

# G-CSF-stimulated Neutrophils Are a Prominent Source of Functional BLyS

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## Abstract

B lymphocyte stimulator (BLyS) is a novel member of the TNF ligand superfamily that is important in B cell maturation and survival. We demonstrate that human neutrophils, after incubation with G-CSF or, less efficiently, IFN $\gamma$ , express high levels of BLyS mRNA and release elevated amounts of biologically active BLyS. In contrast, surface expression of the membrane-bound BLyS was not detected in activated neutrophils. Indeed, in neutrophils, uniquely among other myeloid cells, soluble BLyS is processed intracellularly by a furin-type convertase. Worthy of note, the absolute capacity of G-CSF-stimulated neutrophils to release BLyS was similar to that of activated monocytes or dendritic cells, suggesting that neutrophils might represent an important source of BLyS. In this regard, we show that BLyS serum levels as well as neutrophil-associated BLyS are significantly enhanced after *in vivo* administration of G-CSF in patients. In addition, serum obtained from two of these patients induced a remarkable accumulation of neutrophil-associated BLyS *in vitro*. This effect was neutralized by anti-G-CSF antibodies, indicating that G-CSF, present in the serum, stimulated neutrophils to produce BLyS. Collectively, our findings suggest that neutrophils, through the production of BLyS, might play an unsuspected role in the regulation of B cell homeostasis.

Key words: IFN $\gamma$  • inflammation • B lymphocytes • monocytes • dendritic cells

## Introduction

The B lymphocyte stimulator (BLyS;\* also known as B cell activator factor belonging to the TNF family [BAFF], TNF homologue that activates apoptosis, NF- $\kappa$ B, and JNK [THANK], TNF- and ApoL-related leukocyte-expressed ligand 1 [TALL-1], and zTNF4), is a recently identified novel member of the TNF ligand superfamily that is important in B cell differentiation, survival, and regulation of immunoglobulin production (1–3). Its requirement for the humoral immune response is evident in mice lacking BLyS, which exhibit profound deficiencies in peripheral B cell development and maturation (2, 3). In addition, mice transgenic for BLyS develop autoimmune disorders such as increased germinal center formation, production of autoantibodies, and Ig deposition in kidneys (2, 3).

BLyS exists as a type II membrane protein as well as a soluble protein derived from the membrane-bound form by cleavage with a putative furin family protease (4, 5). The expression of BLyS protein seems to be mainly, but not exclusively, restricted to cells of myeloid origin, as demonstrated by studies showing the capacity of monocytes, macrophages, monocyte-derived dendritic cells (mono-DC), and leukemia myeloid cell lines (HL-60, U937, and THP-1) to produce both the membrane-bound and the soluble forms (5). However, whether mature neutrophils, which are known to express a variety of ligands of the TNF family (6), also produce BLyS has never been investigated. The aim of this paper is to analyze whether neutrophils synthesize and release BLyS, and to investigate how a possible BLyS production by these cells could be regulated.

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\*Abbreviations used in this paper: BLyS, B lymphocyte stimulator; HD, Hodgkin's; mono-DC, monocyte-derived dendritic cells.

## Materials and Methods

*Cell Culture and Analysis.* Highly purified granulocytes (neutrophils > 96.5%; eosinophils < 3%), PBMC, monocytes,

and monocyte-derived dendritic cells (mono-DC) were isolated under endotoxin-free conditions as described previously (5, 7). Immediately after purification, cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (<0.009 ng/ml; Seromed Biochrom KG), usually treated with 1,000 U/ml G-CSF (lenograstim; Chugai Pharmaceutical Co., Ltd.) or 200 U/ml IFN $\gamma$  (Hoffmann-La Roche) and subsequently incubated at 37°C, 5% CO $_2$  atmosphere. In selected experiments, G-CSF- or IFN $\gamma$ -stimulated neutrophils were cultured in the presence or absence of the furin convertase inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK; Qbiogene).

**ELISA.** BLyS-specific ELISA was conducted as described previously (5). IL-1ra and G-CSF were measured by using ELISA commercial kits from Biosource International and R&D Systems, respectively.

**RNA Isolation and Northern Blot Analysis.** Total RNA from neutrophils and PBMC was extracted and analyzed by Northern blot for BLyS, IL-1ra, CXCL-11, and actin mRNA expression, as described previously (7).

