Transcriptional control of human plasmacytoid dendritic cell and B cell differentiation

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Abstract

The terminal differentiation of B cells into antibody-secreting plasma cells is tightly regulated by a complex network of transcription factors. Here we evaluated the role of the Ets factor Spi-B during terminal differentiation of human B cells. All mature tonsil and peripheral blood B cell subsets expressed Spi-B, with the exception of plasma cells. Overexpression of Spi-B in CD19⁺ B cells inhibited, similar to the known inhibitor BCL-6, the expression of plasma cell-associated surface markers and transcription factors as well as immunoglobulin production, i.e. in vitro plasma cell differentiation. The arrest in B cell differentiation enforced by Spi-B was independent of the transactivation domain, but dependent on the Ets-domain. By chromatin immunoprecipitation and assays utilizing an inducible Spi-B construct BLIMP1 and XBP-1 were identified as direct target genes of Spi-B mediated repression. We propose a novel role for Spi-B in maintenance of germinal center and memory B cells by direct repression of major plasma cell factors and thereby plasma cell differentiation.
Introduction

Germinal centers (GC) are specialized areas in the follicles of lymphoid organs, where B cells upon antigen challenge undergo multiple rounds of proliferation, accompanied by somatic hypermutation (SHM) and Ig class-switch recombination (CSR), generating memory B cells, or, alternatively, plasma cells (PC). Memory B cells retain a high-affinity B cell receptor (BCR) at their cell surface, do not secrete antibody and have the intrinsic ability to respond rapidly and proliferate strongly on secondary encounter with antigen. The formation of non-dividing antibody-producing PCs is controlled by a complex network of transcription factors. BLIMP1, encoded by the PRDM1 gene, is essential for PC formation and Ig secretion by initiating a gene regulation cascade which leads to cessation of the cell cycle, repression of genes that are required for the identity of mature and GC B cells, and induction of the Ig secretory program. Furthermore XBP-1, that is controlling the secretory machinery of PCs, and IRF-4 play an essential role in PC differentiation. Induction of PC differentiation requires an active suppression of the B cell phenotype, i.e. of factors that are expressed in GC B cells, most importantly BCL-6 and PAX-5. These factors have been shown to inhibit differentiation of activated B cells, allowing sufficient time for affinity maturation and class switch recombination to occur in response to antigen and T cell signals. The proteins act predominantly by repression of the factors required for PC differentiation resulting in a double-negative feedback mechanism that ensures maintenance of different developmental states in a mutually exclusive manner.

In addition to BCL-6 and PAX-5 the Ets factor Spi-B is directly repressed by BLIMP1 in murine B cells, suggesting that the regulation of Spi-B is important in PC differentiation. Spi-B deficient mice, which have normal B cell numbers, show a defect in GC formation and maintenance, precluding the assessment of the role of Spi-B during later stages of B cell differentiation. Other cells that express Spi-B include early T lineage cells and plasmacytoid dendritic cells (pDCs). Spi-B is crucial for development of human pDCs but not for human B cell development, consistent with data from Spi-B deficient mice. Furthermore, it was recently shown that the Spi-B locus is translocated in the activated B cell-like (ABC) diffuse large B cell lymphoma (DLBCL) cell line OCI-Ly3, leading to increased expression of the transcription factor. To determine whether the overexpression of Spi-B is linked to the pathophysiology of this lymphoma subtype it is required to understand the function of Spi-B in human B cell differentiation.

Our data suggest a role for Spi-B in controlling differentiation of human B cells by repressing the induction of the plasma cell gene expression program. Spi-B bound the regulatory elements of PRDM1 and XBP1, encoding BLIMP1 and XBP-1, respectively, and directly repressed expression of these two factors.
Materials and Methods

**B cell isolation.** Tonsillectomies were performed at the Department of Otolaryngology at the Academic Medical Center, Amsterdam. The use of this tissue was approved by the medical ethical committees of the institution and was contingent on informed consent. Buffy coats were obtained from Sanquin bloodbank, Amsterdam. CD19+ B cells were isolated by positive selection using MACS CD19-coupled microbeads (Mitenyi Biotec) resulting in purities of >98%. Tonsil and peripheral blood subpopulations were separated by FACS Aria sorting.

**Flow cytometry.** Monoclonal antibodies against human CD3, CD19, CD20, CD27, CD38, CD40, CD123, IgD, IgG and NGFR directly conjugated with FITC, PE, APC, PE-Cy7 or APC-Cy7 were purchased from BD Pharmingen, CD138-PE from Dako, BDCA2-APC from Miltenyi Biotec, CD40-PE from Immunotech. Samples were analyzed by flow cytometry on a LSRII (BD) and analyzed using FlowJo software (TreeStar).

**B cell cultures.** For PC differentiation assay, B cells were co-cultured on irradiated CD40L expressing, stably transfected L cells, together with IL-2 (40 U/ml, R&D Systems) and murine (m) IL-21 (50 ng/ml, R&D Systems) in IMDM with 8% FCS. After 72h, L cells were withdrawn and B cells were cultured with cytokines only.

**pDC cultures.** CD34+CD1a- human postnatal thymus progenitors were cultured on a layer of OP9 stromal cells in the presence of 5ng/ml IL-7 (PeproTech) and Flt3L (PeproTech)23 and analyzed after 7 days.

