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Hyporesponsiveness of Donor Cells to Lipopolysaccharide Stimulation Reduces the Severity of Experimental Idiopathic Pneumonia Syndrome: Potential Role for a Gut-Lung Axis of Inflammation


Idiopathic pneumonia syndrome (IPS) is a major complication of allogeneic bone marrow transplantation (BMT). We have shown that experimental IPS is associated with increased levels of LPS and TNF-α in the bronchoalveolar lavage (BAL) fluid. We hypothesized that the deleterious effects of these inflammatory mediators in the lung may be linked to gut injury that develops after BMT. To test this hypothesis, we used mouse strains that differ in their sensitivity to LPS as donors in an experimental BMT model. Lethally irradiated C3FeB6F1 hosts received BMT from either LPS-sensitive or LPS-resistant donors. Five weeks after BMT, to account for insults in various forms occurring in 25–55% of transplanted patients, host disease (GVHD) and acute pulmonary toxicity. Pulmonary diseases. Unfortunately, the utility of allogeneic BMT is limited by serious side effects, including pulmonary fibrosis, graft-versus-host disease (GVHD), and acute pulmonary toxicity. Pulmonary insufficiency in various forms is a common problem (4).

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The Journal of Immunology, 2000, 165: 6612–6619.

During the last several decades, allogeneic bone marrow transplantation (BMT) has emerged as an important therapy for a number of malignant and nonmalignant diseases. Unfortunately, the utility of allogeneic BMT is limited by various serious side effects, including the development of graft-versus-host disease (GVHD) and acute pulmonary toxicity. Pulmonary insufficiency in various forms is a common problem (4).

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The Journal of Immunology, 2000, 165: 6612–6619.
To further investigate the relationship between LPS, TNF-α, and IPS, we have tested the effect of donor responsiveness to LPS on the development of this process by using mice known to be sensitive or resistant to the effects of LPS as bone marrow (BM) donors. C3H/Hej and C3Heb/Fej are closely related substrains of mice that differ in their response to the lethal effects of LPS. C3Heb/Fej animals exhibit normal murine sensitivity to LPS challenge, whereas a genetic mutation in the Tlr 4 gene of C3H/Hej mice has made this strain resistant to LPS stimulation as manifest by decreased cytotoxicity and cellular cytokine production (19–21). These defects are specific to LPS stimulation because macrophages and B cells can respond to other stimuli and, importantly, T cells from LPS-resistant (LPS-r) mice respond normally to mitogen and alloantigen (22, 23). Using these genetic mutants, our data show that recipients of LPS-r BM develop significantly less lung histopathology by week 5 after transplant and uncover a potential etiologic link between gut and lung damage after BMT.

Materials and Methods

Mice

Female C3H/Hej (H-2k), C3Heb/Fej (H-2k), and (C3FeB6)F1 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used for BMT and in vitro experiments between the ages of 12 and 20 wk. The C3H/Hej and C3Heb/Fej substrains have a common origin. C3Heb/Fej is a result of a transfer of C3H/Hej ova to C57BL/6J (eb) performed by Dr. Fekete (Fe) at The Jackson Laboratory in 1948. Despite their separation of 50 years, the mice have very few differences at the DNA level. Histocompatibility between these substrains has been confirmed by the absence of rejection during skin grafting experiments (J. Sharp, unpublished observation) and of GVHD after BMT (our unpublished observation).

