Inhibition of Nitric Oxide Production Is Associated With Enhanced Weight Loss, Decreased Survival, and Impaired Alloengraftment in Mice Undergoing Graft-Versus-Host Disease After Bone Marrow Transplantation

By William R. Drobyski, Carolyn A. Keever, Gerald A. Hanson, Timothy McAuliffe, and Owen W. Griffith

The pathophysiologic role of nitric oxide (NO) in graft-versus-host disease (GVHD) was investigated in a murine bone marrow (BM) transplantation model where donor and recipient were H-2-matched but differed at multiple minor histocompatibility antigens. Host AKR/J (H-2^d) mice received lethal total body irradiation as pretransplant conditioning followed by transplantation of donor B10.BR (H-2^k) BM cells with or without spleen cells as a source of GVH-reactive T cells. NO production, as assessed by serum nitrate and nitrite levels, was increased for up to 3 weeks posttransplant in animals undergoing both moderate and severe GVHD. Administration of N^6^-methyl-L-arginine (L-NMA), an inhibitor of nitric oxide synthase, to animals undergoing GVHD resulted in effective suppression of NO production when compared with saline-treated GVHD control animals. Suppression of NO production by L-NMA in GVHD animals was associated with enhanced weight loss early posttransplant and decreased overall survival. Histologic analysis of tissues from L-NMA–treated and saline-treated GVHD animals showed that early weight loss was not because of an exacerbation of GVHD, indicating that NO did not appear to play an immunosuppressive role in this experimental model. L-NMA–treated animals with enhanced weight loss were observed to have splenic atrophy, decreased extramedullary hematopoiesis, and a reduction in BM cellularity when compared with GVHD control mice that were weight-matched before transplant. Analysis of T-cell chimerism in the spleen showed that L-NMA treatment impaired donor T-cell repopulation. In vitro colony-forming unit (CFU) assays were performed to further assess the role of NO on BM progenitor cell growth. L-NMA added directly into culture had no effect on CFU-granulocyte/macrophage (CFU-GM) formation in normal murine BM. In contrast, total CFU-GM from L-NMA–treated animals were significantly reduced when compared with GVHD controls or BM control animals who did not develop GVHD. Collectively, these data indicate that inhibition of NO impairs hematopoietic reconstitution and support the premise that NO appears to play a novel role in the facilitation of alloengraftment posttransplant.

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stimulated by cytokines such as IL-1, TNF, γ-IFN, and IL-2, resulting in the augmentation of NO production both in vitro and in vivo. Enhanced NO production has been recently documented in animals undergoing a GVH reaction, but the pathophysiological significance of this molecule is unknown. Because cytokine dysregulation is thought to play an important role in GVHD, this raises the possibility that NO might be a more proximate mediator of a cytokine-facilitated GVH response. Conversely, other studies have shown that NO has potent immunosuppressive properties in vitro, suggesting that the molecule might play a role in the downregulation of immune reactivity. To evaluate the role of NO in GVHD, we used a specific NOS inhibitor (Nω-methyl-L-arginine [L-NMA]) in a murine model of allogeneic BMT where donor and recipient are H-2–matched but differ at minor histocompatibility antigens.

MATERIALS AND METHODS

Mice. AKR/J (H-2b, Mls1, Thy 1.1+) and B10.BR/SgSn (H-2d, Mls2, Thy 1.2+) mice were purchased from Jackson laboratories (Bar Harbor, ME). All mice were housed in the American Association for Laboratory Animal Care-accredited Animal Resource Center of the Medical College of Wisconsin (Milwaukee, WI). Mice received regular mouse chow and acidified tap water ad libitum.

Reagents. L-NMA was prepared from L-ornithine (Sigma Chemical Co, St Louis, MO) and N,S-dimethylthioureaunitulinum iodide (Aldrich Chemical Co, Milwaukee, WI) by adaptation of the procedure of Corbin and Reporter. The flavinate salt was convened with acetic acid. Nω-methyl-D-arginine (D-NMA) was synthesized in an identical fashion with the exception that D-ornithine (Sigma) was used in place of L-ornithine. Both L-NMA and Nω-methyl-D-arginine (D-NMA) were dissolved in sterile distilled water and used at a concentration of 35 mg/mL.

