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Flow Based Enumeration of Plasmablasts in Peripheral Blood After Vaccination as a Novel Diagnostic Marker for Assessing Antibody Responses in Patients with Hypogammaglobulinaemia

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1. Introduction

Hypogammaglobulinaemic patients are often started on immunoglobulin substitution therapy before antibody production is adequately evaluated. In such a situation, it is difficult to segregate transferred from antigen-induced specific antibody. Therefore we characterized changes in B-cell subpopulations in hypogammaglobulinaemic patients, including plasmablasts, in peripheral blood by flow cytometry after in vivo antigen challenge. We investigated the specificity of antibody production on the B-cell level by ELISPOT, which is independent of substitution therapy.

Common variable immunodeficiency (CVID) is characterized by low serum levels of IgG, IgA, normal or low levels of IgM and impaired antibody responses after vaccination (Conley et al. 1999). The clinical presentation of CVID includes recurrent respiratory tract infections by encapsulated bacteria, autoimmunity, granuloma formations, enteropathy and increased risk of malignancies. The diagnosis is established by exclusion and elimination of other disorders affecting B-cell differentiation. Although standard treatment include long-term immunoglobulin replacement and antimicrobial therapy, the mortality rate of CVID patients is higher than that of the general population (Chapel et al. 2008; Cunningham-Rundles & Bodian 1999).

Despite intensive research the immunopathogenesis of CVID has not yet been elucidated. It has been suggested that CVID is caused by defects in T cells, B cells, insufficient T-B cell interactions or impaired signaling required for B or T-cell maturation and function, but the characterization of the genetic defects remains unclear in the majority of patients. Molecular defects involving mutations in CD19 (van Zelm et al. 2006), ICOS (Grimbacher et al. 2003; Salzer et al. 2004), CD81 (van Zelm et al.), Msh5 (Sekine et al. 2007) and TACI (Castigli et al. 2005; Mohammadi et al. 2009; Salzer et al. 2005) were found in less than 10% of CVID

patients (Cunningham-Rundles & Bodian 1999; Schaffer et al. 2007). CVID, therefore, is a heterogeneous group of patients expected to have multiple etiologies, all sharing similar immunologic and clinical characteristics.

Although the precise pathogenesis of CVID remains unknown, a number of common abnormalities involving peripheral blood lymphocytes were described including differences in the number of naïve B cells (follicular B cells), $CD21^{low}$ B cells, transitional B cells, non-class-switched IgM/IgD memory B cells (marginal zone-like B cells) (Klein et al. 1997; Shi et al. 2003; Tangye & Tarlinton 2009), class-switched memory B cells and plasmablasts (Carsetti et al. 2004; Sanchez-Ramon et al. 2008; Warnatz & Schlesier 2008; Weller et al. 2004). Specifically, CVID patients have reduced populations of $CD27^+$ memory B cells (class-switched memory B cells and marginal zone-like B cells) and increased percentages of undifferentiated B cells (immature $CD21^{low}$ B cells (Rakhmanov et al. 2009) and naïve $CD27^-$ B cells) associated with impaired class switching (Piqueras et al. 2003; Warnatz et al. 2002) and poor differentiation into plasma cells (Taubenheim et al. 2005) when compared to a control population (Ferry et al. 2005; Litzman et al. 2007).

In addition, a vast array of T-cell abnormalities has been described in CVID patients, including defects in TCR-dependent T-cell activation (Thon et al. 1997), reduced frequency of antigen-specific T cells, impaired IL-2 release in $CD4^+$ T cells (Funauchi et al. 1995), decreased lymphocyte proliferation to mitogens and antigens (Chapel et al. 2008), lack of generation of antigen-primed T cells after prophylactic vaccination (Bryant et al. 1990; Fischer et al. 1994; Giovannetti et al. 2007), impaired cytokine production (Fischer et al. 1994; Thon et al. 1997), reduced expression of CD40L on activated T cells (Farrington et al. 1994; Piqueras et al. 2003; Thon et al. 1997; Warnatz et al. 2002), significant decrease in Treg cells in CVID patients with granulomatous manifestations and immune cytopenias (Horn et al. 2009), significant reduction of frequency and absolute counts of $CD4^+$ T cells, percentage increase in $CD8^+$ T cells, decrease in distribution of $CD4^+$ and $CD8^+$ naïve T cells in comparison to healthy controls (Giovannetti et al. 2007; Mouillot et al. 2010). This complex list of T-cell abnormality likely plays a major role in determining the clinical course of CVID patients.

In spite all of these multiple T-cell defects proposed as possible cause of CVID, the classification schemes presently in use are based on functional or phenotypic characteristics of B cells (assessment of immunoglobulin synthesis in vitro and phenotypic subsets of peripheral blood B cells): Bryant British classification (Bryant et al. 1990), Freiburg classification (Warnatz et al. 2002), Paris classification (Piqueras et al. 2003) and the recent EUROclass classification (Wehr et al. 2008). A few authors, however, suggested T-cell phenotyping as an aditional parameter for classifying CVID, and current efforts aim at the definition of combined T and B-cell phenotyping for the classification of CVID (Mouillot et al. 2010; Warnatz & Schlesier 2008).

Although a lot is known about B cell subsets in of CVID patients, the way their B-cell subpopulations change in response to vaccination compared to normal individuals is largely unknown. Specifically, there are limited data as to antibody responses to protein or polysaccharide antigens and the quantity and quality of antibodies produced by patient from different groups of CVID patients.

