B Lymphocytes Are Required During The Early Priming Of CD4 T Cells For Clearance Of *Pneumocystis* Infection In Mice

By: **Michael M. Opata**, Melissa L. Hollifield, Frances E. Lund, Troy D. Randall, Robert Dunn, Beth A. Garvy, & David J. Feola

Abstract

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B Lymphocytes Are Required during the Early Priming of CD4$^+$ T Cells for Clearance of *Pneumocystis* Infection in Mice

Michael M. Opata,*[,1] Melissa L. Hollifield,* Frances E. Lund,[†] Troy D. Randall,[‡] Robert Dunn,[§] Beth A. Garvy,* and David J. Feola[¶]

B cells play a critical role in the clearance of *Pneumocystis*. In addition to production of *Pneumocystis*-specific Abs, B cells are required during the priming phase for CD4$^+$ T cells to expand normally and generate memory. Clearance of *Pneumocystis* was found to be dependent on Ag specific B cells and on the ability of B cells to secrete *Pneumocystis*-specific Ab, as mice with B cells defective in these functions or with a restricted BCR were unable to control *Pneumocystis* infection. Because *Pneumocystis*-specific antiserum was only able to partially protect B cell–deficient mice from infection, we hypothesized that optimal T cell priming requires fully functional B cells. Using adoptive transfer and B cell depletion strategies, we determined that optimal priming of CD4$^+$ T cells requires B cells during the first 2–3 d of infection and that this was independent of the production of Ab. T cells that were removed from *Pneumocystis*-infected mice during the priming phase were fully functional and able to clear *Pneumocystis* infection upon adoptive transfer into Rag1$^{-/-}$ hosts, but this effect was ablated in mice that lacked fully functional B cells. Our results indicate that T cell priming requires a complete environment of Ag presentation and activation signals to become fully functional in this model of *Pneumocystis* infection. *The Journal of Immunology*, 2015, 195: 611–620.

Pneumocystis jirovecii is an opportunistic fungal pathogen that causes severe disease in immunocompromised individuals. *Pneumocystis* pneumonia (PCP) is an AIDS-defining illness and a significant contributor to morbidity and mortality in this population (1, 2). As such, the role of CD4$^+$ T lymphocytes in the defense against this organism has been extensively studied, as these cells are essential for the clearance of the pathogen (3, 4). It is presumed that effector T cells that are induced to activation through interactions with APCs in the lymph nodes (LN) then migrate to the lungs and activate alveolar macrophages, stimulating them to kill *Pneumocystis* organisms (5). Additionally, activated CD4$^+$ T cells interact with B cells, inducing them to produce *Pneumocystis*-specific Abs that opsonize the organisms, assisting the alveolar macrophages in phagocytosis (6, 7).

Although understudied, the role of B lymphocytes in the defense against *Pneumocystis* infection is critically important. Clinically, the increased incidence of *Pneumocystis* infection in patients receiving anti-CD20 Ab therapy underscores the significance of the B lymphocyte population in host defense against *Pneumocystis* (8–10). Although mice deficient in functional B cells are unable to clear *Pneumocystis* from the lungs (11, 12), the mechanisms by which B cells promote the clearance of *Pneumocystis* are still largely unknown. We previously demonstrated that mice with CD40-deficient B cells can clear *Pneumocystis* infection, suggesting that production of class-switched Ab against *Pneumocystis* is not required for the clearance of the organism (11). Additionally, mice with mutations targeted to Fcγ and Fcε receptors are also able to clear *Pneumocystis* infections, albeit at a slower rate than wild-type (WT) controls (11). Therefore, whereas class-switched *Pneumocystis*-specific Ab enhances clearance of the organism, it does not appear to be required for clearance. This conclusion is consistent with adoptive transfer studies, as CD4$^+$ T cells from *Pneumocystis*-infected WT donors will clear the organisms when transferred to *Pneumocystis*-infected SCID mice (3, 13). Collectively, these studies suggest that the requirement for B cells in the clearance of *Pneumocystis* infection may be independent, at least in part, of their ability to produce class-switched Ab.

Our previous work suggests that the activation of CD4$^+$ T cells in response to *Pneumocystis* is altered in mice that lack B cells. The number of activated CD4$^+$ cells present in both the lungs and draining LN of *Pneumocystis*-infected B cell–deficient (μMT) mice are reduced as compared with that of normal mice, based on surface marker expression and cytokine production (11). Importantly, we have shown that T cells that are primed in B cell–deficient mice fail to expand in response to *Pneumocystis* infection upon adoptive transfer to SCID mice (14). This suggests that B cells must provide some form of activation or proliferation signal to T cells during priming. The influence that B cells exert on T cells during CD4$^+$ T cell priming has also been demonstrated in other murine models of Ag challenge (15, 16). Although we found that the signals provided by B cells to CD4 T cells during *Pneumocystis* infection required interactions through either MHC class II or costimulatory molecules (11, 14), soluble factors including cytokines and secreted Ab may also be important. In support of this hypothesis, we reported recently that B cell–derived TNF is important for driving the

*Division of Infectious Diseases, Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky College of Medicine, Lexington, KY 40536; [†]Department of Microbiology, The University of Alabama at Birmingham, Birmingham, AL 35294; [‡]Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL 35294; [§]Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121; and [¶]Department of Pharmacy Practice and Science, University of Kentucky College of Pharmacy, Lexington, KY 40536

[1]Current address: Division of Infectious Diseases, University of Texas Medical Branch, Galveston, TX.

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Address correspondence and reprint requests to Dr. Beth A. Garvy, Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky Chandler Medical Center, College of Medicine, Room MS411, 800 Rose Street, Lexington, KY 40536-0298. E-mail address: bgarv0@email.uky.edu

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Abbreviations used in this article: AID, activation-induced deaminase; BALF, bronchoalveolar lavage fluid; BM, bone marrow; DC, dendritic cell; i.t., intratracheal(ly); LN, lymph node; PCP, *Pneumocystis* pneumonia; TBLN, tracheobronchial LN; WT, wild-type.

T cell response to *Pneumocystis* (17). However, we still do not know whether the interactions between B and T cells are critical during the early stages of response, or whether B cells are needed to initiate or maintain *Pneumocystis*-specific memory T cells. Therefore, our focus has turned to investigating whether B cell–T cell interactions during *Pneumocystis* infection alter the development or maintenance of the T cell compartment.

In the present study, we have addressed whether *Pneumocystis*-specific B cells play a role in the activation and survival of $CD4^+$ T cells, thereby governing their ability to clear *Pneumocystis* infection. We used genetically engineered mouse models and bone marrow (BM) chimeric mice that were unable to produce or secrete *Pneumocystis*-specific Ab. We demonstrate that in the absence of secreted Ab, T cell activation and *Pneumocystis* clearance is impaired. Furthermore, when T cell priming occurred in an environment devoid of *Pneumocystis*-specific Ab, the ability of CD4 cells to confer clearance of *Pneumocystis* was ablated and supplementation of Ab only partially rescued the mice from PCP. B cell interactions with $CD4^+$ T cells were critical early after infection as demonstrated by adoptive transfer and B cell depletion studies. T cells primed in mice that lacked a *Pneumocystis*-specific BCR did not expand or produce IFN-γ as well as T cells primed in WT mice upon adoptive transfer into host $Rag1^{-/-}$ mice. T cells primed in mice with B cells that lacked a *Pneumocystis*-specific BCR were also unable to control *Pneumocystis* infection. These results suggest that B cell–T cell interactions during priming require specificity for T cells to reach full functionality.

