Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12660

Poor memory B cell generation contributes to non-protective responses to DTaP vaccine antigens in otitis-prone children

S. Basha and M. E. Pichichero
Rochester General Hospital Research Institute, Rochester, NY, USA

Summary

We recently identified a cohort of children with recurrent episodes of acute otitis media (AOM) who fail to generate protective antibody titres to otopathogens and several vaccine antigens. In this study we determined the antibody levels against DTaP vaccine antigens, diphtheria toxoid (DT), tetanus toxoid (TT) and acellular pertussis toxoid (PT) in sera from 15 stringently defined otitis-prone (sOP) children and 20 non-otitis-prone (NOP) children. We found significantly lower concentrations of immunoglobulin (Ig)G antibodies against vaccine antigens in the serum of sOP children compared to age-matched NOP children. To elucidate immunological cellular responses to the vaccines in these children, we investigated memory B cell responses to DTaP vaccination. We used fluorescently conjugated vaccine antigens to label antigen receptors on the surface of memory B cells and examined the frequency of antigen-specific $CD19^+$ $CD27^+$ memory B cells in the peripheral blood. sOP children showed a significantly lower percentage of antigen-specific $CD19^+$ $CD27^+$ memory B cells than NOP children. We also found a linear correlation between the frequencies of memory B cells and circulating IgG titres for DT, TT and PT proteins. To our knowledge, this is the first study to show significant differences in memory B cell responses to DTaP vaccine antigens and their correlation with the circulating antibodies in young children with recurrent AOM.

Keywords: acute otitis media, diphtheria toxoid, memory B cells, pertussis toxoid, tetanus toxoid

Accepted for publication 28 May 2015
Correspondence: M. E. Pichichero, Rochester General Hospital Research Institute, 1425 Portland Avenue, Rochester, New York 14621, USA.
E-mail: Michael.Pichichero@rochesterregional.org

Introduction

Acute otitis media (AOM) is one of the most common infectious diseases affecting 6–30-month-old children, and can lead to hearing impairment and delayed speech development [1,2]. Approximately 80% of children experience at least one episode of AOM and approximately 30% experience three to four episodes diagnosed clinically within a 6–12-month time-span during their first 3 years of life, termed otitis-prone (OP). We have recently defined a subset of OP children, representing 5% of the total population of children, who meet the above definition and whose diagnosis was based on tympanocentesis confirmation of otopathogens in the middle ear fluid; we termed these children stringently defined otitis-prone (sOP) [3–5]. These sOP children have recurrent ear infections associated with elevated bacterial burden, and higher proinflammatory cytokine levels within the middle ear space despite tympanocentesis and individualized antibiotic treatment [6,7].

Predominant bacterial otopathogens for otitis media include *Streptococcus pneumoniae* (*Spn*), non-typeable *Haemophilus influenzae* (*NTHi*) and *Moraxella catarrhalis* (*Mcat*) [6]. Previous work has shown an association of respiratory syncytial virus, rhinovirus, parainfluenza virus, and in one instance a culture-confirmed case of *Bordetella pertussis* with AOM [8–10]. Circulating antibodies in the serum that transudates to mucosal surfaces and/or mucosal immunoglobulin (IgA) antibodies play a role in blocking adherence of these pathogens to mucosal epithelial cells and/or interfere with microbial invasion of the bloodstream [11,12]. Diminished cellular immunity and cytokine secretion could also affect the level of protection from infections leading to frequent AOMs. We previously

showed that sOP children generate low humoral and cellular immune responses to otopathogens following nasal colonization and AOM caused by *Spn* and *NTHi* resembling a neonatal immune profile [6].

The Center for Disease Control (CDC) immunization schedule for children aged 0–18 years recommends primary doses of DTaP vaccine at ages 2, 4 and 6 months, followed by a booster at 15–18 months, and a fifth dose at age 4–6 years. Despite these multiple vaccine doses, pertussis remains poorly controlled, resulting in morbidity and mortality in vaccinated and non-vaccinated children. Recent reports of pertussis outbreaks show that this disease remains dangerous in the United States and other countries [13,14]. In our recent studies, sOP children failed to generate protective antibody responses to many common vaccine antigens, including DTaP components [6,10]. In this study, to delineate a more precise immunological mechanism for the lower antibody levels in sOP children, for the first time to our knowledge we describe an evaluation of the memory B cell ($CD19^+$ $CD27^+$) responses to DTaP vaccine antigens in age-matched sOP and non-otitis-prone (NOP) children and correlated the observations with serum IgG levels.

Material and methods

Subjects

Subjects in this study are from our 7-year, prospective, longitudinal study funded by the National Institutes of Health, National Institute on Deafness and Other Communication Disorders (NIDCD R0108671) to study immunological dysfunction in OP children. For the studies reported here, all 35 children were aged 9–18 months (mean age 10·5 months) from a middle-class, suburban sociodemographic population in Rochester, New York, who had received three doses of DTaP vaccine (Sanofi Pasteur, Swiftwater, PA, USA) at 2, 4 and 6 months of age. A written informed consent was obtained from parents of the children in accordance with a protocol approved by the Rochester General Hospital institutional review board.

