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Excessive Production of IFN-γ in Patients with Systemic Lupus Erythematosus and Its Contribution to Induction of B Lymphocyte Stimulator/B Cell-Activating Factor/TNF Ligand Superfamily-13B

Masayoshi Harigai,1*,† Manabu Kawamoto,‡ Masako Hara,¶ Tetsuo Kubota,†§ Naoyuki Kamatani,¶ and Nobuyuki Miyasaka‡

Expression and immunological significance of IFN-γ, a pivotal cytokine in murine lupus, have not been clearly demonstrated in human systemic lupus erythematosus (SLE). In the present study we investigated the expression of IFN-γ in peripheral blood T cells from patients with SLE and its role in the production of the soluble B lymphocyte stimulator (sBLyS). Peripheral blood T cells from patients with SLE expressed significantly larger amounts of IFN-γ in response to stimulation with anti-CD3 mAb plus anti-CD28 mAb than those from normal controls as shown by three analytical methods, including ELISA, flow cytometry, and quantitative RT-PCR. The ratio of IFN-γ-producing T cells to effector memory T cells in CD3+/CD4+ and CD3+/CD8+ populations in patients with SLE was significantly higher than that of normal controls. The T-box-activated effector memory T cells from patients with SLE contained significantly larger amounts of sBLyS-inducing activity than normal controls; this was almost completely inhibited by the addition of anti-human IFN-γ mAb. Percentages of BLyS-expressing peripheral blood monocytes in patients with SLE were significantly higher than those of normal controls. Monocytes from patients with SLE produced significantly larger amounts of sBLyS in response to IFN-γ than those from normal controls. Taken together, these data strongly indicate that the overexpression of IFN-γ in peripheral blood T cells contributes to the immunopathogenesis of SLE via the induction of sBLyS by monocytes/macrophages, which would promote B cell activation and maturation. The Journal of Immunology, 2008, 181: 2211–2219.

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that is characterized by the production of various kinds of autoantibodies by activated B lymphocytes in vivo. Although the immunopathogenesis of SLE is not completely understood, the altered production of and/or response to cytokines and chemokines have been proposed to be key phenomena.

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which are classified according to the expression of the CD45RA Ag found on all human B and NK cells and subsets of human T cells and CCR7. Central memory T cells (T<sub>EM</sub>), CD3<sup>+</sup> CD45RA<sup>-</sup>CCR7<sup>+</sup> cells, home to T cell areas of secondary lymphoid organs and have only minimal cytokine-secreting capacity, whereas effector memory T cells (T<sub>EM</sub>)<sub>TEM</sub>, CD3<sup>+</sup> CD45RA<sup>-</sup>CCR7<sup>+</sup> cells, migrate to inflamed peripheral tissues and display immediate effector functions, including the secretion of cytokines such as IFN-γ, IL-4, and IL-5 within hours following antigen stimulation. CD8<sup>+</sup> T cells possess another effector memory subset named T<sub>EM</sub>RAM, which is CD3<sup>+</sup> CD8<sup>+</sup> CD45RA<sup>-</sup>CCR7<sup>-</sup> (14). A deviation in memory T cell differentiation has been demonstrated in patients with SLE (15–17), but its relationship to the secretion of cytokines has not been determined in this disease.

B lymphocyte stimulator (BlyS; also known as B cell-activating factor (BAFF)/TNF ligand superfamily-13B (TNFSF-13B)), a type II transmembrane protein and a member of the TNF family, is a critical regulator of B cell proliferation, differentiation, and survival (18–23). The molecule is expressed on the surface of monocytes, dendritic cells, neutrophils, stromal cells, and activated T cells and forms homotrimers or heterotrimers with a proliferation-inducing ligand (24, 25). The extracellular domain of BlyS, which is cleaved by a furin proteinase and released into body fluids such as serum or cerebral spinal fluid, is called soluble BlyS (sBlyS) (21). BlyS transgenic mice developed a lupus-like syndrome (24, 26, 27), whereas the blockade of the BlyS signal provided a therapeutic benefit to lupus model mice (24, 28, 29). Increased levels of serum sBlyS (30–32) as well as BlyS mRNA (31) of peripheral blood leukocytes have been reported in patients with SLE. Although the expression of BlyS is enhanced by treatment with IFN-α, IFN-γ, or CD154 in dendritic cells or macrophages (33, 34), the mechanism of the overexpression of BlyS in SLE has not been completely understood.

