Diagnostic Indications


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Diagnostics for Common Variable Immunodeficiency Syndrome

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Introduction

Common variable immunodeficiency (CVID) is the most common, clinically relevant immunodeficiency disorder. In the western world, the incidence of this disease ranges between 1:20,000 and 1:200,000 [1]. CVID is an antibody deficiency syndrome with decreased serum concentrations of at least two of the three antibody classes IgG, IgM and IgA of at least two standard deviations below the age-appropriate mean value (www.esid.org). According to the diagnostic criteria of the European Society of Immunodeficiency (ESID), the immunodeficiency begins after the 2nd year of life, and immunization has been unsuccessful in most patients. Since CVID is a diagnosis by exclusion, well-known genetic, infectious and toxic causes of hypogammaglobulinemia have to be excluded (www.esid.org). Approximately 20% of the cases [2, 3] are familial and are assumed to have a genetic origin. There is a genetic association between CVID and the more frequent, but typically clinically inconspicuous selective IgA deficiency [4]. Clinically, CVID patients are frequently diagnosed as adolescents or young adults, particularly due to recurrent infections of the upper and lower respiratory tracts. Untreated, these lead to progressive destruction of the airways and lungs. In contrast to patients with severe combined immunodeficiency, CVID patients are particularly susceptible to infections with encapsulated bacteria, while atypical infections are rare. Additionally, many of the patients suffer from gastrointestinal infections during the course of their illness. Further symptoms include autoimmune phenomena, lymphoproliferation (splenomegaly, lymphadenopathy), lymphomas and granulomatous disease [1]. To a large extent, the pathogenesis of this syndrome is unclear and certainly heterogeneous in origin. Both disturbed
T-cell and B-cell differentiation and function have been described in the literature [5–7]. In addition to a thorough medical history and physical examination of the patient, the diagnosis of CVID is based on the determination of total and specific serum antibody titers, the exclusion of paraproteins, protein-loosing enteropathy and renal disease, bone marrow biopsy as well as functional and imaging procedures for the analysis of organ damage. Flow cytometry supplements the diagnostic work-up by assessing peripheral blood lymphocyte populations and investigating the presence of defined genetic defects. A detailed analysis of the lymphocyte population allows the exclusion of severe immune disturbances, the identification of large monoclonal populations and disturbances in early B-cell differentiation which result in very low numbers of peripheral blood B-cells as in X-chromosomal agammaglobulinemia (Bruton’s disease) caused by a defect in the Bruton tyrosine kinase (BTK) [8].

Flow-cytometric analysis can point to specific primary immunodeficiency syndromes such as class switch recombination defects due to activation-induced cytidine deaminase (AID) or uracil-DNA glycosylase (UNG) deficiency which presents with absent IgM− IgD− CD27+ B-cells and concomitant expansion of IgM-only CD27+ B-cells [9]. Additional tests allow the exclusion of genetic defects abrogating the expression of CD40 ligand (CD40L) and CD40 [9]. So far, four genetic defects have been associated with CVID: ICOS, TACI, CD19, and BAFF-R deficiency [10–12]. Since 2003, 9 patients have been identified with a homozygous deletion in the gene encoding for the inducible costimulator (ICOS) [10]. ICOS belongs to the CD28 family and is exclusively expressed on activated T-cells [13]. This defect is detected by flow-cytometric analysis of ICOS expression on activated T-cells. TACI deficiency represents the most common monogenetic defect in CVID [14]. The flow-cytometric analysis of TACI, however, is unreliable, and genetic testing is required. Nevertheless, the few patients with CD19 deficiency and BAFF-R deficiency were detected by flow cytometry (see below).

The differential diagnosis of CVID includes some subforms of X-linked lymphoproliferative syndrome (XLP) [15]. Flow-cytometric analysis of peripheral blood mononuclear cells (PBMCs) of XLP patients show reduced class-switched memory B-cells, expanded transitional B-cells and low intracellular SAP expression [16].

