anticryptococcal antibodies compared to mice with normal complement, demonstrating the role that complement plays with antibody for protection against some fungal pathogens. This was, in fact, what we observed. While both specific anti-*Pneumocystis* antibody and SSZ produced some treatment benefit in mice with PcP, the combination of the two treatment modalities showed a much more pronounced effect. That the benefit noted in our experiment was due to the effect of specific anti-*Pneumocystis* antibodies and not to some nonspecific immune-modulatory effect from nonspecific high-dose immune globulin is supported by the observation that the irrelevant antibody-treated mice in the control group displayed none of the beneficial effects noted in the specific anti-*Pneumocystis* antibody-treated group. An important feature of our experimental approach is that we waited for the mice with PcP infection to become clinically symptomatic. This more closely mimics how patients with PcP present for medical care. Additional studies will be necessary to determine whether there is a relationship between the specific antigen or antigens targeted for opsonization and the beneficial effects that we observed.

In a previous study, we demonstrated that sulfasalazine reduced PcP-related immunopathogenesis, while it enhanced macrophage phagocytosis of *Pneumocystis* (15). SSZ has been shown to exert immunomodulatory effects via the blockade of inflammatory transcription factor nuclear factor kappa B (NF- κ B) (50), and NF- κ B-dependent proinflammatory responses to *Pneumocystis* have been reported for both alveolar macrophages and lung epithelial cells (51–53). NF- κ B also regulates the polarization program of T cells, B cells, and macrophages, and we hypothesize that NF- κ B inhibition is a major mechanism by which SSZ attenuates immunopathogenesis in our treatment model of PcP. It is possible that the additive effects of SSZ on inflammation and pathogen clearance in our model could be caused by the elicitation of a proresolution, M2 phenotype in alveolar macrophages. Although it is still a matter of some debate (54), the ability of macrophages to differentiate into transcriptionally distinct functional states (e.g., M1, M2a, M2b, etc.) in response to different cytokines and other environmental cues is well documented (55, 56). While M1 macrophages are associated with proinflammatory and immune-activating stimuli (i.e., IFN- γ , TNF- α , LPS), alternatively activated (M2) macrophages are skewed toward promoting the resolution of immune responses by producing IL-10 while suppressing proinflammatory mediators like IFN- γ , TNF- α , and IL-12. Interestingly, M2-polarized macrophages have also been reported to show increased levels of the β -glucan receptor Dectin-1 (57, 58), increased complement-mediated phagocytosis (59), and increased phagosomal degradation activity (60). Consistent with this idea, the bioactive metabolite of SSZ, 5-aminosalicylic acid, is an activating ligand for the pro-M2 transcription factor peroxisome proliferator-activated receptor gamma (61). Thus, it is possible that anti-*Pneumocystis* antibody and SSZ together elicit a specific M2-like program in alveolar macrophages that not only results in the reversal of immunopathogenesis but may also augment the phagocytic

TABLE 2 Specific anti-*Pneumocystis* monoclonal antibody pool

Antibody	Isotype	Epitope	Reference
4F11 (G1)	IgG1	A12	67
1G12	IgG2b	40–60 kDa	66
1F2	IgG2a	40–60 kDa	None
1F5	IgG1	40–60 kDa	None
2B5	IgG1	gpA	66
3D6	IgG1	gpA	None
1G4	IgM	A12	25

clearance of *Pneumocystis* by alveolar macrophages either directly (via Dectin-1 or other c-type lectins [62–65]) or indirectly (via increased antibody-mediated clearance).

In conclusion, this is the first time that passively administered anti-*Pneumocystis* antibody plus SSZ has been used to treat mice with existing PcP. This combination treatment achieved a dramatic degree of improvement in the severity of PcP. Further studies need to be completed to evaluate the specific mechanisms by which anti-*Pneumocystis* antibody and SSZ function in concert to attain this level of rescue. Importantly, the physiological improvement and decreased fungal burden were achieved without standard-of-care antibiotic treatment with trimethoprim-sulfamethoxazole (TMP-SMX). Future studies will need to determine whether the inclusion of TMP-SMX affects this treatment regimen. These findings open the possibility that PcP treatment can be significantly improved with better and more specific immunomodulatory strategies to limit immunopathogenesis and enhance fungal clearance.