Bone marrow transplantation

Mice were transplanted according to a standard protocol as described previously (22). BM was harvested from the femurs and tibias of donor mice and depleted of T cells using an anti-Thy 1.2 mAb (American Type Culture Collection, Manassas, VA) and Low-Tox-M rabbit complement (Accurate Chemical & Scientific, Westbury, NY). Cell mixtures of 5 × 10⁶ T cell-depleted BM cells supplemented with 0.25 × 10⁶ nylon wool nonadherent splenic T cells from either syngeneic (C3FeB6F1) or allogeneic (C3H/Hej/C3Heb/Fej) donors were resuspended in Leibovitz’s L-15 medium (Life Technologies, Gaithersburg, MD). The lungs were then removed, placed in a mixture containing 0.1% collagenase B and dispase (1 U/ml; Boehringer Mannheim, Indianapolis, IN). The lungs were then washed twice with airway lavage for 3 times with 5 ml of heparinized PBS (1 U/ml). This process was repeated with 5 cc of a digestion mixture containing 0.1% collagenase B and dispase (1 U/ml; Boehringer Mannheim, Indianapolis, IN). The lungs were then placed in a sterile dish, and sectioned into small pieces using a scalpel. Minced lungs were then placed in media containing 0.1% collagenase B (5 ml per mouse lung total volume) and incubated for 90 min at 37°C. Digested lung tissue was vigorously mixed to form single cell suspensions, which were subsequently filtered over a 70-μm screen. Cells were then washed twice and resuspended in 10% FCS/DMEM supplemented with 50 μ/ml penicillin, 50 μg/ml streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, 0.02 mM 2-ME, and 10 mM HEPES (pH 7.45). Cell suspensions were then reinfused for 90 min at 37°C, and nonadherent cells were collected and resuspended, counted, and stained for FACS analysis, and prepared for cell culture. Single cell splenocyte suspensions were prepared using our standard protocol. Lung and spleen cells (2 × 10⁶) were normalized for total T cell number (see below) and then cultured in flat-bottom 96-well Falcon plates (Becton Dickinson, Franklin Lakes, NJ) in the presence of 1 × 10⁶ irradiated naive C3FeB6F1 peritoneal cells at 37°C in a humidified incubator supplemented with 7.5% CO₂. Proliferative response to host Ag was measured by a 1×10⁶ Bifidobacterium plate reader (Wallac, Turku, Finland) after 72 h by incubation of [3H]thymidine (1 μCi) for the last 24 h of incubation.

For studies of TNF-α secretion, BM and BAL cells from naive C3H/Hej and C3Heb/Fej animals or BAL cells from transplanted mice 5 wk after BMT were suspended in 10% FCS/DMEM supplemented as above, and plated at 2 × 10⁶ cells per well in flat-bottom 96-well Falcon plates (Becton Dickinson) with 10 μ/ml of LPS. Supernatants were collected for TNF-α analysis by ELISA after 4 h.

Cell surface phenotype analysis

To analyze cell surface phenotype, cells from BAL fluid and whole lung were harvested from transplanted mice as described above, resuspended in PBS, and stained with FITC-conjugated mAbs to CD4 (PharMingen, San Diego, CA) and Mac-1 (Caltag, Burlingame, CA) or PE-conjugated CD8 (PharMingen) for flow cytometric analysis. Cells (0.5 × 10⁶) were incubated for 20 min at 4°C with mAb 2.4G2 to block nonspecific staining via Fc receptors and then with the appropriate FITC- or PE-conjugated mAbs for 30 min at 4°C. The cells were subsequently washed twice with PBS/0.2% BSA before fixation in 1% paraformaldehyde. Two-color flow cytometric analysis of 1 × 10⁶ cells was performed using a FACSscan (Becton Dickinson, Mountain View, CA). The FACSscan was calibrated using PE- and FITC-conjugated, nonspecific IgG Abs. Using this data, lymphocyte (CD4⁺ and CD8⁺) and macrophage (Mac-1⁺) populations were normalized within allogeneic groups for MLR and LPS stimulation experiments, respectively.

Cytokine ELISA

Concentrations of TNF-α and IFN-γ were measured in BAL fluid and cell culture supernatants by sandwich ELISA using specific anti-murine mAbs for capture and detection and the appropriate standards purchased from
PharMingen (IFN-γ) and Genzyme (Cambridge, MA) (TNF-α). Assays were performed according to the manufacturer’s protocol. BM samples and tissue culture supernatants were analyzed as previously described (5, 22). Assay sensitivity was 16–20 pg/ml for TNF-α and either 0.25 U/ml or 7.5 pg/ml for IFN-γ. ELISA plates were read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

**Determination of endotoxin levels**

For determination of endotoxin concentration in serum and BAL fluid, the Limulus amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD) was performed according to the manufacturer’s protocol as previously described (5). Briefly, serum and BAL fluid samples were collected and analyzed using pyrogen-free materials, diluted 10% (v/v) in LAL reagent water, and heated to 70°C for 5 min to remove any nonspecific inhibition to the assay. Samples were then incubated with equal volumes of LAL for either 10 min (serum) or 30 min (BAL fluid) at 37°C and developed with equal volumes of substrate solution for 6 min. The absorbance of the assay plate was read at 405 nm using the same microplate reader used in cytokine assays. Samples and standards were run in duplicate and the lower limit of detection was 0.15 U/ml (serum) and 0.03 U/ml (BAL fluid). All units expressed are relative to the U.S. reference standard EC-6.

