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PD-1 Blockade in Rhesus Macaques: Impact on Chronic Infection and Prophylactic Vaccination

Adam C. Finnefrock,¹* Amin Tang,*, Fengsheng Li,*, Daniel C. Freed,*, Meizhen Feng,† Kara S. Cox,*, Kara J. Sykes,*, James P. Guare,‡ Michael D. Miller,† David B. Olsen,† Daria J. Hazuda,§ John W. Shiver,§ Danilo R. Casimiro,* and Tong-Ming Fu†*

Programmed Cell Death 1 (PD-1)² (1), a member of the CD28 gene family, is expressed on activated T, B, and myeloid lineage cells (2, 3). Two ligands for PD-1 have been identified, PD ligand 1 (PD-L1) and ligand 2 (PD-L2); both belong to the B7 superfamily (2). PD-L1 is expressed on many cell types, including T, B, endothelial, and epithelial cells. In contrast, PD-L2 is narrowly expressed on professional APCs, such as dendritic cells and macrophages. PD-1 negatively modulates T cell activation through an ITIM of its cytoplasmic domain (2, 4). PD-1 is recognized as a key target for immune modulation (2, 3). Physiologically, this pathway plays important roles in the maintenance of peripheral tolerance. Experiments have shown that PD-L1 knock-out or blockade in mice can induce onset of autoimmune diseases (5, 6). Furthermore, experimental disruption of PD-1 leads to autoimmunity, exemplified by PD-L1 knockout mice (Pdcd1<sup>−/−</sup>) with spontaneous development of a lupus-like syndrome (7) or dilated cardiomyopathy (8), depending on their genetic background. Intriguingly, the reverse scenario of PD-1 pathway up-regulation has been implicated in mechanisms of host T cell dysfunctions exploited in tumor immune evasion and persistent viral infections (reviewed in Refs. 2 and 3).

The importance of host T cells in clearing viral infections is well known. However, new lines of evidence indicate that sustained exposure to high concentrations of viral Ags can drive T cells to terminal differentiation, e.g., functional “exhaustion”, a phenomenon first described in chronic infection by lymphocytic choriomeningitis virus (LCMV) in mice (9). The exhausted T cells were shown to have reduced capacities to proliferate and perform cytotoxic functions (10–12). A high level of expression of PD-1 in viral-specific CD8 cells is associated with T cell dysfunctions in the LCMV model (13), and this correlation was further recognized in clinical contexts of persistent viral infections with HIV (14–16), hepatitis C virus (17), and hepatitis B virus (18–20). Treatment of LCMV-infected mice with a mAb specific for PD-L1, presumably blocking inhibitory signaling by PD-1, has restored polyfunctionality in formerly exhausted T cells (13). This finding has been extended to HIV and hepatitis C virus T cells in vitro, using Ab to block PD-1/PD-L1 interaction in cultures (14–17). Thus, blocking PD-1 inhibitory signaling by PD-1-specific mAbs has potential clinical application in enhancing host antimicrobial immunity to treat chronic infections, alone or with anti-infective agents. Another therapeutic mode was recently demonstrated (21) in the LCMV model, where therapeutic vaccination in conjunction with PD-1 blockade by anti-PD-L1 mAb increased epitope-specific responses in chronically infected mice.

Recent studies have shown that T cells from rhesus macaques chronically infected with SIV or SHIV also highly express PD-1 (22–24). To evaluate the therapeutic potential of PD-1 blockade in this nonhuman primate model, a panel of mAbs specific to human PD-1 that block interaction with human PD-L1 was generated. The efficacy of such PD-1 blockade was evaluated in vitro based on affinity to PD-1, cross reactivity to rhesus PD-1, and capacity to block PD-L1 interaction. More importantly, the lead mAb was tested in two in vivo rhesus experiments. In the first experiment, PD-1 blockade was demonstrated to alter the viral load kinetics in rhesus macaques chronically infected with SIV. In the second experiment, PD-1 blockade exhibited an adjuvant-like property in a...
prophylactic vaccine model. These studies demonstrate potential approaches for evaluating PD-1 blockade in prophylactic and therapeutic rhesus macaque models, a critical step on the path to evaluating the modulation of the PD-1 pathway in clinical studies.

