Depletion or dysfunction of CD4+ T lymphocytes profoundly perturbs host defenses and impairs immunogenicity of vaccines. Here, we show that plasmid DNA vaccination with a cassette encoding antigen (OVA) and a second cassette encoding full-length CD40 ligand (CD40L), a molecule expressed on activated CD4+ T lymphocytes and critical for T cell helper function, can elicit significant titers of antigen-specific immunoglobulins in serum and Tc1 CD8+ T cell responses in CD4-deficient mice. To investigate whether this approach leads to CD4+ T cell–independent vaccine protection against a prototypic AIDS-defining infection, Pneumocystis (PC) pneumonia, we used serum from mice vaccinated with PC-pulsed, CD40L-modified DCs to immunoprecipitate PC antigens. Kexin, a PC antigen identified by this approach, was used in a similar DNA vaccine strategy with or without CD40L. CD4-deficient mice receiving DNA vaccines encoding Kexin and CD40L showed significantly higher anti-PC IgG titers as well as opsonic killing of PC compared with those vaccinated with Kexin alone. Moreover, CD4-depleted, Kexin-vaccinated mice showed a 3-log greater protection in a PC challenge model. Adoptive transfer of CD19+ cells or IgG to SCID mice conferred protection against PC challenge, indicating a role of humoral immunity in […]

Find the latest version:

http://jci.me/26306-pdf
Depletion or dysfunction of CD4\(^+\) T lymphocytes profoundly perturbs host defenses and impairs immunogenicity of vaccines. Here, we show that plasmid DNA vaccination with a cassette encoding antigen (OVA) and a second cassette encoding full-length CD40 ligand (CD40L), a molecule expressed on activated CD4\(^+\) T lymphocytes and critical for T cell helper function, can elicit significant titers of antigen-specific immunoglobulins in serum and Tc1 CD8\(^+\) T cell responses in CD4-deficient mice. To investigate whether this approach leads to CD4\(^+\) T cell–independent vaccine protection against a prototypic AIDS-defining infection, Pneumocystis (PC) pneumonia, we used serum from mice vaccinated with PC-pulsed, CD40L-modified DCs to immunoprecipitate PC antigens. Kexin, a PC antigen identified by this approach, was used in a similar DNA vaccine strategy with or without CD40L. CD4-deficient mice receiving DNA vaccines encoding Kexin and CD40L showed significantly higher anti-PC IgG titers as well as opsonic killing of PC compared with those vaccinated with Kexin alone. Moreover, CD4-depleted, Kexin-vaccinated mice showed a 3-log greater protection in a PC challenge model. Adoptive transfer of CD19\(^+\) cells or IgG to SCID mice conferred protection against PC challenge, indicating a role of humoral immunity in the protection. The results of these studies show promise for CD4-independent vaccination against HIV-related or other opportunistic pathogens.

**Introduction**

Patients with defects in CD4\(^+\) T cell number and function, whether due to HIV infection, malignancy, or other immunosuppression, are an increasing risk group in modern medicine (1, 2). For example, despite current strategies to treat HIV infection and its complications, Pneumocystis (PC) pneumonia remains a common clinical problem (1). In a recent epidemiological study performed after the initiation of highly active antiretroviral therapy (HAART), the incidence of PC infection has declined; however, the rate of decline has been greater for other AIDS opportunistic infections such as Toxoplasma or CMV infection (1). Since subpopulations of HIV-infected patients remain at risk despite HAART (1, 3, 4), and as there is an increasing patient population on immunosuppressive medical regimens, there is need to develop CD4\(^+\) T cell–independent therapeutic strategies to prevent infection (2). We and others have previously shown that bone marrow–derived DCs can be genetically modified to express CD40 ligand (CD40L) (5, 6), which leads to DC activation, and when pulsed with PC antigens, elicit significant anti-PC antibody titers in CD4-deficient mice as well as conferring protection in SCID mice upon adoptive transfer of immune serum (7). A drawback of this technology is the scalability of a DC approach. However, a potential strength of the DC-based technology was that the protective humoral antibody response was restricted to a few PC antigens (7). Based on these data, we proposed 2 hypotheses: first, that the scalability of the CD4-independent DC approach could be improved by incorporation of CD40L in a DNA vaccine or DNA/adenovirus prime-boost vaccination strategy that would result in antigen-specific immunity in CD4-deficient mice; and second, that serum from mice vaccinated with PC-pulsed, CD40L-modified DCs could be used to identify antigens from PC that would be beneficial when placed in the CD40L DNA prime-boost vaccination protocol.

