

B Cells Are Required for Generation of Protective Effector and Memory CD4 Cells in Response to *Pneumocystis* Lung Infection

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B cell-deficient mice are susceptible to infection by *Pneumocystis carinii* f. sp. *muris* (PC). To determine whether this susceptibility is due to a requirement for B cells to prime T cells, we compared CD4 T cell responses to PC in bone marrow chimeric mice that express MHC class II (MHCII) on all APCs (wild-type (WT) chimeras) and in bone marrow chimeric mice that express MHCII on all APCs except B cells (MHCII^{-/-} chimeras). Although PC was rapidly cleared by WT chimeric mice, PC levels remained high in chimeric mice that lacked MHCII on B cells. In addition, although T cells were primed in the draining lymph nodes of MHCII^{-/-} chimeric mice, the number of activated CD4 T cells infiltrating the lungs of these mice was reduced relative to the number in the lungs of WT chimeras. We also adoptively transferred purified CD4 T cells from the draining lymph nodes of PC-infected normal or B cell-deficient mice into SCID mice. Mice that received CD4 cells from normal mice were able to mount a response to infection in the lungs and clear PC. However, mice that received CD4 cells from B cell-deficient mice had a delayed T cell response in the lungs and failed to control the infection. These data indicate that B cells play a vital role in generation of CD4⁺ memory T cells in response to PC infection in the lungs. *The Journal of Immunology*, 2006, 176: 6147–6154.

The CD4⁺ T cells are required for host defense against the opportunistic fungal pathogen *Pneumocystis carinii* f. sp. *muris* (PC)² (1–3). It is presumed that interactions between CD4 T cells and APCs in the lymph nodes (LN) induce the generation of effector CD4 T cells that migrate to the lung and activate alveolar macrophages, which kill PC organisms. Moreover, the effector T cells also provide help to B cells, resulting in production of PC-specific Abs that can facilitate the clearance of PC by promoting phagocytosis by macrophages (4–6). Although PC-specific Abs can enhance clearance of PC, infected SCID mice are able to clear infection when reconstituted with purified CD4 cells isolated from immunized wild-type (WT) donors (1, 7). However, transfer of purified naive CD4⁺ T cells to SCID mice does not result in control of the PC infection (B. A. Garvy and A. G. Harmsen, unpublished observations), indicating that whereas CD4⁺ effector cells are sufficient for PC clearance, naive T cells do not develop into protective effectors in the absence of other lymphocytes.

In addition to being dependent on CD4⁺ T cells, control of PC pneumonia (PCP) is also dependent on the presence of B cells (5, 8, 9). However, whereas it is clear that B cells are required for elimination of PC, it is not known how B cells mediate their pro-

teective effect. Although PC-specific Ab is likely to play a protective role, we have recently demonstrated that mice containing CD40-deficient B cells can clear PC infections even though these mice were unable to produce PC-specific class-switched Ab (5). In addition, mice with targeted mutations in Fcγ and ε receptors were also able to clear PC infections (5). Together, these data suggested that B cells likely play an Ab-independent role in clearance of PC infection.

One obvious Ab-independent role that B cells may play in immune responses to PC is to present Ag and regulate the priming, expansion, or maintenance of Ag-specific CD4 T cells (10–12). Indeed, we previously showed that the number of activated CD4 cells present in the lungs of PC-infected B cell-deficient (μMT) mice was reduced relative to the number seen in the lungs of infected normal mice (5). Although we were not able to identify PC-specific T cells in our analysis, other groups have shown attenuated Ag-specific CD4 T cell responses to a variety of Ags and pathogens. For example, Christensen et al. (13) found that priming of lymphocytic choriomeningitis virus-specific CD4⁺ cells was significantly impaired in B cell-deficient mice. Likewise, the Bradley and colleagues (14, 15) laboratory demonstrated that B cells are required for the generation and/or maintenance of memory Th2 cells responding to protein Ags.

Although these studies suggest that an intact B cell compartment is important for normal T cell function, they have been difficult to interpret, because B cells are also required during early postnatal development for the development of gp38-expressing stromal cells in the T cell area of the spleen (16). Because B cell-deficient mice have reduced numbers of these stromal cells that may be important for normal CD4 responses, we generated bone marrow (BM) chimeric mice using adult B cell-deficient μMT mice as the recipients so that all groups of mice would be equally defective in the potentially important gp38-expressing stromal cells. Using these chimeric animals, we showed that μMT mice reconstituted with BM from normal C57BL/6 mice made a normal T cell response and

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² Abbreviations used in this paper: PC, *Pneumocystis carinii* f. sp. *muris*; LN, lymph node; WT, wild-type; PCP, PC pneumonia; BM, bone marrow; MHCII, MHC class II; SMX/TMP, sulfamethoxazole/trimethoprim; TBLN, tracheobronchial LN; BALF, bronchial alveolar lavage fluid.

were able to clear PC despite lacking the gp38-expressing stromal cells in the spleen, indicating that these cells are not needed for protection to PC. In contrast, μ MT mice reconstituted with μ MT BM had reduced numbers of activated CD4 T cells present in the lung and were unable to clear PC infection (5), suggesting that B cells play a role in protection independent of their role in regulating the development of spleen. Taken altogether, our data indicated that B cells are necessary for clearance of PC and suggested that B cells may be directly needed for the generation of protective CD4 effector/memory cells.

We have now addressed whether B cells play an important role in CD4 T cell-mediated immunity to PC. Using mixed BM chimeric mice that express MHC class II (MHCII) on all APCs except B cells, we show that Ag presentation by B cells is important in host defense against PC because these mice were unable to clear infection. In addition, using an adoptive transfer model in which we transferred CD4⁺ T cells isolated from the draining LNs of PC-infected WT or B cell-deficient mice into infected SCID mice, we show that CD4⁺ cells primed in B cell-deficient mice were not competent to affect clearance of PC. Furthermore, this appeared to be due to an inability to mount a sustained or memory CD4 response to the organisms. Thus, B cells provide protection to PC not only by producing Ab but also by regulating the size and/or quality of the CD4 T cell-mediated immune response.