**Staining of Membrane-bound and Intracellular BLyS.** Surface expression of membrane-bound BLyS was analyzed by flow cytometry analysis using biotinylated anti-BLyS mAbs (3D4) or biotinylated isotype anti-mouse IgG $_1$  as described previously (5, 7).

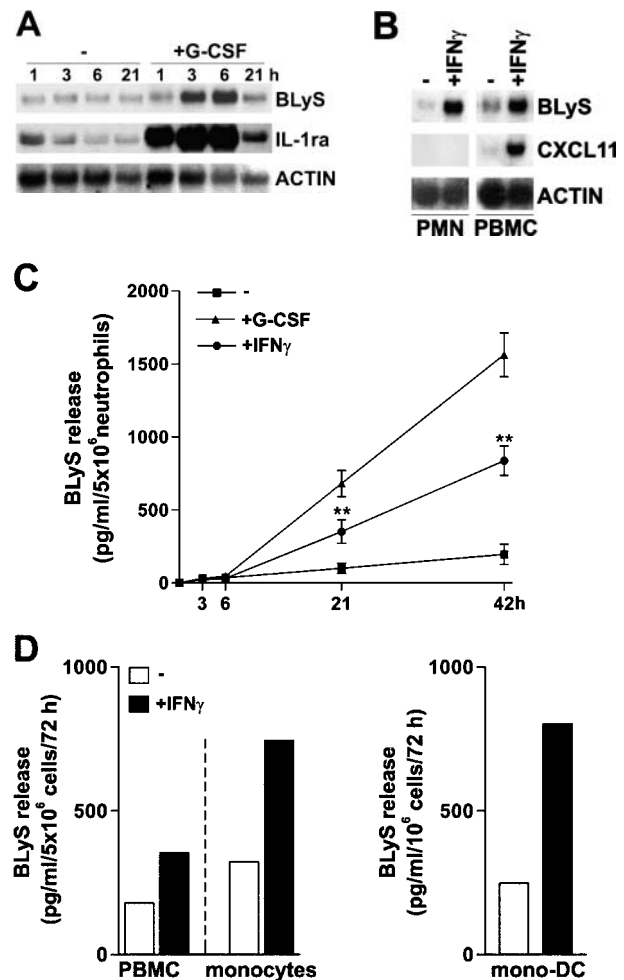
**Immunocytochemistry.** Staining of intracellular BLyS was performed using goat anti-BLyS polyclonal Abs and negative control Abs (R&D) according to Grenier et al. (8).

**Western Blot Analysis.** For the direct immunodetection of BLyS forms, whole-cell lysates from neutrophils were prepared as described previously (7), and then analyzed using a rabbit anti-BLyS polyclonal Abs (Cat. No. 07-167; Upstate Biotechnology). Lysates prepared from HL-60 cells, as well as rBLyS, were also run as positive controls for membrane-bound BLyS and soluble BLyS, respectively.

**Proliferation and Survival of B Cells.** Culture media, conditioned either by neutrophils stimulated with 100 U/ml G-CSF or by G-CSF only, were concentrated (9) and used in a proliferation assay of human tonsillar B cells, as well as in a survival assay of murine splenocytes, exactly as described previously (5, 10).

**Patients.** 10 patients affected by different hematological non-myeloid malignancies (six multiple myeloma, two Hodgkin's [HD], and one non-HD lymphoma), undergoing high dose chemotherapy (CT) and autologous peripheral stem cell transplantation (AP SCT) at the Hematology Unit of the University of Verona, were enrolled for this work. G-CSF (lenograstim) was administered as a daily subcutaneous dose of 300  $\mu$ g from day 2 to day 14 after transplant. BLyS was measured in serum and leukocyte samples collected before conditioning CT (T-0) and from day 12 to day 14 after transplant (T-G-CSF), when neutrophil count resulted >1,000/ $\mu$ l. Three patients (two HD lymphoma and one non-HD lymphoma) who were submitted to standard dose CT and AP SCT without receiving G-CSF support served as controls. In selected experiments, T-0 and T-G-CSF serum samples from two different patients were pre-incubated (at a final 20% vol/vol concentration) for 2 h at 37°C with or without 5  $\mu$ g/ml neutralizing anti-G-CSF polyclonal Abs (R&D Systems) or negative control Abs, and then added to neutrophils isolated from normal donors. After 21 h of culture, neutrophils were lysed and antigenic BLyS was measured in cell-associated pellets.

