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Analysis of the Regulatory Role of BAFF in Controlling the Expression of CD21 and CD23*

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Abstract

The TNF family member BAFF serves to promote the survival and differentiation of maturing splenic B cells. The major receptor for BAFF (BAFF-R) is expressed by the transition 2, marginal zone and follicular, mature conventional B-2 cell populations; functional BAFF/BAFF-R signaling is required for T1 to T2 cell B cell maturation. Induced expression of CD23 and CD21 is also coincident with the T1 to T2 maturation stage. A key question we address in this report is if BAFF signaling directly induces CD21 and CD23 gene transcription and expression at this B cell transition point, or if their expression is simply coincident with B cell maturation and differentiation. We present data that supports the contention that BAFF does not preferentially induce the expression of CD23 or CD21 at the T1 to T2 transition, nor does exogenous BAFF lead to preferential increased expression of these proteins/genes in mature B cell populations. The analysis of LPS-induced splenic B cells from BAFF-R defective (A/WySnJ) mice did not show the preferential induction of expression of CD21 or CD23 that might have been expected if NF- κ B-p52 protein was lacking due to insufficient BAFF-R signaling in cells bearing this mutation. Indeed, chromatin immunoprecipitation analysis demonstrated stable NF- κ B-p52 complexes on CD21 and CD23 genes obtained from both wild type and A/WySnJ B cells. FACS analysis of splenic B cells from 1, 2, 3 and 6 wk old A/WySnJ mice demonstrated a block in differentiation (thus reducing overall B cell numbers) resulting in a failure of such cells to express CD21 but allowing for the expression level of CD23 per cell to reach levels approaching wild type. We have dubbed this CD23^{HI}CD21^{LO} subset as the T1b transition B cell. These data support the recognized role of BAFF as promoting the survival and differentiation of splenic B cells but do not support a model of BAFF signaling directly inducing the expression of the CD21 and CD23 proteins via translocation of NF- κ B-p52 species.

Keywords

Transcription; complement receptor; B cells; BAFF

Introduction

The murine genes encoding the CD21 and CD23 proteins are transcribed during the maturation of splenic B cells from the transition 1 (T1) to transition 2 (T2) stages. The expression of both

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genes is maintained within the splenic B-2 B cell stages of T2, marginal zone (MZ) and follicular, mature (FM), cells although the surface expression of CD23 is severely depressed in the MZ stage (1–3). The expression of both genes is extinguished as B cells mature into terminal plasma cells (4,5). There is no data to suggest that CD21 and CD23 expression is limited in such maturing cells by any other mechanism than mRNA transcription although CD23 can be cleaved from the surface of mature, activated B cells by an ADAM protease (6). The CD21 and CD23 genes share a number of transcriptional control features including conserved binding sites for the B cell specific Pax-5 transcriptional regulator, NF- κ B and NFAT family members, E box proteins such as E2A and Notch family members such as RBP-J κ (Debnath, Roundy, Weis and Weis, submitted). CD21 gene expression is additionally complicated by the apparent contribution of intronic silencer regions that help regulate the cell and stage specificity of expression (7,8). The transcription of the CD21 and CD23 genes can also be coordinately controlled by factors independent of B cell maturation. For example, the human Kaposi's Sarcoma Associated gamma herpes virus possesses a transcriptional control protein, RTA, that dramatically elevates expression of CD21 and CD23 proteins in infected human B cells (9).

The coordinated expression of the CD21 and CD23 genes is coincident with the survival effects of B cell-activating factor (BAFF, BLyS). BAFF is a ligand of the TNF superfamily similar to APRIL, another TNF-family ligand (10–13). Three related receptors (BAFF-R, TACI and BCMA) recognize these ligands; BAFF-R is expressed by maturing and mature splenic B cells and only binds BAFF. TACI and BCMA are also expressed on B cell subpopulations and recognize APRIL with a 100 fold higher binding affinity than BAFF suggesting that APRIL is the biologically relevant ligand for TACI and BCMA (14–17). BAFF signaling serves to increase the survival of T2 and mature B cells; animals lacking BAFF show profound losses in FM and MZ B cell populations, but B-1 B cells, B cell development in the marrow, and migration and establishment of T1 B cells into the spleen are not altered (18,19). The expression of CD21 and CD23 is also severely reduced on splenic B cells obtained from the BAFF-deficient animals. Maturing B cells shift from a dependence upon BAFF and its receptor to APRIL and its receptors, TACI and BCMA (20). For example, animals lacking BCMA lack bone marrow plasma cells indicative of a loss of maintenance of this mature B cell population (21).

