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Murine BAFF-Receptor Residues 168-175 are Essential for Optimal CD21/35 Expression but Dispensable for B cell Survival¹

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Abstract

BAFF-R (B cell-activating factor belonging to the tumor necrosis factor family-receptor) regulates B lymphocyte survival, maturation, homeostasis, and self tolerance through signaling mechanisms that are not completely understood. A spontaneous BAFF-R mutation, *Bcmd-1*, disrupts BAFF-R signaling. However, it is not clear why the *Bcmd-1*-encoded BAFF-R fails to adequately support B cell survival, optimal CD21/35 expression, and B-cell tolerance to dsDNA, since it is 95% identical to the wild-type (wt) BAFF-R and retains the only known signaling motif. A retrotransposon insertion in A/WySnJ strain mice generated the *Bcmd-1* allele, replacing the eight C-terminal BAFF-R residues with 21 retrotransposon-encoded residues. New data reported here show that the displaced residues, previously thought to have no signaling role, are essential for optimal CD21/35 expression but contribute little to B cell survival signaling. Analysis of wt *Baffr* or *Bcmd-1* homozygous (A/WySnJ X B6.*BCL2*)F2 mice confirmed that *BCL2* complemented *Bcmd-1* for B cell survival but not CD21/35 expression. Through *in vivo* retroviral transduction experiments, we show that *Baffr* complemented *Bcmd-1* for B cell survival but not CD21/35 expression, whereas the *Baffr*Δ103-175 deletion mutant lacking the BAFF-R cytoplasmic domain failed to support these functions. Importantly, we show that the *Baffr*Δ168-175 deletion mutant lacking the retrotransposon-displaced residues, and a *Baffr*T170A mutant lacking a critical threonine, supported B cell survival but failed to support optimal CD21/35 expression. These data provide the first evidence for a possible bifurcation at the receptor level in the BAFF-R signaling pathway. We suggest that discrete BAFF-R cytoplasmic domains may interact with distinct downstream pathways to provide fine control over B cell survival, maturation, and tolerance induction.

Keywords

B cell development; BAFF-R; CD21/35; survival signaling

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Introduction

Peripheral B lymphocytes are consumed in the defense against microbial invasion, and replenished through bone marrow B cell hematopoiesis. Although much is known about peripheral B lymphocyte survival, homeostasis, maturation, and self-tolerance induction, questions remain as to the precise signaling mechanisms that control these integrated processes. The cytokine BAFF (B-cell-activating factor belonging to the tumor necrosis factor family), through BAFF-receptor (BAFF-R) signaling, provides indispensable support for transitional B lymphocytes as they migrate from the bone marrow to the spleen and mature into fully immuno-competent peripheral B lymphocytes (Mackay et al., 2007). The evidence supporting this conclusion derives in part from the severe B lymphopenia observed in mice with targeted mutations in either BAFF (Gorelik et al., 2004; Schiemann et al., 2001) or BAFF-R (Sasaki et al., 2004; Schiemann et al., 2001; Shulga-Morskaya et al., 2004; Thompson et al., 2001; von Bulow et al., 2001; Yan et al., 2001). Signals transduced by BAFF-R also enhance B cell expression of the *Cr2* gene encoding CD21/35, completing antigen-independent B cell maturation (Amanna et al., 2003; Rahman and Manser, 2004; Sasaki et al., 2004; Shulga-Morskaya et al., 2004; Tardivel et al., 2004). This is a significant step in B cell maturation, because proper CD19/CD21 complex expression is required for B cell selection into the marginal zone (MZ) pool (Cariappa et al., 2001), and for coupling the innate immune recognition of microbial antigens to B cell activation through the complement system (Fearon and Carroll, 2000).

BAFF binding to BAFF-R propagates an intracellular signal primarily *via* non-canonical nuclear factor-kappaB2 (NF- κ B2) pathway activation (Claudio et al., 2002; Kayagaki et al., 2002; Siebenlist et al., 2005), leading to enhanced expression of the *Bcl-2* and *Bcl-x_L* survival genes (Batten et al., 2000). The BAFF-R cytoplasmic domain motif PVPAT (murine aa 154-158, human aa 162-166) and murine Thr167 (human Thr175) are required for binding of tumor necrosis factor receptor-associated factor 3 (TRAF3) to BAFF-R (Ni et al., 2004; Xu and Shu, 2002). TRAF3 may act as a negative regulator of non-canonical NF- κ B2 by targeting the vital kinase of the pathway, NF- κ B-inducing kinase (NIK), for degradation. Binding of TRAF3 to BAFF-R is thought to sequester TRAF3 so NIK can accumulate and activate the NF- κ B2 pathway (Gardam et al., 2008). Thus, BAFF is required for peripheral B cell homeostasis because it activates the NF- κ B2 pathway leading to survival gene expression.

