Inhibition of pathogenic SHIV replication in macaques treated with antisense DNA of interleukin-4

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Interleukin-4 is implicated in the pathogenesis of HIV-induced AIDS and causes enhancement of replication of virus strains that use the CXCR4 (X4) coreceptor. In this study, we explored the effects of interleukin-4 (IL-4) antisense (AS) DNA on replication of X4, simian human immunodeficiency viruses, SHIVKU-2 and SHIV89.6P. AS IL-4 oligomer caused inhibition of virus replication in cultures of CD4+ T cells and macrophages derived from macaques. Plasmid expressing AS IL-4 DNA was also effective in abrogating virus replication in macrophage cultures. Relevance of these cell culture studies was confirmed in vivo by treating SHIV89.6P-infected macaques with AS IL-4 DNA. Six macaques were inoculated with the virus, and 4 were treated with AS IL-4 DNA. This resulted in a significant decrease in viral RNA concentrations in the liver, lungs, and spleen tissues that are all sites of virus replication in macrophages. This is the first demonstration of effective inhibition of an HIV-like virus in tissues by AS DNA of a cytokine. In the present era of increasing resistance of HIV to antiviral compounds, exploration of adjuvant therapies directed at host responses in combination with antiretroviral drugs may be of value for the treatment of AIDS. (Blood. 2005;105:3094-3099) 

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Introduction

The pathogenesis of HIV infection and the complex disease patterns caused by the virus are known to revolve around the dystrophic effects of the infection first, in helper CD4+ T cells, and later in cells of the macrophage lineage in organs such as the lungs, spleen, and brain.1-4 Loss of virus-infected CD4+ T cells heralds the loss of interferon-γ (IFN-γ)-mediated T-cell responses, and this creates an environment favorable for productive replication of opportunistic pathogens. Some of these agents are powerful inducers of T helper 2 (Th2) immune responses.7,8 Studies on coreceptor usage of HIV have shown that viruses that use the CCR5 (R5) coreceptor are usually associated with virus invasion across mucosal surfaces and highly productive phase of virus replication in activated memory CD4+ T lymphocytes. In many infected individuals, mutant virus strains that use the CXCR4 (X4) coreceptor evolve following the loss of Th1 cellular immune responses. Most of these viruses are capable of replicating only in naive CD4+ T lymphocytes, including T-cell lines,9,10 but some are also known to be dual tropic and capable of replicating productively in macrophages.11,12 Studies on replication of X4 HIV-1 in human macrophage cultures showed that interleukin-4 (IL-4) caused significant enhancement of virus replication.13 Whether IL-4 induced by opportunistic pathogens in immunocompromised HIV-infected persons causes enhancement of X4 virus replication is not known.

We have used the macaque simian–human immunodeficiency virus (SHIV) model of HIV disease to study the role of macrophage-tropic X4 viruses in the pathogenesis of AIDS and neurologic disease and, in particular, the interaction between the virus and macaque macrophages. Pathogenic SHIVs that have the X4 env gene of HIV-1 in a background of SIVmac239 such as SHIV89.6P14 and SHIVKU15 cause acute loss of naive CD4+ T cells, AIDS, and neurologic disease in macaques.11 In earlier reports, we had shown that neuropathologic effects by these viruses were associated with expression of IL-4 in the brain,16 supporting the findings of Gabuzda and Sobel17 that X4 viruses replicating in brain macrophages can cause neurologic disease and of Valentin et al,13 who showed that IL-4 promoted HIV replication in human macrophages. In a subsequent study, we used SHIV-infected macaques to determine whether opportunistic pathogens that induce IL-4 could promote replication of the virus in tissue macrophages of these animals. We injected Schistosoma mansoni eggs, potent inducers of Th2 cytokines, into the portal vein or intratracheally into SHIV-infected macaques. This resulted in the development of granulomas in the liver and lung in a milieu rich in IL-4. Macrophages comprising the granulomas were shown to be productively infected with the virus. In the same study, we also showed that macrophages in Freund adjuvant-induced granulomas that were rich in IFN-γ were poorly permissive for virus replication.18 These data clearly suggested that IL-4 was critical for promoting X4 virus replication. In the present report, we explored the concept of whether antisense (AS) DNA of IL-4 could inhibit replication of pathogenic SHIV in cultures of macaque CD4+ T cells and macrophages and, importantly, in macaques infected with this virus.

