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Clearance of *Pneumocystis carinii* in Mice Is Dependent on B Cells But Not on *P. carinii*-Specific Antibody[1]

Frances E. Lund,* Kevin Schuer,† Melissa Hollifield,† Troy D. Randall,* and Beth A. Garvy[2†‡§]

Both CD4$^+$ T cells and B cells are critical for defense against *Pneumocystis carinii* infection; however, the mechanism by which B cells mediate protection is unknown. We show that *P. carinii*-specific IgM is not sufficient to mediate clearance of *P. carinii* from the lungs since CD40-deficient mice produced normal levels of specific IgM, but were unable to clear the organisms. Using chimeric mice in which the B cells were deficient in CD40 (CD40KO chimeras) we found that clearance of *P. carinii* infection is delayed compared with wild-type controls. These CD40KO chimeric mice produced normal levels of *P. carinii*-specific IgM, but did not produce class-switched IgG or IgA. Similarly, clearance of *P. carinii* was delayed in mice deficient in FcγRI and III (FcγRKO), indicating that *P. carinii*-specific IgG partially mediates opsonization and clearance of *P. carinii*. Opsonization of organisms by complement did not compensate for the lack of specific IgG or FcγR, since C3-deficient and C3-depleted FcγRKO mice were still able to clear *P. carinii*. Finally, μMT and CD40KO chimeric mice had reduced numbers of activated CD4$^+$ T cells in the lungs and lymph nodes compared with wild-type mice, suggesting that B cells are important for activation of T cells in response to *P. carinii*. Together these data indicate that *P. carinii*-specific IgG plays an important, but not critical, role in defense against *P. carinii*. Moreover, these data suggest that B cells also mediate host defense against *P. carinii* by facilitating CD4$^+$ T cell activation or expansion. ***The Journal of Immunology,*** **2003, 171: 1423–1430.**

Pneumocystis carinii pneumonia continues to be a significant problem among immunosuppressed individuals, including patients with AIDS, genetic immunodeficiency diseases, and malignancies requiring chemo- or radiation therapy (1–4). It is widely known that CD4$^+$ T cells are required for clearance of *P. carinii* as CD4$^+$ T cell counts below 200/μl of blood predisposes individuals to *P. carinii* pneumonia (PCP)[3] (5). It has also been shown in murine models of PCP that the absence of CD4$^+$ T cells results in the inability to mount an effective host response to *P. carinii* (6–8). In addition to T cells, alveolar macrophages also play a significant role in defense against *P. carinii* as depletion of alveolar macrophages in rat lungs resulted in the inability to control *P. carinii* infection (9). However, alveolar macrophages, which are believed to be the effector cells that ultimately kill the organisms, are not sufficient for clearance of *P. carinii* since mice deficient in CD4$^+$ T cells have functional macrophages but are still susceptible to PCP (10–12).

Although it is widely accepted that both CD4$^+$ T cells and macrophages are involved in host defense against *P. carinii*, it has been underappreciated that B cells are also required for resolution of *P. carinii* infections. Indeed, several groups have now shown that B cell-deficient (μMT) mice are highly susceptible to *P. carinii* infection (11–13). Although it is not known how B cells contribute to the resolution of *P. carinii* infection, there have been a number of studies indicating that IgG Abs produced by B cells can mediate clearance of *P. carinii* (14–19). First, T cell-depleted mice with large amounts of circulating *P. carinii*-specific IgG either due to previous immunization or vaccination by *P. carinii* Ag-loaded dendritic cells resulted in clearance of organisms after a secondary challenge (14–16). Furthermore, passive immunoprophylaxis using a mAb specific for *P. carinii* was shown to protect immunodeficient animals from PCP (17–19). *P. carinii*-specific IgG is presumed to mediate the clearance of *P. carinii* by opsonizing the organisms which then targets them for phagocytosis via the Fcγ receptors expressed on the alveolar macrophages.

Although these previous studies showed that *P. carinii*-specific Ab can have a positive impact on host defense against PCP, adoptive transfer models have shown that in the total absence of B cells or Ab, *P. carinii*-specific CD4$^+$ effector cells are sufficient to induce the clearance of *P. carinii* from the lungs (8, 20). Thus, since it is still unclear whether Ab is necessary for clearance of primary *P. carinii* infection, it is still not known whether the protection provided by B cells is due to Ab production or to other B cell-dependent immune mechanisms. To directly test whether *P. carinii*-specific IgG is necessary for clearance of *P. carinii*, we have used a mixed bone marrow transplantation approach to generate mice whose B cells are unable to produce class-switched *P. carinii*-specific Ig. We report that chimeric mice that lack CD40 expression on B cells, but retain CD40 on other APC, do not produce *P. carinii*-specific IgG, but are able to clear *P. carinii* from the lungs, albeit with delayed kinetics. Likewise, we show that FcγRKO and C3-depleted FcγRKO mice have delayed resolution to *P. carinii* challenge compared to wild-type (WT) mice, yet eventually resolve the infection. These data suggest that Ab production by B cells promotes, but is not necessary for, the clearance of *P. carinii* from the lungs of infected mice. Furthermore, since B cells are obligate for resolution of *P. carinii* infection, these data strongly suggest that B cells contribute in Ab-independent ways to

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[3] Abbreviations used in this paper: PCP, *P. carinii* pneumonia; KO, knockout; TBLN, tracheobronchial lymph node; WT, wild type; L, ligand.

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the resolution of *P. carinii*, perhaps through Ag presentation, cytokine production, and/or interactions with T cells. In agreement with this hypothesis, we found that $CD4^+$ T cell activation and/or expansion in *P. carinii*-infected B cell-deficient mice was significantly reduced compared with normal animals. Thus, B cells appear to play multiple roles in the resolution of *P. carinii* infection.

