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Prime-Boost Vaccination with HIV-1 Gag Protein and Cytosine Phosphate Guanosine Oligodeoxynucleotide, Followed by Adenovirus, Induces Sustained and Robust Humoral and Cellular Immune Responses

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A prophylactic vaccine for HIV-1 will probably require the induction and maintenance of both humoral and cellular immunity. One current strategy to achieve such long term immune responses is a prime-boost vaccination approach using a DNA priming inoculation, followed by recombinant viral boost. In this report we use a novel prime-boost approach in which the priming injections consist of recombinant HIV-1 Gag protein mixed with cytosine phosphate guanosine oligodeoxynucleotide (CpG ODN), followed by recombinant adenoviral boost expressing HIV-1 Gag. Analysis of the immune responses indicates that HIV-1 Gag protein plus CpG ODN immunization alone induces potent humoral as well as Th1 and CD8+ T cell responses. Boosting with recombinant adenovirus strikingly enhances CD8+, but not Th1, T cell responses, resulting in CD8+ T cell responses far greater in magnitude than Th1 responses. Furthermore, the Th1 and CD8+ T cell responses following prime-boost immunization were seen in both lymphoid and peripheral mucosal organs and were sustained over several months. Together, these data suggest a new immunization approach for elicitation of long term humoral and cellular immune responses. The Journal of Immunology, 2003, 171: 2538–2547.

H uman immunodeficiency virus 1 infection has reached epidemic proportions in many countries around the world. One of the best prospects for the eventual elimination of this disease is the development of a prophylactic HIV-1 vaccine. Successful vaccines against viral pathogens can prevent infection and/or disease through at least two distinct types of immune responses. Humoral immune responses can prevent the virus from entering host cells via direct, Ab-mediated neutralization of virions, thus averting viral replication altogether. By contrast, adaptive cellular immune responses exert their effects on cells already infected with virus and thus are unlikely to completely prevent viral replication. Rather, by eliminating infected cells, these immune responses can potentially control ongoing viral replication, thus averting virus-mediated disease. Each of these mechanisms should be able to contribute to vaccine-induced protection against HIV-1, as evidenced by the prevention of disease in primates pretreated with neutralizing Ab (1, 2) and the association of CD8+ T cell activity with control of HIV infection in both primates and humans (3–7).

While humoral immune responses have been effectively induced by live attenuated, heat-killed, or protein subunit vaccines, live attenuated vaccines have traditionally been the only effective means of inducing robust cellular immune responses (8). More recently, DNA prime-boost vaccines have emerged as an alternate means of inducing both cellular and humoral immune responses (9–13). This approach, in which Ag-encoding DNA is first administered, followed by recombinant virus engineered to express the same Ag, has elicited protection in both mouse and primate models of HIV-1 and malaria (14–18). Furthermore, studies using DNA prime-boost vaccines have shown that the magnitude of the cellular immune response following the recombinant viral boost correlates with the initial response following the priming injections (15). In this regard it is notable that while DNA prime-boost vaccines are immunogenic in primates, the response to DNA immunization alone is relatively modest. Together these data suggest that enhancing the immunogenicity of the initial priming vaccination might substantially affect the magnitude of the immune response following the recombinant viral boost.

One recent strategy for inducing potent humoral and cellular immune responses in mice is the use of cytosine phosphate guanosine oligodeoxynucleotide (CpG ODN)3 as an adjuvant with protein Ag (19, 20). CpG ODN signal through Toll-like receptor 9 (TLR9) (21), inducing both maturation and activation of dendritic cells (DC) and augmenting the production of proinflammatory cytokines (22). This activation of the innate immune response leads to the induction of Ag-specific Ab (23, 24), Th1 (24–26), and CD8+ T cell (22, 24–27, 28)-adaptive immune responses when CpG ODN are coadministered with Ag. In this report we describe

3 Abbreviations used in this paper: CpG ODN, cytosine phosphate guanosine oligodeoxynucleotide; DC, dendritic cell, LN, lymph node; TLR9, Toll-like receptor 9.

