Paradoxical Anti-Inflammatory Actions of TNF-α: Inhibition of IL-12 and IL-23 via TNF Receptor 1 in Macrophages and Dendritic Cells

Maria Zakharova and H. Kirk Ziegler

*J Immunol* 2005; 175:5024-5033; 
doi: 10.4049/jimmunol.175.8.5024

http://www.jimmunol.org/content/175/8/5024
Paradoxical Anti-Inflammatory Actions of TNF-α: Inhibition of IL-12 and IL-23 via TNF Receptor 1 in Macrophages and Dendritic Cells

Maria Zakharova and H. Kirk Ziegler

IL-12 and TNF-α are central proinflammatory cytokines produced by macrophages and dendritic cells. Disregulation of TNF-α is associated with sepsis and autoimmune diseases such as rheumatoid arthritis. However, new evidence suggests an anti-inflammatory role for TNF-α. TNF-α-treated murine macrophages produced less IL-12p70 and IL-23, after stimulation with IFN-γ and LPS. Frequency of IL-12p40-producing macrophages correspondingly decreased as measured by intracellular cytokine staining. IL-12p40 production was also inhibited in dendritic cells. TNFR1 was established as the main receptor involved in IL-12p40 regulation, because IL-12p40 levels were not affected by TNF-α in TNFR1−/−-derived macrophages. Macrophages activated during Listeria monocytogenes infection were more susceptible to inhibition by TNF-α than cells from naive animals, which suggests a regulatory role for TNF-α in later stages of infection. This nonapoptotic anti-inflammatory regulation of IL-12 and IL-23 is an important addition to the multitude of TNF-α-induced responses determined by cell-specific receptor signaling. The Journal of Immunology, 2005, 175: 5024–5033.

The ability of TNF-α to exert such diverse effects stems from the broad expression of TNF-α receptors and the multitude of intracellular responses they are able to initiate. TNFR1 is expressed on most cell types, excluding erythrocytes. The receptor has a death domain in its intracellular region and can initiate the apoptotic pathway in addition to the MAPK pathway, which results in NF-κB translocation. NF-κB activation competes with the apoptotic pathway branch and ensures cell survival. Conversely, TNFR2 has a more restricted expression pattern. It is expressed only on hemopoietic and endothelial cells. TNFR2 can also activate the MAPK pathway (reviewed in Ref. 16). Altered expression of TNF-α has been associated with disease. Autoimmune disorders such as diabetes, RA, MS, and inflammatory bowel disease have all been linked to increased levels of TNF-α. However, in a murine model of autoimmune diabetes, TNF-α was shown to have anti-inflammatory properties depending on the source and timing of TNF-α expression (17, 18). Recent studies suggest that the divergent effects of TNF-α in autoimmunity can be explained based on the location and kinetics of TNF-α expression (19). Kassiotis and Kollias (19) reported that detrimental activity of TNF-α during experimental autoimmune encephalomyelitis (EAE), a murine model of MS, occurs in the acute phase of disease. However, in a later phase, TNF-α is responsible for spontaneous regression of EAE.

As an anti-inflammatory agent, TNF-α can directly limit T cell-mediated responses via apoptosis or indirectly via regulation of IL-12, a major mediator in type 1 inflammatory response (20–22). In several models of inflammation, TNF-α- or TNFR-deficient mice were reported to have severe tissue damage and increased levels of systemic IL-12 (23). In an in vitro model developed by Hodge-Dufour, TNF-α has been shown to directly inhibit IL-12p70 production in both murine and human macrophages (24, 25). TNF-α regulation was shown to affect mRNA levels of IL-12p40 subunit of IL-12 (25). IL-23, a recently identified proinflammatory cytokine, shares the p40 subunit with IL-12 (26). Thus, regulation of IL-12p40 can potentially affect both IL-12 and IL-23 production. Recently, IL-23 was identified as the inducer of pathogenic CD4 T cells involved in demyelination (27). It seems likely...
that TNF-α controls pathogenic effects of EAE via regulation of IL-23.

In this work, we support and expand previous research investigating the regulatory effect that TNF-α exerts on IL-12 in murine myeloid cells. Our original findings show that TNF-α also regulates IL-23. Through the novel approach of intracellular cytokine staining (ICC), we are able to study IL-12p40 regulation on a per-cell basis in both macrophages and dendritic cells, and show that the inhibition is complete in a given macrophage and it is not due to TNF-α-induced apoptosis. More importantly, we show that inhibition is cytokine specific, and that neither IL-6 nor MCP-1 is regulated by TNF-α. Our data indicate that IL-12p40 inhibition in macrophages coincides with stable differentiation events and that TNFR1 is the main receptor responsible for IL-12 regulation. Interestingly, macrophages obtained under inflammatory conditions are more susceptible to TNF-α regulation than macrophages obtained under resting conditions, as was shown in vitro and supported by in vivo data. This finding correlates well with a dual role of TNF-α during an inflammatory response: proinflammatory during the initiation of the immune response and regulating or limiting pathology at a later stage.

