Anti-CD20 Antibody Therapy and Susceptibility to *Pneumocystis* Pneumonia

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Anti-CD20 antibody therapy has been a useful medication for managing non-Hodgkin’s lymphoma as well as autoimmune diseases characterized by autoantibody generation. CD20 is expressed during most developmental stages of B lymphocytes; thus, CD20 depletion leads to B-lymphocyte deficiency. As the drug has become more widely used, there has been an increase in the number of case reports of patients developing *Pneumocystis* pneumonia. The role of anti-CD20 in *Pneumocystis jirovecii* infection is under debate due to the fact that most patients receiving it are on a regimen of multiple immunosuppressive medications. To address the specific role of CD20 depletion in host immunity against *Pneumocystis*, we examined a murine anti-CD20 depleting antibody. We demonstrated that anti-CD20 alone is permissive for *Pneumocystis* infection and that anti-CD20 impairs components of type II immunity, such as production of interleukin-4 (IL-4), IL-5, and IL-13 by whole-lung cells, in response to *Pneumocystis murina*. We also demonstrated that CD4+ T cells from mice treated with anti-CD20 during *Pneumocystis* infection are incapable of mounting a protective immune response when transferred into Rag1−/− mice. Thus, CD20+ cells are critical for generating protective CD4+ T-cell immune responses against this organism.

**Antibody therapy has been a useful medication for managing non-Hodgkin’s lymphoma as well as autoimmune diseases characterized by autoantibody generation.** CD20 is expressed during most developmental stages of B lymphocytes; thus, CD20 depletion leads to B-lymphocyte deficiency. As the drug has become more widely used, there has been an increase in the number of case reports of patients developing *Pneumocystis* pneumonia. The role of anti-CD20 in *Pneumocystis jirovecii* infection is under debate due to the fact that most patients receiving it are on a regimen of multiple immunosuppressive medications. To address the specific role of CD20 depletion in host immunity against *Pneumocystis*, we examined a murine anti-CD20 depleting antibody. We demonstrated that anti-CD20 alone is permissive for *Pneumocystis* infection and that anti-CD20 impairs components of type II immunity, such as production of interleukin-4 (IL-4), IL-5, and IL-13 by whole-lung cells, in response to *Pneumocystis murina*. We also demonstrated that CD4+ T cells from mice treated with anti-CD20 during *Pneumocystis* infection are incapable of mounting a protective immune response when transferred into Rag1−/− mice. Thus, CD20+ cells are critical for generating protective CD4+ T-cell immune responses against this organism.

Anti-CD20 monoclonal antibodies first showed efficacy against chemotherapy-resistant non-Hodgkin’s B-cell lymphomas in 1997 (1, 2). Since then, anti-CD20 has been used therapeutically against hematological cancers, autoimmune diseases, and post-transplant lymphoproliferative disease. CD20 is a B-lymphocyte antigen encoded by a membrane-spanning 4A family member, MS4A1. There is no known ligand for CD20; however, it is believed to play a role in B-cell development and differentiation into plasma cells and in T-cell-independent antibody (Ab) responses (3). With the increased use of anti-CD20 as a treatment, there have been several recent reports of patients receiving anti-CD20 and subsequently developing infection with the opportunistic pathogen *Pneumocystis jirovecii*, which may develop into a fatal pneumonia even with antibiotic therapy (4). However, there is debate about the precise role of anti-CD20 in conferring risk, as many of these patients are also on concomitant immunosuppressive drugs, thus complicating any analyses of clinical studies (5–7).

