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B Cells Moderate Inflammatory Progression and Enhance Bacterial Containment upon Pulmonary Challenge with Mycobacterium tuberculosis

Paul J. Maglione,* Jiayong Xu,† and John Chan2*†

Though much is known about the function of T lymphocytes in the adaptive immune response against Mycobacterium tuberculosis, comparably little is understood regarding the corresponding role of B lymphocytes. Indicating B cells as components of lymphoid neogenesis during pulmonary tuberculosis, we have identified ectopic germinal centers (GCs) in the lungs of infected mice. B cells in these pulmonary lymphoid aggregates express peanut agglutinin and GL7, two markers of GC B cells, as well as CXCR5, and migrate in response to the lymphoid-associated chemokine CXCL13 ex vivo. CXCL13 is negatively regulated by the presence of B cells, as its production is elevated in lungs of B cell-deficient (B cell−/−) mice. Upon aerosol with 100 CFU of M. tuberculosis Erdman, B cell−/− mice have exacerbated immunopathology corresponding with elevated pulmonary recruitment of neutrophils. Infected B cell−/− mice show increased production of IL-10 in the lungs, whereas IFN-γ, TNF-α, and IL-10R remain unchanged from wild type. B cell−/− mice have enhanced susceptibility to infection when aerogenically challenged with 300 CFU of M. tuberculosis corresponding with elevated bacterial burden in the lungs but not in the spleen or liver. Adoptive transfer of B cells complements the phenotypes of B cell−/− mice, confirming a role for B cells in both modulation of the host response and optimal containment of the tubercle bacillus. As components of ectopic GCs, moderators of inflammatory progression, and enhancers of local immunity against bacterial challenge, B cells may have a greater role in the host defense against M. tuberculosis than previously thought. The Journal of Immunology, 2007, 178: 7222–7234.

Tuberculosis (TB)1 has re-emerged as a major global health issue, with ~9 million new cases and 2 million deaths attributed to TB each year (1). The causative agent of TB, Mycobacterium tuberculosis, is an intracellular pathogen that requires a robust type 1 cytokine-mediated immune response for effective containment of acute infectious challenge (reviewed in Refs. 2 and 3). Despite a vigorous defense, M. tuberculosis is able to subvert host immunity and establish a persistent reservoir within the host that can cause reactivation TB when the immune system is compromised, such as by corticosteroids, TNF-α blockade, or HIV infection (4–6).

Granulomatous inflammation is the hallmark of the host response against M. tuberculosis and is thought to provide a physical barrier against bacterial dissemination (7). By focusing the local immune response in contained granulomatous reactions, the host minimizes collateral tissue damage from inflammatory processes initiated to fight infection. Destruction of lung parenchyma by an exorbitant or dysregulated host response can cause lung scarring and subsequent pulmonary disability as well as cavitation, the latter is a process by which large numbers of bacteria breach mucosal barriers resulting in a highly contagious state (8, 9). Consequently, a greater understanding of the factors that regulate the granuloma will be beneficial toward the development of novel TB therapies and vaccines aimed at stimulating protective immunity while minimizing pathologic sequelae of the host response (10).

Adaptive immunity involving sufficient T lymphocyte activity is vital to effective containment of M. tuberculosis (11, 12). However, a definitive role for B lymphocytes in host defense against M. tuberculosis has yet to be as conclusively defined. Recently, the identification of follicle-like B cell dominant structures within TB lungs of humans has suggested that B cells may play a previously unappreciated role in local immunity (13, 14). Moreover, previous work suggests that B cells influence the inflammatory progression in TB lungs (15, 16). Development of granulomatous inflammation is dependent upon regulation of specific cytokines; TNF-α is required for formation and maintenance of granulomatous architecture (5, 7, 17, 18) while overexpression of IL-10 alters the development of typical granulomas in response to mycobacterial infection (19, 20). TNF-α and IL-10 can influence the production of chemokines (17, 21), and the regulation of chemokines consequently influences the recruitment and spatial arrangement of leukocytes in TB lungs (reviewed in Refs. 22 and 23).

We report that ectopic follicles in lungs of mice infected with M. tuberculosis contain B cells expressing markers of germinal centers (GCs). Recruitment and organization of B lymphocytes in TB lungs is likely influenced by CXCL13, as these B cells express CXCR5, regulate CXCL13 production by lung cells, and undergo CXCL13-mediated chemotaxis ex vivo. Upon aerosol infection

1 Department of Microbiology and Immunology and Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461

2 Address correspondence and reprint requests to Dr. John Chan, Albert Einstein College of Medicine, Forchheimer 406, 1300 Morris Park Avenue, Bronx, NY, 10461. E-mail address: jchan@aecon.yu.edu

*Abbreviations used in this paper: TB, tuberculosis; GC, germinal center; PNA, peanut agglutinin; PPD, purified protein derivative.

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with 100 CFU of *M. tuberculosis* Erdman, pulmonary inflammation is exacerbated and corresponds with increased recruitment of neutrophils. The exacerbated inflammatory progression of B cell−/− mice correlates with elevated production of IL-10 in the lungs, while IFN-γ and TNF-α remain unaffected. Increasing the aerosol inoculum to 300 viable bacilli demonstrates enhanced susceptibility of B cell−/− mice associated with increased mortality, elevated bacterial burden in the lungs but not in the liver or spleen, and enhanced immunopathology. Remarkably, phenotypes observed in *M. tuberculosis*-infected B cell−/− mice can be reversed by adoptive transfer of B cells. Together, the data presented herein demonstrate that B cells are intricate players in the progression of TB granulomatous inflammation and can enhance immunity against *M. tuberculosis* challenge.

**Materials and Methods**

**Mice**

Female mice, C57BL/6 (Charles River Laboratories) or B cell-deficient, backcrossed to a ninth generation on C57BL/6 (muMT; The Jackson Laboratory; Ref. 24), 8- to 10-wk old, were used in all experiments. All mice were housed in a biosafety level 3 animal laboratory and maintained pathogen-free by routine serological and histopathological examinations. The Institutional Animal Care and Use Committee has approved the animal protocols used in this study.