Animals deficient in BAFF-R function show many similarities with animals lacking BAFF. The A/WySnJ strain expresses a defective BAFF-R due to a mutation within the cytoplasmic domain that eliminates the ability to bind to the intracellular signaling molecule, TRAF3 (23–27). This animal demonstrates deficiencies in T2, MZ and FM B cells but has normal populations of T1 and B1 B cells. The phenotypes of the BAFF-R deficient animal are virtually identical to those of the A/WySnJ animal (22). B cells obtained from the receptor deficient animal do show some CD23 expression but lack CD21 (22). Over expression of Bcl-2 or Bcl-xl rescues maturing B cells in the BAFF-R deficient/defective animal suggesting that BAFF normally functions to rescue maturing cells from apoptosis (22,24).

BAFF-R activation leads to an alternate pathway of NF- κ B activation that is Nemo independent, requires RIP and NIK activation and results in the production and migration into the nucleus of NF- κ B-p52 (28,29). TRAF3 is implicated as the principal intracellular signaling partner for BAFF-R (30). The comprehensive identification of targets of the BAFF-R signaling pathway has not been reported. A recent report demonstrated that the cell surface expression of CD21 and CD23 on B cells was elevated when splenocytes were incubated in the presence of exogenous BAFF (31). Similarly, another report demonstrated that peritoneal B-1 cells treated with BAFF also increased the cell surface expression of CD21 (32), however, neither of these reports also analyzed the expression level of other B cell specific proteins following BAFF treatment. We have recently shown that NF- κ B-p52 proteins are found associated with

CD21 and CD23 transcriptional control elements; each of these promoters possesses canonical NF- κ B binding sites (Debnath, Roundy, Weis and Weis, submitted). Defective BAFF signaling (either from a BAFF deficient animal or from a BAFF-R defective/deficient animal) could be proposed to depress CD21 and CD23 expression by blocking movement of the NF- κ B-p52 species into the nucleus of the cell and thus suppressing CD21 and CD23 promoter function.

A focus of our laboratory has been upon the transcriptional control of the mouse CD21 (*Cr2*) gene, therefore, we were intrigued with the concept that BAFF could directly control transcription and cell surface expression of CD21. In this report we have examined the expression of CD21 and CD23 in wild type and BAFF-R defective A/WySnJ animals. We find that the expression of CD21 and CD23, either as gene transcripts or cell surface proteins, are not preferentially induced on mature B cells following BAFF treatment since the expression of other B cell surface proteins, such as CD19 and B220, are also elevated. Translocation of NF- κ B-p52 into the nucleus of A/WySnJ B cells, via LPS activation, does not lead to increased CD21/CD23 expression as might be expected if insufficiency of BAFF signaling suppressed NF- κ B-p52 levels. Chromatin immunoprecipitation analysis of B220+ splenocytes obtained from A/J and A/WySnJ mice does not show any differential loss of NF- κ B-p52 binding to the CD21 and CD23 genetic elements suggesting that this protein is not limiting in the BAFF-R defective cells. The analysis of CD21 and CD23 expression by splenocytes derived from 1, 2, 3, and 6wk old A/J and A/WySnJ mice demonstrate that CD23 expression is induced prior to that of CD21 and reaches similar expression levels, per cell, in the A/J and A/WySnJ strains. CD21 expression, however, is nearly undetectable in B cells from the BAFF-R defective strain. These data in total suggest that the induction of expression of CD23 is at a step earlier in B cell maturation than that of CD21 and that the expression of neither of these genes is directly controlled by BAFF-dependent translocation of NF- κ B-p52 into the cell nucleus.

Materials and Methods

Animals and Cell culture conditions

A/J, A/WySnJ, C57/BL6, BALB/c mouse strains were purchased from Jackson Laboratories. Splenocytes and bone marrow cells were isolated and maintained at 37°C/5% CO₂ in RPMI (GIBCO) with 10% BGS (Bovine Growth Serum, Hyclone) and 1% Penicillin-Streptomycin (Pen-Strep; Life Technologies). Where mentioned, cells were treated with 2 μ g/ml recombinant human BAFF (Peprotech), or LPS (1mg/ml, E.coli 0111:B4, Sigma) for indicated times.

Antibodies

Mouse monoclonal anti YY1, NFATc3, NF- κ B-p52, Pax5, E2A, rabbit polyclonal anti Oct1, RBP-Jk, normal mouse IgG and normal rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC conjugated anti-CD19 or anti-CD21, PE conjugated anti-B220 and biotinylated anti-CD23 antibodies were purchased from PharMingen.

