HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8$^+$ T cell responses in nonhuman primates

Ulrike Wille-Reece*, Barbara J. Flynn*, Karin Loré†, Richard A. Koup†, Ross M. Kedl‡§, Joseph J. Mattapallil¶, Walter R. Weiss‖, Mario Roederer¶, and Robert A. Seder*,**

*Cellular Immunology Section, †Immunology Laboratory, and ¶ImmunoTechnology Section, Vaccine Research Center, National Institutes of Health, Bethesda, MD 20892; ‡3M Pharmaceuticals, St. Paul, MN 55144-1000; and ‖Malaria Program, Naval Medical Research Center, Silver Spring, MD 20910

Communicated by William E. Paul, National Institutes of Health, Bethesda, MD, August 31, 2005 (received for review June 23, 2005)

Induction and maintenance of antibody and T cell responses will be critical for developing a successful vaccine against HIV. A rational approach for generating such responses is to design vaccines or adjuvants that have the capacity to activate specific antigen-presenting cells. In this regard, dendritic cells (DCs) are the most potent antigen-presenting cells for generating primary T cell responses. Here, we report that Toll-like receptor (TLR) agonists and ligands that activate DCs *in vitro* influence the magnitude and quality of the cellular immune response in nonhuman primates (NHPs) when administered with HIV Gag protein. NHPs immunized with HIV Gag protein and a TLR7/8 agonist or a TLR9 ligand [CpG oligodeoxynucleotides (CpG ODN)] had significantly increased Gag-specific T helper 1 and antibody responses, compared with animals immunized with HIV Gag protein alone. Importantly, conjugating the HIV Gag protein to the TLR7/8 agonist (Gag-TLR7/8 conjugate) dramatically enhanced the magnitude and altered the quality of the T helper 1 response, compared with animals immunized with HIV Gag protein and the TLR7/8 agonist or CpG ODN. Furthermore, immunization with the Gag-TLR7/8 conjugate vaccine elicited Gag-specific CD8$^+$ T responses. Collectively, our results show that conjugating HIV Gag protein to a TLR7/8 agonist is an effective way to elicit broad-based adaptive immunity in NHPs. This type of vaccine formulation should have utility in preventive or therapeutic vaccines in which humoral and cellular immunity is required.

vaccine | dendritic cell | cross-presentation | cellular immunity

Tuberculosis, malaria, and AIDS are three major causes of death worldwide. Effective vaccines against such infectious diseases will require antibody and T cell responses or both. At present, live-attenuated vaccines are the only licensed formulation to elicit potent and sustained T cell responses in people. Live vaccines, however, may be limited to use against pulmonary tuberculosis and HIV because of lack of efficacy and potential safety constraints, respectively. Hence, there is an urgent need to develop nonlive vaccine formulations capable of generating T helper 1 (Th1) and CD8$^+$ T cell responses in humans. In a variety of experimental mouse models of intracellular infection, vaccines that activate dendritic cells (DCs) (1) via specific Toll-like receptors (TLRs) (2) markedly enhance T cell responses (3) sufficient to mediate protection (4). DCs are heterogeneous and, in humans and nonhuman primates (NHPs), are comprised of CD11c$^+$ conventional (c)DCs and CD123$^+$ plasmacytoid (p)DCs (5, 6). cDCs have higher expression of MHC class II and costimulatory molecules and are more efficient antigen-presenting cells than are pDCs for initiating primary immune responses (3, 4, 7, 8). In addition, cDCs enhance the differentiation of Th1 cells through production of IL-12 (9, 10). pDCs are notable for their capacity to secrete IFN-α (11–14). IFN-α can induce Th1 differentiation in humans (15), enhances expansion of CD8$^+$ T cell responses, and is required for cross-presentation in mice (16). Based on studies *in vitro* with human cells (10, 17) and *in vivo* in mice (4, 7, 11), it has been speculated that these DC subsets have unique but complementary roles for initiating and maintaining cellular immune responses. Their potential role, however, in generating primary T cell responses in NHPs or humans *in vivo* remains to be determined. Because NHP and human DCs express TLR7 and TLR9 (12, 18, 19), whereas cDCs express TLR7 and TLR8 (12, 19), TLR agonists or ligands selective for such receptors may help delineate the potential contribution these DC subsets have for generating primary cellular immune responses *in vivo*. Here, we show how TLR7/8, TLR8 agonists, or a TLR9 ligand affect the magnitude and quality of the humoral and cellular immune response, when used as vaccine adjuvants with HIV Gag protein in NHPs. Importantly, these studies show that conjugating the HIV Gag protein to the TLR7/8 agonist enhanced Th1 immunity, mediated cross-presentation, and altered the quality of such responses.

Materials and Methods

Animals. Indian rhesus macaques were stratified into comparable groups based on age, weight, sex, and frequency of naïve T cells. Animals were maintained at the animal facility of the Walter Reed Army Institute of Research/Naval Medical Research Center (Silver Spring, MD). All experiments were conducted according to the guidelines of the National Research Council, under protocols approved by the Institutional Animal Care and Use Committee at the Walter Reed Army Institute of Research/Naval Medical Research Center, and the National Institutes of Health.