**Systemic and histopathologic analysis of GVHD**

The degree of systemic GVHD was assessed by a standard scoring system that incorporates five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity (5, 22, 25). Transplanted mice were ear punched, and individual weights were obtained and recorded on day 0 and weekly thereafter. At the time of analysis, mice from coded cages were evaluated and graded from 0 to 2 for each criterion. A clinical index was subsequently generated by summation of the five criteria scores (maximum index = 10). We have found this index to be a more sensitive index of GVHD severity than weight loss alone, a parameter which has been found to be a reliable indicator of systemic GVHD in multiple murine models (26).

Acute GVHD was also assessed by detailed histopathologic analysis of liver and intestine, two primary GVHD target organs. Sections of large bowel (transverse) and liver (right lobe) were harvested from animals at the time of analysis and placed in 10% buffered formalin. Specimens were then embedded in paraffin, cut into 5-μm thick sections, and stained with hematoxylin and eosin for histological examination. Slides were coded without reference to mouse type or prior treatment status and examined systematically by a single pathologist (Dr. J. M. Crawford) using a semiquantitative scoring system as previously described (22, 27).

**Recombinant human TNF receptor:Fc (rhTNFR:Fc) treatment**

In some experiments, BMT recipients were treated with a soluble dimeric TNF-α binding protein where two human TNF receptors are bound to the Fc portion of human Ig molecule (rhTNFR:Fc; Immunex; Seattle, WA) (28). Mice were injected i.p. with 100 μg of rhTNFR:Fc daily from BMT injection. In each set of experiments, mice in the control group received 100 μg of human IgG similarly diluted.

**Statistical considerations**

All values are expressed as the mean ± SEM. Statistical comparisons between groups were completed using the nonparametric, unpaired Mann-Whitney U test, except for analyzing survival data when the Wilcoxon rank test was used.

**Results**

**Naive C3H/Hej BAL cells and BM are resistant to LPS stimulation but T cells respond normally to host allo-Ags**

Before determining the effects of donor responsiveness to LPS stimulation on the severity of lung injury after BMT, we measured in vitro TNF-α production to LPS stimulation by bronchialveolar cells and BM from either naive LPS-resistant (LPS-r; C3H/Hej) or LPS-sensitive (LPS-s; C3Heb/Fej) BMT donors. Cells were harvested and cultured in vitro with LPS, and cell supernatants were analyzed for TNF-α concentration by ELISA as described in Materials and Methods. Table I demonstrates that LPS-s cells from each compartment responded normally to LPS and produced significant amounts of TNF-α after 4 h in culture, whereas similarly stimulated cells obtained from LPS-r mice produced little (30-fold less) or no TNF-α (p < 0.01). These findings suggested that donor accessory cells ultimately repopulating the hemopoietic and pulmonary compartments should remain resistant to LPS stimulation after BMT. Next, naive nylon wool-purified T cells were cultured with irradiated host (C3FeB6F1) stimulator cells as described in Materials and Methods. In contrast to the above findings, we found no difference between groups with respect to naive T cell responses to alloantigens; proliferation and IFN-γ secretion of LPS-r and LPS-s T cells were equivalent (Table I).

**Recipients of LPS-r BMT develop less severe lung injury after allogeneic BMT**

In light of these results, we used LPS-r and LPS-s mice as allogeneic BMT donors as a strategy to evaluate the effects of sustained down-regulation of TNF-α secretion to LPS stimulation on the development of acute lung injury after transplant while keeping donor T cell responses to host Ags constant. Lethally irradiated C3FeB6F1 mice received BMT from LPS-r or LPS-s donors as described in Materials and Methods. Transplant parameters were chosen so that a significant number of animals would be alive and available for analysis by 5 wk after BMT, a time point associated with significant lung injury in our BMT systems (5, 7, 16). In all experiments, recipients of syngeneic (F1) BM and T cells served as controls. To evaluate the extent of pulmonary toxicity that developed during this time interval, we first analyzed lung histopathology in surviving animals. Consistent with previous studies, mice receiving syngeneic BM maintained essentially normal histology, whereas two major histopathologic patterns were present in the lungs of mice receiving allogeneic BM and T cells. First, a mononuclear cell infiltrate was noted around both pulmonary vessels and bronchioles, and second, an acute pneumonitis was apparent involving both the interstitial and alveolar spaces (5, 7, 16).