Here we show, using the model antigen chicken OVA, that the addition of CD40L in both the prime and boost phase of vaccination in CD4-depleted mice results in antibody responses similar to those in CD4-replete mice. Moreover, using 1D and 2D gel electrophoresis of immunoprecipitated PC antigens (using serum from CD40L-DC vaccinated mice), we were able to identify immunodominant epitopes of PC. Kexin, an antigen identified by amino-terminal sequencing and tandem mass spectroscopy (8), which has been reported to be on the surface of PC (9), was used to validate DNA vaccination in CD4-deficient hosts.

These studies demonstrate, for the first time to our knowledge, a scalable, therapeutic vaccine strategy in a CD4-deficient mouse model against a CD4 T lymphocyte–dependent pathogen, PC pneumonia, using a defined PC antigen. The results of these studies show promise for advances in CD4-independent vaccines in high-risk hosts with defective CD4\(^+\) T lymphocyte function.

**Results**

*Evaluation of a prime-boost vaccine approach to achieve CD4-independent vaccination.* Based on the fact that CD40L modification of murine bone marrow–derived DCs can result in CD4-independent B cell
class switching in vitro and protective antibody responses in vivo, we examined whether this could be exploited for in vivo vaccina-
tion approaches. We chose prime-boost vaccination (Figure 1A) with DNA vaccines and adenovirus vectors as a platform to test this due to the relative ease of plasmid DNA manipulation and the efficacy of this approach in eliciting both humoral and T cell responses (10). We chose OVA as a model antigen due to the avail-
bility of reagents to assess both humoral and T cell responses effi-
ciently in vivo. Initially, CD4-depleted (with GK1.5) or CD4-replete (treated with rat IgG2b or PBS as a control) mice (C57BL/6) were vaccinated with 3 doses, 3 weeks apart, with a plasmid encoding the chicken OVA cDNA (pOVA) or pOVA/CD40L, and anti-OVA antibodies were measured 14 days after each vaccination (Figure 1A). CD4-replete mice developed both anti-OVA IgG2a (Figure 1B) and anti-OVA IgG1 (Figure 1C) with either pOVA or pOVA/CD40L, and anti-OVA antibodies were measured 14 days after each vaccination (Figure 1A). CD4-replete mice developed both anti-OVA IgG2a (Figure 1B) and anti-OVA IgG1 (Figure 1C) with either pOVA or pOVA/CD40L, and anti-OVA antibodies were measured 14 days after each vaccination (Figure 1A). However, vaccination with pOVA/CD40L resulted in levels of anti-OVA IgG2a and IgG1 comparable to those in CD4-replete mice. Mice vaccinated with empty vector or pCD40L alone failed to mount an anti-OVA response (data not shown).

To investigate the ability of CD40L to induce CD4-independent CD8+ T cells, CD4-depleted or CD4-replete C57BL/6 mice underwent similar prime-boost vaccination with pOVA or pOVA/ CD40L as described above, and SIINFEKL-specific (11) CD8+ T cells were analyzed after 3 DNA immunizations or after AdOVA or AdOVA/CD40L boosting (Figure 3). CD4-depleted mice primed with 3 doses of pOVA alone had 2% SIINFEKL-dimer–positive cells in the spleen, which is near background. Moreover, there was no statistically significant increase in this population of CD8+ T cells in the spleen after AdOVA or AdOVA/CD40L boosting, although there was a trend toward a higher percentage of SIINFEKL-positive CD8 T cells after AdOVA boosting (Figure 3A). In contrast, CD4-depleted mice primed with pOVA/CD40L showed a significant increase in SIINFEKL-positive CD8 T cells after AdOVA...
or AdOVA/CD40L boosting, demonstrating the critical need to have CD40L in the priming vector (Figure 3A). Importantly, the percentage of SIINFEKL-positive cells in CD4-depleted mice was similar to that in CD4-replete mice undergoing a prime-boost protocol with either pOVA or pOVA/CD40L as the priming vector (Figure 3B). Furthermore, IFN-γ secretion by CD8+ T cells in CD4-depleted mice after CD40L prime-boosting was similar to that of CD4-replete mice (Figure 3C).

