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Lung Injury Induced by Alloreactive Th1 Cells Is Characterized by Host-Derived Mononuclear Cell Inflammation and Activation of Alveolar Macrophages

Joan G. Clark, David K. Madtes, Robert C. Hackman, Wei Chen, Martin A. Cheever, and Paul J. Martin

We have investigated a murine model of acute lung injury caused by i.v. administration of a T cell clone (CD4+, Th1 phenotype) that recognizes Ly5, a polymorphic cell surface glycoprotein expressed on hemopoietic cells. Alloreactive cloned T cells, specific for host Ly5 Ag, cause a mononuclear cell pulmonary vasculitis and interstitial pneumonitis. In further studies of the cellular mechanisms involved in this model, we found that mature host T cells or B cells are not required, since lung injury was comparable in transgenic host mice that lack these cells (RAG-1 knockout). Cloned T cells labeled in vitro with bromodeoxyuridine were localized in inflammation foci in lung, but the majority of cells in the foci were not labeled. Using transgenic mice that constitutively express lacZ, we determined that the mononuclear cell vasculitis is of host cell origin. Alveolar macrophages (AM) from T cell-treated mice spontaneously secreted TNF-α in culture, whereas TNF-α was not detected in AM cultures from control mice. TNF-α production in response to LPS stimulation was significantly higher in AM cultures derived from T cell-treated mice than in those from control mice. Challenge with sublethal doses of LPS resulted in 50% mortality in T cell-treated mice and was associated with augmented AM TNF-α production and protein in bronchoalveolar lavage fluid. We conclude that immune activation of T cells of the Th1 phenotype can initiate lung injury characterized by a host-derived mononuclear cell inflammation and activation of AM.


Acute lung injury is a common sequela to bone marrow transplantation (1–5). Acute graft-vs-host disease (GVHD),3 an immune response generated by donor T cells and involving both cellular and cytokine components, is a risk factor for nonfectious lung injury after transplant (1, 6–9). The mechanisms by which this immune response culminates in lung inflammation and injury are not understood. It is well established that mature, immunocompetent, alloreactive donor T lymphocytes are necessary for the generation of GVHD (10). CTLs with the ability to recognize and kill target cells in a MHC class I- or II-restricted manner, have long been viewed as a likely mechanism of cellular injury in the recipient. The GVH reaction is complex, and recent research supports the involvement of both cellular and cytokine components (11, 12). An important advance in the understanding of GVHD has occurred with knowledge of the roles of various T cell subsets in the regulation of systemic immune responses. One important development has derived from studies using cloned CD4+ Th cells, which suggesting that this T cell pool can be divided into functional subsets based on the types of cytokines they produce (13, 14). Th1 cells are characterized by IL-2 and IFN-γ production, whereas Th2 cells are characterized by IL-4, IL-5, and IL-10 production. Th1 cells are strongly associated with T cell-mediated proinflammatory responses.

Studies of cytokine expression in murine models of acute GVHD as well as in humans undergoing allogeneic marrow transplantation have implicated Th1-type responses in the generation of severe GVHD (11, 12). Direct evidence of a role for Th1 cells derives from the demonstration that in vitro generated alloreactive CD4+ T cells of the Th1 type result in the acute lethality characteristic of GVHD (weight loss and diarrhea) in a murine transplant model (11). Moreover, Lehmann et al. have reported that cloned alloreactive Th1 cells administered to untransplanted mice resulted in lung and liver toxicity characterized by vascular leak (15).

While donor T lymphocytes are essential for the initiation of GVHD, the pathologic consequences may be mediated by distal non-T cell immune cells of the monocyte/macrophage lineage. Nestel and colleagues (16) have recently provided experimental evidence for this mechanism, advancing the concept that immune-activated macrophages are important effector cells in murine GVHD. Although this mechanism has not been directly studied in lung, lung injury associated with GVHD in murine marrow transplant models has been previously described (17–19), and Th1-like responses have been suggested as possible effector mechanism (20).

