

Anti-PD-1 antibodies recognizing the membrane-proximal region are PD-1 agonists that can downregulate inflammatory diseases

Authors: Kensuke Suzuki^{1,2†}, Masaki Tajima^{1,3†}, Yosuke Tokumaru^{1,2}, Yuya Oshiro^{1,2}, Satoshi Nagata⁴, Haruhiko Kamada⁴, Miho Kihara⁵, Kohei Nakano⁵, Tasuku Honjo⁶ and Akio Ohta^{1*}

Affiliations:

¹Department of Immunology, Institute of Biomedical Research and Innovation, Foundation for Biomedical Research and Innovation at Kobe, Kobe 650-0047, Japan

²Pharmaceutical R&D Division, Meiji Seika Pharma Co. Ltd., Tokyo 104-8002, Japan

³Division of Integrated High-Order Regulatory Systems, Center for Cancer Immunotherapy and Immunobiology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

⁴Laboratory of Antibody Design, Center for Drug Design Research, National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki 567-0085, Japan

⁵Laboratory for Animal Resources and Genetic Engineering, RIKEN Center for Biosystems Dynamics Research, Kobe 650-0047, Japan

⁶Department of Immunology and Genomic Medicine, Center for Cancer Immunotherapy and Immunobiology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

*Correspondence: ohta-a@fbri.org

†These authors contributed equally.

One Sentence Summary: Agonistic anti-PD-1 antibodies that bind the membrane-proximal extracellular region of PD-1 suppress T cell activity in vivo.

Abstract

The PD-1 receptor triggers negative immunoregulatory mechanism which prevent overactivation of immune cells and subsequent inflammatory diseases. Because of its biological significance, PD-1 has been a drug target for modulating immune responses. Immunoenhancing anti-PD-1 blocking antibodies have become a widely used cancer treatment; however, little is known about the required characteristics for anti-PD-1 antibodies to be capable of stimulating immunosuppressive activity. Here, we show that PD-1 agonists exist in the group of anti-PD-1 antibodies recognizing the membrane-proximal extracellular region (MPER) in sharp contrast to the binding of the membrane-distal region by blocking antibodies. This trend was consistent in an analysis of 81 anti-human PD-1 monoclonal antibodies. Since PD-1 agonist antibodies trigger immunosuppressive signaling by crosslinking PD-1 molecules, Fc engineering to enhance Fc γ RIIB binding of PD-1 agonist antibodies notably improved human T cell inhibition. A PD-1 agonist antibody suppressed inflammation in murine disease models, indicating its clinical potential for treatment of various inflammatory disorders including autoimmune diseases.

Introduction

The immune system features a variety of autonomous regulatory mechanisms that modulate the intensity of immune responses. Imbalance in immune responses can increase the vulnerability to diseases: infectious diseases and cancer in an immunocompromised state, and inflammatory disorders in the presence of excessive immune responses. These endogenous immunoregulatory mechanisms, also known as immune checkpoints, are potential therapeutic targets to rebalance the immune response (1). A well-known successful example of this type of rebalancing is application of immune checkpoint inhibitors to cancer immunotherapy. Since immune checkpoints in the tumor microenvironment contribute to inactivation of anti-tumor immune effectors, intervention with immune checkpoint inhibitors is effective in many cancer patients, including some who are not responding to conventional therapies. The clinical successes achieved by checkpoint blockade broke the stalemate of cancer immunotherapy.

The success of PD-1 blockade in cancer immunotherapy underscores the importance of PD-1-mediated immunoregulation in the human immune system. PD-1 is upregulated in activated immune cells and provides a negative co-stimulatory signaling upon interaction with its ligands, PD-L1 and PD-L2 (2, 3). Stimulated PD-1 recruits phosphatases to downregulate tyrosine phosphorylation in the T cell receptor signaling cascade, resulting in the suppression of T cell activities (4-6). PD-1-dependent immunoregulation is indispensable to prevent self-reactive immune responses as demonstrated by the spontaneous development of inflammatory disorders in PD-1-deficient mice. A variety of inflammatory symptoms in PD-1-deficient mice suggest its critical role in controlling immune responses, ranging from direct tissue damage by cellular immunity to autoantibody production by humoral immunity (2). Since PD-1 expression is induced on activated immune cells, treatments that can induce PD-1 signaling can

downregulate pathogenic effector cells but not PD-1-negative bystander immune cells. Along with its potent immunosuppressive activity, this relative specificity for effector T cells makes agonistic PD-1-targeted therapy an attractive approach for the development of anti-inflammatory treatment.

Several different approaches have been developed to enforce PD-1 stimulation. PD-L1-Fc fusion protein has been used for PD-1 stimulation in vitro and sometimes given in vivo with an expectation to downregulate inflammation (7, 8). A synthetic protein designed to target the PD-L1-binding domain of PD-1 was found to mimic PD-L1 function (9). Since CD80 binding to PD-L1 inhibits its interaction with PD-1, the blockade of CD80-PD-L1 binding promoted PD-1-dependent immunoregulation by increasing PD-L1 availability (10). A bispecific T-cell engager-like approach was also undertaken in the MHC-oriented delivery of PD-L1 to inhibit PD-1-expressing T cells (11). Compared to PD-L1, which interacts with PD-1 with moderate affinity (12-15), high affinity binding of antibodies to PD-1 has potential advantages as a pharmacological PD-1-stimulatory agent. Several anti-PD-1 monoclonal antibodies (mAbs) were reported to inhibit T cells and inflammation (16-18), but their mode of action was undisclosed. Anti-inflammatory antibodies to PD-1 may not be always agonistic antibodies because the depletion of PD-1-expressing effector cells could also downregulate inflammation (19). Agonist antibodies to PD-1 have been proposed as immunomodulatory agents (20), but requirements for agonistic anti-PD-1 mAbs, e.g. the epitope recognition, need to be clarified.

Here, we report a group of anti-PD-1 agonist mAbs that can suppress T cell activities by triggering the inhibitory signaling of PD-1. A shared feature of these agonist mAbs is recognition of the membrane-proximal extracellular region (MPER) and their immunosuppressive activity depends on Fc receptor-supported crosslinking of PD-1 molecules. MPER recognition is

characteristic and crucial to the agonistic activity since PD-1 crosslinking by other antibodies did not induce any immunosuppressive activity. The anti-inflammatory efficacy of agonistic anti-PD-1 mAbs in vivo suggests their clinical potential as immunosuppressants.

Results

Immunosuppressive activity of PD-1 is inducible by immunoglobulins

First, we determined the appropriate conditions where immunoglobulins could stimulate PD-1. A soluble chimeric protein of PD-L1, PD-L1-Fc, has been frequently used to render immunosuppression by the co-ligation of PD-1 and TCR, e.g. co-immobilization of PD-L1-Fc and anti-CD3 mAb (21). Crosslinking of PD-1 alone did not exert immunosuppressive effect since PD-L1-Fc in a soluble form was unable to inhibit T cells even after the crosslink with secondary antibody (Figure S1A). Similarly, immobilized PD-L1-Fc on microbeads, separate from anti-CD3 and CD28 mAbs on the plastic surface, did not inhibit T cells (Figure S1B).

Although co-ligation of PD-1 and TCR has been widely used to stimulate PD-1 in vitro, we found that this experimental system was suboptimal due to nonspecific inhibition. We observed PD-1-independent T cell inhibition by immobilization of anti-CD3 and anti-CD28 mAbs and PD-L1-Fc onto a plastic surface in which the inhibition of IFN- γ production was equally strong in PD-1^{+/+} and PD-1^{-/-} T cells (Figure S1C). The reason for this nonspecific inhibition was related to an impaired immobilization of anti-CD3 mAb in the presence of PD-L1-Fc, resulting in the suboptimal T cell activation. It was possible to overcome this problem by optimizing the concentration and ratio of each component. Using a streptavidin-coated plastic plate and biotin-labeled components at the proper ratio, co-immobilized PD-L1-Fc with anti-CD3 and CD28 mAbs inhibited IFN- γ production from PD-1^{+/+} T cells, but not PD-1^{-/-} T cells

(Figure S1D). When this in vitro system is to be used, PD-1-dependent inhibition may be validated with the use of PD-1^{-/-} T cells as a control.

As the importance of co-ligation with TCR in stimulating PD-1 was confirmed, we tested if anti-PD-1 mAb would be able to replace PD-L1 in triggering the immunosuppressive activity. Anti-mouse PD-1 mAb clones, 29F.1A12 and RMP1-14, were co-immobilized with anti-CD3 and anti-CD28 mAbs, but neither of these anti-PD-1 mAbs could inhibit T cell activity (Figure S1E). Simple co-ligation of TCR and PD-1 using arbitrary antibodies failed to trigger PD-1 stimulation, suggesting the presence of specific epitope recognition requirements for functional anti-PD-1 agonist antibodies. Given the fact that both 29F.1A12 and RMP1-14 are blocking antibodies, we suspected that anti-PD-1 agonistic mAbs might be recognizing a separate epitope from the ligand-binding domain.

To test this possibility, we introduced the FLAG sequence (DYKDDDDK) into human PD-1 and examined if guided binding of an anti-FLAG mAb could stimulate immunosuppressive activity. PD-L1-binding domain has been identified within the IgV domain of PD-1 molecule (15, 22, 23). To guide antibody binding outside the ligand-binding domain, we introduced the FLAG tag in tandem with the N-loop (FLAG-hPD-1) or replaced a part of the stalk region with the FLAG tag (hPD-1 stalk-FLAG) (Figure 1A). When transfected into PD-1-knockout DO11.10 T cell hybridoma cells (DO11.10 cells), FLAG-hPD-1 and hPD-1 stalk-FLAG were normally expressed on the cell surface, and the introduced FLAG tag was accessible for anti-FLAG mAb (Figure 1B).

The $\alpha\beta$ TCR of DO11.10 cells was used to generate the TCR-transgenic mouse with the same name. This I-A^d-restricted T cell line retains antigen specificity and produces IL-2 upon interaction with OVA₃₂₃₋₃₃₉-pulsed IIA1.6 B lymphoma cells. PD-1-dependent

immunosuppression is intact in DO11.10 cells as shown by the PD-L1-dependent inhibition of IL-2 (Figure S2) (24).

The DO11.10-IIA1.6 system provides an excellent platform for analyzing the PD-1 activity in the antigen-specific T cell activation. The transfected FLAG-hPD-1 and hPD-1 stalk-FLAG as well as the wild-type hPD-1, were able to suppress T cells upon interaction with PD-L1-expressing IIA1.6 cells (Figure 1C). The simple addition of anti-FLAG mAb did not change IL-2 production from these hPD-1 transfectants (Figure 1D). However, as in the case with PD-L1-Fc, co-immobilization of anti-FLAG mAb with anti-CD3 and anti-CD28 mAbs did downregulate IL-2 production in the cells expressing hPD-1 stalk-FLAG, but not FLAG-hPD-1 (Figure 1E). Anti-FLAG mAb inhibited T cell activation through binding to a distinct region from the PD-L1 binding site (Figure 1F). This result indicates that the immunosuppressive activity of PD-1 can be stimulated with antibodies and that the recognition of a particular region may be important for agonistic antibodies.

Fc receptor-dependent crosslinking mediates agonist activity of anti-PD-1 mAb

Although immobilized antibodies may stimulate immunosuppressive activity of PD-1, this form of drug will be too complex for practical usage. To enable a treatment in a soluble form, one option is to employ Fc receptors for the engagement of antibodies. Fc receptors have been utilized to crosslink antibodies to other surface molecules such as CD3 and TNF receptor family in order to exert agonistic signaling (25-27). The crosslinking of PD-1 agonists with Fc receptors on APC might enable co-ligation of PD-1 with TCR. We replaced IIA1.6 cell line, which is an Fcγ receptor-defective mutant of A20 B lymphoma (28), with the parental A20 cells and examined the role of Fc receptor on antibody-dependent PD-1 stimulation (Figure 2A). As

expected, the addition of anti-FLAG mAb decreased IL-2 production from the hPD-1 stalk-FLAG-expressing DO11.10 cells, but not the FLAG-hPD-1-expressing cells (Figure 2B). The immunosuppressive effect of anti-FLAG mAb was based on its interaction with the FLAG tag because it did not stimulate wild-type PD-1 (Figure 2B), and the inhibitory effect was reversed by the addition of excess FLAG peptide (Figure 2C). Blocking antibody to CD16/CD32 abrogated the immunosuppression by anti-FLAG mAb, confirming the importance of Fc receptor (Figure 2D).

It should be noted that the binding of anti-FLAG mAb to hPD-1 stalk-FLAG initiated the same PD-1 signaling pathway as PD-L1 does. The replacement of Tyr248 in the intracellular domain of human PD-1 with phenylalanine is known to critically impair the PD-1 signaling (4, 5). When the Y248F mutation was introduced to hPD-1 stalk-FLAG, the DO11.10 cells were unable to respond to PD-L1 and anti-FLAG mAb (Figure 2E, F).

The principle of PD-1 stimulation with antibodies was reproducible in mouse PD-1. When FLAG tag was introduced to the same positions as in human PD-1, anti-FLAG mAb suppressed IL-2 production in DO11.10 cells expressing mPD-1 stalk-FLAG, but not FLAG-mPD-1 (Figure S3). However, co-ligation of truncated PD-1 in which FLAG tag was introduced to the similar position as mPD-1 stalk-FLAG but lacking N-loop and IgV domain (FLAG-mPD-1 Δ N+IgV) did not decrease IL-2 production, suggesting the importance of N-loop and/or IgV domain in the immunosuppressive activity.

Recognition of membrane-proximal region is characteristic in anti-PD-1 agonist mAbs

Next, we sought to discover anti-human PD-1 mAbs that can stimulate immunosuppressive activities. Considering the importance of Fc receptor-mediated crosslinking, we set up the

screening system for agonistic activities using a combination of hPD-1-expressing DO11.10 cells and PD-L1-deficient IIA1.6 cells that were transfected with murine FcγRIIB (Figure 3A). Blocking activities were also monitored by coupling hPD-1-expressing DO11.10 cells with human PD-L1-transduced IIA1.6 cells lacking Fc receptors.

Given that binding epitopes used by individual anti-PD-1 mAbs might be relevant to whether they exhibit agonistic activity, a monoclonal antibody panel with a wide variety of binding epitopes would be suitable for our unbiased search for PD-1 agonists. The binding intensity-based conventional screening of hybridoma clones gives rise to high affinity antibodies; however, the binding sites of selected antibodies may be biased toward specific epitopes, e.g. a highly immunogenic domain. Instead, we used an anti-human PD-1 mAb panel, which focused on the preservation of diverse binding epitopes. Eight segments of the extracellular domain of the human PD-1 molecule were chosen as representative ones forming the molecular surface (Figure 3B, C). These segments were replaced by the corresponding mouse PD-1 segments individually, and anti-human PD-1 mAb clones were classified by the binding capacity to each of these human PD-1 mutants. The anti-human PD-1 mAbs tested showed multiple patterns of binding to the eight segments; the only segment that was not part of an antibody binding site was segment #4.

