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J Immunol 2008; 180:7394-7403; doi: 10.4049/jimmunol.180.11.7394
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B Lymphocyte Stimulator Regulates Adaptive Immune Responses by Directly Promoting Dendritic Cell Maturation

Sook Kyung Chang, Stephen A. Mihalcik, and Diane F. Jelinek

B lymphocyte stimulator (BLyS) is a well-known direct costimulator of adaptive immune cells, particularly B lineage cells. However, we have reported recently that BLyS is also able to activate monocytes. Other innate immune cells, such as dendritic cells (DCs), play a key role in the initiation of adaptive immune responses and the purpose of the current study was to assess whether there is a direct role for BLyS in modulating human DC functions. In this study, we show that BLyS induces DC activation and maturation. Thus, BLyS strongly induced up-regulation of surface costulatory molecule expression and secretion of specific cytokines and chemokines in DCs. BLyS-stimulated DCs (BLyS-DCs) were also able to augment allogeneic CD4 T cell proliferation to a greater extent than control DCs. BLyS-DCs secreted elevated levels of the major Th1-polarizing cytokine, IL-12p70, and they promoted naive CD4 T cell differentiation into Th1 T cells. Regarding BLyS receptor expression, DCs primarily express cytoplasmic transmembrane activator and CAML interactor; however, low levels of cell surface transmembrane activator and CAML interactor are expressed as well. Collectively, our data suggest that BLyS may modulate adaptive immune cells indirectly by inducing DC maturation.

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Received for publication August 20, 2007. Accepted for publication March 28, 2008.

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1 This work was supported by National Institutes of Health Grants CA105258 and CA062242 (awarded to D.F.J.).

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3 Abbreviations used in this paper: DC, dendritic cell; BLyS, B lymphocyte stimulator; BCMA, B cell maturation Ag; TACI, transmembrane activator and CAML interactor; SS, Sjogren’s syndrome; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MNC, mononuclear cell; rh, recombinant human; PB, polymyxin b; CBA, cytometric bead array; RT, room temperature; GRO, growth-related oncogene; FDC, follicular DC.

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of a unique intracellular pool of preformed TACI in monocyte-derived DCs, and present evidence that this pool is located within the Golgi apparatus. Thus, our data extend our understanding of the cellular targets of BLYS and suggest one more mechanism by which BLYS may play a role in modulating the adaptive immune system, i.e., indirectly through DC maturation.

Materials and Methods

Generation of human monocyte-derived DCs

Mononuclear cells (MNCs) were isolated from peripheral blood of healthy donors by Ficoll-Paque density gradient centrifugation. All patients provided written informed consent in accordance with the Declaration of Helsinki. The Mayo Clinic Rochester Institutional Review Board approved the protocol to obtain blood from volunteers. Monocytes were purified from MNCs by positive selection using the StemSep Ab enrichment mixture (Stemcell Technologies) and then cells were stained. For BLyS binding on DCs, cells were cocultured with 0.2 × 10^6 naive DCs in flat-bottom 96-well plates (DC:T cells = 1:5). On day 5, cells were stimulated with 50 ng/ml PMA plus 1 µg/ml ionomycin in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich) for the last 4 h of culture. Cells were fixed and permeabilized using a fixation/permeabilization kit (Caltag Laboratories). Cells were stained with FastImmune IFN-γ, IL-10, IL-17, TNF-α, and IL-12 (BD Biosciences) and alaffocytocyanin-CD3 (BD Biosciences) in permeabilization solution. CD3-positive cells were analyzed for levels of cytokine-specific cytokine expression. FcγR-blocking reagent (Miltenyi Biotec) was used in all staining procedures.

Human cytokine array

The profile of cytokines secreted by DCs (2 × 10^5/0.2 ml in 24-well plates) was determined by using a protein microarray (RayBio Human Cytokine Ab Array V; RayBiotech). After a 24-h incubation with or without stimuli, cell culture supernatants were harvested and stored at −80°C until they were analyzed. Experiments were performed according to the manufacturer’s instructions.

Quantification of cytokine secretion by ELISA

IL-6, IL-1β, IL-23, TNF-α, IL-10, (R&D Systems), and IL-12p70 (BD Bioscience) levels were determined in cell-free culture supernatants using cytokine-specific ELISA kits. Supernatants of dendritic cell cultures were stored at −80°C until they were analyzed. Results are shown in picograms per milliliter or nanograms per milliliter (as mean ± SD).

