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_J Immunol_ 2007; 178:5116-5123; doi: 10.4049/jimmunol.178.8.5116

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Mannose-Binding Lectin A-Deficient Mice Have Abrogated Antigen-Specific IgM Responses and Increased Susceptibility to a Nematode Infection

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To investigate the role of mannose-binding lectin-A (MBL-A) in protection against infectious disease, MBL-A−/−-deficient mice were generated. Using a well-characterized mouse model of human filarial nematode infection, nematode survival and protective immune responses were tested in vivo. Blood-borne Brugia malayi microfilariae survived for significantly longer time periods in MBL-A−/− than in wild-type (WT) mice. However, no differences in either splenic cytokine responses or induction of leukocytes in the blood were observed. A profound abrogation of Ag-specific IgM levels was measured in B. malayi-infected MBL-A−/− mice, and some IgG isotypes were higher than those observed in WT animals. To establish whether there was a defect in Ab responses per se in MBL-A−/− mice or the effect was specific to filarial infection, we immunized these mice with OVA or a carbohydrate-free protein. Significantly, Ag-specific IgM responses were defective to both of these Ags, and Ag-specific IgG responses were largely unaffected. Furthermore, in naive mice, total IgM levels did not differ between MBL-A−/− and WT mice. This article describes the first demonstration that MBL-A may function independently of MBL-C and suggests that MBL-A, like other C-type lectins and members of the complement cascade, is intimately involved in the priming of the humoral Ab response. The Journal of Immunology, 2007, 178: 5116–5123.

Mannose-binding lectin (MBL)1 is part of the first line of immunological defense against invading pathogens. Its essential role in pathogen detection in humans is highlighted by the fact that individuals with MBL deficiency are susceptible to severe and repeated infections (1). MBL is a pattern recognition receptor present in sera, which directly binds a variety of sugars (mannose, l-fucose, N-acetylglucosamine, N-acetylmannosamine, or glucose) on the pathogen surface and triggers a third pathway of complement activation. Most mammalian carbohydrate structures are terminated by galactose or sialic acid and do not present the same molecular patterns characteristic of microorganisms, thus MBL is able to distinguish between self and non-self.

MBL is complexed with MBL-associated serine proteases. Upon MBL recognition of sugars, MBL-associated serine proteases are activated and split the C4 and subsequently C2 components of complement to form the enzyme C3 convertase, which triggers the remainder of the complement (C′) cascade. Thus, the MBL complement pathway is essentially similar to the classical pathway, with MBL replacing C1q in the cascade. Importantly, however, the MBL complement pathway uses direct innate recognition without the need for adaptive immunity. In addition to its role in the complement cascade, binding of MBL to the pathogen surface enhances uptake via receptor-mediated phagocytosis. Thus, MBL may also be an important mediator in Ag processing and presentation.

In humans, only one MBL molecule has been identified. However, in mice, two MBL molecules exist, MBL-A and MBL-C (2). MBL-A and MBL-C are 50% homologous, they are both found in sera and have overlapping binding specificities, and they are both able to activate complement C4 (3). MBL-A has a higher monosaccharide affinity and appears to have a five times greater complement-activating ability (3). Serum concentrations of MBL-A in naive mice (5–40 μg/ml) increase ~3-fold in the acute phase response. At present, it is not known whether MBL-A and MBL-C differ in their pathogen specificity. Indeed, to date it has been shown that absence of MBL-A does not affect either development of the mouse malarial parasite Plasmodium yoelii in the liver, or resistance to disseminated infection with Candida albicans (4). A recent study showed that MBL-A and MBL-C are largely redundant in enhancing the opsonization of the Gram-positive bacteria Staphylococcus aureus, whereas MBL (A + C)−/− mice are susceptible to i.v. but not i.p. infection (5).

In this study, we generated MBL-A-deficient (MBL-A−/−) mice and analyzed the immune responses of MBL-A−/− and wild-type (WT) mice in a well-characterized mouse model of parasitic nematode infection. We found that the blood-borne microfilarial (MF) stage of the nematode, Brugia malayi, has significantly enhanced survival in MBL-A−/− mice. In addition, we have obtained some novel observations, suggesting a role for MBL-A in the development of humoral immunity. As yet, the link between innate recognition by MBL and development of the adaptive immune response has not been investigated. This is the first clear phenotype

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Received for publication November 21, 2006. Accepted for publication January 26, 2007. 
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 
1 This work was supported by the Wellcome Trust. 
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4 Abbreviations used in this paper: MBL, mannose-binding lectin; WT, wild type; MF, microfilariae; MfAg, soluble MF extract; p.i., postimmunization; GC, germinal center; Sn, sialoadhesin. 
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reported in these mice and the first study in which a single deletion in MBL has been shown to render mice more susceptible to a particular infection.

