Patrick M. Meyer Sauteur

# Antibody responses to *Mycoplasma pneumoniae*: protecting against and triggering disease

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Patrick M. Meyer Sauteur, MD

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# Antibody responses to *Mycoplasma pneumoniae*: protecting against and triggering disease

## Antistoffen tegen *Mycoplasma pneumoniae*: beschermen tegen én veroorzaken ziekte

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# Contents

## ABBREVIATIONS

| | |
|---|---|
| AIDP | acute inflammatory demyelinating polyneuropathy |
| AMAN | acute motor axonal neuropathy |
| AMSAN | acute motor sensory axonal neuropathy |
| ASC | antibody-secreting cell |
| AU | arbitrary unit |
| BALF | bronchoalveolar lavage fluid |
| BBB | blood-brain barrier |
| BBE | Bickerstaff brain stem encephalitis |
| BCR | B cell receptor |
| Btk | Bruton tyrosin kinase |
| $Btk^-$ mice | partially B cell-deficient mice (see Chapter 3) |
| BTS | British Thoracic Society |
| CAP | community-acquired pneumonia |
| CARDS | community-acquired respiratory distress syndrome toxin |
| CD | cluster of differentiation |
| CFSE | carboxyfluorescein diacetate succinimidyl ester |
| CFT | complement fixation test |
| CFU | colony-forming unit |
| CLN | cervical lymph node |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| CSR | class switch recombination |
| CT | computed tomography |
| CVID | common variable immunodeficiency |
| DC | dendritic cells |
| DNA | deoxyribonucleic acid |
| EEG | electroencephalogram |
| ELISA | enzyme-linked immunosorbent assay |
| ELISpot | enzyme-linked immunospot |
| FACS | fluorescence-activated cell sorting |
| GalC | galactocerebroside |
| GBS | Guillain-Barré syndrome |
| GC | germinal center |
| GM/GD/GT/GQ | abbreviations for gangliosides (see legend of Table 1, Part 2.2) |
| H&E | hematoxylin and eosin |
| HPS | histopathology score |
| HRP | horseradish peroxidase |

| | |
|---|---|
| Ig | immunoglobulin |
| IL | interleukin |
| IQR | interquartile range |
| IVIg | intravenous immunoglobulin |
| LOS | lipooligosaccharides |
| LRT | lower respiratory tract |
| LRTI | lower respiratory tract infection |
| M129 | *M. pneumoniae* reference strain subtype 1 (ATCC 29342) |
| MFI | mean fluorescence intensity |
| MFS | Miller Fisher syndrome |
| MHC | major histocompatibility complex |
| MLN | mediastinal lymph node |
| μMT mice | B cell-deficient mice (see Chapter 2) |
| MPAM | *M. pneumoniae*-associated mucositis |
| MRI | magnetic resonance imaging |
| MRMP | macrolide-resistant *M. pneumoniae* |
| NA | not available |
| NALT | nasal-associated lymphoid tissue |
| NCS | nerve conduction study |
| ND | not done |
| NK cell | natural killer cell |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| P1 | *M. pneumoniae* adhesion protein P1 |
| P30 | *M. pneumoniae* adhesion protein P30 |
| PCR | polymerase chain reaction |
| PLN | peripheral lymph node |
| PNS | peripheral nervous system |
| RepMP | repetitive DNA elements of *M. pneumoniae* |
| RP14 | recombinant peptide incl. C-terminal part of *M. pneumoniae* adhesion protein P1 |
| rRNA | ribosomal ribonucleic acid |
| RTI | respiratory tract infection |
| SCID | severe combined immunodeficiency |
| SD | standard deviation |
| SHM | somatic hypermutation |
| SPSU | Swiss Pediatric Surveillance Unit |
| TCR | T cell receptor |
| TI | thymus-independent |

| | |
|---|---|
| TLR | Toll-like receptor |
| TMB | tetramethylbenzidine |
| URT | upper respiratory tract |
| URTI | upper respiratory tract infection |
| WT | wild-type |
| WBC | white blood cell |
| XLA | X-linked agammaglobulinemia |
| *xid* | X-linked immunodeficiency |

# Chapter 1

## General introduction and outline of the thesis

Parts of this chapter are published in:

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P.M. Meyer Sauteur
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*Mycoplasma pneumoniae* infections – does treatment help?
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P.M. Meyer Sauteur
C. Vink
A.M.C. van Rossum
*J Infect 2014;69:S42–S46*

Things that could be *Mycoplasma pneumoniae*

R.C.A. de Groot
P.M. Meyer Sauteur
W.W.J. Unger
A.M.C. van Rossum

*J Infect 2017;74:S95–S100*

## INTRODUCTION TO *MYCOPLASMA PNEUMONIAE*

Pneumonia is the single largest infectious cause of death in children worldwide, and caused by a large number of infectious agents, including viruses, bacteria, and fungi.[1] *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* are the two major bacterial causes of pneumonia in hospitalized children.[2,3] Over the past three decades, *S. pneumoniae* conjugate vaccines have markedly reduced the incidence of pneumococcal pneumonia.[4] This thesis focuses on *M. pneumoniae* that was recently reported as the most common bacterial cause of community-acquired pneumoniae (CAP) in hospitalized children in the U.S. (Figure 1).[5]

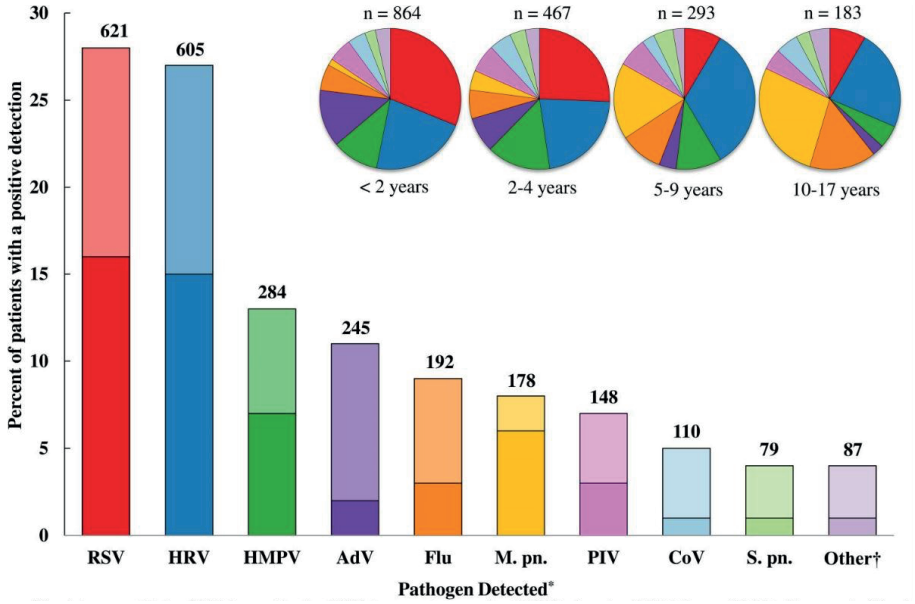


**Figure 1.** Pathogens detected in U.S. children with community-acquired pneumonia (CAP) requiring hospitalization (2010–2012).

*For detection method see Jain et al.[5] Darker and lighter shading in the bar graph indicates single and co-pathogen detection, respectively. Proportions of detections (single and co-detection) by age group are depicted on the pie graphs. Abbreviations: AdV, adenovirus; CoV, coronavirus; Flu, influenza A or B virus; HMPV, human metapneumovirus; HRV, human rhinovirus; M.pn., *M. pneumoniae*; PIV, parainfluenza virus; RSV, respiratory syncytial virus; S.pn., *S. pneumoniae*. Reproduced with permission from Jain et al.,[5] Copyright Massachusetts Medical Society.

### *M. pneumoniae* infection: respiratory tract disease and extrapulmonary manifestations

The clinical entity of "atypical" pneumonia was recognized in the 1930s many years before *M. pneumoniae* was discovered.[6] The term separated this entity of pneumonia from

classical pneumococcal pneumonia due to its lack of response to available antibiotics and the distinct clinical presentation without typical lobar pneumonia and a less severe disease course. That is why the term "walking pneumonia" has been introduced to denote this mild form of pneumonia.

It was in a patient with "atypical" pneumonia in 1944, where *M. pneumoniae* was first isolated from sputum in tissue culture by Eaton et al.[7] At that time, it was believed to be a virus because it was resistant to penicillin and sulfonamides and passed through bacteria-retaining filters. Experiments with Marine recruits and adult prisoners demonstrated that the so-called Eaton agent caused lower respiratory tract infections (LRTIs) in humans.[8,9] In 1963, it was first cultured on cell-free medium and classified as *M. pneumoniae*.[10,11] Today we know that mycoplasmas are prokaryotes that lack a cell wall and represent the smallest self-replicating organisms (Figure 2). With a size of 816,394 base pairs, the genome of *M. pneumoniae* is at least five times smaller than that of *Escherichia coli*.[13] The absence of a cell wall and the specialized attachment organelle facilitate close contact with the host respiratory epithelium, which supplies the bacterium with the necessary nutrients for its growth and proliferation.

*M. pneumoniae* causes both upper respiratory tract infections (URTIs) and LRTIs, with CAP as the major burden of disease. Although *M. pneumoniae* infections are generally

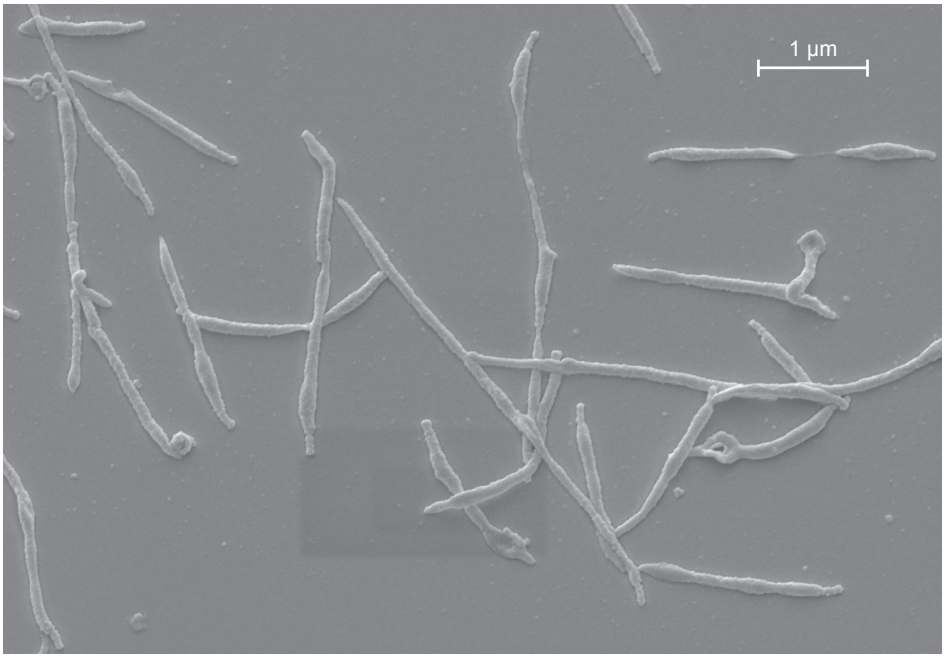


**Figure 2.** *M. pneumoniae* morphology *in vitro*.
Scanning electron micrograph of *M. pneumoniae* strain Mac (subtype 2), performed by Steve Gschmeissner (Bedford, UK). Reproduced with permission from Meyer Sauteur et al.,[12] Frontiers.

mild and self-limiting, patients of every age can develop severe and fulminant disease.[14] *M. pneumoniae* can also cause extrapulmonary manifestations that affect almost every organ.[15]

### *Epidemiology*

*M. pneumoniae* is transmitted by respiratory droplets through close contact. The incubation period can be as long as 1 to 3 weeks. Outbreaks have been reported within families, schools, universities, institutions, camps, and military bases. Family members of index patients with acute respiratory infection and detection of *M. pneumoniae* in the upper respiratory tract (URT) were found positive in 15% by polymerase chain reaction (PCR).[16] Thereof, 75% were <16 years of age and 44% did not develop any respiratory symptoms. At universities, the largest outbreak within 35 years in the U.S. was observed during September 1–December 4, 2012, where a total of 83 CAP cases were identified among students, and 12 out of 19 tested cases (63%) were positive for *M. pneumoniae* by real-time PCR.[17]

Outbreaks appear mainly during *M. pneumoniae* epidemics that occur in 3–7 year cycles, in addition to a background endemic pattern.[18] Data on the most recent epidemic in Europe (2015/2016) have not been published yet; however, the epidemic before had a peak incidence in Finland of 145/100,000 cases in 2011.[19,20] The cyclic occurrence of epidemics may be facilitated by a decreasing herd immunity and different *M. pneumoniae* genotypes circulating in the human population.[18] The two major circulating genotypes, or subtypes, of *M. pneumoniae* are indicated as subtype 1 and 2. Differences between these subtypes in the amino acid sequence of the major adhesion protein P1 are believed to play a role in the epidemiology of infections with *M. pneumoniae*.[21,22] The differences between the 169 kDa P1 proteins of subtype 1 and 2 isolates were found to be concentrated in two specific amino acid stretches within the protein. These regions are encoded by two DNA elements within the P1 gene, i.e., repetitive elements RepMP2/3 and RepMP4. The RepMP2/3 and RepMP4 are not unique to the P1 gene, but are also found at other sites within the bacterial genome.[23] Homologous recombination events between these repetitive elements, which are similar to each other, but not identical, may form the basis of antigenic variation of the P1 protein of *M. pneumoniae*.[21] While such recombination events may induce antigenic variation within subtype 1 or subtype 2 strains, *M. pneumoniae* strains cannot switch from one subtype to the other, as the entire set of RepMP elements found in one subtype differs significantly from those found in the other subtype. Moreover, changes in the proportion of the two subtypes of *M. pneumoniae* were not observed between 2003–2012 in Europe.[19]

## *Infection*

### Respiratory disease

Although CAP is the major burden of disease, milder clinical presentations of *M. pneumoniae* respiratory infections may be much more common than CAP. These include acute bronchitis and URTIs.[24,25] *M. pneumoniae* could be detected by PCR and/or serology in 24% of non-streptococcal pharyngitis cases.[25]

It is estimated that 3–10% of children with *M. pneumoniae* respiratory infection develop CAP and that <5% of CAP cases are severe enough to require hospitalization.[3] Between 1963–1975, *M. pneumoniae* was detected by culture of respiratory specimens and/or a 4-fold rise in complement fixation test (CFT) in 15–20% of radiologically confirmed CAP cases in Seattle, U.S.[26] In subsequent etiological studies, *M. pneumoniae* accounted for 4–39% of the isolates identified by PCR and/or serology in children with CAP admitted to the hospital.[2,27-29] *M. pneumoniae* was first reported as the most common bacterial cause of CAP in children requiring hospitalization in a U.S. multicenter study from 2011–2012 in Nashville and Salt Lake City.[5] In this study, *M. pneumoniae* could be detected by PCR in 178 (8%) out of 2179 cases with CAP, whereas *S. pneumoniae* was found in 79 (4%) cases. Co-existence of *M. pneumoniae* with other pathogens was found in 28% of the patients.[5]

Manifest URTIs and/or LRTIs with *M. pneumoniae* occur at all ages.[26] Recent observations have indicated that *M. pneumoniae* has also a relatively high prevalence in the respiratory tract of children <5 years.[28,30] *M. pneumoniae* CAP, however, was reported to be most frequent among school-aged children from 5–15 years of age, with a decline after adolescence and tapering off in adulthood.[26] This notion was corroborated in the recent CAP study in the U.S., where *M. pneumoniae* was detected significantly more frequent in children ≥5 years of age than in younger children (19% vs. 3%; Figure 1).[5]

In addition to the presentation at school-age, children with CAP due to *M. pneumoniae* have been found to present with a significantly longer duration of fever compared to other children with CAP.[31] Other symptoms that may be associated with *M. pneumoniae* CAP are the absence of wheeze and the presence of chest pain.[32] However, there is still a paucity of high quality data regarding clinical signs and symptoms associated with *M. pneumoniae* infections. A recent Cochrane review[32] therefore concluded that the absence or presence of individual clinical symptoms or signs cannot be used to help clinicians accurately diagnose *M. pneumoniae* in children and adolescents with CAP.

Pathogenic effects in the respiratory tract may be caused by *M. pneumoniae* either directly (by active infection), indirectly (by infection-induced immune mechanisms), or both (Figure 3).[33] *M. pneumoniae* causes direct injury through the generation of activated oxygen. A potential candidate protein of *M. pneumoniae* that may be involved in causing direct damage to the respiratory tract is a pertussis toxin-like protein termed community-acquired respiratory distress syndrome (CARDS) toxin.[34,35] A recombinant version of the

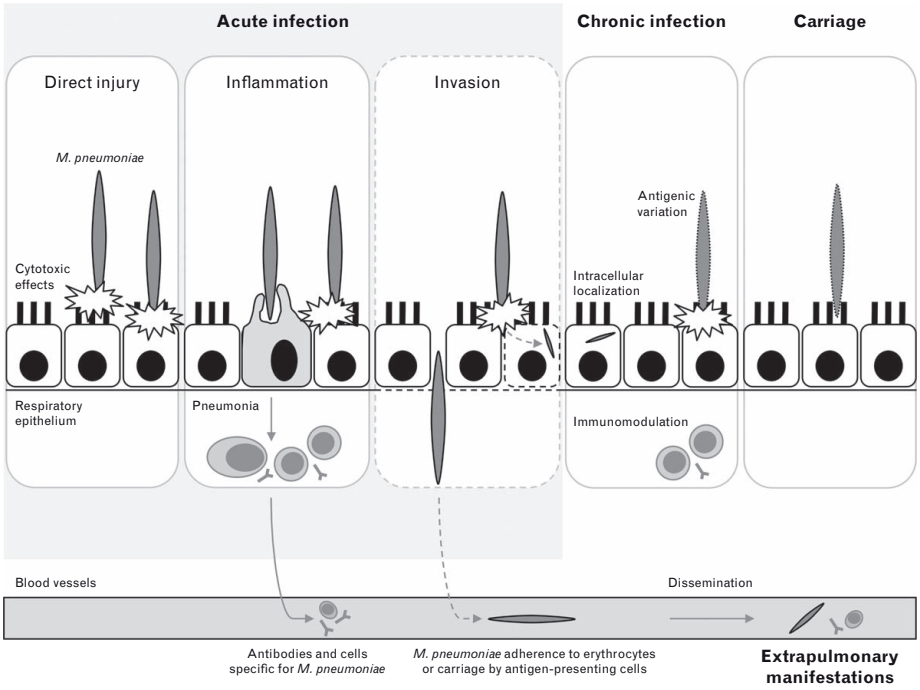


**Figure 3.** Proposed pathogenic states of *M. pneumoniae* at the respiratory tract.
Acute infection: *M. pneumoniae* attaches to the respiratory surfaces, resides mostly extracellular, and produces direct injury by a variety of local cytotoxic effects. Furthermore, it can induce inflammatory responses that result in pneumonia, and even cause extrapulmonary manifestations. The detection of *M. pneumoniae* at extrapulmonary sites (by culture and PCR) as well as intracellular in epithelial cells (*in vitro*) suggests that *M. pneumoniae* may be able to invade the host. Chronic infection and carriage: antigenic variation, immunomodulation, and intracellular localization are hypothesized ways to achieve chronic disease state or carriage. Reproduced with permission from Meyer Sauteur et al.,[33] Wolters Kluwer Health.

CARDS toxin has been shown to bind with high affinity to surfactant protein A and to exhibit mono-ADP ribosyltransferase and vacuolating activities, which causes disruption of the respiratory epithelium in animal models.[34]

In addition to the direct damage resulting from infection by *M. pneumoniae*, the immunological response following infection generates inflammatory reactions that may cause pulmonary and extrapulmonary symptoms. More severe symptoms of CAP have been observed in older children and adolescents.[3] This suggests that the age-dependent magnitude and nature of inflammatory responses in childhood may be a major factor contributing to the development of *M. pneumoniae*-associated disease, similar to what is observed e.g. in infectious mononucleosis or rheumatic fever. In fact, the severity of *M. pneumoniae* CAP in children was closely associated with increased concentrations of interleukin (IL)-8 and IL-18 in acute phase serum and pleural fluid samples.[36] In addition, it has been demonstrated that cell-mediated immunity contributes to the pathogenesis of

*M. pneumoniae* CAP, as it was shown that the severity of CAP correlated positively with the size of a cutaneous induration following intradermal injection of *M. pneumoniae* antigens.[37] This study from 1971 described 20 patients with CAP, of which 19 were children 4–15 years of age, diagnosed by a significant rise in antibody titers against *M. pneumoniae* with CFT. The strongest skin reactions were seen in patients with severe CAP.

### Asthma

*M. pneumoniae* and other "atypical" bacteria have long been implicated in the pathogenesis of asthma.[38] There are many studies that have addressed this issue in the recent past. In an observational study on children and adults with asthma, *M. pneumoniae* infection was diagnosed in 9% of children with asthma ($n$=24/256) and was found more frequent in patients with chronic asthma (14%) than in those with asthma exacerbations (7%; $p$=0.10).[39] The diagnosis of *M. pneumoniae* infection in this study was performed by PCR and/or serology. Another recent study diagnosed *M. pneumoniae* in children with acute asthma (64%, $n$=34/53) and refractory asthma (65%, $n$=17/26), as well as in healthy controls (56%, $n$=36/64), but did not find significant differences between these three groups.[40] The high detection rates reported in this study were obtained using novel diagnostic methods (CARDS toxin enzyme-linked immunosorbent assay [ELISA] and CARDS gene-specific PCR).[40] In a recent Taiwanese study,[41] 1591 children and adults with *M. pneumoniae* infection, diagnosed by positive immunoglobulin (Ig) M or 4-fold IgG titer increase, but without prior asthma history were included from 2000–2008 and followed until the diagnosis of asthma or the end of 2011. Compared to matched 6364 patients without *M. pneumoniae* infection, the cumulative incidence of asthma was significantly higher in the *M. pneumoniae* cohort than in the control cohort ($p$<0.0001). Patients with *M. pneumoniae* infection were at higher risk of having early-onset asthma (age at asthma diagnosis <12 years) and late-onset asthma (age at asthma diagnosis ≥12 years). These most recent findings suggested that *M. pneumoniae* can induce airway inflammation and contribute to incident asthma. Interestingly, exposure to recombinant CARDS toxin resulted in an allergic-type inflammatory response and airway hyperreactivity in mice and baboons.[42,43]

#### *Extrapulmonary manifestations*

Apart from respiratory tract infections (RTIs), *M. pneumoniae* can cause extrapulmonary manifestations in almost every organ, including the skin and the hematologic, cardiovascular, musculoskeletal, and nervous systems.[15] These manifestations may be caused either by direct local effects of *M. pneumoniae*, after dissemination of the bacteria throughout the body, or indirect effects, such as autoimmune reactions (Figure 3). The most frequent manifestations are diseases of the dermatologic and nervous system.

Skin manifestations occur in up to 25% of all *M. pneumoniae* infections, including mainly non-specific exanthems, urticaria, and (less commonly) erythema nodosum. There are also rare but distinct pediatric *M. pneumoniae*-associated skin disorders such as erythema multiforme, Stevens-Johnson syndrome, and a rare but distinct disorder with prominent mucous membrane involvement denominated as *M. pneumoniae*-associated mucositis (MPAM).[44,45] This condition was first described by Fuchs in 1876,[46] and therefore also referred to as Fuchs syndrome.[47] We previously reviewed all published MPAM cases[45]: 32 patients with MPAM were identified, with a median age of 13.5 years at presentation (range 3–38 years, 23 children or young adolescents ≤18 years). All patients presented with prodromal respiratory symptoms with a median duration of 7 days, and pneumonia was found in chest radiograph in 79%. Oral lesions were present in all cases, ocular lesions in 97%, and urogenital lesions in 78%. There were no skin lesions in 69%. Although 12% of the patients were admitted to the intensive care unit, no one suffered from long-term sequelae.

Encephalitis and Guillain-Barré syndrome (GBS) constitute the most common and severe neurologic manifestations, where *M. pneumoniae* infection is established in up to 10% and 15% of patients, respectively.[48,49] Encephalitis is a disorder of the central nervous system (CNS), which presents with an altered mental status in most cases.[50] GBS is predominantly a disease of the peripheral nervous system (PNS) and manifests mainly as acute neuromuscular paralysis (polyneuropathy).[51] Encephalitis can be caused by both direct infection and an immune-mediated process,[50] while GBS is considered the prototype of a postinfectious immune-mediated disorder.[51] *M. pneumoniae* expresses adhesion proteins and glycolipids that share structural homology with a variety of host cells (molecular mimicry) and may induce cross-reactive antibodies.[3] Antibodies against *M. pneumoniae* have been found to cross-react with galactocerebroside (GalC) in encephalitis and GBS patients.[52,53] GalC is a major glycolipid antigen in the myelin sheath of both the PNS and CNS neurons.[54] In this thesis, we investigated the role of antibodies against *M. pneumoniae* in the pathogenesis of encephalitis and GBS **(Part 2)**.

### *M. pneumoniae carriage*

Like many other respiratory pathogens, *M. pneumoniae* can be carried asymptomatically in the respiratory tract (Figure 3).[55] Recent studies have demonstrated that asymptomatic carriage of *M. pneumoniae* is highly prevalent. Detection rates of *M. pneumoniae* DNA in the respiratory tract of healthy children without respiratory symptoms were 21% in a Dutch study (2008–2011, Rotterdam, The Netherlands)[56] and 56% in a U.S. study (2009–2011, San Antonio, U.S.).[40] Longitudinal sampling of *M. pneumoniae*-positive asymptomatic children demonstrated that *M. pneumoniae* can be present in the URT without causing disease, for up to 4 months.[56] The prevalence of *M. pneumoniae* in the URT of asymptomatic children varied considerably between years and seasons. For

example, asymptomatic carriage rates of 3% and 58% were reported in the spring of 2009 and the summer of 2010, respectively.[56] These data suggest that carriage follows an epidemic pattern. It is tempting to speculate that this fluctuation in prevalence is related to the cyclic epidemics of *M. pneumoniae* infections. Apart from *M. pneumoniae*, children were found to simultaneously carry many pathogens in their nose and throat.[56] These pathogens included the bacteria *S. pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Haemophilus influenzae*, and the viruses influenza virus A, human metapneumovirus, respiratory syncytial virus, parainfluenza virus, rhinovirus, coronavirus, bocavirus, and adenovirus. The simultaneous presence of two or more of these pathogens was detected in 56% of asymptomatic children.[56]

## The challenge to diagnose *M. pneumoniae* infection

### *Diagnostic tests*

Because the mere presence of *M. pneumoniae* in the URT is neither indicative nor predictive for respiratory disease, the routine diagnostic procedures to detect acute RTIs with *M. pneumoniae* need to be reconsidered. An overview of diagnostic tests with their advantages and drawbacks is shown in **Chapter 6**.

Current guidelines[57,58] recommend PCR and single-sample serological tests to diagnose *M. pneumoniae* infections. The sensitivity of specific serological tests depends on the time point of the first serum sample and on the availability of paired sera collected ≥2 weeks apart to evaluate seroconversion and/or ≥4-fold antibody titer increase as "gold standard".[59] Specific serum IgM can be detected within 1 week after initial infection and about 1–2 weeks before IgG.[60] Reinfection in adults can lead directly to an IgG response and may lack production of IgM. Specific serum IgA rises, peaks, and decreases earlier than IgM, but is less frequently detected (only in 2% of PCR-positive children with symptomatic RTI).[56] Cross-reactions with other pathogens and non-infectious diseases have been described for CFT and particle agglutination assay, but also some ELISAs lack the required sensitivity and specificity.[61] Further, it is intriguing that the detection of specifc IgM, as well as IgG and IgA by ELISA could not discriminate between the *M. pneumoniae* PCR-positive asymptomatic and symptomatic groups of children.[56] Thus, it is questionable whether or not a positive result in these tests actually indicates the etiological role of *M. pneumoniae* in all cases. Clinicians therefore need to be aware of the implications and clinical significance of a positive PCR and/or serology test result for *M. pneumoniae*.

### *Clinical assessment*

While diagnostic tests may not be reliably predictive for a symptomatic *M. pneumoniae* infection, the clinical assessment of this entity is being revisited. The British Thoracic Society (BTS) guidelines[57] recommend that bacterial pneumonia should be considered in

children when there is persistent or repetitive fever >38.5°C together with chest recession and a raised respiratory rate. A chest radiograph is not a routine investigation in children thought to have CAP. In fact, the radiographic presentation of "atypical" pneumonia due to *M. pneumoniae* is extremely variable. Bilateral, diffuse interstitial infiltrates are common, pleural effusions can occur, but none of the radiographic findings associated with *M. pneumoniae* CAP are specific.[62]

A fast-and-frugal clinical decision tree provided a rapid probability estimate of the cause of CAP in 253 children (1 months–16 years; 1997–1999, Zurich, Switzerland).[31] *M. pneumoniae* infection was diagnosed in 13% ($n$=32) of these children by PCR in respiratory specimens and serology (seroconversion and/or 4-fold rise in antibody titer). Compared with other children with CAP, patients with *M. pneumoniae* were older and had a longer duration of fever ($p$<0.001). Asking the simple question regarding the age of the child and the duration of fever allowed identification of the following group at high risk for CAP due to *M. pneumoniae*: children with CAP who have had fever >2 days and who were >3 years of age. The decision tree placed 72% of all patients with *M. pneumoniae* infection into the high-risk group. These simple rules may further aid physicians in prescribing appropriate first-line antibiotics.

## Treatment and antibiotic resistance of *M. pneumoniae*

### *Antibiotics*

In consequence of the diagnostic uncertainty for *M. pneumoniae* infections, the BTS guidelines[57] suggest empiric macrolide treatment at any age if there is no response to first-line β-lactam antibiotics or in the case of very severe disease. The lack of a cell wall makes *M. pneumoniae* resistant to cell wall synthesis inhibitors such as β-lactam antibiotics. Antibiotics effective against *M. pneumoniae* include macrolides, tetracyclines, and fluoroquinolones.[3] Although the latter two have a good *in vitro* inhibitory effect against *M. pneumoniae*, tetracyclines may cause teeth discoloration[3] and fluoroquinolones may affect the developing cartilage in young children.[63] Thus, they are not recommended by current guidelines in young children; the age limit for tetracyclines is ≥8 years, while that of fluoroquinolones is adolescence with skeletal maturity.[58] The occurrence of arthropathy due to fluoroquinolones, however, is uncertain, and all musculoskeletal adverse effects reported in the literature had been reversible following withdrawal of treatment.[63] The protein synthesis inhibitors of the macrolide class have a more favorable side effect profile and are therefore the first-line antibiotics for *M. pneumoniae* infections in children.[58]

Although antibiotics are effective against *M. pneumoniae in vitro*,[64] there is lack of evidence on their *in vivo* efficacy. Observational data indicated that children with CAP due to *M. pneumoniae* have a shorter duration of symptoms and fewer relapses when treated with an antibiotic active against *M pneumoniae*.[3,65] A recent Cochrane review[59]

evaluated seven studies on the effectiveness of antibiotic treatment for *M. pneumoniae* LRTIs in children. However, the diagnostic criteria, the type and duration of treatment, inclusion criteria, and outcome measures differed significantly, making it difficult to draw any specific conclusions – although one trial suggested that macrolides may be efficacious in some cases.[66] It is clear that studies on the efficacy of antibiotics rely on a correct diagnosis of *M. pneumoniae* infections. Given the aforementioned shortcomings of current diagnostic tests, conclusions on the efficacy of antibiotic treatment will have to be re-examined.

### *Antibiotic resistance*

Since 2000, the extensive macrolide use led to an alarming worldwide increase in the prevalence of macrolide-resistant *M. pneumoniae* (MRMP) strains.[64] Resistance is based on specific point mutations in domain V of the *23S rRNA* (at positions 2063, 2064, and 2617), which reduce the affinity of macrolides to the large subunit (50S) of the bacterial ribosome.[64] MRMP has been observed during macrolide treatment as a result of antibiotic selective pressure.[67-69] To date, macrolide resistance has been detected on a worldwide scale. MRMP had developed in Asia,[70] where MRMP rates have risen as high as 97% in China.[71] MRMP has now also been reported from North America and Europe, and we recently reported the first MRMP infection in a child in Switzerland (Figure 4).[72]

The clinical relevance of macrolide resistance in hospitalized children with CAP may be a longer duration of symptoms.[73,82,83] Zhou et al.[83] found that an increase in MRMP may also have serious clinical consequences in children, leading to more severe radiological findings of CAP and even an increase in extrapulmonary manifestations. In this study, hospitalized children with CAP due to MRMP developed more often extrapulmonary disease than children with CAP caused by macrolide-sensitive strains (30% vs. 10%; $p$=0.03).[83] These manifestations included skin diseases and nervous system complications in 18% and 7%, respectively, of the MRMP-infected children. Serum inflammatory cytokine levels (INF-γ, IL-6, and IP-10) were higher in patients infected with MRMP than in patients infected with macrolide-sensitive strains.[84] This suggests that the higher and more persistent inflammatory stimulation by MRMP may increase the possibility of severe lung lesions and extrapulmonary complications.

### *Vaccines*

Apart from the discovery of novel antibiotics against *M. pneumoniae*, the development of vaccines against *M. pneumoniae* may play a crucial role in the control and/or eradication of this pathogen. While previous attempts to produce vaccines on the basis of inactivated bacteria resulted in limited efficacy against pneumonia and various adverse effects,[85] the recent use of recombinant proteins as potential vaccines was found to be promising. Specifically, the immunization of mice with a recombinant peptide encompassing the

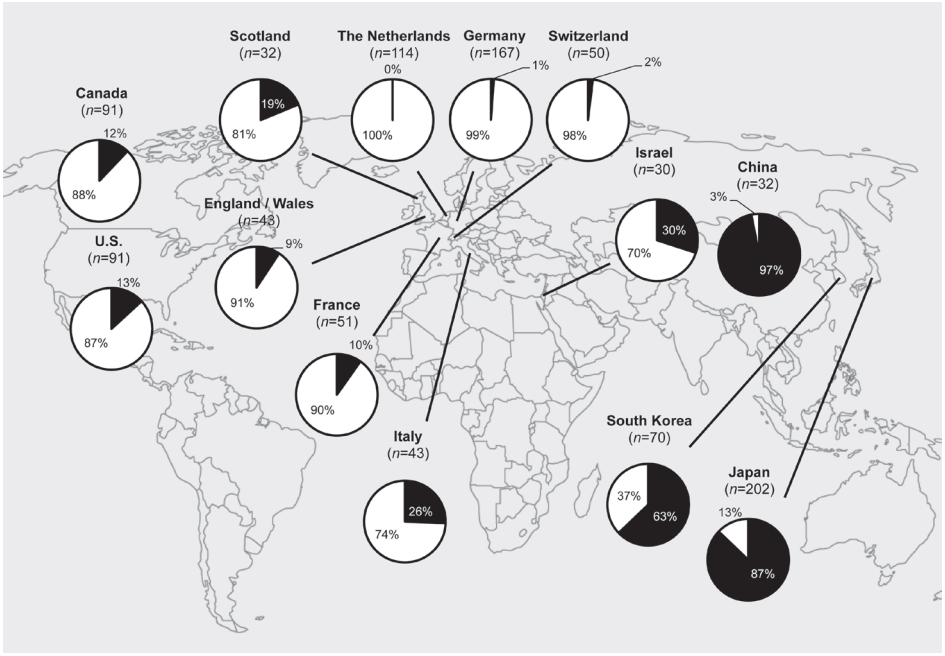


**Figure 4.** Worldwide macrolide-resistant *M. pneumoniae* (MRMP) rates.
Actual MRMP rates are punctually depicted in pie charts (in black) over the world map.[12] Asia: Japan (2011): 87% (*n*=176/202),[73] South Korea (2011): 63% (*n*=44/70),[70] China (2012): 97% (*n*=31/32),[71] Israel (2010): 30% (*n*=9/30);[74] North America: U.S. (2012–2014): 13% (*n*=12/91),[75] Canada (2010–2012): 12% (*n*=11/91);[76] Europe: The Netherlands (1997–2008): 0% (*n*=0/114),[77] Germany (2003–2008): 1% (*n*=2/167),[78] France (2005–2007): 10% (*n*=5/51),[79] Italy (2010): 26% (*n*=11/43),[67] Scotland (2010–2011): 19% (*n*=6/32),[80] Switzerland (2011–2013): 2% (*n*=1/50),[72] England and Wales (2014–2015): 9% (*n*=4/43).[81] Reproduced with permission from Meyer Sauteur et al.,[12] Frontiers.

C-terminal part of the immunodominant P1 protein (RP14) induced strong mucosal and systemic antibody responses against *M. pneumoniae* as well as reduced lung inflammation.[86] In another study, it was shown that immunization of guinea pigs with a chimeric protein consisting of RP14 and the P30 adhesion protein of *M. pneumoniae* resulted in a robust antibody response, and, subsequently, in lower bacterial loads in the respiratory tract.[87] These studies showed that vaccination indeed present a future alternative to antibiotics in the combat against *M. pneumoniae* **(Chapter 3)**. Development of a *M. pneumoniae*-specific vaccine for high-risk individuals such as young children and elderly people may help to reduce morbidity from pneumonia and secondary complications as well as reduce horizontal transmission. Furthermore, the development of antibiotic-resistant strains will be reduced.

## INTRODUCTION TO ANTIBODY RESPONSES

B cell responses are of crucial importance for the host defense against multiple pathogens and have a variety of functions during the development of immune responses to infection. While B cells are known to be involved in clearance of *M. pneumoniae* from the lungs, their role in *M. pneumoniae* carriage and *M. pneumoniae*-associated extrapulmonary disease is unknown.

### Role of B cells in defense against pathogens

B cells produce antibodies after being triggered and activated by antigen and having undergone differentiation into antibody-secreting cells (ASCs). Antibody-mediated immunity is achieved by binding of antibodies to the surface of pathogens resulting in (i) neutralization (inhibiting growth), (ii) opsonisation (phagocytic recognition), (iii) complement activation (deposition of complement and subsequent lysis), (iv) tagging (destruction through antibody-dependent cell-mediated cytotoxicity), (v) coating (prevention of bacterial adherence), and (vi) agglutinating (more effective recognition and mechanical clearance).[88]

### B cell development

B lymphocytes develop in bone marrow (and in fetal liver during gestation), independent of antigen contact, from a multipotent progenitor cell to an immature B cell (Figure 5).[89] The different developmental stages of B cell development (pro-B cell, pre-B cell, and immature B cell) are determined by the functional rearrangement of the Ig heavy and light chain segments of the B cell receptor (BCR) genes (see below).

Defects in early B cell development result in agammaglobulinemias, which are rare antibody deficiencies characterized by low numbers or absence of B cells, marked hypogammaglobulinemia, and increased susceptibility to infections (see below).[89]

B cells leave the bone marrow (primary lymphoid organ) at the transitional B cell stage and complete their final development into mature B cells in the periphery (secondary lymphoid organs).[89] Mature B cells can be divided into three major subsets: follicular B cells, marginal zone B cells, and B-1 cells.[90] Follicular B cells are the dominant subset and they are located in the lymphoid follicles of the spleen and lymph nodes. Marginal zone B cells, by contrast, border on the marginal sinus of the spleen, where they are ideally located to encounter blood-borne pathogens and particulate antigens. B-1 cells are located mostly in the peritoneal and pleural cavities and at mucosal sites, which facilitates their surveillance of the tissues that are the most susceptible to environmental pathogens. B-1 cells produce non-specific antibodies (natural antibodies) that are synthesized early in the immune response and are predominantly of the isotype IgM.[91] They can be distin-

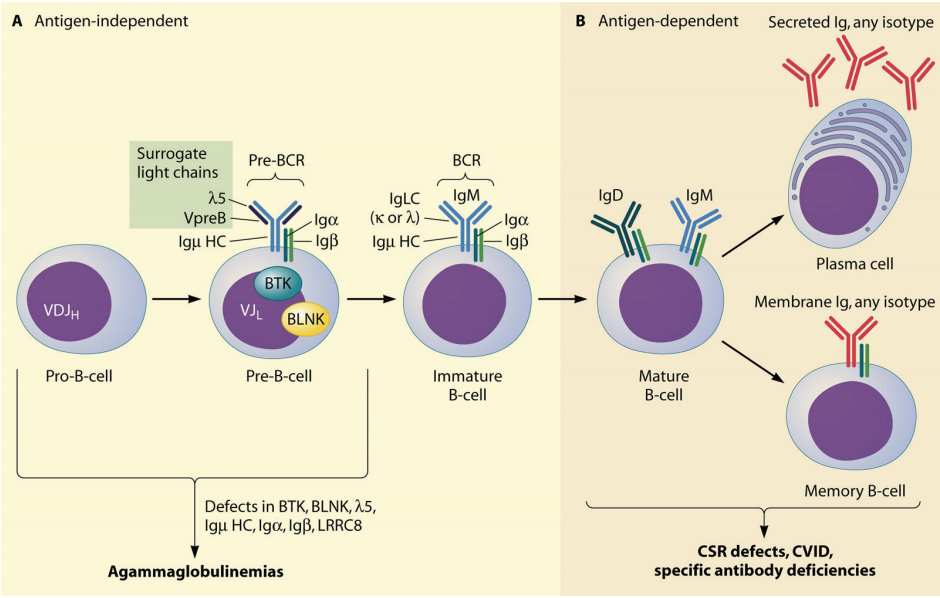


**Figure 5.** Overview of B cell development and defects causing antibody deficiency.
(A) Antigen-independent B cell development, through (i) $VDJ_H$ rearrangement of the IgM (μ) heavy chain (by which the pre-BCR is assembled together with components of the surrogate light chains VpreB and λ5 and signaling molecules Igα and Igβ) and (ii) $VJ_L$ rearrangement of the IgM (μ) light chain (by which the BCR is formed). (B) Antigen-dependent B cell differentiation, after which B cells exit the germinal centers of secondary lymphoid organs as antibody-secreting plasma cells or memory B cells. Abbreviations: BCR, B cell receptor; BLNK, B cell linker protein; BTK, Bruton tyrosine kinase; CSR, class switch recombination; CVID, common variable immunodeficiency; HC, heavy chain; Ig, immunoglobulin; LC, light chain; LRRC8, leucine-rich repeat containing 8 protein. Reproduced with permission from Fried et al.,[89] American Society for Microbiology.

guished by their recognition of self-antigens and those with repetitive epitopes such as carbohydrates.

Defects in later stages of B cell differentiation result in diseases with different degrees of disrupted antibody production, e.g., class switch recombination (CSR) defects, common variable immunodeficiency (CVID), and specific antibody deficiencies.[89]

## B cell activation

### *T cell-dependent*

After BCR stimulation by antigen, marginal zone and follicular B cells become activated B cells and migrate to the border of the follicle.[92] At this site the activated B cells receive signals from antigen-specific $CD4^+$ T cells (e.g., via CD40 and various cytokine receptors) (Figure 6), which trigger proliferation of B cells. Subsequently, B cells either migrate out of the follicle and become short-lived ASCs (plasmablasts) or migrate into the follicle and

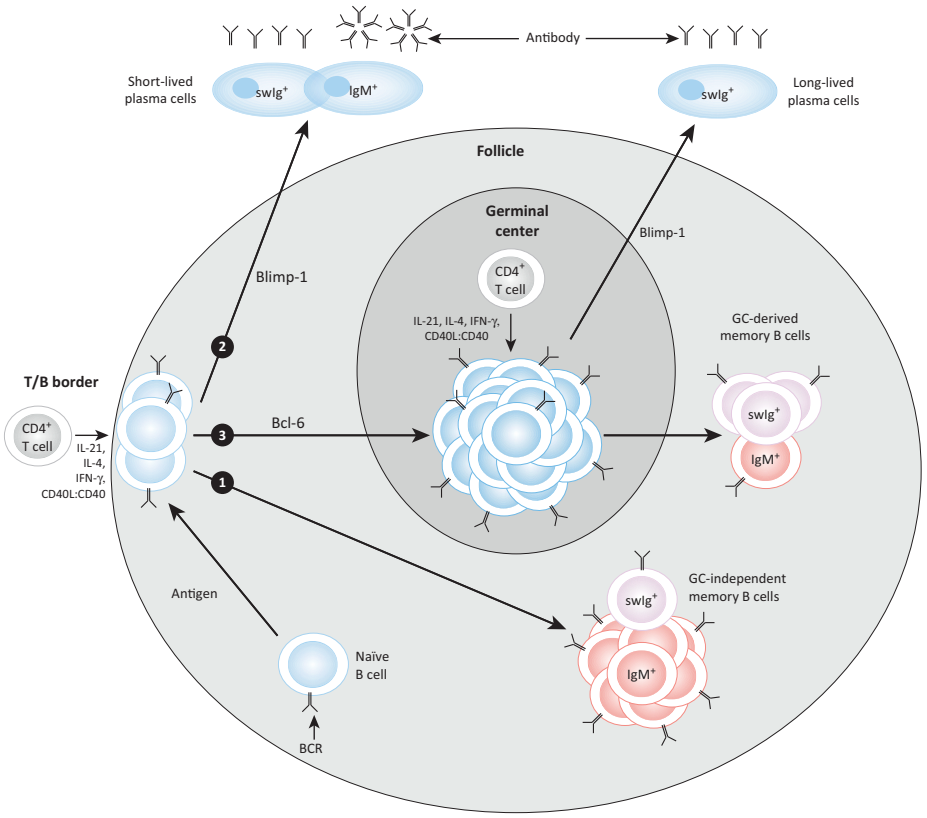


**Figure 6.** B cell differentiation in response to antigen in secondary lymphoid organs.
Abbreviations: Bcl-6, B cell lymphoma 6 protein; BCR, B cell receptor; Blimp-1, B lymphocyte maturation protein-1; Ig, immunoglobulin; swIg, isotype switched Ig. Reproduced with permission from Taylor et al.,[92] Elsevier.

become long-lived plasma cells or memory B cells.[90] In the germinal center (GC), the site where CSR and somatic hypermutation (SHM) occurs, follicular B cells proliferate and undergo $CD4^+$ T cell-dependent affinity-maturation. Cells that receive $CD4^+$ T cell signals exit the GC as long-lived plasma cell or memory B cell.[90] Failure to receive signals from $CD4^+$ T cells results in apoptosis. Long-lived plasma cells reside in survival niches in the bone marrow, even after the antigen has been eliminated, and continuously secrete protective antibodies of a given specificity. Reactivation by antigen leads to differentiation of memory B cells into plasmablasts that are competent to become long-lived plasma cells.[90]

### *T cell-independent*

Non-protein antigens (lipo-/polysaccharides, glycolipids, and nucleic acids) stimulate B cell responses in the absence of helper T cell activation (thymus-independent [TI]

antibody responses) as they cannot be presented to $CD4^+$ T cells in the context of major histocompatibility complex (MHC) molecules.[92] There are two classes of TI antigens (i.e., TI-1 and TI-2). TI-1 antigens (e.g., bacterial lipopolysaccharide or nucleic acids) can trigger B cell activation through their Toll-like receptor (TLR).[93] TI-2 antigens (e.g., bacterial polysaccharides or glycolipids) extensively crosslink BCRs and deliver a prolonged and persistent signal to the B cell, transmitted via Bruton tyrosin kinase (Btk).[94] TI antibody responses are characterized by a fast production of lower-affinity antibodies with limited isotype switching.

## B cell receptor and antibodies

The BCR is located on the outer surface of B cells and is composed of a membrane-bound Ig molecule (mature naïve B cells: IgM, IgD; memory B cells: IgM, IgG, IgA, or IgE) and a signal transduction moiety (Igα/Igβ heterodimer, CD79) (Figure 5). Apart from the presence of the integral membrane domain, the Igs of the BCR are identical to their secreted soluble forms (i.e., antibodies).

### *VDJ recombination*

The Ig molecule consists of paired heavy chains (IgH) and light chains (IgL). Both IgH and IgL contain variable (V) and constant (C) regions (Figure 7). The amino-terminal V

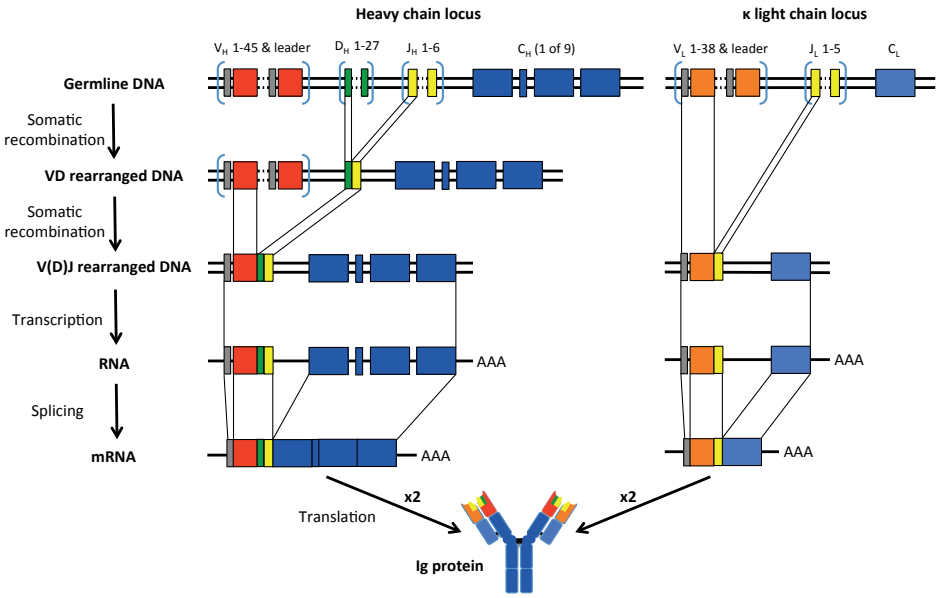


**Figure 7.** Overview of formation and structure of antibodies.
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region of IgH is encoded by variable ($V_H$), diversity ($D_H$), and joining ($J_H$) gene segments on chromosome 14. The V region of IgL contains only variable ($V_L$) and joining ($J_L$) gene segments. There are two types of IgL in humans, kappa (κ) and lambda (λ), which are determined by the $C_L$ region. Only one type of IgL (κ or λ) is present in a particular antibody. Genes encoding the V region of IgL are located on chromosome 2 (κ) and chromosome 22 (λ), respectively. V regions of both IgH ($V_H$, $D_H$, and $J_H$) and IgL ($V_L$ and $J_L$) form the antigen-binding part of the antibody (Fragment antigen-binding, Fab).[89]

During B cell development in bone marrow, initial diversity is generated by VDJ recombination. Gene rearrangement follows a sequential order, starting with recombination of the $V_H$, $D_H$ (IgH only), and $J_H$ gene segments of the IgH locus, followed by $V_L$ and $J_L$ rearrangement of the IgL locus. In addition to randomly assembling different V to D to J segments, which form the basis of antibody function to bind foreign antigen, further diversity develops by random removal and addition of nucleotides at the recombination sites (junctional diversity).

*B cell receptor (BCR)*

Productive IgM (μ) heavy chain recombination leads to the formation of the pre-BCR, which is covalently associated with an IgL-like molecule called the surrogate light chain.[95] The expression of the pre-BCR induces progression from the pro-B cell stage to the pre-B cell stage (Figure 5).[96] Immature B cells subsequently express a BCR.[97]

In the absence of the μ heavy chain the pre-BCR cannot be formed, resulting in a block in B cell development at the pro-B cell stage. This is shown in μMT mice that have an inactivating mutation of the membrane exon of the IgM (μ) heavy chain gene.[98] **μMT mice** were used in **Chapter 2** to investigate the outcome of *M. pneumoniae* infection and carriage in the absence of mature B cells.

BCR signals are directly linked to B cell survival via Btk (Figure 5), through activation of the transcription factor nuclear factor (NF)-κB.[99-101] Mutations in the *Btk* gene result in X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice.[102] The resulting pre-B cell developmental block is almost complete in XLA, but limited in *xid*. **$Btk^-$ mice**, which we used in **Chapter 3**, have severely reduced (>50%) mature B cells, absent B-1 cells, and low serum IgM and IgG3 levels.[103,104] Despite being less in numbers, the mature B cells in $Btk^-$ mice show normal T cell-dependent antibody responses to model antigens.[94] Moreover, the lack of Btk in B cells makes these mice hyporesponsive to certain TI antigens.[94]

*Class switch recombination (CSR) and somatic hypermutation (SHM)*

Contact with antigen and T cells in peripheral lymphoid organs initiate further diversification of antibodies through CSR for the acquisition of different Ig isotypes, SHM for increased antigen specificity, and selection of B cells for improved antigen binding.[105]

The GCs are the primary sites for CSR, SHM, and B cell memory and plasma cell generation (Figure 6). CSR targets the $C_H$ region, which is organized by distinct exon clusters in the order Cμ, Cδ, Cγ, Cε, and Cα. CSR results in DNA replacement of the expressed $C_H$ exon cluster (e.g., Cμ for IgM) with Cγ for IgG, Cα for IgA, or Cε for IgE. The antigen-binding V region is unaltered after CSR. IgM and IgD is generated not through CSR, but through alternative splicing of the primary (germline) transcripts encoded by the Cμ and Cδ genes.

SHM inserts point mutations into the V region of IgH and IgL at a high rate to provide structural substrate for positive selection of higher affinity antibodies by antigen.

*Immunoglobulin (Ig) isotypes*

Ig classes or isotypes have distinct tissue distribution and efficacy against different types of pathogens. IgM (pentamer or hexamer, 960 kDa) proteins have a large size and cannot pass the vascular endothelium into the extravascular space, whereas IgG (monomer, subclasses IgG1–4, 150 kDa), IgE (monomer, 200 kDa), and IgA (monomer or dimer, subclasses IgA1–2, 320 kDa) molecules can be distributed systemically to tissues. During infections in humans, IgM is part of the primary immune response, mediates complement activation, and is effective against most microbial pathogens (high avidity for antigens with repetitive patterns), whereas IgE is effective against large extracellular parasites, and IgA predominates in the combat against mucosal pathogens. The expressed subclass (IgG1–4) of the IgG isotype depends on the signals that pathogens provide to dendritic cells and/or B cells.[105]

*Antibody-mediated (autoimmune) disease*

B and T cells are rendered non-reactive to self by central tolerance in bone marrow (B cells) or thymus (T cells) and by peripheral tolerance in secondary lymphoid organs. Negative selection eliminates avid B and T cells reactive with self-antigens in bone marrow and thymus, respectively.[106] Autoimmune diseases are a diverse group of disorders characterized by abnormal immune reactivity in association with autoreactive B and T cell responses. Multiple factors are thought to contribute to the development of immune response to self, including genetic susceptibility, environmental factors, and hormonal milieu.[107] Of all environmental factors infectious pathogens have the most potential to trigger autoimmunity. There are several mechanisms through which pathogens can initiate or perpetuate autoimmunity (Figure 8).