![Image](http://www.jimmunol.org/DownloadedfromHttp://www.jimmunol.org/ByGuestOnApril16,2017)

**Table I. In vitro TNF-α production to LPS stimulation and T cell responses to host Ags by naive LPS-s and LPS-r cells**

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Responder Only</th>
<th>SI</th>
<th>IFN-γ (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF-α secretion</strong></td>
<td>BAL cells</td>
<td>BM</td>
<td>Host Ag</td>
<td>Responders Only</td>
</tr>
<tr>
<td>LPS-s</td>
<td>8910 ± 98*</td>
<td>240 ± 14*</td>
<td>9015 ± 1772</td>
<td>313 ± 74</td>
</tr>
<tr>
<td>LPS-r</td>
<td>214 ± 10</td>
<td>0 ± 0</td>
<td>8657 ± 1327</td>
<td>242 ± 74</td>
</tr>
</tbody>
</table>

*Naive BAL cells and bone marrow were harvested from LPS-s (C3Heb/Fej) and LPS-r (C3H/Hej) mice, stimulated in vitro (2 × 105 cells per well) with 10 ng/ml of LPS, and TNF-α production was measured by ELISA from cell culture supernatants obtained 4 h thereafter as described in Materials and Methods. Cells from two to three mice were pooled per group. The number of LPS-s and LPS-r BAL cells cultured was normalized per the percentage of Mac-1+ cells present in each cell population. Of note, >90% of cells in the BAL fluid from each group were macrophages as determined by microscopic analysis of cytospin preparations. Next, nylon wool-purified splenic T cells were harvested from naive, irradiated, and then cultured (2 × 105 responders per well) with irradiated host stimulator cells. Proliferation and IFN-γ production after primary MLR were determined as described in Materials and Methods. Data are expressed as mean ± SD of triplicate wells and are representative of one of two similar experiments. *, p < 0.01.
Semiquantitative evaluation of lung sections demonstrated that pulmonary damage was significantly worse after allogeneic BMT compared with syngeneic controls (3.4 ± 0.5 vs 0.8 ± 0.3; p < 0.01). Further analysis demonstrated that mice receiving LPS-r BMT developed significantly less lung pathology compared with LPS-s BMT recipients (Fig. 1A; p = 0.01). This finding was associated with a significantly better pulmonary dynamic compliance and a reduction in BAL fluid cellularity (Fig. 1, B and C; p < 0.05). Thus, transplantation of LPS-r cells resulted in a physiologically significant reduction in lung pathology 5 wk after allogeneic BMT.

**Reduction of lung injury after LPS-r BMT is associated with a down-regulation of TNF-α production, decreased T cell infiltration in the lung, and equivalent pulmonary T cell responses to host Ags**

We have previously shown that both TNF-α and donor-derived, host-reactive T cells are associated with the development of IPS (5, 7). Therefore, we hypothesized that the reduction in pulmonary injury seen 5 wk after LPS-r BMT would be associated with decreased TNF-α secretion in the lungs of these animals. Furthermore, because TNF-α has been shown to enhance the activation, expansion, and survival of T cells both in vivo and in vitro (30, 31), it was possible that the reduction in lung injury after LPS-r BMT could also be mediated by diminished alloreactivity despite equivalent responses of naive LPS-r and LPS-s T cells to host Ags pre-BMT. To test these hypotheses, we examined the BAL fluid compartment of BMT recipients for TNF-α secretion and evaluated pulmonary T cell responses to host Ags. BAL cells were harvested 5 wk after BMT and cultured with LPS as described above. As shown in Table II, BAL cells from LPS-r BMT recipients produced ~30-fold less TNF-α to LPS stimulation than cells obtained from recipients of LPS-s BMT (p < 0.01). This finding correlated with the naive phenotype of C3H/HeJ BAL cells (Table I) and was consistent with our findings that >90% of BAL cells are of donor origin by week 2 after transplant (data not shown). The decrease of TNF-α production in vitro was confirmed in vivo; animals transplanted with LPS-r cells had significantly lower BAL levels of TNF-α compared with LPS-s BMT recipients (Table II; p = 0.02).