In recent years, flow cytometry has been introduced as a diagnostic tool for the classification of CVID. Three schemes (the Freiburg, Paris and EUROClass Classifications) are based on the phenotyping of circulating B-cell subpopulations [17–19] and two on T-cell phenotypes [20, 21]. The classification schemes were introduced to permit a clinically and pathogenetically relevant discrimination of distinct patient groups.
**Table 1.** Antibody mixture for the basic lymphocyte panel

<table>
<thead>
<tr>
<th></th>
<th>FITC 100 µl</th>
<th>PE 100 µl</th>
<th>PerCP 50 µl</th>
<th>APC 25 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>CD8</td>
<td>CD4</td>
<td>CD45</td>
<td>CD3</td>
</tr>
<tr>
<td>K2</td>
<td>CD19</td>
<td>CD56/16b</td>
<td>CD45</td>
<td>CD3</td>
</tr>
</tbody>
</table>

* a 15 µl/50 µl whole blood.

b Per 50 µl.

**Protocol**

**Principles**

These protocols are appropriate for four-color flow cytometry. Both surface-only staining and combinations of stainings for various surface molecules and intracellular proteins can be used. All protocols are compiled on the basis of analyses using the FACSCalibur (BD Biosciences, Heidelberg, Germany) with 488- and 635-nm diode lasers and CellQuest software (BD Biosciences). Appropriate adaptation to other cytometers must be performed if necessary. The reader is expected to be familiar with the prerequisites for the operation, instrument settings, compensation, dot plot analyses and the setting of evaluation windows (region, gates).

**Basic Lymphocyte Status**

In whole-blood staining, the respective proportions of CD4+, CD8+ T-cells, B-cells and NK cells are determined (table 1). This serves as preliminary information as well as for the determination of absolute counts by simultaneous differential blood cell count.

**Exclusion of Confounding Diagnoses**

It is essential to understand that descriptive flow-cytometric analysis can only detect quantitative expression defects, rather than functional or minimum structural changes (such as point mutations). To rule out a hyper-IgM syndrome with defects in CD40 (former type 3), B-cells are stained for CD40. Both CD40L and ICOS defects can be proven only by analyzing activated T-cells. While ICOS induction can be examined in PBMNCs after stimulation, CD4+ T-cells should be isolated beforehand to optimize the determination of surface expression of CD40L. Further functional analyses are performed in specialized laboratories and are not dealt with in this chapter.
**Classification of CVID**

In the last 5 years, three classification schemes based on the quantification of B-cell subpopulations have been introduced: the Freiburg Classification [18], the Paris Classification [17] and a European consensus classification EUROclass [19], which overlap in several aspects (fig. 1). Patients can be classified according to all three schemes combining two stainings (table 2). The first staining, B1, allows differences to be determined between naive (CD19+ CD27– IgM+ IgD+), IgM IgD double-positive memory (CD19+ CD27+ IgM+ IgD+) and class-switched memory B-cells (CD19+ CD27+ IgM– IgD–). The often very low levels of ‘IgM-only’ memory B-cells (CD19+ CD27+ IgM+ IgD–) can also be calculated from the analysis.

In a second staining, B2, the proportion of transitional (CD19+ CD21\textsuperscript{int} CD38++ IgM++) B-cells, plasmablasts (CD19\textsuperscript{low} CD21\textsuperscript{low} CD38+++ IgM– or IgM\textsuperscript{low}) and an activated CD21\textsuperscript{low} population (CD19\textsuperscript{high} CD21\textsuperscript{low} CD38\textsuperscript{low} IgM+) is determined.

In addition, two groups have suggested a classification of CVID patients according to T-cell subpopulations [20, 21] based on staining lymphocytes for CD3, CD4, CD45R0 and CD45RA. The latter distinguishes three groups of patients according to the percentage of circulating naive CD3+ CD4+ CD45R0 and CD45RA. The group with the strongest reduction of naive peripheral CD4 T-cells was associated with an increased incidence of splenomegaly and inflammatory changes [21]. There was a significant correlation to the Freiburg B-cell-based classification scheme.

**Material**

**Sample Material**

8 ml EDTA blood (not older than 24 h); for the determination of ICOS and CD40L on activated T-cells, an additional 8 ml EDTA blood is required.

**Reagents**

- Ficoll separation medium (1,077 g/ml; Biochrom, Berlin, Germany)
- RPMI-1640 (Biochrom)
- FCS (PAN Biotech, Aidenbach, Germany)
- FACSFlow (BD Biosciences)
- Optilyse B (Beckman-Coulter, Krefeld, Germany)
- IntraPrep (Beckman-Coulter)
- Deionized water.

**Equipment**

- FACSCalibur with a 488-nm laser and a 635-nm diode laser
- standard pipettes
transitional B-cells or CD21 B-cells above 2% (smB+). Further subgroups can be differentiated according to the expansion of
with severely decreased switched memory B-cells (smB- deficiency (B--)).

