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Protein vaccines, if rendered immunogenic, would facilitate vaccine development against HIV and other pathogens. We compared in nonhuman primates (NHPs) immune responses to HIV Gag p24 within 3G9 antibody to DEC205 ("DEC-HIV Gag p24"), an uptake receptor on dendritic cells, to nontargeted protein, with or without poly ICLC, a synthetic double stranded RNA, as adjuvant. Priming s.c. with 60 μg of both HIV Gag p24 vaccines elicited potent CD4$^+$ T cells secreting IL-2, IFN-γ, and TNF-α, which also proliferated. The responses increased with each of three immunizations and recognized multiple Gag peptides. DEC-HIV Gag p24 showed better cross-priming for CD8$^+$ T cells, whereas the avidity of anti-Gag antibodies was ∼10-fold higher with nontargeted Gag 24 protein. For both protein vaccines, poly ICLC was essential for T- and B-cell immunity. To determine whether adaptive responses could be further enhanced, animals were boosted with New York vaccinia virus (NYVAC)-HIV Gag/Pol/Nef. Gag-specific CD4$^+$ and CD8$^+$ T-cell responses increased markedly after priming with both protein vaccines and poly ICLC. These data reveal qualitative differences in antibody and T-cell responses to DEC-HIV Gag p24 and Gag p24 protein and show that prime boost with protein and adjuvant followed by NYVAC elicits potent cellular immunity.

viral vector | Pox virus

Most current vaccines mediate protection primarily through induction of antibodies (1). However, in many infections such as HIV, malaria, and tuberculosis, there remains a need for protective T-cell immunity. A promising approach under evaluation for such infections is to use prime-boost immunization regimens with heterologous vectors. These include attenuated viral vectors (2) and protein-based vaccines.The focus of this study is to assess how different protein vaccine platforms influence adaptive immunity in nonhuman primates (NHPs) when used with an immune adjuvant with a viral boost.

For certain infections, protein-based vaccines have been underemphasized due to their limited ability to induce strong Th1 immunity and through cross-presentation, CD8$^+$ T cells. This can be greatly improved by enhancing uptake by dendritic cells (DCs), and by using as adjuvants newly recognized, chemically defined agonists for pattern recognition receptors. These agonists convert vaccine-capturing DCs from their normal tolerogenic role to a mature highly immunogenic state (3, 4). One approach to improve delivery of protein vaccines to DCs is to introduce the protein into monoclonal antibodies (mAbs) that efficiently target DC receptors, and then coadminister the fusion mAb with an appropriate agonist for DC maturation (5, 6). The outcome of antigen presentation is also influenced by the particular DC subset that presents the antigen (7, 8). In mice, presentation through the DEC205 DC receptor induces strong Th1 responses and mediates cross-presentation to CD8$^+$ T cells when administered with an adjuvant. Among the adjuvants tested, poly ICLC, a synthetic double-stranded RNA, is a superior innate stimulus for inducing such T-cell responses in mice (4). To advance this platform, due to greater similarities in innate immunity, NHPs may provide a more predictive preclinical model for vaccination of humans.

In this report, we first compared the magnitude and quality of both antibody and T-cell responses in NHPs to the HIV protein, Gag p24, using nontargeted protein or protein delivered within a new human mAb against monkey and human DEC205 (9). Our second major focus was to determine whether T-cell responses induced by the protein vaccines could be enhanced after boosting with a viral vector. In choosing a viral vector, a number of attenuated poxviruses have been developed and used in prime-boost regimens with HIV (10–13). Among these recombinant modified vaccinia virus Ankara (MVA) and New York vaccinia virus (NYVAC) are attenuated forms of vaccinia virus that boost HIV and simian immunodeficiency virus (SIV) Gag and Env responses in NHPs (14–20). Here all animals were boosted with a replication defective, recombinant NYVAC-HIV Gag/Pol/Nef vector (21). We observed a striking and durable boost in Th1 and CD8$^+$ T-cell responses in animals primed with either HIV Gag p24 protein or DEC-HIV Gag p24 and poly ICLC, compared with responses at the time of the boost or with NYVAC-HIV Gag/Pol/Nef immunization alone.

