Plasmacytoid dendritic cells: how to control the good, the bad, and the ugly at the molecular level

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DIRECT INTERACTION BETWEEN THE ETS TRANSCRIPTION FACTOR SPI-B AND NF-KB SUBUNITS REGULATES PLASMACYTOID DENDRITIC CELL ACTIVATION AND SURVIVAL

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Abstract

Selective expression of toll like receptor (TLR)-7 and TLR9 enables plasmacytoid dendritic cells (pDCs) to sense viruses and bacteria. Ligation of these receptors results in the production of high amounts of type I interferons (IFN)-α/β. This, together with the production of the pro-inflammatory cytokines IL-6 and TNF-α, facilitates the priming of conventional (c)-DCs, T, B, and NK cells, thereby bridging innate and adaptive immune responses. This is further enforced by the notion that activated pDCs can mature and acquire antigen-presenting capacity to activate antigen-specific T cells. In pDCs, TLR7 and TLR9 signal mainly through activation of interferon response factor (IRF)-7 and the NF-κB pathway. While the crucial function of the Ets transcription factor Spi-B during pDC development is well established, its role in the course of pDC activation and maturation is poorly understood. Here we demonstrate that ectopic expression of Spi-B in human progenitor cells generated pDCs with a more mature phenotype as shown by high expression of CD40, CD80, and CD86 compared to control pDCs. Accordingly, decreasing Spi-B levels by shRNA impaired the TLR induced expression of CD40, CD80, and CCR7. Furthermore, we observed that Spi-B interfered with the NF-κB pathway. Interestingly, Spi-B did not only act as a co-activator of NF-κB induced genes, but also demonstrated co-repressor activity in the regulation of NF-κB response genes. We show that this was likely attributed to direct interaction of Spi-B and the NF-κB subunit RelA. Collectively, our data reveal an extended role for Spi-B in the regulation of pDC phenotype and function.
INTRODUCTION

Human plasmacytoid dendritic cells (pDCs) form a rare subtype of immune cells that selectively express Toll like receptor (TLR)-7 and TLR9. TLR-induced activation is mediated by engagement of viral single strand RNA and bacterial DNA, respectively. This leads to rapid secretion of high amounts of type I Interferons $\alpha$ and - $\beta$ (IFN-$\alpha$/IFN-$\beta$), which are directly involved in inhibition of viral replication and in controlling innate and adaptive immune responses. Following activation, pDCs also secrete pro-inflammatory cytokines, such as IL-6 and TNF-$\alpha$, and differentiate into so-called pDC-derived mature DCs. These mature pDCs express high levels of the co-stimulatory molecules CD40, CD80, and CD86 together with MHC class I and II and have upregulated the expression of the chemokine receptor CCR7, thereby promoting the localization to the lymph node where pDCs can induce antigen-specific T cell responses.

PDCs differentiate from CD34$^+$ hematopoietic progenitor cells (HPCs) in the bone-marrow. We previously showed that pDC development depends on the Ets transcription factor (TF) family member Spi-B, partially through direct induction of the anti-apoptotic gene BCL2A1. Spi-B shares with the other members of the Ets family an 85 amino acids conserved DNA-binding Ets domain, which is required to regulate expression of a variety of genes by binding to a purine-rich GGAA/T core DNA sequence. Ets family members can act in cooperation with other transcriptional factors such as AP1, but also with transcriptional cofactors including the TATA-binding protein (TBP) and CREB-binding Protein (CBP). Despite the key role of Spi-B in pDC development and survival, little is known about its role during TLR-induced activation and maturation of pDCs.

Signaling downstream of TLR7 and TLR9 involves the recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor molecule in a complex together with IL-1 receptor-associated kinase (IRAK)-1 and IRAK-4, tumor necrosis factor receptor-associated 6 (TRAF6) and TRAF3, and the transcription factors interferon response factor (IRF)-7 and IRF-5 (reviewed in Gilliet et al.). Activation of this multi-protein complex, which was previously described as the cytoplasmic transductional–transcriptional processor, promotes nuclear translocation of IRF-7 and IRF-5, which are responsible for the induction of type I IFNs gene expression. In addition, the complex activates transcription factor nuclear factor–kappaB (NF-κB), which controls the expression of genes encoding inflammatory cytokines, co-stimulatory molecules and chemokine receptors, and supports pDC survival via induction of anti-apoptotic genes. Structurally, NF-κB members share the rel homology domain (RHD), and the family includes RelA (also known as p65), RelB, c-Rel, p52 and p50. Rel proteins can form homo- or heterodimers, of which the most frequently activated form after TLR signaling is the RelA/p50 heterodimer.
TLR activation triggers the rapid phosphorylation of specific serine residues of IκB proteins mediated by two kinases, IkappaB kinase alpha (IKKα) and IKKβ, leading to ubiquitin-dependent degradation of IκB proteins. This unmasks the nuclear localization signal of RelA/p50 allowing its nuclear translocation. Upon phosphorylation, RelA (phospho-p65) is able to bind DNA and induce gene expression.