We recently provided direct evidence for a Th1-mediated mechanism of acute lung injury in a murine model induced by alloreactive Th1 cell clones that recognize Ly5 (CD45), a polymorphic cell surface glycoprotein expressed on hemopoietic cells (21). In mice, two Ly5 alleles (Ly5a, which encodes the Ly5.1 cell surface molecule, and Ly5b, which encodes the Ly5.2 cell surface molecule) have been defined. Alloreactive cloned T cells, specific for host

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3 Abbreviations used in this paper: GVHD, graft-vs-host disease; AM, alveolar macrophage; BrdUrd, bromodeoxyuridine; BAL, bronchoalveolar lavage.
Ly5 Ag, caused a mononuclear cell pulmonary vasculitis and interstitial pneumonitis.4 At high T cell doses, lethal pulmonary hemorrhage was observed. The only other notable organ involvement was hepatic vasculitis. In vitro studies established that the cloned T cells were not cytolytic, and after stimulation with allogeneic splenocytes or specific Ly5 peptide, they produced IFN-γ, but not IL-2, IL-4, or IL-10.

The apparently selective lung injury lead us to question whether Ly5 might be expressed in lung parenchymal cells as well as hematopoietic cells. To test this, we created chimeras by transplantation of Ly5+ marrow into irradiated Ly5− mice (21). Lung injury was induced by anti-Ly5+ T cells but not by anti-Ly5− cells, indicating that allogeneic hematopoietic cells provide sufficient stimulation for eliciting lung injury. Thus, lung injury initiated by an allogeneic immune effector cell does not require T cell recognition of a specific Ag in lung parenchyma.

These observations prompted us to investigate further the mechanisms by which alloreactive T cell activation can lead to inflammatory lung injury. In this report we present evidence that immune activation of T cells of the Th1 phenotype initiates an inflammatory reaction in lung that is amplified by host-derived mononuclear cells, characterized by activation of alveolar macrophages (AM), and associated with greatly increased susceptibility to sublethal LPS challenge.

Materials and Methods

Mice

C57BL/6, Ly5+, RAG-1-deficient (22), and lacZ transgenic (23) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Some Ly5+ mice were bred at the Fred Hutchinson Cancer Research Center (Seattle, WA). Mice were housed in microisolator cages under specific pathogen-free conditions with free access to sterile chow and water.

T cell clones and culture

T cell clones specific for Ly5+ (clone 8F5) and Ly5− (clone 1A4) alleles were developed and maintained in culture as previously described. In brief, Ly5+ mice and congenic Ly5− mice were cross-immunized with 13 mer Ly5+ and Ly5− peptides identical with a polymorphic region (p257–269) differing by three amino acids. CD4+ Th cells specific for the 13 mer peptides were elicited and cloned by limiting dilution. The clones used in these studies were 1A4 (specific for Ly5−) and 8F5 (specific for Ly5+) (21). Both were of the Th1 phenotype and produced IFN-γ. One of the clones selected for study (1A4) did not produce IL-2. In previous studies no qualitative differences in biologic activity (i.e., induction of lung injury) were detected. T cell clones were expanded by periodic stimulation with peptide (Ly5+) or allogeneic irradiated splenocytes in the presence of congenic irradiated splenocytes (allogeneic to congenic cells in a ratio of 1:10) and were maintained in the presence of IL-2 (10 U/ml).

In one experiment, T cells were stimulated and maintained for 2 wk in the presence of 100 μM bromodeoxyuridine (BrdUrd; Boehringer Mannheim, Indianapolis, IN).

Induction of lung injury

Cloned T cells (5, 7.5, or 10 × 106 cells in 0.5 ml of sterile PBS) were injected i.v. into the lateral tail vein of recipient mice. Resting T cells had undergone in vitro stimulation 2 to 3 wk before transfer into mice. Stimulated T cells were stimulated in vitro with allogeneic splenocytes 24 h before transfer into mice. In one experiment, T cells were stimulated by incubation for 24 h in wells precoated with Ab to CD3 (clone 145-2C11, PharMingen, San Diego, CA).

In some experiments, LPS (Escherichia coli O111: B4, List Laboratories, Campbell, CA) was administered i.p. to mice 24 h after T cell transfer at a dose of 5 mg/kg. This dose (100 μg of LPS in a 20-g mouse) was estimated to be 20% of a lethal dose of LPS in B6 mice (24).