Proteomic approach to identify protein antigens from PC. We have previously reported that PC antigen–pulsed, CD40L-modified DC-based vaccination results in an oligoclonal protective B cell response in CD4-deficient mice recognizing a 55-kDa antigen (7). This antibody was used in immunoprecipitations followed by 1D (Figure 4A) and 2D gel electrophoresis (Figure 4B). For 1D gels, bands were either transferred to nitrocellulose, stained with Ponceau S and subjected to N-terminal sequencing (12), or excised and subjected to enzyme digestion followed by TOF/TOF mass spectrometry (MS) (13). Spots from 2D gels were picked and underwent TOF/TOF MS analysis as well. Tandem MS and Edman degradation of the excised 55-kDa antigen revealed heavy-chain mouse IgG (as expected) but also a peptide, GSVYVFAS, with a high homology to Kexin from *Saccharomyces cerevisiae*, using the BLAST search algorithm for short, nearly exact matches in the yeast database. Based on these data, the full-length Kexin cDNA from PC muris was cloned as vaccine candidate.

CD4-independent vaccination of PC infection using Kexin DNA vaccination. To further evaluate CD40L in CD4-independent vaccination regimen against an opportunistic infection that is critically dependent on intact CD4+ T cell function, we investigated the full-length Kexin cDNA from PC muris was cloned as vaccine candidate.

**Figure 3**
Antigen-specific CD8+ T cell responses. SIINFEKL-specific CD8+ T cell responses in CD4-depleted (A) and CD4-replete (B) mice after OVA and OVA/CD40L prime-boost regimens. n = 3–4 per group. *P < 0.05 compared with no-boost control. (C) IFN-γ secretion determined by ELISA in OVA-stimulated CD8+ T cells from both CD4-replete and CD4-depleted mice. n = 3–4 per group. *P < 0.05 compared with pOVA control. All data are mean values ± SEM.
organism burdens, with nearly a 3-log greater protection compared with control CD4-depleted mice. Of note, $10^4$ rRNA copy number is near the limit of detection of organisms by histological staining with Gomori methenamine-silver (GMS). Similar trends were observed when infection was also scored blindly on GMS-stained lung sections (7), with mice immunized with pKexin/CD40L showing the greatest protection (Figure 6B).

**Kexin DNA vaccination results in protective antibody responses.** Due to the fact that we observed protection in the primary challenge with PC, we investigated whether this was antibody mediated. We initially determined whether the antibody generated by pKexin/CD40L was capable of mediating opsonic killing of PC. Organisms were incubated with immune serum from pKexin/CD40L-vaccinated or control mice (CD40L vaccination alone) in SCID mice followed by PC challenge. SCID mice receiving either serum or CD19+ splenocytes from pKexin/CD40L-vaccinated or control mice (CD40L vaccination alone) in SCID mice followed by PC challenge. SCID mice receiving either serum or CD19+ splenocytes from pKexin/CD40L-vaccinated or control mice showed significantly lower PC burdens by quantitative real-time PCR 28 days after PC challenge compared with control mice (Figure 7B), showing that both serum CD40L-modified DCs (Figure 4) (7). Naive mouse serum failed to stain mouse or monkey PC (Figure 7D), whereas serum from mice vaccinated with pKexin/CD40L stained PC derived from either mouse (red staining) or monkey (yellow staining).

**Discussion**

There is a critical need to develop CD4-independent vaccine approaches for infections, as the numbers of immunocompromised hosts are increasing (16). One strategy toward this end is to define the factors that mediate CD4+ T cell help and provide therapeutic replacement of these factors. Toward this end, we have previously demonstrated that overexpression of IFN-γ, a potent Th1 cytokine produced by CD4+ T cells, can result in eradication of PC in the absence of CD4+ T cell help (17), in part through the augmentation of IFN-γ-secreting type I (Tc1) CD8+ T cell response (18).

Another molecule expressed on activated CD4+ T cells that is critically important for costimulation and CD4+ T cell help is CD40L. CD40L is expressed on activated T cells and activates DCs through the augmentation of IFN-γ secretion (19, 20) and B cell (21) immune responses. Kikuchi and colleagues have demonstrated that CD40L gene-modified DCs pulsed with *Pseudomonas aeruginosa* (PA) could stimulate naive B cells to produce anti-PA antibodies (22) and confer protection against PA challenge. Furthermore, we recently showed that CD40L-modified DCs pulsed with PC could result in a protective antibody response in CD4-depleted mice and protect them against a PC challenge.

