Mannose-binding lectin deficiency alters the development of fungal asthma: effects on airway response, inflammation, and cytokine profile

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Abstract: ***Aspergillus fumigatus* is a major fungal pathogen that may be fatal to immunocompromised individuals and causes airway hyperreactivity and remodeling in sensitized individuals. Herein, we examined the role of mannose-binding lectin (MBL), a complement-activating plasma protein, during pulmonary innate and allergic immune responses directed against *A. fumigatus* spores or conidia. Neither group of nonsensitized MBL-A-sufficient (MBL-A+/+) nor -deficient (MBL-A−/−) mice challenged with an intravenous or intratracheal (i.t.) bolus of *A. fumigatus* spores experienced fungus-induced mortality, but marked airway remodeling was observed in MBL-A−/− mice challenged i.t. with conidia. In a model of chronic fungal asthma, MBL-A+/+ and MBL-A−/− *A. fumigatus*-sensitized mice were examined at days 4 and 28 after an i.t. challenge with *A. fumigatus* conidia. Airway hyperresponsiveness in sensitized MBL-A−/− mice was significantly decreased at both times after conidia challenge compared with the sensitized MBL-A+/+ group. In the sensitized MBL-A−/− mice, whole lung T helper cell type 2 cytokine levels were significantly decreased at day 4 after conidia, and whole lung interferon-γ levels were significantly increased at day 28 after conidia when compared with controls. However, histological evidence showed similar airway remodeling at day 28 after conidia (i.e., subepithelial fibrosis and goblet cell metaplasia) in the two groups of mice. Thus, these findings show that MBL-A is not required for mouse survival following exposure to *A. fumigatus* conidia, and this murine collectin isoform contributes to the development and maintenance of airway hyperresponsiveness but not chronic airway remodeling during chronic fungal asthma. *J. Leukoc. Biol.* 75: 805–814; 2004.**

Key Words: *infectious immunity-fungi · allergy · lung · inflammatory mediators*

INTRODUCTION

Mannose-binding lectin (MBL) is a pattern-recognition molecule that recognizes distinct pathogen-associated molecular patterns (PAMPs) comprised of repeating mannose based on the surface of microorganisms, and when MBL binds to these microorganisms, it initiates the activation of the complement cascade [1, 2]. MBL is a member of a family of collagenous lectins or collectins, which possesses many of the functional characteristics of immunoglobulin (Ig)M, IgG, and Clq, such as an ability to trigger the activation of proserine protease complexes that cleave C4 and C2 of the classical complement pathway [3]. This response is a critical component of innate immunity against a number of clinically relevant pathogens including *Aspergillus fumigatus*, *Candida* species, *Staphylococcus aureus*, β-hemolytic group A streptococci, *Escherichia coli*, *Klebsiella* species, and *Haemophilus influenzae* type b [4]. In humans, serum levels of this collectin are quite variable, such that an individual may exhibit low, intermediate, or high levels, and variability in circulating MBL levels, aside from some notable exceptions [5], has been shown to correlate with susceptibility, resistance to infection [6, 7], and possibly other diseases [8, 9]. In addition, individuals with structural mutations of the MBL gene appear to have an increased incidence of infections [3].

Whereas one form of MBL is found in humans, mice contain two homologous isoforms, MBL-A and MBL-C [10, 11]. MBL-C (which was originally identified as the liver form, although recent evidence indicates that both isoforms are generated by hepatocytes and are found in the circulation [12]) is found in greater abundance in mouse serum than MBL-A; both are typically found in microgram quantities, and the introduction of lipopolysaccharide into mice induces MBL-A but not MBL-C [13]. The recent generation of MBL-A null mice that were MBL-C-sufficient has facilitated the examination of the role of MBL-A in innate immune responses to bacteria and fungus. One study led to the interesting observation that murine MBL-A is involved in the regulation of cytokine generation during bacterial-induced septic peritonitis [14]. As a result of the ability of MBL-A to modulate the inflammatory response, MBL-A-deficient (MBL-A−/−) mice showed an enhanced sur-

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Received July 14, 2003; revised December 4, 2003; accepted January 6, 2004; doi: 10.1189/jlb.0703325.

vival response in this model of sepsis [14]. Thus, MBL is a physiological, significant molecule in the innate immune response.

The spores or conidia of *A. fumigatus* are a constant airborne challenge to humans and animals alike, particularly in developed countries [15]. This ubiquitous airborne saprophytic fungi is normally contained and eliminated by neutrophil and macrophage-driven innate immune events in the immunocompetent host by innate immune mechanisms [16]. *A. fumigatus* is major clinical concern in immunocompromised patients, as this fungus can grow to the point of fatal invasive aspergillosis. Non-immunocompromised individuals can also be adversely affected by pulmonary exposure to this fungus, as those sensitized to this fungus are at risk for allergic bronchopulmonary aspergillosis, fungal asthma, or chronic necrotizing pulmonary aspergillosis (CNPA) [15]. Recently, it was shown that individuals deficient in MBL were susceptible to CNPA [17], but aside from this study, little in known about the in vivo role of MBL in the immune response to *A. fumigatus*.

Thus, in the present study, we investigated the role of MBL-A in pulmonary innate and allergic responses to *A. fumigatus* in MBL-A-sufficient (MBL-A+/+) and MBL-A−/− mice. Specific pathogen-free (SPF) MBL-A−/− mice did not succumb to an intravenous (i.v.) or intratracheal (i.t.) bolus challenge with live *A. fumigatus* conidia. In a well-established model of chronic fungal asthma [18], MBL-A−/− mice, in contrast with their wild-type counterparts, exhibited airway remodeling following the i.t. challenge of conidia. The allergic airway response to *A. fumigatus* conidia also differed between MBL-A+/+ and MBL-A−/− mice; airway hyperreactivity, but not airway remodeling, was attenuated in the deficient mice compared with their wild-type counterparts during the course of chronic fungal asthma.

MATERIALS AND METHODS

MBL-A–/– mice

The generation of the MBL-A–/– mice used in the following experiments has been described in detail previously [14]. Briefly, mice were generated using the 129SvJ MBL-A on a C57Bl/6J background. Embryonic stem clones were injected into C57Bl/6J blastocysts. The resulting chimeras were crossed to C57Bl/6J mice, genotyped by Southern, and crossed five times to discern the stability of the genotype, and the colony was expanded. All MBL-A−/− mice were shown to be completely deficient in MBL-A but sufficient in the other form of MBL found in mice, MBL-C [14]. The disruption of the MBL-A gene did not influence the genetic expression of surfactant protein A, a collectin with known regulatory functions in the lung that has a close genetic link to MBL-A on chromosome 14 (data not shown). MBL-A−/− and similarly derived MBL-A+/+ mice were bred in a SPF facility at the University of Michigan Medical School (Ann Arbor).

