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Article

STAT1-dependent IgG cell-surface expression in a human B cell line derived from a STAT1-deficient patient

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ABSTRACT

STAT1 is a key effector of cytokines involved in the resistance to pathogens; its identified transcriptional targets mediate the innate immune response involved in the defense against viruses and bacteria. Little is known about the role of STAT1 in adaptive immunity, including its impact on BCR or surface Ig expression. Analysis of this point is difficult in humans, as STAT1 deficiency is extremely rare. SD patients die early in childhood from a severe immunodeficiency. Herein, a SD B cell line obtained from a SD patient was compared with a B cell line from a STAT1-proficient subject in search of differences in surface Ig expression. In this SD B cell line, a complete absence of surface IgG was noted. The mRNA encoding the surface form of IgG was detected only in STAT1-proficient B cells; the mRNAs encoding the secreted and the surface forms were detected in SD and STAT1-proficient B cells. Re-expression of STAT1 in SD B cells restored surface IgG expression and a functional BCR. Conversely, shRNA silencing of STAT1 in B cells reduced considerably the expression of the surface IgG. Although limited to one B cell line, these results suggest that STAT1 may play an essential role in surface IgG expression in human B cells. Possible mechanisms involve regulation of mRNA splicing, transcription, or both. These observations extend the role of STAT1 further in adaptive immunity, including the regulation of BCR expression. *J. Leukoc. Biol.* 87: 000–000; 2010.

Introduction

STAT1 belongs to a family of transcription factors that comprises seven genes: STAT1, -2, -3, -4, -5A, -5B, and -6, whose products mediate the cellular responses to cytokines [1]. STAT1 is activated by phosphorylation on tyrosine 701 and serine 727 [2–4], dimerizes, and subsequently accumulates in the nucleus, where it regulates the transcription of target genes [5]. STAT1 is a crucial element of the immune response and of the defense against pathogens, including viruses, bacteria, and parasites [6, 7].

STAT1 is the central component of several signaling pathways, including ILs, growth factors, and hormones [8]. It is a key component of the cellular response to α/β, λ, and ω IFNs [9–11] and is the major component of IFN-γ signaling [12]. A majority of the proteins that are induced by IFN-γ includes transcriptional targets of STAT1 [12].

The transcriptional targets of STAT1 are concerned mostly with cellular immunity; they include proteins that are active against pathogens such as double-stranded RNA-regulated protein kinase, 2′-5′ oligodenylate synthetase, inducible NO synthase, NADPH oxidase, cathepsins (see ref. [12]), proteins with an antiproliferative effect such as the cyclin-dependent inhibitors p21 and p27 [13], proteins with an apoptotic effect such as caspases [14, 15], proteins involved in the immunological response including several components of the antigen-processing machinery such as the family of MHCI proteins [16], and the high-affinity FcγR [17]. The involvement of STAT1 in adaptive immunity is less documented, although some components of the antigen-processing system are STAT1 targets, such as the LMP2 [18, 19], the TAP1, and the MHC class II transactivating protein [20]. Regarding the action on

Abbreviations: CFS=chronic fatigue syndrome, CD=cell-surface antigen; ΔCt=Δ comparative threshold, LMP2=low molecular mass polypeptide 2, NGFR=nerve growth factor receptor, PBS-T=PBS-0.1% Tween, qPCR=quantitative PCR, SD=STAT1-deficient, shRNA=short hairpin RNA, siRNA=small interfering RNA, TAP1=transporter associated with antigen processing 1

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Igs at the surface of B cells in humans, nothing is known about the role of STAT1, and SD human B cells are in effect impossible to obtain, as the disease is extremely rare. The few patients (three, to this day) who were diagnosed suffered from a severe immunodeficiency, resulting in a high sensitivity to mycobacterial and viral infection, and they died early in childhood [21–23]. EBV-transformed B cells were obtained from these patients. One of these cell lines originated from mature B cells that had been in contact with the antigen and has been used in the present study. In mice, the expression of STAT1 can be manipulated, but apart from a high susceptibility to infections [24–26], little is known about the effect of STAT1 deficiency on Ig expression, except that the orientation of class-switching is affected [27, 28]. In this paper, characterization of cell-surface Igs in an EBV-immortalized human SD B cell line showed a complete defect in the surface expression of IgG. Expression of surface IgG was found to be STAT1-dependent in these cells; in addition, the absence of a functional BCR in the SD B cells documents the participation of STAT1 in adaptive immunity.

MATERIALS AND METHODS

Cell culture

SD EBV-B cells (kindly provided by Jean-Laurent Casanova, INSERM U550, Necker, Paris, France), control EBV-B cells (Ct1: Pri; Ct2: 1602), Jurkat (T lymphoma), and K562 (erythroid) cell lines were grown at 37°C in a humidified 5% CO_2 atmosphere at 1×10^6 cells/ml in RPMI-1640 medium [with L-glutamine (Invitrogen, Carlsbad, CA, USA), 20% or 10% heat-inactivated FCS (PAN Biotech, Germany), and 100 U/ml penicillin and 10 μg/ml streptomycin (Gibco-BRL-Life Technologies, Carlsbad, CA, USA)]. Activation of cells was performed by incubating 10^7 cells in 500 μl serum-free culture medium at 37°C, with or without 40 μg/ml antibody to human IgG or IgM (Jackson ImmunoResearch, West Grove, PA, USA; both goat antibodies); the cells were then washed and lysed in sample buffer.