Histology

Mice were anesthetized with Avertin (Aldrich, Metuchen, NJ) and exanguinated by renal artery transection. For histopathologic evaluation, lungs were excised at various times after T cell transfer, inflated with 25% H2O pressure with 10% formalin, fixed overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The histopathology was scored (0–4+) according to the extent and severity of vasculitis and inflammatory cell infiltration as follows: 0 = no abnormality; 1 = minimal inflammation involving <10% of venules; 2 = mild vasculitis involving 10 to 25% of venules; 3 = moderate vasculitis and perivascular inflammation involving >25% of venules; and 4 = severe vasculitis and perivascular lesions with alveolar and interstitial inflammation. All slides were coded and read by a pathologist without knowledge of the experimental treatment.

For immunohistochemical detection of T cells, excised lungs were inflated and embedded in Tissue-Tek OCT compound (Cryotome, IFC, Needham Heights, MA), snap-frozen in liquid nitrogen, and stored at ~70°C. Cryosections were fixed in acetone and stained with Ab to CD3ε (clone 145-2C11, PharMingen). Ab was detected with biotinylated goat anti-hamster Ig followed by streptavidin-peroxidase complex and then diaminobenzidine and NiCl2. Primary Ab was omitted in control sections. Sections were counterstained with acridine orange/safronin O. Control tissue included normal mouse spleen.

For immunohistochemical detection of BrdUrd-labeled T cells, excised lungs were inflated, fixed in fresh 4% paraformaldehyde, and embedded in paraffin. Sections were incubated in proteinase K solution and then in 4 N HCl. BrdUrd was detected with an alkaline phosphatase-conjugated mouse mAb, F(ab′)2, and Fast Red substrate according to the manufacturer’s directions using an in situ cell proliferation kit (AP, Boehringer Mannheim, Indianapolis, IN). Sections were counterstained with hematoxylin. In control slides, mAb was omitted. A cyto centrifugation preparation of the BrdUrd-labeled T cells was fixed in 4% paraformaldehyde, air-dried, and immunostained as described for the tissue sections. Control tissues included lung from a normal mouse and testes from a mouse that received BrdUrd in vivo.

For detection of bacterial β-galactosidase activity, lungs were excised from C57BL/6 mice and ROSA26 transgenic mice that constitutively express the lacZ gene (23). The procedure for in situ β-galactosidase activity in whole lung has been described in detail previously (25). In brief, lungs were fixed by inflation with 1% glutaraldehyde for 60 min. Fixative was removed by aspiration, and the airways were rinsed with PBS. A solution containing 0.2% X-galactosidase (Sigma), 5 mM potassium ferricyanide, 2 mM MgCl2, and 100 mM Tris-base, pH 8.0, was instilled into the trachea at 30-cm H2O pressure. The inflated lungs were immersed in X-galactosidase solution overnight at 37°C. The X-galactosidase solution was then aspirated. The lungs were fixed in 10% formalin and embedded in paraffin as described above. Deparaffinized sections were stained with nuclease Fast Red.

In preliminary studies we determined that endogenous (mammalian) β-galactosidase activity was not detected under these staining conditions in either the T cell clone (8F5) 24 h after in vitro stimulation or in lung or spleen from C57BL/6 mice, but β-galactosidase activity was readily detected in lung and spleen of the transgenic mice.

