CD4$^+$ T cell–independent vaccination against *Pneumocystis carinii* in mice

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Host defenses are profoundly compromised in HIV-infected hosts due to progressive depletion of CD4$^+$ T lymphocytes. Moreover, deficient CD4$^+$ T lymphocytes impair vaccination approaches to prevent opportunistic infection. Therefore, we investigated a CD4$^+$ T cell–independent vaccine approach to a prototypic AIDS-defining infection, *Pneumocystis carinii* (PC) pneumonia. Here, we demonstrate that bone marrow–derived dendritic cells (DCs) expressing the murine CD40 ligand, when pulsed ex vivo by PC antigen, elicited significant titers of anti-PC IgG in CD4-deficient mice. Vaccinated animals demonstrated significant protection from PC infection, and this protection was the result of an effective humoral response, since adoptive transfer of CD4-depleted splenocytes or serum conferred this protection to CD4-deficient mice. Western blot analysis of PC antigen revealed that DC-vaccinated, CD4-deficient mice predominantly reacted to a 55-kDa PC antigen. These studies show promise for advances in CD4-independent vaccination against HIV-related pathogens.

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Introduction

Despite current strategies to treat HIV infection and its complications, *Pneumocystis carinii* (PC) pneumonia remains a common clinical problem. Although there is a clear relationship between CD4$^+$ lymphocyte count and the risk of PC infection (1, 2), the roles of mononuclear phagocytes, CD8$^+$ cells, natural killer (NK) cells, and their secreted cytokines in host defense against this infection are far less clear. Since it remains unclear whether highly active antiretroviral therapy will result in long-term immune reconstitution of patients with AIDS (3, 4), CD4$^+$ T cell–independent host defense mechanisms operative in opportunistic infections may be critical. There is evidence that B cells can be protective against PC, since uMT knockout mice, which are deficient in B cells, are permissive for PC (5). Although some PC vaccines have shown protection (6–9), to date these data are largely confined to animal models where the vaccine was administered to animals with intact immune systems, followed by immunosuppression and challenge with PC. Recently it has been shown that bone marrow–derived dendritic cells (DCs), which can be activated through CD40 signaling (10, 11), can be genetically modified to express CD40 ligand (CD40L), which activates them and allows them to directly activate B cells in a CD4$^+$ T cell–independent fashion (12). Pulsing these genetically modified DCs with *Pseudomonas aeruginosa* leads to CD4-independent production of anti–*P. aeruginosa* antibody, which is protective against a subsequent bacterial challenge (12). Although this strategy looks promising for bacteria, it remains unclear whether this approach could be used for opportunistic or fungal infections such as PC, where CD4$^+$ T lymphocytes are likely more important.

Here, in a mouse model of PC pneumonia, we tested the ability of bone marrow–derived DCs to be activated through transduction with a recombinant adenovirus encoding murine CD40L; we subsequently pulsed them with PC antigen to induce CD4-independent vaccine responses. These pulsed, activated DCs are capable of inducing levels of anti-PC IgG in CD4-depleted mice that are comparable to the levels observed with PC antigen in CFA, followed by a boost 2 weeks later in incomplete Freund's adjuvant (IFA) in CD4-replete mice. Moreover, vaccination with PC-pulsed, CD40L-modified DCs resulted in significant protection to PC pneumonia in a CD4-independent fashion, and this protection was transferable with both splenocytes and serum from vaccinated mice. These studies demonstrate, for the first time to our knowledge, therapeutic vaccination in a CD4-deficient mouse model, effective against PC pneumonia, a prototypic AIDS-related infection.

These studies show promise for advances in CD4-independent vaccines against HIV-related pathogens.

Methods

Adenovirus vectors. AdCD40L is an E1-E3 replication-deficient recombinant Ad5-based vector containing and expressing the full-length murine CD40L cDNA under the cytomegalovirus immediate early promoter. The control AdLuc vector is identical to this but encoded firefly luciferase as previously described (13). Viruses were propagated in 293 cells, purified as previously described (13, 14).