Here we investigated the contribution of Spi-B to activation, maturation and survival of pDCs. We observed that ectopic expression of Spi-B by retroviral transduction in human CD34+ HPC-derived pDCs resulted in a more mature pDC phenotype demonstrated by higher levels of co-stimulatory and MHC class II molecules. When these in vitro generated pDCs were stimulated with the TLR9 agonist CpG-B this further upregulated expression of these proteins. We confirmed these results in the pDC cell line CAL-1, and enforced our findings by demonstrating that decreasing Spi-B levels by shRNAs in CAL-1 cells inhibited TLR induced upregulation of CD40, CD80 and CCR7. We further observed that CAL-1 cells depend on NF-κB activity for their survival as the selective IKK inhibitor Bay 11-7082 induced apoptosis. Notably, cell death was rescued by overexpression of Spi-B, which was most likely attributed to increased expression of the anti-apoptotic gene BCL2A1. Further insight into selective genes co-regulated by Spi-B and NF-κB was gained by employing a tamoxifen inducible Spi-B-ER system in combination with TLR triggering of CAL-1 cells. Interestingly, we identified 2 distinct groups of “TLR-induced genes” of which expression was either synergistically upregulated or inhibited by co-expression of Spi-B. This, together with our results obtained by immunoprecipitation and Proximity Ligation Assays (PLA) revealing physical binding between Spi-B and RelA, provide evidence that Spi-B may act as an activator or as a repressor of the NF-κB signaling pathway.

MATERIAL AND METHODS

Cell culture and reagents
The pDC cell line CAL-1 was kindly provided by Prof. Dr. T. Maeda (Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan). Cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with penicillin [50 U/ml] and streptomycin [50 μg/ml] [P/S], and 8 % fetal calf serum (FCS). Cells were maintained at 37°C, 5% CO₂. To induce nuclear translocation of ER tagged Spi-B, cells were treated with 0.5µM 4-hydroxytamoxifen (4HT; Sigma-Aldrich, St Louis, MO, USA). The NF-κB inhibitor Bay 11-7082 (Calbiochem, San Diego, CA) and 4HT were reconstituted in dimethylsulfoxide (DMSO) as a 100 mM stock solution and stored at -20°C.

Antibodies
For analysis, single cell suspensions were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy7, allophycocyanin APC, APC-cyanine (Cy)7 coupled
anti-human monoclonal antibodies (Abs) targeting the following cell surface markers: CD123, BDCA2, CD45RA, CD14, CD19, CD1a, CD3, CD40, CD80, CD86, CCR7, HLA-DR, or isotype controls (BD Bioscience). For detection of phosphorylated P65 (phospho-S529; pP65) protein, cells were fixed using cytofix/cytoperm buffer, permeabilized in ice-cold methanol and washed with Perm/Wash buffer [BD PharMingen] before incubation with APC-conjugated pP65 Ab (BD biosciences). Samples were analyzed on a LSRII fluorescence-activated cell sorter (FACS) analyzer [BD Bioscience] and analyzed using FlowJo software [TreeStar].

Human cell isolation
Postnatal thymus tissue was obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery (LUMC, Leiden, The Netherlands), and its use was approved by the medical ethical committee of the AMC. Thymocytes were isolated from a Ficoll-Hypaque density gradient [Lymphoprep; Nycomed Pharma, Oslo, Norway]. For pDCs isolation, BDCA4+cells were enriched by immunomagnetic bead selection, using the BDCA4 cell separation kit [Miltenyi Biotec, Bergisch Gladbach, Germany]. CD123+CD45RA+pDCs were sorted by flow cytometry on a FACSARia [BD Biosciences]. For HPCs isolation, CD34+c cells were enriched using the CD34 cell separation kit [Miltenyi Biotec] and Lin−[CD3−CD11c−CD14−CD19−CD56−BDCA2−]−CD34+CD1a− were sorted by flow cytometry [FACSARia, BD Biosciences]. Purity was ≥99% and confirmed by reanalysis of sorted cells.

