Proinflammatory mediators elicit the secretion of the intracellular B-lymphocyte stimulator (BLyS) pool that is stored in activated neutrophils: implications for inflammatory diseases.

Short Title: Inducible BLyS secretion by activated neutrophils

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ABSTRACT

We have recently shown that G-CSF- and IFNγ-activated human neutrophils accumulate and release remarkable amounts of soluble B-lymphocyte stimulator (BLyS) in vitro. In this study, we provide evidence that neutrophils migrating into skin window exudates (SWE), developed in both healthy volunteers and rheumatoid arthritis (RA) patients, synthesize and release BLyS in response to locally produced G-CSF. Accordingly, the concentrations of soluble BLyS in SWE were significantly more elevated than in serum. Because the levels of SWE BLyS, but not SWE G-CSF, were higher in RA patients than in healthy subjects, we examined the effect of CXCL8/IL-8, C5a and other proinflammatory mediators that dramatically accumulate in RA SWE and/or in inflamed synovial fluids. We show that CXCL1/GROα, CXCL8/IL-8, C5a, immune complexes, TNFα, leukotriene B4, fMLP and LPS, which by themselves do not induce BLyS de novo synthesis, act as potent secretagogues for BLyS, which is mainly stored in Golgi-related compartments within G-CSF-treated neutrophils, as determined by immunogold electron microscopy. This action is pivotal in greatly amplifying neutrophil-dependent BLyS release in SWE of RA patients relative to healthy donors. Collectively, our data uncover a novel mechanism that might dramatically exacerbate the release of BLyS by neutrophils during pathological inflammatory responses.
INTRODUCTION

B-lymphocyte stimulator (BLyS) is a recently identified member of the Tumor Necrosis Factor (TNF) ligand superfamily that is important in B-cell differentiation, survival and regulation of immunoglobulin production.\textsuperscript{1,2} BLyS exists as a type II membrane protein as well as a soluble protein\textsuperscript{3,4} and its expression seems to be mainly, but not exclusively, restricted to cells of myeloid origin, including activated monocytes, macrophages, myeloid dendritic cells and neutrophils.\textsuperscript{5,6} BLyS is also known as BAFF (B-cell activator factor belonging to the TNF family), THANK (TNF homologue that activates apoptosis, NF-κB, and JNK), TALL-1 (TNF- and ApoL-related leukocyte-expressed ligand 1), and exerts its effect by binding to three receptors: transmembrane activator and CAML interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R).\textsuperscript{1,2} The requirement of BLyS for humoral immune response has been clearly evidenced in mice lacking BLyS, which exhibit profound deficiencies in peripheral B cell development and maturation as well as a strong impairment in either T cell-dependent and T cell-independent antibody responses.\textsuperscript{1,2} On the other hand, studies reported in the literature suggest a role of soluble BLyS in the pathogenesis of autoimmune diseases. For instance, transgenic mice overexpressing soluble BLyS develop a syndrome with similarities to systemic lupus erythematosus (SLE) in humans.\textsuperscript{1,2} In line with these observations, serum BLyS levels have been found elevated in human patients suffering not only of SLE and Sjogrens’ syndrome, but also of rheumatoid arthritis (RA).\textsuperscript{1,2} In addition, elevated BLyS levels have been detected in patients with non-Hodgkin’s lymphoma (NHL), while other studies have shown that BLyS promotes survival of both NHL-derived B lymphoma cells and B cell chronic lymphocytic leukemia (B-CLL) cells\textsuperscript{7,8} and also acts as a myeloma cell growth factor.\textsuperscript{9} BLyS may therefore promote survival and growth of certain B cell cancers; should this be the case, specific targeting of BLyS could provide a novel treatment for these diseases.

Recently, we have shown that G-CSF-treated neutrophils release soluble BLyS \textit{in vitro} and \textit{in vivo}\textsuperscript{6}, indicating that these cells might play an unsuspected role in the regulation of B-cell
dependent immune responses, and, indirectly, of B cell malignancies or autoimmune disorders such as RA itself. The latter pathology is a systemic disease characterized by chronic inflammation of synovium and destruction of articular cartilage and juxtaarticular bone. Neutrophils are frequently found in abundance in rheumatoid joint effusion, and are supposed to play a pivotal role in the production of articular damage/degradation, especially via release of enzymes contained in their granules.\textsuperscript{10} Interestingly, neutrophils isolated from synovial fluids have been also shown to produce dysregulated levels of IL-1\textbeta, IL-1ra, VEGF, CXCL1, CXCL8 and TGF\textbeta\textsubscript{1} as compared to peripheral blood neutrophils.\textsuperscript{11,12} In this regard, RA patients have levels of BLyS in synovial fluid that exceed their serum levels.\textsuperscript{13} Whether BLyS accumulation in such exudates may derive from inflammatory neutrophils, however, has yet to be investigated.