Materials and Methods

Mice

Adult C57BL/6J, BALB/cJ, B6.129S2-*Igh-6*^{tm1Cgn}/J (μMT), C.129S7(B6)-*Rag1*^{tm1Mom}/J ($Rag^{-/-}$), and C57BL/6-Tg(IghelMD4)4Ccg/J (MD4) mice were purchased from The Jackson Laboratory. C.129(B6)-IgH-JhD^{tm1Dhu} (Jh) and C.B-Igh-1^b/ICR Tac-Prkdc^{Scid} (SCID) mice were purchased from Taconic. Mice doubly deficient in activation-induced deaminase (AID) and μS were crossed in the laboratory of Hidde Ploegh (AID-$\mu S^{-/-}$) and fail to secrete Ab but have a polyclonal BCR repertoire (18). All experimental mice were housed in the Veterans Administration Medical Center veterinary medical unit or University of Kentucky Division of Laboratory Animal Resources units in sanitized cages and given food and water ad libitum. *Pneumocystis* organisms were maintained in a colony of $Rag2^{-/-}$ mice (originally from The Jackson Laboratory) as a source for all infections. All procedures were approved by the Lexington Veterans Administration or University of Kentucky Institutional Animal Care and Use Committees.

Generation of MD4 chimeras

Chimeric mice that were capable of only generating monoclonal B cells specific for hen egg lysozyme, an irrelevant Ag, were created by transplanting MD4 mouse BM into B cell–deficient μMT mice (14, 19). BM chimeric mice were generated by lethally irradiating μMT recipients with 900 rad from a [^{137}Cs] source and reconstituting them with 10^7 BM cells. Mice either were reconstituted with 75% μMT BM plus 25% C57BL/6 BM (WT chimera), 100% μMT BM (μMT chimera), or 75% μMT BM plus 25% MD4 BM (MD4 chimera). Mice were then allowed to rest for 8–12 wk before infection with *Pneumocystis*. Chimerism was confirmed by determining the phenotype of PBLs by flow cytometry prior to experiments and then confirmed at each time point.

Enumeration of inoculated Pneumocystis *organisms*

To prepare organisms for inoculation, lungs were removed from *Pneumocystis*-infected $Rag2^{-/-}$ or SCID mice, pushed through stainless steel mesh in HBSS, and debris was removed by centrifugation at 100 × *g* for 2 min. Aliquots of lung homogenates were spun onto glass slides, fixed with methanol, and stained using DiffQuik (Dade International). The number of *Pneumocystis* nuclei present were counted using microscopy. To infect mice, animals were anesthetized lightly with isoflurane gas, and 10^5–10^7 *Pneumocystis* organisms, depending on the experiment, were injected intratracheally (i.t.) in 100 μl HBSS. Organisms were freshly isolated for all of the experiments shown. For detection of *Pneumocystis* burden, the right lung lobes of each animal were excised, minced, and digested in RPMI 1640 supplemented with 2% FCS, 1 mg/ml collagenase A, and 50 U/ml DNase for 1 h at 37°C. Digested fractions were pushed through mesh screens, and aliquots were spun onto glass slides and stained with DiffQuik for microscopic enumeration. Lung burden is expressed as $\log_{10}$ *Pneumocystis* nuclei per right lung lobes, and the limit of detection was 3.23.

Isolation of cells from alveolar space, lungs, and LN

Mice were killed by exsanguination under deep anesthesia. The lungs were lavaged with 5× 1-ml washes using HBSS containing 3 mM EDTA. Bronchoalveolar lavage fluid (BALF) was obtained from the first wash by spinning out cells and was retained for subsequent analyses. Cells from the first wash were added to the remaining washes and volumes were adjusted for cell enumeration. Lungs were minced and digested in RPMI 1640 containing 3% heat-inactivated FCS, 50 U/ml DNase (Sigma-Aldrich, St. Louis, MO), and 1 mg/ml collagenase A (Sigma-Aldrich) for 1 h at 37°C prior to pushing through mesh screens to acquire single-cell suspensions. After removing an aliquot for enumeration of *Pneumocystis* organisms as described, erythrocytes were lysed from the lung digests by exposure to a hypotonic buffer. The remaining single-cell suspensions were washed and counted. Tracheobronchial LN (TBLN) were excised and pushed through mesh screens to create single-cell suspensions. Erythrocytes were lysed and cells enumerated.

Adoptive transfer of $CD4^+$ T cells

Donor WT (BALB/c and C57BL/6) and B cell–deficient $Jh^{-/-}$ or μMT mice were given i.t. inoculations of 10^7 *Pneumocystis* organisms. Draining LN were isolated 10–14 d postinfection. $CD4^+$ cells were isolated from the TBLN by employing negative selection columns from R&D Systems according to the manufacturer's instructions. We routinely obtained >95% pure $CD4^+$ T cell fractions as determined by flow cytometry. Adoptive host $Rag^{-/-}$ mice received retro-orbital injections of 10^5 purified $CD4^+$ cells and 4 d later were infected i.t. with 10^5–10^6 *Pneumocystis* organisms, depending on the experiment. Note that mice on a C57BL/6 or BALB/c background were used for these experiments because of the availability of the $Rag2^{-/-}$ and $Jh^{-/-}$ mice bred in our colony. We had performed similar experiments using mice from both backgrounds and obtained similar results (14, 17).

Flow cytometric analysis of lung and LN lymphocytes

Lung lavage, lung digest, and TBLN cells were washed in PBS containing 0.1% BSA and 0.02% sodium azide and stained with appropriate concentrations of fluorochrome-conjugated Abs specific for murine CD4, CD8, CD19, CD44, CD62L, CD86, and CD80. Abs were purchased from eBioscience or BD Biosciences. Expression of these molecules on the surface of lymphocytes was determined by multiparameter flow cytometry using a FACSCalibur cytofluorimeter (BD Biosciences) and analyzed using either WinList (Verity Software House) or FlowJo (Tree Star) software.

Generation of and treatment with Pneumocystis *antisera*

B cell–deficient μMT mice were infected with 10^7 *Pneumocystis* organisms. Three days later and twice per week thereafter, mice were given i.p. injections of 100 μl sera collected and pooled from *Pneumocystis*-infected C57BL/6 or μMT mice. Pooled antisera were stored for up to 2 y at –80°C and were not heat treated. Alternatively, *Pneumocystis* organisms were incubated in *Pneumocystis*-immune or μMT sera from *Pneumocystis*-infected mice (diluted 1:5) for 30 min on ice and then washed prior to i.t. inoculation into recipient mice.

Depletion of B cells

B cells were depleted from BALB/c or C57BL/6 mice by injecting them i.p. with 10 mg/kg 18B12 anti-mouse CD20 IgG2a Ab (Biogen Idec) either 2 d before or 2–3 d after being infected i.t. with 10^7 *Pneumocystis* organisms (20). Control groups alternatively received 10 mg/kg 2B8 msIgG2a anti-human CD20 Ab. Mice treated with anti-mouse and anti-human CD20 Ab after infection continued to receive doses of Ab (10 mg/kg) every 7 d until sacrifice, whereas mice receiving the Ab before infection received only the single dose.

Analysis of Pneumocystis*-specific Ab*

Blood obtained at euthanasia by severing the abdominal aorta was clotted and centrifuged at 400 × *g* to obtain sera, which were frozen in aliquots at –80°C for subsequent analysis. A sonicate of *Pneumocystis* organisms (10 μg protein/ml) was coated onto 96-well plates and then plates were blocked with 5% dry milk in HBSS containing 0.05% Tween 20 as previously described

(21, 22). After extensive washing, sera and BALF samples (first washes) were diluted and incubated on plates overnight. Plates were extensively washed and bound IgG, IgM, or IgA was detected using alkaline phosphatase–conjugated anti-mouse IgG, IgM, or IgA (Sigma-Aldrich) followed by incubation with *p*-nitrophenyl phosphate at 1 mg/ml in diethanolamine buffer. OD was read at 405 nm using a plate reader equipped with KC Junior software (Bio-Tek Instruments, Winooski, VT).

Statistical analysis

Differences between experimental groups were determined using ANOVA, followed by a Dunn post hoc test where appropriate. Differences were considered statistically significant when $p < 0.05$. SigmaStat statistical software (SPSS) was used for all analyses.

