Expression of CD154 by a Simian Immunodeficiency Virus Vector Induces Only Transitory Changes in Rhesus Macaques

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Human immunodeficiency virus infection is characterized by dysregulation of antigen-presenting cell function and defects in cell-mediated immunity. Recent evidence suggests that impaired ability of CD4+ T cells to upregulate the costimulatory molecule CD154 is at the core of this dysregulation. To test the hypothesis that increased expression of CD154 on infected CD4+ T cells could modulate immune function, we constructed a replication-competent simian immunodeficiency virus (SIV) vector that expressed CD154. We found that this recombinant vector directed the expression of CD154 on the surface of infected CD4+ T cells and that expression of CD154 resulted in activation of B cells present in the same cultures. Experimental infection of rhesus macaques resulted in very low viral loads for the CD154-expressing virus and the control virus, indicating that expression of CD154 did not result in increased viral replication. Analyses of the anti-SIV immune responses and the phenotype of lymphocytes in blood and lymphoid tissues showed changes that occurred during the acute phase of infection only in animals infected with the CD154-expressing SIV, but that became indistinguishable from those seen in animals infected with the control virus at later time points. We conclude that the level of expression of CD154 in itself is not responsible for affecting the immune response to an attenuated virus. Considering that the CD154-expressing SIV vector and the virus control did not carry an active nef gene, our results suggest that, in CD4+ T cells infected with wild-type virus, Nef is the viral factor that interferes with the immune mechanisms that regulate expression of CD154.

The CD154 protein, also known as CD40 ligand (CD40L), is a type II membrane glycoprotein of 39 kDa with an extracellular domain homologous to tumor necrosis factor alpha (TNF-α) and -β (1). This protein is expressed transiently on CD4+ T cells after activation in vitro or in vivo and weakly on CD8+ T cells after in vitro activation with anti-CD3 or phorbol myristate acetate (PMA)-ionomycin (2). CD154 binds to CD40, a 50-kDa member of the TNF receptor superfamily expressed on several cells of the immune system, including immature and mature B lymphocytes, monocytes, and dendritic cells (DCs) in the T-cell areas of secondary lymphoid organs, follicular dendritic cells (FDCs), and thymic epithelium (45). CD154-CD40 interactions are required for the priming and expansion of antigen-specific CD4+ T cells and CD8+ cytotoxic T lymphocytes (CTLs) and in the induction of costimulatory activity on antigen-presenting cells (APCs) (4–6). CD154-CD40 interactions are also critical in B-cell proliferation, immunoglobulin (Ig) production, Ig class switching, rescue of B cells from apoptotic death, germinal center formation, and generation of B-cell memory (7). Cell-cell contact via CD40-CD154 is required for macrophages to be activated and produce nitric oxide and interleukin-12 (IL-12) (8). The importance of CD154 expression in vivo was revealed by studies of patients suffering from hyper-IgM syndrome (HIGM1), a human X-linked immunodeficiency caused by mutations in the CD154 gene that result in lack of functional expression of CD154 on the surface of activated T cells (9). HIGM1 patients suffer from recurrent upper respiratory tract infections and certain opportunistic infections such as cryptosporidial diarrhea and Pneumocystis carinii pneumonia. Studies of CD154-deficient mice have shown that their CD4+ T cells proliferate very poorly in vivo in response to protein antigens; however, in vitro proliferation in response to polyclonal activators was not affected (23).

CD154-CD40 contacts are important for the development of immune responses to viral infections, although innate defense mechanisms, some CD8+ CTL responses, and short-lived neutralizing antibody responses can be induced in a CD154-CD40-independent fashion (22). However, there is in vivo and in vitro evidence that CD154 signaling is dysregulated during human immunodeficiency virus (HIV) infection (9, 29). For example, early in HIV-1 infection, B-cell responses to T-cell-dependent antigens are impaired, and this deficiency has been associated with a defective T-cell function (30, 48). Among the APC functions that have been reported to be defective in HIV infection is a marked impairment of production of IL-12 by peripheral blood mononuclear cells (PBMCs) and macrophages from HIV-infected patients (7), which can be corrected by addition of soluble trimeric CD154 (10, 42). Similarly, reduced pathogen-specific CD154 expression on CD4+ T cells has been observed in HIV-infected individuals (41). Finally, there are striking similarities between opportunistic infections and hypergammaglobulinemias seen in HIGM1 and HIV-infected patients. The association of HIV gp120 with CD4 has been implicated in the reduced expression of CD154 and the consequent reduced costimulation activities of the APCs (20, 21).
In this study, we report for the first time the construction of a replication-competent SIV vector that expresses a biologically active CD154 molecule. We demonstrate that CD154 is presented on the surface of infected CD4+ T cells and that it can stimulate neighboring B cells. Finally, we show that infection of rhesus macaques with this virus results in early and transient phenotypic changes that do not affect, in the long term, the outcome of the antiviral immune response.