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a novel prime-boost vaccine regimen using HIV-1 Gag protein and CpG ODN as the prime, and recombinant adenovirus expressing HIV-1 Gag as the boost, and characterize the immune responses elicited by the vaccine. Our data show that this regimen induces potent and sustained humoral and cellular immune responses.

Materials and Methods

Mice

Female BALB/c mice, 6–10 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Vaccine Research Center Animal Care Facility (Bethesda, MD) under pathogen-free conditions. All experiments were approved by the Vaccine Research Center animal care and use committee.

Reagents

Purified recombinant HIV-1 Gag p24 Ag (SF-2 isolate) produced in yeast cells was purchased from Austral Biologicals (San Ramon, CA). Endotoxin levels in different experiments ranged between <5 and 250 EU/mg by Limulus lysate assay. Variations in the endotoxin level within this range had no effect on immune responses to the vaccine.

Phosphothiorate-modified oligodeoxynucleotide sequence 1826 (CpG ODN) (25) was provided by Coley Pharmaceutical Group (Ottawa, Canada).

Mixtures of 15-mer peptides overlapping by 11 spanning the entire HIV-1 Gag protein were prepared as previously described (29).

Construction and purification of the recombinant adenoviruses

The adeno-virus-Gag vector was prepared by introduction of a Gag insert into a recombinant adenovector deleted in the E1 and E3 regions (30). A Gag gene coding for HIV-1 Gag polyprotein was described previously (31). Briefly, the Gag gene was subcloned into the SalI and BamHI sites of the shuttle plasmid, pAdCMVmcs. The vector expresses HIV-1 Pr55Gag protein (aa 1–432 from HIVB2 GenBank accession no. K03455, and 433-500 from NL4-3, accession no. M19921). To prepare the adenovirus-Gag vectors, 293 cells were plated onto six-well plates and cultured to ~30% confluence, followed by cotransfection with 2 μg of linearized shuttle plasmid and adenovirus cosmid using calcium phosphate as previously described (30). After 7–12 days the supernatant containing recombinant adenovirus was collected. Production of purified recombinant adenovirus was performed by infection of 293 cells with this virus-containing supernatant. The viruses were purified by cesium chloride, aliquoted at 1 × 10^14 particles/ml, and stored in PBS with 13% glycerol at −20°C.

Antibodies

Anti-CD16/CD32, anti-CD28, anti-CD49d, FITC-anti-CD4, PE-anti-IFN-γ, PerCP-anti-CD8a, allophycocyanin-anti-IL-2, and isotype-matched control Ab were obtained from BD PharMingen (San Diego, CA).

Injections

HIV-1 Gag protein (10 μg/mouse) with or without CpG ODN (25 μg/mouse) were mixed in sterile, endotoxin-free PBS and injected s.c. in the rear footpads in a volume of 40 μl/footpad according to the schedule depicted in Fig. 1. Reducing the number of priming injections from three to two sharply decreased the CD8+ T cell responses to the prime-boost vaccine. Adeno-virus-Gag or empty adenovirus (10^10 particles/10 μl) was given i.m. in the rear quadriceps. All injections were administered with a 28-gauge insulin syringe.

Intracellular cytokine staining

Single-cell suspensions were prepared from the spleens, lungs, and draining (popliteal) lymph nodes (LN) of mice at various times postvaccination. Fresh cells from individual spleens or pooled LN or lungs within a vaccine group were stimulated with or without a single pool of HIV-1 Gag peptides spanning the entire protein and 1 μg/ml of costimulatory Ab (anti-CD28 and anti-CD49d) for a total of 5 h (32), adding 10 μg/ml brefeldin A 2 h into the incubation. Identical results were obtained when the anti-CD49d Ab was omitted. The cells were then washed, blocked for 10 min with 0.5 μg/ml anti-CD16/CD32 Ab in PBS/5% powdered milk, and stained with 2.5 μg/ml FITC-anti-CD4 and 3 μg/ml PerCP-anti-CD8a for 30 min at 4°C. The cells were then stained, fixed with 4% paraformaldehyde, and permeabilized with PBS/0.1% saponin buffer containing 5% powdered milk for 1 h at 4°C. The cells were then stained with 2 μg/ml PE-anti-IFN-γ and 1 μg/ml allophycocyanin-anti-IL-2, and analyzed on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ). Typically, between 100,000 and 200,000 total cells were analyzed per sample. FACS data were analyzed using FlowJo software (Tree Star, San Carlos, CA). Isotype-matched controls for cytokines stained <0.06% of CD8+ cells and <0.03% of CD4+ cells. Gating on CD3+ cells before analysis did not alter the percentages of Ag-specific CD8+ and CD4+ T cells.