In contrast to survival, the molecular details that explain how BAFF enhances CD21/35 expression are unknown. Dependence of *Cr2* gene expression on NF- κ B2 pathway activation has not been reported (Martin, 2007). BAFF might regulate CD21/35 expression and survival independently, since a brief blockade of BAFF *in vivo* reduced CD21/35 expression without reducing B cell survival (Gorelik et al., 2004). Long-term blockade of BAFF through TACI-Ig-transgene expression reduced B cell survival, CD21/35 expression, and MZ B cell development (Tardivel et al., 2004). However, introducing the *BCL2* transgene corrected the survival defect, but not the CD21/35 or MZ B cell defects (Tardivel et al., 2004). These BAFF blockade studies do not address which of the three BAFF receptors, BAFF-R, transmembrane activator and CAML interactor (TACI), or B cell maturation antigen (BCMA), were involved in controlling survival and CD21/35 (Mackay et al., 2007). Our data suggested that the BAFF-R might control both traits, because BAFF-R-mutant A/WySnJ mice had suboptimal B cell survival, CD21/35 expression, and MZ B cell development (Amanna et al., 2003). Enforced *bcl-x_L* gene expression in A/WySnJ B cells restored B cell longevity and follicular B cell development, but did not correct the CD21/35 and MZ B cell defects (Amanna et al., 2003). Similar findings were reported for *BCL2* transgene expression in A/WySnJ mice (Rahman and Manser, 2004). Together, these data support a role for the BAFF-R in B cell survival and CD21/35 control.

The interpretation of the A/WySnJ data is confounded by the complex nature of the BAFF-R mutation. A spontaneous retrotransposon insertion into the *Baffr* gene generated the *Bcmd-1* (*B cell maturation defect-1*) allele (Amanna et al., 2003). The insertion replaced the eight C-terminal amino acid (aa) residues 168-175 of the BAFF-R with 21 retrotransposon-encoded residues (Thompson et al., 2001; Yan et al., 2001). Although these eight residues are part of a 25-aa region that is completely conserved between human and mouse (Thompson et al., 2001), they were disordered in electron density maps and are considered to have no signaling role (Ni et al., 2004). The *Bcmd-1*-encoded BAFF-R is 95% identical to wt BAFF-R at the amino acid level, is correctly expressed in B cell development, and binds BAFF (Shulga-Morskaya et al., 2004; Thompson et al., 2001).

The *Bcmd-1* mutation decreased peripheral B cell survival causing B lymphopenia (Amanna et al., 2001; Miller and Hayes, 1991), diminished the relative maturity of the surviving B cells (Amanna et al., 2003; Rahman and Manser, 2004), and reduced the T-dependent IgG responses (Miller et al., 1992) through an adverse effect on the germinal center reaction (Rahman et al., 2003). Although *Bcmd-1* was widely considered to be a complete loss-of-function allele (Kayagaki et al., 2002; Mackay et al., 2005), we documented phenotypic and functional differences between the *Bcmd-1*-mutant A/WySnJ mice and congenic AW.*Baffr*^{-/-} mice that must be due to retention of partial function and an absence of dominant-negative effects (Mayne et al., 2009). The most striking difference was development of a systemic lupus erythematosus-like syndrome in the *Bcmd-1*-mutant A/WySnJ mice, but not the congenic AW.*Baffr*^{-/-} mice (Mayne et al., 2008).

The present research sought to define the signaling capabilities of the BAFF-R cytoplasmic domain, particularly the C-terminal 8 aa residues, to better understand why the *Bcmd-1*-expressed mutant receptor failed to support CD21/35 expression (Amanna et al., 2003), and drove a lupus-like autoimmune syndrome in A/WySnJ mice (Mayne et al., 2008). Since B lymphopenic and CD21/35-deficient A/WySnJ mice lack aa 168-175, we hypothesized that the C-terminal 8 aa residues control CD21/35 expression and B cell survival. We tested this hypothesis by performing complementation studies in A/WySnJ mice. Wild-type and mutated *Baffr* genes were retrovirally transduced into A/WySnJ HSC, and the transduced HSC-derived B lymphocytes were compared to *Bcmd-1* mutant B lymphocytes within A/WySnJ host mice. Our results show unequivocally that CD21/35 control resides within the C-terminal 8 aa domain, whereas this region is largely dispensable for B cell survival.

Materials and Methods

Mice

Male and female A/J, A/WySnJ, and (A/WySnJ X BCL2)F2 mice were produced in our specific pathogen-free mouse colony in the Department of Biochemistry, University of Wisconsin-Madison. The mice were maintained at 23°C with 40–60% humidity and 12 h light-dark cycles. Mice were age 7–10 wks. The protocols were approved by the Institutional Animal Care and Use Committee. A/WySnJ mice were bred to C57BL/6-TgN(BCL2)22Wehi mice (The Jackson Laboratory, Bar Harbor, ME), which carry a *BCL2* transgene expressed in B lineage cells (Strasser et al., 1991). F1 mice were intercrossed to produce (A/WySnJ X BCL2)F2 mice. DNA isolated from tail sections was genotyped for the presence of the *BCL2* transgene and *Baffr* genotype through PCR as previously described (Mayne et al., 2008; Strasser et al., 1991).