We focused on (1) specific *in vitro* antibody production by individual B cells following vaccinations by T-dependent (protein) and T-independent (polysaccharide) antigens and (2)

changes of B-cell subpopulation after vaccination in peripheral blood of CVID patients and healthy donors (Chovancova et al. 2011).

2. Methodological approach

2.1 Flow cytometry and assessment of plasmablasts

Blood samples from examine subjects were collected between 7 and 12 a.m. to exclude diurnal variation of lymphocyte subsets. Lymphocytes and B-cell subpopulations were analyzed directly from peripheral blood or from isolated PBMC (Litzman et al. 2007). The main B cell subpopulations identified in PBMCs were $CD21^{low}$ B cells characterized as $CD21^{low}CD38^{low}$, naïve B cells ($IgD^{+}CD27^{-}$), marginal zone-like B cells ($IgD^{+}CD27^{+}$), switched memory B cells ($IgD^{-}CD27^{+}$) and plasmablasts ($IgD^{-}CD27^{++}CD38^{++}$). Cells were identified using monoclonal antibodies (mAbs): FITC-conjugated anti-CD38, PE-conjugated anti-IgD, PE-conjugated anti-CD21, PC5-conjugated anti-IgM (all from *Pharmingen International, San Diego, CA, USA*) and PC5-conjugated anti-CD27 *(Beckman Coulter Miami, FL, USA)*. The B-cell subpopulations were analyzed by gating on $CD19^{+}$ cells (PC7-conjugated anti-CD19, *Beckman Coulter, Marseille, France*). Immunophenotyping of B lymphocytes was performed by five-colour cytometry Cytomix FC500 (*Beckman Coulter Miami, FL, USA*). The relative numbers of $CD19^{+}$ B cells are showed as mean ± SD.

2.2 Enzyme-linked immunosorbent spot assay (ELISPOT)

The ELISPOT assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information (Czerkinsky et al. 1983). We have modified the ELISPOT technique for the detection of specific antibody responses to TET and PPS.

96 wells microtitre plates (*MultiScreen™-HA, Millipore Corporation, Billerica, USA)* were coated with tetanus toxoid (*10 Lf/ml, ÚSOL, Prague, Czech Republic*) and PPS (*0.5 μg/ml, PNEUMO 23, Sanofi Pasteur, Lyon, France*) antigens in carbonate buffer (pH = 9.6) overnight at 4 °C. Plates were washed 3 times with PBS containing 0.05% Tween 20 and subsequently incubated for 30 minutes at 37 °C with 100 μl per well of blocking buffer (1% *solution of bovine serum albumin in PBS; Sigma Aldrich, Stenheim, Germany*). Plates were then stored at 4 °C until use. Peripheral blood mononuclear cells (PBMC), obtained from peripheral blood by gradient centrifugation (*Lymphoprep, Axis-Shields PoC AS, Oslo, Norway*) were added to the coated microtitre plates in RPMI 1640 medium (*Sigma Aldrich*) containing 10% heat-inactivated FCS (*LabMediaServis, Jaromer, Czech Republic*) at 4 different dilutions (1.25×10^5; 2.5×10^5; 5×10^5 and 1×10^6 in 100 μl/well for CVID patients and 0.625×10^5; 1.25×10^5; 2.5×10^5; 5×10^5 cells in 100 μl/well for controls and cultured overnight at 37 °C in 5% CO_2. After cells were washed off the plates 100 μl/well rabbit anti-human IgG, IgA or IgM conjugated to horseradish peroxidase (*Dako Cytomation, Glostrup, Denmark;* diluted 1:500 in PBS/Tween) were added to each well and incubated for 1h in the dark at room temperature. Plates were washed 3 times with PBS containing 0.05% Tween 20 followed by the addition of 100 μl/well of 3-amino-9-ethylcarbazole substrate solution (*AEC, Sigma Aldrich*) and incubated for 15 minutes at room temperature in the dark. Plates were rinsed with water and dried overnight at room temperature.

The red-coloured spots were counted with the AID ELISPOT reader (*AID, Autoimmun Diagnostika GmbH, Strassberg, Germany*). This provided accurate recognition and calculation

of the spots and allowed objective differentiation between background and "real" spots. The results were expressed as a number of SFC per million B cells.

2.3 Immunization of subjects

Thirty-seven patients with established CVID (14 males, 23 females, age range 20 – 74 years) were examined. Twenty-six patients were treated with regular infusions of intravenous immunoglobulin (IVIG), six patients received regular subcutaneous immunoglobulin (SCIG) injections and one patient intramuscular immunoglobulin therapy (IMIG). Four patients were newly diagnosed and not yet on immunoglobulin replacement therapy at the time of the study.

All CVID patients were vaccinated simultaneously with tetanus toxoid (TET) vaccine (*ALTEANA, Sevapharma, Prague, Czech Republic*) and unconjugated pneumococcal polysaccharide (PPS) antigens (*PNEUMO 23, Sanofi Pasteur, Lyon, France*), except patient no. 34, who received PPS one year after TET. All patients on IVIG were vaccinated one week prior to administration of replacement therapy.