IgG antibody levels

To measure IgG antibody levels to diphtheria toxoid (DT), tetanus toxoid (TT) and pertussis toxoid (PT) in the samples, an enzyme-linked immunosorbent assay (ELISA) was performed as described previously [15,16]. Briefly, 96-well ELISA plates (Nalge Nunc International, Naperville, IL, USA) were coated with 100 μl/well of DT, TT or PT antigen (1·6 Lf or 1 Lf or 0·6 μg/ml, respectively) diluted in coating buffer (0·01 M sodium phosphate/0·14 M sodium chloride, pH 7·4 for DT and TT antigen or 0·05 M sodium carbonate, pH 9·6 for PT antigen) and incubated for 16–18 h at 37°C. The plates were washed [1 × phosphate-buffered saline (PBS)/0.1% Tween-20] and blocked with 1 × PBS/1% gelatine for 1 h at room temperature. After five washes, 100 μl of serum was added at a starting dilution of 1 : 50 to plates containing 1 × PBS/0·05% Tween-20/0.1% gelatine for DT and TT assays and 1 × PBS/0.5% bovine serum albumin (BSA)/0·05% Tween for PT assays. Reference standards were calibrated to NIBSC 00/496 (DT), TE-3 (TT) and lot3 (PT) were also added to the plate. The mixture was incubated at room temperature for 1 h followed by the addition of 100 μl of goat anti-human IgG antibody conjugated with alkaline phosphatase (Invitrogen, Carlsbad, CA, USA). The plates were added with the secondary antibody at room temperature for 1 h, followed by the addition of 100 μl of substrate solution (1 mg of p-nitrophenyl phosphate/ml in 1 M diethanolamine, pH 9·8, containing 1 mM $MgCl_2$). The plates were incubated for 1 h at room temperature and the reaction was stopped by the addition of 50 μl of 3N NaOH. The plates were read on an automated plate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm with a 630 nm reference filter. Sample antibody titres were calculated relative to the endpoint titre with respective reference curve for each assay. An in-house positive control serum (mixture of human sera) was run on each plate to control for plate-to-plate variability. The levels of IgG in the reference serum were measured quantitatively using a four-parameter method (Molecular Devices, Soft MaxPro, Sunnyvale, CA, USA).

Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by differential centrifugation on Ficoll-Isopaque (Pharmacia, Piscataway, NJ, USA) and sera were stored at −80°C for further use in immunological assays. PBMCs were washed in phosphate-buffered saline (PBS) suspended at a concentration of 10^7 cells/ml in cell recovery freezing media (Gibco, Grand Island, NY, USA) and frozen in liquid nitrogen until used.

Labelling vaccine antigens with a fluorochrome

Purified DT, TT and PT proteins obtained from Sanofi Pasteur (Swiftwater, PA, USA) were conjugated with fluorescein isothiocyanate (FITC), according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Briefly, the protein solution suspended in 1 M sodium bicarbonate solution was incubated with reactive FITC dye (10 mg/ml) at a molar ratio of 40 times the dye compared to protein concentration for 1 h at room temperature. The mixture was centrifuged at 1100 ***g*** for 3 min and the supernatant was purified over spin columns, according to the manufacturer's instructions. The labelling efficiency was checked by measuring the absorbance at a wavelength of 280 and 494 nm.

Phenotypical analysis of B cell subsets

Before the assay, frozen PBMCs were thawed rapidly in a 37°C water bath, washed and suspended in warm complete media [RPMI-1640 plus L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 000 IU/ml each of penicillin and streptomycin, and 50 μM β-mercaptoethanol (all media reagents from Invitrogen)]. To determine the proportion of total B cells and memory B cells in the frozen lymphocyte samples, the cell culture was washed with 1 × PBS and incubated with Fc receptor blocking solution and live–dead fixable aqua dead cell stain (Life Technologies, Carlsbad, CA, USA) for 20 min at 4°C. The cell culture was further stained with cell surface antibodies for 20 min in the dark at 4°C. The cell surface antibodies include mouse monoclonal antibodies (mAbs) specific for human: anti-CD19 Brilliant Violent 605 (clone HIB19) and phycoerythrin (PE)-conjugated anti-CD27 (clone M-T271). Labelled cells were fixed with 2% paraformaldehyde at room temperature for 10 min, acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) with doublet discrimination, and analysed using FlowJo software (version 10·0·7; TreeStar Inc., Ashland, OR, USA).

PBMC stimulation

Following earlier published protocol, PBMCs were plated in 96-well U-bottomed plates (Corning Life Sciences, Corning, NY, USA) at 10^6 cells/ml in complete media with 3 μg/ml deoxycytidyl-deoxyguanosine–oligodeoxynucleotide (CpG-ODN) 2006 (Invivogen, San Diego, CA, USA) in the presence of 10 ng/ml interleukin (IL)-2, IL-10, IL-15 and 100 ng/ml IL-21 (Cell Sciences, Canton, MA, USA) and cultured for 5 days at 37°C in a 5% CO_2 humidified incubator [17]. The cultured cells were harvested by centrifugation at 330 ***g***, washed with 1 × PBS and incubated with Fc receptor blocking solution, live–dead fixable aqua dead cell stain and cell surface antibodies, as described above. To determine the frequency of DTaP vaccine antigen-specific memory B cells in the cell cultures of sOP and NOP children, the cell suspension was incubated with a mixture of FITC-labelled proteins and cell surface antibodies for 20 min in the dark at 4°C. PBMCs from selected samples were added with FITC fluorochrome or unlabelled antigen in separate wells to serve as a negative control. Labelled cells were fixed and assessed using a flow cytometer, as described above.

Statistics

All data were analysed using Graph Pad Prism software (version 6·02). Fisher's exact test was used to compare the clinical characteristics of two cohorts of children. Two-tailed *P*-values for the data were calculated using the Mann–Whitney *U*-test and correlations were analysed by Spearman's rank test. The statistical level of significance was defined as $P < 0{\cdot}05$.