Immunofluorescence analysis

In vitro-generated DCs were placed into 12 × 75-mm FACS tubes and fixed with 4% paraformaldehyde for 20 min at room temperature (RT). After washing, cells were permeabilized with 0.1% Triton X-100 for 5 min at RT and washed with 3 ml of FACS buffer. After blocking with 5% normal rabbit serum in PBS for 1 h at RT, cells were incubated with FcγR blocking reagent (Miltenyi Biotec) in 5% normal rabbit serum in PBS for 10 min. Goat anti-TACI Ab (C-20; Santa Cruz Biotechnology) and mouse GM130 Ab (BD Biosciences) were then added and cells were incubated for an additional 1 h at RT. After washing with 3 ml of FACS buffer, cells were stained with FITC-labeled F(ab')2 rabbit anti-mouse Ab (Jackson ImmunoResearch Laboratories) for 40 min at RT. Cells were then washed with 4 ml of FACS buffer and fixed again with 4% paraformaldehyde for 20 min at RT. Cytospin preparations of the cells on glass slides were made using a Thermo Shandon cytospin 2 (350 rpm, 4 min). When slides were dry, a mounting solution containing 4',6-diamidino-2-phenylindole (Vector Laboratories) was applied, and images were taken using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss).

Statistical analysis

Statistical analysis was performed using the Student t test. Values of p < 0.05 were considered to be significant.

Results

BLYS induces DC maturation

We first determined whether BLYS has any biological effect on DCs. To accomplish this goal, we generated monocyte-derived
DCs using an in vitro system and DCs generated in this manner were then cultured with or without BLyS or LPS. When DC cultures were analyzed by light microscopy, BLyS was observed to induce significant cell clustering (Fig. 1, bottom right panel). In addition, BLyS-stimulated DCs (BLyS-DCs) were larger than unstimulated cells and displayed more dendrites (Fig. 1, bottom left panel). To further characterize the activation of DCs by BLyS, cell surface phenotype was characterized using flow cytometry following incubation of DCs with BLyS or LPS as a control. As shown in Fig. 2A, surface expression of the costimulatory molecules, CD80 and CD86, and the maturation marker, CD83, greatly increased upon stimulation with BLyS or LPS compared with unstimulated cells. BLyS also strongly induced CD40 expression, however, BLyS down-regulated expression of the mannose receptor and DC-SIGN (Fig. 2B). Fig. 2C demonstrates that the ability of BLyS to significantly increase CD80 and CD86 levels and induce CD83 is reproducible across multiple, independent experiments, and that it occurred in a dose-dependent manner (Fig. 2D). In data not shown, HLA-DR expression was not significantly changed by BLyS stimulation. The DC maturation process is also accompanied by a decrease in phagocytic activity. We next assessed the ability of BLyS to attenuate phagocytosis by measuring DC uptake of FITC-conjugated dextran. Compared with unstimulated DCs, BLyS-DCs showed significantly reduced phagocytosis (Fig. 3). Finally, to confirm the effects of BLyS on DCs, we also analyzed directly isolated DCs from peripheral blood. Following isolation, purified blood DCs were cultured with BLyS. As shown in Fig. 4, BLyS also strongly induced CD83 expression on CD11c-positive myeloid DCs.
BLyS stimulates DCs to secrete cytokines and chemokines

To further characterize the effects of BLyS on DCs, we used a cytokine/chemokine Ab-based array to measure protein secretion by DCs cultured in the presence or absence of BLyS or LPS. BLyS induced secretion of a variety of chemokines and cytokines to varying degrees, including IL-6, IL-8, growth-related oncogene (GRO)-α, IL-1α, IL-1β, TNF-α, monocyte chemoattractant protein 1 (MCP-1), MCP-2, MCP-4, macrophage inflammatory protein 1β (MIP-1β), MIP-3α, and RANTES (Fig. 5B). Compared with unstimulated cells, secretion of IL-6, IL-8, MCP-1, and GRO was highly induced by BLyS (Fig. 5B). MCP-1 and RANTES secretion was notable in that greater induction was observed in the BLyS-stimulated cells than in the LPS-stimulated positive control cultures. To a lesser degree, MCP-2, MCP-4, MIP-3α, and MIP-1β were also increased by BLyS stimulation. Of interest, BLyS-treated DCs produced high amounts of the proinflammatory cytokines IL-6, IL-1α, IL-1β, and the Th1 chemokine, IFN-γ-inducible protein 10, but not the chemokines that recruit Th2 type immune cells, such as thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309 (Fig. 5B).

