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μ -Chain–Deficient Mice Possess B-1 Cells and Produce IgG and IgE, but Not IgA, following Systemic Sensitization and Inhalational Challenge in a Fungal Asthma Model

Sumit Ghosh, Scott A. Hoselton, and Jane M. Schuh

Allergic bronchopulmonary aspergillosis is often difficult to treat and results in morbidity associated with chronic airway changes. This study assessed the requirement for B cells and their products in the allergic pulmonary phenotype in a murine model of fungal allergic asthma that mimics allergic bronchopulmonary aspergillosis. C57BL/6 and μ MT mice (assumed to lack peripheral B cells) were sensitized with *Aspergillus fumigatus* extract and challenged with two inhalation exposures of live conidia to induce airway disease. Airway hyperresponsiveness after methacholine challenge, peribronchovascular inflammation, goblet cell metaplasia, and fibrotic remodeling of the airways was similar between μ MT mice and their wild-type counterparts (C57BL/6). Surprisingly, even in the absence of the μ -chain, these μ MT mice produced IgE and IgG Abs, although the Abs induced did not have specificity for *A. fumigatus* Ags. In contrast, IgA was not detected in either the lavage fluid or serum of μ MT mice that had been exposed to *A. fumigatus*. Our findings also reveal the existence of CD19⁺CD9⁺IgD⁺ B-1 cells in the lungs of the μ MT animals. These data show the μ MT mice to have a developmental pathway independent of the canonical μ -chain route that allows for their survival upon antigenic challenge with *A. fumigatus* conidia, although this pathway does not seem to allow for the normal development of Ag-specific repertoires. Additionally, this study shows that IgA is not required for either clearance or containment of *A. fumigatus* in the murine lung, as fungal outgrowth was not observed in the μ MT animals after multiple inhalation exposures to live conidia. *The Journal of Immunology*, 2012, 189: 000–000.

Allergic asthma is characterized by reversible airway obstruction due to the recruitment of leukocytes to the lung in response to an inhaled allergen (1). Increased mucus production in the airways, smooth muscle mass around the large airways, and peribronchial collagen deposition further narrow the airway lumen and restrict normal airway compliance, contributing to airway obstruction (2–6). Sensitization to fungi with production of IgE and/or colonization by fungal species often signals a disease course that is particularly difficult to treat and results in chronic architecture changes in the lung, causing long-term morbidity (7).

Aspergillus fumigatus has a number of characteristics that make it an ideal aeroallergen and opportunistic pathogen of humans. Its small conidia are ubiquitous in indoor and outdoor environments and can remain airborne for long periods of time (8). The size and shape of the conidia are such that they may be inhaled deep into

the lung tissue, past the mucociliary elevator that clears many particulates from the airways (9). Holding an environmental niche as a carbon and nitrogen recycler in compost piles, it can take advantage of a wide range of substrates and can grow at the high internal body temperature that discourages most fungal species (10).

Resident plasma cells have been observed in the lungs of both human asthma sufferers and mice under experimental allergic airways protocols (11). Secretory IgA is recognized as an integral part of the innate mucosal response that protects the upper respiratory tract (12–14), and selective IgA deficiency in clinic patients is associated with an increased prevalence of atopy (15, 16). The IgG subtype IgG1, which is a Th2-elicited Ab, is cytophilic to mast cells (17), and IgG2a, which is produced by Th1-activated B cells, plays a role in host protection against fungal growth (18). As instigators of humoral immunity, B lymphocytes provide specificity to allergens in the production of IgE Abs that enable mast cell degranulation (19). IgE has long been recognized as a perpetrator of asthma exacerbations, and anti-IgE therapies have been used successfully for treatment (20–22). During asthma exacerbations, B cells in all stages of activation and differentiation are found in increased numbers in the blood of asthmatic patients (23). B cells are also present in the bronchial mucosa of asthmatics (24). Although allergen-specific Abs are recognized as contributing factors in the immunopathology of an aberrant response against an innocuous allergen such as pollen or animal dander (25), they have also been suggested to be part of the successful clearance of fungus from the airways (26, 27).

The aim of the present study was to determine the extent to which B cells impact the development and maintenance of the phenotype of the allergic lung. We used mice that, owing to a homozygous targeted disruption of the membrane exon of the Ig μ -chain, are deficient of peripheral B cells, known as μ MT (28). Using an *A. fumigatus* murine inhalation model developed in our laboratory to mimic human fungal asthma (29), we compared the effects of

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of North Dakota State University.

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; WT, wild-type.

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repeated *A. fumigatus* inhalation in C57BL/6 wild-type (WT) controls and μ MT animals. We found that the absence of the μ -chain did not alter the pulmonary pathology that results from inhalation of *A. fumigatus* in allergic animals: airway hyper-responsiveness (AHR), peribronchial inflammation, epithelial changes, and collagen deposition were equivalent to WT controls. Surprisingly, we found that repeated *A. fumigatus* conidia exposure resulted in elevated IgE, IgG1 (in bronchoalveolar lavage [BAL]), and IgG2a production in sensitized μ MT mice, although IgA was undetectable in the μ MT animals. This has implications both for the role of the B cell in the allergic lung and for IgA in the response to fungal allergens. To our knowledge, we report for the first time that, even in the absence of the Ig μ -chain, IgG1 (only in BAL), IgG2a, and IgE isotypes were produced in animals after exposure to fungal Ags, but IgA was not made. The Abs produced after fungal exposure showed no Ag specificity for *A. fumigatus*. Our findings also reveal the previously unreported presence of B-1 cells (CD19⁺CD9⁺IgD⁺) in the lungs of μ MT mice, even in the complete absence of B-2 cells. Taken together, our data demonstrate that μ MT mice have B-1 cells in the lungs and that these animals produce selected isotypes through a μ -independent pathway in the context of the fungal allergen-exposed lung.

