Published in final edited form as:

Leuk Lymphoma. 2014 August ; 55(8): 1884–1892. doi:10.3109/10428194.2013.862619.

BAFF-R Specific Activation of TRAF6 and the PI3K-Pathway in Lymphoma B Cells

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Abstract

BAFF-R is the primary BAFF receptor that is responsible for promoting B-cell development and survival. Malignant B-cells exploit the BAFF/BAFF receptor system and high serum BAFF levels or genetic alterations in BAFF receptors have been found in B-cell cancers. BAFF signaling impacts pro-survival pathways, however, other than NF- κ B2, little is known about the specific pathways activated by individual BAFF receptors. Using a novel BAFF-R expression model we now demonstrate that activation of BAFF-R, independent of TACI and BCMA, can induce phosphorylation of Akt and GSK3 β . Expression of an activated form of BAFF-R also enhanced a pro-survival gene expression pattern, including the novel BAFF-regulated gene Pin1, whose expression was PI3K-dependent. Additionally, we show that TRAF6 is essential for mediating BAFF-R-dependent activation of Akt. Together these data describe a novel role for TRAF6 in BAFF-R-specific activation of the PI3K pathway and provide evidence suggesting a new role for Pin1 in BAFF-R signaling.

Keywords

BAFF-R; BAFF; lymphoma; PI3-kinase

Introduction

Altered regulation of B-cell proliferation and survival is the basis for the onset of B-cell disorders, including lymphoma. After the B-cell receptor (BCR), B-cell activating factor (BAFF, BLyS) signaling ranks as the single most critical pathway for B-cell growth and development [1]. BAFF is a member of the TNF family of cytokines (*TNFSF13B*) and functions by binding to one of three receptors, B-cell maturation antigen (BCMA), transmembrane activator and cyclophilin ligand interactor (TACI) and BAFF-R [2–4]. The

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Author Contribution

F.J.S and A.J.N designed the study, performed experiments, and wrote the manuscript. M.K.M, T.P.T, S.Z., and L.S.H. performed experiments and analyzed data. J.R.C. and S.M.A. helped design the study and reviewed the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

expression pattern of these receptors varies in normal and pathological B-cell populations, and the presence of any combination of BAFF receptors is known to be dependent upon the state of B-cell maturation [5–8]. Mouse studies indicate, however, that BAFF-R is the receptor that is essential for B-cell maturation, clearly illustrated by the near complete loss of mature B-cells in both BAFF-R^{-/-} and BAFF^{-/-} mice [9–11]. Moreover, patients harboring an inactivating BAFF-R mutation display mild hypogammaglobulinemia, which was akin to the reduction in IgG observed in BAFF-R^{-/-} mice [12].

Studies conducted in our laboratory and elsewhere have demonstrated a significant role for BAFF in non-Hodgkin lymphoma (NHL), as BAFF is highly expressed in the NHL tumor microenvironment and elevated BAFF levels correlated with aggressive disease [8,13–16]. We also recently reported the discovery of a mutant form of BAFF-R, BAFF-R_{H159Y}, which is associated with NHL and whose expression increases overall NF-κB activity and IgM secretion [17]. Additionally, auto activation of BAFF-R is commonly observed in diffuse large B-cell lymphoma (DLBCL), and inhibition of BAFF signaling was demonstrated to result in a significant reduction in the growth of DLBCL derived tumors in xenograft model [7,18]. Together, these data support a central role for BAFF-R in malignant B-cell survival. BAFF-R mediated effects that augment normal and malignant B-cell survival have largely been attributed to activation of the non-canonical NF-κB2 pathway. However, BAFF has been shown to activate additional signaling pathways suggesting that BAFF-R may promote B-cell survival through additional mechanisms [19,20]. The ability of BAFF to bind and signal through three independent receptors has hampered our ability to decipher receptor-specific signaling. However, our recently generated BAFF-R specific cell line model confirms a role for BAFF-R mediated activation of NF-κB and now provides us with a useful tool for further characterization of BAFF-R signaling [17].