**MATERIALS AND METHODS**

Cloning of the human and rhesus CD154 genes. The CD154 gene was cloned from RNA from human or rhesus macaque PBMCs stimulated by plating cells on plastic flasks coated with anti-CD3 antibodies (clone UCHT1 from Beckman-Coulter for human PBMCs, and clone FN-18 from U-Cytech for rhesus PBMCs). After 6 h of stimulation, cells were harvested and lysed by 1 ml of Trizol in order to extract intact RNA cellular. A first-strand cDNA was prepared with oligo(dT) (Amersham, Piscataway, N.J.) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif.). Two sets of primers were designed based on the human CD154 sequence: FCD154 (5'CAGCCCTGCAATTGCTGTTGA/CTTTTTGACTGGTTTTGAAATGACATCTG-3') and RCD154 (5'-ACACACCGTGTCACGTCGACTGTTGGA-3'), which bind outside the coding sequence, were used to amplify the full-length gene; and (ii) primers sFCD154 (5'-ATGATTGCGTCTGACCTTTTTGACTGGTTTTGAAATGACATCTG-3') and RCD154 were used to amplify the extracellular domain of the CD154 protein. These primers contained mutations (in italic lowercase) that introduced a SalI cloning site (underlined). The PCR products were isolated from agarose gel and cloned into the pCR 2.1 plasmid (Invitrogen), and three different clones were sequenced. Finally, the human CD154 gene was purified as a SalI DNA fragment and cloned into the SalI site of pSVII (18).

**Cells and viruses.** SIVmac239, which contains a nef gene with an open reading frame, was generously provided by R. Desrosiers (New England National Primate Research Center, Southborough, Mass.). CEM-x-174 cells, rhesus PBMCs, and lymph node (LN) cells were used for SIV isolation and propagation. These cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 mg of streptomycin per ml, and 100 U of penicillin per ml (Cellgro Mediatech, Herndon, Va.) (RPMI 10). The herpesvirus saimiri-transformed rhesus T-302 cell line was maintained in RPMI 10 with 25 IU of IL-2 per ml.

To prepare the recombinant viruses, the CD154 gene, as a Sall fragment, was cloned into the SalI cloning site of the plasmid pSVIIh, which contains the whole SIVmac239 proviral genome with a deletion of the nef gene and a unique Sall cloning site (18). Plasmids containing the CD154 gene in the sense (pSIVCD154) and antisense (pSIVCNTL) orientation were electroporated into CEM-x-174 cells to generate SIVCD154 and SIVCNTL, respectively. Virus replication was monitored by the SIV major core protein (p27) by ELISA (33). Cultures were recorded as positive for virus when p27 antigen was detected at two consecutive time points. End-point cultures were maintained and tested for 4 weeks before being scored as negative. Virus levels were calculated according to the method of Reed and Muench (38) and expressed as TCID50/106 cells. Virus stocks were titrated at the 50% tissue culture infective dose (50% TCID50).

**Virus isolation and propagation.** CEM-x-174 cells in 24-well plates (24). Twice weekly, culture media were assayed for the presence of the SIV major core protein (p27) by ELISA (33). Cultures were recorded as positive for virus when p27 antigen was detected at two consecutive time points. End-point cultures were maintained and tested for 4 weeks before being scored as negative. Virus levels were calculated according to the method of Reed and Muench (38) and expressed as TCID50/106 cells. Virus production, length of cultivation, and criterion for positivity were determined as described above for cell-associated viral loads.

**Plasma viral RNA loads.** The determination of viral RNA loads in heparinized plasma was performed by Quality Biological, Inc. (Gaithersburg, Md.), utilizing a nucleic acid sequence-based amplification (NASBA) assay that has a limit of detection of 2,000 genome equivalents.

**Isolation of proviral DNA.** Proviral DNA was extracted from LNCM-x-174 cell cultures that were positive by SIV Gag ELISA. DNA was amplified by PCR with primers specific for the SIV 3' region CCGTCTCGAGATCTCCAC CAGAGACT (forward) and GACTGTAACAGCGGCAAATG GCC (reverse) (27). Nucleic acids were denatured at 94°C for 1 min, annealed at 65°C for 1 min, and extended at 72°C for 2 min; this cycle was repeated 35 times. DNA fragments containing the intact CD154 gene insert were cloned into 1,160 nucleotides in length, whereas wild-type SIV originated 820 nt DNA fragments.

