Memory CD4$^+$ T Cells Are Required for Optimal NK Cell Effector Functions against the Opportunistic Fungal Pathogen Pneumocystis murina

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Memory CD4⁺ T Cells Are Required for Optimal NK Cell Effector Functions against the Opportunistic Fungal Pathogen Pneumocystis murina

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Little is known about the role of NK cells or their interplay with other immune cells during opportunistic infections. Using our murine model of Pneumocystis pneumonia, we found that loss of NK cells during immunosuppression results in substantial Pneumocystis lung burden. During early infection of C57Bl/6 CD4⁺ T cell–depleted mice, there were significantly fewer NK cells in the lung tissue compared with CD4⁺ T cell–intact animals, and the NK cells present demonstrated decreased upregulation of the activation marker NKP46 and production of the effector cytokine, IFN-γ. Furthermore, coinoculation studies revealed a significant increase in fungal killing when NK cells were combined with CD4⁺ T cells compared with either cell alone, which was coincident with a significant increase in perforin production by NK cells. Finally, we found through adoptive transfer that memory CD4⁺ T cells are required for significant NK cell upregulation of the activation marker NK group 2D and production of IFN-γ, granzyme B, and perforin during Pneumocystis infection. To the best of our knowledge, this study is the first to demonstrate a role for NK cells in immunity to Pneumocystis pneumonia, as well as to establish a functional relationship between CD4⁺ T cells and NK cells in the host response to an opportunistic fungal pathogen. The Journal of Immunology, 2013, 190: 285–295.

Natural killer cells are lymphocytes of the innate immune system and are important effectors in host defense against pathogens and tumors (1). NK cells effect their activity through granule-mediated killing and effector cytokine production (2). NK cells have also been implicated in protection against fungal and parasitic infections through IFN-γ release (3–5) and through direct microbicidal activity (6–8). Being innate lymphocytes, NK cells were thought to effect their activity against pathogens by controlling early infection until an adaptive immune response was generated. However, that long-standing theory was recently challenged by studies demonstrating NK cells’ ability to directly regulate adaptive immune responses through their interaction with dendritic cells (9) and, most recently, CD4⁺ T cells (10–13). CD4⁺ T cells play a central role in the immune system by mediating effector functions of other immune cells, and Horowitz et al. (11) recently described direct cross-talk between NK cells and CD4⁺ T cells during Plasmodium falciparum infection. The investigators found that IL-2 produced by CD4⁺ T cells was essential for activation of NK cells, resulting in significant IFN-γ production. Similarly, it was shown that priming of the CD4⁺ T cells was necessary for NK cell activation in defense of Leishmania major infection (14). These studies indicate that NK cells are not just early effector cells, but they continue to work in concert with the adaptive immune response. This was supported most recently by Shimizu et al. (15), who demonstrated that an effector memory phenotype for CD4⁺ T cells was necessary to sustain prolonged antitumor reactivity of NK cells. Together, these findings indicate that, in an immunocompetent host, CD4⁺ T cells can use the effector functions of NK cells to protect the host against infection; they further hint to the possibility that during immunocompromised states in which CD4⁺ T cells are depressed, NK cells may have reduced function. Thus, this led us to our hypothesis that cross-talk between CD4⁺ T cells and NK cells is an important component of immune defense against opportunistic fungal infections, and we used our murine model of Pneumocystis pneumonia (PCP) to test this.

Pneumonia due to the opportunistic human fungal pathogen Pneumocystis jirovecii is an AIDS-defining illness, and there is a direct inverse relationship between CD4⁺ T cell count in the peripheral blood and risk for infection (16, 17). Animal models of immunodeficiency clearly demonstrated that the loss of CD4⁺ T cells renders mammals susceptible to Pneumocystis lung infection (18); however, the mechanisms of CD4⁺ T cell protection are not fully understood (16, 19). A role for NK cells in host resistance to Pneumocystis was suggested by the occurrence of PCP in patients with HIV infection or other immunodeficiencies with low numbers of NK cells or impaired NK cell function (20). Pneumocystis organisms are species specific, and in experimental infection with the type that infects laboratory mice, Pneumocystis murina, NK cell recruitment to the lung was observed in healthy animals (21). In vitro, it was shown that, in the absence of adaptive immunity, NK cells can be activated by macrophages in response to P. murina organisms (9). Additionally, our laboratory demonstrated previously that exogenous IFN-γ treatment resulted in increased recruitment of NK cells into the lungs of CD4-depleted,
**P. murina**-infected mice, resulting in enhanced clearance of infection (22). However, there has been very little research focused on how NK cells interact with CD4+ T cells in host defense against fungal infections. In these experiments, we sought to elucidate the function of NK cells against *Pneumocystis*. Our data demonstrate that NK cells are an important element of defense against this opportunistic fungal pathogen. Further, we reveal a functional interaction between NK and CD4+ T cell lymphocytes in which optimal NK function requires Ag-specific memory CD4+ T cells.

**Materials and Methods**

**Mice**

Female 6–8 wk-old C57BL/6 wild-type (WT) mice were obtained from National Cancer Institute/Charles Rivers Breeding Laboratories (Wilmington, MA). Male 6–8 wk-old CB17-Prkdcsnd/szJ (SCID) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and B10.B6-Rag2IL2rg

**P. murina inoculation**

**P. murina** organisms for inoculation were isolated from lung homogenates from chronically infected SCID mice, as previously described (18). The animals were kept in the animal care facility at the Louisiana State University Health Sciences Center (LSUHSC) for ≥2 d before any treatment was begun. All animal manipulations were performed under a laminar flow hood. These experiments were approved by the Institutional Animal Care and Use Committee for LSUHSC.

