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

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Programmed Cell Death 1 (PD-1) plays a crucial role in immunomodulation. Binding of PD-1 to its ligand receptors down-regulates immune responses, and published reports suggest that this immune modulation is exploited in cases of tumor progression or chronic viral infection to evade immune surveillance. Thus, blockade of this signal could restore or enhance host immune functions. To test this hypothesis, we generated a panel of mAbs specific to human PD-1 that block PD ligand 1 and tested them for in vitro binding, blocking, and functional T cell responses, and evaluated a lead candidate in two in vivo rhesus macaque (Macaca mulatta) models. In the first therapeutic model, chronically SIV-infected macaques were treated with a single infusion of anti-PD-1 mAb; viral loads increased transiently before returning to, or falling below, pretreatment baselines. In the second prophylactic model, naive macaques were immunized with an SIV-gag adenovirus vector vaccine. Induced PD-1 blockade caused a statistically significant ($p < 0.05$) increase in the peak percentage of T cells specific for the CM9 Gag epitope. These new results on PD-1 blockade in nonhuman primates point to a broader role for PD-1 immunomodulation and to potential applications in humans. The Journal of Immunology, 2009, 182: 980–987.

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 vaccination 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 in i.m. three times at 3 wk intervals. Mice were boosted with an i.p. injection of 2 × 10⁷ cells of a viable 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 countercurrent 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’-(dimethylamino)propyl)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-1/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. 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 (low-binding conditions).

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 in 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 binding of PD-L1 to the PD-1.

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.
were incubated with mAb IgGs at 0.2 and 2.0 ng/ml IgG at 37°C, as described in Materials and Methods.

Second, all mAbs bound to human CD4 T cells, activated in vitro to express a high level of PD-1 (data not shown). Third, their cross reactivities to rhesus PD-1 and mouse PD-1 were determined using cells expressing a high level of PD-1 (data not shown). Most clones reacted to rhesus PD-1 and mouse PD-1 were determined using to express a high level of PD-1 (data not shown). In general, the high frequency of anti-PD-1 mAbs with cross-species binding to PD-1 and blocking of PD-L1 is not surprising given the high amino acid sequence homology (≥96%) between human and Old World nonhuman primates (22). The frequency of cross-species reactivity may be expected to be less for anti-PD-L1 mAbs given the lower sequence homology (≥91%) of PD-L1 (22).

To characterize the kinetics of Ab binding to PD-1 in a 1:1 stoichiometry, monomeric Fabs were made of the PD-L1 binding 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 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. In fact, a subsequent SPR experiment confirmed that 3H4 does not intercompete for binding to PD-1 with the PD-L1 blocking Fab, whereas 1B8 blocks both PD-L1 and PD-1 with equal affinity. This is consistent with a previous report (38) that 1B8 blocks PD-L1 and PD-1 with similar affinity.

Materials and Methods. The affinity 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. In fact, a subsequent SPR experiment confirmed that 3H4 does not intercompete for binding to PD-1 with the PD-L1 blocking Fab, whereas 1B8 blocks both PD-L1 and PD-1 with equal affinity. This is consistent with a previous report (38) that 1B8 blocks PD-L1 and PD-1 with similar affinity.
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. None had suffered any episode of immunodeficiency in the previous 18 mo, except for one incidence of diarrhea in monkey 01D298, who also represented the lowest total CD4 count (56 CD4+/mm³) at the start of the experiment.

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 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 (26). Monkey 01D160 that began the protocol with the highest viral load (1600 copies/ml) during ART had no discernible effect on CM9-specific tetramer staining with a mAb that bound PD-1 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

![Figure 4](http://www.jimmunol.org/)
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-γ, TNF-α, IL-2, and MIP-1β 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
monkeys in groups 3 and 4 received no 1B8 treatment in the priming phase (before day 49), we analyzed the effects of 1B8 treatment by comparing the peak immune responses (mean of percent CM9 tetramer positive on days 10 and 14) of groups 1 and 2 together as one group vs groups 3 and 4 together as another group. The peak CM9-tetramer response was significantly higher (*p* = 0.030) for the 1B8-treated group vs the nontreated group by a nonparametric Wilcoxon rank sum two-sided test. Also of note, tetramer staining for two monkeys in group 2 was sustained through day 40.

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 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.

**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 contrast, 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 pathology (35–38). Although sooty mangabeys are natural hosts for SIV, the underlying immunological reasons for this disparity with humans and rhesus macaques are undoubtedly 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 similar in the early phase, activation and proliferation were decreased in sooty mangabeys by day 30 and thereafter. The number of lymph node PD1⁺ T cells in sooty mangabeys increased dramatically during the early phase (rhesus macaque PD1 expression did not) and this was found to correlate with lower activation, proliferation, and ultimately lower viral replication and higher CD4 counts. The same mechanism may be causing the transient increase in viral loads in our experiment (Fig. 4). This points to a more complex role for PD-1 modulation in SIV (and HIV) infection than has been previously appreciated.

PD-1 blockade was found to have an adjuvant-like effect in naïve rhesus macaques vaccinated with adenovirus vectors encoding SIVgag (Fig. 6). This confirms in a nonhuman primate model an increase in epitope-specific T cell responses due to anti-PD-L1 mAb previously reported in the therapeutic vaccination LC MV mouse model (21). Furthermore, this experiment in naïve monkeys indicates that PD-1 blockade can also have a functional impact during the primary 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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