Pneumocystis carinii infection sensitizes lung to radiation-induced injury after syngeneic marrow transplantation: role of CD4+ T cells

Lauren Bruckner,1 Francis Gigliotti,2,5 Terry Wright,2,5 Allen Harmsen,3 Robert H. Notter,4 Patricia Chess,4 Zhengdong Wang,4 and Jack Finkelstein4

Divisions of 1Hematology/Oncology/Bone Marrow Transplant, 2Infectious Diseases, and 4Neonatology, Departments of Pediatrics and of 3Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, New York; and 5Department of Veterinary Molecular Biology, Montana State University, Bozeman, Montana

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Although the specific mechanisms remain speculative, IPS involves the influx of large numbers of inflammatory cells into the lungs leading to an excessive production of proinflammatory cytokines and/or chemokines, reactive oxygen and nitrogen species, and a depletion of antioxidant potential (3, 24). A variety of potentially interacting lung insults have been implicated in the pathogenesis of IPS, including occult infection with Pneumocystis carinii and total body irradiation (TBI) (9, 15).

P. carinii is a commonly encountered, ubiquitous organism that causes a self-limiting pneumonitis in the normal, immunocompetent host (2). In contrast, alterations in immune integrity increase the susceptibility to overt P. carinii pneumonia (PCP), likely by affecting the balance between cellular immune effector mechanisms and the cytokines they elaborate (37). Using a severe combined immunodeficient (SCID) mouse model of PCP, we have previously shown that P. carinii infection causes little direct pulmonary damage in the absence of a host inflammatory response until very late in the course of disease (36). However, when P. carinii-infected SCID mice are immunologically reconstituted with congenic spleen cells, the resulting inflammatory response causes significant pulmonary compromise before the resolution of the infection (36). Both CD4+ and CD8+ T cells appear capable of generating detrimental effects on lung in this model, but the specific mechanisms are not clear (36). The present study addresses the specific importance of CD4+ and CD8+ T cells in lung injury associated with P. carinii infection alone and in combination with TBI and syngeneic BMT.

TBI has long been associated with the development of lung toxicity in both allogeneic and autologous BMT (25, 29). TBI has been reported to be a primary contributor to the high incidence (31%) of interstitial pneumonitis observed in patients receiving an autologous BMT for myeloma (5). In addition to direct radiation-induced parenchymal damage, it is clear that inflammatory cells are involved in mediating radiation pneumonitis (23). Several studies have shown that radiation induces the recruitment of inflammatory cells to the lungs and generates a lymphocytic alveolitis with increased levels of proinflammatory cytokines and chemokines including TNF-α (6, 17, 28). Within the first few days after TBI, mononuclear cells significantly decrease in number, but a population of host T cells and macrophages survive conditioning, are able to produce TNF-α, and may contribute to this inflammatory response.

PULMONARY TOXICITY is the leading cause of early bone marrow transplant (BMT)-related mortality and remains the major dose-limiting factor in administering radiotherapy and chemotherapeutic agents used in conditioning regimens for BMT (30). Reported incidences of post-BMT lung injury typically range from 40 to 60%, with associated mortality rates of up to 85% (40). Clinical and animal studies have suggested that a noninfectious type of post-BMT lung injury, termed idiopathic pneumonia syndrome (IPS), results from excessive inflammation and resultant immune-mediated lung damage (19, 20).
were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were exposed to lethal TBI using a 137C irradiator to generate a Medical Center.

The hypothesis states that the presence of a relatively mild P. carinii infection can exacerbate the severity of inflammation and pulmonary dysfunction induced by TBI in mice, leading to an IPS-like condition in the post-BMT period. Moreover, we hypothesize that lung injury and surfactant dysfunction resulting from the combined insult of P. carinii/TBI will be more severe than that caused by either P. carinii infection or TBI alone and will correlate with increased levels of TNF-α in bronchoalveolar lavage fluid (BALF). These hypotheses are tested in a murine model of syngeneic BMT in the presence and absence of different types of immune reconstitution.

MATERIALS AND METHODS

Mice. C57BL/6J mice, used for both BMT donors and recipients, were purchased from Jackson Laboratories (Bar Harbor, ME). For P. carinii propagation, CB.17 scid/scid mice were purchased from Charles River Laboratories (Wilmington, MA) and were infected with Pneumocystis-containing lung homogenates by direct intranasal inoculation as previously described (39). Mice were housed in microisolator cages under pathogen-free conditions and were routinely used at 6–8 wk of age. All animal protocols were preapproved by University Committee for Animal Research at the University of Rochester Medical Center.

