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Mechanistic Assessment of PD-1H Coinhibitory Receptor–Induced T Cell Tolerance to Allogeneic Antigens

Dallas B. Flies,1 Tomoe Higuchi,1 and Lieping Chen

PD-1H is a recently identified cell surface coinhibitory molecule of the B7/CD28 immune modulatory gene family. We showed previously that single injection of a PD-1H agonistic mAb protected mice from graft-versus-host disease (GVHD). In this study, we report two distinct mechanisms operate in PD-1H–induced T cell tolerance. First, signaling via PD-1H coinhibitory receptor potently arrests alloreactive donor T cells from activation and expansion in the initiation phase. Second, donor regulatory T cells are subsequently expanded to maintain long-term tolerance and GVHD suppression. Our study reveals the crucial function of PD-1H as a coinhibitory receptor on alloreactive T cells and its function in the regulation of T cell tolerance. Therefore, PD-1H may be a target for the modulation of alloreactive T cells in GVHD and transplantation. The Journal of Immunology, 2015, 194: 000–000.

Targeting cell surface immune modulatory pathways, including costimulatory and coinhibitory molecules, has been widely investigated as a strategy for inducing T cell tolerance (1). Costimulatory and coinhibitory molecules on the surface of T cells could deliver positive and negative signals, respectively, following interaction with specific ligands or counter-receptors primarily expressed on APCs and stromal cells (2). Costimulatory receptors such as CD28 are expressed on naive T cells and function as a second signal in coordination with TCR signaling to activate T cells in most physiological settings (3). Meanwhile, expression of coinhibitory receptors such as PD-1 is characteristically upregulated on T cells following activation and suppresses T cell responses (2). Therefore, manipulating these pathways to maintain and actively induce tolerance has been investigated in detail (4–10). However, to date, strategies targeting known cosignaling molecules have proven less successful to fully inhibit T cell–mediated graft-versus-host disease (GVHD) (5, 6, 11–13).

GVHD is a reaction of donor-derived T cells that is directed against host tissues. GVHD is a major complication of hematopoietic cell transplantation for the treatment of hematologic malignancies, but it can also occur in the setting of solid organ poietic cell transplantation for the treatment of hematologic malignancies against host tissues. GVHD is a major complication of hematopoietic cell transplantation for the treatment of hematologic malignancies, but it can also occur in the setting of solid organ transplantation (1, 6, 14). In GVHD, allogeneic or MHC-mismatched T cells derived from the donor transplant are primed and activated in both lymphoid tissues and in peripheral sites, and they are largely dependent on the interaction of donor T cells with APCs displaying peptide–MHC complexes recognized by foreign donor T cells (15–21). Activation of allogeneic T cells results in broad tissue damage and even death, and it typically requires immune suppression, which then increases the risk of opportunistic infection, malignancy, and metabolic disorders (14). Therefore, developing methods of inducing hematopoietic chimerism, or the stable coexistence of host and donor blood cells, and long-term transplantation tolerance, while avoiding broad immunosuppression, remain necessary for transplantation success and improved quality of life for transplant recipients.

PD-1 homolog (PD-1H, also called VISTA) is a recently identified and broadly expressed coinhibitory molecule with dual functions as both a receptor and a ligand (22–26). PD-1H functions as a coinhibitory receptor on T cells to limit naive T cell activation, whereas PD-1H expressed on APCs interacts with an unknown receptor on T cells to suppress T cell responses (23, 24). We have previously identified agonist PD-1H–specific mAbs that potently inhibit T cell responses (22, 23), whereas another group has developed distinct PD-1H mAbs that enhance T cell immunity (24–26). In the present study, we use both PD-1H knockout (KO) mice and MH5A agonist mAb to demonstrate that PD-1H expression specifically on donor T cells is crucial for regulating alloantigen responses through initial arrest of donor T cell activation and selective expansion of donor regulatory T (Treg) cells.

Materials and Methods

Animals

All mouse procedures were performed in accordance with institutional guidelines at Yale University. Mice were maintained according to National Institutes of Health guidelines, and experimental protocols described in this study were approved by the Yale University Institutional Animal Care and Use Committee. Wild-type (WT) C57BL/6 (B6) C57BL/6 × DBA/2 F1 (BDF1), and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). PD-1H KO mice (gene symbol 4632428N05Rik) were purchased from the Mutant Mouse Regional Resource Center (University of California–Davis) as described in Flies et al. (23). B6.albino β-actin luciferase mice (stock no. 10500) were purchased from Taconic Biosciences (Hudson, NY).