Results

Ag-specific BCR is necessary for clearance of Pneumocystis

To better define the role of B cells in host defense against *Pneumocystis*, we sought to determine whether B cells must express an Ag-specific BCR or be able to secrete Ab to clear *Pneumocystis*. To address whether B cells specific for *Pneumocystis* are required for clearance, we transferred BM from BCR transgenic MD4 mice into irradiated μMT B cell–deficient mice to generate chimeras that contain B cells specific for a single irrelevant Ag (hen egg lysozyme). As controls, we transferred WT BM or μMT BM into the irradiated μMT hosts to generate mice with a complete B cell repertoire (WT chimeras) or mice lacking B cells (μMT chimeras). Two months after reconstitution, we i.t. infected MD4, WT, and μMT chimeras with 10^7 *Pneumocystis* organisms. In one experiment the MD4 chimeras did not clear *Pneumocystis* (Fig. 1A) and had *Pneumocystis* burdens that were indistinguishable from the chimeras lacking B cells. In a second experiment, *Pneumocystis* lung burden was significantly reduced in the MD4 chimeras compared with the μMT chimeras; however the MD4 chimeras, unlike the WT chimeras, were unable to clear the *Pneumocystis* (Fig. 1B). Next, we examined $CD4^+$ cell population dynamics over time after infection in the BALF (Fig. 1C), TBLN (Fig. 1D), and lung digest (Fig. 1E) samples from the chimeras.

Although there were no significant differences among groups of mice in numbers of $CD4^+$ or $CD8^+$ T cells in the BALF, there was a reproducible trend toward lower numbers of $CD4^+$ T cells in the BAL and lung digest at the earlier time points in the μMT and MD4 chimeras. Serum concentrations of *Pneumocystis*-specific IgG Ab are shown over time after infection in Fig. 1F. Neither the μMT chimeric nor the MD4 chimeric mice mounted Ab responses against *Pneumocystis*, as expected, whereas the WT chimeras had detectable specific Ab by day 10 postinfection, with a peak response by day 20. Fig. 1G demonstrates that μMT recipients were appropriately reconstituted with B cells expressing either the IgM^a allotype (WT chimeras), IgM^b (MD4 chimeras), or no B cells at all (μMT chimeras).

Pneumocystis-specific serum and BALF IgM and IgA were assessed at each time point as well. Very low levels of *Pneumocystis*-specific IgA or IgM were detected in either serum or BALF samples for the μMT and MD4 chimeric mice, as dilutions of 1:10 caused the signal to be undetectable (Supplemental Fig. 1). Taken together, these data indicate that the lack of *Pneumocystis*-specific Ab production decreases the ability of the animal to efficiently clear *Pneumocystis*, suggesting that Ag-specific B cells play a role in providing protection following *Pneumocystis* infection.

Absence of secreted Ab impairs clearance of Pneumocystis*, T cell activation*

It has long been known that specific Ab can effectively reduce the burden of *Pneumocystis* in murine models of infection (23, 24). To eliminate the Ab-secreting function of B cells and determine whether B cells can provide protection from *Pneumocystis* independently of *Pneumocystis*-specific secreted Ab, we used AID-$\mu S^{-/-}$ transgenic mice. The AID-$\mu S^{-/-}$ mice cannot class switch or secrete Ab (25) but they do exhibit competent BCR surface expression with a reduced repertoire (18). AID-$\mu S^{-/-}$ and WT (C57BL/6) mice were infected with 10^7 *Pneumocystis* organisms i.t. As shown in Fig. 2A, AID-$\mu S^{-/-}$ mice did not clear *Pneumocystis* by day 32 postinfection, in contrast to the WT mice, which were able to reduce the *Pneumocystis* burden to below the limit of detection by day 21 postinfection. To confirm that the AID-$\mu S^{-/-}$ mice cannot class switch or secrete Ab, we measured IgG titers in the BALF through day 32 postinfection in these mice. As expected, the *Pneumocystis*-specific IgG titers remained at background levels throughout the 32-d time course (Fig. 2B). Interestingly, the AID-$\mu S^{-/-}$ mice had increased numbers of B cells in the lung digest as compared with the WT control animals, with the difference on day 14 postinfection reaching statistical significance (Fig. 2C). Additionally, a larger proportion of B cells in the lung digest of the knockout animals expressed the activation markers CD80 and CD86 as compared with the WT animals (Fig. 2D).

Because our earlier data suggested that class-switched B cells and Abs are not required for *Pneumocystis* clearance and that $CD4^+$ T cells primed in a normal host are sufficient to induce clearance of *Pneumocystis* when adoptively transferred into SCID mice, we hypothesized that the inability of the AID-$\mu S^{-/-}$ mice to clear *Pneumocystis* may be due to a role for Ab on $CD4^+$ cell activation. We therefore examined the characteristics of the $CD4^+$ T cell infiltration into the infected lungs of these mice. At day 7, alveolar infiltration by $CD4^+$ T cells was evident in both mouse strains, but by day 21 the AID-$\mu S^{-/-}$ mice had higher $CD4^+$ cell infiltration that persisted at higher levels through day 32 postinfection (Fig. 2E). Despite this, the percentage of $CD4^+$ T cells in the alveolar space that were activated ($CD44^{hi}CD62L^{lo}$) was reduced over time after infection as compared with the WT strain, with the difference becoming more pronounced late in the response (Fig. 2F). Additionally, the concentrations of TNF-α and IFN-γ in the lavage fluid were lower from days 14–25 postinfection (Fig. 2G, 2H) in the AID-$\mu S^{-/-}$ mice. Taken together, these data suggest that secreted Ab may be required for T cells to reach normal levels of activation in response to *Pneumocystis* infection and facilitate efficient organism elimination from the lungs of the infected host.

Ab can partially rescue B cell–deficient mice and facilitate control of Pneumocystis

Because we found that specific B cells and secreted Ab are necessary for clearance of *Pneumocystis*, we asked whether Ab can control *Pneumocystis* infection in the absence of B cells. μMT mice were infected with *Pneumocystis* and then given twice-weekly injections of serum either from infected C57BL/6 donors or from infected μMT donors. As shown in Fig. 3A and 3B, B cell-deficient mice given passive immunization with serum from *Pneumocystis*-infected WT donors had a significantly lower *Pneumocystis* burden at day 21 postinfection than did B cell–deficient mice given sera from *Pneumocystis*-infected μMT mice. Immunization using serum from naive mice did not affect the clearance of *Pneumocystis* from μMT mice through day 28 postinfection (data not shown). We used a second approach of incubating *Pneumocystis* organisms with either immune serum from *Pneumocystis*-infected C57BL/6 mice or from infected μMT mice prior to instillation in the lungs. Mice that received Ab-opsonized organisms had a lower lung burden during the day 10 and day 20 time points than did the mice that received *Pneumocystis* incubated with serum from B cell–deficient μMT mice (Fig. 3B). *Pneumocystis*-specific Ab isotypes were assessed from the serum of infected mice, and most were shown to be IgG,

