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Immune opsonins modulate BLyS/BAFF release in a receptor-specific fashion*

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Abstract

TNF ligand superfamily member 13B (B-lymphocyte stimulator (BLyS), B cell activating factor (BAFF)) promotes primary B cell proliferation and immunoglobulin production. While the soluble form of BLyS/BAFF is thought to be the primary biologically active form, little is known about the regulation of its cleavage and processing. We provide evidence that Fcγ receptor cross-linking triggers a rapid release of soluble, biologically active BLyS/BAFF from myeloid cells. Surprisingly, this function is primarily mediated by FcγRI, but not FcγRIIa as defined by specific mAb, and can be initiated by both IgG and C reactive protein (CRP) as ligands. The generation of a B cell proliferation and survival factor by both innate and adaptive immune opsonins through engagement of an Fcγ receptor, which can also enhance antigen uptake and presentation, provides a unique opportunity to facilitate antibody production. These results provide a mechanism by which Fcγ receptors can elevate circulating BLyS levels and promote autoantibody production in immune complex mediated autoimmune diseases.

Key Words

Fc Receptors; Monocytes/Macrophages; Human; Autoimmunity

Introduction

B lymphocyte stimulator (BLyS), also known as BAFF (B cell-activating factor of the TNF family)/TNFSF13B, TALL-1/zTNF4/THANK, is an important member of the tumor necrosis factor (TNF) ligand superfamily (reviewed in (1–4)). BLyS/BAFF regulates B cell proliferation and differentiation and regulates immunoglobulin production (5). In facilitating B cell maturation, BLyS/BAFF preferentially supports T2 cell survival compared to T1-T2 differentiation (6, 7).

Studies in both model systems and humans have suggested that BLyS/BAFF is involved in humoral immune responses and in the pathogenesis of autoantibody mediated autoimmune diseases. BLyS^{−/−} mice show deficiencies in peripheral B cell development and maturation (8–10). Additionally, overproduction of BLyS/BAFF in transgenic mice leads to increased

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germinal center formation, high levels of autoantibody production (rheumatoid factor, anti-DNA) and immunoglobulin deposition in the kidney (11). Similar to observations of increased BLyS in autoimmune mice, elevations in circulating BLyS/BAFF are found in the serum of patients with a variety of autoimmune diseases including SLE, RA, Sjögrens Syndrome and WG (12–14).

BLyS/BAFF is expressed in cells of both hematopoietic and non-hematopoietic origins. Hematopoietic cells expressing BLyS/BAFF include monocytes/macrophages, some lymphocytes and dendritic cells. In these cells, BLyS/BAFF is initially expressed as a cell bound transmembrane protein that is cleaved upon cell activation and is typically found in soluble form as a BLyS/BAFF trimer. In the presence of soluble APRIL (a proliferation-inducing ligand), a trimer of BLyS/APRIL has also been demonstrated (15). Neutrophils express BLyS/BAFF, but unlike other myeloid and lymphoid cells, BLyS/BAFF is cleaved intracellularly into its truncated, soluble form (16). Non-hematopoietic cells such as astrocytes and fibroblast-like synoviocytes have been shown to express BLyS/BAFF (17–19). While the regulation of expression of BLyS/BAFF is not completely understood, both IFN γ and G-CSF upregulate total BLyS/BAFF protein in monocytes and neutrophils (20), and treatment of monocytes with LPS also induces an increase in soluble BLyS/BAFF levels in culture (15, 20).

Although the membrane associated form of BLyS/BAFF has potential for biological effects, the soluble form of BLyS/BAFF appears to be the primary biologically active form (21). We demonstrate rapid receptor-specific induced release of soluble biologically active BLyS/BAFF from myeloid cells. Surprisingly, this release is triggered primarily by crosslinking Fc γ RI with C reactive protein (CRP) or IgG, opsonins of the innate and adaptive immune systems. Fc γ receptor ligation, especially that of Fc γ RI, is known to enhance antigen uptake and presentation (22, 23). Thus the concomitant elaboration of a factor promoting B cell survival and Ig production in response to either IgG or CRP presumably provides a microenvironment that facilitates the humoral immune response. Furthermore, these observations suggest a potentially important link between innate and adaptive immune responses and between elevations in circulating BLyS/BAFF and immune complex mediated autoimmune disease.