We assessed the anti-human PD-1 mAb panel and found quite a few agonists along with blockers (Figure 3D, E). Interestingly, these agonist mAbs were part of the group recognizing the MPER of human PD-1, i.e. segments #6, 7 and 8 (Figure 3C, E). The mAb clones binding to segment #7 offered especially strong immunosuppressive activities, while those recognizing segment #8 were less effective. Binding affinity to human PD-1 showed a trend of positive correlation with the agonistic activity of mAbs at least in clones of mouse IgG1 subclass (Figure

S4). The binding domains of agonist mAbs were in a sharp contrast with those for the blocking mAbs, which interacted with the membrane-distal region of human PD-1 molecule as defined by segments #1, 2 and 5.

Anti-PD-1 agonist mAb triggers the immunosuppressive signaling of PD-1

We selected an agonist clone HM266, which had the strongest immunosuppressive activity (Figure 3D), for the subsequent studies. HM266 is a mouse IgG1, which binds to mouse Fc γ RIIB well. Due to the recognition of MPER, HM266 did not compete with PD-L1 or PD-L2 for the binding to PD-1 (Figure S5). The stimulation of PD-1 activity by this anti-PD-1 agonist mAb was additive to that of PD-L1 (Figure S5). Therefore, PD-1 agonist mAb treatment may further enhance PD-1-dependent immunosuppression on top of the pre-existing induction by PD-1 ligands.

The effect of HM266 on T cells expressing different levels of PD-1 showed that the degree of immunosuppression was dependent on PD-1 levels (Figure S6). While IL-2 production from T cells with intermediate PD-1 levels could not be reduced more than 60%, HM266 achieved almost 100% inhibition in T cells with PD-1 overexpression at high levels. Similarly, the increase of Fc γ RIIB expression on IIA1.6 cells also promoted the immunosuppressive activity of HM266 (Figure S6). In contrast, pretreatment of HM266 with endoglycosidase S, which hydrolyzes glycans on IgG and reduces antibody binding to Fc γ receptors, strongly impaired PD-1 agonistic activity of HM266 (Figure S7). These results suggest that the intensity of PD-1 stimulation by PD-1 agonist antibodies is dependent on their engagement on the cell surface.

HM266 induces the same type of signaling events as PD-L1. Stimulation with PD-L1 has been shown to induce PD-1 phosphorylation, thereby recruiting SHP-2, which interrupts TCR signaling cascade with its phosphatase activity (4, 5, 29). We examined PD-1 phosphorylation using DO11.10 cells expressing human PD-1-GFP. Western blotting after immunoprecipitation with anti-GFP antibody indicated PD-1 phosphorylation by HM266 (Figure 4A). To further confirm the downregulation of TCR signaling by HM266, we examined the extent of ERK phosphorylation in stimulated DO11.10 cells. HM266 strongly prevented the induction of phosphorylated ERK in T cells (Figure 4B, C).

Enhanced FcγRIIB binding improves immunosuppressive effect of PD-1 agonist antibodies on human T cells

To validate HM266 in the human immune system, we converted the mouse IgG1 to chimeric immunoglobulins with human IgG1 or IgG4-derived Fc region. Primary-cultured human CD4⁺ T cells were stimulated in a co-culture with human monocytic cell line THP-1 (Figure 5A). In this co-culture, chimeric HM266 with human Fc moderately inhibited IFN-γ production thanks to basal FcγRIIB expression in normal THP-1 cells (hFcγRIIB^{low}; Figure 5B). When THP-1 cells were made to overexpress human FcγRIIB (hFcγRIIB^{high}), the chimeric HM266 antibodies strongly suppressed IFN-γ production from T cells.

Previously, Ravetch's group has shown that anti-CD40 agonist mAb exerts its effect upon engagement on FcγRIIB and that Fc variants with high affinity to FcγRIIB significantly enhanced its efficacy (27, 30). To improve the activity of PD-1 agonist antibodies, we examined human IgG1 Fc variants with enhanced affinity to human FcγRIIB. Within the tested chimeric antibodies, X2 (G236D/H268D), X3 (S239D/H268D) and X4 (S239D/H268D/L328Y/I332E)

variants demonstrated notably promoted engagement on THP-1 cells (Figure 5C) and significant increases in their immunosuppressive activity in the co-culture of human CD4⁺ T cells and THP-1 cells (Figure 5D). Anti-human PD-1 agonist mAbs with enhanced FcγRIIB binding were further confirmed to suppress allogeneic human T cell responses to primary B cells from other individuals. The addition of HM266-hIgG1-X2, X3 and X4 to the mixed lymphocyte reaction strongly suppressed IFN-γ production (Figure 5C).

Anti-PD-1 agonist mAbs suppressed inflammatory diseases

To evaluate in vivo efficacy of the anti-human PD-1 agonist mAb, we employed human PD-1-knock-in (hPD-1-KI) mice in which mouse PD-1 gene was replaced by human PD-1 gene (Figures 6A and S8). To adapt to the mouse Fc receptor system, the HM266 antibody used in the in vivo studies was the original mouse IgG1 form. Acute graft-versus-host disease was induced by the transfer of spleen cells from C57BL/6-background hPD-1-KI mice into BDF1 mice (Figure 6B). As the result of allogeneic T cell activation, H-2^{b+} H-2^{d-} donor T cells were the predominant mononuclear cells in the peripheral blood, and many of them expressed human PD-1 (Figure 6C). However, treatment with HM266 prevented body weight loss in acute GVHD (Figure 6D) and largely reduced expansion of donor-derived T cells in the recipients (Figure 6E, F).

We confirmed the anti-inflammatory efficacy of HM266 in another T cell-dependent inflammation model. The transfer of naïve CD4⁺ T cells from hPD-1-KI mice into RAG2^{-/-} mice induced colitis in several weeks (Figure 7A). HM266 treatment prevented colitis induction as indicated by significant reversal of body weight loss (Figure 7B) and of shortened colon length (Figure 7C). In the lamina propria, HM266 reduced CD4⁺ T cell expansion (Figure 7D).

Decreases in IFN- γ - and IL-17-producing T cells suggested that PD-1 agonist mAb inhibited development of pathogenic effector cells (Figure 7E-G).

We further examined how HM266 treatment affects antibody production. hPD-1-KI mice were immunized with 4-hydroxy-3-nitrophenyl acetyl-ovalbumin (NP-OVA) in alum to monitor anti-NP antibody production. HM266 reduced antigen-specific antibody levels in the plasma (Figure S9), suggesting its efficacy on not only cellular immune responses but also humoral immunity. Anti-PD-1 mAb was previously reported to reduce antibody production in NZBWF1 mice by acting as cell-depleting antibody (19). HM266 is mouse IgG1, which is not considered a very strong mediator of cell depletion; however, cell-depleting activity may contribute to the anti-inflammatory outcome in vivo along with agonistic activity. To discriminate these two mechanisms, we used another anti-human PD-1 mAb HM255, which is also mouse IgG1 subclass and has a similar level of binding affinity (slightly stronger than HM266) but is a weak antagonist that completely lacks agonistic activity. In contrast to HM266, HM255 did not reduce anti-NP antibody levels (Figure S9). This result suggests that the PD-1-stimulatory activity makes a difference to the efficacy of anti-PD-1 antibodies as immunosuppressants.

Discussion

The idea of delivering anti-inflammatory treatment via PD-1 stimulating agents has been examined using various approaches. However, not much was known about what features are needed for anti-PD-1 agonist antibodies to have suppressive activity. In the present study, we first questioned how it would be possible to stimulate PD-1 using immunoglobulins and found two important requirements for agonistic antibodies: MPER recognition and PD-1 crosslinking. This discovery led us to establish the screening system for the agonistic activity of anti-PD-1

mAb. The system took advantage of the antigen-specific cytokine production by a T cell line that was coupled with an MHC-matched antigen-presenting cell line. To function as agonists, antibodies had to be engaged by a Fc receptor that enables PD-1 crosslinking. We did not observe any agonist clone working without crosslinking to Fc receptors. Other forms of PD-1 stimulators, such as recombinant T cell receptor conjugated with PD-L1 or anti-PD-1 antibody fragment, were also designed to work at the interface between T cells and APC (11). When T cells meet with APC, the spatial segregation of membrane proteins has been reported (31, 32). TCR on T cells and MHC on APC were concentrated at the cell-cell interface, and PD-1 needs to be adjacent to TCR to enable dephosphorylation of TCR signaling components by the recruited phosphatases. A cell imaging study has demonstrated co-localization of TCR and PD-1 at the immune synapse in the PD-L1-induced T cell inhibition (5). When PD-1 molecules were not able to stay close to the TCR in the immune synapse, those PD-1 molecules failed to inhibit TCR signaling cascade. We speculate that Fc receptors may keep agonist antibodies at the cell-cell interface (20) and induce PD-1/TCR co-ligation.

To engage agonist antibodies, we used Fc γ RIIB-expressing cells. Fc γ RIIB has been utilized to provide a crosslinking scaffold for agonist antibodies to TNF receptor family members: Fas (33), death receptor 4/5 (25, 34), CD40 (26, 27) and OX40 (35). In these studies, crosslinking via Fc γ RIIB was critically important for the biological activity of agonist mAbs, but Fc γ RIIB's own function as an inhibitory Fc γ receptor was dispensable (34). Other Fc γ receptors were found to be less effective or sometimes detrimental to the function of agonist mAbs (26, 30). We have observed different degrees of immunosuppression between anti-PD-1 agonist mAb clones, but their IgG subtype may have affected agonistic activities due to the difference in mouse Fc γ RIIB binding. Most of the powerful agonist mAb clones were found in mouse IgG1

and IgG2b subtypes, but the potential of IgG2a and IgG3 clones might be underrated due to their lower affinity to FcγRIIB than IgG1 and IgG2b.

As the importance of Fc receptors is increasingly recognized in the therapeutic application of antibodies, Fc region became a focus in developing advanced pharmacological antibody design (36, 37). Such efforts involve modification of the amino acid sequence to modulate affinity and specificity to particular Fc receptors. Anti-CD40 and anti-DR5 agonist mAbs have demonstrated sufficient anti-tumor efficacy in mouse studies but did not reach expectations in human clinical trials, suggesting a substantial species difference in Fcγ receptors that may critically affect the performance of agonist antibodies in the human immune system. In an effort to improve anti-CD40 and anti-DR5 agonist mAbs, Fc variants with enhanced FcγRIIB affinity significantly improved anti-tumor efficacy (30, 34). Our anti-human PD-1 agonist antibody HM266-hIgG1 showed only moderate to no inhibition in primary human CD4⁺ T cells most likely due to low FcγRIIB expression. We intentionally increased the affinity to FcγRIIB, and such modification of Fc region strikingly improved the immunosuppressive activity to primary human T cells. The adaptation to the human immune system will be a key to optimize therapeutic value of anti-PD-1 agonist mAbs.

Although crosslinking of antibodies is essential to the optimal immunosuppression by anti-PD-1 agonist mAbs, the binding epitope is also crucial as shown by a clear trend of agonist binding to MPER. We first identified the relationship between MPER binding with agonistic activity in hPD-1 stalk-FLAG with which anti-FLAG mAb was guided to the segment #8. Later, the antibody group interacting with segment #7 emerged as potent agonists after the screening of the anti-human PD-1 mAb panel. Since MPER is distant from the PD-L1-binding site, anti-PD-1 agonist mAbs were able to downregulate immune response without interfering with PD-1/PD-L1

interaction. In a sharp contrast, none of the blocking antibodies were associated with a binding site in MPER. Blocking antibodies specifically bound to the membrane-distal segments #1, 2 and 5. This area overlaps with the PD-L1 binding site at residues 66-78 (the gap between segments #2 and 5) and 128-134 (segment #1) (22), indicating the direct competition of blocking antibodies with natural ligands. Nivolumab and pembrolizumab, therapeutic anti-PD-1 blocking antibodies, were also shown to interact with human PD-1 at regions #1/2/3 and #1/2/5, respectively (22, 23).

Although MPER binding is important to anti-PD-1 agonist mAbs, MPER is not always the binding epitope of agonistic antibodies to other target molecules. Analyses of 9 anti-human CD40 and 2 anti-4-1BB mAb clones showed that agonist antibodies bound to the distal domain, and antibodies targeting MPER were found to be blockers (38, 39). They speculated that steric constraints may prevent Fc γ RIIB accessibility of MPER-binding antibodies. However, unlike other TNF receptor family proteins, binding epitopes of anti-OX40 agonist mAbs existed in the middle part of MPER in the analysis of 4 representative clones (40). Our analysis of anti-human PD-1 mAb clones provides a comprehensive insight on the epitope specificity that defines biological functions, ranging from blocker to agonist. The clear agonist activity of MPER-binding anti-PD-1 antibodies indicates their capability of engaging Fc γ RIIB in the T cell-APC interface without steric constraints.

PD-1-dependent immunoregulation is involved in a wide range of immune responses including T cell-dependent tissue injury and autoantibody production. Therefore, as shown in animal experiments in this study, anti-PD-1 agonist mAbs may be effective to a variety of inflammatory disorders. The inhibitory effect of HM266 on antibody induction may involve the suppression of PD-1-expressing T follicular helper (Tfh) cells. CD4⁺ cells play an important role

in antibody production (41), and the expansion of Tfh cells by the interruption of PD-1 signaling indicates that Tfh cells are controlled by PD-1 (42, 43). In these papers, PD-L1 and PD-L2 on B cells were suggested to play different roles in the regulation of antibody responses. PD-L1 and PD-L2 are expressed on different cell types and are induced in different cytokine milieu (44). It is not known if PD-1 stimulation by PD-L1 and PD-L2 induces any different outcomes. Nonetheless, HM266 does not interfere with PD-1 binding by both PD-1 ligands and exerts its own agonistic effect independently.

In conclusion, we uncovered specific requirements for agonist anti-PD-1 mAbs and identified PD-1 agonists that inhibit T cells by triggering immunosuppressive signaling. Epitope-specificity plays an important role in defining anti-PD-1 mAb functions. To co-ligate PD-1 with TCR, Fc γ RIIB works as an excellent platform for crosslinking of anti-PD-1 mAbs. PD-1 agonists may be further improved by optimizing the Fc region to promote Fc γ RIIB binding. Since PD-1 can affect various types of immune activities, PD-1 agonist antibodies may have versatile uses as part of an anti-inflammatory strategy. In cancer immunotherapy with immune checkpoint inhibitors, an overcompensated immune response often causes various forms of immune-related adverse events (irAE) that are occasionally life-threatening (45). Anti-PD-1 agonist mAbs may also be useful in rebalancing the intensity of immune response when irAE are encountered.

