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TNF Enhances CD4⁺ T Cell Alloproliferation, IFN- γ Responses, and Intestinal Graft-Versus-Host Disease by IL-12-Independent Mechanisms¹

Geri R. Brown,^{2*‡} Edward L. Lee,^{†‡} and Dwain L. Thiele*

Inhibition of TNF/TNFR2 interactions ameliorates intestinal graft-vs-host disease (GVHD) and Th1 cytokine responses induced by transfer of B6 CD4⁺ spleen cells into irradiated MHC class II disparate B6.C-H-2^{bm12} (bm12) \times B6 F₁ recipients. The present studies examined whether these effects of TNF are IL-12 dependent. T cell proliferative responses of B6.129S1-IL-12rb2^{tm1Jm} (B6.IL-12R^{-/-}) responder spleen cells were found to be comparable to those of control B6 spleen cells. TNF inhibition reduced T cell proliferation and IFN- γ production in supernatants of MLC using either B6.IL-12R^{-/-} or control B6 responder cells. GVHD induced wasting disease in recipients of B6.IL-12R^{-/-} CD4⁺ spleen cells that received a TNF inhibitor-encoding adenovirus (5.4 \pm 6.5% weight loss ($n = 7$)) was significantly reduced compared with levels of weight loss observed in recipients that had received a control adenovirus (25.7 \pm 12.2% weight loss ($n = 11$), $p = 0.001$). Furthermore, TNF inhibition was associated with a reduction in colonic GVHD scores ($p = 0.039$) and in the percentage of the splenic CD4⁺ T cells that expressed IFN- γ (16 vs 6%). These findings indicate that TNF promotes CD4⁺ T cell alloproliferation, IFN- γ responses, and intestinal GVHD by IL-12-independent mechanisms. *The Journal of Immunology*, 2003, 170: 5082–5088.

Allogeneic bone marrow transplantation (BMT)³ is the treatment of choice for many malignant conditions. Acute graft-vs-host disease (GVHD) is a major obstacle to successful outcomes after allogeneic BMT (1). Gastrointestinal involvement is a major cause of morbidity and mortality in human GVHD. Animal models of GVHD have been used in determining the pathogenesis of intestinal GVHD. In many murine models of intestinal GVHD, including the DBA/2J \rightarrow B6D2F₁ and the B6 \rightarrow B6 \times bm12 F₁ GVHD models, CD4⁺ T cells play a prominent role in the pathogenesis (2–4).

Multiple clinical and experimental observations indicate that cytokine dysregulation occurs during acute GVHD (5). A general hypothesis offered as an explanation for this phenomenon is that donor T cells encounter allogeneic histocompatibility Ags on host tissues and, in the presence of IL-12, secrete the Th1 cytokines, IFN- γ and IL-2 (6). IFN- γ primes monocytes and macrophages to secrete large amount of proinflammatory cytokines, including TNF- α . Prevention of acute GVHD while retaining the mature T cells in the bone marrow graft may be possible if the amplification of inflammatory cytokine effectors is disrupted. Randomized clinical trials using novel cytokine inhibitors that neutralize TNF- α are currently in progress as adjuncts to GVHD prophylaxis (7). Im-

portantly, Holler et al. (8) has reported that pretreatment with an anti-TNF Ab that neutralizes TNF during the pretransplant conditioning regimens significantly postpones development of acute GVHD. This finding suggests that blocking early release of TNF may affect a cascade that includes the up-regulation of host cell surface receptors and activation of donor IL-2- and IFN- γ -producing T cells.

TNF/TNFR interactions appear to be important in the development of GVHD (9–13). In previous studies, TNF blockade has been noted to ameliorate the development of intestinal GVHD in the DBA \rightarrow B6D2F₁ MHC class I and II disparate model (11). TNF and its family members appear to influence intestinal inflammation induced by GVHD by promoting a Th1 cytokine profile (IL-2, IFN- γ) (12). Furthermore, TNF/TNFR2 interactions are important for the development of intestinal inflammation and activation/differentiation of Th1 cytokine responses by intestinal lymphocytes in MHC class II disparate GVHD (13). The current studies were undertaken to understand the mechanisms responsible for the reduction in Th1 cytokines by TNF blockade during GVHD.

Polarized Th1 and Th2 cytokine responses can largely be explained by production of subset-specific cytokines. The hallmark cytokine of Th1 cells is IFN- γ . Th1 polarization is initially signaled by the TCR/CD3, after its interaction with Ag/MHC on APCs. Importantly, besides these initiating signals, the critical cytokine postulated to control Th1 differentiation is IL-12 (14–23). IL-12 is a heterodimeric molecule consisting of p35 and p40 subunits, which is secreted predominantly by activated professional APCs such as dendritic cells and activated macrophages. IL-12 signaling through the IL-12R on T cells induces high levels of IFN- γ production through STAT4 signaling (14). Importantly, not all IFN- γ production by T cells is STAT4 dependent. STAT6/STAT4 double-deficient T cells produce some IFN- γ (24). In addition, IL-18, an IL-1-related factor (25–27), has been shown to be a selective activator of IFN- γ responses in Th1, but not Th2, cells.

The relationship between the TNF and polarization of T cells toward Th1 cytokine profile is thought to be indirect. TNF activates macrophages and induces IL-12 release, which favors Th1

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³ Abbreviations used in this paper: BMT, bone marrow transplantation; GVHD, graft-vs-host disease.

response (6). In addition, it has been observed that preincubation of APCs with rTNF enhances APC-induced T cell IFN- γ production, while TNF synthesis inhibitors decrease IFN- γ production (28).

