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.

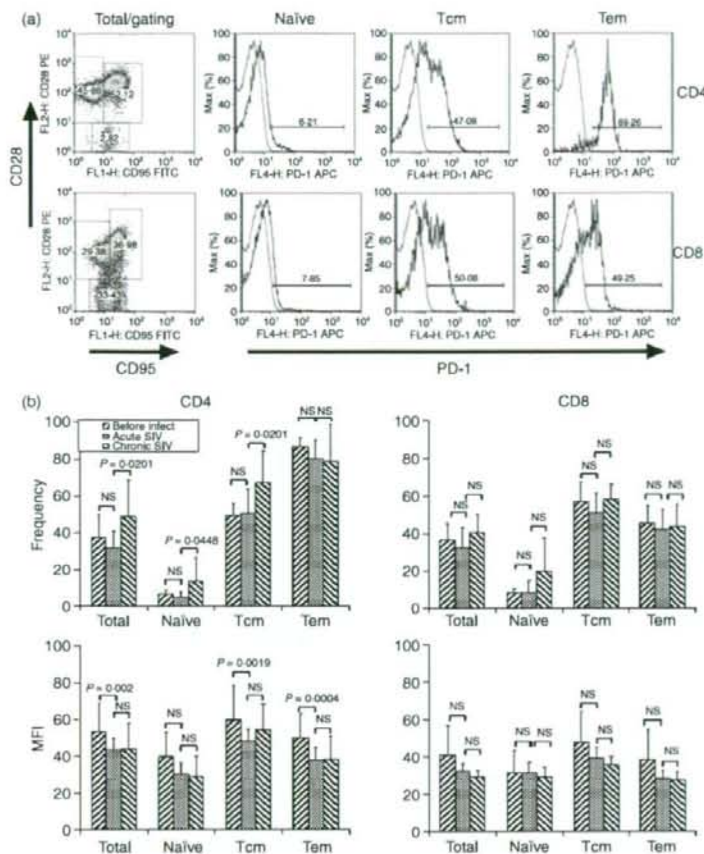

Figure 2. (a) Representative gating and analysis of frequency and mean fluorescence intensity (MFI) of PD-1 expression by CD4^{+} and CD8^{+} T cells from rhesus macaques. (b) Effects of plasma viral loads on the frequency and MFI of PD-1 expression by total, naive, central and effector memory CD4^{+} 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^{+} naive 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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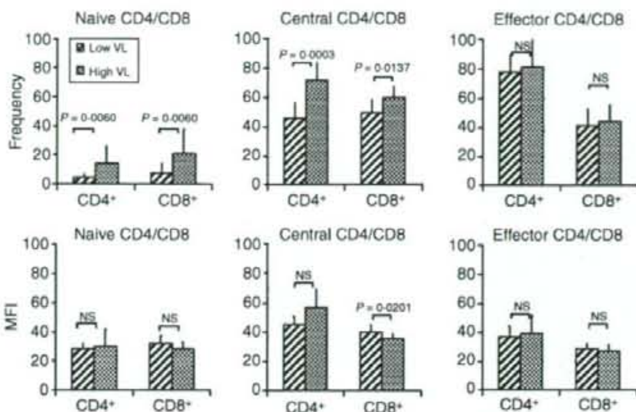

Figure 3. Influence of plasma viraemia on the frequency and mean fluorescence intensity (MFI) of PD-1 expression by naive, central and effector memory CD4^{+} and CD8^{+} T cells from rhesus macaques chronically infected with SIV.

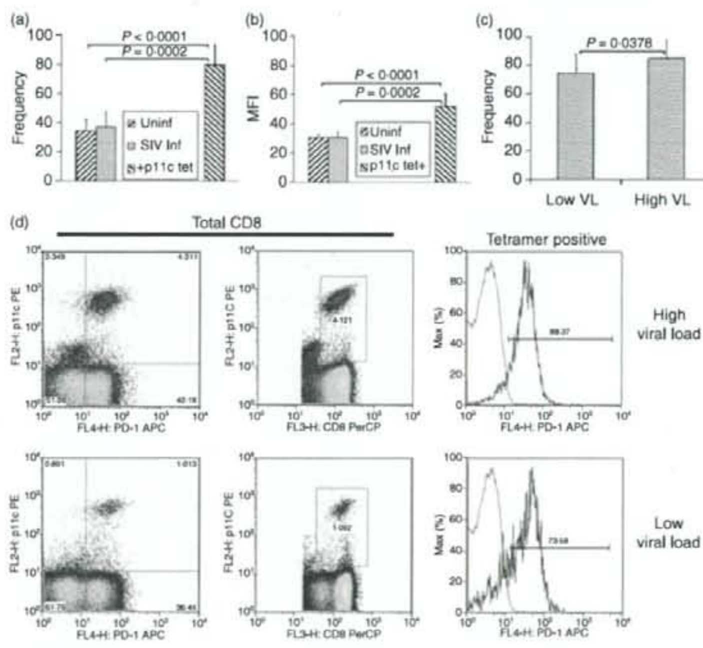

Figure 4. Increased frequency (a) and density (b) of PD-1 expression by Mamu A.01/p11C tetramer^{+} CD8^{+} T cells from SIV-infected Mamu-A01^{+} rhesus macaques (n = 21). (c) Effect of high (n = 11) and low (n = 10) plasma viraemia on the frequency of p11c tetramer^{+} cells expressing PD-1. (d) Typical 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 $> 1 \times 10^{6}$/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

