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Generation of switched memory B cells in response to vaccination in Down syndrome children and their siblings

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ABSTRACT

Background: Immunodeficiency is an integral aspect of Down syndrome, as demonstrated by the increased susceptibility to infection of affected. Mortality is still higher than in general population, with respiratory infections among the major causes of death. As more people with Down syndrome are living today than ever before, it is indispensable to develop strategies to prevent and cure the associated disorders. Vaccination is the most successful instrument of preventive medicine. Special seasonal influenza and pneumococcal vaccination strategies have been designed for individuals with risk conditions of all ages. Down syndrome individuals are not included in the high-risk categories.
Methods: We enrolled in our study 15 children with Down syndrome and their siblings, vaccinated for the first time with seasonal influenza vaccine and receiving a booster dose of a glyco-conjugated pneumococcal vaccine. We compared the immunological features and response to vaccination measuring serum antibody titers and frequency of specific memory B cells.
Results: We confirm that a severe reduction of switched memory B cells is always associated to Down syndrome. After primary vaccination Down syndrome children generate significantly less specific switched memory B cells than their siblings. The response to a booster dose of vaccine is instead comparable in both groups. The production of specific antibodies was equally effective in Down syndrome and controls both after primary and secondary immunization.
Conclusions: Down syndrome individuals should be considered a high risk group, because of their increased susceptibility to infection and reduced number of switched memory B cells. Tailored vaccination protocols are needed in order to reduce their burden of infections throughout life.

1. Introduction

Down syndrome (DS) is the most frequent genetic condition occurring in 1/600 to 1/1000 live birth. Improved medical care and the successful treatment of congenital heart defects have led to an extraordinary increase of life expectancy, from 10 years in 1960 to 60–65 years today [1]. As more people with DS are living today than ever before, novel strategies to prevent and cure the associated disorders are needed. Mortality in DS is still higher than in general population, with respiratory infections (RI) among the major causes of death. Viruses such as influenza virus, respiratory syncytial virus and parainfluenza viruses are frequently responsible for RI [2,3] whereas *Streptococcus pneumoniae* is the major cause of pneumonia [4] in this group of patients. A study has demonstrated that during the outbreak of the pandemic influenza A (H1N1) 2009 virus in Mexico, likelihoods of hospitalization, intubation, and death were respectively 16-fold, 8-fold, and 335-fold greater for DS than non-DS infected individuals [5]. Although anatomic and functional ear–nose–throat abnormalities [6] may favor microbial colonization in DS, the alterations of the immune response [7–14] play an important role in the development and evolution of infections. It has been suggested that the increased incidence of respiratory

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infections and autoimmune disorders in DS is reminiscent of common variable immunodeficiency [15].

Vaccination is the most successful instrument of preventive medicine. Special seasonal influenza and pneumococcal vaccination strategies have been designed for individuals with risk conditions of all ages [16–18]. DS individuals are not included in the high-risk categories [19,20].

Vaccines prevent infectious diseases by inducing the adaptive immune response to deliver two final products: long-lived plasma cells and switched memory B cells. Whereas long-lived plasma cells constantly secrete antibodies thus maintaining a stable level of pre-formed specific immunoglobulins in the serum, switched memory are able to sense antigen and react to a secondary challenge by rapidly proliferating and producing antibodies [21]. In humans, along with switched memory B cells, another type of memory B cells, called IgM memory, ensure the first-line defense against infection by producing natural antibodies [22]. Maintenance of memory B cells occurs through re-stimulation either by re-encounter with antigen or through TLR9 engagement [23,24].

We [25] and others [13,26] have recently shown that DS is associated with a primary defect of the B-cell compartment, characterized by a reduced number of all B-cell populations in the peripheral blood and especially of switched memory B cells. We have also shown that memory B cells of DS children respond to TLR9 with increased proliferation and differentiation [25].

The low number of switched memory B cells in DS could be explained by their insufficient production by the germinal center or by their rapid exhaustion upon TLR9 engagement. The identification of the mechanisms responsible for the low number of switched memory B cells is a pre-requisite for the development of tailored vaccine protocols for the increasing DS populations.

The aim of our study was to investigate the ability of DS children to generate switched memory B cells in response to vaccination.