Materials and Methods

Mice

Adult C57BL/6J, B6.129S2-Tnfsf5^{tm1Imx} (CD40KO), B6.129S4-C3^{tm1Crr} (C3KO), and B6.129S2-Igh-6^{tm1Cgn} (μMT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Cby.129(B6)-FcerIg^{tm1} (FcγRKO) mice were obtained from Taconic Farms (Germantown, NY) and BALB/cBy mice were obtained from the National Cancer Institute. C3-deficient BALB/c mice were generated by i.p. injection of 25 μg of cobra venom factor from *Naja naja* (Sigma-Aldrich, St. Louis, MO) every 3 days as has been previously described (21). For some experiments, chimeric mice were generated as described below at the Trudeau Institute and shipped to the University of Kentucky before infection with *P. carinii*. All experimental mice were housed in the Lexington, KY Veterans Administration Medical Center veterinary medical unit in sterile, filter-topped cages and were given sterile food and water ad libitum. A colony of C.B17 SCID mice originally obtained from Taconic Farms were used to maintain a source of *P. carinii* (a gift from A. Harmsen, Montana State University, Bozeman, MT) for infection of experimental mice. Mice used for the generation of chimeras were maintained on sulfamethoxazole/trimethoprim in the drinking water on a 3-day on, 4-day off schedule. The drug was withdrawn a full 2 wk before infection with *P. carinii*.

Generation of mixed chimeras

To generate mice whose B cells were deficient in CD40, mixed chimeras were made. Recipient μMT mice were lethally irradiated with 9.5 Gy from a ^{156}Cs source and 10^7 bone marrow cells injected i.v. on the same day. Recipient mice received injections of either 75% μMT plus 25% C57BL/6 bone marrow cells, 100% μMT cells, or 75% μMT plus 25% CD40KO bone marrow cells. This regimen resulted in mixed chimeric mice whose B cells lacked CD40 but whose macrophages and dendritic cells were largely CD40 positive. Mice were checked for reconstitution of the B and T cell compartments 10 wk posttransplant by staining PBL for FACS analysis as described below. Mice that did not have reconstituted B lymphocytes were eliminated from further experimentation.

Enumeration and inoculation of P. carinii *organisms*

For isolation of organisms for inoculation, lungs were excised from *P. carinii*-infected SCID mice and pushed through stainless steel mesh in HBSS. Cell debris was removed by centrifugation at $100 \times g$ for 2 min. Aliquots of lung homogenates were spun onto glass slides, fixed in methanol, and stained with Diff-Quik (Dade International, Miami, FL). *P. carinii* nuclei were enumerated by microscopy. Mice to be infected were anesthetized lightly with halothane gas and 5×10^6–10^7 *P. carinii* organisms injected intratracheally in 100 μl of PBS. For determination of lung *P. carinii* burden, right lung lobes were excised, minced, and digested in RPMI 1640 medium supplemented with 2% FCS, 1 mg/ml collagenase A, and 50 U/ml DNase for 1 h at 37°C. Digested lung fragments were pushed through mesh screens and aliquots were spun onto glass slides and stained with Diff-Quik for microscopic enumeration as previously described (7). Lung burden is expressed as $\log_{10}$ *P. carinii* nuclei per right lung lobes and the limit of detection was 3.23.

Isolation of cells from alveolar spaces, lungs, and lymph nodes

Mice were killed by exsanguination under deep halothane anesthesia. The lungs were lavaged with HBSS containing 3 mM EDTA. After removing an aliquot for enumeration of *P. carinii* organisms as described above, erythrocytes were removed from lung digests using a hypotonic lysing buffer, cells were washed, and single-cell suspensions were enumerated. Tracheobronchial lymph nodes (TBLN) were pushed through mesh screens in HBSS and enumerated.

Flow cytometric analysis of lung and lymph node lymphocytes

Lung lavage, lung digest, and TBLN cells were washed in PBS with 0.1% BSA and 0.02% NaN_3 and stained with appropriate concentrations of fluorochrome-conjugated Abs specific for murine CD4, CD44, CD62 ligand (L), IgM, and CD40. Abs were purchased from BD PharMingen (San Diego, CA). Expression of these molecules on the surface of lymphocytes was determined by multiparameter flow cytometry using a FACSCalibur cytofluorometer (BD Biosciences, Mountain View, CA).

P. carinii-*specific ELISA*

Blood was collected from the abdominal aorta under halothane anesthesia and sera were frozen at −80°C. A crude sonicate of *P. carinii* (10 μg/ml) was coated onto microtiter plates for 2 h and coated wells were blocked with 5% dry milk in HBSS supplemented with 0.05% Tween 20 for 1 h. Test sera were serially diluted and incubated in plates overnight (4°C). Plates were washed extensively and bound Ab was detected using appropriate dilutions of alkaline phosphatase-conjugated specific Abs (anti-IgM, IgG, IgA). After 4 h at 37°C, plates were washed and developed using *p*-nitrophenyl phosphate at 1 mg/ml in diethanolamine buffer. A_{405} or end point titer expressed as the $\log_{10}$ inverse dilution at which the A_{405} was <0.1 is reported.

Statistical analysis

Differences between experimental groups were determined using Student's *t* test or ANOVA, followed by Student-Neuman-Keuls post hoc test where appropriate. Differences were considered statistically significant when $p < 0.05$. SigmaStat statistical software (SPSS, Chicago, IL) was used for all analyses.