TBI, bone marrow/splenocyte reconstitution, and T cell depletion. Mice were exposed to lethal TBI using a 137C irradiator to generate a total dose of 13 Gy, delivered in split doses separated by 4 h. Bone marrow was obtained by flushing the femurs and tibias of donor mice. Splenocytes were harvested by passing the spleens over a wire mesh and resuspending them in HBSS. For bone marrow/splenocyte (BMS) reconstitution studies, cells were enumerated and resuspended to a concentration of 10^7 bone marrow cells and 10^6 splenocytes in a total volume of 200 µl of sterile HBSS and injected via tail veins into recipients immediately after they received TBI. T cell depletion studies were carried out by giving mice intraperitoneal injections of either 250 µg of control rat IgG (Sigma), 250 µg of a CD4+ T cell-depleting MAb [clone GK1.5, American Type Culture Collection (ATCC) TIB 207], or 250 µg of CD8+ T cell-depleting MAb (clone 2.43, ATCC TIB 210). Antibody injections for T cell depletion started 1 day before TBI and BMS reconstitution, and antibodies were administered every 4 days for the duration of the experiment. The efficacy of T cell depletion was confirmed by flow cytometry of BALF and splenocytes using anti-CD4-fluorescein (clone RM4-4) and anti-CD8α-peridinin chlorophyll-a protein (clone 53-6.7) from BD Biosciences (San Diego, CA; see flow cytometry studies below).

Isolation and enumeration of mouse P. carinii organisms and P. carinii burden. P. carinii-infected CB.17 scid/scid mice were treated with dexamethasone (4 mg/kg) and tetracycline (500 mg/kg) in drinking water 3–7 days before death to increase pulmonary P. carinii burdens. The lungs were removed, and P. carinii organisms were isolated from pulmonary tissue as previously described (36). Final P. carinii preparations were stained with amoebicidal silver to enumerate cysts, diluted to a final concentration of 1 x 10^7 cysts/80 µl aliquots, and used to intranasally inoculate additional animals. Lung total P. carinii burdens were quantified by real-time polymerase chain reaction (PCR) as previously described (39). Briefly, entire right lung lobes were excised, and the tissues were homogenized with 1 ml of PBS/150 mg of lung tissue in a mechanical homogenizer. Homogenates were boiled for 15 min, vigorously vortexed for 2–3 min, and then centrifuged for 5 min at 12,000 g. The supernatant was boiled and assayed by quantitative PCR using TaqMan primer/fluorogenic probe chemistry and an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). A primer/probe set specific for a 96-nucleotide region of the mouse Pneumocystis kexin gene was designed using Primer Express software (Applied Biosystems). Quantitation was determined by extrapolation against standard curves constructed from serial dilutions of known copy numbers of plasmid DNA containing the target kexin sequence. Data were analyzed using the ABI Prism 7000 SDS version 1.0 software (Applied Biosystems) and are reported as total kexin DNA copies per entire right lung.

Lung injury severity and pulmonary mechanics. Physiological measures of lung injury severity in this study were assessed as: 1) weight loss (percent of body wt lost from the onset of the experiment), 2) increased respiratory rates, and 3) decreased specific dynamic lung compliance (Cdyn mL/cmH2O kg^-1 body wt). Weight loss as a percentage of body weight change was calculated as: % body wt change = 100 starting body wt - weight at time x/starting body wt. Cdyn, and respiratory rates in live mice were measured by plethysmography as previously described (36). Briefly, phenobarbital-anesthetized mice underwent careful neck dissection, and the trachea was surgically cannulated through an anterior incision with a 20-gauge cannula. Cannulated animals were then placed in a mouse plethysmograph (Buxco Electronics) connected to a Harvard rodent ventilator (Harvard Apparatus, South Natick, MA). Mice were ventilated with a tidal volume of 0.01 ml/g body wt at a rate of 150 breaths/min. Respiratory rate was measured, and Cdyn was determined based on respiratory flows and pressures measured using transducers attached to the plethysmography chamber. Data were collected and analyzed using the Biosys Systems XA software package (Buxco Electronics).

Bronchoalveolar lavage and lung tissue preparation. Bronchoalveolar lavage (BAL) and lung tissue were obtained after Cdyn measurements. The chest cavity was surgically opened, and the entire left lobe of the lung was securely tied off at the bronchus with surgical silk, removed with sterile scissors, immediately snap frozen in liquid nitrogen, and stored at -80°C for RNA assessments. The remaining lung lobes were then lavaged by infusing and withdrawing four 1-ml aliquots of normal saline through the tracheal cannula. Recovered BALF was centrifuged at 250 g for 5 min to obtain the cellular fraction, and the supernatant was removed and frozen at -80°C for subsequent analysis of cytokine/chemokine content and/or lung surfactant assessments (see Measurements of TNF-α, phospholipid and protein content, and large surfactant aggregate content). Cells were resuspended in HBSS, enumerated, centrifuged onto glass slides, and stained with Diff-Quick (Dade, Didingen, Switzerland) to obtain differential counts. Multiparameter flow cytometry was also performed on BAL cells after staining with fluorochrome-conjugated specific MAbs distinct from those used to deplete CD4+ and CD8+ T cells in vivo [anti-CD4-fluorescein (clone RM4-4) and anti-CD8α-
peridinin chlorophyll-a protein (clone 53–6.7) from BD Biosciences as noted earlier. Cytometric studies used a FACSCalibur cell sorter (BD Biosciences) with a minimum of 5,000 events analyzed per BAL cell sample.

