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Research Article

# Analysis of therapeutic potential of monocytic myeloid-derived suppressor cells in cardiac allotransplantation

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ABSTRACT

*Background:* Myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) are attractive immune cells to induce immune tolerance. To explore a strategy for improving the efficacy of MDSC therapies, we examined the impact of adoptive transfer of several types of MDSCs on graft rejection in a murine heart transplantation model.

*Methods:* We analyzed the effects of induced syngeneic and allogeneic bone marrow-derived MDSCs (BM-MDSCs) on graft survival and suppressive capacity. We also compared the ability of syngeneic monocytic MDSCs (Mo-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) to inhibit graft rejection and investigated the suppression mechanisms.

*Results:* Both syngeneic and allogeneic donor- or allogeneic third-party-derived BM-MDSCs prolonged graft survival, although syngeneic BM-MDSCs inhibited anti-donor immune responses most effectively *in vitro*. Syngeneic Mo-MDSCs, rather than PMN-MDSCs, were responsible for immune suppression through downregulating inducible nitric oxide synthase (iNOS) and expanded naturally occurring thymic originated Treg (nTreg) *in vitro*. Adoptive transfer of Mo-MDSCs, but not PMN-MDSCs, prolonged graft survival and increased Treg infiltration into the graft heart.

*Conclusion:* Recipient-derived Mo-MDSCs are most effective in prolonging graft survival *via* inhibiting T cell response and nTreg infiltration.

*Abbreviations:* BM, bone marrow; CFSE, carboxyfluorescein succinimidyl ester; DNA, deoxyribonucleic acid; FoxP3, Forkhead box protein P3; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; IL, interleukin; IL-4Rα, interleukin 4 receptor alpha; iNOS, inducible nitric oxide synthase; L-NMMA, $N^G$-monomethyl-L-arginine; MDSCs, myeloid-derived suppressor cells; MNCs, mononuclear cells; Mo-MDSC, monocytic myeloid-derived suppressor cells; mRNA, messenger ribonucleic acid; nor-NOHA, $N^{\omega}$-hydroxy-nor-L-arginine; PMN, polymorphonuclear; qRT-PCR, quantitative reverse-transcript polymerase chain reaction; RPMI, Roswell Park Memorial Institute; SEM, standard error of the mean; Treg, regulatory T cell.

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## 1. Introduction

Myeloid-derived suppressor cells (MDSCs) are heterogeneous immunosuppressive cells derived from progenitor myeloid cells that expand in various pathological environments including cancer, infection, and inflammation [1–5]. In mice, MDSCs, which are defined by the phenotype $CD11b^+$ $Gr\text{-}1^+$, are composed of two major subsets: $Ly\text{-}6G^+$ $Ly\text{-}6C^{low}$ polymorphonuclear (PMN)-MDSCs and $Ly\text{-}6G^-$ $Ly\text{-}6C^{hi}$ monocytic (Mo)-MDSCs. Although both MDSC subsets migrate into the inflammatory environment and inhibit local immune responses, various mechanisms have been reported to contribute to the suppressive functions of MDSCs. PMN-MDSCs mainly regulate the immune response by producing reactive oxygen species (ROS) whereas inducible nitric oxide synthase (iNOS) upregulation has been reported to be critical for Mo-MDSC-induced immune suppression [6–9].

MDSCs are inducible from peripheral monocytes [10], macrophages, stem cells [11] or bone marrow (BM) cells [12] by incubation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-6 *in vitro* [13] and are regarded as an attractive cell population for treating autoimmune and allergic diseases [14–15]. In organ transplantation, MDSCs have been explored as potential therapeutics to attenuate the alloreactive immune response and maintain transplanted organs [16]. GM-CSF, lipopolysaccharide, IL-33, rapamycin, or dexamethasone treatment has been reported to induce MDSCs in the recipient and significantly prolonged allograft survival by inhibiting $CD8^+$ T cell priming [13,14,17–21]. However, clinical trials to apply MDSCs to inhibit graft rejection have not yet demonstrated sufficient benefit for the transplanted patients [22]. In this study, we investigated the potent population of *ex vivo* induced MDSCs suitable for infusion cell therapy to inhibit allogeneic immune responses and prolong transplanted heart survival in an experimental mouse model.

## 2. Material and methods

### 2.1. Mice and reagents

BALB/c, C57BL/6 J (B6), and CBA mice were purchased from Sankyo Laboratory Service Corporation (Nishiichinoe, Japan). $FoxP3^{hCD2}$ reporter mice were kindly provided by Prof. Shohei Hori [23]. All mice were housed under specific pathogen-free conditions in accordance with the institutional guidelines of Juntendo University. Mice were used at 7–12 weeks of age. All protocol was approved by Medicine Animal Ethics Committee of Juntendo University (Approved number 2020131). All animal experiments were performed in compliance with the applicable national laws and regulations and the institutional guidelines on animal experimentation. All the experimental protocols involving animals are reviewed by the institutional animal care and use committee.

$N^G$-Monomethyl-L-arginine (L-NMMA) (50 μmol/mL) and $N^{\omega}$-hydroxy-nor-L-arginine (nor-NOHA) (500 μmol/mL) were purchased from EMD Millipore (Billerica, MA, USA; cat. 475,886) and Calbiochem (San Diego, CA, USA; cat. 399,275), respectively.

### 2.2. Induction of bone marrow-derived MDSCs (BM-MDSCs)

BM-MDSCs were prepared following a previously reported method [13]. Briefly, BM cells were obtained from tibias, femurs, and red blood cells lysed using a red blood cell lysis buffer (BioLegend; San Diego, CA, USA; cat. 420,301). Cells were then washed and passed through a 40 μm strainer (Greiner Bio-One; Kremsmünster, Austria; cat. 542,040). BM cells ($3 \times 10^6$) were cultured in 15 mL Roswell Park Memorial Institute (RPMI)-1640 (Merck; Darmstadt, Germany; cat. R8758) supplemented with 10% fetal bovine serum (Biosera; Marikina, Philippines; cat. FB-1380), 50 μM 2-mercaptoethanol (Fuji Film; Tokyo, Japan; cat. 137–06862), 10 mM 4- (2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, 1 μg/mL streptomycin sulfate (Meiji Seika Pharma; Tokyo, Japan; cat. 6,161,400), 1 mM sodium pyruvate (Merck; cat. S8636), 60 ng/mL GM-CSF (Peprotech; Rocky Hill, NJ, USA; cat. 315–03), and 60 ng/mL IL-6 (Peprotech; cat 216–16) for 96 h in 100 mm dishes (Greiner Bio-One; cat. 664,160–13) at 37 °C with 5% $CO_2$. PMN-MDSCs ($Ly\text{-}6G^+$/$Ly\text{-}6C^{low}$/$CD11b^+$) and Mo-MDSCs ($Ly\text{-}6G^-$/$Ly\text{-}6C^{hi}$/$CD11b^+$) were isolated from BM-MDSCs using a JSAN flow cytometry cell sorting system (Bay Bioscience; Kobe, Japan).