In the present study we demonstrated the increased expression of IFN-γ, an imbalance of master transcriptional factors for production of this cytokine, the contribution of T<sub>EM</sub> subsets to the increased production of IFN-γ, the involvement of IFN-γ in the induction of BlyS by monocytes, and the enhanced production of BlyS by monocytes in response to IFN-γ in patients with SLE. The present study strongly indicated the increased production of IFN-γ and its association with the immunopathogenesis of human SLE.

**Materials and Methods**

**Patients**

We enrolled 50 patients with SLE (47 females and three males) and 35 normal controls (32 females and three males) into the present study. All SLE patients fulfilled the SLE classification criteria of the American College of Rheumatology (Atlanta, GA). The disease activity of the SLE patients was evaluated using the systemic lupus erythematous disease activity index (SLEDAI). The SLEDAI scores (mean ± SE) and the disease duration (mean ± SE) of the patients with SLE were 5.5 ± 6.9 and 7.4 ± 7.2 years, respectively. Twenty-five patients with SLE had active disease (a SLEDAI score > 4) (16). Treatments for the patients with SLE were prednisolone (n = 39, mean ± SE, 11.1 ± 11.3mg/day) and azathioprine (n = 4). Peripheral blood samples were obtained with the written informed consent from all participating individuals. The Helsinki Declaration and the ethical guidelines for clinical research in Japan were followed throughout the study and the study protocol was approved by the ethical committee of the Tokyo Medical and Dental University Hospital (Tokyo, Japan).

**Abs and other reagents**

Anti-CD3 mAb and anti-CD28 mAb were purchased from Immunotech. Allophycocyanin-anti-CD3 mAb, allophycocyanin-anti-CD4 mAb, PE-cyanine 7 (PC7)-anti-CD4 mAb, PC7-anti-CD8 mAb, PE-anti-CD8 mAb, allophycocyanin-anti-CD14 mAb, FITC-anti-CD45RA mAb, FITC-anti-human IFN-γ mAb, PE-anti-human IL-4 mAb, and isotype-matched fluorochrome-conjugated control mAbs of the above mAbs were purchased from Beckman Coulter. PE-anti-CCR7 mAb, anti-CCR7 mAb, goat anti-human IFN-γ Ab, and normal goat IgG were purchased from R&D Systems. Anti-IFN-α mAbs were purchased from PBL Biomedical Laboratories. FITC-conjugated anti-human BlyS mAb and anti-human IL-10 mAb were purchased from Abcam.

**Cell preparations**

PBLs were separated by the centrifugation of heparinized blood over a Ficoll-Conray gradient. T cells were isolated by negative selection from PBLs using the Pan T cell isolation kit (Miltenyi Biotec) according to the manufacturer’s protocol. Monocytes were further depleted from negatively selected T cells using anti-CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer’s protocol. Monocytes were isolated by positive selection from PBL using anti-CD14 MicroBeads.

**Stimulation of T cells**

Peripheral blood T cells were stimulated with immobilized anti-CD3 mAb plus anti-CD28 mAb (CD28 costimulation). Anti-CD3 mAb (0.1 μg/ml in PBS) was bound to flat-bottom, 96-well microtiter plates (Iwaki) or 24-well culture plates (Iwaki) by incubation overnight at 4°C. Anti-CD28 mAb (2 μg/ml) was added directly to medium. Preliminary experiments showed that immobilized anti-CD3 mAb alone at 0.1 μg/ml induced a modest proliferation of peripheral blood T cells, whereas immobilized anti-CD3 mAb plus anti-CD28 together provided full-activation (data not shown). T cells (2 × 10<sup>5</sup> cells/well) were cultured in flat-bottom, 96-well microtiter plates with CD28 costimulation for 72 h in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). Culture supernatants were collected to determine IFN-γ production and BlyS-inducing activity (see below). T cells (5 × 10<sup>3</sup> cells/0.5ml/well) were also cultured in 24-well culture plates to quantify IFN-γ mRNA and intracellular IFN-γ (see below).

