B cell development is critically dependent on NFATc1 activity

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Recommended Citation
Giampaolo, S., Wójcik, G., Klein-Hessling, S., Serfling, E., & Patra, A. (2018) 'B cell development is critically dependent on NFATc1 activity', Cellular and Molecular Immunology, . Available at: https://doi.org/10.1038/s41423-018-0052-9
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B cell development in bone marrow is a precisely regulated complex process. Through successive stages of differentiation, which are regulated by a multitude of signaling pathways and an array of lineage-specific transcription factors, the common lymphoid progenitors ultimately give rise to mature B cells. Similar to early thymocyte development in the thymus, early B cell development in bone marrow is critically dependent on IL-7 signaling. During this IL-7-dependent stage of differentiation, several transcription factors, such as E2A, EBF1, and Pax5, among others, play indispensable roles in B lineage specification and maintenance. Although recent studies have implicated several other transcription factors in B cell development, the role of NFATc1 in early B cell developmental stages is not known. Here, using multiple gene-manipulated mouse models and applying various experimental methods, we show that NFATc1 activity is vital for early B cell differentiation. Lack of NFATc1 activity in pro-B cells suppresses EBF1 expression, impairs immunoglobulin gene rearrangement, and thereby preBCR formation, resulting in defective B cell development. Overall, deficiency in NFATc1 activity arrested the pro-B cell transition to the pre-B cell stage, leading to severe B cell lymphopenia. Our findings suggest that, along with other transcription factors, NFATc1 is a critical component of the signaling mechanism that facilitates early B cell differentiation.

Keywords: Differentiation; EBF1; NFATc1; Pro-B; Pre-B

INTRODUCTION

B cell development in bone marrow (BM) follows a series of differentiation stages through which hematopoietic stem cell (HSC)-derived common lymphoid progenitor (CLP) cells give rise to mature B lymphocytes. Similar to early T cell development in the thymus, early B cell development in BM is also critically dependent on interleukin-7 (IL-7) signaling.1,3 Upon IL-7 signaling, CLP-derived B220<sup>+</sup> CD43<sup>+</sup> pro-B cells differentiate into B220<sup>+</sup> CD43<sup>-</sup> pre-B cells, which subsequently differentiate into B220<sup>+</sup> IgM<sup>+</sup> IgD<sup>-</sup> immature B cells to finally give rise to B220<sup>+</sup> IgM<sup>+</sup> IgD<sup>-</sup> mature B cells. Pro-B cells can also be characterized as B220<sup>+</sup> IL-7R<sup>+</sup> because they respond to IL-7 signals or B220<sup>+</sup> CD19<sup>+</sup> IgM<sup>-</sup> c-Kit<sup>+</sup> because they express stem cell factor receptor (c-Kit). Similarly, pre-B cells are otherwise characterized as B220<sup>+</sup> IL-7R<sup>+</sup> because these cells are independent of IL-7 signaling or B220<sup>+</sup> CD19<sup>+</sup> IgM<sup>-</sup> CD25<sup>-</sup> because they express the IL-2 receptor alpha chain (CD25). During the pro- to pre-B cell transition, developing B cells rearrange their immunoglobulin (Ig) heavy and light chains, which are essential for the formation of the pre-B cell receptor (pBCR). PreBCR signaling imparts independence from IL-7 signaling and is critical for further differentiation to later developmental stages.5,7

Apart from IL-7 signaling, a host of lineage-specific transcription factors (TF) have been implicated in the regulation of early B cell differentiation in BM. Among these TFs are the ets-family TF PU.1, Ikaros, zinc finger TF Bcl11a, helix-loop-helix TF E2A, EBF1 (early B cell factor 1), and paired box TF Pax5.8,9 Both E2A and EBF1 have been reported to regulate B lineage specification and commitment, and Pax5 activity is vital for B lineage maintenance.10-15 Genetic ablation of these TFs has revealed serious abnormalities in B cell development, leading to severe B cell lymphopenia.9

EBF1 activity has been suggested to influence several aspects of early B cell differentiation.16,17 EBF1 activity has been shown to regulate Pax5, Pou2af1 (BOB-1/EBF1), and Foxo1 activity during B cell development.15,18-21 The master regulatory function of EBF1 in B lineage specification and commitment was evident from the analysis of Ebf1<sup>−/−</sup> mice, in which development was blocked at the pro-B cell stage.12 EBF1 in association with E2A, Runx1, and other TFs regulates a number of B lineage-specifying genes that are involved in early B cell survival, proliferation, and Ig gene locus accessibility and rearrangement.16,22,23 Although the functional aspects of EBF1 in B cell development have been extensively studied, the mechanism that induces Ebf1 expression is less well understood. IL-7 signaling has been shown to regulate Ebf1 expression in pro-B cells.24 Additionally, E2A and FOXP1 appear to regulate Ebf1 expression.25-27 However, a clear picture of the regulation of Ebf1 during early B cell development has yet to emerge.

Nuclear factor of activated T cell (NFAT) family TFs play important roles in the development and function of many hematopoietic cell lineages.28-30 Among five family members (NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5), NFATc1, NFATc2, and NFATc3 are predominantly active in lymphocytes, where they regulate processes as diverse as cell survival, proliferation, cytokine gene expression, various T helper cell differentiation, apoptosis, and immune effector function.31,32 In T or B cells, cell
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surface T cell receptor (TCR) or BCR ligation induces increased calcium (Ca\(^{2+}\)) flux in the cytoplasm, which requires phospholipase C\(\gamma\)2 (PLC\(\gamma\)2) activity. Increased Ca\(^{2+}\) subsequently activates the serine/threonine phosphatase calcineurin, which by dephosphorylating multiple serine residues in cytoplasmic NFAT proteins facilitates their activation and nuclear translocation. We have recently described an alternative mechanism of NFAT activation in pTCR-negative thymocytes, wherein IL-7-JAK3 activity activates NFATc1 in a tyrosine phosphorylation-dependent manner.\(^{33}\) NFAT activity in pTCR-negative thymocytes is not only critical for their survival and differentiation into T cells but is also indispensable in preventing the pathogenesis of T acute lymphoblastic leukemia.\(^{34}\)

Furthermore, we and others have previously reported the involvement of NFAT proteins in B cell development and functions.\(^{35-38}\) Although NFAT proteins regulate multiple events in B cell function, their role in early stages of B cell differentiation in BM has not yet been investigated. Here we show that NFAT is active in pBCR-negative B cells and that NFATc1 plays an indispensable and non-redundant role very early during B cell differentiation. The serious defects in early B cell differentiation in mice lacking NFATc1 activity in the hematopoietic system led to severe B cell lymphopenia, which further underscores the critical necessity of NFATc1 in facilitating B cell development.