Microbial antigens sometimes resemble self and therefore trigger BCRs and T cell receptors (TCRs). Such cross-reactions underlie the concept of molecular mimicry, which is defined as dual recognition of structures of a microorganism and host by a single BCR or TCR.[108] Hence, molecular mimicry is a potential mechanism by which infections can trigger cross-reactive antibodies or T cells that cause autoimmune disease, e.g., acute

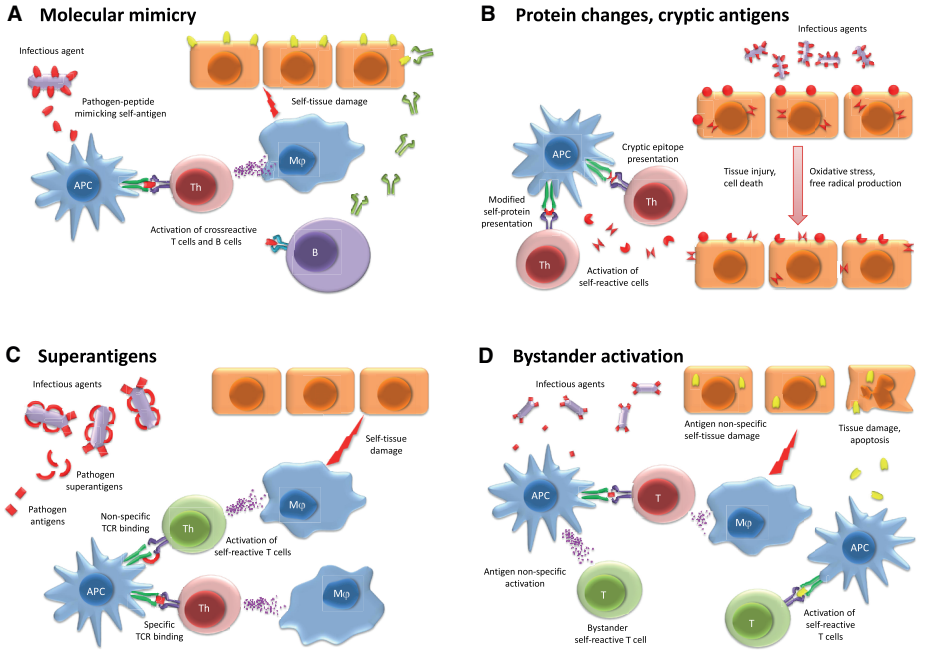


**Figure 8.** Autoimmune mechanisms triggered by infections.
(A) Molecular mimicry: Microbial structures share similarity in amino acid sequence or structure to self-antigen. As a result of cross-reactivity, leading to the activation of naïve, autoreactive T cells specific to the corresponding self-molecule, the immune response can eventually turn toward the self-peptide/-tissue. (B) Protein changes and cryptic antigens: Infection causes tissue injury (cell death, oxidative stress, free radical production, and reparative changes) and proteins that are usually recognized as self can become antigenic and induce an autoimmune response. In addition, proteins that are normally sequestered and shielded from immune recognition ("cryptic") can be exposed to the immune system. These antigens become accessible to (self-reacting) T cells as they had not been presented appropriately to induce central and peripheral tolerance. (C) Superantigens: Superantigens are proteins produced by a variety of microorganisms, or virus-infected cells that can bind a TCR irrespective of its antigenic specificity, resulting in the activation of a large number of T cells of different antigenic specificity, thus behaving as a potent immune-stimulating molecule. (D) Bystander activation: Infection can result in enhanced processing and presentation of self-antigens, which induces the expansion and/or spreading of the immune response toward different self-antigens ("epitope spreading"). Abbreviations: APC, antigen-presenting cell; Mø, macrophage; Th, T helper cell. Reproduced with permission from Sfriso et al.,[107] John Wiley and Sons.

rheumatic fever and GBS. We also investigated if *M. pneumoniae* epitopes trigger the production of antibodies that react with host antigens (glycolipids) and cause disease (encephalitis and GBS) **(Part 2)**.

## HYPOTHESES, OBJECTIVES, AND AIMS

Despite the often described benign nature of *M. pneumoniae* infections, cases of fulminant pneumonia[14] and/or severe extrapulmonary manifestations[3] have been reported. In light of the global increase in antibiotic resistance of *M. pneumoniae*, other strategies in the combat against *M. pneumoniae* are needed. Vaccination may be a promising alternative to antibiotics. However, to develop optimal approaches to vaccination against *M. pneumoniae* it is critical to understand the immune mechanisms that may contribute to protection and/or immunopathology. In particular, it is important to clarify the role of B cells and antibodies in *M. pneumoniae* carriage and *M. pneumoniae*-associated disease as (i) no data exists regarding humoral immunity against *M. pneumoniae* carriage, and (ii) antibody-mediated pathogenesis has been suggested in extrapulmonary diseases.

### Hypotheses

*Overall*

We hypothesized that antibodies to *M. pneumoniae* protect against pulmonary infection but trigger extrapulmonary nervous system disease.

*1. Antibody responses to M. pneumoniae: protecting against disease*

We hypothesized that antibodies to *M. pneumoniae* are essential for resolution of pulmonary infection but are not effective in clearing carriage from the URT. Given its essential role in protection against disease the detection of B cell responses may be a diagnostic indicator for pulmonary infection.

*2. Antibody responses to M. pneumoniae: triggering disease*

We hypothesized that antibodies against galactocerebroside (GalC), a major myelin glycolipid of both the PNS and CNS, are critical for development of nervous system disease following *M. pneumoniae* infection, and investigated whether encephalitis and GBS after this type of preceding infection presented with distinct clinical features and outcome.

### Aims and objectives

*Overall*

To determine the role of antibodies to *M. pneumoniae* in respiratory tract carriage, pulmonary infection, and extrapulmonary nervous system disease.

*1. Antibody responses to M. pneumoniae: protecting against disease*

– To develop a C57BL/6 mouse model for *M. pneumoniae* infection and carriage, which resembles the human situation;

- To investigate the role of antibodies in carriage and clearance of *M. pneumoniae* from the respiratory tract of mice;
- To determine which antigenic structures of *M. pneumoniae* are recognized by specific antibodies in mice and children;
- To investigate if specific B cell responses (antibodies and/or antibody-secreting cells [ASCs]) differentiate *M. pneumoniae* infection from carriage, and thereby optimize current PCR-based diagnostic testing.

### *2. Antibody responses to M. pneumoniae: triggering disease*

#### 2.1 Encephalitis

- To investigate the role of antibodies to *M. pneumoniae* in pathogenesis and diagnosis of childhood encephalitis;
- To assess the intrathecal antibody response to *M. pneumoniae* in children with encephalitis;
- To identify potential myelin targets of antibodies to *M. pneumoniae* in childhood encephalitis;
- To evaluate the presence of pediatric *M. pneumoniae*-associated encephalitis in a national prospective surveillance study;

#### 2.2 Guillain-Barré syndrome

- To determine the relation of *M. pneumoniae* with GBS in a large case-control study, the first study in both adults and children;
- To assess clinical features and outcome in adult and pediatric GBS triggered by *M. pneumoniae*;
- To investigate the antibody response to *M. pneumoniae* in a series of children with severe and complicated disorders within the GBS spectrum after *M. pneumoniae* infection;
- To identify potential myelin targets of antibodies to *M. pneumoniae* in adult and pediatric patients with GBS triggered by *M. pneumoniae*;
- To demonstrate cross-reactivity of anti-GalC antibodies found in adult and pediatric patients with *M. pneumoniae* GBS;
- To determine the role of anti-GalC antibodies in serum and cerebrospinal fluid (CSF) in adult and pediatric GBS.

## OUTLINE

This thesis contains two general parts, i.e., **Part 1** on protective antibody responses and **Part 2** on antibody responses triggering disease. **Part 1** contains studies about kinetics and targets of *M. pneumoniae*-specific antibodies following *M. pneumoniae* infection in mice and children. **Chapter 1** introduces current knowledge about *M. pneumoniae* and B cell responses. In **Chapter 2** we describe the role of B cells and antibodies in *M. pneumoniae* respiratory tract carriage and infection in a newly developed mouse model. This mouse model provided an excellent tool to study *M. pneumoniae*-specific immune responses in the URT, which has not been addressed in previous *in vivo* studies. In **Chapter 3**, target structures of *M. pneumoniae*-specific antibodies are determined and the contribution of such particular antibodies in protecting against RTI is described. In **Chapter 4** we investigated specific B cell responses (antibodies and ASCs) as diagnostic indicator for pulmonary infection in children and evaluated its potential to optimize *M. pneumoniae* diagnosis.

In **Part 2**, the focus is shifted from the respiratory tract (protecting antibody response) towards the nervous system (disease-triggering or pathogenic antibody response). **Part 2.1** addresses the extrapulmonary CNS manifestation encephalitis. **Chapter 5** describes first the intrathecal antibody response to *M. pneumoniae* in encephalitis patients. **Chapter 6** is a review that discusses the role of antibodies in pathogenesis and diagnosis of *M. pneumoniae*-associated encephalitis. Based on the discussion in Chapter 6, potential myelin targets of antibodies to *M. pneumoniae*, including GalC, were assessed in encephalitis in **Chapter 7**. As final step of Part 2.1 about encephalitis, **Chapter 8** presents epidemiological and clinical data of *M. pneumoniae*-associated encephalitis and its pathogenesis in a national prospective surveillance study.

**Part 2.2** is about the PNS manifestation GBS. **Chapter 9** describes a first case series of children with severe and complicated disorders within the GBS spectrum after infection with *M. pneumoniae*, presenting clinical data, outcome, and antibody responses to *M. pneumoniae*. **Chapter 10** covers our primary case-control study, the first study in both adults and children, in which we determine the relation of *M. pneumoniae* with GBS in a large number of cases. This study also investigated whether GBS after this type of preceding infection presents with characteristic clinical features, outcome, and antibody responses to glycolipids, including GalC. At the end of Part 2.2, **Chapter 11** and **Chapter 12** present data about intrathecal antibodies to GalC in patients of the case-control study (Chapter 10) and the case series (Chapter 9).

Finally, in **Chapter 13**, the overall findings and implications of the studies described in this thesis are discussed. **Chapter 14** provides a summary of the thesis.

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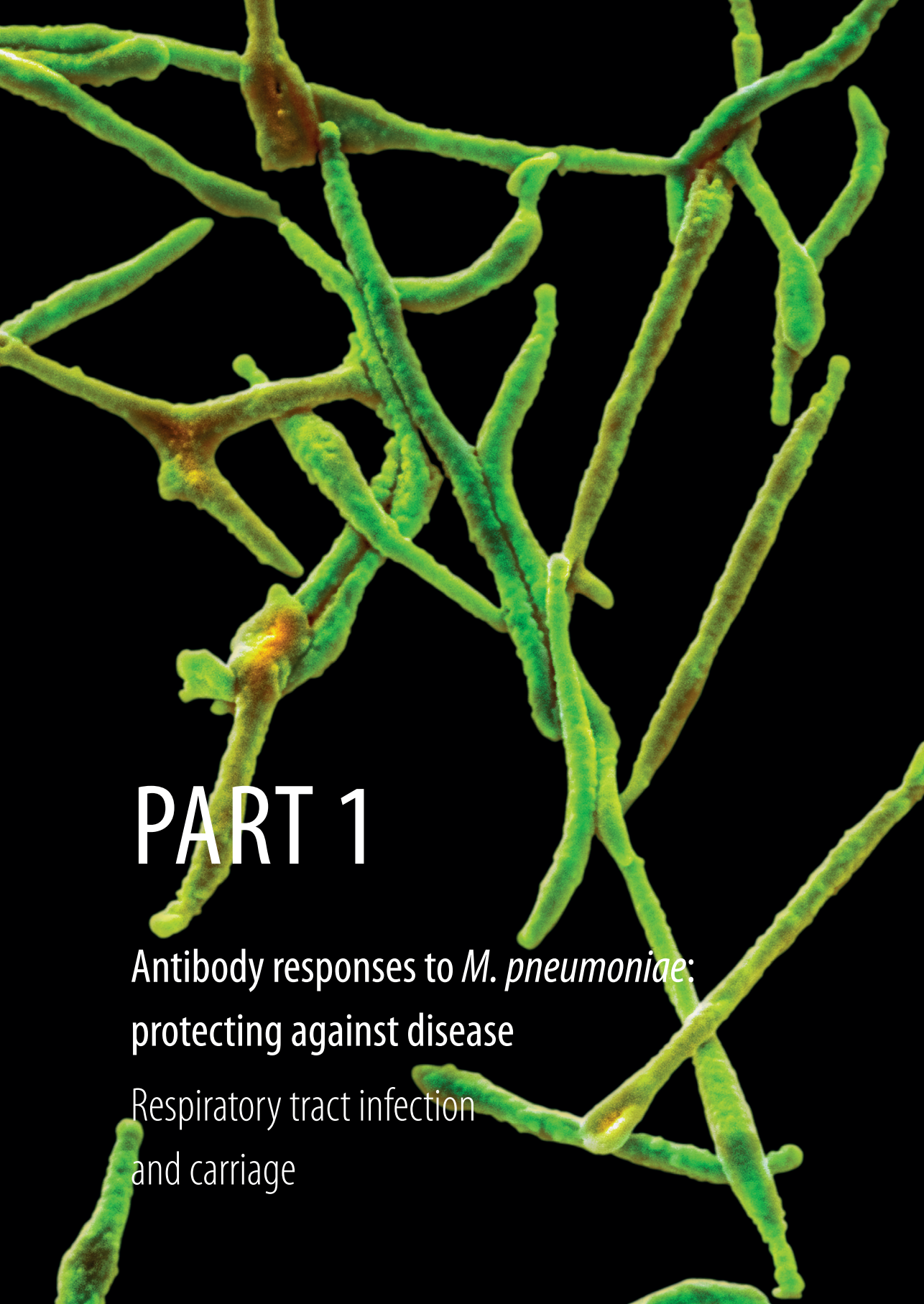

# PART 1

## Antibody responses to *M. pneumoniae*: protecting against disease

Respiratory tract infection and carriage

# Chapter 2

## The role of B cells in carriage and clearance of *Mycoplasma pneumoniae* from the respiratory tract of mice

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## ABSTRACT

### Background

Carriage of *Mycoplasma pneumoniae* in the nasopharynx is considered a prerequisite for pulmonary infection. It is interesting to note that *M. pneumoniae* carriage is also detected after infection. Although B cells are known to be involved in pulmonary *M. pneumoniae* clearance, their role in *M. pneumoniae* carriage is unknown.

### Methods

In this study, we show in a mouse model that *M. pneumoniae* persists in the nose after pulmonary infection, similar to humans.

### Results

Infection of mice enhanced *M. pneumoniae*-specific immunoglobulin (Ig) M and IgG levels in serum and bronchoalveolar lavage fluid. However, nasal washes only contained elevated *M. pneumoniae*-specific IgA. These differences in Ig compartmentalization correlated with differences in *M. pneumoniae*-specific B cell responses between nose- and lung-draining lymphoid tissues. Moreover, transferred *M. pneumoniae*-specific serum Igs had no effect on nasal carriage in B cell-deficient μMT mice, whereas this enabled μMT mice to clear pulmonary *M. pneumoniae* infection.

### Conclusions

We report the first evidence that humoral immunity is limited in clearing *M. pneumoniae* from the upper respiratory tract.

## INTRODUCTION

*Mycoplasma pneumoniae* is a frequent cause of childhood community-acquired pneumonia (CAP) worldwide,[1] and currently is the most commonly detected bacterial cause of CAP in hospitalized children in the U.S.[2] We and others observed that besides causing upper respiratory tract (URT) and lower respiratory tract (LRT) infections, *M. pneumoniae* is also carried in the URT of asymptomatic children.[3-7] Carriage is considered a prerequisite for infection.[3,8-10] Interestingly, carriage also occurs following symptomatic infection.[11] Several studies in humans demonstrated the presence of *M. pneumoniae* in the nasopharynx up to eight months after respiratory tract infection.[3-7]

B cells are of crucial importance for the host defense against multiple pathogens, as B cell activation can result in the production of specific immunoglobulins (Igs) that neutralize the pathogen.[12] Following an initial *M. pneumoniae* respiratory tract infection *M. pneumoniae*-specific IgM is detectable in serum within one week, and increased amounts of *M. pneumoniae*-specific IgG can be found from two weeks postinfection (p.i.).[13,14] *M. pneumoniae*-specific IgA is produced early after infection and its levels both peak and decrease earlier than IgM or IgG.[15] Indeed, B cells are known to be involved in pulmonary mycoplasma clearance.[16-20] Furthermore, patients with hypogammaglobulinemia are at greater risk for *M. pneumoniae* pulmonary disease and/or extrapulmonary manifestations.[21-25] It is likely that carriage of *M. pneumoniae* by these patients make them susceptible to the development of symptomatic *M. pneumoniae* infection.

In contrast to the known important role of B cells in clearance of a pulmonary infection, limited knowledge exists on the role of B cells in the control of *M. pneumoniae* carriage in the URT. Therefore, we set out to determine whether humoral immunity contributes to clearance of *M. pneumoniae* carriage from the URT. Since this is difficult to study in human subjects, we further developed a C57BL/6 mouse model to study *M. pneumoniae* (strain M129) infections and the role of humoral immunity on control of these infections.

## METHODS

### Mice

C57BL/6 mice were purchased from Charles River Laboratories and used at 8–12 weeks of age. μMT transgenic mice (C57BL/6×FVB×129)[26] were bred and housed in the animal facilities of the Erasmus MC under specific pathogen-free conditions. All experiments were approved by the Animal Experiments Committee of the Erasmus MC, Rotterdam, The Netherlands.

### Bacteria

*M. pneumoniae* reference strain M129 (subtype 1, ATCC 29342) was cultured at 37°C/5% $CO_2$ in SP4 medium (pH 7.8–8.0) for 72 hours and concentrated in fresh SP4 medium to $1\times10^{9-10}$ colony-forming units (CFUs)/ml (Supplementary Data). To track *M. pneumoniae*, bacteria were labeled with 10 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions (Molecular Probes).

### *M. pneumoniae* infection and carriage mouse model

Mice were inoculated intranasally with $1\times10^9$ CFU of M129 diluted in 50 µl SP4 medium. Control mice were inoculated with 50 µl SP4 medium. On indicated days after *M. pneumoniae* inoculation, body weight was measured and mice were sacrificed. Lung washes and nasal fluid samples (washes and brushes) were collected. Cells from lungs, lymph nodes, and nasal passages (mucosa and nasal-associated lymphoid tissue [NALT][27]) were isolated. Detailed methods for sample procedures are included in Supplementary Data.

### Serum transfer

Sera were collected and pooled from wild-type (WT) and µMT mice on day 14 p.i., diluted with PBS (1:1.3), and 200 µl was injected intravenously into each WT and µMT recipient mice at day 15, 16, and 22 p.i. One week after the last serum transfer, recipient mice were sacrificed. Sera were devoid of *M. pneumoniae* as confirmed by a negative PCR for *M. pneumoniae*.[28]

### *M. pneumoniae* quantification

The presence of *M. pneumoniae* was detected either by PCR[28] or culture of bronchoalveolar lavage fluids (BALFs) and nasal fluid samples (100 µl undiluted and 10-fold diluted) on SP4 agar plates at 37°C. Suspensions of spleen, liver, and immersed cotton swabs of articular surfaces were processed in a similar fashion.

### *M. pneumoniae* phagocytosis and killing

*M. pneumoniae* M129 was incubated with sera from infected WT mice. As control, sera from uninfected mice were used. Murine RAW 264.7 macrophages were incubated with the respective bacteria for 20 min, washed, and at time point 0 and 180 min, cells were lysed to determine bacterial load (CFU/$10^5$ RAW cells) by culture on SP4 agar plates.

### Histopathology scoring

Histopathology scoring (HPS) of lung sections was performed without knowledge of the treatment or time after infection of mice. The modified HPS system[29] assigns values from 0 to 10 by grading immune cell infiltrates in alveoli and bronchi for distribution, severity

of inflammation, presence of necrosis, and presence of perivascular, peribronchial, and peribronchiolar cuffing (Supplementary Data).

### Quantification of antibodies

Total antibody concentrations (μg/ml) and *M. pneumoniae*-specific antibodies (arbitrary units [AU]/ml) were quantified by ELISA as described in Supplementary Data.

### IgA ASC ELISpot

The frequency of *M. pneumoniae*-specific and total IgA-producing antibody-secreting cells (ASCs) in mediastinal lymph nodes (MLNs), cervical lymph nodes (CLNs), and nasal passages was measured by enzyme-linked immunospot (ELISpot) assay using a commercially available kit (Mabtech), as described in Supplementary Data.

### Flow cytometry

Aliquots of single-cell suspensions were incubated with appropriate dilutions of antibodies for 30 min on ice. Cells were subsequently analyzed using a FACS Canto II (BD Biosciences) and FlowJo 10 software (Tree Star). Monoclonal antibodies and the gating strategy are described in Supplementary Figure S1.

### Cytokine analysis

Cytokines were measured in BALF and nasal lavage fluid by a multiplex fluorescent-bead-based immunoassay (Procarta multiplex, eBioscience).

### Statistical analysis

R software environment (version 3.4.0) and GraphPad Prism (version 5.01) were used for statistical analysis. The Welch's *t*-test, Mann-Whitney *U* test, Kruskal-Wallis test with post hoc Dunn's multiple comparisons test of selected pairs, Fisher exact test or $\chi^2$ test were used to determine statistical significance. Family-wise error rates were controlled using the Holm-Bonferroni Method. Association between paired samples was tested using the Pearson product-moment correlation coefficient. Statistical significance was defined as $p<0.05$.

## RESULTS

### *M. pneumoniae* persists in the nose after pulmonary infection

To unravel the role of B cells in *M. pneumoniae* pulmonary infection and nasal carriage, we further developed a mouse model for *M. pneumoniae* infection using the *M. pneumoniae* reference strain M129 (subtype 1) in C57BL/6 mice.[30-32] Intranasal inoculation of anaesthetized C57BL/6 mice with $1\times10^9$ CFU of *M. pneumoniae* M129 resulted in a mean

relative body weight loss of 4% within three days (Figure 1A). After two weeks, infected mice returned to their initial weight and from then on were comparable to control mice, which did not lose weight at any time point. BALF cultures of infected mice, but not control mice, were positive for *M. pneumoniae* with the highest median bacterial load of $3\times10^4$ CFU/ml on day three p.i. (Figure 1B). Bacterial loads in BALF gradually declined within two weeks, and were undetectable at four weeks after infection. In contrast to the lung, *M. pneumoniae* persisted in the nose. During the first two weeks of infection, median bacterial loads were $5–9\times10^4$ CFU/ml, and at day 42 p.i., *M. pneumoniae* was still detected in the nasal lavage fluid of 5 out of 6 mice, with a median bacterial load of $6\times10^2$ CFU/ml (Figure 1C). The only mouse in which *M. pneumoniae* was not detected in the

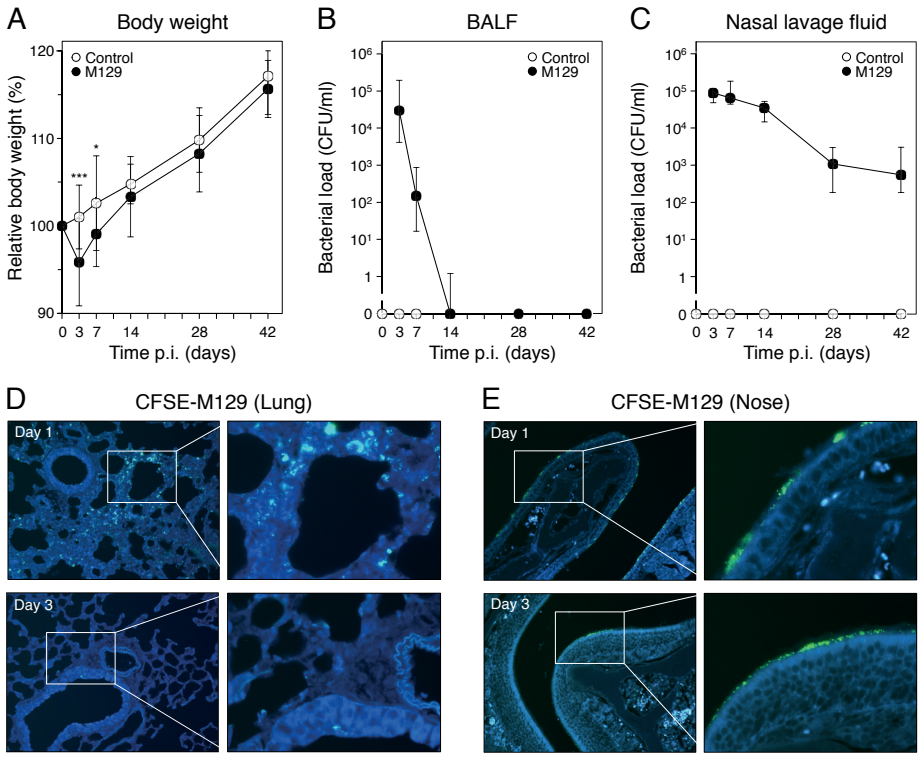



**Figure 1.** *M. pneumoniae* persists in the nose after pulmonary infection.
C57BL/6 WT mice were infected intranasally with $1\times10^9$ CFU *M. pneumoniae* M129 (black circles) or mock-infected with SP4 medium (white circles). (A) Body weight changes were monitored at indicated time points p.i. and expressed as percentage of body weight at day 0; the mean ± SD of each group ($n$=6–16 mice/time point) is shown. *$p$<0.05 and ***$p$<0.001 (Welch's $t$-test with Holm-Bonferroni method correction). (B–C) Bacterial loads as CFU/ml in BALF and nasal lavage fluid; values are expressed as median with interquartile range (IQR). (D–E) Immunofluorescent images of lung and nasal sections of C57BL/6 WT mice ($n$=3 mice/time point) at indicated time points after intranasal inoculation with $1\times10^9$ CFU CFSE-labeled *M. pneumoniae* M129 (image: 200× magnification; inset 630× magnification).

nasal lavage fluid at day 42 p.i. did show a positive culture for *M. pneumoniae* when nasal brush samples were analyzed (data not shown).

These findings were corroborated using CFSE-labeled *M. pneumoniae* M129: CFSE-labeled bacteria were detected on the bronchial epithelium and in alveoli forming islets in the lungs at day one p.i. (Figure 1D). The loss of fluorescence from the lungs of infected mice at day three p.i. likely resulted from bacterial replication, as high bacterial loads were detected in the lungs at this same time point (Figure 1B) and CFSE signals are reduced two-fold at each bacterial division.[33] This was confirmed when sections were incubated with an anti-*M. pneumoniae* antibody: *M. pneumoniae* was detected in the lungs both at day one and day three p.i. (data not shown). By contrast, CFSE-labeled *M. pneumoniae* was detected on the nasal respiratory epithelium at both time points analyzed, without an apparent loss of fluorescence intensity between these time points (Figure 1E). In contrast to previous studies showing invasion and intracellular survival of *M. pneumoniae in vitro*,[34,35] we did not observe CFSE-labeled *M. pneumoniae* within respiratory epithelial cells (Supplementary Figure S2).

### Specific IgG antibodies in serum and BALF coincide with clearance of *M. pneumoniae* from the lungs

To define the course of pneumonia following infection with *M. pneumoniae* and identify immune mechanisms that contribute to pulmonary clearance, we examined pulmonary inflammation by HPS as well as immune cells and antibodies in BALF at the indicated time points after inoculation of mice with *M. pneumoniae*. Pulmonary inflammation, demonstrated by both inflammatory infiltrates and necrosis in HPS (Figure 2A) and increased BALF leukocytes (Supplementary Figure S3A), was most severe on day three p.i., and resolved at day 28 p.i. At day three p.i., the infected lungs showed pulmonary consolidation at gross pathology and histopathology showed thickened alveolar septa characterized by infiltration of inflammatory infiltrates and presence of perivascular and peribronchiolar cuffing (Figure 2B). Using flow cytometry, we detected a major influx of granulocytes, monocytes, and dendritic cells (DCs) into BALF at day three p.i. (Supplementary Figure S3B), which corroborated the inflammation seen histologically at that time point (Figure 2). Additionally, increased numbers of $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, and B cells were detected in infected lungs, albeit at much lower frequencies than observed for the innate immune cells (Supplementary Figure S3C).

Since B cells only marginally infiltrated the lungs, we evaluated the systemic and local humoral immune response against *M. pneumoniae*. Both *M. pneumoniae*-specific IgM and IgG were detected in serum from day seven p.i. (Figure 3A). While specific IgM levels declined after day 28 p.i., specific IgG persisted at high levels. Further analysis revealed that both IgG1 and IgG2a subclasses were present in the *M. pneumoniae*-specific IgG pool (Figure 3B), which indicates neutralizing and complement-dependent killing of *M.*

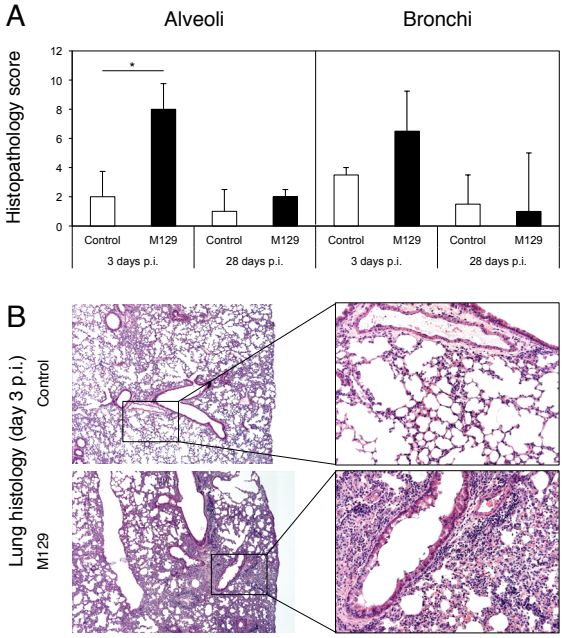



**Figure 2.** *M. pneumoniae* is characterized by alveolar and bronchiolar immune cell infiltrates at day 3 p.i. C57BL/6 WT mice were *M. pneumoniae*-infected (black bars) or mock-infected (white bars). (A) HPS for mice at indicated time points p.i.; bars represent the median ± IQR of each group ($n$=6 mice/time point). *$p$<0.05 (Kruskal-Wallis test with post hoc Dunn's multiple comparisons test). (B) Representative lung paraffin sections from *M. pneumoniae*-infected and control mice on day 3 p.i., stained with H&E (image: 50× magnification; inset: 200× magnification).

*pneumoniae*. Indeed, we found that the serum of *M. pneumoniae*-infected WT mice had the capacity to opsonize *M. pneumoniae* resulting in enhanced internalization and killing of the bacteria by macrophages *in vitro* (Figure 3C).

Notably, *M. pneumoniae*-specific IgG levels in the nasal lavage fluid were far below those detected in BALF on day 42 (Figure 4A). The opposite was seen for specific IgA: the levels were highly increased in nasal lavage fluid but not in BALF. Total IgG and IgA levels were similar in BALF and nasal lavage fluid (Supplementary Figure S4). ELISpot analysis revealed that *M. pneumoniae*-specific IgA ASCs were present in CLN and nasal passages seven days p.i., with highest numbers in CLN (Figure 4B). In contrast and in line with the data obtained by ELISA, *M. pneumoniae*-specific IgA ASCs were not present in MLN.

This prompted us to investigate the activation and expansion of B cells in the CLN and MLN that drain the URT and LRT, respectively. B cell expansion and activation was observed in both MLNs and CLNs, but not in peripheral (inguinal) lymph nodes (PLNs), which indicates that *M. pneumoniae* infection induced a B cell response only locally and not systemically (Figure 4C and 4D). It is interesting to note that the expression levels of CD86 were higher on B cells in MLN than on those in CLN (Figure 4E). Notably,

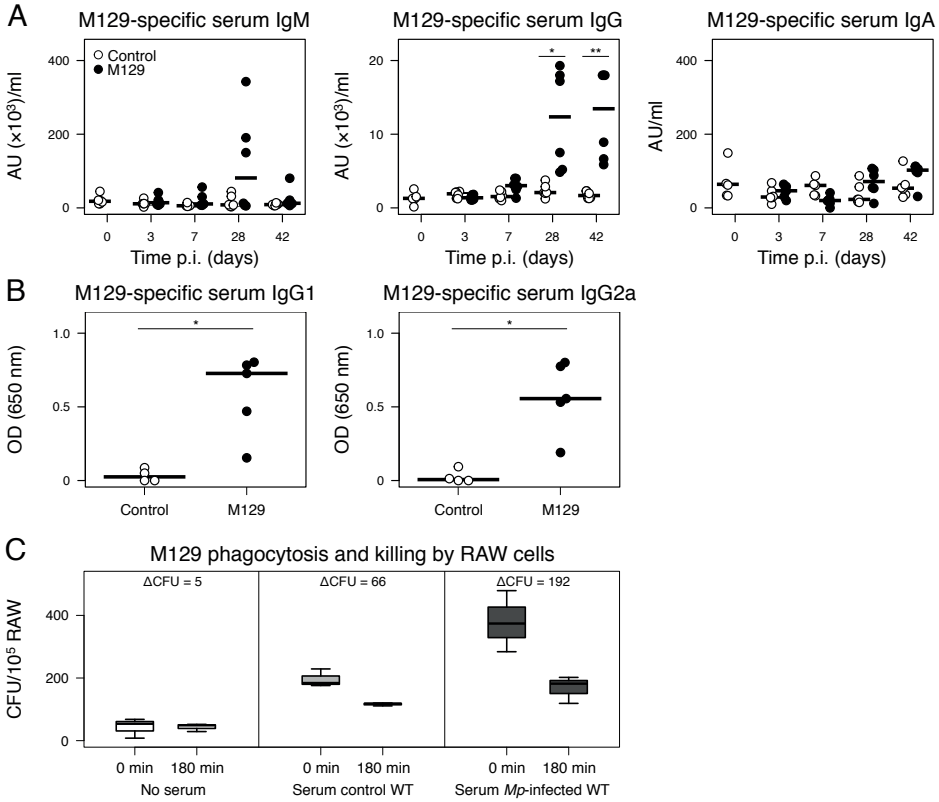



**Figure 3.** *M. pneumoniae*-specific serum antibodies have neutralizing capacities.
(A–B) *M. pneumoniae*-specific antibody levels as AU in serum of *M. pneumoniae*-infected (black circles) and control treated (white circles) C57BL/6 WT mice ($n$=6–16 mice/group per time point): (A) *M. pneumoniae*-specific IgM, IgG, and IgA at indicated time points p.i.; (B) *M. pneumoniae*-specific IgG subclasses IgG1 and IgG2a at day 42 p.i. Dots represent individual mice, and the horizontal line in each graph represents the median. (C) *M. pneumoniae* was incubated with sera from infected (dark gray box) or uninfected (light gray box) C57BL/6 WT mice or with medium (white box) and subsequently co-cultured with RAW cells for 20 minutes. After washing, phagocytosis and killing of *M. pneumoniae* was determined based on the number of CFU/$10^5$ RAW cells recovered. The box represents the lower and upper quartiles, and the median is shown as a line across the box. Whiskers extend to the maximum or minimum values within 1.5 times the IQR above and below the 3rd and 1st quartile, respectively. *$p$<0.05 and **$p$<0.01 (A: Kruskal-Wallis test with post hoc Dunn's multiple comparisons test; B: Mann-Whitney $U$ test).

increased numbers of CD86$^+$ B cells were still found in CLN, but not in MLN, on day 28 p.i., albeit not significant from controls. Together, these findings demonstrate a distinct difference in the B cell response to *M. pneumoniae* between LRT and URT lymphoid tissues.

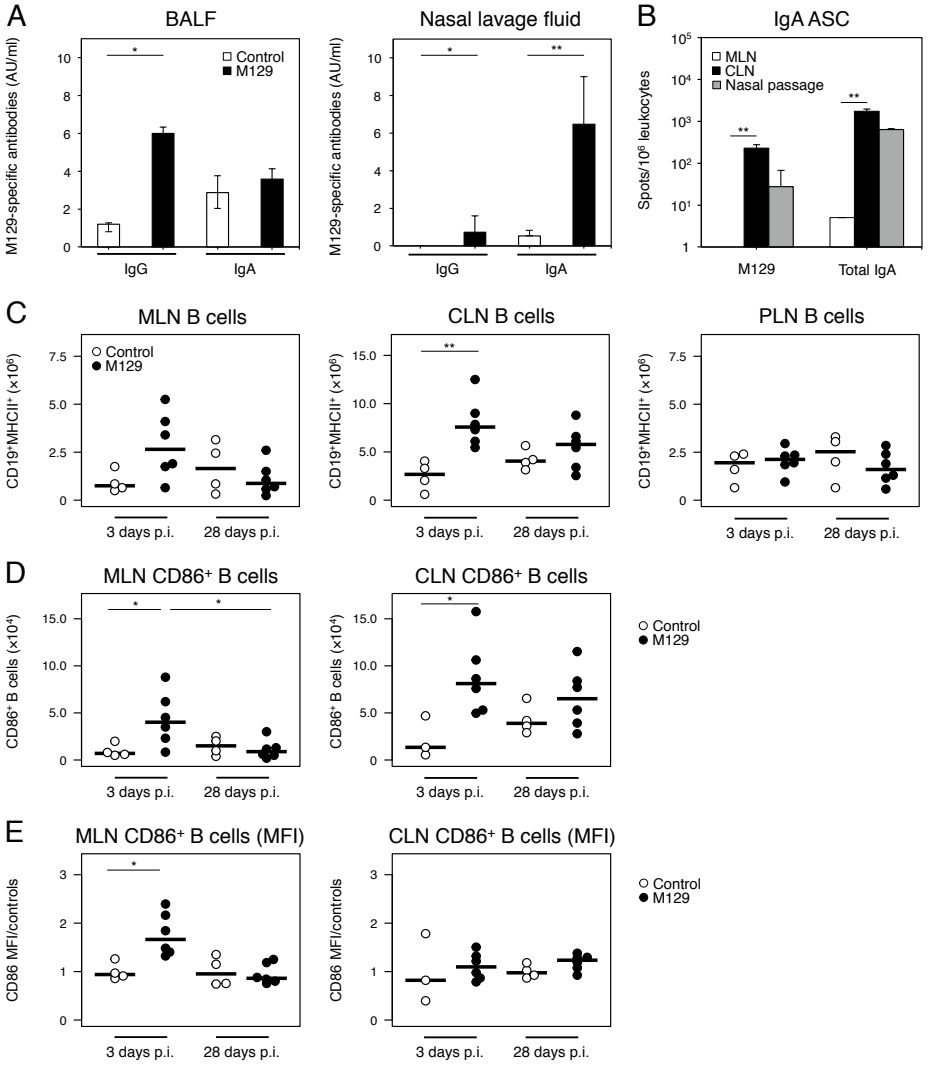



**Figure 4.** Differential B cell activation in upper versus lower respiratory tract.
(A) Analysis of *M. pneumoniae*-specific IgG and IgA antibody levels (AU/ml) in BALF and nasal lavage fluid at 42 days p.i. (IgG) and 28 days p.i. (IgA) of *M. pneumoniae*- and mock-infected C57BL/6 WT mice ($n$=4–6 mice/group per time point). (B) Analysis of *M. pneumoniae*-specific and total IgA ASCs in MLNs, CLNs, and nasal passages at 7 days p.i. of *M. pneumoniae*-infected C57BL/6 WT mice ($n$=3–6 mice). (C) Number of $CD19^+MHCII^+$ B cells in MLNs, CLNs, and PLNs of C57BL/6 WT mice. (D–E) $CD86^+$ B cell numbers (D) and mean fluorescence intensity (MFI) as ratio to medium controls (E) in MLNs and CLNs of C57BL/6 WT mice. Bars and horizontal lines indicate the median and dots individual mice. *$p$<0.05 and **$p$<0.01 (A: Mann-Whitney $U$ test; B–E: Kruskal-Wallis test with post hoc Dunn's multiple comparisons test).

## B cells are essential for pulmonary clearance of *M. pneumoniae* and affect *M. pneumoniae* carriage

To further evaluate the contribution of B cells to protective immunity against *M. pneumoniae*, we assessed whether bacterial replication in the URT and LRT is altered in μMT mice. Similar to WT mice, an initial body weight loss at day three p.i. was measured in μMT mice. Yet, while infected WT mice were back at their initial weight within two weeks and from then on continued to gain weight, the weight of μMT mice remained lower than that of WT mice at all time points analyzed (Figure 5A). Interestingly, while *M. pneumoniae* was no longer detectable in BALF of WT mice at day 28 p.i., bacteria were still detectable at day 42 in μMT mice, albeit at relatively low levels (Figure 5B). In the absence of antibodies mycoplasma is suggested to be more prone to disseminate extrapulmonary,[16-18] however, spleen, liver, and articular surfaces of μMT and WT mice on day 14 and 28 p.i. were negative for *M. pneumoniae* by culture and PCR (data not shown).

In line with the long-term presence of *M. pneumoniae* in the lungs of μMT mice, both the HPS of alveoli (Figure 5D) and the lung leukocyte number (Figure 5E) of μMT mice was higher than those of WT mice on day three p.i. Moreover, in comparison with WT mice, the lungs of μMT mice showed even increased numbers of leukocytes on day 42 p.i. (Figure 5E and 5F).

Interestingly, also significantly reduced control of bacterial replication was noted in the URT of μMT mice (Figure 5C). *M. pneumoniae* was detected in nasal lavage fluid of all μMT mice and the median bacterial loads remained at similar levels from day 14 to 42 p.i. ($2\times10^4$ CFU/ml), while median bacterial loads in WT mice decreased from $4\times10^4$ to $6\times10^2$ CFU/ml during this time period. In the absence of B cells, significantly higher levels of the proinflammatory mediators IL-6 and monocyte chemoattractant protein (MCP)-1 were detected in nasal lavage fluid of μMT mice at day 3 p.i. (Figure 5G).

## Serum transfer from WT infected mice to infected μMT mice controls bacterial replication only in the lungs

To specifically examine the contribution of the IgG and IgM antibodies in serum to clearance of bacteria from the nose, we passively immunized μMT mice with serum from infected WT mice. Therefore, donor and recipient mice were both intranasally inoculated with $1\times10^9$ CFU of *M. pneumoniae* M129. Two weeks later, when recipient WT and μMT mice still had similar median levels of bacterial loads in the lungs (Figure 5B), donor serum was transferred (Figure 6A). As shown in Figure 6B, μMT mice were only able to control *M. pneumoniae* replication in the lungs upon transfer of serum from infected WT mice, as indicated by the similar bacterial load in the lungs of μMT and WT mice at day 28 p.i. Transfer of μMT serum, however, did not reduce *M. pneumoniae* loads in the lungs of infected μMT recipient mice; pulmonary *M. pneumoniae* loads in these μMT recipients

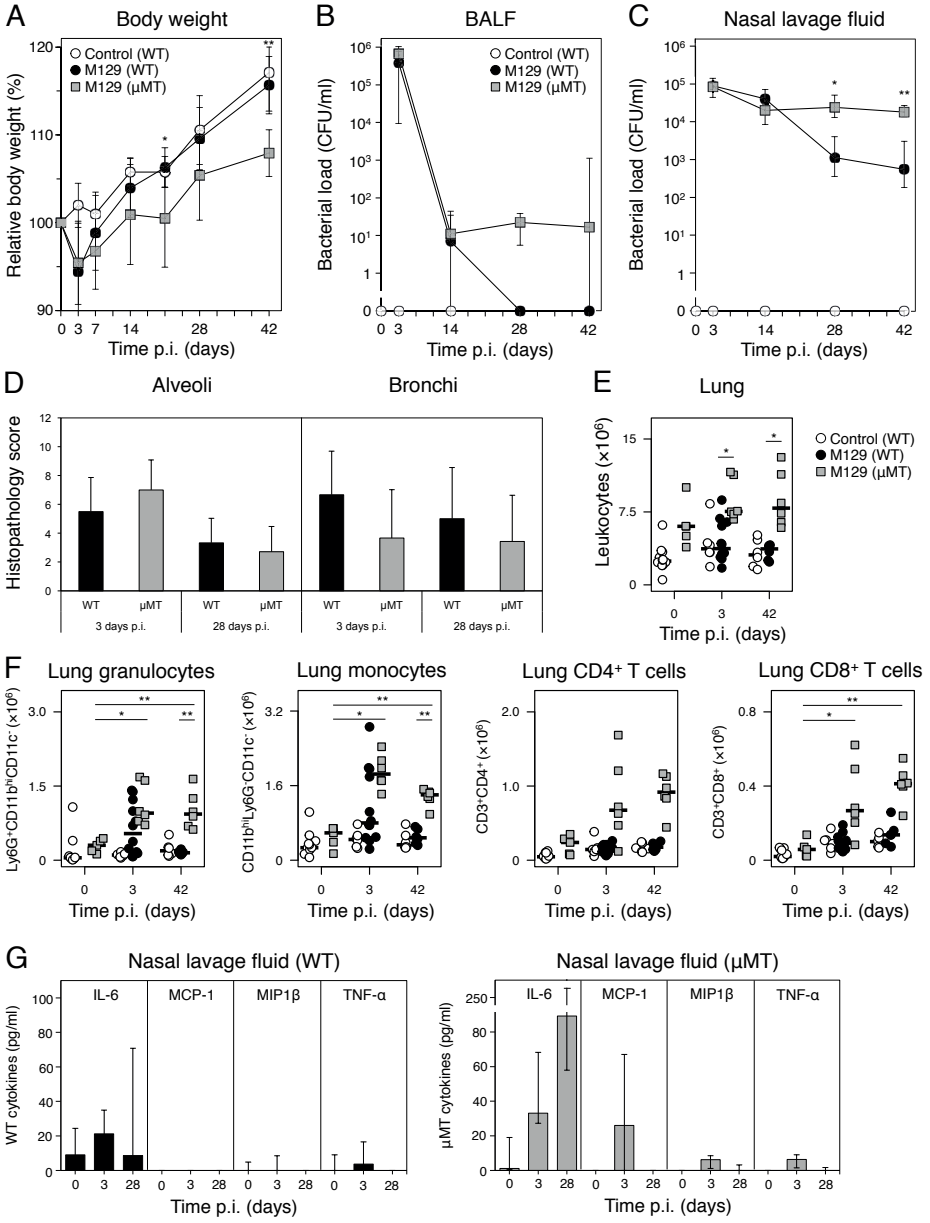
**Figure 5.** B cell-deficient μMT mice show limited pulmonary clearance of *M. pneumoniae* and higher nasal carriage rates.
C57BL/6 WT mice (black circles) and μMT mice (gray squares) were infected intranasally with *M. pneumoniae*. Control mice (white circles) received SP4 medium alone. (A) Body weight changes were monitored at indicated time points p.i. and expressed as percentage of body weight at day 0; the mean ± SD of each group is shown (*n*=6–13 mice/time point). (B–C) Bacterial loads as CFU/ml in BALF and nasal lavage fluid; values are expressed as median with IQR. (D) HPS for mice at indicated time points p.i.; the mean ± SD of each group (*n*=6–7 mice/time point) is shown. (E) Absolute number of leukocytes in lungs at different days p.i. (F) Numbers of lung granulocytes ($Ly6G^+CD11b^{hi}CD11c^-$), monocytes ($CD11b^{hi}Ly6G^-CD11c^-$), $CD4^+$ T cells ($CD3^+CD4^+$), and $CD8^+$ T cells ($CD3^+CD8^+$). (G) IL-6, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP) 1β, and tumor necrosis factor (TNF)-α in nasal lavage fluids of *M. pneumoniae*-infected WT and μMT mice were determined by multiplex fluorescent-bead-based immunoassay. Median levels with IQR are shown. **p*<0.05 and **p*<0.01 (A: Welch's *t* test with Holm-Bonferroni method correction; B–C: Mann-Whitney *U* test with Holm-Bonferroni method correction; D–F: Kruskal-Wallis test with post hoc Dunn's multiple comparisons test; A–C: μMT vs. infected WT mice).

were similar to those of untreated controls at day 28 p.i. (Figure 6B). Moreover, these findings were corroborated by the detection of *M. pneumoniae*-specific IgG in the BALF of four out of six μMT recipient mice two weeks after WT serum transfer (Figure 6D). *M. pneumoniae* persisted in the lungs of the two μMT recipient mice, whose BALF did not contain *M. pneumoniae*-specific IgG after serum transfer (Figure 6E).

By contrast, the bacterial loads in the nose were comparable for all recipient μMT mice (with a median of $2\times10^4$ CFU/ml), irrespective of the specific source of the serum (Figure 6C). The median concentration of *M. pneumoniae*-specific and total IgG in nasal lavage fluid of infected μMT mice after serum transfer was 5 AU/ml and 0.005 μg/ml, respectively, at day 28 p.i. Notably, there was no significant difference between *M. pneumoniae*-specific IgG levels in BALF and nasal lavage fluid of μMT mice receiving WT serum (median 6 vs. 5 AU/ml). In addition to nasal lavage fluid and BALF, *M. pneumoniae*-specific and total IgG antibodies were also detected in serum of μMT mice after WT serum transfer (Supplementary Figure S5).

## DISCUSSION

In this study, we show *M. pneumoniae* persistence after infection in a further developed mouse model for *M. pneumoniae* infection.[30-32] B cells in lung-draining MLNs become activated and expand early after *M. pneumoniae* infection, resulting in elevated levels of *M. pneumoniae*-specific IgM and IgG antibodies in serum and BALF. This B cell activation in MLNs may be key to clear the infection as shown by our observations in μMT mice, in which *M. pneumoniae* infection leads to chronic pulmonary disease, characterized by failure to thrive, more severe pneumonia, and long-lasting innate and adaptive compensatory immune cell infiltrates, due to bacterial persistence in the lungs. Conversely, μMT mice do clear *M. pneumoniae* infections in the lungs when passively immunized


A
Body weight
Relative body weight (%)
Time p.i. (days)
Control (WT)
M129 (WT)
M129 (μMT)
B
BALF
Bacterial load (CFU/ml)
Time p.i. (days)
C
Nasal lavage fluid
Bacterial load (CFU/ml)
Time p.i. (days)
D
Alveoli
Bronchi
Histopathology score
WT
μMT
3 days p.i.
28 days p.i.
E
Lung
Leukocytes (×10⁶)
Time p.i. (days)
F
Lung granulocytes
Lung monocytes
Lung CD4⁺ T cells
Lung CD8⁺ T cells
Time p.i. (days)
G
Nasal lavage fluid (WT)
Nasal lavage fluid (μMT)
IL-6
MCP-1
MIP1β
TNF-α
WT cytokines (pg/ml)
μMT cytokines (pg/ml)
Time p.i. (days)

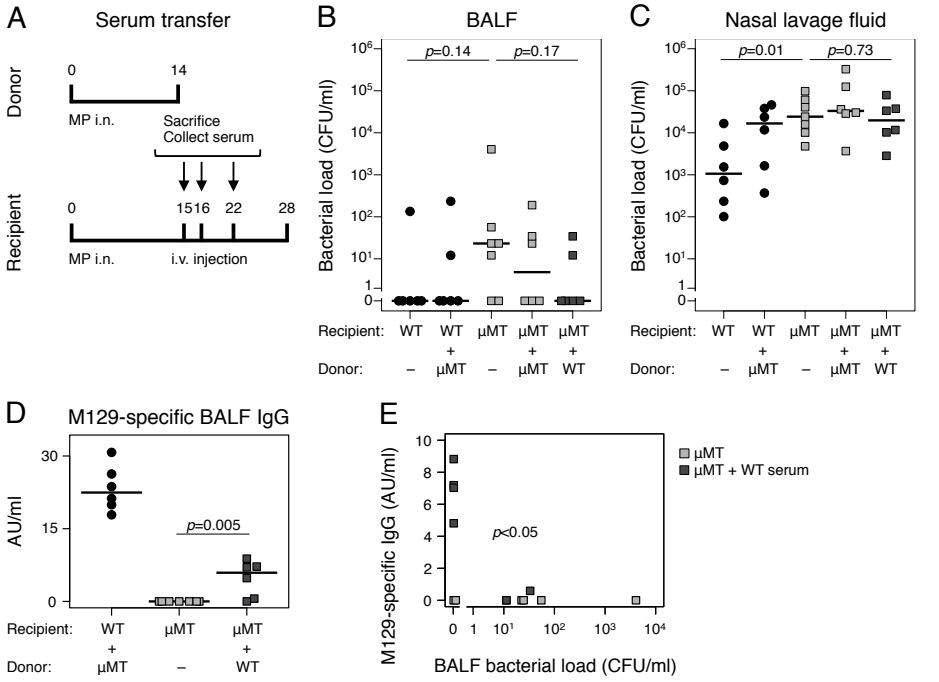



**Figure 6.** Adoptive transfer of wild-type (WT) serum allows B cell-deficient µMT mice to control *M. pneumoniae* infection in the lungs.
(A) Experimental setup of the serum transfer experiment (*n*=6 mice/group). (B–C) Bacterial loads as CFU/ml in BALF and nasal lavage fluid of WT and µMT mice. (D) *M. pneumoniae*-specific IgG levels (AU/ml) in BALF of WT and µMT mice. (E) Correlation between *M. pneumoniae*-specific IgG and bacterial load in BALF of µMT mice that were untreated or passively immunized with WT serum. The *p* value is indicated in the graphs (B–D: Mann-Whitney *U* test; E: Pearson product-moment correlation coefficient).

with serum from infected WT mice. Additionally, we showed that the induced antibodies enhance phagocytosis and killing by macrophages *in vitro*, which suggests that they can contribute to resolve *M. pneumoniae* infections *in vivo*.