In this study, we examined BLyS expression in neutrophils from healthy volunteers and RA patients, either isolated from the peripheral blood or migrating into an acute inflammatory site \textit{in vivo}. For this latter purpose, we have developed the Senn’s skin window model\textsuperscript{14} that, by deliberately inducing a local inflammation on the forearm, permits to collect and analyse the mediators contained in the exudative fluid and to evaluate the effector functions of migrated neutrophils as well. We report that local levels of G-CSF play a crucial role in stimulating BLyS production by neutrophils accumulated at inflammatory sites \textit{in vivo} and that classical proinflammatory mediators such as immune complexes, CXCL8/IL-8, C5a, TNF\alpha, and leukotriene B\textsubscript{4} promote BLyS release by G-CSF- or IFN\gamma-activated neutrophils. Our data not only uncover an unsuspected mechanism whereby the release of BLyS may be greatly amplified during pathologic inflammatory responses, but further emphasize the potential role of neutrophil-derived BLyS in the onset of autoimmune disorders, such as RA and related diseases.

**METHODS**

**Patients.** Fifteen RA patients (3 male and 12 females, range between 31 and 65 years, mean age 47.2 ± 10.2 years) selected according to the ARA proposals\textsuperscript{15} were enrolled for this study after
their signed informed consent. All these patients were medication-free and in the active phase of RA disease (presence of 6 or more swelling joints and at least 2 of the following features: 9 or more tender joints, morning stiffness duration > 45 minutes, erythrocyte sedimentation rate > 28 mm/h). Fifteen age and sex matched healthy subjects were also enrolled as controls. Cells, sera and skin window exudates (SWE) from these individuals were collected as described below and stored at –80°.

**Cell purification and culture.** Highly purified neutrophils (> 96.5%) and Percoll-purified monocytes were isolated under endotoxin-free conditions from either buffy coats or whole blood of healthy donors and RA patients, as previously described.16 Immediately after purification, neutrophils were suspended in RPMI-1640 medium, supplemented with 10% low endotoxin FBS (< 0.5 EU/ml, Biowhittaker Europe, Verviers, Belgium), plated in 24-well tissue culture plates (Orange, Trasadingen, Switzerland) at 5 x 10⁶/ml, and subsequently cultured for the indicated times. Unless specifically indicated, 400 µl of neutrophil suspensions were incubated for 20 h with or without 1000 U/ml G-CSF (Lenograstim, Chugai Pharmaceutical Co., Ltd., Tokio, Japan) or 200 U/ml IFNγ (R&D Systems, Minneapolis, MN) prior to the treatment with the following stimuli for additional 4 h: 100 ng/ml LPS (from E. Coli, serotype 026:B6; Sigma, St. Louis, MO), 5 ng/ml TNFα, 500 ng/ml CXCL1/GROα (Peprotech, Rocky Hill, NJ), 500 ng/ml CXCL8/IL-8 (R&D Systems), 500 ng/ml C5a (Calbiochem, San Diego, CA), 100 nM fMLP, 100 nM leukotriene-B₄ (LTB₄) (Sigma) and 60 µg/ml immune complexes (IC), the latter prepared as described by Brunkhorst et al.17 In other experiments, G-CSF-preincubated neutrophils were treated with or without 6 mM pentoxifylline (PTF), 10 µM jasplakinolide (JK), 3.5 µM monensin, or 25 µM Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK, Alexis, San Diego, CA)⁶, before addition of the stimuli mentioned above. Monocytes (10⁶/well) were cultured in 24-well tissue culture plates for 68 h in the presence or not of 200 U/ml IFNγ followed by a further 4 h period with or without 5 ng/ml TNFα, 500 ng/ml C5a or 100 nM fMLP. Monocyte-derived dendritic cells were
prepared as described. Langerhans cells were obtained by culturing purified monocytes for 7 d in the presence of GM-CSF (100 ng/ml), IL-13 (10 ng/ml) and TGFβ (10 ng/ml). TNFα (10 ng/ml) was added on day 5. At the end of the culture, cells were E-cadherin\textsuperscript{+}, Langerhin\textsuperscript{+}, MHC\textsuperscript{II bright}, CD1\textsubscript{a}\textsuperscript{+}, CD1\textsubscript{4}.\textsuperscript{18} Anti-G-CSF polyclonal Abs (R&D Systems), anti-IFN\textgamma\textsuperscript{+} monoclonal Abs (kindly provided by Dr. F. Gerosa, Dept. Pathology, Verona, Italy) and isotype-matched control Abs were used for cytokine neutralization experiments. Cells, either freshly isolated or treated as indicated above, were collected, spun at 350 g for 5 min and the resulting supernatants and pellets immediately frozen in liquid nitrogen and stored at -80\textdegree. Cell pellets were thawed in PBS containing 0.5 % NP40, 5 mM EDTA, 1 mM PMSF, and 5 \mu g/ml leupeptin and pepstatin A, and then centrifuged to remove cell debris. All reagents were of the highest available grade and were dissolved in pyrogen-free water for clinical use.\textsuperscript{16}

