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Transitional B Cells Exhibit a B Cell Receptor-Specific Nuclear Defect in Gene Transcription

Sarah F. Andrews* and David J. Rawlings2*†

The signaling programs that enforce negative selection in early transitional (T1) B cells in response to BCR engagement remain poorly defined. We conducted a comprehensive comparison of BCR signaling in T1 vs follicular mature splenic B cells. T1, in contrast to follicular mature B cells, failed to express key NF-κB target genes in response to BCR engagement and exhibited a striking defect in assembly of an active transcriptional complex at the promoter of the survival and proliferative genes A1 and c-Myc. Surprisingly, and contrary to previous models, classical protein kinase C and IkB kinase activation, NF-κB nuclear translocation and DNA binding were intact in T1 B cells. Furthermore, despite a marked reduction in NFAT1 expression, differential NFAT or AP-1 activation cannot explain this transcriptional defect. Our combined findings demonstrate that T1 B cells are programmed for signal- and stage-specific “nuclear nonresponsiveness” upon encounter with self-Ags. The Journal of Immunology, 2009, 182: 2868–2878.

B cell development begins within the bone marrow, starting from common lymphoid progenitor cells. Development proceeds through discrete stages that ultimately lead to the generation of immature B cells expressing a functional BCR on the cell surface. Immature B cells subsequently emigrate to the periphery where progression through transitional developmental stages within the spleen leads to the formation of naive mature B cells capable of activation and differentiation into Ab-secreting cells upon encounter with cognate Ag (1). During this developmental sequence, tolerance mechanisms progressively shape the BCR repertoire and are critical to ensuring a self-tolerant B cell pool (2). In the bone marrow, encounter with self-Ag can lead to one of three outcomes: deletion by apoptosis, induction of anergy or receptor editing, and reexpression of a new BCR. Deletion primarily occurs when receptor editing fails to produce a self-tolerant BCR (3). Although the precise events that govern the decision to undergo receptor editing vs anergy remain incompletely defined, high-avidity Ags appear to preferentially promote deletion or deletion, while weaker interactions more commonly induce anergy (2).

B cell tolerance mechanisms are less well characterized in the periphery. However, detailed analysis of BCR specificity in human peripheral B cells has demonstrated a ~50% reduction in self-reactivity in mature vs newly formed B cells, indicating that tolerance mechanisms are also active at this stage (4). In addition, deletion of self-reactive B cells occurs in vivo when self-Ag is specifically expressed in the periphery (5). In contrast to bone marrow B cells, however, transitional B cells in the periphery lose the capacity to receptor edit and instead undergo deletion upon encounter with high-avidity self-Ags in vivo (5, 6). Based on the abundance of autoimmune diseases associated with Abs that recognize multimeric, high-avidity self-Ags such as DNA and RNA, it is critical to better define the signaling events that regulate the generation of naive mature B cells capable of recognizing such Ags.

Deletion-mediated tolerance to self-Ag can be mimicked in vitro by stimulation of early transitional B cells with a BCR cross-linking Ab that recognizes surface IgM (sIgM).3 Mature B cells enter cell cycle and proliferate in response to stimulation with anti-IgM. In contrast, early transitional B cells rapidly die by apoptosis and fail to proliferate following an identical stimulus (7–10). Several lines of evidence indicate that this response in early transitional B cells is not secondary to a global defect in BCR signaling. Indeed, tonic BCR signals are required for cell survival even at the transitional stage (11) and, contrary to the response of anergic cells, transitional cells exhibit robust phosphotyrosine (pY) signals and calcium flux in response to BCR engagement (12–14). These data and other findings have supported the idea that developmentally programmed differences in BCR signal transduction function to specifically modulate peripheral tolerance.

Correlated with the strong apoptotic response and lack of proliferation, early transitional B cells fail to up-regulate key NF-κB target genes that express protein products required for B cell survival and division (7, 10). This observation, in conjunction with an apparent inability to produce inositol-1,4,5-trisphosphate (InsP3) in response to BCR engagement and other indirect evidence, has led to the hypothesis that early transitional B cells fail to activate proximal signals that trigger PKCβ-mediated NF-κB activation (15, 16). This model, however, has remained incompletely tested. In addition, it has remained unclear whether deficient NF-κB target gene expression is due primarily to abortive signaling caused by the strong apoptotic response or instead represents a unique difference in the downstream signaling capacity of the BCR.

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1 Support for this work has included funds from Cancer Research Institute Training Grant (to S.F.A.) and National Institutes of Health Grants HD37091 and CA81140.

2 Abbreviations used in this paper: sIgM, surface IgM; pY, phosphotyrosine; InP3, 1,4,5-trisphosphate; PKCβ, protein kinase Cβ; Tg, transgenic; KO, knockout; PLCγ2, phospholipase Cy2; IkB, IκB kinase; 7-AAD, 7-aminoactinomycin D; NP40, Nonidet P-40; f.p, forward primer; r.p, reverse primer; ChIP, chromatin immunoprecipitation; WT, wild type; FM, follicular mature; PIP3, phosphatidylinositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; cPKC, classical PKC; CsA, cyclosporin A; Pol II, polymerase II.