**Stimulation of monocytes**

Monocytes were cultured in 96-well, flat-bottom plates for 72 h with various concentrations of recombinant human IFN-γ (rhIFN-γ; 0, 2, 10 ng/ml) (R&D Systems) or with rhIFN-α (100 IU/ml) in RPMI 1640 supplemented with 10% FCS, penicillin (100U/ml), and streptomycin (100 μg/ml). sBlyS in these culture supernatants from human monocytes or THP-1 cells was measured using an ELISA system (R&D Systems) according to the manufacturer’s instructions. The sensitivity of the ELISA kit was 0.73 pg/ml.

**Flow cytometry**

Multicolor analysis was performed using the FACScanLibur flow cytometry system (BD Biosciences). Cells were washed three times in ice-cold FC M buffer (PBS, 0.1% BSA, and 0.1% sodium azide) and incubated on ice for 5 min with 10 μg of purified mouse IgG (Chemicon) to block nonspecific IgG binding. Cells were then incubated at 4°C with saturating amounts of the fluorochrome-conjugated (i.e., FITC-, PE-, PC7-, or allophycocyanin-conjugated) mAbs. For simultaneous staining of cell surface molecules and intracellular cytokines, T cells (5 × 10<sup>3</sup> cells/0.5ml/well) were stained with CD28 costimulation in the presence of brefeldin A (GolgiPlug; BD Biosciences) for 6 h and stained with fluorochrome-conjugated mAbs against cell surface molecules, then the cells were permeabilized using the IntraPrep kit (Beckman Coulter) and stained with FITC-anti-IFN-γ mAb and/or PE-anti-IL-4 mAb. To detect expression of BlyS on the cell surface of peripheral blood monocytes, PBLs were stained with PE-conjugated anti-CD14 mAb and FITC-conjugated anti-BlyS mAb.

**ELISA for IFN-γ**

IFN-γ production in the culture supernatants was measured using an ELISA kit according to the manufacturer’s protocol (GE Healthcare). The sensitivity of the ELISA kit was 0.63 pg/ml.

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Quantitative RT-PCR

Total RNA was extracted using TRizol (Invitrogen) from freshly isolated T cells (1 x 10^6) or T cells (5 x 10^5 cells/0.5 ml/well) stimulated with CD28 costimulation for 4 h and reverse transcribed to cDNA using SuperScript II RNase H (Invitrogen). One microliter of the cDNA was used as a template for quantitative PCR, which was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems) with the comparative threshold cycle (ΔΔCt) method according to the instructions of the manufacturer. Primers and probes for quantitative RT-PCR of human GAPDH and IFN-γ and the transcription factors GATA-binding protein-3 (GATA-3) and T-box expressed in T cells (T-bet), were also purchased from Applied Biosystems.

sBLyS-inducing activity

Human monocytes or cells of the human monocytic cell line THP-1 (1 x 10^6/200 μl/well) were cultured in 96-well, flat-bottomed plates for 72 h with various concentration of rhIFN-γ (0–10 ng/ml) (R&D Systems) or T cell culture supernatants (0–30%) used in 10% FCS-RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-ME (50 μM) (Invitrogen). In some experiments, T cell culture supernatants were incubated with goat anti-human IFN-γ IgG or control goat IgG (both used at 5.0 μg/ml), (R&D Systems) at 4°C for 30 min before being added to human monocytes or THP-1 cells. sBLyS in these culture supernatants from human monocytes or THP-1 cells was measured using an ELISA.

Statistics

Differences between groups were evaluated using the Mann-Whitney U test. Paired samples were analyzed using the Wilcoxon rank sum test. Correlations were analyzed using the Pearson’s correlation coefficient. Values of p ≤ 0.05 were considered significant. Statistical analysis was performed using SPSS 15.0.