Materials and Methods

Experimental animals

C57BL/6 and μ MT mice (5–9 wk of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed on Alpha-dri paper bedding (Shepherd Specialty Papers, Watertown, TN) in microfilter-topped cages (Anicare, Bellmore, NY) in a specific pathogen-free facility with ad libitum access to food and water. The study described was performed in accordance with the Office of Laboratory Animal Welfare guidelines and was approved by the North Dakota State University Institutional Animal Care and Use Committee.

Ag preparation and *A. fumigatus* culture

Soluble *A. fumigatus* Ag extract was purchased from Greer Laboratories (Lenoir, NC), and fungal culture stock (strain NIH 5233) was purchased from the American Type Culture Collection (Manassas, VA). The *A. fumigatus* culture was reconstituted in 5 ml PBS, and 60- μ l aliquots were stored at 4°C until use. All experiments that used *A. fumigatus* were conducted with prior approval of the Institutional Biological Safety Committee of North Dakota State University.

Allergen sensitization and challenge by airborne delivery system

Animals were sensitized per Hogaboam et al.'s (30) published protocol, with the exception that aluminum hydroxide was used as the adjuvant. Mice were sensitized globally with 10 μ g *A. fumigatus* Ag (Greer Laboratories) in 0.1 ml normal saline mixed with 0.1 ml Imject Alum (Pierce, Rockford, IL), which was injected s.c. (0.1 ml) and i.p. (0.1 ml). After 2 wk, mice were given a series of three intranasal, weekly 20- μ g doses of *A. fumigatus* Ag in 20 μ l normal saline. Animals were challenged as previously described with a 10-min nose-only aerosol exposure to live *A. fumigatus* conidia (29). Each anesthetized mouse was placed supine with its nose in an inoculation port inhaling the live fungal conidia for 10 min. Two weeks after the first allergen challenge, mice were subjected to a second 10-min aerosol fungal challenge. Naive animals from both groups were neither sensitized nor challenged. After the second allergen exposure, the mice were separated into groups of five for analysis at day 0 (sensitized, but not challenged) or days 7 or 28 after the second aerosol challenge. Day 7 after challenge had been previously determined to be the peak of B cell recruitment into the allergic lungs, and leukocyte inflammation was assessed at this time point. Airway wall remodeling can be seen as early as 7 d after the second aerosol challenge in this model, and the changes to the lung architecture continue to accrue through at least day 28 after the second inhalation of fungal conidia. The day 28 time point was chosen to assess epithelial changes as well as peribronchial fibrosis. The experimental protocol is depicted in Fig. 1.

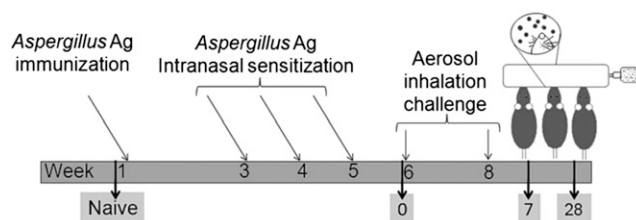


FIGURE 1. Sensitization, challenge, and analysis schedule for the *A. fumigatus* murine model of allergic asthma. Mice are first sensitized with immunizations and intranasal inoculations of fungal Ags. They are then exposed to two nose-only inhalation doses of live *Aspergillus* conidia 2 wk apart. Groups of animals are assessed at various time points after allergen challenge (by convention, time points are named for their day after challenge, represented here by days 7 and 28 time points). Day 0 denotes sensitized animals that did not receive the inhalation exposure to fungus.

AHR measurement

Mice were anesthetized using sodium pentobarbital (Butler, Columbus, OH; 0.1 mg/0.01 kg of mouse body weight), intubated, and ventilated with a Harvard pump ventilator (Harvard Apparatus, Reno, NV) to assess allergic airway responses. Restrained plethysmography (Buxco, Troy, NY) was used to assess AHR. Before performing readings, the system was first calibrated and the stroke volume set at 225 with the strokes per minute set at 150. The value for baseline airway resistance was measured for each animal before an i.v. injection of acetyl- β -methacholine (420 μ g/kg) was administered to determine AHR at each time point.

Sample collection

Approximately 500 μ l blood was removed from each mouse via ocular bleed and centrifuged at 13,000 $\times$ g for 10 min to yield serum. Serum was stored at -20°C until use. BAL was performed on each mouse with 1.0 ml sterile normal saline. The BAL contents were centrifuged at 2000 $\times$ g for 10 min to separate cells from fluid. The BAL fluid was stored at -20°C until use, and cells were used immediately for morphometric analysis. Left lungs were harvested and fixed in 10% neutral buffered formalin for histological analysis.

Morphometric and histological analyses

BAL cells were cytospun (Shandon Scientific, Runcorn, U.K.) onto microscope slides and differentially stained (Quick-Dip stain; Mercedes Medical, Sarasota, FL). Cells from five random high-powered fields were counted to determine the mean number of each cell type per high-powered field in the airway lumen of each mouse.

Formalin-fixed, paraffin-embedded lungs were cut longitudinally across the coronal plane in 5- μ m sections and stained with H&E to assess inflammation and with periodic acid-Schiff stain (Richard-Allan Scientific, Kalamazoo, MI) for the analysis of goblet cells.

Evaluation of collagen thickness

Gomori trichrome (Richard-Allan Scientific) was used to stain histological sections to assess collagen deposition in naive and allergic mice as described previously by Hoselton et al. (29). For each sample, at least 50 discrete points were measured at 50- μ m intervals along the largest lateral bronchiolar branch visible on the histological section (the second or third lateral branch). A perpendicular line was drawn from the point on the basement membrane through the full thickness of the collagen immediately below. The mean collagen thickness was reported for each sample, and the mean of the means was reported for each group.

