Classification of common variable immunodeficiencies using flow cytometry and a memory B-cell functionality assay

Amelia L. Rösel, MD,[a] Carmen Scheibenbogen, MD,[a,b,c] Ulrike Schliesser, ScD,[b] André Sollwedel, ScD,[a] Bodo Hoffmeister, MD, PhD,[c] Leif Hanitsch, MD,[c] Horst von Bernuth, MD,[d] Renate Krüger, MD,[d] Klaus Warnatz, MD, PhD,[e] Hans-Dieter Volk, MD,[a,b]* and Sybill Thomas, ScD[a]* *Berlin and Freiburg, Germany*

Background: The population of patients with common variable immunodeficiency (CVID) comprises a heterogeneous group of patients with different causes of hypogammaglobulinemia predisposing to recurrent infections, higher incidence of autoimmunity, and malignancy. Although memory B cells (memBcs) are key players in humoral defense and their numbers are commonly reduced in these patients, their functionality is not part of any current classification.
Objective: We established and validated a memBc enzyme-linked immunosorbent spot (ELISpot) assay that reveals the capacity of memBcs to develop into antibody-secreting cells and present an idea for a new classification based on this functional capacity.
Methods: The memBc ELISpot assay, combined with flow cytometry, was applied to patients with confirmed CVID in comparison with age-matched healthy control subjects.
Results: *Ex vivo* frequency of IgG-, IgM-, and IgA-secreting plasmablasts was significantly diminished by 27.2-, 2.4-, and 23.3-fold, respectively, compared with that seen in healthy control subjects. Moreover, *in vitro* differentiation of memBcs into antibody-secreting cells was 6.1-, 2.6-, and 3.7-fold significantly reduced for IgG-, IgM-, and IgA-secreting cells, respectively. Proliferation of memBcs correlates inversely to immunoglobulin-secreting capacity, suggesting compensatory hyperproliferation. Furthermore, patients with no serum IgA can still have a detectable IgA ELISpot assay result *in vitro*. Most importantly, the large heterogeneity of memBc function in patients with CVID homogenously grouped by means of fluorescence-activated cell sorting allowed additional subclassification based on memBc/plasmablast function.
Conclusion: These data suggest almost normal memBc/immunoglobulin-secreting plasmablast functionality in some patients if sufficient stimulatory signals are delivered, which might open up opportunities for new therapeutic approaches. (J Allergy Clin Immunol 2015;135:198-208.)

Key words: *Memory B cell, enzyme-linked immunosorbent spot assay, common variable immunodeficiency subtyping, flow cytometry, antibody-secreting cells*

Common variable immunodeficiency (CVID) is a primary immunodeficiency presenting as a heterogeneous phenotype associated with recurring infections, mainly of the respiratory tract, potentially leading to chronic lung disease, autoimmunity, lymphoid infiltration, enteropathy, and a higher risk of malignancies.[1-5] With an incidence of about 1:25,000 to 1:50,000 in Western countries, CVID is the most common symptomatic primary immunodeficiency.[1,6,7] For probable CVID, current diagnostic criteria according to the European Society for Immunodeficiencies are a marked reduction in IgG levels (≥2 SDs less than the age mean) and a clear decrease in levels of at least 1 of the isotypes IgA and IgM, absent isohemagglutinins, and/or poor response to vaccines and onset of immunodeficiency at more than 2 years of age when defined causes of hypogammaglobulinemia have been excluded (www.esid.org).[3,6] Genetic analysis has identified defects in inducible costimulator,[8] CD19,[9] CD20,[10] CD21,[11] CD81,[12] B cell–activating factor receptor,[13] and LPS-responsive beige-like anchor[14] in less than 10% of patients with CVID, leaving more than 90% of the cause unknown.[2,7,15-18] Memory B cells