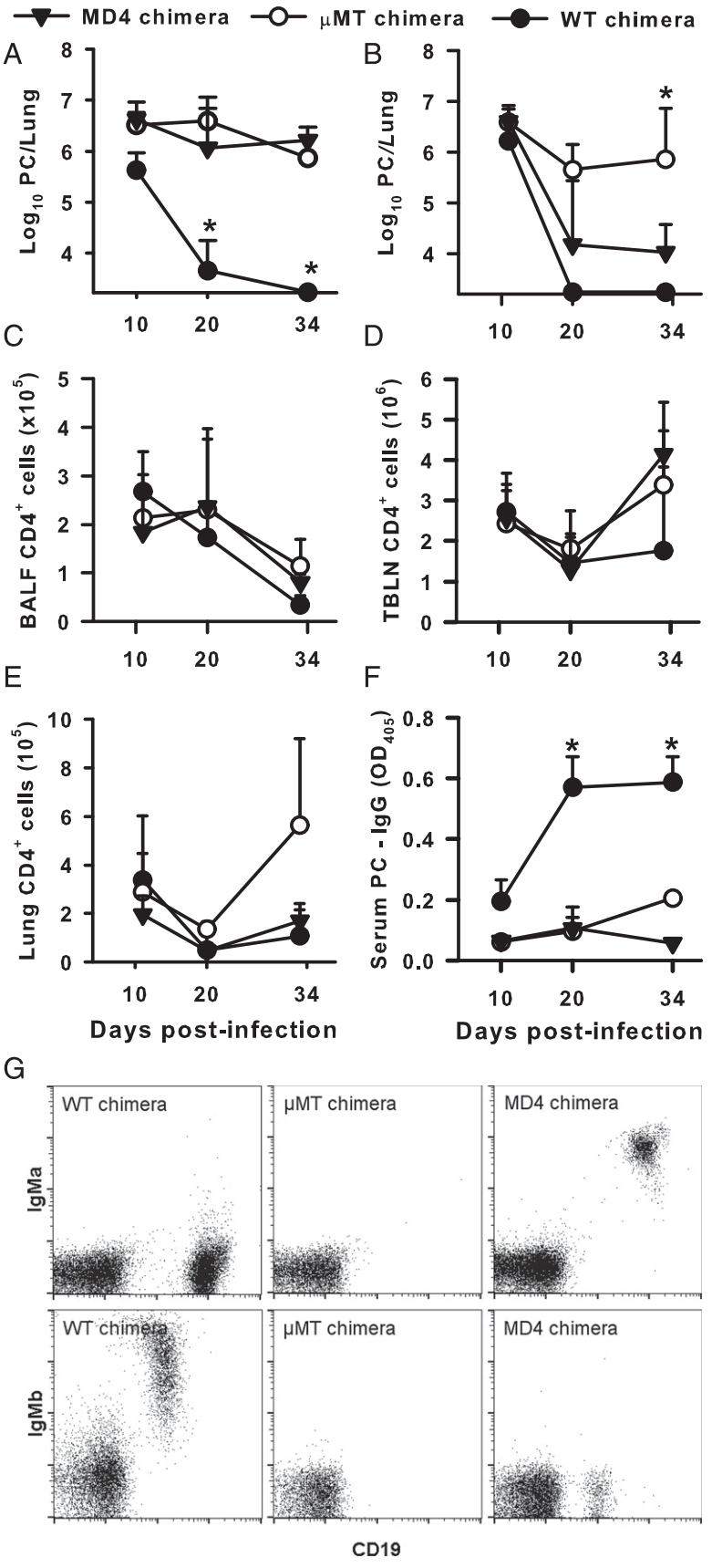

FIGURE 1. Ag-specific BCR is necessary for clearance of *Pneumocystis*. BCR transgenic MD4 mice and B cell–deficient μMT mice were used to generate mixed chimeras whose BCR was specific for hen egg lysozyme. μMT mice were irradiated and then reconstituted with a mix of BM cells from MD4 transgenic and WT (C57BL/6) animals, as described in *Materials and Methods*, to generate WT chimera, μMT chimera, and MD4 chimera groups. After reconstitution, mice were infected with *Pneumocystis* organisms and then humanely killed at postinfection time points. (**A** and **B**) *Pneumocystis* lung burden was assessed in lung digest samples and expressed as $\log_{10}$ *Pneumocystis* organisms per lung in two replicated experiments. (**C**–**E**) T cells were identified by CD4 staining using flow cytometry and enumerated in the BALF (C), TBLN (D), and digested lung (E). (**F**) *Pneumocystis*-specific IgG in the serum was assessed by ELISA. (**G**) Flow cytometry dot plots for representative samples depicting chimerism of each strain. B cells from C57BL/6 mice express IgM^b whereas MD4 mice express the IgM^a allotype. Data are presented as mean ± SD for groups of four to six mice per time point per group and are representative of two replicated experiments. Note that in (A) mice died in the μMT chimera and MD4 chimera groups between days 20 and 34 postinfection, mostly likely due to the high organism burden. $*p < 0.05$ for data compared with WT chimeras at each time point.

with only trace amounts of *Pneumocystis*-specific IgM present (Supplemental Fig. 2). Importantly, despite lowering *Pneumocystis* burden in the lung, the presence of Ab, either injected or through preopsonization, did not result in clearance of organisms within 3 wk as we routinely see in WT mice (Figs. 1, 2). The infiltration of $CD4^+$ T cells into the alveolar spaces was not altered by either the treatment with *Pneumocystis* immune serum or by infection with opsonized *Pneumocystis* (Fig. 3C, 3D), nor were the activation

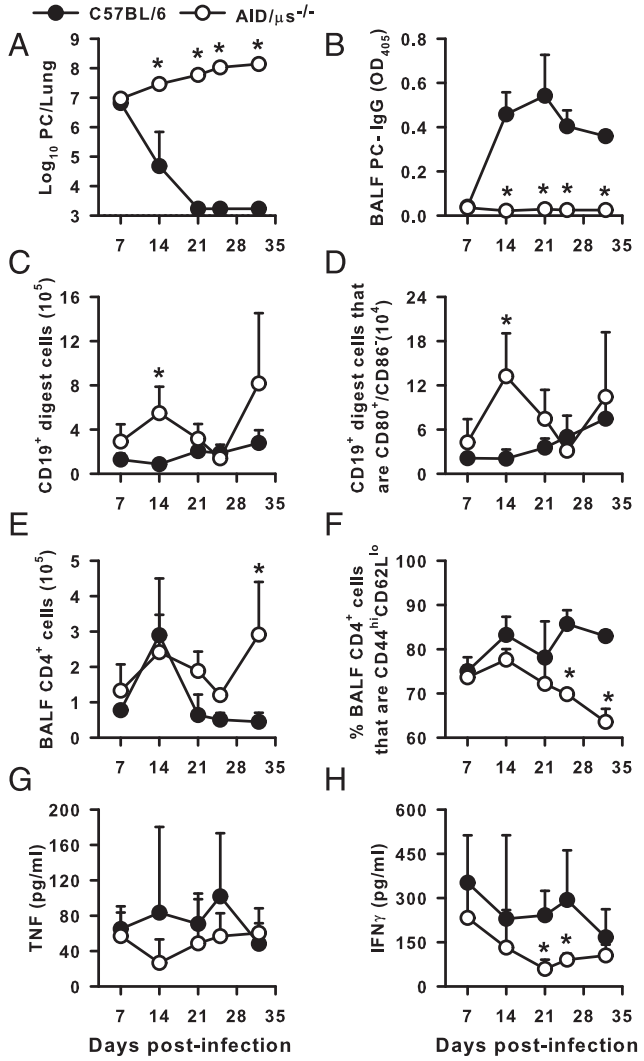

FIGURE 2. Failure to clear *Pneumocystis* infection in the absence of secreted Ab. AID-$\mu S^{-/-}$ mice, which are unable to secrete or class switch Ab, and WT (C57BL/6) mice were infected with *Pneumocystis* organisms. Mice were sacrificed at postinfection time points, and BALF, lung, and TBLN specimens were collected for analysis. (**A**) *Pneumocystis* lung burden clearance kinetics over time after infection, expressed as $\log_{10}$ *Pneumocystis* organisms per lung. (**B**) *Pneumocystis*-specific IgG titers in the BALF confirming that the AID-$\mu S^{-/-}$ mice cannot secrete Ab. Numbers of (**C**) B cells ($CD19^+$) and (**D**) activated B cells ($CD19^+CD80^+CD86^-$) depicted in lung digest samples over time after infection. The number of $CD4^+$ T cells (**E**) and the percentage of $CD4^+$ T cells that were $CD44^{hi}CD62L^{lo}$ (**F**) in the BALF over time after *Pneumocystis* infection are shown. The concentrations of TNF-α (**G**) and IFN-γ (**H**) in BALF over time after infection are shown. Data are presented as mean ± SD for groups of four mice per time point per group. $*p < 0.05$ for data compared with WT mice at each time point.

