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Circulating Antibody-Secreting Cell Response During *Mycoplasma pneumoniae* Childhood Pneumonia

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Background. We recently demonstrated that the measurement of *Mycoplasma pneumoniae* (*Mp*)-specific immunoglobulin (Ig) M antibody-secreting cells (ASCs) improved diagnosis of *Mp* infection. Here, we aimed to describe *Mp* ASC kinetics and duration in comparison to conventional measures such as pharyngeal *Mp* deoxyribonucleic acid (DNA) and serum antibodies.

Methods. This is a prospective longitudinal study of 63 community-acquired pneumonia (CAP) patients and 21 healthy controls (HCs), 3–18 years of age, from 2016 to 2017. *Mycoplasma pneumoniae* ASCs measured by enzyme-linked immunospot assay were assessed alongside *Mp* DNA and antibodies during 6-month follow-up.

Results. *Mycoplasma pneumoniae* ASCs of the isotype IgM were found in 29 (46%), IgG were found in 27 (43%), and IgA were found in 27 (43%) CAP patients. *Mycoplasma pneumoniae* ASCs were detected from 2 days to a maximum of 6 weeks after symptom onset, whereas *Mp* DNA and antibodies persisted until 4 months ($P = .03$) and 6 months ($P < .01$). *Mycoplasma pneumoniae* ASCs were undetectable in HCs, in contrast to detection of *Mp* DNA in 10 (48%) or antibodies in 6 (29%) controls for a prolonged time. The *Mp* ASC response correlated with clinical disease, but it did not differ between patients treated with or without antibiotics against *Mp*.

Conclusions. *Mycoplasma pneumoniae*-specific ASCs are short-lived and associated with clinical disease, making it an optimal resource for determining *Mp* pneumonia etiology.

Keywords. antibiotic; B cell; carriage; diagnosis; vaccination.

Mycoplasma pneumoniae (*Mp*) is a common bacterial pathogen of community-acquired pneumonia (CAP) in children [1]. The "gold standard" for diagnosing *Mp* infection is a ≥4-fold increase in antibody levels [2] but has low sensitivity [3] and is not helpful in acute clinical management because it requires acute and convalescent sera [4]. Single immunoglobulin (Ig) M levels against *Mp* and *Mp*-specific polymerase chain reaction (PCR) on upper respiratory tract (URT) samples are currently used to diagnose *Mp* CAP [5, 6]. However, these diagnostic tests are also positive in asymptomatic carriers, and therefore they are unable to differentiate between *Mp* infection and carriage [7]. We recently demonstrated that the measurement of *Mp*-specific IgM antibody-secreting cells (ASCs) by enzyme-linked immunospot (ELISpot) assay improved diagnosis of *Mp* infection: *Mp*-specific IgM ASCs were detectable in children with *Mp* CAP but not in *Mp* carriers suffering from CAP caused by other pathogens or asymptomatic *Mp* carriers [8].

Previous work in vaccine studies has established that circulating antigen-specific B-cell responses are more rapid and shorter lived than antibody responses [9, 10]. Antigen-specific B cells proliferate and differentiate after antigen exposure into ASCs and memory B cells [11, 12]. Antibody-secreting cells then circulate in the peripheral blood for up to 2 weeks before (1) migrating to secondary lymphoid organs or bone marrow or (2) undergoing apoptosis [12, 13]. However, data on the human B-cell response during infection rather than following controlled antigen challenge via vaccination are scarce. In a longitudinal follow-up of our recent study [8], we aimed to describe the onset, kinetics, duration, and isotype of the antigen-specific plasmablast response after *Mp* infection in children in comparison to more conventional measures such as *Mp* deoxyribonucleic acid (DNA) in the URT and serum antibody responses. We further evaluated the effect of different treatment regimens against *Mp* on the ASC response and these other measures.

METHODS

Ethics Statement

The ethics committee of Zurich, Switzerland, approved the protocol for this study (no. 2016-00148). Written informed consent was obtained from all parents and from children from 14 years of age.

Participants

We consecutively enrolled patients between May 1, 2016 and April 30, 2017, at University Children's Hospital Zurich. In all children, pharyngeal swab specimens were taken. Blood samples were collected if additional consent was given. All participants were invited to participate in this longitudinal follow-up study with additional visits between enrollment–2 weeks, 2 weeks–2 months, and/or 2–6 months after presentation.

Community-Acquired Pneumonia Patients

Community-acquired pneumonia was defined as a clinical diagnosis of pneumonia in previously healthy children aged 3–18 years, as detailed elsewhere [8, 14]. Trained physicians identified cases at the emergency department and on pediatric wards. Community-acquired pneumonia patients <3 years of age were excluded to reduce the probability of viral infection, because it is highest in this age group [1, 15–17]. Community-acquired pneumonia patients were managed by treating physicians, who were not aware of the study test results, according to current guidelines [5, 6].

Healthy Controls

Healthy controls (HCs) included healthy children undergoing elective surgical procedures and siblings of CAP patients. Healthy children undergoing elective surgical procedures were age-matched and excluded if there was a history of a recent (≤1 week) respiratory tract infection. Samples were collected at enrollment by the attending anesthesiologist before surgery while the child was under general anesthesia. We also recruited and followed siblings of CAP patients without evidence of recent respiratory tract infection.

Study Procedures

Collection of Biological Samples

Swabs were taken from the posterior pharynx using flocked nylon fiber tip swabs (Copan Diagnostics, Murrieta, CA). Blood samples were collected in anticoagulated lithium heparin blood collection tubes, and fresh peripheral blood mononuclear cells (PBMCs) were isolated ≤4 hours after sampling to avoid poor assay performance due to decreased ASC viability. The PBMCs were isolated by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden), and viability was assessed by trypan blue exclusion. Serum was stored at –80°C.

Mycoplasma pneumoniae Real-Time Polymerase Chain Reaction

Deoxyribonucleic acid isolation was performed on pharyngeal swab samples with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). A quantitative TaqMan (Applied Biosystems, Foster City, CA) real-time PCR assay was used to detect and quantify *Mp* DNA as described previously [18].