2. Materials and methods

2.1. Study population

The study protocols and consent form were approved by the Ethical Committee of Ospedale Pediatrico Bambino Gesù, Rome. Written informed consent was obtained from the families of the persons included in this study according to the principles expressed in the Declaration of Helsinki.

We enrolled in the study 15 children with DS and 15 controls matched for age (±12 months) and sex. In order to minimize the variables caused by environmental exposure to pathogens and genetic factors other than trisomy 21, siblings of DS children were recruited as controls.

Inclusion criteria for DS children were: diagnosis of DS proven by chromosome analysis, age between 3 and 12 years and no symptoms of infection at the time of blood sampling. Sibling of DS children enrolled as controls had a similar age and no signs of infection.

Exclusion criteria for controls and DS children were: evidence of malignancy, chemotherapy, post-chemotherapy and immunosuppressive treatment; contraindications to split influenza vaccine or pneumococcal vaccine administration.

The clinical history of the patients was obtained from medical records and interviews by the physician. A standard questionnaire for the occurrence of infections and any related hospitalization was also administered. The medical records of all participants were reviewed for frequency of infections, presence of congenital heart disease, previous heart surgery, occurrence of autoimmune diseases and hematologic disorders.

Table 1
Clinical and demographic features of Down syndrome (DS) patients and controls.

	DS patients *N* (%)	Controls *N* (%)
Total	15 (50.00)	15 (50.00)
Male	8 (53.33)	8 (53.33)
Female	7 (43.75)	7 (43.75)
Age (median) ys	6.58	7.75
Recurrent infections total	15/15 (100.00)	4/15 (26.66)
Infections of the superior respiratory tract	14/15 (93.33)	2/15 (13.33)
Pneumonia	7/15 (46.66)	2/15 (13.33)
Gastroenteritis	0/15 (0.00)	0/15 (0.00)
Urinary tract infections	0/15 (0.00)	1/15 (6.66)
Hospitalization for infections	8/15 (53.0)	1/15 (6.66)
Hospitalization for gastroenteritis	1/15 (6.66)	1/15 (6.66)
Hospitalization because of lower respiratory tract infection	7/15 (46.66)	0/15 (0.00)
Hospitalization because of upper respiratory tract infection	0/15 (0.00)	0/15 (0.00)
Congenital heart disease	10/15 (66.66)	1/15 (6.66)
Heart surgery	5/15 (33.33)	1/15 (6.66)
Thymectomy	1/15 (6.66)	0/15 (0.00)
Autoimmune diseases	3/15 (20.00)	0/15 (0.00)
Thyroiditis	3/15 (20.00)	0/15 (0.00)
Hematologic problems	2/15 (13.33)	0/15 (0.00)
Thrombocytopenia	1/15 (6.66)	0/15 (0.00)
Anemia	1/15 (6.66)	0/15 (0.00)

All DS patients and their siblings underwent venipuncture and 5 ml EDTA-venous blood were collected for baseline immunological evaluation (cell blood count, IgA, IgG and IgM concentration, salivary IgA, T and B lymphocyte subsets) and baseline influenza and pneumococcal antibody titers.

The response to a first vaccine dose was evaluated after a single injection of the seasonal influenza vaccine (Inflexal V® (Crucell) containing surface antigens of A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2) and B/Massachusetts/2/2012 virus strains). Administration of a second vaccine dose was recommended for all children, but it was impossible to obtain the consent for a second blood withdrawal.

The recall response was measured after a booster dose of anti-pneumococcal glyco-conjugated vaccine (Prevenar 13-valent®, Pfizer). We calculated the frequency of specific memory B cells and the concentration of specific serum antibodies before vaccination and two weeks after.

2.2. Demographic characteristics and clinical history

The median age of DS children included in this study was 6.58 years (range 3.66–11), and the median age of the controls was 7.75 years (range 3.33–12.83). The sex distribution was the same between DS children and controls (8 males and 7 females).

