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Article

Memory B cell compartment constitution and susceptibility to recurrent lower respiratory tract infections in young children

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ABSTRACT

A proportion of children have recurrent LRTIs, mostly as a result of Spn, which persist after 2 years of age. Here, we investigate, by flow cytofluorometry, the constitution of the memory B cell compartment in 90 healthy children and 49 children with recurrent LRTIs to determine if an increased susceptibility to recurrent LRTIs results from a delayed or abnormal ontogeny with poor antibody-mediated protection. Total IgA, IgM, IgG, and IgG subclasses were measured by nephelometry, as well as antipneumococcal antibodies by ELISA. Pneumococcal vaccination status was obtained. We show that the memory B cells increase between birth and 2 years of age (1.6% vs. 21.1%, $P<0.001$) without further significant increase noted per additional years (3–4 years old: 23.3%; 4–5 years old: 22.2%, $P>0.40$) to reach adult-like values (31.8±11.8%, $P=0.08$). Proportions of switched and IgM memory B cells were similar in children and adults. Comparatively, LRTI children had no delay in the constitution of their memory B cell compartment (2–3 years old: 26.9%; 3–4 years old: 18.2%; 4–5 years old: 26.8%, $P>0.05$). Their switched and IgM memory B cells were similar among age categories, and the distribution was overall similar to that of healthy controls. LRTI children had normal total and pneumococcal serotype-specific antibody values but showed a rapid waning of antipneumococcal antibody levels after vaccination. In summary, our results show that the memory B cell compartment is already similarly constituted at 2 years of age in healthy and LRTI children and thus, cannot explain the increased susceptibility to bacterial pneumonia. However, the waning of antibodies might predispose children to recurrent infections in the absence of revaccination. *J. Leukoc. Biol.* 93: 000–000; 2013.

Abbreviations: APC=allophycocyanin, CB=cord blood, CVID=common variable immune deficiency, GMC=geometric mean concentration, grp=group, Hib=Haemophilus influenzae type b, IgAD=selective IgA deficiency, LRTI=lower respiratory tract infection, MenACWY-CRM=meningococcal polysaccharide-diphtheria conjugated vaccine, MPSV4=meningococcal polysaccharide vaccine, PB=peripheral blood, PC=plasma cell, PCV7=seven-valent conjugate pneumococcal vaccine, PID=primary immune deficiency, PPS=pneumococcal polysaccharide, PPV23=23-valent pneumococcal polysaccharide vaccine, Spn=Streptococcus pneumoniae, WHO=World Health Organization

Introduction

Unlike acute viral LRTIs, which are more frequently identified in children below 2 years of age [1, 2], recurrent LRTIs are predominantly caused by bacteria [3]. Worldwide, Spn and Hib are the major causative pathogens for bacterial pneumonia under 5 years of age [4–6]. In developed countries, however, the widespread use of the Hib conjugate vaccine has decreased the incidence of Hib pneumonia [7]. Consequently, Spn appears today as the most common bacterial cause of community-acquired pneumonia in childhood [2, 8]. It reportedly accounts for as high as 50–73% of cases [3, 9, 10] and consequently, is the preferred candidate when focusing on bacterial pneumonia in young children aged 2–5 years, as evidence increasingly shows it to be a major killer of children.

Despite a high prevalence of nasopharyngeal pneumococcal colonization, the immature immune system of young children responds poorly to the polysaccharidic capsules of Spn [11]. This is demonstrated by a high incidence of invasive pneumococcal disease in young children [6, 12]. After the 2nd year of life, Spn infections become rarer, reflecting immune maturation, including of the spleen [13]. Part of this maturation and protection against pathogens results from antibody-mediated immunity and the development of the memory B cell compartment. However, recurrent LRTIs persist in a subset of children [14]. An impaired memory B cell compartment may result in poor humoral immunity and increased risks of recurrent infections by encapsulated bacteria, such as Spn [15–17]. An abnor-

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mal maturation of this compartment may therefore be found in children with recurrent LRTIs.

The peripheral B cell compartment can be divided in mature, naïve and memory B cells, according to the expression of CD19 and CD27 [13]: (1) mature, naïve B cells ($CD19^+CD27^-$) are antigen-inexperienced cells that have not undergone antigen-driven responses, and (2) memory B cells ($CD19^+CD27^+$) are antigen-experienced cells that can be subdivided into four subpopulations: (a) classical $CD19^+CD27^+IgM^-IgD^-$, class-switched memory B cells; (b) $CD19^+CD27^+IgM^+IgD^+$, unswitched IgM memory B cells, usually called IgM memory B cells; (c) $CD19^+CD27^+IgM^+IgD^-$, IgM-only, memory B cells; and (d) $CD19^+CD27^+IgM^-IgD^+$ IgD-only, memory B cells.

Switched memory B cells are involved in the response to thymus-dependent antigens, typically proteins, and in long-term immunological memory. They circulate in the PB compartment for years, independently of antigenic stimulation [18, 19]. When in contact with specific antigens, they quickly proliferate and differentiate into PCs, secreting high-affinity IgG antibodies. IgM memory B cells collect in the spleen, where blood-borne pathogens are preferentially filtered. Based on surface-molecule and gene-expression analyses, these cells were suggested to be recirculating, splenic, marginal-zone B cells [11]. Extremely reduced in splenectomized and asplenic patients, their presence in PB reflects, at least in part, their production in the spleen or their requirement for an intact splenic microenvironment to survive and to recirculate [20–22]. These IgM memory B cells are mainly involved in response to thymus-independent type 2 T cell antigens (typically polysaccharides), undergo somatic hypermutations but not Ig isotype-switching [20, 23], and do not elicit long-term immunological memory. The absence of IgM memory B cells correlates with poor humoral immunity and an increased risk of recurrent infections with encapsulated bacteria in elderly patients or patients with CVID [24, 25]. Before the age of 2 years, IgM memory B cells are present but possibly unable to populate the splenic marginal zone, thus failing to contribute to the initial control of encapsulated bacteria [13, 24, 26].

To our knowledge, the memory B cell compartment has not been studied in children with recurrent LRTIs. The objective of this study was to determine whether their increased susceptibility to infection results from an immature or abnormal memory B cell compartment. This prospective case-control study thus compares the maturation of the memory B cell compartment between 2 and 5 years of age in children with or without recurrent LRTIs.

MATERIALS AND METHODS

The study was conducted between February 2006 and May 2009 at the Children's Hospital of the University Hospitals of Geneva. It is a primary and tertiary care center with 2300 yearly pediatric admissions. It was approved by the Institutional Ethics Committee, and written consent was obtained from parents, legal guardians, or participants. It was conducted in accordance with the principles of the Declaration of Helsinki, the standards of Good Clinical Practice, and Swiss regulatory requirements.