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In the current study, we show that even in near absence of the apoptotic response, early transitional B cells fail to transcribe genes necessary for survival and progression through the cell cycle. This BCR-driven transcriptional defect is not due to altered proximal NF-κB signaling, NF-κB nuclear translocation, or DNA binding. In addition, altered activation of other key transcription factors, including NFAT and AP-1, is unlikely to account for this deficit. However, despite intact proximal signals and transcription factor activation, T1 B cells fail to assemble an active transcriptional complex at the A1 and c-Myc gene promoters in response to BCR engagement. Taken together, these data support a model wherein developmentally controlled, nuclear events limit BCR-specific transcriptional activity and thereby control the fate of autoreactive transitional B cells.

**Materials and Methods**

**Mice**

BALB/c, MD4 Hel-Ig transgenic (Tg), and Bim knockout (KO) mice were obtained from The Jackson Laboratory. NFAT1 KO mice were provided by A. Rao (Harvard Medical School, Boston, MA). All animals were maintained in the specific pathogen-free animal facility at Seattle Children’s Research Institute (Seattle, WA) and handled according to Institutional Animal Care and Use Committee-approved protocols. Mice were used at 5–8 wk of age.

**Abs and reagents**

Abs specific for phospholipase C (PLC)/2, ERK, pERK1/2, JNK1, p65, c-Rel, IkB kinase (IKK)/1, HDAC1, RNA polymerase II (Pol II), NFAT1, and NFAT2 were purchased from Santa Cruz Biotechnology; AKT, p-AKT, p-JNK1/2, p-PKD, p38, p-p38, p-p65 S536, p-IKK, p-IκB, and p-Ser PKC substrates from Cell Signaling Technologies; pY from Millipore (Upstate Biotechnology); CD21, B220, and anti-active caspase 3 from BD Biosciences; CD24 from BioLegend; and Cy5-labeled nonstimulatory Fab and unlabeled cross-linking F(ab')2 anti-IgM from Jackson ImmunoResearch Laboratories. CpG was purchased from Alexis and Indo 1-AM was purchased from Invitrogen.

**Cell sorting**

Splenic B cells were initially isolated by depletion of CD43+ cells using magnetic beads conjugated with anti-CD43 Abs according to the manufacturer’s instructions (Miltenyi Biotec). CD43+ cells were then surface stained with fluorescently labeled Abs recognizing CD21, CD24, and B220 or IgM Fab and sorted using a FACS Aria cell sorter with Diva software (BD Biosciences). Postsort purities were consistently >95%.
Cell culture, apoptosis, and proliferation assays

Sorted cells were cultured in RPMI 1640 with 10% FCS, 4 μM l-glutamine, 50 μM 2-ME, 10 mM HEPES, and antibiotics at 37°C at 1 × 10^6 cells/ml with or without 10 μg/ml anti-IgM or 500 nM CpG. Proliferation assays were conducted for 48 h with addition of 1 μCi of [3H]thymidine for the final 8 h before harvesting and analyzing using a scintillation counter. For the CFSE assay, cells were labeled with 0.1 μM CFSE for 10 min at 37°C followed by extensive washing. After 48 h in culture, 7-aminoactinomycin D (7-AAD) was added to distinguish live/dead cells and dilution of CFSE was analyzed on a FACSCalibur flow cytometer (BD Biosciences). To detect active caspase 3 levels, cells were stained with a fluorescently labeled Ab specific to the active form of caspase 3 according to the manufacturer’s instructions.

Western blotting

Sorted cells were stimulated at 1 × 10^7/ml with 10 μg/ml anti-IgM or left unstimulated. Cells (1–2 × 10^6) were lysed in 1% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 10% glycerol and lysates were run on an 8–10% acrylamide gel. Blots were incubated with primary Abs overnight, then HRP-conjugated secondary Abs for 1 h followed by detection by ECL. For cytoplasmic and nuclear factions, 3 × 10^6 cells were lysed in hypotonic buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2% NP40) for 10 min, and the supernatant was collected after spinning for 30 s at 14,000 rpm. The pellet was washed one time with the hypotonic buffer, followed by incubation with frequent vortexing for 45 min in a high salt buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT). All lysis buffers were supplemented with 1 μg/ml aprotinin, 10 μg/ml leupeptin, 80 mM N-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, and 2 μM Na_3VO_4.