B cell staining and FACS analysis

Flow cytometric analysis was performed as previously reported (Mayne et al., 2008). In short, duplicate samples (10⁶ cells/sample) of RBC-depleted splenocytes were stained with titrated

amounts of mAb conjugates. Stained samples were analyzed on a FACScalibur™ using CELLQuest™ software (BD Biosciences, Franklin Lakes, NJ). The streptavidin-APC and streptavidin-PercP-Cy-5.5 secondary labels, the PE- and biotin-coupled rat mAb to mouse CD23 (Clone B3B4), and the FITC-coupled rat mAb to mouse CD21/35 (Clone 7G6) were obtained from BD Biosciences. The APC-goat polyclonal Ab to mouse IgM, and the FITC-, PE-, APC- and biotin-rat IgG_{2B} and IgG_{2A} isotype control mAb were obtained from Caltag Laboratories (Burlingame, CA). PE-coupled rat mAb to mouse CD21/35 was a generous gift from Dr. John Kearney (University of Alabama, Birmingham, AL).

MigRI retroviral vectors

The wild-type *Baffr* gene was PCR amplified from an A/J mouse splenic cDNA library. Deep Vent DNA polymerase (New England Biolabs, Beverly, MA) was used to amplify basepairs 8 to 589 of the BAFF-R mRNA sequence (GenBank ID 15208476; forward primer 5'-GCCCAGACTCGGAAGTGT-3' and reverse primer 5'-AGCCTCCACTGCTGCTATTG-3'). This fragment was cloned into the pCRII-TOPO vector using the TOPO TA PCR Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated and sequenced at the UW-Madison Biotechnology Center's DNA Sequence Laboratory. Inserts from error-free clones were excised with EcoRI and subcloned into the EcoRI site of the MigRI vector's multiple cloning site (Pear et al., 1998). Site-directed mutations of *Baffr* were created using PCR and a Quick-Change PCR kit (Stratagene).

Retroviral transduction and chimeric mice

The various MigRI retroviral particles were produced and A/WySnJ hematopoietic stem cells (HSC) were transduced as previously described (Aman et al., 2003; Cariappa et al., 2001). Briefly, purified vector DNA (Wizard *Plus* Maxipreps; Promega, Madison, WI) was CaPO₄ precipitated onto 80–90% confluent monolayers of the Bosc23 or Phoenix-Eco packaging cells grown in IMDM supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 100 U/ml penicillin/streptomycin, and 2 mmol/L L-glutamine. Retroviral supernatants were collected 48 to 72 hrs later, stored at –70 °C, and titered on NIH/3T3 cells.

To produce chimeric mice, the A/WySnJ donors were injected intravenously with 5-fluorouracil (5 mg in 400 µl PBS; Sigma-Aldrich, St. Louis, MO). Five days later, bone marrow cells were collected and cultured (10–20×10⁶ cells) in IMDM (4 mL) supplemented with 15% heat-inactivated FBS, 5% WEHI-3B conditioned medium, 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine, recombinant mouse IL-3 (6 ng/mL), recombinant mouse IL-6 (10 ng/mL), and recombinant mouse stem cell factor (100ng/mL; all from Peprotech, Rocky Hill, NJ). After 24 h of culture, the medium was removed, and two sequential retroviral transductions separated by 24 h were performed by inoculation and centrifugation as described (Pear et al., 1998). The transduced cells were collected 4 h after the second spinoculation, resuspended in PBS, and 0.5×10⁶ to 1×10⁶ cells were injected i.v. into each lethally irradiated A/WySnJ mouse. Lethal irradiation (1100 Rads in two doses, separated by 4 h) was done just prior to bone marrow cell transfer. Splenocytes were collected and analyzed 11–12 wks later.

Results

Survival gene expression failed to restore optimal CD21/35 expression in *Bcmd-1* B cells

The *Bcmd-1* mutation consists of a truncation and an addition of 21 retrotransposon-encoded residues as illustrated in Fig. 1A. The mutant BAFF-R signaling defects might be due to the added residues, including a stretch of Arg, His, and Lys residues that could disrupt protein interactions through charge-charge repulsion. Alternatively, the 8 truncated residues might harbor an unidentified signaling motif.

As a first step toward determining why the *Bcmd-1* mutant receptor failed to support CD21/35 expression, we confirmed that BAFF-R-mutant A/WySnJ B cells expressed less CD21/35 than B cells from the closely-related A/J strain. IgM-stained splenic B cells were gated on the FO B cell population and evaluated for CD21/35 expression (Fig. 2). As expected, the mature wt B cells had a CD21/35 mean fluorescence index (MFI) that was 118 ± 38 units higher than the *Bcmd-1* B cells. We also confirmed that complementing *Bcmd-1* with a B cell survival signal was sufficient to restore B cell development, but not sufficient to restore optimal CD21/35 expression (Amanna et al., 2003; Rahman and Manser, 2004; Tardivel et al., 2004). Mice from an (A/WySnJ X *BCL2*)F2 intercross were genotyped and analyzed for CD21/35 expression. In the absence of the *BCL2* transgene, the B cell subset proportions were similar between homozygous *Baffr* mice and A/J mice, and between homozygous *Bcmd-1* mice and A/WySnJ mice, as expected (Fig. 3A, Table I). In the presence of the *BCL2* transgene, the homozygous *Bcmd-1* mice had increased FO B cells, decreased NF B cells, and decreased MZ B cells, as reported (Rahman and Manser, 2004). Also as reported, the *BCL2* transgene decreased the MZ B cells, did not change the FO or NF B cells in the homozygous *Baffr* mice. The homozygous *Baffr* B cells had a 2-fold higher CD21/35 MFI than homozygous *Bcmd-1* B cells (Fig. 3B, Table I).