Nitrate and nitrite levels were determined by the Griess reaction as described by Green et al. Nitrate was reduced to nitrite using a copper-coated cadmium column. Quantitation of biologic samples was by reference to a linear standard curve. Nitrate and nitrite standards gave peaks of equal area showing that nitrate was fully reduced.

BMT. AKR recipient mice were treated with 900 cGy total body irradiation (TBI) within 4 to 8 hours before transplantation. TBI was administered in a single dose using a Shepherd Mark I cesium irradiator (JL Shepherd and Associates, San Fernando, CA). The dose rate was 83.3 cGy/min. BM was flushed from donor femurs with complete Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) plus 5% fetal bovine serum. The BM plugs were passed through a sterile mesh filter to obtain single cell suspensions. BM cells were then washed, resuspended in fresh medium, and counted. Spleens were passed through sterile mesh screens to obtain single cell suspensions. Spleen cells were then treated with sterile distilled water to eliminate erythrocytes. BM and spleen cells were always greater than 90% viable by trypsin blue dye exclusion. Irradiated recipient mice received a single intravenous injection containing 10 × 10^6 BM cells with or without 5 to 20 × 10^6 spleen cells.

Flow cytometric analysis. Two-color immunofluorescence was used in some experiments to determine the extent of donor T-cell chimerism in the spleens of transplanted animals. Fluorescein isothiocyanate-conjugated anti-Thy 1.2 and phycoerythrin-conjugated anti-L3T4 (CD4) and Lyt 2 (CD8) were obtained from Becton Dickinson (Mountain View, CA). Donor-derived T cells were defined as Thy 1.2+L3T4+ and Thy 1.2+/Lyt 2+. Host T cells were defined as Thy 1.2-/L3T4- and Thy 1.2-/Lyt 2-.

For the evaluation of GVHD, tissue sections were screened with the examiner blinded to the treatment received by each animal. Preliminary studies had indicated that little histologic evidence of GVHD was observed in the skin or liver early posttransplant in animals receiving 5 × 10^6 spleen cells (GVHD control mice). Therefore, to facilitate a semiquantitative assessment of GVHD in control and experimental animals, grading of GVHD was confined to analysis of the colon.

Histologic studies. Tissues were obtained from control and experimental animals, fixed in 10% neutral buffered formalin, and processed into paraffin blocks. Four-micron-thick sections were prepared from each block and were cut at three levels to optimize sampling.

For the evaluation of GVHD, tissue sections were screened in both axial and longitudinal sections. Glands were selected that had the basal portion visibly resting in proximity to the muscularis mucosa. The integrity of the glandular epithelium and the degree of cellular infiltration in the lamina propria were evaluated. The degree of changes in the epithelium was graded on a scale of 1 to 4 with 1 being no abnormality, 2 being mild change consisting of occasional necrobiotic cells without significant gland injury, 3 being moderate change with several glands having one or more necrobiotic cells without gland injury, and 4 being severe change with several glands having injury and/or destruction. Inflammatory cell infiltrates in the lamina propria were graded on a scale of 0 to 4 reflecting no, mild, moderate, or marked infiltration. GVHD scores were normalized to that of BM controls to account for the effects of the conditioning regimen. The scores for epithelial integrity and degree of infiltration in the lamina propria were added to yield a total GVHD score for individual mice.