Results

HIV Gag p24 and DEC-HIV Gag p24 Plus Poly ICLC Induce Strong and Durable IFN-γ Producing T-Cell Responses in NHPs. To establish the activity of our protein vaccines in NHPs, a small pilot study was initiated in rhesus macaques already immunized with HIV Gag DNA followed by rAd5-HIV Gag, or rAd5-HIV Gag alone. At the time of this boosting, there were low levels of HIV Gag-specific CD4$^+$ and CD8$^+$ T cells, and we verified that exposure to poly ICLC did not boost the low levels of these cytokine secreting memory cells in blood. Nontargeted HIV Gag p24 or DEC-HIV Gag p24 plus poly ICLC each boosted CD4$^+$ T-cell responses (∼0.5–2.5%) and also, the DEC-targeted vector boosted CD8$^+$ responses in all NHPs tested (Fig. S1).

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The authors declare no conflict of interest.

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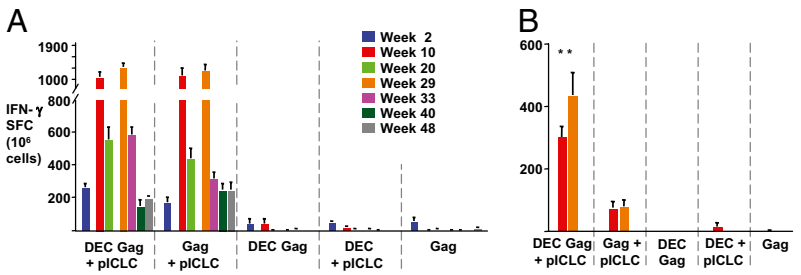

Fig. 1. Immunization with DEC HIV Gag p24 or nontargeted HIV Gag p24 protein and poly ICLC elicits IFN-γ–producing T cells. (*A*) NHPs (four per group) were immunized s.c. three times at wks 0, 8, and 27 with DEC Gag p24 or HIV Gag p24 and poly ICLC. Additional groups were DEC Gag p24 only (n = 2), HIV Gag p24 protein only (n = 3), or DEC "empty" and poly ICLC (n = 2). IFN-γ elispots (SFC)/10^6 PBMCs were quantified after stimulation by HIV Gag p24 pooled peptides. (*B*) As in *A* but isolated, $CD8^+$ T cells were analyzed after the second and third immunizations (means of four NHPs analyzed in triplicate ± SE from a single experiment. $^{**}P < 0.05$ from animals immunized with HIV Gag p24 protein and poly ICLC.

To test the efficacy of protein immunization, we used the s.c. route to deliver DEC-HIV Gag p24 or nontargeted HIV Gag p24 protein with poly ICLC as adjuvant. Poly ICLC is the synthetic double-stranded RNA, poly IC, formulated together with poly L-lysine and carboxymethylcellulose, which induces activation of TLR 3 and MDA-5 (22). Prior studies in mice have shown that this approach elicits strong Th1 responses and a low frequency of $CD8^+$ T cells (3). NHPs were immunized three times with both forms of HIV Gag p24 with or without poly ICLC, and "empty" DEC plus poly ICLC as an additional control.

To initially assess the magnitude of the HIV Gag p24-specific IFN-γ responses, we used ELISPOT analysis of peripheral blood mononuclear cells (PBMCs) (Fig. 1*A*). IFN-γ–producing cells were detectable [~200 spot-forming cells (SFC)/10^6] 2 wk after the first immunization with either HIV Gag p24 protein or DEC-HIV Gag p24 and poly ICLC. There was a marked increase in the responses 2 wk after the second immunization (wk 10) followed by a modest decrease by wk 20. Additional boosting was observed at wk 29, 2 wk after the final or third immunization. Both vaccine groups had sustained IFN-γ responses at wk 48, ~5 mo after the third immunization. At the dose we studied, 60 μg of Gag p24, the numbers of IFN-γ–producing cells were comparable to HIV Gag p24 plus poly ICLC or DEC-HIV Gag p24 plus poly ICLC (Fig. 1*A*), indicating that protein vaccines induce T-cell immunity that can be boosted and is durable.

To assess cross-priming, we measured IFN-γ by ELISPOT in $CD8^+$ T cells enriched from PBMCs 2 wk after the second and third immunizations. DEC targeting was more effective at cross-priming $CD8^+$ cells ($P < 0.05$) in all four animals tested (Fig. 1*B*).

HIV Gag p24 or DEC-HIV Gag p24 Plus Poly ICLC Generates IFN-γ Responses to Multiple Gag Epitopes. To address the breadth of the HIV Gag p24 responses, PBMCs were stimulated with individual 15-mer peptides spanning the entire HIV Gag p24 protein. Most animals had IFN-γ responses to at least three HIV Gag epitopes (Fig. 2).

