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Parenchymal Cell TNF Receptors Contribute to Inflammatory Cell Recruitment and Respiratory Failure in \textit{Pneumocystis carinii}-Induced Pneumonia$^1$

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The opportunistic organism \textit{Pneumocystis carinii} (Pc) produces a life-threatening pneumonia (PcP) in patients with low CD4$^+$ T cell counts. Animal models of HIV-AIDS-related PcP indicate that development of severe disease is dependent on the presence of CD8$^+$ T cells and the TNF receptors (TNFR) TNFRsf1a and TNFRsf1b. To distinguish roles of parenchymal and hematopoietic cell TNF signaling in PcP-related lung injury, murine bone marrow transplant chimeras of wild-type, C57BL6/J, and TNFRsf1a/1b double-null origin were generated, CD4$^+$ T cell depleted, and inoculated with Pc. As expected, C57 $\rightarrow$ C57 chimeras (donor marrow $\rightarrow$ recipient) developed significant disease as assessed by weight loss, impaired pulmonary function (lung resistance and dynamic lung compliance), and inflammatory cell infiltration. In contrast, TNFRsf1a/1b$^{-/-}$ $\rightarrow$ TNFRsf1a/1b$^{-/-}$ mice were relatively mildly affected despite carrying the greatest organism burden. Mice solely lacking parenchymal TNFRs (C57 $\rightarrow$ TNFRsf1a/1b$^{-/-}$) had milder disease than did C57 $\rightarrow$ C57 mice. Both groups of mice with TNFR-deficient parenchymal cells had low bronchoalveolar lavage fluid total cell counts and fewer lavageable CD8$^+$ T cells than did C57 $\rightarrow$ C57 mice, suggesting that parenchymal TNF signaling contributes to PcP-related immunopathology through the recruitment of damaging immune cells. Interestingly, mice with wild-type parenchymal cells but TNFRsf1a/1b$^{-/-}$ hematopoietic cells (TNFRsf1a/1b$^{-/-}$$\rightarrow$ C57) displayed exacerbated disease characterized by increased MCP-1 and KC production in the lung and increased macrophage and lymphocyte numbers in the lavage, indicating a dysregulated immune response. This study supports a key role of parenchymal cell TNFRs in lung injury induced by Pc and a potential protective effect of receptors on radiosensitive, bone marrow-derived cells. \textit{The Journal of Immunology}, 2008, 181: 1409–1419.

$^1$Abbreviations used in this paper: \textit{Pneumocystis carinii} (Pc); bronchoalveolar lavage fluid (BALF); bone marrow transplantation (BMT); lymphocytic immune function (LIF); lung resistance (RL); macrophage (MΦ); pulmonary function (PF); \textit{Pneumocystis carinii}-induced pneumonia (PcP); ribosomal protein L32 (rPL32); soluble TNFR (sTNFR); transmembrane TNFR (TMF); TNF, tumor necrosis factor; wild type (WT).

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reported in animal models (8). However, TNF signaling through its receptors is also directly involved in the immunopathogenesis of PCp. During PCp, peak lung concentrations of TNF correlated temporally to rapid progression of lung injury. The importance of TNF signal transduction in PCp was further supported by observations of Pc infection in mice genetically null for both TNF receptors (TNFRs). CD4+ T cell-depleted TNF receptor sf1a/1b−/− mice displayed reduced inflammatory cell recruitment to the lung when compared with CD4-depleted wild-type (WT) mice similarly infected with Pc (9). Quantitatively, the TNFR-deficient mice had significantly reduced pulmonary RANTES, MCP-1, MIP-2, and cytokine-induced neutrophil chemoattractant responses, as well as reduced histological evidence of PCp-related alveolitis as compared with infected WT mice. Less severe pulmonary inflammation correlated with improved surfactant activity and improved pulmonary function in the TNFR-deficient mice.

While robust TNF responses have been documented in both bronchoalveolar lavage fluid (BALF) from PCp-infected mice and culture supernatants of PCp-stimulated alveolar macrophages, the relevant cellular target of TNF action during PCp has not been determined. TNFRsf1a and TNFRsf1b are ubiquitously expressed at varying, cell type-dependent ratios. Ligand binding of the TNFRs has pleiotropic effects depending on cell type and cellpriming, ranging from induction of apoptosis or proliferation to induction of secondary cytokines. TNFRs are present on most cells, and lung parenchymal (including epithelial) cells, as well as classical immune cells, respond to both Pc and TNF. The present study was performed to determine whether PCp-related lung injury is dependent on TNFFR function on lung parenchymal cells, which are largely radio-resistant at low dosage, or in radiosensitive, bone marrow-derived immune cells. Sublethal total body irradiation followed by bone marrow transplantation was used to create chimeric mice in which either hematogenous cells (radio-sensitive, donor marrow), parenchymal cells (radio-resistant, recipient), or both are null for TNFRs. The chimeric mice were CD4+ T cell depleted before Pc infection, and the progression of disease was assessed.

Materials and Methods

Generation of C57BL/6J TNFRsf1a/1b double-null mice

The original studies of PCp in TNFR-null mice were performed in commercially available TNFRsf1a−/− sf1b−/− double-null mice generated as intercrosses of TNFRSF1A−/− sf1b−/−, created by homologous recombination in C57BL/6-derived ES cells, and TNFRsf1h−/− generated in AB1 (129S5/SvEvBrd) ES cells (The Jackson Laboratory). C57BL/6.129SF2 hybrids were the recommended controls (10). To reduce potentially confounding genetic differences in the current experiments, TNFRsf1a/1b double-null mice were regenicated in a C57BL/6 background. TNFRsf1a−/− sf1b−/− and TNFRSF1A−/− sf1b−/− single-knockout (KO) mice were each backcrossed 12 generations onto C57BL/6J and then interbred to produce double-deficient mice. Wild-type C57BL/6J mice were used as controls (The Jackson Laboratory). Mice used in the current protocol were bred and maintained in microisolator cages in specific pathogen-free rooms in the animal care facility at the University of Rochester Medical Center (Rochester, NY), supplied animals maintained in the same rooms, on bedding mixed with bedding taken from other cages within the room, routinely tested negative for common murine pathogens, including murine hepatitis, pinworm, and Sendai virus. All animal care and experimental protocols were approved by the University of Rochester Committee on Animal Research and follow the guidelines of IUCAC.

