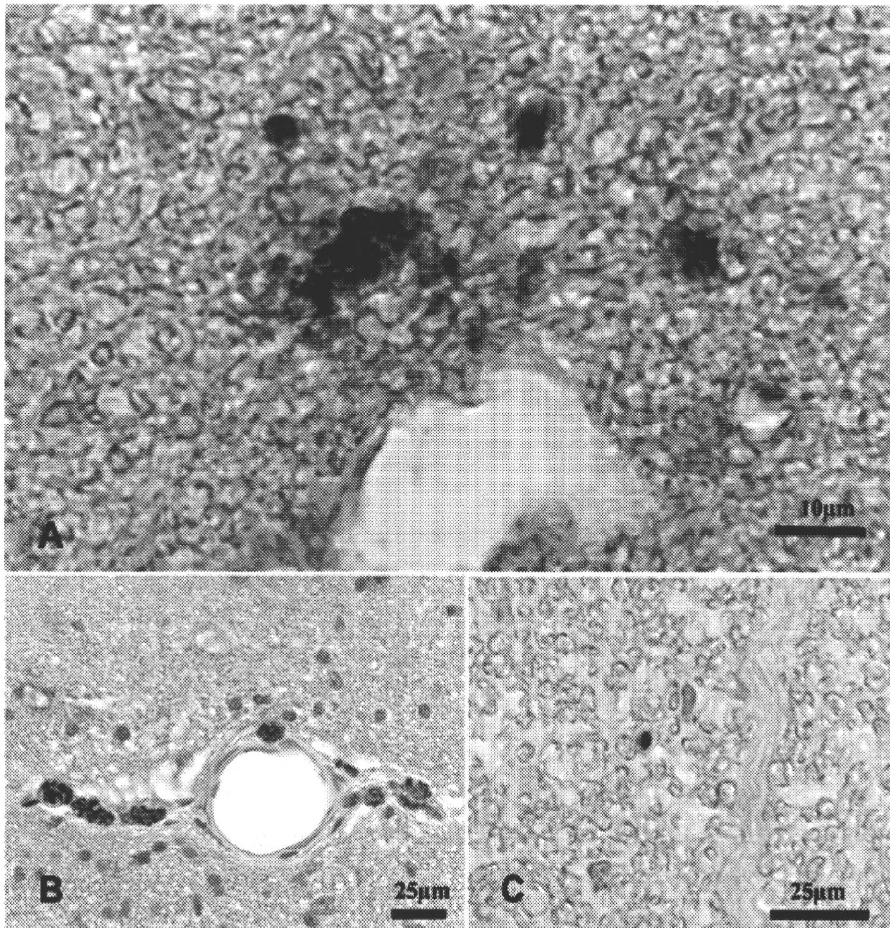


**Fig. 2** Expression of a simian immunodeficiency virus (SIV) envelope protein by the immunostaining of SIVenvgp160/gp32 in macaque #531. SIV envelope protein is demonstrated in an inflammatory lesion in the frontal white matter (A), some perivascular macrophages in the basal ganglia (B), and a few in the spinal cord (C).

(Figs 2A,4B), and seemed to be microglia/macrophages and MNGCs based on their morphology. Some perivascular macrophages were also positive in basal ganglia (Fig. 2B). We also detected a few positive cells in the cerebellum and the spinal cord (Fig. 2C) as well as meningeal mononuclear cells. In another macaque, #626, SIVenvgp160/gp32 positive cells were limited to a few perivascular and meningeal mononuclear cells.

The lymph nodes of two virus-infected animals showed hyperplasia of follicles and their germinal centers showed irregular shapes. Decrease of CD3+ T-cells in the paracortical region was not evident.

All control macaques showed no abnormality in both brains and lymph nodes.

## The expressions of TNF-α and IL-1β in inflammatory lesions

Since macaque #531 showed typical inflammatory lesions with MNGCs, we further examined expression of proinflammatory cytokines by immunohistochemistry. IL-1β-positive cells showed intracytoplasmic labeling. Positive cells were detected only in inflammatory lesions with MNGCs of the frontal white matter, that is to say, we could not detect IL-1β-positive cells in the parenchyma of basal ganglia as well as in the spinal cord. In order to investigate the relation between expression of IL-1β and virus infection, we performed double-label immunohistochemistry for IL-1β and SIVenvgp160/gp32. Interestingly, the IL-1β positive cells were found around the SIVenvgp160/gp32-positive cells, but not SIVenvgp160/gp32-positive cells (Fig. 3). The brain parenchyma of macaque #626 did not showed any IL-1β-positive cells.

TNF-α was also labeled as cytoplasmic staining. Positive cells were detected in some mononuclear cells of inflammatory lesions in the frontal white matter and basal ganglia, as well as a few perivascular macrophages. We could not detect TNF-α-positive cells in the spinal cord. TNF-α-positive cells seemed to be SIVenvgp160/gp32-negative cells in comparison with distribution of SIVenvgp160/gp32-positive cells stained using a serial section (Fig. 4). In macaque #626, a few TNF-α-positive cells were detected in perivascular and meningeal mononuclear cells.

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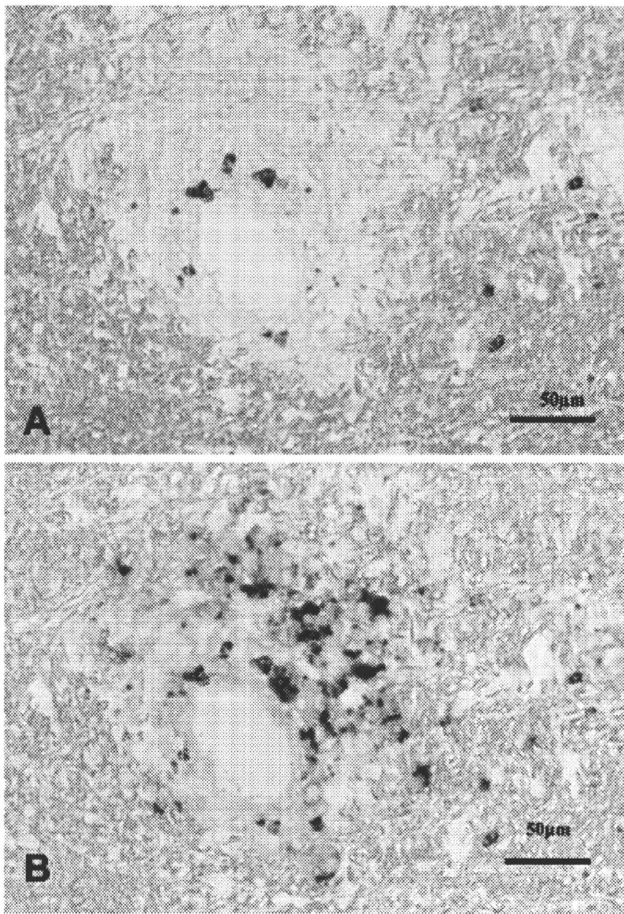


**Fig. 3** Expression of IL-1β and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A) IL-1β-positive cells are detected only in an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B) IL-1β positive cells were found around the SIVenvgp160/gp32-positive cells but not SIVenvgp160/gp32-positive cells demonstrated by double-labeling immunohistochemistry performed using the same section of (A). A: anti-IL-1β; B: double-label immunohistochemistry for IL-1β (red) and SIVenvgp160/ gp32 (dark blue).

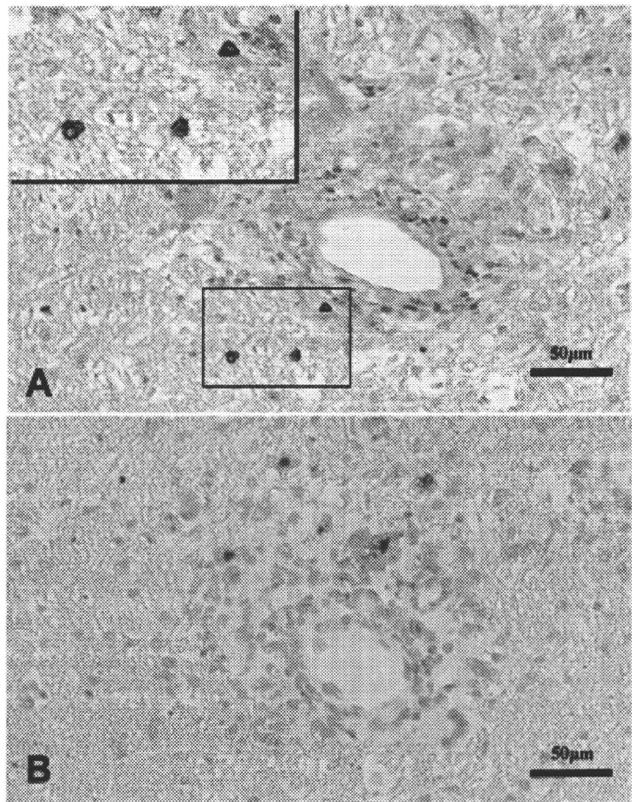


**Fig. 4** Expression of TNF-α and SIVenvgp160/gp32 in an inflammatory lesion with microglial nodule with multinucleated giant cells (MNGCs). (A): TNF-α-positive cells are detected in mononuclear cells of an inflammatory lesion with MNGCs seen in the frontal white matter of macaque #531. (B): Distribution of SIVenvgp160/gp32-positive cells differs from that of TNF-α-positive cells demonstrated by SIVenvgp160/gp32 immunohistochemistry of serial section. A: anti-TNF-α, B: anti-SIVenvgp160/gp32.

## DISCUSSION

Cytokines such as TNF-α and IL-1β may have toxic effects on CNS cells and have been postulated to contribute to the pathogenesis of the neurological complications of human immunodeficiency virus (HIV) infection.[22] However many of such studies were done by in vitro experiments; exposure of macrophages and microglia to either gp120 or Tat resulted in up-regulation of TNF-α expression,[23,24] and exposure of microglia to gp120 resulted in the production of IL-1β.[25,26] In contrast, there are only a few reports which demonstrated proinflammatory cytokines in the AIDS brain tissues directly in vivo. Tyor *et al.*[11] reported that there were significant increases in IL-1β and TNF-α in HIV-positive patients compared with HIV-negative brains, but no correlation was found between levels of cytokines and the presence or absence of CNS disease among HIV-positive individuals. In addition, in vivo expression of these cytokines in the microenvironment of HIV encephalitis, microglial nodules with MNGCs, was not demonstrated in their study. Zhao *et al.*[27] reported that IL-1β was expressed at high levels in areas of microglial nodules in HIV encephalitis. Because some MNGCs were positive for IL-1β in their report, they suggested that IL-1β was induced by HIV-1 infection.