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**Materials and methods**

**Viruses**

SHIVVU-2, an X4 virus, was derived by sequential passage of the molecular clone, SHIV-4, which has the env, tat, rev, and vpu genes of HIV-1 HXBc2. SHIVSHIVVU-2, a dual tropic X4/R5 virus, was kindly provided by Dr. N. Letvin (Harvard University, Cambridge, MA). Both viruses were propagated in macaque peripheral blood mononuclear cell (PBMC) cultures. Stock preparations had infectivity titers between 10^4 and 10^5 tissue culture infectious dose 50%. Cell cultures were inoculated with either virus at a multiplicity of 0.01.

**Cell cultures**

PBMCs from macaques were obtained by Ficoll-Hypaque (Sigma, St Louis, MO) gradient centrifugation as described earlier and prepared as suspension cultures grown in RPMI + 10% fetal bovine serum + 50 U/mL IL-2 (R10). Briefly, suspensions of PBMCs at a concentration of 1 × 10^6 cells/mL R10 medium were treated with staphylococcal enterotoxin A (Sigma) at 5 μg/mL for 24 hours. Cultures were then washed twice with RPMI and resuspended in R10 and inoculated with SHIV at a multiplicity (MOI) of 0.01 for 4 hours at 37°C. Cells were then extensively washed and replenished with fresh R10 and rmIL-4 or AS IL-4 oligodeoxynucleotide (ODN). Every third day, 0.5 mL spent medium was removed from the cultures and replenished with fresh R10 and rmIL-4 or AS IL-4 ODN. Supernatant fluids were collected at regular intervals and monitored for viral p27 by enzyme-linked immunosorbent assay (ELISA). Monocyte-derived macrophage (MDM) cultures were obtained from PBMCs by incubation in macrophage differentiation medium consisting of RPMI medium supplemented with 20% heated human serum (56°C for 30 seconds), 5 U/mL macrophage colony-stimulating factor, 100 U/mL granulocyte-macrophage colony-stimulating factor, and 5% heat-inactivated rhesus monkey serum at 37°C for 7 days to allow adherent monocytes to differentiate into mature macrophages. Cells were plated in 96-well tissue culture dishes (Costar, Cambridge, MA) or as chamber slide preparations at a concentration of 8 × 10^5 cells/200 μL per well and incubated overnight at 37°C. Cultures were then rinsed to remove nonadherent cells, refed with the same medium, and maintained for 7 days. Macrophages were then inoculated with the virus at an MOI of 0.01 for 4 hours at 37°C. Cells were then extensively washed and replenished with fresh medium containing rmIL-4 or AS IL-4 ODN.

**IL-4**

rmIL-4 was provided by the Resource for Nonhuman Primate Immune Reagents, Emory University (Atlanta, GA) and was used at a concentration of 20 U/mL tissue culture medium. Cultures were replenished with fresh rmIL-4 every 3 days.

**Antisense, sense, and scrambled ODNs**

ODNs used in this study were custom-synthesized by Invitrogen Life Technologies (Carlsbad, CA). The sequences of the ODNs are as follows: antisense IL-4, 5’-GGGGCTGCTGTTGTTTGC-3’; sense IL-4, 5’-AAGCAGAAGCAGACGAGCC-3’; and scrambled IL-4, 5’-GGGGTATGCCGCTTTTGGC-3’. The ODNs were used at a concentration of 80 μM in tissue culture medium and replenished every 3 days. This concentration was not toxic as tested by trypan blue staining (data not shown).