We next examined the effect of donor responsiveness to LPS on pulmonary T cell responses to host Ags by analyzing BAL IFN-γ and whole lung T cell phenotypes in vivo and T cell proliferation in vitro. As shown in Table II, BAL fluid IFN-γ levels were equivalent 5 wk after LPS-s and LPS-r BMT. Similarly, when whole lung T cells were harvested and analyzed as described in Materials and Methods, the average CD4/CD8 ratio of T cells infiltrating the lung were comparable between allogeneic groups, 7.7 vs 7.0 in LPS-s and LPS-r recipients, respectively, while being significantly greater than the normal ratio of 2.5 found after syngeneic BMT. Although the average number of CD4+ and CD8+ cells present in the lungs of LPS-s BMT recipients was significantly greater compared with animals receiving LPS-r BMT (5.2 ± 0.5 × 10^6 vs 2.1 ± 0.1 × 10^6 and 0.67 ± 0.09 × 10^6 vs 0.32 ± 0.08 × 10^6 for CD4+ and CD8+ cells, respectively), when equal numbers of pulmonary T cells (CD4+ plus CD8+) were stimulated in vitro with irradiated host Ags, proliferation was modest and no differences were observed between allogeneic groups (Table II). Taken together, these data suggest that donor hyporesponsiveness to LPS attenuates the severity of IPS in this model by reducing TNF-α-mediated injury. Although the majority of our data suggests that genetic resistance of donor cells to LPS does not significantly affect T cell responses to host Ags, we cannot rule out that the reduction in T cell numbers seen in the lungs after LPS-r BMT was not a direct consequence of decreased T cell function.

**Donor resistance to LPS results in decreased BAL levels of LPS and reduced intestinal toxicity after BMT**

Endotoxin or LPS is a potent enhancer of inflammatory cytokine release, and experimental IPS is associated with elevated BAL fluid levels of this molecule (5). Therefore, we next measured LPS concentrations in the BAL fluid of LPS-s and LPS-r BMT recipients 5 wk after transplant. Table III shows that recipients of LPS-r BMT had significantly lower BAL fluid LPS levels compared with mice receiving LPS-s BMT (p < 0.05). This reduction in BAL fluid LPS levels was intriguing and suggested that damage to other target organs may also be decreased after LPS-r BMT. In particular, the intestinal tract is also known to be susceptible to injury mediated by TNF-α after allogeneic BMT (22, 25, 32, 33). Although acute damage to bowel early after BMT is directly related to subsequent systemic GVHD severity (22, 27, 33), a clear association between gut injury and the development of pulmonary toxicity has yet to be established. To evaluate a possible link between the toxicity that occurs in each of these two organs, samples of large bowel were scored semiquantiatively after BMT as described in Materials and Methods. At week 5 after transplantation, the mean bowel pathology index was significantly lower in mice receiving LPS-r BMT compared with recipients of LPS-s donor cells (Table III; p = 0.02). Furthermore, intestinal toxicity was already significant by week 1 after allogeneic BMT when similar differences in histopathology and serum LPS levels were present between LPS-s and LPS-r BMT recipients even though lung disease had not yet developed (Table III). Thus, significant damage to the GI tract preceded peak lung injury in this model. Consistent with previous observations (22), this reduction in intestinal injury seen after LPS-r BMT correlated with less severe systemic GVHD in this group as demonstrated by decreased GVHD-related mortality and lower clinical scores from week 2 onward (Fig. 2; p < 0.01). Similar differences between allogeneic groups were also seen with respect to target organ GVHD; LPS-r BMT recipients had less hepatic injury compared with animals receiving LPS-s BMT (8.2 ± 0.8 vs 4.9 ± 0.4; p < 0.05). It is important to note that the higher mortality seen in the LPS-s group ultimately resulted in a selection of “surviving animals” for analysis at 5 wk. Although this may introduce some selection bias to the analysis, dead animals may have developed even more severe target organ injury, making the differences seen between allogeneic groups even more compelling.
Systemic neutralization of TNF-α reduces intestinal and pulmonary injury after LPS-r BMT