*Pneumocystis* is an opportunistic fungal pathogen that was originally a very strong indicator that a patient had human immunodeficiency virus (HIV). Depletion of CD4+ T cells to levels below a count of 200 per μl of blood was the primary risk factor for susceptibility to *Pneumocystis jirovecii* pneumonia (PJP) (8, 9). The role of CD4+ T cells has been validated several times in a variety of animal models, from selective depletion of CD4+ cells to the use of knockout mice (10, 11). The clearance process typically occurs either through the generation of effector CD4+ T cells that recruit and activate phagocytes, such as macrophages, to clear the infection or by helping B cells to mature into *Pneumocystis*-specific plasma cells, which promote antibody-mediated phagocytosis (12, 13). The importance of B cells was first observed when it was demonstrated that B-cell-deficient mice (μMT mice) were permissive for *Pneumocystis* infection. At the time, this effect was suggested to be due to the lack of serum immunoglobulins in these mice (14). However, subsequent studies demonstrated that B cells play a larger role than just antibody generation, as Lund et al. showed that B cells were required for priming of CD4+ T cells and for generating protective effector and memory CD4+ T cells in response to *Pneumocystis* lung infection in mice (15). This suggested that depletion of CD20+ B cells would also lead to CD4+ T-cell dysfunction and susceptibility to *Pneumocystis* infection. To experimentally test this hypothesis, we administered a murine anti-CD20 depleting antibody (5D2) to mice, followed by subsequent infection with *P. murina*. We found that administration of anti-CD20 conferred susceptibility to primary *Pneumocystis* infection.

Furthermore, it has been reported that some patients receiving anti-CD20-containing treatment regimens for lymphoma develop immune reconstitution inflammatory syndrome (IRIS) after receiving the last treatment (16). Thus, we next investigated the effects of CD20 depletion on the development of IRIS in our murine model. We concluded that although the pathology/lung injury associated with CD4+ T-cell reconstitution was not influenced by the presence or absence of B cells, the ability of the CD4+ T cells to mount a protective immune response against *Pneumocystis murina* was in fact dependent on CD20+ B cells. CD20 depletion did not affect the recruitment of CD4 cells to the lung, but infected lungs had reduced type II immune responses. This study sheds some light on how anti-CD20 treatment in patients may...
Mice were infected with an inoculum of complete DMEM with 10% FBS. Asci were quantified microscopically, and the inoculum was added to sterile Dulbecco’s phosphate-buffered saline (PBS) at 80°C. To process the inoculum, frozen lungs were thawed, strained through a 70-μm filter, and pelleted by centrifugation (800 × g, 10 min, 4°C). The pellet was resuspended in 1 ml of PBS. A 5-μl aliquot was diluted 1:10, heat fixed on a slide, and stained with Hema-3 modified Wright-Giemsa stain (Fisher Scientific, Pittsburgh, PA), followed by ascus counting.

Pneumocystis isolation, inoculum, and antigen preparation. Pneumocystis murina organisms were administered by oral-pharyngeal delivery to Rag2<sup>−/−</sup> Il2rg<sup>−/−</sup> mice, propagated for 10 to 12 weeks in vivo, and isolated from mouse lung tissue as previously described (17). Briefly, Rag2<sup>−/−</sup> Il2rg<sup>−/−</sup> mice with Pneumocystis pneumonia were sacrificed, and the lungs were aseptically harvested and frozen in 1 ml of sterile Dulbecco’s phosphate-buffered saline (PBS) at −80°C. To process the inoculum, frozen lungs were thawed, strained through a 70-μm filter, and pelleted by centrifugation (800 × g, 10 min, 4°C). The pellet was resuspended in 1 ml of PBS. A 5-μl aliquot was diluted 1:10, heat fixed on a slide, and stained with Hema-3 modified Wright-Giemsa stain (Fisher Scientific, Pittsburgh, PA), followed by ascus counting. Pneumocystis murina asci were quantified microscopically, and the inoculum was adjusted to 2 × 10<sup>5</sup> asci per ml. Mice were administered 100 μl (2 × 10<sup>5</sup> ascis) of the inoculum by oral-pharyngeal aspiration as previously described (18). Pneumocystis protein antigen was prepared by differential centrifugation of the inoculum as previously described, followed by sonication of 1 mg of inoculum per ml for 5 min (19).