MATERIALS AND METHODS

Mice

C57BL/6 wild-type (WT), Rag1\(^{-/-}\), Nfatc2\(^{-/-}\), Nfatc3\(^{-/-}\), Nfatc1-EGFP-Bac, tg Vav-CreNfatc1\(^{fl/+}\), Vav-CreNfatc2\(^{fl/+}\), II\(\gamma\)2\(\delta\), or Vav-CreNfatc3\(^{fl/0}\), Vav-CreNfatc1A\(\alpha/\)in, and Vav-CreNfatc1A\(\alpha/\)in mice, all in the C57BL/6 background and of 3–8 weeks age unless mentioned otherwise, were used in the study. All animals were housed in the University of Würzburg central animal facility (ZEMM) following standard animal care procedures. All animals had ad libitum access to food and water. In each experiment, age-matched mice were used without any gender bias, and animals were handled humanely throughout the study.

Flow cytometry and cell sorting

All antibodies (Abs) used in flow cytometry and for isolation of B220 CD43\(^+\) or B220 IL-7R\(\alpha\) pro-B and B220 CD43\(^-\) or B220 IL-7R\(\alpha\) pre-B cells from BM were purchased either from BD Pharmingen or eBioscience. Anti-B220 (RA3 6B2), anti-NFATc1 (S7), anti-NFATc2 (PC61), anti-NFATc3 (B12-1), anti-CD3 (145-2C11), anti-CD24 (M1/69), anti-CD127 (B12-1), and isotype-matched control Abs (S7), anti-CD25 (PC61), anti-CD127 (B12-1), anti-CD3 (145-2C11), anti-CD24 (M1/69), and isotype-matched control Abs were used throughout the study. Biotinylated Abs were revealed with secondary streptavidin-conjugated allophycocyanin or through secondary streptavidin-conjugated allophycocyanin or phycoerythrin-Cy5 (PE-Cy5) Abs. Flow cytometry and data analysis were performed following standard procedures using the FACSCalibur and FlowJo software.

For BM pro- and pre-B cell sorting, a single-cell suspension of BM cells from both hind limbs was prepared. WT BM cells were either stained with B220 and CD43 or with B220 and IL-7R Abs to sort B220 CD43\(^+\) or B220 IL-7R\(\alpha\) pro-B and B220 CD43\(^-\) or B220 IL-7R\(\alpha\) pre-B cells. Similarly, B220 CD43\(^+\) pre-B and B220 CD43\(^-\) pre-B cells were sorted from Rag1\(^{-/-}\) mice BM. Cell sorting was performed using a FACSaria (BD Biosciences) flow cytometer and FACS DIVA software.

Immunofluorescence staining and immunoblotting

Sorted BM B220 CD43\(^+\) pro-B and B220 CD43\(^-\) pre-B cells from WT or Rag1\(^{-/-}\) mice or B220 IL-7R\(\alpha\) pro-B and B220 IL-7R\(\alpha\) pre-B cells from WT mice were immunostained with NFATc1, NFATc2 (both from ImmunoGlobe), or NFATc3 (Santa Cruz) Abs following a previously published protocol.\(^{39}\) Counterstaining with 4,6-diamidino-2-phenylindole was performed to confirm nuclear staining. Image acquisition and data analysis were performed using a TCS SP2 Leica confocal microscope and software.

Whole-cell protein extracts from the WT pro- and pre-B cells were prepared as mentioned previously.\(^{40}\) Fifty micrograms of total protein was analyzed in an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting for detection of calcineurin (Cell Signaling Technology) and PLC\(\gamma\)2 (Santa Cruz) levels. Actin was used as a loading control.

In vitro kinase assays

Rag1\(^{-/-}\) pro-B cells (10\(^7\)) were lysed in modified RIP buffer, and the cells lysates were incubated with 5 µg JAK3 Ab (Santa Cruz Biotech) overnight (on) at 4 °C to immunoprecipitate JAK3. Subsequently, 20 µl protein A agarose beads were added to the cell lysate and incubated for 1 h at 4 °C to capture the immunoprecipitated JAK3. The beads were washed once in 1× tyrosine kinase buffer (60 mM HEPES, 5 mM MgCl\(_2\), 5 mM MnCl\(_2\), 3 mM Na\(_2\)VO\(_4\), and 2.5 µM DTT), and kinase reactions were set up by adding 10 µg of glutathione S-transferase (GST)-NFATc1 or GST-S/TATS (signal transducer and activator of transcription factor 5) protein with 20 µM ATP and 10 nM γATP in radioactive assays at 22 °C for 30 min. For the non-radioactive assays, kinase reactions were performed without addition of γATP to the reaction mixture. Samples were analyzed by 6–8% SDS-PAGE followed by autoradiography for γ-S incorporation or immunoblotted to detect phospho-tyrosine.