A. fumigatus culture and conidia challenge of nonsensitized MBL-A+/+ and MBL-A−/− mice

A. fumigatus American Type Culture Collection (Manassas, VA) strain 13073 was used in the present studies and was grown according to our previously published protocol [18, 19]. Conidia were extensively washed to remove all debris such as mycelium before quantification and injection into MBL-A+/+ and MBL-A−/− mice. In the present study, groups of five MBL-A+/+ mice were challenged i.t. with 5.0×10^6 conidia suspended in 30 μl 0.1% Tween-80 in phosphate-buffered saline (PBS). Groups of five MBL-A−/− mice received 1.0×10^6 or 5.0×10^6 conidia via the i.t. route. In separate experiments, MBL-A+/+ mice (n=5) received 5.0×10^5 conidia suspended in 500 μl 0.1% Tween-80 in PBS. MBL-A−/− mice also received conidia via the i.v. route; 5.0×10^5 or 1.0×10^5 conidia was administered to groups of five MBL-A−/− mice. Each experimental treatment was conducted once. Mouse survival was monitored in all groups of challenged mice. At day 28 after conidia challenge, whole lung tissue was removed from each surviving mouse to assess histological changes (see below).

Sensitization of MBL-A+/+ and MBL-A−/− mice to *A. fumigatus* and establishment of chronic fungal asthma

Age-matched, SPF male and female MBL-A+/+ and MBL-A−/− mice were maintained in a SPF facility for the duration of this study. Prior approval for mouse use in the development of this allergic fungal asthma model was obtained from the University Laboratory Animal Medicine facility at the University of Michigan Medical School. Sensitization of mice to a commercially available preparation of soluble *A. fumigatus* antigens (Greer Laboratories, Lenoir, NC) was performed as described previously [18]. Briefly, mice received a total of 10 μg *A. fumigatus* antigens dissolved in 0.2 ml incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO), distributed equally between an intraperitoneal (i.p.) and a subcutaneous injection. Two weeks later, and for 3 subsequent weeks, mice received a total of 20 μg *A. fumigatus* antigen, dissolved in normal saline, by intranasal (i.n.) installation. One week after the third i.n. challenge, each mouse received 5.0×10^6 live *A. fumigatus* conidia suspended in 30 μl 0.1% Tween-80 via the i.t. route.

Measurement of bronchial hyperresponsiveness

At days 4 and 28 after the i.t. *A. fumigatus* conidia challenge, bronchial hyperresponsiveness in *A. fumigatus*-sensitized MBL-A+/+ and MBL-A−/− mice was assessed in a Buxco™ plethysmograph (Buxco, Troy, NY) [18]. Mice were anesthetized with sodium pentobarbital (40 mg/ml i.p.; Butler Co., Columbus, OH) before their intubation and ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno, NV). Airway resistance was calculated online via computer software (Buxco) and was determined by the division of the transpulmonary pressure by the change in inspiratory volume. Following a baseline period in the Buxco plethysmograph, anesthetized and intubated mice received a dose of 210 and 420 μg/kg methacholine by tail vein injection, and airway responsiveness to this nonselective bronchoconstrictor was again calculated. At day 4 when acute inflammation and airway hyperresponsiveness are most pronounced, the lower 210-μg dose of methacholine was used for the airway hyperresponsiveness calculation, and the 420-μg dose was used at day 28. At the conclusion of the assessment of airway hyperresponsiveness, 500 μl blood was collected from each mouse and transferred to a microcentrifuge tube. Sera were obtained after the sample was centrifuged at 15,000 *g* for 10 min. A bronchoalveolar lavage (BAL) was performed with 1 ml normal saline, and fluid was removed from cells by centrifugation. BAL cells were subsequently cytospun onto coded microscope slides for differential analysis (see below). Finally, whole lungs were dissected from each mouse and snap-frozen in liquid N_2 or prepared for histological analysis.

Morphometric analysis of leukocyte accumulation in BAL samples

Neutrophils, macrophages, eosinophils, and lymphocytes were quantified in BAL samples cytospun onto coded microscope slides using a cytospin (Shandon Scientific, Runcorn, UK). Identification of each cell type was facilitated by differential staining with Wright-Giemsa differential stain and expressed as average cells per high-powered field (1000×).

Enzyme-linked immunosorbent assay (ELISA) analysis

Murine interleukin (IL)-4, IL-5, IL-13, interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), IL-10, IL-12, CXC chemokine ligand (CXCL)2, CXCL3, CXCL9, CCL3, CCL5, CCL11, and CCL22 protein levels were determined in 50 μl whole lung homogenates, using a standardized sandwich ELISA technique described previously in detail [20]. Whole lungs were homogenized in 1 ml normal saline containing 1 mg protease inhibitor (Complete™, Boehringer

Mannheim, Indianapolis, IN). Recombinant murine cytokines and chemokines were used to generate the standard curves from which the concentrations present in the samples were derived. The limit of ELISA detection for each cytokine and chemokine was consistently below 50 pg/ml, and the ELISA was screened to ensure the specificity of each antibody used.

Whole lung histological analysis

Whole lungs from nonsensitized MBL-A+/+ and MBL-A−/− mice at day 28 after *A. fumigatus* conidia challenge and from *A. fumigatus*-sensitized MBL-A+/+ and MBL-A−/− mice at days 4 and 28 after conidia were fully inflated by i.t. perfusion with 4% paraformaldehyde. Lungs were then dissected and placed in fresh paraformaldehyde for 24 h. Routine histological techniques were used to paraffin-embed this tissue, and 5-μm sections of whole lung were stained with hematoxylin and eosin (H/E), periodic acid Schiff (PAS), and Gomori methanamine silver (GMS).

Statistical analysis

All results are expressed as mean ± SEM. A Student's *t*-test was used to determine statistical significance between the MBL-A+/+ and MBL-A−/− groups at various times after conidia challenge; $P < 0.05$ was considered statistically significant.