Retroviral and lentiviral transductions
For overexpression of Spi-B we used either the retroviral construct pLZRS-SpiB-IRES-green fluorescent protein (GFP) or pLZRS-SpiB~ER-IRES-GFP. To knockdown Spi-B under the control of the the polymerase III H1-RNA promoter [pol3] in CAL-1 cells, we used the Spi-B short hairpin [sh]RNA expressing lentiviral vector pTRIP-H1-Spi-B shRNA/EF1α-GFP, in which GFP is driven independently by the EF1α promoter. For virus production, constructs were transfected into the Phoenix-GalV packaging cells [retroviral] or 293T cells [lentiviral]. Control cells were transduced with empty pLZRS-IRES-GFP constructs or pTRIP expressing an irrelevant shRNA targeting Renilla mRNA [pTRIP-H1-Renilla shRNA/EF1α-GFP]. For transduction of CD34+HPCs or CAL-1 cells, 3 x 10⁵ or 10⁶ cells, respectively, were transferred to non-tissue treated culture plates coated with 30 µg/mL retronectin [Takara, Kyoto, Japan] and incubated with virus supernatants for 6 hours.

In vitro differentiation assay
Sorted CD34+CD1a−HPCs from postnatal thymus were transduced as described previously. To differentiate HPCs into pDCs, transduced progenitors were cultured on a layer of mouse OP9 stromal cells in the presence of 5ng/ml Flt3L and 5ng/ml IL-7 [PeproTech]. In vitro generated GFP+CD123+BDCA2+ pDCs were analyzed by
flow cytometry on a fluorescence-activated cell sorter (FACS) analyzer (Becton Dickinson) after 7 days of co-culture.

**PCR**

For quantitative (Q)PCR, total RNA was extracted using Trizol reagent (Invitrogen). RNA concentration and quality was determined using the Nanodrop spectrophotometer (Thermo Fisher Scientific). Equal amounts of total RNA were reverse transcribed into cDNA using the RNA-to-cDNA kit (Roche) according to the manufacturer’s instructions. cDNA was amplified with an iCycler using SYBR green supermix (BioRad) and specific primer sets (supplementary table 1). Each sample was analyzed in triplicates and expression levels were normalized to the three housekeeping genes β-Actin, GAPDH and HPRT.

**Immunoprecipitation and Western blot**

For co-immunoprecipitation, 20 x 10⁶ Spi-B~ER transduced CAL-1 cells were cultured 3h in the presence or absence of CpG-B and 4HT. Cells were then lysed with NP-40 lysis buffer. Protein supernatants were pre-cleared by incubation with an anti-IgG rabbit Ab (Dakocytomation) and 40 μL protein A agarose solution (Millipore). Unspecific immune-complexes were eluted by centrifugation. An aliquot of the supernatants (100μL) were taken as the input control. Supernatants were subsequently incubated overnight with an anti-ER Ab or with an anti-RelA Ab crosslinked to protein A agarose beads. Immuno-complexes bound to the beads were collected by centrifugation, washed in cold PBS and analyzed by western blot as described below.

For western blotting, cell lysates were prepared in NP-40 lysis buffer plus protease inhibitor (Roche, Mannheim, Germany) and equal amounts of protein was analyzed by 12.5 % SDS-PAGE, transferred onto nitrocellulose membrane (Millipore) and immunoblotted with rabbit polyclonal antibody against RelA (Santa Cruz biotechnology, CA) or Spi-B (kindly provided by Lee Ann Sinha, State University of New York, Buffalo, USA), and a goat polyclonal Ab against actin (Santa Cruz biotechnology, CA). Blots were incubated with secondary antibodies: 800nm labeled donkey anti-rabbit or 800nm labeled donkey anti-goat (Li-cor, Lincoln, NE, USA). Proteins were visualized using the Odyssey infrared imaging system (Li-cor).

**Cell survival and apoptosis assays**

Transduced cells (2 x 10⁵) were seeded in 6-well plates and incubated under normal growth condition in medium for 5h. Cells were first treated with or without 4HT (0.5μM) for 4h and cultured in the presence of Bay 11–7082 as indicated. Cells were washed with Annexin binding buffer and stained with PE-conjugated AnnexinV in a buffer containing 7-amino-actinomycin-D (7-AAD) (BD pharmingen) and analyzed by flow cytometry on a fluorescence-activated cell sorter (FACS) analyzer (Becton Dickinson).
Proximity ligation assay