HIV Gag p24 or DEC-HIV Gag p24 Plus Poly ICLC Generates Multifunctional T Cells. To further characterize the vaccine-induced $CD4^+$ and $CD8^+$ T cells, PBMCs were stained for markers denoting central and effector memory cells and simultaneously analyzed by multiparameter flow cytometry for IFN-γ, IL-2, and TNF-α by intracellular cytokine staining after a 6-h in vitro stimulation with HIV Gag peptides (Fig. S2*A*). Cytokines were detected in both central and effector memory T cells (Fig. S2 *B* and *C*).

To detail response kinetics, intracellular cytokine staining was done after the second (wks 10 and 20) and third (wks 29 and 33) immunizations. Comparable and robust $CD4^+$ Gag-specific frequencies were observed in NHPs immunized with HIV Gag p24 or DEC-HIV Gag p24 plus poly ICLC (Fig. 3*A*, *Top*). In addition, animals immunized with DEC-HIV Gag p24 plus poly ICLC in-

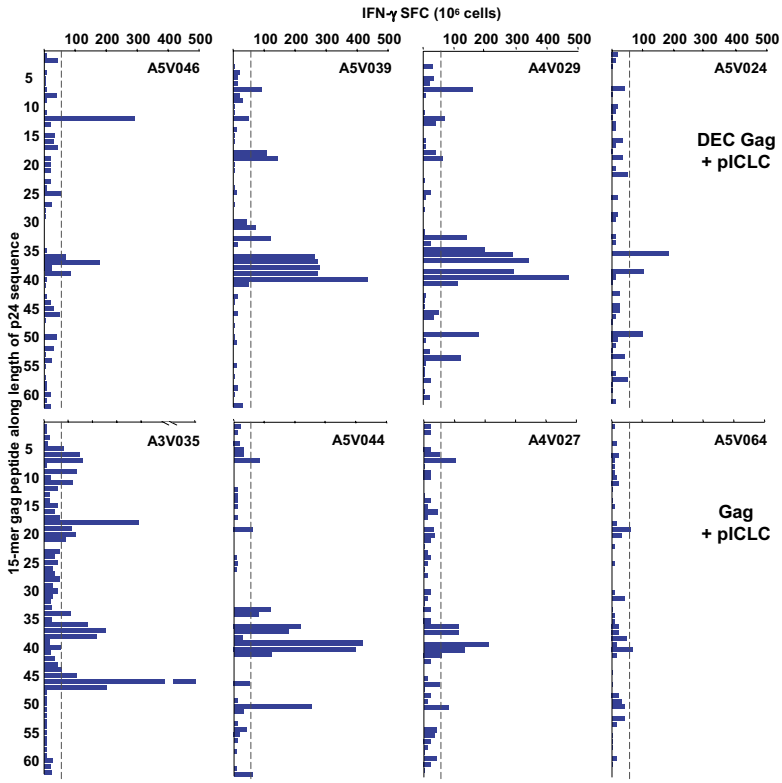

Fig. 2. Breadth of HIV Gag p24 responses. As in Fig. 1*A*, but IFN-γ responses to individual peptides spanning the entire HIV Gag p24 protein at wk 14 by ELISPOT with each animal as a separate plot. The number of HIV Gag peptides with >55 SFC/10^6 cells (dotted line) is noted for each animal.

duced a small but clearly detectable frequency of Gag-specific $CD8^+$ T cells relative to all other vaccine groups (Fig. 3*A*, *Bottom*).

To assess the quality of the responding T cells, the relative proportion of cells making different cytokines were depicted by pie charts, representing the means for the four animals. Both vaccines with poly ICLC induced a high frequency of multifunctional IL-2–, TNF-α–, and IFN-γ–producing $CD4^+$ cells (Fig. 3*B*). Importantly, DEC-HIV Gag p24 with poly ICLC also induced a high proportion of multifunctional $CD8^+$ T-cell responses (Fig. 3*B*, *Right*, red section of pie chart). To extend this analysis, PBMCs at wk 33 after vaccination were stimulated with HIV Gag peptides and supernatants assessed for cytokines and chemokines. Both vaccines induced IL-2, IFN-γ, and MIP 1-β but no detectable IL-4, IL-10, or IL-17 (Fig. S3). The Gag-specific $CD4^+$ and $CD8^+$ T-cell responses were also detected in bronchoalveolar lavage. Hence both vaccines induced strong and durable, multifunctional Th1 responses with DEC-HIV Gag p24 and poly ICLC being more effective to generate $CD8^+$ T cells in blood.

