CD4+ T cell–independent DNA vaccination against opportunistic infections

Mingquan Zheng,[1] Alistair J. Ramsay,[2] Myles B. Robichaux,[2] Karen A. Norris,[3] Corrine Kliment,[1] Christopher Crowe,[1] Rekha R. Rapaka,[3] Chad Steele,[1] Florencia McAllister,[1] Judd E. Shellito,[2] Luis Marrero,[2] Paul Schwarzenberger,[4] Qiu Zhong,[2] and Jay K. Kolls[1]

[1]Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [2]Louisiana State University Health Sciences Center Section of Pulmonary/Critical Care and Louisiana State University Health Sciences Center Gene Therapy Program, Louisiana State University Medical Center School of Medicine, New Orleans, Louisiana, USA. [3]Department of Immunology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA. [4]Louisiana State University Health Sciences Center Section of Hematology/Oncology, Louisiana State University Medical Center School of Medicine, New Orleans, Louisiana, USA.

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-modifed 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-defeicent 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-modifed 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-indepdendent B cell

Nonstandard abbreviations used: CD40L, CD40 ligand; GMS, Gomori methenamine-silver; MS, mass spectrometry; PC, *Pneumocystis*; pOVA, plasmid encoding the chicken OVA cDNA; TOF, time-of-flight.

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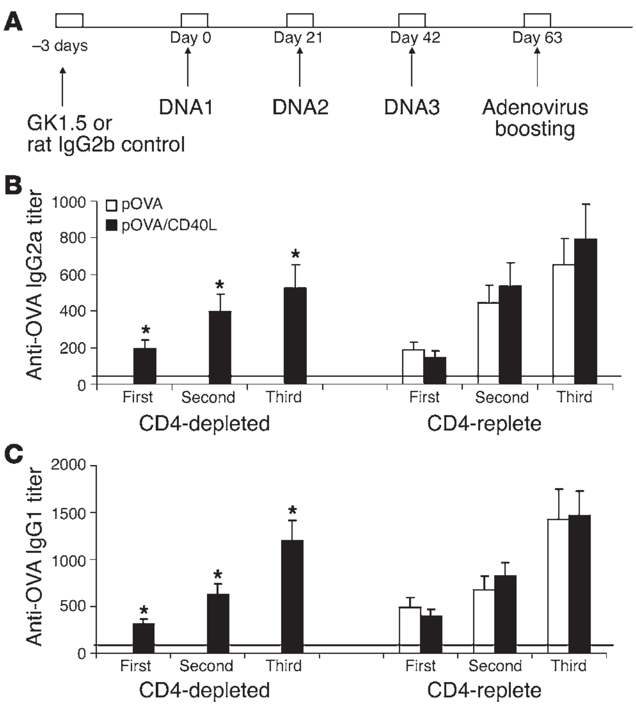

Figure 1

Humoral responses after pOVA or pOVA/CD40L DNA vaccination. (**A**) Schema of prime-boost vaccination protocol. DNA vectors used were pOVA, pOVA/CD40L, pBUDCE4 (as empty vector), or pCD40L. Adenovirus boosting was carried out with AdOVA, AdOVA and AdCD40L, AdLuc, or AdLuc and AdCD40L. Anti-OVA IgG2a (**B**) and IgG1 (**C**) responses after 3 successive intramuscular DNA vaccinations with pOVA or pOVA/CD40L in CD4-depleted and CD4-replete mice n = 6–8 per group. All data are mean titer ± SEM. *P < 0.05 compared with control. A line denotes the limit of detection in the ELISA.