Quantification of serum and BAL IgE, IgG1, IgG2a, and IgA

The total IgE (BD OptEIA; BD Biosciences, San Diego, CA), IgG1 (Immunology Consultants Laboratory, Portland, OR), IgG2a (BD OptEIA), and IgA (Bethyl Laboratories, Montgomery, TX) in serum and BAL were quantified via specific ELISA according to manufacturers' guidelines. Serum samples were diluted in PBS at 1:100 for IgE, at 1:500 and 1:5000 for IgG1 and IgG2a, and at 1:500 for IgA. BAL samples were pooled and diluted at 1:5 for IgE and IgG2a, at 1:2 for IgG1, or undiluted for IgA. The detection limits for the kits were 1.6 ng/ml for IgE, 6.25 ng/ml for IgG1, 3.1 ng/ml for IgG2a, and 15.625 ng/ml for IgA.

A. fumigatus-specific Ab detection

ELISA plates were coated with 100 μ l/well of a 20- μ g/ml sample of *A. fumigatus* Ag (Greer Laboratories) diluted in coating buffer (pH 9.6; 15 mM Na_2CO_3 , 35 mM NaHCO_3) and incubated overnight at 4°C. The next day, the plates were washed three times with PBS containing 0.05% Tween 20, and 200 μ l blocker (3% BSA in coating buffer) was added to each well. Plates were incubated in the dark for 2 h at room temperature and washed three times with PBS-Tween 20. After blocking, 100 μ l/well serially diluted serum or BAL from C57BL/6 and μ MT mice diluted in PBS-Tween 20/1% BSA (10^{-1} – 10^{-8} for serum and 1:2 to 1:64 for BAL fluid) was added to each well and incubated for 1 h. Plates were washed five times with PBS-Tween 20, and 100 μ l/well diluted goat anti-mouse Ig-HRP (SouthernBiotech, Birmingham, AL) secondary Ab was added. Following a 1-h incubation, the plates were again washed five times, and 100 μ l/well tetramethylbenzidine substrate (BD Biosciences) was added. The absorbance was read at 650 nm using a Synergy HT microplate reader (BioTek, Winooski, VT). Additionally, serum and BAL samples were tested to check the specificity of individual subclasses of Ab (IgG1 and IgE) for *A. fumigatus*. For this, rat anti-mouse IgG1-AKP (1:5000 dilution; BD Pharmingen, San Jose, CA) and goat anti-mouse IgE-HRP (1:500 dilution; Thermo Scientific, Rochester, NY) secondary Abs were used in place of Ig-HRP. The absorbance was read at 650 nm when tetramethylbenzidine was used as a substrate and at 405 nm when *p*-nitrophenyl phosphate (disodium salt, hexahydrate) (Sigma-Aldrich, St. Louis, MO) was used as a substrate using a Synergy HT microplate reader.

Flow cytometry

Minced lungs from naive animals and at days 0 (sensitized, but not challenged) and 7 were subjected to collagenase IV (Sigma-Aldrich) digestion and RBC lysis. For collagenase digestion, minced lung sections were treated with 0.04% collagenase IV in DMEM and incubated at 37°C for 1 h with gentle agitation. For flow cytometry analysis, the cells were suspended in PBS with 1% BSA (Sigma Aldrich) to a final concentration of 1×10^7 cells/ml. Fc receptors were blocked with anti-mouse CD16/CD32 (1 μ g/ 1×10^6 cells) for 10 min on ice. The following Abs were used for phenotypic characterization of B lymphocytes using flow cytometry: PerCP-Cy5.5-anti-CD19, PE-anti-CD23, Alexa Fluor 647-anti-CD9, FITC-anti-IgD, FITC-anti-IgM, FITC-anti-IgG, FITC-anti-IgE, FITC-anti-IGA (all Abs were purchased from eBioscience, San Diego, CA). The samples were preincubated with combinations of directly labeled Abs for 30 min in the dark and then washed with PBS/1% BSA twice before the samples were analyzed using an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI) or a FACSCalibur flow cytometer (BD Biosciences). A minimum of 50,000 events were acquired and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Allergic C57BL/6 WT and μ MT animals were compared with each other and to their respective naive controls at each time point. An unpaired Student two-tailed *t* test with a Welch correction was used to determine statistical significance with GraphPad Prism software (GraphPad Software, San Diego, CA). For each of the mouse strains compared with its naive control, statistical significance is indicated as follows: **p* = 0.01–0.05, ***p* = 0.001–0.01, ****p* < 0.001. Where appropriate, # indicates statistical difference between the C57BL/6 and μ MT mice (#*p* < 0.05). All results are expressed as the means $\pm$ SEM.

Results

Airborne fungal challenge results in AHR in μ MT mice after sensitization to *A. fumigatus*

In the present study, airway physiology of both murine groups (i.e., C57BL/6 and μ MT animals) was monitored before allergen challenge at day 0 and at days 7 and 28 after the second conidia inhalation (Fig. 2). Airway response measurements from all study animals were used to determine the baseline mean for AHR prior to methacholine challenge (Fig. 2, dotted line, 1.78 ± 0.05 cmH₂O/ml/s). Sensitized animals from WT and μ MT groups that were not challenged with spore inhalation showed no difference in airway resistance values when compared with naive animals in their respective groups. At day 7 after two conidia challenges, AHR was significantly increased in both murine groups as compared with naive controls (Fig. 2); however, there was no difference in the AHR values of μ MT

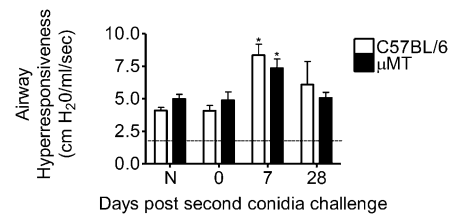


FIGURE 2. Inhalation of *A. fumigatus* increases AHR in C57BL/6 and μ MT mice. Baseline response was obtained prior to methacholine challenge (mean value of 1.78 ± 0.05 cmH₂O/ml/s is indicated by the dotted line). Peak increases in airway resistance were recorded after i.v. methacholine injection (420 μ g/kg). AHR was increased following allergen challenge, and the trend was quite similar in both the murine groups throughout the course of the study. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as the means $\pm$ SEM (*n* = 3–5 mice/group). **p* < 0.05 when compared with the respective naive controls.

animals as compared with the C57BL/6 WT animals. By day 28 after the second conidia challenge, AHR values for both murine groups returned to baseline, irrespective of the presence or absence of the μ gene.