GVHD models

For GVHD models, mice were irradiated with 12 Gy (BDF1) or 10 Gy (B6) 12 h prior to adoptive transfer of MACS-isolated splenic and lymph node (LN) pan T cells (Miltenyi Biotec) and T cell–depleted bone marrow (TCD-...
BM) MACS Thy1.2 microbeads as previously described (22). For the B6 to BDF1 GVHD/CTL model, mice were not irradiated, and $2.5 \times 10^7$ total splenocytes per mouse were adoptively transferred. For CD25 depletion, pan T cells were isolated with CD25-biotin (BD Biosciences) and anti-biotin microbeads (Miltenyi Biotec) and passed over LD depletion columns (Miltenyi Biotec). Two hundred micrograms per mouse MHS A or control Ab was injected via tail vein, and mice were monitored daily for all experiments.

**GVHD model for in vivo imaging analysis**

BALB/c recipients were lethally irradiated (9 Gy) 12 h prior to adoptive transfer. Donor T cells were isolated from B6.albino-β-actin-luciferase (B6.luc) mouse LNs and purified with a mouse pan T cell negative selection kit (Miltenyi Biotec). BM was isolated as above from WT B6 mice and T cells were depleted as described above. T cells ($2 \times 10^7$) and $5 \times 10^7$ TCD-BM cells were adoptively transferred with 200 μg MHS A or control Ab via tail vein ($t = 0$ h) in a total volume of 300 μl PBS.

Prior to imaging, mice were i.p. injected with 300 μg luciferin substrate ~5 min prior to being anesthetized using the XRT-8 gas (isoflurane) anesthesia system according to protocol. Anesthesia was maintained while mice were imaged for bioluminescence in the right lateral and ventral positions using an IVIS Lumina XR in vivo imaging system (Caliper/Life Science, Waltham, MA) according to protocol. Briefly, luminescent detection was set to automatic with a minimum detection level of 3000 photons. Mice were imaged on stage D at 1.5 cm height from stage. Units were set to radiance (photons/s). Imaging and analysis were performed using Living Image software (Caliper/Life Science). For analysis, binning was set to 4, and minimum and maximum radiance levels were determined for optimal view and comparison between groups at each time point. Calculation of total flux was determined by selecting regions of interest (e.g., specific gating whole mouse, cervical LN) and measuring the total intensity in the region calculated as total flux (radiance, or photons/s) in each pixel summed or integrated over the region of interest area ($cm^2 \times 4\mu m$) by Living Image software.

**Abs, cytokines, kits, and reagents**

MHS A was previously developed by immunizing Armenian hamsters with a mouse PD-1H Ig fusion protein and Freund’s adjuvant as previously described in detail (22). Control hamster IgG was purchased from Rockland Immunochemicals (Gilbertsville, PA). Fluorescently labeled Abs for flow cytometry, including CD4, CD8, H-2Kd, IFN-γ, CD19, CD25, Ge-1, CD11b, DX5, and anti-hamster IgG-biotin, were purchased from BD Biosciences (San Jose, CA). CD3 and CD28 mAbs for cell culture were also purchased from BD Biosciences. Annexin V and 7-amanotoaminomycin D (7-AAD) were purchased from BD Biosciences. Foxp3-allophyococyanin, Foxp3-FITC, streptavidin-allophyococyanin and PerCP, and TNF-α ELISA were purchased from eBioscience (San Diego, CA). K67-allophyococyanin and Zombie NIR were purchased from BioLegend. CFSE (Vybrant CFDA SE, Life Technologies) and R1-labeled CFDA (Vybrant CFDA-D1 cell tracer kit) was purchased from Life Technologies. TGF-β and IL-2 were purchased from R&D Systems (Minneapolis, MN).

