

A BAFF-R mutation associated with non-Hodgkin lymphoma alters TRAF recruitment and reveals new insights into BAFF-R signaling

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The cytokine B cell activating factor (BAFF) and its receptor, BAFF receptor (BAFF-R), modulate signaling cascades critical for B cell development and survival. We identified a novel mutation in *TNFRSF13C*, the gene encoding human BAFF-R, that is present in both tumor and germline tissue from a subset of patients with non-Hodgkin lymphoma. This mutation encodes a His159Tyr substitution in the cytoplasmic tail of BAFF-R adjacent to the TRAF3 binding motif. Signaling through this mutant BAFF-R results in increased NF- κ B1 and NF- κ B2 activity and increased immunoglobulin production compared with the wild-type (WT) BAFF-R. This correlates with increased TRAF2, TRAF3, and TRAF6 recruitment to His159Tyr BAFF-R. In addition, we document a requirement for TRAF6 in WT BAFF-R signaling. Together, these data identify a novel lymphoma-associated mutation in human BAFF-R that results in NF- κ B activation and reveals TRAF6 as a necessary component of normal BAFF-R signaling.

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Abbreviations used: BAFF, B cell activating factor; BAFF-R, BAFF receptor; BCMA, B cell maturation antigen; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; MALT, mucosal-associated lymphoid tissue lymphoma; NHL, non-Hodgkin lymphoma; SNP, single nucleotide polymorphism; TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor.

B cell activating factor (BAFF; Schneider et al., 1999), which is also called BLyS (Moore et al., 1999), THANK (Mukhopadhyay et al., 1999), TALL-1 (Shu et al., 1999), or zTNF4 (Gross et al., 2000), is a TNF family member (*TNFRSF13B* [A000383]) critical for maintenance of normal B cell development and homeostasis (Thompson et al., 2000; Schiemann et al., 2001; Schneider et al., 2001). Three receptors, B cell maturation antigen (BCMA; *TNFRSF17* [A000374]; Madry et al., 1998), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI; *TNFRSF13B* [A002248]; von Bülow et al., 2000), and BAFF receptor (BAFF-R; Thompson et al., 2001; also called BR3 or *TNFRSF13C* [A002648]) have been identified as receptors for BAFF. BCMA and BAFF-R are predominantly expressed on B lymphocytes, whereas TACI can be found on B cells and activated T cells (von Bülow and

Bram, 1997). Initial studies of the effects of BAFF on B cell physiology demonstrate that it co-stimulates B cell proliferation and immunoglobulin secretion (Moore et al., 1999; Schneider et al., 1999). Transgenic overexpression of BAFF in mice results in elevated Bcl-2 levels and prolonged survival of B cells, manifesting in increased numbers of mature B cells in the spleen and periphery and development of autoimmune-like characteristics reminiscent of systemic lupus erythematosus (Mackay et al., 1999; Gross et al., 2000; Khare et al., 2000). Peripheral B cell maturation is arrested in BAFF-deficient mice, resulting in almost complete loss of follicular and marginal zone B lymphocytes (Schiemann et al., 2001).

The underlying mechanism responsible for the effect of BAFF on B cells remains poorly

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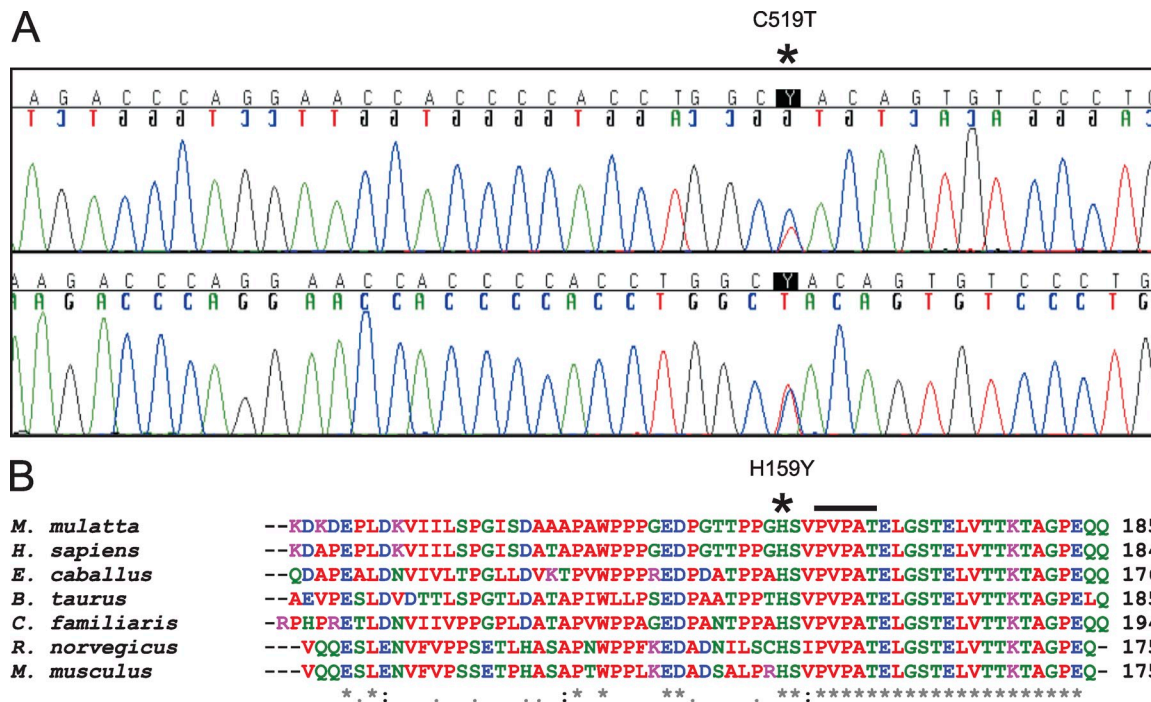


Figure 1. Identification of the BAFF-R His159Tyr SNP. (A) TNFRSF13C was sequenced on both sense (bottom chromatogram) and antisense (top chromatogram) strands, and a heterozygous cytosine-to-thymine transition at position 519 (C519T) was identified. (B) A multiple sequence alignment of a sample of mammalian BAFF-R amino acid sequences, showing evolutionary conservation of the cytoplasmic portion and the location of the missense substitution of tyrosine (Y) for histidine (H) in codon 159. Symbols below the sequence alignment indicate when residues across species encode for identical (*), conserved (:), or semiconserved (.) amino acids.

understood, in part because of the complexity introduced by multiple BAFF-binding receptors. However, a central role for BAFF-R in BAFF biology has been suggested by studies demonstrating that A/WySnJ mice, which carry a mutation in BAFF-R, and BAFF-R deficient mice, show a loss of mature peripheral B cells, a phenotype similar to BAFF-deficient mice (Gross et al., 2001; Thompson et al., 2001; Yan et al., 2001). Current models suggest that BAFF binding to BAFF-R results in a coordinated assembly of a regulatory complex consisting of cIAP1, cIAP2, TRAF2, TRAF3, and NIK. Formation of this complex results in ubiquitin-mediated proteasomal degradation of TRAF3 followed by NIK stabilization and NF- κ B2 activation (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). In addition to NF- κ B2 activation, BAFF-R has been shown to be essential for BAFF-mediated phosphorylation of AKT and Erk1/2 through IKK1 (Otipoby et al., 2008). However, the proximal events that mediate activation of this signaling cascade are not fully characterized.

