Clinical Immunology 175 (2017) 69–74

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Brief Communication

Patterns of constitutively phosphorylated kinases in B cells are associated with disease severity in common variable immunodeficiency

Eli Taraldsrud [a,b,c,d], Pål Aukrust [b,c,d,e], Silje Jørgensen [b,c,d,e], Ole Christian Lingjærde [f,g], Johanna Olweus [a,b,c,1], June H. Myklebust [a,g,1], Børre Fevang [b,c,d,e,*,1]

[a] Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital, Radiumhospitalet, Oslo, Norway
[b] K.G. Jebsen Centre for Cancer Immunotherapy, Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[c] K.G. Jebsen Inflammation Research Centre, Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[d] Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway
[e] Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, Oslo, Norway
[f] Department of Computer Science, University of Oslo, Oslo, Norway
[g] Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, Norway

ARTICLE INFO

Article history:
Received 26 September 2016
Received in revised form 23 November 2016
accepted with revision 27 November 2016
Available online 3 December 2016

Keywords:
Phospho-flow cytometry
B cell
Cell signaling
Constitutive phosphorylation
Common variable immunodeficiency
Hierarchical clustering

ABSTRACT

Patients with common variable immunodeficiency (CVID) constitute a clinically and immunologically heterogeneous group characterized by B-cell dysfunction with hypogammaglobulinemia and defective immunoglobulin class switch of unknown etiology. Current classification systems are insufficient to achieve precise disease management. Characterization of signaling pathways essential for B-cell differentiation and class switch could provide new means to stratify patients. We evaluated constitutive and induced signaling by phospho-specific flow cytometry in 26 CVID patients and 18 healthy blood donors. Strong responses were induced both in CVID and healthy donor B cells upon activation. In contrast, constitutive phosphorylation levels of STAT3,-5,-6, Erk, PLC-γ and Syk were significantly increased in CVID B cells only. Hierarchical clustering revealed a subgroup of CVID patients with elevated constitutive phosphorylation of Syk and PLC-γ. All these patients had non-infectious complications, indicating that a distinct phosphorylation pattern of kinases in B cells identifies a clinically important subgroup of CVID patients.

1. Introduction

Common variable immunodeficiency (CVID) is a clinically and immunologically heterogeneous disease, characterized by impaired B-cell function with defective antibody production [1]. While an increased risk of respiratory and other infections dominates, many CVID patients also have non-infectious complications such as splenomegaly, enteropathy, interstitial lung disease and granulomata, associated with higher mortality, and increased susceptibility for lymphoid malignancies [2]. A majority of the patients have normal or slightly decreased levels of B cells, consisting primarily of naïve B cells [3], indicating defective immunoglobulin (Ig) class switch recombination (CSR) and an impaired germinal center reaction. Monogenic forms of CVID have been associated with mutations in genes encoding proteins involved in B cell receptor (BCR) complex formation and signaling, CSR, or B-cell differentiation, -proliferation and -survival [4,5]. However, these known mutations affect only a small fraction of patients, and the pathogenic process driving this immunodeficiency remains enigmatic in most cases. Current classification schemes are based on alterations in B cell subsets such as frequencies of class switched- and $CD21^{low/-}$ expressing B cells, but they have been criticized for low association to clinical phenotypes and limited usefulness [6,7]. Additional parameters for stratification of patients are required for improved disease management. We reasoned that a thorough characterization of signaling pathways in resting and activated CVID B cells might provide new tools for patient classification. Here, we measured B-cell phosphorylation levels of kinases during steady-state and following activation by stimuli of importance for immunoglobulin class switch and the germinal center reaction, including CD40 ligand (CD40L), interleukin (IL)-4, IL-21, activation of B-cell receptor (BCR) and toll-like receptor (TLR)9 [8].

Abbreviations: BCR, B-cell receptor; Btk, Bruton's tyrosine kinase; CD40L, CD40 ligand; CVID, Common variable immunodeficiency; Erk, extracellular signal-related kinase; IκBα, NFκB inhibitor α; Ig, immunoglobulin; IVIG, intravenous immunoglobulin; PCA, principle component analysis; PLC-γ, phospholipase C-γ; PMA, phorbol 12-myristate acetate; SCIG, subcutaneous immunoglobulin; Syk, spleen tyrosine kinase; STAT, signal transducers and activators of transcription; TLR, toll-like receptor.