Materials and Methods

Isolation of peripheral blood mononuclear cells (PBMCs) and B cells

Blood from healthy donors was collected into heparinized tubes (Becton Dickinson, Franklin Lakes, NJ). PBMCs were isolated by Ficoll-Hypaque gradient centrifugation as previously described (24). CD19⁺ cell were purified using Magnetic Micro Beads (Miltenyi Biotec, Auburn, CA) on positive-selection columns. All studies were approved by the Institutional Review Board for Human Use.

Cell cultures

Human U937, THP-1, and HL-60 cell lines were maintained as suspension culture in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μ g/ml). 293T cells were stably transfected with a cDNA-encoding BLyS and were maintained as adherent cultures in DMEM (Invitrogen Life Technologies, Carlsbad, CA) medium with 10% FBS and 0.5 mg/ml G418.

Antibodies and Reagents

Hybridoma clones producing monoclonal anti-BLyS antibodies were generated by immunizing BALB/c mice with 200 μ g recombinant human BLyS (extracellular domain, aa 71 to aa 285) before fusion of lymph node cells with NS1 myeloma cells. The primary hybridoma clones were screened by ELISA for binding to the recombinant BLyS. Positive clones were further screened with the transfected 293T cells expressing the membrane form of human BLyS. Cell surface expression of human BLyS was confirmed by flow cytometry using TACI-Fc fusion protein (10 μ g/ml) followed by incubation with PE-conjugated goat-anti-human IgG (Southern Biotech) (25). The monoclonal anti-BLyS antibody (mAb 7D4) was chosen for flow cytometry and immunoprecipitation.

A blotting polyclonal anti-BLyS antibody (pAb) was obtained from Alexis Biochemicals, San Diego. Monoclonal anti-CRP antibody HD2-4 (26) and anti-Fc γ RIIb mAb 4F5 (27) were used as described. Anti-Fc γ RI mAb 32.2 and anti-Fc γ RII mAb IV.3 hybridoma cell lines were purchased from American Type Culture Collection (Manassas, VA). Anti-CD14 and anti-CD19 antibodies were purchased from Caltag Laboratories, CA. MAb Fab/F(ab')₂ fragments were prepared by Rockland Immunochemicals and were free of detectable Fc region as assessed by blotting. F(ab')₂ goat anti-mouse IgG (G α M) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit IgG, human IgG isotype controls, IFN γ , and human CRP were from Sigma. Aggregated human IgG was prepared by heating 20mg/ml human IgG for 30 minutes at 55°C followed by removal of insoluble aggregates by spinning at 2000xg for 10 minutes. In addition, highly purified CRP (sodium azide free, low endotoxin) was prepared from donor sera using a standard affinity chromatography protocol (28). Recombinant BAFF-R was purchased from BioVision, Inc (Mountain View, CA), and was labeled by using Alexa Fluor 488 Microscale Protein Labeling Kit from Molecular Probes.

Detection and quantification of BLyS

Soluble BLyS levels were assayed in supernatants of U937 or THP-1 cells by immunoprecipitation and Western blot. Cell associated BLyS was assessed by blotting whole cell lysates (WCL) or by immunoprecipitation. Cells were lysed at 5x10⁷ cells/ml in PBS/1% NP-40 lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics, IN). Lysates or culture supernatants were then preincubated with protein G-Agarose beads at 4°C for 1h followed by incubation with 7D4-conjugated protein G-agarose beads (Roche Diagnostics, IN) at 4°C overnight. After 3 washes with PBS containing 0.1% Tween 20, the beads were boiled in SDS loading buffer. The samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% nonfat dry milk, blots were probed with anti-BLyS pAb followed by HRP-conjugated mouse anti-rabbit IgG and detected with ECLTM Western Blotting Detection Reagents (GE Healthcare).