The present studies examined whether TNFRs can independently signal IFN- γ production in donor T cells during the development of MHC class II disparate GVHD or in MHC class II disparate MLC. The need for IL-12 during TNF-mediated effects on alloresponses and IFN- γ production was determined by assessing IFN- γ , IL-12, and IL-18 levels after TNF blockade in a MHC class II disparate MLC and by use of control vs IL-12R-deficient donor cells in GVHD models. Of note, TNF blockade decreased alloproliferation and IFN- γ responses in MLC using IL-12R-deficient responder T cells, but had little or no effect on IL-12 or IL-18 responses. In addition, TNF blockade ameliorated GVHD wasting disease, colonic disease, and the percentage of T cells expressing IFN- γ in the recipients of IL-12R-deficient donor T cells. Thus, the potentiating effects of TNF on Th1 cytokine responses during GVHD appear to be mediated by IL-12R-independent mechanisms.

Materials and Methods

Mice

C57BL/6J (B6) and B6.C-H-2^{bm12} (bm12) and B6.129S1-IL-12rb2^{tm1Jm} (B6.IL-12R^{-/-}) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free environment. B6 males and bm12 females were bred to produce an F₁ strain (bm12 \times B6 F₁).

Isolation of spleen cells

Spleen cells were harvested by surgically removing the spleen and then mincing and disrupting the spleen over a funnel covered with nylon mesh and washing repeatedly with HBSS into a 50-ml conical tube (29). After the suspension was centrifuged at 600 \times g for 10 min, the cell pellet was resuspended in complete medium and counted.

Mixed lymphocyte cultures

Triplicate wells of control B6 or B6.IL-12R^{-/-} responder spleen cells (3×10^5) were cultured with irradiated MHC class II disparate bm12 (3×10^5) or irradiated syngeneic stimulator cells (3×10^5) in the presence or absence of a chimeric TNF inhibitor protein (0.5 μ g/ml) for 3 days before assay of [³H]thymidine incorporation, as previously described (30).

ELISA

IL-12, IL-18, and IFN- γ levels in the MLC supernatants were assessed. Seventy-two hours after initiation of the culture, supernatants from triplicate MHC class II disparate MLC were transferred to anti-CD3 Ab (0.5 μ g/ml)-coated plates for overnight stimulation. Supernatants were then removed and assayed using ELISA for IL-12, IL-18, and IFN- γ (BD Pharmingen, San Diego, CA). Plates were coated with 100 μ l/well of the diluted capture Ab for the cytokine during overnight incubation at 4°C, and then after washing to remove excess capture Ab, between 50 and 100 μ l of the supernatants or medium was added to triplicate wells and incubated for 2 h. After washing, the enzyme reagent was added to the wells and incubated, substrate solution was added and incubated, and the stop solution was added. The OD was read at 450 nm, as previously described (30). The recommended lowest dilution of the IL-4 standard used in the ELISA from BD Pharmingen assay is 7.8 pg/ml. Standard dilutions were above background and demonstrated linearity to 1.95 pg/ml.

Transplantation

On the day of transplantation, the age- and sex-matched B6 \times bm12 F₁ (H-2^{b/bm12}) recipients were irradiated (900 cGy) and 2 h later were injected via the lateral tail vein with donor cells. A total of 4–5 $\times 10^6$ CD4⁺ T cell-enriched spleen cells and 2–3 $\times 10^6$ T cell-depleted bone marrow cells from B6.IL-12R^{-/-} mice was injected into the lateral tail vein of these lethally irradiated allogeneic B6 \times bm12 F₁ mice, as previously described (31). Within 1 h after transplantation, 1 $\times 10^{10}$ PFU of the TNF inhibitor-encoding or β -galactosidase-encoding adenovirus was injected into the lateral vein of the transplant recipients, respectively (11). Recipients were maintained on acidified (pH 2) antibiotic (100 mg/L neomycin and 10 mg/L polymyxin B) H₂O for 7 days after transplantation. The TNF inhibitor adenoviral system generates an endogenous chimeric TNF inhibitor protein, consisting of the extracellular domain of the human 55-kDa TNFR

fused to the H chain of mouse Ig (32, 33). The soluble, secreted TNF inhibitor protein binds TNF at high affinity with specific neutralizing activity 100-fold higher than that mediated by known Abs (33). Following infusion of the TNF inhibitor adenovirus, the plasma TNF inhibitor concentration is maintained at high levels for 60 days (32).

Splenic B6.IL-12R^{-/-} CD4⁺ T cells were enriched, as previously described. Briefly, spleen cells were incubated with anti-CD8 Abs, anti-RBC, and B cell Abs (J11D.2) for 30 min at 4°C, then incubated with adsorbed rabbit complement at 37°C for 30 min, and passed over a nylon wool column (11–13). Weight loss was evaluated at 10–14 days after transplantation.

Histological scoring of intestinal GVHD

Individual recipients of the B6.IL-12R^{-/-} CD4⁺ spleen cells and bone marrow cells were evaluated according to the scoring system used by Snover (34, 35). The large intestine was isolated by removing all of the intestine distal to the appendix, and 2-cm sections were cut, fixed in Formalin, and then stained with H&E. Peyer's patches were removed from the small intestine, and then 2-cm sections from entire small intestine were cut from the duodenum to the ileum, fixed in Formalin, and stained with H&E. Specifically, colonic GVHD was reported as grade 1 with evidence of increased apoptosis, grade 2 with evidence of cystically dilated crypts containing necrotic debris with individual crypt loss, grade 3 with loss of multiple crypts with preservation of surface epithelium, and grade 4 with complete loss of epithelium (not observed in the study). The pathologist reviewing the slides was blinded to which animals were assigned to each of the experimental protocols.

