Decreased Production of TGF-β by Lymphocytes from Patients with Systemic Lupus Erythematosus

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TGF-β has marked inhibitory effects on the immune system but also serves as a costimulatory factor in the development of T cells with down-regulatory activities. This cytokine is secreted as a latent complex and converted extracellularly to its active form. We have recently learned that anti-CD2 is a potent inducer of lymphocyte-derived TGF-β and that NK cells are the predominant source. The objective of this study was to compare levels of constitutive, anti-CD2-induced and cytokine-regulated TGF-β produced by blood lymphocytes from patients with systemic lupus erythematosus (SLE) in comparison with healthy controls. Using a highly sensitive and specific bioassay to assess TGF-β, we report that unstimulated PBL from SLE patients, especially the NK cell subset, produced decreased levels of active TGF-β. In response to anti-CD2, concentrations of active and total TGF-β were also decreased in SLE. After learning that IL-2 and TNF-α enhance lymphocyte production of active TGF-β, we found that the addition of these cytokines was unable to increase active TGF-β to normal concentrations. Although we observed that IL-10 inhibited the production of active TGF-β, antagonism of this cytokine was unable to completely correct the defect. In two SLE patients with B cell hyperactivity, spontaneous IgG production was almost abolished by the combination of TGF-β and IL-2. Therefore, decreased production of each of these cytokines in SLE could be important in the perpetuation of B cell hyperactivity.

clinic. Most of the hospitalized patients were untreated before admission and were studied before they received corticosteroids. Outpatients were receiving <20 mg of prednisone, and none were receiving cytotoxic drugs. Disease activity was assessed with the SLAM (20) and SLEDAI (21) indices with mean values of 9.2 and 10.2, respectively. Healthy donors served as controls and were matched as closely as possible for age, sex, and ethnic group.

Reagents
Antibodies used were anti-CD2 (OKT11, American Type Culture Collection (ATCC), Rockville, MD, and GT2 made available by Dr. Alain Bernard, Nice, France) (22); anti-CD3 (454, a gift from Dr. William Stohl, Los Angeles, CA) (23); anti-CD74 (L243, ATCC, MD); rTGF-β and anti-TGF-β (1D11.16), a murine IgG1, were kindly provided by Dr. Bruce Pratt (Genentech, South San Francisco, CA) (24). Anti-CD16 (3G8) was provided by Dr. Jay Unkeless, New York, NY. TNF-α and IFN-γ were purchased from R&D Systems, Minneapolis, MN. IL-2 was purchased from Cetus, Emeryville, CA. IL-10 was kind gift from Satwant Narula (Schering Plough Pharmaceuticals, Kenilworth, NJ), as was anti-IL-10 (JES3–19F1) (25) and control rat IgG2a.

Isolation of blood mononuclear cells
PBMC were prepared from heparinized venous blood by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. The mononuclear cells were washed in PBS with 5 mM EDTA (Life Technologies, Grand Island, NY) to remove platelets, which are a rich source of TGF-β (26). Lymphocytes and monocytes were separated from PBMC by centrifugation through a continuous Percoll (Pharmacia) density gradient (27). The percentage of monocytes remaining in the high density, lymphocyte-enriched fraction was somewhat higher in SLE (8.5% vs 4.3%). The percentage of lymphocytes remaining in the monocyte-enriched low density cells was similar in SLE and controls (20.4 vs 21.3%).

NK and T cells were prepared as described previously (4). PBL were immediately rosetted with 2-aminoethylisothiouronium bromide-treated SRBC (28). The nonrosetting cells were then incubated with anti-CD3 and anticyCD74 (anti-HLA-DR) Abs on ice and depleted of reacting cells using immunomagnetic beads (Dynal, Great Neck, NY). The percentage of CD56+ cells in this fraction was similar in SLE and controls (84.0 vs 83.2%). T cells were prepared from rosetting cells by negative selection following depletion of CD16+ and CD74+ cells also using immunomagnetic beads (Dynal). The percentage of CD3+ cells in this fraction was usually >95%.