Materials and Methods

Mice

Adult C57BL/6J and B6.129S2-Igh-6^{tm1Cgn} (μ MT) mice were purchased from The Jackson Laboratory. B6.129-H2-Ab1^{tm1Gru} (MHCII^{-/-}), C.129(B6)-Igh-JhD^{tm1Dhu} (Jh), and C.B-Igh-1^b/ICR Tac-Prkdc^{Scid} (C.B-17 SCID) mice were obtained from Taconic Farms. BALB/cBy mice were obtained from the National Cancer Institute. For some experiments, chimeric mice were generated at Trudeau Institute and shipped to the University of Kentucky before infection with PC. All experimental mice were housed in the Lexington, Kentucky Veterans Administration (VA) 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 was used to maintain a source of PC for infection of experimental mice. Mice used for generation of chimeras were maintained on sulfamethoxazole/trimethoprim (SMX/TMP) in the drinking water on a 3-day on, 4-day off schedule. The drug was withdrawn 2 wk before infection with PC. All animal procedures were approved by the Lexington VA and Trudeau Institute Institutional Animal Care and Use Committees.

Generation of mixed chimeras

To generate mice with B cells that lacked MHCII, radiation-mixed BM chimeras were made as described previously (5). Briefly, recipient μ MT mice were lethally irradiated with 9.5 Gy from a ¹³⁶Cs source, and 10⁷ total BM cells were injected i.v. on the same day. Flow cytometric analysis on peripheral blood 10 wk posttransplantation indicated that no B cells were present in mice transplanted with 100% μ MT BM cells. In contrast, normal numbers of B cells were present in recipient mice receiving a mixture of 75% μ MT plus 25% WT BM (WT chimeras) or a mixture of 75% μ MT plus 25% MHCII^{-/-} BM (MHCII^{-/-} chimeras). Although B cells as well as the rest of the APCs present in WT chimeras expressed detectable levels of class II, 100% of the B cells from the MHCII^{-/-} chimeras were derived from the MHCII^{-/-}-deficient BM and therefore were unable to express MHCII (Fig. 1).

Enumeration and inoculation of *Pneumocystis* organisms

For isolation of organisms for inoculation, lungs were excised from PC-infected SCID mice and pushed through stainless steel mesh in HBSS. Cell debris was removed by centrifugation at 100 × g for 2 min. Aliquots of lung homogenates were spun onto glass slides, fixed in methanol, and stained with DiffQuik (Dade International). PC nuclei were enumerated by microscopy. Mice to be infected were anesthetized lightly with halothane gas, and 10⁶–10⁷ PC organisms were injected intratracheally in 100 μ l of PBS. For determination of lung PC burden, right lung lobes 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 lung frag-

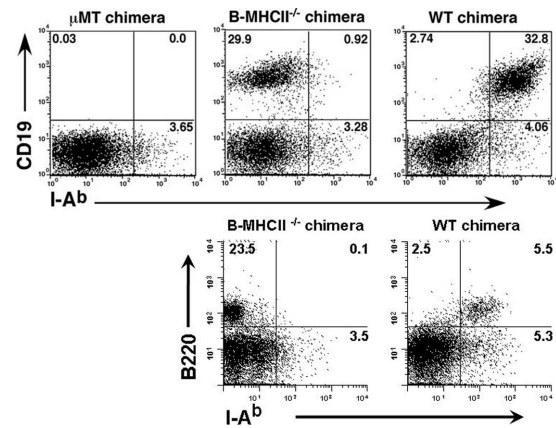


FIGURE 1. Generation of MHCII^{-/-} chimeric mice. WT, MHCII^{-/-} and μ MT-mixed BM chimeras were generated as described in *Materials and Methods*. Ten to 12 wk after lethal irradiation of μ MT recipient mice and transfer of BM (75% μ MT plus 25% either WT or MHCII^{-/-}), peripheral blood leukocytes were analyzed for reconstitution of CD4, CD8, and CD19⁺ lymphocytes by flow cytometry. CD19⁺ B cells from the spleen and lung digest cells were further analyzed for expression of MHCII at experimental time points. Data are representative dot plots showing lymphocyte-gated CD19 or B220 vs MHCII of spleen (*top panels*) and lung (*bottom panels*) cells. Data are representative of 15–20 mice per experimental group and two separate experiments.

ments were pushed through mesh screens, and aliquots were spun onto glass slides and stained with DiffQuik for microscopic enumeration. Lung burden is expressed as log₁₀ PC nuclei per right lung lobes, and the limit of detection was 3.23.

Isolation of cells from alveolar spaces, lungs, and LNs

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 PC 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 LNs (TBLN) were pushed through mesh screens in HBSS followed by removal of erythrocytes and then enumerated.

Adoptive transfer of CD4⁺ T cells

Donor BALB/c or Jh mice were given intratracheal inoculations of 10⁷ PC organisms. For some experiments, draining LNs were isolated at day 10–14 postinfection. For other experiments, 4 wk after primary infection mice were placed on SMX/TMP in drinking water for 4 wk then placed on acidified water for two additional weeks. Mice were given a booster inoculation of PC organisms, and 4 days later draining LNs were removed. CD4⁺ cells were isolated from single-cell suspensions of TBLN using negative selection columns from R&D Systems according to the manufacturer's protocol. We routinely obtain >95% pure CD4⁺ cells using this protocol. SCID mice were injected with 10⁵-purified CD4⁺ cells and 4 days later received intratracheal inoculations of 10⁶ PC organisms.

Assessment of T cell proliferation *in vitro*

CD4 T cells were isolated from the draining LNs of BALB/c or Jh mice at 14 days postinfection using R&D isolation columns as described above (R&D Systems). Cells were cultured in 96-well plates coated with anti-CD3 for 3 days, and aliquots of medium were removed for cytokine analysis. Alamar blue (BioSource International) was added at 10% of the culture volume. Alamar blue contains an oxidation-reduction indicator that changes color in proportion to cell proliferation. Color change was quantitated using a μ Quant plate reader (Bio-Tek Instruments).