Immunizations. Animals were injected s.c. at 4-week intervals with HIV Gag protein (200 μg), with or without CpG oligodeoxynucleotides (CpG ODN) (2 mg), the TLR7/8 agonist (2 mg) (20), the TLR8 agonist (2 mg) (20), or HIV Gag protein conjugated to the TLR7/8 agonist (Gag-TLR7/8 conjugate) (200 mg), with PBS as a diluent. Injections were done in a total volume of 0.5 ml in two sites on the back separated by 6 cm. As negative controls, three animals were treated with PBS, the TLR8 agonist, or CpG ODN only.

Reagents. Cytosine phosphate guanosine oligodeoxynucleotides "C" class (CpG ODN, 2395) were purchased from Coley Pharmaceutical Group (Ottawa, Canada). The TLR7/8 agonist (3M-012), a structural analogue of 3M-003, and the TLR8 agonist (3M-002) were provided by 3M Pharmaceuticals (20).

Abbreviations: CpG ODN, cytosine phosphate guanosine oligodeoxynucleotides; DC, dendritic cell; cDC, conventional DC; NHP, nonhuman primate; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; Th, T helper; TLR, Toll-like receptor.

§Present address: Integrated Department of Immunology, University of Colorado Health Sciences Center, Denver, CO 80202.

**To whom correspondence should be addressed. E-mail: rseder@mail.nih.gov.

www.pnas.org/cgi/doi/10.1073/pnas.0507484102

Purified recombinant HIV Gag p41 protein (HXB2 isolate) was made by the Protein Purification Group at the National Cancer Institute (Frederick, MD). Endotoxin levels were <0.05 endotoxin units. The conjugation of the HIV Gag protein to the TLR7/8 agonist (Gag-TLR7/8 conjugate) was performed at 3M Pharmaceuticals, as outlined below. An extensive biochemical and functional analysis excluded the presence of contaminating Gag peptides in the HIV Gag protein and the Gag-TLR7/8 conjugate preparation. Clinical-grade rADV-Gag was obtained from GenVec (Gaithersburg, MD) (21).

Conjugation of the TLR7/8 Agonist to HIV Gag Protein. The TLR7/8 agonist (3M-012) and HIV Gag protein were mixed at approximately a 10:1 drug-to-protein ratio in a 96-well polypropylene plate at pH 9–9.5 and exposed for 2–5 min to UV light. The resulting conjugate was extensively dialyzed in PBS by using a 10-kDa membrane, effectively removing any nonconjugated TLR7/8 agonist and peptides ≤30 amino acids. There were no peptides detected by MALDI analysis. The conjugation procedure was performed under endotoxin-free conditions. A functional *in vitro* assay to determine the immunogenicity of the Gag-TLR7/8 conjugate showed that comparable amounts of IFN-α were elicited from human pDCs in response to 1 μg/ml of either the Gag-TLR7/8 conjugate or the free TLR7/8 agonist (data not shown). Similarly, IL-12p40/p70 production from NHP peripheral blood mononuclear cells (PBMCs) was comparable when the same concentration of the Gag-TLR7/8 conjugate and free TLR7/8 agonist (see Fig. 5, which is published as supporting information on the PNAS web site) were used.

Preparation of PBMCs. PBMCs were isolated from fresh blood by Ficoll density centrifugation by using Accuspin tubes (Sigma) according to the manufacturer's instructions. Cells were used immediately or after cryopreservation for ELISPOT analysis or intracellular FACS staining. Similar results were seen when fresh or cryopreserved cells were used.

Detection of Gag-Specific IFN-γ- and IL-2-Producing Cells by ELISPOT Assay. The frequency of IFN-γ- and IL-2-producing cells from PBMCs was determined by ELISPOT assay. Briefly, 2×10^5 PBMCs were added in triplicate to 96-well plates coated with anti-human IFN-γ (Bender MedSystems, Vienna) or IL-2 (BD Biosciences Pharmingen). HIV Gag pooled peptides (2 μg of 15-mer peptides overlapping by 11 amino acids spanning the entire protein) were added per well and incubated for 18 h at 37°C. The number of spot-forming cells was determined by using the Axioplan 2 imaging system (Zeiss).