Limitations of this study include the difference in Fc γ receptors between mice and humans. PD-1 agonist antibodies showed anti-inflammatory efficacy in mice, which express Fc γ RI, Fc γ RIIB, Fc γ RIII and Fc γ RIV, but humans have a different set of Fc γ receptors consisting of Fc γ RI, Fc γ RIIA/B/C and Fc γ RIIIA/B. Binding affinities of these receptors to IgG isotypes are variable even when sharing the same name between mice and humans. For example, human Fc γ RIIB interacts with human IgG at approximately a 10 times lower affinity than the interaction

between Fc γ RIIB and IgG of the murine system (46, 47). Therefore, the in vivo efficacy of PD-1 agonist antibodies in mice may not directly reflect that of humanized antibodies in humans. As shown in the significant improvement in function associated with the enhanced Fc γ RIIB binding, some Fc engineering may be beneficial for PD-1 agonist antibodies to effectively downregulate proinflammatory activities in humans. The requirement for crosslinking in activating PD-1 agonistic antibody suggests that its therapeutic efficacy may be dependent on the Fc γ receptor availability in the local environment. Superagonist-type agonist antibody, which does not demand Fc γ receptor-mediated crosslinking, may overcome this limitation, but we did not find any such clones in our screening of anti-PD-1 mAbs. Although PD-1 agonist antibodies can suppress T cell activities, they will not directly inhibit immune effectors lacking PD-1 expression such as most granulocytes and macrophages. Combination of PD-1 agonist antibody with other treatments may be considered to further improve treatment of inflammatory diseases. Since excess suppression of proinflammatory response may increase the risk of infection and cancer, the use of PD-1 agonists in patients will need attention as in the case of other immunosuppressants.

Materials and methods

Study Design

The objective of this study is to define anti-PD-1 agonist antibodies that elicit the immunosuppressive activity of PD-1. To this end, we established the screening system of PD-1 agonist antibodies based on the physiological antigen-dependent T cell activation scheme using a combination of DO11.10 T cell hybridoma and MHC-matched B lymphoma cells. The anti-human PD-1 mAb panel, which includes diverse mAb clones recognizing various domains of

human PD-1 molecule, was subjected to the evaluation of the agonistic activity along with the immunoenhancing blocking activity. Characterization of binding site and the screening for biological activities were conducted by separate labs in a blinded manner. Fc variants of PD-1 agonist antibodies were designed to enhance Fc γ RIIB binding and to improve the immunosuppressive activity in human T cells. To examine in vivo efficacy, PD-1 agonist antibody was given to mouse models of acute graft-versus-host disease and colitis and to NP-OVA-immunized mice. The size of mice cohorts was determined from previous experience so that a desired difference would be detected in power analysis ($\alpha=0.05$, a power of 0.9). Experimental endpoints were determined from disease progression in our prior experience and has been approved by the institutional committee. The mice were randomly assigned to groups, but the experiments were not blinded. The in vivo anti-inflammatory efficacy of PD-1 agonist antibody was evaluated by body weight loss, tissue damage, T cell expansion, and production of cytokine and antibody. The experiments were repeated at least twice.

Mice

Female C57BL/6 mice and BDF1 mice (8-10 weeks-old) were purchased from Japan SLC (Hamamatsu, Japan) or CLEA Japan (Tokyo, Japan). PD-1^{-/-} mice and RAG2^{-/-} mice with C57BL/6-background were obtained from RIKEN Bioresource Research Center (Tsukuba, Japan) and were bred in the animal facility in RIKEN Center for Biosystems Dynamics Research or in Oriental BioService Ltd. All mice colonies and experimental animals were housed in specific pathogen-free condition. All animal experiments were conducted in accordance with the institutional animal care guidelines. For the generation of human PD-1 knock-in mice (Accession No. CDB0117E: <http://www2.clst.riken.jp/arg/mutant%20mice%20list.html>), human PD-1

cDNA sequence followed by bovine poly A signal was inserted into the start codon of mouse PD-1 genome by CRISPR/Cas9 system into C57BL/6N embryos as previously described (48) (Figure S8). The gRNA site (5'-GCC AGG GGC TCT GGG CAT GT-3') was designed by using the Zhang lab website (49). For the homologous recombination-mediated knock-in, the donor vector consisting of homology arms and human PD-1-bpA (bovine growth hormone poly A signal sequence) was generated to insert the human PD-1-bpA cassette at the 4 base upstream of the PAM sequence. The mixture of crRNA (CRISPR RNA), tracrRNA (trans-activating crRNA), donor vector and Cas9 protein was injected into the pronucleus of one-cell stage zygote. Following primer pairs were used for genotyping: wild-type FW 5'- AGG AGA CTG CTA CTG AAG GC -3' and REV 5'- CCA ATC CGT GTA ACC AGG -3' (245 bp); knock-in FW 5'- CAG GCC TCG ACA CCC ACC -3' and REV 5'- CAG CCC AGT TGT AGC ACC -3' (536 bp).

Cell Lines

Parental lines of DO11.10 T cell hybridoma (RRID: CVCL_4163), IIA1.6 (RRID: CVCL_0J27), and A20 cells (RRID: CVCL_1940) were provided by Dr. Tasuku Honjo (Kyoto University). We developed hPD-1⁺ DO11.10 T cell hybridoma and PD-L1⁻ mFcγRIIB⁺ IIA1.6 cells for the assay of agonistic activity based on PD-1-knockout DO11.10 T cell hybridoma and PD-L1-knockout IIA1.6 cells, which were obtained from Dr. Taku Okazaki (Tokyo University). These cells were cultured in RPMI1640 supplemented with 10% FBS, 6.25 mM HEPES, 2.5 mM L-glutamate, 0.625 mM sodium pyruvate, 0.625x non-essential amino acids solution, 62.5 μM 2-mercaptoethanol, 125 U/ml penicillin, 125 μg/ml streptomycin and 6.25 μg/ml gentamycin. To produce the agonistic anti-human PD-1 mAb HM266, the hybridoma cells were grown in CD

hybridoma medium (Gibco Cat#11279023) supplemented with 8 mM L-glutamine, 20 U/ml penicillin and 20 µg/ml streptomycin in a CELLline bioreactor flask (Duran Wheaton Kimble Cat#WCL1000).

Antibodies

See Table S1 for antibodies and recombinant proteins. Flow cytometry was performed using FACSFortessa X-20 (BD Biosciences) and analyzed with FlowJo software.

PD-1 stimulation with PD-L1-Fc

At first, purified streptavidin (10 µg/ml; Biolegend Cat#280302) was immobilized in a 96-well flat-bottomed plastic plate. After blocking with Blocking One reagent (Nacalai Tesque Cat#03953-95), biotinylated antibodies to mouse CD3 and mouse CD28 (1 µg/ml each) and biotinylated mouse PD-L1-Fc (2 or 6 µg/ml; BPS Bioscience Cat#71119) were added as a mixture in PBS. The total molar amount of Fc proteins was adjusted by the compensatory addition of biotinylated mouse IgG2a (MOPC-173). The plate was used for stimulation of mouse T cells after extensive wash with PBS.

Retroviral transduction

Human PD-1, GFP-fused human FcγRIIB and mouse FcγRIIB retroviral plasmids were generated by inserting each cDNA into MSCV-IRES-Thy1.1 DEST (Addgene Cat#17442). Point mutations were introduced by using QuikChange II site-directed mutagenesis kit (Agilent Cat#200523). FLAG-mutant human PD-1 constructs were generated by insertion of the FLAG sequence into N-terminus of hPD-1 (FLAG-hPD-1) or by replacing 8 amino acids of stalk region

to FLAG sequence (position 145-152 TERRAEVP -> DYKDDDDK; hPD-1 stalk-FLAG). Plat-E cells (RRID: CVCL_B488; Cell Biolabs) were transfected with the retroviral plasmids using Eugene HD (Promega, Cat#E2311). Retroviral supernatant was collected 48 and 72 h post-transfection, pooled and passed through 0.45 μ m Minisart Syringe Filter (Sartorius Cat#16533). The retroviral supernatant was centrifuged in Retronectin (Takara Bio Cat#T100A)-coated culture plates for 2 h at 32°C. Subsequently, DO11.10 or IIA1.6 cells were added to the retrovirus-coated culture plates and were centrifuged at 800 x g for 10 minutes at 32°C. This procedure was repeated the next day. After several days of culture, transduced cells were cloned by limiting dilution for further experiments.

Stimulation of DO11.10 T hybridoma cells

DO11.10 T cell hybridoma (5×10^4 cells) were stimulated with 2 μ g/ml of OVA₃₂₃₋₃₃₉ peptide (Eurofins Genomics) using A20 or IIA1.6 B lymphoma cells (1×10^4 cells) as antigen-presenting cells (24). Antibodies were added at 5 μ g/ml unless otherwise indicated. Culture supernatants were collected after 18 h and IL-2 levels were determined by ELISA (Mouse IL-2 DuoSet ELISA; R&D Systems Cat#DY402). For the screening of anti-human PD-1 agonist mAbs, human PD-1-expressing DO11.10 cells were co-cultured with hPD-L1⁻ mFc γ RIIB⁺ IIA1.6 cells to detect IL-2 decrease. Blocking activity of antibodies was monitored as the reversal of immunosuppression conferred by hPD-L1⁺ mFc γ RIIB⁻ IIA1.6 cells.

Generation of anti-human PD-1 antibody panel

The anti-human PD-1 antibody panel was established in the Laboratory of Antibody Design, Center for Drug Design Research, National Institutes of Biomedical Innovation, Health and

Nutrition (Ibaraki, Japan). Anti-human PD-1 mouse monoclonal antibodies were produced by DNA-immunization of A/J mice and a conventional hybridoma method. In brief, a pcDNA3-based expression plasmid encoding human PD-1 was injected intramuscular 6 times followed by a boost immunization with 293T cells transiently expressing human PD-1. After three days, the spleen cells were harvested and fused with P3U1 mouse myeloma cells to produce hybridomas. Monoclonal antibodies in the culture supernatants were screened by flow cytometry using HEK293 cells transiently expressing human PD-1 and Alexa Fluor 647-labeled goat anti-mouse IgG (Jackson ImmunoResearch Cat#115-605-071) as the secondary antibody. After several times of cell cloning by the limiting dilution method, the finally established hybridomas were cultured in a CELLline bioreactor flask to harvest the antibody. HM266 mAb in the culture supernatant was purified using protein A (Ab-Capture ExTra; Protenova Cat#P-003).

To classify anti-human PD-1 mAbs by binding sites, eight segments in the extracellular domain of human PD-1 putatively forming molecular surface were chosen (see Figure 3B, C). Each of the eight segments were replaced by the corresponding murine PD-1 sequence, and these eight different human PD-1 mutants were individually expressed on HEK cells. Anti-human PD-1 mAbs were characterized for their binding capabilities to each of the mutants by flow cytometry and classified in groups.

Western blotting

Retroviral plasmid for bicistronically transducing hPD-1/GFP fusion protein and mouse SHP2 (C459S point mutation) protein was generated by replacing IRES-Thy1.1 sequence to hPD1/GFP-P2A-mSHP2-C459S sequence of MSCV-IRES-Thy1.1 plasmid. The lack of phosphatase activity in SHP-2(C459S) has been shown to intensify the phosphorylated signal of

PD-1 (5). DO11.10 naïve CD4⁺ T cells were retrovirally transduced with hPD-1 using the plasmid indicated above. Transduced T cells (5×10^6 cells) were then cocultured with OVA₃₂₃₋₃₃₉ peptide-pulsed A20 cells (2.5×10^6 cells) in the presence of HM266 or PD-L1-Fc (5 µg/mL) for 5 minutes in 37 °C. Cells were immediately processed for lysate preparation and immunoprecipitation using anti-GFP agarose (MBL International Cat#D153-8). Input and immunoprecipitated samples were denatured by LDS sample buffer and reducing agent (Invitrogen) and processed for Western blotting using anti-phospho-tyrosine mAb (4G10) and anti-human PD-1 mAb (NAT105), followed by HRP-conjugated goat-anti-mouse IgG antibody (Jackson ImmunoResearch Cat#115-035-072).

ERK phosphorylation

DO11.10 cells expressing human PD-1 (5×10^4 cells) were stimulated with 2 µg/ml of OVA₃₂₃₋₃₃₉ peptide in the presence of hPD-L1⁻ mFcγRIIB⁺ IIA1.6 cells (1×10^4 cells). HM266 or control mouse IgG1 (MOPC-21) was added at 5 µg/ml. After culturing in a CO₂ incubator, cells were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.1% Triton X-100 for additional 15 min. Cells were washed with 0.5% BSA-PBS, resuspended in 50% methanol-PBS and kept in a freezer overnight. After extensive wash, Alexa488-anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mAb, PE-anti-DO11.10 TCR mAb and APC-anti-B220 mAb were added for subsequent flowcytometric analysis of phosphorylated ERK in DO11.10 cells.

Interaction of PD-L1-Fc and PD-L2-Fc with PD-1

DO11.10 cells expressing human PD-1 or hPD-1 stalk-FLAG were labeled with human PD-L1-Fc or human PD-L2-Fc (10 µg/ml) for 15 min. After wash, BV421-anti-human IgG Fc mAb was

added to detect PD-L1/PD-L2 on the cells by flow cytometry. To test competitive binding, DO11.10 cells were preincubated with anti-PD-1 mAb (EH12.2H7 or HM266; 5 µg/ml), or anti-FLAG mAb (L5; 5 µg/ml) for 15 min and subsequently labeled with PD-L1-Fc or PD-L2-Fc.

Endoglycosidase treatment of HM266

To inactivate Fc receptor binding, HM266 (1 mg/ml) was deglycosylated by incubating with IgGZERO (1 U/ml; Genovis Cat#A0-IZ1-010) for 4 h at 37 °C. The mixture was incubated with Ni-NTA agarose (Wako Chemical Industries Cat#143-09763) in 50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole (pH 8.0) for 30 min at 4 °C to remove the endoglycosidase. After centrifugation at 500 x g for 5 min, the supernatant was used for an experiment.

Generation of chimeric HM266 with human IgG1 Fc and its variants

The Fc region of HM266, which is originally mouse IgG1, was replaced with human IgG1(K322A). In addition, to enhance the antibody binding to hFcγRIIB, we also generated several chimeric HM266 with variants of human IgG1 Fc. The Fc variants are as follows: L235R (X1), G236D/H268D (X2), S239D/H268D (X3), S239D/H268D/L328Y/I332E (X4). DNA of HM266 heavy and light chains were inserted to pcDNA3.4 (Invitrogen Cat#A14697) separately. Fc-humanized HM266 were generated by the transfection of 1:1 mixture of the plasmids to CHO cells using ExpiFectamine CHO transfection kit (Gibco Cat#A29129). Antibodies in the culture supernatant was purified using protein A followed by gel filtration (Superdex 200 Increase 10/300 GL; Cytiva Cat#28990944). Antibody engagement to human hFcγRIIB was analyzed by incubating antibodies with hFcγRIIB-expressing IIA1.6 cells and subsequent staining with Alexa Fluor 647-anti-mouse F(ab')₂ antibody.