Results

Overproduction of IFN-γ by peripheral blood T cells in SLE patients with CD28 costimulation

As an initial experiment, we compared the expression of IFN-γ at the protein level in the patients with SLE and in normal controls and found a significant difference. Peripheral blood T cells were cultured with CD28 costimulation for 72 h and IFN-γ in the culture supernatants was measured using an ELISA. A–C, T cells (5 x 10^5 cells/0.5 ml/well) from patients with SLE (n = 27) and normal controls (NC) (n = 21) were cultured with CD28 costimulation in the presence of brefeldin A for 6 h, stained with the mAbs allophycocyanin-anti-CD3, PC7-anti-CD4, PC7-anti-CD8, PE-anti-CD8, and FITC-anti-IFN-γ, and analyzed using flow cytometry. The percentages of IFN-γ-producing cells among CD3^+ T cells (A), CD3^+CD4^+ T cells (B), or CD3^+CD8^+ T cells (C) were calculated. Lines inside the boxes represent the median value for each group. The boxes include the 25th–75th percentiles, bars outside the boxes represent the 10th and 90th percentiles, and open circles represent values beyond the 10th and 90th percentiles. p values were calculated using the Mann-Whitney U test.

Using intracellular cytokine staining as described in Materials and Methods, we next determined which T cell subset, CD4^+ or CD8^+, produced IFN-γ in response to CD28 costimulation. Percentages of IFN-γ-producing CD3^+ T cells in patients with SLE

Both CD4^+ and CD8^+ T cells contribute to the overproduction of IFN-γ in patients with SLE

FIGURE 2. Contribution of both CD4^+ and CD8^+ T cells to the over-production of IFN-γ in patients with SLE. A–C, T cells (5 x 10^5 cells/0.5 ml/well) from patients with SLE (n = 27) and normal controls (NC) (n = 21) were cultured with CD28 costimulation in the presence of brefeldin A for 6 h, stained with the mAbs allophycocyanin-anti-CD3, PC7-anti-CD4, PC7-anti-CD8, PE-anti-CD8, and FITC-anti-IFN-γ, and analyzed using flow cytometry. The percentages of IFN-γ-producing cells among CD3^+ T cells (A), CD3^+CD4^+ T cells (B), or CD3^+CD8^+ T cells (C) were calculated. Lines inside the boxes represent the median value for each group. The boxes include the 25th–75th percentiles, bars outside the boxes represent the 10th and 90th percentiles, and open circles represent values beyond the 10th and 90th percentiles. p values were calculated using Mann-Whitney U test in A–C. D, Correlation between the concentration of IFN-γ in the T cell culture supernatants (Fig. 1B) and the percentage of IFN-γ-producing cells among CD3^+ T cells were shown (SLE, n = 23; normal controls, n = 20; total, n = 43). The correlation was analyzed using the Pearson correlation coefficient.
Table I. Percentages of memory T cell subsets in patients with SLE

<table>
<thead>
<tr>
<th>Memory T Cell Subsets</th>
<th>SLE (n = 27)</th>
<th>Normal Controls (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺CD4⁺CD45RA⁺CCR7⁻/CD3⁺CD4⁺</td>
<td>32.3 (25.1–41.1)</td>
<td>29.9 (25.1–37.0)</td>
</tr>
<tr>
<td>CD3⁺CD4⁺CD45RA⁺CCR7⁻/CD3⁺CD4⁺</td>
<td>26.1 (15.2–40.8)</td>
<td>22.1 (18.6–28.3)</td>
</tr>
<tr>
<td>CD3⁺CD8⁺CD45RA⁺CCR7⁻/CD3⁺CD8⁺</td>
<td>3.9 (3.0–7.7)</td>
<td>4.6 (2.0–6.2)</td>
</tr>
<tr>
<td>CD3⁺CD8⁺CD45RA⁺CCR7⁻/CD3⁺CD8⁺</td>
<td>24.1 (14.6–37.1)</td>
<td>27.3 (18.9–33.2)</td>
</tr>
<tr>
<td>CD3⁺CD8⁺CD45RA⁺CCR7⁻/CD3⁺CD8⁺</td>
<td>30.3 (14.9–38.1)</td>
<td>18.3 (10.9–23.5)</td>
</tr>
<tr>
<td>CD3⁺CD8⁺CCR7⁻/CD3⁺CD8⁺</td>
<td>57.5 (34.9–77.2)</td>
<td>51.5 (36.4–57.4)</td>
</tr>
</tbody>
</table>