Patients and subjects

Cases were, at enrollment, children aged between 2 and 5 years with recurrent LRTIs, defined as two or more episodes of pneumonia in a single year or at least three episodes since birth [14]. The diagnosis had to be confirmed clinically, according to the WHO criteria [27], or by radiology, with complete clinical resolution between occurrences [14]. Patients were referred through their pediatrician, the infectious diseases or the pulmonology outpatient clinic, or the laboratory of vaccinology or were recruited at time of hospitalization for pneumonia. Exclusion criteria were: anatomic anomalies of the respiratory tract, primary ciliary dyskinesia, cystic fibrosis, foreign body in the respiratory tract, asthma or severe atopic disease, known congenital or acquired immunodeficiencies, lymphopenia, or i.v. Ig substitution therapy. No immune deficiencies or history of recurrent infections were reported in patients' first-degree relatives. Cases were followed prospectively and had yearly blood sampling. Samples were taken outside of acute infectious episodes to avoid measurement biases, such as Ig consumption. Healthy controls were age-matched children referred for elective surgery, who provided a single blood sample at enrollment. Additional controls included healthy adults, full-term newborns, and children <2 years.

Blood sampling and leukocyte isolation

Fresh PB samples were placed into heparinized-tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) and processed on the day of collection. Plasma was isolated by centrifugation and stored at −80°C. PBMCs were isolated using isotonic succinylated 4% gelatin solution (1/2 v/v; Physiogel, B. Braun Medical, Switzerland) sedimentation for 30 min at 4°C, followed by washing with PBS-0.01% azide. Contaminating erythrocytes were lysed. Remaining cells were washed, and naïve mouse serum was added to prevent Fc-mediated interactions. PBMCs were added in a conical-bottom 96-well plate (10^6 cells/well) and centrifugated (1500 rpm, 5 min, 4°C). Supernatant was decanted, and the cell pellet was resuspended by 100 μl PBS-0.01% azide containing specific antibodies for 30 min in the dark prior to washing. All experiments were conducted on ice, and flow cytometry analyses were performed on the day of blood collection.

Flow cytometry analysis

Purified PBMCs were stained with appropriate combinations of PE-Cy-7, APC, PE, and FITC mAb. PE-Cy7 anti-CD19 (clone SJ25C1), APC-anti-CD27 (clone L128), and PE-anti-IgD (clone IA6-2) mouse anti-human mAb were of the IgG1 class (BD PharMingen, Allschwil, Switzerland). FITC anti-IgM (clone M15/8) mouse anti-human mAb was of the IgG2a class (AbD Serotec, Dusseldorf, Germany). Cells were all analyzed using a FACSCalibur flow cytometer interfaced to CellQuest software (BD Biosciences, Becton Dickinson). A side/forward-scatter dot plot was used to exclude dead cells and to gate the live lymphocyte population. Fifty-thousand live cells/sample were analyzed.

Total IgA, IgG, and IgM titers

Total serum IgA, IgG, and IgM were measured by rate nephelometry (Beckman Coulter, Brea, CA, USA) in the first specimen available, i.e., at time of enrollment. IgG subclasses (IgG1, IgG2, IgG3, IgG4) were also measured by immunonephelometry assays.

Pneumococcal serotype-specific antibodies

To confirm past recurrent pneumococcal infection diagnoses and to assess actual immune status, serotype-specific IgG2 pneumococcal antibodies were measured by ELISAs (with 22F-inhibition), using antigen-coated plates, as described previously [28]. Serotypes 1, 5, and 7F were not included in the PCV7 (Prevenar or Prevnar 7; Pfizer, formerly Wyeth, New York, NY, USA) and therefore, reflect the capacity to raise antibodies against polysaccharide antigens. Antibodies to serotypes 14, 19F, and 23F, included in PCV7, were also measured. Based on our in-house-modified WHO reference ELISA, a protective immunity was considered present if serotype-specific,

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anticapsular serum antibody levels were ≥0.30 μg/ml [29]. Undetectable antibody concentrations were arbitrarily given a value of 0.15 μg/ml to enable analysis. All serological analyses were performed in the accredited Laboratory of Vaccinology of the University Hospitals of Geneva.

Statistical analysis

We planned a study of independent cases and controls. Expecting an arbitrarily, clinically significant 25% difference in B cell subsets between healthy and LRTI children, we estimated that we needed 43 patients in each group to reject the null hypothesis with a power of 0.8. Type I error probability used was 0.05. For group comparisons, Student's *t*-test or Wilcoxon test with a threshold of 5% was used to compare continuous variables, depending on their distribution. X^2 and Fisher's exact test were used for dichotomic variables. Linear regression was used to analyze evolution of B cell subsets with age. The GMCs of antibody levels with SDs were calculated. Student's *t*-test was used to compare the GMCs of antibodies among groups. Data analyses were performed on SPSS (version 15.0; SPSS, Chicago, IL, USA).

RESULTS

Study cohort

Forty-nine children with recurrent LRTIs and 61 healthy controls, aged 2–5 years, were enrolled in this prospective case-control study. Cases were followed during mean 20.3 ± 9.5 months (shortest: 2 months; longest: 40.5 months). Age categories were distributed as follows: grp1 (24–35 months), 29 cases and 22 controls (mean age 30 vs. 29 months, respectively, $P=0.29$); grp2 (36–47 months), nine cases and 18 controls (mean age 41 vs. 43 months, $P=0.13$); and grp3 (48–72 months), 11 cases and 21 controls (both: mean age 54 months). There was a gender predominance of 69 males over 41 females, as a result of a selection bias among control patients (elective surgery for circumcision). However, genders were distributed equally among the different groups (type of patient, age group, and ethnicity). Seventy-eight children were Caucasian, seven North African, three South American, five Asian, four Middle Eastern, two from sub-Saharan Africa, and 11 of plural ethnic origin.

Maturation of the B cell memory compartment in healthy children

To define the maturation of the peripheral B cell compartment, we studied the circulating B cell subsets of 61 healthy children, including, as controls, the CB of seven healthy newborns and seven healthy adults (mean age 32.6 years; SD 8.8). According to our flow cytometry protocol, the B cell compartment was divided into mature, naïve and memory B cells (**Fig. 1**). The proportion of peripheral B cells in the total lymphocyte population was low in CB (**Table 1** and **Fig. 2**). It was significantly higher (21.3%) in 2- to 3-year-old children ($P<0.001$). This proportion decreased progressively to 10.1% (4–5 years) and 4.9% (adults; Table 1 and Fig. 2, box a, grp1 and grp2 vs. CB and vs. adults: $P<0.001$; grp3 vs. CB and vs. adults: $P=0.002$; grp3 vs. grp1 and grp2: $P=0.001$ and 0.018, respectively). Mature, naïve B cells represented 98.2% of CB B cells, decreasing to 78.9 ± 8.1% (Table 1) in young children (CB vs. grp1, grp 2, and grp 3: $P<0.001$; Fig. 2, box b) and 68.1% in adults (adults vs. CB: $P=0.001$; adults vs. grp1 and grp3: $P=0.011$ and 0.018, respectively). As expected, memory B cells were undetectable in CB (Fig. 2, box c). Their proportion was significantly higher in 2- to 3-year-old children (21.1%, CB vs. grp1, grp2, and grp3: $P<0.001$), to eventually reach 31.8% in adults (adults vs. CB: $P=0.001$; adults vs. grp1: $P=0.011$; adults vs. grp3: $P=0.08$). The memory B cells were present in similar proportions in healthy children, grp1–grp3 ($P>0.40$), with a mean percentage of memory B cells in the three groups of 22.7% ± 10.1. This peripheral memory B cell