Polychromatic Flow Cytometry. PBMCs (5×10^6) were stimulated in complete RPMI medium 1640 for 6 h with αCD28, αCD49d, and Brefeldin A (10 μg/ml each), with or without 2 μg/ml HIV Gag peptides. After stimulation, cells were washed twice in FACS buffer and surface-stained with anti-CD4 Cascade blue (CB), anti-CD8 phycoerythrin (PE) Cy5.5, anti-CD95 allophycocyanin, and anti-CD45RA Texas red PE (TRPE). In addition, during this staining, ethidium monoazide bromide (EMA) (1 μg/ml) was included to label dead cells. Cells were incubated for 15 min in the dark at room temperature (RT) and exposed for 10 min to fluorescent light to photolink the EMA to the DNA. After washing, fixing, and permeabilization, cells were stained with anti-IFN-γ FITC, anti-IL-2 PE, anti-TNF-α PE Cy7, and anti-CD3 allophycocyanin Cy7 for 20 min at RT. Cells were washed twice, resuspended in 1% paraformaldehyde, and analyzed by FACS. Cells (6×10^5 to 1×10^6) were acquired on a LSR II flow cytometer (BD Bioscience Pharmingen), and FACS data were analyzed by using FLOWJO software (Tree Star, Ashland, OR). All mAb reagents, either purified or preconjugated, except for CD45RA TRPE (Immunotech/Beckman Coulter), were obtained from BD Bioscience Pharmingen. Antibodies that were not preconjugated (anti-CD8 PE Cy5.5 and anti-CD4 CB) were conjugated in the laboratory of M. Roederer (Vaccine Research Center, National Institutes of Health) by using standard protocols (http://drmr.com/abcon/index.html). All reagents were validated by titration and comparison with commercial conjugates of the same clone.

Detection of Serum Antibodies. HIV Gag-specific antibodies were detected in serum obtained from animals after the fourth immunization injection. Ninety-six-well plates were coated with HIV Gag protein, serum samples were added in serial dilutions, and, after incubation with horseradish-peroxidase-conjugated anti-IgG (BD Bioscience Pharmingen), plates were developed by using 3,3′,5,5′-tetramethylbenzidine substrate-chromogen (DAKO).

Statistics. All group comparisons were done by using *t* tests for unequal variances on log-transformed data. P values < 0.05 were considered significant.

Results

The Frequency of IFN-γ- and IL-2-Producing Cells After Immunization. Based on their potent and selective stimulatory effects on IL-12 and IFN-α production from NHP PBMCs and human pDCs and cDCs *in vitro* (Fig. 5), the ability of the TLR7/8, TLR8 agonist, or CpG ODN to elicit adaptive immune responses in NHPs when administered with HIV Gag protein was assessed. In addition, based on our recent mouse study showing that immunogenicity for adaptive responses was markedly improved if the HIV Gag protein was physically conjugated to the TLR7/8 agonist (Gag-TLR7/8 conjugate), a group of NHPs also received this conjugate vaccine (22). The animals were immunized four times at 4-week intervals and followed for up to 20 weeks. Animals injected with HIV Gag protein plus the TLR7/8 agonist or CpG ODN, but not the TLR8 agonist, had significantly higher frequencies of IFN-γ- (Fig. 1*A*) and IL-2- (Fig. 1*B*) producing cells, compared with immunization with HIV Gag protein alone. Animals immunized with the Gag-TLR7/8 conjugate had the highest frequencies of both IFN-γ- (Fig. 1*A*) and IL-2- (Fig. 1*B*) producing cells, compared with the other vaccine groups. These responses increased with each injection and were sustained for up to 8 weeks after the final immunization (week 20). Of note, the frequency of IFN-γ-producing cells elicited by the Gag-TLR7/8 conjugate was substantially higher than those observed from another study after immunization of NHPs with plasmid DNA encoding HIV Gag (23). Finally, we did not detect any Gag-specific production of IL-4 or IL-10 from PBMCs stimulated *ex vivo* in any of the vaccine groups after immunization (data not shown). These data show that a protein-based vaccine is, indeed, capable of eliciting strong cellular immune responses in NHPs when administered with a TLR7/8 agonist or a TLR9 ligand. Importantly, conjugation of the HIV Gag protein to the TLR7/8 agonist had a striking effect on increasing the magnitude of these responses.

Characterization of T Cell Responses by Multiparameter Flow Cytometry. To extend the analysis of the cellular immune responses generated after immunization, nine-color flow cytometry was used to characterize the phenotype and enumerate the frequency of memory $CD4^+$ and $CD8^+$ T cells producing IL-2, IFN-γ, and TNF-α after *ex vivo* stimulation. Such cytokines are important for sustaining memory (IL-2) and mediating effector function (IFN-γ and TNF-α) and, thus, provide an assessment of the quality of the response. A gating tree from a representative animal immunized with the Gag-TLR7/8 conjugate is shown in Fig. 2. $CD4^+$ and $CD8^+$ T cells were further separated into $CD45RA^+CD95^-$ and $CD45RA^-CD95^+$,

