IMMUNOTHERAPY

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

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The PD-1 receptor triggers a negative immunoregulatory mechanism that prevents 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 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. Because PD-1 agonist antibodies trigger immunosuppressive signaling by cross-linking PD-1 molecules, Fc engineering to enhance FcγRIIB 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 the application of immune checkpoint inhibitors to cancer immunotherapy. Because immune checkpoints in the tumor microenvironment contribute to inactivation of antitumor immune effectors, intervention with immune checkpoint inhibitors is effective in many patients with cancer, 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 programmed cell death 1 (PD-1) blockade in cancer immunotherapy underscores the importance of PD-1–mediated immunoregulation in the human immune system. PD-1 is up-regulated in activated immune cells and provides negative costimulatory signaling upon interaction with its ligands, PD-L1 and PD-L2 (*2*, *3*). Stimulated PD-1 recruits phosphatases to down-regulate tyrosine phosphorylation in the T cell receptor (TCR) 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*). Because PD-1 expression is induced on activated immune cells, treatments that can induce PD-1 signaling can down-regulate 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 is sometimes given in vivo with an expectation to down-regulate 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*). Because 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 major histocompatibility (MHC)–oriented delivery of PD-L1 to inhibit PD-1–expressing T cells (*11*). Compared with 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 always be agonistic antibodies because the depletion of

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PD-1–expressing effector cells could also down-regulate inflammation (*19*). Agonist antibodies to PD-1 have been proposed as immunomodulatory agents (*20*), but the requirements for agonistic anti–PD-1 mAbs, e.g., their epitope recognition specificity, 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 cross-linking of PD-1 molecules. MPER recognition is characteristic and crucial to the agonistic activity because PD-1 cross-linking 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 coligation of PD-1 and TCR, e.g., coimmobilization of PD-L1–Fc and anti-CD3 mAb (*21*). Cross-linking of PD-1 alone did not exert an immunosuppressive effect because PD-L1–Fc in a soluble form was unable to inhibit T cells even after the cross-link with secondary antibody (fig. S1A). Similarly, immobilized PD-L1–Fc on microbeads, separate from anti-CD3 and anti-CD28 mAbs on the plastic surface, did not inhibit T cells (fig. S1B).

Although coligation of PD-1 and TCR has been widely used to stimulate PD-1 in vitro, we found that this experimental system was suboptimal because of 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 interferon-γ (IFN-γ) production was equally strong in PD-$1^{+/+}$ and PD-$1^{-/-}$ T cells (fig. 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 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, coimmobilized PD-L1–Fc with anti-CD3 and anti-CD28 mAbs inhibited IFN-γ production from

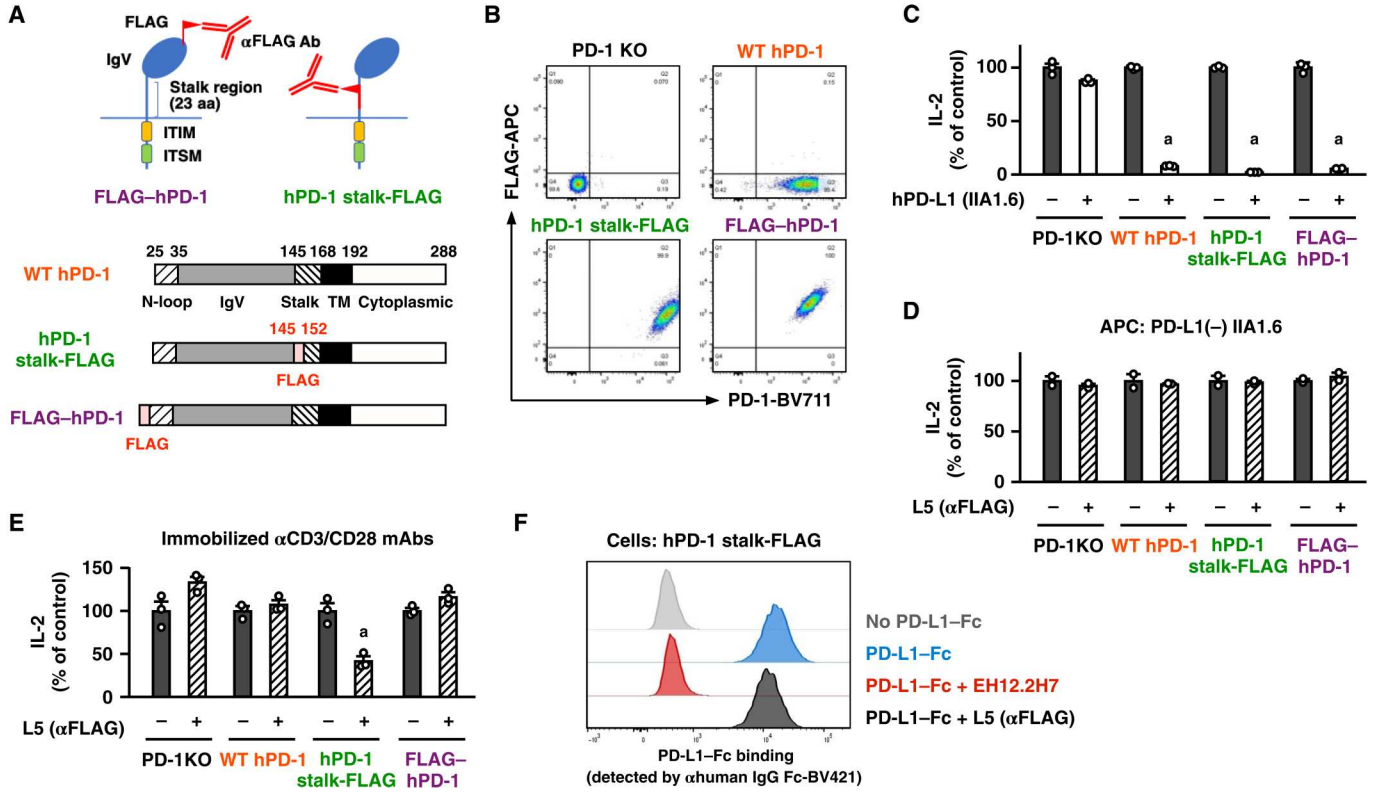

Fig. 1. PD-1 signaling was inducible by guided antibody binding to the stalk region of the PD-1 molecule. (**A**) Introduction of FLAG tag to hPD-1: replacement of a part of the stalk region (hPD-1 stalk-FLAG) or FLAG addition to the N terminus (FLAG–hPD-1). (**B**) FLAG tag detection by anti-FLAG mAb L5 in DO11.10 cells transduced with wild-type hPD-1 (WT hPD-1) or FLAG-tagged hPD-1. (**C**) Immunosuppressive potential of FLAG-tagged PD-1. PD-1–transduced DO11.10 cells were stimulated with $OVA_{323–339}$ in the presence of IIA1.6 cells either devoid of or expressing PD-L1 (see also fig. 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. The antibody concentration was 5 μg/ml. EH12.2H7 is an anti–hPD-1 blocking mAb. Data represent average ± SE of triplicate (C and E) or duplicate (D) samples. a, $P < 0.05$ versus controls (two-tailed Student's *t* test). αFLAG, anti-FLAG; aa, amino acids; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; TM, transmembrane segment; WT, wild type, KO, knockout.