Mycoplasma pneumoniae-Specific Enzyme-Linked Immunosorbent Assay

Mycoplasma pneumoniae-specific IgM, IgG, and IgA antibody levels were determined using a commercially available enzyme-linked immunosorbent assay (Virion\Serion, Würzburg, Germany), according to the manufacturer's instructions.

Mycoplasma pneumoniae-Specific Antibody-Secreting Cell Enzyme-Linked Immunospot Assay

The frequency of circulating *Mp*-specific IgM, IgG, and IgA ASCs was measured by ELISpot assay using fresh PBMCs, as described previously [19], with some modifications. In brief, 96-well ELISpot filter plates (Millipore, Billerica, MA) were coated for 90 minutes at 37°C with the different antigens diluted in sterile phosphate-buffered saline (PBS). The antigens were as follows: detergent extract of *Mp* enriched for highly specific adhesion protein P1 (2 µg/mL; Virion\Serion); Fluarix Tetra quadrivalent influenza A and B virus vaccine (6 µg/mL; GlaxoSmithKline, Middlesex, UK); and affinity-purified antibodies to human Ig light chains (λ and κ, 10 µg/mL; SouthernBiotech, Birmingham, AL) 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 minutes at 37°C. Coated plates were incubated at 37°C for 16–20 hours with 100 000 or 10 000 viable PBMCs, and each dilution was used in triplicate. Plates were then washed, incubated with biotinylated anti-IgM, -IgG, -IgA and alkaline phosphatase (AP)-conjugated streptavidin (all SouthernBiotech), and spots visualized using an AP substrate kit (Bio-Rad Laboratories, Hercules, CA), with each spot appearing at the former location of a single ASC. Spots were counted by an ELISpot reader (AID, Strassberg, Germany) using predefined 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 3 wells minus the mean number of spots in PBS wells. Data were expressed as ASCs per 10^6 viable PBMCs [19].

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 Fisher exact test was used to compare proportions. Spearman rank correlation was used to evaluate relationships between variables. Kinetics were plotted with smooth curves, fitted by loess (with a span of 0.67) using the scatter. smooth formula [20]. Study design and sample size considerations were described in detail previously [8]. All reported *P* values are 2-tailed with statistical significance defined as *P* < .05. Data were analyzed using the R software environment (version 3.6.0) [20].

RESULTS

Study Population

During the 12-month study period, we screened 450 CAP patients and 156 HCs (Figure 1). A total of 152 CAP patients and 156 HCs were enrolled (Table 1). *Mycoplasma pneumoniae*-specific PCR and serological test results of the enrolled population are shown in Table 2. We included 63 CAP patients and 21 HCs with fresh (isolated ≤4 hours) PBMCs available for additional testing with the *Mp*-specific ASC ELISpot assay, as previously reported [8]. There were no statistically significant differences between included and overall enrolled CAP patients and HCs in regards to age, sex, and season at enrollment (Table 1). However, the proportion of siblings of index patients among HCs was higher in the included group of controls compared with enrolled controls (43% vs 12%, $P < .01$). Clinical, radiological, and laboratory characteristics of included CAP patients have been described separately [14, 21].

Kinetics and Duration of the Antibody-Secreting Cell Response

We first assessed the ASC response at presentation and found *Mp*-specific ASCs in CAP patients ($n = 63$) of the isotype IgM, IgG, and IgA in 29 (46%), 27 (43%), and 27 (43%), respectively (Table 2). These first samples were collected at a median of 12 days after onset of symptoms (IQR, 11–16; range, 2–29). Then, the median number of *Mp* IgM ASCs was 690 (IQR, 200–1933) spots per 10^6 PBMCs, which was higher than that of *Mp* IgG ASCs (median 300; IQR, 166–533; $P = .07$) and *Mp* IgA ASCs

(median 167; IQR, 67–333; $P < .01$). *Mycoplasma pneumoniae*-specific IgM ASCs correlated with *Mp* IgG ASCs (rho 0.63; $P < .01$) and *Mp* IgA ASCs (rho 0.62; $P < .01$) but not with *Mp* antibody (any isotype) or *Mp* DNA levels (Supplementary Table 1). Time since onset of symptoms positively correlated with *Mp* IgM and *Mp* IgG antibody levels (rho 0.56 and 0.53; $P < .01$), but not with *Mp* ASCs (any isotype), in first measured sample (Supplementary Table 2). Among HCs ($n = 21$), no *Mp* ASCs were detected, but *Mp* IgM serology was positive in 6 (29%) individuals, 1 (5%) of whom showed seroconversion to *Mp* IgG with a >4-fold increase in *Mp* IgG (Table 2). All 29 *Mp* IgM ASC-positive CAP patients were also *Mp* PCR-positive and *Mp* IgM-positive, and the 3 (5%) *Mp* PCR-positive patients, who were *Mp* IgM ASC- and *Mp* IgM-negative, were identified as *Mp* carriers suffering from CAP caused by other pathogens, as detailed elsewhere [8]. The specificity of the *Mp* IgM ASC ELISpot assay was further demonstrated by the absence of *Mp* IgM ASCs in influenza virus [14] and Epstein-Barr virus (EBV) [22] infected patients (Supplementary Figure 1). The 2 patients who tested positive for influenza virus in pharyngeal swab samples [14] had influenza-specific but not *Mp*-specific ASCs detectable during CAP.