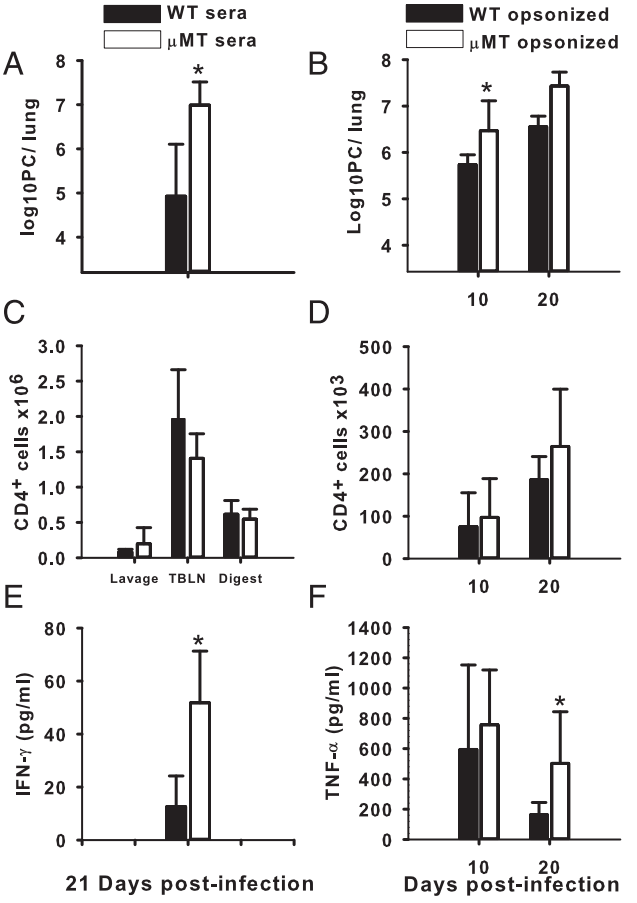

FIGURE 3. Exogenous *Pneumocystis*-specific Ab in B cell–deficient mice partially protects against infection. B cell–deficient mice (μMT) were infected with *Pneumocystis* and then given twice-weekly injections of serum either from infected C57BL/6 donors or from infected μMT donors (**A**, **C**, and **E**) or were infected with *Pneumocystis* preopsonized with antiserum from infected C57BL/6 or μMT donors (**B**, **D**, and **F**). (A and B) *Pneumocystis* lung burden was assessed in lung digest samples and expressed as $\log_{10}$ *Pneumocystis* organisms per lung. (C and D) T cells were identified by CD4 staining using flow cytometry and enumerated in the BALF, digested lung, and TBLN. (E and F) Concentrations of IFN-γ and TNF-α in the BALF were assessed by ELISA. Data are presented as mean ± SD for groups of five mice per time point per group. $*p < 0.05$ for data compared with WT mice at each time point.

levels different (data not shown). However, the levels of IFN-γ in the BALF of mice given WT immune serum (Fig. 3E) or TNF in the BALF of mice infected with *Pneumocystis* serum–opsonized organisms (Fig. 3F) were significantly lower at 3 wk postinfection than in mice without specific Ab.

B cell–T cell interactions are important in the priming phase

We previously found that the presence of B cells during the first 2 wk of infection with *Pneumocystis* was sufficient for expansion of $CD4^+$ T cells in adoptive hosts (14, 17), so we sought to determine whether B cells are critical early during infection at the time $CD4^+$ T cells are primed. To address this, B cells were depleted in WT mice using anti-CD20 Ab (Fig. 4). Anti-mouse CD20 Ab (18B12) was injected i.p. 3 d after i.t. infection of BALB/c mice with 10^7 *Pneumocystis* organisms, and every 7 d thereafter. Administration of this Ab was compared with exposure to an anti-human CD20 Ab as a control. The anti-mouse CD20 Ab was effective at depleting nearly all $CD19^+$ lymphocytes (B cells) in the draining LN on days 16 ($p < 0.001$) and 32 ($p = 0.029$) postinfection (Fig. 4A). Additionally, treatment with anti-mouse CD20 significantly reduced *Pneumocystis*-specific IgG concentrations in both the BALF and in the serum postinfection as compared with the anti-human CD20 control treatment (Fig. 4B, 4C). Although there was a small decrease in the number of $CD4^+$ T cells infiltrating the alveolar space on day 16 postinfection in the B cell–depleted group, the difference did not reach statistical significance ($p = 0.285$) (Fig. 4D). We also observed a nonsignificant decrease in IFN-γ concentration on day 32 postinfection in the B cell–depleted animals (Fig. 4F), despite a significantly higher *Pneumocystis* burden ($p = 0.018$) at that time point (Fig. 4E). Although the *Pneumocystis* burden was higher on day 32 postinfection in the group that received anti-mouse CD20 Ab,

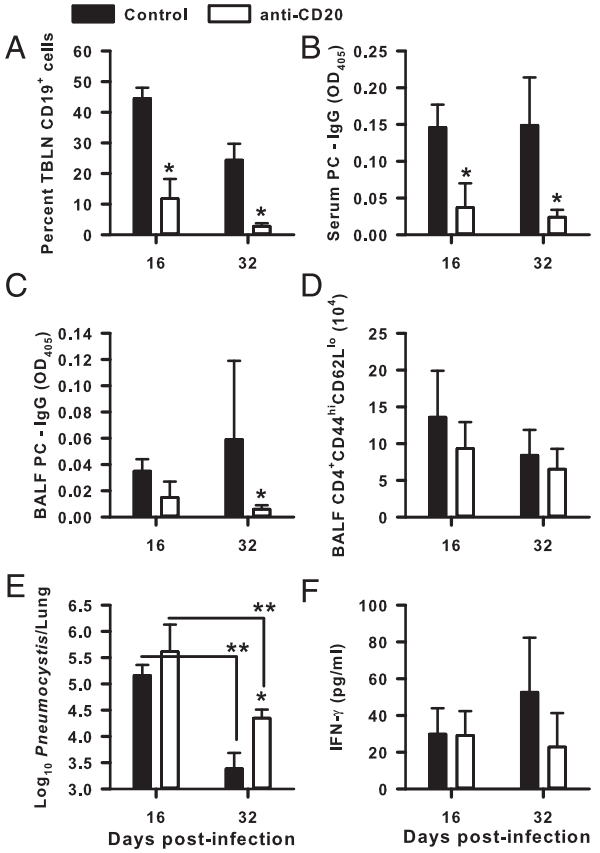

FIGURE 4. Depletion of B cells after infection hinders specific IgG production and clearance of *Pneumocystis*. BALB/c mice were infected with *Pneumocystis* organisms. Anti-mouse CD20 Ab was injected i.p. at a dose of 10 mg/kg starting on day 3 postinfection and every 7 d thereafter. Control mice received anti-human CD20 Ab at the same dose. Mice were sacrificed on days 16 and 32 postinfection, and BALF, lung, and TBLN specimens were collected for analysis. Lungs were homogenized and digested, and TBLN were pushed though mesh to create single-cell suspensions. B cell populations were defined by CD19 expression, and T cells were identified by CD4, CD44, and CD62L staining using flow cytometry. *Pneumocystis*-specific IgG and IFN-γ concentrations were assessed by ELISA, and *Pneumocystis* lung burden was assessed in lung digest samples. (**A**) Percentage of TBLN cells that were $CD19^+$ over time. *Pneumocystis*-specific IgG titers in the serum (**B**) and BALF (**C**) over time postinfection are shown. (**D**) Percentage of $CD4^+$ T cells in the BALF that were activated, defined as $CD44^{hi}CD62L^{lo}$. (**E**) The *Pneumocystis* lung burden over time after infection, expressed as $\log_{10}$ *Pneumocystis* organisms per lung. (**F**) IFN-γ concentrations in the BALF samples after infection. Data are presented as mean ± SD for groups of four mice per time point per group. $^*p < 0.05$ for data compared with control group at the same time point, $^{**}p < 0.05$ for data compared between time points within the same treatment group (both p values determined using ANOVA and a Dunn post hoc test).

both groups reduced *Pneumocystis* significantly as compared with the burdens on day 16 postinfection.