PD-1$^{+/+}$ T cells, but not PD-1$^{-/-}$ T cells (fig. S1D). When this in vitro system is used, PD-1–dependent inhibition may be validated with the use of PD-1$^{-/-}$ T cells as a control.

Because the importance of coligation with TCR in stimulating PD-1 was confirmed, we tested whether anti–PD-1 mAb would be able to replace PD-L1 in triggering the immunosuppressive activity. Anti–mouse PD-1 (mPD-1) mAb clones, 29F.1A12 and RMP1-14, were coimmobilized with anti-CD3 and anti-CD28 mAbs, but neither of these anti–PD-1 mAbs could inhibit T cell activity (fig. S1E). Simple coligation 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 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 (hPD-1) and examined whether guided binding of an anti-FLAG mAb could stimulate immunosuppressive activity. The PD-L1 binding domain has been identified within the immunoglobulin V (IgV) domain of the PD-1 protein (*15*, *22*, *23*). To guide anti-FLAG mAb binding away from this 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; Fig. 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 (Fig. 1B).

The αβ TCR of DO11.10 cells was used to generate the TCR transgenic mice with the same name. This I-A^d–restricted T cell line retains antigen specificity and produces interleukin-2 (IL-2) upon interaction with OVA$_{323–339}$-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 (fig. 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 (Fig. 1C). The simple addition of anti-FLAG mAb did not change IL-2 production from these hPD-1 transfectants (Fig. 1D). However, as in the case with PD-L1–Fc, coimmobilization of anti-FLAG mAb with anti-CD3 and anti-CD28 mAbs did down-regulate IL-2 production in the cells expressing hPD-1 stalk-FLAG, but not FLAG–hPD-1 (Fig. 1E). Anti-FLAG mAb inhibited T cell activation through binding to a distinct region from the PD-L1 binding site (Fig. 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 cross-linking mediates the 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 use Fc receptors for the engagement of antibodies. Fc receptors have been used to cross-link antibodies to other surface molecules such

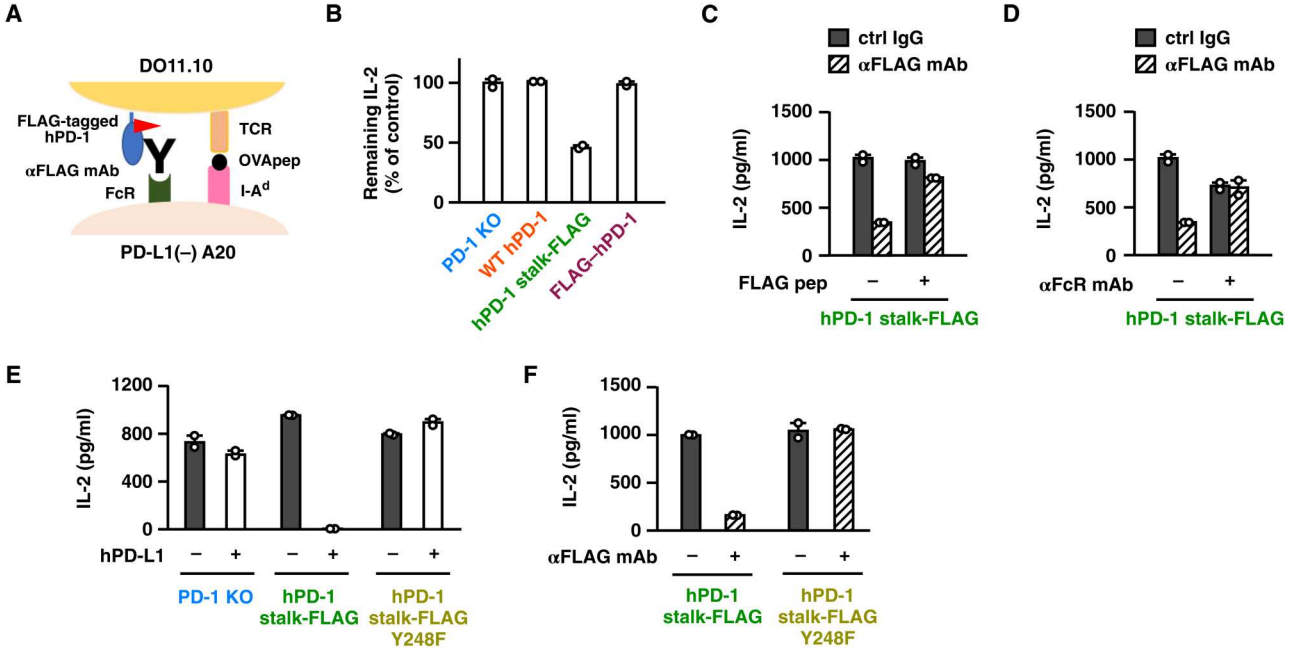

Fig. 2. The utilization of Fc receptor engagement enabled PD-1–mediated immunosuppression by the simple addition of agonist antibody. (**A**) The experimental system. DO11.10 cells expressing hPD-1 were stimulated with OVA$_{323–339}$ peptide using PD-L1–deficient A20 B lymphoma as antigen-presenting cells. (**B**) IL-2 production from PD-1–transduced DO11.10 cells in the presence of anti-FLAG mAb L5 (5 μg/ml). (**C** and **D**) The addition of excess FLAG peptide (3 μg/ml) (C) or anti-CD16/32 mAb (10 μg/ml) (D). (**E** and **F**) PD-1 stimulation with PD-L1–expressing antigen-presenting cells (E) or L5 (5 μg/ml) using PD-L1–deficient A20 cells (F). The Y248F mutant lacks PD-1 signal transmission. Data represent average ± SE of duplicate samples. FcR, Fc receptor.

as CD3 and tumor necrosis factor (TNF) receptor family to exert agonistic signaling (*25*–*27*). The cross-linking of PD-1 agonists with Fc receptors on antigen-presenting cells (APCs) might enable coligation of PD-1 with TCR. We replaced the 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 receptors on antibody-dependent PD-1 stimulation (Fig. 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 (Fig. 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 (Fig. 2B), and the inhibitory effect was reversed by the addition of excess FLAG peptide (Fig. 2C). Blocking antibody to CD16/CD32 abrogated the immunosuppression by anti-FLAG mAb, confirming the importance of Fc receptors (Fig. 2D).

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 Tyr^{248} in the intracellular domain of hPD-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 (Fig. 2, E and F).