**Constructs and retroviral transductions.** Retroviral constructs Spi-B and BCL-6 LZRS-IRES-GFP were described previously19,24. Spi-B/ΔEts-ER and Spi-B/ΔTAD fusion protein vectors were constructed by fusing the C-terminus of full length or ΔEts Spi-B, respectively, to a truncated murine estrogen receptor24. Spi-B/ΔTAD was obtained from Spi-B PCR amplification of cDNA generated from CD34+CD38+ fetal liver cells. By sequencing the location and length of the truncation (215bp) of Spi-B/ΔTAD was determined. For virus production, the constructs were transfected into the Phoenix-GalV packaging cells24. Control cells were transduced with empty LZRS-IRES-GFP constructs. Before transduction, isolated CD19+ B cells were co-cultured with L cells and mIL-21 (50ng/ml) for minimal duration of 36h. Cells were transferred to plates coated with fibronectin (30μg/ml, Takara Biomedicals) and incubated with virus for 6 to 8h. To induce translocation of ER fusion proteins, cells were treated with 0.5uM 4HT (Sigma). De novo protein translation was inhibited by pre-incubation with 2.8μg/ml of CHX (Sigma).

**Immunoblot analysis.** Western blotting was performed as described19. Membranes were incubated with antibodies against human Spi-B20,25 (kindly provided by Lee Ann Sinha, State University of New York, Buffalo, USA and F. Moreau-Gachelin, Institute Curie, France), BLIMP126 (kindly provided by R. Tooze, Leeds Institute of Molecular Medicine, UK), PAX-527 (kindly provided by Stephen Nutt, WEHI, Australia), BCL-6 (C-19), IRF-4 (M-17), estrogen receptor (ER) (MC-20) or Actin (I-19, all Santa Cruz Biotechnology).

**Quantitative real time PCR.** Total mRNA was isolated from cells using RNeasy mini kit (Qiagen) and reverse transcribed into cDNA with first strand buffer, superscript II reverse transcriptase (Invitrogen), DNTP (Promega). For qPCR we used an iCycler and SYBR green Supermix (BioRad). Each sample was analyzed in duplicates and expression levels were normalized to β-actin expression. Primer sequences for BCL-6, BLIMP1 and β-Actin27 and Spi-B25 are published, other primers are listed in supplemental data.

**Chromatin immunoprecipitation (ChIP).** 8 x 10^6 SpiB-ER-GFP+ RAJI cells were incubated with or without 4HT for 4h. ChIP was performed according to an adapted version of the Upstate Chip kit protocol (Upstate, Charlottesville, USA). IP was performed with either 3μg of polyclonal anti-ER antibody (Santa Cruz) or 3μg of normal rabbit IgG (Caltag). Precipitated chromatin was purified with QIAmp DNA mini kit (Qiagen) analyzed by icycler PCR. Primers are listed as supplemental data. Each ChIP was performed in triplicates and each PCR reaction in duplicates.

**ELISA.** Plates were coated with capture Abs anti-human IgG or IgM (Dako) at 10μg/ml washed in ELISA wash buffer. 10% FCS in PBS was used as blocking agent and diluent for cell supernatants and for enzyme-conjugated detection antibodies. TMB substrate/stop solution (Biosource) was used for development of IgG and IgM ELISAs.
Results

Spi-B is expressed in all human B cell subsets but not in PCs.

Human B cells express Spi-B\textsuperscript{19}, but its role during terminal B cell differentiation has not been investigated. Analysis of the role of Spi-B in terminal differentiation of Spi-B deficient B cells was precluded due to impaired BCR signaling leading to defects in maturation and maintenance of GCs\textsuperscript{17}. Here we analyzed Spi-B protein expression in various mature B cell subsets from blood and tonsil representing different stages in B cell development. Peripheral blood (PB) CD19\textsuperscript{+}CD3\textsuperscript{−} B cells, including naïve (IgD\textsuperscript{+}CD27\textsuperscript{−}) and switched memory (IgD\textsuperscript{−}CD27\textsuperscript{+}) B cells, were analyzed by immunoblotting. Spi-B protein was expressed both in naïve and memory B cells, but with higher expression in memory B cells (Figure 1A). In line with our earlier observation, Spi-B was not detected in CD3\textsuperscript{+} T cells from the same blood donor\textsuperscript{19}. Human CD19\textsuperscript{+}CD3\textsuperscript{−} tonsil B cells were discriminated as described previously\textsuperscript{28} into naïve B cells (IgD\textsuperscript{+}CD38\textsuperscript{−}), class-switched memory cells (IgD\textsuperscript{−}CD38\textsuperscript{−}), early non-switched germinal center (GC) B cells (IgD\textsuperscript{−}CD38\textsuperscript{−}), GC B cells (IgD\textsuperscript{−}CD38\textsuperscript{+}), and PCs (PC, IgD\textsuperscript{−}CD38\textsuperscript{++}). Spi-B protein was expressed in all tonsil subsets except in PCs (Figure 1B). Higher expression levels were observed in GC B cells and resting memory cells than in naïve cells. The same lysates were also analyzed for expression of BCL-6 and BLIMP1 protein. As expected, BCL-6 protein was highly expressed in early and late GC B cell subsets and only weakly in PCs (Figure 1B and ref. 29), while BLIMP1 was highly expressed in IgD\textsuperscript{−}CD38\textsuperscript{++} PCs only (Figure 1B and ref. 30).

