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HOST RESPONSE AND INFLAMMATION

Antibodies to Protein but Not Glycolipid Structures Are Important for Host Defense against *Mycoplasma pneumoniae*

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ABSTRACT Antibody responses to *Mycoplasma pneumoniae* correlate with pulmonary *M. pneumoniae* clearance. However, *M. pneumoniae*-specific IgG antibodies can cross-react with the myelin glycolipid galactocerebroside (GalC) and cause neurological disorders. We assessed whether antiglycolipid antibody formation is part of the physiological immune response to *M. pneumoniae*. We show that antibodies against *M. pneumoniae* proteins and glycolipids arise in serum of *M. pneumoniae*-infected children and mice. Although antibodies to *M. pneumoniae* glycolipids were mainly IgG, anti-GalC antibodies were only IgM. B-1a cells, shown to aid in protection against pathogen-derived glycolipids, are lacking in Bruton tyrosine kinase (Btk)-deficient mice. *M. pneumoniae*-infected Btk-deficient mice developed *M. pneumoniae*-specific IgG responses to *M. pneumoniae* proteins but not to *M. pneumoniae* glycolipids, including GalC. The equal recovery from *M. pneumoniae* infection in Btk-deficient and wild-type mice suggests that pulmonary *M. pneumoniae* clearance is predominantly mediated by IgG reactive with *M. pneumoniae* proteins and that *M. pneumoniae* glycolipid-specific IgG or IgM is not essential. These data will guide the development of *M. pneumoniae*-targeting vaccines that avoid the induction of neurotoxic antibodies.

KEYWORDS Bruton tyrosine kinase, Guillain-Barré syndrome, *Mycoplasma pneumoniae*, antibodies, autoimmunity, encephalitis, galactocerebroside, molecular mimicry, pneumonia, vaccine

Mycoplasma pneumoniae is a major cause of community-acquired pneumonia (CAP) and can trigger immune-mediated neurological complications such as Guillain-Barré syndrome (GBS) and encephalitis (1). *M. pneumoniae* belongs to the smallest self-replicating microorganisms, in terms of both cellular dimensions and genome size (1). Unlike other bacteria, *M. pneumoniae* lacks a peptidoglycan layer and is therefore naturally resistant to cell wall synthesis inhibitors such as β-lactams. Macrolide antibiotics are recommended to treat *M. pneumoniae* infections in children (2). Extensive macrolide use led to an alarming worldwide increase of macrolide-resistant *M. pneumoniae* (MRMP) strains, with rates of over 90% in some regions (3, 4). This emergence of MRMP highlights the importance of implementing control strategies to prevent infection, such as vaccines. Vaccination primarily induces antibody responses capable

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of neutralizing infection (5), but attempts to develop such vaccines against *M. pneumoniae* using inactivated bacteria in humans (reviewed in reference 6) and live attenuated strains in an animal model (7) have been complicated by limited efficacy against respiratory disease. No serious adverse effects and only mild local reactions were reported in humans (6). However, it has been observed that reinfection or challenge after vaccination with inactivated or live attenuated strains led to exacerbation of disease in some anecdotal reports (8, 9) and animal experiments (10–14). Thus, to develop optimal approaches to vaccination against *M. pneumoniae*, it is critical to understand the immune mechanisms that contribute to resistance and immunopathology of *M. pneumoniae* disease (15).

Immune responses against *M. pneumoniae* have been intensively investigated in various animal models (e.g., see references 14 and 16–23). B cells are known to be involved in pulmonary *M. pneumoniae* clearance (22, 24–27), and we recently showed that in B cell-deficient μMT mice, *M. pneumoniae* infection led to chronic pulmonary disease, characterized by higher histopathology scores (28). The observed compensatory immune responses by both innate (granulocytes and monocytes) and adaptive ($CD4^+$ and $CD8^+$ T cells) immune cells were not able to clear *M. pneumoniae* infection in the absence of antibodies. In contrast, μMT mice cleared *M. pneumoniae* infections in the lungs when passively immunized with *M. pneumoniae*-specific immunoglobulin G (IgG)-containing serum from infected wild-type (WT) mice 2 weeks after infection. These findings indicate that B cells and *M. pneumoniae*-specific antibodies are crucial for *M. pneumoniae* clearance in the lungs. Furthermore, these data suggest that they may not contribute to immunopathology following primary infection given the less severe pulmonary inflammation and better outcome in WT mice than in B cell-deficient μMT mice (28).

M. pneumoniae is covered only with a cell membrane containing antigenic protein and glycolipid structures (29). The membrane-anchored proteins at the cell pole form an attachment structure important for initiating respiratory infection (30). Proteins constitute over two-thirds of the *M. pneumoniae* membrane mass, with the rest being membrane lipids, i.e., cholesterol, phospholipids, and glycolipids (29). *M. pneumoniae* glycolipid subfractions have been shown to be highly immunogenic in mice and humans (31). Their strong immunogenicity has been leveraged in diagnosis of *M. pneumoniae* infection, whereby antigens derived from crude culture extracts that contain large amounts of glycolipids were used in serological assays (32, 33). However, because of cross-reactions with other mycoplasmas or Gram-negative bacteria, current diagnostic assays focus on specific adhesion proteins (e.g., protein P1) rather than glycolipids (32). Importantly, *M. pneumoniae* glycolipids also exhibit homology with mammalian tissue compounds, which trigger cross-reactive antibodies that may target cells of multiple host organ systems (34). GBS and encephalitis constitute the most common and severe neurological diseases of *M. pneumoniae* extrapulmonary manifestations in which an underlying postinfectious antibody-mediated process has been proposed (33). In fact, it has been shown that galactocerebroside (GalC)-specific antibodies bind to a lipid structure present in *M. pneumoniae*, indicative of molecular mimicry between the major myelin glycolipid GalC and *M. pneumoniae* (35).

