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In Vivo Neutralization of TNF-α Promotes Humoral Autoimmunity by Preventing the Induction of CTL

Charles S. Via,¹ Andrei Shustov,* Violeta Rus,* Thomas Lang,* Phuong Nguyen,* and Fred D. Finkelman†

Neutralization of TNF-α in humans with rheumatoid arthritis or Crohn’s disease has been associated with the development of humoral autoimmunity. To determine the effect of TNF-α neutralization on cell-mediated and humoral-mediated responses, we administered anti-TNF-α mAb to mice undergoing acute graft-vs-host disease (GVHD) using the parent-into-F1 model. In vivo neutralization of TNF-α blocked the lymphocytopenic features characteristic of acute GVHD and induced a lupus-like chronic GVHD phenotype (lymphoproliferation and autoantibody production). These effects resulted from complete inhibition of detectable antihost CTL activity and required the presence of anti-TNF-α mAb for the first 4 days after parental cell transfer, indicating that TNF-α plays a critical role in the induction of CTL. Moreover, an in vivo blockade of TNF-α preferentially inhibited the production of IFN-γ and blocked IFN-γ-dependent up-regulation of Fas; however, cytokines such as IL-10, IL-6, or IL-4 were not inhibited. These results suggest that a therapeutic TNF-α blockade may promote humoral autoimmunity by selectively inhibiting the induction of a CTL response that would normally suppress autoreactive B cells. The Journal of Immunology, 2001, 167: 6821–6826.

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2 Address correspondence and reprint requests to Dr. Charles S. Via, Division of Rheumatology and Clinical Immunology, University of Maryland School of Medicine, Medical School Teaching Facility 8-34, 10 South Pine Street, Baltimore, MD 21201. E-mail address: cvia@umaryland.edu

3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; GVHD, graft-vs-host disease; CCCA, Cincinnati cytokine capture assay; FasL, Fas ligand.

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To test this hypothesis, we examined TNF-α regulation of graft-vs-host disease (GVHD) in the parent-into-F1 murine model. This system, in which homozygous parental T cells are inoculated into unirradiated heterozygous mice, can lead to the development of either a cell-mediated (acute GVHD) or an Ab-mediated (chronic GVHD) antihost response, depending upon the parental mouse strain used. For example, inoculation of (C57BL/6 × DBA/2)F1 mice with C57BL/6 parental T cells induces acute GVHD while inoculation of the same F1 hosts with DBA/2 parental T cells induces chronic lupus-like GVHD (13). Both acute and chronic GVHD are characterized initially by B cell hyperactivity and autoantibody production. In acute GVHD, however, donor cells develop within 7 days into antihost CTL that eliminate most host B cells, including autoreactive B cells, during the subsequent 5–7 days (14). In chronic GVHD, in contrast, antihost CTL fail to develop and continued autoantibody production results in a lupus-like immune complex glomerulonephritis. Previous studies demonstrating that selective in vivo inhibition of CD8⁺ T cell-CTL development prevents acute GVHD and leads to the development of chronic lupus-like GVHD support the view that CD8⁺ CTL control autoreactive B cell hyperactivity and that the absence of such CTL plays a permissive role in humoral autoimmunity development (15). We now demonstrate that TNF-α is required to suppress humoral autoimmunity in GVHD and does so by inducing CTL development rather than by contributing to CTL effector function.

Materials and Methods

Mice

C57BL/6J (B6) and C57BL/6 × DBA/2 (BDF₁) male mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME).

Induction of GVHD

Single-cell suspensions were prepared in HBSS from the spleens of normal B6 parental donors. Cell suspensions were filtered through sterile nylon mesh, washed, and diluted to a concentration of 10⁹ viable (trypan blue excluding) cells/ml. Acute GVHD was induced by injecting 50 × 10⁶ B6 splenocytes i.v. into the tail veins of normal unirradiated BDF₁ recipients (16). Chronic GVHD was induced using 50 × 10⁶ CD8-depleted B6 splenocytes...
Flow cytometry analysis and engraftment studies

Spleen cells were prepared as described in Ref. 16. Following incubation with the anti-murine FcγRI/RIII mAb, 2.4G2, (21) for 10 min, cells were stained with saturating concentrations of FITC-, biotin-, or PE-conjugated mAb against CD4, CD8, B220, Fas, or H-2Kb purchased from BD Biosciences (Mountain View, CA.) or BD Pharmingen. Two-color flow cytometry was performed using a FACScan (BD Biosciences), lymphocytes were gated by forward and side scatter, and fluorescence data were collected on 10,000 cells. Donor T cells were defined as CD4+ or CD8+ and stained negatively for MHC class I expressed by the recipient, but not the donor, cells. Host B cells were identified as B220-positive host I-A-positive cells. Monocyte populations were excluded on the basis of forward and side scatter.