**Materials and Methods**

**Preparation of knockout mice**

We isolated mouse MblA genomic clones by screening a λ Fix II mouse 129/Sv genomic library (Stratagene) with a full-length MblA cDNA probe (6). Two phage clones, L and GA1, were restriction mapped and found to include 24.5 kb of genomic DNA encompassing the entire MblA locus (Fig. 1A). An 8-kb HindIII fragment from AL and a 3-kb HindIII fragment from AG1 were subcloned into pBluescript II KS (Stratagene). A targeting vector was constructed with the 8-kb HindIII fragment as the upstream homologous arm and the 3-kb HindIII fragment as the downstream homologous arm (Fig. 1A). Because the mutations causing human MBL deficiency are located in the collagenous domain, which is essential for MBL polymerization, we disrupted the murine exons encoding this domain. The 2-kb region of the upstream arm containing exons 2 and 3, which encode the translation start codon and the collagenous domain, was replaced with a neomycin resistance cassette inserted in the opposite orientation to the endogenous locus.

Gene targeting was conducted using 129/Sv XY ES cells RW4 (Incyte Genomics). ES cells (10^7) were electroporated (400 V, 125 μF) with 10 μg of linearized targeting vector RNA and grown for 10 days with G418 (250 μg/ml). Surviving clones were screened for homologous recombination with the MblA allele by Southern blot analysis of genomic DNA and digested with Stul to detect the novel Stul restriction site introduced by gene targeting (see Fig. 1A), using a radiolabeled probe derived from a sequence external to the targeting construct. To exclude additional random integration of the targeting construct, homologous recombinant clones were screened bySouthern blot analysis with a radiolabeled probe derived from a sequence internal to the targeting construct. Nine of the 109 neomycin-resistant clones were identified as gene targeted. Targeted ES cells were injected into C57BL/6J blastocysts that were implanted into C57BL/6J pseudopregnant foster mothers. Transmission of the targeted MblA allele was determined by Stul digestion and Southern blot analysis of genomic DNA prepared from day 21 tail biopsies from F1 mice. Male and female heterozygous mice were bred to obtain mice homozygous for the null allele (Mbl-A^-/-) on a 129/Sv × C57BL/6 hybrid background. Autosomes were performed on 4-mo-old homozygous mice to investigate whether absence of MBL-A caused any gross anatomical or histopathological changes. Histological tissue sections were taken from the liver, kidney, spleen, lung, and heart of Mbl-A^-/- and WT mice and examined for any deletion-associated structural differences following staining with H&E. All animal experiments were performed in accordance with the ethical review committee.

**PCR genotyping**

Genotyping of mice was performed by PCR of DNA obtained by tail biopsy. PCR used the primers neo forward CTTCAATTGTGTACGGCTCT, exon 3 forward CAAGGGGAGCAGAATAG, and reverse TG GAAATGTAAATGAGGAAAATACC. PCR amplification conditions were as follows: denaturation at 94°C for 5 min, 35 cycles of 94°C for 45 s, 61°C for 30 s, 72°C for 1 min, and a final cycle of 72°C for 10 min.

**RNA extraction and Northern blot analysis**

RNA was extracted from tissue using TRIZol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. Northern blots of RNA (25 μg) from tissue of mice were probed with MblA mRNA using a 550-bp PstI/BamH1 fragment from the 3’ end of MblA cDNA (6), whereas MblC was probed with 300 bp of exon 6 from an EcoRI digest of a MblC clone (our unpublished data). A 245-bp mouse β-actin probe was obtained from pTRI-β-actin mouse (Ambion). Probes were labeled using the Megaprime random priming protocol (Amersham Biosciences).

**SDS PAGE and Western blot analysis**

Mouse serum was run on a polyacrylamide gel, electrophoresed onto a nitrocellulose membrane (Bio-Rad), and blocked overnight at 4°C in PBS, 0.05% Tween 20, and 5% milk powder (PBS-T). Rabbit anti-mouse MBL-A (6) was diluted 1:5000 in PBS-T with 5% milk powder and the membrane was incubated at room temperature for 2 h. The membrane was washed with PBS-T and incubated with HRP-conjugated swine anti-rabbit IgG diluted 1:5000 in PBS-T. Chemiluminescence was detected with HRP-L substrate (National Diagnostics) and exposure to x-ray film.

**Assay for MBL pathway of complement activation**

Microtiter plates were coated with 5 μg of mannan (Sigma-Aldrich) in 100 μl of 15 mM Na2CO3, 35 mM NaHCO3, and 1.5 mM NaNO3 (pH 9.6). After incubation overnight at 37°C, the plate was washed three times with PBS-T, followed by overnight blocking (3% BSA in PBS) at 4°C. Serum was serially diluted in either Veronal-buffered saline (4.4 mM diethybarbiturate acid, 1.8 mM sodium barbiturate, 145 mM NaCl, 5 mM CaCl2, and 5 mM MgCl2 (pH 7.3)) or Veronal-buffered saline plus 20 nM EDTA. The blocked plate was washed with PBS-T and PBS plus 50 μl of serum and incubated at 37°C for 1 h to allow complement activation to proceed. Plates were washed and HRP-conjugated goat anti-mouse C3 (1/5000 in PBS-T; Chemicon International) was added for 1 h at 37°C. HRP was detected by the addition of o-phenyldiamine (Sigma-Aldrich) and phosphate buffer (pH 5.0). Absorbance was measured at 490 nm.