**Statistical Analysis.** Data are expressed as means  $\pm$  SEM. Statistical evaluation was performed by the Student's *t* test for paired data and considered significant if *P* < 0.05.



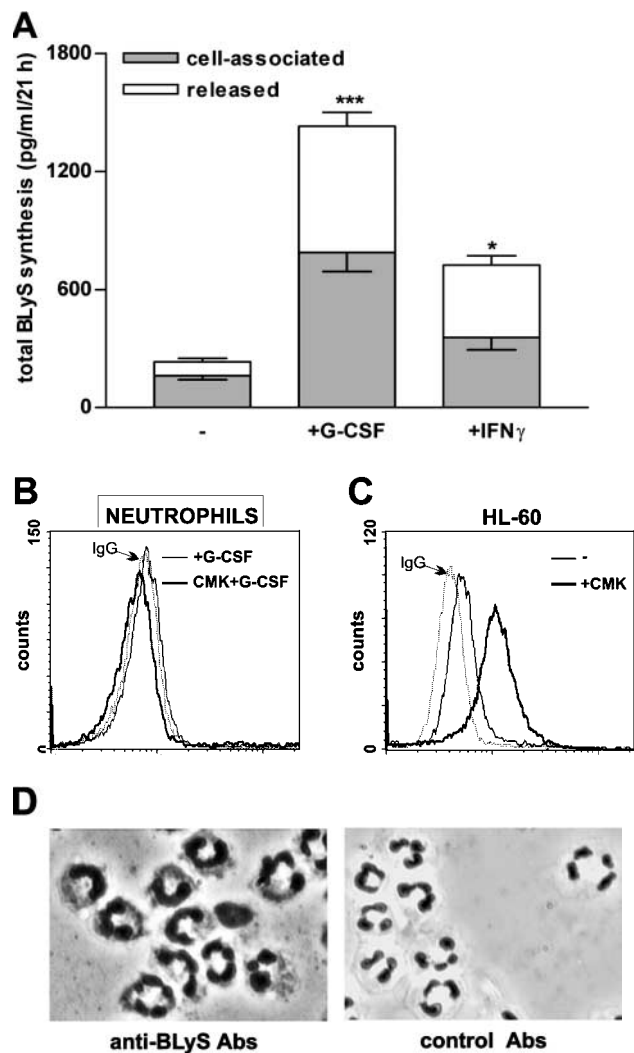
**Figure 1.** BLyS mRNA expression and release by activated neutrophils. (A) Purified populations of neutrophils were incubated with 1,000 U/ml G-CSF. At the time points indicated, total RNA was extracted and analyzed for BLyS, IL-1ra, and actin mRNA expression by Northern blotting. (B) Neutrophils and PBMC purified from the same donor were cultured for 21 h with or without 200 U/ml IFN $\gamma$ , and then were subjected to Northern blot analysis for BLyS, CXCL-11/I-TAC, and actin mRNA expression. Data are representative of results from at least two independent experiments for each panel. (C) Neutrophils were incubated for up to 42 h at 37°C with 1,000 U/ml G-CSF and 200 U/ml IFN $\gamma$ . Culture supernatants were harvested and processed for BLyS detection by a specific ELISA. Values represent means  $\pm$  SEM of duplicate determinations calculated from four independent experiments. The asterisks represent significant differences between stimulated and resting neutrophils. \*\*, *P* < 0.01. (D) PBMC, monocytes, and mono-DC were cultured in the presence or the absence of 200 U/ml IFN $\gamma$  for 3 d. Culture supernatants were collected, and the levels of BLyS were measured by ELISA. Values represent the means of duplicate determinations calculated from two independent experiments.