Materials and Methods

BALB/c mice were immunized with 100 μg DNA vaccine-expressing human PD-1 i.m. three times at 3 wk intervals. Mice were boosted with an i.p. injection of 2 × 10⁴ cells of a stable cell line expressing high levels of human PD-1 diluted in 0.5 ml PBS. Splenocytes were collected 3 days later and hybridoma fusions were generated and screened by ELISA, using recombinant PD-1/Fc (R&D Systems). Clones were expanded and concentrations of hybridoma supernatants were determined by a mouse IgG quantitation ELISA.

Biophysical screening of hybridoma supernatants was conducted under high-binding conditions on a Biacore 2000 (GE Healthcare) instrument at 25°C with HBS-EP buffer (0.01 M HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20). A single-cycle surface plasmon resonance (SPR) assay was developed to determine binding to human PD-1, blocking of PD-L1, and to screen for the human Fc portion of the recombinant PD-1/Fc fusion protein used. PD-1/Fc was covalently immobilized with EDC/NHS (N-ethyl-N’-(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide) to one surface of a CM5 dextran chip (GE Healthcare) at a level of 450 response units, homologous Fc, and mouse PD-1/Fc to others, and one surface was capped and left blank. Hybridoma supernatants were flowed at 5 μl/min over all surfaces, and then recombinant 100 nM PD-L1/Fc protein was flowed over all surfaces. Time-resolved signals were acquired from all surfaces simultaneously, and the signal from the blank control surface was subtracted as a negative control. Recombinant human PD-1/Fc, human PD-L1/Fc, mouse PD-1/Fc, and homologous human Fc proteins were purchased from R&D Systems. For kinetic analysis by SPR, monomeric Fabs were generated from IgGs (1B8, 8A10, and 3H4) by enzymatic digestion. Fractions from the purification column were collected and tested in the Caliper Labchip system. Only fractions containing pure monomeric Fabs were selected for SPR analysis. From concentrations determined with the Caliper Labchip, dilution series were made for each Fab and tested on a Biacore T100 with the standard kinetic affinity wizard against covalently immobilized PD-1/Fc at 25°C under low-binding conditions (response unitsmax < 50) to avoid mass-transfer limitations. Data were fit in the BIACore evaluation 1.1.1 software (GE Healthcare) with a 1:1 binding model under varying starting assumptions to obtain a range of best-fit Kd values.

The in vivo experiments were conducted in rhesus macaques (Macaca mulatta) housed at New Iberia Research Center, New Iberia, LA, in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). Both studies were approved by the Institutional Animal Care and Use Committee of both University of Louisiana and Merck Research Laboratories. For the therapeutic experiment, individual monkeys were assigned to two groups to equalize geographic mean body masses and sex distribution where possible. All administered Abs were purified by standard protein A affinity chromatography, and IgGs were reconstituted in PBS and confirmed for low endotoxin levels by the Limulus amebocyte lysate method. IgGs were administered by i.v. infusion using a peristaltic pump or injected i.m. with a tuberculin syringe. Plasma SIV viral load and circulating CD4 levels were determined as described previously (25). One infected but untreated monkey and one uninfected and untreated monkey were followed as controls to monitor assay consistency. Merck investigational compound L-870812, shown to inhibit HIV-1 and SIV integrase activity (26), was orally dosed at 20 mg/kg daily. All monkeys in both studies were typed Mamu-A*01, then CD4 and CD8 T cell counts were identified by gating on lymphocytes and CD4³ and CD8³ T cells were identified by the same procedure, gating on CD4³.

Statistical analyses were performed in the R language (version 2.7.1 2008-06-23, R Foundation for Statistical Computing). Due to the small sample sizes available in nonhuman primate studies, nonparametric tests were used, specifically Wilcoxon paired or unpaired two-sided tests (as appropriate) with exact computation of p values without assumptions of normality.