Materials and Methods

Mice

C57BL/6, B6.129S2-Tnfrsf1a (TNFR1 knockout (KO)), B6.129S2-Tnfrsf1b (TNFR2 KO), and B6.129S-Tnfrsf1a tm1Imx Tnfrsf1b tm1Imx (TNFR1,2 KO) mice were obtained from The Jackson Laboratory. OT-II TCR-transgenic mice (H-2d) were generously donated by Dr. B. Evavold (Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA). Mice were housed in filter-topped microisolator cages with laboratory chow and water available ad libitum. All mice were handled in accordance with protocols approved by the Internal Animal Care and Use Committee at Emory University.

Reagents

Recombinant mouse rmTNFα was purchased from R&D Systems; rmGM-CSF was purchased from BioSource; Annexin VFITC-conjugated rmIFN-γ and rmIL-4 were purchased from BD Pharmingen. LPS from Salmonella minnesota R595 was obtained from Ribi ImmunoChem Research. Streptococcus pyogenes lysoproteinic acid, Staphylococcus aureus peptidoglycan, and poly(I:C) were purchased from Sigma-Aldrich. Cpg oligodeoxynucleotide (TCG ATG ACG TTC CTG ACG TT) was purchased from Coley Pharmaceutical Group.

Antibodies

For ELISA, specific sources were as follows: anti-mouse IL-12p40 hybridomas C15.6.7.6 and C17.8.20.15 were provided by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA), anti-mouse IL-12p70 9A5 and anti-mouse IL-10 JES-2A5 and SXC-1-biotin were purchased from BD Pharmingen. Purified anti-IL-12p40 Abs were directly conjugated to biotin for use in ELISA using standard techniques. IL-23 sandwich ELISA kit was purchased from eBioscience. For FACS analysis, the sources of Abs were as follows: anti-mouse F4/80 was purchased from Caltag Laboratories. Anti-mouse CD4, CD11c, CD14, CD40, CD44, CD54 (ICAM-1), CD80, CD86, Gr1, MHC class II (I-Ab), and IL-12p40 were purchased from BD Pharmingen.

Flow-cytometric data analysis

Cells were labeled with fluorescent Abs for surface molecules and intracellular cytokines as described previously (28). Samples were analyzed on a FACScan (BD Biosciences). FlowJo software was used for data analysis. A myeloid population was defined based on forward (FSC) and side scatter (SSC) layout that was confirmed by backgating with myeloid markers. A second gate was set to define macrophages based on F4/80− cells. A third gate was set for the cytokine expression. Similarly, IL-12 production by dendritic cell was analyzed by gating on CD11c+ cells. All gates (surface and intracellular cytokine) were set based on isotype control Abs coupled to the appropriate fluorescent label. Negative isotype control gates were set at or below 0.5%.

Protein microarray

For IL-6 and MCP-1 cytokine analysis, we used protein microarray services provided by Allied Biotech. Briefly, the services used a sandwich Ab-based protein detection multiplex assay. A streptavidin-Cy5 conjugate was used for assay detection. The assay was done in quadruplicate with the positive and negative controls spotted on each microarray. The assay was validated using conventional ELISA.

Macrophage cell cultures

Thioglycolate-induced macrophages were collected via peritoneal lavage on day 4 after 2.5-ml injection of thioglycolate broth i.p. Less than 3% of all cells were B220− and CD3+ lymphocytes. In the myeloid population (defined by FSC/SSC), up to 85% of cells were F4/80+ macrophages and 2–5% were CD11c+ F4/80+ dendritic cells. DCs expressed CD80, CD40, and high levels of class II (98% of cells were positive for I-Ab).

Thioglycolate-induced peritoneal exudate cells (PECs) were cultured in tissue culture-treated plates (1.5 × 106/ml) at 37°C and 5% CO2 in 10% FCS RPMI 1640, 5 × 10−5 M 2-ME, 0.5 mM sodium pyruvate, 10 mM HEPES buffer, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM l-glutamine. For ELISA, thioglycolate-induced peritoneal cells were allowed to adhere for 2 h, after which nonadherent cells were washed off to achieve a ~95% purity of macrophages. Adherent macrophages were preincubated with TNF-α followed by stimulation with IFN-γ (100 pg/ml) and LPS (1 ng/ml) for 16 h. Supernatants were collected and assayed for cytokine production via sandwich ELISAs. For ICC assay, cells were incubated in Costar low-adherence culture plates. Cells were preincubated with TNF-α for 16 h, followed by incubation with IFN-γ (100 pg/ml) and LPS (1 ng/ml) in the presence of monensin (1.5 μM) for another 10 h. Cells were fixed, permeabilized, and labeled with fluorescence Abs to surface molecules and intracellular cytokines as previously reported (28).