Agonistic activity on human CD4⁺ T cells

Purified human CD4⁺ T cells (Lonza Cat#2W-200) were stimulated for 3 days by immobilized anti-human CD3 mAb (3 µg/ml). The activated CD4⁺ T cells (5 x 10⁴ cells) were restimulated with Cytostim (Miltenyi Biotec Cat#130-092-172) in the co-culture with THP-1 monocytic leukemia cells (2.5 x 10⁴ cells; RRID: CVCL_0006). HM266 chimeric with human Fc was added at the indicated concentrations. Culture supernatants after 18 h were used for quantification of IFN-γ (Human IFN-γ ELISA MAX; BioLegend Cat#430104). Alternatively, human CD4⁺ T cells (2 x 10⁵ cells) were subjected to mixed lymphocytes culture with allogenic human CD19⁺ B cells (1 x 10⁵ cells; Precision for Medicine Cat#84400-1.0) for 7 days. Activated CD4⁺ T cells (5 x 10⁴ cells) were restimulated with the same allogenic B cells (2 x 10⁵ cells) in the presence of HM266 chimeric antibody. IFN-γ levels in the culture supernatants were determined after 18 h.

Acute graft-versus-host disease

hPD-1 KI mice (C57BL/6-background; H-2^{b+} H-2^{d-}) and BDF1 mice (H-2^{b+} H-2^{d+}) were used as donor and recipient, respectively. Female BDF1 mice received i.p. injection of cyclophosphamide (100 mg/kg; Wako Pure Chemical Industries Cat#030-12953) prior to cell transfer. After 24 h, spleen cells from female hPD-1-KI mice (5 x 10⁷ cells/mouse) and were injected intravenously into BDF1 mice. Recipient mice received i.p. injection of HM266 (0.5 mg/mouse) or control mouse IgG1 (MOPC-21) twice a week starting on the day of cell transfer. The intensity of graft-versus-host response was monitored by the increase of donor cells in recipient PBMC.

Colitis induction

Sorted CD25⁻ CD4⁺ T cells from the spleens of hPD-1 KI mice (5×10^5 cells) were injected intravenously into sex-matched RAG2^{-/-} mice. The mice received i.p. injection of HM266 (0.5 mg/mouse) or control mouse IgG1 (MOPC-21) immediately after the cell transfer and twice a week thereafter. Development of colitis was monitored by measuring body weight. Mesenteric lymph nodes and colon tissues were recovered after 8 weeks, and cells in the lymph nodes and lamina propria were subjected to flowcytometric analysis. Lamina propria mononuclear cells were prepared as described previously (50). The dissected colon tissues were incubated with HBSS containing 10% FBS, 15 mM HEPES, 5 mM EDTA and 1 mM dithiothreitol for 15 min at 37 °C. After wash, the tissue samples were digested with IMDM containing 10% FBS, 5% NCTC-109 (Gibco Cat#21340039), 0.5 mM 2-mercaptoethanol, 0.17 mg/ml Liberase TL (Roche Diagnostics Cat#05401020001) and 30 µg/ml DNase I for 60 min at 37 °C. Mononuclear cells were obtained from the interface of 30% and 70% Percoll after discontinuous density centrifugation.

For intracellular staining, cells were stimulated with plate-bound anti-CD3 mAb for 6 hours and treated with BD GolgiPlug (BD Biosciences Cat#555029) for the final 2 hours of culture. The stimulated cells were stained with PerCP/Cy5.5-anti-mouse CD4 and BV711-anti-human PD-1 mAbs, followed by fixation in 4% paraformaldehyde-PBS for 15 min. After wash, cells were incubated with a permeabilizing buffer (50 mM sodium chloride, 5 mM EDTA, 0.5% Triton X-100, 10 mM Tris-HCl) for 15 min, washed, and then stained with FITC-anti-mouse IFN-γ mAb and APC-anti-mouse IL-17 mAb.

Immunization with NP-OVA

hPD-1-KI mice were immunized i.p. with NP-OVA (10 µg/mouse; Biosearch Technologies Cat#N-5051) in alum. The mice received i.p. injection of HM266, HM255 (500 µg/mouse) or control mouse IgG1 (MOPC-21) on day 0, 3, 7 and 10. Blood samples were collected on day 7 and 14 for the determination of anti-NP IgM levels by ELISA. The plasma samples were added to 96-well plastic plate precoated with NP-BSA (3 µg/ml; Biosearch Technologies Cat#N-5050XL). After wash, IgM binding was detected by biotin-anti-mouse IgM mAb (0.6 µg/ml) followed by HRP-streptavidin (1:200 dilution; R&D Systems Cat#DY998).

Statistical analysis

Data represent mean $\pm$ SEM. Statistical significance was calculated by two-tailed Student's t-test for the comparison between two groups and Tukey-Kramer test for the multiple comparison of more than two groups. Comparison of natural logarithm of IgM numbers was calculated by Holm-Bonferroni method. P values less than 0.05 was considered significant in the present experiments. Data shown in figures are representative of 2 or more experiments, which essentially demonstrated similar results.

List of Supplementary Materials

Figure S1. Conditions for PD-1 agonists to stimulate PD-1.

Figure S2. The functional assay of PD-1-dependent inhibition in the antigen-specific T cell activation.

Figure S3. Guided antibody binding to the stalk region resulted in the immunosuppressive activity of mouse PD-1.

Figure S4. Correlation of binding affinity of anti-PD-1 agonist mAbs with their immunosuppressive activity.

Figure S5. Induction of PD-1-dependent immunosuppression by anti-PD-1 agonist antibody is additive to PD-1 ligands.

Figure S6. The agonistic activity of HM266 increases dependent on the levels of PD-1 expression in T cells and Fc receptor expression in antigen-presenting cells.

Figure S7. HM266 induces PD-1-mediated immunosuppression based on Fc receptor binding.

Figure S8. Generation of human PD-1-knock-in mice.

Figure S9. The inhibition of antibody production by HM266 is mediated by its PD-1 agonist activity but not cell-depleting activity.

Table S1. List of antibodies and recombinant proteins.

References

1. S. Grebinoski, D. A. Vignali, Inhibitory receptor agonists: the future of autoimmune disease therapeutics? *Curr Opin Immunol* **67**, 1-9 (2020).
2. T. Okazaki, S. Chikuma, Y. Iwai, S. Fagarasan, T. Honjo, A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat Immunol* **14**, 1212-1218 (2013).
3. F. A. Schildberg, S. R. Klein, G. J. Freeman, A. H. Sharpe, Coinhibitory Pathways in the B7-CD28 Ligand-Receptor Family. *Immunity* **44**, 955-972 (2016).
4. J. M. Chemnitz, R. V. Parry, K. E. Nichols, C. H. June, J. L. Riley, SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* **173**, 945-954 (2004).
5. T. Yokosuka, M. Takamatsu, W. Kobayashi-Imanishi, A. Hashimoto-Tane, M. Azuma, T. Saito, Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* **209**, 1201-1217 (2012).
6. N. Patsoukis, J. S. Duke-Cohan, A. Chaudhri, H. I. Aksoylar, Q. Wang, A. Council, A. Berg, G. J. Freeman, V. A. Boussiotis, Interaction of SHP-2 SH2 domains with PD-1 ITSM induces PD-1 dimerization and SHP-2 activation. *Commun Biol* **3**, 128 (2020).
7. E. Ozkaynak, L. Wang, A. Goodearl, K. McDonald, S. Qin, T. O'Keefe, T. Duong, T. Smith, J. C. Gutierrez-Ramos, J. B. Rottman, A. J. Coyle, W. W. Hancock, Programmed death-1 targeting can promote allograft survival. *J Immunol* **169**, 6546-6553 (2002).
8. M. Y. Song, C. P. Hong, S. J. Park, J. H. Kim, B. G. Yang, Y. Park, S. W. Kim, K. S. Kim, J. Y. Lee, S. W. Lee, M. H. Jang, Y. C. Sung, Protective effects of Fc-fused PD-L1 on two different animal models of colitis. *Gut* **64**, 260-271 (2015).
9. C. M. Bryan, G. J. Rocklin, M. J. Bick, A. Ford, S. Majri-Morrison, A. V. Kroll, C. J. Miller, L. Carter, I. Goreshnik, A. Kang, F. DiMaio, K. V. Tarbell, D. Baker, Computational design of a synthetic PD-1 agonist. *Proc Natl Acad Sci U S A* **118**, (2021).
10. D. Sugiura, I. M. Okazaki, T. K. Maeda, T. Maruhashi, K. Shimizu, R. Arakaki, T. Takemoto, N. Ishimaru, T. Okazaki, PD-1 agonism by anti-CD80 inhibits T cell activation and alleviates autoimmunity. *Nat Immunol* **23**, 399-410 (2022).
11. A. P. Curnock, G. Bossi, J. Kumaran, L. J. Bawden, R. Figueiredo, R. Tawar, K. Wiseman, E. Henderson, S. J. Hoong, V. Gonzalez, H. Ghadbane, D. E. Knight, R. O'Dwyer, D. X. Overton, C. M. Lucato, N. M. Smith, C. R. Reis, K. Page, L. M. Whaley, M. L. McCully, S. Hearty, T. M. Mahon, P. Weber, Cell-targeted PD-1 agonists that mimic PD-L1 are potent T cell inhibitors. *JCI Insight* **6**, (2021).
12. D. Y. Lin, Y. Tanaka, M. Iwasaki, A. G. Gittis, H. P. Su, B. Mikami, T. Okazaki, T. Honjo, N. Minato, D. N. Garboczi, The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. *Proc Natl Acad Sci U S A* **105**, 3011-3016 (2008).
13. X. Cheng, V. Veverka, A. Radhakrishnan, L. C. Waters, F. W. Muskett, S. H. Morgan, J. Huo, C. Yu, E. J. Evans, A. J. Leslie, M. Griffiths, C. Stubberfield, R. Griffin, A. J. Henry, A.

- Jansson, J. E. Ladbury, S. Ikemizu, M. D. Carr, S. J. Davis, Structure and interactions of the human programmed cell death 1 receptor. *J Biol Chem* **288**, 11771-11785 (2013).
14. R. Magnez, B. Thiroux, S. Taront, Z. Segaula, B. Quesnel, X. Thuru, PD-1/PD-L1 binding studies using microscale thermophoresis. *Sci Rep* **7**, 17623 (2017).
 15. K. M. Zak, P. Grudnik, K. Magiera, A. Domling, G. Dubin, T. A. Holak, Structural Biology of the Immune Checkpoint Receptor PD-1 and Its Ligands PD-L1/PD-L2. *Structure* **25**, 1163-1174 (2017).
 16. M. E. Keir, Y. E. Latchman, G. J. Freeman, A. H. Sharpe, Programmed death-1 (PD-1):PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes. *J Immunol* **175**, 7372-7379 (2005).
 17. Y. Seko, H. Yagita, K. Okumura, M. Azuma, R. Nagai, Roles of programmed death-1 (PD-1)/PD-1 ligands pathway in the development of murine acute myocarditis caused by coxsackievirus B3. *Cardiovasc Res* **75**, 158-167 (2007).
 18. D. G. Helou, P. Shafiei-Jahani, R. Lo, E. Howard, B. P. Hurrell, L. Galle-Treger, J. D. Painter, G. Lewis, P. Soroosh, A. H. Sharpe, O. Akbari, PD-1 pathway regulates ILC2 metabolism and PD-1 agonist treatment ameliorates airway hyperreactivity. *Nat Commun* **11**, 3998 (2020).
 19. S. Kasagi, S. Kawano, T. Okazaki, T. Honjo, A. Morinobu, S. Hatachi, K. Shimatani, Y. Tanaka, N. Minato, S. Kumagai, Anti-programmed cell death 1 antibody reduces CD4+PD-1+ T cells and relieves the lupus-like nephritis of NZB/W F1 mice. *J Immunol* **184**, 2337-2347 (2010).
 20. C. Paluch, A. M. Santos, C. Anzilotti, R. J. Cornall, S. J. Davis, Immune Checkpoints as Therapeutic Targets in Autoimmunity. *Front Immunol* **9**, 2306 (2018).
 21. G. J. Freeman, A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser, L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood, T. Honjo, Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* **192**, 1027-1034 (2000).
 22. S. Horita, Y. Nomura, Y. Sato, T. Shimamura, S. Iwata, N. Nomura, High-resolution crystal structure of the therapeutic antibody pembrolizumab bound to the human PD-1. *Sci Rep* **6**, 35297 (2016).
 23. S. Tan, H. Zhang, Y. Chai, H. Song, Z. Tong, Q. Wang, J. Qi, G. Wong, X. Zhu, W. J. Liu, S. Gao, Z. Wang, Y. Shi, F. Yang, G. F. Gao, J. Yan, An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. *Nat Commun* **8**, 14369 (2017).
 24. D. Sugiura, T. Maruhashi, I. M. Okazaki, K. Shimizu, T. K. Maeda, T. Takemoto, T. Okazaki, Restriction of PD-1 function by cis-PD-L1/CD80 interactions is required for optimal T cell responses. *Science* **364**, 558-566 (2019).
 25. N. S. Wilson, B. Yang, A. Yang, S. Loeser, S. Marsters, D. Lawrence, Y. Li, R. Pitti, K. Totpal, S. Yee, S. Ross, J. M. Vernes, Y. Lu, C. Adams, R. Offringa, B. Kelley, S. Hymowitz, D. Daniel, G. Meng, A. Ashkenazi, An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell* **19**, 101-113 (2011).
 26. A. L. White, H. T. Chan, A. Roghanian, R. R. French, C. I. Mockridge, A. L. Tutt, S. V. Dixon, D. Ajona, J. S. Verbeek, A. Al-Shamkhani, M. S. Cragg, S. A. Beers, M. J. Glennie,