Next we analyzed Spi-B expression levels during in vitro PC generation. We adapted previously described culture conditions that drive proliferation and differentiation of human B cells into antibody-secreting PCs\textsuperscript{31,32}, accompanied by downregulation of cell surface BCR, CD19 and CD20 expression and upregulation of the activation marker CD38 as well as the PC marker Syndecan-1 (CD138) on B cells\textsuperscript{33}. Naïve and memory PB B cells were cultured for 3 days on CD40-ligand transfected (CD40L)-L cells in the presence of IL-2 and IL-21, followed by 4 days of culture only in the presence IL-2 and IL-21. In line with previous findings\textsuperscript{34,35}, we observed that the generation of CD38\textsuperscript{+}CD20\textsuperscript{−} PCs from memory B cells was much faster than from naïve B cells (Figure 1C). Less than 15% of naïve B cells differentiated into PCs within 7 days, and consistent with this we observed only low levels of BLIMP1 protein. Within two days naïve B cells strongly increased Spi-B protein levels after activation in vitro with CD40L and cytokines (Figure 1C). When starting from memory B cells, BLIMP1 was readily and strongly induced, consistent with the observation that the majority of B cells acquired a CD38\textsuperscript{+}CD20\textsuperscript{−} PC phenotype within 7 days of culture. Notably, Spi-B protein levels rapidly decreased during PC differentiation. During PC differentiation B cells completely and irreversibly switch their gene expression profile. To get further insight in Spi-B regulation during PC differentiation, cultured memory PB cells were analyzed at consecutive days for Spi-B mRNA levels by quantitative PCR. In addition, we analyzed expression of factors known to be regulated during B cell differentiation, including PAX-5 and BCL-6, which are downregulated, and BLIMP1, IRF-4 and XBP-1, which are induced and mediate PC formation\textsuperscript{3}. Consistent with results obtained by immunoblotting, in vitro PC differentiation of PB memory B cells was associated with a decrease in Spi-B transcript levels (Figure 1D). Interestingly, the kinetics in reduction of Spi-B expression levels paralleled that of the repressors PAX-5 and BCL-6. As expected, BLIMP1, XBP-1 and IRF-4 expression levels increased during in vitro PC differentiation. Taken together these results show that Spi-B is expressed in all human PB and tonsil B cell subsets but not in PCs. Spi-B mRNA and protein levels decreased during in vitro differentiation of PB memory B cells into PCs. The differential expression of this transcription factor in memory and plasma cells suggests an active regulation and a role for Spi-B during human B cell differentiation.
Spi-B inhibits plasma cell differentiation

Figure 1. Spi-B is expressed in human B cell subsets, but not in plasma cells. Equal numbers of CD19⁺ B cells of (A) peripheral blood or (B) tonsil were sorted into subpopulations as indicated by gating and analyzed by immunoblotting. Actin levels were determined as loading control. One representative experiment of three is shown. (C+D) CD19⁺ B cells purified from peripheral blood were co-cultured under plasma cell promoting conditions. (C) Naïve (left panel) or memory (right panel) B cells were cultured for 7 days and analyzed for CD20 and CD38 expression by flow cytometry. Numbers in the quadrants indicate percentages of cells. For immunoblotting, cells were collected at the indicated time points and analyzed for Spi-B and BLIMP1 expression. Actin levels were determined to ensure equal loading of the samples. (D) Memory B cells were cultured for the time periods indicated and expression levels were assessed by quantitative real-time PCR. Averages ± SD of PCR duplicates are shown. One representative experiment of two is shown.

Ectopic overexpression of Spi-B arrests differentiation of PB B cells into immunoglobulin-secreting PCs.
To elucidate the reason for downregulation of Spi-B in PCs, Spi-B was constitutively expressed in human B cells, using the LZRS retroviral vector harboring the Spi-B coding sequence upstream of the IRES-GFP cassette (Figure S1 and ref. 19), and cultured under plasma cell inducing conditions. In parallel we transduced B cells with BCL-6, which arrests human PC differentiation in vitro as previously reported. Seven days after transduction a vast proportion of control transduced cells had differentiated into PCs (Figure 2A) of which a proportion expressed CD138. Strikingly, ectopic expression of Spi-B prevented PC differentiation, as we observed significantly less formation of CD38⁺CD20⁻ and CD19⁺CD138⁺ cells compared to control cultures (Figure 2A and 2B). As expected, also BCL-6-GFP⁺ cells were blocked in PC differentiation. AnnexinV / 7-AAD labeling of transduced cells showed that Spi-B did not affect cell survival (data not shown). Transduction of sorted naïve as well as memory B cells with Spi-B revealed that Spi-B abrogates
PC differentiation of both B cell subsets (data not shown). Also, we observed that Spi-B was not only able to arrest in vitro plasma cell differentiation induced by IL-21, but also by IL-10 (data not shown). Terminal differentiation of B cells is intimately associated with increased Ig secretion. Based on the strong inhibitory effect of Spi-B on phenotypically-defined PC differentiation, we speculated that ectopic expression of Spi-B would inhibit antibody secretion under PC permissive conditions. Conform to this hypothesis, PB B cells transduced with Spi-B secreted 4-6 fold less IgM and IgG than controls as determined by ELISA in the supernatant of transduced cell cultures (Figure 2C). As expected, also BCL-6 transduced B cells were impaired in Ig production, similar to Spi-B transduced B cells.

