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Increased STAT3 phosphorylation on CD27$^+$ B-cells from common variable immunodeficiency disease patients

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ABSTRACT

Maturation and differentiation of B-cells are driven by T-cells' help through IL-21/STAT3 axis in GC centers or through extrafollicular pathways, in a T-independent manner. B-cell differentiation is defective in common variable immunodeficiency disease (CVID) patients. We investigated if IL-21/STAT3 axis alterations could influence B-cell fate. We activated purified CVID B-cells with surrogate T-dependent (anti-CD40), T-independent (TLR-9 ligand) stimuli or through B-cell receptor engagement (anti-IgM) with or without IL-21. IL-21 mediated STAT3 activation was greater on CD27$^-$ than CD27$^+$ B-cells depending on the stimulus. IL-21 alone induced STAT3 phosphorylation (pSTAT3) only on CD27$^-$ B-cells and IL-21 induced higher pSTAT3 levels on CD27$^-$ than CD27$^+$ B-cells after anti-IgM or anti-CD40 activation. CVID CD27$^+$ B-cells showed selective STAT3 hyperphosphorylation after activation with anti-IgM or anti-CD40 alone and anti-IgM, anti-CD40 or ODN combined with IL-21. Increased STAT3 activation during immune responses could result in B-cell differentiation defects in CVID.

1. Introduction

Humoral immunological memory is mediated by the combined effects of long lived plasma cells (PC) and memory B-cells generated from naïve B-cells after exposure to antigen (Ag) along with T-dependent help during germinal center (GC) reactions [1,2]. Alternatively, memory B-cells or PC can be generated independently of GC in a T-independent manner via toll like receptors (TLR) or transmembrane activator and CAML interactor (TACI) activation by BAFF/APRIL [3,4]. Heterogeneity among responding memory B-cells has been shown greater than previously appreciated and their generation requirements remain obscure.

T-cells provide help to B-cells through co-stimulatory molecules or by secreting interleukins that interact with their B-cell counterparts. Co-stimulation with IL-21 is essential in the maturation and differentiation of human B-cells to antibody-secreting cells (ASC), but T-cell interaction is mandatory and the molecular mechanism mainly depends on the activation of the IL-21/signal transducer and activator of transcription 3 (STAT3) axis in the GC [5,6,7]. However, the role of STAT3 for T-independent extrafollicular ASC generation is not clear. IL-21 co-stimulation can induce several outcomes on B-cells depending on their maturation status and the stimulus used to activate them: (i) in the presence of stimulation through B-cell receptor (BCR) with cooperation of T-cells (CD40–CD40L interaction), IL-21 induces B-cell differentiation to ASC [8], whereas (ii) in the absence of T-cell cooperation, IL-21 and BCR ligation can induce apoptosis [8] or Granzime B and IL-10 producing B-cells, with cytotoxic and regulatory capabilities, respectively [9]. Although TLR ligands can provide additional signals for the generation of ASC and even substitute T-cell derived signals [10,11], the interaction between IL-21 and TLR pathways has not been widely addressed.

STAT3 is a transcriptional factor that regulates target genes' expression involved in many immunological functions such as memory acquisition [12]. STAT3 mediates B-cell responses working as intracellular

Abbreviations: AICDA, activation induced cytidine deaminase; APRIL, a proliferation-inducing ligand; ASC, antibody-secreting cell; BAFF, B-cell activating factor; BCR, B-cell receptor; BLIMP-1, B lymphocyte induced maturation protein-1; CAML, calcium modulator and cyclophilin ligand; CFSE, carboxyfluorescein succinimidyl ester; CVID, common variable immunodeficiency disease; GC, germinal center; GOF, gain of function; HIES, hyper-IgE syndrome; Ig, immunoglobulins; MFI, median fluorescence intensity; ODN, oligodeoxynucleotides; PC, plasma cell; PID, primary immunodeficiency; TACI, transmembrane activator and CAML interactor; TLR, toll like receptor; TRAIL, TNF related apoptosis inducing ligand; STAT3, signal transducer and activator of transcription 3.