Real-time PCR and statistical analysis

Sorted cells (3 × 10^7) were stimulated at 1 × 10^7/ml with 10 μg/ml anti-IgM or 500 nM CpG, pelleted, and frozen at −80°C. RNA isolation and detection of transcript levels were performed as previously described (8). Primers used were: c-Myc forward primer (fp), 5′-TCTCCACTCTACCCAGCAACAATACG and c-Myc reverse primer (rp), 5′-CGTGCATGCTGGGCTTT; NFAT2 fp, 5′-CTGGGACACTTTTGTGGA and NFAT2 rp, 5′-GGAAGGTATCCCG; NFAT1 fp, 5′-TTGGGAGATGGAAGCTACGGTG and NFAT1 rp, 5′-CCACACAGCAACTCTGCTTTG; NFAT4 rp, 5′-TGGTTCATACCTCTACCTGG and NFAT4 fp, 5′-TTTCGCCCTGTTGGTGGCG; and c-Myc forward primer (fp), 5′-TCTCCACTCTACCCAGCAACAATACG and c-Myc reverse primer (rp), 5′-CGTGCATGCTGGGCTTT; NFAT2 fp, 5′-CTGGGACACTTTTGTGGA and NFAT2 rp, 5′-GGAAGGTATCCCG; NFAT1 fp, 5′-TTGGGAGATGGAAGCTACGGTG and NFAT1 rp, 5′-CCACACAGCAACTCTGCTTTG; NFAT4 rp, 5′-TGGTTCATACCTCTACCTGG and NFAT4 fp, 5′-TTTCGCCCTGTTGGTGGCG. Statistical significance was determined using a two-tailed t test of independent sample means.
EMSA

A double-stranded oligonucleotide spanning the NF-κB-binding region of the Il2r promoter 5'-CAACGGCACAGGAATCCCTCTCTTT-3', AP-1-binding region of the metallothionein promoter 5'-TTCGTGACACGCAGCTCAGGCCCAGTTTCTCTCTCTTCT-3', NFAT-binding region of the A1 promoter 5'-TGACTGCTCGCTCGAGT-3', and Sp1-binding site of the A1 promoter 5'-CCTCTTCTCGGTTCTGTGGGTTTC-3' was end-labeled with [γ-32P]ATP and 32P-labeled DNA was used as a probe. The specificity of the binding was assessed by competition with unlabeled oligonucleotides and by supershift analysis with Abs that recognize: A, total pY; B, phosphorylation of Akt at S473; C, phosphorylation of S6; D, phosphorylation of ERK1/2, p38, or JNK1/2 (short time points); or E, ERK1/2 and p38 (longer time points). Blots were stripped and reprobed with anti-PLCγ2, anti-AKT, or anti-ERK1/2 to evaluate protein loading. Data shown are representative of at least three independent experiments.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays with nuclear extracts prepared from cells sorted from wild-type (WT) BALB/c mice were performed as described previously, with some modifications (20). Cells were incubated at 3 × 106/ml with 10 μg/ml anti-IgM or 500 nM CpG and fixed with 1% formaldehyde for 10 min at room temperature. Chromatin from lysed cells was sheared by sonication, diluted three times in dilution buffer (50 mM Tris (pH 8.0), 0.5% NP40, 0.5 mM EDTA, and 0.2 M NaCl), precleared for 1 h at 4°C with salmon sperm-saturated protein A beads followed by overnight incubation with 1 μg of Ab. Beads were added to immunoprecipitates for 2 h and washed twice with high salt buffer I (20 mM Tris (pH 8.0), 0.1% SDS, 1% NP40, 2 mM EDTA, and 500 mM NaCl), twice with high salt buffer II (250 mM LiCl, 1% Tris, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris (pH 8.0)), and twice with TE. After extraction with TE plus 1% SDS, immune cross-linking was reverted at 65°C for 4–6 h and DNA was ethanol precipitated overnight. DNA was resuspended in TE, digested with proteinase K, and purified with QIAquick columns (Qiagen). PCR (30 cycles) was then performed on one-sixth of the DNA obtained using primers spanning a 300- to 400-bp region near the NF-κB and NFAT binding sites of the A1 promoter: fp, 5'-CCCTCTCTGGTTTGCTCGGGGGTTCTC and rp, 5'-AGAG GGCCTTCACAGGCAGCCTCGTG; Bcl-xL promoter: fp, 5'-CTCTCAACTGCTAGGGAATCAC-3' and rp, 5'-TTCACTGCTGCCATGGGAATCAC-3' or start site of the c-Myc gene: fp, 5'-CGCACCACACACACACAGGGATGAC and rp, 5'-AGGACGCACACACATAGGATGGAG-3'.