To exclude that our observations are merely due to high levels of Spi-B overexpression we used a Spi-B~ER fusion construct, which allows nuclear transport of the ER-fusion protein in a 4-hydroxytamoxifen (4HT) concentration-dependent manner. Immunoblot analysis confirmed the presence of the fusion protein, which was detectable by antibodies directed against Spi-B and ER (Figure S2 and data not shown). While PB B cells transduced with a control vector were not affected by 4HT, in Spi-B~ER transduced cell cultures the percentage of CD38^−CD20^− PCs generated correlated with the 4HT concentration, indicating a Spi-B dose-dependent regulation of PC formation. Thus, forced expression of Spi-B during in vitro PC generation inhibited the differentiation of PB B cells into PCs in a dose-dependent manner. Spi-B blocked PC formation as efficient as BCL-6, a known PC repressor. Consequently, antibody production of the pre-GC isotype IgM as well as the switched isotype IgG was strongly reduced after forced expression of Spi-B in PB B cells.

**Figure 2.** Impaired human B cell differentiation into Ig-secreting plasma cells by ectopic expression of Spi-B. CD19^+^ B cells isolated from peripheral blood were transduced with Spi-B, BCL-6, Spi-B~ER or control vectors and cultured in conditions promoting plasma cell differentiation (as in Figure 1). (A+B) After 7 days of culture GFP^+^ cells were analyzed for CD19, CD20, CD38 and CD138 surface expression by flow cytometry. (A) Contour plots of one representative experiment out of ten are shown. Numbers in the quadrants indicate percentages of cells. (B) Percentages of CD38^−CD20^− and CD19^+CD138^ cells in Spi-B and BLC-6 transduced cultures were normalized to control cultures. Averages ± SD of ten independent experiments are shown. Student’s t-test, ** p < 0.01. (C) Five days after transduction, GFP^+^ cells were sorted and equal numbers of cells (50,000) were cultured with IL-2 and IL-21 for an additional 48h. The supernatants were collected and IgM and IgG protein levels were analyzed by ELISA. Averages ± SD of ELISA triplicates are shown. One representative experiment out of three is displayed. (D) After 7 days of culture the percentages of CD38^+CD20^− cells within the GFP^+^ population in Spi-B~ER and control transduced cultures supplemented with the indicated concentrations of 4HT were assessed. One representative experiment out of two is shown.
Spi-B represses the induction of the PC gene expression program.

To gain insight into the mechanism of the Spi-B enforced PC differentiation block, we assessed the gene expression levels of BCL-6, BLIMP1, XBP-1, IRF-4 and PAX-5 in B cells following ectopic Spi-B expression (Figure 3A). In parallel we analyzed the expression levels of these genes in BCL-6-transduced B cells. Five days after culture in PC inducing conditions, which induced the expression of PC genes (Figure 1D), GFP+ cells were sorted and analyzed by real time PCR. Notably, forced expression of Spi-B did not affect BCL-6 levels in PB B cells and vice versa overexpression of BCL-6 had no effect on Spi-B expression levels (Figure 3A), which suggests that these genes do not act in a sequential pathways. However, 6-10 fold lower levels of BLIMP1, IRF-4 and XBP-1 mRNA were detected both in Spi-B and BCL-6 transduced cells compared to controls (Figure 3A). As assessed by conventional RT-PCR, the reduction of XBP-1 expression resulted in decreased levels of the spliced form of XBP-1 mRNA that gives rise to the transcriptionally active XBP-1(s) protein (Figure S3). Expression levels of PAX-5 did not significantly change in cells overexpressing Spi-B or BCL-6 compared to controls. Protein levels in transduced cells reflected mRNA levels of the analyzed transcription factors as forced expression of Spi-B inhibited expression of BLIMP1 and IRF-4 protein to a similar extend as forced expression of BCL-6, while Spi-B did not affect BCL-6 or PAX-5 protein levels (Figure 3B). Decreasing Spi-B expression levels by shRNA21 did not increase PC formation or affect expression levels of the PC transcription factors, leaving open the possibility that multiple transcriptional repressors have to be downregulated in parallel (data not shown). Thus, forced expression of Spi-B prevents the induction of the PC gene program under culture conditions that permit PC differentiation.

Figure 3. Repression of the plasma cell gene expression program by Spi-B. CD19+ B cells were retrovirally transduced with constructs expressing Spi-B, BCL-6 or control-GFP. Five days after transduction and culturing in conditions promoting plasma cell differentiation (as in Figure 1), GFP+ cells were sorted. (A) Gene expression levels of Spi-B, BCL-6, BLIMP-1, XBP-1, IRF-4 and PAX-5 were analyzed by quantitative RT-PCR. Expression levels in Spi-B and BCL-6 transduced cells were normalized to expression levels in control transduced cells. Averages ± SD of four independent experiments are shown. Student’s t test, **p < 0.01. (B) Cell lysates from sorted GFP+ cells were subjected to immunoblot analysis using antibodies directed against Spi-B, BCL-6, BLIMP-1, IRF-4 or PAX-5. Actin levels were determined as loading control. Representative results of two independent experiments are shown.
The Spi-B transactivation domain is not required to arrest PC differentiation.