Plasmid constructs, transfections, and cell sorting

Vectors (STAT1α/NGFR), containing a bidirectional tetracycline-inducible promoter driving the expression of STAT1α and a truncated version of the NGFR, were derived from the CKR-516 vector described previously [29]. Transfection, antibiotic selection, and induction of vectors by tetracycline were as described [29]. Cell sorting was performed by magnetic separation (Miltenyi Biotec, Auburn, CA, USA) using iron-coupled anti-NGFR. Induction of NGFR was verified by flow cytometry and induction of STAT1α by Western blotting.

shRNA constructs, lentiviral production, and cell infection

The following three 19-nt-long sequences were chosen to fit the coding region of the human STAT1 gene: 1, 5′-ttcctggagcaggttcacc-3′ (shRNA-STAT1) targeting nt 389; 2, 5′-tgcttgcttggatcagctg-3′ (shRNA-STAT1) targeting nt 1108; and 3, 5′-ttggaacagaaatacacct-3′ (shRNA-STAT1) targeting nt 1196. Of the three oligonucleotides tested, only 1 and 2 were found to reduce the expression of STAT1; oligonucleotide 3 had no effect on STAT1 expression and was therefore used as a negative control. For the design of the shRNA sequence, a 9-nt spacer loop (5′ ttcaagaga 3′) was added at the 3′ end of each 19 nt siRNA, followed by the inverted sequence of the corresponding siRNA. For example, the sequence of the first (1) sense oligonucleotide was: 5′-ttcctggagcaggttcaccttcaagagaggtgaacctgctccaggaa-3′. Each double-stranded oligonucleotide was cloned into the TRIP IZI-GFP vector [30] with a polymerase III H1 promoter [31] to allow constitutive expression of the shRNA in infected cells. The TRIP IZI-GFP/shRNA lentiviral vector was packaged into lentiviral particles by transfection of the packaging human cell line 293T. STAT1 shRNA lentiviral particles obtained were used to infect the Ct1 cell lines. Efficiency of infection was verified by measuring GFP by flow cytometry, and efficacy of the inhibition of STAT1 expression by the shRNA was verified by Western blotting using a STAT1-specific antibody.

Reporter gene assays

Cells were transfected with pm7-TAP1-luciferase reporter plasmid and pCMV-β-galactosidase plasmid [16]. Transfected cells were incubated (24 h) and lysed in Promega's reporter lysis buffer, and luciferase activity was measured using a luminometer (Bio-Tek Clarity, Bio-Tek Instruments, Winooski, VT, USA) and normalized for protein and β-galactosidase activity.

Flow cytometry

NGFR induction was measured with a PE-labeled NGFR antibody (Miltenyi Biotec). In some cases, enrichment of transfected cells was performed using NGFR induction (MACSelect™, Miltenyi Biotec). Cell-surface CDs and Igs and total Igs were measured by flow cytometry (XL Beckman-Coulter counter). For membrane expression, 0.1×10^6 cells were washed and incubated (15 min in the dark) with 5 μl antibody in PBS, washed, resuspended in binding buffer (BD Biosciences, San Jose, CA, USA), and analyzed. FITC-coupled antibodies (anti-IgG, anti-IgM, anti-IgD, anti-IgA, anti-IgE, and rabbit IgG or human IgA for isotypic labeling) were from Dako (Denmark).

Calcium release into the cytoplasm is one of the events reflecting B cell activation through triggering of the BCR, which can be monitored easily by flow cytometry and was measured using a fluorescent probe as described previously [32]. Cells (10^6) were loaded with 2 μM Fluo-4 acetoxymethylester (Molecular Probes, Eugene, OR, USA) in HBSS without Ca^{2+} and Mg^{++} and incubated for 30 min at 37°C. Cells were washed twice with HBSS and then suspended in 1 mL RPMI. Fluorescence of the cellular suspension was observed with a flow cytometer (Becton Dickinson, San Jose, CA, USA). Ionomycin was added to release all calcium. Cells were kept at 37°C for IgG or IgM stimulation. Basal fluorescence was acquired during 50 s, then cells were stimulated with anti-IgG (AffiniPure goat anti-human IgG, Fc fragment specific at 218 mg/ml, or rabbit anti-human IgG, Fc fragment specific at 80 mg/ml, Jackson ImmunoResearch) or anti-IgM (rabbit anti-human IgM, Fc 5 μ fragment specific at 80 mg/ml, Jackson ImmunoResearch) antibodies, and fluorescence was acquired for 250 s prior to ionomycin treatment (2 μg/ml, Sigma Chemical Co., St. Louis, MO, USA). To measure the change in calcium influx, the mean fluorescence intensity for interval times of 10 s was determined and the mean intensity values plotted as a function of time. The fold increase in calcium mobilization following stimulation was determined as follows: For each dot, the mean basal intensity (=mean of all basal intensity dots) was substracted, and the value obtained was then divided by the mean basal intensity and multiplied by 100. Control experiments were conducted with STAT1-proficient B cells in which calcium release was detected following anti-IgG antibody stimulation but not with control antibody (not shown).

Immunofluorescence microscopy

Cells (8×10^4) were centrifuged (5 min, 800 rpm) onto coverslips, dried, and incubated with FITC-labeled anti-IgG antibody (two different clones were used; Dako; 15 min, 37°C), washed in PBS-T. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (Molecular Probes) at 1/250. After three washes with PBS-T, the coverslips were mounted using Dako fluorescent mounting medium (DakoCytomation, Denmark) and examined on a fluorescence microscope (Zeiss, Thornwood, NY, USA).

