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STAT3-Mediated Up-Regulation of BLIMP1 Is Coordinated with BCL6 Down-Regulation to Control Human Plasma Cell Differentiation

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STAT family members have been implicated in regulating the balance between B cell lymphoma (BCL)6 and B lymphocyte induced maturation protein (BLIMP)1 to control plasma cell differentiation. We previously showed that STAT5 induces BCL6 to block plasma cell differentiation and extend the life span of human B cells. The heterogeneity in STAT activation by cytokines and their effects on B cell differentiation prompted us to investigate the effect of STAT3 activation in plasma cell differentiation. First stimulation with IL-21, which promotes plasma cell differentiation, induced robust and prolonged STAT3 activation in primary human B cells. We then investigated effects of direct STAT3 activation on regulation of plasma cell genes, cellular phenotype, and Ig production. Activation of a tamoxifen-regulated STAT3-estrogen receptor fusion protein triggered BLIMP1 mRNA and protein up-regulation, plasma cell phenotypic features, and Ig secretion. When STAT3 was activated by IL-21 in B cells ectopically expressing BCL6, BLIMP1 was up-regulated, but only partial plasma cell differentiation was achieved. Lastly, through coexpression of BCL6 and STAT3-ER, we verified that STAT3 activation functionally mimicked IL-21 treatment and that STAT3-mediated BLIMP1 up-regulation occurred despite high BCL6 expression levels indicating that BCL6 is not the dominant repressor of BLIMP1. Thus, up-regulation of BLIMP1 alone is not sufficient for differentiation of primary human B cells into plasma cells; concomitant down-regulation of BCL6 is absolutely required for completion of the plasma cell differentiation program. The Journal of Immunology, 2008, 180: 4805–4815.

In the germinal center (GC),§ B and T cells communicate via cytokines and cell surface molecules. The GC reaction involves activation of CD4+ T cells leading to up-regulation of surface molecules and cytokine production, which help activated B cells to grow and differentiate. CD40L is expressed on the surface of activated T cells and binds to CD40 expressed on B cells to promote B cell activation (1). Following migration of GC B cells from the dark into the light zone, they differentiate either into memory or Ig-secreting plasma cells to establish humoral immunity (2). Several factors are thought to play a role in the development of memory or plasma cells from the GC. Interaction of B cells with cell surface molecules such as OX40 or ICOS on T cells have been shown to regulate memory B cell formation (3, 4). The strength of initial BCR signaling has also been implicated in differentiation of GC B cells into plasma cells (5) where high affinity BCR or high Ag load favor plasma cell differentiation (6).

It is appreciated that cytokines produced by activated T cells promote proliferation and/or differentiation of B cells into plasma cells. IL-2 and IL-4 promote B cell proliferation (7–9), while IL-10 and IL-21 drive proliferation and differentiation of B cells into plasma cells (10, 11). These cytokines signal through Jak-STAT pathways (12). There are four Jaks (Jak1, Jak2, Jak3, and Tyk2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6). Many groups have shown in various cell types that a given cytokine can activate multiple STATs. IL-2 activates STAT5 and STAT3 (13), IL-4 activates STAT6 and STAT5 (14), IL-10 activates STAT3 and STAT1 (15), and IL-21 has been shown to activate mainly STAT3, and to a lesser extent, STAT5 and STAT1 (16). We have recently shown that activation of STAT5 in human B cells blocks plasma cell differentiation and promotes proliferative self-renewal (17).

Transcriptional regulation plays an important role in plasma cell differentiation. B cell lymphoma (BCL)6 is a transcriptional repressor expressed in GC B cells (18) and has been shown to be required for GC formation in mice (19–21). It has been proposed that BCL6 blocks plasma cell differentiation due the fact that it negatively regulates expression of B lymphocyte induced maturation protein (BLIMP)1 (22). Gene targeting has shown that BLIMP1 is necessary for plasma cell differentiation in vivo (23) and is sufficient to drive plasma cell differentiation in B cell lines (24, 25). BLIMP1 expression has been correlated with plasma cell commitment in mice and humans (26–28). BLIMP1 initiates

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3 Abbreviations used in this paper: GC, germinal center; BCL, B cell lymphoma; BLIMP, B lymphocyte induced maturation protein; XBP, X-box-binding protein; CD40L-L, L cell fibroblasts stably expressing CD40L; 4-HT, 4-hydroxytamoxifen; p, phospho; WT, wild-type; LZRS, Lazarus; ER, estrogen receptor; IRES, internal ribosomal entry site; ivPC, in vitro-derived plasma cell; NGFR, nerve growth factor receptor.

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plasma cell differentiation by extinguishing MHC class II expression, loss of B cell identity, cessation of proliferation, and an increase in the cellular machinery required for high-level protein production, respectively. Recent data have also shown that IFN regulatory factor (IRF)4 plays a crucial role in plasma cell differentiation (33, 34).

Although the molecules involved in regulating commitment to the plasma cell fate have been well studied (for review see Ref. 35), the initiating stimuli which affect these regulatory circuits are less clear. We showed previously that STAT5 signaling up-regulated BCL6 expression to inhibit plasma cell differentiation in primary human B cells (17). STAT3, in contrast, has been shown to up-regulate BLIMP1 gene expression and promote plasma cell differentiation of murine B cell lines (36). In this study, we show in primary human B cells that inductive activation of STAT3 triggered BLIMP1 expression, and promoted plasma cell differentiation and Ig production. With these findings, we expand the BCL6/BLIMP1 axis model of plasma cell differentiation to incorporate the influence of STAT3 activation on BLIMP1 regulation and initiation of plasma cell differentiation.