Dendritic cell cultures

Dendritic cells were cultured from bone marrow of C57BL/6 mice as described previously (29). Briefly, bone marrow cells were cultured in 15% FCS DMEM with GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) at 37°C and 10% CO2 for 7 days. Media was gently changed at days 2 and 4. On day 7, cells were cultured in tissue culture-treated plates (1.5 × 106/ml) (in the absence of GM-CSF and IL-4) and exposed to TNF-α, IFN-γ, and LPS as described for macrophages. Based on CD11c expression monitored by flow-cytometric analysis, dendritic cells comprised 60–70% of all cells in the culture. CD11c+ cells had low expression of class II (~40% of CD11c+ cells expressed I-Ab+), Bone marrow-derived dendritic cells expressed CD80 (74%) and CD86 (57%) costimulatory molecules. CD3− and B220− lymphocytes were present at <1% frequency. F4/80− macrophages were present in bone marrow culture at 1% as measured by flow-cytometric analysis.

Tg T cell cultures

CD4 T cells were enriched from spleens of OT-II TCR transgenic animals by using a naïve CD4 T cell mouse selection column (R&D Systems). Cells were incubated with thioglycolate–induced APCs at 1:1 ratio for 120 h in the presence of OVA323–339 peptide at 1 μg/ml. OVA peptide was generously donated by Dr. B. Evavold. Supernatant was collected and analyzed for IFN-γ by ELISA.

Listeria monocytogenes infection

In some experiments (see Fig. 6), peritoneal macrophages from infected mice were used. C57BL/6 mice were infected i.p. with an optimizing dose of 2 × 109 CFU/mouse (LD50 ≈ 9 × 106 CFU/mouse) L. monocytogenes wild-type strain 43251 (American Type Culture Collection). Bacterium were grown overnight in brain heart infusion broth (Difco Laboratories) at 37°C and then washed three times in PBS. PECs were collected on day 0 (naive), day 2, day 6, and day 10 postinfection. Cells were stained with macrophage maturation markers F4/80 and Gr1 (30). PECs were cultured as described above in the presence or absence of TNF-α and stimulated with IFN-γ and LPS. ELISA and ICC assays were used to measure IL-12p40 production. In one experiment (see Fig. 8a), mice were injected with 1 mg of ampicillin on day 1 postinfection and were also provided ampicillin in drinking water at 2 mg/ml for the remainder of the experiment (31).
Results

TNF-α inhibits the amount of IL-12p40 protein production as well as the frequency of IL-12p40-producing macrophages

TNF-α was added at a range of concentrations to the thioglycolate-induced macrophages and incubated for 16 h. After preincubation, macrophages were stimulated with IFN-γ and LPS for another 16 h. IL-12p40 production was measured by ELISA. IL-12p40 was inhibited dramatically at 30 ng/ml TNF-α from 12 to 2 ng/ml (Fig. 1A). TNF-α was also able to inhibit IL-12p40 induced by IFN-γ alone and LPS alone (Table I). This dose-dependant inhibitory effect confirmed the findings previously reported by Hodge-Dufour and Ma (24, 25).

To measure IL-12p40 regulation on a per-cell basis, cytokine production was measured via ICC. Macrophages were preincubated with TNF-α as described above and then stimulated with IFN-γ and LPS in the presence of monensin to prevent cytokine secretion. Cells were collected and stained for surface F4/80 and intracellular IL-12p40. TNF-α effectively reduced the frequency of IL-12p40-producing macrophages by as much as 70% (Fig. 1B).

TNF-α also lowered the IL-12p40 mean fluorescent intensity (MFI) of the macrophages that were still expressing the cytokine. The intracellular staining technique was informative, because it allowed us to largely prevent cross talk between cells present in culture. Protein secretion (i.e., cytokines) was prevented with monensin that was present in culture during the IFN-γ and LPS stimulation period. This system allowed us to conclude that the regulatory effect of TNF-α was clearly taking place in the first 16 h, before the addition of IFN-γ and LPS, and that it was not due to a secondary agent induced by LPS and IFN-γ.

The frequency of IL-12p40-expressing macrophages measured by ICC was directly related to the amount of IL-12p40 secreted measured by ELISA. Combined data from at least four independent experiments showed that, at 30 ng/ml TNF-α, both the amount of IL-12p40 secreted and the number of IL-12p40-producing macrophages was reduced by 70% (Fig. 1C). These data suggest that there are all-or-nothing cell differentiation events that lead to the cessation of IL-12p40 production.

Biologically active IL-12p70 is a heterodimer consisting of
IL-12p35 and IL-12p40 subunits. IL-12 shares its p40 subunit with proinflammatory cytokine IL-23 (26). IL-12p70 levels were reduced to a similar degree as IL-12p40 by TNF-α (Fig. 1E; Ref. 24), and, for the first time, we show that IL-23 production is regulated by TNF-α similarly to IL-12 (Fig. 1, E and F).

**TNF-α action is time dependent**

To examine the duration of TNF-α preincubation necessary to inhibit IL-12p40 production, macrophages were preincubated with TNF-α for various times, after which cells were stimulated with IFN-γ and LPS for an additional 16-h period. No inhibition was observed when IFN-γ and LPS were added at the same time with TNF-α. Some inhibition of IL-12p40 was seen after 8-h preincubation with TNF-α, and optimal inhibition was observed after exposure to TNF-α for 12–16 h before stimulation with IFN-γ and LPS (Fig. 2A).