Type I can be differentiated into type Ia and type Ib based on the expansion of
the CD21 B-cell population to over 20% of the B-cells in type Ia and less than 20% in type Ib

The Paris Classification distinguishes group MB0, with a decrease in all CD27+ B-cells, from
a group MB1 with a rather selective decrease in class-switched memory B-cells and a group of
patients with nearly normal numbers of non-class-switched as well as class-switched B-cells (MB2)

The most recent classification, EUROClass, first of all distinguishes patients with severe B-cell
deficiency (B--–) from patients with B-cells above 1% (B+). The latter group is divided into patients
with severely decreased switched memory B-cells (smB--) and patients with switched memory
B-cells above 2% (smB+). Further subgroups can be differentiated according to the expansion of
transitional B-cells or CD21 B-cells.

Fig. 1. Flow charts for the classification of patients with CVID. The classification is based on the
analysis of different B-cell subpopulations. a Freiburg Classification: Type I is characterized by a
decrease in class-switched B-cells to below 0.4% of lymphocytes while type II patients have a nearly
normal number. Type I can be differentiated into a type Ia and a type Ib based on the expansion of
the CD21 B-cell population to over 20% of the B-cells in type Ia and less than 20% in type Ib
[18]. b The Paris Classification distinguishes a group MB0, with a decrease in all CD27+ B-cells, from
a group MB1 with a rather selective decrease in class-switched memory B-cells and a group of
patients with nearly normal numbers of non-class-switched as well as class-switched B-cells (MB2)
[17]. c The most recent classification, EUROClass, first of all distinguishes patients with severe B-cell
deficiency (B--) from patients with B-cells above 1% (B+). The latter group is divided into patients
with severely decreased switched memory B-cells (smB--) and patients with switched memory
B-cells above 2% (smB+). Further subgroups can be differentiated according to the expansion of
transitional B-cells or CD21 B-cells.
centrifuge
- counting chamber
- vortex
- 50-ml Falcon polypropylene test tubes with caps (BD Biosciences)
- 15-ml Falcon polypropylene test tubes with caps (BD Biosciences)
- 12- to 75-mm Falcon polystyrene test tubes (BD Biosciences)
- 0.5-ml sample test tubes with caps (Biozym, Hessisch Oldendorf, Germany).

Antibodies
The antibodies utilized are listed in table 3.

Protocol

Whole-Blood Staining for ‘Basic-Lymphocyte Status’
The major lymphocyte population is analyzed by whole-blood staining from EDTA blood samples in lyse-no-wash assays, which are available from various manufacturers. One possible antibody panel is listed in table 1.

Staining of Peripheral Blood Mononuclear Cells (Classification, CD40 Expression)
The staining to exclude a CD40 defect or monoclonal populations and to classify CVID is carried out on PBMNCs following purification over Ficoll density gradients. The technical procedure for staining PBMNCs which have been isolated by Ficoll gradients is described in ‘Characterization of B-Lymphocytes; ‘Procedures’ (pp. 214). The antibody mixtures of the Freiburg classification are summarized in table 2. It should be mentioned that B-cell phenotyping can also be performed in whole-blood assays and that the results are comparable. For a protocol, see Ferry et al. [22].

Determination of CD40L and ICOS Expression on Activated T-Cells/Activation of T-Cells
For the determination of ICOS expression, $1 \times 10^6$ Ficoll-isolated PBMNCs are plated into 24-well plates in RPMI-1640 with 10% (v/v) FCS and incubated for 16–20 h

Table 2. Antibody mixture for the Freiburg Classification

<table>
<thead>
<tr>
<th></th>
<th>FITC 100 μl</th>
<th>PE 100 μl</th>
<th>PC7 25 μl</th>
<th>FL4 25 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>CD27 (1:5)</td>
<td>anti-IgD (1:40)</td>
<td>CD19</td>
<td>anti-IgM Cy5 (1:40)</td>
</tr>
<tr>
<td>B2</td>
<td>CD38</td>
<td>CD21</td>
<td>CD19</td>
<td>anti-IgM Cy5 (1:40)</td>
</tr>
<tr>
<td>B3</td>
<td>anti-κ (1:2)</td>
<td>anti-λ (1:2)</td>
<td>CD19</td>
<td>anti-IgM Cy5 (1:40)</td>
</tr>
<tr>
<td>B4$^b$</td>
<td>CD40</td>
<td>??</td>
<td>CD19</td>
<td>anti-IgM Cy5 (1:40)</td>
</tr>
</tbody>
</table>

$^a$ 10 μl/50 μl cell suspension.