V-D-J recombination

For analysis of rearranged Ig heavy and light chain genes, CDNA was synthesized from sorted B220 CD43\(^+\) pro-B cells from WT and Vav-CreNfatc1\(^{fl/+}\) mice using the Miltenyi Biotech cDNA Synthesis Kit and protocol. Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed to measure the expression of various rearranged heavy and light chain genes as well as germ line transcripts, using previously described primer pairs.\(^{41}\)

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (NEs) prepared from freshly isolated BM B220 \(^{+}\) B cells from Rag1\(^{-/-}\) mice were analyzed in EMSA for DNA binding of NFATc1 at the Ebf1 promoter. The following oligonucleotides were used as EMSA probes: Ebf1 I: 5′-ATATCACCGACGGAACGAAAGAAACACATCTTTTTGTT-3′, Ebf1 II: 5′-TGAGAGAGGAGGAGGAAGAAAAGAGGAGGAA-3′, and Ebf1 III: 5′-CTTGGCTGGTTGGAAGATGACATCTTGAAGTT-3′, representing conserved NFAT sites I, II, and III at the Ebf1 promoter. An oligonucleotide containing the distal NFAT site at the murine il2 promoter (122 bp) was used as a positive control: (−292) 5′-CCCAAGGAGAGAAAATTTGTTTTCACAGAAGCCG-3′ (−261). Five micrograms of nuclear proteins was incubated with 1 µg poly-dIdC and 1 µl γ-32P-labeled il2pu-bd or Ebf1 probes for 20 min on ice. For competition, a 100-fold molar excess of unlabeled cold probe was used, and in the supershift assays, 3 µg NFATc1, NFATc2, or NFATc3 Abs were used to evaluate the specificity of the NFAT–DNA complexes. DNA-bound NFAT complexes were resolved in 5% non-denaturing gels using 0.5× TBE buffer at 140 V for 2 h. Subsequently, the gel was transferred to Whatman paper and vacuum-dried for 2 h. Dried gels were exposed to photographic film on/n at −20 °C, and NFAT–DNA complexes were visualized in the autoradiogram. Free probe: labeled oligos without NE, in each probe set, lane 1: NFAT–DNA complexes, lane 2: competition with cold oligos, lane 3: supershift with NFATc1 Abs, lane 4: supershift with NFATc2 Abs, and lane 5: supershift with NFATc3 Abs.

Chromatin immunoprecipitation (ChIP)

For each ChIP protocol, 5–8×10\(^6\) WT pro-B cells were used following the Miltenyi Biotech ChIP protocol. Eight micrograms of NFATc1
Luciferase reporter assays

Murine Ebf1a or Ebf1b luciferase reporter constructs (100 ng) were co-transfected independently along with 1000 ng control vectors, expression vectors for a constitutively active STAT5a (STAT5a*) or NFATc1 (Nc1) alone, or with both STAT5a and NFATc1 together into 293 HEK cells using TurboFect Transfection Reagent (Thermo Scientific™, # R0531). At 24 h post-transfection, the cells were left unstimulated or stimulated with 12-O-tetradecanoylphorbol-13-acetate plus ionomycin (100 ng/ml each, Calbiochem) in the absence or presence of cyclosporin A (CsA, 100 ng/ml) for 16 h. Subsequently, luciferase activity depicting Ebf1a and Ebf1b promoter transactivation was measured using a MicroLumat LB 96P (EG&G Berthold) luminometer.

Photographs

Photographs of the thymus, spleen, and lymph nodes (LNs) from WT, Vav-Cre Nfatc1fl/fl+, and Vav-Cre Nfatc1P2fl/fl mice were obtained using a Nikon Coolpix 4500 digital camera. Image processing was performed using the Adobe Photoshop software.
Semiquantitative RT-PCR
Sorted B220⁺ CD43⁺ pro- and B220⁺ CD43⁻ pre-B cells from WT mice, pro-B cells from WT, Vav-CreNfatc1Δ/- and Vav-CreNfatc3Δ/- mice, sorted WT double-negative 3 (DN3) thymocytes, and Vav-CreNfatc1P2fl/+ mouse thymocytes and pro-B cells were used to synthesize cDNA using the Miltenyi Biotec cDNA Synthesis Kit and protocol. Semiquantitative RT-PCR was performed to estimate the expression levels of the indicated genes. Primer sequences are available in the supplementary information online.

Statistics
Data are presented as the mean ± s.d. Statistical significance was assessed using Student’s t test for comparison between two groups and analysis of variance for differences among groups.

RESULTS
NFAT activation in pro-B cells
To investigate whether TFs of the NFAT family play any role in B cell development in BM, we analyzed WT pro- and pre-B cells for the presence of NFAT proteins. Immunofluorescence analysis of WT B220⁺ CD43⁺ IgM⁺ pro-B cells revealed many fold higher nuclear NFAT levels compared with B220⁺ CD43⁺ IgM⁺ pre-B cells (Fig. 1a, b). Confirming this finding, analysis of pro-B (B220⁺ IL-7R⁺) and pre-B (B220⁻ IL-7R⁻) cells isolated based on IL-7R expression revealed a similar NFAT distribution pattern (Supplementary Figure S1a and b). Irrespective of the markers used to identify pro- and pre-B cells, NFAT activation in pBCR-negative pro-B cells was higher for NFATc1, NFATc2, and NFATc3 compared with that in pBCR-positive pre-B cells (Fig. 1a, b and Supplementary Figure S1a and b). This observation was further consolidated by analyses of Rag1⁻⁻ pro-B cells. Similar to WT pro-B cells, Rag1⁻⁻ pro-B cells also exhibited high levels of nuclear NFATc1 (Fig. 1c, d), suggesting that NFAT proteins most likely play a role at the preBCR-negative stages of B cell development in BM.