The longitudinal follow-up study included 52 (62%) children (41 CAP patients and 11 HCs) with >2 visits in 42 (81%) and >3 visits in 27 (52%) children, performed at <2 weeks, 2 weeks–2 months, and 2–6 months in 43 (83%), 38 (73%), and 38 (73%) individuals, respectively. *Mycoplasma pneumoniae*
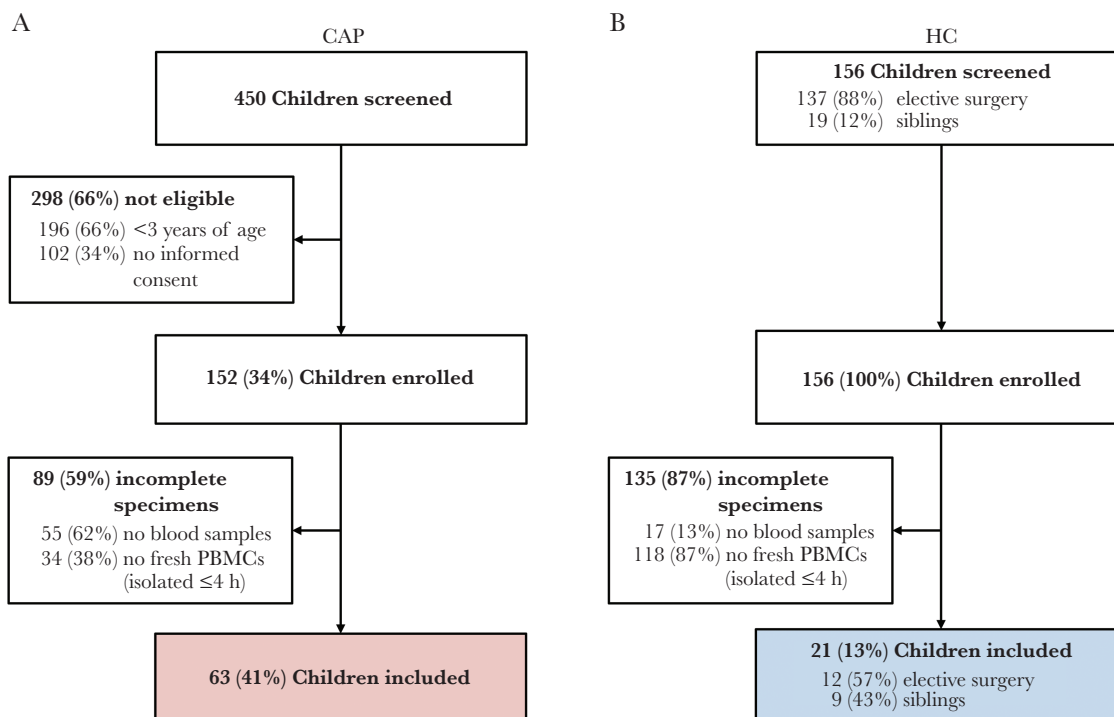
Figure 1. Study profile. Recruitment and flow of (A) community-acquired pneumonia (CAP) patients and (B) healthy controls (HC). PBMC, peripheral blood mononuclear cells.

Table 1. Baseline Characteristics of CAP Patients and Controls[a]

Characteristics	CAP		HC		OR (95% CI)	P
	Included (n = 63)	Enrolled (n = 152)	Included (n = 21)	Enrolled (n = 156)		
			n = 12 (57) Elective Surgery	n = 137 (88) Elective Surgery		
			n = 9 (43) Siblings	n = 19 (12) Siblings		
Age (years), median (IQR)	6.0 (4.4–10.2)	5.7 (4.3–8.9)	6.1 (4.9–7.9)	5.9 (4.3–8.1)	–	.91
Sex (male), n (%)	39 (62)	84 (55)	17 (81)	102 (65)	.4 (.1–1.4)	.18
Season at enrollment, n (%)						
Spring (March–May)	11 (17)	21 (14)	5 (24)	43 (28)	.7 (.2–2.9)	.53
Summer (June–August)	13 (21)	30 (20)	9 (43)	22 (14)	.4 (.1–1.2)	.08
Autumn (September–November)	17 (27)	37 (24)	4 (19)	17 (11)	1.6 (.4–7.3)	.57
Winter (December–February)	22 (35)	64 (42)	3 (14)	74 (47)	3.2 (.8–18.7)	.10
Day-care or preschool attendance, n (%)	63 (100)	NA	20 (95)	NA	NA	.25
Immunizations[b], n (%)	52/55 (95)	NA	14/14 (100)	NA	NA	1.00
Underlying disease, n (%)	10 (16)	32 (21)	12 (57)	137 (88)	.1 (.0–.5)	**<.01**
ENT, n	0	4	12	137[c]		
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	0		

Abbreviations: CAP, community-acquired pneumonia; CI, confidence interval; ENT, ear, nose, and throat; HC, healthy control; IQR, interquartile range; NA, not available; OR, odds ratio.

[a]Differences between included CAP and included HC children (in bold) were determined by the Mann-Whitney U test (medians) and Fisher exact test (proportions). P < .05 are indicated in bold.

[b]"Immunizations" refer to being immunized per the national immunization schedule in Switzerland.

[c]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).

ASCs were detected only within 6 weeks after symptom onset (Figure 2), in contrast to Mp DNA (P = .03) and Mp IgM and Mp IgG (P < .01) persisting at 4 and 6 months, respectively (Supplementary Table 3). The Mp IgA levels in CAP patients were low (median 11 U/mL, IQR 6–25) and above the cutoff in only 13 (21%) (Table 2) and differed significantly from Mp IgM (P < .01) and Mp IgG (P < .01) levels. However, Mp antibody levels and kinetics correlated between isotypes (Supplementary Table 1 and Figure 2). Detecting a ≥4-fold Mp IgG increase in Mp IgM ASC-positive patients was significantly more likely with Mp IgG <20 U/mL (91%, n = 10 of 11) compared with Mp IgG ≥20 U/mL (0%, n = 0 of 18; P < .01) at presentation.

It is interesting to note that 3 siblings of CAP patients developed CAP during follow-up and were then also tested positive for Mp IgM ASCs. One of the 3 siblings was sampled (as asymptomatic sibling of a CAP patient) 3 days before the onset of symptoms, and at this time point, the sibling tested negative by the Mp ASC ELISpot assay, followed by a positive Mp ASC ELISpot assay result 6 days after developing symptoms and CAP diagnosis. Representative Mp IgM ASC ELISpot assay responses in this patient are shown in Figure 3. The kinetics of Mp DNA, Mp antibodies, and Mp ASCs of this patient are indicated in black in Supplementary Figure 2.

