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## Immune evaluation and vaccine responses in Down syndrome: Evidence of immunodeficiency?

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### Abstract

**Background**—Patients with Down syndrome (DS) appear to be at a greater risk for serious infections, but it is unclear whether this is due to anatomic variations or intrinsic immune defects.

**Objective**—We assessed a cohort of pediatric subjects with DS to determine if immunological abnormalities indeed account for the excess infections.

**Methods**—We performed quantitative assessment of T-independent (type 2 – pneumococcal polysaccharide vaccine) and T-dependent Ab responses (with inactivated seasonal influenza vaccine) along with numerical quantitation of lymphocyte subpopulations and thymic output in a random population sample of children with DS (cases) along with family-matched sibling or community controls.

**Results**—Median serum IgG levels were significantly higher in cases (1090 mg/dL) as compared with controls (808 mg/dL,  $P = 0.02$ ). Cases had significantly lower median CD4 T cell counts than the controls (636 cells/ $\mu$ L,  $P = 0.01$ ). Cases had reduced CD19 B cell counts and CD19% than the controls ( $P = 0.009$  and  $0.006$  respectively). Cases also showed decreased total memory (CD19+CD27+,  $P = 0.002$ ) and class-switched memory (CD19+CD27+IgM–IgD–,  $P = 0.004$ ) B cells.

The median CD4 recent thymic emigrant (RTE) in females and males cases was lower than controls ( $P = 0.007$  and  $0.07$  respectively). Cases had a lower median T cell receptor excision circle (TREC) count of 2556 as compared to the controls count of 5216,  $P < 0.006$  although both the cases and controls were within the established reference range. There were no differences in the percentage of cases and controls who responded to inactivated influenza vaccine, but the response to polysaccharide pneumococcal vaccine was suboptimal in cases.

**Conclusions**—Our study suggests that there are subtle abnormalities in both humoral and cellular arms of the immune response in children with DS as compared to the control subjects.

### Keywords

Infections; Immunity; Humoral immunity; Cellular immunity; Vaccine; Influenza; Pneumococcal polysaccharide vaccine; Immunoglobulin

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## 1. Introduction

The life expectancy of children with DS has increased over time [1–3], but their risk of premature mortality is substantial [4–6]. Although the median age of death has doubled to about 50 years over the past 2 decades, mortality is still higher than in the general population, with respiratory tract infections amongst the major cause of death [2,6]. Viruses such as influenza virus, respiratory syncytial virus (RSV) and parainfluenza viruses are responsible for much of the respiratory tract infections [4,7]. *Streptococcus pneumoniae* is the leading bacterial cause of respiratory illness. It is unclear whether the increased risk of infections in DS subjects is due to primary immune deficiency (PID).

Support for an intrinsic immune deficiency has been provided by a cross-sectional study, in which immunophenotyping was used to evaluate lymphocyte subpopulations in 96 subjects with DS who ranged in age from one to 20 years [8]. Compared with previously published data on healthy children without DS [9], children with DS had a diminished expansion of T and B cell lymphocytes in the first years of life. Although T lymphocytes eventually approximated normal levels, B lymphocytes remained diminished (with 88 percent of values below the 10th percentile).

Abnormal proportions of peripheral blood lymphoid subsets, cellular dysfunction, and autoimmune phenomena have also been described in subjects with DS [10–14]. It is unclear whether these are age-related changes or are clear evidence of immunodeficiency. There are limited studies evaluating various arms of the immune system in patients with DS, including T cell subsets [14,15] and immunoglobulin subclasses [16] concurrently. Comprehensive immunological evaluation with baseline immune parameters and thymic output in the same cohort have not been reported.

Thymic function has also been assessed in subjects with DS [17] to evaluate whether there is a component of precocious aging, and there has been some evidence of thymic and T cell aberrations but firm conclusions on B cell compartment are yet to found.

This study was proposed to evaluate if PID is indeed an underlying mechanism for the excess morbidity and mortality seen in patients with DS. The objectives of the study were to undertake a comprehensive immunologic evaluation in patients with DS and in a group of referent subjects and to evaluate the immune response to two vaccines (pneumococcal polysaccharide vaccine and inactivated influenza vaccine) in patients with DS and referent subjects.