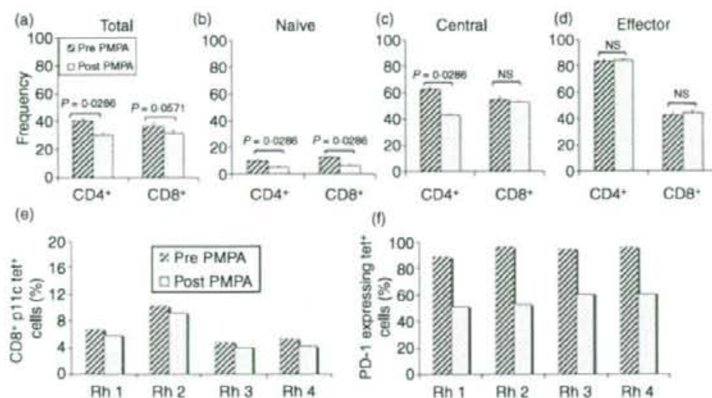

Figure 5. Modulation of PD-1 (%) expression on total (a), naive (b), central (c) and effector memory (d) CD4$^+$ and CD8$^+$ T cells sampled from rhesus macaques treated with effective antiretroviral chemotherapy (PMPA) during chronic SIV infection. (e, f) Modulation of frequency and MFI of PD-1 expression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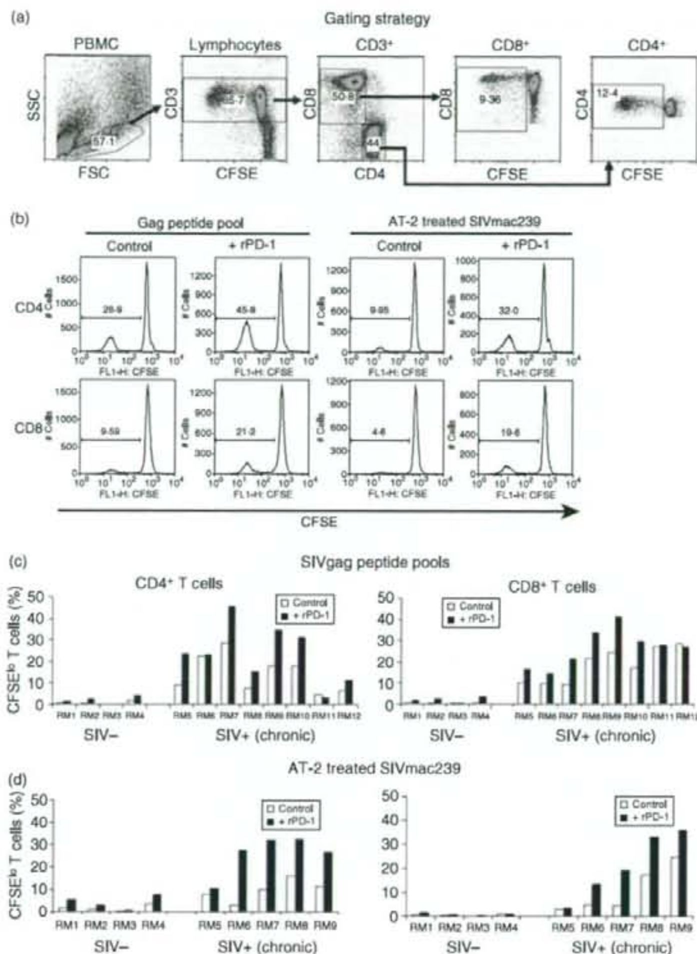

Figure 6. Augmentation of SIV-specific *in vitro* $CD4^+$ and $CD8^+$ T-cell proliferative responses by recombinant soluble rhesus macaque PD-1 (rPD-1). Aliquots of peripheral blood mononuclear cells (PBMC) labelled with CFSE were restimulated with either media (control), 100 ng/ml a pool of overlapping 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

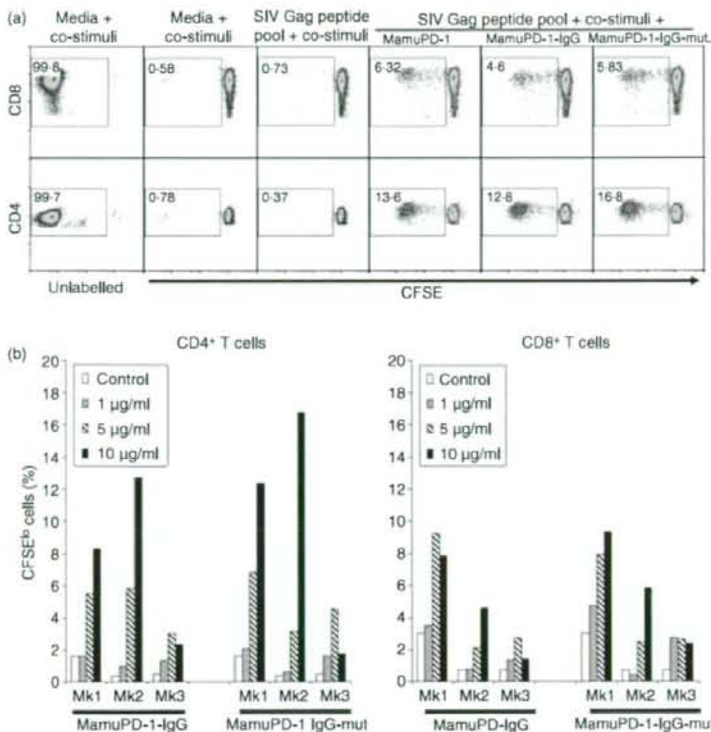

Figure 7. Comparison of soluble rMamuPD-1, rMamuPD-1–IgG and rMamu-PD-1–IgG mutants for their capacity to enhance the SIVgag-specific proliferation of CD4$^+$ and CD8$^+$ T cells from rhesus macaques chronically infected with SIVmac239. (a) Representative flow cytometric profile of CFSE-expressing CD4$^+$ 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.