$^b$ Only if hyper-IgM is suspected.
at 37°C and 5% CO₂ with and/or without 1 µg/ml PHA. The cells are subsequently transferred into a FACS tube, washed once in FACS medium (5 min, 300 × g) and stained with the antibody mixture according to the protocol for PBMNCs. To confirm that the T-cells are activated, they are additionally stained for CD69 (table 4, A1 + A2). The methodology to exclude a CD40L defect is described in ‘Primary Immunodeficiency Diseases; CD40L and CD40 Defect (Hyper-IgM Syndromes, HIGM Syndromes)’ (pp. 531) (table 4, A1 + A3).

### Table 3. List of antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Order No.</th>
<th>Provider</th>
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<tbody>
<tr>
<td>CD3</td>
<td>SK7</td>
<td>APC</td>
<td>345767</td>
<td>BD</td>
</tr>
<tr>
<td>CD4</td>
<td>13B8.2</td>
<td>PE</td>
<td>A07751</td>
<td>BC</td>
</tr>
<tr>
<td>CD8</td>
<td>B9.11</td>
<td>FITC</td>
<td>A07756</td>
<td>BC</td>
</tr>
<tr>
<td>CD16</td>
<td>3G8</td>
<td>PE</td>
<td>A07766</td>
<td>BC</td>
</tr>
<tr>
<td>CD19</td>
<td>J4,119</td>
<td>FITC</td>
<td>A07768</td>
<td>BC</td>
</tr>
<tr>
<td>CD19</td>
<td>J4,119</td>
<td>PC7</td>
<td>IM3628</td>
<td>BC</td>
</tr>
<tr>
<td>CD21</td>
<td>B-ly4</td>
<td>PE</td>
<td>555422</td>
<td>BD</td>
</tr>
<tr>
<td>CD27</td>
<td>M-T271</td>
<td>FITC</td>
<td>F7178</td>
<td>Dako</td>
</tr>
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<td>CD38</td>
<td>HIT2</td>
<td>FITC</td>
<td>555459</td>
<td>BD</td>
</tr>
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<td>CD40</td>
<td>5C3</td>
<td>FITC</td>
<td>555588</td>
<td>BD</td>
</tr>
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<td>CD40L</td>
<td>TRAP1</td>
<td>PE</td>
<td>555700</td>
<td>BD</td>
</tr>
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<td>CD45</td>
<td>2D1</td>
<td>PerCP</td>
<td>345809</td>
<td>BD</td>
</tr>
<tr>
<td>CD56</td>
<td>NKH-1</td>
<td>PE</td>
<td>A07788</td>
<td>BC</td>
</tr>
<tr>
<td>CD69</td>
<td>FN50</td>
<td>FITC</td>
<td>555530</td>
<td>BD</td>
</tr>
<tr>
<td>Anti-BAFF-R</td>
<td>goat IgG</td>
<td>purified</td>
<td>AF1162</td>
<td>R and D</td>
</tr>
<tr>
<td>Anti-goat-IgG</td>
<td>swine IgG</td>
<td>PE</td>
<td>G50004</td>
<td>Caltag</td>
</tr>
<tr>
<td>Anti-ICOS</td>
<td>5A-3</td>
<td>PE</td>
<td>12–9948–71</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>goat F(ab)2</td>
<td>Cy5</td>
<td>109–176–129</td>
<td>JIR</td>
</tr>
<tr>
<td>Anti-IgD</td>
<td>goat F(ab)2</td>
<td>PE</td>
<td>2032–09</td>
<td>SB</td>
</tr>
<tr>
<td>Anti-κ</td>
<td>G20–193</td>
<td>FITC</td>
<td>555791</td>
<td>BD</td>
</tr>
<tr>
<td>Anti-λ</td>
<td>JDC-12</td>
<td>PE</td>
<td>555797</td>
<td>BD</td>
</tr>
<tr>
<td>Isotype control</td>
<td>mouse-IgG1</td>
<td>PE</td>
<td>IM0670</td>
<td>BC</td>
</tr>
</tbody>
</table>

BD = BD Biosciences; BC = Beckman Coulter; SB = SouthernBiotech; JIR = Jackson ImmunoResearch Laboratories; R and D = R and D Systems; Caltag = Caltag/Invitrogen.  
a The antibodies listed are proven and recommended by the authors, but can be replaced by suitable alternative ones.