## 2. Methods

### 2.1. Study population

We prospectively enrolled 24 subjects (12 with DS and 12 subjects without DS) who were living in Olmsted County and were not institutionalized.

Inclusion criteria for Case subject:

1. Diagnosis of Down syndrome.
2. Age  $\geq 2$  years and  $< 18$  years as of November 1, 2009 and requiring only one dose of influenza vaccine for the 2009–2010 influenza season.

Exclusion criteria for case subject:

1. Age  $< 2$  years or  $> 18$  years as of November 1, 2009.

2. Evidence of malignancy, chemotherapy, post-chemotherapy, receipt of immunosuppressive therapy in the past 28 days, or other known secondary immune suppressive condition.
3. Previous pneumococcal polysaccharide vaccination.
4. Any contraindications to inactivated influenza vaccine or polysaccharide pneumococcal vaccine.

One matched subject was selected from Olmsted County.

Inclusion criteria for Referent (Control) subject:

1. Age  $\geq$  2 years and <18 years as of November 1, 2009 and requiring only one dose of influenza vaccine for the 2009–2010 influenza season.

Exclusion criteria for Referent (Control) subject:

1. Age <2 years or >18 years as of November 1, 2009.
2. Evidence of malignancy, chemotherapy, post-chemotherapy, receipt of immunosuppressive therapy in the past 28 days, or other known secondary immune suppressive condition.
3. Previous pneumococcal polysaccharide vaccination.
4. Any contraindications to inactivated influenza vaccine or polysaccharide pneumococcal vaccine.

## 2.2. Immune assessment

**2.2.1. CBC**—Baseline CBC was obtained in the Coulter LH750 instrument that provided a white cell count, red cell count, and platelet count using impedance counting. Automated white blood cell differentials were performed on the instrument using VCS technology, which combined volume, conductivity and light-scatter measurement of individual cells to provide a 3 dimensional plot of cell populations and sub-populations (Coulter Systems Reference Guide, Beckman-Coulter Corporation, Miami, FL, USA).

**2.2.2. Immunoglobulins**—Immunoglobulin (Ig) – IgG, IgM, IgA were obtained using nephelometry.

**2.2.3. T and B lymphocyte subsets**—Lymphocyte subset (T, B and NK cells) quantitation was performed using a 6-color flow cytometric assay with absolute quantitation using Trucount<sup>®</sup> tubes (BD BioSciences, San Jose, CA, USA) [18].

**2.2.4. B cell immune assessment**—Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using a Ficoll gradient and used for multiparametric flow cytometry. The assay involved a multicolor 5-tube panel for the following antibodies: CD45, CD19, CD20, CD27, IgD, IgM, CD38, and CD21. After the staining with specific antibody, the cells were washed and fixed with paraformaldehyde and then analyzed by flow cytometry on a BD FACSCanto instrument. The cell-surface expression was denoted as the percent of CD19+ B cells expressing each of the specific markers. CD19+ and CD20+ B cells were expressed as a percent of the total lymphocytes (CD45+). The absolute counts for the B-cell subsets are derived from flow cytometry analysis of whole blood using the BD Multitest TBNK Panel (BD Bio-Sciences) kit, which contains monoclonal antibodies for CD45, CD3, CD4, CD8, CD19, and CD16+CD56+ (Table 1).

**2.2.5. Influenza vaccine responses**—Serum from each subject was diluted and placed in wells of substrate slides containing influenza virus-infected cells (influenza A and B in separate well). A fluorescent antibody conjugate was used to react with the virus-infected cells.

**2.2.6. Pneumococcal vaccine responses**—IgG antibodies to all 23 *S. pneumoniae* serotypes were measured by microsphere photometry. Purified pneumococcal polysaccharides coupled covalently to polystyrene microspheres bind IgG antibodies in patients' sera during the first incubation. After incubation, the microspheres were washed and incubated with phycoerythrin-conjugated antihuman IgG antibody. The concentration of IgG antibodies to each polysaccharide was determined by comparison to dose–response curves calculated from serial dilutions of a serum pool from immunized adults with known concentrations of antibodies to each polysaccharide (secondary standard). The secondary standard was traceable to a standard reference preparation (FDA 89-SF) that contains known concentrations of IgG antibodies to 23 different *S. pneumoniae* serotypes. Dose–response curves prepared from serial dilutions of the secondary standard parallel the dose–response curves of the primary reference preparation for all polysaccharides [19,20].

