Original Paper

Blocking CD226 Promotes Allogeneic Transplant Immune Tolerance and Improves Skin Graft Survival by Increasing the Frequency of Regulatory T Cells in a Murine Model

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Key Words

CD226 • Transplant immunity • Treg

Abstract

Background/Aims: Regulatory T cells (Tregs) play key roles in maintaining peripheral tolerance and preventing autoimmune disease. Treg modulation could be helpful in treating malignancies, autoimmune disease, and allergies, as well as to facilitate organ transplantation. Signals transduced by co-stimulatory molecules are essential for Treg differentiation, homeostasis, and function. One well-known active receptor, CD226, also known as DNAM-1 or PTA1, is an adhesion molecule that interacts primarily with CD155 and is involved in Treg differentiation and immune tolerance to transplanted tissue. **Methods:** Anti-CD226 monoclonal antibody (mAb) and truncated recombinant CD226 proteins were employed to manipulate the CD226 signal. Various T cell markers on freshly isolated splenocytes and T lymphocytes were characterized by flow cytometry. Cell proliferation was measured by carboxyfluorescein succinimidyl ester dye, mRNA transcripts by q-RT PCR, and protein expression by western blotting. A BALB/c-to-C57BL/6 skin allograft model was used to determine the effects of CD226 blocking treatment. **Results:** We observed that both intact extracellular domains of CD226 were necessary for functional interaction of the receptor with its ligand CD155, even though one domain was shown to bind CD155 with lower affinity in a solid binding assay. Importantly, CD226 mAb promoted Treg expansion in a mixed lymphocyte culture and inhibited the cytotoxicity of effector cells. In allogeneic skin transplant mice, administering

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CD226 mAb reduced inflammation and prolonged allogeneic graft survival, with an increase in the frequency of Tregs. **Conclusions:** Our results reveal the mechanism underlying CD226-CD155 interactions and indicate that CD226 signals can be manipulated to promote Treg expansion. Moreover, we provide new evidence that suggests the therapeutic potential of anti-CD226 with allogeneic transplantation.

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Introduction

The basis of an immune response is associated with the ability to purge autoreactive lymphocytes, impose peripheral tolerance, and improve responses against damaging or dangerous signals. T cell responses are controlled by multiple co-stimulatory and co-inhibitory receptors. T cell responses, activation, differentiation, and proliferation are regulated through the activation of various stimulating or inhibiting receptors. Among the varieties of T cells, CD4⁺CD25⁺ Tregs, which express the forkhead family transcription factor (Foxp3), are a key component in maintaining peripheral tolerance that protects us from autoimmune disease and prevents allograft rejection. Tregs induction, proliferation and function were modulated by various microenvironment factors, such as cytokine, chemokine, hormone, and hypoxia [1]. The balance of co-stimulatory and coinhibitory receptors is critical for the production and maintenance of Tregs, and their expression on Tregs regulates their homeostatic and suppressor functions [2]. Understanding the function of co-stimulatory receptors in Tregs is at the forefront of strategies to treat tumors and chronic inflammation diseases and prevent transplant rejection.

Over the past couple of years, CD226 has emerged as an important co-stimulatory receptor. In previous research, CD226 was mainly considered an active receptor expressed on natural killer (NK) cells and activated T cells [3] that played a role in immune synapse formation during cell cytotoxicity. Its antigen-presenting cell (APC) ligands are CD155 and CD112/CD113, which are shared with the principal co-inhibitory receptor T cell Ig and ITIM domain (TIGIT). Together with TIGIT, CD226 comprises a pathway that closely parallels the CTLA-4/CD28 pathway. Similar to CD28 and CTLA-4, CD226 and TIGIT mainly share CD155 ligands, and CD112/CD113 in particular situations [4]. Interactions between CD226/TIGIT and its ligands are involved in many immunological activities, including NK cell education and cytotoxicity, naive T cell differentiation and proliferation, and platelet activation and aggregation [5, 6].

Tregs play an essential role in maintaining immune stability and preventing autoimmune diseases [7-9]. Recent studies have shown that the absence of CD226 promotes Treg proliferation and the suppressive function of Tregs in a murine graft-versus-host disease (GVHD) model [10, 11]. Anti-CD226 treatment was shown to reduce the development of GVHD and alleviate the severity of developed GVHD, which is dependent on an increase in Foxp3⁺CD4⁺ Tregs. Expression of CD226 on Tregs and T conventional (Tconv) cells is upregulated after bone marrow transplantation. Furthermore, CD226 divides human Treg cells into different groups [12]. CD226⁻ Tregs are more stable than CD226⁺ Tregs during *in vitro* expansion, indicating that CD226 expression affects Treg function. However, the mechanism underlying CD226 function and its effects on Tregs in solid organ transplantation are largely unknown.