We recently showed that both IgM and IgG anti-GalC antibodies are present in the serum of GBS patients and that the presence of anti-GalC IgG correlates with GBS (36). Anti-GalC IgM was also found in 18% of anti-*M. pneumoniae*-seropositive control patients without neurological diseases (36). Interestingly, all anti-GalC IgM-positive individuals within this control cohort were children. This raises the question of whether the formation of antibodies to *M. pneumoniae* glycolipids is part of the physiological immune response and necessary to clear *M. pneumoniae* in children.

Antibody responses against glycolipids are thought to be driven by B-1a cells, splenic marginal zone B cells, and nodal marginal zone B cells (i.e., thymus independent [TI]) or by the help of natural killer T (NKT) cells (i.e., thymus dependent [TD]) (37–40). An important role for B-1a cells in producing antibodies to pathogen-derived glycolipid structures has been shown for *Mycobacterium tuberculosis* and *Francisella tularensis*

(41–43). Interestingly, priming of Bruton tyrosine kinase-deficient (Btk$^-$) mice with *F. tularensis*-derived glycolipids did not result in protection against a lethal challenge with an *F. tularensis* live vaccine strain (44). The lack of a protective antibody response in the Btk-deficient mice was attributed to the absence of B-1a cells (44, 45). Whether TI B cell responses, and in particular B-1a cells, are also important for protection against *M. pneumoniae* infection is unknown.

We set out to investigate in children which antigenic structures of *M. pneumoniae* are recognized by antibodies, using a well-defined cohort of children with CAP diagnosed with *M. pneumoniae* infection. Furthermore, employing WT and Btk$^-$ mice, we unraveled the role of TI B cell responses in the resolution of pulmonary *M. pneumoniae* infection.

RESULTS

IgM but not IgG to GalC is induced during *M. pneumoniae* infection in children. In light of our previous findings (36), we assessed whether anti-GalC IgM develops in all children with *M. pneumoniae* CAP. To this end, we examined the serum of children with *M. pneumoniae* CAP for the presence of anti-GalC antibodies by an enzyme-linked immunosorbent assay (ELISA), and *M. pneumoniae*-negative asymptomatic healthy control (HC) children were tested as controls. We detected anti-GalC IgM at significantly higher levels in sera of children with *M. pneumoniae* CAP than in sera of HC children (Fig. 1A). Moreover, anti-GalC IgG was detectable only at very low levels in both children with *M. pneumoniae* CAP and HC children (Fig. 1B). These findings confirm and extend previous observations that during childhood, *M. pneumoniae* CAP IgM against GalC develops, whereas anti-GalC IgG does not.

IgM and IgG recognize *M. pneumoniae* protein and glycolipid structures during *M. pneumoniae* infection in humans. In addition to the antiglycolipid response to GalC, we next investigated the antibody response against the complete *M. pneumoniae* glycolipid as well as *M. pneumoniae* protein fractions. First, *M. pneumoniae* proteins and glycolipids were separated from *M. pneumoniae* cultures using chloroform-methanol (MeOH) extraction (2:1, vol/vol). The separation of proteins and glycolipids from the *M. pneumoniae* lysate was analyzed by SDS-PAGE followed by silver staining (Fig. 1C) and by thin-layer chromatography (TLC) followed by orcinol staining (Fig. 1D), respectively. The reactivity and kinetics of *M. pneumoniae*-specific IgM and IgG antibodies toward these structures were subsequently determined with an ELISA. To this end, we incubated sera of children with *M. pneumoniae* CAP with either *M. pneumoniae* glycolipid or *M. pneumoniae* protein fractions and an *M. pneumoniae* lysate as a control (referred to as total *M. pneumoniae*). As for total *M. pneumoniae*, a specific antibody response of both IgM and IgG isotypes against *M. pneumoniae* proteins and glycolipids could be detected in all *M. pneumoniae* CAP patients within 9 days after the onset of the first CAP symptoms and peaked at around 1 month (20 to 35 days) (Fig. 1E to G). The specific IgM and IgG response returned to baseline values around 3 months after the onset of CAP symptoms. Analysis of IgG subclasses revealed that both IgG1 and IgG2 subclasses were present in the anti-*M. pneumoniae* glycolipid IgG pool (Fig. 1H). These findings show that the human IgM and IgG response to *M. pneumoniae* is directed against both *M. pneumoniae* glycolipids and *M. pneumoniae* proteins.

IgG against glycolipids also predominates during *M. pneumoniae* infection in mice. We previously showed that passive immunization of B cell-deficient μMT mice with serum of WT mice containing *M. pneumoniae*-specific IgG enabled μMT mice to clear pulmonary *M. pneumoniae* infection (28). In fact, the detection of *M. pneumoniae*-specific IgG in bronchoalveolar lavage fluids (BALFs) correlated with bacterial clearance in the lungs of μMT recipient mice after WT serum transfer. Here, we demonstrate that upon *M. pneumoniae* infection, antibodies are generated against both *M. pneumoniae* protein and glycolipid structures. However, it is unclear whether both the anti-*M. pneumoniae* protein and glycolipid antibodies are important for clearance of *M. pneumoniae* from the lungs. To evaluate this, we examined pulmonary *M. pneumoniae*