**Skin window technique.** The skin window (SW) was performed as already described\textsuperscript{19}, according to the method previously developed\textsuperscript{14}, with minor modifications. Briefly, an abrasion of 1 cm\textsuperscript{2}, produced with a rotating sterile abrasive cylinder operated by a milling cutter (minidrill, Saint Julien en Genevois, France) was developed on an area of volar surface of forearm, without skin lesions. A bell-shaped, sterile and disposable plastic skin chamber with a circular adhesive base (FAR Italia, Verona, Italy), having on its top a 5 mm-wide hole equipped with a plug, was put on the skin abrasion and fixed with a fenestrated sticking plater. 1 ml of autologous serum was then injected into the chamber and 24 h later exudates were collected by aspiration and spun at 350 g for 5 min. The resulting supernatants (SW exudates, SWE) were divided into working aliquots and stored at –80\textdegree until use, whereas cell pellets (> 95 % neutrophils, < 2 % monocytes, < 3 % lymphocytes, as revealed by MayGrunwald-Giemsa staining) were washed twice and lysed as described above.

**Analysis of mediator concentration.** Cytokine concentrations in SWE, sera, cell-free supernatants and cell-associated pellets were measured by specific ELISA for: BLyS, as described\textsuperscript{5}; IL-1ra (kit from Biosource International, Camarillo, CA, USA); G-CSF and TNFα
(kits from R&D Systems); CXCL8 (kit from Euroclone Ltd., Wetherby West Yorkshire, UK). C5a was detected by a commercial kit purchased from IBL-Hamburg GmbH (Hamburg, Germany). Detection limits were: 40 pg/ml for BLyS, 50 pg/ml for IL-1ra, 15 pg/ml for G-CSF, CXCL8 and TNFα and 100 pg/ml for C5a.

**Degranulation of neutrophils.** Cell-free supernatants from neutrophils treated as described above were harvested and their content of antigenic MMP-9 and albumin measured by specific commercial ELISAs. Determination of β-glucuronidase and lactoferrin was performed as already described.20,21

**Western blot analysis.** Detection of BLyS in neutrophil lysates was determined by utilizing a rabbit anti-BLyS polyclonal Abs (#07-167, Upstate Biotechnology, Lake Placid, NY, USA) recognizing membrane-bound (32 kDa) and soluble BLyS (17 kDa) forms, as already described.6

**Postembedding Immunoelectron Microscopy.** Neutrophils, incubated with 1000 U/ml G-CSF or, as negative control, 10 ng/ml GM-CSF (R&D Systems), were fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4° C. After rinsing in the same buffer, cells were dehydrated in graded concentrations of acetone and embedded in Unicryl™ resin (British BioCell International, Cardiff, UK). The resin was polymerized by UV irradiation at 4°C. Ultrathin sections (70-80 nm thick) were collected on 200-mesh Formvar –coated gold grids and processed for immunogold labelling.22 Sections were treated with TRIS buffer containing 5 % BSA and then incubated overnight in either anti-golgin (#A-21270, Molecular Probes, Leiden, The Netherlands) diluted 1:40, or anti-BLyS monoclonal antibodies (3D4)6 diluted 1:100 with TRIS buffer containing 5 % BSA, 1 % Tween 20 and 1 % normal goat serum. After several washes, sections were subsequently placed for 1 h in goat anti-mouse immunoglobulins labelled with 15-nm gold particles (Amersham), diluted 1:40 in TRIS buffer containing 1 % Tween 20. They were then thoroughly washed with TRIS, rinsed in distilled water and air dried. Gold labelled sections were stained with uranyl acetate and lead citrate or viewed directly on a Zeiss EM 10 electron
microscope (Zeiss). Controls for the specificity of the immunoreaction were performed by omitting the primary antibody.

**Statistical analysis.** Data are expressed as means ± SEM. Statistical evaluation was performed by the Student's t-test for paired data and considered significant if p values were < 0.05.

**RESULTS**

**BLyS levels in SWE and sera of normal subjects and rheumatoid arthritis (RA) patients.** In a first series of experiments, we measured BLyS levels in SWE and serum samples that were harvested from healthy volunteers and RA patients. Table 1 shows that serum BLyS was significantly higher in RA patients than in normal donors, confirming and extending previous findings.\textsuperscript{13,23} Interestingly, the concentrations of BLyS augmented approximately two-fold in SWE as compared to serum (Table 1), being significantly more elevated in SWE of RA patients than of healthy donors (Table 1). Such enhancement was genuine, as demonstrated by the calculation of the net BLyS production occurring at the SW site (Table 1), and by the fact that the concentrations of TNFα or IL-1ra in SWE of healthy volunteers and RA patients were not significantly different (Table 1). In addition, the number of leukocytes recruited after 24 h into SWE of healthy donors and RA patients were similar (55.6 ± 4 and 63.5 ± 5 x 10\textsuperscript{6}, respectively), thus excluding the possibility that BLyS levels are more elevated in RA SWE because neutrophils are recruited at higher levels. Collectively, these results demonstrate that during an acute inflammatory response, BLyS is locally produced, and that this BLyS production is more elevated in RA patients.