RESULTS

MBL-A−/− mice did not succumb to a bolus of i.t. or i.v. *A. fumigatus* conidia

Immunocompetent mice are extremely resistant to the development of invasive aspergillosis as a result of the potent antifungal properties of recruited neutrophils and monocytes as well as resident alveolar macrophages (reviewed in ref. [15]). Typically, some mutation and/or chemical alteration of the innate immune response is necessary before *Aspergillus* can grow in the lung environment, thereby causing invasive aspergillosis [16]. In the present study, we saw no evidence that the bolus introduction of live *A. fumigatus* conidia, by the i.t. or i.v. route, had a deleterious effect on the survival of MBL-A−/− mice, as all challenged mice survived for the duration of this study. One hundred percent survival was also observed in all MBL-A+/+ groups. Thus, MBL-A deficiency did not affect mouse survival following a pulmonary or systemic bolus of *A. fumigatus* conidia.

Nonsensitized MBL-A−/− mice exhibited peribronchial inflammation and goblet cell metaplasia at day 28 after i.v. challenge with *A. fumigatus* conidia

As the i.v. or i.t. administration of *A. fumigatus* conidia into nonsensitized MBL-A−/− mice did not adversely affect the survival of these mice, we next examined whether the presence of the conidia in these mice altered the histological appearance of various tissues at day 28 after the conidia challenge. In MBL-A+/+ and MBL-A−/−mice that received an i.v. bolus of 1.0–5.0 × 10^5 *A. fumigatus* conidia, we observed no evidence of tissue damage in kidney, lung, liver, and brain (not shown) nor was there any histological evidence that *A. fumigatus* was retained in any of the above tissues in MBL-A−/− mice (i.e., GMS staining was absent), and this observation was similar to that seen in MBL-A+/+ mice (GMS staining is not shown for either group of mice). However, the i.t. challenge of 5.0 × 10^6 *A. fumigatus* conidia into MBL-A−/− mice appeared to cause major changes to the architecture of airways in these mice. In contrast to MBL-A+/+ mice at day 28 after conidia (**Fig. 1, A** and **C**), the lungs of MBL-A−/− mice exhibited marked peribronchial inflammation (Fig. 1B) and goblet cell metaplasia (Fig. 1D). Thus, this histological analysis revealed that the i.t. administration of *A. fumigatus* in nonsensitized MBL-A−/− mice promoted the maintenance of airway inflammation and remodeling.

A. fumigatus-sensitized MBL-A−/− mice did not develop significant bronchial hyperresponsiveness following *A. fumigatus* conidia challenge

We next examined whether MBL-A deficiency altered the course and/or magnitude of chronic fungal asthma. We have extensively studied the allergic airway response invoked by the introduction of *A. fumigatus* conidia into *A. fumigatus*-sensitized mice, and airway hyperresponsiveness is a cardinal feature of this model [21]. Following i.t. challenge with live *A. fumigatus* conidia, *A. fumigatus*-sensitized mice exhibit airway hyperresponsiveness that is present several weeks after i.t. challenge [18]. To assess the role of MBL-A in the initiation and maintenance of airway hyperresponsiveness, i.v. injections of 210 and 420 μg/kg methacholine were administered to *A. fumigatus*-sensitized MBL-A−/− and MBL-A+/+ mice. Neither of these doses of methacholine caused a significant increase in airway hyperresponsiveness above basal levels in *A. fumigatus*-sensitized wild-type mice before the conidia challenge (data not shown). At day 4, the airway response was acute in the MBL-A+/+ mice, and the lower 210-μg/kg methacholine dose was required to show the magnitude of the difference in airway responses between the two groups. At day 28, the result from the 420-μg/kg dose is reported. At days 4 (**Fig. 2,** upper panel) and 28 (Fig. 2, lower panel) after conidia, metha-

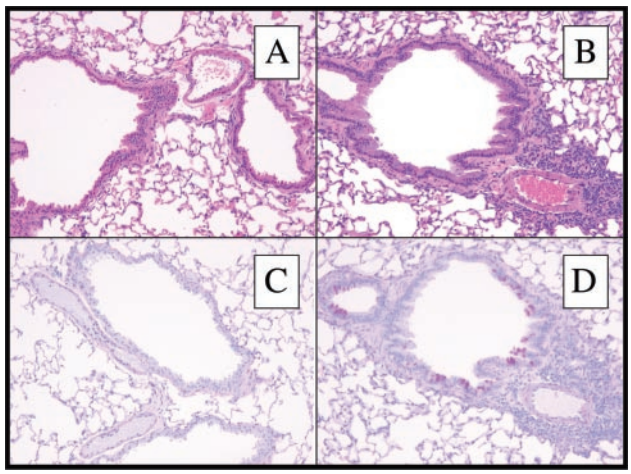

Fig. 1. Representative photomicrographs of H/E (A and B)- and PAS (C and D)-stained whole lung sections from nonsensitized MBL-A+/+ (A and C) and MBL-A−/− (B and D) mice at day 28 after conidia challenge. Both groups of mice (n=five/group) were injected i.t. with 5.0 × 10^6 conidia. The peribronchial inflammatory response was markedly enhanced in the MBL-A−/− group (B) compared with the MBL-A+/+ (A) group. Although PAS-stained goblet cells were absent in the MBL-A+/+ group (C), several goblet cells were evident in the airways of MBL-A−/− mice (D). Original magnification, 200×.