Bronchoalveolar lavage (BAL) and AM culture

Mice were anesthetized with Avertin and exanguinated by renal artery transection. The thorax was opened by a midline sternotomy, and the trachea was excised by a midline incision and cannulated with a polypropylene catheter. The lungs were lavaged with 1.5-ml aliquots of sterile Ca2+- and Mg2+-deficient PBS supplemented with 0.6 mM EDTA. The first milliliter of BAL was placed in a separate tube and centrifuged at 500 × g to remove cells. The BAL supernatant was frozen at −70°C for analysis of IFN-γ, TNF-α, and protein (26). The next 10 ml of lavage fluid was centrifuged to recover cells. The cell pellet was combined with the cell pellet from the first milliliter in cold HBSS, centrifuged, and resuspended in 1 ml of HBSS. Cells from control or experimental animals (n = 3 or 6) were pooled and counted using trypan blue exclusion as a measure of viability. Differential cell counts were performed on Wright-stained cytocentrifuge preparations. More than 95% of the cells were AM. Cells were then centrifuged again and resuspended at a concentration of 2 × 106 cells/ml in RPMI with 5% glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were plated in 96-well culture dishes and cultured at 37°C in a 5% CO2 atmosphere for 60 min. Nonadherent cells and media were removed, and adherent cells were washed once with HBSS. Adherent cells were then incubated in RPMI with 5% glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FCS. In some experiments, LPS (E. coli O111: B4, List Laboratories, Campbell, CA) at different concentrations was added.
development and progression of lung injury, we stimulated cell clone activation and proliferation were required for the development and progression of lung injury. We previously showed that in vitro activation of Ly5α-specific T cells induced lung injury in vivo in Ly5α mice, but not Ly5β mice, and vice versa (21). To determine whether continuous specific Ag presentation and T cell clone activation and proliferation were required for the development and progression of lung injury, we stimulated Ly5α-specific T cells in vitro with Ly5α peptide and congenic splenocytes. Resting or stimulated Ly5α-specific T cells (5 × 10⁶ cells) were administered i.v. to Ly5α or Ly5β mice, and lung histopathology was evaluated 1, 3, and 6 days later. As shown in Table I, the administration of resting anti-Ly5α T cells resulted in lung injury to Ly5α mice, but not Ly5β mice, but stimulated anti-Ly5α T cells caused lung injury in both Ly5α and Ly5β mice. The extent of involvement, based on semiquantitative histopathology score, was similar throughout the period of observation. Moreover, the evolving histopathologic features were similar in all affected mice (Fig. 1). Similar results were obtained with T cells that were stimulated in vitro with Ab to CD3 for 24 h before adoptive transfer in vivo (data not shown). As previously shown in the chimera experiments, these results indicate that Ag localization in lung is not required (i.e., a lung-specific target). These results also suggest that the initial activation of the T cell clone promotes a sustained bystander inflammatory injury in lung.

The T cell clone-induced inflammatory response in lung is composed of both transferred T cells and host-derived monocellular cells

Immunohistochemical identification of T cells. Lung tissue for frozen sections was obtained from C57BL/6 (Ly5α) mice 1 day after transfer of 5 × 10⁶ resting anti-Ly5α T cells. By immunohistochemistry, a subpopulation of cells in the mononuclear inflammatory cell reaction around vessels was lightly stained by a T cell Ab (not shown). We estimated that approximately 20% of the cells were CD3e positive. Lymphocytes in control spleen tissue were strongly positive.

To determine whether the T cells in lung were derived from the cloned T cells, we labeled the anti-Ly5α T cells in vitro with BrdUrd (100 µM) before transfer to Ly5α mice. By immunohistochemistry, BrdUrd-labeled cells were detected in the vascular inflammatory foci 1, 2, 3, and 7 days after transfer of the T cells (Fig. 2), but not in normal lung from control animals. Rare labeled cells were seen in apparently normal areas of lung from T cell–treated mice. It appeared that the number of labeled T cells decreased over time. This could result from either T cell disappearance (death) or loss of label. These results suggest that Th1 cell localization in lung is involved in the initial steps of the inflammatory response.

T cell transfer to T and B cell-deficient mice. To further characterize the cells in the inflammatory lesions, we transferred anti-Ly5α T cells to RAG-1 knockout (C57BL/6 background) mice. These mice have a deletion in the V(D)J recombination activation gene (RAG-1) and have no mature B or T cells (22). Lung tissue was obtained 1, 3, and 7 days after T cell transfer and examined histologically. The characteristic vascular lesion (illustrated in Fig. 1) was present in both control Ly5α mice and mutant RAG-1-deficient mice. Semiquantitative pathologic scoring of the lesions, performed in a blinded manner, indicated no difference between control and mutant mice at any of the time intervals tested (Table II). These results indicate that host-derived T or B cells are not required for the clone-induced inflammatory reaction in lung.