Intracellular staining

For assessment of intracellular IFN- γ , GVHD spleen cells were harvested 10–14 days after BMT and incubated in CD3⁺ Ab-coated plates in a 5% CO₂, 37°C incubator overnight. Brefeldin A was added for 4 h before harvest. Cells were labeled with the FITC-labeled CD4⁺ or control Abs and then fixed with 4% formaldehyde at room temperatures for 10 min. After fixation, the cells were incubated on ice for 1 h with saponin-containing medium to permeabilize the membranes. The PE-labeled, anti-IFN- γ (XMG1.2) Ab or the control Ab was added and incubated at 4°C for 1 h (36). This was followed by two washes with saponin-containing medium and one final wash with normal staining medium. The cells were analyzed by fluorescence-activated flow cytometry on FACSscan. In preliminary experiments, <2% of splenic CD4⁺ T cells from control mice without GVHD exhibited IFN- γ after overnight anti-CD3 stimulation.

Statistical analysis

GVHD induced weight loss, and survival data were analyzed by the Mann-Whitney rank sum nonparametric test, while the cellular experiments were analyzed by Student's *t* test.

Results

Decreased proliferation of B6.IL-12R^{-/-} splenic T cell is observed in MHC class II disparate MLC with the addition of a chimeric TNF inhibitor protein

B6.IL-12R^{-/-} or B6 responder spleen cells were cultured with irradiated MHC class II disparate bm12 spleen cells or syngeneic stimulator cells with or without a TNF inhibitor protein (0.5 μ g/ml) for 3 days before assay of [³H]thymidine incorporation. As detailed in Fig. 1, there was a significant (*p* < 0.05 in all experiments) decrease in proliferation after the addition of the TNF inhibitor protein to MLC using B6 responder cells (mean inhibition 68%, range 44–80%) or to MLC using B6.IL-12R^{-/-} responder cells (mean inhibition 51%, range 41–58%). These results indicate that TNF plays a role in potentiating MHC class II alloantigen-stimulated proliferation of both IL-12R-deficient and wild-type B6 spleen cells.

TNF inhibition diminishes IFN- γ production by B6.IL-12R^{-/-} responder spleen cells in MHC class II disparate MLC

TNF blockade in alloresponses by wild-type mouse T cells is associated with a decrease in Th1 responses (12, 30). Additional studies were conducted to determine whether the IFN- γ production is decreased after TNF blockade in MHC class II disparate MLC

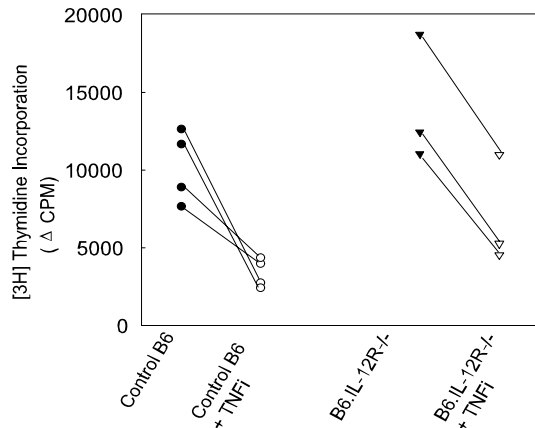


FIGURE 1. TNF inhibition decreases B6.IL-12R^{-/-} T cell alloproliferative responses in MHC class II disparate MLC. A total of 3×10^5 IL-12R^{-/-} splenic T cells was cultured with irradiated MHC class II disparate bm12 or syngeneic stimulator cells for 3 days in the presence or absence of the TNF inhibitor protein before assay of [³H]thymidine incorporation. The concentration of the chimeric TNF inhibitor was $0.5 \mu\text{g/ml}$. $\Delta \text{cpm} = \text{mean } [^3\text{H}] \text{thymidine incorporation allogeneic} - \text{mean } [^3\text{H}] \text{thymidine incorporation syngeneic}$.

using B6.IL-12R^{-/-} splenic T cells. As illustrated by the results of the representative experiment detailed in Fig. 2, TNF blockade lowers the levels of IFN- γ in the supernatants of MHC class II disparate MLC using B6 splenic T cells (58% decrease). Of note, relative to levels observed in MLC containing wild-type responder T cells, there is significantly less IFN- γ production in MLC containing B6.IL-12R^{-/-} responder spleen cells (77% reduction). Importantly, TNF blockade further reduced the IFN- γ production (81%) in MLC using B6.IL-12R^{-/-} responder T cells. These data suggest that TNF/TNFR interactions stimulate IFN- γ production in MHC class II disparate MLC independent of IL-12/IL-12R interactions on responder T cells.