Results

To evaluate the effects of IgG blockade of the PD-1/PD-L1 interaction, a panel of mouse mAbs was generated by hybridoma technology. Greater than 50 clones were initially identified through vitro binding to recombinant human PD-1 in ELISA, and the hybridoma lines were expanded in culture for IgG production. mAbs were diluted to equal IgG concentration and then screened by SPR to evaluate their ability first to bind specifically to PD-1, and subsequently to block PD-L1/PD-1 interaction, in the same single-cycle assay. Representative sensograms of SPR-determined binding and blocking are shown in Fig. 1. In these examples, all mAbs bind to PD-1 conjugated on the sensor chip, indicated by an increase in SPR signal over the time interval spanned by the red arrow. The black line (“0”) indicates a medium control with no binding to PD-1. After a brief pause, recombinant PD-L1 was injected during the time interval spanned by the blue arrow. The medium control showed additional SPR signal due to uninhibited binding of PD-L1 to the PD-1. Three mAbs (3H4, 2.3A9, and 6D10) also failed to block; 6D10 also has a low affinity to PD-1 (low equilibrium binding, fast desorption during pause interval). Other mAbs blocked PD-L1 binding to PD-1 as indicated by flat SPR signal during PD-L1 injection. The binding/blocking levels were quantified for each curve by averaging the SPR signals over the regions shown in the gray rectangles; the PD-L1/PD-1 binding is plotted vs the mAb IgG/PD-1 binding in Fig. 2A following the color scheme in the legend of Fig. 1 (other mAbs are colored gray).

A series of experiments were performed to determine the binding and blocking characteristics of the mAbs at the cellular level by flow cytometry. First, all mAb IgGs bound to human PD-1 on the surfaces of stably transfected human embryonic kidney 293 cells (data not shown), consistent with the SPR data in Fig. 2A.
Second, all mAbs bound to human CD4 T cells, activated in vitro to express a high level of PD-1 (data not shown). Their reactivities to rhesus PD-1 and mouse PD-1 were determined using surface binding to activated rhesus and mouse CD4 T cells. Third, their cross-reactivities to rhesus PD-1 and mouse PD-1 were determined using in vitro activated rhesus and mouse CD4 T cells. Most clones bound to rhesus CD4 T cells; unsurprisingly for mAbs derived from mouse hybridomas, no clone bound to mouse CD4 T cells. In vitro activated human or rhesus CD4 T cells

![Figure 2](https://example.com/figure2.png)

**Figure 2.** A. Calculations from Figure 1. IgGs were considered to bind PD-1 if SPR (x-axis) > 100 RU and to block PD-L1 binding PD-1 if SPR (y-axis) < 10 RU. Colors correspond to the legend in Fig. 1; additional clones are gray. B. In vitro flow cytometry data for blocking IgGs in A. Percentage of PD-L1 prevented from binding to PD-1 on activated human CD4s. In general, the high frequency of anti-PD-1 mAbs with cross-species binding to PD-1 and blocking of PD-L1 is not merely a function of affinity, but also epitope specificity. In fact, a subsequent SPR experiment confirmed that 3H4 does not intercompete for binding to PD-1 with the PD-L1 blocking mAbs. Comparing 1B8 and 8A10, the relatively poor blocking does not intercompete for binding to PD-1; the apparent affinity of the bivalent IgG forms is expected to be better by avidity considerations alone. By comparing 1B8 and 3H4, the ability to block PD-L1 is not merely a function of affinity, but also epitope specificity.

To characterize the kinetics of Ab binding to PD-1 in a 1:1 stoichiometry, monomeric Fabs were made of the PD-L1 blocking mAbs 1B8 and 8A10 and the PD-L1 nonblocking mAb 3H4, and tested by SPR for kinetic binding to PD-1 as described in the previous biophysical and in vitro binding/blocking experiments alone. By comparing 1B8 and 3H4, the ability to block PD-L1 is not merely a function of affinity, but also epitope specificity.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effect of PD-1 blockade by anti-PD-1 1B8 IgG in vitro. Human PBMCs from six volunteers who received tetanus toxoid booster vaccinations were cultured for 3 days with tetanus toxoid at 5 µg/ml and with either 1B8 IgG (20 µg/ml) or with an isotype-matched control IgG. IFN-γ and IL-10 cytokine concentrations in supernatants were measured by ELISA. PBMCs that were cocultured with 1B8 produced more IFN-γ than those cocultured with the control IgG (p = 0.031, paired Wilcoxon signed rank two-sided test). Production of IL-10 was not significantly enhanced. Identical symbols represent the same volunteer.