Analysis of Intracellular Ca²⁺ Concentrations

Calcium influx was measured as previous described (29). Briefly, U937 cells were incubated at 37 °C for 40 min with 5 μ M Indo-1/AM (Molecular Probes, Eugene, OR). After loading, the cells were washed once with modified HBSS (1.1mM CaCl₂, 1.6mM MgCl₂ and 1mg/mL BSA, pH7.3) and incubated with different anti-Fc γ R mAbs for 30 min at 4°C followed by one cold wash. The cells were then resuspended to 5 × 10⁶/ml and analyzed on an SLM 8000C Spectrofluorometer (SLM-Aminco, Urbana, IL). Cells were stimulated with goat anti-mouse F(ab')₂ at 60 seconds.

Specific FcγR Cross-linking

U937 cells or human PBMC were first incubated with anti-FcγRI IgG or F(ab')₂ (mAb 32.2), with anti-FcγRIIa IgG or Fab (mAb IV.3), and/or with anti-FcγRIIb IgG or F(ab')₂ (mAb 4F5) at saturating concentrations (10 μg/ml) for 30 min at 4°C, washed twice, and resuspended in modified HBSS followed by cross-linking with GaM F(ab')₂ (35 μg/ml) at 37°C for 15 min. Cells were then washed and the remaining GaM binding sites were blocked with mouse IgG1/IgG2b mixture (final concentration 10 μg/ml) before FACS analysis.

Flow cytometry

Cells were prepared for flow cytometric analysis by incubating cells with saturating concentrations of specific mAb or an isotype control for 30 min on ice, followed by incubation with goat anti-mouse F(ab')₂ conjugated with FITC for 30 min on ice. Alternatively, cells were stained with biotinylated 7D4 followed by FITC-conjugated streptavidin. In isolated PBMC, monocytes were identified with anti-CD14 and B cells were identified with anti-CD19. Cells were analyzed on a FACScan flow cytometer and data were analyzed using Cell Quest software (Becton Dickinson Immunocytometry, CA).

B cell viability assay

Survival of CD19⁺ B cells was assessed as previously described (20). Briefly, B cells at a density of 1x10⁶/ml were seeded in the upper chamber of transwell (Corning Coster, NY) in RPMI-1640 medium supplemented with 10% fetal bovine serum. Medium, or 5x10⁶/ml CD19-depleted mononuclear cells (MNC), or 1x10⁶/ml U937 cells were placed in the lower chambers with or without IgG pre-coating. After seeding, the cells were cultured for 3 days. Surviving B cells were quantified by light microscope in the presence of trypan-blue.

Data analysis and statistics

Results are presented as mean ± SEM, and differences between means were analyzed using the Student's t test (GraphPad Prism). A probability of 0.05 was used to reject the null hypothesis that there is no difference between the experimental and control groups.

Results

CRP induces decrease of membrane BLyS/BAFF expression on U937 cells

To examine the regulation of cell surface BLyS expression, we prepared and characterized a monoclonal anti-BlyS antibody (clone: 7D4, IgG2b, κ) that was selected based on its ability to recognize both the soluble and the membrane forms of BLyS. Activity of mAb 7D4 was determined using 293T cells transfected with full length human BLyS (Fig. 1A, upper panel). TACI-Fc fusion protein was used as a positive control for detection of membrane BLyS expression (Fig. 1A, lower panel). No binding of mAb 7D4 to the empty vector transfected control 293T cells was observed. Detection of soluble BLyS was performed using a previously established BLyS ELISA with mAb 7D4 as the capture antibody (12). Soluble BLyS was detected in the supernatant of cells transfected with BLyS, but no soluble BLyS was detected in culture supernatants of cells transfected with empty vector (Fig. 1B). Immunoprecipitation of supernatant derived BLyS with mAb 7D4 followed by Western blot confirmed that the released BLyS from the transfected cells was the predicted 17kd cleaved form of BLyS, and BLyS in transfected 293T cell lysates was the full length 34kd form (data not shown). These results indicate that mAb 7D4 is able to bind both cell surface and released soluble BLyS.