Owing to the high levels of NFATc1, NFATc2, and NFATc3 in pro-B cells, we next investigated which of the three NFAT family members is critical for early B cell development. Analysis of the B cell population in the BM of Nfatc2Δ/- or Nfatc3Δ/- mice revealed comparable proportions of B220⁺ cells as in WT mice (Fig. 1e). Even the combined loss of both NFATc2 and NFATc3 (Nfatc2Δ/Δ-Nfatc3Δ/Δ) did not alter B220⁺ population in BM (Fig. 1e), suggesting that NFATc2 and NFATc3 activity was most likely dispensable and that NFATc1 was the key NFAT protein regulating early B cell differentiation. This observation was supported by an earlier report that did not reveal any abnormality in early B cell development in Nfatc2Δ/Δ-Nfatc3Δ/Δ, or the double-mutant mice, although mature splenic B cell function was affected. NFATc1 expression in early B cell developmental stages was further confirmed, as B220⁺ CD19⁺ IgM⁺ c-Kit⁺ pro-B cells from Nfatc1-eGfp-Bac transgenic (tg) reporter mice showed high GFP levels compared with those from WT mice (Fig. 1f and Supplementary Figure S1c). In fact, varying levels of GFP representing NFATc1 expression in pro-B, B220⁺ CD19⁺ IgM⁺ CD25⁺ pre-B, B220⁺ CD19⁺ IgM⁺ IgD⁻ immature B, and B220⁺ CD19⁺ IgM⁺ IgD⁺ mature B cells in BM (Fig. 1f and Supplementary Figure S1c) and in splenic B cells (Supplementary Figure S1d) suggest that NFATc1 activity plays an essential role in B cell development and function.

To gain insight into how NFAT is activated in pro-B cells, we analyzed the expression of components of the classical NFAT activation pathway, i.e., the PLCγ2–calcium–calcineurin pathway. RT-PCR as well as immuno blot analysis showed that, compared with pre-B cells, pro-B cells expressed very low levels of Plcg2 and calcineurin (Pp2b) (Fig. 1g, h). Considering the high levels of NFAT (Fig. 1a–d and Supplementary Figure S1a and b) and insignificant expression of Plcg2 and calcineurin, it was apparent that NFAT activation was calcineurin independent in pro-B cells. This finding was supported by observations from previous reports showing normal early B cell development in the BM of calcineurin-deficient mice. To understand the mechanism of NFAT activation, we investigated whether IL-7–JAK3 signaling also activates NFATc1 in pro-B cells as we have observed in thymocytes. Pro-B cells depend on IL-7 signaling for survival and differentiation. Accordingly, we detected high levels of IL-7R expression on pro-B compared with pre-B cells (Fig. 1i). To characterize the involvement of JAK3, we performed kinase assays with JAK3 immunoprecipitated from pro-B cells. In kinase assays, JAK3 phosphorylated NFATc1 as well as STAT5, indicating that, similar to observations in thymocytes, JAK3 plays the role of NFAT kinase, resulting in NFAT activation and nuclear translocation in pro-B cells (Fig. 1j). JAK3-mediated tyrosine phosphorylation of NFATc1 was confirmed by immunoblotting the kinase assay products with pTyr Abs (Fig. 1k). These observations suggest that NFAT activation in pro-B cells is dependent on IL-7–JAK3 signaling.

Impaired B cell development in the absence of NFATc1 NFATc1-deficient mice are embryonic lethal. To circumvent this problem and investigate the influence of NFATc1 on early B cell developmental stages, we analyzed Vav-CreNfatc1Δ/- mice, in which NFATc1 activity was ablated in the hematopoietic system. Analysis of Vav-CreNfatc1Δ/- mice showed a consistent and strong reduction in the size of primary lymphoid organs, such as the thymus, LNs, and spleen (Fig. 2a). A reduction of the thymus size in Vav-CreNfatc1Δ/- mice due to developmental defects in thymocyte differentiation has been reported. The small LNs and spleen in Vav-CreNfatc1Δ/- mice suggest possible defects in B cell development, as B cells constitute a major cell population in these organs. Accordingly, spleen cellularity was drastically reduced in Vav-CreNfatc1Δ/- mice compared with their littermate controls (Fig. 2b). Analysis of T and B cell distributions in the spleen revealed a strongly reduced B220⁺ B cell population in Vav-CreNfatc1Δ/- mice (Fig. 2c, d). Further assessment of IgM and IgD-expressing B cells in the spleen confirmed that the loss of NFATc1 activity exerted a strong negative influence on B cell development (Fig. 2e, f).

In the absence of NFATc1 activity, very few CD21⁺ CD23⁺ follicular and CD21⁻ CD23⁻ marginal zone B cells, as well as B220⁺ CD5⁺ B-1 B cells, developed in Vav-CreNfatc1Δ/- mice (Fig. 2g–n). Additionally, IgM⁺ B cells in the peritoneum were markedly reduced in Vav-CreNfatc1Δ/- mice compared with WT control mice (Fig. 2k, l). This strong phenotype in the absence of NFATc1 activity further confirmed that NFATc1 is the key NFAT protein essential for B cell development, and NFATc2 or NFATc3 cannot compensate for its loss. The reduced B220⁺ CD5⁺ population in Vav-CreNfatc1Δ/- mice is in agreement with previous reports concerning the requirement for NFATc1 for the development of these cells. Taken together, these abnormalities in B cell populations in the peripheral lymphoid organs of Vav-CreNfatc1Δ/- mice indicate a defect in B lymphopoiesis in the BM.