Table 1 summarizes the clinical findings in DS children and controls. In particular all DS children had recurrent infections (100%) more frequently of the upper respiratory tract (93.3%; 14/15) and 46.6% (7/15) of them suffered from recurrent pneumonia. Furthermore, 8 of the 15 DS children included in the study (53%) had required hospitalization because of infection. Ten DS patients had CHD (66.6%). Half of them had undergone heart surgery, because of atrioventricular canal (two children) and ventricular septal defect (three children). Only one child had been thymectomized. Patients who did not require surgery had patent forame ovale (one child), atrial septal defect (two children), ventricular septal defect (two children). All DS children with CHD were hemodynamically stable and did not need any therapy at the time of enrollment and during the study.

Three patients (20%) had autoimmune thyroiditis. Two DS children had hematologic disorders: one child had thrombocytopenia and one had iron deficiency anemia.

2.3. Cell isolation and flow cytometry analysis

Heparinized PBMCs were isolated by Ficoll Paque™ Plus (Amersham Pharmacia Biotech) density-gradient centrifugation, counted and stained with the appropriate combination of fluorescent labeled antibodies and analyzed by flow cytometry [27,28]. Dead cells were excluded from analysis by side/forward scatter gating. All analyses were performed on a FACSCanto (BD Biosciences) interfaced to PC FACSDiva software. A total of 500,000 events per sample were analyzed. Identification of B- and T-cell subsets is described in Supplementary materials and methods.

2.4. Cell cultures

For polyclonal stimulation of memory B cells $1–2 \times 10^6$ PBMCs were cultured for 5 days with CpG as described before [27].

2.5. ELISPOT

ELISPOT for total and pneumococcal polysaccharide specific IgM, IgA and IgG was performed as described before [27]. Antibody-producing cells against influenza were evaluated on plates coated with Inflexal V® (Crucell).

2.6. Haemagglutination-inhibition (HAI) tests

Specific anti-influenza antibodies against the same antigens included in 2013/2014 influenza vaccines (A/California/7/2009, A/Texas/50/2012, and B/Massachusetts/2/2012) were measured by HAI tests, according to standard procedures [29]. The HAI titer was the reciprocal of the highest dilution of serum that inhibited virus-induced haemagglutination.

Geometric mean titers (GMTs), seroprotection rate (the percentage of vaccine recipients with a post-vaccination HAI titer of at least 1:40) and seroconversion rate (the percentage of vaccine recipients with a ≥4-fold increase in serum HAI titers after vaccination) were calculated. A seroprotection rate exceeding 70%, a seroconversion rate exceeding 40%, and a GMT ratio (post-/pre-vaccination) exceeding 2.5 were considered as cut-off levels of vaccine immunogenicity, according to the guidelines of the European Committee for Proprietary Medical Products (CPMP) [30].

2.7. ELISA immunoassay

ELISA for the detection of total IgM, IgA and IgG and for anti-PnPS serum IgG was performed as previously described [27].

2.8. Statistical analysis

Comparison of lymphocyte population counts and total immunoglobulin levels was performed using the Mann Whitney *U* test. ELISPOT results and level of specific immunoglobulin between controls and DS children were statistically analyzed using the Kruskall–Wallis test followed by Dunn's multiple comparison test.

HAI titers <10 were assigned an arbitrary value of 5. Comparison of GMTs before and after vaccination were carried out using the Wilcoxon signed rank test. *P* values lower than 0.05 were considered statistically significant.

All statistical analyses were performed using the GraphPad InStat Software.

3. Results

3.1. Immunological features

We confirmed that DS children have normal numbers of all T cell populations with the exception of naïve $CD4^+$ T cells that are significantly reduced, to roughly 70% of the control numbers (Fig. 1A and B). As compared to their siblings, DS children have a significant reduction of total, mature-naïve and transitional B cells. The size of each population is diminished to about 30% of controls (Fig. 1C). The memory pool is the most dramatically affected: DS children have ¼ of the memory B cells of their siblings. The two populations of memory B cells are not equally reduced: IgM memory B cells are 30% and switched memory B cells only 15% of the controls (Fig. 1D). The immunological parameters are reported in Table 2.

Notwithstanding the contraction of the B-cell compartment, serum immunoglobulin are normal, with the exception of IgM that is around 40% less than in controls (Fig. 1 E–G). IgA in the saliva is present in normal amounts (Fig. 1H).