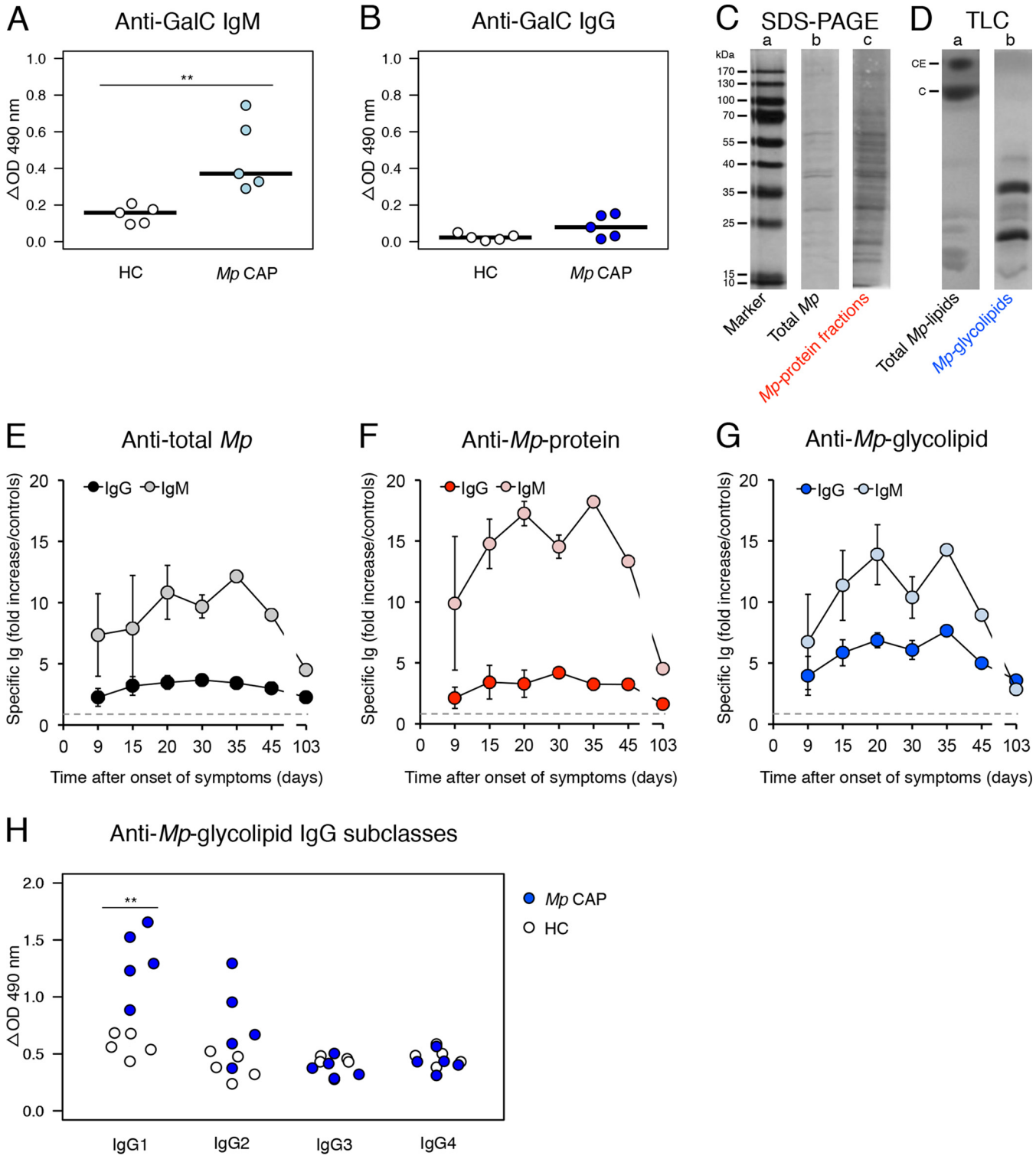

FIG 1 The antibody response induced by pulmonary *M. pneumoniae* infection in children is directed against GalC and *M. pneumoniae* protein and glycolipid fractions. (A and B) The presence of antigalactocerebroside (anti-GalC) IgM (A) and anti-GalC IgG (B) in serum of children with *M. pneumoniae* (*Mp*) community-acquired pneumonia (CAP) and healthy controls (HC) was determined by an ELISA. Sera of children with *M. pneumoniae* CAP were obtained at a median of 34 days (interquartile range, 30 to 42 days) after the onset of symptoms ($n = 5$). Dots represent data for individual children, and the horizontal line in each graph represents the median. OD, optical density. (C) Total *M. pneumoniae* and purified *M. pneumoniae* protein fractions were analyzed by SDS-PAGE followed by silver staining. Lane a, marker; lane b, total untreated *M. pneumoniae* M129 (125 ng); lane c, *M. pneumoniae* protein fractions (125 ng). (D) Thin-layer chromatography (TLC) was used to analyze purified *M. pneumoniae* lipids. Lane a, cerium(IV) sulfate stain for total *M. pneumoniae* lipids; lane b, orcinol stain for *M. pneumoniae* glycolipids. C, cholesterol; CE, cholesterol esters. (E to G) IgG and IgM levels in sera of children with *M. pneumoniae* CAP ($n = 5$) at the indicated time points after the onset of symptoms and reactivity against total *M. pneumoniae* (E) or the isolated *M. pneumoniae* proteins (F) and *M. pneumoniae* glycolipids (G). Not all children had sera available at each time point beyond 30 days. Data are expressed as fold increases over controls (levels in sera of HC