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signal transducer not only for IL-21 but also for several ILs as IL-2, IL-4, IL-6 or IL-10, which are well-known B-cell growth and/or switch regulatory factors [13,14,15,16,17]. In particular, STAT3 driven IL-21 co-stimulation modulates B-cell differentiation initiated through T-dependent stimulus [5,6,7], BCR engagement [18] or TLR-9 ligands [19,20]. Thus, fine tuning of IL-21/STAT3 axis is important and defective STAT3 expression and/or phosphorylation can lead to immunodeficiency and neoplasia [21].

Several human PIDs are characterized by diminished memory B-cells, decreased Ig levels and/or defective Ag specific antibody responses. Analysis of PIDs has identified several molecular defects of the IL-21/STAT3 axis affecting memory cell generation. For instance, low circulating memory B-cells contribute to impaired Ag specific antibody responses in autosomal dominant hyper-IgE syndrome (HIES), caused by STAT3 mutations and characterized by high serum IgE levels and mucocutaneous or pulmonary infections [22,23]. STAT3 deficiency in HIES patients severely compromises memory B-cell generation in vivo and prevents naïve B-cell differentiation to PC lineage in vitro, although isotype switch is preserved [24]. Moreover, the threshold of activation required for B-cell differentiation is significantly lower in memory compared to naïve B-cells [18].

CVID is the most common symptomatic PID, with an estimated prevalence of 1 in 25,000–50,000, characterized by hypogammaglobulinaemia and defective response to vaccination. Patients suffer from respiratory and/or gut recurrent infections but additional noninfectious features, including autoimmune and autoinflammatory processes or lymphoproliferative disorders, may also be present. Genetic mutations have been described in only 5–10% CVID patients, being TACI variants the most commonly identified [25,26,27,28]. Although the pathogenesis of the disease remains unknown, most CVID patients show a failure on final B-cell differentiation into memory B-cells or ASC. Furthermore, a low percentage of circulating memory B-cells in CVID patients has been associated with a worse clinical presentation and poor response to vaccines [29,30,31]. We have previously demonstrated that B-cells from CVID patients stimulated with anti-CD40 or oligodeoxynucleotides (ODN), fail to produce normal levels of immunoglobulins (Igs) upon co-stimulation with IL-21, even in the presence of BCR stimulation. This can imply a signaling defect in either signaling pathway or alternatively, that B-cells die in culture upon stimulation [32]. In keeping with this, memory B-cells from a subgroup of CVID patients characterized by impaired memory B-cell differentiation were less prone to be rescued from apoptosis after activation regardless of IL-21 signaling. Their memory B-cells also showed an increase of the pro-apoptotic TRAIL molecule, which supports the role of apoptosis in their memory B-cell survival defects [33].

The aim of this study was to investigate if there were differences in STAT3 expression and/or phosphorylation in naïve ($CD27^-$) or memory ($CD27^+$) B-cells that accounted for the deficiencies previously described in CVID patients with a more compromised memory B-cell compartment.

2. Material and methods

2.1. Patients

20 CVID patients were selected according to diagnostic criteria of the International Union for Immunological Societies scientific group for primary immunodeficiency diseases. Patients were recruited from the Immunology Department at Son Espases Hospital, the Balearic Islands' reference hospital that covers a total population of 1,101,794. Patients were classified into two groups according to the European consensus classification for CVID (EUROclass) [34]: (i) CVID patients with ≤2% of IgD^-CD27^+ (switched memory phenotype) B-cells or smB−; and (ii) patients with >2% of IgD^-CD27^+ B-cells or smB+. Patients with <1% of peripheral B-cells were not included. Patients received intravenous gamma globulin therapy every 21 days and did not suffer from infections at the time of the study. Peripheral blood samples were collected before gamma globulin replacement. Table 1 summarizes the patients' age, gender, percentages of B-cell subpopulations and *TNFRSF13B* gene (encoding TACI) variants. Age and sex matched healthy blood donors were included as controls. The study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by CEIC (Balearic Islands Clinical Research Ethics Committee; IB 1564/11 PI). Informed consent was obtained from all subjects.