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

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

Next, we sought to find anti–hPD-1 mAbs that can stimulate immunosuppressive activities. Considering the importance of Fc receptor–mediated cross-linking, 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 (Fig. 3A). Blocking activities were also monitored by coupling hPD-1–expressing DO11.10 cells with hPD-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 mAb 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–hPD-1 mAb panel, which focused on the preservation of diverse binding epitopes. Eight segments of the extracellular domain of the hPD-1 molecule were chosen as representative ones forming the molecular surface (Fig. 3, B and C). These segments were replaced by the corresponding mPD-1 segments individually, and anti–hPD-1 mAb clones were classified by the binding capacity to each of these hPD-1 mutants. The anti–hPD-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–hPD-1 mAb panel and found quite a few agonists along with blockers (Fig. 3, D and E). These agonist mAbs were part of the group recognizing the MPER of hPD-1, i.e., segments #6, #7, and #8 (Fig. 3, C and E). The mAb clones binding to segment #7 offered especially strong immunosuppressive activities, whereas those recognizing segment #8 were less effective. Binding affinity to hPD-1 showed a trend of positive correlation with the agonistic activity of mAbs, at least in clones of a mouse IgG1 subclass (fig. S4). The binding domains of agonist mAbs were in sharp contrast with those for the blocking mAbs, which interacted with the membrane-distal region of hPD-1 molecule as defined by segments #1, #2, and #5.

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

We selected the agonist clone HM266, which had the strongest immunosuppressive activity (Fig. 3D), for the subsequent studies. HM266 is a mouse IgG1, which binds to mouse FcγRIIB well. Because of the recognition of MPER, HM266 did not compete with PD-L1 or PD-L2 for the binding to PD-1 (fig. S5). The stimulation of PD-1 activity by this anti–PD-1 agonist mAb was additive to that of PD-L1 (fig. S5). Therefore, PD-1 agonist mAb treatment may further enhance PD-1–dependent immunosuppression on top of the preexisting 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 (fig. S6). Although 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 FcγRIIB expression on IIA1.6 cells also promoted the immunosuppressive activity of HM266 (fig. S6). In contrast, pretreatment of HM266 with endoglycosidase S, which hydrolyzes glycans on IgG and reduces antibody binding to Fcγ receptors, strongly impaired the PD-1 agonistic activity of HM266 (fig. 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 Src homology 2 domain–containing protein tyrosine phosphatase-2 (SHP-2), which interrupts the TCR signaling cascade with its phosphatase activity (*4*, *5*, *29*). We examined PD-1 phosphorylation using DO11.10 cells expressing hPD-1–green fluorescent protein (GFP). Western blotting after immunoprecipitation with anti-GFP antibody indicated PD-1 phosphorylation by HM266 (Fig. 4A). To further confirm the down-regulation of TCR signaling by HM266, we examined the extent of extracellular signal–regulated kinase (ERK) phosphorylation in stimulated DO11.10 cells. HM266 strongly prevented the induction of phosphorylated ERK in T cells (Fig. 4, B and C).

Enhanced FcγRIIB binding improves the 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

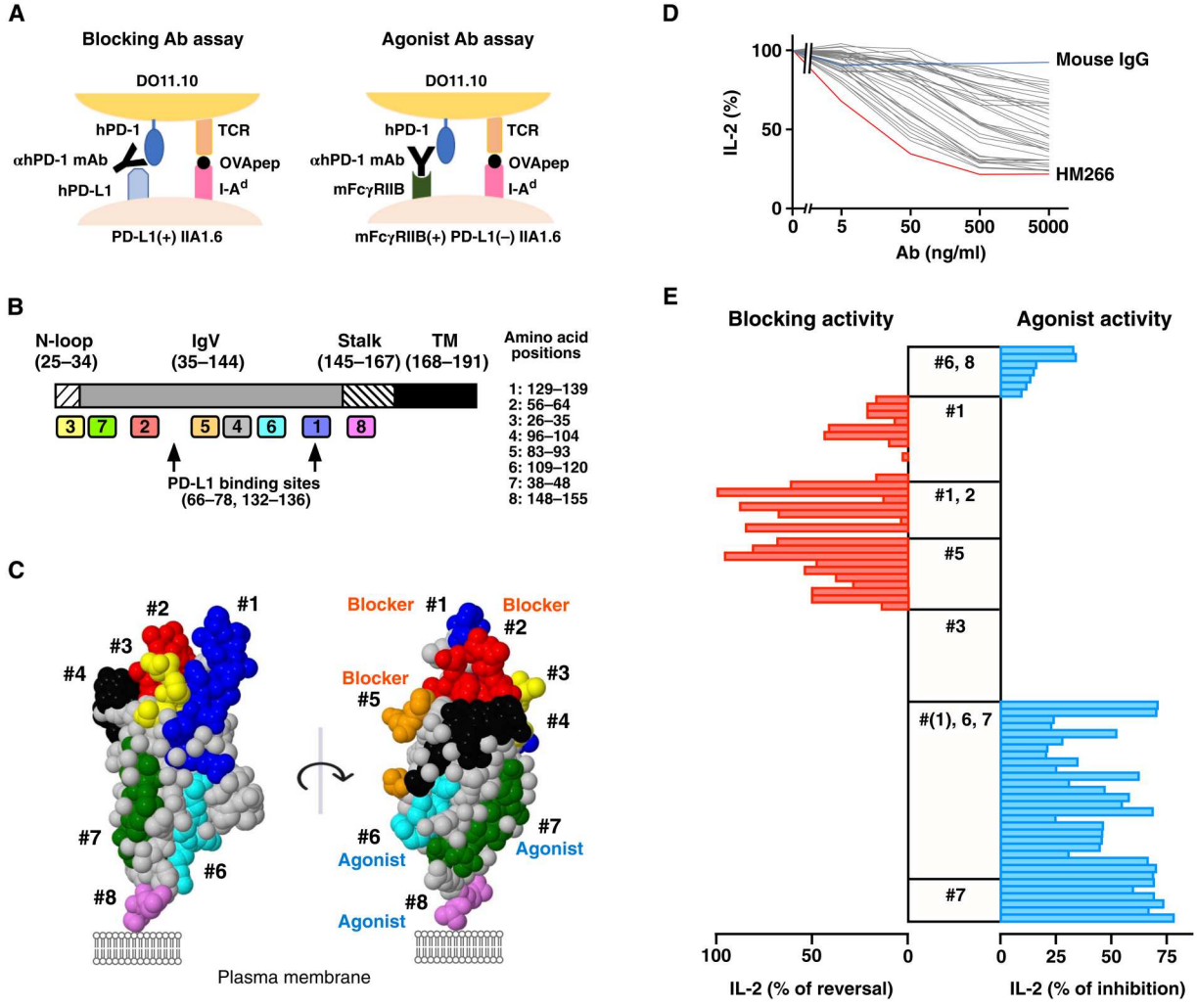

Fig. 3. Binding epitopes of anti–PD-1 agonist mAbs are specifically located in MPER, which is distinct from the region recognized by blocking mAbs. (**A**) The screening system for blocking and agonistic activities of anti–hPD-1 mAbs. (**B**) Eight segments that were used to classify anti–hPD-1 mAbs by their binding capabilities. (**C**) The putative locations of the eight segments on the molecular surface of hPD-1. A three-dimensional (3D) model depicts the extracellular part of hPD-1. This 3D model was created using Jmol software based on the structure of hPD-1 from Protein Data Bank (accession code 3RRQ). (**D**) Activities of anti–hPD-1 agonist mAbs ($n = 2$). (**E**) Blocking (red) and agonistic (blue) activities of individual anti–hPD-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 the #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.

were stimulated in a coculture with human monocytic cell line THP-1 (Fig. 5A). In this coculture, chimeric HM266 with human Fc moderately inhibited IFN-γ production thanks to basal FcγRIIB expression in normal THP-1 cells (hFcγRIIB^{low}; Fig. 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 (*27*, *30*) 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. 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 (Fig. 5C) and significant increases in their immunosuppressive activity in the coculture of human CD4$^+$ T cells and THP-1 cells (Fig. 5D). Anti–hPD-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, HM266-hIgG1-X3, and HM266-hIgG1-X4 to the mixed lymphocyte reaction strongly suppressed IFN-γ production (Fig. 5C).