Magnetic Cell Separation and FACS

Splenic B cells were purified using Miltenyi magnetic beads according to manufacturer's protocol. FITC conjugated or PE conjugated antibodies (PharMingen) were incubated with cells at 4° C for 20 minutes in presence of 10% mouse and rat serum. Incubations were followed by two washes in the staining buffer (0.1% BSA in 10% PBS). Samples were analyzed with a FACScan flow cytometer and data were analyzed using Cell Quest software (Becton Dickson, Mountain View, CA)

RNA preparation, cDNA synthesis and RT-PCR

Total RNA from cells was isolated using the Trisol reagent (Invitrogen). 2.0µg of total RNA was used for cDNA synthesis and the cDNA was isolated using the Qiagen PCR purification kit. RT-PCR using Light Cycler (Roche) was performed as previously described (33) using splenic DNA as a negative control. Primer sequences for RT-PCR analysis are listed in Table 1.

Chromatin Immunoprecipitation (ChIP)

This protocol was adapted from published procedures (34–36). Splenocytes were isolated from A/J or A/WySnJ mice and erythrocytes lysed using ACK RBC lysis buffer. B220 positive cells were separated using Miltenyi microbeads following manufacturer's protocol; cells were then washed and resuspended in PBS with 0.1% BSA. 1×10^6 cells were pelleted for each ChIP reaction and PBS was removed. DNA/protein crosslinking was done by resuspending cell pellets in 1% formaldehyde and incubated for 15 minutes at room temperature. 50µl of 2.5 M glycine was added to each reaction and incubated for 5 minutes with rotation and then centrifuged to pellet cells. Pellet was washed once with 1X PBS with 0.1% BSA and cells were resuspended in RIPA (plus the protease inhibitor "Complete Minipill": Roche) and incubated for 20 minutes on ice with intermittent hard vortexing. Lysed samples were sonicated 7 times for 30 seconds each time. Sonicate was centrifuged for 10 min at 4° C. Supernatant was taken and centrifuged again to pellet debris out. For the input sample, 50µl of the supernatant was mixed with 450µl of ChIP elution buffer (10mM EDTA, 1%(w/v) SDS, 50mM Tris-HCl PH8.0) and crosslinking was resolved by heating at 65° C overnight and then purified with PCR purification columns. For all other samples, 400µl of the remaining supernatant was supplemented with 100µl of RIPA with protease inhibitors for each immunoprecipitation reaction. For immunoprecipitation, 1µg of mouse monoclonal or rabbit polyclonal antibody was used (except where indicated otherwise). Immunoprecipitations were done for 2 hours rotating at 4°C. Sheep anti-mouse or sheep anti-rabbit IgG conjugated dynabeads (DynaL Biotech) that had been previously blocked and washed with 1X PBS with 1 mg/mL BSA, were added to the reactions and incubated overnight. The complex-bound Dynabeads were washed using a magnetic rack, 3 washes with RIPA, 2 washes with RIPA plus 500mM NaCl, 2 washes with RIPA plus 250mM LiCl, 1 wash with RIPA plus 500mM NaCl, 1 wash with RIPA plus 250mM LiCl and, finally, 3 washes with TE. DNA was eluted from the bound beads by adding 250µl of ChIP elution buffer to each reaction and incubating overnight at 65° C with gentle shaking. Supernatant was then separated from Dynabeads using a magnetic rack and soluble DNA was purified using PCR column purification. PCR amplification in the presence of ^{32} P-dCTP was done for each sample (input, isotype control, and immunoprecipitated samples) using primers specific for different CD21 and CD23 promoter and intronic elements for 30 cycles with 6 seconds elongation and 55° C annealing temperature (33). PCR products were subjected to electrophoresis within a sequencing gel, the gel was dried and exposed to X-ray film overnight. Primer sequences are listed in Table 1.

Results

Addition of exogenous BAFF does not preferentially lead to enhanced expression of CD21 or CD23 on mature B cells

BAFF-dependent increased expression of CD21 and/or CD23 on mature B cells was evaluated by incubating normal BALB/c total splenocytes in the presence or absence of exogenous BAFF for 48 and 72hrs. As shown in Fig.1A, prolonged incubation of cells in the presence of BAFF led to a slight increase in CD21 and CD23 transcripts however those for CD19 were also elevated in the presence of BAFF while T cell expression of CD3 was unaffected.

BAFF treated cells were then analyzed by FACS analysis, measuring the mean fluorescence intensity of CD21, CD19 and B220 after culture for 72hrs in the presence or absence of exogenous BAFF (Figure 1,B). B cells staining for these three markers demonstrated slightly elevated levels of all three proteins in the presence of BAFF. The percentage of B cells in these cultures did not rise indicating that BAFF was not simply leading to a proliferation of B cells. The relative mean fluorescence intensity of B220, CD19 and CD21 from spleen cultures held for 24 and 48hrs in the presence or absence of exogenous BAFF was also analyzed (Figure 1,C). A marked increase in staining intensity for CD19 was noted with a lesser induction of CD21 and B220 cell surface expression.