3.2. Total memory function

We measured the function and size of the B-cell memory repertoire in vitro by stimulating PBMCs with the TLR9 ligand CpG [23,24]. Notwithstanding the significant reduction of IgM and switched memory B cells in the peripheral blood, the number of total IgM-, IgA- and IgG-secreting cells was comparable in DS and non-DS children confirming that memory B cells of DS individuals have an increased ability to generate antibody-producing cells in response to CpG in vitro [25]. In both controls and DS children the total number of memory B cells able to generate antibody producing cells did not change significantly after vaccination (Fig. 2A).

3.3. Response to the first dose of influenza vaccine

We compared the ability of children with or without DS to generate switched memory B cells in response to the first dose of vaccine. All children received one injection of the 2013–2014 seasonal influenza vaccine containing the surface antigens of the A/California/7/2009 A/H1N1, A/Texas/50/2012 AH3N2 and B Massachusetts/2/2012 virus strains. None of the controls had been previously vaccinated against influenza. Among DS children only two had received the seasonal vaccine before (one in 2007 and the other in 2004), thus with vaccines containing viral strains other than those contained in the 2013–2014 Inflexal. We measured the number of memory B cells specific for the influenza 2013–2014 vaccine by ELISPOT before (pre) and two weeks after (post) vaccination.

The number of B cells producing IgM and IgA antibody able to bind the influenza antigens contained in the vaccine was comparable in controls and DS children before immunization and did not change significantly after vaccination. Influenza-specific memory B cells changed in response to vaccination. In the controls the median number of B cells producing IgG against influenza vaccine antigens increased from 75 cells/million in the pre-vaccination sample to 392 after vaccination. The increase, however, did not reach statistical significance probably because of the low number of subjects. In DS children the number of influenza specific memory B cells secreting IgG was not influenced by vaccination (median values were 20 and 39, pre- and post-vaccination, respectively). For this reason, in comparison to their siblings DS children had significantly less specific IgG producing cell, after vaccination (Fig. 2B).