Materials and Methods

B cell isolation

B cells were obtained from buffy coats prepared from the peripheral blood of adults (Sanguin Bloodbank) by Ficoll-Paque separation and CD19 MACS microbeads (Miltenyi Biotec). Tonsils were obtained from routine tonsillectomies (AMC Department of Otolaryngology). Use of these tissues was approved by the medical ethical committee of the AMC and was contingent upon informed consent. Purity was ≥ 97% as determined by flow cytometry. For long-term culture (>2 wk) CD19-MACS-selected B cells were sorted to 99.9% purity for CD19^+CD3^- cells on a FACS Aria (BD Biosciences). Purity was confirmed by reanalysis of sorted cells.

B cell phenotyping

The following mAbs against the human molecules CD3 (SK7), CD19 (4G7, SJ25C1, ID3, or HIB19), CD20 (2H7), CD25 (2A3), CD27 (L128), CD38 (HB7), CD86 (In2.2), HLA-DR (L243); Igk (TB28–2, JDC-12); and Igλ (G20–19) were directly conjugated to FITC, PE, PerCP, allophycocyanin, PE-indotricarbocyanine, allophycocyanin-indotricarbocyanine, or to biotin and were purchased from BD Pharmingen. Streptavidin-PE-indotricarbocyanine conjugate (BD Pharmingen) was used to detect biotin staining. From DakoCytomation, we purchased PE-labeled anti-CD138 (MI15), FITC- and PE-conjugated anti-κ L chain (polyclonal rabbit Fab’), and PE-conjugated anti-λ L chain (polyclonal rabbit Fab’; DakoCytomation). Stained cells were analyzed on an LSR II (BD Biosciences) and flow cytometry data were processed using FlowJo software version 6 (TreeStar) on Apple computers. Histograms and dot plots use log10 fluorescence intervals.

Cell culture

B cells (5 × 10^5) were cocultured on γ-irradiated (100Gy) mouse L cell fibroblasts stably expressing CD40L (CD40L-L cells, 10^5 cells/mL). Cytokines used were IL-2 (20 U/mL), IL-4 (10 ng/mL R&D Systems), or rMIL-21 (50 ng/mL; R&D Systems). The 4-hydroxytamoxifen (4-HT, 1 μM) was from Sigma-Aldrich. Optimal cytokine concentrations were determined empirically by [3H]thymidine incorporation assay. Cells were routinely tested for mycoplasma and were found to be negative by PCR.

Induction and determination of STAT phosphorylation

For acute cytokine stimulations (15 or 60 min) CD19^- B cells (freshly isolated or CD40L-activated) were rested 2 h at 37°C. Cells were then stimulated with medium alone or with rMIL-21 (200 ng/mL) for 15 or 60 min at 37°C. For long-term phospho-p-STAT analysis (24–144 h) cells were cultured with CD40L stimulation in the presence of 50 ng/mL rMIL-21 and directly assayed. Cells were fixed with ice-cold methanol and permeabilized with the CytoPerm/Fix kit (BD Biosciences) and stained intracellularly with mAb against phospho-STAT1 (Y701) conjugated to Alexa-Fluor 488, phospho-STAT3 (Y705) conjugated to Alexa-Fluor 647, and phospho-STAT5 (Y694) conjugated to Alexa-Fluor 647 (all from BD Biosciences).

Retroviral constructs

We obtained wild-type (WT) human STAT3 from E. Caldenhoven (Erasmus University, Rotterdam, The Netherlands) and generated Lazarus (LZRS)-WT-STAT3 internal ribosomal entry site (IRES)-GFP. To fuse the WT STAT3 with C terminus of estrogen receptor (ER), we performed PCR targeting the C terminus of STAT3 and modified the stop codon to introduce a BglII site using the primer set 5’-CGGCCGATGCTGGAG and 5’-CGAAGATCTAGGGAATGGCGCCTC. This PCR product was digested with BanHI-BglII and ligated into BanHI-linearized pBluescript ER-C-terminal (provided by H. Kurata, DNAX Institute). The C terminus STAT3ER fragment was released with BanHI/EcoRV and ligated into BanHI/SalI-digested LZRS-WT-STAT3-IRES-GFP to generate full length LZRS-STAT3ER-IRES-GFP. LZRS-BCL6-IRES-YFP (yellow fluorescent protein) and LZRS-BCL6-IRES-ANGFR (signaling-incompetent version of the nerve growth factor receptor) were made by isolating BCL6 from LZRS-BCL6-IRES-GFP (17). Generated vectors were verified by sequencing. LZRS-STAT3ER-IRES-GFP and LZRS-BCL6-IRES-GFP were previously described (17).

Retroviral transduction

Transductions and virus production by Phoenix GaV packaging cells were performed essentially as described (17) except that B cells were activated on CD40L-L cells in the presence of rmIL-21 (50 ng/mL) for 36 h before transduction.

Confocal microscopy

STAT3ER-transduced B cells were cultured overnight with or without 1 μM 4-HT and were plated onto poly-L-lysine-coated cover slips, fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 45 s at room temperature. Blocking and AB incubations were conducted in 2% BSA. Anti-ERα (MC-20, Santa Cruz Biotechnology, 1:1000) and Texas red-conjugated donkey anti-rabbit (Molecular Probes, 1/400) was used for detection. The 4’-6’-diamidino-2-phenylindole was used at 300 nM. Images were captured with a Leica TCS-SP2 confocal microscope. Localization results were similar over multiple fields. Staining with secondary Ab alone did not display any signal in the Texas red channel.