Generation of chimeras by bone marrow transplantation (BMT)

To distinguish the role of parenchymal cell TNFRSF1A in Pc-induced lung injury, BMT chimeras (donor→recipient) C57→C57, C57→TNFRsf1a/1b−/−, TNFRsf1a/1b−/−→C57, and TNFRsf1a/1b−/−→TNFRsf1a/1b−/− were generated by radioablation of female recipients (total body irradiation, 6 Gy × 2 doses, Shepherd irradiator, 6000 Ci 137Cs source) followed by reconstitution with male donor bone marrow. The radiation dose was previously demonstrated to induce granulocytopenia with evidence of radiation-induced injury at ∼3–4 wk. Bone marrow was extracted from a minimum of three donors of the appropriate mouse strain by flushing femurs and tibias into HBSS with 1% FCS, dispersing through a 21-gauge needle, and pooling. Erythrocytes were removed by hypotonic lysis. The cells were counted, resuspended in media at 5 × 10^6/ml, and delivered to recipient mice by tail vein injection (1 × 10^7 per mouse). Following BMT, mice were allowed to reconstitute for 8 wk under microisolator conditions, supplied with high efficiency particulatefiltered air, sterilized food, acid water, and bedding. To confirm chimerism, fluorescent in situ hybridization with a probe directed against a region of Sry, the sex-determining gene of the Y chromosome, was used on cells obtained by bronchoalveolar lavage (BAL) in some mice to confirm the male origin of cells transplanted to female recipients (11). Additionally, analysis of soluble TNFR present in peripheral blood and FACS analysis of cells isolated from spleens and BAL were consistent with the chimeric design (Table I).

Induction of PCp in mice after BMT

Eight weeks after reconstitution, the BMT chimeras were made susceptible to PCp infection by twice-weekly i.p. injections of anti-CD4+ mAb (0.25 mg, clone TIB 207; American Type Culture Collection) as previously described (9). Injections were begun at least 4 days before PCp inoculation and were continued for the duration of the experiment. After initiation of CD4 depletion, each experimental mouse was anesthetized with a mixture of ketamine (68 mg/kg body weight) and xylazine (6 mg/kg body weight) and then intranasally inoculated with PCp (5 × 10^6 as determined by cyst count) in 100 μl of sterile saline. Mice were then housed under standard, microisolator technology until sacrifice at ∼28 days after PCp inoculation. The timing of end-point analysis was determined by appearance of illness and elevated respiratory rates. Despite close observation, 10 mice died before harvest, as detailed in the Results. The results of four independent experiments, each with all chimeric groups included, were combined and are presented in aggregate.

Physiologic assessment of pulmonary compliance and resistance

Dynamic lung compliance and resistance were measured in live mice, anesthetized by sodium pentobarbital injection (100 mg/kg body weight, i.p.), using a previously described method with modifications (5). A tracheotomy was performed and a 20-gauge cannula was inserted 3 mm into an anterior nick in the exposed trachea. To ensure that the mice tolerated the procedure, they were examined for spontaneous respirations before proceeding further. Mice were immediately placed into a plethysmograph designed for

Table 1. Surface markers of cells in BAL and spleen confirm TNFR chimera constitution and CD4 ablation

<table>
<thead>
<tr>
<th>Marker</th>
<th>WT</th>
<th>KO</th>
<th>WT to WT</th>
<th>KO to KO</th>
<th>KO to WT</th>
<th>WT to KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFRsf1b−/−</td>
<td>30.1 ± 8.6</td>
<td>0.8 ± 0.3</td>
<td>41.2 ± 11.6</td>
<td>0.4 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>33.3 ± 8.6</td>
</tr>
<tr>
<td>CD4</td>
<td>0.05 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.02 ± 0.02</td>
<td>0.1 ± 0.04</td>
<td>0.04 ± 0.04</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>CD8−/−</td>
<td>21.8 ± 5.6</td>
<td>10.0 ± 6.4</td>
<td>22.2 ± 15.7</td>
<td>19.5 ± 6.6</td>
<td>13.5 ± 4.8</td>
<td>23.6 ± 9.7</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNFRsf1b−/−</td>
<td>9.8 ± 1.2</td>
<td>0.5 ± 0.0</td>
<td>8.3 ± 2.2</td>
<td>0.4 ± 0.5</td>
<td>0.6 ± 0.14</td>
<td>11.4 ± 4.1</td>
</tr>
<tr>
<td>CD4−/−</td>
<td>0.1 ± 0.0</td>
<td>0.01 ± 0.03</td>
<td>0.05 ± 0.06</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>CD8−/−</td>
<td>7.1 ± 2.8</td>
<td>7.8 ± 2.3</td>
<td>6.6 ± 1.7</td>
<td>8.0 ± 2.2</td>
<td>6.5 ± 2.7</td>
<td>6.1 ± 3.5</td>
</tr>
</tbody>
</table>

* n = 3–8, mean ± SD; # p < 0.05 vs WT donor chimeras.
anesthetized mice (Buxco Electronics) and connected to a Harvard rodent ventilator (Harvard Apparatus). Mice were ventilated with a tidal volume of 0.01 ml/g body weight at a rate of 150 breaths per minute. Data were collected and analyzed using the Biosysstems XA software package (Buxco Electronics). Dynamic lung compliance (ml/cm H₂O) normalized for peak body weight, and lung resistance (ml/cm H₂O/sec) were calculated by the method of Amurad and Mead (12) from air flow and pressure signals transduced from the chamber and passed through an analog-to-digital converter.