MATERIALS AND METHODS

Mouse model of *Pneumocystis* pneumonia. Severe combined immune-deficient (SCID) mice on a C.B-17 background were maintained in our specific-pathogen-free colony at the University of Rochester Medical Center. For these studies, 6- to 8-week-old female mice were infected with *Pneumocystis* by intratracheal (i.t.) inoculation of 1×10^5 *Pneumocystis* cysts and then also cohoused with other *Pneumocystis*-infected SCID mice to ensure infection. The mice were fed acidified water and sterile food. Immune reconstitution (IR) was initiated 3 weeks after infection by giving mice an intraperitoneal (i.p.) injection of 5×10^7 splenocytes from wild-type C.B-17 mice as described previously (6, 15). All animals involved were treated ethically according to the guidelines from the Association for Assessment and Accreditation of Laboratory Care International (AAALAC International), and the protocols used were approved by the University of Rochester Committee on Animal Resources.

Anti-*Pneumocystis* antibody and sulfasalazine administration. The anti-*Pneumocystis* antibodies have been developed and characterized by our laboratories (Table 2) (25, 66, 67). We pooled several *Pneumocystis*-specific IgG and IgM antibodies and injected mice i.p. with this antibody combination (0.1 mg of each antibody per dose) every 3 days after immune reconstitution. Control mice were given an equal amount of nonspecific whole-mouse immunoglobulin purified from naive animals (ChromPure; Jackson ImmunoResearch Laboratories, Inc.). Sulfasalazine (SSZ; Sigma, St. Louis, MO) was prepared fresh daily and given via i.p. injections at a dose of 200 mg/kg of body weight, as described previously (15). Each group within an experiment contained at least 6 mice, and each experiment was replicated 1 to 2 times. For the experiments assessing the effect of treatment with anti-*Pneumocystis* monoclonal antibody on the macrophage phenotype, control mice received an equivalent amount of a mouse MAb raised against endotoxin or the *Haemophilus influenzae* type b capsular polysaccharide, both of which were produced in our laboratories.

Physiological assessment of pulmonary function. Lung resistance and compliance were measured on live ventilated mice using a Harvard rodent ventilator (Harvard Apparatus, Holliston, MA) and a whole-body plethysmograph (Buxco Electronics Inc., Wilmington, NC) as previously described (41). Briefly, mice were anesthetized, a tracheostomy was performed, and a 20-gauge cannula was inserted 3 mm into an anterior nick in the exposed trachea. To ensure that the mice survived the procedure, spontaneous respirations were observed before proceeding. The mice were immediately placed into a plethysmograph (Buxco Electronics Inc.) and connected to a Harvard rodent ventilator (Harvard Apparatus). The mice were ventilated with a tidal volume of 0.01 ml/g of body weight at a rate of 150 breaths per minute. Respiratory flow and pressure measurements were taken from the plethysmograph chamber. Dynamic lung compliance was calculated in milliliters per centimeter of H₂O from the flow and pressure signals and then normalized for body weight. Resistance values were calculated in centimeters of H₂O per milliliter per second from the same signals. Pulmonary function was normalized to body weight. The Biosystems XA software package (Buxco Electronics Inc.) was used to collect and analyze the data.

Determination of *Pneumocystis* lung burden. The *Pneumocystis* lung burden in lung homogenates from mice was assessed using Gomori's methenamine silver (GMS) staining of cyst forms (39) and real-time quantitative PCR (qPCR) to quantify the total *Pneumocystis* burden, as described previously (41). Primers specific for the single-copy *Pneumocystis kexin* gene (68) and a Bio-Rad CFX96 real-time PCR

detection system (Bio-Rad, Hercules, CA) were used for qPCR determination of the *Pneumocystis* burden, as described previously (41).

Cytokine analyses in BAL fluid. Bronchoalveolar lavage (BAL) was performed on whole lung with four, 1-ml volumes of ice-cold $1\times$ Hanks' balanced salt solution (HBSS). The concentrations of the cytokines TNF- α , IFN- γ , CCL2, IL-4, IL-5, and IL-17 were measured with DuoSet enzyme-linked immunosorbent assays (ELISAs; R&D, Minneapolis, MN) per the manufacturer's instructions. The ELISAs were performed on cell-free supernatant from BAL fluid to measure cytokine protein concentrations.