The C4 assay was virtually identical. Rat anti-mouse C4 Ab (Connex) was added to BSA-blocked mammalian plates coated with serum as above and incubated for 1 h at 37°C. The plate was washed and goat anti-rat Ig-HRP (Chemicon International) was used to detect the anti-mouse C4.

**Parasite material**

*B. malayi* Mf were obtained by peritoneal lavage of infected gerbils (*Meriones unguiculatus*) with RPMI 1640. Gerbil host cells were removed by centrifugation over lymphocyte separation medium (Flow Laboratories). Mf were washed, counted, and resuspended in RPMI 1640 for i.v. injection. Soluble Mf extract (MfAg) was prepared by homogenization of Mf in PBS on ice, followed by centrifugation at 10,000 × g for 20 min at 4°C. The supernatant was passed through a 0.2-μm filter, and the concentration of Ag was determined using the Bio-Rad protein assay. MfAg was stored at −20°C until use.

**Infection protocol**

Groups of five 8-wk-old MBL-A^-/- mice (F2, C57BL/6 × 129) and their WT counterparts were injected with 2 × 10^5 Mf of *B. malayi* i.v. Mice were bled at intervals from the lateral tail vein. Mf survival was monitored in −50 μl of blood during the experimental time course. Blood was also used for plasma collection and total and differential white blood cell counts. At experiment termination, MF were counted in 200 μl of cardiac blood.

**Measurement of binding of MBL-A and the level of MBL-A-dependent C3 deposition on the surface of Mf**

One hundred Mf were incubated in WT or MBL-A^-/- sera with or without EDTA diluted 1/2 in 10 mM Tris, 0.5 M NaCl, 5 mM CaCl2, and 0.05% Tween 20 for 2 h at 4°C. Mf were washed in 20 mM Tris, 0.5 M NaCl, 5 mM CaCl2, and 0.05% Tween 20. Mf were incubated with polyclonal rabbit anti-MBL-A (6) diluted 1/50 in the same incubation buffer for 1 h at 4°C. Mf were washed before incubation with swine anti-rabbit IgG FITC (DakoCytomation) for 45 min at 4°C. Mf were counterstained with Evans blue and mounted in Vectashield mounting medium and analyzed beneath the fluorescent microscope. C3 deposition on the Mf surface following MBL-A binding was measured by incubating Mf in either WT or MBL-A^-/- sera with or without EDTA diluted 1/2 in 10 mM Tris, 0.5 M NaCl, 5 mM CaCl2, and 0.05% Tween 20 for 2 h at 4°C, followed by washing and incubation with goat anti-mouse C3 (1/5000; MP Biomedicals). Mf were washed and incubated with rabbit anti-goat FITC (DakoCytomation) at a concentration of 1/300. Fluorescent intensity levels were quantified using Leica Qwin V3 software at the same magnification, light settings, and detection filter 480/560 for each specimen. Intensity levels of 20 Mf from each sample were measured.

**Immunization with OVA and KpsD**

Groups of five 8-wk-old MBL-A^-/- mice (F2, C57BL/6 × 129) and their WT counterparts were immunized with 100 μg of OVA (Sigma-Aldrich) in CFA s.c. in the inguinal region. On days 14 and 28 of the immunization protocol, mice were given a boost of 100 μg of OVA in IFA s.c. Two further groups of five MBL-A^-/- (F2, C57BL/6 × 129) and WT mice were immunized with 100 μg of OVA precipitated in aluminum hydroxide i.p. on days 0, 14, and 28. Mice were sacrificed on day 42 postimmunization (p.i.). In other experiments, groups of five 8-wk-old MBL-A^-/- and WT mice were room temperature with 100 μg of KpsD (7) s.c. on day 0 and challenged on day 21 p.i., and mice were bled on days 0, 4, 7, and 11 after primary immunization and then at days 0, 7, 14, and 21 after secondary immunization.
Total and differential white blood cell counts

Total white blood cells were counted using a hemocytometer after diluting whole blood 1:10 with Türk’s solution (3% acetic acid and 0.01% gentian violet). Blood smears were made on glass slides, allowed to air dry for 20 min, and fixed in 100% methanol. Slides were stained with May-Grunwald (Sigma-Aldrich) and 5% Giemsa solution. The numbers of lymphocytes, neutrophils, eosinophils, and monocytes were counted under a light microscope, and the total cell numbers per milliliter of blood were calculated.