T Cells Induced by HIV Gag p24 or DEC-HIV Gag p24 Plus Poly ICLC Proliferate Following Restimulation. A hallmark of T-cell function is to proliferate following stimulation. Using carboxyfluorescein succinimidyl ester (CFSE) labeling of PBMCs, animals immunized with DEC-HIV Gag p24 and poly ICLC (Fig. S4*A*), and to a lesser extent HIV Gag p24 protein and poly ICLC (Fig. S4*B*), had increased frequencies of CFSE low cells and production of IFN-γ (Fig. 3*C*) from $CD4^+$ T cells following the 5-d culture with HIV Gag. Consistent with the previous data on cross-priming, only the DEC-HIV Gag p24 and poly ICLC immunized animals had consistent proliferation and IFN-γ production from divided $CD8^+$ T cells (Fig. S4*A* and Fig. 3*C*).

HIV Gag p24 Plus Poly ICLC Generates High Titer and Avidity IgG Antibodies. To assess antibody responses, we carried out ELISAs on the sera (Fig. 4*A*). NHPs immunized with both forms of the protein vaccine had robust total Gag-specific IgG titers that were comparable and increased after each immunization. To determine avidity, surface plasmon resonance (SPR) binding analyses was used to measure the kinetics of association and dissociation of serum antibodies with HIV Gag p24 rather than binding at equilibrium in the ELISAs. Remarkably, whereas high avidity responses were observed in all animals immunized with HIV Gag p24 plus poly ICLC, only moderate to low avidity responses were induced by DEC-HIV Gag p24 plus poly ICLC (Fig. 4*B*).

Boosting with Recombinant NYVAC Strongly Enhances IFN-γ–Producing T Cells Following Priming with DEC-HIV Gag p24 or HIV Gag p24 Plus Poly ICLC. Heterologous prime-boost immunization with a variety of vaccine vector platforms can substantially increase immunity compared with homologous prime boosting. Here, 31 wk after the third immunization with the HIV Gag p24 protein vaccines, NHPs were boosted with NYVAC-HIV Gag/Pol/Nef (21). To control for the influence of the NYVAC-HIV Gag/Pol/Nef immunization alone, a group of six naïve animals was added to the study. There were still sizeable (~1%) $CD4^+$ IFN-γ–producing T cells 31 wk after the last immunization with DEC-HIV Gag p24 or HIV Gag p24 protein + poly ICLC, which was the time of the NYVAC-HIV Gag/Pol/Nef boost (Fig. 5*A*, *Top Left*). After a single boost, all primed animals had an approximately two- to four-fold increase in the frequency of $CD4^+$ IFN-γ–producing cells at the peak, which remained stable up to 16 wk (Fig. S5 *A* and *B*). By contrast, there were low to undetectable $CD4^+$ IFN-γ responses in animals boosted with NYVAC after priming with only HIV Gag p24 or DEC-HIV Gag p24 protein alone, and empty DEC and poly ICLC (Fig. 5*A*). There also was a striking increase in the frequency of $CD8^+$ IFN-γ–producing cells in animals primed with DEC-HIV Gag p24 or HIV Gag p24 protein and poly ICLC followed by the NYVAC-HIV Gag/Pol/Nef boost, compared with the other primed animals or those immunized with NYVAC-HIV Gag/Pol/Nef alone (Fig. 5*B*, *Right*). When a second boost with NYVAC-HIV Gag/Pol/Nef boost was given 16 wk later, it did not further enhance the robust memory T-cell responses in both protein vaccine groups (Fig. 5*C*). These data show that a single immunization of replication defective recombinant NYVAC-HIV Gag/Pol/Nef elicits low to undetectable HIV Gag-specific T-cell immunity but as a boost, it leads to a major increase in the magnitude of $CD4^+$ and $CD8^+$ IFN-γ–producing T cells in animals primed with protein vaccine and poly ICLC.

