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B and T Lymphocyte Attenuator Regulates B Cell Receptor Signaling by Targeting Syk and BLNK

Andrew C. Vendel,* Jill Calemine-Fenaux,* Anita Izrael-Tomasevic,* Vandana Chauhan,† David Arnott,* and Dan L. Eaton1*  

B and T lymphocyte attenuator (BTLA) functions as a negative regulator of T cell activation and proliferation. Although the role of BTLA in regulating T cell responses has been characterized, a thorough investigation into the precise molecular mechanisms involved in BTLA-mediated lymphocyte attenuation and, more specifically, its role in regulating B cell activation has not been presented. In this study, we have begun to elucidate the biochemical mechanisms by which BTLA functions to inhibit B cell activation. We describe the cell surface expression of BTLA on various human B cell subsets and confirm its ability to attenuate B cell proliferation upon associating with its known ligand, herpesvirus entry mediator (HVEM). BTLA associates with the BCR and, upon binding to HVEM, recruits the tyrosine phosphatase Src homology 2 domain-containing phosphatase 1 and reduces activation of signaling molecules downstream of the BCR. This is exemplified by a quantifiable decrease in tyrosine phosphorylation of the protein tyrosine kinase Syk, as measured by absolute quantification mass spectrometry. Furthermore, effector molecules downstream of BCR signaling, including the B cell linker protein, phospholipase Cγ1, and NF-κB, display decreased activation and nuclear translocation, respectively, after BTLA activation by HVEM. These results begin to provide insight into the mechanism by which BTLA negatively regulates B cell activation and indicates that BTLA is an inhibitory coreceptor of the BCR signaling pathway and attenuates B cell activation by targeting the downstream signaling molecules Syk and B cell linker protein.

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The role of BTLA appears to be limited to lymphocyte activation as shown in BTLA-deficient mice, which present normal lymphoid organ development and near wild-type lymphocyte numbers. However, these mice display hyperproliferative T and B cell responses to TCR- and BCR-mediated activation, respectively (9, 10, 11). Furthermore, loss of BTLA function leads to increased susceptibility to experimental autoimmune encephalomyelitis and MHC-mismatched allograft rejection, supporting the role of BTLA in modulating T cell activation and effector responses (11, 12).

Surface expression analysis of BTLA indicates that it is expressed on a wide number of lymphocytes in mice. It is most highly expressed on B cells, followed by CD4+ T cells, and lower expression on CD8+ T cells, macrophages, dendritic cells, and NK cells (9, 13). During murine B cell development, BTLA expression first appears in pre-B cells and shows increased expression through B cell maturation, with the highest expression on mature B cells (9). In this study, we show a detailed cell surface expression profile of BTLA during human B cell development. We demonstrate, as in mice, human B cells present relatively low BTLA expression on pro-, pre-, and immature B cells and highest expression on memory peripheral blood B cell subsets.

Thus far, the majority of work on BTLA has focused on understanding its role in regulating T cell function in mice, and little is known about its role in regulation of B cells or its functional impact in human lymphocyte regulation. In this work, we evaluate the role and functional mechanism by which BTLA regulates human B cell activation. We show activation of BTLA through HVEM attenuates human B cell proliferation and induces phosphorylation of the ILT domains, resulting in Src homology 2 domain-containing phosphatase (SHP) 1, but not SHP2 recruitment. We also found that BTLA associates with the BCR members IgM, CD79a, and CD79b. Although BTLA is associated with the BCR, it does not impact immediate activation of the BCR complex. However, we found that BTLA reduces the activity of protein tyrosine kinase, Syk, as observed by reduced Syk phosphorylation levels upon HVEM treatment of human B cells. Downstream of Syk, B cell linker protein (BLNK) and phospholipase Cγ2 (PLCγ2) also show lower phosphorylation levels upon BTLA signaling, indicating reduced activity of the BCR signal transduction cascade.
Lastly, BTLA activity reduces nuclear localization of the transcription factor NF-κB. Together, our results detail a possible mechanism by which BTLA functions in attenuating B cell activation.