The control group consisted of 80 healthy individuals. Fifty (16 males, 34 females, age range 22 – 72 years) were vaccinated with TET; ten (4 males, 6 females, age range 15 – 46 years) were given PPS alone; twenty (8 males, 12 females, age range 14 – 50 years) received both TET and PPS. The study was approved by the Ethics Committee of Masaryk University, Brno and signed informed consent was obtained from each participant.

2.4 Enzyme-linked immunosorbent assay (ELISA) and immunoglobulin quantification

Commercially available kits were used for measuring specific IgG antibody levels against tetanus toxoid (*VaccZyme™ Human Anti Tetanus Toxoid IgG EIA Kit, The Binding Site Group Ltd, Birmingham, United Kingdom*) and IgG antibodies titers against IgA (*Human Anti-IgA isotype IgG ELISA, BioVendor, Brno, Czech Republic*) in serum.

Trough serum levels of immunoglobulins IgG, IgA and IgM were measured in CVID patients prior to the IVIG infusion by nephelometry using the BN2 Nephelometer *(Dade Behring, Marburg, Germany)* according to the manufacturer's instructions.

2.5 Statistical analysis

Data were analyzed using the STATISTICA software [*StatSoft, Inc. (2007), STATISTICA (data analysis software system), version 8.0.*; www.statsoft.com]. Mann-Whitney U-test and Wilcoxon matched pairs test were used for analyses of dependencies between particular parameters in studied groups; $p < 0.05$ was regarded as statistically significant.

3. Laboratory findings

3.1 Kinetics and optimal timing for detection of specific spot forming cells isolated from peripheral blood after vaccination

The kinetics of anti-TET (T-dependent) specific antibody production by peripheral blood B cells was tested by ELISPOT assay in healthy volunteers from day 5 to day 9 after antigenic challenge. The same strategy was used in the assessment of anti-PPS (T-independent)

specific antibody production in healthy controls from day 1 to day 8 after antigen challenge. Day 7 was found to be optimal for the detection of specific antibody producing B-cells in peripheral blood for both antigens and all tested immunoglobulin isotypes (IgG, IgA, and IgM). Our findings are in agreement with previous studies (Kodo et al. 1984; Stevens et al. 1979; Thiele et al. 1982).

3.2 Kinetics and specific antibody responses against protein (T-dependent) and polysaccharide (T-independent) antigens in healthy individuals

The group of healthy controls was vaccinated with protein antigen (tetanus toxoid, TET), unconjugated PPS antigens (PNEUMO 23) either separately or in combination. We found no significant difference in the number of SFC (IgG, IgA, IgM) against vaccinated antigens whether they were administered separately or simultaneously (Mann-Whitney U-test, p with range between 0.56 to 0.98). The number of specific SFC against both types of vaccines in the cohort of healthy controls is shown in Table 1.

	SFC/10^6 B cells		
	MEDIAN	MINIMUM	MAXIMUM
IgG anti-TET	10 371	964	86 747
IgA anti-TET	532	24	9 707
IgM anti-TET	0	0	0
IgG anti-PPS	3 843	812	76 880
IgA anti-PPS	33 935	3 200	186 384
IgM anti-PPS	9 540	2 165	52 994

Table 1. The number of spot forming cells against protein (n=70) and polysaccharide (n= 30) antigens in a group of healthy controls. SFC/10^6 B cells (spot forming cells per million CD19+ B cells); IgG, IgA, IgM anti-TET (IgG, IgA, IgM antibodies specific spot forming cells against tetanus toxoid); IgG, IgA, IgM anti-PPS (IgG, IgA, IgM antibodies specific spot forming cells against pneumococcal polysaccharides)

3.3 Specific antibody response in subgroups of CVID patients

CVID patients (n = 37) were classified according to the Freiburg (Warnatz et al. 2002) and EUROclass classification (Wehr et al. 2008) (Table 2), allowing a comparative analysis of antibody production and clinical phenotype. As we had expected, the majority of our well-defined CVID patients did not mount a specific humoral immune response against the two vaccines but several patients produced low numbers of vaccine-specific SFC (see below).

As for EUROclass classification scheme, 3 patients of group smB+21^{norm} (n = 7, patient no. 18, 19, 20), 1 patient of group smB+21^{low} (n = 6, patient no. 13) and 1 patient of group smB-21^{low} (n = 12, no. 1) had detectable IgG antibody responses against tetanus toxoid. In group smB+21^{low} there was 1 patient (no. 14) who secreted IgM and another patient (no. 12) who formed IgA and IgM antibodies against PPS. The latter patient is the only one among the CVID group who formed specific antibodies of 2 different immunoglobulin isotypes. Regarding the group smB-21^{norm} (n = 12), no specific antibody production was detected. In the Freiburg classification all patients with detectable antibody responses (no. 12, 13, 14, 18, 19 and 20) were from group II the exception (no. 1) being a group Ia patient.