### 2.3. Thymic assessment

**2.3.1. CD4 and CD8 recent thymic emigrants (RTEs)**—CD4 RTEs were assessed in peripheral blood drawn in EDTA tubes and was performed as a single-tube multicolor assay. A panel of antibodies were used for the assay: CD3, CD4, CD31, CD45RA, CD45RO, conjugated to various fluorochromes.

CD8 RTEs were assessed in PBMCs obtained from peripheral blood drawn in EDTA tubes. The assay was performed as a 2-tube assay with the first tube serving as the background control for the CD103 antibody. A panel of antibodies were used for the assay: CD3, CD8, CD45RO, CD62L, and CD103, conjugated to various fluorochromes.

The blood was incubated with the antibodies in the dark for 20 min, followed by RBC lysis for 10 min. Absolute counts were obtained using BD TruCount tubes. The sample was then centrifuged and resuspended in 500  $\mu$ L of flow cytometry staining buffer for analysis on a BD FACS Canto A or Canto II flow cytometer. The data analysis was performed using BD FACS Diva software.

**2.3.2. T-cell receptor excision circle (TREC) analysis**—The PBMCs were treated with proteinase K to prepare cell lysates. The resulting cell lysates containing genomic DNA were used for amplification in the real-time quantitative PCR assay. Each sample was amplified using a probe specific for the TCR delta-deletion TREC signal joint and a probe for housekeeping gene, albumin. The TREC and albumin counts obtained were calculated as copies per million cells using a standard curve for both TREC and albumin [21].

Blood samples were collected before and 4–6 weeks after vaccination. An adequate response to influenza vaccine was considered a post vaccine titer  $\geq$ 1:40 or a 4-fold rise in hemagglutination inhibition titers [22].

An adequate response to pneumococcal polysaccharide vaccine was arbitrarily defined as a post vaccine antibody concentration of  $\geq$ 1.3 mcg/mL or a minimum 2-fold rise from baseline value [23–25]. In addition, we used the expert panel recommendation to define an adequate response in children as a response to at least 50% of the serotypes present in Pneumovax [24,26–28].

**2.4. Statistical analyses**—Standard descriptive statistics (mean, standard deviation, median, interquartile range) were used to analyze the immune parameters and vaccine responses.

For each subject, we also determined and compared seroconversion for both vaccines. The seroconversion rate was compared between cases and controls using Mc Nemar's test for comparing correlated proportions. All calculated *P*-values were two-sided, and *P*-values less than 0.05 were considered statistically significant. Analyses were performed using the SAS software package (Version 9.0, SAS Institute, Inc., Cary, NC, USA).

### 2.5. Power—

	Control – seroprotection	Control – no seroprotection	Total
Cases – seroprotection	<i>a</i>	<i>b</i>	<i>a + b</i>
Cases – no seroprotection	<i>c</i>	<i>d</i>	<i>c + d</i>
Total	<i>a + c</i>	<i>b + d</i>	<i>N = a + b + c + d</i>

We assumed 90% seroprotection in controls ( $((a + c)/N)$  and 60% seroprotection in cases ( $((a + b)/N)$ ), and assuming correlation between matched cases and controls of 0.07 and the proportion of discordant pairs being 40% ( $((b + c)/N)$ ), the study would have 80% power to detect this 30% difference based on 30 cases and 30 controls. This calculation was based on McNemar's test with a 0.05 two-sided significance level using nQuery Advisor, version 6.0.

**2.6. Ethical conduct**—This study was conducted in conformity with applicable national and local requirements for protecting the rights and welfare of human subjects participating in biomedical research. The study protocol was approved by the Institutional Review Board (IRB) at the Mayo Clinic. Subjects gave informed consent/assent by signing approved consent forms.

## 3. Results

**3.1. Demographic characteristics**—The study population consisted of 12 cases and 12 controls. The median ages of the cases and controls were very similar. The median age amongst the cases was 9.2 years (mean age: 9.4 years) and 9.7 years in controls (mean age: 9.4 years). The sex distribution was even amongst the controls (6 males and 6 females) while there were 10 females and 2 males amongst the cases.