CD226 can transmit costimulatory signals and then induce LFA-1 aggregation. The N-terminal extracellular region of CD226 consists of two immunoglobulin V-like domains (D1 and D2), which allow binding to CD155 and CD226 function. A recent structural study indicated that CD226 binds its ligands mainly via its first N-terminal IgV-like domain [13]. Another functional study revealed that the first extracellular domain of CD226 (CD226-D1) primarily mediates NK cell recognition, adhesion, immune synapse formation, and cytotoxicity against target cells [14]. However, for other cell types, whether CD226-D1, the first extracellular domain of the protein, is fully functional remains unknown.

In our study, we explored the role of CD226 in allogeneic transplantation and the mechanism underlying this function. We examined the effects of anti-CD226 treatment

on lymphocyte reactions both *in vitro* and in a transplantation model, and measured the percentage of Tregs. We also determined the roles of the two ectodomains of CD226 regarding Treg suppressive function.

Materials and Methods

Mice

Wild-type C57BL/6 (B6, H-2^b), and BALB/c (H-2^d) mice were provided by the animal center of Fourth Military Medical University and housed under specific pathogen-free (SPF) conditions. All experimental procedures were performed on 6- to 8-week-old sex- and age-matched female mice. Animal care and experimental protocols were approved by the Fourth Military Medical University Animal Experimental Committee.

Antibodies and reagents

The following flow cytometry antibodies used in this experiment were purchased from BD Biosciences, eBioscience, or BioLegend. FITC: CD4 (Clone: RM4-5), H-2^b (Clone: AF6-88.5.5.3); PE: Foxp3 (Clone: FJK-16s), CD226, (Clone: 10E5); PerCp-Cy5.5: CD4 (Clone: GK1.5); APC: CD25 (Clone: PC61.5), H-2^b (Clone: AF6-88.5.5.3), CD155 (Clone: TX56), and CD226 (Clone: 10E5). Other reagents used in this experiment were anti-mouse CD226 functional grade (Clone: 10E5, eBioscience, Cat #: 16-2261), rat IgG2b κ isotype control functional grade purified (eBioscience, Cat #: 16-4031), BrdU cell proliferation ELISA (Roche, Cat #: 11647229001), Carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (Thermo Fisher, Cat #: C34544), Mitomycin C (MP Biomedicals, Cat #: 02194532), Dynabeads mouse T activator CD3/CD28 (Thermo Fisher, Cat #: 11452D), and mouse CD4⁺CD62L⁺ T cell isolation kit (Miltenyi, Cat #: 130-106-643). Mouse CD226-Fc recombinant protein was custom made by Chimerigen (Taizhou, China, Lot #: CHI-MF-111CD226-C500).

Vector construction and recombinant CD226/CD155 expression

All sequences of human and mouse CD226 and CD155 were accessed in the National Center for Biotechnology Information (NCBI) Database. The sequences of primers for the mouse CD226 extracellular domains were: m226ECD1-F, 5'-GAAGATCTAGTGCTTATGTTACT-3', and m226ECD1-R, 5'-GGGGTACCTCTGACCAACTAC-3'; and m226ECD2-F, 5'-GGGGTACCAGATAGTTTTGAGATAGCA3', and m226-R, 5'-GGGGATCCCGAGTCTTGGTTTGG-3', which amplified the first and second extracellular domains of mouse CD226, respectively. Two different deletion mutants were cloned by RT-PCR from CTLL cell total mRNA. The sequences of primers for full-length mouse CD155 were: m155-F, 5'-GAAGATCTATGGCCCCACTCGCC-3', and m155-R, 5'-CGGAATTCGCCTGTGCTGTTTGG-3'. Mouse CD226D1 and CD226D2 were constructed using the eukaryotic expression vector pEGFP-N1, which encodes the target extracellular domain linked with transmembrane and cytoplasmic sequences with a C-terminal GFP tag. Mouse CD155 was constructed using the eukaryotic expression vector pDsRed, which encodes mouse CD155 protein with a C-terminal RFP tag. The other sequences of primers for mouse CD226 extracellular domain were: m226ECD-F, 5'-GGGGTACCTGAAGAGACATTGTGGGAC-3', and m226ECD-R, 5'-CGGGATCCATAAAATGTTTATTGGTTCC-3'. The target fragment was constructed using the eukaryotic expression vector p p3C-Ig/m226ECD, which encodes the mouse CD226 extracellular domain with a C-terminal human Fc tag. The sequences of primers for the human CD226 extracellular domain were: h226ECD1-F, 5'-AAGCTTGAAGAGGTGCTTTGGCAT-3', and h226ECD1-R, 5'-CGGGATCCACAGCTGCCTCAAAACT-3'; and h226ECD2-F, 5'-AAGCTTCCATCAAATAGCCACATT3', and h226ECD2-R, 5'-GGGGTACCGTATATTGGTT-3'. Two different deletion mutants containing the first and second extracellular domains of human CD226, respectively, CD226D1 (19–134 amino acids) and CD226D2 (135–251 amino acids), were cloned by RT-PCR from total mRNA from Jurkat cells. The sequences of primers for the human CD155 extracellular domain were: h155ECD-F, 5'-GGGGTACCGTCGTGTCAGCGCCACC-3', and h155ECD-R, 5'-CGGGATCCAAGATGGCGTTACGGGACATGCC-3'. Human CD226D1, CD226D2, and CD155ECD were constructed using the eukaryotic expression vectors pSectag2B/Fc, which encodes the target extracellular domain protein with a C-terminal human Fc tag. CHO, COS-7, or 293T cells that were successfully transfected with these expression plasmids were grown in DMEM. The Fc-tagged recombinant

proteins were harvested from culture supernatants and purified with a house-made CNBr-activated Sepharose-4B (Amersham Biosciences) column coupled with anti-Fc Monoclonal Ab (mAb) (Clone: WT.6) [15]. A 10–12% SDS-PAGE was conducted to separate the purified products.