Circulating monocytes and monocytic cell lines constitutively express BLyS/BAFF on the cell surface and as previously reported we found that stimulation of these cells with IFN γ induced a marked increase in the level of surface BLyS expression (Fig. 2). Because CRP, an acute phase reactant and opsonin of the innate immune system, may be an important modulator of acquired immune responses, we determined whether CRP could alter cell surface BLyS/BAFF expression. Indeed, membrane BLyS/BAFF expression on both constitutive and IFN γ stimulated human monocytic cell line U937 is decreased by cross-linking of CRP with F(ab')₂ fragments of a monoclonal anti-CRP antibody HD2-4. In cells with constitutive BLyS expression, cross-linked CRP induced a 16.2 $\pm$ 5.8% decrease ($p=0.026$, $n=8$) (Fig. 2A) and in IFN γ stimulated cells cross-linked CRP induced a 23.1 $\pm$ 5.6% decrease in surface BLyS expression ($p=0.014$, $n=5$) (Fig. 2B).

Fc γ R cross-linking causes reduced membrane BLyS/BAFF expression

CRP can exert biological effects on myeloid cells through engagement of Fc γ receptors as cognate receptors (30). To directly determine if the decrease in BLyS/BAFF surface expression can be modulated by Fc γ receptors, we incubated U937 cells with plate bound IgG. In contrast to the induction of surface BLyS expression by IFN γ , overnight culture of U937 on IgG coated wells resulted in a marked decrease in cell surface BLyS/BAFF expression (52.2 $\pm$ 3.0%, $p=0.003$, $n=3$) (Fig. 2C). In addition to surface bound IgG, soluble aggregated human IgG also resulted in a marked decrease in cell surface BLyS/BAFF expression (Fig. 2D) demonstrating that adherence to a surface is not necessary for the IgG-induced decrease in surface BLyS expression. To confirm that the Fc portion of the anti-BLyS antibody 7D4 was not contributing to the apparent IgG-induced decrease in cell surface BLyS, we utilized a soluble recombinant BLyS receptor (TNFSF13C or BAFF-R) corresponding to the extracellular domain for detection of surface BLyS. Indeed, using the soluble BAFF-R we observed the same IgG induced decrease in surface BLyS expression after IgG stimulation (54.7% $\pm$ 5.8%, $p=0.016$, $n=4$) (Fig. 2E). Comparable results with surface bound IgG were observed in two other human monocytic cell lines, THP-1 and HL-60 (data not shown). Overnight culture of U937 cells on mouse IgG F(ab')₂ coated wells did not change BLyS/BAFF expression (data not shown).

To examine whether the effect of Fc γ R cross-linking on monocytic cell lines could be extended to primary human cells, peripheral blood mononuclear cells were isolated and stimulated with aggregated human IgG, and surface BLyS/BAFF expression on CD14⁺ monocytes was measured by flow cytometry. In concert with the cell line data, monocyte cell surface BLyS was consistently decreased by Fc γ R engagement (Fig. 2F). These results indicate that Fc γ R stimulation of both monocytes and monocytic cell lines induces a marked decrease in cell surface BLyS/BAFF.

Kinetic Analysis of membrane BLyS/BAFF expression

The kinetics of loss of cell surface BLyS were determined (Fig. 3). In contrast to the transcriptional regulation of BLyS expression by IFN γ which peaks at 24 hours (Fig. 3A) (15, 31), Fc γ R cross-linking resulted in a rapid loss of cell surface BLyS/BAFF from the surface of U937 cells. Indeed, stimulation with surface IgG resulted in the rapid down regulation of membrane BLyS/BAFF expression within 5 min, reaching a maximum by 15 min (Fig 3B). The decreased levels of cell surface BLyS/BAFF did not rebound during continued exposure to surface IgG over a period of 48 hrs (Fig. 3A). We did not detect any change in BLyS mRNA levels by quantitative PCR during the 8–24hr incubation with IgG (results not shown). These results, together with the kinetic pattern of BLyS/BAFF loss from the cell surface, rule out the possibility that the Fc γ R induced change on BLyS/BAFF expression is transcriptional.