**Lymphocyte phenotyping.** Phenotypic characterization of lymphocytes in peripheral blood (PB) and LN was performed by flow cytometry with three-color direct immunofluorescence. Surface staining was performed by incubating whole blood or LN cells with monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or Tricolor (PE-Cy5) for 30 min at room temperature. The antibodies were purchased from BD Pharmingen (clone CD3/OKT4, clone CD4/OKT3, clone CD8/OKT8, clone CD14/OKT14, clone CD43/OKT4, clone CD86/OKT18, clone CD20/OKT19, clone CD56/OKT18, clone CD16/OKT3, clone CD57/OKT15, clone CD154/OKT1), and data were analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems).

**In vitro analysis of retroviral infection.** CEM-x-174, rhesus T-302 cells, and unstimulated PBMCs from uninfected rhesus macaques were infected at a multiplicity of infection of 0.1 with SIVCD154, SIVmac239, or SIVCNTL. Expression of CD154 was monitored by flow cytometry with direct staining with anti-CD154-PE (clone TRAP1; Beckman-Coulter). Other markers detected were CD3 (clone FN-18, Biosource, Camarillo, Calif.), CD4 (clone OKT4; Ortho Diagnostic Systems, Raritan, N.J.), CD8 (clone 3B5; Caltag, Burlingame, Calif.), and CD20 (clone B1; Beckman-Coulter). Samples were acquired in a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.), and data were analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems).

**Cytokines and viral antigens in plasma.** Levels of IL-12, IL-18, and alpha interferon (IFN-α) in plasma were determined with commercially available ELISA kits (Cytoscreen monkey IL-12 from BioSource; Hu-IL-18 ELISA from MBL, Nagoya, Japan; and Hu-IFN-α ELISA from PBL, New Brunswick, N.J.). The limits of detection were 4 pg/ml for IL-12, 12.5 pg/ml for IL-18, and 10 pg/ml for IFN-α. Plasma IFN (IFN-α/β) activity was also measured by monitoring inhibition of the cytopathic effect caused by encephalomyocarditis virus infection in A549 cells (23).

**Cell-associated viral loads and virus isolation.** Cell-associated virus, latent or productive, was measured by limiting dilution assay of cocultures of LN cells and CEM-x-174 cells in 24-well plates (24). Twice weekly, culture media were assayed for the presence of the SIV major core protein (p27) by ELISA (33). Cultures were recorded as positive for virus when p27 antigen was detected at two consecutive time points. End-point cultures were maintained and tested for 4 weeks before being scored as negative. Virus levels were calculated according to the method of Reed and Muench (38) and expressed as TCID50/106 cells. Virus production, length of cultivation, and criterion for positivity were determined as described above for cell-associated viral loads.

**Anti-SIV antibody detection.** Plasma samples were analyzed for the presence of antibodies reactive to SIV envelope glycoproteins and the p27 core protein. Antigens for ELISA plates were obtained from a viral preparation of SIVmac239 concentrated by 20% sucrose cushion centrifugation. The protein content of the viral preparation was determined by the BCA Protein Quantification kit (Pierce). Antibodies were quantitated with 96-well ELISA plates coated with concanavalin A and SIV gp160 as previously described (11, 39). For the anti-p27 antibody ELISA, the disrupted, envelope-depleted viral preparation was added to ELISA plates previously coated with anti-p27 antibodies (33) and incubated overnight at 4°C.

**IFN-γ ELISPOT.** Rhesus PBMCs were seeded at 2 x 10^5 cells in 150 μl of RPMI 10 in IFN-γ enzyme-linked immunospot (ELISPOT) plates (U-Cytech, Utrecht, The Netherlands), and incubated with pools of 15-mer peptides that
encompassed the SIVmac239 Gag protein (obtained from the NIH AIDS Research and Reference Reagent Program) at 2 μg/ml. After 18 h of incubation at 37°C, cells were lysed and plates were developed according to the manufacturer’s instructions. Spots were counted, and the difference between the number of spots for the reactive peptide minus the number of spots for the peptide control was adjusted by the percentage of CD8+ T cells in the samples and expressed as spot-forming cells (SFC)/10^6 CD8+ T cells.

Statistical analyses. The two-sample t test for samples with equal variances was performed with Excel (Microsoft Corporation). Statistical significance was given for P < 0.05.