In our present study, expression of IL-1β and TNF-α were detected in the inflammatory lesions with MNGCs, and these positive cells were found to be negative for SIVenvgp160/gp32. There were a few TNF-α positive cells and almost no IL-1β positive cells in the area other than inflammatory lesions including microglial nodules. Our findings indicate that virus-infected microglia/ macrophages do not always express IL-1β and TNF-α. The

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findings seen in macaque #531 might indicate a limited role of IL-1β and TNF-α in the very early stage of ADC. In order to understand a precise role of proinflammatory cytokines in ADC, further studies are required focusing on origin or nature of the cells expressing proinflammatory cytokines.

The differences between previous reports[22,27] and our present data about in vivo expression of cytokines might be explained by complexities of brain pathology in patients who have died from terminal AIDS. Human autopsies were usually performed in the advanced stages of AIDS. In such conditions, the brains may contain a variety of pathologic conditions other than HIV encephalitis such as diffuse poliodystrophy, another pathologic event of ADC, many kinds of opportunistic infections and tumors, and/or effects of anti-viral agents. Our macaque #531 with typical pathologic findings of SIV encephalitis was not in the stage of AIDS, and opportunistic diseases or diffuse poliodystrophy were not observed. We can also exclude the effects of chemotherapy.

In the present study, macaque #531 with typical SIV encephalitis did not show obvious neurological symptoms or behavior abnormality. This reminded us of a previous report in which the brains of asymptomatic HIV-1-positive individuals who died accidentally revealed HIV-1 infection and inflammatory response in the cerebral white matter.[28] These observations indicate that histopathologic findings of HIV encephalitis might be subclinical in many individuals infected with HIV-1. Another macaque (#626) did not show microglial nodules with MNGCs. The plasma viral load of this animal was much lower than that of macaque #531. This suggested that presence or absence of HIV encephalitis might simply depend on the value of plasma viral load.

Our macaque infected with SIV239env/MERT induced typical microglial nodules with MNGCs as a model of HIV encephalitis, and this macaque model may be useful for the better understanding of HIV encephalitis pathogenesis.

## ACKNOWLEDGMENTS

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# Soluble PD-1 rescues the proliferative response of simian immunodeficiency virus-specific CD4 and CD8 T cells during chronic infection

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## Summary

**Phenotypic and functional studies of the programmed death-1 (PD-1) molecule on CD4⁺ and CD8⁺ T cells were performed on peripheral blood mononuclear cells from uninfected and simian immunodeficiency virus (SIV)-infected rhesus macaques. These data demonstrated a rapid upregulation of PD-1 expression on tetramer-positive CD8⁺ T cells from MamuA.01⁺ SIV-infected macaques upon infection. Upregulation of PD-1 on total CD8⁺ T cells was not detectable. In contrast, CD4⁺ T-cell PD-1 expression was markedly higher in total CD4⁺ T cells during chronic, but not acute, infection and there was a correlation between the level of PD-1 expression on naive and central memory CD4⁺ T cells and the levels of viral loads. Such association was emphasized further by a marked decrease of PD-1 expression on tetramer-positive CD8 T cells as well as on CD4⁺ T cells on longitudinal samples collected before and after the initiation of antiretroviral therapy and downregulation of viral replication *in vivo*. Cloning of PD-1 and its two ligands from several non-human primate species demonstrated > 95% conservation for PD-1 and PD-L2 and only about 91% homology for PD-L1. Functional studies using soluble recombinant PD-1 protein or PD-1–immunoglobulin G fusion proteins induced marked increases in the SIV-specific proliferative responses of both CD4⁺ and CD8⁺ T cells from rhesus macaques. The results of these studies serve as a foundation for future *in vivo* trials of the use of rMamu-PD-1 to potentially enhance and/or restore antiviral immune responses *in vivo*.**

**Keywords:** immune response; PD-1; PD-L1; simian immunodeficiency virus; T cells

## Introduction

Both human and select non-human primate species infected with human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), respectively, demonstrate a sequential progression of immune dysfunction which ultimately contributes to increased susceptibility to opportunistic infections and death.[1,2] While the time line for the appearance of such immune dysfunction varies, it is clear from following the initial depletion of CD4⁺ T cells, primarily within the gastrointestinal tract,[3,4] that there is a gradual loss of CD28 expression, a failure of antigen-specific memory T cells to synthesize interleukin-2 (IL-2), to proliferate, to synthesize proinflammatory cytokines such as tumour necrosis factor-α, perforin, and interferon-γ (IFN-γ), and to degranulate, and a development of anergy followed by senescence and death.[5–12] The administration of antiviral chemotherapy in a variety of combinations has led to a dramatic decrease in viral loads and has prolonged the life of HIV-1-infected humans.[13–15] However, such chemotherapy is required for the lifetime of the patient; it is not without side effects and issues involving quality of life dictate that alternative strategies are desperately needed.[13,15–18] More importantly, such chemotherapy, while resulting in low to undetectable levels of plasma viraemia, does not appear to rapidly or fully reverse immune dysfunction.[17,19,20] Strategies for reconstituting immune function to a reasonably functional level have therefore been identified as one of the major research targets in lentiviral research.

Studies utilizing murine models of acute versus chronic infection, such as those that have used the lymphocytic choriomeningitis virus (LCMV) model system,

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have provided significant new insights into the potential mechanisms of dysfunction of T-cell responses that occur during chronic viral infection.[21,22] Measurement of the kinetics of virus-specific immune function in such a model has shown that while robust virus antigen-specific functional T-cell responses can be measured during the acute infection period, this is soon followed by the progressive loss of such function during the chronic viraemia period and the T cells during such chronic viraemia have been termed 'immune exhausted'.[23,24] Such a lack of functional T-cell responses has also been described when antigen-specific T cells are repeatedly challenged with their cognate antigen, such as those described using the murine model of influenza virus infection.[25,26] These findings of 'immune exhaustion' in the murine system were remarkably similar to the observations made in a variety of human clinical infections, such as in patients with chronic hepatitis B or C virus infections and, more relevant to the present study, in HIV-1- and SIV-infected humans and non-human primates.[27–29] Until recently, the general consensus view was that such T-cell exhaustion was essentially non-reversible and the only avenue for successful replenishment of T-cell function was by the generation of newly minted T cells that should be provided with the right environment and growth potential *in vivo*. The only clinically applicable alternative reported so far has been the isolation of T cells from patients and non-human primates, followed by expansion *in vitro* and autologous adoptive transfer protocols.[30–32] Thus, the recent findings that blocking of the interaction between programmed death-1 (PD-1; CD279) and its cognate ligand PD-L1 can restore function in otherwise 'immune-exhausted' T cells has opened a new vista for the potential therapeutic utilization of such strategies *in vivo*.[21,33,34] It is important to note that before the discovery of the potential to reverse immune function by utilizing PD/PD-L1 blockade, similar strategies were explored for the blockade of cytotoxic T-lymphocyte antigen-4 with its ligands CD80/CD86,[35,36] blockade of B- and T-lymphocyte attenuator[37,38] and strategies aimed at manipulating regulatory T-cell function[39] in a variety of human diseases and animal models.

Our laboratory has been exploring strategies aimed at immune reconstitution in SIV-infected non-human primate models of acquired immune deficiency syndrome (AIDS).[40,41] Thus, rhesus macaques (*Macaca mulatta*) are susceptible to infection with SIV, a lentivirus that is highly related to HIV-1 and HIV-2, leading to chronic high viral loads, progressive immune dysfunction and immune exhaustion similar to human HIV infection.[42] A number of laboratories, including ours, have thus been contemplating studies aimed at determining whether the *in vivo* blocking of the PD/PD-L1 pathway leads to more robust SIV-specific immune responses and reversal of immune exhaustion in the rhesus macaques. However, it was reasoned that before embarking on such *in vivo* studies, a more detailed examination of the constitutive and induced expression of this molecule was warranted because the *in vivo* blocking of this pathway is likely to interfere with the normal physiological function of such interactions. The fact that SIV infection leads to the generation of a wide spectrum of autoantibodies,[43] which are known to be influenced by interactions between PD-1/PD-L1,[44,45] makes it important to take this issue into account if such *in vivo* studies are initiated. Finally, because the repeated use of monoclonal murine or even humanized antibodies against PD-1 or its ligand is bound to induce immune responses against the foreign antibodies in the monkeys and limit its use *in vivo* , our laboratory has prepared several recombinant forms of rhesus macaque PD-1 and herein report the efficacy of this reagent to enhance immune function in antigen-specific macaque peripheral blood mononuclear cells (PBMC) *in vitro*.

## Materials and methods

### *Non-human primates*

Ten adult healthy uninfected Indian rhesus macaques and 21 SIV-infected rhesus macaques served as blood donors for this study. All animals were housed at the Yerkes National Primate Research Center (YNPRC) of Emory University and were cared for in conformance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Health and Human Services guidelines 'Guide for the Care and Use of Laboratory Animals'. The SIV-infected animals in these studies were inoculated intravenously with 200 50% tissue culture infective dose ($TCID_{50}$) of SIVmac239. The blood samples from these SIV-infected rhesus macaques were obtained at various times post-infection (p.i.). A sub-group of these rhesus macaques were treated with 9-(2-phosphonyl-methoxypropyly)adenine (PMPA) (20 mg/kg subQ daily for 28 days) after reaching viral load set-point and served as a source of samples for the study of the effect of antiviral drug therapy on the expression of PD-1. RNA samples from other species were obtained through the Resource for non-human primate immune reagents (http://pathology.emory.edu/Villinger/index.htm).

### *Viral load determination*

Plasma viral load was routinely monitored in each of the SIV-infected monkey species by the NIAID, NIH CFAR sponsored Virology Core Laboratory of Emory University School of Medicine.

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*Flow cytometric analysis of PD-1*

The PBMC were isolated from freshly obtained peripheral blood samples from each monkey using standard Ficoll–Hypaque gradient centrifugation. The cells at the interface were washed twice with RPMI-1640 supplemented with penicillin/streptomycin, L-glutamine and 10% fetal calf serum (hereafter referred to as media). The PBMC were adjusted to $10 \times 10^6$/ml media and dispensed in 100-µl aliquots into individual 5-ml sterile test tubes. Aliquots of the PBMC were then incubated with a predetermined optimum concentration of fluorescein iosthiocyanate (FITC)-conjugated anti-CD95, phycoerythrin (PE)-conjugated anti-CD28, Peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (or PerCP-conjugated anti-CD8) and biotinylated anti-PD-1 followed by allophycocyanin (APC)-conjugated streptavidin. Controls consisted of a tube that was incubated with similarly conjugated identical isotype immunoglobulin (background control), tubes with each of the above reagents alone, and a tube containing the same combination of reagents except biotinylated clone SP34 (anti-CD3) was utilized instead of biotinylated anti-PD-1 (to make sure that the frequencies of PD-1-expressing $CD4^+$ $CD8^+$ T cells reflected accurate frequencies of the CD4 and CD8 subsets). All the stained tubes were incubated for 30 min at 4°, washed three times with media and then resuspended in 0·5 ml cold phosphate-buffered saline (PBS) pH 7·4 containing 2% fetal bovine serum and then subjected to flow cytometric analysis using the fluorescence-acitvated cell sorting (FACS) Caliber (B-D immunocytometry Division, Mountain View, CA). Following standard gating using forward and side scatter for lymphoid cells, data on a minimum of 20 000 events were collected and the data obtained on the subsets were analysed utilizing CELL QUEST and FLOWJO software.