**3.2. Complete blood count and baseline immunoglobulin levels**—CBC with differential was available for 12 cases and 12 control subjects. Table 2 summarizes the results in the two groups. Total WBC count was statistically lower in the cases as compared to control group ( $P = 0.03$ ), but both were within the normal range. Serum IgG levels were higher in the cases than the controls ( $P = 0.02$ ), but both groups were within the normal range established for age using a separate cohort of healthy donors.

**3.3. Peripheral blood T and B lymphocyte subsets**—Table 3 summarizes the T and B lymphocyte subsets. CD3 T cell percentage was lower in cases than controls (Wilcoxon rank sum test;  $P = 0.023$ ). CD19 B cell count and percentage both were lower in cases as compared with the controls (Wilcoxon rank sum test,  $P < 0.001$  and 0.006 respectively). Absolute CD4 T cell (helper T cell) count was lower in cases ( $P = 0.019$ ) and CD8% was

higher in cases ( $P = 0.002$ ). The helper/suppressor ratio was  $> 1.0$  in both the groups but was statistically higher in controls ( $P = 0.003$ ).

**3.4. Thymic output and recent thymic emigrants (RTEs)**—Recent thymic emigrants (RTEs) refer to those populations of naive T cells that have not diluted their T-cell receptor excision circles (TRECs) [21,29] levels with cell division. The median CD4 RTE in females with DS was 202 compared with 494 in females without DS ( $P = 0.007$ , ref. range: 170–1007 cells/ $\mu\text{L}$ ). The median CD4 RTE in males with DS was 267 compared with 715 in males without DS ( $P = 0.07$ , ref. range: 50–926 cells/ $\mu\text{L}$ ). The median CD4 RTE% in females with DS was 35% compared with 58% in females without DS ( $P = 0.003$ , ref. range: 26–68%). The median CD4 RTE% in males with DS was 25% compared with 54% in males without DS ( $P = 0.04$ , ref. range: 19–61%). There was no difference in the CD8 RTE counts or CD8 RTE% between cases and controls ( $P > 0.5$ ).

Similar to the CD4 RTE results, TREC counts were also significantly lower in patients with DS, although results from both groups were within the reference range ( $>801$  TREC copies per million PBMCs, Table 4). Cases had a median TREC count of 2556 whereas the controls had a median TREC count of 5216 ( $P < 0.006$ ).

**3.5. Advanced immune assessment: B cell phenotyping**—B cell phenotyping data was available for 8 cases and 6 controls. Total memory B cells (CD19+CD27+) were lower in cases ( $P = 0.028$ ). In addition, class-switched memory B cells (CD19+CD27+IgD–IgM–) were lower in cases ( $P = 0.009$ ). CD21 low (“immature” CD19+CD21–) B cell% was higher in cases ( $P = 0.005$ ) and mature B cells (CD19+CD21+) was lower in cases ( $P = 0.006$ ). Transitional B cells and plasmablasts were lower in cases but this difference did not reach statistical significance (Table 5).

### 3.6. Vaccine responses

**3.6.1. Influenza vaccine:** Pre and post influenza vaccine serology was available for 12 cases and 12 controls.

**3.6.2. Influenza A:** Five cases had a pre-vaccine IgG serology  $\leq 1:10$  and 7 cases had a pre-vaccine serology of 1:40. Amongst the controls, six subjects had a pre-vaccine IgG serology of  $\leq 1:10$ , 2 subjects had a serology of 1:40, four subjects had a titer of 1:160 and one subject had a titer of 1:640. All cases and controls had a pre-vaccine and post-vaccine IgM  $< 1:10$ . Adequate post vaccine response was seen in all controls with one subject with a titer of 1:40 while the rest had titers  $\geq 1:160$ . Amongst the cases, two subjects failed to show a rise in titer  $\geq 1:40$  while the rest showed either a 4-fold rise in titer or a post vaccine titer  $\geq 1:40$ .