**Plasmids**

Sense and AS IL-4 plasmids were constructed from the rhesus (Rh) IL-4 clone provided by the Resource for Nonhuman Primate Immune Reagents. The RhIL-4 plasmid was cut with either BstXI/ApoI (antisense) or Ncol/ApoI (sense), and the restriction fragments obtained were then cloned into the EcoRV restriction site of pcDNA 3.1 (+) (Invitrogen, Carlsbad, CA) by blunt-end ligation to generate a cytomegalovirus-driven RhIL-4 AS and sense construct, respectively. A reporter plasmid gWIZ green fluorescent protein (GFP) supplied by GTS (San Diego, CA) was also used in our studies. Plasmids were amplified in Escherichia coli (DH10B from Gibco BRL, Carlsbad, CA) and extracted by endofree Mega/Giga plasmid purification kit from Qiagen (Valencia, CA). For in vitro experiments, primary macrophages grown in 24-well plates were transfected with 1 μg plasmid DNA per well complexed with the cationic lipid, in vitro jetPEI-Man (Qbiogene, Irvine, CA) as per the manufacturer’s instructions.

**Studies in macaques**

Cynomolgus macaques, approximately 3 years of age, were used in these studies. One animal was used for determination of biodistribution of the gWIZ GFP, and 6 others were used for virus and AS DNA studies. The first animal was inoculated intravenously with 500 μg GFP plasmid complexed with the liposome in vivo MegaFectin (Qbiogene) by using the enhancer-1, according to the manufacturer’s instructions. This animal was killed 2 days later, and lungs, liver, and spleen were harvested to assess localization of GFP. The other 6 animals were inoculated intravenously with 1 mL stock SHIVVU-2. Seven days later, 4 of the macaques were injected intravenously with 1 mg AS IL-4 DNA complexed with MegaFectin. Two of these animals were given a second injection of the AS DNA 2 days later. All 6 animals were killed 7 days later for assessment of virus replication in different tissues. The macaques were tranquillized with ketamine and then deep anaesthetized with pentobarbitals. Laparotomies were performed, and the animals were exsanguinated from the abdominal aorta. A portion of the plasma was collected in ethylene diamine tetra-acetic acid and used for determination of viral RNA content. The animals were then perfused via the left ventricle of the heart with 1 L saline, and perfusate from the right atrium was discarded. Tissues were then harvested rapidly. Portions of each organ were fixed in either 4% paraformaldehyde for histologic assays or in Streck fixative (Streck Laboratories, Omaha, NE) for immunofluorescence assays. Other portions of the tissues were snap-frozen in isomethyl butane chilled with liquid nitrogen. These tissues were used as sources of RNA.

**Virus assays**

Supernatant fluids from virus-inoculated cultures were examined for virus content by using the reverse transcriptase assay or an ELISA measuring viral Gag p27. Viral RNA concentrations in tissues were assessed by using real-time reverse transcription (RT)–PCR as previously described. Briefly, total RNA isolated from frozen tissues was treated with DNase and subjected to real-time RT-PCR (ABI, Foster City, CA) using gag primers and a Taqman Probe with thermal cycling conditions as described. Levels of hypoxanthine phosphoribosyl transferase (HPRT) mRNA, a housekeeping gene, were also measured by real-time RT-PCR to normalize the viral load. The amplification efficiencies of the gag and HPRT targets can be considered essentially equal, as the difference in their slopes (∆S) of the standard curves was within 0.2.

**Immunocytochemistry and histochemistry**

Immunocytochemical analysis was performed on chamber slide preparations of macrophage cultures or on sections from paraffin-embedded tissues. Slides were treated with murine monoclonal antibody to p27, the Gag protein of simian immunodeficiency virus (SIV; ABI, Columbia, MD) followed by treatment with biotinylated goat anti-mouse immunoglobulin G (DAKO, Carpinteria, CA), peroxidase-conjugated streptavidin (DAKO), and NovaRed substrate (Vector Laboratories, Burlingame, CA), which yields a reddish reaction product. For fluorescence microscopy, Streck-fixed tissues were frozen, embedded at −25°C in orinithine carbamoyltransferase compound (OCT) (Miles, Elkhart, IN), and cut into 5- to 10-μm thick sections. Micrographs were captured on a Nikon Eclipse E600 microscope equipped with a 4 ×/0.13 or 10 ×/0.30 objective lens, opemt 1 × DC10 NN camera and analysis image processing acquisition software (Nikon Instruments, Melville, NY). Confocal images were captured on LSM 510 Laser Scanning Microscope with a Plan-Neo 25 ×/0.80 IK DIC objective lens and LSM software (Carl Zeiss Microscopy, Jena, Germany).
Semiquantitative RT-PCR analyses