T cell transfer to ROSA-26 mice. The origin (i.e., recipient mouse vs clone) of cells in the inflammatory foci was further examined in ROSA-26 transgenic mice (B6, 129 background) that constitutively express the lacZ gene (23). Therefore, cells originating from these mice can be identified by histochemical detection of β-galactosidase activity. Lung tissue was collected from these transgenic mice or from control C57BL/6 mice 1, 3, and 7 days after transfer of anti-Ly5α T cells stimulated in vitro as previously described. Histochemical analysis revealed β-galactosidase activity in lung parenchymal cells and in the majority of inflammatory cells present in vascular lesions in the transgenic mice 3 and 7 days after T cell transfer, indicating that they were of recipient origin (Fig. 3). Consistent with the BrdUrd data, some (20–30%) β-galactosidase-negative inflammatory cells (presumably T cells) were present at 1 day. No β-galactosidase activity was detected in the lungs of control mice that had been similarly treated or in cytosin slides of activated cloned T cells. The results of these experiments collectively suggest that the lung inflammatory response initiated by Th1 cells is amplified by recipient mononuclear cells.

Proinflammatory cytokines and increased protein levels are present in BAL after T cell transfer. To determine whether proinflammatory cytokines were increased in association with Th1 cell–induced lung inflammation, we measured IFN-γ and TNF-α in BAL 24 h after i.v. transfer of 5 × 10⁶ Ly5α-specific T cells (clone 1A4) to Ly5α mice. Neither IFN-γ nor TNF-α was detected in control mice, while in T cell–treated mice, BAL contained both TNF-α (mean ± SD, 29 ± 8 pg/ml; n = 6) and IFN-γ (2287 ± 752 pg/ml; n = 3). After 48 or 72 h, IFN-γ and TNF-α were not consistently detectable in BAL.

To determine whether the histopathologic inflammatory response was associated with vascular leak, we measured total protein in BAL at intervals after the administration of 10³ Ly5α-specific T cells to Ly5α mice. As shown in a representative experiment (Table III), total protein was significantly increased 24 h after T cell administration and remained elevated at 72 h.

### Table I. Anti-Ly5α T cells activated in vitro cause lung injury in Ly5β mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>T Cell (activation)</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly5β</td>
<td>Anti-Ly5α (resting)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ly5α</td>
<td>Anti-Ly5α (activated)</td>
<td>2+, 1+</td>
<td>2+, 3+</td>
<td>2+, 3+</td>
</tr>
<tr>
<td>Ly5α</td>
<td>Anti-Ly5β (resting)</td>
<td>3+, 3+</td>
<td>3+, 3+</td>
<td>2+, 2+</td>
</tr>
<tr>
<td>Ly5α</td>
<td>Anti-Ly5β (activated)</td>
<td>2+, 3+</td>
<td>4+, 3+</td>
<td>4+, 4+</td>
</tr>
</tbody>
</table>

Ly5α-specific T cells were activated in vitro with Ly5α peptide and congenic splenocytes. Resting or stimulated Ly5α-specific T cells (5 × 10⁶ cells) were administered i.v. to Ly5α or Ly5β mice, and lung histopathology was scored on a scale of 0 to 4+.
T cell transfer results in activation of AM. Macrophage production of proinflammatory mediators has been implicated in tissue injury due to GVHD. To determine whether Th1 cell-induced lung injury involves macrophage activation, we analyzed AM production of TNF-α in vitro. Twenty-four hours after transfer of anti-Ly5b T cells (5 x 10⁶ cells), AM from treated and control Ly5a mice (n = 6 in each group) were obtained and cultured in parallel for 24 h. Conditioned medium from the AM cultures was assayed by ELISA for in vitro release of TNF-α (Fig. 4). AM lavaged from control animals did not release detectable TNF-α in the absence of LPS, whereas AM lavaged from T cell-treated mice spontaneously released TNF-α. Addition of LPS to cultured AM from control mice stimulated release of TNF-α into the culture supernatants. In mice given T cells, the capacity of AM to release TNF-α in response to LPS stimulation was increased two- to threefold.