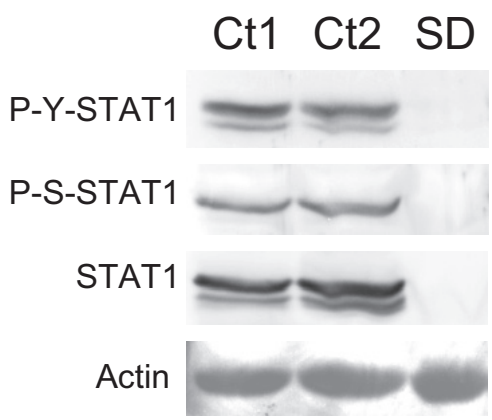

Figure 1. Expression and phosphorylation of STAT1 in deficient and proficient B cells. Expression and phosphorylation (P) on the tyrosine 701 (Y) and serine 727 (S) residues of STAT1 in STAT1-proficient B cells (Ct1 and Ct2) and absence of STAT1 in the SD B cells as analyzed by Western blotting.

Electron microscopy

Cells (1×10^6) were washed with PBS and resuspended in phosphate buffer (0.1 M, pH 7.2) containing glutaraldehyde 0.2% and paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) 4% and incubated for 1 h at room temperature. Cells were washed three times in PBS at room temperature and analyzed as described [33]. IgG molecules were detected using anti-human IgG (Sigma Chemical Co.) as a primary antibody and gold-conjugated secondary antibody (electron microscopy, Grade Gold conjugate, BBInternational, UK). The images were captured using a CM10 transmission electron microscope (Philips, Andover, MA, USA).

Gel electrophoresis

The IgG were detected and identified using the hydragel Bence Jones kit (SEBIA, Issy les Moulineaux, France); proteins (15 μg/sample) were separated on gels and revealed by a semi-automated system (Hydrasis, SEBIA). Human serum was used as a control to localize the IgG.

Western blotting

Cells were washed in PBS, lysed in sample buffer [50 mM Tris-HCl, pH 6.8 (Bio-Rad, Hercules, CA, USA), 2% SDS (Sigma Chemical Co.), 20% glycerol (Prolabo, VWR, Fontenay Sous Bois France), 1 mM sodium vanadate ($NaVO_3$, Labosi, Fisher Scientific, Elancourt, France), 1 mM DTT (Merck, Rahway, NJ, USA), and 0.01% bromophenol blue (Sigma Chemical Co.)], sonicated, heated, and stored at –70°C. Proteins (50 μg) were separated on SDS-PAGE (10%) and transferred onto nitrocellulose membranes, and membranes blocked with 5% dry skimmed milk in TBS were incubated with antiboby overnight at 4°C. Antiphosphotyrosine 701-STAT1 (1/1000) and anti-STAT1 (1/1000) were from Cell Signaling (Beverly, MA, USA); antiphosphoserine 727-STAT1 (1/1000), antiactin (1/1000), anti-STAT3 (1/500), anti-STAT5 (1/500), and anti-β-tubulin (1/500) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blots were washed in TBS-T, incubated with peroxidase-coupled goat anti-mouse (Santa Cruz Biotechnology) or goat anti-rabbit (Upstate, Lake Placid, NY, USA) secondary antibody (1/5000), washed in TBS-T, and revealed by chemiluminescence (LumiGLO reagent and peroxide, Cell Signaling) and autoradiography (X-Omat R film, Kodak, Rochester, NY, USA). When necessary, membranes were stripped with the Blot Restore kit (Chemicon International, El Segundo, CA, USA) and reprobed. Prestained molecular weight standards (Fermentas, St-Rémyles Chevreuse, France) were used.

Real-time qPCR

Enrichment of transfected cells was performed using NGFR induction (MACSelect™, Miltenyi Biotec). The TaqMan® gene expression Cells-to-CT™ kit (Applied Biosystems, Foster City, CA, USA) was used to extract total RNA and to perform RT and gene amplification. For the transmembrane domain of IgG, an Applied Biosystems custom TaqMan gene expression assay was used; the sequences were chosen to cover exons 5 and 6 to avoid detecting genomic DNA: sense primer, 5′-ccatcttcatcacactcttcctgtt; antisense primer, 5′-accaccgaggagaagatcca; 5′-FAM probe, 5-ctacagtgccaccgtcacc. For the region that is common to the secreted and the transmembrane forms, the TaqMan gene expression assay (Applied Biosystems; ref. Hs00941525_g1) was used. For cyclophilin A (peptidylprolyl isomerase A), used as a reference, the TaqMan gene expression assay (ref. Hs99999904_m1) was used. All steps were performed following the recommendations of the manufacturer. Relative expression levels of each gene were calculated as reported already.

RESULTS

Cell-surface markers in SD B cells

To study the expression of Igs in B cells from SD humans, EBV-B cell lines that had been obtained from severely immunodeficient SD patients were used [23]. From the three patients described [23], one cell line obtained consisted of mature B cells. Two control, STAT1-proficient EBV-B cell lines (Ct1 and Ct2) were also used in this study. Analysis of STAT1 in the Ct1 and Ct2 controls showed that it was expressed and phosphorylated on tyrosine 701 and serine 727 (**Fig. 1**). It has been shown previously that this basal activation of STAT1 results from the LMP1-induced, NF-κB-driven secretion of IFNs [34]. In the SD cells, STAT1 was not expressed (Fig. 1). The three cell lines were analyzed for genomic Ig rearrangement by genomic PCR and were found to be Ig heavy-chain variable region-rearranged (not shown). Phenotypic analysis by flow cytometry of the SD and the Ct cells showed that although they displayed a slightly different maturation status, they were, on the whole, similar, presenting a mature, activated B cell phenotype with no difference in the CDs analyzed except in CD 138 (**Table 1**).