- Interaction with FcγRIIB is critical for the agonistic activity of anti-CD40 monoclonal antibody. *J Immunol* **187**, 1754-1763 (2011).
27. F. Li, J. V. Ravetch, Inhibitory Fcγ receptor engagement drives adjuvant and anti-tumor activities of agonistic CD40 antibodies. *Science* **333**, 1030-1034 (2011).
 28. B. Jones, J. P. Tite, C. A. Janeway, Jr., Different phenotypic variants of the mouse B cell tumor A20/2J are selected by antigen- and mitogen-triggered cytotoxicity of L3T4-positive, I-A-restricted T cell clones. *J Immunol* **136**, 348-356 (1986).
 29. T. Okazaki, A. Maeda, H. Nishimura, T. Kurosaki, T. Honjo, PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci U S A* **98**, 13866-13871 (2001).
 30. R. Dahan, B. C. Barnhart, F. Li, A. P. Yamniuk, A. J. Korman, J. V. Ravetch, Therapeutic Activity of Agonistic, Human Anti-CD40 Monoclonal Antibodies Requires Selective FcγRI Engagement. *Cancer Cell* **29**, 820-831 (2016).
 31. K. Choudhuri, D. Wiseman, M. H. Brown, K. Gould, P. A. van der Merwe, T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature* **436**, 578-582 (2005).
 32. J. R. James, R. D. Vale, Biophysical mechanism of T-cell receptor triggering in a reconstituted system. *Nature* **487**, 64-69 (2012).
 33. Y. Xu, A. J. Szalai, T. Zhou, K. R. Zinn, T. R. Chaudhuri, X. Li, W. J. Koopman, R. P. Kimberly, FcγRs modulate cytotoxicity of anti-Fas antibodies: implications for agonistic antibody-based therapeutics. *J Immunol* **171**, 562-568 (2003).
 34. F. Li, J. V. Ravetch, Apoptotic and antitumor activity of death receptor antibodies require inhibitory Fcγ receptor engagement. *Proc Natl Acad Sci U S A* **109**, 10966-10971 (2012).
 35. D. Zhang, M. V. Goldberg, M. L. Chiu, Fc Engineering Approaches to Enhance the Agonism and Effector Functions of an Anti-CD40 Antibody. *J Biol Chem* **291**, 27134-27146 (2016).
 36. X. Wang, M. Mathieu, R. J. Brezski, IgG Fc engineering to modulate antibody effector functions. *Protein Cell* **9**, 63-73 (2018).
 37. A. M. Chenoweth, B. D. Wines, J. C. Anania, P. Mark Hogarth, Harnessing the immune system via FcγRI function in immune therapy: a pathway to next-gen mAbs. *Immunol Cell Biol* **98**, 287-304 (2020).
 38. X. Yu, H. T. C. Chan, C. M. Orr, O. Dadas, S. G. Booth, L. N. Dahal, C. A. Penfold, L. O'Brien, C. I. Mockridge, R. R. French, P. Duriez, L. R. Douglas, A. R. Pearson, M. S. Cragg, I. Tews, M. J. Glennie, A. L. White, Complex Interplay between Epitope Specificity and Isotype Dictates the Biological Activity of Anti-human CD40 Antibodies. *Cancer Cell* **33**, 664-675 e664 (2018).
 39. S. M. Chin, C. R. Kimberlin, Z. Roe-Zurz, P. Zhang, A. Xu, S. Liao-Chan, D. Sen, A. R. Nager, N. S. Oakdale, C. Brown, F. Wang, Y. Yang, K. Lindquist, Y. A. Yeung, S. Salek-Ardakani, J. Chaparro-Riggers, Structure of the 4-1BB/4-1BBL complex and distinct binding and functional properties of utomilumab and urelumab. *Nat Commun* **9**, 4679 (2018).
 40. P. Zhang, G. H. Tu, J. Wei, P. Santiago, L. R. Larrabee, S. Liao-Chan, T. Mistry, M. L. Chu, T. Sai, K. Lindquist, H. Long, J. Chaparro-Riggers, S. Salek-Ardakani, Y. A. Yeung, Ligand-

- Blocking and Membrane-Proximal Domain Targeting Anti-OX40 Antibodies Mediate Potent T Cell-Stimulatory and Anti-Tumor Activity. *Cell Rep* **27**, 3117-3123 e3115 (2019).
41. G. V. Zuccarino-Catania, S. Sadanand, F. J. Weisel, M. M. Tomayko, H. Meng, S. H. Kleinstein, K. L. Good-Jacobson, M. J. Shlomchik, CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. *Nat Immunol* **15**, 631-637 (2014).
 42. K. L. Good-Jacobson, C. G. Szumilas, L. Chen, A. H. Sharpe, M. M. Tomayko, M. J. Shlomchik, PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* **11**, 535-542 (2010).
 43. R. S. Herati, D. A. Knorr, L. A. Vella, L. V. Silva, L. Chilukuri, S. A. Apostolidis, A. C. Huang, A. Muselman, S. Manne, O. Kuthuru, R. P. Staupé, S. A. Adamski, S. Kannan, R. K. Kurupati, H. C. J. Ertl, J. L. Wong, S. Bournazos, S. McGettigan, L. M. Schuchter, R. R. Kotecha, S. A. Funt, M. H. Voss, R. J. Motzer, C. H. Lee, D. F. Bajorin, T. C. Mitchell, J. V. Ravetch, E. J. Wherry, PD-1 directed immunotherapy alters Tfh and humoral immune responses to seasonal influenza vaccine. *Nat Immunol* **23**, 1183-1192 (2022).
 44. T. Yamazaki, H. Akiba, H. Iwai, H. Matsuda, M. Aoki, Y. Tanno, T. Shin, H. Tsuchiya, D. M. Pardoll, K. Okumura, M. Azuma, H. Yagita, Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* **169**, 5538-5545 (2002).
 45. A. Young, Z. Quandt, J. A. Bluestone, The Balancing Act between Cancer Immunity and Autoimmunity in Response to Immunotherapy. *Cancer Immunol Res* **6**, 1445-1452 (2018).
 46. R. H. Vonderheide, M. J. Glennie, Agonistic CD40 antibodies and cancer therapy. *Clin Cancer Res* **19**, 1035-1043 (2013).
 47. T. Castro-Dopico, M. R. Clatworthy, IgG and Fcγ Receptors in Intestinal Immunity and Inflammation. *Front Immunol* **10**, 805 (2019).
 48. T. Abe, K. I. Inoue, Y. Furuta, H. Kiyonari, Pronuclear Microinjection during S-Phase Increases the Efficiency of CRISPR-Cas9-Assisted Knockin of Large DNA Donors in Mouse Zygotes. *Cell Rep* **31**, 107653 (2020).
 49. P. D. Hsu, D. A. Scott, J. A. Weinstein, F. A. Ran, S. Konermann, V. Agarwala, Y. Li, E. J. Fine, X. Wu, O. Shalem, T. J. Cradick, L. A. Marraffini, G. Bao, F. Zhang, DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**, 827-832 (2013).
 50. B. A. David, S. Rubino, T. G. Moreira, M. A. Freitas-Lopes, A. M. Araujo, N. E. Paul, R. M. Rezende, G. B. Menezes, Isolation and high-dimensional phenotyping of gastrointestinal immune cells. *Immunology* **151**, 56-70 (2017).

Acknowledgements

The authors thank to Dr. Naoko Ikuta for advising stats calculations and to Miwa Okada, Kumiko Yonezaki and Yukako Kamita for their technical support and maintenance of mice colonies. We developed the screening system for PD-1 agonist antibodies based on the

DO11.10/IIA1.6 system that was provided by Dr. Taku Okazaki (University of Tokyo). We are grateful to Takayoshi Fukushima (Meiji Seika Pharma) for preparing antibody variants. We thank Drs. Kenji Chamoto, Norimichi Nomura (Kyoto University) and Takashi Masuko (Kindai University) for useful discussions.

Funding

This work was supported by Meiji Seika Pharma Co. Ltd. and the Foundation for Biomedical Research and Innovation at Kobe.

Author Contributions

Conceptualization: KS, MT, TH, AO

Methodology: KS, MT, AO

Investigation: KS, MT, YT, YO

Resources (anti-human PD-1 mAb panel): SN, HK

Resources (human PD-1-knock-in mice): MK, KN

Writing – Original Draft: AO

Writing – Review and Editing: KS, MT, SN, TH, AO

Supervision: TH, AO

Funding Acquisition: TH

Competing Interests

K.S., M.T., Y.T., Y.O., S.N., H.K., T.H. and A.O. are inventors on patent applications (WO2021/241523A1, WO2022/239820) submitted by Foundation for Biomedical Research and Innovation at Kobe, National Institutes of Biomedical Innovation, Health and Nutrition, and Meiji Seika Pharma Co. Ltd. that cover anti-human PD-1 agonist antibodies and their therapeutic

application. K.S., Y.T. and Y.O. are employees of Meiji Seika Pharma Co. Ltd. The remaining authors declare no competing interests.

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. The human PD-1 knock-in mouse strain and the anti-human PD-1 antibodies used in this study are available upon request from the corresponding author through a material transfer agreement with the Foundation for Biomedical Research and Innovation at Kobe.

Figure legends

Figure 1. PD-1 signaling was inducible by guided antibody binding to the stalk region of PD-1 molecule. (A) Introduction of FLAG tag to human PD-1: replacement of a part of the stalk region (hPD-1 stalk-FLAG) or FLAG addition to the N-terminus (FLAG-hPD-1). (B) FLAG tag detection by anti-FLAG mAb L5 in DO11.10 cells transduced with wild-type human PD-1 (WT hPD-1) or FLAG-tagged human PD-1. (C) Immunosuppressive potential of FLAG-tagged PD-1. PD-1-transduced DO11.10 cells were stimulated with OVA₃₂₃₋₃₃₉ in the presence of IIA1.6 cells either devoid of or expressing PD-L1 (see also Figure S2). (D) The addition of L5 in the soluble form (5 µg/ml) to PD-1-transduced DO11.10 cells. (E) Stimulation of PD-1-transduced DO11.10 cells by anti-CD3 mAb (0.5 µg/ml), anti-CD28 mAb (0.5 µg/ml) and L5 (3 µg/ml). (F) PD-L1-Fc (10 µg/ml) binding to hPD-1 stalk-FLAG. Antibody concentration was 5 µg/ml. EH12.2H7 is anti-hPD-1 blocking mAb. Data represent average ± SE of triplicate (C, E) or duplicate (D) samples. a, $p < 0.05$ vs controls (two-tailed Student's t-test).

Figure 2. The utilization of Fc receptor engagement enabled PD-1-mediated immunosuppression by the simple addition of agonist antibody. (A) The experimental system. DO11.10 cells expressing human PD-1 were stimulated with OVA₃₂₃₋₃₃₉ peptide using PD-L1-deficient A20 B lymphoma as antigen-presenting cells. (B) IL-2 production from PD-1-transduced DO11.10 cells in the presence of anti-FLAG mAb L5 (5 µg/ml). (C, D) The addition of excess FLAG peptide (3 µg/ml) (C) or anti-CD16/32 mAb (10 µg/ml) (D). (E, F) PD-1 stimulation with PD-L1-expressing antigen-presenting cells (E) or L5 (5 µg/ml) using PD-L1-deficient A20 cells (F). Y248F mutant lacks PD-1 signal transmission. Data represent average ± SE of duplicate samples.

Figure 3. Binding epitopes of anti-PD-1 agonist mAbs are specifically located in MPER, which is distinct from the region recognized by blocking mAbs. (A) The screening system for blocking and agonistic activities of anti-human PD-1 mAbs. (B) Eight segments that were used to classify anti-human PD-1 mAbs by their binding capabilities. TM: transmembrane segment. (C) The putative locations of the eight segments on the molecular surface of human PD-1. The 3D model depicts the extracellular part of human PD-1. The 3D model was created using Jmol software based on the structure of human PD-1 from PDB (accession code 3RRQ). (D) Activities of anti-human PD-1 agonist mAbs (n = 2). (E) Blocking (red) and agonistic (blue) activities of individual anti-human PD-1 mAb clones (n = 2). Numbers in the center column represent the eight segments in (B). For example, #1 group denotes mAbs that lost binding only when #1 segment was mutated. Similarly, #(1), 6, 7 indicates that the mutation in #6 and #7 segments critically impaired the binding and the #1 segment affected the binding to different degrees dependent on mAb clones.

Figure 4. Anti-PD-1 agonist mAb exerts its immunosuppressive activity by triggering PD-1 signaling. (A) Western blotting of phosphorylated human PD-1. DO11.10 cells transduced with human PD-1-GFP and SHP-2(C459S) were stimulated with OVA₃₂₃₋₃₃₉-pulsed A20 cells for 5 min. PD-1 was stimulated with PD-L1-expressing A20 cells or HM266-mIgG1. (B, C) ERK phosphorylation in stimulated DO11.10 cells for indicated time. Antibody concentration was 5 µg/ml. Data represent average ± SE (n = 3). c, p < 0.001 (two-tailed Student's t-test).

Figure 5. Anti-PD-1 agonist mAb inhibits primary human T cells. (A) Human primary CD4⁺ T cells were stimulated with CytoStim in the presence of human FcγRIIB-expressing THP-1 cells. (B) T cells were stimulated using parental (FcγRIIB^{low}) or FcγRIIB-overexpressing THP-1 cells (FcγRIIB^{high}) in the presence of either HM266-hIgG1(K322A) or HM266-hIgG4(S228P). Data represent average ± SE (controls without antibody, n = 8; with antibodies, n = 3). a, p < 0.05; b, p < 0.01; vs control Ab; Tukey-Kramer test. (C) Binding of Fc variants of HM266-hIgG1 to FcγRIIB^{high} THP-1 cells. (D, E) The addition of HM266-hIgG1(K322A) and its Fc variants to human CD4⁺ T cells stimulated with Cytostim (D) or mixed lymphocyte reaction (E). Data represent average ± SE (controls without antibody, n = 8; with antibodies, n = 3). a, p < 0.05; b, p < 0.01; vs HM266-hIgG1; Tukey-Kramer test.

Figure 6. Anti-PD-1 agonist mAb suppressed GVHD in vivo. (A) PD-1 expression in stimulated T cells from human PD-1-knock-in (hPD-1-KI) mice. (B) Acute GVHD induction in BDF1 mice. (C) hPD-1 expression in donor T cells in PBMC on day 8. (D) Body weight change. (E, F) Proportions of donor-derived (H-2K^b H-2K^d) cells in PBMC. Data represent average ± SE (ctrl Ab, n = 10; HM266-mIgG1, n = 5). b, p < 0.01, c, p < 0.001; vs control Ab; two-tailed Student's t-test.

Figure 7. Anti-PD-1 agonist mAb suppressed the induction of colitis. (A) Colitis induction by the transfer of CD4⁺ CD25⁻ T cells from hPD-1-KI mice. HM266-mIgG1 (0.5 mg/mouse) was given twice a week. (B) Body weight change after cell transfer. (C) Macroscopic picture of the colon and cecum on day 58. (D) CD4⁺ T cell numbers in the lamina propria. (E-G) CD4⁺ T cells producing IFN-γ (E), IL-17 (F) or both (G) in the lamina propria and mesenteric lymph nodes on

day 58. Data represent average $\pm$ SE of 7 mice. a, $p < 0.05$, b, $p < 0.01$, c, $p < 0.001$; vs control Ab; two-tailed Student's t-test.