Results

T1 B cells exhibit a BCR signal-dependent transcriptional defect

Previous studies have reported that early transitional murine B cells, in contrast to mature B cells, fail to up-regulate expression of proteins required for cell cycle progression and survival including: c-Myc, Cdk2, A1, Bcl-xL, and cyclin E upon BCR engagement (7, 10). However, in these earlier studies, protein expression was measured at relatively late time points (12–24 h) after anti-IgM stimulation when the majority of T1 B cells have undergone apoptosis (7–10). To ensure that this lack of protein expression was not due simply to cell death, we measured the transcriptional expression of a subset of these genes 3 h after BCR stimulation, a time point at which anti-IgM-induced apoptosis is not evident in T1 B cells (Fig. 1A, left panel and data not shown). T1 (B220+CD24highCD21low) and follicular mature (FM) (B220+CD24lowCD21high) B cells from WT mice were isolated by cell sorting as described previously (8). Sort purity was typically >95%. BCR engagement with anti-IgM led to a 15- to 25-fold increase in A1, Bcl-xL, and c-Myc mRNA levels in FM B cells (Fig. 1B). In contrast, minimal transcriptional expression occurred in T1 B cells during this time period. A detailed kinetic analysis demonstrated that transcriptional expression of all three genes and an additional NF-κB target gene, IκBα, was detectable as early as 60–90 min after BCR engagement in FM, but remained minimal in T1 B cells (Fig. 1C). IκBα transcript levels did not increase in T1 B cells at later time points, similar to c-Myc, Bcl-xL, and A1 (data not shown).

To verify that these findings did not simply reflect a delay in the transcriptional activity of T1 B cells, we also measured transcription at later time points after BCR engagement using cells purified from Bim-deficient (Bim KO) mice. The proapoptotic Bcl-2 family member Bim is critical for the apoptotic program in immature B
and T cells (21). Apoptosis upon BCR engagement, as measured by up-regulation of active caspase 3, was largely abrogated in Bim KO T1 B cells (Fig. 1A, right panel) and cell survival was nearly identical in Bim KO T1 vs WT FM B cells 48 h after anti-IgM stimulation (Fig. 1E). However, even at 12–15 h after receptor engagement, transcriptional activity was minimal in T1 compared with FM B cells (Fig. 1D). Curtailing anti-IgM-induced apoptosis also had no effect on the proliferative capacity of T1 B cells (Fig. 1E). Thus, in addition to the relatively well-characterized BCR-induced apoptotic program, T1 B cells exhibit a cell-intrinsic, stage-specific transcriptional defect that limits the expression of gene products previously shown to play critical roles in BCR-dependent survival and proliferation (22–24). In contrast, T1 B cells were able to up-regulate each of these gene products to high levels upon stimulation with the TLR9 ligand CpG (Fig. 1F). These findings also correlated with the ability of T1 cells to survive and proliferate in response to this signal (data not shown). Together, these data demonstrate that T1 B cells exhibit a BCR signal-specific transcriptional defect.

**BCR signaling in highly purified slgM-matched T1 vs FM B cells**

The near absent transcriptional response in T1 B cells strongly implies that this subset has differences in the activation of a key signal(s) downstream of BCR engagement. Consistent with this idea, a number of previous studies have reported altered BCR-proximal signals in immature vs mature primary B cells (7, 9, 10, 12–14, 25–28). These studies, however, have also led to contradictory findings likely due to differences in cell source and purity, slgM expression profile, and cell activation methods.

We directly compared BCR signaling using highly purified, functionally, and phenotypically well-defined T1 vs FM B cells with matched slgM levels. To achieve this goal, we sort purified CD24<sup>high</sup>/CD21<sup>low</sup> T1 vs CD24<sup>int</sup>/CD21<sup>int</sup> FM B cells derived from MD4 Hel-Ig Tg mice (29) which express nearly identical slgM levels (Fig. 2A). Recent work from our laboratory has extensively characterized the functional responses and developmental capacity of primary B cells purified according to these and related gating criteria (8). As shown in Fig. 2, B and C (and data not shown), sorted Tg B cell subsets exhibited identical proliferative and transcriptional responses compared with the equivalent WT B cell subsets. Unless otherwise stated, all subsequent biochemical analyses were performed using purified Hel-Ig Tg B cell subsets. Importantly, preincubating with the cell surface Abs used to isolate these subsets by cell sorting had no appreciable effect on downstream signaling as assessed by total pY levels, JNK, PKD, or AKT phosphorylation (Fig. 2, D and E).

As an initial assessment of proximal tyrosine kinase activity, we compared total protein tyrosine phosphorylation (pY) in response to anti-IgM engagement. As shown in Fig. 3A, T1 B cells exhibited an equivalent or slightly increased pY signal compared with FM B cells. A critical signal initiated by BCR engagement is the production of phosphatidyl-inositol-1,4,5-trisphosphate (PIP<sub>3</sub>) via activation of PI3K. PIP<sub>3</sub> is essential for membrane recruitment of pleckstrin homology domain-containing adapters and enzymes and strongly promotes activation of Tec family kinases and AKT. Although direct measurement of PI3K activity was not possible due to limited cell numbers, we assessed <em>trans</em>-phosphorylation of AKT at S473 as a surrogate PIP<sub>3</sub>-dependent BCR signal (30, 31). T1 and FM B cells exhibited identical levels and kinetics of p-AKT induction at early time points (Fig. 3B). In addition, based on equivalent activation of an AKT downstream target, S6, at later time points, sustained AKT and/or mTOR activity is also intact in T1 B cells (Fig. 3C).