**3.6.3. Influenza B:** Three cases had a pre-vaccine IgG serology  $\leq 1:10$ , three subjects had a titer of 1:40 and eight subjects had titers  $\geq 1:160$ . Amongst the controls, three subjects had titers  $\leq 1:10$ , three additional subjects had titers of 1:40 and seven subjects had titers  $\geq 1:160$ . All cases and controls had a pre-vaccine IgM  $< 1:10$ . All except one case and all controls had a post-vaccine IgM  $< 1:10$ . All cases and controls had a post vaccine IgG titer  $\geq 1:40$ . In assessing 4-fold rise in titer, five cases and two controls failed to show an adequate response, which was not statistically significant.

**3.7. Pneumococcal vaccine**—Pre- and post-vaccine pneumococcal serologies were available for 10 cases and 12 controls. Amongst the cases, no subject showed an adequate response to 7F, 10A, 12F, 17F and 22F serotypes. Nine of 11 subjects (81%) showed an adequate response to serotype 14. Eight subjects (72%) showed an adequate response to serotypes 4 and 18C, and seven subjects (63%) showed an adequate response to serotype 9N

and 9V. Six subjects each (54%) showed an adequate response to serotypes 1, 3 and 15B, five subjects (45%) showed an adequate response to 11A and four subjects each (36%) showed a response to 2, 6B, 19F and 33F serotype. Three subjects each (27%) showed a response to serotypes 8, 20 and 23F while two subjects (18%) showed an adequate response to 19A and one subject (9%) showed a response to serotype 5. In assessing individual subjects, 7 cases responded to 10 or fewer of the 23 serotypes in Pneumovax. Three subjects responded to 11 serotypes while one subject responded to 12.

Amongst the controls, no subject showed an adequate response to 7F, 17F, 19A and 22F serotypes. Nine of 11 subjects (81%) showed an adequate response to serotypes 4, 9N, 9V and 18C. Eight subjects (72%) showed an adequate response to serotype 14 and seven subjects (63%) showed an adequate response to serotype 15B. Six subjects each (54%) showed an adequate response to serotypes 1, 6B, 11A, and 19F. Three subjects each (27%) showed a response to serotypes 2, 3, 20, 23F and 33F while two subjects (18%) showed an adequate response to serotype 8 and one subject (9%) showed a response to serotype 5, 10A and 12F.

In assessing individual controls, 11 controls responded to 10 or fewer of the 23 serotypes in Pneumovax while one subject responded to 12 serotypes.

#### 4. Discussion

This prospective study demonstrated that children with DS had higher serum IgG despite decreased CD19+ B cells. They also had reduced class-switched memory B cells and an increase in the activated CD21 low B cell population. In addition, they had decreased CD4 T cells. Children with DS had lower thymic output and function as compared with controls. CD4 RTE and TREC counts showed close linear correlation both in children with and without DS. However, we found no correlation between CD8 RTE and TREC levels in both children with and without DS, suggesting that homeostasis in the CD8 T-cell compartment is probably maintained more by peripheral expansion than by thymic output.

The lower CD4 count in cases has been previously described and does not fit severe T cell immunodeficiency, but does point more towards a partial failure of T lymphocyte generation, an intrinsic T lymphocyte defect, an increased apoptosis, or a combination of these [30]. Further studies looking at T cell phenotyping and regulatory T cell subsets would help decipher this phenomenon.

TREC output is known to decline with age in healthy adults [29]. Our results suggest that a component of the immune deficiency and susceptibility to infection that is seen in patients with Down syndrome is related to defects in thymic function. Whether the thymic dysfunction is related to precocious aging (early senescence [10,31]) or intrinsic defect (as in DiGeorge syndrome [8,30,32–36]) is still debated. With the constellation of defects in both B and T cell compartment, the possibility of this being an intrinsic defect is more likely but further longitudinal studies are needed to validate this claim.

The inactivated influenza vaccine was immunogenic in both cases and controls, though the responses were marginally lower in cases than controls (Fischer's exact test,  $P = 0.3$ ). More than 50% of the cases and controls had pre-vaccine titers to both influenza A and B in the protective range suggestive past vaccination or exposures.

The response to pneumococcal vaccine in both cases and controls was suboptimal. The response to Pneumovax in DS adults was found to be lower than controls [37] but a similar study in Brazilian children found adequate response [23]. Our cohort had higher titers even before vaccination which could be attributed to previous contact, especially the effects of

pneumococcal conjugate vaccination, both direct (50% subjects had received Prevnar in both cases and controls) and herd immunity. There are studies in adults showing a priming effect of Prevnar on subsequent Pneumovax administration [38], but most studies have not followed the 4-fold rise in titers strictly or looked at all 23 serotypes in the pneumococcal polysaccharide vaccine. This maybe an indication that the polysaccharide vaccine may have lower immunogenicity not only in children less than age two, but children in general.