**Materials and Methods.** The affinities at 25°C were: $K_D = 6.2 \pm 0.8$ nM for 1B8 Fab, $K_D = 38 \pm 5$ nM for 8A10 Fab, and $K_D = 17 \pm 4$ nM for 3H4 Fab. All of these tested monovalent Fabs have relatively high affinity (low $K_D$) to PD-1; the apparent affinity of the bivalent IgG forms is expected to be better by avidity considerations alone. By comparing 1B8 and 3H4, the ability to block PD-L1 is not merely a function of affinity, but also epitope specificity.

PD-L1/PD-L1 blockade has been shown to enhance Ag-specific T cell proliferation and cytokine production in culture (30–32). To confirm this effect ex vivo, recall T cell responses to tetanus toxoid in culture with or without 1B8-induced PD-1 blockade were evaluated. Six human volunteers received tetanus toxoid booster vaccinations, and their PBMCs were isolated 3–4 wk later and cultured with tetanus toxoid in the presence of 1B8 or an isotype-matched control IgG. Supernatants were collected 3 days later and concentrations of IFN-γ and IL-10 were measured by quantitative ELISA. As plotted in Fig. 3, production of IFN-γ was significantly enhanced in the cultures with 1B8 compared with those with isotype control IgG (p = 0.031, paired Wilcoxon signed rank two-sided test). Production of IL-10 in these cultures was not altered by 1B8 compared with the isotype control IgG (p = 0.44, paired Wilcoxon signed rank two-sided test).

From the preceding biophysical and in vitro binding/blocking data, and elevated IFN-γ production upon Ag stimulation, 1B8
was selected for PD-1 blockade experiments in vivo. Recent reports in cell cultures have shown that the proliferative and functional capacity of HIV-specific T cells can be restored through PD-1 manipulation (14–16). We sought to evaluate whether these findings could be extended to an in vivo chronic infection model in nonhuman primates. Mamu-A*01 positive rhesus macaques from a previous experiment were chronically infected with SIVmac239 for over 18 mo. All subjects were in stable health status at the start of the experiment and their viral load and CD4 counts were monitored over 18 mo. Group A received daily ART (integrase inhibitor) from day 19–47. Group B received daily ART on days 0–47 and a single infusion of 1B8 on day 12. One monkey (Δ 01D160) in group A was euthanized for humane reasons. A and B, Viral loads in group A and group B. The lower limit of quantification of 10^3 copies/ml is indicated by the dashed lines.

The experiment was designed to assess whether a single infusion of 1B8 IgG could affect viral loads with or without synergy from integrase inhibitor antiretroviral therapy (ART). In group A, monkeys received a single infusion of 1B8 at 5 mg/kg on day 0, and then on day 19 daily dosing of ART was begun. Accordingly, the effects on viral load due to PD-1 blockade alone can be seen through day 19 (Fig. 4A). Although the viral loads of group A on day 0 span over two orders of magnitude, their viral kinetics followed remarkably parallel trajectories. Relative to each monkey’s viral load on day 0, viral loads rose 3.4 ± 1.1-fold on day 2 and peaked at 14 ± 6-fold on day 5 before declining to 3.5 ± 1.6-fold on day 12. On day 19, the final day before ART, the viral loads diverged; monkey 01C021 rose to 13.2-fold over day 0, 01C043 returned to 1.8-fold, while 01D160 fell to 0.4-fold and 99X019 to 0.2-fold. Note that the viral load for monkey 99X019 fell below the limit of quantification (1000 copies/ml) after PD-1 blockade but before ART. After sample collection on day 19, daily ART was begun and continued until day 47. The viral loads declined rapidly as previously observed with this integrase inhibitor compound (26). Monkey 01D160 that began the protocol with the highest viral load reached a low viral load (1600 copies/ml) during ART but unfortunately rebounded to 4.0 × 10^7 copies/ml on day 56 and was subsequently euthanized. We hypothesize that this may be due to an escape mutation similar to viral variants previously reported (26), although this was not directly assessed. Monkey 99X019 maintained a viral load below 1000 copies/ml before and throughout ART and for at least 9 days after cessation of ART.