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is one of the most commonly dysregulated pathways described in human tumors [21]. BAFF-dependent activation of PI3K signaling in normal B-cells has been previously described and aberrant PI3K activity plays a fundamental role in many B-cell disorders including lymphoma [22–25]. However, little is known about the ability of BAFF-R to specifically activate this pathway independent of TACI and BCMA. Therefore, in order to elucidate the potential effects that BAFF-R may exert on the PI3K pathway, we investigated the role of BAFF-R in BAFF-mediated PI3K pathway activation. Our studies provide novel findings and show that BAFF-R specifically induces activation of Akt and GSK3β and we identify TRAF6 as a new component of the BAFF-R signaling pathway. Our studies also identify Pin1 as a novel target of BAFF-R activation. These studies contribute to our understanding of how BAFF-R functions in normal B cells and also suggests a potential mechanism of how malignant B cells exploit the BAFF/BAFF-R pathway.

Materials and Methods

Cell Lines

The Karpas 422 cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. The KAS-6/1 cell line was a gift from Dr. Diane Jelinek and described previously [26].

Cell Culture

HEK 293 empty vector (EV) and BAFF-R expressing cell lines were maintained in DMEM media (Gibco Life Technologies, San Francisco, CA) supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma Life Science, St. Louis, MO). KAS-6/1 BAFF-R_{WT} and Karpas 422 cells were maintained in RPMI supplemented with 10% FBS and 1% penicillin-streptomycin. KAS-6/1 BAFF-R_{WT} cultures were additionally supplemented with puromycin (300 ng/ml, Gibco Life Technologies) and IL-6 (1 ng/ml, PeproTech, Rocky Hill, NJ). Cells were placed in serum-free media (base media supplemented with 0.5% endotoxin free BSA, Sigma Life Science) for ~16 hrs. prior to treatment with inhibitors or BAFF. Specific cell culture conditions are described in the Results section and/or figure legends.

Inhibitors and Cytokines

Halt phosphatase inhibitor was purchased from Thermo Scientific (Waltham, MA) and protease inhibitor tablets were purchased from Roche Diagnostics GmbH (Indianapolis, IN). Wortmannin was purchased from Millipore (Billerica, MA) and BAFF from Enzo (Farmingdale, NY). Rapamycin was purchased from Sigma Aldrich (St. Louis, MO) and Torin 1 was purchased from Cayman Chemical (Ann Arbor, MI). Puromycin was purchased from Gibco Life Technologies.

Antibodies

HRP conjugated rabbit and mouse secondary antibodies were purchased from GE Healthcare (Waukesha, WI), while goat-HRP and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Akt, pAkt_{S473}, pAkt_{T308}, GSK3 β , pGSK3 β , MTOR and pMTOR_{S2481}, and pMTOR_{S2488} were purchased from Cell Signaling (Beverly, MA). HA-HRP was purchased from Roche (Indianapolis, IN), while anti-TRAF6 and BAFF-R FITC antibodies were purchased from Abcam (Cambridge, UK). IgG1 FITC was purchased from Becton Dickinson (Franklin Lakes, NJ), while IgG1 PE and TACI PE were purchased from R&D Systems (Minneapolis, MN).

Western Blots

Cells were stimulated with BAFF (200 ng/ml) for the indicated times prior to lysis. For the inhibitor assays, wortmannin treatment was initiated 1 hr. prior to stimulation with BAFF, and torin 1 and rapamycin treatment were started 24 hr. prior to BAFF stimulation. Western blots were performed as previously described [17]. Briefly, whole cell lysates were isolated in ice-cold RIPA buffer, quantified using the Bio-Rad (Hercules, CA) D_C Protein Assay and 50 μ g of protein was separated on Bio-Rad Criterion pre-cast gels. SDS-PAGE gels were transferred to Immobilon P 0.45 μ m PVDF (Millipore), blocked in 5% milk/TBST and incubated O/N with primary antibody at 4 °C. On the following day, blots were washed and incubated against secondary HRP conjugated antibodies for 45 minutes, washed and developed using the SuperSignal West Dura ECL kit (Pierce Protein Research Products, Thermo Scientific). Densitometry was performed on scanned blots using AlphaImager 3400 software (ProteinSimple, Santa Clara, CA).