RNA was extracted from tissue samples by using Trizol reagent (Life Technologies, Grand Island, NY). Semiquantitative RT-PCR analyses were performed on the RNA by using the Access RT-PCR kit (Promega, Madison, WI) in a Perkin-Elmer (Emeryville, CA) DNA Thermal Cycler 480 as described earlier by using macaque IL-4 primers or CXCR4 primers.

Real-time RT-PCR analysis for IL-4

For quantification of cellular IL-4 mRNA levels, total RNA isolated from macrophages treated with rmIL-4 or AS IL-4 was subjected to real-time RT-PCR by using IL-4 primers and Taqman probe (5’-TTC CAC AGG CCC AAV GTT-3’) and Taqman probe (5’-6FM-CCG ATT CCT GAA AGG GCT CGA CAG-TAMRA) labeled at the 5’ end with the reporter dye FAM (6-carboxyfluorescein) and at the 3’ end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine) using ABI Prism 7700. Primers and the Taqman probe were designed by using the Primer Express software (ABI). Thermal cycling conditions consisted of 50°C for 2 minutes for uracil-N-glycosylase, 60°C for 30 minutes and 95°C for 10 minutes, followed by 44 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Prime RNAse inhibitor was used in the reactions (7.5 U; Brinkmann-Eppendorf, Westbury, NY), and reaction volumes were 25 µL. Standard curves were performed by using 6- to 10-dilutions of nuclear runoff IL-4 RNA. Samples were analyzed twice, in duplicate. As a measure of cellular mRNA levels, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA copy numbers in the RNA samples were also determined by a real-time RT-PCR Taqman assay over 40 cycles (ABI). The IL-4 mRNA levels were normalized to the cellular GAPDH mRNA number to express the results from an equivalent cell number.

Results

IL-4 enhanced X4 virus replication in macaque PBMCs and macrophages

Since the pathogenesis of HIV-1 infection in humans and SHIV infection in macaques is associated with infection in CD4+ T cells and macrophages, we investigated whether IL-4 would modulate SHIV replication in these 2 cell lineages. Infection with either SHIVKU2 or SHIV89.6P yielded similar results in cell culture systems. As seen in Figure 1, IL-4 caused significant increases in viral titers of SHIVKU2 in PBMCs (Figure 1A) and macrophage cultures (Figure 1B), compared with untreated cultures.

Furthermore, IL-4 also mediated enhancement of viral replication in a macaque CD4+ T-cell line immortalized by herpes virus Saimiri (data not shown). This enhanced viral replication was also demonstrated by immunocytochemical analyses, showing that IL-4 increased the numbers of virus-positive macrophages and enhanced viral cytopathic effects characterized by multinucleated giant cell formation (Figure 2).

Specific inhibition of SHIV replication by AS IL-4 ODNs

We next inquired whether AS IL-4 ODN treatment would abrogate virus replication in macaque PBMC and macrophage cultures. The cultures were inoculated at an MOI of 0.01 and 4 hours later treated with either AS DNA of IL-4 or various control ODNs. In these experiments, antisense IL-4 DNA was added to virus-inoculated cultures, and supernatant fluids collected on days 3, 6, and 9 after infection were assayed for SIV Gag. The cultures were replenished with fresh AS IL-4 ODN every third day. As seen in Figure 1A-B, AS IL-4 DNA specifically and markedly inhibited virus replication (80%-90%) as measured by reverse transcriptase assays or ELISA. In contrast to cultures treated with IL-4, AS IL-4 ODN decreased the numbers of virus-positive macrophages and the extent of viral cytopathogenicity characterized by multinucleated giant cell formation (Figure 2). This effect was again confirmed also in the CD4+ T-cell line (data not shown). The effect of AS IL-4 ODN on virus replication was sequence-specific since sense and scrambled IL-4 oligos used at similar concentrations did not inhibit virus replication (Figure 1A). To further rule out nonspecific effects of IL-4, we selected an irrelevant cytokine, IL-8, as a control. AS IL-8 ODNs when used at the same concentration as AS IL-4 failed to inhibit virus replication (data not shown).

