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Inhibition of Th1 Polarization by Soluble TNF Receptor Is Dependent on Antigen-Presenting Cell-Derived IL-12

Burkhard Becher, Manon Blain, Paul S. Giacomini, and Jack P. Antel

Th1-polarized CD4+ T cells are considered central to the development of a number of target-directed autoimmune disorders including multiple sclerosis. The APC-derived cytokine IL-12 is a potent inducer of Th1 polarization in T cells. Inhibition of IL-12 in vivo blocks the development of experimental allergic encephalomyelitis, the animal model for multiple sclerosis. Based on previous work that suggests that the production of IL-12 by activated human central nervous system-derived microglia is regulated by autocrine TNF-α, we wanted to determine whether inhibition of TNF could induce a reduction of Th1 responses by its impact on systemic APCs. We found that soluble TNFR p75-IgG fusion protein (TNFR:Fc) inhibited production of IFN-γ by allo-Ag-activated blood-derived human CD4 T cells. We documented reduced IL-12 p70 production by APCs in the MLR. By adding back recombinant IL-12, we could rescue IFN-γ production, indicating that TNFR:Fc acts on APC-derived IL-12. Consistent with an inhibition of the Th1 polarization, we found a decreased expression of IL-12Rβ1 subunit on the T cells. Furthermore, the capacity of T cells to secrete IFN-γ upon restimulation when previously treated with TNFR:Fc is impaired, whereas IL-2 secretion was not altered. Our results define a TNF-dependent cytokine network that favors development of Th1 immune responses. The Journal of Immunology, 1999, 162: 684–688.
Isolation of peripheral blood-derived cells

PBMC were isolated from healthy adult volunteer donors by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Baie D’Urfé, Canada). After the isolation of enriched APCs, the PBMC were washed twice with PBS and cultured for 1 h in RPMI 1640 medium (Life Technologies, Burlington, ON, Canada) supplemented with 10% FCS, 2.5 mg/ml penicillin, 2.5 mg/ml streptomycin, and 2 mM glutamine (all from Life Technologies) in 75-cm² tissue culture flasks (Falcon, VWR Scientific, Montreal, Canada). The nonadherent cells were removed by gentle shaking. The adherent cells consisted of 95% HLA-DR/B7-2 positive monocytes.

CD4⁺ T cells were isolated from PBMC using anti-CD4 mAbs conjugated to magnetic beads (Dynal, Great Neck, NY). The beads were detached from the cells after isolation following the supplied protocol. The cells were then washed with PBS and their purity was of ≥96% as assessed by flow cytometry (23).

Semiquantitative PCR analysis

Total RNA was isolated using TRIZOL Reagent (Life Technologies). To transcribe into cDNA, 3 μg RNA, 3.3 mM random hexamer primers (Boehringer Mannheim, Manheim, Germany), reverse transcriptase buffer, 3 mM dNTPs, 400 U Maloney murine leukemia virus reverse transcriptase (all from Life Technologies), 0.6 μg RNA guard, and 3 mM DTT (both from Pharmacia) were added to a total volume of 32 μl. The reaction mixture was incubated for 1 h at 42°C followed by a 10-min incubation at 75°C. Primers used for PCR were obtained from Life Technologies and had the following sequences: IL-12Rβ₁ forward, 5'-ACAGGACACACTCTCTGGAC-3' and β-actin forward, 5'-AGAGGGACCTGTGTGTCACC-3'; and β-actin reverse, 5'-ATGCCATCCTGCGTCTGGACCTGGC-3'. The primers for IL-12Rβ₁ and β-actin were constructed to generate fragments of 281 bp and 378 bp, respectively. cDNA 200 ng was added to the reaction mixture containing PCR buffer, 0.2 mM dNTPs (Life Technologies), 50 pMol of either primer set for IL-12Rβ₁ or β-actin, and 0.5 μl Taq polymerase (Life Technologies). The reaction mixture was completed with H₂O to a total volume of 50 μl. Samples were placed in a Gene Amp PCR system 9600 (Cetus, Perkin-Elmer, Norwalk, CT) for 25 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min followed by a 10-min extension at 72°C. After amplification, 15 μl of each sample was electrophoresed on a 1.5% agarose gel (Life Technologies). The bands were visualized with ethidium bromide. For quantification purposes, 2.5 μl of ³²PdCTP (DuPont/NEN, Mississauga, ON, Canada) was added to the reaction mixture before PCR. The gels were dried and the bands were analyzed using a phosphorimaging and Image Quant software (Molecular Dynamics, Sunny Valley, CA).