Production and Isolation of Lentivirus Particles

Lentivirus particles were generated by transfecting HEK 293T cells with 9 µg of pGIPZ non-silencing shRNAmir, TRAF6 shRNAmir or pGIPZ BAFF-R_{WT} expressing plasmids and 28.5 µg of Trans-Viral Packaging Mix purchased from Open BioSystems, Thermo Scientific. The plasmid mixtures were combined with 187.5 µg of Arrest-In Transfection Reagent (Open BioSystems, Thermo Scientific, 5:1, Arrest-In to DNA), incubated for 20 minutes at RT and added drop-wise to 6 million logarithmically growing HEK 293T cells in 3 ml. of serum-free DMEM. Following a 6 hr. incubation, 3 ml. of DMEM supplemented with 20% FBS was added to the cultures. Culture media was harvested following a 48 hr. incubation and spun down (3,000g/15 min/4 °C) to remove cellular contamination. Lentiviral particles were concentrated (1:100) using the PEG-it Virus Precipitation Solution according to manufacturer's instructions (System Biosciences). The estimated lentiviral concentration (infectious units (ifu)/ml) was calculated using the Lentivector Titer Kit (Mellgen Laboratories, Surrey, BC, Canada) according to the manufacturer's instructions, except that the DNA intercalating agent EvaGreen (Biotium, San Francisco, CA) was used in place of syber green. Quantified lentiviral particles were either used immediately or stored at -80 °C.

Construction of Stable HEK 293 BAFF-R shRNAmir Cell Lines

The construction of HEK 293 cells stably transduced with pcDNA3.1 BAFF-R_{WT}, BAFF-R_{H159Y} and EV was previously described [17]. BAFF-R expressing HEK 293 cell lines were transduced with 10,000 ifu of lentiviral particles containing either non-silencing or TRAF6 shRNAmir. Briefly, 100,000 cells were plated per well of a 12-well plate and allowed to adhere O/N. On the following day, media was replaced with serum-free DMEM and lentiviral particles were added drop-wise to cells. Following a 6 hr. incubation in serum-free DMEM, FBS was added to a final concentration of 10%. GFP expression was detected within 24 hr. of transduction, and puromycin selection (1 µg/ml) began 72 hr. post-transduction. All HEK BAFF-R shRNAmir cell lines were passed 3 times following selection prior to being used for further experimentation.

Construction of Stable KAS-6/1 BAFF-R_{WT} Cell Line

HA-tagged BAFF-R_{WT} was subcloned into the pGIPZ empty vector (Thermo) expression plasmid, and subsequently used to generate BAFF-R_{WT} expressing lentiviral particles as described above. Two million KAS-6/1 cells were transduced with pGIPZ-BAFF-R_{WT} lentiviral particles (100,000 ifu) in serum-free RPMI supplemented with IL-6 (1 ng/ml) and 8 µg/ml polybrene (Sigma Life Sciences). Following a 6 hr. incubation in serum-free RPMI, FBS was added to a final concentration of 10% and replenished after 24 hr. (polybrene omitted). Puromycin selection (300 ng/ml) was initiated 72 hr. post-transduction. KAS-6/1 BAFF-R_{WT} expressing cell lines were passed 3 times following selection prior to being used for further experimentation.