RT-PCR

Total RNA was isolated with the RNeasy kit (Qiagen) and reverse transcribed using oligo (dT) (Promega) and Superscript II reverse transcriptase (Invitrogen Life Technologies). Primer sequences for BCL6, BLIMP1, and ACTB (17) are published. Other primers used were: PAX-5: forward GGAAGGGTGAATGCCTGG, reverse GGCTTGATGCTTCCTGTCTC; IRF-4: forward ACCGAAGCTGGAGGGACTAC, reverse GTG GGAGGAGTGAATCAGCTTGG, reverse GGCTTGATGCTTCCTGTCTC; IRF-4: forward ACCGAAGCTGGAGGGACTAC, reverse GTG GGAGGAGTGAATCAGCTTGG, reverse GGCTTGATGCTTCCTGTCTC. Quantitative RT-PCR was conducted with a Bio-Rad iCycler and relative mRNA expression levels were normalized to HPRT and calculated using the 2^-ΔΔCT method.

Immunoblotting

Whole cell extracts were isolated using Triton Lysis Buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA (pH 7.4), 1% Triton X-100, 10% glycerol) supplemented with HALT protease inhibitor mixture (Roche) and 1 mM Na3VO4. In brief, 15–30 μg of lysate was separated on acrylamide gels and transferred to nitrocellulose (Schleicher & Schuell). Blots were developed by ECL and exposed to x-ray film (Amersham Hyperfilm).

ELISA

Plates were coated with capture Abs anti-human IgG, IgM, or IgA (DakoCytomation) at 5 μg/mL in 0.1 M NaHCO3 (pH 9.6) for 2 h at 37°C and washed in ELISA wash buffer (PBS, 0.5% Tween 20). Ten percent FCS in

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FIGURE 1. Sustained STAT3 phosphorylation after IL-21 activation in primary human B cells. A, Peripheral blood (PB) CD19<sup>+</sup> B cells were freshly isolated and rested at 37°C for 2 h. Cells were then stimulated for 15 min with either medium alone (–, thin histogram), or mIL-21 (200 ng/ml, shaded histogram). Levels of phospho-STAT3 (Y705), phospho-STAT1 (Y701), or phospho-STAT5 (Y694) were determined by FACS. B, CD19<sup>+</sup> B cells were activated for 36 h on CD40L-transfected mouse L cell fibroblasts (CD40L-L cells). Cells were then rested followed by stimulation with medium alone (thin line) or IL-21 (shaded) for 15 or 60 min and levels of phospho-STAT3, STAT1, and STAT5 were determined by flow cytometry. C, CD19<sup>+</sup> B cells were cultured with CD40L stimulation with medium alone (thin line) or with IL-21 (shaded) and STAT activation was measured at the indicated times.

ELISPOT for total IgG- and IgM-secreting cells

Immobilon-P membrane filter plates (Millipore) were coated overnight with either 5 μg/ml anti-human IgM mAb (Sanquin Reagents) or 5 μg/ml anti-human IgG mAb (Bethyl Laboratories) in PBS. Plates were blocked using IMDM containing 10% FCS. Cells were washed in IMDM with 10% FCS and incubated on the filter plates for 20 h at 37°C for the anti-IgG ELISPOT and 75 h for the anti-IgM ELISPOT. Bound IgG was detected with a mix of HRP-labeled IgG1 (1/1000), IgG2 (1/2000), IgG3 (1/2000) and IgG4 (1/1000) (Sanquin Reagents). Bound IgM was detected with HRP-labeled anti-human IgM polyclonal Ab (1/1000) (Bethyl Laboratories). Spots were developed using TMB-substrate for PELIspot (Sanquin Reagents) and analyzed using an AELvis reader (A.EL.VIS GmbH) and eli.analyze software version 4.0.

Results

STAT3 activation is sufficient to promote plasma cell differentiation

To investigate the role of STAT3 in control of plasma cell differentiation we first chose to examine the STAT activation spectrum elicited by IL-21, a potent promoter of plasma cell differentiation. We exposed resting human peripheral blood CD19<sup>+</sup> B cells to IL-21 and assessed activation of STAT3, STAT5, and STAT1 by flow cytometry with phospho-STAT (p-STAT)-specific Abs. In resting cells, IL-21 induced STAT3 activation, but failed to induce STAT1 or STAT5 activation (Fig. 1A). As positive controls IFN-γ induced p-STAT1, and IL-4 stimulation led to increased p-STAT5 (data not shown). Because B cells generally receive cytokine signals in the context of T cell help we examined STAT activation in CD40L-stimulated B cells. Acute (15 min) exposure to IL-21 induced STAT3 and STAT5 activation but not STAT1 in CD40L-stimulated B cells (Fig. 1B). After 60 min, p-STAT5 signal returned to baseline while p-STAT3 remained elevated (Fig. 1B). This prompted us to examine STAT activation during the period in which IL-21 induces plasma cell differentiation. B cells were cultured with CD40L and in the presence or absence of continuous IL-21 and p-STAT3, p-STAT5, and p-STAT1 were determined in time. STAT3 activation was apparent at 24 h, peaking at 36 h and still present at 96 h (Fig. 1C). Activation of STAT5 and STAT1 above baseline were not observed at any time during the culture period (Fig. 1C). Note that B cells activated for 36 h did not lose their capacity to activate STAT5 as they did activate STAT5 after acute exposure to IL-21 (Fig. 1B). These results showed that STAT3 signaling was more sustained than the brief STAT5 signal induced upon IL-21 triggering of primary human B cells. Given the ability of IL-21 to induce plasma cell differentiation, these results suggested that STAT3 may mediate this process.