PLA detection was performed according to the manufacturer’s protocol [Olink Bioscience, Uppsala, Sweden]. In short, 2 x 10^6 Spi-B-ER transduced GFP+ CAL-1 cells were stained with 7-AAD, and transferred on coverslips using a cytocentrifuge for 1 min at 200 rpm [Cytospin2, Shandon]. Cells were subsequently fixed using 3% paraformaldehyde for 10 min at 22°C. Cells were washed three times with PBS and blocked for 30 min at 22°C in PBS containing 10% normal donkey serum, 0.5% bovine serum albumin [BSA, Sigma Aldrich], and 0.5% saponin. After blocking, cells were incubated for 60 min at 22°C with rabbit anti-ER and mouse anti-RelA antibodies in PBS containing 10% normal donkey serum, 0.5% BSA, and 0.5% saponin. Cells were washed three times with PBST (0.05% Tween in PBS) and incubated with the secondary mouse PLUS and rabbit MINUS antibodies for 1.5 hours at 37°C in the dark. Cells were washed three times in PBST before detection of the probe using the in situ PLA detection kit (Abnova, Walnut, USA). Cells were analyzed with a 63x objective on a Zeiss LSM 710 fluorescence microscope. The PLA signal, which is represented by the number of 633 nm dots per cell and directly proportional to the number of protein-protein interaction per cell, was quantified by analysis of the confocal images using the Java-based ImageJ open-source freeware [version 1.46].

RESULTS

Spi-B is required for pDC activation and maturation

We previously showed that ectopic expression of Spi-B by retroviral transduction in CD34+ progenitor cells promotes commitment to the pDC lineage, leading to the development of higher percentages and absolute numbers of pDCs in Flt3L supplemented cultures compared to control transduced cell cultures. In order to further investigate the role of Spi-B on the phenotype of pDCs, human HPCs were retrovirally transduced with the Spi-B overexpression LZRS construct expressing GFP as a marker gene for transduced cells and cultured in Flt3L containing culture medium on OP9 stromal cells. After 7 days of culture, GFP+BDCA2+CD123hi pDCs were phenotypically characterized by flow cytometry. Consistent with our previous findings, ectopic Spi-B expression increased the percentage of BDCA2+CD123+ pDCs 2-10 fold [data not shown]. As compared to in vitro generated pDCs transduced with the control LZRS vector, Spi-B-overexpressing pDCs showed a significant increase in surface expression of the co-stimulatory molecules CD40 and CD80, and of MHC class II, while expression levels of CD86 were similar in both conditions [Figure 1A-B]. When Spi-B transduced pDCs were activated with the TLR9 agonist CpG-A, this resulted in significantly higher up-regulation of CD40 and CD80 as compared with control transduced cells [Figure 1B]. After CpG-A activation, CD86 expression levels were now also higher on Spi-B transduced pDCs as compared to control transduced pDCs [Figure 1B]. However, a significant difference in expression of HLA-DR between Spi-B and control TLR9 activated pDCs was not detectable.
To enforce our data, we also wanted to analyze the effect on pDC phenotype after impairing Spi-B expression. However, the development of HPCs into pDCs is severely hampered when knocking down Spi-B, which precluded us to test this in this setting. Therefore, we employed the leukemic pDC cell line CAL-1, which we previously validated as a useful model to study molecular mechanisms in pDCs, to address the role of Spi-B on pDC phenotype. Consistent with the data obtained using CD34-derived pDCs, overexpression of Spi-B in CAL-1 cells increased surface expression of CD40 and CD80 as compared to control transduced CAL-1 cells, both in medium and TLR9 ligand (CpG-B) activated conditions. When knocking down Spi-B expression in CAL-1 cells using retroviral mediated transfer of Spi-B shRNAs that effectively decrease Spi-B protein levels in CAL-1 upon transduction, we observed that CD40 and CD80 expression were not efficiently upregulated after CpG-B induced activation as compared to control shRNA (targeting Renilla luciferase) transduced CAL-1 cells. Also steady state levels of these receptors were reduced in medium cultured cells that express Spi-B shRNAs, which is in accordance with previous studies showing CD40 as a direct target of Spi-B.

Furthermore, it is noteworthy that CCR7 expression levels were only affected by Spi-B overexpression or knockdown after TLR activation, but not in medium cultured CAL-1 cells. This suggests that additional signals downstream of TLR9 are required to regulate CCR7 levels, which can be co-regulated by Spi-B. Collectively, our observations indicate that Spi-B is critically involved in the maturation and possibly the homing of pDCs after TLR ligation.