NYVAC Boosting Generates Multifunctional $CD4^+$ and $CD8^+$ Cytokine-Producing T Cells with a Similar Breadth to Those Elicited by Protein Priming. The quality of the prime-boosted responses showed that both $CD4^+$ and $CD8^+$ T cells (Fig. S6 *A* and *B*) were highly poly functional and remained stable over 10 wk after the two protein

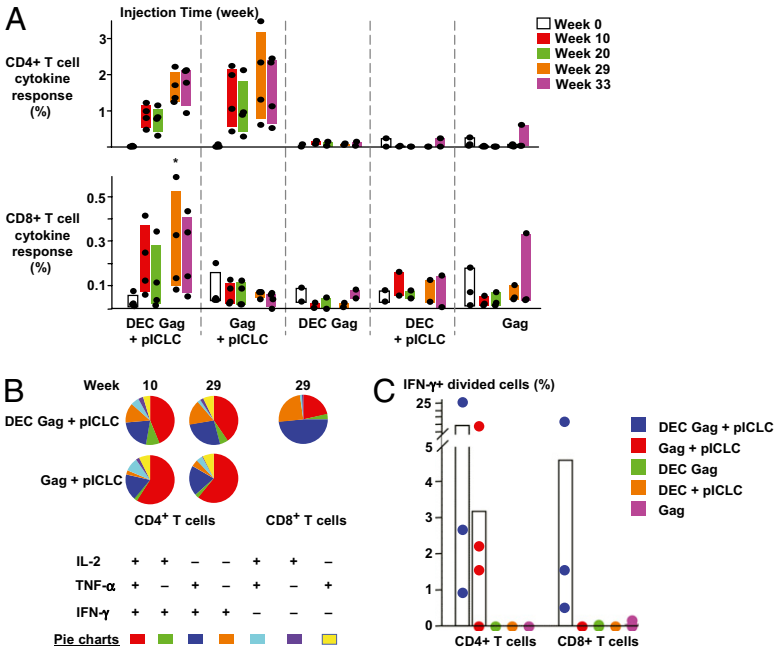

Fig. 3. Features of HIV Gag-specific $CD4^+$ and $CD8^+$ T-cell cytokine responses. (*A*) Frequency of Gag-specific cytokine (IFN-γ, IL-2, or TNF-α) producing $CD4^+$ and $CD8^+$ T cells in PBMCs by multiparameter flow cytometry at indicated times. (*B*) As in *A*, but polyfunctionality of the cytokine responses in frozen PBMCs in a batch analysis, was assessed by SPICE analysis and depicted as pie charts to show relative proportions of seven populations producing different combinations of IFN-γ, IL-2, or TNF-α. Mean of each population's percentages from four NHPs per group at wks 10 and 29. # denotes a difference from animals immunized with HIV Gag p24 protein and poly ICLC ($P < 0.05$) at wk 29. (*C*) CFSE-labeled PBMCs were cultured 5 d ± the entire pool of HIV Gag p24 peptides. The entire pool of HIV Gag peptides was added for 12 h and the frequency of IFN-γ–producing cells assessed by flow cytometry. Dots are individual animals and bars the interquartile range.

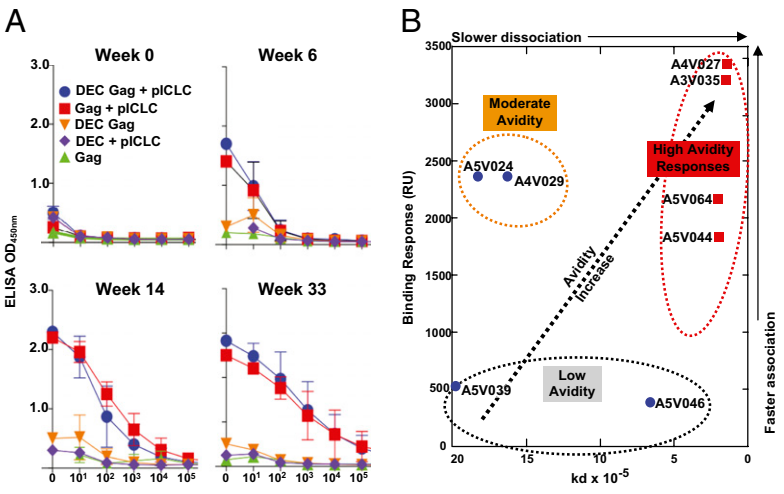

Fig. 4. Antibody binding titers and avidity following protein vaccines. (*A*) HIV Gag-specific antibodies in serum at various times postimmunization. (*B*) Surface plasmon resonance binding avidity map to Gag p24 in RU versus dissociation rate (kDa, s-1). Ranking of relative avidity is based on response magnitude (binding response in RU) and dissociation rate (kDa, s-1). Binding RU and kDa of the control antibody HIVIG at 10 mg/mL is the red circle. Higher avidity antibody responses show higher binding RU and slower kDa values. Ranking of groups based on their relative avidity is outlined in clusters.

vaccines. Furthermore, the breadth of T-cell responses was comparable before and after boosting (Fig. 6). Thus, a robust, broad, durable, and polyfunctional $CD4^+$ and $CD8^+$ T-cell response is generated by boosting a relatively low frequency of cross-primed $CD8^+$ T cells induced by a protein vaccine with a single immunization with NYVAC-HIV Gag/Pol/Nef.