Preparation of whole-lung cells (WLC) and antigen stimulation. Mice were infected with an inoculum of P. murina for 2 weeks. At the time of euthanasia, mice were anesthetized by intraperitoneal injection of a ketamine-xylasine cocktail and euthanized by exsanguination. Immediately after, mice were perfused vascularity by 5 ml of heparinized PBS injected into the right ventricle. The right superior and inferior lung lobes were then harvested, minced with razor blades, and digested in 5 ml serum-free medium with 2 mg/ml collagenase for 90 min in a 37°C shaking incubator. The cell suspension was strained through a 70-μm filter and then washed and resuspended in complete Dulbecco’s modified Eagle’s medium (DMEM). Red blood cells were then lysed with ammonium chloride solution, washed, resuspended in 5 ml of DMEM, and counted. A total of 10<sup>6</sup> cells per well were plated in a 96-well round-bottom plate in complete DMEM with 1 μg/ml P. murina antigen and 20 U/ml interleukin-2 (IL-2). An aliquot from each group was taken for cell analysis by flow cytometry. Platlets were stained at 37°C and 5% CO<sub>2</sub> for 72 h. Finally, supernatants were harvested for multiplex (Millipore) cytokine analysis on a Bioplex reader (Bio-Rad).

Flow cytometric analysis. A total of 10<sup>6</sup> single cells from the mouse lung were stimulated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma) and 750 ng/ml ionomycin (Sigma) for 5 to 6 h. One hour after the start of stimulation, cells were given 1 μl/ml GolgiStop (BD Pharmingen, San Diego, CA) to block cytokine secretion. Cells were surface stained with T-cell receptor beta (TCRB), CD4, CD8, and B220 for 15 to 30 min in PBS supplemented with 1% bovine serum albumin (BSA). Cells were then fixed in 1% formalin and acquired for flow cytometry by an LSR-II flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo (Treestar).

RNA isolation and Pneumocystis quantification by RT-PCR. The right middle lobe of the lung was harvested in 1 ml of TRIzol and homogenized. RNA was purified and quantified as previously described (17). Briefly, cDNA was synthesized from 1 μg whole-lung RNA via iScript reverse transcription reagents (Bio-Rad, Hercules, CA), and real-time PCR (RT-PCR) was performed using primers and probes for the Pneumocystis murina large-subunit (LSU) rRNA transcript and SsoFast/ SsoAdvanced probe supernix (Bio-Rad). The threshold cycle values were converted to copy numbers by use of a premade standard of known Pneumocystis murina LSU rRNA, as previously described (20).

Serum collection and Pneumocystis murina antigen enzyme-linked immunosorbent assay (ELISA). Blood was collected periodically by tail bleed and/or at the time of sacrifice by syringe from the vena cava. Coag-
ulated blood was then centrifuged for 10 min at 10,000 g. The serum supernatant was collected and stored at 80°C. Maxisorb plates were coated with 1 µg P. murina antigen in 100 µl bicarbonate coating buffer per well overnight at 4°C. Plates were blocked with 5% blotting-grade blocker (Bio-Rad) and 1% BSA. Plates were first stained with sample serum in a dilution series from 26 to 213 overnight at 4°C and then stained with murine Ig-specific horseradish peroxidase (HRP)-conjugated antibodies. Plates were then developed with tetramethylbenzidine (TMB) substrate for 5 to 30 min, depending on the control serum, and the reaction was stopped with an equal volume of 2 N H2SO4. The optical density at 450 nm (OD450) was read using a Synergy H1 Hybrid reader (BioTek, Winooski, VT).

Antibody-mediated cell depletion. CD4+ cells were depleted using an anti-CD4 monoclonal antibody, GK1.5, as previously described (17). Mice were injected in the intraperitoneal space weekly with a 0.3-mg dose of Ab in 200 µl sterile PBS. CD4+ cell depletion efficiency was assessed by flow cytometry with anti-CD4 clone RM4-5, which does not compete with GK1.5. CD20+ cells were depleted using a mouse anti-mouse CD20 monoclonal antibody (clone 5D2, murine IgG2a; Genentech). Mice were given intraperitoneal injections with 0.1-mg doses of Ab every 5 days. CD20+ cell depletion efficiency was assessed by flow cytometry with anti-B220.