Flow cytometric analysis of lung and LN lymphocytes

Lung lavage, lung digest, and TBLN cells were washed in PBS with 0.1% BSA and 0.02% NaN₃ (PBA) and stained with appropriate concentrations of fluorochrome-conjugated Abs specific for murine CD4, CD8, CD44, CD62L, IgM, and MHCII. Abs were purchased from BD Biosciences.

Expression of these molecules on the surface of lymphocytes was determined by multiparameter flow cytometry using a FACSCalibur cytofluorimeter (BD Biosciences).

Pneumocystis-specific ELISA

Blood was collected from the abdominal aorta under halothane anesthesia and sera frozen at -80°C . A crude sonicate of PC ($10\ \mu\text{g}/\text{ml}$) 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). After 4 h at 37°C , plates were washed and developed using *p*-nitrophenylphosphate at 1 mg/ml in diethanolamine buffer. OD_{405} or endpoint titer expressed as the \log_{10} inverse dilution at which the OD_{405} was <0.1 is reported.

Determination of cytokine concentrations in bronchial alveolar lavage fluid (BALF) and culture supernatants

Culture supernatants and BALF collected from the first wash were frozen for subsequent determination of cytokine levels using cytokine bead arrays and flow cytometry according to the manufacturer's directions (CBA; BD Biosciences). A Luminex system (Luminex) using a BeadLyte fluorescent bead kit (Upstate Biotechnology) was used to evaluate cytokines for some experiments using protocols provided by the manufacturer.

Statistical analysis

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

Results

MHCII expression on B cells is required for clearance of Pneumocystis

We have previously shown that both B cells and T cells are necessary for clearance of PC (5). To determine whether B cells have a central role in presenting Ag to CD4^{+} T cells in the context of PC infection, we assessed whether MHCII-expressing B cells are required for the clearance of PC. Therefore, we lethally irradiated B cell-deficient μMT mice and reconstituted them with BM from μMT , C57BL/6 (B6) or $\text{MHCII}^{-/-}$ mice (see *Materials and Methods* for details). B cells in chimeric animals reconstituted with a mixture of μMT BM and $\text{MHCII}^{-/-}$ BM are all derived from the $\text{MHCII}^{-/-}$ BM and are class II deficient ($\text{MHCII}^{-/-}$ chimeras). Importantly, the majority ($>75\%$) of all other APCs present in these mice are derived from the μMT BM and are competent to express MHCII and present Ag to CD4 T cells (Fig. 1). As controls, we also reconstituted mice with a mixture of μMT BM and B6 BM. All APCs, including the B cells, are competent to express MHCII (WT chimeras) (Fig. 1). Finally, we reconstituted μMT mice with μMT BM to produce chimeric mice that completely lacked B cells but contained other MHCII-expressing APCs (μMT chimeras). The reconstituted chimeric mice were given intratracheal inoculations of 10^7 PC organisms, and PC burden in the lungs was determined over time. As shown in Fig. 2A, neither $\text{MHCII}^{-/-}$ chimeras nor μMT chimeras were able to clear PC from the lungs by day 29 postinfection. In contrast, lung PC burden was below the limit of detection in WT chimeras by day 29 postinfection (Fig. 2A). These data indicate that MHCII-expressing B cells are necessary for resolution of PC infection.

MHCII^{-/-} chimeras do not produce isotype-switched PC-specific Ab

Because cognate B-T cell interactions are important for activation of B cells and for stimulating production of specific class-switched Ab (5), we quantitated PC-specific IgM and IgG in sera of chimeric mice. As shown in Fig. 2B, no PC-specific IgG was detected in the sera of $\text{MHCII}^{-/-}$ chimeric mice. In addition to failing to