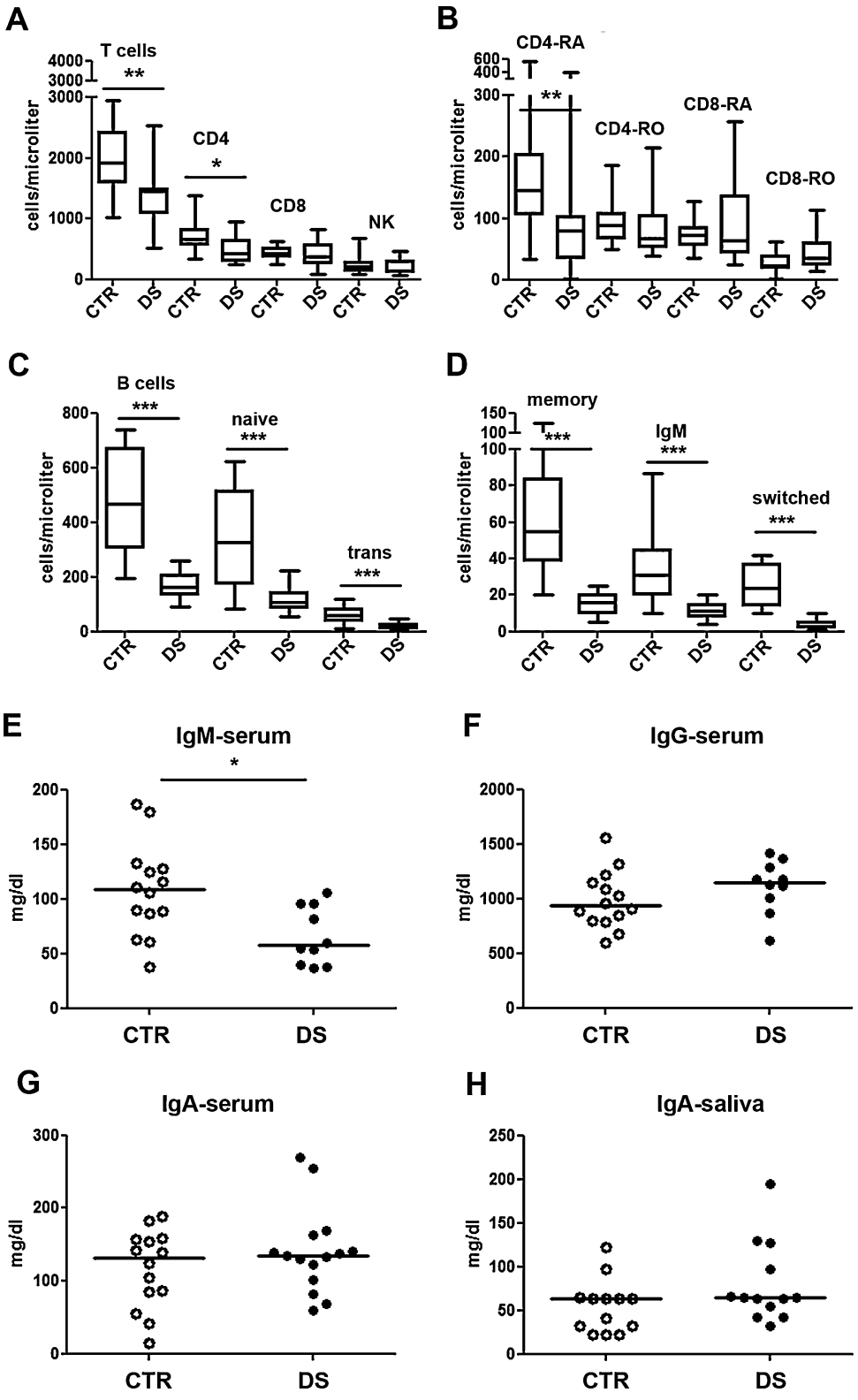

Fig. 1. T and B cells numbers in the peripheral blood of DS and control (CTR) children and immunoglobulin concentration. B and T cell populations were identified by flow-cytometry and calculated based on blood counts of the same day. (A, B) The box plots show the absolute numbers of the indicated T-cell populations. Statistical significance was calculated by the Mann Whitney U test on the values measured in all 15 DS and 15 CTR children. (C, D) Box plots represent the absolute numbers of the indicated B-cell populations in DS and CTR children. Mann Whitney U test was used to calculate significance on the values measured in all 15 DS and 15 CTR children. (E–G) Total serum IgM, IgG and IgA levels in the serum of CTR and DS children were measured by ELISA. Statistical significance was calculated by the Mann Whitney *U* test on the Ig levels of all 15 DS and 15 CTR children. The concentration of serum IgM was significantly different in DS children compared to CTR. (H) The concentration of IgA in the saliva, measured by ELISA, is comparable in CTR and DS children. Mann Whitney *U* test was used to calculate significance on the values measured in all 15 DS and 15 CTR children. Open circles represent the values measure in CTR and close circles in DS children.

Table 2
Immunological parameters.

	DS median (range)	CTR median (range)	Mann–Whitney *P*-value*
Lymph	1725 (1100–3400)	2620 (1500–4500)	**0.00596**
B cells	183 (100–400)	466 (200–700)	**<0.0001**
Transitional	15 (2–94)	57 (12–118)	**0.00072**
Mature	107 (53–242)	327 (84–623)	**0.0001**
Memory B	16 (9–44)	54 (20–126)	**<0.0001**
IgM mem.	11 (5–35)	31 (10–87)	**0.00014**
Sw. mem.	4 (2–10)	24 (10–42)	**0.00116**
T cells (CD3)	1445 (796–2538)	1915 (1026–2941)	**0.0056**
CD4	424 (262–959)	663 (342–1376)	**0.0136**
CD8	370 (243–830)	423 (248–620)	0.3270
NK	134 (60–470)	202 (79–686)	0.1467
CD4-RA	80 (1–408)	145 (34–567)	**0.0016**
CD4-RO	67 (39–215)	88 (50–187)	0.4284
CD8-RA	63 (25–257)	71 (35–128)	0.9549
CD8-RO	35 (14–114)	22 (2–61)	0.1463
Serum IgM	57.5 (37–106)	108.5 (38–187)	**0.01078**
Serum IgA	134 (60–270)	131.5 (15–188)	0.65994
Serum IgG	1149 (621–1419)	933 (592–1558)	0.16758
Salivary IgA	64.8 (32.4–364.5)	63 (21.6–121.5)	0.409027778

* Statistically significant values are shown in bold.