Increased soluble BLyS/BAFF is released into supernatants upon FcγR aggregation

To determine the mechanism of loss of cell surface BLyS/BAFF, we considered the possibility that membrane BLyS/BAFF was released from the cell surface upon cleavage. Accordingly, we immunoprecipitated soluble BLyS from cell free supernatants collected after FcγR ligation. Both THP-1 cells (Fig. 4A) and U937 cells (Fig. 4B) constitutively express the 32kD membrane BLyS as seen by immunoprecipitation from cell lysates. In contrast, only the 17kD soluble form could be immunoprecipitated from the cell-free culture supernatants after overnight IgG stimulation, demonstrating the appearance of a cleaved form of soluble BLyS/BAFF. The kinetics of the appearance of detectable cleaved soluble BLyS/BAFF demonstrated increasing protein at 8 hrs and 20 hrs after stimulation (Fig. 4B). A low level of cleaved BLyS/BAFF protein was observed in the non-stimulated cells after overnight culture consistent with a low level of constitutive BLyS/BAFF shedding. While we cannot formally exclude the possibility that some cell surface BLyS/BAFF is also internalized after FcγR cross-linking, these results clearly show the FcγR activation results in the generation of cleaved soluble BLyS/BAFF.

FcγRI (CD64) is the major inducer of BLyS/BAFF release

Among different families of FcγRs, two activating receptors FcγRI, FcγRIIa and the inhibitory FcγRIIb, are expressed on the surface of U937 cells, THP-1 cells and circulating monocytes. To determine which of those FcγRs is responsible for BLyS/BAFF release, receptor-specific activation of cells with specific anti-receptor mAbs was performed. To our surprise, using U937 cells, cross-linking of FcγRI but not FcγRIIa induced loss of BLyS/BAFF expression. FcγRI (CD64) cross-linking with mAb 32.2 IgG or F(ab')₂ resulted in a rapid and significant loss of surface BLyS/BAFF expression while cross-linking of FcγRIIa (CD32A) with mAb IV.3 IgG or Fab induced little to no change in surface BLyS/BAFF expression (Fig. 5A). Heterotypic cross-linking of both FcγRI and FcγRIIa with anti-receptor mAbs resulted in similar loss of surface BLyS/BAFF expression relative to cross-linking of FcγRI alone. As a positive control for mAb IV.3 IgG and Fab activity, we tested the ability of cross-linked IV.3 and of 32.2 to elicit a rise in intracellular calcium levels. Homotypic ligation of either FcγRI or FcγRIIa was capable of inducing a calcium influx with FcγRIIa generating a stronger response than FcγRI (Fig. 5C). Heterotypic cross-linking of both FcγRI and FcγRIIa enhanced the calcium influx to levels higher than FcγRI alone (Fig. 5D). Together, these results document a unique role for FcγRI over FcγRIIa in the induction of BLyS/BAFF release despite of the fact that both receptors utilize an ITAM motif and that FcγRIIa resulted in a stronger Ca²⁺ influx than FcγRI.

U937 cells and THP-1 cells also express the inhibitory Fc receptor, FcγRIIb. Using the recently described anti-FcγRIIb mAb 4F5 IgG or F(ab')₂ (27), we cross-linked FcγRIIb alone or in combination with the anti-FcγRI mAb 32.2 F(ab')₂. Homotypic cross-linking of FcγRIIb did not result in any change in cell surface BLyS expression (Fig. 5A). Surprisingly, though heterotypic cross-linking of FcγRI and FcγRIIb resulted in diminished rise of intracellular Ca²⁺ (Fig. 5D), there was no change in the magnitude of BLyS loss from the cell surface. These results suggest that BLyS cleavage is resistant to FcγRIIb mediated inhibition.

To confirm the unique role of FcγRI in inducing BLyS/BAFF shedding on primary myeloid cells, we performed the anti-receptor mAb cross-linking studies using peripheral blood monocytes. Rapid loss of BLyS/BAFF expression was observed after stimulation of the cells through FcγRI. There was little reduction in BLyS/BAFF expression after stimulation of cell through FcγRIIa (Fig. 5B). Consistent with the data in the U937 cell line, heterotypic ligation of FcγRI with FcγRIIa or FcγRIIb showed little alteration of the level of FcγRI induced BLyS/BAFF reduction (results not shown).