Results

Study population

From the study populations, age-matched samples were used for comparative analysis from 15 children who were sOP and 20 children who were NOP. The clinical characteristics of these children are shown in Table 1.

sOP children have lower antibody titres than NOP children

All sOP and NOP plasma samples were tested for serological IgG levels to DT, TT and PT proteins (Fig. 1). We found sOP children had significantly lower IgG antibodies to DT ($P = 0{\cdot}03$), TT ($P = 0{\cdot}001$) and PT ($P = 0{\cdot}0002$) compared to NOP children. A greater percentage of sOP *versus* NOP subjects showed undetectable antibody titres to DT (33 *versus* 13%), TT (47 *versus* 13%) and PT (33 *versus* 0%) antigens (Table 2).

Phenotypical analysis of B cell subsets

In a preliminary study, flow cytometric analysis of freshly thawed PBMC cultures of five young children (mean age 12 months) showed a minimal frequency of $CD19^+$ B cells (mean 1·68%), $CD19^+CD27^+$ B cells (mean 0·44%), among which the antigen-specific B cells were imperceptible. To be able to detect the DTaP vaccine antigen-specific memory B cells, we expanded the PBMC cultures with a Toll-like receptor (TLR)-9 agonist CpG-ODN 2006 for 5 days in the presence of a mixture of different cytokines. Stimulation of cell cultures with CpG and cytokines for 5 days resulted in an $8{\cdot}4 \pm 2{\cdot}7$-fold proliferation of $CD19^+$ B cells and an $8{\cdot}2 \pm 3{\cdot}1$-fold increase in $CD19^+CD27^+$ memory B, cells as shown in a representative plot from a single sample (Fig. 2). Further addition of antigen in culture or incubation after 5 days did not result in an additional increase in B cell proliferation (data not shown).

Table 1. Clinical characteristics of stringent otitis-prone (sOP) and non-otitis-prone (NOP) children involved in the study.

Characteristic	sOP (n = 15)	NOP (n = 20)	*P*-value
Sex			
Male	11	10	0·29
Female	4	10	0·29
Mean age (months)	10.8	10.1	0·24
AOM episodes (no.)			
>3 in 6 months	11	0	<0·0001
>4 in 12 months	4	0	0·03
Atopy	3	6	0·70
Breast feeding	3	1	0·29

P-values were calculated using Fisher's exact test.

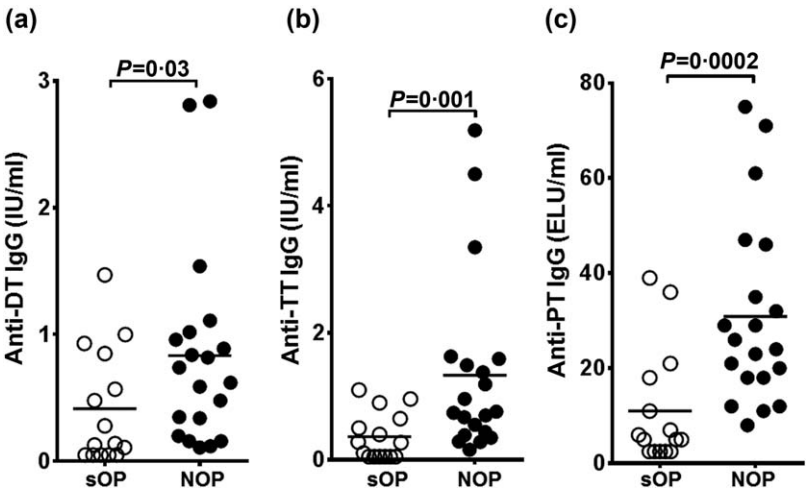

Fig. 1. Serological antigen-specific immunoglobulin (Ig)G responses after three doses of acellular DTaP vaccine in stringent otitis-prone (sOP) ($n = 15$) and non-otitis-prone (NOP) ($n = 20$) children. sOP children showed a significantly lower concentration of IgG antibodies to (a) DT (diphtheria toxoid), (b) TT (tetanus toxoid) and (c) PT (pertussis toxoid) compared to NOP children. Bars represent geometric mean values. DT and TT antibody titres were measured as international units (IU) and PT-specific antibodies were measured as enzyme-linked immunosorbent assay (ELISA units) (ELU). *P*-values were calculated using the Mann–Whitney *U*-test.

sOP children have reduced a number of memory B cells

PBMC cultures of sOP and NOP children stimulated with CpG-ODN 2006 and cytokines for 5 days were used to measure the frequencies of B cell subsets. A schematic of the B cell subsets as determined by flow cytometric analysis is shown in Fig. 3. We found the proportion of $CD19^+$ B cells in the lymphocyte population was comparable in both groups of children, with a mean percentage of $11{\cdot}8 \pm 2{\cdot}18$ in sOP children and $12{\cdot}2 \pm 1{\cdot}62$ in NOP children (Fig. 4a). Further, within the B cell population the percentage of $CD19^+CD27^+$ memory B cells was significantly higher in NOP children ($P = 0{\cdot}008$), with a mean of $12{\cdot}20 \pm 1{\cdot}62$ when compared to sOP children, mean $3{\cdot}38 \pm 0{\cdot}50$ (Fig. 4b). The frequencies of DT, TT or PT-specific memory B cells in sOP children were relatively lower in sOP children compared to NOP children ($P = 0{\cdot}37$, $P = 0{\cdot}027$, $P = 0{\cdot}015$, respectively), as shown in Fig. 5.

Correlations between antigen-specific memory B cells and circulating IgG antibody titres

The circulating antigen-specific IgG titres were analysed for their correlation with frequency of corresponding antigen-specific memory B cells. A significant correlation between IgG levels and memory B cell frequencies was found for DT, TT and PT proteins in both NOP and sOP children (Fig. 6a–c). Assessing all the data in response to DT, TT or PT proteins from all subjects collectively, the highest correlation was found for DT, followed by TT and PT in both groups of children.

