Enhanced Antibody Responses Elicited by a CpG Adjuvant Do Not Improve the Protective Effect of an Aldrithiol-2-Inactivated Simian Immunodeficiency Virus Therapeutic AIDS Vaccine\textsuperscript{\textregistered}

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The potential benefit of using unmethylated CpG oligoribodeoxynucleotides (ODN) as an adjuvant in a therapeutic simian immunodeficiency virus (SIV) vaccine consisting of AT2-inactivated SIVmac239 was evaluated in SIV-infected rhesus macaques receiving antiretroviral therapy (ART). We hypothesized that using CpG ODN as an adjuvant in therapeutic vaccination would enhance SIV-specific immune responses and suppress SIV replication after ART was stopped. To test our hypothesis, we immunized chronically SIV-infected rhesus macaques receiving ART with one of the following therapeutic vaccines: (i) AT2-inactivated SIVmac239, (ii) CpG10103 plus AT2-inactivated SIVmac239, (iii) CpG10103, and (iv) saline. While immunization with CpG plus AT2-SIVmac239 significantly increased SIV-specific immunoglobulin G (IgG) antibody titers, the mean plasma viral RNA (vRNA) level in these animals after ART did not differ from those of saline-treated animals. The AT2-inactivated SIVmac239-immunized animal group had a significantly higher mean SIV-specific gamma interferon T-cell response after three immunizations and lower plasma vRNA levels for 6 weeks after ART was withdrawn compared to the saline-treated animal group. Compared to the saline control group, the animal group treated with CpG alone had a significantly higher mean SIV-specific lymphocyte proliferation index and a higher rate of plasma vRNA rebound after ART. These results demonstrate that while the use of CpG as an adjuvant enhances SIV-specific antibody responses, this does not improve the control of SIV replication after ART is stopped. The lack of benefit may be related to the high levels of SIV-specific lymphocyte proliferation in the CpG adjuvant group.

Antiretroviral therapy (ART) is effective in suppressing human immunodeficiency virus (HIV) replication and maintaining a symptom-free stage of HIV infection for extended periods in many patients (33). Despite the considerable efficacy of ART, the long-term benefits of ART are limited by the emergence of drug-resistant strains (29), drug toxicity (38), and the inability to eradicate viral reservoirs (10, 11, 13). In very early stages of HIV infection, virus-specific CD4\textsuperscript{+} T cells are dramatically depleted (16, 36, 44). Although the total CD4 T-cell counts of many patients rise after the initiation of ART, the persistent depletion and/or anergy of HIV-specific CD4\textsuperscript{+} T cells often does not improve (7, 20, 34). In HIV and simian immunodeficiency virus (SIV) infections, antiviral CD8\textsuperscript{+} T-cell immune responses play critical roles in controlling viral replication (5, 6, 35, 40). Thus, improvement of the anti-HIV/SIV T-cell immunity during ART by immunotherapeutic intervention might be an effective adjunct strategy to treat this chronic viral infection.