The CAL-1 cell line depends on NF-κB activity and Spi-B for its survival

TLR9 mediated signal transduction is known to depend on NF-κB activity leading to nuclear translocation and phosphorylation of RelA/p65 and consecutive transcription of NF-κB dependent target genes. In agreement with this, we observed that TLR mediated activation of CAL-1 cells using either CpG-B or the TLR7 ligand R848 increased the levels of phosphorylated RelA/p65 as detected by phospho-flow cytometry. Strikingly, we observed that addition of the NF-κB inhibitor Bay 11-7082, which selectively and irreversibly inhibits the phosphorylation of IκB thereby decreasing the nuclear translocation of active NF-κB dimers, to CAL-1 cells induced apoptosis in a dose-dependent manner as shown by the appearance of AnnexinV+ cells. As apoptosis induction by Bay 11-7082 occurred in the absence of TLR ligation this most likely reflects that CAL-1 cell survival critically depends on constitutive NF-κB activity. To gain more insight in the role of Spi-B in the context of NF-κB activation we used an inducible Spi-B-estrogen receptor (ER) fusion construct that concomitantly expresses GFP to trace transduced cells by flow cytometry. Nuclear translocation of Spi-B-ER can be induced in the presence, but not in the absence of 4HT. Spi-B-ER transduced CAL-1 cells were cultured either in the absence or presence of 0.5μM 4HT and with different concentrations
Interestingly, apoptosis induction was significantly inhibited when Spi-B~ER transduced CAL-1 cells were cultured in the presence of 4HT at all concentrations of Bay 11-7082 tested as compared to cells cultured in the absence of 4HT (Figure 2C-D). This strongly suggests that Spi-B is able to rescue CAL-1 cells from apoptosis induced by blocking constitutive NF-κB activity. Based on the our previous findings that Spi-B directly targets the anti-apoptotic gene BCL2A1, we were interested to analyze the levels of the BCL2A1 transcript in Bay/4HT treated CAL-1 cells. Interestingly, the levels of BCL2A1 mRNA were significantly higher in all
Figure 2. Spi-B inhibits apoptosis induced by blocking NF-κB pathway activation. (A) CAL-1 cells were activated with the TLR9 ligand CpG-B (10 µg/mL) or the TLR7 ligand R848 (10 µg/mL) for different time periods as indicated. Levels of phosphorylated p65 (phospho-p65) were analyzed by phosho-flow cytometry. This is a representative experiment out of 3. RCN, relative cell number. (B) Flow cytometric analysis of apoptotic CAL-1 cells after incubation with different concentrations of the NF-κB inhibitor Bay 11-7082 for 15 hours. Shown are percentages of AnnexinV⁺ apoptotic cells of a representative experiment out of 4. (C-E) Spi-B~ER transduced CAL-1 cells were incubated with the NF-κB inhibitor Bay 11-7082 at the indicated concentrations in the presence or absence of 4HT (0.5 µM). (C, D) After 11 hours, Spi-B~ER CAL-1 cells were analyzed by flow cytometry. (C) Representative experiment showing the percentages of AnnexinV⁺ apoptotic cells in the absence of 4HT (open circles) or presence of 4HT (closed circles). (D) Results of 4 independent experiments showing relative fold differences in the percentages of AnnexinV⁺ apoptotic cells in the presence of 2 µM of Bay 11-7082 and with or without 4HT. ** P < 0.01. (E) QPCR analysis was performed to determine the levels of BCL2A1 mRNA, which were compared to the mean levels of β-Actin, GAPDH and HPRT. The levels of BCL2A1 mRNA were normalized to the level of BCL2A1 mRNA in Spi-B~ER transduced CAL-1 cells without Bay 11-7082 and in the absence of 4HT, which was set to 1. * P < 0.05. ND, not determined.
conditions in which CAL-1 cells were cultured in the presence of 4HT independent of the dose of Bay 11-7082 used (Figure 2E). Taken together, these results demonstrate that CAL-1 cells depend on NF-κB and Spi-B-induced activities for their survival, which are likely attributed to regulation of BCL2A1 mRNA levels.

**Spi-B and NF-κB synergize to regulate BCL2A1 gene expression**

In addition to Spi-B, also NF-κB directly targets the BCL2A1 gene. Consistent with this, ligation of TLR9 in freshly isolated pDCs by either CpG-A or CpG-B induced BCL2A1 gene transcription as determined by QPCR (Figure 3A). To gain more insight in the relative contribution of Spi-B and TLR induced NF-κB activity to upregulate BCL2A1 mRNA levels, we stimulated Spi-B-ER transduced CAL-1 cells with either 4HT alone or CpG-B alone or 4HT and CpG-B together. As expected from our earlier findings, we observed that BCL2A1 mRNA levels were upregulated 6-fold in Spi-B-ER transduced CAL-1 cells in the presence of 4HT after 4h, as compared to medium cultured cells (Figure 3B). Activation of the cells with CpG-B alone resulted in a more pronounced 10-fold upregulation in BCL2A1 mRNA levels. Strikingly, concomitant activation of the NF-κB pathway with CpG-B together with 4HT to induce Spi-B nuclear translocation further increased BCL2A1 mRNA levels (150-fold) as compared to medium cultured cells (Figure 3B). These findings suggest that NF-κB and Spi-B synergistically regulate BCL2A1 mRNA levels during pDC activation.