Biological activities of Fc γ R cross-linking derived BLyS

The functional significance of Fc γ receptor BLyS/BAFF shedding was determined by testing B cell viability as previously described (20) in co-cultures with either U937 cells or primary MNC. In these experiments, the U937 cells or MNC were cultured in wells in the presence or absence of pre-coated IgG and the isolated B cells were co-cultured in the same well separated by a 0.4 μ m transwell membrane. As previously shown (18), B cells cultured alone had poor survival with nearly 60% of cells non-viable after 3 days in culture while addition of soluble rBLyS maintained 100% cell viability (Fig. 6). In the absence of Fc γ R ligation, U937 cells and primary MNC increased B cell survival (Fig. 6A&B), presumably due in part to constitutive BLyS shedding (see Fig. 4). However, with IgG stimulation, both U937 cells and MNC significantly enhanced B cell viability above levels observed in the absence of IgG stimulation ($p < 0.002$). Not only was viability maintained, the higher levels of BLyS/BAFF shedding from U937 cells resulted in a slight increase in B cell numbers suggesting proliferation. Enhanced B cell survival after U937 cell/MNC Fc γ R stimulation was reversed with the inclusion of the neutralizing anti-BLyS antibody 7D4 (Fc γ R stimulation vs Fc γ R stimulation plus 7D4 neutralization: $p < 0.018$) (Fig. 6A/B). These results confirm that biologically active soluble BLyS/BAFF is released from monocytes by Fc γ R cross-linking.

Discussion

Our study reports for the first time that the shedding of soluble BLyS/BAFF from myeloid cells is regulated in an Fc γ receptor specific manner by opsonins of both innate and acquired immune origins. Both CRP and IgG triggered rapid and significant shedding of soluble BLyS/BAFF from the surface of myeloid cells. A unique role for Fc γ RI (CD64) over Fc γ RII is shown by direct anti-receptor mAb engagement. The shed BLyS/BAFF is biologically active and directly promotes B cell survival.

Of interest, our results show that Fc γ RI (CD64) is much more effective in IgG induced shedding process compared to Fc γ RIIa (CD32A). Both Fc γ RI and Fc γ RIIa are ITAM-bearing activating Fc γ Rs, although the ITAM in the cytoplasmic domain of Fc γ RIIa differs in primary sequence from the ITAM-containing common FcR γ -chains that associates with Fc γ RI. There are now several lines of evidence suggesting that the ligand binding α -chain of Fc γ RI can modulate FcR γ -chain associated receptor functions such as phagocytosis, regulation of Th2 cytokines production (IL-6 and IL-10), and antigen presentation (32–34). Receptor-induced shedding of BLyS/BAFF is not a property of all FcR γ -chain associated receptors because we observed no change in BLyS/BAFF expression after crosslinking of another γ -chain associated receptor CD89 (results not shown).

It is known that ITAM-dependent activation can be inhibited by concomitant ligation of ITIM bearing receptors such as Fc γ RIIb (35, 36). However, when we cross-linked Fc γ RI with Fc γ RIIb, the level of membrane BLyS/BAFF expression was indistinguishable from ligation of Fc γ RI alone despite significant reduction in the Fc γ RI induced Ca²⁺ flux. This result suggests that Fc γ R mediated BLyS/BAFF shedding may be an ITAM-independent process (as shown in Fig. 4A). While we cannot exclude the possibility that myeloid cells do not express sufficient Fc γ RIIb to inhibit the cleavage signaling pathway, these levels of Fc γ RIIb were sufficient to mediate over 70% inhibition of the Fc γ RI triggered Ca²⁺ influx. Thus, either BLyS shedding is independent of ITAM signaling or it requires minimal levels of ITAM activation. Indeed, the tyrosine kinase inhibitor genistein achieved less than 50% reduction of the IgG induced membrane BLyS/BAFF release (results not shown), suggesting that γ -chain tyrosine phosphorylation is not the only event contributing to BLyS/BAFF shedding.