**FIGURE 1.** Lung histopathology after i.v. T cell administration of Ly5b-specific T cells to Ly5a mice. Control (resting) T cells (5 x 10⁶ cells) caused minimal lung inflammation at 1 day (A, x40; B, x250). Administration of T cells (5 x 10⁶ cells) that were activated in vitro resulted in prominent vasculitis at day 1, with mononuclear cells adherent to vascular endothelium and present in the perivascular space (C and D). The vasculitis was more extensive 3 days after activated T cell administration (E and F). At 6 days, lung inflammation was more generalized, with mononuclear cells and macrophages present in alveoli and interstitium, residual perivasculitis, and edema (G and H).
FIGURE 2. Immunohistochemical detection of BrdUrd-labeled T cells. T cells (Ly5b specific) were cultured in the presence of BrdUrd (100 μM) before transfer of 5 × 10⁶ cells to Ly5b mice. At intervals of 1 to 7 days after T cell administration, lungs were excised, fixed in 4% paraformaldehyde, immunostained with mAb to BrdUrd conjugated with alkaline phosphatase, and developed with Fast Red substrate. Labeled T cells (red) were detected in inflammatory foci in lungs of treated mice (magnification, ×400). The negative control was normal lung; the positive control was in vivo BrdUrd-labeled testes (not shown).

(7.5 × 10⁶ SF5 cells). The LPS dose was estimated to be 20% of a lethal dose of LPS in B6 mice (24). There were no deaths among eight mice that received LPS alone or eight mice that received T cells alone. However, among eight mice that received T cells followed by LPS, four died within 24 h of LPS challenge. Lethargy, weight loss (30% of baseline), and diarrhea were observed in the survivors. Mice that received T cells only were not visibly affected, but mice that received LPS only were lethargic and lost 20% of baseline weight. Lung pathology in the surviving mice (treated with both T cells and LPS) revealed no increase in interstitial and alveolar mononuclear infiltrate 24 and 48 h after LPS challenge compared with mice that received T cells only. In fact, the vascular inflammatory foci were less intense. Mild alveolar edema and increased cellularity was present in mice that received LPS only.

In another similar experiment, Ly5b mice (n = 3) were similarly challenged with anti-Ly5b T cells (10⁷ cells) followed by LPS. Control mice (n = 3 in each group) received no treatment, T cells only, or LPS only. Twenty-four hours after LPS, mice were laved for analysis of BAL protein and cultures of AM. After 24 h in culture, TNF-α levels in the AM culture media were measured (Fig. 5). TNF-α production by AM from mice that received T cells or LPS only was significantly increased compared with that in untreated controls (p < 0.01). However, even higher TNF-α production was observed in AM from mice that received T cells or LPS only was significantly increased compared with that in untreated controls (p < 0.01). However, even higher TNF-α production was observed in AM from mice that received T cells followed by LPS challenge (p < 0.01). Similarly, BAL protein was mildly increased (not significantly) in mice treated with either T cells or LPS alone, whereas mice that received T cells followed by LPS challenge had substantially increased BAL protein concentration (p < 0.05) (Fig. 6). Thus, increased susceptibility to sublethal LPS challenge after Th1 cell activation is associated with increased AM TNF-α production and lung protein leak into alveoli. Even though the increased mortality under these conditions may not be directly related to the lung, an enhanced macrophage inflammatory response to LPS may be an important mechanism of lung injury in the setting of immune cell activation.

Discussion

We have described a murine model of acute lung inflammation that is induced by alloreactive Th1 cells. This model establishes the principle that lung injury can occur as part of a proinflammatory immune response even in the absence of a lung-specific Ag. Our work indicates that immune-activated Th1 cells are localized in lung at foci of inflammation, but the inflammatory response is comprised mainly of recipient mouse mononuclear cells. As a consequence of Th1 cell activation, AM are activated to produce proinflammatory mediators including TNF-α. In addition, Th1 cell-mediated lung injury results in greatly increased susceptibility to sublethal LPS challenge, with associated increases in AM production of TNF-α and increases in BAL protein.