Although these studies demonstrate the proof-of-principle that CD40L is an excellent adjuvant to elicit CD4-independent immune responses, DC approaches suffer from significant issues of scalability. Second, vaccine approaches to organisms such as PC that have not been successfully adapted to large-scale ex vivo culture have been hampered by a lack of good candidate antigens. We sought to overcome these shortcomings by (a) using CD40L-
modified DCs to identify vaccine candidates and (b) using CD40L in a prime-boost regimen to overcome the lack of immunogenicity of DNA vaccines in CD4-deficient hosts.

We have previously shown that IgG responses generated by AdCD40L-modified DC technology were largely restricted to a 55-kDa antigen of PC that is protective upon adoptive transfer (7). To further define this and other antigens, we used a combined approach of 1D and 2D gel electrophoresis followed by transfer to nitrocellulose and N-terminal sequencing or spot excision, enzyme digestion, and tandem MS. N-terminal sequencing of 1 of the 55-kDa bands revealed a peptide sequence with a high degree of homology to Kexin, a furin-like protease (7). We also identified major surface glycoprotein–A (14); however, due to significant divergence in this protein across PC species, we focused on investigating Kexin. Furthermore, a monoclonal antibody to this antigen has shown protection in passive immunization experiments in mice (23).

Despite the identification of Kexin as a vaccine candidate, a platform was required to achieve a CD4-independent vaccine approach in vivo. We chose DNA vaccination, as DNA vaccines have been shown to transduce DCs in muscle (24, 25) but also allow for cross-presentation of antigens in muscle (26), and CD40L transduction would aid in DC maturation as well as potentially activation and class-switching of B cells (27). The addition of CD40L in the priming regimen, along with eliciting the above-described humoral responses, also allowed for the generation of antigen-specific IgG1 and IgG2a independently of CD4+ T cells. Moreover, DNA vaccines, like certain replication-defective viral vectors, appear to have the important advantage of safety for use in immune-deficient individuals, unlike replicating vector systems. The antibody titer after DNA vaccination could be boosted significantly with recombinant adenovirus vectors encoding the same antigen. Whether direct transduction of DCs or cross-presentation of antigen by myocytes is occurring in vivo is unknown at present but is being investigated. The addition of CD40L in the priming regimen, along with eliciting the above-described humoral responses, also allowed for the generation of antigen-specific CD8+ T cells after adenovirus boosting. Based on these data, we investi-

Figure 5
Humoral responses after pKexin or pKexin/CD40L DNA vaccination. (A) Schema of PC Kexin vaccination protocol. DNA vectors used were pKexin, pKexin/CD40L, pBUDCE4 (as empty vector), or pCD40L. (B) Anti-PC IgG1 and IgG2a responses after pKexin or pKexin/CD40L after intramuscular DNA immunization in CD4-depleted and CD4-replete mice. n = 6–8. All data are mean titer ± SEM. *P < 0.05 compared with control. (C) Anti-PC IgG1 and IgG2a responses after pKexin or pKexin/CD40L after intramuscular DNA immunization in CD4+/− and CD4+/+ mice. n = 4–6. All data are mean titer ± SEM. **P < 0.05 compared with CD4+/+ mice or pKexin/CD40L in CD4−/− mice. A line denotes the limit of detection in the ELISA.

Figure 6
Protection against a primary PC challenge in CD4-depleted mice after DNA immunization. CD4-depleted mice were vaccinated with 3 rounds of control (pCD40L), pKexin, or pKexin/CD40L followed by challenge with PC. PC was quantified by quantitative real-time PCR (A) or by histology (B). n = 4–6. All data are mean values ± SEM. *P < 0.05, **P < 0.01 compared with control.
gated whether this approach could yield therapeutic vaccination against a prototypic AIDS-defining illness, PC pneumonia.