Leukocytes are recruited to the allergic airways after fungal conidia challenge in μ MT mice

Leukocyte recruitment to the lungs of allergen-sensitized animals that had inhaled conidia was evaluated using H&E-stained lung sections and morphometric analysis of BAL cells. Naive animals from both groups exhibited no pulmonary inflammation (Fig. 3A, 3B). Similarly, sensitized C57BL/6 and μ MT animals that did not inhale spores (day 0) showed no evidence of inflammation (Fig. 3C and 3D, respectively). However, upon allergen challenge, both C57BL/6 and μ MT animals actively recruited inflammatory cells to the lungs. Allergic animals exhibited prominent perivascular and peribronchial leukocyte inflammation 7 d after the second spore challenge (Fig. 3E, 3F). The pattern of perivascular and peribronchial inflammation was similar in the C57BL/6 and μ MT animals at day 7, and inflammation was largely resolved in both strains by day 28 after challenge (Fig. 3G, 3H).

Morphometric analysis of monocyte/macrophage lineage cells, neutrophils, eosinophils, and lymphocytes was performed to estimate the relative makeup of the cellular inflammation and to monitor leukocyte egress into the airway lumen (Fig. 4). In naive and sensitized animals that were not challenged (day 0), alveolar macrophages were the dominant cell type (Fig. 4A). Neutrophils, lymphocytes, and particularly eosinophils were prominent cell types identified in the BAL 7 d after the second conidia challenge (Fig. 4B–D). Eosinophils were the most numerous cell type counted (Fig. 4C) in the BAL of both C57BL/6 and μ MT mice at day 7 after the second conidia exposure, emphasizing the polarization of the immune response in favor of allergy after multiple inhalations of conidia. At day 28 after challenge, macrophages were again the major cellular component of the BAL compartment with very few neutrophils (Fig. 4A, 4B). The inflammation pattern was similar between C57BL/6 and μ MT animals, with eosinophils dominating at day 7 in both murine groups when they were compared with their naive controls.

Inhalation of fungal conidia changes the airway architecture in allergic C57BL/6 mice and allergic μ MT mice

In the present study, goblet cells were assessed by counting periodic acid-Schiff–stained cells and representing them as a percentage of total epithelial cells lining the second or third lateral airways in each histological section. Goblet cell metaplasia was not observed

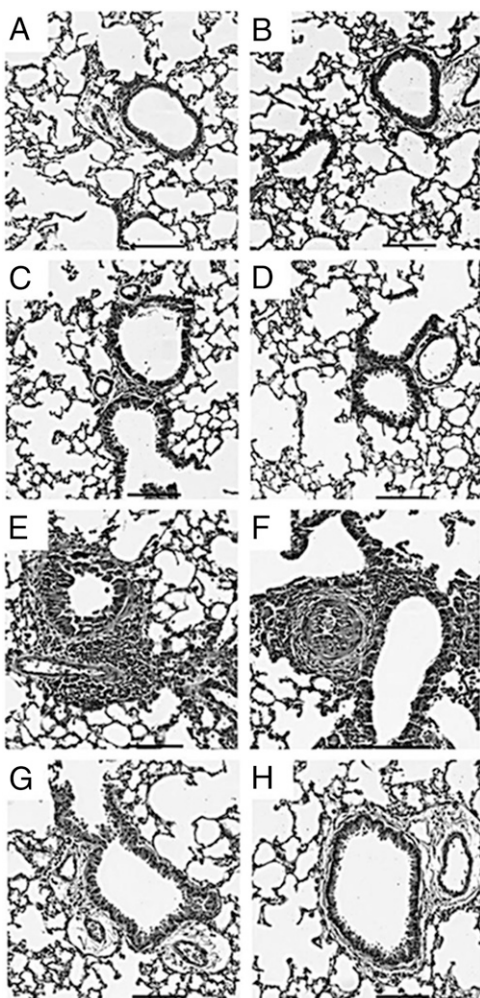


FIGURE 3. Inhalation of *A. fumigatus* conidia increases pulmonary inflammation in C57BL/6 and μ MT mice. Representative photomicrographs of H&E-stained lung sections of allergen-challenged C57BL/6 (left column) and μ MT (right column) mice. Naive and day 0 mice in both groups did not show inflammation (A–D). Peribronchovascular inflammation was prominent at day 7 after the second conidia challenge in both groups (E, F) and subsided well into day 28 (G, H). Scale bars, 100 μ m.

in the day 0 (sensitized, but not challenged) animals of either the C57BL/6 or μ MT groups (Fig. 5C). Challenge with *A. fumigatus* conidia resulted in a marked increase in the percentage of goblet cells lining the airways (Fig. 5C). As compared with sensitized animals that did not receive inhaled conidia, the number of goblet cells was increased dramatically (~65% of total) but equally in both groups 7 d after challenge (Fig. 5). By day 28 after the second conidia challenge, fewer goblet cells were noted in the allergic lungs of both the C57BL/6 and the μ MT group as compared with the day 7 time point, although there was no difference in the number of goblet cells between the WT and the μ MT groups (Fig. 5C; ~27% of the total epithelial cells for each).

Collagen accumulation in the peribronchial space of allergic C57BL/6 or μ MT animals was significantly increased at both day 7 and day 28 after the second conidia inhalation as compared with sensitized animals that had not been exposed to inhaled conidia (Fig. 6). In contrast to the pattern seen in goblet cell metaplasia, collagen accumulation did not diminish over the time course of this study. This phenomenon has been seen and extended in other studies by our laboratory in both BALB/c and C57BL/6 mice (29, 31–33).