In contrast to the lungs, B cells in the nose-draining CLN did not become activated by the presence of *M. pneumoniae*. As the draining lymph nodes are the major sites of B cell induction following RTI,[36] the observed differences in B cell expansion and activation between the nose-draining CLN and lung-draining MLN suggest a different control of bacterial replication in the URT compared to that in the LRT. Indeed, *M. pneumoniae* persisted in the nose of WT mice while the bacterium was cleared from the lungs during the course of infection. Importantly, these data mimic the findings in humans, where *M. pneumoniae* is carried in the URT of children up to eight months after symptomatic infection.[3-7] The observed difference in B cell responses between LRT and URT lymphoid tissues is in line with the low levels of *M. pneumoniae*-specific IgG and IgM in the nasal

lavage fluid. It is unlikely that *M. pneumoniae*-specific IgG does not reach the URT, as total IgG levels were similar in nasal lavage fluid and BALF, and we observed comparable bacterial loads in the URT of WT mice as in that of $Btk^-$ mice, which have reduced peripheral B cell numbers (data not shown). In contrast to *M. pneumoniae*-specific IgG, *M. pneumoniae*-specific IgA was more abundant in nasal lavage fluid than in BALF of *M. pneumoniae*-infected mice. While this has not been shown before for bacterial infections, similar observations were made in influenza virus-infected mice.[37] The vast majority of ASCs in the URT (NALT) of such mice were found to produce IgA, whereas B cell responses in the LRT (lungs) mainly generated IgG and IgM.[38] In fact, we also detected *M. pneumoniae*-specific IgA ASCs in CLN and nasal passage, but not in MLN, which is in line with observations from Hodge and Simecka,[39] showing mycoplasma-specific IgA ASCs in nasal passages but not in the lungs from mice following nasal immunization. Furthermore, these data also corroborate our findings on the presence of IgA in nasal washes but not in BALF. Detection of *M. pneumoniae*-specific IgA-producing ASCs in the nasal passage, albeit at lower numbers than in CLN, may indicate that some of the activated B cells have migrated into the nasal passage.

The locally induced immune responses upon *M. pneumoniae* carriage in the URT and infection of the LRT are distinct. We show that *M. pneumoniae*-specific IgG controls *M. pneumoniae* in the lungs and does not impact on carriage in the URT. By contrast, *M. pneumoniae*-specific IgA and IgA ASCs in the URT affect *M. pneumoniae* carriage, but do not mediate complete resolution of the bacteria. Using μMT mice and serum transfer studies we further assessed the relative roles of immunity in the URT and LRT of *M. pneumoniae* infected mice. Transfer of WT immune serum, containing *M. pneumoniae*-specific IgG, controlled *M. pneumoniae* pulmonary infection in lungs of μMT mice but did not lead to a reduction of *M. pneumoniae* loads in the nose. Together, these observations demonstrate the importance of local immunity. Yet, why IgA cannot clear bacterial carriage in the URT is unknown.[40] *M. pneumoniae* is not known to possess IgA protease activity.[41] Also, increasing local specific IgA responses by immunization did not lead to a corresponding reduction of mycoplasma bacterial loads in the nose,[39] which suggests that enhancing IgA responses alone does not control carriage in the URT.

Our observation that *M. pneumoniae* infections are more severe – and chronic – in μMT mice may resemble the findings in patients that suffer from B cell deficiencies,[42] such as common variable immunodeficiency, X-linked agammaglobulinemia, or hypogammaglobulinemia, who have been reported to be at increased risk for *M. pneumoniae* pulmonary disease and/or extrapulmonary manifestations, e.g., arthritis.[21-25] It can be speculated that if these patients experience *M. pneumoniae* infections, they may benefit from intravenous immunoglobulin (IVIg) treatment, as our data demonstrate that administration of serum containing *M. pneumoniae*-specific IgG protected μMT mice from

pulmonary *M. pneumoniae* infections. In fact, high amounts of *M. pneumoniae*-specific IgG can be detected in commercial human IVIg preparations.[43]

While the *M. pneumoniae* mouse model provides a useful tool to investigate the immunological processes involved in controlling infection of the lungs and carriage in the nose, the use of the human pathogen *M. pneumoniae* instead of *M. pulmonis*[44] can be a limitation of our study considering the genomic differences between these pathogens.[45]

## CONCLUSIONS

Together, we show for the first time that, like in humans, *M. pneumoniae* is carried in the URT of mice after symptomatic infection. In addition, we demonstrate that B cells and *M. pneumoniae*-specific antibodies are crucial for *M. pneumoniae* clearance in the lungs of mice, but are limited in clearing *M. pneumoniae* from the URT. These novel aspects of *M. pneumoniae*-host interactions will aid the understanding of *M. pneumoniae* pathogenesis and promote the development of *M. pneumoniae*-targeting vaccines to prevent the progress of *M. pneumoniae* pulmonary disease in individuals that carry *M. pneumoniae*.

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## SUPPLEMENTARY DATA

## METHODS

### Bacteria

*M. pneumoniae* reference strain M129 (subtype 1, ATCC 29342) was cultured at 37°C/5% $CO_2$ in SP4 medium (1.4% Difco PPLO broth [Becton Dickinson, Sparks, MD, USA] supplemented with 0.15% Difco TC Yeastolate, UF [Becton Dickinson], 1.4% glucose, 20% horse serum, 0.02 mg/ml phenol red, 1000 U/ml penicillin, 500 U/ml polymyxin B, 3 µg/ml amphotericin B, and 5 µg/ml voriconazole; pH 7.8–8.0) for 72 hours, harvested, washed and concentrated in fresh SP4 medium to $1\times10^9$ CFU/ml. Aliquots were stored at −80°C until use.

### *M. pneumoniae* infection and carriage mouse model

At indicated time points, mice were sacrificed and BALF samples were obtained by flushing the lungs 2 times with 1 ml of PBS containing 0.5 mM EDTA. The same tracheal puncture was used to flush the nasal cavity 2 times with 0.2 ml PBS containing 0.5 mM EDTA. All BALF and nasal lavage fluid samples were kept on ice until further processing. Next, the lungs were removed and right lung lobes were cut into grain size pieces and incubated in PBS supplemented with 20 µg/ml Liberase-TL (Roche Diagnostics GmbH, Manheim, Germany) and 2 U/ml DNase I (Sigma, St. Louis, MO, USA) for 30 min at 37°C. Lung cell suspensions were passed through a 100 µm gauze (BD Falcon, Franklin Lakes, NJ, USA). Erythrocytes in single cell suspensions of lungs and LNs were lysed with ACK lysis buffer. Subsequently, the immune composition was analyzed by flow cytometry upon staining with specific antibodies. The left lobes of the lungs were placed in 4% PFA for 24 h, washed in 70% ethanol, dehydrated in series of alcohols, and embedded in paraffin. The heads of mice were placed in acetic acid-zinc-formalin for 24 h, washed in distilled water for 30 min, decalcified in Gooding and Stewart's decalcification fluid (10% formic acid and 5% formaldehyde) for 6 h, before being processed and embedded in paraffin similar as for lung tissue. Four µm-thick paraffin-embedded sections were stained with H&E.

### *M. pneumoniae* quantification

To analyze the presence and amount of *M. pneumoniae ex vivo*, 100 µl of undiluted and 10-fold diluted BALF and nasal lavage fluid were cultured on SP4 agar plates at 37°C. Sample suspensions of homogenized spleen and liver tissue, as well as from immersed cotton swabs of articular surfaces, were processed in a similar fashion. Quantification was performed by counting CFUs. Alternatively, the presence of *M. pneumoniae* was detected using quantitative PCR as previously described.[28]

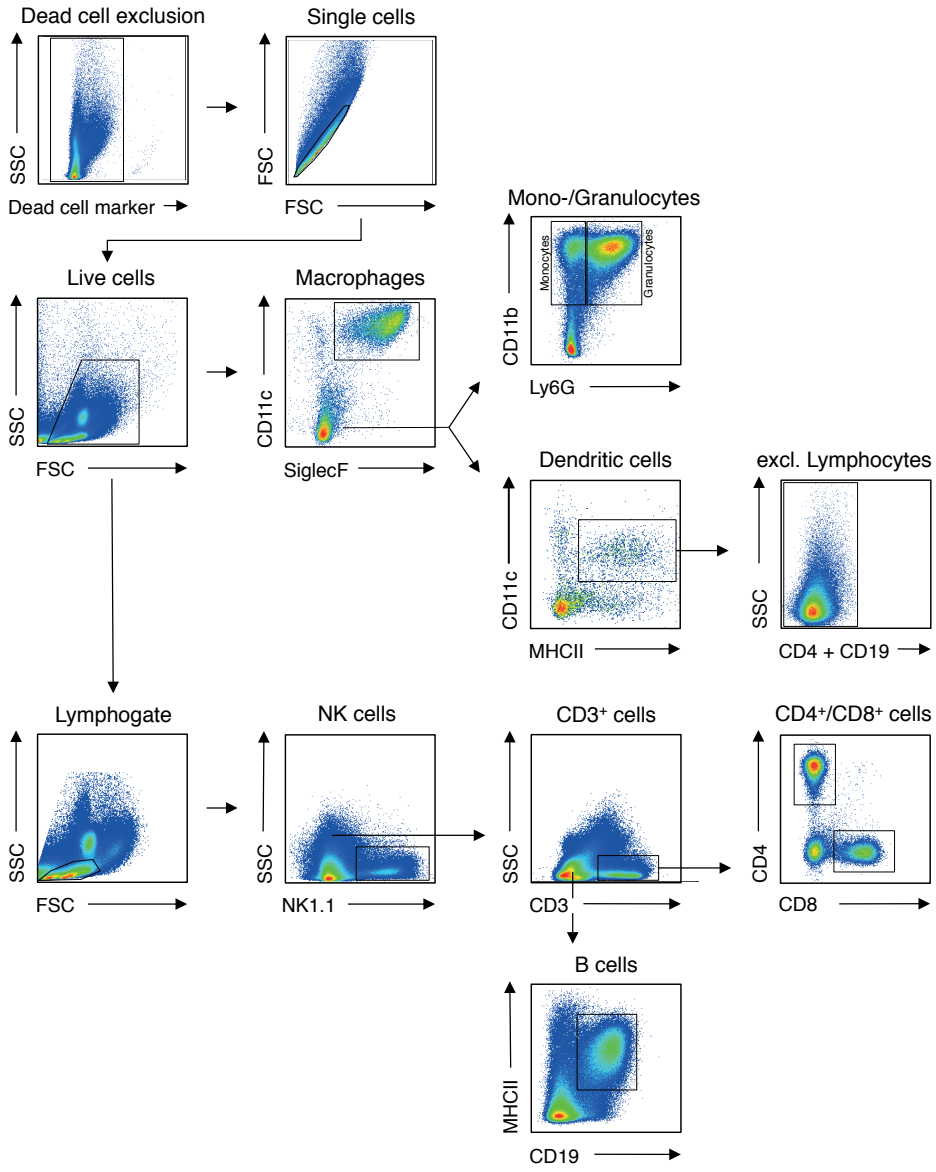
## Quantification of antibodies

*Detection of total antibody concentration*

Concentrations of IgM, IgG, and IgA were determined in serum, BALF or nasal lavage fluids by sandwich ELISA. In brief, diluted sera or lavage fluids were added in duplicate onto 96-well polystyrene plates (Corning Costar, Corning, NY, USA) that were pre-coated with polyvalent rabbit anti-mouse IgM (1000 ng/ml), goat anti-mouse IgG (2200 ng/ml) or goat anti-mouse IgA (350 ng/ml). The bound antibodies in sera and lavage fluids were detected by addition of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgM, goat anti-mouse IgG or goat anti-mouse IgA. Binding was visualized using tetramethylbenzidine (TMB; Sigma) as substrate and optical density was measured at 650 nm with a VersaMax ELISA microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The results were analyzed by microplate data collection and analysis software (VersaMax). Purified mouse IgM, IgG or IgA were used as standard. All antibodies and standards were obtained from Thermo Scientific (Waltham, MA, USA).

*Detection of M. pneumoniae-specific antibodies*

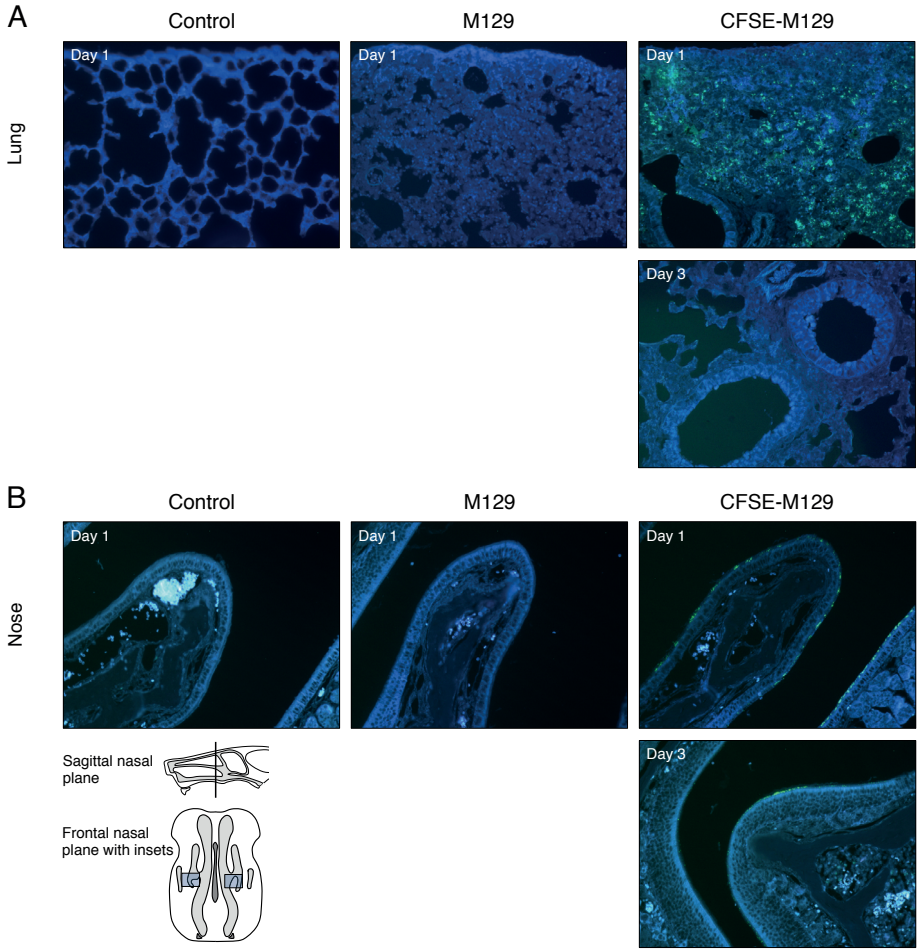
For assessment of specific antibodies against *M. pneumoniae*, 96-well half-area polystyrene plates (Corning Costar) were coated o/n at 4°C with 10 µg whole cell lysates of *M. pneumoniae* M129 in 100 mM sodium carbonate-bicarbonate buffer (pH 9.6) containing 1 M urea. The next day, non-specific binding was blocked by incubation with PBS/1% BSA (Sigma) for 2 h at RT. The samples were diluted in PBS/0.1% BSA (1:100 and 1:300 for IgM; 1:50 and 1:100 for IgG; 1:10 and 1:30 for IgA) and 100 µl were incubated in duplicate o/n at 4°C. Bound antibodies were detected by HRP-conjugated antibodies as described above. The same Ig isotype standards as for the detection of the total antibody concentration was used to determine the AU of antibodies against *M. pneumoniae* M129 as follows. At a given optical density value in the linear range, the reciprocal dilutions of the serum samples were divided by the reciprocal dilution of the standard antibodies.




**Supplementary Figure S1.** Flowcytometric gating strategy for innate and adaptive immune cells in lung tissue.

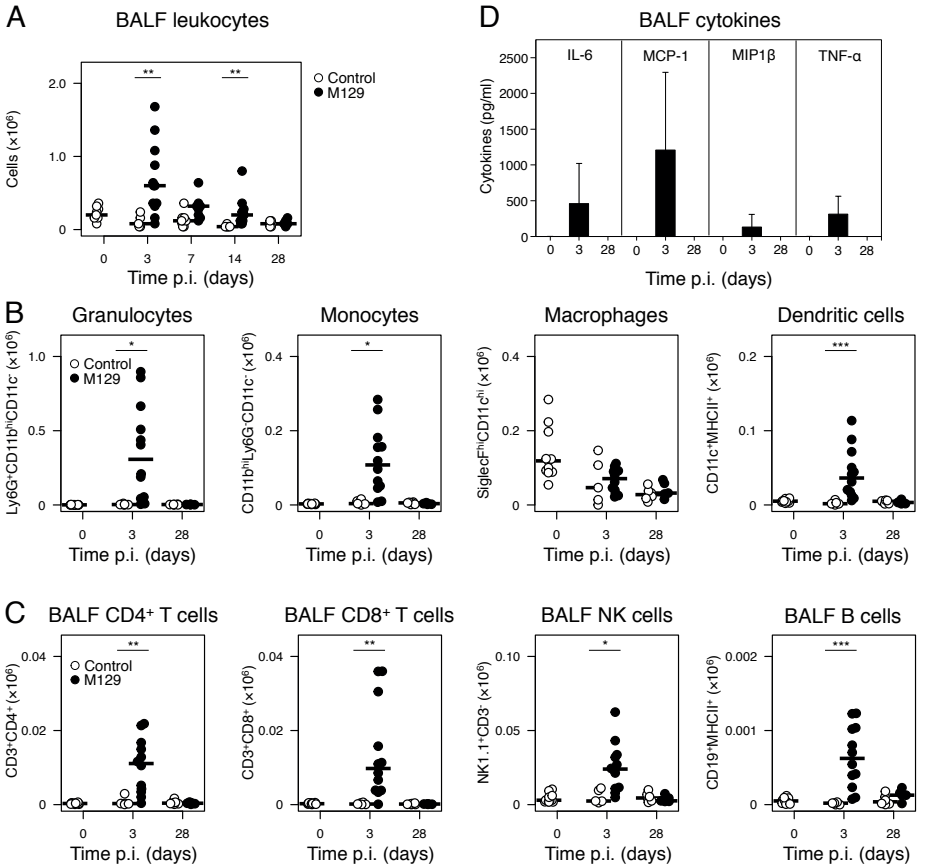
The following anti-mouse monoclonal antibodies were used: PerCP-Cy5.5-conjugated anti-CD19 (clone 1D3; eBioscience) and anti-Ly-6G (1A8; BD Biosciences), APC-conjugated anti-CD4 (RM4-5; BD Biosciences) and anti-CD11c (HL3; BD Biosciences), PE-conjugated anti-CD8a (53-6.7; Biolegend, San Diego, CA, USA) and anti-SiglecF (E50-2440; BD Biosciences), biotinylated anti-CD3e (145-2C11; Biolegend) and anti-Ly-6C (AL-21; BD Biosciences), APC-Cy7-conjugated anti-MHCII (M5/114.15.2; Biolegend), PE-Cy7-conjugated anti-NK1.1 (PK136; Biolegend) and anti-CD11b (M1/70; BD Biosciences), and BV510-conjucated anti-CD5 (53-7.3; BD Biosciences) and anti-CD19 (1D3; BD Biosciences). The secondary antibody was FITC-conjugated streptavidin (Biolegend). Controls included isotype controls (Biolegend and eBioscience), single positive,

and unstained cells. Dead cells were excluded using the live/dead fixable dead cell stain kit (Pacific blue dye, Invitrogen) and tandem dyes were protected with stabilizing fixative (BD Biosciences). Gating strategy: viable single cells were analyzed by excluding dead cells. Subsequently, single cells were gated (FSC-W and FSC-H) and viable single innate cells or lymphocytes were analyzed by gating on the basis of FSC-A and SSC-A. Granulocytes and monocytes were identified as $Ly6G^{+}CD11b^{hi}CD11c^{-}$ and $CD11b^{hi}Ly6G^{-}CD11c^{-}$, respectively; alveolar macrophages as $SiglecF^{hi}CD11c^{hi}$ and DCs as $CD11c^{+}MHCII^{+}$ (excluding lymphocytes by CD4 and CD19 negativity); CD3 positivity discerned $CD4^{+}$ and $CD8a^{+}$ T cells ($CD3^{+}CD4^{+}$ and $CD3^{+}CD8a^{+}$ cells) from $NK1.1^{+}CD3^{-}$ NK cells; additionally, $CD3^{-}CD19^{+}MHCII^{+}$ B cells were distinguished.



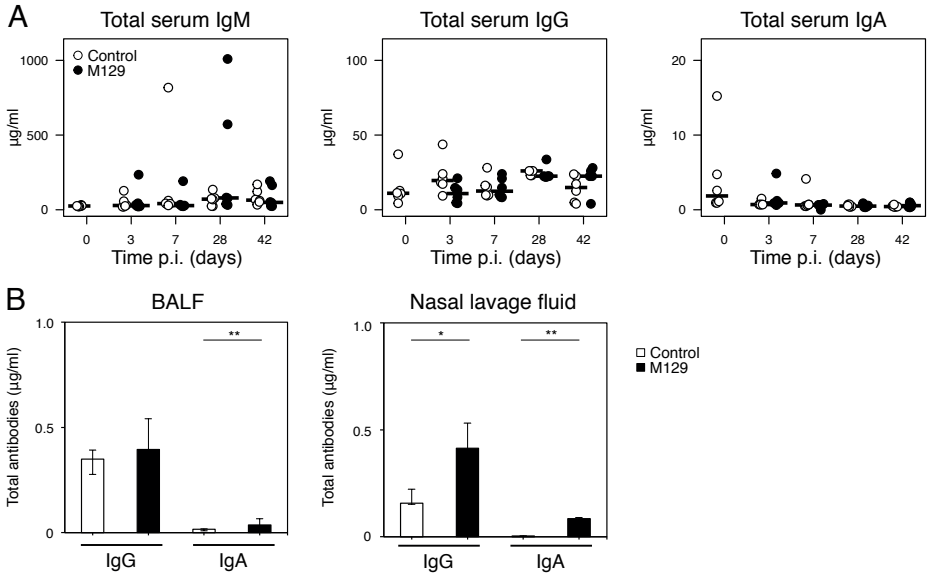

**Supplementary Figure S2.** Immunofluorescent images of lung and nasal sections of mice infected with CFSE-labeled *M. pneumoniae* and of controls.

Experimental setup as described in the legend to Figure 1D–E: (A) Lung sections. (B) Nasal sections. Images at 200× magnification of C57BL/6 mice at indicated time points after intranasal administration of SP4 medium (control), $1\times10^{9}$ CFU of *M. pneumoniae* (M129), and $1\times10^{9}$ CFU of CFSE-labeled *M. pneumoniae* (CFSE-M129) ($n$=3 mice/group/time point). The vertical line in the scheme of the nasal cavity indicates the anterior surface of the frontal nasal tissue blocks, which were selected for microscopic examination (inset indicates the location for images).



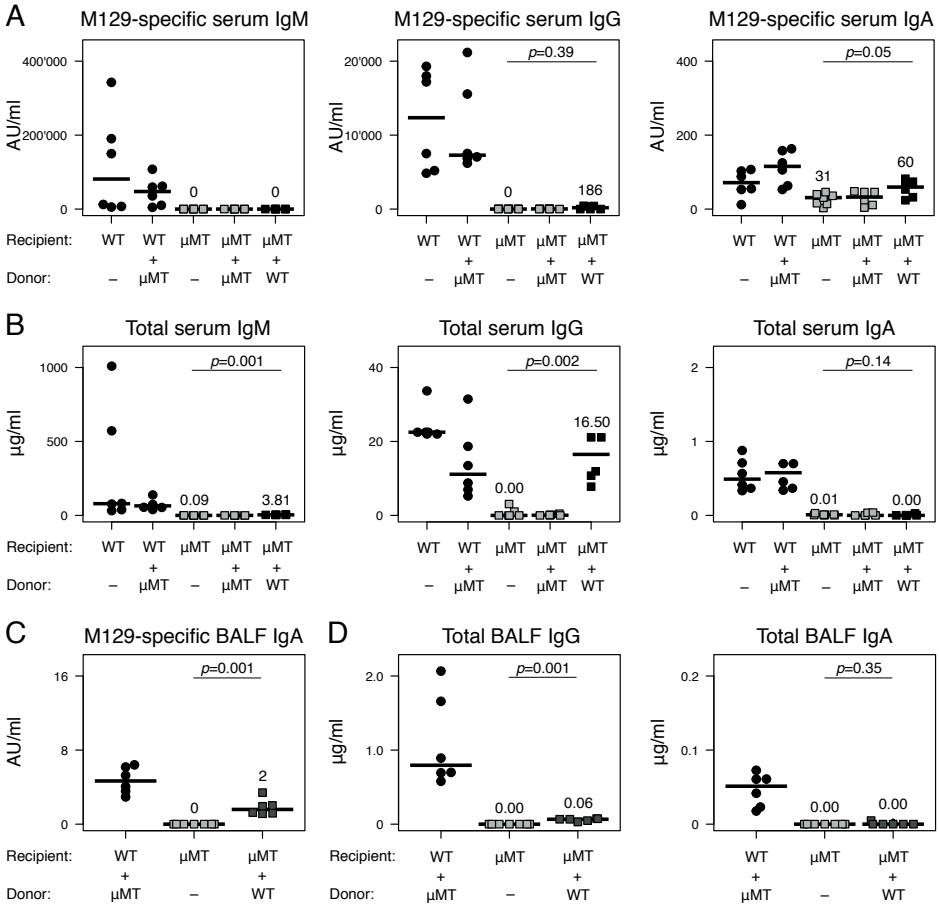

**Supplementary Figure S3.** Immune cells and pro-inflammatory mediators in BALF during *M. pneumoniae* infection in WT mice.

Experimental setup as described in the legend to Figure 1 (*n*=6–16 mice/group/time point): (A) Absolute leukocyte number in BALF at indicated time points p.i. (B–C) Frequency of innate (B) and adaptive (C) immune cell subsets in BALF. (D) IL-6, MCP-1, MIP1β, and TNF-α levels in BALF. Data are expressed as median (with IQR). **p*<0.05, ***p*<0.01, ****p*<0.001 (Mann-Whitney *U* test).




**Supplementary Figure S4.** Kinetics of antibodies in serum, BALF, and nasal lavage fluids of *M. pneumoniae*-infected mice.

Experimental setup as described in the legends to Figure 3 and 4: (A) Total serum concentrations of IgM, IgG, and IgA at indicated time points p.i. (B) Total IgG and IgA concentrations in BALF and nasal lavage fluid at day 42 p.i. (IgG) and day 28 p.i. (IgA), respectively. Data are expressed as median (with IQR). *$p<0.05$, **$p<0.01$ (Mann-Whitney *U* test).




**Supplementary Figure S5.** Specific and total antibodies in serum and BALF of WT and µMT mice after serum transfer.

Experimental setup as described in the legend to Figure 6: (A–B) *M. pneumoniae*-specific and total IgM, IgG, and IgA in serum. (C) *M. pneumoniae*-specific IgA in BALF. (D) Total IgG and IgA in BALF. The horizontal line represents the median. The absolute median value is specified above for µMT mice without serum transfer and µMT mice receiving WT serum. The *p* value is indicated in the graphs (Mann-Whitney *U* test).

# Chapter 3

## 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 neurologic disorders. We assessed whether anti-glycolipid 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 of 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 as 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 development of *M. pneumoniae*-targeting vaccines avoiding induction of neurotoxic antibodies.

## INTRODUCTION

*Mycoplasma pneumoniae* is a major cause of community-acquired pneumonia (CAP) and can trigger immune-mediated neurologic 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] The 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 of neutralizing infection,[5] but attempts to develop such vaccines against *M. pneumoniae* using inactivated bacteria in humans (reviewed in [6]) and live attenuated strains in the 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.[14,16-23] 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 did clear *M. pneumoniae* infections in the lungs when passively immunized with *M. pneumoniae*-specifc IgG-containing serum from infected wild-type (WT) mice two 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 only covered 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-reaction 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 neurologic 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 immunoglobulin (Ig) M and IgG anti-GalC antibodies are present in 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 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 (Btk)-deficient mice with *F. tularensis*-derived glycolipids did not result in protection against a lethal challenge with *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 the protection to *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 for *M. pneumoniae* infection. Further, employing WT and Btk$^-$ mice we unraveled the role of TI B-cell responses in the resolution of pulmonary *M. pneumoniae* infection.

## METHODS

### Mice

C57BL/6 mice were purchased from Charles River Laboratories and used at 8–12 weeks of age. Btk$^-$ mice (Btk$^{-/-}$ or Btk$^{-/Y}$, on C57BL/6 background[46]) and CD19-hBtk mice (backcrossed on Btk$^-$ C57BL/6 mice for >10 generations[47]) were bred and housed in the animal facilities of the Erasmus MC under specific pathogen-free conditions. All experiments were conducted according to the Dutch guidelines for animal experimentation and approved by the Animal Experiments Committee of the Erasmus MC, Rotterdam, The Netherlands (protocol number 103-13-05 and 103-14-02).

### Patients

Children with CAP and asymptomatic controls, admitted for a planned elective surgical procedure, from 3–18 years of age were enrolled from May 1, 2016 to April 30, 2017, during a CAP study at the University Children's Hospital Zurich (P.M. Meyer Sauteur, submitted for publication). Diagnosis was based on detection of *M. pneumoniae* DNA in pharyngeal swab specimens by polymerase chain reaction (PCR), specific serum IgM antibodies by enzyme-linked immunosorbent assay (ELISA), and circulating IgM antibody-secreting cells by enzyme-linked immunospot (ELISpot) assay. Asymptomatic controls were 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-No. 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 $1\times10^9$ colony-forming units (CFUs) 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 either by PCR or culture of BALF on SP4 agar plates.[48]

### Lipid extraction

Lipids were extracted from *M. pneumoniae* M129 culture by adding chloroform/methanol/water 2:1:1 (*V*/*V*/*V*). After 1 h incubation at 4°C, the mixture was vortexed and centrifuged at 2,000 × *g* for 1 min. After repeating the extraction procedure, cells were sonicated for 30 min and centrifuged at 10,000 × *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 thin-layer chromatography (TLC). Hereto, 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/MQ (60/35/8, *V*/*V*/*V*). Total lipids were visualized using 10% cerium(IV) sulfate in 15% $H_2SO_4$, and glycolipids using 0.1% orcinol in 5% $H_2SO_4$, and heating up to 110°C.

### Protein extraction

After delipidation of the *M. pneumoniae* lysate by chloroform/methanol/water 2:1:1 (*V*/*V*/*V*) 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 × *g* for 5 min. Pellets were washed twice with acetone, air-dried on ice, and dissolved in 4 M urea/250 mM ammonium bicarbonate. Protein concentration was determined by bicinchoninic acid assay. Additionally, proteins (125 ng) were analyzed by SDS-PAGE, followed by Silver staining (Merck).

### Quantification of 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 o/n at 4°C with *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. Non-specific binding was blocked using PBS/1% BSA (Sigma). Serum samples, diluted in PBS/0.1% BSA, and undiluted bronchoalveolar lavage fluid (BALF) samples were added and incubated o/n at 4°C. Bound antibodies were detected by 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 IgG1–4 (Sanquin), respectively. Reactions were visualized using tetramethylbenzidine (TMB; Sigma) as substrate, and stopped using 1 M $H_2SO_4$. 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

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

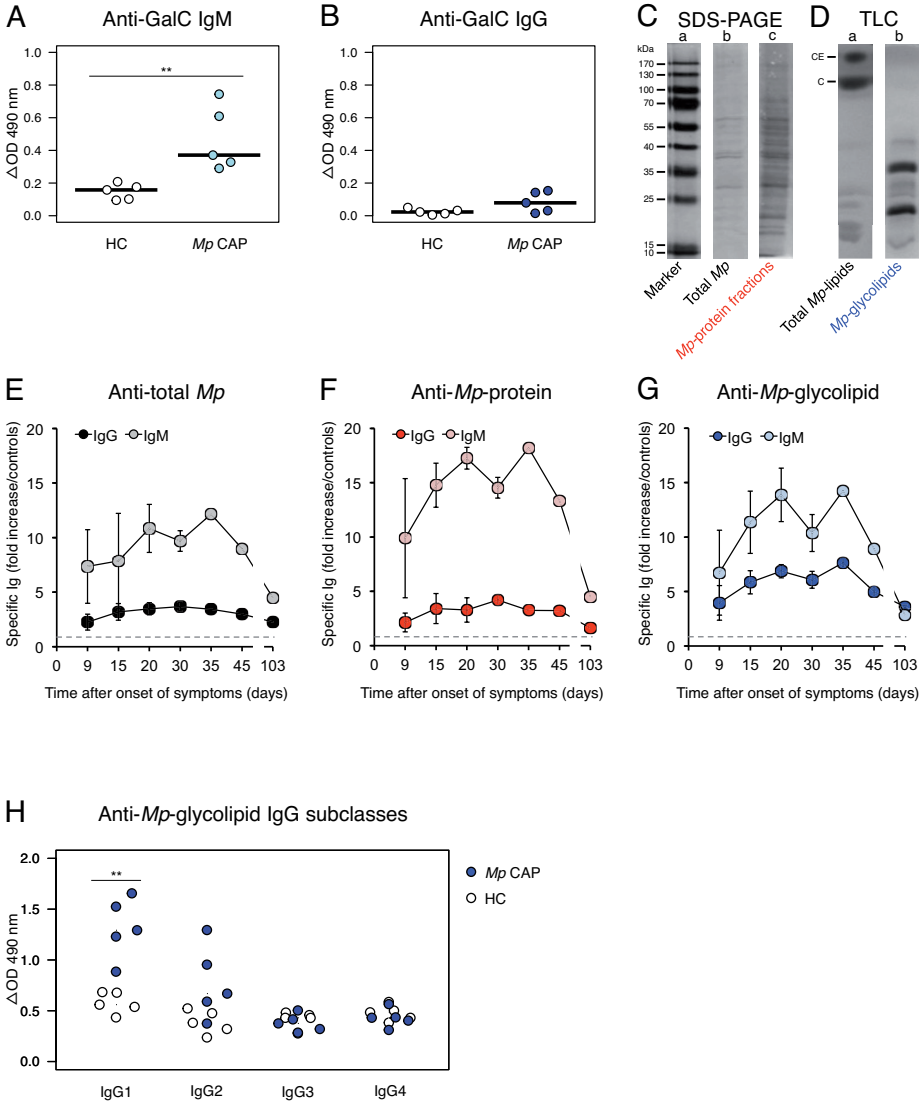
## RESULTS

### 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 *M. pneumoniae* CAP children for the presence of anti-GalC antibodies by 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 *M. pneumoniae* CAP than of HC children (Figure 1A). Moreover, anti-GalC IgG was detectable only at very low levels in both *M. pneumoniae* CAP and HC children (Figure 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 anti-glycolipid response to GalC, we next investigated the antibody response against the complete *M. pneumoniae*-glycolipid as well as *M. pneumoniae*-protein fraction. First, *M. pneumoniae*-proteins and –glycolipids were separated from *M. pneumoniae* cultures using chloroform/methanol extraction (2:1 *V*/*V*). The separation of proteins and glycolipids from the *M. pneumoniae* lysate was analyzed by SDS-Page followed by Silver staining (Figure 1C) and TLC followed by orcinol staining (Figure 1D), respectively. The reactivity and kinetics of *M. pneumoniae*-specific IgM and IgG antibodies towards these structures was subsequently determined with ELISA. To this end, we incubated sera of *M. pneumoniae* CAP children with either *M. pneumoniae*-glycolipid or *M. pneumoniae*-protein fractions and *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 first CAP symptoms and peaked around 1 month (20–35 days) (Figure 1E–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 (Figure 1H). These findings show that the human IgM and IgG response to *M. pneumoniae* is directed against both *M. pneumoniae*-glycolipids and –proteins.

**Figure 1.** The antibody response induced by pulmonary *M. pneumoniae* infection in children is directed against GalC and *M. pneumoniae*-protein and –glycolipid fractions.
(A–B) The presence of anti-galactocerebroside (GalC) IgM (A) and anti-GalC IgG (B) in serum of children with *M. pneumoniae* community-acquired pneumonia (CAP) and healthy controls (HCs) was determined by ELISA. Sera of children with *M. pneumoniae* CAP was obtained at median 34 days (interquartile range 30–42) after onset of symptoms (*n*=5). Dots represent individual children and the horizontal line in each graph the median. (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–G) IgG and IgM levels in serum of children with *M. pneumoniae* CAP (*n*=5) at indicated time points after onset of symptoms and reactive 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 >30 days. Data are expressed as fold increase/controls (levels in serum of HC, *n*=5). Control levels are indicated by a dashed line. The mean ± SD is shown. (H) IgG subclasses against *M. pneumoniae*-glycolipids in *M. pneumoniae* CAP and HC children (serum samples as used in A). Dots represent individual children. ***p*<0.01 (A–B, H: Mann-Whitney *U* test).

## IgG against glycolipids predominates also 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 BALF 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 the anti-*M. pneumoniae*-protein and –glycolipid antibodies are both important for clearance of *M. pneumoniae* from the lungs. To evaluate this we examined pulmonary *M. pneumoniae* 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 *M. pneumoniae* CAP children. 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 p.i. and peaked around 28 p.i. (Figure 2A–C). These findings in mice parallel the data from children by demonstrating a specific antibody response targeting both *M. pneumoniae*-proteins and –glycolipids.

Further, 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

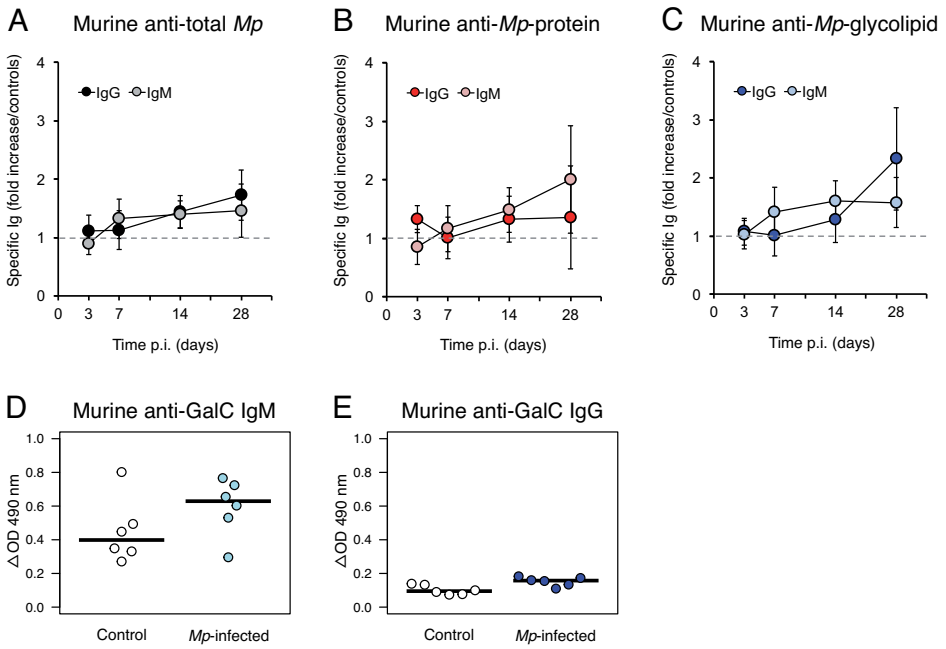



**Figure 2.** Antibody response against GalC and *M. pneumoniae*-protein and –glycolipid fractions during pulmonary *M. pneumoniae* infection in mice.
(A–C) IgG and IgM levels in serum of *M. pneumoniae*-infected C57BL/6 WT mice at indicated time points postinfection (p.i.) and reactive against total *M. pneumoniae* (A) or *M. pneumoniae*-proteins (B) and *M. pneumoniae*-glycolipids (C). Data are expressed as fold increase/controls (i.e., levels in serum of mock-infected control mice). Control levels are indicated by a dashed line. The mean ± SD is shown ($n$=6–12 mice/time point). (D–E) Presence of anti-galactocerebroside (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 individual mice and the horizontal line in each graph the median.

mice, levels of IgM antibodies to GalC were higher in *M. pneumoniae*-infected WT mice, although significance was not reached ($p$=0.19; Figure 2D). In agreement with the data from children, anti-GalC IgG was detectable at equal very low levels after pulmonary *M. pneumoniae* infection as in uninfected control WT mice (Figure 2E).

## IgG against *M. pneumoniae*-proteins but not –glycolipids is crucial to resolve *M. pneumoniae* infection in mice

We next evaluated whether a potential reduction in anti-glycolipid antibodies in Btk$^-$ mice affects *M. pneumoniae* clearance from the lungs. We thus compared Btk$^-$ mice with WT mice in terms of outcome of *M. pneumoniae* infection and anti-glycolipid antibody responses. Surprisingly, CFU counts of *M. pneumoniae* in BALF of Btk$^-$ mice were not different from those in infected WT mice (Figure 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. pneumoniae*-specific antibody levels increased over time in both WT and Btk⁻ mice, much more pronounced for IgG than IgM, but the levels in Btk⁻ mice were significantly lower than those in WT mice (Figure 3B and C). The same pattern was observed for total IgG and IgM antibody levels in Btk⁻ mice (data not shown). Although

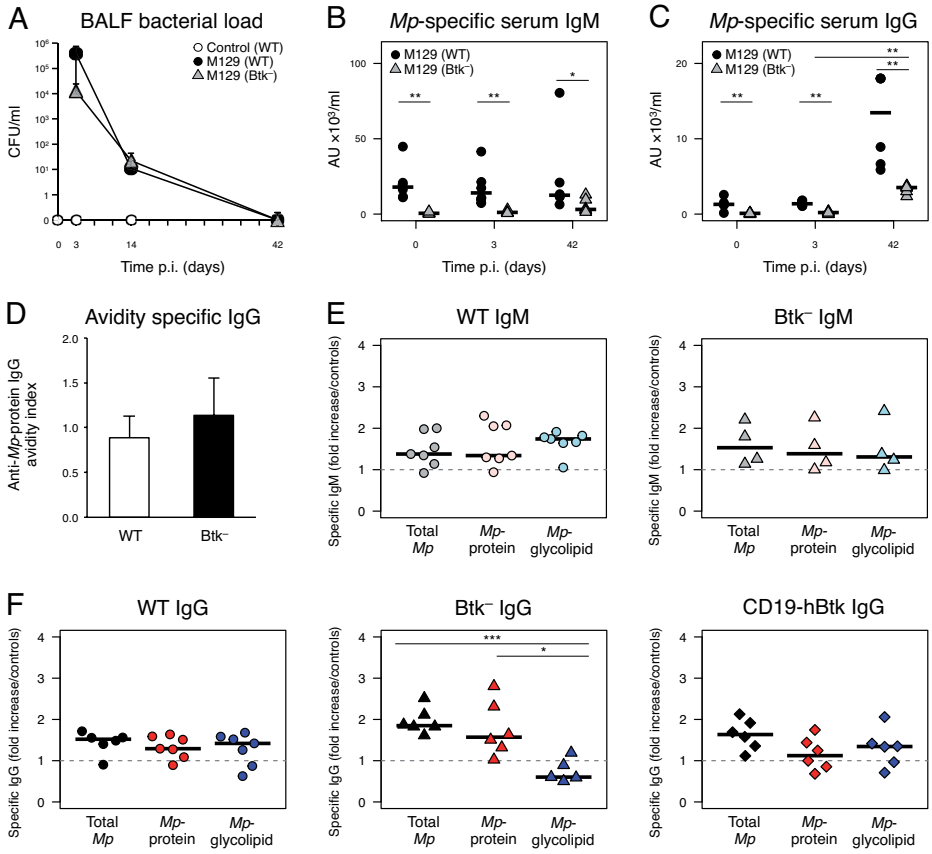



**Figure 3.** IgG against *M. pneumoniae*-proteins but not *M. pneumoniae*-glycolipids is crucial to resolve *M. pneumoniae* infection in mice.

C57BL/6 WT mice (black circles; *n*=6–12 mice/time point) and Btk⁻ mice (triangles; *n*=6–12 mice/time point) were infected intranasally with *M. pneumoniae*. Control WT mice (white circles) received SP4 medium alone. Dots represent individual mice and the horizontal line the median. (A–C) At 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/ml with interquartile range. (D) Avidity index of IgG against *M. pneumoniae*-proteins at day 42 p.i. The bars represent the mean ± SD. (E–F) Serum levels of *M. pneumoniae*-specific IgM (E) and IgG (F) against total *M. pneumoniae* (gray/black) or the separated *M. pneumoniae*-proteins (light red/red) and *M. pneumoniae*-glycolipids (light blue/blue) in serum of *M. pneumoniae*-infected C57BL/6 WT mice (circles), Btk⁻ mice (triangles), and additionally IgG in serum of CD19-hBtk mice (diamonds) at day 14 p.i. **p*<0.05, ***p*<0.01, ****p*<0.001 (A–C, E–F: Kruskal-Wallis test with post hoc Dunn's multiple comparisons test; D: Welch's *t*-test).

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 those in WT mice (Figure 3D).

The increase in IgM against *M. pneumoniae*-proteins or –glycolipids compared to controls was similar between WT and Btk⁻ mice (Figure 3E). Also comparable IgG responses to *M. pneumoniae*-proteins were measured in sera from WT and Btk⁻ mice (Figure 3F, left). In contrast, the IgG response to *M. pneumoniae*-glycolipids in Btk⁻ mice was strikingly different compared to WT mice: no IgG antibodies against *M. pneumoniae*-glycolipids were detected (Figure 3F, center). The generation of IgG antibodies against *M. pneumoniae*-glycolipids was partially restored in CD19-hBtk mice (Figure 3F, right), in which Btk is selectively rescued in B cells and only lacks in myeloid cells.[47] 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 (Figure 4A). As in serum, the IgG antibody levels to *M. pneumoniae*-

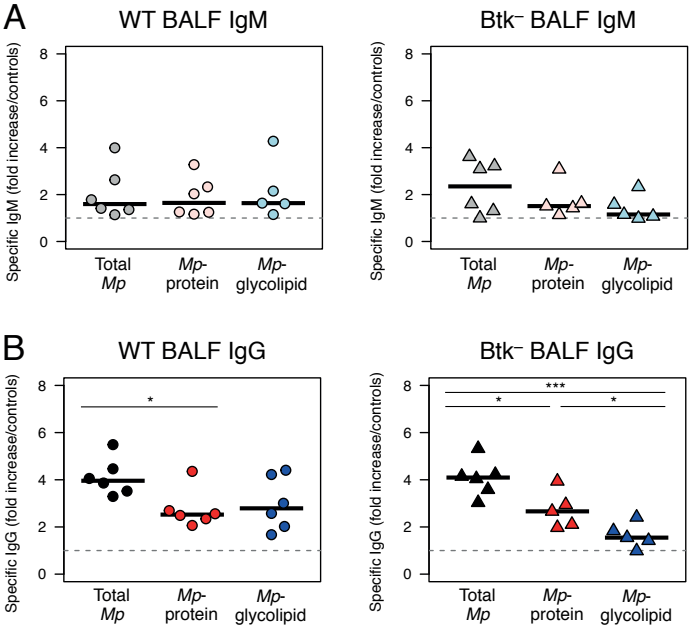



**Figure 4.** Local antibody response against *M. pneumoniae*-protein and –glycolipid fractions during pulmonary *M. pneumoniae* infection in mice.

(A–B) BALF levels of *M. pneumoniae*-specific IgM (A) and IgG (B) against total *M. pneumoniae* (gray/black) or the separated *M. pneumoniae*-proteins (light red/red) and *M. pneumoniae*-glycolipids (light blue/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 individual mice and the horizontal line the median. *$p$<0.05, ***$p$<0.001 (Kruskal-Wallis test with post hoc Dunn's multiple comparisons test).

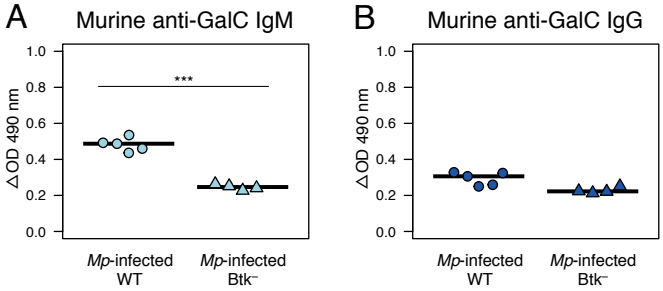



**Figure 5.** Comparison of the antibody response against GalC between WT and Btk⁻ mice during pulmonary *M. pneumoniae* infection.
(A–B) Serum anti-galactocerebroside (GalC) IgM (A) and anti-GalC IgG (B) of *M. pneumoniae*-infected WT mice ($n$=5 mice) and Btk⁻ mice ($n$=4 mice) at 42 days p.i. ***$p$<0.001 (Mann-Whitney $U$ test).

glycolipids in Btk⁻ mice were significantly lower than those in BALF of WT mice, and close to control levels (Figure 4B). Serum anti-GalC IgM and IgG antibodies were not produced by *M. pneumoniae*-infected Btk⁻ mice (Figure 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 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 comparable 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 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 compared to WT mice (data not shown). B-1a cells have been shown to inhibit the macrophage–NK cell cross-talk.[49] The observed improved control of *M. pneumoniae* replication in Btk⁻ mice may thus result from the absence of B-1a cells in these mice.[46,50] We speculate that in WT mice *M. pneumoniae* triggers 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 sufficient and/or long lasting as *M. pneumoniae* is cleared from the lungs of WT mice within 4–6 weeks.

In addition to their important role in clearance of *M. pneumoniae* in the lungs, the induced *M. pneumoniae*-protein-specific antibodies may also prevent re-infection or reduce horizontal transmission. It was shown 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., school children 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 increased risk for *M. pneumoniae* pulmonary disease and/or extrapulmonary manifestations.[51-55]

Our data reveal that the humoral response against *M. pneumoniae*-glycolipids is redundant for clearance of *M. pneumoniae* in the lungs of mice. This is rather unexpected given the fact that IgG levels to *M. pneumoniae*-glycolipids were higher than to *M. pneumoniae*-proteins and were of the IgG1 and IgG2 subclasses, which indicates potential neutralizing and complement-dependent killing of *M. pneumoniae*. Furthermore, within the pool of *M. pneumoniae*-glycolipid-specific IgM antibodies also some 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, we here confirmed that in absence of neuropathy anti-GalC IgG was not formed during pulmonary *M. pneumoniae* infection. By 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 re-challenge 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 like 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.[46,50] Notably, in contrast to *M. pneumoniae* infection, antibodies to pathogen-derived glycolipid structures had been shown 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 *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 non-classical 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 poly-reactive 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.[43,56] 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,[37,57] 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 anti-GalC IgG are removed from the circulation by target-mediated clearance.[58] 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 switch in rare cases, and produce potentially neurotoxic anti-GalC IgG. However, the exact events that lead to induction of autoreactive B cells and anti-GalC IgG remain to be identified.

## CONCLUSIONS

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 not only of importance 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 anti-glycolipid antibodies.

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# Chapter 4

## Diagnosis of *Mycoplasma pneumoniae* childhood pneumonia with measurement of specific antibody-secreting cells

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## ABSTRACT

### Background

*Mycoplasma pneumoniae* is a frequent cause of community-acquired pneumonia (CAP) in children. Timely and reliable identification of the causative pathogen is critical for initiation of effective and tailored antimicrobial treatment. Current diagnostic tests for *M. pneumoniae* infection, including polymerase chain reaction (PCR) and serology, are unable to differentiate infected patients and carriers suffering from CAP caused by other pathogens. We investigated the measurement of specific antibody-secreting cells (ASCs) by enzyme-linked immunospot (ELISpot) assay as a new diagnostic test for *M. pneumoniae* CAP.

### Methods

From a prospective longitudinal study of 152 children with CAP and 303 controls enrolled from May 2016 to April 2017, we included 63 CAP patients and 30 healthy controls based on the availability of fresh (isolated ≤4 h) peripheral blood mononuclear cells for an *M. pneumoniae*-specific IgM ASC ELISpot assay, as well as PCR on pharyngeal specimens. Results were compared using an exact McNemar test.

### Results

*M. pneumoniae* DNA was detected by PCR in 32 CAP patients (50.8%) and IgM ASCs in 29 patients (46.0%; $p$=0.72). The three CAP patients who were PCR-positive but IgM ASC-negative were diagnosed with another pathogen. *M. pneumoniae* DNA was detected in 12 controls (40.0%), all of whom tested negative for IgM ASCs ($p$<0.001). IgM ASC detection re-classified one out of six positive PCR test results (16.1%, $n$=15/93; $p$=0.021).

### Conclusions

The measurement of specific IgM ASCs by ELISpot improves the diagnosis of *M. pneumoniae* CAP. Extending this method to other pathogens may pave the way for timely and reliably determining disease etiology in childhood CAP.

## INTRODUCTION

Community-acquired pneumonia (CAP) is a common serious infection and a leading cause of hospitalization in children.[1,2] Knowledge about the underlying pathogen is a major unmet clinical need, particularly in CAP caused by *Mycoplasma pneumoniae*. Timely and reliable identification is critical for initiating effective and tailored antimicrobial treatment. However, determining the causative pathogen of childhood CAP is complicated by the low yield of blood cultures and difficulty obtaining specimens from the lower respiratory tract of children.[3,4] Therefore, clinicians attempt to detect potential pathogens in upper respiratory tract (URT) specimens,[3,4] knowing that children carry viruses and bacteria in their URT that may or may not be causative for the current pneumonia episode.[5-7] Consequently, the interpretation of diagnostic tests performed with URT specimens is limited and may lead to unnecessary antimicrobial prescriptions.[3,4,8,9]

The hurdle in differentiating infection from carriage was documented recently for *M. pneumoniae*,[10,11] a frequently reported pathogen underlying CAP in children worldwide (up to 20–40% during epidemics).[1,4,12] Current diagnostic tests, including polymerase chain reaction (PCR) of URT specimens or serology, do not differentiate between *M. pneumoniae* infection and carriage.[10] *M. pneumoniae* is found in the URT in up to 56% of healthy children.[1,10,11,13-16] These findings challenge recent epidemiological data indicating *M. pneumoniae* as the most common bacterial cause of CAP, in up to 23% of hospitalized children aged 10–17 years in the U.S.[1] In the absence of an accurate test for diagnosing *M. pneumoniae* CAP in children, it is not surprising that studies and meta-analyses on the efficacy of antibiotics are inconclusive for this condition[17,18] and that the Infectious Diseases Society of America (IDSA) guidelines target the use of antibiotics in this situation as an area needing additional research.[3] A ≥4-fold increase in antibody levels is still considered the gold standard for diagnosing *M. pneumoniae* infection,[19] but such a definition is not helpful in acute clinical management, as it requires acute and convalescent sera.

