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Decreased nematode clearance and anti-phosphorylcholine specific IgM responses in mannose-binding lectin deficient mice.

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Running Head: PC-specific IgM is reduced in *mbl*$^{-/-}$ mice

independent antigens were not greatly altered in MBL-deficient mice, however, absence of MBL-A and/or MBL-C resulted in a reduced IgM response to phosphorylcholine which is a major constituent of both filarial nematode and bacterial antigens.

ABSTRACT

Brugia malayi is a nematode that causes human lymphatic filariasis. Previously, we showed that mannose binding lectin (MBL) -A is necessary for clearance of *B. malayi* microfilariae in mice and presence of MBL-A is linked with maximal levels of parasite-specific IgM. Common human MBL gene polymorphisms result in low MBL expression and lead to recurring bacterial infections. Furthermore, these low-expressing human MBL polymorphisms result in greatly increased susceptibility to lymphatic filarial infection. Indeed, gain of new filarial infections over a 30-year period are 10-fold higher in people with low, compared to high, MBL-expression phenotypes. Human MBL closely resembles mouse MBL-C, rather than MBL-A, therefore we examined the role of mouse MBL-C in clearance of microfilariae. Absence of MBL-C alone, or both MBL-A and -C, resulted in delayed clearance of microfilariae and reduced parasite-specific IgM in mice. There were few profound changes in B cell sub-populations or in the ability of MBL-deficient mice to respond to T-dependent or T-independent antigens. However, absence of MBL-A and/or MBL-C resulted in reduced IgM to phosphorylcholine, a constituent of filarial and bacterial antigens, suggesting that inability to form proficient antibody responses to this moiety leads to lack of microfilarial clearance and overall susceptibility to filariasis.

INTRODUCTION

Mannose binding lectin is critical for innate recognition and clearance of *Brugia malayi,* a parasitic nematode that causes the major human tropical disease, lymphatic filariasis 1. In mice, we previously revealed an intriguing connection between the presence of mannose binding lectin-A (MBL-A), removal of *B. malayi* microfilariae and induction of parasite-specific IgM antibody 1. In addition, common human MBL gene polymorphisms that result in low MBL expression give rise to greatly increased susceptibility to human filarial infections caused by *Wuchereria bancrofti* 2. Indeed, the gain of new filarial infections in the same individuals followed-up 30 years later, was found to be approximately 10-fold higher in individuals with low, rather than high, serum MBL expression phenotypes 2.

The importance of MBL in defence against several bacterial, viral and protozoal pathogens, including malaria, is well-known 3. However, the necessity of just a single pattern recognition receptor (PRR) - glycan interaction for the induction of protective immunity during a helminth infection is a novelty to our knowledge. Interestingly, induction of T helper 2 (Th2) responses to another helminth, *Schistosoma mansoni*, is glycan-mediated, and while protective immunity to schistosome infection has yet to be directly linked to PRR recognition of glycan structures, recognition of glycan structures by antibody is capable of killing schistosomes as well as other helminths 4-6. Despite the well-known importance of anti-polysaccharide responses in bacterial vaccines, induction of anti-glycan immunity is poorly understood.

MBL is an acute phase protein, manufactured by the liver and present in the serum. Humans have one type of MBL, while mice have two isoforms, MBL-A and MBL-C; human MBL most closely resembles mouse MBL-C 7, 8. Primarily, MBL is known to trigger complement activation via the lectin pathway. Upon recognition of glycans on the pathogen surface, MBL-associated serine protease 2 cleaves the complement components, C4 and C2, to form the enzyme C3 convertase. MBL-A has approximately five times greater C'-activating ability than MBL-C 7. In addition, binding of MBL to pathogen/antigen enhances receptor-mediated phagocytosis, and clearance of apoptotic cells 9. Human and mouse MBL have overlapping monosaccharide specificities and they bind methyl-D-mannose, D-mannose, N-acetyl-D-glucosamine, L-fucose, *N*-acetyl-D-mannosamine, α-methyl-D-glucose and D-glucose. However, mouse MBL-A and MBL-C (but not human MBL) also bind D-mannosamine and D-fucose, and only mouse MBL-A can bind D-galactose and D-glucosamine. The fundamental importance of MBL in immunity to microbial pathogens is highlighted by the fact that humans with MBL deficiencies are susceptible to severe recurrent infections against a range of bacteria, viruses, fungi and protozoa 10. Interestingly, the aforementioned genetic mutations that result in MBL-deficient or low-producing phenotypes are present at high frequency in the population (worldwide ~35%) which has stimulated debate as to whether possession of these phenotypes confers an evolutionary advantage 11.

Mice that lack MBL-A and MBL-C, *mblac*$^{-/-}$, have enhanced susceptibility to *Staphylococcus aureus* i.v. and also to postburn infection with *Pseudomonas aeruginosa* 12, 13. In contrast, MBL-A deficient mice do not differ in their susceptibility to either *Candida albicans* or *Plasmodium yoelii* 14. However, our previous studies showed that *mbla*$^{-/-}$ mice are unable to clear *B. malayi* microfilariae (Mf) and that, in addition, these mice had reduced levels of Mf-specific IgM, while Mf-specific IgG1, IgG2a and IgG3 were increased. Our results were in accord with several previous studies in mice with IgM deficiencies, for example, both *xid* mice, which lack Bruton's tyrosine kinase, and B-cell deficient µMT mice have a reduced ability to kill Mf 15-17. Additionally, both *xid* and secIgM$^{-/-}$ mice have an enhanced susceptibility to *B. malayi* infective larvae. Overall, we hypothesise that if MBL-A regulates

the production of antigen (Ag)-specific IgM responses, this may explain the increased susceptibility of *mbla*$^{-/-}$ mice to Mf.

Building on the two critical observations, that host MBL-A is necessary for protection in mice and that susceptibility to human filariasis is correlated with a reduction in MBL level, in this paper, we have used the mouse model to explore the interaction between host MBL, parasite clearance and MBL-influenced induction of antibody responses. Our studies show that clearance of *B. malayi* Mf is delayed and Mf-specific IgM responses are decreased in both *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice.

RESULTS

MBL-A and MBL-C differ in their ability to bind *B. malayi* microfilarial and adult antigen and activate C3

Absence of MBL-A leads to a decreased ability of mice to clear *B. malayi* microfilariae 1. To elucidate whether both MBL-A and MBL-C are efficient at binding microfilarial antigens, we investigated their ability to bind to the control polysaccharide, mannan, as well as to microfilarial and adult extracts.

Both MBL-A and MBL-C bound efficiently to the control polysaccharide, mannan, as binding was present in *mbla*$^{-/-}$, *mblc*$^{-/-}$ and C57Bl/6, but not *mblac*$^{-/-}$, sera (Figure S1a,d) and this binding was significantly abrogated in the presence of EDTA ($P < 0.001$). MBL-A from *mblc*$^{-/-}$ and C57Bl/6 mouse sera bound microfilarial and adult worm antigens and this was also calcium dependent ($P < 0.001$) (Figure S1a-c). Surprisingly however, MBL-C was relatively weak at binding microfilarial antigen (Figure S1e) while MBL-C bound to adult *Brugia* extract effectively (Figure S1f), suggesting that MBL-A may bind to *B. malayi* microfilarial glycans more efficiently than MBL-C. Unsurprisingly no MBL-A was detected in sera from naïve *mbla*$^{-/-}$ or *mblac*$^{-/-}$ (Figure S1a-c) and equally no MBL-C was detected in sera from *mblc*$^{-/-}$ or *mblac*$^{-/-}$ mice (Figure S1d-f).