It has been well accepted that Spi-B is able to act as a transcriptional activator\textsuperscript{37-42}. We were, therefore, intrigued by the findings described above suggesting a repressor role for Spi-B. Structurally, Spi-B possesses an amino-terminal transactivation domain (TAD), which is required for activating transcription\textsuperscript{39}. Next we aimed to assess the contribution of this domain to the regulatory activity of Spi-B by transducing cells with a truncated Spi-B protein lacking TAD (Spi-B/ΔTAD) (Figure S1). As a positive control we determined the expression level of CD40, a direct target of Spi-B transactivation activity\textsuperscript{37}. Overexpression of Spi-B full length (fl) in hematopoietic precursors cultured on OP9 cells with Flt3L, which drives development of plasmacytoid DCs (pDCs)\textsuperscript{19}, resulted in higher surface expression levels of CD40 on pDCs (Figure 4A), whereas Spi-B/ΔTAD did not alter CD40 levels compared to controls. This indicates that the TAD domain of Spi-B is crucial for the induction of CD40 gene transcription. Interestingly, both Spi-B/fl as well as Spi-B/ΔTAD transduced B cells were arrested in differentiation into CD38\textsuperscript{−}CD20\textsuperscript{−} and CD19\textsuperscript{−} CD138\textsuperscript{+} PCs (Figure 4B) when analyzed at day 5 of a PC differentiation assay. These data support the notion that transactivation activity by TAD is largely dispensable for the repression of PC differentiation by Spi-B. Collectively, these data suggest that Spi-B employs distinct mechanisms to promote expression of CD40 on one hand and to regulate expression of PC genes on the other, and that different Spi-B domains are required to either exert transactivation or repression, respectively.

**Figure 4.** The transactivation domain of Spi-B is not required for regulation of plasma cell differentiation. (A) CD34\textsuperscript{+}CD1a\textsuperscript{−} progenitors isolated from human postnatal thymus were transduced with control, Spi-B/full-length (fl) or Spi-B/ΔTAD constructs and cultured on OP9 with Flt3L and IL-7. The histogram shows surface expression level of CD40 on CD123\textsuperscript{+}BDCA2\textsuperscript{+} pDCs transduced with control (shaded histogram), Spi-B/fl (solid line) and Spi-B/ΔTAD (dashed line) constructs. (B) Peripheral blood CD19\textsuperscript{+} B cells were transduced with control, Spi-B/fl or Spi-B/ΔTAD constructs and cultured in conditions promoting plasma cell differentiation (as in Figure 1). After 7 days of culture GFP\textsuperscript{+} cells were analyzed for CD19, CD20, CD38 and CD138 surface expression. Contour plots of one representative experiment out of two are shown. Numbers in the quadrants indicate percentages of cells.
Spi-B inhibits plasma cell differentiation

Spi-B binds to the promoter of human PRDM1 and XBP-1.

Spi-B transduced B cells expressed lower levels of the plasma cell genes BLIMP1, XBP-1, and IRF-4 (Figure 3), but whether these were direct target genes was not resolved. Therefore we investigated whether Spi-B directly binds to the promoter regions of these genes by using chromatin immunoprecipitation (ChIP) assays. IP was performed using an antibody directed against ER (α-ER) or unspecific rabbit Ig on lysates derived from Spi-B~ER transduced Raji cells that were either left untreated or incubated with 4HT for 4 hours. Chromatin abundance was measured by quantitative PCR using primer pairs in the promoter regions of BLIMP1, XBP-1, and IRF-4. Primers were designed to randomly amplify several regions within the potential regulatory elements of PRDM1, XBP1 and IRF4 (Figure 5A and not shown). Based on previous reports15,43,44 we assessed Spi-B binding to the human PRDM1 locus upstream of the transcription start as well as in intron 4, which corresponds to intron 5 of the murine PRDM1 locus. Furthermore, we analyzed Spi-B binding to a region up to 3 kb upstream of the transcription start and the proximal -200 to -700 region of the human XBP1 and IRF4 loci, respectively, which have been reported to control promoter activity12,45,46. As a positive and negative control we analyzed binding of Spi-B to the CD40 promoter37 and the open reading frame (ORF) of Spi-B, respectively (Figure 5B). Notably, we observed binding of Spi-B to the PRDM1 promoter at two out of the three regions investigated (Figure 5C). For these regions, termed region a (-1660 to -1467 relative to the BLIMP1 transcription start) and region c (-388 to -234), chromatin levels after ChIP with α-ER were 10-fold and 6-fold increased, respectively, upon treatment with 4HT, and 40-fold and 20-fold higher compared to nonspecific Ig precipitation.

Figure 5. Binding of Spi-B to the promoter regions of the human PRDM1 and the XBP-1 locus. (A) Schematic representation of the 5’ region of the human PRDM1 and XBP1 locus. Binding sites of primers used for analysis of chromatin immunoprecipitation (ChIP) are indicated relative to the transcription start site. (B-D) ChIP analysis for Spi-B binding. SpiB~ER-GFP+ RAJI cells were cultured in the presence or absence of 4HT for 4h and subjected to ChIP using α-ER antibody or, as control, normal rabbit IgG (Ctrl Ig). (B) As positive control for Spi-B binding, precipitated chromatin was analyzed by real time PCR for abundance of CD40 promoter DNA. The Spi-B open reading frame (ORF) served as irrelevant gene control for Spi-B binding. (C) Precipitated chromatin was analyzed for abundance of three different regions (regions a, b, c) of the PRDM1 promoter. (D) Precipitated chromatin was analyzed with primers binding to regions b and locus c upstream of the transcription start in the XBP1 gene. Values are normalized to chromatin levels in control Ig samples. Averages ± SD of precipitation triplicates are shown. One representative experiment out of two is shown.
No binding of Spi-B to any of the regions tested for intron 4 of the PRDM1 locus was detected (Figure 5A and data not shown). Furthermore, binding of Spi-B was observed at two out of five regions analyzed within the XBP1 promoter (Figure 5D and data not shown). Using primers amplifying region b (-1897 to -1737) and region c (-1475 to -1332), chromatin abundance was 15-fold and 10-fold increased in IP samples with α-ER versus unspecific Ig, respectively. Binding to the IRF-4 promoter was analyzed with three primer sets (region a (-103 to -330), region b (-834 to -1064) and region c (-1212 to -1350), however no binding of Spi-B was detected (data not shown).