Next, we compared the effects of B cell depletion either 2 d before or 2 d postinfection to confirm the timing of when B cells are required for clearance of *Pneumocystis*. Normal BALB/c mice were treated with anti-mouse CD20 either 2 d before infection or every 7 d starting 2 d postinfection with 10^7 *Pneumocystis* organisms. Depleting B cells 2 d before infection impaired the clearance of *Pneumocystis* to a more significant degree as compared with mice whose B cells were depleted after inoculation (Fig. 5A). *Pneumocystis* burden in the mice receiving anti-mouse CD20 prior to infection was as high on day 16 postinfection as in $Jh^{-/-}$ mice that lacked B cells altogether. By day 31 postinfection both sets of anti-mouse CD20-treated mice were able to clear the organisms to levels of those of the control group (Fig. 5A). Depletion either before or after *Pneumocystis* infection resulted in very low numbers of B cells in the draining LN (Fig. 5B). *Pneumocystis*-specific IgG was decreased to a greater degree on day 16 postinfection in the mice receiving B cell depletion before infection, although mice with depleted B cells after infection also had a significantly lower *Pneumocystis*-specific IgG concentration in the serum and BALF on day 31 (Fig. 5C, 5D). Additionally, there were very low levels of *Pneumocystis*-specific IgM present in the mice that received anti-CD20 Ab at day 16 postinfection, with a small amount of recovery at the later time point (Supplemental Fig. 3). Consistent with the results presented in the previous figure, mice depleted of B cells with anti-CD20 had similar numbers of activated $CD4^+$ T cells, as defined by CD44 and CD62L expression, in the BALF compared

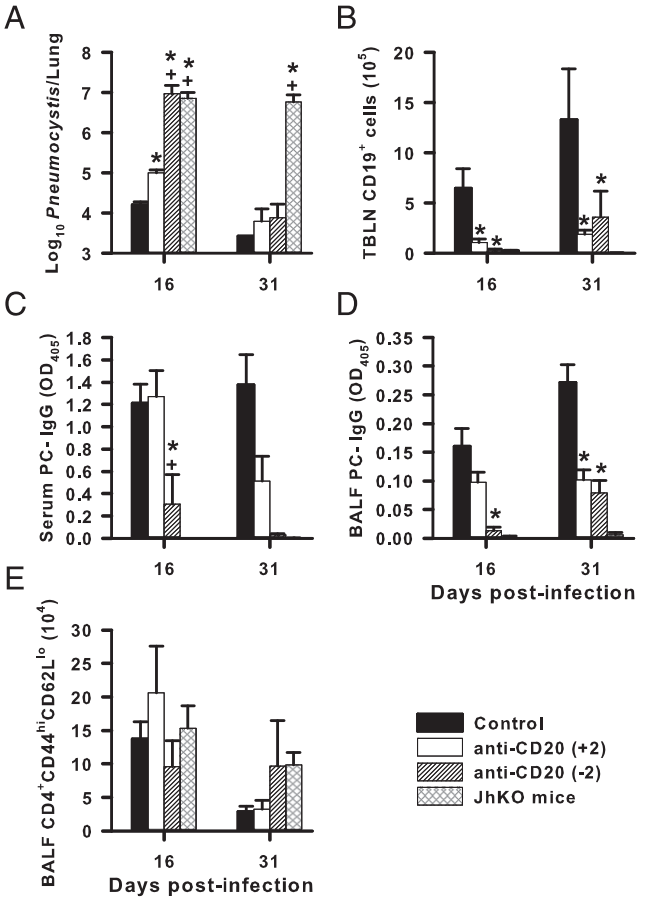

FIGURE 5. Depletion of B cells by anti-CD20 Ab prior to infection impedes the early clearance of *Pneumocystis* as well as Ab responses. BALB/c mice were infected with 10^7 *Pneumocystis* organisms. To deplete B cells, mice received either a one-time i.p. injection of 10 mg/kg anti-mouse CD20 Ab 2 d before infection (−2), or a series of injections every 7 d starting at day 2 postinfection (+2). Control groups consisted both of BALB/c mice that received anti-human CD20 Ab and $Jh^{-/-}$ mice that lack B cells. (**A**) The *Pneumocystis* lung burden at each time point postinfection, expressed as $\log_{10}$ *Pneumocystis* organisms per lung. (**B**) Depletion of B cells ($CD19^+$) in the TBLN depicted over time postinfection. Serum (**C**) and BALF (**D**) *Pneumocystis*-specific IgG levels at postinfection time points assayed at a 1:10 dilution and neat, respectively, are shown. (**E**) Number of $CD4^+$ T cells that were activated ($CD44^{hi}CD62L^{lo}$) in the BALF after infection. Data are presented as mean ± SD for groups of four mice per time point per group. $^*p < 0.05$ using ANOVA and a Dunn post hoc test for data compared with control group, $^+p < 0.05$ to the +2 anti-CD20 treatment group.

with mice with an intact B cell compartment at the time of infection (Fig. 5E).

T cells primed in the absence of B cells fail to expand upon adoptive transfer

Data to this point confirm that Ag-specific B cells are required for host defense against *Pneumocystis* and that *Pneumocystis*-specific Ab does not completely compensate for the absence of B cells. We have previously demonstrated that $CD4^+$ T cells primed for 14 d in the absence of B cells fail to expand and control *Pneumocystis* infection upon adoptive transfer to immunodeficient SCID recipients (14). Because dendritic cells (DCs) are thought to be the most important cells for presenting Ag to naive T cells, we asked the question of whether B cells are required early after infection to prime $CD4^+$ T cells. To address this we isolated $CD4^+$ T cells during the first 3 d postinfection to determine whether they would clear *Pneumocystis* in adoptive hosts. Donor WT BALB/c or B cell–deficient $Jh^{-/-}$ mice were infected with *Pneumocystis*, and $CD4^+$ T cells were isolated from the draining TBLN at 3 or 14 d postinfection. T cells were injected into $Rag^{-/-}$ recipients followed by infection 4 d later. As shown in Fig. 6, $CD4^+$ T cells from $Jh^{-/-}$ mice failed to expand in the TBLN, lungs, or alveolar spaces of adoptive hosts and failed to control lung *Pneumocystis* burden. In contrast, $CD4^+$ T cells isolated from WT mice did control infection (Fig. 6D). Importantly, $CD4^+$ cells from mice infected for 3 d were as good as or better than those from mice infected for 2 wk in terms of expansion or migration to the lungs. These data suggest that B cells at the initial priming stage confer some sort of survival or proliferation signal to $CD4^+$ T cells, which is required for expansion upon secondary challenge.