The requirement for BAFF and BAFF-R in normal human and mouse B cells is well studied, but there is also significant evidence to suggest that BAFF plays an important role in malignant B cell proliferation and survival (Briones et al., 2002; He et al., 2004; Novak et al., 2004; Fu et al., 2006). Serum BAFF levels are elevated in patients with non-Hodgkin lymphoma (NHL), and high BAFF levels correlate with aggressive disease and a poor response to therapy (Novak et al., 2004). There is also increasing genetic evidence, suggesting

an association between the development of human disease with genetic variation in genes encoding BAFF and its receptors. Mutations in *TNFRSF13B* (TACI) were identified in patients with familial common variable immunodeficiency (CVID) and IgA deficiency (Castigli et al., 2005; Salzer et al., 2005), and we have found that single nucleotide polymorphisms (SNPs) in *TNFRSF13B* (BAFF) are associated with elevated BAFF levels and risk for developing NHL (Novak et al., 2006, 2009). To build upon these findings, we sequenced the *TNFRSF13B*, *TNFRSF13*, *TNFRSF13B*, *TNFRSF17*, and *TNFRSF13C* genes in NHL patients to identify novel genetic variants that may be associated with NHL risk. This approach identified a mutation in *TNFRSF13C* that results in a histidine 159-to-tyrosine substitution located in the cytoplasmic tail of BAFF-R. Analysis of cells expressing H159Y BAFF-R demonstrates that this mutation results in increased BAFF-R-mediated NF- κ B1 and NF- κ B2 activation. The enhanced signal activated by BAFF-R H159Y is coupled with a severalfold increase in TRAF3, TRAF2, and TRAF6 recruitment to BAFF-R. We demonstrate that recruitment of TRAF6 to BAFF-R is not unique to the mutant H159Y BAFF-R but is also an important and necessary feature of normal BAFF-R signaling.

RESULTS

Identification of the BAFF-R His159Tyr mutation

We sequenced the *TNFRSF13B*, *TNFRSF13*, *TNFRSF13B*, *TNFRSF17*, and *TNFRSF13C* genes to identify additional

Table I. Identification of the BAFF-R H159Y mutation in biopsy specimens from patients with NHL

NHL subtype	Number screened	Number positive for BAFF-R H159Y	Percent positive for BAFF-R H159Y
			%
DLBCL	<i>n</i> = 42	<i>n</i> = 2	4.8
FL	<i>n</i> = 41	<i>n</i> = 4	10
Lymphoplasmacytic lymphoma	<i>n</i> = 22	<i>n</i> = 1	4.5
MALT	<i>n</i> = 24	<i>n</i> = 1	4.1

NHL subtype was determined by a board-certified Mayo Clinic Hematopathologist.

novel genetic variants that may be associated with NHL. Among 40 individual samples (20 controls and 20 follicular lymphoma [FL] cases) that were bidirectionally sequenced, we identified a heterozygous cytosine-to-thymidine transition in one patient specimen at position 519 (C519T, rs61756766, National Center for Biotechnology Information [NCBI] reference sequence NM_052945.3) of *TNFRSF13C* (Fig. 1 A). The C519T transition predicts a missense substitution of tyrosine for histidine in codon 159 (H159Y) in the highly conserved cytoplasmic tail of BAFF-R, adjacent to the TRAF3 binding motif PVPAT (Fig. 1 B). This genetic variation was previously identified in 1/48 patients studied with CVID; however, it was not found to be associated with immunodeficiency disease or a change in BAFF-R mRNA or protein expression (Losi et al., 2005). We next expanded our analysis of BAFF-R H159Y and analyzed NHL tumor biopsies for the presence of the mutation (Table I). 4/41 FLs, 2/42 diffuse large B cell lymphomas (DLBCLs), 1/22 lymphoplasmacytic lymphomas, and 1/24 mucosal-associated lymphoid tissue lymphomas (MALTs) carried the heterozygous mutation. When feasible, we examined matched tumor and germline tissue from patients and found that the BAFF-R mutation was present in both germline and tumor tissue in 2/2 FLs and 2/2 DLBCLs tested. The BAFF-R H159Y mutation was not detected in any of the normal control tissues (*n* = 100). These data identify a novel lymphoma-associated mutation in BAFF-R, and the presence of the mutation in germline tissue suggests that the BAFF-R mutation may be associated with lymphoma risk.

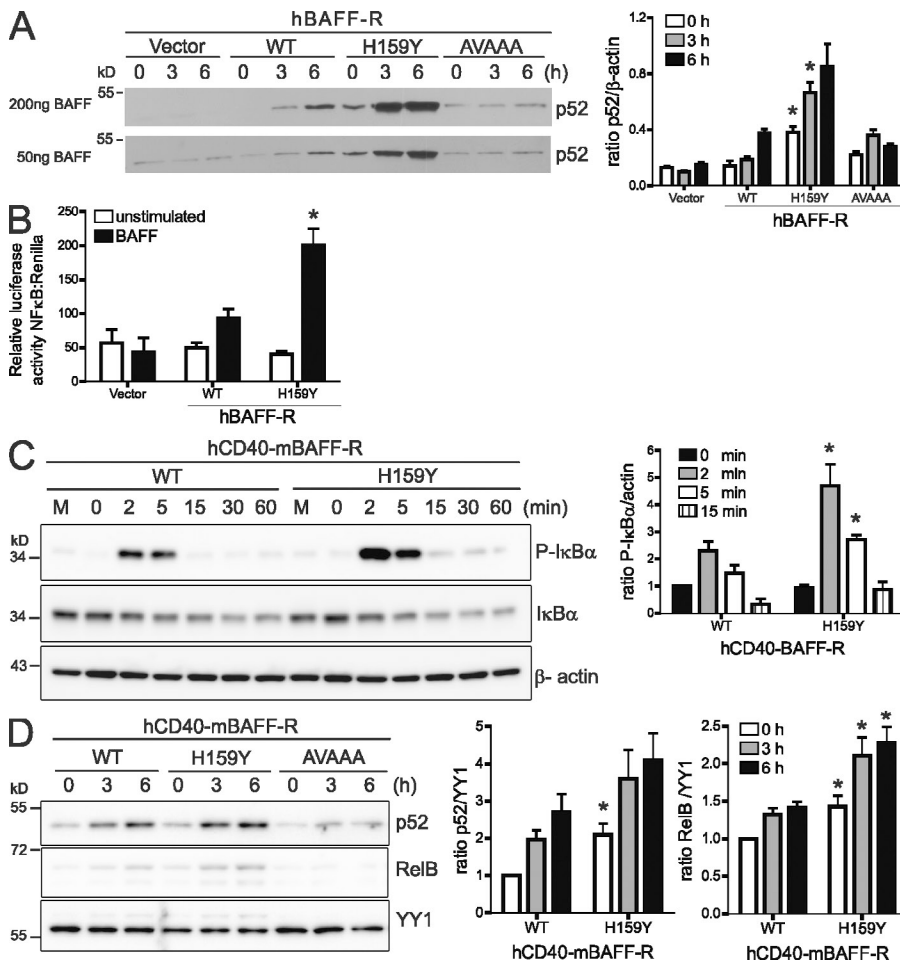
Increased NF- κ B activation by BAFF-R His159Tyr

Given its close proximity to the TRAF3 binding site in the cytoplasmic domain of BAFF-R, we first wanted to determine if the H159Y mutation altered BAFF-induced signaling. We generated HEK293 cells that express HA-tagged WT BAFF-R (WT), BAFF-R with the H159Y mutation (H159Y), or BAFF-R with an ablated TRAF3 binding site as a negative control (AVAAA; Fig. S1 A; Morrison et al., 2005). Expression of BAFF-R protein was confirmed by Western blot analysis and flow cytometry (Fig. S1 B and C).

