Thymopoietic and Bone Marrow Response to Murine *Pneumocystis* Pneumonia

Xin Shi,1 Ping Zhang,2 Gregory D. Sempowski,3 and Judd E. Shellito1*

Section of Pulmonary/Critical Care Medicine, Department of Medicine,1 and Department of Physiology,2
Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112, and
Duke Human Vaccine Institute, Duke University School of Medicine,
Durham, North Carolina 277103

Received 15 November 2010/Returned for modification 13 December 2010/Accepted 4 February 2011

CD4⁺ T cells play a key role in host defense against *Pneumocystis* infection. To define the role of naïve CD4⁺ T cell production through the thymopoietic response in host defense against *Pneumocystis* infection, *Pneumocystis murina* infection in the lung was induced in adult male C57BL/6 mice with and without prior thymectomy. *Pneumocystis* infection caused a significant increase in the number of CCR9⁺ multipotent progenitor (MPP) cells in the bone marrow and peripheral circulation, an increase in populations of earliest thymic progenitors (ETPs) and double negative (DN) thymocytes in the thymus, and recruitment of naïve and total CD4⁺ T cells into the alveolar space. The level of murine signal joint T cell receptor excision circles (msjTRECs) in spleen CD4⁺ cells was increased at 5 weeks post-*Pneumocystis* infection. In thymectomized mice, the numbers of naïve, central memory, and total CD4⁺ T cells in all tissues examined were markedly reduced following *Pneumocystis* infection. This deficiency of naïve and central memory CD4⁺ T cells was associated with delayed pulmonary clearance of *Pneumocystis*. Extracts of *Pneumocystis* resulted in an increase in the number of CCR9⁺ MPPs in the cultured bone marrow cells. Stimulation of cultured bone marrow cells with ligands to Toll-like receptor 2 ([TLR-2] zymosan) and TLR-9 ([ODN M362]) each caused a similar increase in CCR9⁺ MPP cells via activation of the Jun N-terminal protein kinase (JNK) pathway. These results demonstrate that enhanced production of naïve CD4⁺ T lymphocytes through the thymopoietic response and enhanced delivery of lymphopoietic precursors from the bone marrow play an important role in host defense against *Pneumocystis* infection.

*Pneumocystis jirovecii* is an opportunistic fungal pathogen causing severe pneumonia and pulmonary complications in immunocompromised hosts, particularly in individuals infected with the human immunodeficiency virus (HIV). CD4⁺ T cells are known to play a key role in host defense against *Pneumocystis* infection (43). During HIV infection, activated memory CD4⁺ T cells are the primary target cells destroyed by the virus (51). With the depletion of memory CD4⁺ T cells, generation of naïve T lymphocytes from the thymus becomes a critical mechanism for the host to sustain enhanced T cell turnover (27, 42). Clinical investigations have shown that the thymic output of naïve CD4⁺ T cells is activated in HIV-infected patients following highly active antiretroviral therapy (HAART) treatment (11, 38). This thymic activation is correlated with the increase in the number of naïve CD4⁺ T cells and restoration of total CD4⁺ T cell counts in the peripheral circulation (10, 16). Accumulated evidence suggests that the thymic output of naïve CD4⁺ T cells may play an important role in maintaining or restoring immune function in immunocompromised hosts. At this time, knowledge concerning the role of thymic production of naïve CD4⁺ T cells in the host defense against *Pneumocystis* infection is lacking.

Thymopoiesis requires continuous replenishment of lymphoid progenitors from the bone marrow. In mouse bone marrow, the most primitive hematopoietic stem cells (HSCs) with long-term (LT) self-renewal potential are enriched in the Lin⁻c-Kit⁺ Sca-1⁻ (LKS) cell fraction with the Thy1.1⁻ (producing low levels of Thy1.1) or CD34⁻ profile (2). These cells give rise to the short-term HSCs (ST-HSCs) or multipotent progenitors (MPPs) which are enriched in the Thy1.1⁻ or CD34⁻ LKS cell population. Lineage commitment has been considered to occur after the ST-HSC stage. Common lymphoid progenitors (CLPs) are among cells bearing the IL-7Rα⁻ IL-7Rα⁺ (interleukin-7 receptor α) Thy1.1⁻ phenotypic markers, which may serve as the precursors of both B and T cell lineages (20). A subset of marrow MPPs bearing CCR9⁻ VCAM-1⁻ (vascular adhesion molecule 1) surface markers has been identified as the lymphoid-specific precursors upstream of CLPs (24). Since CCR9⁻ MPPs can more competitively home to the thymus than CLPs, they are considered the major marrow precursors of the earliest T lineage progenitors in the thymus (ETPs) (24).

Upon homing to the thymus, the marrow precursors initially give rise to double negative (DN) thymocytes. These DN cells differentiate to express both CD4⁺ and CD8⁺ antigens, followed by acquisition of the CD3⁺ antigen (double positive thymocytes). Passing through positive and negative selection processes, the mature T lymphocytes become single CD4⁺ or CD8⁺ cells. These matured single positive naïve T cells finally exit the thymus via efferent lymphatics.