MLR

A total of 10⁵ T cells was cocultured with either 2 × 10⁵ or 5 × 10⁵ allogeneic APC. For proliferation and cytokine assays, primary MLRs were conducted in 96-well plates. After 5 days, unless indicated otherwise, 1 μCi [³H]thymidine was added to the wells for 5 h. The cells were harvested and thymidine uptake was determined using a beta-scintillation counter. Culture medium was recovered from sister cultures to determine the cytokine concentrations. For secondary MLRs, T cells were recovered from the primary MLRs and cocultured with freshly isolated allogeneic APC from the same donor as in the primary MLR. Secondary MLRs were conducted for 3 more days at which time cytokine release was measured.

L929 cytotoxicity assay

L929 cells (10⁴) were cultured in RPMI 1680 medium with 10% FCS. We added either 50 or 100 U/ml (1.7 or 0.85 ng/ml) of TNF-α in the presence of different concentrations of TNFR:Fc (Genzyme, Cambridge, MA) or carrier buffer. The cells were incubated for 24 h and supernatants were analyzed for lactate dehydrogenase (LDH) content as previously described (24).

Cytokine ELISA

IL-12 ELISA kits were obtained from R&D Systems. IL-2, IL-10, and IFN-γ ELISA kits were obtained from BioSource International (Camarillo, CA). Tissue culture supernatants were stored at −80°C until analysis. ELISA assays were performed following manufacturers instructions.

Results

TNFR:Fc inhibits TNF-α-mediated cytotoxicity

In initial studies, we established that TNFR:Fc could specifically inhibit the cytotoxic effect of TNF-α by exposing TNF-sensitive L929 cells to TNF-α and TNFR:Fc or carrier control. Fig. 1A shows the toxicity of different concentrations of rTNF-α on L929 cells as assessed by LDH release assay. In a subsequent experiment, we have used 50 or 100 U (0.85 or 1.7 ng/ml) of TNF-α that results in maximum LDH release. We can block cytolytic activity mediated by 100 U of recombinant TNF-α using 140 pg/ml of TNFR:Fc, indicating that engagement of membrane TNFR on L929 cells is completely ablated by addition of TNFR:Fc (Fig. 1B).

TNFR:Fc inhibits IFN-γ production in an allogeneic MLR

To analyze the effect of TNFR:Fc on immunoregulatory functions, we have performed MLRs in the presence of TNFR:Fc. CD4⁺ T cells were isolated from healthy donors and mixed with allogeneic APC at different ratios (5:1–2:1) in 96-well plates. TNFR:Fc or irrelevant IgG1 mAb was added to the reaction. The MLR was conducted for 5 days, and T cell proliferation was assessed by [³H]thymidine uptake (23). Supernatants from sister cultures were harvested and analyzed for IFN-γ by ELISA.

As shown in Fig. 2A, addition of TNFR:Fc to the primary MLRs inhibited IFN-γ production in a dose-dependent fashion. Maximum inhibition was first observed with 140 pg/ml of TNFR:Fc. For subsequent experiments we used 2.8 μg/ml of TNFR:Fc and 4 μg/ml of irrelevant IgG1 in vehicle buffer (ctrl). After 5 days, TNFR:Fc inhibited IFN-γ production by 76 ± 7% SEM, on average (Table I). As shown in Fig. 2B, levels of IL-2 and IL-10 were not altered by TNFR:Fc. We did not detect IL-4 or IL-5 under any culture condition used. In time course studies, we observed that TNFR:Fc inhibits IFN-γ production from the earliest time point this cytokine could be detected (Fig. 2C). TNFR:Fc does not alter proliferation at the concentrations used over the 5-day time period of the primary MLR.
Table I. Inhibition of IFN-γ production by TNFR:Fc

<table>
<thead>
<tr>
<th>IFN-γ Production (pg/ml)</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
<th>Expt. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLR + ctrl</td>
<td>171</td>
<td>399</td>
<td>284</td>
<td>&gt;1000</td>
<td>563</td>
</tr>
<tr>
<td>MLR + TNFR:Fc</td>
<td>23</td>
<td>134</td>
<td>72</td>
<td>296</td>
<td>93</td>
</tr>
<tr>
<td>% decrease</td>
<td>87</td>
<td>66</td>
<td>75</td>
<td>70</td>
<td>83</td>
</tr>
</tbody>
</table>

*Data indicate results of five individual experiments in which an MLR was carried out for 5 days in the presence of 2.8 μg/ml anti-CD3 or 4 μg/ml irrelevant IgG1 isotype control in carrier buffer (ctrl). For each data point, culture media from triplicate wells was pooled and analyzed for IFN-γ by ELISA. Supernatants from APCs alone were used as background control. (% decrease = 100 × (1 - [IFN-γ]TNFR:Fc/[IFN-γ]ctrl)).