number	Freiburg classification	EURO classification	sex	age	replacement therapy	IgG	IgA	IgM	IgG anti-IgA	IgG anti-TET	IgA anti-TET	IgM anti-TET	IgG anti-PPS	IgA anti-PPS	IgM anti-PPS
						g/l			titer	SFC/10^6 B cells					
1	Ia	smB-21^{low}	F	40	IMIG	2.78	<0.01	< 0.04	neg	344	0	0	0	0	0
2	Ia	smB-21^{low}	F	74	IVIG	5.39	<0.01	< 0.05	neg	0	0	0	0	0	0
3	Ia	smB-21^{low}	M	47	SCIG	3.92	0.06	0.19	neg	0	0	0	0	0	0
4	Ia	smB-21^{low}	M	50	IVIG	3.37	<0.01	< 0.05	neg	0	0	0	0	0	0
5	Ia	smB-21^{low}	M	36	IVIG	7.35	<0.01	0.05	neg	0	0	0	0	0	0
6	Ib	smB-21^{norm}	F	66	IVIG	5.82	<0.01	0.10	n. d.	0	0	0	0	0	0
7	Ib	smB-21^{norm}	F	34	no	2.01	<0.01	< 0.05	neg	0	0	0	0	0	0
8	Ib	smB-21^{norm}	M	30	IVIG	5.77	<0.01	< 0.05	neg	0	0	0	0	0	0
9	Ib	smB-21^{norm}	F	20	no	0.66	<0.01	0.13	1:50	0	0	0	0	0	0
10	Ib	smB-21^{norm}	F	44	IVIG	3.16	<0.01	0.10	neg	0	0	0	0	0	0
11	Ib	smB-21^{norm}	M	55	IVIG	5.09	<0.01	0.32	neg	0	0	0	0	0	0
12	II	smB+21^{low}	F	71	IVIG	6.29	0.08	0.29	neg	0	0	0	0	2562	659
13	II	smB+21^{low}	M	19	IVIG	4.49	<0.01	0.07	neg	407	0	0	0	0	0
14	II	smB+21^{low}	M	24	no	4.99	<0.01	0.20	neg	0	0	0	0	0	104
15	II	smB+21^{low}	F	54	IVIG	6.95	<0.01	0.15	1:100	0	0	0	0	0	0
16	II	smB+21^{low}	F	41	SCIG	6.14	<0.01	0.05	neg	0	0	0	0	0	0
17	II	smB+21^{low}	M	57	SCIG	5.66	0.02	< 0.05	neg	0	0	0	0	0	0
18	II	smB+21^{norm}	F	19	SCIG	6.75	0.05	0.75	neg	185	0	0	0	0	0
19	II	smB+21^{norm}	M	44	IVIG	5.91	0.22	< 0.05	neg	713	0	0	0	0	0
20	II	smB+21^{norm}	M	44	IVIG	6.37	<0.01	0.10	neg	231	0	0	0	0	0
21	II	smB+21^{norm}	M	31	IVIG	3.80	0.02	0.07	n. d.	0	0	0	0	0	0
22	II	smB+21^{norm}	F	68	IVIG	6.35	<0.01	< 0.05	neg	0	0	0	0	0	0
23	II	smB+21^{norm}	M	59	IVIG	6.86	0.04	0.06	neg	0	0	0	0	0	0
24	II	smB+21^{norm}	F	41	IVIG	5.26	<0.01	0.08	neg	0	0	0	0	0	0

Table 2. Continues on next page

number	Freiburg classification	EURO classification	sex	age	replacement therapy	IgG	IgA	IgM	IgG anti-IgA	IgG anti-TET	IgA anti-TET	IgM anti-TET	IgG anti-PPS	IgA anti-PPS	IgM anti-PPS
						g/l			titer	SFC/10^6 B cells					
25	II	smB-21^{low}	F	42	IVIG	8.15	<0.01	0.50	neg	0	0	0	0	0	0
26	II	smB-21^{low}	F	58	IVIG	5.98	<0.01	< 0.05	neg	0	0	0	0	0	0
27	II	smB-21^{low}	F	57	IVIG	8.15	<0.01	< 0.05	neg	0	0	0	0	0	0
28	II	smB-21^{low}	M	34	IVIG	6.21	<0.01	0.05	neg	0	0	0	0	0	0
29	II	smB-21^{low}	F	43	IVIG	6.27	<0.01	< 0.05	neg	0	0	0	0	0	0
30	II	smB-21^{low}	F	50	SCIG	4.48	<0.01	< 0.05	neg	0	0	0	0	0	0
31	II	smB-21^{low}	F	28	IVIG	5.62	<0.01	< 0.05	neg	0	0	0	0	0	0
32	II	smB-21^{norm}	F	44	IVIG	6.75	<0.01	0.05	neg	0	0	0	0	0	0
33	II	smB-21^{norm}	F	61	IVIG	7.54	0.09	< 0.05	neg	0	0	0	0	0	0
34	II	smB-21^{norm}	F	27	no	2.35	<0.01	0.45	neg	0	0	0	0	0	0
35	II	smB-21^{norm}	F	40	IVIG	7.10	<0.01	0.09	neg	0	0	0	0	0	0
36	II	smB-21^{norm}	F	40	SCIG	8.51	<0.01	< 0.05	neg	0	0	0	0	0	0
37	II	smB-21^{norm}	M	19	IVIG	7.00	<0.01	< 0.05	neg	0	0	0	0	0	0

Table 2. (continued) Results of the ELISPOT assay in group of CVID patients. F (female); M (male); n.d. (not done); SFC/10^6 B cells (spot forming cells per million $CD19^+$ B cells); IgG, IgA, IgM anti-TET (IgG, IgA, IgM specific spot forming cells against tetanus toxoid); IgG, IgA, IgM anti-PPS (IgG, IgA, IgM specific spot forming cells against pneumococcal polysaccharides); IgG anti-IgA (IgG antibodies against IgA)

The decreased production of SFC in CVID patients was independent of replacement immunoglobulin treatment: four CVID patients without substitution therapy showed the same defect in the production of SFC and specific antibodies after vaccination as CVID patients on replacement therapy.