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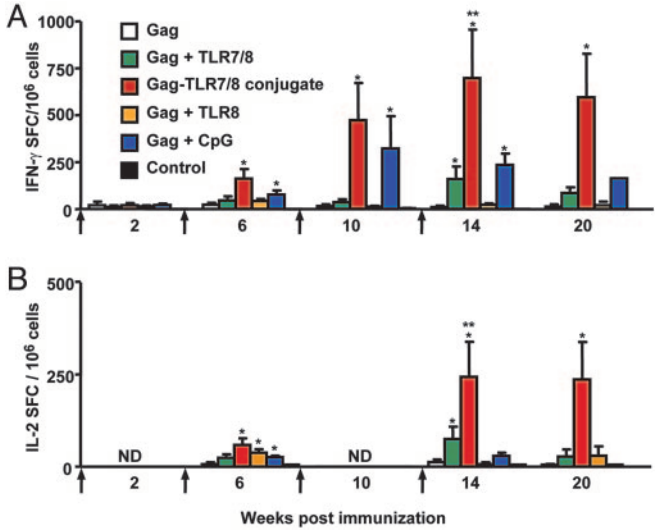

Fig. 1. IFN-γ- and IL-2-producing cells are increased after immunization with HIV Gag protein and a TLR7/8 agonist or CpG ODN. NHPs were immunized four times at 4-week intervals with HIV Gag protein, with or without CpG ODN, TLR7/8, or TLR8 agonist. An additional group was immunized in a similar manner with HIV Gag protein conjugated to the TLR7/8 agonist (Gag-TLR7/8 conjugate). PBMCs were harvested at various times postimmunization, and the frequency of IFN-γ- (*A*) and IL-2- (*B*) producing cells was determined by ELISPOT assay. Data show the means (±SD) of five animals for all time points except week 20 (Gag plus CpG ODN = 1 animal only). $P < 0.05$, compared with Gag group (*) or compared with all other groups (**). ↑ indicates time of immunization; ND, not determined.

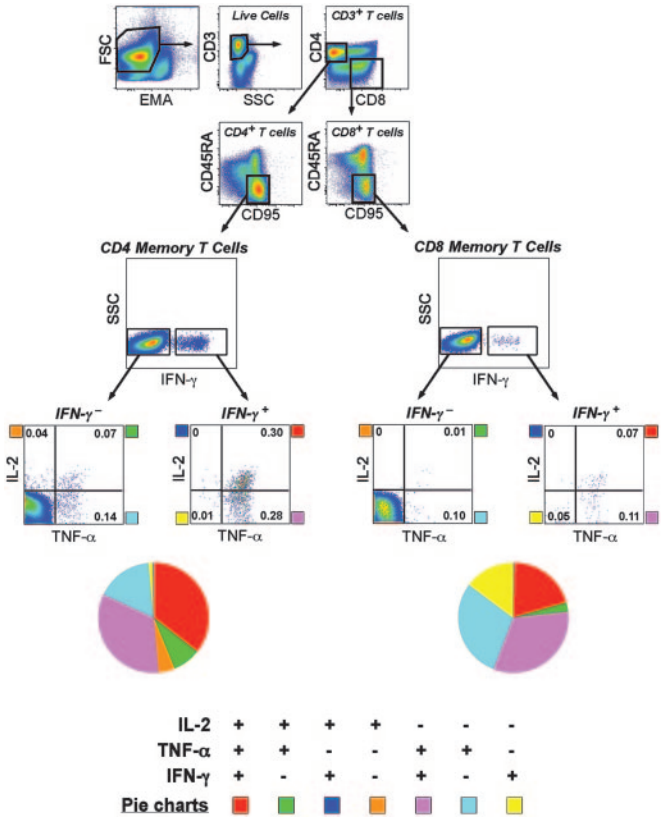

Fig. 2. Functional characterization of IL-2-, IFN-γ- or TNF-α-producing memory T cells by multiparameter flow cytometry. PBMCs were analyzed after the fourth immunization by nine-color flow cytometry. The gating strategy is shown on a representative animal immunized with Gag-TLR7/8 conjugate. The pie charts show the quality of the cytokine response, comprised of seven functionally distinct populations producing IL-2, IFN-γ, and TNF-α, individually or in any combination. The percentages shown are based on the production of the respective cytokines within the $CD45RA^{-}CD95^{+}$ $CD4^{+}$ or $CD8^{+}$ population. FSC, forward scatter; EMA, ethidium monoazide bromide; SSC, side scatter.

which have been shown in NHPs to distinguish between naïve and memory cells, respectively (24). Essentially all cytokine-producing T cells were $CD45RA^{-}CD95^{+}$. This population was used to calculate the total magnitude and quality of the cytokine response and will be referred to as memory cells. Within the gated $CD4^{+}$ or $CD8^{+}$ $CD45RA^{-}CD95^{+}$ memory T cell population, cells were then segregated into IFN-γ^{-} or IFN-γ^{+} cells and further assessed for production of IL-2 and TNF-α or both. This analysis revealed seven functionally distinct populations producing IL-2, IFN-γ, and TNF-α, individually or in any combination. Together, such populations comprise 100% of the total CD4 or CD8 memory cytokine response, and are represented pictorially by the pie charts in Figs. 2 and 3.