Modulation of IL-4 and CXCR4 RNAs by exogenous IL-4 and AS IL-4 ODN

To explore whether the enhancing effect of IL-4 was associated with modulation of endogenous IL-4 production and whether AS ODN designed against IL-4 truly inhibited endogenously produced IL-4 in treated cultures, we performed an IL-4–specific RT-PCR analysis of RNA extracted from infected cultures that were treated with IL-4 and AS IL-4 DNA. As shown in Figure 3A, infected macrophages treated with IL-4 showed a marked increase in IL-4 mRNA, while cultures treated with AS DNA showed significantly reduced levels of IL-4 mRNA. These findings were confirmed by real-time RT-PCR (Figure 3B). We and others had shown previously that the virus-enhancing effect of IL-4 correlated with
Increased expression of the viral coreceptor, CXCR4, in treated cultures. Analysis of CXCR4 message by RT-PCR demonstrated a down-regulation of CXCR4 mRNA in SHIV-infected CD4+ T cells treated with AS IL-4 ODN as opposed to an up-regulation in IL-4–treated cultures, emphasizing the specificity of the AS IL-4 effect (Figure 3C).

**SHIV replication in the presence of sense or AS IL-4 plasmid DNA**

AS IL-4 ODNs provided a tool to assess the role of endogenously produced IL-4 on SHIV replication. The observed inhibition of virus replication in virus-infected cultures of lymphocytes and macrophages by AS IL-4 ODNs suggested that this mechanism may provide a novel therapeutic approach. However, the prohibitive cost of ODNs prompted us to explore whether recombinant AS IL-4 DNA could be substituted with synthetic ODNs for the purpose of inhibiting endogenous IL-4 production. Plasmids expressing AS or sense IL-4 were constructed and transfected into virus-infected macrophages with the use of the cationic lipid, in vitro jetPEI-Man (Qbiogene). A single treatment of the infected cultures with AS IL-4 resulted in marked and sustained reduction of virus replication by as much as 85% for the next 9 days. (Figure 4; P ≤ .001). In contrast, no significant inhibition of virus replication was observed in control cultures administered a sense IL-4 plasmid. These results confirmed that the AS IL-4–expressing vector had the same effect as the ODNs and, therefore, could be substituted for the latter in therapeutic attempts.

**Biodistribution of GFP in macaque tissues**

As a preliminary investigation prior to intravenous delivery of AS DNA to infected macaques, we examined the biodistribution of a reporter gene, GFP, in 1 macaque. The animal was injected intravenously with the GFP plasmid and killed 2 days later. As shown in Figure 5, direct visualization of fixed tissue sections by using confocal microscopy revealed colocalization of MegaFectin (red fluorescence) and GFP (green fluorescence) in lungs, liver, and spleen. These 3 organs are primarily involved in the clearance of particulate foreign matter from the blood. This experiment was a prerequisite for new follow-up experiments that used liposomes containing AS IL-4 DNA as a therapy for infected macaques.