To determine the ability of MBL-A and MBL-C to activate the complement cascade when exposed to parasite antigens, the level of C3 bound to mannan, microfilarial antigen, or adult antigen, following incubation with sera from the different strains of mice was measured (Figure S1g-i). C3 deposition is the point in the complement cascade at which the three complement activation pathways converge and the cascade of other complement effector proteins is initiated. Any C3 activation via the classical pathway is likely due to natural antibody as there would be no other specific antibody present in naïve mouse sera. C3

activation could also occur spontaneously via the alternative complement pathway which is calcium-independent. Thus, any C3 binding seen after the addition of EDTA can be attributed to the natural antibody or the alternative pathway.

C3 deposition on mannan due to MBL-A presence was significant in sera from *mblc*$^{-/-}$ and C57Bl/6 mice ($P < 0.001$) and this was calcium-dependent (Figure S1g). MBL-C in *mbla*$^{-/-}$ sera initiated only a low level of C3 deposition on mannan (Figure S1g). Similarly, MBL-A presence lead to C3 deposition on microfilarial and adult antigens, while MBL-C initiated lower, C3 deposition (Figure S1h-i). MBL-C appeared to be less efficient than MBL-A at activating C3 following mannan or microfilarial antigen binding, particularly in light of the fact there is approximately 6-fold higher levels of MBL-C than MBL-A in mouse serum 7. The abrogation of C3 activation by the addition of EDTA suggests that the alternative pathway plays a minimal role in C3 deposition on mannan or microfilarial antigen. Adult antigen did not elicit a significant level of C3 deposition in any of the sera. Figure S2 shows that similar to microfilarial extract, MBL-A binds to live microfilariae and activates C3 more efficiently than MBL-C.

Delayed clearance of *Brugia malayi* microfilariae in *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice

Previous data from our lab has shown that *mbla*$^{-/-}$ mice take longer to clear *B. malayi* microfilariae from the bloodstream than wild-type mice 1. To examine whether this is also true for *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice, groups of 5 individual *mbl*-deficient and C57Bl/6 mice were infected with 250,000 microfilariae i.v. Blood microfilarial numbers were counted on days 7, 14, 21 and 28 post infection (p.i.).

At day 7 p.i., all three strains of mice had similar microfilarial loads (Figure 1a). However, by day 14, C57Bl/6 mice showed significantly greater clearance of microfilariae than either *mblc*$^{-/-}$ or *mblac*$^{-/-}$ mice ($P < 0.05$), and by day 21, C57Bl/6 mice had nearly cleared infection whereas *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice still had significant numbers of microfilariae in the blood ($P < 0.01$). At day 28 p.i. all microfilariae were cleared from the C57Bl/6 mice ($P < 0.001$) while the *mblac*$^{-/-}$ mice had a persistent microfilarial load ($P < 0.05$ *mblc*$^{-/-}$ versus *mblac*$^{-/-}$ day 28).

Microfilariae-specific IgM is reduced in *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice

Our previous work showed that *mbla*$^{-/-}$ mice have a distinct defect in microfilarial-specific IgM production 1. To determine whether infected *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice also have altered antibody responses, we measured the levels of microfilarial-specific antibody isotypes in *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 mice following microfilariae infection. Both *mblc*$^{-/-}$ and *mblac*$^{-/-}$

mice had significantly less microfilarial-specific IgM responses compared to infected C57Bl/6 mice at day 28 p.i. (Figure 1b). However, there were no significant differences in the levels of microfilarial-specific IgG1, IgG2b, IgG2c or IgG3 between *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 mice at 28 days following infection with microfilariae (Figure 1c-f).

Lack of MBL-A or MBL-C leads to alteration in basal antibody isotype levels

To investigate whether the reduction in microfilarial-specific IgM seen in the absence of MBL, in this and previous studies 1, is due to a deficiency in the basal level of immunoglobulin, the total concentration of each isotype in the sera of naïve WT and MBL-deficient mice was compared using BindaRID radial immunodiffusion plates. Basal levels of IgG1 were similar in the C57Bl/6 wild-type, *mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice (Figure S2a). Interestingly, mice lacking either MBL-A, MBL-C or both had significantly higher basal levels of IgG2b than wild type mice ($P < 0.001$) (Figure S2b). IgG3 was significantly elevated in the absence of MBL-A ($P < 0.05$) (Figure S2c). Contrary to expectation, basal IgM was significantly higher in the absence of MBL-A, while absence of MBL-C alone did not alter the basal IgM concentration (Figure 2d).

Analysis of B cell populations in naive *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 mice

In order to analyse whether a decrease in natural-IgM producing B cell populations could explain the lower Mf-binding IgM observed in the absence of MBL-A or-C, B cell subset populations were measured in naïve *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and wild-type mice. In the bone marrow, B220^{+}IgM^{-} pre-B and B220^{+}IgM^{+} transitional B cells were counted. In the spleen, B220^{+}CD21^{+}CD23^{-} MZB cells, B220^{+}CD21^{-}CD23^{-} immature B cells and B220^{+}CD21^{+}CD23^{+} follicular B cells (FBC) were identified and in the peritoneal cavity, B220^{+}IgM^{+}CD5^{+} B1a and B220^{+}IgM^{+}CD11b^{+} B1b cells were counted.

In the bone marrow, the absence of MBL-A or -C did not appear to alter the number of pre-B cells or transitional B cells significantly ($P > 0.05$) (Figure 3a-b). However, in the spleen, absence of either MBL-A or -C resulted in significantly higher numbers of B220^{+}CD21^{+}CD23^{+} follicular B cells compared to C57Bl/6 mice ($P < 0.05$) (Figure 3c), while the absence of MBL-A resulted in a ~66% reduction in the total number of immature B220^{+}CD21^{-}CD23^{-} B cells at (650,000±200,000 to ~200,000±42,000 cells) ($P < 0.05$) (Figure 3d). This suggested that MBL-A, in particular may alter the rate of splenic B cell maturation. Interestingly, the percentage of marginal zone B (MZB) cells (B220^{+}CD21^{+}CD23^{-}) in the spleen was lower in all mice lacking MBL-A or-C and this was significant in the absence of MBL-A (Figure 3e).

In the peritoneal cavity, B1a cells were detected in very low numbers, and while the total number of B1a cells in *mbla*$^{-/-}$ was higher than in all three of the other groups (4700 ± 1600), B1a cells did not differ significantly in total numbers between any of the mouse strains (Figure 3f). B1b cells were more abundant in strains lacking MBL but the number of cells did not differ significantly between the groups (Figure 3g).

Antibody isotype responses to the T-dependent antigen TNP-KLH

To determine whether T-dependent responses are altered in mice lacking MBL-A and/or MBL-C, groups of *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 mice were immunised with the T-dependent antigen TNP-KLH at day 0 and given a secondary boost at day 28. KLH-specific isotype responses were measured at various time-points.

Levels of circulating TNP-specific IgM in mice lacking MBL were generally similar to wild-type mice ($P > 0.05$). Anti-TNP-KLH IgG1, IgG2c and IgG3 antibody responses were not significantly different between wild type mice and mice lacking either MBL-A or MBL-C (Figure 4a). IgG2c levels in all mice were very low following primary immunisation and were only detectable after secondary immunisation. TNP-KLH specific IgG2b was produced at lower levels in mice lacking MBL-A (*mbla*$^{-/-}$ and *mblac*$^{-/-}$) at day 14 post-immunisation ($P > 0.05$). However, following secondary challenge, all mice elicited similar IgG2b responses. Taken together, these results suggest that neither lack of MBL-A nor MBL-C lead to major problems in mounting antibody responses against the T-dependent antigen, TNP-KLH.