Acknowledgements

The help of the Yerkes National Research Primate Research Center in terms of care and handling of the animals and the help of the Emory CFAR Virology Core in performing plasma viral load analyses are gratefully acknowledged. We would like to thank Dr Rama Amara and Dr John Altman (Emory VRC, Atlanta) for helpful discussion and sharing of reagents and the NIH AIDS Reagent Program for the provision of peptide reagents. The authors would also like to thank Dr M. Miller and Gilead for their generous provision of the antiretroviral agent PMPA utilized in these studies. This work was supported by NIH RO1 AI 27057, R01 HL075833, R24 RR016988 and RR00165. K. P. was also supported by a Senior Research Scholarship Program from the Thailand Research Fund.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. ELISPOT analysis of SIVgag restimulated PBMC from two SIV-infected rhesus macaques in the presence or absence of 10 µg/ml goat anti-PD1 antibody. The polyclonal activation of PBMC irrespective of the antigenic stimulus used is shown.

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Genetic factors that confer sensitivity to HAART in HIV-infected subjects: implication of a benefit of an earlier initiation of HAART

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Evaluation of: Ahuja SK, Kulkarni H, Catano G *et al.*: *CCL3L1-CCR5* genotype influences durability of immune recovery during antiretroviral therapy of HIV-1-infected individuals. *Nat. Med.* 14(4), 413–420 (2008). It is widely accepted that the effect of highly active antiretroviral therapy (HAART) varies widely among HIV-infected individuals. Host genetic factors are thought to be linked to the sensitivity to HAART in HIV-infected individuals. Ahuja *et al.* attempted to identify the genes that determine the sensitivity to HAART in HIV-infected subjects. Based on the hypothesis that CD4^{+} depletion and the recovery process in HIV-infected subjects are under the control of specific common genetic pathways, they evaluated the associations of genetic variations, such as *CCR5* genotype, *CCL3L1* copy number variation and *HLA* alleles, with the sensitivity to HAART in two cohorts from the USA. They found that the *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, is apparently a good predictor of the recovery rate of CD4^{+} T cells during HAART. In particular, the recovery rate of CD4^{+} T cells during HAART has the most sensitive association with the copy number of *CCL3L1*. Furthermore, Ahuja *et al.* studied the impact of *CCL3L1-CCR5* genetic risks in HIV-infected individuals initiating HAART during acute or early infection. They suggested that *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART in HIV-infected subjects with a level of 350 CD4^{+} T cells/mm^3 or more. This study has provided a critical breakthrough in predicting the response to HAART in HIV-infected subjects.

The combination of antiretroviral drugs known as highly active antiretroviral therapy (HAART) has drastically improved the prognosis for great numbers of HIV-infected patients in a very short period of time. Ahuja *et al.* have identified a new genetic mechanism controlling susceptilibility to HAART [1]. The AIDS death rate in the USA declined by more than two-thirds within 2 years of the appearance of the protease inhibitors [2]. However, it is widely reported that the effect of HAART, such as the level of viral replication suppression and the recovery rate of CD4^{+} T-cell counts, varies widely among HIV-infected individuals. Host genetic factors in all likelihood are linked to the sensitivity to HAART in HIV-infected individuals. In fact, there have been several studies performed to evaluate the impact of genetic factors on the sensitivity to HAART. The main features and outcomes of these studies are summarized in Table 1. The majority of these studies have been focused on sequence variations in *HLA-B* [3,4] and *CCR5* [5–9].

Ahuja *et al.* sought to identify the genes that determine the sensitivity to HAART in chronic HIV-infected subjects. They hypothesized that CD4^{+} depletion and the recovery process are under the control of specific common genetic pathways, and that genetic factors determining HIV-1/AIDS susceptibility are tightly linked to the sensitivity to HAART. In the study, they focused on three genetic variations, *CCR5* genotypes, *CCL3L1* copy number variations and *HLA-B*57*. These three genes have been reported to be tightly linked to HIV/AIDS susceptibility. Sequence variations in *CCR5*, which result in reduced or absent cell-surface expression of the HIV coreceptor CCR5, decrease the susceptibility to HIV-1 infection [10–13]. CCL3L1, a natural ligand for CCR5, is a potent HIV-1-suppressive chemokine. *CCL3L1* copy number variation is tightly linked to HIV/AIDS susceptibility, and a lower copy number is associated with both an enhanced risk for acquiring HIV-1 and also progressing more rapidly to AIDS and death [14,15]. *HLA-B*57* has also been reported to be associated with a better prognosis in HIV-infected individuals [10,11,16,17].

Furthermore, the report has shed new light on the benefits of an earlier initiation of HAART. In the current guidelines for the starting of HAART [101], the earlier initiation of HAART, especially in HIV-infected individuals with a level of 350 CD4^{+} T cells/mm^3 or more, remain controversial. The authors studied the impacts of

Keywords: CCL3L1, CCR5, HAART, HIV-1, HLA

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Table 1. The main features and outcomes of seven studies performed to evaluate the effects of HLA genotypes and *CCR5Δ32* on the clinical course after HAART.