Materials and Methods

Protein reagents

Proteins were expressed in Chinese hamster ovary cells as detailed previously (4). Briefly, C-terminal Fc-fusion proteins were purified by affinity chromatography using MahSure select Sepharose (Amersham Pharmacia), eluted with acetate (pH 3.0), and neutralized with a 1:10 dilution of 1 M Tris (pH 8.0). After dialyzing against PBS overnight at 4°C, final purification of Fc-tagged proteins was accomplished by S200 gel filtration chromatography. Protein identity and purity were evaluated by SDS-PAGE and mass spectrum analysis. Endotoxin levels were measured for all protein samples and were below 0.5 of endotoxin units/mg.

Cell culture and stable cell line

The human BJAB cell line was cultured in RPMI 1640 medium supplemented with 10% FBS (HyClone) and 10 mM glutamine at 37°C in 5% CO₂. A stable BJAB cell line constitutively expressing human BTLA (BTLA-BJAB) was generated as described previously (4). In short, BJAB cells were infected with a murine stem cell virus-human BTLA construct (BD Biosciences) and BTLA expression was confirmed by flow cytometry (supplemental Fig. 1).^1^

Flow cytometry analysis for B cell surface expression of BTLA

Before staining, all cell suspensions were incubated with human IgG to block Fc receptors. Whole bone marrow cells (AliCells) were stained with fluorescein isothiocyanate (anti-CD19 and anti-CD50). Pro-pre-immature B cells were identified as CD19⁺ CD10⁻ and mature B cells were identified as CD19⁺ CD10⁺. Tonsil tissue (AliCells) was dissociated and a single-cell suspension was stained with anti-CD19, anti-IgD, and anti-CD38. Naïve tonsillar B cells were identified as CD19⁺ IgD⁺ CD38⁻/low. Germinal center B cells were identified as CD19⁺ IgD⁺ CD38⁺ and memory B cells were identified as CD19⁺ CD10⁺ IgD⁺ CD38⁺. Ficol-separated PBMC from whole blood were stained with anti-CD19, anti-CD50, and anti-CD27. Naïve B cells were identified as CD19⁺ IgD⁺ CD27⁻. Transition B cells were CD19⁺ IgD⁺ CD27⁺ and memory B cells were CD19⁺ IgD⁺ CD27⁺. BTLA expression was detected with biotinylated monoclonal 3B1 (Genentech) followed by allophycocyanin-conjugated streptavidin (BD Biosciences). All fluorochrome Abs were purchased from BD Biosciences.

Human B cell proliferation assays

Following the manufacturer’s protocol, B cells were purified from human whole blood using a B Cell Isolation Kit II (Miltenyi Biotec). B cell proliferation assays were conducted using plate-bound HVEM-Fc or control-Fc protein and soluble Abs. Flat-bottom 96-well plates (Costar) were coated with mouse anti-human IgG1-Fc clone MH1015 (Invitrogen) at 20 µg/ml for 2 h at 37°C. Wells were aspirated before adding 10 µg/ml goat F(ab')₂, anti-human IgM (Jackson ImmunoResearch Laboratories) and human B cells (100,000 cells/well) in complete medium with or without soluble BTLA mAbs (3B1, 5F5; Genentech) at 10 µg/ml. Cells were incubated in supplemented medium for a total of 72 h at 37°C/5% CO₂ and pulsed with [3H]thymidine for the last 18 h of culture. Proliferation was detected with biotinylated monoclonal 3B1 (Genentech) followed by a 1 ml of 5% sucrose in lysis buffer but not shown. C-terminal Fc-fusion proteins were purified by affinity chromatography using MahSure select Sepharose (Amersham Pharmacia), eluted with acetate (pH 3.0), and neutralized with a 1:10 dilution of 1 M Tris (pH 8.0). After dialyzing against PBS overnight at 4°C, final purification of Fc-tagged proteins was accomplished by S200 gel filtration chromatography. Protein identity and purity were evaluated by SDS-PAGE and mass spectrum analysis. Endotoxin levels were measured for all protein samples and were below 0.5 of endotoxin units/mg.