When we measured activation of MAPK pathways, we found that site-specific phosphorylation of p38 and ERK was comparable.
in T1 and FM B cells even at late time points after receptor engagement (Fig. 3, D and E). In contrast, we reproducibly observed a significant increase in relative JNK phosphorylation in T1 B cells compared with FM B cells (Fig. 3D). The expression level of all three MAPK family members was identical between T1 and FM B cells (data not shown).

Activation of PLCγ2 and DAG signaling is intact in T1 B cells

A1, c-Myc, and Bcl-xL are each direct NF-κB target genes in BCR-triggered primary B cells (22–24, 32, 33). Therefore, to address whether BCR proximal events leading directly to PKCβ-mediated NF-κB activation are impaired in T1 B cells, we first assessed the relative activity of PLCγ2, which is responsible for the generation of DAG and InsP3, required for classical PKC activation. We used a phospho-specific Ab that recognizes pY759, a Btk-dependent phosphorylation site crucial for PLCγ2 activity (34). Although phosphorylation at this site increased in response to BCR engagement in both T1 and FM B cells, the relative level of pY759 was consistently lower in T1 cells at all time points, suggesting a partial deficit in PLCγ2 activation (Fig. 4D). To evaluate the initial release of Ca2+ from InsP3 receptor-gated endoplasmic reticulum stores, we measured the BCR Ca2+ response in the absence of extracellular Ca2+ and observed no appreciable differences (Fig. 4B, lower panel). Ca2+ flux in the presence of extracellular Ca2+ was also similar between T1 and FM B cells (Fig. 4B, upper panel). Thus, BCR-induced InsP3 production in T1 cells triggers equivalent levels of endoplasmic reticulum Ca2+ release compared with FM B cells.

As a surrogate measure for proximal DAG-dependent signaling, we evaluated activation of PKD. DAG directly binds to PKD, promoting its membrane localization and subsequent PKC-mediated PKD trans-phosphorylation (35). We observed equivalent levels of pPKD in T1 vs FM B cells, indicating that initial, BCR-triggered, DAG-mediated signals are similar in T1 vs FM B cells (Fig. 4D). Interestingly, while PKD levels were identical in resting cells, the protein level of the more slowly migrating pPKD species decreased in T1 B cells. Although the significance of this latter finding remains unclear, these data strongly support the conclusion that initial, BCR-triggered, DAG-mediated signals are similar in T1 vs FM B cells.

Because BCR-triggered Ca2+ flux and DAG signaling were intact and largely indistinguishable in T1 vs FM B cells, we next sought to assess the relative activity of cPKC in these subsets. Based upon the limited cell numbers available in our experiments, we used an Ab that specifically recognizes consensus serine residues phosphorylated by cPKC as a surrogate for cPKC enzymatic activity. Activated T1 cells consistently exhibited a greater number of phosphorylated species, as well as higher relative signal intensity, compared with FM B cells (Fig. 4C). These findings indicate that activation of cPKC is intact in T1 B cells. Furthermore, because cPKC expression levels are identical in these subsets (data not shown) and Ca2+ flux and DAG signaling are also similar, the increase in cPKC substrate phosphorylation in T1 B cells may reflect differences in relative phosphatase activity. A similar mechanism might also explain the increase in JNK activity in this subset.
In summary, these data indicate that although PLCγ2 site-specific phosphorylation is modestly reduced in T1 cells, PLCγ2 activity is sufficient to promote subsequent downstream signals including activation of cPKC.

**Activation of the NF-κB signaling cascade is intact in T1 B cells**

Since cPKC activation was not impaired in T1 B cells, we next directly evaluated the status of protein modifications required for canonical NF-κB signaling. Interestingly, phosphorylation of both IKKα/β and IκBα was higher and more accelerated in T1 B cells compared with FM B cells (Fig. 5A). Consistent with this, IκBα degradation proceeded more rapidly in T1 compared with FM B cells (Fig. 5A), although the basal level of total IκBα was consistently lower in T1 B cells. Despite this increase in IKKα/β and IκBα activity, trans-phosphorylation of p65 at S536, an IKK-mediated modification that enhances p65 transcriptional activity (36, 37), was similar in T1 and FM B cells (Fig. 5A). These data indicate that the composite signals required to release NF-κB subunits from sequestration within the cytosol are comparable or somewhat increased, in T1 vs FM B cells.

We next evaluated whether any differences existed with regard to nuclear translocation of p65 or c-Rel in these developmental subsets. As shown in Fig. 5B, both the kinetics of nuclear import and the relative levels of c-Rel and p65 were indistinguishable in T1 and FM B cells using cells from Hel-Ig Tg (Fig. 5B) or WT mice (data not shown). We then measured the DNA-binding ability of nuclear p65 and c-Rel in anti-IgM-stimulated T1 and FM B cells from WT mice. An EMSA showed equivalent protein binding to a κB binding site upon anti-IgM stimulation in T1 vs FM B cells (Fig. 5C). Supershift studies also demonstrated equal binding of both c-Rel and p65 (Fig. 5D). Thus, surprisingly, despite the lack of transcription of NF-κB target genes in T1 B cells, the BCR-driven signaling events leading to NF-κB subunit nuclear import and DNA binding are equivalently activated in T1 and FM B cells.