Ag-specific BCR is required for adequate priming of T cells

We demonstrated in Fig. 2 that an Ag-specific BCR is required for complete clearance of *Pneumocystis*. The next experiments were designed to determine whether T cells primed in mice that lacked *Pneumocystis*-specific BCR would be able to confer *Pneumocystis* clearance upon adoptive transfer into $Rag1^{-/-}$ hosts. WT, μMT, and MD4 chimeras were infected with *Pneumocystis*, and after 3 d T cells were isolated and adoptively transferred into $Rag1^{-/-}$ mice, which were infected with *Pneumocystis* 4 d later. By day 28 postinfection, the mice receiving transferred cells from the WT chimeras began to clear the infection (Fig. 7A). Fig. 7B shows that the number of $CD4^+$ T cells as well as the number of activated $CD4^+$ T cells, based on surface protein expression and IFN-γ expression, tended to be lower when primed in MD4 chimeras as compared with WT and μMT chimeras. Interestingly, the mice that received cells primed in the μMT chimeric animals did show expansion of T cells, and these T cells did show signs of activation (Fig. 7B). However, transfer of these cells could not confer control of *Pneumocystis* (Fig. 7A). These results are similar to those that we published previously showing that T cells from μMT mice can expand and become activated, but are also unable to clear the pathogen (17). These results demonstrate that the mere presence of B cells is not sufficient for efficient priming of T cells. Rather, *Pneumocystis*-specific B cells are required for the early priming of T cells against *Pneumocystis*.

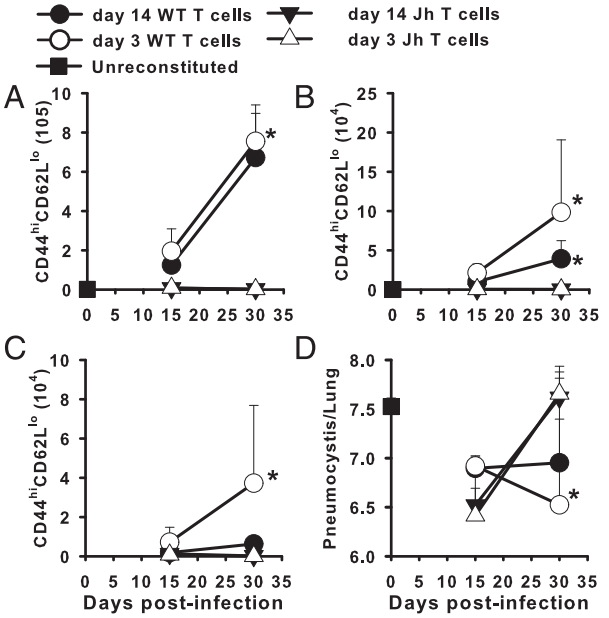

FIGURE 6. $CD4^+$ T cells primed for 3 d in B cell–sufficient donors are capable of affecting clearance of *Pneumocystis* in adoptive $Rag^{-/-}$ hosts. Donor WT BALB/c or B cell–deficient $Jh^{-/-}$ mice were infected with *Pneumocystis* and $CD4^+$ T cells were isolated from the draining TBLN at 3 or 14 d postinfection. T cells were adoptively transferred into $Rag^{-/-}$ recipients followed by infection with *Pneumocystis* nuclei 4 d later. T cells were identified by CD4, CD44, and CD62L staining using flow cytometry, with the number of activated $CD4^+$ T cells ($CD44^{hi}CD62L^{lo}$) depicted over time in the (**A**) lung digest, (**B**) BALF, and (**C**) TBLN. (**D**) *Pneumocystis* lung burden was assessed in lung digest samples and expressed as $\log_{10}$ *Pneumocystis* organisms per lung. Data are presented as mean ± SD for groups of four mice per time point per group and are representative of three replicated experiments. $*p < 0.05$ for data generated in the $Jh^{-/-}$ recipients compared with recipients of T cells from WT mice at each time point.

Discussion

There are increasing reports that *Pneumocystis* colonizes the lungs of individuals with immunosuppressing conditions such as HIV infection and chronic obstructive pulmonary disease, corresponding with worsening pathology (26, 27). Although much is known about the elements of the immune response necessary for host defense against *Pneumocystis*, there is still a lack of understanding of how these immune factors interact with each other. We have previously reported that B cells provide expansion and/or survival signals to $CD4^+$ T cells that contribute significantly to their function (11, 14). In the data presented in the present study, we demonstrate that B cells need to be present during the first few days after infection to provide signals to T cells and for an optimal Ab response. We further confirmed that B cells need to have a BCR with specificity to *Pneumocystis* and be able to secrete Ab to clear the infection. Taken together, these data indicate that B cells are multifunctional in the response to *Pneumocystis*. Specific Ab production is important for control of *Pneumocystis* lung burden. Consistent with our previously published data showing that mice with B cells that do not express MHC class II do not clear *Pneumocystis* (14), we found in the present study that B cells are required early during the response to *Pneumocystis*, suggesting a role in the initial priming of T cells.

Although it is important to define these basic immunologic functions, the importance of the interaction between B cells and T cells is underscored by the clinical observation that B cell depletion with anti-CD20 Ab therapies impairs T cell activation (28, 29). This is associated with an increased incidence of PCP (8–10), in addition to other T cell–mediated infections, including hepatitis B (30, 31). Stroopinsky et al. (32) examined this phenomenon and in 2012 reported that activation of T cells isolated from lymphoma patients treated with rituximab was reduced as defined by ex vivo cytokine secretion. Interestingly, this study also demonstrated direct inhibitory effects upon T cells with rituximab, indicating that clarity is needed as to the mechanism of this interaction (32).

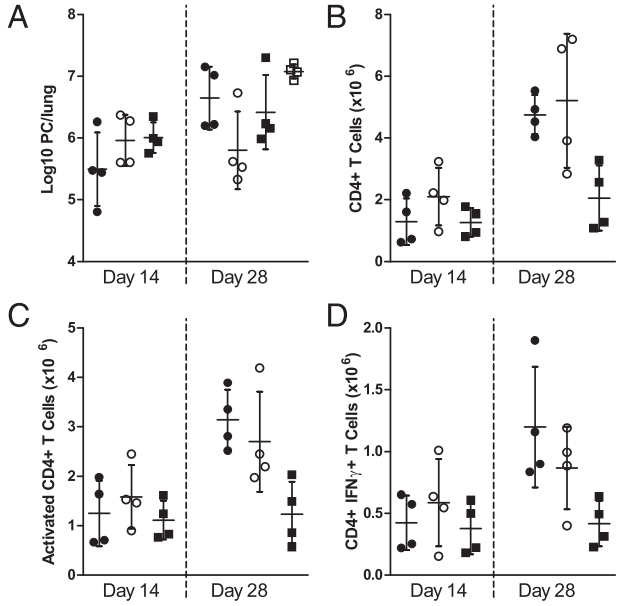

FIGURE 7. Ag-specific BCR is required for proper $CD4^+$ T cell priming. WT, μMT, and MD4 chimeras were infected with *Pneumocystis*, and after 3 d T cells were adoptively transferred into $Rag1^{-/-}$ mice, which were then infected with *Pneumocystis* 4 d later. Mice receiving T cells from μMT chimeras (●), WT (B6 + μMT) chimeras (○), MD4 chimeras (■), and mice that were unreconstituted (□) were then humanely killed at postinfection time points. (**A**) *Pneumocystis* lung burden was assessed in lung digest samples and expressed as $\log_{10}$ *Pneumocystis* organisms per lung on days 14 and 28 postinfection. The numbers of (**B**) $CD4^+$ T cells, (**C**) activated $CD4^+$ T cells (based on high CD44 and low CD62L expression), and (**D**) $CD4^+$ T cells producing IFN-γ (assessed via intracellular cytokine staining and flow cytometry) are depicted at the corresponding time points. Data are presented as mean ± SD along with symbols representing each individual mouse. No statistically significant differences were observed defined at a $p < 0.05$ using ANOVA and a Dunn post hoc test for data compared with the WT chimera (B6 + μMT) group at each time point.