## Results and Discussion

**BLyS Production by Activated Neutrophils.** Having observed that G-CSF- or IFN $\gamma$ -treated neutrophils express high levels of BLyS mRNA (Fig. 1, A and B), and having excluded that this response might reflect contamination by PBMC (Fig. 1 B), we next investigated whether neutrophils can also produce the BLyS protein. Fig. 1 C shows that untreated cells constitutively release small but detect-

able amounts of BLYS, whereas significant quantities of BLYS were secreted in response to either G-CSF or IFN $\gamma$  and progressively accumulated into the supernatants up to 42 h. Estimation of neutrophil apoptosis by propidium iodide staining and flow cytometric analysis substantiated the notion that G-CSF and IFN $\gamma$  possess a strong capacity to significantly maintain neutrophil survival (9). Dose-response experiments confirmed that 1,000 U/ml of G-CSF and 200 U/ml of IFN $\gamma$  represented optimal stimulatory concentrations for BLYS release (unpublished data). In contrast, other mediators such as IL-4, TGF $\beta$ , IL-10, IL-13, TNF $\alpha$ , GM-CSF, and fMLP, used at optimal stimulatory concentrations, failed to increase the basal release of BLYS. Secretion of BLYS by neutrophils, and not by contaminating cells, was substantiated by the findings that PBMC, on a per cell basis, secreted equivalent amounts of BLYS in response to IFN $\gamma$  (Fig. 1 D), and that G-CSF-stimulated eosinophils were completely unable to release BLYS (unpublished data). Importantly, experiments using stimulated monocytes and mono-DC from additional donors showed yields of BLYS similar to those detected in the supernatants from G-CSF-stimulated neutrophils (Fig. 1 D).

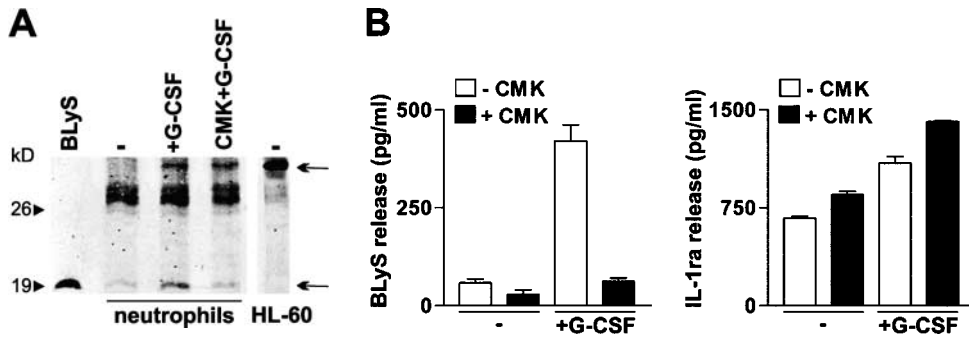
Subsequently, we measured total BLYS production in activated neutrophils, i.e., released BLYS in parallel with cell-associated BLYS. These experiments demonstrated that treatment of neutrophils for 21 h with either G-CSF or, less efficiently, IFN $\gamma$  induced a dramatic increase of total BLYS synthesis relative to cells treated with medium only (Fig. 2 A). Time course studies revealed that total BLYS synthesis started 6 h after cytokine treatment and linearly increased up to 42 h (unpublished data). Interestingly, the percentage of secreted BLYS relative to the total was higher in neutrophils stimulated with both G-CSF and IFN $\gamma$  ( $45 \pm 12$  and  $51 \pm 10\%$ , respectively) than in untreated cells ( $30 \pm 2\%$ ; Fig. 2 A), suggesting that G-CSF and IFN $\gamma$  enhance not only the synthesis, but also the rate of secretion of BLYS. Because our ELISA did not permit us to discriminate between the membrane-bound and the secreted BLYS forms, G-CSF-treated neutrophils were analyzed by flow cytometry and immunocytochemistry. G-CSF was chosen as the stimulus to be used in this and all subsequent assays, not only because of its highest capacity to trigger BLYS production and secretion, but also because of its specificity as a neutrophil agonist. As reported in Fig. 2 B, both IFN $\gamma$ - (not depicted) and G-CSF-treated neutrophils were negative in FACS<sup>®</sup> analysis for surface BLYS expression, whereas membrane-bound BLYS was readily detectable on HL-60 (Fig. 2 C). However, immunocytochemical analysis (Fig. 2 D), as well as intracellular staining of permeabilized neutrophils (unpublished data) confirmed that G-CSF (but not GM-CSF) induced a specific increase of intracellular BLYS (unpublished data), supporting the ELISA findings on a BLYS de novo synthesis in G-CSF-treated neutrophils (Fig. 2 A). In the experiments performed for the detection of membrane-bound BLYS, neutrophils and HL-60 cells were also cultured in the presence of CMK, a specific furin convertase inhibitor. The treatment dramatically increased surface-bound BLYS in HL-60 (Fig. 2 C), which is consistent with the fact that, in these cells, cleavage of membrane-bound BLYS by furin convertase is required for BLYS production (4, 5). In contrast, membrane-bound BLYS did not appear on neutrophils cultured in the presence of the inhibitor (Fig. 2 B).