We next extended our analysis of FO *Bcmd-1* B cells expressing the *bcl-x_L* survival gene from a MigRI retroviral vector in AW.*bcl-x_L* mice (Amanna et al., 2003). We previously reported that the retrovirally transduced *bcl-x_L* gene complemented *Bcmd-1* with respect to FO B cell development. Here, we found that *bcl-x_L* failed to complement *Bcmd-1* with respect to optimal CD21/35 expression, since the FO B cells from AW.*bcl-x_L* mice had significantly lower CD21/35 expression than the FO B cells from A/J mice, whether they were transduced with the MigRI virus carrying the *bcl-x_L* survival gene or the empty vector MigRI control virus (data not shown). These data show that restoring *Bcmd-1* B cell development through *BCL2* or *bcl-x_L* survival gene expression was not sufficient to enable optimal CD21/35 expression.

BAFF-R residues 103-175 regulate B cell survival and CD21/35 expression

To define the signaling capabilities of BAFF-R cytoplasmic domains more precisely *in vivo*, we used the MigRI retrovirus system to transduce *Baffr* genes with defined mutations into A/WySnJ HSC, transplanted the HSC into lethally-irradiated A/WySnJ recipients, and evaluated B lymphocyte development and CD21/35 expression. Using A/WySnJ mice as recipients for these experiments raises a concern about interference from the endogenous mutant BAFF-R proteins in the retrovirally-transduced B cells. We attempted to use B6.*Baffr*-ko mice lacking endogenous BAFF-R proteins as recipients (Sasaki et al., 2004), but the combination of extremely poor MigRI transduction efficiencies, coupled with the virtual absence of B lymphocytes in the mice, made this approach untenable. Since we had previously shown that *Bcmd-1* is a hypomorphic partial loss-of-function allele and does not act in dominant-negative fashion (Mayne et al., 2009), we used A/WySnJ recipients where retroviral transduction efficiency was typically ~5–20% and each recipient had some B cells for analysis. This approach allowed us to evaluate each mutant *Baffr* construct for complementation of *Bcmd-1*, because recipient mice had B cells that were a mixture cells expressing only *Bcmd-1* and cells expressing *Bcmd-1* plus the desired *Baffr* mutation.

The MigRI retroviral vector used in these experiments expresses the gene of interest on a bicistronic mRNA containing an internal ribosomal entry site (IRES) and encoding green fluorescent protein (GFP) as a marker of successful gene transfer (Fig. 1B) (Pear et al., 1998). We first created retroviral vectors representing the full-length transcript (MigRI.*Baffr*), and a naturally occurring mRNA splice variant (MigRI.*Baffr* Δ 118-128), a full cytoplasmic domain truncation mutant (MigRI.*Baffr* Δ 103-175), and an empty MigRI vector to use as a negative control (Fig 1C). Each of these vectors was used to transduce A/WySnJ

HSC, which were then transferred into irradiated recipient A/WySnJ mice. After 11–12 wk of hematopoietic cell reconstitution, the splenocytes were immunostained and analyzed by flow cytometry for IgM, GFP, and BAFF-R protein expression. The AW.*Baffr*, AW.*Baffr* Δ 118-128, and AW.*Baffr* Δ 103-175 splenic B cells all showed a robust correlation between increasing GFP and increasing BAFF-R immunofluorescence, indicating that these vectors encoded a BAFF-R protein that was correctly expressed on the B-cell surface (data not shown).

The CD21/23 gating scheme was then used to analyze the IgM⁺GFP⁺ and IgM⁺GFP[−] B cell populations (Oliver et al., 1999). For all retroviral constructs tested, B cell phenotypes were confirmed using the IgM/IgD (Cariappa et al., 2001), and IgM/C1qRp/CD23 (Allman et al., 2001) gating schemes (data not shown). The data from the CD21/23 gating scheme are shown to highlight changes in CD21/35 expression (note that a CD21-PE antibody was used for Table II in contrast to a CD21-FITC antibody for Table I, thus absolute MFI values between the tables differ). There were no significant differences in the B cell subset percentages or the FO B cell CD21/35 MFI between GFP⁺ and GFP[−] B cells from the recipients of MigRI-transduced HSC, indicating that expression of the empty retroviral vector had no impact on B cell development (Fig. 4, Table II). However, there was a 58% decline in the NF B cell percentage, a 1.5-fold increase in the FO B cell percentage, and a 1.8-fold increase in FO B cell CD21/35 MFI between GFP⁺ and GFP[−] B cells in the recipients of HSC transduced with the MigRI.*Baffr* vector (Fig. 4, Table II). Similar results were obtained with the MigRI.*Baffr* Δ 118-128 vector (Table II). These changes were not observed with the MigRI.*Baffr* Δ 103-175 vector (Fig. 5, Table II), which encoded a mutant protein lacking a cytoplasmic domain, even though this protein attained cell surface expression and retained the regions necessary to bind BAFF. Collectively these data establish that retroviral-mediated transfer of the *Baffr* gene (and the splice variant) complemented *Bcmd-1* for B cell development and CD21/35 expression, and further, that the BAFF-R residues 103-175 were essential for these survival and maturation signals.