Antibody isotype responses to the T-independent type I antigen TNP-LPS

Lipopolysaccharide (LPS) is a major component of the membrane of gram-negative bacteria and is characterised as a T-independent antigen. The antibody responses to immunised LPS conjugated to haptenated TNP (TNP-LPS) were measured in *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 mice.

IgM responses did not appear to differ significantly following immunisation with TNP-LPS in mice lacking MBL-A or -C, while mice lacking MBL-A had significantly lower IgG1 responses than wild-type mice at 14 days post- TNP-LPS immunisation (Figure 4b)($P < 0.01$). The IgG2b and IgG2c responses were generally similar between all mouse strains, though mice lacking MBL-A appeared to have a more sustained IgG2c response (Figure 4b). Interestingly, there were marked differences in IgG3 responses to TNP-LPS between *mblc*$^{-/-}$

and *mblac*$^{-/-}$ mice and the wild-type C57Bl/6 mice (Figure 4b) suggesting that presence of MBL-C is necessary for maximal IgG3 production following immunisation with TNP-LPS (P < 0.001).

Overall, lack of MBL-A lead to decreased IgG1 responses, while lack of MBL-C lead to reduced IgG3 responses, following immunisation with the T-independent type I antigen, LPS.

Mbl$^{-/-}$ mice have reduced IgM responses to phosphorylcholine following infection with microfilariae

Phosphorylcholine (PC) is a well described component in *B. malayi* excretory-secretory (ES) products and *N*-acetylglucosaminyltransferase (GlcNAcT) has been identified as the major PC-bearing protein in *B. malayi* ES 15. PC is frequently recognised by natural IgM antibody. We measured the PC-specific IgM and IgG isotypes against PC-BSA, in *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and wild-type C57Bl/6 mice infected with microfilariae.

Intriguingly, absence of MBL-A or –C lead to significantly lower levels of PC-specific IgM following infection with *B. malayi* microfilariae compared to wild-type (C57Bl/6) mice (*mblc*$^{-/-}$ P < 0.01, *mblac*$^{-/-}$ P < 0.05 and *mbla*$^{-/-}$ P < 0.001) (Figure 5a). There were no significant differences in the levels of basal PC-specific natural IgM in naïve mice of any of the strains (Figure 5a). PC-specific IgG1, IgG2b and IgG2c responses were similar between wild-type mice and those lacking MBL following infection. However, MBL-A or -C deficient mice had significantly decreased PC-specific IgG3 response (P < 0.05) (Figure 5b). Thus, while lack of MBL-A or -C does not appear to lead to a deficiency in natural IgM *per se*, either MBL-A or -C-deficiency, leads to inefficient production of PC-specific natural IgM and a reduced IgG3 response following infection.

Mbla$^{-/-}$ mice also have reduced IgM responses to phosphorylcholine following infection with *Streptococcus pneumoniae* or immunisation with conjugate vaccine

Phosphorylcholine is also a cell wall component of many commensal and pathogenic bacteria. PC in *S. pneumoniae* decorates techoic acid; it acts as an adhesin and is a key molecule enabling invasion. We immunised mice with intact *S. pneumoniae* (Pn14) or conjugate vaccine, which are known to be differentially processed by antigen presenting cells. Figure 6 shows the differential responses of IgM and IgG in *mbla*$^{-/-}$ mice to intact bacteria or conjugate vaccine. There were no differences in the IgG response to T-

dependent protein PSP-A or the IgG anti-PPS14 (capsular polysaccharide type 14) response or the IgG anti-PC response. However, the IgM anti-PC response to both intact and conjugate vaccine was significantly reduced in *mbla*$^{-/-}$ mice immunised either with intact bacteria or with conjugate. Furthermore, the anti-PPS14 IgM response was also significantly reduced in *mbla*$^{-/-}$ mice immunised with conjugate.

DISCUSSION

Our previous work showed that the mouse MBL isoform, MBL-A, is vital for clearance of blood-borne microfilariae of the filarial nematode, *Brugia malayi* 1. However, MBL-A deficiency does not alter clearance or egg production of the nematode *Trichuris muris* or the digenean *Schistosoma mansoni* in mice 18. A possible mechanism for abrogated clearance of microfilariae in *mbla*$^{-/-}$ mice was indicated by their distinct defect in the ability to produce either microfilarial-specific IgM 1, 15. Furthermore, in previous studies, we and others, have shown that mice with IgM deficiencies, such as *xid* (which lack Bruton's tyrosine kinase) and µMT mice (which lack B cells) are unable to kill microfilariae 15-17. Incidentally, both *xid* and secretory IgM$^{-/-}$ mice are also more susceptible to the infective larval stage of *B. malayi*. We therefore surmised that the resultant lowered IgM may explain the increased susceptibility of *mbla*$^{-/-}$ mice to microfilariae. In the current study, we show that mice deficient in MBL-C (*mblc*$^{-/-}$), as well as mice deficient in both MBL isoforms, MBL-A and MBL-C (*mblac*$^{-/-}$), have a similar phenotype and display both delayed clearance of *B. malayi* microfilariae and decreased microfilarial-specific IgM responses.

Intriguingly, our mouse studies have been paralleled in human lymphatic filariasis and a significant negative association between MBL expression and circulating filarial antigen (a comparative measure of adult worm burden) has been shown 2, 15. In addition, ten-fold more new infections were attained among individuals with a low MBL expression phenotype over a thirty-year period 2. These studies indicate that MBL deficiency, in addition to preventing clearance of microfilariae in mice, is also likely to facilitate infective larval establishment in humans. The level of anti-filarial antibody was not measured in these human studies so it has yet to be established whether any anti-filarial antibody isotypes are also altered in low human MBL phenotypes.

Our investigations found lower numbers of marginal zone B cells (MZB) which generate natural IgM against T-independent antigens in mice lacking MBL-A or -C but we did not find substantial defects in B1 cell populations. No major differences in the ability of mice lacking MBL to make antibody responses to either T-dependent (T-D) or T-independent (T-I) type 1 antigens were revealed which could account for lowered parasite-specific IgM in MBL-

deficient mice 15. However, our previous work showed that *mbla*$^{-/-}$ mice also had reduced anti-parasite IgM responses against another nematode *Trichuris muris,* in addition to *B. malayi*, but not against the unrelated digenean helminth *Schistosoma mansoni* 18. We therefore surmised that the IgM defect may be particular to an antigen that is abundant in nematodes, but less common in other helminth species. Phosphorylcholine (PC) was a likely candidate, as nematodes in particular, elaborate PC-containing glycans 19, 20 and PC is a target of T-independent natural IgM. Our investigations of the ability of MBL-deficient mice to produce anti-PC responses when infected with microfilariae, showed that *B. malayi*-infected MBL-deficient mice do indeed have an impaired ability to produce both IgM against filarial PC. Furthermore, the anti-PC IgG3 response was also impaired.