Anti–PD-1 agonist mAbs suppressed inflammatory diseases

To evaluate in vivo efficacy of the anti–hPD-1 agonist mAb, we used hPD-1 knock-in (hPD-1 KI) mice in which an mPD-1 gene was replaced by an hPD-1 gene (Fig. 6A and fig. S8). To adapt to the mouse Fc receptor system, the HM266 antibody used in the in

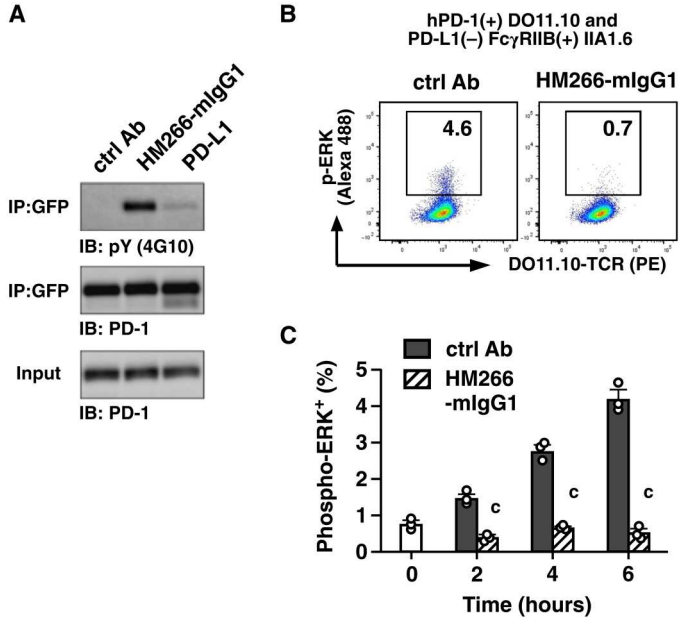

Fig. 4. Anti–PD-1 agonist mAb exerts its immunosuppressive activity by triggering PD-1 signaling. (**A**) Western blotting of phosphorylated hPD-1. DO11.10 cells transduced with hPD-1-GFP and SHP-2(C459S) were stimulated with $OVA_{323–339}$-pulsed A20 cells for 5 min. PD-1 was stimulated with PD-L1–expressing A20 cells or HM266-mIgG1. (**B** and **C**) ERK phosphorylation in stimulated DO11.10 cells for the indicated time. Antibody concentration was 5 μg/ml. Data represent average ± SE ($n = 3$). c, $P < 0.001$ (two-tailed Student's t test). IP, immunoprecipitation; PE, phycoerythrin.

vivo studies was the original mouse IgG1 form. Acute graft-versus-host disease (GVHD) was induced by the transfer of spleen cells from C57BL/6 background hPD-1 KI mice into B6D2F1 (BDF1) mice (Fig. 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 hPD-1 (Fig. 6C). However, treatment with HM266 prevented body weight loss in acute GVHD (Fig. 6D) and largely reduced expansion of donor-derived T cells in the recipients (Fig. 6, E and 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 (Fig. 7A). HM266 treatment prevented colitis induction, as indicated by significant reversal of body weight loss (Fig. 7B) and of shortened colon length (Fig. 7C). In the lamina propria, HM266 reduced $CD4^+$ T cell expansion (Fig. 7D). Decreases in IFN-γ– and IL-17–producing T cells suggested that PD-1 agonist mAb inhibited development of pathogenic effector cells (Fig. 7, E to 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 (fig. 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 a 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–hPD-1 mAb HM255, which is also a 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 (fig. S9). This result suggests that the PD-1 stimulatory activity makes a difference in 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 cross-linking. 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 an Fc receptor that enables PD-1 cross-linking. We did not observe any agonist clone working without cross-linking to Fc receptors. Other forms of PD-1 stimulators, such as recombinant TCR conjugated with PD-L1 or anti–PD-1 antibody fragment, were also designed to work at the interface between T cells and APCs (*11*). When T cells meet with APCs, the spatial segregation of membrane proteins has been reported (*31*, *32*). TCR on T cells and MHC on APCs were concentrated at the cell-cell interface, and PD-1 needs to be adjacent to the TCR to enable dephosphorylation of TCR signaling components by the recruited phosphatases. A cell imaging study has demonstrated colocalization 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 the TCR signaling cascade. We speculate that Fc receptors may keep agonist antibodies at the cell-cell interface (*20*) and induce PD-1/TCR coligation.

To engage agonist antibodies, we used FcγRIIB-expressing cells. FcγRIIB has been used to provide a cross-linking 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, cross-linking via FcγRIIB was critically important for the biological activity of agonist mAbs, but FcγRIIB's own function as an inhibitory Fcγ receptor was dispensable (*34*). Other Fcγ 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 because of the difference in mouse FcγRIIB 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 because of their lower affinity to FcγRIIB than IgG1 and IgG2b.

As the importance of Fc receptors was increasingly recognized in the therapeutic application of antibodies, the Fc region became a