To test the hypothesis that STAT3 activation could promote plasma cell differentiation, we generated an inducible system to specifically activate STAT3. We fused the mutated hormone-binding domain of the ERα (39) to the C terminus of full-length human STAT3. STAT3ER was cloned into the LZRS-IRES-GFP retroviral vector (40) for overexpression in human cells. Proper synthesis of STAT3ER fusion protein was verified by immunoblotting in 293T cells transfected with LZRS-IRES-STAT3ER. An anti-STAT3 Ab revealed two bands; the lower band representing endogenous STAT3 and the upper band representing STAT3ER (Fig. 2A), which was confirmed by blotting with an anti-ERα Ab, which showed only the upper band (Fig. 2A). Upon administration of 4-HT, STAT-ER proteins translocate from the cytosol to the nucleus to activate target genes (17, 41). 4-HT-dependent nuclear translocation of STAT3ER was verified in transduced primary B cells by confocal microscopy (Fig. 2B).

In mice, expression of STAT3 in B cells is important for T cell-dependent plasma cell differentiation (42). STAT3 activating cytokines such as IL-10, and IL-21 also promote this process in human B cells (10, 11, 43). In contrast STAT5 activation leads to long-term self-renewal of human B cells which are blocked in plasma cell differentiation (17). For this reason, we directly compared the effect of STAT3 and STAT5 activation on long-term proliferative capacity of B cells. Total CD19<sup>+</sup> cells were transduced with LZRS-IRES-control GFP, LZRS-STAT3ER-IRES-GFP, or LZRS-STAT5ER-IRES-GFP and cultured on CD40L-L cells in the presence of IL-2, IL-4, and with or without 4-HT as described (17). The frequency and number (Fig. 2C) of STAT3ER-GFP<sup>+</sup> cells peaked at week two and then progressively declined by the end of the culture period. In sharp contrast, STAT5ER-transduced cells exhibited an increase in frequency and in absolute number (Fig. 2C), apparent after approximately 3 wk in culture, consistent with previous findings (17).
As activation of STAT3ER did not lead to long-term self-renewal of B cells, we questioned whether these cells were differentiating into plasma cells. During plasma cell differentiation expression of CD38 and CD138 (Syndecan-1) increase, while expression of CD20, HLA-DR, and CD19 are decreased on plasma cells (2). Compared with GFP⁺ control cells and

FIGURE 2. STAT3 activation leads to acquisition of plasma cell phenotype in vitro. A, Immunoblot analysis of 293T cells transfected with LZRS-IRES-STAT3ER, probed with anti-ERα or anti-STAT3 Abs. B, STAT3ER-transduced CD19⁺ B cells were cultured overnight with or without 1 μM 4-HT and then analyzed with anti-ERα Ab and 4',6'-diamidino-2-phenylindole to show nuclear localization by confocal microscopy. C, Total PB CD19⁺ B cells were transduced with LZRS-control-IRES-GFP (Control GFP, ○), LZRS-STAT3ER-IRES-GFP (STAT3ER, □), or LZRS-STAT5b-ER-IRES-GFP (STAT5b-ER, □). Transduced cells were cultured on CD40L-L cells in the presence of IL-2 (40U/ml) and IL-4 (10 ng/ml) and in the presence (closed symbols) or absence (open symbols) of 1 μM 4-HT. Upper panel, Percentages of transduced cells were determined over time and the relative increase in GFP from the start of the culture is expressed. Lower panel, Absolute numbers of GFP⁺ cells were determined and the cumulative expansion is shown. D, At day 17 GFP⁺ cells were gated and analyzed for CD38, CD20, CD19, HLA-DR, and CD138 expression. Data are representative of three independent experiments using different donors. E, Tonsil CD19⁺ B cells were transduced with LZRS-control-IRES-GFP or LZRS-STAT3ER-IRES-GFP and cultured on CD40-L cells with IL-2 plus IL-4 in the presence of 4-HT (1 μM). The percentage of GFP⁺ cells was determined continuously throughout the culture period. Data are means ± SD of two individual donors. F, At day 17 after transduction tonsil STAT3ER-B cells exhibited increased CD38 and decreased CD19 expression.
with STAT3ER-transduced B cells cultured without 4-HT, STAT3ER cells cultured with 4-HT exhibited an increase in cells that were CD38highCD20−/HLA-DRlow and CD138+ (Fig. 2D). This phenotype is consistent with plasma cell differentiation. STAT3bER cells cultured with 4-HT did not exhibit phenotypic changes associated with plasma cell differentiation (Fig. 2D) in accordance with previous observations (17). We observed the same effect of STAT3 activation in tonsil CD19+ B cells: lack of long-term growth, up-regulation of CD38, and down-regulation of CD19 (Fig. 2, E and F). Thus, STAT3 activation, in contrast to STAT5, did not confer a long-term growth advantage to primary human B cells, but rather, induced a phenotype consistent with plasma cell differentiation.