Measurement of Mf-specific serum Ig isotypes

Mf-specific and OVA-specific IgM and IgG isotypes were both measured by ELISA. In brief, 96-well plates were coated overnight at 4°C with 1 µg/ml MfAg or 30 µg/ml OVA in 50 µl of carbonate buffer (pH 9.6). After blocking with 100 µl/well of 10% FCS in carbonate buffer, the plates were incubated with individual mouse sera serially diluted in PBS and 0.5% Tween 20 from 1/100 to 1/6400. Ag-specific Abs were detected using peroxidase-conjugated goat anti-IgM (1/4000, SBA 1080-05), anti-IgG1 (1/6000, SBA 1070-05), anti-IgG2a (1/4000, SBA 1080-05), anti-IgG2b (1/4000, SBA 1090-05), or anti-IgG3 (1/10000, SBA 1100-05). 2,2’-azino-bis[3-ethylbenz-thiazoline-6-sulfonic acid] (Sigma-Aldrich) at 1 mg/ml in citrate buffer plus 0.003% hydrogen peroxide was used as the substrate. Plates were read at 405 nm. Nonsaturating serum dilutions were compared for analysis.

Cell culture and cytokine ELISA

Spleen cells were cultured at 5 × 10⁵ cells/ml in RPMI 1640 plus 10% FCS and 10 µg/ml MfAg or 1 µg/ml anti-CD3 as previously described (8). Cells were incubated for 66 h at 37°C, after which supernatants were removed for cytokine analysis. The concentration of the cytokines, IL-4, IL-5, IL-10, and IFN-γ in the recovered supernatants, was determined by sandwich ELISA. Purified and biotinylated mAb pairs, 11B11 and BVD6-24G2 (IL-2), KL-1 and BVD6-24G2 (IL-4), TRFK5 and TRFK4 (IL-5), JES5-2A5 and SXC-1 (IL-10), and R46A2 (IL-12) were purchased from BD Pharmingen. Cytokine concentrations were measured against recombinant murine cytokine standards as previously described (8). Streptavidin alkaline-phosphatase (1/4000, SBA 1080-05), anti-IgG2b (1/4000, SBA 1090-05), or anti-IgG3 peroxidase-conjugated goat anti-IgM (1/4000, SBA 1020-05; Southern Biotechnology Associates) were used for detection, and plates were read at 405 nm.

Statistical analysis

All statistics were calculated from individual values using the Mann-Whitney U test for small sample sizes. A result of p < 0.05 was considered statistically significant.

Results

Generation and characterization of MBL-A−/− mice

MBL-A−/− mice were generated by introducing a neomycin resistance gene into exons 2 and 3 of the MblA gene (Fig. 1A). To confirm the lack of MblA transcripts in these mice, Northern blot analysis was performed using RNA isolated from tissues taken from MBL-A knockout homozygous, heterozygous, and WT mice. MBL-A and MBL-C mRNA were expressed in liver, but not in kidney or lung, of both heterozygous and WT mice (Fig. 1B). In MBL-A−/− mice, mRNA for MBL-A was absent. In Western blots of whole mouse serum probed with a rabbit anti-mouse MBL-A Ab under nonreducing conditions, high molecular mass bands corresponding to MBL-A homopolymers were present in WT mice, but were absent in MBL-A−/− mice (Fig. 1C). The ability of mice to activate complement via the MBL pathway was measured by both C3 (Fig. 1D) and C4 (Fig. 1E) activation. WT and heterozygote mice showed strong C3 binding, and WT mice were also shown to activate C4. In the presence of EDTA, both C3 and C4 binding was abrogated. MBL-A−/− mice showed low levels of both C3 and C4 binding (in the absence of EDTA), presumably due to the presence of MBL-C, which also activates the MBL complement pathway. MBL-A−/− mice bred normally and appeared fit. Autopsies and histopathology on 4-mo-old mice did not reveal any gross anatomical or histopathological differences between MBL-A−/− and WT mice.

Survival of B. malayi Mf is significantly enhanced in the absence of MBL-A

Mice deficient in MBL-A were infected with B. malayi Mf i.v. MBL-A−/− mice maintained high and sustained Mf levels in the peripheral bloodstream and, within 7 days, Mf levels in MBL-A−/− mice were significantly higher than in WT mice (p > 0.01; Fig. 2A). In contrast, WT mice rapidly cleared their Mf infections within 20 days p.i. At experiment termination 28 days p.i., mice were bled from the cardiac cavity to attain a greater quantity of
MBL-A<sup>−/−</sup> mice retained significant numbers of Mf (p > 0.01), whereas WT mice had completely cleared their Mf (Fig. 2B).

**FIGURE 2.** Enhanced survival of *B. malayi* Mf in MBL-A<sup>−/−</sup> mice. Groups of five individual MBL-A<sup>−/−</sup> and WT mice were injected with 2.5 × 10<sup>5</sup> Mf i.v. A, Blood was taken from the tail vein and the Mf/ml blood counted at various time intervals p.i. in MBL-A<sup>−/−</sup> mice (■) and WT mice (□) B. At experiment termination on day 28, blood was taken from the cardiac cavity and the number of Mf/ml counted in MBL-A<sup>−/−</sup> mice (■) and WT mice (□). Results are shown as mean ± SE of Mf counts from individual mice per group, where * shows a statistical difference between MBL-A<sup>−/−</sup> and WT mice when p < 0.05, and **, p < 0.01.