Semi-Quantitative RT-PCR

We performed semi-quantitative RT-PCR analysis to evaluate the steady state levels of Bcl-2, Bcl-xL, Bim, and Pin1 mRNA using a Bio-Rad CFX96 Real Time machine. Primers

were designed using Primer3 Input on-line software [27] and the sequences for each primer pair are listed as follows; Bcl-2 for ATGTGTGTGGAGAGCGTCAA, rev GGCCGTACAGTTCCACAAAG, Bcl-xL for CTTGGATGGCCACTTACCTG, rev TGCTGCATTGTTCCCATAGA, Bim for TCTCGATCCTCCAGTGGGTA, rev TCTTGGGCGATCCATATCTC, Pin1 for AGTCGGGAGAGGAGGACTTT, rev TCAAATGGCTTCTGCATCTG and TBP for AGTTGTACAGAAGTTGGGTTTTTC, rev AACAAATTCTGGGTTTGATCATTC. Total RNA was isolated from HEK 293 cells treated w/wo BAFF (200 ng/ml) for 24 hr. using RNAlater (Ambion, Austin, TX) and RNeasy Plus Mini Kit (Invitrogen, Carlsbad, CA). cDNA was generated with 1 µg of total RNA using oligo(dT) and the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). Two µl of cDNA reaction was used as template. cDNA was amplified with HotStarTaq (Qiagen, Germantown, MD) with the addition EVA Green. The amplification conditions consisted of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, an annealing temperature of 60 °C for 30 s, and an extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Relative quantification of amplicons was performed using the CT (relative to TBP) method as previously described [28].

Flow Cytometry and Analysis

Briefly, one million cells were washed 1X in ice-cold PBS, incubated with a combination of either IgG1 FITC and PE or BAFF-R FITC and TACI PE for 30 min. on ice, and washed again in ice-cold PBS. Flow cytometry was performed using a FACSCalibur (Becton Dickinson) cytometer. Data was analyzed using FloJo software (Tree Star Inc., Ashland OR).

Statistical Analysis

Statistical analyses were performed using the Student's t-test of variance. Where indicated, a combined analysis of 2–3 independent experiments is shown graphically and error bars represent the standard error of the mean. In all analyses, values were calculated from the mean of the treatment group divided by the corresponding untreated control as describe in the figure legends. In all figures, an asterisks indicates a significant ($p < 0.05$) p-value. Values that did not reach significance are not reported unless discussed in the results section.

Results

BAFF-R Mediated Activation of Akt and GSK3β

In previous studies, we generated BAFF-R_{WT} and BAFF-R_{H159Y} expressing cell lines to characterize BAFF-R signaling [17]. BAFF-R_{H159Y} essentially functions as a hyperactivated BAFF receptor, mimicking the effect of an elevated and/or constitutive BAFF signaling environment. Using these cell lines, we evaluated the activation status of PI3K signaling by performing western blot analyses of phosphorylated Akt, GSK3β and MTOR. Compared to untreated controls (UT), BAFF-induced phosphorylation of Akt_{S473} and GSK3β was detected in cells expressing both BAFF-R_{H159Y} (Figure 1A, left panel) and BAFF-R_{WT} (Figure 1A, right panel). However, a much higher level of detectable pAkt_{S473} was observed in the BAFF-R_{H159Y} expressing cells and a longer exposure time was needed to detect it in the BAFF-R_{WT} cells. No difference in BAFF-R protein levels was observed when