Colon-forming unit (CFU) assays. Femurs were harvested from mice, and BM was flushed from the BM cavity with Dulbecco's modified Eagle's medium. Cells were counted and resuspended at a concentration of 1.5 × 10^6 cells/mL in Iscove's modified Dulbecco's medium. Viability always exceeded 95% by trypsin blue dye exclusion. BM cells were plated in 35-mm tissue culture dishes containing 2.8% methylcellulose, (Fisher Scientific, Itasca, IL) 30% fetal calf serum, erythropoietin (15 U/mL; Toyobo, New York, NY), hemin (6.5 μg/mL; Sigma), metacaptopelthanol (3.2 × 10^-3 M), and pokeweed mitogen-stimulated spleen cell conditioned medium. Conditioned medium was obtained on the sixth day of culture and used at a concentration of 5%. This has been shown to be optimal for promoting progenitor cell growth in BM from normal, untreated AKR mice. Cultures were incubated at 37°C in 5% CO2 for 14 days. Colonies (greater than 50 cells) were scored at day 7 and 14 under an inverted microscope. Data were expressed as CFU-granulocyte/macrophage (CFU-GM) per 10^6 plated cells and total CFU-GM per two femurs.

Experimental design. AKR recipients were administered 900 cGy TBI as pretransplant conditioning. Based on the fact that 15 of 16 (94%) irradiated control mice (without BMT) died at a median of 16 days after irradiation (range, 13 to 22 days), this was considered a lethal dose. Animals were transplanted with B10.BR BM (10 × 10^6 cells) with or without graded doses of spleen cells (5 to 20 × 10^6). Irradiated mice receiving BM only were considered experimental controls, because these animals do not develop GVHD because of an insufficient number of T cells in the BM inoculum (typically 2%
transplanted with 10 x 10^6 B10.BR BM and were then treated with saline IN administration in normal and transplanted AKR mice. Normal unirradiated AKR mice IN L-NMA on GVHD and hematopoietic reconstitution. A dose of 250 mg/kg twice per day). Irradiated (900 cGy TBI) AKR animals were administered with saline were designated as GVHD controls. BMS chimeras re-

treatment with either phosphate buffered saline (PBS) or L-NMA to animals for a total of 40 days. Data are derived from four experiments. The group mean weights were based on decreasing numbers of

<table>
<thead>
<tr>
<th>Cohorts</th>
<th>Nitrate/Nitrite Levels (µmol/L)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
</tr>
<tr>
<td>BM only (N = 3)</td>
<td>28.3 ± 2.2</td>
</tr>
<tr>
<td>BMS-20 (N = 6)</td>
<td>127.5 ± 18.2</td>
</tr>
<tr>
<td>BMS-5 (N = 4)</td>
<td>190.2 ± 24.5</td>
</tr>
</tbody>
</table>

Cohorts of mice were alternately bled at the defined intervals for determination of nitrate/nitrite levels. A total of 6 and 10 mice were in the BM and BMS-20 groups, respectively (Experiment no. 1), whereas 8 mice each were in the BM and BMS-5 groups (Experiment no. 2).

Abbreviations: BMS-20, BM plus 20 x 10^6 spleen cells; BMS-5, BM plus 5 x 10^6 spleen cells.

to 3%). Animals receiving BM plus spleen cells (BMS) received treatment with either phosphate buffered saline (PBS) or L-NMA (250 mg/kg twice per day, intraperitoneally) to assess the effect of L-NMA on GVHD and hematopoietic reconstitution. A dose of 250 mg/kg was chosen based on a prior report that had shown that this dose prevented histopathologic evidence of intestinal GVHD in a parent→F1 murine model.7 L-NMA or saline was administered to animals for a total of 10 consecutive days, with the first dose administered 4 to 6 hours before transplant. All dosing was based on pretransplant weights of individual mice. BMS chimeras treated with saline were designated as GVHD controls. BMS chimeras receiving either saline or L-NMA were stratified before transplant by weight so that the mean weight of each experimental group was similar. Weights were obtained for every animal at least twice weekly for 60 days. All mice were ear punched before transplant so that individual animals could be serially assessed and to allow for comparisons between L-NMA--treated and weight-matched GVHD control animals. In some experiments, AKR hosts were preconditioned with 900 cGy TBI and then transplanted with AKR BM (10 x 10^6) with or without AKR spleen cells (20 x 10^6) to assess the effect of L-NMA in a syngeneic transplant model.