The nucleocapsid (NC) protein of retroviruses contains a zinc finger sequence (Cys-X2-Cys-X4-His-X4-Cys) that is essential for the recognition and packaging of the genomic RNA during virion particle assembly. Inactivation of the zinc finger domain of NC by the compound 2,2'-dithiodipyridine (aldrithiol-2 [AT2]) eliminates HIV-1 and SIV infectivity, while viral and host cell-derived proteins on virion surfaces retain conformational and functional integrity (4, 30). In macaque studies, AT2-inactivated SIV appears to be a promising vaccine immunogen (9). AT2-inactivated SIV- and HIV-pulsed dendritic cells, when used as a therapeutic vaccine, induce profound virus-specific T-cell responses that are closely associated with a decrease in plasma viral RNA (vRNA) levels (25, 26). When combined with CpG oligodeoxyribonucleotides (CpG ODN), a Toll-like receptor 9 (TLR9) agonist, SIV-specific T-cell gamma interferon (IFN-\(\gamma\)) production induced by AT2-inactivated SIV-presenting dendritic cells is dramatically augmented in vitro (31). This study sought to test the hypothesis that using CpG ODN as an adjuvant in AT2-inactivated SIVmac239 therapeutic vaccination would further enhance SIV-specific immune responses leading to improved suppression of SIV replication after ART was stopped. We found that while CpG significantly enhanced SIV-specific immunoglobulin G (IgG) antibody titers after AT2-inactivated SIVmac239 immunization, there was no significant control of viral replication after the ART was stopped in these animals. In contrast, the animal group immunized with AT2-inactivated SIVmac239 alone had a significantly lower mean plasma vRNA level after ART was stopped than the saline-treated control animals.
with a SIVmac239 p27 frozen PBMC samples were thawed, washed with AIM V medium (Invitrogen, Cht University, Utrecht, The Netherlands) as previously described (1). Briefly, monkey cytokine enzyme-linked immunospot (ELISPOT) kit (U-CyTech; Utrecht) blood mononuclear cell (PBMC) sample was determined by using an IFN-γ ELISPOT assay. The number of IFN-γ-secreting cells was in a peripheral blood mononuclear cell (PBMC) sample determined by using an IFN-γ monkey cytokine enzyme-linked immunospot (ELISPOT) kit (U-CyTech; Utrecht University, Utrecht, The Netherlands) as previously described (1). Briefly, frozen PBMC samples were thawed, washed with AIM V medium (Invitrogen, Grand Island, NY) supplemented with 20% fetal bovine serum (Invitrogen) (complete medium), and cultured overnight in 24-well tissue culture plates in complete medium. After overnight culture, 2 million cells/ml were stimulated with a SIVmac239 p27mem peptide pool at a concentration of 1 μg of each peptide/ml in a 96-well flat-bottom tissue culture plate and incubated for 18 h at 37°C. Negative controls consisted of cells that were cultured in medium only and cells from uninfected animals. Positive control wells were stimulated with phorbol myristate acetate-ionomycin (Sigma), as suggested in the U-CyTech protocol. The next day, cells were transferred directly to an anti-IFN-γ-coated ELISPOT plate and incubated for 5 h. After the incubation, cells were washed off and all remaining steps were performed in accordance with the manufacturer’s protocol. The developed plates were read by using the Zeiss ELISPOT reader (Carl Zeiss, Inc., Jena, Germany) and KS ELISPOT software (Zeiss). A sample was considered positive only if the number of IFN-γ-secreting cells/well exceeded 50 cells per 1 x 10^5 PBMC. For reporting purposes, the background IFN-γ spot numbers observed in medium-only wells were subtracted from the IFN-γ spot numbers of SIV peptide-stimulated wells. By these criteria, PBMC samples taken from study animals before the initial immunization were consistently negative for SIV p27-specific IFN-γ secretion (data not shown). In every assay, PBMC from SIV-negative monkeys and SIV-positive responder animals were included as controls.

**SIV antibody ELISA.** Prior to determination of antibody titers, plasma samples were screened for the presence of anti-SIV antibodies using a 1:100 dilution of plasma in the same enzyme-linked immunosorbent assay (ELISA) protocol used to determine antibody titers, as described below. Results of the screening assay were calculated using the following ratio: change in optical density (ΔOD)/cutoff (CO), where ΔOD is defined as the difference between the mean OD of a dilution of sample tested in two antigen-coated wells and the mean OD of the same dilution of sample tested in two antigen-free (control) wells. The CO value is the mean ΔOD + 3 standard deviations of duplicate wells containing plasma from 12 randomly selected seronegative adult female rhesus macaques. If the ΔOD ratio for a sample was greater than 2.0, the sample was considered to be positive.

An additional analysis was used to determine anti-SIV antibody titers in antibody-positive plasma samples. Ninety-six-well microtiter plates (Nunc Immunoplate II; Maxisorp, Applied Scientific, South San Francisco, CA) were coated with whole pelleted SIVmac251 (Advanced Biologics, Inc., Columbia, MD) at 5 μg/ml in 0.1 M NaCO3-NaHCO3 buffer (pH 9.6) and blocked with 4% nonfat powdered milk. Plasma samples were serially diluted (1:4 in duplicate, and the plates were incubated overnight at 4°C. The initial dilution of plasma was 1:100 for SIV-specific immunoglobulin G (IgG) assay. Antibody binding was detected using a 1:2,000 dilution of peroxidase-conjugated goat anti-monkey IgG (H+L) (Janssen Scientific Affiliates, Los Angeles, CA) at 0.1 mg/ml in 0.1 M NaCO3-NaHCO3 buffer (pH 9.6). Plates were developed with peroxidase substrate (50 mg/ml in 0.1 M NaCO3-NaHCO3 buffer) for 30 min at 37°C. The color reaction was measured at 492 nm before reading the OD at 620 nm. The OD of each plate was corrected for the mean OD of the zero-dilution control, and antibody titers were expressed as the reciprocal of the last dilution giving a ΔOD value greater than 0.1, where ΔOD is defined as the difference between the mean ODs of two antigen-coated and two antigen-free (control) wells.