Previously it had been shown that co-culture of normal mouse total splenocytes with exogenous BAFF led to an increased B cell surface expression of CD21 and CD23 (31). In that assay, however, no other B cell surface proteins were evaluated to determine if the CD21 and CD23 genes were being selectively induced, or if total B cell fitness and survival was increased. The data in Figure 1 agree with this previous report but further suggest that long-term culture of normal mature B cells with BAFF up-regulates the expression of many B cell markers including but not limited to CD21 and CD23.

Analysis of CD21 and CD23 expression in marrow and spleen of mice lacking BAFF signaling

The previous experiment required a response from resting, mature splenic B cells (the vast majority of which are within the T2, MZ and FM stages) to the addition of BAFF. An alternative experiment would be to analyze resting and activated cells from mice either lacking the cytokine itself (BAFF deficient animals) or animals lacking the receptor that recognizes the cytokine (either an engineered deficiency or natural mutation). Our requests to analyze the BAFF deficient mice (18,19) were not granted thus we chose to utilize the A/WySnJ BAFF receptor model. This animal was first described as displaying a peripheral B cell deficiency (25–27). Further analysis demonstrated that the BAFF-R of this animal possessed a mutation within the cytoplasmic tail that precluded BAFF signaling. These mice demonstrate a significant block in B cell differentiation at the T1 to T2 splenic B2 transition stage.

Total bone marrow and spleen cells from A/J and A/WySnJ mice as well as B220+ positive populations from these tissues were analyzed by quantitative RT-PCR for expression levels of CD21, CD23, Pax-5 and CD19. Total spleen and bone marrow samples from C57BL/6 mice were also included. The paucity of mature splenic B cells in the A/WySnJ animals is evident in the reduction of all four of these transcripts, compared to wild type (Figure 2). As expected, there is also a sharp decline in CD21 and CD23 transcript levels in the B220+ splenic cells from the A/WySnJ mice, compared to the control, consistent with a reduction of T2, MZ and FM cells in the A/WySnJ animal. The level of expression of Pax-5 and CD19 in the B220+ splenic cells of both strains is, as expected, essentially the same although both of these gene's transcripts are slightly elevated in the A/WySnJ animal marrow samples, compared to A/J, perhaps out of compensation for the paucity of mature B cells in the periphery.

The previous figure and data from others indicate that the A/WySnJ animal possesses a significant blockade at the T1 to T2 transition stage that reduces the production of T2, MZ and FM B cells. The expression of CD21 and CD23 is coincident with this step in maturation. The binding of BAFF to BAFF-R induces an alternative NF- κ B pathway that does not require NEMO mediated degradation of the I κ B inhibitory complex. The effect of this alternative pathway is to create the NF- κ B-p52 subunit that is then translocated into the nucleus for the transcriptional activation of target genes. Others have shown that this pathway can be duplicated in B cells by adding LPS to cultures thus making use of the classical pathway utilized by adjuvants (Tlr signaling) and CD40 ligation (29,32) to liberate the NF- κ B-p52 protein. We have shown elsewhere by chromatin immunoprecipitation that the NF- κ B-p52 protein does bind to the CD21 and CD23 promoters. Therefore we rationalized that if the BAFF/BAFF-R

block in the A/WySnJ mouse limits the production of the NF- κ B-p52 protein (resulting in a lack of CD21 and CD23 expression) then by incubating such cells in the presence of LPS, we should release the p52 subunit into the nucleus and induce the expression of CD21 and CD23. Alternatively, if LPS treatment would not induce CD21 and CD23 transcription and expression in A/WySnJ B cells, then other factors required for CD21 and CD23 expression must be missing.

Total splenocytes were taken from the A/WySnJ and control A/J mouse and cultured for 48hrs with LPS. As controls, marrow cells from these animals were also analyzed. Activation of cells with LPS was evident by an increase of staining with CD80 (data not shown). B220+ and CD19+ cells from each cultured sample were examined for CD21 and CD23 expression. As shown in Figure 3, LPS treatment alone in the control marrow cells shows an enhanced expansion of CD19+, B220+ and CD23+ B cells but little effect is seen on increasing the level of CD21 expression. CD21 expression by the A/WySnJ marrow also shows little response to LPS treatment.