**Mycobacteria and mouse infection**

Bacterial stock of *M. tuberculosis* strain Erdman (Dr. F. Collins, Trudeau Institute, Saranac Lake, NY) was prepared by passage through mice to maintain virulence, expansion once by culture in 7H9 liquid medium (Difco), and storage in 3 × 108 bacilli/ml aliquots at −80°C. Before infection, the aliquot was thawed, diluted 1/10 in PBS with 0.05% Tween 80 (Sigma-Aldrich), and sonicated to achieve uniform suspension. Mice were infected by aerosol using the Lovelace nebulizer (In-Tox Products) with *M. tuberculosis* diluted to a concentration calibrated to deliver −100 or 300 bacilli to the lungs. The 100 CFU dose is considered the "conventional" inoculum used in the murine tuberculosis model. Inoculum dose was confirmed by colony counts on 7H10 agar plates (Difco) of whole lung homogenates at 16–24 h post aerosol for each experiment.

**CFU enumeration**

At indicated intervals after infection, tissue bacterial burden was quantified by plating serial dilutions of lung, liver, and spleen homogenates onto 7H10 agar plates. In all experiments, two right lung lobes or approximately one-third of the lung, one-half to one-eighth of the liver, and approximately one-half of the spleen were used for enumerations of tissue bacterial burden. Bacterial burden was assessed as CFU, determined by the number of colonies on plates after incubation at 37°C for 21 days.

**Histopathological and immunohistochemical studies**

Tissue samples from lung and spleen were fixed in 10% buffered formalin and subsequently embedded in paraffin. For histopathological and immunohistochemical studies, serial 5–6 μm sections were stained with H&E or reserved for immunohistochemical staining. For immunohistochemistry, Ags were exposed using a citrate unmasking solution (Vector Laboratories). Subsequently, sections were treated with 3% H2O2 to quench endogenous peroxidase activity. Sections were then incubated at room temperature in diluted serum and avidin/biotin blocking reagents to limit nonspecific binding according to the manufacturer’s protocols (Vector Laboratories). Samples were incubated with rat anti-mouse B220 IgG2a (BD Pharmingen) or isotype control Ab, in a humidified container overnight at 4°C. For peanut agglutinin (PNA) staining, samples were incubated with biotinylated PNA (Vector Laboratories) at room temperature for 60 min. Primary Ab was detected using the Vectastain ABC kit (Vector Laboratories) and was labeled with diaminobenzidine substrate (Sigma-Aldrich). Slides were coverslipped and mounted using VectaMount permanent mounting medium (Vector Laboratories).

**Preparation of single-cell suspension of lung cells**

In all experiments, left lungs were aseptically removed, minced using sterile razor blades (Fisher Scientific), and incubated in 1 mg/ml collagenase and 30 μg/ml DNase (Sigma-Aldrich) at 37°C for 60 min. To achieve a single-cell suspension, lung fragments were pressed through a 70-μm pore nylon cell strainer using the flat end of a sterile 3-ml syringe plunger. Cells were washed twice in complete RPMI (RPMI 1640 with 1-glutamine and 25 mM HEPES, 10% FBS, and 55 μM 2-ME), and RBC were lysed by incubation in ACK lysis solution (0.156 M NH4Cl, 10 mM KHCO3, and 0.1 mM Na2EDTA (pH 7.2)) for 5 min at room temperature. Cells were then washed again in complete RPMI 1640 and counted, using trypan blue to exclude dead cells.

**Ex vivo lung cell culture and ELISA**

For ex vivo cell culture, single-cell suspension lung cells were cultured in complete RPMI alone or in complete RPMI supplemented with 10 μg/ml purified protein derivative (PPD; Statens Serum Institute, Copenhagen, Denmark). RPMI was supplemented at 1.0 × 107 cells/ml. After 48 h of culture, supernatants were harvested, filter sterilized, and stored at −20°C. Matched Ab pairs for CXCL13, IFN-γ, IL-10, and TNF-α used for ELISA were purchased from R&D Systems and used according to manufacturer’s protocol. Where indicated, B cells (>97% CD19+) purified from splenocytes from *M. tuberculosis*-infected C57BL/6 mice using CD19+ MACS beads (Miltenyi Biotec) were added per well at the initiation of culture.

**Flow cytometry**

Lung cells were washed in FACS buffer (PBS, 2% heat-inactivated FBS and 10 mM NaCl) and incubated in Fc block (BD Biosciences) to prevent nonspecific binding. Samples were immunostained using fluorescently labeled Abs for CD3, CD4, CD8, CD19, CD45, β2CR, CXCR5, GL7, IL-10R, and Ly6G (all from BD Pharmingen) and F4/80 (Caltag Laboratories). Flow cytometry data were acquired using a FACSCalibur cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Chemotaxis assay**

Suspensions of single lung cells, obtained as explained above, were washed twice in MACS buffer (PBS, 0.5% FBS, and 2 mM EDTA (pH 7.2)) and Fc-blocked on ice for 10 min. Samples were then incubated on ice with CD19 magnetic beads (Miltenyi Biotech), and B cells were purified in accordance with the manufacturer’s protocol. Purified lung B cells were re-suspended to 107 cells/ml in complete RPMI and 100 μl of this cell suspension was added to the upper chamber of a Transwell plate (Corning Costar). Lower chambers were filled with 600 μl of medium alone, medium with 1 μg/ml CXCL13 (R&D Systems), or medium with 1 μg/ml CXCL13 and 15 μg/ml anti-CXCL13 Ab (R&D Systems). After incubation of 3 h at 37°C, the number of cells in the lower well was counted by hemocytometer and confirmed to be B cells by dual expression of CD45 and CD19 on flow cytometry.

**B cell adoptive transfer study**

For each of the two adoptive transfer studies (one using an inoculum of 300 CFU and the other 100 CFU), age-matched B cell−/− mice as well as wild-type mice used as controls and for procurement of B cells for transfer were simultaneously infected in the aerosolization apparatus to ensure delivery of equal infection inoculum to all experimental groups. B cells used for adoptive transfer were immunomagnetically purified from spleens using MACS columns (Miltenyi Biotech) of wild-type *M. tuberculosis*-infected mice as explained above. Cells thus obtained were >97% CD19−, B cells were adoptively transferred into B cell−/− mice i.p. (6 × 107 to 1.5 × 108 B cells on days 7, 14, and 22 postinfection). These time points were chosen because our previous studies have shown that the number of B cells infiltrating the lungs of *M. tuberculosis*-infected mice increases rapidly during the first 4 wk of infection (14).