**Analysis of BAL and cells isolated from spleen and BALF**

Pretreatment serum was collected by mandibular bleed. At peak Pc in the most severely affected mice, all mice were euthanized by injection with pentobarbital (130 mg/kg, i.p.). Serum was collected by cardiac puncture and allowed to clot in serum separator microtainers. Single-cell suspensions from spleen were obtained by homogenization in DMEM medium and successive filtration through 100- and 40-μm cell strainers and through 25-μm nylon gauze. For BAL, the trachea was intubated, the anterior chest wall removed, and the lungs were lavaged with normal saline (room temperature, 2 ml). BALF was centrifuged at 250 × g for 10 min at 4°C. The supernatant was placed on ice before assay for lactate dehydrogenase and then frozen at −80°C until assayed for total protein by bichinonic acid assay (Pierce), as well as for MIP-2, MCP-1, TNF, and soluble TNFRs by ELISA (Duoset, R&D Systems), using commercial reagents and protocols. The pelleted BALF cells were resuspended in 1 ml 0.15 M NH₄Cl, 0.01 M NaHCO₃ solution for 10 min to lyse RBC, then washed twice with HBSS and resuspended in DMEM. A total cell count of resuspended BALF cells was performed by hemocytometer. BALF cellular differential was determined on 50 μl cytospins stained with Diff-Quik (Dade Behring) as previously described. MIP-2, MCP-1, KC, and TNF were also assessed by ELISA in lung homogenate prepared in antiprotease buffer and normalized to total protein concentration of the samples.

**Flow cytometry**

CD4, CD8, and TNFRs1b-positive leukocyte populations in BAL and spleen were identified by multiparameter flow cytometry as previously described (13). Cells obtained from lung lavage were washed, resuspended in PBS containing 1% BSA-0.1% sodium azide, and stained for 30 min at 4°C with anti-CD4-FITC (clone RM4-4), anti-CD8a-peridinin chlorophyll-a protein (clone 53-6.7, PerCP), and anti-TNFRs1b-PE (BD Pharmingen). The anti-CD4 clone RM4-4 was used to confirm that CD4, CD8, and TNFRsf1b-positive leukocyte populations in BAL and histological evidence of PcP in the global absence of TNFRs (9). To begin to define the cells that, via the TNFRs, mediate the inflammatory response leading to lung injury, chimeric WT/ TNFRs1a/sf1b double-null mice, expressing receptors either on high- and low-power fields are presented from three mice for each chimera group.

**Statistical analysis**

Where appropriate, statistical significance was determined by Student’s t test or by ANOVA with Fisher’s protected least significant difference post hoc test (StatView, SAS Institute).

**Histological assessment of Pc-induced lung injury**

At peak injury, ~28 days after Pc instillation, the left lung was inflation fixed with 10% buffered formalin overnight, dehydrated to 70% ethanol, and paraffin embedded. Paraffin sections (4–5 μm thick) were deparaffinized and rehydrated through graded ethanol and stained with H&E. Photomicrographs were taken by a SPOT RT digital camera. Representative low- and high-power fields are presented from three mice for each chimera group.

**Results**

Previous study of TNFR-null mice demonstrated a significant reduction in inflammatory cell recruitment, chemokine production, and histological evidence of PcP in the global absence of TNFRs (9). To begin to define the cells that, via the TNFRs, mediate the inflammatory response leading to lung injury, chimeric WT/TNFRs1a/sf1b double-null mice, expressing receptors either on radio-resistant parenchymal cells or on radiosensitive bone marrow donor-derived cells, were generated. Following reconstitution, the bone marrow chimeras were treated with anti-CD4 Ab to induce susceptibility to Pc. Flow cytometry of the BALF cells demonstrated that <1% of the recruited lymphocytes were CD4⁺positive, confirming the uniform CD4 depletion state of the chimeras (Table I). The absence of CD4⁺ T cells was confirmed in FACS analysis of splenocytes. FACS analysis of splenocytes and BALF cells for TNFRs1b receptor also confirmed the design and success of the BMT-induced chimera. Less than or equal to 1% of lymphocytes in BALF or spleen were TNFRs1b-positive in KO to WT and KO to KO chimeras where these cells should have originated from donor receptor-null bone marrow.

The percentage of spleen and BALF cells that were CD8⁺positive did not differ significantly between the chimera groups and did not correlate with severity of illness.
worsened as compared with noninfected mice either transplanted or not.

Maximal \( P. c. \)-induced impairment of pulmonary function and weight loss requires TNFRs on radio-resistant cells