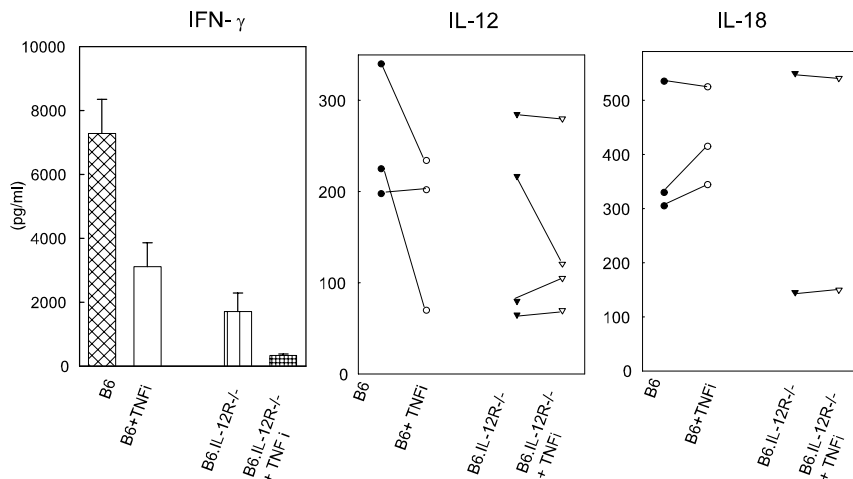


FIGURE 2. TNF inhibitor decreases IFN- γ production in MHC class II disparate MLC using either control B6 or B6.IL-12R^{-/-} responder spleen cells. However, IL-12 production is minimally reduced and IL-18 production is unchanged after TNF blockade. Control B6 or B6.IL-12R^{-/-} spleen cells were initially stimulated with irradiated bm12 spleen cells for 72 h and then stimulated with immobilized anti-CD3 for 12 h. The supernatants were harvested and assessed for IFN- γ content, IL-12, and IL-18. IFN- γ production by IL-12R-deficient responder cells was significantly lower than that of wild-type B6 responders ($p = 0.010$). TNF blockade significantly reduced both B6 and B6.IL-12R^{-/-} spleen cell IFN- γ production ($p = 0.016$ and 0.05 , respectively). The results of this experiment are representative of three additional experiments. The supernatants were harvested and assessed for IL-12 and IL-18 content. Data points for the IL-12 and IL-18 data reflect mean values from triplicate determinations in individual experiments.

IL-18 production is unchanged after TNF blockade in MHC class II disparate MLC using B6 or B6.IL-12R^{-/-} responder spleen cells

The critical cytokine that has been reported to control Th1 differentiation and IFN- γ production is IL-12 (14–23). In addition, IL-18, an IL-1-related factor (25–27), has been shown to be a selective activator of IFN- γ in Th1, but not Th2, cells. To assess whether IL-12 or IL-18 production was modulated by TNF blockade, we assessed the concentrations of these two cytokines in the supernatants of these MHC class II disparate MLC.

As illustrated by the results of experiments detailed in Fig. 2, TNF blockade decreased IL-12 content modestly in some, but not all experiments using either control B6.IL-12R^{-/-} or B6 spleen cells. In contrast, IL-18 production was unchanged after TNF blockade in MHC class II disparate MLC. These data suggest that TNF/TNFR interactions have a modest effect on IL-12 production, but no effect on IL-18 production in MHC class II disparate MLC.

In other experiments, ELISA were performed to assess the effect of TNF blockade on IL-4 content in the supernatants of MHC class II disparate MLC using control B6 or B6.IL-12R^{-/-} spleen cells. Minimal levels of IL-4 (all less than 5 pg/ml) were noted in the supernatant of MLCs using control B6 or B6.IL-12R^{-/-} spleen cells with or without TNF blockade.

TNF inhibition prevents GVHD in MHC class II disparate recipients of IL-12R-deficient donor cells

To determine whether the role of TNF in MHC class II disparate GVHD is IL-12 dependent, we assessed the effect of TNF blockade in a MHC class II disparate GVHD model involving transfer of donor cells that are IL-12R deficient (B6.IL-12R^{-/-}) to bm12 \times B6 F₁ mice. Splenic CD4⁺ T cells and T cell-depleted bone marrow cells from B6.IL-12R^{-/-} were infused into lethally irradiated semiallogeneic MHC class II disparate bm12 \times B6 F₁ mice. A total of 1×10^9 PFU of TNF inhibitor-encoding or control adenovirus was injected into the lateral tail vein of the bone marrow transplant recipients at the time of transplantation. Ten to fourteen days after transplant, bm12 \times B6 F₁ recipients of the B6.IL-

12R^{-/-} CD4⁺ T cells and bone marrow cells that received the adenovirus encoding the TNF inhibitor exhibited less weight loss than recipients of the control adenovirus ($5.4 \pm 6.5\%$ ($n = 7$) vs $25.7 \pm 12.2\%$ ($n = 11$), $p = 0.001$) (Fig. 3). In addition, GVHD mortality was delayed by TNF blockade in bm12 \times B6 F₁ recipients of IL-12R^{-/-} CD4⁺ T cells (Fig. 4).

In additional studies, H&E-stained sections of colon were obtained 14 days after bm12 \times B6 F₁ mice were injected with the B6.IL-12R^{-/-} donor CD4⁺ splenic T cells and bone marrow cells, and histopathology was graded, as previously described (13). Intestinal GVHD in bm12 \times B6 F₁ recipients of control B6.IL-12R^{-/-} donor CD4⁺ T cells and the control adenovirus was manifested by evidence of apoptosis, cystically dilated crypts containing necrotic debris with individual crypt loss, and damaged epithelium (Fig. 5A). In contrast, the large intestine from bm12 \times B6 F₁ recipients of the TNF inhibitor-encoding adenovirus had nearly normal intestinal pathology (Fig. 5B). The large intestines isolated from the bm12 \times B6 F₁ recipients of the B6.IL-12R^{-/-} CD4⁺ T cells and the control adenovirus had evidence of a higher grade of colonic GVHD (1.5 ± 0.188 , $n = 10$) than recipients of the B6.IL-12R^{-/-} CD4⁺ T cells and the TNF inhibitor adenovirus (0.333 ± 0.051 , $n = 11$, $p = 0.039$) (Fig. 6). These results indicate that TNF/TNFR interactions are important for the development of MHC class II disparate intestinal GVHD induced by B6.IL-12R^{-/-} CD4⁺ T cells.