Thymic production of naïve T cells can be evaluated by the abundance of T cell receptor excision circles (TRECs) in peripheral T cells (41). Murine signal joint TRECs (msjTRECs) are the episomal DNA circles generated during the rearrangement of the VDJ genes of the T cell receptor (TCR) α and β.
chains in the thymus. These circles are stably retained during cell division but do not replicate and therefore become diluted among daughter cells in peripheral lymphoid tissue.

To identify the significance of thymopoietic and bone marrow activity in the host defense against Pneumocystis infection, we conducted experiments using both an in vivo model of murine Pneumocystis infection and in vitro cell cultures. Our results show that thymopoietic activity is enhanced following intrapulmonary inoculation of Pneumocystis in adult mice. This thymopoietic response is supported by enhanced marrow generation and delivery of thymopoietic precursor cells. Appropriate bone marrow support and thymic output of naïve CD4+ T cells constitute important components of the host immune defense against Pneumocystis infection.

**MATERIALS AND METHODS**

**Animals.** Specific-pathogen-free male C57BL/6 mice were purchased at 5 weeks of age from Harlan Charles River Breeding Labs (Wilmington, MA). Animals were housed in filter-topped cages and fed autoclaved clove and water ad libitum. All caging procedures and surgical manipulations were done under a laminar flow hood. These experimental protocols were performed in adherence to the National Institutes of Health guidelines on the use of experimental animals and with approval of the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center.

**Thymectomy.** Thymectomy was performed using a previously described procedure (37) which allows complete visualization of the entire thymus and its complete removal. Control mice received a sham operation.

**Experimental design.** Three weeks after thymectomy or sham operation, pulmonary infection with Pneumocystis was induced via intratracheal injection of Pneumocystis murina at a dose of 2 × 10^6 cysts per mouse (43). Animals were sacrificed at 1, 2, 3, 4, 5, and 6 weeks after the inoculation of Pneumocystis. Control mice were challenged with phosphate-buffered saline (PBS) alone. For analysis of thymocytes, BAL fluid cells, splenocytes, and lymphocytes of lung-associated lymph nodes, cells were suspended in RPMI 1640 medium containing 2% fetal calf serum (FCS) (2 × 10^6 cells in 100 μl of medium). The cell suspension was added with a mixed panel of biotinylated anti-mouse lineage markers (clone A 19-3, R35-95, and A95-1) (BD PharMingen, San Diego, CA). Following incubation for 20 min at 4°C, phycoerythrin (PE)-conjugated streptavidin (10 μg/ml of each) specific for mouse CD3e (clone 145-2C11; BD Biosciences), CD4 (clone RM4-5; Invitrogen), IL-7Rα (clone 1D7; eBio-Science), anti-mouse/rat CD90.1 (Thy1.1, clone HS151), anti-mouse CD127 (IL-7Rα, clone A7R34), anti-mouse CCR9 (CD199, clone CW-1.2), anti-mouse CD106 (VCAM-1, clone 429), and anti-mouse Ly-6A/E (Sca-1, clone D7) or the matched isotype control antibodies were added into the incubation system. The suspensions were further incubated in the dark for 20 min at 4°C. After washing with cold PBS, they were suspended in 0.5 ml of PBS containing 1% paraformaldehyde.

For analysis of thymocytes, BAL fluid cells, splenocytes, and lymphocytes of lung-associated lymph nodes, cells were suspended in RPMI 1640 medium containing 2% FCS (2 × 10^6 cells in 100 μl of medium) and fluorochrome-conjugated antibodies (10 μg/ml of each) specific for murine CD3ε (clone 145-2C11; BD Biosciences), CD4 (clone RM4-5; Invitrogen), IL-7Rα (clone 1D7; eBio-Science), anti-mouse/rat CD90.1 (Thy1.1, clone HS151), anti-mouse CD127 (IL-7Rα, clone A7R34), anti-mouse CCR9 (CD199, clone CW-1.2), anti-mouse CD106 (VCAM-1, clone 429), and anti-mouse Ly-6A/E (Sca-1, clone D7) for 20 min at 4°C, the cells were washed with cold PBS and then fixed in 0.5 ml of PBS containing 1% paraformaldehyde.

Analysis of cell phenotypes was performed on a FACSARia flow cytometer with FACS DIVA software (Becton Dickinson, San Jose, CA). Gating of each cell type was defined by the expression of CD3ε and CD4. Our results show that thymopoietic activity is enhanced following intrapulmonary inoculation of Pneumocystis in adult mice. This thymopoietic response is supported by enhanced marrow generation and delivery of thymopoietic precursor cells. Appropriate bone marrow support and thymic output of naïve CD4+ T cells constitute important components of the host immune defense against Pneumocystis infection.
a carboxy vinyl reaction. The reaction was terminated by heating at 95°C for 5 min. The PCR amplification was performed for 40 cycles, with each cycle at 94°C for 20 s and 60°C for 1 min. Data were converted to mRNA copy number using a standard curve of known copy number. Next generation sequencing (NGS) to quantify the presence of mRNA and to estimate the expression levels.

**Real-time PCR of msjTREC DNA expression.** Murine splenic T cell receptor gene expression was determined using the absolute quantitative real-time PCR protocol developed by Gregory D. Sempowski’s laboratory at Duke University (41). The results are expressed as copies of msjTREC copies per million (CPM) of DNA.