*Analysis of PD-1 expression by p11c-tetramer-reagent-positive cells*

Basically, the protocol was the same as above, except aliquots of the PBMC from Mamu-A01$^+$ monkeys were incubated with predetermined optimum concentrations of FITC-conjugated anti-CD3 (clone SP34, BD, Mountain View, CA), PE-conjugated p11c tetramer complex (courtesy of Dr John Altman, Emory University VRC, Atlanta, GA), PerCP-conjugated anti-CD8 and biotinylated anti-PD-1 followed by APC-conjugated streptavidin. Again, the tubes were washed twice in media and then resuspended in 0·5 ml cold PBS pH 7·4 + 2% fetal calf serum vortexed gently and then subjected to flow cytometric analysis as described above. The frequency of p11c-tetramer-positive cells that stained with the anti-PD-1 reagent was determined.

*Quantification of cell proliferation*

The effects of blocking the interaction between PD-1 and its ligand PD-L1 on virus antigen-specific T cells were analysed using a cell proliferation assay. Briefly, $10^7$ PBMC were labelled with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE), washed twice in PBS with 5% fetal bovine serum. The CFSE-labelled cells were adjusted to $1 \times 10^6$ cells/ml and incubated with either a mixture of SIV gag peptides covering the entire SIV gag sequence (20-mers overlapping by 12) at 0·1 µg/ml of each peptide or AT-2-treated SIVmac239 (containing 0·5 µg SIV p27) in media containing anti-CD28/CD49d monoclonal antibodies (1 µg/ml of each antibody) in the presence or absence of recombinant rhesus macaque PD-1 (rMamu-PD-1) at a predetermined optimal concentration of 6·7 µg/ml or 1–10 µg rMamu-PD-1–immunoglobulin G (IgG) or rMamu-PD-1–IgG mutant. The cells were cultured for 6 days and were maintained by the addition of fresh media on days 2 and 4. On day 6, the cells were stained with PE-conjugated anti-CD3,, PerCP-conjugated anti-CD4 and APC-conjugated anti-CD8. Proliferation was evaluated via dilution of CFSE on a FACS Calibur flow cytometer. Data from at least 50 000 cells gated on the lymphocyte population were analysed by FLOWJO software (Tree Star, Ashland, OR). $CD3^+$ cells were selected and the percentage of cells with diluted CFSE ($CFSE^{+low}$) was determined in gated populations of total $CD4^+$ or $CD8^+$ T cells.

*Enumeration of SIV-specific T cells via IFN-γ ELISPOT*

The enzyme-linked immunosorbent spot-forming cell (ELISPOT) assay was performed as previously described.[46] Briefly, the IFN-γ ELISPOT kit was a kind gift of Dr N. Ahlborg (Mabtech, Nacka, Sweden); this kit utilizes the clones GZ-4 and 7-B6-1 for the capture and detection of the cytokine. The assay was performed using unfractionated PBMC from the monkeys. Individual wells of the assay kit were incubated with 250 000 PBMC in complete media alone (negative control), 5 µg/ml concanavalin A (positive control), or 10 µg/ml of individual pools of overlapping SIV gag (each pool containing eight peptides which were 20-mers overlapping by 12) or env peptides (each pool containing eight peptide 25-mers overlapping by 13, courtesy of the NIH AIDS Reagent Program) and the plates were incubated overnight at 37° in a 7% $CO_2$ humidified incubator. Each culture was performed in the absence or presence of 10 µg/ml goat polyclonal anti-PD-1 (AF1086, R&D Systems, Minneapolis, MN) or the anti-PD-L1 (clone MIH1, eBioscience, San Diego, CA) monoclonal antibody. The wells were washed extensively, which was followed by the addition of the biotinylated detector antibody and incubation for 2 hr. The wells were once again washed and streptavidin-conjugated alkaline phosphatase was added, followed by the addition of the substrate nitro-

blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate buffer (Mabtech). An automated spot reader system (CTL, Shaker Heights, OH) was used to determine the developed spots and the mean number of spots in the experimental wells was deducted from the media control to derive the net spots which were then used to calculate the average spot-forming cells per $10^6$ input cells. In limited experiments, once a distinct pool of SIVenv or SIVgag was found to induce significant responses, PBMC from the same monkey were obtained at a later time interval and the assay was repeated using the same protocol with the cells being incubated with individual peptides of the pool in an effort to more clearly identify the role of PD/PD-L1 blockade on specific SIV peptide-specific responses.

### *Cloning and characterization of non-human primate PD-1, PD-L1 and PD-L2*

Cloning of the PD-1 and its two ligands was performed by reverse transcription–polymerase chain reaction (RT-PCR) from RNA isolated from activated PBMC, lymph node cells or splenocytes from rhesus macaques (*Macaca mulatta*), sooty mangabeys (*Cercocebus atys*) and, for purposes of phylogenetic comparison, pigtailed macaques (*Macaca nemestrina*), cynomolgus macaques (*Macaca fascicularis*), baboon (*Papio anubis*) and the common marmoset (*Callithrix jacchus*) as previously described.[47] The primers were designed after blasting the human cDNA with the rhesus genome (http://www.hgsc.bcm.tmc.edu). The primers utilized included the following: (sense PD-1: CWCTGSTGGRGCTGCTCCAGG; anti-sense PD-1: TGA AGCAGTGACTGCATCTGG and CTCATGGTGGAGGG TCTGCAG; sense PD-L1: CGAGGCTCCGCACCAGCCG and TGCAGGGCATTCCAGAAGAT; anti-sense PD-L1: TTTCGCCAGGTTCCATTTTCAGTG and AATCCCTGC TTGAAGATCAGAAGT; sense PD-L2: CAGCTAGAAAG AATCCTGGGTT and GGCTGT TCATTTTGGTGGCTA; anti-sense CARGTTTCAGATTAAAGTGCTGG and CTG GCTCCCAAGACCACAGGT.

The amplified fragments were ligated into pGEM-T (Promega; Madison, WI) vectors. The ligated plasmids were transfected and amplified into competent JM109 cells. Plasmids were then purified and the various clones were sequenced (Agencourt, Beverly, MA). Several clones obtained from multiple animals from the same species were analysed and shorter amplicons were analysed for the evaluation of potential splice variants. The sequences were compared and aligned using the GCG package.

### *Construction and expression of recombinant soluble macaque PD-1 and soluble macaque PD-1–IgG fusion protein*

In an effort to create antagonists to the PD-1–ligand pathways without the capacity to signal through either PD-1 or its ligands, recombinant soluble PD-1 was expressed. A fragment corresponding to the mature extracellular domain of rhesus macaque PD-1 was amplified with sense primer TGC*CCATGG*ACCAGGATGGTTCT TAGACTCC introducing an *Nco*I site for fusion with the macaque IL-4 leader peptide, and the anti-sense primer AGCATGC*GAATTC*AGTGGTGATGGTGATGGTGCACCA GGGCTTGGAACTGG binding to the C terminus of the extracellular domain of PD-1 fused to a hexahistidine tag. The amplified fragment was then ligated in frame with the macaque IL-4 leader sequence[47] and the entire insert was then subcloned into the pcDNA3.1 Neo(–) using *Sal*I and *Eco*RI. Expression was performed in transfected T293 with purification of the PD-1 released in the supernatants using a nickel activated column (Amersham Bioscience, Piscataway, NJ). After elution with 250 mM imidazole, the protein was dialysed against PBS and tested for purity by polyacrylamide gel electrophoresis and Coomassie staining as well as by Western blot.

In addition, because these antagonists were to be used *in vivo* with the need for longer half-lives and bioavailability, soluble rhesus PD-1 was also fused to either the native macaque IgG2 heavy chain Fc (consisting of the hinge region, CH2 and CH3 domains) or a mutant Fc. For the mutant Fc two amino acid substitutions were introduced to eliminate potential interactions with Fc receptors and complement, L235A and P331S, respectively.[48,49] Each construct was introduced into the pcDNA3.1 Neo(–) expression vector and production of the PD-1/Fc proteins was carried out in 293T cells transfected using the Amaxa system (Gaithersburg, MD). Expression vector construction was carried out using a series of PCRs to fuse the various fragments. Briefly, the extracellular domain of rhesus PD-1 in frame with the rhesus IL-4 leader sequence was amplified from pGEM rh4sPD-1 (his) with the primers T7 and PD-1Ig2b (5′-GTAGATCTACCCACCAGGGCTTGGA ACTG-3′) and the IgG2 Fc encoding fragment was produced by amplifying from the pGEM PAmIL15IgG plasmid using primers PAmigg2b (5′-GGTAGATCTACGTGC CCACCGTGCCCAGCTGAA-3′) and IgG6ae (5′-TATG ACGTCGAATTCTCATTTACCCGGAGACACGGAGA-3′). These two fragments were then joined by PCR using primers T7 and IgG6ae. The resulting product was digested with *Spe*I and *Eco*RI and ligated into pcDNA3.1 Neo(–) previously digested with *Nhe*I and *Eco*RI, resulting in pcDNA PD-1/Fc.

The mutant PD-1/Fc was similarly produced except that the pcDNA3.1 insert was constructed by the following series of PCRs to create the desired mutations. First, three overlapping PCR fragments were amplified: the 5′ end (encoding PD-1 fused to the N-terminal portion of the Fc) using primer T7 and G2L235AR (5′-TGACGG TCCCCCCGCGCGTTCAGCTGGGCA-3′); an internal 316-base pair (bp) fragment was amplified with primers G2L234AF (5′-AGCTGAACTCGCGGGGGGACCGTCAG

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TCTT-3′) and P331SR (5′-ACAGTTTTCTGCCTTGAG GCCGGGAGGCCT-3′), and a 3′ end 383-bp fragment amplified with primers P331SF (5′-AGGCCTCCCGGCC TCAAGGCAGAAAACTGT-3′) and IgG6ae. The two fragments comprising the C terminus of the mutated IgG Fc were then reassembled using primers G2L234AF and IgG6ae and the resulting product was joined to the 5′ end containing the IL-4 leader, PD-1 soluble fragment fused to the hinge regions of the immunoglobulin using primers T7 and IgG6ae. All constructs were verified by sequencing.