### 2.3. Flow cytometric analysis

Flow cytometric analyses were performed on a FACS Verse (BD Biosciences; Franklin Lakes, NJ, USA) following immunofluorescence staining. Before staining, the cells were pre-incubated with anti-mouse CD16/32 (2.4G2) monoclonal antibodies (mAb) (Mouse BD Fc Block, BD Biosciences) to avoid non-specific binding of mAbs to Fcγ receptors. Cells were then stained with fluorescence dye-conjugated antibodies according to the manufacturer's protocol. To assess the MDSC population, the following mAbs were used: PerCP/Cyanine5.5-conjugated anti-mouse/human CD11b mAb (BioLegend M1/70 cat. 101,227), PE-conjugated anti-mouse Gr-1 (Ly-6G/Ly-6C) mAb (BioLegend RB6-8C5 cat. 108,407), FITC-conjugated anti-mouse Ly-6C mAb (BioLegend HK1.4 cat. 128,005), PE-conjugated anti-mouse Ly-6G mAb (BioLegend 1A8 cat. 127,607), PE-conjugated anti-mouse F4/80 mAb (BioLegend BM8 cat 123,109), PE-conjugated anti-mouse CD115 mAb (BioLegend AFS98 cat 135,505), and PE-conjugated anti-mouse IL-4Rα mAb (BioLegend 15F8 cat. 144,803). To examine regulatory T cells (Tregs), the following antibodies were used: PerCP-conjugated anti-mouse CD4 mAb (BioLegend GK1.5 cat. 100,431), FITC-conjugated anti-mouse CD8 mAb (BioLegend 53–6.7 cat. 100,705), PerCP-conjugated anti-mouse CD45 mAb (BioLegend 30-F11 cat. 103,129), PE-conjugated anti-human CD2 mAb (BioLegend RPA-2.10 cat. 300,207), Alexa Fluor® 647-conjugated anti-FoxP3 mAb (BioLegend MF-14 cat. 126,408) and APC-conjugated anti-mouse Ki-67 mAb (BioLegend 16A8 cat. 652,405). FoxP3 was stained using fixation and permeabilization buffers in a FoxP3 kit (BioLegend cat. 136,803). Data were analyzed using Flowlogic software (Inivai Technologies; Victoria, Australia).

### 2.4. Mixed lymphocyte reaction (MLR) culture

Splenocytes ($2 \times 10^6$/ml) of B6 mice were co-incubated with 30 Gy irradiated splenocytes ($2 \times 10^6$/ml) from BALB/c mice in the 96-well round bottom (200 μL/well; Corning; Corning, NY, USA; cat. 3595) or 24-well (500 μL/well; Greiner Bio-One; cat. 662,160) plates for 3–5 days. The induced BM-MDSCs were added to the MLR at graded ratios.

### 2.5. CD3 stimulation

Anti-mouse CD3 mAb (BioLegend; 145-2C11 cat. 100,340) was immobilized by overnight incubation at 1 μg/mL in 200 μL phosphate-buffered saline (PBS) on a 96-well flat-bottom plate. The wells were washed five times with PBS, and $2 \times 10^6$ B6 spleen cells/mL were co-cultured with graded ratios of MDSCs in the culture medium at 37 °C with 5% $CO_2$ for 72 h or 7 days. In some experiments, $FoxP3^{hCD2}$ reporter mice splenocytes were incubated with PE-conjugated anti-human CD2 mAb and $hCD2^+$ cells were sorted using anti-PE microbeads magnet separation (BioLegend; cat. 480,080) according to the manufacturer's instructions.

### 2.6. Thymidine uptake

Cells stimulated by MLR or anti-mouse CD3 mAb were pulsed with $^3$H-thymidine (37,000 Bq/well) for 18 h and harvested using a Micro 96 Harvester (Skatron; Lier, Norway). $^3$H-thymidine incorporation into DNA was measured using a microplate counter (Micro Beta Plus; Wallac; Tarku, Finland).

### 2.7. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeling

Responder cells were labeled with CFSE (Thermo Fisher Scientific; Waltham, MA, USA) according to previously reported methods [24]. A total of $2 \times 10^6$ spleen cells from B6 mice were stained with 5 μM CFSE for 5 min at 37 °C and labeling was stopped by addition of RPMI 1640 containing 10% fetal bovine serum. After washing twice, the CFSE-labeled cells were immediately counted and used for *in vitro* experiments.

### 2.8. Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were collected 72 h after MLR or CD3 stimulation, and the interferon (IFN)-γ concentration was measured using a DuoSet ELISA Development System (R&D Systems; Minneapolis, MN, USA) according to the manufacturer's instructions.

### 2.9. Quantitative reverse-transcript polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the cell pellets using the RNeasy Micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA quality was assessed using a ND-1000 Spectrophotometer (Thermo Fisher Scientific, Watham, MA, USA). Reverse transcription was performed by using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) and DNA Engine Tetrad 2 Thermal Cycler (Bio-Rad; Hercules, CA, USA) to synthesize complementary DNA. qRT-PCR was performed with the Step OnePlus Real-Time PCR System (Thermo Fisher Scientific) using TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression products for *Ifng* (Thermo Fisher Scientific Mm01168134), *Gzmb* (Thermo Fisher Scientific Mm00442837), *Foxp3* (Thermo Fisher Scientific Mm00475162), *Tgfb* (Thermo Fisher Scientific Mm00436960), *Il10* (Thermo Fisher Scientific Mm01288386), and *Prf1* (Mm00812512) and TaqMan Endogenous Control (Thermo Fisher Scientific 4352339E) for mouse *Gapdh*; the thermal profile included 45 cycles on a Step One Plus Real Time PCR System (Applied Biosystems; Foster City, CA, USA).

### 2.10. Isolation of mononuclear cells (MNCs) from grafts

Transplanted heart allograft was perfused with cold saline, rinsed, and resected. Single-cell suspensions were generated in Multi-Tissue Dissociation Kit-1 on a gentleMACS Octo Dissociator (Miltenyi Biotec) according to the manufacturer's instructions. Suspensions were then filtered through 70-μm nylon strainers, rinsed in saline, and fractionated across 5 ml Ficoll-Paque premium (Cytiva) density gradient. Separate fractions were then applied for FACS analysis or RNA extraction for qPCR.

### 2.11. Histopathology

Allografts were harvested on 7 day after transplantation from 5 mice treated with MDSCs were fixed in 10% formalin solution and then embedded in paraffin, hematoxylin and eosin (H&E) staining was performed. Immuno-fluorescent staining were performed in the allograft isolated, washed, and then embedded in Tissue-Tek OCT compound (Sakura Fintek USA, Inc., Torrance, CA). Cryostat sections of 3 mm were fixed with 4% paraformaldehyde (Merck, Darmstadt, Ger-many) in 0.1 M phosphate buffer (pH 7.4) for 30 min at 4 °C, followed by incubation with FITC-conjugated rat-anti-mouse CD4 mAb (Clone RM4–5, BD Pharmingen) or isotype-matched control FITC-conjugated rat IgG2a (CloneR35–95, BD Pharmingen) for 1 h at 37 °C. Endogenous biotin was blocked using avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. After blocking, the sections were incubated with biotin-conjugated rat anti-mouse FoxP3 mAb (Clone FJK—16S, eBioscience) or isotype-matched control biotin-conjugated rat IgG2a (Clone R35–95, BD Pharmingen) for 1 h at 37 °C, and then labeled with Alexa Fluor 594-conjugated streptavidin (Invitrogen, Eugene, OR). The fluorescence images were captured with AxioPlan2 (Zeiss, Jena, Germany). The number of infiltrating $CD4^+$ $FoxP3^+$ cells in cardiac myocyte in each section was analyzed using KS400 Image analysis system (Zeiss). A total of 5–7 typical areas were examined in each specimen, and positively stained cell density was calculated per square millimeter.