The enhanced production of IL-6, IL-1β, and TNF-α by BLyS was confirmed by direct ELISA. Particularly, IL-6 and TNF-α production was greatly enhanced and IL-1β was moderately increased by BLyS (Fig. 5C). Because IL-6 and IL-1β, in concert with IL-23, have been shown to induce differentiation of Th17 cells (47, 48), we next examined the ability of BLyS to induce IL-23 secretion. As shown in Table I, we observed that BLyS stimulation resulted in only very low levels of IL-23 secretion as revealed by ELISA.
cell proliferation. Data reflect the mean ± SD of triplicates from one experiment and are representative of six independent experiments.

and it was quite variable between donors. Despite the variable and low levels of IL-23 secretion, we did investigate whether BLyS stimulation induced the appearance of Th17 cells, characterized by the intracytoplasmic expression of IL-17. However, in results not shown, we failed to detect cells expressing intracellular IL-17 following BLyS stimulation.

**BLyS-DCs enhance allogeneic CD4 T cell proliferation**

The results described above clearly demonstrate that BLyS induces DC maturation. To address the functional activity of BLyS-DCs, we next performed allogeneic MLR. BLyS-DCs were comparable to LPS-DCs in their ability to strongly augment CD4 T cell proliferation (Fig. 6). DCs stimulated with 100 ng/ml CD40L had only a moderate effect on CD4 T cell proliferation (Fig. 6); however, when a higher concentration of CD40L was used (200 ng/ml), the ability of DCs to enhance CD4 T cell proliferation was comparable to BLyS-DCs (data not shown).

**BLyS-DCs induce Th1 immune responses**

Next, we questioned whether BLyS-DCs are involved in naïve CD4 T cell differentiation. To address the question, we first determined whether BLyS induces DCs to produce IL-12, a well-known Th1-driving cytokine. Although BLyS-DCs did not appear to secrete IL-12 as assessed using the cytokine array (Fig. 5B), the sensitivity and sample size of this assay are limited. We next used a more sensitive ELISA that would allow us to directly measure IL-12p70, the active form of IL-12. Even though the levels of IL-12 were variable among 10 independent experiments, IL-12p70 was clearly induced by BLyS with at least a 2-fold induction observed when compared with cells cultured with medium alone (Nil; Fig. 7A). However, BLyS failed to induce IL-10 secretion in four independent experiments (Fig. 7B).

**BLyS-induced DC maturation is not caused by LPS contamination**

Because DCs are very sensitive to LPS stimulation, we needed to exclude the possibility that endotoxin contamination in the recombinant BLyS accounted for the ability of BLyS to stimulate DC maturation. Even though we excluded LPS contamination in the recombinant BLyS in our previous study by using PB and boiling (33), we subjected both BLyS and LPS, as a control, to heat inactivation by boiling for 30 min. We then assessed the ability of the heat-inactivated BLyS and LPS to induce surface marker expression as well as IL-12p70 cytokine expression as compared with control, non-heat-inactivated reagents. Heat-inactivation completely blocked BLyS-induced expression of surface markers, CD80, CD86, and CD83, whereas it had no effect on the ability of LPS to enhance surface marker expression (Fig. 9A). Similarly, pretreatment of BLyS with PB did not impair its ability to up-regulate CD80 expression whereas it did, as expected, impair LPS-mediated induction of DC CD80 expression (Fig. 9B). Similar results were obtained when cytokine secretion was assayed instead. Thus, IL-12p70 was still induced by boiled LPS, but not by boiled BLyS (Fig. 9C). In summary, the direct effects of BLyS on DC maturation do not result from LPS contamination.