Prdm1, the gene encoding murine Blimp-1, is indispensable for plasma cell differentiation (23). Its homologue, BLIMP1, is highly expressed in human plasma cells (28). In murine B cell lines STAT3 has been shown to regulate prdm1 (36). Because the phenotypic changes brought on by STAT3 activation were consistent with plasma cell differentiation, we tested the effect of STAT3 activation on expression of genes involved in control of plasma cell differentiation. CD19+ B cells were transduced with STAT3ER or control virus. GFP+ cells were then sorted and cultured in the presence or absence of 4-HT and expression of PRDM1, IRF4, and XBP1 (44) were determined by quantitative (q)RT-PCR. For comparison, we also examined gene expression levels in in vitro-derived plasma cells (ivPC), which were generated by culture of peripheral blood B cells for 3 days on CD40L-L cells with IL-2 and IL-21, followed by 4 days of culture in the absence of CD40L-L cells, but with IL-2 and IL-21. Similar culture conditions have been shown to drive proliferation and differentiation of human B cells into Ab-secreting PCs (11, 45). As expected, BLIMP1, XBP1, and IRF4 mRNA levels were high and BCL6 and PAX5 levels were low in ivPC (Fig. 3A). BLIMP1 levels were strongly induced by IL-21 while IRF4 and XBP1 expression were also induced, but to a lesser extent (Fig. 3A). Consistent with the principal activation of STAT3 by IL-21 (Fig. 1C), we found that specific STAT3 activation induced BLIMP1 to a similar extent as IL-21 (Fig. 3A). Moreover, IRF4 and XBP1 mRNA expression were increased by STAT3ER activation (Fig. 3A). IL-21 induced BCL6 gene expression (Fig. 3A), but specific STAT3 activation did not significantly increase BCL6 mRNA levels (Fig. 3A). IL-21-mediated up-regulation of BCL6 has been also seen by others (11, 46), but the significance of this effect is not clear. Except for the IL-21-stimulated cells, BCL6 mRNA levels were uniformly low in agreement with down-modulation of BCL6 gene expression by CD40L (47) and were not further decreased by STAT3ER activation. PAX5 also inhibits plasma cell differentiation (48, 49), but neither IL-21 nor activation of STAT3ER affected PAX5 mRNA levels (Fig. 3A).

We also determined protein expression of BLIMP1, IRF4, BCL6, and PAX5 in response to specific STAT3 activation. In agreement with the mRNA profile, IL-21 induced expression of BLIMP1, IRF4, and BCL6 protein, while STAT3ER activation induced BLIMP1 and IRF4, but not BCL6 (Fig. 3B). PAX5 protein was decreased by IL-21 stimulation, but not appreciably affected by STAT3ER activation. Our results suggest that specific STAT3 activation was sufficient to initiate the plasma cell gene expression program by inducing BLIMP1.

Plasma cell differentiation leads to secretion of Ig. To determine the effect of STAT3ER activation on Ig production, naive (CD19+ IgM+CD27−) and switched memory B cells (CD19+ IgM− CD27+) cells were transduced with control-IRE-S-GFP or STAT3ER-IRE-S-GFP. GFP+ cells were sorted and cultured in the presence or absence of 4-HT. For comparison nontransduced cells were also cultured in the presence or absence of IL-21. Five days later IgM and IgG secretion were measured by ELISA. We found that activation of STAT3ER with 4-HT in naive B cells resulted in enhanced IgM secretion (Fig. 3C). Low amounts of IgG were detected in IL-21-treated naive cells (data not shown), consistent with a role for IL-21 in isotype switching (50), but no IgG was detected in supernatants of control- or STAT3ER-transduced cultures cultured with 4-HT (data not shown), suggesting that specific STAT3 activation did not induce isotype switching. In switched memory cells, IgG secretion was strongly induced upon STAT3ER activation as well as by IL-21 treatment (Fig. 3D). Increased Ig secretion in response to STAT3ER activation was not due to increased cell number (Fig. 3E). To confirm these results we performed ELISPOT analysis for total IgM and IgG in IL-21-treated and STAT3ER-activated B cells. Naive (CD19+ IgM+CD27−) and switched memory B cells (CD19+ IgM− CD27+) expressing control-IRE-S-GFP or STAT3ER-IRE-S-GFP were stimulated for 7 days and IgM and IgG production were determined. IL-21 induced strong increases in the frequency (8 ± 1% for IL-2 vs 32 ± 8% for IL-21) and intensity (100 ± 10 arbitrary units (AU) for IL-2 vs 715 ± 23 AU for IL-21) of IgM-secretion in naive B cells, while STAT3ER activation more mildly increased the frequency (3 ± 1% for −4-HT vs 7 ± 1% for +4-HT) and spot intensity (48 ± 5 AU for −4-HT vs 103 ± 25 AU for +4-HT) of IgM-producing cells (Fig. 3F). Of note specific STAT3 activation, unlike IL-21 stimulation did not induce IgG secretion in naive B cells (data not shown), in support of results obtained by ELISA. In switched memory B cells, IL-21 increased the frequency (7 ± 1% for IL-2 vs 18 ± 2% for IL-21) and intensity (372 ± 50 AU for IL-2 vs 824 ± 54 AU for IL-21) of IgG-secretion in naive memory B cells, while STAT3ER activation more mildly increased the frequency (9 ± 1% for −4-HT vs 15 ± 2% for +4-HT) and spot intensity (288 ± 21 AU for −4-HT vs 740 ± 120 AU for +4-HT) of IgG-producing cells (Fig. 3G). Taken together these results demonstrate that specific activation of STAT3 in primary human B cells resulted in BLIMP1 up-regulation and enhanced Ig secretion but not to isotype switching.