Discussion

In the present study we examined the magnitude of memory B cell responses to DTaP vaccine antigens and correlated antigen-specific memory B cell responses with IgG antibody levels in sOP and NOP children. We found that sOP children showed a significantly lower percentage of antigen-specific $CD19^+CD27^+$ memory B cells than NOP children. We also found a weak but linear correlation between the frequencies of memory B cells and circulating IgG titres for DT, TT and PT proteins. This is the first study to show significant differences in memory B cell responses to DTaP vaccine antigens and their correlation with the circulating antibodies in young children with recurrent AOM.

In our previous studies, we showed that sOP children have significantly lower serum and mucosal antibody titres to *Spn* and *NTHi* proteins compared to age-matched NOP children [15,18]. We also found recently that sOP children have a deficiency in functional $CD4^+$ T cell responses and experience recurrent AOM episodes with higher respiratory viral burden [10,16]. Further, our studies have

Table 2. Geometric mean concentrations (GMC) and 95% confidence interval (CI) of the serological immunoglobulin (Ig)G responses to DTaP vaccine antigens; DT (diphtheria toxoid), TT (tetanus toxoid) and PT (pertussis toxoid) in stringent otitis-prone (sOP) ($n = 15$) and non-otitis-prone (NOP) ($n = 20$) children.

DTaP antigen	DT		TT		PT	
Subjects	sOP ($n = 15$)	NOP ($n = 20$)	sOP ($n = 15$)	NOP ($n = 20$)	sOP ($n = 15$)	NOP ($n = 20$)
GMC	0·21	0·56	0·19	0·86	6·79	25·70
(95% CI)	(0·10–0·42)	(0·35–0·87)	(0·09–0·38)	(0·56–1·34)	(3·93–11·7)	(19·2–34·5)
Median	0·14	0·68	0·27	0·75	5·00	25·00
(range)	(0·05–1·47)	(0·11–2·84)	(0·05–1·10)	(0·16–5·19)	(2·5–39·0)	(8·0–75·0)
% of subjects with undetectable IgG	0(0/20)	0(0/20)	0(0/20)	0(0/20)	0(0/20)	0(0/20)

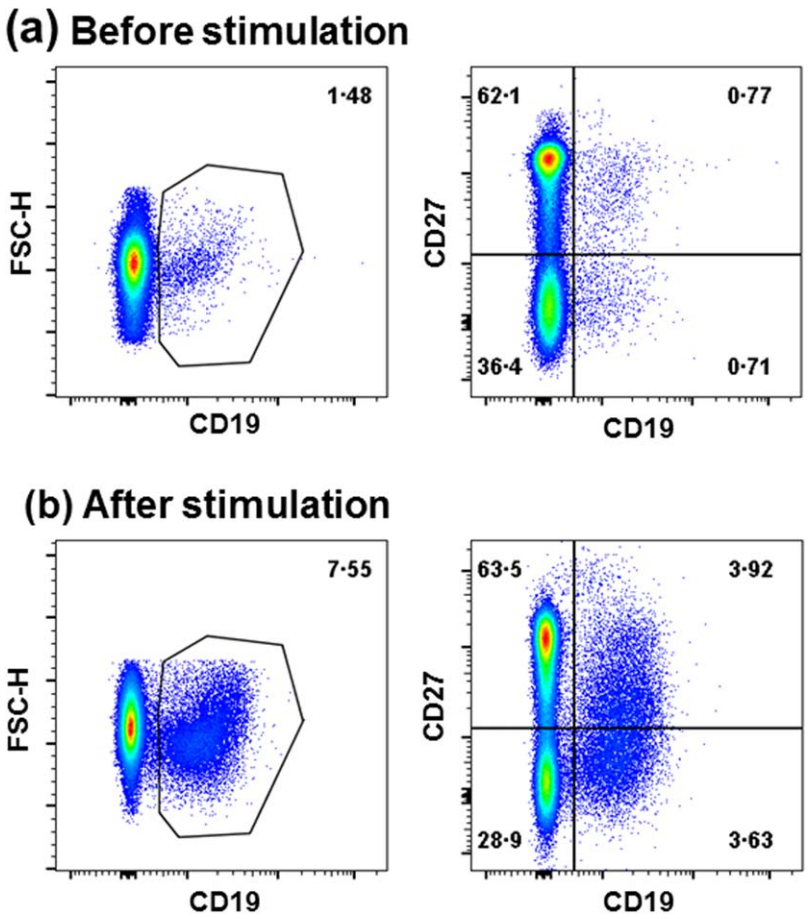

Fig. 2. The percentage frequencies of B cells (out of total lymphocytes) with surface staining of CD19 and CD27 markers (a) before and (b) 5 days after stimulation with deoxycytidyl-deoxyguanosine–oligodeoxynucleotide (CpG-ODN) 2006, interleukin (IL)-2, IL-10, IL-15 and IL-21.

demonstrated that sOP children have a dysfunctional immune system, as evidenced by failure to generate antibody responses to multiple routine paediatric vaccinations, indicating that this population may be exceptionally vulnerable to vaccine-preventable infections [19,20]. The poor antibody responses observed in this study to DT, TT and PT proteins in sOP children supports our central hypothesis that these children have an immunological deficiency in antibody-producing cells. Previous studies in the past 25 years reported mixed results on the vaccine antibody titres of OP and non-OP children [21,22]. Prellner *et al.* found lower antibody responses to rubella vaccine antigens but