B cell development in BM is critically dependent on NFATc1 activity
To elucidate the precise stage at which B cell development is affected owing to the absence of NFATc1 activity, we analyzed B cell differentiation in the BM of Vav-CreNfatc1Δ/- mice. BM cellularity as well as BM B220⁺ B cell numbers were dramatically reduced in Vav-CreNfatc1Δ/- mice, although an increase in the percentage of distributed B220⁺ cells was observed (Fig. 3a, b). Further analysis revealed a strong reduction in both the percentage of distribution and in the absolute numbers of the BM B220⁺ CD43⁺ pro-B cells (Fig. 3c, d), as well as an increase in the percentage but a marked reduction in the absolute numbers of B220⁺ CD43⁻ pre-B cells (Fig. 3c, e) in Vav-CreNfatc1Δ/- compared with WT mice. The B220⁺ CD43⁺ population can be further subdivided into three subsets based on the surface expression of heat-stable antigen (CD24) and B1 molecules, i.e.,...
B220^+^CD43^-^CD24^-^BP1^- pre-pro-B cells, B220^+^CD43^-^CD24^-^BP1^- pro-B cells, and B220^+^CD43^-^CD24^-^BP1^- large pre-B cells. Our analysis revealed that, in Vav-Cre\(Nfatc1^{fl/fl}\) mice, B cell development was arrested at the B220^+^CD43^-^CD24^-^BP1^- pre-pro-B cell stage (Fig. 3f, g). Very few cells differentiated to the later stages, resulting in a dramatically reduced B cell population in the BM and peripheral lymphoid organs (Figs. 3b and 2d). Similar to the B220^+^CD43^- population, the B220^+^CD43^- population can be divided into three subsets based on the expression of IgM and IgD molecules: B220^+^CD43^-IgM^-IgD^- small pre-B,
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Fig. 2 Impaired B cell development in Vav-CreNfatc1fl/fl mice. a Photographs of the thymus, spleen, and LNs from Vav-CreNfatc1fl/fl mice compared with littermate Vav-CreNfatc1fl/+ and WT mice. b Total splenocyte numbers in WT (n = 8), Vav-CreNfatc1fl/fl (n = 9), and Vav-CreNfatc1+/− (n = 9) mice. c Flow cytometry showing the distribution of CD3+ T and B220+ B cells in the spleen from Vav-CreNfatc1fl/fl mice compared with WT controls. d Absolute numbers of B220+ B cells in the spleen from Vav-CreNfatc1fl/fl and WT mice. e, f Distribution of IgM+ and IgD+ B cells in the spleen (e) and quantification of the percentage of cells (f) from WT and Vav-CreNfatc1fl/fl mice. g, h Follicular and marginal zone B cell distribution in the spleen (g) and quantification of the percentage of cells (h) from Vav-CreNfatc1fl/fl mice compared with littermate control mice as revealed by CD21 and CD23 staining. i, j Distribution of splenocytes according to CDS and IgM expression (i) and quantification of the percentage of cells (j) in WT and Vav-CreNfatc1fl/fl mice. k, l Distribution of CD5+ and IgM+ cells (k) and quantification of the percentage of cells (l) in the peritoneal of Vav-CreNfatc1fl/fl mice compared with WT controls. m, n Distribution of follicular and marginal zone B cells (m) and quantification of the percentage of the respective population. Data represent one of the three independent experiments (n = 3 per group) and are shown as the mean ± s.d., one-way ANOVA (b, d), and unpaired t-test (f, h, j, l, n).

B220+ CD43+ IgM+IgD− immature B, and B220+ CD43+ IgM+IgD+ mature B cells.49 In Vav-CreNfatc1fl/fl mice compared with their littermate control mice, there were very few B220+ CD43+ IgM−IgD− immature and B220+ CD43+ IgM+IgD+ mature B cells, confirming a developmental arrest at the pro-B cell stage due to the absence of NFATc1 activity (Fig. 3, h).

We have previously shown that the abolition of NFATc1 activity in B cells (Mbl1-CreNfatc1fl/fl) leads to defects in mature B cell function, but it does not influence the early B cell differentiation stages in BM.32 Correlating our observations with that from Mbl1-CreNfatc1fl/fl mice, it is apparent that NFATc1 activity at a stage where the immature B cells still have not acquired the pBCR is critical for B cell development. To investigate the basis behind the differentiation block, we assumed that most likely lack of NFATc1 activity is impairing the IL-7-responsive stages of B cell development in Vav-CreNfatc1fl/fl mice. Furthermore, analysis of II7r−/− and II7r+/− mice revealed a similar developmental arrest at the early B cell differentiation stage, as observed in Vav-CreNfatc1fl/fl mice (Fig. 3i and Supplementary Figure S2a). These observations suggest that NFATc1 activation is indispensable for B cell development in BM.

Loss of NFATc1 dysregulates Ebf1 and the expression of other B lineage-specific genes. To gain insight into the defects in pro-B cell development in the absence of NFATc1 activity, we analyzed the expression of various molecules implicated to regulate B cell development. RT-PCR analysis revealed NFATc1 was specifically and efficiently deleted in pro-B cells of Vav-CreNfatc1fl/fl mice (Fig. 4a). However, normal expression of NFATc2 and reduced expression of NFATc3 were not sufficient to restore the B cell phenotype in Vav-CreNfatc1fl/fl mice, ruling out any compensation by these NFAT family members (Fig. 4a). Interestingly, analysis of molecules that are critically involved in B lineage commitment and specification did not reveal any alterations in the expression of Spi1 (PU.1), Tcf3 (E47), Tcf12 (HEB), Oct1, Oct2, Id1, Id2, and Id3, ruling out any involvement of NFATc1 in the regulation of these genes (Fig. 4a). In fact, all these genes were slightly upregulated in Vav-CreNfatc1fl/fl pro-B cells. Several NF-kB family members have been shown to regulate B cell development, as evidenced in studies examining gene-deficient mice.51 However, we observed an elevated level of Rela (P65), Relb, and Nkb2 expression in Vav-CreNfatc1fl/fl compared with WT mouse pro-B cells (Fig. 4a). Interestingly, we observed a strong downregulation of Ebf1 expression as well as reduced expression of Foxo1 and Pax5 in Vav-CreNfatc1fl/fl mice compared with WT mouse pro-B cells (Fig. 4a). Reduced Pax5 expression could be due to a downregulation of Ebf1 activity, as it has been reported that Pax5 expression is regulated by Ebf1.16 Thus EBF1 seems to be the prime target of NFATc1 during pro-B cell development.