\( P. c. \)-induced pulmonary inflammation has been shown to correlate well with surfactant dysfunction and physiologic impairment of lung function as measured by lung resistance and dynamic compliance in mice (9). Lung resistance and compliance were slightly altered by the BMT procedure, with both resistance and compliance tending to be lower in transplanted WT to WT and KO mice, respectively. These subtle \((p > 0.05)\) BMT effects were not TNFR dependent. In the present study, resistance and compliance measures in all \( P. c. \)-infected chimeric mice were significantly impaired as compared with noninfected, nontransplanted WT and KO mice, as well as with noninfected, transplanted WT to WT and KO to KO controls (Fig. 1, gray bars in C and D vs noninfected represented in A and B), consistent with a TNFR-independent component of PcP. However, pulmonary function of WT to WT mice was significantly more compromised by \( P. c. \) infection compared with KO to KO mice, reinforcing the importance of TNFR signal transduction in the development of PcP. Consistent with the hypothesis that parenchymal cell TNFR function has an important role in PcP, the greatest decrement in lung function, measured as reduced compliance and increased resistance, was noted in the KO to WT chimeras, trending greater than that measured in WT to WT chimeras and significantly greater than all other chimera groups (Fig. 1, C and D). The obtained measurements on WT donor to KO recipient chimeras showed slight improvement in lung function over WT to WT animals that did not reach statistical significance \((p > 0.05)\). However, the mortality rate in the WT to WT group was significantly greater than in either KO recipient group, consistent with more severe disease. While overall mortality rate was fairly low in these experiments, 10 mice died, all within days of the 4-wk experimental end-point. Five were WT to WT chimeras out of 20 tested \((25\%)\), 4 were KO to WT chimeras out of 19 tested \((21\%)\), only 1 was a WT to KO chimera out of 14 \((7\%)\) tested, and none was from a KO to KO chimera, reinforcing the increased severity of disease in mice whose parenchymal cells expressed TNFRs as compared with recipient mice who were receptor null. When average lung function measurements of all surviving mice with WT parenchymal cells (WT to WT and KO to WT) were compared with the average measurements of all mice with KO parenchymal cells (WT to KO and KO to KO), the role of parenchymal TNFRs was unmistakable. The mean lung compliance for mice with WT parenchymal cells was 0.68 ± 0.04 as compared with 0.93 ± 0.05 for the mice with KO parenchymal cells \((p < 0.0001)\). Similarly, mean lung resistance values for the two groups were 2.36 ± 0.9 and 1.84 ± 0.04, respectively \((p < 0.0001)\). These data are also supported by the overall mortality rates of ~23% for mice with WT parenchymal cells and 3% for mice with KO parenchymal cells.

Weight loss following \( P. c. \) infection is another clinically significant marker of pneumonia severity. As seen in Fig. 2, the mice expressing TNFRs only on radio-resistant, parenchymal cells demonstrated significantly greater weight loss than any of the other \( P. c. \)-exposed chimeric mice \((p < 0.01)\). Importantly, both groups of mice with TNFR expressing parenchymal cells (WT to WT and KO to WT) exhibited significantly greater weight loss than did mice lacking parenchymal TNFRs \((KO to KO and WT to KO)\), again highlighting the role of parenchymal TNFRs during PcP-related injury. As with the pulmonary function measurements, accounting for those mice that died before harvest at 4 wk of infection, average weight loss would be expected to be even greater in the groups with WT parenchymal cells. Thus, considering morbidity and mortality, the presence of TNFRs on only radio-resistant cells was sufficient to induce clinically significant PcP.

No significant difference was measured in resistance, compliance, or weight loss in WT to KO as compared with WT to WT chimeras. The values for these measures fell between those of the WT to WT and KO to KO chimeras, demonstrating that TNFRs restricted to marrow-derived cells are capable of generating PcP.

FIGURE 1. Pulmonary function in TNFR WT, KO, and BMT chimeric mice. Lung resistance \((A \) and \( C) \) and specific dynamic lung compliance \((B \) and \( D) \) measurements were made in live, intubated mice 4 wk after \((A \) and \( B) \) saline instillation or \((C \) and \( D) \) \( P. c. \) infection. Black bars, nontransplanted, \( n = 7 – 12 \); gray bars, transplanted \((donor \to \) recipient), \( n = 4 \) per noninfected group, \( n = 16 – 26 \) per \( P. c. \)-infected group. Values are means \(\pm SEM\); \( p \) values are in brackets. NS = \( p > 0.05 \). There were no significant differences among saline-treated controls. Resistance and compliance of \( P. c. \)-infected chimeras were all significantly worsened as compared with noninfected mice either transplanted or not.
similar to but milder than globally TNF responsive mice (when considering the differences in mortality.

Accumulation of inflammatory cells, lactate dehydrogenase (LDH), and protein in the bronchoalveolar space was TNFR location dependent

Total cell count and differential of cells available by BAL were determined to quantify differences in the alveolar inflammatory cell recruitment in the presence or absence of parenchymal TNFRs (Fig. 3). There was a marked recruitment of lavageable TNFRs in the WT to WT chimeras, as well as a significantly greater increase in the lavaged cell count of the KO to WT mice. In comparison, the remaining two chimera groups with receptor-null parenchymal cells had significantly reduced total BALF cell counts, not differing from saline-treated chimeric controls. Differential cell counts on lavaged cells demonstrated a small, TNFR-independent increase in lymphocytes in saline control chimeras as compared with nontransplanted, noninfected mice $(p < 0.05)$. The increase in total cell number in Pc-infected mice with WT parenchymal cells was accounted for mainly by increases in lymphocytes and neutrophils (Fig. 3B). Furthermore, KO to WT mice showed a dramatic increase in the number of lavageable AMs compared with all other Pc-infected groups. That the numbers of BALF alveolar macrophages in all other chimeras were reduced from baseline (saline treated) suggests margination of resident and, potentially, recruited macrophages. Although the percentage of BALF cells that were CD8$^+$ did not differ between the chimeras (Table I), the absolute numbers of CD8$^+$ T cells recruited by the WT to WT and KO to WT mice were significantly greater than those recruited by the WT to KO or the KO to KO mice, suggesting that the presence of TNFRs on radio-resistant, parenchymal cells is sufficient for CD8$^+$ T cell recruitment and is necessary for peak recruitment.