### 2.12. Statistical analyses

Statistical analyses were performed with Prism 6 software (GraphPad; La Jolla, CA, USA) using the *t*-test. Survival rates were compared using the log-rank test. Statistical significance was defined as $p < 0.05$.

## 3. Results

### 3.1. Recipient syngeneic BM-MDSCs demonstrated potent immune suppression

The immunosuppressive mechanism of the induced BM-MDSCs is major histocompatibility complex (MHC) non-restricted. Thus, both recipient syngeneic BM-MDSCs derived from C57BL/6 mice and allogeneic BM-MDSCs derived from the BALB/C donor or the CBA third-party would inhibit anti-allogeneic donor immune responses and prolong graft survival. When we adoptively transferred $5 \times 10^6$ BM-MDSCs into the recipient mice with allogenic cardiac transplantation, treatment with all three types of BM-MDSCs prolonged graft survival (Fig. 1A). In contrast, recipient BM-MDSCs significantly inhibited proliferation and IFN-γ production than other donor or 3rd party BM-MDSCs when the recipient splenocytes were stimulated with irradiated donor splenocytes *in vitro*, although all three types of MDSCs exerted immune suppression (Fig. 1B). Thus, recipient syngeneic BM-MDSCs exerted more potent immune suppressive function than other donor or 3rd party allogeneic BM-MDSCs, although the long-term graft acceptance was comparable when the recipients were treated with $5 \times 10^6$ MDSCs.

### 3.2. BM-MDSCs inhibited effector T cell infiltration into the graft

We then examined the infiltrating mononuclear cells (MNCs) in the graft of recipient BM-MDSC-treated mice and found that the graft infiltrating $CD4^+$ and $CD8^+$ T cells were significantly decreased (Fig. 2A). There were no differences in the number of infiltrating MDSCs and the ratio of $FoxP3^+$ Tregs/CD4 T cells between the control and recipient BM-MDSC treatment (Fig. 2A and B). mRNA expression level of *Ifng* and *Gzmb* mRNA as well as the ratio of mRNA expression level of *Ifng* to *Foxp3* and the ratio of mRNA expression level of *Gzmb* to *Foxp3* in the infiltrating mononuclear cells (MNCs) were significantly reduced by the recipient BM-MDSC treatment (Fig. 2C). Within the allograft infiltrating effector CD8 T cells, Ki-67, which is a cell proliferation antigen, was not significantly reduced by MDSCs treatment (Fig. 2D). These results suggest that adoptive transfer of recipient BM-MDSCs significantly prolonged allograft survival, possibly due to the inhibition of effector T cell infiltration into the graft.

### 3.3. iNOS dependent inhibition by Mo-MDSCs is the major mechanism of induced BM-MDSCs

Decreased effector T cells and a lack of difference in suppressor cell infiltration in the graft suggested that the transferred recipient BM-MDSCs inhibited the priming of anti-donor effector T cells before infiltration. Thus, we examined the inhibitory effect of the induced BM-MDSCs on naïve T cell activation *in vitro*. We identified Mo-MDSCs ($CD11b^+$ Ly-$6G^-$ Ly-$6C^{hi}$), rather than PMN-MDSCs ($CD11b^+$ Ly-$6G^+$ Ly-$6C^{low}$), as the major subset of induced BM-MDSCs (Fig. 3A and B). Since it has been reported that Mo-MDSCs inhibit immune responses *via*

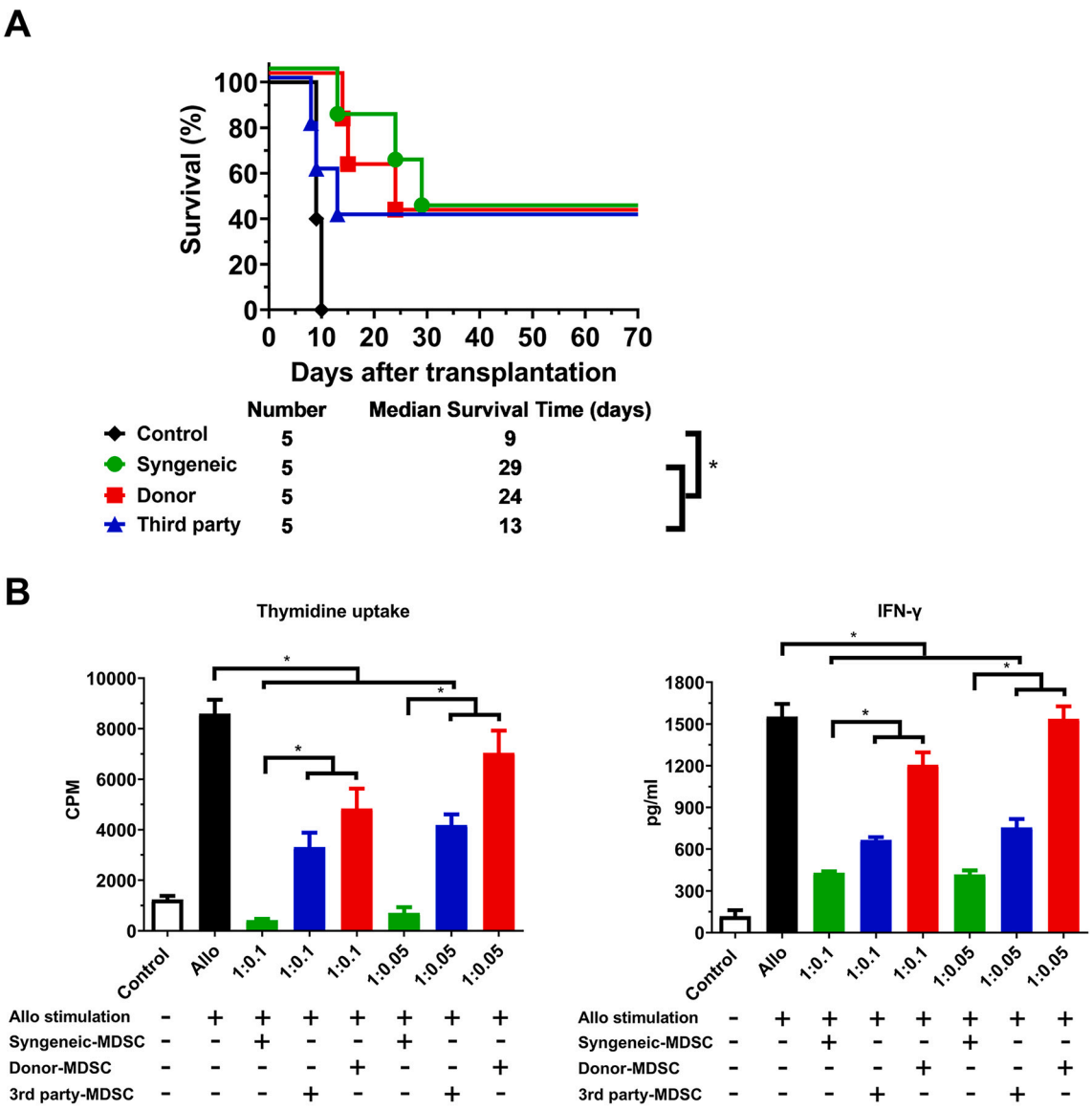


**Fig. 1.** Recipient syngeneic BM-MDSCs potently inhibited anti-donor immune responses. (A) Cardiac allograft transplantation was performed with adaptive transfer of the indicated syngeneic or allogeneic BM-MDSCs, and graft survival was observed more than 70 days. ($n = 5$ in each group) *, $p < 0.01$ by log-rank test. (B) Splenocytes of B6 mice were co-incubate with 30 Gy irradiated BALB/c splenocytes in the 96-well plate. Some MLRs were performed with syngeneic (B6), donor (BALB/C) or 3rd party (CBA) BM-MDSCs at the indicated ratios. Then, thymidine uptake for the last 18 h and IFN-γ concentration in the supernatants were examined. Mean ± SEM of 3–5 mice in each group are presented. * $p < 0.05$. Similar results were obtained from three independent experiments.