**Determination of plasma mediators.** Plasma levels of interleukin-3 (IL-3), IL-7, and IL-9 were measured using a Milliplex MAP Kit (Millipore, Billerica, MA). The plasma level of Fms-related tyrosine kinase-3 (Flk-3) ligand was measured by enzyme-linked immunosorbent assay (ELISA) using a murine Flk-3 ligand kit (R&D Systems, Minneapolis, MN).

**Preparation of Pneumocystis extracts.** Pneumocystis extracts were prepared using a previously reported protocol (30) with some modifications. Sterility was maintained throughout the procedure. Pneumocystis-infected mouse lungs were homogenized in ice-cold NPCK buffer (2.68 mM KCl, 1.47 mM KH₂PO₄, 51.1 mM Na₂HPO₄, 7.43 mM NaH₂PO₄, 62 mM NaCl, 0.05 mM CaCl₂, 0.05 mM MgCl₂) containing 100 mM dithiothreitol. The homogenate was centrifuged at 50 × g for 5 min at room temperature to remove cell debris. Pneumocystis cells in the supernatant were collected by centrifugation at 10,000 × g for 10 min at 4°C. Pneumocystis cell pellets were then resuspended in 5 mL of 0.85% NaCl-NPCK buffer and incubated at 37°C for 5 min to lyse erythrocytes. After samples were washed three times with cold NPCK buffer, isolated Pneumocystis organisms were quantified by enumeration of nuclei stained with Giemsa stain. The host DNA was removed by incubating Pneumocystis extracts in 10 mL of NPCK buffer containing 0.02% (2 U) of DNase I type IV (Invitrogen, CA) at 37°C for 10 min. After samples were washed three times with cold NPCK buffer, isolated Pneumocystis organisms were suspended in NPCK buffer and subjected to ultrasonication for 20 s at 40 W and a 70% duty cycle (Heat Systems-Ultrasonics Inc., Plainview, NY). The nuclei and cell ghosts were removed by centrifugation at 1,000 × g for 3 min. The supernatant representing the extract of 1 × 10⁶ Pneumocystis cysts/ml was aliquoted and stored at −80°C.

**In vitro culture of bone marrow cells.** Nucleated bone marrow cells isolated from naive mice were plated into a 24-well tissue culture plate with 1 × 10⁵ cells per well in a total volume of 0.5 mL of StemSpan serum-free medium (StemCell Technologies, Vancouver, BC, Canada). The cells were cultured at 37°C in an atmosphere of 5% CO₂ for 16 h with 0.4 mL Pneumocystis extracts or ligands to TLR-2 (zymosan), TLR-4 (Escherichia coli lipopolysaccharide [LPS]), or TLR-9 (ODN M362) (Invivogen, San Diego, CA) in the presence and absence of specific c-Jun kinase (JNK) inhibitor SP600125 (Sigma-Aldrich, St. Louis, MO).

**Table 1. Markers of cell types**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Surface marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>LKS</td>
<td>Lin c-Kit⁺ Sca-⁺</td>
</tr>
<tr>
<td>HSC</td>
<td>Lin c-Kit⁺ Sca-⁺ Thy-1⁻ CD34⁻</td>
</tr>
<tr>
<td>MPP</td>
<td>Lin c-Kit⁺ Sca⁻ Thy-1⁻ CD34⁺</td>
</tr>
<tr>
<td>CCR9⁺ MPP</td>
<td>Lin c-Kit⁺ Sca⁻ Thy-1⁺ CD34⁺</td>
</tr>
<tr>
<td>CLP</td>
<td>Lin c-Kit⁺ Sca⁻ Thy-1⁻ IL-7Rx⁺</td>
</tr>
<tr>
<td>CMP</td>
<td>Lin c-Kit⁺ Sca⁻ IL-7Rx⁻</td>
</tr>
<tr>
<td>ETP</td>
<td>(CD19 CD3 CD4 CD8 F4-80 Gr-1⁻)</td>
</tr>
<tr>
<td>DN1</td>
<td>CD4⁻ CD8⁻ CD25⁻</td>
</tr>
<tr>
<td>DN2</td>
<td>CD4⁻ CD8⁻ CD25⁺</td>
</tr>
<tr>
<td>DN3</td>
<td>CD4⁺ CD8⁻ CD25⁻</td>
</tr>
<tr>
<td>DN4</td>
<td>CD4⁺ CD8⁺ CD25⁻</td>
</tr>
<tr>
<td>T cells</td>
<td></td>
</tr>
<tr>
<td>CD4⁺ CD8⁻</td>
<td></td>
</tr>
<tr>
<td>Naïve CD4⁺</td>
<td>CD3⁻ CD4⁺ CD25⁻</td>
</tr>
<tr>
<td>Central memory CD4⁺</td>
<td>CD3⁻ CD4⁺ CD25⁺</td>
</tr>
<tr>
<td>Effect memory CD4⁺</td>
<td>CD3⁻ CD4⁺ CD25⁺</td>
</tr>
</tbody>
</table>

