Interferon-γ Suppresses T-Cell Proliferation to Mitogen Via the Nitric Oxide Pathway During Experimental Acute Graft-Versus-Host Disease

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The development of graft-versus-host disease (GVHD) is associated with long-lasting and profound deficits in immune function that lead to increased morbidity and mortality after bone marrow transplantation (BMT). We investigated a mechanism of T-cell immunodeficiency in response to mitogen or alloantigen in an experimental model of acute GVHD by analyzing the roles of two immunosuppressive moieties: interferon gamma (IFN-γ) and nitric oxide (NO). Splenocytes from mice with GVHD did not proliferate either to the T-cell mitogen, concanavalin A (Con A), or to host alloantigens, but only mitogen-activated cultures produced increased levels of NO. The abrogation of NO synthesis with L-arginine (NMMA) restored mitogen-induced proliferation but not the response to host antigens. The mechanism of impaired proliferation to mitogen was dependent on IFN-γ because blockade of this cytokine in culture inhibited NO production and restored proliferation to Con A to levels similar to those in transplanted control mice without GVHD. NMMA did not substantially reduce IFN-γ levels, demonstrating that NO acted distally to IFN-γ in the pathway of immunosuppression in response to mitogen. Furthermore, the prevention of IFN-γ production in vivo after allogeneic BMT, by transplantation of polarized type 2 donor T cells (secreting interleukin-4 but not IFN-γ), also prevented NO production and restored splenocyte responses to mitogen. Our data demonstrate the existence of NO-dependent and NO-independent pathways involved in suppression of T-cell proliferation during acute GVHD. Excess NO synthesis appears to be one mechanism by which IFN-γ induces immunodeficiency after allogeneic BMT.

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of IFN-γ in vitro. Prevention of IFN-γ production in vivo after BMT, by transplantation of polarized donor T cells with a type 2 cytokine phenotype (ie, cells producing IL-4 but not IFN-γ26), also inhibited mitogen-induced NO production and prevented unresponsiveness of host lymphocytes to Con A. These data therefore support the hypothesis that NO acts distal to IFN-γ in one of at least two pathways that control proliferative responses to mitogen by T cells from mice with GVHD.

**MATERIALS AND METHODS**

**Mice.** Female C57BL/6 (B6, H-2b) and B6.C-H2m^12^ (bm12, K^D^T^-^A^-^M^12^-^E^-^) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The donors and recipients in this study were between ages 8 and 12 weeks, and were housed in sterilized microisolator cages in which they received autoclaved food and autoclaved acidified water for 2 weeks after BMT.

**Reagents.** The following monoclonal antibodies (MoAbs) were used for cell-surface analysis: phycoerythrin (PE)-conjugated anti-CD8 MoAb and fluorescein (FITC)-conjugated anti-CD4 MoAb (Pharmingen, San Diego, CA). For neutralization of bioactivity and enzyme-linked immunosorbent assay (ELISA) capture MoAb for murine IFN-γ, purified R4-6A227 was used, and for detection, biotin-conjugated XMG1.228 Anti-IL-4 MoAbs for detection of murine IL-4 were 11B1129 and biotin-conjugated BV6-24G226 (Pharmingen, San Diego, CA). Murine rIL-4 with a specific activity of 7 × 10^4 U/mg by FDCP-2 assay was obtained from Immunex Corp (Seattle, WA). Murine rIFN-γ was purchased from Amgen (Thousand Oaks, CA) and human rIL-2 from Pharmacia Diagnostics Inc (Silver Spring, MD). The anti-Thy-1.2 MoAb used for T-cell depletion was isolated from tissue culture supernatants from hybridoma clone HO-13-4 (American Type Culture Collection, Rockville, MD).25 Low-Tox-M rabbit complement was obtained from Accurate Corp (Westbury, NY). Con A and the alkaline phosphatase substrate solution were obtained from Vector Laboratories (Burlingame, CA), and a standard curve was generated to determine cytokine concentration in the sample. The detection limit of both cytokine assays was 2.0 U/mL.