Gene	Study	Sample size	Cohort and ethnic backgrounds	Effects on the clinical course after HAART	Ref.
HLA-B	Brumme *et al.* (2007)	n = 765	HOMER cohort, British Colombia, Canada	Impaired CD4⁺ T-cell recovery in HLA class I homozygosity Slower virologic suppression in subjects with uncommon HLA alleles	[3]
	Rauch *et al.* (2008)	n = 265	Swiss HIV cohort study and Western Australian HIV cohort study	Impaired CD4⁺ T-cell recovery in HLA-Bw4 homozygosity	[4]
CCR5Δ32	Bratt *et al.* (1998)	n = 147	Subjects from Sweden	No significant effect on the efficacy of HAART	[5]
	Valdez *et al.* (1999)	n = 113	White subjects from Cleveland, OH, USA	A better response (viral load and CD4⁺ T cells) in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes	[6]
	O'Brien *et al.* (2000)	n = 273	AIDS Clinical Trial Group 343 study. Caucasians with CD4⁺ T-cell ≥200/mm³ and plasma HIV RNA ≥1000 copies/ml	No significant effect on the efficacy of HAART	[7]
	Kasten *et al.* (2000)	n = 107	Subjects from Germany	A better response (viral load and CD4⁺ T cells) in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes	[8]
	Laurichesse *et al.* (2007)	n = 565	French Agence Nationale de Recherrche SERCO/HEMOCO cohort	A better virological response in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes No significant effect on survival or AIDS-free survival	[9]

HAART: Highly active antiretroviral therapy; HEMOCO: Hemophiliacs HIV-1 infection; HLA: Human leukocyte antigen; HOMER: HAART Observational Medical Evaluation and Research SERCO: Seroconversion; wt: Wild-type.

CCL3L1-CCR5 genetic risks in HIV-infected individuals initiating HAART during acute or early infection.

Genes responsible for HAART sensitivity in chronic HIV-infected subjects

In the study by Ahuja *et al.*, the Wilford Hall Medical Center (WHMC; TX, USA) cohort was studied to evaluate the associations of genetic variations with the sensitivity to HAART in chronic HIV-infected subjects. The WHMC cohort is a component of the USA Military Tri-Service AIDS Clinical Consortium Natural History Study, which is one of the largest cohorts of HIV-positive patients followed prospectively at a single medical center. In the WHMC cohort, 502 HIV-infected subjects received HAART.

Ahuja *et al.* classified a large cohort of HIV-1-infected subjects into three *CCL3L1-CCR5* genetic risk groups on the basis of the copy number of *CCL3L1* and *CCR5* genotype [1]. The high-risk group possessed both a population-specific low *CCL3L1* copy number (*CCL3L1*^{low}) and detrimental *CCR5* variations (*CCR5*^{det}). The low-risk group had both a population-specific high *CCL3L1* copy number (*CCL3L1*^{high}) and non-detrimental *CCR5* variations (*CCR5*^{nondet}). The moderate risk group harbored either one or the other of the two risk factors, *CCL3L1*^{low} *or CCR5*^{det}. Their cohort was also categorized based on the levels of viral load suppression after HAART to investigate whether, among subjects with a similar viral load suppression, the rate and extent of CD4⁺ T-cell recovery differed according to the *CCL3L1-CCR5* genetic risk status. They also categorized the cohort based on the levels of CD4⁺ T cells with which HAART was started. They found that the *CCL3L1-CCR5* genetic risk status is apparently a good predictor of the recovery rate of CD4⁺ T cells during HAART, especially in those who attained viral load suppression after HAART and those who started

HAART with levels of less than 350 CD4+ T cells/mm^3. They also reported that the recovery rate of CD4+ T cells during HAART has the most sensitive association with the copy number of *CCL3L1*.

On the other hand, possession of *HLA-B*57* had no impact on the recovery rate of CD4+ T cells in HIV-infected subjects receiving HAART. Surprisingly, individuals possessing *HLA-B*57*, which was associated with a delayed disease progress, had an impaired CD4+ T-cell recovery during the first 2–3 years after starting HAART. Similar findings have also been reported by others. Rauch *et al.* have reported that *HLA-Bw4* homozygosity, which has been reported to be linked to a better prognosis in HIV-infected subjects, was associated with an impaired CD4+ T-cell recovery after HAART [4]. They also reported that the possession of *HLA-B*57* was likely to be associated with an impaired CD4+ T-cell recovery. The basis for *HLA-B*57* protection seems to be related, in part, to a highly conserved immunodominant epitope in Gag, a response to which seems to confer early protection during acute infection [18]. HIV-1 specific CD8+ T-cell response restricted by *HLA-B*57* provides a potential mechanism for epidemiological protection, even though it might have no beneficial effects on CD4+ T-cell recovery during HAART.

Interestingly, *HLA-B*57* has been reported to be associated with a hypersensitivity reaction to adacavir, a nucleotide reverse-transcriptase inhibitor [19,20]. The current treatment guidelines recommend screening for *HLA-B*5701* before starting patients on an abacavir-containing regimen, to reduce the risk of a hypersensitivity reaction [101]. *HLA-B*57*-related mechanisms for HAART resistance and hypersensitivity to abacavir are not clear; however, we must pay extra attention to HIV-infected subjects possessing *HLA-B*57* during the course of HIV treatment.

Genetic risk status & earlier initiation of HAART in acute or early HIV-infected subjects

Despite the possible benefits of HAART in HIV-infected subjects with CD4+ T-cell counts over 350 cells/mm^3, there are a couple of reasons which mitigate against the earlier initiation of HAART [101]. However, the earlier initiation of HAART in HIV-infected subjects with a high risk of disease progression appears to be indicated.