Statistical analysis. Data are reported as mean values ± 1 SD. Descriptive statistics for weight profiles are reported as group means. The group mean weights were based on decreasing numbers of measurements over time because of expiring animals. Repeated measures analysis of variance was performed using individual animal weight profiles. Analysis examined the questions of whether the observed weight profiles showed a group difference, variation over time, and a group-time interaction (ie, different time variation across study groups). Analyses were performed at the two-sided 0.05 level of significance. The Greenhouse-Geisser significance level adjustment was used for testing differences within animals over time. Repeated measures analysis of variance was also performed to compare the effect of L-NMA on CFU-GM in normal murine BM and

Table 1. Effect of Varying Intensity of GVH Reactivity on NO Production

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Nitrate/Nitrite Levels (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
</tr>
<tr>
<td>BM only (N = 4)</td>
<td>104 ± 18.4</td>
</tr>
<tr>
<td>BMS-5 (N = 4)</td>
<td>190.2 ± 24.5</td>
</tr>
</tbody>
</table>

Fig 1. Serial weight curves showing lack of toxicity of L-NMA administration in normal and transplanted AKR mice. Normal unirradiated AKR mice (N = 4; △) received a 10-day course of L-NMA (250 mg/kg twice per day). Irradiated (900 cGy TBI) AKR animals were transplanted with 10 x 10^6 B10.BR BM and were then treated with saline (N = 3; □) or L-NMA (N = 4; ■) for 10 consecutive days.

Fig 2. Actuarial survival of B10.BR/AKR chimeras. AKR mice were conditioned with 500 cGy TBI and were then administered either 10 x 10^6 B10.BR BM cells alone (N = 10; ○) or BM admixed with 5 x 10^6 B10.BR spleen cells (BMS-5). BMS-5 chimeras received treatment with either saline (N = 28; ○) or L-NMA (N = 35; □) for 10 consecutive days. Data are derived from four experiments.
formed to determine the effect of GVHD on NO production. Cytotoxic curves were compared using the log rank test. Pair-wise comparison having significantly different population means. Actuarial survival 5 chimeras were treated with either PBS (e) or L-NMA

Student's t-test to compare L-NMA-treated animals with saline-treated were performed at the 95% confidence level, to identify groups having significantly different survival distributions. Three groups. All animals survived through day 70, at which time they were killed. As a more objective parameter of clinical well-being, serial weights were obtained in animals over the course of this experiment (Fig 1). Irradiated animals in both the L-NMA and saline groups showed a characteristic decrease in weight, with nadirs occurring approximately 5 to 6 days after transplant. There was no difference in the weight curves between the two groups. Unirradiated animals treated with L-NMA slowly gained weight over the course of the experiment.

The effectiveness of this dose of L-NMA in inhibiting NO production was assessed in a pilot study. Because a more intense GVH reaction (20 × 10^6 spleen cells versus 5 × 10^6 spleen cells) was associated with higher mortality (see above), the efficacy of L-NMA in animals receiving 20 × 10^6 spleen cells was initially tested. Nitrate/nitrite levels were measured on day 9, near the end of L-NMA administration, and again at a time when levels were expected to be elevated (Table 1). Blood samples were obtained by retroorbital venipuncture before the morning L-NMA dose so that the nitrate/nitrite levels would be reflective of a trough L-NMA level. The control group consisted of animals transplanted with BM cells only. Nitrate/nitrite levels were higher in saline-treated mice (mean, 52.9 ± 24.5 μmol/L; n = 4) than in L-NMA-treated animals (mean, 22.8 ± 15.7 μmol/L; n = 5) or animals receiving BM only (mean, 33.0 ± 19.4 μmol/L; n = 3), indicating that L-NMA was able to suppress NO production in animals undergoing an intense GVH reaction to the level of BM controls.