**BMT and GVHD induction.** This protocol has been described previously.26 Briefly, for BMT across MHC class II barriers, the donor inoculum was adjusted for BMT hosts to receive 5 × 10^7 polarized type 1 or type 2 CD4+ T cells and 5 × 10^4 T-cell–depleted bone marrow cells (TCD-BM) from bm12 donor mice. This cell mixture was injected intravenously (in 0.25 mL Leibovitz’s L-15 media; GIBCO-BRL, Grand Island, NY) into sex-matched B6 recipient mice that had received a lethal radiation dose of 1,100 cGy (15Cs source) in two dosages separated by 3 hours. For positive GVHD controls, nylon-wool–purified splenocytes containing 5 × 10^4 naive CD4+ T cells were injected together with TCD-BM. Non-GVHD controls received TCD-BM only. For the coinjection experiments, B6 recipient mice were transplanted with TCD-BM cells and nylon-wool–purified bm12 splenocytes containing 5 × 10^4 naive CD4+ T cells with the addition of either polarized type 1 or type 2 cells (5 × 10^4 CD4+).

**Assessment of GVHD.** Body weight and mortality rate were assessed on a weekly basis for 50 days after transplantation. To measure lymphocyte responsiveness in GVHD, splenocytes were isolated from BMT recipient mice 13 days after transplantation and cytotoxicity were lysed with NH4Cl. Cells were cultured at 4 × 10^5/well in flat-bottomed 96-well plates in the absence or presence of Con A (2.5 μg/mL). Proliferation of GVHD splenocytes to Con A was measured after 72 hours by incorporation of [3H]thymidine (1 μCi) for the last 20 hours of incubation. Proliferation to alloantigen was measured by culture of responder splenocytes from transplanted mice (4 × 10^5 well) with irradiated stimulator cells from B6 mice (4 × 10^5/well) for 96 hours. In control cultures, syngeneic stimulator cells from the bm12 donor strain were used. In certain experiments, splenocytes were also cultured in the presence of titrating doses of a MoAb against murine IFN-γ (R4-6A2) and NMMA was added at a final concentration of 40 μg/mL. Supernatants were collected after 48 hours of culture and assayed for NO2, IFN-γ, and IL-4 content as described later.

To analyze cell-surface phenotype, splenocytes from recipient mice (day 13 post-BMT) were resuspended in PBS for flow-cytometric analysis. Cells (0.5 × 10^6) were incubated for 20 minutes at 4°C with MoAb 2.4G2 to block nonspecific staining via Fc receptors,27 and then incubated with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 MoAbs for 30 minutes at 4°C. The cells were then washed twice with PBS before fixation in 1% paraformaldehyde. Two-color flow-cytometric analysis of 10^6 labeled cells was performed using a FACSScan (Becton Dickinson, Mountain View, CA). The FACSScan was calibrated using PE- and FITC-conjugated, nonspecific IgG antibodies. The splenic cell population containing mainly lymphocytes (including GVHD blasts) was gated by forward- and side-scatter parameters.

**Cytokine assays.** Levels of IFN-γ and IL-4 in tissue culture supernatants were determined by sandwich ELISA using specific antimmune MoAbs for capture and detection (Pharmingen). An alkaline phosphatase ABC-AP kit was purchased from Vector Laboratories (Burlingame, CA), and a standard curve was generated to determine cytokine concentration in the sample. The detection limit of both cytokine assays was 2.0 U/mL.

**Determination of NO production.** NO rapidly oxidizes to both nitrate (NO3−) and nitrite (NO2−). To convert NO3− into NO2−, tissue culture supernatants (50 μL) were incubated for 15 minutes at room temperature with nitrate reductase (0.08 U/mL) in the presence of NAPDH (30 μg/mL) in a 96-well microtiter plate. To detect NO2− accumulation, 50 μL freshly prepared Griess reagent (a 1:1 solution of 0.1% N-naphthylethenediamine and 1% sulfanilamide/5% H3PO4) was subsequently added to samples and incubated for 5 minutes at room temperature as previously described.28 The Griess reagent reacts with NO2− to form a purple azo dye, and the absorbance...
combined mean

**Clinical evidence of GVHD was demonstrated by sustained**

Body weights were measured for 50 days post-BMT. Statistical analysis was performed using the nonparametric Wilcoxon rank-sum test. The indicated groups were significantly different from GVHD mice transplanted with TCD-BM cells and nylon-wool-purified splenocytes from recipient mice were analyzed on day 13 post-BMT for expression of T-cell surface markers. FACS analysis values are expressed as total positive cells per spleen (x10^6). Approximately 75% of splenic T cells were CD4+ in both groups. Naïve splenocytes contained, on average, 20.8 ± 0.3 x 10^5 CD4+ and CD8 cells. Data represent the combined mean ± SEM of individual mice from 3 experiments (n = 6 to 9 mice per group). *P < .001 versus mice without GVHD (Student's t-test).