BAFF-R residues 168-175 are essential for optimal CD21/35 expression but not B cell survival

To test the hypothesis that the 8 truncated aa residues might have an essential role in BAFF-R signaling, we produced AW.*Baffr* Δ 168-175 mice using retroviral gene transduction as before (Fig. 1C), and analyzed their splenocytes by flow cytometry. The close correlation between increasing GFP expression and increasing BAFF-R protein expression in B cells from these mice again verified that vector-encoded BAFF-R proteins were expressed on the B-cell surface (data not shown). Surprisingly, transduction of the *Baffr* Δ 168-175 mutant yielded a 35% decline in the NF B cell percentage and a 1.2-fold increase in the FO B cell percentage, but no change whatsoever in FO B cell CD21/35 MFI when GFP⁺ B cells were compared to GFP[−] B cells (Fig 5, Table II). This result was unexpected, because a previous report did not implicate this region in TRAF3 binding (Ni et al., 2004). We conclude that aa 168-175 have an essential role in BAFF-R-mediated signaling for optimal CD21/35 expression, although these residues appear to contribute little to B cell survival signaling.

BAFF-R residues 168-175 may harbor a signaling motif

Because previous experiments did not implicate BAFF-R residues 168-175 in TRAF3 binding (Ni et al., 2004; Xu and Shu, 2002), and TRAF3 was the only adapter protein involved in BAFF-R survival signaling (Claudio et al., 2002; Kayagaki et al., 2002), we reasoned that the displaced BAFF-R residues 168-175 might harbor a previously unidentified signaling motif for CD21/35 upregulation. We noted that this conserved BAFF-R region shares some homology with the CD40 receptor (Amanna et al., 2003), another member of the tumor necrosis factor receptor (TNFR) family that is critical for B cell antigen responses. Among the homologous residues were several Thr, known to be common phosphorylation targets, and a Pro, known to be important in protein folding. Accordingly, to look for an unidentified

signaling motif, we elected to mutate Thr170 and Pro173, and evaluate the mutants for possible disruption of BAFF-R-mediated signaling. We hypothesized that one or both of these point mutants would fail to complement *Bcmd-1* for optimal CD21/35 expression.

The MigRI.*BaffrT170A* and MigRI.*BaffrP173A* retroviral vectors were generated through site-directed mutagenesis, AW.*BaffrT170A* and AW.*BaffrP173A* mice were created, and flow cytometry was performed on GFP⁺ and GFP⁻ splenic B cells as described above. The robust correlation between increasing GFP fluorescence and increasing BAFF-R protein expression indicated proper surface expression of these mutant BAFF-R proteins on splenic B cells (data not shown). The *BaffrT170A* and *BaffrP173A* mutants yielded 31% and 45% declines, respectively, in the NF B cell percentages, and 1.3-fold increases in the FO B cell percentages (Fig. 6, Table II). The *BaffrP173A* mutant also supported a highly significant 1.7-fold increase in FO B cell CD21/35 intensity, similar to the increase seen with wt *Baffr* transduction. Contrastingly, the *BaffrT170A* mutant supported a very weak 1.2-fold increase in CD21/35 intensity (Fig. 6, Table II). Thus, both point mutants supported B cell survival, but only the *BaffrP173A* mutant fully supported optimal CD21/35 expression. We interpreted these data as suggesting that the displaced BAFF-R residues 168-175 harbor a previously unidentified signaling motif for CD21/35 upregulation, and suggest that Thr170 is part of the motif.

The effects of all the expressed mutations on B cell survival and CD21/35 expression are summarized in Fig. 7. For this summary, we first calculated the difference between the GFP⁺ and GFP⁻ cell populations within each mouse, and subsequently calculated the mean values and S.D. shown in Fig. 7. This allows a direct comparison to be made between transduced and untransduced cells within the same experimental animal. Overall, our data suggest that the BAFF-R signal bifurcation occurs at the level of the receptor, with aa 103-167 signaling primarily B cell survival and aa 168-175, particularly Thr170, signaling CD21/35 upregulation.

Discussion

The new data presented here provide the first direct evidence that the BAFF-R signal transduction pathway may be bifurcated at the receptor level to regulate B cell survival and CD21/35 expression independently. Analysis of B cell development and CD21/35 expression in *Baffr* or *Bcmd-1* homozygous (A/WySnJ X B6.*BCL2*)F2 mice and in AW.*bcl-x_L* mice confirmed that the survival gene expression complemented the signaling-impaired *Bcmd-1* allele with respect to B cell survival, but not CD21/35 expression (Amanua et al., 2003; Rahman and Manser, 2004; Tardivel et al., 2004). These results are consistent with a bifurcation in the BAFF-R pathway, but the complexity of the *Bcmd-1* mutation and the fact that *BCL2* transgene expression altered FO and MZ B cell development in normal mice confounds this interpretation.

We extended the analysis by performing retroviral gene transduction studies in *Bcmd-1* mice. This approach allowed us to test specific mutations *in vivo*, and to compare transduced with untransduced cells within individual animals. Our *in vivo* experiments established that the survival and CD21/35 enhancement signals emanated from the BAFF-R cytoplasmic domain, since the wt *Baffr* gene and the natural *BaffrΔ118-128* splice variant supported B cell development and optimal CD21/35 expression, whereas the *BaffrΔ103-175* deletion mutant did not. The *BaffrΔ168-175* variant, which lacks the residues that were displaced by the retrotransposon in the *Bcmd-1* allele, failed to support optimal CD21/35 expression. This was surprising since the deleted residues were previously thought to have no signaling role (Ni et al., 2004). The *BaffrT170A* mutant similarly failed to support optimal CD21/35 expression. Thus, our data establish that the C-terminal 8 residues of the BAFF-R, particularly Thr170,

contribute significantly to optimal CD21/35 expression, although they appear to be less important for survival signaling.