Ahuja *et al.* studied the Acute Infection and Early Disease Research Program (AIEDRP) cohort to evaluate the impacts of *CCL3L1-CCR5* genetic risks on the sensitivity to HAART in HIV-infected individuals initiating HAART during acute or early infection [1]. In this prospective study, 315 HIV-infected subjects with signs or symptoms of an acute retroviral syndrome or evidence of recent HIV infection were enrolled. Among the subjects who received HAART during acute infection and who attained viral load suppression, a low genetic risk of *CCL3L1-CCR5* was associated with a greater recovery rate of CD4+ T cells in comparison with a moderate or high genetic risk. They suggested that *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART in HIV-infected subjects with a level of 350 CD4+ T cells/mm^3 or more.

Future perspective

Ahuja *et al.* provided critically important information regarding the genes that determine the sensitivity to HAART in HIV-infected subjects. However, further studies are required to assess the following four issues. First, the association between genetic variations and the HAART sensitivity should be replicated by using other population samples. The authors evaluated two cohorts, WHMC and AIEDRP, both of which were from the USA. Since subtype B of HIV-1 is the most prevalent in the USA [2], it remains possible that the genotype–phenotype interactions observed in this study might not be identified in the context of other HIV-1 subtypes.

Second, additional genes might be linked to the HAART sensitivity in HIV-infected subjects. Other HIV/AIDS susceptible genes [10,11,15] and/or genes linked to drug metabolism might be good candidates. Recently, genome-wide association studies have proven to be a powerful approach to identify the genes responsible for human common diseases [21,22]. Scanning the entire human genome by a genome-wide association study could serve an alternative approach to identifying the genes tightly linked to the HAART sensitivity in HIV-infected subjects.

Third, what is the mechanism by which the *CCL3L1-CCR5* genetic risk status influences the sensitivity to HAART? The HIV-1 entry-dependent effect through the interaction among HIV-1, *CCR5* and *CCL3L1* has been widely accepted as a key mechanism of inhibiting HIV-1 infection [10,11], because CCR5 is a HIV co-receptor and *CCL3L1* is a natural ligand of *CCR5*. Alternatively, viral entry-independent mechanisms have been suggested as the major protective

mechanisms acting through the *CCL3L1* and *CCR5* axis in their previous study [23]. These two mechanisms might have synergistic and/or additive effects with HAART-induced viral load suppression. However, the HIV-1 entry-independent mechanisms remain to be elucidated. Shalekoff *et al.* reported that *CCL3L1* copy numbers were tightly linked to CD4+ and CD8+ T-cell responses to the HIV-1 Gag protein [24]. *CCL3L1-CCR5* genetic risk status might influence cell-mediated immunity such as HIV-1-specific CD4+ and CD8+ T-cell responses. Understanding the HIV-1 entry-independent mechanisms would be useful for establishing new strategies of HIV treatment and to develop new types of HIV drugs.

Finally, the long-term effect of earlier initiation of HAART on survival and/or AIDS-free survival should be evaluated. Several studies have demonstrated that an earlier initiation of HAART is associated with a greater recovery rate of CD4+ T cells, but not with a significant reduction in the risk of non-AIDS events/morbidity [101]. Long-term and prospective studies in HIV-infected subjects with high genetic risk are needed to definitively evaluate the benefit of the earlier initiation of HAART.

It is widely accepted that genetic variations are tightly linked to the sensitivity to HAART in HIV-infected subjects. Ahuja *et al.* provided a critical breakthrough to predict the response to HAART in HIV-infected subjects. They opened the door of 'personalized medicine' in HIV therapy. It is to be hoped that further progress will provide great insights into an understanding of the contribution of genetic factors to the response to HIV treatment.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Study design

- The Wilford Hall Medical Center (TX, USA) cohort was studied to evaluate the associations of genetic variations with the sensitivity to highly active antiretroviral therapy (HAART) in chronic HIV-infected subjects.
- The Acute Infection and Early Disease Research Program cohort was studied to evaluate the impacts of genetic risks on the sensitivity to HAART in HIV-infected subjects initiating HAART during acute or early HIV infection.

Analyses performed

- The associations of *CCR5* genotypes, *CCL3L1* copy number variations and *HLA* alleles with HAART sensitivity (viral load suppression and CD4+ T-cell recovery rate) were evaluated.
- HIV-1 infected subjects were classified into three *CCL3L1-CCR5* genetic risk groups – low, moderate and high – on the basis of the copy number of *CCL3L1*, as well as *CCR5* genotype.

Results

- *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, were associated with the recovery rate of CD4+ T cells during HAART.
- The recovery rate of CD4+ T cells during HAART is the most sensitive activity associated with the copy number of *CCL3L1*.
- Among the subjects who received HAART during acute infection and who attained viral load suppression, a low genetic risk of *CCL3L1-CCR5* was associated with a greater recovery rate of CD4+ T cells in comparison with a moderate or high genetic risk.

Conclusion

- *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, is apparently a good predictor of the recovery rate of CD4+ T cells during HAART.
- *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART during acute or early HIV infection.

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Immunogenetics
DOI 10.1007/s00251-008-0332-0

ORIGINAL PAPER

Natural selection in the *TLR*-related genes in the course of primate evolution

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Received: 4 June 2008 / Accepted: 4 September 2008
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Abstract The innate immune system constitutes the front line of host defense against pathogens. Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. Here, we provide evidence that the TLR-related genes have come under natural selection pressure in the course of primate evolution. We compared the nucleotide sequences of 16 TLR-related genes, including *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14*, among seven primate species. Analysis of the non-synonymous/synonymous substitution ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. The genomic segments encoding the intracellular Toll/interleukin 1 receptor domains, which exhibited lower rates of non-synonymous substitution, have undergone purifying selection. In contrast, *TLR4*, which carried a high proportion of non-synonymous substitutions in the part of extracellular domain spanning 200 amino acids, was found to have been the suggestive target of positive Darwinian selection in primate evolution. However, sequence analyses from 25 primate species, including eight hominoids, six Old World monkeys, eight New World monkeys, and three prosimians, showed no evidence that the pressure of positive Darwinian selection has shaped the pattern of sequence variations in *TLR4* among New World monkeys and prosimians.