Dendritic cells. Bone marrow–derived DCs were obtained from hematopoietic progenitors from the femurs of 6- to 8-week-old male BALB/c mice (Charles River Laboratories, Worcester, Massachusetts, USA), and grown in complete RPMI 1640 medium (10% FBS, 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin) supplemented with 100 units/ml recombinant mouse GM-CSF and 20 ng/ml recombinant mouse IL-4 (both from R&D Systems Inc., Minneapolis, Minnesota, USA) (12). Loosely adherent cells were harvested on day 6 by gentle pipetting. DC preparations were over 90% positive for class II MHC (I-A) and CD11c with less than 1% of the cells staining for CD4, CD8, CD19, or DX-5, an NK-cell marker (BD PharMingen, San Diego, California, USA). Transduction efficiencies were carried out by infecting DCs with varying moi of a recombinant adenovirus expressing enhanced green fluorescent protein (eGFP), followed by flow cytometry 24 hours later. An moi of 100 was found to be optimal and resulted in over 90% of the DCs being eGFP-positive. For in vivo vaccination, DCs were modified with adenovirus vectors (AdCD40L or AdLuc, at an moi of 100; Figure 1a). To assess DC activation, culture medium was collected after 24 hours, and the level of mouse IL-12 p70 in the culture medium was determined by ELISA (R&D Systems).

PC antigen vaccine 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 (15), and protein antigen was produced by sonication for 5 minutes. For vaccine preparation, day 6 DCs were transduced with AdCD40L or AdLuc or mock-transduced followed by pulsing with or without PC antigen, for 4 hours at 37°C at a ratio of 1 μg protein to 10^6 DCs. The PC inoculum was prepared as previously described (13, 16). 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, Cincinnati, Ohio, USA), 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, McGaw Park, Illinois, USA). The number of PC cysts was quantified microscopically (16), and the inoculum concentration was adjusted to 2×10^6 cysts per milliliter. Gram stains were performed on the inoculum to exclude contamination with bacteria.

Vaccination protocol and PC challenge. Male 6- to 8-week-old BALB/c mice were depleted of CD4 cells as previously described (13, 16) by administration of 0.3 mg GK1.5, a depleting anti-CD4 monoclonal antibody (17). This dose of GK1.5 results in over 97% depletion of CD4$^+$ T cells in the spleen, thymus, and lung, as measured by staining with RM4-4 (BD Pharmingen), an anti-CD4 antibody that is not blocked by GK1.5. Three days after CD4 depletion, subgroups of mice were vaccinated with 5×10^4 DCs in 100 ml PBS injected intravenously. Mice received PBS DCs only, DCs pulsed with PC, DCs transduced with AdLuc and unpulsed, DCs transduced with AdLuc and pulsed with PC, DCs transduced with AdCD40L, or DCs transduced with AdCD40L and pulsed with PC (n = 8–12 per group, split into two separate experiments).

To assess in vivo B cell responses, mice were bled at 4 weeks for serum anti-PC antibody titers. As a positive control, a subgroup of normal 6- to 8-week-old BALB/c mice were immunized subcutaneously with 25 μg of PC antigen in CFA (Sigma Chemical Co., St. Louis, Missouri, USA) followed by injection of 25 μg of PC antigen in IFA. Two weeks after vaccination, mice were challenged with 2×10^5 PC cysts intratracheally. Mice were sacrificed at 2–4 weeks to assess intensity of PC infection by measuring PC organism burden by TaqMan PCR (Applied Biosystems, Foster City, California, USA), which measures copy number of PC ribosomal RNA (see below), as well as by Gomori methanamine silver (GMS) stain, as previously described (18–20). For adoptive transfer studies, 6- to 8-week-old male BALB/c mice were CD4-depleted with GK1.5 and 3 days later received 300 μl of immune serum or splenocytes (from mice previously immunized with ADCD40L-modified DCs pulsed with PC), or unimmunized serum or splenocytes (from mice receiving DCs only or AdLuc-modified DCs pulsed with PC). 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 stain.