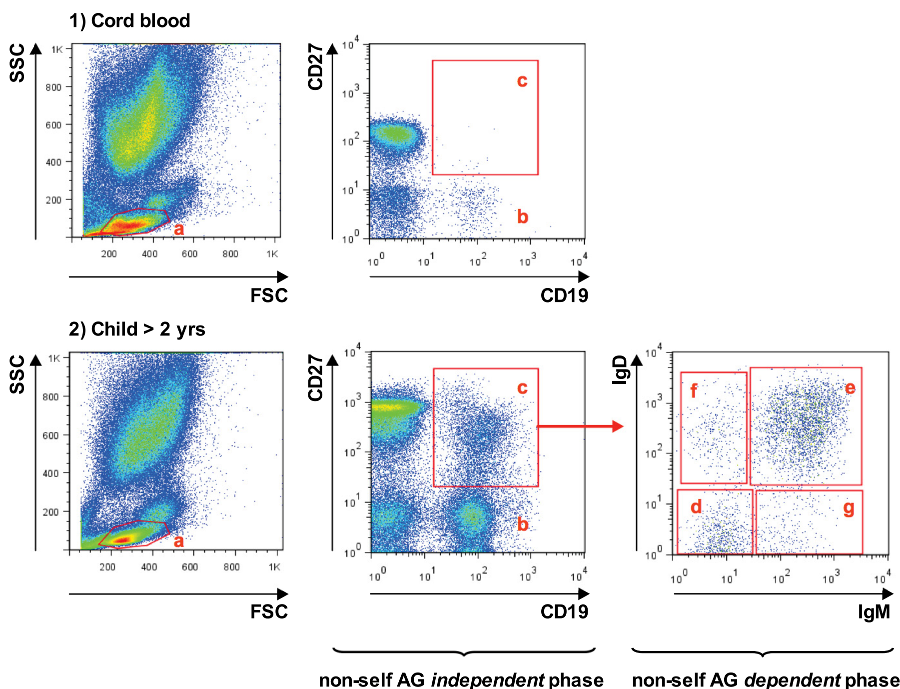

Figure 1. Phenotyping human circulating B cells by four-color fluorescence. In (A) CB and (B) children >2 years old, B cells were acquired, according to their forward- and side-scatter (FSC and SSC, respectively) characteristics and gated on $CD19^+$ (box a). CD27 discriminates between (box b) mature $CD19^+CD27^-$ and (box c) memory $CD19^+CD27^+$ B cells, subdivided into (box d) switched ($CD19^+CD27^+IgM^-IgD^-$), (box e) IgM memory ($CD19^+CD27^+IgM^+IgD^+$), (box f) IgD-only ($CD19^+CD27^+IgD^+IgM^-$), and (box g) IgM-only ($CD19^+CD27^+IgM^+IgD^-$). AG, Antigen.

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TABLE 1. Distribution of B Cell Populations in Human PB among Patient Categories

	Distribution of B cell populations in human PB				
	CD19$^+$	CD19$^+$CD27$^-$	CD19$^+$CD27$^+$	CD19$^+$CD27$^+$IgM$^-$ IgD$^-$	CD19$^+$CD27$^+$IgM$^+$ IgD$^+$
Age	B cells mean *(SD)* [95% CI] (a)	Mature B cells[a] mean *(SD)* [95% CI] (b)	Memory B cells[a] mean *(SD)* [95% CI] (c)	Switched memory B cells[a] mean *(SD)* [95% CI] (d)	IgM memory B cells[a] mean *(SD)* [95% CI] (e)
Healthy patients					
CB	4.9 *(2.4)* [1.5–8.6]	98.2 *(0.5)* [97.3–99.0]	1.6 *(0.5)* [0.7–2.2]	Below detectable value	Below detectable value
<2 years old	21.3 *(6.8)* [11.3–31.2]	75.8 *(14.3)* [41.4–85.8]	24.1 *(14.3)* [14.2–58.3]	10.0 *(8.8)* [4.1–27.0]	11.4 *(5.7)* [3.9–20.2]
2–3 years old	14.7 *(4.8)* [7.5–21.0]	78.9 *(8.1)* [67.9–90.8]	21.1 *(8.1)* [8.9–32.0]	9.5 *(4.8)* [4.0–16.3]	9.1 *(4.8)* [4.0–19.4]
3–4 years old	13.2 *(4.2)* [6.2–19.2]	76.7 *(9.1)* [49.7–86.6]	23.3 *(9.1)* [13.2–50.2]	11.7 *(7.1)* [4.3–30.9]	8.4 *(2.8)* [5.0–16.8]
4–5 years old	10.1 *(3.7)* [5.4–16.8]	77.7 *(7.5)* [65.3–88.8]	22.2 *(7.4)* [11.1–34.6]	9.3 *(3.6)* [5.2–14.5]	9.6 *(3.8)* [4.3–15.1]
Adults	4.9 *(2.9)* [2.6–9.5]	68.1 *(11.8)* [54.0–88.9]	31.8 *(11.8)* [11.1–46.0]	17.7 *(9.2)* [5.6–30.6]	11.8 *(4.6)* [3.7–18.5]
Recurrent LRTI patients					
2–3 years old	17.1 *(7.2)* [8.6–29.5]	73.1 *(16.4)* [31.7–92.4]	26.9 *(16.3)* [7.6–68.1]	12.2 *(10.3)* [2.6–31.9]	10.8 *(7.6)* [3.4–23.7]
3–4 years old	16.3 *(6.2)* [6.0–24.4]	81.7 *(8.8)* [68.6–94.0]	18.2 *(8.8)* [6.0–31.4]	6.9 *(3.7)* [1.9–13.2]	8.3 *(4.6)* [2.5–15.5]
4–5 years old	10.9 *(2.4)* [7.4–14.5]	73.1 *(9.5)* [52.0–88.1]	26.8 *(9.5)* [11.8–48.0]	12.3 *(5.8)* [5.8–26.8]	10.9 *(4.4)* [4.0–16.5]

Means expressed in percent. CI, Confidence interval for the mean. The letters at the bottom of each cell population refer to their corresponding box plots (see Figs. 1–3). [a]Expressed as percentage of total B cells (B cells=100%).

compartment of 2- to 5-year-old children was constituted by switched memory (45%), IgM$^+$ memory (42%), IgM$^+$-only (9%), and IgD$^+$-only (3%) B cells. Further analyses thus focused on switched and IgM memory B cells.

As expected, switched and IgM$^+$ memory B cells were not identified in CB. However, they were present in adult-like proportion already in the blood of 2- to 3-year-old healthy children with no influence of gender or ethnicity (both $P>0.20$). This unexpected observation led to the evaluation of 22 healthy, younger children [mean age: 13.4±2.56 months; 17 boys (77.3%); 14 Caucasian (63.4%)]. The proportion of peripheral B cells was significantly higher at 13 months than in CB ($P=0.001$; Table 1 and Fig. 2). Mature, naïve B cells represented two-thirds of peripheral B cells, significantly lower than in CB ($P<0.001$). Inversely, memory B cells, whether switched or IgM$^+$, were more frequent than in CB ($P<0.001$). The total peripheral memory B cell compartment of 1- to 2-year-old children was essentially constituted of switched memory (40%) and IgM$^+$ memory (49%) B cells. The proportion of switched and IgM$^+$ memory B cells was thus similar when comparing <2-year-olds with older, healthy children (Table 1; all $P>0.05$, except with grp2: $P=0.013$).