For CD107a/CD107b staining, 10 μl/ml each of FITC-anti-CD107a and FITC anti-107b (BD PharMingen) was added to cells at the beginning of peptide stimulations together with 0.7 μl/golgiStop (BD Pharmingen) as described previously (32a). After 5 h, the cells were washed and stained as described above.

Detection of HIV-1 Gag-specific Ab

Serum was obtained from mice at various times before and after vaccination. Plates (96-well) coated overnight with 1 μg/ml HIV-1 Gag protein were washed, blocked for 2 h in 10% FBS, and then washed again. The diluted serum samples were added to the wells in duplicate for 1 h at room temperature, the plates were washed, and alkaline phosphatase-conjugated anti-mouse IgG (total; Protos Immunoresearch, Burlingame, CA), anti-mouse IgG1 (BD Pharmingen), or anti-IgG2a (BD Pharmingen) was added at 1/2000 dilution for 1 h at room temperature. After washing, the plates were developed with nitrophenyl phosphate (Southern Biotechnology Associates, Birmingham, AL) and read using a SpectraMax Plus machine (Molecular Devices, Sunnyvale, CA). All washes were performed five times.

Serum from mice vaccinated with HIV-1 Gag protein and CpG ODN was used to generate a standard curve and as a positive control. Only readings within the linear range of the curve were used to determine Ab titers of individual samples. The titer of the positive control was arbitrarily set as 1000 U. This corresponded to a titer of ~100,000 when a reading of 2× background was used to calculate titers. Anti-mouse IgG (total) was always used as the secondary Ab in samples used to generate standard curves. The higher titers in the ELISAs for IgG subtypes relative to the total IgG ELISAs probably results from different specific activities of the different secondary Abs used.

Results

CD8+ T cell cytokine responses elicited by prime-boost immunization with HIV-1 Gag protein and CpG ODN, followed by adenovirus expressing HIV-1 Gag

The aim of our studies was to assess the humoral and cellular immune responses elicited by a novel prime-boost immunization protocol. This protocol uses HIV-1 Gag protein and CpG ODN as the priming immunization, followed by a boost with recombinant adenovirus expressing HIV-1 Gag (henceforth referred to as protein + CpG ODN prime-boost vaccination).
We first assessed CD8⁺ T cell responses following the priming injections. A schematic depicting the experimental design is shown in Fig. 1. BALB/c mice were injected three times with HIV-1 Gag protein and/or CpG ODN (injections 1, 2, and 3). Ten days after the third priming injection (pre-adeno boost), mice from each group were sacrificed, and splenocytes were prepared and stimulated with a pool of overlapping HIV-1 Gag peptides and anti-CD28 costimulatory Ab to assess HIV Gag-specific immune responses. The frequency of IFN-γ⁺ cells within the CD8⁺ T cell population was determined using intracellular cytokine staining as depicted in Fig. 2A. Initially, a lymphocyte-enriched population was selected from total spleen cells by forward/side scatter gating, then CD8⁺/CD4⁻ T cells were selected and analyzed for their production of IFN-γ. As depicted in Fig. 2B (left side), two of three individual mice vaccinated with protein + CpG ODN exhibited potent Ag-specific CD8⁺ T cell responses, while the third mouse exhibited a lower, but still detectable, response of 0.27%, following stimulation with HIV-1 Gag peptides and anti-CD28. In several additional experiments the CD8⁺ IFN-γ responses to immunization with protein + CpG ODN ranged between 0.3–1.8% (data not shown). In marked contrast, mice vaccinated with HIV-1 Gag protein or CpG ODN alone did not exhibit detectable responses to stimulation (Fig. 2C). As a negative control, cells stimulated with anti-CD28 Ab alone did not produce IFN-γ in any of the groups, indicating that cytokine production was Ag specific (Fig. 2B, right side).