With our small sample size, we were unable to conclude if the pneumococcal vaccine responses were different between the cases and controls, but further studies are needed before recommending this vaccine in the DS population as the immunologic benefits may still be unknown. The possibility of using a protein conjugate vaccine may hold promise.

The clinical implications of our findings are multifold: Reduced class switched memory B cells have previously not been described in children with DS but they were still within the reference range. This along with increase in CD21low B cells do indicate a possible maturation defect, but our sample size was small to make any firm conclusions. When comparing our cases to the established reference ranges, most of the studies were within the normal limits but there were difference when compared to the control population.

Our study suggests that there are subtle abnormalities in both humoral and cellular arms of the immune response, in children with DS as compared to the controls.

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## Abbreviations

<b>DS</b>	Down syndrome
<b>TREC</b>	T-cell receptor excision circle
<b>RTE</b>	recent thymic emigrant
<b>PID</b>	primary immunodeficiency
<b>CBC</b>	complete blood count

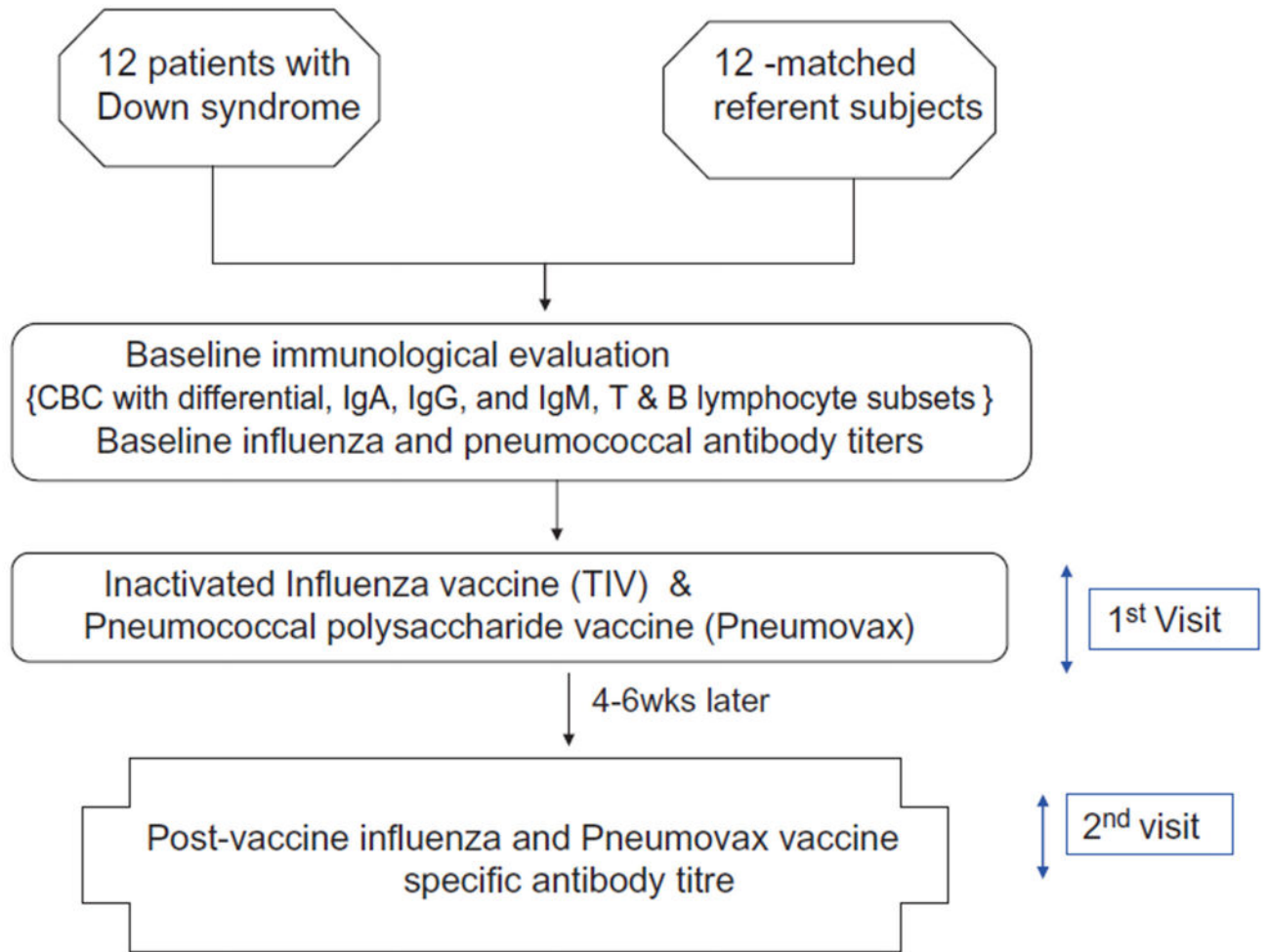
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**Table 1**