Table II. Inhibition of APC-derived IL-12 in allogeneic MLR by TNFR:Fc

<table>
<thead>
<tr>
<th></th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>12</td>
<td>14</td>
<td>81</td>
</tr>
<tr>
<td>TNFR:Fc</td>
<td>1</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>% inhibition</td>
<td>93</td>
<td>71</td>
<td>68</td>
</tr>
</tbody>
</table>

*Data indicate the results of three individual experiments in which an MLR was carried out in the presence of 1 μg/ml anti-CD3 for 24 h. A total of 2.8 μg/ml TNFR:Fc or 4 μg/ml irrelevant IgG1 isotype control in carrier buffer (ctrl) were added. For each data point, culture media from triplicate wells were pooled and analyzed for IL-12 p70 by ELISA. The results are expressed in pg/ml IL-12 p70 (inhibition = 100 − ([IL-12]TNFR:Fc/[IL-12]ctrl) × 100)).

to determine whether TNFR:Fc-mediated inhibition of IFN-γ production involved either an APC-dependent network and/or a direct effect on the T cells. Regarding the former, we have previously shown that TNFR:Fc can inhibit IL-12 production by activated-human adult microglial cells (18). When microglial cells are activated with LPS, they produce TNF-α before IL-12. When we then block the action of autocrine TNF-α by using TNFR:Fc, we could significantly inhibit IL-12 production. In the current study, the levels of IL-12 p70 production in the MLR were at the lower levels of detectability (7.8 pg/ml). To overcome this limitation and to directly determine whether IL-12 p70 production is dependent on TNF, we added anti-CD3 mAbs (0.1 μg/ml) to the MLR to activate nonalloresponsive T cells, which in turn results in more robust IL-12 levels. Table II shows the decrease in IL-12 p70 production by APC in MLRs treated with TNFR:Fc measured by ELISA for IL-12 p70.

We then went on to establish whether the decrease in IL-12 production by TNFR:Fc-treated APCs is responsible for the decrease of IFN-γ in an allogeneic MLR. We added human rIL-12 (R&D Systems) to the cells, to determine whether IFN-γ production can be recovered. When 100 pg of IL-12 was added to the cultures, we were able to completely recover IFN-γ production by the T cells (Fig. 3). Addition of IL-12 did not rescue IFN-γ secretion (data not shown). We could also mimic the effect of TNFR:Fc and decrease IFN-γ production by the addition of 0.5 μg/ml anti-IL-12 mAbs (Fig. 3).

To establish whether the depletion of TNF during the primary stimulation of alloreactive T cells influences their ability to secrete IFN-γ during a secondary MLR in the absence of the antagonist, we isolated and extensively washed the T cells after the initial 5-day primary MLR. These cells were then incubated with fresh allogeneic APC for 3 more days, at which time the culture medium was collected and analyzed for IFN-γ. Fig. 4 shows that T cells...
isolated from TNFR:Fc-treated MLR cultures produce significantly less IFN-γ than control cultures when restimulated by fresh APCs under regular culture conditions. As expected, the levels of IFN-γ were generally higher in the secondary response. The levels of IL-2 in secondary MLRs were also increased compared with primary MLR cultures but not altered due to the depletion of TNF-α to these cultures restores normal IFN-γ levels, indicating specificity of TNFR:Fc. In contrast to the MLR studies, we could not mimic the effect of TNFR:Fc by the use of anti-IL-12 mAbs (not shown). However, when recombinant IL-12 was added, we could again increase IFN-γ levels (Fig. 5B). These results demonstrate that exogenous IL-12 can override the inhibitory effect of TNFR:Fc on IFN-γ production.

As mentioned above, in addition to polarized cytokine profiles, Th1 cells can also be distinguished from Th2 cells by other phenotypic markers. During the course of Th2 polarization induced by anti-IL-12 and IL-4 treatment in vitro, the Th0 and Th1 cells down-regulate IL-12Rβ2 subunit and display a decreased responsiveness to IL-12. Treatment with IFN-γ or IL-12 can restore higher levels of this receptor (14). After stimulation for 24 h with anti-CD3, we performed a radiolabeled semiquantitative PCR for IL-12Rβ2. The gel was analyzed by phosphorimaging. Fig. 6 shows that anti-CD3-activated T cells in TNFR:Fc-treated cultures express significantly less IL-12Rβ2 message than control cultures, indicating that TNF-α can mimic some of the functions ascribed to IFN-γ.

