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Review Article

# Flowcytometric Phenotyping of Common Variable Immunodeficiency

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**Background: Common variable immunodeficiency (CVID) comprises heterogeneous antibody deficiency disorders. To classify this heterogeneous syndrome, clinical as well as immunologic parameters have been combined. Flowcytometric analysis of circulating T and B lymphocyte subpopulations has become an important tool in this endeavor of disease classification.**

**Methods: Multicolor flowcytometric analysis of circulating lymphocytes.**

**Results: The flowcytometric analysis of B and T cell subpopulations in the blood of CVID patients has contributed significantly to the identification of separate groups within the CVID population. In addition, the flowcytometric analysis of the inducible costimulator on activated T cells, CD19 and BAFF-R on B cells are valid screening methods for three of the four known genetic defects associated with CVID. Only TACI deficiency can not be sufficiently detected by flowcytometric measures.**

**Conclusions: Flowcytometric classification of patients with CVID has become a standard procedure during the diagnostic work up. This should be performed according to common guidelines to guarantee world wide comparability between different immunodeficiency centers.** © 2008 Clinical Cytometry Society

**Key terms: CVID; B cell; T cell; flow cytometry; classification; ICOS; TACI; BAFF-R; CD19+**

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## COMMON VARIABLE IMMUNODEFICIENCY

Common variable immunodeficiency (CVID) comprises various antibody deficiency disorders. It usually manifests during the second and third decade by recurrent bacterial infections of the upper and lower respiratory tract. The diagnosis is defined by the severe reduction of at least two immunoglobulin isotypes, a poor response to vaccination, the onset after the second year of life, and the exclusion of defined differential diagnosis (www.esid.org) (Table 1).

The term was first coined in 1973 (1) differentiating CVID from other defined primary antibody deficiency syndromes including the class-switch recombination deficiency syndromes (2), comprising CD40 ligand (3), CD40 (4), AID (5), UNG (6) deficiency, and a subgroup with undefined forms of class-switch recombination defects, agammaglobulinemia, including X-linked agammaglobulinemia Bruton (XLA) (7) and autosomal recessive forms affecting the pre B cell receptor expression or function (8), and selective IgA deficiency (sIgAD) (9). This definition rendered CVID a very heterogeneous mixture of antibody deficiency disorders. Therefore classification schemes of pathogenetic and clinical relevance for a better comprehension of CVID were warranted from the beginning. Already these were based on phenotyping of B and T lymphocytes (10), but also functional assays examining the capacity of B cells to proliferate or/and produce immunoglobulins in vitro (11,12) or of T cells to produce cytokines in vitro (13).

Since functional assays in clinical settings are laborious and often difficult to standardize, only few centers performed the assays on a routine basis. With the increasing availability of flowcytometry in the clinical diagnostic laboratories, increasing choice of antibodies for staining

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Table 1
*Diagnostic Criteria of Common Variable Immunodeficiency (CVID)*

| |
|---|
| Male or female patient who has a marked decrease of IgG (at least 2 SD below the mean for age) and a marked decrease in at least one of the isotypes IgM or IgA, and fulfills all of the following criteria: |
| 1. Onset of immunodeficiency at greater than 2 years of age |
| 2. Absence of isohemagglutinins and/or poor response to vaccines |
| 3. Defined causes of hypogammaglobulinemia have been excluded |
| Differential diagnosis of hypogammaglobulinemia |
| *Drug induced* |
| Antimalarial agents, Captopril, Carbamazepine, Glucocorticoids, Fenclofenac, Gold salts, Penicillamine, Phenytoin, Sulfasalazine |
| *Genetic disorders* |
| Ataxia Telangiectasia |
| Autosomal or X linked forms of SCID |
| Class Switch recombination defects (CD40L, CD40, AID, UNG) |
| Transcobalamin II deficiency and hypogammaglobulinemia |
| Agammaglobulinemia (btk, μ chain, a.o.m.) |
| X-linked lymphoproliferative disorder (XLP) |
| Some metabolic disorders |
| Chromosomal anomalies (Chr. 18q- Syndrome, Monosomy 22, Trisomy 8 or 21) |
| Myotonic dystrophy (DM1 and DM2) |
| *Infectious diseases* |
| Congenital infection with HIV or rubella |
| (Congenital infection with) CMV or *Toxoplasma gondii* |
| Epstein-Barr virus |
| *Malignancy* |
| Chronic Lymphocytic Leukemia |
| Immunodeficiency with Thymoma |
| Non Hodgkin's lymphoma |
| *Systemic disorders* |
| Immunodeficiency caused by hypercatabolism of immunoglobulin |
| Immunodeficiency caused by excessive loss of immunoglobulins (nephrosis, severe burns, lymphangiectasia, severe diarrhea) |

The table is adapted from http://www.esid.org/workingparty.php?party = 3&sub = 2&id = 73#Q5.

of surface markers permitting precise discrimination of lymphocyte subpopulations and our better understanding of the immune system flowcytometric characterization of circulating lymphocytes became the primary classification scheme for CVID.

## B CELL DEVELOPMENT—FROM PRECURSOR TO ANTIBODY PRODUCING PLASMA CELL

B lymphocytes are generated via several sequential steps of differentiation (Fig. 1).

### Central B Cell Development

The early development of human pro and pre B cells from pluripotent stem cells is antigen independent and takes place in the bone marrow.