Maturation of the B cell compartment in children with recurrent LRTIs

We next asked if the maturation of memory B cell compartment differed in children with recurrent LRTIs compared with healthy children (Table 1 and **Fig. 3**). B cells represented 15.6% ± 6.6% of the total lymphocyte population compared with 12.7% ± 4.7% in healthy children ($P=0.011$). The highest proportion of B cells was again observed in 2- to 3-year-old children and decreased progressively over time (grp1 vs. grp3: $P<0.001$; grp2 vs. grp3: $P=0.034$; Fig. 3, box a). The memory B cells were present in similar proportions in LRTI children, grp1–grp3 ($P>0.05$), with a mean percentage of memory B cells in the three groups of 25.3% ± 14.1. The distribution of mature, naïve and memory B cells was similar to that of healthy children (Fig. 3, boxes b and c): switched (43%) and IgM$^+$ memory B cells (43%) were again the most common compartments, followed by IgM$^+$-only (12%) and IgD$^+$-only (3%) B cells. Switched and IgM$^+$ memory B cells were present in similar proportions in the various age groups (Fig. 3, boxes d and e). The proportion of switched and IgM$^+$ memory B cells was thus similar when comparing children with or without recurrent LRTIs. By linear regression, the proportion of B cells decreased similarly with age in LRTI ($P=0.001$) and healthy children ($P<0.001$). There was no influence of age on the other B cell compartments, neither in LRTI nor in healthy patients.

As cross-sectional analyses may overlook maturation kinetics, we followed the individual maturation of the B cell memory pool in 42 LRTI children. One year after enrollment, the proportion of IgM$^+$ memory B cells had already increased significantly (10% vs. 13%, $P=0.031$). The comparison of the first

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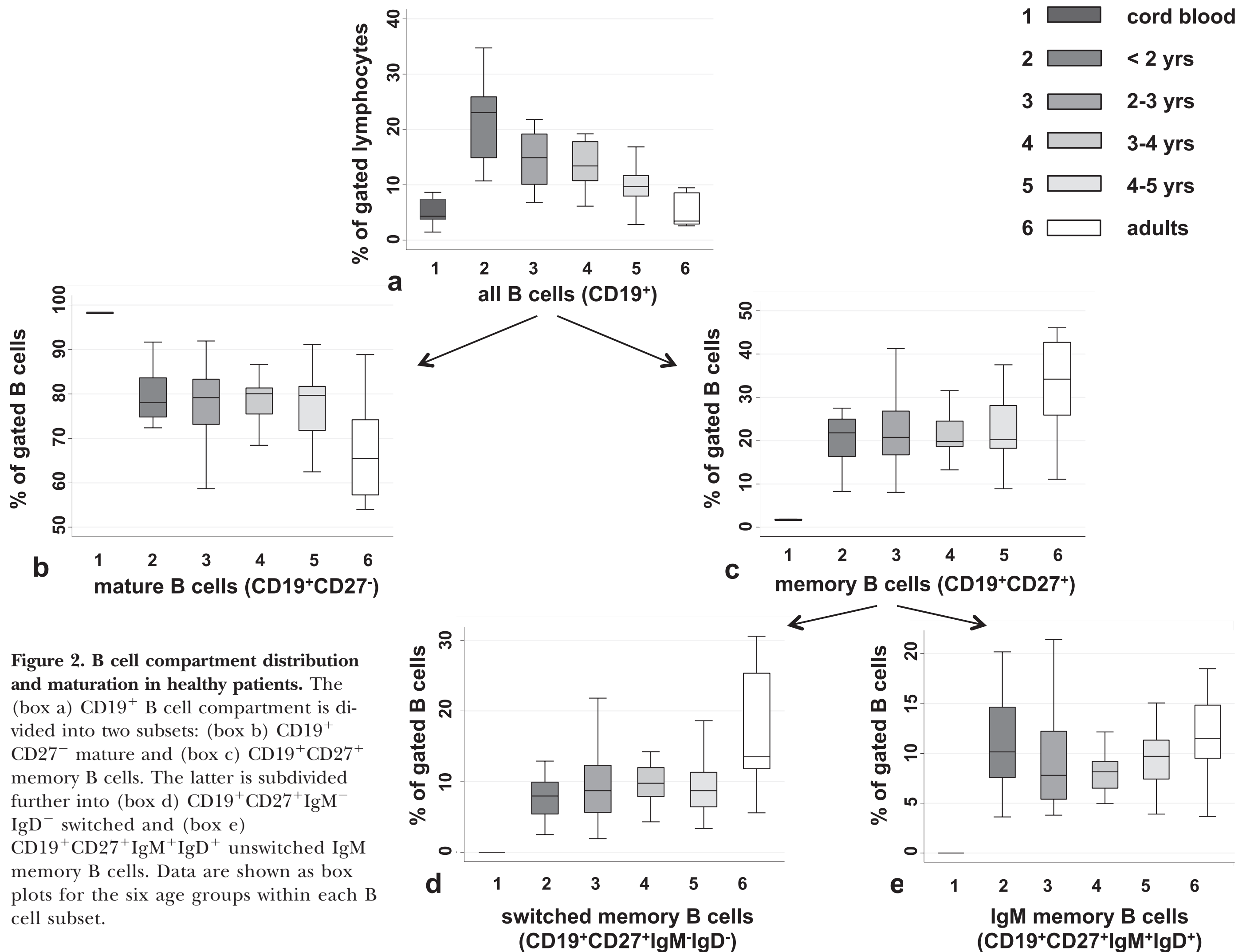

Figure 2. B cell compartment distribution and maturation in healthy patients. The (box a) $CD19^+$ B cell compartment is divided into two subsets: (box b) $CD19^+CD27^-$ mature and (box c) $CD19^+CD27^+$ memory B cells. The latter is subdivided further into (box d) $CD19^+CD27^+IgM^-IgD^-$ switched and (box e) $CD19^+CD27^+IgM^+IgD^+$ unswitched IgM memory B cells. Data are shown as box plots for the six age groups within each B cell subset.

with the last available samples (mean interval 19.6±8.1 months; range 8–41 months) was sufficient to identify a significant decrease in mature, naïve B cells (75% vs. 70%, P=0.039) and an increase in memory B cells (25% vs. 30%, P=0.044), whether switched (11% vs. 13%, P=0.022) or IgM (10% vs. 13%, P=0.015). Again, this maturation was not influenced by gender or ethnicity (all P>0.50).

Pneumococcal vaccination status

Healthy controls had no history of LRTIs and had not been immunized against Spn prior to enrollment, in accordance with Swiss recommendations in 2006–2009. Six LRTI children had prior vaccination within 1 year with PCV7 and six others with the PPV23 (Pneumovax23; Merck, Whitehouse Station, NJ, USA).