RESULTS

In vitro characterization of recombinant viruses. We cloned the human and rhesus CD154 genes from stimulated T cells. Our studies of the kinetics of expression of CD154 on rhesus lymphocytes, stimulated with plate-bound anti-CD3 antibodies, showed that maximum expression on CD4+ T cells was achieved after 4 to 6 h of stimulation. The levels of expression were maintained up to 12 h poststimulation, after which they diminished (data not shown). This regulated expression is similar to the observed for the human CD154 gene (17). Comparison of the nucleotide sequences coding for the extracellular region of human (GenBank accession no. L07414) and rhesus CD154, amplified with PCR primers sFCD154 and RCD154, showed only two amino acid differences among 218 residues (Fig. 1). Although we were able to amplify the full-length human CD154 gene with primers FCD154 and RCD154, we did not succeed with the rhesus CD154 gene, which indicates that there may be more differences in nucleotide sequences for the 5’ end region between the two genes. Considering the 99% homology between the extracellular portions of human and rhesus CD154 proteins, we decided to use the human CD154 gene for all our constructs. The CD154 gene was then inserted in place of nef into the genome of SIVmac239. Viruses carrying the CD154 gene in the sense (SIVCD154) and antisense (SIVCNTL) orientations were generated in CEM-x-174 cells. Only cells infected with SIVCD154 expressed CD154 on their surface, as detected by flow cytometry (data not shown).

We analyzed the effects of SIV infection on the level of expression of CD154 on the cell surface of a continuous T-cell line derived in our laboratory from rhesus macaque PBMCs by infection with herpesvirus saimiri. Infection of T-302 cells with viruses that lacked nef, such as SIVCD154 and SIVCNTL, resulted in a slight CD4 downregulation, whereas infection with SIVmac239 induced marked downregulation of both CD3 and CD4 proteins (data not shown). These data combined indicated that the recombinant viruses SIVCD154 and SIVCNTL indeed do not have a functional nef gene.

We also studied the phenotypic changes induced by infection of rhesus PBMCs with SIV. Because we were interested in studying the capacity of the different viruses to induce cell activation, we infected unstimulated PBMCs with SIVmac239, SIVCD154, or SIVCNTL and cultured them in the presence of IL-2. Analysis of cells on day 9 showed that infection with SIV reduced the level of expression of the IL-2 receptor α chain (CD25) on T cells; however, PBMCs infected with SIVCD154 had significantly higher levels of CD25 than cells infected with SIVCNTL or SIVmac239 (Fig. 2A). Expression of CD154 also showed that only T cells infected with SIVCD154 had detectable levels of this surface protein (Fig. 2B). Identification of which T cells expressed CD154 revealed that the CD8+ population, that is the CD4+ T cells, were the ones presenting the protein on their surface (Fig. 2C): the few CD8+ T cells that expressed CD154 were CD4+ CD8−, which are regularly present in macaques (data not shown). This experiment indicated that SIVCD154 was able to infect rhesus CD4+ T cells and directed the expression of CD154 on the surface of the infected cells.

Because B cells are one of the cell types that interact with CD154-expressing CD4+ T cells, we also analyzed activation markers and costimulatory molecules on these cells in the infected PBMC cultures. The percentage of B cells expressing CD40, the ligand of CD154, was significantly augmented only in cultures infected with SIVCD154 (Fig. 3A). Similarly, only cultures infected with SIVCD154 had elevated percentages of B cells expressing the early activation marker CD69 (Fig. 3B). Finally, only B cells from cultures infected with SIVCD154 showed upregulation of CD86, a costimulatory molecule whose expression is increased after CD40-CD154 interaction. These data clearly showed that the CD154 molecule expressed on CD4+ T cells infected with SIVCD154 interacted with CD40 on B cells and that this CD40-CD154 contact induced activa-

FIG. 1. Comparison of the amino acid sequence of the extracellular domain of the human (GenBank accession no. L07414) and rhesus CD154 gene products. The first methionine (M in boldface) residue in both sequences was introduced by PCR. Differences in sequence are underlined in boldface italic.

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<td>Rhesus</td>
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tion and up-regulation of other costimulatory molecules on B cells. Thus, SIVCD154 induced expression of CD154 on infected cells, and this newly synthesized molecule was biologically active.

Infection of rhesus monkeys with recombinant SIV vectors. After demonstrating the in vitro capacity of SIVCD154 to induce a biologically active CD154 on the surface of infected CD4$^+$ T cells, we designed an experiment to study the real relevance of this CD154 expression within the context of a retroviral infection. Therefore, we inoculated two groups of four rhesus macaques each with a high dose of SIVCD154 or SIVCNTL. Measurement of viral loads in plasma or in LNs indicated low levels of replication during the acute phase of infection but did not show any significant difference between the two groups of animals. In general, virus in plasma was detectable by 2 weeks postinfection, and it peaked then or at 4 weeks postinfection. By 12 weeks postinfection, virus in plasma was below the limit of detection (2,000 SIV copies) in almost all of the animals (Fig. 4A). Cell-associated viral loads in plasma or in LNs were measured at 4 weeks postinfection. Virus isolation from rhesus lymphocytes by long-term culture demonstrated that some animals were able to control infection efficiently after primary infection (rhesus macaques 37, 44, 38, 40, and 41), whereas other animals had persistent, low-level viremia (rhesus macaques 35, 36, and 42); however, both patterns were seen in either group (data not shown). Thus, infection of rhesus macaques with an SIV that expressed CD154 did not result in higher viral loads when compared with animals infected with an attenuated SIV that did not express this molecule.