**Spi-B co-regulates expression of other NF-κB-dependent genes induced by TLR activation in CAL-1 cells**

These results prompted us to investigate whether Spi-B may play a role in the regulation of other NF-κB-dependent genes as well. We evaluated expression of

![Figure 3: Spi-B and NF-κB participates to regulate BCL2A1 mRNA in CAL-1 cells. (A) Freshly isolated pDCs were cultured for 6h in medium or with 10μg/ml CpG–A or 10μg/ml CpG–B B, and expression of BCL2A1 mRNA in stimulated vs. unstimulated cells was assessed by QPCR. (B) BCL2A1 mRNA levels were measured by QPCR in Spi-B-ER transduced CAL-1 cells stimulated with 0.5μM 4HT for 4h in the presence or absence of 10μg/ml CpG–B. Values were normalized to Spi-B-ER transduced cells cultured in medium only, which was set to 1.](image)
genes, which have been previously described to be under the control of NF-κB and/or Spi-B, including CD40, the cytokines IL-6, TNF-α, and IFN-β, and the chemokine receptor CCR7. In addition, we analyzed expression of the calcitonin receptor-like receptor (CALCRL), which we identified in a micro-array analysis as a potential Spi-B regulated gene (data not shown, 4). Therefore, we activated Spi-B-ER transduced CAL-1 cells with CpG-B in the presence or absence of 4HT. In addition, as we were interested to understand the relative contributions of Spi-B and NF-κB to induction of gene expression, we either added 4HT first and 2 hours later CpG-B or CpG-B first and 2 hours later 4HT. After a total incubation time of 6 hours we performed QPCR analysis (Figure 4). It was notable that regulation of gene expression could be divided into two groups. The first group included CD40, TNF-α and IFN-β, which genes were upregulated by Spi-B alone, but more pronounced by CpG-B alone. However, when Spi-B and CpG were combined, gene expression was increased to even higher levels as compared to either stimulus alone independent of whether cells were incubated first with 4HT or with CpG-B (Figure 4A). The second group of genes, which included IL-6, CCR7 and CALCRL, were induced by CpG-B alone, but not by 4HT alone. More strikingly, when CAL-1 cells were stimulated with CpG-B followed after 2 hours by 4HT we observed strong inhibition in CpG-B induced gene expression (Figure 4B). Even when 4HT was added first, followed 2 hours later by CpG-B, expression of IL-6, CCR7 and CALCRL was not upregulated to the levels observed when cells were stimulated with CpG-B alone. While the CCR7 results here appear in contrast to the CCR7 levels we detected on activated Spi-B transduced CAL-1 cells (Figure 1C), this may reflect differences in regulation of CCR7 at the mRNA and protein level, which we currently are unable to explain. Taken together, these results suggest a bimodal role of Spi-B in CAL-1 cells, either acting in synergy with NF-κB to enhance gene expression, or conversely acting to repress NF-κB induced gene expression.

**Spi-B physically interacts with the NF-κB subunit RelA**

To increase our understanding on the interplay between Spi-B and NF-κB we took advantage of the notion that physical interactions between RHD and Ets domains have been documented.21 We hypothesized that Spi-B and RelA may physically interact to regulate gene expression in pDCs. Therefore, we performed co-immunoprecipitation (IP) experiments using cell lysates of Spi-B-ER transduced CAL-1 cells that were either left unstimulated, or stimulated with CpG-B and 4HT for 4 hours. When IP was performed on lysates with an anti-RelA/p65 antibody we clearly detected the Spi-B protein, both in unstimulated as well as in CpG-B plus 4HT stimulated cells, as visualized by immunoblotting using an anti-Spi-B Ab, suggesting that RelA and Spi-B interact (Figure 5A). CAL-1 cells in which we overexpressed Spi-B or knocked down Spi-B using shRNAs served as positive and negative controls, respectively, for detection of the Spi-B protein using our anti-Spi-B Ab. In addition, we analyzed CAL-1 cell lysates obtained before the IP
[input] as a control. Notably, we did not detect binding of Spi-B~ER with one of the other NF-κB subunits c-Rel or RelB (data not shown).