We have previously reported that BM chimeric mice with B cells lacking MHC class II were unable to clear *Pneumocystis* infection, confirming that Ag presentation by B cells is important for production of class-switched Ab as well as activation of Th cells (14). In the present study, we determined that Ag-specific B cells are required for clearance of *Pneumocystis*. Transgenic mice expressing the irrelevant MD4 BCR specific for hen egg lysozyme were unable to clear infection, indicating that specific responses, and not merely a populated B cell compartment, is required for host defense against *Pneumocystis*. A similar result was reported in *Salmonella*-infected MD4 transgenic mice, although it was reported that the specific BCR was required for memory Th1 cell development (33). To determine whether specific Ab secretion is required for clearance of *Pneumocystis*, we used the AID-s$\mu^{-/-}$ mice that have B cells unable to class switch or secrete Ab. These mice also failed to clear *Pneumocystis*, which is consistent with a previous report in which s$IgM^{-/-}$ mice, able to class switch but not secrete IgM Ab, had a defective ability to control *Pneumocystis* infection (34). IgM in the absence of IgG is not sufficient for clearance of *Pneumocystis*, suggesting that complete B cell responses, including secreted IgG and IgM, work together to control *Pneumocystis* burden (11, 14). In the absence of class-switched IgG, as well as in the absence of IgM, *Pneumocystis* clearance does not occur, suggesting that both isotypes are important in this process. Importantly, the AID-$\mu s^{-/-}$ mice have a relatively normal repertoire of B cell receptors (18), and so these data confirm that secretion of specific Ab is critical for controlling *Pneumocystis* infection. Interestingly, the AID-$\mu s^{-/-}$ mice had reduced proportions of activated $CD44^{hi}CD62L^{lo}$ $CD4^+$ T cells in alveolar spaces, which corresponded to reduced BALF IFN-γ levels compared with WT mice.

The numbers of total and activated B cells in the lungs of AID-$\mu s^{-/-}$ mice were increased, suggesting that in the absence of secreted Ab and in the face of increasing lung *Pneumocystis* burden, T and B cells migrate to the lungs in increasing numbers to try to deal with the infection. Alternatively, it may be that AID-$\mu s^{-/-}$ mice cannot adequately control immune responses owing to a lack of Ab available to bind to inhibitory Fc receptors (35). Multiple groups, including us, have reported that passive immunization of immunodeficient mice with *Pneumocystis*-specific Ab reduces organism lung burden (3, 24, 36). We now show in the present study that injection of antiserum or infection with preopsonized *Pneumocystis* organisms significantly reduced the *Pneumocystis* lung burden in B cell–deficient μMT mice, but did not result in complete clearance of organisms by the end of the experiment. These data are consistent with the conclusion that although specific Ab plays an important role in reducing lung *Pneumocystis* burden, B cells also contribute to clearance of *Pneumocystis* via Ab-independent mechanisms.

We hypothesized that if B cells are important APCs, then the timing of the exposure of T cells to B cells would be important in activating $CD4^+$ T cells to effector cells. Adoptive transfer of $CD4^+$ T cells from WT or B cell–deficient mice at 3 d postinfection had little effect on clearance of *Pneumocystis* compared with the 14 d at which we usually take LN T cells for transfer (14). We had previously found that T cells isolated from mice that lack B cells ($Jh^{-/-}$) at day 14 postinfection failed to expand in adoptive immunodeficient hosts. Interestingly, 3 d postinfection was enough time for T cells from WT mice to become activated and to expand and migrate to the lungs to the same extent as did T cells isolated at day 14 postinfection. We did find that the number of $CD4^+$ T cells in the draining LN of the adoptive hosts that received cells from WT mice infected for 3 d was near the limit of detection, which was similar to what we saw in mice that received T cells from $Jh^{-/-}$ donors. It is possible that had we taken our time points out past 30 d postinfection, the cells from WT mice infected for 3 d would have become exhausted. This would be consistent with the possibility that B cells provide survival signals to $CD4^+$ T cells. We have preliminary data showing that a high proportion of $CD4^+$ T cells in *Pneumocystis*-infected B cell–deficient mice undergo apoptosis (data not shown), and we are working at determining whether this contributes to failure of these mice to clear infection.

Our adoptive transfer approach has the advantage of examining $CD4^+$ T cell function after priming with or without B cells in the absence of Ab, but it is not a physiological model. Utilizing anti-CD20, we were able to deplete the naive pool of B cells 2 d before infection, or alternatively 2 d postinfection, which allows for some specific B cell expansion. We found that depletion of B cells prior to infection had more serious consequences for clearance of *Pneumocystis* than did depletion 2 or 3 d postinfection. However, depletion of B cells prior to infection had only minor effects on $CD4^+$ T cell numbers in the BALF and a more significant effect on *Pneumocystis*-specific IgG than did depletion of B cells 2 d postinfection. This may imply that in the presence of DCs, naive $CD4^+$ T cells are not optimally primed and in the absence of specific Ab are thereby unable to effect clearance of *Pneumocystis*. We did not find a difference in the TNF or IFN-γ levels in the BALF between the groups, suggesting that B cell depletion had no effect on local cytokine responses. TNF can be made by multiple cell types in response to *Pneumocystis*, including T cells. IFN-γ was likely produced mostly by T cells, which suggests that the T cell effector responses are intact in the anti-CD20–treated mice. A recent report

showed that B cells are necessary for the generation of an optimal Th2 type of response to the intestinal nematode *Heligmosomoides polygyrus* (37). In mice treated with anti-CD20, T cells and DCs failed to colocalize, thereby affecting the development of a normal T cell response. Interestingly, the investigators used influenza as a control and found that in the primary Th1 response to the virus, B cells were dispensable, suggesting that the role of B cells in the priming phase may be specific to Th2-type responses that require Ab for clearance, much like *Pneumocystis* (6, 37).

One caveat to the B cell depletion experiments is that we did not assess whether B1 cells were depleted by the anti-CD20 treatment. Based on other models in the literature, it is likely that B1 cells were still present. In a study involving lupus-prone mice, anti-CD20 Ab treatment showed depletion of B1 cells ($CD11b^+IgM^+$) to be incomplete (38). The production of natural Abs from B1 cells could be contributing to the clearance of *Pneumocystis* in our mice. Rapaka et al. (34) demonstrated that mice lacking natural Abs specific for carbohydrate moieties present on fungi, including *Pneumocystis*, had defects in Ab isotype switching and T cell subset differentiation, resulting in a reduced ability to control infection. Our IgM and IgG isotype assessments at both time points by *Pneumocystis*-specific ELISA show very low levels of IgM present in the serum of mice on day 16 postinfection, with some recovery in both anti-CD20–treated groups on day 31. Despite the possibility of natural Ab being present, the mice undergoing anti-CD20 Ab depletion of B cells had a decreased ability to clear *Pneumocystis*. Our results are also corroborated by a recently published work that shows treatment with anti-CD20 Ab impaired the clearance of *Pneumocystis* infection, and in addition made $CD4^+$ T cells defective upon later transfer into $Rag1^{-/-}$ mice (39).

DCs have long been considered the professional APC required for optimal stimulation of naive T cells. This has been confirmed in other infection models using the CD11c-DTR mice. Depletion of DCs during the priming stage of infection with *Schistosoma mansoni* resulted in a significant reduction in Th2 cells and skewing toward a Th1 response (40). Depletion of DCs has also been shown to abrogate priming of $CD8^+$ cells in response to *Listeria monocytogenes* or *Plasmodium yoelii* (41) as well as to compromise the recall response to viral and bacterial pathogens (42). We have preliminary data suggesting that depletion of DCs just prior to *Pneumocystis* infection has no negative impact on expansion and activation of $CD4^+$ T cells. Our ongoing work will address whether B cells can compensate for the absence of DCs in this model.

The results presented in the present study confirm that B cells play a significant role in host defense against *Pneumocystis*. In addition to secreting specific Ab, B cells are involved in the expansion and survival of $CD4^+$ T cells, a function that can provide protection, potentially in the absence of DCs. This may infer that B cells have a nonredundant role in promoting presentation of *Pneumocystis* Ag to $CD4^+$ T cells in the process of responding to infection with *Pneumocystis*.

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Disclosures

The authors have no financial conflicts of interest.

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