Remarkably, although the Ig types IgM, IgA, IgD, and IgE were absent from the surface of SD or control B cells (**Fig. 2A**), the IgG type was present at the surface of the control B cells but not at the surface of the SD B cells (Fig. 2, B and C, for quantitative analysis).

TABLE 1 *Surface Markers of STAT1-Proficient (Ct1 and Ct2) and SD B Cell Lines*

	Ct1	Ct2	SD
CD			
CD5	–	–	–
CD19	+	+	+
CD20	+	+	+
CD22	+	+	+
CD23	+	+	+
CD27	+	+	+
CD38	+	+	+
CD138	+	+	–

Immunophenotype analysis was performed by flow cytometry for cell-surface antigens (+, detected; –, not detected).

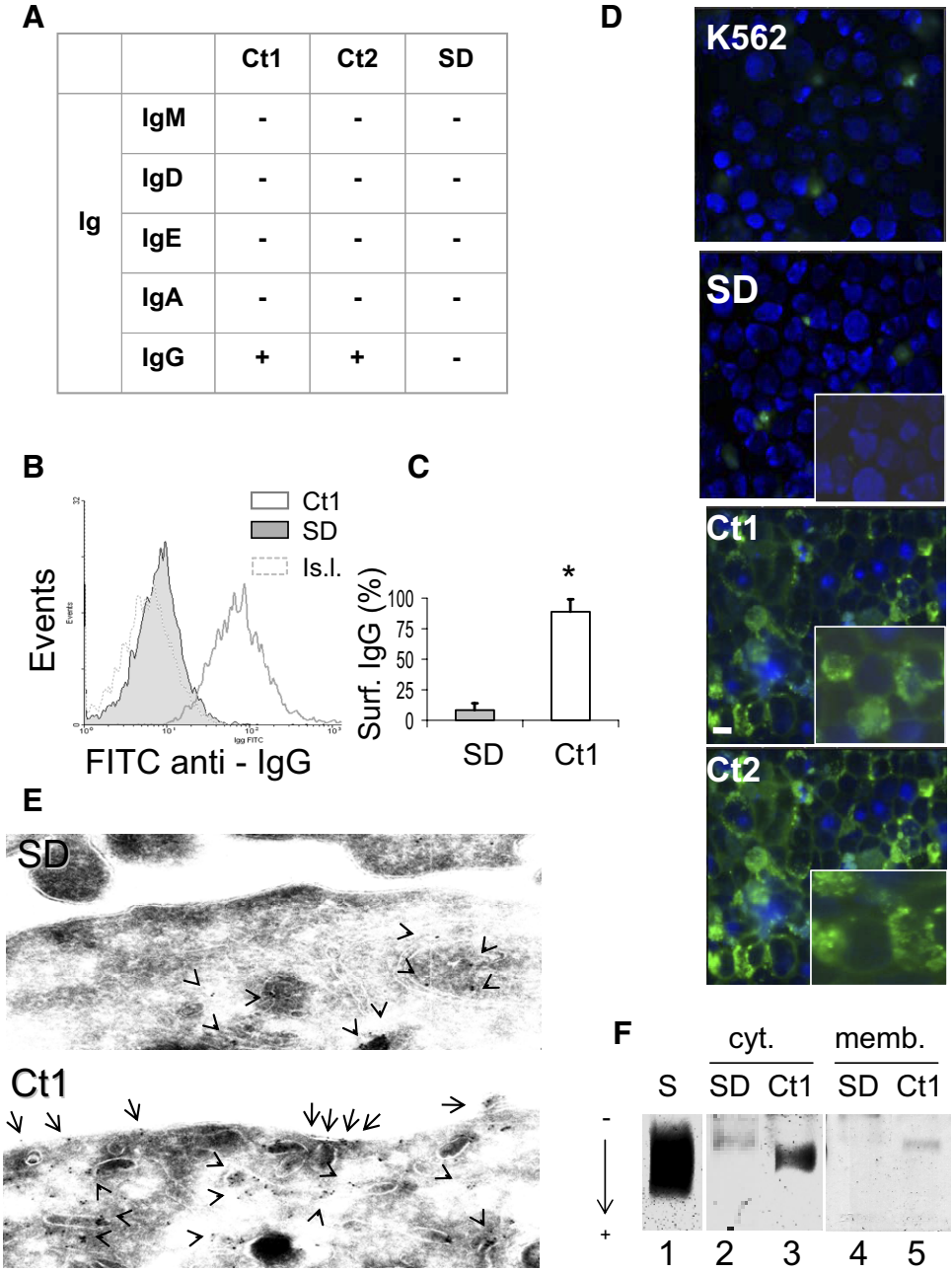

Figure 2. IgG is not detected at the cell surface of SD B cells and is low but present in the intracellular compartment. (A) Expression of the Ig types at the surface of STAT1-proficient B cells (Ct1 and Ct2) and SD B cells. (B) Flow cytometry analysis using FITC-labeled anti-IgG showing the absence of labeling of the surface of SD cells (shaded area) and labeling of the Ct1 cells (open area); the area with the dotted line represents isotypic labeling (Is. l.). (C) Quantitative analysis of three experiments as shown in B using SD B cells and control B cells (Ct1). *, Significantly different, *t*-test: $P < 0.01$; mean of at least four experiments performed in duplicate. Surf., Surface. (D) Analysis by immunofluorescence microscopy of the expression of IgG at the surface of SD B cells and control B cells (Ct1 and Ct2) using a FITC-labeled anti-IgG antibody; a control, negative cell line was included (K562; original scale bar, 2 μm; insets, 4 μm). (E) Electron microscopy study of the expression of IgG in Ct1 B cells and SD B cells. (F) Semi-automated agarose gel electrophoresis and specific IgG detection in the cytoplasm (cyt.; lane 2) and the membrane (memb.; lane 4) of SD and in the cytoplasm (lane 3) and the membrane (lane 5) of STAT1-proficient (Ct1) B cells. Identical amounts of protein (15 μg/lane) were used; human serum (S) was used as a positive control of IgG (lane 1).

To confirm the absence of expression of IgG at the surface of the SD B cells, immunofluorescence microscopy on nonpermeabilized cells was used. Cells were labeled with two different FITC anti-IgG antibodies originating from a different clone. The IgG was detectable at the surface of Ct1 and Ct2 cells but not at the surface of SD B cells or on the erythroid K562 cells used as a negative control (Fig. 2D). Electron microscopy also confirmed the absence of IgG on the surface of SD B cells (Fig. 2E, upper panel) and their presence at the surface of Ct1 B cells (Fig. 2E, lower panel). Agarose gel electrophoresis, using a semi-automatic device combined with antibody detection, showed the absence of IgG in membrane extracts from SD B cells (Fig. 2F, lane 4) and their presence in Ct1 B cell membrane extracts (Fig. 2F, lane 5). Thus, in SD B cells, there was a complete absence of surface IgG. However, although intracellular IgG was clearly detected in SD B cells, it was in considerably lower amounts than in Ct1 cells (Fig. 2F, compare lanes 2 and 3).

Expression of the IgG in B cells is STAT1-dependent

To establish STAT1 dependence of the surface expression of IgG, expression of STAT1 was restored by transfecting SD B cells with an episomal, tetracycline-inducible, vector-expressing STAT1. The vector also contained the sequence of the extracellular domain of the NGFR, allowing magnetic sorting of STAT1-expressing transfected cells with anti-NGFR antibodies. More than 80% of the cells present in the positive-sorted frac-