**Flow cytometry**

Splenocytes and/or LNs were disaggregated with frosted class slides, passed through a 100-μm screen, and RBCs were lysed with ACK lysis buffer. Livers were disaggregated using a gentleMACS system (Miltenyi Biotec) and lymphocytes were isolated in Percoll diluted to 35% in PBS (Sigma-Aldrich) and centrifugation at 1000 x g for 20 min at room temperature. For flow cytometry analysis, donor cells were differentiated from recipient cells based on H-2Kd staining (B6, H-2Kd+: BALB/c, H-2Kb+). Foxp3 and K67 staining was performed using an eBioscience Foxp3 staining kit according to instructions. In some experiments, cells were restimulated ex vivo with PMA (Sigma-Aldrich) at 1 μg/ml, ionomycin at 50 ng/ml (Sigma-Aldrich), and GolgiStop (BD Biosciences) for 5 h prior to intracellular staining for IFN-γ using a BD Biosciences intracellular staining kit according to protocol. Annexin V and 7-AAD staining were performed according to BD Biosciences protocols. Zombie NIR staining was performed according to BioLegend protocol. Splenocytes for tolerance studies were labeled with 2 μM CFSE for 10 min in RPMI complete medium in a 37°C water bath followed by two washes in excess RPMI complete medium. CFSE-labeled cells were then added to 48-well plates at a 1:1 ratio with 4 Gy irradiated B6 splenocytes and restimulated for 72 h followed by staining of cells with H-2Kd, CD4, and CD8 for flow cytometry analysis of CFSE dilution. Flow cytometry was performed using a FACSCalibur or LSRFortessa (BD Biosciences).

**In vitro Treg cell induction, cell proliferation, and Treg cell suppression in an MLR assay**

Treg cells were induced in 24-well plates with coated anti-CD3 (5 μg/ml coated overnight at 4°C in PBS), soluble anti-CD28 (1 μg/ml), IL-2 (5 ng/ml), and TGF-β at 5 ng/ml or concentrations indicated. Control Ab or MHS A was added at 10 μg/ml where indicated. For Treg cell proliferation, Treg cell cultured for 5 d as above were MACS isolated by CD25 positive selection and plated in 96-well plates precoated with anti-CD3 at indicated concentrations and IL-2 (5 ng/ml). [3H]thymidine (PerkinElmer) was added to wells during the final 8 h of culture and cells were analyzed for DNA incorporation (proliferation) using a TriLux MicroBeta (PerkinElmer). Treg cell suppressive function in the presence of MHS A was analyzed in an MLR assay using induced Treg cells as above. Treg cells were incubated with either control Ab or MHS A for 1 h and then washed and added to 96-well round-bottom plates containing B6 T cells (responders) and lethally irradiated BDF1 target splenocytes. Ratio indicates the Treg cells/responder cells, with responder cells remaining consistent in all wells, and Treg cells decreasing along the x-axis of the graph. Cells were cultured for 5 d. [3H] thymidine was added during the final hours of culture and analyzed as above.

**Graphs and statistical analysis**

Graphs and statistical analyses were generated with GraphPad Prism (GraphPad Software) and Microsoft Excel. Statistical analyses of survival experiments were performed using a log rank (Mantel–Cox) test; all other analyses were performed by a one-tailed, equal variance t-test. A p value < 0.05 was considered significant.

**Results**

**PD-1H mAb prevents damage of alloreactive T cells in GVHD target organs**

We previously showed in mouse models of GVHD that a single dose of anti–PD-1H mAb (clone MH5A) on day 0 protects nearly 100% of mice from lethality (22). MH5A was demonstrated to be effective at a range of doses (50–250 μg) when administered on day 0, without any observed toxicity at this dose range (Supplemental Fig. 1). To examine whether MHS A functionally inhibited both donor T cell expansion and cytoxic toxicity, we used a GVHD/CTL model in which total splenocytes from B6 (H-2d) WT mice were adoptively transferred to partially MHC-mismatched, or semiallogeneic, BDF1 (H-2b/k) mice and treated with MHS A or control IgG on day 0. In this model, donor cells recognize the recipient cells as foreign because of MHC H-2d expression, whereas BDF1 mice recognize B6 cells as self because of MHC H-2b expression. Therefore, only donor cells will attack recipient cells, but not vice versa. Importantly, because recipients are not irradiated, we are able to monitor donor-derived cytotoxic killing of recipient B cells and T cells, which are major targets in acute GVHD, while simultaneously monitoring the expansion of alloreactive donor T cells. Mice treated with MHS A had significantly reduced expansion of donor CD4+ and CD8+ T cells, whereas there was no loss of recipient CD19+ B cells, CD4+ T cells, and CD8+ T cells in comparison with control IgG-treated mice, which had significant losses in all three cell populations (Fig. 1). This finding indicates that MHS A directly inhibits alloreactive T cell expansion and cytokotoxic function.