3.4. Response to a glyco-conjugated pneumococcal vaccine booster dose

All DS children and the majority of the controls (13/15) had received either two or three doses of anti-pneumococcal glyco-conjugated vaccines before enrollment in our study. The last dose had been administered two to three years before booster.

As observed for influenza neither specific IgM nor IgA numbers changed after vaccination. The booster dose was able to significantly increase the number of specific memory B cells in DS children (Fig. 2C). In the controls the increase of IgG producing cells was not significant probably because of a strong pre-existing immunity.

3.5. Vaccine-induced antibodies

Specific antibodies in the serum are the most accepted biological read-out of vaccine efficacy. Although all controls and the majority of DS children had never been vaccinated before against influenza, serum HAI antibody titers to H1N1 and H3N2 $\geq$ 1:40 were detectable at T0 in controls (71% and 57% of individuals, respectively) and DS children (50% and 36% of individuals, respectively) (Table 3), indicating a previous natural exposure to these viral strains. A different scenario was observed for the B strain for which none or limited seroprotection rate (14% of individuals) was observed in DS and controls, respectively. No specific HAI titers against the current viral antigens were found at T0 in 2 DS children who received a seasonal influenza vaccine in 2007 and 2008, respectively.

Influenza vaccination increased significantly the HAI antibody titers to influenza antigens in both DS and control children (*P*-values $\leq$0.002). Although GMTs were higher in controls than in DS, this group met CPMP criteria, with seroconversion rates between 71% and 78%, and seroprotection rates between 71% and 93% for the three viral antigens (Table 3). Finally, the GMT ratio exceeded 2.5 for the three antigens both in DS and controls.

IgG antibodies against pneumococcal polysaccharides were present in the serum before challenge at comparable concentrations in DS and non-DS children and increased significantly in both groups (Fig. 3).

4. Discussion

Vaccination is the most effective and least expensive tool of preventive medicine. Vaccines have been designed and vaccine schedules have been implemented with the aim of achieving optimal protection for most of the population. There are, however, individuals with special needs belonging to high risk categories because of medical conditions, occupational exposures, or risk behaviors [31–33]. Targeted indications have been developed for these categories in order to increase vaccine induced protection during the highest risk periods. Individuals with DS are not included in high-risk groups notwithstanding their increased susceptibility to infections, mostly of the respiratory tract.

It is important to precisely identify the immunological impairment typical of trisomy 21 in order to develop strategies for the prevention of infection and its consequences in the growing DS population.

To this aim we have compared a group of DS children to their siblings of similar age and living in the same household. We confirmed that the B-cell compartment is severely compromised in DS children [25,26,34,35]. Transitional, mature-naïve and memory B cells are all significantly reduced in percentages and absolute numbers.

Whereas the numbers of transitional and mature-naïve B cells are regulated by bone marrow production and space in the lymphoid organs, respectively, memory B cells are generated in the periphery in response to T-independent and dependent cues. In particular, switched memory B cells are produced by the germinal center reaction to infection or vaccination and are the main actors of recall responses. The low number of switched memory B cells could be either explained by reduced production or increased consumption.

Here we show that DS children vaccinated for the first time against influenza generate significantly less switched memory B cells specific for the vaccine than their siblings.

As all children had previously received a complete vaccination cycle with a glyco-conjugated vaccine against *S. pneumoniae*, we were able to evaluate the effectiveness of a secondary immune response by administering a booster dose of glyco-conjugated vaccine. The secondary response was successful, generating a number of specific memory B cells similar to controls. DS children, however, had a lower number of polysaccharide-specific switched memory B cells in the pre-booster sample, although all children had completed the recommended vaccination schedule. This observation may reflect the exhaustion of memory B cells in DS individuals due to their increased potential to terminally differentiate into plasma cells [25].

The production of specific antibodies was equally effective both after a primary and a secondary immunization.