Results

Clearance of P. carinii *is dependent on B cells and CD40-CD40L interactions*

It has been previously reported that mice deficient in B cells (μMT mice) are susceptible to PCP (11, 12). Furthermore, it is known that CD40L expression on T cells is necessary for resolution of *P. carinii* infection since individuals with mutations in the CD40L gene (X-linked hyper-IgM syndrome) are susceptible to opportunistic infections including *P. carinii, Cryptosporidium,* and *Candida* (22, 23). In addition, blocking the CD40-CD40L interaction in *P. carinii*-infected mice with CD40L-specific Ab resulted in the inability to resolve PCP (20). Since CD40L expression on T cells is required for *P. carinii* clearance and B cell activation, germinal center formation, and class switching to IgG, we first examined whether CD40-deficient mice were as susceptible as μMT mice to intranasal inoculations of *P. carinii*. As shown in Fig. 1, WT C57BL/6 mice control the infection by day 20 and have either cleared or have very low lung burdens of *P. carinii* by day 35 postinfection. In some experiments, WT mice cleared the infection even faster than 5 wk postchallenge (data not shown). In contrast, μMT and CD40KO mice had steadily increasing lung *P. carinii* burdens over the 5 wk of the experiment (Fig. 1). These mice

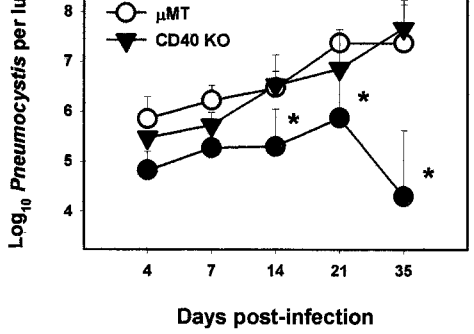

FIGURE 1. B cell-deficient and CD40-deficient mice are unable to clear *P. carinii* organisms from the lungs. WT, μMT, or CD40KO mice were given intratracheal inoculations of 10^7 *P. carinii* organisms and lung burden was quantitated days 4 through 35 postinfection. Data represent the mean ± SD of four to five mice per group. *, $p < 0.05$ compared with μMT or CD40KO.

succumb to infection when the lung *P. carinii* burden approaches $\log_{10}$ 8.0. To test whether the class-switched Ab response was normal in the CD40KO mice, serum was collected from the mice at various times postinfection and the levels of *P. carinii*-specific IgM and IgG were determined. As expected, the B cell-deficient mice did not produce detectable quantities of *P. carinii*-specific Ab of any isotype (Fig. 2). In contrast, the CD40KO and WT mice produced comparable levels of serum *P. carinii*-specific IgM (Fig. 2). However, as predicted, the CD40KO mice were unable to produce any *P. carinii*-specific IgG (Fig. 2).

Chimeric mice whose B cells are deficient in CD40 have delayed clearance of P. carinii

The previous results indicate that both B cells and CD40 are required for resolution of *P. carinii* infection. The results also indicated that *P. carinii*-specific IgM produced by CD40KO mice was not sufficient to provide protection nor was it able to reduce the severity of infection. In addition, since neither CD40KO nor μMT mice are able to produce class-switched *P. carinii*-specific Ab, the results could suggest that class-switched IgG Ab is obligate for clearance of primary *P. carinii* infection. However, CD40 is expressed on a number of cell types, including B cells, macrophages, and dendritic cells, and is important for the activation of all of these cells types (24, 25). Furthermore, CD40-CD40L interactions between APC and T cells are critical for optimal $CD4^+$ T cell priming (24–28).

To separate the requirement for CD40 expression on B cells to induce activation and class switching from its functional role(s) on other cell types, three different types of mixed bone marrow chimeric mice were generated according to the protocol described in *Materials and Methods*. The first group consisted of μMT hosts that were reconstituted with μMT bone marrow. These μMT chimeric mice were completely B cell deficient but produced all other cell types (data not shown). The second group consisted of μMT hosts that were reconstituted with a 3:1 ratio of μMT and CD40KO bone marrow. B cells were present in these CD40KO

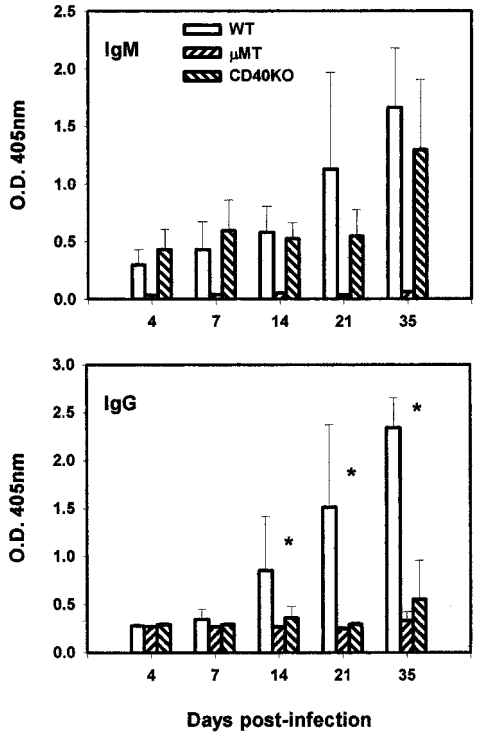

FIGURE 2. CD40KO mice fail to produce class-switched *P. carinii*-specific IgG in response to infection. Mice were infected with *P. carinii* as described for Fig. 1. Serum was collected at the indicated times postinfection and relative *P. carinii*-specific IgM or IgG levels were determined by ELISA. Data represent the mean ± SD A_{405} of sera from four to five mice per time point per group. Note that background levels are represented by serum samples from μMT mice that do not have B cells. *, $p < 0.05$ compared with CD40KO mice.