In a subset of studies, lung tissue was fixed for histological examination instead of being lavaged. After the left pulmonary lobe was removed for RNA isolation as noted above, remaining lung lobes were inflated at 15-cm gravity flow pressure with 10% formalin fixative (Sigma-Aldrich, St. Louis, MO). The lungs were fixed in situ for 10 min under gravity flow pressure and then carefully excised and placed in fixative for at least an additional 16 h in vitro. Fixed lung tissue was rinsed and stored in 70% ethanol, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin to visualize architecture and inflammatory infiltrates.

**Measurements of TNF-α, phospholipid and protein content, and large surfactant aggregate content.** Recovered BALF was centrifuged at 250 g for 5 min to remove cells, and levels of TNF-α in the cell-free supernatant were quantified by using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions. The total phospholipid content of cell-free BALF was determined by the colorimetric phosphatase assay of Ames (1). Total protein was assessed by the method of Lowry et al. (22) modified by the addition of 15% SDS to allow accurate quantization in the presence of lipid. Cell-free BALF was subsequently centrifuged at 12,500 g for 30 min to obtain large surfactant aggregates for further analysis. The percentage of total phospholipid in the large aggregate pellet from cell-free BALF was determined by a second phosphate assay, and large aggregate surface activity was determined using a pulsating bubble surfactometer as described below.

**Surface activity measurements.** Centrifuged large surfactant aggregates were evaporated under nitrogen and resuspended with hand vortexing in 0.15 M NaCl plus 2 mM CaCl2 at a uniform phospholipid concentration of 2.0 mg/ml for surface activity measurements on a pulsating bubble surfactometer (General Transco, Largo, FL). Based on the design of Enhorning (12), this instrument assesses a physiologically relevant combination of overall surface tension lowering ability that includes the effects of both adsorption and dynamic film compression. Surfactant samples were initially injected into a washed plastic sample chamber mounted on the precision pulsator unit of the surfactometer. A small air bubble, communicating with ambient air, was formed in the sample and pulsed between minimum and maximum radii of 0.4 and 0.55 mm at 37 ± 0.5°C. Bubble size was monitored through a microscope during continuous cycling at a rate of 20 cycles/min. The pressure drop across the air-liquid interface in the bubble (∆P) was measured with a pressure transducer, and surface tension (γ) was calculated from the Laplace equation for a sphere: ∆P = 2γ/radius. Surface activity data are reported as γ at minimum bubble radius as a function of time from the start of bubble pulsation.

**Statistical analysis.** Results are expressed as means ± 1 SE. Data were analyzed by ANOVA or Student’s t-test using the Analyse-it software package for Microsoft Excel (Analyse-it Software, London, UK). P values ≤ 0.05 were considered to be statistically significant.

**RESULTS**

Pulmonary compromise and surfactant dysfunction were most severe with the combination of P. carinii infection and TBI compared with either insult alone. Immunocompetent C57BL/6 mice were intranasally inoculated with P. carinii (a lung homogenate containing \(1 \times 10^8\) P. carinii cysts/mouse) or were sham infected 7 days before receiving TBI and syngeneic BMS reconstitution. A third group of mice was inoculated with P. carinii but did not receive TBI or BMS reconstitution (P. carinii only). The degree of pulmonary compromise was then compared between these three groups of mice (P. carinii only, TBI only, and P. carinii/TBI). Specific physiological measures of pulmonary dysfunction were weight loss, increased respiratory rates, and reduced Cdyn (Fig. 1). The relatively low doses of P. carinii and TBI used here resulted in only minimal, if any, lung dysfunction in P. carinii-only and TBI-only mice compared with unmanipulated control mice (data not shown).

In contrast, P. carinii/TBI mice that received the combination of P. carinii infection and TBI/BMS reconstitution lost as much as 25% of their initial body wt (P = 0.0015, day 21 post-BMT) and exhibited significantly increased respiratory rates (P = 0.025, day 21 post-BMT) and decreased lung compliance (P < 0.0001, day 17 post-BMT) that was evident as early as 11 days post-BMT (18 days after P. carinii inoculation). Histopathological examination of fixed lung tissue from P. carinii/TBI mice revealed increased cellularity with increased perivascular and peribronchial edema, airway epithelial cell metaplasia, and septal thickening compared with lung tissue from P. carinii-only mice [Fig. 2, C and B (×100) and F and E (×400), respectively]. Lungs from TBI-only mice showed minimal histopathological differences compared with control (unmanipulated) mice (Fig. 2, D and A, respectively).