Serological studies

Mice were bled at the times indicated and sera were tested by ELISA for the presence of IgG antibodies to ssDNA as described in Ref. 16. Briefly, microtiter plates were coated with heat-denatured salmon sperm DNA, blocked with 2% BSA-PBS and incubated with 2-fold serial dilutions of experimental mouse sera beginning at a dilution of 1/40. The plates were then incubated with alkaline phosphatase-labeled anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) and OD quantitated at 405 nm. For each experiment, pooled MRL/lpr sera were tested in parallel and a standard curve constructed for conversion of experimental sera OD values to units. An arbitrary value of 1000 U was assigned to MRL/lpr sera at a dilution of 1/2000.

Statistical Analysis

Data were examined for normality and equal variance (Kolmogorov-Smirnov test). If satisfactory, groups were compared by two-tailed Student’s t test, if not they were compared by the Mann-Whitney rank sum test.

Results

Neutralization of TNF-α in vivo inhibits the development of acute GVHD and enhances anti-DNA Ab production in a dose-dependent fashion

To determine the role of TNF-α in the development of acute GVHD, BDF1 mice were inoculated with B6 spleen cells and received either no additional treatment or 0.125–2 mg of anti-TNF-α mAb twice a week, beginning on the day of parental cell transfer (Table I). Treatment with the highest dose of anti-TNF-α mAb (2 mg) not only blocked the reduction in total splenocytes and host B cells typically seen in acute GVHD, but also resulted in lymphoproliferation, as evidenced by an ~40–50% increase in both total spleen cells and host B cells compared with normal untreated F1 mice (p < 0.005 for both). Treatment with 0.125 mg of anti-TNF-α mAb did not significantly alter GVHD-associated splenic lymphopenia or B cell elimination. Intermediate doses (0.5 mg) of anti-TNF-α mAb resulted in a trend toward less severe acute GVHD; however differences were not statistically significant when compared with untreated or low-dose mAb-treated acute GVHD mice.

The lymphoproliferation and B cell expansion observed in mice injected with the highest dose of anti-TNF-α mAb was associated with a significant increase in serum anti-ssDNA Ab levels compared with either untreated acute GVHD mice (2.5-fold) or with normal F1 mice (8-fold; p < 0.05 for both; Table I). Mice treated for 10 days with high-dose anti-TNF-α mAb still exhibited increased spleen cell numbers and anti-DNA Ab titer 1 month later (data not shown). Taken together, these data indicate that stringent neutralization of TNF-α induces features of chronic GVHD in mice that would otherwise develop acute GVHD and suggest that less complete TNF-α neutralization can block features of acute GVHD without inducing lupus-like GVHD.
Neutralization of TNF-α in vivo blocks antihost CTL activity in acute GVHD

Lymphopenia in acute GVHD is mediated, in large part, by elimination of host cells by host-specific donor CTLs (16, 22). Because selective inhibition of donor antihost CTL activity can permit the development of chronic GVHD (14, 23), the above results were consistent with the possibility that neutralization of TNF-α promotes chronic GVHD by inhibiting donor antihost CTL development. To test this idea, mice received 2 mg of anti-TNF-α mAb control mAb twice a week and antihost CTL responses were assessed at the time of maximal CTL activity, 10 days after parental cell transfer (14). As shown in Fig. 1, in vivo anti-TNF-α mAb treatment completely inhibited antihost CTL activity, as compared with mice that received parental cells but either no mAb or control mAb. In contrast to its ability to inhibit the induction of CTL activity when administered in vivo before the development of acute GVHD, anti-TNF-α mAb did not block the antihost CTL effector function as evidenced by an inability to: 1) block anti-ssDNA cytolytic activity when added to IL-2-stimulated cultures of spleen cells from day 10 acute GVHD mice (data not shown) or 2) block antihost CTL effector function when added during the 4-h assay phase (data not shown).