**Figure 2.** Lack of membrane-bound BLYS expression in neutrophils. (A) Neutrophils ( $5 \times 10^6$ /ml) were incubated for 21 h at 37°C with 1,000 U/ml G-CSF and 200 U/ml IFN $\gamma$ . Cell-free supernatants and the corresponding pellets were harvested and antigenic BLYS determined in each compartment. The mean values  $\pm$  SEM of the total production of BLYS (depicted as cell-associated and released) from four independent experiment are shown. Asterisks represent significant differences between stimulated and resting neutrophils. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . (B and C) Neutrophils were cultured for 21 h with 1,000 U/ml G-CSF, in the absence or presence of 25  $\mu$ M CMK. HL-60 cells were cultured for 21 h in the absence or presence of 50  $\mu$ M CMK. Membrane-bound BLYS expression was examined by FACS<sup>®</sup> analysis using biotinylated 3D4 mAbs followed by PE-conjugated streptavidin. (D) Immunocytochemical staining of intracellular BLYS performed in cytospin preparations of neutrophils incubated for 21 h with 1,000 U/ml G-CSF. Data are representative of results from at least two independent experiments for each panel.

ment with the fact that, in these cells, cleavage of membrane-bound BLYS by furin convertase is required for BLYS production (4, 5). In contrast, membrane-bound BLYS did not appear on neutrophils cultured in the presence of the inhibitor (Fig. 2 B).

**BLYS Processing Takes Place at the Intracellular Level.** To unequivocally identify the intracellular BLYS specie(s) present in neutrophils, we performed immunoblots of whole-cell extracts, using anti-BLYS Abs recognizing the



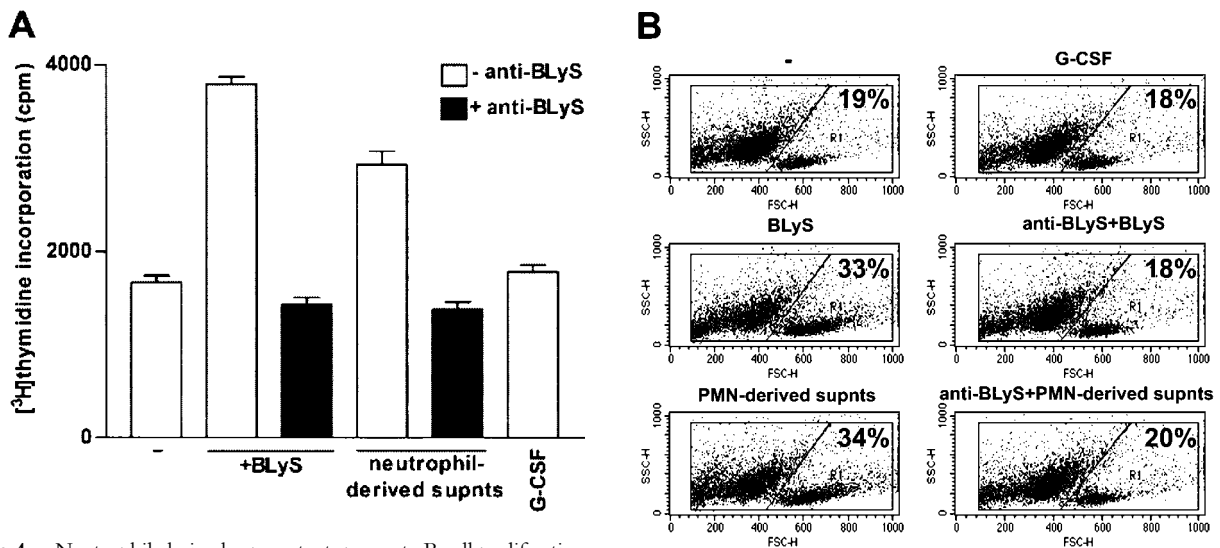
**Figure 3.** BLYS processing takes place at the intracellular level. (A) 1 ng/lane rBLYS and whole-cell extracts prepared from 40  $\mu$ g unstimulated HL-60 cells, and from 150  $\mu$ g neutrophils incubated for 21 h with or without 1,000 U/ml G-CSF, in the absence or presence of 25  $\mu$ M CMK, were electrophoresed, blotted, and analyzed for BLYS protein expression using a specific anti-BLYS polyclonal Abs. The top arrow indicates the non-processed 32-kD BLYS, whereas