**Relationship between G-CSF levels and BLyS production in SWE.** Neutrophils treated with G-CSF and IFNγ are known to produce amounts of soluble BLyS that are comparable to those produced by monocytes or monocyte-derived dendritic cells.\textsuperscript{6} Because neutrophils constitute the majority (> 95 %) of the cell populations that accumulates into SWE, we assumed that locally migrated neutrophils were the source of BLyS in SWE. To verify our assumption, we ascertained whether SWE could induce BLyS production by neutrophils. For this purpose, peripheral blood
neutrophils of healthy subjects were cultured for 20 h in medium containing either 20 % SWE or serum obtained from both RA patients and normal donors, in the presence or the absence of neutralizing anti-G-CSF and anti-IFNγ Abs. As control, neutrophils were also cultured with G-CSF or IFNγ, in the presence or the absence of their respective neutralizing Abs. These experiments demonstrated that SWE, but not serum, from both RA patients and healthy donors (Figure 1), induce neutrophils to de novo synthesize and accumulate intracellular BLyS. The effects of these SWE were almost completely blocked by neutralizing anti-G-CSF Abs only (by 87 ± 8 % in healthy donors and by 83 ± 8 % in RA patients, n= 3 for each group), as anti-IFNγ Abs (Figure 1) and isotype matched Abs (data not shown) resulted ineffective. Accordingly, G-CSF was present at very high levels in most SWE samples, even though, surprisingly, identical G-CSF concentrations were found in SWE of both RA patients and healthy donors (Table 1). In contrast, G-CSF was undetectable in serum (Table 1), in agreement with the inability of serum to induce BLyS (Figure 1) and with the fact that the amounts of cell-associated BLyS in circulating neutrophils of RA patients were comparable to those of healthy donors (128 ± 15 pg/2x10^6 cells and 158 ± 19 pg/2x10^6 cells, respectively, n=10). Taken together, our data demonstrate that G-CSF is produced at very high levels during the development of the local SW inflammatory process, in turn promoting the production of BLyS by migrated neutrophils. They do not, however, explain why the amounts of soluble BLyS found in SWE of RA patients were higher than those of healthy donors.

**Proinflammatory mediators promote the secretion of BLyS by G-CSF-treated neutrophils.**

Because CXCL8 and C5a (in addition to BlyS) were found to accumulate at significantly greater levels in SWE of RA patients than healthy donors (Table 1), we measured total BLyS production (i.e. released BLyS in parallel with cell-associated BLyS) in neutrophils preincubated or not with G-CSF, and then treated with CXCL8, C5a or other proinflammatory mediators, including TNFα, immune complexes (IC) or LTB4, that typically augment in SWE or inflammatory synovial fluids. In line with previous studies, G-CSF induces a time-dependent increase of de novo
BLyS synthesis that leads to a maximal intracellular accumulation of antigenic BLyS after 20 h (not shown) and that is, however, only partially secreted into the external milieu (Figure 2A). In contrast, CXCL8 (500 ng/ml), TNFα (5 ng/ml) C5a (500 ng/ml) and IC (60 µg/ml), added for up to 24 h to either freshly purified (data not shown) or to 20-h cultured neutrophils, were unable to stimulate the synthesis/release of BLyS (Figure 2A). By comparison, addition of CXCL8, TNFα, C5a and IC to both G-CSF- and IFNγ-preincubated neutrophils triggered a remarkable secretion of soluble BLyS (Figures 2A and 3). Other classical inflammatory stimuli, for instance 100 nM fMLP (Figure 2A and 3), 500 ng/ml CXCL1, 100 nM LTB4 or 100 ng/ml LPS (data not shown), had a similar effect on BLyS secretion in G-CSF-treated neutrophils. Notably, the capacity of proinflammatory mediators to activate the secretion of BLyS in G-CSF-treated neutrophils appeared to be rapid (clearly evident within 15 min, data not shown), and selective, as the same phenomenon was not observed in the case of IL-1ra (Figure 2B). Additional studies revealed that concentrations of G-CSF as low as 10 U/ml were effective in stimulating neutrophils to synthesize and accumulate an intracellular BLyS storage ready to be released upon CXCL8, TNFα or IC treatment (Figure 4A). Furthermore, dose-response experiments revealed that concentrations of CXCL8, C5a and TNFα as low as 5 ng/ml, 5 ng/ml and 0.1 ng/ml, respectively - which approximately correspond to the amounts detected in SWE (Table 1)25,26 -, displayed a remarkable BLyS-secretagogue effect in G-CSF-treated neutrophils (Figure 4B). G-CSF/IFNγ-treated neutrophils, stimulated with proinflammatory agonists, remained negative for surface BLyS expression (not shown).6