comparing cells expressing BAFF-R_{WT} or BAFF-R_{H159Y} (Figure 1A). A graphical representation of the BAFF-induced activation of Akt_{S473} and GSK3 β in BAFF-R_{H159Y} and BAFF-R_{WT} is shown in Figure 1B. BAFF-induced phosphorylation of Akt_{T308} was also detected in BAFF-R_{H159Y} cells (Supplemental Fig. 1A). Phosphorylation of MTOR was constitutive in all cell lines, and BAFF did not increase pMTOR_{S2448} (Figure 1A) or pMTOR_{S2481} (Supplemental Figure 1B) levels. To demonstrate that phosphorylation of Akt and GSK3 β was dependent upon PI3K signaling, we treated BAFF-R_{H159Y} cells with BAFF in the presence or absence of the PI3K inhibitor wortmannin. Phosphorylation of Akt was completely eliminated, and a significant reduction in the level of pGSK3 β and pMTOR was observed (Figure 1C). Lastly, we sought to determine whether the high level of pAkt_{S473} displayed by BAFF-R_{H159Y} expressing cells resulted from activation of mTORC1 or mTORC2. Treatment of cells with the dual mTORC1/mTORC2 inhibitor torin 1 (Figure 1D), as opposed to the mTORC1 inhibitor rapamycin (Figure 1E), reduced the level of BAFF-R_{H159Y}-mediated phosphorylation of Akt_{S473}, GSK3 β and pMTOR_{S2448} (Figure 1D, 1E), suggesting that BAFF-R_{H159Y}-mediated phosphorylation of Akt at S473 is primarily dependent upon mTORC2. Taken together, these data suggest that stimulation of wildtype and mutant BAFF-R results in activation of the PI3K pathway, and that BAFF-R_{H159Y} constitutively activates the PI3K pathway yet remains responsive to BAFF.

BAFF-R Mediated Regulation of Gene Expression

BAFF signaling primarily functions to prevent cells from undergoing apoptosis and to control entry into the cell cycle [29]. We were interested in determining whether BAFF-R signaling would affect the expression of genes instrumental in regulating these pathways. Using a semi-quantitative real time PCR approach, we examined the steady state mRNA levels of Mcl-1, Pim-2, Bcl-2, Bcl-xL, and Bim, all genes known to regulate apoptosis and the cell cycle. We also investigated the impact of BAFF-R on the expression of Pin1, a gene that plays a critical role in mediating cell-cycle progression and is known to be regulated by Akt [30,31]. Compared to BAFF-R_{WT}, the constitutively active BAFF-R_{H159Y} cells displayed increased expression of the pro-growth and survival genes Bcl-2 (1.8 fold, $p=0.033$), Bcl-xL (1.6 fold, $p=0.047$) and Pin1 (1.6 fold, $p=0.043$), and decreased expression of apoptosis inducing gene Bim (0.8 fold, $p=0.038$) (Figure 2A). BAFF-R_{H159Y}-dependent changes in Mcl-1 and Pim-2 mRNA levels were not observed (data not shown). To determine if any of these genes were upregulated in a PI3K-dependent manner, BAFF-R_{H159Y} cells were treated with wortmannin prior to PCR analysis. Our studies show that only Pin1 was reduced (4.5 fold, $p=.028$) in wortmannin treated BAFF-R_{H159Y} expressing cells, suggesting that Pin1 expression is regulated in part by PI3K (Figure 2B).

TRAF6 Contributes to BAFF-R-Mediated Activation of PI3K

In previous work we have shown that BAFF-R associates with and requires TRAF6 for activation of NF- κ B [17]. TRAF6 has also been shown to play a role on CD40-mediated activation of Akt [32]. The potential role TRAF6 plays in mediating the ability of BAFF-R to activate PI3K signaling was evaluated by constructing stable TRAF6 shRNA_{mir} knock-down cell lines via lentiviral transduction of our original HEK BAFF-R_{H159Y} cell line. We were able to nearly eliminate TRAF6 protein expression in the BAFF-R_{H159Y} cell line (TRAF6) as demonstrated in Figure 3A. When TRAF6 knock down cells were stimulated

with BAFF, near significant reductions in the phosphorylation of Akt (~2 fold, $p=0.087$) and GSK3 β (2 fold, $p=0.086$) were observed in the TRAF6 knock down cells at the 3 hr. time point compared to their respective non-silenced (NS) controls (Figure 3A and 3B). These data suggest that TRAF6 contributes to the BAFF/BAFF-R-mediated activation of the PI3K pathway.