**T1 B cells have lower BCR-induced NFAT activity**

Although the expression of A1, Bcl-xL, and c-Myc each requires canonical NF-κB signaling, the lack of transcription in the face of an apparently intact NF-κB signaling cascade suggested that additional transcription factors might impact these events. In silico analysis of the promoter region of each of these genes identified conserved binding sites for the transcription factor NFAT (data not shown) and NFAT activity has recently been shown to be critical for A1 transcription in specific cell lineages (19). Indeed, treatment of FM B cells with the calcineurin inhibitor, cyclosporin A (CsA), led to reduced BCR-induced transcription of each of these genes, with nearly complete inhibition of both A1 and Bcl-xL compared with untreated cells (Fig. 6A, left panel). In contrast, CsA treatment did not limit CpG-induced gene expression (Fig. 6A, right panel).

Given the apparent BCR-specific role for Ca2+ signaling in A1, Bcl-xL, and, to a lesser extent, c-Myc transcription, we hypothesized that NFAT activation might be impaired in T1 B cells. Consistent with the nearly identical calcium signals, T1 and FM B cells were stimulated with anti-IgM (left panel) or CpG (right panel) with or without 2 μM CsA for 3 h. Gene transcript levels were determined and shown as relative fold increase with anti-IgM stimulation relative to unstimulated cells or cells treated with CsA alone. Representative of four independent experiments.
cells exhibited similar rates of BCR-triggered NFAT1 and 2 dephosphorylation (Fig. 6B); while NFAT activation was not observed upon CpG stimulation (data not shown). However, although both T1 and FM B cells expressed similar NFAT2 levels, NFAT1 expression was significantly lower in T1 B cells. This correlated with an ~6- to 8-fold reduction in NFAT1 transcript levels, consistent with stage-specific transcriptional regulation rather than altered processing or protein stability (Fig. 6C). NFAT4 transcript levels were equivalent in both subsets (Fig. 6C).

To evaluate the potential functional significance of reduced NFAT1 in T1 B cells, we first measured relative NFAT binding via EMSA. Using a probe containing the NFAT binding site within the A1 promoter, we observed significantly decreased NFAT binding in WT T1 B cells (Fig. 6D). Supershift studies demonstrated equivalent levels of NFAT2 containing DNA-binding complexes (Fig. 6D). In contrast, NFAT1 complexes were almost undetectable in T1 B cells. Next, to determine whether reduced NFAT1 activity might account for the transcriptional defect in T1 B cells, we analyzed the function and transcriptional profile of NFAT1 KO B cells. Sorted WT and KO FM B cells exhibited similar proliferation and survival responses upon anti-IgM or CpG stimulation (Fig. 7, A and B) and similar levels of A1, Bcl-xL, and c-Myc transcription upon BCR engagement (Fig. 7C). Together, these observations indicate that despite altered NFAT1 expression and DNA binding, this difference alone is unlikely to limit BCR-dependent transcription of A1, Bcl-xL, and c-Myc in T1 B cells.

AP-1 has also been strongly implicated in mediating transcription of A1 (38). We therefore measured AP-1 activation via EMSA as well. WT T1 and FM B cells exhibited equivalent BCR-dependent AP-1-binding activity (Fig. 8A). In addition, BCR stimulation led to similar nuclear levels of both c-Jun and c-Fos (Fig. 8B).

Thus, BCR-triggered AP-1 activity is equivalent in these developmental subsets.

**Deficient RNA Pol II recruitment at the A1 promoter in T1 B cells**

Transcriptional activation via NF-κB requires its association with histone acetyltransferases including, most notably, p300/CBP (39, 40). This greatly facilitates recruitment of the Pol II transcription complex and subsequent target gene transcription. This process has been extensively characterized for mitogen-induced transcriptional activation at the A1 promoter (38). Given the fact that lower NF-κB, NFAT, or AP-1 activity cannot explain the transcriptional defect observed in T1 B cells, we wished to directly test whether the deficit in BCR-driven transcription in T1 cells correlated with alterations in Pol II recruitment. To do so, we used a ChIP-based assay to evaluate protein recruitment to the A1, c-Myc, and Bcl-xL gene promoters in WT T1 vs FM B cells. BCR engagement led to a significant (5- to 8-fold) increase in Pol II binding at both the A1 and c-Myc promoters in FM B cells upon BCR engagement (Fig. 9, A and B). Strikingly, significantly less recruitment was observed in T1 B cells (~2-fold). We were unable to detect any recruitment of Pol II to the Bcl-xL promoter in T1 or FM B cells with anti-IgM stimulation (Fig. 9C). In contrast, CpG stimulation led to a strong accumulation of Pol II at the A1, c-Myc, and Bcl-xL promoters in both T1 and FM B cells (Fig. 9, C–E). Thus, despite equivalent activation of NF-κB, NFAT, and AP-1 in BCR-stimulated T1 B cells, these cells exhibit a marked, signal-specific deficit in the capacity to assemble transcriptional complexes essential for A1, c-Myc, and Bcl-xL expression.