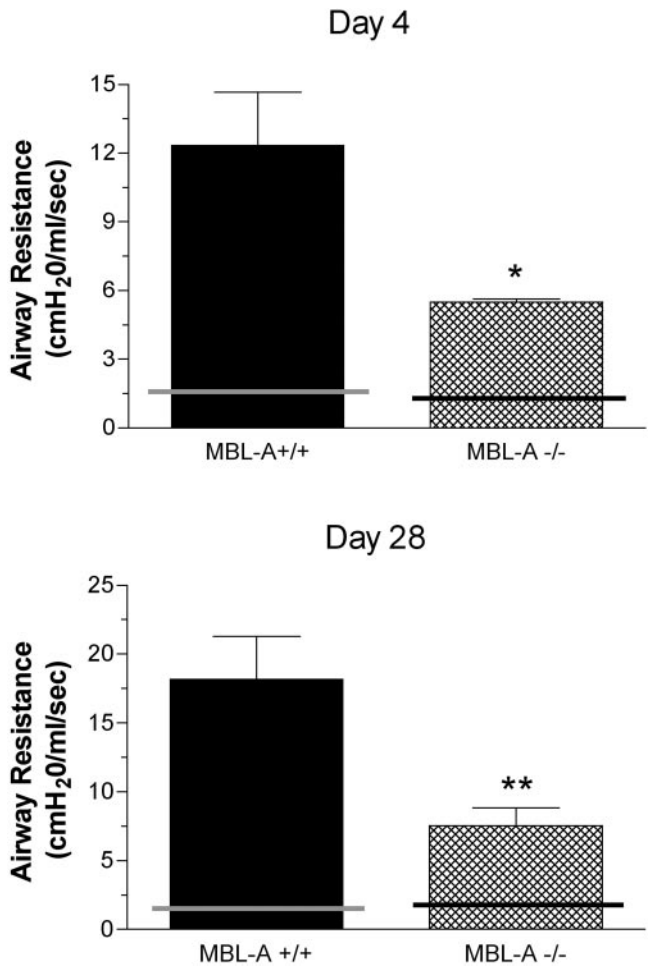

Fig. 2. Airway resistance in *A. fumigatus*-sensitized MBL-A+/+ and MBL-A−/− mice at days 4 (upper) and 28 (lower) after i.t. challenge with 5.0×10^6 *A. fumigatus* conidia. Hyperresponsiveness, as measured by changes in airway resistance (units=cm H_2O/ml/s), was measured in MBL-A+/+ and MBL-A−/− mice at days 4 and 28 after conidia challenge. A baseline reading was taken for each animal before methacholine injection (represented as solid lines across methacholine-challenged response bars). At day 4, 210 μg/kg methacholine was used to assess the airway response, and 420 μg/kg methacholine was used at day 28. Values are expressed as mean ± SEM; n = five/group/time point. *, $P \leq 0.05$; **, $P \leq 0.01$, compared with methacholine-induced airway hyperresponsiveness in MBL-A+/+ mice.

choline induced significant airway hyperresponsiveness in the MBL-A+/+ group. In contrast, the *A. fumigatus*-sensitized MBL-A−/− mice exhibited significantly less airway hyperresponsiveness when compared with the MBL-A+/+ controls (Fig. 2, upper and lower panels). These data show that MBL-A is necessary for the development of allergic airway hyperresponsiveness in the context of *A. fumigatus*.

MBL-A deficiency significantly alters lymphocyte recruitment into the airways of *A. fumigatus*-sensitized mice after live conidia challenge

The accumulation of leukocytes in and around the airways is a hallmark of allergic asthma. Therefore, leukocyte recruitment into the lung was monitored at days 4 and 28 following conidia challenge in *A. fumigatus*-sensitized MBL-A+/+ and MBL-A−/− mice (**Fig. 3**). The number of macrophages and neutrophils counted in the BALs from *A. fumigatus*-sensitized MBL-A−/− mice was significantly lower than that counted in the BALs of similarly sensitized MBL-A+/+ mice at day 4 after conidia (Fig. 3, upper panel). In contrast, BAL eosinophil counts were significantly higher in the knockout group compared with the wild-type group at day 4 (Fig. 3, upper panel). BAL lymphocyte counts did not differ between the two groups of mice at this time point. At day 28 after conidia challenge (Fig. 3, lower panel), eosinophil and lymphocyte numbers within the BAL were significantly decreased in MBL-A−/− mice compared with BAL samples from the MBL-A+/+ groups at both times after conidia (Fig. 3). Although these cells were present in BAL samples from the MBL-A+/+ group, no

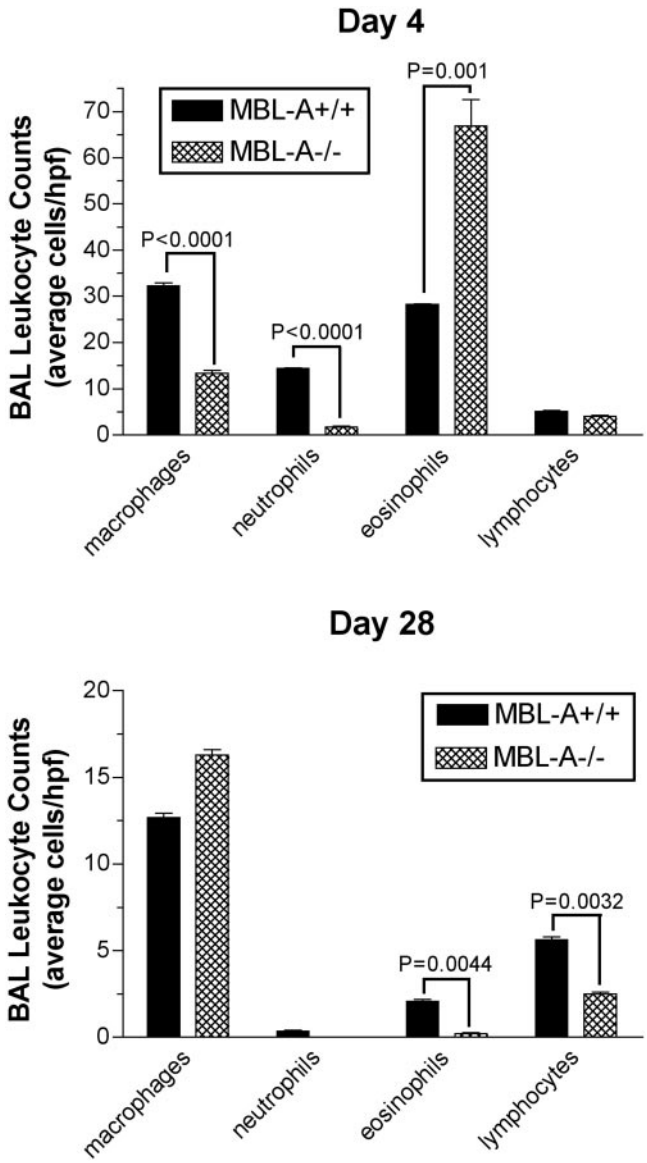

Fig. 3. Leukocyte counts in BAL samples from *A. fumigatus*-sensitized MBL-A+/+ and MBL-A−/− mice at days 4 and 28 after i.t. challenge with 5.0×10^6 *A. fumigatus* conidia. BAL cells were dispersed onto microscope slides using a cytospin; neutrophils, eosinophils, lymphocytes, and macrophages were differentially stained with Wright-Giesma stain. A minimum of 20 high-powered fields was examined in each cytospin. Values are expressed as mean ± SEM of the total cells counted/high-powered field (1000×).

neutrophils were present in the BAL of the MBL-A−/− group at day 28. Taken together, these data suggested that the lack of MBL-A significantly altered the recruitment of the major cell types that have been implicated in *Aspergillus*-induced allergic airway disease.