Based on our analysis, the B cell developmental defect in Vav-CreNfatc1fl/fl mice clearly occurs at a similar stage as that in IL-7- or IL-7R-deficient mice (Fig. 3) and Supplementary Figure S2a).34 To exclude the possibility that the absence of NFATc1 activity impairs IL-7-STAT5 signaling, we investigated the expression of all components of the IL-7 signaling pathway. Normal expression of II7r, Jak3, Stat5a, and Stat5b genes in Vav-CreNfatc1fl/fl compared with WT mouse pro-B cells (Fig. 4b) excluded the possibility that the absence of STAT5 activity was responsible for the arrested B cell development in Vav-CreNfatc1fl/fl mice. The B cell development stage in IL-7- or IL-7R-deficient mice has been attributed to the absence of EBF1 expression.44 The lack of EBF1 expression in Vav-CreNfatc1fl/fl mice (Fig. 4a) resembles to that in IL-7 and IL-7R-deficient mice and suggests that NFATc1 plays an essential role in IL-7 signaling.

In addition to the defect in Ebf1 expression, pro-B cells in Vav-CreNfatc1fl/fl displayed a strong downregulation of the Rag1, Rag2, and Tdt (Dntt) genes involved in the recombination and rearrangement of Ig genes. Furthermore, Vav-CreNfatc1fl/fl pro-B cells lacked expression of X5 (Iglf1), VpreB, Igα (Cd79a), and Igβ (Cd79b), all of which are components of the pBCR complex. These observations suggest that NFATc1 is a critical player in B cell development, and the absence of NFATc1 can have profound effect on B lymphopoiesis. We also observed similar defects in early-B cell differentiation-specific gene expression in IL-7 signaling-deficient pro-B cells, which was in agreement with previously published reports (Supplementary Figure S2b).

EBF1 expression in pro-B cells is regulated from two distinct promoters, leading to the expression of EBF1α and EBF1β isoforms.46 It has been reported that EBF1β is the major isof orm expressed in pro-B cells and regulates the expression of EBF1α. In investigations to determine which EBF isoform was affected by the absence of NFATc1, we observed that both EBF1α and EBF1β were strongly downregulated in Vav-CreNfatc1fl/fl mouse pro-B cells (Fig. 4c), suggesting that EBF1 is a target of NFATc1 in pro-B cells.

NFATc1 regulates EBF1 expression in pro-B cells. To investigate whether NFATc1 transcriptionally regulates EBF1 expression, we searched for the NFAT DNA-binding consensus sequence 5'-GGAAA-3' approximately 1 kb upstream of the Ebf1b transcriptional start site. We detected five NFAT consensus sequences in this upstream sequence, four of which were conserved in mouse and human (Fig. 5a). To verify NFATc1 binding to these conserved sites, we performed EMSAs with NE prepared from WT pro-B cells. As shown in Fig. 5b, NFATc1 binding was detected in sites I, II, and III, with the second and third conserved sites showing a higher affinity in comparison to site I. NFATc1 binding to site IV was weaker than to the other three conserved sites (data not shown). NFATc1 binding to these sites was abolished specifically by an Ab against NFATc1, whereas the complex remained unaltered in the presence of Abs against either NFATc2 or NFATc3 (Fig. 5b). Furthermore, strengthening our observations regarding NFATc1-mediated regulation of Ebf1 expression, ChIP assays revealed the in vivo NFATc1 binding at the Ebf1 promoter in WT pro-B cells (Fig. 5c). Supporting these observations, in reporter assays, we observed minimal transactivation of the Ebf1b promoter by NFATc1 (Fig. 5d). However,
NFATc1 in combination with STAT5 increased Ebf1b promoter activity by several fold, and this effect was reduced when NFATc1 activity was blocked by CsA treatment (Fig. 5d). Interestingly, NFATc1 exerted a similar influence on the Ebf1a promoter, suggesting that both Ebf1a and Ebf1b are transcriptionally regulated by NFATc1 (Fig. 5d), which supports our observations of strongly reduced expression of both isoforms in Vav-Cre Nfatc1fl/fl pro-B cells (Fig. 4c). These observations suggest that, downstream of IL-7 signals, NFATc1 and STAT5 synergistically regulate Ebf1 activity and critically influence early B cell differentiation in BM.

NFATc1 regulates Ig gene rearrangement in pro-B cells As B cell development in Vav-Cre Nfatc1fl/fl mice was arrested at the pro-B cell stage, we analyzed the rearrangement status of the IgH and IgL chain locus. RT-PCR analysis revealed that, compared with WT pro-B cells, transcripts for either κ- or λ-light chains as well as the proximal and distal VH segment containing the IgH chain were negligible in Vav-Cre Nfatc1fl/fl pro-B cells (Fig. 6). Correspondingly, germline transcripts for distal VH segments were absent in Vav-Cre Nfatc1fl/fl pro-B cells, indicating a defect in Ig heavy chain locus accessibility (Fig. 6) and raising the possibility of NFATc1 acting as a facilitator of Ig gene locus accessibility and...
rearrangement. This result further supports a role for NFATc1 downstream of IL-7 signaling in the regulation of B cell developmental events, as IL-7 signaling has been reported to regulate IgH locus accessibility.\(^{52,53}\)

A threshold level of NFATc1 activity is essential for B cell differentiation

NFATc1 is expressed from two distinct promoters. NFATc1\(^\alpha\) isoforms are directed from the distal P1 and NFATc1\(^\beta\) isoform expression is directed from the proximal P2 promoter.\(^{54}\) We have recently demonstrated distinct developmental stage-specific Nfatc1 promoter activity during thymocyte differentiation with NFATc1\(^\beta\) isoforms expressed in pTCR-negative cells and both NFATc1\(^\alpha\) and NFATc1\(^\beta\) activity in pTCR-positive thymocytes.\(^{34}\) To assess whether a similar pattern of NFATc1 promoter activity occurs during B cell development, we evaluated Nfatc1\(^\alpha\) and Nfatc1\(^\beta\) isoforms in pro-B cells in the absence of pBCR signaling, and pBCR-bearing B cells require both NFATc1\(^\beta\) and NFATc1\(^\alpha\) for further differentiation into mature B cells.