3.4 Changes of B-cell subpopulations in peripheral blood one week after vaccination

The mean percentage of $CD19^+$ B cells was 11 ± 4 % in healthy controls and 13 ± 7.6 % in CVID patients before vaccination. One week after vaccination the percentages were unchanged (12 ± 5 % in healthy controls and 13 ± 6.7 % in CVID patients).

We then examined the changes of absolute and relative numbers of plasmablasts and other B lymphocyte subpopulations in the peripheral blood one week after antigen challenge (Fig. 1, 2). In healthy controls no statistically significant changes in absolute and relative numbers of switched memory B cells were found between the two measurement time points, before

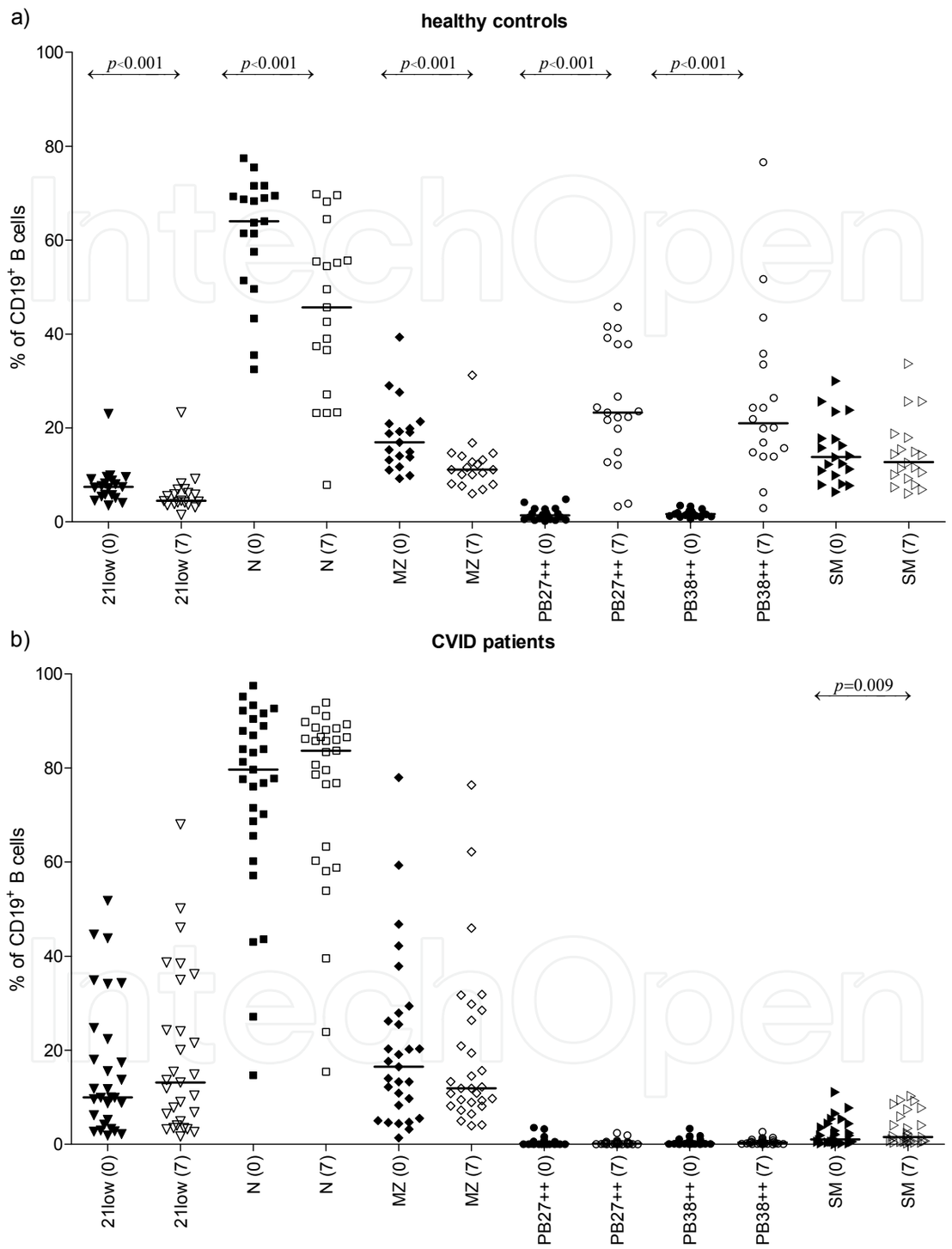

Fig. 1. Changes of relative numbers of B-cell subpopulations in healthy controls (HC; n = 19; a) and CVID patients (CVID; n = 29; b) before (0) and one week (7) after antigen challenge. 21^{low} ($CD21^{low}$ B cells), N (naïve B cells), MZ (marginal zone - like B cells), $PB27^{++}$ and $PB38^{++}$ (plasmablasts), SM (class-switched memory B cells)

and one week after vaccination. However, a highly significant increase in absolute as well as relative numbers of plasmablasts gated as IgD^{-}CD27^{++} (PB CD27^{++}) cells and IgM^{-}CD38^{++} (PB CD38^{++}) cells ($p<0.001$ in both cases) occurred (Fig. 3), while the absolute and relative numbers of CD21^{low} B cells ($p<0.02$), naïve B cells ($p<0.001$) and MZ-like B cells ($p<0.001$) decreased. In contrast, among the cohort of CVID patients no statistically significant changes of examined cellular subpopulations, including plasmablasts (Fig. 1, 2 and 3) were observed except for a slight increase in smB cells to a level still well below the levels of healthy controls. This increase was statistically significant in Wilcoxon matched pairs test.