Circulating antigen-specific B cell responses have been investigated in vaccine studies and demonstrated to be more rapid and shorter lived than antibody responses.[20,21] After exposure, antigen-specific B cells proliferate and differentiate into antibody-secreting cells (ASCs) and memory B cells.[22,23] ASCs transiently circulate in the peripheral blood in the first days after an antigen encounter.[21,24] Previous preliminary studies demonstrated high sensitivity and specificity in determining disease etiology during infection using the enzyme-linked immunospot (ELISpot) assay to enumerate ASCs (reviewed in [21]). In view of these promising first results, we aimed to investigate *M. pneumoniae*-specific ASCs using ELISpot in children with CAP and controls, and to evaluate its detection as a new test for diagnosing *M. pneumoniae* CAP in a prospective clinical research set-up.

## METHODS

### Ethics statement

The ethics committee of Canton Zurich, Switzerland, approved the protocol for this study. Written informed consent was obtained from all parents and from children over 14 years of age.

### Participants

We enrolled consecutive patients between May 1, 2016, and April 30, 2017, at University Children's Hospital Zurich.

#### *CAP patients*

CAP was defined as the presence of fever >38.5°C and tachypnea according to the British Thoracic Society guidelines.[4] Trained physicians identified cases at the emergency department and on pediatric wards. Eligible participants were inpatients or outpatients aged 3–18 years. CAP patients <3 years of age were excluded to reduce the probability of viral co-existence in the URT, as it is highest in this age group.[1,4,12] Patients with hospital-acquired pneumonia, cystic fibrosis or other chronic lung disorders (excluding asthma), or known primary or secondary immunodeficiency were excluded. From this CAP cohort, we selected a series of CAP patients based on the availability of fresh (isolated ≤4 h) peripheral blood mononuclear cells (PBMCs), serum samples, and pharyngeal swabs to undergo a complete diagnostic work-up.

#### *Healthy controls*

Controls included healthy children undergoing elective surgical procedures and family members of CAP patients. Healthy children undergoing elective surgical procedures were age-matched weekly and excluded in the case of recent (≤1 week) respiratory tract infections. Pharyngeal swab and blood samples were collected at enrollment by the attending anesthesiologist before the surgical procedures while the child was under general anesthesia. In order to identify asymptomatic *M. pneumoniae* carriers, we also recruited and followed siblings and/or parents of CAP patients. Family members were also excluded in case of respiratory tract infection. From this control cohort, we selected a series of healthy controls according to the same criteria as the CAP series.

### Study procedures

#### *Data collection*

We systematically collected demographic, epidemiological, and clinical data at enrollment and follow-up visits using a standardized questionnaire.

*Collection of biological samples*

Swabs were taken from the posterior pharynx using flocked nylon fiber tip swabs (Copan Diagnostics, Murrieta, CA, USA). Blood samples were collected in anticoagulated lithium heparin blood collection tubes and fresh PBMCs isolated ≤4 h after sampling to avoid poor assay performance due to decreased ASC viability. PBMCs were isolated by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and viability assessed by trypan blue exclusion. Serum was stored at −80°C. Participants were sampled at enrollment (day 0) and at short (<2 weeks), long (2 weeks–2 months), and/or late intervals (2–6 months).

*M. pneumoniae real-time PCR*

DNA isolation was performed using pharyngeal swab sample medium and the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). A quantitative TaqMan (Applied Biosystems, Foster City, CA, USA) real-time PCR assay was used to detect and quantify *M. pneumoniae* DNA as described previously.[25]

*M. pneumoniae-specific enzyme-linked immunosorbent assay (ELISA)*

Serum samples were tested for the presence of *M. pneumoniae*-specific IgM and IgG antibodies using a commercially available ELISA (Virion\Serion, Würzburg, Germany).

*M. pneumoniae-specific ASC ELISpot*

The frequency of circulating IgM ASCs was measured by ELISpot assays using fresh PBMCs, to avoid poor performance due to reduced ASC recovery after a freeze-thaw cycle, without further culture as described previously,[26] with some modifications. Briefly, 96-well ELISpot filter plates (Millipore, Billerica, MA, USA) were coated for 90 min at 37°C with the different antigens diluted in sterile phosphate-buffered saline (PBS). The antigens were: detergent extract of *M. pneumoniae* enriched for highly specific adhesion protein P1 (2 μg/ml; Serion Immunologics, Würzburg, Germany); Fluarix® Tetra quadrivalent influenza virus vaccine (6 μg/ml; GlaxoSmithKline, Middlesex, UK); and affinity-purified antibodies to human Ig light chains (λ and κ, 10 μg/ml; Southern Biotech, Birmingham, AL, USA) as the positive control. The negative control consisted of PBS only in uncoated wells. After washing, coated plates were blocked with medium for another 90 min at 37°C. Coated plates were incubated at 37°C for 16–20 h with 100,000 or 10,000 viable PBMCs, and each dilution was used in triplicate. Plates were then washed, incubated with biotinylated anti-IgM and alkaline phosphatase (AP)-conjugated streptavidin (all Southern Biotech), and spots visualized using an AP substrate kit (Bio-Rad Laboratories, Hercules, CA, USA), with each spot appearing at the former location of a single ASC. Spots were counted by an ELISpot reader (AID, Strassberg, Germany) using pre-defined settings. The spots identified by the machine were manually inspected for the

presence of artifacts. Antigen-specific spot counts were calculated as the mean of three wells minus the mean number of spots in PBS wells. Data were expressed as ASCs per $10^6$ PBMCs.[26]

## Statistical analysis

We report dichotomous variables as percentages and continuous variables as medians with interquartile ranges (IQRs). The Mann-Whitney *U* test was used to compare medians, and the $\chi^2$ or Fisher's exact test to compare proportions. To test differences in the classification accuracy of PCR and IgM ASC ELISpot, we performed the exact McNemar test. All reported *p*-values are 2-tailed with significance at <0.05. Analyses were performed using the Stata 14.2 statistics software package (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

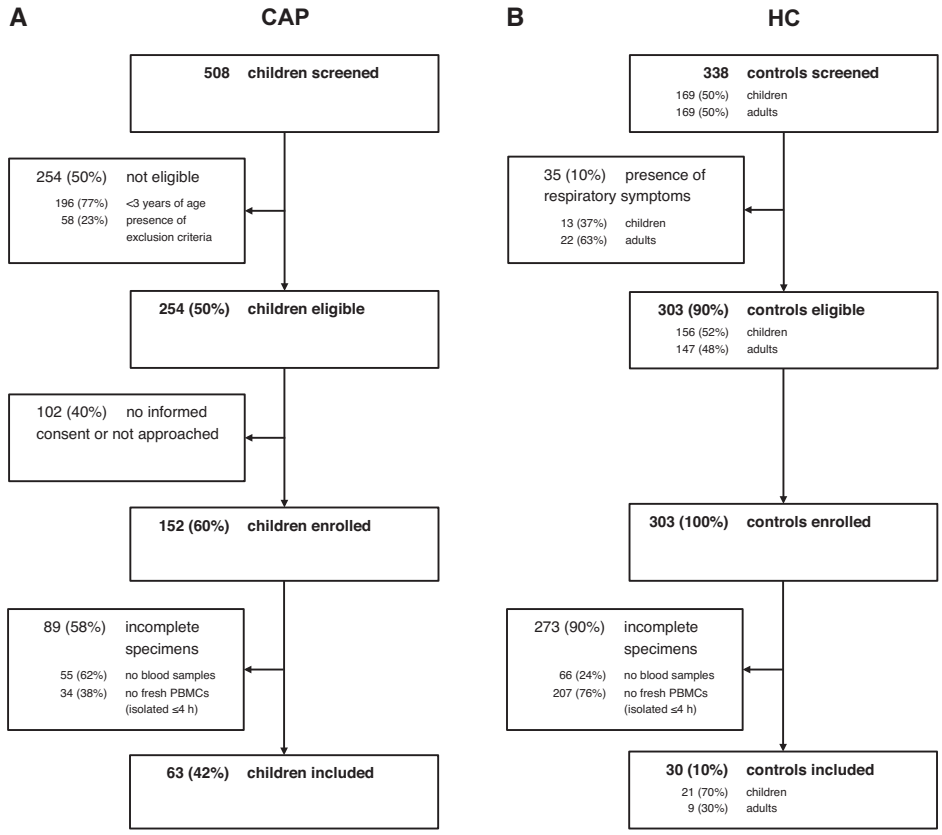


**Figure 1.** Study profile.
Recruitment and flow of (A) CAP patients and (B) controls. Abbreviations: CAP, community-acquired pneumonia; HC, healthy control; PBMC, peripheral blood mononuclear cell.

## RESULTS

During the 12-month study period, we screened a total of 508 CAP patients and 338 controls (Figure 1). A total of 254 CAP patients who were not eligible (i.e., <3 years of age or presence of exclusion criteria) and 102 patients who did not provide informed consent or were not approached were excluded. Of the enrolled population (Figure 1), we included 63 CAP patients and 30 healthy controls who were able to undergo a complete diagnostic work-up specific for *M. pneumoniae*, including PCR of pharyngeal swab samples, IgM ASC ELISpot assay of fresh (isolated ≤4 h) PBMCs, and ELISA of serum samples. The baseline characteristics of the included patients are shown in Table 1. There were no differences between included and enrolled CAP patients and controls in regards to age, sex, and season at enrolment (Table 1).

In the CAP series, *M. pneumoniae* DNA was detected by PCR in 32 patients (50.8%) and *M. pneumoniae*-specific IgM ASCs were detected by ELISpot in 29 patients (46.0%; $p$=0.72). Representative IgM ASC ELISpot responses are shown in Figure 2. These samples were collected at disease presentation a median 12 days after the onset of symptoms (IQR

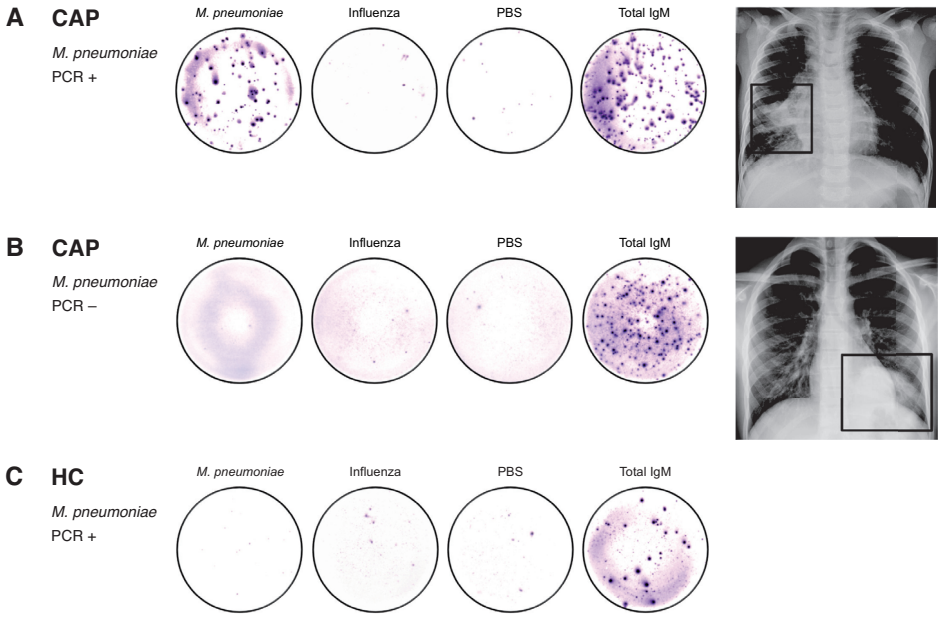


**Figure 2.** *M. pneumoniae*-specific IgM ASC ELISpot response.
Assays were specific for the following antigens: *M. pneumoniae*, influenza A and B virus, and total IgM. The negative control consisted of PBS only in uncoated wells. Representative patterns of ELISpot wells with 10,000 PBMCs per well are shown. Corresponding chest radiographs of CAP patients are shown on the right. The pulmonary infiltrate is indicated with a frame. (A) *M. pneumoniae* PCR-positive CAP. (B) *M. pneumoniae* PCR-negative CAP. (C) *M. pneumoniae* PCR-positive HC (carrier). Abbreviations: CAP, community-acquired pneumonia; HC, healthy control; Ig, immunoglobulin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

**Table 1.** Baseline characteristics of CAP patients and controls.

| | **CAP** | | **HC** | | Differences between included CAP patients and included HCs |
|---|---|---|---|---|---|
| | **Included** | Enrolled | **Included** | Enrolled | |
| | **(*n*=63)** | (*n*=152) | **(*n*=30; *n*=21 children; *n*=9 adults)** | (*n*=303; *n*=156 children; *n*=147 adults) | *p* |
| Age, median (IQR) | 6.0 (4.4–10.2) | 5.7 (4.3–8.9) | Children: 6.1 (4.9–7.9)<br>Adults: 40.2 (39.1–42.1) | Children: 5.9 (4.3–8.1)<br>Adults: 39.1 (34.6–43.2) | 0.90<br>– |
| Sex (male), *n* (%) | 39 (62) | 84 (55) | Children: 17 (81)<br>Adults: 1 (11) | Children: 102 (65)<br>Adults: 56 (38) | 0.18<br>– |
| Season at enrollment, *n* (%): | | | | | |
| - Spring (March–May) | 11 (17) | 21 (14) | 9 (30) | 61 (20) | 0.27 |
| - Summer (June–August) | 13 (21) | 30 (20) | 13 (44) | 56 (19) | 0.04 |
| - Autumn (September–November) | 17 (27) | 37 (24) | 4 (13) | 43 (14) | 0.17 |
| - Winter (December–February) | 22 (35) | 64 (42) | 4 (13) | 143 (47) | 0.05 |
| Day-care or pre-/school attendance, *n* (%) | 63 (100) | NA | Children: 20 (95) | NA | 0.56 |
| Immunizations[1], *n* (%) | 50/51 (98) | NA | Children: 14/14 (100) | NA | 1.00 |
| Preexisting disease, *n* (%) | 10 (16) | 32 (21) | Children: 12 (57)<br>Adults: 0 (0) | Children: 137 (88)<br>Adults: 1/34 (3) | <0.001<br>– |
| - ENT, *n* (%) | 0 | 4 | 12 | 137[2] | – |
| - Asthma or history of wheezing, *n* (%) | 2 | 8 | 0 | 0 | – |
| - Cardiovascular, *n* (%) | 0 | 2 | 0 | 0 | – |
| - Gastrointestinal, *n* (%) | 2 | 2 | 0 | 0 | – |
| - Neurological, *n* (%) | 2 | 5 | 0 | 0 | – |
| - Other, *n* (%) | 4 | 11 | 0 | 1 | – |

Abbreviations: CAP, community-acquired pneumonia; HC, healthy control; ENT, ear, nose, and throat; IQR, interquartile range; NA, not available.
Differences between groups were determined by the Mann-Whitney *U* test (medians) and Fisher's exact test (proportions).
[1] "Immunizations" refers to being immunized per the national immunization schedule in Switzerland;
[2] Children with elective surgery at the division of otolaryngology (*n*=137): hyperplasia of adenoids (*n*=62); eustachian catarrh (*n*=26); cysts, fistulae, and sinuses (*n*=10); protruding ears (*n*=8); and others (*n*=31).

11–16, range 2–29) and yielded a median *M. pneumoniae*-specific IgM ASC frequency of 443 spots per $10^6$ PBMCs (IQR 176–1933, range 27–9133; Figure 3). Three siblings of CAP patients developed CAP during follow-up and also tested positive for *M. pneumoniae*-specific IgM ASCs. Interestingly, one of the three siblings tested negative by ELISpot 3 days prior to the onset of symptoms followed by a positive IgM ASC ELISpot result 6 days after developing symptoms.

In the three PCR-positive CAP patients who tested negative for IgM ASCs, another pathogen was found based on the results of multiplex PCR from pharyngeal swab samples and specific serology: adenovirus (patient 1), rhinovirus (patient 2), and respiratory syncytial virus (patient 3) (Table 2). These three patients had significantly lower pharyngeal *M. pneumoniae* DNA levels than IgM ASC ELISpot-positive CAP patients (Figure 3). Though all IgM ASC-positive patients were IgM-seropositive for *M. pneumoniae*, specific IgM antibodies were also found in 10.7% of CAP patients negative for IgM ASCs.

We compared clinical characteristics between *M. pneumoniae*-specific IgM ASC-positive and –negative CAP patients (Table 3). *M. pneumoniae*-positive patients were older than *M. pneumoniae*-negative patients ($p<0.001$) and presented less frequently in winter ($p=0.04$) and had less frequent preexisting diseases ($p=0.02$). The duration of respiratory symptoms and fever prior to CAP diagnosis was longer in *M. pneumoniae*-positive patients compared to *M. pneumoniae*-negative patients ($p<0.001$). Other clinical characteristics, hospitalization rates, length of hospital stay, and outcomes were similar between the two groups.

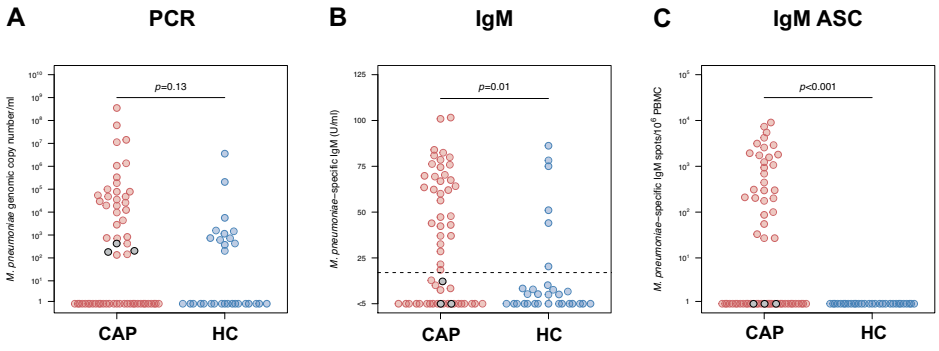


**Figure 3.** Comparison of diagnostic test results between CAP patients and controls.
(A) *M. pneumoniae* DNA levels in pharyngeal swab samples. (B) *M. pneumoniae*-specific IgM levels. The dashed horizontal line represents the cut-off for the test (17 U/ml), and the lower limit of quantification is 5 U/ml. (C) *M. pneumoniae*-specific IgM ASC responses. PCR-positive CAP patients testing negative for IgM ASCs are indicated in gray. Differences in medians between series are indicated with the corresponding *p* value (Mann-Whitney *U* test). Abbreviations: ASC, antibody-secreting cell; CAP, community-acquired pneumonia; HC, healthy control; Ig, immunoglobulin; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; U, unit.

**Table 2.** Characteristics and final diagnosis of CAP patients PCR-positive for *M. pneumoniae* but negative by IgM ASC ELISpot.

| | **Patient 1** | | | **Patient 2** | **Patient 3** | |
|---|---|---|---|---|---|---|
| **Demographic characteristics** | | | | | | |
| Age (years) | 4.5 | | | 5.9 | 3.4 | |
| Sex | M | | | M | M | |
| Season at enrollment | October | | | November | March | |
| Preexisting disease | No | | | No | No | |
| **Clinical characteristics** | | | | | | |
| Prodrome: | | | | | | |
| - Respiratory symptoms (days) | 1 | | | 4 | 4 | |
| - Fever (days) | 2 | | | 5 | 4 | |
| Symptoms and signs: | | | | | | |
| - Fever (°C) | 40.2 | | | 39.0 | 38.7 | |
| - Runny nose | Yes | | | Yes | No | |
| - Sore throat | Yes | | | Yes | Yes | |
| - Cough | No | | | Yes | Yes | |
| - Chest pain | No | | | No | No | |
| - Wheezing | No | | | No | No | |
| - Abnormal auscultatory findings | Yes | | | No | Yes | |
| - Oxygen saturation <93% | No | | | No | No | |
| Hospitalization | No | | | No | No | |
| Outcome with full clinical recovery | Yes | | | Yes | Yes | |
| **Microbiological characteristics** | | | | | | |
| PCR: | | | | | | |
| *M. pneumoniae* genomic copy number/ml | 415 | | | 213 | 177 | |
| Other pathogens detected by multiplex PCR: | Adenovirus<br>Rhinovirus<br>*Chlamydia pneumoniae* | | | Rhinovirus | RSV A<br>Human bocavirus | |
| Serology: | | | | | | |
| - Time point of serum sample collection after onset of symptoms (days) | 1 | 7 | 20 | 12 | 19 | 40 |
| *M. pneumoniae*-specific antibodies: | negative | negative | negative | negative | negative | negative |
| - IgM (≤17 U/ml) | 5 | 13 | 12 | 2 | 4 | 3 |
| - IgG (≤15 U/ml) | 1 | 3 | 2 | 1 | 2 | 2 |
| *C. pneumoniae*-specific antibodies: | negative | negative | negative | – | – | – |
| - IgM (<10 U/ml) | 5 | 8 | 9 | – | – | – |
| - IgG (<10 U/ml) | <4 | <4 | <4 | – | – | – |

**Table 2.** Characteristics and final diagnosis of CAP patients PCR-positive for *M. pneumoniae* but negative by IgM ASC ELISpot. (*continued*)

| | **Patient 1** | | | **Patient 2** | **Patient 3** | |
|---|---|---|---|---|---|---|
| Adenovirus-specific antibodies: | positive | positive | positive | – | – | – |
| - IgM (<1 Index) | <1 | <1 | <1 | – | – | – |
| - IgG (<13 U/ml) | 19 | 23 | 24 | – | – | – |
| RSV-specific antibodies: | – | – | – | – | positive | positive |
| - IgM (<1 Index) | – | – | – | – | <1 | <1 |
| - IgG (<15 U/ml) | – | – | – | – | 27 | 16 |
| **Final diagnosis:** | Adenovirus | | | Rhinovirus | RSV A | |

Abbreviations: CAP, community-acquired pneumonia; Ig, immunoglobulin; M, male; PCR, polymerase chain reaction; RSV, respiratory syncytial virus.



Among controls, *M. pneumoniae* DNA was detected in pharyngeal swab samples from 12 subjects (40.0%). Bacterial DNA levels varied between $1.8\times10^2$ and $3.6\times10^6$. All of these subjects tested negative for IgM ASCs ($p<0.001$; Figure 3). Thus, IgM ASC detection re-classified one out of six positive PCR test results over the total sampled population (16.1%, $n=15/93$; $p=0.021$). In contrast to the negative result with IgM ASC ELISpot, some healthy controls showed a combination of *M. pneumoniae*-specific serum IgM and PCR (33.3%), and even exhibited seroconversion to IgG (28.6%) or a ≥4-fold increase in IgG (8.3%). Though some participants were serologically or PCR-positive for *M. pneumoniae* up to 3 months following enrollment, specific ASC responses were undetectable throughout the entire follow-up period. These results were in line with the lack of respiratory symptoms during follow-up visits.

## DISCUSSION

In this prospective longitudinal study, the measurement of specific peripheral blood IgM ASCs by ELISpot improved the diagnosis of *M. pneumoniae* CAP in children. Specific IgM ASC detection re-classified one out of six positive PCR test results, and thus corrected misdiagnosis of *M. pneumoniae* infection in CAP patients, in which the detection of *M. pneumoniae* DNA in pharyngeal samples reflected carriage but not disease-causing infection in the absence of specific IgM ASCs. These findings help resolve a major, longstanding obstacle in the clinical management of pneumonia, but also in research. Accurate identification of the causative pathogen in CAP from a peripheral blood sample during acute infection rectifies the hitherto overstated prevalence of *M. pneumoniae* CAP and allows targeted therapeutic management. In the context of research, this test could be useful for selecting patients for clinical investigations assessing the efficacy of antimicrobial treatment for this disease entity.

**Table 3.** Clinical characteristics of *M. pneumoniae*-specific IgM ASC ELISpot-positive vs. negative CAP patients.

| | **IgM ASC ELISpot + (*n*=29)** | **IgM ASC ELISpot – (*n*=34)** | OR (95% CI) | *p* |
|---|---|---|---|---|
| **Demographic characteristics** | | | | |
| Age (years), median (IQR) | 8.6 (6.3–11.0) | 4.7 (3.9–6.2) | – | <0.001 |
| Sex (male), *n* (%) | 16 (55) | 23 (68) | 0.6 (0.2–1.9) | 0.43 |
| Season at enrollment, *n* (%): | | | | |
| - Spring (March–May) | 5 (17) | 6 (18) | 1.0 (0.2–4.4) | 1.00 |
| - Summer (June–August) | 9 (31) | 4 (12) | 3.3 (0.8–16.8) | 0.07 |
| - Autumn (September–November) | 9 (31) | 8 (23) | 1.5 (0.4–5.2) | 0.58 |
| - Winter (December–February) | 6 (21) | 16 (47) | 0.3 (0.1–1.0) | 0.04 |
| Preexisting disease, *n* (%) | 1 (3) | 9 (26) | 0.1 (0.0–0.8) | 0.02 |
| - Asthma or history of wheezing, *n* (%) | 1 (3) | 1 (3) | 1.2 (0.0–95.2) | 1.00 |
| **Clinical characteristics** | | | | |
| Prodrome: | | | | |
| - Respiratory symptoms (days), median (IQR) | 9.0 (7.0–10.5) | 4.0 (3.0–7.0) | – | <0.001 |
| - Fever (days), median (IQR) | 8.0 (7.0–10.0) | 4.0 (3.3–6.0) | – | <0.001 |
| Symptoms and signs: | | | | |
| - Fever (°C), median (IQR) | 39.1 (39.0–39.7) | 39.2 (39.0–39.5) | – | 0.89 |
| - Runny nose, *n* (%) | 7 (24) | 14 (41) | 0.5 (0.1–1.5) | 0.19 |
| - Sore throat, *n* (%) | 4 (14) | 4 (12) | 1.2 (0.2–7.1) | 1.00 |
| - Cough, *n* (%) | 27 (93) | 30 (88) | 1.8 (0.2–21.2) | 0.68 |
| - Chest pain, *n* (%) | 3 (10) | 7 (21) | 0.5 (0.1–2.2) | 0.32 |
| - Wheezing, *n* (%) | 0 (0) | 1 (3) | NA | 1.00 |
| - Abnormal auscultatory findings, *n* (%) | 19 (66) | 24 (71) | 0.8 (0.2–2.6) | 0.79 |
| - Oxygen saturation <93%, *n* (%) | 5 (17) | 8 (24) | 0.7 (0.2–2.8) | 0.76 |
| Hospitalization, *n* (%) | 9 (31) | 19 (56) | 0.4 (0.1–1.1) | 0.07 |
| - LOS (days), median (IQR) | 4.0 (3.0–7.0) | 2.5 (2.0–5.8) | – | 0.31 |
| - ICU admission, *n* (%) | 0 (0) | 1 (3) | NA | 1.00 |
| Outcome with full clinical recovery at 6 months, *n* (%) | 27[1] (93) | 34 (100) | NA | 0.21 |

Abbreviations: CAP, community-acquired pneumonia; IQR, interquartile range; ICU, intensive care unit; LOS, length of hospital stay; NA, not available.

Differences between included CAP patients and controls were determined by the Mann-Whitney *U* test (medians) and Fisher's exact test (proportions).

[1] Abnormal outcomes: bronchiolitis obliterans with decreased lung function (*n*=1), exertional dyspnea without physical findings, i.e., normal lung and cardiac function (*n*=1).

We corroborate the results of the only previous study on *M. pneumoniae*-specific ASCs, which included 12 *M. pneumoniae*-seropositive children with CAP[27]; IgM ASCs were detected within 5 days after symptom onset and declined rapidly upon resolution of CAP. In our study, we detected specific IgM ASCs as early as 2 days after the presentation of clinical symptoms, whereas a peak occurred at presentation of CAP. In contrast to this previous study, we included a series of asymptomatic controls. This addition is essential when assessing the usefulness of specific IgM ASCs as a diagnostic test that differentiates between carriage and infection.

A strength of the current study is the extensive investigation of a well-defined study cohort of children, both patients and controls, using pharyngeal swabs and fresh blood samples at several time points up to 6 months following CAP diagnosis. Some of the siblings of index patients were included as controls, which made it possible to investigate the pattern symptom acquisition and/or changes in the results of the diagnostic tests. The high detection rate of *M. pneumoniae* by PCR in this study is related to the inclusion age of 3–18 years, the range in which *M. pneumoniae* is most frequently detected,[1,4,12] and the coinciding *M. pneumoniae* epidemic in Europe during the study period (data not shown). We were able to assess the extent of misclassification in pharyngeal PCR-guided clinical management of children with CAP using the new specific IgM ASC ELISpot. The new test re-classified one out of six positive PCR test results. However, the study also has several limitations. First, though sampling was performed in a relevant population of clinical CAP and healthy controls, the study population represents a convenience sample, and we cannot rule out that selection occurred. Nevertheless, the baseline characteristics of both CAP patients and controls did not deviate from the enrolled population. Second, we are unable to provide information on co-colonization or co-infection in both CAP patients and healthy controls, as we did not systematically test for other pathogens. However, *M. pneumoniae* was recently shown to frequently coexist with other bacterial and viral pathogens in the URT of both symptomatic and asymptomatic children.[1,10] Therefore, detection of other pathogens would not likely have changed the conclusions of this study. Finally, children <3 years of age were excluded and, therefore, performance of the IgM ASC ELISpot in children of that age is unclear.

The ASC ELISpot is a robust technique,[21,28] and the protocol described here could be translated directly into the clinical setting to diagnose *M. pneumoniae* infection by using only a small volume of peripheral blood (≥1 ml). Yet, the ASC ELISpot is quite labor-intensive requiring fresh or frozen PBMCs[26,28] and has a rather long overall turnaround time (~24 h), but alternative protocols were developed recently that suggest more rapid (~6–8 h) ASC detection.[26] Optimizing such protocols in the future may help translate the IgM ASC ELISpot assay into routine clinical care.

To date, the specific IgM ASC ELISpot assay is the only test that timely and reliably differentiates *M. pneumoniae* infection from carriage. Improving the early diagnosis of *M.*

*pneumoniae* infection in CAP patients is of paramount importance, as it offers the opportunity to avoid the overuse of and resistance against empirical antimicrobial treatment; a positive test result will justify ceasing empirical administration of β-lactams, to which *M. pneumoniae* is intrinsically resistant, and starting the administration of macrolides once their effect is proven.[3,4] As pneumonia accounts for more days of antibiotic use in U.S. children's hospitals than any other condition,[29] targeted antimicrobial management efforts and cost-effectiveness considerations are substantial.[8,9,30] Accurate early diagnosis provided by the specific IgM ASC ELISpot assay is also essential for future interventional studies assessing the effect of antimicrobial treatment in the management of *M. pneumoniae* CAP.[17,18,30]

## CONCLUSIONS

Our findings on the specific ASC response in childhood CAP are promising, supporting the need for implementation research to examine the added clinical utility of the IgM ASC ELISpot assay to diagnose *M. pneumoniae* infection in the clinical care of children with CAP. Extending this method to other pathogens may pave the way for timely and reliably determining disease etiology in childhood CAP.

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## ACKNOWLEDGEMENTS

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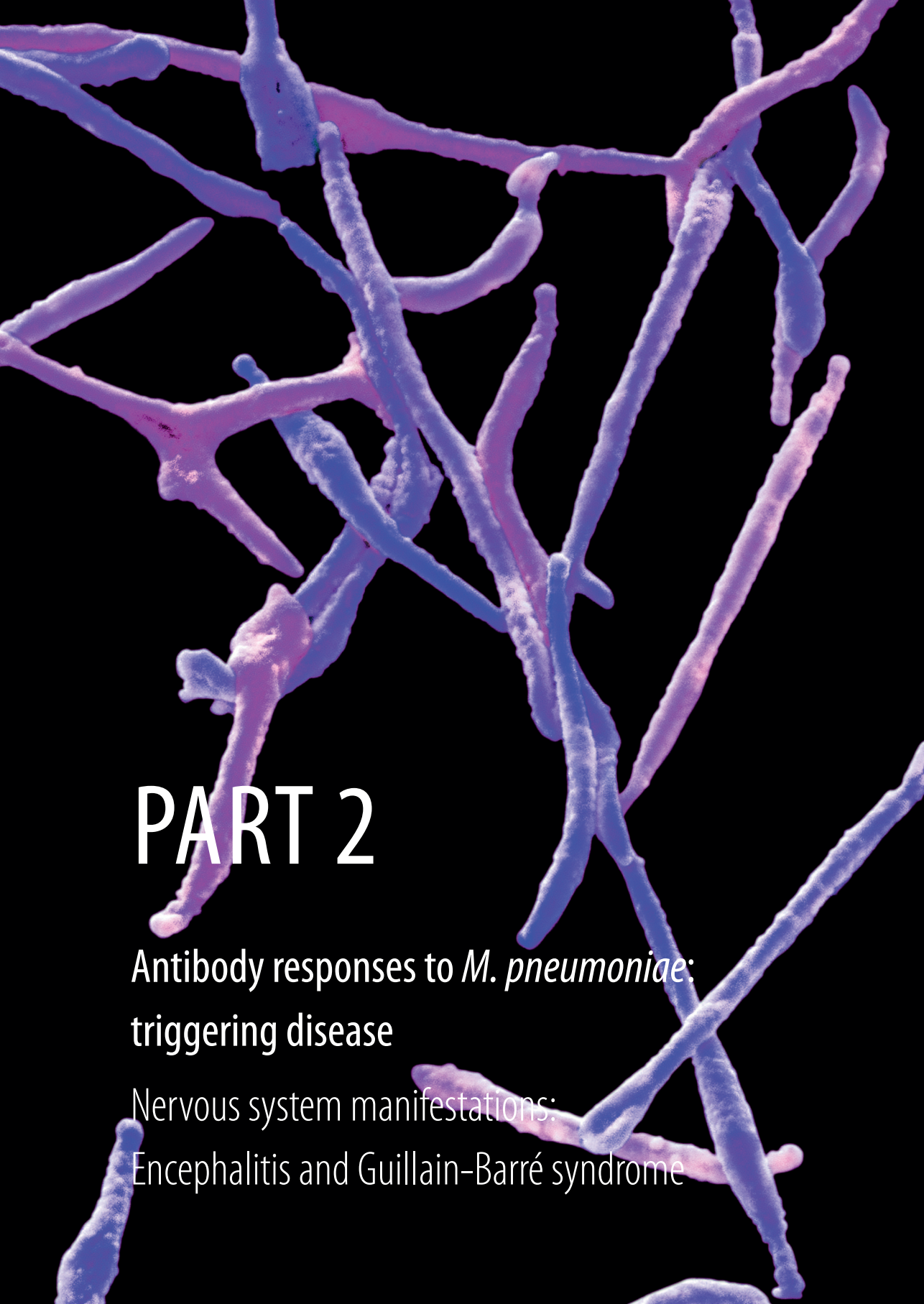
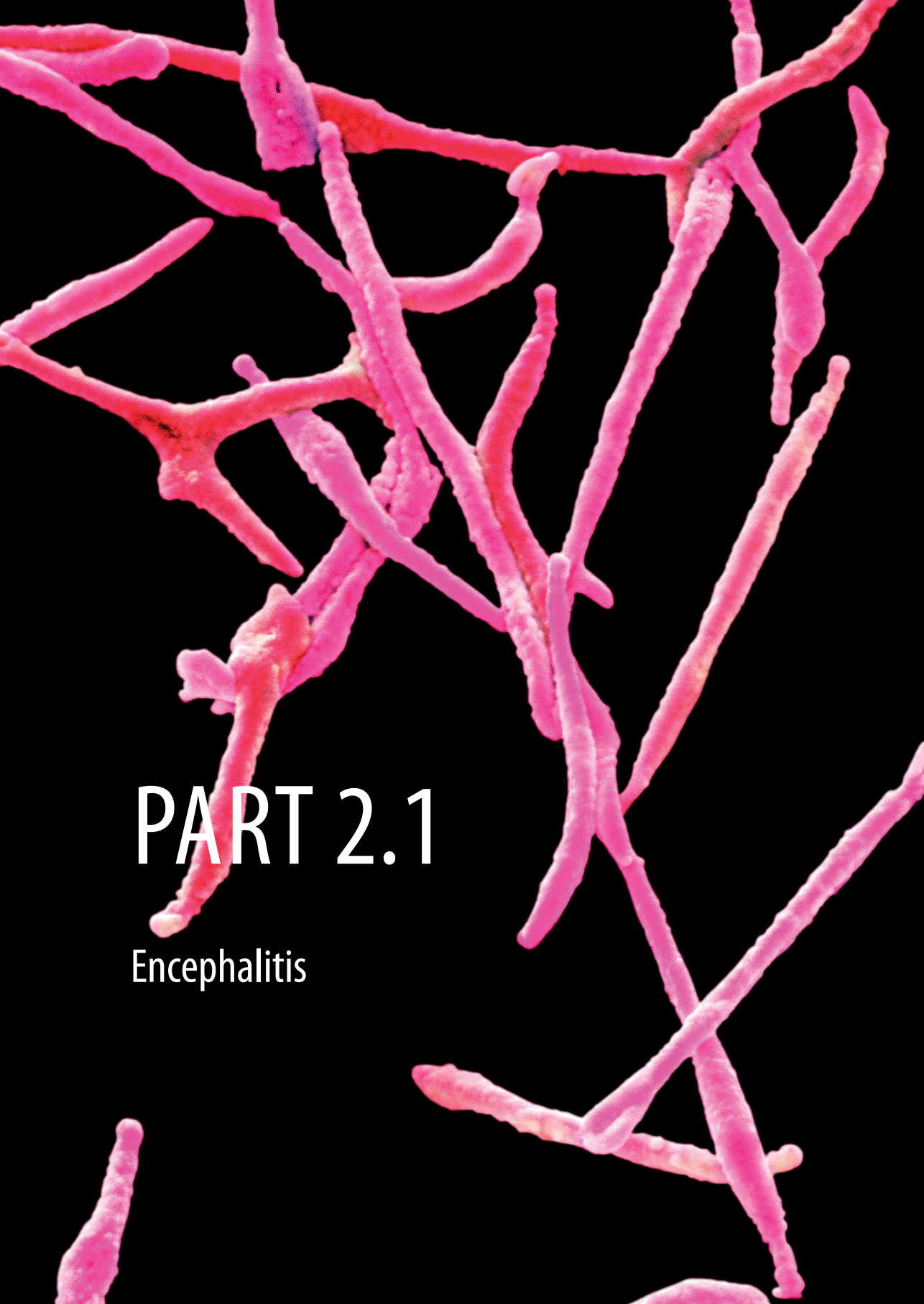


# PART 2

## Antibody responses to *M. pneumoniae*: triggering disease

Nervous system manifestations: Encephalitis and Guillain-Barré syndrome



# PART 2.1

## Encephalitis

## INTRODUCTION TO ENCEPHALITIS

Encephalitis is a severe neurologic disorder characterized by inflammation of the brain parenchyma.[1] It results in substantial morbidity and mortality worldwide. The clinical diagnosis is complicated as the symptoms and signs are similar to many other serious neurological diseases. There are many infectious and immune-mediated etiologies, but specific causes are identified in only about 50% of the cases.

### Clinical definition

Encephalitis is defined as inflammation of the brain parenchyma associated with neurologic dysfunction.[2] Pathologic examination and testing of brain tissue are considered to be the "gold standard" diagnostic test. Because of the risk of invasive neurosurgical procedures, the clinical definition of encephalitis is based on selected clinical, laboratory, electroencephalographic, and neuroimaging features in most cases. Current clinical diagnostic criteria for encephalitis are listed in Table 1. The clinical definition is viewed to be complementary to the etiological definition, since diagnosis of an infection with an organism that is strongly associated with encephalitis would confirm a clinical diagnosis of encephalitis.[1]



**Table 1.** Diagnostic criteria for encephalitis and encephalopathy of presumed infectious or autoimmune etiology.

| **Major criterion (required):** |
|---|
| Patients presenting to medical attention with altered mental status (defined as decreased or altered level of consciousness, lethargy or personality change) lasting 24 h with no alternative cause identified. |
| **Minor criteria (2 required for *possible* encephalitis; 3 required for *probable* or *confirmed** encephalitis):** |
| - Documented fever ≥38°C (within the 72 h before or after presentation); |
| - Generalized or partial seizures not fully attributable to a preexisting seizure disorder; |
| - New onset of focal neurologic findings; |
| - CSF WBC count ≥5/mm$^3$; |
| - Abnormality of brain parenchyma on neuroimaging suggestive of encephalitis that is either new from prior studies or appears acute in onset; |
| - Abnormality on EEG that is consistent with encephalitis and not attributable to another cause. |

Abbreviations: CSF, cerebrospinal fluid; EEG, electroencephalogram; WBC, white blood cell. Table adapted from Venkatesan et al.[1]

* Confirmed encephalitis requires one of the following: (1) pathologic confirmation of brain inflammation consistent with encephalitis; (2) defined pathologic, microbiologic, or serologic evidence of acute infection with a microorganism strongly associated with encephalitis from an appropriate clinical specimen;[3] or (3) laboratory evidence of an autoimmune condition strongly associated with encephalitis.[7]

### Etiological definition

The most commonly demonstrated etiology in encephalitis is infection, predominantly caused by viruses. However, defining the causal relationship between a microbe and en-

cephalitis is complex.[3] Over 100 different infectious agents may cause encephalitis, often as one of the rarer manifestations of infection. Further, the failure to identify a pathogen does not exclude the diagnosis. In fact, a higher proportion of studies with children compared to with adults had 50% of cases with unknown etiology (62% vs. 20%).[4]

The "gold standard" techniques to detect causative infectious agents in encephalitis depend on brain biopsy material. Because this is not possible in most cases, there exists a hierarchy of sample locations to attribute causality (Figure 1).[3]

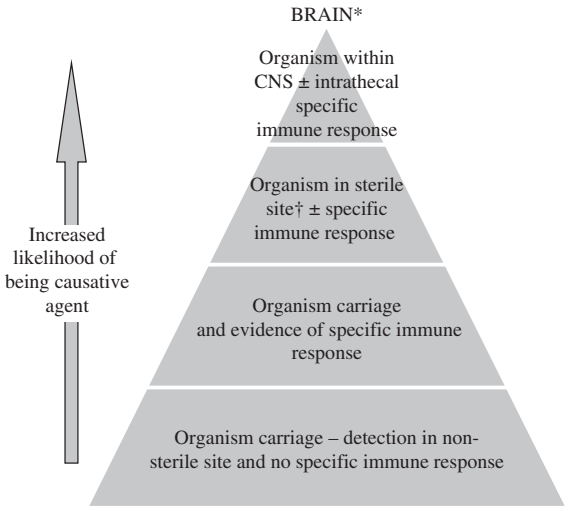



**Figure 1.** Hierarchy of diagnostic tests for defining causal relationship between a pathogen and the syndrome encephalitis.
*Hierarchy not relevant for all bacteria and viruses, e.g., rabies virus. †Normally sterile site: blood, cerebrospinal, joint, pleural, or pericardial fluid. Reproduced with permission from Granerod et al.,[3] Cambridge University Press.

## Specific etiologies

The multiple etiologies in pediatric encephalitis of a recent study are shown in Table 2. Overall, *Mycoplasma pneumoniae* infection is reported in 5–10% of pediatric encephalitis patients.[5,6] In this thesis, we investigate the association of *M. pneumoniae* with pediatric encephalitis (**Chapter 5–8**).

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**Table 2.** Etiology of pediatric encephalitis.

| **Etiology of encephalitis** | **Total** | |
|---|---|---|
| | *n* | % |
| **Infectious** | **49** | **30** |
| - Enterovirus | 20 | 12 |
| - *M. pneumoniae* | 11 | 7 |
| - Herpes simplex virus | 9 | 5 |
| - Cytomegalovirus | 3 | 2 |
| - other* | 6 | 4 |
| **Infection-associated encephalopathy** | **13** | **8** |
| - Influenza virus | 5 | 3 |
| - Rotavirus | 5 | 3 |
| - Acute necrotizing encephalopathy** | 3 | 2 |
| **Immune-mediated or autoantibody-associated** | **56** | **34** |
| - Acute disseminated encephalomyelitis (ADEM) | 35 | 21 |
| - N-methyl-D-aspartate receptor (NMDAR) antibody | 10 | 6 |
| - Voltage-gated potassium channel complex (VGKC-complex) antibody | 7 | 4 |
| - Dopamine D2 receptor antibody | 4 | 2 |
| **Unknown** | **46** | **28** |
| **Total** | **164** | **100** |

Table about etiology of pediatric encephalitis in Australia, 1998–2010, adapted from Pillai et al.[8]
* Group A *Streptococcus* (*n*=1), adenovirus (*n*=1), Epstein-Barr virus (*n*=1), parainfluenza virus 3 (*n*=1), parvovirus B19 (*n*=1), varicella zoster virus (*n*=1);
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# Chapter 5

## *Mycoplasma pneumoniae* intrathecal antibody responses in Bickerstaff brain stem encephalitis

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## ABSTRACT

The pathogenesis of *Mycoplasma pneumoniae* encephalitis is not established. We report, for the first time, the case of a patient with severe Bickerstaff brain stem encephalitis in whom we detected intrathecal production of *M. pneumoniae*-specific antibodies, contrasting the findings in another patient with less severe encephalitis in whom we detected intrathecal *M. pneumoniae* DNA but no specific antibodies. Our observations suggest that intrathecal *M. pneumoniae*-specific antibody responses may contribute to a more severe course of *M. pneumoniae* encephalitis.

## INTRODUCTION

*Mycoplasma pneumoniae* is a leading cause of encephalitis in children.[1] The paucity of reports on *M. pneumoniae* isolation from or detection in the central nervous system (CNS) favors the hypothesis that *M. pneumoniae*-associated encephalitis is caused by an immune-mediated inflammation.[2] The inflammation may be induced by molecular mimicry between *M. pneumoniae* and neuronal cell components.[3]

We recently reported on the case of a 15-year-old girl with self-limiting *M. pneumoniae*-associated encephalitis showing microbial CNS invasion but no intrathecal specific antibody responses (Table 1: Case 1). Therefore, we speculated that intrathecal antibody responses might be present in more severe *M. pneumoniae*-associated encephalitis.[4] Here, we report on a contrasting case of a patient with *M. pneumoniae*-associated encephalitis with neurologic sequelae who indeed showed detectable intrathecal specific antibody responses.



**Table 1.** Microbiological and clinical data for two patients with confirmed *M. pneumoniae*-associated encephalitis.

| **Diagnosis** | Case 1[4] | Case 2 |
|---|---|---|
| | **Meningoencephalitis** | **Bickerstaff brain stem encephalitis** |
| Age (years) and sex | 15, F | 9, M |
| Prodromal respiratory symptoms (duration) | Cough and fever (2 weeks) | Cough and runny nose (3 weeks) |
| Pulmonary infiltrate (chest radiograph) | Yes | Yes |
| White blood cell count (4.5–13.5×$10^9$/l) | 11.7×$10^9$/l (neutrophils 79%) | 6.8×$10^9$/l (neutrophils 70%) |
| C-reactive protein (<10 mg/) | 2.7 mg/l | 4.0 mg/l |
| **Clinical findings** | | |
| GCS score | 15 | 5 |
| Symptoms | Neck pain, headache, diplopic images, and irritability | Meningism, ataxia, ophthalmoplegia, hemiplegia, and coma |
| Magnetic resonance imaging | Normal | Brain stem encephalitis |
| Electroencephalography | Normal | Mild diffuse and moderate focal slowing (right hemisphere and occipitotemporal) |
| **CSF** | | |
| White blood cells (0–5 cells/µl) | 39 cells/µl (95% mononuclear) | 11 cells/µl |
| Protein (150–450 mg/l) | 200 mg/l | 320 mg/l |
| Glucose (2.5–4.2 mmol/l) | 3.7 mmol/l (blood: 5.3 mmol/l) | 3.0 mmol/l (blood: 5.0 mmol/l) |
| ***M. pneumoniae* real-time PCR** | | |
| Pharyngeal swab | Negative | Positive |
| CSF | Positive | Negative |

**Table 1.** Microbiological and clinical data for two patients with confirmed *M. pneumoniae*-associated encephalitis. (*continued*)

| **Diagnosis** | Case 1[4] | Case 2 |
|---|---|---|
| | **Meningoencephalitis** | **Bickerstaff brain stem encephalitis** |
| ***M. pneumoniae*-specific antibodies** | | |
| *M. pneumoniae* ELISA (serum) (≤11 U/mL) | IgM: 31.7, IgG: 15.5, IgA: 40.7 U/ml | IgM: 67.0, IgG: 48.7, IgA: 34.3 U/ml |
| Intrathecal antibody synthesis | No | Yes |
| Reiber index[5] (cutoff: 1.5) | – | IgM: 15.5, IgG: 7.2, IgA: 5.4 |
| **Treatment and outcomes** | | |
| Treatment | Azithromycin PO (5 days) | Doxycycline IV (7 days)<br>IVIg (1 g/kg once)<br>Prednisolone IV (5 days) |
| Sequelae | No | Yes |

Abbreviations: CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; GCS, Glasgow Coma Scale; Ig, Immunoglobulin; IV, intravenous; PCR, polymerase chain reaction; PO, orally.

## CASE PRESENTATION

A 9-year-old boy was admitted in November 2012 to our hospital with a 3-week history of respiratory symptoms followed by headache and drowsiness (Table 1: Case 2). He manifested meningism, ataxia, ophthalmoplegia, left-sided hemiplegia, and eventually coma within 24 hours. The analysis of cerebrospinal fluid (CSF) revealed 11 white blood cells/µl, normal protein and glucose concentrations, and no growth in conventional bacterial cultures. Magnetic resonance imaging (MRI) showed pronounced brain stem encephalitis that worsened within 1 week (Figure 1). Because *M. pneumoniae* DNA was detected by real-time polymerase chain reaction (PCR) from a pharyngeal swab, we searched for intrathecal *M. pneumoniae*-specific antibodies. Indeed, we detected intrathecal specific antibody synthesis of three-isotype immunoglobulin (Ig) reaction according to Reiber[5] with dominant IgM response, as observed in neuroborreliosis[5]: index (cutoff 1.5) was 15.5 (IgM), 7.2 (IgG), and 5.4 (IgA), respectively. In addition, antigen-specific immunoblotting of CSF and serum IgG revealed positive responses in CSF against highly specific *M. pneumoniae* adhesion proteins P1 and P90 (insufficient CSF to test for IgM and IgA). *M. pneumoniae* was not detectable by PCR in CSF. Chest radiograph showed left-sided paracardial opacities. Other investigations for an infective cause were negative, including PCR for herpes simplex virus types 1 and 2, enterovirus, and Epstein-Barr virus in CSF; PCR for influenza A/B of a pharyngeal swab; and serology for *Borrelia burgdorferi*. Metabolic analysis included normal arterial blood gas, blood lactate level (1.3 mmol/l), and ammonia (24 µmol/l). The empirical treatment with ceftriaxone and acyclovir was changed to intravenous doxycycline (4 mg/kg once daily for 7 days), and the patient additionally

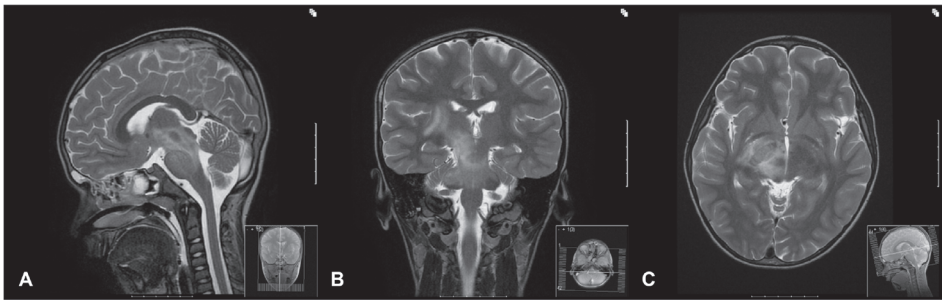


**Figure 1.** Magnetic resonance imaging findings in the brain of a 9-year-old boy with extensive Bickerstaff brain stem encephalitis during *M. pneumoniae* infection.
(A) Sagittal, (B) coronal, and (C) axial anatomy with T2-weighted cranial magnetic resonance imaging showing hyperintensity and generalized edema of the brain stem with craniocaudal extension from crus posterior of capsula interna to the pons.



received intravenous Ig (1 g/kg body weight once) and prednisolone (2 mg/kg twice daily for 5 days). The patient had to be intubated for 4 days. After 2 weeks, he was discharged into rehabilitation with mild residual ataxia, ophthalmoplegia, and hemiparesis.

## DISCUSSION

Ophthalmoplegia and ataxia with disturbance of consciousness together with MRI findings of brain stem encephalitis lead to the diagnosis of Bickerstaff brain stem encephalitis (BBE)[6] that is considered a parainfectious autoimmune disorder similar to Miller Fisher syndrome and Guillain-Barré syndrome.[7] All three disorders have been associated with *M. pneumoniae* infection but only two cases of BBE associated with *M. pneumoniae* infection have been reported.[8,9] Nevertheless, the criteria to diagnose *M. pneumoniae* CNS infection[10] were fulfilled in our case for the first time in BBE. Possible reasons for the negative PCR in CSF may be "spontaneous" clearance of *M. pneumoniae* from CSF as in our previous case[4] or absent influx of CSF to the active inflammation site in the brain stem. The latter may be reflected by the low pleocytosis in our and the reported BBE cases.[8,9] An alternative explanation could be that the pathogen may have been immunologically cleared from the CNS. We detected an immune response, however, when the patient was symptomatic. This suggested that the mere infection process without immune reaction did not cause any symptoms that, in turn, is in line with *M. pneumoniae* not to harm or destroy cells.[4] Notably, the late-onset disease, defined as the onset of CNS symptoms more than 1 week after the onset of respiratory symptoms,[11] is consistent with the negative PCR in CSF[1,11] and further supports an immune-mediated process.[12] Thus, if clearance took place, then immunity was involved, and the immune reaction may have also caused immune-mediated symptoms. Anti-ganglioside GQ1b antibodies, frequently

detected in BBE (in up to 68%[6,7]), were not detectable in our patient. Anti-GM1, anti-GD1a, and anti-GD1b antibodies were negative, too.