The stability of the CD154 gene in virus isolated from the infected macaques was analyzed by PCR and flow cytometry. As seen in other experiments with recombinant retroviruses, the genetic stability varied from animal to animal, and it was similar for SIVCD154 and SIVCNTL. For example, the CD154 gene was lost as early as 2 to 4 weeks postinfection in rhesus macaques 35 and 36, whereas the full-length CD154 gene was still detectable at 12 weeks postinfection in rhesus macaques 42 and 44 (Fig. 5A). Confirming the presence of the full-length CD154 gene, virus isolated from rhesus macaques 42 and 44 at 12 weeks postinfection was still able to direct expression of CD154 on the surface of infected cells (Fig. 5B). By 16 weeks postinfection, we were not able to detect full-length CD154 inserts in any of the animals. These measurements demonstrated that the stability of the CD154 gene in the recombinant vectors was not dependent on the orientation of the insert with respect to the SIV genome; they also showed that SIVCD154 directed the expression of CD154 on infected cells in vivo.

Phenotypic changes in lymphocytes from rhesus monkeys infected with recombinant SIV vectors. We analyzed the phenotypic changes in lymphocytes from rhesus monkeys infected with recombinant SIV vectors.
notypic changes induced by infection with SIVCD154 and SIVCNTL in LN and blood lymphocytes during the acute phase of the viral infection. Particularly in LNs, we studied expression of CD154 on CD4\(^+\) T cells, and activation markers and costimulatory molecules that are present on B cells and that linked to CD40-CD154 interactions. Variations in the percentages of CD4\(^+\) T, CD8\(^+\) T, and B cells were not statistically different between both groups. LN lymphocytes from all animals showed a gradual, slight reduction in the percentage of CD4\(^+\) T cells and an increase in the number of CD8\(^+\) T cells;

FIG. 3. Analysis of costimulatory molecules and activation markers on rhesus B cells after infection with SIVs. Unstimulated rhesus PBMCs were infected with SIVCD154, SIVmac239 (nef open), or SIVCNTL at a multiplicity of infection of 0.1; uninfected cells were included as controls. After 9 days of infection, cells were harvested; stained with fluorescent antibodies against CD20, CD40, CD69, and CD86; and analyzed in a FACScan. (A) CD20 versus CD40, gated on lymphocytes; (B) CD20 versus CD69, gated on lymphocytes; (C) CD20 versus CD86, gated on lymphocytes. The experiment was repeated with three different donors, and the same representative example is provided.

FIG. 4. Viral loads in plasma and LN cells in rhesus macaques after infection with SIV vectors. Rhesus macaques were infected with 10\(^4\) TCID\(_{50}\) of SIVCD154 (solid symbols, straight lines) or SIVCNTL (open symbols, dotted lines). (A) SIV RNA loads in heparinized plasma were determined by NASBA; the limit of detection was 2,000 SIV copies/100 \(\mu\)l of plasma. (B) Cell-associated viral loads were determined in LN cells by limited dilution and cocultivation with CEM-x-174 cells.
A similar increase was observed for LN B cells (Fig. 6A). Levels of expression of CD154 on LN CD4+ T cells were similar for both groups before infection (0.42% ± 0.23% for animals in the SIVCD154 group versus 0.41% ± 0.26% for animals in the SIVCNTL group), but increased to higher levels in animals infected with SIVCD154 by 2 weeks postinfection (1.11% ± 0.25% for animals in the SIVCD154 group versus 0.73% ± 0.34% for animals in the SIVCNTL group); however, this difference was not statistically significant (P = 0.16; Fig. 6B). Analysis of costimulatory molecules and activation markers in LN B cells did not reveal differences that were statistically significant between the two groups either. However, it was interesting to observe in all animals a transient increase in the percentage of HLA-DR+ B cells, whereas the percentage of

FIG. 5. Characterization of virus isolated from macaques infected with SIVCD154 or SIVCNTL. (A) Stability of the CD154 insert. The 3’ long terminal repeat region of the viruses isolated from infected rhesus macaques was amplified by PCR as described in Materials and Methods, using primers that encompassed the region where the CD154 gene had been inserted. PCR-amplified DNA fragments were separated by agarose electrophoresis. FL, full-length CD154 insert, 1,500 bp; M, 100-bp-ladder molecular weight marker; PC, positive control for extended insert, 1,340 bp; wt, wild-type SIVmac239, 880 bp. WPI, weeks postinfection. (B) Expression of CD154 by virus isolated from infected macaques. CEM-x-174 cells used for isolation of SIV from SIVCD154- or SIVCNTL-infected macaques were stained with an anti-CD154 antibody and analyzed by flow cytometry. SIVCD154 represents CEM-x-174 cells directly infected with SIVCD154.
CD86+ B cells dropped significantly to low and stable levels after infection (Fig. 6B).