Toward this end, DNA immunization with Kexin/CD40L resulted in significant anti-PC antibody titers, which were protective in primary challenge experiments as well as in adoptive transfer experiments. It is important to note that the efficacy was greater in the primary challenge experiments compared with the adoptive transfer experiments, likely due to the dilution effect of serum transfer and the half-life of the antibody. In support of this, after transfer of immune serum with an anti-PC titer of greater than 1:1,500, the remaining anti-PC IgG1 titer 28 days later was 1:346. Moreover, Kexin/CD40L immunization resulted in antibody capable of mediating opsonic phagocytosis of PC, which may be critical for its therapeutic affect. Western blot analysis demonstrated that mice immunized with Kexin/CD40L recognized a protein of the predicted full-length Kexin (105 kDa) as well as a 55-kDa protein that may be a proteolytic cleavage product of Kexin in the sonicated PC antigen. As this was the same antigen lot used to pulse DCs, this lends further support for using antigen-pulsed DCs for epitope identification/prioritization.

We did not examine adenovirus boosting with PC, as antibody generation correlates well with protection in this model (7); however, based on the ability to induce a Tc1 CD8\(^+\) T cell response, prime boosting may be the preferred approach where a CD8\(^+\) T cell response — as, for example, against tumors (28, 29) or intracellular pathogens (30) — is critical. Ultimately a CD8\(^+\) T cell response, in addition to an antibody response, may also be important in PC, as there is evidence that polarized, IFN-\(\gamma\)–producing CD8\(^+\) T cells are effective in mediating host defenses against this pathogen (18). Although the safety and efficacy of this novel vaccine approach will need to be validated in other systems, the data presented here show promise in eliciting strong cellular and humoral responses in the absence of CD4\(^+\) T cells.
Antigen IP and 2D electrophoresis. We have previously reported that CD4-deficient mice vaccinated with DCs expressing CD40L pulsed with PC develop an oligoclonal antibody response, with an immunodominant response being against a 55-kDa antigen (7). As PC is at present poorly characterized in terms of potential vaccine antigens, we used this DC-vaccine serum to identify immunodominant antigens expressed by PC. Purified PC was lysed in 1 ml Triton X-100 lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl [pH 8.0]) for 30 minutes on ice. Samples were centrifuged for 10 minutes at 10,000 g at 4 °C, and the supernatant was collected for immunolabeling. PC antigens were labeled using 0.5 µl specific anti-PC sera (obtained from DC-vaccinated mice as previously described (7) and 100 µl of both protein A and G magnetic microbeads and incubated for 30 minutes on ice. A magnetic separating column (M column; Miltenyi Biotec) was prepared by rinsing with 200 µl Triton X-100 lysis buffer then eluting the labeled lysate. The column was washed with 800 µl Triton X-100 and 100 µl 20 mM Tris-HCl (pH 7.5). The filtrate was discarded. Antibodies bound to the column were released by incubating the column for 5 minutes with 20 µl lysis buffer (7 M urea, 2 M thiourea, 4% 3-[{(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], 1% Triton X-100, 100 mM dithiothreitol [DTT]) then eluting with 50 µl CTT/10. Precipitated antigens were separated using 1D and 2D electrophoresis. For 2D gel electrophoresis, each sample was labeled with Cy5 fluorescent dye for 30 minutes in the dark on ice. Antigens were isoelectrically focused PC organisms were purified by differential centrifugation as previously described (7), and protein antigen was produced by sonication for 5 minutes. The PC inoculum for infectious challenge was prepared as previously described (7). Briefly, C.B-17 SCID mice with PC pneumonia were injected with a lethal dose of pentobarbital, and the lungs were aseptically removed and frozen for 30 minutes in 1 ml PBS at ~70 °C. Frozen lungs were homogenized in 10 ml PBS (model 80 Stomacher; Tekmar Instruments), filtered through sterile gauze, and pelleted at 500 g for 10 minutes at 4 °C. The pellet was resuspended in PBS, and a 1:4 dilution was stained with modified Giemsa stain (Diff-Quik; Baxter). The number of PC cysts was quantified microscopically, and the inoculum concentration was adjusted to 2 × 10⁵ cysts/ml. Gram stains were performed on the inoculum to exclude contamination with bacteria.