### Table 4. Antibody panel to ascertain activation of T-cells

<table>
<thead>
<tr>
<th>FITC</th>
<th>PE</th>
<th>PerCP</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>CD69</td>
<td>isotype G1</td>
<td>CD3</td>
</tr>
<tr>
<td>A2</td>
<td>CD69</td>
<td>ICOS</td>
<td>CD3</td>
</tr>
<tr>
<td>A3</td>
<td>CD69</td>
<td>CD40L (CD154)</td>
<td>CD3</td>
</tr>
</tbody>
</table>

a 5 µl of each of the pretitrated antibodies is administered to every 0.5–1.0 × 10⁶ cells.
Measurements

Accurate instrument settings for photomultipliers and compensation are the preconditions for B-cell determination. For well-titrated antibodies, the compensation is uniform within the panel. Usually, the instrument settings are very stable over time, and a control with beads of any color is sufficient. It is not necessary to prepare each measurement with single-color stainings.

Measurement of ‘Basic Lymphocyte Status’ (fig. 2)
The acquisition protocol contains a two-dimensional acquisition window with the FL3 (CD45) and side scatter (SSC) axes. The R1 analysis region defines the lymphocyte population through the expression of CD45 and low scatter. As further windows, the lymphocytes in R1 are represented as FL1 vs. FL4 and FL2 vs. FL4 plots. Data of at least 5,000 lymphocytes should be collected.

Measurements for the Classification of CVID (fig. 3)
The analysis of the B-cell subpopulation is described in detail in ‘Characterization of B-lymphocytes’ (pp. 211). Table 2 shows the recommended minimum staining supplemented by a staining for CD40 (see below). Staining B1 (fig. 3a–c, panel 1) enables a distinction between naïve B-cells (UL, CD27– IgD+ IgM+), non-class-switched (UR, CD27+ IgD+ IgM+) and class-switched (LR, CD27+ IgD– IgM–) memory B-cells [23]. The second staining, B2 (fig. 3, panel 2), shows the markers CD21 and CD38 for further B-cell differentiation levels. CD38 is strongly expressed
Fig. 3a, b

**healthy control**

R1 Lymphocytes
R2 B-cells

**Panel 1:**
UL) Naive B-cells
UR) Marginal zone like B-cells
LR) Class-switched memory B-cells

**Panel 2:**
R3) CD21* B-cells
R4) Transitional B-cells
R5) Plasmablasts

a

**CVID patient type Ia**

R1 Lymphocytes
R2 B-cells

**Panel 1:**
UL) Naive B-cells
UR) Marginal zone like B-cells
LR) class switched memory B-cells

**Panel 2:**
R3) CD21* B-cells
R4) Transitional B-cells
R5) Plasmablasts

b
on all B-cell precursors (also see ‘Characterization of B-Lymphocytes’, pp. 211). Naive B-cells show an intermediate CD38 surface expression, while memory B-cells are mostly negative for CD38. The low levels of expression of CD21 and CD38 (R3) defines the CD21\textsuperscript{low} B-cell population, which is included in the Freiburg and EUROClass Classification schemes. In addition, the high expression of IgM and CD38 defines transitional B-cells (R4) [24] whereas missing or low IgM expression and the highest CD38 expression are found on plasmablasts (R5) [25]. In case of a detectable plasmablast population, the gate for CD27+ IgM and memory B-cells in B1 needs to be adapted to exclude CD27++ plasmablasts.

**Measurements to Detect ICOS and CD40L Expression on Activated T-Cells**

The cells are defined first by a two-dimensional data collection window, which deliberately includes larger cells in the FSC/SSC display and subsequently by gating on CD4+ cells (*cave* after stimulation by CD3). CD3 is only weakly detectable on the surface of activated cells. To confirm activation, the expression of CD69 (x-axis) and

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**Fig. 3.** Analysis of peripheral B-cell subpopulations for the classification of CVID. A lymphocyte region (R1) and a CD19+ region (R2) are combined to a B-cell gate, in which B1 and B2 stainings are analyzed. Panel 1 permits the distinction between naive (UL), IgM memory (UR) and class-switched B-cells. In panel 2 the CD21\textsuperscript{low} B-cells (R3) can be defined in the first window (CD38/CD21). To identify transitional B-cells (R4) and the plasmablasts (R5), the representation CD38/IgM is preferred. The evaluation is partially done by means of quadrant statistics and partially by means of regional statistics. **a** Results in a normal person. **b** Typical findings in a CVID patient with expansion of CD21\textsuperscript{low} B-cells. **c** The uniqueness of the third example is the expansion of plasmablasts in the peripheral blood of a patient during an infection.
CD40L and/or ICOS (y-axis) on CD4+ T-cells is represented in a second window (not shown). Data of at least 5,000 CD4+ cells should be collected.