TNF inhibition decreases the number of infiltrating donor T cells in the large intestine in recipients of the B6.IL-12R^{-/-} CD4⁺ T cells

In addition to the analysis of colonic GVHD detailed in Figs. 5 and 6, other studies were performed to assess the degree of T cell infiltration into the intestine. Intestinal lymphocytes were removed from the small and large intestines of bm12 \times B6 F₁ recipients of control or IL-12R-deficient donor cells 10–14 days after BMT.

As demonstrated by the data displayed in Fig. 7, no statistically significant difference was noted in the total number of CD3⁺ T cells in the spleen ($p = 0.653$) or small intestine ($p = 0.48$) of recipients of the control or the TNF inhibitor-encoding adenovirus. However, significantly fewer CD3⁺ were obtained from the large intestine of the bm12 \times B6 F₁ recipients of B6.IL-12R^{-/-} CD4⁺

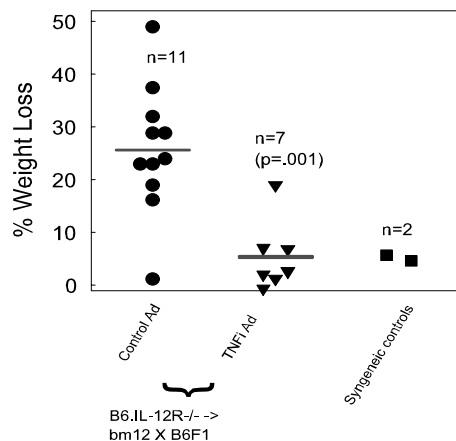


FIGURE 3. TNF inhibition in vivo reduces wasting disease in B6.IL-12R^{-/-}→bm12 \times B6 F₁ GVHD. Irradiated B6 \times bm12 F₁ mice were infused with 2×10^6 T cell-depleted B6.IL-12R^{-/-} bone marrow cells and 5×10^6 CD4⁺ B6.IL-12R^{-/-} spleen cells and either the β galactosidase ($n = 11$)-encoding (Control Ad) or TNF inhibitor ($n = 7$)-encoding adenoviral vector (TNFi Ad). Mice were weighed 10–14 days after transplantation.

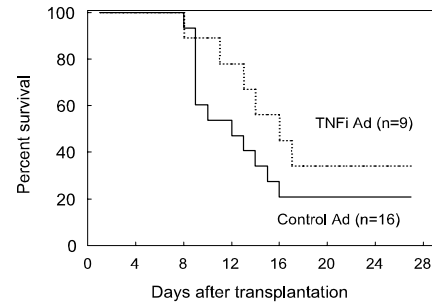


FIGURE 4. TNF inhibition in vivo delays mortality in B6.IL-12R^{-/-}→B6 \times bm12 F₁ mice. Irradiated B6 \times bm12 F₁ mice were infused with 2×10^6 T cell-depleted B6.IL-12R^{-/-} bone marrow cells and 5×10^6 CD4⁺ B6.IL-12R^{-/-} spleen cells and either the α galactosidase ($n = 11$)-encoding (Control Ad) or TNF inhibitor ($n = 7$)-encoding adenoviral vector (TNFi Ad). Survival of recipients of TNFi Ad was significantly different from that of recipients of control Ad ($p < 0.05$).

T cells that had received the TNF inhibitor-encoding adenovirus ($0.048 \pm 0.076 \times 10^6$) than from recipients of the control adenovirus ($.35 \pm 0.47 \times 10^6$, $p = 0.0468$).

TNF blockade decreases in vivo generated CD4⁺ T cell IFN- γ responses in bm12 \times B6 F₁ recipients of B6.IL-12R^{-/-} CD4⁺ T cells

In additional experiments, splenic lymphocytes from bm12 \times B6 F₁ recipients that had received either the TNF inhibitor or the control adenovirus were assessed for cytokine responses. The percentage of CD4⁺ T cells that expressed IFN- γ were less in the B6.IL-12R^{-/-}→bm12 \times B6 F₁ recipients of the TNF inhibitor-encoding adenovirus ($n = 4$) than in recipients of the control adenovirus mice ($n = 7$) ($10.97 \pm 3.6\%$ vs $32 \pm 3.5\%$) (Fig. 8). Of note, in bm12 \times B6 F₁ recipients of control B6 bone marrow and spleen cells ($n = 7$) ($61 \pm 11.3\%$) than in recipients of B6.IL-12R^{-/-} donor cells (Fig. 8). As detailed in Fig. 7, similar numbers of CD4⁺ T cells were recovered from the spleens of B6.IL-12R^{-/-}→bm12 \times B6 F₁ recipients of either the control adenovirus or the TNF inhibitor-encoding adenovirus. Thus, there was a significant decrease in both the percentage and the absolute number of CD4⁺, IFN- γ -expressing splenic lymphocytes after TNF blockade.