Preparation of splenocytes and purified CD4+ and purified B220 cells for adoptive transfer. Spleens harvested from C57BL/6 mice were harvested, diced, and strained through a 70-µm filter to create a single-cell splenocyte suspension. CD4+ cells and B220 cells were purified using a Stem Cell EasySep negative-selection mouse CD4+ T-cell isolation kit and mouse B220 B-cell isolation kit, respectively. Cells were enumerated and resuspended in sterile PBS. Cells were resuspended at 2.5 x 10^6 cells/ml, and each mouse received 5 x 10^5 cells (200 µl) via intravenous (tail vein) injection. In testing the capacity of CD4 for clearance, cells were adoptively transferred into mice 2 weeks prior to infection and sacrificed 4 to 6 weeks after infection with Pneumocystis murina. To induce IRIS, mice were originally infected for 21 days prior to cell adoptive transfer and then sacrificed 10 days after transfer.

Pulse oximetry and lung injury qualification from BALF. The mouse blood oxygen saturation, respiratory rate, and heart rate were measured from the tail by using a MouseOx pulse oximeter (Starr Life Sciences, Oakmont, PA) as previously described (18). Briefly, mice were anesthetized with 100 mg of intraperitoneal ketamine/kg of body weight, and the tail clip sensor was placed at the base of the tail, scanning the lateral vein. One-minute measurements were recorded, and the average values for 20 stable readings were reported. The first milliliter of bronchoalveolar lavage fluid (BALF) was harvested by repeated 0.5-ml washes with a single full syringe with 1 ml PBS. The BALF was centrifuged at 400 x g for 10 min at 4°C to remove live cells and debris. Total protein was measured by use of a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Lactate dehydrogenase (LDH) was analyzed with an LDH activity assay kit (BioVision, Milpitas, CA), and readouts were recorded every 5 min for 1 h.
Statistical analysis. GraphPad Prism (GraphPad Software, La Jolla, CA) one-way analysis of variance (ANOVA) with the Holm-Sidak multiple-comparison posttest was used to calculate P values. Nonparametric data for three or more groups were analyzed with the Kruskal-Wallis test and the Dunn multiple-comparison posttest. Two group comparisons were done with the multiple t test. P values are annotated as follows: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; and ****, P ≤ 0.0001.

RESULTS
Anti-CD20 treatment induces susceptibility to *Pneumocystis*. First, we independently validated that 5D2 (murine anti-CD20) was capable of depleting B cells in mice. Prior to depletion (day 0), over half of the splenocytes were IgM⁺, and the majority were also CD23⁺. By day 7 postdepletion, this population of cells was reduced by approximately 90% (Fig. 1A). To investigate if anti-CD20 conferred susceptibility to primary *Pneumocystis* infection, we administered anti-CD20 to mice, followed by *Pneumocystis murina* infection (Fig. 1B). We measured *P. murina* burdens both 2 and 4 weeks after infection, and at 2 weeks, 5D2-treated mice and isotype control mice had no differences in infectious burden. However, 4 weeks after infection, control mice began clearing infection, whereas 5D2-treated mice had approximately 2-log larger *P. murina* burdens in the lung (Fig. 1C). Thus, CD20⁺ cells are crucial for a protective immune response, and depletion results in high susceptibility to primary *Pneumocystis* infection. We also repeated this experiment by assessing infection at 6 and 8 weeks postinoculation and observed that the infection burden was maintained in anti-CD20-treated mice but was cleared in control mice (data not shown).

**CD20 depletion blocks type II immune responses in the lung.** To determine mechanisms by which anti-CD20 was permissive for sustained *Pneumocystis* infection, we assessed cellular immune responses in whole-lung cells (WLCs). We harvested lungs from...
5D2-treated, GK1.5-treated (CD4-depleted), and isotype control mice 2 weeks after infection. We chose the 2-week time point because the infection loads were approximately equal among all the groups at this time, so any difference in immune responses could not be attributed to the presence of more antigen in vivo (Fig. 2A). The lungs were digested and strained to generate WLC cultures, which were analyzed by flow cytometry or stimulated with P. murina antigen. Total WLCs were counted so that absolute lymphocyte counts could be calculated. There was no difference in the number of recovered WLCs; however, there was a significant reduction in the total lymphocyte count in CD20-depleted animals (Fig. 2Band C). Furthermore, flow cytometry analysis showed that there was a 98% CD4 depletion with GK1.5 administration and an approximately 90% B220 depletion with 5D2 (Fig. 2D).