**Western blot analysis.** A subset of cultured bone marrow cells was lysed with a lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 2 mM sodium orthovanadate [Na₅VO₃], 1 mM phenylmethylsulfonyl fluoride [PMSF], 50 mM sodium fluoride [NaF], 5 mg/mL aprotinin, 5 mg/mL pepstatin, and 5 mg/mL leupeptin, pH 7.6) to prepare cell lysates. Western blot analysis of phospho-JNK levels in the cells was performed as described previously by our group (55). Protein concentrations of cell lysates were determined using a bicinchoninic (BCA) protein assay kit (Pierce, Rockford, IL). Western blots were performed using 12% SDS-PAGE ready gel (Bio-Rad Laboratories, Hercules, CA). Blots were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% fat-free milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% [vol/vol] Tween 20, 0.02% sodium azide, pH 7.4) and hybridized sequentially with primary antibody against mouse phospho-JNK monoclonal IgG1 antibody (diluted 1:1,000 with blocking buffer; Santa Cruz, CA) and horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:1,000 with blocking buffer; Cell Signaling, CA). Bound antibodies were detected by an ECL Plus Western blotting detection kit (GE Healthcare, NJ). The blot was stripped with Re-Blot Plus mild antibody stripping solution (Millipore, Temecula, CA) following protocols supplied by the manufacturer. The membrane was rebolted with rabbit anti-β-actin antibody (Cell Signaling Technology, Danvers, MA) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) to determine β-actin content in the sample loaded in each lane of the gel. Semi-quantification of the positive band in the images was performed using a Kodak Gel Logic 2,200 Imaging System. Data are presented as the net intensity ratio (NIR) of the phospho-JNK protein band versus the corresponding reference protein (β-actin) band.

**Statistical analysis.** Data were presented as means ± standard errors of the means. The sample size is indicated in the legend to each figure. Statistical analysis was performed using GraphPad Prism, version 5, software (GraphPad Software, La Jolla, CA). Two-way analysis of variance and one-way analysis of variance followed by a Student-Newman Keuls test were used for comparisons among multiple groups. An unpaired Student’s t test was used for comparison of two different groups. Differences were considered statistically significant at a P value of <0.05.

**RESULTS**

**Changes in pulmonary Pneumocystis burden.** To assess the role of the thymus in host defense against murine Pneumocystis infection, clearance of Pneumocystis from the lung was examined in mice without and with prior thymectomy. Healthy mice housed in filter-top cages have no detectable Pneumocystis rRNA in whole-lung tissue. As shown in Fig. 1, after Pneumocystis infection, sham-operated mice showed an elevated level of Pneumocystis rRNA in the lung at weeks 1 and 2 after Pneumocystis infection. Pneumocystis rRNA was then no longer detectable by week 3 post-Pneumocystis inoculation, which...
indicates effective pulmonary clearance of the pathogen in these animals. In contrast, mice with thymectomy showed a progressive increase in *Pneumocystis* burden in the lung during the first 3 weeks of the infection. The level of *Pneumocystis* rRNA in the lung then declined but remained elevated at week 4 after inoculation. These results indicate that thymus activity plays an important but not essential role in host defense against *Pneumocystis* infection in adult mice.

**Changes in BAL fluid CD4**$^+$**T cell subtypes.** In response to *Pneumocystis* inoculation, lymphocytes are recruited into lung tissue (47). Due to changes in thymic output of naïve CD4$^+$ T cells and peripheral clonal expansion of memory T cells following *Pneumocystis* infection, recruited CD4$^+$ T cell subpopulations in the alveolar space of animals without and with thymectomy may be altered accordingly. Therefore, we analyzed changes in total numbers of CD4$^+$ T cells as well as CD4$^+$ T cell subtypes in the alveolar space as reflected by cells recovered from bronchoalveolar lavage (BAL) fluid. As shown in Fig. 2, very few CD4$^+$ T cells were recovered from BAL fluid of uninfected mice. At weeks 3 and 5 post-*Pneumocystis* infection, the proportions of total CD4$^+$ T cells as well as naïve, central memory, and effector memory CD4$^+$ T cells in recovered BAL fluid cells were significantly increased in sham-operated mice. Thymectomy attenuated the increase in the numbers of total CD4$^+$ T cells, naïve CD4$^+$ T cells, and effector memory CD4$^+$ T cells recovered by BAL at weeks 3 and 5 post-*Pneumocystis* inoculation. The fraction of central memory CD4$^+$ T cells in BAL fluid at week 5 post-*Pneumocystis* infection was also lower in mice with thymectomy than in sham-operated animals.

**Changes in CD4$^+$ T cell subtypes in peripheral lymphoid tissues and blood.** In order to further understand the role of thymic activity in the host defense response, we analyzed changes in CD4$^+$ T cell populations as well as CD4$^+$ T cell subtypes in the lung-associated lymph nodes, spleen, and systemic circulation.