Natural IgM recognises PC on the cell-surface of many bacteria or as an autoantigen when it is exposed during cell membrane breakdown. We therefore investigated whether *mbla*$^{-/-}$ mice have a defect in IgM production to bacterial PC or whether this deficiency is restricted to PC-nematode antigens. Intriguingly, we revealed a deficiency in the ability of *mbla*$^{-/-}$ mice to make IgM to *Streptococcus pneumoniae* PC, and to capsular polysaccharide type 14 if immunised with conjugate. Pneumococcal conjugate vaccine is processed in the spleen by dendritic cells (DC), while, intact bacteria are processed by macrophages 21, 22. These data suggest that MBL-deficient mice are unlikely to have an absolute antibody repertoire defect, and that the defect is dependent not only on antigen type, but may also depend on the cell type that processes and/or presents the antigen. The IgG anti-PPS14 response to conjugate, and the IgG anti-PspA response to both Pn14 and conjugate which appear to derive from follicular B cells were unaffected in MBL-deficient mice 22, 23. This was also reflected in the fact that MBL-deficient animals did not appear to have a defect in follicular B cells or B1 cells. The majority of the PPS14 IgM response is thought to come from MZB cells which appeared to be lower in number in the absence of MBL 24. Overall these results suggest that there may be some differences in the ability of MBL-deficient mice to produce natural IgM, however this ability is improved if the antigen is processed by macrophages rather than dendritic cells.

MBL may recognize a wide range of novel glycoconjugates expressed by nematodes. These glycoconjugates can be linked through asparagine residues to protein (N-linked), through hydroxyamino acids to protein (O-linked) or to lipids (glycosphingolipids) and in addition to PC, they often contain fucose 25, 26. Parasitic nematodes and the free-living nematode *Caenorhabditis elegans* share most of the glycosylation forms so far reported. Indeed, previous studies have shown that nematode glycans induce type 2 responses, and it is specifically the fucosylated glycoprotein fraction of extracts from both *B. malayi* and *C. elegans* that contain this Th2-inducing activity 26. In addition, in another nematode model a

protective IgM response has been linked to Th2 induction thus providing a bridge between glycan induction of both Th2 and IgM responses 27.

A key aspect of eliciting optimal immunity, which is frequently overlooked in immunization studies with nematodes is the role of the APC. MBL presence can alter the immune response elicited to antigen/pathogen, by opsonising antigen and enhancing receptor-mediated capture. Importantly human MBL-bound *S. aureus* has been shown to co-localise with TLR2 in the early phagosome of macrophages, and, MBL presence fundamentally enhances the TLR2/6-dependent cytokine responses elicited 28. Thus the role of MBL in immunity to *B. malayi* may lie in its ability to complex with *Brugia* antigen and direct it for recognition by dendritic cells (DC) and/or other APC. This interaction with MBL-antigen complexes may involve TLR2 or other recognition molecules; all APC, including B cells, possess TLR and receptors for MBL (CR1) as well as receptors for downstream-activated C3 29, 30. It is now accepted that accessory cells (either APC or T cells) are necessary for the generation of all antibody responses. This is because efficient B cell activation and differentiation even to T-independent-antigen requires two signals, one via the BCR and the second via either cytokines and/or anti-apoptotic factors, which may be produced by T-independent-antigen activated accessory cells or T-dependent/CD40L+ activated T cells 31-33. In addition, TLR stimulation of B cells can either synergise with T cell help and BCR cross-linking, or, TLR ligands themselves can allow T-independent B cell responses 29, 30. Thus, differential processing of antigen in the absence of MBL may lead to a poor second signal from the T-independent-antigen activated accessory cell (or a poor primary signal if MBL/TLR-association is required) and therefore a reduced natural antibody response to particular T-independent targets. B cells also respond to native antigen held by Fc receptor, complement receptors and/or C-type lectins on the surface of follicular DC and marginal-zone macrophages. Thus, absence of MBL could critically alter activation and signalling responses to *Brugia* glycoprotein antigen.

Clearly the MBL molecule is important for protection against lymphatic filariasis in humans and in a mouse model of filariasis. As human genetic polymorphisms resulting in low expression of this molecule have been linked to filariasis, confirmation of the link between MBL-deficiency and filarial-susceptibility could identify groups of patients in most need of chemotherapy. Elucidation of the MBL-glycan moiety recognition in nematodes and clarification of the influence of MBL upon humoral immunity could have direct use in the broader context of rethinking vaccine delivery systems, and have widespread consequences for both medical and veterinary communities.

METHODS

Ethics statement

Animal experiments were conducted in accordance with our project licence granted by the Home Office under the Animal Scientific Procedures Act (1986). The project was approved by the local Ethical Review Committee at the Royal Veterinary College.

Mice

Mbla$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice were obtained from Prof Kazue Takahashi (Harvard Medical School) and bred at the Royal Veterinary College 12, 34. Wild type C57Bl/6 mice were purchased from Harlan UK at 6-8 weeks of age. All mice used in experiments were male, age-matched between groups and housed in individually- ventilated cages.

Parasites

B. malayi-infected gerbils (*Meriones unguiculatus*) were purchased from TRS Labs (Georgia, USA) and were housed in standard conditions. Mf were obtained by peritoneal lavage with RPMI 1640 and gerbil cells were removed by centrifugation over lymphocyte separation media (Flow Labs, McLean, VA, USA). Soluble Mf extract (MfAg) was prepared as described previously 1.

Infection protocol

Groups of four to six individual eight week old male C57Bl/6 wild-type (WT) mice and/or gene-targeted mice were either left uninfected (naïve), or they were injected with 2 x 10^5 *B. malayi* Mf i.v. (primary (1°) infection). Parasitaemia was monitored as previously described, in blood collected from the tail vein. Sera were collected for measurement of antibody. The experiment was repeated twice.

Immunisations

Groups of 5 individual male mice of the strains C57Bl/6, *mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice were immunised either with 100µg TNP-KLH in 1mg alhydrogel adjuvant (InvivoGen, Europe) in saline i.p. or 50µg TNP-LPS in saline i.p. Five individuals males of each strain were left unimmunised. TNP-KLH immunised mice were boosted on day 28 p.i. Mice were bled from the tail vein for serum collection. The experiment was repeated twice.

Groups of 7 individual six week old *mbla*$^{-/-}$ and C57Bl/6 female mice were immunised either with 1 x 10^9 CFU intact heat-killed *Streptococcus pneumoniae* capsular type 14 (Pn14) in saline i.p., or with conjugate vaccine (PPS14-PspA plus C-PS-PspA) i.p. The conjugate vaccine comprised pneumococcal surface protein (PspA) covalently linked to pneumococcal capsular polysaccharide type 14 (PPS14), PPS14-PspA, plus PspA covalently linked to the phosphorylcholine determinant of the cell wall C-polysaccharide (C-PS), teichoic acid, C-PS-PspA. 1µg PPS14-PspA plus 1µg C-PS-PspA adsorbed on 13µg alum and mixed with 25µg of stimulatory 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN) were injected i.p 23. Mice were boosted similarly on day 26, with either 1 x 10^9 CFU Pn14 or conjugate vaccine i.p. Mice were bled from the tail vein for serum collection. The experiment was repeated twice.

Measurement of basal levels of antibody isotypes

Radial immunodiffusion (BindaRID) kits (The Binding Site Ltd., Birmingham, UK) were used to detect basal levels of the antibody isotypes, IgG1, IgG2b, IgG3 and IgM, in sera from uninfected naïve mice according to the manufacturer's instructions. Sera from 8 naïve *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 mice were diluted with 7% BSA in PBS and added to each well. The concentration of antibody isotype was determined by measurement of the diffusion ring diameter against a calibration curve, drawn using dilutions of the manufacturer's standard immunoglobulin.