Using these cell lines, we examined the ability of BAFF-R_{WT} and BAFF-R_{His159-Tyr} to activate processing of p100 NF- κ B2 to the active p52 subunit. Stimulation of both BAFF-R_{WT} and BAFF-R_{His159-Tyr} with 200 ng/ml BAFF for 3 or 6 h resulted in p100 processing (Fig. 2 A, top). We observed

increased p100 processing at baseline and upon BAFF stimulation in BAFF-R_{His159-Tyr}-expressing cells. Increased NF- κ B2 signaling was also evident at lower ligand concentrations. Using 50 ng/ml BAFF (Fig. 2 A, bottom), we saw significant increases in p100 processing (*P* = 0.0009 and *P* = 0.0008 at the 0- and 3-h time points, respectively; *n* = 3). p100 processing was not detected in the vector control cells and minimal p100 processing was detected in cells expressing the TRAF3 binding-deficient BAFF-R_{AVAAA}. Data from three independent experiments are shown graphically in Fig. 2 A (right). In parallel experiments, we tested the ability of BAFF-R_{WT} and BAFF-R_{His159-Tyr} to activate transcription of a NF- κ B reporter gene plasmid (Fig. 2 B). Consistent with the p100 processing results, BAFF-R_{His159-Tyr} induced increased NF- κ B activation compared with BAFF-R_{WT} (*P* = 0.01; *n* = 3). These results are not a result of variation in BAFF-R expression between the cell lines (Fig. S1, B and C).

Because signaling by TNFR family members can show cell type-specific features (Bishop, 2004), it was important to determine whether BAFF-R_{His159-Tyr} could induce increased NF- κ B activation in physiologically relevant B lineage cells. Mature B cells can express both BAFF-R and TACI, making it difficult to ascribe BAFF-induced signaling events in B cells to BAFF-R alone. We therefore used a chimeric receptor approach and generated A20.2J mouse B cell lines that express a receptor that consists of the extracellular domain of human CD40 fused with the transmembrane and cytoplasmic domain of mouse BAFF-R (hCD40-mBAFF-R; Fig. S1 D). This approach allows us to specifically study BAFF-R responses in B lineage cells in the absence of TACI signaling using hCD154 (the natural ligand for hCD40) or an agonistic anti-hCD40 antibody (G28.5). This chimera was previously demonstrated to activate NF- κ B2 and TRAF3 degradation (Morrison et al., 2005) and can therefore serve as a valid model to study BAFF-R signals. A20.2J B cells were stably transfected to express hCD40-BAFF-R_{WT} or hCD40-BAFF-R_{His159-Tyr}, and clones with matched surface expression of chimeric receptor (Fig. S1 E) were incubated with Hi5 insect cells expressing hCD154 (Rowland et al., 2007). Early phosphorylation of I κ B α was significantly enhanced (*P* = 0.05 and *P* = 0.018 at 2 and 5 min, respectively; *n* = 3) after stimulation of hCD40-BAFF-R_{His159-Tyr} (Fig. 2 C). Likewise, hCD40-BAFF-R_{His159-Tyr} induced a significant increase in basal levels of p52 and RelB nuclear localization (*P* = 0.009 and 0.02, respectively; *n* = 4) and in nuclear RelB levels at



3 and 6 h after stimulation ($P = 0.02$ and 0.005 ; $n = 4$; Fig. 2 D), confirming the increase in NF- κ B activation induced by BAFF-R_{His159-Tyr} in HEK293 cells. Collectively, these data demonstrate that stimulation of BAFF-R_{His159-Tyr}, either as full-length or chimeric receptor, results in significantly increased NF- κ B activity compared with BAFF-R_{WT}.

BAFF-R His159Tyr drives increased immunoglobulin production

The results in the previous section clearly indicate that the His159Tyr mutation alters signaling by BAFF-R. We next addressed if this alteration in early signals has biological impact on B cell function. BAFF-R plays an important role in early B cell maturation from transitional to mature stages (Schneider et al., 2001), but we could not test this function as we do not currently have access to B cells from individuals bearing this mutation. However, BAFF also plays a central role in regulation of immunoglobulin secretion by B cells (Moore et al., 1999; Schneider et al., 2001). To determine whether BAFF-R_{His159-Tyr} affected antibody production, we used a CH12.LX mature B cell line that has been previously shown to secrete phosphatidyl choline-specific IgM in response to CD40 stimulation in a NF- κ B-dependent manner (Hsing and Bishop, 1999). We stably transfected CH12.LX cells with hCD40-BAFF-R_{WT} or hCD40-BAFF-R_{His159-Tyr}

(Fig. S2), stimulated the cells with anti-hCD40 to activate the chimeric receptor, and measured IgM secretion (Fig. 3). As a control, cells were also stimulated with an isotype control antibody or anti-mCD40, which activates the endogenous full-length mCD40 expressed by these cells. Upon stimulation, cells expressing hCD40-BAFF-R_{His159-Tyr} produced fourfold more IgM compared with hCD40-BAFF-R_{WT}-expressing cells ($n = 3$, $P = 0.0401$), which is consistent with the increase in I κ B α phosphorylation observed earlier. Both types of subclones exhibited equal IgM secretion when stimulated via endogenous mouse CD40. These data show that the BAFF-R His159Tyr mutation has biological impact on B cell function.

Increased TRAF recruitment by BAFF-R His159Tyr

To define the mechanism by which BAFF-R_{His159-Tyr} activates increased NF- κ B pathway activation, we examined BAFF-R signaling events upstream of NF- κ B activation. One of the most proximal signaling events to occur upon BAFF stimulation is the recruitment of TRAF3 to the BAFF-R cytoplasmic tail. TRAF2 and TRAF6 have also been reported to associate with TACI (Xia et al., 2000) and BCMA (Hatzoglou et al., 2000), so we also analyzed their ability to associate with BAFF-R. Using A20.2J B cells expressing hCD40-BAFF-R_{WT} and hCD40-BAFF-R_{His159-Tyr},