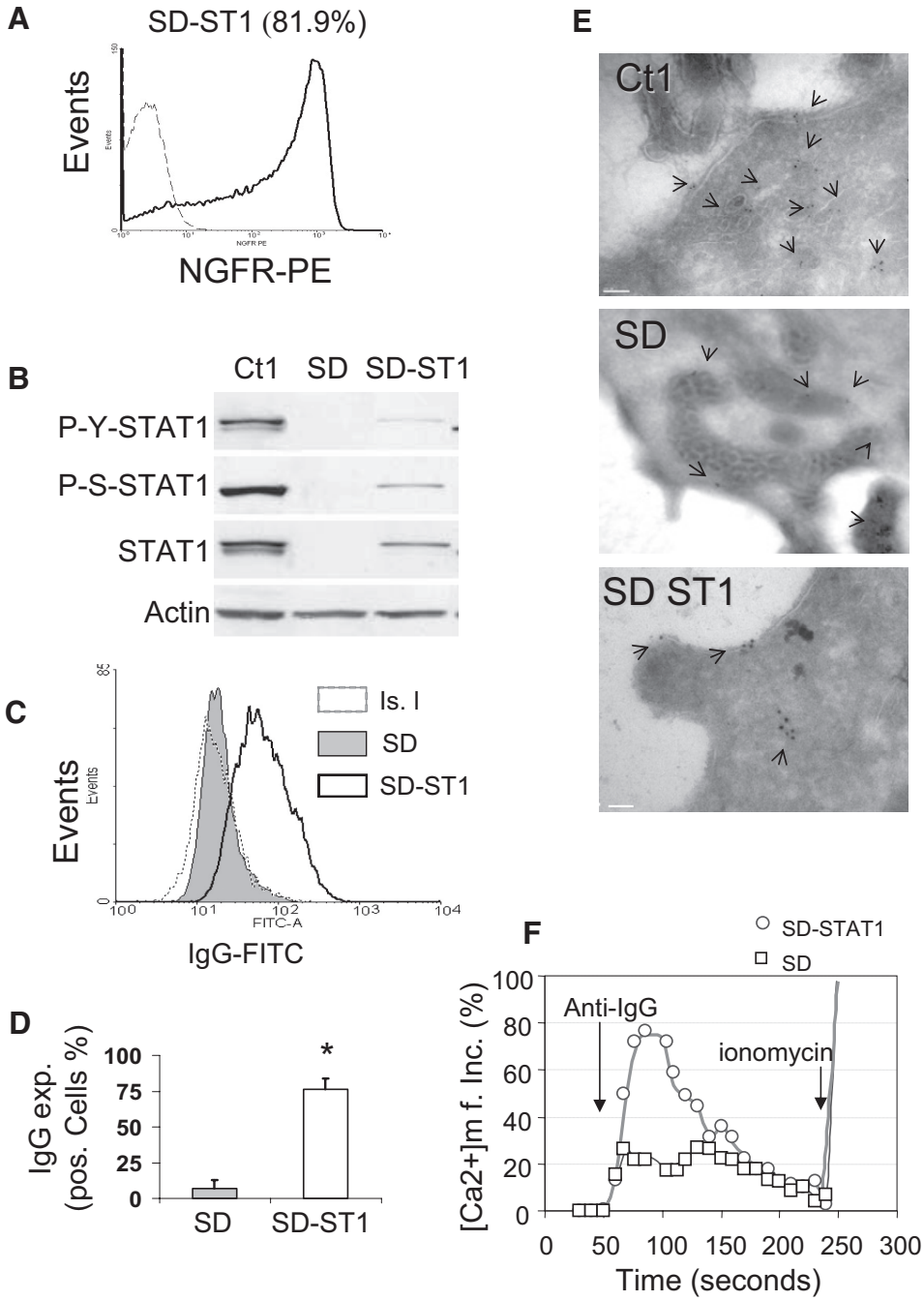

Figure 3. Restoration of the expression of STAT1 in the SD B cells rescues the expression of IgG at their surface. (A) Flow cytometry analysis of transfected and sorted SD B cells. More than 80% positive-sorted cells were NGFR-positive. Dotted line, Negative-sorted cells; solid line, positive-sorted cells. ST1, STAT1. (B) Restoration of the expression of STAT1 as verified by Western blotting using appropriate antibodies. (C) Rescue of IgG expression at the surface of SD B cells measured by flow cytometry following STAT1 transfection (shaded area, nontransfected SD B cells; open area with bold line, STAT1-transfected SD B cells; open area with dotted line, isotypic labeling. (D) Quantitative analysis of three experiments as shown in C using SD B cells and STAT1-transfected SD B cells (SD-ST1). *, Significantly different, *t*-test: $P < 0.01$; $n = 3$. (E) Detection of IgG in STAT1-transfected SD B cells using electron microscopy: Ct1, Control STAT1-proficient cells (original scale bar, 0.1 μm); SD cells (original scale bar, 0.2 μm); STAT1-transfected SD cells (original scale bar, 0.2 μm); arrows denote IgG. (F) Restoration of cytoplasmic calcium release in response to anti-IgG stimulation in STAT1-negative STAT1-transfected B cells (SD). Cytoplasmic calcium was measured after stimulation with anti-IgG (40 μg/ml) or ionomycin added after 280 s in all samples (40 μg/ml), in SD B cells (□) and in STAT1-transfected SD B cells (○). $[Ca^{2+}]$m represents the fold increase (f. Inc.) in calcium mobilization expressed as percent of the mean basal intensity.

tion were NGFR-positive (**Fig. 3A**). The expression of an active STAT1 was verified (Fig. 3B). Flow cytometry showed a restored surface expression of IgG in STAT1-transfected SD cells (Fig. 3, C, and D, for quantitative analysis). Analysis by electron microscopy also showed the recovery of cell-surface IgG in STAT1-transfected SD cells (Fig. 3E). Experiments were repeated several times with similar results.

STAT1 restores a functional BCR in SD B cells