Detection of C3 deposition on live microfilariae

To measure C3 deposition on live microfilariae in the presence and absence of MBL-A, -C or both, 200 live microfilariae were incubated with 100µl sera from naive *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ or C57Bl/6 mice diluted 1:4 in PBS with 0.5% Tween-20 ± 20mM EDTA for 2hours at 4°C. Microfilariae were washed in PBS/Tween C3 deposition and C3 was detected using 160ng/ml goat anti-mouse C3 (MP Biomedicals Inc., UK) for 1 hour at 4°C. Following a further PBS/Tween wash, incubation with 60ng/ml rabbit anti-goat FITC for 0.5 hour 4°C (DAKO UK Ltd.), and a final wash, fluorescence was quantified using LeicaQWin software. Ten individual microfilariae were quantified per group and the level of fluorescence was measured from on a scale of zero to maximum fluorescence (0-255). The experiment was repeated four times.

Measurement of Mf-specific immunoglobulin isotypes

Mf-specific immunoglobulin levels were measured by ELISA 35. Briefly 96-well plates were coated overnight at 4°C with 1µg/ml soluble MfAg in 50µl carbonate buffer (pH 9.6). After blocking each well with 10% FCS in carbonate buffer, the plates were incubated with individual mouse sera diluted 1:50 in PBS/Tween. Antigen-specific antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-IgM (Southern Biotechnology Associates, Birmingham, AL, USA; SBA 1020-05), anti-IgG1 (SBA1070-05), anti-IgG2a (SBA 1080-05), anti-IgG2b (SBA 1090-05) or anti-IgG3 (SBA 1100-05). 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma) was used as the substrate. Plates were read at 405nm. Total IgE was measured by ELISA as previously described 15.

Detection of TNP-specific antibody isotype levels following immunisation with TNP-Ficoll, TNP-LPS or TNP-KLH

96-well plates were coated overnight at 40C with 100µl of 2µg/ml TNP-BSA diluted in carbonate buffer (pH 9.6). Wells were blocked for 1 hr at 37^0C with 10% BSA in carbonate buffer (pH 9.6). Individual mouse sera diluted 1:100 in PBS 0.5% Tween-20 was incubated overnight at 4°C. Levels of anti-TNP specific IgM, IgG1, IgG2b, IgG2c and IgG3 were then detected using the anti-isotype antibodies and substrate mentioned above.

MBL-A and MBL-C binding ELISA

96-well plates were coated with the control antigen 1µg/ml mannan, MfAg or adult antigen in carbonate buffer (pH9.6) overnight at 4°C. Plates were incubated with individual mouse sera 1:100 diluted in PBS/Tween either in the presence or absence of 20mM EDTA. Binding of MBL to glycans is calcium-dependent and EDTA is a calcium chelator which prevents calcium signalling and therefore activation of the lectin pathway of complement. Rat anti-MBL-A or anti-MBL-C antibodies (Cell Sciences, Newburyport, MA, USA) were incubated at 0.4µg/ml for 1.5h to detect the levels of serum lectin that bound to the antigens. Polyclonal rabbit anti-rat HRP 0.4µg/ml was used as a secondary antibody. In assays for the detection of C3 deposition, goat anti-C3 (Sigma, Poole, UK) was used to detect binding of C3 to the antigens and polyclonal rabbit anti-goat HRP was used as the detection antibody.

Detection of antibody to phosphorylcholine

To measure levels of anti-phosphorylcholine (PC) antibodies in sera from *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 following infection with *B. malayi* microfilariae, 96-well plates were coated with 5μg/ml of PC-BSA diluted in carbonate buffer. The protocol outlined above was then followed to measure levels of anti-PC specific IgM, IgG1, IgG2b, IgG2c and IgG3.

Measurement of serum titres of Pn Ag-specific Ig isotypes

Serum titres of PPS14- and PspA-specific IgM and IgG were determined by ELISA as described previously 36.

Flow cytometric analysis of B cell populations

Lymphocytes were retrieved from spleen, bone marrow and peritoneal cavity from four individual mice of *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and WT strains. 1 x 10^6 cells per sample were re-suspended in PBS/ 1% FCS / 0.01% azide buffer. Cells were incubated with 0.25mg/ml α-FCR antibody (clone 24G2) for 10 minutes. Samples were incubated with 0.25μg/ml of each relevant fluorescent antibody diluted in PBS/ 1% FCS / 0.01% azide buffer on ice in the dark for 30 minutes. Follicular, marginal zone and immature B cells were distinguished in the spleen using anti-CD23-FITC, anti-CD21-PE and anti-B220-PE antibodies. In the bone marrow, immature and mature B cells were identified using anti-B220-FITC and anti-IgM-PE, while in the peritoneal cavity B1a cells were identified with anti-B220-FITC, anti-IgM-PE and anti-CD5-PECy5 and B1b cells with anti-IgM-FITC, anti-CD11b-PE and antiB220-PECy5. Cell samples were acquired using a FACS Canto II flow cytometer (BD Pharmingen, UK) and analysed using FlowJo flow cytometry analysis software.

Statistical analysis

All data are expressed as the arithmetic mean ± SE. One-way ANOVA with Bonferroni's post-hoc analysis was used for intergroup comparisons between infected and uninfected *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ and WT mice. *P*-values lower than 0.05 were considered statistically significant. Prism (Graphpad Software Inc., San Diego, CA, USA) statistical analysis software was used to determine significance.

CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

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FIGURE LEGENDS

Figure 1. Increased *B. malayi* microfilarial survival and anti-filarial antigen antibody responses in *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice. Groups of 5 individual mice from each of the strains *mblc*$^{-/-}$, *mblac*$^{-/-}$ and C57Bl/6 were infected with 200,000 microfilariae i.v. **(a)** Data represents the mean ± S.E. the number of microfilariae counted in blood collected from the

tail vein on days 7, 14, 21 and 28. **(b-f)** Mf-specific immunoglobulins were measured by ELISA in sera collected from mice 28 days post-infection. Data represents the mean ± S.E. of microfilarial-specific **(b)** IgM, **(c)** IgG1, **(d)** IgG2b, **(e)** IgG2c and **(f)** IgG3 and in the sera for each mouse strain. The experiment was repeated twice and data shown is from one representative experiment. One-way ANOVA with Bonferonni's post-test was used to analyse the statistical differences where * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$.

Figure 2. Total basal serum immunoglobulin isotype levels in naive C57Bl/6, *mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice. Individual sera were applied to BindaRID plates pre-coated with antibody to each isotype (n=8 mice per group). The size of diffused circles was measured and concentration of each antibody (ng/ml) determined **(a)** IgG1, **(b)** IgG2b, **(c)** IgG3 and **(d)** IgM according to the manufacturers standard curve. The experiment was repeated twice and data shown is from one representative experiment. One-way ANOVA with Bonferonni's post-test was used to analyse the statistical difference in concentration of antibody between the different strains of mice where * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$.

Figure 3. Comparison of B cell populations in naïve *mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ and C57Bl/6 mice. Lymphocytes were separated from femur bone marrow, spleen and peritoneal cavity of n=6 mice per group. 10^6 cells were incubated with αFcR (1µg/ml) and stained with conjugated antibodies B220-Pe-Cy5, CD23-FITC and, CD21-PE or IgM-PE (0.25µg/ml). Cells were analysed by flow cytometry and defined as **(a)** Bone marrow $B220^+IgM^-$ pre-B cells, **(b)** bone marrow $B220^+IgM^+$ transitional B cells, **(c)** splenic follicular $B220^+CD21^+CD23^+$ B cells, **(d)** splenic immature $B220^+CD21^-CD23^-$ B cells, **(e)** splenic marginal zone $B220^+CD21^-CD23^+$ B cells, **(f)** Peritoneal $B220^+IgM^+CD5^+$ B1a cells pooled from 3 separate samples of 2 individual mice, **(g)** Peritoneal $B220^+IgM^+CD11b^+$ B1b cells pooled from 3 separate samples of 2 individual mice. **(h)** Gating strategy used for CD21 and CD23 on $B220^+$ cells to distinguish MZB, follicular B cells and immature B cells. One way ANOVA with Bonferonni's post-test was used to statistically analyse data where * represents $P < 0.05$.