**FIGURE 1.** In vivo blockade of TNF-α eliminates ex vivo detection of antihost CTL activity in acute GVHD mice. B6×BDF1 mice were treated with 2 mg of anti-TNF-α (MP6-XT-22) or control mAb (GL113) i.v. at days 0, 4, and 7 after parental cell transfer. At 10 days, mice were sacrificed and splenocytes tested for ex vivo killing of H-2 b targets as described in Materials and Methods. No significant killing of H-2 b targets was observed. Results are shown as the group mean ± SE at a given E:T ratio (n = 5 mice/group). Similar results were seen in two additional experiments.

**TABLE I.** Anti-TNF-α mAb blocks the lymphocytopenic features of acute GVHD in a dose-dependent manner

<table>
<thead>
<tr>
<th>Group</th>
<th>Splenic Yield</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>B cells</th>
<th>Anti-DNA⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal F1</td>
<td>91.8 (7.2)</td>
<td>ND</td>
<td>ND</td>
<td>48.9 (3.9)</td>
<td>6.4 (0.9)</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td>57.7 (10.2)</td>
<td>6.2 (0.4)</td>
<td>10.5 (0.8)</td>
<td>15.8 (5.4)</td>
<td>20.4 (8.8)</td>
</tr>
<tr>
<td>Acute GVHD + anti-TNF (2 mg)</td>
<td>133.0 (3.5)</td>
<td>2.4 (0.5)</td>
<td>2.1 (0.6)</td>
<td>65.7 (5.1)</td>
<td>55.5 (10.2)</td>
</tr>
<tr>
<td>Acute GVHD + anti-TNF (0.5 mg)</td>
<td>72.3 (8.8)</td>
<td>5.7 (1.3)</td>
<td>7.4 (1.0)</td>
<td>27.3 (5.3)</td>
<td>18.2 (6.0)</td>
</tr>
<tr>
<td>Acute GVHD + anti-TNF (0.125 mg)</td>
<td>47.8 (10.3)</td>
<td>4.5 (0.8)</td>
<td>3.8 (0.4)</td>
<td>13.0 (2.5)</td>
<td>9.7 (1.1)</td>
</tr>
</tbody>
</table>

*Acute GVHD was induced as described in Materials and Methods and mice received anti-TNF-α mAb (MP6-XT-22) i.v. at the indicated dose on days 0, 3, 7, and 10 after parental cell transfer. Values for total splenocytes and donor or host lymphocyte subsets are shown as group mean (±SE) n = 4 mice/group for all groups except untreated acute GVHD (n = 3). Similar results were observed in two independent experiments.

Anti-ssDNA Ab levels are expressed as units per milliliter (see Materials and Methods).
<table>
<thead>
<tr>
<th>Segment</th>
<th>Anti-TNF-α mAb</th>
<th>Anti-ssDNA Ab</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>B cells</th>
<th>Anti-DNA⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td>0.05</td>
<td>Not detectable above background.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Increased serum levels of TNF-α have been reported in acute GVHD mice as early as day 10 after parental cell transfer but were not seen at day 8 (24). Although the foregoing data strongly argue that TNF-α is present in the first few days of acute GVHD, we have been unable to detect increased serum TNF-α at days 3, 5, or 7 by standard ELISA (data not shown). Recent modifications of the CCl3 assay significantly increase the sensitivity of this assay (19). Using this approach, we have determined that as early as day 6, both...
Acute and chronic GVHD mice exhibit significant increases in several major cytokines. In particular, acute GVHD mice exhibit significantly elevated serum TNF-α levels which are ≥3-fold greater than control mice and ≥2-fold greater than chronic GVHD mice (Table II). Moreover, striking elevations in serum IFN-γ levels are seen in acute GVHD which are several logs greater than those of either chronic GVHD mice or control F1 mice. It should be noted that serum IFN-γ levels in chronic GVHD mice, while significantly less than those of acute GVHD mice, are nevertheless significantly greater than control mice. By day 9 after parental cell transfer, an ~5-fold elevation in serum IL-2 levels is seen for both acute and chronic GVHD mice compared with control F1 mice (p < 0.01, acute GVHD or chronic GVHD vs normal; p = NS, acute vs chronic). Additionally, chronic GVHD mice exhibited an approximately 4-fold elevation in serum IL-4 compared with control F1 mice (p < 0.01); however, acute GVHD mice exhibited even greater elevations in serum IL-4 levels compared with either control F1 mice (≥9-fold, p < 0.01) or chronic GVHD mice (2-fold, p < 0.01). These data indicate that although significant increases in cytokine production are present in both forms of GVHD, acute GVHD mice make greater amounts of TNF-α, IL-4, and most notably IFN-γ compared with chronic GVHD mice.