Pro B cells ($CD19^-CD10^{+/-}CD20^-CD22^+CD24^-$ $vpreB^-Ig\alpha^{-/+}$) are characterized by the expression of the first B cell specific surface marker CD22. Late pro and early pre B cells commence assembling the immunoglobulin surface receptor (BCR) and its signaling components. The completed rearrangement of the heavy chain and pairing with the surrogate light chain on the surface as the Pre B cell receptor (preBCR) marks the pre B cell stage (14). Pre B cells ($CD19^+$ $CD10^+CD20^-CD24^{++}vpreB^+Ig\alpha^+$ intracellular $\mu^+$) expressing correctly assembled preBCR on the surface are positively selected for proliferation and further differentiation. Deletion of B cell specific transcription factors BSAP, E2A, EBF, responsible for the transcription of the BCR, the recombinase-activating genes RAG1 and -2, responsible for the rearrangement of the μ-heavy (and later light) chains, pre BCR components (μ-chain, Igα, Igβ, λ5, vpreB) and downstream signaling components, like Syk, Slp65, and btk, cause severe blocks at the pro-/pre B cell transition (15). Defects of some of these signaling molecules have been identified in immunodeficient humans (8).

After the successful assembly of the preBCR and several rounds of expansion, kappa or lambda light chains replace the surrogate light chain. The pairing of the μ heavy chain and a light chain finally leads to the surface expression of IgM, the hallmark of immature B cells. Immature B cells ($CD19^+CD10^+CD20^+CD24^{++}IgM^+$) are most likely positively selected for the successful recombination of heavy and light chain. Simultaneously, the expression of the BCR on the surface allows for the first time antigen-specific negative selection. At this stage the central development of B cells is accomplished and so-called transitional B cells leave the bone marrow for full maturation in secondary lymphoid organs, especially the spleen. Neither pro-, pre-, nor immature B cells are found in the circulation of healthy humans.

### Peripheral B Cell Development

Only 10–20% of immature B cells produced in the bone marrow reach the spleen (16). A large proportion (17) is deleted due to the expression of autoreactive BCRs. After emerging from the bone marrow murine peripheral B cells pass through several consecutive developmental stages termed transitional B cell T1-2 or 3 (18,19). Transitional type 1 (T1) B cells ($IgM^{hi}IgD^-$ $CD21^-CD23^-$) resemble the immature B cells of the bone marrow. In the splenic environment T1 B cells differentiate into T2 B cells ($IgM^{hi}IgD^+CD21^{int}CD23^+$). Beside BCR-derived signals, BAFF (synonym: Blys) and its receptor BAFF-R (synonym: BR3) (20) have been shown to be key regulators for the survival at this stage. BAFF-R belongs to the TNF receptor family and is almost exclusively expressed on B cells. While early precursors have no detectable expression, transitional T2 B cells are the first to express BAFF-R on the surface (21,22). The development of human transitional B cell seems to resemble the described murine differentiation at least in part (23).

After passing the transitional stage, the human mature B cell compartment consists of follicular B cells and mar-

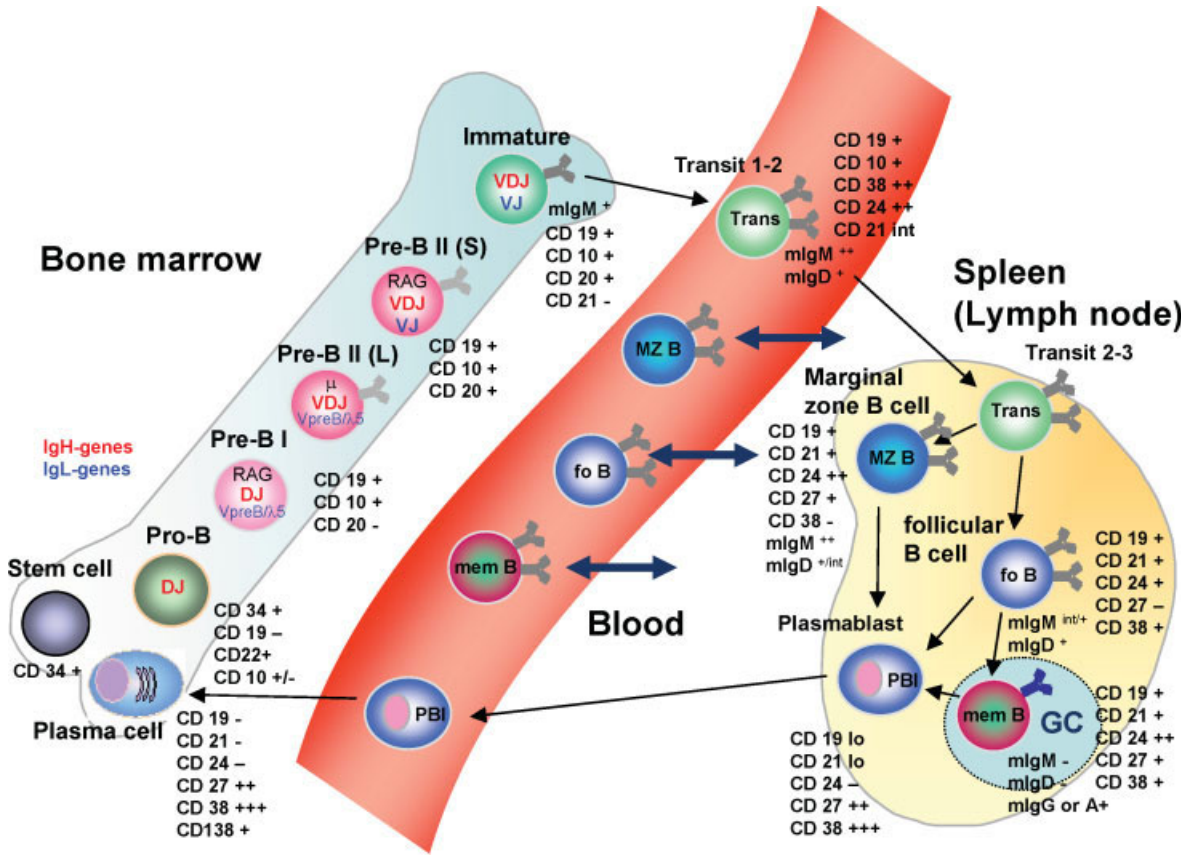


FIG. 1. Scheme of B cell development. The central and peripheral B cell developments are outlined. "Blood" represents circulating B cell populations. Relevant (not all!) surface markers for each differentiational step are listed. Please refer to text for more details. fo B cell, follicular B cell; GC, germinal center; mem B, memory B cells; MZ B cell, marginal zone B cell; Pbl, plasmablast; Trans or Transit, transitional B cell.

ginal zone B cells. It is not known whether a human equivalent of the murine B1 B cell compartment exists. The murine marginal zone of the spleen contains a sessile $IgM^{hi}CD21^{hi}CD23^{lo}CD9^{+}$ B cell population (MZ B cells) which is a crucial player of the early antibody response, especially to blood-borne, T independent but also dependent antigens (24).