In HCs, Mp DNA and/or Mp IgM and IgG were detected for up to ≥2 months, whereas Mp ASCs and Mp IgA levels were negative during the complete 6-month follow-up period (Figure 4). These results were in line with the lack of respiratory symptoms during follow-up visits.

Relation of the Antibody-Secreting Cell Response With Clinical Disease

We next compared the ASC response with clinical disease. As previously demonstrated in this cohort [14], Mp IgM ASC-positive CAP was statistically associated with prolonged prodromal symptoms and extrapulmonary manifestations, predominantly skin disorders [21]. However, the magnitude of the Mp-specific ASC response did not correlate with any of these clinical features (Supplementary Table 2). Furthermore, lower levels of C-reactive protein (CRP), white blood cell (WBC) count, absolute neutrophil count (ANC), and procalcitonin were statistically associated with IgM ASC-positive Mp infection [14]. Mycoplasma pneumoniae IgM ASC responses correlated positively with CRP levels (rho 0.45; P = .01), and negatively with WBC count (rho −.61; P < .01) and ANC (rho −.66; P < .01), but not with other laboratory parameters (Supplementary Table 2).

Next, we evaluated the effect of different treatment regimens against Mp on the ASC response. Ten (34.5%) patients received

Table 2. *Mp*-Specific Test Results of Enrolled and Included CAP Patients and Controls[a]

Diagnostic Test	CAP	HC	OR (95% CI)	P
Enrolled Cohort	**(n = 152)**	**(n = 156)**		
***Mp*-specific DNA**	44 (29)	12 (8) 6/137 (4) elective surgery 6/19 (32) siblings	4.9 (2.4–10.6)	**<.01**
***Mp*-specific Antibodies**				
IgM+	39/97* (40)	15/139* (11)	5.5 (2.7–11.7)	**<.01**
IgG+	39/97 (40)	16/139 (12)	5.1 (2.6–10.7)	**<.01**
IgA+	24/97 (25)	0/139 (0)	NA	**<.01**
IgM+ IgG+	37/97 (38)	7/139 (5)	11.5 (4.7–32.3)	**<.01**
IgM+ IgA+	24/97 (25)	0/139 (0)	NA	**<.01**
IgG+ IgA+	23/97 (24)	0/139 (0)	NA	**<.01**
IgM+ IgG+ IgA+	23/97 (24)	0/139 (0)	NA	**<.01**
Included Cohort	**(n = 63)**	**(n = 21)**		
***Mp*-specific DNA**	32 (51)	10 (48) 4/12 (33) elective surgery 6/9 (66) siblings	1.1 (.4–3.5)	1.00
***Mp*-specific Antibodies**				
IgM+	32 (51)	6 (29)	2.6 (.8–9.1)	.08
IgG+	27 (43)	5 (24)	2.4 (.7–9.4)	.19
IgA+	13 (21)	0 (0)	NA	**.03**
IgM+ IgG+	26 (41)	5 (24)	2.2 (.7–8.8)	.20
IgM+ IgA+	13 (21)	0 (0)	NA	**.03**
IgG+ IgA+	11 (17)	0 (0)	NA	.06
IgM+ IgG+ IgA+	11 (17)	0 (0)	NA	.06
Seroconversion:				
IgM	0/38** (0)	0/11** (0)	–	1.00
IgG	5/38 (13)	1/11 (9)	1.5 (.1–78.7)	1.00
IgA	5/38 (13)	0/11 (0)	NA	.57
Class switch from IgM to IgG	7/38 (18)	1/11 (9)	2.2 (.2–111.7)	.66
Titer Increase:				
IgM ≥2-fold [3]	3/38 (8)	0/11 (0)	NA	1.00
IgG ≥4-fold [2]	10/38 (26)	1/11 (9)	3.5 (.4–170.1)	.41
***Mp*-specific ASC**				
IgM ASC+	29 (46)	0 (0)	NA	**<.01**
IgG ASC+	27 (43)	0 (0)	NA	**<.01**
IgA ASC+	27 (43)	0 (0)	NA	**<.01**
IgM ASC+ IgG ASC+	27 (43)	0 (0)	NA	**<.01**
IgM ASC+ IgA ASC+	27 (43)	0 (0)	NA	**<.01**
IgG ASC+ IgA ASC+	26 (41)	0 (0)	NA	**<.01**
IgM ASC+ IgG ASC+ IgA ASC+	26 (41)	0 (0)	NA	**<.01**

Abbreviations: ASC, antibody-secreting cells; CAP, community-acquired pneumonia; CI, confidence interval; DNA, deoxyribonucleic acid; HC, healthy control; Ig, immunoglobulin; *Mp*, *Mycoplasma pneumoniae*; NA, not available; OR, odds ratio.

[a]Data are presented as no. (%). Differences between groups are indicated by the Fisher exact test. *P* < .05 are indicated in bold.

*Sera were available in 97 (64%) CAP patients and 139 (89%) controls.