Mixed lymphocyte culture (MLC)

Balb/c splenocytes (H-2^d) were treated with mitomycin C (MMC) for 30 minutes at 37°C before they were added into the culture system as stimulator cells. Freshly isolated C57BL/6 mice (H-2^b) lymphocytes were used as responder cells. Stimulator cells and responder cells were mixed at a ratio of 3:1 in 96-well cell culture plates. Anti-CD226 mAb was administered in three doses: low (0.5 µg/mL), medium (2 µg/mL), and high (5 µg/mL). In the BrdU cell proliferation assay, BrdU was added to the culture medium following a 3-day incubation. Another 16 h later, proliferative response was measured using a microplate reader. To analyze Treg percentages in culture, cells were collected for flow cytometry between days 1 and 4. MLC were also used in the proliferation assay. For CFSE staining and proliferation assays, lymphocytes were stained with CFSE at a concentration of 1 µmol/L at 37°C for 15 minutes. The reaction was stopped by adding complete RPMI 1640 medium with 15% fetal bovine serum for 5 minutes. After 4 days, the percentages of proliferating recipient lymphocytes were analyzed by flow cytometry.

Cytotoxicity assay

Activated C57BL/6 splenocytes harvested from MLC were employed as cytotoxic T lymphocytes (CTLs). The cytotoxic activity of CTL against target P815 cells was measured using a standard 4-h ⁵¹Cr release assay as described previously [14]. Briefly, P815 cells were labeled with 3.7 × 10⁶ Bq of sodium chromate (Na₂⁵¹CrO₄) (PerkinElmer) per 10⁶ cells for 2 h at 37°C. CTL cells were incubated with these P815 cells in 96-well round-bottom plates for 4 h at 37°C and 5% CO₂. ⁵¹Cr release was evaluated by determining counts per minute (cpm). The percentage of specific ⁵¹Cr release was calculated using the following formula: (cpm in the presence of effector cells – cpm of spontaneous release) / (cpm in the total ⁵¹Cr release – cpm of spontaneous release) × 100%. The cpm in the total ⁵¹Cr release was detected in target cells incubated with 1% Triton X-100. Spontaneous release did not exceed 10% of the maximum release.

Generation of in vitro induced Treg cells

C57BL/6 CD4⁺CD62L⁺ cells were separated using a CD4⁺CD62L⁺ T cell isolation kit and stimulated with CD3/CD28 Dynabeads at a ratio of 1:3. The cytokines IL-2 and TGF-β were added to the culture system to a final concentration of 100 U/mL and 5 ng/mL, respectively. Other reagents included all-trans retinoic acid (atRA) (10 nmol/L) and anti-CD226 mAb (2 µg/mL). Cells were incubated for 4 days before analyzing Foxp3 expression.

Skin transplantation and in vivo treatment

BALB/c and C57BL/6 mice were chosen as donors and recipients, respectively. All surgical procedures were performed under general anesthesia (isoflurane), and no pain-control medication was provided afterward. Skin allograft protocols and rejection criteria were used in accordance with a previous report [16, 17]. Briefly, full-thickness skin from donor tails was grafted on beds prepared on the lateral flanks of C57BL/6 recipients. Graft sites were protected under sterile gauze and bandages, which were removed 10 days after transplantation. The skin allografts were monitored daily after the bandages were removed. Anti-CD226 mAb (clone #: 10E5) or isotype Rat IgG2b were injected through the retro-orbital sinus on days 0, 2, 4, and 6, and afterwards every 3 days at a dose of 50 µg/mouse. In the Isotype and anti-CD226 groups, 3 mg/kg/d Rapa (Calbiochem) was given intraperitoneally (i.p.) from days 0 to 10 [18]. Skin allografts were considered rejected if ≥ 70% of the surface was necrotic. Treg percentages in spleens, draining lymph nodes (DLNs), and non-draining lymph nodes (NDLNs) were analyzed at the end of the experiment.

Flow cytometry analysis of absolute number of Treg and Teff cells

The absolute number of Treg and Teff cells were calculated based on flow cytometry assisted with flow-count microbeads. Briefly, the cultured cells in the MLC system or 100 µL peripheral blood cells collected from recipient mice after skin allograft rejection were surface stained with monoclonal antibody against H-2^b and CD4. Then, the cells were fixed and permeabilized (eBioscience, Cat #: 00-5523) followed by staining with antibodies against Foxp3. After washing twice, 10 000 Flow-Count microbeads were added to the cells and mixed thoroughly before flow cytometry analysis. For each sample, 5 000 microbeads were acquired, facilitating the calculation of absolute numbers of CD4⁺Foxp3⁺ Treg cells and CD4⁺Foxp3⁺ Teff cells.