Survival signals emanating from the BAFF-R are transmitted by the non-canonical NF- κ B2 pathway (Claudio et al., 2002; Kayagaki et al., 2002). This pathway begins with NF- κ B-inducing kinase (NIK) and leads ultimately to enhanced transcription of the *Bcl-2* and *Bcl-x_L* pro-survival genes (Mackay et al., 2007). BAFF binding causes BAFF-R monomers to undergo oligomerization and bind TRAF3 (Claudio et al., 2002; Kayagaki et al., 2002). TRAF3 binding to the BAFF-R cytoplasmic domain prevents TRAF3-mediated NIK degradation, allowing NIK to activate non-canonical NF- κ B2 (Liao et al., 2004; Sasaki et al., 2008). Previous studies reported TRAF3 binding to BAFF-R via a PVPAT motif (murine aa 154-158, human aa 162-166) (Ni et al., 2004), so it was not surprising that the *Baffr* Δ 168-175 variant supported B cell survival. However, our data present a paradox. The *Bcmd-1*- and *Baffr* Δ 168-175-encoded receptors retain the PVPAT motif, but only the *Baffr* Δ 168-175-encoded receptor signaled B cell survival. The explanation for this paradox may relate to the fact that the partial loss-of-function protein expressed by *Bcmd-1* contains a region of 8 retrotransposon-encoded amino acids (RPHIRRHK) with a high positive charge density at the C-terminus (Amanna et al., 2003). We speculate that the *Bcmd-1*-encoded protein signals survival poorly because this positively-charged region of the retrotransposon-encoded tail impedes receptor oligomerization or TRAF3 binding.

An important question remaining to be answered is how the C-terminal 8 residues of the BAFF-R, particularly murine Thr 170, contribute to optimal CD21/35 expression. Our retroviral *Baffr* gene transfer data show unequivocally that optimal CD21/35 expression is under BAFF-R-mediated control, confirming *Baffr* gene ablation data from others (Sasaki et al., 2008; Sasaki et al., 2004). Recent research has shown that TRAF2 and TRAF3 cooperate in BAFF-R-mediated signaling, with TRAF2 signaling degradation of TRAF3, eliminating TRAF3-mediated suppression of NIK (Gardam et al., 2008). In those studies, B cell-specific deletion of either TRAF2 or TRAF3 resulted in BAFF-R-independent survival and maturation of B lymphocytes, including enhancement of CD21/35 expression. Thus, from the perspective of non-canonical NF- κ B2 signaling, the B cell survival and CD21/35 maturation phenotypes appear linked. It is possible that BAFF-R residues 168-175 are required for full NF- κ B2 pathway activation, and that suboptimal levels of NF- κ B2 signaling in the *Baffr* Δ 168-175 mutant support survival but not optimal CD21/35 expression. This single pathway hypothesis is consistent with our data comparing the wt *Baffr* to the *Baffr* Δ 168-175 mutant with respect to B cell development.

An alternative possibility is that the BAFF-R-derived signals for optimal CD21/35 expression depend on a second pathway that utilizes aa 168-175. The finding that NF- κ B proteins are located at the CD21/35 (*Cr2*) gene promoter of the CD21-deficient A/WySnJ mouse suggests that non-NF- κ B signals may be necessary for full CD21/35 expression (Debnath et al., 2007). In this context, it is intriguing that a motif search of the BAFF-R cytoplasmic domain identified the TTKT sequence (murine aa 167-170, human aa 175-178) within the deleted region as a potential target site for protein kinase C (PKC)-mediated phosphorylation. It is possible that phosphorylation of the TTKT sequence, possibly by PKC, may be needed for optimal CD21/35 protein expression. Interestingly, PKC-beta interacts with components of the NF- κ B pathway, influencing NF- κ B signaling (Guo et al., 2004). It is also noteworthy that B cell receptor signal transduction activates PKC-delta, and that PKC-delta is specifically required for transitional B cell tolerance induction (Guo et al., 2004), because the *Bcmd-1*-encoded BAFF-R lacks the TTKT motif and drives the development of a lupus-like systemic autoimmune syndrome in A/WySnJ mice (Mayne et al., 2008; Mayne et al., 2009). The TTKT motif may also play a role in BAFF-R binding to TRAF3, since murine BAFF-R Thr167 (human Thr175) was necessary for this binding (Ni et al., 2004). We suggest that the BAFF-R cytoplasmic TTKT sequence

may be a target site for PKC-delta or PKC-beta-mediated phosphorylation, and further, that such phosphorylation may be part of the cross-talk between the B cell receptor and the BAFF-R receptor that determines transitional B cell tolerance induction *via* fine control of B cell survival.