To investigate whether MBL-A can bind directly to the surface of Mf, live Mf were incubated with sera from WT or MBL-A<sup>−/−</sup> mice in the presence or absence of EDTA. Polyclonal Ab to MBL-A was used to detect bound MBL-A (6). MBL-A serum strongly bound to the surface of Mf, but in the presence of EDTA, MBL-A binding was abrogated to the level measured using sera (Fig. 3C). C3 was detected on the surface of Mf following incubation with WT sera (Fig. 3, A and D). The C3 binding was abrogated in the presence of EDTA, which prevents C3 activation following MBL-A and MBL-C binding (but does not prevent any C3 binding via the alternative complement pathway) (Fig. 3B). C3 binding following incubation with MBL-A<sup>−/−</sup> sera was significantly reduced compared with WT sera (p > 0.05; Fig. 3, B and D), showing that the majority of C3 deposited by WT sera was activated via MBL-A and the lectin pathway. C3 binding after incubation with MBL-A<sup>−/−</sup> sera did not differ whether EDTA was present or not (p < 0.05), suggesting that MBL-C contributes little to C3 activation on the Mf surface.

**FIGURE 3.** MBL-A can bind to the surface of Mf and activate C3. A and *B. malayi* Mf were incubated with naive sera from either WT mice or (B) MBL-A<sup>−/−</sup> mice, binding of C3 was detected using a goat anti-mouse C3 Ab. C. *B. malayi* Mf were incubated with naive sera from either WT mice or MBL-A<sup>−/−</sup> mice with or without EDTA, and the amount of MBL-A binding was detected using a rabbit polyclonal anti-MBL-A Ab followed by anti-rabbit Ig FITC. (The level of FITC was quantified using Leica Qwin version 3 software on 20 Mf/assay). KO, Knockout.

**FIGURE 4.** Mf-specific Ig isotype responses in MBL-A<sup>−/−</sup> mice. Groups of five MBL-A<sup>−/−</sup> (■) and five WT (□) mice were injected with 2 × 10<sup>5</sup> *B. malayi* Mf. Mice were bled at various intervals p.i., and Mf-specific Ig was measured by ELISA. Data are presented as mean ± SE of individual mice per group, where * shows a statistical difference between MBL-A<sup>−/−</sup> and WT mice when p < 0.05, and **, p < 0.01.

**MBL-A binds the surface of *B. malayi* Mf and activates C3**

Complement activation is responsible for a plethora of immunological responses, and activation of the membrane-attack complex alone is unlikely to affect a large nematode bound by a thick collagenous cuticle. We therefore analyzed the immune responses of *B. malayi* Mf-infected MBL-A<sup>−/−</sup> and WT mice (F2, C57BL/6 × 129).
Altered levels of Mf-specific Ig isotypes in MBL-A−/− mice infected with Mf (data not shown).

Cytokine responses in MBL-A−/− or mice infected with Mf (data not shown).

Spleen cells removed from MBL-A−/− and WT mice were analyzed during the course of Mf infection in MBL-A−/− and WT mice. No significant differences in the levels of lymphocytes, monocytes, eosinophils, or neutrophils were found between MBL-A−/− and WT mice in either uninfected mice or mice infected with Mf (data not shown).

Cytokine responses in MBL-A−/− mice following Mf infection

Spleen cells from MBL-A−/− and WT mice 28 days after Mf infection were restimulated in vitro with either Mf extract or anti-CD3 to determine whether lack of MBL-A leads to a significant skewing of the cytokine immune response. IFN-γ, IL-4, IL-5, and IL-10 responses did not significantly differ between spleenocytes from groups of MBL-A−/− and WT mice (data not shown).

Altered levels of Mf-specific Ig isotypes in MBL-A−/− mice

To establish whether there were alterations in the development of other immune responses in MBL-A−/− mice. Total and differential white blood cells were analyzed during the course of Mf infection in MBL-A−/− and WT mice. No significant differences in the levels of lymphocytes, monocytes, eosinophils, or neutrophils were found between MBL-A−/− and WT mice in either uninfected mice or mice infected with Mf (data not shown).

To establish whether MBL-A−/− mice were defective in their ability to generate IgM to Ags other than live nematode infection, we assessed the ability of these mice to produce Ab responses to the model Ag OVA. Repeated immunization of MBL-A−/− and WT mice with OVA in either Freund’s adjuvant or alum revealed profound differences in the induction of IgM between the two strains compared with WT mice. The levels of Mf-specific IgG2b induced in MBL-A−/− mice did not differ significantly from those of WT animals (p < 0.05). Thus, the data suggest that MBL-A plays a critical role either in the level of IgM production and/or in the regulation of the degree of class switching to IgG isotypes.