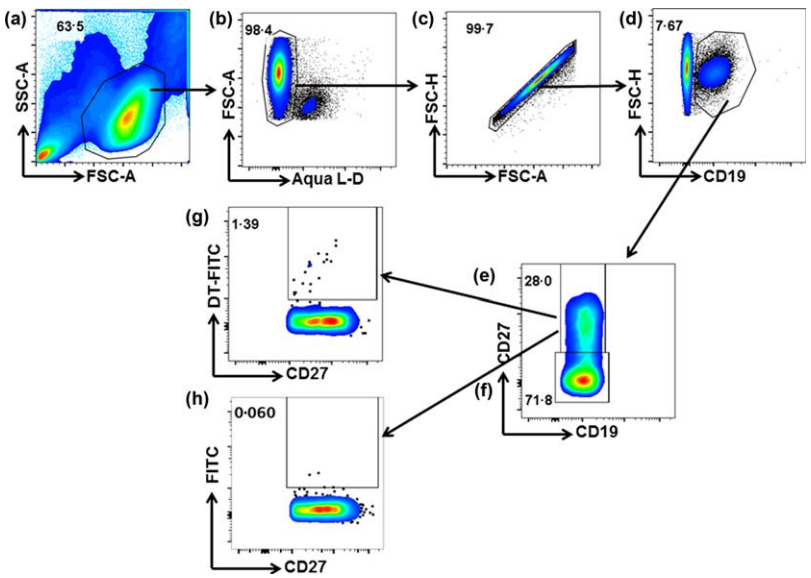

Fig. 3. Gating strategy for enumerating antigen-specific memory B cells among stringent otitis-prone (sOP) and non-otitis-prone (NOP) children. The dead cell debris and clumps were excluded first and gated as total selected lymphocytes (a), followed by gating on live cells (b) and singlet cells (c) based on their forward- and side-scatter properties. Total B cells (d), memory B cells (e) and naive B cells (f) were gated based on the expression of $CD19^+$ $CD27^+$. The antigen-specific memory B cells (g) were gated based on the antigen-bound fluorochrome-fluorescein isothiocyanate [Ag-$FITC^+$ conjugated to diphtheria toxoid (DT), tetanus toxoid (TT) or pertussis toxoid (PT)]. A negative control without antigen-labelled free FITC molecules was added to check the background (h). Preliminarily, the gating was standardized and compared with all antibody fluorescence minus one (FMO) controls.

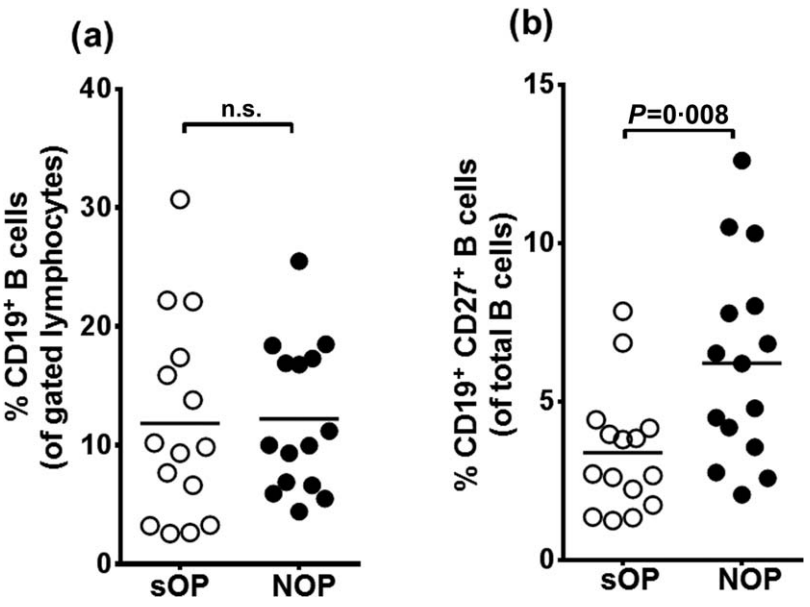

Fig. 4. The percentage frequencies of B cell subsets in peripheral blood mononuclear cell (PBMC) cultures of stringent otitis-prone (sOP) and non-otitis-prone (NOP) children after stimulation with deoxycytidyl-deoxyguanosine–oligodeoxynucleotide (CpG-ODN) 2006 (3 μg/ml), interleukin (IL)-2, IL-10 and IL-15 (10 ng/ml) and IL-21 (100 ng/ml). The percentage of (a) total B cells (of gated lymphocyte population) and (b) memory B cells (of gated B cell population) in sOP ($n = 15$) and NOP ($n = 20$) children. Bars represent mean values. *P*-values were calculated using the Mann–Whitney *U*-test.

not to tetanus and diphtheria antigens in OP children [21], while Wiertsma *et al.* found no difference in antibody levels against diphtheria, tetanus and *Haemophilus influenzae* type b (Hib) protein in OP and non-OP children [22]. The latter studies are not directly comparable to ours, because they defined otitis-prone as any child who experienced at least two AOM episodes in a given year [22]. Our study rests on a rigorous definition of OP, requiring that the child experience at least three episodes of AOM within a single 6-month period, or at least four within a year, and all cases must be confirmed by microbiological culture of middle ear fluid obtained by tympanocentesis [19].