Electronic supplementary material The online version of this article (doi:10.1007/s00251-008-0332-0) contains supplementary material, which is available to authorized users.

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Keywords Toll-like receptor · Natural selection · Primate evolution

Introduction

Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains with varying numbers of leucine-rich-repeat motifs (LRR) and a cytoplasmic signaling domain termed the Toll/interleukin 1 receptor (TIR) domain (Akira et al. 2006; Bowie and O'Neill 2000). Different TLRs recognize a variety of

pathogen-associated molecules, including lipids and nucleic acids, and all TLRs transduce signals through TIR domains to activate immune cells (Akira et al. 2006; Bowie and O'Neill 2000). Stimulation with TLR ligands recruits adaptor proteins such as Myeloid differentiation factor 88 (MYD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor molecule 1 (TICAM1), and TICAM2, all of which also have a TIR domain, to the cytoplasmic portion of the TLRs and activate signaling cascades to produce proinflammatory cytokines and chemokines (Akira et al. 2006; Bowie and O'Neill 2000).

Viral, bacterial, and parasitic infections have been postulated to be among the strongest selective pressures on primate evolution. It is also widely accepted that the susceptibility to infectious pathogens, such as *Mycobacterium tuberculosis* bacilli and HIV-1, are different among primate species (Isaza 2003; Stremlau et al. 2004). Given that TLRs play crucial roles in the innate immune system, the intriguing hypothesis that TLRs have emerged under the intense pressure of natural selection in the course of primate evolution is rising. Actually, it is suggested that *TLR1*, *TLR6*, and *TLR10* have come under particular natural selection pressures in the human population, because the sequence variations of these three genes display considerable geographical diversity in the British population (Wellcome Trust Case Control Consortium 2007). Moreover, it has been reported that natural selection has acted on *TLR4* in humans, since excess of rare non-synonymous polymorphisms in *TLR4* are observed in humans (Smirnova et al. 2001).

To investigate the natural selection hypothesis, we analyzed the nucleotide sequences of 16 TLR-related genes, including ten *TLRs* (TLR1–10), four genes linked to signal transduction (*MYD88*, *TILAP*, *TICAM1*, and *TICAM2*), and two genes linked to *TLR4* (*MD2* and *CD14*) in primates. MD2 and CD14 are key molecules of the LPS signaling through TLR4 (Poltorak et al. 1998; Shimazu et al. 1999; Nagai et al. 2002). Our study shows that the genomic segments encoding the intracellular TIR domains have undergone purifying selection and that the extracellular domain of *TLR4* has been the suggestive target of positive Darwinian selection in the course of primate evolution. We concluded that natural selection has indeed shaped the sequence patterns of TLR-related genes in primate evolution.

Materials and methods

DNA sequences

DNA samples from 25 primates, including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), black gibbon (*Hylobates concolor*), white-handed gibbon (*Hylobates lar*), siamang (*Hylobates syndactylus*), crab-eating macaque (*Macaca fascicularis*), rhesus macaque (*Macaca mulatta*), hamadryas baboon (*Papio hamadryas*), black and white colobus (*Colobus guereza*), silvered lutong (*Trachypithecus cristatus*), dusky lutong (*Trachypithecus obscurus*), common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), red-handed tamarin (*Saguinus midas*), lion tamarin (*Leontopithecus rosalia*), common squirrel monkey (*Saimiri sciureus*), tufted capuchin (*Cebus apella*), long-haired spider monkey (*Ateles belzebuth*), and Central American spider monkey (*Ateles geoffroyi*), tarsiers (*Tarsius* spp.), lesser galago (*Galago senegalensis*), and ring-tailed lemur (*Lemur catta*) were analyzed. Overlapping primer sets covering all coding exons of 16 genes including the *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were designed on the basis of size and overlap of PCR amplicons. Genomic DNA was subjected to PCR amplification followed by sequencing using the BigDye Terminator cycling system. Sequencing analysis was performed in an ABI3130x automated DNA sequencer (Applied Biosystems).

Statistical analysis

Sequence alignments were performed by the Clustal X program (Thompson et al. 1997). All values for Ka, Ks, Ka/Ks, %GC, and Codon Bias Index (CBI) were evaluated by DnaSP (Rozas et al. 2003). The Bn–Bs program (Zhang et al. 1998) was applied to evaluate the Ka/Ks ratio in individual branches of the primate phylogenetic tree. We studied positive Darwinian selection for the target region of TLR4 by using the MEGA version 4.0 program (Tamura et al. 2007). Ancestral amino acid sequence was estimated by a parsimony method using PROTPARS program in PHYLIP (Felsenstein 1989).

Results

The nucleotide sequences of tn *TLRs* (TLR1–10), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were determined among seven primates, including human, chimpanzee, bonobo, gorilla, orangutan, crab-eating macaque, and rhesus macaque. All sequences were newly determined in the study, and all accession numbers were shown in Table S1. The lengths of the deduced coding sequences for each gene differed among the seven primates, as summarized in Table 1.