*Peripheral blood T cells were stained with the allophycocyanin-anti-CD3, PC7-anti-CD4, PC7-anti-CD8, PE-anti-CCR7, and FITC-anti-CD45RA mAbs and analyzed using flow cytometry. Data were expressed as median and 25–75 percentiles (in parentheses) for each subset. There was no statistical difference between the two groups.

(27) Normal Controls (n = 21) (p = 0.001; Mann-Whitney U test), which was compatible with the results of Fig. 1 (Fig. 2A). Percentages of IFN-γ-producing CD4⁺ T cells as well as CD8⁺ T cells in patients with SLE were significantly higher than those in normal controls (p = 0.028 for CD4⁺ T cells and p = 0.001 for CD8⁺ T cells, Mann-Whitney U test) (Fig. 2, B and C). We found a significant and strong positive correlation between the percentages of IFN-γ-producing CD3⁺ T cells and IFN-γ in the culture supernatants of T cells (R² = 0.518; p < 0.001) (Fig. 2D).

Memory T cell subsets in patients with SLE

Recent studies have identified several memory T cell subsets. Among them, TEM cells are considered to produce IFN-γ, IL-4,
and IL-5 within hours following antigenic stimulation. Because our flow cytometry analysis revealed an increased percentage of IFN-γ-producing T cells at 6 h after CD28 costimulation in patients with SLE, we postulated that there was a skewed distribution of memory T cell subsets; this was investigated in the same patients (n = 27) and normal controls (n = 21) that were used for the intracellular cytokine-staining experiments described above. As has been previously reported (15), the patients with SLE showed a significantly increased percentage of a CD3+CD4+ memory T cell population (CD3+CD4+CD45RA−/CD3−CD4+) than normal controls (median (25–75 percentiles): SLE, 66.3 (52.6–75.2); normal control, 55.8 (47.3–60.3); p = 0.014). However, there was no significant difference between the patients with SLE and normal controls in the percentages of CD3+CD4+CD45RA−CCR7+/CD3−CD4+, CD3+CD4−CD45RA−CCR7−/CD3−CD4+, CD3+CD8−CD45RA−CCR7−/CD3−CD8−, CD3+CD8−CD45RA−CCR7+/CD3−CD8−, and CD3−CD8−CCR7+/CD3+CD8− cells (Table I).

We next analyzed the correlation between the percentages of IFN-γ-producing T cells and TEM cells. We found a significant positive correlation between the percentages of IFN-γ-producing CD3+CD4+ T cells and CD3+CD4+CD45RA−CCR7− T cells (i.e., TEM), as well as between those of IFN-γ-producing CD3+CD8+ T cells and CD3+CD8−CCR7+ T cells (i.e., TEM plus TEmRA), (R² = 0.62, p < 0.001 for CD4+ T cells; R² = 0.27, p < 0.001 for CD8+ T cells) (Fig. 3, A and B); this was compatible with the strong and major contribution of memory T cells to the expression of IFN-γ immediately after stimulation. There was no significant correlation between the percentages of IFN-γ-producing CD3+CD4+ T cells and CD3+CD4+CD45RA−CCR7+ T cells or those of IFN-γ-producing CD3+CD8+ T cells and CD3+CD8−CD45RA−CCR7+ T cells (i.e., TEmRA) (data not shown). We therefore calculated the ratio of the percentage of IFN-γ-producing T cells to that of the TEmRA cells (i.e., TEm for CD3+CD4+ T cells, TEm plus TEmRA for CD3+CD8+ T cells) as an indicator for the fraction of IFN-γ-producing T cells among the TEM cells. The ratios in the patients with SLE were significantly higher than those of normal controls in both CD4+ and CD8+ T cells (p = 0.006 for CD4+ T cells, p = 0.002 for CD8+ T cells; Mann-Whitney U test) (Fig. 3, C and D). These differences remained statistically significant when we excluded the SLE patients who had a ratio >1.0 from Fig. 3, C and D (one and three patients, respectively; p = 0.010 and p = 0.009 for Fig. 3, C and D, respectively). Increased expression of IFN-γ in the TEm cells was confirmed in negatively selected CCR7+ T cells. CCR7+ T cells were stained with allophycocyanin-anti-CD4 mAb, PC7-anti-CD8 mAb, FITC-anti-IFN-γ mAb, and PE-anti-IL-4 mAb and analyzed using flow cytometry. Representative results, which were in concordance with Fig. 3, C and D, were shown in Fig. 3, E–J.