From [a]the Institute for Medical Immunology, Charité University Medicine Berlin; [b]the Berlin-Brandenburg Centre for Regenerative Therapies, Berlin; [c]the Out-patients Clinic for Immunodeficiencies, Charité University Medicine Berlin; [d]the Clinic for Pediatrics, Department of Pneumology and Immunology, Charité University Medicine Berlin; and [e]the Centre for Chronic Immunodeficiency, University Clinic and University of Freiburg.
*These authors contributed equally to this work.
Supported by BMWi-ZIM (Federal Ministry of Economics and Technology), Cooperation Project KF2088107FR9, for the grant to partially finance this project.
Disclosure of potential conflict of interest: A. L. Rösel has received research support from the BMWI-ZIM (Federal Ministry of Economics and Technology) Cooperation Project. C. Scheibenbogen has consultant arrangements with CureVac, Baxter, and Biotest; is employed by Charité; has received research support from Deutsche Forschungsgemeinschaft, EFRE, Baxter, and Behring; has received payment for lectures from Baxter; and has received travel support from Octapharma and Behring. H. von Bernuth has received research support from Deutsche Forschungsgemeinschaft and Bundesministerium für Bildung und Forschung and has received payment for lectures from CSL Behring. K. Warnatz has received payment for lectures from Baxter, GlaxoSmithKline, CSL Behring, Pfizer, Biotest, Novartis Pharma, Stallergenes AG, Roche, Meridian Health Communications, Octapharma, and the American Academy of Allergy, Asthma & Immunology; has received payment for manuscript preparation from UCB Pharma; and has received payment for development of educational presentations from the European Society for Immunodeficiencies. H.-D. Volk has received kits for enzyme-linked immunosorbent spot (ELISpot) assay free of charge from AID; has consultant arrangements with Bayer, Boehringer, Novo Nordisk, CRO, and Charité; has received research support from Bayer, Novo Nordisk, and AID; and has received license fees for patents. S. Thomas has received research support from the BMWI-ZIM (the Federal Ministry of Economics and Technology) Cooperation Project; is employed by Federal Joint Committee, PSF 120606, D-10596; and has received payment for lectures from Bundeskongress Pathologie Berlin. The rest of the authors have declared that they have no relevant conflicts of interest.
Received for publication September 14, 2013; revised June 9, 2014; accepted for publication June 11, 2014.
Available online August 10, 2014.
Corresponding author: Hans-Dieter Volk, MD, Institute for Medical Immunology, Charité Berlin, Augustenburger Platz 1, 13353 Berlin. E-mail: hans-dieter.volk@charite.de.
0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2014.06.022

Abbreviations used
ASC: Antibody-secreting cell
CVID: Common variable immunodeficiency
ELISpot: Enzyme-linked immunosorbent spot
FACS: Fluorescence-activated cell sorting
HC: Healthy control
Ig-sPb: Immunoglobulin-secreting plasmablast
memBc: Memory B cell
PB: Pacific Blue
PE: Phycoerythrin

(memBcs) are formed during T cell–dependent antigen encounter and ensure subsequent rapid immune responses with vast amounts of high-affinity antibodies,[19-22] and their numbers are often greatly reduced in patients with CVID.[5,18,23,24]

Many ways to improve the diagnostics of CVID have been proposed over the past decades,[2,16,17,23,25,26] sometimes without sufficiently reflecting the heterogeneity of the disease,[2] which still constitutes a major challenge for classification.[5,17] Currently, serum immunoglobulin levels, particularly representing long-lived plasma cell function, and flow cytometry (fluorescence-activated cell sorting [FACS]), showing numeric B-cell subgroup deficiencies, are the gold standard for CVID diagnostics and classification.[6,23,26] The most recent scheme, EUROclass, combines the Freiburg[26] and Paris[27] classifications. Patients are separated into subgroups according to their B-cell counts, then based on memBc counts, and finally based on further B-cell subpopulations, such as activated and transitional B cells, to be correlated with clinical data.[23,28] Memory B-cell function (ie, the residual capacity to respond to rechallenge with pathogens) has been described[29-31] but is not part of any commonly used clinical test, even though these cells are a vital part of humoral defense and their numbers are known to be reduced in many patients with CVID.[5,23] Therefore, we established a well-standardized and validated memBc enzyme-linked immunosorbent spot (ELISpot) assay. The *in vitro* ELISpot assay detects functioning antibody-secreting cells (ASCs)[32] attained from reactivated memBcs during stimulation culture,[20] allowing us to distinguish between several functional deficits and thereby facilitating a more accurate classification when used in combination with FACS. Additionally, the *ex vivo* memBc ELISpot assay can reveal *in vivo*–activated immunoglobulin-secreting plasmablasts (Ig-sPbs). A more detailed classification might be the basis of a personalized therapy for CVID.[6,33] Identifying patients with remaining functional memBcs might reveal a group of patients needing less IgG substitution.[1,34] In addition, almost normal *in vitro* memBc function in the presence of sufficient costimuli might indicate options for new therapeutic approaches to partly replace IgG substitution therapy.