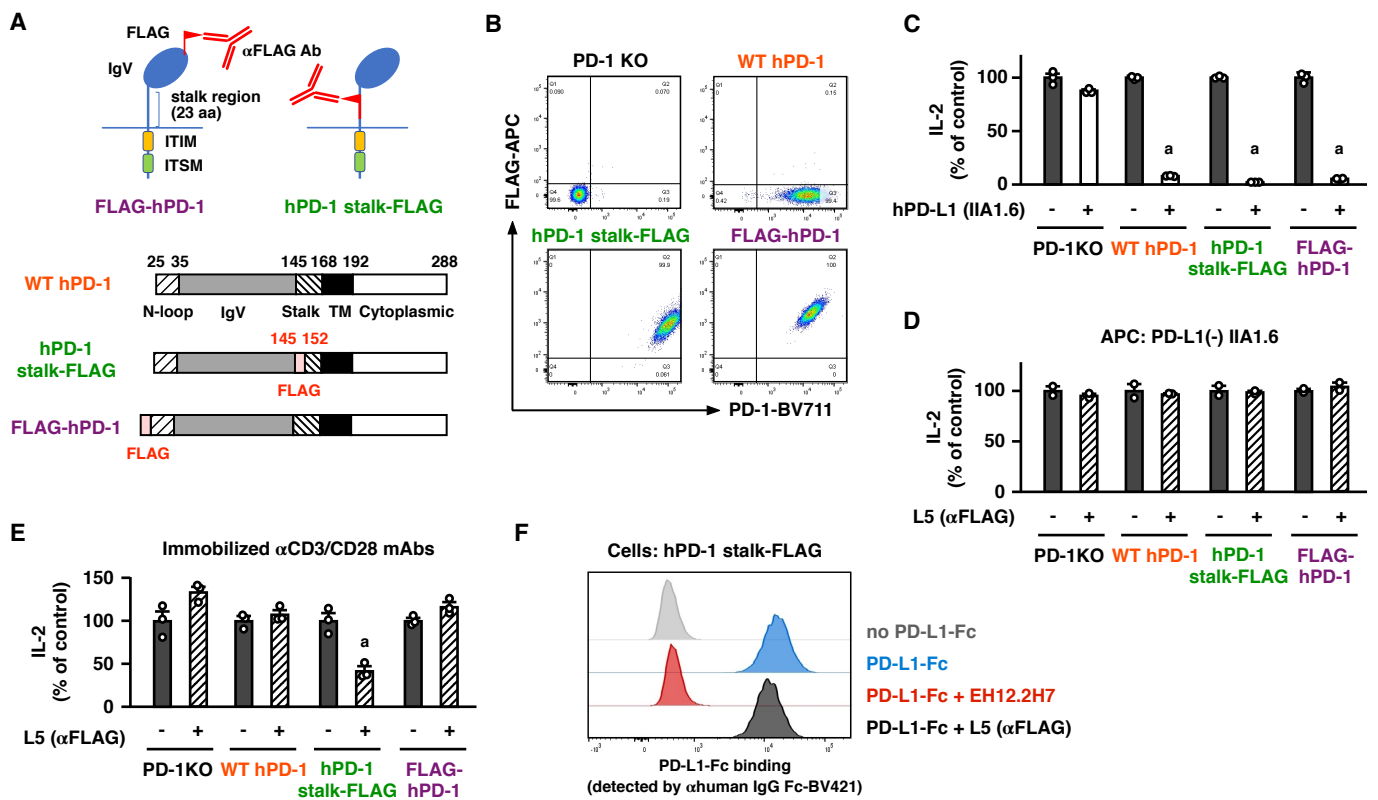


Figure 1

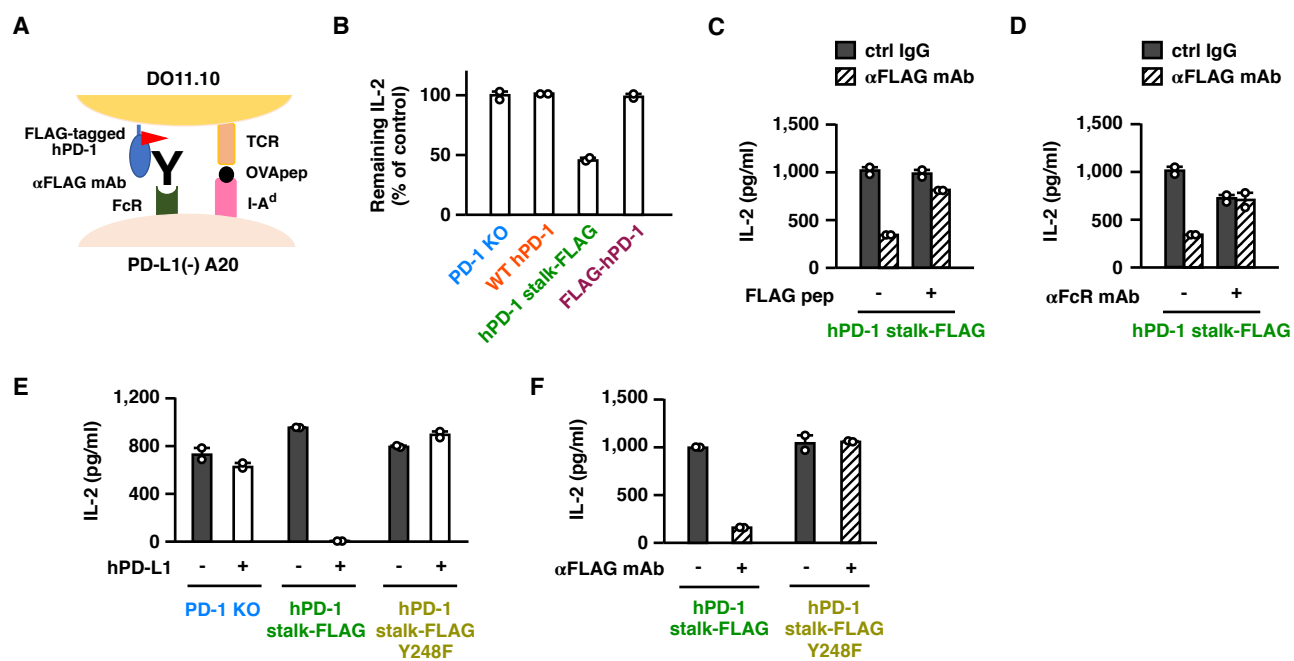


Figure 2

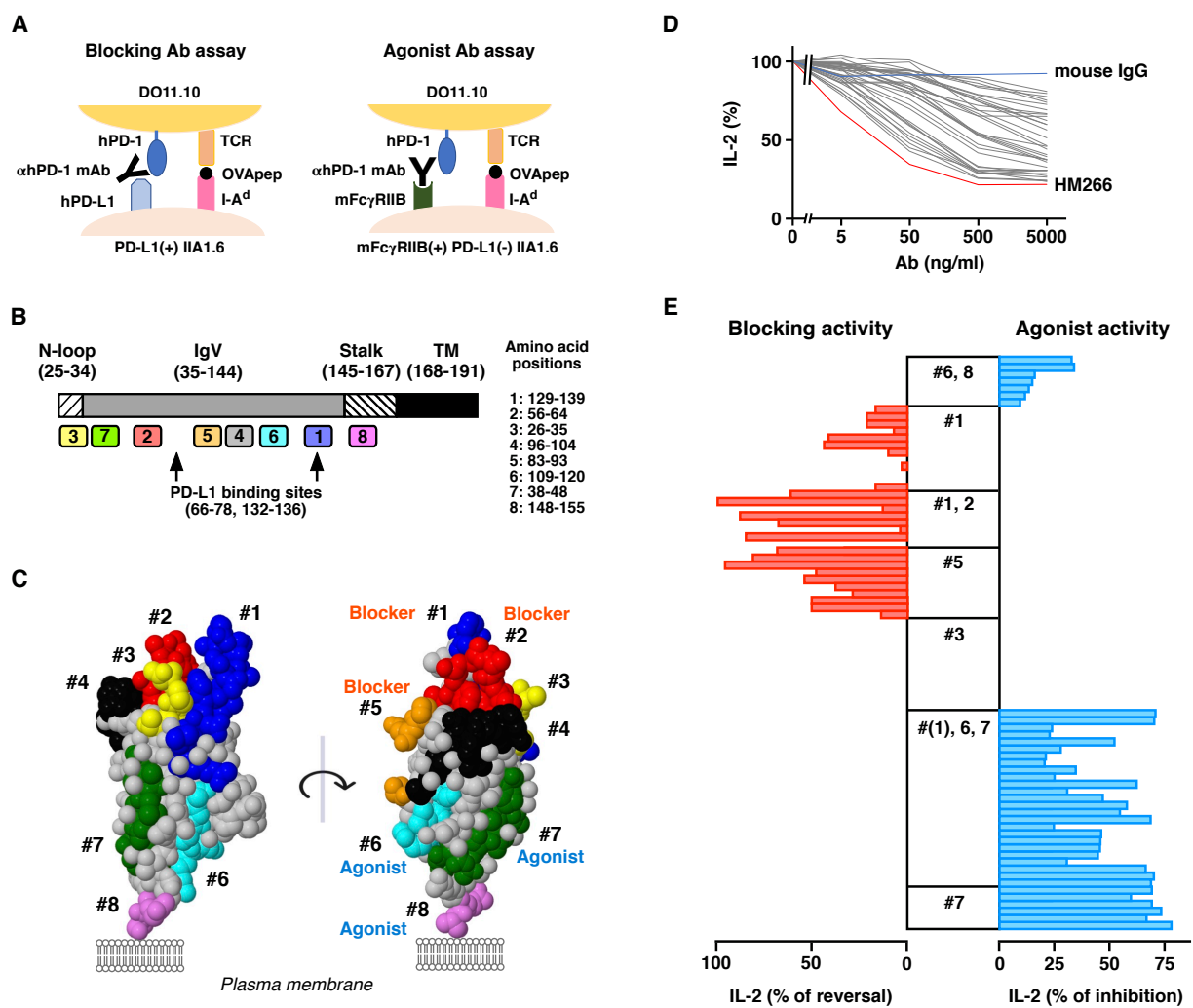


Figure 3

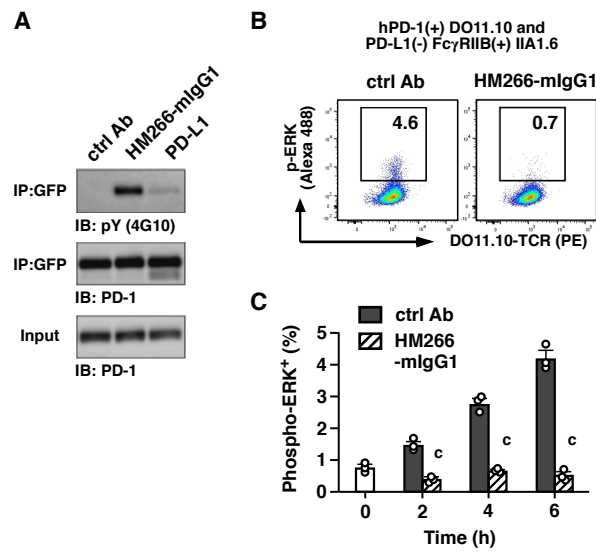


Figure 4

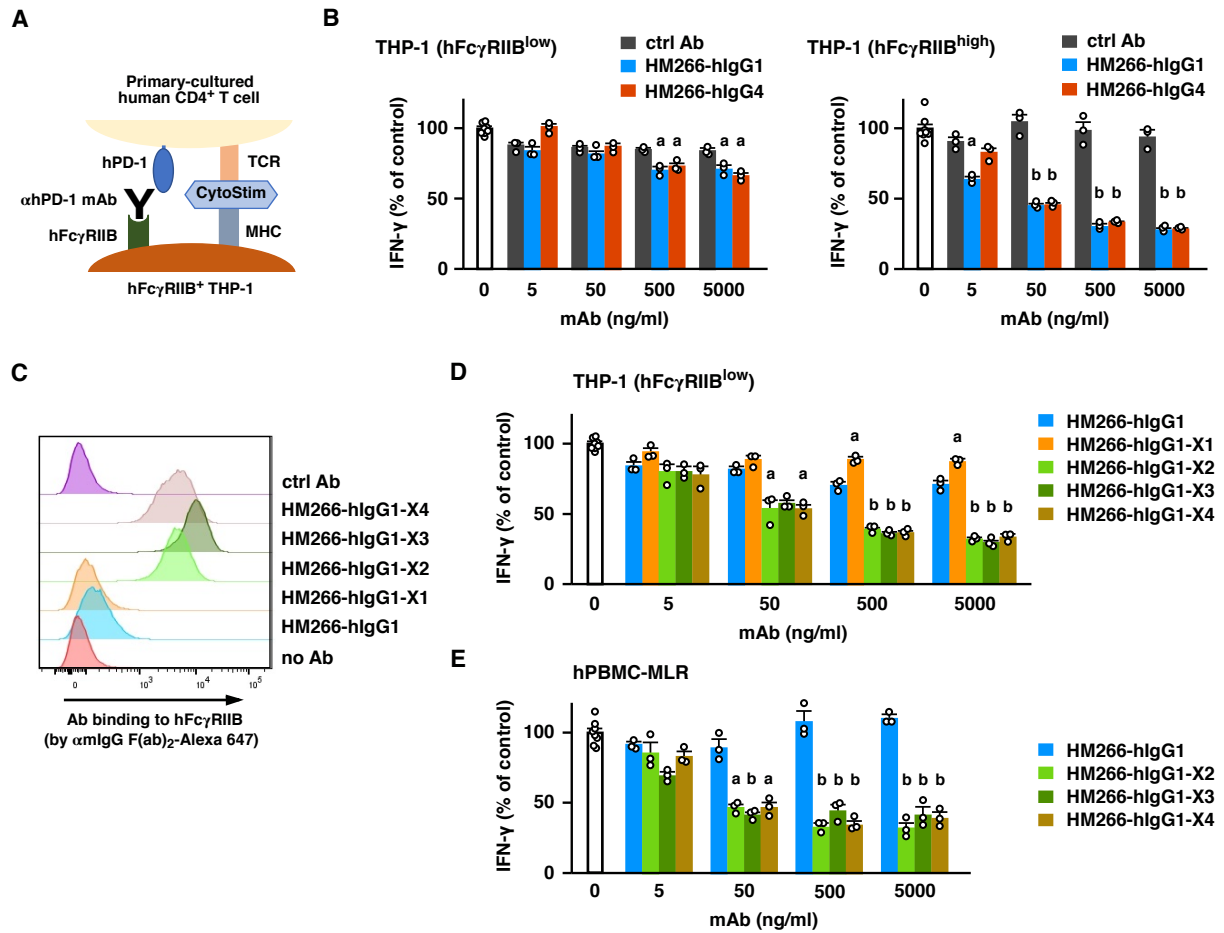


Figure 5

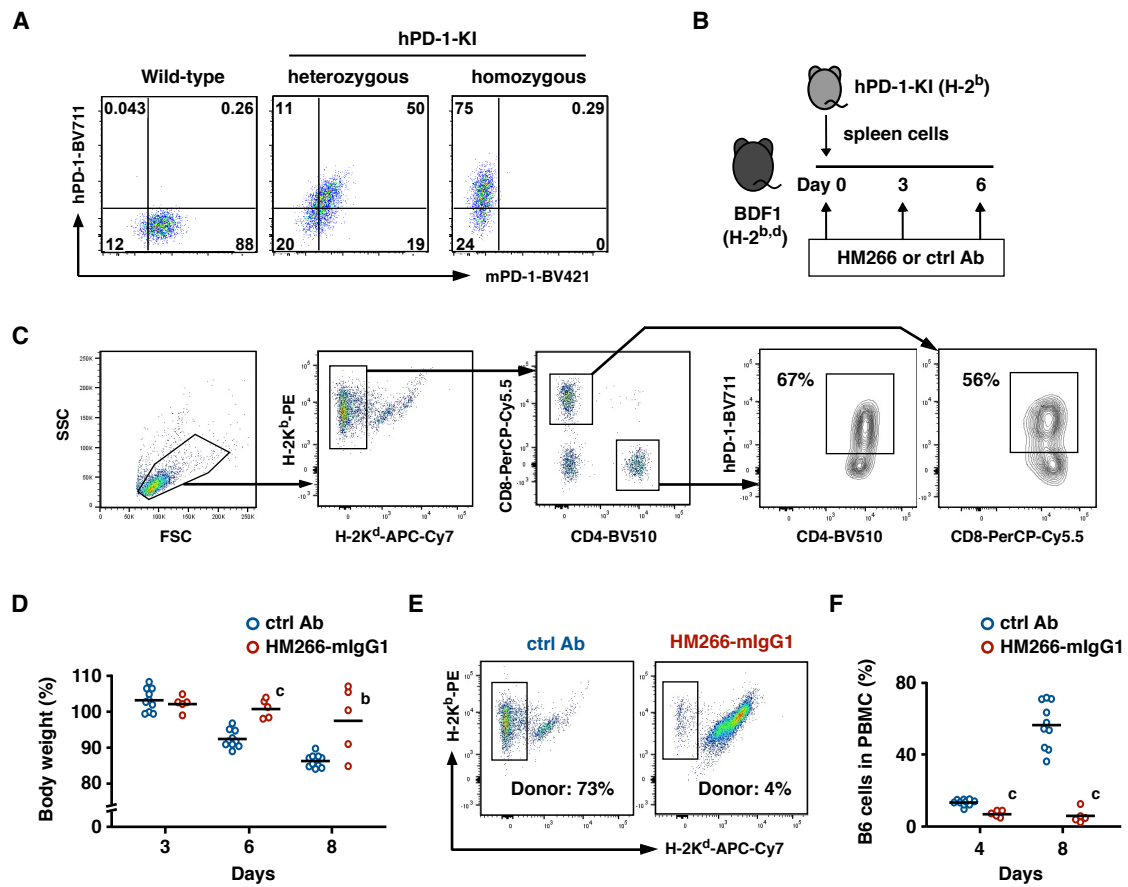


Figure 6

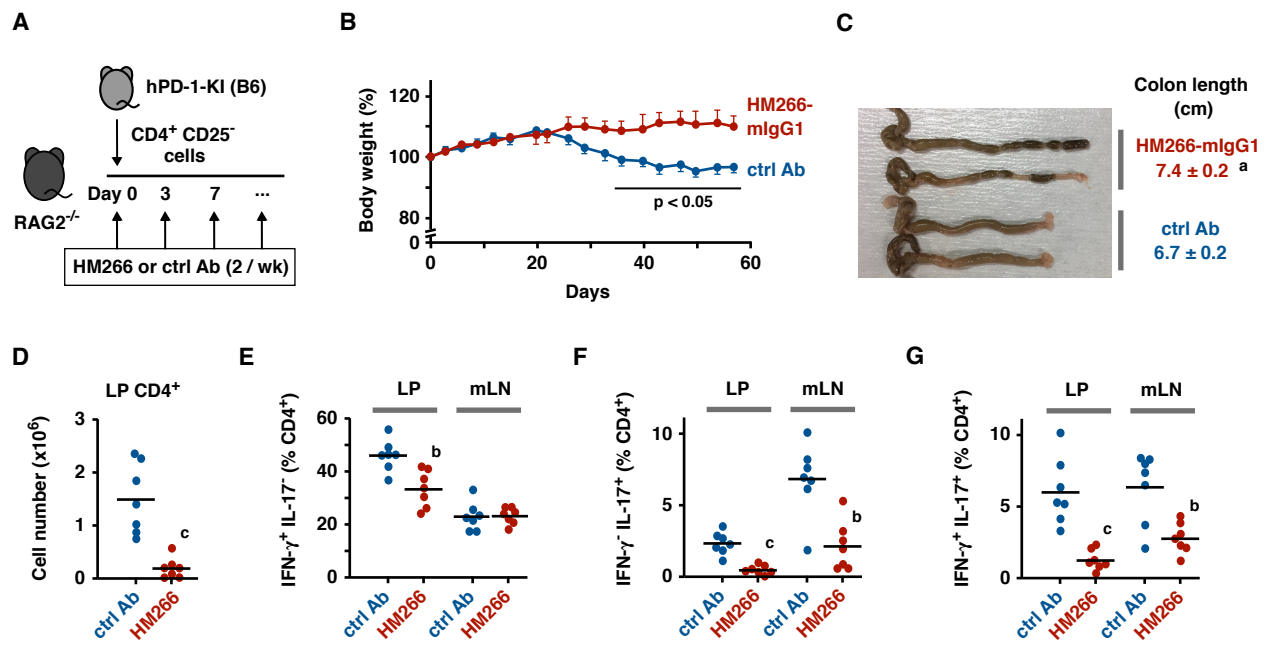


Figure 7

SUPPLEMENTARY MATERIALS

Supplementary Figures

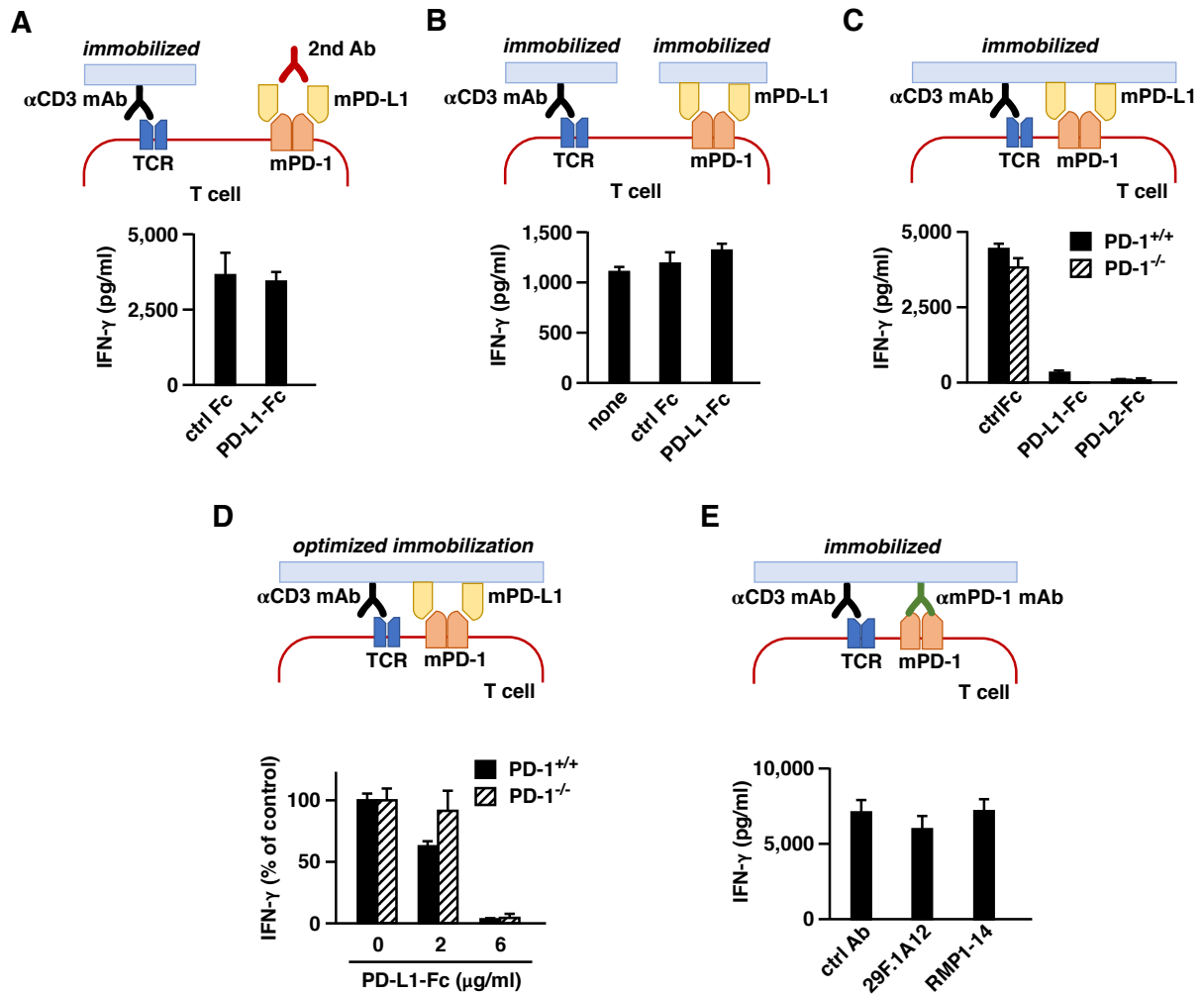


Figure S1. Conditions for PD-1 agonists to stimulate PD-1. To determine the condition where pharmacological stimulation of PD-1 would be possible, mouse PD-L1-Fc was added in different forms to mouse T cell culture. (A) Soluble PD-L1-Fc was crosslinked by anti-human IgG Ab. T cell were stimulated with immobilized anti-mouse CD3 mAb (3 μg/ml) on the plastic surface.

(B) PD-L1-Fc (3 $\mu\text{g/ml}$) was immobilized on a plastic surface. T cells were stimulated with Dynabeads mouse T-activator CD3/CD28 (Gibco Cat#11452D). (C) Anti-CD3 mAb (3 $\mu\text{g/ml}$) was co-immobilized with murine PD-L1-Fc or PD-L2-Fc (6 $\mu\text{g/ml}$) onto the plastic surface. (D) The optimized co-immobilizing condition for PD-1-dependent delivery of immunosuppressive signal. Biotinylated anti-CD3 and anti-CD28 mAbs (1 $\mu\text{g/ml}$) and biotinylated murine PD-L1-Fc (2 or 6 $\mu\text{g/ml}$) were bound to the streptavidin-immobilized plastic surface. (E) Co-immobilization of anti-CD3 mAb (3 $\mu\text{g/ml}$) and anti-PD-1 mAb (29F.1A12 or RMP1-14; 12 $\mu\text{g/ml}$) to the plastic surface.