In humans, MZ B cells have been identified as $CD27^{+}IgM^{hi}$ B cells and represent about 15–25% of the circulating B cell pool (25). BCR specificities for polysaccharides like pneumococcal polysaccharides (PnPS) are enriched among the MZ B cells and a decrease of circulating MZ B cells is associated with a poor PnPS vaccination response (26). In humans, $CD27^{+}IgM^{+}$ B cells are present already during the first 2 years of life when children still fail to respond to PnPS vaccination (25).

Follicular B cells are named after their localization in follicles of the spleen or lymph node. Before antigen encounter, follicular naive B cells express CD23, CD21, $IgM^{int}$, and IgD, but no CD27. Follicular B cells represent the main B cell population in the blood and secondary lymphoid tissues of mice and men. Most of their BCRs recognize proteins and therefore follicular B cells are typically recruited in T cell dependent responses. This allows for the participation in germinal center reaction and differentiation into long-lived memory B cells or plasma cells.

### Antigen Driven B Cell Differentiation of Mature B Cell Compartments

Depending on BCR signal strength, activation of costimulatory receptors, and other factors, antigen activation of mature B cell leads either to death or further differentiation into the memory or plasma cell pool.

When successfully activated, B cells enter the FDC network inside the B cell follicles and give rise to germinal centers around day 3–5 of T dependent antibody responses (27). Germinal centers are a highly specialized niche, crucial for class switch recombination, affinity maturation, selection, and expansion of antigen-specific B cell clones (28). Additional T cell derived signals, foremost by CD40L, allow these cells to survive and acquire characteristics of memory B cells. Other stimuli including IL10, IL21 (29) induce a part of germinal center cells to enter an alternative pathway of differentiation generating plasma cells (30). During the germinal center phase B cells go through several stages (BM1-5) (31), of which only the pre (naive, BM1-2) and post germinal center (memory, BM5) stages can be found in the circulation.

### B Cell Memory

While the identification of murine B cell memory suffered from the lack of specific surface markers, human memory B cells can be identified by the expression of CD27 (32).About 16–55% (5–95% confidence interval) of circulating B cells are $CD27^+$ B cells of which about 50% express IgM and IgD while the rest has undergone class switch recombination with only a very low percentage of post germinal center IgM-only ($IgM^+IgD^-CD27^+$) B cells in the peripheral blood. Several lines of evidence point out that the $IgM^+IgD^+CD27^+$ B cells represent marginal zone B cells (see above) and only $IgD^{neg}$, mostly class-switched $IgM^-IgD^-$ $CD27^+$ B cells are true post germinal center memory B cells. Humans with defective germinal center reaction due to CD40L− or ICOS defect lack this population completely (see below).

### Plasma Cell

Plasma cells emerge from extrafollicular as well as germinal center responses. During primary antigen encounter plasmablasts form antibody forming foci (AFC) in the medullary cords of lymph nodes and in the red pulp of the spleen (33). These plasma cells can switch to IgG1, carry only few SHM and are short lived (33). In contrast, postgerminal center responses can create a long lasting humoral immunity. Plasma cells are not circulating and only few plasmablasts are detectable in the peripheral blood. The number depends on recent activation and increases around day 6 after vaccination to return soon back to below 2% (34).

## B CELL PHENOTYPING OF CVID

Based on the rationale that in CVID intrinsic or extrinsic disturbances of B cell differentiation underlie the failure of differentiation into plasma cells and therefore antibody production a flowcytometry based phenotyping of circulating B cells was suggested for the classification of this syndrome. Before extensive B cell phenotyping, a preliminary screening by a small lymphocyte panel including the enumeration of CD4 and CD8 T cells, CD19 B cells, as well as NK cells is highly recommended. This also serves the calculation of absolute numbers of the various subpopulations. Flowcytometric B cell phenotyping distinguishes up to six different circulating subpopulations by the combination of surface markers as indicated in Table 2. Further technical details are described in (35) (English version in press).

B cells can be identified in the lymphocyte gate by the expression of different surface markers like CD19, CD20, or CD22. The most commonly used marker is CD19 which is present on all circulating B cell populations (Fig. 2a).

After gating on CD19 positive cells the detection of IgM, IgD, and CD27 expression is used to distinguish naïve ($CD19^+CD27^-IgM^+IgD^+$), marginal zone like ($CD19^+CD27^+IgM^{++}IgD^+$), class-switched ($CD19^+$ $CD27^+IgM^-IgD^-$), and IgM only ($CD19^+CD27^+IgM^{++}$ $IgD^-$) memory B cells (Fig. 2b). The combined staining for CD19, CD21, CD38, and IgM permits the additional distinction of transitional B cells ($CD19^+CD21^{int}$ $CD38^{++}IgM^{++}$), plasmablasts ($CD19^{low}CD21^{int}CD38^{+++}$ $IgM^{-(+)}$), and an unusual $CD21^{low}$ expressing B cell ($CD19^{++}CD21^{low}CD38^{low}IgM^+$) population (Fig. 2c) which is rare in healthy donors. While transitional B cells and $CD21^{low}$ B cells are part of the CD27 negative "naïve" B cell population, plasmablasts express high levels of CD27 (36) and fall into the CD27 positive "memory" gate. They need to be adjusted for while determining the percentage of memory B cells. But usually only very few plasmablasts are present in the circulation, most of the time no adjustment is necessary. Normal ranges of the percentage (% of B cells) of each B cell subpopulation are indicated in Table 3.