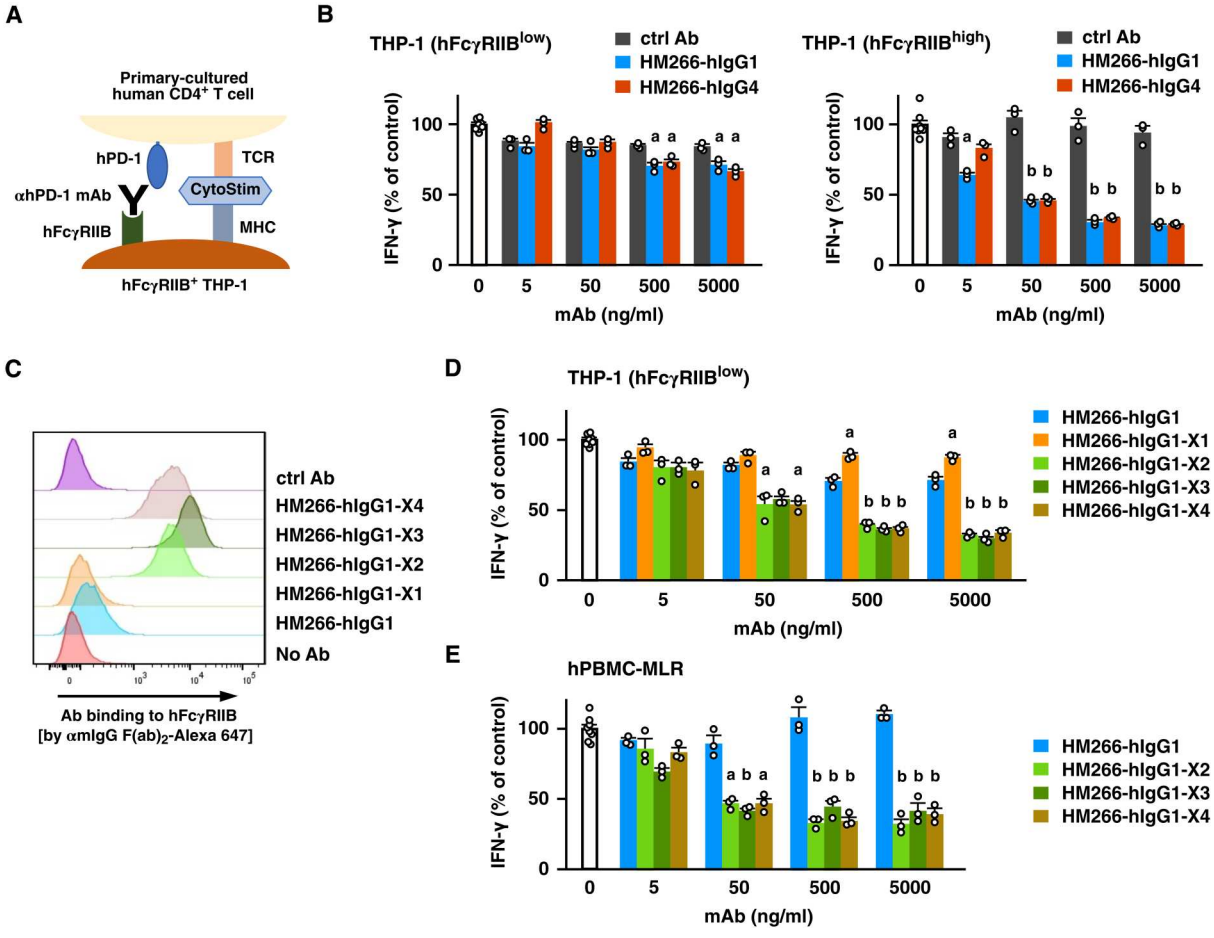

Fig. 5. Anti–PD-1 agonist mAb inhibits primary human T cells. (**A**) Human primary CD4+ T cells were stimulated with CytoStim in the presence of human FcγRIIB-expressing THP-1 cells. (**B**) T cells were stimulated using parental (FcγRIIB^{low}) or FcγRIIB-overexpressing THP-1 cells (FcγRIIB^{high}) 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, $P < 0.01$; versus control Ab; Tukey-Kramer test. (**C**) Binding of Fc variants of HM266-hIgG1 to FcγRIIB^{high} THP-1 cells. (**D** and **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, $P < 0.01$; versus HM266-hIgG1; Tukey-Kramer test.

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 antitumor 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 antitumor efficacy (*30*, *34*). Our anti–hPD-1 agonist antibody HM266-hIgG1 showed only moderate to no inhibition in primary human CD4+ T cells most likely because of low FcγRIIB expression. We intentionally increased the affinity to FcγRIIB, and such modification of the Fc region notably improved the immunosuppressive activity to primary human T cells. The adaptation to the human immune system will be a key to optimizing the therapeutic value of anti–PD-1 agonist mAbs.

Although cross-linking 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–hPD-1 mAb panel. Because MPER is distant from the PD-L1 binding site, anti–PD-1 agonist mAbs were able to down-regulate immune response without interfering with PD-1/PD-L1 interaction. In 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 to 78 (the gap between segments #2 and #5) and 128 to 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