Lipid raft isolation

BTLA-BJAB cells were stimulated as described above and resuspended in lysis buffer on ice for 30 min. Cell lysates were further disrupted by sonication using 3 × 10 s 6-W pulses. A total of 500 µl of cell lysates was mixed with 85% sucrose in lysis buffer and 750 µl of these samples was transferred to the bottom of a Beckman 13 x 51-mm centrifuge tube. The diluted lyses were overlaid with 2 ml of 30% sucrose in lysis buffer and centrifuged at 4°C followed by 1 ml of 5% sucrose in lysis buffer and finally 250 µl of lysis buffer. Samples were centrifuged in a SW-55Ti swinging bucket rotor at 200,000 × g for 17 h at 4°C. Four hundred-microliter fractions were collected from top-down and 50-µl aliquots from each fraction were mixed with 35 µl of SDS sample buffer plus 1 mM DTT, boiled for 10 min, and subjected to SDS-PAGE and Western blot analysis. Lipid rafts were identified by probing for Lyn using an anti-Lyn Ab (Santa Cruz Biotechnology). BTLA and IgM were detected using Abs described above.

In-gel digestion

Syk immunoprecipitates were resolved by SDS-PAGE and bands corresponding to Syk were excised for in situ digestion. Gels were destained, reduced with DTT, and free cysteine side chains were alkylated with iodoacetamide. Excised gel pieces were washed, dehydrated in undiluted acetonitrile, and then rehydrated with 50-ng/µl solution of modified porcine trypsin (Promega) in 50 mM NH₄HCO₃. Digestion proceeded overnight at 37°C. Digested supernatants were transferred to sample vials. Gel pieces were washed twice with 5% formic acid to extract the remaining peptides and added to sample vials to a final volume of 30 µl.

Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry was performed using a hybrid triple quadrupole-linear ion trap mass spectrometer (Q-Trap 4000; Applied Biosystems) equipped with a microelectrospray ionization source. Samples were introduced by microcapillary reverse-phase liquid chromatography (C₁₈:3 50-µm inside diameter) in the “vented column” configuration (14). Samples were loaded at 2.5 µl/min for 6 min and eluted at 250 nl/min in a 40-min program (solvent A: 0.1% v/v formic acid in water; solvent B: 0.1% v/v formic acid in acetonitrile).

Syk phosphopeptide quantitation

Selected reaction monitoring (SRM) experiments were implemented and optimized for the Syk tryptic peptide (residues 339–361) EALPMDVFQGK (GlycoIDE) equipped with a microelectrospray ionization source. Samples were introduced by microcapillary reverse-phase liquid chromatography (C₁₈:3 50-µm inside diameter) in the “vented column” configuration (14). Samples were loaded at 2.5 µl/min for 6 min and eluted at 250 nl/min in a 40-min program (solvent A: 0.1% v/v formic acid in water; solvent B: 0.1% v/v formic acid in acetonitrile).
demonstrate that mature peripheral B cells exhibit the highest subsets using a previously described BTLA-specific mAb (4). We analyzed BTLA expression on human primary B cell lineages, we analyzed BTLA expression on human primary B cell subsets. BTLA surface expression varies depending on the developmental stage of the B cell. Subsets were identified as bone marrow-derived, pro/pre/immature (CD19+CD10−) and mature (CD19+CD10+). Tonsillar B cells were identified as naive (CD19+IgD−CD38−/low), germinal center (CD19+IgD−CD38+), and memory (CD19+IgD−CD38+). Circulating B cells were identified as naive (CD19+IgD−CD27+), transition (CD19+IgD−CD27+), and memory (CD19+IgD−CD27+). Histograms are presented as log shifts compared with the isotype control (shaded).

**FIGURE 1.** BTLA expression on human B cell subsets. BTLA surface expression varies depending on the developmental stage of the B cell. Subsets were identified as bone marrow-derived, pro/pre/immature (CD19+CD10−) and mature (CD19+CD10+). Tonsillar B cells were identified as naive (CD19+IgD−CD38−/low), germinal center (CD19+IgD−CD38+), and memory (CD19+IgD−CD38+). Circulating B cells were identified as naive (CD19+IgD−CD27+), transition (CD19+IgD−CD27+), and memory (CD19+IgD−CD27+).