Figure 2. BAFF-R His159Tyr exhibits enhanced signaling ability. (A) HEK-293 cells expressing BAFF-R_{WT}, BAFF-R_{His159Y}, BAFF-R_{A4A4A}, or a vector control were stimulated with 200 or 50 ng/ml BAFF for 0, 3, or 6 h. NF- κ B p100 processing to p52 was analyzed by immunoblotting. Levels of p52 and actin were quantified by densitometry, and combined analysis of three independent experiments is shown in the bar graph. (B) NF- κ B luciferase reporter assay in HEK-293 cells expressing BAFF-R_{WT}, BAFF-R_{His159Y}, or BAFF-R_{A4A4A}. (C) A20.2J B cells expressing hCD40-BAFF-R_{WT} or hCD40-BAFF-R_{His159Y} chimeras were incubated with media alone (M), uninfected Hi5 cells (0), or Hi5-hCD154 cells for the indicated times. Levels of phospho-I κ B α , total I κ B α , and actin were examined by Western blotting and quantified by densitometry. Combined analysis of four independent experiments is shown in the bar graph. (D) CH12.LX B cells expressing hCD40-BAFF-R_{WT}, hCD40-BAFF-R_{His159Y}, or hCD40-BAFF-R_{A4A4A} were incubated with anti-human CD40 (G28.5) or isotype control (0). Levels of p52, RelB, and YY1 in the nuclear cell fraction were examined by Western blotting and quantified by densitometry. Combined analysis of four independent experiments is shown in the bar graph. In all analyses, error bars represent the \pm SEM and statistical comparisons were made between BAFF-R_{WT} and BAFF-R_{His159Y}. *, $P < 0.05$.

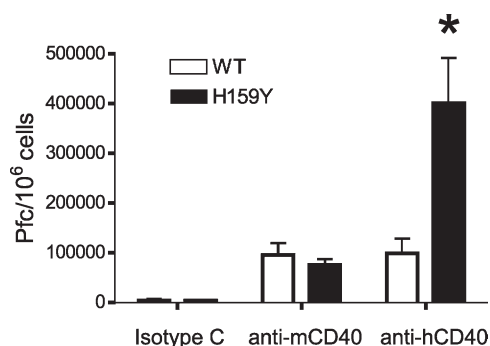


Figure 3. BAFF-R His159Tyr stimulates enhanced immunoglobulin production. CH12.LX cells stably expressing hCD40-BAFF-R_{WT} or hCD40-BAFF-R_{H159Y} were stimulated for 72 h with 2 μ g/ml of agonistic mCD40-specific antibody, hCD40-specific antibody, or isotype control. Numbers of IgM-secreting B cells (\pm SEM) from three independent experiments are shown. *, $P < 0.05$. Pfc, plaque forming cell.

we analyzed the ability of each receptor to recruit TRAF2, TRAF3, and TRAF6 after stimulation (15 or 60 min) and immunoprecipitation with the anti-hCD40 antibody G28.5 (Fig. 4 A). Compared with BAFF-R_{WT}, BAFF-R_{H159Y} conferred a fivefold increase in recruitment of TRAF2 ($P = 0.001$), a fourfold increase in TRAF3 ($P = 0.058$), and a 25-fold increase in TRAF6 ($P = 0.043$; 60-min time point, $n = 4$; Fig. 4 B). The increased TRAF association was not a result of differences in BAFF-R or TRAF expression by each cell line (Fig. S1 E and Fig. S3, respectively). Data from four independent experiments are shown graphically in Fig. 4 B. To verify that the increase in TRAF association was not an artifact of using a chimeric form of BAFF-R_{H159Y-Tyr}, we performed immunoprecipitations of full-length BAFF-R_{WT} or BAFF-R_{H159Y-Tyr} expressed in HEK293 cells after stimulation with BAFF. Consistent with our observations using hCD40-BAFF-R in B cells, there was an increase in TRAF2 and 6 associations with BAFF-R_{H159Y-Tyr} versus BAFF-R_{WT} (Fig. 4 C). The increased association was not a result of increased BAFF-R or TRAF expression (Fig. 4 C, bottom, lysate). These results suggest that the increased NF- κ B activity induced by BAFF-R_{H159Y-Tyr} may be, in part, a result of increased TRAF recruitment. Additionally, these results also reveal for the first time that TRAF6 can associate with BAFF-R and thus potentially participate in BAFF-R signaling.

TRAF6 associates with BAFF-R and is required for BAFF-R signaling

TRAF6 has not previously been shown to interact with BAFF-R or play a role in BAFF-R-mediated signaling. The studies in the previous section clearly demonstrate that TRAF6 is recruited to BAFF-R; however, both of these models used exogenous expression of BAFF-R. To determine if TRAF6 is recruited to endogenous full-length BAFF-R, unmodified mouse A20.2J B cells (WT) were treated with recombinant BAFF, and endogenous BAFF-R was immunoprecipitated and analyzed for TRAF recruitment (Fig. 5 A, left). TRAF6

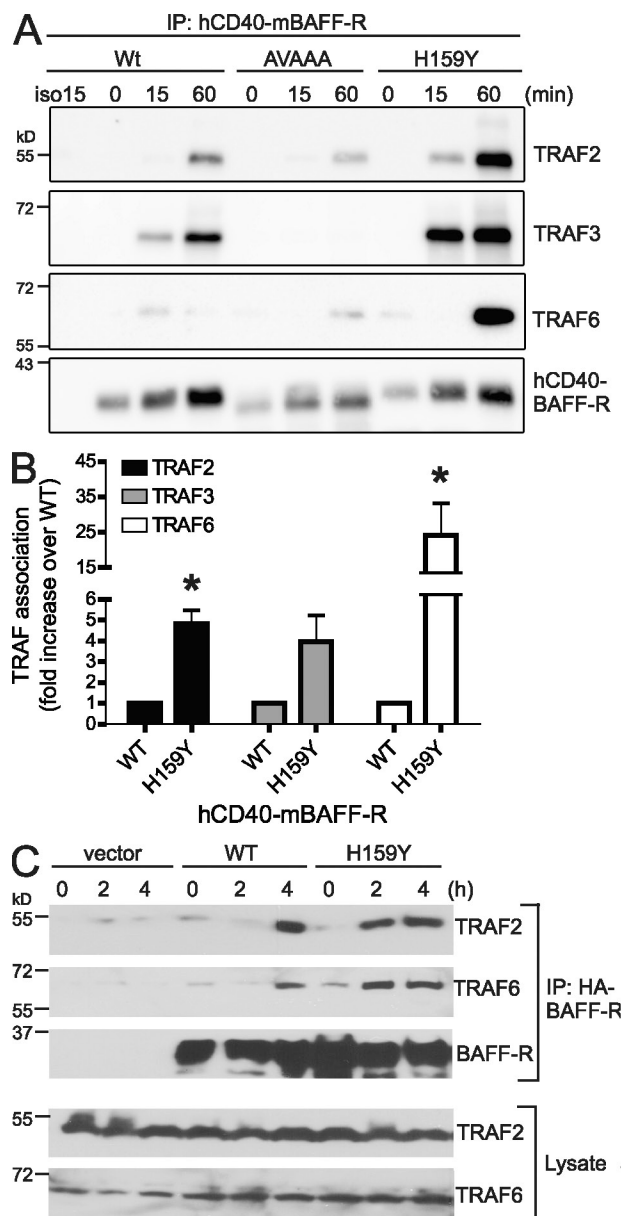


Figure 4. The His159Tyr BAFF-R mutant recruits increased amounts of TRAF2, 3, and 6. (A) A20.2J B cells expressing hCD40-BAFF-R_{WT} or hCD40-BAFF-R_{H159Y} chimeras were subjected to combined stimulation and immunoprecipitation with the hCD40-specific antibody G28.5. (iso 15, 15 min with isotype control). The immunoprecipitate was probed for the presence of TRAF2, TRAF3, TRAF6, and hCD40-BAFF-R. (B) Levels of co-immunoprecipitated TRAF2, 3, and 6 were normalized to the amount of hCD40-BAFF-R immunoprecipitated. Histogram represents the mean \pm SEM of four independent experiments. *, $P < 0.05$ (BAFF-R_{H159Y} vs. BAFF-R_{WT}). (C) HEK-293 cells expressing HA-tagged BAFF-R_{WT}, BAFF-R_{H159Y}, BAFF-R_{AVAAA}, or a vector control were stimulated with BAFF and subjected to immunoprecipitation using an HA-specific antibody. Associated protein or total lysates were probed for the presence of TRAF2 or TRAF6 by Western blotting.