This case of BBE with detectable intrathecal specific antibody responses suggests that the latter may contribute to augmented severity of CNS disease and supports our speculated immune-mediated pathogenesis of *M. pneumoniae*-associated encephalitis. Moreover, intrathecal specific antibody responses of three-isotype Ig reaction according to Reiber[5] may be essential to ascertain the diagnosis of *M. pneumoniae*-associated encephalitis.

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# Chapter 6

## Antibody responses to *Mycoplasma pneumoniae*: role in pathogenesis and diagnosis of encephalitis?

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The pathogenesis of encephalitis associated with the respiratory pathogen *Mycoplasma pneumoniae* is not well understood. A direct infection of the central nervous system (CNS) and an immune-mediated process are discussed.[1] Recent observations suggest that intrathecally detectable antibodies against the bacterium, which can serve to establish the etiology of encephalitis, may indeed mediate the disease.

*M. pneumoniae* is a major cause of upper and lower respiratory tract infections in humans worldwide, particularly in children.[2,3] Up to 40% of community-acquired pneumonia in children admitted to the hospital are attributed to *M. pneumoniae* infection.[4-7] Although the infection is rarely fatal, patients of every age can develop severe and fulminant disease. Apart from the respiratory tract infection, *M. pneumoniae* can cause extrapulmonary manifestations. They occur in up to 25% of manifest *M. pneumoniae* infections and may affect almost every organ, including the skin as well as the hematologic, cardiovascular, musculoskeletal, and nervous system.[8] Encephalitis is one of the most common and severe complications.[1] *M. pneumoniae* infection is established in 5–10% of pediatric encephalitis patients,[9,10] and up to 60% of them show neurologic sequelae.[10,11]



It is important to early establish the cause of encephalitis in order to specifically treat what can be treated and to defer from unnecessary treatment. The diagnosis of *M. pneumoniae* encephalitis is challenging. The current diagnostic algorithm of the "Consensus Statement of the International Encephalitis Consortium"[12] recommends for the diagnosis of *M. pneumoniae* infection in children with encephalitis serology and polymerase chain reaction (PCR) from throat samples (routine studies), and for children with positive results and/or additional respiratory symptoms PCR in cerebrospinal fluid (CSF) (conditional studies).

However, *M. pneumoniae* serology and PCR in the respiratory tract cannot discern between colonization and infection in a clinically relevant time frame.[13] The main reason for this is the relatively high prevalence of *M. pneumoniae* in the upper respiratory tract of healthy children (up to 56%).[13,14] The demonstrated positive serological results in such asymptomatic PCR-positive children (positive immunoglobulin [Ig] M in 17%, IgG in 24%, and IgA in 6% of 66 cases)[13] may simply reflect one or more previous encounters with *M. pneumoniae* and are not necessarily related to the presence of *M. pneumoniae* in the respiratory tract. It is clear that this may give rise to an overestimation of the *M. pneumoniae*-related disease burden. A more reliable diagnosis of *M. pneumoniae* infection may be achieved by using paired patient sera in order to detect seroconversion and/or a four-fold increase in antibody titers in addition to PCR (Table 1). However, such procedures are time-consuming and are, therefore, neither practicable nor useful in an acutely ill patient.

**Table 1.** Overview of diagnostic tests for *M. pneumoniae*.

| Method | Test | Target/antigen | Antibodies | Specimen | Performance[a] | Value | Comments |
|---|---|---|---|---|---|---|---|
| **Direct identification of *M. pneumoniae*** | Polymerase chain reaction (PCR) | Different target genes (e.g., P1 gene, 16S rDNA, 16S rRNA, RepMP elements etc.) | – | Respiratory specimen (nasopharyngeal secretion, pharyngeal swab, sputum, bronchoalveolar lavage), cerebrospinal fluid (CSF), other bodily fluids or tissues | High sensitivity, high specificity | RD[b] | - Validation and standardization required for routine diagnostic<br>- Nucleic acid amplification tests (NAATs) provide fast results (in less than a day) and may be earlier than serology (because antibody production requires several days) |
| | Culture | – | – | Respiratory specimen (see above) | Low sensitivity, high specificity | AD | - Special enriched broth or agar media<br>- Isolation takes up to 21 days |
| **Non-specific serological tests for *M. pneumoniae*** | Cold-agglutinin test ("bedside test") | Erythrocytes (I antigen) | Cold agglutinins (IgM) | Serum | Low sensitivity, low specificity | –[c] | - Cold agglutinins target the I antigen of erythrocytes (alternative theory: cold agglutinins directly target *M. pneumoniae* adhered to erythrocytes)<br>- Positive in only about 50% and in the first week of symptoms<br>- Less well studied in children<br>- Cross-reactivity with other pathogens and non-infectious diseases |
| **Specific serological tests for *M. pneumoniae*** | Complement fixation test (CFT) | Crude antigen extract with glycolipids and/or proteins | Igs (no discrimination between isotypes) | Serum | Sensitivity and specificity comparable to EIA | –[c] | - Positive criteria: 4-fold titer increase between acute and convalescent sera or single titer ≥1:32<br>- Cross-reactivity with other pathogens and non-infectious diseases |

**Table 1.** Overview of diagnostic tests for *M. pneumoniae*. (*continued*)

| Method | Test | Target/antigen | Antibodies | Specimen | Performance[a] | Value | Comments |
|---|---|---|---|---|---|---|---|
| | Particle agglutination assay (PA) | | IgM and/or IgG | Serum | Sensitivity and specificity comparable to EIA | –[c] | - See above |
| | Immuno-fluorescent assay (IFA) | | IgM and/or IgG | Serum | Less sensitive and less specific than EIA | –[c] | - Subjective interpretation |
| | Enzyme immuno-assay (EIA) | Proteins (e.g., adhesion protein P1) and/or glycolipids | IgM, IgG,[d,e] IgA[f] | Serum[d], CSF[e,g], other bodily fluids[7] | Moderate-high sensitivity, moderate-high specificity | RD | - The sensitivity depends on the time point of the first serum and on the availability of paired sera (for seroconversion and/or rise in titer)<br>- "Gold standard": 4-fold titer increase as measured in paired sera |
| | Immunoblotting | | IgM, IgG, IgA | | High sensitivity, high specificity[h] | AD | - Confirmatory assay |

Abbreviations: AD, advanced diagnostic test; CFT, complement fixation test; CNS, central nervous system; CSF, cerebrospinal fluid; EIA, enzyme immunoassay; IFA, immunofluorescent assay; Ig, immunoglobulin; NAATs, nucleic acid amplification tests; PA, particle agglutination assay; PCR, polymerase chain reaction; RD, routine diagnostic test; RepMP, repeated *M. pneumoniae* DNA. References: [13,19,38-46]

[a] Qualitative statements included because of the wide range of test performances, which depend on the assay, the patient cohort (children and/or adults), the reference standard (PCR, culture, and/or serology), the respiratory specimen (for PCR), and the time point of the sample collection after disease onset (for EIA); e.g., sensitivities and specificities for PCR[40,41]: 79–100% and 96–99%; IgM EIA (in relation to PCR)[42]: 35–77% and 49–100%; IgG EIA[40,42]: 37–100% (no indication on specificity because of missing information on previous *M. pneumoniae* infections). [b] Epidemiological differentiation of clinical strains on the basis of differences in the P1 gene by PCR or in the number of repetitive sequences at a given genomic locus by multilocus variable-number tandem-repeat analysis (MLVA).[45] [c] Largely replaced by EIA. [d] Kinetics of antibody responses in blood: IgM: onset: within 1 week after the onset of symptoms; peak: 3–6 weeks; persistence: months (to years). IgG: onset and peak: 2 weeks after IgM; persistence: years (to lifelong); reinfection in adults may lead directly to an IgG response in the absence of an IgM response. IgA: onset, peak, and decrease earlier than IgM. [e] Antibody responses in the CNS differ from blood: There is no switch from an IgM to an IgG response, the pattern of IgM, IgG, and IgA synthesis remains rather constant and depends on the cause, and there is a long-lasting and slow decay of intrathecal antibody synthesis.[19] In *M. pneumoniae* encephalitis, a dominant IgM response has been observed.[20] [f] The prevalence of serum IgA determined by EIA has been shown to be very low in PCR-positive children with symptomatic respiratory tract infection (2.0%).[13] [g] To our knowledge no validated test available. [h] Immunoblotting with a combination of at least 5 specific *M. pneumoniae* proteins showed sensitivities (in relation to PCR) of 83% (IgM), 51% (IgG), and 64% (IgA), and specificities of 94–100% (IgM), 98–100% (IgG), and 93–97% (IgA).[46]

The detection rate of *M. pneumoniae* by PCR in CSF of *M. pneumoniae* encephalitis patients is relatively low (0–14%).[9,10,15,16] Moreover, various cases with *M. pneumoniae* encephalitis in which bacterial DNA could not be detected in CSF had a more prolonged duration of respiratory symptoms before the onset of encephalitis (>5–7 days).[10,15,17] These cases indicate that *M. pneumoniae* encephalitis may represent a post-infectious phenomenon, which manifests after clearance of the bacteria from the CNS or respiratory tract by the immune system. The immune response to *M. pneumoniae* in the CNS or other sites may also contribute to the encephalitis (Figure 1).

Interestingly, a promising diagnostic marker for *M. pneumoniae* encephalitis has recently emerged from a few case studies. In one study, intrathecal synthesis of antibodies to *M. pneumoniae* was reported in 14 cases of *M. pneumoniae* encephalitis (74%).[18] The intrathecal production of antibodies is generally considered a highly specific marker for infection of the CNS.[19] All cases who underwent PCR testing (93%) indeed had a positive

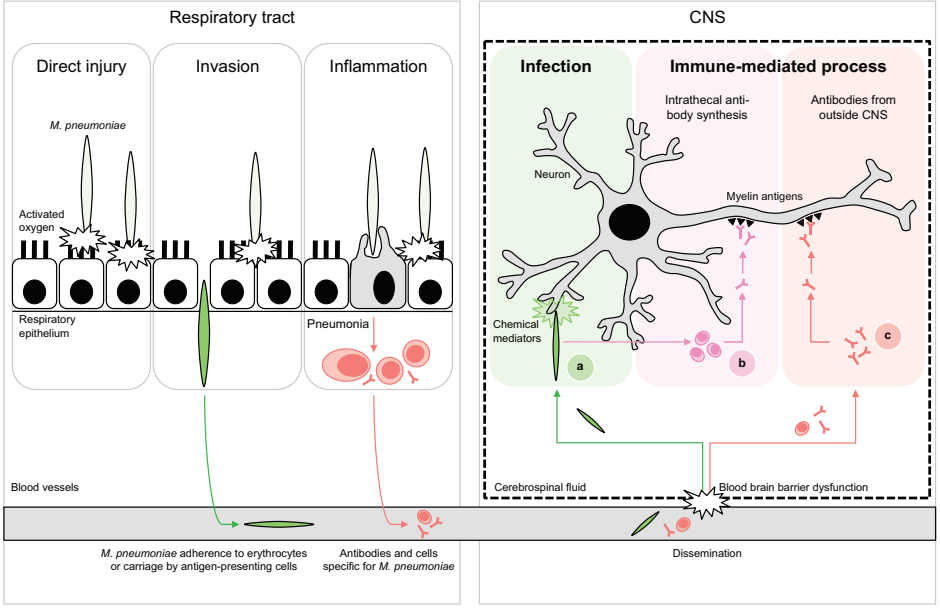


**Figure 1.** Proposed schematic pathomechanisms in *M. pneumoniae* encephalitis.
(Left) Respiratory tract infection. *M. pneumoniae* resides mostly extracellular on epithelial surfaces. Its close association allows producing direct injury by a variety of local cytotoxic effects. Furthermore, it can induce inflammatory responses, elicited by both adhesion proteins and glycolipid epitopes that result in pneumonia. (Right) Encephalitis. Extrapulmonary disease of the central nervous system (CNS) is characterized by systemic dissemination with resultant direct infection and local tissue injury (a), or immune-mediated injury (b–c). The latter may occur as a result of cross-reactive antibodies against myelin components, e.g., gangliosides and galactocerebroside (GalC). These antibodies could theoretically be originated from intrathecal synthesis (b) or from outside the CNS (c). Figure adapted from [1]; see references in the text.

PCR targeting *M. pneumoniae* in CSF,[18] even though it has been recently demonstrated that intrathecal antibody responses to *M. pneumoniae* but not bacterial DNA can be present at the onset of clinical encephalitis.[20] In another study, it was reported that intrathecal antibodies to *M. pneumoniae* were found to cross-react with galactocerebroside (GalC) in 8 out of 21 (38%) of *M. pneumoniae* encephalitis cases.[21] All these 8 cases showed a negative PCR targeting *M. pneumoniae* in CSF. The cross-reactivity in these cases is likely induced by molecular mimicry between bacterial glycolipids and host myelin glycolipids, including GalC and gangliosides (Figure 2). Cross-reactive, anti-GalC antibodies have previously been detected in patients with Guillain-Barré syndrome (GBS) that suffered from a preceding *M. pneumoniae* infection.[22-26] GBS is a typical post-infectious immune-mediated peripheral neuropathy.[27] In GBS, cross-reactive antibodies cause complement activation and formation of a membrane attack complex at the peripheral nerves, resulting in neuromuscular paralysis. Anti-GalC antibodies have been associ-



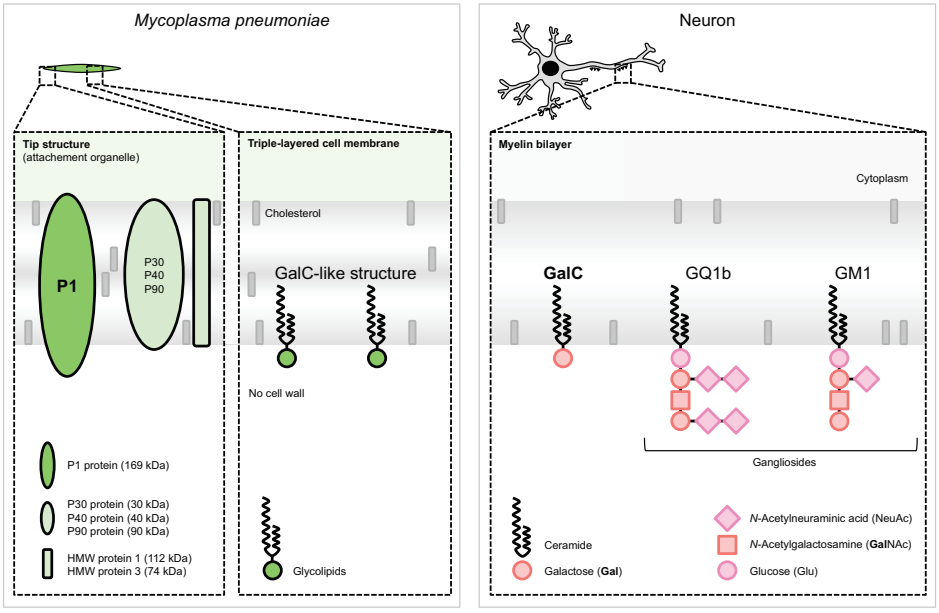


**Figure 2.** Schematic structures responsible for molecular mimicry between *M. pneumoniae* and neuronal cells.

(Left) *M. pneumoniae* adhesion proteins and glycolipids. The immunogenic and major cytadherence proteins P1 and P30 are densely clustered at the tip structure. The P1 protein[47] and glycolipids, e.g., forming a GalC-like structure,[22] elicit cross-reactive antibodies induced by molecular mimicry. (Right) Host myelin glycolipids, to which antibodies were found in patients with *M. pneumoniae* encephalitis. Glycolipids are organized in specialized functional microdomains called "lipid rafts", and play a part in the maintenance of the cell membrane structure. Abbreviations: GalC, galactocerebroside; GQ1b, ganglioside quadrosialo 1b; GM1, ganglioside monosialo 1 (the numbers stand for the order of migration on thin-layer chromatography, and the lower cases for variations within basic structures). Structures of *M. pneumoniae* adhesion proteins and host glycolipids are adapted from [48] and [49], respectively. HMW, high-molecular-weight.

ated with demyelination in patients with GBS.[23,26] Moreover, these anti-GalC antibodies cause neuropathy in rabbits that are immunized with GalC.[28] Such antibodies may also be involved in demyelination of central nerve cells in *M. pneumoniae* encephalitis, as a significant correlation was found between the presence of anti-GalC antibodies in the CSF and demyelination ($p$=0.026).[21]

Anti-GalC antibodies have not only been detected in CSF, but also in serum of *M. pneumoniae* encephalitis patients,[21,24,29-31] including rates from 13% (2/15)[21] to 100% (3/3)[29], respectively. It is possible that the blood-brain barrier (BBB) can become permeable during inflammation, such that antibodies can cross the BBB and cause disease. As a consequence, the cross-reactive antibodies in the CSF of *M. pneumoniae* encephalitis patients do not necessarily have to be produced intrathecally (Figure 1).

*M. pneumoniae* infections may also be followed by the production of antibodies to gangliosides, both in patients with GBS and encephalitis. In *M. pneumoniae* encephalitis, such antibodies were directed against GQ1b[32,33] or GM1[34] (Figure 2). Interestingly, anti-GQ1b antibodies are associated with a distinct and severe encephalitis variant, referred to as Bickerstaff brain stem encephalitis.[35]

In conclusion, while PCR and serology may be of limited value in the diagnosis of *M. pneumoniae* encephalitis, the detection of intrathecal antibodies to *M. pneumoniae*, including cross-reactive antibodies against GalC and gangliosides, may be regarded as a promising new diagnostic tool.

The routine diagnostic work-up of *M. pneumoniae* encephalitis should therefore aim for the detection of *M. pneumoniae* antibodies in both CSF and serum, in addition to *M. pneumoniae* PCR in CSF. Intrathecal antibodies can be detected by widely accessible enzyme immunoassays (EIAs) or immunoblotting (Table 1), while intrathecal antibody synthesis can be established either by calculation of an antibody index[19] or through parallel immunoblotting of simultaneously collected CSF and serum samples.[36,37] Anti-ganglioside antibodies can be detected routinely by some specialized laboratories, but their detection together with cross-reactive antibodies against GalC primarily serve for scientific purposes and may help to clarify *M. pneumoniae* antibodies' immune target(s). Furthermore, their hypothesized role in the pathogenesis might provide a basis for immunomodulatory treatment in *M. pneumoniae* encephalitis.

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# Chapter 7

## Intrathecal anti-GalC antibodies in Bickerstaff brain stem encephalitis

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## LETTER TO THE EDITOR

We recently reported on a 9-year-old boy with ophthalmoplegia, ataxia, left-sided hemiplegia, and eventually coma diagnosed as Bickerstaff brain stem encephalitis (BBE) following *Mycoplasma pneumoniae* infection.[1] The etiologic diagnosis in this case was confirmed by the detection of an intrathecal synthesis of *M. pneumoniae*-specific antibodies.[2] Because we could not detect bacterial DNA in the cerebrospinal fluid (CSF) of the patient at disease onset,[1] we suggested a postinfectious immune-mediated process that manifested after clearance of the bacteria from the central nervous system (CNS).

We here aimed to identify a potential myelin target of cross-reactive antibodies elicited by *M. pneumoniae* in this BBE case.

For the analysis, the same serum and CSF samples used for the analysis of *M. pneumoniae*-specific antibodies taken at onset of neurologic symptoms and stored at −80°C were investigated.

IgM and IgG antibodies to GM1, GM2, GD1a, GD1b, GQ1b, AGM1, and galactocerebroside (GalC) (all from Sigma-Aldrich, Zwijndrecht, the Netherlands) were measured as described previously.[3,4] To determine anti-GalC antibodies, half-area 96-wells plates (Costar, Corning B.V. Life Sciences, Amsterdam, the Netherlands) were coated with 450 pmol of glycolipid per well. All sera were diluted 100-fold. The optical densities (ODs) from uncoated (only ethanol containing wells) were subtracted from the glycolipid-coated wells. Cutoff values were either predefined (i.e., a background-subtracted OD of 0.2 for IgG and 0.3 for IgM) or obtained by measuring 30 healthy control sera (mean OD plus three times the standard deviation). Positive samples were titrated using two-fold serial dilution series starting at a 1:100 dilution. The titer was defined as the reciprocal of the highest dilution that resulted in an OD higher than the cutoff value.

Since patients with Guillain-Barré syndrome (GBS) may also produce antibodies to complexes of two glycolipids instead of a single glycolipid,[5] we additionally tested antibodies to glycolipid complexes as described previously,[5] with the modification that 225 pmol/well was used for GalC and 75 pmol/well for other glycolipids in half-area plates.

Our analysis demonstrated the isolated presence of anti-GalC IgM and IgG antibodies in both CSF and serum (Figure 1). In serum, the binding activity of IgG antibodies to GalC was inhibited by the presence of GM1, GD1a, GD1b, or GQ1b and attenuated by the presence of GM2 and asialo-GM1. The presence of serum antibodies against N-methyl-D-aspartate receptor, voltage-gated potassium channel complex, and aquaporin-4 had previously been excluded.

To our knowledge, this is the first case of BBE with the detection of anti-GalC antibodies.

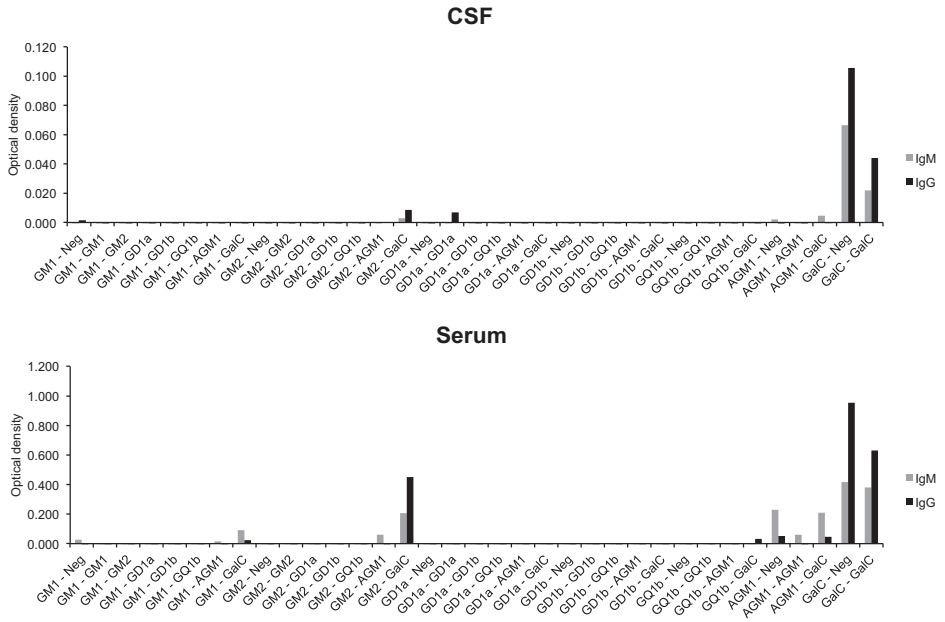


**Figure 1.** Antibody profile against various glycolipids in a patient with Bickerstaff brain stem encephalitis following *M. pneumoniae* infection.

Both IgM and IgG antibodies against single glycolipids (x – Neg) and glycolipid complexes (x – y) in cerebrospinal fluid and serum were determined by enzyme-linked immunosorbent assay. Abbreviations: AGM1, asialo-GM1; GalC, galactocerebroside.

BBE is closely related to GBS, forming a continuous spectrum.[6,7] This is supported by immunological findings in which antibodies against the myelin glycolipid GQ1b were strongly associated with BBE.[8] Anti-GQ1b antibodies were also found in the serum of the two previously reported BBE cases associated with *M. pneumoniae* infection.[9,10]

In GBS, antibodies against *M. pneumoniae* infection have been found to cross-react with the myelin glycolipid GalC.[11,12] GalC is a major glycolipid antigen in the myelin of both the peripheral and CNS, and accounts for 32% of the CNS myelin lipid content.[13] Anti-GalC antibodies caused demyelinating neuropathy in rabbits immunized with GalC[14] and have also been associated with demyelination in GBS,[12] encephalitis,[15] and encephalomyelitis.[16] Anti-GalC antibodies were not tested in the other two BBE cases associated with *M. pneumoniae* infection.[9,10]

Because we detected an intrathecal antibody synthesis of *M. pneumoniae*-specific antibodies in this case and because it has been demonstrated that anti-GalC antibodies cross-react with *M. pneumoniae*,[11,12] there is evidence that these anti-GalC antibodies were induced by *M. pneumoniae*. In fact, we detected anti-GalC antibodies in CSF and serum, although the mere presence of anti-GalC in CSF may have a pivotal role for the pathogenesis of BBE. However, the mechanisms of *M. pneumoniae*-driven antibody

responses within the CNS are unclear. It is also possible that during inflammation the blood-brain barrier (BBB) can become permeable, which would thereby enable serum antibodies to cross the BBB and cause disease.

In conclusion, the presence of antibodies to GalC (in absence of antibodies to GQ1b) in this patient may suggest that these antibodies are involved in the development of BBE. Their hypothesized role in the pathogenesis may provide a basis for immunomodulatory treatment in BBE associated with *M. pneumoniae* infection.

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# Chapter 8

## Swiss national prospective surveillance of pediatric *Mycoplasma pneumoniae*-associated encephalitis

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## SUMMARY

### Objective

To assess the presence of *Mycoplasma pneumoniae*-associated encephalitis in children in Switzerland and its likely pathogenesis.

### Methods

*M. pneumoniae*-associated encephalitis cases seen at a single-center during 2010–2013 were reviewed, and the Swiss Pediatric Surveillance Unit (SPSU) prospectively conducted a nationwide surveillance 2013–2015. Case definition included confirmed, probable, and possible cases.

### Results

Seven patients (median age 8.7 years, range 4.7–10.1 years) with confirmed or possible *M. pneumoniae*-associated encephalitis were observed. All patients manifested prodromal respiratory symptoms over at least 5 days and five out of the six who had a chest radiograph, showed pulmonary infiltrates. *M. pneumoniae* DNA in cerebrospinal fluid was negative in all patients. Intrathecally synthesized *M. pneumoniae*-specific immunoglobulin (IgM and IgG) were investigated and found positive in one patient (confirmed case). *M. pneumoniae* DNA in respiratory specimens and/or *M. pneumoniae*-specific IgM and IgG in serum were detected in the other six patients (possible cases). One confirmed and two possible cases had neurological sequelae at 4–19 months follow-up.

### Conclusion

The lack of detectable *M. pneumoniae* DNA in cerebrospinal fluid of our encephalitis patients suggests a likely immune-mediated pathogenesis ignited by a respiratory inflammatory process including pneumonia.

## INTRODUCTION

Encephalitis is the most severe extrapulmonary manifestation of *Mycoplasma pneumoniae* infection.[1] Its pathogenesis is not understood.[2] Detecting *M. pneumoniae* in the central nervous system (CNS) may be most straightforward to confirm *M. pneumoniae*-associated encephalitis.[3] However, two large recent national encephalitis studies were unable to detect *M. pneumoniae* DNA by means of polymerase chain reaction (PCR) in cerebrospinal fluid (CSF) (United Kingdom, 2005–2006,[4] and France, 2007[5]). In contrast, another national encephalitis study reported *M. pneumoniae* as a possible cause, detected either by serology or positive PCR in respiratory specimens, in 6% (96/1570; US, 1998–2005[6]), compared with two cases confirmed by PCR in CSF (0.1%). Thus, CNS infection or immune-mediated pathology are debated. These divergent pathogenetic hypotheses create uncertainty in diagnostic procedures and lead to differences in case definition criteria.[7]

We aimed to assess the presence of pediatric *M. pneumoniae*-associated encephalitis in Switzerland and its likely pathogenesis.

## METHODS

### Study design

*Preparatory study*

To evaluate whether a nationwide surveillance was worth being conducted we retrospectively reviewed pediatric *M. pneumoniae*-associated encephalitis cases in the frame of our community-acquired pneumonia study at the University Children's Hospital of Zurich[8] between July 1, 2010 and June 30, 2013.

*National prospective surveillance study*

Between July 1, 2013 and June 30, 2015, the Swiss Pediatric Surveillance Unit (SPSU), a national network of 33 hospitals,[9,10] prospectively collected reports of pediatric *M. pneumoniae*-associated encephalitis. Anonymized demographic, clinical, and microbiological data were sent to the SPSU central, which forwarded the forms to the principal investigator who verified the case definition. An outcome evaluation questionnaire was dispatched to the caring practitioner half a year after case presentation.

### *M. pneumoniae*-associated encephalitis case definition

Children ≤16 years of age fulfilling (1) the clinical case definition for acute encephalitis (Table S1),[11] and (2) the etiological case definition for acute encephalitis caused by *M. pneumoniae*,[3] i.e., (2a) "confirmed" (detection of *M. pneumoniae* by PCR in CSF or of intrathecal synthesis of specific antibodies), (2b) "probable" (≥4-fold rise in specific serum

antibody titer), or (2c) "possible" (detection of *M. pneumoniae* by PCR in respiratory specimens and/or single raised specific serum antibody titer), were considered cases.

## RESULTS

One "confirmed" and three "possible" pediatric *M. pneumoniae*-associated encephalitis cases were observed during the 3-year preparatory study and three "possible" cases during the 2-year national prospective surveillance study (Table 1). All patients were male (median age 8.7 years, range 4.7–10.1). Pleocytosis was present in six cases (86%; median cell number 30/µl, range 8–96) and neuroimaging was consistent with encephalitis in five cases (71%) (Table S1). Preceding respiratory symptoms were reported in all patients (median duration 7 days, range 5–21 days) and pneumonia was radiologically confirmed in five of six patients (83%). *M. pneumoniae* DNA was detected in the respiratory specimens of six patients, but not in CSF, and *M. pneumoniae*-specific immunoglobulin (IgM and IgG) in serum of all five tested patients and in CSF of one tested patient. Three patients (43%) still had neurological signs and symptoms at 4–19 months follow-up (Table 1).

## DISCUSSION

Here, we have documented for the first time the presence of pediatric *M. pneumoniae*-associated encephalitis in Switzerland and gathered indications of an immune-mediated process.

Our three *M. pneumoniae*-associated encephalitis cases observed during the 2-year national surveillance represent an estimated yearly incidence of 0.09 cases per 100,000 population ≤19 years of age,[12] although underreporting is likely. Nevertheless, the figure is comparable to that in Finland, estimated after a national surveillance in 1993–1994 (0.1 cases per 100,000).[13] The incidence, however, depends on the presence or absence of epidemics. Indeed, we retrospectively identified four patients in a single-center over a prior 3-year period and three of them coincided with the *M. pneumoniae* epidemic in Europe from 2010–2011.[8,14]

*M. pneumoniae* was found as the cause of 9% (84/906; USA, 1998–2006[15]) and 31% (50/159; Canada, 1994–1998[16]) of cases of pediatric encephalitis, whereby 2% (1/62)[15] and 12% (6/50)[16] of the cases, respectively, were confirmed by PCR in CSF. The yearly incidence of pediatric encephalitis in developed countries is about 10.5 cases per 100,000 children.[13] Since no data on pediatric encephalitis exist for Switzerland we cannot estimate the relative frequency of our *M. pneumoniae*-associated encephalitis cases.

We found no *M. pneumoniae* DNA in CSF of any patient but it was present in throat specimens of all patients except one, and specific serum antibodies were found in all patients tested. This suggests that their encephalitis was likely immune-mediated follow-

**Table 1.** Characteristics of seven cases with pediatric *M. pneumoniae*-associated encephalitis.

| No. | Inclusion | Age (y), sex | Prodrome (d), symptoms | Chest radiograph | Etiological case definition for *M. pneumoniae*-associated encephalitis[a] | | | | | Treatment (duration [d]) | Outcome (last follow-up [m]) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **CSF** | | **RS** | **Serum** | **Case definition**[3] | | |
| | | | | | PCR | Antibodies (IgM, IgG) | PCR | Antibodies (IgM, IgG) | | | |
| **Preparatory study (2010–2013)** | | | | | | | | | | | |
| 1 | 12/2010 | 4.7, M | 5<br>Fever, rhinitis, pharyngitis, conjunctivitis, mucositis, erythema multiforme, cervical adenopathy[b] | Infiltrate | – | ND | – | +[c] | Possible | Cefaclorum (3)<br>Acyclovir (3)<br>Ceftriaxone (3)<br>Clarithromycin (7)<br>IVIg (1)<br>Prednisolone (9) | Normal (4) |
| 2 | 01/2011 | 8.3, M | 6<br>Fever, cough, rhinitis | Infiltrate | – | ND | + | +[c] | Possible | Ceftriaxone (3)<br>Doxycycline (7) | Normal (2) |
| 3 | 12/2011 | 10.1, M | 5<br>Fever, cough | ND | – | ND | + | +[c] | Possible | Acyclovir (3)<br>Ceftriaxone (3) | Normal (1) |
| 4[18] | 11/2012 | 8.7, M | 21<br>Cough, rhinitis | Infiltrate | – | +<br>(intrathecal synthesis)[d] | + | + | Confirmed | Doxycycline (7)<br>IVIg (1)<br>Prednisolone (5) | Mild residual hemiparesis of the upper extremities, incomplete facial palsy (19) |
| **National prospective surveillance study (2013–2015)** | | | | | | | | | | | |
| 5 | 04/2014 | 9.3, M | 15<br>Fever, cough, rhinitis | Infiltrate | – | ND | + | ND | Possible | Acyclovir (1)<br>Ceftriaxone (1)<br>Ciprofloxacin (1)<br>Vancomycin (1)<br>Moxifloxacin (8) | Optic disc swelling (6) |

**Table 1.** Characteristics of seven cases with pediatric *M. pneumoniae*-associated encephalitis. (*continued*)

| No. | Inclusion | Age (y), sex | Prodrome (d), symptoms | Chest radiograph | Etiological case definition for *M. pneumoniae*-associated encephalitis[a] | | | | | Treatment (duration [d]) | Outcome (last follow-up [m]) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **CSF** | | **RS** | **Serum** | **Case definition**[3] | | |
| | | | | | PCR | Antibodies (IgM, IgG) | PCR | Antibodies (IgM, IgG) | | | |
| 6 | 09/2014 | 7.8, M | 9<br>Fever, cough | Infiltrate | – | ND | + | ND | Possible | Amoxicillin (3)<br>Clarithromycin (3)<br>Acyclovir (3)<br>Ceftriaxone (3)<br>Ciprofloxacin (14) | Aggressive behavior, headache (4) |
| 7 | 05/2015 | 8.8, M | 7<br>Fever, rhinitis, pharyngitis | – | – | ND | + | + | Possible | Acyclovir (3)<br>Ceftriaxone (1)<br>Methylprednisolone (3)<br>Prednisolone (14) | Normal (hospital discharge) |

Abbreviations: CSF, cerebrospinal fluid; Ig, immunoglobulin; IVIg, intravenous immunoglobulin; ND, not done; PCR, polymerase chain reaction; PS, pharyngeal swab; RS, respiratory specimens.

[a] No case met confirmed or probable etiological case definition for another infectious agent[3]; negative investigations as follows:

Case 1: PCR CSF: Herpes simplex virus 1/2, enteroviruses; PCR PS: Influenza A, respiratory syncytial virus; Serology: Herpes simplex virus 1/2; CSF and blood: conventional bacterial cultures;

Case 2: PCR PS: respiratory viruses (multiplex PCR); PS antigen test: *Streptococcus pneumoniae*; Serology: Tick-borne encephalitis; CSF and blood: conventional bacterial cultures; Tuberculin skin test;

Case 3: PCR CSF: Herpes simplex virus 1/2, enteroviruses; Serology: Tick-borne encephalitis; CSF and blood: conventional bacterial cultures;

Case 4: PCR CSF: Herpes simplex virus 1/2, enteroviruses, Epstein-Barr virus; PCR PS: Influenza A/B; Serology: *Borrelia burgdorferi*; CSF and blood: conventional bacterial cultures;

Case 5–7: no other potential etiologic agents reported, but no further information according to study design.

[b] Patient 1 developed pericarcial effusion during the course of the disease. PCR assays of pericardial fluid and tissue biopsy for *M. pneumoniae* were negative.

[c] Persisting elevated IgM and IgG titers over 4 weeks (patient 1), 8 weeks (patient 2), or 6 weeks (patient 3).

[d] Three-isotype immunoglobulin reaction (IgM, IgG, and IgA).

ing respiratory infection. Indeed, all patients had prodromal respiratory symptoms over at least 5 days. Interestingly, a recent study[17] reported that *M. pneumoniae*-associated encephalitis patients with negative PCR for *M. pneumoniae* in CSF showed radiological signs of pneumonia with infiltrates on chest radiograph more frequently than patients with positive PCR in CSF (77% vs. 33%). We found infiltrates on chest radiograph in 83%.

Pleocytosis was present in most of our patients and neuroimaging was compatible with an inflammatory process in 71%. This further underscores the likelihood of an immune-mediated pathogenesis in our *M. pneumoniae*-associated encephalitis cases, which was proven by the detection of an intrathecal antibody synthesis in one investigated patient (patient 4).[18] Antibodies against *M. pneumoniae* have been shown to cross-react with galactocerebroside (GalC).[19] GalC is a major glycolipid antigen in the myelin of both the peripheral and central nervous systems. We previously demonstrated, in this specific case, antibodies against GalC in serum and CSF,[20] which suggests that these antibodies are involved in the development of *M. pneumoniae*-associated encephalitis.[2] Intriguingly, one patient showed extensive extrapulmonary multiorgan inflammation with additional involvement of skin and heart (patient 1), which required prolonged anti-inflammatory treatment.

In conclusion, we suspect an immune-mediated process in our observed *M. pneumoniae*-associated encephalitis cases. Pneumonia may be an indicator for the remote inflammatory process. The association of encephalitis with *M. pneumoniae* may be increasingly established by expanding diagnostic procedures through the analysis of intrathecal antibodies.



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## SUPPLEMENTARY MATERIAL

**Table S1.** Clinical case definitions for seven cases with pediatric *M. pneumoniae*-associated encephalitis.

| No. | Inclusion | Age (y), sex | **Major criterion**[11]: | **Minor criteria** (≥3 required)[11]: | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Fever | New onset of neurologic signs and symptoms | Pleocytosis (CSF WBC ≥5/µl) | CSF WBC count (cells/µl) | Abnormality on neuroimaging suggestive of encephalitis | Abnormality on EEG consistent with encephalitis |
| **Preparatory study (2010–2013)** | | | | | | | | | |
| 1 | 12/2010 | 4.7, M | Decreased level of consciousness | + | Ataxia | – | 3 | + (MRI) | + |
| 2 | 01/2011 | 8.3, M | Decreased level of consciousness | + | Ataxia<br>Cerebellar mutism<br>Nuchal rigidity<br>Headache | + | 38 | – | + |
| 3 | 12/2011 | 10.1, M | Decreased level of consciousness | + | Altered verbal communication<br>Incontinence (urine and stool)<br>Nuchal rigidity<br>Headache | + | 96 | + (MRI) | + |
| 4[18] | 11/2012 | 8.7, M | Coma | – | Ataxia<br>Ophthalmoplegia<br>Hemiplegia<br>Nuchal rigidity<br>Headache | + | 11 | + (MRI) | + |
| **National prospective surveillance study (2013–2015)** | | | | | | | | | |
| 5 | 04/2014 | 9.3, M | Decreased level of consciousness | + | Nuchal rigidity<br>Headache<br>Vomiting | + | 8 | + (MRI, CT) | ND |

**Table S1.** Clinical case definitions for seven cases with pediatric *M. pneumoniae*-associated encephalitis. (*continued*)

| No. | Inclusion | Age (y), sex | **Major criterion**[11]: | **Minor criteria** (≥3 required)[11]: | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 6 | 09/2014 | 7.8, M | Decreased level of consciousness<br>Personality change | + | Nuchal rigidity<br>Headache<br>Vomiting | (+) | 25 (traumatic) | – | + |
| 7 | 05/2015 | 8.8, M | Decreased level of consciousness | + | Ataxia<br>Dysarthria<br>Headache<br>Vomiting | + | 35 | + (MRI) | ND |

Abbreviations: CSF, cerebrospinal fluid; CT, computed tomography; EEG, electroencephalography; MRI, magnetic resonance imaging; ND, not done; WBC, white blood cells.

# PART 2.2

## Guillain-Barré syndrome

Parts of the introduction are published in:

The spectrum of preceding infections in Guillain-Barré syndrome
– beyond *Campylobacter*
P.M. Meyer Sauteur
A.M.C. van Rossum
B.C. Jacobs
In: *GBS100 Monograph*, Peripheral Nerve Society 2016;181–195 (ISBN 978-0-9975103-0-0)

## INTRODUCTION TO GUILLAIN-BARRÉ SYNDROME

Guillain-Barré syndrome (GBS) is the most common cause of acute neuromuscular paralysis in countries where poliomyelitis has been eliminated,[1] characterized by symmetrical weakness of the limbs, and hyporeflexia or areflexia, which reaches a maximum severity within four weeks.[2] GBS is considered the prototype of a postinfectious immune-mediated disorder. About two-thirds of GBS patients experience respiratory or gastrointestinal symptoms days to weeks before the onset of neurologic symptoms.[3] A specific pathogen can be identified in about half of patients with GBS. *Campylobacter jejuni* accounts for around one-third of these infections.[1,4]

Over the last 10 years, there is accumulating evidence on the role of preceding infections and cross-reactive anti-glycolipid antibodies in the immunopathology of GBS. Most notably, there is now convincing evidence that *C. jejuni* is a potential trigger of GBS and is associated with the acute motor axonal neuropathy (AMAN) subtype and IgG antibodies against GM1, GM1b, GD1a, or GalNAc-GD1a (Figure 1).[5] Much less is known about the role of preceding infections and the pathogenesis in the patients with GBS without a preceding *C. jejuni* infection.

### Detecting a pathogen in GBS – what does it mean?

The demonstration of a causative pathogen in GBS is rather complex. First, there is usually a delay of days to weeks between the infection and the diagnosis of GBS. The detection rate of a pathogen by culture and/or polymerase chain reaction (PCR) at GBS onset is low.[3] It is possible that the pathogen has already been cleared at the infectious site by the immune reaction that may have also caused GBS.

Second, demonstrating the presence of a pathogen in a GBS patient does not necessarily implicate that there is an infection that triggered an immune response to the nerves. Many pathogens, apart from *C. jejuni*, persist or are carried in healthy asymptomatic individuals without any symptoms.[6] As discussed in **Chapter 1 and 4**, no single diagnostic test or combination of tests is capable of differentiating carriage from infection with respiratory pathogens, some of them also associated with GBS. Caution should be taken in the interpretation of such diagnostic test results, also because some serological assays may lack sensitivity and specificity.

Third, GBS is a highly heterogeneous disorder. Various subtypes of GBS have been reported that differ in their clinical, electrophysiological, and histological features (Table 1). This applies also to the type of preceding infection. Additionally, some infections may have triggered the onset of disease only in a small minority of the cases.

Forth, some patients may have already received intravenous immunoglobulin (IVIg) before blood samples were obtained to investigate infection serology, and such serology may then be false positive.

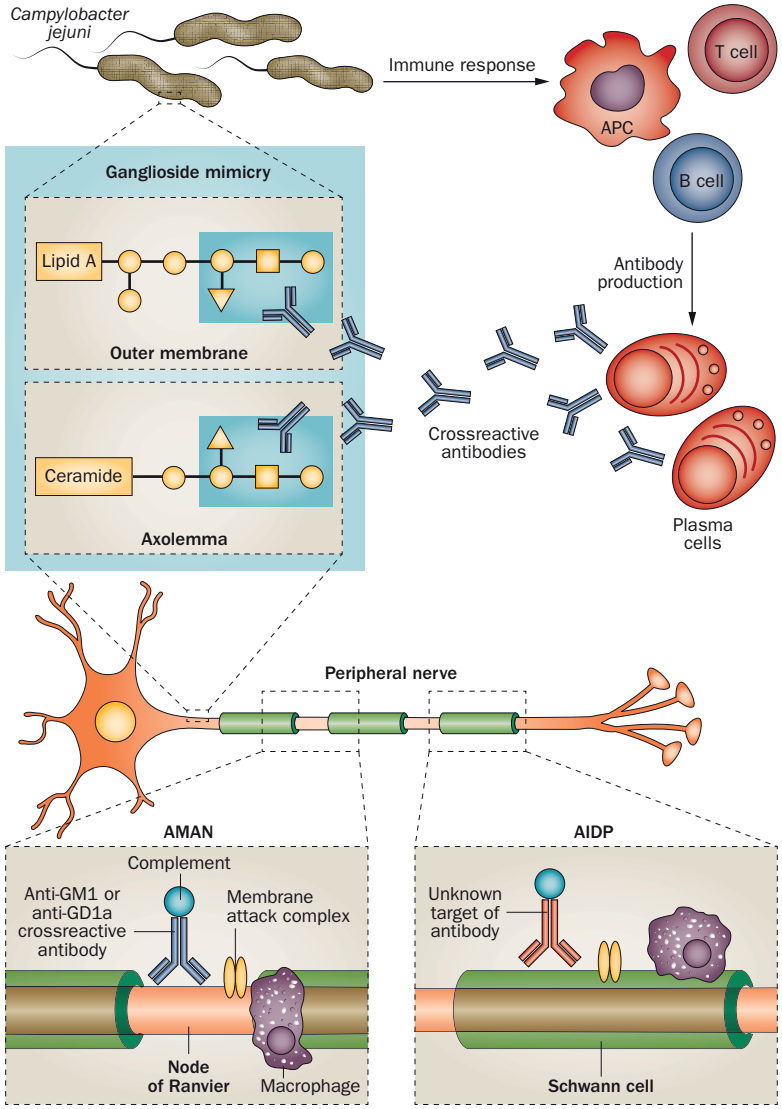


**Figure 1.** Immunopathogenesis of GBS.
*C. jejuni* lipooligosaccharides (LOS) can trigger antibodies that cross-react with gangliosides on peripheral nerves. Anti-ganglioside antibodies can bind to nodal axolemma (AMAN) or Schwann cells (AIDP) and activate complement followed by membrane-attack complex formation, resulting in nerve-conduction failure (AMAN) or vesicular degeneration with invasion of myelin by macrophages (AIDP). It is not excluded that for other GBS patients cross-reactive T cells or very different mechanisms than cross-reaction may play a role in the pathogenesis. Abbreviations: AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; APC, antigen-presenting cell. Reproduced with permission from van den Berg et al.,[5] Springer Nature.

**Table 1.** GBS subtypes, clinical features and anti-glycolipid antibodies.

| GBS subtypes | Clinical features | NCS findings | Antibodies[a] |
|---|---|---|---|
| **Acute inflammatory demyelinating polyneuropathy (AIDP)** | Sensorimotor GBS, often combined with cranial nerve deficits and frequent autonomic dysfunction | Demyelinating polyneuropathy | Various[b] |
| **Acute motor axonal neuropathy (AMAN)** | Pure motor GBS, cranial nerves rarely affected | Axonal polyneuropathy, sensory action potential normal | **GM1a**, **GD1a**, GM1b, GalNAc-GD1a |
| **Acute motor sensory axonal neuropathy (AMSAN)** | Resembles severe AMAN, but sensory fibres are affected, leading to sensory deficits | Axonal polyneuropathy, sensory action potential reduced or absent | **GM1**, **GD1a** |
| **Pharyngeal-cervical brachial variant** | Prominent weakness of oropharyngeal, facial, neck and shoulder muscles | Normal in most patients | **GT1a**, GQ1b, GD1a |
| **Miller Fisher syndrome (MFS)** | Ataxia, ophthalmoplegia, areflexia | Normal in most patients | **GQ1b**, GT1a |
| **Bickerstaff brain stem encephalitis (BBE)** | Ataxia, ophthalmoplegia, altered level of consciousness (CNS variant of MFS) | Normal in most patients | **GQ1b**, GT1a |

Abbreviations: NCS, nerve conduction study. Gangliosides: GalNAc, *N*-Acetylgalactosamine; GD, ganglioside disialo; GM, ganglioside monosialo; GQ, ganglioside quadrosialo; GT, ganglioside trisialo (numbers stand for the order of migration on thin-layer chromatography, and lower-case letters for variations within basic structures). Table adapted from van den Berg et al.[5]

[a] Antibodies are predominantly IgG, but IgM and IgA antibodies have also been demonstrated;

[b] Association with GBS and role in pathogenesis unknown.



## Infectious association – or cause?

When an infectious diagnosis can be made, how sure are we that there is a causal relationship between the identified pathogen and GBS?

Despite the association between many infections and GBS, the overall risk of developing GBS is very small. For example, only one in 1,000–5,000 patients with *C. jejuni* enteritis will develop GBS in the subsequent two months.[7] A fact which certainly also applies to other infectious pathogens, where the acute disease is much more frequent than this severe postinfectious complication.

One of the critical steps in GBS pathogenesis is the generation of antibodies that cross-react with specific gangliosides. It has been shown for *C. jejuni* that such cross-reactive antibodies are not produced during uncomplicated *C. jejuni* enteritis.[5] Apart from *C. jejuni*, many other identified pathogens have also been reported in association with GBS: *M. pneumoniae*, *Haemophilus influenzae*, cytomegalovirus, Epstein-Barr virus, and influenza virus (reviewed in [8]) (Figure 2). There are even more pathogens reported in single GBS case reports or series. The involvement of these pathogens in GBS is less clear, and has not been determined in controlled larger studies.

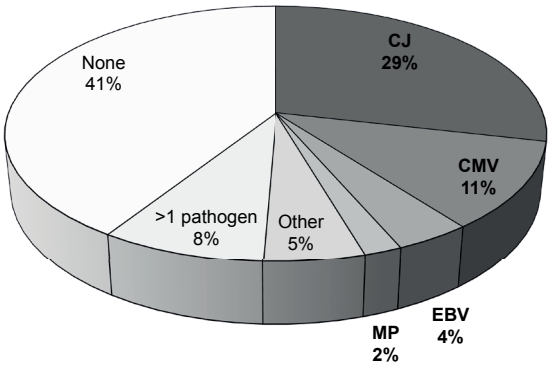


**Figure 2.** Incidence of positive infection serology in 154 adult GBS patients.
"Other" pathogens were *H. influenzae*, influenza virus A/B, parainfluenza virus, adenovirus, herpes simplex virus, and varicella-zoster virus. Most of GBS patients with >1 pathogen detected had positive EBV and/or CMV serology in addition to other pathogens. Abbreviations: CJ, *C. jejuni*; CMV, cytomegalovirus; EBV, Epstein-Barr virus; MP, *M. pneumoniae*. Reproduced from Meyer Sauteur et al.[8]

Interestingly, the variety in types of preceding infection may be related to the diversity in clinical presentation,[9] outcome,[10] and also to the specificity of antibodies to glycolipids (Table 2).[4] Therefore, the detection of a distinct anti-glycolipid antibody type could give an indication on the preceding infectious agent that triggered the antibody response.[11] In this thesis, we focus on the role of *M. pneumoniae* in GBS (**Chapter 9–12**).