Data Analysis

Peripheral Lymphocyte Population
To assess the basic lymphocyte status, the lymphocyte region (R1) is drawn according to figure 2a with the FL3 (CD45) and SSC axes. The proportions of CD4+ and CD8+ T-cells (UR, fig. 2b, c), NK cells (UL, fig. 2d) and B-cells (UL, fig. 2e) are determined from the respective quadrants. The absolute values are calculated by multiplication of total lymphocyte counts from the differential blood cell count and the relative proportion of the individual populations of lymphocytes. The CD45 staining used in whole blood is used to define a lymphocyte window which excludes erythrocytes and basophils, from which the proportion of lymphocyte subpopulations can be accurately calculated.

Results
The patient’s clinical symptoms and medication should be taken into account when evaluating the analytical data. Reference values for the different B-lymphocyte subpopulations for adults are specified in ‘Characterization of B-Lymphocytes’; table 7 (pp. 222). The reference values for other lymphocyte populations in adults have been reported by Bisset et al. [26]. A clear reduction in B-cell number (<1%) gives an indication of the different forms of agammaglobulinemia. Clear inversions of the CD4+/CD8+ relationship can be a sign of viral infection. In particular in the presence of low CD4+ cell levels, an HIV infection should be ruled out. The diagnosis of an EBV infection should also be kept in mind, in particular in the case of male patients with positive family histories (XLP). Such a shift in lymphocyte profile without a clear cause is found more frequently in CVID patients. Lymphomas are an important possible diagnosis with regard to abnormal (e.g. immunoglobulin negative) B-cell populations or even monoclonal populations (characterized by a clearly shifted \( \kappa/\lambda \) relationship) (see ‘Flow Cytometry in the Diagnosis of Non-Hodgkin’s Lymphomas’, pp. 642).

Classification
All classification schemes based on B-cell phenotype group patients according to the reduction in class-switched memory B-cells. The Paris classification includes non-class-switched memory B-cells, the Freiburg Classification includes CD21\(^{\text{low}}\) B-cells and the EUROClass includes CD21\(^{\text{low}}\) B-cells and transitional B-cells as additional discriminating parameters (fig. 1).
Results

For details on B-cell phenotyping, also see ‘Characterization of B-Lymphocytes’ (pp. 211).

The evaluation and reference values for the B-cell subpopulations are described in ‘Characterization of B-Lymphocytes’ (pp. 211). In general, it is meaningful to indicate the proportion of the entire B-cell population as percent of the lymphocytes and the subpopulation in percentage of B-cells. From these data, lymphocyte-specific or absolute values can be calculated if necessary.

**Freiburg Classification** (fig. 1a): Following the Freiburg Classification, the proportion of class-switched memory B-cells of the total lymphocyte population is determined first (fig. 3, panel 1). If the proportion is below 0.4%, the patient is diagnosed as type I (about 75% of the patients). Patients with more than 0.4% belong to type II (about 25% of the patients). Type I patients are subdivided according to the proportion of CD21^low^ B-cells into type Ia with an expansion of CD21^low^ B-cells of greater than 20% and type Ib with less of an expansion (fig. 3, panel 2). Freiburg type Ia patients present significantly more often with splenomegaly and granulomatous disease [18].

**Paris Classification** (fig. 1b): The division of CVID patients according to the Paris Classification [17] is based on the same staining procedure as the B1 staining of the Freiburg Classification. The groups are differentiated into MB2 with a normal percentage of memory B-cells, MB1 with decreased class-switched and normal non-class-switched memory B-cells and MB0 with a decrease in both CD27+ B-cell populations. ‘Decreased’ values are defined as 2 standard deviations below the mean. In the cohorts examined, the proportion of CD27+ B-cells for the MB0 group was <11%; the proportion of class-switched B-cells was <8% for the MB1 group. With this classification scheme, significant levels of splenomegaly and granulomatous inflammation were found in the MB0 group [17].