**Fluorescence microscopy**
To monitor NF-κB nuclear translocation, human BJAB B cells stably expressing BTLA, under different stimulating conditions, were incubated for at least 20 min at 37°C on chemically coated chambered slides (Lab-Tek II-CC; Nunc). Cells were washed in PBS before a 10-min 2% paraformaldehyde fixative treatment followed by permeabilization in ice-cold methanol for 5 min. Cells were washed and incubated with Alexa Fluor 647-conjugated anti-p65 Ab (Santa Cruz Biotechnology) in PBS plus 5% FBS for 1 h at room temperature. Cells were washed and mounted in PBS before microscopic analysis. Images were obtained using an Axiovert 200M inverted microscope (Zeiss). Fractional NF-κB nuclear occupancy was calculated by measuring volumetric fluorescence intensity of p65 found in the nucleus compared with whole-cell p65 intensity. A minimum of 100 cells per time point (0, 15, 30, 60, and 240 min) was analyzed for each condition.

**NF-κB ELISA**
BJAB cells expressing BTLA were serum starved in PBS for 30 min before anti-IgM stimulation, 10 μg/ml in 1 ml of PBS, in the presence of control-Fc or HVEM-Fc (10 μg/ml) for 4 h at 37°C. Cells were washed in PBS before nuclear extraction per the manufacturer’s protocols (Panomics). A transcription factor ELISA kit (Panomics) was used to measure NF-κB activity with the provided reagents and protocols. Briefly, nuclear extracts (0.5 mg/μl) were incubated with biotinylated NF-κB-specific DNA probe (Panomics) before capture on a streptavidin-coated plate provided. Complexes were washed and probed for NF-κB using an anti-p65-specific Ab followed by incubation with a HRP-conjugated secondary Ab and detection using provided chromagenic substrates. The presence of NF-κB was evaluated with a Safire2 plate reader (Tecan) measuring relative absorbance at 450 nm. Samples were run in triplicate and compared with unstimulated controls.

**Results**

**BTLA surface expression on human B cell subsets**
BTLA is expressed on a wide variety of murine immune cells with varying degrees of surface expression on B, T (CD4+, CD8+, and regulatory T), macrophages, dendritic cells, and NK cells (9, 13, 16). To aid in our understanding of BTLA function in human cell lineages, we analyzed BTLA expression on human primary B cell subsets using a previously described BTLA-specific mAb (4). We demonstrate that mature peripheral B cells exhibit the highest BTLA expression and lowest expression was observed on bone marrow-derived precursor B cells (Fig. 1). Uniform BTLA expression was observed on naive, transitional, and memory peripheral B cells, while tonsillar and bone marrow-derived B cells showed an increase in BTLA expression from the pro/pre/immature to memory stage. The restriction of BTLA expression to only mature B cells in the bone marrow suggests that BTLA does not have a pivotal role in B cell development in humans, which agrees with normal lymphocyte development observed in BTLA-deficient mice (9, 11).

**HVEM binding leads to BTLA activation, SHP1 recruitment, and attenuation of human primary B cell proliferation in a dose-dependent manner**
In T cells, HVEM induces tyrosine phosphorylation within the ITIM domains of BTLA, leading to protein tyrosine phosphatase recruitment, attenuation of T cell proliferation, and effector responses (5, 6, 9–11). Similarly, we showed that recombinant HVEM attenuates BCR-mediated human B cell proliferation (Fig. 2A), which was prevented with soluble, BTLA-specific Ab that blocks HVEM binding (3B1; Fig. 2A), but not by a nonblocking anti-BTLA Ab (5F5; Fig. 2B), demonstrating that the inhibitory activity of HVEM-Fc is mediated through BTLA. Flow cytometry verified on B cells that 3B1 competes with HVEM for BTLA binding, while 5F5 does not (Fig. 2B). Although HVEM has been shown to bind other proteins, including LIGHT, lymphotoxin α, glycoprotein D, and CD160, these proteins are not expressed on the surface of human B cells (17–21) (supplemental Fig. 1). LIGHT expression on B cells can be induced under certain co-stimulatory conditions, but these conditions were not used in this study, supporting the inhibitory role of HVEM on B cells is directed through BTLA. As seen in T cells, HVEM induced tyrosine phosphorylation of BTLA and SHP1 recruitment to BTLA (Fig. 2C). However, we did not observe SHP2 recruitment to BTLA upon HVEM binding (data not shown). Together, these results...
demonstrate that HVEM binding to human B cells leads to activation of the inhibitory receptor, BTLA and attenuation of B cell proliferation.