Histology

Grafted skin and inguinal lymph nodes were harvested and fixed in 10% buffered formalin and embedded in paraffin at the time point of graft rejection. Tissues were sectioned and stained with hematoxylin and eosin (H&E) to determine morphological changes according to standard protocols. Rejection criteria by the Banff 2007 Working Classification were applied [19].

Statistical analysis

Data were analyzed using GraphPad Prism software (version 5.0, CA, USA). Student t test, one-way ANOVA, and two-way ANOVA were used as indicated. Differences in animal survival (Kaplan-Meier survival curves) were analyzed with the log-rank test. A value of $p < 0.05$ was considered statistically significant.

Results

Blockade of CD226 suppresses proliferation and cytotoxicity of lymphocytes in MLC

MMC-treated BALB/c splenocytes and C57BL/6 lymphocytes were used in the MLC. Lymphocyte proliferation was measured by the absorbance of BrdU in an ELISA. Anti-CD226 mAb treatment blocked CD226 binding with its ligand when added into the MLC. The absorbance of the MLC was reduced in groups with anti-CD226 treatment compared to that in the group to which no reagent was added (blank group) (Fig. 1A). Different doses of anti-CD226 mAb showed different suppressive effects. The inhibitory effects of the reagents on the proliferation of MLC were dose-dependent. The higher the dose administered to the MLC, the lower the proliferation rate detected in the BrdU proliferation assay (Fig. 1A). Secondly, the cytotoxicity of effector CTL on P815 cells was significantly inhibited by anti-CD226 mAb at 5 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$, as compared to isotype antibody-treated controls, and this effect was dose-dependent (Fig. 1B). In addition, we addressed how anti-CD226 mAb affects CTL differentiation and cytotoxicity at different time points, with 2 $\mu\text{g/mL}$ of anti-CD226 mAb added from day 1 to 4 after MLC setup. The earlier the mAb was added, the more obvious the CTL inhibition (Fig. 1C).

Furthermore, we analyzed the mechanism of anti-CD226 mAb and the dynamic of Tregs in MLC. We determined the ratio of Foxp3⁺ cells to Foxp3⁻ cells at different time points by flow cytometry. The time-ratio graph shows an increase in the ratio of Foxp3⁺/Foxp3⁻ cells in the α -CD226 mAb group as compared to the isotype group at every time point (Fig. 1D), which were involved in the suppression of CTL proliferation and cytotoxicity in MLC. Typical scatter diagrams of each group are presented (Fig. 1E). The absolute number of Tregs was calculated based on flow cytometry assisted by flow-count microbeads. The time-ratio graph shows an increase in the absolute number of Treg cells in the α -CD226 mAb group as compared to that in the isotype group (Fig. 1F), which indicated that blocking the CD226 signal could promote the production of Foxp3⁺ Treg cells.

Blockade of CD226 promotes iTreg production in vitro

To generate *in vitro*-induced Tregs (iTregs), CD4⁺CD62L⁺ cells were isolated and stimulated with CD3/CD28 Dynabeads for four days. In the presence of IL-2 and TGF- β , 52.6% of total CD4⁺CD62L⁺ cells were converted into Foxp3⁺ cells (Fig. 2A). Other than IL-2 and TGF- β , we also added atRA or anti-CD226 mAb into the culture system and measured the percentage of Foxp3⁺ cells under different conditions. The use of atRA increased the percentage of Foxp3⁺ cells to 72.1%, which was consistent with previous reports. Blockade of CD226 by mAb also resulted in an increased percentage of Foxp3⁺ cells (Fig. 2, A and B), suggesting that CD226 blockage can facilitate the generation of iTregs *in vitro*.

CD3/CD28 stimulation upregulates CD155, CD226, and TIGIT expression in CD4⁺ cells

CD155, CD226, and TIGIT expression on CD4⁺ T cells was measured by flow cytometry. After stimulation with CD3/CD28 Dynabeads, CD155 expression on CD4⁺T cells was upregulated over time (Fig. 2C). Upregulation of CD226 and TIGIT expression on both CD4⁺Foxp3⁺ cell and CD4⁺Foxp3⁻ cells was detected at days 0 and 4 (Fig. 2D).