**Inhibition of virus replication in macaques treated with liposomes containing AS IL-4 DNA**

Next, we chose to treat monkeys with AS IL-4 DNA during the acute phase of SHIV infection, because significant levels of systemic viral replication are observed during the first 2 to 4 weeks after infection. The animals were, therefore, inoculated with SHIV_89.6P; 2 animals were given AS IL-4 on day 7 after infection, and 2 others received the AS IL-4 on days 7 and 9 after infection. The 2 virus control animals were not treated. All 6 animals were killed at 2 weeks after infection. Blood from the 4 treated and 2 untreated animals was analyzed on days 7, 10, and 14 for viral RNA content in plasma and CD4+ T-cell counts in the mononuclear cells in peripheral blood. Figure 6A shows that by day 7 after inoculation, all 6 animals had viral RNA concentrations varying from 10^7 to 10^9 copies/mL plasma. Titers remained essentially at this level by day 10 and declined slightly by day 14. In 3 of the 4 AS IL-4–treated animals there was a 1 log reduction in the plasma viral RNA concentration compared with the control animals. CD4+ T-cell numbers declined precipitously in both the treated and untreated animals following virus inoculation, as shown in Figure 6B. There was no significant difference between the CD4+ T-cell counts from the control or treated animals.

Having determined that intravenous administration of liposomes would be cleared from blood by cells in the liver, lungs, and
spleen, these tissues coincidentally being the site of virus replication predominantly in macrophages.\textsuperscript{3,6} we then investigated whether AS DNA of IL-4 would inhibit virus replication in these organs in vivo. Tissue samples of organs in which the AS IL-4 DNA was expected to accumulate were examined for virus content. Liver, lung, and splenic tissues from the 4 treated and the 2 untreated infected macaques were examined for presence of full-length viral gag mRNA by real-time RT-PCR. Gag RNA copy numbers were normalized to the copy number of the cellular mRNA for HPRT, also measured by real time RT-PCR. The gag/HPRT ratio showed that the tissues from treated animals exhibited markedly less viral RNA than those of the 2 control animals. Further, the tissues from the 2 animals that received 2 injections of liposome/AS IL-4 DNA had even less viral RNA than the 2 that received only a single treatment (Figure 7A). Specifically, animals receiving a single injection of AS DNA had 60% less viral RNA, whereas the 2 that were treated twice had about 80% to 90% reduction in the viral RNA in spleen and lungs compared with the 2 untreated controls. Immunohistochemical studies on spleen sections that used a Gag-specific antibody corroborated the viral RNA quantification data since only a few small foci of viral-infected cells were found in the tissue sections of the AS-treated animals compared with many large foci of viral antigen-positive cells in splenic sections of the untreated animals (Figure 7B). The virus-positive cells in the spleen were primarily macrophages in the red pulp area of the spleen (data not shown).

Discussion

The pathogenesis of X4 SHIV infection in macaques reproduces the late phase of HIV pathogenesis in which development of X4 virus mutants is associated with rapid loss of CD4\(^+\) T cells\textsuperscript{23} and, in some cases, intense replication of these viruses in tissue macrophages. In the macaque model, pathogenic X4 SHIVs, SHIV\textsubscript{KR}\textsuperscript{15}, SHIV\textsubscript{Ba-0.6,P}\textsuperscript{14}, and SHIV\textsubscript{H122R} are dual tropic for CD4\(^+\) T cells and macrophages. Infection in macaques with these viruses results in dramatic elimination of CD4\(^+\) T cells within the first 2 weeks of infection,\textsuperscript{11} and, as shown by Igarashi et al,\textsuperscript{4,6} this is followed by intense replication of the virus in macrophages in the spleen, liver, and lungs. For this pilot study, we used the SHIV\textsubscript{Ba-0.6,P} model to provide proof of concept that AS DNA of IL-4 could inhibit virus replication in tissue macrophages in vivo. The rationale for these in vivo studies was predicated on 2 sets of preliminary data. First, in cell culture studies, IL-4 caused enhanced replication of SHIV replication in cultured macaque CD4\(^+\) T cells and macrophages. Second, intratracheal and portal vein injection of SHIV-infected macaques with S. mansoni eggs, potent inducers of IL-4,\textsuperscript{24,25} led to highly productive viral replication in macaques in granulomas induced by the eggs.\textsuperscript{18} These findings led to the hypothesis that lentivirus replication in macrophages may be modulated by endogenously produced IL-4 and that inhibition of this endogenous IL-4 production may result in decreased viral replication in the macrophage cell lineage. This hypothesis was clearly confirmed by our cell culture experiments, not only in macrophage host cells but also in CD4\(^+\) T cells. This begged the question whether this effect could be reproduced in vivo in organs in which infected macrophages can be readily found. No data are currently available on the biodistribution of liposome-mediated plasmid delivery via the intravenous route in macaques. We, therefore, performed a preliminary experiment to address the biodistribution of liposomes containing the GFP reporter gene in macaque tissues and showed that the reporter transgene was concentrated in the major clearance organs of the body, namely, the liver, lungs, and spleen. Since macrophages in these organs become predominant host cells for virus replication following elimination of CD4\(^+\) T cells, we elected to take advantage of this physiologic phenomenon by administering liposomes containing the AS DNA into SHIV\textsubscript{Ba-0.6,P}-infected macaques via the intravenous route. The rationale for giving AS treatment 1 week following virus inoculation was based on the fact that peak viremia in cynomolgus macaques occurs 1 week following intravenous injection of the virus. Viral RNA and immunohistochemical analyses of tissues from the untreated infected animals showed intense replication of virus in the 3 tissues. These findings were matched by significantly less viral RNA and viral antigen concentrations in all 3 tissues from the 4 animals that were given the AS IL-4 DNA. Studies on the viremia in the 6 animals showed that there was a 1 log\textsubscript{10} reduction in the titers of plasma viral RNA