class switching in vitro and protective antibody responses in vivo, we examined whether this could be exploited for in vivo vaccination 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 availability of reagents to assess both humoral and T cell responses efficiently 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, whether PBS or rat IgG2b was used as the control. In contrast, CD4-depleted mice failed to mount an antibody responses to pOVA alone. 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 examine the effect of DNA/adenovirus prime-boosting, mice were randomized to be boosted with an adenovirus encoding OVA (AdOVA) or firefly luciferase (AdLuc, as an irrelevant antigen) or the combination AdOVA/AdCD40L or AdLuc/AdCD40L. Mice boosted with the irrelevant antigen firefly luciferase, with or without CD40L, showed no increase in pre-boosting anti-OVA IgG1 (Figure 2A) or anti-OVA IgG2a titers (Figure 2B). In contrast, in both CD4-replete mice and in CD4-depleted mice, anti-OVA titers could be significantly boosted with the administration of AdOVA or AdOVA and AdCD40L (Figure 2, A and B). Moreover, in CD4-depleted mice, the addition of CD40L in the boosting regimen resulted in an additional increase in anti-OVA IgG1 and IgG2a that was not observed in CD4-replete mice, resulting in anti-OVA titers comparable to those in the CD4-replete group. Administration of AdOVA or AdOVA/CD40L alone to naive mice resulted in levels that were comparable to those in mice vaccinated with DNA alone, with detectable anti-OVA titers but with anti-OVA IgG1 and IgG2a titers less than 1:1,000 (see supplemental data; supplemental material available online with this article; doi:10.1172/JCI26306DS1).

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

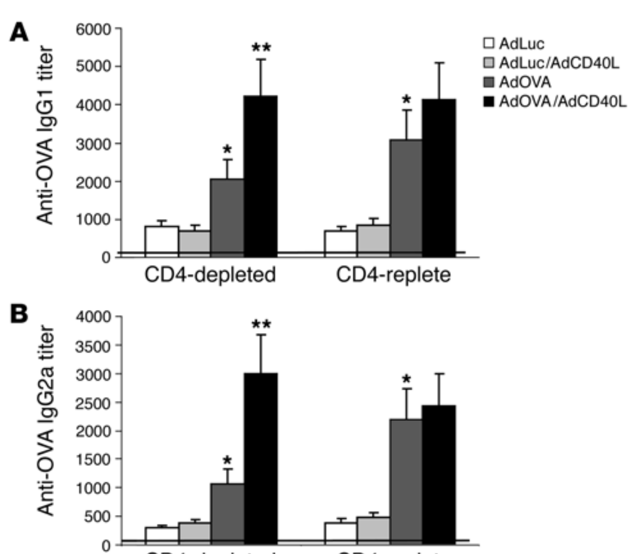

Figure 2

Anti-OVA IgG1 (**A**) and IgG2a (**B**) responses after AdOVA or AdOVA/CD40L boosting after intramuscular DNA priming with pOVA or pOVA/CD40L in CD4-depleted and CD4-replete mice. n = 6–8 per group All data are mean titer ± SEM. *P < 0.05 compared with control; **P < 0.01 compared with control. A line denotes the limit of detection in the ELISA.