Figure 4. Ig isotype levels in C57Bl/6, *mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice following immunisation with the T-dependent antigen, TNP-KLH. Groups of 5 individual C57Bl/6, *mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice were immunised at day 0 with 50μg of **(a)** TNP-KLH or **(b)** TNP-LPS and groups of 5 individual mice of each strain were left as naïve unimmunised controls. A secondary immunisation of 100μg TNP-KLH was given to mice previously immunised with TNP-KLH at day 28 (indicated by arrow). Mice were bled via the tail vein 1 day before primary immunisation (day -1) and then at various time-points post-immunisation and TNP-specific antibody isotypes were detected by ELISA. The experiment was repeated twice. Differences between each of the knock-out strains and C57Bl/6 were statistically analysed by two-way ANOVA with Bonferonni's post-test at each time point. $P < 0.05$ is denoted by * and $P < 0.01$ by **.

Figure 5. *Mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice infected with *B. malayi* microfilariae have decreased anti-PC IgM and IgG3 responses compared to C57Bl/6 mice. Groups of 5 individual C57Bl/6, *mbla*$^{-/-}$, *mblc*$^{-/-}$ and *mblac*$^{-/-}$ mice were either infected with 250,000 *B. malayi* microfilariae (I) or kept as uninfected controls (N). Mice were killed at day 28, sera was collected and ELISA was used to determine the concentration of PC-specific IgM and IgG3. Responses in naïve mice were used as the base-line. One-way ANOVA with Bonferonni's post-test was used to analyse the statistical difference in concentration of antibody between the mouse strains, where * represents $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 6. IgM, but not IgG, antibody responses to PC in *mbla*$^{-/-}$ mice immunised with intact *S. pneumoniae* or conjugate vaccine are reduced compared to C57Bl/6 mice. Groups of 7 C57Bl/6 (open circles) and *mbla*$^{-/-}$ (closed circles) mice were immunised with either intact Pn14 bacteria or with conjugate vaccine and boosted on day 26. The antibody responses to PSP-A, PPS14 and PC were measured by ELISA. either infected with 250,000 *B. malayi* microfilariae (I) or kept as uninfected controls (N). Mice were killed at day 28, sera was collected and ELISA was used to determine the concentration of PC-specific IgM and IgG. Responses in naïve mice were used as the base-line. The experiment was repeated twice and data shown is from one representative experiment. One-way ANOVA with Bonferonni's post-test was used to analyse the statistical difference in concentration of antibody between the mouse strains, where * represents $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

SUPPLEMENTARY MATERIAL

Figure S1. MBL-A and MBL-C bind and activate C3 on *B. malayi* adult antigen, but MBL-A is more efficient at binding microfilarial antigen. The ability of MBL-A and MBL-C to detect mannan, *B. malayi* microfilarial extract and adult extract was measured by ELISA. Plates were coated with either 0.1μg per well of **(a,d,g)** Mannan, **(b,e,h)** microfilarial extract, or **(c,f,i)** adult extract and incubated with sera from four individual naive *mbla*$^{-/-}$, *mblc*$^{-/-}$,

mblac$^{-/-}$ or C57Bl/6 mice in the presence or absence of EDTA. **(a-c)** Anti-mouse MBL-A was used to detect the binding of MBL-A in the sera to the different antigens. **(d-f)** Anti-mouse MBL-C was used to detect the binding of MBL-C to the different antigens. **(g-i)** Anti-mouse C3 was used to detect C3 deposition following incubation with the different sera. The experiment was repeated twice and data shown is from one representative experiment. Statistical analysis was carried out using one-way ANOVA and Bonferonni's post-test where * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$.

Figure S2. C3 deposition on live microfilariae. (a) Live microfilariae were incubated with sera from naive *mbla*$^{-/-}$, *mblc*$^{-/-}$, *mblac*$^{-/-}$ or C57Bl/6 mice in the presence or absence of EDTA. C3 deposition was detected using goat anti-mouse C3 followed by incubation with rabbit anti-goat FITC. Fluorescence was quantified using LeicaQWin software (n=10). One-way ANOVA was used to test statistical significance where * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$. Mean ± SE fluorescence are shown for 10 individual microfilariae representing data from four similar experiments. Examples of C3 deposition following microfilarial incubation with **(b)** MBL-C$^{-/-}$ sera and C. MBL-A$^{-/-}$ sera.