BCL6 expression prevents IL-21-induced plasma cell differentiation

BCL6 is expressed in GC B cells and functions to repress BLIMP1 and plasma cell differentiation. It has been suggested that BCL6 and STAT3 compete for binding at the murine prdm1 promoter (36), which may imply that BLIMP1 expression could not be induced by STAT3 signaling in GC B cells expressing high BCL6. We then asked whether BLIMP1 could be induced by STAT3 signaling if BCL6 expression was already established and, if so, what are the consequences in terms of cellular differentiation, function, and growth? To address this we made use of the known induction of BLIMP1 expression by treatment with IL-21 in combination with BCL6 overexpression. CD19+ B cells were transduced with an LZRS-BCL6-IRE-S-YFP (yellow fluorescent protein) vector, which was verified by culturing all cells with CD40L stimulation in the presence of IL-2 and IL-4 until the transduced cells reached 100% of the culture (data not shown) as previously shown for BCL6-IRE-S-GFP (17, 51). These BCL6+ cells were then cultured in the presence of IL-2 and IL-4 or IL-21 alone and BLIMP1 levels were measured by qRT-PCR. We observed that BLIMP1 mRNA levels were rapidly increased and maintained by IL-21 in BCL6+ cells over a 3-day period (Fig. 4A) and for more prolonged periods (up to 21 days, data not shown). Identical results were obtained with cells transduced with other BCL6-IRE-S overexpression vectors with either GFP (51), or signaling incompetent nerve growth factor receptor (ΔNGFR) as transduction markers. BCL6 can...
undergo posttranslational modifications that result in degradation (52), so it was possible that BLIMP1 induction by IL-21 in BCL6-transduced cells was due to decreased functional BCL6 protein. We assessed BCL6 and BLIMP1 protein expression in these cells and found that IL-21 strongly induced BLIMP1 protein, and even a slight increase in BCL6 protein after 7 days in culture (Fig. 4B).
We next determined whether the IL-21-mediated BLIMP1 increase in BCL6-transduced cells had an effect on their cell surface phenotype. CD19+ B cells were transduced with BCL6-IRES-ΔNGFR and cultured in the presence of IL-2 and IL-4 or with IL-21 alone. In the presence of IL-21 a proportion of the ΔNGFR- cells were CD38highCD20+ and CD38+CD20− (Fig. 4C), which correspond to in vitro-derived plasmablasts and plasma cells, respectively (45, 53). Compared with ΔNGFR- cells in either IL-2 and IL-4 or IL-21 cultures, the frequency of CD38+CD20− among BCL6-ΔNGFR+ cells was reduced (Fig. 4C). Despite the proportion of high BCL6 expression, IL-21 elicited an increase in the proportion of CD38+CD20− cells among BCL6-ΔNGFR+ cells (Fig. 4C). However, these BCL6+ cells cultured with IL-21 did not differentiate to the CD38+CD20− stage (Fig. 4C). Similar results were obtained when using ΔNGFR-transduced cells instead of BCL6-ΔNGFR- cells. The percentage of CD38+CD20− cells reached a plateau of around 30% in the prolonged presence of IL-21 (Fig. 4D). Surface BCR expression was equivalent with similar distributions of IgM and IgG on IL-21-cultured cells compared with those in IL-2 and IL-4 cultures, which further supports the notion that functional BCL6 protein persisted in IL-21-treated BCL6-transduced cells. Given the BLIMP1 up-regulation and phenotypic changes induced by IL-21, we expected that IL-21 treatment would lead to increased Ig production in BCL6+ B cell cultures. Indeed in CD19+ B cells expressing BCL6 IgM and IgG production were increased on a per cell basis after culture with IL-21 as measured by ELISA (Fig. 4F) and in frequency as measured by ELISPOT (Fig. 4G). In total, CD19+ cells transduced with BCL6, IL-21 increased the frequency of IgG and IgM-producing cells (IgG: 842 A.U. for IL-21 vs 346 A.U. for IL-2 and IL-4; IgM: 17 % for IL-21 vs 1 % for IL-2 and IL-4; IgG: 157 ± 17 A.U. for IL-2 and IL-4 vs 364 ± 90 A.U. for IL-21+). Also, IL-21 increased the intensity of both IgG (157 ± 17 A.U. for IL-2 and IL-4 vs 364 ± 90 A.U. for IL-21) and IgM (28 ± 5 A.U. for IL-2 and IL-4 vs 540 ± 60 A.U. for IL-21) secretion in BCL6-transduced B cells. These results show that IL-21 promotes BLIMP1 expression, an intermediate plasma cell phenotype, and increased Ig production even in cells expressing high levels of the differentiation inhibitor BCL6.
next, we found a robust increase in IgG (Fig. 5B, left panel) and IgA secretion (Fig. 5B, right panel) in BCL6-YFP and IL-21, and tubulin protein levels were determined by immunoblot analysis. E and F, Raji cells expressing control-IRES-GFP or STAT3ER-IRES-GFP were cultured for 4 days in the presence or absence of 4-HT. 4-HT was not detected in these cultures. C. Semi-quantitative RT-PCR for BLIMP1 and HPRT1 mRNA expression in BCL6-YFP/STAT3ER-GFP cells cultured for 4 days in the presence or absence of 4-HT. D, BLIMP1 and BCL6 (F) mRNA levels were determined by qRT-PCR and normalized to ACTB expression.

Functional mimicry of IL-21 by STAT3 activation

To further establish the link between the effects of IL-21 and STAT3 activation, we tested whether STAT3 activation could functionally substitute for the effects of IL-21 in BCL6-overexpressing cells. To address this, BCL6-YFP cells were transduced with LZRS-STAT3ER-IRES-GFP or LZRS-control-IRES-GFP. Double transduced cells were then sorted and cultured in the presence of either medium alone, IL-2 and IL-4, or only 4-HT. First, we observed that activation of STAT3ER in BCL6-transduced result in a cumulative increase in cell number over a 3 wk period (Fig. 5A). This is in agreement with the proliferative effect of IL-21 in BCL6-transduced cells (Fig. 4G). Next, we found a robust increase in IgG (Fig. 5B, left panel) and IgA secretion (Fig. 5B, right panel) in BCL6/STAT3ER cells treated with 4-HT for 4 days compared with those cultured in the absence of 4-HT. Although the STAT3ER-mediated proliferative increase was modest after 4 days, we also verified an increase in Ig production at the per cell level. Finally, we examined whether STAT3ER activation was sufficient to induce BLIMP1 in GC-like BCL6-expressing cells. This was indeed the case, as addition of 4-HT to BCL6/STAT3ER cells cultured on CD40L-L cells alone induced a clear increase in BLIMP1 mRNA levels while, without 4-HT, BLIMP1 expression was undetectable (Fig. 5C). At the protein level, 4-HT induced BLIMP1 expression, while not substantially affecting BCL6 protein expression in BCL6/STAT3ER-double transduced cells (Fig. 5D). We confirmed these findings in Raji B cells that were stably transduced with STAT3ER, and induced with 4-HT. Also in these lymphoma cells which express high levels of endogenous BCL6, activation of STAT3ER was sufficient to up-regulate BLIMP1 (Fig. 5E), but did not significantly affect BCL6 levels (Fig. 5F). Taken together these results demonstrate that STAT3 activation alone is sufficient to induce BLIMP1 expression in both primary and transformed human B cells, even in the presence of high BCL6 expression.