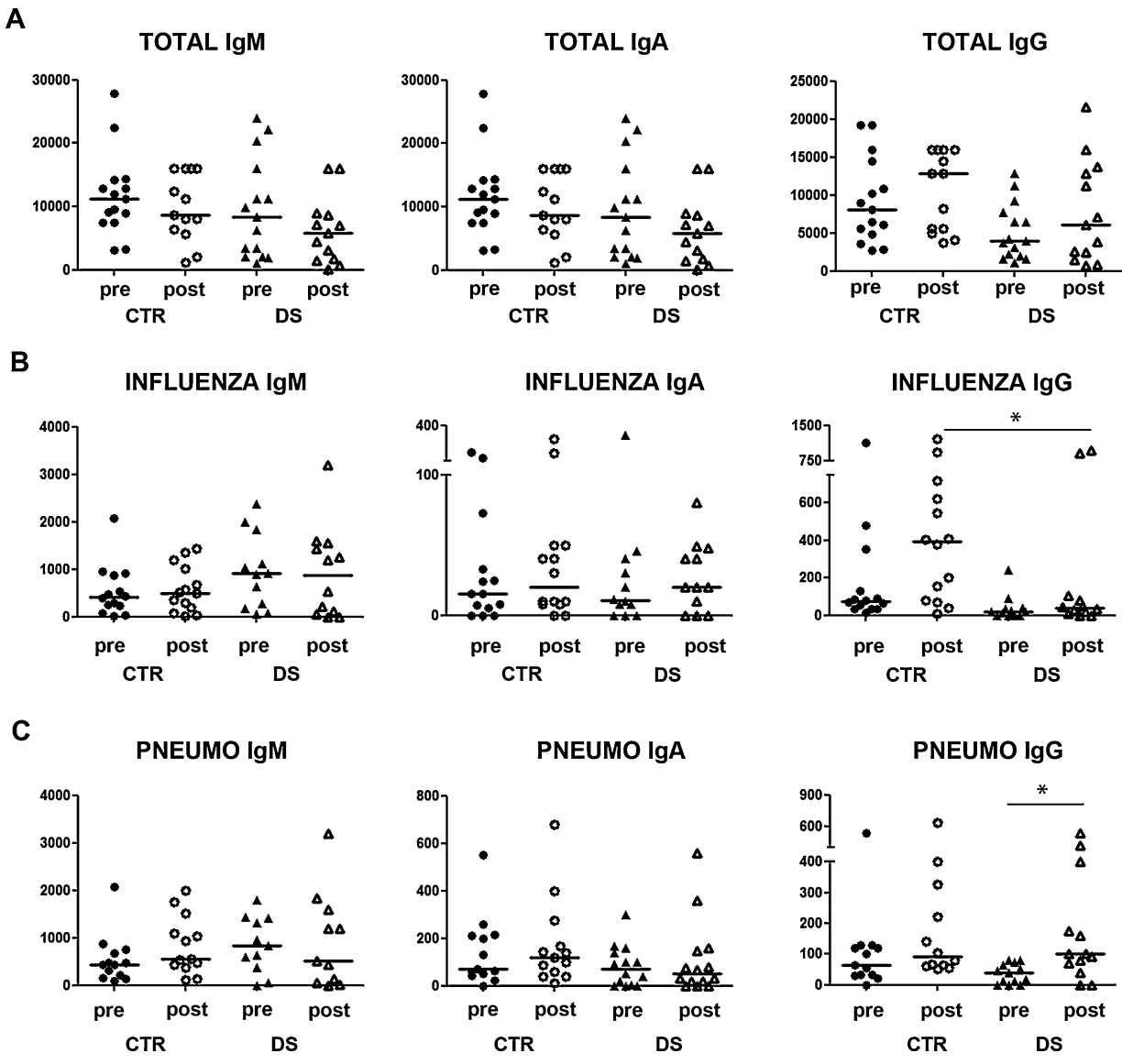

Fig. 2. (A) The ability of memory B cells to generate antibody producing cells after CpG stimulation was measured by ELISPOT in 15 CTR and 15 DS children. The number of spots obtained from 10^6 cells of CTR and DS children, secreting either IgM, IgA or IgG is indicated. No significant differences between DS and CTR pre- and post-immunization values were found. (B) Numbers of memory B cells producing IgM, IgA and IgG antibodies specific for influenza in the peripheral blood of CTR and DS children were measured by ELISPOT before and after vaccination. (C) Numbers of memory B cells producing IgM, IgA and IgG antibodies specific for pneumococcal polysaccharides in the peripheral blood of CTR and DS children were measured by ELISPOT before and after vaccination. Kruskall–Wallis test followed by Dunn's multiple comparison test were used to evaluate significant differences between all samples. Data are shown as scatter plots. The numbers of antibody secreting cells detected in the pre-vaccination samples are indicated by close circles for CTR children and by close triangles for DS children. The numbers of antibody secreting cells detected in the post-vaccination samples are indicated by open circles for CTR children and by open triangles for DS children.

Table 3
Antibody response to influenza vaccination in DS children and CTR measured by haemagglutination inhibition assay.

	A/California/7/2009 (H1N1pdm)		A/Texas/50/2012 (H3N2)		B/Massachusetts/2/2012	
	T0	T1	T0	T1	T0	T1
Geometric mean titer						
DS	32.8	452	17.2	131.2	5.8	44
CTR	36.2	609	22.1	262.4	7.8	138
Geometric mean titer ratio						
DS		13.8		7.6		7.6
CTR		16.8		11.9		17.7
Seroconversion rate (%)						
DS		78		78		71
CTR		78		86		86
Seroprotection rate (%)						
DS	50	93	36	86	0	71
CTR	71	86	57	93	14	86

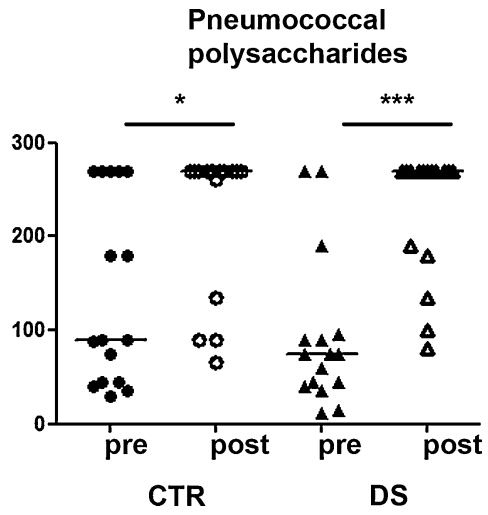

Fig. 3. Specific antibodies increase in the serum after vaccination. Serum IgG antibodies directed against pneumococcal polysaccharides was measured in the serum by ELISA before and after vaccination. Kruskall–Wallis test followed by Dunn's multiple comparison test were used to calculate significance on the values measured in all 15 DS and 15 CTR children. Pre-vaccination values of serum IgG in CTR children are indicated by close circles. Open circles represent specific antibody concentrations detected after vaccination. For DS children pre-immunization values are indicated by the close triangles and the open triangles show the post-vaccination levels.