Fig. 1. Blockade of CD226 suppresses lymphocyte proliferation. Freshly isolated C57BL/6 lymphocytes were cultured with MMC-treated splenocytes for 4 days. Autogenous MMC-treated C57BL/6 splenocytes were used as control stimulator cells in the auto group, whereas allogeneic BALB/c splenocytes were used as stimulator cells in the allo group. (A) Cells of the allo group were cultured in the absence or presence of different doses of anti-CD226 mAb or Rat IgG2b isotype. Data are shown as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$; (unpaired t test). (B) The cytotoxicity of effector CTL on P815 cells was significantly and dose-dependently inhibited by anti-CD226 mAb as compared to that of the isotype antibody-treated control (isotype group). (C) Anti-CD226 mAb (2 μ g/mL) was added from days 1 to 4 after MLC setup, and the earlier the mAb was added, the more obvious its inhibitory effect on CTL cytotoxicity. (D) the ratio of Foxp3⁺ to Foxp3⁻ cells in CD4⁺ cells were analyzed by flow cytometry every day after MLC setup. Cells in the isotype group were co-cultured with C57BL/6 lymphocytes and MMC-treated BALB/c splenocytes, plus 2 μ g/mL isotype IgG2b. Cells in the α -CD226 group were cultured in the presence of 2 μ g/mL anti-CD226 mAb 10E5. Graph of the time-ratio is shown as the mean $\pm$ SD.

* $p < 0.05$, ** $p < 0.01$; (unpaired t test). (E) Typical scatter diagrams of each group in D are presented. (F) Absolute number of Tregs was calculated based on flow cytometry assisted with flow-count microbeads. The time-ratio graph shows an increase of Tregs number in the α -CD226 mAb group as compared to the isotype group.

CD226 ligand functional binding requires two intact ectodomains

To analyze the molecular mechanism of CD226 interactions with its ligands, we used a series of mutants to determine whether the two CD226 ectodomains are involved in the interactions. First, we used a solid phase adsorption assay to measure the binding of truncated recombinant CD226 proteins with the CD155 ligand. Recombinant CD155 protein or control bovine serum albumin (BSA) were coated on 96-well ELISA plates, and then CD226-Fc, CD226D1-Fc, and CD226D2-Fc in Tris-NaCl buffer were each incubated with the test or control. After that, the HRP-conjugated anti-Fc mAb (house made, Clone: FMU-Fc7) [15] and TMB substrate were applied to show binding activity. As shown in Fig. 3B, CD226-Fc has a strong binding affinity for CD155, whereas CD226D1-Fc showed a lower binding affinity, and its affinity with CD226D2-Fc was even lower than that of CD226D1-Fc. Next, we analyzed the functional interactions of CD226 truncated mutants with CD155 by comparing the cytotoxicity of CD226-positive NK cells (NK92) to the ligand-positive cell line K562. K562 cells were incubated with CD226-Fc, CD226D1-Fc, or CD226D2-Fc for 30 min before co-culture to block endogenous CD155-CD226 interactions. We found that cytotoxicity for NK92 cells was blocked significantly by adding CD226-Fc, and blocked less by CD226D1-Fc or CD226D2-Fc (Fig. 3C). The adhesion of CFSE-labeled NK92 cells to a cultured ECV304 cell

Fig. 2. Blockade of CD226 facilitates the generation of iTregs. CD4⁺CD62L⁺ cells were cultured in the presence of CD3/CD28 Dynabeads and different reagents for 4 days. Other reagents added to the culture system were 100 U/mL IL-2, 5 ng/ml TGF- β , 10 nmol/L atRA, and/or 2 μ g/mL anti-CD226 mAb. (A) The average percentages of Foxp3⁺ cells in CD4⁺ cells under different conditions. The reagents added in different groups are presented in B. (B) The Foxp3 percentage of iTreg in different groups is presented as the mean $\pm$ SD. ** $p < 0.01$ (unpaired t test). (C, D) Lymphocytes were stimulated with CD3/CD28 Dynabeads. The expression of CD155 on CD4⁺ cells was analyzed by flow cytometry daily, and the expressions of CD226 and TIGIT on both CD4⁺Foxp3⁺ cells and CD4⁺Foxp3⁻ cells were analyzed by flow cytometry at days 0 and 4. All experiments were repeated at least three times. The percentage of CD155⁺ cells (C), CD226⁺ and TIGIT⁺ cells are shown (D).

monolayer was detected by a Tecan fluorescence plate reader. As shown in Fig. 3D, the presence of the full length of the two ectodomains of CD226 disturbed the adhesion of NK92 to ECV304 cells markedly, whereas CD226D1-Fc and CD226D2-Fc could not inhibit adhesion.

Previous research has shown that CD226 plays a key role in immune synapse formation in the binding of NK cells with target cells. We constructed a series of C-terminal GFP-tagged truncates, namely CD226FL, CD226D1, and CD226D2, and the CD155-RFP vector to determine whether CD226 binds to its ligand (Fig. 4A). CD226-GFP truncated transfected COS-7 cells and CD155-RFP transfected COS-7 cells were mixed at a 1:1 ratio for 4 h at 37°C in chamber slides and fixed with 4% polyoxymethylene. Using a laser scanning confocal microscope (LSCM), we observed significant “capping” of CD226FL-GFP and co-localization with CD155-RFP, which showed the efficiency of binding between CD226 and CD155. In the interacting region, the CD226 and CD155 molecules accumulated locally, then the green and red fluorescence merged to form a yellow junction. Truncated CD226D1-GFP or CD226D2-GFP were evenly distributed and no “capping” could be found, which indicated that CD155 did not bind effectively (Fig. 4B). When an anti-CD226 antibody was added to the CD226FL-GFP transfected COS-7 cells in the co-culture group, the aggregation of CD226 and CD155 was totally blocked (Fig. 4C).