**Table 2.** The spectrum of preceding infections in GBS.

| Pathogen | Detection rate (%) | | | Anti-glycolipid antibodies | | | Microbial mimic structure |
|---|---|---|---|---|---|---|---|
| | **GBS** | **HC** | **NC** | **GBS** | **Controls** | | |
| **Bacteria** | | | | | | | |
| *C. jejuni* | **26%**[12,◊] | 1–2% | – | – | – | – | **GM1**-, **GD1a**-, **GD3**-, **GT1a**-like LOS[13,14] |
| | **32%**[4,◊◊] | 8%* | 12%* | anti-GM1 41% and anti-GD1b 29%[4,◊◊] | – | – | |
| | **57%**[1,◊◊] | 8%* | 3%* | anti-GM1/GD1a 56%[1,◊◊] | anti-GM1/GD1a 6%* | anti-GM1/GD1a 1%[1]* | |
| | – | – | – | *C jejuni* +: anti-GM1 29%[15,◊◊] | – | – | |
| *M. pneumoniae* | **5%**[4,#] | 0% | 1%* | *M. pneumoniae* +: anti-GalC 73%[16,#] | *M. pneumoniae* +: anti-GalC 58%[16,#], anti-GalC 18%[17,#] | *M. pneumoniae* –: anti-GalC 0%[16,#*], anti-GalC 0%[17,#] | **GalC**-like[18] |
| | **15%**[19,#] | 1%* | 2%* | | | | |
| | **21%**[20,#] | 14% | 14% | | | | |
| | **3%**[21,○] | – | – | | | | |

**Table 2.** The spectrum of preceding infections in GBS. (*continued*)

| Pathogen | Detection rate (%) | | | Anti-glycolipid antibodies | | | Microbial mimic structure |
|---|---|---|---|---|---|---|---|
| | **GBS** | **HC** | **NC** | **GBS** | **Controls** | | |
| *H. influenzae* | **9%**[22,#] | 0% | 8% | *H. influenzae* +: anti-GM1 100% and anti-GQ1b 25%[22,#] | – | – | **GM1**-like,[23] **GT1a**/**GQ1b**-like[22] |
| | **7%**[24,#] | 6% | 7% | *H. influenzae* +: anti-GQ1b and anti-GT1a 86%[24,#] (GBS + MFS) | – | – | |
| | MFS: **11%**[24,#] | 6%* | 7%* | | | | |
| | **1%**[4,#] | – | – | – | – | – | |
| *Salmonella enterica* species | **0%**[25,&] | – | – | S. Paratyphi A +: anti-GQ1b[26,&,a] | – | – | – |
| | **1%**[27,&] | – | – | | | | |
| **Viruses** | | | | | | | |
| CMV | **13%**[4,#] | 0%* | 2%* | CMV +: anti-GM2 25%[4,#] | CMV +: – | CMV –: – | **GM2**-like, expressed by CMV-infected fibroblasts[28] |
| | **6%**[19,§] | 1% | 2% | – | – | – | |
| | **8%**[29,#] | 0%* | – | anti-GM2[$] 100%[29,#] | anti-GM2[$] 60% | anti-GM2[$] 0%* | |
| | MFS: **6%**[29,#] | 0% | – | | | | |
| | **12%**[21,#,§] | – | – | anti-GM2[$] 42%[21,#,§] | anti-GM2[$] 47% | anti-GM2[$] 0% | |
| | **15%**[30,#] | – | – | anti-GM2[$] 22%[31,#] | anti-GM2[$] 2%* | anti-GM2[$] 0%* | |
| | **12%**[32,#,§,†] | – | – | | | | |
| | **8%**[33,#,§] | – | – | | | | |
| EBV | **10%**[4,#] | 0%* | 1%* | No antibodies against known glycolipids detected[4,21] | | | – |
| | **4%**[19,#] | 0% | 0% | | | | |
| | **1%**[21,#] | – | – | | | | |
| | **2%**[33,#,§] | – | – | | | | |
| HSV-1/2 | **1%**[4,o] | – | – | – | – | – | – |
| VZV | **1%**[4,o] | – | – | – | – | – | – |
| | HZ: **0.025%**[34,††] | HZ: 0.001%* | – | | | | |
| Influenza virus (A and B) | **2%**[4,o] | – | – | Influenza A +: anti-GD1b[35,#,a], anti-GD1b, -GM1, and -GD1a[36,•,a] | – | – | No structural homologies with known gangliosides[37] |
| | **3%**[38,o] | – | – | | | | |

**Table 2.** The spectrum of preceding infections in GBS. (*continued*)

| Pathogen | Detection rate (%) | | | Anti-glycolipid antibodies | | | Microbial mimic structure |
|---|---|---|---|---|---|---|---|
| | **GBS** | **HC** | **NC** | **GBS** | **Controls** | | |
| HIV | UK: **0%**[39,·] | – | – | – | – | – | – |
| | Africa: **55%**[40,·] | 4%* | – | | | | |
| HEV | **5%**[41,#] | 0.5%* | – | No antibodies against known glycolipids detected[41] | | | – |
| | **11%**[42,#] | 0%* | 2%* | | | | |

Abbreviations: AIDS, acquired immunodeficiency syndrome; bp: base pairs; CMV; cytomegalovirus; EBV, Epstein-Barr virus; MFS: Miller Fisher syndrome; HC, healthy controls; HEV, hepatitis E virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HZ, herpes zoster; LOS, lipooligosaccharide; NC, controls with other neurological diseases; VZV, varicella-zoster virus. *$p \leq 0.05$; [a]data from a single case report; –: no data available. Table adapted from Meyer Sauteur et al.[8]

Evidence for infection: to compare an acute serological response against a pathogen between different studies we collected data of positive specific IgM results (if available); [◊]Isolation of *C. jejuni* in stool, or ≥2 positive isotypes by ELISA (IgM, IgG, IgA), or positive IgA or IgG by ELISA and history of diarrhea within the previous 12 weeks; [◊◊]ELISA: IgM, IgG, or IgA; [#]ELISA: IgM; [°]CFT: Igs (no discrimination between isotypes); [&]Isolation of *Salmonella* Typhi in blood, stool, and/or urine; [§]ELISA: IgG avidity; [†]Plasma CMV DNA was positive in 62% of cases with CMV infection[#§]; [$]Anti-GM2 IgM; [††]Clinically defined HZ; [·]PCR.

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# Chapter 9

## Severe childhood Guillain-Barré syndrome associated with *Mycoplasma pneumoniae* infection: a case series

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## ABSTRACT

We report seven children with recent *Mycoplasma pneumoniae* infection and severe Guillain-Barré syndrome (GBS) that presented to two European medical centers from 1992 to 2012. Severe GBS was defined as the occurrence of respiratory failure, central nervous system (CNS) involvement, or death. Five children had GBS, one Bickerstaff brain stem encephalitis (BBE), and one acute-onset chronic inflammatory demyelinating polyneuropathy (A-CIDP). The five patients with severe GBS were derived from an original cohort of 66 children with GBS. In this cohort, 17 (26%) children had a severe form of GBS and 47% of patients with *M. pneumoniae* infection presented with severe GBS. Of the seven patients in this case series, five were mechanically ventilated and four had CNS involvement (two were comatose). Most patients presented with non-specific clinical symptoms (nuchal rigidity and ataxia) and showed a rapidly progressive disease course (71%). Antibodies against *M. pneumoniae* were detected in all patients and were found to be intrathecally synthesized in two cases (GBS and BBE), which proves intrathecal infection. One patient died and only two patients recovered completely. These cases illustrate that *M. pneumoniae* infection in children can be followed by severe and complicated forms of GBS. Non-specific clinical features of GBS in such patients may predispose a potentially life-threatening delay in diagnosis.

## INTRODUCTION

Nervous system disease is one of the most common and severe extrapulmonary manifestations of infections with the respiratory pathogen *M. pneumoniae*.[1] Preceding *M. pneumoniae* infection has been reported in 3–15% of Guillain-Barré syndrome (GBS) in adults[2-4] and also in GBS variants[5-7] and related neuropathies.[8]

At present, it is unclear if *M. pneumoniae* infections may also precipitate the development of childhood GBS. In children, the incidence of *M. pneumoniae* infections is higher than in adults.[9] Previous studies showed that the type of preceding infection may influence the clinical presentation and outcome of GBS.[10] We present a case series of children who developed severe and complicated disorders within the GBS spectrum after infection with *M. pneumoniae*.

## MATERIALS AND METHODS

### Patients

A group of previously healthy children (≤16 years of age) was diagnosed with severe GBS and related disorders following a recent *M. pneumoniae* infection in two European centers from 1992 to 2012 (patient 1–6: Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands; patient 7: University Children's Hospital Zurich, Switzerland). All patients fulfilled the diagnostic criteria for GBS.[11] The case definition for severe GBS included respiratory failure requiring mechanical ventilation, additional involvement of the central nervous system (CNS), and death. Total numbers for children diagnosed with GBS were available for Rotterdam ($n$=66, excluding GBS-related disorders), but not for Zurich. Some of the patients had previously been included in other studies.[12-14] Informed consent was given by the parents and/or the patients if 12 years or older. The study was approved by the Erasmus MC medical ethics board.

### *M. pneumoniae* diagnostics

#### *M. pneumoniae DNA*

Detection of *M. pneumoniae* in the respiratory tract and in cerebrospinal fluid (CSF) was performed by real-time polymerase chain reaction (PCR) as previously described.[15]

#### *Antibodies to M. pneumoniae*

A recent infection with *M. pneumoniae* had previously been defined by detection of immunoglobulin (Ig) M antibodies to *M. pneumoniae*.[16] Patients were only included in this study if serum and/or CSF samples were available to confirm the serology. All serum samples were retested for further comparative analysis of IgM, IgG, and IgA antibodies to *M. pneumoniae* adhesion protein P1 using a commercially available enzyme-linked

immunosorbent assay (ELISA) (Genzyme, Virotech GmbH, Rüsselsheim, Germany). Test results were confirmed with a recently developed and validated line immunoblot containing a combination of at least five *M. pneumoniae*-specific proteins, including P1 and P90.[17] Where available, CSF samples were also tested. Intrathecal antibody synthesis was determined either by calculation of an antibody index or through parallel immunoblotting of simultaneously collected CSF and serum samples.[18]

## RESULTS

We identified seven children diagnosed with severe GBS and a positive IgM serology to *M. pneumoniae*. The disease spectrum included five patients with GBS and two with GBS-related disorders. One patient with the clinical variant Bickerstaff brain stem encephalitis (BBE) and one patient who at first appeared to have GBS, but was later diagnosed with acute-onset chronic inflammatory demyelinating polyneuropathy (A-CIDP).

Excluding GBS-related disorders (BBE and A-CIDP), severe GBS occurred in 17 of the total cohort of 66 children (26%) diagnosed with GBS in Rotterdam. In this cohort, GBS after *M. pneumoniae* infection was classified in 47% as severe GBS ($n$=7, from which only five had residual samples and were thus included in this study).

An overview of the patient characteristics is shown in Table 1. The median age was 7.2 years (range, 4.9–14.5 years). Most patients presented with non-specific clinical features at onset of disease (headache [57%], unsteady gait [43%], and pain [43%]) and at admission (nuchal rigidity [57%] and ataxia [29%]). The disease was rapidly progressive in most cases (Figure 1). Consistent with the case definition for severe GBS, one patient died, four patients had clinically defined CNS involvement, and five patients required mechanical ventilation.

Respiratory tract symptoms and/or fever were reported in 57% (median duration 11 days, range, 2–21 days). *M. pneumoniae* DNA was detected in the respiratory tract in two of four (50%) patients, both with respiratory tract symptoms and infiltrates on chest radiograph. *M. pneumoniae* DNA was not observed in CSF of five tested patients. The detection of IgM antibodies to *M. pneumoniae* at the time of diagnosis could be verified for this study in all cases by both ELISA and immunoblotting. CSF from all patients showed a white blood cell count <50/µl and an increased protein concentration ("cyto-albuminologic dissociation"). Intrathecal antibody production was demonstrated in two cases. Poor outcome occurred in two cases (patient 5 and 7). Two patients (29%) showed full recovery (GBS disability score 0).

**Table 1.** Overview of seven children with severe GBS spectrum disease associated with *M. pneumoniae* infection.

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Diagnosis | **GBS with hydrocephalus** | **GBS** | **GBS** | **GBS** | **GBS** | **A-CIDP** | **BBE** |
| Age and Sex | 13, M | 7, M | 6, M | 7, F | 5, M | 14, M | 9, M |
| Previous medical history | Panophthalmitis of the left eye at 3 years of age | – | – | – | – | – | – |
| **Clinical signs and symptoms** | | | | | | | |
| Prodromal symptoms | GIT, fever | RT, GIT, fever | RT, fever | GIT | – | NA | RT |
| Neurologic signs and symptoms at onset | Headache, nausea, vomiting, unsteady gait, paraesthesias | Weakness of the extremities, dysarthria | Headache, pain in both arms | Headache, neck pain | Pain in the neck and legs, unsteady gait, difficulty in swallowing, drooling | Unsteady gait | Headache, drowsiness |
| Neurologic signs and symptoms at admission | Nuchal rigidity, hyporeflexia, allodynia of toes | Weakness of the extremities, areflexia | Nuchal rigidity, weakness and pain of the extremities | Nuchal rigidity, swallowing difficulty, unable to walk | NA[a] | Ataxia, ataxic dysarthria, tremor capitis, nystagmus | Altered level of consciousness, nuchal rigidity, ataxia, ophthalmoplegia, hemiplegia, |
| CNS involvement | + | – | – | – | (+) (Postanoxic coma) | + | + (Coma) |
| Facial weakness (n. VII) | + | + | + | + | NA | – | + |
| Ocular motor weakness and ptosis (n. III, IV, VI) | – | + | – | – | NA | – | + |
| Bulbar weakness (n. IX, X, XI) | + | + | – | + | NA | + | + |

**Table 1.** Overview of seven children with severe GBS spectrum disease associated with *M. pneumoniae* infection. (*continued*)

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Sensory disturbance | + | – | – | – | NA | + | + |
| Paraesthesias | + | + | – | – | NA | + | + |
| Areflexia | + | + | + | + | NA | + | + |
| Pain | + | + | + | + | NA | + | + |
| Autonomic dysfunction | GIT | Cardiovascular | Urinary retention | Cardiovascular | Cardiovascular | – | – |
| GBS disability score[b] (admission) | 2 | 4 | 4 | 4 | NA | 3 | 4 |
| GBS disability score[b] (nadir) | 3 | 5 | 5 | 5 | 6 | 3 | 5 |
| MV (days) | – | 20 | 11 | 10 | (4)[a] | – | 5 |
| **Additional studies** | | | | | | | |
| Chest radiograph (pulmonary infiltrate) | NA | + | NA | + | – | – | + |
| Brain MRI | Mild dilatation of the ventricular system | NA | NA | NA | Postanoxic damage and edema | – | Hyperintensity and generalized edema of the brain stem with extension from crus posterior of capsula interna to the pons |
| Nerve conduction studies | Equivocal | – | AIDP | AIDP | Inexitable motor nerves, normal sensory nerve conduction | Equivocal | NA |
| CSF white blood cell count (<10/µl) | 1 | 4 | 11 | 17 | 18 | 17 | 11 |

**Table 1.** Overview of seven children with severe GBS spectrum disease associated with *M. pneumoniae* infection. (*continued*)

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| CSF protein (g/l[c]) | 0.64 | 0.91 | 2.10 | 1.70 | 3.34[a] | 1.38 | 0.32 |
| ***M. pneumoniae* diagnostics** | | | | | | | |
| PCR pharyngeal swab | – | + | NA | – | NA | NA | + |
| PCR CSF | – | – | – | NA | – | NA | – |
| IgM serum[d] (≤11.0 VU) | + (19.1) | + (45.5) | + (12.4) | + (59.5) | + (45.7) | + (14.2) | + (67.0) |
| IgG serum (≤8.0 VU) | + (23.0) | + (26.5) | + (10.9) | NA[e] | + (11.6) | – (0.0) | + (48.7) |
| IgA serum (≤11.0 VU) | + (13.5) | + (24.0) | – (0.0) | + (17.0) | – (0.0) | – (0.0) | + (34.3) |
| IgM CSF[f] (<1.3 AI) | + (2.4) | NA | NA | NA | NA | (–) | + (15.5) |
| IgG CSF (<1.3 AI) | NA | NA | NA | NA | NA | NA | + (7.15) |
| IgA CSF (<1.3 AI) | NA | NA | NA | NA | NA | NA | + (5.4) |
| **Treatment** | | | | | | | |
| Treatment agent (duration [days]) | IVIg[g] (5) | IVIg[g] (5), methyl-prednisolone (5), cefuroxime (3), clarithromycin (11) | IVIg[g] (5), methyl-prednisolone (5), cefotaxime[h], gentamicin[h] | IVIg[g] (5), dexamethasone (5), cefuroxime[h], gentamicin[h] | – | IVIg[g] (5) | IVIg[g] (1), prednisolone (5), doxycycline (7) |
| **Outcome** | | | | | | | |
| Unable to walk unaided (days) | 6 | 35 | 87 | 27 | (Deceased) | 28 | 200 |
| Long-term residual complaints and symptoms (last follow-up) | – (1.7 years) | – (2.4 years) | Tandem gait disturbed (16.8 years) | Mild weakness of the extremities (28 days) | (Deceased) | Unable to walk >200 m, balance disorder, mild weakness of the lower extremities (6.7 years) | Mild residual hemiparesis of the upper extremities, incomplete facial palsy (1.6 years) |

**Table 1.** Overview of seven children with severe GBS spectrum disease associated with *M. pneumoniae* infection. (*continued*)

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Brain MRI (last follow-up) | Residual dilatation of the ventricular system (1.2 years) | NA | NA | NA | NA | NA | Mild residua and new focal signal alteration in the ventral mesencephalon (0.3 years) |

Abbreviations: A-CIDP, acute-onset chronic inflammatory demyelinating polyneuropathy; AI, antibody index; AIDP, acute inflammatory demyelinating polyneuropathy; BBE, Bickerstaff brain stem encephalitis; CSF, cerebrospinal fluid; GBS, Guillain-Barré syndrome; GIT, gastrointestinal tract; IVIg, intravenous immunoglobulin; MRI, magnetic resonance imaging; MV, mechanical ventilation; n., cranial nerve; NA, not available; RT, respiratory tract; VU, Virotech unit; –, negative; +, positive.

[a] Neurological exam not performed; the diagnosis was not suspected until after the intubation. The patient developed cardiac arrest after intubation at day 2 after onset. After cardio-pulmonary resuscitation, he was comatose (Glasgow Coma Scale 3) and had postanoxic damage and edema in the brain MRI. After 4 days, the EEG was flat and life support withdrawn. Lumbar puncture was performed post mortem.

[b] GBS disability score[28]: 0: healthy state; 1: minor symptoms and capable of running; 2: able to walk 10 m or more without assistance but unable to run; 3: unable to walk unaided; 4: bedridden or chairbound; 5: requiring MV; 6: dead. Poor outcome is defined as the inability to walk unaided 10 m across an open space at 6 months (GBS disability score ≥3).

[c] Reference range: 0.16–0.31 g/l (1–10 years) and 0.24–0.49 g/l (10–18 years).

[d] ELISA test results were confirmed by a line immunoblot with a specificity of 93%–100% compared to real-time PCR results in respiratory tract samples.[17]

[e] Blood sample taken 3 days after IVIg treatment, serum IgG could therefore not be used for the analysis.

[f] Intrathecal antibody synthesis was determined by an antibody index (AI) as ratio between *M. pneumoniae*-specific antibody CSF/serum quotient and albumin CSF/serum quotient (according to Reiber)[29] in patient 7 (confirmed with immunoblotting) and anti-tetanus toxoid IgG CSF/serum quotient[30] in patient 1 (because of limited sample volume). In patient 6, an AI could not have been determined because serum and CSF were not simultaneously collected. An AI ≥1.3 was considered positive.[29]

[g] 0.4 g IVIg/kg.

[h] Duration of treatment not clear.

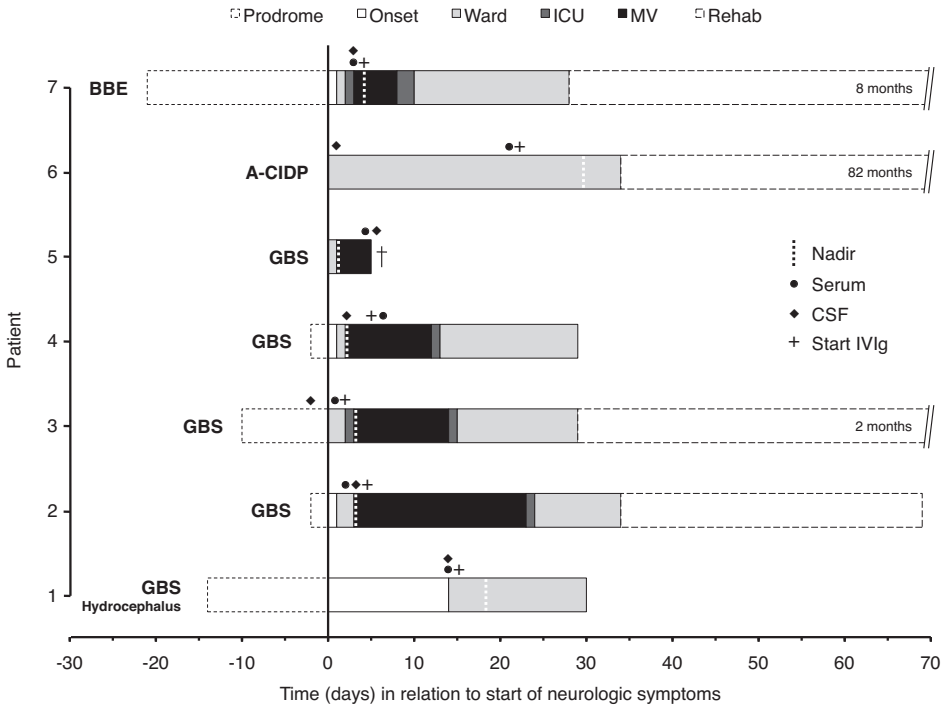


**Figure 1.** Disease course of seven children with severe GBS spectrum disease associated with *M. pneumoniae* infection.

The bars indicate the number of days at home and at various care facilities for each patient after first signs of disease. The vertical line through all cases at day 0 reflects the start of neurologic symptoms. "Prodrome" is defined as time from start of preceding symptoms of infection to start of neurologic symptoms and "onset" as time from start of neurologic symptoms to hospital admission. The final diagnosis is specified on the left. In addition, the time points of taking blood (serum), cerebrospinal fluid (CSF), and the start of intravenous immunoglobulin (IVIg) are assigned on top of the bars. Disease nadir defined as the time point of lowest GBS disability score[28] is indicated with a dashed vertical line (nadir). Abbreviations: A-CIDP, acute-onset chronic inflammatory demyelinating polyneuropathy; BBE, Bickerstaff brain stem encephalitis; ICU, intensive care unit; MV, mechanical ventilation; Rehab, rehabilitation center.



## DISCUSSION

This study demonstrates that *M. pneumoniae* infection in children can precipitate a spectrum of severe disorders of both the peripheral and CNS. *M. pneumoniae* is a major cause of respiratory tract infections in children[9] and a wide spectrum of extrapulmonary complications may develop, including encephalitis and GBS.[1] Serology and PCR are still the most accurate methods to determine the involvement of *M. pneumoniae* in GBS.[19] Serological testing in GBS is usually based on single pretreatment samples because intravenous immunoglobulin may influence the test results. Some commercially available serological tests lack the required sensitivity and specificity.[20] We therefore confirmed all

IgM responses previously detected by ELISA with a highly specific immunoblot.[17] These specific IgM responses reflect a recent activation of the immune system elicited by *M. pneumoniae* at the respiratory surface, which is essential in the context of GBS where specific preceding infections may precipitate an immune response against neural targets.[10]

In a considerable proportion of patients with GBS, *M. pneumoniae* infection was followed by a severe form of GBS. In patients with GBS, serum antibodies to *M. pneumoniae* have been found to cross-react with the myelin glycolipid galactocerebroside (GalC).[21] These anti-GalC antibodies caused demyelinating neuropathy in rabbits that were immunized with GalC[22] and have also been associated with demyelination in GBS,[23] encephalitis,[24] and acute disseminated encephalomyelitis.[25]

Given the pathogenic role of cross-reactive antibodies in GBS,[10] one might speculate that such antibodies would also be present in the CNS of the four patients with CNS involvement. Interestingly, we indeed demonstrated intrathecal synthesis of antibodies against *M. pneumoniae* in two patients with CNS involvement (BBE and GBS), which proves intrathecal infection.[18] Both patients did also show radiological evidence of CNS involvement.

GBS with CNS and prominent cranial nerve involvement can present with a short interval from onset to nadir.[26] This study additionally indicates that GBS after *M. pneumoniae* infection may present with non-specific clinical features of GBS, especially nuchal rigidity and ataxia. These clinical signs have already been reported in children with GBS.[12,27] Non-specific symptoms in patient 5 led to a misdiagnosis, which had fatal consequences because of the rapid disease progression. The awareness of a non-specific presentation of severe subtypes of GBS associated with *M. pneumoniae* infection should prevent a potentially life-threatening delay in diagnosis and lead to earlier treatment.

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# Chapter 10

## *Mycoplasma pneumoniae* triggering the Guillain-Barré syndrome: a case-control study

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## ABSTRACT

### Objective

Guillain-Barré syndrome (GBS) is an acute postinfectious immune-mediated polyneuropathy. Although preceding respiratory tract infections with *Mycoplasma pneumoniae* have been reported in some cases, the role of *M. pneumoniae* in the pathogenesis of GBS remains unclear. We here cultured, for the first time, *M. pneumoniae* from a GBS patient with antibodies against galactocerebroside (GalC), which cross-reacted with the isolate. This case prompted us to unravel the role of *M. pneumoniae* in GBS in a case-control study.

### Methods

We included 189 adults and 24 children with GBS and compared them to control cohorts for analysis of serum antibodies against *M. pneumoniae* ($n$=479) and GalC ($n$=198).

### Results

Anti-*M. pneumoniae* immunoglobulin (Ig) M antibodies were detected in GBS patients and healthy controls in 3% and 0% of adults ($p$=0.16) and 21% and 7% of children ($p$=0.03), respectively. Anti-GalC antibodies (IgM and/or IgG) were found in 4% of adults and 25% of children with GBS ($p$=0.001). Anti-GalC-positive patients showed more frequent preceding respiratory symptoms, cranial nerve involvement, and a better outcome. Anti-GalC antibodies correlated with anti-*M. pneumoniae* antibodies ($p$<0.001) and cross-reacted with different *M. pneumoniae* strains. Anti-GalC IgM antibodies were not only found in GBS patients with *M. pneumoniae* infection, but also in patients without neurological disease (8% vs. 9%; $p$=0.87), whereas anti-GalC IgG was exclusively found in patients with GBS (9% vs. 0%; $p$=0.006).

### Interpretation

*M. pneumoniae* infection is associated with GBS, more frequently in children than adults, and elicits anti-GalC antibodies, of which specifically anti-GalC IgG may contribute to the pathogenesis of GBS.

## INTRODUCTION

Guillain-Barré syndrome (GBS) is a postinfectious immune-mediated peripheral neuropathy.[1] First symptoms of GBS typically occur within 1 to 4 weeks after the clinical manifestations of a preceding infection, with *Campylobacter jejuni* the most frequently identified.[2] It is hypothesized that the immune response against the infectious agent generates antibodies that cross-react with glycolipids present in peripheral nerves and nerve roots. This results in nerve damage or functional blockade of nerve conduction.

Two thirds of GBS patients report preceding symptoms of a gastrointestinal or respiratory tract infection before onset of neurological symptoms.[1] A common cause of respiratory tract infections is *Mycoplasma pneumoniae*, where incidence of infections is higher in children than adults.[3] Apart from respiratory tract infections, *M. pneumoniae* can also cause nervous system disease.[4,5] Preceding *M. pneumoniae* infection has been serologically reported in adult and pediatric GBS with frequencies of 2% to 21%, compared with 0% to 14% in healthy adults and children and 1% to 14% in adults and children with neurological diseases other than GBS.[2,6-9] Patients with GBS after *M. pneumoniae* infection may produce antibodies against galactocerebroside (GalC),[10-14] one of the major glycolipids of both the peripheral nervous system and central nervous system.[15] Anti-GalC antibodies have been demonstrated to cross-react with *M. pneumoniae* antigens[10,13] and to cause demyelination *in vitro*[16] and in rabbits immunized with GalC.[17]

However, there are several limitations in the current knowledge about the relationship between *M. pneumoniae* infections and GBS. First, to our knowledge, *M. pneumoniae* has not been isolated from patients with GBS to test for cross-reactivity with anti-glycolipid antibodies. Second, highly variable rates of positive *M. pneumoniae* serology have been reported in GBS. Third, there is a lack of proper case-control studies, and fourth, anti-GalC antibodies have been also found in patients with *M. pneumoniae* infections without neurological symptoms.[10,12,18]

Here, we present, for the first time, an *M. pneumoniae* culture-positive GBS patient with anti-GalC antibodies that cross-reacted with the patient-derived *M. pneumoniae* strain. This prompted us to determine the relation of *M. pneumoniae* with GBS in a large case-control study, the first study in both adults and children. We hypothesized that anti-GalC antibodies are critical for development of GBS following *M. pneumoniae* infection and investigated whether GBS after this type of preceding infection presented with distinct clinical features and outcome.

## PATIENTS AND METHODS

### Ethics Statement

Ethical approval for this study was granted by the institutional review board of the Erasmus MC (Rotterdam, The Netherlands).

### Patients

The derivation set contained 462 patients with GBS (Figure 1). All patients fulfilled the current diagnostic criteria for GBS.[19] Excluded were 165 adults and 61 children because there was insufficient serum available for testing, or the sample was taken after intravenous immunoglobulin (IVIg) treatment or without exact information on the time point of collection. Further excluded were 19 adults and 4 children with either a GBS variant or with another diagnosis during follow-up. In total, 189 adults and 24 children (<18 years of age) were included (Table 1). All samples were stored at −80°C before testing.

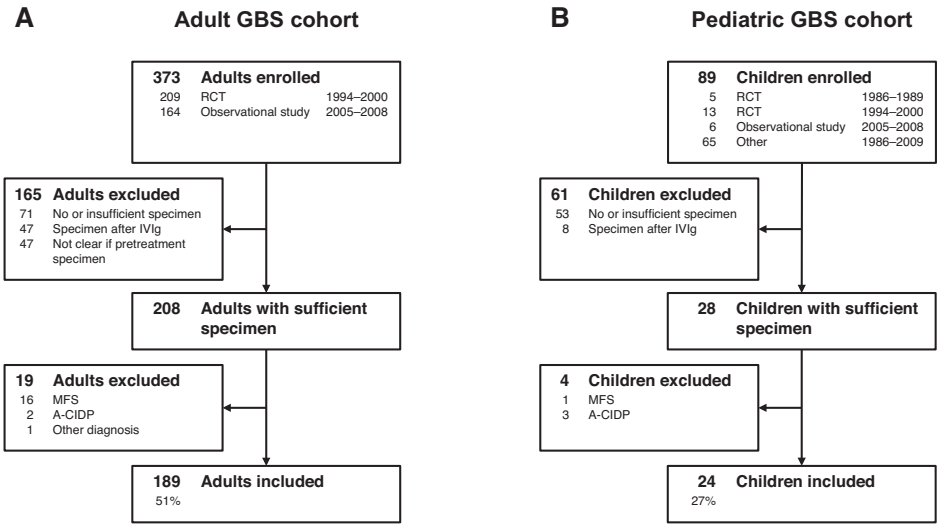


**Figure 1.** Enrollment flow diagram of GBS patients.
(A) Adult GBS cohort. (B) Pediatric GBS cohort. Abbreviations: A-CIDP, acute onset chronic inflammatory demyelinating polyneuropathy; GBS, Guillain-Barré syndrome; IVIg, intravenous immunoglobulin; MFS, Miller Fisher syndrome; RCT, randomized controlled trial.

### Controls

The control derivation set contained extensive separate cohorts for comparison of GBS patients with both the *M. pneumoniae*-specific enzyme-linked immunosorbent assay (ELISA) and the glycolipid ELISA (Table 1). In addition, 16 out of 19 *M. pneumoniae*-

**Table 1.** Demography and origin of patients and controls for anti-*M. pneumoniae* and anti-GalC antibody testing in serum.

| Anti-*M. pneumoniae* antibodies | *n* | Population (region; year) | Sex (male), % | Age, median (IQR) | Reference |
|---|---|---|---|---|---|
| Adult GBS | 189 | Adult GBS patients (The Netherlands, Belgium, Germany[a]; 1994–2000 and 2005–2008) | 58% | 52 (37–66) | [40,41] |
| Pediatric GBS | 24 | Pediatric GBS patients (Rotterdam, The Netherlands; 1986–2009) | 67% | 11 (9–14) | [40-42] |
| Adult HC[b] | 104 | Adult blood donors (South Germany; 2010–2011) | NA[2] | 20–60[b] | Serion GmbH, Würzburg, Germany (unpublished data) |
| Pediatric HC[c] | 339 | Asymptomatic children (Rotterdam, The Netherlands; 2009–2011) | 36% | 4 (1–9) | [29] |
| Pediatric NC[d] | 36 | Children with non-infectious neurological diseases (Rotterdam, The Netherlands; 2012):<br>- Auto-immune disease 11 (31%)<br>- Epilepsy 17 (47%)<br>- Neuromuscular disease 2 (5%)<br>- Other non-inflammatory diseases 6 (17%) | 50% | 7 (4–10) | Erasmus MC–Sophia Children's Hospital |
| **Anti-GalC antibodies** | | | | | |
| Adult GBS | 189 | See above | | | |
| Pediatric GBS | 24 | See above | | | |
| Adult NNC[e] | 82 | Non-neurologic adult patients (Rotterdam, The Netherlands; 2009–2013) | 60% | 48 (34–60) | Department of Viroscience, Erasmus MC |
| Pediatric NNC[e] | 80 | Non-neurologic pediatric patients (Rotterdam, The Netherlands; 2009–2014) | 51% | 12 (9–15) | Department of Viroscience, Erasmus MC |
| Pediatric NC | 36[f] | See above | | | |

Abbreviations: GalC, galactocerebroside; HC, healthy controls; Ig, immunoglobulin; IQR, interquartile range; NA, not available; NC, neurologic controls; NNC, non-neurologic controls.

All patients were tested with the Serion ELISA classic *M. pneumoniae* (Serion GmbH, Würzburg, Germany).

[a] Most patients were randomized in Dutch centers; the others in two Belgian centers and two German centers;[40,41] [b] Adult blood donor samples taken from 2010–2011 in South Germany and collected by the Serion GmbH (Würzburg, Germany). Detailed information on sex and age had not been collected; [c] Children without respiratory tract disease enrolled from 2009–2011 during admission for a planned elective surgical procedure at the short-stay department of the Erasmus MC–Sophia Children's Hospital;[29] [d] Children with non-infectious neurological diseases enrolled from January to May 2012 during routine check-ups at the pediatric neurology out-patient clinic of the Erasmus MC–Sophia Children's Hospital; [e] In-patients and out-patients without neurological diseases tested for anti-*M. pneumoniae* antibodies at the Erasmus MC between 2009–2013/2014. With the exception that patients from the neurology out-patient clinics and the neurology wards, as well as the intensive care units were excluded, no information on disease characteristics were available. The samples of these patients were stored at the department of Viroscience and selected for this study for *M. pneumoniae*-specific isotype constellations, as well as matched for sex, age, and isotype titers. The *M. pneumoniae*-specific isotype constellations are IgM+IgG- (*n*=22), IgM-IgG+ (*n*=30), and IgM-IgG- (*n*=30) for the adult NNC and IgM+IgG- (*n*=10), IgM+IgG+ (*n*=19), IgM-IgG+ (*n*=27), and IgM-IgG- (*n*=24) for the pediatric NNC, respectively; [f] Sufficient serum samples for anti-GalC antibody testing were available in 25 (69%) of 36 patients of the pediatric NC.

positive control samples of a prior study[10] were available for further testing with the glycolipid ELISA.

## Data collection

### *Clinical and laboratory data*

Clinical and laboratory characteristics of GBS patients are shown in Table 2.

**Table 2.** Clinical and laboratory characteristics of adult and pediatric GBS patients.

| | **Adult GBS** (*n*=189)[a] | **Pediatric GBS** (*n*=24)[a] |
|---|---|---|
| **Demographic characteristics** | | |
| - Sex (male), *n* (%) | 110/189 (58%) | 16/24 (67%) |
| - Age, median (IQR) | 52 (37–66) | 11 (9–14) |
| **Characteristics of GBS** | | |
| - Preceding respiratory symptoms, *n* (%) | 69/186 (37%) | 11/23 (48%) |
| - Cranial nerve involvement, *n* (%) | 64/188 (34%) | 1/21 (5%) |
| - Pain, *n* (%) | 110/185 (59%) | 14/24 (58%) |
| - CSF white blood cell count/µl, median (IQR) | 1 (1–3) | 3 (1–3) |
| - CSF protein (g/l), median (IQR) | 0.70 (0.47–1.26) | 0.64 (0.40–1.75) |
| - Electrophysiology: | | |
| - AIDP, *n* (%) | 92/176 (52%) | 9/15 (60%) |
| - AMAN, *n* (%) | 6/176 (4%) | NA[b] |
| - Equivocal, *n* (%) | 72/176 (41%) | NA[b] |
| - Inexcitable, *n* (%) | 4/176 (2%) | NA[b] |
| - Normal, *n* (%) | 2/176 (1%) | 0/15 (0%) |
| - GBS disability score at nadir, median (IQR) | 4 (3–4) | 4 (3–4) |
| - Mechanical ventilation, *n* (%) | 36/189 (19%) | 4/23 (17%) |
| **Specific treatment** | | |
| - IVIg, *n* (%) | 172/189 (91%) | 20/23 (87%) |
| - Methylprednisolone, *n* (%) | 84[c] | 3[c] |
| - Plasma exchange, *n* (%) | 1[c] | 1/23 (4%) |
| - None, *n* (%) | 17/189 (9%) | 2/23 (9%) |

Abbreviations: AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; CSF, cerebrospinal fluid; IQR, interquartile range; IVIg, intravenous immunoglobulin; NA, not available.

[a] All patients fulfilled the current diagnostic criteria for GBS.[19] Data were collected prospectively for all adults and children enrolled in the studies.[40-42] For other children, hospital records were reviewed;

[b] Other electromyography patterns than AIDP were reported in 6/15 (40%) of children, but not further specified;

[c] Combined treatment together with IVIg.

### *M. pneumoniae-specific ELISA*

All serum samples were tested for the presence of anti-*M. pneumoniae* IgM and IgG antibodies using a commercially available ELISA (Serion ELISA classic *M. pneumoniae*, Serion GmbH, Würzburg, Germany) according to the manufacturer's instructions. This assay includes *M. pneumoniae* whole-cell lysate as antigen, enriched for the highly specific adhesion protein P1. The balanced accuracy for IgM and IgG is declared with 93.4% and 97.3%, respectively (Serion GmbH).

### *Glycolipid ELISA*

IgM and IgG antibodies to GM1, GM2, GD1a, GD1b, GQ1b, and GalC (all from Sigma-Aldrich, Zwijndrecht, The Netherlands) were measured as described previously.[20,21] To determine anti-GalC antibodies, half-area 96-well plates (Costar, Corning B.V. Life Sciences, Amsterdam, The Netherlands) were coated with 450 pmol of glycolipid per well. All sera were diluted 100-fold. The optical densities (ODs) at 490 nm from uncoated wells (containing ethanol) were subtracted from the glycolipid-coated wells. Cut-off values were either predefined (i.e., a background-subtracted OD of 0.2 for IgG and 0.3 for IgM) or obtained by measuring 30 healthy control sera (mean optical density plus three times the standard deviation). Positive samples were titrated using two-fold serial dilution series starting at a 1:100 dilution. The titer was defined as the reciprocal of the highest dilution that resulted in an OD higher than the cut-off value. Antibodies to glycolipid complexes were determined as described before[22] with the modification that 225 pmol/well was used for GalC and 75 pmol/well for other glycolipids in half-area plates.

### *Inhibition ELISA*

To determine whether anti-GalC antibodies cross-react with *M. pneumoniae*, the strains M129 (subtype 1 reference strain, ATCC 29342), FH (subtype 2 reference strain, ATCC15531), A16, A58, A103, ST (all clinical strains from patients with respiratory tract infection), and the strain of the index case were grown in tissue culture flasks at 37°C/5% $CO_2$ in SP4 medium (pH 7.8). Before the medium was acidified, *M. pneumoniae* was harvested with a cell scraper, followed by centrifugation (10,000 g for 20 min). Aliquots were stored at −80°C until use. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Sera were incubated for 3 h at 4°C with increasing concentrations of *M. pneumoniae* antigen mixture 1% BSA in PBS, centrifuged, and supernatants were tested for binding to GalC as described above. As control, sera were incubated with 1% BSA in PBS alone. To assess the specificity of anti-GalC antibodies, *C. jejuni* lipooligosaccharides (LOS) GB2 and GB19 were used as controls as previously described.[23]

### Statistical analysis

Data were analyzed using the R software environment (version 3.2.1; R Foundation for Statistical Computing). The Mann-Whitney *U* test was used to compare medians, and the $\chi^2$ test or Fisher's exact test to compare proportions. The log-rank test and Cox regression was used to compare the time to outcome. Two-sided *p* values <0.05 were considered to be statistically significant. Correction for multiple testing was performed using the Bonferroni method, separately for analyses within adults and within children.

## RESULTS

### Index case presentation

A 34-year old woman presented with a 3-day history of progressive weakness of the legs with increasing difficulty in climbing stairs without weakness of the arms or sensory symptoms. She had complained about fever, cough, and shortness of breath two weeks before admission. She received no treatment for the respiratory tract infection. Except for migraine, her personal and family medical history was unremarkable.

The patient was in a slightly reduced general condition and coughed, but had no fever or other abnormal vital signs (body temperature 37.0°C, respiratory rate 19/min, oxygen saturation 95%). Neurological assessment revealed walking difficulty (GBS disability score[24] 3) and weakness of the proximal muscles of both legs (iliopsoas, hamstring, and quadriceps; Medical Research Council (MRC) sum score[25] 4). There were no sensory or cranial nerve deficits, ataxia, or weakness of the arms. Areflexia was present in both the upper and lower extremities, but plantar reflexes were present on both sides. Nerve conduction studies four days after onset of neurological symptoms were unremarkable, except for absent H-reflexes for the m. soleus on both sides. White blood cell count and C-reactive protein were normal, while the erythrocyte sedimentation rate was elevated with 71 mm/h. Cerebrospinal fluid (CSF) analysis revealed a mild pleocytosis with 39 white blood cells/µl and a slightly raised protein concentration (0.53 g/l), which is compatible with the diagnosis of GBS.[19] No chest radiograph was performed.

*M. pneumoniae* was cultured from a pharyngeal swab of the patient at presentation (Figure 2A). Typing of *M. pneumoniae* by multiple-locus variable-number tandem repeat analysis[26] revealed a subtype 1 strain. Anti-*M. pneumoniae* serum IgM, IgG, and IgA antibodies were 145, 14, 150 U/ml at presentation and 74, 34, 12 U/ml after 3 months, respectively (norm IgM ≤17, IgG ≤30, and IgA ≤14 U/ml) (Figure 2B). There was no intrathecal production of these anti-*M. pneumoniae* antibodies. Serum anti-glycolipid antibodies were found exclusively against GalC of both IgM (ΔOD 490 nm: 1.6) and IgG (0.5) isotypes (Figure 2C). Anti-GalC antibodies were absorbed by incubation with *M. pneumoniae*, most strongly by the cultured isolate, but not by *C. jejuni* LOS (Figure 2D).

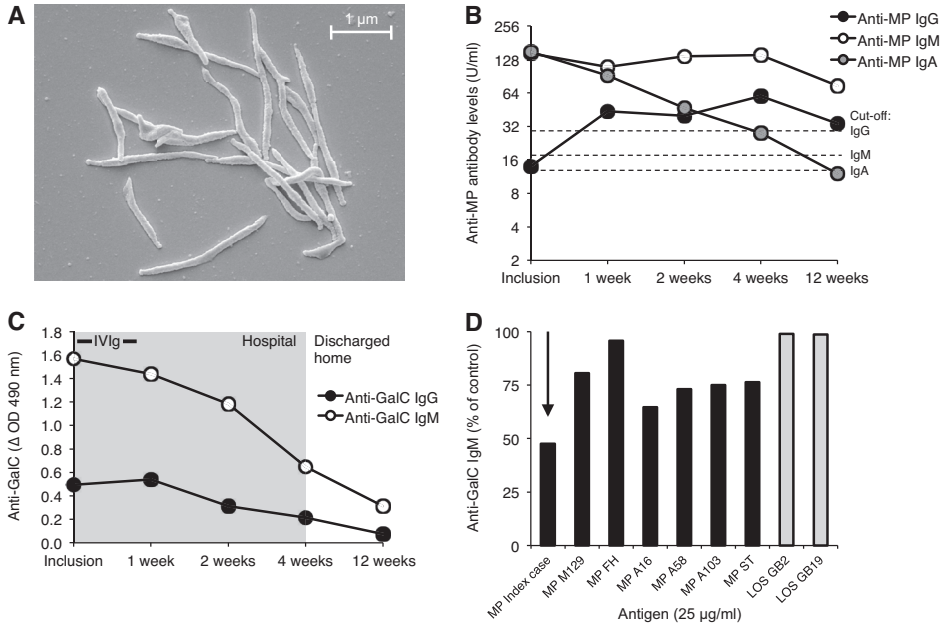


**Figure 2.** An *M. pneumoniae* culture-positive GBS case.
(A) Scanning electron microscopy picture of the *M. pneumoniae* strain cultured from respiratory specimen of the index case. (B) Serum IgM, IgG, and IgA antibody levels (U/ml) at indicated time points. The dotted lines represent the cut-off for IgM (17 U/ml), IgG (30 U/ml), and IgA (14 U/ml). (C) Kinetics of serum anti-GalC IgM and IgG at indicated time points, with length of hospital stay (gray shaded) and IVIg treatment (black bar). (D) Cross-reactivity of anti-GalC IgM assessed by incubating anti-GalC reactive serum samples of the index case at inclusion with 25 µg/ml of the specific *M. pneumoniae* isolate (arrow), as well as other *M. pneumoniae* strains (M129, FH, A16, A58, A103, ST) and *C. jejuni* lipooligosaccharides (GB2 and GB19). The mean is shown as percentage of control (incubation of serum samples without antigen) of two representative experiments. Abbreviations: GalC, galactocerebroside; Ig, immunoglobulin; IVIg, intravenous immunoglobulin; LOS, lipooligosaccharides; MP, *M. pneumoniae*; OD, optical density.

The patient was treated with a standard single course of IVIg of 0.4 g/kg for 5 days. Notably, the IVIg brand administered to the patient as well as 3 other IVIg brands did not contain anti-GalC antibodies (IgG or IgM; 4 different lot numbers of each IVIg brand tested, data not shown). Additionally, the patient received codeine cough suppressant at admission. No respiratory symptoms were reported during hospitalization. During the first two days, the muscle weakness progressed slightly with additional involvement of both arms. Thereafter, the patient improved and was discharged home after 4 weeks, with remaining muscle weakness of the triceps (MRC sum score 4/4), finger extensor (4.5/5), iliopsoas (4/4), and hamstring (4/4). Reflexes remained absent in the arms and legs. At discharge, anti-GalC IgM and IgG were hardly detectable. At a 3-month follow up, the neurological exam was normal, but the patient complained about slight muscle ache in the arms and legs.

## Increased frequency of antibodies against *M. pneumoniae* in children with GBS compared to adults

This case prompted us to investigate the frequency of IgM and IgG antibodies against *M. pneumoniae* in adult and pediatric GBS and appropriate controls. To this end, all cohorts were tested with the same *M. pneumoniae*-specific ELISA. Anti-*M. pneumoniae* IgM antibodies were demonstrated in 3% (5/189) of the adults and 21% (5/24) of the children with GBS, compared to none (0/104, $p$=0.16) in the adult healthy controls (HC) and 7% (24/339, $p$=0.03) in the pediatric HC (Table 3 and Figure 3). Anti-*M. pneumoniae* IgG antibodies were found in 34% (64/189) of the adult and 58% (14/24) of the pediatric GBS patients, in contrast to 12% (12/104, $p$<0.0001) of the adult HC and 19% (64/339, $p$<0.0001) of the pediatric HC. A positive serology was significantly more frequent in pediatric GBS patients than in adult GBS patients for both IgM ($p$=0.0005) and IgG ($p$=0.03). Children with non-infectious neurological diseases (neurologic controls, NC) had positive anti-*M. pneumoniae* IgM and IgG antibodies in 19% (7/36, $p$=1.00) and 39% (14/36, $p$=0.19), respectively. Of note, the enrollment of this control group from January to May 2012 coincided with the peak of a nationwide *M. pneumoniae* epidemic (personal communication, Adam Meijer, Institute for Public Health and the Environment (RIVM), The Netherlands[27,28]). Of the GBS patients who were positive for anti-*M. pneumoniae* IgM, all adults and 60% of the children were also positive for the IgG isotype. The median level of anti-*M. pneumoniae* IgM and IgG antibodies was higher in the GBS cohorts compared to controls, although significance was reached only for IgG in adults ($p$<0.0001) (Figure 3).

**Table 3.** Anti-*M. pneumoniae* serum antibodies in GBS patients and controls.

| **Adults** | **Adult GBS** | **Adult HC** | | | | | |
|---|---|---|---|---|---|---|---|
| | (*n*=189) | (*n*=104) | OR (95% CI) | $p_1$ | | | |
| **Anti-MP IgM+** | 5 (3%) | 0 (0%) | NA | 0.16 | | | |
| **Anti-MP IgG+** | 64 (34%) | 12 (12%) | 3.9 (1.9–8.4) | 0.00002* | | | |
| **Anti-MP IgM+IgG+** | 5 (3%) | 0 (0%) | NA | 0.16 | | | |
| **Children** | **Pediatric GBS** | **Pediatric HC** | | | **Pediatric NC** | | |
| | (*n*=24) | (*n*=339) | OR (95% CI) | $p_1$ | (*n*=36) | OR (95% CI) | $p_2$ |
| **Anti-MP IgM+** | 5 (21%) | 24 (7%) | 3.4 (0.9–10.7) | 0.03 | 7 (19%) | 1.1 (0.2–4.7) | 1.00 |
| **Anti-MP IgG+** | 14 (58%) | 64 (19%) | 6.0 (2.3–15.8) | 0.00005* | 14 (39%) | 2.2 (0.7–7.2) | 0.19 |
| **Anti-MP IgM+IgG+** | 3 (13%) | 21 (6%) | 2.2 (0.4–8.1) | 0.20 | 7 (19%) | 0.6 (0.1–3.0) | 0.72 |

Abbreviations: CI, confidence interval; HC, healthy controls; Ig, immunoglobulin; MP, *M. pneumoniae*; NA, not available; NC, neurologic controls; OR, odds ratio.

All patients were tested with the Serion ELISA classic *M. pneumoniae* (Serion GmbH, Würzburg, Germany). The differences between GBS and control groups are indicated by Fisher's exact test; $p_1$: $p$ value for GBS vs. HC; $p_2$: $p$ value for GBS vs. NC. *Significant $p$ values after correcting for multiple testing by the Bonferroni method.

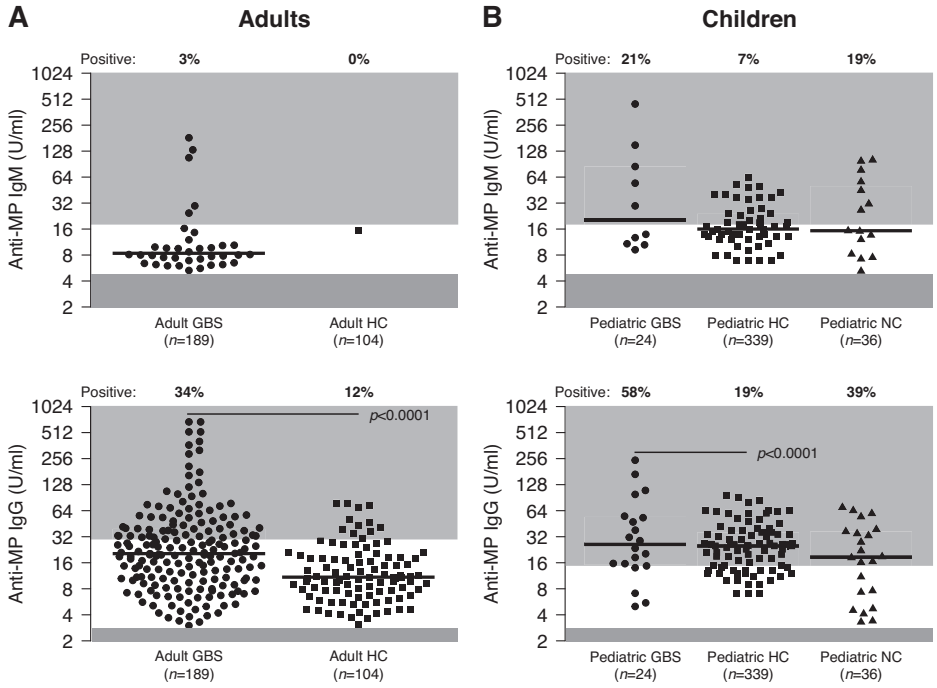


**Figure 3.** Anti-*M. pneumoniae* serum antibody levels in GBS patients and controls.
Serum IgM and IgG antibody levels (U/ml) are compared between adult GBS (A), pediatric GBS (B), and controls. The horizontal lines represent the median. The light gray area assigns positive levels above the cut-off of the test (IgM: 17 U/ml for adults and children, IgG: 15 U/ml for children and 30 U/ml for adults) and the dark gray area levels below the lower limits of quantification (IgM: 5 U/ml, IgG: 3 U/ml; note that samples below these limits are not indicated in the graphs) as indicated by the manufacturer (Serion GmbH, Würzburg, Germany). Proportions of positives are indicated on top of each graph. Significant differences in proportions of positives between GBS patients and controls after correcting for multiple testing by the Bonferroni method are indicated with the corresponding *p* value (Fisher's exact test). Abbreviations: GBS, Guillain-Barré syndrome; HC, healthy controls; Ig, immunoglobulin; MP, *M. pneumoniae*; NC, neurologic controls.



These data suggest that there is an association of a positive IgM and IgG serology for *M. pneumoniae* and GBS both in adult and pediatric patients, and that a positive serology is much more frequent in children with GBS than in adults.

## Anti-GalC antibodies are more frequent in pediatric than adult GBS and are associated with better outcome in adults and children

We determined next IgM and IgG antibodies to the glycolipids GM1, GM2, GD1a, GD1b, GQ1b, and GalC in the GBS cohorts by ELISA. Anti-glycolipid serum antibodies against any type of glycolipid were found in 33% (*n*=62) of adults and 42% (*n*=10) of children with GBS (Table 4). Antibodies were found of both isotypes IgM and IgG. The most frequent type was anti-GM1 in adult GBS, and anti-GalC in pediatric GBS.

**Table 4.** Anti-glycolipid serum antibodies in GBS patients.

| | **Adult GBS** | | | **Pediatric GBS** | | | |
|---|---|---|---|---|---|---|---|
| | (*n*=189) | IgM | IgG | (*n*=24) | IgM | IgG | *p* |
| **Anti-GalC** | 7 (4%) | 2 | 6 | 6 (25%) | 4 | 3 | 0.001* |
| **Anti-GM1** | 32 (17%) | 24 | 21 | 4 (17%) | 4 | 4 | 1.00 |
| **Anti-GM2** | 16 (8%) | 4 | 13 | 0 (0%) | NA | NA | 0.23 |
| **Anti-GD1a** | 10 (5%) | 1 | 9 | 0 (0%) | NA | NA | 0.61 |
| **Anti-GD1b** | 17 (14%[a]) | 4 | 16 | 2 (8%) | 2 | 2 | 0.74 |
| **Anti-GQ1b** | 7 (4%) | 2 | 5 | 1 (4%) | NA | 1 | 1.00 |
| **Total** | **89** | **37** | **70** | **13** | **10** | **10** | |
| **Patients with ≥1 antibody** | 62 (33%) | | | 10 (42%) | | | 0.49 |
| **Patients with ≥2 antibodies** | 19 (10%) | | | 3 (13%) | | | 0.73 |

Abbreviations: GalC, galactocerebroside; GBS, Guillain-Barré syndrome; GD1a, ganglioside disialo 1a; GD1b, ganglioside disialo 1b; GM1, ganglioside monosialo 1; GM2, ganglioside monosialo 2; GQ1b, ganglioside quadrosialo 1b; Ig, immunoglobulin; NA, not available.
The differences between adult and pediatric GBS are indicated by Fisher's exact test. *Significant *p* values after correcting for multiple testing by the Bonferroni method.
[a] Anti-GD1b antibodies were tested only in 122 (65%) of 189 adult GBS patients.