PD-1–Ig was purified onto Sepharose Protein G columns, eluted with 0·2 N acetic acid/0·15 M NaCl pH 2·5, and neutralized with one-tenth 2 M Tris–HCl pH 9·5. The protein was dialysed against PBS and tested for purity by polyacrylamide gel electrophoresis and Coomassie staining as well as by Western blot.

### *Analysis of PD-1 mRNA by Northern blot*

Briefly, aliquots of PBMC from human, rhesus macaque and sooty mangabey donors were either left unstimulated or stimulated overnight with beads coated with anti-CD3 (clone SP34 for human PBMC or FN18 for monkey PBMC) and anti-CD28 (clone L293) monoclonal antibodies or with 2 μg/ml of concanavalin A. After incubation the cells were washed and lysed and RNA was obtained using the Trizol reagent (Invitrogen, Carlsbad, CA). Ten-microgram aliquots of the various RNA samples were then run on an agarose gel and hybridized to a $^{32}$P-labelled probe generated from the rhesus macaque PD-1 full-length clone via random primer labelling. After extensive washes, the blots were exposed to film for various lengths of time.

### *Statistical analyses*

Data are presented as mean ± SD of the number of samples that were utilized for each of the assays or analyses. The Mann–Whitney *U*-test was utilized to calculate statistical significance using the STATVIEW software program (Cary, NC).

## Results

### Sequence analysis of PD-1, PD-L1 and PD-L2

In attempts to verify the homologies of PD-1 and its ligands PD-L1 and PD-L2 in our study model, each molecule was cloned from RNA isolated from the PBMC of several rhesus macaques and sooty mangabeys as well as from RNA samples isolated from the PBMC of a number of other non-human primate species. Several PD-1 cDNA clones were obtained from the Old World primate species *M. mulatta*, *M. fascicularis*, *M. nemestrina* and sooty mangabeys and sequenced (Fig. 1a). Overall, these sequences shared > 96% homology at both the nucleotide and amino acid levels. Most amino acid changes were conserved among the four Old World primate species analysed. Our cloning attempts for New World marmoset PD-1 was not successful. One clone each from a rhesus and a mangabey was missing a lysine residue at the beginning of Exon 5, which was reminiscent of a previously reported splicing event for the T-cell receptor zeta transcript, in which a G-protein motif appeared disrupted by the insertion of an additional residue at the junction right before an immunoreceptor tyrosine-based activation motif.[50] The deletion in these PD-1 clones does not *a priori* induce any detectable functional alteration. In fact both the intracellular immunoreceptor tyrosine-based inhibition and switch motifs were clearly conserved among the various primate species. Of interest also was the finding that a number of shorter open reading frames were identified that curiously did not conform to a strict exon/intron splicing event because both of the clones were missing part of exon 1, the entire exon 2 and part of exon 3, resulting in a deletion of most of the extracellular domain of PD-1. Such large deletions have been noted before for other molecules (e.g. T-cell immunoglobulin and mucin domain protein-3 (TIM-3) and refs 51,52) although the functional relevance of such transcripts remains unknown. Although alternative splice variants of human PD-1 have been reported,[51,53] the variants identified herein from both Old World primate species represent novel variants. To address the potential presence of alternative splice variants, Northern blot analysis of resting and activated PBMC was performed. Representative data from several such analyses are shown in Fig. 1b. There appeared to be at least two major variants that were detectable for humans and mangabeys and potentially three variants in rhesus macaques. It is not clear which of these variant(s) were finally expressed on the cell membrane in a functional form.

In addition to PD-1, its cognate ligands PD-L1 and PD-L2 were also cloned and sequenced. While the degree of conservation of PD-L1 was relatively high at the nucleotide level (96%) across the various primate species tested, at the protein level, 7–10% divergence was noted between monkey and human PD-L1 (Fig. 1c). Of note, and as previously reported,[47] the New World marmoset sequence was approximately equidistant in divergence between human and Old World primates. The sequences derived from cynomologus macaques and from a baboon exhibited an early termination signal and may not have been representative of the species. Also of interest was the isolation of a deletion construct comprising essentially only the extracellular immunoglobulin-like V-type domain. It is possible that if such a sequence is synthesized it is likely to be in a soluble form and has the potential to bind to PD-1. This clone was however only obtained from a single animal and it remains to be

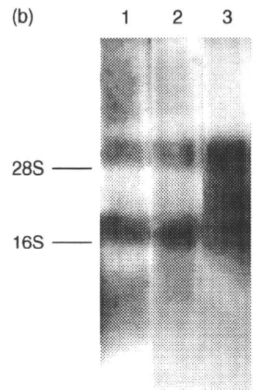
(a)

```
            Signal peptide   --|  Exon 1                 /Exon 2       | |          |
Human       MQIPQAPWPVVWAVLQLGWRPGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRVT
Rhesus      -------------------------E----------------L---------------A--------------------------------R------
Cynomolgus  -------------------------E-------A--------L---------------A---------------------------------------
Mangabey    -------------------------E----------------L---------------A---------------------------------------
Mang Var    -------------------------E........................................................................
Pigtail     -------------------------E----------------L---------------A---------------------------------------
Pigtail Var1 ------------------------E........................................................................
Pigtail Var2 -------C----E----------L-E........................................................................

            Exon 3    Extra-cellular  | || |  | |   Ig-like V-type                  Transmembrane
Human       QLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLVVGVVGGLLGSLVLLVWVLAVICSRAAR
Rhesus      ---------------------------------------------------------------------A----------------------------Q
Cynomolgus  R--------------------------------------------------------------------A----------------------------Q
Mangabey    ---------------------------------------------------------------------A----------------------------Q
Mang Var    ...............................................----------------------A----------------------------Q
Pigtail     ---------------------------------------------------------------------A----------------------------Q
Pigtail Var1 ..............................................----------------------A----------------------------Q
Pigtail Var2 ..............................................----------------------A--------------------------D-Q

            Exon 4     /Exon 5     xxxx  Cytoplasmic tail   ++++
Human       GTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPL *
Rhesus      ---E-------------------------------------A-----------------L----------------PR------------- *
Cynomolgus  ---E-------------------------------------A-----------------L----------------PR------------- *
Mangabey    ---E-------------------------------------A-----------------L----------------PR------------- *
Mang Var    ---E-------------------------------------A-----------------L----------------PR------------- *
Pigtail     ---E-------------------------------------A-----------------L----------------PR------------- *
Pigtail Var1 ---E--------------------E------------Q---A-----------------L----------------PR------------- *
Pigtail Var2 ---E-------M------------E------------Q---A-----------------L--------------W-PR---H--------- *
                       &
```

(b)




(c)

```
            Signal Peptide -|           Extracellular Ig-like V-type                              100
Human       MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQ
Rhesus 1    ---------TI----------------------------R-------G-TS----------------------------N-----Q-------------R
Rhesus 2    ---------TI--------------------------V-----------TS----------------------------N-----Q-------------R
Pig-tailed  ---------TI--------------------------------------TS----------------------------N-----Q-------------R
Cynomolgus  ---------TI-----------------------K--------------TS----------------------------N-----Q-------------R
Mangabey 1  ---------TI--------------------------------I-----TS----------------------------N-----Q-------------R
Mangabey2.1 ---------TI---S----------------------------------TS----------------------------N-----Q-------------R
Mangabey2.2 ---------TI--------------------------------------TS----------------------------N-----Q-------------R
Baboon      ---------TI--------------------------------------TS----------------------------N-----Q-------------R
Marmoset    ---------------------------------------------R-E-TS----------------------------------Q---N-----I----

            101                                         Extracellular Ig-like C2-type             200
Human       ITDVKLQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTTTTNSKREEKLFNVTSTLRIN
Rhesus 1    -------------------------------------------------------------------------------------------L--------
Rhesus 2    -------------------------------------S-----------------------------------------------------L--------
Pig-tailed  ----------------------------------------------------------------------------------A--E-----L--------
Cynomolgus  ------------------------------GD*
Mangabey 1  ---------------------------------------------------------------V---------------------------L--------
Mangabey2.1 -------------------------------------------------------------------------------------------L--------
Mangabey2.2 -------------------------------.....................................................................
Baboon      -----------------------------------------------------------------------------D*
Marmoset    --------------------------------------------------Y--------------------------------E--K-------A-----

            201                                  Transmembrane           Cytoplasmic tail   290
Human       TTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNERTHLVILGAILLCLGVALTFIFRLRKGRMMDVKKCGIQDTNSKKQSDTHLEET *
Rhesus 1    --A------I----G-----------------L--------------F-L---------Y--------M--S--R-------R--Q---- *
Rhesus 2    --A------I----------------------L--------------F-L---------Y--------M--S--R-------R--Q---- *
Pig-tailed  --A------I----------------------L--------------F-L---------Y--------M-----R-------R--Q---- *
Mangabey 1  --A------I----------------------L----------------L---------Y--------M-----R-------R--Q---- *
Mangabey2.1 --A------I----------------------L----------------L---------Y--------M-----R-------R--Q---- *
Mangabey2.2 ...................................................................................--Q---- *
Marmoset    --A-------------------------Q--VL----------------F---------Y--------L-I---R-------N--Q---- *
```

(d)

```
            1 Signal peptide -|                      Extracellular Ig-like V-type                  100
Human       MIFLLLMLSLELQLHQIAALFTVTVPKELYIIEHGSNVTLECNFDTGSHVNLGAITASLQKVENDTSPHRERATLLEEQLPLGKASFHIPQVQVRDEGQY
Rhesus 1    ----------------T--------------------A------------------------------------------S-------------------
Rhesus 2    ----------------T--------------------AI-----------------------------------------S-------------------
Pigtail 1   ----------------T--------------------A------------------------------------------S-------------------
Pigtail 2   ----------------T--------------------A------------------------------------------S-------------------
Mangabey 1  ----------------T---------------------------------------------------------------S-------------------
Mangabey 2  ----------------T---------------------------------------------------------------S-------------------
Mangabey 3  ----------------T---------------------------------------------------------------S-------------------
Marmoset    ----------------T-..................................................................................

            101                                              Extracellular Ig-like C-type         200
Human       QCIIIYGVAWDYKYLTLKVKASYRKINTHILKVPETDEVELTCQATGYPLAEVSWPNVSVPANTSHSRTPEGLYQVTSVLRLKPPPGRNFSCVFWNTHVR
Rhesus 1    ---------------------------------------------------------I--------------------------H-----------AQ--
Rhesus 2    ---------------------------------------------------------I--------------------------H-----------AQ--
Pigtail 1   ---------------------------------------------------------I--------------------------H-----------AQ--
Pigtail 2   --------------------................................................................................
Mangabey 1  ---------------------------------------------------------I--------------------------H-----------AQ--
Mangabey 2  ------------------------------------------------------R--I--------------------------H-----------AQ--
Mangabey 3  ---------------------------------------------------------I--------------------------H--------S--AQ..
Marmoset    ....................------------------------------------DI-----G------------------NLH-----------AP--

            201                    Transmembrane              Cytoplasmic tail
Human       ELTLASIDLQSQMEPRTHPTWLLHIFIPSCIIAFIFIATVIALRKQLCQKLYSSKDTTKRPVTTTKREVNSAI *
Rhesus 1    ------------I-----------------------------------------------S---K-------- *
Rhesus 2    ---------R--I-----------------------------------------------S---K-------- *
Pigtail 1   ------------I-----------------------------------------------S---K-------- *
Pigtail 2   .......................................................-----S---K-------- *
Mangabey 1  ------------I-----------------------------------------------S---K-------- *
Mangabey 2  -----N------I-----------------------------------------------S---K-------- *
Mangabey 3  .........................................................................TQQKDL SPRKKGK *
Marmoset    --------P-------AS---------------------M------H------T------T------------ *
```



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determined whether such a molecule is more widely produced and functional *in vivo*.