The BCR is functional only if all of its constituants are present and assembled. Here, in the SD B cells studied, the Igα/β chains were expressed (not shown), suggesting that the absence of IgG per se was the cause of a nonfunctional BCR. To analyze B cell activation following the specific triggering of the BCR by an antibody that recognizes it, measurement of calcium release was used. In the control B cells (Ct1), triggering of the BCR resulted in the rapid rise of cytoplasmic calcium as documented elsewhere [35] (not shown), and similarly, an antibody to IgM or of a nonspecific IgG had no effect on cytoplasmic calcium levels (not shown). By contrast, stimulation of the SD B cells with anti-IgG (anti-γ-chain antibody) had no effect on cytoplasmic calcium levels (Fig. 3F). Upon transfection of STAT1, the anti-IgG-triggered cytoplasmic calcium release was restored (Fig. 3F), clearly indicating the recovery of a functional

BCR. Calcium mobilization by ionomycin indicated that the calcium-regulating channels were functional in the SD cells (Fig. 3F).

STAT1 regulates surface IgG by modulating mRNA expression in B cells

To determine whether the deficiency in the cell-surface IgG in SD B cells is the result of impaired processing at the protein or transcript level, mRNA levels were measured by qPCR. In B cells, the surface and secreted forms of Igs are known to be translated from a long and a short mRNA, respectively. This is the result of an alternate polyadenylation, a process that has been well described for the IgM subtype (see **Fig. 4A**, long and short) [36]. As the SD B cells contain no detectable surface IgG, we sought to determine whether the corresponding mRNA was detectable by using specific primer pairs (primers a and b; see Fig. 4A). The mRNA for the surface form was undetected in the SD B cells, and it was present in the Ct1 B cells (Fig. 4B), thus accounting for the absence of surface IgG in the SD B cells. Importantly, IgG surface mRNA was rescued in STAT1-transfected SD B cells (Fig. 4C), in agreement with the restoration of IgG at their surface. To gain insight into the mechanism underlying the absence of expression of surface IgG in STAT1-negative B cells, the total IgG mRNA was quantified in the SD and the Ct1 B cells using the c and d primers pair (see Fig. 4A). The results show that the amounts of mRNA were clearly detectable in the SD cells, although much lower than in the Ct1 cells (Fig. 4D).