BLyS/BAFF is critical for maintaining homeostasis of primary B cells. Deficiency of BLyS in the mouse causes a block in B cell development, and blocking of the BLyS/APRIL receptor TACI can reverse the autoantibody mediated autoimmune phenotype in the NZB/WF1 mouse (9, 37). Conversely, recent reports suggested that excess amount of BLyS can rescue self-reactive B cells from peripheral deletion and allow them to enter forbidden follicular and marginal zone niches (38) and over expression of BLyS/BAFF in the mouse causes autoimmune symptoms characteristic of SLE (39). It is likely that a furin-like protease plays a role in the cleavage of both cell surface BLyS/BAFF from myeloid cells and of intracellular BLyS/BAFF in neutrophils (16), but the exact enzyme or other factors that regulate proteolytic activity towards BLyS/BAFF remain unclear. Indeed, we observed that the furin inhibitor CMK and the metalloprotease inhibitor 1,10-phenanthroline demonstrated only marginal inhibition of Fc γ R induced BLyS/BAFF shedding (results not shown). Interestingly, a similar protease-resistant Fc γ RI induced cleavage of CR1 (CD35) has been observed (40–42).

CRP circulates at a very low level in most healthy individuals, but in response to inflammation or infection, CRP levels rise rapidly and to a high level (43–45). CRP is traditionally regarded as an innate immune opsonin that acts in part through activation of the classical complement cascade and also interacts with myeloid receptors including Fc γ R. Previous work indicates that CRP can induce secretion of IL-6 and matrix metalloproteinase 1 (MMP-1) (46), triggers shedding of L-selectin and upregulation of the β 2 integrin CD11b expression in myeloid cells (47), and triggers IL-6 receptor shedding from granulocytes (42). It is now recognized that many of these events are due to CRP engagement of Fc receptors on myeloid cells. Our observation raises the possibility that CRP could link innate responses to acquired immune responses by engaging Fc γ Rs and modulating BLyS/BAFF cleavage resulting in promotion of B cell maturation.

Together, our studies demonstrate that the level of soluble functional BLyS/BAFF can be up-regulated by myeloid Fc γ Rs, with a primary role for Fc γ RI. A central role of Fc γ RI allows for the regulation of soluble-cleaved BLyS/BAFF by both IgG and by the innate acute phase reactive CRP. Furthermore, a unique role for Fc γ RI in regulation of antibody production is suggested through enhancement of antigen presentation (23) and through cleavage of membrane BLyS making soluble BLyS available for B cell activation. This role for myeloid Fc γ RI contrasts with the ability of B cell Fc γ RIIb to inhibit BCR signaling and antibody production. The production of an antibody/autoantibody promoting B cell survival factor by Fc γ receptors provides a possible mechanism for known elevations in BLyS/BAFF in patients with autoantibody-associated autoimmune disease and supports current therapeutic BLyS/BAFF blockade in these diseases.

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Figure 1. Characterization of the anti-BLyS antibody 7D4

(A) Flow cytometry analysis of 293T cells transfected with human BLyS or empty vector. Cells were stained with 10 μ g/ml TACI-Fc fusion protein followed by PE-conjugated anti-human IgG (lower panel) or 5 μ g/ml 7D4 followed by PE-conjugated anti-mouse IgG2b (upper panel). (B) Expression levels of BLyS in culture supernatants of 293T cells (5×10^5 /ml) cultured in fresh medium for 24 hours. Soluble BLyS in culture supernatants was measured by sandwich ELISA using mAb 7D4 as capture antibody and rabbit anti-human BLyS polyclonal antibody as the detection antibody.