was coimmunoprecipitated with BAFF-R within 10 min of BAFF stimulation. We also examined TRAF2 and TRAF3 recruitment to BAFF-R. Similar to results seen in Fig. 4,

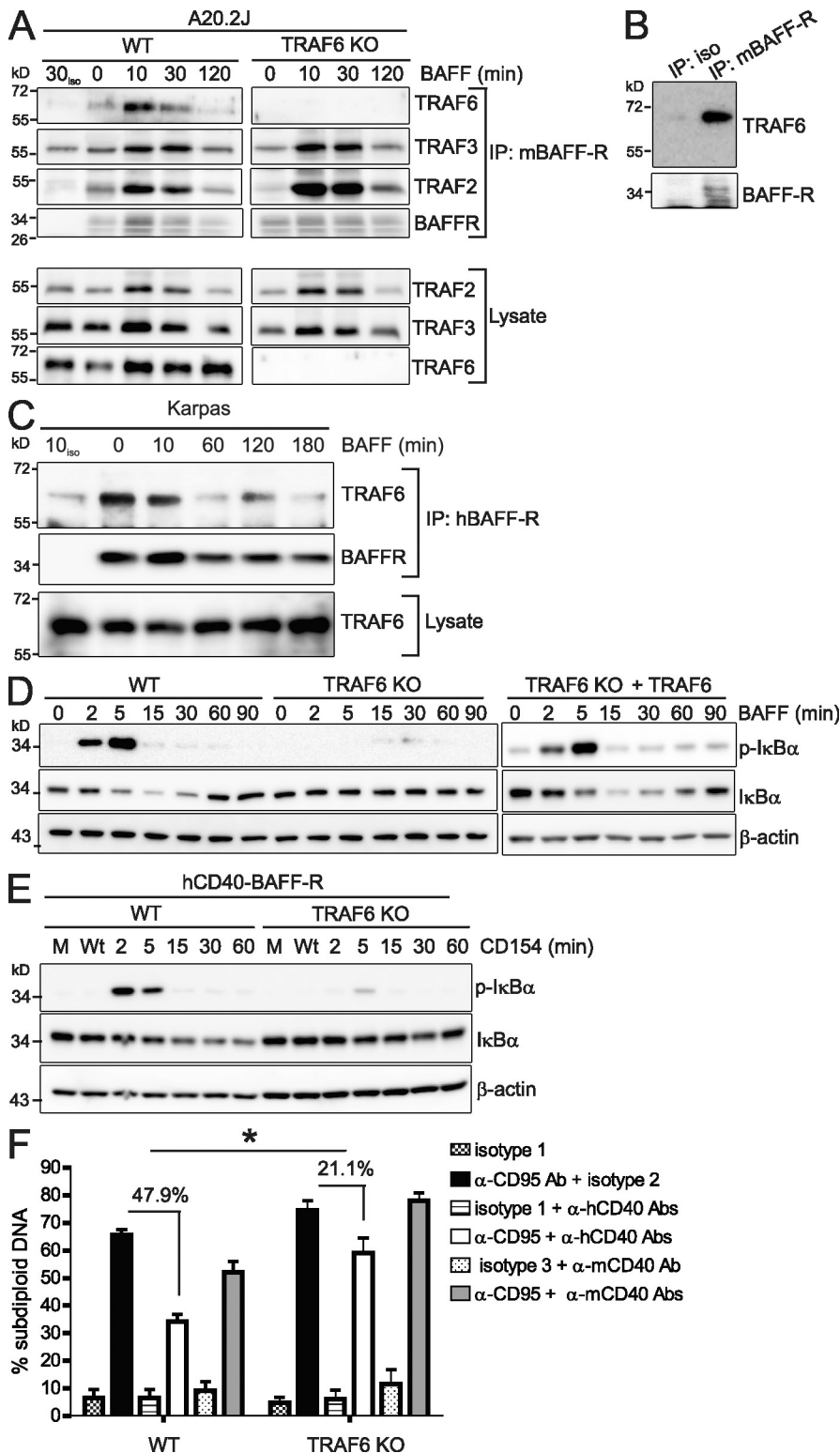


Figure 5. TRAF6 is recruited to BAFF-R in B cells and is required for BAFF- and BAFF-R-induced prosurvival signals.

(A–C) Mouse A20.2J and A20.2J TRAF6 KO cells (A), primary mouse T cell-depleted splenocytes (B), or human Karpas B cells (C) were stimulated with BAFF, and the Brij58 insoluble (lipid raft enriched) fraction (lysate) was subjected to immunoprecipitation with a mouse or human BAFF-R-specific antibody or isotype control (iso). Immunoprecipitated sample was probed for the presence of TRAF6, 2, or 3. A and C are representative of three independent experiments and B is representative of two independent experiments. (D) A20.2J WT, A20.2J TRAF6 KO, and A20.2J TRAF6 KO cells expressing full-length TRAF6 were incubated with BAFF for the indicated times. (E) A20.2J WT and A20.2J TRAF6 KO cells expressing the hCD40-BAFFR chimera were incubated with hCD154 for the indicated times. Western blots were performed to detect phosphorylated IκBα, total IκBα, and actin. D and E are representative of three independent experiments with similar results. (F) A20.2J WT and A20.2J TRAF6 KO cells expressing the hCD40-BAFFR chimera were incubated with agonistic mCD95/Fas-specific antibody to induce apoptosis. Agonistic hCD40-specific antibody was used to activate the hCD40-BAFF-R chimera. Agonistic mouse CD40-specific antibody rescues in a TRAF6-dependent manner and was used as a control (Benson et al., 2006). Isotype controls for each of these antibodies are named isotype 1, 2, and 3, respectively. Subdiploid (apoptotic) cells were measured by flow cytometric analysis after propidium iodide staining. Data are plotted as the mean ± SEM of five independent experiments, with the mean percentage of rescue calculated as described in Materials and methods. *, $P < 0.05$ (percentage of rescue in WT vs. TRAF6 KO cells).

we detected recruitment of both TRAFs to endogenous BAFF-R. The requirement of TRAF6 for TRAF2 and TRAF3 recruitment to BAFF-R has not been explored and we therefore performed the same experiment in TRAF6-deficient A20.2J B cells (TRAF6 KO; Rowland et al., 2007). In the absence

of TRAF6, TRAF2 and TRAF3 were recruited normally to BAFF-R, indicating that TRAF6 is not required for association of TRAF2 and TRAF3 with BAFF-R. Lack of TRAF6 expression and equivalent BAFF-R expression by WT and TRAF6 KO cells was confirmed by Western blot analysis and flow cytometry (Fig. S4, A and B). The association of TRAF6 with BAFF-R was also evident in primary mouse splenic B cells (Fig. 5 B) and in the human Karpas B cell line (Fig. 5 C), indicating that this novel interaction is not restricted to a specific cell line or species. The recruitment of TRAF6 to hBAFF-R in the absence of exogenous BAFF is attributed to endogenous BAFF production by the Karpas

B cell line, a common feature of Epstein-Barr virus-positive B cell lymphomas (He et al., 2003).