In other experiments, spleen lymphocytes from bm12 \times B6 F₁ recipients that had received B6.IL-12R^{-/-} spleen cells and either the TNF inhibitor or the control adenovirus were assessed for IL-4 responses. In two separate experiments, the percentage of CD4⁺ IL-4-expressing T cells was similar in control mice ($n = 6$) and in recipients of the TNF inhibitor-encoding adenovirus ($n = 6$) ($1.5 \pm 2.3\%$ vs $1.3 \pm 2.7\%$ ($p = 0.93$)).

Discussion

This is the first report indicating that IL-12- and IL-18-independent TNF/TNFR interactions on CD4⁺ T cells promote optimal stimulation of IFN- γ production and alloresponses during MHC class II disparate MLC and GVHD. This conclusion is based on results of experiments using responder T cells that lack the IL-12R β 2 subunit of the IL-12R (37). In cells from the B6.IL-12R^{-/-} mouse strain used in these studies, the IL-12 β 1 subunit of the IL-12R still binds the IL-12 with both high and low affinity receptors, but no IL-12 biological function can be detected (35). Con A-activated splenocytes from both control B6 and B6.IL-12R^{-/-} mice proliferate equally well when stimulated with IL-2. However, a marked reduction in the production of IFN- γ by the B6.IL-12R^{-/-} spleen

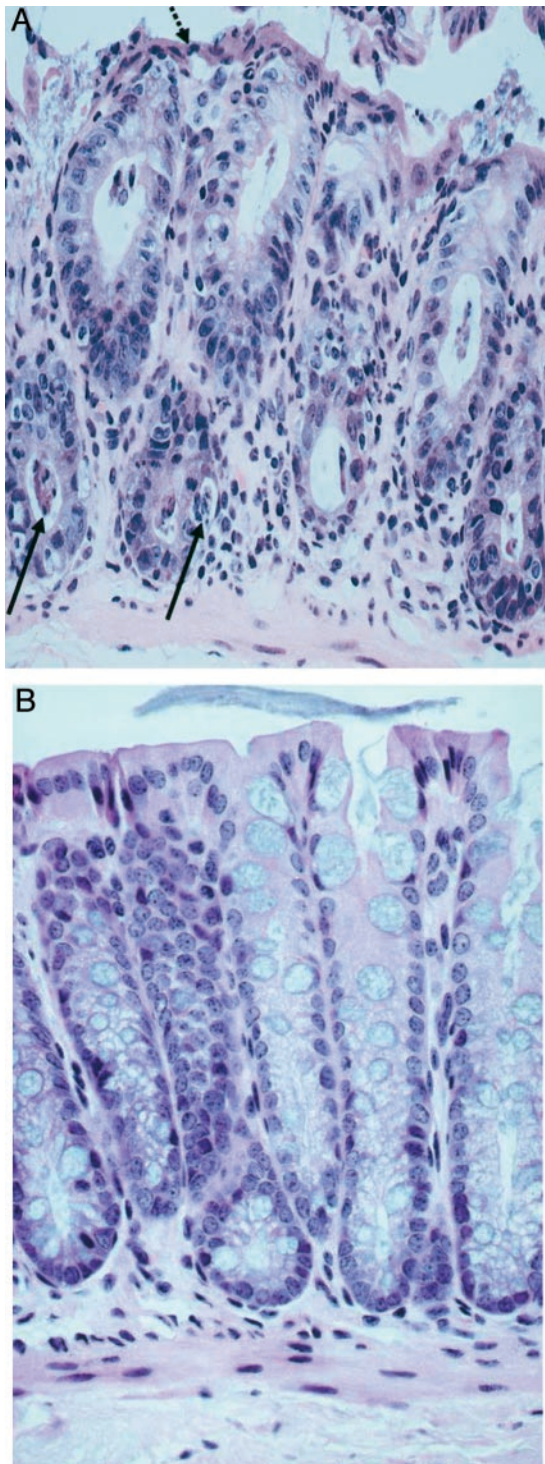


FIGURE 5. TNF blockade ameliorated the histological features of intestinal GVHD in $\text{bm12} \times \text{B6 F}_1$ recipients of $\text{B6.IL-12R}^{-/-} \text{CD4}^+$ T cells. Histological features of apoptosis of crypt cells (filled arrow), cystically dilated crypts containing necrotic debris (dashed arrow), and damaged epithelium (dotted arrow) were observed in colons obtained 9–15 days after transplantation in recipients of the $\text{B6.IL-12R}^{-/-} \text{CD4}^+$ T cells and the control Ad (A), but normal colonic histology was noted in the recipients of the $\text{B6.IL-12R}^{-/-} \text{CD4}^+$ T cells and the TNF inhibitor-encoding adenovirus (TNFi Ad) (B).

cells occurs (37). As illustrated by the results of the present studies, alloantigen-induced proliferative responses of $\text{B6.IL-12R}^{-/-}$ spleen cells are similar to those of B6 spleen cells, but IFN- γ