**Paired sera were available in 38 (60%) CAP patients and 11 (52%) controls.

clarithromycin, 10 (34.5%) patients received doxycycline, and 9 (31%) patients were not treated with any antibiotic in vitro active against *Mp* (ie, amoxicillin [n = 5], amoxicillin-clavulanate [n = 1], ceftriaxone [n = 1], no antibiotic treatment [n = 2]). Kinetics of pharyngeal *Mp* DNA, *Mp* antibodies, and *Mp* ASCs according to different treatment management are shown in Figure 5. There were no statistically significant differences between patients with or without treatment in vitro active against *Mp* with regards to ASC and antibody responses (Supplementary Table 4). In contrast, *Mp* DNA was detected in patients treated with clarithromycin and doxycycline in 58% and 90% at 1- to 2-month follow-up (*P* = .01) and 38% and 0% at 3- to 4-month follow-up (*P* = .20), respectively. It is notable that clinical outcome (length of hospital stay and long-term sequelae) and fever duration was not statistically different between treatment groups (Supplementary Figure 3). Five patients who received antibiotics against *Mp* were additionally treated with corticosteroids because of severe extrapulmonary mucocutaneous
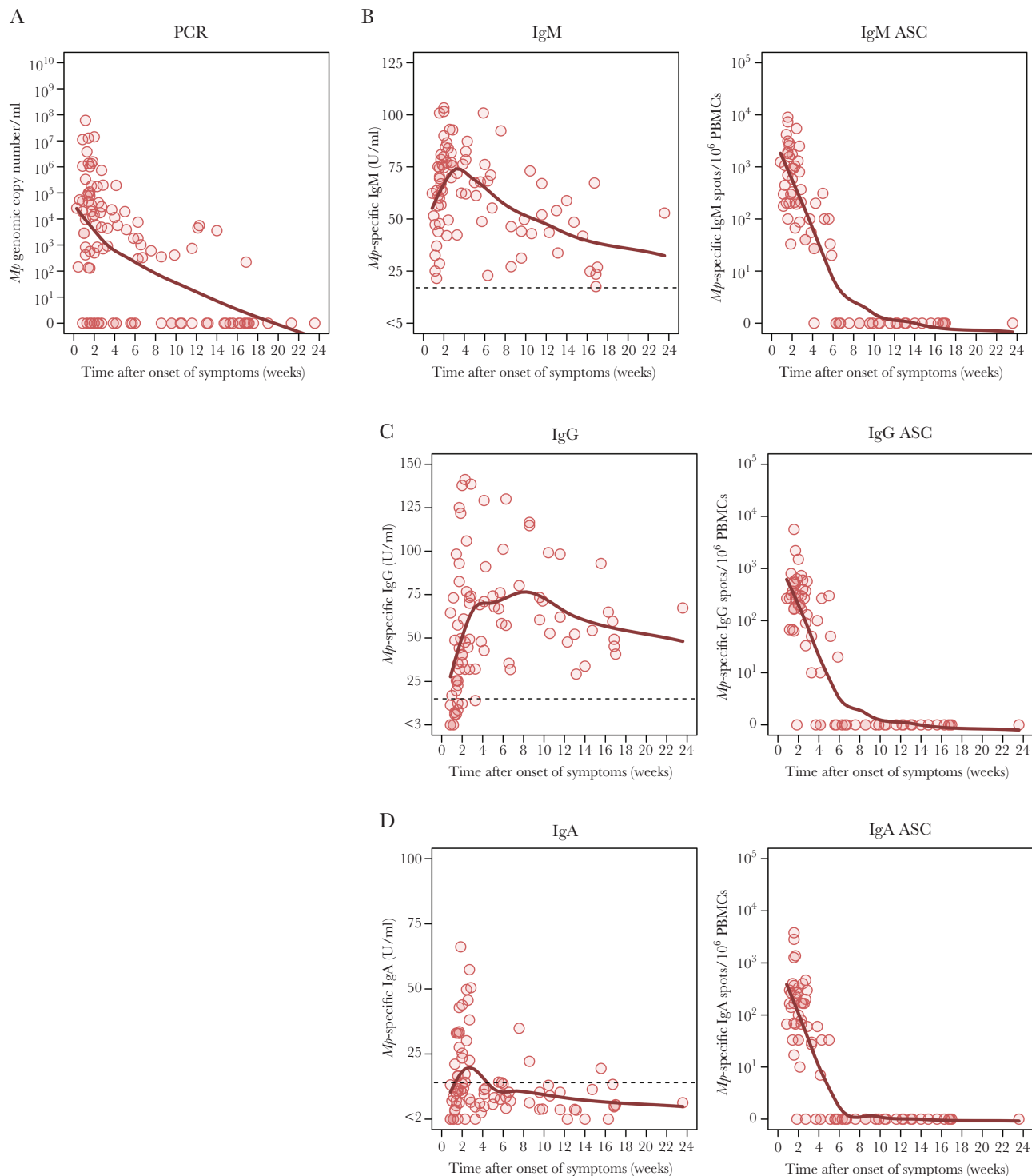
Figure 2. Kinetics of pharyngeal *Mycoplasma pneumoniae* (*Mp*) deoxyribonucleic acid (DNA) levels, *Mp* antibodies, and *Mp* antibody-secreting cells (ASCs) in *Mp* immunoglobulin (Ig) M ASC-positive community-acquired pneumonia patients. (A) *Mycoplasma pneumoniae* DNA levels in pharyngeal swab samples as genomic copy number per milliliter. (B–D) *Mycoplasma pneumoniae* antibodies and *Mp* ASCs of the isotype IgM (B), IgG (C), and IgA (D). Smooth curves fitted by loess (with a span of 0.67) and original data as dots are shown. The dashed horizontal lines represent the cutoff for the enzyme-linked immunosorbent assay, and lower limits of quantification for IgM, IgG, and IgA are 5, 3, and 2 U/mL. PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction.