Administration of L-NMA exacerbarates weight loss and adversely affects survival in mice with GVHD. The effect of L-NMA administration on survival of mice undergoing a GVH reaction was investigated. AKR recipients were conditioned with 900 cGy TBI and administered 10 × 10^6 BM
Table 2. Effect of L-NMA on NO Production and Survival After Syngeneic (AKR—AKR) BMT

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of Mice</th>
<th>Spleen Cell Inoculum</th>
<th>L-NMA Administration (250 mg/kg twice per day)</th>
<th>Nitrate/Nitrite (μmol/L)</th>
<th>60-Day Survival (% Surviving Animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>20 \times 10^6</td>
<td>—</td>
<td>12.3 ± 1.8</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>20 \times 10^6</td>
<td>—</td>
<td>26.4 ± 12.2</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>—</td>
<td>+</td>
<td>11.8 ± 7.1</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td>12.8 ± 0.7</td>
<td>100</td>
</tr>
</tbody>
</table>

AKR mice were conditioned with 900 cGy TBI and transplanted with AKR BM (10 \times 10^6 cells) with or without 20 \times 10^6 AKR spleen cells. Nitrate/nitrite levels were determined on day 9 posttransplant. All animals were then followed up for survival.

Table 3. Effect of L-NMA on Spleen Size in BMS Chimeras Undergoing GVHD

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Total Spleen Size (no. of cells \times 10^{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretransplant</td>
<td>Day 9 to 10</td>
</tr>
<tr>
<td>Saline—treated (n = 9)</td>
<td>24.8 ± 0.8</td>
</tr>
<tr>
<td>L-NMA—treated (n = 9)</td>
<td>24.2 ± 1.8</td>
</tr>
</tbody>
</table>

Weights and spleen sizes reflect the mean values for each group. Results are derived from three separate experiments.

Fig 4. Serial weight curves for the first 60 days posttransplant. AKR mice were conditioned with 900 cGy TBI and transplanted with AKR BM alone or AKR BM plus 20 \times 10^6 spleen cells (BMS-20). Mice transplanted with BM only were treated with either saline (C) or L-NMA (B) for 10 consecutive days. BMS-20 chimeras also received treatment with either saline (B) or L-NMA (C) over the same interval. Values represent mean weights of surviving animals at each time-point.

NO PRODUCTION IN GVHD AFTER ALLOGENEIC BMT

cells plus 5 \times 10^6 spleen cells from B10.BR donors. Mice received treatment for 10 days with either PBS or L-NMA (250 mg/kg twice per day, intraperitoneally). A control group received 900 cGy TBI plus 10^7 BM cells. Nitrate/nitrite values on the ninth day posttransplant were determined in all experimental groups. Saline-treated animals (n = 28) had significantly higher nitrate/nitrite levels (mean, 55.6 ± 12.6 μmol/L) compared with those of L-NMA—treated mice (n = 33; mean, 42.2 ± 12.4 μmol/L; (P = .001). Nitrate/nitrite levels in control animals (n = 10) averaged 43.6 ± 7.5 μmol/L, which did not differ from L-NMA—treated animals.

Survival of these animals is shown in Fig 2. Sixty-day survival for saline-treated animals was significantly better than for L-NMA—treated animals (50% vs 17%; P < .001). Poorer survival in L-NMA—treated animals was primarily because of substantial early mortality (median survival, 12 days in L-NMA—treated animals versus 58 days in saline-treated animals). L-NMA—treated animals were observed to have significantly greater weight loss (Fig 3) within the first 10 days posttransplant when compared with saline-treated mice (P < .01). After the initial 2 weeks, saline-treated animals and surviving L-NMA—treated animals were both able to regain weight and did not differ significantly from each other thereafter. In contrast, L-NMA—treated animals who received BM only (without spleen cells) showed no disproportionate loss in weight when compared with saline-treated BM controls (data not shown).

To confirm that the effect of L-NMA administration was enantiomer-specific, identically transplanted mice (n = 10) received a 10-day course of D-NMA (250 mg/kg twice per day, intraperitoneally). Nitrate/nitrite levels in this group averaged 54.1 ± 10.6 μmol/L, indicating that D-NMA was unable to inhibit NO production. Weight curves were similar to those of saline-treated animals (data not shown). Sixty-day survival was 80% with no early mortality, showing that the effect observed with L-NMA was enantiomer-specific.