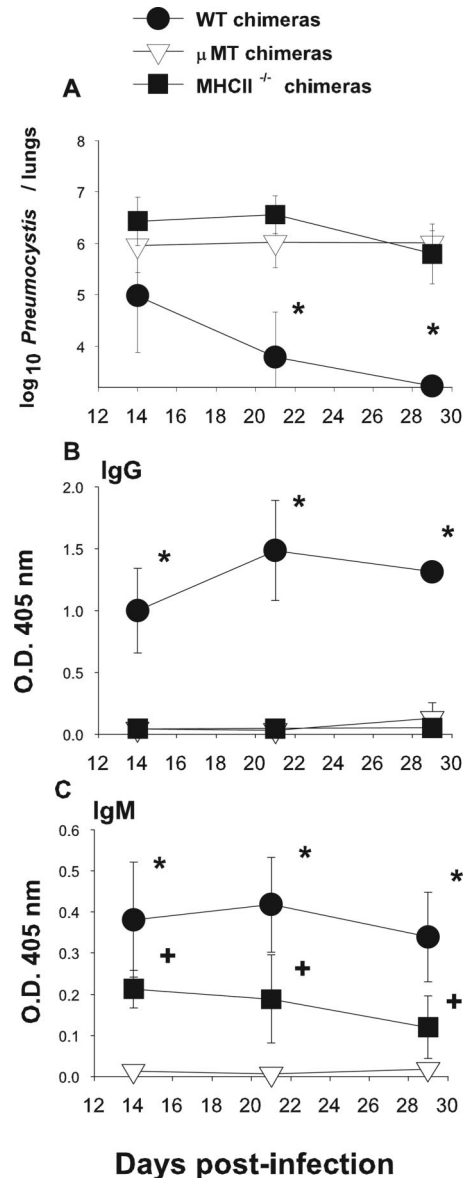


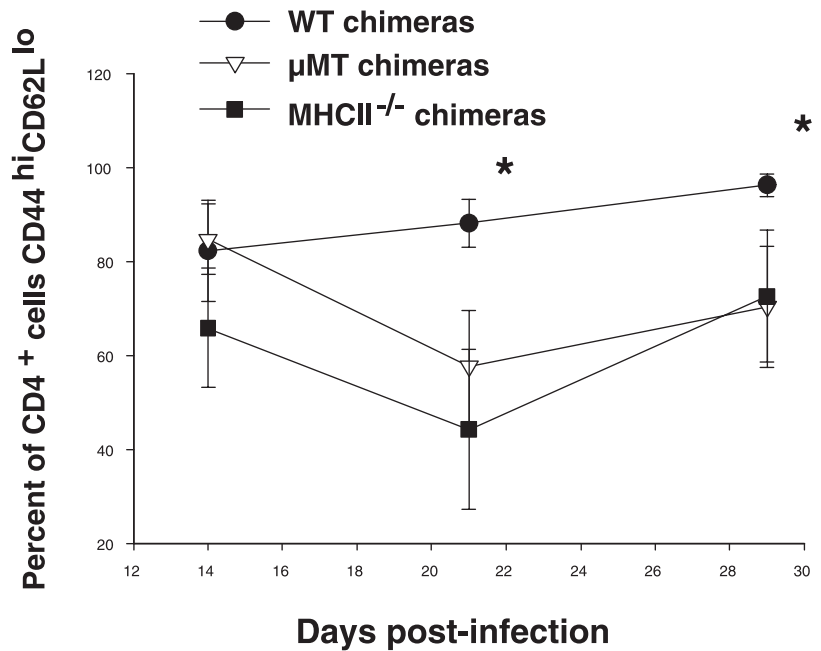
FIGURE 2. $\text{MHCII}^{-/-}$ chimeras fail to clear *Pneumocystis* infection. WT, $\text{MHCII}^{-/-}$, and μMT -mixed BM chimeras were generated as described in *Materials and Methods*. Mice were given intratracheal inoculations of 10^7 PC organisms and examined for lung organism burden (A), PC-specific serum IgG (B), or PC-specific IgM (C) at the indicated time points postinfection. PC-specific IgM and IgG were measured in sera by ELISA. Data represent the mean \pm SD of 4–5 mice per group and are representative of two separate experiments. *, $p < 0.05$ compared with μMT or $\text{MHCII}^{-/-}$ chimeras; +, $p < 0.05$ compared with μMT chimeras using ANOVA and Student-Newman-Keuls post hoc test.

isotype switch, B cells from the $\text{MHCII}^{-/-}$ chimeras produced significantly reduced levels of PC-specific IgM (Fig. 2C) and failed to up-regulate CD86 and CD80 (data not shown). Finally, there were significantly reduced numbers of germinal center B cells in the draining TBLN of $\text{MHCII}^{-/-}$ chimeras as determined by binding to peanut agglutinin (data not shown). Thus, these data show that a cognate T-B interaction is needed for B cell activation, Ab production, and protection in a PC infection.

Effector CD4 T cells are reduced in number in the lungs of infected MHCII^{-/-} chimeras

To follow up on our previously published observation that percentage of functional effector CD4 T cells was slightly reduced at

FIGURE 3. The proportion of activated CD4⁺ cells in the alveolar spaces decreases over time in μ MT and MHCII^{-/-} chimeras. Chimeric mice were generated as per Fig. 1 and infected with PC at day 0. BALF cells were stained for CD4, CD44, and CD62L and analyzed by flow cytometry. Data are expressed as the proportion of CD4⁺ cells in the BALF that express the activation markers CD44^{high}CD62L^{low}. Data represents the mean \pm SD of 4–5 mice per group per time point and are representative of two separate experiments. *, $p < 0.05$ compared with μ MT or MHCII^{-/-} chimeric mice using ANOVA and Student-Newman-Keuls post hoc test.



early time points in the lungs of PC-infected B cell-deficient mice (5), we used flow cytometry to examine the activation status of the T cells in the alveolar spaces, lung parenchyma, and TBLNs of chimeras that were infected with PC. There were no statistically significant differences in the absolute numbers of CD4⁺ cells with an activated phenotype (CD44^{high}CD62L^{low}) in the TBLN of WT, μ MT, or MHCII^{-/-} chimeras at any of the time points examined (data not shown). However, there were reduced numbers of activated CD4⁺ cells in the lungs of the MHCII^{-/-} chimeras compared with WT chimeras at later time points postinfection. For example, the proportion of CD4 cells with an activated phenotype was significantly higher in the BALF isolated from WT chimeric mice compared with either μ MT or B-MHCII^{-/-} chimeras at days 21 and 29 postinfection (Fig. 3). Thus, these data suggested that MHCII-expressing B cells are needed for the generation of effector CD4 T cells capable of migrating to the infected lung and mediating clearance of PC.

The proportion of cytokine-producing CD4 cells is normal in B cell-deficient mice

Because we have previously shown that production of class-switched PC-specific Ab is not necessary for clearance of PC (5), we asked whether B cells are required to present Ag and activate CD4⁺ T cells during the course of a PC infection. We first determined whether CD4⁺ T cells are activated in the draining LN of B cell-deficient mice after PC infection. Therefore, we purified CD4⁺ cells from the TBLN of BALB/c or B cell-deficient Jh mice 10–14 days postinfection and restimulated the T cells with plate-bound anti-CD3 for 5 days. (Note that we changed mouse background to BALB/c for these experiments due to the increased availability of Jh mice. We have not found any differences between C57BL/6 and BALB/c mice in susceptibility to PC.) As shown in Fig. 4A, the CD4⁺ T cells isolated from Jh mice proliferated even better than T cells from the normal BALB/c mice. Similarly, IL-2 and IFN- γ production 5 days after culture initiation were elevated in the restimulated CD4 T cells isolated from the TBLNs of B cell-deficient Jh mice compared with BALB/c mice (Fig. 4, B and C). To determine whether the differences in cytokine production and proliferation of CD4 cells from BALB/c compared with those from Jh mice was a kinetic difference, we also measured proin-

flammatory cytokines in cultures at 24 and 48 h after stimulation with plate-bound anti-CD3. As shown in Fig. 4D, there were no differences in the production of IFN- γ by BALB/c CD4 cells compared with Jh CD4 cells at either time point. Similar results were found for proliferative responses (data not shown). Together these