IFN-γ did not reverse the inhibitory activity of TNF-α, nor did it have the same regulatory effect. Both cytokines were added to the culture at time 0; after 16-h incubation with TNF-α and IFN-γ, cells were stimulated with LPS for an additional 16 h. IL-12p40 production was again reduced in cells treated with TNF-α (Fig. 2B). Sustained TNF-α incubation was necessary for optimal inhibition of IL-12p40, and this inhibition was dominant over IFN-γ when the two cytokines were present at the same time.

**TNF-α specifically inhibits IL-12p40**

The effect of TNF-α preincubation on the production of other macrophage-derived inflammatory mediators was also investigated. TNF-α did not inhibit IL-6 or MCP-1 production as measured by cytokine multiplex assay (Fig. 2C). Previous studies done by Hodge-Dufour et al. (24) have shown that TNF-α can inhibit IL-6 production. The disparity of our findings could be attributed to the different stimuli used. In our system, thioglycolate-induced macrophages were preincubated with TNF-α for 16 h before stimulation with IFN-γ and LPS for another 16 h. In the previous study, macrophages were incubated with both TNF-α and IFN-γ for 16 h before stimulation with low m.w. hyaluronan for 8 h.

**IL-10 was not detectable in any of the treatment groups as measured by ELISA, cytokine microarray, and ICC (data not shown), suggesting that, in our system, IL-10 is not responsible for TNF-α-induced IL-12p40 inhibition. These data are consistent with findings by Hodge-Dufour et al. (24), where the authors showed that IL-12 production by macrophages derived from IL-10 KO mice were inhibited equally to wild type.**

**TNF-α inhibits IL-12p40 induced by various stimuli**

IL-12 can be induced by various stimuli, including bacterial Ags that bind pattern recognition receptors, such as TLRs, or by cellular inflammatory products, such as IFN-γ. To examine whether TNF-α inhibits various IL-12-inducing pathways selectively, we stimulated macrophages with several TLR ligands. TNF-α preincubation inhibited the IL-12p40 induced by all stimuli tested (Table I). These data suggest that TNF-α is a potent regulator of IL-12p40 and has a broad inhibitory effect on all Toll-like (signaling) pathways examined.

**IL-12p40 inhibition is not due to apoptosis caused by TNF-α**

To determine whether cell death played a role in IL-12p40 inhibition by TNF-α, the number of viable cells after TNF-α incubation was measured by an MTT assay. In keeping with other studies (24, 25), no significant difference in the number of viable cells was noted (data not shown). To more closely evaluate the possible role of apoptosis at a single-cell level, macrophages were stained with annexin V and propidium iodide (PI). Annexin V binds phosphatidylserine exposed on cell membranes of dead or apoptotic cells. PI labels the cell nucleus and is excluded from viable cells. The combined stain allowed us to distinguish between apoptotic, dead, and live macrophages. As shown in Fig. 3A, there was no significant difference in the number of dead cells (annexin V+PI+) or cells that were undergoing apoptosis (annexin V+PI+) in the various treatment groups. To address the possible early apoptosis induced shortly after the addition of TNF-α or immediately after the addition of IFN-γ and LPS, macrophages were stained with annexin V and PI at various times during the experiment. There was

### Table I. Inhibition of IL-12p40 induced by various stimuli

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Concentration (µg/ml)</th>
<th>Percent Inhibition of IL-12p40 (%)</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Ave ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γR</td>
<td>IFN-γ</td>
<td>10</td>
<td>88</td>
<td>68</td>
<td>55</td>
<td>70</td>
<td>16.6</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>2</td>
<td>29</td>
<td>55</td>
<td>17</td>
<td>34</td>
<td>19.4</td>
</tr>
<tr>
<td>TLR4</td>
<td>LTA</td>
<td>10</td>
<td>55</td>
<td>64</td>
<td>30</td>
<td>50</td>
<td>17.6</td>
</tr>
<tr>
<td>TLR2</td>
<td>Peptidoglycan</td>
<td>10</td>
<td>60</td>
<td>56</td>
<td>29</td>
<td>48</td>
<td>16.9</td>
</tr>
<tr>
<td>TLR3</td>
<td>Poly(I:C)</td>
<td>100</td>
<td>33</td>
<td>93</td>
<td>61</td>
<td>62</td>
<td>30.0</td>
</tr>
<tr>
<td>TLR9</td>
<td>Cpg DNA</td>
<td>1</td>
<td>40</td>
<td>25</td>
<td>49</td>
<td>38</td>
<td>12.1</td>
</tr>
</tbody>
</table>

* Thioglycolate macrophages were preincubated with 30 ng/ml TNFα followed by 16-h incubation with various stimuli. Ave, Average.

**FIGURE 2.** Time dependency and specificity of TNF-α action. A. Thioglycolate macrophages were preincubated with 30 ng/ml TNF-α for various times followed by 16-h incubation with IFN-γ and LPS. B. Macrophages were preincubated with both IFN-γ and TNF-α before the addition of LPS. A representative of three independent experiments is shown. C. MCP-1 and IL-6 production was measured via cytokine multiplex assay. An average of three independent experiments is shown. Arrow bars represent SD.
no significant increase in dead or apoptotic cells in the cells treated with TNF-α at any times during the experiment (Fig. 3B). Even if TNF-α selectively killed the IL-12p40-expressing cells, the sensitivity of the assay would allow us to detect this effect. For example, at least 14% of all cells would have to be eliminated by apoptosis, because TNF-α reduces the frequency of IL-12p40⁺ cells by at least this amount (14% based on all cells in culture, not gated on macrophages). Thus, these data allowed us to convincingly rule out apoptosis as the mechanism for TNF-α-induced inhibition of IL-12p40.