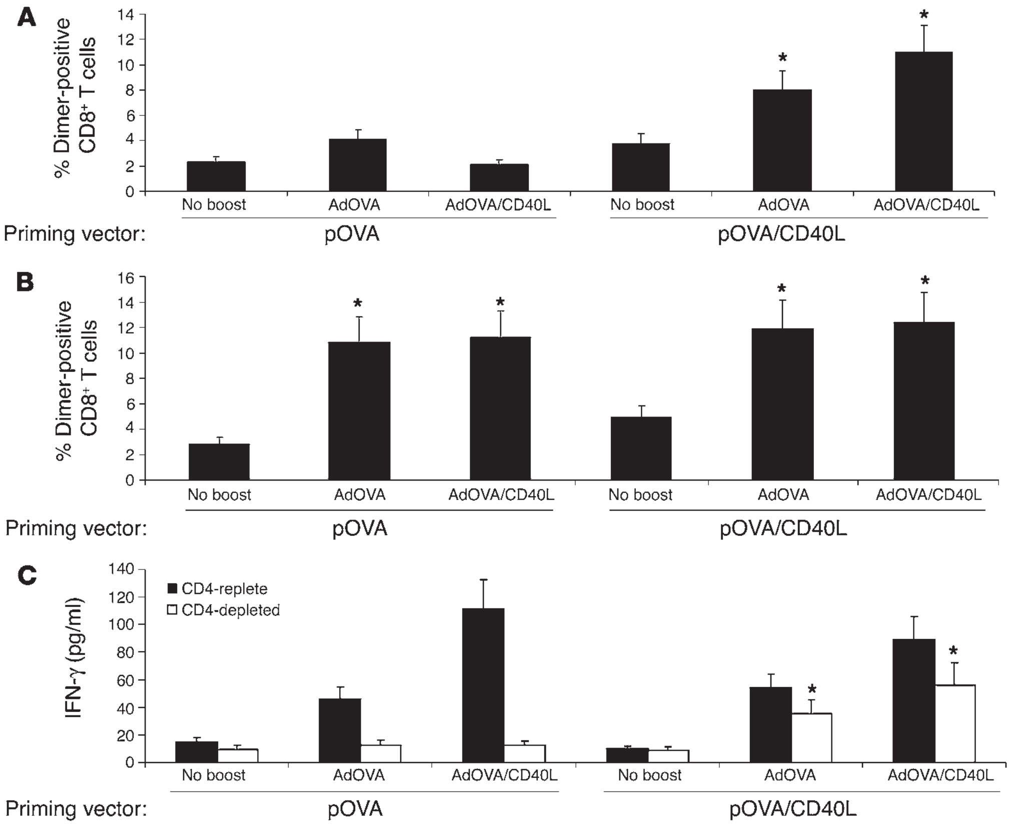

Figure 3
Antigen-specific CD8+ T cell responses. SIINFEKL-specific CD8+ T cell responses in CD4-depleted (**A**) and CD4-replete (**B**) mice after OVA and OVA/CD40L prime-boost regimens. $n = 3–4$ per group. $^*P < 0.05$ compared with no-boost control. (**C**) IFN-γ secretion determined by ELISA in 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.

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-defcient 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 opportunist infection that is critically dependent on intact CD4+ T cell function, we investigated the full-length Kexin cDNA. We chose this antigen over others identified in the proteomic screen, such as major surface glycoprotein–A, due to the fact that Kexin shows greater conservation (see supplemental data) across PC species than major surface glycoprotein–A (14). Immunization with pKexin (schema depicted in Figure 5A) resulted in significant anti-PC IgG1 and IgG2a titers in CD4-replete mice, whereas titers were significantly lower in CD4-depleted mice (Figure 5B). In comparison, CD4-depleted mice immunized with pKexin/CD40L demonstrated significantly higher titers of anti-PC IgG1 compared with CD4-depleted mice immunized with pKexin alone (Figure 5B). Mice immunized with pCD40L or empty vector alone demonstrated no detectable anti-PC titer (data not shown). Moreover, the anti-PC titers observed after Kexin immunization were higher than those observed after DNA vaccination with OVA alone (Figure 1). To verify the CD4-independence of Kexin vaccination, we performed 3 rounds of pKexin versus pKexin/CD40L vaccination in wild-type or $CD4^{-/-}$ mice. We observed that, similarly to what occurred in mice depleted with GK1.5, anti-PC IgG1 and IgG2a levels significantly increased in $CD4^{-/-}$ mice when vaccinated with pKexin/CD40L as opposed to pKexin alone (Figure 5C).

Since we observed significant anti-PC titers after Kexin DNA immunization in CD4-depleted mice, to determine whether this response was protective, we performed a PC challenge experiment. PC organism burden was assessed 28 days after challenge by quantitative PCR as well as by histological scoring, as previously described (7). Quantitative PCR analysis showed that mice immunized with pKexin compared with control mice, receiving pCD40L alone, had approximately one-half log lower PC-specific ribosomal RNA (rRNA) content in lung tissue (Figure 6A). Furthermore, mice immunized with pKexin/CD40L showed significantly lower