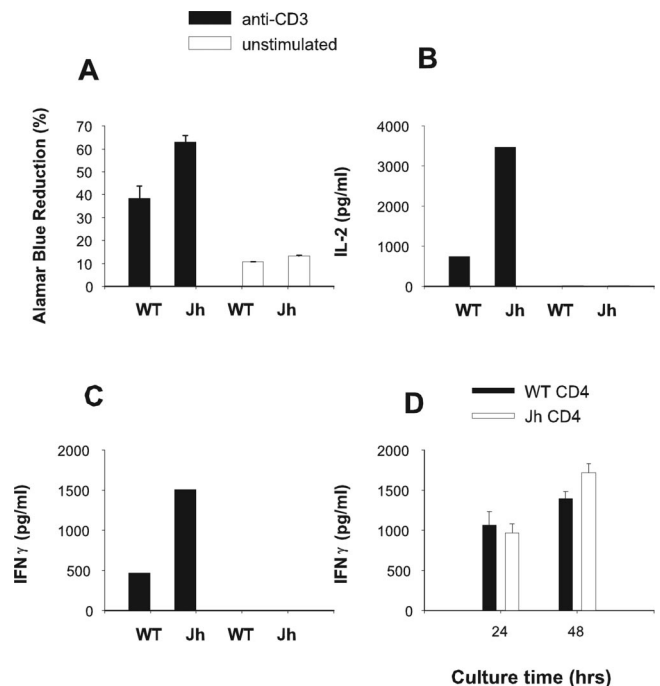


FIGURE 4. Cytokine production and proliferation are comparable or greater in Jh compared with WT CD4⁺ cells. Jh and BALB/c WT mice were given intratracheal inoculations of 10^7 PC organisms. CD4⁺ cells were purified from the draining TBLN at days 10 or 14 postinfection and were cultured with plate-bound anti-CD3 for 1–5 days. A, Proliferation was measured by Alamar blue reduction using a plate reader (top). IL-2 (B) and IFN- γ (C) concentrations in supernatants 5 days after culture and IFN- γ (D) concentrations in supernatants at 24 and 48 h after culture initiation were quantitated using cytokine bead array kits and FACS. Data are expressed as the mean of triplicate wells and are representative of three separate experiments.

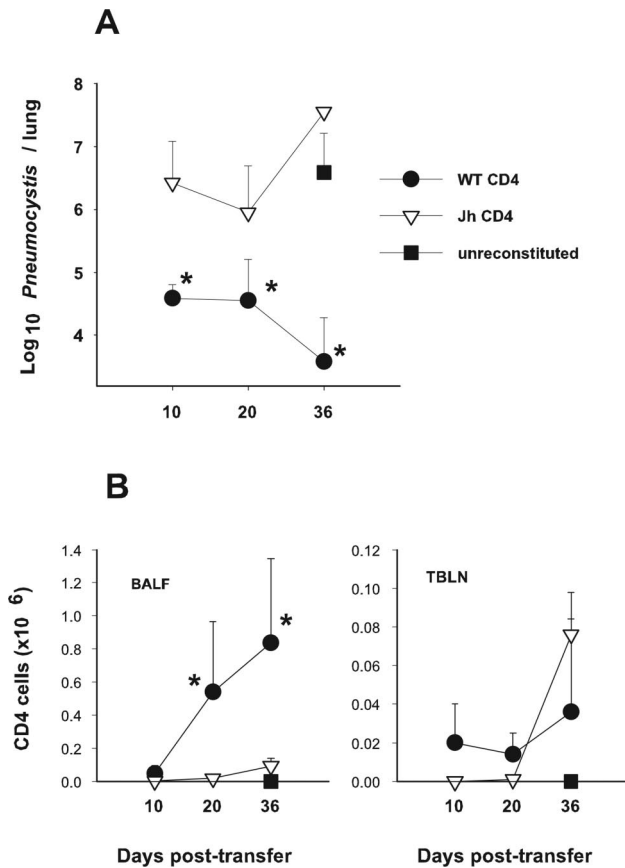


FIGURE 5. CD4⁺ cells from infected Jh mice are unable to expand in SCID adoptive hosts or clear PC. CD4⁺ cells were purified from PC-infected Jh and BALB/c WT at day 10 postinfection and adoptively transferred into SCID hosts. SCID mice were infected with 10⁶ PC organisms 4 days after adoptive transfer, and lung organism burden was determined at the indicated time points. *A*, Lung PC burden was determined microscopically. *B*, Total CD4⁺ cells in the BALF and TBLN were quantitated using flow cytometry. Data represent the mean \pm SD of 3–5 mice per group and are representative of four separate experiments. *, $p < 0.05$ compared with Jh data at the same time point as determined by ANOVA and Student-Newman-Keuls post hoc test.

data suggest that the initial activation of CD4 T cells in the TBLN in response to PC is not dependent on the presence of B cells, but that B cells may play a role after the initial priming of T cells.