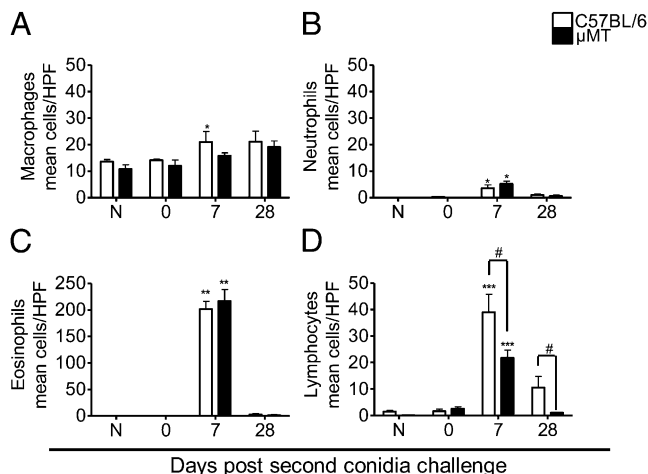


FIGURE 4. Effect of *A. fumigatus* conidia inhalation on inflammatory leukocytes in the allergic lung. Airway inflammation was marked by the presence of (A) macrophages, (B) neutrophils, (C) eosinophils, and (D) lymphocytes in naive, allergic C57BL/6, and μ MT mice. The inflammation pattern was similar in both C57BL/6 and μ MT mice throughout the course of the study. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as the means $\pm$ SEM ($n = 4$ –5 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with the respective naive controls; # $p < 0.05$ when μ MT animals were compared with C57BL/6 animals. HPF, High-powered field.

Fungal inhalation resulted in increased serum IgA, IgG1, IgG2a, and IgE levels in allergic C57BL/6 mice, whereas μ MT mice exhibited elevated IgG1 in BAL and IgG2a and IgE in serum

In the present study, inhalation of *A. fumigatus* conidia resulted in an increase in the BAL IgA from C57BL/6 mice at day 7 after two conidia inhalations. IgA Abs were not detected in either serum or the BAL fluid of μ MT mice (Fig. 7A, 7B). IgG1 was detected in the BAL fluid of allergic μ MT mice 7 d after two exposures to conidia, but it was not found in the serum (Fig. 7C, 7D). Although serum IgG2a levels in naive μ MT animals were significantly lower than in WT animals, sensitization with fungal Ags stimulated its

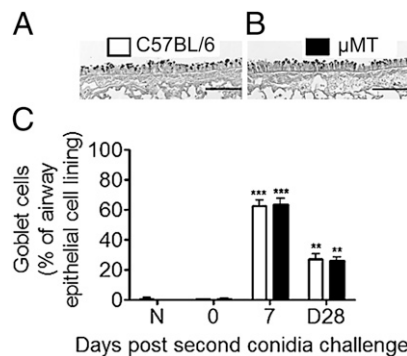


FIGURE 5. Inhalation of *A. fumigatus* conidia increases goblet cell metaplasia in C57BL/6 and μ MT mice. Representative photomicrographs of periodic acid-Schiff–stained whole lung sections of C57BL/6 and μ MT mice show that goblet cells and mucus were evident in the airways at day 7 after the second conidia challenge (A, B). Scale bars, 100 μ m. Goblet cell numbers were reported as the percentage of total epithelial cells along segments of airway epithelium lining the large lateral branches of the bronchi (C). Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as the means $\pm$ SEM ($n = 3$ –5 mice/group). ** $p < 0.01$, *** $p < 0.001$ when compared with the respective naive controls.

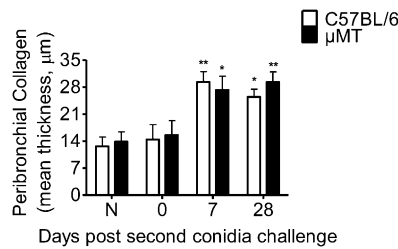


FIGURE 6. Effect of *A. fumigatus* conidia inhalation on peribronchial collagen thickness. Gomori trichome stain was used to visualize subepithelial collagen deposition in histological sections. Peribronchial collagen thickness was similar in both C57BL/6 and μ MT mice throughout the course of the study. Approximately 50 discrete points were measured at 50- μ m intervals along the largest lateral bronchiolar branch visible on the histological section (L2 or L3). A perpendicular line was drawn from the point on the basement membrane through the full thickness of the collagen immediately below. The mean collagen thickness was reported for each sample. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as the means $\pm$ SEM ($n = 3$ –5 mice/group). * $p < 0.05$, ** $p < 0.01$ when compared with their respective naive controls.

production to levels equivalent to those of WT animals, and the μ -deficient animals matched IgG2a levels throughout the rest of the study (Fig. 7E). IgG2a was also detected in the BAL fluid of the μ MT mice (Fig. 7F). IgE was elevated in *A. fumigatus*-sensitized and challenged C57BL/6 and μ MT animals (Fig. 7G, 7H). Although there was a significant difference in the IgE levels