Antibodies in children with recurrent LRTIs compared with healthy controls

To evaluate the functional capacity of B cells, we next measured total serum IgA, IgM, and IgG in LRTI (n=47) and healthy children (n=61). Normal values above 0.07 g/l were found in all children for total IgA and were similar between LRTI (0.71±0.05 g/l) and healthy patients (0.87±0.07 g/l; P=NS). In LRTI patients, IgA levels were similar in different age groups (grp1: 0.67±0.05; grp2: 0.71± 0.15; grp3: 0.82±0.11 g/l). IgM titers were not significantly different between cases (mean 1.00±0.39 g/l) and controls (mean 1.06±0.43 g/l) or among age groups and did not correlate with the proportion of IgM^+ memory B cells. IgG titers were lower in LRTI (mean 7.40±1.85 g/l) than healthy children (mean 8.32±2.14 g/l, P=0.021; **Fig. 4**). Correlations between IgG titers and switched memory B cells were observed in the younger, healthy patients (grp1, P=0.013) and in LRTI children (P=0.029) but disappeared when analyzing by age group. IgG subclasses were within normal values for LRTI and healthy children (sample not available for five healthy children) [30]. The youngest LRTI children (grp1) had statistically lower IgG1 values (mean 5.81±0.28 g/l) than same-aged, healthy children (mean 6.13±0.3 g/l; P=0.01). However, for both groups, the values were clearly within the normal values for age (2.7–9.4

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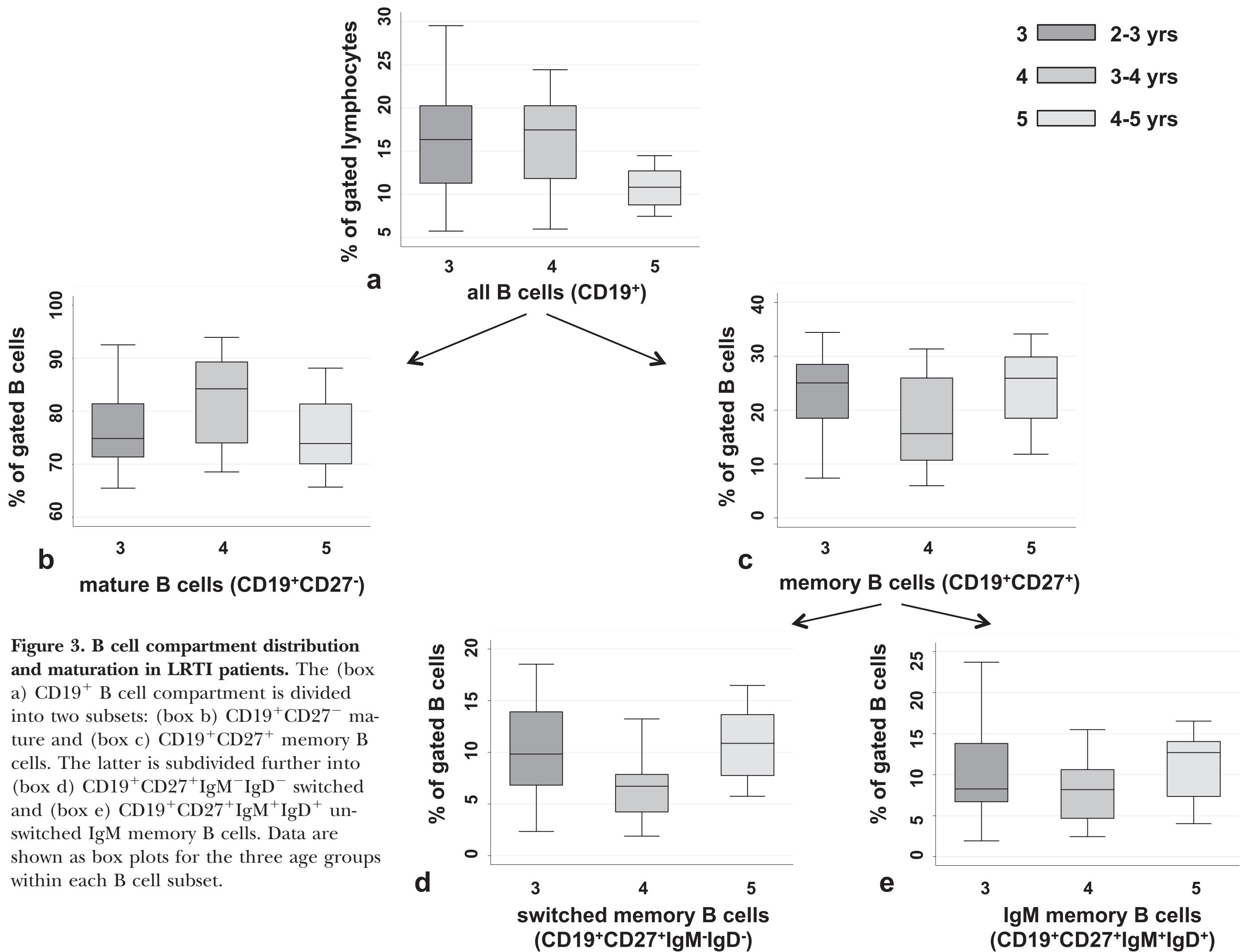

Figure 3. B cell compartment distribution and maturation in LRTI patients. The (box a) $CD19^+$ B cell compartment is divided into two subsets: (box b) $CD19^+CD27^-$ mature and (box c) $CD19^+CD27^+$ memory B cells. The latter is subdivided further into (box d) $CD19^+CD27^+IgM^-IgD^-$ switched and (box e) $CD19^+CD27^+IgM^+IgD^+$ unswitched IgM memory B cells. Data are shown as box plots for the three age groups within each B cell subset.

g/l). All other comparisons were statistically nonsignificant for IgG subclasses.

We then measured antibody responses against the 1, 5, and 7F Spn serotypes, as surrogates of the response capacity to bacterial polysaccharides. Thirty-two LRTI children (65%) had protective antibody levels against at least two-thirds of these serotypes, at enrollment, as a result of prior immunization/exposure, or after a single injection of the PPV23. Among the 17 remaining LRTI children, eight (16%) had no detectable antibodies, and nine (18%) had protective antibody levels against only one-third of serotypes. However, all 17 children reached protective antibody levels after a single dose of PPV23. Thus, the vulnerability of certain young children to LRTIs was not associated with a limitation in Ig-secreting PC function. Clinical history or severity of disease—according to medical charts, pediatricians' reports, or parents' recall—did not correlate with different antibody levels (from normal to absent).

We next assessed the same LRTI patients for antibody responses against the 14, 19F, and 23F Spn serotypes (n=43/49, 88%). Among them, six children with prior PCV7 vaccination showed protective antibody levels against all three serotypes and thus were not compared with healthy patients. Thirty-six LRTI children (84%) had protective antibody levels against at least two-thirds of these serotypes, five children (12%) against one-third, and two children (4%) had no detectable antibodies. Among the eight children described previously with undetectable antibodies against Spn serotypes 1, 5, and 7F, only one (aged 28.3 months) had no antibodies against serotypes 14, 19F, and 23F. Interestingly, this patient produced antibodies against five of six serotypes after a single dose of PPV23, 2 months later, ruling out an impaired response to polysaccharidic antigens. For all three serotypes, antibody response was significantly lower in healthy than LRTI children in the younger age groups (PPS 14, healthy grp1: 0.23 ± 0.95 μg/ml vs. LRTI grp1: 1.33 ± 5.59 μg/ml, P=0.0058; healthy grp2: 0.50 ± 1.64 μg/ml vs. LRTI grp2: 1.15 ± 7.06 μg/ml, P=0.039. PPS 19F, healthy grp1: 0.67 ± 1.33 μg/ml vs. LRTI grp1: 1.58 ± 6.00 μg/ml, P=0.020. PPS 23F, healthy grp1: 0.23 ± 1.00 μg/ml vs. LRTI grp1: 0.83 ± 3.20 μg/ml, P=0.0067; **Fig. 5**). An increase in antibody concentration was only present for Spn 19F in healthy children, grp1–grp3 (P=0.041).