Table 2
*Antibody Panel for 4-Color B Cell Phenotyping*

| Panel | FITC | PE | PE-Cy7 | Cy5/APC |
|---|---|---|---|---|
| B1 | CD27 | Anti-IgD | CD19 | Anti-IgM |
| B2 | CD38 | CD21 | CD19 | Anti-IgM |

The following antibodies are tested by the authors, but can be replaced by suitable alternatives. CD19 (J4.119, IM3628, Beckman-Coulter, Krefeld, Germany), CD21 (B-ly4, 555422), CD38 (HIT2, 555459, BD-Biosciences, Heidelberg, Germany), CD27 (M-T271, F7178, DakoCytomation, Hamburg, Germany), anti-IgD (Goat F(ab′)2, 2032-09, SouthernBiotech, Biozol, Eching, Germany), anti-IgM (Goat F(ab′)2, 109-176-129, Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany).

The staining for CD21 and CD38 surface expression should be performed on a regular basis for the evaluation of transitional and $CD21^{low}$ B cells, which play an essential part in the classification (see below). The interpretation of all findings has to acknowledge the available clinical information including age, previous and current medical therapy, comorbidities, and the potential mode of inheritance.

It also needs to be mentioned that the classification was originally developed on Ficoll-isolated peripheral blood mononuclear cells (PBMC). A recent publication using a whole blood method found an excellent correlation between these methods (37). For technical details see Ferry et al. (37).

### Freiburg Classification

The original Freiburg classification (Fig. 3a) was developed to discriminate patients with disturbed germinal center dependent differentiation of memory B cells as well as patients with defects of early peripheral B cell differentiation by analyzing CD21 expression. Therefore the Freiburg classification (38) groups patients with more than 1% $CD19^+$ B cells within the lymphocyte gate (CD45 vs. SSC) into type I patients with severely reduced class-switched memory $CD27^+IgM^-IgD^-$ B cells (<0.4% of lymphocytes), and type II patients with nearly normal number of class-switched memory B cells (≥0.4% of lymphocytes). The calculation of