**Statistical analysis**

Statistical significance was assessed using the unpaired Student *t* test, calculated using Prism 4 software (GraphPad). Values of *p* < 0.05 were considered significant.

**Results**

*B* cells in the lungs express markers of ectopic GCs during pulmonary TB

The goal of this study was to gain greater understanding of how B lymphocytes participate in the host response during pulmonary *M. tuberculosis* infection. Recently, follicle-like arrangements of B cells that appear to be local sites of immune proliferation have been identified in lungs of both humans and mice with pulmonary
TB (13, 14, 16). Comparably, B cell follicles develop in the lungs of mice with respiratory influenza virus infection (25). The follicles that emerge in response to influenza challenge contain GC reactions and are a component of ectopic lymphoid tissue that primes successful influenza immunity even in the absence of secondary lymphoid organs (25). The development of lymphoid tissue in nonlymphoid organs, known as ectopic or tertiary lymphogenesis, which occurs in a broad range of autoimmune and infectious diseases (26), has yet to be conclusively identified as a characteristic of pulmonary TB.

FIGURE 1. Markers of ectopic GCs are expressed in TB lungs. Wild-type or B cell−/− mice were infected aerogenically with 100 CFU of *M. tuberculosis* Erdman. A, Sections from the same experiment taken 90 days after aerosol were stained for the B cell marker B220, left column, and corresponding serial sections were stained for the GC marker PNA, middle column, or with H&E, right column. *, B220+ aggregates; #, PNA+ GC B cells. B cell−/− lung and wild-type spleen sections are provided as negative and positive controls, respectively. B, Using flow cytometry, GL7+ βTCR+ cells were detected in the lungs and spleens of wild-type mice but were markedly diminished in B cell−/− mice.
CXCL13 influences B cell chemotaxis in TB lungs. A, CXCR5 is expressed on the surface of B cells in TB lungs. The percentage of CXCR5 expressing CD45<sup>−</sup>CD19<sup>+</sup> B cells in TB lungs (left) of wild-type C57BL/6 mice infected with 100 CFU of M. tuberculosis Erdman is similar to that found in the spleen at the same time point (right). B, B cells isolated from TB lungs migrate in response to CXCL13. Values expressed as percentage of total B cell input (***, p < 0.001 between samples). C, Production of CXCL13 is elevated in ex vivo lung supernatants of B cell<sup>−/−</sup> mice and acts as a chemoattractant for B cells isolated from TB lungs.

CXCL13 is produced at elevated levels in M. tuberculosis-infected lungs of B cell<sup>−/−</sup> mice and acts as a chemoattractant for B cells isolated from TB lungs.

It has been previously demonstrated that the B cell chemoattractant CXCL13 is required for proper recruitment and organization of B cells in the follicles of lymph nodes and spleen (29). In addition, ectopic expression of CXCL13 is sufficient to promote B cell migration and the development of organized lymphoid tissue outside the secondary lymphoid organs (30). Therefore, the interaction of CXCR5 and CXCL13 in B cell chemotaxis is a characteristic component of the GC reaction. For these reasons, we turned our focus upon the B cell chemoattractant CXCL13 to examine whether this chemokine could be influencing the development of B cell follicles in the lungs during pulmonary TB.

First, we examined whether the CXCL13 receptor, CXCR5, was expressed on B cells in TB lungs. At 30 days after infection, B cells in TB lungs expressed CXCR5 at levels similar to that of B cells in the spleen (Fig. 2A). No significant difference in CXCR5 expression was observed on B cells isolated from TB lungs 150 days after infection as compared with 30 days (data not shown). We then tested whether migration of these B cells can be triggered by CXCL13 ex vivo. Using a Transwell assay, we noted that B cells isolated from TB lungs migrated to CXCL13 and chemotaxis by CXCL13 ex vivo. Using ex vivo lung cell culture, we found that cells from TB lungs 30 days after infection produce CXCL13 and the levels of this chemokine are elevated in B cell<sup>−/−</sup> mice (Fig. 2C). Addition of B cells to ex vivo cultures of B cell<sup>−/−</sup> lung cells appeared to diminish levels of CXCL13 slightly after 48-h coincubation, but receiving medium only or CXCL13 in the bottom well and between those with CXCL13 plus neutralizing Ab or CXCL13 alone in the bottom well).

D, Expression of CXCR5 is elevated on the surface of CD45<sup>+</sup> total leukocytes and CD45<sup>−</sup>CD4<sup>+</sup> double-positive leukocytes in B cell<sup>−/−</sup> lungs compared with wild type. Data in this figure are representative of two independent experiments.

The presence of GC reactions outside the secondary lymphoid organs is a hallmark of tertiary lymphogenesis. To assess whether the follicle-like structures of pulmonary TB are the product of tertiary lymphogenesis, we looked for the emergence of B220<sup>−</sup>PNA<sup>−</sup> GC B cells in the lungs. PNA is a lectin that binds to a carbohydrate moiety expressed by GC B cells (27). B220<sup>−</sup>PNA<sup>−</sup> GC B cells were detected in lymphoid follicles within the lungs as well as the spleen 90 days after infection, while both B220 and PNA staining was absent from lungs of B cell<sup>−/−</sup> mice at the same time point (Fig. 1A).

The Ab GL7 is used to detect a surface protein expressed by GC B cells (28). To further test whether ectopic GCs formed in TB lungs, we looked for expression of the GC B cell marker GL7 by flow cytometry. We excluded T lymphocytes, another cellular population that can express GL7, from our analysis on the basis of βTCR expression. 30 days after aerosol infection, GL7<sup>+</sup>βTCR<sup>−</sup> cells were detected in the lungs and spleens of wild-type mice but were absent from B cell<sup>−/−</sup> mice at this same time point (Fig. 1B). The GL7<sup>+</sup>βTCR<sup>−</sup> cells were further characterized as expressing the B cell marker CD19 (data not shown). The presence of both PNA<sup>−</sup> and GL7<sup>+</sup> B cells in TB lungs strongly suggests that ectopic GCs form in the lungs of mice upon pulmonary M. tuberculosis infection.
analysis did not reveal statistical significance. To examine whether the elevation of CXCL13 in lung cell culture supernatants could be attributed to a reduced availability of its receptor in the absence of B cells, we investigated whether CXCR5 was expressed in the lungs of B cell−/− mice. Surprisingly, CXCR5 is expressed by CD45+ leukocytes in the lungs of B cell−/− mice at levels even greater than in wild-type mice (Fig. 2D). Similarly, CD45+CD4+ cells also express CXCR5 at higher levels in wild-type lungs than B cell−/− (Fig. 2D). Thus, the increased production of CXCL13 in B cell−/− lungs is not the result of diminished availability of CXCR5. Collectively, these results revealed that the CXCR5-expressing B cells in the lungs of M. tuberculosis-infected mice are responsive to CXCL13, the latter produced by cells from pulmonic tuberculous granulomas. The in vivo role of the CXCL13-CXCR5 interaction in the formation of the lymphoid nodules in the lungs of tuberculous mice remains to be evaluated.