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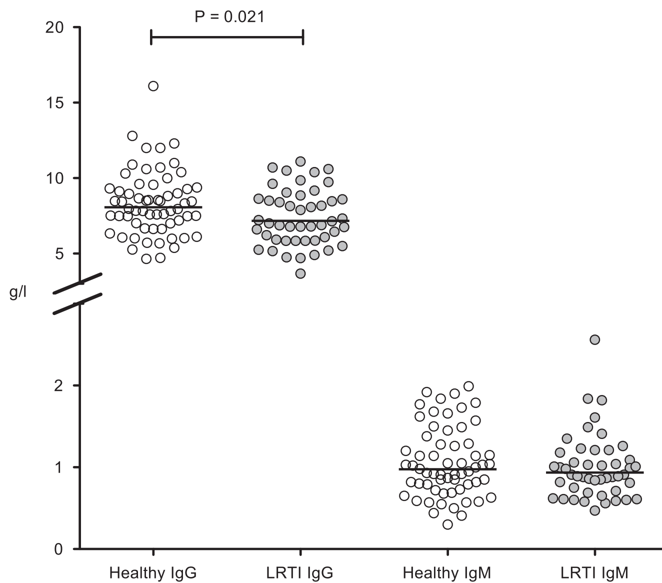

Figure 4. Total IgG and IgM antibody compared between healthy children and children with recurrent LRTIs. The horizontal lines represent the GMCs.

Serotype-specific, anti-PPS antibody persistence in LRTI children

When available, we followed the individual persistence of the 1, 5, 7F, 14, 19F, and 23F pneumococcal serotype-specific antibodies, 1–2 months (mean follow-up 1.08 months) and >8 months (mean follow-up 13.08 months) postvaccination in 17 LRTI children. For all serotypes, the mean antibody concentrations increased more than fourfold at 1–2 months postvaccination (PPS 1 prevaccination: 0.17±0.017 vs. postvaccination: 3.04±0.61, P<0.0001. PPS 5 pre: 0.53±0.095 vs. post: 3.23±0.48, P<0.0001. PPS 7F pre: 0.40±0.12 vs. post: 4.56±0.52, P<0.0001. PPS 14 pre: 0.61±0.15 vs. post: 3.97±1.18, P=0.0076. PPS 19F pre: 0.87±0.13 vs. post: 4.39±1.04, P=0.0019. PPS 23F pre: 0.92±0.42 vs. post: 2.56±0.66, P=0.045; **Fig. 6**). After 8 months postvaccination, the mean antibody concentrations decreased for four serotypes (PPS 1, 1–2 months postvaccination: 3.04±0.61 vs. >8 months postvaccination: 0.92±0.20, P=0.0049. PPS 5, 1–2 months post: 3.23±0.48 vs. 8 months post: 1.43±0.18, P=0.0032. PPS 7F, 1–2 months post: 4.56±0.52 vs. 8 months post: 1.71±0.24, P<0.0001. PPS 23F, 1–2 months post: 2.56±0.66 vs. 8 months post: 0.73±0.18, P=0.023) but not significantly for PPS 14 and PPS 19F (PPS 14, 1–2 months post: 3.97±1.18 vs. 8 months post: 1.90±0.41, P=0.14. PPS 19F, 1–2 months post: 4.39±1.04 vs. 8 months post: 2.16±0.94, P=0.13; Fig. 6).

DISCUSSION

Recurrent LRTIs in children possibly results from immune maturation delay, as children's infection rate usually improves spontaneously after the age of 5 years. Several authors postulated that a smaller memory B cell compartment may increase the risk of bacterial infections, including invasive Hib diseases [31] and pneumococcal infections [22, 32, 33]. This hypothesis is not valid for children with recurrent LRTIs in our study. We show that memory B cells, which are undetectable at birth, increase rapidly over the first few months of life to reach adult-like values, ~2 years of age, in both cohorts of healthy and LRTI children; this might reflect the maturation of the spleen. Age-dependent B cell subpopulations in the PB of our healthy children were identical to reference values established by several authors [34–37]. In particular, the proportions of the $CD19^{+}CD27^{+}$ memory B cells across age groups were similar than those recently described by Piatosa et al. [36], representing ~20% of the total B cell pool, split between 10% of IgM memory and 10% of switched memory B cells. Schatorjé et al. [38] have also described comparable age-matched proportions of memory B cell subpopulations, although they described a continuing four- to 12-fold increase in percentages of switched and unswitched memory B cells, respectively, that extended after 2 years of age. However, given the large tolerance intervals obtained on the means and the lack of statistical tests, the extent of this increase should be interpreted with caution. Indeed, we also reported a several-fold, age-related increase of the memory B cell compartment from birth to adulthood. Yet, our results with narrower confidence intervals showed that the increase at the level of the switched and unswitched memory B cell subpopulations was accomplished mostly during the first 2 years of life, remaining at adult-like proportions thereafter without further significant increase. We report only a trend

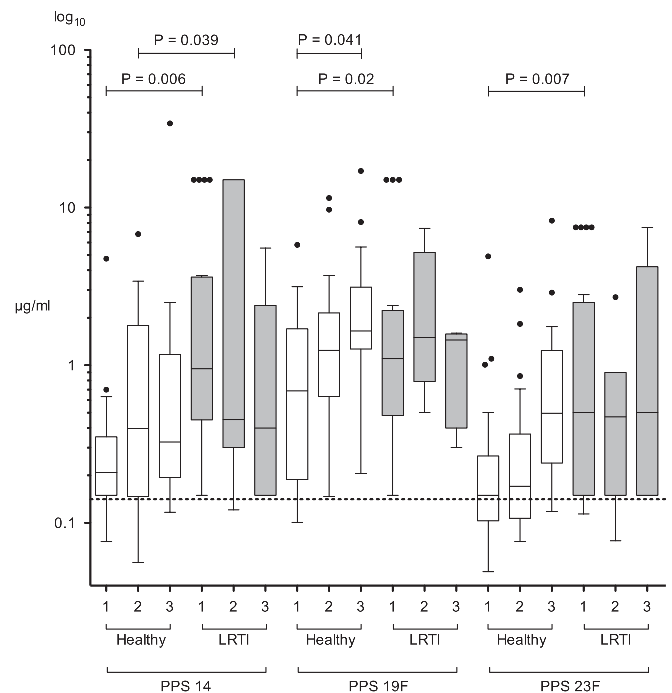

Figure 5. Comparison of 14, 19F, and 23F serotype-specific anti-PPS IgG concentrations in healthy children and children with recurrent LRTIs. Data are shown as box plots for the three age groups (grp1: 2–3 years, grp2: 3–4 years, grp3: 4–5 years) on a y-axis logarithmic scale. The horizontal, dotted line represents the cut-off value of 0.15 μg/ml, below which antibody concentrations are undetectable.