As with the pulmonary function tests, there was a small but in this case significant increase in BALF protein content, as well as a trend toward increased LDH, in noninfected control chimeras compared with noninfected, nontransplanted controls, which was TNFR independent (Fig. 4, A and B). Consistent with enhanced capillary leak and cell death, the BAL total protein was elevated $>10$-fold in the WT (see Ref. 9) and chimeras after Pc infection; no distinction could be made between chimeras based on protein concentration in the alveolar space, although the trend was lower in KO recipient lungs as compared with WT recipients (Fig. 4C).
LDH concentrations in BALF were also elevated in all Pc-treated mice (Fig. 4); in this case, LDH was significant higher in the WT to WT chimeras and trended higher in the KO to WT mice, suggesting enhanced cellular necrosis and release of LDH dependent on parenchymal cell response to TNF (Fig. 4). The average BALF protein and LDH concentrations were greater in the TNFR WT recipients than in the receptor-null recipients: 1.45 ± 0.55 vs 1.08 ± 0.58 (p = 0.01) and 304.5 ± 46.5 vs 132.1 ± 20.8 (p < 0.005), respectively.

Severe Pc-induced alveolitis occurred in TNFR WT recipient chimeras in comparison to TNFR-null recipient chimeras. Analysis of lung sections from the Pc-infected TNFR chimeras by light microscopy demonstrated differences in degree of lung injury that were consistent with those suggested by the physiologic and cellular indices (Fig. 5). Both the WT to WT and KO to WT lungs demonstrated dense, mononuclear cell infiltration that was maximal around distal bronchioles and pulmonary vessels; the pattern was somewhat more intense, interstitial, and diffuse in the absence of TNFRs in the marrow-derived cells of the KO to WT mice. Inflammation was also present in the absence of parenchymal cell TNFRs, but cellularity was markedly reduced when compared with parenchymal WT lungs. The architecture of the WT to KO chimera lungs, and especially the alveolar spaces, were relatively spared in comparison to all other chimeras. Interestingly, the TNFR-null mice (KO to KO) were not free of injury but demonstrated a relatively disorganized cellular infiltration and large amounts of bland alveolar debris that may be related to direct injury from the high Pc burden.

Increased soluble TNFRs (sTNFRs) measured in BAL and serum of WT animals with Pc

TNFRs, active as cell surface transmembrane receptors, are solubilized by enzyme cleavage in response to inflammatory stimuli (for review, see Refs. (16, 17). Elevated serum sTNFRs in patients with acute respiratory distress syndrome correlated with mortality (18). In the current study, sTNFRsf1a and sTNFRsf1b were analyzed in serum and BALF to test the hypothesis that TNFRs are also shed and accumulate in response to Pc infection. As controls, sTNFR levels were measured in serum harvested 8 wk after bone marrow reconstitution, just before beginning CD4+ T cell depletion, and were consistent with the desired chimeric composition.
In these pretreatment samples, sTNFRsf1a and sTNFRsf1b concentrations in WT to WT transplants were comparable to WT, nonirradiated, nontransplanted mice, demonstrating that solubilization was not an artifact of bone marrow transplantation. TNFRsf1a/1b−/− to TNFRsf1a/1b−/− chimeras had no detectable sTNFRs in serum. Serum sTNFR concentrations of the mixed chimeras were, as expected, intermediary between WT and KO animal levels. The relative levels of the two receptors were dependent on the genotype of the donor and recipient mice. Comparable serum sTNFRsf1a levels were detected in noninfected WT, WT to WT, and KO to WT chimeras. The receptor was very low in the WT to KO chimeras, suggesting that the primary origin of this soluble receptor, constitutively in circulation, is the parenchyma. Serum TNFRsf1b was comparable to WT control in the WT to KO chimeras but reduced in the KO to WT chimeras. The receptor was very low in the WT to KO chimeras, suggesting that the primary origin of this soluble receptor, constitutively in circulation, is the parenchyma. Serum TNFRsf1b was comparable to WT control in the WT to KO chimeras but reduced in the KO to WT chimeras. The receptor was very low in the WT to KO chimeras, suggesting that the primary origin of this soluble receptor, constitutively in circulation, is the parenchyma. Serum TNFRsf1b was comparable to WT control in the WT to KO chimeras but reduced in the KO to WT chimeras. The receptor was very low in the WT to KO chimeras, suggesting that the primary origin of this soluble receptor, constitutively in circulation, is the parenchyma. Serum TNFRsf1b was comparable to WT control in the WT to KO chimeras but reduced in the KO to WT chimeras. The receptor was very low in the WT to KO chimeras, suggesting that the primary origin of this soluble receptor, constitutively in circulation, is the parenchyma.

Severity of lung injury was not directly related to Pneumocystis burden

Although KO to KO mice were the healthiest of the TNFR chimera mice, determination of Pc burden in the lungs at 4 wk after infection demonstrated the highest number of organisms in this group (10.1 ± 2.3 × 10^6 per lung vs 6.6 ± 1.8 × 10^6 per lung in WT to WT mice averaged across four experiments), a 2-fold difference when normalized to WT to WT burden in each experiment (Fig. 7). These data are consistent with previous reports that TNF exerts...
some control over Pc growth in SCID mice (19) and in CD4-depleted mice (8), and they also demonstrate that the host’s immune response, and not Pc burden, is the critical factor in determining the severity of PcP. Interestingly, while KO to WT mice had the most intense pulmonary inflammatory response and most severe PcP, they did not have reduced Pc burden (6.4 ± 2.0 × 10⁶ per lung). This indicates that an inappropriate immune response can exacerbate disease without the beneficial effect of killing Pc.

The lowest Pc burden was detected in the mice with WT bone marrow but TNFR-deficient parenchymal cells, the WT to KO chimeras (1.6 ± 0.5 × 10⁶ per lung). These mice consistently maintained a significantly lower Pc burden than did the remaining chimeras.