the bottom arrow indicates the cleaved 17-kD BLYS. The experiment shown is representative of three experiments. (B) Neutrophils ( $5 \times 10^6$ /ml) were cultured for 21 h with 1,000 U/ml G-CSF, in the absence or presence of 25  $\mu$ M CMK. Culture supernatants were harvested and examined for BLYS and IL-1ra content. Values represent means  $\pm$  SEM of duplicate determinations calculated from three independent experiments.

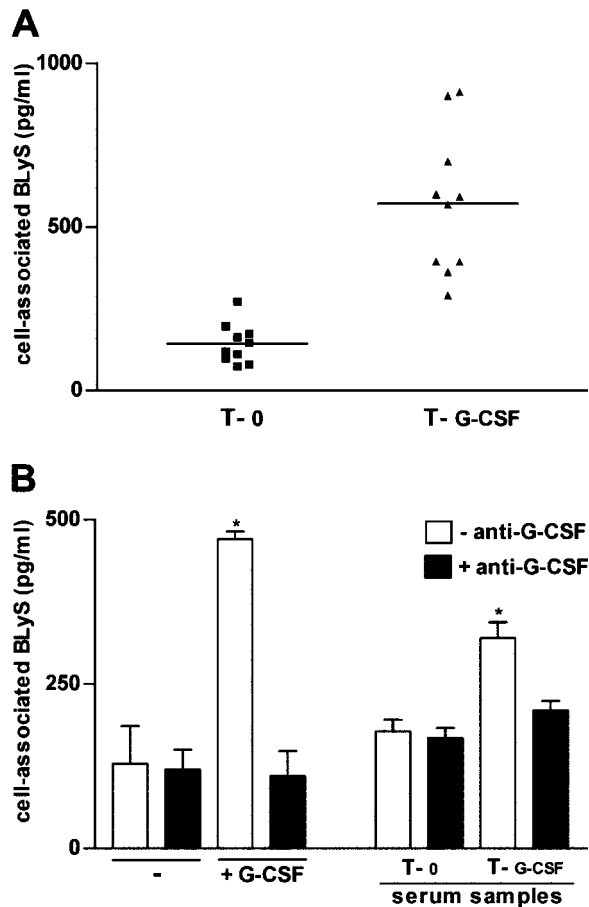
nonprocessed 32-kD protein, as well as the cleaved 17-kD form (reference 4; Fig. 3 A, arrows). These experiments confirmed that HL-60 cells (Fig. 3 A), THP-1 cells, monocytes, and mono-DC (not depicted) constitutively synthesize the 32-kD BLYS protein (corresponding to the membrane-bound BLYS), which is consistent with the notion that release of BLYS by these cell types results by proteolytic cleavage of the membrane-bound protein (5). In contrast, lysates from unstimulated neutrophils contained the 32- and 17-kD (corresponding to rBLYS) proteins (4, 5), which both dramatically increased on cell treatment with G-CSF (Fig. 3 A). Treatment with CMK completely inhibited the expression of the 17-kD protein, but not of the 32-kD one (Fig. 3 A). Accordingly, CMK suppressed the release of BLYS by G-CSF-treated neutrophils (Fig. 3 B). The inhibition was neither caused by drug toxicity—because IL-1ra production was unaffected (Fig. 3 B)—nor exerted at the transcriptional level—because G-CSF-induced BLYS mRNA accumula-

tion was not influenced by CMK (not depicted). Together, our data indicate that in neutrophils, BLYS is released after intracellular processing of the longer form by a furin convertase-like enzyme. In this context, APRIL/TRDL-1 $\alpha$ , another recently identified TNF family member closely related to BLYS, was shown to be processed in a similar manner (11). The fact that neutrophils, but not PBMC or DC, process BLYS intracellularly indicates that the mechanisms regulating BLYS expression are cell-type characteristic and are subjected to distinct regulatory pathways.