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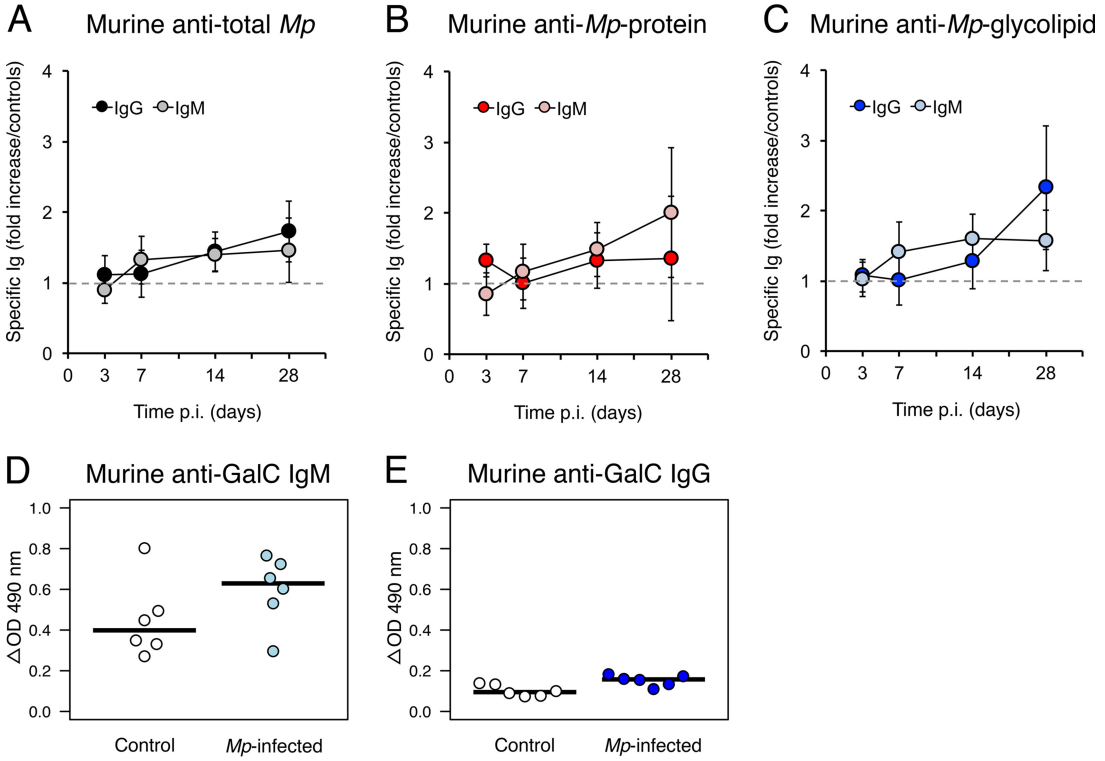

FIG 2 Antibody response against GalC and *M. pneumoniae* protein and glycolipid fractions during pulmonary *M. pneumoniae* infection in mice. (A to C) IgG and IgM levels in sera of *M. pneumoniae*-infected C57BL/6 WT mice at the indicated time points postinfection (p.i.) and reactivity against total *M. pneumoniae* (A) or *M. pneumoniae* proteins (B) and *M. pneumoniae* glycolipids (C). Data are expressed as fold increases over controls (i.e., levels in sera of mock-infected control mice). Data for control levels are indicated by a dashed line. The means ± SD are shown ($n = 6$ to 12 mice/time point). (D and E) Presence of anti-GalC IgM (D) and absence of anti-GalC IgG (E) in sera of *M. pneumoniae*-infected and mock-infected mice obtained at 28 days p.i. ($n = 6$). Dots represent data for individual mice, and the horizontal line in each graph represents the median.

infection in WT and Btk$^-$ mice, as it has been shown that Btk$^-$ mice cannot mediate protective antibody responses to pathogen-derived glycolipid structures (43).

First, we investigated whether *M. pneumoniae* infection also results in a specific antibody response against *M. pneumoniae* proteins and glycolipids in WT mice. We therefore tested the reactivity and kinetics of murine serum antibodies of *M. pneumoniae*-infected C57BL/6 WT mice against *M. pneumoniae* proteins or glycolipids as for children with *M. pneumoniae* CAP. Consistent with our data from children, a specific antibody response of both IgM and IgG isotypes against *M. pneumoniae* proteins and glycolipids could be detected in all mice within 7 days postinfection (p.i.) and peaked at around day 28 p.i. (Fig. 2A to C). These findings in mice parallel the data from children by demonstrating a specific antibody response targeting both *M. pneumoniae* proteins and glycolipids.

Furthermore, we determined whether anti-GalC IgM is also detectable during the course of *M. pneumoniae* infection in mice. Sera of *M. pneumoniae*-infected WT mice, isolated at 28 days p.i., were incubated with GalC. Indeed, compared to mock-infected control WT mice, levels of IgM antibodies to GalC were high in *M. pneumoniae*-infected WT mice, although significance was not reached ($P = 0.19$) (Fig. 2D). In agreement with the data from children, anti-GalC IgG was detectable at equally very low levels after pulmonary *M. pneumoniae* infection as in uninfected control WT mice (Fig. 2E).

FIG 1 Legend (Continued)
children; $n = 5$). Control levels are indicated by a dashed line. The means ± standard deviations (SD) are shown. (H) IgG subclasses against *M. pneumoniae* glycolipids in children with *M. pneumoniae* CAP and HC children (serum samples as the ones used for panel A). Dots represent data for individual children. **, $P < 0.01$ (A, B, and H) (by a Mann-Whitney U test).