Intracellular localization of BLyS in neutrophils. All proinflammatory agonists used functioned like classical secretagogues, insofar as they only augmented the percentage of secreted BLyS (approximately by 40 %, Figure 3), without upregulating its synthesis (Figure 2A). Accordingly, the use of optimal concentrations of PTF and JK, two actin-polymerization disturbing agents inhibiting degranulation27,28 which suppressed BLyS release induced in neutrophils by 5 ng/ml
TNFα (63 ± 2 %, PTF; 46 ± 3 %, JK), 100 nM fMLP (45 ± 3 %, PTF; 48 ± 3 %, JK) (Figure 5), or 500 ng/ml C5a (75 ± 4 %, PTF), confirmed that a secretory pathway underlies BLyS exocytosis. Under the same conditions, however, the secretion of specific granules (lactoferrin), tertiary granules (MMP-9/gelatinase) and secretory vesicles (albumin) in response to TNFα and fMLP was completely inhibited by both PTF and JK (Figure 5), whereas the release of azurophil granules (β-glucuronidase) was absent (data not shown), as expected. Furthermore, stimuli such as C5a and CXCL8, other than TNFα at very low doses (0.5 ng/ml), did not induce the secretion of β-glucuronidase, lactoferrin, MMP-9, or albumin in G-CSF-activated cells (data not shown), yet retained the capacity to activate BLyS release. Collectively, these results excluded primary, secondary, tertiary, and secretory granules as the intracellular compartments containing BLyS. In this regard, we have recently demonstrated that in G-CSF-treated neutrophils, unlike in other myeloid cells, BLyS is released following its intracellular processing catalyzed by furin, an enzyme known to be predominantly localized within the trans-Golgi network (TGN)/endosomal system. We therefore reasoned that also the pool of mobilizable BLyS might be preferentially stored in the Golgi apparatus. To verify this hypothesis, we examined the effect of monensin, a specific inhibitor of post-Golgi secretion. Monensin inhibited BLyS release induced by TNFα (by 62 ± 4 %), fMLP (by 53 ± 2 %) (Figure 5), and C5a (by 76 ± 5 %) (data not shown), without substantially affecting lactoferrin, MMP-9/gelatinase or albumin secretion (Figure 5). To unequivocally determine the localization of BLyS in G-CSF-treated neutrophils, we took advantage of immunogold labelling by electron microscopy (Figure 6), using monoclonal antibodies against BLyS and golgin, the latter molecule being a specific marker for the Golgi apparatus. In G-CSF-treated neutrophils - additionally stained with uranyl acetate and lead citrate -, Golgian fields appear as isolated vesicles with irregular morphology, dispersed in the cytoplasm, mainly in perinuclear areas (Figure 6, panels A, B and C). By this technique, BLyS labelling was found only in perinuclear vesicles resembling Golgi-like structures but not in the cytoplasm, the granules or the nucleus (Figure 6D, showing a magnification of a Golgian field), similarly to the
labelling pattern displayed by the anti-golgin antibodies. No labelling was found in control sections where the primary Ab was omitted (not shown) or in GM-CSF-treated neutrophils (data not shown). Furthermore, BLyS immunostaining by confocal microscopy confirmed that it partially colocalizes with labelling with furin- the latter being another Golgi marker - when both images were superimposed and fluorescence merged (data not shown), demonstrating that BLyS is preferentially localized in the Golgi compartment.

**TNFα and fMLP do not activate BLyS intracellular processing.** To further clarify whether proinflammatory agonists induced the secretion of soluble BLyS via acceleration of its intracellular processing or, alternatively, by acting on the intracellular cleaved BLyS, already present in G-CSF-treated neutrophils, we performed immunoblots of neutrophil extracts. In this regard, both forms of intracellular BLyS - the non-processed 32 kDa and the cleaved 17 kDa, corresponding to the membrane-bound protein and to the soluble cytokine, respectively - dramatically increase in response to G-CSF (Figure 7A). Remarkably, only the 17 kDa BLyS form disappears upon stimulation with TNFα or fMLP (Figure 7A, lanes 4 and 5), demonstrating that these agonists do not trigger the cleavage of the non-processed 32 kDa form but, rather, favor a complete secretion of the soluble BLyS pool available inside the cell. Accordingly, treatment with CMK, a specific furin convertase inhibitor, did not influence the capacity of CXCL8, TNFα, fMLP and IC to activate the secretion of BLyS in G-CSF-treated neutrophils (Figure 7B). Taken together, the data support the concept that proinflammatory mediators selectively mobilize the intracellular BLyS fraction that has been already processed by furin, and that, for this reason, can be promptly secreted.