To evaluate the non-synonymous/synonymous substitution ratio, we applied the Bn–Bs program (Zhang et al. 1998). This program uses a modified Nei–Gojobori method (Nei and Gojobori 1986) to estimate pairwise synonymous

Table 1 The non-synonymous and synonymous substitution ratio for 16 TLR-related genes among seven primates

Gene	Chromosome (Human)	Size of coding region (bp)	Entire coding sequence			TIR domain			Non-TIR region		
			Σbn	Σbs	Σbn/Σbs	Σbn	Σbs	Σbn/Σbs	Σbn	Σbs	Σbn/Σbs
TLR1	4p14	2,358	0.041	0.095	0.429	0.019	0.103	0.164	0.046	0.093	0.491
TLR2	4q32	2,352, 2,349[Orangutan]	0.025	0.086	0.290	0.009	0.058	0.157	0.028	0.091	0.311
TLR3	4q35	2,712	0.032	0.121	0.267	0.018	0.070	0.259	0.035	0.130	0.270
TLR4	9q32–33	2,517, 2,490[Orangutan], 2,478[Crab-eating, Rhesus]	0.038	0.085	0.447	0.006	0.118	0.053	0.045	0.079	0.566
TLR5	1q41–42	2,574	0.030	0.108	0.282	0.018	0.173	0.105	0.032	0.097	0.339
TLR6	4p14	2,388, 2,385[Bonobo]	0.030	0.120	0.240	0.021	0.199	0.105	0.031	0.105	0.293
TLR7	Xp22.3–p22.2	3,147	0.014	0.069	0.202	0.003	0.087	0.034	0.016	0.066	0.236
TLR8	Xp22.3–p22.2	3,123, 3,120[Crab-eating, Rhesus]	0.020	0.095	0.209	0.003	0.056	0.054	0.023	0.101	0.224
TLR9	3p21.3	3,096	0.029	0.153	0.187	0.003	0.202	0.016	0.032	0.145	0.224
TLR10	4p14	2,433	0.024	0.106	0.228	0.018	0.045	0.402	0.026	0.118	0.216
MYD88	3p22–p21.3	888	0.009	0.096	0.094	0.000	0.087	0.000	0.017	0.102	0.165
TIRAP	11q23–q24	663, 660[Crab-eating, Rhesus]	0.035	0.164	0.216	0.032	0.254	0.126	0.037	0.110	0.341
TICAM1	19p13.3	2,139[a]	0.039	0.171	0.227	0.007	0.163	0.041	0.046	0.172	0.269
TICAM2	5q23.1	705	0.020	0.119	0.167	0.000	0.132	0.000	0.033	0.110	0.300
MD2	8q21.11	480	0.015	0.054	0.269	–	–	–	–	–	–
CD14	5q31.1	1,125	0.013	0.040	0.332	–	–	–	–	–	–

[a] *TICAM1* has a CCT(Pro)-repeat variation

and non-synonymous distances among the sequences and then estimates the branch lengths in terms of synonymous (bs) and non-synonymous substitutions (bn) per site by using the ordinary least-squares method, while the tree topology is given. Σbn and Σbs indicate the value summing up bn and bs, respectively, in the lineages. When the value of Σbn and Σbs and the ratio of Σbn/Σbs were evaluated for the entire coding sequences from each gene, there was no evidence to support that these genes have come under the pressure of positive natural selection. All of the values of the Σbn/Σbs ratio from the analyzed genes were much lower than 1.0, which suggested that these genes have been under the pressure of negative selection (Table 1).

To identify the genomic segments, which have undergone natural selection, a sliding window plot analysis (600-bp window with 30-bp steps) was performed throughout these genes. Analysis of the Σbn/Σbs ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. Three candidate segments, where the pressure of negative or positive natural selection might have operated, were identified in *TLR7*, *MYD88*, and *TLR4* (Fig. 1a).

Two target segments showed little non-synonymous nucleotide difference among the seven primates (Fig. 1a and Supplementary material, Fig. S1). One was located at the coding segment encoding the C-terminal of TLR7 and the other at the segment encoding the C-terminal of MYD88, both of which encode the TIR domain (Fig. 1a). Phylogenetic comparisons from 14 human sequences encoding TIR domains reveal no obvious similarity between *TLR7* and *MYD88* (Fig. 2a). We then evaluated the Σbn/Σbs ratios for the TIR domains for 14 genes with TIR domains. The sizes of the genomic segments encoding TIR domains [average 393 bp (249–426 bp)] were smaller than the window size (600 bp) used in our analysis so that our window analysis would underestimate the Σbn/Σbs ratios for TIR domains. The values of Σbn and Σbn/Σbs ratio for TIR domains displayed lower values when compared with those of the non-TIR coding sequences except for Σbn/Σbs ratio from *TLR10* (Table 1 and Fig. 2b). In particular, *TLR7*, *TLR8*, *TLR9*, *MYD88*, and *TICAM2* have much lower values for Σbn at the TIR domains. Taken together, it is suggested that the TIR domains have been under the control of negative/purifying selection.

On the other hand, sequence comparisons among the seven primates support the positive Darwinian selection at the extracellular domain of TLR4, for which the Σbn/Σbs ratios were much higher than 1.0 (the highest value in the 600-bp window is 2.37, with a statistical significance in *Z* test; Zhang et al. 1998, Tamura et al. 2007; *Z* score 2.16; p value <0.01; Fig. 1). Among analyzed windows fro TLR-related genes, *TLR4* and *TICAM1* have extreme high values of Σbn. However, the windows of *TICAM1* harboring high values of Σbn also have the high value of Σbs. The high values of %GC seem to be associated with the high-nucleotide substitution rate in *TICAM1* (Fig. 1a and b). A lower value of CBI was also correlated with a lower synonymous nucleotide substitution rate (data not shown).