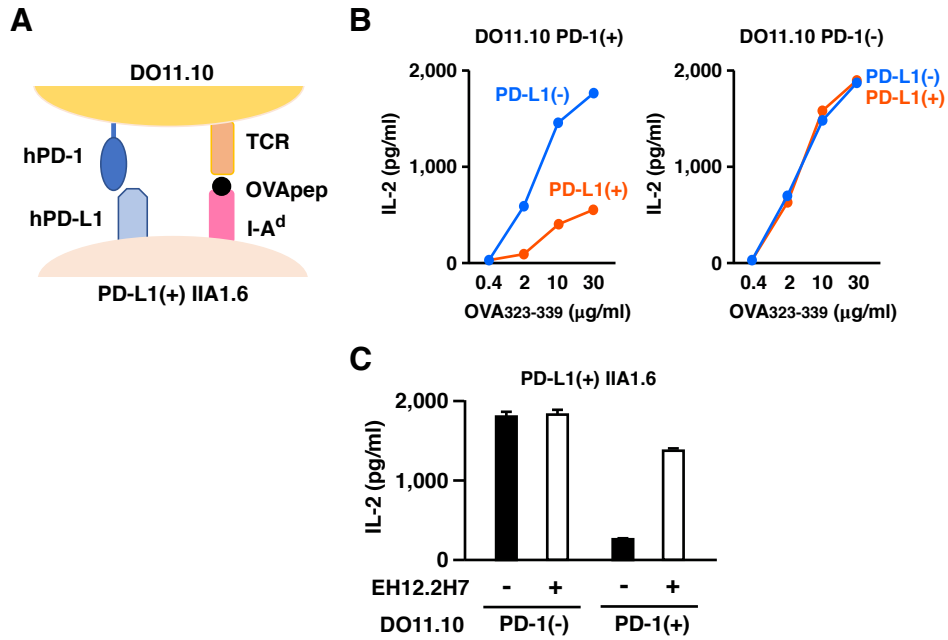


Figure S2. The functional assay of PD-1-dependent inhibition in the antigen-specific T cell activation. (A) DO11.10 cells expressing human PD-1 instead of mouse PD-1 were stimulated with its intrinsic antigen OVA₃₂₃₋₃₃₉ peptide in the presence of IIA1.6 B lymphoma as antigen-presenting cells. (B, C) IL-2 production from the stimulated DO11.10 cells in the presence of human PD-L1⁺ or PD-L1⁻ IIA1.6 cells as APC (B). The addition of anti-human PD-1 blocking mAb EH12.2H7 (5 μg/ml) (C).

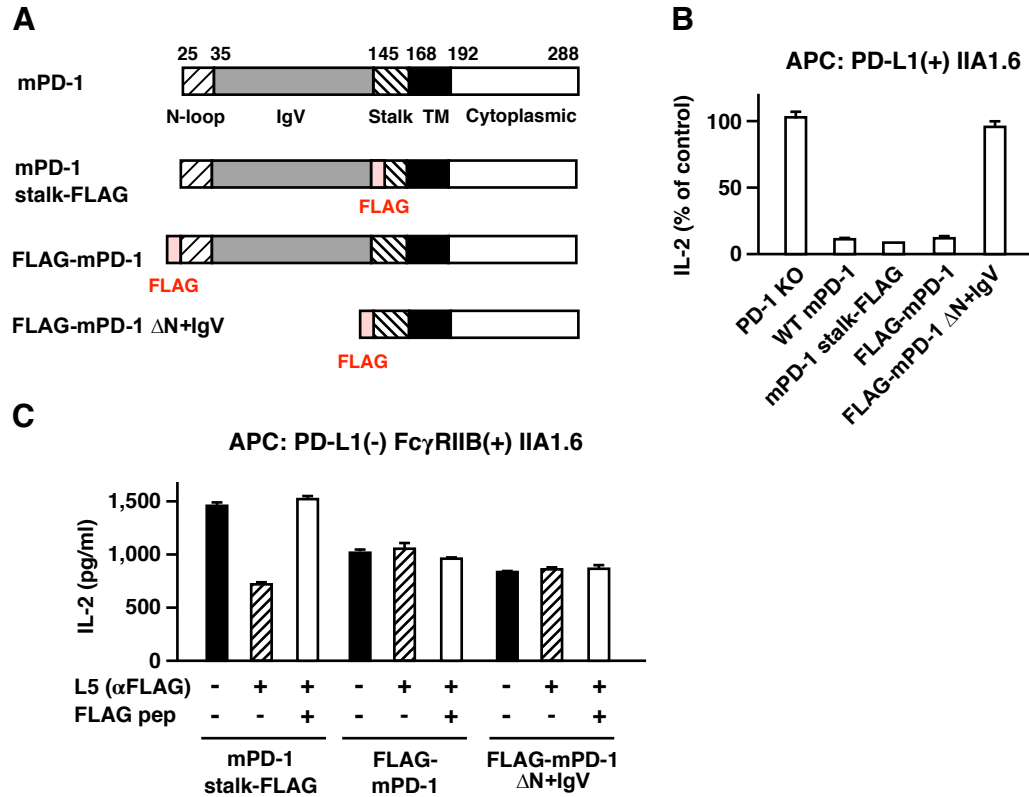


Figure S3. Guided antibody binding to the stalk region resulted in the immunosuppressive activity of mouse PD-1. (A) mPD-1 stalk-FLAG and FLAG-mPD-1 were produced in the same way as in Figure 1. In addition, FLAG tag was introduced in front of the stalk region to mouse PD-1 lacking N-loop and IgV domains (FLAG-mPD-1 ΔN+IgV). (B) Immunosuppressive activity in response to PD-L1 by mouse PD-1 and its FLAG-tagged variants. Wild-type mouse PD-1, mPD-1 stalk-FLAG and FLAG-mPD-1 or FLAG-mPD-1 ΔN+IgV, which lacks PD-L1 binding site, were expressed in DO11.10 cells. (C) Immunosuppressive activity of anti-FLAG mAb in mPD-1 FLAG-tagged variants.