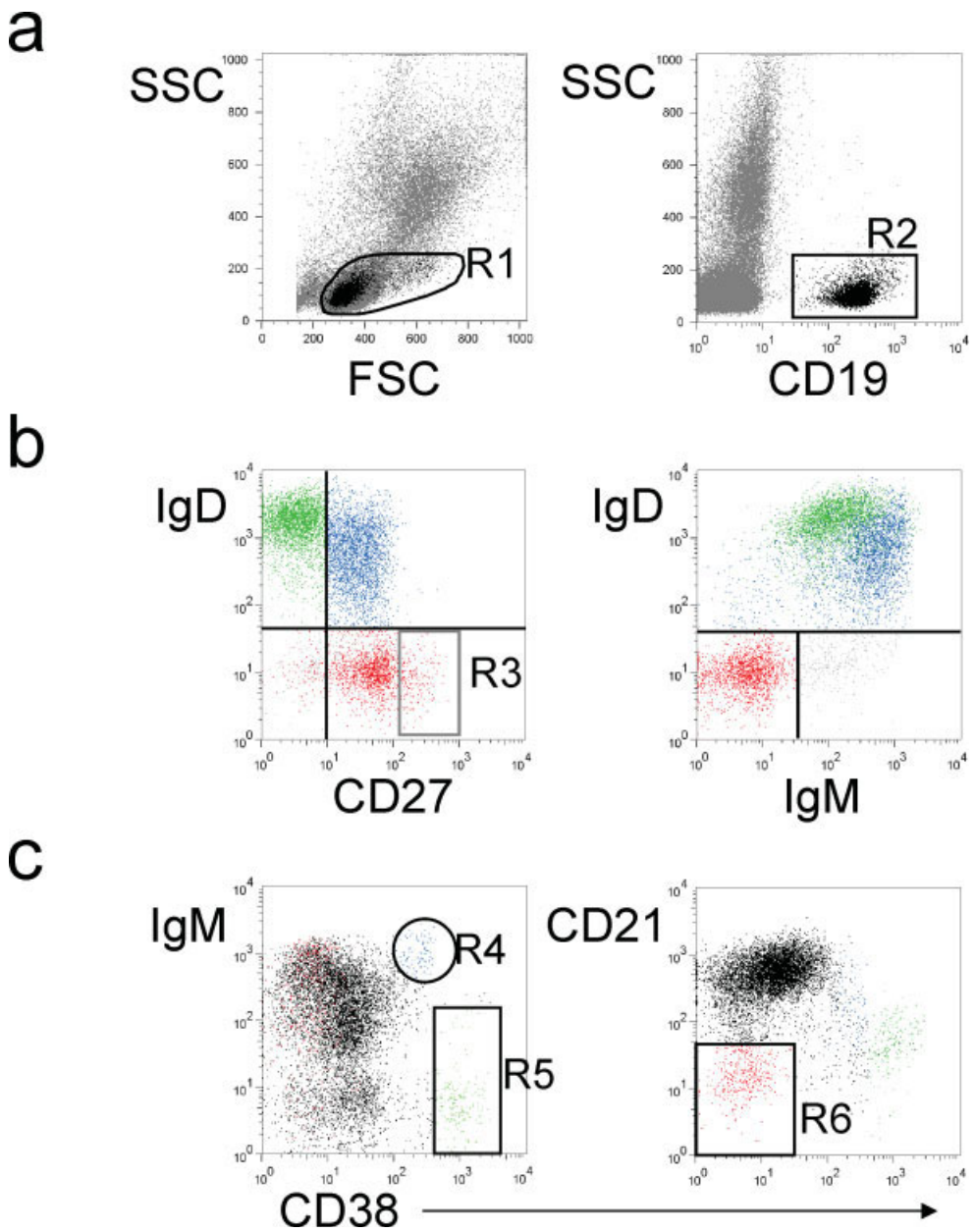


FIG. 2. B cell phenotyping of a healthy donor. (**a**) $CD19^+$ B cells (R2) are gated within the lymphocyte scatter region (R1). Gating should include large lymphocytes and $CD19^{low}$ B cells comprising plasmablasts. (**b**) Using panel B1 from Table 2, B cells can be subdivided into several subpopulations: naïve (green, $CD19^+CD27^-IgM^+IgD^+$), marginal zone like (blue, $CD19^+CD27^+IgM^{++}IgD^+$), class switched (red, $CD19^+CD27^+IgM^-IgD^-$), and IgM only (gray, $CD19^+CD27^+IgM^{++}IgD^-$) memory B cells. $CD27^{high}$ B cells in region R3 represent plasmablasts. (**c**) Staining B cells with panel B2 from Table 2 distinguishes transitional B cells (R4, blue, $CD19^+CD21^{int}CD38^{++}IgM^{++}$), plasmablasts (R5, green, $CD19^{low}CD21^{int}CD38^{+++}IgM^{-(+)}$), and $CD21^{low}$ B cells (R6, red, $CD19^{++}CD21^{low}CD38^{low}IgM^+$).

$CD19^+CD27^+IgM^-IgD^-$ B cells as percentage of lymphocytes acknowledges changes of total B cells and therefore represents more closely changes in absolute numbers. Type I patients were subdivided into a group with highly expanded $CD21^{low}$ B cells (type Ia, $\geq$20% of $CD19^+$ B cells) and a group with less than 20% of $CD21^{low}$ B cells (type Ib).

According to the recent analysis of over 300 European CVID patients (39), 77% of CVID patients belong to type I patients (21% type Ia and 56% type Ib) and 23% to type II corroborating the original findings in the Freiburg cohort (38). The decreased percentage of type Ia compared to the original publication is due to limiting $CD21^{low}$ B cells to B cells with a low expression of CD38 separating $CD21^{low}$ B cells from transitional B cells. The original description of $CD21^{low}CD19^{++}$ cells as immature B cells turned out to be wrong and further examination clarified that the B cell population with a low CD21 expression consists of three different populations ($CD21^{low}$ B cells, transitional B cells, and plasmablasts) as described earlier. The association between expanded $CD21^{low}$ B cells and splenomegaly in type Ia patients was confirmed for the $CD21^{low}CD38^{low}$ B cell subpopulation.

### Paris Classification

Piqueras et al. (40) suggested a classification of CVID patients based on the assessment of class-switched mem-

Table 3
*Reference Values of B-Cell Subpopulations in Peripheral Blood*

| B-cell population | | Reference range[a] (%) |
|---|---|---|
| CD19+ in CD45+ lymphocytes | B cells | 4.9–8.4 |
| IgM− IgD− in CD19+ B cells | Class switched B cells | 7.6–31.4 |
| IgD+ CD27− in CD19+ B cells | Naive B cells | 42.6–82.3 |
| IgD+ CD27+ in CD19+ B cells | Marginal zone-like B cells | 7.4–32.5 |
| IgM−IgD− CD27+ in CD19+ B cells | Class switched memory B cells (and Plasmablasts) | 6.5–29.1 |
| $CD21^{low}$ CD38− in CD19+ B cells | $CD21^{low}$ B cells | 0.9–7.6 |
| M++ D++ CD24++ CD38++ in CD19+ B cells | Transitional B cells | 0.6–3.4 |
| IgM-(+) CD24-CD38+++CD27++ in CD19+ B cells | Plasmablasts | 0.4–3.6 |

[a]5. to 95. percentile, based on 54 healthy donors (age range 19–61 years).

ory B cells and total $CD27^+$ B cells including the marginal zone like B cells (Fig. 3b).

Using the same panel of surface markers as the Freiburg classification including CD19, CD27, IgD, and IgM, the classification distinguishes a group MB2 with a nearly normal percentage of CD27 B cells, a group MB1 with decreased class switched but normal marginal zone like B cells and a group MB0 with reduced class switched and marginal zone like B cells. The cut off values were defined at two standard deviations below the normal values of a control group. In the control cohort these cut off values were 11% for total $CD27^+$ B cells and 8% for class switched memory $CD27^+IgM^-IgD^-$ B cells of $CD19^+$ B cells. There was a significant association of splenomegaly and granulomatous inflammation with group MB0 (40).

### EUROClass Classification

The EUROClass classification (39) was born out of the effort to define a consensus classification based on the evaluation of a multicenter trial of over 300 European CVID patients (Fig. 3c). The analysis confirmed several previous findings of the Freiburg and Paris classification. By combining aspects of both classifications and adding new criteria EUROClass distinguishes patients with less than 1% of B cells of lymphocytes ($B^-$) from patients with a higher percentage ($B^+$). $B^+$ patients are divided into patients with severe deficiency of class-switched memory B cells (<2% of $CD19^+$ B cells, $smB^-$) and patients with more than 2% of class-switched memory B cells ($smB^+$). A small subgroup of $smB^-$ patients is characterized by the expansion of transitional B cells ($smB^-Tr^{hi}$). Most of these patients present with lymphadenopathy. On the other hand, the expansion of $CD21^{low}$ B cells again represented the strongest marker for splenomegaly. Therefore EUROClass also discriminates patients according to the expansion of $CD21^{low}$ B cells above or below 10% within $CD19^+$ B cells ($CD21^{lo}$ vs. $CD21^{norm}$). This classification allows overlapping grouping of patients with expansion of transitional and $CD21^{low}$ B cells.