Discussion

Here we show that our selected adjuvant, poly ICLC, was essential to generate antibody and T-cell immunity to nontargeted and DEC-targeted protein vaccines, highlighting the efficacy of poly ICLC as an adjuvant in NHPs with only two immunizations. We uncovered differences in the way DC-targeted and -nontargeted protein vaccines influence the magnitude and quality of the T-cell and antibody response with the same adjuvant in NHPs. With poly ICLC as an adjuvant, and a 60-μg dose of protein, both nontargeted and DEC-targeted HIV Gag p24 protein induced potent multifunctional Th1 responses that also had considerable breadth and durability. Such multifunctional responses prospectively correlated with protection against *Mycobacterium tuberculosis*, *Leishmania major*, and vaccinia virus models of infection in mice (23–25).

In contrast to $CD4^+$ T-cell responses, DEC targeting of HIV Gag p24 allowed for the better induction and recall of $CD8^+$ T-cell immunity. The cross-priming by DEC-targeted HIV Gag appeared more effective as assessed by assays for cytokine-producing $CD8^+$ T cells, proliferative capacity, and for long-lived memory responses that could be boosted by recombinant NYVAC-HIV Gag/Pol/Nef. Conceivably the value of DEC targeting results from improved intracellular traffic and/or processing of HIV Gag in DCs via the DEC receptor or superior cross-presenting features in the DC subsets that express DEC. $CD8^+$ T-cell responses to protein vaccines have been observed in other NHP studies but required conjugation of the protein to a TLR7/8 agonist (26) or the protein needed to be emulsified in montanide with the TLR 7/8 agonist (27); but in these studies, DC targeting was not assessed. $CD8^+$ T-cell immunity has also been generated in NHPs using a hepatitis C virus (HCV) core protein adsorbed onto immune-stimulating complexes (ISCOMs) (28) or a trimeric HIV Env protein given with ISCOMS and CpG (29). These data suggest that various aspects of formulation, which include the nature of the protein itself and DC targeting, will significantly influence cross-presentation.

In terms of adjuvants, a common feature between TLR 7/8 agonists and poly ICLC is the efficacy in inducing type I IFN, which enhances cross-presentation (30) and is essential for poly IC to render DC immunogenic (4). The formulation of poly ICLC whereby poly IC is complexed with poly L-lysine and carboxymethylcellulose was designed to prolong its effects in vivo (31). Recent studies in mice with DEC-targeted HIV Gag (3) or in NHPs with nontargeted keyhole limpet hemocyanin (KLH) (32), showed that poly ICLC was effective in generating $CD4^+$ T-cell responses. In a separate NHP study using the malaria circumsporozoite (CSP) protein and poly IC rather than poly ICLC, we did not observe