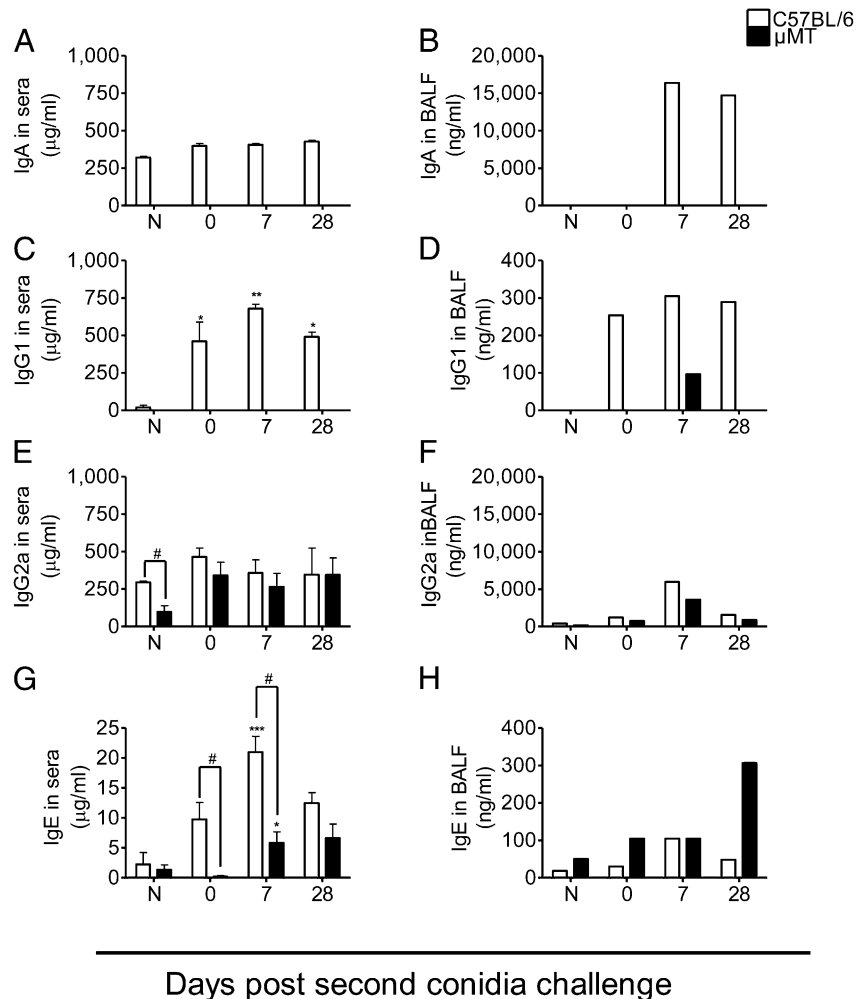
of C57BL/6 and μ MT mice, the production of IgE was significantly higher at day 7 after the second conidia challenge in both the murine groups when they were compared with their respective naive controls, suggesting that isotype switching to an allergic phenotype was possible even in the μ MT mice. However, IgE production in C57BL/6 mice was 4-fold higher than μ MT levels at day 7 after the second inhalation.

To investigate the extent to which the Abs produced as a result of fungal sensitization and inhalation exposure were specific to *A. fumigatus*, serial dilutions of serum and BAL samples from C57BL/6 and μ MT mice were collected at day 7 after the second conidia exposure and analyzed against the sensitizing Ag. The specificity of Abs to *A. fumigatus* at day 7 after the second conidia challenge (when the Ab levels are higher in serum and BAL) of both C57BL/6 and μ MT mice are shown in Fig. 8. The serum and BAL Abs produced in the C57BL/6 mice were specific to *A. fumigatus* (filled square), whereas the ones produced in μ MT mice (filled circle) appeared to be nonspecific and the values were comparable to those of naive control animals (Fig. 8). When the specificity of individual subclasses of Abs (IgE and IgG1) for *A. fumigatus* was tested, we observed similar results (data not shown).

CD19⁺CD9⁺IgD⁺ B-1 cells are present in the lungs of μ MT mice despite a lack of the Ig μ -chain

It has previously been shown that μ MT mice on the BALB/c, but not C57BL/6, background display an incomplete block in B cell development and harbor mature B cells in secondary lymphoid organs (34, 35). Although all μ MT animals used in this study were

FIGURE 7. Inhalation of *A. fumigatus* conidia induces μ MT mice to produce IgG1 (only in BAL), IgE, and IgG2a in serum and BAL fluid (BALF). (A and B) IgA Ab levels in serum and BAL fluid of C57BL/6 and μ MT mice. (C–F) IgG1 and IgG2a Ab levels in serum and BAL of naive and allergic C57BL/6 and μ MT mice. (G and H) IgE Ab levels in serum and BAL of C57BL/6 and μ MT mice. The Ab levels of C57BL/6 and μ MT mice were compared with naive animals and to each other at each time point. ELISAs indicated that the μ MT mice produced Abs in response to *A. fumigatus* allergen challenge. Data were analyzed using an unpaired Student two-tailed *t* test with a Welch correction. All values are expressed as the means $\pm$ SEM ($n = 4$ –5 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with naive controls, # $p < 0.05$ when μ MT animals were compared with C57BL/6 animals. No statistics are shown for Ig ELISAs for BAL fluid, as the samples from each time point were pooled and run as a single sample.



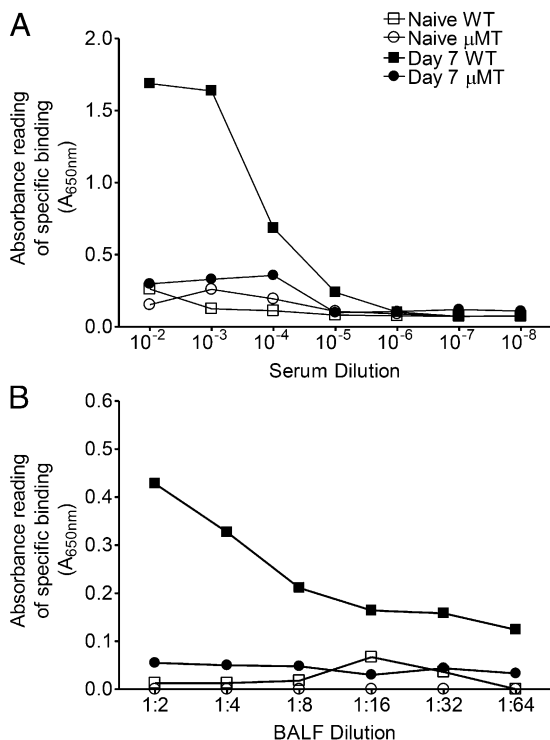


FIGURE 8. Inhalation of *A. fumigatus* conidia induces specific serum and BAL Ab production in C57BL/6 mice, but not in μ MT mice. Serum and BAL samples from the C57BL/6 and μ MT mice were pooled and serial dilution was used to evaluate Ab titers. **(A)** The specificity of total serum Ab from C57BL/6 (day 7 WT) and μ MT (day 7 μ MT) animals for *A. fumigatus* was evaluated against *A. fumigatus* Ag. **(B)** The specificity of total BAL Ab from C57BL/6 (day 7 WT) and μ MT (day 7 μ MT) animals for *A. fumigatus* Ag. C57BL/6 mice produced specific Abs for *A. fumigatus*, whereas none was detected in μ MT mice.