**BLyS-binding receptors**

To determine how BLyS activates DCs, we first looked at BLyS binding using flow cytometry. BLyS binding was consistently detected; however, this occurred in a temperature dependent manner. BLyS binding was detectable when cells were incubated at 37°C (Fig. 10A), but not at 4°C (data not shown). Next, we tried to identify which receptor is responsible for BLyS binding on DCs. There are three known BLyS-binding receptors: TACI, BCMA, and BAFFR. None of the receptors were detectable on the cell surface using flow cytometry (Fig. 10B). Because we have previously shown that each Ab for the three receptors does detect their
targets on normal human B cells (47), the results were not simply from Ab failure to detect their targets. Using normal TACI-expressing B cells as a tool, we demonstrated that preincubation of cells with exogenous BLyS did not block the ability of the anti-TACI Ab to bind to receptor (data not shown), thereby excluding the possibility that our inability to detect TACI by flow cytometry resulted from epitope blocking. In addition, DCs lacked cytoplasmic BAFFR by flow cytometry, and BCMA expression at the mRNA level was very heterogeneous among different donors (data not shown). In contrast, TACI expression was consistently detected by RT-PCR (data not shown), intracellular flow cytometry, and immunofluorescence (Fig. 10C). Even though surface TACI was not detectable by flow cytometry, some surface TACI was detected by confocal microscopy in nonpermeabilized cells (Fig. 10D). Of interest, intracellular TACI was principally localized in the perinuclear area, which colocalized with the staining observed using the cis-Golgi matrix protein (GM130)-specific mAb. Punctate forms of TACI were also detected in the cytoplasm albeit to varying degrees among different donors (Fig. 10, D and E). In addition, the same staining pattern of TACI was also detected in DCs freshly isolated from peripheral blood (data not shown). Upon incubation of cells at 37°C before staining, there was a marked increase in levels of punctate TACI expression (data not shown). This observation is consistent with our flow cytometric assessment.

FIGURE 8. BLyS-DCs induce naive CD4 T cell differentiation into Th1 T cells. After DCs (0.5 × 10³/well) were preactivated with or without 200 ng/ml BLyS, 20 ng/ml LPS, or 100 ng/ml CD40L for 24 h, cells were cocultured with freshly isolated allogeneic naive CD4 T cells (2.5 × 10⁵/well) for 2 days in round-bottom 96-well plates. Culture supernatants were collected and Th1/Th2 cytokine production was measured using a human CBA (A and B). Data are representative of four independent experiments. C, DCs were preactivated with or without 200 ng/ml BLyS, 100 ng/ml LPS, or 200 ng/ml CD40L for 24 h. After three washes, DCs (0.2 × 10³/well) were cocultured with purified allogeneic naive CD4 T cells (1 × 10⁵/well) for 5 days in flat-bottom 96-well plates. On day 5, cells were restimulated with PMA plus ionomycin in the presence of brefeldin A for the last 4 h of culture, and cells were analyzed for intracellular IFN-γ and IL-4 staining using flow cytometry.
of BLyS binding, i.e., ligand binding was observed at 37°C. Finally, we also investigated receptor expression following stimulation of DCs with CD40L, BLyS, or LPS; however, none of these stimuli induced surface expression of any of the three BLyS binding receptors (data not shown).

Discussion

It has been shown that BLyS levels are high in certain autoimmune diseases, such as SS, SLE, and RA (34–38). Here, we show that BLyS induces DC activation and maturation as revealed by enhanced expression of costimulatory molecules and cytokine production, but reduced phagocytic activity. Moreover, BLyS-DCs promoted naive CD4 T cell proliferation and differentiation into Th1 T cells. Our observations support the proposal that high BLyS levels in patients with autoimmune diseases may result in the activation of innate immune cells to secrete large amounts of proinflammatory cytokines, and subsequently modulate adaptive immune cells, which may be involved in the initiation or perpetuation of autoimmune diseases.

BLyS induced the expression of surface costimulatory molecules, CD40, CD80 and CD86, however, MHC class II expression was not up-regulated by BLyS stimulation. Although activated DCs typically do acquire higher levels of HLA class II expression, there is precedence for stimuli that do not. Thus, Radhakrishnan et al. (49) demonstrated that cross-linking the B7-DC molecule on DCs dramatically enhanced their ability to present MHC class II-peptide complexes in the absence of up-regulation of MHC class II surface expression (49, 50). It is also interesting to note that there is evidence that signaling through BCMA renders B lymphocytes more efficient APCs. Thus, Yang et al. (51) demonstrated APRIL or agonistic BCMA Abs activated the JNK pathway which resulted in increased expression of CD40, CD80, CD86, MHC class II, and CD54. Therefore, even though the expression of MHC class II was not up-regulated on DCs by BLyS stimulation, BLyS signaling may augment Ag presentation in DCs by up-regulating costimulatory molecule expression through TACI.