**BLyS detection in SW neutrophils.** A final group of experiments revealed that SW neutrophils of RA patients contained amounts of cell-associated BLyS that were approximately 40 % lower than those detected in SW neutrophils of healthy donors (83 ± 15 versus 148 ± 24 pg/ml, p = 0.03, n=10). Substantiating these findings, cells extracts prepared from SW neutrophils of RA patients and analysed by immunoblot revealed an almost total absence of the intracellular 17 kDa soluble
BLyS (Figure 7C). Similarly to circulating neutrophils, SW neutrophils did not express
membrane-bound BLyS (data not shown). Taken together, these observations are consistent with a
complete extracellular discharge of soluble BLyS by SW neutrophils of RA patients. This is likely
to stem from a stronger stimulation of RA neutrophils by proinflammatory mediators, that are
present at higher levels in RA SWE (Table 1).

DISCUSSION

The data presented in this study identify a novel mechanism regulating BLyS release by human
neutrophils that might have important implications for the pathogenesis of inflammatory
conditions (such as rheumatoid arthritis or infections), or of hematological diseases involving a
dysregulation of B cell homeostasis. We report, for the first time, that typical proinflammatory
stimuli, such as chemotactic factors (CXCL1 and CXCL8), cytokines (TNFα), immune complexes
(IC), anaphylatoxin (C5a), eicosanoids (LTB₄), and bacterial-derived products (LPS and fMLP),
which in freshly isolated or cultured neutrophils fail to induce BLyS de novo synthesis, trigger and
greatly amplify the release of BLyS by inflammatory neutrophils. These stimuli act as classic
secretagogues, insofar as they appear to only mobilize the pre-existing pool of soluble BLyS that
accumulates in G-CSF- or IFNγ-pretreated neutrophils and that preferentially localizes to Golgi-
like perinuclear structures. Although it was not our goal to characterize the molecular mechanisms
underlying this secretagogue effect, a possible explanation could involve the well-known ability of
G-CSF/IFNγ to up-regulate the expression of many of the cognate receptors for inflammatory
stimuli, such as CXCR1 and CXCR2 (the receptors for CXCL8)33, the fMLP-R34 and
CD64/FcγRI.35-37 Whatever the case may be, the fact that the release of BLyS was equally evoked
by mediators belonging to so many different classes (chemokines, cytokines, chemotactic factors,
eicosanoid and FcRs), suggests that their secretagogue properties ultimately depends on the
availability of a mobilizable pool of intracellular BLyS in neutrophils. The findings that CMK
failed to block BLyS release by proinflammatory stimuli, and that TNFα and fMLP promoted the
complete secretion of the intracellular fraction corresponding to the soluble form of BLyS, without activating any cleavage of the remaining 32 kDa BLyS form, further highlight the peculiarity of neutrophils as cells committed to exclusively secrete soluble BLyS. Accordingly, monocytes, which also are known to produce soluble BLyS, but only after cleavage of the membrane-bound form, failed to release soluble BLyS in response to TNFα, C5a, IC and fMLP, even if preincubated with IFNγ for up to 3 days prior to stimulation (P. Scapini et al., unpublished observations). In any instance, our data highlight that BLyS release may derive not only following its transcription and de novo synthesis induced by selected stimuli (including IFNγ, IFNα, IL-10, LPS and CD40L in monocytes, and monocyte-derived dendritic cells or G-CSF/IFNγ in neutrophils), but also after mobilization of its intracellular stores.

The ability of IC, CXCL1, CXCL8, TNFα, C5a, LPS, LTB4 and fMLP to trigger the secretion of BLyS by G-CSF-treated neutrophils provides an explanation for the other findings presented in this study. With the aim to evaluate whether neutrophils produce soluble BLyS in human inflammatory diseases, we developed, in normal individuals and RA patients, the Senn’s skin window (SW) model, which allows to study the dynamics of the inflammatory response and compare the effector functions of neutrophils collected in the exudate with those of neutrophils isolated from peripheral blood. Our experiments revealed that in SWE of normal individuals and, more markedly, in RA patients, the concentrations of soluble BLyS were significantly higher than those found in blood serum, indicating that BLyS is produced by locally migrated neutrophils. We also found that G-CSF was absent in serum of both RA patients and normal donors but detectable at very high concentrations in SWE. Despite the fact that there was no difference in the yields of G-CSF found in the two groups of SWE samples, the functional importance of G-CSF was demonstrated by the use of neutralizing anti-G-CSF Abs that inhibited the capacity of SWE from both RA patients and normal donors to stimulate the production of BLyS by neutrophils. The data corroborated our previous observations on the capacity of G-CSF to act as a very potent agonist for neutrophil-derived BLyS in vivo, but did not explain why the levels of BLyS were higher in SWE.
of RA patients than in SWE of healthy subjects. In this regard, we have excluded a BLyS hypersecretion by other known BLyS-producing cells, such as Langherans cells (LC) or mono-DC, because, at least in vitro, monocyte-derived LC and mono-DC do not respond to G-CSF, and do not produce amounts of soluble BLyS in response to IFNγ that are comparable to those produced by neutrophils (P. Scapini et al., unpublished observations). On the other hand, the levels of SWE CXCL8 and C5a, like those of BLyS, were much higher in RA patients than in control subjects. Therefore, it is plausible that the generation of soluble BLyS in SWE of RA patients reflects a more potent stimulation of these neutrophils by CXCL8, C5a and the other agonists. Our observation that SWE neutrophils of RA patients contained lower amounts of cell-associated BLyS than SWE neutrophils of healthy donors would indeed support such a notion.