### Interpretation of Deregulated B Cell Homeostasis

About 90% of all CVID patients display near normal B cell numbers in their peripheral blood. A minority of CVID patients (5–10%) have very low peripheral B cell counts (<1% of lymphocytes), suggestive of either defects during early B cell differentiation in the bone marrow, failure of essential survival factors in the periphery or the presence of negative regulators thereof.

Class-switched memory B cells are a sensitive marker for a sufficient germinal center function. All patients with known abrogated germinal center function as in CD40L deficiency and ICOS deficiency (41) (see below) fall into type I, MB1/MB0, and $smB^-$, respectively, confirming the discriminating function of evaluating the class-switched memory B cell population.

CVID patients with near normal number of class-switched memory B cells (Type II, MB2, $smB^+$) need to be carefully examined for other underlying disease. We identified two patients within our cohort with PROMM (proximal myotonic myopathy, also called DM2 myotonic dystrophy type 2) a muscle dystrophic disease due to an expansion of CCTG repeats in intron 1 of the *ZNF9* gene (42). As in myotonic dystrophy type 1, the coexisting hypercatabolism of antibodies leading to hypogammaglobulinemia is not understood. In the subgroup II of CVID the disruption of B cell differentiation affects post-germinal center plasma cell differentiation without affecting memory differentiation, rendering plasma cell specific genes prime candidates.

While the absence of switched memory B cells is highly suggestive of disturbed germinal center reaction, the selective reduction of IgM memory B cells most likely reflects a disrupted marginal zone B cell differentiation. No defects have been identified causing a selective absence of $CD19^+CD27^+IgM^+IgD^+$ B cells in humans. The expansion of circulating $CD21^{low}$ B cells is a manifestation of a chronic, possibly IFN type I driven immune activation (manuscript submitted) and is significantly associated with splenomegaly and granulomatous disease.

Expansion of transitional B cells is associated with lymphadenopathy (39). This association is not understood. In addition, one genetic defect has been identified