BTLA complexes with the BCR

BTLA recruits the protein tyrosine phosphatase SHP1 in T and B cells, but beyond this, it is unclear how BTLA activity leads to suppressed lymphocyte activation (6, 9, 11). To determine ablated downstream signaling mechanisms following BTLA activation, BTLA was immunoprecipitated from human primary B cells after various stimulating conditions and was probed for protein interactions by Western blot analysis. BTLA was found to associate with members of the BCR signaling complex, including IgM, CD79a, and CD79b (Fig. 3A). BTLA appears constitutively associated with the BCR and the level of association is not altered following B cell activation or binding of HVEM to BTLA. Furthermore, this interaction appeared to be specific to the BCR complex since BTLA failed to associate with the costimulatory receptor CD19. The association of BTLA with the BCR was confirmed by immunoprecipitation of the CD79a/b complex and probing for BTLA (Fig. 3B). Although the BCR coprecipitated with BTLA, it is unclear if this is due to direct or indirect protein-protein interactions.
To assess BTLA inhibitory axis in altering BCR-mediated B cell activation, the phosphorylation of the signaling arm of the BCR complex, CD79a/b, upon BTLA activation was determined using a pan anti- phosphory-tyrosine Ab. As expected, BCR stimulation results in significant CD79a/b phosphorylation (Fig. 3B). However, CD79a/b phosphorylation was not significantly decreased when cells were treated with HVEM, indicating that BTLA does not impact the activation of the CD79a/b signaling complex. We also observed no change in CD79a/b phosphorylation when the amount of immunoprecipitation sample loads were titrated and analyzed by Western blot analysis (supplemental Fig. 2). Furthermore, BTLA showed no ability to alter CD19 activation monitored by Tyr319 phosphorylation within the ITAM of CD19. Together, these results suggest that the co-inhibitory signaling activity of BTLA may lay downstream of the BCR.

Previous studies in T cells indicate that BTLA can translocate to lipid rafts by its association with CD3ζ (22). Therefore, we assessed the ability of BTLA to transport to lipid rafts in B cells upon BCR activation in the presence and absence of HVEM. Lysates from unstimulated or stimulated (with or without HVEM) BTLA-BJAB cells were subjected to discontinuous sucrose gradient centrifugation. Lyn, a known lipid raft marker, was used to identify lipid rafts in these cells (23). Lyn was found to be present in fractions 2 and 3 and excluded from the bottom fractions (Fig. 3C). BTLA was not found to be present within the lipid raft fractions under all stimulation conditions, whereas the BCR was detected in lipid rafts only upon ligation of the BCR with anti-IgM. Subsequent BCR localization with lipid rafts was unaffected upon HVEM treatment. These results suggest that although BTLA associates with the BCR it lacks the properties required for incorporation into lipid rafts in B cells and imparts its function peripheral to these densely occupied membrane signaling scaffolds.

**BTLA is a negative regulator of Syk, BLNK, and PLCγ2**

Because the inhibitory activity of BTLA does not appear to be directed toward the primary BCR signaling complex, we evaluated its ability to alter the activity of signaling molecules downstream of the BCR. Once phosphorylated, the CD79a/b ITAM domains recruit the protein tyrosine kinase Syk, which is subsequently phosphorylated by Lyn and mediates critical downstream signaling pathways, leading to proliferation and differentiation of B cells (24, 25). B cells were stimulated and the ability of BTLA to regulate Syk tyrosine phosphorylation was assessed by immunoprecipitation followed by Western blot analysis. Ligation of the BCR leads to substantial Syk phosphorylation, which was decreased upon BTLA activation by HVEM, as observed by Western blot analysis using a pan anti-phospho-tyrosine Ab, indicating that BTLA inhibits BCR signaling by targeting the protein tyrosine kinase Syk (Fig. 4A).