Because recipient T cell numbers were unchanged in the in vivo GVHD/CTL assay, it was unlikely that MHS A was cytotoxic or depleting in vivo. However, we performed several studies to rule out these potential effects. Using a B6 to BDF1 GVHD model in which recipient BDF1 mice were lethally irradiated to mimic the setting of human GVHD, we analyzed splenocytes 2–72 h after adoptive T cell transfer for CD4+ and CD8+ cell accumulation. Early accumulations of donor CD4+ and CD8+ T cell numbers were decreased in MH5A-treated mice compared with control IgG-treated mice (Supplemental Fig. 2A), which confirmed our previous studies (22). However, when viable donor cells were assayed for PD-1H expression, we found that...
PD-1H was greatly enhanced in the presence of MH5A, suggesting that MH5A did not deplete PD-1H+ T cells (Supplemental Fig. 2B). Additionally, there was no difference in the percentage of apoptotic/dead donor CD4+ and CD8+ T cells, as measured by 7-AAD staining, in MH5A- and control IgG-treated mice, further suggesting that MH5A was not cytotoxic in vivo (Supplemental Fig. 2C). To further validate that MH5A did not deplete PD-1H+ T cells, we determined that MH5A treatment of normal WT mice had no effect on T cell numbers (Supplemental Fig. 2D) and that MH5A did not induce complement-mediated lysis of PD-1H+ T cells in vitro (Supplemental Fig. 2E). Taken together, these data suggest that MH5A does not induce cytotoxic, depleting, or apoptotic mechanisms of donor T cell death.

MH5A mediates early and systemic control of alloreactive T cells in vivo

To visually examine MH5A-mediated modulation of allogeneic T cell responses in vivo, we used a bioluminescent imaging model of GVHD. Bioluminescent imaging has proven very successful in GVHD studies for monitoring cellular responses (27). In this model, T cells from albino B6 transgenic mice expressing luciferase under the ubiquitous β-actin promoter (B6.luc) (28, 29) were purified and adoptively transferred together with WT B6 TCD-BM cells (to avoid bioluminescent interference from BM cells) to lethally irradiated BALB/c (H-2d) recipients, thus demonstrating that PD-1H expression on recipient cells has little effect on the regulation of allogeneic T cell responses. To examine whether the absence of PD-1H expression on recipient cells (non–T cells) would also exacerbate GVHD, we adoptively transferred BALB/c WT T cells (PD-1H+) and TCD-BM cells to lethally irradiated PD-1H KO or WT B6 recipients. Interestingly, we observed a slightly increased rather than decreased survival time in PD-1H KO recipients, thus demonstrating that PD-1H expression on recipient cells had little effect on the regulation of allogeneic T cell responses (Fig. 3D). Taken together, these results show that PD-1H expression on T cells, but not other cell subsets, such as APCs, in which PD-1H could potentially function as an inhibitory ligand (24–26), is crucial for modulating T cell responses in GVHD.

PD-1H expression on T cells is essential for MH5A-directed suppression of murine GVHD

Our results indicate that PD-1H expression on T cells is crucial for controlling donor T cell responses in GVHD. However, it was unknown whether MH5A-mediated inhibition required PD-1H expression on donor T cells. To investigate this, we used both WT (PD-1H+) and PD-1H KO T cells in combination with MH5A treatment. First, PD-1H+ T cells from BALB/c mice were adoptively transferred to lethally irradiated PD-1H KO recipients on a B6 background with either MH5A treatment or control IgG. Spleens and livers were then analyzed 6 d later. In this setting, in which PD-1H was expressed on donor T cells but not on recipient
FIGURE 2. MH5A inhibits systemic expansion of alloreactive donor T cells. B6.luc transgenic donor T cells and WT B6 TCD-BM cells were adoptively transferred to lethally irradiated BALB/c recipients with control Ab (A and C) or MH5A (B and D). Mice were imaged for bioluminescence at 2, 24, 48, 96, and 120 h for T cell accumulation and expansion measured as radiance for relative comparison between groups. Ventral (A and B) and right lateral (C and D) imaging of two mice per treatment group are shown as representatives of five mice per group. Minimum and maximum radiance levels and range are indicated at each time point for optimal view. Gating of whole body (E), cervical LN region (F), and abdominal region (G) in the ventral position was performed for calculation of total flux. *p < 0.05, **p < 0.025, ***p < 0.005.
cells, MH5A potently inhibited WT (PD-1H) donor T cell accumulation in the spleen and liver compared with control Ab, showing that PD-1H expressed specifically on donor T cells was sufficient for MH5A-mediated T cell inhibition (Fig. 4A). Second, we adoptively transferred PD-1H KO T cells from B6 mice to PD-1H-sufficient BDF1 recipients with either MH5A or control IgG.