The fact that the number of plasmablasts corresponds with the number of SFC strongly suggest that the examination of peripheral blood plasmablasts on day 7 after vaccination can be used as a surrogate marker for specific antibody responses in normal controls and as a diagnostic procedure to identified CVID and other patients with defect in terminal B-cell differentiation (Chovancova et al. 2011).

4. Consequences

4.1 Hypogammaglobulinaemic patients and diagnostic vaccination

Poor vaccination responses to protein and polysaccharide antigens is essential for definition-based diagnosis of CVID (Conley et al. 1999). Quantitative assessment of specific antibody in serum is routinely performed by ELISA assay. However CVID patients are often started on immunoglobulin substitution therapy before antibody production is adequately evaluated. In such a situation, it is difficult to segregate transferred from antigen-induced specific antibody. We have designed a *in vitro* functional measurement of antibody production on the B-cell level using the ELISPOT technique, which is independent of substitution therapy (Chovancova et al. 2011). In addition, we monitored changes in B-cell subpopulations, including plasmablasts, in peripheral blood by flow cytometry after in vivo antigenic challenge.

The defect in the antibody production and SFC reduction observed in a cohort of CVID patients are not secondary to Ig substitution since the same defects were also seen in four CVID patients before starting Ig replacement therapy. IVIG treated CVID patients were vaccinated exactly one week before administration of immunoglobulin substitution. In this manner the theoretically possible influence of immunoglobulin replacement therapy on the generation of SFC was reduced.

Prior to this study, specific antibody production in substituted CVID patients following vaccination had been evaluated in serum. Goldacker et al. (Goldacker et al. 2007) measured specific antibodies in serum by ELISA assay. The contribution of parallel immunoglobulin substitution on antibody titers was difficult to correct and required a relatively complicated vaccination formula. Based on these calculations the authors reported a decrease in serum antibody levels against T-dependent and T-independent antigens in CVID patients between IVIG infusions. Using a meningococcal polysaccharide vaccine, Rezaei et al. described decreased vaccination response against meningococcal polysaccharide measured in serum of CVID patients while on IVIG (Rezaei et al. 2008; Rezaei et al. 2010). Immunization with a protein neoantigen, e.g. bacteriophage, and investigation of immune response with neutralization assay brought similar results (Ochs et al. 1971). Nevertheless, there is very little quantitative data correlating individual vaccination responses to proposed functional