Our findings not only confirm previous observations on a more elevated BLyS concentration in the serum of RA patients than in healthy controls,¹³,²³ but are consistent with, and further extend, the work of Tan and coworkers,³⁸ who reported that the levels of soluble BLyS are higher in synovial fluids of RA patients than in synovial fluids of patients with degenerative articular diseases. These authors hypothesized that soluble BLyS derives from the cleavage of membrane-bound BLyS expressed by inflammatory monocytes present in synovial fluids.³⁸ Our data suggest that neutrophils, which are massively present in inflamed synovial fluids of RA,¹⁰ could also significantly contribute to soluble BLyS accumulation in such exudates. Neutrophils, in fact, not only produce and release BLyS much more rapidly than monocytes and/or monocyte-derived DC,⁵,⁶ but according to the findings presented in this study, could represent ideal target cells for G-CSF and/or IFNγ acting in combination with CXCL1, CXCL8, TNFα, immune complexes, C5a, and LTB₄, which are all present at high levels in inflammatory synovial fluids.¹⁰,³⁹,⁴⁰ Therefore, neutralization of the capacity of neutrophils to release soluble BLyS might offer a novel target to halt the activation of B cells and, consequently, interfere with the maintenance of an inflammatory state in several chronic inflammatory diseases.
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REFERENCES


FIGURE LEGENDS

Figure 1. Skin-window exudates induce the production of BLyS by neutrophils: role of G-CSF. Serum samples and SWE obtained from RA patients and healthy donors were preincubated for 2 h at 37° C in the presence or absence of neutralizing anti-G-CSF or anti-IFNγ Abs (5 µg/ml) and then added (at a final 20 % v/v concentration) to neutrophils (2x10⁶/400 µl) isolated from normal donors. As control, neutrophils were also incubated with 100 U/ml G-CSF or IFNγ. The mean values ± SEM of BLyS cell-associated to neutrophils cultured for 20 h are shown (n = 3). Asterisks indicate significant inhibitory effects of anti-G-CSF Abs. ** p, < 0.001.

Figure 2. CXCL8, C5a, fMLP, insoluble immune complexes (IC) and TNFα induce the secretion of BLyS from the intracellular pool accumulated in G-CSF-treated neutrophils. Neutrophils (2x10⁶/400 µl) were incubated with or without G-CSF for 20 h prior to addition of CXCL8, C5a, fMLP, IC or TNFα. After additional 4 h, cell-free supernatants and the corresponding cell pellets were then harvested for determination of antigenic BLyS and IL-1ra. The mean values ± SEM of the total production (depicted as cell-associated and released) of BLyS (A), and IL-1ra (B), from 6 and 3 donors, respectively.

Figure 3. Secretagogue effect on BLyS by proinflammatory mediators. Neutrophils were incubated with or without G-CSF or IFNγ for 20 h prior to addition of CXCL8, C5a, fMLP, IC or TNFα. Cell-free supernatants and the corresponding cell pellets were harvested after 4 h and analysed for the content of antigenic BLyS. The percentages of secreted BLyS were calculated from the total BLyS levels. Data report the mean values ± SEM of the percentages of BLyS release under the various conditions (calculated from 6 and 3 experiments for G-CSF- and IFNγ-treated neutrophils, respectively). n.d. = not done.
Figure 4. Characterization of BLyS secretion by G-CSF-treated neutrophils stimulated with proinflammatory mediators. (A), Neutrophils were incubated with doses of G-CSF ranging from 10 to 1000 U/ml for 20 h prior to addition of CXCL8, TNFα or IC. After additional 4 h, cell-free supernatants and the corresponding cell pellets were harvested for determination of antigenic BLyS. The mean values of the total production of BLyS (depicted as cell-associated and released) are shown. The experiment depicted in this figure is representative of two. (B), Neutrophils were preincubated with 100 U/ml G-CSF for 20 h prior to addition of CXCL8, C5a and TNFα at the doses indicated. After additional 4 h, cell-free supernatants were harvested for determination of antigenic BLyS. The mean values of released BLyS are shown. The experiment depicted in this figure is representative of three.