Antigen IP and 2D electrophoresis. We have previously reported that CD4-deficient mice vaccinated with DCs expressing CD40L pulsed with PC develop an oligoclonal antibody response, with an immunodominant response being against a 55-kDa antigen (7). As PC is at present poorly characterized in terms of potential vaccine antigens, we used this DC-vaccine serum to identify immunodominant antigens expressed by PC. Purified PC was lysed in 1 ml Triton X-100 lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl [pH 8.0]) for 30 minutes on ice. Samples were centrifuged for 10 minutes at 10,000 g at 4 °C, and the supernatant was collected for immunolabeling. PC antigens were labeled using 0.5 µl specific anti-PC sera (obtained from DC-vaccinated mice as previously described (7) and 100 µl of both protein A and G magnetic microbeads and incubated for 30 minutes on ice. A magnetic separating column (M column; Miltenyi Biotec) was prepared by rinsing with 200 µl Triton X-100 lysis buffer then eluting the labeled lysate. The column was washed with 800 µl Triton X-100 and 100 µl 20 mM Tris-HCl (pH 7.5). The filtrate was discarded. Antibodies bound to the column were released by incubating the column for 5 minutes with 20 µl lysis buffer (7 M urea, 2 M thiourea, 4% 3-[{(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], 1% Triton X-100, 100 mM dithiothreitol [DTT]) then eluting with 50 µl CTT/10. Precipitated antigens were separated using 1D and 2D electrophoresis. For 2D gel electrophoresis, each sample was labeled with Cy5 fluorescent dye for 30 minutes in the dark on ice. Antigens were isoelectrically focused PC organisms were purified by differential centrifugation as previously described (7), and protein antigen was produced by sonication for 5 minutes. The PC inoculum for infectious challenge was prepared as previously described (7). Briefly, C.B-17 SCID mice with PC pneumonia were injected with a lethal dose of pentobarbital, and the lungs were aseptically removed and frozen for 30 minutes in 1 ml PBS at ~70 °C. Frozen lungs were homogenized in 10 ml PBS (model 80 Stomacher; Tekmar Instruments), filtered through sterile gauze, and pelleted at 500 g for 10 minutes at 4 °C. The pellet was resuspended in PBS, and a 1:4 dilution was stained with modified Giemsa stain (Diff-Quik; Baxter). The number of PC cysts was quantified microscopically, and the inoculum concentration was adjusted to 2 × 10⁵ cysts/ml. Gram stains were performed on the inoculum to exclude contamination with bacteria.

Methods
PC antigen and inoculum preparation. PC organisms were isolated from lung tissue of C.B-17 SCID mice that were previously inoculated with PC. PC organisms were purified by differential centrifugation as previously described (7), and protein antigen was produced by sonication for 5 minutes. The PC inoculum for infectious challenge was prepared as previously described (7).

Antigen IP and 2D electrophoresis. We have previously reported that CD4-deficient mice vaccinated with DCs expressing CD40L pulsed with PC develop an oligoclonal antibody response, with an immunodominant response being against a 55-kDa antigen (7). As PC is at present poorly characterized in terms of potential vaccine antigens, we used this DC-vaccine serum to identify immunodominant antigens expressed by PC. Purified PC was lysed in 1 ml Triton X-100 lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl [pH 8.0]) for 30 minutes on ice. Samples were centrifuged for 10 minutes at 10,000 g at 4 °C, and the supernatant was collected for immunolabeling. PC antigens were labeled using 0.5 µl specific anti-PC sera (obtained from DC-vaccinated mice as previously described (7) and 100 µl of both protein A and G magnetic microbeads and incubated for 30 minutes on ice. A magnetic separating column (M column; Miltenyi Biotec) was prepared by rinsing with 200 µl Triton X-100 lysis buffer then eluting the labeled lysate. The column was washed with 800 µl Triton X-100 and 100 µl 20 mM Tris-HCl (pH 7.5). The filtrate was discarded. Antibodies bound to the column were released by incubating the column for 5 minutes with 20 µl lysis buffer (7 M urea, 2 M thiourea, 4% 3-[{(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS], 1% Triton X-100, 100 mM dithiothreitol [DTT]) then eluting with 50 µl CTT/10. Precipitated antigens were separated using 1D and 2D electrophoresis. For 2D gel electrophoresis, each sample was labeled with Cy5 fluorescent dye for 30 minutes in the dark on ice. Antigens were isoelectrically focused PC organisms were isolated from lung tissue of C.B-17 SCID mice that were previously inoculated with PC. PC organisms were purified by differential centrifugation as previously described (7), and protein antigen was produced by sonication for 5 minutes. The PC inoculum for infectious challenge was prepared as previously described (7).
To assess whether samples containing anti-
RNA isolated and cloned (10). The peptide of OVA-SIINFEKL (synthesized at Louisiana State University Health Sciences Center Core Laboratories) was loaded onto PE-labeled H-2K<sup>B</sup> MHC-Ig–T cell direct binding assay (Applied Biosystems). The PCR amplification was performed for 40 cycles, with each cycle at 94°C for 20 seconds and 60°C for 1 minute, in triplicate using the ABI Prism 7700 SDS (Applied Biosystems). The threshold cycle values were averaged from the values obtained from each reaction, and data were converted to rRNA copy number using a standard curve of known copy number of PC rRNA.