BAFF-R provides the predominant survival signal to B cells in the periphery and also drives expression of the B cell co-receptor CD21/35 on these cells. This receptor thus promotes B cell survival for maintenance of the B lymphocyte pool, and also provides essential signals for coupling the complement system of the innate immune response to B cell activation (Cariappa et al., 2001; Fearon and Carroll, 2000). Our new data show that the survival signals and CD21/35 upregulation signals clearly derive from the cytoplasmic domain of the BAFF-R, but may emanate from distinct regions. Moreover, our new data raise the possibility that a previously unrecognized signaling motif resides in the C-terminal 8 residues that were previously thought to be structurally and functionally insignificant (Ni et al., 2004). We hypothesize that murine residues 167-170, TTKT, constitute a potential PKC phosphorylation site that is linked to BAFF-R signaling for CD21/35 maturation, and may also be linked to the systemic autoimmune phenotypes we observed in A/WySnJ mice (Mayne et al., 2008; Mayne et al., 2009). Further experiments are underway to evaluate the phosphorylation status of the TTKT motif and the TRAF binding capabilities of wild-type and mutant BAFF-R proteins in the expectation that the results will provide insights into how B cell signaling pathways are integrated to exert fine control over B cell survival and tolerance induction.

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Abbreviations used in this paper

aa	amino acid(s)
BAFF	B cell activating factor belonging to the TNF family
BAFF-R	BAFF receptor
Bcmd	B cell maturation defect
FO	follicular
MZ	marginal zone
MFI	mean fluorescent intensity
NF	newly formed
SLE	systemic lupus erythematosus
TNF	tumor necrosis factor
TNFR	TNF receptor

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Figure 1.

The MigRI retroviral vector enables *in vivo* dissection of BAFF-R cytoplasmic domains. **A**, Amino acid sequence of the cytoplasmic domain of murine wild-type BAFF-R and mutant BCMD-1 protein. Bold type represents residues added by the retrotransposon insertion. Underlined text represents the TRAF3-binding motif. Boxes outline the regions 118–128 and 168–175 targeted in deletion mutants. The T170 and P173 residues replaced with alanine in the point mutations are denoted with arrows. **B**, Schematic representation of the MigRI retroviral vector used for the *in vivo* gene transduction experiments. **C**, The panel of *Baffr* cytoplasmic domain mutations tested.

Figure 2.

BAFF-R-mutant A/WySnJ B cells are fewer, more immature, and express lower levels of CD21/35 than A/J B cells. *A*, A/WySnJ and control A/J splenocytes were stained with antibodies to IgM, CD21/35, and CD23. Total B cell percentage was determined by the IgM⁺ live lymphocytes. *B*, IgM⁺ splenocytes from A were gated into NF, FO, and MZ B cell subsets on the basis of their CD21/35 and CD23 expression as described [Aman, 2003 #27]. *C*, The CD21/35 surface expression on mature FO B cells in the CD21/35⁺CD23⁺ gate was quantified as fluorescence intensity. Shown are representative results from 12–15 mice per strain.

Figure 3.

The wild-type *Baffr* gene controls optimal FO B cell CD21/35 surface expression independently of FO B cell survival. **A**, Splenocytes from (A/WySnJ X *BCL2*)F2 mice were stained with antibodies to IgM, CD21/35, and CD23, and the IgM⁺ live lymphocytes were gated into NF, FO, and MZ B cell subsets as in Figure 1. *Baffr^{+/+}* indicates mice homozygous for the wild-type allele, whereas *Bcmd-1* indicates mice homozygous for the A/WySnJ mutant allele. The *BCL2⁺* indicates *BCL2* transgene expression, whereas *BCL2⁻* indicates no *BCL2* transgene expression. **B**, The CD21/35 surface expression on mature FO B cells was quantified as fluorescence intensity. The results shown are representative of 4–7 mice per strain.

Figure 4.

Expressing a wild-type BAFF-R in the BAFF-R-mutant A/WySnJ B cells restored FO B cell survival and increased the CD21/35 surface expression. *A*, Splenocytes from A/WySnJ mice that were given bone marrow transduced with the empty MigRI retroviral vector (*AW.MigRI* mice) or with MigRI carrying the wild-type *Baffr* gene (*AW.Baffr* mice) were stained with antibodies to IgM, CD21/35, and CD23, and gated into IgM⁺GFP⁻ (untransduced) and IgM⁺GFP⁺ (transduced) B cell subsets. *B*, The IgM⁺GFP⁻ and IgM⁺GFP⁺ B cells were gated into NF, MZ and FO B cell subsets as in Figure 1. *C*, The CD21/35 surface expression on mature FO B cells in the GFP⁻ and GFP⁺ gates was quantified as fluorescence intensity. Shown are representative results from 4–5 mice per strain analyzed in three separate experiments.

Figure 5.

BAFF-R residues 168-175 are necessary to signal optimal CD21/35 expression but not FO B lymphocyte development. *A*, Splenocytes from *AW.Baffr Δ 103-175* and *AW.Baffr Δ 168-175* retroviral mice were stained for flow cytometry and gated into transduced (IgM⁺GFP⁺) and untransduced (IgM⁺GFP⁻) B cell subsets as in Figure 4. *B*, The IgM⁺GFP⁻ and IgM⁺GFP⁺ B cells were gated into NF, MZ and FO B cell subsets as in Figure 1. *C*, The CD21/35 surface expression on mature FO B cells in the GFP⁻ and GFP⁺ gates was quantified as fluorescence intensity. Shown are representative results from 5–6 mice per strain analyzed in three separate experiments.