Analysis of peripheral blood lymphocytes (PBLs) showed some transient differences between animals infected with SIVCD154 or SIVCNTL that were statistically significant. For example, animals infected with SIVCD154 had a drastic reduction in the number of CD4+ and CD8+ T cells, B cells, and NK cells at 2 weeks postinfection. The reduction in absolute number of NK cells was even more pronounced, lasting until 8 weeks postinfection (Fig. 7). This reduction in absolute numbers at 2 weeks postinfection in animals infected with SIVCD154 was also accompanied by a reduced percentage of activated (cells expressing CD69) CD4+ T, B, and NK cells (Fig. 8). Interestingly, only CD8+ T cells from animals infected with SIVCD154 had a peak of activation by 8 weeks postinfection. In general, after the acute phase of infection, absolute numbers of blood lymphocytes in all animals remained stable, but the general state of activation of CD4+ and CD8+ T cells remained higher than the preinfection levels.

Immunological response of rhesus macaques to infection with recombinant SIV vectors. We studied changes in plasma cytokine concentration induced by infection with SIVCD154 and SIVCNTL, as well as the cellular and humoral immune response elicited by these viruses. In contrast to what is observed after infection with pathogenic SIV (19), analysis of levels of IFN-α, IL-12, and IL-18 in plasma before and after infection with these recombinant SIV vectors did not show consistent and significant changes for any of these cytokines in either group (data not shown).

The antibody response against SIV Gag and Env was determined by specific ELISA. Similar to what was observed throughout this study, there were no statistically significant differences between the levels of antibodies produced by animals infected with SIVCD154 and those produced by animals infected with SIVCNTL. The patterns were similar for both groups of animals and consisted of very low antibody titers for those animals that controlled viremia efficiently, whereas viremic animals such as rhesus macaques 35 and 36 had high anti-SIV gp160 and Gag antibody titers (Fig. 9A).

The cell-mediated immune response was studied by IFN-γ ELISPOT using 15-amino-acid peptides that encompassed the SIV Gag protein; these SFC were identified as being CD8+ T cells by IFN-γ intracellular staining (data not shown). Although by 3 weeks postinfection we were able to identify SIV Gag-specific SFC in three out of four animals in the SIVCD154-infected group, as compared to one out of four for the SIVCNTL-infected animals, these differences disappeared by 8 weeks postinfection (Fig. 9B). Interestingly, the pattern that was observed for the cell-mediated immune response was the opposite one to that seen for the humoral response; that is, animals that controlled the infection efficiently had high levels of SIV Gag-specific SFCs, whereas viremic animals had very low numbers.

In summary, both SIVCD154 and SIVCNTL behaved like attenuated viruses, and there was no evidence that the level of
expression of CD154 induced by SIVCD154 altered the development of the immune response to the virus.

**DISCUSSION**

It has been observed that infection with HIV leads to dysregulation of CD154 signaling in CD4+ T cells and impairment of the generation of T-cell-dependent immune responses in infected patients (28, 29, 42). It was first suggested by Chirmule et al. (8) that interaction between HIV gp120 and the CD4 receptor might play a role in the mechanisms underlying CD154 dysregulation. Recently, Zhang et al. (50) demonstrated that, indeed, interactions between native, virion-associated HIV gp120 and the CD4 receptor were responsible for reducing CD154 mRNA and protein levels on T cells. Consequently, it has been suggested that this reduced CD154 expression on CD4+ T cells, in turn, impairs the ability of the APCs to express cytokines and deliver other costimulatory signals, leading to the deficiencies in both APC and CD4+ T-cell function observed during HIV and SIV infection. As a result of these findings, we hypothesized that this vicious cycle could be broken with a recombinant SIV expressing its own CD154, because SIV-infected CD4+ T cells would have the ability to express CD154 even if the association of gp120 with CD4 blunted the normal expression of CD154.