RNA isolation and TaqMan probes and primers for PC rRNA. Total RNA was isolated from the right lungs of infected mice by a single-step method using TRIZOL reagent (Life Technologies Inc., Rockville, Maryland, USA). As a standard for the assay, a portion of PC muris rRNA (GenBank accession no. AF257179) was cloned into PCR2.1 (Invitrogen Corp., Carlsbad, California, USA), and PC rRNA was produced by in vitro transcription using the T7 RNA polymerase. The template was digested with RNase-free DNase, quantitated by spectrophotometry, and aliquoted at –80°C until further use. The TaqMan PCR primers for mouse PC rRNA are 5′-ATG AGG TGA AAA GTC GAA AGG G-3′ and 5′-TGA TTG TCT CAG ATG AAA AAC CTC TT-3′. The probe was labeled with a reporter fluorescent dye, 6-carboxyfluorescein (6FAM), and the sequence was 6FAM-AACAGCCCAGAATAAT-

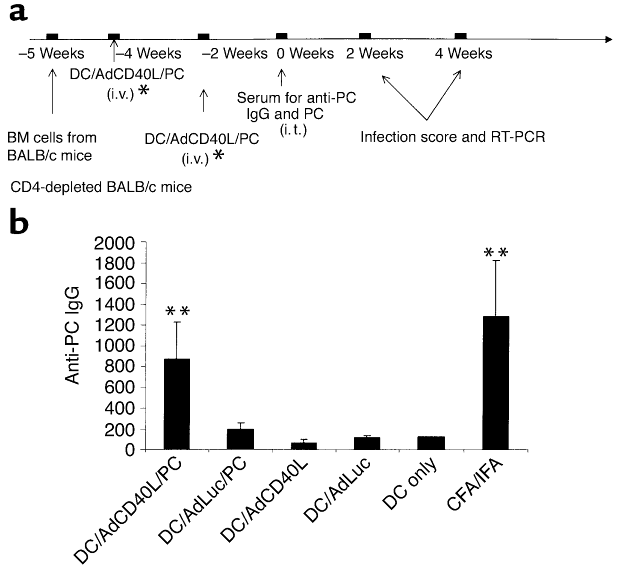

Figure 1

(**a**) Outline of double vaccine protocol. *Vaccine groups: PBS only, DCs only, DC/AdCD40L, DC/AdLuc, DC/AdCD40L/PC, DC/AdLuc/PC. (**b**) Induction of anti-PC IgG after two rounds of DC-based PC vaccine. Mice were vaccinated as outlined in **b**, and serum was obtained at 4 weeks to measure induction of anti-PC IgG. CD4-intact mice were used as a positive control and received PC antigen in CFA followed by IFA 2 weeks later. This serum was harvested at 4 weeks for anti-PC IgG determination (n = 5–6 per group; $^{**}P < 0.05$). BM, bone marrow; i.t., intratracheal; i.v., intravenous.

GAATAAAGTTCCTCAATTGTTAC-TAMRA. Real-time PCR was carried out using one-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 by using a standard curve of known copy number of PC rRNA. This assay has a correlation coefficient higher than 0.98 and over eight logs of PC RNA concentration and correlates with viable PC since both heat killing and exposure to trimethoprim/suflamethoxazole ablate the signal.

PC ELISA and Western blot. To determine anti-PC IgG titers, ELISA plates (Corning Inc., Corning, New York, USA) were coated with 100 ng of PC antigen per well in carbonate buffer pH 9.5, overnight. Plates were washed with PBS + 0.05% Tween-20 (wash buffer) and blocked with BSA and 2% milk. After washing, serial dilutions of serum were added to each well and incubated for 1 hour at room temperature. After washing, 100 μl of 1:1000 alkaline phosphatase–conjugated goat anti-mouse IgG (Bio-Rad Life Science Research Group, Hercules, California, USA) was added and incubated for 1 hour at room temperature. After washing, the plates were developed using Sigma 104 substrate tablets (Sigma Chemical Co.) in diethanolamine buffer and the absorbance at 490 nm was determined. For Western blotting, 100 μg of PC antigen per lane was separated on SDS-PAGE and transferred to PVDF membrane (Millipore Corp., Bedford, Massachusetts). The membrane was blocked with skim milk and cut into strips followed by incubation with 1:100 dilution of mouse serum from the different groups of vaccinated mice. The blot was developed by incubation with a secondary alkaline phosphatase–conjugated goat anti-mouse IgG (Bio-Rad Life Science Research Group) and BCIP/NBT reagent (Bio-Rad Life Science Research Group).