Inhibition of NO production is associated with weight loss but not with exacerbation of GVHD early in the posttransplant period. Weight loss is known to be a sensitive indicator of GVHD in mice. Therefore, it was possible that the observed early weight loss in L-NMA—treated animals was because of an exacerbation of GVHD resulting from the suppression of NO production. To evaluate this possibility, tissues from saline- and L-NMA—treated mice that had received BM plus 5 \times 10^6 spleen cells were analyzed at 9 to 10 days posttransplant for histologic evidence of GVHD. L-NMA—treated animals showing the most profound weight loss were selected along with a group of saline-treated animals (GVHD controls) that had similar pretransplant weights.

None of the animals in either group were found to have significant pathologic changes of GVHD in the skin or liver on the ninth day posttransplant (data not shown). Therefore, histologic analysis was confined to the colon. Saline-treated animals were observed to have pathologic changes in the colon (eg, crypt cell necrosis and lymphocytic infiltration in
the lamina propria) consistent with GVHD. Although L-NMA-treated animals overall had less evidence of GVHD, differences in the histologic GVHD scores for saline (mean, 2.7 ± 1.9) and L-NMA mice (mean, 1.2 ± 0.9; N = 8 mice per group) were statistically nonsignificant (P = .10). These findings suggested that administration of L-NMA did not exacerbate GVHD and that the enhanced weight loss in L-NMA-treated animals could not be ascribed to GVHD. No other histologic abnormalities were observed in the colon, skin, or liver of L-NMA-treated animals to account for weight loss. The majority of surviving saline-, L-NMA-, and D-NMA–treated animals transplanted with BMS had clinical evidence of GVHD by day 60, indicating that L-NMA, when administered over a 10-day period, was not able to abrogate the eventual development of GVHD.

Weight loss was observed only in irradiated AKR recipients that had received BMS from histoincompatible B10.BR donors. To determine whether spleen cells alone, in the absence of histocompatibility differences between donor and recipient, could effect weight loss, experiments were performed using a syngeneic model. AKR recipients were irradiated with 900 cGy and transplanted with AKR BM with or without spleen cells (20 × 10^7). Control animals received BM only and were treated with either saline or L-NMA. The results of this experiment are shown in Table 2. L-NMA–treated recipients of AKR BMS had identical survival compared with that of saline-treated animals. Moreover, there was no difference in weight curves posttransplant between any of the groups (P = .90; see Fig 4). NO production at 9 days posttransplant was not increased above that of unirradiated AKR controls. These data indicated that a histocompatibility difference between donor and recipient plus the presence of spleen cells in the donor inoculum were both necessary to augment NO production and to effect enhanced weight loss in the postransplant period.

L-NMA treatment impairs hematopoietic reconstitution in animals undergoing GVHD. During histologic studies for analysis of GVHD, it was observed that the spleens of L-NMA–treated chimeras showing marked weight loss were noticeably smaller than those of saline-treated animals, suggesting that L-NMA might interfere with postransplant hematopoietic reconstitution. To quantify this observation,
Because it was possible that L-NMA might indirectly affect hematopoietic reconstitution posttransplant, in vitro assays were performed. Addition of L-NMA to haemopoietic progenitor cell growth. Suppression was shown to be enantio-mer-specific, because treatment with D-NMA did not inhibit NO production. Nitrate/nitrite measurements were deliberately taken immediately before L-NMA dosing. This was done so that measurements would be performed when L-NMA levels in individual mice would presumably be lowest and would, therefore, correspond to a period of least NOS suppression. Thus, the fact that NO production was suppressed at this point makes it likely that suppression was continuous during the period of dosing.

Prior studies in rodents have shown that NO is able to suppress immune reactivity in vitro. This conclusion is based on observations that inhibition of NO production with specific NOS inhibitors enhanced mitogen- and alloantigen-induced lymphocyte proliferation. Suppression of proliferation by NO appeared to be mediated in part by macrophages, because macrophage depletion before culture was able to
NO has also been implicated as having a role in mediating some of the immune suppression observed in in vitro studies performed in animals undergoing GVHD. Although these data support an immunosuppressive role for NO in vitro, other studies have implied that NO may be able to potentiate immune reactivity in vivo. Lander and coworkers showed that NO donor compounds were able to augment selected activation parameters in peripheral blood mononuclear cells, suggesting that NO might facilitate recruitment of other effector populations thereby enhancing immune reactivity. Similarly, NO has been shown to potentiate islet cell destruction caused by IL-1 administration in an autoimmune model of diabetes. More recently, McCartney-Francis et al. reported that inhibition of NO ameliorated the manifestations of inflammatory arthritis. These latter studies suggest that NO plays a proinflammatory role and serves as a more proximate mediator of tissue damage in vivo.