IFN-γ, the defining proinflammatory cytokine produced by Th1 cells, has been implicated in the pathogenesis of GVHD. Serum levels are increased in patients and in murine GVHD (27–29). IFN-γ mRNA is increased in target organs, including the lung in murine GVHD (30). In our experiments, Th1 clone cells were present selectively in inflammatory foci in lung, and IFN-γ was increased in BAL fluid after Th1 cell administration. Prominent among the effects of IFN-γ is the activation of macrophages to secrete cytokines such as TNF-α (31, 32). In our model, AM from Th1 cell-treated mice spontaneously secreted TNF-α in vitro, and TNF-α levels were increased in BAL. Similar spontaneous TNF-α production by peritoneal macrophages has been reported in murine GVHD and has been attributed to IFN-γ, although a causative role has yet to be directly established (16). Other Th cell-derived cytokines, such as IL-2, macrophage inflammatory factor, and TNF-β might also play an important activating role, having potentially overlapping function with IFN-γ (32).

While Th1 cells and their cytokine products may be critical in initiating an inflammatory response in lung, our experiments in lacZ transgenic mice indicate that the inflammatory cell population is comprised largely of host-derived cells. A similar preponderance of recipient-derived cells has been shown in lung and other target organs in an unirradiated murine GVHD model induced by transfer of CD3 cells (33). Host-derived macrophages were also increased in lungs of irradiated mice with GVHD (29). Our experiments in
FIGURE 3. Histochemical detection of β-galactosidase activity (blue) in lungs from ROSA-26 mice. Activated T cells (10⁷ cells) were given to control (C57BL/6) mice or ROSA-26 transgenic mice that constitutively express the lacZ gene. Three days after T cell administration, lungs of control and ROSA-26 mice were excised and processed for histochemical detection of β-galactosidase as described in Materials and Methods. In ROSA-26 mice, positive blue granular staining was detected in lung parenchymal cells and mononuclear cells in inflammatory foci. No β-galactosidase activity was detected in lungs of similarly treated C57BL/6 mice. Left panel, ×400; right panel, ×1000.

Table III. Bronchoalveolar lavage protein is increased after Th1 cell administration

<table>
<thead>
<tr>
<th>Time After T Cells (h)</th>
<th>n</th>
<th>BAL Protein Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>6</td>
<td>395 ± 45</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>877 ± 66</td>
</tr>
<tr>
<td>48</td>
<td>5</td>
<td>573 ± 168</td>
</tr>
<tr>
<td>72</td>
<td>5</td>
<td>991 ± 184</td>
</tr>
</tbody>
</table>

*At intervals after T cell (Ly5.5⁰-specific) administration (10⁷ cells), mice (Ly5.5⁰) were sacrificed, and total protein in bronchoalveolar lavage was determined. Values are mean ± SEM.

Values are significantly different from control (p ≤ 0.05, one-way ANOVA with Tukey multiple comparison test).

RAG-1-deficient transgenic mice, which lack T and B cells, showed that the inflammatory response to Th1 cell administration was not diminished in these mice. This result suggests that monocytes are a major component within the inflammatory cell population. The mechanisms involved in inflammatory cell recruitment to lung are not known, but the striking localization of mononuclear cells in the pulmonary vasculature strongly suggests that vascular adhesion molecules are up-regulated during the development of Th1 cell-induced lung injury. The exclusively mononuclear cell inflammatory response points to a possible role for VCAM-1.

Both in vitro experimental data and animal studies suggest that VCAM-1 may be of particular relevance in our model (34–36). VCAM-1 is a member of the Ig supergene family of cell adhesion molecules expressed on vascular endothelial cells and is induced by proinflammatory cytokines such as IL-1 and TNF-α (37). Cell adhesion to VCAM-1 is mediated by the integrin α4β1 (very late Ag-4), which is expressed on most mononuclear cells but not neutrophils. Other studies of animal models of immune and inflammatory disease suggested that the α4 integrin-dependent pathway plays a central role (35).

The selective recruitment of mononuclear cells may be further refined by localized expression of chemotactic molecules. Recently, a superfamily of proinflammatory cytokines with chemotactic activity has been characterized (38, 39). The β-chemokine family, also known as C-C chemokines, attract primarily monocytes and T lymphocytes (40). C-C chemokines are produced by T lymphocytes as well as a variety of other cell types, including endothelial cells, macrophages, and airway epithelial cells (39–42). C-C cytokine production is stimulated by proinflammatory cytokines (43). In addition to their chemotactic activity, C-C cytokines are now known to modulate cytokine production and adhesion molecule expression (44, 45). Clearly, this family of C-C chemokines, by virtue of selective cell recruitment and their stimulatory activity, could function in conjunction with vascular adhesion molecules and integrins as important modulators of inflammatory responses such as that observed in our model.