Activated CD4⁺ cells from B cell-deficient mice do not infiltrate the lungs when transferred into SCID mice

Because we observed a reduction in the frequency of activated CD4 T cells present in the lungs of PC-infected B cell-deficient mice, we next determined whether CD4 T cells primed in B cell-deficient mice are able to migrate to the lungs and clear PC. We therefore used an adoptive transfer model in which we isolated CD4⁺ cells from the TBLN of BALB/c and Jh B cell-deficient mice 14 days postprimary infection with PC and transferred these T cells into SCID mice. For these experiments we used mice on a BALB/c background, because we and others have found that SCID mice on the C57BL/6 background are significantly more susceptible to PC-induced inflammation and death in this adoptive transfer model than are SCID mice on the BALB/c background (data not shown and Ref. 1). Four days after transfer the SCID mice were infected with 10⁶ PC organisms, and clearance of the organism was monitored over the next month. As expected, PC-specific ELISAs confirmed that no Ab was produced in the infected SCID

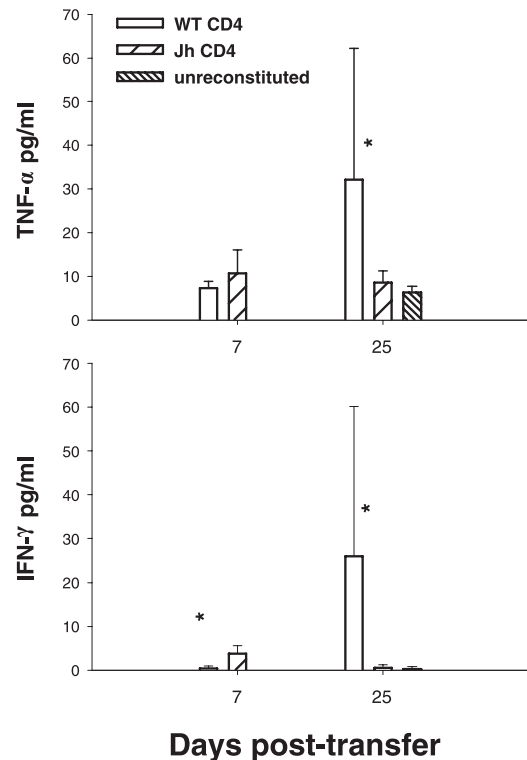


FIGURE 6. Proinflammatory cytokines in the BALF of SCID mice reconstituted with Jh CD4⁺ cells are significantly lower than that of mice reconstituted with WT cells. SCID mice were reconstituted with CD4⁺ T cells from the TBLN of Jh or BALB/c donors at day 10 postinfection as per Fig. 5. BALF was assayed for TNF- α (*top panel*) or IFN- γ (*bottom panel*) using cytokine bead arrays. Data represent the mean \pm SD of 3–5 mice per group and are representative of two separate experiments. *, $p < 0.05$ compared with mice reconstituted with CD4 cells from Jh mice or unreconstituted SCIDs using ANOVA and Student-Newman-Keuls post hoc test.

recipients and no CD8⁺ cells were detected in the reconstituted mice (data not shown). Notably, the phenotype of the transferred cells was similar between those isolated from BALB/c and those isolated from Jh mice. Approximately 15–20% of the CD4⁺ cells expressed low levels of CD62L, high levels of CD44, and CD69 (data not shown). Moreover, >90% expressed CCR2 while only 1–4% expressed CCR5, both chemokine receptors associated with migration to sites of inflammation (data not shown). Similar to previous studies showing that adoptive transfer of CD4 cells from PC-primed WT mice into infected SCID mice facilitates clearance of PC (1, 7, 17), we found that CD4 T cells transferred from previously infected BALB/c mice mediated PC clearance in the recipient mice within 36 days (Fig. 5*A*). In striking contrast, when CD4⁺ cells from infected Jh mice were transferred into SCID mice they failed to protect the SCID mice, and PC burdens remained at very high levels even out to day 36 (Fig. 5*A*). Although by day 36 postinfection, SCID mice that had received T cells primed in the Jh mice had significant numbers of donor CD4⁺ cells in the TBLN, the number present in the lungs remained negligible at all time points (Fig. 5*B*). This reduction in the number of T cell effectors present in the lungs of mice receiving T cells from the Jh mice corresponded with the low levels of IFN- γ and TNF- α in the BALF of the recipient SCID mice (Fig. 6). Therefore, CD4 T cells primed in a B cell-deficient environment do not develop into effector cells that are capable of migrating to the infected lungs and mediating clearance of PC.

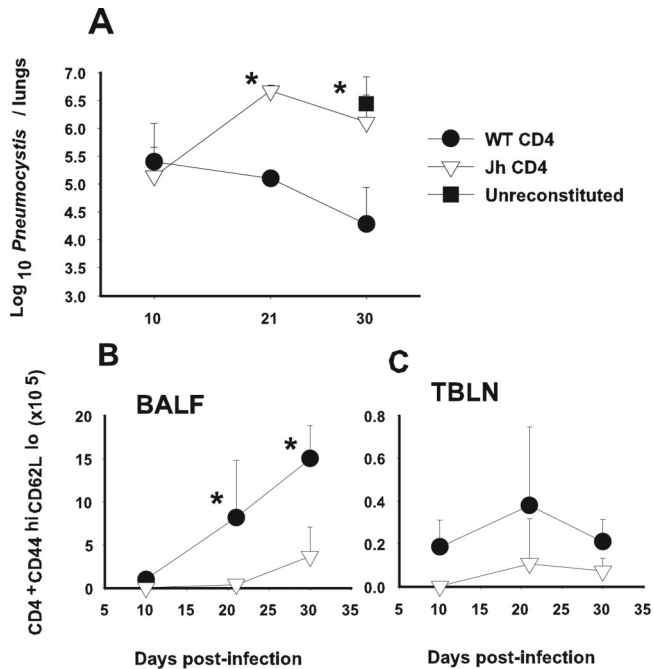


FIGURE 7. TBLN CD4⁺ cells from Jh mice undergoing a secondary response fail to clear PC in adopted SCID hosts. BALB/c WT or Jh mice were infected with PC, treated with SMX/TMP, and then rechallenge with PC. CD4⁺ cells were purified from Jh and BALB/c WT mice 4 days after secondary challenge of PC and were then transferred in SCID hosts. Four days later, SCID mice were infected with 10⁶ PC organisms. **A**, Lung organism burden was determined at the indicated time points. Flow cytometry was used to determine the number of activated CD4⁺ cells in BALF (**B**) and TBLN (**C**) at the indicated time points postinfection. Data represents the mean \pm SD of 3–4 mice per group. *, $p < 0.05$ compared with mice reconstituted with WT cells using ANOVA and Student-Newman-Keuls post hoc test.