**Regulation of macrophage cell surface markers by TNF-α**

We next characterized the effect of TNF-α on macrophage function by measuring a panel of associated cell surface molecules. Macrophages were incubated with TNF-α for 16 h and then labeled with Abs to cell surface markers. Cell surface marker expression was analyzed based on MFI and expressed as a ratio of cells treated with TNF-α to cells incubated in medium alone (Table II). F4/80 MFI was slightly lower after incubation with TNF-α (reduced by 0.89 ± 0.04). The frequency of F4/80⁺ macrophages decreased from 67 to 59%. In contrast, CD11c MFI was induced about 2-fold after TNF-α incubation (1.89 ± 0.11). The frequency of CD11c⁺ dendritic cells increased from 1.6 to 3.9%. CD11b expression was reduced in two of three experiments (0.82 ± 0.21).

There was no considerable change in CD80 or I-Ab expression (1.12 ± 0.10 and 1.17 ± 0.24, respectively). However, CD40 and CD86 costimulatory molecule expression was induced by TNF-α (2.33 ± 0.13 and 1.5 ± 0.17). CD14, a receptor for LPS, expression was increased by TNF-α by 2-fold (2.22 ± 0.83). ICAM-1 expression was significantly increased by TNF-α (5.36 ± 2.62), in keeping with a known TNF-α-induced component of ICAM-1 promoter (32). Considering these data collectively, TNF-α appeared to cause a phenotypical shift in macrophages toward a dendritic cell phenotype. This shift in cell differentiation toward a dendritic lineage and the fact that dendritic cells are another important source of IL-12 production, prompted us to compare the IL-12 regulation by TNF-α in a dendritic cell population.

**IL-12p40 is inhibited by TNF-α in dendritic cells**

Thioglycolate-derived PECs contained up to 5% CD11c⁺F4/80⁻ cells. Approximately 20% of cells in this subset (CD11c⁺F4/80⁻) expressed IL-12p40 after 26 h incubation without exogenous stimuli. IFN-γ and LPS stimulation increased the frequency of IL-12p40⁺ cells to 60%. TNF-α preincubation significantly lowered the frequency of IL-12p40⁺ cells in this population by ~20% (Fig. 4A). This result prompted us to look at IL-12p40 production in a better-characterized DC population. Bone marrow-derived dendritic cells were preincubated with TNF-α and stimulated with IFN-γ and LPS. IL-12p40 protein production was measured by ELISA and the frequency of IL-12p40-producing cells was assayed by gating on CD11c⁺ cells. Both the protein secretion and the frequency of IL-12p40-producing cells were reduced by TNF-α in bone marrow-derived dendritic cells (Fig. 4B).

---

**Table II. Surface phenotype of macrophages treated with TNFα**

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Fraction Increase of MFI - MFI TNFα Treated / MFI Untreated</th>
<th>Ave ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80 Gated on F4/80⁺ macrophages</td>
<td>0.9 / 0.92 / 0.85</td>
<td>0.89 ± 0.04</td>
</tr>
<tr>
<td>CD11c</td>
<td>2.01 / 1.86 / 1.8</td>
<td>1.89 ± 0.11</td>
</tr>
<tr>
<td>CD11b</td>
<td>1.02 / 0.84 / 0.6</td>
<td>0.82 ± 0.21</td>
</tr>
<tr>
<td>CD40</td>
<td>2.22 / 2.47 / 2.3</td>
<td>2.33 ± 0.13</td>
</tr>
<tr>
<td>CD80</td>
<td>1.23 / 1.09 / 1.04</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>CD86</td>
<td>1.7 / 1.42 / 1.38</td>
<td>1.5 ± 0.17</td>
</tr>
<tr>
<td>I-Ab</td>
<td>1.43 / 1.13 / 0.96</td>
<td>1.17 ± 0.24</td>
</tr>
<tr>
<td>CD14</td>
<td>3.04 / 1.39 / 2.24</td>
<td>2.22 ± 0.83</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>7.97 / 2.73 / 5.38</td>
<td>5.36 ± 2.62</td>
</tr>
</tbody>
</table>

*Thioglycolate-induced PECs were treated with 30 ng/ml TNFα for 16 h or medium alone and then stained for surface markers. Ave, Average.
IL-12p40 is inhibited by TNF-α via TNFR1

Macrophages from C57BL/6 and TNFR-deficient mice were exposed to TNF-α for 16 h and then stimulated with IFN-γ and LPS, as described above. IL-12 was inhibited by TNF-α in both C57BL/6 and TNFR2−/−-derived macrophages (Fig. 5). However, macrophages deficient in TNFR1 were not susceptible to TNF-α-mediated inhibition of IL-12. At high concentrations of TNF-α, IL-12p40 inhibition was less in TNFR2−/− macrophages compared with C57BL/6 macrophages. Although this finding suggests that TNFR2 may also play a minor role in TNF-α-induced IL-12p40 inhibition, it is clear that TNFR1 is the main receptor responsible for IL-12p40 regulation.