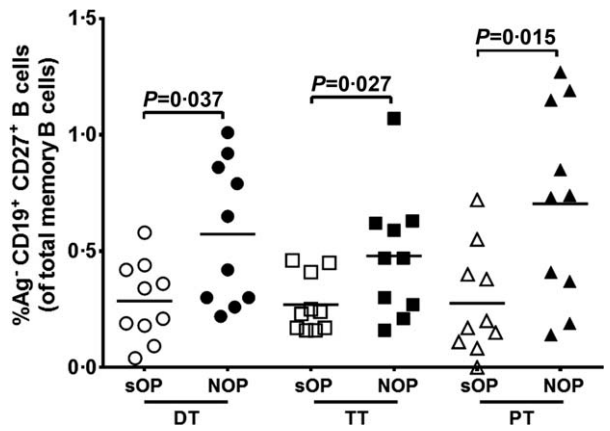

Fig. 5. The percentage frequencies of antigen-specific memory B cells (of gated B cell population) in cell cultures after 5 days of stimulation of lymphocytes with deoxycytidyl-deoxyguanosine–oligodeoxynucleotide (CpG-ODN) 2006 (3 μg/ml), interleukin (IL)-2, IL-10 and IL-15 (10 ng/ml) and IL-21(100 ng/ml) in stringent otitis-prone (sOP) ($n = 10$) and non-otitis-prone (NOP) ($n = 10$) children. Bars represent mean values. *P*-values were calculated using the Mann–Whitney *U*-test.

In our preliminary experiments using freshly thawed PBMCs from young children (mean age 12 months), we observed a very low frequency of $CD19^+$ B cells (mean 1.68%) with minimal frequency of $CD19^+$ $CD27^+$ B cells and imperceptible antigen-specific memory B cells. Several earlier reports have shown a variety of methodologies to characterize the B cell phenotypes by prolonged stimulation of the B cell receptor (BCR) or CD40 engagement and used a cocktail of B cell activating cytokines to determine the frequency of antigen-specific memory B cells [15,23–26]. However, prolonged B cell activation using CD40 ligands influences cell viability and may cause inconsistent clonal distribution. Bernasconi *et al.* showed that in the absence of antigen, memory B cells proliferate and differentiate into antibody-secreting cells in response to TLR agonists, while naive B cells require BCR ligand stimulation [27]. Further, it was also demonstrated that the TLR-9 receptor was highly up-regulated in memory B-cells [15,27]. In accordance with earlier reports, we observed a five to 10-fold proliferation of the total B cells and memory B cells after 5 days of incubation [15,28]. Buisman *et al.* demonstrated a similar number of antigen-specific memory B cells with both cryopreserved cells and freshly isolated cells and detected a comparable number of IgG antibody-secreting cells (ASC) [15]. Using enzyme-linked immunospot (ELISPOT) assay, enrichment of B cells prior to stimulation increased the sensitivity of antigen-specific memory B cell detection [15]. However, enriching pure culture requires a higher volume of venous blood or a higher number of PBMCs. Due to the limitation of sample numbers and the minimal frequency of these cells in the collected small amounts of venous blood from young children with the sOP or NOP condition, we could not enrich the B cells or perform both flow cytometry and ELISPOT analysis on the same sample; instead, we cultured PBMCs