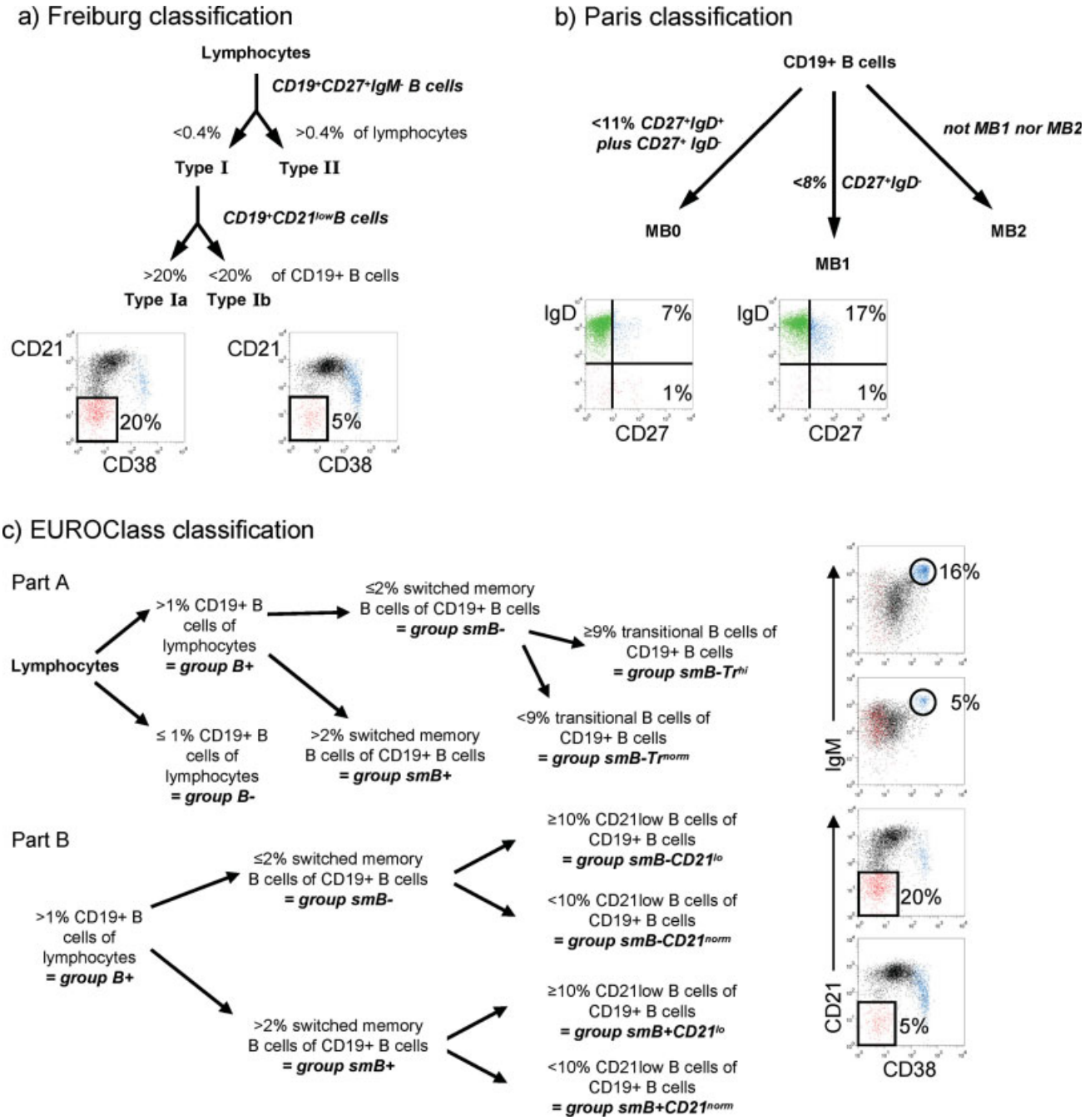


FIG. 3. B-cell phenotype based classifications of CVID. (**a**) The Freiburg classification first discriminates CVID patients on the basis of class switched $CD19^+CD27^+IgM^-IgD^-$ memory B cells: patients with class switched B cells below 0.4% of lymphocytes are assigned to type I, patients with class switched B cells equal or above 0.4% are classified to type II. Patients of type I are subclassified on the basis of B cells expressing low amounts of CD21: patients exhibiting equal or more than 20% $CD21^{low}$ cells out of CD19+ B cells are grouped into type Ia and patients with less than 20% CD21low B cells are classified to type Ib (representative dot plots are shown). (**b**) The Paris classification groups patients according to the percentage of memory B cell within $CD19^+$ B cells: patients with less than 11% of total $CD27^+$ cells of $CD19^+$ B cells are classified in group MB0, patients with more than 11% of $CD27^+$ B cells but less than 8% of class switched B cells are grouped into MB1 and all patients who do not fulfill one of these criteria are assigned to group MB2. (**c**) The EUROClass classification discriminates patients with more than 1% $CD19^+$ B cells of lymphocytes (group $B^+$) and equal or less than 1% (group $B^-$). The $B^+$ group is subdivided into patients with more than 2% class switched memory $CD27^+IgM/IgD^-$ B cells within the $CD19^+$ B cell compartment (group $smB^+$) and patients with equal or less then 2% class switched cells (group $smB^-$). The $smB^-$ group can be further subdivided based on the expansion of transitional B cells (part A): patients with equal or more than 9% of transitional B cells of total $CD19^+$ B cells are assigned to group $Tr^{hi}$ and patients with less than 9% transitional B cells are classified into $Tr^{norm}$. As shown in part B, the groups $smB^+$ and $smB^-$ both are further subdivided into groups $CD21^{norm}$ (less than 10% $CD21^{low}$ B cells of $CD19^+$ B cells) or $CD21^{lo}$ (equal or more than 10% $CD21^{low}$ B cells). Representative flowcytometric plots of relevant B cell subpopulations are shown.

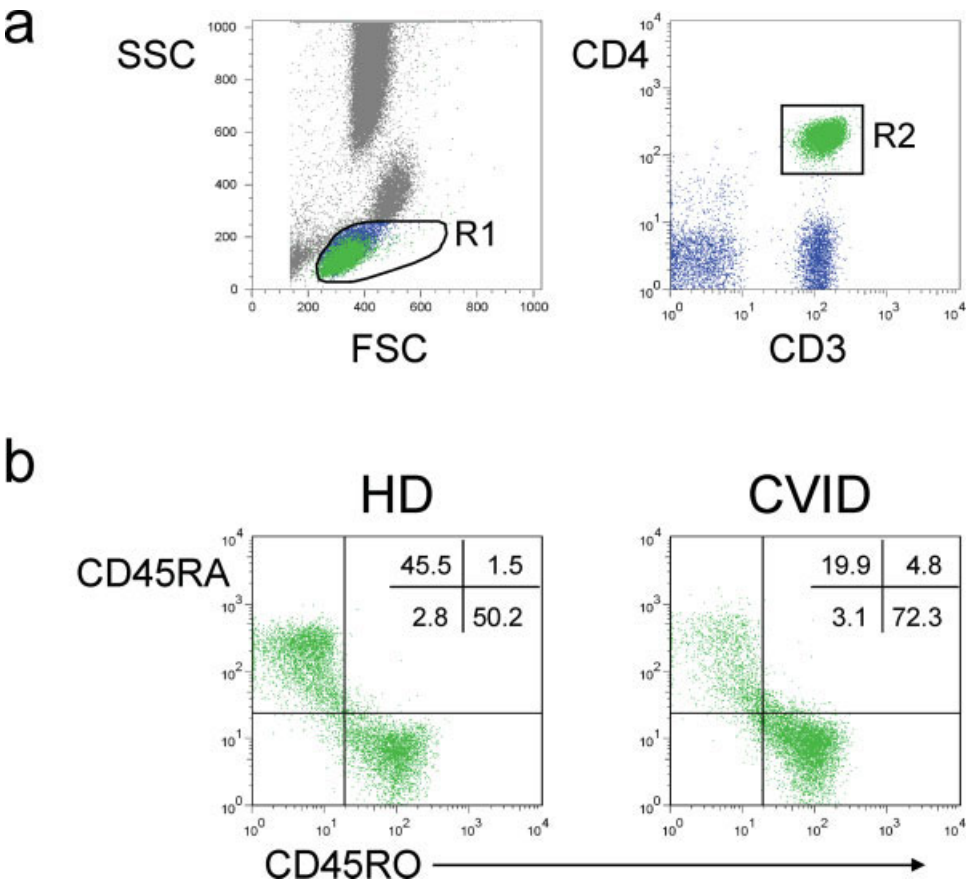


FIG. 4. Phenotyping of naive and memory CD4 T cells. (**a**) CD4 T cells are gated by combining a lymphocyte scatter region (R1) and $CD3^+CD4^+$ cells (R2) using a whole blood lyse-no-wash protocol (Optilyse B, Beckman-Coulter, Krefeld, Germany). (**b**) Within the CD4 T cell population the distribution of peripheral $CD45RA^+$ naïve and $CD45RO^+$ memory CD4 T cells is shown for an age-matched healthy donor (left) and a CVID patient with severe reduction of naïve CD4 T cells (right).

causing a severe expansion of transitional B cells (see below).

A recent article (43) suggests the examination of circulating $IgA^+$ B cells as a marker for increased risk of infusion reaction to Ig replacement therapy in completely IgA deficient CVID patients.