In group B (Fig. 4B), monkeys were under ART from day 0 to 47. The rapid decline of viral loads through day 5 is consistent with the previous publication on this integrase inhibitor (26), and with group A while on ART. However, on day 12, the viral loads of three monkeys continued to decline (monkey 01D298 to below 1000 copies/ml), while the viral load of monkey 99X025 rebounded. After sampling on day 12, each monkey in group B received a single infusion of 1B8 at 5 mg/kg. Treatment with 1B8 IgG on day 12 led to a transient rise in two monkeys, no discernable effect in 01D298 that maintained an undetectable viral load, and no effect on 99X025. On day 47, all ART ceased for both groups; all monkeys were monitored for viral loads and CD4 counts through day 124. Of note, 01D298 that started the protocol with the lowest CD4 count was also the best responder in group B, maintaining a viral load below 1000 copies/ml far longer than the other monkeys, followed by a detectable rise on the last day of ART (day 47) and exceeding 10^7 copies/ml only 9 days later but returning to its preprotocol level on day 124.

To characterize the parameters that may underlie the complex viral load kinetics, several assays were performed in parallel. First, the degree of in vivo blockade by the single infusions of anti-PD-1 1B8 mAb was measured. Lymphocytes in peripheral blood were stained with a mAb that bound 1B8 and competed with 1B8 for the same epitope (demonstrated by competitive binding in unpublished SPR data). The results are shown in Fig. 5A for group A (treated on day 0), and in Fig. 5B for group B (treated on day 12). As can be seen in the figures, 1B8-mediated blockade at least 90–95% was maintained for at least 7 to 12 days after 1B8 infusion depending upon the individual. Partial blockade (~50%) continued for most monkeys until 19 to 21 days after 1B8 infusion. The high efficiency and persistence of PD-1 blockade was consistent with the high affinity and low off-rate for 1B8 in SPR analysis and the efficient blockade in vitro of PD-L1 × 1B8 (Fig. 2B) even at a low concentration of 0.2 μg/ml. Thus, infusion of 1B8 at the 5 mg/kg dose, even as a mouse IgG, was able to achieve effective and persistent PD-1 blockade in monkeys.

Second, the epitope-specific T cell responses of these Mamu-A*01 positive monkeys were evaluated by the fraction of CD3+ CD8+ lymphocytes positively stained by tetramer specific for the dominant SIV Gag CM9 tetramer. These data are shown in Fig. 5C (group A) and in Fig. 5D (group B). In group A, the fraction of CD8 T cells that were CM9-tetramer positive were generally unchanged. In group B, after starting ART, three monkeys show a detectable rise in CM9 responses on day 5, but this subsided on day 12. The transient nature may be due to the rapid reduction in viral loads upon starting ART. PD-1 blockade during ART had no discernible effect on CM9-specific tetramer fractions.

Third, the number of total number CD4+ T cells was assayed frequently during the protocol; data are shown in Fig. 5E (group A) and in Fig. 5F (group B). PD-1 blockade did not have a profound effect on CD4+ levels. In group A, although a transient rise was seen 12 days after 1B8 infusion for the three monkeys with highest CD4 counts, this rise was not sustained and did not extend to...
monkey 99X019, which had the lowest absolute number of CD4\(^+\) T cells. In group B, CD4\(^+\) T cells clearly increased upon ART; the apparent increase after 1B8 infusion cannot be separated from the underlying trend due to continuing ART and must be discounted. Finally, multicytokine intracellular staining (ICS) for IFN-\(\gamma\), TNF-\(\alpha\), IL-2, and MIP-1\(\beta\) was performed on PBMC samples collected at days -29, 12, 33, and 47 of the protocol, but no significant differences were seen after 1B8 treatment, and the impact of 1B8 treatment on viral loads was not correlated with the number of polyfunctional T cells (data not shown). One limitation of this stringent model is the duration of chronic viral disease, \(18\) mo. This may mask some immunological correlates due to the depth and duration of immune dysfunction.