Cell culture procedures
Procedures for cell cultures have been described previously (4). In brief, 1 × 10⁶ of the various mononuclear cell populations were added to the wells of 96-well flat-bottom microtiter plate (Greiner Rocky Mountain Scientific, Salt Lake City, UT). These plates were selected following a comparison of the nonspecific binding of TGF-β to commonly used tissue culture plates. The lymphocytes were suspended in AIM V serum-free medium (Life Technologies) since serum contains significant amounts of TGF-β. Some lymphocytes were stimulated with the optimal concentrations of anti-CD2 to induce TGF-β production (GT2 1:40 and T11 1:80) hydridoma culture supernatants.

TGF-β assay
Mink lung epithelial cells (MLEC) that had been transfected with an expression construct containing a plasmagen activator inhibitor (PAI-1) promoter fused to luciferase reporter gene were kindly provided by D. B. Rifkin, New York, NY. The procedure described by this group was used to assay TGF-β (28). MLEC (2 × 10⁶/well) were incubated with 200 µl of supernatant for 18 h at 37°C. To assay for luciferase activity, MLEC were lysed by a cell lysis reagent (Analytical Luminescence, Ann Arbor, MI). Cell lysates were then reacted with assay buffer and luciferin solution (both from Analytical Luminescence) immediately before being measured in a luminometer (Lumat, Berthold Analytical Instruments Inc., Nashua, NH). To measure total TGF-β activity, the samples were heated at 80°C for 3 min to release the active cytokine from the latent complex. To measure active TGF-β activity, the supernatants were examined without heating. In all assays, several concentrations of rTGF-β were included to generate a standard curve. The variability of triplicate cultures was <10% of the mean value.

Table I. Specificity of the TGF-β assay

<table>
<thead>
<tr>
<th>Exp</th>
<th>Anti-CD2</th>
<th>Medium (pg/ml)</th>
<th>Anti-TGF-β (pg/ml)</th>
<th>mIgG1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>229</td>
<td>&lt;5</td>
<td>279</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>593</td>
<td>&lt;5</td>
<td>284</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>210</td>
<td>&lt;5</td>
<td>300</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>445</td>
<td>&lt;5</td>
<td>288</td>
</tr>
</tbody>
</table>

* PBL (1 × 10⁵) were incubated with or without anti-CD2 for 48 h, and the supernatants were harvested. Anti-TGF-β (10 µg/ml) or an equivalent amount of control mouse IgG1 was added, and the supernatant was examined for total TGF-β activity using mink cells (MLEC) with a luciferase construct.

Possible effects of TGF-β and IL-2 on spontaneous Ig production in SLE
PBMC (2 × 10⁵) from patients with active SLE were cultured in serum-free (AIM V) culture medium in the wells of a 96-well flat-bottom microtiter plate. For the first 3 days, the PBMC were cultured at 37°C in 5% CO₂ in a humidified incubator with or without IL-2 (10 U/ml) and/or TGF-β (10 pg/ml). The medium was then removed, and after the cells were washed, fresh serum-free medium was added, and the cells were cultured for 7 more days. The supernatants were harvested and assayed for IgG content by an ELISA, as described previously (29).

Statistical analysis
The significance of the results was analyzed by Student’s t test or the Mann-Whitney test performed using GBSTAT software (Professional Statistics and Graphics Computer Program, Dynamic Microsystems Inc., Silver Spring, MD).

Results
Production of TGF-β by SLE and control lymphocytes
To verify the specificity of the TGF-β bioassay used, we added a neutralizing anti-TGF-β mAb to the culture supernatants of unstimulated and anti-CD2-stimulated PBL. In the four experiments shown in Table I, this procedure abolished >98% of the mink cell response.

We first compared lymphocytes from control donors and SLE patients for their ability to produce total TGF-β either constitutively or after stimulation with anti-CD2 (Fig. 1A). Constitutive production of total TGF-β by control PBL was somewhat more than that of patient lymphocytes (623 ± 107 vs 385 ± 98 pg/ml). The response to anti-CD2 by control PBL was significantly more vigorous than SLE PBL (1289 ± 224 vs 586 ± 99, p = 0.03). NK cells were the principal source of this cytokine. During this 48-h culture period, production of total TGF-β by T cells was barely detectable. Although we had an insufficient amount of blood from SLE patients to prepare B cells, the amount of total TGF-β produced by this lymphocyte subset from healthy donors was generally <200 pg/ml (see Ref. 10, Table IV).