MBL-A−/− mice exhibited a muted T helper cell type 2 (Th2) cytokine response at day 4 after *A. fumigatus* conidia challenge, followed by an increase in IFN-γ at day 28

Numerous clinical [22, 23] and basic [24–27] studies have shown that Th2 cytokines are present and participate in the allergic inflammatory response elicited by *A. fumigatus*. Therefore, we examined whether whole lung Th2 cytokine levels were altered in MBL-A−/− mice during the course of chronic fungal asthma. As shown in **Figure 4**, whole lung levels of IL-13 (Fig. 4A), IL-4 (Fig. 4B), and IL-5 (Fig. 4C) were significantly lower in *A. fumigatus*-sensitized MBL-A−/− mice compared with the wild-type group at day 4 after conidia. At day 28 after conidia, no differences in whole lung Th2 cytokine levels were detected between the two groups of *A. fumigatus*-sensitized mice; however, the level of the Th1 cytokine IFN-γ was significantly higher in the knockout mice compared with the wild-type controls (Fig. 4D). Although Th2 cytokines enhance allergic responses to *Aspergillus*, Th1 cytokines, particularly IFN-γ, are potent inhibitors of this response (reviewed in ref. [21]). Other cytokines relevant to immune responses elicited by *Aspergillus*, including TNF-α [28], IL-10 [29], and IL-12 [30], were not affected by MBL-A deficiency at either time after conidia challenge in *A. fumigatus*-sensitized mice (data not shown). These findings demonstrate that MBL-A deficiency has a major effect on the early elaboration of Th2 cytokines in the lungs of *A. fumigatus*-sensitized mice during chronic fungal asthma, and this may have implications in the balance of Th1/Th2 cytokine production in the chronic stages of the disease.

MBL-A deficiency modulated the whole lung levels of CXCL9, CCL5, and CCL2 during the course of chronic fungal asthma

In our previous studies, we have examined the role of various chemokines in the initiation and maintenance of chronic fungal asthma [21]. These data have shown that CC and CXC chemokines have prominent but distinct roles in the physiologic,

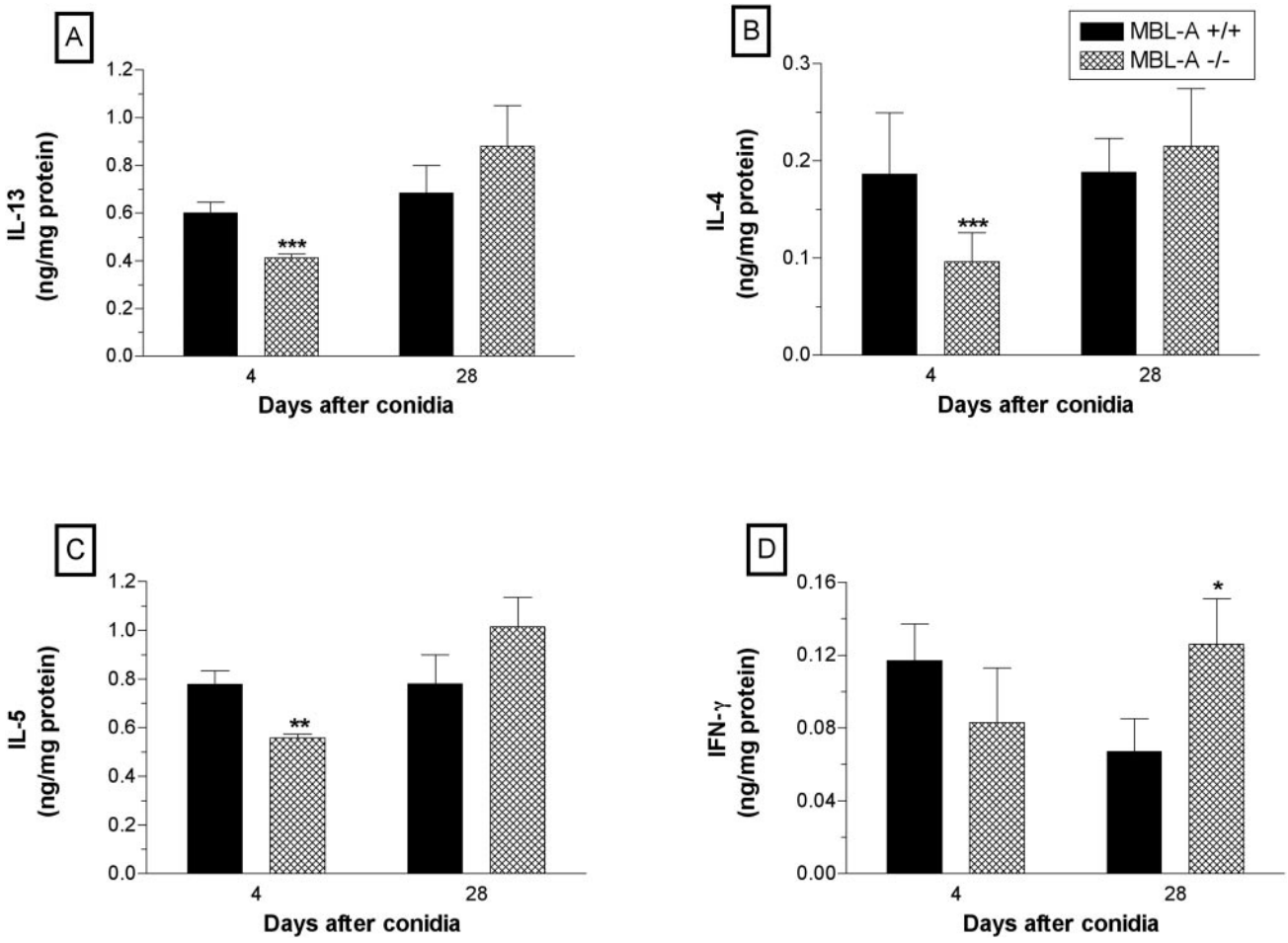

Fig. 4. Whole lung cytokine levels in *A. fumigatus*-sensitized MBL-A+/+ and MBL-A−/− mice at days 4 and 28 after i.t. challenge with 5.0×10^6 conidia. Cell-free supernatants from whole lung samples by specific ELISA measured IL-13 (A), IL-4 (B), IL-5 (C), and IFN-γ (D). Specific cytokine levels were normalized to total protein in each sample. Values are expressed as mean ± SEM; n = five/group/time point. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with whole lung cytokine levels in MBL-A+/+ mice.