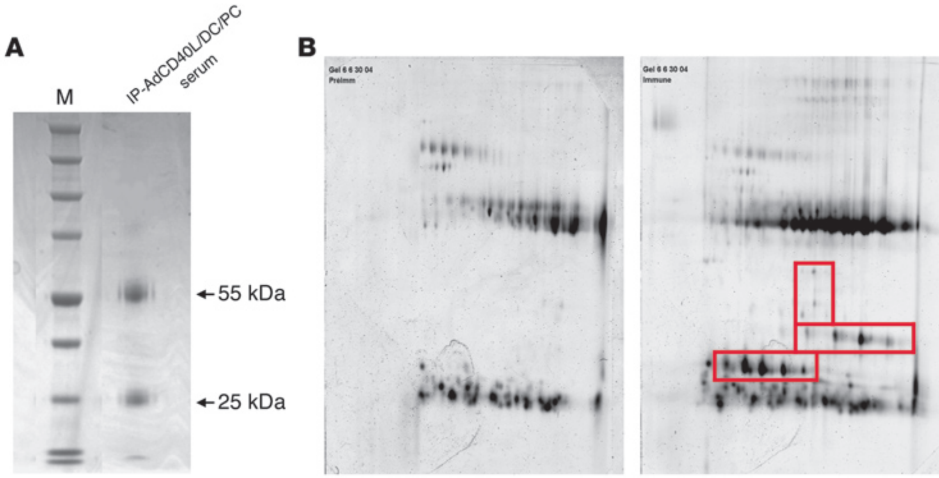

Figure 4
Proteomics of PC antigens. (**A**) Immunoprecipitation of PC antigens after DC-based vaccination in mice with 1 antigen identified at 55 kDa. N-terminal sequencing and tandem MS analysis of this antigen showed greater than 85% homology with Kexin. M denotes molecular weight marker. (**B**) Typhoon images of 2D gels of Cy5-labeled naive serum (left) or immunoprecipitated PC antigens (right). Proteins were separated within pH 5–8 for the first dimension. Boxes indicate proteins that were analyzed by matrix-assisted laser desorption/ionization (MALDI) TOF/TOF mass spectrometry.

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 mice or control serum followed by incubation with peritoneal macrophages for 16 hours. Remaining viable PC was quantified by assessing the integrity of PC rRNA using real-time PCR as previously described (7, 15). Incubation with serum from pKexin-immunized mice showed opsonic killing, with an increase in macrophage-mediated killing of PC from 24% in naive mice to 38% in pKexin-immunized mice. However, mice immunized with pKexin/CD40L had the greatest increase in killing, to nearly 80%. This serum-mediated increase in killing was not attenuated by heat inactivation but was significantly abrogated by incubation with protein A and G, suggesting it was due to opsonic anti-PC immunoglobulin in the serum (Figure 7A).

To further investigate whether antibody induction was critical to protection after pKexin/CD40L vaccination, we performed adoptive transfer experiments with 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 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 and B cells were capable of conferring protection against PC. Last, we investigated the antigen specificity of serum from mice given pKexin/CD40L compared with naive serum or serum from CD4-depleted mice for 28 days in a Western blot assay against sonicated PC antigen, as well as staining of mouse and monkey PC. Neither naive serum nor serum from CD4-depleted mice infected with PC for 28 days showed any immunoreactivity against sonicated PC antigen (Figure 7C), demonstrating the critical need for CD4 cells to generate antibody against PC. In contrast, serum from pKexin/CD40L-immunized mice demonstrated robust reactivity to a 105-kDa antigen (the predicted molecular weight of full-length Kexin) as well as to a 55-kDa antigen that migrates a similar distance as the antigen observed in mice immunized with PC-pulsed, 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-indpendent 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 to influence CD8$^+$ cytotoxic T cell (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-modifed 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-