**P. murina chronic-infection studies**

For burden analysis of severely immunocompromised animals, SCID, RAG2−/−, or RAG2−/−γ−/− mice were infected as described above. After chronic infection was established, animals were euthanized by i.p. anesthesia with ketamine/xylazine (200 mg per kg/10 mg per kg), followed by exsanguination via dorsal vein transection. Lung tissue was aseptically removed, and *P. murina* copy number was measured as described.

**For depletions studies, WT mice were depleted of CD4+ T cells by i.p. injection of 0.3 mg anti-CD4+ MAb (hybridoma GK1.5; Tacomic) in 0.05 ml PBS 3 d prior to infection. Depletion was maintained by i.p. injection every 6 d. This treatment protocol results in >97% sustained depletion of CD4+ lymphocytes from blood and lymphoid tissue for up to 14 wk (18). For NK cell depletions, mice were infected i.p. with 0.05 mg of anti-Asialo GM1 (Cedarlane, Burlington, NC) in 0.05 ml of PBS every 4 d for 2 wk prior to infection, and depletion of NK cells was maintained thereafter with i.p. injection of 0.02 mg in 0.02 ml every 4 d, as per the manufacturer’s recommendations. This treatment protocol resulted in >87% reduction in NK cell numbers in lung tissue, as measured by flow cytometry. Chronic infection was allowed to develop prior to sacrifice and fungal burden measurement. For doubly depleted animals, the Ab regimen was the same as for single depletion, which resulted in an 80% decrease in NK cells and an 85% decrease in CD4+ T cells, as measured by flow cytometry.

**Flow cytometric analysis and intracellular staining of lymphocytes from lung tissue**

Left lung tissue of each animal was minced; suspended in 10 ml PBS 3 d prior to infection. Depletion was maintained by i.p. injection of 0.3 mg anti-CD4+ MAb (hybridoma GK1.5; Tacomic) in 0.05 ml PBS 3 d prior to infection. Depletion was maintained by i.p. injection every 6 d. This treatment protocol results in >97% sustained depletion of CD4+ lymphocytes from blood and lymphoid tissue for up to 14 wk (18). For NK cell depletions, mice were infected i.p. with 0.05 mg of anti-Asialo GM1 (Cedarlane, Burlington, NC) in 0.05 ml of PBS every 4 d for 2 wk prior to infection, and depletion of NK cells was maintained thereafter with i.p. injection of 0.02 mg in 0.02 ml every 4 d, as per the manufacturer’s recommendations. This treatment protocol resulted in >87% reduction in NK cell numbers in lung tissue, as measured by flow cytometry. Chronic infection was allowed to develop prior to sacrifice and fungal burden measurement. For doubly depleted animals, the Ab regimen was the same as for single depletion, which resulted in an 80% decrease in NK cells and an 85% decrease in CD4+ T cells, as measured by flow cytometry.

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NK cells or CD4+ T cells were purified from naive splenocytes via negative bead selection using an NK cell isolation kit or a CD4+ T cell isolation kit, as per the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Purity of NK and CD4+ T cells was found to be 87.8 ± 1.5%.

**Preparation of lung homogenates and multiplex analysis of cytokine levels**

Left lung tissue was weighed, placed in 1 ml PBS, and homogenized using an Omni TH homogenizer (Omni International, Warrenton, VA). Debris was removed from homogenates by centrifugation at 10,000 × g for 10 min, and samples were stored at −80˚C for future analysis. Concentrations of the analytes IFN-γ, IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-10, IL-15, IL-17, IP-10, LIF, MCP-1, MIP-1α, MIP-1β, MG, RANTES, TNF-α, and IL-12p40 were determined using the Milliplex mouse cytokine/chemokine 96-well plate assay, as per the manufacturer’s instructions (Millipore, Billerica, MA). Plates were read using a Luminex 100/200 (Austin, TX), and analysis was performed using Bioplex Manager 3.0 software (Bio-Rad, Hercules, CA).

**RNA isolation and real-time RT-PCR for *P. murina* rRNA quantification**

Total RNA was isolated from the right lung tissue of infected mice by the TRizol method (Invitrogen, Grand Island, NY) reverse transcribed, and real-time PCR for *P. murina* quantification was assayed as previously described (24).

**In vitro microbicidal assays**

Naïve animals were euthanized by i.p. anesthesia with ketamine/xylazine (200 mg per kg/10 mg per kg), followed by exsanguination via dorsal vein transection. Spleens were aseptically removed, and splenocytes were suspended in culture medium by mechanical disruption of tissue through a 40-μm nylon cell strainer (BD Biosciences). RBCs were lysed using RBC lysis buffer, as per the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO).

NK cells or CD4+ T cells were purified from naive splenocytes via negative bead selection using an NK cell isolation kit or a CD4+ T cell isolation kit, as per the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Purity of NK and CD4+ T cells was found to be 87.8 ± 1.2% and 90.5 ± 3.1%, respectively, by flow cytometry (Supplemental Fig. 1). For NK microbicidal assays, NK cells in complete medium (RPMI 1640 with 10% FBS and 1% penicillin-streptomycin) were seeded in 96-well plates at 104, 105, or 106 cells/well, so that the number of each lymphocyte subset remained constant (i.e., 105 NK cells versus 104 CD4+ T cells versus 105 NK cells combined with 104 CD4+ T cells). Pneumocystis organisms were added at a 1:E:T ratio of 10:1. Contents of wells were gently mixed at 1000 rpm for 30 s on a plate mixer and then incubated for 24 h at 37˚C, 5% CO2. Total RNA was extracted from final *P. murina* inoculum using a Total RNA mini kit (Bio-Rad). Viable *P. murina* organisms were assessed using real time RT-PCR, as described. Data are presented as *P. murina* rRNA copy number. For NK cytotoxicity assay, lungs were homogenized by a Total RNA mini kit (Bio-Rad). Viable *P. murina* organisms were assessed using real time RT-PCR, as described. Data are presented as *P. murina* rRNA copy number. For NK cytotoxicity assay, lungs were homogenized by a Total RNA mini kit (Bio-Rad). Viable *P. murina* organisms were assessed using real time RT-PCR, as described.
Adoptive-transfer studies