Altered levels of OVA-specific Ig isotypes in MBL-A−/− mice

To establish whether MBL-A−/− mice were defective in their ability to generate IgM to Ags other than live nematode infection, we assessed the ability of these mice to produce Ab responses to the model Ag OVA. Repeated immunization of MBL-A−/− and WT mice with OVA in either Freund’s adjuvant or alum revealed profound differences in the induction of IgM between the two strains.

FIGURE 5. OVA-specific Ig isotype responses in MBL-A−/− mice. Groups of five MBL-A−/− (■) and five WT (○) mice were immunized with OVA on days 1, 14, and 28 in either Freund’s adjuvant (A) or alum (B). Mice were bled at various intervals p.i., and OVA-specific Ig was measured by ELISA. The figure shows OVA-specific isotypes 14 days after the last immunization. C, OVA-specific IgM on day 14 following primary immunization with OVA in CFA. Data are presented as mean ± SE of individual mice per group, where * shows a statistical difference between MBL-A−/− and WT mice when p < 0.05, and **, p < 0.01.

FIGURE 6. OVA-specific IgM responses in MBL-A−/− mice. A, Groups of five MBL-A−/− (■) and five WT (○) mice were immunized with OVA on day 0 in Freund’s adjuvant. Mice were bled at various intervals p.i., and OVA-specific IgM was measured by ELISA. B, Total IgM levels in naive MBL-A−/− (■) and WT (○). Data are presented as mean ± SE of individual mice per group, where * shows a statistical difference between MBL-A−/− and WT mice when p < 0.05, and **, p < 0.01.
Production of OVA-specific IgM in MBL-A−/− mice was not detectable following immunization in either adjuvant, while high levels of OVA-specific IgM were induced in WT mice ($p > 0.01$). OVA-specific IgM responses in MBL-A−/− mice were also significantly decreased during primary responses to OVA (Fig. 6A). However, levels of total serum IgM isotypes in naive MBL-A−/− (158 ± 53 ng/ml; $n = 7$) did not differ significantly from the levels in WT mice (208 ± 32 ng/ml, $p < 0.05$; Fig. 6B). This suggests that MBL-A plays a critical role in the induction and/or regulation of Ag-specific IgM.

High levels of the OVA-specific IgG isotypes, IgG1, IgG2a, and IgG2b, were induced in both MBL-A−/− and WT mice (Fig. 5). IgG3 production was not induced in either mouse strain by this immunization protocol (data not shown). MBL-A−/− mice produced higher levels of both IgG1 and IgG2a when immunized with OVA in either Freund’s or alum adjuvant, but the differences were not significant ($p < 0.05$; Fig. 5, A and B). Intriguingly, however, the levels of OVA-specific IgG2b induced in MBL-A−/− mice were significantly lower than those of WT animals in animals immunized with OVA in either Freund’s or alum adjuvant ($p > 0.05$; Fig. 5). Overall, IgG isotypes induced by repeated OVA immunization were similar between MBL-A−/− and WT mice, although MBL-A may play a role in the regulation of IgG2b production.

**Induction of Ag-specific Ig isotype responses to carbohydrate-free Ag**

OVA is known to possess a number of carbohydrate residues (9) that can be recognized by MBL-A. Thus, to investigate whether the Ig responses in MBL-A−/− mice differ from WT because Ags with carbohydrate residues are recognized or processed differently in the presence of MBL-A, we immunized both MBL-A−/− and WT mice with an Escherichia coli bacterial protein (KspD), which is likely to be glycosylation free (7).

Following primary and secondary immunization with KspD, Ag-specific isotypes were measured in the sera of both MBL-A−/− and WT mice (Fig. 7). Only very low levels of KspD-specific IgM were detected in WT mice on day 11 after primary immunization; however, in MBL-A−/− mice, no IgM was induced following KspD immunization ($p > 0.05$). In contrast, relatively high levels of KspD-specific IgG1 were induced in both MBL-A−/− and WT mice following primary and secondary immunization ($p < 0.05$). Low levels of KspD-specific IgG2a and IgG2b were consistently detected in both groups of mice, and after secondary immunization, the KpsD-specific IgG2b levels were augmented in WT compared with MBL-A−/− ($p < 0.05$; Fig. 7).

**Discussion**

In this study, we generated mice that were deficient in MBL-A, but not MBL-C. Using a well-characterized model of parasitic nematode infection (10, 11), we showed that the blood-borne Mf stage of the parasitic nematode, *B. malayi*, greatly enhanced survival in MBL-A−/− mice. Thus, we report a clear phenotype in mice that lack MBL-A alone and demonstrate that MBL-A is functionally nonredundant in immunity to certain pathogens.