In cells isolated from lung-associated lymph nodes, the proportions of total CD4$^+$ T cells, naïve CD4$^+$ T cells, and central memory CD4$^+$ T cells were persistently reduced in the 6-week-period post-*Pneumocystis* inoculation in sham-operated animals (Fig. 3). Thymectomy caused an additional and significant decrease in the naïve CD4$^+$ T cell subtype as well as total CD4$^+$ T cells and central memory CD4$^+$ T cells in lung-associated lymph nodes compared to cell populations of the sham-operated controls. The effector memory CD4$^+$ T cell subtype in lung-associated lymph node cells of the sham-operated mice was initially reduced following *Pneumocystis* infection but recovered by week 6 postinfection. Mice with thymectomy showed the same level of effector memory CD4$^+$ T cells in lung-associated lymph nodes and a change in this subtype of cells following *Pneumocystis* infection similar to that observed in sham-operated mice.

In the spleens of sham-operated mice, the fractions of total CD4$^+$ T cells, naïve CD4$^+$ T cells, and central memory CD4$^+$ T cells were initially reduced following *Pneumocystis* infection. These reductions were recovered at week 6 post-*Pneumocystis* inoculation (Fig. 3). Mice with thymectomy showed significant decreases in the fraction of total CD4$^+$ T cells, naïve CD4$^+$ T cells, and central memory CD4$^+$ T cells in splenocytes compared to the sham-operated mice. These decreases were persistent throughout the 6-week-period following *Pneumocystis* infection. In sham-operated mice, the number of effector memory CD4$^+$ T cells in splenocytes was moderately reduced between 2 and 4 weeks of *Pneumocystis* infection but recovered at week 5 following the infection. In mice with thymectomy, the proportion of effector memory CD4$^+$ T cells in splenocytes was maintained at a level similar to that of the sham-operated mice. *Pneumocystis* infection was not associated with a reduction of effector memory CD4$^+$ T cells in splenocytes of thymectomized mice.

The numbers of total CD4$^+$ T cells, naïve CD4$^+$ T cells, and
central memory CD4+ T cells remained stable in the systemic circulation of sham-operated mice following Pneumocystis infection (Table 2). The level of effector memory CD4+ T cells in the circulation was also stable during the initial 5 weeks after Pneumocystis inoculation in sham-operated mice. At week 6 post-Pneumocystis inoculation, the circulating level of effector memory CD4+ T cells in sham-operated mice was significantly elevated, suggesting that the influx of these effector cells into the bloodstream exceeds their extravasation at this stage. In mice with thymectomy, the number of total CD4+ T cells in the systemic circulation was markedly reduced compared to the level in the sham-operated animals. This reduction of total CD4+ cells in the circulation was persistent throughout the 6-week-period post-Pneumocystis inoculation. The reduction of total CD4+ T cells in the circulation of thymectomized mice primarily resulted from the lack of naïve and central memory CD4+ T cells in the bloodstream (Table 2). In the meantime, the level of effector memory CD4+ T cells in the circulation was maintained in thymectomized mice compared to the level in the sham-operated animals (Table 2).

Changes in ETP and DN cell populations in the thymus. In order to understand thymic functional activity following Pneu-
### TABLE 2. Circulating lymphocytes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sham operation</th>
<th>Thymectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total CD4&lt;sup&gt;+&lt;/sup&gt; cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>166,840±12,986</td>
<td>142,486</td>
</tr>
<tr>
<td>Week 2 p.i.</td>
<td>174,825±10,702</td>
<td>122,379</td>
</tr>
<tr>
<td>Week 4 p.i.</td>
<td>167,127±10,493</td>
<td>154,891</td>
</tr>
<tr>
<td>Week 5 p.i.</td>
<td>189,010±14,391</td>
<td>166,637</td>
</tr>
<tr>
<td><strong>Naïve CD4&lt;sup&gt;+&lt;/sup&gt; cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>84,880±5,997</td>
<td>58,979</td>
</tr>
<tr>
<td>Week 2 p.i.</td>
<td>102,007±7,641</td>
<td>79,967</td>
</tr>
<tr>
<td>Week 4 p.i.</td>
<td>107,841±7,341</td>
<td>91,447</td>
</tr>
<tr>
<td>Week 5 p.i.</td>
<td>123,912±9,542</td>
<td>108,896</td>
</tr>
<tr>
<td><strong>Central memory CD4&lt;sup&gt;+&lt;/sup&gt; cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>31,162±2,408</td>
<td>19,382</td>
</tr>
<tr>
<td>Week 2 p.i.</td>
<td>39,327±2,764</td>
<td>25,572</td>
</tr>
<tr>
<td>Week 4 p.i.</td>
<td>32,891±2,288</td>
<td>22,430</td>
</tr>
<tr>
<td>Week 5 p.i.</td>
<td>34,491±2,568</td>
<td>26,120</td>
</tr>
<tr>
<td><strong>Effector memory CD4&lt;sup&gt;+&lt;/sup&gt; cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10,009±1,3041</td>
<td>7,071</td>
</tr>
<tr>
<td>Week 2 p.i.</td>
<td>14,520±1,5413</td>
<td>9,294</td>
</tr>
<tr>
<td>Week 4 p.i.</td>
<td>12,520±1,8214</td>
<td>8,762</td>
</tr>
<tr>
<td>Week 5 p.i.</td>
<td>14,013±1,9437</td>
<td>10,248</td>
</tr>
</tbody>
</table>

* Mice with or without thymectomy were challenged with intratracheal *Pneumocystis* (2 × 10<sup>10</sup> cysts/mouse). A blood sample was collected each week after the *Pneumocystis* infection. CD4<sup>+</sup> cell types were determined by flow cytometry. * and †, P < 0.05 versus the corresponding control group (uninfected mice); ‡, P < 0.05 versus sham-operated mice at the same time point.