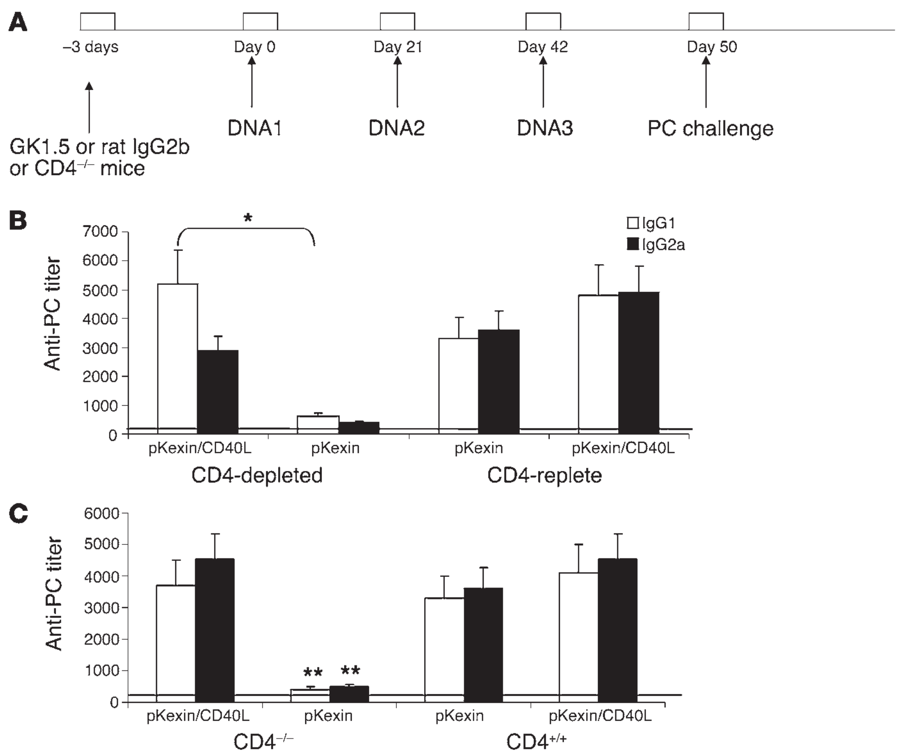

Figure 5
Humoral responses after pKexin or pKexin/CD40L DNA vaccination. (**A**) Schema of PC Kexin vaccination protocol. DNA vectors used were pKexin, pKexin/CD40L, pBUDCE4 (as empty vector), or pCD40L. (**B**) Anti-PC IgG1 and IgG2a responses after pKexin or pKexin/CD40L after intramuscular DNA immunization 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.

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-modifed 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 traduce 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 OVA vaccination described here clearly allows 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-

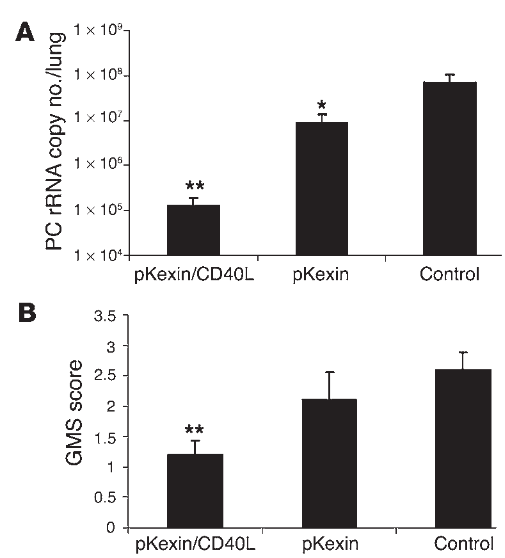

Figure 6
Protection against a primary PC challenge in CD4-depleted mice after DNA immunization. CD4-depleted mice were vaccinated with 3 rounds of control (pCD40L), pKexin, or pKexin/CD40L followed by challenge with PC. PC was quantified by quantitative real-time PCR (**A**) or by histology (**B**).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