Previous studies have shown that MBL-A deficiency does not alter resistance to either disseminated candidiasis or to hepatic invasion by *Plasmodium yoelii* (4). However, studies using MBL null mice, which lack both MBL-A and C, report some enhanced susceptibilities to certain pathogens. For example, MBL-A and C null mice are more susceptible to i.v. but not i.p. *Staphylococcus aureus* infection (5). MBL null mice are also more susceptible to postburn infection with *Pseudomonas aeruginosa* (12). However, in these studies, loss of both MBL-A and MBL-C is needed for the increased susceptibility, and it has yet to be determined whether MBL-A and MBL-C can differ in their pathogen specificity.

Thus, our finding that survival of *B. malayi* is enhanced in MBL-A−/− mice is surprising. We have shown that MBL-A binds to the surface of Mf and that C3 can be measured as a result of MBL-A binding. *B. malayi* Mf are entirely enclosed by a carbohydrate-rich sheath that expresses D-galactose, N-acetyl-d-glucosamine, d-mannose, and L-fucose (13–16). Intriguingly, differential analysis of the carbohydrate specificities of MBL-A and MBL-C reveals an explanation for MBL-A-dependent, but not MBL-C-dependent, C3 binding to the Mf surface; MBL-A has a significantly higher potency than MBL-C for binding both L-fucose and N-acetyl-d-glucosamine, while MBL-C does not bind D-galactose (3). However, the thick collagenous cuticle of MF is unlikely to be susceptible to the pore-forming qualities of the membrane-attack complex of complement. In addition, although MBL-A is known to augment opsonization and phagocytosis of certain microbes (17), Mf are too large (170–230 μm in length) to be phagocytosed and therefore enhanced survival of MF in the absence of MBL-A is unlikely to result from reduced phagocytosis. We therefore examined several immunological parameters in the MBL-A−/− mice to determine whether absence of MBL-A interfered with the induction of other immune responses.

Products of the complement cascade, such as C5a and C5a, are crucial for phagocyte recruitment; nevertheless, absence of MBL-A did not alter either total or differential white blood cell counts. However, the possibility that MBL-A enhances cellular attachment to parasites and increased trapping in the spleen or other organs cannot be excluded. Although, MBL-A deficiency may cause complement-mediated changes in cellular activation status, we found no profound differences in splenic T cell cytokine responses to either parasites or OVA in the absence of MBL-A (although in some experiments we did observe increased IFN-γ in...
MBL-A−/− mice). Previous work has shown that enhanced survival of MBL-A−/− mice in an acute septic peritonitis model correlated with decreased levels of both TNF-α and IL-6 in the blood and peritoneal cavity (18), while in contrast, MBL-A−/− mice had decreased Th2 responses and increased IFN-γ during fungal-induced asthma (19). Human MBL suppresses the monocyte proinflammatory cytokines IL-1α and IL-1β and increases IL-10, IL-1R antagonist, MCP-1, and IL-6 following LPS stimulation in vitro (20). However, splenic dendritic cells and peritoneal macrophages taken from MBL-A and C−/− mice express similar amounts of costimulatory molecules and MHC to WT mice (21). Thus, in different contexts, MBL has been shown to induce or modulate proinflammatory responses, and it has yet to be definitively determined whether MBL performs different roles depending on whether it recognizes carbohydrates on the pathogen surface or it recognizes altered self in the context of necrotic and apoptotic cells (12).

Interestingly, a distinct defect in Ag-specific IgM production was found during Mf infection. This defect alone could account for the increased survival of B. malayi MF in MBL-A−/− mice, as a number of previous studies in rodent models of filarial disease have suggested that IgM is critical for MF clearance (22–24). Indeed, CBA/N mice, which lack Bruton’s tyrosine kinase and peritoneal B1 cells and are deficient in T-independent type II IgM and IgG3 responses, support both greater MF survival and increased development of Brugia sp. infective larvae (L3) than their WT counterparts (22, 23). In addition, mice deficient in circulating IgM (secIgM−/−) have abrogated clearance of primary and secondary L3 infections (25). IgM is also needed in mice for protection against secondary infection of the nematode Strongyloides stercoralis (26). Overall, therefore, the lower levels of parasite-specific IgM found in B. malayi MF-infected MBL-A−/− mice could account for the enhanced MF survival. Indeed, the mutual ability of IgM and MBL-A to fix complement, and the known ability of MBL to bind Ig, could have additive effects if complement-mediated effector mechanisms are protective against these parasites. Interestingly, a preliminary study in human filarial disease has associated the MBL2 polymorphism in humans with susceptibility to lymphatic filariasis (27).

In contrast to IgM, MF-specific IgG1, IgG2a, and IgG3 were greatly enhanced in MBL-A−/− mice. Levels of total serum IgM were not fundamentally deficient in naïve MBL-A−/− mice and did not differ from those of WT mice. However, Ag-specific IgM levels to a model Ag, OVA, were also severely abrogated in the absence of MBL-A. Thus, MBL-A appears to regulate the production of Ag-specific IgM responses. As IgM is produced primarily in response to carbohydrate moieties on proteins (and it is known that OVA has carbohydrate structures capable of binding capable of binding MBL (9)), we also examined Ag-specific IgM levels to a bacterial protein, KpsD, that is carbohydrate free (7). MBL-A−/− mice were unable to produce any Ag-specific IgM to carbohydrate-free protein (while very low levels were induced in WT mice), suggesting that MBL-A maybe involved in tagging foreign Ag for antigenic processing. Intriguingly, IgG responses were generally unaffected, although IgG2b responses to both OVA and KpsD were significantly lower than in WT animals.