To evaluate the benefit of PD-1 blockade on T cell responses in monkeys with an unimpaired immune system, PD-1 blockade was combined with prophylactic vaccination, using a nonreplicating adenovirus vector type 5 encoding SIV Gag (25). Eleven naive Mamu-A\(^*\)01 positive rhesus macaques were allocated into four groups; all groups received the adenovirus vector vaccine at a dose of \(10^{10}\) viral particles in 0.5 ml into the right deltoid at day 0 and 49. Monkeys in group 1 (\(n = 2\)) and group 2 (\(n = 3\)) were simultaneously treated with 1B8 at 5 mg/kg respectively delivered i.v. or i.m. (left deltoid) at the time of vaccination. Monkeys in group 3 (\(n = 3\)) received no Ab treatment at day 0 and 1B8 treatment at 5 mg/kg on day 49 i.v. Monkeys in group 4 (\(n = 3\)) were given an i.v. isotype-matched IgG negative control at 5 mg/kg at days 0 and 49.

The percent of CD8\(^+\) T cells positively stained by CM9 tetramer for each monkey group is shown in Fig. 6. Overall tetramer positive staining exceeded 1% on day 10 post vaccination, and peaked on either day 10 or 14; these kinetics are consistent with previous experience with this vector. There is substantial variability among monkeys in each group, expected from the suboptimal vaccination dose. However, enhancement by PD-1 blockade with 1B8 treatment was observed for monkeys in groups 1 and 2. Because both groups were treated with 1B8, albeit by different routes, while
The peak CM9-tetramer response was significantly higher (together as one group vs groups 3 and 4 together as another group) of groups 1 and 2 compared by comparing the peak immune responses (mean of percent CM9 tetramer positive on days 10 and 14) of monkeys in groups 3 and 4 received no 1B8 treatment in the priming phase (before day 49), we analyzed the effects of 1B8 treatment. Group 3 received no mAb treatment at day 0 and 1B8 i.v. at day 49. Group 4 received isotype-matched IgG negative control i.v. at days 0 and 49.

Because 1B8 is a fully murine IgG, its persistence in rhesus monkeys, and hence its efficacy, will be ultimately limited by emergence of rhesus anti-mouse IgG Abs (RAMAs). In this experiment, the presence of RAMAs in serum by ELISA was detectable as early as 10–14 days after 1B8 treatment, and RAMA titers increased by two orders of magnitude when given a second 1B8 treatment (data not shown). The highest tetramer levels (Fig. 6) were generally associated with low concentrations or delayed appearance of RAMAs. The observed effective blockade of PD-1 × 1B8 on cell surfaces, lasting 7–12 days but incomplete at 19–21 days is compatible with time scale for RAMA responses to develop. This suggests that the delay of RAMA could sustain the occupancy of 1B8 on PD-1 at the cell surface, and hence prolong the period of effective PD-1 blockade. One would expect enhanced blockade efficiency and immunological benefit through repeated administrations, ideally with a human-Fc chimeric or fully humanized mAb for optimal pharmacokinetics.

**FIGURE 6.** Enhancement of T cell response by PD-1 blockade in rhesus monkeys vaccinated with adenovirus vector. Percent of CD3+/CD8+ lymphocytes that were positive for the SIV Gag CM9 tetramer in naive rhesus macaques; all macaques were vaccinated with an adenovirus vector encoding SIV Gag at days 0 and 49. Groups 1 and 2 were also treated with anti-PD-1 Ab 1B8 at 5 mg/kg, either i.v. (group 1) or i.m. (group 2) at the time of vaccination. Group 3 received no mAb treatment at day 0 and 1B8 i.v. at day 49. Group 4 received isotype-matched IgG negative control i.v. at days 0 and 49.