The homology between human and Old World primate PD-L2 was 97% at the nucleotide level and 95% at the protein level (Fig. 1d). The marmoset clone diverged slightly more than 95% at the nucleotide level and 92% at the protein level, although only a truncated clone was obtained from this species, similar to a splice variant reported in humans but for PD-L1.[52] Of interest again was the finding of splice variants in pigtail macaques (deletion of the immunoglobulin-like C-type extracellular domain), marmoset (deletion of the extracellular immunoglobulin-like V-type domain) and in a mangabey (deleted of the transmembrane domain and intracellular tail). The functional relevance of these splice variants is currently under study.

### Modulation of PD-1 expression by CD4$^+$ and CD8$^+$ T cells following SIV infection of rhesus macaques

The specific antibodies listed in the methods section were selected based on cross-reactivity with non-human primate cell samples (data not shown) and subsequently utilized at a predetermined optimum concentration for all the studies reported herein (Fig. 2a). Since previous reports have characterized PD-1 expression both by frequency analysis and also documented global increases in density on CD8$^+$ T cells in chronic infections, similar studies were carried out here. The PBMC samples from 10 rhesus macaques were analysed from time-points before infection, during the acute viraemia period (4–6 weeks post-infection, plasma viral loads ranging from $10^4$–$10^7$ vRNA/ml) and during the chronic phase (>6 months post-infection, but clinically healthy, plasma viral loads from < 100 to > $10^6$ vRNA/ml). The major changes noted were in the frequency of total CD4$^+$ T cells that express PD-1 ($P = 0{\cdot}02$) which was restricted to the naïve ($P = 0{\cdot}045$) and central ($P = 0{\cdot}02$, Fig. 2b) memory subsets, while CD4$^+$ T effector memory or CD8$^+$ T-cell subsets did not exhibit significant differences over the chosen time periods before and after SIV infection. Density of PD-1 expression also did not seem to markedly differ on CD4$^+$ or CD8$^+$ T cells examined from the different time-points, although a slight decrease in the density of PD-1 expression on CD4$^+$ T cells was observed during acute infection (Fig. 2b). These data suggested that in the context of chronic SIV infection with sustained viraemia, the CD4 compartment most prominently expressed higher levels of PD-1, as a reflection of immune activation and a potentially higher susceptibility to undergo apoptosis.

### Effect of plasma viral loads on PD-1 expression

When the level of viraemia was taken into consideration relative to the duration of the chronic infection, attempts to establish a direct correlation between viral loads and frequency of PD-1 expression on the surface of T cells from rhesus macaques chronically infected with SIV were not successful. However, as an alternative approach 21 chronically infected monkeys were grouped according to their respective viral load set points as high viral loads (> 50 000 vRNA /ml plasma, $n = 11$) or low viral loads (< 10 000 vRNA/ml, $n = 10$) and both groups were compared for frequency and density of PD-1 expression (Fig. 3). As seen, naïve and central memory subsets of both CD4$^+$ and CD8$^+$ T cells from rhesus macaques with high viral loads (> 50 000 viral copies/ml) showed higher global frequencies of cells expressing PD-1 than similar subsets of CD4$^+$ and CD8$^+$ T cells from rhesus macaques with lower viral loads ($P = 0{\cdot}006$ for CD4 and CD8) (Fig. 3). Effector memory CD4$^+$ and CD8$^+$ T-cell subsets did not show any detectable differences. Except for a slight decrease in the density of PD-1 expression by central memory CD8$^+$ T cells, no other detectable differences in PD-1 density expression were noted.

### Expression of PD-1 by p11c-tetramer$^+$ CD8 cells from Mamu-A01 rhesus macaques

Although our studies suggest a more profound modulation of PD-1 expression on CD4$^+$ T cells during chronic

**Figure 1.** (a) Alignment of PD-1 amino acid sequences from human and non-human primates including alternative transcripts from sooty mangabey (Mang Var) and pig-tailed macaque (Pigtail Var). The transmembrane region is shaded. Additionally, transcripts were isolated from rhesus macaque and sooty mangabey in which the lysine (&) at the start of exon 5 was absent. 'N' indicates potential N-linked glycosylation sites, '–' residues identical to human PD-1, '.' deleted residues, 'xxxx' immunoreceptor tyrosine-based inhibition motif (ITIM), '++++' immunoreceptor tyrosine-based switch motif (ITSM), '|' indicates residues reported to be critical for binding to the ligand for the murine PD-1. (b) Analysis of PD-1 mRNA by Northern blot shows two bands. The figure is representative of analysis performed on mRNA extracted from the peripheral blood mononuclear cells (PBMC) of six different monkeys of each of the two species and humans. Ten micrograms of total mRNA from the PBMC of human (lane 1), sooty mangabey (lane 2) and rhesus macaque (lane 3) underwent electrophoresis and blotted with a randomly labelled $^{32}$P probe consisting of the entire rhesus macaque PD-1 cDNA ORF. (c) Alignment of PD-L1 amino acid sequences from human and non-human primates. Sequences of two animals from both rhesus macaque and sooty mangabey are represented as well as an alternative transcript from the second mangabey. '–' residue identical to human PD-L1, '.' deleted residues, 'N' indicate potential N-linked glycosylation sites. (d) Alignment of PD-L2 amino acid sequences from human and nonhuman primates. Numbers following monkey names designate different animals. '–' residues identical to human PD-L2, '.' deleted residues, 'N' indicate potential N-linked glycosylation sites.

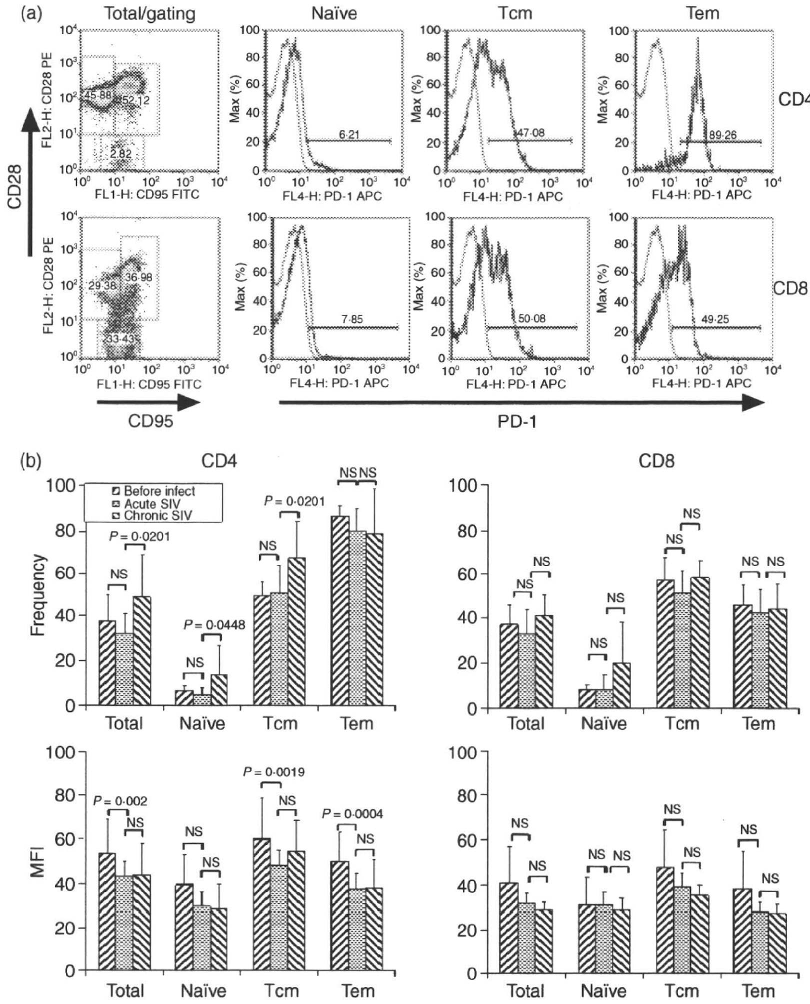


**Figure 2.** (a) Representative gating and analysis of frequency and mean fluorescence intensity (MFI) of PD-1 expression by $CD4^+$ and $CD8^+$ T cells from rhesus macaques. (b) Effects of plasma viral loads on the frequency and MFI of PD-1 expression by total, naive, central and effector memory $CD4^+$ and $CD8^+$ T cells from rhesus macaques sampled before infection and either during acute (4–6 weeks postinfection) or during chronic (>6 month postinfection) SIV infection. Statistical evaluation used the Mann–Whitney *U*-test.

SIV infection, other studies have reported markedly elevated levels of PD-1 on antigen-specific T cells during HIV or SIV infection.[54–58] In an effort to determine the frequency and density of PD-1 expression on SIV antigen-specific $CD8^+$ T cells in our animals, advantage was taken of using PBMC samples from a total of 21 chronically infected asymptomatic Mamu-$A01^+$ rhesus macaques and the p11c tetramer reagent. As seen in Fig. 4a,b, there was a marked increase in both the frequency ($P < 0{\cdot}01$) and density ($P < 0{\cdot}01$) of PD-1 expression by the p11c-$tetramer^+$ cells when compared to total $CD8^+$ T cells from SIV-infected or uninfected monkeys, confirming data reported by others. Furthermore, when the effect of viral load was examined in these Mamu-$A01^+$ monkeys, there also appeared to be a higher frequency of PD-1-expressing p11C-$tetramer^+$ cells in the monkeys with a high viral load compared with those with lower viral loads (Fig. 4c, $P = 0{\cdot}038$). Dot plots and profile of PD-1 expression by $CD8^+$ p11c-$tetramer^+$ cells of two representative monkeys with low and high plasma viral loads, respectively, are depicted in Fig. 4d.