A number of auto- and transphosphorylation events contribute to the overall activity of Syk (26–28). After ligation of the BCR signaling complex, Syk is phosphorylated on Tyr348 and Tyr352 by the protein tyrosine kinase Lyn, leading to its initial activation, which is followed by autophosphorylation to reach maximum signaling activity of Syk (28, 29). This phosphoregulated activation of Syk is balanced by further phosphorylation of Tyr253 by Lyn, which has been shown to down-regulate Syk activity (28). To assess whether BTLA alters the phosphorylation state of residues critical for initial activation of Syk (Tyr348/Tyr352), absolute quantification (AQUA) mass spectrometry was used. A synthetic phospho-peptide corresponding to residues 339–361 of Syk was generated and spiked into Syk immunoprecipitation samples from human B cells. Known concentrations of synthetic peptide were used to quantify the level of Syk (Tyr348/Tyr352) phosphorylation from BCR-stimulated B cells after BTLA activation. Cross-linking of the BCR led to an increase in Syk Tyr348/Tyr352 phosphorylation, which was decreased following BTLA activation by HVEM (Fig. 4B). Quantification of mass spectrometry (MS) data shows that HVEM treatment leads to a 30% decrease in total phosphate incorporation at Tyr348/Tyr352 of Syk (Fig. 4C). These data suggest that BTLA inhibitory activity is directed, in part, toward Syk.

Next, we determined whether the targets of the protein tyrosine kinase activity of Syk, including BLNK and PLCγ2, showed reduced phosphorylation following BTLA activation. BLNK, a central adaptor molecule, is phosphorylated by Syk and recruits
FIGURE 5. BTLA signaling leads to reduced phosphorylation of BLNK and PLCγ2. Human primary B cells were treated for 15 min at room temperature with no stimulation, anti-IgM plus control protein, or anti-IgM plus HVEM stimulation followed by BLNK or PLCγ2 immunoprecipitation and western blot analysis with a pan anti-phosphotyrosine Ab. Blots were stripped and reprobed with anti-BLNK or PLCγ2Abs to show equal loading. Fold change in BLNK and PLCγ2 phosphorylation was calculated from the ratio of pBLNK:BLNK or pPLCγ2:PLCγ2 band intensities. Images of scanned blots for each experiment are shown as insets.

PLCγ2, bridging the kinase activity of Syk with downstream effector pathways (30–32). Both BLNK and PLCγ2 Immunoprecipitated from human primary B cells showed decreased levels of phosphorylation following HVEM treatment, indicating that BCR signaling downstream of Syk is attenuated (Fig. 5). Although the activity of BTLA leads to reduced phosphorylation of Syk, BLNK, and PLCγ2, none of these proteins interact with BTLA directly (supplemental Fig. 3). Therefore, the impact of BTLA on these signaling molecules is likely due to recruitment of the protein tyrosine phosphatase SHP1 to the cytoplasmic tail of BTLA upon HVEM treatment. Collectively, these results demonstrate that BTLA targets BCR signaling by reducing Syk activity, which leads to a reduction in activation of downstream signaling molecules.

BTLA reduces NF-κB nuclear translocation

NF-κB is an indispensable transcriptional activator required for driving B cell activation, proliferation, and effector immune responses (33–36). NF-κB is retained in the cytosol by the regulatory protein IkB in resting B cells, and BCR signaling leads to IKK-mediated degradation of IkB and translocation of active NF-κB to the nucleus (24, 37). We used the BTLA-BJAB cell line to measure the effect of BTLA inhibitory signaling on regulating NF-κB activation and nuclear localization. BTLA-BJAB cells showed an increase in NF-κB nuclear translocation after BCR stimulation with anti-IgM, which was decreased in cells treated with HVEM, indicating that BTLA blocks NF-κB activation (Fig. 6A).

BTLA-mediated decrease in NF-κB nuclear localization was consistent over an extended BCR stimulation time course, suggesting that B cell activation is significantly muted by BTLA inhibitory activity (Fig. 6B). Furthermore, DNA-binding activity of NF-κB was reduced upon HVEM treatment as shown by ELISA analysis of p65 DNA-binding activity (Fig. 6B, inset). This result further supports the role of BTLA as a suppressor of BCR-mediated signal transduction leading to B cell activation.