We engineered a recombinant SIV that lacked the nef gene and had the capacity to replicate and express CD154 on the surface of the infected cells. It could be argued that all the studies performed in HIV-infected individuals that suggest a CD154 dysfunction upon infection have been performed with individuals infected with nef-expressing viruses. In fact, infection of macaques with nef-deficient SIV resulted in mild immune alterations and lack of disease progression during the first several years of infection (26), with similar outcomes found in humans infected with nef-deficient HIV (13). However, long-term studies in infected humans and rhesus macaques have demonstrated that nef-deficient HIV and SIV are still pathogenic and able to induce immune dysfunction and AIDS, although in a protracted manner (reviewed in reference 47). Thus, considering that nef is not the only viral gene involved in immune dysfunction of the host and that experimental evidence suggests that gp120-CD4 interactions impair CD154 expression, our experimental approach was valid. We then demonstrated in vitro that this virus, SIVCD154, could infect rhesus PBMCs, induce the expression of CD154 (Fig. 2), and stimulate the B cells present in the same culture (Fig. 3). For these experiments, we had to infect rhesus PBMCs that were not stimulated before infection. Mitogenic stimulation of PBMCs prior to infection is a routine technique employed for the amplification of retroviral cultures; however, the resulting global upregulation of activation and stimulation markers on lymphocytes would mask any effect due to the expression of CD154 by SIVCD154. Thus, considering that nef is not the only viral gene involved in immune dysfunction of the host and that experimental evidence suggests that gp120-CD4 interactions impair CD154 expression, our experimental approach was valid. We then demonstrated in vitro that this virus, SIVCD154, could infect rhesus PBMCs, induce the expression of CD154 (Fig. 2), and stimulate the B cells present in the same culture (Fig. 3). For these experiments, we had to infect rhesus PBMCs that were not stimulated before infection. Mitogenic stimulation of PBMCs prior to infection is a routine technique employed for the amplification of retroviral cultures; however, the resulting global upregulation of activation and stimulation markers on lymphocytes would mask any effect due to the expression of CD154 by SIVCD154. Thus, our in vitro PBMC studies resulted in a low number of CD4+ T cells being infected, but the unique outcome of B-cell activation and upregulation of CD154 seen after infection with SIVCD154 was clearly distinguishable from what was observed for SIVmac239 and SIVCNTL. For final proof of our hypothesis, we performed in vivo experiments, infecting rhesus macaques with SIVCD154. As a control, we infected a second group of animals with SIVCNTL, a virus that underwent similar genetic manipulations as SIVCD154 but did not express CD154. Surprisingly,
infection of macaques with SIVCD154 did not result in remarkable differences from what was observed in animals infected with SIVCNTL.

The role of CD154 in cell activation and retroviral dissemination has been controversial, and one of our concerns was whether expression of CD154 would alter the infectivity of the attenuated SIV\textit{\text{\text{\text{H9004}}}} nef vectors. Several investigators have reported that activation of macrophages or dendritic cells (DCs) with trimeric CD154 resulted in release of β-chemokines and TNF-α, reduction of cell surface expression of CD4 and CCR5, and resistance to HIV and SIV infection (12, 14, 30, 32, 34, 35). However, other authors reported either completely opposite results (6) or that this CD154-mediated macrophage activation rendered these cells highly susceptible to infection with CXCR4-dependent viruses (3). Similarly, increased levels of HIV infection have been reported after CD154-mediated activation of Langerhans cell-like DCs (25) and plasmacytoid DCs (15). In the context of our experiments, both SIVCD154 and SIVCNTL behaved like attenuated viruses that induced low viral loads and, depending on the animal, transient viremic episodes (Fig. 4). Although virus isolated from SIVCD154-infected macaques was shown to direct expression of CD154 during the acute phase of the infection (Fig. 5), viral titers were similar to those seen for the macaques infected with the control virus. Thus, expression of active CD154 during acute SIV infection with a \textit{\text{\text{H9004}} nef}-deficient virus did not result in increased viral replication. The higher viral loads observed in animals 35 and 36, which were infected with SIVCD154 and SIVCNTL, respectively, are a consequence of the loss of the CD154 gene at a very early time point. This loss of “superfluous” genetic material results in viral species more fitted for replication and is a phenomenon that has been observed before, both in vivo and in vitro for recombinant SIVs that lack a \textit{\text{\text{H9004}} nef} gene (2, 18, 20, 21, 27, 40).