## Effect of antiviral chemotherapy on the expression of PD-1

Samples of PBMC taken from a group of four Mamu-A01 SIV-infected rhesus macaques before and shortly after a 28-day daily course of antiviral PMPA chemotherapy were analysed for the frequency and density of PD-1 expression. All four monkeys responded to the chemotherapy with a decrease of their viral loads from > 50 000 plasma viral copies/ml to undetectable levels. As seen in Fig. 5a, antiviral chemotherapy resulted in a significant decrease in the frequency of PD-1-expressing $CD4^+$ and to some extent also $CD8^+$ T cells. The difference was primarily the result of a decrease in the frequency of $CD4^+$ central memory T cells as well as of $CD4^+$ and $CD8^+$ naïve T cells but not effector memory T cells (Fig. 5b–d). With respect to antigen-specific $CD8^+$ T cells, while PMPA therapy did not have a detectable effect on the frequency of p11C-$tetramer^+$ cells (Fig. 5e), antiviral therapy induced a marked decrease in the frequency of p11c-$tetramer^+$ cells that expressed PD-1 (Fig. 5f). Interestingly,

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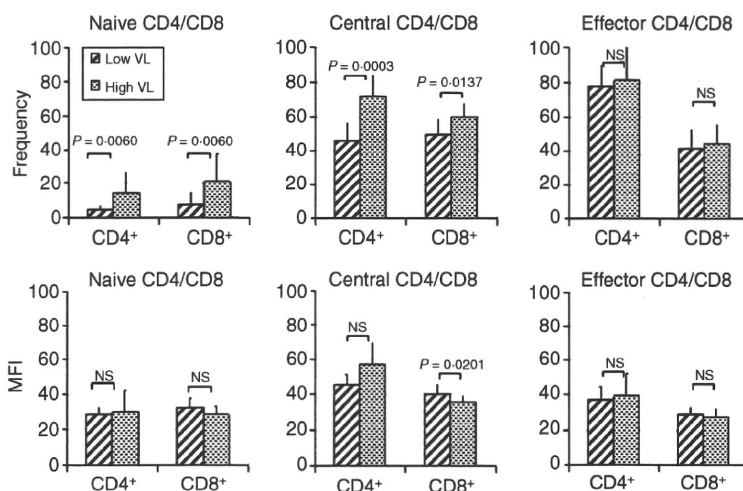


Figure 3. Influence of plasma viraemia on the frequency and mean fluorescence intensity (MFI) of PD-1 expression by naive, central and effector memory CD4$^+$ and CD8$^+$ T cells from rhesus macaques chronically infected with SIV.

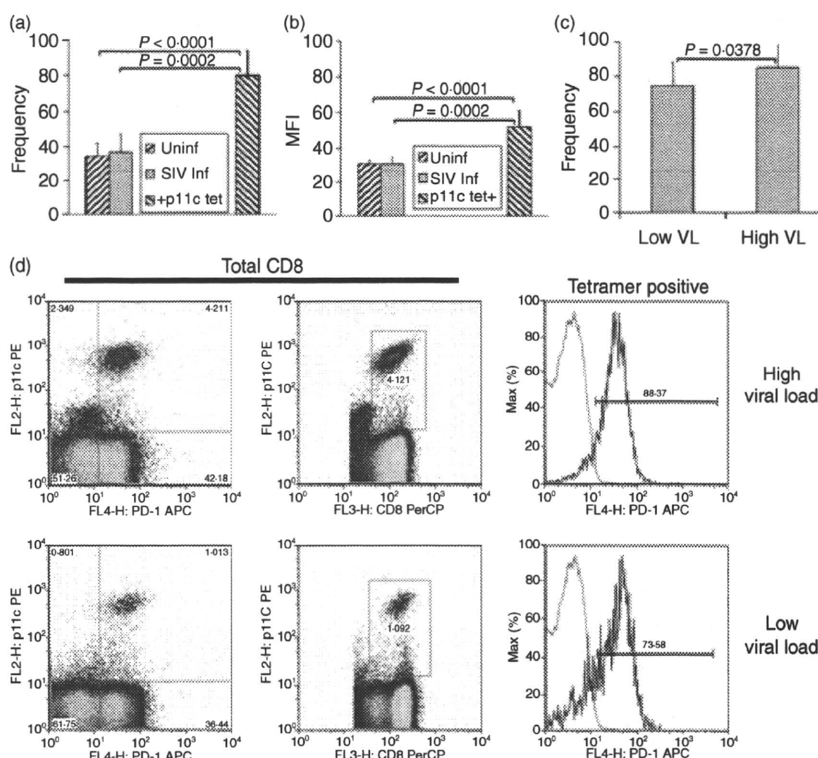


Figure 4. Increased frequency (a) and density (b) of PD-1 expression by Mamu A.01/p11C tetramer$^+$ CD8$^+$ T cells from SIV-infected Mamu-A01$^+$ rhesus macaques ($n$ = 21). (c) Effect of high ($n$ = 11) and low ($n$ = 10) plasma viraemia on the frequency of p11c tetramer$^+$ cells expressing PD-1. (d) Typical profile of PD-1 expression by p11C tetramer$^+$ CD8$^+$ T cells from PBMC of Mamu-A01$^+$ rhesus macaques with high and low plasma viral loads. Most p11C tetramer$^+$ CD8$^+$ T cells express PD-1 on their surface (left panel). Gated are the positive signals versus the isotype control staining.

PMPA therapy of two Mamu-A01$^+$ rhesus macaques relatively late during infection (> 2 years postinfection) with plasma viral loads of > 1 × 10$^6$/ml did not decrease the density of PD-1 expression on the p11c-tetramer$^+$ cells (data not shown), which has important implications for potential PD-1-based therapies in SIV-infected rhesus macaques and HIV-1-infected humans.

### Attempts to reverse the inhibitory function of PD-1/PD-L1 interaction

Several strategies were tested to study the effect of blocking the interaction between PD-1 and its ligands during SIV antigen-specific immune restimulation *in vitro*. First, PBMC from a total of 12 chronically infected rhesus macaques were assayed *in vitro* by the ELISPOT assay utilizing a series of overlapping pools of SIVenv and SIVgag peptides and for purposes of control media alone (negative) and concanavalin A (positive). The cultures also contained either 10 μg/ml anti-PD-L1 antibody (clone MIH1, eBiosciences) or an isotype control antibody. Data obtained showed low levels of increases in the responses to select pools of peptides but importantly gave inconsistent findings in samples from the same monkey obtained at different time intervals (data not shown). The reasons for such inconsistent data are discussed below. The

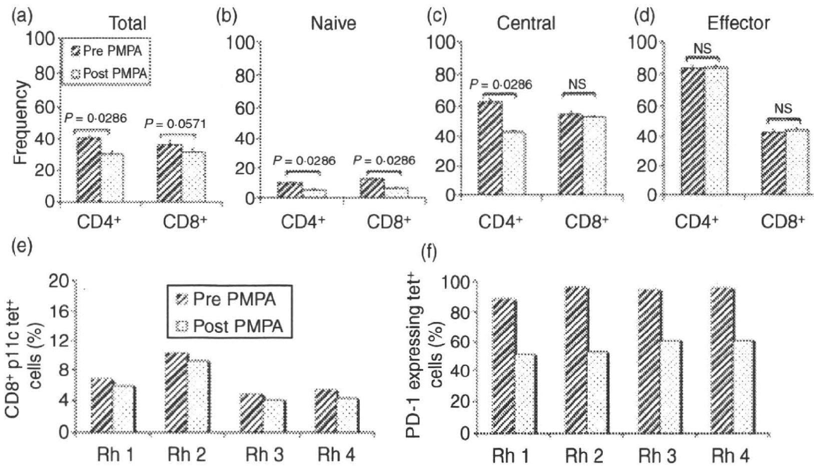


**Figure 5.** Modulation of PD-1 (%) expression on total (a), naive (b), central (c) and effector memory (d) $CD4^+$ and $CD8^+$ T cells sampled from rhesus macaques treated with effective antiretroviral chemotherapy (PMPA) during chronic SIV infection. (e, f) Modulation of frequency and MFI of PD-1 expression on p11C tetramer$^+$ $CD8^+$ T cells from four Mamu A.01$^+$ rhesus macaques following effective (PMPA) chemotherapy.

second strategy used a polyclonal antibody against PD-1 (goat anti-human PD-1 that cross-reacts with rhesus PD-1, R & D Systems) to block interactions between PD-1 and PD-L1. Use of such an antibody at varying concentrations (1–25 μg/ml) consistently led to polyclonal activation of both $CD4^+$ and $CD8^+$ T cells, a finding that has important implications for *in vivo* therapy discussed below (Supplemental Fig. S1). The third strategy utilized a series of our laboratory prepared recombinant rhesus macaque soluble PD-1 molecules derived from the above mentioned cloning effort in a 6-day proliferation assay similar to the one reported by others.[54–57] Use of these reagents provided the most consistent results. Thus, CFSE-labelled PBMC from a total of eight SIVmac239-infected rhesus macaques were analysed for their proliferative response to either a pool of SIVgag peptides (100 ng/ml), 0·5 μg p27/ml of AT-2-treated SIVmac239 or for purposes of control, media alone in the presence or absence of an optimal concentration of 6·7 μg rMamu-PD-1. As seen in Fig. 6a,b, $CD4^+$ T cells from six of eight and five of five rhesus macaques showed enhanced proliferative responses when cultured with the pool of SIVgag peptides or whole AT-2-inactivated SIVmac239, respectively, in the presence of rMamu-PD-1 as compared with controls. Similarly, $CD8^+$ T cells from six of eight and five of five rhesus macaques showed enhanced proliferative responses when incubated with the SIVgag pool of peptides and the AT-2-treated SIVmac239, respectively, in the presence of rMamu-PD-1. Control incubation of PBMC from four uninfected RM showed no proliferation of either $CD4^+$ or $CD8^+$ T cells both in the absence of presence of rMamu-PD-1, denoting that the enhancement observed were not secondary to non-specific activation. Of interest was the fact that data shown in Fig. 6(a,b) reflecting donor rhesus macaques 5–7 in this assay exhibited high viral loads (> $10^5$ copies/ml plasma), rhesus macaques 8 and 9 had intermediate viral loads ($10^3$–$10^4$ copies/ml plasma) and rhesus macaques 10–12 had barely detectable loads (approximately 100 copies). Although not absolute, enhancement of the antigen-specific $CD8^+$ and $CD4^+$ T-cell responses was more readily detectable from viraemic monkeys than from controls, supporting the notion that chronic high antigen exposure promotes the role of PD-1 in limiting effector responses.