Opsonization/killing assay. To assess whether samples containing anti-PC antibody by ELISA contained opsonic activity against PC, we used an in vitro killing assay that detects both non-opsonic and opsonic macrophage-mediated killing of PC, as previously described (15). Alveolar macrophages were obtained from male BALB/c mice by bronchoalveolar lavage. Cell preparations were greater than 98% enriched macrophages. Macrophages (1 x 10<sup>6</sup>/ml) in a volume of 100 μl were cocultured with 100 μl PC (1 x 10<sup>4</sup> cysts/ml) for 16 hours at 37°C, 5% CO<sub>2</sub>. Controls for 100% viability included PC incubated with medium alone. The contents of each well were collected and pelleted at 800 g for 5 minutes. The supernatants were discarded, and total RNA was isolated from the cell pellets using TRIzol reagent (Invitrogen Corp.). PC viability was analyzed through real-time PCR measurement of rRNA copy number and quantified by employing a standard curve of known copy number of PC rRNA, as previously described (15). For opsonization studies, PC was incubated with 10 μl of serum from control or vaccinated mice prior to incubation with macrophages. In certain experiments, serum was immunodepletes of IgG using Protein A/G beads (Miltenyi Biotec).

**MHC-Ig–T cell direct binding assay.** The peptide of OVA-SIINFEKL (synthesized at Louisiana State University Health Sciences Center Core Laboratories) was loaded onto PE-labeled H-2K<sup>B</sup>-Ig dimers (BD Biosciences) at 1:160 in PBS. Total splenocytes from OVA prime-boost mice and control mice were stained with the peptide:dimer complex following manufacturer’s protocol. Cells were measured by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). The percentage and mean channel fluorescence of the dimer-stained T cells were calculated.

**IFN-γ secretion.** Secretion of IFN-γ by CD8<sup>+</sup> T cells was analyzed with an IFN-γ ELISA (R&D Systems) kit according to the manufacturer’s instructions. CD8<sup>+</sup> T cells were purified using CD8<sup>+</sup> beads (Miltenyi Biotec) from vaccinated mice and were cultured for 48 hours at 37°C and 5% CO<sub>2</sub> with bone marrow–derived DCs in medium alone or with OVA antigen (10 μg/ml). Supernatants were harvested for IFN-γ by ELISA.

**Immunofluorescence staining of mouse and monkey PC with anti-Kexin serum.** Mouse or monkey PC was stained with anti-Kexin antibody raised in mice after pKexin/CD40L DNA vaccination. Naive mouse serum served as a negative control. After primary staining, organ samples were extensively washed and stained with anti-mouse Alexa 488 (Molecular Probes; Invitrogen Corp.). For monkey PC experiments, organs were also stained with FITC anti-human PC (BIODESIGN International). Organisms were then fixed with 1% paraformaldehyde and mounted on positively charged slides with ProLong with DAPI (Molecular Probes; Invitrogen Corp.).

**Statistics.** All data are presented as the mean ± SEM. Statistical analysis was performed with a commercially available statistical software package (GraphPad Prism; GraphPad Software Inc.). Data were tested for differences using ANOVA for mixed and random effect models followed by the Tukey-Kramer range test. P values less than 0.05 were considered statistically significant.

**Acknowledgments**

This work was supported by Public Health Service grants P01HL076100 and R01HL061271.

Received for publication July 18, 2005, and accepted in revised form October 4, 2005.

Address correspondence to: Jay K. Kolls, Children’s Hospital of Pittsburgh, Suite 3765, 3705 Fifth Avenue, Pittsburgh, Pennsylvania 15213, USA. Phone: (412) 648-7475; Fax: (412) 692-6645; E-mail: jay.kolls@chp.edu.

Pneumocystis carinii pneumonia. *Infect. Immun.* **70:**1069–1074.


29. Helmich, B.K., and Dutton, R.W. 2001. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. *J. Immunol.* **166:**6500–6508.