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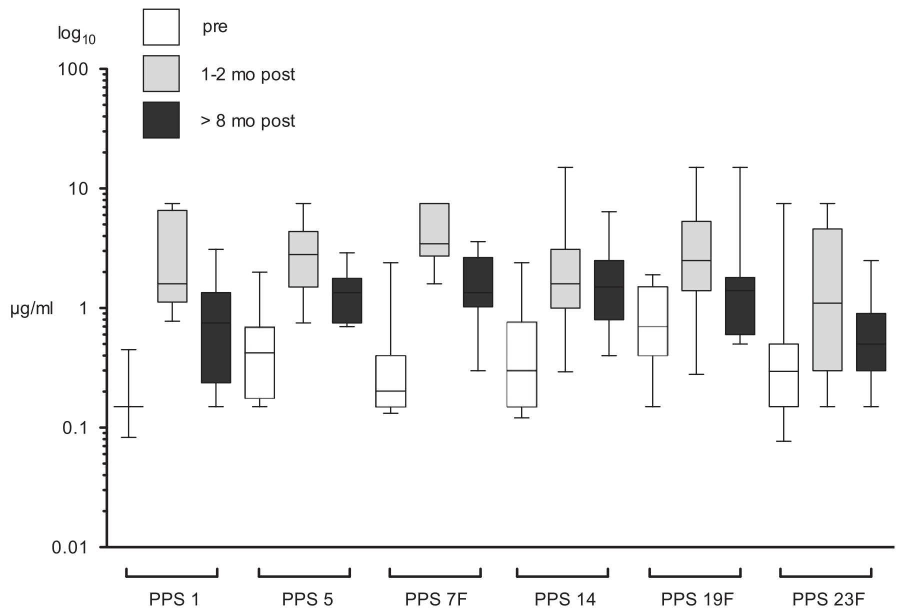

Figure 6. Serotype-specific anti-PPS antibody waning in LRTI children. Comparison of 1, 5, 7F, 14, 19F, and 23F serotype-specific anti-PPS antibody concentrations prevaccination (pre), 1–2 months postvaccination (1–2 mo post), and >8 months postvaccination (>8 mo post) in LRTI children on a *y*-axis logarithmic scale.

toward an increase in the relative proportion of the switched memory B cells after 5 years of age. These results were consistent with previous reports from Luning Prak et al. [39]. On the other hand, we did not assess the proportion of memory B cells between 5 years of age and adulthood. It would therefore be possible that we missed a transient increase of these cell populations during this time period. Indeed, van Gent et al. [37] observed such a transient increase in the proportions of IgM and the switched memory B cell subsets after 5 years of age, which returned to 2- to 5-year-old values by 18 years of age, in accordance with our results. Altogether, our study shows a quantitative regression of the B cell pool during the first years of life, while undergoing an intense maturation of the memory B cell compartment. Adult-like values were reached at 2–5 years of age, which seems to correlate with the progressive decline in the incidence of PID before school years.

In adult patients with CVID and HIV, an increased risk of infections caused by encapsulated bacteria is correlated with the lack or loss of IgM memory B cells [24, 40, 41]. In splenectomized patients with recurrent pneumococcal infections, depletion in IgM memory B cells has been described [22]. Weller et al. [42] showed that IgM memory B cells display no sign of antigen-driven clonal expansion, leading to the proposition that these cells develop without T-dependent or -independent immune stimulation. Recently, however, IgM memory B cells were shown to be generated upon TLR ligation of transitional B cells, generating "primitive memory B cells" that may produce natural IgM antibodies as a first line of defense [43]. Their detection at adult levels, already in infants younger than 2 years, suggests that the increased susceptibility to encapsulated bacterial infections in healthy or LRTI, 2- to 4-year-old children does not result from their absence/delayed maturation.

Our results showed an intact B cell maturation process, including in the splenic compartment. Indeed, most of the LRTI children showed an increase of their B cell memory pool during a 1-year follow-up, including in IgM memory B cells. This maturation was not apparent across age groups, as the proportion of switched and IgM memory B cell pools did not change significantly between 2 and 5 years of age. We can therefore postulate that the memory B cell compartment undergoes an initial, rapid growth phase during the first years of life, during which, the initially naïve immune system undergoes intense antigen exposure [39, 44]. Thanks to the plasticity of the memory pool, it can quickly expand and differentiate into PCs in response to antigenic stimuli. A small fraction of the newly formed memory B cells may expand the pre-existing pool [45]. As demonstrated previously by Bernasconi et al. [46], memory B cells proliferate and differentiate at a low rate into PCs in response to TLR-mediated polyclonal stimuli or bystander T cell help and continually replace those during their turnover in the bone marrow. Thus, rapid changes within the memory pool can be observed at the individual level, according to the antigenic pressure that drives its expansion or contraction. Another part of the immune maturation against encapsulated pathogens is related to the acquisition of serum-specific antibodies to polysaccharides. A quantitative, humoral defect may be a result of a delayed immune maturation or to an immunodeficiency condition, such as polysaccharide antibody deficiency [47] or common variable immunodeficiency. Carsetti et al. [48] reported in 54 CVID adult patients with LRTIs that a reduced frequency of peripheral IgM memory B cells correlated to an absence of anti-PPS IgM. However, a puzzling result reported by this group is that this correlation was only found for anti-PPS serotype 14, 1, and 4 antibodies, whereas these serotypes are usually immunogenic [12, 49]. It is possible that other serotypes were less prevalent in their "healthy" population, and therefore, the difference between the two groups didn't reach significance. In contrast to adult CVID patients, we did not find a decreased proportion of peripheral IgM memory B cells in young LRTI children.

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Moschese et al. [50] reported in 32 children, aged between 13 and 36 months, with suspected transient hypogammaglobulinemia of infancy, a subgroup of patients with significantly lower values of IgM and switched memory B cell subsets than in age-matched controls: it was associated with persistent hypogammaglobulinemia. In our study, we observed no correlation between IgM memory B cells and total IgM levels, and total IgG and IgM levels, as well as IgG subclasses, were within normal ranges (for age) in LRTI and healthy children.

Selective IgAD is known as the most common PID and is associated frequently with recurrent sino-pulmonary infections [51]. IgAD is defined as serum IgA levels of <0.07 g/l in the presence of normal serum IgG and IgM levels in otherwise immunocompetent patients older than 4 years of age [52]. Below 4 years, the immaturity of the IgA secretory system can make the diagnosis difficult. In our study, a IgAD was ruled out for all patients, even below 4 years of age.