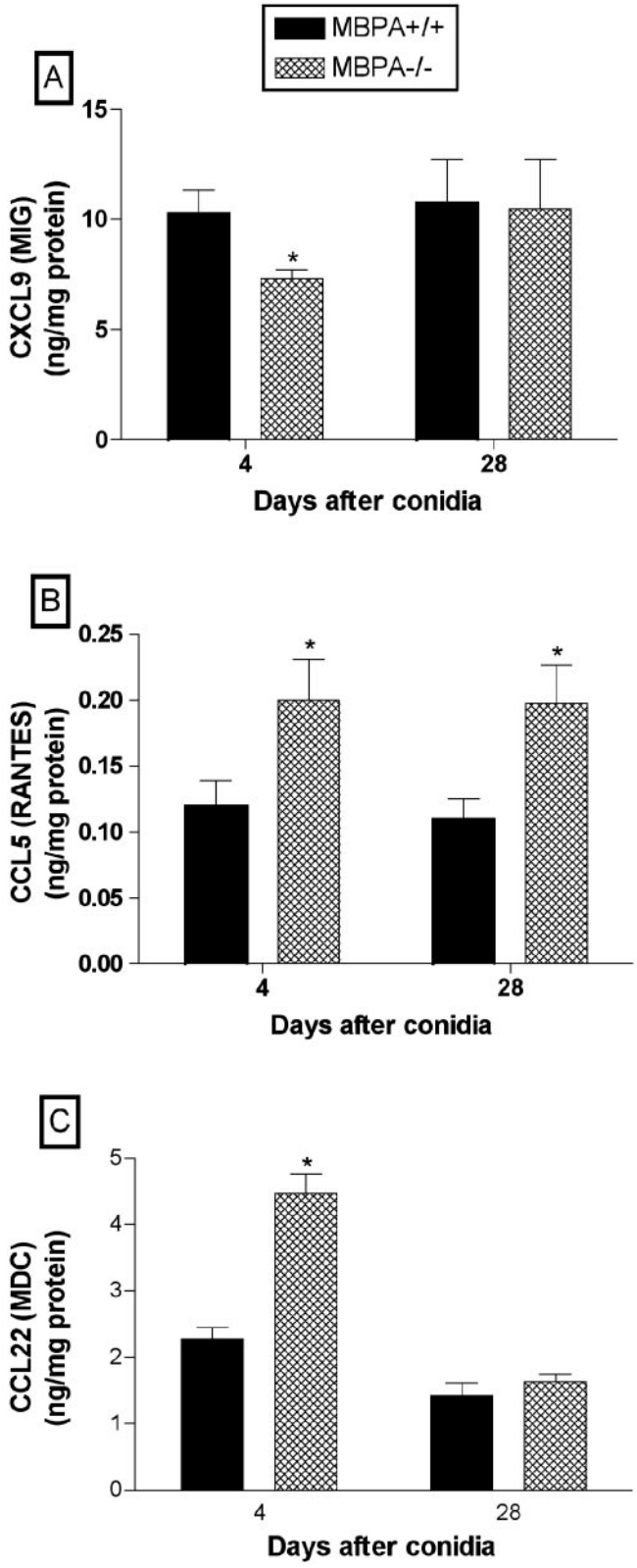

Fig. 5. Whole lung chemokine levels in *A. fumigatus*-sensitized MBL-A+/+ and MBL-A−/− mice at days 4 and 28 after i.t. challenge with 5.0×10^6 conidia. Cell-free supernatants from whole lung samples by specific ELISA measured CCL5, CCL22, and CXCL9. Specific levels were normalized to total protein in each sample. Values are expressed as mean ± SEM; n = five/group/time point. *, $P \leq 0.05$, compared with whole lung chemokine levels in MBL-A+/+ mice. MIG, Monokine induced by IFN-γ; RANTES, regulated on activation, normal T expressed and secreted; MDC, macrophage-derived chemokine.

inflammatory, and remodeling responses in this model of chronic fungal asthma. In the present study, ELISA measured whole lung levels of the CXC chemokines relevant to chronic fungal asthma including CXCL2, CXCL3, and CXCL9 [31]. Whole lung levels of CXCL2 and CXCL3 were not altered by MBL-A deficiency, but the level of the Th1 cytokine-induced CXCL9 was significantly lower in whole lung samples from the MBL-A−/− group at day 4 after conidia compared with the MBL-A+/+ group at the same time (**Fig. 5A**). Major CC chemokines relevant to this chronic fungal asthma model were also measured, and they include CCL3 [32], CCL5 [33], CCL11 [34], and CCL22 [35]. Compared with the appropriate MBL-A+/+ group, significantly higher levels of CCL5 (Fig. 5B) and CCL22 (Fig. 5C) were detected in whole lung samples from the MBL-A−/− group at day 4 after conidia, and CCL5 remained significantly higher in this group at day 28 after conidia. Together, these data suggest that MBL-A negatively regulates the elaboration of pro-allergic chemokines in the airways of mice experiencing chronic fungal asthma.

Elimination of fungal material from the lungs of *A. fumigatus*-sensitized mice was not impaired by MBL-A deficiency

The presence of fungal material in tissues, such as the lung, is readily identified using a GMS stain. In our previous studies of this chronic fungal asthma model, we have observed that certain gene-deficient mice are clearly impaired in their ability to clear fungal material and conidia from their airways. One example is the CCR2-deficient mouse, which shows persistent evidence of GMS-positive fungal material in mononuclear cells within and around major airways [36]. In the present study, we observed some distinct differences in GMS staining in the MBL-A+/+ and MBL-A−/− groups (**Fig. 6**). In the wild-type group, GMS-positive mononuclear cells were prominent at day 4 (Fig. 6A) after conidia challenge, whereas few GMS-positive cells were present in the lungs of this group at day 28 (Fig. 6C). In the MBL-A−/− group, GMS-positive mononuclear cells were present at day 4, but the majority of the GMS staining was present in and around the pulmonary vasculature (Fig. 6B). Although much less GMS staining was present in the lungs of MBL-A−/− mice at day 28, GMS staining was still prominent in the vasculature. Thus, although the elimination of fungal material from the airways did not appear to be impaired by MBL-A deficiency, it was evident that the distribution of GMS staining in the lungs of MBL-A differed from that observed in the MBL-A+/+ mice.

Airway remodeling was not altered by MBL-A deficiency during the course of chronic fungal asthma

Further histological analysis of whole lung samples from MBL-A+/+ and MBL−/− mice revealed some differences between these groups of mice during the course of fungal asthma. It was apparent that the peribronchial inflammatory response was less pronounced in the MBL-A+/+ group (**Fig. 7A**) at day 4 after conidia compared with the MBL-A−/− group (Fig. 7B). The degree of peribronchial inflammation did not appear to differ between the MBL-A+/+ (Fig. 7C) and the MBL-A−/− (Fig.