To further investigate whether exclusive NFATc1\(^\beta\) activity is really critical, and whether B cell differentiation is impaired in the absence of P2 promoter activity, we analyzed Vav-Cre*Nfatc1\(^{fl/fl}\) mice.\(^{34}\) However, preliminary analysis of the LN, spleen, and BM in these mice did not reveal any perturbations in size or cellularity, suggesting that B cell differentiation was normal in the absence of P2-derived NFATc1\(^\beta\) (Fig. 7b, c). Further characterization revealed a normal distribution of B220\(^+\)B cells in the BM and spleen of these mice (Fig. 7e and Supplementary Figure S3a). Additionally, the unaffected distribution of B220\(^-\)IgM\(^-\) and B220\(^-\)IgD\(^-\) B cells and of the follicular (CD21\(^+\)CD23\(^+\)) and marginal zone (CD21\(^+\)CD23\(^-\)) B cells in the spleen of Vav-CreP2\(^{fl/fl}\) mice revealed that B cell development was comparable to that in WT mice (Fig. 7e and Supplementary Figure S3b). These observations are in stark contrast to the arrested B cell development in Vav-Cre*Nfatc1\(^{fl/fl}\) mice, which led us to investigate whether NFATc1 activity was still present in pro-B cells in Vav-Cre*Nfatc1\(^{P2}\) mice. RT-PCR analysis revealed a complete loss of P2 promoter activity, but surprisingly, in the absence of Nfatc1\(^\beta\), robust expression of Nfatc1\(^\alpha\) directed from the P1 promoter was observed in Vav-Cre*Nfatc1\(^{P2}\) mice (Fig. 7f). Thus the normal B cell development...
in Vav-CreNfatc1P2fl/fl mice was clearly due to compensation for the lost NFATc1β activity by the NFATc1α isoforms. These observations further underline the importance of NFATc1 in B cell development.

To gain insight concerning the absence of NFATc1α expression at the pro-B cell stage, when it is present in pre-B cells along with NFATc1β (Fig. 7a) and is able to replace NFATc1β activity during early B cell development (Fig. 7f), we analyzed Vav-CreNfatc1αAfl/fl mice in which a constitutively active version of Nfatc1αA was knocked-in in the Rosa-26 locus flanked by a floxed stop cassette.34 Surprisingly, analysis of the spleen and BM revealed an NFATc1αA dose-dependent decrease in cellularity, suggesting a possible defect in B cell development in Vav-CreNfatc1αAfl/fl mice (Fig. 7g). Characterization of the B cell populations revealed a drastic reduction of B220+ cells in the BM and spleen (Fig. 7h and Supplementary Figure S4a). Furthermore, we observed a severe lack of IgM+ and IgD+ B cells in the BM and spleen, as well as follicular and marginal zone B cells in the spleen of Vav-CreNfatc1αAfl/fl mice compared with their littermate controls (Fig. 7j and Supplementary Figure S4b and c). Additionally, the development of B220+CD19+CD5+B cells in the spleen was also impaired in Vav-CreNfatc1αAfl/fl mice.

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**Fig. 5** NFATc1 binds the Ebf1 promoter and regulates its activity. a Conserved NFAT-binding consensus sequence (5′-GGAAA-3′) in the Ebf1b promoter region 1-kb upstream of the transcriptional start site in mice and humans. b Electrophoretic mobility shift assay (EMSA) with nuclear extracts from BM pro-B cells showing NFATc1 binding at the conserved NFAT consensus sites in the Ebf1 promoter region. Ebf1 I, Ebf1 II, and Ebf1 III indicate the oligos used in EMSA containing the first, second, and third NFAT-binding sites. In each set, lane 1 represents NFAT–DNA complexes; lane 2, competition with 100-fold excess corresponding cold oligos; lane 3, supershift of NFAT–DNA complexes with NFATc1 Abs; lane 4, supershift of NFAT–DNA complexes with NFATc2 Abs; and lane 5, supershift of NFAT–DNA complexes with NFATc3 Abs. In the case of il2Pu box-distal (il2Pu-bd), lanes 1–3 represent NFAT–DNA complexes, cold oligo competition, and supershift with NFATc1 Abs, respectively. c In vivo NFATc1 binding at the Ebf1 promoter as revealed in the ChIP assays. d Transactivation of Ebf1a and Ebf1b promoters in response to NFATc1 or STAT5 activity alone or both together as revealed by luciferase reporter assays. Data are representative of three independent experiments and are shown as the mean ± s.d., one-way ANOVA (d).

**Fig. 6** NFATc1 deficiency impairs V-D-J recombination in Vav-CreNfatc1P2fl/fl pro-B cells. RT-PCR analysis showing V-J and V-D-J recombination at the light and heavy chain locus, respectively, in pro-B cells from Vav-CreNfatc1P2fl/fl mice compared with littermate control mice. Data are representative of two independent experiments with cDNA prepared from sorted cells collected from pooled BM cells from multiple mice for each genotype in each experiment.
severely affected in Vav-CreNfatc1αAfl/fl mice (Fig. 7k and Supplementary Figure S4d). To locate the precise stage at which B cell development was arrested as a result of NFATc1αA co-expression along with NFATc1β, we analyzed the distribution of B220+CD19+IgM−CD25+ pre-B cells in the BM of Vav-CreNfatc1αAfl/fl mice. A drastically reduced pre-B cell population in the BM revealed that, similar to the Vav-CreNfatc1fl/fl mice, B cell development in Vav-CreNfatc1αAfl/fl mice was blocked at the pro-B cell stage (Fig. 7l and Supplementary Figure S4e). These observations suggest that NFATc1α activity in addition to NFATc1β activity is critically dependent on NFATc1...
NFATc1β activity at the pro-B cell stage is detrimental for B cell development, and a certain threshold of NFATc1 activity irrespective of the α or β isoforms is indispensable for normal B cell differentiation.