Recombinant viral vectors have been shown to greatly enhance CD8⁺ T cell responses when used as a boost following initial immunization.

**FIGURE 2.** The frequency of HIV-1 Gag-specific, IFN-γ⁺ cells within CD8⁺ T cells following protein + CpG ODN immunization. A, Gating analysis. Cells were initially gated on lymphocytes using forward/side scatter. CD4⁺, CD8⁺ cells were then gated (left side) and the frequency of IFN-γ⁺ cells within this population was determined (right side). B, Representative FACS plots of preboost responses. Mice were vaccinated as indicated in Fig. 1. Splenocytes were prepared and stimulated with HIV-1 Gag pooled peptides and costimulatory Ab (anti-CD28) or anti-CD28 alone. CD8⁺ and IFN-γ⁺ cells were detected by intracellular cytokine staining as described in Materials and Methods. C and D, Graphic depiction of pre-boost (C) and postboost (D) responses. E, Individual mice; horizontal bars, group averages. The background (dotted line) is defined as the percentage of cells responding to costimulatory Ab alone. E, Representative FACS plots of cells isolated from mice vaccinated with protein + CpG ODN prime-boost vaccination (top panel) or adenovirus-Gag alone (bottom panel) and stained with anti-CD107a and anti-CD107b during peptide stimulation.
immunization with plasmid DNA (14, 15, 17). We thus assessed the ability of such vectors to enhance immunity induced by protein + CpG ODN vaccination. Mice boosted with recombinant adenovirus expressing HIV-1 Gag exhibited a 7-fold increase in the frequency of Ag-specific, CD8+ IFN-γ-producing cells (compare Fig. 2, C and D). It should be noted that boosting with an empty adenovirus vector did not enhance the CD8+ T cell responses following protein + CpG ODN vaccination (data not shown). Mice primed with HIV-1 Gag protein alone, CpG ODN alone, or PBS exhibited much smaller, but detectable, CD8+ IFN-γ responses following the adenovirus boost. In five separate experiments, all 15 mice that received protein + CpG ODN prime-boost vaccination exhibited CD8+ T cell responses of >3.6%, with an average of 7.9% (data not shown), while the responses to HIV-1 Gag protein, CpG ODN, or PBS followed by adenovirus boost ranged from 1–2%. Together, these data demonstrate the striking enhancement of CD8+ T cell responses to this prime-boost vaccine with respect to either vaccine modality alone.

Secretion of IFN-γ is one mechanism by which CD8+ T cells mediate their effector function. To ascertain whether the CD8+ T cells generated by our vaccine had additional effector function, we assessed the expression of CD107a (lysosomal associated membrane protein-1) and CD107b (lysosomal associated membrane protein-2) from CD8+ T cells following immunization. These markers are only expressed on the surface of CD8+ T cells that are actively degranulating (65) and are inversely correlated with presence of perforin in actively degranulating cells. Thus, expression of CD107 is directly correlated with cytotoxic activity. Thus, in a separate experiment, splenocytes were isolated from mice immunized with protein + CpG ODN prime-boost vaccination or adenovirus alone at 1 mo following the adenovirus boost. The cells were then stimulated with HIV-1 Gag peptides and anti-CD28 in the presence of Ab to CD107a and CD107b, fixed, and stained for intracellular IFN-γ. FACS analysis revealed that essentially all CD8+ T cells positive for IFN-γ+ also expressed CD107a and/or CD107B (Fig. 2E). These data demonstrate that indeed all IFN-γ-producing CD8+ T cells also degranulate in response to HIV-1 Gag peptide stimulation in vitro.