We next investigated whether TRAF6 is required for BAFF-mediated activation of NF- κ B. WT and TRAF6 KO A20.2J B cells were incubated with recombinant BAFF and phosphorylation and degradation of I κ B α was analyzed (Fig. 5 D). BAFF stimulation of WT cells resulted in phosphorylation of I κ B α , followed by degradation. In the absence of TRAF6, cells were unresponsive to BAFF and little I κ B α phosphorylation was detected. To ensure the specificity for TRAF6, TRAF6 KO cells were reconstituted with full-length TRAF6 (TRAF6 KO + TRAF6) and analyzed for their ability to respond to BAFF (Fig. 5 D, right; and Fig. S4 A). Similar to WT cells, TRAF6 reconstituted cells were able to phosphorylate and degrade I κ B α upon BAFF stimulation.

Because BAFF can bind both BAFF-R and TACI in the A20.2J B cells, we used the hCD40-BAFF-R chimera to confirm that BAFF-R specifically could induce TRAF6-dependent I κ B α phosphorylation in B cells. WT and TRAF6-deficient A20.2J cells were stably transfected with hCD40-BAFF-R_{WT} (Fig. S4 C) and incubated with hCD154. I κ B α phosphorylation was observed at 2 and 5 min, as is the case with a BAFF-induced signal, and phosphorylation was significantly reduced in TRAF6-deficient cells (Fig. 5 E). We did not detect any reproducible difference in BAFF-R-mediated NF- κ B2 activation in these cell lines (unpublished data), which is consistent with observations made using BAFF stimulation of TRAF6-deficient primary mouse B cells (Kobayashi et al., 2009).

To demonstrate a functional consequence of this defective I κ B α phosphorylation, the previous observation that BAFF can rescue B cells from Fas/CD95-induced apoptosis (Hancz et al., 2008) was examined in TRAF6-deficient A20.2J B cells expressing equal amounts of hCD40-BAFF-R and CD95 (Fig. S1 E and Fig. S4 B, respectively). Incubation of A20.2J cells with agonistic anti-CD95 antibody induces apoptotic cell death in ~70% of cells after 8 h as measured by staining with propidium iodide. Concurrent incubation of cells with agonistic antibodies that activate both CD95 and hCD40-BAFF-R inhibits CD95-mediated apoptotic cell death in ~50% of A20.2J B cells (Fig. 5 F). This BAFF-R-mediated rescue from apoptosis is reduced over twofold ($P = 0.0019$, $n = 5$) in the absence of TRAF6. Together, these data reveal TRAF6 as a newly identified member of the BAFF-R-proximal signaling complex, with important roles in BAFF-R-induced I κ B α activation and BAFF-R-mediated rescue from Fas-induced apoptosis.

DISCUSSION

Some of the genetic changes that lead to malignant transformation of B cells have been discovered, but many of the other contributing mechanistic details underlying transformation events are not yet known. Recent publications highlight the role for gene mutations in the pathogenesis of NHL. Specifically, mutations in CARD11 (Lenz et al., 2008) and CD79B (Davis et al., 2010) are present in tumor biopsies from patients with activated B cell (ABC)-like DLBCL. The ability of mutations in CARD11 to activate NF- κ B, and

those in CD79b to chronically activate signaling through the B cell receptor, suggests that dysregulation of signaling pathways contribute to NHL development. In accordance with this data, we have identified a novel lymphoma-associated mutation in BAFF-R that results in increased basal and BAFF-induced NF- κ B activation. Our studies suggest that the mutation is not specific to one subtype of NHL, although incidence was highest in FL (10%). Unlike DLBCL or MALT lymphoma, FL has not been shown to have constitutive NF- κ B activation. However, FL is a heterogeneous disease and gene set enrichment analysis suggests that t(14;18) negative FLs are enriched for NF- κ B and ABC-like gene signatures (Leich et al., 2009). Although this study focuses on the ability of BAFF-R_{His159-Tyr} to activate NF- κ B, we have preliminary data suggesting that BAFF-R_{His159-Tyr} also activates additional BAFF-R-mediated pathways, including Akt, which is activated in both FL (Gulmann et al., 2005) and DLBCL (Gupta et al., 2009). Therefore, the specific mechanism of how BAFF-R_{His159-Tyr} regulates tumor cell growth and development may be through activation of multiple signaling cascades. An expanded analysis of the presence of the mutation across the spectrum of all B cell malignancies and correlations with other clinical and biological parameters is currently ongoing and will hopefully lend insight into the function of the BAFF-R_{His159-Tyr} mutation.

In our initial analysis, we identified the BAFF-R_{His159-Tyr} mutation in NHL tissue, suggesting that it was a somatic mutation acquired during tumor formation. However, further analysis on available matched patient tumor and germline DNA revealed that it was a germline mutation in four of four cases. Overall, in our sample, frequency of the BAFF-R_{His159-Tyr} mutation was 6.2% in NHL tumor DNA compared with 0% in our 100 normal controls and 1.4% reported in the NCBI SNP database. Collectively, the increased frequency of the BAFF-R_{His159-Tyr} mutation in NHL compared with normal controls suggests that it may be a rare variant that confers risk to development of B cell lymphomas. In all cases analyzed, the mutation in BAFF-R was found to be heterozygous. The absence of a homozygous mutation in BAFF-R does not exclude it from having functional consequence as gain-in-function, haploinsufficiency, or dominant-negative effects are possible disease-causing mechanisms in heterozygous carriers. An example of this has been seen for *TNFRSF13B* (TACI), where C104R heterozygosity increases the risk for common variable immunodeficiency disorders and influences clinical presentation (Salzer et al., 2009). The ability of BAFF-R_{His159-Tyr} to activate increased levels of NF- κ B suggests that it models an oncogenic gain-in-function mutation. Although these types of mutations have not been clearly identified in NHL, heterozygous germline gain-in-function mutations in *KIT* and *PDGFR*A have been shown to be associated with development of gastrointestinal stromal tumors (Beghini et al., 2001; Chompret et al., 2004).