Figure 1

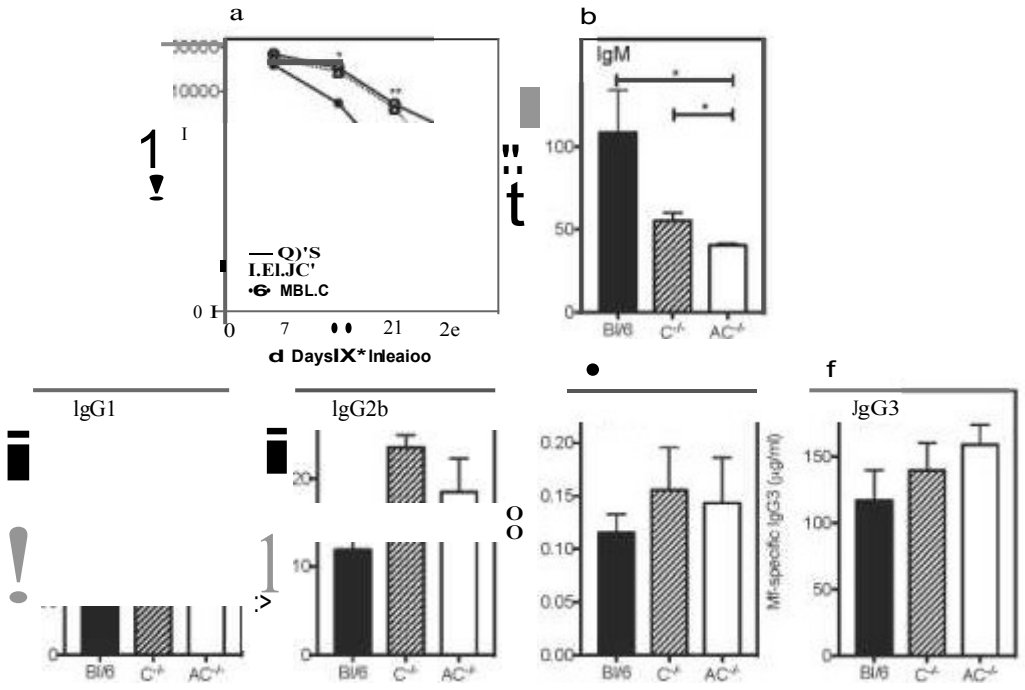

Figure 2

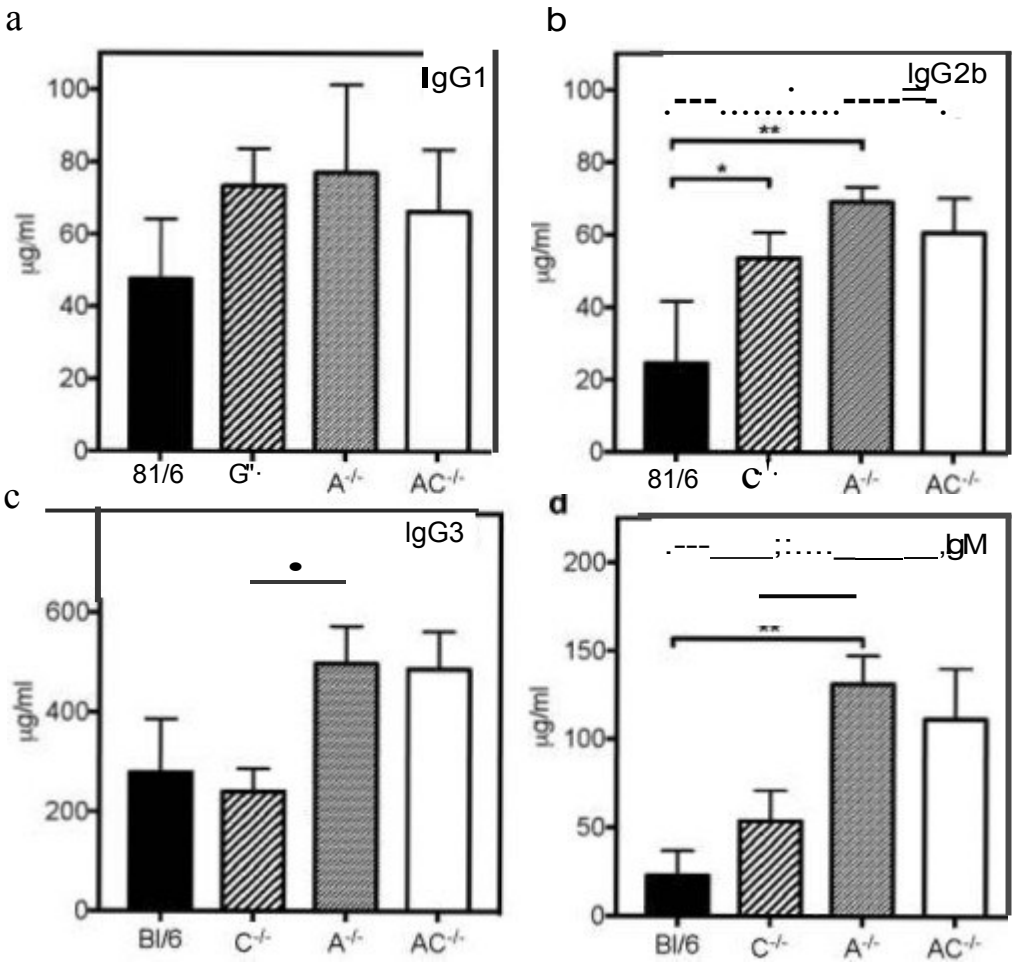
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Figure 3

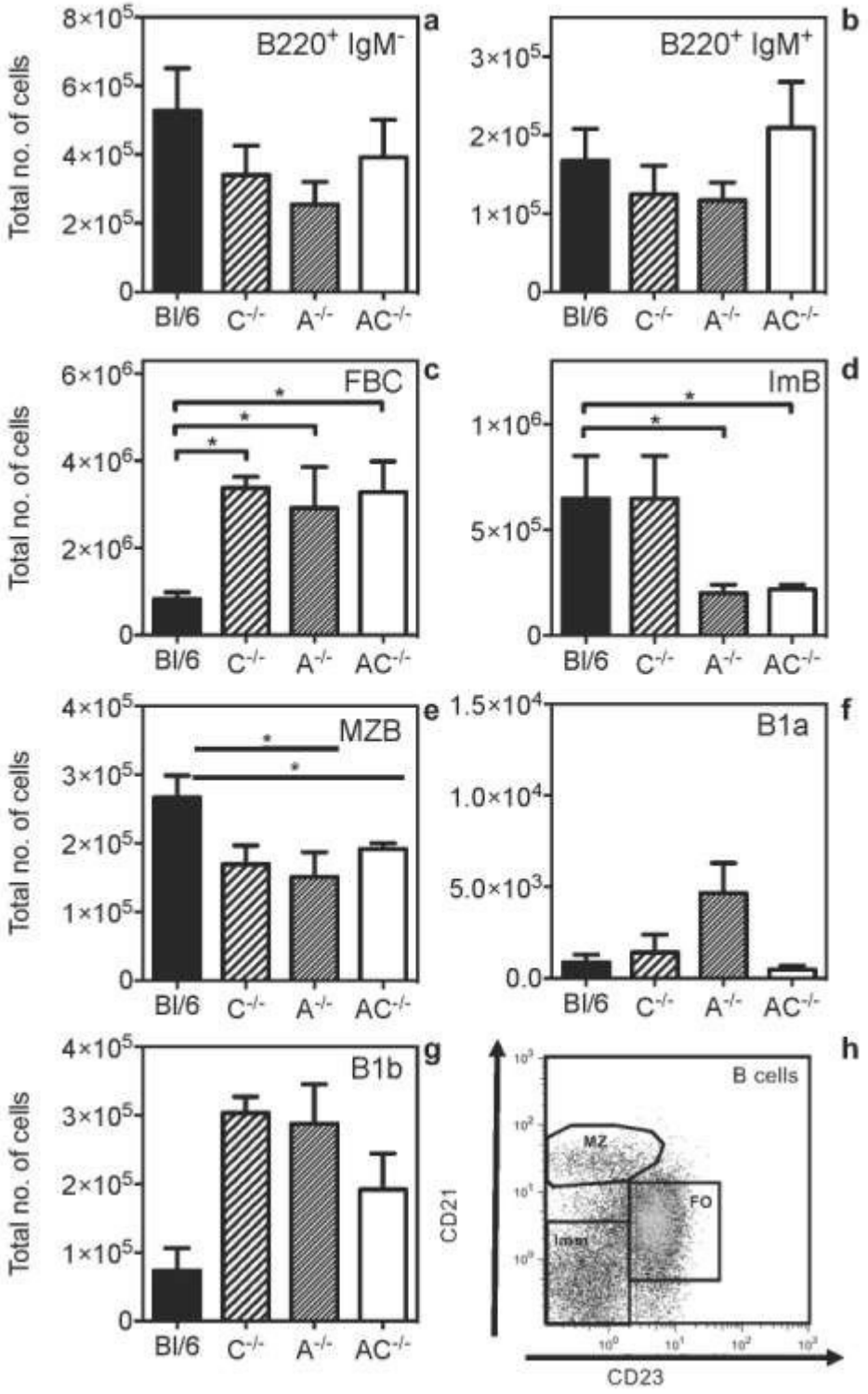
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Figure 4

