High-content cytometry and transcriptomic biomarker profiling of human B-cell activation

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Background: Primary antibody deficiencies represent the most prevalent, although very heterogeneous, group of inborn immunodeficiencies, with a puzzling complexity of cellular and molecular processes involved in disease pathogenesis.
Objective: We aimed to study in detail the kinetics of CD40 ligand/IL-21–induced B-cell differentiation to define new biomarker sets for further research into primary antibody deficiencies.
Methods: We applied high-content screening methods to monitor B-cell activation on the cellular (chip cytometry) and transcriptomic (RNA microarray) levels.
Results: The complete activation process, including stepwise changes in protein and RNA expression patterns, entry into the cell cycle, proliferation and expression of activation-induced cytidine deaminase (AID), DNA repair enzymes, and post–class-switch expression of IgA and IgG, was successfully monitored during *in vitro* differentiation. We identified a number of unknown pathways engaged during B-cell activation, such as CXCL9/CXCL10 secretion by B cells. Finally, we evaluated a deduced set of biomarkers on a group of 18 patients with putative or proved intrinsic B-cell defects recruited from the European Society for Immunodeficiencies database and successfully predicted 2 AID defects and 1 DNA repair defect. Complete absence of class-switched B cells was a sensitive predictor of AID deficiency and should be further evaluated as a diagnostic biomarker.
Conclusion: The biomarkers found in this study could be used to further study the complex process of B-cell activation and to understand conditions that lead to the development of primary antibody deficiencies. (J Allergy Clin Immunol 2014;133:172-80.)

Key words: *Primary immunodeficiency, primary antibody deficiency, B-cell immunology, chip cytometry*

Primary immunodeficiencies (PIDs) are prototypic for rare diseases: they are notoriously difficult to diagnose, with long diagnostic delays[1] resulting in both considerable morbidity and high health care costs. Hypogammaglobulinemias are the most prevalent subgroup of PIDs. They represent a very heterogeneous collection of diseases with the shared hallmark of decreased serum immunoglobulin levels and predisposition to severe and chronic bacterial infections. However, the individual disease entities differ significantly regarding their accompanying clinical symptoms, including propensity to opportunistic infections (eg, CD40/CD40 ligand [CD40L] defects[2]), malignancies in CD40L and DNA repair defects,[3,4] neurologic abnormalities (as in ataxia telangiectasia),[5] or autoimmunopathies (eg, activation-induced cytidine deaminase [AID] defect[6]).

Misdiagnosis can have fatal consequences,[7,8] and reliable and comprehensive diagnostic tests are urgently needed. The Online Mendelian Inheritance of Man (OMIM) database currently lists 71 distinct diseases,[9] all resulting in hypogammaglobulinemia but attributed to a wide range of different molecular defects.[4,10-16]

Most of the disease-causing genes of hypogammaglobulinemias are not identified yet, and the genotype-phenotype correlations are often week, and therefore genotyping might not be the diagnostic method of choice for this disease group in the near future.[17] Cytometric diagnostic approaches analyze resting peripheral B cells and deduce functional defects from the phenotype of a resting cell.[18] However, a recent study demonstrates that the diagnostic value of this approach is limited to subgroups of hypogammaglobulinemias only.[19] Another weakness of this approach is based on the fact that memory B-cell levels are physiologically low[20,21] during early childhood, which compromises the important early diagnosis of class-switch defects.

Functional *in vitro* assays are the diagnostic method of choice for a number of immunodeficiencies (eg, degranulation in patients with hemophagocytic lymphohistiocytosis, oxidative burst in patients with chronic granulomatous disease, or CH50 [total complement activity] in patients with complement defects), and it might be useful to include a cytometry-based, functional B-cell

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Abbreviations used
AID: Activation-induced cytidine deaminase
CD40L: CD40 ligand
CSR: Class-switch recombination
CVID: Common variable immunodeficiency
MACS: Magnetic-activated cell sorting
PID: Primary immunodeficiency

assay in the repertoire of PID diagnosis in the future. We recently reported on a patient with hypogammaglobulinemia in whom we successfully identified an intrinsic B-cell differentiation block through *in vitro* activation of B cells by means of stimulation of CD40L and IL-21.[22] CD40L and IL-21 mimic the main effector molecules of T follicular helper cells.[23] Therefore activation of these molecules allows the analysis of B cell–intrinsic, T cell–dependent B-cell differentiation *in vitro*.[24] One limitation of this approach is that B cells of patients with common variable immunodeficiency behave normally when stimulated with CD40L/IL-21,[25] so that these patients cannot profit from this approach.

The aim of the present study was to perform high-content biomarker profiling of human B-cell activation both in health and disease to find potential cellular biomarkers for later diagnostic use. We identified a set of 10 biomarkers for discrimination of different B-cell differentiation steps. Next, we used this biomarker set to test 18 patients with putative or proved intrinsic B-cell defects recruited from the European Society for Immunodeficiencies database and successfully predicted 2 AID defects and 1 DNA repair defect.

METHODS

The use of patient material was approved by the institutional review boards of the relevant centers.