Although NO production has been documented to be increased in GVHD, the pathophysiologic role of NO in GVHD has not been extensively studied. Garside and coworkers showed that treatment with L-NMA ameliorated the pathologic manifestations of intestinal GVHD after haploidentical BMT in mice. However, this study did not verify that L-NMA effectively blocked NO production, nor was the effect of L-NMA on survival of animals undergoing GVHD assessed. In the present study, L-NMA administration was associated with enhanced weight loss, initially suggesting that inhibition of NO production might exacerbate GVHD. However, histologic analyses of colonic tissue samples from L-NMA- and saline-treated GVHD controls clearly indicated that L-NMA did not exacerbate GVHD in this experimental model. This finding is consistent with previous in vivo observations in other disease states and supports the premise that NO does not appear to play an immunosuppressive role in GVHD. Amelioration of GVHD by inhibition of NOS early after BMT was suggested by our data, but the study was not conclusive on this point. Assessment of a potential salutary effect of L-NMA on GVHD was confounded by the fact that L-NMA adversely affected allograftment which is a prerequisite for the induction of GVHD. The observation that the majority of sur-
Table 4. Effect of In Vitro L-NMA Administration on CFU-GM

<table>
<thead>
<tr>
<th>L-NMA Treatment</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>No L-NMA</td>
<td>151 ± 16.5</td>
<td>322 ± 34.8</td>
</tr>
<tr>
<td>1 mmol/L L-NMA</td>
<td>134 ± 18.6</td>
<td>300 ± 18.2</td>
</tr>
<tr>
<td>Experiment no. 2</td>
<td>199 ± 12.4</td>
<td>191 ± 21.3</td>
</tr>
<tr>
<td>No L-NMA</td>
<td>122 ± 25.4</td>
<td>199 ± 34.5</td>
</tr>
<tr>
<td>1 mmol/L L-NMA</td>
<td>139 ± 15.8</td>
<td>161 ± 21.8</td>
</tr>
<tr>
<td>Experiment no. 3</td>
<td>159 ± 20.0</td>
<td>176 ± 22.4</td>
</tr>
</tbody>
</table>

BM was obtained from normal AKR mice and assayed in vitro for the formation of CFU-GM in the presence or absence of 1 mmol/L L-NMA. Assays were scored on days 7 and 14. Data are presented as CFU-GM per 1 × 10⁶ plated cells ± 1 SD. Each experiment represents a replicate assay.

Vivinh L-NMA–treated animals went on to develop clinical signs of GVHD indicated that L-NMA, as administered in this study, was not able to abrogate the eventual development of GVHD. Whether alternative routes of administration or more prolonged therapy might mitigate GVHD without resulting in early mortality requires further study.

The observation of enhanced weight loss in L-NMA–treated animals was unexpected and appeared to be a major contributing factor to early mortality. The finding of weight loss was consistent with prior studies by Evans and colleagues who reported a wasting syndrome in Leishmania-infected animals treated with L-NMA. These investigators attributed this finding to a reduction in food intake and speculated that elevated levels of cytokines such as TNF may have played a role in the development of cachexia. In this study, weight loss was not observed in syngeneic recipients or allogeneic recipients of BM. Weight loss was observed only in irradiated animals transplanted with histoincompatible BMS, indicating that an allogeneic immune response was a prerequisite for the induction of enhanced weight loss. Although GVHD was not the cause of pronounced weight loss, the intensity of the GVHD reaction appeared to influence the degree of weight loss and early mortality in L-NMA–treated animals. Specifically, we have observed that, in very mild GVH reactions, weight loss and early mortality are mitigated (unpublished observations). Therefore, a threshold level of GVHD appears to be necessary for this outcome. It appears that TBI is also a critical factor, because weight loss occurred early posttransplant, after which time, some L-NMA–treated animals were able to regain weight as they recovered from the conditioning regimen. The fact that this occurred despite an ongoing GVH reaction suggests that GVHD alone was insufficient to effect continued weight loss. Because both TBI and GVHD are able to cause enhanced cytokine production, this would be consistent with the hypothesis that weight loss was cytokine-mediated. Studies are ongoing to define this mechanism that may represent a novel approach for the study of the phenomenon of cachexia in inflammatory conditions.