Although most constitutive, lymphocyte-derived TGF-β is in the latent form, the biologically active form was also detectable. The amount of active TGF-β from control donors’ and SLE patients’ lymphocytes is shown in Fig. 1B. Similar to levels of the latent complex, active TGF-β detected in culture supernatants from controls was approximately twice as much as that in SLE patients. As with total TGF-β, NK cells were the principal source of active TGF-β, and control NK cells produced significantly more than those from SLE patients (98 ± 37 vs 39 ± 13 pg/ml, p = 0.01). Active TGF-β from T cells was not detectable, and that from
control B cells was only barely detectable (results not shown; see Ref. 10, Table V).

The response to stimulation with anti-CD2 was significantly greater in controls ($p < 0.001$). Again NK cells appeared to be the predominant source of this cytokine. However, since the relative increase by NK cells was less than that of PBL, other lymphocyte populations might contribute to the extracellular conversion of latent to active TGF-β. Production of this cytokine by T cells was negligible during this interval. Thus, defects in both total and active TGF-β were documented in SLE that could be largely attributed to NK cells.

Since monocytes are a major source of TGF-β, we compared the production of the total and active form in SLE and controls (8, 9). As shown in Table II, there were no significant differences in the two groups.

We next investigated the kinetics of lymphocyte TGF-β production by measuring TGF-β activity over several days. The results in Figure 2 show that SLE production of TGF-β was always lower than that of control donors. Most striking was the difference in the production of total and active TGF-β following stimulation with anti-CD2, a difference that increases with time.

### Cytokine effects on the production of active TGF-β

It has been established that blood mononuclear cell production of IL-2, TNF-α, and IFN-γ is decreased in SLE and that IL-10 production is increased (14–18). We selected, accordingly, these four cytokines for study. PBL from healthy controls were incubated for 48 h with doses over a 3-log concentration range. Figure 3 shows that IL-2 and TNF-α increased the production of active TGF-β in a dose-dependent manner. IFN-γ, however, had no effect, and IL-10 was suppressive.
On the basis of these results, we selected five SLE patients with active disease who previously demonstrated decreased TGF-β production in response to anti-CD2. As before, the response to anti-CD2 in SLE was markedly less than that by controls (Table III). In this small group, constitutive production of active TGF-β was significantly decreased in SLE. The addition of IL-2 and TNF-α proportionally increased levels of active TGF-β in both groups but was unable to correct the defect.

Since IL-10 decreased levels of active TGF-β and production of this cytokine is increased in SLE (17, 18), we investigated the effect of antagonizing spontaneously produced IL-10 with a neutralizing mAb. In this subgroup of SLE patients, constitutive active TGF-β was again significantly decreased (Fig. 4). The addition of anti-IL-10 increased active TGF-β in both SLE and controls, and this effect was relatively greater in SLE so that the differences between these two groups were no longer significant. Following anti-CD2 stimulation of PBL, anti-IL-10 had no effect on TGF-β activity. Since monocytes are the principal source of IL-10, PBMC were cultured in parallel with PBL in three experiments with similar results (not shown).

Possible significance of decreased TGF-β on B cell hyperactivity in SLE

In view of recent evidence that TGF-β is an important costimulatory factor in the development of T suppressor cells (4), the decreased amounts of active TGF-β in SLE we have documented might contribute to the inability of CD8+ T cells to down-regulate B cell activity. We have recently observed that a brief exposure of CD8+ T cells from healthy individuals to TGF-β and IL-2 enables them to down-regulate Ig production (Figure 6 in Ref. 10). We considered that similar exposure of SLE lymphocytes to these cytokines might condition them to down-regulate spontaneous IgG production. Examples of this cytokine-mediated suppression of Ig production are shown in Figure 5. PBMC from two SLE patients were exposed to IL-2 (10 U/ml), TGF-β (10 pg/ml), or both of these cytokines for 72 h and subsequently cultured for an additional 7 days. In case 1, neither IL-2 nor TGF-β alone had significant effects, whereas in case 2 each of these cytokines appeared to have some effect. In both cases, a brief exposure of PBMC to both IL-2 and TGF-β resulted in the suppression of spontaneous IgG production by 85%. The mechanism responsible for this effect is the subject of current investigation.

Discussion

This is the first report, to our knowledge, documenting the capacity of circulating blood lymphocytes from patients with an autoimmune disease to produce active TGF-β. Levels of both total and active TGF-β could not correct the defect in active TGF-β production in SLE (Table III).