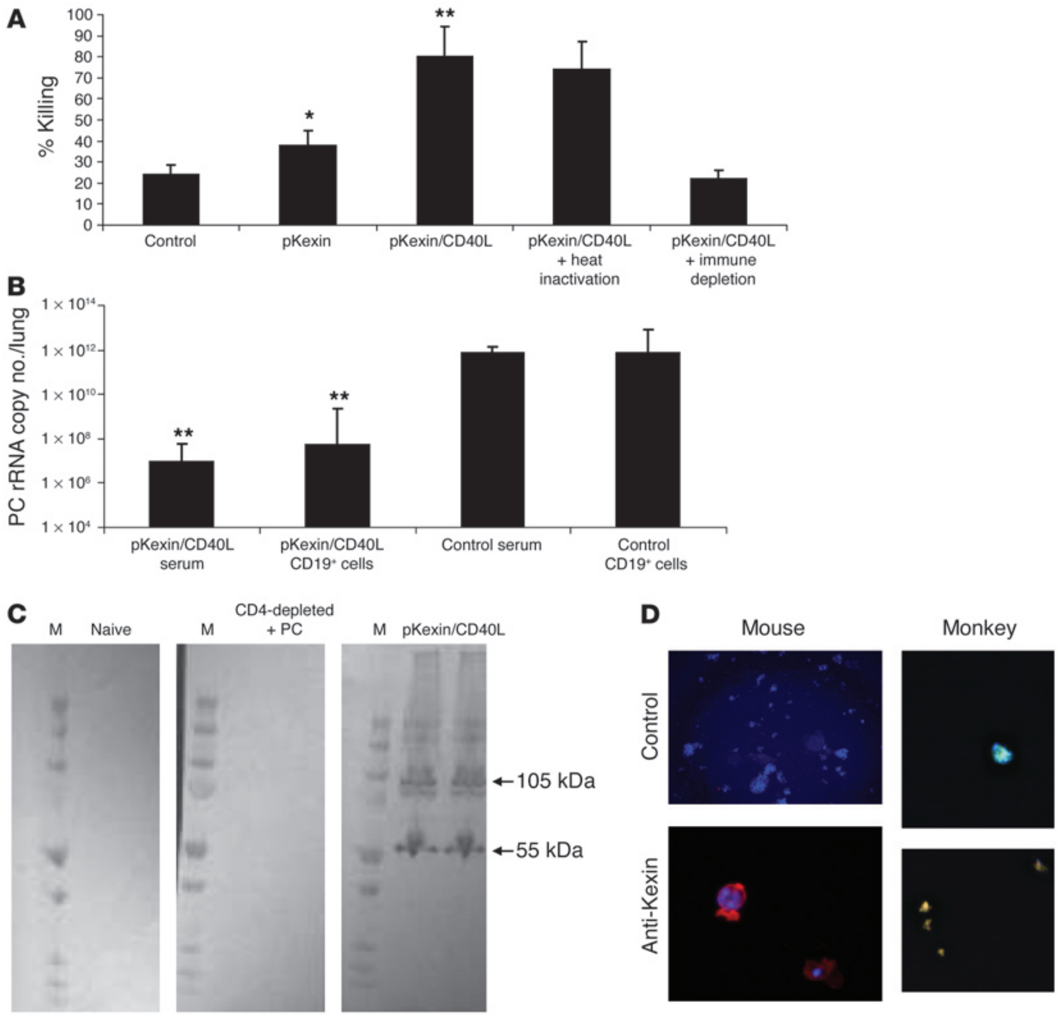

Figure 7

Functionality and antigen specificity of serum after pKexin/CD40L vaccination in mice. (**A**) Opsonic activity of serum after DNA immunization with control, pKexin, or pKexin/CD40L vectors. (**B**) Passive transfer of serum or $CD19^+$ splenocytes confers protection against a PC challenge in SCID mice. $n = 4–6$. All data are mean values ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ compared with control. (**C**) Representative Western blot analysis of serum after Kexin vaccination against sonicated PC antigen. Neither naive serum nor serum from CD4-depleted mice with active PC infection gave any activity on Western blotting. Serum from mice undergoing 3 rounds of pKexin/CD40L vaccination (far right 2 lanes) reacted with a 105- and 55-kDa protein. (**D**) Representative immunofluorescent staining of mouse and monkey PC with anti-Kexin serum.

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-γ–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.