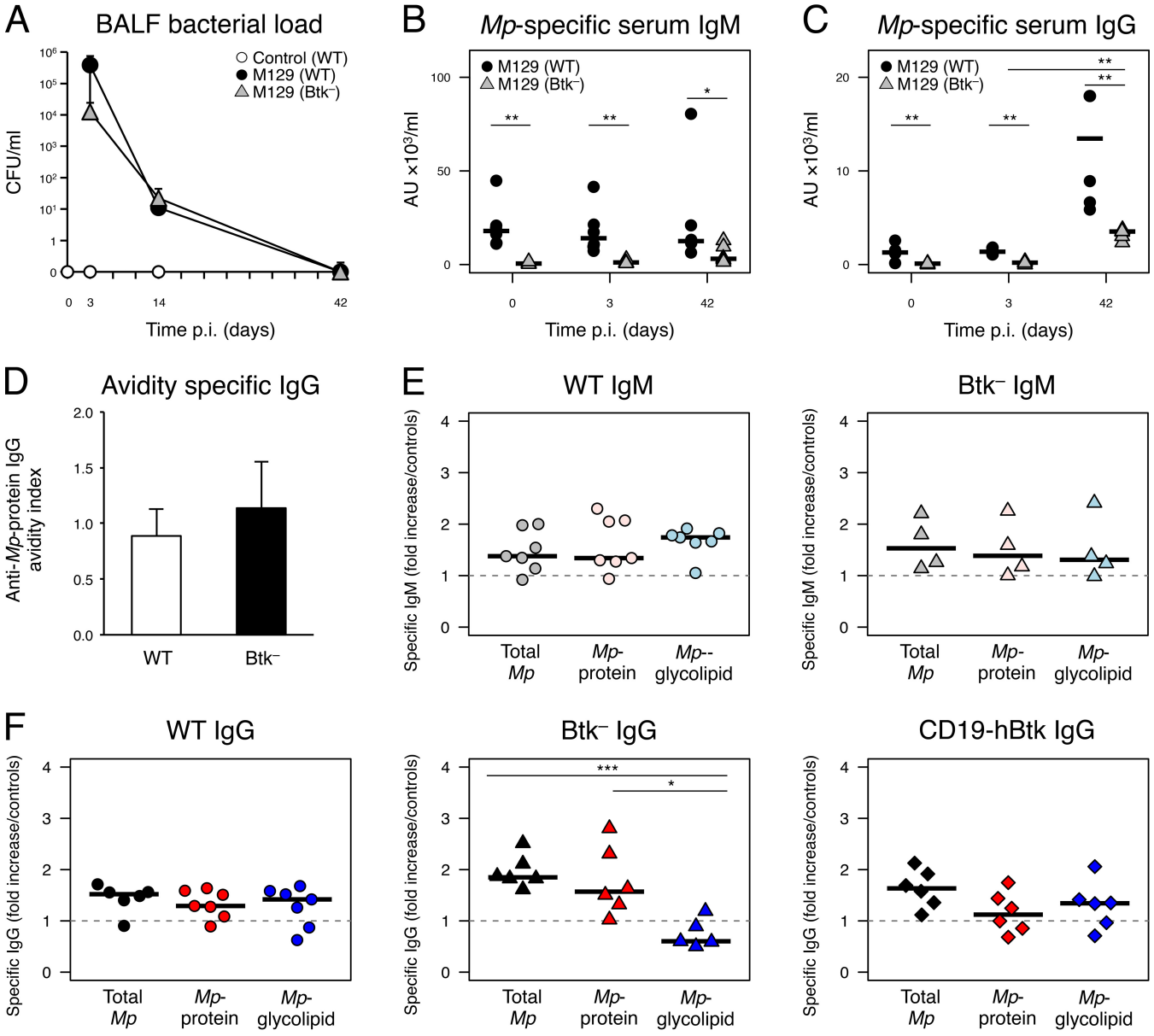

FIG 3 IgG against *M. pneumoniae* proteins but not *M. pneumoniae* glycolipids is crucial to resolve *M. pneumoniae* infection in mice. WT C57BL/6 mice ($n = 6$ to 12 mice/time point) and Btk$^-$ mice ($n = 6$ to 12 mice/time point) were infected intranasally with *M. pneumoniae*. Control WT mice received SP4 medium alone. Dots represent data for individual mice, and the horizontal lines represent the medians. (A to C) At the indicated time points, bacterial loads in BALF (A) and serum levels of *M. pneumoniae*-specific IgM (B) and IgG (C) were determined. Bacterial loads are expressed as median CFU per milliliter with interquartile ranges. AU, arbitrary units. (D) Avidity index of IgG against *M. pneumoniae* proteins at day 42 p.i. The bars represent the means ± SD. (E and F) Serum levels of *M. pneumoniae*-specific IgM (E) and IgG (F) against total *M. pneumoniae* (gray and black) or the separated *M. pneumoniae* proteins (light red and red) and *M. pneumoniae* glycolipids (light blue and blue) in sera of *M. pneumoniae*-infected C57BL/6 WT mice (circles) and Btk$^-$ mice (triangles) and additionally IgG in sera of CD19-hBtk mice (diamonds) at day 14 p.i. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (determined by a Kruskal-Wallis test with Dunn's *post hoc* multiple-comparison test [A to C, E, and F] or Welch's *t* test [D]).

IgG against *M. pneumoniae* proteins but not *M. pneumoniae* glycolipids is crucial to resolve *M. pneumoniae* infection in mice. We next evaluated whether a potential reduction in antiglycolipid antibodies in Btk$^-$ mice affects *M. pneumoniae* clearance from the lungs. We thus compared Btk$^-$ mice with WT mice in terms of the outcomes of *M. pneumoniae* infection and antiglycolipid antibody responses. Surprisingly, CFU counts of *M. pneumoniae* in BALF of Btk$^-$ mice were not different from those in infected WT mice (Fig. 3A). Btk$^-$ mice showed even better control of pulmonary infection than WT mice at day 3 p.i. Nevertheless, both WT and Btk$^-$ mice were able to clear *M. pneumoniae* within 42 days. Analysis of serum antibodies revealed that *M.*