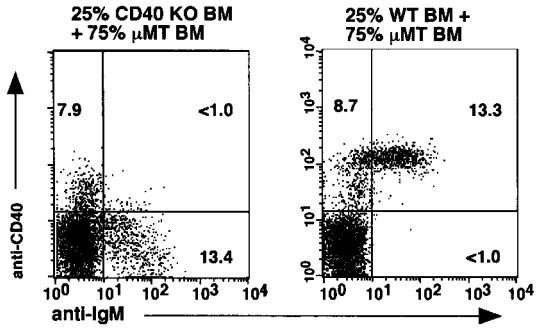

FIGURE 3. B cells from CD40KO chimeric mice are deficient in CD40 expression. Mixed chimeric mice were generated as detailed in *Materials and Methods*. Peripheral blood cells of chimeric mice were examined by FACS for expression of CD40 and IgM at 10 wk posttransplant. A representative dot plot from the peripheral blood of a single WT chimeric (*right panel*) or CD40KO chimeric (*left panel*) mouse is shown. The percentage of live cells in each quadrant is indicated.

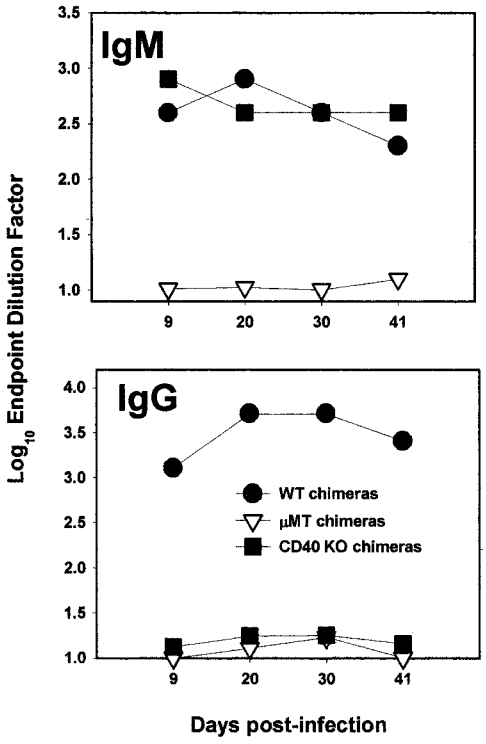

FIGURE 4. B cells from CD40KO chimeric mice do not class switch to form *P. carinii*-specific IgG. Mixed bone marrow chimeric mice were generated as detailed in *Materials and Methods*. WT, μMT, and CD40KO B cell chimeras were infected with intratracheal inoculations of *P. carinii* and serum was collected at the indicated time points. ELISA was used to determine relative serum levels of *P. carinii*-specific IgM or IgG of individual mice. IgM and IgG end point titers were determined using serial dilutions of sera. Data represent the $\log_{10}$ inverse of the end point dilutions on pooled sera from four to five mice per group per time point. The end points were determined by the dilution at which A_{405} was <0.1. Data are representative of three separate experiments.

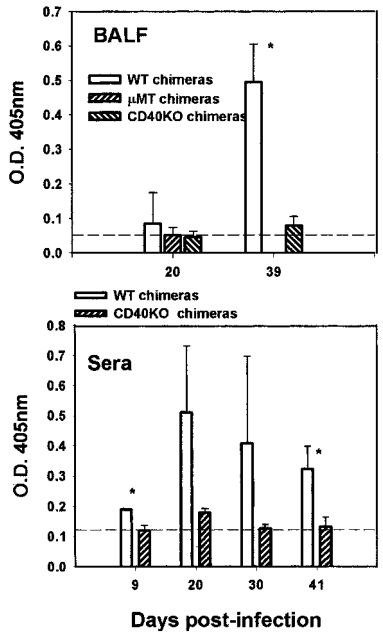

FIGURE 5. *P. carinii*-specific IgA is not produced in CD40KO chimeric mice. Mixed bone marrow chimeric mice were generated as detailed in *Materials and Methods*. WT, μMT, and CD40KO B cell chimeras were infected with intratracheal inoculations of *P. carinii* and bronchial alveolar lavage fluid (BALF, *top panel*) and serum (*bottom panel*) collected at the indicated time points. ELISA was used to determine relative serum or BALF levels of *P. carinii*-specific IgA of individual mice. Data represent the mean ± SD of A_{405} of sera (1/10 dilution) or BALF (undiluted) from two to three mice per time point per group and are representative of two separate experiments. Note that background levels are represented by a dotted horizontal line. *, $p < 0.05$ compared with CD40KO chimeric mice.

chimeric mice, but the B cells in these mice were derived from CD40KO bone marrow and were unable to express CD40 (Fig. 3). In contrast, the majority (75%) of all other cell types in the CD40KO chimeric mice, including macrophages and dendritic cells, were derived from CD40-sufficient bone marrow and were competent to express CD40 (Fig. 3). In the final group, μMT hosts were reconstituted with a 3:1 ratio of μMT and WT bone marrow. The B cells as well as all other cell types in these WT chimeric mice were derived from CD40-sufficient bone marrow (Fig. 3). Ten weeks postreconstitution, the chimeric mice were infected with *P. carinii* and *P. carinii*-specific Ab titers were determined at various times postinfection. As expected, the μMT chimeras did not make any detectable Ab of any isotype while the WT chimeras produced *P. carinii*-specific IgM, IgG, and IgA Abs (Figs. 4 and 5). In contrast, the CD40KO B cell chimeras were unable to generate *P. carinii*-specific IgG or IgA, despite making *P. carinii*-specific IgM (Figs. 4 and 5). Indeed, *P. carinii*-specific IgG levels in the CD40KO B cell chimeras never rose above the background level seen with the μMT chimeric mice at any time point postinfection (Fig. 4).