**Lymphocyte proliferative responses to SIV p27 antigen.** Antigen-specific lymphocyte proliferation assays were performed by modification of an assay that was described in a previous publication (27). The cells were suspended at 2 x 10^5/ml in RPMI 1640 medium supplemented with 10% fetal calf serum and plated in triplicate at 50 μl per well in 96-well round-bottom microtiter plates. Antigen dilutions or control reagents were plated at 50 μl per well. One hundred microliters of fresh medium was added after 48 h, and the plates were incubated for 7 days in a CO2 incubator. The wells were pulsed with [3H]thymidine (1 μCi per well; NEN-DuPont Co., Wilmington, DE) overnight prior to harvest. The plates were treated with 1 N NaOH before harvesting the plates.
were aspirated onto fiberglass filters and washed with a cell harvester (Inotech Biosystems International, Lansing, MI). The filters were saturated with scintillation cocktail and sealed, and cpm in the \(^3\)H window were measured using a 96-well scintillation counter (Microbeta 1450; Wallac Biosystems, Gaithersburg, MD). The SIV antigen used for this assay was whole AT2-inactivated SIVmac239 (provided by the AIDS Vaccine Program, SAIC-Frederick, Inc., National Cancer Institute, Frederick, MD). Results reported from this assay represent the highest estimate observed obtained using 125 or 12.5 ng of p27 per well. For each sample, only the highest stimulation index (SI) in the dilution series is reported. An SI, calculated as the mean cpm of replicate antigen wells divided by the mean cpm of control wells, was scored positive if \(\geq 2.5\). This assay was optimized in a series of preliminary experiments, and the cutoff was established by testing PBMC from eight healthy, SIV-uninfected rhesus macaques. In every assay, PBMC from an uninfected animal are included as a control. The proliferative responses to SIV antigens of 20 monkeys that had not been exposed to SIV were tested, and it was determined that a level of 200 cpm in the negative control wells was required to eliminate false-positive SIs. Thus, proliferation cultures were excluded from analysis if the negative control counts were < 200 cpm.

**Statistics.** Group differences in plasma vRNA copies post-ART initiation were tested (two-tailed tests) by fitting an exponential function based on a nonlinear mixed-effects model using the NLMIXED procedure in SAS version 9 (42). All other data were analyzed using Prism software (GraphPad Software, Inc.). The area under the concentration-time curve (AUC) of plasma vRNA, the mean number of IFN-γ-secreting cells of each group, and the mean anti-SIV IgG antibody titer of each group were compared using a one-way analysis of variance (ANOVA), and if the mean values were significantly different \((P < 0.05)\), Tukey’s multiple comparison post hoc test was used for pairwise comparisons of the groups.

**RESULTS**

**Effect of immunization of post-ART plasma vRNA levels.** By 4 weeks after ART initiation, vRNA levels had decreased to between 125 and 1,250 copies per ml of plasma in all of the animals, and these low levels were maintained throughout the 32 weeks of ART treatment (Fig. 1A). After week 32, the point when ART was stopped, the plasma vRNA levels of the different groups rebounded at different rates. Applying a nonlinear mixed-effects model, the estimated mean rates of rebound in plasma vRNA levels from week 32 through week 38 were compared. The mean rate of change in the plasma vRNA levels of animals immunized with AT2-inactivated SIVmac239 alone was significantly lower than that of saline-treated animals \((P < 0.0001)\), and the mean rate of change in the plasma vRNA levels of the animals immunized with CpG alone was significantly higher than that of saline-treated animals \((P < 0.019)\). Differences in the estimated mean plasma vRNA levels at asymptote between animals in the saline-treated group versus other groups were not statistically significant (Fig. 1A).