Constitutive activation of NF- κ B is a hallmark of NHL (Vallabhapurapu and Karin, 2009), and the ability of BAFF-R_{His159-Tyr} to activate this signaling cascade could be

a contributing factor to enhanced NF- κ B activation in a subset of patients. Although addressing the oncogenic capabilities of BAFF-R_{His159-Tyr} is beyond the scope of this initial study, data presented in this paper do suggest that this mutation contributes to dysregulated signaling through BAFF-R. The presence of the mutation may be of particular consequence in individuals who also have high serum BAFF levels, as the combined effect of elevated BAFF and the BAFF-R_{His159-Tyr} mutation may create a scenario where B cells have constitutive and dysregulated signaling through BAFF-R. The enhanced activation of both NF- κ B1 and NF- κ B2 pathways detected in cells transfected with BAFF-R_{His159-Tyr} was consistent across cell lines and using two different receptor models. The contribution of BAFF-R_{His159-Tyr} to cell survival and proliferation could not be analyzed in the human or mouse B cell lines used for our studies as a result of their naturally high proliferative capacity. To address these issues, we are generating a transgenic BAFF-R_{His159-Tyr} mouse to better model how this mutation contributes to B cell signaling defects and lymphomagenesis in the intact animal.

Our current understanding of BAFF-R is that it interacts with TRAF3 and Act1 adapter proteins in B cells, and the mechanistic role for TRAF3 in BAFF-R-induced NF- κ B2 activation has been the focus of several recent studies. Although others show that TRAF2 and cIAP1/2 are required for BAFF-R-induced degradation of TRAF3 and stabilization of NIK, the recruitment of TRAF2 or TRAF6 to a BAFF-R-containing protein complex has not been demonstrated to date (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Our data showing that TRAF2 coimmunoprecipitates with the cytoplasmic portion of BAFF-R is consistent with previous investigations showing that direct recruitment of TRAF2 to another TNFR superfamily member CD40 is an essential step in CD40-induced cIAP-mediated TRAF3 degradation (Hostager et al., 2003; Moore and Bishop, 2005; Vallabhapurapu et al., 2008).

The necessity of TRAF6 for BAFF-R-induced I κ B α phosphorylation and degradation clarifies a major question posed by the recent generation of the first B cell-specific TRAF6 knockout mouse. TRAF6 deficiency reduces mature B cell populations in the bone marrow and spleen (Kobayashi et al., 2009). The authors posit that this deficiency cannot be fully attributed to the impaired signaling of TLRs or CD40, implying the involvement of a different receptor. Based on the results presented in this paper and the established role of BAFF-R in B cell survival and homeostasis, we suggest that impaired signaling by BAFF-R contributes to the B cell deficiency exhibited by the B cell-specific TRAF6-deficient mouse. TRAF6 has been shown to be necessary for IL-1-induced phosphorylation of I κ B α by acting as an E3 ubiquitin ligase for IKK- γ /NEMO (Lamothe et al., 2007), and TRAF6 may perform a similar role in the context of BAFF-R signaling. The recruitment of TRAF6 by BAFF-R also complements the recent finding that the protein Act1 acts as an E3 ubiquitin ligase for TRAF6 in the context of IL-17 signaling (Liu et al., 2009). Act1 was shown to be recruited to the cytoplasmic portion of BAFF-R after stimulation with BAFF (Qian et al., 2004) and, in this context, could

also be required for the ubiquitination of TRAF6. It is not clear at this time whether the association of TRAF2 and TRAF6 with BAFF-R is through a direct interaction with the BAFF-R cytoplasmic tail or indirectly through TRAF3. Heteromeric TRAF2-TRAF3 complexes have been shown to form in B cells (Vallabhapurapu et al., 2008; Zarnegar et al., 2008) so it is quite feasible that the association is via this mechanism. However, the ability of the TRAF3 binding-deficient BAFF-R_{AVAAA} mutant to recruit TRAF2 and TRAF6, although diminished (Fig. 4A), suggests that TRAF3 binding may not be required for BAFF-R/TRAF2 and TRAF6 associations. The requirements within the cytoplasmic tail of BAFF-R for TRAF2 and TRAF6 associations are currently being analyzed.

The crystal structure of a 24-residue fragment from the cytoplasmic domain of BAFF-R bound in complex with TRAF3 has been determined (Ni et al., 2004) and the structure revealed that the 162-PVPAT-166 sequence in BAFF-R serves as the recognition motif for binding to TRAF3. Contacts observed in the crystal structure were confirmed by protein binding studies, and critical distal residues were also identified that mediate TRAF3 recognition, for example, T175. The substitution at BAFF-R residue 159 is just three residues N-terminal to the PVPAT recognition motif, and this residue in BAFF-R may represent an additional contact site for TRAF3 binding that has not yet been identified. Inspection of the atomic model of the BAFF-R-TRAF3 complex suggests that substitution of a tyrosine residue for histidine at this site may introduce the potential for formation of a stronger hydrogen-binding network, which is consistent with the increased binding observed in the present study (K. Ely and S. Mylvaganam, personal communication). Predicted contact residues in TRAF3 will be tested in future binding assays.

The increased amount of TRAFs recruited to BAFF-R_{His159Y} likely contributes to the increased NF- κ B activation detected in cells expressing the mutant receptor. Our data suggest that TRAF6 contributes to activation of the NF- κ B1 pathway, although TRAF2 and 3 likely activate NF- κ B2. However, we did not detect any difference in TRAF3 degradation between the BAFF-R_{His159Y} and BAFF-R_{WT}. Therefore, there may be additional as yet unidentified factors that interact with BAFF-R or TRAF2, 3, and/or 6 that mediate the enhanced B cell signaling shown by BAFF-R_{His159Y}.

Collectively, our data identify a novel lymphoma-associated mutation in BAFF-R and describe exciting new aspects of BAFF-R signaling that are highly relevant to human B cell biology. Building upon these results to obtain a more complete understanding of how this signaling is regulated will provide valuable information about normal B cell homeostasis and function and pathogenic BAFF-R contributions to human disease.

MATERIALS AND METHODS

Patient material. The Institutional Review Board at the Mayo Clinic reviewed and approved this study. DNA and tumor tissue from NHL patients and normal controls was acquired in an ongoing clinic-based case-control study at the Mayo Clinic upon providing written informed consent. Details of patient specimens are available elsewhere (Cerhan et al., 2007; Novak et al., 2009).

Sequencing of *TNFSFR13C* and mutation analysis. Genomic germline DNA was isolated from 20 FL patients and 20 normal controls and used for the initial sequencing of *TNFSFR13C*. Purified DNA was amplified by PCR using primer pairs that span the *TNFSFR13C* promoter and each exon. PCR fragments were sequenced at the Mayo Clinic DNA Sequencing Core Facility and analyzed using Mutation Surveyor software (SoftGenetics). Identification of the C519T mutation in DNA isolated from NHL tumor biopsies ($n = 129$) and tissue (PBMC) from normal controls ($n = 100$) was done by restriction fragment length polymorphism (RFLP) analysis and/or direct sequencing. For RFLP analysis, a PCR fragment including position 519 was subjected to restriction enzyme digest with MscI (New England Biolabs, Inc.) to confirm the presence or absence of the mutation.

Multiple sequence alignment. BAFF-R amino acid sequences were aligned using the ClustalW program. Default parameters were used (Larkin et al., 2007).

Cell lines. The HEK-293 and Karpas-422 cell lines were obtained from the American Type Culture Collection and Deutsche Sammlung von Mikroorganismen und Zellkulturen, respectively. The mouse B cell lines CH12.LX, A20.2J, and A20.2J TRAF6 KO have been described previously (Hsing and Bishop, 1999; Rowland et al., 2007). Hi5 insect cells infected with WT or hCD154-expressing baculovirus were produced as described previously (Rowland et al., 2007) and used at a ratio of 1 insect cell to 10 B cells.