Results

Activation of DCs by AdCD40L. To assess viability and function of AdCD40L, bone marrow was harvested and DCs were derived by culturing the cells in GM-CSF and IL-4 for 6 days as outlined in Methods. DCs that were transduced with AdCD40L secreted significantly greater amounts of IL-12 p70 24 hours after transduction (1,110 ± 198 pg/ml, $n = 4–6$, $P < 0.001$) compared with AdLuc- or mock-transduced DCs (< 15 pg/ml, $n = 4–6$). Moreover, more than 90% of these cells were shown to express CD40L by flow cytometry (data not shown). These data are similar to those reported by Kikuchi and colleagues (12).

AdCD40L-modified DCs as a CD4-independent vaccine against PC. We next assessed in vivo protection of AdCD40L-modified DCs pulsed with PC by administering a single dose of 5×10^4 DCs intravenously. Vaccinated or control mice were then challenged with PC 2 weeks after vaccine administration and infection was scored by both TaqMan and GMS stain.

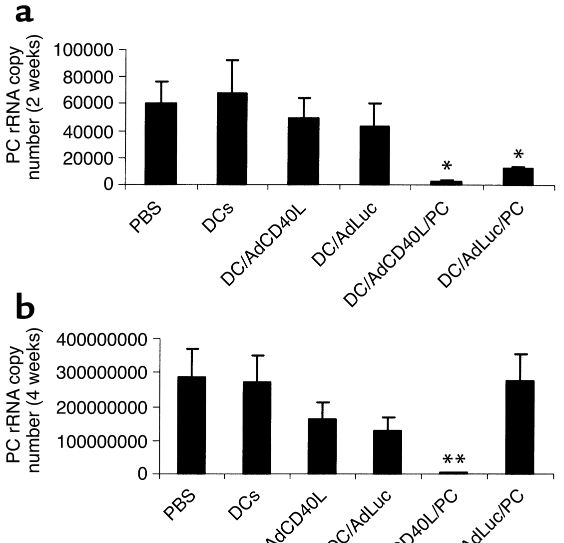

Figure 2

Protection of DC-vaccinated mice to in vivo challenge with PC. Mice were vaccinated as outlined in Figure 1b and then challenged with 2×10^5 PC cysts intratracheally and sacrificed at 2 weeks (**a**) or 4 weeks (**b**) after PC challenge (n = 5–6 per group). $^*P < 0.05$, $^{**}P < 0.001$ versus controls.