**EUROClass** (fig. 1c): The EUROClass classification [19] uses the same staining procedure and assessment as the Freiburg Classification and was the fruit of common efforts of several European immunodeficiency centers. It discriminates a B− group with equal or less than 1% B-cells and B+ group with more than 1% of B-cells. The latter group is divided into smB+ (>2% IgM− IgD− CD27+ B-cells) and smB− (< = 2% IgM− IgD− CD27+ B-cells) patients. In addition, patients with expansion of CD21^low^ B-cells ≥10% are classified as ‘smB− or smB+ CD21^low^ compared to ‘CD21^norm^’; patients with an expansion of transitional B-cells ≥9% are designated ‘smB-Tr^hi^’ compared to ‘Tr^norm^’.

The evaluation of more than 300 patients confirmed the association of severely reduced class-switched memory and CD21^low^ B-cells with splenomegaly and granulomatous disease. In addition, it demonstrated an association between expanded transitional B-cells and lymphadenopathy.

**T-Cell-Based Classification:** A recent suggestion for the classification of CVID is based on the relative reduction of naive CD4 T-cells [21].
Diagnosing Common Variable Immunodeficiency

**Exclusion of Monoclonal B-Cell Populations**
The B3 staining gives insight into monoclonal B-cell population distributions (fig. 4). After gating on lymphocytes and B-cells, the $\kappa$ versus $\lambda$ light chain expression is represented. This relationship should come to approximately 50:50. Clear deviations or the presence of a substantial proportion of light-chain-negative B-cells can be considered ambiguous clues for atypical B-cell populations after the exclusion of plasmablasts (stain B2, R5). The presence of such abnormal populations should be clarified more precisely in cooperation with the department of hematology.

**Exclusion of Class Switch Recombination Defect due to CD40 Deficiency**
For this analysis, CD40 expression is examined after gating on B-cells. Normally, almost 100% of B-cells are positive for CD40. As previously mentioned, this analysis only allows the confirmation of severe structural defects in this molecule. The hyper-IgM syndrome, which is due to a defect in the CD40 receptor, is extremely rare (<1% the patients with hyper-IgM syndrome) and so far only early-childhood onset has been described. The investigation is easily accomplished and can be carried out without excessive expenditures. Thus far, none of the CVID patients we examined manifested abnormal CD40 expression. Thus, even with a positive result, further genetic and functional analysis would be necessary to confirm the diagnosis.

**Exclusion of Class Switch Recombination Defect due to CD40 Ligand Deficiency**
The exclusion of CD40L defects is complex. Therefore, this procedure should be used only if the suspicion is strong, i.e. in male patients, especially with a positive
family history, whose serum IgM value is in the upper range of normal or is increased. After stimulation, activated CD4+ T-cells and nonactivated control cells should be evaluated according to figure 5. As an internal control for successful activation, activated CD4+ T-cells are evaluated for increased surface expression of CD69. Nonactivated T-cells should be CD40L+.

**Exclusion of AID and UNG Deficiency**

AID and UNG deficiency cannot be excluded by flow cytometry, but the absence of class-switched B-cells combined with increased IgM-only memory B-cells (IgM+ IgD− CD27+) strongly suggests intrinsic B-cell defects of class switch recombination.

**Exclusion of ICOS Deficiency**

In CVID patients with autosomal recessive transmission of the illness and severely reduced class-switched memory B-cells, a defect in ICOS expression should be investigated. ICOS is expressed on activated T-cells within a few hours and, in contrast to nonstimulated cells, is readily detectable after 16 h (fig. 5). Due to its T-cell-specific expression, other lymphocyte populations can serve as negative controls. As positive control, PBMNCs from a healthy person must be tested in parallel. We carry out this evaluation after gating CD4+ T-cells as for the analysis of CD40L. The exclusive analysis of CD4+ T-cells is necessary since the expression of the ICOS receptor is higher on CD4+ T-cells than on CD8+ T-cells.