Gag-TLR7/8 Conjugate Immunization Generates Polyfunctional Th1 and $CD8^{+}$ T Cell Responses. The magnitude of the total Gag-specific memory $CD4^{+}$ T cell cytokine responses comprising the combined frequency of IL-2-, IFN-γ-, or TNF-α-producing cells are presented for individual animals from each vaccine group after the fourth immunization (Fig. 3*A*). Consistent with the above data, NHPs immunized with the Gag-TLR7/8 conjugate had the highest total cytokine responses, compared with all other vaccine groups. Moreover, animals immunized with HIV Gag protein and the TLR7/8 agonist or CpG ODN had higher responses than animals that received HIV Gag protein, with or without the TLR8 agonist (Fig. 3*A*). With regard to the quality of such responses, the extremely low frequency of total memory-cytokine-producing cells in NHPs immunized with HIV Gag protein, with or without the TLR8 agonist, precluded a consistent determination of the relative frequencies of distinct cytokine-producing populations among the four animals in these vaccine groups. The quality of the memory $CD4^{+}$ T cell cytokine responses derived from the average of the individual animals after immunization with HIV Gag protein plus CpG ODN or the TLR7/8 agonist was similar (Fig. 3*B*), with ≈40% of cells producing IFN-γ only. Interestingly, the composition of the cytokine response was dramatically altered when the HIV Gag protein was conjugated to the TLR7/8 agonist with ≈40% of memory CD4 T cells producing all three cytokines (IL-2, IFN-γ, and TNF-α) and a second major population secreting IFN-γ and TNF-α. Collectively, these data illustrate the heterogeneity of Th1 responses and show that conjugating the HIV Gag protein to the TLR7/8 agonist has a profound effect on both the magnitude and the quality of such responses.

Although there is clear evidence for cross-presentation of protein antigens in mice (16, 25), there are no reports that directly demonstrate this finding in NHPs. Remarkably, four NHPs immunized with the Gag-TLR7/8 conjugate vaccine had clearly detectable $CD8^{+}$ T cell cytokine responses (Fig. 3*C*). Approximately 40% of the total cytokine responses were comprised of IFN-γ- and TNF-α-producing cells, and ≈25% were polyfunctional, secreting all three cytokines (Fig. 3*D*). We would note that the ability of $CD8^{+}$ T cells to secrete IL-2 may be important for their capacity to expand and could enhance their effector function *in vivo*. In the other vaccine groups, the low number of positive events collected precluded a consistent qualitative assessment. Because the generation of $CD8^{+}$ T cell responses by the Gag-TLR7/8 conjugate vaccine

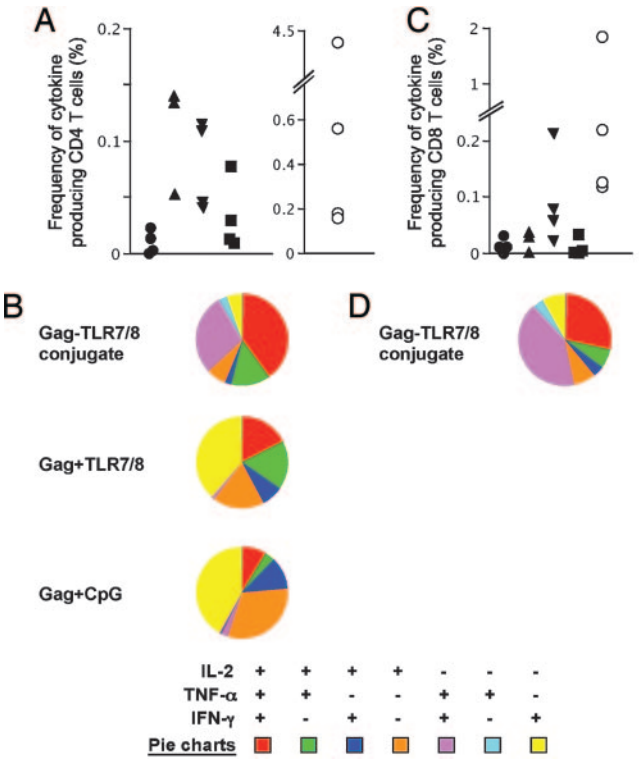

Fig. 3. Gag-TLR7/8 conjugate immunization enhances the magnitude and alters the quality of Th1 and CD8$^+$ T cell responses. Nine-color flow cytometry was performed on PBMCs from NHPs immunized with the TLR7/8, TLR8 agonist, CpG ODN, or Gag-TLR7/8 conjugate after the fourth immunization. Cytokine analysis for IL-2, IFN-γ, or TNF-α was performed on CD45RA$^-$CD95$^+$ CD4$^+$ or CD8$^+$ T cells, as described in Fig. 2. (*A*) The frequencies of the total memory CD4$^+$ T cell cytokine response from four individual animals in all vaccine groups, except CpG ODN (n = 3 animals). (*B*) Quality of CD4 memory response. The total memory CD4$^+$ T cell cytokine response is further divided into seven distinct subpopulations producing any combination of IFN-γ, IL-2, or TNF-α; these data are shown as the means of their respective percentages from the four animals per group (except CpG ODN; n = 3 animals). (*C*) The frequencies of the total memory CD8$^+$ T cell cytokine response from four individual animals (except CpG ODN; n = 3 animals). (*D*) The quality of the memory CD8$^+$ T cell cytokine response is shown as the means from the four animals postimmunization with Gag-TLR7/8 conjugate Vaccine groups (*A* and *C*), ●, Gag; ▲, Gag plus CpG; ▼, Gag plus TLR7/8; ■, Gag plus TLR8; ○, Gag-TLR7/8 conjugate.