In vivo blockade of TNF-α in acute GVHD selectively blocks production of IFN-γ

Activation of donor CD4+ T cells is a common feature of both acute and chronic GVHD and initially results in the production of IL-2, IL-4, and IL-10 (14). In acute GVHD, activation of donor CD4+ T cells leads to donor CD8+ T cell activation which results in IFN-γ production and the development of an antihost CTL response (14). In contrast, donor CD8+ T cell activation and marked IFN-γ production are not features of chronic GVHD. Because early administration of anti-TNF-α mAb inhibits CTL development (Fig. 2) and IFN-γ contributes to CTL development in this model (14, 18), it was possible that anti-TNF-α mAb blocked CTL development, in part, by inhibiting an IFN-γ response. To determine whether a TNF-α blockade alters cytokine production in acute GVHD, splenic mRNA was assessed for cytokine gene expression by semi-quantitative RT-PCR. As shown in Fig. 3A, in vivo treatment with anti-TNF-α mAb resulted in a ~3-fold inhibition of IFN-γ mRNA expression as compared with untreated or control mAb-treated BDF1 mice that had been inoculated with B6 spleen cells. In contrast, anti-TNF-α mAb treatment did not significantly inhibit IL-4 or IL-10 mRNA expression. These results were confirmed at the level of serum cytokine protein. As shown above in Table II, acute GVHD mice exhibit very high serum levels of IFN-γ at day 6 after parental cell transfer. Anti-TNF-α treatment completely inhibits the acute GVHD-associated rise in serum IFN-γ but does not significantly alter the serum levels of a B cell stimulatory cytokine such as IL-6 (Fig. 3B).

TNF-α blockade impairs IFN-γ-mediated Fas up-regulation in acute GVHD

We have previously shown that elimination of host B cells in acute GVHD by donor CTL involves both a Fas/FasL pathway and a perforin pathway (18). Moreover, significant Fas up-regulation on host B cells is characteristic of acute GVHD, but not chronic GVHD, and is largely IFN-γ dependent (18). To determine whether the striking reduction in serum IFN-γ in anti-TNF-α-treated acute GVHD mice results in functional consequences, IFN-γ-dependent Fas expression on host B cells was examined by flow cytometry at 10 days after parental cell transfer. As shown in Fig. 4, A and B, the characteristic up-regulation of Fas on host B cells in acute GVHD is mostly, but not completely, down-regulated in mice receiving anti-TNF-α mAb. These results are consistent with previous work demonstrating a comparable degree of Fas down-regulation in chronic GVHD mice or in anti-IFN-γ mAb-treated acute GVHD mice (18).

Discussion

Although controversial, human and murine studies suggest a link between reduced TNF-α production and the development of humoral autoimmunity. For example, lupus-prone New Zealand Black/White (NZB/W) mice express an allelic form of the TNF-α gene that is associated with decreased TNF-α production (25). Treatment of NZB/W mice with anti-TNF-α mAb exacerbates renal disease and mortality (25), while administration of rTNF-α at

Table II. Acute GVHD mice exhibit elevations of serum IFN-γ and TNF-α compared to chronic GVHD mice at day 6 after parental cell transfer

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>IL-2</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal F1</td>
<td>≤40</td>
<td>69 (4)</td>
<td>57 (6)</td>
<td>12 (1)</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td>154,476 (7,815)</td>
<td>265 (46)</td>
<td>344 (72)</td>
<td>104 (14)</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td>297 (35)</td>
<td>118 (13)</td>
<td>298 (35)</td>
<td>51 (4)</td>
</tr>
</tbody>
</table>