manifestations [21]. *Mycoplasma pneumoniae* IgM ASC numbers did not differ between those patients before corticosteroid treatment and other CAP patients at an early time point (<2 weeks after onset of symptoms) (*P* = .49), but they were significantly lower at a later time point (2–4 weeks after onset of symptoms) after the administration of corticosteroids compared with
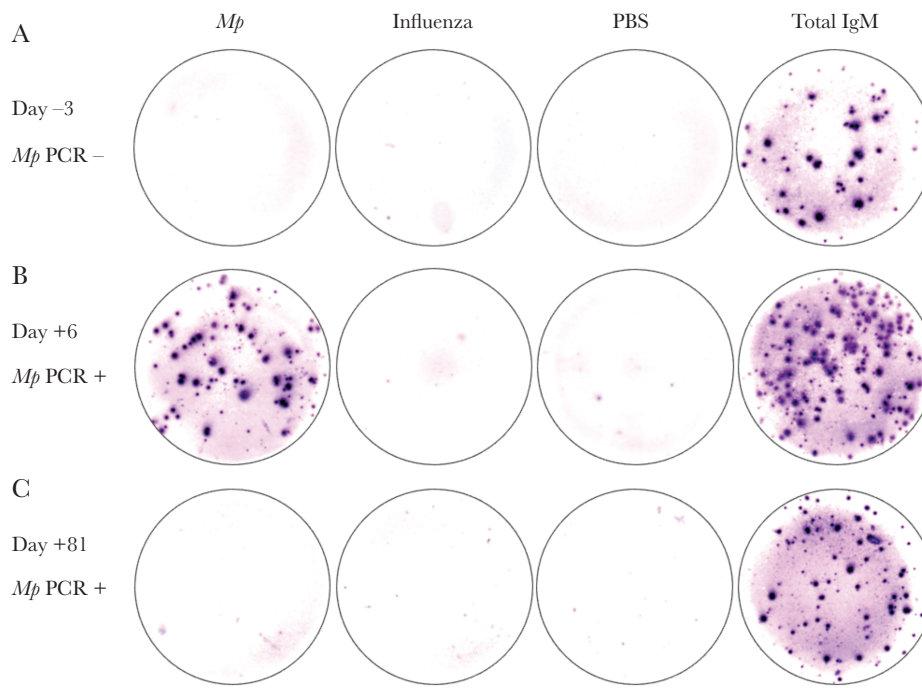
Figure 3. Enzyme-linked immunospot (ELISpot) assay for antibody-secreting cells in a patient who developed *Mycoplasma pneumoniae* (*Mp*) community-acquired pneumonia. Representative patterns of ELISpot assay wells 3 days before the onset of symptoms (A) and on day 6 (B) and day 81 (C) after symptom onset (100 000 peripheral blood mononuclear cells per well). Ig, immunoglobulin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

CAP patients without corticosteroid treatment sampled at the same time period (*P* = .04) (Supplementary Figure 4).

DISCUSSION

In this longitudinal follow-up study, we demonstrate that *Mp*-specific ASCs are short-lived and associated with clinical disease, in contrast to pharyngeal *Mp* DNA and serum antibodies that can persist for months and may be present also during *Mp* carriage. This study adds to our recent work [8] by detailing the onset, kinetics, duration, and isotype (IgM/IgG/IgA) of the antigen-specific plasmablast response after *Mp* infection in children and investigating the effect of different treatment regimens against *Mp* on the specific immune response and pharyngeal *Mp* DNA load. These findings expand our current knowledge on specific B-cell responses to infection and provide an explanation for the high specificity and sensitivity of the *Mp* ASC ELISpot assay during *Mp* CAP [8]. The data presented give insight into disease pathophysiology and can therefore serve as a model for developing better diagnostic tests for other childhood infectious diseases.

We detected *Mp* ASCs as early as 2 days after the presentation of clinical symptoms, which was in line with the only previous study on *Mp*-specific ASCs including 12 *Mp*-seropositive children with CAP, in which *Mp* ASCs were detected within 5 days after symptom onset [23]. To our knowledge, we assessed also for the first time B-cell responses in a patient shortly before the development of symptoms and could thereby exemplarily

demonstrate a specific ASC response upon CAP onset, which disappeared again with disease resolution, whereas *Mp* DNA and antibodies persisted for prolonged time. *Mycoplasma pneumoniae*-specific ASCs peaked approximately 1–2 weeks after onset of symptoms, similar to previous work: we recently reviewed the literature on the ASC response to infection [9] and found that the timing of ASC appearance in the blood is highly consistent after infection across several pathogens. Antibody-secreting cells are first detectable in peripheral blood at approximately 4 days after onset of symptoms before peaking at days 6–8 [9, 24]. In contrast to the conserved timing of ASC appearance, the magnitude of ASC responses varies widely between different bacterial and viral pathogens as well as between ASC isotypes [9]. Our results are consistent with previous work studying the immune responses to both vaccination [13] and infection [9], and they demonstrate a synchronized response of B cells with different isotypes during infection. In contrast, serum antibody responses showed a sequential appearance in our study: IgM and IgA were first detectable followed by IgG with concentrations that were still rising in the first 4 weeks after symptom onset and lasting for several months.