CD4+ T cell cytokine responses elicited by prime-boost immunization with protein + CpG ODN and adenovirus-Gag

We next assessed CD4+ T cell responses following immunization with protein + CpG ODN, in this case assessing both IFN-γ and IL-2 production. Again, responses were initially assessed before the adenovirus boost. The gating used to quantify IFN-γ-secreting CD4+ T cells is shown in Fig. 3A. As shown in Fig. 3B, HIV-1 Gag-specific, CD4+ IFN-γ-producing cells were detected in the spleens of mice immunized with protein + CpG ODN, but not in any of the other groups. Furthermore, in five separate experiments, 12 of 15 mice exhibited detectable IFN-γ responses above background levels, ranging from 0.19–0.7% of CD4+ cells and averaging 0.4%. Following adenovirus-Gag boost, the CD4+ IFN-γ responses to the vaccines increased only slightly to 0.55%, as shown in Fig. 3, C and D. The average CD4+ IFN-γ response following the adenovirus-Gag boost over five experiments was 0.52%, with 15 of 15 mice exhibiting detectable responses (data not shown). In the experiment depicted, mice primed with Gag protein alone, CpG ODN alone, or PBS exhibited postboost responses only slightly lower than those seen in mice primed with protein + CpG ODN. In other experiments, however, these groups exhibited responses significantly lower than the protein + CpG ODN group, ranging from 0.15–0.3%.

The percentage of IL-2-producing cells in the CD4+ T cell population was analyzed in a similar manner. Before the adenovirus boost, IL-2+ cells were detected in the protein + CpG ODN group at a frequency of 0.55% of CD4+ cells, but were not detected in the other groups (Fig. 3E). Following the adenovirus boost, the frequency of CD4+IL-2+ cells in the protein + CpG ODN group did not appreciably change; other groups exhibited lower percentages of ~0.3% (Fig. 3F). Additional analysis of CD4+ cells revealed detectable quantities (0.15–0.3%) of IFN-γ/IL-2+, IFN-γ/IL-2-, and IFN-γ/IL-2+ cells in all responding mice; thus, the IFN-γ- and IL-2-producing cells constitute distinct, but overlapping, subsets of the CD4+ T cell population (data not shown). All 15 mice exhibited detectable CD4+/IL-2 responses to protein + CpG ODN prime-boost vaccination. Ag-specific IL-4 secretion by CD4+ T cells was also examined and was determined to be <0.05% in all mice (data not shown). In summary, protein + CpG ODN vaccination is capable of inducing both Ag-specific CD8+ T cells and Th1 responses; the adenovirus-Gag boost dramatically increases the frequency of cytokine-producing CD8+, but not CD4+ T cells.

Organ distribution of HIV-1 Gag-specific T cells following immunization

The responses described above were assessed using splenocytes. Because effector CD4+ and CD8+ T lymphocytes preferentially reside in nonlymphoid organs (33, 34), we assessed the relative proportions of Ag-specific lymphocytes in a nonlymphoid organ such as lung. In addition, the local immune responses in the draining (popliteal) LN were assessed. When interpreting the data from the LN, it should be noted that the popliteal LN drains the site of the priming injections (administered s.c. in the footpad), but not the site of the adenovirus boost (administered i.m. in the quadriceps). The organ distribution of HIV-1 Gag-specific, IFN-γ-producing CD8+ T cells following the primary injections is depicted in Fig. 4A. Roughly equal numbers of these cells were detected in the spleen and lungs, whereas the numbers in the LN were modestly lower. Ag-specific CD8+ T cells were undetectable in any of the organs examined in the other groups. Following the adenovirus-Gag boost, the overall frequencies of Ag-specific CD8+ T cells in the protein + CpG ODN group sharply increased in both spleen and lungs. The frequency of Ag-specific CD8+ T cells in the LN, while significantly higher than before the boost, was strikingly lower than that in the other organs (Fig. 4B).