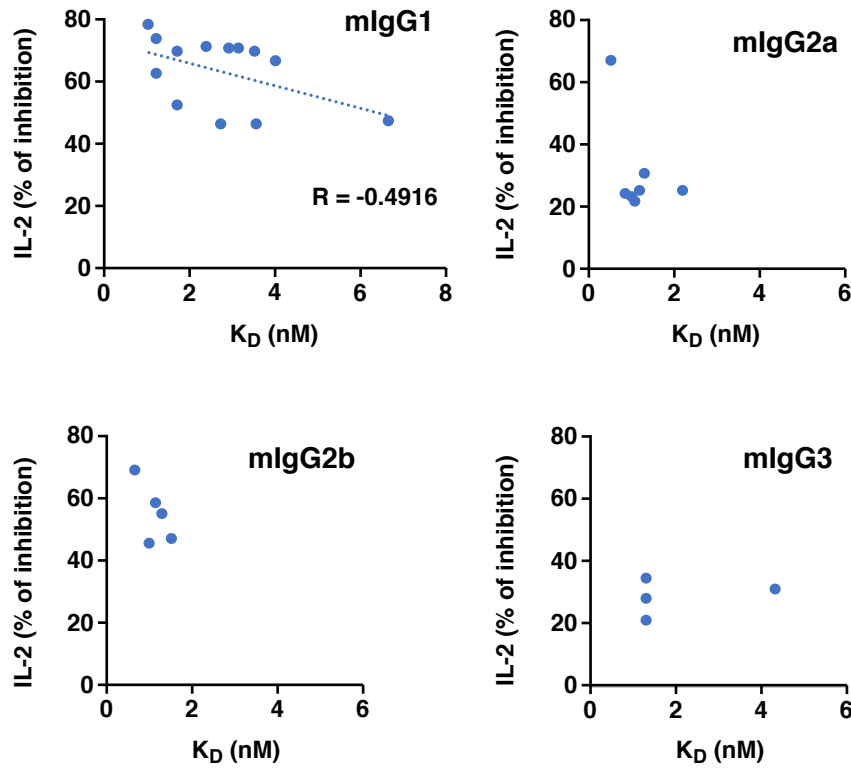


Figure S4. Correlation of binding affinity of anti-PD-1 agonist mAbs with their immunosuppressive activity. Strong agonist mAb clones recognizing domain #7 (groups #(1), 6, 7 and #7 in Figure 3) were analyzed for their binding affinity to human PD-1. Since binding to Fc γ RIIB significantly affects the agonist activity, the antibody group was separately analyzed by IgG subtypes. The agonistic activity of each clone was plotted with K_D value to human PD-1.

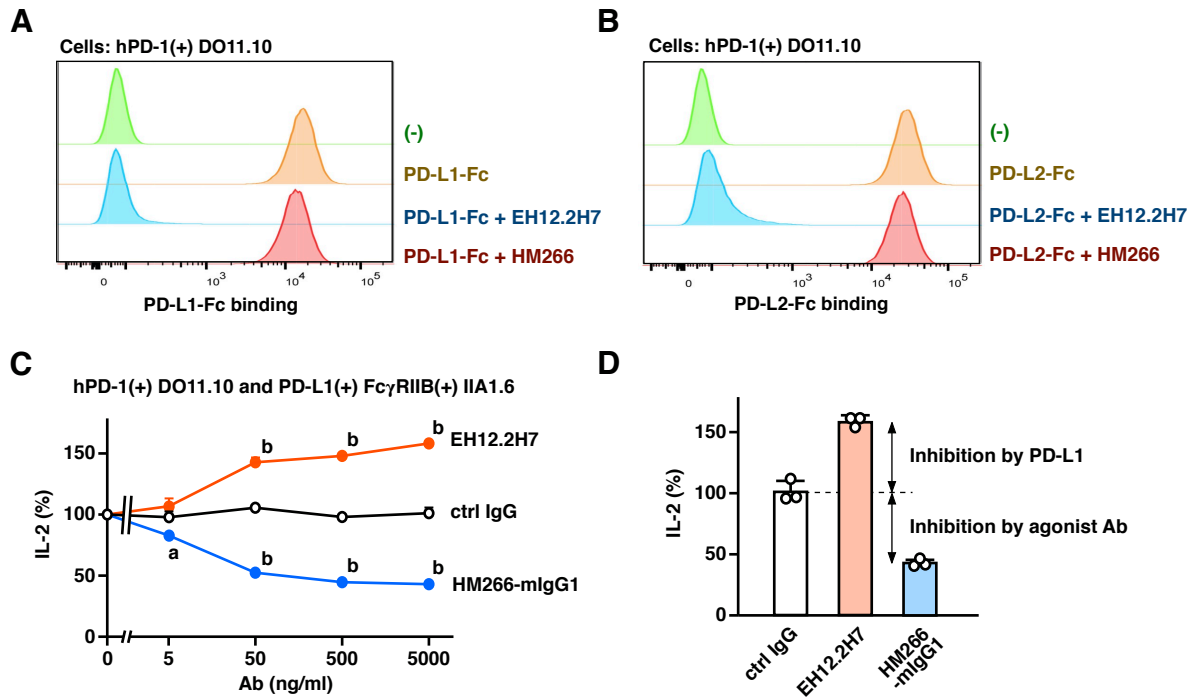


Figure S5. Induction of PD-1-dependent immunosuppression by anti-PD-1 agonist antibody is additive to PD-1 ligands. (A, B) Non-competitive PD-1 binding of its ligands with anti-PD-1 antibody. DO11.10 cells expressing human PD-1 were incubated with PD-L1-Fc (A) or PD-L2-Fc (B). Anti-human PD-1 antibodies, EH12.2H7 (blocker) and HM266-mIgG1 (agonist), were added at 5 μ g/ml. The binding of PD-L1-Fc and PD-L2-Fc was detected using Alexa Fluor 647-labeled anti-human IgG Fc antibody. (C, D) Co-inhibition of IL-2 production from the stimulated DO11.10 cells by PD-L1 and HM266-mIgG1. DO11.10 cells expressing human PD-1 were stimulated with human PD-L1⁺ IIA1.6 cells in the presence or absence of EH12.2H7 or HM266-mIgG1 (C). The changes in IL-2 levels by anti-PD-1 mAbs (5 μ g/ml) indicate suppression by PD-L1 and agonist antibody (D). Data represent average $\pm$ SE (n = 3). a, $p < 0.05$, b, $p < 0.01$; vs control IgG; Tukey-Kramer test.

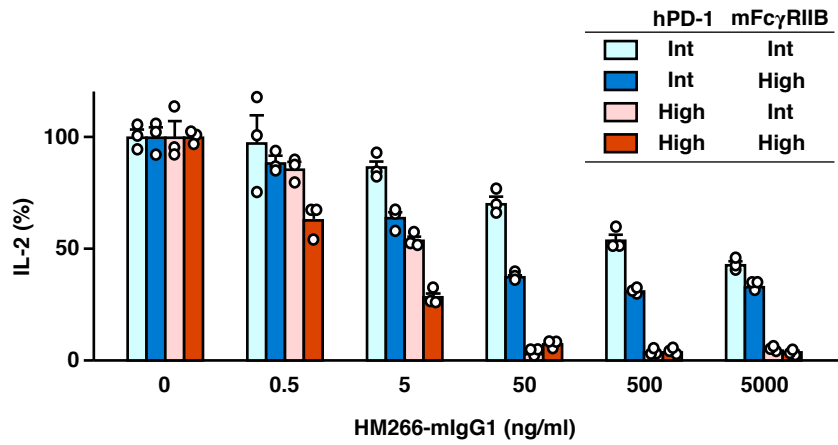


Figure S6. The agonistic activity of HM266-mIgG1 increases dependent on the levels of PD-1 expression in T cells and Fc receptor expression in antigen-presenting cells. DO11.10 hybridoma cells expressing human PD-1 at high or intermediate levels were co-cultured with IIA1.6 cells expressing human FcγRIIB at high or intermediate levels. IL-2 levels were determined after the stimulation with antigenic peptide OVA₃₂₃₋₃₃₉. Data represent average ± SE (n = 3).

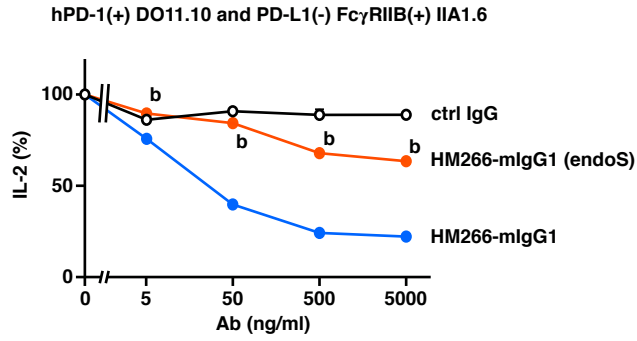


Figure S7. HM266 induces PD-1-mediated immunosuppression based on Fc receptor binding.

To prevent Fc receptor binding, HM266-mIgG1 was pretreated with endoS, which hydrolyzes Fc glycan. DO11.10 cells expressing human PD-1 were stimulated with FcγRIIB⁺ IIA1.6 cells. IL-2 levels in response to OVA₃₂₃₋₃₃₉ were determined. Data represent average ± SE (n = 3). b, p < 0.01; vs HM266-mIgG1; Tukey-Kramer test.

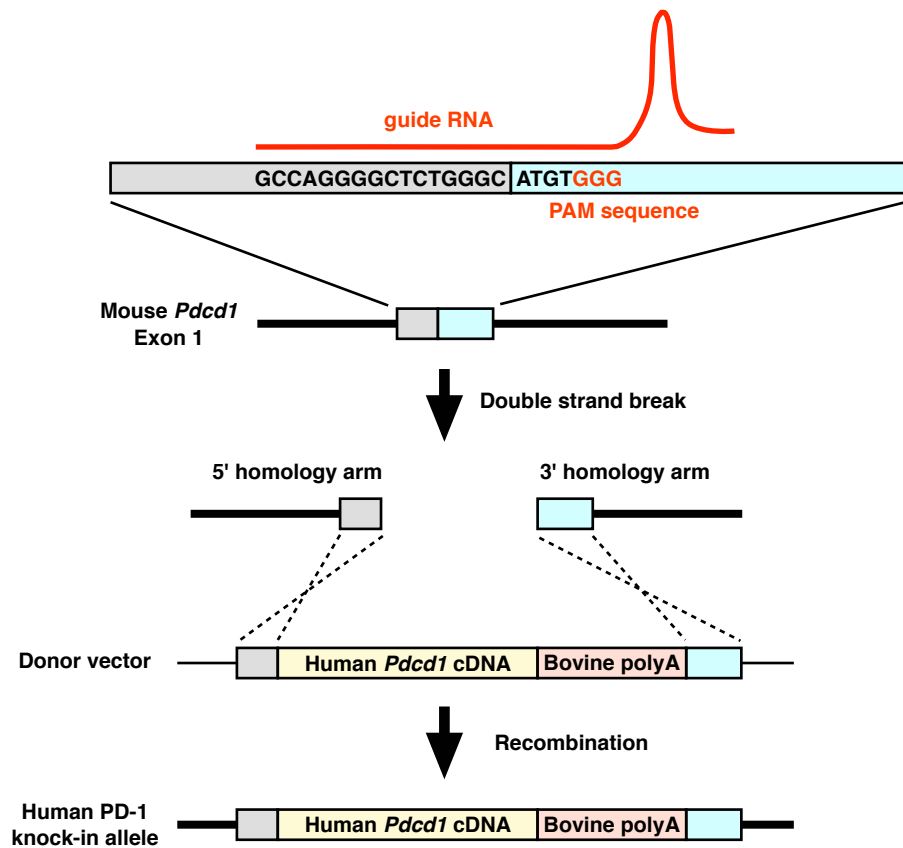


Figure S8. Generation of human PD-1-knock-in mice. Guide RNA was microinjected together with human PD-1 cDNA-bovine poly A sequence-containing donor vector and Cas9 protein. For the homologous recombination-mediated knock-in, the donor vector consisting of homology arms and human PD-1-bpA (bovine growth hormone poly A signal sequence) was generated to insert the human PD-1-bpA cassette at the 4 base upstream of the PAM sequence.

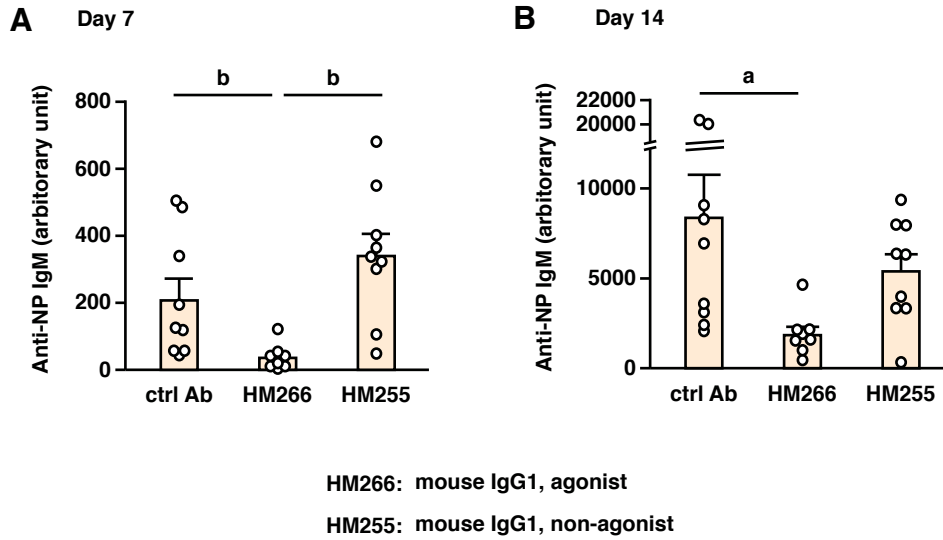


Figure S9. The inhibition of antibody production by HM266 is mediated by its PD-1 agonist activity but not cell-depleting activity. Human PD-1-knockin mice were immunized with NP-OVA/alum, and plasma anti-NP IgM levels were monitored by ELISA on day 7 and 14. HM266 and HM255 are anti-human PD-1 mAbs of the same mouse IgG1 subclass, but HM255 is not an agonist antibody. The treatment with these antibodies (500 μ g) started at the time of NP-OVA immunization and was repeated on day 3, 7 and 10. Data represent average $\pm$ SE (n = 8 or 9). a, $p < 0.05$, b, $p < 0.01$; Holm-Bonferroni method to natural logarithm of numbers.