BAFF Stimulates PI3K Activity in Malignant B Cells Expressing BAFF-R

All of the data presented thus far was generated using HEK 293 cells expressing BAFF-R. Since BAFF-R expression is primarily limited to B-cells, we wanted to ensure that our assays conducted in HEK cell lines accurately reflected BAFF-dependent modulation of PI3K signaling in a cell line derived from a B-cell lineage. We first determined that Karpas 422 lymphoma cells, which express BAFF-R [33], were BAFF responsive by demonstrating that BAFF stimulation activates NF- κ B2 signaling, indicated by the significant increase in p52 protein levels within 6 hr. of treatment (Figure 4A). BAFF-dependent activation of PI3K was more rapid, as maximal phosphorylation of Akt_{S473} and GSK3 β was observed around 30 min post stimulation (Figure 4B and 4C). Because Karpas 422 cells express TACI,[34] we could not fully attribute BAFF-mediated Akt and GSK3 β phosphorylation specifically to BAFF-R. All lymphoma cell lines that we have analyzed express BAFF-R in combination with TACI or BCMA posing a significant challenge to specific analysis of BAFF-R. Therefore, we used the KAS-6/1 myeloma cell line, which express no surface BAFF-R [35], to generate a BAFF-R expressing B cell line model in which we could study BAFF-R signaling. To date, there have been no reports indicating differential BAFF-R signaling between myeloma and lymphoma cell lines suggesting this would be a representative model. KAS-6/1 cells were transduced with WT BAFF-R (Supplemental Figure 3). As expected, BAFF-R expressing KAS-6/1 cells exhibited enhanced p52 activation compared to untransduced KAS-6/1 cells, and additionally showed a marked increase in the levels of Akt_{S473} phosphorylation following stimulation with BAFF (Figure 4D and E). Similar to the results seen in 293 cells transduced with BAFF-R (Figure 1A), we did not see a BAFF-induced increase in phosphorylation of MTOR_{S2481/S2488} (Supplemental Fig. 3). Taken together, these results show that BAFF-R induces activation of the PI3K pathway in B cells.

Discussion

The impact of BAFF on Akt signaling was initially described in the context of multiple myeloma (MM), in which high levels of APRIL/BAFF were associated with enhanced Akt activation via TACI and BCMA [36]. BAFF was also shown to activate Akt in human acute lymphoblastic leukemia cells in vitro, and the ability for BAFF to inhibit B-cell apoptosis was significantly diminished following the loss of the PI3K catalytic subunit p110 δ in cultured B-cells [37–39]. However, none of these studies associated the BAFF-dependent activation of PI3K/Akt to a specific BAFF receptor. BAFF-R is the primary BAFF receptor in B-cells, and has been demonstrated to be critically important in the regulation of Akt signaling in normal mouse B-cells [40]. However, to the best of our knowledge, no published information exists concerning the role BAFF-R plays in PI3K/Akt signaling in human cells. Using cells stably expressing either BAFF-R_{WT} or BAFF-R_{H159Y}, we now

demonstrate that BAFF-R can regulate the PI3K/Akt signaling cascade in the absence of any other BAFF receptor. We were able to observe phosphorylation of Akt at S473 in cells expressing both WT and mutant BAFF-R, however, the level of phosphorylation was significantly greater in cells expressing BAFF-R_{H159Y}. Given that phosphorylation of Akt at S473 is reported to require the activation of mTORC2, we used a dual mTORC inhibitor (torin 1) in combination with the mTORC1 inhibitor rapamycin to conclusively demonstrate that BAFF-R_{H159Y}-phosphorylation of S473 was primarily dependent upon mTORC2 [41]. Western analysis indicated no difference in BAFF-R protein levels suggesting that the enhanced PI3K activity exhibited in cells expressing BAFF-R_{H159Y} may be specific to the mutant receptor. In our previous publication [33] we showed that signaling through mutant BAFF-R results in increased NF-κB1 and NF-κB2 activity and increased immunoglobulin production compared with the wild-type (WT) BAFF-R. This correlated with increased TRAF2, TRAF3, and TRAF6 recruitment to BAFF-R_{H159Y}. We hypothesize that constitutive association of BAFF-R with TRAF6 or constitutive activation of the NF-κB2 pathway contribute to the more potent activation seen by mutant BAFF-R as both TRAF6 [32] and IKK1[40] have been shown to activate the Akt pathway.