To test whether the loss of class-switched Abs in the CD40 chimeric mice resulted in increased susceptibility to *P. carinii* infection, *P. carinii* lung burdens were determined. As shown in Fig. 6, B cell-deficient μMT chimeric mice were unable to control PCP and all died or were humanely killed within 30 days postinfection. In contrast, WT chimeric mice cleared infection within 30 days (Fig. 6). Interestingly, the lung *P. carinii* burdens of the CD40KO B cell chimeras were 10-fold higher than the WT chimeras at days 10 and 20 postinfection; however, unlike the μMT chimeras, the CD40KO B cell chimeras eventually resolved the infection (Fig. 6). Since the clearance of *P. carinii* from the lungs was slower in CD40KO B cell chimeric mice than in WT chimeras, CD40 expression on B cells is important for the rapid resolution of *P. carinii* infection. In addition, the data showed that CD40 expression on B cells is required for production of class-switched *P. carinii*-specific IgG, but that *P. carinii*-specific IgG is not obligate for clearance of *P. carinii* from the lung.

Clearance of P. carinii *infection is delayed in FcγR1 and III knockout (KO) mice*

The delayed clearance of *P. carinii* infection observed in the CD40KO chimeras could have been due to the absence of *P. carinii*-specific IgG. Alternatively, the delay may have been due to some other B cell function that is dependent on CD40 expression, such as costimulation of T cells via CD80 or CD86 or Ag presentation after up-regulation of MHC class II expression (24, 26). To test whether *P. carinii*-specific IgG facilitates rapid clearance of infection, mice deficient in Fcγ receptors I and III and Fcε receptors (FcγRKO) were infected with *P. carinii*. It is known that Fc receptor expression on phagocytes allows the phagocyte to bind, phagocytose, and kill organisms that have been opsonized by specific IgG, thus FcγRKO mice provide a good model to test the efficacy of IgG in eliminating *P. carinii*. As shown in Fig. 7, there was a delay in the clearance of *P. carinii* in FcγRKO mice compared with WT controls. WT BALB/c mice had reduced *P. carinii* burden 100-fold compared with FcγRKO mice by day 20 postinfection (Fig. 7). However, by day 31, all mice had undetectable lung *P. carinii* burdens. The delayed clearance of *P. carinii* in FcγRKO mice was not due to differences in Ab production as the relative amounts of *P. carinii*-specific IgM and IgG found in the sera were not significantly different in the FcγRKO mice compared with the WT mice (Fig. 7). Together these data suggest that the delay in clearance of the organisms in the FcγRKO mice was due to the inability to phagocytose opsonized *P. carinii.* However, it is clear that in the absence of FcγR-dependent opsonization of *P. carinii*, other compensatory mechanisms must stimulate phagocytosis and clearance of *P. carinii*.

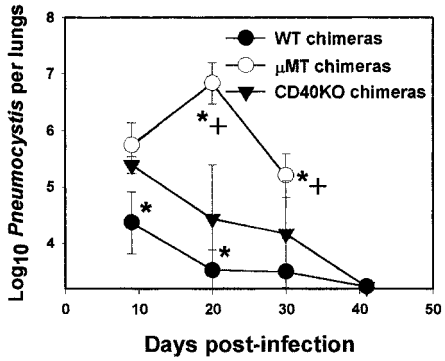

FIGURE 6. Clearance of *P. carinii* from the lungs is delayed in CD40KO B cell chimeras compared with WT chimeras. Mixed bone marrow chimeric mice were generated as detailed in *Materials and Methods*. WT, μMT, and CD40KO B cell chimeras were infected with intratracheal inoculations of *P. carinii* and lung burdens were determined microscopically at the indicated time points. Data represent the mean ± SD of four to five mice per group per time point and are representative of three separate experiments. Note that μMT chimeric mice began to die due to infection between days 20 and 30 and the data shown for day 30 represent μMT chimeric mice that had survived to that point. *, $p < 0.05$ compared with WT mice at the same time point. †, $p < 0.05$ compared with CD40KO B cell chimeras.