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). 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 2×10^6 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. Antigens 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, 10 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 on 11-cm gel strips with 2% ampholytes at pH 3–10 or 5–8 (Amersham Biosciences and Bio-Rad Laboratories) for the first dimension. The strips were equilibrated in DTT and iodoacetic acid equilibration solutions for 15 minutes each to ensure the dissociation of disulfide bonds within the proteins. The strips were placed in 10% acrylamide gel plates. The 2D gels were run at 25 mA/gel for 30 minutes, then 50 mA/ gel for 4–5 hours. The gels were placed into 40% MeOH/1% acetic acid overnight. Once gels were scanned using a Typhoon fluorescence imager (Amersham Biosciences), they were placed in Bio-Safe Coomassie Blue stain (Bio-Rad Laboratories). Protein spots were extracted and underwent tandem matrix-assisted laser desorption/ionization (MALDI) time-of-flight/TOF (TOF/TOF) analysis (Integrated Biotechnology Laboratories). In addition, some 1D gels were transferred to nitrocellulose, stained with Ponceau S (Sigma-Aldrich), and underwent N-terminal sequencing (Louisiana State University Health Sciences Center Core Laboratories). Sequence data was used to query a yeast database (BLAST; http://www.ncbi.nlm.nih.gov/blast/) using rat PC, and *S. cerevisiae* and *S. pombe* databases.

Plasmids and adenovirus vectors. To investigate the efficiency of DNA vaccination with and without CD40L as an adjuvant, we performed some initial studies with the model antigen the chicken ovalbumin (OVA) cDNA. 1D and 2D gel electrophoresis of PC antigens revealed several antigens (Figure 1), and N-terminal sequence analysis showed 1 antigen with a greater than 80% homology to Kexin from *S. cerevisiae* (GenBank accession number CAA93360). This antigen was chosen for further study, as $CD8^+$ T cell responses against this antigen have correlated with non-progression in an SIV-infect monkey model of PC pneumonia (31). The PC muris *Kexin* cDNA (amplified by PCR) and murine CD40L were cloned into pBUDCE4.1 (Invitrogen Corp.), which contains 2 expression cassettes (1 driven by CMV and 1 driven by the human elongation factor-1α [EF-1α] promoter), allowing both genes to be effectively expressed in transduced cells. CMV was used to express the antigen, and EF-1α was used to express CD40L. These plasmids were termed pOVA/CD40L, pKexin/CD40L, or pOVA, or pKexin as control vectors lacking CD40L. All open reading frames were sequenced using chain termination methodology, and expression of the cassettes was confirmed by transient transfection into 911 cells (32) followed by immunohistochemistry or FACS. Endotoxin-free plasmid DNA for vaccination was produced in *E. coli* (DH5α) and purified using EndoFree Mega kits (QIAGEN). Adenovirus *OVA* (AdOVA) was generated from pCMVOVA after homologous recombination in *E. coli* with AdEasy-1 (Stratagene) (33).

Vaccination protocol and PC challenge. All animal procedures were approved by the Children's Hospital of Pittsburgh Institutional Animal Care and Use Committee. Male 6- to 8-week-old C57BL/6 mice were used for OVA experiments. Male 6- to 8-week-old BALB/c or $CD4^{-/-}$ (B6;129S-$Cd4^{tm1Mak}$) mice and their respective C57BL/6 controls were used for PC Kexin experiments. In the case of antibody depletion of $CD4^+$ T cells, mice were CD4 replete (injected with rat IgG2b or PBS) or depleted of CD4 cells by administration of 0.3 mg GK1.5, a depleting anti-CD4 monoclonal antibody, as previously described (7). This dose of GK1.5 results in greater than 97% depletion of $CD4^+$ T cells in the spleen, thymus, and lung, as measured by staining with RM4-4 (BD Biosciences — Pharmingen), an anti-CD4 antibody that is not blocked by GK1.5. Three days after CD4 depletion, subgroups of mice were vaccinated with 100 μg of pOVA, pOVA/CD40L, pKexin, pKexin/CD40L, or pCD40L DNA intramuscularly into the tibialis anterior muscle every 3 weeks for a total 3 doses (Figures 1A and 5A). To assess in vivo B cell responses, mice were bled every 3 weeks for serum anti-OVA and anti-PC antibody titers. To investigate the effect of boosting after DNA priming, mice vaccinated with OVA were randomized 3 weeks after the third DNA vaccination, to be boosted with AdOVA or AdOVA/AdCD40L.