iNOS or arginase upregulation [12], we examined the inhibitory effect of BM-MDSCs on anti-CD3 mAb-induced T cell activation with the iNOS inhibitor L-NMMA and the arginase 1 inhibitor nor-NOHA. Proliferation of splenic T cells, including both CD8 and CD4 cells, which is substantially induced by anti-CD3 mAb stimulation, was significantly inhibited by BM-MDSCs (Fig. 3C and D). This inhibitory effect was significantly diminished by L-NMMA but not by nor-NOHA (Fig. 3C and D).

Examination of the expression of monocytic surface markers on Mo-MDSCs and PMN-MDSCs isolated using a cell sorter, such as F4/80, CD115, and IL-4Rα, showed upregulation of these markers on Mo-MDSC (Supplementary Fig. 1). When splenocytes were stimulated with anti-CD3 mAb in the presence of PMN-MDSCs or Mo-MDSCs, Mo-MDSCs significantly inhibited T cell proliferation and IFN-γ production than PMN-MDSCs (Fig. 4A). Moreover, L-NMMA significantly diminished the inhibitory effect of Mo-MDSCs on CD4 and CD8 proliferation, but nor-NOHA did not (Fig. 4B and C). Besides, the inhibitory effect by PMN-MDSCs was not diminished by L-NMMA or nor-NOHA (Fig. 4B and C). These results suggested that Mo-MDSCs, rather than PMN-MDSCs, were the major subsets in the induced BM-MDSCs and that the iNOS dependent pathway was the main mechanism for inhibiting naïve T cell activation.

### 3.4. Naturally occurring Tregs increased when T cells were activated in the presence of Mo-MDSCs

When we examined the expression level of mRNA for inhibitory cytokines to induce Treg, such as IL-10 and TGF-β, in Mo-MDSCs or PMN-MDSCs that were co-cultured with naïve $CD4^+$ T cells under the anti-CD3 mAb stimulation, expression of mRNA for *Il10*, but not *Tgfb*, was significantly increased in both Mo-MDSCs and PMN-MDSCs after co-culture (Fig. 5A). Then, we examined Treg expansion by co-culture with Mo-MDSCs or PMN-MDSCs using $FoxP3^{hCD2}$ reporter mice that co-express hCD2 on the cell surface and FoxP3 in the cells [23]. When the naïve $CD4^+$ T cells of $FoxP3^{hCD2}$ reporter mice were stimulated with anti-CD3 mAb in the presence of MDSCs, $CD4^+$ $hCD2^+$ cells, which are FoxP3 positive Tregs, increased in the co-culture with BM-MDSCs,

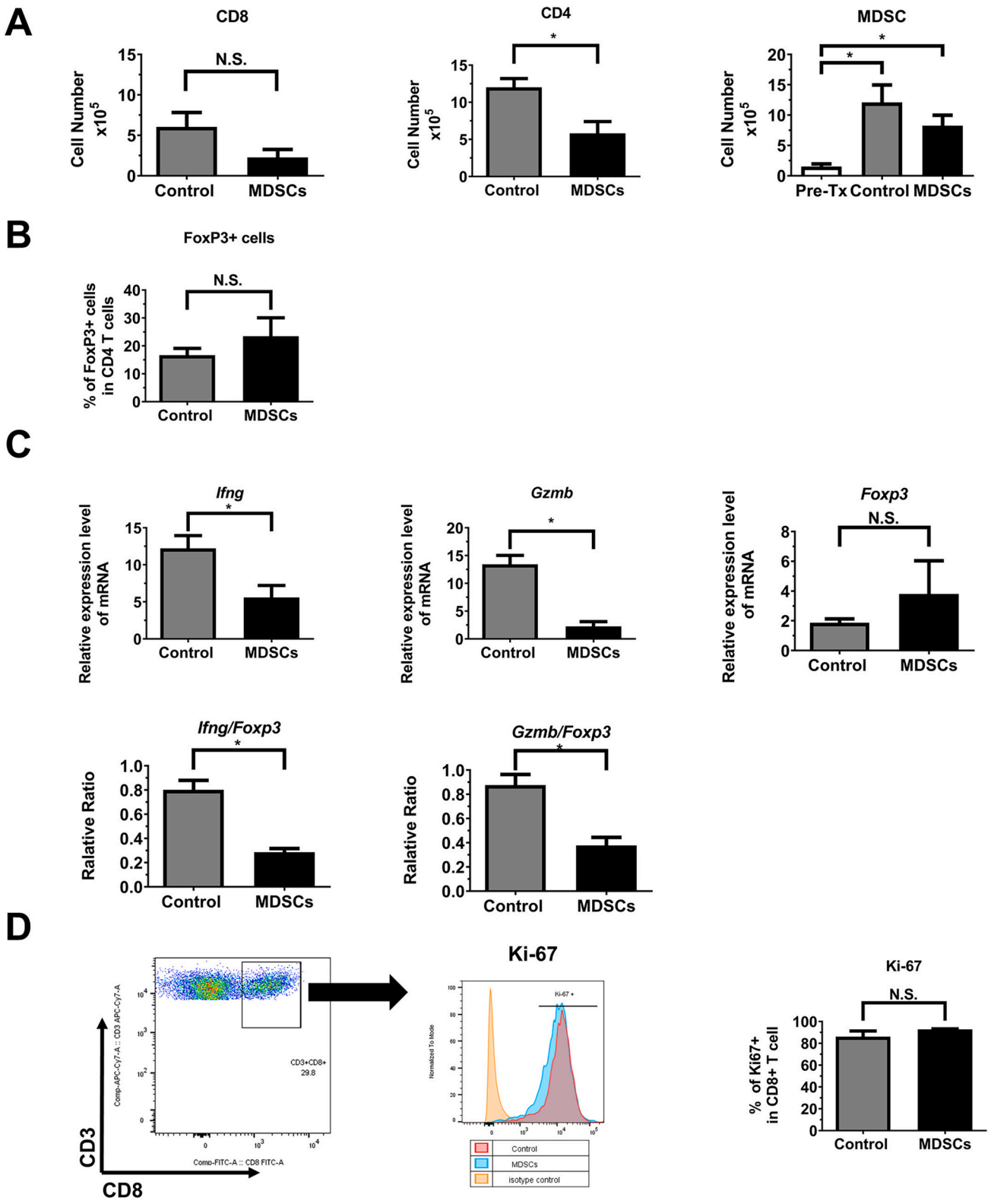


**Fig. 2.** Reduced effector T cell infiltration, but augmented infiltration of suppressor cells, into the heart graft. (A and B) Heart grafts were obtained 9 days after transplantation. Then, graft infiltrating MNCs s were prepared and the number of CD8 T cells, CD4 T cells and CD11b Gr-1$^{+}$ MDSCs (A) and FoxP3-expressing cell population in CD4 T cells (B) were examined as described in Methods. (C) mRNA was obtained from graft infiltrating MNCs harvested from the grafts 9 days after transplantation, then expression level of *Ifng*, *Gzmb*, and *Foxp3* mRNA was examined by RT-PCR. Data are presented as the relative expression level compared with that of syngeneic graft. The ratio of *Ifng* to *Foxp3* and *Gzmb* to *Foxp3* were calculated and presented. (D) Ki-67 expression on infiltrated CD8$^{+}$ T cells in graft was investigated and presented. Mean ± SEM of 3–5 mice in each group are presented (A - D). * $p < 0.05$. Similar results were obtained from three independent experiments (A - D).