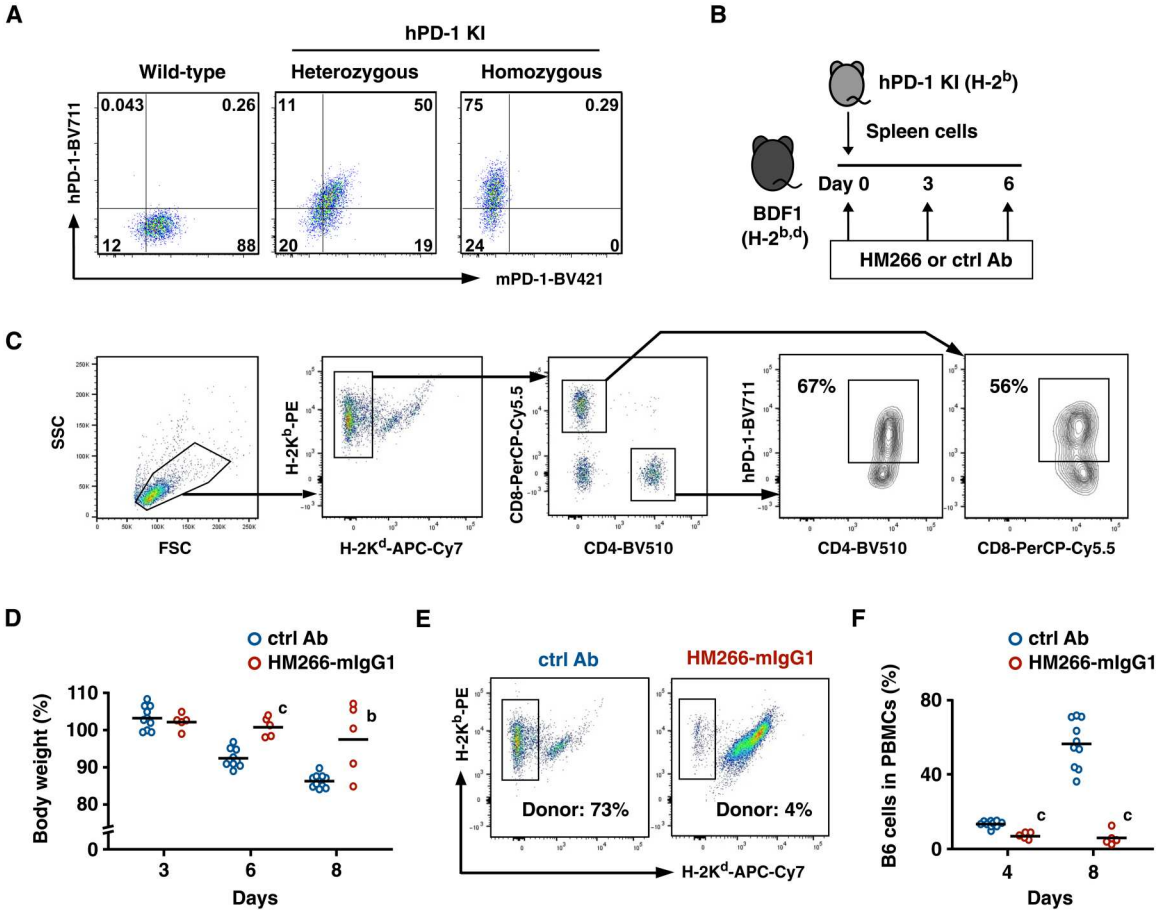

Fig. 6. Anti–PD-1 agonist mAb suppressed GVHD in vivo. (**A**) PD-1 expression in stimulated T cells from hPD-1 KI mice. (**B**) Acute GVHD induction in BDF1 mice. (**C**) hPD-1 expression in donor T cells in PBMCs on day 8. (**D**) Body weight change. (**E** and **F**) Proportions of donor-derived (H-2K^{b+} H-2K^{d-}) cells in PBMCs. Data represent average ± SE (ctrl Ab, n = 10; HM266-mIgG1, n = 5). b, $P < 0.01$; c, $P < 0.001$; versus control Ab; two-tailed Student's t test. SSC, side scatter; FSC, forward scatter.

to interact with hPD-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 nine anti-human CD40 and two 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 FcγRIIB 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 four representative clones (*40*). Our analysis of anti–hPD-1 mAb clones provides comprehensive insight into 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 FcγRIIB 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 for a variety of inflammatory disorders. The inhibitory effect of HM266 on antibody induction may involve the suppression of PD-1–expressing T follicular helper (T_{FH}) cells. CD4$^+$ cells play an important role in antibody production (*41*), and the expansion of T_{FH} cells by the interruption of PD-1 signaling indicates that T_{FH} 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 milieus (*44*). It is not known whether 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 coligate PD-1 with TCR, FcγRIIB works as an excellent platform for cross-linking of anti–PD-1 mAbs. PD-1 agonists may be further improved by optimizing the Fc region to promote FcγRIIB binding. Because 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 (irAEs)

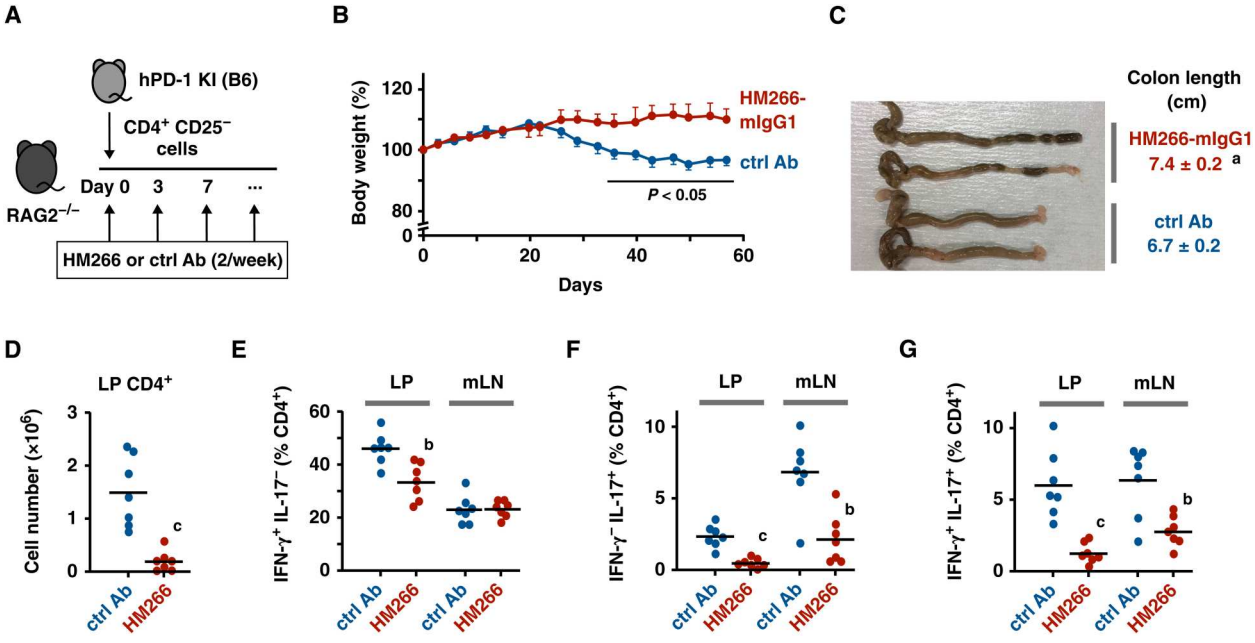

Fig. 7. Anti–PD-1 agonist mAb suppressed the induction of colitis. (**A**) Colitis induction by the transfer of $CD4^+$ $CD25^-$ T cells from hPD-1 KI mice. HM266-mIgG1 (0.5 mg per mouse) was given twice a week. (**B**) Body weight change after cell transfer. (**C**) Macroscopic picture of the colon and cecum on day 58. (**D**) $CD4^+$ T cell numbers in the lamina propria (LP). (**E** to **G**) $CD4^+$ T cells producing IFN-γ (E), IL-17 (F), or both (G) in the lamina propria and mesenteric lymph nodes (mLN) on day 58. Data represent average ± SE of seven mice. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$; versus control Ab; two-tailed Student's *t* test.

that are occasionally life-threatening (*45*). Anti–PD-1 agonist mAbs may also be useful in rebalancing the intensity of immune response when irAEs are encountered.