**RESULTS**

**Induction of acute GVHD.** Acute GVHD was induced in irradiated B6 hosts by transplantation of a mixture of 5 x 10^6 freshly isolated TCD-BM cells and nylon-wool-purified splenocytes containing 5 x 10^6 naive CD4+ T cells from bm12 donor mice. Transplanted mice injected with TCD-BM but no added T cells served as non-GVHD controls. Clinical evidence of GVHD was demonstrated by sustained loss in body weight starting as early as 10 days post-BMT (Fig 1A). Excess mortality was not characteristic of this GVHD model, with 20% to 30% of mice dead by 2 months post-BMT (data not shown).

The number of T cells in transplanted mice was assessed by analysis of the expression of CD4 and CD8 surface molecules on splenocytes isolated on day 13 post-BMT (Fig 1B). In hosts with GVHD, the total number of splenic T cells was increased, as expected, and showed significantly greater numbers of CD4+ and CD8+ cells (P < .001) than in the group without GVHD. Approximately 75% of T cells were CD4+ in both groups (data not shown). To examine the effect of GVHD on donor T-cell engraftment, experiments with congenic B6.PL-Thy1a (Thy1.1+) mice as recipients were performed. Splenic T cells were at least 97% donor origin (Thy1.2+) in all BMT groups 4 weeks after transplantation (data not shown).

**Impairment of mitogen-induced but not alloantigen-induced splenocyte proliferation during GVHD is associated with excess NO production.** We first assessed GVHD in our BMT system in terms of the proliferative response to the T-cell mitogen, Con A. We observed a profoundly suppressed proliferative response to Con A by GVHD splenocytes isolated on day 13 post-BMT (<300 cpm thymidine incorporation; Fig 2A, top). Con A–stimulated splenocytes of the mixture was measured at 550 nm using a Microplate Reader (Bio-Rad Laboratories, Hercules, CA). The quantity of NO2 was calculated using a dilution curve of a standard solution of NaNO2. The curve was linear in the range from 0 to 200 μmol/L, and the detection limit of this assay was 2.0 μmol/L, NO2-. NO2 and NO3 in GVHD splenocyte cultures were always recovered at a ratio of 3 to 2 (65.3% ± 11.3% NO2), a finding that is in agreement with published data of NO catabolism in activated macrophages. We have accordingly limited our analysis to the measurement of NO2 in these studies.

**Statistical analysis.** Values for cell-surface molecules (total positive cells) and cytokine production, NO2 production, and proliferation to Con A were obtained from individual mice. Data are the combined mean ± SEM from at least two separate experiments per figure. For statistical analysis of parametric data, Student's t-test was used, except for the weight loss data, for which P values were determined using the nonparametric Wilcoxon rank-sum test.
from mice without GVHD showed a relative proliferative response 2 orders of magnitude greater than for cells from GVHD mice. We also measured proliferation of these splenocytes to host alloantigens. Proliferation of GVHD splenocytes was suppressed twofold compared with splenocytes from non-GVHD mice (Fig 2B; \( P < .05 \)). As expected, the decrease was not as dramatic as with Con A, because proliferation to alloantigen by splenocytes from non-GVHD control mice was only slightly enhanced above their response to bm12 self-antigens (1.5 \times 10^4 \text{ cpm}).

We next investigated whether impaired proliferation to both stimuli was associated with enhanced levels of NO, a strong inhibitor of lymphocyte responses in other systems. Indeed, after stimulation with Con A, splenocytes isolated on day 13 post-BMT from mice with GVHD produced significant amounts of NO as assessed by measurement of the metabolite NO\(_2\)-\(\gamma\) (Fig 2A, bottom). In contrast, levels of NO\(_2\)-\(\gamma\) in supernatants of splenocytes from TCD-BM control mice were less than 5 \(\mu\text{mol/L}\). In these experiments, the impaired response of GVHD splenocytes to B6 alloantigen was not associated with enhanced levels of NO (Fig 2B, bottom).