Discussion

To balance effective immune response and self-tolerance, lymphocytes employ a myriad of costimulatory and coinhibitory molecules. The coinhibitory receptor BTLA is expressed on various lymphocytes and has been shown to attenuate T cell activation and proliferation (9, 11). This inhibitory activity was confirmed in BTLA-deficient mice, which display normal lymphatic development, but present a hyperproliferative phenotype in response to T or B cell activation (9, 11). The ligand for BTLA is the TNF receptor HVEM, which is unique in that it is the first example of an Ig superfamily receptor interacting with a TNF family member (4, 5). In T cells, activation of BTLA leads to tyrosine phosphorylation of ITIM domains located within its cytoplasmic tail, which leads to recruitment of the phosphatase SHP1 and inhibition of undefined positive signaling molecules (6, 8, 11). Although BTLA recruits SHP1, the precise mechanisms involved in BTLA-mediated lymphocyte engagement of p65 DNA-binding activity (Fig. 6).

NF-κB nuclear localization was consistent over an extended BCR stimulation time course, suggesting that B cell activation is significantly muted by BTLA inhibitory activity (Fig. 6B). Furthermore, DNA-binding activity of NF-κB was reduced upon HVEM treatment as shown by ELISA analysis of p65 DNA-binding activity (Fig. 6B, inset). This result further supports the role of BTLA as a suppressor of BCR-mediated signal transduction leading to B cell activation.

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attenuation are unclear (6, 8). Furthermore, little work has been done to elucidate the role of BTLA in other lymphocytes, including B cells. In this study, we describe mechanisms involved in BTLA-mediated attenuation of human B cells.

Using B cells isolated from human primary tissues, we show that BTLA displays the highest cell surface expression on peripheral blood B cells compared with tonsillar or bone marrow-derived subsets. Expression of BTLA only on mature, compared with precursor, bone marrow B cells suggests it does not have a profound role in human B cell development. This agrees with mice lacking the btlA gene, which develop normally and have intact lymphocyte numbers, but present a hyperproliferative phenotype upon lymphocyte activation, supporting its role as a co-inhibitory receptor (9, 11).

The inhibitory activity of BTLA is mediated by its interaction with the TNF receptor HVEM. First described as a host cell receptor for HSV by its interaction with HSV-1 glycoprotein D and later as a costimulatory receptor due to its interaction with the TNF ligands, lymphotixin α and LIGHT, HVEM has most recently been characterized as a co-inhibitory molecule due to its binding to BTLA and hyperproliferative phenotype seen in HVEM-deficient mice (4, 5, 38–40). In this study, we confirm its inhibitory role in regulating B cell activation by its ability to illicit BTLA-mediated attenuation of human B cell proliferation (Fig. 2, A and B). Recently, CD160 was identified as another co-inhibitory receptor that binds HVEM resulting in the inhibition of CD4+ T cell activation. However, it is unlikely that this interaction plays a role in B cell physiology, as CD160 is not expressed on B cells. Furthermore, the only HVEM binding partner that is present on mature, naive B cells appears to be BTLA (17–21).

In T cells it is unclear whether both SHP1 and SHP2 are recruited following activation of BTLA (5, 6). Pervanadate treatment of murine T cells leads to recruitment of both SHP1 and SHP2 to BTLA (5, 11), while in human T cells stimulated with artificial APCs only SHP1 is recruited (6). In human B cells, we have found that only SHP1 is recruited following direct activation of BTLA with HVEM (Fig. 2C) or when B cells are treated with pervanadate (data not shown). Not only do these results, and those published previously (6), caution against using pervanadate as an artificial stimulus, but they also point toward a potential difference in BTLA recruitment of effector proteins in mice vs humans. If so, this suggests subtle differences in BTLA function between mice and humans that may manifest in differences in BTLA-mediated lymphocyte regulation between species and warrants careful dissection of BTLA function in human, as well as murine lymphocytes. Although SHP1 and SHP2 are both involved in regulating cell signaling, their physiological roles can lie on divergent signaling pathways (41–43). Whereas SHP1 counteracts positive cell signaling pathways, SHP2 has been shown, in some cells, to activate NF-κB signaling leading to cellular activation (42), further suggesting a difference in BTLA-mediated signaling between species and cell types.