**Biological Activities of Neutrophil-derived Supernatants.** To ascertain whether BLYS released by neutrophils was biologically active, we initially tested the ability of neutrophil-derived supernatants in a standard B cell costimulation assay. As shown in Fig. 4 A, BLYS contained in conditioned medium obtained from G-CSF-stimulated neutrophils (used at the final concentration of 5 ng/ml) induced an approximately twofold increase of B cell [ $^3$ H]thymidine uptake, whereas



**Figure 4.** Neutrophil-derived supernatants promote B cell proliferation and survival. [ $^3$ H]Thymidine incorporation by human tonsillar B cells (A) and flow cytometry analysis on FSC/SSC plots of splenocytes from C57BL/6J mice (B) were assessed after a 4- or 3-d culture, respectively, with 5 ng/ml rBLYS or conditioned medium prepared from G-CSF-stimulated neutrophils, in the absence or presence of 0.3  $\mu$ g/ml of neutralizing anti-BLYS mAbs.  $10^{-5}$  dilution SAC was added as a costimulus for the proliferation assay. Cell-free culture medium containing G-CSF was used as a control. Values in A are expressed as the mean number  $\pm$  SEM of triplicate determinations calculated from three independent experiments. The plots in B are representative of three separate experiments.



**Figure 5.** BLYS production by neutrophils isolated from G-CSF-treated patients. (A) Neutrophils were collected from patients either before G-CSF infusion (T-0) or at day +12 to +14 after treatment (T-G-CSF), lysed, and examined for cell-associated BLYS by ELISA. The plot shows the mean values of averaged duplicate determinations obtained from the number of subjects indicated. (B) Serum samples obtained from two different patients (both at T-0 and T-G-CSF) were preincubated for 2 h at 37°C in the presence or absence of 5  $\mu$ g/ml neutralizing anti-G-CSF and added to neutrophils isolated from normal donors (at a final 20% vol/vol concentration). As a control, neutrophils were also incubated with 100 U/ml G-CSF ( $\pm$  anti-G-CSF Abs). The mean values  $\pm$  SEM of BLYS levels associated to neutrophil pellets are shown. The asterisks represent significant differences between stimulated and resting neutrophils. \*,  $P < 0.05$  ( $n = 3$ ).

medium containing G-CSF only was substantially ineffective. Similar findings were subsequently obtained in a survival assay of murine splenocytes evaluated either by morphology (Fig. 4 B) or by annexin V/PI staining (unpublished data), as reported previously (10). Proliferation and survival of B cells were BLYS-specific because they were completely abrogated by the neutralizing anti-BLYS mAbs (but not by isotype matched control Abs; unpublished data), and were comparable to that induced by 5 ng/ml BLYS (1, 5).

**BLYS Production by Neutrophils Isolated from G-CSF-treated Patients.** Finally, we examined the capacity to produce BLYS by neutrophils purified from patients undergoing administration of rG-CSF. Under these conditions, G-CSF is known to selectively promote the proliferation and the differentiation of neutrophil progenitors in vivo and to