Anti-GalC antibodies (IgM and/or IgG) were present in serum of 4% (7/189) adults and 25% (6/24) children with GBS ($p$=0.001). In these anti-GalC-positive patients, antibodies against other glycolipids were only present in one adult (anti-GM1 and anti-GD1b) and one child (anti-GQ1b). No antibodies were found against complexes of two glycolipids[22] instead of a single glycolipid (data not shown).

The median age for anti-GalC-positive and –negative GBS patients was 45 years (interquartile range [IQR] 26–51) and 55 years (IQR 38–66, $p$=0.11) in the adult cohort, and 14 years (IQR 13–16) and 10 years (IQR 9–14, $p$=0.22) in the pediatric cohort, respectively. There was no gender predominance within these groups. Anti-GalC-positive GBS patients presented with intriguing clinical characteristics: anti-GalC-positive adult GBS patients more often reported preceding respiratory symptoms (86%) than did patients without anti-GalC (35%, $p$=0.02). In addition, in adults, anti-GalC-positive GBS patients tended to have more cranial nerve involvement than anti-GalC-negative patients (71% vs. 33%, $p$=0.08). In children, we did not detect significant differences between anti-GalC-positive and –negative groups, most likely because the group sizes were too small. The presence of anti-GalC antibodies was not associated with electrophysiological subtypes. In general, the outcome was better for children than adults. In children, no deaths occurred and all were able to walk unaided within 4 months (median 2 weeks). There were no significant differences in treatment regimens between anti-GalC-positive and –negative adults (IVIg, 86% vs. 91%; IVIg + methylprednisolone, 29% vs. 45%; plasma exchange, 0% vs. 1%) and children (80% vs. 83%; 20% vs. 11%; 0% vs. 6%). GBS patients with anti-GalC antibodies showed a trend for a better outcome compared to anti-GalC negative patients (Table 5).

**Table 5.** Outcome analysis of GBS patients with or without anti-GalC antibodies.

| | **Adult GBS** | | | **Pediatric GBS** | | |
|---|---|---|---|---|---|---|
| | **Anti-GalC+** | **Anti-GalC-** | | **Anti-GalC+** | **Anti-GalC-** | |
| | (*n*=7) | (*n*=182) | *p* | (*n*=6) | (*n*=18) | *p* |
| **Time to reach the ability to walk unaided** (weeks), median (IQR) | 3.0 (2.0–10.0) | 5.0 (2.0–14.0) | 0.33[a] | 1.6 (0.9–2.3) | 2.6 (1.2–5.8) | 0.18[a] |
| **Poor outcome** (GBS disability score >2 at 6 months),[45] *n* (%) | 0/7 (0) | 30/178[b] (17) | 0.51[c] | 0/6 (0) | 0/18 (0) | NA |
| **Deaths**, *n* (%) | 0/7 (0) | 7/182 (4) | 1.00[c] | 0/6 (0) | 0/18 (0) | NA |

Abbreviations: GBS, Guillain-Barré syndrome; GalC, galactocerebroside; IQR, interquartile range; NA, not available.

[a] Log-rank test. Survival analysis combined for adults and children after adjustment for age by Cox regression: $p$=0.22; [b] GBS disability score at 6 months was available only for 178 (98%) of 182 anti-GalC- adult GBS patients; [c] $\chi^2$ test.

## Anti-GalC antibodies are related to *M. pneumoniae* infections in GBS and are specifically absorbed dose-dependent by several *M. pneumoniae* strains

Next, we looked at the putative association between anti-GalC and anti-*M. pneumoniae* antibodies. Serological evidence for a recent *M. pneumoniae* infection was more often found in GBS patients with anti-GalC antibodies than in patients without anti-GalC antibodies, which was the case for both anti-*M. pneumoniae* isotypes IgM and IgG (Table 6). In fact, there was no such relation between other anti-glycolipid antibodies and anti-*M. pneumoniae* antibodies (Figure 4). Further, there was also no relation between anti-GalC antibodies and a positive *C. jejuni* serology (data not shown).

**Table 6.** Anti-*M. pneumoniae* antibodies in GBS patients with or without anti-GalC antibodies.

| **GBS** | **Anti-GalC+** | | **Anti-GalC-** | | |
|---|---|---|---|---|---|
| **Anti-MP IgM+** | **6/13** | **(46%)** | **4/200** | **(2%)** | **<0.00001*** |
| - Adults | 3/7 | | 2/182 | | 0.0003* |
| - Children | 3/6 | | 2/18 | | 0.08 |
| **Anti-MP IgG+** | **11/13** | **(85%)** | **67/200** | **(34%)** | **0.0004*** |
| - Adults | 5/7 | | 59/182 | | 0.05 |
| - Children | 6/6 | | 8/18 | | 0.02 |
| **Anti-MP IgM+IgG+** | **6/13** | **(46%)** | **2/200** | **(1%)** | **<0.00001*** |
| - Adults | 3/7 | | 2/182 | | 0.0003* |
| - Children | 3/6 | | 0/18 | | 0.01* |

Abbreviations: GalC, galactocerebroside; GBS, Guillain-Barré syndrome; Ig, immunoglobulin; MP, *M. pneumoniae*.

The differences between anti-GalC+ and anti-GalC- GBS are indicated by Fisher's exact test. *Significant $p$ values after correcting for multiple testing by the Bonferroni method.

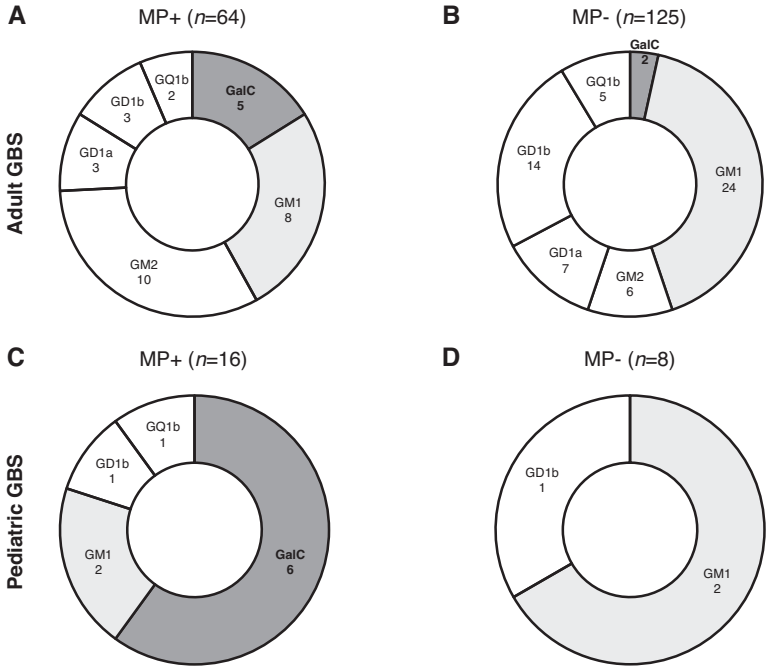


**Figure 4.** Distribution of anti-glycolipid serum antibodies in adult and pediatric GBS in relation to a positive *M. pneumoniae* serology.

In total, 89 anti-glycolipid antibodies were detected in the 189 adult GBS cases (A and B) and 13 in the 24 pediatric GBS cases (C and D). *M. pneumoniae* positivity (MP+) indicates positive for specific IgM and/or IgG (both isotypes have been associated with GBS). Each segment is provided with the target glycolipid antigen and the correspondent absolute number of detected anti-glycolipid antibodies. Anti-GD1b antibodies were tested only in 122 of 189 adult GBS patients. Abbreviations: GalC, galactocerebroside; GBS, Guillain-Barré syndrome; GD1a, ganglioside disialo 1a; GD1b, ganglioside disialo 1b; GM1, ganglioside monosialo 1; GM2, ganglioside monosialo 2; GQ1b, ganglioside quadrosialo 1b; Ig, immunoglobulin; MP, *M. pneumoniae*.

To demonstrate cross-reactivity of anti-GalC antibodies found in adult and pediatric GBS with *M. pneumoniae*, patient sera were first incubated with increasing antigen concentrations (1.66, 6.25, and 25 μg/ml) of *M. pneumoniae* subtype 1 (M129) and subtype 2 (FH) reference strains, as well as with four clinical strains from patients with respiratory tract infection. The supernatants were subsequently tested for the presence of anti-GalC antibodies by ELISA. Anti-GalC antibodies were absorbed in a dose-dependent manner by *M. pneumoniae* (Figure 5). The absorption was more effective for anti-GalC IgG than IgM by all *M. pneumoniae* strains. In a subset of patient sera, the *M. pneumoniae* FH strain showed less effective absorption of IgG compared to the other strains. Anti-GalC antibodies were not absorbed by *C. jejuni* LOS (GB2 and GB19; data not shown).

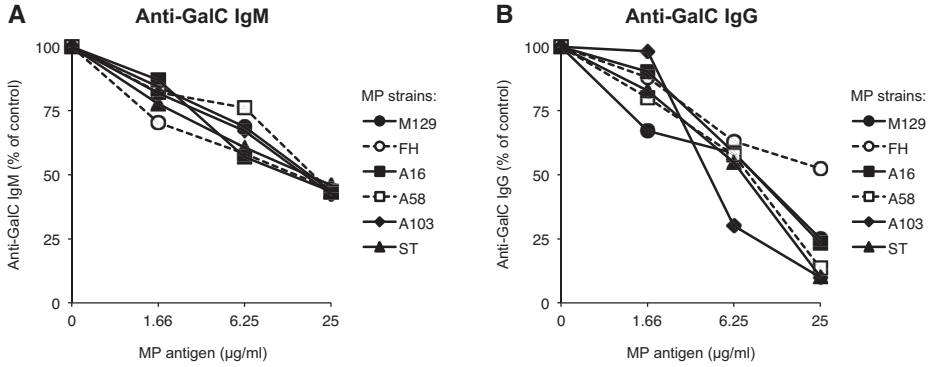


**Figure 5.** Absorption of serum anti-GalC IgM and IgG antibodies by different *M. pneumoniae* strains. Cross-reactivity of anti-GalC IgM (A) and anti-GalC IgG (B) with *M. pneumoniae* was assessed by incubating anti-GalC reactive serum samples with increasing doses of different *M. pneumoniae* strains, i.e., M129 (subtype 1 reference strain), FH (subtype 2 reference strain), A16, A58, A103, and ST (all clinical strains from patients with respiratory tract infection). Anti-GalC antibody concentrations remained unaffected with *C. jejuni* lipooligosaccharides (GB2 and GB19; data not shown). Data shown are the means as percentage of control (incubation of serum samples without antigen) and are derived from two GBS patient serum samples for each isotype. Abbreviations: GalC, galactocerebroside; Ig, immunoglobulin; MP, *M. pneumoniae*.

## Anti-GalC IgG but not IgM antibodies are strongly associated with GBS following *M. pneumoniae* infection

To determine whether anti-GalC antibodies were specifically associated with the occurrence of neurological symptoms in GBS, we also tested patients without neurological symptoms (non-neurologic controls, NNC) and patients with non-infectious neurologic diseases (NC) for the presence of anti-GalC antibodies (Table 1).

Anti-GalC antibodies could be detected in 10 patients of the pediatric NNC, of which all had a positive *M. pneumoniae* serology (anti-*M. pneumoniae* IgM $n$=8, IgG $n$=8). These patients accounted for 18% (10/56) of the control children with positive anti-*M. pneumoniae* antibodies. Anti-GalC antibodies were not detected in the adult NNC and pediatric NC.

Interestingly, all these anti-GalC-positive control children had exclusively anti-GalC antibodies of the isotype IgM. There was no difference for this isotype between *M. pneumoniae*-positive GBS and control patients ($p$=0.87). These findings were supported by retesting samples of previously reported anti-*M. pneumoniae* IgM-positive control patients without neurological symptoms,[10] in which we found anti-GalC antibodies in 38% (6/16) of the patients (5 children and 1 adult). In fact, the majority (83%, 5/6) of these control patients also had only anti-GalC IgM in serum.

In contrast, serum anti-GalC IgG were specifically associated with GBS. We found anti-GalC IgG only in *M. pneumoniae*-positive GBS patients, but not in *M. pneumoniae*-positive control patients ($p$=0.006) (Figure 6). In addition, anti-GalC IgG was also more common in *M. pneumoniae*-positive GBS than *M. pneumoniae*-negative GBS ($p$=0.03).

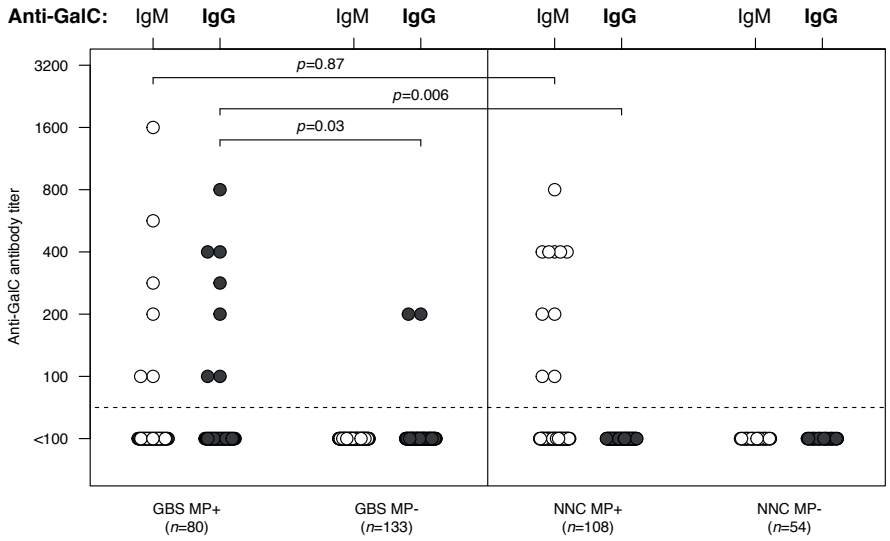


**Figure 6.** Anti-GalC isotype distribution and antibody titers in GBS patients and controls.
*M. pneumoniae* (MP+) indicates positive for specific IgM and/or IgG (both isotypes have been associated with GBS). Some patients have both IgM and IgG antibodies against GalC and are represented by two data points in the graph. Differences in proportions are indicated with the corresponding *p* value ($\chi^2$ test). Abbreviations: GalC, galactocerebroside; GBS, Guillain-Barré syndrome; Ig, immunoglobulin; MP, *M. pneumoniae*; NNC, non-neurologic controls.

These data demonstrate that *M. pneumoniae* elicits anti-GalC antibodies in a certain proportion of patients, predominantly in children, and suggest that anti-GalC IgG, but not IgM, is critical for the development of GBS following *M. pneumoniae* infection.

## DISCUSSION

The role of the respiratory pathogen *M. pneumoniae* in GBS was previously reported to be unclear. This was mainly due to the variable frequency with which anti-*M. pneumoniae* antibodies were detected in GBS patients and the presence of anti-GalC antibodies in controls. However, in this study, we demonstrate that there is a strong association between preceding *M. pneumoniae* infections and the development of GBS.

We found that the highly variable frequency of a positive anti-*M. pneumoniae* IgM serology among GBS patients is related to the age of investigated patients. The higher frequency of anti-*M. pneumoniae* IgM in children than adults (21% vs. 3%) is in line with the higher incidence of *M. pneumoniae* infections in children.[3] This is also reflected by the relatively high seroprevalence in healthy controls of children in contrast to adults (7% vs. 0%). The prevalence fluctuated significantly between year and season of sampling,[29] which was suggested to be related to the known cyclic epidemic pattern of *M. pneumoniae*

infections that occurs at intervals of 3–7 years, in addition to a background endemic pattern.[30] In fact, the increased frequency of anti-*M. pneumoniae* IgM in our pediatric controls with neurologic diseases other than GBS compared to pediatric healthy controls (19% vs. 7%) may be explained by its 5-month enrollment during a nationwide epidemic peak in European countries from 2010–2012.[30] In terms of the epidemic pattern of *M. pneumoniae* infections, we did not observe clusters of GBS cases associated with *M. pneumoniae* over the entire study period in adults and children (data not shown). The discrepancy between the high frequency of *M. pneumoniae* infections and the rarity of GBS indicates that probably not all *M. pneumoniae* strains are similarly effective in inducing GBS and, or, that not all infected individuals are equally susceptible to produce the immune response causing GBS.[31]

The anti-*M. pneumoniae* IgM and IgG responses in our GBS patients reflect a recent activation of the immune system elicited by *M. pneumoniae* at the respiratory surface, whether induced by infection or carriage.[5,29] This is essential in the context of GBS where specific preceding infections may precipitate an immune response against neural targets. An immune response against GalC was detected in 4% of adult GBS and 25% of pediatric GBS, where it was the most frequent type of the detected anti-glycolipid antibodies. In previous studies, anti-GalC antibodies were reported in 1% (2/136)[14] and 12% (16/130)[10] of GBS patients (predominantly adults). The rare detection of antibodies against other glycolipids or complexes of glycolipids in anti-GalC-positive GBS patients suggests that the presence of anti-GalC antibodies may be pathogenic in those cases. This is supported by the kinetics of anti-GalC antibodies in the index case, where decreasing levels correlated with clinical improvement. Anti-GalC antibodies indeed caused demyelination *in vitro*[16] and in experimental allergic neuritis,[17] and have also been associated with demyelination in GBS,[10] encephalitis,[32] and acute disseminated encephalomyelitis.[33] In the current study, there was no significant difference in demyelinating features between anti-GalC-positive and –negative patients possibly because demyelination was the major electrophysiological feature.[34] In comparison with the anti-GalC-negative GBS patients, anti-GalC-positive adults presented more frequently with cranial nerve involvement and a better outcome. The better outcome was most probably related to the younger age of the anti-GalC-positive patients.[35] Although their neurological illness dominated the clinical picture, they more frequently reported preceding symptoms of a respiratory tract infection than controls. This finding was congruent with the clear relation between anti-GalC and anti-*M. pneumoniae* antibodies (IgM and IgG). Although GBS following *M. pneumoniae* infection has been reported also in association with antibodies against GM1,[14] we did not find a relation between anti-glycolipid antibodies other than anti-GalC with *M. pneumoniae* serology, in both adults and children.

As in our index case, we were able to demonstrate cross-reactivity of anti-GalC antibodies with *M. pneumoniae* in adult and pediatric GBS. This cross-reactivity was

more effective for anti-GalC IgG than IgM. The specificity of anti-GalC antibodies was demonstrated by a lack of cross-reactivity with *C. jejuni* LOS. It is unclear, however, why the *M. pneumoniae* FH strain led to less effective absorption in a subset of GBS patients compared to the other strains. Differences between *M. pneumoniae* strains in their glycolipid structures (GalC-like structure) may be responsible for the observed less effective absorption.[36]

Until now, it has been difficult to explain the presence of anti-GalC antibodies in individuals with *M. pneumoniae* infection without neurological symptoms.[14] Anti-GalC antibodies were previously detected in the serum of 8% (1/12)[14], 18% (6/33)[18], and 25% (8/32)[12] of patients with *M. pneumoniae* infection without neurological disease. Indeed, we also detected anti-GalC antibodies in 18% of our non-neurologic control patients who were positive for anti-*M. pneumoniae* antibodies. Interestingly, all these patients were children, and most surprisingly, all exclusively had antibodies against GalC of the isotype IgM. These findings were confirmed by retesting other previously found *M. pneumoniae*-positive controls without neurological symptoms,[10] where the vast majority of anti-GalC-positive patients were also children and again exclusively had anti-GalC IgM antibodies. Of note, such anti-GalC IgM+IgG- patients without neurological symptoms already had an isotype class switch to anti-*M. pneumoniae* IgG antibodies in 80% of the cases (8/10), which suggested that GalC-specific B cells did not switch as opposed to *M. pneumoniae* protein-specific B cells. The amount and variety of isotypes within a B cell pool is determined by the priming condition,[37] and glycolipids and proteins have already been shown to elicit different isotype responses during helminth infections.[38] Thus, one might speculate that the anti-GalC class switch to IgG is causing GBS. We indeed found anti-GalC IgG specifically associated with GBS, and anti-GalC IgG was also present in the vast majority of other anti-GalC-positive GBS cases.[10,11,13] Further, our results are in line with the study of Susuki et al.,[14] who also found that the anti-GalC antibodies in controls were of the IgM isotype, whereas in patients with GBS, these antibodies were of the IgG isotype. In fact, an unregulated class switch has been shown to favor the onset of autoimmune disorders, such as systemic lupus erythematosus.[39] We therefore propose that an aberrant class switch of GalC-specific B cells to IgG may be critical for the development of GBS. Apart from a class switch and the titer of isotypes, also the physiological activity (fine specificity and affinity) of anti-GalC antibodies and its ability to penetrate the inflamed blood-nerve barrier may play a role in GBS.

There are several limitations of our study. First, different control cohorts were used to compare the presence of anti-*M. pneumoniae* and anti-GalC antibodies in GBS. Second, control cohorts were tested only for anti-GalC but not for other anti-glycolipid antibodies. Third, treatment modalities were systematically assessed for IVIg, methylprednisolone, or plasma exchange,[40-42] but not for antibiotics. We thus cannot consider with certainty that indeed no antibiotics were administered that may have influenced the outcome. However,

it is well-known that *M. pneumoniae* respiratory tract infection can be self-limiting[43] or respond to anti-inflammatory treatment,[44] which was also shown by the full recovery of the index case without antibiotic treatment.

In conclusion, this study demonstrates a strong relation between *M. pneumoniae* infection and GBS, more frequently in children than adults, and that anti-GalC IgG, but not IgM, is associated with GBS. These results suggest that the development of anti-GalC IgG is a critical step in the pathogenesis of GBS after an infection with *M. pneumoniae*.

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# Chapter 11

## Intrathecal antibody responses to GalC in Guillain-Barré syndrome triggered by *Mycoplasma pneumoniae*

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## ABSTRACT

*Mycoplasma pneumoniae* triggers Guillain-Barré syndrome (GBS) and elicits anti-galactocerebroside (GalC) antibodies. Specifically anti-GalC immunoglobulin (Ig) G is associated with *M. pneumoniae*-GBS, possibly because of its better ability to cross the blood-nerve barrier (BNB). We here investigated cerebrospinal fluid (CSF) for the presence of anti-GalC in GBS. Intrathecal anti-GalC was found in 46% of *M. pneumoniae*-GBS patients ($n$=6/13), in contrast to 16% of GBS controls ($n$=4/25) and 0% of non-GBS controls ($n$=0/7). The antibodies most likely originated from increased BNB permeability and/or intrathecal synthesis. Intrathecal anti-GalC IgG was specifically associated with *M. pneumoniae*-GBS, further supporting that anti-GalC IgG contributes to the pathogenesis of GBS.


## HIGHLIGHTS

- Although GBS is a peripheral neuropathy anti-GalC antibodies are found in CSF.
- Intrathecal anti-GalC IgG is specifically associated with *M. pneumoniae*-GBS.
- Anti-GalC IgG in CSF may be derived from blood and related to increased BNB permeability.
- In one patient with additional central nervous system involvement, anti-GalC IgG most likely derived from intrathecal synthesis.

## INTRODUCTION

Guillain-Barré syndrome (GBS) is a post-infectious immune-mediated peripheral neuropathy.[1] We previously showed that *M. pneumoniae* is associated with GBS[2,3] and found evidence for a recent *M. pneumoniae* infection by serology in 3% of adults and 21% of children with GBS.[3] *M. pneumoniae* elicits antibodies against galactocerebroside (GalC),[4] one of the major glycolipids of both the peripheral and central nervous system (CNS).[5] Anti-GalC antibodies have been demonstrated to cross-react with *M. pneumoniae* antigens in adult and pediatric GBS,[3] and to cause demyelination *in vitro*[6] and in rabbits immunized with GalC.[7]

A recent intriguing finding was that specifically anti-GalC immunoglobulin (Ig) G was associated with GBS after this type of infection.[3] A possible hypothesis for this observation may be the better ability of anti-GalC IgG to cross the inflamed radicular blood-nerve barrier (BNB) because of its smaller size than IgM. The nerve roots connect the peripheral and CNS, and the intrathecal segment is surrounded by cerebrospinal fluid (CSF) and covered by a BNB consisting of elastic root sheath derived from the arachnoid.[8] Based on this hypothesis and given that *M. pneumoniae* infection can also result in GBS spectrum disorders involving the CNS[9] we here investigated CSF for the presence of anti-GalC antibodies in *M. pneumoniae*-GBS and different control cohorts.

## MATERIALS AND METHODS

### Patients

Thirteen *M. pneumoniae*-GBS patients along with 25 *M. pneumoniae*-negative GBS controls and 7 non-GBS controls were selected for the study. All GBS patients fulfilled the current diagnostic criteria for GBS.[10] The *M. pneumoniae*-GBS patients were selected out of GBS patients[3] according to the following criteria: (1) positive for anti-*M. pneumoniae* IgM, and/or (2) positive for anti-GalC IgM and/or IgG (these anti-GalC antibodies had been shown to be specifically absorbed in a dose-dependent manner by *M. pneumoniae*), and (3) sufficient CSF available for further testing. The control patients were randomly selected *M. pneumoniae*-negative (IgM and IgG) GBS controls[11-13] and non-GBS controls with other neurological diseases and unknown *M. pneumoniae* serology. These non-GBS controls were patients who underwent a lumbar puncture for routine diagnostics at the Department of Neurology, Erasmus MC (Rotterdam, The Netherlands). Except for gender and age, no further information about these controls was available. The serum and CSF samples were stored at −80°C before testing. Ethical approval was granted by the institutional review board of the Erasmus MC (Rotterdam, The Netherlands).

### *M. pneumoniae*-specific antibodies

Serum anti-*M. pneumoniae* IgM and IgG antibodies were tested using a commercially available ELISA (Serion ELISA classic *M. pneumoniae*, Serion GmbH, Würzburg, Germany) as previously described.[3]

### Intrathecal antibody synthesis

Intrathecal antibody synthesis was analyzed by calculation of an antibody index of simultaneously collected CSF and serum samples. Because of the limited amount of remaining CSF in our cohort we used the tetanus toxoid-specific antibody CSF to serum concentration quotient as previously described[14] instead of the albumin concentration quotient[15] to calculate the intrathecal fraction of *M. pneumoniae*-specific IgG antibodies.

### Anti-GalC antibodies

Sera were diluted 100-fold and previously tested as described.[3] CSF was used 1:10. Optical densities (ODs) at 490 nm from uncoated wells (containing ethanol) were subtracted from glycolipid-coated wells. Cut-off values (0.05 for IgG and 0.03 for IgM) were obtained by measuring an independent set of 14 control CSF of patients with other neurological diseases (mean OD plus 3 times the standard deviation).

### Statistical methods

Data were analyzed using the R software environment (version 3.4.1). Mann-Whitney *U* test was used to compare medians, and $\chi^2$ test or Fisher's exact test to compare proportions. Spearman rank correlation was used to evaluate relationships between variables. Two-sided $p$ values <0.05 were considered to be statistically significant. Correction for multiple testing was performed using the Bonferroni method.

## RESULTS

Anti-GalC IgG and/or IgM were detected in CSF in 46% (6/13) of *M. pneumoniae*-GBS patients, i.e., 2 adults and 4 children (Figure 1). Demographic, clinical, and laboratory characteristics are shown in Table 1. All 6 patients were tested positive for anti-*M. pneumoniae* serum antibodies, and showed higher anti-*M. pneumoniae* serum antibody levels than the other *M. pneumoniae*-GBS patients. Among the clinical characteristics, *M. pneumoniae*-GBS patients with intrathecal anti-GalC reported more frequently pain ($p$<0.01). CSF analysis revealed significantly higher protein concentrations (median 1.81 g/l, interquartile range [IQR] 1.22–1.88). CSF anti-GalC IgG and IgM levels correlated with CSF protein concentration (anti-GalC IgG: $p$=0.05; IgM: $p$<0.00001) and serum anti-GalC IgG and IgM antibody titers (anti-GalC IgG: $p$=0.03; IgM: $p$=0.03).

Intrathecal antibody synthesis was tested in *M. pneumoniae*-positive GBS patients by

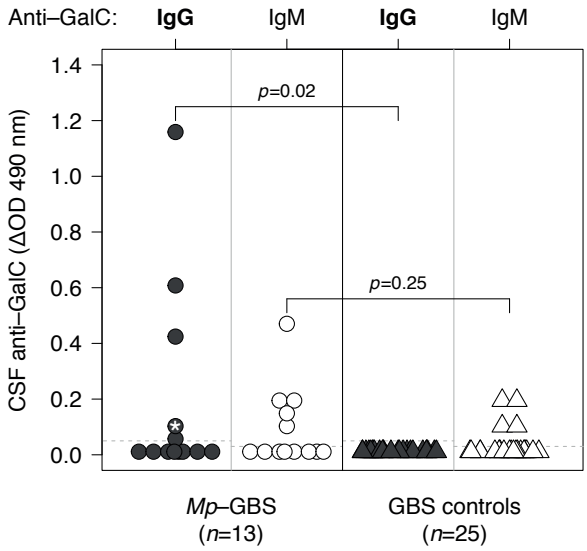


**Figure 1.** Anti-GalC antibodies in CSF of patients with GBS.
CSF anti-GalC antibody isotype and level in *M. pneumoniae*-GBS (dots) vs. GBS controls (triangles). The sample of the patient with intrathecal production of *M. pneumoniae*-specific antibodies is indicated with an asterix (CSF anti-GalC IgG+ and IgM-). Cut-off values (0.05 for IgG and 0.03 for IgM) were obtained by measuring an independent set of 14 control CSF of patients with other neurological diseases (mean OD plus 3 times the standard deviation) and indicated by a gray dotted line. Three patients have both CSF anti-GalC IgM and IgG antibodies and are represented by two data points in the graphs. The $\chi^2$ test was used to compare proportions. Abbreviations: CSF, cerebrospinal fluid; GalC, galactocerebroside; GBS, Guillain-Barré syndrome; Ig, immunoglobulin; *Mp*, *M. pneumoniae*; OD, optical density.

calculation of an antibody index of *M. pneumoniae*-specific IgG where sufficient CSF was available (*n*=3/6, 50%). Antibody indexes were 0.98, 1.22, and 2.40, respectively (cut-off <1.3). These results show that intrathecal antibody synthesis occurred in one patient (Figure 1). He was positive for intrathecal anti-GalC IgG, but not IgM. This was also the only patient with CNS involvement.

In contrast, 16% of GBS controls had anti-GalC in CSF (4/25, *p*=0.11). These were all adults. No anti-GalC was found in non-GBS controls (57% male, median age 51 years, IQR 40–68). The 4 GBS controls with anti-GalC in CSF were all tested negative for anti-GalC in serum, and exclusively had intrathecal anti-GalC IgM. They also differed from the other GBS controls by a significantly higher CSF protein concentration (median 2.33 g/l, IQR 1.97–2.46 vs. 0.65, 0.48–0.80; *p*=0.01). Intrathecal anti-GalC IgG was specifically associated with *M. pneumoniae*-GBS (*p*=0.02) (Figure 1).

Nerve conduction studies in the 10 patients with the detection of intrathecal anti-GalC antibodies (6 *M. pneumoniae*-GBS patients and 4 GBS controls) included also testing of the late electrophysiological responses and/or reflexes (F waves or H reflexes) in 7 patients, and were found to be abnormal or absent in 86% (6/7).

**Table 1.** Demographic, clinical, and laboratory characteristics of *M. pneumoniae*-GBS patients and GBS controls.

| | ***M. pneumoniae*-GBS CSF anti-GalC+** | ***M. pneumoniae*-GBS CSF anti-GalC-** | | **GBS controls** | |
|---|---|---|---|---|---|
| | (*n*=6) | (*n*=7) | $p_1$ | (*n*=25) | $p_2$ |
| **Demographic characteristics** | | | | | |
| - Sex (male), *n* (%) | 2 (33%) | 5 (71%) | ns | 10 (40%) | ns |
| - Age (years), median (IQR) | 14 (13–26) | 22 (22–58) | ns | 46 (33–68) | **0.02** |
| - Children, *n* (%) | 4 (67%) | 1 (14%) | ns | 3 (12%) | **0.02** |
| **Characteristics of GBS** | | | | | |
| - Pain, *n* (%) | 6 (100%) | 4 (57%) | ns | 8 (32%) | **0.01*** |
| - Sensory disturbances, *n* (%) | 4 (67%) | 3 (43%) | ns | 14 (56%) | ns |
| - Cranial nerve involvement, n (%) | 1 (17%) | 4 (57%) | ns | 11 (44%) | ns |
| - CNS involvement, *n* (%) | 1 (17%) | – | ns | – | ns |
| - GBS disability score[a] at nadir, median (IQR) | 4 (3–4) | 4 (3–5) | ns | 4 (3–4) | ns |
| - Mechanical ventilation, *n* (%) | 1 (17%) | 2 (29%) | ns | 5/23 (22%) | ns |
| **Electrophysiology** | | | | | |
| - AIDP, *n* (%) | 5/6 (83%) | 2/6 (33%) | ns | 12/21 (57%) | ns |
| - AMAN, *n* (%) | – | – | ns | – | ns |
| - Equivocal, *n* (%) | 1/6 (17%) | 3/6 (50%) | ns | 7/21 (33%) | ns |
| - Inexcitable, *n* (%) | – | 1/6 (17%) | ns | 2/21 (10%) | ns |
| - Normal, *n* (%) | – | – | ns | – | ns |
| **Laboratory characteristics** | | | | | |
| - Serum anti-*M. pneumoniae* IgM (U/ml),[b] median (IQR) | 90 (18–175) | 27 (14–88) | ns | 3 (2–6) | **<0.001*** |
| - Serum anti-*M. pneumoniae* IgG (U/ml),[c] median (IQR) | 207 (125–453) | 72 (30–197) | ns | 7 (5–9) | **0.004*** |
| - Time onset weakness to LP (days), median (IQR) | 6 (4–12) | 3 (2–5) | ns | 3 (2–6) | ns |
| - CSF protein (g/l), median (IQR) | 1.81 (1.22–1.88) | 0.46 (0.40–0.48) | **0.005*** | 0.71 (0.49–1.18) | ns[d] |
| - CSF white blood cell count/µl, median (IQR) | 2 (1–4) | 5 (2–7) | ns | 2 (1–2) | ns |
| - Intrathecal *M. pneumoniae*-specific antibody synthesis, *n* (%) | 1/3 (33%) | NA | – | NA | – |

Abbreviations: AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; CNS, central nervous system; CSF, cerebrospinal fluid; GalC, galactocerebroside, IQR, interquartile range; LP, lumbar puncture; NA; not available; ns, not significant. The differences between groups are indicated by Fisher's exact test (proportions) or Mann-Whitney *U* test (medians); $p_1$: *p* value for *M. pneumoniae*-GBS CSF anti-GalC+ vs. *M. pneumoniae*-GBS CSF anti-GalC-; $p_2$: *p* value for *M. pneumoniae*-GBS CSF anti-GalC+ vs. GBS controls. *Significant *p* values after correcting for multiple testing by the Bonferroni method. [a] GBS disability score: 0=healthy; 1=minor symptoms and capable of running; 2=able to walk 10 m or more without assistance but unable to run; 3=able to walk 10 m across an open space with help; 4=bedridden or wheelchair-bound; 5=requiring assisted ventilation for at least part of the day; and 6=death; [b] Cut-off: 17 U/ml for children and adults; [c] Cut-off: 15 U/ml for children and 30 U/ml for adults; [d] *p*=0.01 excluding the GBS controls with detection of intrathecal anti-GalC IgM (*n*=4).

## DISCUSSION

Here, we demonstrate the presence of intrathecal anti-GalC antibodies in a considerable proportion of GBS cases. Possible explanations for the presence of anti-GalC in CSF may be dysfunction of the BNB,[8,16] endocytosis at the neuromuscular junction with delivery to the spinal cord and brainstem,[17] or intrathecal synthesis.[16]

In *M. pneumoniae*-GBS patients with anti-GalC in CSF, intrathecal antibody synthesis was demonstrated in one case by calculation of an antibody index of *M. pneumoniae*-specific antibodies, which proves CNS infection.[16,18] Notably, this was the only case with CNS involvement.

In the other *M. pneumoniae*-GBS patients anti-GalC in CSF most likely originated from the peripheral blood caused by an increased permeability of the radicular BNB since CSF anti-GalC levels correlated with CSF protein concentrations and serum anti-GalC titers. These antibodies may be induced by *M. pneumoniae* infection outside the CNS and entrance of these antibodies to the CSF is probably made possible after breakdown of the radicular BNB. The role of intrathecal anti-GalC antibodies in the pathogenesis of GBS is unknown but they may affect the nerve roots. This is supported by a significantly increased CSF protein concentration in those *M. pneumoniae*-GBS patients with intrathecal anti-GalC antibodies, even in the first few days of the clinical course, which is characteristic for involvement of nerve roots as a result of BNB dysfunction.[8] Nerve conduction studies in the patients with intrathecal anti-GalC antibodies also showed abnormal late electrophysiological responses and/or reflexes (F waves or H reflexes), providing further evidence for the dysfunction of proximal nerve roots.[19,20]

Notably, all 6 *M. pneumoniae*-GBS patients with anti-GalC in CSF complained about pain. Although numbers are small, this finding was significantly more frequent in *M. pneumoniae*-GBS patients than in GBS controls. Pain is a very complex symptom during GBS and may be neuropathic or the result of affected nerve roots (radicular pain),[13] but we lack the clinical information to discriminate these types of pain.

The 4 GBS controls with anti-GalC IgM in CSF were all tested negative for anti-GalC in serum. In fact, it is still unclear why serum antibodies to nerve glycolipids are not found in all patients with GBS.[21] Possible reasons why they are not present anymore in the circulation at onset of GBS may be that they are all bound to nerves and/or removed from the circulation by uptake at the neuromuscular junctions with delivery to the spinal cord and brainstem.[17] Notably, all these 4 patients had significantly higher CSF protein concentration compared to other GBS controls, which may further suggests that they are washed out from the circulation into the CSF by an increased BNB permeability.

Interestingly, intrathecal anti-GalC IgG was specifically associated with *M. pneumoniae*-GBS. These findings resemble our previous observation of anti-GalC in serum: We found that the anti-GalC antibodies in controls without neurological disease were of the IgM

isotype, whereas in patients with GBS, these antibodies were of the IgG isotype.[3] These observations led to the hypothesis that anti-GalC IgM may represent an epiphenomenon in GBS. The fact that we here found anti-GalC IgG also in CSF specifically associated with *M. pneumoniae*-GBS further supports that anti-GalC IgG contributes to the pathogenesis of GBS. However, since anti-GalC IgM has also been found in CSF, other properties of anti-GalC IgG than the better ability to cross the inflamed radicular BNB may be critical for the development of GBS, like isotype titer, physiological activity (fine specificity and affinity), and different ability to activate complement.

## CONCLUSION

Our observations demonstrate that intrathecal anti-GalC IgG, but not IgM, is associated with *M. pneumoniae*-GBS, and corroborate our hypothesis that the development of anti-GalC IgG is a critical step in the pathogenesis of GBS after *M. pneumoniae* infection. The finding of an intrathecal production of anti-*M. pneumoniae* antibodies in a patient with GBS and additional CNS symptoms indicates that CNS infection may occur preceding or during GBS development and warrants further investigation.

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# Chapter 12

## Antibody responses to GalC in severe and complicated childhood Guillain-Barré syndrome

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## LETTER TO THE EDITOR

Dear Editor,

We recently presented a case series of seven children who developed severe and complicated Guillain-Barré syndrome (GBS) after infection with *M. pneumoniae*.[1] The disease was rapidly progressive and severe: one died, four had clinically defined central nervous system (CNS) involvement, and five required mechanical ventilation. Given the often relatively benign disease course of GBS after this specific type of infection[2] the severe and complicated GBS in this series was rather unexpected.

Since we recently demonstrated that pediatric and adult GBS after *M. pneumoniae* infection is associated with antibodies against galactocerebroside (GalC)[2] we investigated if anti-GalC antibodies were also present in these children with severe GBS.

Sera were tested for IgM and IgG antibodies to GM1, GM2, GD1a, GD1b, GQ1b, and GalC (all from Sigma-Aldrich, Zwijndrecht, the Netherlands) as previously described.[2-4] CSF was tested for IgM and IgG to GalC at 1:10 dilution. ODs at 490 nm from uncoated wells (containing ethanol) were subtracted from glycolipid-coated wells. Cut-off values (0.05 for IgG and 0.03 for IgM) were obtained by measuring 14 CSF samples of patients with other neurological diseases (mean OD plus 3 times the standard deviation). Some patients had previously been included in other studies.[2,5,6]

Data were analyzed using the R software environment (version 3.4.1). The $\chi^2$ test was used to compare proportions. Two-sided $p$ values <0.05 were considered to be statistically significant. The study was approved by the Erasmus MC Medical Ethics Board.

Anti-GalC IgG and/or IgM were found in six out of seven patients (86%). Antibodies against other glycolipids were present in three of those six (anti-GM1). No antibodies were found against complexes of two glycolipids (data not shown).

Since anti-GalC IgG is specifically associated with GBS after *M. pneumoniae* infection,[2] we next compared the frequency of anti-GalC IgG in severe *M. pneumoniae*-positive pediatric GBS to (1) *M. pneumoniae*-positive pediatric GBS patients (cohort as previously described[2]), who did not fulfill the criteria for severe GBS[1] (defined as "less-severe" GBS), and (2) *M. pneumoniae*-negative pediatric GBS.[2] The presence of anti-GalC IgG was significantly more frequent in serum of severe *M. pneumoniae*-positive GBS patients (43%, $n$=3/7) than in "less-severe" *M. pneumoniae*-positive GBS (17%, $n$=2/12; $p$=0.04, Figure 1) or *M. pneumoniae*-negative GBS (0%, $n$=0/8; $p$=0.03).

Sufficient CSF was available in three patients with severe *M. pneumoniae*-positive GBS. CSF anti-GalC IgG and IgM was found in three (100%) and one (33%), respectively. All these three did show signs and symptoms of CNS involvement.

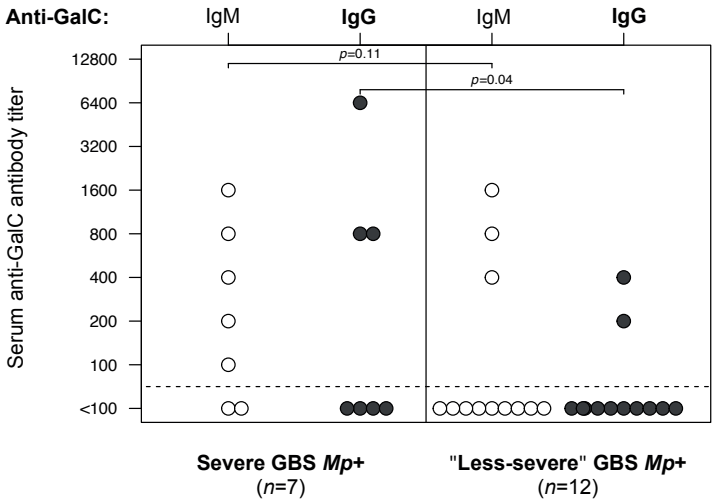


**Figure 1.** Anti-GalC isotype distribution in children with severe and "less-severe" *M. pneumoniae*-associated GBS.

Serum anti-GalC antibodies were determined in severe *M. pneumoniae*-positive childhood GBS of the case series[1] and compared to its presence in "less-severe" *M. pneumoniae*-positive childhood GBS of our previous GBS case-control study.[2] *Mp*+ indicates positive for anti-*M. pneumoniae* IgM ± IgG (both isotypes have been associated with GBS[2]). Differences in proportions are indicated with the corresponding *p* value ($\chi^2$ test). Abbreviations: GalC, galactocerebroside; GBS, Guillain-Barré syndrome; Ig, immunoglobulin; *Mp*, *M. pneumoniae*.

The increased presence of anti-GalC IgG in severe *M. pneumoniae*-positive GBS compared to "less-severe" *M. pneumoniae*-positive GBS may suggest that these antibodies are also involved in the development of severe and complicated GBS. Anti-GalC IgG was also detected in another recent study in GBS as most frequent anti-glycolipid antibody, identified in 37%.[7] The reason for the poor outcome in our series (one patient died and only two patients recovered completely) remains unclear. We[2] and Samukawa and coworkers[8] found previously no significant difference in the outcomes between anti-GalC-positive and anti-GalC-negative GBS. In both studies, the anti-GalC-negative group consisted of different subgroups of GBS patients including *Campylobacter jejuni*-related anti-GM1-positive patients who are known to have a poor outcome.[9] Here, all comparisons were performed within the *M. pneumoniae*-positive GBS group. Another risk factor for poor outcome in GBS is more severe disease at entry.[10] The development of GBS may also depend on patient-related factors that influence the susceptibility to produce cross-reactive anti-glycolipid antibodies triggered by infections.[11] The differential outcome between severe *M. pneumoniae*-positive GBS patients of our series and "less-severe" *M. pneumoniae*-positive GBS may suggest that our patients were more prone to produce a stronger immune response causing GBS. Indeed, the titers of anti-GalC IgG in these patients were higher compared to "less-severe" *M. pneumoniae*-positive GBS. Apart

from the role of anti-GalC antibodies, also other host factors may account for distinct outcomes in GBS.[12,13]

Of the seven GBS patients in this series, four had CNS involvement (two were comatose). The relatively frequent CNS involvement in neurological diseases associated with *M. pneumoniae* may be common in children. Kuwahara and coworkers[7] observed that patients with CNS diseases were younger than those with GBS and variants, and hypothesized that this could possibly be a result of the relatively undeveloped blood-brain barrier in children.

In conclusion, the correlation of anti-GalC IgG with a severe and complicated disease course and presence in CSF of patients with CNS involvement suggests that these antibodies may contribute to the pathogenesis of severe *M. pneumoniae*-associated childhood GBS.

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# Chapter 13

## Discussion

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## THE POSITION OF THE THESIS IN THE FIELD OF PEDIATRIC INFECTIOUS DISEASE

Antibiotic resistance is a major health threat worldwide and there is a strong link between the magnitude of antibiotic prescription and the selection of antibiotic resistant pathogens. Tailored prescription of antibiotic treatment is needed to minimize selection of antibiotic resistant pathogens. Suspected bacterial pneumonia is the most frequent reason to prescribe antibiotics in children.[1] Unfortunately, there is so far no single non-invasive diagnostic tool that establishes the etiology of pneumonia or that can discern between carriage and infection (except for blood cultures in e.g. pneumococcal pneumonia, where bacteremia occurs in <5% of the cases). *M. pneumoniae* is a major bacterial cause of CAP in hospitalized children and is even becoming a more important pathogen since the introduction of the pneumococcal conjugate vaccine.[2-4] In light of the global increase in antibiotic resistance of *M. pneumoniae*,[5] the **main goal is to clarify the pathogenesis** of this infection (i) to better understand the need and efficacy of antimicrobial treatment for this CAP and (ii) to develop diagnostic and therapeutic strategies for *M. pneumoniae*-associated disease. Vaccination may be a promising alternative to antibiotics. However, to develop optimal approaches to vaccination against *M. pneumoniae* it is critical to understand the immune mechanisms that may contribute to protection and/or immunopathology. The main objective of this thesis was to determine the role of antibodies to *M. pneumoniae* in (i) respiratory tract carriage, (ii) pulmonary infection, and (iii) extrapulmonary nervous system disease, where an antibody-mediated pathogenesis has been suggested. In this chapter, the main findings of our studies are reported and put in broader perspective for daily clinical routine and further research.

## IMPLICATIONS OF THE RESULTS OF THE THESIS

### Antibody responses to *M. pneumoniae*: protecting against disease

We and others observed that besides causing URTI and LRTI, *M. pneumoniae* is also carried in the URT of asymptomatic children **(Chapter 4)**.[6-10] Carriage is defined as the state where a pathogen appears in the normal flora without causing disease,[11] and is considered a prerequisite for infection.[6,12-14] Interestingly, carriage also occurs following symptomatic infection.[11]

We aimed to investigate *M. pneumoniae* carriage and infection and the role of humoral immunity on these microbial stages of pathogenesis. Since this is difficult to study in human subjects, we developed a new *M. pneumoniae* mouse model. Using this *M. pneumoniae* mouse model, we showed that *M. pneumoniae* was carried in the nose after pulmonary infection **(Chapter 2)**. These observations mimic our findings in humans, where *M. pneumoniae* was carried in the URT of children ≥4 months after symptomatic

infection **(Chapter 4)**. Our findings are of major importance for clinical practice since they show that the mere presence of a putative pathogen in the URT is not indicative for (concurrent) respiratory disease. Thus, it is likely that *M. pneumoniae* PCR-positive children with respiratory tract disease receive antibiotics against *M. pneumoniae* although the infection may be caused by other respiratory pathogens, which have been shown to co-exist frequently with *M. pneumoniae* in the respiratory tract.[6]

We further showed that B cells and antibodies are crucial for *M. pneumoniae* clearance in the lungs of mice. Our serum transfer experiments demonstrated that transferred *M. pneumoniae*-specific serum antibodies enabled B cell-deficient μMT mice to clear pulmonary *M. pneumoniae* infection **(Chapter 2)**. These data supported previous findings on the essential role of humoral immunity in clearance of *M. pneumoniae* pulmonary infection. Importantly, our observation that *M. pneumoniae* infections are more severe and chronic in μMT mice resemble the findings in patients that suffer from B cell deficiencies,[15] such as CVID, XLA, or hypogammaglobulinemia, who have been reported to be at increased risk for *M. pneumoniae* pulmonary disease and/or extrapulmonary manifestations, e.g., arthritis.[16-20] Thus, it can be speculated that if these patients experience *M. pneumoniae* infections, they may benefit from IVIg treatment, as our data demonstrate that administration of serum containing *M. pneumoniae*-specific IgG protected μMT mice from pulmonary *M. pneumoniae* infections. Notably, we have demonstrated that high amounts of *M. pneumoniae*-specific IgG are present in commercial human IVIg preparations **(Chapter 10)**. These data are in line with current guidelines on the management of patients with B cell deficiencies, which recommend the administration of IVIg to treat and/or prevent respiratory tract diseases.[21]

However, in contrast to the lungs, *M. pneumoniae*-specific IgG did not clear carriage in the URT. Transfer of WT murine serum, containing *M. pneumoniae*-specific IgG, controlled *M. pneumoniae* pulmonary infection in the lungs of μMT mice but did not lead to a reduction of *M. pneumoniae* loads in the nose. Unlike *M. pneumoniae*-specific IgG, *M. pneumoniae*-specific IgA was more abundant in nasal lavage fluid than in BALF of *M. pneumoniae*-infected mice. The induction of IgA ASCs and IgA production in the URT affected *M. pneumoniae* carriage, but did not mediate complete resolution of the bacteria **(Chapter 2)**. Notably, it has been shown for *S. pneumoniae* that higher density of pneumococcal carriage has been associated with increased risk of pneumonia.[22] Thus, although the induced antibodies do not mediate complete resolution of *M. pneumoniae* in the URT the reduction in bacterial loads may reduce at least the risk for progression to disease by local spread along the respiratory tract. Progression to pulmonary disease may be prevented by the induction of *M. pneumoniae*-specific IgG, which cleared *M. pneumoniae* in the lungs of mice. Thus, carriage may not need to be completely resolved considering the fact that progression to disease can be prevented by specific antibody responses. These findings are of major importance for the current development of *M. pneumoniae*-targeting vaccines.

Unlike other bacteria, *M. pneumoniae* lacks a peptidoglycan layer and is only covered with a cell membrane containing immunogenic proteins and lipids **(Chapter 3 and 6)**.[23] Importantly, *M. pneumoniae* glycolipids exhibit sequence homology with mammalian tissues, which may trigger cross-reactive antibodies that target cells of multiple organ systems.[24] It is therefore of major importance for the development of vaccines against *M. pneumoniae* to know which antigenic membrane structures are targeted by induced antibodies. We showed that during *M. pneumoniae* infection in mice and humans specific antibodies arise that are directed against *M. pneumoniae*-derived proteins and glycolipids **(Chapter 3)**. However, we found that clearance of *M. pneumoniae* from the lungs is predominantly mediated by antibodies reactive with *M. pneumoniae* proteins. This is supported by other studies showing that anti-*M. pneumoniae* protein antibodies may not only clear but also prevent infection: Antibodies of *M. pneumoniae*-immunized guinea pigs targeting recombinant P1 and P30 protein prevented adherence of *M. pneumoniae* to human bronchial epithelial cells *in vitro*.[25] These findings illustrate that vaccine strategies against *M. pneumoniae* should aim to target *M. pneumoniae* proteins to protect against disease. Potential *M. pneumoniae* vaccine candidates may consist of a recombinant peptide of the P1 (RP14)[26] and the P30[27] adhesion protein, which both induced a strong antibody response and reduced pulmonary disease in the animal model **(Chapter 1)**. Target groups for such vaccines would include young children without treatment alternatives to macrolides in countries with high MRMP rates and elderly people. The vaccine may help to reduce morbidity from pneumonia and secondary complications, as well as to reduce horizontal transmission to individuals at high risk for *M. pneumoniae* infection, i.e., patients that suffer from B cell deficiencies as mentioned above.

### Antibody responses to *M. pneumoniae*: triggering disease

Using $Btk^-$ mice, we observed that anti-*M. pneumoniae* glycolipid antibodies seem redundant for clearance of pulmonary *M. pneumoniae* infection **(Chapter 3)**. We detected antibodies against the glycolipid GalC of the isotype IgM in all *M. pneumoniae*-positive pneumonia patients, which demonstrated that the formation of these antibodies is part of the physiological immune response to *M. pneumoniae*. As opposed to anti-GalC IgG, which were not found in patients with *M. pneumoniae* CAP. Notably, it cannot be excluded that these seronegative patients may produce anti-GalC IgG that cause neuropathy but, after binding to the nerve terminal, are cleared from the circulation and can thus not be found in serum.[28] However, none of these patients displayed neurologic symptoms. The anti-GalC IgM response in these patients may indeed be redundant. These observations are in contrast to the findings in patients with *M. pneumoniae*-associated nervous system diseases: We found that anti-GalC IgG, but not IgM, is associated with encephalitis **(Chapter 7)** and GBS **(Chapter 10, 11, and 12)**. These results suggest that the development of anti-GalC IgG is a critical step in the pathogenesis of *M. pneumoniae*-associated nervous

system disease. These findings further support the use of immunomodulators in these disorders (corticosteroids and/or IVIg in encephalitis and IVIg in GBS) as recommended by current guidelines. Corticosteroids (such as prednisolone) reduce inflammation[29] and IVIg has pleiotropic immune-modulatory effects.[30] Importantly, we also showed that commercial human IVIg preparations did not contain anti-GalC antibodies (IgG or IgM) **(Chapter 10)**.