Contrary to the results with the CD8+ T cells, the adenovirus-Gag boost did alter the distribution of Ag-specific CD4+ T cells. Specifically, the percentages of cytokine-producing CD4+ T cells decreased in the lungs, increased in the LN, and remained approximately the same in the spleens. As a result, while the lungs had higher percentages than spleens of IFN-γ-secreting cells before the boost, the percentages in the two organs were similar following the boost (Fig. 4, C and D). In the case of IL-2 secretion, spleens and lungs had similar percentages of cytokine-producing cells before the adenovirus-Gag boost, while spleens had a greater percentage following the boost (Fig. 4, E and F). LN generally had lower percentages of Ag-specific CD8+ T cells than the other organs. The lower percentages of CD4+ and CD8+ T cells in LN may be due to the propensity of effector T lymphocytes to migrate out of LN and into the periphery (34). The other experimental groups had similar postboost CD4+ T cell responses as the protein + CpG ODN group in all organs. All these trends were reproducibly observed over several experiments, except that the postboost, CD4+ T cell responses were lower in the groups that had not been primed with protein + CpG ODN (data not shown).
Memory T cell responses following prime-boost immunization

In the previous figures, HIV-1 Gag-specific immune responses were assessed 10 days following the primary immunization or boost, a point at or near the peak level of the responses. To assess the durability of such responses, we performed a separate experiment in which immune responses were analyzed 6 and 11 wk after the adenovirus boost. The results of this experiment are depicted in Fig. 5A. The CD8+ T cell responses in the protein + CpG ODN group following the priming injections were lower than usual in this experiment, but the peak responses following the adenovirus boost were comparable to those in other experiments. Assessment of immune responses at later time points indicated that while the CD8+ T cell responses in the protein + CpG ODN group declined somewhat from peak levels, they remained robust over the course of the experiment (average responses of 5.1 and 4.4% at 6 and 11 wk, respectively). Moreover, CD4+ T cell responses were stable throughout the course of the experiment (Fig. 5B).

In a separate experiment T cell responses were assessed at 16 wk postadenovirus boost. The mice exhibited CD8+ IFN-γ, CD4+ IFN-γ, and CD4+ IL-2 responses of 3.6 ± 0.9, 0.40 ± 0.11, and 0.37 ± 0.10%, respectively (data not shown). Further analysis of the phenotypic markers revealed that 60–90% of cytokine-producing CD8+ and CD4+ cells were CD62Llow, consistent with a memory T cell phenotype (data not shown). Together, these data suggest that cellular immune responses induced by protein + CpG ODN prime-boost vaccination are sustained for several months following the adenovirus boost.

FIGURE 3. Frequency of HIV-1 Gag-specific, IFN-γ+ and IL-2+ cells within CD4+ cells following protein + CpG ODN immunization. A, Gating for CD4+ T cells is similar as that described in Fig. 2. B, Ag-specific CD4+ IFN-γ+ T cells were detected as described for Fig. 2. C and D, Graphic representation of the frequency of IFN-γ+ cells within the CD4+ T cell population before (C) and after (D) adenovirus boost. Values for individual mice (●) and group averages (horizontal bars) are depicted. E and F, Graphic representation of the frequency of IL-2+ cells within the CD4+ T cell population before (E) and after (F) adenovirus boost.
Humoral immune responses following prime-boost immunization

Finally, we characterized the Ab responses elicited by protein + CpG ODN prime-boost immunization. For these experiments mice were bled at various time points, and anti-HIV-1 Gag Ab titers were assessed by ELISA. As shown in Fig. 6A, the mice in the protein + CpG ODN group produced Ab titers several orders of magnitude higher than the other groups. Moreover, the responses were sustained throughout the experiment. Remarkably, anti-HIV-1 Gag Ab responses from mice immunized with protein + CpG ODN were not enhanced following the adenoviral boost. By contrast, anti-HIV-1 Gag Ab titers were enhanced by the adenoviral boost in the other groups. In a separate experiment, we also examined the IgG subtype distribution of the anti-HIV-1 Gag Ab response. As shown in Fig. 6B, protein + CpG ODN immunization elicited demonstratively higher titers of both IgG1 and IgG2a anti-HIV-1 Gag Ab relative to protein immunization alone, both before and after the adenovirus boost.