Antibodies. Anti-hCD40 (G28.5) and anti-mCD40 (1C10) were produced from a hybridoma, and isotype control MOPC21 was purchased from Sigma-Aldrich. Anti-BAFF-R (ab5965) was purchased from Abcam. Anti-NF- κ B p52 (05–361) was purchased from Millipore. Anti-TRAF2 (592) and anti-TRAF6 (1B1–2) were purchased from MBL International. Anti-TRAF3 (H122) and anti-YY1 (H-10) were purchased from Santa Cruz Biotechnology, Inc. Anti-human CD40 was produced by Elmira Biologicals. Anti-p52 (4882), anti-phospho-I κ B α (2859), anti-I κ B α (9242), anti-TRAF2 (4724), and anti-TRAF6 (4743) were purchased from Cell Signaling Technology. Anti- β -actin (600–501) was from Novus Biologicals. HA-HRP was purchased from Roche. HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Plasmid construction. BAFF-R H159Y and AVAAA were generated by site-directed mutagenesis (Invitrogen) and subcloned into pcDNA3.1. The hCD40–BAFF-R chimeric construct was subcloned into the plasmid pRSV5.neo. The His159Tyr transition was introduced to this chimera by overlap extension PCR.

Transfection and stable cell line generation. HA-tagged WT, H159Y, or AVAAA BAFF-R constructs were transfected into HEK293 cells with Lipofectamine (Invitrogen). Cells were selected in 500 μ g/ml G418 (Cellgro) and subcloned to generate stable cell lines. A20.2J and CH12.LX B cell lines were transfected with pRSV5.neo hCD40–BAFF-R constructs using a BTX Electro Square Porator (Harvard Apparatus). Stable clones were maintained in 400 μ g/ml G418.

Flow cytometry. FITC-conjugated mouse anti-BAFF-R (ab38977) was purchased from Abcam. FITC-conjugated anti-hCD40, anti-mBAFF-R, and anti-mCD95 and isotype control antibodies were purchased from eBioscience. Flow cytometry was performed using FACSCalibur (BD) or Guava EasyCyte (Millipore) instruments. FlowJo software (Tree Star, Inc.) was used for analyses.

p52 and RelB assays. 3×10^6 serum-starved HEK293 cells expressing human WT, H159Y, or AVAAA BAFF-R were incubated with 50 or 200 ng/ml BlyS. Cells were lysed in RIPA buffer and levels of p52 and β -actin were measured by immunoblotting. For mouse B cells, 3×10^6 A20.2J cells were incubated with 10 μ g/ml of agonistic anti-human CD40 antibody (G28.5). Nuclear extracts were prepared as previously described (Rowland et al., 2007) and levels of p52, RelB, and YY1 were measured by immunoblotting.

NF- κ B reporter assay. HEK293 cells expressing human WT, H159Y, or AVAAA BAFF-R were transiently transfected with 1 ng Renilla and 10 μ g pNF- κ B luciferase reporter plasmid or a control reporter plasmid that lacks the NF- κ B DNA sequence. 6 h after transfection, cells were treated with 200 μ g/ml of recombinant human BAFF for 24 h. Luciferase activity was measured in cell extracts and normalized against Renilla with the Dual Luciferase kit (Promega).

I κ B α phosphorylation assay. A20.2J and A20.2J TRAF6 KO cell lines were stimulated with 250 ng/ml of recombinant human BAFF (PeproTech) at 37°C. A20.2J and A20.2J TRAF6 KO cells expressing hCD40–BAFF-R were stimulated with hCD154. Whole cells were lysed for SDS-PAGE and Western blot analyses.

TRAF recruitment to hCD40–BAFF-R. A20.2J B cells expressing hCD40–BAFF-R chimeras were immunoprecipitated using the dual stimulation-immunoprecipitation method described previously (Rowland et al., 2007). In brief, cells were incubated with 20 μ l of protein G Dynabeads (Invitrogen) coated with 10 μ g G28.5 at 37 or 4°C. Bead-bound cells were lysed and cell debris removed by washing to leave only G28.5-associated hCD40–BAFF-R and any associating proteins. Levels of hCD40–BAFF-R, TRAF2, TRAF3, and TRAF6 were measured by immunoblotting.

IgM secretion assay. CH12.LX cells expressing hCD40–BAFF-R_{WT} or hCD40–BAFF-R_{His159-Tyr} were incubated with 2 μ g/ml anti-mCD40 (1C10), anti-hCD40 (G28.5), or isotype control for 72 h to induce secretion of the phosphatidyl choline-reactive IgM. IgM-secreting cells were quantified by direct plaque-forming cell assay as previously described (Haxhinasto et al., 2002).

TRAF recruitment to BAFF-R. Mouse A20.2J, human Karpas, or primary T cell-depleted mouse splenocytes were stimulated with 500 ng/ml BAFF at 37°C. Enrichment of the lipid raft fraction was performed by lysing cells in 1% Brij58, 150 mM NaCl, 20 mM Tris, 50 mM β -glycerophosphate, and EDTA-free mini-complete protease inhibitor mix (Roche). The Brij58 insoluble fraction was solubilized in 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 20 mM Tris, 50 mM β glycerophosphate, and protease inhibitors. This fraction was subjected to immunoprecipitation with anti-mouse or anti-human BAFF-R (AF1357 and AF1162, respectively; BD). $10\text{--}17 \times 10^6$ serum-starved HEK293 cells expressing human WT, H159Y, or AVAAA BAFF-R were incubated with 200 ng/ml BAFF. Cells were lysed and HA–BAFF-R was immunoprecipitated using the ProFound HA tag IP/Co-IP kit (Thermo Fisher Scientific).

Apoptosis assays. A20.2J cells were stimulated with anti-mouse CD40 (1C10), anti-human CD40 (G28.5) or isotype controls at 10 μ g/ml, or anti-mouse CD95 (BD) or isotype control at 100 ng/ml for 8 h. Subdiploid nuclei were detected by PI staining and flow cytometry as previously described (Benson et al., 2006). Percentage of rescue was calculated by subtracting the number of subdiploid cells after dual anti-mouse CD95 and anti-human CD40 stimulation from the number of subdiploid cells after anti-mouse CD95 treatment alone. This difference was expressed as a proportion of the total number subdiploid cells after anti-mouse CD95 treatment alone.

Statistical analysis. All statistical comparisons were done using an unpaired Student's t test. Two-sided p -values < 0.05 were considered statistically significant.

Online supplemental material. Fig. S1 shows schematics of the BAFF-R constructs used to transfect HEK293T cells and A20.2J B cells and expression of the BAFF-R constructs by FACS and immunoblotting. Fig. S2 shows cell surface expression of hCD40–BAFF-R chimeras in CH12.LX B cells. Fig. S3 shows expression of TRAF1, 2, 3, and 6 in A20.2J B cell transfected with hCD40–BAFF-R chimeras. Fig. S4 shows total cellular TRAF6 expression and cell surface expression of endogenous mBAFF-R and mCD95 in A20.2J WT and A20.2J TRAF6 KO cell lines. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20100857/DC1>.

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