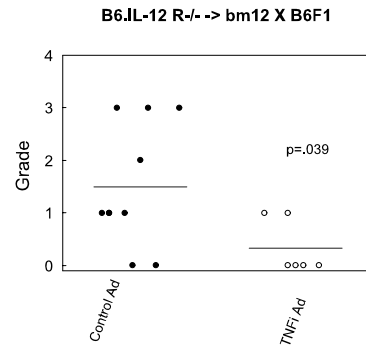


FIGURE 6. TNF inhibition decreases the histological grade of GVHD in the large intestine in MHC class II disparate intestinal GVHD induced by $\text{B6.IL-12R}^{-/-} \text{CD4}^+$ T cells. Colonic tissue was harvested 10–14 days after transplantation from nine recipients of $\text{B6.IL-12R}^{-/-} \text{CD4}^+$ splenic T cells and control Ad, from six recipients of $\text{B6.IL-12R}^{-/-} \text{CD4}^+$ splenic T cells and TNFi Ad, and two recipients of syngeneic donor cells ($\text{B6} \times \text{bm12 F}_1$). The colonic tissue was fixed, sectioned, stained with H&E, and scored, as detailed in *Materials and Methods*.

responses are reduced, as previously reported, in studies assessing both alloantigen- and Ag-induced T cell responses.

We have previously observed that TNF/TNFR2 interactions on responder CD4^+ T cells are critical for optimal alloproliferation and Th1 cytokine responses by T cells in response to alloantigenic stimuli in vitro or in vivo during intestinal GVHD (30). As TNF has been reported to stimulate IL-12 production by macrophages (6), these earlier results suggested that TNF may amplify Th1 cytokine responses by an indirect effect on IL-12 production. However, the present experiments demonstrated that TNF inhibition decreases alloproliferative responses and IFN- γ production in MHC class II disparate MLC using B6 spleen cells that lack the $\beta 2$ subunit of the IL-12R. Thus, these findings indicate that TNF promotes IFN- γ responses via an IL-12-independent mechanism.

IL-18, an IL-1-related factor (38–40), also has been shown to be a selective activator of IFN- γ in Th1, but not Th2, cells. Both IL-1 and IL-18 activate a IRAK (a kinase associated with IL-1R) (36) and NF- κB in Th1 cells (37–38) and TNFR-associated factor 6 (38). IRAK-deficient mice have defective IL-18-mediated Th1-type responses in vivo (41). Finally, a powerful synergy between IL-12 and IL-18 for IFN- γ production has been found (42). In the present studies, using in vitro MHC class II disparate MLC with either control B6 or $\text{B6.IL-12R}^{-/-}$ responder T cells, IL-18 production was stable during TNF blockade. These results suggest that TNF/TNFR effects on IFN- γ production are not mediated via modulation of IL-18 responses.

The present studies also indicate that TNF/TNFR interactions are critical for IL-12R-independent, MHC class II disparate GVHD. Previous investigators have suggested that the mechanism of the actions of TNF during acute GVHD was as an effector of host cytotoxicity (9, 43). More recent studies have demonstrated that the absence of TNFR1 on host cells was associated with a delay in mortality from acute GVHD, but had no effect on histologic patterns of GVHD in the intestinal tract or liver (42). Other studies have focused on the role of TNFR signaling in donor T cells in the $\text{B6} \rightarrow \text{B6D2 F}_1$ and $\text{B6} \rightarrow \text{B6C3F}_1$ class I + II MHC disparate mouse GVHD models (9) in which CTL effector mechanisms play a prominent role in determining GVHD mortality rates (9, 45, 46). In these GVHD models, TNFR1 deficiency, but not TNFR2 deficiency, on donor T cells was associated with decreased GVHD mortality and clinical severity that correlated with decreased T cell proliferative, IL-2, and CTL responses. Effects on gut GVHD in these studies were not reported. Studies from our

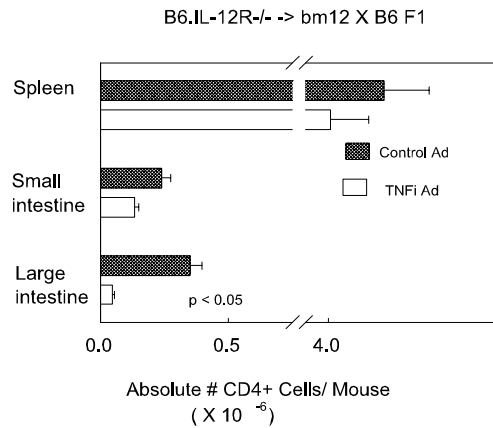


FIGURE 7. TNF inhibition decreases the number of infiltrating donor T cells in the large intestine in recipients of the B6.IL-12R^{-/-} CD4⁺ T cells. T cells were isolated from the spleens, small intestines, and large intestines of the recipients of the B6.IL-12R^{-/-} CD4⁺ T cells and either the control Ad or the TNFi Ad. Flow cytometry assessed the percentage of CD4⁺ T cells. Absolute number was derived from the percentage of CD4⁺ T cells × the total number of cells.

laboratory have demonstrated that TNFR2 signaling in donor CD4⁺ T cells is important for development of intestinal GVHD. Moreover, TNF/TNFR2 (and/or lymphotoxin α /TNFR2) interactions amplify allospecific T cell responses to MHC class II differences and play a critical role in the activation and differentiation of Th1 cytokine responses during MHC class II disparate GVHD. TNF blockade might also alter donor T cell migratory patterns, and this effect may account for diminished T cell expansion in some, but not all, GVHD target organs following TNF blockade.