Table III. IL-2 and TNF-α could not correct the defect in active TGF-β production in SLEa

<table>
<thead>
<tr>
<th>TGF-β (pg/ml)</th>
<th>Healthy donors (n = 5)</th>
<th>SLE (n = 5)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>41 ± 12</td>
<td>9 ± 2</td>
<td>0.032</td>
</tr>
<tr>
<td>Anti-CD2</td>
<td>290 ± 81</td>
<td>31 ± 7</td>
<td>0.013</td>
</tr>
<tr>
<td>IL-2 (10 U/ml)</td>
<td>69 ± 7</td>
<td>13 ± 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF-α (10 ng/ml)</td>
<td>77 ± 19</td>
<td>22 ± 8</td>
<td>0.037</td>
</tr>
<tr>
<td>IL-2 and TNF-α</td>
<td>131 ± 21</td>
<td>38 ± 14</td>
<td>0.006</td>
</tr>
</tbody>
</table>

a PBML (1 × 10⁶) were cultured for 48 h with the above additives, and the supernatants were tested for active TGF-β.
active TGF-β in short term culture supernatants of unstimulated and stimulated lymphocytes from SLE patients were decreased in comparison with normal controls. Previously, a relative decrease in active TGF-β produced by T cell lines from patients with active multiple sclerosis had been reported (30). Although the decreased levels of TGF-β activity in SLE most likely reflect decreased production, these differences could also be due to increased consumption by TGF-β receptors. Further studies will be needed to exclude this possibility.

In an accompanying report, we have found that NK cells are the principal source of both total and active TGF-β produced by unstimulated lymphocytes (10). In SLE and healthy controls, NK cells produced substantially more TGF-β than T cells (Fig. 1). Decreased amounts, however, of both constitutive and induced NK cell-derived TGF-β were found in SLE, and these defects were found in amounts of the total and active form of this cytokine. Thus, in addition to the well-known defect of NK cell cytotoxic activity in SLE (31, 32), production of TGF-β appears to be decreased as well.

While resting human T cells produced trivial amounts of TGF-β during the first 48 h of culture, stimulated T cells can produce significant quantities of this cytokine (33). Although many T cells appear to be chronically stimulated in SLE (34, 35), they produced only minimal amounts of TGF-β.

Other sources of TGF-β include B cells and monocytes. Although we also reported that resting B cells from healthy donors produce small amounts of TGF-β (10), these lymphocytes have the capacity to produce this cytokine (36, 37). Moreover, IgG secreted by Ag-activated B cells has been found to be complexed latent TGF-β (38). When this complex is bound to FcR on macrophages, latent TGF-β is converted to its active form and may be immunosuppressive (39). Monocytes have been considered to be the principal hemopoietic source of TGF-β (8, 9). Differences in mono-ocyte-derived total and active TGF-β between SLE patients and controls were not found (Table II).

It is important to emphasize that this report concerns short term production of TGF-β. We have focused our attention on this interval because CD8+ T cells require the presence of picogram per milliliter quantities of active TGF-β coincident with activation for development of down-regulatory function (10).

Although stimulated T cells do not produce much TGF-β during the initial 72 h, they can produce considerable quantities of latent TGF-β at later periods (33; K. Ohtsuka and D. A. Horwitz, unpublished observations).

Decreased concentrations of TGF-β in SLE did not appear to correlate with disease activity. To date, TGF-β production has been measured in a group of 10 subjects with rheumatoid arthritis, and a modest decrease of anti-CD2-induced total and active TGF-β has been observed. Larger numbers of RA patients are required to determine the significance of this finding.

Unlike anti-CD3, anti-CD2 mAbs strongly stimulate lymphocytes to produce total and active TGF-β (10). Following stimulation with anti-CD2, both production of total and active TGF-β was markedly reduced in SLE. Because the lymphocyte mitogenic response to anti-CD2 in SLE is also reduced (40, 41), decreased TGF-β production might reflect decreased signaling through the CD2 pathway rather than a decreased capacity of SLE lymphocytes to produce TGF-β. We, therefore, turned our attention to cytokine regulation of TGF-β production.

Concentrating on cytokines known to be abnormally produced in SLE, we have shown that both TNF-α and IL-2 increase lymphocyte-derived active TGF-β and that IL-10 has the opposite effect. To our knowledge, these findings have not been previously
DECREASED TGF-B PRODUCTION IN SLE