FIGURE 3. Adoptive transfer of PD-1H-deficient donor T cells exacerbates GVHD. (A) PD-1H KO donor T cells plus TCD-BM cells or WT littermate donor T cells plus TCD-BM cells from B6 background were adoptively transferred to lethally irradiated WT BDF1 recipients and monitored for survival. (B) Splenocytes and liver lymphocytes were isolated at indicated time points and absolute number of T cells was determined. (C) Total splenocytes or liver lymphocytes were restimulated ex vivo for 5 h with PMA, ionomycin, and GolgiStop and intracellularly stained for IFN-γ production. (D) WT BALB/c LN cells and BM were adoptively transferred to lethally irradiated B6 WT or PD-1H KO recipients and monitored for survival. Five mice per group were used for survival experiments, and three mice per group were used for time point analyses. Experiments were repeated two to three times. *p < 0.05, **p < 0.025.
treatment. In this setting, MH5A did not inhibit, but actually slightly enhanced, T cell accumulation in the spleen or liver compared with control-treated mice (Fig. 4B). We next performed a GVHD survival study in which either purified PD-1H KO or WT PD-1H+ T cells from a B6 background were adoptively transferred to lethally irradiated BDF1 recipients with MH5A or control Ab. Splenocytes and liver lymphocytes were isolated on day 7 for determination of absolute number of donor CD4+ and CD8+ T cells. (C) PD-1H KO T cells plus TCD-BM cells or WT littermate T cells plus TCD-BM cells from B6 mice were adoptively transferred to lethally irradiated BDF1 recipients with MH5A or control Ab and mice were monitored for survival. Three mice per group were analyzed for T cell numbers, and five mice per group were used for survival studies. Experiments were repeated two to three times. *p < 0.05, **p < 0.025, ***p < 0.005.

MH5A mAb promotes tolerance in donor T cells

Our results clearly demonstrated that MH5A treatment inhibited PD-1H+ alloreactive T cell expansion and function. However, mechanisms of donor T cell tolerance remained undefined. To investigate donor T cell tolerance induction, we used the fully MHC-mismatched model of GVHD in which BALB/c donor T cells were adoptively transferred to lethally irradiated B6 recipient mice with MH5A or control IgG treatment. As positive and negative controls for tolerance induction experiments, syngeneic adoptive transfers of BALB/c to lethally irradiated BALB/c mice and B6 to lethally irradiated B6 mice were included. In the BALB/c to B6 GHVD model, we have previously shown that MH5A treatment inhibits early donor T cell accumulation and function and promotes long-term survival (22). Paradoxically, we noticed that by day 14 total splenocyte numbers were increased in MH5A-treated mice compared with controls (Fig. 5A). This was also evident by visual observation of enlarged spleens (Fig. 5B). Initial examination of T cell numbers on day 14 showed that MH5A-treated mice had increased numbers of donor CD4+ T cells but no change in CD8+ T cells compared with control IgG-treated mice (Fig. 5C, 5D). Interestingly, despite the splenomegaly in MH5A-treated mice, the spleen appeared healthy, similar to syngeneic controls. Meanwhile, the control-treated spleens were fibrotic and discolored, indicating
Donor T cell tolerance is induced by prophylactic MH5A treatment. BALB/c T cells plus TCD-BM cells were adoptively transferred to lethally irradiated B6 recipients with MH5A or control Ab. Syngeneic adoptive transfers of BALB/c to lethally irradiated BALB/c and B6 to lethally irradiated B6 were also performed for controls in T cell tolerance studies. (A) Spleens were isolated on day 14 and analyzed for total splenocytes. (B) Images of spleens isolated from indicated mice on day 14. (C and D) Donor CD4+ and CD8+ T cell numbers on day 14. (E) Day 14 total splenocytes were labeled with CFSE and restimulated ex vivo with irradiated B6 splenocytes for 72 h. Donor CD4+ and CD8+ T cells were analyzed by flow cytometry for cell division as determined by CFSE dilution. Syngeneic BALB/c to BALB/c splenocytes were used as positive controls. (Figure legend continues)
damage that we have routinely observed in the spleens of mice with acute GVHD. The spleen appearance correlated with the overall health of MHSA- versus control IgG-treated mice.