provides strong evidence for cross-presentation, it was of interest to compare such responses with those elicited from NHPs immunized with a clinical-grade replication-defective adenovirus expressing HIV Gag (rADV-Gag). The magnitude of the total memory CD8$^+$ T cell cytokine responses from three NHPs immunized with rADV-Gag ranged from 0.1–0.9% and were comprised of cells producing IFN-γ only (≈25%), IFN-γ and TNF-α (≈30%), or IFN-γ, TNF-α, and IL-2 (≈15%) (data not shown). Together, these data show that multiple immunizations with the Gag-TLR7/8 conjugate are required to generate CD8$^+$ T cell responses of comparable magnitude and similar quality, when compared with a single injection of the most immunogenic replication-defective viral vector. Despite the greater efficiency of rADV for eliciting CD8$^+$ T cell responses, preexisting immunity from prior adenoviral infection may limit the immunogenicity of rADV vaccines. In addition, rADV immunization itself will induce antibody responses that may limit its ability to be used for repeated boosting. Thus, the Gag-TLR7/8 conjugate offers a vaccine modality that can be administered repeatedly to boost and/or sustain cellular immune responses.

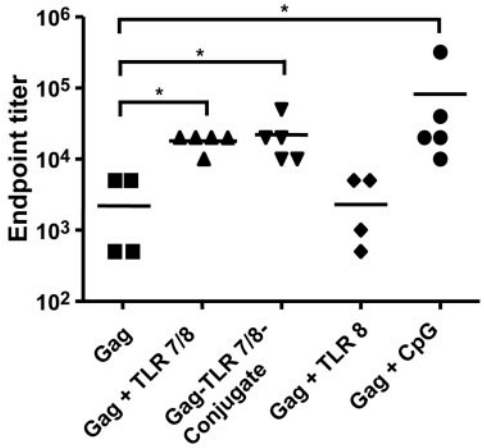

Fig. 4. Immunization with HIV Gag protein and the TLR7/8 agonist, CpG ODN or Gag-TLR7/8 conjugate elicits high-titer antibody responses. HIV Gag-specific antibodies were assessed in serum obtained from NHPs after the fourth immunization. Data are shown from four to five individual animals per group. *, $P < 0.05$.

Immunization with Gag Protein and TLR Agonists Increases Antibody Responses *in Vivo*. The final aspect of this study was to assess the humoral immune responses after immunization. Significant increases in endpoint Gag-specific antibody titers of >10,000 were observed in NHPs immunized with HIV Gag protein and TLR7/8 agonist, CpG ODN, or the Gag-TLR7/8 conjugate, compared with HIV Gag protein alone (Fig. 4). These data are consistent with the expression of TLR9 and TLR7 on B cells (26) and demonstrate that such vaccines induce strong humoral and cellular immune responses in NHPs.

Discussion

A rational approach to vaccine development is to define the immune correlates of protection and then design vaccines to elicit such responses. This report attempts to address both of these issues. The findings that immunization with HIV Gag protein and CpG ODN or the TLR7/8 agonist, but not the TLR8 agonist, elicited significantly greater Th1 responses than did HIV Gag protein suggests that activation of pDCs may be sufficient for generating Th1 responses in NHPs. pDCs could mediate their effects on Th1 differentiation through IFN-α (27) acting directly on the CD4$^+$ T cells. In addition, IFN-α could influence maturation of cDCs, thereby indirectly altering the response. Because pDCs (7, 28) and IFN-α (27) have little direct role for eliciting primary Th1 responses in mice, these data highlight the importance of using NHPs for modeling vaccines in which such responses may be important in people. With regard to the potential role of using TLR agonists to activate cDCs, the TLR8 agonist elicited substantial production of functional IL-12 *in vitro* from NHP PBMCs and human cDCs but was a poor adjuvant for generating Th1 responses *in vivo*. These data suggest a discrepancy between the *in vitro* immunogenicity and the ability of the TLR8 agonist to be an effective adjuvant for eliciting T cell responses. It is possible that increasing the amount of the TLR8 agonist or conjugating it to the HIV Gag protein would have elicited better Th1 responses. Overall, these data provide a baseline for the type of cellular responses that can be generated by mixing these specific TLR adjuvants with the HIV Gag protein.