Anti-CD226 mAb prolongs skin allograft survival

A BALB/c to C57BL/6 skin allograft model was employed to determine the effects of CD226 blockage on transplantation rejection. Recipients treated with anti-CD226 mAb plus rapamycin (α -CD226 group) showed longer allograft survival than recipients treated with isotype Rat IgG2b plus rapamycin (isotype group) or recipients without any immunosuppressive treatment (Blank group) (Fig. 5A). Skin allografts in the Blank group were totally rejected at day 11 post-transplantation, whereas allografts in the α -CD226 group and isotype group showed no rejection or only minor rejection. At day 13, most allografts in the isotype group were considered rejected, whereas grafts in the α -CD226 group only showed minor rejection (Fig. 5, A and B). Finally, all the allografts in the α -CD226 group

Fig. 3. CD226 with intact ectodomains binds to CD155 more efficiently. (A) Schematic diagram of the secondary structure of the recombinant Fc-tagged CD226 truncated mutants. (B) CD226-Fc, CD226D1-Fc, and CD226D2-Fc showed different binding affinities to CD155. The full-length CD226 ectodomain showed an obviously higher binding affinity for CD155 than that of truncated CD226D1 or CD226D2. (C) The cytotoxicity of NK92 cells for K562 cells was decreased by CD226-Fc recombinant protein, but not by CD226D1-Fc or CD226D2-Fc mutants. (D) Adhesion of NK92 cells to an ECV304 cell monolayer was disturbed by the two full-length CD226 ectodomains, whereas CD226D1-Fc and CD226D2-Fc did not inhibit adhesion.

Fig. 4. The two intact CD226 ectodomains are required for the capping of CD226-CD155 complexes. (A) Schematic diagram of the secondary structure of the recombinant GFP-tagged CD226 truncated mutants and RFP-tagged CD155 molecule. (B) Recruitment of CD226 to CD155 requires the two intact ectodomains. COS-7 cells were transiently transfected with CD226-GFP truncated mutants and CD155-RFP and were imaged by laser scanning confocal microscopy. We observed significant aggregations of CD226-FL-GFP and CD155-RFP, showing the efficiency of trans-interactions. Truncated CD226-D1-GFP and CD226-D2-GFP were evenly distributed on the surfaces of transfected cells, and no “capping” could be found. (C) An anti-CD226 antibody interrupted the capping of CD226-CD155 complexes.

were rejected by day 17 post-transplantation. The Kaplan-Meier survival analysis showed statistically significant differences in survival time between mice in the isotype group and α -CD226 group (Fig. 5B).

H&E staining showed results similar to the skin allograft observations. Disintegration of skin structure, necrosis of the epidermis, and mononuclear cell infiltration were observed in skin allografts in the Blank group at day 11 post-transplantation, whereas skin allografts in the Isotype and α -CD226 group showed structural integrity. Signs of rejection in skin allografts in the Isotype and α -CD226 group were observed on days 13 and 17 post-transplantation (Fig. 5C). Mice in the Blank group also exhibited lymphadenovariex at day 11 post-transplantation, which was more obvious in draining lymph nodes (DLNs) than in non-draining lymph nodes (NDLNs) (Fig. 5D).

Anti-CD226 treatment increases Treg percentages in secondary lymphoid organs of recipients

To investigate the association between anti-CD226 mAb treatment and Tregs, we analyzed Treg percentages in spleens, DLNs, and NDLNs of recipient mice when skin allografts were considered rejected. Treg percentages were higher in the α -CD226 group

Fig. 5. Anti-CD226 treatment prolongs skin allograft survival. BALB/c skin was transplanted to C57BL/6 mice, and bandages were removed at day 10 post-transplantation. Blank group recipient mice (n = 5) received transplanted allografts from BALB/c mice without immunosuppressive therapy, whereas mice in the isotype group (n = 5) received rapamycin by intraperitoneal injection at a dose of 3 mg/kg/d plus Rat IgG2b isotype. Mice in the α -CD226 group (n = 5) received rapamycin plus anti-CD226 mAb (Rat IgG2b). (A) Photos were taken at different time points post-transplantation as shown. (B) Kaplan-Meier survival curve of mice in the three groups by day of post-transplantation and showing percent survival. **p<0.01; (Kaplan-Meier survival analysis). (C) For biopsy, the allografts were cut off and stained with hematoxylin-eosin at different time points post-transplantation. (D) Mice in each group were euthanized on day 11 post-transplantation and dissected for draining lymph nodes and non-draining lymph nodes. Blank group mice exhibited obvious lymphadenovari-

ix, and the sizes of draining lymph nodes were bigger than those of non-draining lymph nodes.