B cell panel subset assessment.

Surface markers	Identified population
CD19+	B cells expressing CD19
CD19+CD27+	Total memory B cells
CD19+CD27+IgD+IgM+	Marginal zone or non-switched memory B cells
CD19+CD27+IgD-IgM+	IgM-only memory B cells
CD19+CD27+IgD-IgM-	Class-switched memory B cells
CD19+IgM+	IgM+ B cells
CD19+CD38+IgM+	Transitional B cells
CD19+CD38+IgM-	Plasmablasts
CD19+CD21-	CD21 low B cells
CD19+CD21+	CD21+ (high) B cells

**Table 2**

Complete blood count (CBC) and baseline immunoglobins in cases and controls.

	Cases (N = 12) Median (IQR)	Controls (N = 12) Median (IQR)	P value
Hemoglobin (g/dL)	14.3(13.1–14.9)	13.9(13.5–14.5)	0.29
Total WBC count (×10 <sup>9</sup> /L)	5.9(4.8–6.9)	6.8(6.4–8.0)	0.03 <sup>*,a</sup>
Absolute lymphocyte count, ALC (×10 <sup>9</sup> /L)	1.86(1.58–2.27)	2.3(1.7–3.0)	0.13
Platelet count (×10 <sup>9</sup> /L)	278(241–355)	278(230–306)	0.59
IgG (mg/dL)	1090(849–1390)	807(619–1021)	0.028 <sup>*,b</sup>
IgM (mg/dL)	56.5(51.7–95.0)	87.5(67.3–105)	0.07
IgA (mg/dL)	131(71–192.3)	124(65.2–161.5)	0.7

\* Statistically significant,  $P < 0.05$ .

<sup>a</sup> Cases lower than controls, statistically significant.

<sup>b</sup> Cases higher than controls, statistically significant.

**Table 3**

T and B lymphocyte markers in cases and controls.

Lymphocyte markers	Cases (N = 12) Median (IQR)	Controls (N = 12) Median (IQR)	P value
T cell {CD3 (thou/ $\mu$ L)}	1262 (1088–1680)	1602 (1284–2280)	0.11
CD3%	76 (67–80)	66 (63–69.5)	0.029 <sup>*,b</sup>
Natural killer {CD16+CD56+ (cells/ $\mu$ L)}	224 (166–315)	290 (208–351)	0.23
Natural killer%	12 (10–17)	12 (7.5–15)	0.56
B cell {CD19+ (cells/ $\mu$ L)}	190 (111–342)	493 (351–845)	<0.001 <sup>*,a</sup>
CD19%	12 (6.0–19.0)	21 (17.5–24)	0.006 <sup>*,a</sup>
Helper cells (CD4) (cells/ $\mu$ L)	636 (486–786)	1028 (759–1370)	0.019 <sup>*,a</sup>
CD4%	37 (30–42)	40 (35.5–45.5)	0.06
Suppressor cells (CD8) (cells/ $\mu$ L)	582 (414–788)	580 (424–861.5)	0.74
CD8%	37 (27–39)	24 (18.5–27.5)	0.0025 <sup>*,b</sup>
Lymphocyte ratio Helper/suppressor ratio	1.09 (0.36–1.33)	1.79 (1.22–2.42)	0.003 <sup>*,a</sup>

\* Statistically significant,  $P < 0.05$ .<sup>a</sup> Cases lower than controls, statistically significant.<sup>b</sup> Cases higher than controls, statistically significant.