To test whether changing the vaccine formulation would alter the immunogenicity, a second focus of this study was to determine whether conjugating the HIV Gag protein to the TLR7/8 agonist enhanced immunity *in vivo*. Indeed, immunization with

the Gag-TLR7/8 conjugate vaccine resulted in higher Th1 responses, compared with all other vaccine groups. Furthermore, the generation of $CD8^+$ T cell responses after immunization with Gag-TLR7/8 conjugate is direct evidence that a protein vaccine with a TLR agonist can lead to cross-presentation in NHPs. Potential mechanisms to explain such findings include increased antigen uptake by DCs (29), duration of antigen/adjuvant stimulation, or synchronized delivery of protein and adjuvant to the same cell (30). Moreover, because TLR7 and -8 are expressed intracellularly on endosomes, the conjugate vaccine may alter the magnitude or duration of TLR signaling within the endosome, resulting in better activation (31) and may facilitate processing of the protein into the MHC/class I pathway. Finally, the conjugation process itself may have increased the immunogenicity of the HIV Gag protein through formation of multimeric aggregates

In terms of additional mechanisms that would influence the generation of Gag-specific $CD8^+$ T cell responses, IFN-α has been shown to be required for mediating cross-presentation in mice (16). Here, we show that the TLR7/8 agonist and CpG ODN induced IFN-α from human pDCs *in vitro* and enhanced the generation of Th1 responses *in vivo* when used as adjuvants with HIV Gag protein, suggesting that IFN-α is also induced *in vivo*. However, animals immunized with HIV Gag protein and the TLR7/8 agonist or CpG ODN did not elicit appreciable $CD8^+$ T cell responses. Thus, whereas IFN-α is necessary for cross-presentation in mice, it may not be sufficient in primates with a protein vaccine. Because the TLR7/8 agonist has the capacity to activate both cDCs and pDCs, it remains an open question as to whether either or both of the subsets are required to elicit $CD8^+$ T cell responses. Studies using protein conjugated to CpG ODN or a TLR8 agonist should provide further insight into the specific role that direct activation of plasmacytoid cDCs or cDCs has in this process.

The final aspect of these studies focused on the quality of the T cell responses. The ability to delineate seven functionally distinct T cell populations based on production of IL-2, IFN-γ, and TNF-α emphasizes the heterogeneity of the T cell responses generated after immunization and should be useful for more accurately defining immune correlates of protection for diseases requiring such responses. The ability of the Gag-TLR7/8 conjugate to induce a high frequency of IL-2-, IFN-γ-, and TNF-α-producing memory $CD4^+$ and $CD8^+$ T cells may provide the optimal functional cell. In this regard, IL-2 would be important to sustain memory and mediate expansion of both $CD4^+$ and $CD8^+$ T cells, whereas IFN-γ and TNF-α mediate effector functions. A critical question is whether these polyfunctional cytokine responses would confer protection against a challenge. This cannot be assessed in this study, because the immunogen used was HIV and not simian immune virus (SIV) Gag protein. We used HIV Gag protein so that any promising results could be readily translated into a vaccine regimen in humans. Because of the limited cross-reactivity of HIV to SIV Gag and the fact that HIV does not infect macaque species, there was no utility in challenging animals in this study with SIV. Studies are now underway in which SIV Gag protein will be conjugated to the TLR7/8 agonist to determine whether this formulation alone or in combination with a boost with rADV-Gag will confer protection against challenge. In conclusion, this report shows that a protein-based vaccine may be useful for preventive and therapeutic vaccines for infections and tumors in which humoral and cellular immunity is required. Future studies using protein-TLR7/8 conjugate vaccines alone or in prime-boost regimens with replication-defective viral vaccines will establish whether such an approach will confer protection in NHP models of HIV, malaria, and tuberculosis.

We thank Stephen P. Perfetto and Richard Nguyen for their technical assistance with the LSR, Dr. James Arthos for the biochemical analysis of the Gag-TLR-7/8 conjugate, Stephen Felt for expert husbandry and technical assistance with the vaccinations, and Martha Nason for statistical analysis. This work was supported, in part, by the Naval Medical Research Center Work Unit Science and Technology Objective F 6.2.622787A.0101.870.EFX.