*Acute and chronic GVHD were induced and serum cytokines measured by CCCA as described in Materials and Methods. Mice were injected with 10 μg of biotin anti-INF-γ and anti-TNF-mAb on day 5 and anti-IL-4 and anti-IL-2 mAbs on day 8 and bled on days 6 and 9. Results are shown as mean picograms per milliliter per group with SE in parentheses; n = 4–5 mice/group.

b p < 0.01 compared to control.

p < 0.01 compared to chronic GVHD.
NZB/W mice (29). In humans, defective TNF-response in these mice and results in severe renal diseases similar to lupus Black mice enhances the otherwise mild autoimmune retnity (28), whereas breeding a defective accelerates the development of lymphadenopathy and autoimmu-
ty in parent

more, deletion of the type I TNFR gene from C57BL/6.lpr mice accelerates the development of lymphadenopathy and autoimmu-

The present study demonstrates that TNF-α is critical for the induction of CTL in vivo and suggests a mechanism by which reduced TNF-α activity may contribute to the development of humoral autoimmunity in both mouse and human. In the parent→F1, GVHD model, both acute and chronic GVHD are initiated by the activation of donor CD4+ T cells that produce IL-2 and mature into effector T helper cells that activate B cells to proliferate and secrete Ig. As a result, polyclonal B cell activation and autoantibody production can be observed 7–10 days after parental cell transfer in both forms of GVHD (14). Activation of donor CD8+ T cells, which mature into antihost CTL, eliminates autoantibody-secreting host B cells and serves to differentiate acute GVHD from chronic GVHD. Actions that selectively impair CD8+ T cell differentiation into antihost CTL, such as in vivo treatment with anti-IL-2 mAb (15), depletion of donor CD8+ T cells before parental cell transfer (16), or deletion of the perforin gene from donor CD8+ T cells (11), convert acute GVHD to chronic lupus-like GVHD. Thus, CTL likely prevent lupus-like humoral autoimmunity in parent→F1 GVHD by eliminating autoreactive host B cells.

Our results indicate that neutralization of TNF-α during the first few days after parental cell transfer selectively inhibits CD8+ T cell maturation into CTL effectors, in association with suppression of IFN-γ production and decreased Fas/FasL up-regulation. Because anti-TNF-α mAb treatment does not inhibit all cytokine production or B cell hyperactivity, mice then develop chronic lupus-like GVHD.

Although TNF-α is well known to be involved in the CTL effector function, a role for TNF-α in CTL generation has only recently been suggested. T cells from TNFR I-deficient mice exhibit reduced in vitro production of IFN-γ and IL-2 in response to alloantigen (35) and anti-TNF-α Ab has been described to decrease CTL generation, reduce splenomegaly and gastrointestinal pathol-ogy, decrease weight loss, and improve survival in an irradiated recipient model of bone marrow transplantation and GVHD and in a parent→F1 model of GVHD (36–39). In addition, impaired CTL function and reduced Th1 cytokine production were observed in GVHD when donor cells were obtained from TNFR p55-deficient
mice (35). However, previous studies did not investigate the connection between anti-TNF-α treatment and the development of autoimmunity.

Our finding that TNF-α is critical for CTL induction and subsequent control of autoreactive B cells ties together previous unlinked clinical and experimental observations that: 1) TNF-α suppresses humoral autoimmunity (26, 27); 2) TNF-α can induce IFN-γ production (40, 41); 3) IFN-γ enhances CTL function by up-regulating Fas and FasL expression (18); and 4) CTL suppresses humoral autoimmunity by killing autoreactive B cells (11, 16). Our studies do not eliminate the possibility that TNF-α may also contribute to CTL activation by up-regulating perforin expression, either through an IFN-γ-dependent or independent mechanism.

Lastly, our results establish a mechanism by which treatment of autoimmune disease patients with TNF-α antagonists could induce or exacerbate disorders of humoral autoimmunity, such as SLE. Although our results by no means argue against the clinical use of TNF-α antagonists which have been remarkably effective therapies for many patients with rheumatoid arthritis or Crohn’s disease, they underscore the need to carefully monitor patients and to identify factors that might predispose patients to develop autoimmune pathology when treated with TNF-α antagonists. In addition, the possibility that TNF-α may be a general requirement for CTL development should promote caution in using TNF antagonists in patients with conditions in which CTL appear to limit severity, such as viral infections. Conversely, TNF antagonists may be of benefit in conditions in which CTL are detrimental, such as acute allograft rejection.

References