Adoptive transfer of memory CD4⁺ cells from B cell-deficient mice failed to result in control of *Pneumocystis lung* burden

Because the generation of functional *Pneumocystis*-specific CD4 effectors is defective in the B cell-deficient mice, we speculated that the generation or maintenance of CD4 memory cells specific for PC may also be compromised in B cell-deficient mice. To address whether B cells are required for generating memory CD4⁺ T cells, we again used an adoptive transfer model. BALB/c or B cell-deficient Jh mice were given intratracheal infections with PC. After allowing time (4 wk) for a primary immune response to take place, mice were treated with SMX/TMP for an additional 4 wk to ensure that all PC organisms were eliminated from the susceptible Jh mice. The mice were then taken off SMX/TMP for 2 wk and rechallenge with live PC. Four days after the challenge infection, the CD4⁺ T cells were isolated from the draining LNs of both groups of mice and were transferred into SCID mice, which were then challenged with PC. The phenotype of the transferred cells isolated from BALB/c or Jh donors was similar with $\sim 15\%$ of the CD4⁺ cells expressing high CD44 and low CD62L (data not shown). Expression of the early activation marker CD69 was also the same between the two groups and represented 6–7% of the CD4⁺ cells (data not shown). Similar to our previous results, SCID mice that received CD4⁺ cells from Jh mice failed to control PC infection (Fig. 7A). In contrast, SCID mice that were reconstituted with CD4⁺ memory T cells from BALB/c mice clearly controlled the infection and had significantly fewer organisms in the lungs 4 wk postinfection compared with mice given effector memory T cells isolated from Jh mice (Fig. 7A).

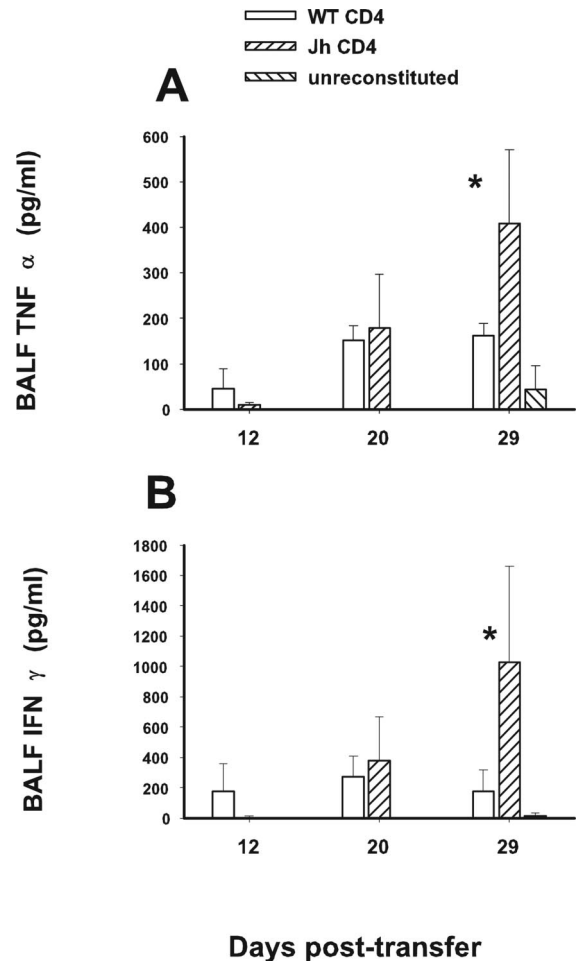


FIGURE 8. Production of proinflammatory cytokines are delayed in the lungs of SCID mice reconstituted with CD4⁺ cells isolated from Jh mice undergoing a secondary response to PC. CD4⁺ cells were purified from Jh and BALB/c WT mice during a secondary response to PC as per Fig. 7 and transferred into SCID hosts. Four days later, SCID mice were infected with 10⁶ PC organisms. Cytokine bead arrays were used to quantitate the concentration of TNF- α (**A**) and IFN- γ (**B**) in SCID mice reconstituted with Jh or WT cells. Data represent the mean \pm SD of 3–4 mice per group. *, $p < 0.05$ compared with WT cell reconstituted or unreconstituted SCID mice using ANOVA and Student-Newman-Keuls post hoc test.

CD4⁺ memory cells transferred from B cell-deficient mice do not infiltrate the lungs efficiently

Failure to control PC infection corresponded with failure of CD4⁺ cells from Jh mice to infiltrate the lungs or alveolar spaces. As shown in Fig. 7C, the total numbers of activated CD4⁺ cells isolated from the TBLNs of both groups of reconstituted SCID mice were similar. However, the number of activated CD4 T cells present in the BALF of reconstituted SCID mice was consistently reduced in SCID mice reconstituted with T cells from the Jh memory mice (Fig. 7B). Indeed, between 3 and 4 wk postinfection, $>10^6$ activated CD4⁺ cells were found in the alveolar spaces of mice reconstituted with BALB/c cells, whereas 5- to 10-fold fewer CD4⁺ cells transferred from the memory Jh mice infiltrated the alveoli (Fig. 7B).

To determine whether any functional CD4 memory cells arrived in the lungs of recipient mice, we measured cytokine levels in the BALF of the infected recipients at various times after infection. As expected, unreconstituted SCID animals had very low levels of either TNF- α or IFN- γ , confirming that CD4 cells are necessary for driving the proinflammatory cytokine response to PC (Fig. 8).