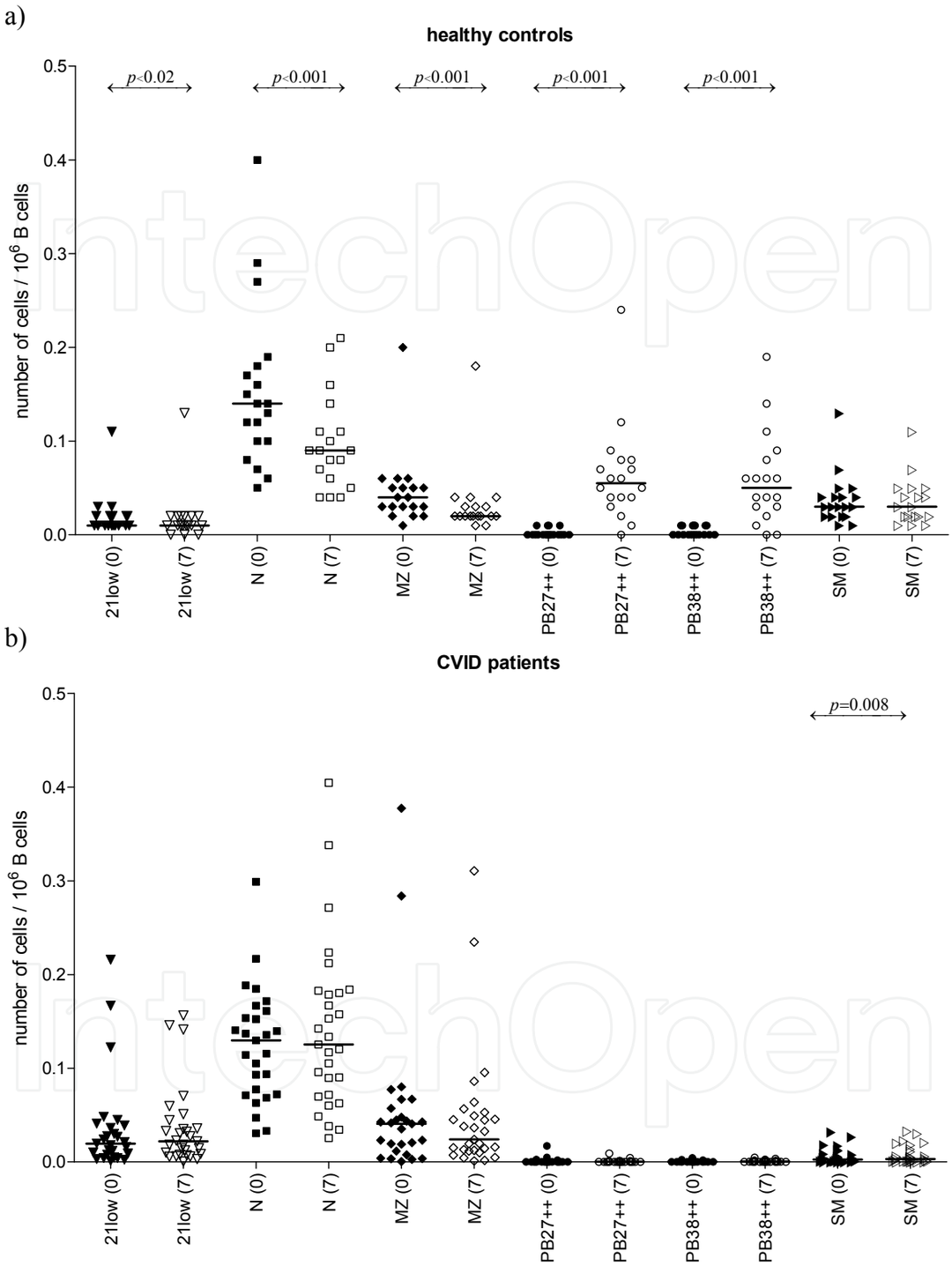

Fig. 2. Changes of absolute numbers of B-cell subpopulations in healthy controls (HC; n = 19; a) and CVID patients (CVID; n = 29; b) before (0) and one week (7) after antigen challenge. 21^{low} ($CD21^{low}$ B cells), N (naïve B cells), MZ (marginal zone - like B cells), $PB27^{++}$ and $PB38^{++}$ (plasmablasts), SM (class-switched memory B cells)