B cells influence the progression of granulomatous histology and leukocyte recruitment in TB lungs, without influencing the capacity to make IFN-γ

The development of lung pathology is delayed in the absence of B cells after aerosol infection with CDC 1551, a clinical isolate of M. tuberculosis (15). Using the virulent Erdman strain of M. tuberculosis maintained in our laboratory, we challenged B cell−/− mice.

### Table I. Grading of pulmonary inflammation

<table>
<thead>
<tr>
<th>Inflammatory Grade</th>
<th>Description</th>
<th>Wild Type 50–100 CFU</th>
<th>B Cell−/− 50–100 CFU</th>
<th>Wild Type 300 CFU</th>
<th>B Cell−/− 300 CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>Inflammation diffusely distributed throughout the lung, inflammatory reactions exceeding 5 μm in diameter, significant areas of uninfiltred lung parenchyma</td>
<td>3/36 (8.3%)</td>
<td>8/30 (27%)</td>
<td>12/47 (26%)</td>
<td>34/55 (62%)</td>
</tr>
<tr>
<td>II</td>
<td>Inflammation contained within lesions ≤5 μm or less in diameter, significant areas of uninfiltred lung parenchyma</td>
<td>21/36 (58%)</td>
<td>21/30 (70%)</td>
<td>33/47 (70%)</td>
<td>20/55 (36%)</td>
</tr>
<tr>
<td>I</td>
<td>Minimal inflammation, sparse lymphocytes, absence of apparent granulomatous lesions</td>
<td>12/36 (33%)</td>
<td>1/30 (3.3%)</td>
<td>2/47 (4.3%)</td>
<td>1/55 (1.8%)</td>
</tr>
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*H&E-stained lung sections cut 30 days after aerosol with the indicated dose were used to grade inflammatory progression. Incidence of inflammatory grade for individual 10× fields as a fraction of total from three identical experiments, three to four mice per group.

**FIGURE 3.** Granulomatous inflammation is dysregulated in the absence of B cells, but IFN-γ production in the lungs is unaffected. A, H&E-stained lung sections 30 days after an aerosol infection with 100 CFU of M. tuberculosis Erdman demonstrating exacerbated pulmonary inflammation in the absence of B cells. Representative sections from wild type demonstrating typical granulomatous progression and sections from B cell−/− mice demonstrating severe grade inflammation. Sections displayed in this figure are representative of three similar experiments conducted with 3–4 mice per group. B, Quantitation of leukocyte infiltration of lungs by flow cytometry 30 days after aerosol infection. B cell−/− mice had significant elevations in the recruitment of total CD3+ T cells, CD3+CD8+ T cells, and Ly6G+CD4+80− neutrophils (*, p < 0.05) but not CD3+CD4+ T cells. C, Measurement of IFN-γ production by ELISA of ex vivo lung cell culture 30 days after aerosol infection. Data in this figure are representative of three identical experiments with similar results, three to four mice per group.
mice by aerosol with 100 bacilli to examine whether the progression of lung pathology was similar to that observed for the CDC 1551 study. Extensive examination of lung sections from three independent experiments revealed that, upon 100 bacilli aerosol challenge, B cell−/− lungs showed exacerbated immunopathology characterized by greater number of infiltrative lesions exceeding 5 μm in diameter (wild-type mice, 8.3%; B cell−/− mice, 27%), categorized as grade III lesions in our system (Table I), as well as higher instances of grade II lesions (infiltrate diameter of 5 μm or less) (wild-type mice, 58% vs B cell−/− mice 70%, Table I). In contrast, wild-type lungs display a higher number of grade I inflammation, defined as minimal pulmonary immunopathology without apparent granulomatous reactions (wild-type mice, 33% vs B cell−/− mice, 3.3%). After airborne challenge with M. tuberculosis Erdman, B cell−/− mice have a greater propensity toward severe pulmonary inflammation than wild-type mice (Fig. 3A).

Concurrently, we used flow cytometry to examine whether overall leukocyte recruitment into lungs was altered in B cell−/− mice infected with M. tuberculosis Erdman. We quantified numbers of total CD3+ T cells, as well as the CD3+CD4+ and the CD3+CD8+ T cell subtypes. Neutrophils were quantified as those cells expressing the granulocyte marker Ly6G but not the macrophage marker F4/80, as the Ly6G Ab may cross-react with Ly6C expressed by activated macrophages (31). At 30 days after infection, we found significant increases in the numbers of total CD3+ T cells and CD3+CD8+ T cells, but not CD3+ CD4+ T cells in the lungs of B cell−/− mice (Fig. 3B). B cell−/− mouse lungs also had significantly elevated Ly6G+, F4/80− neutrophils when compared with wild type (Fig. 3B). These findings demonstrate a significant increase in the proportion of CD8+ T cells and neutrophils in TB lungs in association with the granulomatous dysregulation observed histologically in the absence of B cells.

To examine cytokine production inherent in the local immune response of TB lungs, we used ex vivo culture of equalized numbers of lung cells stimulated with M. tuberculosis PPD. Ex vivo culture demonstrated that IFN-γ productive capacity remains unaltered in lungs of B cell−/− mice (Fig. 3C), a finding in support of a previous study which reported no difference in IFN-γ mRNA in the lungs of M. tuberculosis-infected B cell−/− mice (32).

The presence of B cells influences IL-10, but not TNF-α, production in TB lungs

Previous work has implicated TNF-α as an important mediator of granuloma formation during M. tuberculosis infection (5, 7, 17, 18). Thus, we were curious to test whether changes in TNF-α production in B cell−/− lungs correlated with the dysregulated granulomatous histology observed in these mice. No significant difference in TNF-α productive capacity was detected between wild-type and B cell−/− mice 30 days after infection using equalized numbers of lung cells (Fig. 4A).