**Discussion**

Immature B cells are developmentally programmed to undergo deletion in response to strong receptor cross-linking via Ag, a response essential for the maintenance of B cell tolerance. Despite efforts by many investigators, the mechanisms that orchestrate the differential responsiveness to BCR engagement in early transitional vs mature B cells remain incompletely defined. In the current study, we provide data that challenge existing models and suggest a new view of these events.
Our data clearly demonstrate that T1 B cells manifest a BCR-specific defect in transcriptional activation. T1 B cells essentially fail to activate proliferative and antiapoptotic genes in response to BCR engagement, and this deficit proceeds independently of the apoptotic program initiated in response to BCR engagement. In contrast, efficient transcription of the identical gene products is triggered in response to the TLR ligand CpG. This observation prompted us to attempt to identify a signal-specific control mechanism that might be operative specifically within T1 B cells. Strikingly, our findings demonstrate that T1 B cells manifest a BCR-specific deficit in the capacity to assemble active transcriptional machinery at the BCR target genes AI and c-Myc. T1 B cells exhibit this deficit despite normal proximal BCR signal transduction, as assessed via multiple biochemical parameters and, much to our surprise, despite intact, inducible activation of key transcription factors including NF-κB, NFAT, and AP-1. Thus, our combined findings imply that T1 B cells are specifically programmed for nuclear nonresponsiveness in the face of a strong Ag cross-linking signal.

Previous studies have suggested that altered activation of AKT, MAPK, and/or PLCγ2 signals might account for the differential responsiveness of transitional B cell following anti-IgM stimulation (9, 10, 12–14, 26–28). In addition, both InsP3 (12, 28) and DAG levels (13) have been shown to be significantly lower in immature B cells following BCR stimulation. These various data, in association with indirect findings based upon pharmacological manipulation of PKC, have led to a long-standing hypothesis that immature B cells fail to activate the DAG-dependent, proximal PKCβ/NF-κB signaling cascade, thereby explaining their reduced survival and aborted cell cycle entry (13, 15).

We directly tested this hypothesis using highly purified T1 vs FM B cells that were identically matched for relative sIgM expression levels. In contrast to previous reports, we identified only minor differences in proximal BCR signaling in T1 vs FM B cells. In our system, AKT and MAPK signaling was intact in T1 B cells and despite a modest reduction in Btk-dependent PLCγ2 phosphorylation, subsequent PLCγ2-driven signals were nearly identical in T1 and FM B cells. Our findings are also supported by data derived from previously published work. As shown herein and in other studies (13, 14), immature B cells exhibit no defect in the release of InsP3 and DAG (26) and likely reflects use of more heterogeneous populations.

We demonstrate that T1 B cells manifest a BCR-specific defect in transcriptional activation. Although T1 B cell failed to activate proliferative and antiapoptotic genes in response to BCR engagement, efficient transcription of these same genes was triggered in response to CpG. Although c-Myc, AI, and Bcl-x_L each comprise well-characterized NF-κB target genes (22–24, 32, 33), additional transcription factors are likely to coordinately regulate their expression in T1 B cells. Consistent with this idea, BCR-, but not TLR-, triggered Bcl-x_L, c-Myc, and AI transcription were each inhibited by CsA. Notably, calcineurin B1-deficient B cells fail to

FIGURE 9. Deficient transcriptional activation at the AI and c-Myc promoter in T1 B cells upon BCR cross-linking. A and B, Chromatin from WT T1 and FM B cells stimulated 60 or 90 min with anti-IgM was immunoprecipitated with Abs that recognize Pol II or polyclonal rabbit IgG as a control. PCR was performed on DNA isolated from immunoprecipitates or 5% of input DNA (loading control) to amplify a DNA region within the AI promoter (A) or c-Myc promoter (B). Shown is a representative figure of three independent experiments (A and B) and mean with SD of the fold increase in PCR product over unstimulated cells (right panel). C–E, Chromatin from WT T1 and FM B cells stimulated for 90 min with anti-IgM or CpG was immunoprecipitated as above and PCR was performed as indicated. C, Representative of four independent experiments. D and E, Mean with SD of the fold increase in PCR product over unstimulated cells obtained using primers recognizing the AI promoter (D) or c-Myc promoter (E).
proliferate in response to anti-IgM stimulation yet retain proliferative responses to LPS, consistent with a signal-specific requirement for Ca\(^{2+}\) signaling in BCR-driven proliferation (41). Although both calcium flux and dephosphorylation of NFAT1 and NFAT2 were intact in T1 B cells, NFAT1 binding to an exogenous A1 promoter was markedly reduced in T1 cells and correlated with a specific deficit in NFAT1 transcript and protein levels in this developmental subset. To our knowledge, this is the first report of differential NFAT family member expression within B2 B cell subsets.