and isotype group than in the Blank group. Higher Treg percentages in the spleens and DLNs were shown in the α -CD226 group than in the isotype group, whereas no statistically significant difference was observed in NDLNs (Fig. 6, A and B). Similar to the *in vitro* results, the absolute numbers of CD4⁺Foxp3⁺ Tregs and CD4⁺Foxp3⁻ Teff cells were calculated based on flow cytometry. An obvious increase of Treg cells in the α -CD226 group was presented, as compared to the numbers observed in Isotype and Blank groups, which indicated that blocking the CD226 signal could promote the production of Foxp3⁺ Treg cells *in vivo* (Fig. 6C). When skin allografts were considered rejected, C57 recipients' lymphocytes were isolated from spleens and labeled with CFSE. BALB/c lymphocytes were treated with MMC and co-cultured with these CFSE-labeled splenocytes. On day 4, we analyzed the percentage of proliferated lymphocytes by flow cytometry. The percentage of proliferated lymphocytes in the α -CD226 group was lower than that in the other two groups (Fig. 6, D and E).

Discussion

Tregs are crucial in controlling immunity and self-tolerance. Consequently, Tregs play a central role in inhibiting rejection and promoting allograft tolerance during transplantation. A more complete understanding of Treg biology may lead to novel therapeutic approaches to enhance Treg numbers and function [20]. In recent years, CD4⁺Foxp3⁺ Treg-based immunotherapy has emerged as a promising strategy to promote operational tolerance for MHC-mismatched allogeneic tissue transplantation. Treg cells have been highlighted among allogeneic hematopoietic stem cells and in tissue transplantation immune tolerance. However, a main hurdle for their therapeutic usage is their low frequency and unstable functional phenotype. Many previous studies have shown that many experimental agents require Tregs for tolerance induction and/or maintenance. Tregs play critical roles in establishing an immunological set point, and their depletion enhances immune responses. The proliferation, differentiation, and homeostasis of Tregs are critical for their clinical

Fig. 6. Anti-CD226 treatment increases the Treg percentage in recipient mice. Recipient mice were euthanized after skin allograft rejection. Spleens, draining lymph nodes, and non-draining lymph nodes were analyzed for Treg percentage by flow cytometry. (A) Tregs percentage determined by Fopx3⁺ cells among CD4⁺ T cells. All experiments were repeated three times, and the typical contour diagram and percentage of Fopx3⁺ cells are shown. (B) Graph of data in A presented as the means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$; (paired t test). Lymphocytes were separated from recipients' spleens, labeled with CFSE, and cultured under similar conditions as MLC. Proliferation of lymphocytes was analyzed by flow cytometry at day 4 and was presented as the CFSE dilution. (C) Absolute number of CD4⁺Fopx3⁺ Tregs and CD4⁺Fopx3⁻ Teff cells were calculated with the assistance of flow-count microbeads. The histogram shows an obvious increase in Treg number in the α -CD226 group as compared to those in the Isotype and Blank group. (D) Proliferated lymphocytes are expressed as the CFSE dilution. (E) Percentages of proliferated cells are shown as mean $\pm$ SD. ** $p < 0.01$; (unpaired t test).

usage in allograft rejection therapies. Treg proliferation and function are regulated via multiple molecules [21, 22]. One of these receptors, CD226, a costimulatory molecule in various systems, is an important receptor for Tregs. Thus, we investigated the effect of CD226 blockage on Treg proliferation, as well as its contribution to improving transplant tolerance *in vitro* and *in vivo*. In this study, we found that blocking the CD226 signal by mAb promoted Treg proliferation and differentiation, and suppressed Tconv proliferation and CTL cytotoxicity in an allogeneic MLC system. Furthermore, blockage of CD226 by mAb efficiently inhibited immune responses and promoted the tolerance of MHC mismatched allogeneic graft transplantation. This inhibition was associated with a significant increase in the percentage and absolute number of Tregs. Our study therefore reveals the critical role of CD226 in pathogenesis and potentially therapy for allogeneic transplantation by promoting Tregs and suppressing Tconv functions.

It has been reported that manipulating CD226 can divide thymus-derived Tregs (tTregs) into different subpopulations [12]; however, whether CD226 functions in *in vitro* induced Treg (iTreg) differentiation was still unclear. We claimed that anti-CD226 mAb played a predominant role in introducing Tregs in the MLC system. Although MLC is a well-known and classical model commonly used to analyze MHC compatibility before tissue transplantation, lymphocytes can be activated and proliferate when stimulated by allogeneic antigens on APC or by co-stimulating or inhibiting molecules. We thought that splenocytes stimulated by CD3/CD28 antibodies represented a relatively simple system compared with MLC. In our study, we also determined whether anti-CD226 treatment could increase the percentage of CD4⁺Fopx3⁺ cells in an iTreg induction experiment. Upon stimulation with immobilized anti-CD3 and anti-CD28 mAbs (CD3/CD28 Dynabeads) and in the presence of anti-CD226 mAb, the percentage of Fopx3⁺ Tregs significantly increased relative to that in the CD3/CD28 stimulation groups with or without IL-2/TGF- β stimulation and in the group with atRA added. However, it remains unclear whether the increase in Treg percentage upon blockade of CD226 in MLC was due to the expansion of tTregs or conversion of iTregs. Further experiments are needed to reveal how CD226 participates in Treg differentiation and/or proliferation. Moreover, the data clearly show the requirement for the CD226 costimulatory signal to be blocked to initiate proliferation of Treg cells, which was sustained by the observation that

CD226 mAb increased the number of peripheral Treg cells in a mouse skin transplant model *in vivo*.