We then wished to determine whether NO was causally linked to the suppression of mitogen-induced proliferation of GVHD lymphocytes. Splenocytes from the same BMT groups were cultured in the presence of Con A and with or without NMMA, a specific antagonist for the enzyme, NO synthase. Addition of NMMA restored the Con A–induced proliferative response of splenocytes from GVHD mice to that of cells from TCD-BM control mice (Fig 2A, top). Simultaneously, treatment of cultures with NMMA decreased NO production by GVHD splenocytes to levels secreted by non-GVHD control cultures. Not surprisingly, addition of NMMA to MLR cultures did not increase proliferation of GVHD splenocytes to alloantigen (Fig 2B), confirming the distinct nature of this defect.

**IFN-\(\gamma\) induces T-cell immunosuppression during GVHD.** We next attempted to define the mechanism leading to enhanced NO secretion in mitogen-stimulated cultures. Increased production of IFN-\(\gamma\) has been reported to be characteristic in the pathophysiology of acute GVHD in several experimental BMT models. Splenocytes from GVHD mice (day 13 post-BMT) secreted high levels of IFN-\(\gamma\) in response to Con A despite an inability to proliferate. In contrast, IFN-\(\gamma\) production by control mice without GVHD was not significant (Fig 3A).

Because IFN-\(\gamma\) has previously been shown to inhibit mitogen-driven proliferative responses of splenocytes during GVHD, we next analyzed whether neutralization of IFN-\(\gamma\) in vitro would prevent suppression of mitogen-induced proliferation of GVHD splenocytes in our system. GVHD was induced as described earlier, and recipient splenocytes (day 13) were cultured with Con A and an antibody to murine IFN-\(\gamma\). Neutralization of IFN-\(\gamma\) restored the Con A–driven proliferation of T cells from GVHD mice to the magnitude observed in non-GVHD controls (Fig 3B). The same approach was tested in a GVHD model to minor HA barriers (B10.BR → CBA), where addition of anti–IFN-\(\gamma\) MoAb restored proliferation to mitogen to levels found in syngeneic BMT control groups (data not shown).

**IFN-\(\gamma\) mediates T-cell immunosuppression in response to mitogen and induces NO production during GVHD.** B6 recipient mice were transplanted as in Fig 1. Splenocytes were isolated on day 13 post-BMT from mice with (A) or without (B) GVHD. (A) Levels of IFN-\(\gamma\)-induced by Con A. Splenocytes were cultured with Con A for 48 hours, and supernatants were analyzed by ELISA. Naïve splenocytes produced, on average, 238 ± 45.5 \(\mu\text{mol/mL}\) IFN-\(\gamma\)-\(\gamma\) with Con A stimulation. Data represent the mean ± SEM of individual mice from 2 or 3 combined experiments (n = 6 to 9 mice per group). \( P < .001 \) versus non-GVHD controls (Student’s t-test). (B) Proliferative responses of splenocytes to Con A at 72 hours. (C) Production of NO by Con A–activated splenocytes. Cells from the same transplant groups were cultured in the presence of increasing concentrations of anti–IFN-\(\gamma\) MoAb, and levels of NO\(_2\)–\(\gamma\) were expressed in \(\mu\text{mol/L}\). Data in B and C represent the mean ± SEM from individual mice from 2 combined experiments (n = 5 mice per group). \#\( P < .002 \) versus cultures without addition of anti–IFN-\(\gamma\) MoAb (Student’s t-test).

**Fig 3. IFN-\(\gamma\) mediates T-cell immunosuppression in response to mitogen and induces NO production during GVHD.** The data above demonstrate that increases in both IFN-\(\gamma\) and NO can inhibit splenocyte proliferation to Con A during acute GVHD. IFN-\(\gamma\) is known to induce NO synthesis from mononuclear cells in vitro, and we therefore hypothesized that IFN-\(\gamma\) mediated the immunodeficiency during GVHD via induction of NO. Neutralization of IFN-\(\gamma\) with anti–IFN-\(\gamma\) MoAb resulted in a significant inhibition of NO\(_2\)-\(\gamma\) production at antibody concentrations of 300 ng/mL or higher (Fig 3C). In contrast, NO\(_2\)-\(\gamma\) production by splenocytes from non-GVHD mice was unaffected by this treatment. Although NO levels in cultures of GVHD mice with the maximum concentration of MoAb used (10\(^{5}\) ng/mL) were higher than levels produced by non-GVHD cultures, proliferation in these cultures was restored to levels of non-GVHD controls when NO\(_2\)-\(\gamma\) levels decreased below a level of approximately 20 \(\mu\text{mol/L}\).