![Figure 6](image-url)  
**Figure 6.** Plasma viral load and CD4\(^+\) T cell counts in the peripheral blood of AS-IL-4 treated and untreated macaques. (A) Cell-free plasma viral RNA load of the treated and untreated macaques at multiple time points was determined by real-time RT-PCR by using standards covering 6 orders of magnitude. Viral loads are shown as viral RNA copies per milliliter plasma. (B) CD4\(^+\) T-cell profile in the control and AS IL-4-treated SHIV\textsubscript{Ba-0.6,P}-infected macaques. The absolute number of CD4\(^+\) T cells per microliter blood was calculated by multiplying the percentage of lymphocyte subset with the absolute number of lymphocytes per microliter from complete blood count.

![Figure 7](image-url)  
**Figure 7.** Viral RNA load in AS IL-4 DNA treated and untreated SHIV-infected macaques as seen by real-time RT-PCR and immunohistochemistry. (A) Viral RNA levels in the lungs (top), liver (middle), and spleen (bottom) in SHIV\textsubscript{Ba-0.6,P}-infected macaques (blue bars); viral RNA in infected macaques given a single dose of AS IL-4 (green bars); and viral RNA in the animals receiving 2 injections of the AS DNA (black bars). Viral gag mRNA/million HPRT ratios obtained after real-time RT-PCR are presented. RNA was extracted from 3 different regions of each tissue and analyzed individually. (B) p27 Staining in the spleen sections of control (top) and treated animals (bottom).
concentrations in the treated, compared with the untreated animals. Despite this, there was no significant change in CD4+ T-cell numbers between the 2 groups. This could be explained by a recent report that demonstrated that in X4-SHIV–infected animals, all animals that expressed more than 10^6 copies of viral RNA/mL blood, lost peripheral CD4+ T cells.26 A number of DNA ODNs against several regulatory and structural27-30 gene products of the virus have been designed as anti-HIV therapeutic agents. This approach, however, has limited clinical feasibility because of the high mutation rate of the HIV genome and the genetic variation between different strains of the virus.31 The novelty of the approach used in the present study was that the AS DNA was directed to a host factor that regulates virus replication. Although only 4 animals were used in the therapeutic study, this report clearly provides proof of concept that this form of therapy is highly effective in curtailing virus replication in tissues in which macrophage-tropic virus replication is known to occur. Thus, targeting of AS IL-4 plasmid may be used in future therapeutic attempts as adjunct therapy for defined and organ-specific symptoms caused by virus replication in the macrophages. One such organ could be the brain. These small inhibitory molecules may not only be effective in macrophages in systemic tissues but may also have a better chance of crossing the blood-brain barrier than the antiretroviral drugs currently in use, with the potential for quelling virus replication in macrophages in the brain.

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References

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