To investigate whether donor T cells were tolerized in MHSA-treated mice, we performed a modified MLR assay in vitro. In this experimental setting, total splenocytes from day 14 GVHD mice (as described above) were labeled with CFSE and cultured with irradiated B6 splenocytes for 72 h. For controls, T cells isolated from the BALB/c to BALB/c adoptive transfer mice would serve as a positive control for responsiveness to irradiated allogeneic B6 splenocytes, whereas T cells from the B6 to B6 adoptive transfer mice should have no response against syngeneic B6 cells (negative control). Division of donor CD4+ and CD8+ T cells was then determined at 72 h by CFSE dilution (Fig. 5E, 5F). Whereas donor CD4+ and CD8+ T cells isolated from control IgG-treated mice proliferated vigorously, donor T cells from MHSA-treated mice had significantly reduced proliferation.

This finding suggested that early restraint of alloreactive T cell activation by MHSA resulted in T cell tolerance or suppression. In support of this notion, we found that the time of MHSA administration was crucial for GVHD inhibition. In the BALB/c to lethally irradiated B6 GVHD survival model, MHSA was administered 1 d before, the same day, or 3 d after adoptive transfer of BALB/c T cells + TCD-BM cells. We found that treatment with MHSA on day −1 or day 0 effectively prevented GVHD lethality, whereas treatment on day 3 was not protective (Fig. 5G). These findings supported the notion that regulation of alloreactive T cells through PD-1H signaling on T cells during T cell priming and activation was required for subsequent tolerance. However, mechanisms through which MHSA treatment maintained long-term tolerance despite the expansion of donor T cells required additional study.

**T cell expansion maintains tolerance in MHSA-treated mice**

Because donor CD4+ T cells were significantly increased in MHSA-treated mice on day 14 (Fig. 5C) along with the induction of long-term tolerance and survival, we postulated that CD4+ Treg cells may be increased as a mechanism of alloreactive T cell suppression. Indeed, we found significantly increased Foxp3+ Treg cells in MHSA-treated mice on day 14 (Fig. 6A). Moreover, CD4+Foxp3+ Treg cell percentages increased in MHSA-treated mice, whereas CD4+Foxp3− Treg cell percentages actually decreased over time in control IgG-treated mice (Fig. 6B). The ratio of both CD8+ T cells and CD4+ T cells to Treg cells was significantly reduced in MHSA-treated mice on day 14 (Fig. 6C, 6D). However, the absolute number of Treg cells in the spleen was only transiently increased in MHSA-treated mice compared with controls (Fig. 6E), whereas a time course analysis of the ratio of donor CD8+ cells to donor Treg cells clearly illustrated an altered cell composition in MHSA-treated mice compared with controls (Fig. 6F). These findings supported the direct suppression of effector T cells by PD-1H signaling, rather than direct effects on Treg cells. Concomitantly, IFN-γ production was reduced in CD4+ and CD8+ T cells on day 20 in MHSA-treated mice (Fig. 6F, FG), whereas serum TNF-α levels were also significantly reduced by day 20 in MHSA-treated mice (Fig. 6I). These findings support a model in which donor effector T cells are directly suppressed by PD-1H signaling, thus allowing for the selective expansion of donor Treg cells, which further suppresses alloreactive donor T cells to maintain tolerance.