Macrophages obtained under inflammatory conditions are more susceptible to TNF-α inhibition

IL-12p40 regulation by TNF-α was studied in macrophages obtained at various stages of maturation during Listeria infection. PECs were collected on days 0, 2, 6, and 10 after infection with L. monocytogenes. Based on cell surface marker analysis of peritoneal cells, there were dramatic changes in macrophage populations (30). Specifically, by day 2, most macrophages present in peritoneal cavity exhibited an immature, highly activated phenotype of F4/80−Gr1−, unlike in naive mice, where macrophages preferentially exhibited a mature F4/80+Gr1+ phenotype (Fig. 6A). By day 6, mature F4/80+Gr1+ macrophages began accumulating in the peritoneal cavity, and by day 10 the macrophage phenotype was largely similar to that of the naive mouse. Adherent PECs collected at different times during infection were preincubated with TNF-α and stimulated with IFN-γ and LPS. We found that TNF-α inhibited IL-12p40 production in all macrophage populations (Fig. 6B).

The difference in response of activated and mature macrophages prompted us to investigate IL-12p40 regulation in vivo under infectious conditions. Listeria-infected and naive mice were treated with TNF-α or PBS. Twenty-five hours subsequent to initiation of treatment, mice were injected with IFN-γ and LPS, to mimic the in vitro system. Mice that were previously infected with Listeria had higher levels of IL-12p40 in both serum and peritoneal fluid. However, only in infected mice, IL-12p40 levels were inhibited by TNF-α injections. Naive mice were resistant to TNF-α-induced IL-12p40 regulation (Fig. 7, A and B). Our in vivo data supporting the inhibition of IL-12 by TNF-α provides strong evidence for the biological significance of our in vitro macrophage studies. This is the first time that direct injection of exogenous TNF-α has been shown to result in the decrease of IL-12 in vivo. Previous in vivo studies showing a decrease in IL-12 were performed in TNF-α or TNFR KO animals, where lymphoid organogenesis was impaired and could influence cellular function.

FIGURE 4. TNF-α inhibits IL-12p40 production by DCs. A, Thioglycollate-induced peritoneal cells were preincubated with TNF-α (30 ng/ml) for 16 h and later stimulated with IFN-γ (100 pg/ml) and LPS (1 ng/ml) in the presence of monensin for 10 h. Cells were collected and labeled with Abs against surface CD11c, F4/80, and intracellular IL-12p40. The analysis is gated on CD11c+ F4/80+ cells. An average of three experiments is shown; error bars represent SD. One-way ANOVA and Newman-Keuls multiple comparison test were used for statistical analysis. B, Bone marrow-derived dendritic cells were preincubated with TNF-α (30 ng/ml) and stimulated with IFN-γ (100 pg/ml) and LPS (1 ng/ml). IL-12p40 production was measured by ELISA and ICC. A representative experiment of three is shown.

FIGURE 5. IL-12p40 is inhibited by TNF-α via TNFR1. Thioglycolsate-induced adherent macrophages from C57BL/6, TNFR1−/−, and TNFR2−/− mice were incubated with TNF-α, followed by stimulation with IFN-γ (100 pg/ml) and LPS (1 ng/ml). Supernatants were analyzed for IL-12p40 via ELISA. A representative experiment of three is shown; error bars represent variation in three ELISA replicate wells.
this problem, we injected mice with ampicillin on day 1 postinfection and supplied the antibiotic in drinking water for the following 9 days (31). Consistent with other findings, we have observed an increase in the activation markers in TNFR-deficient mice at day 10 postinfection compared with wild-type controls (34, 35). CD4 T cells from TNFR-deficient animals had an increased expression of CD44 and a decrease in CD62L expression (Fig. 8A).

TNF-α pretreatment reduced the effectiveness of macrophages as APCs. Thioglycolate-induced macrophages were incubated for 16 h with 30 ng/ml TNF-α pretreatment before the addition of OT-II cells and OVA323–339 peptide. On day 5 of T cell expansion, IFN-γ levels produced by T cells that were cultured in the presence of TNF-α pretreated APCs were reduced by >50% (Fig. 8B).

**Discussion**

The current study demonstrates, both in vivo and in vitro, the acquired sensitivity of macrophages and dendritic cells to TNFR1-mediated regulation by TNF-α. The inhibitory effect of TNF-α on IL-12 production serves as a dampening influence on the otherwise-strong bidirectional amplification loop of IL-12 and IFN-γ that is initiated by macrophages and NK cells, and then further amplified by T cells. We argue that TNF-α, a cytokine that is produced by activated macrophages and T cells during a Th1-type response, is a key feedback regulator of IL-12 and, indirectly, IFN-γ. This view is supported by the findings of unchecked inflammation and immunopathology in mice lacking TNF-α or TNFRs (13, 23, 33).