Figure 6.

The P173A but not the T170A BAFF-R mutant signals optimal CD21/35 expression. *A*, Splenocytes from *AW.BaffrT170A* and *AW.BaffrP173A* retroviral mice were stained for flow cytometry and gated into transduced (IgM⁺GFP⁺) and untransduced (IgM⁺GFP⁻) B cell subsets as in Figure 4. *B*, The IgM⁺GFP⁻ and IgM⁺GFP⁺ B cells were gated utilizing the CD21/CD23 gating scheme as in Figure 1. *C*, The CD21/35 surface expression on mature FO B cells in the GFP⁻ and GFP⁺ gates was quantified as fluorescence intensity. Shown are representative results from 6–7 mice per strain analyzed in three separate experiments.

Figure 7.

BAFF-R residues 168-175 are dispensable for B cell development, but essential for optimal CD21/35 expression. *A*, Change in FO B cell percentages (GFP⁺ - GFP⁻) signaled *in vivo* by BAFF-R cytoplasmic domain mutants. *B*, Change in CD21/35 expression (GFP⁺ - GFP⁻) signaled *in vivo* by BAFF-R cytoplasmic domain mutants. Change in FO B cell percent or CD21/35 expression was calculated by the difference between the GFP⁺ and GFP⁻ population of cells within each mouse. The values represent a direct comparison of effects of transduced mutations (GFP⁺) as compared to the internal control cells (GFP⁻) of the same experiment. Shown are combined results from all 39 retroviral mice analyzed for this study.

Table I

The *Baffr* gene controlled optimal CD21/35 surface expression independently of B cell survival in (A/WySnJ X *BCL2*)F2 mice.

	Percentage of IgM ⁺ splenic B cells and CD21/35MFI as a function of (A/WySnJ X <i>BCL2</i>)F2 genotype ^a			
	<i>BCL2</i> ⁻		<i>BCL2</i> ⁺	
	<i>Bcmd-1</i>	<i>Baffr</i>	<i>Bcmd-1</i>	<i>Baffr</i>
B cell subset % ^b				
NF	41.2±10 ^{*,#}	17.8±1.4 [*]	31.4±8.1 [#]	28.8±5.5
FO	45.8±8.9 ^{*,#}	70.4±5.2 [*]	64.8±8.9 [#]	70.2±5.3
MZ	9.9±3.1 [#]	10.8±4.1	1.6±1.6 [#]	1.2±0.4
CD21/35 MFI ^c				
FO	22±7 [*]	52±8 [*]	19±8	32±2

^aThe (A/WySnJ X *BCL2*)F2 mice were genotyped for the *BCL2* transgene and *Baffr* allele (*Bcmd-1* = A/WySnJ allele, *Baffr* = wild-type allele). Flow cytometry was performed. IgM⁺ B cells were gated and subdivided according to the IgM/CD23/CD21) gating scheme.

^bThe values represent the mean percentage (± SD) of the IgM⁺ B cells (4–11 mice/group).

^cThe values represent the mean CD21/35-FITC MFI (± SD) for FO B cells (4–7 mice/group).

^dThe Mann-Whitney test gave $p < 0.05$ for comparisons between *BCL2*⁻ *Bcmd-1* and *BCL2*⁻ *Baffr* (^{*}) and *BCL2*⁻ *Bcmd-1* and *BCL2*⁺ *Bcmd-1* ([#]) B cell subsets.

Table II

Summary of mutational analyses of BAFF-R in A/WySnJ retroviral mice^a

Retroviral Construct	NF B cell %		FO B cell %		FO B cell CD21 MFI ^b	
	GFP ⁻	GFP ⁺	GFP ⁻	GFP ⁺	GFP ⁻	GFP ⁺
empty MigRI	41.1±1.8	37.5±1.8	51.1±2.7	54.2±2.9	114±10	119±7
MigRI.Baffr	50.6±3.5	21.3±2.7**	40.8±3.3	61.2±2.3**	110±20	197±41*
MigRI.BaffrΔ118-128	31.8±1.2	7.7±1.2**	61.0±2.0	82.8±3.1**	152±6	213±15**
MigRI.BaffrΔ103-175	34.0±3.7	31.6±4.2	57.8±3.0	58.8±2.5	103±5	99±2
MigRI.BaffrΔ168-175	33.9±1.5	22.2±1.2**	56.6±2.3	67.8±2.6**	100±5	106±6
MigRI.BaffrT170A	43.1±2.8	29.6±3.7**	47.2±3.6	59.4±4.3**	172±18	201±19*
MigRI.BaffrP173A	47.2±4.7	26.0±4.8**	45.5±5.6	60.2±6.5**	165±27	280±45**

^aThe * indicates that the GFP⁺ and GFP⁻ cell populations differed significantly (p<.05) and

** indicates that the GFP⁺ and GFP⁻ cell populations differed significantly (p<.01) as in Table I

^bNote: a CD21-PE antibody was used for these studies, whereas a CD21-FITC antibody was used for Table I experiments. Thus, CD21 MFI values cannot be compared directly between the two experiments, only expression patterns.