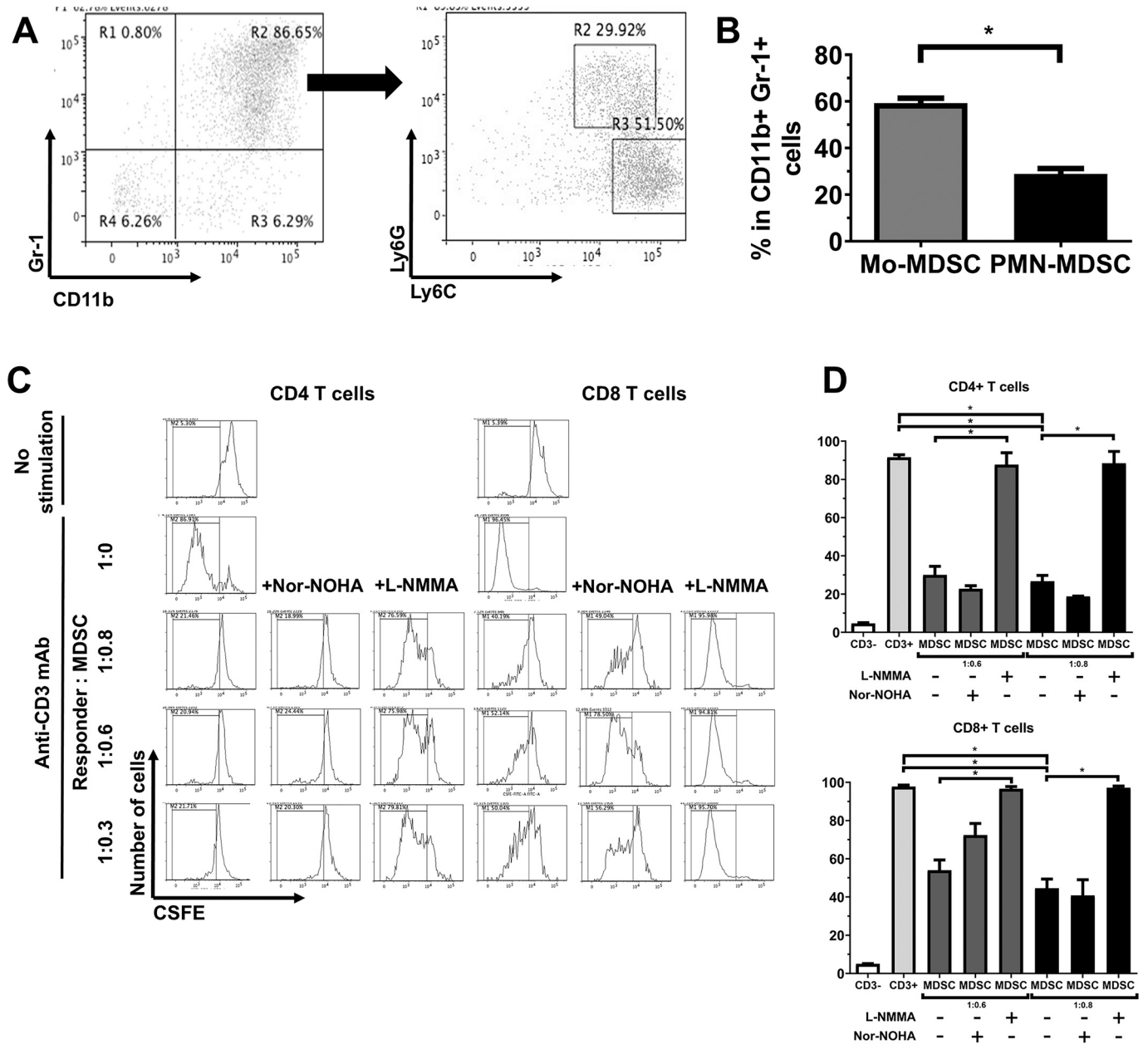


**Fig. 3.** BM-MDSCs inhibited T cell activation depending on iNOS synthase. (A and B) Population of monocytic MDSCs (Mo-MDSC) (Ly-6G$^-$Ly-6C$^{hi}$) and PMN-MDSCs (Ly-6G$^+$Ly-6C$^{low}$) in BM-MDSCs (CD11b$^+$Gr-1$^+$) were examined by flow cytometry, and the representative dot plot panels (A) and Means ± SEM of 3–5 mice in each group (B) are presented. (C and D) CFSE-labeled splenocytes were stimulated with anti-CD3 mAb in the presence of BM-MDSCs at the indicated ratio. Proliferation of CD4 and CD8 T cells was examined by flow cytometry as described in Methods. In some experiments, L-NMMA or Nor-NOHA was added to inhibit iNOS syntheses or arginase 1 respectively. Representative histograms of CFSE expression in CD4 and CD8 T cells (C) and mean ± SEM of 3–5 mice in each group (D) are presented. * $p < 0.05$. Similar results were obtained from three independent experiments (A - D).

particularly with Mo-MDSCs, but not in co-culture with PMN-MDSCs (Fig. 5B). Notably, hCD2$^+$ FoxP3-expressing CD4$^+$ Tregs did not increase when naïve hCD2$^-$ CD4$^+$ T cells of FoxP3$^{hCD2}$ reporter mice were stimulated with anti-CD3 mAb in the presence of Mo-MDSCs (Fig. 5C). These results suggest that Mo-MDSCs, but not PMN-MDSCs, increased FoxP3-expressing cells in CD4 T cells activated by anti-CD3 mAb stimulation, and that was due to the expansion of naturally occurring thymic-originated Tregs (nTregs) that originally express FoxP3, but not due to the induction of peripheral Tregs from naïve CD4$^+$ T cells.