**T cells are selectively promoted by MHSA in vivo**

Because the most CD4+Foxp3− naive T cells and CD4+Foxp3+ Treg cells express PD-1H (Fig. 7A), it is possible that MHSA directly regulates both naive and Treg cells. To examine the possibility that MHSA could induce Treg cells, we performed an in vitro Treg cell conversion assay. CFSE-labeled naive CD4+ T cells were cultured with IL-2 and titrated doses of TGF-β in the presence of MHSA or control IgG and monitored for proliferation and Foxp3 expression. We observed a slight but insignificant increase in Foxp3+ Treg cells in the presence of MHSA (Fig. 7B), thus suggesting that MHSA does not enhance Treg conversion in vitro. Next, we examined whether the proliferative potential of induced Treg cells was changed in the presence of MHSA mAb. To do this, Tregs were induced for 5 d in the presence of TGF-β and were then added to 96-well plates coated with anti-CD3 in the presence of 5 ng/ml IL-2, and with either control IgG or MHSA. No significant differences in proliferation were found 72 h later (Fig. 7C). B6 Treg cells induced in vitro were also used in an MLR assay in which 5-d-induced Treg cells were preincubated with either control IgG or MHSA and then washed and added at various ratios to an MLR of B6 responder cells and irradiated BDF1 splenocytes as targets. Treg cells in this assay were capable of suppressing equally well in the presence of control IgG or MHSA (Fig. 7D). These findings suggest that MHSA does not directly induce the conversion or expansion of Treg cells in vivo or alter the suppressive function of Treg cells, although it remains unknown whether the presence of additional factors in vivo that are not present in vitro may enhance MHSA effects on Treg cells in vivo.

To investigate whether MHSA promoted Foxp3+ Treg cell expansion and/or conversion in vivo, total T cells or CD25-depleted naive T cells were adoptively transferred with TCD-BM cells from B6 donors to lethally irradiated BDF1 mice. Mice receiving total T cells or CD25-depleted T cells were treated with MHSA or control IgG on day 0. Spleens of these mice were examined on days 5, 10, and 15 for the number of CD4+Foxp3+ Treg cells and CD8+ T cells. We found that MHSA treatment resulted in enhanced expansion of donor Treg cells in both adoptive transfer models (Fig. 7E, 7F). Concordantly, MHSA treatment led to a significant decrease in the ratio of CD8+ T cells to Treg cells in both settings (Fig. 7G, 7H). These in vivo data showed that MHSA selectively promotes Treg cell expansion, possibly through Treg cell conversion in vivo through direct or indirect mechanisms. In support of Treg cell conversion, we found little difference in proliferation or viability in Treg cells on days 10, 15, and 20 as measured by Ki67 and a fixable cell viability marker, respectively (Supplemental Fig. 3).

**Discussion**

We have previously shown that engagement of the PD-1H coinhibitory receptor by agonistic mAb has a profound effect in suppressing various types of T cell responses, including those to alloreactive T cell responses, and ameliorates GVHD in mouse models. The underlying mechanism, however, remains to be elucidated. Our studies reveal two possible immunological mecha-
FIGURE 6. Treg cells are transiently increased and promote tolerance in MH5A-treated mice. BALB/c T cells plus TCD-BM cells were adoptively transferred to lethally irradiated B6 mice with MH5A or control Ab. (A) Day 14 splenocytes were stained for surface CD4 and CD25 and for intracellular Foxp3 expression. (B) Representative dot plots of splenocyte Treg cell percentages in spleen. Plots are gated on CD4. On the far left are cells adoptively transferred, and on the right is analysis of Foxp3 and CD25 at indicated time points. (C and D) The ratio of the absolute number of donor CD8+ T cells to CD4+CD25+Foxp3+ T cells (C) and CD4+CD25+Foxp3+ T cells to CD4+CD25+Foxp3+ T cells (D) was calculated on day 14. (E) Treg absolute cell numbers in the spleen on days 5, 10, 15, and 20. (F) The ratio of the absolute number donor CD8+ T cells to CD4+CD25+Foxp3+ T cells was calculated at the indicated time points. Red lines show trends over time. (G and H) Total splenocytes harvested on days 15 and 20 were restimulated ex vivo with PMA, ionomycin, and GolgiStop for 5 h and stained for intracellular levels of IFN-γ. Cells were gated on CD4+ or CD8+ T cells as indicated. (H) Serum from GVHD mice was isolated at the indicated time points and analyzed by ELISA for TNF-α levels. *p < 0.05, **p < 0.025, ***p < 0.005.
nisms: prevention of early T cell priming upon engagement of allogeneic Ag, and subsequent induction of Treg cells in vivo. In the GVHD models described in the present study, cellular analysis and in vivo imaging demonstrate that engagement of PD-1H results in arrest of T cell expansion, an important prerequisite for the induction of T cell tolerance/anergy. Subsequently increased Treg cells in lymphoid organs provide another mechanism in the maintenance of long-term tolerance for allogeneic Ags. Overall, these findings support a two-stage model of PD-1H coinhibitory receptor–directed tolerance induction.