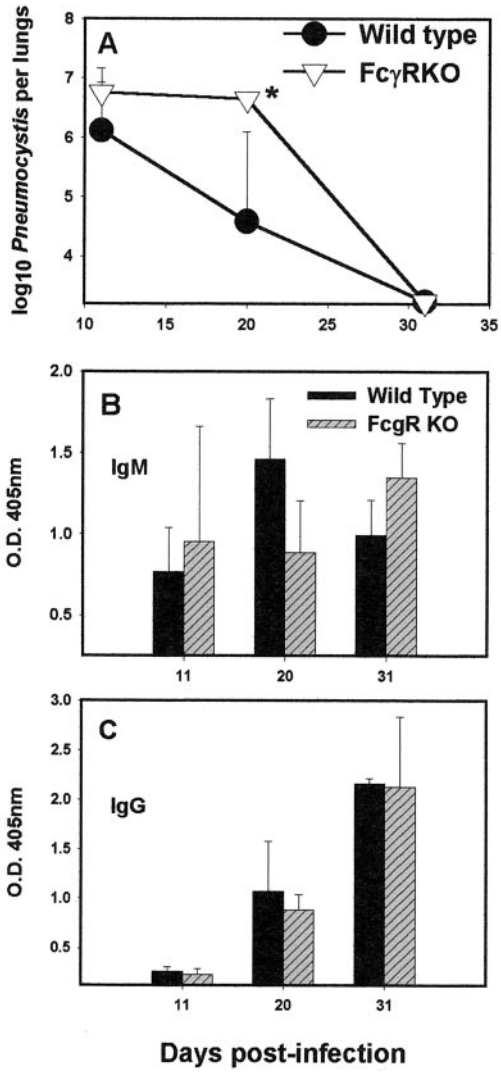

FIGURE 7. FcγRKO mice clear *P. carinii* infection with delayed kinetics compared with WT mice. Mice deficient in FcγRI, III, and FcεR (FcγRKO) and WT BALB/c mice were infected with intranasal inoculations of *P. carinii* and lung burden (*A*) and sera (*B*) *P. carinii*-specific IgM and IgG were determined at the indicated time points. Lung *P. carinii* burden was determined microscopically and Ab levels were determined by ELISA using 1/100 dilutions of sera. Data represent the mean ± SD of five mice per group per time point and are representative of three separate experiments. *, $p < 0.05$ compared with WT mice at the same time point.

Complement deficiency does not alter clearance of P. carinii

B cells are obligate for protection from PCP, yet a deficiency in *P. carinii*-specific IgG or in the FcγR necessary for binding IgG-opsonized organisms delays clearance of *P. carinii* but does not prevent resolution of the infection. These data indicate that B cells must participate either directly or indirectly in the killing of *P. carinii* via additional immune mechanisms. One additional Ab-dependent mechanism in which B cells could contribute to *P. carinii* resolution is to produce *P. carinii*-specific IgM that could be used to activate complement, resulting in opsonization of the *P. carinii*. This possibility seemed unlikely since CD40KO mice produce *P. carinii*-specific IgM, yet die with equivalent kinetics as mice that completely lack B cells (Fig. 1). However, C3 activation can also take place via Ab-independent mechanisms. Therefore, to further rule out the possibility that the complement component C3 or its downstream effectors can compensate for the absence of IgG

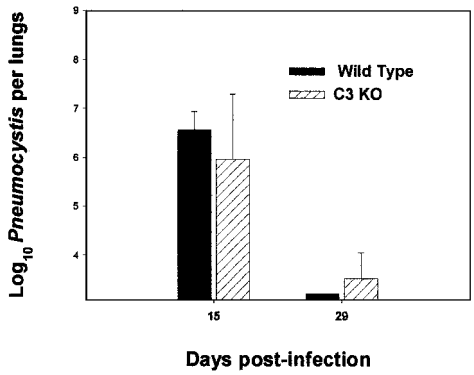

FIGURE 8. Mice deficient in the C3 component of complement are not susceptible to PCP. C3KO and WT mice were infected with intranasal inoculations of *P. carinii* and lung burdens assessed microscopically at the indicated time points. Data represent the mean ± SD of three mice per group per time point and are representative of two separate experiments.

or FcγR, we tested whether C3 is required for host defense against *P. carinii*. C3KO mice were infected intratracheally with 10^7 organisms and *P. carinii* lung burden was determined. As shown in Fig. 8, there was no difference in the *P. carinii* lung burdens of WT and C3KO mice at days 15 and 29 postinfection. These data indicate that C3 is not necessary for clearance of *P. carinii*. Finally, to test whether opsonization of organisms by C3 cleavage products in combination with *P. carinii*-specific IgM or IgG is necessary for *P. carinii* clearance from the lung, C3 was depleted from FcγRKO mice using cobra venom factor. As shown in Table I, all mice were able to clear *P. carinii* infections from the lungs by day 28 postinfection. Together, these data demonstrate that C3 and FcγRs as well as *P. carinii*-specific IgG are not obligate for the clearance of *P. carinii* from the lungs of infected mice.

Activated CD4$^+$ T cells are reduced in the lung airways and TBLN of B cell-deficient and CD40KO B cell chimeric mice

The results described above indicate that *P. carinii*-specific Ab, particularly class-switched Ab, contributes to, but is not obligate for, the clearance of *P. carinii* from the lungs. However, B cells are clearly required, suggesting that B cells must also mediate clearance of *P. carinii* by mechanisms independent of Ab production. To test whether B cells might also regulate the CD4$^+$ T cell response, we determined the number of activated T cells in the draining TBLN and lungs of *P. carinii*-infected WT, μMT, and CD40KO B cell chimeras. As shown in Fig. 9, the number of activated CD4$^+$CD62L^{low} T cells increased in the draining TBLN of WT chimeric animals over the course of infection, peaking at day 20. In contrast, the μMT and CD40KO chimeric mice had reduced numbers of activated CD4$^+$ T cells in the TBLN from day 10 through day 40 (Fig. 9). The percentage of CD4$^+$ T cells with an activated phenotype was considerably lower in the TBLN of

Table I. *Resolution of PCP in FcγRKO mice is not dependent on complement*

Experimental Group	*P. carinii* Lung Burden ($\log_{10}$)[a]
WT	<3.2, <3.2, <3.2, <3.2
WT + cobra venom factor[b]	<3.2, <3.2, <3.2, <3.2
FcγRKO	<3.2, <3.2, <3.2, <3.2
FcγRKO + cobra venom factor	<3.2, <3.2, <3.2, <3.2

[a] Lung burden was determined microscopically day 28 postinfection.
[b] Cobra venom factor was administered i.p. every third day starting with the day of infection.