WT C57Bl6 mice were infected with *P. murina*, as described above, and allowed to resolve infection and develop a memory immune response. After 6 wk, animals were sacrificed, and CD4\(^+\) T cells were isolated from splenocytes from infected and naïve mice, as described above. One million isolated CD4\(^+\) T cells from naïve or infected mice or RPMI 1640 medium were then gently injected into the jugular vein of RAG2\(^{-/-}\) mice sedated with ketamine/xylazine, and transferred cells were allowed to circulate for 5 d. Mice were then infected with 2 \( \times 10^5 \) *P. murina* cysts intratracheally (i.t.). Mice were sacrificed 5 d postinfection (p.i.), and lung tissue was removed for flow cytometry measurement, as previously described.

Statistical analysis

Analysis of data was performed using GraphPad Prism statistical software (La Jolla, CA). Data are reported as mean ± SEM, as noted. Comparisons between groups that were normally distributed were made using the Student *t* test, and comparisons between multiple groups were made with analyses of variance with appropriate follow-up testing. Significant differences were accepted at *p* values < 0.05.

Results

Loss of NK cells contributes to *Pneumocystis* burden

*Pneumocystis* organisms cannot be cultured; therefore, propagation for study is routinely performed by infection of severely immunosuppressed mouse strains. We previously noted that experimental infection of mouse strains lacking T, B, and NK lymphocytes results in significantly higher organism burden than infection of mice lacking only T and B lymphocytes. To confirm this observation, C57BL/6 SCID, and RAG2\(^{-/-}\) mice which lack T and B lymphocytes, or RAG2\(^{-/-}\)-γc\(^{-/-}\) mice, which lack all lymphocytes including NK cells, were inoculated i.t. with 2 \( \times 10^5 \) *P. murina* cysts, and infection was allowed to develop. At 6 wk p.i., animals displayed morbidity and were sacrificed to assess fungal burden in lung tissue by real-time RT-PCR. As expected, RAG2\(^{-/-}\)- and SCID mice had substantial *Pneumocystis* burden in lung tissue (2.4 \( \times 10^{10} \) ± 8.5 \( \times 10^9 \) and 4.7 \( \times 10^9 \) ± 3.6 \( \times 10^8 \), respectively) (Fig. 1A), whereas RAG2\(^{-/-}\)-γc\(^{-/-}\) knockout mice displayed a 3-log higher burden (1.2 \( \times 10^{13} \) ± 5.7 \( \times 10^{12} \)) compared with both RAG2\(^{-/-}\)- and SCID mice, suggesting that NK cells control overwhelming organism burden during severe immunosuppression.

Our laboratory previously demonstrated an initial increase in NK cells in the lungs of CD4\(^+\)-depleted mice during *Pneumocystis* infection, followed by a sharp decrease after the first week (22). To further study the relationship between NK cells and CD4\(^+\) T cells in our model of PCP, we inoculated 2 \( \times 10^5 \) *P. murina* cysts into the lungs of WT C57BL/6 mice depleted of CD4\(^+\) T cells using GK1.5, depleted of NK cells using anti-Asialo GM1, or depleted of both CD4\(^+\) T cells and NK cells. Mice were sacrificed at 4 wk p.i., and fungal load in lung tissue was assayed by real-time RT-PCR. As expected, mice depleted of CD4\(^+\) T cells showed a considerable increase in fungal burden compared with WT control infected mice (Fig. 1B). Surprisingly, however, NK-depleted mice also had substantial *P. murina* burden, similar to the CD4\(^+\)-depleted animals. However, mice depleted of both NK and CD4\(^+\) T cells displayed a 3-log higher fungal burden in the lung compared with WT mice. Together, these results indicate that the NK lymphocyte subset is involved in host immunity to *Pneumocystis* by controlling overwhelming fungal burden during PCP, and they suggest a cooperative interaction between NK and CD4\(^+\) T lymphocytes.

**NK cell microbicidal activity and perforin production in vitro are enhanced by the addition of CD4\(^+\) T cells**

Our in vivo data suggest that NK cells have direct microbicidal activity against *Pneumocystis* organisms. To measure this in a controlled environment, NK cells were isolated from naive splenocytes and serially diluted in a 96-well cell culture plate. A fixed number of *P. murina* organisms was added to wells so that the resultant effector (NK)/target (*Pneumocystis*) ratios were 0:1:1, 1:1, and 10:1; incubation took place at 37°C, and *P. murina* organisms alone were used as a control. After 24 h, fungal burden was assessed by quantitative PCR. As shown in Fig. 2A, NK cells are microbicidal to *Pneumocystis* at increasing concentrations, with a significant decrease in fungal viability observed between E:T ratios of 1:1 and 10:1.

Recent studies demonstrate that optimal microbicidal activity of NK cells requires cross-talk with other effector cells (14, 25, 26), and our in vivo results suggest an interaction between NK and CD4\(^+\) T cells. To determine whether CD4\(^+\) T cells have a direct effect on NK microbicidal activity, an in vitro coincubation study was performed. Primary NK cells and CD4\(^+\) T cells were isolated from naïve splenocytes (Supplemental Fig. 1A, 1B, respectively), and NK microbicidal activity against *Pneumocystis* was assayed by real-time RT-PCR of *Pneumocystis* rRNA copy number (n = 4 mice/group). *p < 0.05, **p < 0.01, unpaired Student *t* test.