**DISCUSSION**

The role of NFAT proteins in early B cell developmental stages, during which they do not even have a pBCR, is not known. Our findings concerning NFAT protein expression in pro-B cells are quite intriguing (Fig. 1a–d and Supplementary Figure S1a and b). A previous report investigating the role of NFATC2 and NFATC3 in B cell development did not observe any abnormality in the BM, although the function of mature B cells was affected. This phenomenon could be due to a functional compensation by NFATc2 or NFATc3 (Figs. 2 and 3). The role in pBCR-negative stages of differentiation. Accordingly, signaling might be the cause of this phenotype. EB1F1 deficiency has been reported to be the main factor underlying the developmental arrest at the pro-B cell stage in Il7r−/− and Il7r−/− mice. Regulation of Eb1f1 expression by IL-7 signaling has also been reported. However, the molecular mechanism linking IL-7 and Eb1f1 expression is far from clear. Our observations regarding a strong defect in Eb1f1 expression in pro-B cells from Vav-CreNfatc1fl/fl mice (Fig. 4a) suggested that, similar to Il7r−/− and Il7r−/− mice, the loss of EBF1 activity could be the major factor responsible for the impaired B cell phenotype in these mice. Although we also observed downregulated Pax5 expression (Fig. 4a), it is not likely to be the reason for the observed phenotype because Eb1f1 has been shown to regulate Pax5 expression. Moreover, the strong downregulation in pBCR component expression (Cd79a, Cd79b, Vpreb, Iglf1) and absence of Rag1, Rag2, and Tdt expression in Vav-CreNfatc1fl/fl mice (Fig. 4a) resembled the phenotype of EBF1-deficient mice, in which B cell development is also arrested at the pro-B cell stage. Interestingly, we observed an increase in the expression of Oct1, Oct2, Rela, Relbf, Nfkb2, and Spi1 in Vav-CreNfatc1fl/fl compared with littermate control mice (Fig. 4a); however, increased expression of these genes failed to rescue the B cell defects in Vav-CreNfatc1fl/fl mice. Additionally, the higher levels of Tcf12, Tcf3, and Id protein (Id1, Id2, and Id3) expression in Vav-CreNfatc1fl/fl pro-B cells had no beneficial influence on B cell development (Fig. 4a). Overall, the gene expression defects in Vav-CreNfatc1fl/fl pro-B cells were similar to those observed in IL-7−/−/− and Pax5−/− mice (Supplementary Figure S2b). Most interestingly, despite the block at the pro-B stage and the elevated expression levels of Notch proteins (Fig. 4a), no increases in T lineage cells were observed in Vav-CreNfatc1fl/fl mice. Our analysis clearly demonstrates that the dysregulated Eb1f1 expression in Vav-CreNfatc1fl/fl pro-B cells is the result of a lack of NFATc1 activity (Fig. 5). The defect in V-DJ recombination in Vav-CreNfatc1fl/fl pro-B cells (Fig. 6) again was similar to that reported in Il7r−/−/−, Il7r−/−/−, and Eb1f1−/− mice. IL-7 signaling and Eb1f1 have been shown to regulate Ig gene rearrangement. Again, the similarity in this aspect suggests that IL-7, NFATc1, and EB1F1 all act in a linear axis (Supplementary Figure S5a) and that a deficiency in any one will lead to similar defects in B cell development.

In pro-B cells, Nfatc1 expression occurred exclusively from the P2 promoter, whereas in pre-B cells we could detect Nfatc1 expression from both the P1 and P2 promoters (Fig. 7a), marking a qualitative and quantitative difference in NFATc1 proteins in pro- and pre-B cells. This finding is again similar to our results obtained for pTcR-negative and pTcR-positive thymocytes during T cell development. In addition, this differential promoter-directed NFATc1 expression in pro- and pre-B cells reflects the threshold of NFATc1 activity required for their differentiation, as evidenced in our analysis of Vav-CreNfatc1P2fl/fl mice, in which the absence of P2, P1 promoter activity compensated for the loss of Nfatc1β and signaling.
facilitated normal B cell development (Fig. 7d, e and Supplementary Figure S3). Additionally and stressing the importance of a threshold level of NFATc1 activity during a stage-specific differentiation of B cells, increased NFATc1 levels via the co-expression of NFATc1α along with NFATc1β in pro-B cells led to severe B cell lymphopenia (Fig. 7g–l and Supplementary Figure S4).

In summary, our study underlines the essentiality of an optimal level of NFATc1 activity for B cell development and for preventing B cell lymphopenia (Supplementary Figure S5b). Our study also raises the possibility of manipulating NFATc1 activity to provide relief in clinical cases of B cell immunodeficiency.

ACKNOWLEDGEMENTS

The authors thank C. Linden and E. Schmitt (University of Würzburg) for excellent cell sorting and photographs of mouse organs, respectively, V. Ellenrieder (University of Goettingen) for the NFATc1fl/fl mice, and A. Rao (University of California at San Diego) and L. Glimcher (Harvard Medical School and Brigham and Women’s Hospital) for the Nfatc1−/− mice. This work was supported by a Deutsche José Carreras Leukämie Stiftung e.V. (DJCLS R 15/12) (to A.K.P.), a fellowship from the VanderFerd Foundation, UK (to A.K.P.), Deutsche Forschungsgemeinschaft (DFG) grant TGR2 (to E.S.) and by the Wilhelm Sander-Stiftung (to E.S. and K.S.H.).

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41423-018-0052-9) contains supplementary material.

Competing interests: The authors declare no competing interests.

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