After stimulation of WLCs with P. murina antigen for 72 h, we analyzed effector cytokines in cell supernatants by Luminex assay. Gamma interferon (IFN-γ) production by WLCs did not differ across the groups (Fig. 3A). IL-17a was not highly produced, and although there was a trend toward higher levels in control mice, this was not statistically significant between the groups (Fig. 3B). However, the vehicle control group had a strong type II signature, which was substantially reduced in both the CD4- and CD20-depleted groups. Briefly, IL-4 and IL-13 were substantially reduced in both CD4- and CD20-depleted samples. Although IL-5 expression was also reduced after CD20 depletion, levels were still significantly higher than those in the CD4-depleted samples (Fig. 3C). However, depleting CD4+ cells from CD20-depleted mice further reduced type II responses, which were equivalent to levels with CD4 depletion alone (data not shown). These data show that CD20 depletion leads to a defect in effector immune responses in the lung and that the decrease is primarily in type II immunity of whole-lung cells.

Anti-CD20 treatment eliminates CD4+ T-cell protective responses. To address whether CD20 depletion specifically alters CD4 cell effector function, we then examined whether adoptively

FIG 4 Loss of CD4+ cell-specific protection after anti-CD20 treatment. (A) Mice were CD20 depleted and infected with P. murina for 2 weeks. Splenic CD4+ cells were then purified and adoptively transferred into Rag1-/- mice. After homeostatic cell proliferation, mice were infected with P. murina for 4 weeks. (B) Purified splenic CD4+ cells from CD20-depleted mice were stimulated with CD3/CD28 beads. Cytokines in the supernatants were analyzed simultaneously by Millipore multiplex assay on a Bioplex reader. (C) Total lung RNAs were isolated, and Pneumocystis burdens were measured by real-time PCR analysis of the mitochondrial large-subunit rRNA copy number. Burdens are reported as means ± SEM for 5 mice per group.
transferring purified CD4⁺ T cells from naive C57BL/6 WT mice into Rag1⁻/- mice had any protective effects against *Pneumocystis*. Adoptive transfer of purified CD4⁺ T cells resulted in significant reductions in *P. murina* lung burdens compared to those of vehicle control mice (see Fig. S1 in the supplemental material). We next examined if CD4⁺ T cells from 5D2-treated mice or isotype control mice could clear *Pneumocystis*. Briefly, 5D2-treated C57BL/6 WT mice were infected with the standard inoculum of *Pneumocystis murina* for 2 weeks, after which CD4⁺ cells from these mice were purified and adoptively transferred to Rag1⁻/- mice at the time of infection with *P. murina*. (E) *Pneumocystis* burdens following transfer of convalescent-phase serum as measured by real-time PCR as described above. All data are reported as means ± SEM for 3 to 5 mice per group.

**FIG 5** Humoral immunity is sufficient for protection against *Pneumocystis*. (A) Wild-type C57BL/6 mice were infected with *P. murina* and allowed to convalesce for 6 weeks. They were then CD20 depleted and infected with *P. murina* for 2 weeks. (B) Total lung RNAs were isolated, and *Pneumocystis* burdens were measured by real-time PCR analysis of the mitochondrial large-subunit rRNA copy number. (C) Total *P. murina* antigen-specific IgGs were measured by direct ELISA of a 1:64 dilution of serum as the primary antibody and anti-mouse IgG–HRP as the secondary antibody. (D) Sera harvested from convalescent mice were adoptively transferred into Rag1⁻/- mice at the time of infection with *P. murina*. (E) *Pneumocystis* burdens following transfer of convalescent-phase serum as measured by real-time PCR as described above. All data are reported as means ± SEM for 3 to 5 mice per group.