Overexpression of IL-10 by transgenic mice leads to atypical granuloma development in response to mycobacteria infection (19, 20). To examine whether there was a similar correlation between IL-10 in the lungs and the granulomatous dysregulation of B cell−/− mice, we examined whether IL-10 production was elevated in the absence of B cells. Indeed, B cell−/− lung cells had increased IL-10 productive capacity relative to cell number-matched wild-type cultures 30 days after aerosol (Fig. 4B). To rule out that elevated IL-10 levels were simply the result of diminished availability of the IL-10R resulting from the absence of B cells, we used flow cytometry to compare surface expression of IL-10R in the lungs of wild-type and B cell−/− mice 30 days after aerosol.

Levels of IL-10R expression were not significantly different between the two groups of mice (Fig. 4C), indicating that the elevated IL-10 production of B cell−/− mice was not the result of the reduced availability of IL-10R. The addition of B cells to ex vivo lung cells was significantly elevated compared with controls (*, p < 0.05) as detected by ELISA of ex vivo lung cell culture. C, IL-10R expression by CD45− leukocytes in TB lungs was not significantly different between wild-type and B cell−/−. D, Addition of B cells to B cell−/− lung cell culture ex vivo did not significantly affect IL-10 production (p > 0.05). This figure incorporates data from three identical experiments, three to four mice per group.

Collectively, the data presented thus far have provided evidence that the granulomatous reaction in B cell−/− mice infected with 100 bacilli of M. tuberculosis Erdman is remarkably different from that observed in the wild-type TB host, as assessed by the levels of pulmonary inflammation, the compositions of infiltrating immune cells, as well as the cytokine and chemokine milieu. These observations are not the result of elevated bacterial burden, as pulmonary bacterial numbers at the time of analysis (30 days after infection) are similar between these strains after aerosol with 100 CFU of M. tuberculosis (data not shown).

B cell−/− mice have elevated susceptibility and increased immunopathology upon 300 bacilli aerosol with M. tuberculosis Erdman

Accumulating evidence suggests that the host response to M. tuberculosis in the mouse is more robust than necessary for adequate control of infection (33). In addition, overlapping defense mechanisms may compensate for one another, prohibiting the evaluation of a single immunological component of the host for its relative
FIGURE 5. B cell−/− mice are more susceptible to 300 CFU aerosol challenge and show elevated bacterial burden as well as increased immunopathology in the lungs. A, B cell−/− mice have diminished survival after 300 bacilli aerosol. B, After 300 bacilli aerosol, B cell−/− mice have elevated bacterial burden in lungs 4 wk after infection (∗, p < 0.05) but no significant differences in spleen or liver (ns, p ≥ 0.05). C, Measurement of IFN-γ production by lung cell ELISA 3 wk after aerosol. No significant differences between wild type and B cell−/− were detected. PPD-stimulated cultures are shown. D, Quantitation of neutrophil infiltration of lungs by flow cytometry 3 wk after 300 bacilli aerosol. B cell−/− mice had significant elevations in neutrophils (∗, p < 0.05). E, H&E-stained lung sections 4 wk after 300 bacilli aerosol demonstrating exacerbated immunopathology of B cell−/− lungs. Data in this figure represent three identical experiments with similar results.

Significance in protection against a pathogen. We postulated that elevating the infection dose might be helpful toward exposing the importance of individual immune components that may not be apparent in an infection caused by a lower initial dose, due to compensatory protective functions of host defense (34). In the case of B cells, intensifying pulmonary inflammation by using a higher infection inoculum may also allow better assessment of the role of these lymphocytes in regulating the granulomatous response to *M. tuberculosis*. For these reasons, we decided to evaluate the response of B cell−/− mice after aerogenic challenge with 300 CFU.

Upon 300 CFU aerosol infection with *M. tuberculosis* Erdman, B cell−/− mice are more susceptible than wild-type with approximately one-third of the B cell−/− group succumbing to disease by 6 wk after infection (Fig. 5A). Although the wild-type mice remain well throughout the study, infected B cell−/− animals display signs of illness beginning 3–4 wk postinfection. Correlating with this increased susceptibility, B cell−/− mice have significantly elevated bacterial burden in the lungs, but not the liver or spleen, 30 days after challenge with 300 CFU by aerosol (Fig. 5B). These data, which have been observed in four independent experiments, suggest that B cells are required for optimal containment of *M. tuberculosis* in the lungs during pulmonary TB.

Despite the increased susceptibility of B cell−/− mice, IFN-γ productive capacity in the lungs remained at levels similar to that of wild type (Fig. 5C), indicating that the enhanced susceptibility of this mouse strain was not the result of an inability to secrete adequate levels of IFN-γ. As in the 100 CFU model, pulmonary influx of neutrophils was increased in B cell−/− mice compared with wild type, but to a greater extent after aerogenic challenge with 300 CFU (100% increase; Figs. 3B and 5D). Analysis of the pulmonic response of B cell−/− mice infected with 300 CFU of *M. tuberculosis* revealed histological evidence of granulomatous dysregulation similar to that observed in the 100 CFU infection model, with many B cell−/− mice demonstrating exacerbated immunopathology relative to wild type (Fig. 5E). Thirty days after infection with 300 CFU of *M. tuberculosis* Erdman, B cell−/− lungs are far more likely to demonstrate severe pulmonary inflammation by our grading system (grade III lesions: B cell−/− mice, 62% vs wild-type mice, 26%) while wild-type lungs are more inclined to have typical, contained granulomatous reactions of 5 μm or less (combined grade I and II lesions: B cell−/− mice, 37.8% vs wild type, 74.3%, Table I). Thus, similar characteristics of granulomatous dysregulation were observed in lungs of B cell−/− mice after a 100 or 300 CFU
challenge, albeit with a seemingly more severe phenotype associated with the higher bacterial dose. However, because the lung bacterial burden in the B cell⁻/⁻ mice infected with 300 viable bacilli is significantly higher than that in the wild type, B cell⁻/⁻ mice, the interpretation of the changes in the pulmonic granulomatous response observed in the B cell-deficient mice in this higher inoculum model is not straightforward. Nevertheless, it is clear from the susceptibility of B cell⁻/⁻ mice upon airborne challenge with 300 CFU that B cells can enhance protection against the tubercle bacillus.