However, in some individuals, B-cell responses could be detected up to 6 weeks after onset of symptoms, indicating that *Mp*-specific ASCs may circulate longer compared with other (respiratory) infections, in which antigen-specific ASCs disappeared in most cases already 14 days after symptom onset [9, 25–30]. These findings support the clinical observation of
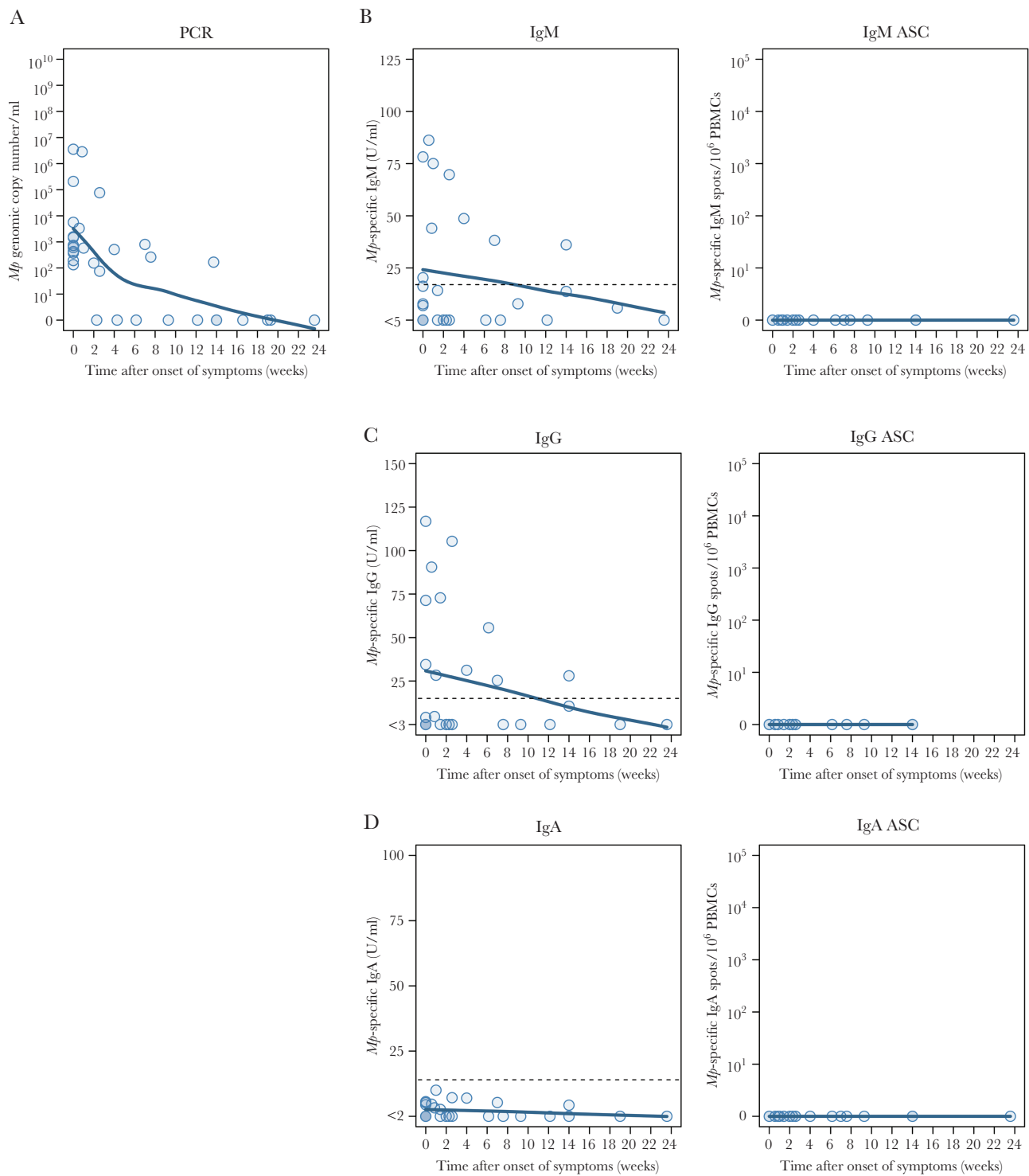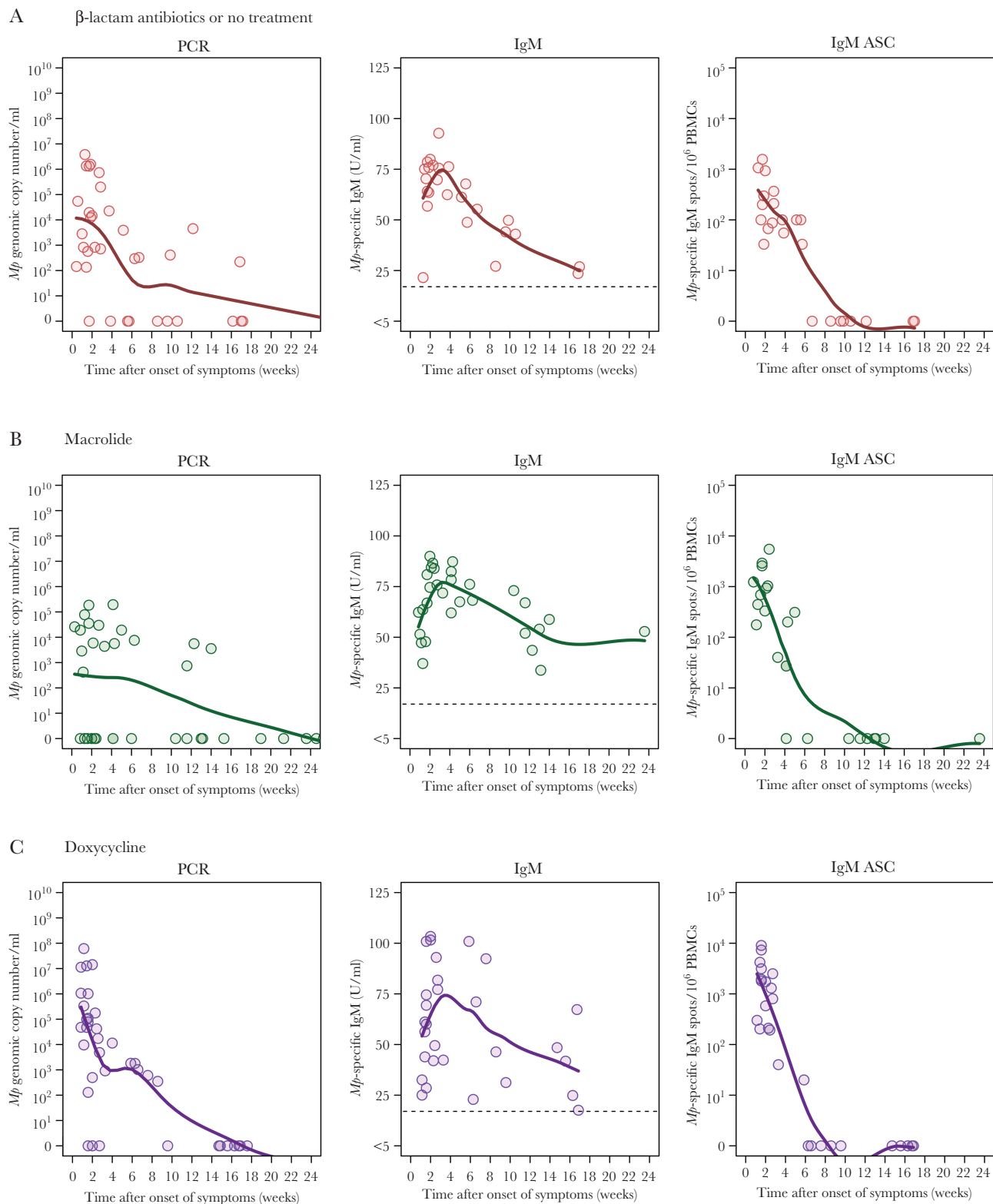
Figure 4. Kinetics of pharyngeal *Mycoplasma pneumoniae* (*Mp*) deoxyribonucleic acid (DNA) levels, *Mp* antibodies, and *Mp* antibody-secreting cells (ASCs) in *Mp* polymerase chain reaction (PCR)-positive controls. (A) *Mycoplasma pneumoniae* DNA levels in pharyngeal swab samples as genomic copy number per milliliter. (B–D) *Mycoplasma pneumoniae* antibodies and *Mp* ASCs of the isotype immunoglobulin (Ig) M (B), IgG (C), and IgA (D). Smooth curves fitted by loess (with a span of 0.67) and original data as dots are shown. The dashed horizontal lines represent the cutoff for the enzyme-linked immunosorbent assay, and lower limits of quantification for IgM, IgG, and IgA are 5, 3, and 2 U/mL. PBMC, peripheral blood mononuclear cell.