To go further, given that the evaluation of long-term protection against several micro-organisms includes measuring IgG antibody levels against a specific micro-organism [53] and as PPS antibodies rely largely on the IgG2 subclass in children [54, 55], we next measured the serotype-specific IgG pneumococcal antibodies. Sanders et al. [56] have reported that $>30\%$ of children aged between 2 and 5 years with recurrent LRTIs and with normal serum IgG levels and antibody responses to protein vaccines had absent or low antibody responses to certain Spn antigens. This was also observed in our population, where we, respectively, report 34% and 16% of LRTI children with minimal or without protective antibody levels against Spn 1, 5, 7, 14, 19F, or 23F antigens. This might reflect a transient maturational delay rather than a persistent, selective, impaired response to polysaccharide antigens. Indeed, although serum IgG titers were lower than in healthy children, all LRTI patients responded to Spn polysaccharide exposure. We also showed that before any vaccination, the serotype-specific pneumococcal antibody titers against PPS 14, 19F, and 23F were higher in younger LRTI than in healthy children. This possibly reflects the higher incidence of pneumococcal exposure in this population. However, most of our LRTI patients showed a rapid antibody waning over time, some decreasing below the putative protective thresholds of 0.3 μg/ml within a few months. Indeed, although it is widely accepted that children over 2 years old are able to produce a protective, serotype-specific antibody response to PPS, our results revealed that IgG2 serotype-specific antibody titers returned to preimmunization levels within 8 months in the LRTI patients in the absence of booster vaccine doses. To our knowledge, this kinetic pattern was never described before in children with recurrent LRTIs. This emphasized the limitations of PPS vaccines that cannot sustain protective serum antibody levels over time, as they lack the ability to induce a T cell-dependent immunologic memory. Interestingly, antibody waning was less pronounced in our LRTI patients for Spn 14 and 19F, which might be explained again by the higher prevalence of both serotypes in Switzerland in the pre-PCV7 era and thus, reflects the continuous "natural" antigenic stimulation. Unfortunately, as our healthy children were not vaccinated with PPV23, we were unable to determine if such a waning was also present in this population.

The waning of polysaccharide-induced antibody response is not a new concept; it was similarly, already described in children vaccinated against meningococcal serogroups A and C [57, 58], with a decrease from 100% protection to 8% in 3 years [59]. Likewise, a decline in vaccine-induced capsular polysaccharide antibodies has been observed 12 months after vaccination with a quadrivalent polysaccharide meningococcal vaccine (serogroups A, C, Y, and W-135) in children younger than 5 years [60]. Such a waning of functional antibody levels, despite persisting immunological memory, has been described following vaccination with protein–polysaccharide conjugate vaccines [11]. Recently, Black et al.[61] have demonstrated, in healthy children, aged 2–5 years old, that bactericidal antibody titers, following a single dose of a quadrivalent MPSV4 or a quadrivalent MenACWY-CRM, both decreased over the first 12 months after vaccination, although the antibody titers remained higher and more persistent in MenACWY-CRM recipients than MPSV4 recipients. Hib type b-MenC conjugate vaccine was also associated with a yearly decline in circulating bactericidal antibodies [62, 63]. Regarding serogroup B meningococcal vaccination, only limited numbers of pediatric studies have assessed the kinetics of immune response following vaccination. Holst et al. [57] and Galloway et al. [59] have described a decline of meningococcal B vaccine effectiveness, beginning ~12 months postvaccination, respectively, from 87% to 30% in adolescents and from 82% to 33% in children aged between 6 months and 5 years. The waning of antibody levels and corresponding loss of vaccine effectiveness are also supported by vaccine studies against Hib in young children [11, 64]. Altogether, these studies raise concern about the preservation of circulating protective antibody levels for long-term protection against encapsulated bacteria. It is especially important, as newly produced, serotype-specific antibodies by primed memory B cells, following a re-encounter with bacteria, take several days to develop, during which bacteria can invade and infect the host. In our study, the rapid antibody waning and the absence of primed immunity by conjugated pneumococcal vaccine, despite a constituted memory B cell compartment, might have contributed to the pneumococcal vulnerability and the recurrent pneumococcal infections in our LRTI patients. Further studies should address whether early antibody waning in LRTI patients is correlated to differences in the establishment/maintenance of the memory B cell pool and/or has clinical significance.

Interestingly, some studies in mice and humans show that the $CD4^+$ Th17 T cells may, in part, protect against mucosal pneumococcal colonization [65]. Lu et al. [66] have shown that the cytokine IL-17A produced by those Th17 T cells, without direct cytotoxic effect on Spn, acts on neutrophils to recruit them to the nasopharyngeal tonsils and activate them to phagocyte Spn. Moreover, authors show that human rIL-17A may also enhance antibody-dependent killing of Spn in vitro in a dose-dependent manner. Collec-

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tively, these data suggest that the pneumococcal immunity may not only rely on the memory B cell compartment at the blood level but also on the acquisition and maturation of a Th17 T cell immunity at the mucosal level. The high prevalence of nasopharyngeal pneumococcal colonization seen in young children leans toward this direction. We think that future studies might focus on this field of research, looking at the Th17 T cell compartment constitution and memory at the mucosal level.

This study has some limitations. LRTI is defined clinically according to the WHO definitions. This may possibly lead to heterogeneity between the patients. For example, LRTI patients may have other etiologies than Spn. However, local and international epidemiological studies recognize Spn as the most prevalent cause of LRTIs in young children [27]. According to this, pneumonia in our LRTI children was probably frequently a result of Spn, as highlighted by the presence of pneumococcal serotype-specific antibodies in all sera, except in six children with antibodies secondary to vaccination with PPV23 prior to enrollment. These antibodies were unlikely to be related to Spn nasopharyngeal carriage, and our LRTI children had no past history of pneumococcal-invasive disease other than pneumonia. The performance of systematically bacterial cultures would not have helped to establish an etiology. Indeed, sputum cultures are difficult in young children, and nasopharyngeal cultures are unreliable because of contamination by upper-respiratory tract noncommensal bacteria. Furthermore, blood cultures have a low sensitivity, given that the prevalence of bacteremia is <2–15% in community-acquired pneumonia [67]. Finally, the detection of pneumococcal antigen or other indirect markers in urine samples is not reliable in children [68]. Indeed, urinary tests may be informative as negative predictors of pneumococcal infection [2] but are not likely to be useful for distinguishing children with true pneumococcal pneumonia from others with another disease and pneumococcal nasopharyngeal colonization [69]. The Pediatric Infectious Diseases Society and the Infectious Diseases Society of America have recently recommended not using the urinary antigen detection tests for the diagnosis of pneumococcal pneumonia in children [8]. This explains why up to 50% of children with pneumonia have no bacterial proof, despite thorough investigations [70].

Our study has some other limitations: as only one blood sample was available for healthy children, it is possible that normal variations were missed. Moreover, it is difficult to establish correlations between the proportion of cell populations and function. Although the children had no total Ig deficiency, we cannot totally exclude that some children had an unrecognized, underlying immune deficiency. Finally, we measured circulating B cell populations; it is possible that it does not reflect central compartments, such as LNs, spleen, or bone marrow. In children, obtaining such types of samples would be difficult and probably unethical.

In conclusion, this study shows that the memory B cell compartment is already constituted at 2 years of age in adult-like proportion. Despite a higher proportion of total B cells, LRTI children do not have a delayed constitution of their B cell memory compartment compared with healthy children. The constitution of this memory B cell compartment essentially occurs in the first 2 years of life, in response to antigen exposure. We also show that a qualitative defect in the memory B cell compartment is absent in LRTI children and thus, cannot explain their increased susceptibility to bacterial pneumonia.

AUTHORSHIP

J.N.S. designed and performed experiments, analyzed the results, and wrote the manuscript. A.G.L. analyzed the results. S.G. performed experiments. C.D. analyzed the results. C-A.S. and K.M.P-B. designed the study, analyzed the results, and wrote the manuscript.

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DISCLOSURES

The authors have no potential financial conflict of interest related to this manuscript.

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