Koyama et al. showed that CD226 is highly expressed on both CD4⁺Foxp3⁺ Tregs and CD4⁺Foxp3⁻ cells post-transplantation [11]. Our study supports this data by showing that CD226 expression is upregulated on Tregs and CD4⁺Foxp3⁻ cells in an *in vitro* MLC experiment. In addition, we showed that expression of CD155, the main ligand of CD226, is upregulated each day in MLC, which agrees with clinical research [23]. The expression levels of CD226 and CD155 continued to increase, and their interaction continued to strengthen, which may have mediated the persistent activation of host's killer cells and the rejection of grafts. At the same time, we also measured the upregulated expression of TIGIT on both CD4⁺Foxp3⁺ cells and CD4⁺Foxp3⁻ cells after 4 days of CD3/CD28 stimulation. TIGIT may play a negative feedback role, such as CTLA-4 in the allogeneic immune responses.

CD226 is a potential target for therapeutic approaches to GVHD, and here we have shown that it also can be used as a treatment in solid tissue or organ transplantation. Administration of anti-CD226 mAb at the time of transplantation resulted in significant Treg expansion, which prolonged allogeneic skin transplant survival time. Although we did not achieve long-term tolerance of allografts with anti-CD226 treatment, the grafts survived significantly longer and the inflammatory responses were reduced compared to allografts without treatment. Lymphocyte proliferation in the grafted mice also decreased after anti-CD226 treatment. In the *in vitro* experiment, lymphocyte proliferation and CTL cytotoxicity were also suppressed by anti-CD226 treatment, along with the increase in Treg percentage. The protective function of Tregs in acute allograft rejection has been confirmed in many studies [16, 24-26], and CTLs are also considered important effector cells in acute allograft rejection [27]. These findings suggested that CD226 blockade might be therapeutic when acute allograft rejection occurs, rather than inducing tolerance.

As a member of the Ig superfamily, CD226 contains two IgV-like functional domains within its extracellular domain [28]. Recently, Hou et al. reported that the first extracellular domain of CD226 (CD226-ECD1) mediates NK cell recognition, immune synapse formation, and cytotoxicity, suggesting that CD226-ECD1 retains almost all of the functionality of the full-length CD226 protein on NK cell surfaces [14]. Some other studies have also shown the trans-interaction of Ig superfamily molecule-mediated intermolecular signaling transduction and even regulation of intercellular signal transmission by binding different domains. In CD96, another member of the Ig superfamily that binds to CD155, the first extracellular Ig-like domain plays a particularly important role in its interaction with CD155 [29]. TIGIT has one Ig domain in its extracellular region, which shares the same ligands as CD226: CD155 and CD112 [30]. The crystal structure of TIGIT-CD155 showed that CD155 interacts with TIGIT via its first ectodomain [31]. However, our study demonstrated that the full function of CD226 relies on the integrity of its two IgV-like extracellular domains. By comparing CD226-D1, CD226-D2, and the full-length CD226 ectodomain, we showed that truncated CD226 mutants were able to bind to CD155, but with obviously lower affinity. When target cells were pre-incubated with recombinant CD226D1-Fc or CD226D2-Fc to inhibit cell-cell conjugation, NK cell-mediated adhesion and cytotoxicity were reduced somewhat, and the full-length CD226-Fc ectodomain significantly suppressed adhesion and cytotoxicity, strongly blocking interactions with CD155. In addition, the full length ectodomain CD226-GFP showed the most "capping" of CD155-RFP. These results suggested that an intact CD226 ectodomain was important for functional interaction.

Under natural conditions, in addition to the 315 amino acid residues in wild-type murine CD226, three mRNA isoforms were found in the mouse thymus (GenBank accession numbers: AF421198, AF421199, and AF421200). Among them, isoform 1 encodes 187 amino acid residues, is lacking most of the second extracellular domain, and terminates at the transmembrane region. Isoform 2 has 220 amino acid residues that are missing after the signal peptide, the first extracellular domain, and that encode an intact second domain with the transmembrane region and cytoplasmic domain. Isoform 3 encodes 129 amino acid residues, and based on comparison with isoform 2, the signal peptide region is

deleted. A study of murine NK and T cells also showed the existence of a naturally occurring CD226 splice variant that lacks the outermost IgV-like domain [32]. The trans-interactions between Ig superfamily molecules are finely regulated, possibly via interactions of different ectodomains.

In summary, our study provided new evidence to support the therapeutic potential of anti-CD226 treatment in allogeneic transplantation and revealed the mechanism underlying CD226 function. The results of this study are valuable for understanding the mechanism by which CD226 recognizes its ligands. However, the downstream molecular pathway activated by the CD226-CD155 interaction is still unclear, and more studies are needed to clarify this.

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Disclosure Statement

The authors declare no conflicting financial interests.

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