**Changes in msJTREC level in CD4<sup>+</sup> splenocytes.** Measurement of msJTRECs in peripheral T cells has been used to study thymic output (41). The level of msJTRECs in CD4<sup>+</sup> splenocytes of sham-operated mice was decreased at week 1 post-*Pneumocystis* infection compared with the control level in uninfected mice (Fig. 4C). This initial decrease in msJTREC level was gradually attenuated between weeks 2 and 4 postinfection. At week 5 post-*Pneumocystis* inoculation, the msJTREC level in CD4<sup>+</sup> splenocytes was markedly increased in sham-operated mice, indicating enhanced homing of newly produced naïve CD4<sup>+</sup> lymphocytes to the spleen at this stage. In thymectomized animals, as expected, the level of msJTRECs in CD4<sup>+</sup> splenocytes was reduced compared with sham-operated mice, indicating impaired thymic output.

**FIG. 4.** The number of earliest thymic progenitors (ETPs) (A) and double negative (DN) cells (B) in the thymus * and †, P < 0.05 versus the corresponding cell type in the control group (uninfected mice) (n = 5). (C) The level of msJTREC DNA in CD4<sup>+</sup> splenocytes. * P < 0.05 versus the corresponding control group (uninfected mice); †, P < 0.05 versus Sham-operated mice at the same time point (n = 5). WBC, white blood cells; p.i., postinfection.
were not increased (or even reduced) following exposure to *Pneumocystis* inoculation.

**Plasma levels of mediators.** Plasma concentrations of IL-3 and IL-7 were below detectable limits. Plasma levels of IL-9 and Flt-3 ligand were similar in sham-operated and thymectomized mice and were not altered throughout the 6-week-period post-*Pneumocystis* infection in both groups (data not shown).

**Changes in hematopoietic precursor populations in the bone marrow and blood.** The enhancement of thymic output requires bone marrow support to provide thymopoietic progenitor cells. In order to understand the role of bone marrow during this response, we examined the alteration of hematopoietic precursor cell production in the bone marrow following *Pneumocystis* infection. As shown in Fig. 5A, the numbers of marrow LKS cells and HSCs increased following *Pneumocystis* infection in mice without and with thymectomy. These increases in marrow LKS and HSC populations reached a peak level at weeks 2 and 3, respectively, post-*Pneumocystis* inoculation. Similarly, the number of marrow CCR9+ MPPs (precursors of ETPs) was significantly increased following *Pneumocystis* infection in both groups. The increase in marrow CCR9+ MPPs reached a peak value at week 4 in sham-operated animals and at week 5 in thymectomized mice. The bone marrow pool of CLPs was slightly reduced in sham-operated mice following *Pneumocystis* infection. In mice with thymectomy, the marrow CLP population was moderately increased at weeks 5 and 6 post-*Pneumocystis* infection. Bone marrow levels of Lin- c-Kit+ Sca-1- cells and CMPs were moderately reduced in both the sham operation and thymectomy groups.

In association with the increase in the marrow pool of LKS cells and CCR9+ MPPs, the number of these precursors was significantly increased in the systemic circulation, with the peak values at weeks 1 and 3, respectively, post-*Pneumocystis* infection in both groups (Fig. 5B). These results indicate that bone marrow plays an important role in supporting the thymopoietic response to *Pneumocystis* infection in mice without and with thymectomy. These results indicate that bone marrow precursor cell response to *Pneumocystis* infection.

**Changes in hematopoietic precursor cell populations in bone marrow cells following *in vitro* culture with *Pneumocystis* extracts and TLR ligands.** Bone marrow hematopoietic precursor cells including primitive hematopoietic stem cells express TLR receptors and respond to TLR ligand stimulation (14, 32, 52). In order to understand possible signaling mechanisms underlying the bone marrow precursor cell response to *Pneumocystis* infection, we performed *in vitro* experiments in which bone marrow cells from naive mice were cultured with *Pneumocystis* extracts, the TLR-2 ligand zymosan, TLR-4 ligand LPS, and TLR-9 ligand ODN M362. As shown in Fig. 6, the numbers of LKS cells, HSCs, MPPs, CCR9+ MPPs, and CLPs were significantly increased in the cultured bone marrow cells following 16 h of exposure to *Pneumocystis* extracts and to ligands of TLR-2, TLR-4, and TLR-9. In contrast, the numbers of Lin- c-Kit+ Sca-1- cells and CMPs in the culture system were not increased (or even reduced) following exposure to these stimulants. The dose-response test showed that zymosan and ODN M362 each caused a dose-dependent increase in LKS and CCR9+ MPP cell types in cultured bone marrow cells (Fig. 7). These data demonstrate that TLR signaling may mediate the bone marrow precursor cell response to *Pneumocystis* infection.