**Spi-B directly represses expression of BLIMP1 and XBP-1.**

We employed a 4HT inducible Spi-B/fl~ER fusion construct to further investigate whether binding of Spi-B to the promoters of BLIMP1 and XBP-1 also directly controls expression of these genes. As an additional negative control we made use of a truncated Spi-B protein lacking the DNA binding domain (ΔEts)29,25, which we also fused to ER (Spi-B/ΔEts~ER, Figure S2) and which did not affect PC differentiation upon induction by 4HT addition (data not shown). Spi-B/fl~ER, Spi-B/ΔEts~ER and control transduced B cells were cultured under conditions permitting PC development and sorted on day 4 after transduction for GFP expression. Cells were cultured for an additional 48h, after which the cells were first preincubated with cycloheximide (CHX), impeding de novo protein translation, before treatment with 4HT for 4h. As expected, we detected higher levels of CD40 expression in Spi-B/fl~ER transduced cells cultured with 4HT compared to untreated cells as measured by qPCR, while 4HT treatment alone did not affect CD40 levels in cells transduced with a control or Spi-B/ΔEts~ER vector (Figure 6). Within 4 hours after 4HT addition, Spi-B/fl~ER reduced the expression levels of both BLIMP1 and XBP-1 mRNA, which were 2.5- to 3-fold lower compared to control cells, whereas induction of the mutated Spi-B/ΔEts~ER by 4HT did not affect the expression of these PC factors (Figure 6). This suggests direct regulation of BLIMP1 and XBP-1 by Spi-B that is dependent on the Ets domain of Spi-B. In contrast, IRF-4 levels were similar in all conditions, indicating that IRF-4 expression is indirectly controlled by Spi-B (Figure 3). Importantly, treatment of cells with 4HT for 4h did not affect the number or percentages of living cells in control, Spi-B/fl~ER or Spi-B/ΔEts~ER transduced cultures, including GFP+ cells, CD38+CD20- cells or CD138+ cells (data not shown), excluding the possibility that 4HT had a toxic effect on Spi-B/fl~ER transduced cells.

Direct binding and repression of the BLIMP1 promoter by Spi-B was confirmed by a luciferase reporter assay (Figure S4). We employed a pGL3 vector containing a 2kb fragment of the PRDM1 locus upstream of the transcription start43, which was co-transfected with pcDNA3.1 control or Spi-B expression vectors into NIH3T3 cells. As expected, treatment of the cells with PMA/Ionomycin for 6 hours induced luciferase expression43. Concomitant expression of Spi-B reduced luciferase expression by 30%, indicating that Spi-B directly repressed BLIMP1 promoter activity. A restrictive DNA consensus sequence for Spi-B binding is not known; Spi-B binds to the short GGAA/T motif47,48 as well as the non-canonical AGAA motif47, with a rule for flanking nucleotides41. Within the 2kb BLIMP1 promoter region used in our experiments we found numerous (58) GGAA and AGAA motifs, which precluded assessment of the exact Spi-B binding site(s).

Taken together, our results show that Spi-B directly binds to the promoters of two important plasma cells factors BLIMP1 and XBP-1 and favor the concept that Spi-B directly represses the transcription of these PC inducing genes.
Figure 6. Direct repression of BLIMP1 and XBP-1 by Spi-B. Peripheral blood CD19* B cells transduced with Spi-B/fl~ER-GFP, Spi-B/ΔEts~ER or control GFP vector were cultured in conditions allowing for plasma cell differentiation (as in Figure 1) and sorted for GFP expression. After sorting, the cells were preincubated with cycloheximide (CHX) before addition of 4-hydroxytamoxifen (4HT) for 4h. Gene expression levels were analyzed by quantitative RT-PCR. Values are normalized to expression in samples without 4HT treatment. Averages ± SD of PCR duplicates are shown. One representative experiment out of two is shown.
Discussion

This report reveals a novel role of Spi-B in late stages of human B cell differentiation. Primary naïve and memory B cells from tonsil and peripheral blood expressed Spi-B at the mRNA and protein levels. In contrast, CD38++CD20+ PCs isolated from tonsils or generated in vitro from PB B cells lacked expression of Spi-B. We demonstrated that downregulation of Spi-B is required for efficient differentiation of human PB B cells into PCs, as enforced expression of Spi-B completely blocked PC differentiation and Ig secretion.