Flow cytometry. Standard surface flow cytometry was performed for the following markers: CD3, CD4, CD8, CD11c, and GR-1 (BD Biosciences, San Diego, CA). The cellular fractions obtained by bronchoalveolar lavage were incubated with Fc Block (BD Biosciences), followed by incubation with fluorescently labeled antibodies that recognize the markers listed above. Fluorescence-minus-one (FMO) controls were used to set the gates for each antibody stain in the panel. At least 10,000 cells were routinely analyzed for each *Pneumocystis*-infected mouse, and at least 5,000 cells were analyzed for uninfected control mice. Multispectral imaging flow cytometry (ImageStream; Amnis Corporation) was used as we have described previously (15) to quantify the number of CD11c⁺ alveolar macrophages in experimental mice that contained internalized *Pneumocystis*. Internalized *Pneumocystis* organisms were identified in permeabilized and fixed macrophages using a pool of specific anti-*Pneumocystis* MAbs created in our laboratories (15). Briefly, the lungs were lavaged, and the recovered cells were washed with phosphate-buffered saline (PBS) and incubated with mouse Fc Block (BD Biosciences). The cells were then surface stained with anti-CD11c-phycoerythrin (clone HL3; BD Biosciences) and washed with PBA (0.5% bovine serum albumin and 0.05% sodium azide in PBS). The cells were permeabilized with BD Cytotfix/Cytoperm fixation and permeabilization solution and incubated with a pool of anti-*Pneumocystis* MAbs (MAbs 4F11, 2B5, 3D6, 1F1, and 1F5). Following a wash step, the cells were incubated with Alexa Fluor 647-conjugated goat anti-mouse IgG (H+L; Invitrogen Molecular Probes, OR). Stained cells were washed, pelleted, and resuspended in 50 μ l of ice-cold 1% paraformaldehyde in PBS. Samples were stored at 4°C in the dark until analyzed. At least 20,000 event image files were collected. Alveolar macrophages without internalized *Pneumocystis* treated in the same manner were used as internal controls to set the threshold for the fluorescence detection of *Pneumocystis*. In addition, isotype controls were used to confirm the binding specificity of the anti-*Pneumocystis* antibodies. Data were analyzed using ImageStream data exploration and analysis software (IDEAS; Amnis Corporation).

Effect of antibody on alveolar macrophage phenotype. *Pneumocystis*-infected SCID mice underwent immune reconstitution (IR) with normal splenocytes as described above. Groups of mice received nonspecific mouse immunoglobulin, anti-*Pneumocystis* antibodies, SSZ, or anti-*Pneumocystis* antibodies plus SSZ. The antibodies were given as described above on days 1 and 3 post-IR, and SSZ was given on days 1, 2, and 3 post-IR. The mice were sacrificed on day 3 postreconstitution, and bronchoalveolar lavage was performed. Aliquots containing 5×10^4 cells were cytospun onto glass slides and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. The cells were permeabilized with 0.02% Triton X-100 in PBS for 15 min at room temperature, rinsed 3 times with PBS, and then blocked with species-specific 5% normal serum in 0.05% Tween 20 in PBS (PBS-T) for 1 h at room temperature. Anti-inducible nitric oxide synthase (anti-iNOS) antibody (Abcam) and anti-YM-1 antibody (Stemcell) were used to distinguish M1 and M2 macrophages. iNOS was used as a prototypical marker of M1 macrophages, and YM-1 was used as a marker of M2 macrophages (16). DAPI (4',6-diamidino-2-phenylindole; Molecular Probes) diluted 1:36 in PBS for 5 min at room temperature and then rinsed 3 times in PBS was used to identify macrophage nuclei. Slides were mounted with antifade solution (Vectashield; Vector Laboratories, CA). A Nikon Eclipse E400 fluorescence microscope was used for photomicroscopy. Control alveolar macrophages from uninfected SCID mice were incubated with the same antibodies and used to set the threshold for the fluorescence detection of iNOS and YM-1 in the experimental samples. All photographs for a given protein were taken with identical exposure settings.

Statistical analysis. Differences between experimental groups were analyzed using analysis of variance with Bonferroni's multiple-comparison posttest. Differences were considered significant at *P* values of <0.05. All data were analyzed using GraphPad Prism (version 8) software (GraphPad Software, San Diego, CA). For statistical purposes, the value used for the *Pneumocystis* burden of animals with zero detectable *Pneumocystis* organisms either by cyst counts or by qPCR was equivalent to the limit of detection of the assay.

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