Surfactant dysfunction during lung injury from P. carinii and/or TBI was assessed by measuring the composition and surfactant activity of cell-free BALF and centrifuged large
aggregates obtained at the time of maximal pulmonary compromise, which occurred in *P. carinii*/TBI mice at 17 days post-BMT (24 days after *P. carinii* inoculation). At this time, cell-free BALF from *P. carinii*/TBI mice had a much higher protein:phospholipid ratio and a much lower content of large surfactant aggregates compared with mice given either *P. carinii* alone or TBI alone (Table 1). In addition, the surface activity of resuspended large aggregates on a pulsating bubble surfactometer was significantly impaired in *P. carinii*/TBI mice compared with the other groups of mice studied (Fig. 3). Large aggregates from mice given *P. carinii* alone or TBI alone had surface tension lowering ability that was similar to untreated control mice.

Post-BMT lung injury induced by *P. carinii* infection and TBI is associated with an increase in BALF lymphocytes. BALF from untreated control C57BL/6 mice had a low average cellular content of 0.3 × 10^5 cells/ml that was composed almost entirely of macrophages (Table 2). Mice treated with

Table 1. Lung compliance and biochemical composition of BALF from day 17 post-BMT

<table>
<thead>
<tr>
<th>Samples</th>
<th>Compliance, ml/cmH_2O·kg^{-1}</th>
<th>PL Content, mg/ml</th>
<th>Protein Content, μg/ml</th>
<th>Pr/PL (%)</th>
<th>Large Aggregates (% of total PL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>1.88 ± 0.04</td>
<td>0.16 ± 0.01</td>
<td>114 ± 6</td>
<td>70 ± 5</td>
<td>60.7</td>
</tr>
<tr>
<td>TBI alone (n = 6)</td>
<td>1.83 ± 0.04</td>
<td>0.17 ± 0.01</td>
<td>141 ± 11</td>
<td>82 ± 5</td>
<td>52.8</td>
</tr>
<tr>
<td>Pc alone (n = 4)</td>
<td>1.78 ± 0.06</td>
<td>0.11 ± 0.00*</td>
<td>148 ± 23</td>
<td>135 ± 17</td>
<td>50.5</td>
</tr>
<tr>
<td>Pc/TBI (n = 6)</td>
<td>0.98 ± 0.09</td>
<td>0.11 ± 0.01*</td>
<td>421 ± 43**</td>
<td>409 ± 61#</td>
<td>30.9</td>
</tr>
</tbody>
</table>

Data are means ± SE with *n* as indicated except for large aggregate category in which *n* = 1 (pooled samples). Large aggregates were pelleted by centrifugation at 12,500 g. PL, phospholipid content; Pr/PL, protein to phospholipid ratio. Control, untreated C57BL/6 mice. *P < 0.005 compared with control mice and TBI alone mice; **P < 0.0005 compared with control, Pc-alone, and TBI-alone groups of mice; #P < 0.005 compared with control, Pc-alone, and TBI-alone groups of mice. Pc, *Pneumocystis carinii*; BALF, bronchoalveolar lavage fluid; BMT, bone marrow transplant; TBI, total body irradiation.
the combination of P. carinii/TBI had an increase in cellularity that was primarily due to the accumulation of lymphocytes, with increases also seen in the absolute numbers of eosinophils and neutrophils. Immunocompetent mice treated with P. carinii alone had a more pronounced and rapid accumulation of eosinophils than did P. carinii/TBI mice at both 11 and 17 days post-BMT in Table 2 (corresponding to 18 and 24 days post-P. carinii infection, respectively). At 17 days post-BMT, eosinophils comprised well over one-half of the BALF cellularity in mice given P. carinii alone. However, the degree of respiratory compromise based on C_{dy}n was significantly worse in P. carinii/TBI mice compared with P. carinii-only mice at both 11 and 17 days post-BMT (Table 2).

FACS analysis of lavaged lymphocytes indicated that P. carinii/TBI mice had a significant increase in the ratio of CD4\(^+\):CD8\(^+\) T cells at 11 days post-BMT compared with mice given P. carinii or TBI alone (Table 2). In contrast, by 17 days post-BMT, P. carinii/TBI mice had a reduced ratio of CD4\(^+\):CD8\(^+\) T cells compared with mice given either insult alone, indicative of a different pattern of pulmonary inflammation in the presence of the combination injury (Table 2). Approximately 50% of P. carinii/TBI-treated mice succumbed to respiratory compromise, and those surviving beyond day 17 post-BMT exhibited a progressive increase in relative CD8\(^+\) T cell numbers in lavage that was temporally associated with the eventual resolution of P. carinii infection and respiratory symptoms. By day 29 post-BMT, lymphocytes accounted for more than two-thirds of total BALF cellularity in these mice (data not shown in Table 2).