Several factors have been reported to regulate PC differentiation, including PAX-5, BCL-6, Bach2, and Mitf (reviewed in ref. 3). PAX-5 represses a number of genes involved in PC differentiation, including BLIMP1 and possibly XBP-111,13,49. Similarly, BLIMP1 gene expression is further repressed by BCL-6 and Bach214,16. It is not likely that Spi-B inhibits PC differentiation through induction of PAX-5, BCL-6, or Bach2 expression, since transcript and protein levels of these genes did not significantly increase upon forced expression of Spi-B (Figure 3 and data not shown). In contrast, expression levels of both BLIMP1 and XBP-1 were reduced shortly after nuclear translocation of Spi-B in the presence of the protein synthesis blocker CHX. This, together with the observation that Spi-B bound to the regulatory elements of PRDM1 and XBP1 as revealed by ChIP analysis, argue in favor of a role for Spi-B in directly repressing transcription of BLIMP-1 and XBP-1 providing an explanation for the observed block in PC formation and Ig secretion in Spi-B transduced B cells.

To date, Spi-B has only been implicated in transactivation of gene expression. Target genes include, in addition to CD4037, the adaptor protein Grap238, the heptahelical receptor P2Y1039, the Rel/NF-kappa B family member c-ref40 and the tyrosine kinases c-fes/c-fps41 and Btk42. To our knowledge this is the first report that attributes repressor activity to Spi-B. Other Ets family members, including PU.1, which is a close homolog of Spi-b50, have been reported to display repressor activity previously51,52. PU.1 negatively regulates expression of its targets by recruiting several proteins, including a histone deacetylase (HDAC)51. Repressor activity of PU.1 depends on the Ets domain53 and was pinpointed to two lysine-rich acetylation motifs, which when mutated strongly affected the repressor, but not the activator function of PU.154. Interestingly, a similar acetylation motif as in PU.1 can be identified in the Ets domain of Spi-B (data not shown). In line with a role for the Ets domain of Spi-B in conferring repressor activity we show here that this domain is required to arrest PC formation, while the TAD domain can be omitted. In contrast, however, the TAD domain of Spi-B is required to positively regulate CD40 expression. Taken together, it is tempting to speculate that a similar mechanism as employed by PU.1, mediating an activator as well as a repressor function, controls the dual role of Spi-B.

All our studies investigating the effects of Spi-B and its DNA binding were done using overexpression of Spi-B. We have conclusively shown that Spi-B can repress BLIMP1 and XBP-1 expression and consequently PC differentiation when ectopically expressed. These expression levels are likely at higher than endogenous levels in B cells. We did observe, however, a block in plasma cell formation already when Spi-B~ER expression was induced at very low concentrations of 4HT, suggesting that even low nuclear levels of Spi-B are sufficient to repress plasma cell formation. Still we cannot exclude the possibility that ectopically expressed Spi-B is binding to low affinity sites, thereby blocking the ability of adjacent activators to bind or induce transcription. Whether these putative low affinity sites are recognized by endogenous levels of Spi-B remains elusive.

It is evident from our results that Spi-B needs to be downregulated for proper PC formation, but it is unclear what regulates Spi-B expression. We exclude the possibility that BCL-6 controls Spi-B levels, since overexpression of BCL-6 did not increase mRNA or protein expression levels of Spi-B. It was reported that in mice Spi-B is a direct target of BLIMP1 suggesting that Spi-B expression is repressed by this factor5. Our data, however, do not support the notion that BLIMP1 is the factor that initiates downregulation of Spi-B during PC differentiation since we observed that Spi-B levels had already decreased before induction of BLIMP1 (Figure 1D). This is in line with recent findings in the mouse indicating that BLIMP1 is not required for the initiation of PC differentiation and that the initial decrease of PAX-5 levels occurs prior to BLIMP1.
expression\textsuperscript{27}. These new findings challenge the current model in which the silencing of the B cell transcription program and the induction of the plasma cell program is initiated by BLIMP1\textsuperscript{55}. It is yet unclear which factor(s) is (are) responsible for the initial decrease of PAX-5 and Spi-B.

We observed that shRNA-mediated downregulation of Spi-B did not promote spontaneous Ig secretion or PC formation. This is in line with a previous report in Spi-B deficient mice, which do not have increased numbers of plasma cells or elevated serum Ig titers\textsuperscript{17}. It suggests that Spi-B may not be required to inhibit the spontaneous differentiation of naïve B cells, but rather to inhibit premature differentiation of proliferating B cells in the GC. If true, then GC B cells deficient in Spi-B expression might prematurely differentiate, which would be consistent with the kinetics of GC reactions seen in the Spi-B knockout mouse\textsuperscript{17}. Such early termination of the GC reaction may be the cause of decreased antigen-specific antibodies produced in Spi-B deficient mice, especially if loss of Spi-B actually promotes plasma cell formation from GC B cells. These data are especially intriguing given the potential role of another Ets family member, Ets-1, in regulating spontaneous Ig secretion from naïve B cells\textsuperscript{56}. Thus, it is possible that Ets-1 may play a dominant role in controlling the premature differentiation of naïve B cells, whereas Spi-B plays a dominant role in regulating the premature differentiation of GC B cells. This hypothesis is supported by the observation that expression of Ets-1 is low in GC B cells\textsuperscript{57} whereas expression of Spi-B is high (ref. 57 and present study). Alternatively, our observations raise the possibility that Spi-B is required for maintenance of the memory B cell fate, consistent with the high expression levels of Spi-B protein in human tonsil and PB memory B cells compared to naïve B cells. Aberrant expression of Spi-B has been implicated in tumorigenesis. It has been shown recently that Spi-B is expressed at higher levels in activated B-cell like (ABC) DLBCL than in germinal center B cell-like (GCB) DLBCL\textsuperscript{58}. The gene profile of ABC DLBCL suggests that this lymphoma subgroup is derived from B cells that are blocked in the process of differentiating from GC B cells to PCs\textsuperscript{59}. In one of these cell lines the Spi-B locus was translocated and inserted in proximity to the Ig 3'α enhancer, resulting in relatively high Spi-B transcription levels\textsuperscript{22}. Therefore, it was suggested that Spi-B might play a role in the pathophysiology of the ABC DLBCL subtype. In line with this notion, we observed here that forced Spi-B expression in primary human B cells prevented plasmacytic differentiation. Thus aberrantly expressed, Spi-B may contribute to the formation of B cell lymphomas by blocking terminal differentiation and in this context represent a valuable marker for diagnosis of this tumor type.