### 3.5. *Mo-MDSC treatment effectively prolonged graft survival with increased Treg infiltration*

We finally examined the effect of adoptive transfer of recipient-derived Mo-MDSCs or PMN-MDSCs on the graft survival in the mice cardiac transplantation model. When Mo-MDSCs, but not PMN-MDSCs, were adoptively transferred into the recipient mice, graft acceptance was increased (Fig. 6A). Histological analyses consistently demonstrated less inflammation and cell infiltration in the graft of Mo-MDSC-treated recipients (Fig. 6B). Moreover, immunohistochemical analysis demonstrated that FoxP3$^+$ Treg infiltration increased in the graft tissue of Mo-MDSC-treated mice (Fig. 6B). When the mRNA expression of effector molecules, such as perforin and granzyme B, as well as FoxP3 in MNCs

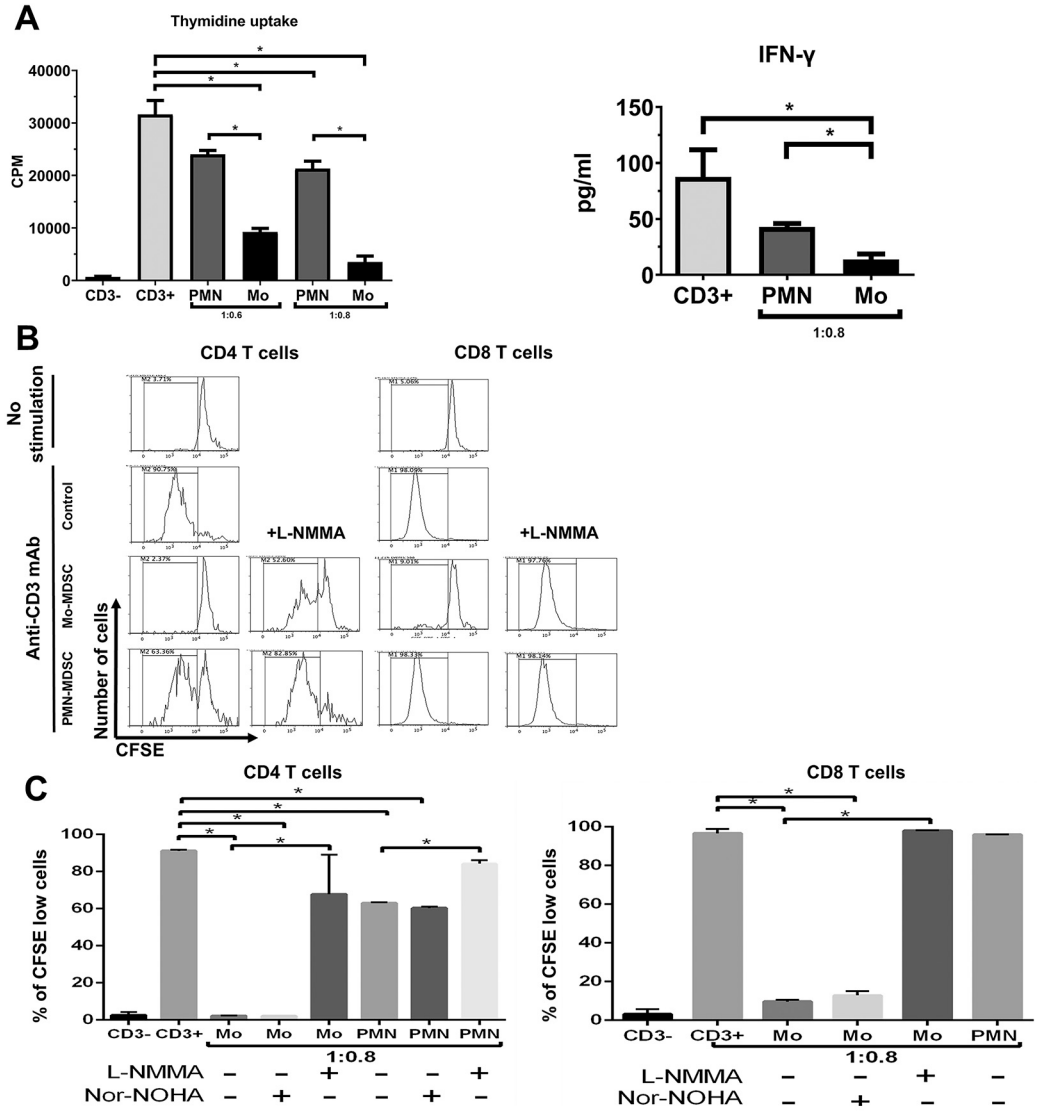


**Fig. 4.** Mo-MDSCs, but not PMN-MDSCs, inhibited T cell activation through iNOS synthase. (A) Splenocytes were stimulated with amti-CD3 mAb in the presence of Mo-MDSCs or PMN-MDSCs at the indicated ratios for 3 days. Then, thymidine uptake for the last 18 h and IFN-γ concentration in the supernatants were examined. Mean ± SEM of 3–5 mice in each group are presented. *, $p < 0.05$. (B and C) CFSE-labeled splenocytes were stimulated with anti-CD3 mAb in the presence of Mo-MDSCs or PMN-MDSCs at Responder: MDSC ratio = 1:0.8 for 3 days. Then, proliferation of CD4 T cells and CD8 T cells was examined by flow cytometry as described in Methods. L-NMMA or Nor-NOHA was added in some experiments. Representative histograms of CFSE expression in CD4 or CD8 cells (B) and mean ± SEM (C) are presented. *, $p < 0.05$.

infiltrating in the allograft were measured, either subset of MDSCs inhibited both *Prf1* and *Gzmb* mRNA expression, however only Mo-MDSCs infusion upregulated the expression of *Foxp3* in the graft (Fig. 6C). Taken together, these results suggested that the adoptive transfer of either Mo-MDSCs or PMN-MDSCs inhibited perforin and granzyme B expression in the graft, and Mo-MDSCs, but not PMN-MDSCs, significantly prolonged heart graft survival possibly due to increasing infiltration of Treg cells into the graft.

## 4. Discussion

Regulatory myeloid immune cells, such as MDSCs and Treg cells, are immune-suppressing cells that are attractive candidates for treatment of autoimmune diseases, allergic diseases, and transplanted patients [5,12,18,20,25–33]. In the present study, we explored the BM-MDSC populations exhibiting the most potent prolongation of transplanted heart graft survival in a mouse model. Mo-MDSCs significantly inhibited naïve T cell activation mainly through iNOS and expanded nTregs *in vitro*, but PMN-MDSCs did not. Consistently, adoptive transfer of Mo-MDSCs effectively prolonged graft survival and increased Treg infiltration into the graft (Supplementary Fig. 2). Thus, recipient-derived Mo-MDSCs would be the most potent therapeutic MDSC population to prolong allograft acceptance.

Both Mo-MDSCs and PMN-MDSCs have been reported to induce a number of pathogenic conditions, accumulate in the inflammatory environment, and inhibit immune responses [20,33,34]. In cancer patients, it was reported that PMN-MDSCs are more expanded and more recruited in potential metastatic lesions to promote cancer expansion than are Mo-MDSCs [1,35–37], Mo-MDSCs are more infiltrated and expanded than PMN-MDSCs at the primary tumor site [38,39]. During organ transplantation, systematic immunosuppressive therapy, such as rapamycin treatment, induced PMN-MDSCs that infiltrated into the targeted allograft [20], however, most reported *ex vivo* induced MDSCs acquired the characteristics of the monocytic phenotype, the same as