![FIGURE 1. Pneumocystis lung burden in the absence of NK cells. (A) SCID, RAG2\(^{-/-}\), and RAG2\(^{-/-}\)-γc\(^{-/-}\) mice on a BL/6 background were infected i.t. with 2 \( \times 10^5 \) cysts of *P. murina* (PC). At 45 d p.i., mice were sacrificed, and fungal burden in lung tissue was assessed by real-time PCR. This experiment was repeated twice with similar results (n = 5 mice/group). (B) WT C57BL/6 mice were depleted of CD4\(^+\) T cells, NK cells, or both CD4\(^+\) T cells and NK cells and infected with *Pneumocystis*, as above. Mice were sacrificed at 30 d p.i., and fungal load in lung tissue was assayed by real-time RT-PCR of *Pneumocystis* rRNA copy number (n = 4 mice/group). *p < 0.05, **p < 0.01, unpaired Student *t* test.](http://www.jimmunol.org/content/ji/287/2/287.f1)

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In an effort to decipher the mechanism of the decreased survival observed during coincubation, NK cells alone or NK and CD4\(^+\)
T cells were incubated with freshly isolated Pneumocystis for 24 h at a 10:1 E:T ratio, as above, and NK cytolytic granules, granzyme B, and perforin were measured by ICS. We found no significant production of granzyme B by NK cells at this time point (data not shown); however, we did find that a small percentage of NK cells produced perforin in response to the fungal organism (Fig. 2C). Interestingly, coincubation of CD4+ T cells with NK cells resulted in significantly greater intracellular perforin production by the NK cells (Fig. 2C, representative graph shift and bar graph comparing means), demonstrating a direct interaction between the two lymphocyte subsets. To determine whether this interaction between the two lymphocyte subsets resulted in altered cytokine production by NK cells was assessed using ICS. Representative graph of fluorescence shift between two groups (left panel). Total perforin+ NK cells in each group (right panel). Purity of isolated lymphocytes is shown in Supplemental Fig. 1. The experiment was repeated twice with similar results (n = 5/experiment). Data in bar graph are mean ± SEM. *p < 0.05, unpaired Student t test.

Influx of NK cells is hampered by the loss of CD4+ T cells during early infection

Being part of the innate-immune response, NK cells are believed to effect their activity early during the infection process. To better define the early kinetics of NK cells in response to Pneumocystis infection in both a normal and immunocompromised setting, WT or CD4+ T cell–depleted mice were infected with P. murina, and NK cell recruitment into the lungs was measured at days 1, 3, and 5 p.i. by flow cytometry. We observed substantial recruitment of NK cells in WT animals at day 1 p.i., which increased through day 5 p.i. (Fig. 3A). In the CD4+ T cell–depleted mice, there was also an increase in NK cells in response to P. murina; however, their numbers were significantly lower than in WT animals, demonstrating the requirement of CD4+ T cells for normal NK cell recruitment to the site of infection. Pneumocystis burden in the lung

FIGURE 2. In vitro NK microbicidal activity. (A) NK cells from naive animals were serially diluted in a 96-well culture plate, and a fixed number of P. murina organisms was added to wells for resultant E:T ratio of 0.1:1, 1:1, and 10:1; they were incubated at 37˚C for 24 h. For control wells, Pneumocystis organisms were added to wells with medium alone. Pneumocystis viability in wells was then assessed using real-time RT-PCR, as previously described (n = 5 wells/group). The experiment was repeated two times with similar results. (B) NK cells and CD4+ T cells were isolated from splenocytes of naive C57BL/6 mice and NK cells or CD4+ T cells were incubated with P. murina organisms for 24 h at 37˚C, 5% CO2 at a fixed E:T ratio of 10:1; NK cells were considered the effector cells. CD4+ T cells with Pneumocystis and Pneumocystis in medium alone were also plated as controls, and P. murina rRNA copy number was assessed, as above (n = 5 wells/group). The experiment was repeated three times with similar results. (C) Coincubation of NK cells and CD4+ T cells. NK cells or NK cells and CD4+ T cells were incubated with P. murina organisms at a 10:1 E:T ratio, as above. After 24 h, intracellular perforin production by NK cells was assessed using ICS. Representative graph of fluorescence shift between two groups (left panel). Total perforin+ NK cells in each group (right panel). Purity of isolated lymphocytes is shown in Supplemental Fig. 1. The experiment was repeated twice with similar results (n = 5/experiment). Data in bar graph are mean ± SEM. *p < 0.05, unpaired Student t test.

FIGURE 3. NK cell kinetics in the presence or absence of CD4+ T cells during early infection. (A) WT and CD4-depleted C57BL/6 mice were infected i.t. with 2 × 105 cysts of Pneumocystis. At days 1, 3, and 5 p.i., mice were sacrificed, left lung tissue was homogenized, and NK cell number was determined by flow cytometry. (B) To assess Pneumocystis lung burden, total RNA was isolated by the phenol-chloroform extraction method and reverse transcribed, and fungal burden was assayed by real-time PCR. This experiment was repeated four times with similar results (n = 3–5/experiment). *p < 0.05, two-way ANOVA with Bonferroni posttest to compare replicate means.
was also assessed at these early time points. In our model, deviation in fungal load between WT and immunocompromised mice is typically observed 9–12 d p.i. Therefore, as expected, we found no significant difference in fungal burden between the two infected groups at these early time points (Fig. 3B).