MHC class II-induced GVHD induced by B6.IL-12R^{-/-} animals has not been described previously. Importantly, a severe weight loss and an evidence of intestinal GVHD were noted. The intestinal GVHD was graded according to the Snover criteria, and the severity in the controls was judged to be 1.1 ± 1.5 , with only 4 of 10 graded 2 or higher. This low severity differed from the colons isolated from the bm12 × B6 F₁ recipients of the control B6 CD4⁺ T cells, in which 7 of 10 had evidence of grade 2 or higher GVHD (13). Thus, colonic GVHD is not as severe in the recipients of the B6.IL-12R^{-/-} CD4⁺ T cells, but the residual IL-12-independent disease is further decreased by TNF blockade.

In contrast, the wasting disease induced by transfer of B6.IL-12R^{-/-} CD4⁺ T cells into MHC class II disparate recipients is more severe than noted previously in the GVHD induced by control B6 spleen cells (13). Thus, the wasting disease initiated by T cells may be secondary to other mechanisms distinct from Th1 cytokine responses. Nevertheless, this severe wasting disease, initiated by IL-12R-deficient T cells, is also significantly decreased by TNF blockade. The increased severity of GVHD-associated wasting disease in the absence of IL-12/IL-12R interactions suggests that IL-12-dependent responses such as IFN- γ production may in some way ameliorate weight loss.

TNF inhibition *in vivo* reduces percentage and absolute number of IFN- γ -producing CD4⁺ splenic T cells and diminishes intestinal disease in B6→bm12 × B6F₁ or B6.IL-12R^{-/-}→bm12 × B6 F₁ GVHD. The close correlation between the severity of colonic disease and levels of IFN- γ expression *in vivo* in the present and previous studies suggests that colonic disease is directly associated with IFN- γ production. Potential explanations for the effect of TNF in GVHD may be found by reviewing TNF signaling pathways. TNF, by binding to either TNFR1 or TNFR2, can cause activation of NF- κ B, which in turn has been reported to up-regu-

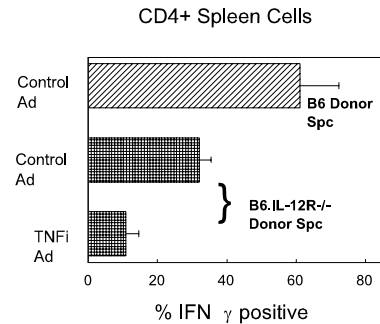


FIGURE 8. TNF blockade decreases the percentage of CD4⁺ splenic T cells expressing IFN- γ in bm12 × B6 F₁ recipients of B6.IL-12R^{-/-} CD4⁺ T cells. Splenic T cells were isolated 12 days after BMT from recipients of B6.IL-12R^{-/-} CD4⁺ T cells and either the TNFi Ad or the control Ad and assessed for intracellular IFN- γ expression.

late IFN- γ (47). In the B6.IL-12R^{-/-} splenic T cells that lack the IL-12R, TNF most likely continues to induce IFN- γ production through NF- κ B signaling, albeit at lower levels than in cells with intact IL-12 signaling pathways, resulting in a less severe colonic GVHD.

Although TNF inhibition significantly prolongs survival in the IL-12R^{-/-}→bm12 × B6 F₁ mice, GVHD mortality is not prevented. In studies conducted in other mouse strain combinations, we have noted previously that severity of intestinal GVHD, especially colonic GVHD, does not correlate well with GVHD mortality (2). In all strain combinations examined to date (11, 13), the most impressive effect of TNF inhibition is its effects on colonic GVHD and associated IFN- γ responses. In contrast, TNF inhibition has minimal effects on alloproliferative responses in the spleen (13) or overall GVHD mortality. These findings suggest that TNF effects on Th1 cytokine responses play a major role in evolution of intestinal GVHD, while alternative immune effector mechanisms are involved in other systemic manifestations of GVHD.

Despite the fact that absence of IL-12/IL-12R interactions has been frequently associated with augmented Th2 cytokine production (48), such Th2 responses were not readily detected during GVHD elicited by either control B6 or B6.IL-12R^{-/-} spleen cells, as only very low numbers of IL-4-expressing T cells were detected in spleens of B6→Bm12 × B6 F₁ or B6.IL-12R^{-/-}→Bm12 × B6 F₁ GVHD. One likely explanation for this observation is that the vigorous TNF response elicited during intestinal GVHD promotes sufficient IFN- γ to allow Th1 polarization and counterregulatory inhibition of Th2 responses. Of note, other investigators have suggested that the IL-12/IL-12R interactions in a fully MHC-mismatched murine model of acute GVHD may actually be important in survival by affecting the production of other inflammatory cytokines (49).

In conclusion, augmentation of CD4⁺ T cell alloproliferation and IFN- γ responses by TNF is, in large part, IL-12 independent and not associated with any apparent modulation of IL-18 levels. Although IFN- γ responses promoted by TNF in the absence of IL-12 stimulation are less vigorous than those observed in the presence of both stimuli, such responses appear sufficient to induce Th1 cytokine-dependent intestinal GVHD. Importantly, in the absence of IL-12 signaling, T cells generate a Th1 response, and this residual Th1 response is TNF dependent. These findings indicate that TNF/TNFR interactions provide an alternative pathway for induction of Th1 cytokine responses and Th1 cytokine-mediated inflammatory disease. In addition, as wasting disease during MHC class II disparate GVHD is also mediated by TNF, but is more severe in the absence of IL-12R signaling, this *in vivo* effect of

TNF appears to be a Th1 cytokine-independent process. Thus, TNF is important in generating two distinct aspects of GVHD that appear to be mediated by both Th1 cytokine-dependent and Th1 cytokine-independent mechanisms.

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