In summary, in this paper we describe two novel findings: First, that Spi-B may function as a transcriptional repressor via a mechanism independent of the protein’s transactivation domain, and second, that Spi-B prevents transcription of two of the main PC factors BLIMP1 and XBP-1, which ultimately results in preserving human B cells in an undifferentiated state and thus preventing PC differentiation.

Acknowledgments

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References

lymphocytes committed to the plasma cell lineage. J Pathol. 2005;206:76-86.
58. Rosenwald A, Wright G, Chan WC et al. The use of molecular profiling to predict survival after

Supplementary figures

Figure S1. Detection of ectopic Spi-B/full length and Spi-B/ΔTAD expression in PB B cells. PB B cells transduced with LZRS-Spi-B/full-length, LZRS-Spi-B/ΔTAD or control virus were sorted for GFP expression three days after transduction and Spi-B expression was assessed by immunoblotting relative to actin levels. Asterisk indicates unspecific band. One representative experiment of two is shown.

Figure S2. Detection of Spi-B/full length~ER and Spi-B/ΔEts~ER fusion protein expression in PB B cells. PB B cells transduced with LZRS-Spi-B/full-length~ER, LZRS-Spi-B/ΔEts~ER or control virus were sorted for GFP expression three days after transduction and Spi-B expression was assessed by immunoblotting relative to actin levels. One representative experiment of two is shown.

Figure S3. Decreased levels of spliced XBP-1 upon Spi-B overexpression. CD19+ B cells were retrovirally transduced with constructs expressing Spi-B, BCL-6 or control-GFP. Five days after transduction and culturing in conditions promoting plasma cell differentiation (as in Figure 1), GFP+ cells were sorted. XBP-1 gene expression levels were analyzed by ‘classical’ RT-PCR with primers spanning over the splicing region (Davies, 2003 434 /id). One representative experiment of two is shown.
Figure S4. The human BLIMP1 promoter is regulated by Spi-B. The BLIMP1 promoter-luciferase construct containing 2kb of the human PRDM1 locus upstream of the transcription start was kindly provided by A. Dent (Vasanwala, 2002 21 /id). NIH3T3 cells were transfected with 0.1ng pCMV Renilla, 0.9μg LUC reporters and 1μg of control or Spi-B overexpression vectors (pcDNA3.1). Cells were harvested after 24h, after addition of PMA (20ng/ml) and Ionomycin (0.3μM) for the last 6h. Lysates were analyzed with the dual-luciferase reporter assay (Promega). Values are normalized to relative luciferase activity in control transfected cells. Averages ± SD of two experiments are shown.

Primers

<table>
<thead>
<tr>
<th>Primers for icycler PCR</th>
<th>forward</th>
<th>reverse</th>
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<tr>
<td>PAX-5</td>
<td>GGAGGAGTGAATCAGCTTGG</td>
<td>GGCTTGATGCTTCTCGTGTC</td>
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<tr>
<td>IRF-4</td>
<td>ACCGAAGCTGGAGGGACTAC</td>
<td>GTGGGGCAACAGCATAAAG</td>
</tr>
<tr>
<td>XBP-1*</td>
<td>TCACCCCTCCAGAACATCTC</td>
<td>AAAGGGAGGCTGGTAAGGAA</td>
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<tr>
<td>CD40</td>
<td>CGGCTTCTTTCTTCAATGTGT</td>
<td>ACCAAGGAGTGGCAACAG</td>
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*Primers recognize unspliced and spliced forms of XBP-1.

<table>
<thead>
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<th>Primers for ChIP</th>
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<td>CD40</td>
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<tr>
<td>PRDM1 locus ‘a’</td>
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<tr>
<td>locus ‘b’</td>
<td>CTTCCATTTTCTCTGCTTC</td>
<td>GTGTTGTTGTTGATTGTTGT</td>
</tr>
<tr>
<td>locus ‘c’</td>
<td>TCAACGCTACTTGGGCTCT</td>
<td>TCCGCTACAGCCAATTTAAG</td>
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<tr>
<td>XBP1 locus ‘a’</td>
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<td>CACCAGTGCCGATTAATTT</td>
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<tr>
<td>locus ‘b’</td>
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<td>locus ‘e’</td>
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Primer pairs were checked for specificity by melting curve analysis and gel electrophoresis. Primer efficiencies were determined by template dilution and were highly similar.