**Lack of CD4+ T cells during early P. murina infection results in reduced NKp46 expression and IFN-γ production but not cytolytic molecules**

NK cells can regulate their function through numerous receptors that initiate effector cell activity. Two major families of activation receptors on NK cells include NKG2D and natural cytotoxicity receptors (NCRs), such as NKp46, both of which have been associated with NK responses to eukaryotic pathogens (29, 30). To examine the NK response to *Pneumocystis* and determine whether loss of CD4+ T cells affects activation of the innate immune cells, WT and CD4+ T cell–depleted mice were infected with *Pneumocystis*, and NK activation markers NKG2D and NKp46 were examined by cell surface staining during early infection. We found that NKG2D expression on total NK cells showed a slight decrease in expression at day 5 p.i., and there were no major differences between WT and CD4+ T cell–depleted infected animals (Fig. 4A). In contrast, we found that a relatively small number of NK cells expressed the NCR NKp46 at days 1–3, but the numbers began to increase at day 5 in WT mice; however, they decreased significantly in CD4-depleted animals at that time point (Fig. 4B). NK cells are characterized by the secretion of the effector cytokines TNF-α and IFN-γ and by cytotoxic ability, through cytolytic molecule production (i.e., granzymes and perforin in mice), as well as by FasL upregulation. As stated previously, IFN-γ is an important cytokine for protection against *Pneumocystis* (22), and the granular molecules perforin and granzyme B have been implicated in protection against other fungal pathogens (31, 32). Our in vitro results suggest that the increased fungal killing during coinoculation was due to an interaction between the two lymphocyte subsets, resulting in increased perforin production by NK cells. Therefore, to determine whether NK-specific effector activity against *Pneumocystis* infection was influenced by the presence of CD4+ T cells, WT or CD4+ T cell–depleted animals were infected as above; NK-specific production of IFN-γ, granzyme B, and perforin was measured by ICS; and FasL cell surface expression was measured at days 1, 3, and 5 p.i. We found that IFN-γ NK cells increased steadily in lung tissue in WT mice. However, in the depleted animals, there was a sharp decrease in IFN-γ production at day 3, which was significantly less than in WT mice by day 5 (Fig. 4C), indicating that priming of CD4+ T cells may be necessary for maintenance of this response. To investigate whether IFN-γ production was associated with up-regulation of one or both of the activation markers, multiparametric analysis of flow data was performed. However, we did not find any significant relationship between NK cell expression of either NKG2D or NKp46 and the production of IFN-γ, indicating that these CD4+ T cell–mediated NK cell responses to *Pneumocystis* are independent of each other early during de novo infection (data not shown).

To determine whether in vivo loss of CD4+ T cells affected NK cell cytotoxic granule production, intracellular levels of granzyme B and perforin were measured in NK cells via ICS. We found that very few NK cells produced either granular molecule, with granzyme B levels consistent over the 5 d (Fig. 4D) and perforin production decreasing significantly after day 1 (Fig. 4E); however, there was no difference in the production of either molecule in
normal versus depleted animals, suggesting little involvement during early infection. Cytokine production by *Pneumocystis* β-glucan-induced dendritic cells was found to be regulated, in part, by FasL expression on CD4+ T cells (33). To determine whether NK cell expression of FasL was involved in the immune response to *Pneumocystis*, as well as whether CD4+ T cells influenced the NK cell response, we measured cell surface expression of FasL on NK cells during the first 5 d of infection. We found a significant increase in expression on day 3 in both WT and CD4-depleted animals, which then decreased by day 5 (Fig. 4F); again, there was no significant difference in expression between normal and immunocompromised mice. Together, these results demonstrate that, in vivo, CD4+ T cells are required for maintenance of NKp46 expression and IFN-γ by NK cells, but not the NK cytotoxic effects of granule production or FasL expression early in response to de novo *P. murina* infection.

**NK-activating chemokines and cytokines are decreased in lungs of CD4+ T cell–depleted, *Pneumocystis*-infected animals**

To determine whether the decreased NK cell recruitment/activation seen with CD4+ T cell loss in response to *Pneumocystis* was associated with changes in the cytokine milieu in the lungs, a multiplex protein-array assay (18-plex) was performed on lung tissue homogenate at days 1, 3, and 5 p.i. The analytes measured included MCP-1, LIF, RANTES, IP-10, MIG, MIP-1α, MIP-1β, IL-1α, IL-1β, TNF-α, IL-2, IL-7, IL-12, IL-17, and IFN-γ. Of these, the CC chemokines MCP-1, LIF, and RANTES have been shown to mediate recruitment of NK cells during fungal infections. For example, MCP-1 was shown to be a critical early host defense mechanism against *Aspergillus* infection (34), and *Pneumocystis* was shown to stimulate MCP-1 production by alveolar epithelial cells (35). We found a significant decrease in MCP-1 protein levels in the infected lungs of animals depleted of CD4+ T cells at day 1 p.i., and even more so at day 5 p.i., relative to WT infected animals (Fig. 5A). Loss of circulating RANTES has been associated with invasive fungal infection (36); in our model, we found that levels of RANTES in WT animals were significantly increased at day 5, whereas levels in CD4-depleted animals remained the same at all three time points: a significant difference from WT mice at day 5 p.i. (Fig. 5B). LIF is associated with a specific subset of human and murine NK cells found at mucosal surfaces, primarily in the tonsil and gut, which produce IL-22 (NK-22), are poorly cytotoxic, and instead are associated with epithelial protection (37, 38) and, possibly, Th-17 cell expansion (39). As shown in Fig. 5C, WT mice produced substantial amounts of LIF at 1 d p.i., which decreased significantly by day 3 and began to increase again at day 5. In contrast, only low and constant levels of LIF were measured in the depleted animals. To

**FIGURE 5.** Chemokine/cytokine milieu in lungs of WT or CD4+ T cell–depleted mice during early *Pneumocystis* infection. WT and CD4+-depleted C57BL/6 mice were infected i.t. with 2 × 10⁵ cysts of *P. murina*. At days 1, 3, and 5 p.i., mice were sacrificed, right lung tissue was homogenized in 1 ml of PBS, and the chemokine/cytokine profile was assessed by multiplex assay. (A–I) Analytes with significant differences between WT and CD4+ T cell–depleted animals are shown (*n = 5 mice/group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA with Bonferroni posttest to compare replicate means.
investigate whether NK-22 cells were involved in the anti-
*Pneumocystis* response. IL-22 was measured via ELISA, yet we found no IL-22 production in our in vitro system or in the lung homogenates. Further, there was no significant difference in IL-17 between WT and CD4+-depleted lung tissue, suggesting that NK-22 cells are not significantly involved in our model at these early time points (data not shown).