Although the nature of the PD-1H signaling pathways involved in suppressing T cell responses has yet to be elucidated, PD-1H engagement appears to “imprint” or program T cells with a tolerant status, which results in alloreactive T cells being unable to fully respond to alloantigens. We noted that MH5A-treated mice had similar radiance levels in the whole body and in lymphoid organs as untreated mice, indicating that PD-1H engagement can effectively suppress T cell responses in vivo.
The observation of T cell imprinting is consistent with the promotion of Treg cells as a major suppressive mechanism. Because we have been unable to show that MH5A directly affects Treg cells in vitro, MH5A may indirectly promote Treg expansion through alloreactive T cell modulation and reduction in IFN-γ and TNF-α levels (Fig. 6). We have found that neutrophils largely outnumbered all other cell subsets following lethal irradiation and BM reconstitution in both control- and MH5A-treated mice, suggesting rapid reconstitution of neutrophils (D.B. Fliies, T. Higuchi, and L. Chen, unpublished observation). We have shown previously that neutrophils constitutively express PD-1H (22). It is tempting to speculate that engagement of PD-1H on neutrophils may suppress their functions and indirectly affect Treg induction. However, because most CD4+Foxp3+ Treg cells also express PD-1H, it remains possible that MH5A may directly regulate CD4+Foxp3+ Treg cells through PD-1H signaling. Interestingly, it has been shown that cohibitory molecules often have differing functions on Treg cells and conventional T cells (31). Our results indicate that PD-1H signaling may use multiple pathways to influence T cell function.

In summary, our studies indicate that targeting of PD-1H on T cells imprint a tolerogenic status on alloreactive T cells and selectively promotes donor Treg cell expansion, which together result in long-term T cell tolerance induction. Therefore, our findings implicate that prophylactic agonist targeting of PD-1H may be an effective therapeutic modality for promoting functional T cell tolerance to alloantigens in human transplantation.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. Titration of MH5A dose in GVHD. In the B6 to lethally irradiated BDF1 model of GVHD, MH5A was administered at the indicated dose at the time of adoptive transfer (day 0). Mice were monitored for survival.
Supplemental Figure 2. MH5A does not deplete T cells in vivo and is not cytotoxic in vitro. (A-C) MACS purified T cells and TCD-BM from wt B6 mice were adoptively transferred to lethally irradiated BDF1 mice with MH5A or control Ab. Splenocytes were isolated at indicated time points for analysis of (A) donor CD4+ and CD8+ T cell numbers, (B) PD-1H surface expression on viable (7-AAD negative) cells, and (C) apoptotic/dead cells by 7-AAD staining. 3 mice/group were examined at each time point repeated three times. (D) Wt B6 mice were intraperitoneally injected with 300 ug of MH5A or control Ab and splenocytes were harvested 72 hours later and counted and stained for various cell subsets for flow cytometry analysis. Absolute cell numbers were calculated by multiplying total viable cell counts with percent of total/100 as determined by flow cytometry (cell count x (% total/100)). 3 mice were analyzed per group. (E) An in vitro complement-mediated lysis assay was performed using CD4+ T cells isolated from the spleen of C57BL/6 wt mouse. CD4+ T cells expressing PD-1H were incubated with MH5A, control hamster IgG or CD4 mAb (clone GK1.5) as a positive control for 30 minutes on ice, followed by the addition of baby-rabbit complement (10:1 dilution) and incubation at 37 degrees C for 15 minutes. Cells were then stained with 7-AAD for 5-10 minutes and immediately analyzed by flow cytometry to determine cell viability.
Supplemental Figure 3. Treg cell expansion and viability are similar in control and MH5A treated mice. In the Balb/c to lethally irradiated B6 GVHD model, splenocytes were isolated on days 10, 15 and 20 and analyzed by flow cytometry. CD4+FoxP3+ donor T cells were stained for the proliferation marker Ki67 and for viability with a fixable viability marker prior to intracellular staining for FoxP3 and Ki67.