One salient limitation to the use of recombinant proteins *in vivo* is their relatively short half-life, in particular for compounds that are supposed to inhibit rather than trigger a response.[46] We therefore generated two additional versions of the rMamu-soluble PD-1 by fusing PD-1 to the Fc domain of a macaque IgG2 or to the same domain in which two amino acids were mutated to incapacitate this Fc binding to complement or Fc receptors respectively. It was therefore relevant to test the capacity of these two fusion recombinants to exert the expected inhibition of the PD-1/PD-ligand pathway. As represented in Fig. 7a, both soluble rMamu-PD-1–IgG proteins appeared equally efficient at enhancing SIVgag-specific proliferation as the soluble rMamu-PD-1 protein used in the assays reported in Fig. 6. Furthermore, a dose-dependent enhancement of the proliferative response was demonstrated with increasing amounts of either soluble rMamu-PD-1–IgG protein (Fig. 7b). This suggests that soluble PD-1–IgG proteins may be used *in vivo* to evaluate the potential benefit of such proteins during chronic SIV infection of rhesus macaques in future studies.

## Discussion

Immunological dialogue at the cell surface among lymphoid cells involves interactions between a series of cell surface receptors and their cognate ligands and, based upon the expression and degree of interaction of these molecules, the immunological dialogue is translated into biological function. Thus, for antigen-specific immune responses, besides the interaction between the T-cell receptor on T cells and the peptide-bearing major histo-

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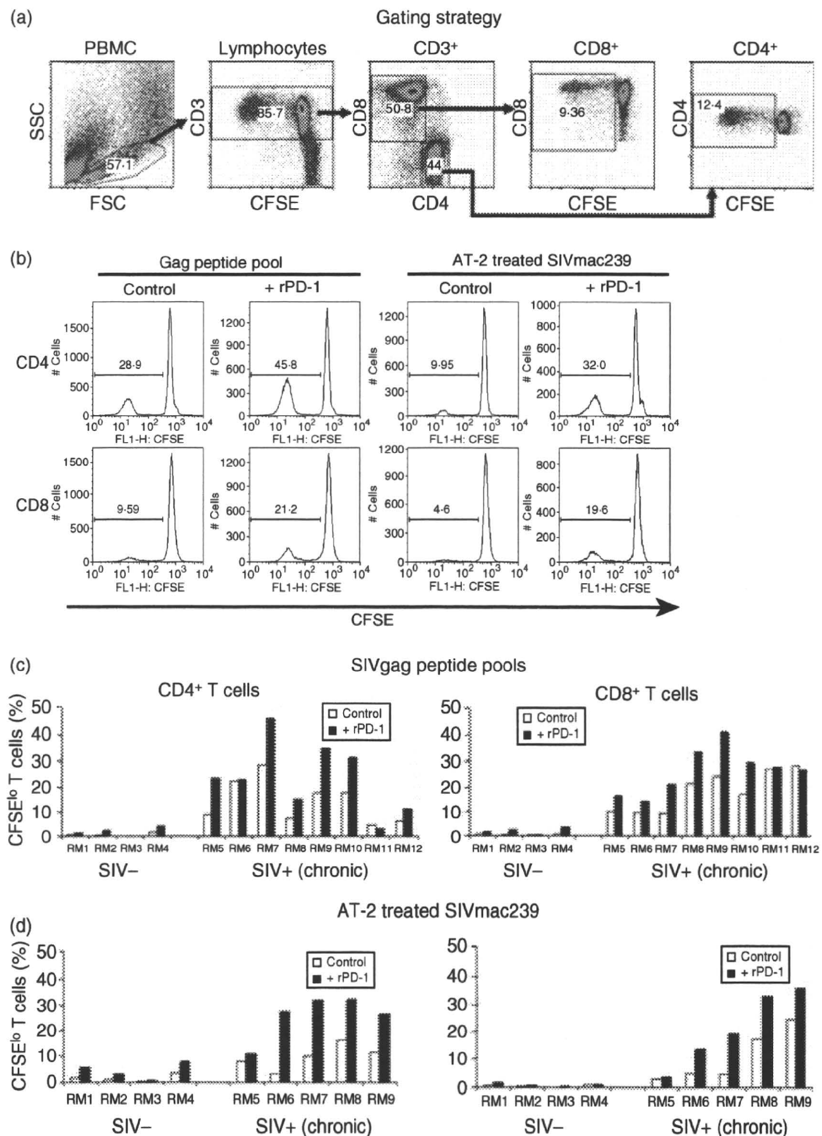


**Figure 6.** Augmentation of SIV-specific *in vitro* $CD4^+$ and $CD8^+$ T-cell proliferative responses by recombinant soluble rhesus macaque PD-1 (rPD-1). Aliquots of peripheral blood mononuclear cells (PBMC) labelled with CFSE were restimulated with either media (control), 100 ng/ml a pool of overlapping SIVgag peptides (Gag peptide pool, a) or 0·5 µg p27/ml of AT-2-treated SIVmac239 (b) in the absence or presence of 6·7 µg/ml of rPD-1 *in vitro* for 6 days. Each aliquot was then stained with anti-CD3, anti-CD4 and anti-CD8 and analysed by flow microfluorometry for the frequency of $CD3^+$ $CD4^+$ T cells and $CD3^+$ $CD8^+$ T cells that express decreased levels of CFSE ($CFSE^{lo}$) as an index of antigen-specific proliferation (c). (a) represents the response of PBMC from four uninfected rhesus macaques (RM 1–4), five rhesus macaques (RM 5–7) chronically infected with SIVmac239 with high viral loads, two (RM 8 and 9) with intermediate viral loads and three chronically infected macaques (RM 10–12) with low viral loads (< 1000 vRNA/ml) to the SIV-gag peptide pool. (b) same as (a) except the response shown is against the AT-2-treated SIV and the low viral load RM 10–12 were omitted.

compatibility complex molecules on the antigen-presenting cells, a series of additional molecules on the T cells and antigen-presenting cells interact and the quality and quantity of the immune response is dictated by such interactions. What is becoming increasingly clear is that there are not only immune enhancing but also immune blunting and regulating signals that result from the interactions between some of these molecules and their ligands. The most extensively studied immune facilitating/enhancing molecules include the CD28 and the CD80/86 and the regulatory molecules, include interaction between cytotoxic T-lymphocyte antigen-4 and CD80/CD86 and PD-1 with PD-L1.[33,59,60] These latter regulatory interactions have been reasoned to contribute to the maintenance of peripheral tolerance, especially because PD-L1 has a broad tissue distribution.[61,62] Results from several lines of study have advanced the concept that repeated challenge of the immune system, including chronic exposure to high levels of antigen brought about following select infection, neoplasm or transplantation, leads to T cells that become progressively dysfunctional and not effective in mediating immune function.[36,45,63] This has been clearly observed in the murine models of LCMV and influenza virus infection[23,25,61,64] and in mice that are immunized in the absence of $CD4^+$ T helper cells,[64,65] which has obvious implications for lentivirus infection of humans and non-human primates.

The demonstration that the functional inactivation of the PD-1/PD-ligand pathway in the context of chronic LCMV infection leads to restoration of T-cell-mediated resolution of viraemia has promoted the concept of functional 'immune exhaustion' of T cells and particularly antigen-specific $CD8^+$ T cells during chronic infection and sustained high viraemia. These observations have led

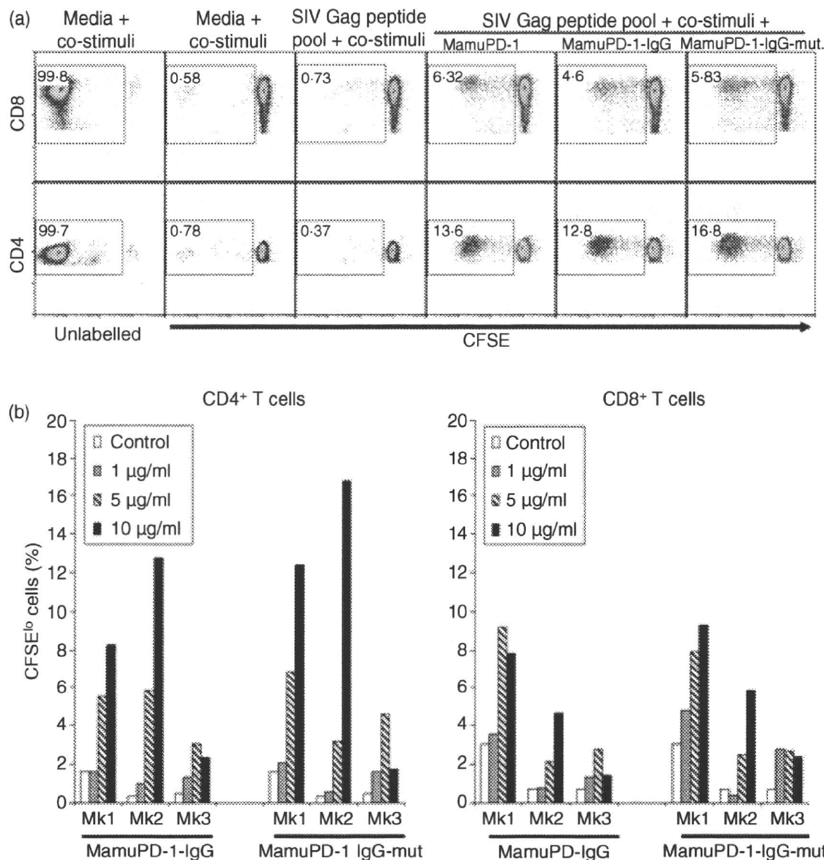


Figure 7. Comparison of soluble rMamuPD-1, rMamuPD-1–IgG and rMamu-PD-1–IgG mutants for their capacity to enhance the SIVgag-specific proliferation of $CD4^+$ and $CD8^+$ T cells from rhesus macaques chronically infected with SIVmac239. (a) Representative flow cytometric profile of CFSE-expressing $CD4^+$ and $CD8^+$ T cells from a SIVmac239-infected rhesus macaque incubated *in vitro* for 6 days 100 ng/ml of a pool of overlapping SIVgag peptides in the absence and presence of increasing doses of rPD-1–IgG. (b) Dose–response of the enhanced SIVgag-specific proliferative response of $CD4^+$ and $CD8^+$ T cells from three rhesus macaques chronically infected with SIVmac239 with increasing doses of rMamuPD-1–IgG and rMamu-PD-1–IgG mutants.