Discussion

In this study, we report two principal findings: 1) activation of STAT3 in human B cells induced BLIMP1 up-regulation and drove plasma cell differentiation and 2) STAT3 activation was sufficient to induce BLIMP1 even in the presence of BCL6. STAT3 activation in these cells led to increased Ig production but not full plasma cell differentiation. Thus, although STAT3 induction by cytokines such as IL-21 may be an initiating step in plasma cell differentiation in GC B cells, BCL6 down-regulation is also required for completion of this process.

Cytokines that have been shown to activate STAT3 in various cell types (such as IL-6, IL-10, and IL-21) have all been implicated in plasma cell differentiation (10, 11, 43, 54). IL-21, which is exclusively T cell-derived, is particularly potent in driving this process (11, 46, 50, 55). IL-21 activates both STAT3 and STAT5 in T cell types (such as IL-6, IL-10, and IL-21) have all been implicated in plasma cell differentiation (10, 11, 43, 54). IL-21, which is exclusively T cell-derived, is particularly potent in driving this process (11, 46, 50, 55). IL-21 activates both STAT3 and STAT5 in T cells (60). Recent work from the Th17 field has also addressed the differential effects of STAT3 and STAT5 activation. IL-21 activates both STAT3 and STAT5 in T cells (60) and has also been shown to play a role in Th17 development (61). However, while STAT3 is essential for Th17 cell
development (62), STAT5 activation itself has been shown to be inhibitory for Th17 development (63). Thus, although IL-21 activates both STAT3 and STAT5 in both mouse and human lymphocytes the STAT3-mediated effects of IL-21 downstream signaling are more prominently manifested. Additionally because we showed that BCL6 is positively regulated by STAT5 (17), this result may also explain why BCL6 levels increased after IL-21 treatment, but not by STAT3 activation (11, 46).

To further explore this dichotomy in STAT3/STAT5 signaling, we directly compared the effects of their individual activation on self-renewal capacity and differentiation in vitro. As we did for STAT5 (17) we generated a tamoxifen-inducible STAT3ER construct. Although STAT5ER activation led to long-term proliferation, STAT3ER activation did not. STAT3 activation alone was sufficient to promote plasma cell differentiation as determined by phenotype, plasma cell cell gene up-regulation, and Ig secretion. In line with our results, inhibition of STAT3 using a dominant negative mutant in murine B cell lines resulted in loss of cytokine-induced terminal differentiation (36). Mice harboring a B cell-specific STAT3 deficiency exhibit a selective defect in development of IgG-secreting plasma cells (42). STAT3 activation is also essential for survival of multiple myeloma cells, a tumor derived from plasma cells (64). Wang et al. (65) found that strong BCR activation led to activation of STAT3, a finding which correlates with recent studies showing that high affinity BCR signaling promotes plasma cell differentiation (6). Our data directly show a positive role for STAT3 in human plasma cell differentiation.

BCL6 and BLIMP1 form a regulatory circuit due to their repression of each other at the level of gene expression to control plasma cell differentiation (22, 31). Mice lacking Bcl6 contain elevated numbers of Blimp-1$^+$ plasma cells (66). This also applies to human B cells as reducing BCL6 expression by RNA interference results in increased BLIMP1 (S.A. Diehl, unpublished data), and BLIMP1 levels are very low in B cells overexpressing BCL6 (17). Although important for controlling plasma cell differentiation, this model does not take into account other inputs such as cytokine signaling that can affect this balance. From this and our previous work (17), we hypothesize that the Jak/STAT pathway may play a role in establishing the BCL6/BLIMP1 balance in B cells.