Our laboratory and other investigators showed that the CXC chemokines IP-10 and MIG are increased during *Pneumocystis* infection, as well as that loss of IP-10 results in decreased NK cell recruitment to lungs of *P. murina*-infected mice (40–42). Additionally, an association between IP-10 production and NK cell cytotoxicity was demonstrated in *Leishmania* infection (43). As shown in Fig. 5D, IP-10 protein levels were significantly increased at day 5 p.i. in WT animals, whereas there was significantly less in the CD4-depleted animals. Similarly, there was a progressive increase in MIG tissue concentration, with a significant difference between the two groups at day 5 p.i. (Fig. 5E). IL-1β is associated with defensin production in other fungal infections (44). In our model, there was a significant difference in IL-1β between the two groups at day 1 p.i. (Fig. 5F). At days 3 and 5, the amount of IL-1β decreased in infected mice.

IL-12 and TNF-α activate NK cells to produce IFN-γ and, as shown in Fig. 5G, WT mice produced a large amount of IL-12 very early (day 1), which decreased at later time points; this spike in IL-12 production was absent in mice lacking CD4+ T cells. Similarly, TNF-α was high at day 1, and it decreased by days 3 and 5 p.i. (Fig. 5H). In the immunocompromised animals, the initial TNF-α level was less than in WT mice, and it also decreased at later time points. IFN-γ levels in the lung tissue spiked on day 5 in normal infected animals, whereas levels in the depleted group remained the same as those on day 1. The difference was significant at day 5 between the two groups (Fig. 5I). Together, these results demonstrate that lack of CD4+ T cells during *Pneumocystis* infection results in diminished NK-specific and associated chemokines and cytokines in lung tissue, leading to decreased numbers and possibly altered function of the innate lymphocyte.

**Ag-specific memory CD4+ T cells are required for optimal NK cell activity against Pneumocystis infection in vivo**

A relationship between memory CD4+ T cells and NK cells was reported (15, 45), and results from our chronic-infection studies indicate that there is continued interaction between CD4+ T cells and NK cells during *P. murina* infection. To determine whether Ag-specific memory CD4+ T cells are required for sustained NK cell effector activity, normal C57BL/6 mice were infected with 2 × 10^5 cysts of *P. murina* and allowed to develop a CD4+ memory T cell response. After 7 wk, CD4+ T cells were isolated from the lungs using a negative-selection column and adoptively transferred into RAG2^−/− mice, which lack T and B lymphocytes but possess NK lymphocytes. Another group was transferred with CD4+ T cells isolated from lungs of naive mice, and a control group was sham transferred with medium. Lymphocytes were allowed to circulate for 5 d and then the mice were infected with *P. murina*. After 5 d, mice were sacrificed, and lung *Pneumocystis* burden and NK cell effector functions were assessed. Effector and memory CD4+ T cell subsets were also measured, as well as their cytokine production. As shown in Fig. 6A, *Pneumocystis* burden was significantly reduced in the memory-transferred group at this early time point p.i. Although total NK cells were similar among the three groups (Fig. 6B), there was a significant increase in NKG2D^+ NK cells in the memory T cell–transferred mice compared with both sham and naive T cell recipients (Fig. 6C). NKP46^+ NK cells were also measured, but we found no expression...
of this activation marker (data not shown). As shown in Fig. 6D, there was a significant increase in the numbers of IFN-γ-producing NK cells in the presence of memory CD4+ T cells compared with sham and naive-transferred mice. Multiparameter analysis was performed to determine whether there was a relationship between NKG2D and IFN-γ production. As shown in Table I, the majority of NKG2D+ NK cells were IFN-γ+, yet there were significantly more NKG2D+IFN-γ+ NK cells in mice that received memory T cells versus naive or sham-transferred mice (1.32 ± 0.1 versus 0.63 ± 0.1 or 0.57 ± 0.1, respectively).

Granzyme B production was evident in all three groups, yet there were significantly more granzyme B+ NK cells with the transfer of memory cells relative to naive cells (Fig. 6E). We also found that perforin expression was significantly increased with transfer of memory CD4+ T cells (Fig. 6F). FasL expression was also measured, but no group expressed this TNF-related ligand (data not shown). When transferred T cells were phenotyped in the lungs of the recipient mice, we found that recipients of cells from previously infected mice had significantly more CD4+ cells in their lungs at 5 d p.i. (Fig. 7A), with the majority being effector memory phenotype (Fig. 7B), and only a small number was central memory or naive (data not shown). As shown in Fig. 7C, there was also a significant amount of IFN-γ-producing CD4+ T cells in the memory-transferred mice, but there was no significant amount of difference with regard to IL-2–producing CD4+ T cells between the groups (data not shown). Together, these results demonstrate that Ag-specific memory CD4+ T cells are necessary for optimal anti-Pneumocystis activity of NK cells, partially through upregulation of the activation marker NKG2D.

**Discussion**

CD4+ T cells are required for optimal host defense against several opportunistic pathogens, as evidenced by disease states resulting from clinical AIDS wherein CD4+ T cells are deficient in numbers and function. Mechanisms of CD4+ T cell protection against infection are not fully elucidated. In this article, we put forth a previously unappreciated function for CD4+ T cells against opportunistic pathogens: utilization of the effector activities of NK cells. In this study, we demonstrate that CD4+ T cells, both in the naive, and particularly in the effector memory state, can positively influence effector mechanisms of NK cells. In vitro, CD4+ T cells can directly augment the antifungal activity of NK cells. In vivo, CD4+ T cells are required for NK cell activation and IFN-γ production during early primary fungal infection. However, we demonstrate through adoptive transfer that optimal NK effector activity, consisting of both effector cytokine and cytolytic molecule production, is achieved through the presence of memory CD4+ T cells.