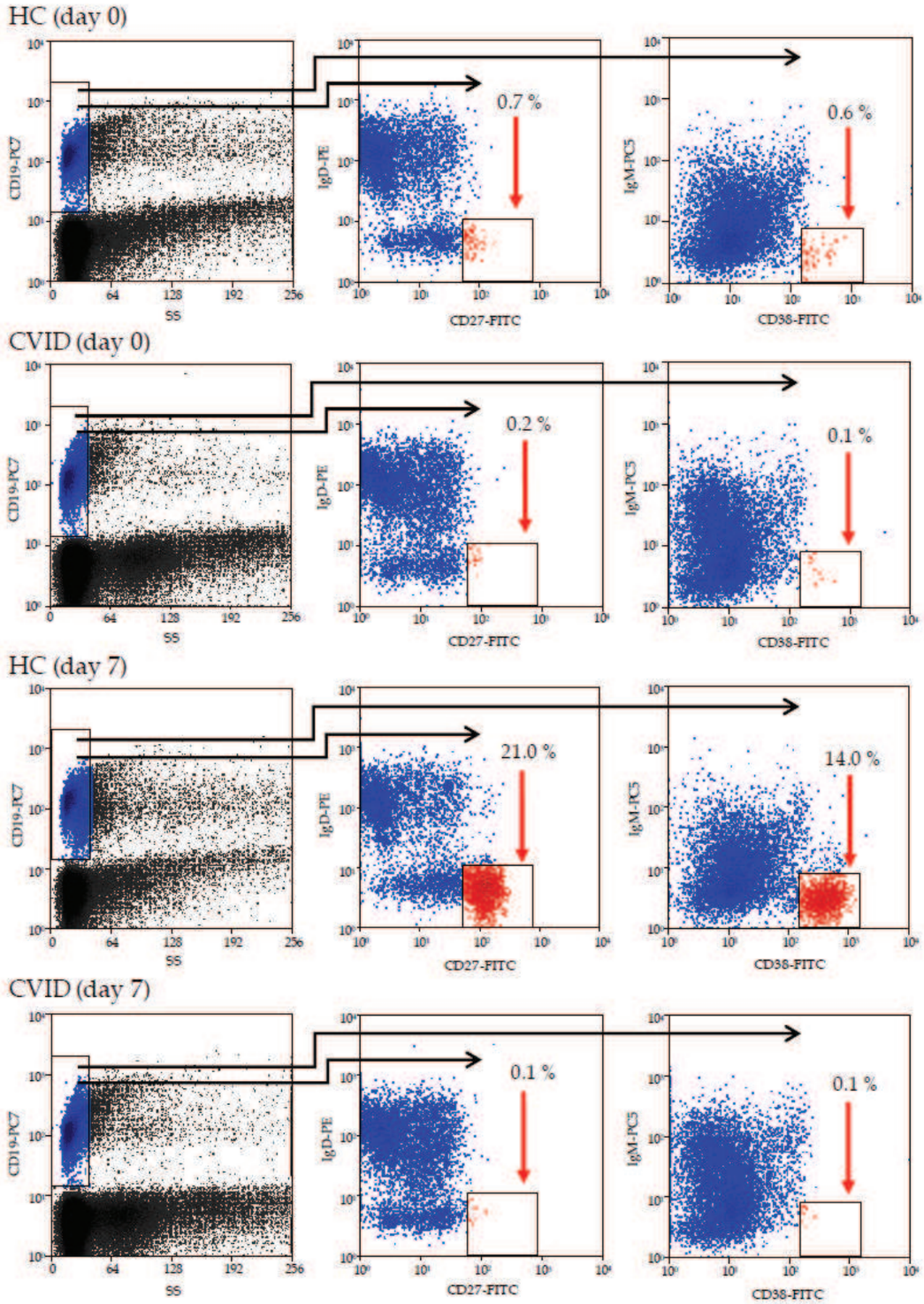

Fig. 3. Development of plasmablasts after vaccination. Plasmablasts (red arrows) were gated from $CD19^{+}$ B cells (gate in column 1) as $IgD^{-}CD27^{++}$ (column 2) and $IgM^{-}CD38^{++}$ (column 3). The cells were investigated before (day 0) and on day 7 after vaccination. HC – healthy control; CVID – CVID patient, $PB27^{++}$ and $PB38^{++}$ – plasmablasts

classifications of CVID (Goldacker et al. 2007; Rezaei et al. 2008). Our group of CVID patients was arranged according to the Freiburg (Warnatz et al. 2002) and EUROclass classification (Wehr et al. 2008). As expected, the majority of our well-defined CVID patients (30/37) failed to mount a specific humoral immune response when analysed by SFCs collected from peripheral blood before and after immunization. The seven CVID patients who responded had much smaller quantities of specific SFC compared to healthy donors. All but one patient with measurable antibody responses belong to group II of the Freiburg classification or EUROclass group smB^+ which represent those CVID patients with nearly normal numbers of class-switched memory B cells. Patients in these groups are characterized by milder complications of the disease compared to other groups (Alachkar et al. 2006; Wehr et al. 2008).

4.2 Novel diagnostic tool using flow cytometry in hypogammaglobulinaemic patients with vaccination

During the last few years a number of studies described differences between B-cell subpopulations of CVID patients and those of healthy volunteers but the kinetics of these changes after encounter with an antigen in vivo (Pinna et al. 2009) has not previously been explored. We investigated the dynamic changes of $CD21^{low}$ B cells, naïve B cells, marginal zone-like B cells, plasmablasts and switched memory B cells of CVID patients compared to healthy donors (Chovancova et al. 2011). Previous studies showed that memory B cells and plasmablasts have different kinetics in peripheral blood (Stevens et al. 1979). Plasmablasts reach their peak on day 7 after encounter with the antigen in peripheral blood while switched memory B cells showed a marked increase in number on day 14 after antigen challenge (Pinna et al. 2009). The absolute number of naïve B lymphocytes is determined by the generation of new naïve B cells from the bone marrow pool (a slow process) and by acute loss of naïve B lymphocytes via further maturation after antigen encounter (Agenes et al. 2000). Statistically significant up-regulation of naïve B cells and its continued accumulation after antigen challenge in CVID patients indicates disturbed conversion of undifferentiated B cells to more mature B-cell stages in germinal centers. Differentiation is crucially dependent on T-lymphocyte help, suggesting that the basic defects in the majority of CVID patients are not in B cells but in helper T-lymphocytes (Borte et al. 2009; Fischer et al. 1994; Fischer et al. 1996; Thon et al. 1997).

The reduced numbers of switched memory B cells which correlate with clinical complications (Ko et al. 2005; Viallard et al. 2006) and failure to increase the number of plasmablasts after antigen challenge may be explained by insufficient signals from helper T cells of CVID patients. In previous studies we and others have shown that B cells of CVID patients are able to produce antibodies if they are exposed *in vitro* to helper T-lymphocyte from healthy donors or to appropriate cytokines (Borte et al. 2009; Fischer et al. 1994; Fischer et al. 1996; Thon et al. 1997), [55]. Taubenheim et al. studied B-cell differentiation in lymph nodes from three CVID patients with splenomegaly and found distinct blocks in terminal plasma cell development but normal expression of a key regulator of terminal plasma cell differentiation, Blimp-1 (Taubenheim et al. 2005). Moreover, the clinically important observation that B cells from CVID patients may produce antibodies under certain circumstances correlates with the fact that CVID patients lacking IgA are able to generate IgG anti-IgA antibodies in vivo (Horn et al. 2007). Among our cohort of vaccinated CVID patients, two patients from subgroups Ib and II (Table 2) produced IgG anti-IgA antibodies in low titers although these two patients did not respond to vaccination.

Our observation that the majority of CVID patients lack antigen specific spot forming B cells and fail to increase circulating plasmablasts following *in vivo* antigen challenge provides a rapid screening test to demonstrate defective antibody responses in CVID patients, even when on replacement IVIG therapy (Chovancova et al. 2011).

5. Conclusion and clinical implications

Identification of circulating plasmablasts after vaccination is a new simple flow based test to assess antibody responses in hypogammaglobulinaemic patients, even if on immunoglobulin (IVIG or SCIG) replacement therapy.

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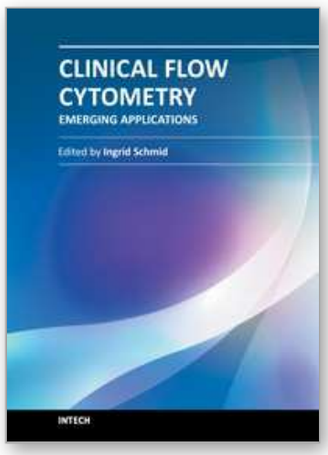

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