### Antibody responses to *M. pneumoniae*: role in diagnosis

This thesis determines the role of antibodies and B cells specific for *M. pneumoniae* in respiratory infection and extrapulmonary nervous system disease. Diagnostic tests targeting essential steps in the pathogenesis of *M. pneumoniae*-associated disease as mentioned above are illustrated in Figure 1. The detection of *M. pneumoniae*-specific B cell responses can be crucial to diagnose these clinical syndromes. Based on our findings, we summarized a new approach for assessment and management of *M. pneumoniae*-associated CAP, encephalitis, and GBS in Figure 2, which will be discussed below.

The humoral immune response is highly specific for the infecting pathogen. In *M. pneumoniae* pulmonary infection, the antigen-specific B cell response may be a sensi-

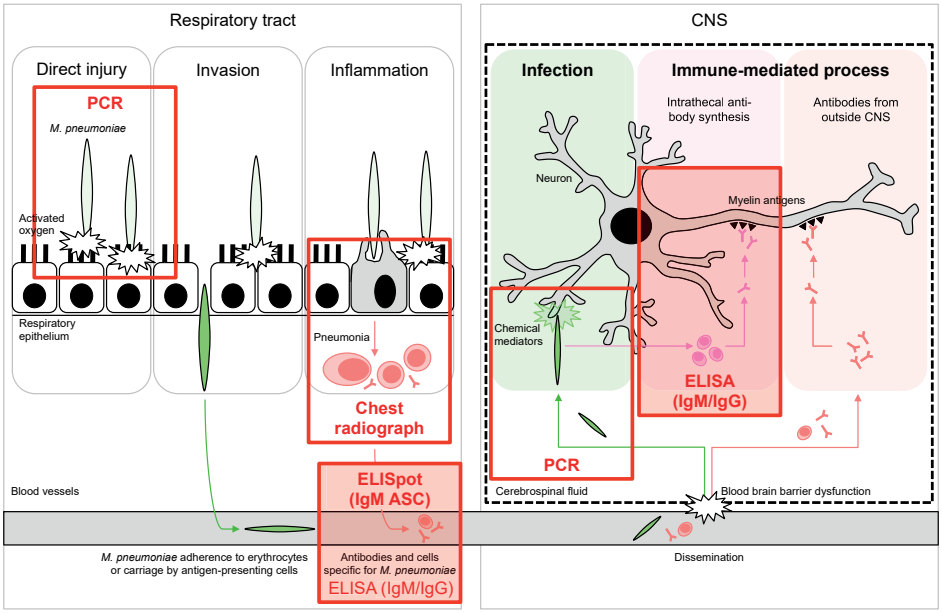


**Figure 1.** Diagnostic tests targeting essential steps in the pathogenesis of *M. pneumoniae*-associated disease.

Abbreviations: ASC, antibody-secreting cell; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot; Ig, immunoglobulin; PCR, polymerase chain reaction. 

tive indicator for infection based on our findings in mice that B cell responses are important for clearance of *M. pneumoniae* from the lungs **(Chapter 2)**. However, the use of convalescent sera is not helpful in clinical settings, because of the time delay that is inevitable when waiting for a titer increase. Notably, a specific B cell response cannot only be detected by measuring antibodies with ELISA but also by measuring ASCs with an ELISpot. We showed that the specific ASC response is more rapid and shorter lived, and thus an optimal target for determining infectious etiology in CAP patients **(Chapter 4)**. ASCs peaked at disease presentation, around 2–3 weeks following onset of symptoms, i.e., 3–6 weeks after exposure. These findings are in line with the clinical observation of slow disease progression in *M. pneumoniae* CAP, where prolonged presence of the pathogen likely results in continuous antigen exposure and, hence, immune activation, allowing to detect *M. pneumoniae*-specific ASCs in all *M. pneumoniae* CAP patients within 2–3 weeks of disease onset. So far, the specific IgM ASC ELISpot was the only test reliably differentiating *M. pneumoniae* infection from carriage. The measurement of ASCs could therefore potentially serve as a future test for the timely diagnosis of *M. pneumoniae* childhood CAP (Figure 2). Improvement in the early diagnosis of *M. pneumoniae* infection in CAP patients is of paramount importance to initiate effective and tailored antimicrobial treatment.

The diagnosis of *M. pneumoniae*-associated encephalitis is even more challenging. The lack of detectable *M. pneumoniae* DNA in CSF **(Chapter 8)** suggested a likely immune-mediated pathogenesis ignited by a respiratory inflammatory process including pneumonia. Also others[31] support our finding that pneumonia may be an indicator for the remote inflammatory process in such cases. A chest radiograph should therefore be performed in encephalitis patients to detect pulmonary infiltrates and to increase the diagnostic likelihood of *M. pneumoniae*-associated disease. This was recently implemented in the guidelines for encephalitis.[32] In addition, we detected for the first time intrathecal synthesis of *M. pneumoniae*-specific antibodies in a patient with severe encephalitis **(Chapter 5)**, which proves CNS infections. These results are of major clinical importance by establishing a causal relationship between *M. pneumoniae* and this severe clinical syndrome. Intrathecal antibody synthesis can be detected either by calculation of an antibody index or through parallel immunoblotting of simultaneously collected CSF and serum samples **(Chapter 6)**. The routine diagnostic work-up of *M. pneumoniae*-associated encephalitis should therefore aim for the detection of *M. pneumoniae*-specific antibodies (IgM and/or IgG) in both CSF and serum, in addition to *M. pneumoniae*-specific PCR in CSF (Figure 2). Further, it will be interesting to investigate the specific intrathecal antibody response additionally by ELISpot.

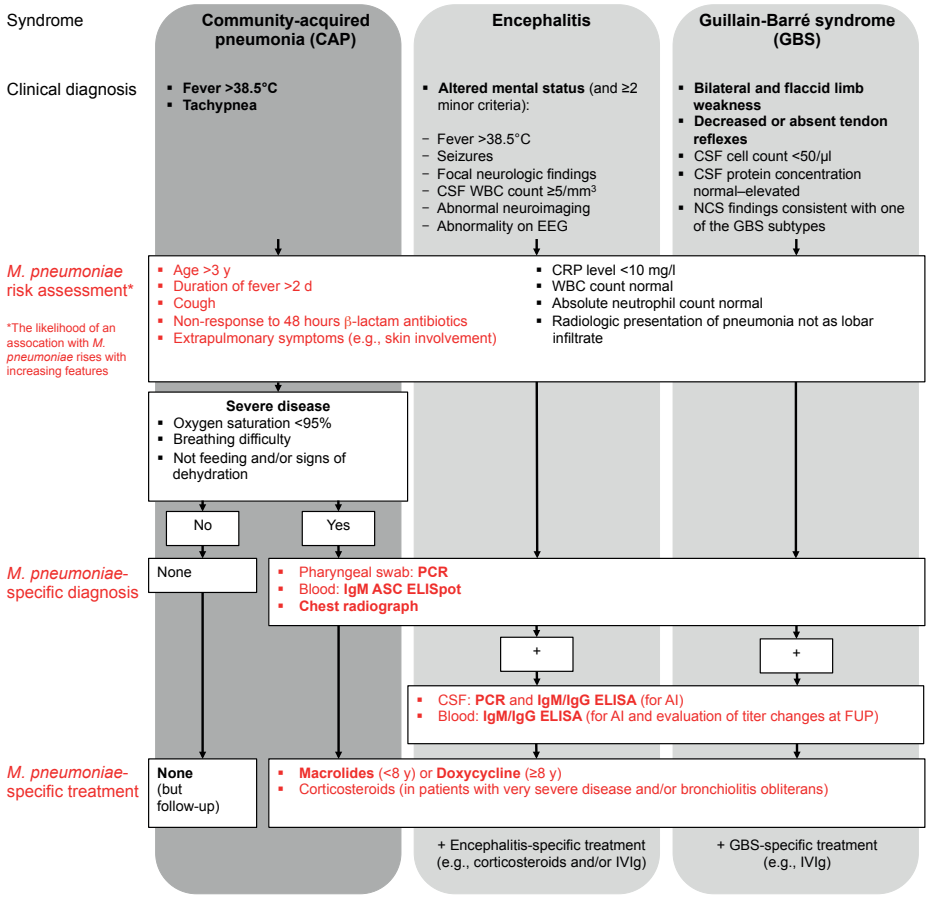


**Figure 2.** Algorithm for assessment and management of CAP and nervous system diseases associated with *M. pneumoniae* infection.
Abbreviations: AI, antibody index; ASC, antibody-secreting cell; CAP, community-acquired pneumonia; CRP, C-reactive protein; CSF, cerebrospinal fluid; EEG, electroencephalogram; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot; FUP, follow-up visit; Ig, immunoglobulin; IVIg, intravenous immunoglobulin; NCS, nerve conduction studies; PCR, polymerase chain reaction; WBC, white blood cell. 

In contrast, in *M. pneumoniae*-associated GBS, it is of lesser importance to know whether or not the detected pathogen is the cause of the infection in this postinfectious immune-mediated peripheral neuropathy. It is largely about the presence of cross-reactive antibodies, induced by either infection with or carriage of a pathogen,[33] that are associated with GBS. However, a causative relationship between an infecting pathogen and GBS may be achieved (in retrospect) by using paired patient sera in order to detect seroconversion and/or a 4-fold increase in antibody titers. However, this approach is mostly hindered by the administration of IVIg in GBS patients. Interestingly, we also established the etiological

diagnosis in two GBS patients by the detection of a specific intrathecal antibody synthesis against *M. pneumoniae* **(Chapter 9)**. The autoimmune response in GBS results in injury and inflammation at peripheral nerves and nerve roots, which are surrounded by CSF. The detection of a specific intrathecal antibody response may therefore be useful as an additional diagnostic tool to further establish the infectious diagnosis in GBS (Figure 2).

### Antibody responses to *M. pneumoniae*: role in treatment

Although *M. pneumoniae* infection is generally mild and self-limiting, patients of every age can develop severe pulmonary and/or extrapulmonary disease **(Chapter 1)**. Current guidelines suggest empiric macrolide treatment at any age if there is no response to first-line β-lactam antibiotics or in the case of very severe disease.[34] Because there is insufficient evidence about the efficacy of antibiotics in the treatment of *M. pneumoniae* LRTI in children,[35] we recommend antibiotic treatment only in children with severe *M. pneumoniae* CAP (Figure 2). In case of no treatment (i.e., not severe CAP as defined in Figure 2), patients should also be followed until they have fully recovered, not to miss a potential (but rare) progression to more severe disease.

The self-limiting course of *M. pneumoniae* infection in most cases suggests that the immunological response following infection generates inflammatory reactions that may cause pulmonary and extrapulmonary symptoms.[3] Although the pathogenesis is incompletely understood, individuals with severe illness may experience a "hyperimmune" response in the lung.[36] Importantly, our findings show that B cell immunity may not contribute to immunopathology since B cell-deficient μMT mice experienced more severe disease and lung pathology. This suggests that other immune reactions apart from B cell responses may contribute to disease. And indeed, other animal models indicate that the severity of pneumonia is increased by an intact T lymphocyte response. T cell-depleted hamsters developed less severe pulmonary disease,[37] and T cell reconstitution of SCID mice increased the severity of disease.[38] In humans, the severity of CAP has been shown to correlate positively with the size of cutaneous induration following intradermal exposition to *M. pneumoniae* antigens.[39] These results suggested a relationship between the severity of CAP and the degree of the skin reaction after intradermal application of *M. pneumoniae* antigens, which is caused by a *M. pneumoniae*-specific type IV delayed-type hypersensitivity reaction mediated by specific CD4+ T cells. In fact, the rapid clinical improvement following corticosteroid administration in children with severe *M. pneumoniae* CAP[40,41] may illustrate the contribution of the immune system to immunopathology of *M. pneumoniae*-associated disease, which may overshadow the direct pathological effects of *M. pneumoniae* on the respiratory tract in some cases. Systematic studies are needed to determine also in *M. pneumoniae* CAP the true benefit of immunomodulators, such as corticosteroids and/or IVIg (Figure 2).

## CONCLUSIONS

We demonstrated that the infection-induced *M. pneumoniae*-specific antibody response is essential to clear pulmonary *M. pneumoniae* infection, but has a limited effect on *M. pneumoniae* carriage in the URT. Our data indicate that the humoral response to *M. pneumoniae*-derived glycolipids is redundant for pulmonary clearance of *M. pneumoniae*. The hypothesis that the *M. pneumoniae*-induced anti-GalC IgG response turns autoimmune is not only of importance to understand *M. pneumoniae*-associated immune-mediated diseases, but also to construct *M. pneumoniae*-targeting vaccines, as based on these findings such vaccines may include *M. pneumoniae*-derived protein antigens rather than lipids thereby avoiding the induction of potential autoimmune anti-glycolipid antibodies.

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# Chapter 14

## Summary

## Samenvatting

## Zusammenfassung

## SUMMARY

Since the introduction of the pneumococcal conjugate vaccine, *M. pneumoniae* has been reported to be the most common bacterial cause of community-acquired pneumonia (CAP) among hospitalized children **(Chapter 1)**. Although *M. pneumoniae* infections are generally mild and self-limiting, patients of every age can develop severe CAP or extrapulmonary manifestations. Unlike other bacteria, *M. pneumoniae* lacks a peptidoglycan layer and is therefore naturally resistant to cell wall synthesis inhibitors such as β-lactams. *M. pneumoniae* is only covered by a cell membrane containing antigenic protein and glycolipid structures, of which some resemble particular host antigens (molecular mimicry) bearing the potential to trigger autoimmune disease by cross-reactive antibodies. Antibiotics *in vitro* effective against *M. pneumoniae* include macrolides, tetracyclines, and fluoroquinolones. However, the *in vivo* efficacy of these antibiotics for *M. pneumoniae* infection is unclear. Macrolides are extensively used worldwide, and this has led to alarming macrolide-resistant *M. pneumoniae* rates.

The main goal of this thesis is to clarify the pathogenesis of this infection to better understand the need and efficacy of antibiotics for CAP in patients with *M. pneumoniae* and to develop diagnostic and therapeutic strategies for *M. pneumoniae*-associated disease. Vaccination may be a promising alternative to antibiotics. However, to develop optimal approaches to vaccination against *M. pneumoniae* it is critical to define immune mechanisms that may contribute to protection or immunopathology. The studies described in this thesis focus on the role of antibodies to *M. pneumoniae* in protecting against or triggering disease.

We hypothesized that antibodies to *M. pneumoniae* protect against pulmonary infection, but trigger extrapulmonary nervous system disease. Specifically, we aimed to determine in mice and human the role of antibodies to *M. pneumoniae* in (i) respiratory tract carriage, (ii) pulmonary infection, and (iii) extrapulmonary nervous system disease, where an antibody-mediated pathogenesis has been suggested.

Summary of the most important findings in this thesis:

**Part 1: Antibody responses to *M. pneumoniae* – protecting against disease**

– A new C57BL/6 mouse model is developed for *M. pneumoniae* carriage in the upper respiratory tract (URT) after infection of the lower respiratory tract (LRT) **(Chapter 2)**, similar to humans **(Chapter 4)**;

– Observed differences in immunoglobulin (Ig) compartmentalization (URT: IgA; LRT: IgM and IgG) correlate with differences in *M. pneumoniae*-specific

B cell responses between nose- and lung-draining lymphoid tissues **(Chapter 2)**;

– The infection-induced *M. pneumoniae*-specific antibody response is essential to clear pulmonary *M. pneumoniae* infection, but has a limited effect on *M. pneumoniae* carriage in the URT **(Chapter 2)**;

– Antibodies reactive with *M. pneumoniae* are directed against both *M. pneumoniae*-proteins and –glycolipids. Pulmonary clearance is predominantly mediated by antibodies reactive with *M. pneumoniae*-proteins, whereas the humoral response to *M. pneumoniae*-glycolipids seems redundant for bacterial clearance in the LRT **(Chapter 3)**;

– Antibodies and *M. pneumoniae* DNA in the URT persist for months after CAP and its detection cannot differentiate between infection and carriage. In contrast, *M. pneumoniae*-specific IgM antibody-secreting cells (ASCs) are detectable in peripheral blood only during CAP. Specific IgM ASC detection re-classifies one out of six positive PCR test results, and thus corrects misdiagnosis of *M. pneumoniae* infection in CAP patients, in which the detection of *M. pneumoniae* DNA in pharyngeal samples reflects carriage but not disease-causing infection in the absence of specific IgM ASCs **(Chapter 4)**;

– *M. pneumoniae*-specific IgM ASCs can be detected by the here established enzyme-linked immunospot (ELISpot) assay. This new test has the potential to become the new gold standard to diagnose *M. pneumoniae* infection in clinical care for children with CAP **(Chapter 4)**.

## Part 2: Antibody responses to *M. pneumoniae* – triggering disease

### Part 2.1: Encephalitis

– The detection of an intrathecal synthesis of antibodies to *M. pneumoniae* **(Chapter 5)** and the lack of detectable *M. pneumoniae* DNA in cerebrospinal fluid (CSF) of encephalitis patients **(Chapter 8)** support an immune-mediated pathogenesis in *M. pneumoniae*-associated encephalitis **(Chapter 6)**;

– Antibodies against *M. pneumoniae* infection have been found to cross-react with the myelin glycolipid galactocerebroside (GalC), one of the major glycolipids of both the peripheral nervous system (PNS) and central nervous system (CNS). The presence of anti-glycolipid antibodies targeting GalC in CSF of encephalitis patients suggests that these antibodies mediate *M. pneumoniae*-associated CNS disease **(Chapter 7)**.

### Part 2.2: Guillain-Barré syndrome (GBS)

– *M. pneumoniae* infection is associated with GBS, more frequently in children than adults, and elicits anti-glycolipid antibodies targeting GalC **(Chapter 10)**;

- Anti-GalC antibodies in adult and pediatric GBS patients cross-react with *M. pneumoniae* **(Chapter 10)**;
- Patients positive for anti-GalC antibodies show more frequent preceding respiratory symptoms, cranial nerve involvement, and a better outcome **(Chapter 10)**;
- Anti-GalC antibodies are also present in patients with *M. pneumoniae* infection without neurological symptoms. All these patients are children and have antibodies against GalC of the isotype IgM **(Chapter 3 and 10)**. These results suggest that the development of anti-GalC IgG is a critical step in the pathogenesis of GBS after an infection with *M. pneumoniae*. We indeed find anti-GalC IgG specifically associated with GBS **(Chapter 10)**;
- The presence of anti-GalC IgG antibodies also in CSF of GBS patients **(Chapter 11)** and in GBS children with additional CNS involvement **(Chapter 9 and 12)** corroborate that these antibodies contribute to the pathogenesis of both PNS and CNS disease.

The results in this thesis pave the way for improved diagnostic and treatment strategies for *M. pneumoniae*-associated disease. We present an algorithm in the **Discussion (Chapter 13)** for the assessment and management of CAP and nervous system diseases associated with *M. pneumoniae* infection. This approach aims to reduce morbidity of pulmonary and/or extrapulmonary disease and to tailor treatment to prevent the emergence of antibiotic resistance.

Overall, our data are of importance not only to understand *M. pneumoniae*-associated immune-mediated diseases, but also for the development of preventive and therapeutic strategies such as *M. pneumoniae*-targeting vaccines. Based on our findings such vaccines may include *M. pneumoniae*-protein antigens rather than *M. pneumoniae*-lipids thereby avoiding the induction of potential autoimmune anti-glycolipid antibodies.

## SAMENVATTING

Vertaald in het Nederlands door Theo Hoogenboezem

Sinds de invoering van de vaccinatie tegen pneumococcen wordt bij kinderen die vanwege longontsteking in het ziekenhuis zijn opgenomen *M. pneumoniae* als belangrijkste bacteriële ziekteverwekker gevonden **(Hoofdstuk 1)**. *M. pneumoniae*-infecties hebben over het algemeen een mild verloop en behoeven dan geen verdere behandeling. Onafhankelijk van leeftijd kan zich echter een ernstiger vorm van longontsteking ontwikkelen. Hierbij kunnen zich ook niet direct met de luchtwegen verbonden ziekteverschijnselen voordoen. In tegenstelling tot andere bacteriën mist *M. pneumoniae* een celwand waarin peptidoglycaan voorkomt. Het vertoont daardoor een natuurlijke resistentie tegen antibiotica die de opbouw van de celwand remmen (β-lactamaseremmers). *M. pneumoniae* is daarentegen omhuld door een celmembraan waarin zich eiwitten- en glycolipidestructuren bevinden. Doordat sommige structuren sterk op die van de mens lijken (moleculaire mimicry) kunnen ze potentieel een auto-immuunreactie oproepen door de vorming van antilichamen die kruis-reageren. Tot de antibiotica die *in vitro* werkzaam zijn tegen *M. pneumoniae* behoren macroliden, tetracyclines en fluoroquinolonen. De doeltreffendheid *in vivo* van deze antibiotica tegen *M. pneumoniae* is niet altijd duidelijk. Macroliden worden wereldwijd veelvuldig ingezet tegen *M. pneumoniae*-infecties. Dit heeft geleid tot een alarmerende toename van ongevoeligheid voor deze antibiotica.

Het belangrijkste doel van dit proefschrift is de ontwikkeling en het verloop van *M. pneumoniae*-infecties op te helderen, en op basis van die kennis nieuwe diagnostische en therapeutische strategieën voor *M. pneumoniae*-infecties te ontwikkelen om te komen tot een efficiënte inzet van antibiotica bij patiënten met longontsteking, veroorzaakt door *M. pneumoniae*. Vaccinatie lijkt een veelbelovend alternatief voor antibioticagebruik. Voor een optimale vaccinatiestrategie tegen *M. pneumoniae* is het essentieel om immunologische mechanismen te begrijpen die bijdragen aan bescherming of juist immunopathologie veroorzaken.

Het beschreven onderzoek in dit proefschrift richt zich op de rol van antilichamen die zowel bij muis als mens beschermen tegen door *M. pneumoniae* veroorzaakte longontsteking, waarbij deze antilichamen buiten de long, bij de mens ongewenste neurologische problemen kunnen veroorzaken.

Samenvatting van de belangrijkste resultaten:

**Deel 1: Antilichaamvorming tegen *M. pneumoniae* – bescherming tegen ziekte**

- Een nieuw C57BL/6 muismodel is ontwikkeld voor *M. pneumoniae*-dragerschap in de bovenste luchtwegen na een door *M. pneumoniae*-infectie veroorzaakte ontsteking in de lagere luchtwegen **(Hoofdstuk 2)**;
- Het immuunsysteem is gecompartimenteerd. De hoeveelheden specifieke, tegen *M. pneumoniae* gerichte antilichamen van Immunoglobuline A correleren met veranderingen in neus-drainerende lymfeklieren. De hoeveelheden van immunoglobulines G en M correleren met veranderingen in long-drainerende lymfeklieren **(Hoofdstuk 2)**;
- Specifieke antilichamen, opgewekt tijdens een *M. pneumoniae*-infectie, zijn essentieel om een longontsteking van deze bacterie te doen verdwijnen maar hebben een beperkt effect op dragerschap in de bovenste luchtweg (neus) **(Hoofdstuk 2)**;
- Het opruimen van een door *M. pneumoniae* veroorzaakte longontsteking komt vooral tot stand door antilichamen gericht tegen eiwitten van *M. pneumoniae*. De humorale respons, veroorzaakt door antilichamen gericht tegen glycolipiden van het membraan van deze bacterie, speelt hierbij een veel geringere rol **(Hoofdstuk 3)**;
- Maanden na een doorgemaakte infectie met *M. pneumoniae* kan het DNA van de bacterie nog in de bovenste luchtwegen worden aangetoond, evenals gevormde antilichamen in het serum. Dit maakt het moeilijk onderscheid te maken tussen infectie en dragerschap. *M. pneumoniae*-specifieke, Immunoglobuline (Ig) M producerende cellen (ASCs) zijn in bloed alleen aan te tonen tijdens een infectie. Aantonen van deze specifieke IgM producerende ASCs her-classificeert een van de zes positieve PCR testresultaten als echte infectie en corrigeert daarmee vijf onterechte, als infectie aangemerkte diagnoses tot dragerschap **(Hoofdstuk 4)**;
- De enzyme-linked immunospot (ELISpot) meting, die zijn waarde inmiddels heeft bewezen bij de detectie van perifere, specifiek tegen *M. pneumoniae* gerichte, IgM producerende ASCs zou de gouden standaard kunnen worden voor de diagnose *M. pneumoniae*-infectie, bij in het ziekenhuis opgenomen kinderen met longontsteking **(Hoofdstuk 4)**.

**Deel 2: Door antilichaamvorming tegen *M. pneumoniae* veroorzaakte ziekten**

**Deel 2.1: Encefalitis**

- Het aantonen van antilichamen gericht tegen *M. pneumoniae* **(Hoofdstuk 5)** in combinatie met niet detecteerbare hoeveelheden *M. pneumoniae*-DNA in hersenvocht van patiënten met encefalitis **(Hoofdstuk 8)** steunen een

betrokkenheid van het immuunsysteem bij het ontstaan en verloop van deze aandoening **(Hoofdstuk 6)**;

– Antilichamen tegen *M. pneumoniae* kruis-reageren met het myeline glycolipid Galactocerebroside (GalC), een van de belangrijkste glycolipiden van zowel het perifere- als het centrale zenuwstelsel. Aanwezigheid van antilichamen gericht tegen het glycolipide GalC in hersenvocht doen vermoeden dat deze antilichamen betrokken zijn bij *M. pneumoniae*-geassocieerde ziekten van het centraal zenuwstelsel **(Hoofdstuk 7)**.

**Deel 2.2: Guillain-Barré Syndrome (GBS)**

– Het GBS is vaker bij kinderen dan bij volwassenen een gevolg van een voorafgaande infectie met *M. pneumoniae.* Dit komt dan door antilichaamvorming gericht tegen glycolipid GalC **(Hoofdstuk 10)**;

– Antilichamen, gevonden in serum van volwassenen en kinderen, gericht tegen GalC, reageren met *M. pneumoniae* **(Hoofdstuk 10)**;

– Patiënten die eerder klachten zoals longontsteking hebben doorgemaakt en die positief scoren op de aanwezigheid van anti-GalC antilichamen hebben vaker klachten van het zenuwstelsel die de hersenen betreffen maar ook een gunstiger klinisch beloop van de ziekte **(Hoofdstuk 10)**;

– Patienten die een *M. pneumoniae* infectie hebben doorgemaakt, echter zonder bijkomende neurologische symptomen, hebben in hun serum ook anti-GalC antilichamen. Bij al deze pediatrische patienten worden antilichamen tegen GalC gevonden van de IgM-klasse **(Hoofdstuk 3 en 10)**. Dit suggereert dat de vorming van anti-GalC antilichamen van de IgG-klasse een kritieke stap is bij de pathogenese van het Guillain-Barré Syndroom na een doorgemaakte infectie met *M. pneumoniae*. Wij hebben vastgesteld dat aanwezigheid van anti-GalC van de IgG-klasse specifiek geassocieerd is met GBS **(Hoofdstuk 10)**;

– De aanwezigheid van anti-GalC antilichamen van de Immunoglobuline G klasse in hersenvocht bij patiënten met het GBS **(Hoofdstuk 11)** en bij GBS kinderen met betrokkenheid van het centraal zenuwstelsel **(Hoofdstuk 9 en 12)** bevestigt de bijdrage van deze antilichamen aan het beloop van de ziekte van zowel het perifere als het centrale zenuwstelsel.

De resultaten in dit proefschrift maken de weg vrij voor verbeterde diagnostiek en behandelstrategieën voor aandoeningen waarbij *M. pneumoniae* betrokken is. Wij presenteren een algoritme voor het vaststellen en de bewaking van zowel longontsteking als aandoeningen van het zenuwstelsel waar een *M. pneumoniae*-infectie bij betrokken is **(Hoofdstuk 13)**. Deze benadering beoogt een verbeterd eindresultaat van de ziekte door

behandeling op maat van zowel longontstekingen als aandoeningen waar de long niet bij betrokken is, om antibiotica-ongevoeligheid te voorkomen.

De in dit proefschrift beschreven resultaten zijn niet alleen van belang voor het begrip van *M. pneumoniae*-infecties, maar ook voor de ontwikkeling van *M. pneumoniae*-vaccins. Afgaand op onze bevindingen zouden deze vaccins vooral moeten bestaan uit eiwit-antigenen van *M. pneumoniae* en niet uit lipiden van deze bacterie, om potentiële autoimmuunreacties te voorkomen.

## ZUSAMMENFASSUNG

Mit Auszügen aus Medienmitteilungen der Universität Zürich vom 01.10.2016 und 21.11.2017 (www.media.uzh.ch/de/medienmitteilungen)

Mykoplasmen (*Mycoplasma pneumoniae*) zählen zu den häufigsten Erregern der bakteriellen Lungenentzündung (Pneumonie) beim Kind. Die weltweite Einführung der Impfung gegen Pneumokokken, den Hauptvertreter der bakteriellen Pneumonie, hat dazu geführt, dass *M. pneumoniae* mittlerweile der häufigste nachgewiesene bakterielle Erreger der ambulant erworbenen Pneumonie (community-acquired pneumonia, CAP) in den USA ist **(Kapitel 1)**. Eine Impfung gegen *M. pneumoniae* gibt es bis jetzt nicht. Obwohl eine Infektion mit *M. pneumoniae* vorwiegend mild und selbstlimitierend verläuft, können in jeder Altersgruppe schwere Pneumonien auftreten sowie verschiedene andere Organsysteme betroffen sein (z.B. Nervensystem, Haut, Blutgefässsystem und Verdauungstrakt). Im Gegensatz zu anderen Bakterien besitzen Mykoplasmen keine Zellwand. Sie sind daher natürlich resistent gegen die bei der Pneumonie primär eingesetzten Penicillin-Antibiotika, welche die Zellwandsynthese hemmen. Anstatt einer Zellwand hat *M. pneumoniae* nur eine Zellhülle, welche aus vielen Eiweiss- und Fettstoffen besteht. Den Mykoplasmen ist eigen, dass gewisse dieser Eiweiss- und Fettstoffe grosse Ähnlichkeit mit körpereigenen Strukturen haben (z.B. mit den Nervenscheiden). Diese Fremdstoffe (Antigene) von *M. pneumoniae* können daher im Körper die Bildung von Antikörpern gegen sich selbst bewirken. Antibiotika, welche das Wachstum von Mykoplasmen *in vitro* (im Reagenzglas) hemmen, sind Makrolide, Tetrazykline und Fluorochinolone. Jedoch ist bis anhin unklar, ob diese Antibiotika auch *in vivo* (im Körper) den Krankheitsverlauf positiv beeinflussen. Trotzdem werden v.a. Makrolide sehr häufig eingesetzt, was zu einer weltweit alarmierenden Zunahme von Antibiotikaresistenzen geführt hat. In gewissen Weltregionen gibt es daher für Kleinkinder oft keine geeigneten Antibiotika mehr gegen Mykoplasmen.

Das Ziel dieser Arbeit ist es, die Krankheitsentstehung dieser Infektion besser zu verstehen, damit man in Zukunft den Nutzen einer Antibiotikatherapie für diese Pneumonie besser evaluieren und weitere diagnostische und therapeutische Strategien gegen *M. pneumoniae* entwickeln kann. Die Entwicklung spezifischer Impfstoffe könnte bei der Bekämpfung der Mykoplasmen eine vielversprechende Alternative zu den Antibiotika sein. Eine erfolgreiche Impfstoffentwicklung setzt jedoch voraus, dass man die Reaktionen des Immunsystems als Antwort auf einen Erreger, welcher ganz oder teilweise im Impfstoff enthalten ist, bestmöglich versteht; d.h. ob die durch Impfung und/oder Infektion ausgelöste Immunreaktion schützt (Verhinderung der Krankheit) oder sogar selbst zur Krankheit beitragen kann (Entzündungsreaktion, Autoimmunkrankheit). Die Studien dieser Arbeit widmen sich der Rolle der Antikörper als Teil der Immunantwort gegen *M. pneumoniae* mit der Frage: Verhindern oder verursachen diese Antikörper Mykoplasmen-bedingte Krankheiten?

Hypothese: Wir nehmen an, dass Antikörper gegen *M. pneumoniae* vor einer Pneumonie schützen, jedoch bestimmte Krankheiten des Nervensystems auslösen können. Im Speziellen möchten wir die Rolle dieser gegen *M. pneumoniae* gerichteten Antikörper untersuchen hinsichtlich (i) Besiedlung im Nasen-Rachen-Raum (Kolonisation), (ii) Infektion in der Lunge (Pneumonie), und (iii) *M. pneumoniae*-assoziierten neurologischen Erkrankungen wie Enzephalitis und Guillain-Barré-Syndrom (GBS), bei welchen eine Antikörper-vermittelte Autoimmunantwort vermutet wird.

Zusammenfassung der wichtigsten Ergebnisse dieser Arbeit:

**Teil 1: Antikörper gegen *M. pneumoniae* – Schutz vor Krankheit**

- Spezifische Immunzellen, sogenannte B-Zellen, sind für die Heilung der Infektion essentiell. Die von ihnen produzierten Antikörper eliminieren die Mykoplasmen in der Lunge. Hingegen bleiben die Bakterien im Nasen-Rachen-Raum wochenlang bestehen. Bakterien wurden mit einem Fluoreszenzstoff kultiviert und dadurch erstmals während der Infektion visuell in der Lunge und den oberen Atemwegen verfolgt. Die Ergebnisse am neu entwickelten Mausmodell (C57BL/6-Mäuse) bestätigen klinische Beobachtungen bei Kindern, deren obere Atemwege im Anschluss an eine Infektion mit Mykoplasmen besiedelt blieben **(Kapitel 2 und 4)**;
- Die Immunabwehr nach der Infektion unterscheidet sich wesentlich zwischen unteren Atemwegen (Lunge) und oberen Atemwegen (Nasen-Rachen-Raum): In der Lunge hat es mehr sogenannte IgM- und IgG-Antikörper sowie eine deutliche Zunahme und Aktivierung von B-Zellen in den lokalen Lymphknoten – wodurch die Erreger innerhalb von Wochen zerstört werden. Im Gegensatz dazu hat es in den oberen Atemwegen IgA-Antikörper, keine Aktivierung von B-Zellen und demzufolge eine Persistenz des Erregers. Experimente mit Mäusen ohne B-Zellen liefern letztlich den Beweis, dass die in die Mäuse transferierten Antikörper die Bakterien in der Lunge effektiv zerstören, diese aber den Erreger in den oberen Atemwegen nicht eliminieren können **(Kapitel 2)**;
- Die Antikörperreaktion richtet sich gegen Eiweiss- und Fettstoffe der Zellhülle von *M. pneumoniae*. Antikörper, welche die Mykoplasmen in der Lunge eliminieren, sind gegen Eiweissstoffe gerichtet. Hingegen scheinen diejenigen gegen Fettstoffe nicht notwendig zu sein für die Heilung der Infektion **(Kapitel 3)**;
- Der Nachweis von spezifischen Antikörpern im Blut und *M. pneumoniae* in den oberen Atemwegen erlaubt es nicht zu unterscheiden, ob die Mykoplasmen den Nasen-Rachen-Raum, also die Pforte zur Lunge, lediglich besiedeln

(Kolonisation) oder ob sie die Ursache der Pneumonie sind (Infektion). Der Nachweis spezifischer B-Zellen, welche die Antikörper produzieren (antibody-secreting cells, ASCs), ist hingegen ein effizienter diagnostischer Indikator einer Infektion mit Mykoplasmen – im Unterschied zu einer Kolonisation, bei der keine solchen B-Zellen im Blut des Patienten vorhanden sind **(Kapitel 4)**;

– Diese spezifischen B-Zellen (ASCs) können mit einem eigens entwickelten Test (enzyme-linked immunospot assay, ELISpot) zuverlässig nachgewiesen werden. Dieser Test könnte in der Zukunft als neuer Standard ("Goldstandard") für die Diagnose der Mykoplasmen-Pneumonie verwendet werden **(Kapitel 4)**.

## Teil 2: Antikörper gegen *M. pneumoniae* – Auslöser der Krankheit

### Teil 2.1: Enzephalitis

– Mykoplasmen sind nicht nur verantwortlich für Atemwegsinfektionen, sondern können bei Betroffenen auch zu Hirnentzündung (Enzephalitis) und Nervenerkrankung mit Empfindungsstörungen und Lähmungserscheinungen (GBS) führen. Verantwortlich dafür sind Antikörper, die nicht nur die Bakterien, sondern gleichzeitig die Hülle der körpereigenen Nervenzellen angreifen. Die grosse Ähnlichkeit von Fettstoffstrukturen der Zellhülle von *M. pneumoniae* mit körpereigenen Strukturen der Nervenscheiden führt dazu, dass sich die Immunabwehr sowohl gegen die Mykoplasmen als auch gegen die umhüllende Myelinschicht von Nervenbahnen richtet. Dabei handelt es sich um Antikörper, die ein bestimmtes Zucker-Fett-Molekül der Zellhülle von Mykoplasmen erkennen. Diese Antikörper binden gleichzeitig an Galactocerebrosid (GalC), einer der häufigsten Bausteine im menschlichen Myelin. Diese fettreiche Substanz stellt die elektrische Leitfähigkeit der Nervenfasern im peripheren Nervensystem (PNS) und zentralen Nervensystem (ZNS) sicher. Wird sie zerstört, kommt es zu Lähmungen und Empfindungsstörungen (PNS) bzw. Bewusstseinseinschränkung (ZNS). Der Nachweis von Antikörpern gegen *M. pneumoniae* und gegen GalC in der Gehirn-Rückenmarksflüssigkeit (Liquor) bei Patienten mit Mykoplasmen-Pneumonie, welche im Verlauf eine Enzephalitis entwickeln, lässt einen Antikörper-vermittelten Krankheitsprozess (Autoimmunkrankheit) der zugrundeliegenden Enzephalitis vermuten **(Kapitel 5–8)**.

### Teil 2.2: Guillain-Barré-Syndrom (GBS)

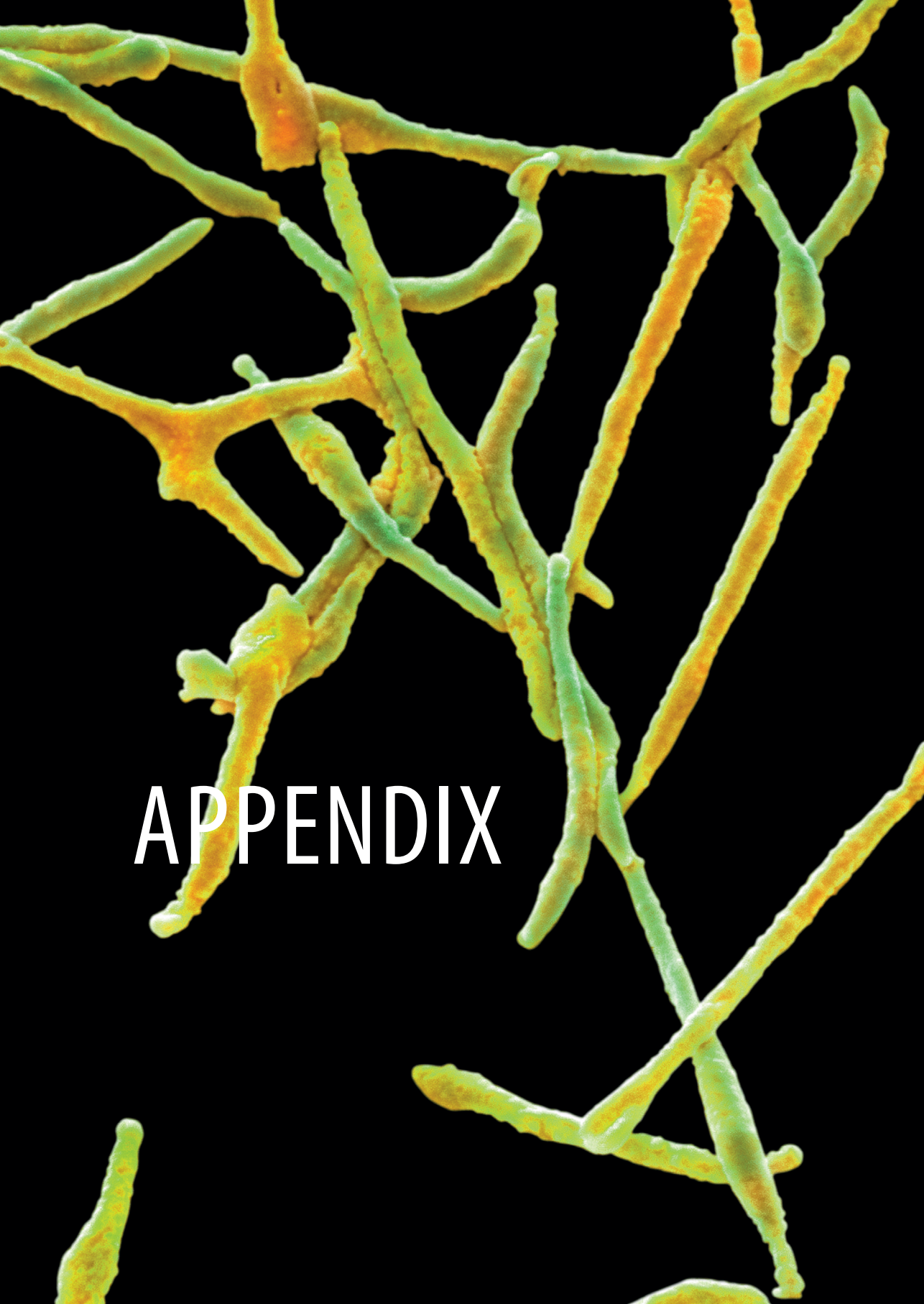
– *M. pneumoniae* löst die Autoimmunkrankheit GBS aus, häufiger bei Kindern als bei Erwachsenen. Das GBS ist eine akute lebensbedrohliche Erkrankung der Nerven des PNS, die zu Lähmungen, Schwäche und Empfindungsstörun-

gen an Armen und Beinen führt. Uns gelingt es erstmals, Mykoplasmen von einem GBS-Patienten im Labor zu kultivieren **(Kapitel 10)**;

– Bereits zuvor wurden bei GBS-Patienten vereinzelt Antikörper gegen GalC nachgewiesen. Auch bei diesem Patienten finden sich solche, und ihre Konzentration im Blut korreliert mit dem Krankheitsverlauf. Tatsächlich reagieren die Antikörper gegen GalC in immunologischen Tests am stärksten mit dem vom Patienten entnommenen und im Labor kultivierten Bakterienstamm. Auch weitere Mykoplasmen-Stämme reagieren, wenn auch schwächer, wohingegen andere Bakterienarten nicht erkannt werden. Somit ist der Nachweis der Kreuzreaktivität des Anti-GalC-Antikörpers erbracht **(Kapitel 10)**;
– Interessanterweise finden sich Anti-GalC-Antikörper auch bei Patienten ohne GBS, die kurz zuvor mit Mykoplasmen infiziert wurden. Allerdings sind diese ausschliesslich vom Antikörper-Isotyp M (Immunglobulin M, IgM), der im Verlauf einer Immunantwort am frühesten gebildete Typ **(Kapitel 3 und 10)**. Die Anti-GalC-Antikörper bei GBS-Patienten sind dagegen vom Typ IgG **(Kapitel 10)**. Dieser Wechsel des Antikörper-Typs könnte für die Entstehung von GBS mitverantwortlich sein;
– Diese Vermutung wird bekräftigt durch den Nachweis von ebendiesen Anti-GalC-Antikörper vom Typ IgG im Liquor von GBS-Patienten **(Kapitel 11)** und Liquor von GBS-Kindern mit zusätzlich ZNS-Symptomen **(Kapitel 9 und 12)**.

Die Resultate dieser Arbeit bahnen den Weg für verbesserte diagnostische und therapeutische Strategien für Erkrankungen verursacht durch *M. pneumoniae*. In der **Diskussion (Kapitel 13)** präsentieren wir einen Algorithmus für die klinische Beurteilung und das Management von Patienten mit Mykoplasmen-Pneumonien und Patienten mit *M. pneumoniae*-assoziierter Enzephalitis oder GBS. Kranke Kinder erhalten damit künftig eine gezielte und optimal wirksame antibiotische Therapie gegen die ursächlichen Bakterien. Als positiver Nebeneffekt wird zudem der Resistenzentwicklung von Bakterien entgegengewirkt.

Die Resultate können weiter helfen, spezifische Impfstoffe zu entwickeln, die das Immunsystem auf die Abwehr vorbereiten und eine Infektion verhindern würden. Basierend auf den Ergebnissen dieser Arbeit sollte ein solcher Impfstoff Eiweiss- und nicht Fettstoffe enthalten, damit sich die Immunabwehr ausschliesslich gegen die Mykoplasmen jedoch nicht gegen die umhüllende Myelinschicht von Nervenbahnen richtet.



# APPENDIX

## ABOUT THE AUTHOR

Patrick M. Meyer Sauteur was born on December 19, 1980 in Zurich, Switzerland. He completed secondary school (Gymnasium) in 2001 at the Kantonsschule Bülach (Mathematics and Natural Sciences). In the same year he started his medical training at the University of Zurich. In 2004, he began his thesis to obtain the doctor of medicine (MD) on chronic achilles tendinopathy and ruptures at the Department of Orthopedics at Balgrist University Hospital Zurich (supervisor: Prof.dr. Christian Gerber). He finished medical school in 2007 and completed his MD thesis in 2008. He did his medical training in pediatrics from 2008 to 2009 at the Triemli Hospital Zurich and from 2010 to 2012 at the University Children's Hospital Zurich, where he became a pediatrician in 2013 (Swiss Board in Pediatrics). In 2012, he started his fellowship in pediatric infectious diseases (PID) at the Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital Zurich (supervision: Prof.dr. D. Nadal, Prof.dr. C. Berger). He was awarded in 2013 a Swiss National Science Foundation (SNSF) grant to start the research presented in this thesis at the Laboratory of Pediatrics, Division of Infectious Diseases and Immunology, Erasmus MC University Medical Center–Sophia Children's Hospital, Rotterdam, The Netherlands (supervision: Prof.dr. A.M.C. van Rossum, Dr. C. Vink, Dr. W.W.J. Unger). In 2015, he continued his training as fellow in PID at the University Children's Hospital Zurich, and became a specialist in infectious diseases in 2016 (Swiss Board in Infectious Diseases and Hospital Epidemiology). Meanwhile he continued working on his PhD research from 2015 to 2018 with protected research time awarded by a Fellowship Award of the European Society for Pediatric Infectious Diseases (ESPID) and a senior physician research position from the Promedica Foundation. In 2017, he became a consultant in PID at the Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital Zurich (supervision: Prof.dr. C. Berger). He is married to Michèle Corinne Sauteur and lives together with his children Loïc and Juline in Hünenberg, Switzerland.

## LIST OF PUBLICATIONS

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## PhD PORTFOLIO

| | |
|---|---|
| **Name:** | Patrick M. Meyer Sauteur |
| **PhD period:** | 2013–2018 |
| **Erasmus MC department:** | Laboratory of Pediatrics, Division of Pediatric Infectious Diseases and Immunology |
| **Research school:** | Erasmus Postgraduate School Molecular Medicine (MolMed) |
| **Promotoren:** | Prof.dr. A.M.C. van Rossum |
| | Prof.dr. B.C. Jacobs |
| **Copromotor:** | Dr. W.W.J. Unger |

| Specification | Time period | ECTS* |
|---|---|---|

*1 ECTS (European Credit Transfer System) = 28 study hours (contact hours + self study + exams + presentations)

### 1. PhD training

*Courses*

| | | |
|---|---|---|
| R (Statistical Package); MolMed | 2013 | **1.4** |
| PubMed Workshop; University of Zurich | 2013 | **0.1** |
| Training Course; European Society for Pediatric Infectious Diseases (ESPID) | 2013 | **0.3** |
| Advanced Immunology; MolMed | 2014 | **1.0** |
| Biomedical Scientific English Writing; MolMed | 2014 | **2.0** |
| Postgraduate Training Course; Swiss Society for Infectious Diseases (SSID) | 2013–2016 | **1.2** |
| Good clinical practice (GCP): module I–III; University of Zurich | 2015–2016 | **0.9** |
| Training Course; Pediatric Infectious Disease Group of Switzerland (PIGS) | 2016 | **0.4** |
| Course for Consultants I–II; University Children's Hospital Zurich | 2017 | **0.2** |

*Seminars*

| | | |
|---|---|---|
| Research Seminars; Laboratory of Pediatrics, EMC | 2013–2016 | **4.0** |
| Research Seminars; Division of Neurology and Immunology, EMC | 2013–2015 | **1.0** |
| Research Seminars; T cell Consortium, EMC | 2013–2014 | **0.4** |

### 2. Scientific presentations and meetings

*Presentations*

| | | |
|---|---|---|
| **Poster**; Annual Conference European Society for Pediatric Infectious Diseases (ESPID) | 2013 | **0.4** |
| **Poster**; Annual Conference Swiss Society of Pediatrics (SSP) | 2013 | **0.4** |
| **Oral**; Joint Meeting Swiss Society for Microbiology (SSM) | 2013 | **0.9** |

| | | |
|---|---|---|
| **Oral**; Annual Conference European Society for Pediatric Infectious Diseases (ESPID) | 2015 | **0.9** |
| **Oral**; Annual Conference Symposium of the Netherlands Respiratory Society (NRS) platform on animal models in pulmonary research | 2015 | **0.9** |
| **Oral**; Colloquium Infectious Diseases and Microbiology University Hospital Zurich | 2015 | **0.9** |
| **Oral**; Training Course Pediatric Infectious Disease Group of Switzerland (PIGS) | 2016 | **0.9** |
| **Poster**; Annual Conference European Society for Pediatric Infectious Diseases (ESPID) | 2017 | **0.4** |
| **Poster**; Annual Conference Swiss Society of Pediatrics (SSP) | 2017 | **0.4** |
| **Poster**; Annual Conference Swiss Society for Infectious Diseases (SSID) | 2017 | **0.4** |
| **Oral**; Training Course for Medical Professionals Cantonal Hospital St. Gallen | 2017 | **0.9** |
| **Oral**; Pediatrics Update Refresher Swiss Society of Pediatrics (SSP) | 2017 | **0.9** |
| **Oral**; Symposium of the German Mycoplasma Section | 2017 | **0.9** |
| **Oral**; Colloquium Neuropediatrics University Children's Hospital Zurich | 2017 | **0.9** |
| **Poster**; Annual Conference European Society for Pediatric Infectious Diseases (ESPID) | 2018 | **0.4** |
| **Oral**; Annual Conference Swiss Society of Pneumology (SSP) | 2018 | **0.9** |
| **Oral**; Annual Conference Swiss Society for Infectious Diseases (SSID) | 2018 | **0.9** |
| **Oral**; Annual Retreat Children's Research Center (CRC) Zurich | 2018 | **0.9** |
| **Poster**; Annual Retreat Children's Research Center (CRC) Zurich | 2018 | **0.9** |
| **Oral**; Training Course for Medical Professionals Cantonal Hospital Lucerne | 2018 | **0.9** |

### *Conference attendance*

| | | |
|---|---|---|
| Annual Conference; European Society for Pediatric Infectious Diseases (ESPID) | 2013–2018 | **7.2** |
| Farewell Symposium Prof.dr. Ronald de Groot; European Society for Pediatric Infectious Diseases (ESPID) | 2013 | **0.1** |
| Annual Conference; Swiss Society for Pediatric Pulmonology (SSPP) | 2015, 2018 | **0.4** |
| Annual Conference; Swiss Society of Pediatrics (SSP) | 2013, 2017 | **0.4** |
| Annual Infectious Diseases Symposium; Swiss Society for Infectious Diseases (SSID) | 2016–2017 | **0.4** |
| Annual Conference; Swiss Society for Infectious Diseases (SSID) | 2016–2018 | **0.6** |

## 3. Writing activities

### *Grants*

| | | |
|---|---|---|
| Swiss National Science Foundation (SNSF) (Grant Nr. PBZHP3_147290) | 2013 | **2.0** |
| Sophia Scientific Research Foundation (SSWO) (Grant Nr. 2014-150/WO) | 2014 | **2.0** |
| Fellowship Award of the European Society for Pediatric Infectious Diseases (ESPID) (2015–2017) | 2015 | **2.0** |
| Promedica Foundation University of Zurich (Grant Nr. 1351/M) | 2015 | **2.0** |
| Starr International Foundation | 2015 & 2017 | **4.0** |

### *Reviewer activities*

| | | |
|---|---|---|
| Acta Paediatr, Acta Clinica Belgica, Arch Dis Child (2x), Biomed Pharmacother, BMC Infect Dis, BMC Pediatrics, BMJ Case Rep (3x), Clin Case Rep, BMJ Open, Eur J Inflamm, Eur J Neurol, Eur J Pediatr (6x), Front Microbiol, Infection (2x), J Eur Acad Dermatol Venereol, J Neurol, Neonatology, Neuropediatrics, Pediatr Infect Dis J (3x), Sci Rep, Ther Clin Risk Manag | 2013–2018 | **5.0** |

## 4. Teaching activities

### *Training*

| | | |
|---|---|---|
| Master's program in Human Biology, Modul BIO 430 Immunology, University of Zurich | 2015–2018 | **4.0** |

### *Supervising*

| | | |
|---|---|---|
| Supervising Ariane Burkhard, MD student University Zurich | 2016–2017 | **10.0** |
| Supervising Selina Krautter, MD student University Zurich | 2017–2018 | **10.0** |
| Supervising Jeremy Wyler, MD student University Zurich | 2018 | **4.0** |

| | |
|---|---|
| **Total** | **82.0** |

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**Figure 1.** EMC experimental animal facility, March 30, 2015.

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**Figure 2.** Limmatquai Zurich, March 6, 2015.

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**Figure 3.** Wilhelminapier Rotterdam, November 4, 2016.

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