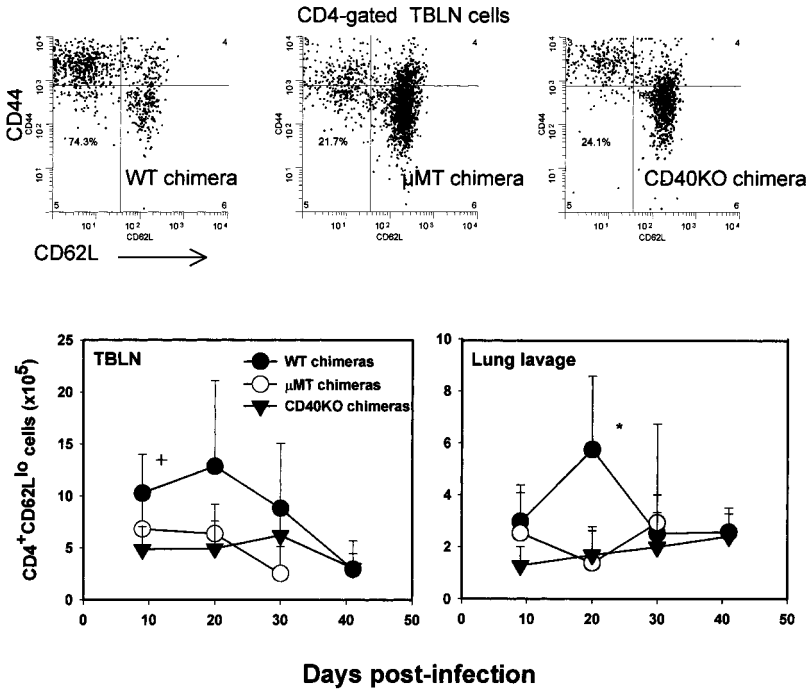

FIGURE 9. μMT and CD40KO chimeras have reduced numbers of activated $CD4^+$ T cells in the alveolar spaces and draining lymph nodes compared with WT chimeras. Mixed chimeric mice were generated as detailed in *Materials and Methods*. WT, μMT, and CD40KO B cell chimeras were infected with intratracheal inoculations of *P. carinii.* Lung alveolar cells and TBLN cells were harvested at the indicated times postinfection. The proportions of activated $CD4^+CD62L^{low}$ T cells were determined using flow cytometry. The *top three panels* show flow cytometry dot plots of CD44 vs CD62L expression on lymphocyte and CD4-gated TBLN cells from individual mice day 20 postinfection. The proportion of $CD4^+$ lymphocytes that are $CD62L^{low}$ is shown. Dot plots are representative of data from four to five mice per group. The *bottom panels* show the absolute numbers of $CD4^+CD62L^{low}$ T cells in the TBLN and lung lavages. Data are expressed as the mean ± SD of four to five mice per group per time point and are representative of three separate experiments. *, $p < 0.05$ compared with WT mice at the same time point. †, $p < 0.05$ for all WT compared with all μMT chimeras or all CD40KO B cell chimeras without consideration of time point effects.

μMT and CD40KO chimeric mice than in the WT chimeras at day 20 (Fig. 9). Indeed, by day 30 postinfection, the number of activated $CD4^+$ T cells in the draining TBLN had actually begun to decline in the B cell-deficient μMT chimeric mice (Fig. 9), despite the fact that the *P. carinii* burden was quite high in these animals (see Fig. 6). In the CD40KO B cell chimeric animals, the number of activated T cells remained low, but relatively constant, from day 10 through day 40 (Fig. 9).

Similar results were observed when the number of activated $CD4^+$ T cells in the lung lavage fluids of the infected chimeric mice was determined (Fig. 9). Again, the peak increase in the number of activated $CD4^+$ T cells in the lung airways was seen at day 20 in the WT chimeras, while the number of activated $CD4^+$ T cells present in the airways of μMT and CD40KO chimeric mice remained low throughout the time course of infection. Importantly, identical results were seen when the number of activated $CD44^{high}$ T cells was determined in each of the groups (data not shown), indicating that CD40-expressing B cells are necessary to induce optimal T cell activation and/or expansion after *P. carinii* infection. Thus, it appears that B cells help to mediate protection to *P. carinii* infection by both the production of opsonizing class-switched Ab as well as by regulating the activation and expansion of $CD4^+$ T cells.

Discussion

In this report, we confirm the findings of others (11, 12) that mice deficient in B cells are more susceptible to PCP and are unable to resolve a primary infection. The increased susceptibility of B cell-deficient mice could be due to the absence of *P. carinii*-specific Ab; however, B cells also function as APC (29–31) and are able to produce a diverse array of cytokines (32, 33), indicating that they might play additional roles in the immune response. We directly tested whether *P. carinii*-specific Ab was required for resolution of *P. carinii* infection by several different approaches. First, we produced mice that are unable to generate a *P. carinii*-specific IgG response (CD40KO B cell chimeric animals). These mice were able to resolve the infection, albeit with delayed kinetics. Likewise, FcγR-deficient mice resolved the infection by day 30. Together, these results suggest that opsonization of *P. carinii* by IgG is not sufficient to mediate the clearance of *P. carinii* from the lungs. Although it has recently been reported that IgA is produced at mucosal surfaces in μMT mice (34), *P. carinii*-specific IgA was not detected in either the lungs or sera of CD40KO chimeras, indicating that specific IgA did not compensate for the lack of IgG in this model.