In these studies, we used pulmonary infection with *P. murina* as a model of opportunistic infection relevant to CD4+ T cell function. CD4+ T cells are well recognized to play an important role in host defense against *Pneumocystis*. One well-studied effector mechanism is the production of IFN-γ by the Th1 subset of CD4+ cells. IFN-γ is critical in activating alveolar macrophages to produce cytokines, such as IL-12 and TNF-α, and to kill *Pneumocystis* organisms (46). We showed previously that overexpression of IFN-γ in the lungs of CD4-depleted mice results in *Pneumocystis* eradication, accompanied by significant recruitment of NK cells into lung tissue during the first week of infection (22); however, those studies were focused on CD8+ T cells, and the role of NK cells was not studied further. NK cells have two major effector functions: cytokine secretion (IFN-γ and TNF-α) and target cell lysis. In this study, we show that NK cells are an important arm of immune defense against *Pneumocystis* infection, with early recruitment to the lungs of *P. murina*-infected WT animals and with production of IFN-γ, granzyme, and perforin.

However, we discovered that, in the absence of CD4+ T cells, NK cell recruitment and activation in lung tissue in response to *Pneumocystis* are significantly decreased. NK-specific IFN-γ production initially is similar between WT and CD4-depleted mice, but it begins to decrease in the absence of CD4+ cells, significantly so by day 5 p.i., suggesting that newly primed Ag-specific CD4+ T cells are necessary to maintain the NK cell response. Our findings are supported by Bihl et al. (14), who demonstrated that newly primed Ag-specific CD4+ T cells directly activate NK cells to produce IFN-γ in response to *L. major* infection. In their system, NK activation was achieved directly through secretion of IL-2 by CD4+ T cells, as well as indirectly through the regulation of IL-12 secretion by dendritic cells. Similarly, we found a spike in IL-2 at day 1 p.i., followed by a spike in IFN-γ at day 5 p.i. The high production of both cytokines was abrogated in the absence of CD4+ T cells (Fig. 5). However, similar levels of IL-2 were measured in WT and CD4+ T cell–depleted, *P. murina*–infected animals (data not shown), suggesting another cell source for IL-2 in the absence of CD4+ T lymphocytes, most likely CD8+ T cells (47). Yet, in vitro coinubation analysis both in the presence and absence of APCs revealed that IL-2 and IFN-γ production were not a factor in the increased microbicidal activity of NK cells. Our system revealed that, in the presence of APCs, production of IL-2 by CD4+ T cells was not necessary for IFN-γ production by NK cells. CD4+ T cells did produce significant IL-2 in response to *P. murina* organisms in the presence of APCs. Yet, in the same experiment, we found that NK cells produced IFN-γ at similar levels in the presence or absence of CD4+ T cells. One explanation for the difference between our findings and those of Bihl et al. (14) is the production of additional NK-activating chemokines. Significant production of IP-10 and RANTES was measured when NK cells were incubated with *P. murina* in vitro in the presence of APCs (data not shown). MCP-1, RANTES, MIP-1α, MIP-1β, and IP-10 were all shown to activate NK cells (43, 48, 49). Indeed, it is well known that the cytokine milieu during infection orchestrates the ensuing immune response (50). Our in vivo results revealed substantial production of MCP-1, RANTES, MIP-1α, MIP-1β, and, especially IP-10 and MIG (ng/g tissue levels) in lung homogenates of WT *Pneumocystis*-infected animals; this was markedly reduced in the lungs of

**Table I. Multiparametric analysis**

<table>
<thead>
<tr>
<th>Transfer</th>
<th>NKG2D+IFN-γ</th>
<th>NKG2D+IFN-γ</th>
<th>NKG2D+IFN-γ</th>
<th>NKG2D+IFN-γ</th>
</tr>
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<tbody>
<tr>
<td>Memory</td>
<td>29.82 ± 1.05*</td>
<td>1.32 ± 0.14***</td>
<td>8.48 ± 0.97***</td>
<td>60.36 ± 1.04***</td>
</tr>
<tr>
<td>Naive</td>
<td>28.06 ± 0.59</td>
<td>0.63 ± 0.07</td>
<td>4.59 ± 0.56</td>
<td>66.72 ± 1.06*</td>
</tr>
<tr>
<td>Sham</td>
<td>25.02 ± 1.35</td>
<td>0.57 ± 0.05</td>
<td>4.5 ± 0.55</td>
<td>69.92 ± 0.87</td>
</tr>
</tbody>
</table>

Multiparametric analysis of percentages of CD3+ NK1.1+ cells from adoptive transfer for measurement of double-positive NKG2D+ IFN-γ+ cells.

*p < 0.05, to sham-transferred animals; **p < 0.05, to naive CD4+ T cell–transferred animals.
CD4-depleted animals. A correlation between NK cell recruitment and IP-10 expression was demonstrated in the early defense against other eukaryotic infections (43, 51, 52). Further, our previous findings demonstrated that CXCR3-deficient, CD4+-intact animals have delayed clearance of Pneumocystis organisms, and exogenous administration of IP-10 accelerated clearance in the CD4+ T cell–depleted mice (41). However, it has not been determined whether a direct connection between NK cells and IP-10 exists in host defense against Pneumocystis.