* Corresponding author at: Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, Rikshospitalet, PO Box 4950, Nydalen, Oslo, Norway.
E-mail address: borre.fevang@rr-research.no (B. Fevang).

[1] JO, JHM and BF contributed equally to this manuscript.

http://dx.doi.org/10.1016/j.clim.2016.11.014
1521-6616/

2. Material and methods

2.1. CVID patients and healthy donors

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Lymphoprep; Axis Shield, Oslo, Norway) and cryopreserved in liquid nitrogen. Twenty-nine CVID patients were included in the study, but three were excluded from further analysis due to very low B cell numbers (<100 B cell events per sample). For patients receiving intravenous immunoglobulin (IVIG) substitutions, blood samples were drawn immediately prior to infusion. None of the patients had ongoing acute infection at the time of sample collection, or received immunosuppressive drugs. The CVID diagnose was based on the criteria from ESID Registry [9]. Mutational analysis of the patients was not performed. Eighteen healthy blood donors were used as controls; among which eight were sex- and age-matched: 4 females, median age = 50 yrs., range: 42–58 yrs. Table 1 shows clinical and immunological characteristics of the patients in the study. Infection only was defined as absence of any non-infectious complication, based on previously defined criteria [10]. The study was conducted according to the Declaration of Helsinki and approved by the Regional Ethical Committee. All participants in the study signed a written consent before donation of blood samples.

2.2. Reagents

Recombinant human (rh) IL-4, and rh IL-21 (eBioscience) were used at a final concentration of 20 ng/mL. The TLR-9 agonist CpG7909 (Life Technologies, Invitrogen, Carlsbad, CA, USA) was used at the final concentration of 10 μg/mL, CD40L (Peprotech, Rocky Hill, NJ, USA) at 10 μg/mL, and PMA and ionomycin (Sigma-Aldrich, St Louis, MO, USA) were used at 1 μg/mL respectively. For BCR stimulation, anti-IgM $F(ab'_2)$ and anti-IgG $F(ab'_2)$ (Life Technologies) were each used at a final concentration of 8.6 μg/mL.

For phospho-flow analysis, the following antibodies were used: p-Erk1/2 (T202/Y204, clone 20a), p-STAT3 (Y705, clone 4/p-STAT3), p-STAT5 (Y634, clone 47), p-STAT6 (Y641, clone 18), p-PLC-γ (Y759, clone K86-689.37), p-Btk (Y223, clone N35-86) and p-Syk/pZap70 (Y319/Y352, clone 17a/P-ZAP70) were from BD Biosciences, San Jose, CA, USA. IκBα (L35A5) was from Cell Signalling Technology, Danvers, MA, USA. Pacific Blue and Pacific Orange esters used for fluorescent barcoding of cells were from Life Technologies, Molecular probes. These reagents were used for immunophenotyping: Alexa750 dead/live cell dye from Life Technologies, Molecular probes, CD3 (OKT-3) from Orthoclone Cilag, CD27 (L128) and CD19 (HIB19) from BD Biosciences, IgM (MHM-88), CD21 (Bu32), CD24 (ML5) and CD38 (HIT2) from Biolegend and IgD (polyclonal) from DAKO.