Differential chemokine induction dependent on cell-specific TNFR expression

Recruitment of inflammatory cells to the lung during PcP has been correlated to chemokine production, in particular to MIP-2, MCP-1, KC, and cytokine-induced neutrophil chemoattractant, in addition to TNF (9). The chemokine elevation was largely dependent on TNFR expression, as it was blunted in TNFR-null mice (9). To determine whether the variation in PcP severity observed in the presence or absence of parenchymal cell TNFRs correlated with altered chemokine production, both lung tissue homogenates and BALF from TNFR chimeric mice were analyzed for chemokine protein. Previous studies demonstrated that MCP-1, MIP-2, KC, and TNF were below the limit of detection in lung and BALF of uninfected mice. No significant difference in homogenized lung tissue TNF was detected between chimeras, while MCP-1 was modestly, and KC was markedly, increased in KO to WT mice in comparison to TNFR-null recipient chimeras (Fig. 8B). MIP-2 protein concentration was significantly increased in all mice expressing TNFRs on either (or both) marrow-derived or parenchymal cells, with relatively reduced levels in fully null chimeras. Measurement of the cytokines in BALF detected chemokine concentration differences dependent on TNFR distribution (Fig. 8A). MCP-1 protein was significantly elevated in BALF of KO to WT chimeras, with those animals also having been documented to have more severe PcP (Figs. 1 and 6) and a dramatic increase in lavageable macrophages (Fig. 3). BALF TNF levels were highest in the KO to KO mice and lowest in the WT to WT mice, with TNF levels in the mixed chimeras falling between. This result likely reflects alteration of TNF-negative feedback loops that require the expression of TNFRs on both compartments for normal control of TNF production. Relatively low concentrations of MIP-2 were detected in the BALF, with the greatest levels found in the WT to TNFR-null mice, suggesting dysregulation of the chemokine with stimulation of marrow-derived cells in the absence of parenchymal TNF response.

Analysis of mRNA for the cytokines MIP-2, MCP-1, and KC in whole lung preparations by quantitative real-time PCR demonstrated patterns of expression similar to the protein concentrations measured in BAL and lung homogenates. MCP-1 and KC mRNA concentrations were significantly elevated in KO to WT chimeras when compared with all Pc-treated chimeras and controls, while MIP-2 mRNA was significantly less concentrated in the TNFR-null chimeras, intermediate in the mixed chimeras, and several-fold greater in the WT to WT chimeras (Fig. 8C). RANTES, also previously shown to be stimulated in response to Pc (9), was increased in all cases from noninfected controls (0.2 ± 0.1 ratio to

FIGURE 8. Cytokines in BALF and whole lung isolated from Pc-infected TNFR chimeric mice 4 wk after infection. TNF, MIP-2, MCP-1, and KC were assessed by ELISA in (A) BAL fluid and (B) homogenized lung tissue. C, MIP-2, MCP-1, KC, RANTES, and IL-10 were measured by quantitative real-time PCR in isolated lung RNA. Values are means ± SEM; n = 7–8, p values in brackets.
rpl32) but with no significant difference between chimeras. The expression of IL-10, an antiinflammatory cytokine, was also tested as a potential explanation for differences in PcP severity. There were significant differences in IL-10 mRNA levels between the chimeras, in part correlated with severity of disease, as highest concentrations were measured in fully TNFR-null chimeras. However, those most injured, the KO to WT mice, had IL-10 mRNA levels comparable to WT animals, while they were significantly lower in moderately injured WT to KO mice.

Discussion

Many lines of evidence support the importance of TNF in the development of PCp. Pc stimulates TNF production and release from alveolar macrophages in both immunocompetent and immunosuppressed mice and from monocytes and macrophages in culture (20, 21). Release of TNF from alveolar epithelial cells in response to the organism has also been demonstrated (22). In the reconstituted SCID model of PCp, the onset of reduced compliance and hypoxia is temporally related to peak TNF mRNA in lung tissue and TNF protein in BALF, in association with the influx of neutrophils, macrophages, and lymphocytes. TNF protein is likewise increased in CD4+ T cell-depleted, Pc-infected mice in a CD8+ T cell-dependent manner (9). Our previous studies using TNFR-deficient mice demonstrated that maximal Pc-induced chemokine production, lung injury, and pulmonary dysfunction required intact TNFR signal transduction (9). The current study demonstrates that TNFRs on cells resistant to split dose irradiation are sufficient to mount an inflammatory response to Pc, comparable to or in excess of that generated when all cells express the receptors. Limitation of TNFR distribution to marrow-derived cells improved control of Pc burden and reduced Pc-induced injury.

Pulmonary function and inflammatory markers were analyzed in the TNFR chimeric mice when the sickest treatment group reached clinically peak disease, ~4 wk after Pc treatment in the chimeric mice; at and beyond this point mortality increased markedly in the KO to WT and WT to WT chimeras. Each of the transplanted mice that expressed TNFRs either on radiosensitive marrow-derived cells or radio-resistant parenchymal cells or both (WT to WT, WT to KO, or KO to WT) demonstrated some evidence of pneumonia at this time point. However, the greatest mortality, decrement in pulmonary function, weight loss, and inflammatory cell recruitment occurred in those mice expressing TNFRs on radio-resistant but not on marrow-derived cells. TNFR-null chimeras (KO to KO) were least injured despite having a relatively greater Pc burden than the remaining chimeras. Interestingly, in vitro Pc-dependent induction of chemokines in alveolar epithelial cells occurs independently of TNFRs (23), which may explain the inflammation and physiologic impairment observed even in the TNFR-null mice. In vivo, TNFR-deficient models indicate that maximal inflammation that correlates with clinically significant loss of weight and pulmonary function and that results in death requires parenchymal TNF responses. TNF expression limited to marrow-derived cells was sufficient to generate Pc-stimulated lung injury and to augment loss of pulmonary function and weight loss but was much less apt to cause mortality, and it was associated with reduced MCP-1, KC, and TNF production, intraalveolar cell death (by LDH), and recruitment of lavageable cells. As previously observed, the severity of lung injury was not directly related to Pneumocystis burden (24, 25). Reduction in lung compliance weakly correlated with Pc burden but only in the presence of parenchymal TNF function, which may reflect inhibition of surfactant production or function (9). The global TNFR-null mice carried the greatest Pc burden but the least evidence of injury, consistent with a role for TNF in clearance of Pc as previously demonstrated (9). In the present study, the parenchymal cell-null, marrow WT animals maintained the lowest burden of organism, suggesting that TNF stimulates immune cells to remove the organism. It has also been suggested that adherence of Pc to lung epithelial cells enhances proliferative growth of the organism (26). The absence of parenchymal TNFRs may reduce Pc-epithelial cell adherence, thereby reducing the organism’s growth potential and, potentially, leaving it more accessible to immune cell clearance. Further study is necessary to clarify this observation.