Additional control experiments were performed to rule out that NMMA restored proliferation via inhibition of IFN-\(\gamma\) secretion. We found that addition of NMMA to GVHD splenocyte cultures reduced IFN-\(\gamma\) production only by 30%, to 64 ± 9 U/mL, a level still 20-fold higher than in non-GVHD controls. Thus, this partial inhibition of cytokine synthesis was not sufficient to explain the complete restoration of proliferation to Con A by NMMA. Moreover, addition of exogenous rIFN-\(\gamma\) (100 U/mL) did not significantly alter Con A–induced proliferation of splenocytes obtained from mice without GVHD (data not shown). The presence of NMMA did not affect proliferation of splenocytes from
IFN-γ AND NITRIC OXIDE IN GVHD

A 150
B
C
D

Fig 4. Transplantation of polarized type 2 allogeneic donor T cells inhibits both IFN-γ and NO production and restores proliferation to mitogen. B6 recipient mice were transplanted with 5 x 10^6 TCD-BM cells containing 5 x 10^5 nylon-wool-purified fresh splenocytes (●), 5 x 10^5 polarized type 1 CD4+ cells (●), 5 x 10^5 polarized type 2 CD4+ cells (●), or no CD4+ cells (●). Splenocytes from recipient mice (day 13 post-BMT) were stimulated with Con A and analyzed for secretion of IFN-γ and IL-4, proliferation, and production of NO; (A and C). Cytokine production by GVHD splenocytes. IFN-γ and IL-4 concentrations (U/ml) in tissue culture supernatants were measured 48 hours after stimulation. (B) Levels of NO; were analyzed after 48 hours of culture. (D) Proliferative response to Con A after 72 hours of culture. Data represent the mean ± SEM from individual mice from 2 (B, D) or 3 (A, C) combined experiments (n = 6 to 8 mice per group). *P < .01, mice receiving polarized type 2 cells versus positive GVHD controls (Student’s t-test).

mice without GVHD, ruling out the possibility that NMMA acted as an independent mitogen (Fig 2A). Thus, IFN-γ acts proximally to NO in the pathway of suppression of mitogen-induced T-cell proliferation in vivo.

Transplantation of polarized type 2 CD4+ T cells inhibits NO production and prevents T-cell immunodeficiency. We have previously shown that transplantation of polarized type 2 T cells abrogates the IFN-γ response (2 weeks post-BMT) during acute GVHD. Downregulation of IFN-γ secretion through this approach was associated with less weight loss compared with mice with GVHD (P < .001) and 100% survival 2 months post-BMT (data not shown). Expansion of splenic T cells by day 13 was not distinguishable from that observed in the GVHD group (Fig 1A and B).

To test whether prevention of IFN-γ production in vivo also prevented NO production and restored T-cell proliferation, B6 mice were transplanted with TCD-BM cells and cultured bm12 cells containing 5 x 10^5 polarized type 2 CD4+ T cells. Splenocytes from transfused mice (day 13 post-BMT) receiving polarized type 2 donor T cells produced high levels of IL-4 but very low amounts of IFN-γ (Fig 4A and C). Levels of IFN-γ were significantly lower than in GVHD mice transplanted with naive T cells (P < .001) or with polarized type 1 donor T cells (P < .001), and were identical to those produced in mice transplanted with TCD-BM only. This lack of IFN-γ secretion occurred despite an approximately fivefold increase in the number of splenic T cells in recipients of polarized type 2 donor cells compared with the TCD-BM group (8.4 ± 1.3 × 1.6 ± 0.2 x 10^6). The accumulation of NO2− by Con A-stimulated splenocytes was decreased by 1 order of magnitude compared with the two GVHD groups (Fig 4B). Importantly, their proliferative response to Con A was increased by almost 2 orders of magnitude (Fig 4D). Addition of anti–IFN-γ antibody in vitro affected neither NO production nor the proliferative response in splenocytes from mice receiving type 2 cells (data not shown).