A1 gene transcription is preferentially regulated by NFAT1 in mast cells (19), suggesting that reduced NFAT1 levels might also account for deficient A1 transcription in T1 B cells. Contrary to this idea, NFAT1-deficient FM B cells efficiently transcribed A1, Bcl-xL, and c-Myc and exhibited no appreciable defects in BCR-induced survival or proliferation. Altered NFAT2 or 4 expression was also unlikely to compensate for NFAT1 deficiency in these cells, because we detected no increase in transcript or protein levels in purified B cells (data not shown). Although it remains formally possible that NFAT1 KO B cells manifest other compensatory events (or that T1 and FM B cells have differential requirement for NFAT1), our combined data suggest that the T1 B cell transcriptional deficit is largely independent of altered NFAT1 activity and that NFAT1 is dispensable for BCR-dependent A1, c-Myc and Bcl-xL transcription. AP-1 has also been shown to bind to the A1 promoter following PMA/ionomycin treatment in Jurkat T cells (38). However, DNA binding of this transcription factor was similar between T1 and FM B cells upon BCR stimulation. Thus, differential activation or expression of AP-1 components also fails to explain the transcriptional defects seen in T1 B cells.

Despite considerable efforts, it proved technically impossible to measure BCR-dependent NF-kB or NFAT binding at endogenous promoter sites by ChIP in primary B cells. Therefore, we focused our efforts on assessment of Pol II recruitment to the promoter. Pol II recruitment was markedly reduced in T1 B cells following anti-IgM stimulation, yet the levels of Pol II recruitment in response to CpG in this subset was similar to that observed for FM B cells. Thus, despite similar “proximal” and more “distal” BCR-dependent signaling events in T1 and FM B cells, transitional B cells exhibit a marked defect in assembly of an active transcriptional complex at the promoter site of a key survival gene, and, based upon our transcriptional profiling are anticipated to manifest similar defects at additional BCR-dependent promoter elements.

Several factors, not addressed herein, might account for the signal-specific nuclear transcriptional defect identified in this study. First, phosphorylation and acetylation of NF-kB subunits, p65 in particular, are necessary for proper association with coactivators CBP/p300 and subsequent transactivation (40, 42). Importantly, mutations of several key phosphorylation/acetylation sites can alter p65 transactivation potential without reducing its nuclear translocation or DNA-binding capacity. Second, developmentally controlled expression of another nuclear transcriptional regulator might impact these events. In particular, the calcineurin-regulated transcription factor MeF2c is expressed at lower levels in transitional B cells and is essential for BCR-induced proliferation and survival (43). These data, in concert with the blockade in BCR-dependent A1 and Bcl-xL transcription mediated by CsA suggest that reduced MeF2c levels might significantly limit gene expression in T1 B cells. Assessment of the effect of enforced MeF2c expression within T1 B cells on BCR transcriptional activity will be required to address this idea. Third, BCR engagement could be activating transcriptional repressors in T1 B cells as opposed to a failure in activating transcription factors. However, this seems unlikely as the addition of anti-IgM to CpG-stimulated T1 B cells did not diminish transcript levels compared with cells stimulated with CpG alone (data not shown). Fourth, epigenetic differences in T1 vs FM B cells might prevent gene transcription. This explanation seems unlikely based upon the rapid (3-h) transcriptional activation of these genes observed in T1 B cells with the TLR ligand CpG, but still remains a possibility. Finally, it has been proposed that initial activation of NF-kB signaling in T1 B cells might be enhanced relative to that present in FM B cells. This might lead to higher initial levels of IkBa transcription and, consequently, more rapid IkBa-mediated removal of NF-kB subunits, thereby limiting NF-kB signaling (44). However, although IKK and IkBa phosphorylation was increased in T1 B cells, we observed no increase in IkBa transcription in T1 B cells even at very early time points after BCR engagement nor did we see differences in p65 nuclear/cytoplasmic shuttling up to 3 h after stimulation. Thus, our data argue strongly for a defect in initiation, rather than premature termination, of transcription.

In summary, our findings provide important new insight into what controls the differential response to BCR engagement observed during peripheral B cell development. In contrast to T1 B cells, late transitional B cell exhibit increased survival and proliferative responses to BCR ligation with and without costimulatory signals (8, 45) and may serve as a target for Ag-driven positive selection (8). These observations, combined with the current study, suggest that transitional cells may gain BCR transcriptional competence during this developmental window. Identification of the microenvironmental signals that mediate this response change, and/or those capable of bypassing it, will lead to a better understanding of the events that shape the mature B cell repertoire.

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**Disclosures**

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**References**