NK cell function is mediated by a myriad of activation and inhibition receptors. We chose to measure the expression of NKp46 and NKG2D, two receptors previously implicated in host response to infection. We found that a very small percentage of NK cells expressed NKp46 in response to P. marina in vivo during nascent infection, yet we observed a significant increase in its expression in WT mice by day 5, which was not observed in CD4+-depleted animals. However, no expression of NKp46 was measured in the adoptively transferred RAG2-/- mice, suggesting that its involvement in Pneumocystis infection is minimal or may be an early response. It is possible that NKp46 and other NCRs may be more involved in intracellular infection, because the majority of reports of upregulation involve viral or intracellular parasitic infection (53–57). Another explanation for the discrepancy in our results is the possibility that the immunodeficient RAG2-/- mice may be functionally deficient in NKP46 expression, although this has not been reported. In contrast, NKG2D+ NK cells were significantly increased in the presence of memory CD4+ T cells, and polyfunctional analysis revealed significantly more IFN-γ+ NKG2D+ NK cells in memory-transferred animals. However, it should be noted that only ~13% of the IFN-γ+ NK cells were also NKG2D+, suggesting only partial involvement of this activation receptor in the NK response to Pneumocystis. Nevertheless, our findings are supported by Guan et al. (7), who demonstrated that IFN-γ production by NK cells is abrogated by anti-NKG2D treatment during Toxoplasma gondii infection. However, in our model it appears that the relationship between CD4+ T cells and NKG2D+IFN-γ+ cells requires the memory CD4+ subset, because we did not see any difference in NKG2D expression between WT and CD4+-depleted animals during de novo infection, and polyfunctional analysis of those results did not reveal any double-positive NKG2D+IFN-γ+ cells (data not shown).

In addition to cytokine secretion, a major effector function of NK cells is lysis of target cells through release of cytolytic proteins, namely granzymes and perforin in mice, and through induction of apoptosis via expression of cell surface molecules, such as FasL. NK FasL expression is critical for defense against intracellular pathogens and antitumor responses (58–60). It was also shown to be upregulated on CD4+ T cells in response to Pneumocystis β-glucans (33). Our results demonstrate that FasL is upregulated on NK cells during early infection but then is rapidly down-regulated, and we found that the presence of CD4+ T cells did not influence this response. Furthermore, no FasL expression was detected in RAG2-/- mice adoptively transferred with sham, naive, or memory CD4+ T cells, indicating little involvement during long-term infection. Perforin and granzyme B have been implicated in the protective immune response against many fungal and parasitic infections (61–64). For example, direct perforin–dependent microbicidal activity by NK cells was demonstrated against Cryptococcus (65, 66), and Müller et al. (67) demonstrated that perforin and granzyme were essential for tissue clearance of Trypanosoma from infected mice. Our in vitro results show that CD4+ T cells from naive mice can directly increase perforin production by NK cells; the mechanism behind this interaction is being examined in our laboratory. In vivo, we demonstrate that memory CD4+ T cells significantly upregulate both granzyme B and perforin production in NK cells, suggesting a role in protection against infection. This is further supported by a study by Meissner et al. (68), who demonstrated that Pneumocystis-infected, perforin-/- mice have more severe Pneumocystis-mediated lung damage than do CD4-/- animals, implicating a protective role for perforin. Further studies are needed to decipher the role of perforin, granzyme B, and other members of the granzyme family in immunity to Pneumocystis.

To our knowledge, our study is the first to establish a functional relationship between CD4+ T cells and NK cells in the host response to P. marina infection and demonstrates the requirement of memory CD4+ T cells for optimal NK activity against this opportunistic fungal pathogen. However, many questions remain to be answered. One potential mechanism behind the lymphocyte cross-talk that we observed is OX40–OX40L interaction. OX40L is an inducible ligand, and OX40 is a member of the TNF superfamily, primarily expressed on activated CD4+ cells and re-activated effector memory cells. A direct interaction between NKG2D-activated NK cells and CD4+ T cells was demonstrated by Zingoni et al. (69), who showed that treatment with IL-2, IL-15, or IL-12 resulted in upregulation of OX40L on NK cells, leading to OX40+ CD4+ T cell proliferation and IFN-γ cytokine production. Another key player in our model may be CD40L.
CD40L interaction with NK cells was described recently in tumor immunity; Shimizu et al. (15) demonstrated that CD40L effector memory CD4+ T cells activate NK cells through IL-2 production and induction of IP-10 from dendritic cells. Indeed, CD40L is essential for resolution of Pneumocystis infection in mice (70). The involvement of CD40L and subsequent NK activation in our model is supported by our previous results demonstrating that adoptive transfer of splenocytes from CD40L-vaccinated, CD4-depleted mice was able to confer protection against PCP in CD4-deficient mice (24). Further experiments are needed to demonstrate the link among CD40L+CD4+ T cells, NK cells, and IP-10.

To conclude, our studies show that:

1) Loss of NK cells during long-term infection results in increased fungal burden in otherwise immunocompetent, as well as, immunodeficient animals.

2) In vitro, NK cells alone are microbicidal to P. murina in a dose-dependent manner.

3) Addition of CD4+ T cells to NK cells in vitro results in increased fungal killing that is coincident with increased intracellular perforin production by NK cells.

4) The presence of CD4+ T cells during early de novo infection is required for upregulation of the NK cell activation marker NKp46, as well as for maintenance of NK cell–specific IFN-γ production.

5) The presence of memory CD4+ T cells (primarily of the effector memory phenotype), rather than naïve CD4+ T cells, significantly augments NK cell activation (NKGD2), effector cytokine production (IFN-γ), and cytolytic molecule production (granzyme B and perforin) during early P. murina infection.

In summary, these findings present a novel mechanism by which CD4+ T cells combat this opportunistic fungal pathogen and provide a previously unappreciated role for NK cells in our animal model of immunity to PCP. Clearly, further studies are needed to better understand this cooperative NK–CD4+ T cell interaction in the hope of ultimately developing CD4+-independent NK cell therapies for immunosuppressed individuals.

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Disclosures
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References