In this study, we present clear evidence that BLyS induces DCs to secrete a variety of cytokines and chemokines. Although the absolute levels of cytokine/chemokine secretion were different, the overall pattern of cytokine induction by BLyS is strikingly similar to LPS stimulation. BLyS-DCs highly expressed GRO-α, MCP-2, MCP-4, MIP-1β, MIP-3α, MCP-1 and RANTES, suggesting that BLyS-DCs may recruit innate immune cells into the sites of inflammation, and thus, induce or amplify the inflammatory immune responses. Of interest, MCP-1 and RANTES were induced to a greater degree in BLyS-DCs than in LPS-DCs, supporting the conclusion that BLyS strongly promotes monocyte and T cell recruitment to aid in proinflammatory responses. BLyS-DCs expressed the T cell chemokine IFN-γ-inducible protein 10, but did not secrete detectable levels of Th2 T cell chemokines, thymus and activation-regulated chemokine,
levels of IL-1

By inducing cytokine production and costimulatory molecule expression, BLyS-DCs promote naïve CD4 T cells to secrete high amounts of IFN-γ and IL-2, but not IL-4, IL-5, or IL-10. The enhanced IFN-γ production by CD4 T cells stimulated with BLyS-DCs was confirmed by intracellular staining. Previous studies showed that BLyS is involved in Th1 related autoimmune diseases, such as SLE, SS, and RA (35–38, 55–57). Another study showed that BLyS transgenic mice showed a much greater degree of Th1-mediated inflammatory immune responses (58). Our data also suggest that BLyS is involved in Th1 immune responses, which may be mediated through DC maturation by BLyS.

Recently discovered Th17 cells are distinguished from Th1 and Th2 cells via their ability to produce IL-17, a cytokine that has been shown to play a critical role in several inflammatory and autoimmune diseases (11, 59–63). The majority of studies to date have been conducted in the murine system, and it has been reported that IL-6 and TGF-β are required to differentiate naïve CD4 T cells into Th17 cells whereas IL-23 is required for the survival and effector function of Th17 cells (64–66). However, two very recent studies demonstrated that IL-23, IL-6, and IL-1β are required for human Th17 differentiation, and only one article studied whether APCs mediate Th17 differentiation (47, 48).

The authors found that activated monocytes and directly purified CD1c (BDCA1) positive myeloid DCs, but not GM-CSF/IL-4 derived DCs, promoted Th17 cell differentiation (47). Because Th17 cells require for human Th17 differentiation, and only one article studied whether APCs mediate Th17 differentiation (47, 48). The authors found that activated monocytes and directly purified CD1c (BDCA1) positive myeloid DCs, but not GM-CSF/IL-4 derived DCs, promoted Th17 cell differentiation (47). Because Th17 cells are involved in autoimmune diseases, we also examined whether BLyS-DCs are also involved in Th17 differentiation. We found that BLyS induced DCs to secrete high levels of IL-6 and moderate levels of IL-1β. In addition, we observed that BLyS stimulation resulted in very low levels of DC IL-23 secretion although it was quite variable between donors. Despite production of these three cytokines, BLyS matured DCs upon coculture with T cells did not induce the appearance of T cells expressing intracellular IL-17. Thus, it is possible that the levels of IL-6, IL-1β, and/or IL-23 induction by BLyS may not be sufficient for driving Th17 differentiation. Alternatively, high IFN-γ production by CD4 T cells activated by BLyS-DCs may inhibit Th17 differentiation as has recently been demonstrated in a study of human Th17 cells (68). However, it is possible that under different culture conditions, such as using directly purified DCs and/or using different lengths of coculture incubation time, a role for BLyS-DC in inducing IL-17 may be discovered. Studies of this nature are currently underway.