slow disease progression in *Mp* CAP [14], which may result in a longer antigen exposure and therefore immune activation, which allows detection of *Mp*-specific ASCs in all patients

during CAP. A more prolonged antigen exposure with slow disease progression may also be the reason for the significantly increased *Mp* IgG levels already in the first serum sample of *Mp*

Figure 5. Kinetics of pharyngeal *Mycoplasma pneumoniae* (*Mp*) deoxyribonucleic acid levels, *Mp* antibodies, and *Mp* antibody-secreting cells (ASCs) in *Mp* immunoglobulin (Ig) M ASC-positive community-acquired pneumonia patients according to different treatment management. (A) β-lactam antibiotics (*n* = 7) or no treatment (*n* = 2). (B) Macrolide (clarithromycin) (*n* = 10). (C) Doxycycline (*n* = 10). Smooth curves fitted by loess (with a span of 0.67) and original data as dots are shown. The dashed horizontal lines represent the cutoff for the enzyme-linked immunosorbent assay (lower limit of quantification, 5 U/mL). PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction.

IgM ASC-positive CAP patients. The development of a further increase of *Mp* IgG in those patients ≥4-fold was therefore very unlikely, which continues to question its value as gold standard for diagnosing *Mp* infection [3, 4]. Its interpretation is also complicated by our observation of a >4-fold increase in *Mp* IgG level in an asymptomatic sibling.

Besides describing *Mp* ASC response kinetics and duration, we also determined antibody specificity and isotype of the plasmablast response to infection, which are key to develop accurate ASC measurements [9]. We showed that *Mp*-specific ASCs were not detectable in patients infected with influenza virus or EBV, whereas patients with influenza virus infection on the other hand developed influenza virus-specific ASC responses during CAP. The detection of *Mp* IgM ASCs was more sensitive than the detection of *Mp* IgG ASCs or *Mp* IgA ASCs for determining *Mp* infection, again in line with previous work [23].

The *Mp*-specific ASC response was associated with clinical disease and correlated with CRP levels and WBC counts. Although *Mp* CAP was associated with lower CRP levels compared with CAP of other origin [14], severe disease defined as the presence of extrapulmonary skin manifestations was associated with increased systemic inflammation and higher CRP levels [21]. This finding suggests that in these children, inflammation may be indeed driven by *Mp* antigens, which warrants further investigation. It is interesting to note that there were no differences in ASC or antibody responses, or clinical outcomes such as fever duration, between groups with different antibiotic treatment regimens. In contrast, corticosteroid treatment significantly decreased ASC responses, although they were still detectable. One third of *Mp* IgM ASC-positive CAP patients were not treated with an antibiotic in vitro active against *Mp*, but all of them equally and fully recovered. Although the study design does not allow conclusions about the effectiveness of treatment, these findings highlight the need for future interventional studies to assess the efficacy of antibiotics for *Mp* CAP [2, 31].

The ASC ELISpot assay is a robust technique [9, 32], and the protocol described here could be translated directly into the clinical setting to diagnose *Mp* infection by using only a small volume of peripheral blood (≥1 mL). However, the ASC ELISpot assay is labor-intensive, requiring the handling of fresh or frozen PBMCs [19, 32], and has a rather long overall turnaround time (~24 hours), but recent alternative protocols suggest more rapid (~6–8 hours) ASC detection [19]. Optimizing such protocols will help to routinely use the *Mp* IgM ASC ELISpot assay for diagnosing *Mp* CAP.

Despite variation in sampling time points, the study population represents a well defined cohort of children, both patients and controls, in whom pharyngeal swabs and fresh blood samples at several time points up to 6 months after inclusion could be obtained. It is challenging to collect blood samples from healthy children and already recovered children, and we can therefore not fully exclude any selection bias that occurred in the included subgroup of study participants. However, baseline characteristics of both included CAP patients and controls were similar compared with the enrolled study population. Some of the siblings of index patients were included as controls, which made it possible to increase the likelihood of detecting asymptomatic *Mp* carriers and to investigate the pattern symptom acquisition and/or changes in the results of the diagnostic tests. *Mycoplasma pneumoniae* was indeed more frequently detected in the URT of siblings compared with controls sampled during elective surgery (32% vs 4%), which is in line with a higher *Mp* transmission rate within families and between other close contacts [14, 33, 34]. Proportionally, more siblings than other controls were included than were initially enrolled into the study (43% vs 12%), which may be partly due to a higher motivation of family members of affected patients agreeing to multiple sampling and follow-up. This may also explain the differences in *Mp* detection rates between included and enrolled study participants. A larger prospective confirmatory study is needed to validate these promising results of the *Mp* ASC ELISpot assay and to examine the added clinical utility of the ASC ELISpot assay to diagnose *Mp* infection in the clinical care of children with CAP.

CONCLUSIONS

In conclusion, *Mp*-specific ASCs are short-lived and associated with clinical disease, in contrast to pharyngeal *Mp* DNA and serum antibodies. There are limited data on the duration of B-cell responses during infection, and our study indicates that *Mp*-specific peripheral blood B cells appear early in the course of illness, last only for several weeks before disappearing completely, and therefore they can be easily detected in a clinical setting with good diagnostic sensitivity and specificity. These findings expand our current knowledge on specific B-cell responses to infection, and they reveal ASCs as an optimal resource for determining disease etiology in *Mp* pneumonia and possibly other childhood infections.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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