Mice with convalescent-phase immunity are resistant to anti-CD20-mediated susceptibility. We next determined if anti-CD20 conferred the risk of secondary infection with *P. murina*. To examine this, we generated convalescent mice by infecting them with *P. murina* and allowing them 8 weeks to clear and recover from the infection. After the recovery period, one group was given 5D2, another group received GK1.5, and a third group received a carrier control (sterile PBS). All groups were then reinfected with the same inoculum of *P. murina*, and fungal burdens were measured 2 weeks after the second infection (Fig. 5A). All groups, including the CD4⁻ and CD20-depleted groups, completely eradicated the infection (Fig. 5B). We wanted to discern the method of clearance, so we assessed the level of *Pneumocystis murina*-specific antibodies in the serum. All groups had approximately equal levels of anti-*P. murina* IgG (Fig. 5C). We attributed the clearance to the presence of anti-*Pneumocystis* IgG. To formally test this, we examined the effector activity of these antibodies by adoptively trans-
ferring 200 µl of either naive or convalescent-phase serum from WT C57BL/6 mice infected with *P. murina* to Rag1<sup>−/−</sup> mice, which lack T and B cells (Fig. 5D). Rag1<sup>−/−</sup> mice that received convalescent-phase serum were protected from infection (Fig. 5E). These studies show that anti-CD20 does not confer susceptibility to infection in the presence of preexisting *Pneumocystis* immunity.

**CD20 depletion does not influence IRIS severity.** We next determined if CD20<sup>+</sup> cells may contribute to IRIS. The first model we used to test this experiment was severe combined immunodeficient (SCID) mice infected with *Pneumocystis murina* followed by adoptive transfer of whole splenocytes to induce IRIS (21). As a negative control for IRIS in this experiment, a group of SCID mice received whole splenocytes and were depleted of CD4<sup>+</sup> T cells *in vivo*. We then examined whether or not anti-CD20 reduced or prevented tissue pathology (Fig. 6A). We first measured body weight and found that mice that received anti-CD20 lost approximately the same percentage of total body weight as the nondepleted mice (Fig. 6B). However, depletion of CD4<sup>+</sup> T cells prevented weight loss (Fig. 6B). Total protein in the BALF, a measure of lung damage, was also significantly reduced in mice that received anti-CD4 antibodies but increased in control and anti-CD20-treated mice (Fig. 6C). Lactate dehydrogenase activity in the BALF, a measure of cell death in the lung, was elevated in mice that received whole splenocytes but was significantly abrogated in mice that received anti-CD4 (Fig. 6D). However, there was slightly higher LDH activity in mice that received anti-CD20 (Fig. 6D). These data show that IRIS requires CD4<sup>+</sup> T cells but that anti-CD20 has little effect on the development of IRIS in this model.

We next examined if CD20<sup>+</sup> cells in conjunction with CD4<sup>+</sup> T cells affected IRIS. We also tested the hypothesis that *Pneumocystis*-experienced B cells or plasma cells would provide a protective effect and mitigate IRIS. Briefly, we adoptively transferred CD4<sup>+</sup> T cells alone, CD4<sup>+</sup> T cells plus B220 cells from naive mice, or CD4<sup>+</sup> T cells plus B220 cells from antigen-experienced (Ag Exp) mice into *P. murina*-infected Rag1<sup>−/−</sup> mice. Ag Exp cells were from mice infected with *Pneumocystis murina* for 14 days. We sacrificed mice at day 10 posttransfer, as *Pneumocystis* burdens were still equal between all the groups at this time point (Fig. 7A) but it was the peak day of lung injury. By day 10, we could detect anti-chitin IgM levels in the sera of the naive and antigen-experienced B220 cell transfer mice and anti-*Pneumocystis* IgG levels.
only in the Ag Exp B220 cell transfer mice (Fig. 7B and C). As in the previous experiment, adding B220 cells, whether naive or Ag Exp, did not affect weight loss, blood oxygen saturation, or BALF lactate dehydrogenase (Fig. 7D to F). These data show that B cells are dispensable for CD4+ T-cell-mediated IRIS in this model.