In the spleen, the wild type A/J cells demonstrate an expansion in total B cell counts with LPS treatment (percentage increase of CD21+CD19+ and CD23+B220+ cells). The relative staining intensity of CD21 and CD23 did not change which is the expected result if such genes are already maximally transcribed. However, if the A/WySnJ splenic cells have depressed expression of CD21 and CD23 due to limiting NF- κ B-p52 subunits, then the staining of these proteins per cell should be dramatically increased following LPS treatment. Instead we observe a slight expansion in numbers of CD19+CD21+ cells but do not see any net increase in mean intensity staining of CD21 expression. Similarly the expression of CD23 is not elevated in such cells following LPS treatment although the total number of B220+CD23+ cells is increased. These data suggest that the absence of adequate BAFF signaling in the A/WySnJ cells does not restrict CD21 or CD23 expression simply due to a lack of NF- κ B-p52 protein function.

NF- κ B-p52 complexes with the CD21 and CD23 genes are not altered in B cells between A/J and A/WySnJ animals

The depressed differentiation of splenic B cell populations in the A/WySnJ animal, coupled with the deficient expression of CD21 and, to a lesser extent, CD23 (22) (see below), suggested key transcription factors important in both T1 to T2 differentiation and CD21 and CD23 expression might be lacking in B cells of A/WySnJ mice. In a recent report, we have documented *in vivo* binding of a number of transcription factors to the CD21 and CD23 genes. These factors include those shared by both CD21 and CD23 (NFAT family members, RBP-J κ , NF- κ B-p52, Pax-5 and E2) and those only bound to the CD21 genetic control elements (Oct-1 and YY1). Therefore, we chose to determine if there was any difference in binding patterns of these factors to the CD21 and CD23 genes in B220+ splenocytes from A/J and A/WySnJ mice. As shown in Figure 4, utilizing a ChIP assay that tests for *in vivo* binding of transcription factors to native chromatin, we were surprised to find that there was no significant difference in factor binding patterns between the cells obtained from the A/J mouse versus those of the A/WySnJ animal. Even though cells from A/WySnJ animals are virtually deficient in CD21 surface expression and have depressed CD21 transcript levels there is no definable difference in transcription factor binding to the CD21 genes in the control and mutant cell types. Most notable is the presence of the NF- κ B-p52 subunit associated with the CD23 and CD21 genetic elements in both wild type and mutant cells suggesting that the lack of a functional BAFF-R is not inhibiting the binding of this transcription factor to these genes. These data suggest that the key regulatory factor(s) that allows for CD21 transcription (and to a lesser extent CD23) in the T1 to T2 transition stage is currently unknown.

CD23 and CD21 are differentially expressed in maturing splenic B cells

The previous analysis of the BAFF and BAFF-R deficient animals indicated a significant block in T1 to T2 cell maturation and a severely depressed level of expression of CD21 and CD23 on the remaining splenic B cells. While most analyses have ascribed these cells as primarily possessing the T1 phenotype, a recent report suggested that the spleen of BAFF deficient animals did possess normal numbers and types of T2, MZ and FM cells, but that such cells were specifically lacking CD21 and CD23 expression (31). The more recent analysis of the BAFF-R deficient animal (whose phenotype parallels that of the A/WySnJ animal) indicated that mature B cell populations in the spleen are lacking and that CD23 may be expressed in a BAFF-independent pathway (22).

We decided to explore this question by scrutinizing the expression of CD21 and CD23 by splenic B cells during development in the A/J and A/WySnJ animals. The spleen of a mouse does not develop mature architecture and B cell phenotypes until 4 to 6 weeks after birth. Until then the B cell component of the spleen is primarily made up of transitional B cells (T1 and T2 cells). Our rationale was that by enriching for these immature cell populations, we might be able to determine distinct maturation steps defined by CD23 and CD21 expression. Spleens were recovered from A/J and A/WySnJ animals 1, 2 and 3 weeks after birth and analyzed for CD21 and CD23 expression. As shown in Figure 5, the wild type animal possesses a low percentage of CD23^{HI}CD21^{LO} staining cells after one week that shifts to a CD23^{HI}CD21^{HI} population as the animal matures (3 weeks of age). The mean fluorescence intensity of CD23 staining is not appreciably altered in this time course; however, the expression of CD21 does increase, per positive cell, in the more mature animal.

The splenic population of maturing A/WySnJ animals shows a different pattern than that of wild type. The total number of maturing B cells is less (as expected), however, those that do develop express appreciable levels of CD23 but virtually no (or very low levels of) CD21. The mean fluorescence intensity of CD23 staining for these cells from the A/WySnJ mice is similar to that of the wild type animal suggesting that normal levels of expression of CD23 are independent of BAFF/BAFF-R signaling.