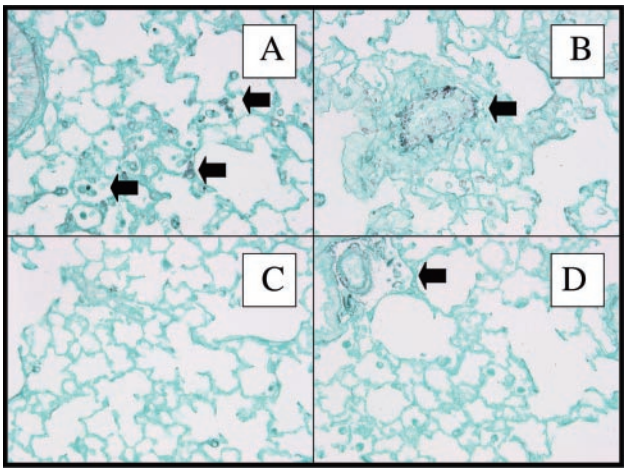

Fig. 6. Representative photomicrographs of GMS-stained whole lung sections from *A. fumigatus*-sensitized MBL-A+/+ (A and C) and MBL-A−/− (B and D) mice at days 4 (A and B) and 28 (C and D) after i.t. challenge with 5.0×10^6 conidia. The arrows highlight fungal material. In MBL-A+/+ mice, the majority of fungal material was observed in mononuclear cells at day 4 (A), and little fungal material was observed at day 28 (C) after conidia challenge. In MBL-A−/− mice, the majority of fungal material was visualized around pulmonary vessels. Original magnification, 200×.

7D) groups at day 28 after conidia. The MBL-A−/− group (**Fig. 8B**) had appreciably more goblet cell metaplasia at day 4 compared with the MBL-A+/+ group (Fig. 8A) at the same time point after conidia, yet no difference could be detected between the wild-type (Fig. 8C) and knockout (Fig. 8D) groups at day 28 after conidia. Finally, the degree of peribronchial fibrosis in the MBL-A+/+ (**Fig. 9A**) and the MBL-A−/− (Fig. 9B) groups at day 28 was indistinguishable, which was confirmed in a biochemical analysis of total collagen in whole lung samples at this time (data not shown). Together, these data suggested that certain airway remodeling features in this model of chronic fungal asthma were accelerated by the absence of

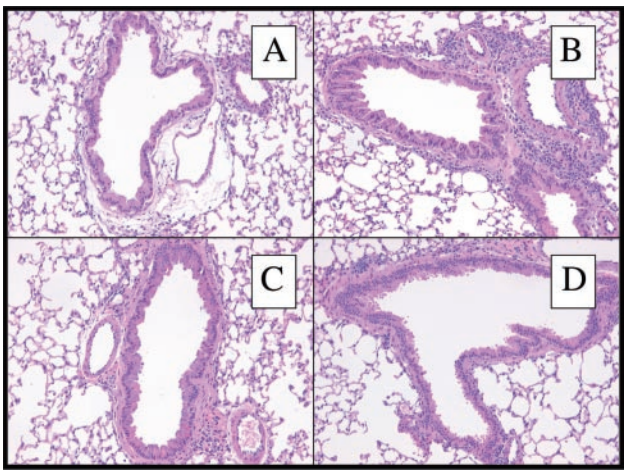

Fig. 7. Representative photomicrographs of H/E-stained whole lung sections from *A. fumigatus*-sensitized MBL-A+/+ (A and C) and MBL-A−/− (B and D) mice at days 4 (A and B) and 28 (C and D) after i.t. challenge with 5.0×10^6 conidia. MBL-A−/− (B) mice exhibited markedly greater peribronchial inflammation compared with the MBL-A+/+ (A) group at day 4 after conidia. Original magnification, 200×.

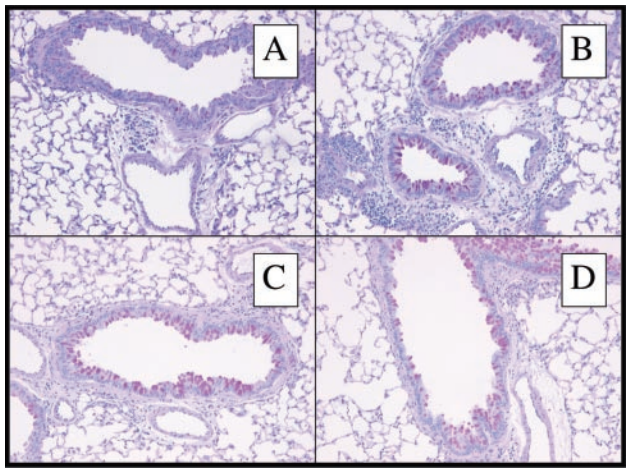

Fig. 8. Representative photomicrographs of PAS-stained whole lung sections from *A. fumigatus*-sensitized MBL-A+/+ (A and C) and MBL-A−/− (B and D) mice at days 4 (A and B) and 28 (C and D) after i.t. challenge with 5.0×10^6 conidia. MBL-A−/− (B) mice exhibited markedly greater goblet cell metaplasia compared with the MBL-A+/+ (A) group at day 4 after conidia. Original magnification, 200×.

MBL-A in *A. fumigatus*-sensitized mice, but at the latter time point, the degree of airway remodeling was similar in both groups of mice.

DISCUSSION

MBL is a liver-derived serum molecule that belongs to the collectin family, a group of glycoproteins whose functions are linked to innate immune responses against viral, fungal, microbial, and parasitic pathogens [2, 37]. The collectins have a collagen-like domain and a C-type lectin domain. The human collectins surfactant protein-A (SP-A), SP-D, and MBL-A are found in a genetic cluster on chromosome 10 [38]. The genetic grouping of these particular collectins is mirrored in the murine systems where they are clustered on chromosome 14 [39]. Recent research has revealed a protective role for SP-A and SP-D in *Aspergillus* infection [40, 41]. The proximity of the MBL-A gene to these important, functionally related, innate molecules suggests a role for MBL-A in the immune response to fungal pathogens.

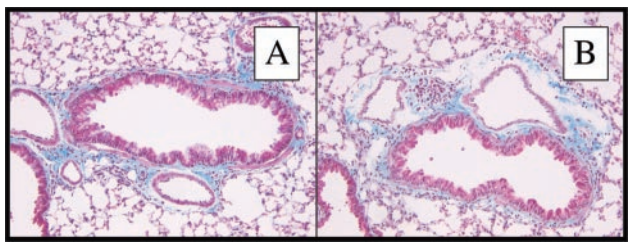

Fig. 9. Representative photomicrographs of trichrome Masson-stained whole lung sections from *A. fumigatus*-sensitized MBL-A+/+ (A) and MBL-A−/− (B) mice at day 28 after i.t. challenge with 5.0×10^6 conidia. No differences in peribronchial fibrosis were observed between the two groups. Original magnification, 200×.