In STAT1-proficient B cells, suppression of STAT1 reduces IgG surface expression

Further confirmation of the dependence to STAT1 of the surface expression of IgG was sought by using RNA silencing of STAT1 in STAT1-proficient Ct1 B cells. Three different shRNA oligonucleotides inserted into a lentivirus vector were transduced in Ct1 cells, and the cells were analyzed for STAT1 expression. Of the shRNA sequences tested, two (1 and 2) were found to inhibit significantly, although not totally, the expression of STAT1 in STAT1-proficient B cells (**Fig. 5A**). The sequence 3, which had no effect on the expression of STAT1, was used as a negative control. To ensure the specificity of the silencing oligonucleotides, the expression of STAT3 and STAT5 was examined by Western blotting and found to be unchanged. In the shRNA-STAT1-transduced cells, the expression of surface IgG was inhibited significantly (Fig. 5B and Supplemental Data), thus corroborating the dependence to STAT1 of IgG cell-surface expression in human B cells.

DISCUSSION

In this paper, we find that in a human B cell line derived from a SD patient, the expression of surface IgG is null, suggesting that STAT1 regulates this process. This extends the notion that STAT1 is not only involved in cellular immunity but also in key functions of the adaptive immunity. A further in-depth analysis of the mechanisms underlying STAT1-regulated IgG surface expression was prevented by the absence of available human SD B cells. The B cell line used in this study was transformed by EBV, known to induce higher levels of STAT1 activation [37], thus raising the question of the actual level of surface IgG in nontransformed SD memory B cells. Further studies with non-EBV-transformed SD human B cells are clearly necessary to clarify this issue. It is known that in humans and mice, STAT1 is not required for the early steps of development. The results described in this paper show that an essential function of STAT1 in cellular immunity is suggested by the profound defects that result from its absence. SD patients and mice respond poorly to pathogens and do not survive infection [21], but the dependence of adaptive immunity on STAT1 has been much less studied. Here, by comparing a B cell line derived from a SD patient [23] and its normal equivalent, we found that all cell-surface markers analyzed were identical, with the notable exception of the IgG. A defect in IgG expression was unquestionnably assessed by using different complementary methods, including immunofluorescence mi-

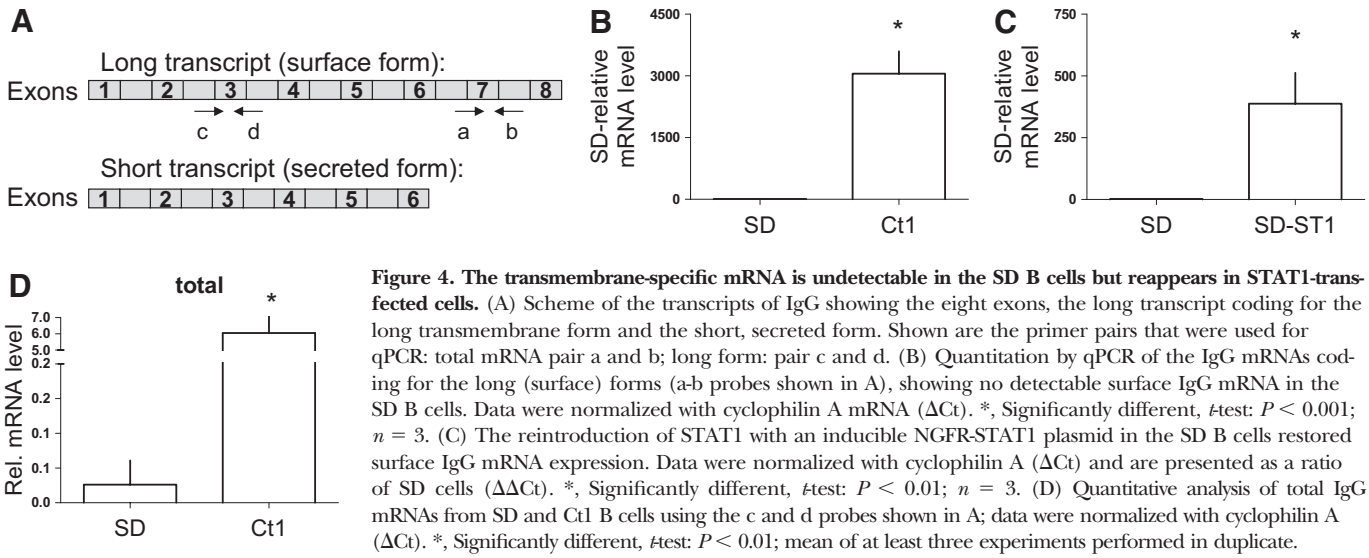

Figure 4. The transmembrane-specific mRNA is undetectable in the SD B cells but reappears in STAT1-transfected cells. (A) Scheme of the transcripts of IgG showing the eight exons, the long transcript coding for the long transmembrane form and the short, secreted form. Shown are the primer pairs that were used for qPCR: total mRNA pair a and b; long form: pair c and d. (B) Quantitation by qPCR of the IgG mRNAs coding for the long (surface) forms (a-b probes shown in A), showing no detectable surface IgG mRNA in the SD B cells. Data were normalized with cyclophilin A mRNA (ΔCt). *, Significantly different, *t*-test: $P < 0.001$; $n = 3$. (C) The reintroduction of STAT1 with an inducible NGFR-STAT1 plasmid in the SD B cells restored surface IgG mRNA expression. Data were normalized with cyclophilin A (ΔCt) and are presented as a ratio of SD cells (ΔΔCt). *, Significantly different, *t*-test: $P < 0.01$; $n = 3$. (D) Quantitative analysis of total IgG mRNAs from SD and Ct1 B cells using the c and d probes shown in A; data were normalized with cyclophilin A (ΔCt). *, Significantly different, *t*-test: $P < 0.01$; mean of at least three experiments performed in duplicate.