1. Steinman, R. M. & Pope, M. (2002) *J. Clin. Invest.* **109,** 1519–1526.
2. Takeda, K., Kaisho, T. & Akira, S. (2003) *Annu. Rev. Immunol.* **21,** 335–376.
3. Pulendran, B. (2004) *Immunol. Rev.* **199,** 227–250.
4. Shah, J. A., Darrah, P. A., Ambrozak, D. R., Turon, T. N., Mendez, S., Kirman, J., Wu, C. Y., Glaichenhaus, N. & Seder, R. A. (2003) *J. Exp. Med.* **198,** 281–291.
5. Shortman, K. & Liu, Y. J. (2002) *Nat. Rev. Immunol.* **2,** 151–161.
6. Coates, P. T., Barratt-Boyes, S. M., Zhang, L., Donnenberg, V. S., O'Connell, P. J., Logar, A. J., Duncan, F. J., Murphey-Corb, M., Donnenberg, A. D., Morelli, A. E., Maliszewski, C. R. & Thomson, A. W. (2003) *Blood* **102,** 2513–2521.
7. Krug, A., Veeraswamy, R., Pekosz, A., Kanagawa, O., Unanue, E. R., Colonna, M. & Cella, M. (2003) *J. Exp. Med.* **197,** 899–906.
8. LeibundGut-Landmann, S., Waldburger, J. M., Reis e Sousa, C., Acha-Orbea, H. & Reith, W. (2004) *Nat. Immunol.* **5,** 899–908.
9. Ito, T., Amakawa, R., Kaisho, T., Hemmi, H., Tajima, K., Uehira, K., Ozaki, Y., Tomizawa, H., Akira, S. & Fukuhara, S. (2002) *J. Exp. Med.* **195,** 1507–1512.
10. Lore, K., Betts, M. R., Brenchley, J. M., Kuruppu, J., Khojasteh, S., Perfetto, S., Roederer, M., Seder, R. A. & Koup, R. A. (2003) *J. Immunol.* **171,** 4320–4328.
11. Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F. & Trinchieri, G. (2001) *Nat. Immunol.* **2,** 1144–1150.
12. Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. & Lanzavecchia, A. (2001) *Eur. J. Immunol.* **31,** 3388–3393.
13. Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z. K., Endres, S., Krieg, A. M. & Hartmann, G. (2001) *Eur. J. Immunol.* **31,** 2154–2163.
14. Kadowaki, N., Antonenko, S. & Liu, Y. J. (2001) *J. Immunol.* **166,** 2291–2295.
15. Cella, M., Facchetti, F., Lanzavecchia, A. & Colonna, M. (2000) *Nat. Immunol.* **1,** 305–310.
16. Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P. & Tough, D. F. (2003) *Nat. Immunol.* **4,** 1009–1015.
17. Fonteneau, J. F., Gilliet, M., Larsson, M., Dasilva, I., Munz, C., Liu, Y. J. & Bhardwaj, N. (2003) *Blood* **101,** 3520–3526.
18. Bauer, M., Redecke, V., Ellwart, J. W., Scherer, B., Kremer, J. P., Wagner, H. & Lipford, G. B. (2001) *J. Immunol.* **166,** 5000–5007.
19. Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. & Liu, Y. J. (2001) *J. Exp. Med.* **194,** 863–869.
20. Gorden, K. B., Gorski, K. S., Gibson, S. J., Kedl, R. M., Kieper, W. C., Qiu, X., Tomai, M. A., Alkan, S. S. & Vasilakos, J. P. (2005) *J. Immunol.* **174,** 1259–1268.
21. Tritel, M., Stoddard, A. M., Flynn, B. J., Darrah, P. A., Wu, C. Y., Wille, U., Shah, J. A., Xu, L., Betts, M. R., Nabel, G. J., and Seder, R. A. (2003) *J. Immunol.* **171,** 2538–2547.
22. Wille-Reece, U., Wu, C. Y., Flynn, B. J., Kedl, R. M. & Seder, R. A. (2005) *J. Immunol.* **174,** 7676–7683.
23. Casimiro, D. R., Chen, L., Fu, T. M., Evans, R. K., Caulfield, M. J., Davies, M. E., Tang, A., Chen, M., Huang, L., Harris, V., *et al.* (2003) *J. Virol.* **77,** 6305–6313.
24. Pitcher, C. J., Hagen, S. I., Walker, J. M., Lum, R., Mitchell, B. L., Maino, V. C., Axthelm, M. K. & Picker, L. J. (2002) *J. Immunol.* **168,** 29–43.
25. Heath, W. R., Belz, G. T., Behrens, G. M., Smith, C. M., Forehan, S. P., Parish, I. A., Davey, G. M., Wilson, N. S., Carbone, F. R. & Villadangos, J. A. (2004) *Immunol. Rev.* **199,** 9–26.
26. Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S. & Hartmann, G. (2002) *J. Immunol.* **168,** 4531–4537.
27. Rogge, L., D'Ambrosio, D., Biffi, M., Penna, G., Minetti, L. J., Presky, D. H., Adorini, L. & Sinigaglia, F. (1998) *J. Immunol.* **161,** 6567–6574.
28. Boonstra, A., Asselin-Paturel, C., Gilliet, M., Crain, C., Trinchieri, G., Liu, Y. J. & O'Garra, A. (2003) *J. Exp. Med.* **197,** 101–109.
29. Shirota, H., Sano, K., Hirasawa, N., Terui, T., Ohuchi, K., Hattori, T., Shirato, K. & Tamura, G. (2001) *J. Immunol.* **167,** 66–74.
30. Sporri, R. & Reis e Sousa, C. (2005) *Nat. Immunol.* **6,** 163–170.
31. Honda, K., Ohba, Y., Yanai, H., Negishi, H., Mizutani, T., Takaoka, A., Taya, C. & Taniguchi, T. (2005) *Nature* **434,** 1035–1040.