This analysis was extended by analyzing 1, 3 and 6 week splenic populations for the expression of B cell markers to both account for B cell numbers as well as to follow the expression of CD21 and CD23 in the more mature spleens. As shown in Figure 6, the percentage of B cells in the spleen (determined by CD19 staining) reaches a steady state in the A/J and A/WySnJ animals between weeks 3 to 6 (about 65% and 25–30% of total splenocytes, respectively); the mean fluorescence intensity of CD19 staining is the same for A/J and A/WySnJ B cells. The specific percentages of positive cells in each plot are summarized in Table 1. The 6 wk old A/WySnJ spleen samples show a subpopulation of B cells that express CD23 at nearly the same mean fluorescence intensity as the wild type cells. The CD23+ population in the 6 wk A/WySnJ samples is about 65% of the total B cells (19.4% CD23 positive cells versus 30% positive CD19 cells) suggesting that the remaining CD23-CD19+ B cells in the A/WySnJ animal are T1 cells. The same cell types were observed previously in the analysis of the BAFF-R deficient animal (22). The expression of CD21 is only minimally, if at all, evident on B cells in the 1, 3 and 6 wk A/WySnJ spleens. These data indicate that the expression of CD19, CD23 and CD21 are differentially regulated on maturing B cells and that CD21 requires an additional induction step, compared to CD23, for efficient cell surface expression.

Discussion

In this report we have analyzed the expression of various B cell proteins in the context of BAFF signaling and the transitional maturation of splenic B cells. A functional BAFF pathway has been determined in many studies to be critical for the development of mature B cell populations,

particularly during the T1 to T2 B cell transition stage when the expression of the BAFF-R is elevated (31). T1 B cells are sensitive to apoptosis following surface IgM crosslinking; however, BAFF activation induces the expression of anti-apoptotic proteins that render T2, FM and MZ cells resistant to apoptosis following BCR activation (37,38). This step is critical for the development of a functional B cell response to foreign antigen. The expression of a number of B cell proteins are induced at the T1 to T2 cell transition including CD21, CD23, ICOS ligand, BAFF-R (39) while others, such as CD38 and CD157, are depressed (40,41).

We chose to examine CD21 and CD23 expression in the context of BAFF signaling with mature splenic B cells and with immature splenic B cells undergoing the T1 to T2 transition stages of differentiation. A previous publication suggested that CD21 and CD23 cell surface expression by mature B cells was directly dependent upon the BAFF signaling pathway, presumably by inducing transcription of these genes via the NF- κ B pathway, and was independent of the B cell survival and differentiation signals promulgated by BAFF activation during the T1 to T2 transition (31). Contrary to their earlier observations and the conclusions of others analyzing BAFF deficient and BAFF-R deficient/defective animals (18,19,22,25,26), this report suggested B cell subsets in BAFF deficient animals were normal but simply lacked normal expression of CD21 and CD23. This was potentially a critical observation in that antibodies against CD21 and CD23 are commonly used to define T1, T2, MZ and FM spleen populations. Incubating splenic B cells obtained from wild type or BAFF deficient animals did show an increase in cell surface staining intensity of these two proteins, but no other B cell specific proteins were used as controls (31). Another recent report also showed that incubation of peritoneal B-1 B cells with BAFF led to an increased level of expression of CD21 (similar in magnitude to that shown in this manuscript for B-2 cells) compared to the media alone control, but, again, the expression level of other B cell surface proteins was not analyzed (32).

We show in this report that the addition of exogenous BAFF to mature wild type splenic B cell populations does increase CD21 and CD23 transcripts and cell surface staining intensity, however, other B cell markers analyzed in this fashion (CD19 and B220) were also elevated. These data indicate that BAFF serves to promote the fitness and vitality of mature B cells in culture and thus can positively impact the expression levels of many, if not all, B cell surface proteins.

To analyze the role of BAFF signaling in CD21 and CD23 expression in transitional B cell maturation we turned to the A/WySnJ animal that possesses a defective BAFF-R. The phenotypes of BAFF deficient animals and the BAFF-R deficient (or defective in the case of A/WySnJ) are very similar in that these animals possess early transitional B cells and lack normal populations of mature splenic B cell populations (19,25,47). Thus the analysis of the A/WySnJ mouse for BAFF-dependent responses such as CD21 and CD23 expression should be very similar, if not identical, to analyses of the BAFF deficient animals.