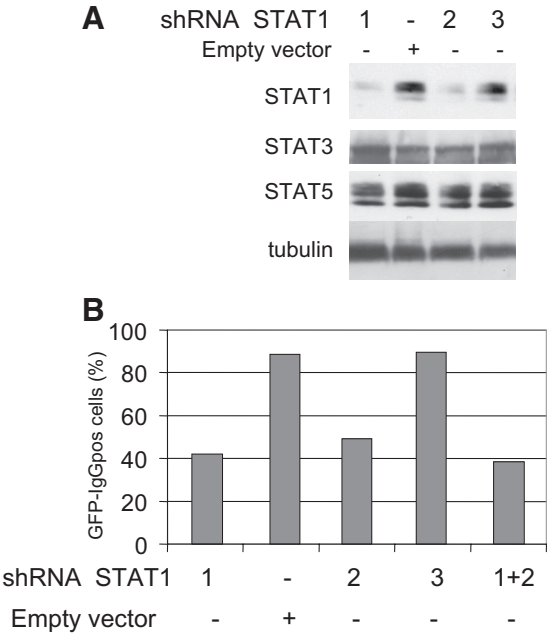

Figure 5. Silencing of STAT1 in STAT1-proficient B cells inhibits the cell-surface expression of IgG. (A) Expression of STAT1, STAT3, and STAT5 in STAT1-proficient B cells transduced with lentivirus carrying STAT1-directed shRNAs with oligonucleotides 1, 2, and 3 or transduced with empty lentivirus; the expression of STAT1, STAT3, STAT5, and tubulin was determined by Western blotting. Oligonucleotide 3 had no effect on the expression of STAT1 and was therefore used as a negative control. (B) Cell-surface expression of IgG in cells transduced with the lentivirus carrying oligonucleotides 1, 2, and 3 or a combination of 1 and 2; cells were also transduced with empty vector. Cell-surface IgG was measured by flow cytometry 9 days after transduction; similar results were obtained after 12 or 13 days. See also Supplemental Data. IgGpos, IgG-positive.

croscopy, flow cytometry, electron microscopy, and electrophoresis. The dependence of IgG expression on STAT1 was strengthened by the recovery of surface IgG in SD cells following transfection of STAT1, also resulting in the recovery of BCR responsiveness. This was reinforced by the reduction in surface IgG observed following inhibition of STAT1 expression by shRNA silencing in STAT1-proficient B cells. Although these results point to a direct involvement of STAT1, they do not exclude the possibility of an indirect mechanism, which would nevertheless be STAT1-dependent. Several experiments were performed to elucidate the mechanism of the dependence of surface IgG expression to STAT1. First, an incorrect processing of IgG from the cytoplasm to the surface was verified by an Endo-H sensitivity assay, the absence of difference allowed to exclude this hypothesis, as there was no change between control and SD B cells (not shown). Second, analysis of the mRNA expression of the secreted and the surface forms was allowed to suggest a transcriptional mechanism. In SD B cells, the mRNA for the surface form was absent, and reintroduction of STAT1 restored it. It is known for IgMs that the surface and secreted forms derive from a transcript that undergoes a complex processing [36] involving alternate use of two 3′-end polyA signals, yielding a short and a long mRNA. RNA polymerase II arrest at the first polyadenylation site of the transcript yields a short mRNA and is stimulated by the cleavage polyA specificity factor/cleavage stimulatory factor complex [38], itself negatively regulated by U1A [36]. Elevated U1A expression is thought to reduce the short transcript in undifferentiated B cells [39]. Indeed, U1A expression is diminished in IFN-γ-treated, differentiating B cells, suggesting that STAT1 could have a role at this level. As gene segments are similarly arranged in all Ig isotypes (IgD, IgA, IgG, and IgE), the processing for IgGs is probably similar to that of IgMs. The cleavage-polyadenylation/RNA splicing of IgG could therefore be affected by STAT1-dependent changes in U1A expression. However, the levels of U1A in STAT1-proficient and SD B cells were not found to be different in our preliminary experiments (not shown). In addition, factors other than U1A may be required, as suggested by previous studies in transfected HeLa cells, showing that the transcription ratio of the two Ig mRNAs is not modified by the overexpression of U1A [40]. As the transcription and the mRNA processing are known to be coordinated and nonindependant processes [41, 42], they might both be regulated by STAT1. This might be the case here, as in addition to an absence of surface IgG, SD B cells have a lower level of secreted IgG. Finally, our results also call to mind the CFS of unknown etiology, in which there is IgG deficiency [43] which may result from STAT1 deficiency or instability, as previously suggested (K. K. Knox and D. R. Carrigan, project proposal, http://www.ncf-net.org/library/Knox-Carrigan-2003.htm). We suggest that the IgG deficiency in patients with CFS is a result of STAT1 deficiency. Indeed, a recent report points to a human γ-retrovirus as the contributing factor in this disease [44], illustrating a possible involvement of STAT1, given its essential role in the response to viral infection. In conclusion, the absence of expression of surface IgG in a SD human B cell line reveals the intriguing possibility that STAT1 regulates IgG expression, resulting in a defect in adaptive immunity, including the absence of a functional BCR. This defect may participate in the immunodeficiency of the SD patients.

AUTHORSHIP

I. N. and R. F. designed the study. I. N., P.-A. D., P. O. S., E. E. F., and R. F. performed experiments. S. B.-D., F. N., I. D.-F., and G. W. B. provided essential reagents. I. N. and R. F. wrote the paper.

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