The above findings revealed an association between early and continued intestinal damage and the eventual development of lung pathology in this model. Next, we further studied the relationship between TNF-α and damage to the gut and lung by direct TNF-α blockade. Previous observations have demonstrated that neutralization of TNF-α could protect the gastrointestinal tract from early damage (22). We hypothesized that neutralization of TNF-α throughout the entire time course of BMT would result in continued protection of the gastrointestinal tract and an ultimate reduction in lung injury. rhTNFR:Fc is a bivalent, soluble form of the p75 TNF-α receptor bound to the heavy chain portion of the human Ig molecule that has potent TNF-α-neutralizing capacity in the mouse (28, 34). In a preliminary experiment, rhTNFR:Fc was administered to C3FeB6F1 recipients of LPS-s BMT for 5 wk as described in Materials and Methods. Similarly transplanted allogeneic BMT controls and all syngeneic BMT recipients were injected with control Ig. TNF-α neutralization resulted in reduced GVHD mortality (50 vs 80%) and reduced lung injury (1.8 ± 1.0 vs 4.8 ± 0.7; p = 0.07) by week 5 after BMT, but the high mortality rate in control-treated animals precluded optimal comparisons of pulmonary toxicity between groups. Therefore, we examined recipients of LPS-r BMT in subsequent experiments, reasoning that the majority of control animals would be alive 5 wk after BMT and thus provide a sufficient number of animals to detect significant differences in lung injury between allogeneic groups. Elevated levels of TNF-α were present in LPS-r BMT recipients both in the BAL fluid at week 5 (Table II) and serum by week 1 after BMT (22). Administration of rhTNFR:Fc after LPS-r BMT resulted in significant reductions in both intestinal injury and pulmonary toxicity compared with similarly transplanted animals receiving control Ig (Fig. 3, A–C; p < 0.05). Protection of the bowel by rhTNFR:Fc administration also resulted in reduced translocation of LPS into the BAL fluid (Fig. 3D; p < 0.05). Systemic neutralization of TNF-α did not reduce the overall liver pathology score compared with control treatment after LPS-r BMT. However, the administration of rhTNFR:Fc did reduce the severity of hepatocellular damage as assessed by the presence of pan lobar necrosis and the number of microabscesses, acidophil bodies, and mitotic figures (0.3 ± 0.2 vs 0.8 ± 0.3; p < 0.05). These findings confirm a causative role for TNF-α in the development of IPS in this system and suggest an etiologic link between gut and lung damage after allogeneic BMT.

Discussion

We have examined the effects of donor responsiveness to LPS on the development of IPS in an irradiated P → F1, murine BMT system. Our data demonstrate that physiologically significant pulmonary histopathology is observed 5 wk after BMT across both major and minor histocompatibility Ags and that the transplantation of LPS-r donor cells significantly reduces the severity of this process. The reduced lung injury seen after LPS-r BMT was associated with decreased production of TNF-α both in vivo and in vitro, whereas pulmonary T cell responses to host Ags were equivalent between allogeneic groups. Recipients of LPS-r BMT also demonstrated decreased intestinal histopathology, less severe systemic GVHD, and reduced translocation of LPS to the BAL fluid. Systemic blockade of TNF-α throughout the entire post-BMT period effectively reduced gut and lung damage as well as BAL fluid levels after LPS-r BMT, further supporting a relationship between gastrointestinal and pulmonary toxicity via this inflammatory pathway. Taken together, these data demonstrate that donor resistance to endotoxin attenuates the severity of pulmonary toxicity by decreasing TNF-α production and reducing the translocation of LPS across the intestinal mucosa in this model. These findings support the hypothesis that LPS-induced cellular activation and cytokine release contribute to lung injury via a gut-lung axis of inflammation.

Several lines of investigation have recently contributed to our understanding of how inflammatory cytokines contribute to complications occurring after allogeneic BMT (33, 35, 36). In particular, TNF-α has been established as an effector of both clinical (35–37) and experimental (22, 25, 32) GVHD. Although critical for the development of early gastrointestinal injury that occurs in this context (22, 25, 32, 33), the role of TNF-α in GVHD-mediated damage to other target organs such as the liver has been less well defined (38). From a pulmonary perspective, TNF-α has been

Table II. BAL fluid cytokine levels and in vitro cellular responses 5 wk after BMT