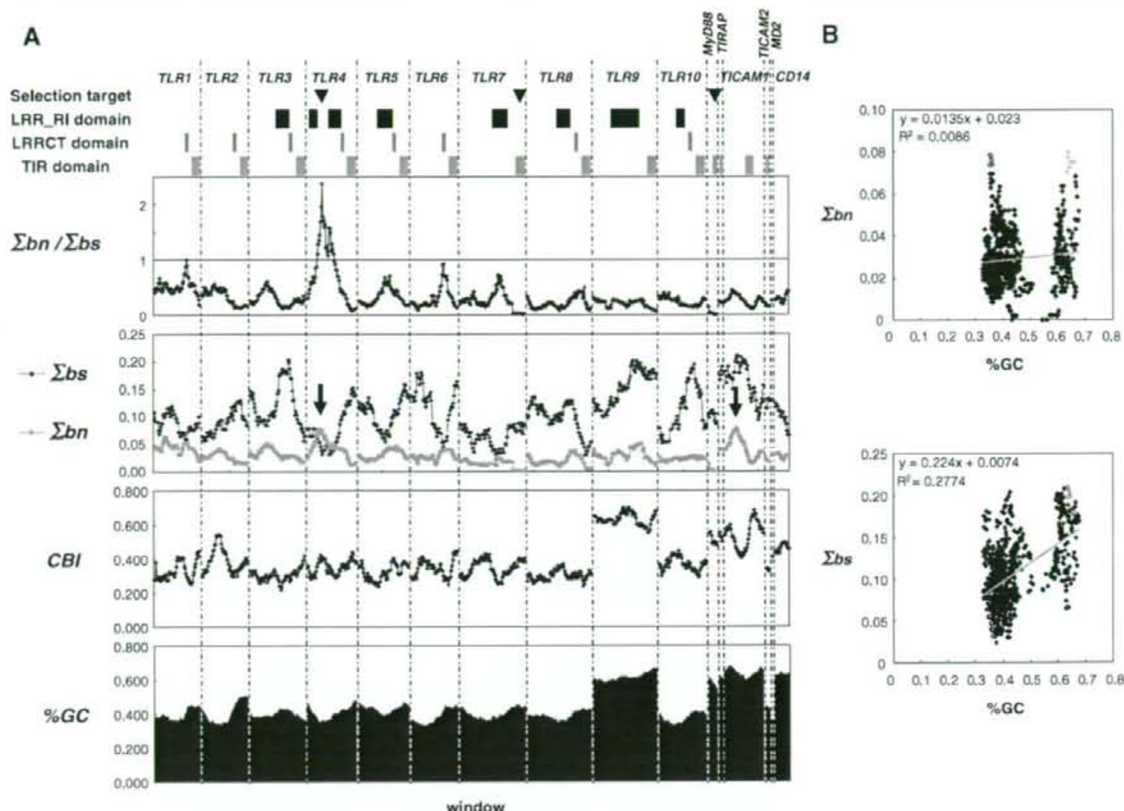

Fig. 1 **a** The values of Σbn/Σbs, Σbn, Σbs, CBI, and %GC based on the sliding window plot analysis for the TLR-related gene (600-bp window with 30-bp steps). The *arrow heads* indicate the candidate segments for the pressure of positive or negative natural selection. The *arrows* indicate that *TLR4* and *TICAM1* have extreme high values of Σbn among analyzed windows. CBI is a measure of the deviation from the equal use of synonymous codons, which indicates the extent to which a gene uses a subset of optimal codons (Bennetzen and Hall 1982). Three conserved domain structures, *LRR_RI* (leucine-rich repeats, ribonuclease inhibitor-like subfamily), *LRRCT* (leucine-rich repeat C-terminal domain), and *TIR* (Toll/interleukin-1 receptor homology domain), are referred from CD-search (Marchler-Bauer and Bryant 2004). **b** Pairwise comparisons between Σbn and %GC and between Σbs and %GC. All values of Σbn, Σbs, and %GC were based on the sliding window plot analysis for the TLR-related gene. *TLR4* (*white lozenge*) and *TICAM1* (*gray lozenge*) have several windows with extreme high values of Σbn

In the window of *TLR4* harboring the highest value of Σbn, its level of CBI (0.398) was almost equivalent to the average level among analyzed windows (0.387±0.114).

The estimated values of bn and bs of each lineage at *TLR4* target region were shown in Fig. 3. The values of bn in three lineages since the emergence of great apes were significantly higher than those of bs. These lineages have a relatively low value of bs; however, the values of bn were much larger than the estimated value of bs for entire TLR4 coding sequences in each lineage except for orangutan lineage (Supplementary material, Fig. S1). These lines of evidence suggested that the extracellular domain of TLR4 has been the possible target of positive Darwinian selection in the course of primate evolution.

To evaluate this finding further, we determined the sequences of a ~600 bp *TLR4* target region from additional 18 primates, including three gibbons (black gibbon, white-handed gibbon, and siamang), four Old World monkeys (hamadryas baboon, black and white colobus, silvered lutong, and dusky lutong), eight New World monkeys (common marmoset, cotton-top tamarin, red-handed tamarin, lion tamarin, common squirrel monkey, tufted capuchin, long-haired spider monkey, and Central American spider monkey), and three prosimians (tarsiers, lesser galago, ring-tailed lemur). Each of target sequences from three gibbons, two lutongs, and two tamarins is identical so that a total of 21 sequences were advanced to further analyses.