Thus, DS individuals in this study are perfectly able to respond to vaccination with antibody secretion. They are, however, less able to produce and maintain switched memory B cells.

Strengths of our study include examination of B cell functions in two populations (DS and their siblings) similar for genetic and environmental background with the exception of trisomy 21.

Limitations of the study are mainly associated with the small sample size. The obtained results may suffer from low power and lack of statistical significance even when clinically significant results were detected. Generalizability may have been also affected by the small sample size, although the enrollment was systematically conducted, and controls were matched among siblings.

A further limitation of our study is that we compare a primary and secondary immunization not to the same vaccine but to two different types of immunizing agents. Although from the immunological point of view, the split inactivated influenza vaccine is essentially a T-dependent vaccine as the pneumococcal glyco-conjugated preparation [36–38], further studies should investigate the response to the first and each subsequent dose of glyco-conjugated pneumococcal vaccine and to a first and second influenza vaccine dose. It is also important to further address the question of the duration of memory, a fundamental information for the development of appropriate vaccine schedules, not only for children but also for DS individuals of all ages.

In conclusion, DS individuals have a major defect in the number and generation of switched B memory cells and therefore should be considered as a group at high risk for infections. Further studies on large populations of DS children are necessary to evaluate whether tailored vaccination schedules may reduce the frequency of infections and their complications thus improving the quality of life of DS individuals.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.10.083.

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