However, transfer of memory CD4⁺ cells from WT mice resulted in production of the proinflammatory cytokines TNF- α and IFN- γ in the lung by day 12 and remained elevated throughout the time course (Fig. 8). In contrast, TNF- α and IFN- γ levels were initially lower in mice receiving memory CD4 cells from the B cell-deficient Jh mice but did rise later in the response, suggesting that the very small number of CD4 cells able to go to the lungs later in the immune response (see Fig. 8) were sufficient to induce production of the inflammatory cytokines (Fig. 8). However, the CD4 T cell-dependent production of these cytokines was not sufficient to mediate clearance of PC from the lung at least out to day 30. Taken altogether, the data indicate that functional B cells are required for developing normal numbers of PC-specific effector or memory CD4 T cells and inducing protective cell-mediated immunity to PC. The potential implications of these results for patients receiving B cell ablation therapies are discussed.

Discussion

The data presented in this study demonstrate that B cells capable of presenting Ag to CD4 T cells are critical for the generation of CD4 effector cells that can mediate clearance of the opportunistic fungal pathogen PC. In addition, B cells are needed for the generation of functional effector memory T cells capable of eliminating PC. We found that mice containing B cells unable to express MHCII did not clear PC infection from the lungs even though T cell activation did appear to take place in the TBLN. However, these data were confounded by the finding that the MHCII^{-/-} chimeras failed to produce PC-specific IgG and produced reduced levels of IgM. Therefore, we used an adoptive transfer approach and found that CD4⁺ T cells primed in the absence of B cells did not migrate to the infection site when transferred into PC-infected adoptive hosts. Moreover, there were significantly reduced numbers of PC-specific memory CD4⁺ T cells in B cell-deficient mice as evidenced by the delayed expansion of these cells upon adoptive transfer into SCID hosts. These data confirm that B-T cell interactions are important for T cell function in the context of a fungal infection in the lungs.

We have previously reported that the CD40-CD40L interaction between B and T cells is important for generating class-switched Ab against PC (5). Likewise, we now show that a cognate interaction between PC-specific B cells and CD4 T cells is necessary to generate PC-specific class-switched Ab. Interestingly, whereas the chimeras expressing CD40-deficient B cells were able to clear PC, albeit with delayed kinetics (5), the MHCII^{-/-} chimeras failed to clear PC from the lungs. Thus, whereas it is possible that the lack of class-switched Ab in the MHCII^{-/-} chimeras could have contributed to delayed clearance of the organisms, Ag-presenting B cells must also play Ab-independent roles in PC clearance. Consistent with this hypothesis, B cells have been shown to be important for generation of CD4 T cell responses to experimental Ags such as hen egg lysozyme or keyhole limpet hemocyanin as well as in several infection models (10–13, 18–27). In support of these data, we also found reduced proportions of activated CD4⁺ cells in the alveolar spaces at days 21 and 29 postinfection. However, despite the reduction in the number of T cells present in the lungs of the infected B cell-deficient mice at the later time points postinfection, the *in vivo* accumulation of CD4⁺ T cells in the TBLN of both WT and Jh mice was equivalent at 3 and 8 days after infection with PC (data not shown), and anti-CD3-restimulated CD4⁺ cells from the TBLN of Jh mice proliferated and produced IL-2 at levels even higher than that seen in T cells isolated from normal mice. Our data were unexpected because Jh mice failed to clear PC, even though it appeared that CD4 T cell priming was as good as or better than WT mice. This was particularly puzzling because sev-

eral groups have reported that primed and purified CD4⁺ cells are sufficient for clearing PC when transferred into infected SCID hosts (1, 7, 17).

Because it was not clear why Jh mice were unable to resolve PCP even though CD4 T cells clearly looked primed during the first 10–14 days postinfection, we reasoned that perhaps these cells were not developing into activated effectors capable of migrating to the lung and eliminating PC. Indeed, whereas adoptive transfer of CD4 T cells isolated from the TBLN of WT mice between days 10 and 14 postprimary infection into infected SCIDs consistently resulted in expansion of cells and resolution of PCP, CD4⁺ T cells primed in Jh mice failed to infiltrate the lungs upon transfer, and SCID mice were unable to clear the infection even though they had a similar phenotype and expressed comparable levels of CCR2 and CCR5 as T cells from WT mice. This suggests that B cells are required for further expansion, survival, and/or migration of effector CD4⁺ cells to nonlymphoid tissues. It has been postulated that after the initial presentation of Ag to T cells by dendritic cells, further expansion requires B cells because dendritic cells become limiting in number (15, 28).

Our data support the recent studies that indicate that the presence of B cells is important for generation of memory T cells (14, 15). These studies showed that the frequency of IL-2-producing CD4⁺ cells that responded to keyhole limpet hemocyanin was significantly diminished over time in B cell-deficient mice compared with WT mice (14, 15). We also found that the generation of functional PC-specific memory CD4⁺ T cells was significantly diminished in B cell-deficient mice. When Jh mice were rechallenged with PC after being cured of a primary infection, the number of CD4⁺ cells with an activated phenotype was significantly reduced compared with WT mice (data not shown). Moreover, CD4⁺ cells from Jh mice failed to accumulate in the lungs when adoptively transferred into infected SCID mice, resulting in an inability to control PC infection. Interestingly, at later time points (day 30) there was a small, but measurable infiltration of activated CD4⁺ cells into the alveolar space of SCID mice reconstituted with CD4⁺ cells from Jh mice. Moreover, this corresponded to significant increases in proinflammatory cytokines including TNF- α and IFN- γ , suggesting that some functional memory cells were generated in Jh mice; however, the frequency of these memory cells was greatly diminished compared with those in WT mice.

Together our data support the model that B cells are required for later expansion and generation of memory CD4 T cells. We also have evidence that cognate interactions between B and T cells are necessary for the generation of protective CD4 effectors as MHCII^{-/-} chimeras failed to resolve PCP. However, this is in contrast to other reports that indicate that CD4 memory generation is dependent on the presence of activated B cells, regardless of specificity (14, 15). Instead, in the context of PC infection, we favor the hypothesis that cognate B-T cell interactions are required for continued expansion and differentiation of dendritic cell-primed CD4 T cells into effector cells capable of migrating to the lung and that memory cells are generated from these expanded effectors.

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Disclosures

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