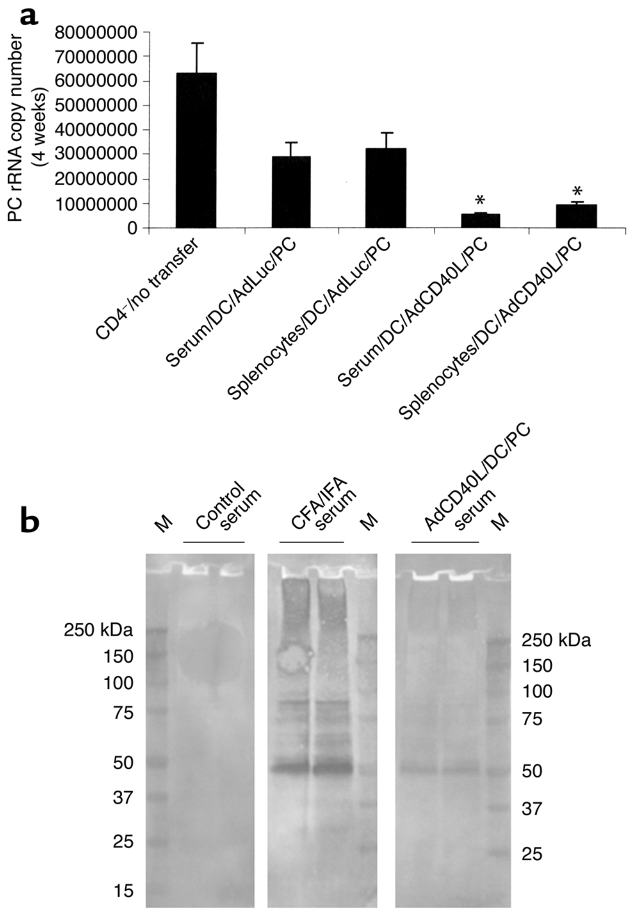

Figure 3

(**a**) Adoptive transfer of serum or splenocytes from DC/AdCD40L/PC-vaccinated mice confers protection to CD4-depleted mice from in vivo challenge with PC. Mice were passively immunized with serum or splenocytes from vaccinated or control CD4-depleted mice. Twenty-four hours later, mice were challenged with PC; they were sacrificed 4 weeks later to assess intensity of PC infection (n = 5–6 per group; $^{*}P < 0.01$ versus controls). (**b**) Immunoreactivity of serum from vaccinated mice. The two lanes of control mice represent one each of DCs alone or AdLuc-modified DCs pulsed with PC. Serum from CFA/IFA-immunized mice resulted in a polyclonal response, whereas serum from two representative AdCD40L/DC/PC mice reacted with a 55-kDa PC antigen.

Although we observed protection, as measured by a decrease in PC rRNA copy number, of approximately one log, there were readily detectable PC cysts in the lung (but in reduced numbers) in the vaccinated group. Since serum antibody titers were only in the 1:128-to-1:256 range, we administered two vaccines 2 weeks apart (Figure 1a).

Administering a series of two vaccines 2 weeks apart to CD4-depleted mice resulted in a significant induction of anti-PC IgG in the group given AdC40L-modified DCs pulsed with PC, compared with respective control groups (Figure 1b). Moreover, these 4-week titers were similar to those achieved in CD4-intact mice vaccinated with PC antigen in CFA and IFA given 2 weeks apart (Figure 1b). To assess in vivo protection, these mice were challenged with 2×10^5 PC cysts intratracheally and sacrificed at 2 and 4 weeks to assess intensity of PC infection. All mice received weekly GK1.5 to ensure continued CD4 depletion. CD4-depleted mice receiving a series of two vaccines with AdCD40L-modified DCs pulsed with PC showed a significant reduction in PC organism burden at both 2 and 4 weeks compared with control mice (Figure 2). Mice vaccinated with AdLuc-modified DCs pulsed with PC showed protection at 2 weeks but not at 4 weeks. This may be due to a low level of antibody, produced by activated DCs alone.

Adoptive transfer of CD4-depleted splenocytes or serum confers protection against PC. In order to determine whether protection from PC infection in the absence of CD4⁺ T cells was mediated by humoral or cellular immunity, we performed adoptive transfer experiments into recipient CD4-depleted mice. BALB/c mice were treated with GK1.5 and 3 days later received 300 μl of serum intravenously from vaccinated mice or the various control groups of mice. Another group of CD4-depleted mice received 10^7 splenocytes by intraperitoneal injection, from the same vaccinated or control mice as used in the serum transfer experiments. Twenty-four hours later mice were challenged with 2×10^5 PC cysts intratracheally; they were sacrificed 4 weeks later to determine intensity of PC infection. The splenocyte preparations were verified to have less than 1% CD4⁺ cells by staining with clone RM4-4 that is not blocked by GK1.5. Mice that received serum or splenocytes from mice previously vaccinated with AdCD40L-modified DCs pulsed with PC demonstrated significant protection compared

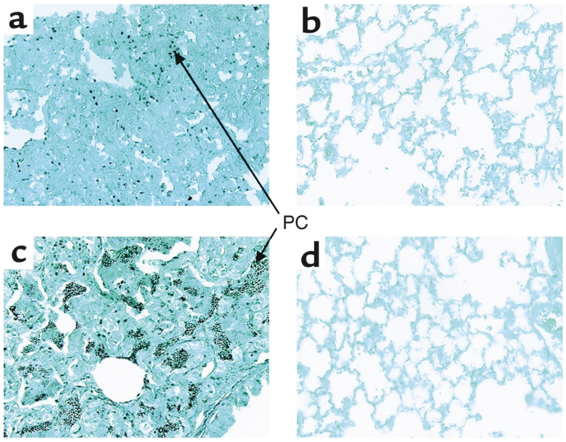

Figure 4

Representative GMS stains from vaccinated or control mice. (**a**) Lung section from a mouse that received splenocytes from CD4-depleted mice vaccinated with AdLuc-modified DCs pulsed with PC. (**b**) Lung section from a mouse that received serum from mice vaccinated with AdCD40L-modified DCs pulsed with PC. (**c**) Lung section of a mouse that received serum from mice vaccinated with AdLuc-modified DCs pulsed with PC. (**d**) Lung section of a mouse that received splenocytes from mice vaccinated with AdCD40L-modified DCs pulsed with PC.