STAT3 activation has been implicated in positive regulation of prdm1 in the mouse (36) and we show in this study that this holds true in human. BCL6 inhibits BLIMP1 expression (17, 22, 67), but not absolutely because we show elevated BLIMP1 in BCL6$^+$ cells exposed to STAT3 activation. Cumulatively, these findings suggested that BCL6 and STAT3 exert opposing functions at the BLIMP1 locus to control plasma cell differentiation. The DNA consensus binding sites for BCL6 (TTC/[C/T][T/A][G/A]GAA) and for STAT5 are similar (TTCTC[A/T]GAA). For this reason, Reljic and coworkers (36) proposed that BCL6 represses limp-1 expression by competing with STAT3 binding at the promoter of PRDM1, the gene encoding limp-1. However, it was subsequently shown that BCL6 and STAT3 do not compete for binding at key intronic regulatory sites of the PRDM1 locus (66). To discern between these two possibilities, we forced expression of BCL6 and asked whether STAT3 activation could turn on BLIMP1 expression. We demonstrate in this study that even in the presence of high BCL6 expression, BLIMP1 expression could be increased by STAT3, either directly or via IL-21 stimulation. These results, therefore, show that BCL6 and STAT3 are not mutually exclusive in their activities, but rather they can be integrated to generate long-lived B cells that maintain BLIMP1 expression and Ig-producing capacity. Our results do not formally exclude the possibility that BCL6 and STAT3 compete for binding at the PRDM1 promoter, but our results strongly indicate that this is not the dominant mechanism for control of this locus because intronic binding of BCL6 in the PRDM1 gene also plays a crucial role in the repression of this locus (66, 67). We also observed an increase in IRF4 and XBP-1 after STAT3 activation. XBP-1 has been shown to be downstream of BLIMP1 (32), but can also be induced in the absence of BLIMP1 (38). IRF4 plays an important role in maintenance of the plasma cell phenotype (33, 34), but whether IRF4 is up- or downstream of BLIMP1 is not entirely clear. Due to unresolved relationships between BLIMP1 and IRF4 or XBP-1, we cannot say whether STAT3-mediated up-regulation of XBP-1 and IRF4 are dependent upon BLIMP1. We do propose, however, that STAT3 strongly promoted a genetic program consistent with plasma cell differentiation.

In this study, and in our earlier work (17), we show that STAT5 activation promotes self-renewal of proliferating B cells which are blocked in their terminal differentiation through up-regulation of the repressor BCL6. In contrast to our results, a recent study has shown a negative role for STAT5 in regulating BCL6 expression in various hematopoietic tumor cell lines including BCL1, NKL, 3D2, and Ba/f3 cells (68). BCL6 is a repressor of differentiation in B cells and our results clearly demonstrated that direct activation of STAT5 activation blocks plasma cell differentiation, supporting a positive relationship between STAT5 and BCL6. In fact, withdrawal of 4-HT from STAT5ER B cells resulted in spontaneous appearance of plasma cells. Thus, it is possible that the regulatory relationship between STAT5 and BCL6 in these transformed cell lines is different from that of primary human B cells. Another study proposed that, via AP-1 and STAT3 activation, IL-21 positively regulated BCL6 expression in mouse B cells (69). Although we and others have also found that IL-21 increased BCL6 expression (11, 46), we did not find that specific STAT3 activation affected BCL6 mRNA or protein levels. STAT3 activation, however, decreased BLIMP1 expression and might therefore be an initiating stimulus to push the BCL6/BLIMP1 balance in favor of BLIMP1, leading to human plasma cell differentiation.

The current model of transcriptional regulation of GC-based plasma cell differentiation states that BCL6 expression is high in GC centroblasts and centrocytes. The mechanism by which this occurs is still unknown. It is well known, however, that BCL6 expression is absent in plasma cells (28) due to a repressive regulatory circuit with BLIMP1 (31), which is high in plasma cells (28). Thus the question arises, how can STAT3 activation promote plasma cell differentiation? To address this question, we investigated whether STAT3 activation promotes plasma cell differentiation by triggering expression of BLIMP1, and that this occurred in cells expressing high levels of BCL6. Two possibilities are as follows: 1) that either BCL6 needs to first decrease to allow BLIMP1 to increase or 2) that BLIMP1 can be increased in BCL6$^+$ GC B cells until BLIMP1 levels reach a critical threshold to tip the balance toward plasma cell differentiation. Our results do not exclude the possibility that first mechanism may occur, for example, through CD40L ligation (47) with GC T cells. In contrast, we provide evidence that the latter mechanism is also feasible. We show that STAT3 activation initiated plasma cell differentiation by triggering expression of BLIMP1, and that this occurred in cells expressing high levels of BCL6, suggesting that this factor is not the dominant repressor of BLIMP1 expression. Using a BCL6 transgenic mouse model, Cattoretti et al. (70) showed that high BCL6 expression occurred in a subset (15%) of Ig-secreting, CD138$^+$ plasma cells, suggesting that forced BCL6 expression could not completely block plasma cell formation in vivo. It is possible that in this system a selection of plasma cells exhibiting weak BCL6 expression occurred. Our data suggest that BCL6 expression nearly completely blocks in vitro plasma cell differentiation. In light of the data of Cattoretti et al., it is possible that in our hands forced BCL6 expression negatively affects the survival of human plasma cells in...
vitro, while promoting survival of nondifferentiated cells. In addition to BCL6, down-regulation of the repression activity of other factors such as PAX5 can lead to plasma cell differentiation (48, 49). Indeed, such a mechanism of “alleviated repression” was recently identified as a mechanism underlying PAX5-mediated blockade of plasma cell differentiation (38). However, we were unable to detect significant changes in PAX5 expression through manipulation of STAT3 activity.

Thus, by induction of STAT3 signaling, either directly or with IL-21, we demonstrate BLIMP1 up-regulation under conditions that may resemble the GC B cell environment-high BCL6 expression by GC B cells, CD40 triggering, and exposure to cytokotpes. Furthermore, we show that while BCL6 did not inhibit induction of plasma cell differentiation, its down-regulation was required for completion of this process. Considering our previous data showing that STAT5 positively controls BCL6 expression (17) together with the results presented in this study, finding the source of STAT5 or STAT3-activating cytokines may be of interest.

CXCR5^+ CD4^+ Follicular helper T cells are a possible candidate as subsets of these cells have been shown an array of cytokotpes such as IL-21, IL-4, and IL-2 (71, 72). We propose that the balance of activated STAT3 and STAT5 is an important factor in determining whether GC B cells differentiate into self-renewing B cells (STAT5 activation) or into plasma cells (STAT3 activation) in the GC.

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Disclosures
The authors have no financial conflict of interest.

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