Regulation of Ab responses by members of the complement cascade is not without precedent; animals deficient in C1q (28), C4 (29), C2/C3 (29), or CR2 (CD21, C3 receptor) (30) all have impaired primary and secondary Ab responses. SecIgM−/− mice are also impaired in their ability to generate T-dependent IgG responses at suboptimal antigenic doses, suggesting that IgM responses are needed for priming the Ab response (31). In addition, secIgM−/− mice are more susceptible to systemic bacterial infection, showing the need for natural IgM in defense (31). In a similar systemic bacterial infection model, absence of MBL-A rendered mice more resistant to infection, suggesting that MBL-A−/− mice may not be defective in natural IgM (18). In our experiments, there was no IgM response to nonglycosylated protein, whereas there is a small response in MBL-A-sufficient mice, suggesting that even for T-dependent Ags, MBL-A aids in priming the response. However, levels of IgG isotypes are not compromised, suggesting that MBL-A is only involved in initial priming of the Ab response. Interestingly, recent work has also shown that mice deficient in sialoadhesin (Sn, Siglec-1, or CD169), a member of the sialic acid-binding Ig-like family of lectins, have reduced levels of total IgM (by ∼50%) while IgG remains unaltered (32). In Sn-deficient mice, there is little alteration in the numbers of cells that produce natural IgM, such as B1-a cells in the peritoneal cavity, although there is a slight increase in marginal zone B cells in the spleen. Sialoadhesin-positive macrophages are known to localize in the primary B cell follicles and prime Ab responses (32). One possibility is that MBL-A acts in a similar manner to sialoadhesin; MBL-A may bind Ag allowing enhanced uptake by APCs such as marginal zone macrophages or dendritic cells that initiate priming of Ab responses in lymphoid organs.

As well as providing an initial response to Ag, serum IgM plays a regulatory role in subsequent immune response development and accelerates the production of high-affinity IgG by B2 cells. Natural IgM enhances the adaptive Ab response by promoting the formation of immune complexes containing activated C3 fragments (33). In addition, it is well known that administration of IgM or IgG around the time of Ag encounter enhances Ab responses to suboptimal Ag doses (34). In the case of IgM, this process requires C’ activation, and both C4 and C2, but alternative pathway components are not important (35). Furthermore, a functioning C system is needed for both localization of Ag to follicles and germinal center (GC) formation. Thus, compromised complement components and/or IgM responses would lead to less Ag localization in follicles and possibly reduced GC formation and IgG responses. Indeed, recent studies have shown that a loss of MBL-A and C leads to a reduction in GC B cell numbers (21). Thus, the defect in IgM found in MBL-A−/− mice combined with lower C’ activation in the absence of MBL-A may compromise the level and affinity maturation of Ab responses. Most importantly, MBL-A may play a role in targeting glycosylated Ag to APCs that bear collectin receptors following MBL-carbohydrate structure binding. Both parasites and OVA (9) are known to possess carbohydrate residues recognized by MBL. Thus, we are currently investigating whether the targeting of Ag to the follicles, the formation of GC, or the speed and avidity of the Ab response to T-I Ag and T-D Ag is compromised in these mice.

Indeed, there is evidence from MBL null mice that MBL-A and/or MBL-C play an important immunoregulatory role in B cell homeostasis and GC formation. B1-b cells are increased in MBL null mice, suggesting that either MBL-A and/or MBL-C may have a negative homeostatic role on B1 cell development or that the B1 cells have simply expanded in response to the delayed apoptotic cell clearance that occurs in these mice (21). Previous work has shown a similar phenotype in secIgM−/− mice which, like MBL null mice, have impaired clearance of dying cells and expanded B1 numbers; however, they are susceptible to accelerated autoimmunity (36, 37). MBL null mice do make autoantibodies but they are at low levels and are not associated with pathology (21). It is possible, therefore, that although MBL deficiency is associated with abnormal disposal of apoptotic cell-derived self-Ag, the total absence of MBL does not allow expansion of self-reactive B cells due to the reduced follicular dendritic cell-B cell interaction or...
impaired GC formation, and ultimately this may protect the mice from developing full autoimmunity. Thus, it is vital to determine the role of MBL in GC reactions and whether this might underlie the different outcomes of failed apoptotic cell clearance in C1q and secrIgM null animals.

Acknowledgments
Dr. Rob Goldin performed autopsies on the mice. We thank Dr. Emma Cadman for critically reviewing this manuscript.

Disclosures
The authors have no financial conflict of interest.

References