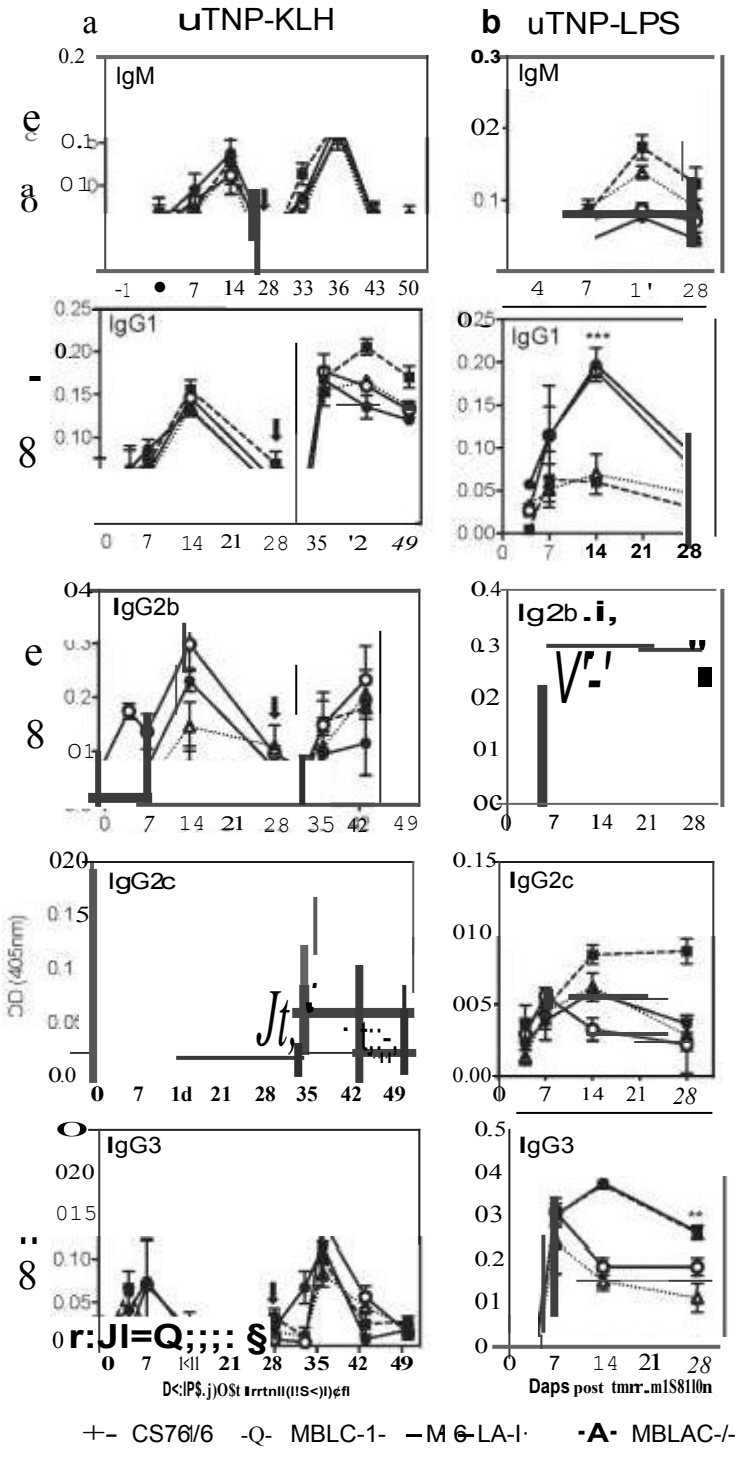
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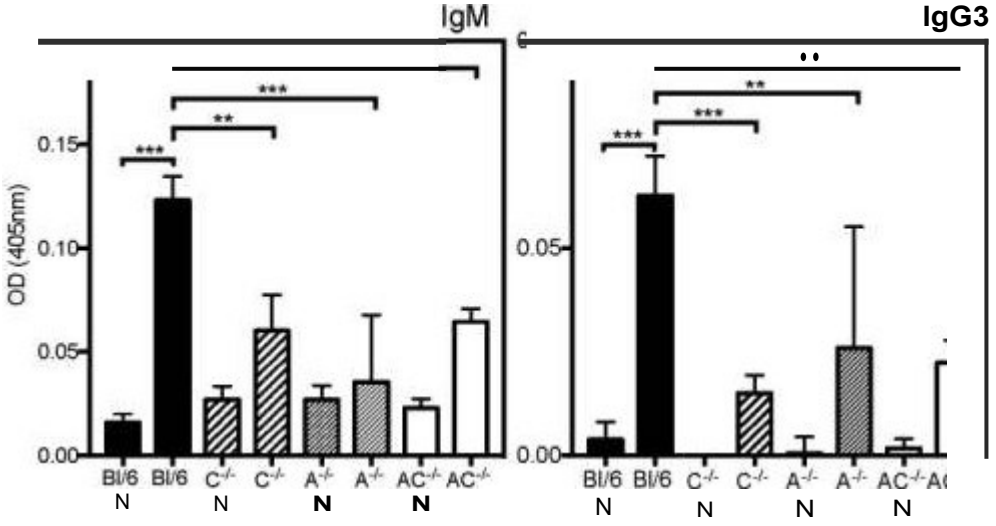
Figure 5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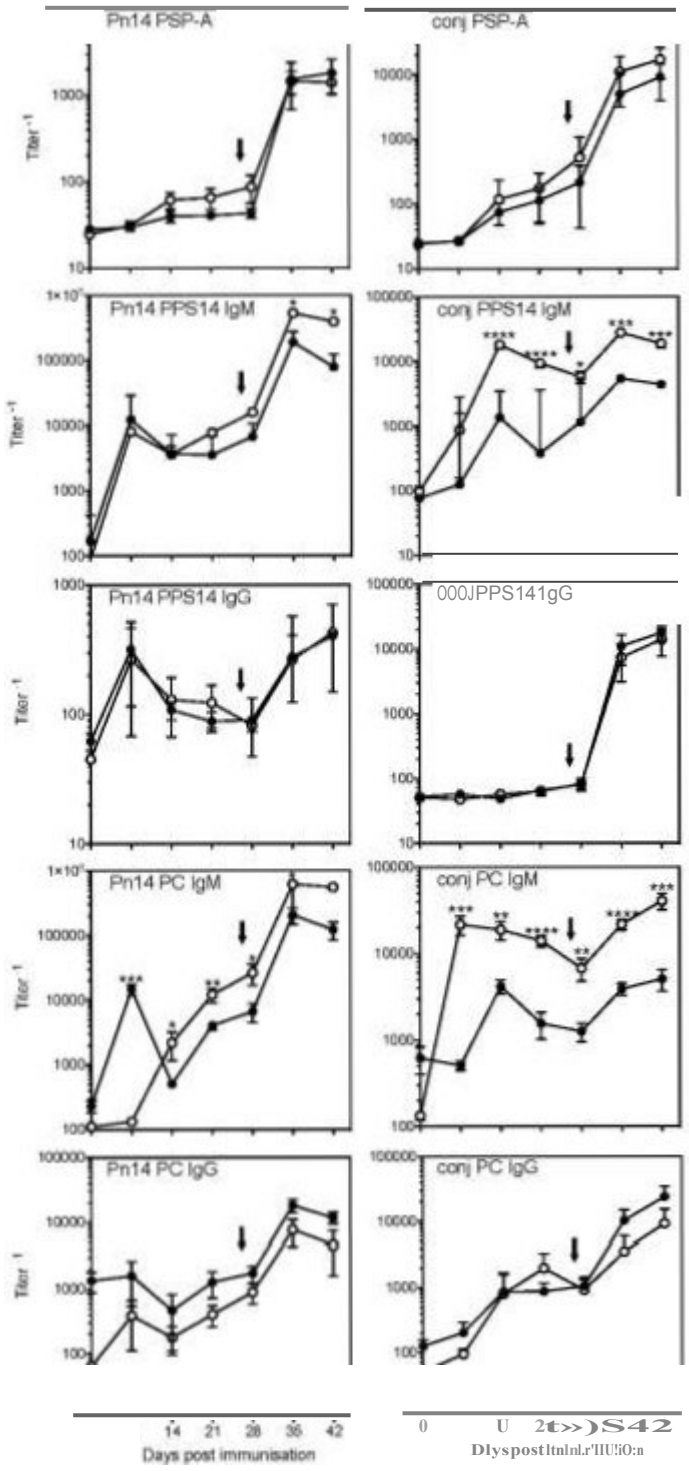
Figuro 6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