Adoptive transfer of B cells into B cell⁻/⁻ mice reverses the enhanced susceptibility and aberrant granulomatous response upon aerosol infection with M. tuberculosis Erdman.

To more conclusively demonstrate the influence of B cells in the protection of mice against pulmonary M. tuberculosis challenge,

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Adoptive transfer of B cells protects B cell⁻/⁻ mice upon 300 CFU aerosol with *M. tuberculosis* Erdman. A, Percent of mice surviving until day 27 after aerosol. B cell⁻/⁻ mice treated with adaptively transferred B cells did not show diminished survival upon 300 CFU challenge as untreated B cell⁻/⁻ mice. B, Adoptively transferred B cell⁻/⁻ mice did not show elevated bacterial burden in the lungs as was present in untreated B cell⁻/⁻ mice. C, Adoptive transfer of B cells diminished elevated neutrophilia of B cell⁻/⁻ mice. D, Pulmonary recruitment of T cells remained largely unchanged upon adoptive transfer of B cells. E, IL-10 production in lungs of wild type, B cell⁻/⁻, and adoptively transferred B cell⁻/⁻ 32 days after 100 CFU aerosol. Data in this figure incorporate data from two experiments, five mice per group.

**Table II. Grading of pulmonary inflammation in adoptive transfer experiments**

<table>
<thead>
<tr>
<th>Inflammatory Grade</th>
<th>Description</th>
<th>Wild Type</th>
<th>B Cell⁻/⁻</th>
<th>B Cell⁻/⁻ + B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>Inflammation diffusely distributed throughout the lung, inflammatory reactions exceeding 5 μm or less in diameter, significant areas of uninfiltired lung parenchyma</td>
<td>4/12 (33%)</td>
<td>16/20 (80%)</td>
<td>6/22 (27%)</td>
</tr>
<tr>
<td>II</td>
<td>Inflammation contained within lesions — 5 mm or less in diameter, significant areas of uninfiltired lung parenchyma</td>
<td>6/12 (50%)</td>
<td>4/20 (20%)</td>
<td>15/22 (68%)</td>
</tr>
<tr>
<td>I</td>
<td>Minimal inflammation, sparse lymphocytes, absence of apparent granulomatosus lesions</td>
<td>2/12 (17%)</td>
<td>0/20 (0%)</td>
<td>1/22 (4.5%)</td>
</tr>
</tbody>
</table>

* H&E-stained lung sections cut 28 days after aerosol with 350 CFU of *M. tuberculosis* Erdman were used to grade inflammatory progression. Incidence of inflammatory grade for individual 10× fields as a fraction of total, four to five mice per group.
FIGURE 7. Adoptively transferred B cells are detected in spleens of B cell−/− mice and result in detectable levels of serum IgM and IgG. A, CD19+ and βTCR− cells were detected in the spleens but not the lungs of adoptively transferred B cell−/− mice. B, B220+ B cells formed miniature follicle-like structures in the spleens of adoptively transferred B cell−/− mice. C, Serum IgM and IgG were detected in adoptively transferred but not untreated B cell−/− mice.
we used an adoptive transfer model. Adoptive transfer of B cells enhanced survival of B cell−/− mice challenged with 300 CFU of *M. tuberculosis*, as none of these mice succumbed to infection at 27 days post aerosol, while there is a 40% mortality observed in the group not receiving B cells (Fig. 6A). This enhanced survival in adoptive transfer recipient B cell−/− mice is associated with decreased lung bacterial burden (Fig. 6B; B cell−/− mice vs adoptively transferred B cell−/− mice; p < 0.05). Additionally, the elevated pulmonary neutrophilia of B cell−/− mice returned to wild-type levels after adoptive transfer of B cells (Fig. 6C). Levels of CD3+ T cells were relatively unchanged in B cell−/− mice after adoptive transfer, indicating that the enhanced protection of these treated mice was not the result of changes in T cell numbers (Fig. 6D). Strikingly, all B cell−/− mice adoptively transferred with B cells had graded granulomatous progression comparable to that of wild type, while untreated B cell−/− mice exhibited exacerbated immunopathology (Table II). In a second adoptive transfer experiment, the effect of B cell transfer upon the phenotype of enhanced IL-10 production by lung cells of *M. tuberculosis*-infected B cell−/− mice was examined. This second study used the more conventional 100 CFU inoculum to evaluate whether B cell transfer can rescue anomalies observed in B cell−/− mice in both the 100 CFU and the 300 CFU models. Adoptively transferring B cells into B cell−/− mice after 100 CFU challenge remarkably lowered the production of IL-10 in the lungs down to levels comparable to those observed in infected wild types (Fig. 6E). Collectively, these results indicate that adoptively transferred B cells could complement phenotypes of tuberculous B cell−/− mice at both the 100 and 300 CFU models. These observations strongly suggest that the enhanced susceptibility and the dysregulated granulomatous reaction observed in the B cell−/− mice is indeed caused by the absence of B cells.

We performed flow cytometry to assess whether the protective effect of adoptively transferred B cells upon B cell−/− mice required the presence of B cells locally within the lungs. CD19+ B cells were not detected in the lungs of untreated or adoptively transferred B cell−/− mice by flow cytometry 27 days postinfection, but were detected in the spleens of adoptively transferred mice while remaining absent in the untreated B cell−/− mice (Fig. 7A). Remarkably, using immunohistochemistry, we noted that B220+ B cells were present in small aggregate clusters within the spleens of recipient mice, appearing as miniature counterparts to the splenic follicles seen in wild-type mice (Fig. 7B). To access whether these adoptively transferred B cells were making Ab, we measured serum levels of IgM and IgG and noted that adoptively transferred B cell−/− mice made detectable levels of both IgG and IgM, albeit at amounts that are ~10% that of wild type (Fig. 7C). Thus, adoptively transferred B cells that reverse the B cell−/− phenotypes in recipient mice were present in the spleens, but not lungs, and produced both IgM and IgG.