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**Fig. 5.** Confirmation of an ICOS defect. After the activation of T-cells over 16 h in vitro (see text regarding details), surface expression of both CD40L and ICOS could be clearly proven after gating on CD4+ T-cells for healthy controls (HD). The increased expression of CD69 is used as control for activation. In the comparison, the patient shows an almost normal CD40L expression, but no ICOS expression. One of the 9 thus far identified CVID patients with a genetic ICOS defect [10] is shown. Additionally, a nonstimulated control should always be run in parallel (not shown here).
Exclusion of TACI Deficiency

TACI deficiency is the most common genetic defect in CVID patients [14]. It is not associated with a specific B- or T-cell phenotype [11]. TACI itself is expressed on memory B-cells and can readily be detected by flow cytometry with several monoclonal antibodies, but only very few TACI-deficient patients present with absent or severely reduced TACI surface expression, rendering the flow-cytometric evaluation unreliable.

Exclusion of CD19 Deficiency

CD19 deficiency [12] was readily detectable by flow cytometry in all five cases reported up to the present date since most laboratories use CD19 for the identification of B-cells. Therefore the discrepancy between the absence of CD19+ cells and the simultaneous detection of other B-cell markers (for example immunoglobulin or CD20) strongly suggests CD19 deficiency. In the standard lymphocyte panel, CD19-deficient B-cells may present as an undefined CD3− CD19− CD16/CD56− lymphocyte population, resulting in a summation of T-, B- and NK cells below 100%.

Exclusion of BAFF-R Deficiency

So far, only 2 patients have been identified with BAFF-R deficiency, thus making it a very rare defect. B-cell phenotyping showed a reduction in total B-cells and a significant accumulation of transitional B-cells in both affected patients (Warnatz et al., in preparation). In healthy donors, BAFF-R is highly expressed on all peripheral B-cells. It should be noted that some monoclonal antibodies such as 11C2 and 8A7 show weaker signals in some patients while this difference is not detectable by the polyclonal antiserum (M. Rizzi and H. Eibel, personal communication).

Comments

We recommend the standard evaluation of patients with hypogammaglobulinemia by the assessment of the basic lymphocyte panel and B-cell phenotyping for classification as well as exclusion of atypical lymphocyte populations. The evaluation of differential diagnoses specified under ‘Diagnosing Common Variable Immunodeficiency’ (see above) remains reserved for special cases. In our laboratory, B-cell staining is performed on Ficoll-separated PBMNCs. Several laboratories carry out B-cell staining with whole-blood assays. This procedure represents an alternative which requires the appropriate controls.

Quality Control

Apart from the staining with appropriate isotype controls, each analysis should be run together with healthy control samples in order to exclude technical errors. The
stimulation experiments should likewise contain both a medium control (nonstimulated) and a healthy control sample. Highly pathological findings should be confirmed by an independent second analysis. All results should be interpreted considering the patient’s clinical history.

Troubleshooting

From our experience, evaluations of B-cell subpopulations in patients with less than 1% B-cells are difficult and must be interpreted with caution. Different specific antibodies and/or conjugates (e.g. CD38-PE vs. CD38-APC) can lead to different results, which must be considered in the conclusions. In particular, the highly variable quality of the anti-ICOS antibodies must be taken into account. The problems of in vitro stimulation of T-cells are numerous (also see ‘Detection of Antigen-Specific T-Cells using MHC Multimers or Functional Parameters’, pp. 476).

Expected Results

The expected results for B-cell subpopulations in peripheral blood and bone marrow are specified in the respective sections and illustrations.

Required Time

The preparation of PBMNCs takes about 1.5 h. The same amount of time must be scheduled for surface staining. T-cell stimulation to confirm CD40L requires 0.5 h on the 1st day for the preparation of the plates, 2 h on the 2nd day for the cleaning and incubation of the PBMNCs, which can be further processed after 16–20 h of overnight culture. The data acquisition of 2,000–5,000 cells requires up to 5 min per tube.

Summary

Common variable immunodeficiency syndrome (CVID) is a heterogeneous antibody deficiency syndrome that manifests clinically by recurrent respiratory and gastrointestinal infections either in childhood (after the 2nd year of life) or in the 2nd–3rd decade. Since CVID represents a diagnosis by exclusion, well-known genetic, infectious and toxic causes must be excluded. The diagnostic criteria were established by the ESID (www.esid.org). Flow cytometry allows the detection of patients with class switch recombination deficiency syndromes and agammaglo-
bulinemia (B-cell numbers <1%). Increasingly, flow cytometry also serves to classify this heterogeneous syndrome and, to a limited extent, to exclude genetic defects associated with CVID. Further flow-cytometric analyses are currently reserved for research.

References