**JNK activation and the increase in CCR9+ MPPs in bone marrow cells.** CCR9+ MPPs are a subtype of LKS cells. Our current results showed that the numbers of LKS cells and CCR9+ MPPs were increased in the bone marrow of mice with *Pneumocystis* infection and in *in vitro* cultured marrow cells following exposure to *Pneumocystis* extracts and TLR ligands. In previous studies, we have observed that phenotypic conversion of Lin- c-Kit+ Sca-1- cells by reexpression of Sca-1 is a major mechanism underlying the rapid expansion of the marrow LKS cell pool in response to infectious stimuli (22). The promoter region of the Sca-1 gene contains multiple binding sites for AP1. c-Jun is the most potent transcriptional activator in the AP1 family (31). Ligand engagement of TLR-2, TLR-4, or TLR-9 activates JNK, leading to enhancement of c-Jun transcriptional activity by phosphorylation of its N-terminal activation domain (6, 14, 28, 31). Therefore, we examined the role of JNK signaling in *Pneumocystis*-mediated increases in LKS and CCR9+ MPP cells. As shown in Fig. 8A, exposure of cultured bone marrow cells to *Pneumocystis* extracts, zymosan, and ODN M362 for 8 h significantly increased JNK phosphorylation in these cells. Specific JNK inhibitor SP600125 profoundly inhibited the increase in the number of LKS cells as well as LKS subtypes including CCR9+ MPPs in cultured bone marrow cells following exposure to *Pneumocystis* extracts, zymosan, and ODN M362 (Fig. 8B).

**DISCUSSION**

Thymic output of naïve T cells declines after puberty in both humans and mice (40, 48, 49). However, a thymic reserve may persist into adulthood (36). In circumstances of accelerated T lymphocyte turnover, such as those caused by chemotherapy or HIV disease, thymic function can be altered (5, 29, 35). Clinical investigations have shown that HIV-1-seropositive adults with abundant thymic tissue exhibit both a higher percentage of naïve CD4+ T cells and a higher total CD4+ T cell count in the circulation (29). Comparing HIV-2 and HIV-1 infections has revealed that HIV-2-infected patients demonstrate enhanced thymic function compared to age-matched healthy individuals (12). This activation of thymopoiesis is implicated in the relative maintenance of CD4+ T cell counts during HIV-2 disease. Administration of growth hormone to HIV-1-infected adults or interleukin-7 to simian immunodeficiency virus (SIV)-infected rhesus macaques under antiretroviral therapy increases the thymic output of naïve T cells in these hosts (4, 15, 33). It is well known that T cell replenishment in the body relies on proliferation of existing T cells in the peripheral tissues and *de novo* naïve T cell production by the thymus. Of these two mechanisms, thymopoiesis is more efficient in restoring or replenishing the peripheral T cell profiles than clonal expansion of existing peripheral T cells since peripheral expansion of T cells restricts the T cell repertoire to preexisting memory T cells, which leads to inefficiency in responding to new antigens (40).
CD4+ T cells are critical for the host defense against *Pneumocystis* infection (43, 44, 45, 46). Our previous studies have shown that during *Pneumocystis* infection, the utilization of peripheral CD4+ T cells increases partially due to increased destruction of these cells through enhanced apoptosis (47). This acceleration of CD4+ T cell turnover requires increased generation of CD4+ T cells by the host. At this time, mechanisms underlying the replenishment of CD4+ T cells remain...
incompletely elucidated with regard to host defense against *Pneumocystis* infection. No information is available regarding the significance of thymopoiesis in the host response to *Pneumocystis* infection.

The results of our current investigation show that blocking thymopoiesis in adult C57BL/6 mice by thymectomy delayed clearance of *Pneumocystis* from the lung following intratracheal inoculation. These data indicate that thymic function constitutes an important component of the host defense against *Pneumocystis* infection. It has been known that HIV infection and corticosteroid therapy both cause thymic toxicity. Thymic suppression in immunocompromised patients, particularly those with AIDS or receiving long-term corticosteroid therapy, may further impede the host defense against *Pneumocystis* infection. Our mice with thymectomy, however, were eventually able to eradicate *Pneumocystis* infection in the lung, which suggests that peripheral clone expansion of existing T cells in normal hosts can still sustain the host defense against *Pneumocystis* infection although in an inefficient manner. Our current study is in agreement with clinical observations in

FIG. 6. Changes in bone marrow cell types following culture with different stimuli. *, $P < 0.05$ versus the corresponding control group ($n = 5$ samples/group). PC, *Pneumocystis*; ZYM, zymosan.

FIG. 7. Changes in the number of CCR9$^+$ MPPs and LKS cells in bone marrow cells following culture with different doses of TLR-2 and TLR-9 ligands. *, $P < 0.05$ versus the corresponding control group ($n = 5$ samples/group).
which no increase in incidence of Pneumocystis infection has been reported in individuals previously thymectomized for myasthenia gravis (17).