on the C57BL/6 background, we considered the possibility that *A. fumigatus* exposure might overcome the B cell developmental block (28, 35). In the present study, after inhalation with *A. fumigatus* conidia, CD19⁺ B cells were detected in the lungs of μ MT mice at day 7 after the second conidia inhalation and their

numbers were fewer than those in WT controls (Fig. 9C–F). When evaluated, CD19⁺ B cells were also detected in the lungs of naive μ MT mice (data not shown).

Given the fact that the Abs produced in the μ MT mice were not specific for *A. fumigatus*, we looked for the presence of B-1 lymphocytes in the lungs, as these cells are known to produce Abs in a nonspecific manner and they predominate in the pleural and peritoneal cavities (36). In addition to B-1 cells, we also looked for B-2 cells in the lungs of μ MT mice, as these conventional B-2 cells form a major population of lymphocytes that is present in the body (37, 38). The CD19⁺ B cell population in the lungs of C57BL/6 WT mice expressed either CD9 (as B-1 cells are CD9⁺) (36, 39) or CD23 (as B-2 cells are CD23⁺ and have low to no expression of CD9) (38, 40), showing the presence of both B-1 and B-2 B cells (Fig. 9C, 9E). Conversely, CD19⁺ B cells that were present in the lungs of μ MT mice did not express CD23, indicating the absence of B-2 lymphocytes (Fig. 9F). Similar to the B-1 population in C57BL/6 WT mice, the μ MT CD19⁺ B cells expressed CD9, illustrating the presence of B-1 lymphocytes (36) (Fig. 9D).

It has been shown that IgD can substitute for IgM when it is expressed early in the B cell development process (41). As such, we looked for the expression of IgD on the CD19⁺CD9⁺ cells that were present in the lungs of C57BL/6 WT and μ MT mice using flow cytometry. IgD was expressed on the CD19⁺CD9⁺ cells present in the lungs of C57BL/6 and μ MT mice at day 7 after the second conidia challenge (Fig. 9G, 9H). As expected, IgM⁺ cells were not detected in either naive or *A. fumigatus*-challenged μ MT mice (data not shown). These data demonstrate that in the μ MT mice, IgD can substitute for IgM early in B-1 cell development.

Discussion

In the present study, we show that the localized production of IgG1, IgG2a, and IgE is elicited in μ MT mice in response to systemic fungal sensitization and inhalational challenge in an experimental allergic asthma model. In addition to the localized production, our work demonstrates that μ -deficient mice produced systemic IgG2a and IgE Abs after exposure to *A. fumigatus* extract Ags followed by inhalation of *A. fumigatus* conidia. However, when tested in binding assays with the *Aspergillus* Ags that were used to sensitize the animals, the Ab isotypes from the μ MT animals proved to be

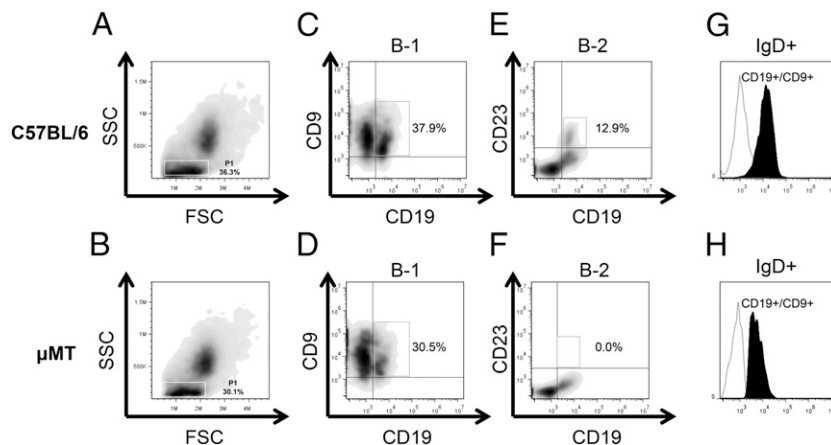


FIGURE 9. CD19⁺CD9⁺IgD⁺ B-1 cells are present in the lungs of μ MT mice despite a sustained block in B cell development. Lungs harvested from C57BL/6 and μ MT mice at day 7 after the second conidia challenge were analyzed for the presence of B-1, B-2, and IgD⁺ B-1 cells. **(A and B)** The forward scatter (FSC) and side scatter (SSC) plots of the cells isolated from the lungs of C57BL/6 and μ MT mice. **(C and D)** The percentage of CD19⁺CD9⁺ B-1 cells in the lungs of C57BL/6 and μ MT mice (gated on population P1 in the FSC-SSC plot). **(E and F)** The percentage of CD19⁺CD23⁺ B-2 cells in the lungs of C57BL/6 and μ MT mice (gated on population P1 in the FSC-SSC plot). **(G and H)** Histogram overlay of CD19⁺CD9⁺ (non B-1 cells, open histogram) and CD19⁺CD9⁺ (B-1 cells, filled histogram) cell populations showing the presence of IgD on the surface of B-1 cells in the lungs of C57BL/6 and μ MT mice.

nonspecific, whereas the Abs produced by the μ -sufficient controls were specific.