Since the CD40KO chimeric mice did clear *P. carinii* infection and were capable of producing *P. carinii*-specific IgM, it was possible that IgM production by B cells is necessary for resolution of infection with *P. carinii*. Indeed, recent experiments have demonstrated that mAbs of the IgM isotype directed against the KEX1 protein of *P. carinii* were able to control growth of *P. carinii* when injected intranasally into SCID mice (19). Additionally, it has been shown that IgM Abs can mediate clearance of another fungal pathogen, *Cryptococcus neoformans* (35). However, our data using CD40KO mice (Figs. 1 and 2) indicated that *P. carinii*-specific IgM is not sufficient to control *P. carinii* infection since these mice produced specific IgM but were unable to clear the pathogen. In addition, although IgM is a powerful activator of the complement system, C3-deficient mice were also able to resolve the *P. carinii* infections, suggesting that *P. carinii*-specific IgM is unlikely to play a significant complement-dependent role in controlling *P. carinii* lung burden. Finally, C3 and FcγR double-deficient mice were able to resolve *P. carinii* infection, although with somewhat delayed kinetics. Taken together, our data strongly suggest that Ab and complement-mediated opsonization of *P. carinii* can facilitate but are not sufficient to mediate immune protection to primary *P. carinii* infection.

Since it is generally accepted that alveolar macrophages are ultimately responsible for clearance of *P. carinii* organisms from the lungs (9), targeting of the organisms to these macrophages must be mediated by additional mechanisms that are independent of FcγRs and complement. As described above, we think that it is unlikely that these alternate clearance mechanisms rely on T cell-dependent Ab production. Alternatively, Ezekowitz et al. (36) reported that in the absence of serum, mannose receptors on macrophages facilitated phagocytosis of *P. carinii* organisms. It was later determined

that macrophage mannose receptors bound to α-mannan residues of gpA (36, 37), the major surface glycoprotein expressed on *P. carinii*. It has also been reported that β-glucan receptors on macrophages interact with *P. carinii* organisms as do host proteins, including fibronectin, vitronectin, and surfactant proteins A and D (38–40). These proteins all have in common the ability to bind to carbohydrate moieties found on the surfaces of many microorganisms and may be responsible for phagocytosis of the organisms in the absence of specific IgG, FcγRs, or complement. However, none of these mechanisms compensate for the lack of B cells since μMT mice were unable to clear *P. carinii* infection.

Given that B cells are obligate for the clearance of a primary infection of *P. carinii* (11, 12) and that *P. carinii*-specific, high-affinity, class-switched Ab does not appear to be necessary for the resolution of infection, it is likely that B cells contribute to the immune response via non-Ab-mediated mechanisms. To explore this possibility, we determined whether the absence of B cells altered the $CD4^+$ T cell response. In the experiments presented here, we show that T cell activation and/or expansion in the draining TBLN and lungs of *P. carinii*-infected mice was largely reduced in B cell-deficient and CD40KO chimeric mice.

There are at least two different hypotheses that could be used to explain why T cell expansion is reduced in the infected μMT and CD40KO chimeric mice. First, it has been recently shown by Cyster and colleagues (41) that splenic T cell zone development is controlled by B cells. They show that lymphotoxin-α-producing B cells promote T cell and dendritic cell accumulation in the spleen during early postnatal development of the spleen. Although it is theoretically possible that this developmental defect in B cell-deficient mice is responsible for the reduced T cell activation in the *P. carinii*-infected chimeric animals, we think that this is highly unlikely. First, B cell-deficient mice have reduced numbers of T cells and dendritic cells in the spleen but not in the lymph nodes (41). We observed a clear difference in the number of activated T cells in the draining lymph nodes of infected μMT and CD40KO chimeric mice (Fig. 8). Second, the number of total $CD3^+$ cells in the lymph nodes of uninfected μMT chimeric mice was equivalent to the WT chimeric mice (data not shown), indicating that the "defect" in the expansion/activation of $CD4^+$ T cells in the μMT chimeric animals occurs after infection and is not due to a preexisting developmental deficiency in the animals. Third, all of the mice in this experiment were bone marrow reconstituted μMT animals and thus all of the chimeric mice have equivalently defective spleens since bone marrow reconstitution does not fix the developmental defect in these mice (41). Finally, and most importantly, we observed the same deficiency in T cell activation/expansion in B cell-sufficient CD40 chimeric animals. Thus, the reduction in activated T cells in the lymph node and lung after *P. carinii* infection cannot simply be explained by the loss of B cell-derived lymphotoxin-α in the spleen. Instead, we propose that CD40-expressing B cells are needed for optimal T cell activation/expansion after *P. carinii* infection. Given that activated B cells can efficiently present Ag to T cells, we hypothesize that B cells might help to regulate the activation or expansion of the primed Ag-specific $CD4^+$ T cells. It has been shown that ligation of CD40 on naive B cells by CD40L on T cells is required for up-regulation of B7 molecules that are necessary for costimulation of T cells during Ag presentation (24, 42). Furthermore, B cells are known to be essential for expansion of T cells in lymph nodes (29–31). Thus, Ag-presenting CD40-expressing B cells might mediate the costimulation of T cells during *P. carinii* infection. Additional experiments will be necessary to determine the molecular mechanism(s) by which B cells assist in the activation and expansion of Ag-specific $CD4^+$ T cells after primary infection.

Despite the unanswered question of how B cells modulate $CD4^+$ T cell responses to *P. carinii* infection, our data strongly suggest that B cells contribute in multiple ways to the ultimate resolution of primary *P. carinii* infection. Surprisingly, we observed that despite the absolute requirement for B cells in the host defense against *P. carinii*, specific IgG, FcγRs, and complement are not necessary for clearance, although these immune mechanisms do facilitate more rapid clearance of the pathogen. Thus, B cells must play additional Ab-independent roles during primary infection, perhaps by regulating the strength or quality of the $CD4^+$ T cell response.

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