Other intriguing aspects of our animal studies were the similarities in lymphocyte phenotypes observed between monkeys infected with SIVCD154 and those infected with SIVCNTL. In LNs, the percentage of CD154+ CD4+ T cells increased to higher levels in SIVCD154-infected macaques during the acute phase of the infection, but the differences were not statistically significant. In this lymphoid tissue, lymphocytes from both groups of animals showed transient rises in HLA-DR+ B cells (Fig. 6B). The lack of a clear difference for the levels of CD154 expression or B-cell activation between both groups of infected animals may be a reflection of the very low levels of viral replication observed for SIVCD154 or SIVCNTL. The remarkable and long-lasting decrease in CD86+ LN B cells may be the consequence of an unidentified antiviral immune response, since it has been shown that activated B cells can induce stimulation of CD4+ T cells via CD86-CD28 interaction and lead to increased HIV replication (31). The only differences that were statistically significant occurred in peripheral blood at 2 weeks postinfection, when absolute numbers of lymphocytes and activation levels were reduced in animals infected with SIVCD154 (Fig. 7 and 8). This phenomenon cannot be explained by levels of viral replication, because viral loads were low and similar for both groups of macaques. One possible explanation is that expression of CD154 on SIVCD154-infected T cells resulted in the activation of CD40-expressing endothelial cells, and activated endothelial cells have been shown to regulate leukocyte trafficking into inflammatory sites by secreting the chemokines IL-8, MCP-1, and RANTES (44, 46). Thus, this reduction in absolute numbers and activation levels for blood lymphocytes may be a reflection.

FIG. 8. Changes in activation state (expression of CD69) of peripheral blood lymphocytes after infection with SIV vectors. Rhesus macaques were infected with SIVCD154 (open diamonds) or SIVCNTL (solid squares). Blood lymphocytes were stained with antibodies to CD3, CD4, CD8, CD16, CD20, and CD69 and analyzed as described in Materials and Methods. Values represent the mean of four animals with the standard deviation. Asterisks indicate a significant difference between both groups ($P < 0.05$, unpaired $t$ test).
of increased extravascular trafficking of activated lymphocytes, which may be rapidly controlled by homeostatic mechanisms once viral loads decline.

As mentioned before, CD154-CD40 interactions are critical for the development of T-cell-dependent immune responses. Nevertheless, analysis of the anti-SIV immune responses induced in macaques infected with SIVCD154 or SIVCNTL did not show any significant difference, except for an early anti-SIV Gag cell-mediated response in SIVCD154-infected monkeys (Fig. 9B). We believe that these similarities in immunogenicity are a consequence of SIVCD154 and SIVCNTL both being negative for Nef expression. SIV Nef has been shown to downmodulate cell surface expression of CD4 (16), CD3 ζ chain (4, 24), major histocompatibility complex (MHC) class I (37), and CD28 (5, 43) molecules, and to induce expression of FAS ligand (49) on infected cells. All these events have the potential to affect the ability of the infected CD4⁺ T cell to interact with an APC via the T-cell receptor (TCR) or the delivery of costimulatory signals. Thus, it is possible that, in the absence of Nef, the disturbances to the CD154-CD40 axis associated with the interaction between gp120 and CD4 (8, 50) are not of a magnitude such as to prevent the development of a strong immune response. Another factor that favors the host immune response over the virus replication, and that has been observed before (1, 18, 20), is that both SIVCD154 and SIVCNTL are recombinant SIV nef viruses that have an additional DNA sequence (the CD154 gene), which reduces the kinetics of viral replication and allows the generation of an early immune response. Nevertheless, this immune response is not completely effec-

FIG. 9. Analysis of the anti-SIV humoral and cellular immune response of rhesus macaques after infection with SIV vectors. Rhesus macaques were infected with SIVCD154 (rhesus macaques 35, 37, 42, and 44) or SIVCNTL (rhesus macaques 36, 38, 40, and 41). (A) Anti-SIV Env and Gag antibody titers were determined in rhesus plasma by antigen-specific ELISA as described in Materials and Methods. (B) SIV Gag-specific IFN-γ SFC were determined by ELISPOT using rhesus PBMCs stimulated with overlapping 15-mer SIV Gag peptides.
tive, since some animals in which the virus reached higher loads remained chronically positive for virus isolation.

In summary, we engineered a replication-competent, nef-deficient SIV that expressed a biologically active CD154 molecule. In vitro studies demonstrated that cells infected with this recombinant virus expressed CD154 on their surface and that this CD154 had costimulatory activity on neighboring CD40-bearing cells. However, in vivo studies in rhesus macaques verified that additional expression of CD154 during infection with an attenuated SIV did not have a detectable influence on the development of anti-SIV immune responses or on viral replication in the absence of an active nef gene. Our results suggest that the level of expression of CD154 in itself may not be responsible for the dysregulation observed during pathogenic infection. However, our studies do not preclude the development of immunotherapeutic approaches that target CD154, but they reinforce the concept that the CD154-related immune defects seen in HIV infection may be caused by events upstream of the signals that lead to the expression of CD154.

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