**Discussion**

Currently, most who study adaptive immunity of TB focus upon T lymphocytes, appropriately so because T cells have been well-established as vital for optimal containment of *M. tuberculosis*. B cells, in contrast, have been generally disregarded as important components within the TB immune response because of controversy regarding the role of Abs in engendering protection coupled with discrepant results observed in various murine TB models involving B cell−/− mice (32, 35). However, recent studies have demonstrated that B cells influence inflammatory progression (15) and are contiguous with sites of cellular proliferation (13) within TB lungs, suggesting that regulation of the local host response in the lungs may be a novel mechanism by which these lymphocytes affect TB immunity. In this study, we investigated the characteristics and the kinetics of the formation and progression of B cell follicles in TB lungs as well as the effects of B cell deficiency on host response to *M. tuberculosis*. The results have provided evidence indicating that B cells, present within infected lungs as tertiary lymphoid nodules, optimize the local immune response in the lungs during *M. tuberculosis* infection.

Previous studies have identified the emergence of GC reactions outside of the secondary lymphoid tissue as an indication of tertiary lymphogenesis in autoimmune and infectious disease (25, 36, 37). Because follicle-like structures consisting predominantly of B cells have been identified in TB lungs of both humans and mice by our laboratory and others (13, 14, 16), we wanted to look for evidence of lymphoid neogenesis in TB lungs through the identification of GC reactions. GC B cells were detected in TB lungs by B220+ PNA+ immunohistochemical staining. Concurrently, B cells in TB lungs express the GC B cell marker GL7, lending further support for a B cell component of tertiary lymphogenesis in TB lungs. Our work also strongly supports a role for CXCL13 in B cell-related granulomatous progression as its receptor, CXCXR5, is expressed by B cells in TB lungs, which migrate in response to CXCL13 ex vivo, and the regulation of this chemokine is altered in B cell−/− mice. The B cell chemoattractant CXCL13 is strongly associated with lymphoid neogenesis, a process that can be induced ectopically by the expression of this chemokine alone (30). Therefore, our data suggest that progressive lymphoid neogenesis occurs in the lungs during chronic pulmonary TB, ultimately resulting in the development of ectopic B cell follicles that contain GCs. Because GCs represent an immunological compartment in which various immune cells interact and Ag-specific B cell maturation occurs (38, 39), B cell follicles in TB lungs likely contribute significantly to the host response to *M. tuberculosis*.

Because most cellular proliferation within TB lungs, as detected by Ki67 staining, occurs in the vicinity of B cell follicles (13), we examined how the architecture of the local immune response can be affected by the absence of B cells. Previously, using the CDC 1551 clinical isolate of *M. tuberculosis*, it was reported that pulmonary inflammatory progression was delayed in B cell−/− mice (15). Using the virulent *M. tuberculosis* Erdman strain, evidence of delayed inflammatory progression was not apparent in infected B cell−/− mice as reported in the study using CDC 1551. Instead, we observed that B cell−/− mice infected with the Erdman strain for 30 days displayed exacerbated inflammatory reactions in the lungs compared with wild type (Fig. 2 and Table I). As lung bacterial burden of the *M. tuberculosis*-infected B cell−/− and wild-type mice upon 100 CFU challenge are comparable, this granulomatous pathology is specific for a deficiency in B cells and not due to inhibited bacterial containment. There is prior evidence suggesting that B cells may limit damage caused by the host response, as the granulomatous pneumonia of *M. tuberculosis*-infected mice upon certain therapeutic vaccination is dramatically worsened in B cell−/− mice (40). The mechanisms underlying the exacerbated pulmonary inflammatory response in *M. tuberculosis*-infected B cell−/− mice are unclear at present. Understanding of such mechanisms may shed light on how B lymphocytes affect the TB host response. The fact that our findings using *M. tuberculosis* Erdman deviate from those reported using CDC 1551 emphasizes that the inflammatory progression can vary dependent upon the strain of *M. tuberculosis* used in the experiment (41) and underscores the complexity of interpreting data from different strains. Indeed, CDC 1551 has been reported to induce a more vigorous host response compared with other strains of *M. tuberculosis*, including Erdman (42). Relevant to the present study, a previous study involving the
CDC 1551 strain has reported that B cells modulate the dissemination of bacilli (15). This dissemination phenotype is not apparent in the present study, which uses the Erdman strain of *M. tuberculosis*. The reason underlying the discrepant dissemination result between the CDC1551 study and ours is unclear but could also be due to the differences in the nature of the two strains (41, 42). Nevertheless, whether infecting with CDC 1551 or Erdman, it remains clear that B cells influence the local host response in the lungs as typical granulomatous progression is disrupted in the absence of B cells during infection with either strain (Ref. 15 and our data).

In conjunction with the exacerbated inflammation observed histologically upon aerosol infection with *M. tuberculosis* Erdman, we found that the absence of B cells elevates the number of particular subsets of leukocytes recruited to the lungs. At 30 days postinfection, there was an elevation of total CD3+ T cells and Ly6G+Ly480- neutrophils within the lungs of B cell−/− mice. Notably, there is a significant increase of CD8+ T cells in B cell−/− lungs, which may be the result of the host response to the exacerbated inflammation observed in the infected B cell−/− mice, as CD8+ T cells have been reported to protect against inflammatory pathology in murine models of *Pneumocystis* and *Myco- plasma* infection (43, 44). An alternative explanation is that in the absence of B cells, there is an increased expansion or migration of neutrophils and CD8+ T cells that manifests as increased numbers in the lungs. Neutrophilia has been reported in infections of B cell−/− mice with other intracellular pathogens, such as *Francisella* and *Leishmania* (45, 46). The role of neutrophils in TB is controversial, though these leukocytes can amply interact with various immune components of the granuloma including B cells (47). The relevance and significance of the elevated neutrophilia in B cell−/− mice during *M. tuberculosis*-infection remain to be evaluated. However, an increase in neutrophils is associated with more susceptible mouse strains as well as TB patients before treatment (13, 48, 49). The lack of any significant differences in IFN-γ production despite these changes in lung histopathology and cellular composition in the absence of B cells indicates that in this model, granuloma regulation may not necessarily influence the capacity to produce IFN-γ and, conversely, levels of this cytokine may not always correlate with alterations in granuloma integrity.