Limitations of this study include the difference in Fcγ receptors between mice and humans. PD-1 agonist antibodies showed anti-inflammatory efficacy in mice, which express FcγRI, FcγRIIB, FcγRIII, and FcγRIV, but humans have a different set of Fcγ receptors consisting of FcγRI, FcγRIIA/B/C, and FcγRIIIA/B. Binding affinities of these receptors to IgG isotypes are variable even when sharing the same name between mice and humans. For example, human FcγRIIB interacts with human IgG at an approximately 10 times lower affinity than the interaction between FcγRIIB 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 FcγRIIB binding, some Fc engineering may be beneficial for PD-1 agonist antibodies to effectively down-regulate proinflammatory activities in humans. The requirement for cross-linking in activating PD-1 agonistic antibody suggests that its therapeutic efficacy may be dependent on the Fcγ receptor availability in the local environment. Superagonist-type agonist antibodies, which do not demand Fcγ receptor–mediated cross-linking, 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. Because 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 was to identify anti–hPD-1 agonist antibodies that elicit the immunosuppressive activity of PD-1. To this end, we established a screening system for PD-1 agonist antibodies based on physiological antigen-dependent T cell activation using a combination of the DO11.10 T cell hybridoma and MHC-matched B lymphoma cells. The anti–hPD-1 mAb panel, which includes diverse mAb clones recognizing various segments of the hPD-1 molecule, was evaluated for agonistic activity along with immunoenhancing blocking activity. Characterization of binding sites and the screening for biological activities were conducted by separate laboratories in a blinded manner. Fc variants of PD-1 agonist antibodies were designed to enhance FcγRIIB binding and to improve the immunosuppressive activity in human T cells. To examine in vivo efficacy, we gave PD-1 agonist antibody to mouse models of acute GVHD and colitis and to NP-OVA–immunized mice. The size of mice cohorts was determined from a previous experience so that a desired difference would be detected in power analysis ($\alpha = 0.05$, a power of 0.9). Experimental end points were determined on the basis of disease progression in our prior experience and were 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 to 10 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) or CLEA Japan (Tokyo, Japan). PD-1$^{-/-}$ mice and RAG2$^{-/-}$ mice with a 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 mouse colonies and experimental animals were housed in specific pathogen–free conditions. All animal experiments were conducted in accordance with the institutional animal care guidelines. For the generation of hPD-1 KI mice (accession no. CDB0117E; www2.clst.riken.jp/arg/mutant%20mice%20list.html), the *hPD-1* cDNA sequence followed by a bovine growth hormone polyadenylate (poly A) signal was inserted into the start codon of the *mPD-1* genome by the CRISPR-Cas9 system into C57BL/6N embryos as previously described (fig. S8) (*48*). The guide RNA site (5′-GCC AGG GGC TCT GGG CAT GT-3′) was designed using the Zhang lab website (*49*). For the homologous recombination-mediated knock-in, the donor vector consisting of homology arms and *hPD-1-bpA* (bovine growth hormone poly A signal sequence) was generated to insert the *hPD-1-bpA* cassette at the 4 base pair (bp) upstream of the PAM sequence. The mixture of crRNA (CRISPR RNA), tracrRNA (transactivating crRNA), donor vector, and Cas9 protein was injected into the pronucleus of one-cell stage zygotes. The following primer pairs were used for genotyping: wild-type, 5′-AGG AGA CTG CTA CTG AAG GC-3′ (forward) and 5′-CCA ATC CGT GTA ACC AGG-3′ (reverse) (245 bp) and knock-in, 5′-CAG GCC TCG ACA CCC ACC-3′ (forward) and 5′-CAG CCC AGT TGT AGC ACC-3′ (reverse) (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 T. 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 T. Okazaki (Tokyo University). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 6.25 mM Hepes, 2.5 mM L-glutamate, 0.625 mM sodium pyruvate, 0.625× nonessential amino acid solution, 62.5 μM 2-mercaptoethanol, penicillin (125 U/ml), streptomycin (125 μg/ml), and gentamycin (6.25 μg/ml). To produce the agonistic anti–hPD-1 mAb HM266, we grew the hybridoma cells in CD hybridoma medium (Gibco, catalog no. 11279023) supplemented with 8 mM L-glutamine, penicillin (20 U/ml), and streptomycin (20 μg/ml) in a CELLine bioreactor flask (Duran Wheaton Kimble, catalog no. WCL1000).

Antibodies

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

PD-1 stimulation with PD-L1–Fc

First, purified streptavidin (10 μg/ml; BioLegend, catalog no. 280302) was immobilized in a 96-well flat-bottomed plastic plate. After blocking with Blocking One reagent (Nacalai Tesque, catalog no. 03953-95), we added biotinylated antibodies to mouse CD3 and mouse CD28 (1 μg/ml each) and biotinylated mPD-L1–Fc (2 or 6 μg/ml; BPS Bioscience, catalog no. 71119) as a mixture in phosphate-buffered saline (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 washing with PBS.

Retroviral transduction

hPD-1, GFP-fused human FcγRIIB, and mouse FcγRIIB retroviral plasmids were generated by inserting each cDNA into MSCV-IRES-Thy1.1 DEST (Addgene, catalog no. 17442). Point mutations were introduced with the QuikChange II site-directed mutagenesis kit (Agilent, catalog no. 200523). FLAG-mutant hPD-1 constructs were generated by insertion of the FLAG sequence into the N terminus of hPD-1 (FLAG–hPD-1) or by replacing eight amino acids of the stalk region with the FLAG sequence (positions 145 to 152 TERRAEVP → DYKDDDDK; hPD-1 stalk-FLAG). Plat-E cells (RRID: CVCL_B488; Cell Biolabs) were transfected with the retroviral plasmids using FuGENE HD (Promega, catalog no. E2311). Retroviral supernatant was collected 48 and 72 hours after transfection, pooled, and passed through a 0.45-μm Minisart syringe filter (Sartorius, catalog no. 16533). The retroviral supernatant was centrifuged in RetroNectin (Takara Bio, catalog no. T100A)–coated culture plates for 2 hours at 32°C. Subsequently, DO11.10 or IIA1.6 cells were added to the retrovirus-coated culture plates and were centrifuged at 800*g* for 10 min 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 cells (5×10^4 cells) were stimulated with $OVA_{323–339}$ peptide (2 μg/ml; Eurofins Genomics) using A20 or IIA1.6 B lymphoma cells (1×10^4 cells) as antigen-presenting cells (*24*). Antibodies were added at 5 μg/ml unless otherwise indicated. Culture supernatants were collected after 18 hours, and IL-2 levels were determined by enzyme-linked immunosorbent assay (ELISA; mouse IL-2 DuoSet ELISA; R&D Systems catalog no. DY402). For the screening of anti–hPD-1 agonist mAbs, hPD-1–expressing DO11.10 cells were cocultured with hPD-L1^{-} mFcγRIIB^{+} IIA1.6 cells to detect IL-2 decrease. Blocking activity of antibodies was monitored as the reversal of immunosuppression conferred by hPD-L1^{+} mFcγRIIB^{-} IIA1.6 cells.

Generation of anti–hPD-1 antibody panel

The anti–hPD-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–hPD-1 mouse mAbs were produced by DNA immunization of A/J mice and a conventional hybridoma method. Briefly, a pcDNA3-based expression plasmid encoding *hPD-1* was injected intramuscularly six times followed by a boost immunization with 293T cells transiently expressing hPD-1. After 3 days, the spleen cells were harvested and fused with P3U1 mouse myeloma cells to produce hybridomas. mAbs in the culture supernatants were screened by flow cytometry using human embryonic kidney (HEK) 293 cells transiently expressing hPD-1 and Alexa Fluor 647–labeled goat anti-mouse IgG (Jackson ImmunoResearch, catalog no. 115-605-071) as the secondary antibody. After several

rounds of cell cloning by the limiting dilution method, the established hybridomas were cultured in a CELLine bioreactor flask to harvest the antibody. HM266 mAb in the culture supernatant was purified using protein A (Ab-Capcher ExTra; ProteNova, catalog no. P-003).

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

Western blotting

A retroviral plasmid for bicistronically transducing hPD-1/GFP fusion protein and mouse SHP2 (C459S point mutation) protein was generated by replacing the IRES-Thy1.1 sequence with an hPD-1/GFP-P2A-mSHP2-C459S sequence in the 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 $OVA_{323–339}$ peptide–pulsed A20 cells (2.5×10^6 cells) in the presence of HM266 or PD-L1–Fc (5 μg/ml) for 5 min in 37°C. Cells were immediately processed for lysate preparation and immunoprecipitation using anti-GFP agarose (MBL International, catalog no. D153-8). Input and immunoprecipitated samples were denatured by NuPAGE LDS sample buffer and reducing agent (Invitrogen) and processed for Western blotting using anti-phosphotyrosine mAb (4G10) and anti–hPD-1 mAb (NAT105) followed by horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, catalog no. 115-035-072).