Discussion

In this report we have demonstrated that vaccination of mice with protein + CpG ODN, followed by adenovirus-Gag, elicits robust Ag-specific Ab and Th1+ and CD8+ T cell responses. The responses can be detected in several organs and persist for at least several months after vaccination. While previous work has shown that CpG ODN can stimulate CTL, Th1, and Ab responses to protein Ag (22–28), this is the first report in which protein + CpG ODN vaccination is combined with a recombinant viral boost. Moreover, the Ag-specific CD8+ T cells elicited by the vaccine were shown to be capable of multiple effector functions, in that they were capable of both cytokine production and degranulation in response to stimulation. These findings provide the first evidence that CD107a/CD107b staining can be used in mouse cells to demonstrate multiple effector functions at the single-cell level.

Previous studies have shown that DNA prime-boost vaccination leads to higher immune responses compared with either modality alone (14, 17). It is particularly notable that the relatively limited CD8+ T cell responses induced by the DNA priming inoculations have such profound effects on responses following a heterologous vaccine boost (15, 17). Similarly, in these studies the CD8+ T cell responses following the adenovirus boost were greatly enhanced by prior priming with protein + CpG ODN. This enhancement was noted even in experiments in which the initial CD8+ T cell responses were relatively low (Fig. 5). The priming injections may accomplish this by influencing the immunodominance hierarchy of the subsequent response to the boost to favor the recombinant Ag (35). The viral boost may selectively expand the small pool of Ag-specific CD8+ T lymphocytes by inducing IFN-α production, leading to IL-15 production (36), which has been shown to enhance CD8+ T cell proliferation and survival (37–40). An alternate mechanism for the effect of the priming injections comes from
the recent finding that Ag-specific CD4⁺ T cells are required for secondary expansion of memory CD8⁺ T lymphocytes (41). Similarly, Ag-specific CD4⁺ T cells induced by the priming injections of protein + CpG ODN prime-boost vaccination may enhance CD8⁺ T cell responses following the adenovirus boost.

While CD8⁺ IFN-γ responses following immunization with protein + CpG ODN were dramatically enhanced following adenoviral boost, Th1 responses were not substantially enhanced (Fig. 2, C and D, and Fig. 3, C and F). Similar results were obtained in a different mouse strain (C57BL/6) and in BALB/c mice primed with Gag DNA and boosted with adenovirus-Gag (data not shown). In related findings, recombinant adenovirus by itself has been shown here (Fig. 2D and Fig. 3, D and F) and by others (42) to prime CD8⁺ T cell responses more strongly than CD4⁺ T cell responses. This may be because adenovirus, which efficiently infects DC in vivo (43, 44), induces production of IFN-α (in the case of E1A-deleted vectors such as the one used here), but not IL-12 p70, by DC (43, 45–47). In mice, IFN-α has a greater effect on inducing CD8⁺ compared with Th1 responses (48, 49). While some studies have reported the efficient production of IL-12 p70 and induction of CD4⁺ T cell responses by adenovirus, the DC in those studies were either manipulated in vitro or infected at a high multiplicity of infection, which might not reflect the in vivo responses to adenovirus administered in a vaccine (50).

It is also notable that CD8⁺ T cell cytokine responses achieved by protein + CpG ODN prime-boost vaccination were substantially higher than Th1 responses. This finding is consistent with other studies showing that CD8⁺ T cell responses to a variety of vaccines and viral and bacterial pathogens are greater than the concurrent CD4⁺ T cell responses (51). This phenomenon may result from the greater replicative capacity and faster replication rate of CD8⁺ T cells following activation (51) and/or the ubiquity of MHC class I molecules relative to MHC class II molecules or may reflect a higher ceiling of the CD8⁺ response as a result of homeostatic mechanisms.