The regulation of genes linked to cell survival constitutes a primary function of normal and pathological BAFF signaling, and BAFF-dependent expression of genes involved in mitochondrial dependent apoptosis remain the most well described function of BAFF signaling. Modest BAFF-dependent increases in the anti-apoptotic genes Bcl-2 and Bcl-xL have been reported in primary mouse splenocytes [42]. Conversely, expression and activity of the pro-apoptotic protein BIM was decreased in murine WEHI-231 B-cells and primary mouse splenocytes treated with BAFF [43]. Our results build upon this data by demonstrating that BAFF-R signaling results in a significant enhancement in the established pro-survival gene expression profile displayed by the BAFF treated mouse splenocytes. BAFF-R_{H159Y} significantly increased Bcl-2 and Bcl-xL expression over levels attributed to BAFF-R_{WT}, while similarly treated cells showed a significant decrease in the expression of Bim. Moreover, we show for the first time that BAFF-R signaling increases the expression of Pin1, a protein which functions in the regulation of cell cycle progression and as a binding partner of the NF-κB transcription factor RelA, promoting RelA stabilization and nuclear translocation [44]. Pin1 has also been implicated in the transformation of primary lymphocytes resulting from elevated levels of nuclear Rel protein and activation of cRel and is over-expressed in a wide variety of human tumors [45,46]. Additionally Pin1 plays an indispensable role in the ability of toll-like receptors to mount an interferon-dependent response in mice, a pathway also regulated by BAFF-dependent activation of TACI [47–50].

Few studies have been published describing the potential crosstalk between BAFF regulated gene expression and PI3K signaling, although BAFF-mediated increases in Mcl-1 protein expression were completely eliminated in mouse splenocytes co-treated with the mTORC1 inhibitor rapamycin [51]. Such data suggests that the PI3K pathway plays a role in modulating BAFF-dependent gene regulation, and therefore, we sought to determine whether PI3K inhibition might affect BAFF-R_{H159Y}-mediated increased gene expression of Bcl-2, Bcl-xL, Bim or Pin1. We did not observe any significant difference in the expression of Bcl-2, Bcl-xL or Bim in BAFF-R_{H159Y} expressing cells following treatment with wortmannin. This is not surprising, as although PI3K signaling has been demonstrated to

synergistically work with Bcl family members in order to promote tumor survival, inhibition of PI3K signaling generally does not alter the expression of such proteins [52,53]. Interestingly, expression of Pin1 was significantly reduced following inhibition of PI3K in BAFF-R_{H159Y} cells. We could find no references linking PI3K activity to Pin1 expression in B-cells, however, inhibition of PI3K signaling has been reported to inhibit Pin1 expression in breast cancer [54,55].