To further characterize the relative levels of SIV replication among the groups, the plasma vRNA levels of each animal from weeks 32 to 38 were transformed into an AUC value (Fig. 1B). The mean plasma vRNA AUC of the four animal groups were significantly different from each other based on a one-way ANOVA test \((P = 0.0184)\), and Tukey’s post hoc multiple comparison test was used to compare the mean plasma vRNA AUC of each animal group to that of the saline group. Thus, the mean plasma vRNA AUC of the AT2-inactivated SIVmac239-immunized group was significantly lower than that of saline-treated group \((P < 0.05)\) (Fig. 1B). These results demonstrate that the animals immunized with AT2 SIV alone were able to control viral replication after ART was stopped significantly better than did the saline control animal group. However, after ART was stopped in the animals immunized with AT2-inactivated SIV plus CpG or CpG alone, the control of viral replication was not significantly different from that in the saline control animal group.

**Changes in SIV-specific IFN-γ T-cell responses induced by immunization.** To assess the magnitude of vaccine-induced SIV-specific T-cell responses, we measured the frequency of IFN-γ SFC in PBMC stimulated by a pool of SIVgag peptides using an IFN-γ ELISPOT assay (Fig. 2). At weeks 12, 20, and 28 after ART initiation (4 weeks after each immunization), the AT2-inactivated SIVmac239-immunized animals had the highest mean frequency of IFN-γ SFC compared to the other three groups. At week 28 (4 weeks after the third immunization), the mean frequencies of IFN-γ SFC among the four groups were significantly different \((P = 0.0086)\), one-way ANOVA). The AT2-inactivated SIVmac239-immunized group had significantly higher mean IFN-γ SFC than the CpG plus AT2-inac-
activated SIVmac239 and saline-immunized groups ($P < 0.05$, Tukey’s multiple comparison tests). At week 32, the time point when the ART was stopped, the mean frequency of IFN-γ SFC in all four groups was increased; however, no significant difference was apparent. Thus, AT2-inactivated SIVmac239 vaccination significantly augmented the SIV-specific IFN-γ T-cell responses of SIVmac239-infected macaques on ART after three immunizations.

Enhanced anti-SIV antibody responses with CpG as an adjuvant. Virus-specific antibody responses were assessed by determining plasma anti-SIV IgG antibody titers in the animals. Before the therapeutic immunization (week 4), SIV-specific IgG antibodies were detectable in all the animals (Fig. 3). Four weeks after the first vaccination (week 12), the antibody titers of CpG plus AT2-inactivated SIVmac239-treated animals increased and the mean anti-SIV IgG antibody titer of this group was significantly higher than the mean titer of the CpG-immunized or AT2-inactivated SIVmac239 group, or the mean titer of the saline-treated group. From week 12 to week 32, the mean SIV-specific IgG antibody titer remained significantly higher in the animal group “immunized” with CpG only compared to the mean value in the animal groups immunized with AT2-inactivated SIVmac239, CpG plus AT2-inactivated SIVmac239 and saline. Thus, multiple CpG immunizations significantly enhanced the SIV-specific T-cell proliferation responses to autologous antigen produced by low-level viral replication.

DISCUSSION

The aim of this study was to determine whether a CpG ODN, used as an adjuvant, could augment the vaccine-induced SIV-specific immune responses in chronically infected monkeys immunized with AT2-inactivated SIV and enhance the...
control of viral replication after the ART is stopped. We found that immunization with CpG plus AT2-inactivated SIVmac239 significantly enhanced SIV-specific IgG antibody responses, but these animals had less control of viral replication after the ART was stopped than the animals immunized with unadjuvanted AT2-inactivated SIVmac239.

CpG ODN is a ligand of TLR9. TLR9 is expressed by human plasmacytoid dendritic cells and B cells. Among the three structurally distinct CpG ODN, CpG-A, -B, and -C, the B-class ODN preferentially acts on B cells to augment the proliferation and generation of plasma cells from naive, memory, and germinal center B cells (19). Consistent with these observations, the B class ODN (CpG10103) significantly enhanced SIV-specific IgG antibody responses in this study when used as an adjuvant for AT2-inactivated SIVmac239. The mechanism of increased SIV-specific antibody responses remains to be determined, but numerous previous studies have shown that CpG mediates B-cell activation (21), inhibits B-cell apoptosis (43), and enhances DNA recombination that results in IgG class switch (17, 24).