Post-BMT lung injury induced by the combination of P. carinii infection and TBI is dependent on CD4\(^+\) T cells. To address in more detail the role of T cell subsets in the development of P. carinii/TBI lung injury, immunocompetent mice were infected with P. carinii and then depleted in vivo of CD4\(^+\) or CD8\(^+\) T cells beginning 1 day before TBI administration and BMS reconstitution. The removal of CD4\(^+\) T cells resulted in significantly reduced levels of pulmonary compromise compared with nondepleted P. carinii/TBI mice or CD8\(^+\)-depleted P. carinii/TBI mice (Fig. 4A). The average C_{dy}n on day 17 post-BMT in CD4\(^+\)-depleted P. carinii/TBI mice was equivalent to control mice and to mice treated with TBI alone and was significantly increased compared with nondepleted P. carinii/TBI mice and CD8\(^+\)-depleted P. carinii/TBI mice (P = 0.0002, Fig. 4 and Table 3). These differences in compliance agreed with data on respiratory rates and weight loss, which also indicated a significant improvement in lung injury in CD4\(^+\)-depleted P. carinii/TBI mice (data not shown). CD4\(^+\)-depleted P. carinii/TBI mice had no weight loss or increase in respiratory rates compared with control mice, whereas nondepleted P. carinii/TBI mice and CD8\(^+\)-depleted P. carinii/TBI mice had significant weight loss (>20% of body wt by day 21 post-BMT, P = 0.0015) and increased respiratory rates (P = 0.02) compared with CD4\(^+\)-depleted P. carinii/TBI mice.

Table 2. Compliance, BALF cellularity, and BALF composition in mice given Pc infection, TBI, or both

<table>
<thead>
<tr>
<th>Day Post-BMT</th>
<th>BALF cells/ml ×10(^5)</th>
<th>% BALF Cell Differential</th>
<th>CD4:CD8 Ratio</th>
<th>Lung Compliance m(H)2O (\cdot) kg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lymphocytes</td>
<td>Neutrophils</td>
<td>Eosinophils</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>TBI only</td>
<td>0.5±0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pc only</td>
<td>0.6±0.2</td>
<td>27±6(\ast)</td>
<td>36±2(\ast)</td>
<td>9±2</td>
</tr>
<tr>
<td>Pct/TBI</td>
<td>2.7±0.8(\ast)</td>
<td>50±5(\ast)</td>
<td>28±1(\ast)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Day 11</td>
<td></td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>TBI only</td>
<td>0.3±0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pc only</td>
<td>10.5±5(\ast)</td>
<td>21±1(\ast)</td>
<td>28±9(\ast)</td>
<td>35±15(\ast)</td>
</tr>
<tr>
<td>Pct/TBI</td>
<td>3.6±2(\ast)</td>
<td>55±7(\uparrow)</td>
<td>31±4(\ast)</td>
<td>&lt;5(\uparrow)</td>
</tr>
<tr>
<td>Day 17</td>
<td></td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>TBI only</td>
<td>0.7±0.2</td>
<td>41±7</td>
<td>5±3</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Pc only</td>
<td>6.4±1.8(\ast)</td>
<td>21±2(\ast)</td>
<td>7±5</td>
<td>64±2(\ast)</td>
</tr>
<tr>
<td>Pct/TBI</td>
<td>18.5±6.5(\ast)</td>
<td>42±3(\uparrow)</td>
<td>26±3(\uparrow)</td>
<td>15±6(\uparrow)</td>
</tr>
<tr>
<td>Control</td>
<td>0.3±0.1</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

Values are means ± SE for n = 4–6 mice/group obtained from 2 different experiments. BALF was obtained at various times post-BMT, the total BALF cellularity was determined by counting on a hemocytometer, the cellular composition was determined by DiffQuik analysis, and the proportion of CD4\(^+\) and CD8\(^+\) T cells was quantified by multiparameter flow cytometry. Normal untreated C57BL/6 mice were used as controls at each time point. *P < 0.05 compared with mice treated with TBI alone or control mice. †P < 0.05 compared with mice treated with Pc alone. ‡Consistent mild respiratory depression occurred 8–10 days post-BMT, but was not statistically significant compared with controls.