Through recognition of distinct PAMPs, MBL triggers complement fixation through a modified classical pathway that involves the activation of C4 and C2 via MBL-associated serine proteases to generate complement components that have C3-convertase activity (reviewed in ref. [1]). Considerable interest in MBL has emerged because of growing clinical evidence that MBL makes an important contribution to innate immunity, with its absence typically increasing individual susceptibility to infectious pathogens and/or affecting the course of underlying disease [7, 9]. Although a strong association between MBL deficiency and susceptibility to bacterial and parasitic infections has been documented [9], far less is known about the importance of this collectin in antifungal responses. MBL appears to be important in containing *A. fumigatus*, given a recent clinical study that showed that decreased circulating concentrations of MBL were significantly associated with the presence of CNPA [17]. The advent of mice lacking one of the two MBL isoforms found in mice [14, 42] allowed us to undertake a detailed examination of the role of MBL in containing a bolus challenge of *A. fumigatus* conidia in the context of nonimmunocompromised mice that lacked prior sensitization to *A. fumigatus* or mice that were systemically sensitized to soluble *A. fumigatus* antigens.

The mode of recognition of *Aspergillus* conidia by phagocytes has been studied previously and yielded the following: Prior opsonization of the spores with normal rabbit serum, rabbit anti-*A. fumigatus* serum, complement, or lung lavage fluid has no profound enhancing effect on the phagocytosis or killing of the spores [43]; optimal association between phagocyte and conidia was dependent on an active, alternative complement pathway [44]; conidia cell wall components can degrade bound C3 [45, 46]; conidia pigmentation regulates C3 binding [47]; and bound MBL was able to promote C4 deposition in a concentration-dependent manner [4]. Although MBL-A−/− mice were not more susceptible to *Candida albicans* or *Plasmodium yoelil* compared with MBL-A+/+ [42], we nonetheless first assessed whether MBL-A−/− mice were similarly resistant to an i.t. or i.v. challenge with live *A. fumigatus* conidia. Our data, combined with the previous study in MBL-A−/− [42], suggest that the lack of MBL-A may not be sufficient to profoundly impair the antifungal innate immune response, given that MBL-C may fully compensate for the lack of A isoform. However, MBL-A deficiency did have an effect on the histological appearance of the lung, possibly suggesting that the clearance of *A. fumigatus* from the lungs of nonsensitized MBL-A−/− mice was not as efficient as that in their wild-type counterparts. The airway features most prominent in the MBL-A−/− group at day 28 after the i.t. introduction of 5.0×10^6 conidia were peribronchial inflammation and goblet cell hyperplasia. Both of these airway features are cardinal signs of persistence of fungal antigens with the lung, possibly in a manner similar to that we have observed in the context of *A. fumigatus*-sensitized mice [18]. One caveat to this hypothesis is that we did not observe GMS-stained material in the lungs of MBL-A−/− mice, but it is possible that this histological technique for fungal antigen detection may not be sufficiently sensitive to detect the minute amounts of fungal antigen that may be driving the airway inflammatory and remodeling events. Thus, the lack of MBL-A did not affect mortality following a bolus conidia challenge, but its absence did affect the lung in a manner reminiscent of *Aspergillus*-induced airway disease [21].

Asthma is a chronic, Th2, cytokine-dominated, inflammatory disease of the lung, resulting in airway hyperreactivity and remodeling. Th2 cytokines, particularly IL-4, IL-5, and IL-13, regulate the airway hyperresponsiveness, eosinophil activation, and mucus production. Although not historically viewed as important in the pathogenesis of asthma [48], recent studies have shown that the complement anaphylatoxins C3a [49–51] and C5a [52] contribute to pulmonary allergy in experimental allergic airway responses. The major features of allergic airway disease that were significantly attenuated by the absence of complement or complement receptors were airway hyperresponsiveness, airway eosinophilia, and Th2 cytokine levels. In the present study, the results obtained from *A. fumigatus*-sensitized MBL-A−/− mice, challenged i.t. with conidia, shared similarities with those observed in the previous studies referenced above [48]. At day 4 after conidia challenge in *A. fumigatus*-sensitized MBL-A−/− mice, we observed a profound decrease in airway hyperreactivity and whole lung Th2 cytokine levels compared with their wild-type counterparts. However, BAL eosinophilia was actually enhanced in the day-4 MBL-A−/− group. This finding is possibly explained by the significant increases in potent eosinophil chemoattractants CCL5 and CCL22 [33, 35] in the lungs of this group. Coinciding with a significant increase in whole lung levels of IFN-γ, airway hyperreactivity and BAL eosinophilia were significantly attenuated at day 28 after conidia challenge in the MBL-A−/− group compared with the MBL-A+/+ group. However, the absence of MBL-A in *A. fumigatus*-sensitized mice did not attenuate the goblet cell metaplasia and peribronchial fibrosis typically associated with chronic fungal asthma [18]. This finding contrasts with that of Drouin and colleagues [51] who found that C3a receptor-deficient mice had significantly reduced mucus production compared with their wild-type controls. Although we have observed a dissociation between airway responsiveness to methacholine challenge and airway remodeling (i.e., goblet cell metaplasia) during chronic fungal asthma [32, 35, 53], it is possible that the presence of MBL-C in MBL-A−/− mice or inflammatory responses modulated by chemokines such as CCL5 [33] and CCL22 [35] may have promoted the airway remodeling features intensely evident at day 28 after conidia challenge in both groups of sensitized mice. Generation of mice deficient in both murine MBL isoforms may facilitate whether airway-remodeling events are driven by MBL-C.

In summary, the present study demonstrates that MBL-A has a nonredundant role in the context of murine innate and allergic pulmonary responses to *A. fumigatus* conidia. Although the mice examined in the present study were sufficient in MBL-C [14], it was clear that the absence of MBL-A modulated airway inflammatory and remodeling events in nonsensitized and *A. fumigatus*-sensitized mice. Taken together, these above findings recapitulate the earlier conclusions of Takahashi et al. [14] in which they demonstrated that the role of MBL-A appeared to extend beyond that of opsonin to modulator of inflammatory processes in the mouse.

ACKNOWLEDGMENT

The National Institutes of Health supported this work.

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