2.2. B lymphocyte purification, B-cell subpopulations sorting and cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation. Purified B-cells were obtained from PBMC by negative selection using the Dynabeads Untouched™ human B-cell separation kit (Invitrogen), according to the manufacturer's instructions. $CD27^-$ and $CD27^+$ B-cells or IgD^+CD27^- and IgD^+CD27^+ B-cells were sorted from $2–4 \times 10^6$ purified B-cells using an Influx sorter cytometer (Becton Dickinson). Purified or sorted B-cells were resuspended in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), glutamine (2 mM) and antibiotics (penicillin and streptomycin). For proliferation assays, purified B-cells or sorted $CD27^-$ and $CD27^+$ B-cells (1×10^6/ml) were labeled during 5 min at room temperature (RT) (25 °C) with 1 μg carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen), following the manufacturer's instructions.

CFSE-free and CFSE-labeled purified B-cells or sorted $CD27^-$ and $CD27^+$ B-cells (5×10^4) were cultured in 96-well plates and stimulated with: cytosine-phosphate-guanosine (CpG)-ODN (type B TLR-9 ligand) (0.6 μg/ml; InvivoGen) or anti-human CD40/TNFRSF5 antibody (1 μg/ml; R&D Systems) with or without $F(ab)_2$ goat anti-human IgM (5 μg/ml; Jackson ImmunoResearch) in the presence or absence of human recombinant IL-21 (100 ng/ml; Biosource). CFSE-free and CFSE-labeled B-cell cultures were incubated, for 24 h or 72 h respectively, at 37 °C in a 5% CO_2 atmosphere. For blotting experiments 2×10^6 purified B-cells were cultured 24 h in 24-well plates and stimulated as already mentioned.

2.3. Flow cytometry

Patients' classification, B-cell purity, surface marker expression, proliferation and intracellular staining to detect STAT3 expression and phosphorylation were analyzed by flow cytometry using an Epics FC500 flow cytometer and the Kaluza software (both from Beckman Coulter).

Whole blood samples were stained with anti-CD19-ECD, anti-CD27-PCy7 (both from Coulter Immunotech) and anti-IgD-FITC (Dako) to phenotypically classify the CVID patients.

Cell purity was assessed using the following monoclonal antibody combinations: anti-CD45-FITC, anti-CD19-PCy5 (both from Coulter Immunotech) and anti-CD3-PE (Becton Dickinson) for purified B-cells and anti-CD19-PCy7 plus anti-CD27-PCy5 (both from Coulter Immunotech) for sorted $CD27^-$ and $CD27^+$ B-cells. Anti-IgD-FITC was added to this combination to assess purity of sorted IgD^+CD27^- and IgD^+CD27^+ B-cells. Purity was always superior to 95%.

Intracellular staining protocol was performed to evaluate STAT3 expression and phosphorylation in tyrosine residue (pY) 705 of STAT3 (pSTAT3) in CFSE-free purified B-cells, sorted $CD27^-$ and $CD27^+$ B-cells or sorted IgD^+CD27^- and IgD^+CD27^+ B-cells following the manufacturer's instructions (IntraPrep Permeabilization Reagent from Beckman Coulter). Briefly, 5×10^4 cultured cells were harvested, stained for 15 min with anti-CD19-PCy7 and anti-CD27-PCy5 and washed with cold phosphate-buffered saline (PBS). After surface staining, cells were fixed with formaldehyde solution for 15 min, washed with cold PBS and permeabilized with saponine solution for 20 min at RT (25 °C) in the dark. Intracellular staining was performed with anti-STAT3-PE or anti-STAT3(pY705)-PE (both from Becton Dickinson) added within