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Fig. 3. Surface activity of lavaged surfactant obtained 17 days post-BMT (24 days post-Pc infection) from mice treated with Pc infection (Pc only), TBI with BMS reconstitution (TBI only), or both (Pc/TBI). Minimum surface tensions are shown as a function of time of pulsation from resuspended lavaged surfactant aggregates. Surface tension measurements were made with a pulsating bubble surfactometer at a uniform low phospholipid concentration of 1 mg/mL. Shown are the mean values ± SE for n = 3–6 mice/group per time point.
Lung injury in this model is not attributable to direct damage caused by *P. carinii*. CD8\(^+\) depletem *P. carinii*/TBI mice had almost identical *P. carinii* burdens to nondepleted *P. carinii*/TBI mice at all time points investigated (Fig. 4B). In contrast, CD4\(^+\) depletem *P. carinii*/TBI mice had significantly higher *P. carinii* lung burdens measured by quantitative PCR. By 17 days post-BMT (24 days after *P. carinii* infection), *P. carinii* lung burdens in CD4\(^+\) depletem *P. carinii*/TBI mice averaged 3.2 \(\times\) 10\(^5\) Kex copies/right lung compared with 0.9 \(\times\) 10\(^5\) Kex copies/right lung for non-depleted *P. carinii*/TBI mice and 1.4 \(\times\) 10\(^5\) Kex copies/right lung for CD8\(^+\) depletem mice (Fig. 4B, *P* = 0.013 and 0.012, respectively). In the *P. carinii*-only group, *P. carinii* clearance was evident by 4 wk after infection (corresponding to day 17 post-BMT, Fig. 4B), and *P. carinii* was undetectable by 6 wk after infection (data not shown). This is in agreement with previously published data on *P. carinii* clearance in normocompetent mice (16). *P. carinii*/TBI mice had *P. carinii* burdens that persisted through day 17 post-BMT (4 wk after infection); however, those mice surviving their lung injury did eventually clear their *P. carinii* infections and had no detectable *P. carinii* and no respiratory symptoms at 5 wk post-BMT (6 wk after *P. carinii* infection, data not shown).

**CD4\(^+\)** T cell-mediated inflammatory responses are key contributors to lung injury in mice given a combination of *P. carinii* infection and TBI. BALF from CD4\(^+\) depletem *P. carinii*/TBI mice was characterized by a 30-fold or more reduction in cellularity compared with CD8\(^+\) depletem and non-T cell-depletem mice that were similarly treated with *P. carinii* infection and TBI (Table 3, *P* < 0.0001 and *P* = 0.015, respectively). Alveolar macrophages were the predominant cell type in the CD4\(^+\) depletem mice, similar to the composition seen in untreated C57BL/6 control mice (Table 3). In contrast, the cellular composition of BALF in CD8\(^+\) depletem *P. carinii*/TBI mice was virtually identical to that of the nondepleted *P. carinii*/TBI mice. The reduced influx of inflammatory cells to the lungs of CD4\(^+\) depletem *P. carinii*/TBI mice was correlated with improved lung compliance measures, further suggesting that the inflammatory lung damage in this model is CD4\(^+\)** T cell dependent (Table 3, Fig. 4A).

**TNF-**\(\alpha\)** is a component of the inflammatory response observed in mice given a combination of *P. carinii* infection and TBI. To begin to identify inflammatory mediators involved in promoting lung injury in this model, we measured levels of the proinflammatory cytokine TNF-\(\alpha\) in cell-free BALF. All groups of treated mice (*P. carinii* only, TBI only, and *P. carinii*/TBI) had at least mild elevations in the levels of TNF-\(\alpha\) in cell-free BALF compared with untreated controls (data not shown). However, *P. carinii*/TBI mice had significantly elevated TNF-\(\alpha\) levels compared with mice receiving *P. carinii* alone or TBI alone (Fig. 5). The greatest differences in TNF-\(\alpha\) levels occurred at day 11 post-BMT (Fig. 5A, *P* < 0.0001), a time when lung injury severity was beginning to increase in *P. carinii*/TBI mice based on weight loss and C\(_{\text{dyn}}\) changes (e.g., Fig. 1). Depletion of CD4\(^+\)** T cells in *P. carinii*/TBI mice abrogated the increased TNF-\(\alpha\) cytokine response (Fig. 5B).

**DISCUSSION**

This study reports a murine model of post-BMT lung injury and demonstrates that even mild infection with *P. carinii* can sensitize the lungs to the damaging effects of TBI. Experiments here utilized *P. carinii* inoculums and irradiation doses associated with minimal acute pulmonary dysfunction when given alone. Mice given a combination of *P. carinii* infection and TBI had significantly increased pulmonary dysfunction based on decreased body weight, increased respiratory rates, and decreased C\(_{\text{dyn}}\) relative to control mice (data not shown) or mice given either *P. carinii* or TBI alone (Fig. 1).Histopathological evaluations of fixed lung tissue indicated that the pulmonary dysfunction in *P. carinii*/TBI mice was associated with increased numbers of inflammatory cells, increased perivascular and peribronchial edema, airway epithelial cell
metaplasia, and septal thickening compared with mice given *P. carinii* or TBI alone. Mice injured with the combination of *P. carinii*/TBI had significantly increased protein-to-phospholipid ratios in cell-free BALF as well as significantly decreased percentages of large surfactant aggregates at 17 days post-BMT (Table 1). In addition, resuspended large surfactant aggregates from *P. carinii*/TBI mice had significantly decreased TNF-α levels in BALF compared with the undepleted and CD8-depleted groups treated with *Pc*/TBI. *P < 0.05 comparing the CD4-depleted group to the non-T cell-depleted or CD8-depleted groups (all 3 groups treated with *Pc*/TBI). †Differences in lung compliance did not reach statistical significance between CD8-depleted and non-T cell-depleted mice treated with *Pc*/TBI. ‡Mice treated with *Pc* alone had significantly higher BALF cellularity than CD4-depleted mice treated with *Pc*/TBI (*P = 0.004). See Table 1 for statistical differences between *Pc* only, TBI only, and *Pc*/TBI treatment groups.