**Table 4**

Thymic output, CD4, and CD8 recent thymic emigrants (RTEs) in cases and controls.

	<b>Cases (N = 12) Median (IQR)</b>	<b>Controls (N = 12) Median (IQR)</b>	<b>P value</b>
CD4 recent thymic emigrant (CD31+CD4 RTEs) cells/ $\mu$ L	201 (201–251)	607 (363–810)	<0.001 <sup>*,a</sup>
CD4 RTE%	35.3 (24.5–38.8)	55.6 (52.9–59.9)	<0.001 <sup>*,a</sup>
CD8 recent thymic emigrant (CD8+CD103+ RTEs) cells/ $\mu$ L	1.4 (0.0–2.3)	0.9 (0.65–1.55)	0.44
CD8 RTE%	0.3 (0.0–0.4)	0.2 (0.15–0.20)	0.15
TREC copies per million CD3 T cells	2556 (789–4415)	5216 (3770–6964)	0.007 <sup>*,a</sup>

\* Statistically significant,  $P < 0.05$ .

<sup>a</sup> Cases lower than controls, statistically significant.

**Table 5**

B cell phenotyping in case and controls.

	<b>Cases (N = 8) Median (IQR)</b>	<b>Controls (N = 6) Median (IQR)</b>	<b>P value</b>
CD19+ (cells/ $\mu$ L)	257.8 (106.4–340)	453 (188–700)	0.14
CD19+CD27+ (cells/ $\mu$ L)	29.8 (25.7–36.8)	55.1 (38–157.3)	0.028 <sup>*,a</sup>
CD19+CD27+%	14.6 (9.4–26.5)	15.0 (12.8–20.7)	0.94
CD19+CD27+IgM+IgD+ (cells/ $\mu$ L)	15.5 (11.2–19.7)	24.3 (15.5–66.3)	0.07
CD19+CD27+IgM+IgD+%	7.9 (4.5–12.2)	6.3 (5.5–8.7)	0.69
CD19+CD27+IgM+IgD– (cells/ $\mu$ L)	2.45 (0.85–4.2)	0.95 (0.67–3.15)	0.36
CD19+CD27+IgM+IgD–%	1.15 (0.32–2.4)	0.35 (0.15–0.55)	0.68
CD19+CD27+IgM–IgD– (cells/ $\mu$ L)	10.4 (7.6–13.5)	22.8 (16.3–53.8)	0.009 <sup>*,a</sup>
CD19+CD27+IgM–IgD–%	5.8 (2.8–10.3)	6.4 (4.2–9.4)	0.74
CD19+IgM+	161 (84.8–233.53)	228 (131–466)	0.15
CD19+IgM+%	73.3 (54.3–83.2)	59.9 (40.4–71.8)	0.16
CD19+CD38+IgM+ (cells/ $\mu$ L)	75.1 (24.1–127.3)	106.95 (53.8–240.9)	0.24
CD19+CD38+IgM+%	25.8 (22.6–42.3)	27.2 (22.9–28.3)	0.89
CD19+CD38+IgM– (cells/ $\mu$ L)	21.1 (7.3–54.8)	60.2 (26.5–184.7)	0.07
CD19+CD38+IgM–%	9.2 (4.4–25.1)	15 (8.5–31.3)	0.27
CD19+CD21– (cells/ $\mu$ L)	9.9 (7.2–18.9)	9.8 (3.1–18.4)	0.56
CD19+CD21–%	5.8 (3.3–6.9)	2.2 (1.3–3.0)	0.005 <sup>*,b</sup>
CD19+CD21+ (cells/ $\mu$ L)	249.6 (97.5–323.3)	447.3 (184.8–934.6)	0.07
CD19+CD21+%	94.3 (93.4–96.8)	97.9 (97–98.9)	0.006 <sup>*,a</sup>

\* Statistically significant,  $P < 0.05$ .<sup>a</sup> Cases lower than controls, statistically significant.<sup>b</sup> Cases higher than controls, statistically significant.