An important emerging theme is that while donor T cells are essential for the generation of GVHD, the pathology may be in large part mediated by distal, non-T cell immune processes, such as the secretion of multiple inflammatory products from cells of monocyte/macrophage lineage (11, 12, 43). In our experiments, TNF-α was spontaneously secreted by AM after Th1 cell administration. TNF-α is directly cytotoxic and also mediates a wide variety of proinflammatory effects, including activation of leukocytes to produce other proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α itself. TNF-α also has been implicated as an important cytokine in inflammatory lung injury models (39).

FIGURE 4. Murine AM TNF-α production in vitro. Twenty-four hours after transfer of anti-Ly5.5⁰ T cells (5 × 10⁶), AM from treated and control mice (n = 6 in each group) were obtained and cultured (10⁶ cells/well in 1 ml of medium) in parallel for 24 h. Conditioned medium from the AM cultures was assayed by ELISA for in vitro release of TNF-α. In the absence of LPS, TNF-α was not detected in AM cultures from control mice, whereas AM from T cell-treated mice spontaneously secreted TNF-α. LPS-stimulated higher levels of TNF-α production in AM from T-cell treated compared with AM from control mice (p ± SEM (n = 3 cultures).
Allergic T cell activation, monocuclear cell recruitment to lung, and monocyte/macrophage activation appear to be important component mechanisms in the pathogenesis of lung injury in our experiments. Tissue injury may be further exacerbated by environmental challenges such as LPS in susceptible mice. Our experiments showed that susceptibility to sublethal doses of LPS was greatly increased after Th1 cell administration. The LPS challenge in Th1 cell-treated mice was associated with increased AM production of TNF-α, increased BAL protein leak, and increased mortality. Others have shown that proinflammatory cytokines such as IFN-γ increase susceptibility to LPS (46).

Others have proposed that gastrointestinal epithelial injury in murine models of GVHD results in translocation of LPS from the intestinal lumen into the bloodstream (16, 20). Nestel and colleagues (16) have advanced the concept that immune-activated monocytes/macrophages are important effector cells in GVHD. They investigated peritoneal macrophage activity and TNF-α production during GVHD. The data provide evidence that during GVHD, macrophages are primed as a result of TNF-α production during the alloimmune reaction, and that endogenous LPS triggers macrophage production of TNF-α, resulting in the symptoms characteristic of acute GVHD. In a subsequent report, these investigators have also shown that peritoneal macrophages from mice with GVHD express elevated mRNA levels of inducible nitric oxide synthetase and nitric oxides (30).

Similar priming of AM could contribute to acute lung injury, but this mechanism has not been directly investigated in murine models of marrow transplantation. Studies of murine transplant models of pneumonitis and GVHD demonstrated elevated BAL levels of LPS, neutrophils, and TNF-α (20). Injection of LPS caused severe hemorrhagic lung injury only in mice with GVHD and was associated with marked increases in BAL TNF-α. These data are consistent with our studies that directly demonstrate AM activation, TNF-α production, and increased sensitivity to LPS challenge both in vitro and in vivo.

Extrapolating these observations to patients with GVHD and noninfectious lung injury is speculative at this time, but the concept of immune-mediated increased susceptibility to LPS and possibly other external factors could help explain the variable association of lung injury with GVHD, the severity of the injury, and the poor response to treatment.

In conclusion, our model of alloreactive Th1-mediated lung injury mimics some features of lung injury encountered in murine models of GVHD. We have established the principle that activated Th1 cells alone can initiate an inflammatory lung injury that is amplified by mononuclear cell recruitment and AM activation and is associated with greatly increased susceptibility to further injury caused by LPS challenge. The cell and cytokine components of immune-mediated lung injury are undoubtedly more complex, but the experimental model we describe offers an opportunity to dissect the cellular and molecular mechanisms involved in this effector pathway.

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References