TRAF proteins play an essential role in regulating a number of pro-survival pathways used by B-cells, including signaling by toll-like receptors, CD40 and BAFF receptors [56–62]. The established model of BAFF-R-dependent activation of the NF- κ B2 pathway is predisposed upon TRAF3 binding to the PVPAT region of BAFF-R, allowing for the subsequent increase in cytoplasmic levels of NF- κ B inducing kinase (NIK), efficient processing of p100 to p52 and translocation of p52/relB to the nucleus [20,63]. Our laboratory was the first to demonstrate the recruitment of TRAF6 in a complex with activated BAFF-R, and more importantly, we elucidated the essential nature of TRAF6-mediated regulation of BAFF-R dependent NF- κ B1 signaling [17]. Our current work expands the essential role TRAF6 plays in mediating BAFF-R activation of pro-survival signaling, demonstrating that BAFF-R-dependent activation of PI3K signaling is, at least in part, dependent upon TRAF6. Although we believe this to be the first time that BAFF-R signaling dependent activation of Akt has been linked to TRAF6, knockdown of TRAF6 expression has been reported to negatively affect Akt activation through the loss of TRAF6-mediated Lys63 ubiquitination activity [64]. Interestingly, knock-down of TRAF6 did not affect constitutive activation of PI3K signaling. Given that TRAF6 recruitment only occurs following BAFF stimulation, BAFF-R_{H159Y} may recruit an as yet unknown factor augmenting BAFF-R-signaling, which itself may require TRAF6 in order to associate with the receptor.

BAFF-R, through activation of NF- κ B, is central to both normal and malignant B-cell biology. Our studies now suggest that BAFF-R also induces activation of the PI3K pathway. Additionally, we demonstrate a novel role for BAFF-R in regulation of Pin1 and suggest that TRAF6 contributes to BAFF-R-mediated PI3K-pathway activation. These studies may have clinical implications, as BAFF levels are commonly dysregulated in both autoimmune disease and B- cell malignancies. Moreover, the BAFF-R_{H159Y} mutation has been detected in a small percentage of NHL patients. Collectively, these data suggest that targeting the PI3K pathway therapeutically may benefit patients with B-cell malignancies characterized by hyperactive BAFF-R signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the National Institutes of Health (P50CA097274 to J.R.C. and A.J.N) and the Lymphoma Research Foundation Follicular Lymphoma Research Grantee program (to A.J.N.).

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Figure 2. Activation of BAFF-R induces expression of pro-survival genes

(A) Expression of Bcl-2, Bcl-xL, Bim, and Pin1 mRNA were measured in BAFF-R_{WT} and BAFF-R_{H159Y} expressing cells as described in Methods. Combined analysis of 3 independent experiments is shown graphically. Statistical comparisons were made between BAFF-R_{WT}, and BAFF-R_{H159Y}. (B) BAFF-R_{H159Y} expressing cells were left untreated or treated with wortmannin (500 nM) and expression of Bcl-2, Bcl-xL, Bim and Pin1 mRNA were measured as described in Methods. Combined analysis of 3 independent experiments is shown graphically. Statistical comparisons were made between (A) WT and H159Y and (B) untreated and treated samples.

Figure 3. TRAF6 contributes to BAFF-R induced PI3K activation

(A) BAFF-R_{H159Y} expressing cells transduced with either a non-silencing shRNAir (NS) or shRNAir targeting TRAF6 (TRAF6) were left untreated or treated with BAFF for the indicated times. Whole cell lysates were analyzed by Western blot for expression of TRAF6, pAkt, total Akt, pGSK3β, total GSK3β or Actin. The Western blot shown is representative of at least 3 independent experiments. (B) Combined analysis of 3 independent experiments is shown graphically. Statistical comparisons were made between untreated and BAFF-treated samples (3 hr).

Figure 4. BAFF-R activates PI3K signaling in B-cells

(A) Karpas 422 cells were left untreated or were treated with BAFF for the indicated times and were analyzed for expression of (A) NF- κ B2 (p100 and p52) or (B) pAkt_{S473}, total Akt, pGSK3 β , total GSK3 β or Actin by Western blot. Combined analysis of 3 independent experiments is shown graphically in (C). (D) KAS-6/1 cells or KAS-6/1 cells expressing BAFF-R were left untreated or treated with BAFF for the indicated times and were analyzed for expression of NF- κ B2 (p100 and p52) or pAkt and total Akt by Western blot. Combined analysis of 2 independent experiments is shown graphically in (E).