Fig. 5. Increased bronchoalveolar lavage fluid (BALF) levels of TNF-α positively correlate with the degree of lung injury and are abrogated by CD4+ T cell depletion. C57BL/6 mice were intranasally inoculated with a lung homogenate containing 1 10⁵ *Pc* cysts/mouse 7 days before receiving (*Pc*/TBI) or not receiving (*Pc* only) lethal TBI and reconstitution with syngeneic bone marrow and splenocytes. Two additional groups of *Pc*/TBI-treated mice were in vivo depleted of either CD4+ or CD8+ T cells by intraperitoneal injections of anti-CD4- or anti-CD8-specific monoclonal antibodies biweekly beginning 1 day before receiving TBI and continuing until the end of the experiment. BALF was obtained at various times post-BMT, the total BALF cellularity was determined by counting on a hemocytometer, and the cellular composition was determined by Diffquick analysis. Normal, age-matched C57BL/6 mice were used as controls. *P < 0.05 comparing the CD4-depleted group to the non-T cell-depleted or CD8-depleted groups (all 3 groups treated with *Pc*/TBI). †Differences in lung compliance did not reach statistical significance between CD8-depleted and non-T cell-depleted mice treated with *Pc*/TBI. **Differences in lung compliance did not reach statistical significance between age-matched C57BL/6 mice treated with *Pc* only, TBI only, or both *Pc*/TBI.
nomenon known as “recall pneumonitis” (32). The postulated mechanism for this phenomenon is that chemotherapy influences the regulation and/or progression of acute lung injury rather than causing further direct damage to radiation-targeted cells. In our model, P. carinii infection may similarly be affecting the processing of radiation injury and/or causing additive damage. P. carinii infection has previously been shown to increase radiation-induced lung injury in animals (15, 27). The results found here indicate that P. carinii infection sensitizes the lungs to the damaging effects of TBI through inflammatory pathways mediated at least in part by CD4⁺ T cells. In addition, the activity of TNF-α may be involved in the severity of the combination P. carinii/TBI injury. Elevated levels of TNF-α were present in cell-free BALF from P. carinii/TBI mice at 11 days post-BMT (Fig. 5), a time when lung injury was beginning to increase in severity in these animals (Fig. 1). This TNF-α increase was abrogated by the depletion of CD4⁺ T cells (Fig. 5), and $C_{\text{ov}}$ was also improved by CD4⁺ T cell depletion in P. carinii/TBI mice (Table 3, Fig. 4A). We have recently reported that TNF receptor signaling (TNFR1 and TNFR2) is important in CD8⁺ T cell-dependent inflammation and lung injury in a CD4⁺ -depleted model of murine PCP (39). Further work is needed to investigate specific mechanisms by which CD4⁺ immunity and TNF-α signaling contribute to combination P. carinii/TBI lung injury. TNF-α was studied here as a representative initial proinflammatory cytokine, and the activities of a much broader spectrum of inflammatory mediators also need to be examined in P. carinii/TBI lung injury in the future.

Surfactant dysfunction in P. carinii/TBI mice involved both large aggregate depletion (Table 1) and a decreased intrinsic surface activity of remaining large aggregates (Fig. 3). Reductions in large surfactant aggregate content have been previously reported in a number of animal models of acute lung injury (Refs. 26 and 35 for review). Our prior work has also reported decreases in the surface activity of lavaged surfactant during the course of severe PCP in murine models (38, 39). In the present study, decreases in surfactant aggregate surface activity were most severe in P. carinii/TBI mice (Fig. 3) and were presumptively important in decreasing compliance and increasing respiratory rates in these animals (Fig. 1). Mechanistically, decreases in surfactant activity in inflammatory lung injury can involve interactions with biophysical inhibitors like plasma proteins or cellular lipids in edema fluid and/or interactions with chemically acting inhibitors such as phospholipases, proteases, or reactive oxygen/nitrogen species (26, 35). Surface activity measurements here did not have plasma proteins from original BALF added back into the medium used to resuspend centrifuged aggregates (Fig. 3). However, recent work in rodents with aspiration lung injury (11) has demonstrated that some plasma proteins in lavage remain associated with surfactant aggregates during centrifugation and impair intrinsic surface activity as found here for P. carinii/TBI mice (Fig. 3). The high levels of total protein originally present in BALF from P. carinii/TBI mice compared with mice given P. carinii or TBI alone (Table 1) would be expected to further impair surfactant activity in vivo. Lavaged surfactant aggregates from animals with acute lung injury can also contain increased levels of inhibitory lysophosphatidylcholine that could potentially contribute to decreases in intrinsic surface activity (11), although this was not specifically examined in P. carinii/TBI mice here.