In the present study, AT2-inactivated SIVmac239 vaccination effectively augmented SIV-specific IFN-γ T-cell responses and enhanced the control of plasma viral replication after ART is stopped. This finding is consistent with numerous previous studies showing that therapeutic vaccination, either with or without ART, can effectively boost virus-specific immunity and decrease viral replication (14, 23, 25, 26, 32, 39). AT2-inactivated SIVmac239 immunization enhanced SIV-specific T-cell proliferation at week 12 post-ART compared to preimmunization levels of SIV-specific T-cell proliferation, but the T-cell proliferation at weeks 20, 28, and 32 post-ART was similar to that at the preimmunization time point. Thus repeated AT2 SIV immunization did not result in strong SIV-specific T-cell proliferative responses. Interestingly, AT2-inactivated SIVmac239 induced SIV-specific IFN-γ T-cell responses were significantly attenuated by CpG adjuvant through unknown mechanisms. Although the issue has not been addressed experimentally, it is possible that CpG lowers the threshold to activation-induced cell death in CD8+ T cells such that the cells die upon in vitro restimulation. As AT2 SIV is itself a relatively strong antigen (25, 26), the immune stimulation elicited by CpG may be sufficient to induce this proapoptotic state in CD8+ T cells. This may provide a mechanistic explanation for the decreased control of SIV replication that was associated with AT2 SIV immunization.

Importantly, animals immunized with CpG alone had significantly stronger SIV-specific T-cell proliferative responses and a significantly faster rate of plasma vRNA rebound than saline control animals. The lack of benefit in controlling viral replication with the use of CpG as an adjuvant may be related to CpG-induced lymphocyte proliferation. We have previously shown that SIV-specific lymphocyte proliferation measured by this assay was due to the expansion of CD4+ T cells and not CD8+ T cells (27). Thus, it seems likely that the proliferation measured in the current study represents the potential for SIV-specific CD4+ T cells to proliferate in the presence of SIV antigens after ART is withdrawn. In addition to the enhanced lymphocyte proliferation documented in the present study, we previously demonstrated that CpG-B induces moderate expression of IFN-α mRNA but strong expression of proinflammatory cytokine mRNAs in rhesus PBMC (3). Furthermore, intravaginal CpG application induces T-cell division in the genital tract of rhesus macaques, and this is associated with increased viral replication after vaginal SIV inoculation (2, 41).

Other examples of the negative effects of lymphocyte proliferation and immune activation on control of viral replication even in the presence of strong adaptive immunity include a study reporting that administration of the proinflammatory cytokine interleukin-15 (IL-15) in acute SIV infection increases plasma vRNA levels at the set point stage despite the fact that the treatment induces stronger SIV-specific T-cell responses (28). In chronically SIV-infected rhesus macaques receiving ART, IL-15 treatment stimulated massive CD4+ T-cell proliferation and eliminated the decreased viral replication induced by therapeutic vaccination (18). Although the detailed mechanisms of IL-15 induction in CpG-stimulated immune responses in rhesus macaques remain unclear, the fact that IL-15−/− mice fail to produce IL-12 and IFN-γ after CpG administration indicates that IL-15 is an essential cytokine in CpG-stimulated immune activation (22). The most direct evidence that immune activation can drive viral replication comes from recently completed studies in SIV-infected rhesus macaques using a CTLA-4 antagonist (8). CTLA-4 is a negative regulatory molecule expressed on activated T cells and a subset of regulatory T cells. Administration of a blocking anti-CTLA-4 monoclonal antibody in acute SIV-infection leads to T-cell activation and enhanced viral replication (8). Furthermore, CTLA-4 blockade in chronic SIV infection decreases the effect of ART therapy on viral replication and abrogates the ability of T-cell vaccines to decrease the viral set point (8).

The present study comprised relatively few animals, and the results need to be confirmed before any general statements regarding the utility of CpG as an HIV vaccine adjuvant can be made. Furthermore, use of CpG with a relatively weak HIV antigen such as the gp160 envelope glycoprotein may not be associated with some of the deleterious effects (lymphocyte proliferation and decreased antiviral IFN-γ T-cell responses) that were documented in the present study. Although a recent publication has shown that a CpG adjuvant can enhance the immunogenicity of DNA vaccines resulting in the suppression of SIV replication (23), T-cell proliferation may be a serious concern with the use of this adjuvant in HIV-infected patients as it will result in more substrate for viral replication (15, 36, 37). CpG ODN are “danger signals” to the innate immune system through which the adaptive immune system is engaged and the host’s protective immune response is triggered. However, CpG-induced lymphocyte proliferation may be counterproductive in the context of an AIDS vaccine.

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