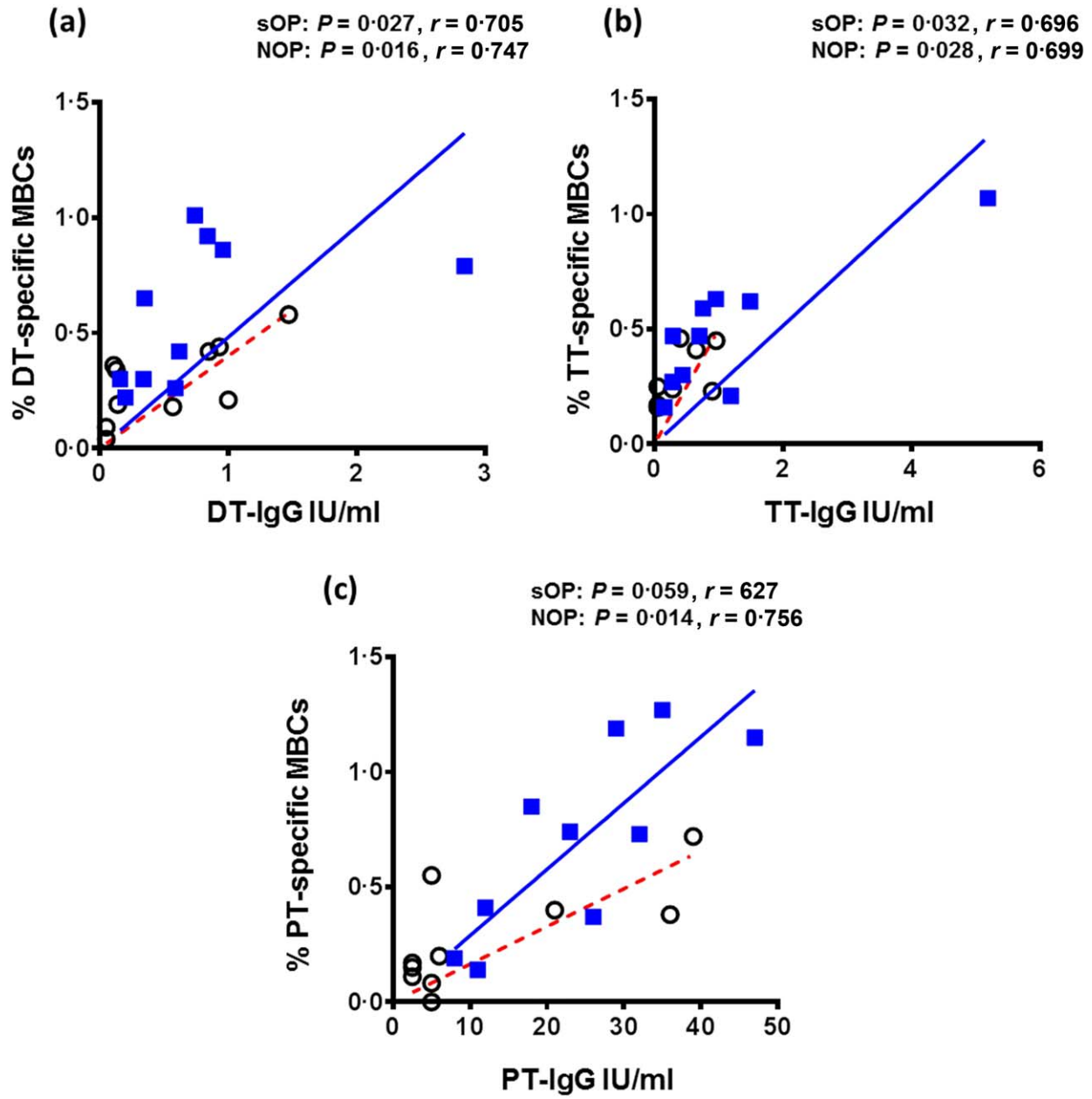

Fig. 6. Correlations between numbers of antigen-specific memory B cells (MBCs, *y*-axis) and serum immunoglobulin (Ig)G levels (*x*-axis) in stringent otitis-prone (sOP) (open red-coloured circles, $n = 10$) and non-otitis-prone (NOP) children (closed blue-coloured squares, $n = 10$). Non-parametric Spearman's analysis indicated a significant correlation between MBCs and serum IgG levels to (a) DT (diphtheria toxoid), (b) TT (tetanus toxoid) and (c) PT (pertussis toxoid) proteins.

and stimulated polyclonally in the presence of cytokines. Despite a more than eightfold expansion of $CD19^+/CD27^+$ cells upon polyclonal stimulation with CpG we were able to identify a minimal frequency of DTaP vaccine antigen-specific memory B cells. We used flow cytometry to detect the fluorescently labelled antigen-binding cells to determine the percentages of antigen-specific memory B cells from the peripheral blood of sOP and NOP children. Leggat *et al.* showed that fluorescently labelled polysaccharide antigens identified antigen-specific memory B cells in response to pneumococcal polysaccharide vaccination [29]. Amanna *et al.* demonstrated the analysis of tetanus and diphtheria protein-specific $CD27^+$ memory B cells using fluorescently labelled protein antigens [30]. Previously, we demonstrated a suboptimal pneumococcal antigen-specific memory B cell responses in a different subset of children in our prospective, longitudinal study and showed that sOP children have poor generation of pneumococcal protein antigen-specific memory B cells [31]. Earlier reports have demonstrated that antigen-specific memory B cells persist

years after vaccination and correlate with humoral immunity [25,32–34]. Our results are in agreement with previous published data, indicating a low but statistically significant linear correlation between the frequencies of memory B cells and IgG antibodies to the vaccine antigens studied. However, the percentage of naive B cells responding to polyclonal stimulation was not statistically different between sOP and NOP children (data not shown).

The overall results of our experiments suggest that, in response to polyclonal stimulation, the B cell population in sOP children has a mechanistic dysfunction, interfering with proliferation or differentiation into antibody-secreting cells. We conclude that sOP children have defective immune responses to vaccine antigens administered parenterally. These sOP children with low vaccine-induced immunity probably do not contract vaccine-preventable infections because they are protected by herd immunity, and it is a great concern if loss of herd immunity occurs. Whether the naive B cells in the secondary lymphoid organs of sOP children are unable to process the BCR signals after vaccine antigen stimulation or fail to getain optimal T follicular cell help for the expression of CD27 receptor to differentiate into memory B cells is now an area of active investigation in our laboratory.

Acknowledgements

This study was supported by NIH NIDCD RO1 08671. We thank Dr Janet R. Casey, the nurses and staff of Legacy Pediatrics, the collaborating pediatricians, the parents who consented and the children who participated in this study. We also thank Dr Anthony Almudevar (Department of Biostatistics and Computational Biology, University of Rochester Medical Center) for data statistical analysis and Jill Mangiafesto for assistant in ELISA assays. Dr Robert Zagursky, Rochester General Hospital Research Institute, provided useful discussion in preparation of this manuscript. The authors were supported by the National Institutes of Health R0108671.

Disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

References

1 Pichichero ME. Otitis media. Pediatr Clin North Am 2013; **60**: 391–407.
2 Pichichero ME, Casey JR. Otitis media. Expert Opin Pharmacother 2002; **3**:1073–90.
3 Thompson D, Oster G, McGarry LJ, Klein JO. Management of otitis media among children in a large health insurance plan. Pediatr Infect Dis J 1999; **18**:239–44.
4 Alho OP, Laara E, Oja H. What is the natural history of recurrent acute otitis media in infancy? J Fam Pract 1996; **43**:258–64.
5 Poehling KA, Szilagyi PG, Grijalva CG *et al.* Reduction of frequent otitis media and pressure-equalizing tube insertions in children after introduction of pneumococcal conjugate vaccine. Pediatrics 2007; **119**:707–15.
6 Casey JR, Kaur R, Friedel VC, Pichichero ME. Acute otitis media otopathogens during 2008 to 2010 in Rochester, New York. Pediatr Infect Dis J 2013; **32**:805–9.
7 Casey JR, Adlowitz DG, Pichichero ME. New patterns in the otopathogens causing acute otitis media six to eight years after introduction of pneumococcal conjugate vaccine. Pediatr Infect Dis J 2010; **29**:304–9.
8 Decherd ME, Deskin RW, Rowen JL, Brindley MB. Bordetella pertussis causing otitis media: a case report. Laryngoscope 2003; **113**:226–7.
9 Stockmann C, Ampofo K, Hersh AL *et al.* Seasonality of acute otitis media and the role of respiratory viral activity in children. Pediatr Infect Dis J 2013; **32**:314–9.
10 Verhoeven D, Xu Q, Pichichero ME. Differential impact of respiratory syncytial virus and parainfluenza virus on the frequency of acute otitis media is explained by lower adaptive and innate immune responses in otitis-prone children. Clin Infect Dis 2014; **59**:376–83.
11 Khan MN, Sharma SK, Filkins LM, Pichichero ME. PcpA of *Streptococcus pneumoniae* mediates adherence to nasopharyngeal and lung epithelial cells and elicits functional antibodies in humans. Microbes Infect 2012; **14**:1102–10.
12 Khan MN, Pichichero ME. Vaccine candidates PhtD and PhtE of *Streptococcus pneumoniae* are adhesins that elicit functional antibodies in humans. Vaccine 2012; **30**:2900–7.
13 Liko J, Robison SG, Cieslak PR. Pertussis vaccine performance in an epidemic year – Oregon, 2012. Clin Infect Dis 2014; **59**: 261–3.
14 Winter K, Glaser C, Watt J, Harriman K. Pertussis epidemic – California, 2014. Morb Mortal Wkly Rep 2014; **63**:1129–32.
15 Sharma SK, Casey JR, Pichichero ME. Reduced serum IgG responses to pneumococcal antigens in otitis-prone children may be due to poor memory B-cell generation. J Infect Dis 2012; **205**:1225–9.
16 Sharma SK, Pichichero ME. Functional deficits of pertussis-specific $CD4^+$ T cells in infants compared to adults following DTaP vaccination. Clin Exp Immunol 2012; **169**:281–91.
17 Buisman AM, de Rond CG, Ozturk K, Ten Hulscher HI, van Binnendijk RS. Long-term presence of memory B-cells specific for different vaccine components. Vaccine 2009; **28**:179–86.
18 Kaur R, Casey JR, Pichichero ME. Serum antibody response to five *Streptococcus pneumoniae* proteins during acute otitis media in otitis-prone and non-otitis-prone children. Pediatr Infect Dis J 2011; **30**:645–50.
19 Pichichero ME, Casey JR, Almudevar A. Nonprotective responses to pediatric vaccines occur in children who are otitis prone. Pediatr Infect Dis J 2013; **32**:1163–8.
20 Pichichero ME, Kaur R, Casey JR, Xu Q, Almudevar A, Ochs M. Antibody response to *Streptococcus pneumoniae* proteins PhtD, LytB, PcpA, PhtE and Ply after nasopharyngeal

colonization and acute otitis media in children. Hum Vaccin immunother 2012; **8**:799–805.
21 Prellner K, Harsten G, Lofgren B, Christenson B, Heldrup J. Responses to rubella, tetanus, and diphtheria vaccines in otitis-prone and non-otitis-prone children. Ann Otol Rhinol Laryngol 1990; **99**:628–32.
22 Wiertsema SP, Sanders EA, Veenhoven RH *et al.* Antibody levels after regular childhood vaccinations in the immunological screening of children with recurrent otitis media. J Clin Immunol 2004; **24**:354–60.
23 Karahan GE, Eikmans M, Anholts JD, Claas FH, Heidt S. Polyclonal B cell activation for accurate analysis of pre-existing antigen-specific memory B cells. Clin Exp Immunol 2014; **177**:333–40.
24 Hendrikx LH, Ozturk K, de Rond LG *et al.* Identifying long-term memory B-cells in vaccinated children despite waning antibody levels specific for Bordetella pertussis proteins. Vaccine 2011; **29**:1431–7.
25 Crotty S, Ahmed R. Immunological memory in humans. Semin Immunol 2004; **16**:197–203.
26 Cao Y, Gordic M, Kobold S *et al.* An optimized assay for the enumeration of antigen-specific memory B cells in different compartments of the human body. J Immunol Methods 2010; **358**:56–65.
27 Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003; **101**:4500–4.
28 Rojas OL, Narvaez CF, Greenberg HB, Angel J, Franco MA. Characterization of rotavirus specific B cells and their relation with serological memory. Virology 2008; **380**:234–42.
29 Leggat DJ, Khaskhely NM, Iyer AS *et al.* Pneumococcal polysaccharide vaccination induces polysaccharide-specific B cells in adult peripheral blood expressing CD19(+)CD20(+)CD3(-)CD70(-)CD27(+)IgM(+)CD43(+)CD5(+)/(-). Vaccine 2013; **31**:4632–40.
30 Amanna IJ, Slifka MK. Quantitation of rare memory B cell populations by two independent and complementary approaches. J Immunol Methods 2006; **317**:175–85.
31 Sharma SK, Casey JR, Pichichero ME. Reduced memory CD4+ T-cell generation in the circulation of young children may contribute to the otitis-prone condition. J Infect Dis 2011; **204**:645–53.
32 Reuveny S, White MD, Adar YY *et al.* Search for correlates of protective immunity conferred by anthrax vaccine. Infect Immun 2001; **69**:2888–93.
33 Bonsignori M, Moody MA, Parks RJ *et al.* HIV-1 envelope induces memory B cell responses that correlate with plasma antibody levels after envelope gp120 protein vaccination or HIV-1 infection. J Immunol 2009; **183**:2708–17.
34 Crompton PD, Mircetic M, Weiss G *et al.* The TLR9 ligand CpG promotes the acquisition of Plasmodium falciparum-specific memory B cells in malaria-naive individuals. J Immunol 2009; **182**:3318–26.