In this study CpG ODN also dramatically enhanced Ab responses to recombinant protein. This property of CpG ODN has been shown for primates (52, 53) and humans (54) as well as mice (23), suggesting that protein + CpG ODN prime-boost vaccination may be an effective strategy for induction of humoral responses in these species as well. Contrary to observations with DNA prime-boost vaccination (13), the Ab responses following administration of protein + CpG ODN were not increased following adenovirus-Gag boost (Fig. 6A). This may reflect the fact that CD4⁺ T cell responses were not boosted by adenovirus-Gag (Fig. 3, D and F). When specific IgG isotypes were assayed, mice immunized with protein alone produced only IgG1 Ab, while mice immunized with protein + CpG ODN produced high titers of both IgG1 and IgG2a Ab (Fig. 6B). This increase in IgG2a is consistent with the induction of Th1 cells induced by CpG ODN.

Similarly to DNA prime-boost vaccination, protein + CpG ODN prime-boost vaccination elicits robust cellular and humoral immune responses without the safety concerns associated with live attenuated virus. This vaccine therefore warrants consideration as an alternative to DNA prime-boost vaccination for pathogens requiring cellular immune responses, such as HIV-1, tuberculosis, malaria, and leishmania. Immune responses to DNA prime-boost vaccines are likely to be limited by inefficient transfection of mammalian cells by DNA (55–57), suggesting that it may be possible to further improve the robust immune responses achieved by these vaccines. Increasing the level of Ag by codon optimization (13, 30) or the addition of adjuvants (17) may substantially improve the immunogenicity of DNA vaccines; however, these measures do not address the low transfection efficiency inherent in DNA formulations currently approved for human use. Increased Ag levels
achieved by protein + CpG ODN administration should increase the number of cells presenting recombinant Ag relative to DNA vaccination, which should, in turn, improve peak responses following the recombinant viral boost (13, 17).

Part of the immunogenicity of DNA vaccination is due to the presence of unmethylated CpG ODN motifs in the injected plasmid (58–60). Protein + CpG ODN vaccination offers an advantage over DNA vaccination in that the CpG ODN motifs present are explicitly defined, and their levels can be precisely modulated. Moreover, specific CpG ODN motifs can be chosen to elicit CTL, Th1, or Ab responses in both rodents and primates (52, 53, 61), depending on the requirements of a particular vaccine.

FIGURE 6. A, HIV-1 Gag-specific Ab responses following protein + CpG ODN prime-boost immunization. Mice were vaccinated as indicated in Fig. 1 and were bled 1.5 wk after the third priming injection or 1.5 or 6 wk after the adenovirus boost. Anti-HIV-1 Gag Ab titers (total IgG) were determined by ELISA. B, In a separate experiment sera from mice bled after the third priming injection or 10 days after the adenovirus boost were assayed for anti-HIV-1 Gag total IgG (top panel), IgG1 (middle panel), or IgG2a (bottom panel) Ab.
Several questions remain regarding the use of protein + CpG ODN prime-boost vaccination in primates and humans. First, the distribution of cells expressing TLR9 molecules is more widespread in mice than primates and humans, potentially limiting the immunogenicity of CpG ODN (19, 61). Another issue is whether CpG ODN are capable of inducing cross-priming in primates or humans. DNA vaccines elicit CD8+ T cell responses by programing transfected cells to express the recombinant Ag in the cytosol, facilitating delivery into the MHC class I Ag-processing pathway (62, 63). Recombinant protein, by contrast, does not tend to elicit CD8+ T cell responses because it is internalized by endosomes, which feed exclusively into the MHC class II processing pathway. Exogenous Ag can in some circumstances be presented on MHC class I molecules, a phenomenon known as cross-presentation (64). This study shows that CpG ODN can induce cross-presentation in BALB/c mice, but CpG ODN have not yet been demonstrated to have this property in primates. Even if CpG ODN cannot induce cross-presentation in primates and humans, protein + CpG ODN prime-boost vaccination should be capable of inducing significant Th1 and Ab responses (via the priming injections) and CD8+ T cell responses (via the adenosine boost), making it a useful vaccine paradigm in the future for diseases requiring cell-mediated immunity.

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