**Increased T-bet mRNA/GATA-3 mRNA ratio of peripheral blood T cells in patients with SLE**

The differentiation of T cells into Th1 or Th2 type is under the control of the master transcriptional factors T-bet and GATA-3 (35). Because IFN-γ is a prototype of a Th1 cytokine, we quantified mRNA of these transcriptional factors in freshly isolated peripheral blood T cells and calculated the T-bet mRNA/GATA-3 mRNA ratio in each patient with SLE (n = 17) or in normal controls (n = 14). The T-bet mRNA/GATA-3 mRNA ratio was significantly higher in patients with SLE; this was compatible with the overproduction of IFN-γ in response to CD28 costimulation (p < 0.0005; Mann-Whitney U test) (Fig. 4).

**FIGURE 4.** Increased T-bet mRNA/GATA-3 mRNA ratio of peripheral blood T cells in patients with SLE. Total RNA was extracted from freshly isolated peripheral blood T cells and reverse transcribed to cDNA. T-bet, GATA-3, and GAPDH mRNA were quantified using the ABI PRISM 7900HT sequence detection system. The ratios of T-bet mRNA to GATA-3 mRNA were compared between the patients with SLE (n = 17) and normal controls (NC) (n = 14). The lines inside the boxes represent the median value for each group. The boxes include 25th–75th percentiles, bars outside boxes represent the 10th and 90th percentiles, and open circles represent values beyond the 10th and 90th percentiles. p values were calculated using the Mann-Whitney U test.

**Increased T cell-derived sBLyS-inducing activity in patients with SLE**

To obtain insight into the pathological significance of IFN-γ in SLE, we investigated its contribution to the production of sBLyS by human monocytes or human monocytic cell line THP-1 cells. rhIFN-γ dose-dependently induced production of sBLyS in human monocytes as well as in THP-1 cells. Therefore, we used THP-1 cells in the following experiments. The sBLyS-inducing activity was significantly inhibited by preincubation of rhIFN-γ with anti-IFN-γ IgG but not by control IgG (Fig. 5A). T cell culture supernatants from a normal control also increased the production of sBLyS by THP-1 cells in a dose-dependent fashion. The sBLyS-inducing activity of the T cell culture supernatant was almost completely inhibited by the addition of anti-IFN-γ IgG but not by control IgG in both normal controls and patients with SLE (Fig. 5, A and B). The addition of anti-IFN-α mAb or anti-IL-10 mAb did not inhibit the sBLyS-inducing activity of the T cell culture supernatant (data not shown). Using this assay system, we measured the sBLyS-inducing activity of T cell culture supernatants from patients with SLE (n = 22) and normal controls (n = 16). T cell culture supernatants were used at 5% in this experiment. We could not perform this assay in some patients with SLE and normal controls because of the shortage of culture supernatants. T cell culture supernatants from the patients with SLE induced significantly larger amounts of sBLyS than those from normal controls (p = 0.048; Mann-Whitney U test) (Fig. 5C). We also measured sBLyS using an ELISA in the T cell culture supernatants diluted at 5% in medium (36), but the concentration of sBLyS was <10 pg/ml and was considered not to affect the measurement of sBLyS-inducing activity in our assay system. There was a significant and strong
correlation between the sBLyS-inducing activity and IFN-γ in the T cell culture supernatants ($R^2 = 0.82; p < 0.001$) (Fig. 5D).