To identify target(s) of BTLA-mediated inhibitory signaling, we examined the effect of BTLA in regulating the activity of members of the BCR signaling pathway. We found BTLA to be constitutively associated with the BCR complex members IgM and CD79a/b, but failed to associate with the BCR costimulatory receptor CD19. Whether this is due to direct or indirect interactions of BTLA with the BCR is unknown and requires further investigation. Although BCR association was observed, BTLA failed to alter the activation of CD79a/b or CD19, suggesting the inhibitory activity of BTLA lies downstream of the immediate BCR signaling complex, which was further supported by the failure of BTLA to transport into lipid rafts along with the BCR upon B cell activation.

Insight into the inhibitory mechanism of BTLA was realized when we observed a decrease in BCR-induced Syk tyrosine phosphorylation upon HVEM treatment in primary B cells. Using AQUA MS analysis, we observed the tyrosine residues (Tyr348/52) critical for Syk activity, and BCR signaling (28), are downstream targets of BTLA. These results indicate that the inhibitory activity of BTLA is directed toward signaling molecules proximal to the BCR complex and suggests a functional similarity to inhibitory BCR coreceptors such as CD22, CD45, CD72, and PIR-B (44–46).

Once activated, Syk phosphorylates the adapter protein BLNK, allowing it to scaffold PLCγ2 and transduce Syk-mediated BCR signaling to downstream effector molecules (30, 31, 47). Since it is a central molecule involved in transmitting BCR signaling, disruption of BLNK activity leads to inhibition of B cell activation (47, 48). Our data show that BTLA signaling leads to decreased BLNK phosphorylation and, to a lesser extent, PLCγ2 phosphorylation in human primary B cells. It is likely that the phosphatase activity of SHP1 recruited by BTLA leads to decreased phosphorylation of both Syk and BLNK, which have previously been shown to be SHP1 targets (44, 49). To further support that BTLA attenuates BCR signaling through Syk and BLNK, we observed that BTLA signaling via HVEM leads to reduction of NF-κB activity, consistent with the observation that NF-κB activation is dependent on Syk and BLNK-mediated BCR signaling (50). SHP1 function in cell signaling has been shown to suppress NF-κB activity, whereas SHP2 promotes its activity, leading to cell activation (42). Therefore, it follows that BTLA stimulation by HVEM and subsequent SHP1 recruitment leads to reduced NF-κB activity in B cells.

Taken together, our results begin to describe the molecular mechanisms involved in attenuation of B cell activation by BTLA. Other co-inhibitory receptors, including CD22, CD45, FcγRIIB, ILT, and PD-1, have been shown to influence the strength and duration of BCR signaling by associating with the BCR complex and attenuating downstream signaling cascades (44–46). With its ability to attenuate B cell proliferation, BTLA has functional similarities to the killer cell inhibitory receptor class of inhibitory receptors like CD22 and PIR-B (44, 45, 51). Defects in CD22 lead to increased B cells numbers, hyperproliferative phenotype, and production of autoantibodies, which parallels the phenotypes observed in BTLA-deficient mice (52–54). Although BTLA and CD22 have some similarities, there are distinct functions of these two receptors that set them apart. For instance, CD22 has been shown to regulate calcium flux by activating the calcium efflux pump PMCA4, which, in turn, down-regulates calcium mobilization in B cells (55). Unlike CD22, BTLA does not appear to have an overall impact on calcium flux in B cells (supplemental Fig. 4). This is likely due to incomplete inhibition of PLCγ2 phosphorylation via BTLA signaling or through Syk-independent BTK-mediated activation of PLCγ2 that would allow for proper calcium mobilization in B cells (56–59). We have also observed that the inhibitory activity of BTLA is not regulated by global calcium flux, indicating that its role is outside of this signaling axis in B cells (supplemental Fig. 5). From this, we maintain that BTLA function is likely to be directed at modulating the activation of Syk and BLNK, suggesting that BTLA has a parallel, yet unique role in regulating B cell activation. Furthermore, based on our observations that BTLA constitutively associates with the BCR, recruits the tyrosine phosphatase SHP1, and regulates B cell activation by targeting the downstream signaling molecules.
Syk, BLNK, PLCγ2, and NF-κB, it is likely that BTLA plays a pivotal role in mediating the activation threshold of B cells, and avoidance of autoimmunity, compared with what has been observed in T cells (9, 11).

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Disclosures

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References