Discussion

Our study delineates a cytokine network involving TNF/LT and IL-12 that regulates the polarization of Th1 T cells. We show that by inhibiting the action of APC- and T cell-derived TNF/LT in an MLR, one also decreases the production of IFN-γ by CD4 T cells. TNFR:Fc does not alter IL-2 or IL-10 levels. This finding supports our conclusion that treatment with TNFR:Fc selectively inhibits IFN-γ secretion without impacting on general lymphocyte function and survival or driving Th2 polarization. We have previously shown that TNF-α and IFN-γ are important costimuli for the induction of IL-12 expression in APC (18). IL-12 is the most potent soluble factor driving the development of a Th1 profile by T cells (16, 25). Here, we demonstrate a significant reduction of IL-12 p70 production by APCs in allogeneic MLRs supplemented with TNFR:Fc. Addition of rIL-12 to the TNFR:Fc-treated cultures restores normal IFN-γ levels, indicating that the dominant effect of TNFR:Fc on this system is the inhibition of IL-12 production by APCs.

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

**FIGURE 4.** MLR was conducted for 5 days in the presence of 2.8 μg/ml TNFR:Fc (▲) or 4 μg/ml irrelevant IgG1 isotype control in carrier buffer (■). The nonadherent cells (T cells) were then harvested, extensively washed, and cocultured with freshly isolated APC under regular culture conditions. After 3 days of secondary stimulation, the culture media from triplicate wells were pooled and analyzed for IFN-γ by ELISA. Each data point represents an individual MLR.

**FIGURE 5.** Dose-dependent inhibition of IFN-γ production in polyclonally activated pure CD4 T cells. A, CD4 T cells were activated with 1 μg/ml anti-CD3 mAbs in the presence of increasing concentrations of TNFR:Fc or 4 μg/ml irrelevant IgG1 isotype control in carrier buffer (ctrl) for 24 h. Culture media were pooled from triplicate wells and analyzed for IFN-γ production by ELISA. Results are expressed as the mean of two experiments ± SEM. B, Activated-CD4 T cells were treated with 2.8 μg/ml TNFR:Fc in the presence or absence of 100 pg/ml IL-12 p70 or 4 μg/ml ml irrelevant IgG1 isotype control in carrier buffer (ctrl) for 24 h. Culture media were pooled from triplicate wells and analyzed for IFN-γ production by ELISA. Results are expressed as the mean of two experiments ± SEM.

**FIGURE 6.** TNFR:Fc decreases IL-12Rβ2 subunit expression in activated CD4+ T cells. CD4 T cells were activated with 1 μg/ml anti-CD3 mAbs in the presence of either TNFR:Fc (2.8 μg/ml) or IgG1 isotype control (4 μg/ml) for 24 h. Background control is derived from nonactivated T cells. A representative autoradiograph from a 25-cycle PCR for IL-12Rβ2 (281 bp) and β-actin (378 bp) is shown.
The APC-dependent mechanism may not be the only modulator of IFN-γ production by T cells. We show that in the absence of APCs, polyclonally activated pure CD4+ T cells decrease IFN-γ production when treated with TNFR:Fc. It is evident that TNF acts directly on T cells to maintain a Th1 phenotype by enhancing IL-12 responsiveness (via IL-12-Rβ2) and up-regulating IFN-γ production. The direct inhibitory effect of TNFR:Fc on T cells can be overridden by exogenous IL-12. The latter finding supports the hypothesis that the inhibition of IFN-γ production by TNFR:Fc is predominantly achieved via its effect on APC, in particular, IL-12 production. Ultimately, IFN-γ itself can also contribute to this cytokine network in a feedback fashion by further stimulating IL-12 production in activated APC.

TNFR:Fc has now been used therapeutically both in experimental and human autoimmune inflammatory disorders. TNF levels produced by monocytes in MS are increased and correlate with disease severity [26]. Animals treated with TNFR:Fc after immunization with myelin basic protein or proteolipid protein but before disease severity [26]. Neither study provided data on whether there was skewing of the T cell cytokine profile in peripheral circulation of patients with MS were treated with TNFR p50-IgG fusion protein, shown to be efficacious for clinical symptoms. However, when patients with MS also are reported to have elevated cerebrospinal fluid and serum IL-12 levels [27, 28].

In our vitro study provides insight as to how TNFR:Fc can influence cytokine networks that regulate the polarization of cytokine patterns. Immune therapy in RA with TNFR:Fc has been shown to be efficacious for clinical symptoms. However, when patients with MS were treated with TNFR p50-lgG fusion protein, there was a reported increase in the relapse rate lesion formation as assessed by magnetic resonance imaging [22]. Neither study provided data on whether there was skewing of the T cell cytokine response, in a manner demonstrated in our study. To understand the in vivo effects of TNF/LT-directed therapy, one needs to consider the complicated pharmacokinetics and the pleiotropic nature of the cytokines TNF and LT. Our current study may provide an approach to determine that desired in vivo effects are occurring and to minimize the risk of clinical toxicity.

Acknowledgments

We thank Dr. A. Trout for the generous supply of TNFR:Fc.

References