with control mice as measured by PC rRNA copy number and GMS staining of lung sections (Figures 3a and 4). Moreover, adoptive transfer of serum was equally as effective as, if not more effective than, CD4-depleted splenocytes alone in providing protection to CD4-depleted mice, demonstrating that humoral antibody is critical for the protective effect.

Protective serum from AdCD40L-modified DC–vaccinated mice demonstrates a restricted IgG response. To assess the specificity of the protective antibody response, serum from vaccinated and control groups was used at a 1:100 dilution to blot against PC antigen separated by SDS-PAGE. Protective serum from AdCD40L/DC/PC-vaccinated mice reacted predominantly with a 55-kDa antigen of PC (Figure 3b), whereas adjuvant immunized mice demonstrated a polyclonal response. No immunoreactivity was detected at a 1:100 dilution in DC control vaccinated mice (Figure 3b and data not shown).

Discussion

There is a critical need to develop CD4-independent vaccines for opportunistic infections, as the number of immunocompromised hosts is increasing due to HIV infection, as well as iatrogenic causes such as chemotherapy. One strategy 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 (13).

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 allows DCs to interact directly with $CD8^+$ cytotoxic T cells (21, 22) or B cells (23). 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 (12) and confer protection against PA challenge. The studies described in this paper extend these studies to the most common AIDS-defining pathogen, PC, which is critically dependent on $CD4^+$ T cell status.

Interestingly, a finding observed in this study that was not observed in the study by Kikuchi and colleagues in the PA model was that there was a low level of anti-PC IgG generated in some of the DC control groups —namely, AdCD40L-modified DCs, which were not pulsed, and AdLuc-modified DCs that were or were not pulsed (Figure 3). However, in the bacterial model, mice succumb to infection in a few days, whereas PC is a chronic infection that can persist for 8–12 weeks in CD4-depleted BALB/c mice. Thus, one possibility is that unpulsed DCs, after undergoing adenovirus transduction that can activate and mature DCs (24, 25), migrate to the lung and pick up antigen in vivo, which could result in increased protection. Toward this end, we have examined migration of DCs modified with an adenovirus expressing eGFP and can detect these cells in the lungs of mice 24 hours after intravenous administration (data not shown).

Furthermore, the IgG response generated by AdCD40L-modified DC technology was restricted to a 55-kDa antigen of PC which is protective upon adoptive transfer. These data suggest that an optimal humoral response, potentially to the 55-kDa antigen of PC (8, 26), is clearly protective against PC. In support of this is the fact that uMT mice, which are deficient in B cells, are susceptible to PC (5). Moreover, immunization with the major surface glycoprotein (6), p55 PC antigen (9), or whole PC given by an intranasal route (8) has been shown to provide protection in animal models after subsequent immunosuppression and PC challenge. However, these are the first data to our knowledge of successful vaccination of CD4-deficient hosts against PC. Furthermore, the restricted antigen response suggests that this technology could be used for in vivo screening of protective antigens in a $CD4^+$ T cell–independent fashion.

Future experiments will determine the isotype of antibody that confers protection, although data from Garvy and colleagues suggest that antibodies derived from either Th1 or Th2 stimulation are protective (27); moreover, it will be important to further characterize immunodominant antigens responsible for this effect. It is likely critical that this technology, to be effective in the setting of HIV infection, requires normal DC function. It has been reported that DC function, particularly chemotaxis and susceptibility to apoptosis, may be deranged in the context of HIV infection (28, 29). However, pox virus–activated DCs can elicit CD4 and CD8 responses in chronically HIV-infected individuals (30). Thus, targeting DCs ex vivo or in vivo with peptide or a DNA vaccine approach with CD40L as an adjuvant may ultimately induce therapeutic vaccine responses in high-risk individuals.

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