Figure 5. Effect of pentoxifylline (PTF), jasplakinolide (JK) and monensin on BLyS secretion. 20 h G-CSF-treated neutrophils were incubated for 1 h with or without PTF, JK or monensin prior to TNFα or fMLP addition. After 3 h, cell-free supernatants were harvested for ELISA determination of antigenic BLyS, lactoferrin, MMP-9 and albumin. Data report the mean values ± SEM of the percentages of inhibition on TNFα/fMLP-induced release of BLyS, lactoferrin, MMP-9 and albumin provoked by PTF, JK and monensin over G-CSF-treated cells (calculated from 4 experiments).

Figure 6. BLyS localization by Immunoelectron Microscopy. The figure shows an ultrastructural immunocytochemistry for golgin (A-C) and BLyS (D) within G-CSF-treated neutrophils, using specific primary monoclonal Abs and gold-labelled secondary Abs (15-nm gold particles). All the images were obtained from the same experiment. Cells in panels A-C are stained with uranyl acetate and lead citrate to enhance the contrast and allow a better visualization of cell morphology and ultrastructure. Panel B illustrates an area of panel A at higher magnification. The areas marked by squares in panels A and B represent vesicles of Golgi complexes. Panels C and D
illustrate some Golgian fields at high magnification and the absence of BLyS immunoreactivity in granules (indicated with “g”). Scale bars correspond to 750 nm in panel A, 350 nm in panel B and 200 nm in panels C and D. All the images were obtained from a representative experiment out of four performed with similar results.

Figure 7. TNFα and fMLP do not activate BLyS intracellular processing. (A), Neutrophils were incubated for 20 h with or without G-CSF and then stimulated with TNFα and fMLP. After 4 h, cells were lysed as described in the material and methods. 180 µg of whole cell extracts were electrophoresed, blotted and analyzed for BLyS protein expression using a specific anti-BLyS polyclonal Abs recognizing membrane-bound (arrow at 32 kDa) and soluble BLyS (arrow at 17 kDa) forms. The experiment shown is representative of two. (B), Neutrophils were cultured for 20 h with G-CSF and then stimulated with CXCL8, TNFα, fMLP and IC in the absence or the presence of CMK. Culture supernatants were harvested and then processed for BLyS determination by specific ELISA. The experiment depicted is representative of three. (C), SW neutrophils isolated from RA patients and control individuals were lysed and subjected to immunoblot analysis for intracellular BLyS as described above. The experiment shown is representative of two.
Table 1. Accumulation of various mediators in sera and skin window exudates (SWE) collected from healthy donors and Rheumatoid Arthritis (RA) patients.

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<th>SERUM</th>
<th>SWE</th>
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<tr>
<td></td>
<td>normal donors</td>
<td>RA patients</td>
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<tr>
<td><strong>BLyS (pg/ml)</strong></td>
<td>2811 ± 140</td>
<td>4550 ± 300 ***</td>
</tr>
<tr>
<td><strong>(BLyS net increase)</strong></td>
<td>2822 ± 180</td>
<td>4200 ± 400</td>
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<tr>
<td><strong>G-CSF (pg/ml)</strong></td>
<td>&lt; 15</td>
<td>&lt; 15</td>
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<tr>
<td><strong>CXCL8 (pg/ml)</strong></td>
<td>&lt; 15</td>
<td>&lt; 15</td>
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<tr>
<td><strong>TNFα (pg/ml)</strong></td>
<td>n.d</td>
<td>n.d</td>
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<tr>
<td><strong>C5a (ng/ml)</strong></td>
<td>8.1 ± 1.3</td>
<td>25 ± 5 *</td>
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<tr>
<td><strong>IL-1ra (ng/ml)</strong></td>
<td>0.18 ± 0.5</td>
<td>0.12 ± 0.4</td>
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Mean values ± SEM of BLyS, G-CSF, CXCL8, TNF, C5a and IL-1ra levels in sera and SWE from RA patients and healthy subjects are shown. The net increase of SWE BLyS is also reported, as calculated by subtracting, for each sample, the amount of BLyS present in the serum from the total amount of BLyS recovered in the corresponding autologous SWE. Asterisks indicate significant differences between RA patients and healthy subjects: *, p < 0.05; **, p < 0.001; ***, p < 0.0001. n.d. = not done.
Figure 1
Figure 2
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Figure 4
Figure 5
Figure 6
Figure 7
Proinflammatory mediators elicit the secretion of the intracellular B-lymphocyte stimulator (BLyS) pool that is stored in activated neutrophils: implications for inflammatory diseases

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