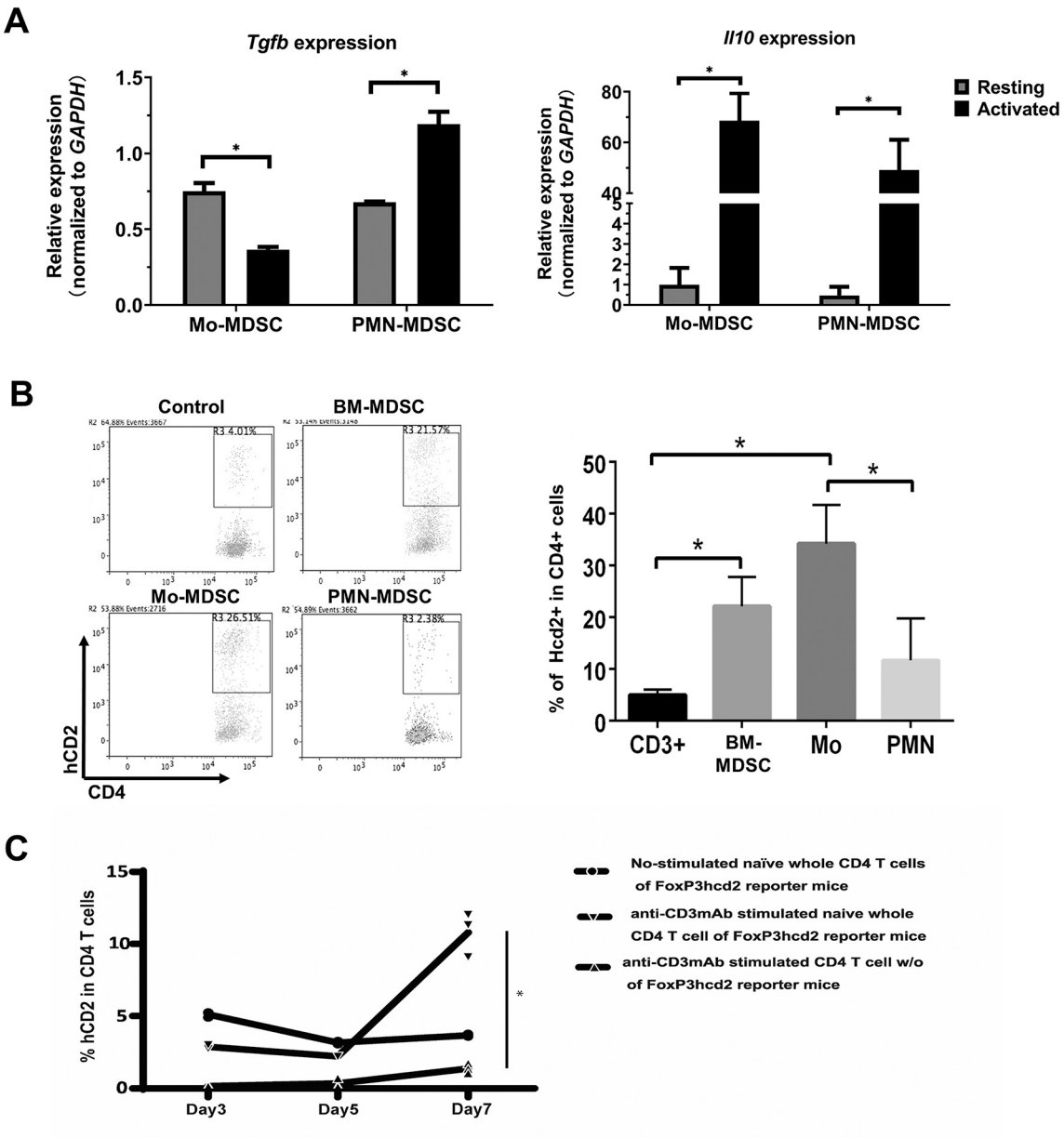


**Fig. 5.** T cell activation with Mo-MSDCs expanded natural Treg. (A) Bone marrow cells (BMC), Mo- and PMN-MDSCs were prepared and co-cultured with splenocytes with anti-CD3 mAb for 7 days. Then, Mo- or PMN-MDSCs were isolated using cell sorter and mRNA was prepared from these cells respectively. mRNA was also prepared BMC, Mo- or PMN-MDSCs before the co-culture. The expression level of *Tgfb* and *Il10* mRNA was examined by RT-PCR. Data are presented as the relative expression level compared with that of BMC. Mean ± SEM of 3–5 mice in each group are presented. * $p < 0.05$. (B) Splenocyte of $Foxp3^{hCD2}$ reporter mice were stimulated with anti-CD3 mAb for 7 days in the presence of BM-MDSC, Mo-MDSCs or PMN-MDSCs. Then, FoxP3/hCD2-expressing CD4 T cells were analyzed by flow cytometry. Representative dot plot and mean ± SEM of 3–5 mice in each group are presented. * $p < 0.05$. (C) Whole or $hCD2^+$ CD4 T cell-depleted splenocyte of $FoxP3^{hCD2}$ reporter mice were stimulated with anti-CD3 mAb for 3, 5 and 7 days in the presence of Mo-MDSCs. Then, percentage of $hCD2^+$ cells in CD4 T cells was analyzed by flow cytometry. Results of individual mice ($n$ = 3–5 in each group) are presented. * $p < 0.05$. Similar results were obtained from three independent experiments (B and C).

was found in the current study [12–14,27,31]. Therefore, PMN-MDSCs and Mo-MDSCs might develop adaptations to pathological and *in vitro* inducing conditions, and the therapeutic role of PMN-MDSCs for rapamycin therapy warrants careful clinical examination.

We here demonstrated that Mo-MDSCs inhibited T cell activation and expanded nTreg more efficiently than PMN-MDSCs. Mo-MDSC-mediated inhibition within 5 days was demonstrated to be iNOS dependent by an *in vitro* MLR, and nTreg expansion was observed at 7 days but was not 5 days after co-incubation. These results suggested that inhibition of T cell activation by Mo-MDSCs does not depend on nTreg expansion, although Treg induction is regarded as a common and important characteristic of regulatory myeloid immune cells [16,27,29,40,41]. Therefore, Mo-MDSC-mediated inhibition of T cell activation and expansion of naturally occurring Treg may be closely related but independent phenomena. Notably, MDSC infiltration was not increased in the heart graft of MDSC-treated recipients. Moreover, even when we traced the infused BM-MDSCs in the recipient mice using green fluorescent protein (GFP)-expressing BM-MDSCs, we were unable to detect GFP-expressing Mo-MDSCs in the graft, second lymphoid organs, or lungs of recipient mice 1 day after transplantation (Fig. S2). In contrast, Treg infiltration was increased in the heart grafts of Mo-MDSC-treated mice even 7 days after Mo-MDSC treatment. These results