Validation of the RelA and Spi-B interaction in primary pDCs is important, but large cell numbers are required for these experiments precluding such analysis. However, to further substantiate our findings that RelA and Spi-B in CAL-1 cells physically interact, we performed in situ proximity ligation (PLA) assays, which allow immunocytochemical visualization, localization, and quantification of protein-protein interactions. Unstimulated or CpG-B/4HT activated CAL-1 cells were first incubated with both anti-RelA and anti-ER antibodies. Species specific secondary antibodies labeled with unique short DNA strands (PLA probes) were then added. Only when the bound probes are in close proximity they can serve to generate circular DNA strands, which in turn serve as a template for a rolling circle amplification reaction. The amplification product is then detected using fluorescently labeled complementary oligonucleotides. The maximum distance between epitopes required for the formation of amplifiable ligation products is estimated to be about 30–40 nm, based on known antibody and oligonucleotide sizes.22 The results of the PLA show abundant small bright fluorescent foci in CAL-1 cells double-stained using anti-RelA and anti-ER only when cells were stimulated

Figure 4: Spi-B participates to NF-κB-mediated gene regulation in activated CAL-1. mRNA levels of (A) CD40, TNF-α, IFN-β, and (B) IL-6, CCR7, CALCRL mRNA were measured by QPCR in Spi-B~ER transduced CAL-1 cells stimulated quantified in Spi-B~ER transduced CAL-1 cells after stimulation with 0.5μM 4HT, in the presence or absence of 10μg/ml CpG-B. Values were normalized to Spi-B~ER transduced cells cultured only with medium, which was set to 1.
Figure 5: Spi-B~ER physically interacts with the NF-κB subunit RelA. (A) Spi-B~ER transduced CAL-1 cells were cultured 4h in the presence of 4HT and CpG-B or left unstimulated. Analysis of Spi-B~ER immune complexes pulled down using anti-RelA Ab cross-linked to protein A agarose beads were analyzed by western blot using anti-Spi-B Ab. Protein lysates of CAL-1 cells transduced with Spi-B or with Spi-B shRNA were analyzed as visual control for detection of the Spi-B protein band. Input is shown as positive control before immunoprecipitation. (B-C) Spi-B~ER GFP~ (green) CAL-1 cells pre-incubated in the presence or absence of CpG-B and 4HT were fixed and permeabilized, and stained with DAPI (blue). Physical interactions between RelA and Spi-B~ER were investigated by Proximity Ligation Assay (PLA), and detected by confocal microscopy analysis [GFP, green channel, 488nm; DAPI, blue channel, 405nm; PLA probes, red channel, 633nm]. (B) PLA signal (red dots) was analyzed in GFP~DAPI~CAL-1 incubated in the absence (left, unstimulated) or in the presence of CpG-B and 4HT (right, CpG-B + 4HT). (C) 100μm stack confocal picture of stimulated DAPI~GFP~CAL-1 cells stimulated with CpG-B and 4HT.
with CpG-B plus 4HT, but not in unstimulated cells (Figure 5B). When the PLA was carried out using cells double-stained with either anti-RelA, or with control rabbit IgG [the control for the anti-ER antibody] or with anti-ER and control donkey IgG [the control for the anti-RelA antibody], hardly any fluorescence was detected [data not shown]. Cells incubated with PLA probes alone also failed to show any fluorescence [data not shown]. In Figure 5C a stack of 96 images is shown. These results suggest that RelA and Spi-B-ER are in close proximity with 40 nm maximum separation and are consistent with the data obtained in our IP experiments.

**DISCUSSION**

Here we extend our earlier findings that the Ets family member Spi-B is crucial for the development of human pDCs, by demonstrating that Spi-B has a key role during TLR-induced maturation of pDCs as well. Ectopic expression of Spi-B in *in vitro* generated pDCs showed significantly higher expression levels of markers associated with a mature pDC phenotype, including CD40, CD80, CD86, and CCR7, as compared to control transduced cells. Consistent with these findings we show that expression of Spi-B shRNAs in the pDC model cell line CAL-1 impaired TLR-induced maturation, as shown by reduced expression of these markers. As it is generally accepted that the NF-κB signaling pathway downstream of TLRs is activated upon engagement by ligand we hypothesized that Spi-B interferes with this pathway. We enforced this notion by demonstrating that known NF-κB regulated target genes, including BCL2A1, CD40, TNF-α and IFN-β, were synergistically upregulated by co-expression of ectopic Spi-B. Conversely, we also observed that transcription of a different set of genes, including IL-6, CCR7 and CALCRL, were inhibited when both NF-κB and Spi-B were co-expressed. This, together with our data showing direct interaction between the NF-κB subunit RelA/p65 and Spi-B, provides further evidence that Spi-B has potential to act either as a co-activator or a repressor of NF-κB controlled gene expression.