ERK phosphorylation

DO11.10 cells expressing hPD-1 (5×10^4 cells) were stimulated with $OVA_{323–339}$ peptide (2 μg/ml) in the presence of hPD-$L1^-$ mFcγ$RIIB^+$ IIA1.6 cells (1×10^4 cells). HM266 or control mouse IgG1 (MOPC-21) was added at 5 μg/ml. After culturing in a CO_2 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 washing, Alexa Fluor 488 anti–phospho-p44/42 mitogen-activated protein kinase (Erk1/2) (Thr^{202}/Tyr^{204}) mAb, PE–anti-DO11.10 TCR mAb, and APC–anti-B220 mAb were added for subsequent flow cytometric analysis of phosphorylated ERK in DO11.10 cells.

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

DO11.10 cells expressing hPD-1 or hPD-1 stalk-FLAG were labeled with hPD-L1–Fc or hPD-L2–Fc (10 μg/ml) for 15 min. After washing, Brilliant Violet 421 (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, we deglycosylated HM266 (1 mg/ml) by incubating with IgGZERO (1 U/ml; Genovis, catalog no. A0-IZ1-010) for 4 hours at 37°C. The mixture was incubated with Ni–nitrilotriacetic acid agarose (Wako Chemical Industries, catalog no. 143-09763) in 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole (pH 8.0) for 30 min at 4°C to remove the endoglycosidase. After centrifugation at 500*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 was originally mouse IgG1, was replaced with human IgG1(K322A). In addition, to enhance the antibody binding to hFcγRIIB, we also generated several chimeric HM266 versions with variants of human IgG1 Fc. The Fc variants are as follows: L235R (X1), G236D/H268D (X2), S239D/H268D (X3), and S239D/H268D/L328Y/I332E (X4). DNA of HM266 heavy and light chains were inserted into pcDNA3.4 (Invitrogen, catalog no. A14697) separately. Fc-humanized HM266 was generated by the transfection of a 1:1 mixture of the plasmids into Chinese hamster ovary (CHO) cells using an ExpiFectamine CHO transfection kit (Gibco, catalog no. A29129). Antibodies in the culture supernatant were purified using protein A followed by gel filtration (Superdex 200 Increase 10/300 GL; Cytiva, catalog no. 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, catalog no. 2W-200) were stimulated for 3 days by immobilized anti-human CD3 mAb (3 μg/ml). The activated $CD4^+$ T cells (5×10^4 cells) were restimulated with CytoStim (Miltenyi Biotec, catalog no. 130-092-172) in a coculture with THP-1 monocytic leukemia cells (2.5×10^4 cells; RRID: CVCL_0006). Chimeric HM266 with human Fc was added at the indicated concentrations. Culture supernatants after 18 hours were used for quantification of IFN-γ (human IFN-γ ELISA MAX; BioLegend, catalog no. 430104). Alternatively, human $CD4^+$ T cells (2×10^5 cells) were subjected to mixed lymphocyte cultures with allogeneic human $CD19^+$ B cells (1×10^5 cells; Precision for Medicine, catalog no. 84400-1.0) for 7 days. Activated $CD4^+$ T cells (5×10^4 cells) were restimulated with the same allogeneic B cells (2×10^5 cells) in the presence of chimeric HM266 antibody. IFN-γ levels in the culture supernatants were determined after 18 hours.

Acute GVHD

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 donors and recipients, respectively. Female BDF1 mice received an intraperitoneal injection of cyclophosphamide (100 mg/kg; Wako Pure Chemical Industries, catalog no. 030-12953) before cell transfer. After 24 hours, spleen cells from female hPD-1 KI mice (5×10^7 cells per mouse) were injected intravenously into BDF1 mice. Recipient mice received intraperitoneal injections of HM266 (0.5 mg per 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 recipients' peripheral blood mononuclear cells (PBMCs).

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 intraperitoneal injections of HM266 (0.5 mg per 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 flow cytometric analysis. Lamina propria mononuclear cells were prepared as described previously (*50*). The dissected colon tissues were incubated with Hanks' balanced salt solution containing 10% FBS, 15 mM Hepes, 5 mM EDTA, and 1 mM dithiothreitol for 15 min at 37°C. After washing, the tissue samples were digested with Iscove's modified Dulbecco's medium (IMDM) containing 10% FBS, 5% NCTC-109 (Gibco, catalog no. 21340039), 0.5 mM 2-mercaptoethanol, Liberase TL (0.17 mg/ml; Roche Diagnostics, catalog no. 05401020001), and deoxyribonuclease I (30 μg/ml) 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, catalog no. 555029) for the final 2 hours of culture. The stimulated cells were stained with peridinin chlorophyll protein/cyanide 5.5 (PerCP/Cy5.5) anti-mouse CD4 and BV711 anti–hPD-1 mAbs, followed by fixation in 4% paraformaldehyde-PBS for 15 min. After washing, cells were incubated with a permeabilizing buffer (50 mM sodium chloride, 5 mM EDTA, 0.5% Triton X-100, and 10 mM tris-HCl) for 15 min, washed, and then stained with fluorescein isothiocyanate anti-mouse IFN-γ mAb and APC anti-mouse IL-17 mAb.

Immunization with NP-OVA

hPD-1 KI mice were immunized intraperitoneally with NP-OVA (10 μg per mouse; Biosearch Technologies, catalog no. N-5051) in alum. The mice received intraperitoneal injections of HM266, HM255 (500 μg per mouse), or control mouse IgG1 (MOPC-21) on days 0, 3, 7, and 10. Blood samples were collected on days 7 and 14 for the determination of anti-NP IgM levels by ELISA. The plasma samples were added to 96-well plastic plates precoated with NP-BSA (3 μg/ml; Biosearch Technologies, catalog no. N-5050XL). After washing, IgM binding was detected by biotin anti-mouse IgM mAb (0.6 μg/ml) followed by HRP-streptavidin (1:200 dilution; R&D Systems, catalog no. DY998).

Statistical analysis

Data represent means ± SEM. Statistical significance was calculated by two-tailed Student's *t* test for the comparison between two groups and by Tukey-Kramer test for the multiple comparisons of more than two groups. Comparison of natural logarithm of IgM numbers was calculated by the Holm-Bonferroni method. *P* values of less than 0.05 were considered significant. Data shown in the figures are representative of two or more experiments that essentially demonstrated similar results.

Supplementary Materials

This PDF file includes:
Figs. S1 to S9

Other Supplementary Material for this manuscript includes the following:
Table S1
Data file S1
MDAR Reproducibility Checklist

View/request a protocol for this paper from *Bio-protocol*.

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

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