<table>
<thead>
<tr>
<th>Group</th>
<th>BAL Fluid IFN-γ (pg/ml)</th>
<th>1° MLR: Proliferation (cpm)</th>
<th>BAL Fluid TNF-α (pg/ml)</th>
<th>In Vitro LPS Stim TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Host Ag</td>
<td>Responders Only</td>
<td>SI</td>
</tr>
<tr>
<td>LPS-s</td>
<td>17.0 ± 4.2</td>
<td>6453 ± 798</td>
<td>2200 ± 127</td>
<td>2.9</td>
</tr>
<tr>
<td>LPS-r</td>
<td>11.9 ± 3.9</td>
<td>5386 ± 400</td>
<td>2480 ± 50</td>
<td>2.1</td>
</tr>
<tr>
<td>Syn</td>
<td>&lt;7.5</td>
<td>1291 ± 83</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* C3FeB6F1 mice received allogeneic BMT from either LPS-s or LPS-r donors as in Fig. 1. At weeks 1 and 5 after BMT, tissue samples (lung and large bowel), and serum or BAL fluid were harvested and analyzed for semi-quantitative histopathology or LPS concentration as described in Materials and Methods. Data are expressed as mean ± SEM and represent a combination of two separate experiments with each time point. n = 6–12 per group; *, p < 0.05; #, p < 0.01 LPS-r vs LPS-s.
shown to be a critical effector of lung disease in several settings (5, 9–11, 17, 34, 39–41). Elevations of TNF-α have also been reported in the serum of patients who develop IPS (17) and in the lungs of animals with GVHD (5, 9–11). In addition, we have recently demonstrated that neutralization of TNF-α from weeks 4–6 after BMT effectively reduced the progression of lung injury in an allogeneic BMT model across multiple minor histocompatibility differences (16).

LPS is a major structural component of the outer membrane found on Gram-negative bacteria and is a potent inducer of TNF-α secretion. When the LPS molecule is shed from bacteria comprising normal bowel flora, it can leak into the systemic circulation and trigger a broad range of deleterious inflammatory responses from LPS-s cells including macrophages, monocytes, and neutrophils. Recently, our group and others have uncovered a critical role for LPS in the development of acute GVHD after allogeneic BMT and have shown that mice with GVHD are extremely sensitive to cellular activation induced by LPS (22, 25, 27, 42). We have also demonstrated that elevations in BAL fluid levels of LPS are associated with noninfectious lung injury that is seen in this context (5). In addition, the injection of exogenous LPS into animals with advanced GVHD exacerbates underlying pulmonary injury and results in a severe inflammatory response including alveolar hemorrhage (5). Pertinent to these experimental data, evidence for cytokine activation and LPS amplification in the bronchoalveolar compartment has been noted in patients with adult respiratory distress syndrome (41) and has recently been demonstrated in patients with IPS after BMT (18, 43). Clark and colleagues examined BAL fluid from patients with IPS and found increased vascular permeability and levels of inflammatory cytokines (IL-1, IL-2, TGFα, and sTNFr) and components of the LPS amplification system (LPS binding protein and CD14). These investigators concluded that proinflammatory cytokine activation contributes to the pathogenesis of IPS and suggested that patients with this complication may be at increased risk of lung injury mediated by LPS (18).

Our results confirm a role for LPS and TNF-α in the development of IPS after experimental BMT and support the hypothesis that TNF-α contributes to the development of this process both directly and indirectly. In addition to being directly cytotoxic, TNF-α likely contributes to lung injury by also promoting leukocyte chemotaxis (44, 45) and by activating endothelial cells (ECs), professional APCs, and lymphocytes (7, 12, 46–48). Therefore, the reduction in lung toxicity seen after LPS-α BMT or TNF-α neutralization with rhTNFR:Fc is likely the result of several factors. Down-regulation of TNF-α secretion in the lung should mitigate, at least in part, its direct toxic effects on both pulmonary parenchyma and endothelium as has been observed in other systems (39, 40, 49). We have found that EC apoptosis precedes the development of experimental IPS, and that neutralization of TNF-α with rhTNFR:Fc significantly decreased the severity of pulmonary EC damage after allogeneic BMT.5 In addition, decreased levels of TNF-α may also modulate endothelial activation and expression of adhesion molecules, thereby down-regulating the ability of ECs to provide costimulatory signals and promote leukocyte trafficking (44, 45, 50–52). Support for the latter is provided by the reduction in lymphocyte numbers after LPS-α BMT seen after whole lung digest.

The partial reduction in lung disease resulting from TNF-α down-regulation in this system is in accord with other reports (9, 16) demonstrating that other proinflammatory cytokines and cellular mechanisms involved in acute GVHD also contribute to the development of IPS (38, 53, 54). Specifically, IL-1β, TGFβ, and nitrating species have been implicated in the generation of early lung toxicity after allogeneic BMT, particularly when cyclophosphamide is included in the conditioning regimen (13, 14). Additionally, donor-derived T lymphocytes contribute to the pathogenesis of experimental IPS as shown by our group and others (5, 10, 12). Evaluation of pulmonary T cell responses in this system did not demonstrate significant differences between allogeneic groups, a finding that is consistent with the equivalent splenic T cell responses seen in this system 2 wk after LPS-s and LPS-α BMT (22) and the inability of short term (2 wk) administration of rhTNFR:Fc to alter pulmonary T cell responses in a murine IPS model to minor Ags (16).