Another major focus of our investigation was to better understand the mechanism by which DCs respond to BLyS. When these studies were initiated, DCs were not known to express any of the three BLyS-binding receptors. Our studies very interestingly demonstrated that DCs constitutively express intracellular TACI, and it is principally localized in the intense perinuclear compartment, which may reflect the Golgi apparatus (Fig. 10E). This TACI localization pattern is strikingly distinct from that observed in B cells, which express the majority of this receptor in the plasma membrane (our unpublished data). Of note, DCs isolated from peripheral blood also showed intracellular localization of TACI within the same compartment (data not shown). Although one previous study demonstrated that BCMA is localized in the Golgi apparatus in the U266 myeloma cell line (67), to our knowledge, this is the first suggestion that preformed TACI exists in the Golgi in DCs. It has been shown that TNFR1 is expressed in the plasma membrane and complexes with the intracellular signaling molecule, TRADD, however, the primary subcellular location of TNFR1 is the trans-Golgi network (68). Recent other reports also showed that TLR4 is localized in the Golgi and recognizes internalized LPS in intestinal epithelial cells (69). In addition, neoplastic non-Hodgkin’s lymphoma and Hodgkin’s lymphoma B cells have been shown to express autocrine BLyS and APRIL, and TACI and BCMA expression may permit enhanced cell proliferation and survival by binding to autocrine or paracrine sources of BLyS and APRIL (70). These authors also showed evidence of intracellular TACI staining colocalized with BLyS, suggesting that intracellular TACI may bind to endogenous BLyS or internalized BLyS inside of the cells and signal in this manner (70). Because DCs exhibit a high level of endocytosis, it is possible that intracellular TACI may bind to internalized BLyS, thus activating DCs in an intracellular manner. However, it is also interesting to note that we were able to detect low level, punctate TACI expression on the cell membrane using a sensitive confocal microscope method. This staining pattern was reproducibly accentuated upon incubation of cells at 37°C (data not shown). The apparent temperature dependent increase in cell surface localized TACI is coincident with our observations that BLyS binding in DCs was only detectable by flow cytometry when we incubated cells with BLyS at 37°C, but not at 4°C (data not shown). Collectively, these two observations suggest the exciting possibility that during cell activation, cell surface TACI levels are rapidly increased by the transit from the Golgi to the cell surface, thereby resulting in an enhanced ability to bind BLyS. Such a process has been previously described for CD40L (71). In addition, previous studies have shown that the mature follicular DC (FDC) network is disrupted in BLyS−/− mice, but not in BAFFR null mice, suggesting FDCs need BLyS for their maturation possibly through TACI or BCMA, but not BAFFR (72, 73). Impaired production of lymphotixin β by B cells could be one possible mechanism accounting for the defective FDC network in BLyS−/− mice. However, our study suggests an alternative explanation that FDC may mature directly in response to BLyS in a TACI-dependent manner.

Because DCs are known to produce BLyS, it raises a question of whether endogenous BLyS binds to TACI and activates DCs in an autocrine manner. However, in the steady state, we did not observe DC activation in the absence of stimulation, implying that baseline levels of endogenous BLyS expression are insufficient to activate DCs. Because we demonstrate that exogenous BLyS activates DCs, it is possible that exogenous BLyS significantly enhances endogenous BLyS expression. To test this possibility, we used RT-PCR to measure BLyS mRNA levels following stimulation of DCs with BLyS, LPS, and IFN-γ as a positive control. In data not shown, although LPS and IFN-γ increased BLyS mRNA levels as expected, treatment with exogenous BLyS did not increase BLyS mRNA levels. Therefore, we suggest that the underlying mechanism is not interaction of endogenous BLyS with TACI, but rather reflects activation by exogenous BLyS.

DCs clearly play a pivotal role in shaping Th-mediated immunity. Environmental signals are also key in determining whether DCs orchestrate a Th1, Th2, and/or Th17 immune response. Moreover, DCs also modulate regulatory T cells to promote their homeostasis and hence induction of immune tolerance (5). In our study, we expand the knowledge of signals regulating DC function by demonstrating that BLyS strongly activates DCs to secrete inflammatory cytokines, IL-6, IL-1β, and TNF-α, as well as instructs DC to induce naïve CD4 T cell proliferation and differentiation into Th1 CD4 T cells. Thus, our observations support the speculation that elevated levels of BLyS may break peripheral tolerance via its ability to activate DCs in a manner that also results in...
promotion of a Th1 response, leading ultimately to Th1-type immune disorders.

Acknowledgments
We thank Dr. A. B. Dietz for discussions about DC generation and Drs. S. Chi and M. McNiven for kindly providing us with the anti-GM130 Ab and helpful discussions.

Disclosures
The authors have no financial conflict of interest.

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