In the present work using a fungal trigger to elicit allergic airways disease, the characteristic signs and symptoms of allergic airway disease were present. AHR, pulmonary inflammation, excessive mucus production, and serum IgE in μ MT mice were comparable to those in C57BL/6 controls. In contrast, studies using a repeated aerosol exposure to OVA showed reduced lung inflammation and mucus hypersecretion in μ MT mice as compared with controls (42, 43). Additionally, OVA-challenged mice failed to develop AHR, suggesting a possible role of B cells in the development of AHR in response to OVA (42, 43). Thus, in addition to the type of Ab that can be elicited in μ MT animals, the type of immune response is also different with different antigenic stimuli, dissecting further the role of B cell activation in response to fungal pathogens/allergens.

IgM and IgA Igs share a number of similarities. IgA is related more closely to IgM than to other isotypes, with the μ - and α -chains sharing a characteristic long secretory segment (44). Additionally, both IgA and IgM can form multimers in conjunction with the J chain and both can be secreted at the mucosal surfaces coupled to the polymeric Ig receptor (45). Some investigators have speculated that expression of IgA may act as a surrogate for membrane IgM in B cell development in some instances (45). Secretory IgA has been recognized as an integral part of the innate mucosal response that protects the upper respiratory tract (12, 13), and selective IgA deficiency in clinic patients has been associated with an increased prevalence of atopy (15, 16). In experimental conditions, μ MT mice infected with *Salmonella* did produce IgA (45), and we expected that if any isotype was made in the μ MT mice in response to fungal stimulation, IgA would be that isotype. However, we found no IgA in the serum or the BAL of μ MT mice. This suggests that: 1) other Ab isotypes can substitute for IgM in B cell development; 2) that the type of antigenic stimulus dictates isotype development in the μ MT mouse, even when the context of the exposure (mucosal delivery) is similar (although not identical); and 3) that IgA is not necessary for fungal containment in this model.

In previous work using μ MT mice on a C57BL/6 background, a very sensitive method to detect low levels of Fc ϵ RI-bound IgE on basophils showed that IgE was made in μ MT mice after prolonged exposure to *Heligmosomoides polygyrus*, *Tricuris muris*, or *Schistosoma mansoni* gut parasites (46). In fungal sensitization and challenge, our results show a similar capacity for an IgE response in μ MT mice after treatment with *Aspergillus* Ags. In the present study, a robust IgE response was readily quantified by ELISA, and elevated IgE levels were sustained throughout the study. Our findings show that a conserved mucosal humoral response, which is not mediated through IgM, may significantly affect the response to not only gut parasites, but to inhaled fungal pathogens as well. Interestingly, in studies in which OVA was used as a surrogate for clinically relevant inhaled allergens, no IgE was produced in μ MT mice (3, 42).

In addition to IgE, we report IgG1 production in the BAL and IgG2a production in the serum and BAL of μ MT mice on a C57BL/6 background. IgG1, which is associated with Th2-type responses elicited by IL-4, was elevated only in the BAL and only after fungal challenge, suggesting a localized production of this Ab isotype. IgG2a, which plays an important role in fungal opsonization and clearance (18), was elevated throughout the study in both C57BL/6 and μ MT mice. This further supports the fact that antigenic stimulus dictates isotype development in the μ MT mice and that without the benefit of the μ gene a small percentage of pre-B cells can escape elimination, switch to downstream Ig H chains, and respond to Ags (35, 47).

The canonical pathway of B cell ontogeny requires surface expression of the μ Ig chain at an early pre-B cell stage (48). Indeed, until recently only B cells that express IgM were thought to migrate from the bone marrow to the peripheral lymphoid organs (35), and membrane-bound IgM expression was thought to be essential for B cell maturation and differentiation to Ab-producing cells. However, recent research using μ MT mice has shown that the expression of the μ Ig chain is not an absolute requirement for B cell survival (35, 45, 46, 49). These genetically altered animals have been useful tools in understanding the complex biological processes associated with different diseases.

Although Ab-binding ELISAs were not attempted in the helminth infection study, the IgE was functional in that it elicited IL-4 production by basophils (46). In that study, the Ab was produced at a low concentration and Ab-producing B cells could not be detected in the central or peripheral lymph organs. In the present study, whereas μ MT mice were able to produce Abs after sensitization to and challenge with fungal Ags, our results show that the Abs produced by the μ -deficient animals had no affinity for *A. fumigatus* Ags as compared with those from WT animals. Taken together, these results suggest a tissue-centric Ab production, which mandated the assessment of B cell populations in the lung.

In investigating the potential source of Abs in μ MT mice, to our knowledge, no other study has examined the presence of CD19⁺ IgD⁺ cells in the lungs. Because we did not find CD19⁺ IgD⁺ or CD19⁺ IgM⁺ cells in the bone marrow or spleens of naive mice (data not shown), we hypothesized that tissue-resident B-1 cells act as the source of Abs in μ MT mice. We were able to detect IgD-expressing CD19⁺CD9⁺ B-1 cells in the lungs of μ MT mice using flow cytometry, supporting a tissue-resident B-1 cell as a source for localized Ab production. These observations are consistent with the notion that B cells can receive switching signals in peripheral sites (45, 46, 50, 51), a process that may occur in the allergic lung.

In summary, we provide conclusive evidence that B-1 cells can impact asthma pathophysiology in the absence of conventional B-2 lymphocytes. From these studies, we report two significant conclusions. First, the route of the pathogenic/allergenic challenge as well as the type of Ag has a significant impact on the generation of Ab responses in μ MT mice lacking the normal pathway for B-2 cell maturation, as different types of Ag yield very different outcomes. The second major finding is that as a B-2 cell knockout mouse, μ MT animals may be very useful to determine the role of B-1 cells in response to various pulmonary insults. Future studies may include elucidating the mechanism for B-1 isotype switching in the lung and the contribution of B-1 cells to protective responses, which would have important implications for experimental analysis and for understanding normal B-1 and B-2 cell activation in health and disease.

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Disclosures

The authors have no financial conflicts of interest.

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