IL-10 and TNF-α influence the development of granulomas, so we hypothesized that the granulomatous dysregulation observed in the absence of B cells would be associated with altered production of one or both of these cytokines. In our study we found that IL-10 production, but not that of TNF-α, is regulated by the presence of B cells. IL-10 production by lung cells obtained from B cell−/− mice is elevated compared with leukocytes from wild type 30 days after infection, but TNF-α remained unchanged. These findings did not necessarily prove a direct relationship between elevated IL-10 and B cells, as the elevation in production of the anti-inflammatory cytokine IL-10 may be a protective reaction to the exacerbated inflammation observed in the absence of B cells rather than a cause of granulomatous dysregulation. Addition of B cells to ex vivo culture of B cell−/− lung cells could not revert the IL-10 phenotype. However, adoptive transfer of B cells into B cell−/− mice diminishes IL-10 production of the recipients to levels comparable to that of wild types. Our finding of elevated IL-10 production in the absence of B cells supports previous work with CBA xid mice, which lack the tyrosine kinase Btk. In this mouse strain that has B cell dysfunction, there is a similar elevation in IL-10 during *M. tuberculosis* infection (50). Because induction of IL-10 is thought to be a mechanism by which *M. tuberculosis* subverts the immune response (51) and IL-10 is associated with TB susceptibility in humans (52, 53), augmenting the putative mechanisms by which B cells down-regulate IL-10 offers an intriguing possibility for therapeutic intervention. Collectively, the data generated from the 100 CFU inoculum model have revealed that the TB granulomatous response in the lungs of B cell−/− mice, compared with that of wild-type animals, is aberrant in terms of histological appearance, cellular composition, and cytokine and chemokine production. As the lung bacterial burden is comparable between the B cell−/− and wild-type mice, the aberrant granulomatous response observed in the B cell−/− mice is not the result of differing bacillary load. Therefore, these results strongly indicate that B cells significantly influence the granulomatous reaction during *M. tuberculosis* infection.

In the attempt to unmask functions of B cells not apparent in the 100 CFU model, we elevated the initial aerosol dose of *M. tuberculosis* Erdman to 300 CFU and found that B cells were required for optimal survival at this dose. By 6 wk after infection with 300 CFU, approximately one-third of B cell−/− mice succumbed to infection. The exact explanation as to why only a subgroup of B cell−/− mice infected with 300 CFU of *M. tuberculosis* Erdman succumbed to the infection, unless adoptively transferred with B cells, is currently unknown. We have found that *M. tuberculosis*-infected B cell−/− mice that survive beyond the first 6 wk of infection enter a period of several months of chronic but stable bacterial persistence (P. J. Maglione and J. Chan, unpublished observations). Eventually, these chronically infected B cell−/− mice succumb sooner than wild-type animals, with a swifter and more severe disease reactivation. These results suggest that the role of B cells during TB infection may be phase specific. Along these lines, it is possible that B lymphocytes may be crucial to limiting the host-damaging immune pathology as a result of the vigorous host response that mounts upon initial *M. tuberculosis* challenge and during the reactivation of chronic TB. Alternatively, B cells may be required for engendering optimal bacterial containment during the acute and reactivation phases of infection, which in turn, limits resultant pathology.

Though, to our knowledge, this is the first report of heightened susceptibility of B cell−/− mice to aerosol infection with *M. tuberculosis*, it has been previously reported that B cell−/− mice have enhanced susceptibility in an i.v. model of infection with a relatively high infection dose (35). The fact that an elevated inoculum was needed to demonstrate the requirement of B cells for optimal survival may indicate that facets of the host response that can compensate for B cell deficiency at a lower dose of infection may be overwhelmed when the host is challenged with a higher inoculum. This dose-dependent phenomenon has been reported previously in mouse models of TB in the absence of particular components of the immune system (33, 54). Curiously, the enhanced susceptibility of B cell−/− mice to 300 CFU aerosol corresponded with elevated bacterial burden in the lungs but not in the liver or spleen, indicating a particular tissue-specific enhancement effect of B cells upon the local immune response of the lungs. The increased susceptibility of B cell−/− mice to *M. tuberculosis* observed in the 300 CFU model is not associated with a diminished capacity to produce IFN-γ in the lungs, demonstrating that the development and pulmonary recruitment of IFN-γ-producing leukocytes was unimpeded in the absence of B cells. These findings indicate that in the 300 CFU model, B cells uniquely enhance local TB immunity of the lungs and are required for the development of optimal protection during the acute phase of infection.

Collectively, the results of B cell−/− mouse studies have provided strong evidence that B cells play a significant role in regulating the TB granulomatous response by influencing the level of inflammation, the nature of the cellular infiltrates, as well as cytokine and chemokine production. This regulatory role of B cells
during TB infection is further reinforced by results of the adoptive transfer studies. Significantly, transfer of B cells derived from spleens of \textit{M. tuberculosis}-infected wild-type C57BL/6 resulted in remarkable reversal of the enhanced susceptibility of B cell/−/− mice (as assessed by mortality and lung bacterial burden), hyperproduction of IL-10, and pulmonic neutrophilia observed in the untreated B cell/−/− strain. Interestingly, the presence of B cells in the lungs within this early time frame of infection (up to 27 days) is not required for protection, as no CD19/− B cells were detected in the lungs of adaptively transferred mice. However, B cells were noted in the spleen upon adoptive transfer in arrangements reminiscent of mature follicles and recipient mice are capable of producing both IgG and IgM at levels in the serum that are ~10% of those detected in infected wild types. These data strongly suggest that B cells can operate at sites distant from the lung to promote optimal protective host response, perhaps in an endocrine manner via the production of circulating Abs, cytokines, or other soluble factors. It is noteworthy that adoptive transfer of B cells using a protocol similar to the one described in the present study may provide a system that allows the replenishment of Ig-producing B cells in B cell/−/− mice. Finally, coupled with data demonstrating unaffected IFN-γ production in the B cell/−/− mice, the adoptive transfer studies strongly indicate a B cell-intrinsic deficit in these mice upon \textit{M. tuberculosis} infection rather than a potential deficiency in B cell-dependent T cell development (55), as noted in studies of other diseases using the JhD B cell-deficient mouse strain (56, 57). In summary, the results of this work support an emerging body of data demonstrating that B cells have a greater contribution to TB immunity than previously thought and play a significant role in optimizing the host response against \textit{M. tuberculosis}. Further experimentation designed to characterize the exact mechanism by which B cells regulate the granulomatous reaction during TB infection may shed light on their requirement for the development of optimal antituberculosis immunity in the host.

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Disclosures

The authors have no financial conflict of interest.

References