The activation of the BAFF-R protein on transitional B cells is known to prevent apoptosis of B cells by up-regulating the expression of anti-apoptotic proteins such as Bcl-2 (37). The cell activation pathway initiated by this event utilizes TRAF3 and the "alternative" pathway of NF- κ B activation leading to the production of the NF- κ B-p52 transcription factor (28,29,48,49). No other transcriptional control pathways have been described as a result of BAFF-R activation. Treatment of such B cells with LPS can also serve to liberate the NF- κ B-p52 subunit (29): the same release of NF- κ B-p52 was observed after treatment of B-1 cells with LPS (32). We have found in a separate study that NF- κ B-p52 can be found associated with the CD21 and CD23 genes via chromatin immunoprecipitation (ChIP) studies; the promoters of both genes possess canonical NF- κ B binding sites (Debnath, Roundy, Weis and Weis, submitted). Therefore if BAFF-R signaling does directly lead to transcriptional induction (and thus cell surface expression) of CD21 and CD23, then it would be anticipated that such control would

be exerted via the NF- κ B-p52 transcription factor. However, CD21⁺ splenic B cells from BAFF-R defective animals (A/WySnJ) treated with LPS did not demonstrate any net increase in CD21 cell surface staining. This observation was underscored when ChIP analysis of the CD21 and CD23 genes between B cells obtained from the A/J and A/WySnJ strains demonstrated no difference in the *in vivo* binding of a variety of transcription factors including NF- κ B-p52. These data suggest that the lack of CD21 expression by transition-arrested B cells in the BAFF-R defective animal is not due to a lack of the NF- κ B-p52 subunit for transcriptional induction and that another transcription factor, whose expression/function is dependent upon B cell maturation, is required for maximal CD21 expression.

The analysis of the BAFF-R defective animal, A/WySnJ, did provide some unique insights into the timing and mechanism of CD21 and CD23 expression. Previously the assumption was that the expression of CD21 and CD23 was at the same time in B cell development and coincident with the T1 to T2 transition. However, as shown in Figures 5 and 6, the analysis of both wild type and the A/WySnJ maturing B cell subsets suggests that CD23 is expressed earlier and is under the control of a distinct pathway compared to CD21. This observation was also noted in studies with the BAFF-R deficient animal (22). This is a surprising finding since the CD21 and CD23 genes share many of the same transcriptional regulatory elements, and there were no obvious differences in the binding of these factors to the CD21 and CD23 genes in the A/J and A/WySnJ cells. While there may be post transcriptional controls on cell surface expression of CD21 and CD23, none have been described except for the release of soluble CD23 from cell surface by members of the ADAM protease family (6). These expression data suggest that BAFF-R signaling is not required for the expression of CD23 agreeing with the conclusions of others (22). However, expression of CD21 does require B cells to mature and survive into the T2 transition state, a stage that does require functional BAFF-R function. Since NF- κ B-p52 binding to CD21 genetic elements is not altered in the splenic B cells of the BAFF-R defective animal, it is probable that CD21 expression is positively regulated by an unknown factor whose function and/or expression is positively regulated by the BAFF-R pathway. Therefore, CD21 expression in splenic B cells is dependent upon the B cell survival and differentiation function of BAFF.

In summary, the expression of CD21 and CD23 (and a number of other proteins) is clearly dependent upon the state of B cell development. BAFF signaling allows maturing T1 cells to enter the T2, MZ and FM subsets of maturing/mature splenic B cells, which, in turn, allows for the expression of these proteins. CD23 expression in the absence of BAFF signaling suggests the presence of a “T1b” stage at which CD23 is expressed but not CD21. Transition from this “T1b” stage to T2 (which allows for CD21 expression) is inhibited in the absence of BAFF signaling (as evidenced in the A/WySnJ, BAFF-R deficient and BAFF deficient animal lines). However, the known end product of BAFF activation, NF- κ B-p52, is not limiting in A/WySnJ cells indicating other factors must be present to allow for CD21 expression. Thus while the expression of CD21 is directly dependent upon BAFF to allow cells to enter the T2 stage, the transcription of the gene is not directly dependent on BAFF signaling.

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Abbreviations

EMSA	electromobility shift assay
ChIP	chromatin immunoprecipitation
F-OH	formaldehyde
T1	transition 1 B cells
T2	transition 2 B cells
MZ	marginal zone B cells
FM	follicular, mature B cells
CsA	cyclosporine
BCR	

	B cell receptor
BAFF-R	BAFF receptor
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor 1
BCMA	B cell activation antigen
APRIL	a proliferation-inducing ligand
BLyS	

Figure 1. BAFF treatment does not preferentially induce expression of CD21 or CD23

Splenocytes were isolated from adult BALB/c mice and incubated for varying time periods in RPMI supplemented with bovine growth sera. Test wells received 2 μ g/ml soluble BAFF; control wells received an equal volume of sterile water. **A.** Real time RT-PCR analysis was performed on RNA samples taken from cells grown for 48hrs (top panel) or 72hrs (bottom panel) in the presence (dark bars) or absence (light bars) of BAFF. Error bars represent the standard deviation of two independent PCR analyses of the same samples. This figure reports a single culture experiment but is reflective of numerous experiments that provided similar results. **B.** FACS analysis of splenocytes cultured for 72hrs in the presence (dark line) or absence (light line) of exogenous BAFF. **C.** Calculation of mean fluorescence intensity (MFI) of CD21, CD19 and B220 expression from cultures grown for 24 and 48 hours in the presence (dotted line) and absence (solid line) of exogenous BAFF.