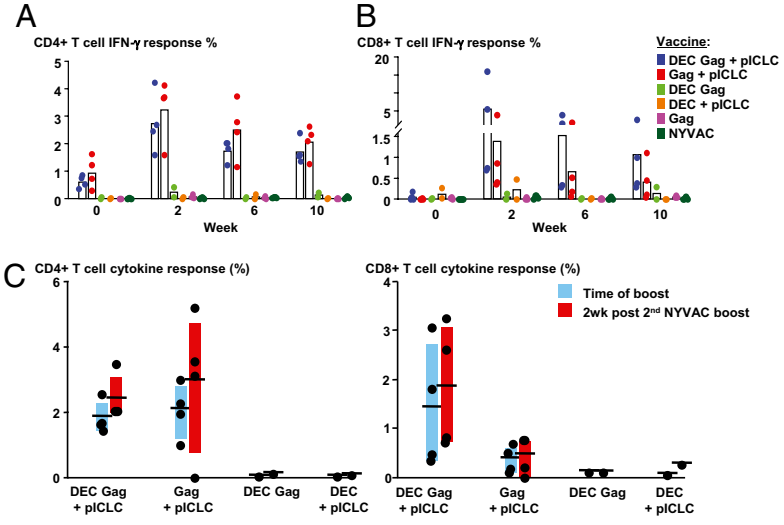

Fig. 5. Magnitude of HIV Gag-specific $CD4^+$ and $CD8^+$ T-cell IFN-γ responses after prime-boost immunization. As in Fig. 3, HIV Gag-specific IFN-γ–producing $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells at 31 wk after the third immunization with the protein vaccines and poly ICLC, which was the time of the NYVAC HIV Gag/Pol/Nef boost (wk 0) and then 2, 6, and 10 wk later. Results are from frozen PBMCs analyzed at the same time. The box plots show the mean of each group. Dots are from individual animals and bars, the interquartile range. (*C*) As in *A* and *B* but responses are shown before and 2 wk after a second boost of NYVAC, which was given 16 wk after the first dose.

$CD8^+$ T-cell responses (33). To assess the safety of poly ICLC, in a separate ongoing NHP vaccine study with an HIV Env protein, we observed no significant adverse events on the basis of blood counts, serum chemistries, and a series of clinical parameters. Moreover, poly ICLC has been administered to humans without major toxicity.

Synthetic double-stranded RNAs lead to enhanced B-cell activation and antibody production in mice (34) and also NHPs (32, 33, 35, 36). We confirmed these findings, but surprisingly, we found that the avidity of Gag-specific antibodies was lower with the DEC-targeted vaccine. This likely results from DEC205 expression by all germinal center B cells, so that processing of Gag by Gag-nonspecific B cells distracts the helper T cells required for high-affinity antibody formation (37).

Our second set of findings was to further optimize HIV Gag-specific T-cell immunity using heterologous prime-boost immunization. Prior studies in NHPs using SIV Gag antigen showed that immunization with DNA followed by MVA (15, 16, 38) or NYVAC (17, 39) strongly enhanced SIV Gag-specific $CD8^+$ responses compared with DNA or poxvirus immunization alone, as detected with Gag-specific tetramer or ELISPOTs. Such responses were functional, as determined by cytotoxic T-lymphocyte (CTL) assays or production of IFN-γ by intracellular cytokine staining (ICS) and correlated with some control of infection following challenge. However, in one study where NHPs were primed with DNA and boosted with either fowlpox, MVA, or vaccinia virus encoding SIV Gag, all of the poxviruses vaccines were comparable in their ability to boost SIV Gag-specific $CD8^+$ T-cell immunity, compared with homologous DNA prime and boost, but there was a rapid decay of the responses at memory time points (20). We suggest that the durability of the $CD8^+$ T-cell responses with our prior prime-boost regimens resulted from the large $CD4^+$ T-cell responses during priming, which could have helped $CD8^+$ responses.

With respect to prior work using HIV Gag antigens to immunize NHPs, DNA vaccines containing full-length HIV Gag or a specific peptide have been used to prime animals followed by recombinant fowlpox virus (rFPV) (14) or MVA (40) boost. Priming led to increased proliferative responses and $CD8^+$ T-cell immunity as determined by CTL assays (14) or by direct staining with a tetramer or ICS following a prolonged (7–10 d) in vitro culture with antigen (40). Here we show that cross-primed HIV Gag-specific, cytokine-producing, effector $CD8^+$ T cells, induced by DEC HIV Gag and to a lesser extent HIV Gag p24 protein together with poly ICLC, are strongly enhanced following a single boost with NYVAC-HIV Gag/Pol/Nef. The magnitude of these responses compare favorably with those observed following prime-boost immunization using DNA prime and Ad-5 or MVA HIV Gag boost (41) or Ad5-HIV Gag prime followed by MVA-HIV Gag boost (19). Although it is difficult to directly compare immunity achieved in different NHP studies with various vectors and inserts, the magnitude, quality, and durability of the $CD4^+$ Th1 and $CD8^+$ T-cell responses after the NYVAC boost in this study are notable.