Figure 2. CRP and IgG induced reduction in membrane BLyS (mBLyS) expression

U937 cells (A), or U937 cells pretreated with IFN γ overnight (B), were incubated with CRP (10 μ g/ml) and then stimulated with anti-CRP F(ab')₂ mAb HD2-4 (10 μ g/ml) followed by crosslinking with GaM F(ab')₂ (35 μ g/ml), and surface BLyS expression was measured with mAb 7D4. (C&D) U937 cells were incubated overnight alone or in the presence of rabbit IgG (rIgG) (200mg/ml pre-coated on the plate surface), IFN γ (200 U/ml), or aggregated human IgG (agg IgG) (200ug/ml) as indicated, and cells were stained for surface mBLyS expression. Data presented are representative of three or more independent experiments. (E) U937 cells were incubated 30 mins alone or in the presence of agg IgG, followed by determination of surface mBLyS expression by Alexa 488 conjugated BAFF-R recombinant protein. Alexa488 conjugated BSA was used as background staining control. (F) Human PBMC were incubated with or without aggregated human IgG for 30 min, followed by determination of surface mBLyS expression on CD14⁺ cells by flow cytometry. Stimulated versus control, $p=0.012$, $n=6$.

Figure 3. Kinetic analysis of mBLyS expression

U937 cells were incubated in the presence or absence of plate-bound rIgG (200mg/ml pre-coated on the plate surface) or IFN γ (200U/ml) for varying times, followed by determination of surface mBLyS expression by flow cytometry. Data are presented relative to control mBLyS expression versus stimulated cells. 3 to 5 independent time course studies were performed.

Figure 4. Soluble BLYS released into supernatants upon Fc γ R cross-linking

Culture supernatants of THP-1 cells (**A**) or U937 cells (**B**) at varying time points were analyzed by immunoprecipitation of soluble BLYS. Cells were incubated with or without plate-bound rIgG for 24hrs (**A**) or for 3 different time points (**B**). BLYS was immunoprecipitated from culture supernatants (**A&B**) and whole cell lysates (**A**) with anti-BLYS mAb 7D4 bound to protein G-agarose. The membrane 32kD and cleaved soluble 17kD forms of BLYS are indicated. Recombinant BLYS (extra cellular domain) is also shown. As a loading control, the membrane in (**A**) was stripped and reprobed with an anti-actin antibody. Data are one representative experiment of three independent experiments.

Figure 5. Fc γ RI is the major inducer of BLyS release from U937 cells

(A) U937 cells ($5 \times 10^6/\text{ml}$) were stimulated by receptor specific cross-linking of Fc γ Rs. Fc γ Rs were cross-linked using anti-Fc γ RI mAb 32.2 ($10 \mu\text{g}/\text{ml}$), anti-Fc γ RIIa mAb IV3 ($10 \mu\text{g}/\text{ml}$) and/or anti-Fc γ RIIb mAb 4F5 ($10 \mu\text{g}/\text{ml}$) followed by GaM F(ab')₂. (B) PBMC were stimulated with anti-Fc γ RI mAb 32.2 F(ab')₂ or anti-Fc γ RIIa mAb IV3 Fab followed by GaM F(ab')₂. CD14 positive cells were analyzed for mBLyS expression by flow cytometry. (C) Homotypic crosslinking of 32.2 IgG or IV3 IgG induced a transient rise in intracellular Ca²⁺ in U937 cells. (D) Calcium influx induced by heterotypic ligation of 32.2 F(ab')₂ (CD64) and either IV3 Fab (CD32A) or 4F5 Fab (CD32B) compared with 32.2 F(ab')₂ alone in U937 cells. Data presented are representative of 3 independent experiments.

Figure 6. Released soluble BLyS by FcγR stimulation supports B cell survival in vitro

Freshly isolated CD19⁺ B cells from healthy adults were cultured in the upper chamber of a transwell chamber. The bottom chamber contained either medium alone, U937 cells ($\pm$ rIgG) (**A**) or primary CD19 depleted MNC ($\pm$ rIgG) (**B**) for 72 hrs. Trypan blue negative B cells were determined. As a positive control, recombinant soluble BLyS (200ng/ml) was used (**A**). BLyS activity was neutralized with a blocking antibody (mAb 7D4, 50 μ g/ml) (**A&B**). (**A**) $n=3-10$ independent determinations; (**B**) $n=4-7$ independent determinations. * $p<0.018$, ** $p<0.002$.