Mice that were vaccinated with the pKexin/CD40L constructs demonstrated significant anti-PC IgG titers, so we elected to perform a challenge with 2×10^5 PC cysts intratracheally rather than boost with additional antigen (Figure 1B). Mice were sacrificed at 2 and 4 weeks to assess intensity of PC infection by measuring PC organism burden by TaqMan PCR (Applied Biosystems), which measures copy number of PC ribosomal RNA (see below), as well as by GMS stain, as previously described (7).

OVA and PC ELISA. To determine anti-OVA and anti-PC IgG1 and IgG2a titers, ELISA plates (Corning Inc.) were coated with 100 ng of OVA and PC antigen per well in carbonate buffer pH 9.5 overnight. Plates were washed with PBS plus 0.05% Tween-20 (wash buffer) and blocked with BSA and 2% milk. After washing, serial 2-fold dilutions of serum (treated with 0.1 M 2-mercaptoethanol to reduce IgM) were added to each well and incubated for 2 hours at room temperature. After washing, 100 μl of 1:1,000 HRP-conjugated goat anti-mouse IgG1 and IgG2a (SouthernBiotech) were added and incubated for 1 hour at room temperature. After washing, the plates were developed using TMB substrate reagent set (BD Biosciences — Pharmingen), and the absorbance at 450 nm was determined. An OD of 0.1 was used as the cutoff, and the limit of detection of both ELISAs was a titer of 1:32.

Adoptive transfer studies. For adoptive transfer studies, 6- to 8-week-old male SCID mice received 300 μl of immune serum or 5×10^4 $CD19^+$ splenocytes (from mice previously immunized with DNA constructs or PBS). Twenty-four hours later, all mice were challenged with 2×10^5 PC cysts. Mice were sacrificed at 4 weeks for intensity of PC infection by TaqMan PCR or GMS staining.

RNA isolation and TaqMan probes and primers to quantify PC rRNA. To more precisely quantify PC organism burdens in vivo, PC rRNA content was measured in total RNA isolated from the right lungs of infected mice as previously described (7, 15). As a standard for the assay, a portion of PC muris rRNA (GenBank accession number AF7179) was cloned into PCR2.1 (Invitrogen Corp.), and PC rRNA was produced by in vitro transcription using the T7 RNA polymerase. Real-time PCR was carried out using 1-step TaqMan RT-PCR reagents (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 (1×10^6/ml) in a volume of 100 μl were cocultured with 100 μl PC (1×10^4 cysts/ml) for 16 hours at 37°C, 5% CO_2. 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 immunodepleted 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^b: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$^+$ T cells was analyzed with an IFN-γ ELISA (R&D Systems) kit according to the manufacturer's instructions. CD8$^+$ T cells were purified using CD8$^+$ beads (Miltenyi Biotec) from vaccinated mice and were cultured for 48 hours at 37°C and 5% CO_2 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, organisms were extensively washed and stained with anti-mouse Alexa 488 (Molecular Probes; Invitrogen Corp.). For monkey PC experiments, organisms 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 program (GraphPad Prism; GraphPad Software Inc.). Data were tested for differences using ANOVA for mixed and random effect models followed by the Tukey-Krammer range test. *P* values less than 0.05 were considered statistically significant.

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Address correspondence to: Jay K. Kolls, Children's Hospital of Pittsburgh, Suite 3765, 3705 Fifth Avenue, Pittsburgh, Pennsylvania 15213, USA. Phone: (412) 648-7457; Fax: (412) 692-6645; E-mail: jay.kolls@chp.edu.

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