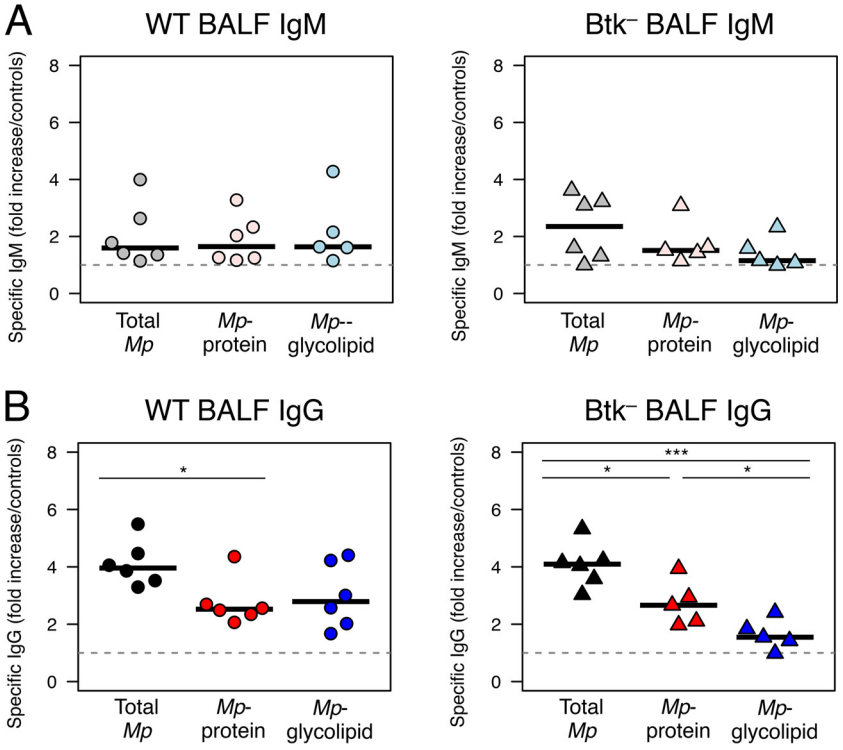

FIG 4 Local antibody response against *M. pneumoniae* protein and glycolipid fractions during pulmonary *M. pneumoniae* infection in mice. Shown are BALF levels of *M. pneumoniae*-specific IgM (A) and IgG (B) against total *M. pneumoniae* (gray and black) or the separated *M. pneumoniae* proteins (light red and red) and *M. pneumoniae* glycolipids (light blue and blue) of *M. pneumoniae*-infected C57BL/6 WT mice (circles) ($n = 6$) and Btk$^-$ mice (triangles) ($n = 6$) at day 14 p.i. Dots represent data for individual mice, and the horizontal lines represent the medians. *, $P < 0.05$; ***, $P < 0.001$ (by a Kruskal-Wallis test with Dunn's *post hoc* multiple-comparison test).

pneumoniae-specific antibody levels increased over time in both WT and Btk$^-$ mice, which were much more pronounced for IgG than for IgM, but the levels in Btk$^-$ mice were significantly lower than those in WT mice (Fig. 3B and C). The same pattern was observed for total IgG and IgM antibody levels in Btk$^-$ mice (data not shown). Although Btk$^-$ mice contained lower IgG levels after *M. pneumoniae* infection, the avidity index of IgG antibodies against *M. pneumoniae*-derived proteins was not different from that in WT mice (Fig. 3D).

The increases in levels of IgM against *M. pneumoniae* proteins or glycolipids compared to controls were similar between WT and Btk$^-$ mice (Fig. 3E). Also, comparable IgG responses to *M. pneumoniae* proteins were measured in sera from WT and Btk$^-$ mice (Fig. 3F, left). In contrast, the IgG response to *M. pneumoniae* glycolipids in Btk$^-$ mice was strikingly different from that in WT mice: no IgG antibodies against *M. pneumoniae* glycolipids were detected (Fig. 3F, middle). The generation of IgG antibodies against *M. pneumoniae* glycolipids was partially restored in CD19-hBtk mice (Fig. 3F, right), in which Btk is selectively rescued in B cells and is lacking only in myeloid cells (46). These findings could be corroborated by measuring IgM and IgG to *M. pneumoniae* proteins or glycolipids of WT and Btk$^-$ mice in BALF. As previously observed (28), we found very low levels of IgM, which were comparable between mouse strains for *M. pneumoniae* proteins and glycolipids (Fig. 4A). As in serum, the IgG antibody levels to *M. pneumoniae* glycolipids in Btk$^-$ mice were significantly lower than those in BALF of WT mice and close to control levels (Fig. 4B). Serum anti-GalC IgM and IgG antibodies were not produced by *M. pneumoniae*-infected Btk$^-$ mice (Fig. 5).

DISCUSSION

Here, we extend previous findings on the essential role of *M. pneumoniae*-specific IgG antibodies in pulmonary clearance (27, 28) by demonstrating that IgG antibodies

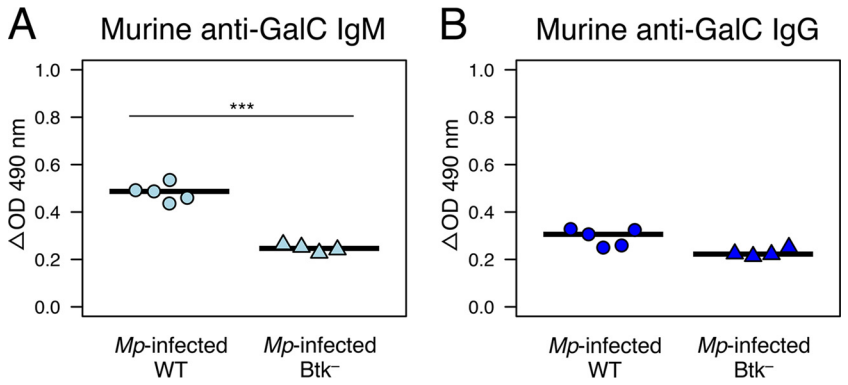

FIG 5 Comparison of the antibody responses against GalC between WT and Btk$^-$ mice during pulmonary *M. pneumoniae* infection. Shown are serum anti-GalC IgM (A) and anti-GalC IgG (B) responses of *M. pneumoniae*-infected WT mice ($n = 5$ mice) and Btk$^-$ mice ($n = 4$ mice) at 42 days p.i. ***, $P < 0.001$ (by a Mann-Whitney U test).

reactive with *M. pneumoniae* proteins alone seem to be sufficient to clear *M. pneumoniae* in the lungs. We show that Btk$^-$ mice clear *M. pneumoniae* infections comparably to WT mice but do not generate a detectable humoral response to *M. pneumoniae* glycolipids. These data also indicate that even low levels of *M. pneumoniae* protein-specific IgG antibodies, albeit of sufficient avidity, are able to mediate protection in the lungs of Btk$^-$ mice. Notably, Btk$^-$ mice showed better control of pulmonary *M. pneumoniae* infection than did WT mice at day 3 p.i. At this time point, significantly higher numbers of alveolar macrophages and NK cells were observed in lungs of Btk$^-$ mice than in WT mice (data not shown). B-1a cells have been shown to inhibit macrophage-NK cell cross talk (47). The observed improved control of *M. pneumoniae* replication in Btk$^-$ mice may thus result from the absence of B-1a cells in these mice (48, 49). We speculate that in WT mice, *M. pneumoniae* triggers the activation of B-1a cells, which dampen *M. pneumoniae* clearance by inhibiting the activation of macrophages and/or NK cells. However, the inhibitory effect of B-1a cells is not as sufficient and/or long lasting, as *M. pneumoniae* is cleared from the lungs of WT mice within 4 to 6 weeks.