To exclude the possibility that the lack of NO induction by polarized type 2 donor T cells was merely due to their functional impairment, we performed mixing experiments of polarized type 1 and type 2 cells with naive donor T cells. We asked whether polarized type 2 cells could regulate normal T cells and prevent NO production and the T-cell immunodeficiency in response to mitogen after BMT. A cell mixture containing 5 x 10^6 TCD-BM cells, 5 x 10^5 naive CD4+ T cells from nylon-wool-purified splenocytes, and 5 x 10^5 polarized type 2 CD4+ T cells was transplanted into irradiated B6 hosts. This 1:10 mixture of polarized type 2 and naive donor T cells, respectively, abrogated the production of IFN-γ by Con A-stimulated splenocytes isolated on day 13 post-BMT (Fig 5A). In contrast, a mixture of polarized type 1 cells with naive T cells did not abrogate IFN-γ secretion. NO2− production was significantly inhibited after transplantation of type 2 cells when compared with mice with GVHD (Fig 5B), and their proliferative response was completely restored to levels of mice without GVHD, as expected (Fig 5C). This potent effect of small numbers of polarized
type 2 T cells within the donor T-cell population confirmed their viability and functional capacity in vivo and demonstrated their active regulatory role in IFN-γ secretion during GVHD. Taken together, the data shown in Figs 4 and 5 demonstrate that inhibition of IFN-γ in vivo by injection of polarized type 2 T cells prevents the increased NO production and subsequent T-cell immunodeficiency in response to mitogen during experimental GVHD.

**DISCUSSION**

Acute GVHD has long been described as "suppressive," whereas chronic GVHD has been called "proliferative." We have studied two mechanisms that may contribute to deficient immune responses of T lymphocytes during acute GVHD: the production of excess IFN-γ and the production of the immunosuppressive molecule, NO. Using a GVHD model to MHC class II antigens, we found that splenocytes from mice with GVHD did not proliferate either to the T-cell mitogen, Con A, or to host alloantigens, but only mitogen-activated cultures produced increased levels of NO. Inhibition of NO synthase with NMMA restored proliferation to mitogen but did not improve proliferation to alloantigen, confirming the existence of both NO-dependent and NO-independent T-cell deficits. We then focused on NO-dependent immunosuppression in response to mitogen, and addressed the question of whether the IFN-γ and NO pathways are independent and parallel or causally linked during acute GVHD. We hypothesized that IFN-γ induces the synthesis of NO, which is an immediate cause of T-cell unresponsiveness to mitogen during acute GVHD. Our hypothesis predicts that blockade of IFN-γ production during acute GVHD should prevent both NO secretion and lymphocyte unresponsiveness, that suppression of T-lymphocyte function by IFN-γ should occur only with excess NO production, and that inhibition of NO should restore lymphocyte function irrespective of IFN-γ production.

Our experimental data support this hypothesis. First, as expected, IFN-γ secretion by splenocytes from mice with GVHD was increased (Fig 3A). Previous studies have demonstrated convincingly that dysregulation of this cytokine is characteristic of acute GVHD. Blockade of IFN-γ production in cultures of splenocytes isolated from GVHD mice on day 13 post-BMT restored proliferative responses to Con A (Fig 3B). These data confirm observations from in vitro studies of other BMT models that IFN-γ mediates immunosuppression during GVHD. Second, the production of NO was dependent on excess IFN-γ secretion by cells from animals with GVHD (Fig 3C). This excess production is consistent with the ability of IFN-γ to induce NO in several in vitro systems and also with data from experiments wherein mice deficient in genes for IFN-γ or its receptor cannot generate NO. Third, abrogation of NO synthesis by addition of the specific NO synthase blocker, NMMA, to cultures restored the proliferation of GVHD lymphocytes to mitogen to the magnitude of non-GVHD splenocytes (Fig 2A) while reducing IFN-γ secretion by only 30% (data not shown). However, it is important to note that although inhibition of the NO pathway increased proliferation by two orders of magnitude in these studies, the proliferation achieved was equivalent to cultures of splenocytes with fivefold fewer T cells (Fig 1B). Thus, proliferation of these T cells, although greatly improved, was still not normal. Finally, in vivo blockade of IFN-γ production during GVHD abolished production of NO in response to Con A in vitro and restored proliferation to this mitogen (Fig 4). Blockade of the IFN-γ response in vivo was achieved by transplantation of polarized alloreactive donor T-cell populations with a type 2 cytokine pattern, ie, cells producing IL-4 but not IFN-γ. Type 2 donor T cells were able to regulate naive T cells, as demonstrated by the inhibition of IFN-γ and NO secretion and restoration of proliferative responses (Fig 5). The same cell-mixing strategy has been successfully used previously, and type 2 donor T cells also prevented other manifestations of the effector phase of GVHD.