to the rapid application of this concept to a number of human chronic infections such as hepatitis and to cancer patients with a continuous high supply of tumour-specific antigens.[29,59,66] While it was previously thought that such immune-exhausted cells were irreversibly dysfunctional, the finding that the blocking of PD-1 interactions with PD-L1 led to a remarkable reversal of the exhausted phenotype in the chronic LCMV model associated with complete control of viraemia[21] has rapidly led to the examination of PD-1 expression in human HIV-1 infection.[54–56] The results from several laboratories provided confirmatory evidence for a role of PD-1 expression and the immune-exhausted phenotype. Thus, there was a correlation noted between increasing viral loads, disease progression and density of levels of PD-1 expression on antigen-specific $CD8^+$ T cells. These series of findings have prompted studies utilizing SIV-infected non-human primates and aimed at determining the *in vivo* efficacy of the administration of agents that can block the interaction between PD-1 and its ligands in efforts to determine if the 'immune exhaustion' phenotype can be reversed and importantly whether such reversal leads to rekindling of virus-specific effector immune responses, lowering of viral loads and slowing down of disease progression. Before such studies are initiated, it was reasoned by our laboratory that a more detailed examination of the constitutive expression of this molecule on lymphoid cells and their subsets, and the influence of SIV infection and antiretroviral drugs have on the expression of this molecule is warranted. This was precisely the objective of the studies reported herein. Thus, it was reasoned that because the constitutive expression of PD-1 and its ligands is known to have a normal physiological role, attempts to block such interactions may have deleterious effects, such as the induction of autoimmunity, that need to be taken into account before large-scale *in vivo* studies using non-human primates. The role of the interaction between PD/PD-L1 or PD-L2 pathways in regulating autoimmunity has been reviewed elsewhere.[44,45] In this regard, our laboratory has already documented the fact that SIV infection of rhesus macaques leads to the induction of a spectrum of autoantibodies but with no detectable clinical consequence.[43] However, inhibiting the interaction between PD-1 and its ligands *in vivo* may have the potential of precipitating autoimmune disease in such SIV-infected animals. A recent study comparing HIV-1-infected patients on highly active antiretroviral therapy (HAART) with untreated patients reported that, similar to our results, a decrease in PD-1 expression was associated with therapy, the expression of PD-L1 on PBMC remained elevated in both HAART and untreated patients compared to healthy controls, suggesting a potential

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maintenance of such immune control mechanisms *in vivo* and a lack of association of PD-L1 expression with immune activation associated with the sustained viraemia.[67]

The results of our studies in the monkey model confirm the reported upregulation of PD-1 by antigen-specific $CD8^+$ T cells in lentivirus-infected hosts,[54–56] and confirm that such elevated expression is related to the level of viraemia,[68] even though a direct correlation between the frequency of PD-1 expression and viral load could not be conclusively established, suggesting individual variations in such upregulation. One difference noted between the data presented herein on monkeys and the human data reported by the team of Dr Walker[54] was the absence of detectable increase in mean fluorescence intensity (MFI) in $CD8^+$ central memory T cells in correlation with viral loads. This may be because all the monkeys analysed in this study were still at the clinical asymptomatic stage, but the issue remains to be fully elucidated.

More striking however, was the observed modulation of PD-1 expression on $CD4^+$ T cells, a finding that has, to our knowledge, only been reported by one other group in the context of HIV infection.[69] Furthermore, the enhancement of proliferation observed following blockade of the PD-1/PD-1 ligand pathways appeared to benefit $CD4^+$ T cells more than $CD8^+$ T cells (Figs 6 and 7). This finding highlights a potential alternative mechanism by which such blockade might operate its 'immune effector rescue' function, indirectly potentially restoring $CD4^+$ T-cell-mediated help to $CD8^+$ T effectors rather than, or in addition to, a direct effect on $CD8^+$ T-cell effectors. At least in the context of chronic infection, and HIV or SIV infection in particular, such a pathway is not without precedent because $CD4^+$-mediated help has been clearly identified to be critical for sustained $CD8^+$-mediated T-cell function.[31,32,70–72] Alternatively blockade of PD-L1 rather than PD-1 alone may additionally prevent the recently reported interaction of PD-L1 with B7.1, which also limits T-cell costimulation and activation, presumably by competition, thereby limiting the recruitment and activation of newly stimulated T cells. Therefore, the competition for B7.1 may contribute to limiting the recruitment of effector T cells while ligation of PD-1 on the chronically stimulated established memory T cells acts to limit T-cell receptor-mediated signalling via SHP1 and SHP2, both mechanisms likely to contribute to immunotolerance.[73]

On the other hand, PD-1 may be primarily a marker of cell activation and the expression of PD-1 is related to the rate of cell activation,[74] a hypothesis which would also correlate well with the early upregulation of this molecule on SIV-specific $CD8^+$ T cells and the higher frequency of PD-1 expression by $CD4^+$ effector memory T cells (Tem) (irrespective of infection status) relative to naive and central memory $CD4^+$ T cells. However, one argument against such a generalized assumption was the finding that neither $CD4^+$ nor $CD8^+$ T cells expressed elevated frequencies or MFI for PD-1 during acute infection, a period of intense immune activation, suggestions that indeed PD-1 expression may be associated with T-cell activation following chronic exposure to antigen.

Second, what are the mechanisms involved in the selective enhancement of PD-1 expression by total $CD4^+$ but not $CD8^+$ T cells following SIV infection in rhesus macaques? One possible explanation for this finding is that PD-1 expression is influenced not only by the rate of cell activation but also by the quantity and/or quality of virus-specific immune responses against the viral antigens. PD-1-expressing $CD4^+$ T cells have also been noted to serve as immune regulatory cells.[75] Thus, following SIV infection, it is possible that immune activation leads to the generation of PD-1-expressing $CD4^+$ T cells to regulate SIV-specific immune responses and apoptosis-mediated contraction of the effector T-cell pool. It is important to keep in mind that SIV infection does lead to depletion of $CD4^+$ T cells in rhesus macaques and there is evidence for clonal depletion of T-cell $V\alpha/\beta$ T-cell receptor-expressing families in this model of human AIDS.[76] Additional studies are clearly needed to address this issue. Third, why is there a relationship between plasma viral loads and PD-1 expression in cells from rhesus macaques and why is there not a clear correlation of viral loads and PD-1 expression on defined T-cell subsets? We submit that this is probably because of the level of chronic immune-mediated cell activation that is characteristic of SIV-infected rhesus macaques (and HIV-infected humans) and there may be a threshold of chronic antigen exposure needed to induce clearly elevated frequencies of PD-1-expressing T cells. Alternatively, individual differences among our outbred macaques may account for the lack of a linear correlation and such a correlative relation may necessitate the inclusion of larger numbers of animals. Higher levels of virus also lead to higher plasma levels of a select spectrum of cytokines in rhesus macaques, which could also influence the level of PD-1 expression. In addition, PD-1 expression following SIV infection may be inversely proportional to the general affinity of the host T cells for their epitope, correlating with our previous data showing poor affinity by both $CD4^+$ and $CD8^+$ T cells from viraemic monkeys compared with high affinity in non-viraemic monkeys.[77]

The effect of antiretroviral therapy on PD-1 expression is an important issue because it clearly has therapeutic implications. Thus, if rPD-1 or other approaches for inhibiting PD-1/PD-L1 interactions are being contemplated, the role of antiretroviral therapy in such studies needs to be taken into account. As observed in the present studies, antiretroviral therapy markedly reduced the levels of PD-1-expressing total $CD4^+$ and total $CD8^+$ T cells and the frequency of p11C tetramer$^+$ cells that

expressed PD-1 (Fig. 5). While reduced exposure to viral antigens could readily account for the decrease noted in the p11C-tetramer$^+$ cells, it is difficult to visualize the decrease in the frequency of total CD4$^+$ and CD8$^+$ cells. This could be related to the level of cell activation and a decrease in the plasma levels of cytokines that are reduced following antiretroviral therapy that influence PD-1 expression. An important issue with regards to the influence of antiretroviral therapy on PD-1 expression was the finding that antiretroviral therapy in rhesus macaques with high plasma viral loads during late chronic infection while reducing plasma the viral load (> 3 logs) did not lower the frequency of PD-1-expressing p11C-tetramer$^+$ cells in two of two monkeys (not shown). Thus, the fact that antiretroviral therapy is effective in reducing PD-1 expression on antigen-specific T cells during early chronic but not late chronic infection suggests that there must be other mechanisms involved in the expression of PD-1 that are not related to viral load. It would be of interest to determine at what time interval postinfection is therapy with agents that inhibit PD/PD-L1 interactions most efficient in reversing the course of disease. The present data suggest that because chemotherapy is effective in not only lowering viral loads but also in decreasing levels of PD-1 expression during the early postinfection period, rPD-1 therapy may be optimal later on, especially during a time interval when chemotherapy lowers plasma viral load but does not decrease the frequency of PD-1-expressing CD4$^+$ and CD8$^+$ T cells.

Finally, the fact that rPD-1 was able to enhance SIV antigen-specific responses in both the CD4$^+$ and CD8$^+$ T-cell lineages in PBMC samples from SIV-infected rhesus macaques at least *in vitro* is an important finding because the data strengthen the rationale for *in vivo* usage of rPD-1 to increase the global SIV-specific immune function, e.g. by allowing costimulation of CD4$^+$ T cells by dendritic cells[44] and to increase the generation of SIV-specific effector immune responses. The finding that functional reversal by blocking PD-1 primarily enhanced the already primed cellular immune response to specific antigens rather than the generation of novel responses to irrelevant neoantigens and potential autoantigens provides hope that a therapeutic window might be exploited, without excessive risk of inducing autoimmune reactions. These data suggest that functionally active PD-1 is expressed only following antigen-specific immune responses followed by repeated antigen exposure and requires such continuous exposure. It is also important to note that if the PD-1/PD-L blockade functions by limiting the action of CD4$^+$ regulatory T cells,[78] the function and protective effect exerted by this cell subset will need to be ascertained in *in vivo* therapeutic trials using rPD-1.

The assays used to show functional reversal of PD-1/PD-L1 blocking presented herein were of interest. Thus, data using pools of overlapping SIVenv and gag peptides and the IFN-γ ELISPOT assay were either low or inconsistent. This failure of the ELISPOT assay was not secondary to testing of a few monkeys because a total of 16 SIVmac239-infected rhesus macaques were analysed at least two or three times each. Of note, similar to reports by Day *et al.* and Petrovas *et al.*,[54,55] intracellular cytokine (ICC) analysis failed to highlight any effect of the PD-1/PD-L1 blockade, suggesting that such blockade on T-cell activity is indirect, which is in agreement with our data. The fact that the 6-day CFSE-assisted proliferation assay provided the most consistent data using either SIVgag peptides or the AT-2-treated SIV suggests that the kinetics of reversal may be key to observing enhancement of antigen-specific responses by blocking the PD/PD-L1 pathway.

We submit that a more detailed study of the consequences of blocking PD-1/PD-L1 interaction *in vitro* is of primary importance in the preparation of a potential therapeutic exploration of PD-1/PD-L1 blockade in the context of pathogenic and chronic lentiviral infection. Given the recent provocative clinical benefit of PD-1 pathway blockade in the murine LCMV model,[21] the current status of HIV immunotherapy clearly dictates that such a promising strategy be given careful consideration. However, given also the recent immunotherapy debacle with the Tegenero anti-CD28 monoclonal antibody,[79] such immunotherapy with potential for the induction of autoimmune responses will require a well-defined animal model in which not only efficacy but also safety can be adequately addressed. We submit that this report may contribute to the definition of such a model.

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