Inflammatory lung injury in PCP has been postulated to involve both direct mechanisms of cytotoxic lung injury and indirect mechanisms involving T cell-directed recruitment of phagocytic cells and release of cytokines and chemokines (34). Results found here for the cellular composition of BALF from P. carinii/TBI mice are in general agreement with published data from a CD4⁺ -depleted murine model of PCP in which a T cell-dependent influx of BALF neutrophils was found to be associated with increased lung injury (36). BALF from P. carinii/TBI mice revealed a disproportionate initial influx of lavaged CD4⁺ T cells at 11 days post-BMT relative to CD8⁺ T cells compared with mice receiving either P. carinii or TBI alone (Table 2).

The skewing of the CD4⁺:CD8⁺ T cell ratio in P. carinii/TBI mice is intriguing. CD4⁺:CD8⁺ T cell ratios and the particular cytokine patterns elaborated by each T cell subset (Th1 vs. Th2) can impact clinical outcomes, such as in patients with methotrexate-induced pneumonitis (13). Whereas acute graft-vs.-host disease (GVHD) has been associated with Th1-like cytokine responses, P. carinii infection has been shown to elicit Th2-type responses (IL-4, IL-5, and IL-13) in hosts with at least partially intact CD4⁺ immunity (31, 33). In an allogeneic model of P. carinii-driven pneumonitis in the setting of GVHD, Th1-like cytokine responses (IL-2, interferon-γ) predominated, and neutralization of interferon-γ led to an exacerbation of lung injury (14). However, these studies did not address how the above cytokine responses differed from the immune response to P. carinii infection in the normal host. In the current syngeneic BMT studies, eosinophils were markedly increased in BALF from mice given P. carinii alone compared with P. carinii/TBI mice (Table 2). The relative reduction in eosinophils in BALF from P. carinii/TBI mice compared with mice given P. carinii alone may reflect an alteration in organism-induced Th1- vs. Th2-type immune responses in the setting of TBI-induced immune dysregulation. Furthermore, TNF-α has been implicated in eosinophil recruitment and activation via elaboration of IL-5 and thus may be involved in promoting this potential Th1/Th2 polarization (41).

Depletion of CD4⁺ T cells in P. carinii/TBI mice was clearly associated with a decreased severity of lung injury in our study (Table 3, Fig. 4). However, it is difficult to determine to what extent this decrease in injury severity reflects the direct actions of CD4⁺ T cells as opposed to secondary alterations in the levels of other inflammatory mediators following CD4 depletion (e.g., decreases in TNF-α production in CD4⁺-depleted P. carinii/TBI mice in Fig. 5). Alternatively or additionally to effects mediated by TNF-α release, depletion of CD4⁺ lymphocytes in P. carinii/TBI mice could also generate a shift from Th2 to Th1 cytokine patterns to affect lung injury severity as described above. Regardless of the mechanism, lung injury in our syngeneic murine BMT model required the presence of CD4⁺ T cells. Mice depleted in CD4⁺ lymphocytes had no respiratory impairment and did not exhibit increased levels of inflammatory mediators (TNF-α) in BALF, whereas severe inflammatory lung injury was present in P. carinii/TBI mice that were not depleted of T cells or were depleted only of CD8⁺ T cells (Table 3, Fig. 4). The abroga-
tion of pulmonary compromise in CD4\(^+\)-depleted animals occurred despite having equal or greater lung \textit{P. carinii} burdens (Fig. 4B). This lack of correlation of \textit{P. carinii} burden with injury agrees with other models of PCP where direct \textit{P. carinii} damage does not appear to play a major role in \textit{P. carinii}-induced lung injury (15, 36).

In summary, the data presented in the current studies suggest that \textit{P. carinii} infection sensitizes the lung to TBI-related damage by a process dependent on nonalloreactive CD4\(^+\) T cells. It is postulated that in this syngeneic post-BMT lung injury model, animals exposed to the combination of \textit{P. carinii} infection and TBI have increased production of inflammatory mediators, including TNF-\(\alpha\), and this abnormal inflammatory response leads to more severe lung injury than found when either insult is present in isolation (27). Specific cellular and inflammatory pathways by which TNF-\(\alpha\) or other cytokine signaling may contribute to \textit{P. carinii}/TBI injury need to be identified and examined in detail in future research.

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**REFERENCES**