In light of the known sensitivity of other GVHD target organs to injury mediated by TNF-α, it is also possible that in addition to directly reducing TNF-α production in the alveolar compartment, transplantation of LPS-α donor accessory cells altered other aspects of the systemic inflammatory response to LPS “upstream” from the lung. From this perspective, the structural and functional integrity of the gastrointestinal tract, which is believed to be a critical target organ for the induction and propagation of systemic GVHD, was likely to be important (33). Both clinical and experimental studies have shown that disruption of the gastrointestinal mucosa by the combined effects of BMT conditioning and GVHD-related injury can facilitate the translocation of endogenous LPS into the bloodstream (22, 25, 27, 55–57). Once in the peripheral circulation, LPS triggers “primed” peripheral mononuclear phagocytes to release cytotoxic amounts of inflammatory cytokines, which, in conjunction with cellular effectors (CTL and NK cells), contribute to target cell apoptosis and target organ injury and dysfunction (33). Our data demonstrate an early and continued reduction in intestinal histopathology after LPS-α BMT. The functional consequence of this protection is highlighted by the reduction in IPS levels measured initially in the serum and later in the BAL fluid of LPS-α BMT recipients and is further supported by the results of our TNF-α blocking experiments, which revealed a potential link between these two critical target organs.

The liver is pivotally located between the intestinal reservoir of Gram-negative bacteria and their toxic byproducts and the rich capillary network in the lung and represents another critical GVHD target organ. Kupffer cells in the liver detoxify and subsequently clear endotoxin from the portal circulation (38) and protect the lung in experimental models of sepsis and adult respiratory distress syndrome (59, 60). However, if the capacity of the liver to

clear an endotoxin challenge is exceeded, both inflammatory cytokines and unprocessed LPS can traverse into the systemic circulation and cause acute end organ damage (61–63). Support for this hypothesis is provided by clinical reports of acute noninfectious pulmonary toxicity associated with severe GVHD and venoocclusive disease (VOD) (2, 64) and by our previous work demonstrating that exogenous LPS challenge of animals with extensive GVHD overwhelmed the capacity of the liver to detoxify the endotoxin surge and resulted in extensive hepatocellular damage and enhanced lung inflammation via a TNF-α-mediated mechanism (16).

In this study, recipients of LPS-r BMT developed less hepatic injury compared with mice receiving LPS-s donor cells. However, the failure of systemic neutralization of TNF-α to further reduce overall liver damage in LPS-r BMT recipients underscores potential differences that may exist in GVHD physiology within various target organs. Although Hattori et al. have clearly shown that Fas-mediated toxicity by CTL plays an important role in hepatic GVHD (38), we have found that the liver is also sensitive, at least in part, to TNF-α-mediated injury (16). TNF-α is also critical to hepatic injury induced by Th-1 cells in a murine hepatitis model (65). In the current system, TNF-α production is decreased after LPS-r BMT; however, when we assessed Fas-Fas ligand-mediated killing using Fas-sensitive LK35.2 targets in an in vitro chromium release assay (66), we found no differences between naive LPS-s and LPS-r T cell effectors. Taken together, our data are consistent with the hypothesis that the liver, like the lung, is susceptible to both inflammatory and cell-mediated damage and suggest that although the liver may be an intermediary organ in the gut-lung axis of inflammation that contributes to IPS, it is the severity of intestinal GVHD that primarily determines the development of lung injury in this system.

This study confirms a significant but not exclusive role for TNF-α in the development of noninfectious lung injury after BMT and reveals a mechanism by which cellular activation and cytokine release induced by LPS may contribute to this process. Importantly, our results demonstrate a potential etiologic link between damage to the gastrointestinal tract and lung after BMT and support the theory that both inflammatory cytokine release involving a gut-lung axis, along with T cell effector mechanisms contribute to the development of IPS after allogeneic BMT. Furthermore, they suggest that methods that reduce cellular inflammatory responses to LPS (via direct inhibition of LPS or TNF-α) and, specifically, those that protect the integrity of the gut mucosa, such as IL-11 or keratinocyte growth factor (27, 67), may represent novel, noncross-reactive adjuncts to standard forms of immunosuppression currently used to prevent or treat noninfectious, immunologically mediated lung injury that develops after allogeneic BMT.

References