**Increased expression of BLyS by peripheral blood monocytes in patients with SLE**

To evaluate relevance of the sBLyS-inducing activity on monocytes in patients with SLE, we quantified the expression of BLyS on freshly isolated peripheral blood monocytes. Representative results of a patient with SLE and a normal control are shown in Fig. 6A. Percentages of BLyS$^{+}$CD14$^+$/CD14$^-$ were significantly higher than those of normal controls ($p = 0.034$; Mann-Whitney U test) (Fig. 6B). Mean fluorescence intensities of BLyS in patients with SLE were also significantly higher than those of normal controls (median (range): SLE, 6.8 (4.1–18.0); normal, 4.6 (1.8–10.8); $p = 0.007$; Mann-Whitney U test). We found no significant difference in production of sBLyS by CD14$^+$ monocytes without stimulation. rhIFN-γ as well as rhIFN-α significantly enhanced production of sBLyS by CD14$^+$ monocytes in both normal control and patients with SLE ($p < 0.0001$ for both stimuli and for both group). CD14$^+$ monocytes from patients with SLE produced

**FIGURE 5.** sBLyS-inducing activity in T cell culture supernatants of patients with SLE. A and B, Peripheral blood T cells were cultured with CD28 costimulation and T cell culture supernatants were collected as described in Fig. 1. Human monocytes or THP-1 cells were cultured with rhIFN-γ (0–10 ng/ml) or T cell culture supernatants (at 0–30% in A and 5% in B) for 72 h; the levels of sBLyS in the culture supernatants were measured using an ELISA. In some experiments rhIFN-γ and T cell culture supernatants were preincubated for 30 min at 4°C with anti-IFN-γ IgG (hatched column) or control IgG (diagonal column) before the addition to the cells. Assays were performed in triplicate and SD values were <10% in all columns. ***, $p < 0.01$ by the Student $t$ test. C, THP-1 cells were cultured with T cell culture supernatants from the patients with SLE ($n = 22$) and normal controls (NC) ($n = 16$) at 5% for 72 h, and levels of sBLyS in these culture supernatants were measured using an ELISA. The lines inside the boxes represent the median value for each group. The boxes include the 25th–75th percentiles, bars outside boxes represent the 10th and 90th percentiles, and the open circles represent values beyond the 10th and 90th percentiles. $p$ values were calculated using the Mann-Whitney U test. D, The correlation of the concentration of sBLyS induced by the T cell culture supernatants in C and the concentration of IFN-γ in the T cell culture supernatants measured using ELISA (Fig. 1B) were plotted. The correlations were analyzed using the Pearson correlation coefficient.
percentages of BLyS-expressing cells in CD14+ blood T cells from patients with SLE expressed significantly larger amounts of sBLyS than those from normal controls; 2) overexpressed IFN-γ contributed to the excessive sBLyS-inducing activity in T cell culture supernatants of patients with SLE; and 3) monocytes from patients with SLE produced significantly larger amounts of sBLyS in response to the stimulation with rhIFN-γ than those from normal controls.

Because several investigators have reported conflicting results (10–13), we addressed the expression of IFN-γ by T cells from patients with SLE with various assay methods; these included an ELISA for IFN-γ protein in T cell culture supernatants, quantitative RT-PCR for IFN-γ mRNA, and an intracellular cytokine assay for IFN-γ. The excessive expression of IFN-γ by peripheral blood T cells after CD28 costimulation was consistently demonstrated in all of these assay systems. The increased T-bet mRNA/GATA-3 mRNA ratio also supported the skewed differentiation of peripheral blood T cells into IFN-γ-producing T cells. The differing results regarding the expression of IFN-γ among several reports, including ours, could be explained to some extent by differences in the methods of stimulation. Because we used CD28 costimulation that mimics stimulation by APCs, our results would better reflect the capacity of cytokine secretion by T cells in vivo than those obtained with artificial stimulation such as that achieved by PMA plus ionomycin stimulation (12), which bypasses the TCR/CD3 complex.