In our murine model, immature thymocyte proliferation in the thymus and thymic output of naïve CD4+ T cells were significantly enhanced following Pneumocystis infection. The numbers of ETPs and DN thymocytes in the thymus of sham-operated mice were significantly increased after intratracheal inoculation of Pneumocystis. Similarly, the level of msjTRECs in CD4+ splenocytes was significantly elevated in sham-operated mice at 5 weeks post-Pneumocystis challenge. These results support an activation of thymopoietic activity in adult mice in response to Pneumocystis infection. Interestingly, the level of msjTRECs in CD4+ splenocytes was not increased during the initial 4 weeks post-Pneumocystis inoculation. A possible explanation is that newly released naïve CD4+ T cells from the thymus may be preferentially recruited into the infected tissue site, i.e., the alveolar space. Our results demonstrated that the numbers of naïve CD4+ T cells and total CD4+ T cells recruited into lung tissue were markedly increased following Pneumocystis infection. During this period of time, peripheral lymphoid tissues and spleen shows a decrease in the numbers of naïve and central memory CD4+ T cells but preservation of effector memory T cells following thymectomy. These alterations of CD4+ T cell subtypes support the important role of the thymus in supporting naïve and central memory cells within lymphoid tissue following a Pneumocystis challenge. These observations also provide supporting evidence for the possible dynamic homing of these cells to an infected tissue site instead of peripheral lymphoid tissues.

The active thymopoietic response to Pneumocystis infection requires thymopoietic progenitors supplied by the bone marrow. Previous studies have shown that marrow CCR9+ MPPs represent a major precursor population of ETPs (24). Our current results show that the marrow pool of CCR9+ MPPs was expanded following Pneumocystis infection. This increase in the number of marrow CCR9+ MPPs was accompanied by the expansion of the upstream HSC pool as well as the entire LKS cell population in the bone marrow. With the increase in marrow CCR9+ MPPs, bone marrow release of these precursors into the systemic circulation was enhanced. As a consequen-
quence, the number of CCR9+ MPPs in the peripheral blood was significantly increased. In contrast to the increase in generation of thymopoietic precursors, the numbers of Lin− c-Kit+ Sca-1− cells and MPPs in the bone marrow were reduced, which suggests the polarization of marrow lineage support toward T cell production following Pneumocystis infection. These data identify the bone marrow as a key component of the thymopoietic response to Pneumocystis infection. In our in vivo experiments, the levels of Lin− c-Kit+ Sca-1− cells and MPPs were elevated in the bloodstream during the early stage of Pneumocystis infection, which suggests an activated mobilization of these cells into the systemic circulation. The significance of this temporarily enhanced mobilization of Lin− c-Kit+ Sca-1− cells and MPPs into the circulation following Pneumocystis infection remains to be elucidated. One possible speculation for release of these precursors from the bone marrow at the early stage of Pneumocystis infection is that it may facilitate appropriate niche space for rapid expansion of the thymopoietic precursor cell population in the bone marrow.

Previous studies have demonstrated that bone marrow hematopoietic precursor cells express TLRs, enabling these cells to respond to TLR ligand stimulation (14, 32, 52). Pneumocystis is a fungal pathogen. Its cell components including glycoprotein and (1–3)-β-d-glucan can be recognized by TLRs, such as TLR-2 and TLR-4 (9, 50, 52, 54). Prior investigations have not addressed TLR-9 in Pneumocystis, although this pattern receptor is important in recognition of other fungal pathogens (39). Clinical investigations and experimental studies have repeatedly shown that circulating levels of Pneumocystis-derived cell wall components are increased following pulmonary infection (39). Clinical investigations and experimental studies have repeatedly shown that engagement of TLR-2, TLR-4, or TLR-9 with their ligands activates JNK, leading to enhancement of c-Jun transcriptional activity by phosphorylation of its N-terminal activation domain (14). Therefore, we determined if the JNK signal pathway was involved in mediating the hematopoietic precursor cell response to Pneumocystis infection. The results of our experiments showed that Pneumocystis extracts, zymosan, and ODN M362 each activated JNK in cultured bone marrow cells. Addition of the specific JNK inhibitor SP600125 to the culture system profoundly inhibited the increase in LKS cells and CCR9+ MPPs in cultured bone marrow cells following exposure to Pneumocystis extracts, zymosan, or ODN M362. These findings demonstrate that the TLR-JNK-AP1 signaling cascade may play a vital role in mediating the enhancement of marrow T cell lineage support during the host defense response to Pneumocystis infection.

In our in vivo experiments, the plasma levels of IL-3, IL-7, IL-9, IFN-γ, and Flt-3 ligand were not altered. Previous investigations have shown that these mediators may modulate thymopoietic activity. However, the role of these mediators (if any) in our model of Pneumocystis infection remains undetermined.

In summary, thymopoietic activity is enhanced following Pneumocystis pneumonia in adult C57BL/6 mice along with increases in thymopoietic precursor cells in the bone marrow. Ready access of Pneumocystis-derived TLR ligands into the systemic circulation may stimulate bone marrow primitive hematopoietic precursor cell reprogramming to enhance T lymphocyte lineage commitment through activation of the JNK-AP1 pathway.

ACKNOWLEDGMENTS

We thank Joseph S. Sblosky, Connie P. Porretta, Jane A. Schexnayder, and Amy B. Weinberg for their technical assistance. This work was supported by National Institutes of Health grants HL076100, AA017494, AA019676, and AG25150.

REFERENCES

2. Akashi, K. 2007. Cartography of hematopoietic stem cell commitment de-