The anti-inflammatory actions of TNF-α have been previously reported where TNF-α was required for limiting the duration of the inflammatory response and preventing tissue damage by inducing apoptosis of activated Ag-specific lymphocytes (20, 33). In several infectious models, mice lacking TNF-α or TNFRs exhibited a disorganized inflammatory response characterized by increased IL-12 serum levels as well as an increase in IL-12 levels (13, 23, 33). TNFR1−/− mice succumb to infection with *Mycobacterium avium* as a direct result of high IL-12 serum levels as well as an increase in CD4 and CD8 T cells at the site of granuloma disintegration (13). Similar immune disregulation, characterized by increased numbers of type 1 CD4 and CD8 T cells in addition to elevated...
The prolonged culture of macrophages in TNF-α required for optimal IL-12 inhibition is indicative of major intracellular events, and, possibly, cellular differentiation. In accordance, exposure of macrophages to TNF-α induces a shift in phenotype toward a distinct differentiation state. After incubation in TNF-α, there is a 2-fold increase in CD11c expression and a decrease in F4/80 (Table II). Recent literature has demonstrated that TNF-α is a differentiation factor for dendritic cells (37, 38). It is possible that the phenotypical shift we observe is due to partial activation of DC differentiation pathway.

Optimal Th1 differentiation in vitro is achieved by mature DCs. Interestingly, completely matured DCs are impaired in IL-12 production. This end maturation state is achieved after prolonged exposure (24 h) of DCs to TNF-α, LPS, or CD40L and is counteracted by IFN-γ (39). Our data show that prolonged 16-h exposure of DCs as well as macrophages to TNF-α confers similar effects on IL-12 production. Another study has reported that prolonged exposure of macrophages to CD40L will also abrogate their ability to produce IL-12 at high levels (40). It seems that a specific cytokine milieu that is present during APC activation is responsible for controlling the degree and duration of IL-12 production (39, 41–43). The fact that this IL-12 regulation pathway is present in both macrophages and dendritic cells and affects multiple Toll-like pathway activation implies the importance of TNF-α as a major regulator of Th1-biased cytokines.

LPS-induced tolerance occurs when cytokine production is inhibited upon secondary restimulation of macrophages with LPS. It has been suggested that secondary mediators produced by monocytes in response to LPS are responsible for tolerization. This idea is supported by the fact that in vivo administrations of TNF-α and IL-1 before injection of LPS will protect mice against sepsis (44, 45). Mice lacking TNFRp55 are resistant to lower doses of LPS (46, 47). The IL-12 inhibition by TNF-α might have a direct correlation to LPS tolerance. In accordance with our data, others have shown that LPS tolerance results in an increase of CD14 and CD54 expression, a decrease in CD11b (48–51). Although there are obvious similarities between LPS tolerance and the IL-12 regulation that we have defined in our studies, there are some mechanistic differences. IL-12 is reduced in TNFR−/− mice upon LPS-induced tolerization, suggesting that TNF-α is not the main or only mediator of LPS-induced tolerance (52). Several studies have suggested that the mechanism of LPS tolerance is due to accumulation of inhibitory NF-κBp50 homodimers at the promoter sites. This is supported by the fact that mice-deficient in p50 are resistant to LPS tolerance (51, 53). However, Ma et al. (25) has shown evidence that, in TNF-α-inhibited macrophages, the NF-κB complexes are equally composed of p50 and p65 subunits.

IL-12p70 is a heterodimer of two subunits that are located under separate transcription controls and are differentially regulated (54). The IL-12p40 promoter has been extensively studied and contains several enhancer and suppressor sites that respond to STAT1/2, MyD88, IL-4, and PGE2 signaling (54). Our laboratory and others have shown that TNF-α inhibits IL-12p70 heterodimer with similar magnitude to IL-12p40 (Fig. 1E and Ref. 24). IL-12p40 homodimer has been shown to act as a IL-12p70 antagonist by binding to IL-12R without inducing biological effects (55). Because TNF-α inhibits IL-12p40 to the same degree as the biologically active IL-12p70, it is unlikely that IL-12p40 homodimer accounts for TNF-α-mediated inhibition.
In our studies, we defined TNFR1 as the main receptor responsible for IL-12p40 inhibition. TNF-α did not affect IL-12p40 production by macrophages deficient in TNFR1 (Fig. 5). IL-12 was inhibited in macrophages lacking TNFR2. In addition, TNF-α concentration resulting in 50% inhibition of IL-12p40 is directly related to K_50 of TNFR1 (TNF-α at 5 ng/ml = 294 pM; TNFR1 K_50 = 300 pM), implying that the degree of receptor occupancy is directly related to the inhibitory effect.