We found significant positive correlations between the percentages of IFN-γ-secreting T cells and Tem cells in both CD3+CD4+ and CD3−CD8+ T cells (Fig. 3, A and B); these results are compatible with the fact that Tem cells, but not Tem cells, have the capacity to secrete cytokines immediately after Ag stimulation. An increased percentage of CD8+ Tem cells (17) as well as that of CD4+ Tem cells (16) have been reported in patients with SLE. Although CD3+CD8+CD45RA−CCR7+ T cells (Temra) showed a tendency to increase (p = 0.07) in our patients with SLE, we could not observe such a drastic difference in the memory T cell population between SLE and normal controls even after separating the patients into active and inactive diseases (data not shown). Nevertheless, the ratio of IFN-γ-secreting T cells to Tem cells in both CD3+CD4+ and CD3−CD8+ T cells was significantly increased in patients with SLE (Fig. 3, C and D), suggesting the skewed differentiation of Tem cells into IFN-γ-secreting cells.

The mechanism of the preferential differentiation into IFN-γ-secreting T cells in patients with human SLE remains to be clarified. T-bet is a master transcriptional factor for the differentiation into IFN-γ-secreting T cells that cooperates with another transcriptional factor, Hlx (35, 37). The promoter analysis of the IFN-γ gene revealed several other relevant transcriptional factors, including AP-1, AP-2, NFAT1, ATF, and NF-κB (38–40). Wong et al. (41) has demonstrated a decreased NF-κB activity of T cells after CD28 costimulation in patients with SLE; therefore, transcriptional factors other than NF-κB might be more important for the overexpression of IFN-γ in SLE. In addition to the increased T-bet mRNA/GATA-3 mRNA ratio that was demonstrated in the present study, the expression of these transcriptional factors should be investigated in the future.

BLyS is a critical regulator of B cell maturation and survival. The immunopathological significance of this cytokine has been implicated by increased levels of serum sBLyS (30–32) and BLyS mRNA (31) in the peripheral blood leukocytes of patients with SLE, which are in concordance with the results of the present study. A recent study also demonstrated that BAFF-R, one of the receptors for BLyS, was occupied in patients with SLE (42), suggesting the contribution of this cytokine to disease mechanisms in SLE. Despite these intriguing findings, how BLyS is overexpressed in SLE has not been completely understood and, to our
knowledge, this is the first study that demonstrated the major contributions of IFN-γ to the sBLyS-inducing activity of T cells. The present data suggest that T cells activated by APCs (i.e., macrophages and dendritic cells) produce excessive amounts of IFN-γ in patients with SLE and stimulate sBLyS production by APCs, which in turn would activate B cells (18–23) as well as monocytes (43). The higher production of sBLyS in response to rhIFN-γ by monocytes from the patients with SLE compared with those from normal controls further support this scenario. Expression of BLyS by PBMCs in response to IFNs has been reported in patients with primary Sjögren’s syndrome (pSS) (44). Expression of BLyS mRNA in PBMCs from patients with pSS after IFN-α stimulation, but not IFN-γ stimulation, was significantly higher than that from normal controls, and a similar trend was observed in peripheral blood monocytes. These data indicate that different IFNs, IFN-α and IFN-γ, are relevant to the production of BLyS in patients with pSS and SLE, respectively. It has recently been shown that BLyS activates T cells as a costimulatory molecule (45). The costimulation of T cells by BLyS indicates that T cell-induced BLyS on APCs further activates T cells and contributes to the vicious cycle activated T cells, these two molecules may cooperate to induce BLyS during T cell-Ag-presenting cell interaction.

In summary, the present study clearly demonstrates the increased expression of IFN-γ in patients with SLE. IFN-γ, together with other soluble factors and surface molecules, would stimulate APCs to produce BLyS. Because this immunological abnormality is a common feature of both human and mouse lupus and because targeting therapy for IFN-γ has been successfully applied to lupus mice (5), treatment with humanized anti-IFN-γ mAb or recombinant IFN-γ-Ig fusion protein would provide a novel therapeutic strategy for this intractable disease.

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Disclosures
The authors have no financial conflict of interest.

References