In addition to their important role in the clearance of *M. pneumoniae* in the lungs, the induced *M. pneumoniae* protein-specific antibodies may also prevent reinfection or reduce horizontal transmission. It was shown previously that antibodies from *M. pneumoniae*-immunized guinea pigs targeting recombinant P1 and P30 adhesion proteins inhibit the adherence of *M. pneumoniae* to human bronchial epithelial cells *in vitro* (23). This indicates that an *M. pneumoniae*-specific vaccine for high-risk individuals, i.e., schoolchildren and elderly people, should aim at inducing potent antibodies directed against *M. pneumoniae* proteins. The data obtained with Btk$^-$ mice suggest that such a vaccine may even be effective in children with common variable immunodeficiency or hypogammaglobulinemia with reduced B cells, who have been reported to be at an increased risk for *M. pneumoniae* pulmonary disease and/or extrapulmonary manifestations (50–54).

Our data reveal that the humoral response against *M. pneumoniae* glycolipids is redundant for the clearance of *M. pneumoniae* in the lungs of mice. This is rather unexpected given the fact that levels of IgG against *M. pneumoniae* glycolipids were higher than those against *M. pneumoniae* proteins and were of the IgG1 and IgG2 subclasses, which indicates potential neutralization and complement-dependent killing of *M. pneumoniae*. Furthermore, within the pool of *M. pneumoniae* glycolipid-specific IgM antibodies, some also bear cross-reactivity to self-tissue (i.e., GalC-like *M. pneumoniae* structure [33]). Indeed, we previously elucidated cross-reactivity between *M. pneumoniae* and GalC and associated the presence of anti-GalC IgG with GBS triggered by *M. pneumoniae* (36). However, here, we confirmed that in the absence of neurop-

athy, anti-GalC IgG was not formed during pulmonary *M. pneumoniae* infection. In contrast, the formation of anti-GalC IgM, which seems not to inflict neurotoxicity, was part of the physiological response.

Cross-reactivity of *M. pneumoniae* glycolipid-specific antibodies with self-tissue, causing immunopathology, may be one possible reason why BALB/c mice vaccinated with live attenuated *M. pneumoniae* developed more-severe pulmonary disease following infection with wild-type *M. pneumoniae* (10). This could also be observed after repeated infections with wild-type *M. pneumoniae* in BALB/c mice but not in C57BL/6 mice (11). It is known that not only rechallenge after vaccination but also primary *M. pneumoniae* infection of BALB/c mice led to worse pulmonary inflammation than in C57BL/6 mice (18), which simply reflects heterogeneity in susceptibility to *M. pneumoniae* infection in mice as in humans (1, 18).

The observation that *M. pneumoniae* glycolipid-specific antibodies are not generated in Btk$^-$ mice suggests that the production of lipid-specific antibodies may be, at least in part, mediated by B-1a cells, as these cells are lacking in Btk$^-$ mice (48, 49). Notably, in contrast to *M. pneumoniae* infection, antibodies to pathogen-derived glycolipid structures were shown previously to protect against infections with *M. tuberculosis* and *F. tularensis* (41–43). Immunization of Btk$^-$ mice with *F. tularensis* glycolipids did not result in protection against a lethal challenge with an *F. tularensis* live vaccine strain, which was attributed to the absence of B-1a cell-produced glycolipid-specific antibodies (44, 45). Apart from TI B cell responses, a potential role for nonclassical T helper cells, the NKT cells, in the production of *M. pneumoniae* lipid antibodies cannot be ruled out.

In fact, cross-linking of the B cell receptor (BCR) on B-1a cells by lipid-based antigens leads to an innate-like polyreactive IgM response (so-called natural antibody response) important for early protection against mucosal pathogens (37). B-1 cells can also provide long-lasting TI IgM memory (43, 55). One might speculate that the presence of anti-GalC IgM but not IgG after *M. pneumoniae* infection in children and mice indicates that a GalC-like structure in *M. pneumoniae* triggered specifically B-1a cells to produce natural IgM. Natural antibodies are characterized by low affinity (37, 56), which may explain why *M. pneumoniae* glycolipid-specific antibodies were redundant for clearance of pulmonary *M. pneumoniae* infection. Apart from affinity, the level of anti-GalC IgG may be critical for the initiation of *M. pneumoniae*-associated neurological disease. It is possible that low levels of GalC IgG are removed from the circulation by target-mediated clearance (57). Interestingly, B-1a cells triggered by glycolipid antigens can also undergo activation-induced deaminase-dependent class switching (43). However, this is a rare event (43), as is the development of GBS following *M. pneumoniae* infection (36). Thus, these findings further support the hypothesis that a GalC-like *M. pneumoniae* structure may trigger B-1a cells, which undergo class switching in rare cases, and produce potentially neurotoxic anti-GalC IgG. However, the exact events that lead to the induction of autoreactive B cells and anti-GalC IgG remain to be identified.

In conclusion, our data extend previous findings on the essential role of *M. pneumoniae*-specific antibodies in clearing *M. pneumoniae* from the lungs (27, 28) by suggesting that the IgG response to *M. pneumoniae*-derived proteins is important for pulmonary clearance of *M. pneumoniae*. The finding that *M. pneumoniae* glycolipid-specific IgM and IgG antibodies are redundant for *M. pneumoniae* clearance but can also target the myelin glycolipid GalC is of importance not only for the understanding of *M. pneumoniae*-associated immune-mediated diseases but also for the design of *M. pneumoniae*-targeting vaccines. Based on our results, such vaccine formulations should include *M. pneumoniae* protein antigens rather than *M. pneumoniae* lipids, thereby avoiding the induction of potential autoimmune antiglycolipid antibodies.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Charles River Laboratories and used at 8 to 12 weeks of age. Btk$^-$ mice (Btk$^{-/-}$ or Btk$^{-/Y}$, on the C57BL/6 background [48]) and CD19-hBtk mice (backcrossed on Btk$^-$ C57BL/6 mice for >10 generations [46]) were bred and housed in the animal facilities of the Erasmus MC under specific-pathogen-free conditions. All experiments were conducted according to

Dutch guidelines for animal experimentation and approved by the Animal Experiments Committee of the Erasmus MC, Rotterdam, The Netherlands (protocol numbers 103-13-05 and 103-14-02).