Workflow

Fig E1 in this article's Online Repository at www.jacionline.org provides an overview of the workflow of the methods used in this article.

Antibodies

The antibodies used in this study are listed in Table E1 in this article's Online Repository at www.jacionline.org. All antibodies used were phycoerythrin labeled, except for anti-AID, which was labeled in house with Alexa Fluor 555 as the dye.

Sample source

PBMCs were prepared from EDTA blood of healthy volunteers or from patients after informed consent. Patients were recruited by searching the European Society for Immunodeficiencies registry database[26] for a presumptive or definitive diagnosis of class-switch defects (except CD40L defects) and by contacting all European immunodeficiency centers caring for such patients. Six centers participated in this study, contributing of total of 18 patients (see Table E2 in this article's Online Repository at www.jacionline.org). All patients are regularly substituted for IgG.

In vitro differentiation of human B cells

PBMCs (2×10^6) or magnetic-activated cell sorting (MACS)–separated subpopulations were incubated on irradiated CD40L-transfected NIH3T3 mouse fibroblasts in IL-21 (4 ng/mL)–containing medium (RPMI 1640; 2mmol/L L-glutamate, 1% penicillin/streptomycin [Biochrome, Berlin, Germany], 10 mmol/L HEPES, and 5% heat-inactivated FCS) for defined durations, as indicated in Fig E1.

In vitro activation of human T cells

PBMCs (2×10^6) were stimulated for 72 hours by using the T Cell Activation/Expansion Kit from Miltenyi Biotec (Bergisch Gladbach, Germany).

Chip cytometry

Chip cytometry was performed, as described before in detail.[27] For surface markers, antibodies were diluted 1:10 in PBS and incubated on the cells inside the chip for 5 minutes at room temperature, followed by washing the channels with 200 μL of PBS. For intracellular markers, antibodies were diluted in permeabilization buffer and incubated for 1 hour at room temperature, again followed by a washing step with 200 μL of permeabilization buffer.

MACS sorting

When necessary, different subpopulations were prepared from PBMCs or, after stimulation with negative or positive selection, by using MACS, according to the manufacturer's recommendations. We used human the B Cell Separation Kit II, CD4 kit, CD19 MACS, and IgD-phycoerythrin combined with anti-PE MicroBeads (all from Miltenyi Biotec). Purity was confirmed by means of flow cytometry (data are shown in the respective Results section).

RT-PCR for sterile and productive immunoglobulin transcripts

Total RNA was prepared by using phenol-chloroform extraction after TRIZOL (Invitrogen Life Technologies, Carlsbad, Calif). cDNA synthesis was conducted in a reaction volume of 50 μL containing (medial) 200 ng of total RNA, 0.01 mol/L dithiothreitol, 0.4 mmol/L dNTPs, and 200 U/mL SuperScript II Reverse Transcriptase (Invitrogen). cDNA templates were subjected to PCR amplification by using the primers and reaction conditions shown in Table E3 in this article's Online Repository at www.jacionline.org. PCR products were separated by means of electrophoresis on 1.5% agarose gels and visualized with ethidium bromide staining. The PCR protocol was 5 minutes of denaturation at 94°C, followed by 30 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 68°C (52°C for Iμ-Cμ and 57°C for VHDJH-Cα), and 1 minute of extension at 72°C. Finally, we performed a final extension at 72°C for 7 minutes.

Microarray

RNA was isolated by using the RNA Isolation Kit from Qiagen (Hilden, Germany). RNA was subjected to microarray analysis by using the Affymetrix Microarray Platform (Affymetrix, Santa Clara, Calif). The Human Gene 1.0 ST Array cDNA Synthesis and Amplification Kit was used to make double-stranded cDNA from total RNA, which was then labeled with biotin (Genechip Terminal Labeling Kit). After chemical fragmentation of the biotin-labeled cRNA targets, they were hybridized to Affymetrix Human Gene 1.0 ST microarrays by using the Fluidics Station 450 and scanned with the Affymetrix Genechip Scanner 3000 with Genechip Operating Software 1.4. Raw data were normalized by using log-transformed data with the Bioconductor package, applying the VSN algorithm.[28] Purity of cell populations subjected to transcriptomic analysis was tested by analyzing the expression of cell lineage–specific transcripts, as shown in Table E4 in this article's Online Repository at www.jacionline.org. Only samples that showed no significant expression of contaminating cell lineages were analyzed subsequently. As an example, control transcripts of sorted $CD4^+$ T cells, sorted B cells, and whole PBMCs are shown.

For Fig 1 and Fig E2, which is available in this article's Online Repository at www.jacionline.org, B cell–specific transcripts were filtered transcriptwise: A gene was considered as being expressed exclusively by B cells if the mean expression of its transcripts from T-cell experiments was lower than the mean transcript from B-cell experiments and if at least 2 B-cell experiments had an expression level of greater than 7.5 (ln, mean median expression of all microarrays after normalization = 7.2). For the filter in Fig 1, *A*, experiments at day 3 were used, and for the filter in Fig 1, *B*, the experiments from day 0 were used, respectively.

For validation of the obtained results, relevant transcripts were compared with NextBio Basic, an ontology-based access portal to high-content