Although there is recent evidence that IFN-γ may reduce the amplitude of T-cell proliferation in an MLR, several observations suggest that IFN-γ alone does not mediate hyporesponsiveness to mitogen in our GVHD model. First, the presence of IFN-γ per se did not affect the response to mitogen in control cultures: naive donor splenocytes secreted high levels of IFN-γ when stimulated with Con A (>200 U/mL), but did not produce detectable levels of NO and proliferated vigorously to Con A. Second, proliferation was not changed by addition of IFN-γ (or anti–IFN-γ MoAb) to any culture in which only background levels of NO were generated. Third, even though addition of NMMA to cultures restored proliferation to Con A to control levels, it did not substantially affect IFN-γ production by GVHD splenocytes, ruling out the possibility that NMMA affected proliferation via inhibition of cytokine synthesis. NMMA did not act as an independent T-cell mitogen, because it had no effect on proliferation when added to cultures of non-GVHD control groups and recipients of polarized type 2 T cells. Taken together, these data are strong evidence that a type 1 cytokine response during acute GVHD regulates T-cell immunosuppression in response to mitogen through the induction of NO. We postulate that IFN-γ primes mononuclear cells during acute GVHD and NO production is triggered by a secondary signal provided by LPS, a mechanism that has been proposed for the secretion of the proinflammatory cytokine, TNF-α, during acute GVHD. It is likely that other cytokines in addition to IFN-γ contribute to the induction of NO and the suppression of a mitogen-driven response during GVHD. We and others have reported that the production of TNF-α appears to be an important event in the generation of lymphocyte immunodeficiency in response to lipopolysaccharide during acute GVHD. It is well known that in other systems TNF-α cannot by itself induce NO production in macrophages, but requires signals provided by several different cytokines. Such cytokine interactions may help to explain differences between our data and those of Hoffman et al., wherein anti–IFN-γ antibody produced little inhibition of NO production by Con A–stimulated splenocytes in murine nonirradiated P → F, GVHD. In contrast, our GVHD model uses irradiated hosts. Total-body irradiation clearly induces inflammatory
cytokines, including TNF-α and IL-1, that influence NO production. The potential synergy among cytokines in the induction of the NO pathway and GVHD is under further investigation.

Our data clearly demonstrate the existence of NO-dependent and NO-independent pathways responsible for defects in T-cell proliferation in this model. Impaired proliferation to host alloantigens appears to be independent of NO, whereas the lack of response to mitogen involves both NO-dependent and -independent pathways. Although prevention of NO production dramatically increased proliferation, the response was equivalent to that in cultures from mice without GVHD that contained fivefold fewer T cells. Impaired proliferation of splenocytes to mitogens is the most profound characteristic of GVHD-associated immunosuppression, but poor proliferation to alloantigen and impaired CTL responses have also been described. At least one of several other mechanisms that are known to lead to GVHD-associated T- and B-cell immunodeficiency is probably operative; such mechanisms include generation of nonspecific suppressor cells, production of the suppressive cytokine, TGF-β, and anergy of mature lymphocytes, and T-cell deficits in the IL-2 response.

At present, the full range of pathophysiologic effects of NO during GVHD is unknown. Although abrogation of NO secretion appears able to prevent defective lymphocyte responses to mitogens, its production may well be critical for other aspects of host defense. NO has been shown to mediate tumoricidal activity by macrophages. In addition, IFN-γ–deficient mice are unable to mediate NO cytotoxicity. Thus, NO may be important for a graft-versus-leukemia effect associated with GVHD, although its inhibition of CTL responses may neutralize such a benefit. Similarly, blockade of NO synthesis might actually increase the risk of infectious complications from BMT despite improving lymphocyte responsiveness. Moreover, engraftment of hematopoietic stem cells may also depend on NO function. A recent report demonstrated that inhibition of NO production was associated with weight loss, decreased survival, and impaired engraftment in a murine model of allogeneic BMT. Analysis from a number of perspectives will therefore be needed to determine whether blockade of the NO pathway will offer any significant therapeutic benefits in our attempts to control GVHD and its complications.

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