**Discussion**

In the chronic SIV-infection rhesus model, viral loads rose after a single infusion of anti-PD-1 mAb, peaking 5 days after treatment and returning to initial levels 19 days after (Fig. 4A). Although the blockade of PD-1 at the cell surface was nearly complete 5 days after treatment and persisted throughout most of this period (Fig. 5A), it was difficult to find an immunological correlation to the viral load kinetics. PD-1 blockade in chronic viral infections was initially hypothesized to act primarily by restoring “functionally exhausted” T cells (33, 34). However, in the chronic SIV infection rhesus model presented here, CD4 and CD8 cells were not functionally improved by PD-1 blockade. First, there was a lack of increase in single-function or multifunctional cytokine responses by ICS. Second, the fraction of CD8 T cells recognizing the immunodominant SIV Gag CM9 epitope did not increase after PD-1 blockade (Fig. 5, C and D). Third, the total number of CD4 cells did not significantly increase after PD-1 blockade (Fig. 5, E and F). This implies that CD4 proliferation, if present, was not a primary factor in the viral load kinetics. Because of the frequent sampling and the number of assays performed, no additional blood was available for retrospective analysis of activation or proliferation markers.

Although the long-term chronic SIV infection model is highly relevant to clinical applications, several limitations of this model may impair the observation of subtle correlations. First, the model may be too stringent. The monkeys had been chronically infected...
for longer than 18 mo, and the prolonged infections could have irreversibly compromised their immune systems and limit the sensitivity of detection. Second, transient cellular cytokine responses might have been missed as a consequence of the less frequent sampling (necessitated by the large blood volume draw required by the assay). Third, an improvement in T cell responses due to subtle phenotypic changes may not have been captured by the tetramer and multicolor ICS assays. Fourth, PD-1 blockade may benefit other components or compartments of the immune system, beyond T cells from peripheral blood, that potentially play a more significant role in this case.

Enhancement of immune responses through PD-1 blockade may also be mediated by dendritic cells. Blockade by Fabs of PD-L1 and PD-L2 on dendritic cells has been demonstrated to increase IFN-γ and IL-10 cytokine production and T cell proliferation in vitro (31). In comparison, blockade by an IgG of PD-1 on human PBMCs in culture increased IFN-γ but not IL-10 cytokine production in our experiment (Fig. 3). Thus, blocking PD-1 interaction with PD-L1 and PD-L2 on dendritic cells (21, 31) by increasing the number of activated DCs could cause the transient rise in viral loads observed in both groups (Fig. 4) in the therapeutic chronic SIV rhesus model. If confirmed, this would lend support to the PD-1 pathway having a complex role both in acting upon particular cell types and in modulating the interactions between different components of the immune system during the course of viral infection.

In the case of SIV, PD-1 up-regulation, if early and of sufficient magnitude, may actually be beneficial in preventing long-term pathogenicity. It is now well-known that sooty mangabeys can be infected with SIV but do not generally develop AIDS-like pathogenesis. It is also undoubtably complicated and not yet fully understood. In a recent publication (24), Estes et al. infected rhesus macaques and sooty mangabeys with SIV. Although SIV replication during the acute phase was similar in both species, and levels of activation and proliferation in lymph nodes were definitively during the acute phase of T cell activation. This is consistent with recent studies demonstrating that PD-1 expression is also associated with early phases of T cell activation (and proliferation). Hokey et al. (39) recently observed that T cells from DNA vaccinated rhesus macaques exhibited elevated PD-1 expression when stimulated ex vivo, and those T cells were mostly activated memory and effector phenotypes with no uncapped telomeres to indicate exhausted proliferation. Similarly, using an adoptive transfer mouse model with transgenic TCR cells, Goldberg et al. (40) demonstrated that PD-1 expression in adoptively transferred CD8 T cells was up-regulated early upon encountering Ag in mice, reaching its maximum within a couple of cell divisions. Blocking PD-1/PD-L1 interaction by administration of mAb enhanced such T cell proliferation in mice. Taken together, these lead to a hypothesis that a PD-1-induced immunomodulation mechanism may exist that is independent of T cell exhaustion.

Because of the complex interaction between cellular components of the immune system, and the different roles of PD-1 signaling in early vs late stages of infection, it is expected that further in vivo experiments will provide a deeper understanding of the role of blocking PD-1 and its ligands PD-L1 and PD-L2. We hope that extending these experiments to nonhuman primates will eventually lead to enhanced prophylactic and therapeutic vaccination modes as well as antiviral and antitumor therapies (41–43).

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Disclosures

The authors are employees of Merck & Co., Inc.

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