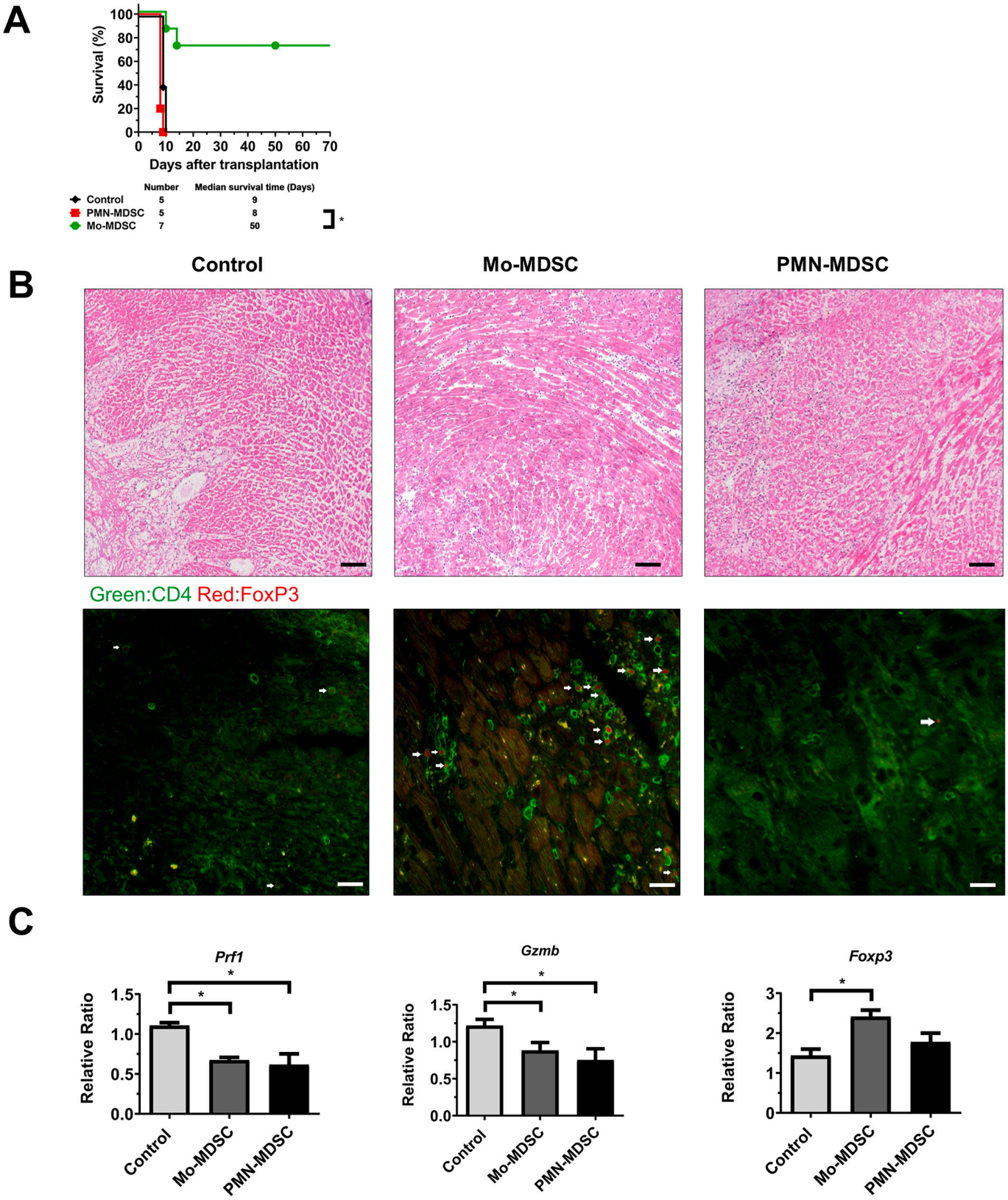


**Fig. 6.** Increased Treg infiltration in the graft accepted in Mo-MDSC-treated recipients. (A) Heart transplantation was performed immediately after injection of Mo-MDSCs or PMN-MDSCs. Then, graft survival was observed more than 50 days. ($n = 5$ in each group) * $p < 0.01$ by log-rank test. (B) Heart grafts were obtained from recipient mice 9 days after transplantation, then histological analysis was performed as described in Methods. Representative allograft tissue images of H&E staining (upper) and immunohistochemistry staining for CD4 (green) and FoxP3 (red) (lower) are presented. $CD4^{+}FoxP3^{+}$ cells were indicated by white arrows. Scale bar is 100 μm in H&E staining and 50 μm in immunohistochemistry staining. (C) Effector cytotoxic molecules (*prf1* and *Gzmb*) and *Foxp3* expression in the allograft were analyzed by qPCR and presented. Mean ± SEM of 4–5 mice in each group are presented. * $p < 0.05$. Similar results were obtained from three independent experiments (B and C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggest that Mo-MDSCs might be deleted promptly after inhibiting the activation of naïve T cells priming and expanding nTregs, and these Mo-MDSC-expanded nTregs would play important roles for maintenance of immune repression to result in tolerance.

We here demonstrated that allogenic, either donor or third-party, BM-MDSCs exerted less inhibitory effect on T cell responses to allogenic donor stimulation in MLR, such as proliferation and IFN-γ production. This would be due to the additional immune reaction of the responder cells against allogenic MHC expressed on BM-MDSCs under the *in vitro* culture conditions. Besides, infusion of allogenic (either donor or third-party) BM-MDSCs prolonged heart graft survival as long as that of recipient BM-MDSCs, and this suggested the possibility that donor or third-party BM-MDSCs were also applicable to prevent the rejection of transplanted graft. MHC non-restricted application in organ transplantation could be an advantage of MDSC-based therapies, since therapeutic MDSC would be inducible from third-party off-the-shelf cell products or induced pluripotent stem (iPS) cells [11,42,43].

We showed here that infusion of recipient-, donor- and third- party-derived BM-MDSC prolonged heart graft survival comparably, but these BM-MDSC administration did not increased FoxP3$^+$ cells in the heart graft. On the other hands, recipient Mo-MDSC, but not PMN-MDSC, treatment prolonged heart graft survival and increased FoxP3$^+$ cells in the heart graft. Interestingly, it was recently reported that administration of donor BM-MDSCs 7 days before transplantation promote graft acceptance by induction of recipients` endogenous MDSCs with donor specific suppressive function [44]. Thus, induction of endogenous inhibitory cells in the recipient is a critical event for the immune inhibitory effect of BM-MDSCs *in vivo*, and these induced cells would contribute to maintain inhibition of alloimmune responses resulting in the long-time graft acceptance. Besides, induced endogenous inhibitory cells might be various depending on cell source of infused BM-MDSCs and treatment protocol.

Generally, Mo-MDSC- and nTreg-mediated immune inhibition are regarded as non-specific. Thus, further studies are needed to investigate whether immune responses to third-party products and pathogens can be maintained in the recipients with MDSC-induced immune tolerance. Moreover, systematic MDSC administration might have limited utility for target delivery. Hence, locoregional application close to the inflammatory site, such that MDSCs were mixed with islet allografts and transplanted into the same location [45], would be required for optimal usage. Further preclinical nonhuman primate studies [46] are required to improve the clinical utility and benefits of MDSC therapies [47].

## 5. Conclusions

*Ex vivo* induced Mo-MDSC therapy has the potential to prevent graft rejection in organ transplantation, and allogenic MDSCs may be clinically applicable.

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## Authors contribution

K. F. participated in writing the manuscript, data analysis, and performance of the research. K. U. participated in data analysis, research design and writing the manuscript. E. Y. participated in data analysis and performance of the research. J. Z. participated in performance of the research. Y. K. participated in performance of the research. M. U. participated in performance of the research. Y. Y. participated in performance of the research. H. B. participated in data analysis. R. M. participated in data analysis and performance of the research. K. T. participated in data analysis and performance of the research. M. H. participated in data analysis and performance of the research. T. I. participated in data analysis. J. K. participated in data analysis. A. M. participated in research design. K. O. participated in research design. K. Takeda participated in data analysis, research design and writing the manuscript.

## Declaration of Competing Interest

All authors declared no competing interests.

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