## T CELL PHENOTYPING OF CVID

Several lines of evidence support a disturbed T cell immunity in patients with CVID (44–46). Therefore some authors suggested T cell phenotyping as a parameter for classifying CVID. The most commonly described abnormality is the reduction of total or naive CD4 T cells (Fig. 4). Thus Aukrust et al. (47) identified a subgroup $CVI^{Hyper}$ of CVID patients characterized by decreased number of circulating CD4 T cells, splenomegaly, and persistent immune activation in vivo, particularly of monocytes/macrophages possibly driven by persistent TNF alpha stimulation. This was associated with B cell lymphopenia and failure of B cell immunoglobulin production in vitro. These data support the view that T cell regulation of B cells plays a role of varying severity in this group of CVID patients.

This finding was recently confirmed by another group (48). Separating patients into three groups based on the percentage of naive $CD45RA^+$ CD4 T cells (<15%; 16–30% and > 30% of CD4 T cells) the group with the most severe reduction of naive CD4 T cells was associated with reduced thymic output of CD4 T cells, reduction of class-switched memory B cells, expansion of $CD21^{low}$ B cells, splenomegaly, and granulomatous disease.

Current efforts aim at the definition of combined T and B cell phenotyping for the classification of CVID.

In addition, regulatory T cells have been demonstrated to be reduced in CVID patients with splenomegaly and signs of chronic inflammation (49). This change is linked with the type Ia/$smB^-CD21^{low}$ subgroup of CVID. In patients with ICOS deficiency (see below) circulating $CXCR5^+$ CD4 T cells are severely decreased (50). This is uncommon in other CVID patients rendering the evaluation of $CXCR5^+$ CD4 T cells a potential surrogate marker for ICOS deficiency.

## FLOW CYTOMETRIC ASSESSMENT OF KNOWN GENETIC DEFECTS ASSOCIATED WITH CVID

Naturally all genetic defects have to be confirmed by genetic sequencing, but all defects except transmembrane activator and CAML interactor (TACI) deficiency have been first discovered by flowcytometric analysis. The following paragraphs indicate flowcytometric hallmarks of the four known genetic defects associated with CVID.

### ICOS Deficiency

The inducible costimulator (ICOS) belongs to the CD28 receptor family and is selectively expressed on activated T cells (51). In the absence of ICOS or its ligand ICOS-L the formation of germinal centers and as a result the differentiation of immunologic memory are severely perturbed (52,53).

In humans a genomic deletion of exons 2 and 3 caused ICOS-deficiency in nine CVID patients (41,54). In accordance with the severe effect on germinal center formation all patients presented with absent class-switched memory B cells. It remained unexplained why in most ICOS deficient patients total B cell numbers and especially the marginal zone like B cells were also reduced (54). Within the T cell compartment the distribution of CD4/CD8 as well as CD45RA/CD45RO T cells was not altered. Interestingly, however, ICOS deficiency is associated with a severe reduction of $CXCR5^+$ memory like CD4 T cells (50).

ICOS deficiency should be excluded in patients with absent class-switched memory B cells (Freiburg type I, PARIS MB0 (or MB1), EUROClass $smB^-$) and with family history of antibody deficiency compatible with autosomal recessive trade, especially when $CXCR5^+$ CD4 T cells are reduced. ICOS deficiency was detectable in all patients by the lack of ICOS upregulation after PHA or CD3/CD28 stimulation of T cells.

### TACI Deficiency

TACI belongs to the TNF receptor family and is predominantly expressed on $CD27^+$ B cell subsets (55). Mutations of TNFRSF13B coding for TACI turned out to be the most frequent genetic defect in CVID known so far (56,57). Genomic alterations can be found in up to 8% of CVID with the impact of heterozygous mutations being less clearly defined. TACI deficiency does not influence B or T cell homeostasis in defined ways and therefore neither B nor T cell phenotyping is helpful in the diagnosis of TACI deficiency. Most mutations are point mutations which do not allow the detection by flowcytometric staining for TACI. Therefore TACI deficiency needs to be functionally and genetically determined.

### CD19 Deficiency

CD19 is part of the B cell costimulatory complex (58) and is expressed on all B cell populations between the pre B cell and the plasma cell stage. So far five CVID patients have been described with CD19 deficiency (59,60). All have been readily recognized by flowcytometry. The absence of $CD19^+$ cells while other B cell specific markers like CD21, IgM, etc. are present is highly suggestive of CD19 deficiency.

### BAFF-R Deficiency

BAFF-R like TACI belongs to the TNF receptor family and is highly expressed on all peripheral B cells and is the main receptor for BAFF (61). The interaction of BAFF with its receptor is a key regulatory mechanism in peripheral B cell homeostasis. The absence of BAFF-R causes a severe decrease in peripheral B cells and expansion of transitional B cells due to a developmental block at the transition from T1 to T2 B cells in the spleen of mice (61). The first human with a deletion of BAFF-R has been identified. Compatible with the murine findings human BAFF-R deficiency also causes a decrease in mature B cells and increase in circulating transitional B cells (manuscript submitted).

## CONCLUSION

Flowcytometric evaluation has become part of the standard diagnostic work up of patients with CVID. It has improved the classification of CVID patients. B cell phenotyping identifies patients with severe defects of germinal center dependent B cell memory formation, early defects of peripheral B cell differentiation, and possibly inflammatory changes. Similarly T cell phenotyping discloses disturbed T cell homeostasis in subgroups of patients. In addition, flowcytometric findings can imply certain underlying genetic defects in some of the patients. Flow cytometry-based functional assessment of lymphocyte activation is part of ongoing research but has not (yet?) become part of advanced diagnostics in CVID. The future task will be the optimized use of flowcytometric phenotypic and functional examination of the immune system of CVID patients to improve our current comprehension of pathogenesis and the availability of clinically relevant predictive markers. To achieve this goal the ESID has founded a working group "Immune phenotyping in immunodeficiency" (IPID) expected to be launched at the upcoming ESID meeting in autumn 2008.

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