Patients. Children with CAP and asymptomatic controls, admitted for a planned elective surgical procedure, from 3 to 18 years of age were enrolled from 1 May 2016 to 30 April 2017 during a CAP study at the University Children's Hospital Zurich (P. M. Meyer Sauteur, unpublished data). Diagnosis was based on the detection of *M. pneumoniae* DNA in pharyngeal swab specimens by PCR, specific serum IgM antibodies by an ELISA, and circulating IgM antibody-secreting cells by an enzyme-linked immunospot (ELISpot) assay (Meyer Sauteur, unpublished). Asymptomatic controls tested negative for *M. pneumoniae* by PCR and serum IgM. Sera of these *M. pneumoniae*-positive CAP patients and *M. pneumoniae*-negative asymptomatic controls were used in this study, since no further information about respiratory disease characteristics was available from previous *M. pneumoniae*-seropositive controls without neurological diseases (36), and *M. pneumoniae* infection can present with a wide range of respiratory tract symptoms apart from CAP. The study was approved by the ethics committee of the Canton Zurich, Switzerland (BASEC number 2016-00148). Written informed consent was obtained from all parents and from children above the age of 14 years.

Bacteria. *M. pneumoniae* reference strain M129 (subtype 1; ATCC 29342) was cultured as previously described (28).

Infection. Mice were inoculated intranasally with 1×10^9 CFU of *M. pneumoniae* M129 diluted in 50 μl SP4 medium. Control mice were inoculated with 50 μl SP4 medium.

***M. pneumoniae* quantification.** The presence of *M. pneumoniae* was detected by either PCR or culture of BALF on SP4 agar plates (58).

Lipid extraction. Lipids were extracted from the *M. pneumoniae* M129 culture by the addition of chloroform-methanol-water (2:1:1, vol/vol/vol). After a 1-h incubation at 4°C, the mixture was vortexed and centrifuged at 2,000 $\times$ *g* for 1 min. After repeating the extraction procedure, cells were sonicated for 30 min and centrifuged at 10,000 $\times$ *g* for 5 min. Lipid extracts contained in the chloroform layer were pooled and evaporated under nitrogen. Lipids were dissolved in ethanol, and extraction was verified by the Liebermann-Burchard reaction. The purification of *M. pneumoniae* glycolipids was confirmed by TLC. Here, extracted lipids (5 μg) were applied to a silica glass plate (Merck Millipore) after activation for 2 h at 110°C. The TLC plate was developed using $CHCl_3$–MeOH–Milli-Q (60:35:8, vol/vol/vol). Total lipids were visualized by using 10% cerium(IV) sulfate in 15% H_2SO_4, and glycolipids were visualized by using 0.1% orcinol in 5% H_2SO_4, with heating up to 110°C.

Protein extraction. After delipidation of the *M. pneumoniae* lysate by chloroform-methanol-water (2:1:1, vol/vol/vol) extraction, cold acetone was added to the aqueous (upper) layer and the insoluble interface, followed by incubation at 4°C for 2 h. Proteins were collected by centrifugation at 10,000 $\times$ *g* for 5 min. Pellets were washed twice with acetone, air dried on ice, and dissolved in 4 M urea–250 mM ammonium bicarbonate. The protein concentration was determined by a bicinchoninic acid assay. Additionally, proteins (125 ng) were analyzed by SDS-PAGE, followed by silver staining (Merck).

Quantification of *M. pneumoniae*-specific antibodies against *M. pneumoniae* antigens and GalC. To assess the presence of specific antibodies against total *M. pneumoniae*, *M. pneumoniae* proteins and glycolipids, and GalC, 96-well half-area polystyrene plates (Corning Costar) were coated overnight at 4°C with an *M. pneumoniae* lysate (0.5 μg) (28) or *M. pneumoniae* proteins (normalized to 1 ng adhesion protein P1 per well present in both total *M. pneumoniae* and *M. pneumoniae* protein fractions) in a 100 mM sodium carbonate-bicarbonate buffer (pH 9.6) containing 1 M urea or with *M. pneumoniae* glycolipids (normalized to 0.6 μg cholesterol per well present in both total *M. pneumoniae* and *M. pneumoniae* glycolipid fractions) and GalC (900 pmol) dissolved in ethanol. Nonspecific binding was blocked using phosphate-buffered saline (PBS)–1% bovine serum albumin (BSA) (Sigma). Serum samples, diluted in PBS–0.1% BSA, and undiluted BALF samples were added and incubated overnight at 4°C. Bound antibodies were detected by the addition of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM or rabbit anti-mouse IgG (Thermo Scientific), biotinylated goat anti-human IgM with HRP-conjugated streptavidin (Thermo Scientific), or HRP-conjugated rabbit anti-human IgG (Invitrogen) and IgG1 to -4 (Sanquin). Reactions were visualized by using tetramethylbenzidine (TMB; Sigma) as the substrate and stopped by using 1 M H_2SO_4. The optical density was measured at 450 nm with an ELISA microplate reader (VersaMax). The results were analyzed by microplate data collection and analysis software (VersaMax).

Statistical analysis. The R software environment (version 3.4.0) was used for statistical analysis. Welch's *t* test, the Mann-Whitney U test, and the Kruskal-Wallis test with *post hoc* Dunn's multiple-comparison test of selected pairs were used to determine statistical significance. Statistical significance was defined as a *P* value of <0.05.

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We have no conflict of interest.

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