We show here for the first time that Spi-B has a regulatory role during TLR-induced maturation of pDCs besides its key role in orchestrating pDC lineage commitment in HPCs. Numerous studies have indicated that the NF-κB pathway activated downstream of TLRs can be regulated by different mechanisms, including protein-protein interaction, conformational changes, phosphorylation, miRNAs, ubiquitylation and proteasome-mediated degradation. Our results show that in CAL-1 cells Spi-B and RelA physically interact. These data are in accordance with previous studies describing physical interaction between the RHD of NF-κB subunits and proteins containing an Ets domain, which is present in Spi-B. Due to limited availability of *ex vivo* human pDCs, however, we were unable to confirm these observations in human primary pDCs. Nevertheless, the data obtained in the CAL-1 cells strongly suggest that interplay between Spi-B and the NF-κB pathway
impacts on pDC activation, maturation and cell survival. In line with this, it is notable that Spi-B and NF-κB have been reported to share several common target genes, including PRDM1 coding for Blimp-1,13,24 CD40,13,17 the Bruton’s tyrosine kinase [BTK],25,26 and BCL2A1.4,19,20 Biochemical studies have revealed that for binding of RHD and Ets domains DNA is required.21 Consensus binding sites for both NF-κB and Spi-B have been identified in the BCL2-A1 promoter region.9,27

Previously, we and others have convincingly demonstrated that Spi-B can act as transactivator but also as repressor of gene expression.4,13,25 In line with this, the results collected in this study show that Spi-B can transactivate or repress NF-κB regulated genes. It remains to be resolved how Spi-B exerts such diametrically opposite functions. In human B cells we showed that inhibition of BLIMP1 and XBP-1 gene expression was dependent on the Ets domain of Spi-B,13 indicating that DNA binding of Spi-B is required for its repressor capability. In contrast, the transactivation domain of Spi-B could be omitted for BLIMP1 driven plasma cell differentiation, but not for proper expression of CD40. These collective findings strongly suggest that other proteins are recruited to determine Spi-B activity. A key determinant of gene activation versus repression by DNA-binding factors is co-activator versus co-repressor recruitment. Based on the known homology of Spi-B with PU.1 it is reasonable to speculate that similar mechanisms are exploited. PU.1 has been shown to interact with either CREB binding protein (CBP) or histone deacetylases (HDACs), which mediate synergistic or antagonistic interactions between other transcription factors, respectively.28-30 Spi-B has been reported to bind to CBP, and when co-transfected increased reporter gene activity.7 This enforces the idea that CBP may increase Spi-B transactivation activity in pDCs, although this requires confirmation.

We show here that the pDC model cell line CAL-1 depends on NF-κB for its survival as inhibition of the NF-κB pathway using the selective and irreversible IKK inhibitor Bay 11–7082 rapidly and dose-dependently induced apoptosis. Notably, ectopic expression of Spi-B could to a certain extent prevent apoptosis of CAL-1, and this coincided with increased expression levels of BCL2A1. In agreement with this, we showed earlier that BCL2A1 is a direct target of Spi-B in pDCs.4 It is noteworthy that Staudt and colleagues showed the importance of NF-κB as a critical cancer-associated survival factor in the activated B-cell-like (ABC) form of diffuse large B-cell lymphoma (DLBCL).31 Comparable to our results obtained using the CAL-1 cell line, inhibition of NF-κB in the ABC-DLBCL cell line OCI-Ly3 lead to the induction of cell death. This was attributed to down-regulated expression of Bcl-xL, Bcl-2, XIAP, and Survivin.31 Interestingly, the same group showed using a shRNA virus library screen that reduced Spi-B expression in OCI-Ly3, but not in a germinal center (GC)-DLBCL cell line, induced cell death.32 Taking into account our results collected here, we propose that it will be of significant interest to examine whether Spi-B regulates BCL2A1 expression in ABC-DLBCL as well. Once confirmed,
BCL2A1 may be considered as an attractive candidate for the development of small molecule inhibitors to sensitize ABC-DLBCL, but also pDC-derived leukemic cells, for apoptosis and thus improve the efficiency of anti-cancer therapy. Taken together, these findings provide new insights in molecular mechanisms controlling the survival, activation and maturation of pDCs mediated by Spi-B and NF-κB interactions that either cooperatively or antagonistically regulate gene expression.
REFERENCES