Recent studies in TNF-α or TNFR-deficient murine infectious models suggest temporally distinct roles for TNF-α. In several models of inflammation, in the absence of TNF-α or its receptor, the inflammatory response is delayed. However, following an infection, there is a disproportionate inflammatory response (56). In a murine model of autoimmune diabetes, the exact timing of TNF-α expression determined whether the cytokine played a protective or a pathologic role (17, 18). It is also becoming increasingly evident that TNF-α exhibits a regulatory function as well as a pathogenic function in the development of MS. In the EAE model of MS, TNF-α-deficient mice developed a delay in the onset of disease; however, once developed, the disease was more severe compared with wild-type control (19). We postulate that TNF-α is involved in initiating the inflammatory response early during an infection. Vasodilation and cell recruitment are some of the early proinflammatory actions of TNF-α. Later in the infection, TNF-α is able to remove the inflammatory T cells through apoptosis and further regulate the adaptive immune response via inhibition of IL-12 and IL-23.

To address the temporal functions of TNF-α, we studied IL-12p40 regulation in macrophages obtained at various times after Listeria infection. We hypothesized that macrophages obtained later in the infection will be predisposed to inhibition by TNF-α. Our data showed that macrophages obtained under various inflammatory conditions are all susceptible to TNF-α (Fig. 6B). However, IL-12p40 production by naive macrophages was significantly less inhibited by TNF-α (Fig. 6C). Similar results were acquired in our in vivo system, where IL-12p40 serum and peritoneal levels were inhibited by TNF-α injections in mice that were previously infected with Listeria, but not in naive mice. Suffice it to say, the mechanism of temporal regulation of IL-12p40 is unclear, it is obvious that TNF-α is a potent regulator of IL-12 and IL-23, and its effect is amplified during inflammatory conditions in various cells of myeloid origin. Clearly, TNF-α inhibitory effect is amplified during an inflammatory response. Our findings support a dual role of TNF-α as an initiator of inflammatory response early in the infection and selective regulator of inflammatory response in the later stages of inflammation.

In this paper, we have defined TNF-α as a potent regulator of APC function during an inflammatory response. TNF-α induces change in cell function as shown by changes in cell surface molecule expression, such as an increase in CD11c expression, and simultaneous reduction in proinflammatory cytokines IL-12 and IL-23. This negative regulatory loop is essential in controlling the inflammatory response and is augmented during infection. These data correlate with a dichotomous role of TNF-α recently revealed as a result of anti-TNF-α treatment in MS and RA patients, further exposing the intricate balance of the inflammatory response.

Disclosures
The authors have no financial conflict of interest.

References
7. Mohan, N., E. T. Edwards, T. R. Cupps, P. J. Oliverio, G. Sandberg, H. Crayton, J. R. Schlessinger, and J. N. Siegel. 2001. Demethylaminating and IL-12p40 serum and peritoneal levels were inhibited by TNF-α in our in vivo system, where IL-12p40 serum and peritoneal levels were inhibited by TNF-α. We postulate that TNF-α is involved in initiating the inflammatory response early during an infection. Vasodilation and cell recruitment are some of the early proinflammatory actions of TNF-α. Later in the infection, TNF-α is able to remove the inflammatory T cells through apoptosis and further regulate the adaptive immune response via inhibition of IL-12 and IL-23.

To address the temporal functions of TNF-α, we studied IL-12p40 regulation in macrophages obtained at various times after Listeria infection. We hypothesized that macrophages obtained later in the infection will be predisposed to inhibition by TNF-α. Our data showed that macrophages obtained under various inflammatory conditions are all susceptible to TNF-α (Fig. 6B). However, IL-12p40 production by naive macrophages was significantly less inhibited by TNF-α (Fig. 6C). Similar results were acquired in our in vivo system, where IL-12p40 serum and peritoneal levels were inhibited by TNF-α injections in mice that were previously infected with Listeria, but not in naive mice. Suffice it to say, the mechanism of temporal regulation of IL-12p40 is unclear, it is obvious that TNF-α is a potent regulator of IL-12 and IL-23, and its effect is amplified during inflammatory conditions in various cells of myeloid origin. Clearly, TNF-α inhibitory effect is amplified during an inflammatory response. Our findings support a dual role of TNF-α as an initiator of inflammatory response early in the infection and selective regulator of inflammatory response in the later stages of inflammation.

In this paper, we have defined TNF-α as a potent regulator of APC function during an inflammatory response. TNF-α induces change in cell function as shown by changes in cell surface molecule expression, such as an increase in CD11c expression, and simultaneous reduction in proinflammatory cytokines IL-12 and IL-23. This negative regulatory loop is essential in controlling the inflammatory response and is augmented during infection. These data correlate with a dichotomous role of TNF-α recently revealed as a result of anti-TNF-α treatment in MS and RA patients, further exposing the intricate balance of the inflammatory response.

Disclosures
The authors have no financial conflict of interest.

References
7. Mohan, N., E. T. Edwards, T. R. Cupps, P. J. Oliverio, G. Sandberg, H. Crayton, J. R. Schlessinger, and J. N. Siegel. 2001. Demethylaminating and IL-12p40 serum and peritoneal levels were inhibited by TNF-α. We postulate that TNF-α is involved in initiating the inflammatory response early during an infection. Vasodilation and cell recruitment are some of the early proinflammatory actions of TNF-α. Later in the infection, TNF-α is able to remove the inflammatory T cells through apoptosis and further regulate the adaptive immune response via inhibition of IL-12 and IL-23.