Accumulation of inflammatory cells and LDH in the bronchoalveolar space was parenchymal cell TNFR dependent. Although each of the chimeras developed some degree of lung injury, there was a marked increase in numbers of inflammatory cells recruited to the lavageable air space in WT recipient mice, particularly when the bone marrow-derived, recruited inflammatory cells did not express the TNFRs. No increase in total lavageable cell counts was detected in the absence of parenchymal TNF signal transduction. The differential of the BALF cells was altered in response to Pc; infiltration by neutrophils and lymphocytes occurred in all chimeras but was exaggerated by the presence of parenchymal TNFRs. Flow cytometry suggested marked increases in the percentage of lymphocytes that are CD8+ in all Pc-exposed groups, with no differences found between chimeras. However, when considered as absolute numbers of lavageable cells, the maximal increase in recruitment of CD8+ T cells was seen in the TNFR WT recipient chimeras. This result is consistent with a significant role for parenchymal TNF signal transduction in the recruitment of these cells, which have been shown to mediate Pc-induced lung injury in this CD4− T cell-depleted model. Note that despite markedly increased inflammatory cell infiltration mediated by parenchymal TNFRs, the KO to WT chimeras were unable to control the Pc burden any better than did the fully WT mice. This is consistent with the failure of sensitized CD8+ T cells to control organism burden; however, the failure of enhanced macrophage numbers in this chimeras to control the Pc again supports the role of TNF stimulation of the immune cells in Pc clearance.

A significant contribution of parenchymal cell TNF responses to lung injury induced by pathogens has been previously demonstrated. For example, CD8+ T cell recognition of alveolar cells expressing a specific viral Ag triggered MCP-1 and MIP-2 expression by the lung epithelial cells in large part due to T cell transmembrane TNF (tmTNF) and the presence of TNFRs on the epithelium (27, 28). Additionally, a study of alveolar macrophages in patients with acute respiratory distress syndrome demonstrated enhanced tmTNF correlated with severity of disease, although soluble TNF concentrations in the BAL did not (29), suggestive of the importance of inflammatory cell-bound TNF interacting with parenchymal receptors. Because tmTNF is an active signaling molecule, the expression of TNFRs on parenchymal cells constitutes a mechanism for intercellular communication with tmTNF-expressing immune cells. In this way, TNF expressed by inflammatory cells may have enhanced capacity to either induce cytotoxicity or to stimulate proinflammatory protein production by the structural cells of the lung. In the case of Pc, the epithelial cells are anchors for the organism and so are best positioned to stimulate or amplify a local host response. Prior studies also suggest that CD8+ T cell mediated Pc-induced lung damage is dependent on MHC class I expression by radio-resistant cells (6). Intracellular Pc Ag processing or presentation by alveolar epithelial cells has not yet been demonstrated but is feasible. Intercellular TNF-TNFR interactions may enhance such lymphocyte-epithelial cell interactions.

As well as TNF-α, the TNFRs also bind and transduce signal of the homotrimeric ligand, lymphoxygenin-α (LT-α), previously known as TNF-β. LT-α also signals by forming heterotrimers with
LT-β that bind the LT-β receptors. Removal of the TNFRs therefore prevents signal transduction by homotrimeric LT-α, as well as by TNF-α. The phenotypes observed in the present study are then the result of manipulating both TNF-α and LT-α activity. Due to the strong relationship previously established between TNF-α and progression of PcP, it is thought likely that it is primarily the effect of TNF-α signal transduction via TNFR activity that has been altered in this study. No regulation or role of LT-α in PcP has been clearly demonstrated to date. Whether PcP is altered by LT-α binding of TNFRs on parenchymal or bone marrow cells, however, remains to be studied potentially by anti-ligand Ab or ligand-specific knockout transgenic models.

The TNF sf1a receptor has been demonstrated to be necessary for normal development and maintenance of splenic B cell follicles and germinal centers (30–32). In mice deficient in either LT-α or the type I TNFR, but not the type II TNFR, germinal centers failed to develop in peripheral lymphoid organs. While the chimeras in the present study have not been tested for germinal centers, it is highly unlikely that a reduction or failure of Ab production in response to Pc accounts for the differences in PcP between the CD4+ T cell-depleted chimeras studied given the known role of these lymphocytes in producing anti-Pc Abs. Even when Pc-infected SCID mice are given Pc-sensitized lymphocytes, CD4+ T cells are required for an Ab response to be generated (25). Additionally, nonimmunized, CD4-depleted mice, directly analogous to the animals tested in the present study, do not make an Ab response to Pc (33). Therefore, none of the chimeras tested in this study would have had an Ab response to Pc, regardless of the presence or absence of TNFRs.