METHODS

Blood samples and clinical data from patients and healthy subjects

After the immunoglobulin ELISpot assay and flow cytometric panels were established and validated, fresh blood samples from 14 patients with confirmed diagnosis of CVID were compared with samples from 47 healthy control (HC) subjects. CVID was defined based on the criteria of the European Society for Immunodeficiencies.[3]

This study was approved by the Institutional Review Board of Charité Berlin for HC subjects in October 2010 (EA1/250/10) and for patients in June 2012 (EA2/046/12). All participants provided written informed consent.

One aliquot of each sample was used for flow cytometry and the *ex vivo* ELISpot assay revealing Ig-sPbs, and the second aliquot was tested for functional capacity to differentiate into ASCs during *in vitro* stimulation (Fig 1).

The HC group (n = 47 adults), consisting of an equal number of male and female subjects in the age groups of 18 to 30, 31 to 40, 41 to 50, 51 to 60, and 61 to 70 years, showed no significant age- or sex-related differences of response (see Fig E1 in this article's Online Repository at www.jacionline.org).

Memory B-cell ELISpot assay before and after stimulation

PBMCs were flow cytometrically analyzed before and after culture under standard incubation settings for 6 days in 6-well plates at 3e6 cells/3 mL complete RPMI with the stimulation cocktail.[35] In brief, we used 6 μg/mL of the Toll-like receptor 9 ligand CpG,[36] 1:10,000 *Staphylococcus aureus* Cowan activating Toll-like receptor 2, 1 ng/mL pokeweed mitogen with a great effect on B-cell proliferation and T-cell activation,[37] and 50 μmol/L β-mercaptoethanol stimulating B- and T-cell help, according to the method of Crotty et al,[35] to achieve optimal antigen-unspecific stimulation.[38-40]

ELISpot assay was performed with standardized 96-well plates commercially coated by our partner, GenID (Straßberg, Germany), to detect total IgG, IgM, and IgA secretion at several concentrations per well (2.5×10^5 *ex vivo* and 1.25×10^4, 6.5×10^3, 3.1×10^3, and 1.5×10^3 *in vitro*). After incubation for 2 hours at standard settings, plates were washed, and biotinylated secondary antibody was applied overnight. Detection was performed with 1 hour of streptavidin, washing, and 3 to 5 minutes of 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt to induce color development. Once the reaction was stopped and the plates dried, they were analyzed with the ELISpot Reader (ImmunoSpot Cellular Technology Ltd, Cleveland, Ohio). The *ex vivo* ELISpot assay from freshly isolated PBMCs detects *in vivo*–activated Ig-sPbs, and the ELISpot assay from *in vitro*–stimulated PBMCs reveals ASCs from reactivated memBcs for the class-switched isotypes IgG and IgA, but we cannot exclude formation of ASCs from both naive B cells and memBcs for IgM.

EUROclass panel FACS staining

Samples were prepared from fresh and cultured PBMCs, as described for the ELISpot assay. Cells were stained with LD-PB (Invitrogen, Carlsbad, Calif) in 100 μL for 20 minutes at 4°C before adding the antibodies for 10 minutes at room temperature. EUROclass panel mix 1 includes anti-CD3–Pacific Blue (PB), anti-CD27–phycoerythrin (PE), anti-IgD–fluorescein isothiocyanate (BD Biosciences, San Jose, Calif), anti-CD14–PB, anti-CD16–PB (Invitrogen), anti-CD19–PE-Cy7, anti-IgM–allophycocyanin (Beckman Coulter, Fullerton, Calif), and mix 2 contains the same antibodies, except for anti-CD21–PE and anti-CD38–fluorescein isothiocyanate (BD Biosciences) replacing anti-CD27 and anti-IgD, respectively. Counting Beads (Invitrogen) were added to a sample of cells from the suspension plated for culture, as well as directly from the culture plate after stimulation, and stained with anti-CD3–PB and anti-CD19–PE-Cy7, as mentioned above.[41] Gating on leukocytes, singlets, and living $CD19^+$ cells, we marked naive cells (IgD^+CD27^-), marginal zone cells (IgD^+CD27^+), memBcs (IgD^-CD27^+), and plasmablasts ($CD19^{dim}38^{high}$), according to the method of Wehr et al.[23]

Software and statistics

For flow cytometric analysis, all samples were measured on an LSR II (BD Biosciences) by using FACSDiva (version 6.1.3, BD Biosciences) and FlowJo (version 9.2; TreeStar, Ashland, Ore) software; for ELISpot assay readout, ImmunoCapture (version 6.0) and ImmunoSpot Academic (version 4.17; Cellular Technology, Shaker Heights, Ohio) software was used, and