Figure 2. Transcript analysis of CD21, CD23, CD19 and Pax-5 in A/J and A/WySnJ cell populations
 Quantitative RT-PCR RNA was performed on splenic cells derived from age and sex matched A/J and A/WySnJ mice. Gene transcripts analyzed are as marked (CD21, CD23, Pax-5 and CD19). All products are presented as relative transcripts compared to 1,000 β -actin transcripts. Error bars are standard deviation of duplicate PCR reactions from the same experiment. A single experiment is shown but is indicative of multiple analyses providing similar results. Cells were obtained from A/J mice (J), A/WySnJ mice (WY) and C57BL/6 mice (C57). Samples analyzed were: Spl, total spleen; BM, total bone marrow; Spl B220, B220+ cells positively selected from the spleen; BM B220, B220+ cells positively selected from the bone marrow.

Figure 3. LPS treatment of A/WySnJ bone marrow and splenocytes does not induce CD21 or CD23 expression

Total bone marrow cells and splenocytes from A/J and A/WySnJ animals were incubated with LPS (or sterile water as control) for 48hrs and then analyzed for expression of B cell surface proteins CD19, CD23, B220 and CD21. Percentage of cells in the significant quadrants are as marked. Results shown are from a single experiment but are representative of multiple similar analyses.

Figure 4. NF- κ B-p52 binding to the CD21 and CD23 genetic elements is not altered in the A/WySnJ mouse

B220+ spleen B cells were analysed by ChIP (see *Materials*). Lanes are: C, water control PCR reaction; I, input of cross-linked and sonicated cell lysate; iso, isotype controls of 1 μ g of normal mouse IgG (m) or normal rabbit IgG (r); and antibodies specific for different transcription factors, YY1 (YY); NF- κ B-p52 (N κ); Pax-5 (P5); E2A (E2); NFATc3 (Na); RBP-Jk (RB); Oct1 (OC). Results of a single ChIP analysis are shown but are indicative of multiple similar analyses.

Figure 5. CD23 expression is earlier in B cell differentiation than CD21 and is independent of BAFF functions

Total splenocytes were obtained from age matched A/J and A/WySnJ mice (1, 2 and 3 weeks after birth) and analyzed for CD21 (X-axis) and CD23 (y-axis) expression. The single black vertical bar is set in each plot as a demarcation between CD21- and CD21+ cells.

Figure 6. Expression of CD19, CD23 and CD21 on maturing wild type and BAFF-R defective B cells

Total splenocytes were obtained from A/J and A/WySnJ 1 week, 3 weeks and 6 weeks after birth and analyzed for the expression of the B cell markers. Specific staining is shown as the solid curve while isotype control antibodies are shown by the light gray line.

Table 1

Oligonucleotides used for Real Time Quantitative RT-PCR and ChIP analysis.

ChIP OLIGOS	FORWARD	REVERSE
Promoter P2 (CD21)	GGTTTGAAACTGAATTAAGTGG	GGAAGTCTGCTTTTGGTTCAGG
Nidogen	CCAGCCACAGAATACCATCC	GGACATACTCTGCTGCCATC
CD23	CAGGGTGAGGACTGTTGTGATGATGCG	GGTGGGCCTTGTGGAGTCACAG
RT-PCR OLIGOS		
CD21	ATGGGATCCTTGGGTTTCGCTC	GCTAGGTGAACAAGGTACCT
β -Actin	CTCCATCGTGGGCCGCTCTAG	GTAACAATGCCATGTTCAAT
CD23	CTCTCCCAGAACCTGAACAGACTC	AGCCCTTGCCAAAATAGTAGCAC
CD19	AGGAAAAGGAAGCGAATGACT	GCCTGGCCAGAGGTAGATGTA
CD5	TTTCTGCCTCGGACAGTCTGGAAG	CTTGACCTGACACTTGGAGTTG
CD3	GCAACAATGCCAAAGACCCTC	ACCTCAGCGAAGATAAAGCCG
PAX5	CGAGTCTGTGACAATGACACTGTGC	CAGGATGCCACTGATGGAGTATG

Table 2

Percentage of CD21, CD23 and CD19 positive cells from A/J and A/WySnJ mice of variable age.

	CD21+	CD23+	CD19+	
1 week	A/J	2.8	9.5	22.8
	A/WySnJ	0.3	2.3	8.8
3 week	A/J	24.1	42.5	63.0
	A/WySnJ	1.3	11.2	25.7
6 week	A/J	56.5	64.2	65.6
	A/WySnJ	3.6	19.4	30.0