2.3. Activation of signaling and phospho-specific flow cytometry

Samples were thawed, resuspended in RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) with 10% heat-inactivated Fetal Calf Serum (FCS, PAN-Biotech GmbH, Aidenbach, Germany) and rested at 37C° for 30 min before distribution into v-bottomed 96-well polystyrene plates at 200 μL per well. A small fraction of the cells were immunophenotyped, whereas the remaining cells were rested for another 20 min before activation with CpG7909 for 60 min, IL-4, IL-21, CD40L and PMA/ionomycin for 15 min and anti-IgG/IgM (α-BCR) for 4 min. Signaling was stopped by fixation with paraformaldehyde (Electron microscopy Service, Hatfield, PA, USA) at a final concentration of 1.6% for five minutes incubation at room temperature (RT). In experiment using the Btk inhibitor Ibrutinib or the Syk inhibitor Fostamatinib/R406 (Selleckchem, Houston, TX, USA), PBMCs were incubated with Ibrutinib at 2 μM, Fostamatinib at 2.5 μM or without drug for 1 h prior to fixation. The PBMCs were permeabilized using >90% freezer-cold methanol, and stored at −80C° before further processing. Rehydration of the cells was performed by washing twice with PBS by centrifugation. The cells were barcoded using the fluorescent esters Pacific Blue and Pacific Orange at following final concentrations: Pacific Blue: L4: 300 pg/μL; L3: 80 pg/μL; L2: 20 pg/μL; L1: 2 pg/μL and Pacific Orange: L1: 9 pg/μL; L2: 90 pg/μL; L3: 500 pg/μL. After incubation with barcoding dyes for 30 min at RT in the dark, the samples were washed twice, and all the different stimulation conditions from one patient were collected into one tube. The cells were then stained with surface and phospho-specific antibodies for 30 min in the dark at RT and washed once by centrifugation at 710 g. The samples were collected on a LSR II flow cytometer, and data were analyzed using Cytobank software (www.Cytobank.org). Each sample was identified by gating on singlets, lymphocytes (by scatter properties) and then $CD3^-$, $CD20^+$ B cells and deconvoluted according to barcode fluorescence, corresponding to the respective sample. Relative phosphorylation changes were calculated using arcsinh transformation of median fluorescence intensity (MFI) of the cell population of interest [11]. Activation-induced phosphorylation was calculated as relative change = arcsinh (MFI of phospho-protein in activated B cells/scale argument)—arcsinh (MFI of phospho-protein in unstimulated B cells/scale argument). The scale argument numbers are channel-specific (ranged between 150 and 400). Basal phosphorylation levels were calculated relative to healthy donor B cells as follows: Basal levels = arcsinh (MFI of phospho-protein in untreated B cells/scale argument)—arcsinh (MFI of phospho-protein in a control donor B cells/scale argument). One healthy donor sample was included in each run of the assay and used for normalization of the data.

2.4. Statistics

Statistical differences between patients and controls were analyzed by non-parametric MannWhitney test as indicated. One-way ANOVA was performed for comparison of clusters, and Dunn's Multiple Comparison Test was used as post-hoc analysis. Calculations were performed with GraphPad Prism 5 version 5.02 software (San Diego, CA, USA). To perform Principal Component Analysis (PCA) and hierarchical cluster analysis, phosphorylation values were represented as an (nxm)—matrix P, where n is the number of samples and m is the number of phosphorylation parameters. Here, n = 43, m = 6 for the basal phosphorylation levels, and n = 36, m = 9 for the activation induced phosphorylation levels. Hierarchical clustering of rows and columns in P was performed using hclust with Euclidean distance and complete linkage. The final arrangement of samples in the clustered heat map was then obtained by the additional application of reorder hclust in the R package gclus. Identification of distinct clusters in the population of samples was performed with Partitioning Algorithm by Recursive Thresholding (PART) using the R package cluster Genomics, and was used with the minimal size of a cluster set to min Size = 10 and the number of reference data sets set to B = 200 [12]. PCA was applied to P. Each sample was then represented by a pair of values (PC1, PC2), where PC1 and PC2 are the score values corresponding to the first and second principal component, respectively.

To formally test the association between our 5-cluster classification and “infection only”, the n = 26 patients were cross-classified by their cluster label and “infection only” state, and a two-sided Fisher Exact test was applied to the resulting 5 × 2 contingency table. The analysis was done using Fisher test in R v3.3.2 for Mac.

3. Results and discussion

3.1. Activation-induced signaling responses in CVID B cells are normal or enhanced

Strong signaling responses were induced in CVID B cells upon activation, including IL-4-induced p-STAT6, IL-21-induced p-STAT3, and α-BCR-induced p-Syk and p-PLC-γ, as well as CD40L-, PMA/