Macrophage predominance persisted and was amplified in the BALF of KO to WT mice, in contrast to the other chimeras. This occurred in association with exaggerated production of the CC chemokine MCP-1, documented by increased tissue mRNA as well as BAL protein concentrations. Because MCP-1 is a chemoattractant for lymphocytes as well as for macrophages, it is possible that parenchymal response to TNF results in enhanced induction of MCP-1 from epithelial cells, for example, that in turn would increase recruitment of these cells. This model would be consistent with the viral model in which CD8+ T cell tmTNF stimulates alveolar epithelial cells to produce MCP-1 (34). It is also possible that activation of parenchymal cells by Pc-induced TNF causes release of other chemoattractants that enhance the recruit of inflammatory cells that become the source of MCP-1. Further analysis of the present model will determine the source of the CC chemokine. Previous studies suggest that CD8+ T cells, even stimulated by specific recognition of alveolar epithelial cells, do not produce MCP-1, whereas the target cells do (28). In contrast, in situ hybridization in the SCID mouse model of PcP demonstrated the primary location of MCP-1 mRNA to be the type II epithelial cells (23). Maximal MCP-1 production occurs in the absence of hematogenous cell TNFRs, and thus direct TNF stimulation of macrophages is not the source.

The alveolar macrophage numbers and MCP-1 induction was not as marked in the WT to WT chimera as in the KO to WT chimera, which is suggestive not only of a role for TNFR stimulation of parenchyma in production of this chemokine in response to Pc, but also of a suppressive effect of TNFRs in the marrow-derived cells. One potential explanation for immune cell TNFR-mediated suppression of Pc-induced lung inflammation is that in the absence of TNFRs, inflammatory cells recruited to the lung fail to undergo TNF-induced apoptosis, an important system of regulation of inflammation. Further studies of cellular turnover in the present model are indicated.

MCP-1 and MIP-2 have both been implicated in the pathogenesis of PcP, having been shown to be induced by Pc and by TNF, as well as correlating with severity of disease in WT and TNFR KO mice. In this Pc chimera model, however, independent regulation of the two chemokines was observed. In vitro studies with Pc-stimulated primary alveolar type II cells suggested that MCP-1 was induced by Pc directly and that this induction was dependent on both NF-xB and JNK activity (23). Preliminary data suggest a synergistic induction of MCP-1 from lung epithelial cells exposed to both Pc and TNF (data not shown). In contrast, MIP-2, similarly induced by direct Pc interaction with epithelial cells, was unaffected by JNK inhibitors, consistent with differential regulation of the two chemokines and perhaps greater dependence of MIP-2 gene expression on NF-xB, of which TNF is a most potent stimulant. Considering the RNA measurements, while maximal MCP-1 induction was dependent on parenchymal cell signaling in the absence of immune cell receptors, MIP-2 induction was maximal when both cell compartments could respond to TNF, inducing a 3-fold increase over that in the mixed chimeras.

One or both of the two distinct receptors, TNFRsf1a and TNFRsf1b, have been identified on most lung cells tested, including type II pneumocytes, although their relative ratio varies by cell type and can be altered by stimulation (35–38). In most studies, TNFRsf1a is constitutively expressed while TNFRsf1b expression is inducible. The predominant receptor, by mRNA, in mouse and human lung is TNFRsf1a, although TNFRsf1b is induced by many stimuli including TNF delivery and Pc infection (9). A differential role of the two receptors in PcP has not yet been clarified. As for TNF, the TNFRs are also targets for matrix metalloproteinases. sTNFR may act as a reservoir of soluble TNF or as circulating inhibitors of both soluble and tmTNF. In this study, we demonstrated that Pc infection induces shedding of these receptors and accumulation of sTNFRsf1a and sTNFRsf1b both in serum and BAL of mice. Whether soluble TNFR levels may be useful as biomarkers reflecting the severity of PcP or may have a physiological role in disease is not yet determined. Interestingly, BMT chimera experiments suggest that in the basal, healthy state, most circulating sTNFRsf1a originates from parenchyma, whereas bone marrow-derived cells appear to be the source of >50% of sTNFRsf1b. In PcP, both sTNFRsf1a and sTNFRsf1b originated from both parenchymal and marrow-derived cells. Although the quantities of sTNFR present in serum or BAL have been shown to mirror the severity of other diseases, the regulation and function of TNF solubilization remain unclear. sTNFRs may be involved in controlling the TNF response during the generation of an immune response. The KO to WT chimera had reduced sTNFR and enhanced inflammatory response relative to WT to WT chimeras. It is possible that the lack of soluble receptors acting as competitive inhibitors contributes to the enhanced injury documented in the mixed chimeras. Alternatively, expression of membrane-bound TNFRs may create a feedback loop that regulates TNF transcription. Increased TNF production was documented in this study but only in the KO to KO mice, suggesting that TNF feedback on either marrow-derived or parenchymal cells is sufficient to regulate the ligand.

The present study demonstrates that TNFRs on parenchymal cells, those resistant to split dose irradiation, are sufficient to mount an inflammatory response to Pc, even if marrow-derived cells are receptor null, comparable to or in excess of that generated when all cells express the receptors. The relative importance of epithelial, endothelial, or mesenchymal cell TNFR responses, as well as the source of the stimulating TNF, remains to be determined. Limitation of TNFR distribution to marrow-derived cells improved control of Pc burden and reduced the injurious host response, best
demonstrated in this study by improved survival and cellular recruitment as compared with normal receptor expression. Limitation of TNFβ distribution to parenchymal cells markedly worsened the inflammatory response and resulted in injury. The results of this study support the notion that therapeutic interventions that inhibit parenchymal cell TNFβ signal transduction, while maintaining or enhancing immune cell TNFβ responses (analogous to the WT to KO chimeras), could be effective both in limiting Pc burden and in limiting the injurious host inflammatory response. Alternatively, global anti-TNF treatment, if given as an adjunctive therapy to the currently used and effective anti-Pc drugs, could also have benefit for patients with severe PcP by reducing the immune aspects of PcP-related lung injury.

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Disclosures
The authors have no financial conflicts of interest.

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