We cannot exclude the possibility that the microbiologic flora of animals was in part responsible for weight loss and early mortality in L-NMA–treated mice. Infection is a major cause of death in mice undergoing GVHD, and the microbiologic status of animals can modulate the intensity of GVH reactivity. This is born out by studies that have shown that GVHD severity can be ameliorated or completely prevented by transplanting animals in germ-free environments. NO is known to be important in the host defense against selected parasites and intracellular organisms, although its full role in the spectrum of host immune responses has not been completely defined. It is possible that early mortality in L-NMA–treated animals may have been because of a reduced ability to eradicate infection or that the process predisposing animals to weight loss may have enhanced host susceptibility to infectious complications. It is unlikely that deaths were exclusively caused by impaired hematopoietic reconstitution, because irradiated control animals survived longer than L-NMA–treated mice.

NO has been postulated to play a role in vitro in the growth and differentiation of leukemia cells, suggesting that NO may be important in hematopoiesis. L-NMA–treated mice with early weight loss were noted to have marked reduction in BM cellularity and extramedullary hematopoiesis when compared with weight-matched saline-treated GVHD controls. Donor T-cell repopulation in the spleens of these animals was also reduced (Fig 6 and Table 3). Collectively, these data suggest that inhibition of NOS was able to compromise hematopoietic reconstitution posttransplant. To further evaluate this observation, we performed in vitro CFU assays to assess the effect of L-NMA on normal murine BM. Addition of L-NMA to colony assays had no effect on CFU-GM, indicating that L-NMA did not directly inhibit progenitor cell growth. In contrast, there was a significant reduction in CFU-GM in BM obtained from cachectic L-NMA–treated animals when compared with either GVHD or BM control animals, indicating that inhibition of NO production indirectly affected BM suppression. The reason inhibition of NO production was able to impair hematopoiesis is not clear. Graft rejection has not been observed in B10.BR/AKR donor-host combinations, because animals transplanted with BM depleted of mature T cells become mixed chimeras. This is presumably because of the lack of mixed lymphocyte culture reactivity in the host-versus-graft
administration. Therefore, impaired hematopoietic reconstitution was not likely to be due to augmentation of a host-antidonor immune response. It is tempting to speculate that L-NMA may have augmented release of cytokines such as TNF and γ-IFN that are known to have BM suppressive effects in vivo. However, there is presently no data to support the premise that inhibition of NO production is able to enhance cytokine release. It is also possible that L-NMA administration may have facilitated the induction of other soluble factors or effector cell populations which played more proximate BM suppressive roles. It should also be noted that these observations may be due in part to the strain combinations used in this study and that other strains will need to be evaluated to confirm the generalizability of these results.

In conclusion, this study showed that NO production is increased in GVHD induced across minor histocompatibility antigens. Administration of L-NMA was able to suppress NO production in animals undergoing GVHD but was associated with pronounced early weight loss and decreased survival. L-NMA treatment did not exacerbate GVHD early in the posttransplant period, indicating that NO does not appear to play a critical immunosuppressive role in this experimental model. Inhibition of NO production was associated with impaired hematopoietic reconstitution, suggesting that NO may play a role in facilitating engraftment in allogeneic BMT recipients. Efforts to understand the mechanisms by which inhibition of NO production impairs hematopoietic reconstitution may provide new insights into understanding the process of alloengraftment and warrants further study.

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