activate mature neutrophils (12). We evaluated BLYS serum levels and BLYS production by leukocytes (measured as cell-associated protein), either before G-CSF infusion (T-0), or at day +12 to +14 after treatment (T-G-CSF), when neutrophil count resulted  $>1,000/\mu$ l (13). Similar to previous reports (12, 13), preinjection endogenous levels of G-CSF, measured in serum, were undetectable, whereas 12–14-d posttreatment levels were, on average,  $1,170 \pm 248$  pg/ml. Fig. 5 A shows that T-0 neutrophils contain amounts of cell-associated BLYS at levels substantially similar to those observed in neutrophils freshly purified from healthy donors ( $171 \pm 40$  pg/ml,  $n = 8$ ). Remarkably, accumulation of cell-associated BLYS in T-G-CSF neutrophils was significantly enhanced, increasing, on average, three- to fourfold (Fig. 5 A). In contrast, analysis of intracellular BLYS in autologous PBMC, performed before and after, G-CSF administration, demonstrated that the accumulation of cell-associated BLYS did not significantly change (resulting  $185 \pm 31$  pg/ml at T-0, and  $267 \pm 27$  pg/ml at T-G-CSF,  $n = 4$ ; unpublished data). Importantly, analysis of serum samples obtained from all these individuals revealed that G-CSF administration was associated with a marked increase in BLYS serum levels, which raised from values of  $3,393 \pm 355$  pg/ml (at T-0) to  $6,494 \pm 757$  pg/ml (at T-G-CSF;  $n = 4$ ,  $P = 0.002$ ). Average values of BLYS serum levels in healthy donors corresponded to  $2,945 \pm 334$  ( $n = 8$ ). Furthermore, in three matched patients without G-CSF support, serum levels of BLYS as well as neutrophil-associated BLYS did not change over time (unpublished data), indicating that the rise of BLYS in treated patients is a specific effect of G-CSF.

To demonstrate that G-CSF administered to the patients is directly responsible for the increased levels of BLYS in the sera, neutrophils from healthy donors were cultured for 21 h in medium containing 20% serum obtained from two of our patients (before and after G-CSF administration), in the presence or the absence of neutralizing anti-G-CSF Abs (Fig. 5 B). The amounts of G-CSF present in these sera corresponded to 0 pg/ml at T-0 and  $\sim 1,200$  pg/ml at T-G-CSF. As a control, neutrophils were also cultured with 100 U/ml G-CSF in the presence or the absence of neutralizing anti-G-CSF polyclonal Abs. As shown in Fig. 5 B, medium containing T-G-CSF serum stimulated a higher expression of BLYS in neutrophils than medium containing T-0 serum. The enhancement of cell-associated BLYS was blocked (80% inhibition) by neutralizing anti-G-CSF Abs (Fig. 5 B), whereas negative control Abs was ineffective (unpublished data).

In this paper, we demonstrate that human neutrophils activated by G-CSF and IFN $\gamma$  display the capacity to produce BLYS. It is worthwhile to remark that, although the capacity of neutrophils to produce cytokines is low compared with mononuclear cells (6), the amount of BLYS release by G-CSF-treated neutrophils was similar to that of IFN $\gamma$ -treated monocytes and mono-DC. Because neutrophils constitute the most numerous leukocytes in the blood (normally 50–70%), and are the predominant infiltrating cells present in acute inflammatory response, they might

represent a major source of BLyS. What could be the physiological or pathological meaning of the ability of neutrophils to produce BLyS? Because neutrophils do not colocalize with naive B cells within secondary lymphoid organs, and do not express membrane-bound BLyS, we exclude that they might have a direct role in the regulation of naive B lymphocyte proliferation under physiological conditions. However, high amounts of BLyS produced by activated neutrophils in pathological conditions, such as chronic infection or inflammation, might produce local effects and/or promote BLyS efflux to local lymphoid tissues via the lymphatics or the blood. In this way, neutrophil-derived BLyS might influence B cell maturation in the spleen and lymph nodes or even promote B cell hyperplasia and the emergence of autoreactive pathogenic B cells.

The potent G-CSF stimulation of BLyS production in neutrophils is a finding that, in light of G-CSF's previously reported poor capacity to modulate neutrophil-derived cytokines (6), is of great interest and needs to be further investigated. Elevated concentrations of G-CSF have been found in the serum and in the synovial fluid of rheumatoid arthritis patients (14), and in the serum of patients during acute stages of bacterial infections (12) diseases with pathogenesis associated with neutrophil activation. In contrast, BLyS serum levels have been shown to be significantly increased in patients with immune-based diseases such as rheumatoid arthritis (15), systemic lupus erythematosus (16), and Sjogren's syndrome (17). Understanding whether the increased BLyS levels result from G-CSF-mediated activation of neutrophils might help to clarify the mechanisms underlying the development of autoimmune diseases. Indeed, it has been proposed a potential link between the activation of innate immunity occurring during infections and the subsequent development of autoimmune phenomena (18).

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