The observed dichotomy in the potency of NYVAC-HIV Gag/Pol/Nef as a boost compared with its limiting priming for T-cell responses is consistent with prior studies in NHPs and humans using a variety of pox virus vectors and antigens (11, 13). Due to the large number of viral peptides potentially presented from a poxvirus, competition for antigen may limit induction of T cells

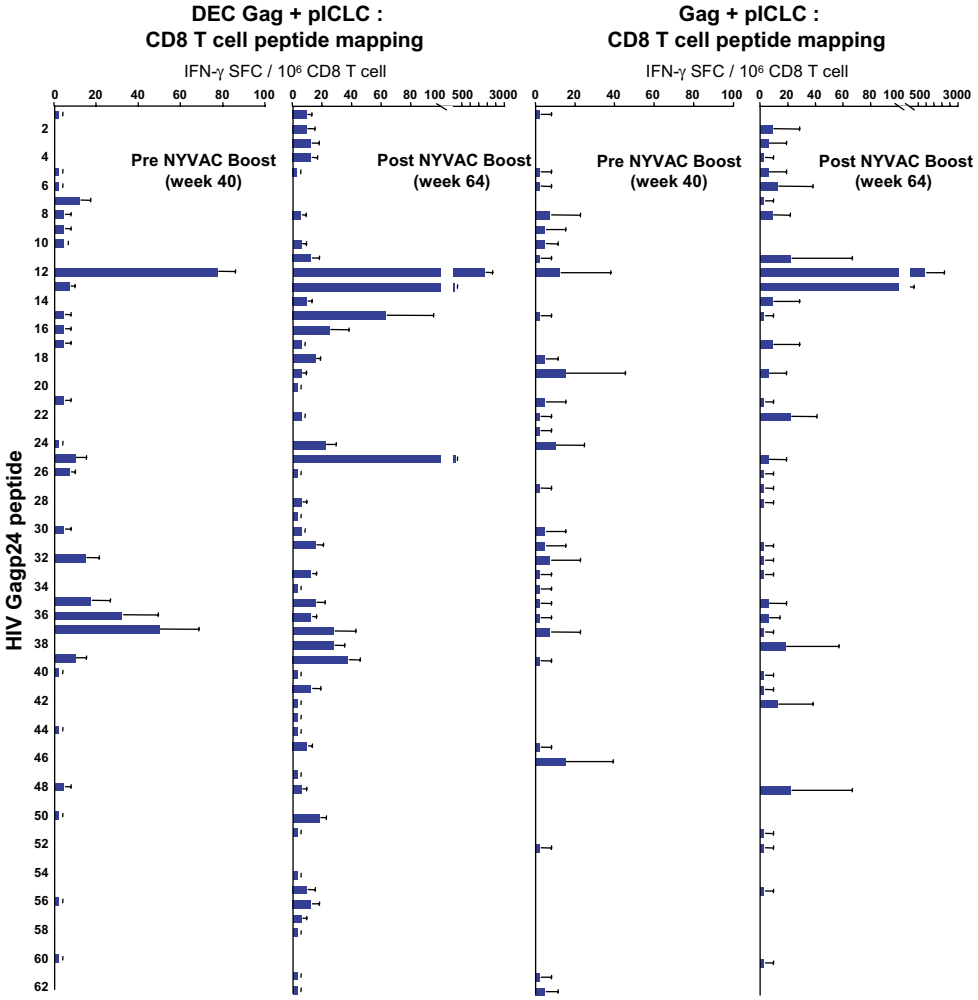

Fig. 6. Breadth of HIV Gag-specific $CD8^+$ T-cell IFN-γ responses before and after boosting with replication defective NYVAC. The breadth analysis is shown by pooling responses by each NHP in each vaccine group before and after NYVAC boost.

specific for the insert. By contrast, the amount of antigen needed to boost memory $CD8^+$ T cells may be less stringent. Innate response induced by NYVAC could also play a critical role in expanding memory T-cell responses.

The studies reported here need to be extended to human subjects to verify whether our heterologous prime-boost immunization with vectors and protein vaccines is a logical vaccine approach to optimize both humoral and cellular immunity and protection against AIDS, malaria, and tuberculosis.

Materials and Methods

Immunizations. To prime NHPs, animals were immunized three times s.c. in the groin with 60 μg HIV Gag p24 protein, with or without 1 mg of poly ICLC, or 200 μg DEC-Gag p24 (containing 60 μg HIV Gag p24) with or without 1 mg of poly ICLC. All injections were given in a total volume of 1 mL in a single site s.c. at wks 0, 8, and 27. For boosting, all primed animals received an injection of NYVAC-HIV Gag/Pol/Nef (10^8 pfu in 0.5 mL) intramuscular (i.m.) 31 wk after the last protein immunization (wk 58).

Additional materials and methods are provided in *SI Materials and Methods*.

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