**DISCUSSION**

As there is an increase in the number of incidents of opportunistic infections involving patients receiving anti-CD20-containing treatment regimens, there is a need to understand the effects of anti-CD20 on the immune response to infection. We pursued this question by developing an in vivo mouse model of CD20 depletion in an attempt to recapitulate the clinical scenario of anti-CD20 treatment. We were first able to observe that anti-CD20 alone was capable of inducing susceptibility to *Pneumocystis* infection by diminishing the immune response to *Pneumocystis* in the lung. WLCs showed reduced type II responses, and CD4+ T cells specifically were not capable of providing protective responses in an adoptive transfer model. These data suggest that anti-CD20 inhibits T-cell priming against *P. murina.* (22). It is still not known why CD20+ B cells specifically are required to prime CD4+ cell responses to *Pneumocystis*. Previous studies have shown that both CD40 and major histocompatibility complex (MHC) class II molecules on B cells are critical for CD4+ T-cell priming; however, why other antigen-presenting cells are not capable is still a question that needs to be addressed (13). Interestingly, the absence of
CD20⁺ cells during Pneumocystis infection may lead to clonal deletion of Pneumocystis-specific CD4⁺ cells, since they are not as capable of clearing Pneumocystis as CD4⁺ cells from naive mice. This may be due to a lack of CD4⁺ T-cell costimulation by B cells during Pneumocystis infection.

While the previous experiments were conducted with naive mice, anti-CD20-treated patients are likely not naive to Pneumocystis jirovecii, as most individuals are seropositive for anti-Pneumocystis antibodies (19, 23). The potential reservoirs for Pneumocystis are still unknown, but some research indicates the possibility that many diseased and healthy patients may be colonized with Pneumocystis. Some studies also suggest that hosts may be transiently exposed or asymptptomatically carry Pneumocystis through their lives (24). We observed that convalescent mice were capable of clearing Pneumocystis without the aid of CD4⁺ or CD20⁺ cells, most likely through antibody-mediated protection. Since memory CD4⁺ cells and antibodies are enough to clear Pneumocystis, these data raise the question of why immunosuppressed patients acquire Pneumocystis jirovecii infections. Anti-CD20 antibody therapy in patients alone has not been shown to decrease immunoglobulin levels; however, if this therapy is combined with other immunosuppressive therapies, hypogammaglobulinemia can occur (25). It has also been demonstrated that Pneumocystis spp. have a dynamic extracellular proteome as a result of varying their major surface glycoproteins, which may be used to evade the host immune response (26, 27). Reports of large genetic variability between Pneumocystis jirovecii isolates also suggest the possibility that patients are simply being exposed to new strains of Pneumocystis (22, 28).

IRIS is a condition that was first observed with the advent of highly active antiretroviral therapy (HAART), which dramatically suppresses HIV replication (29). This suppression leads to an expeditious reconstitution of CD4⁺ T cells, which then react to an accumulation of viable infections and/or residual microbial antigens acquired during immunosuppression. As a result, the host can experience a massive amount of inflammation in multiple organs, primarily the lungs and central nervous system (CNS) (30, 31). This immunopathology is mediated through CD4⁺ T cells (32), but it was previously shown that other cell types, such as CD8 cells, modulate the pathology by increasing the Treg/Teffector ratio (33). Previous studies have shown that natural antibodies may reduce pathology (18); however, the direct role of B cells during IRIS has yet to be studied. Thus, as B cells are required for T-cell effector function in protection, we hypothesized that CD20⁺ cells may also play a role in the inflammatory pathology of T-cell-mediated IRIS. We observed that the absence or presence of CD20⁺ cells did not exacerbate the pathology in the model studied here. We hypothesize that this is due to nonspecific CD4⁺ T-cell activation through homeostatic proliferation. Thus, we conclude that CD20⁺ cells are dispensable for CD4-mediated IRIS.

Thus, in conclusion, anti-CD20 confers a risk of primary infection with P. murina, but we did not observe a risk of secondary infection in mice that had preexisting humoral immunity. In addition, mice treated with anti-CD20 had reduced whole-lung-cell type II responses to Pneumocystis, and CD4 cells from depleted mice had an intrinsic impairment in the ability to clear Pneumocystis in our adoptive transfer model. These data suggest that clinical Pneumocystis jirovecii pneumonia may be due to concomitant immunosuppression or to patients acquiring antigenically distinct strains of Pneumocystis that preexisting humoral immunity is ineffective at preventing. Lastly, anti-CD20 did not affect the severity of CD4 T-cell-mediated IRIS in this model.

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