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Prostaglandin E2 Potentiates Mesenchymal Stem Cell–Induced IL-10+IFN-γ+CD4+ Regulatory T Cells To Control Transplant Arteriosclerosis

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Mesenchymal stem cells (MSCs) are known for their immunomodulatory functions. We previously demonstrated that bone marrow–derived MSCs effectively control transplant arteriosclerosis (TA) by enhancing IL-10+ and IFN-γ+ cells. The objective of this study is to elucidate the mechanism by which MSCs induce IL-10+IFN-γ+CD4+ regulatory T type 1 (T_{R1})–like cells. In an MLR system using porcine PBMCs, MSC-induced IL-10+IFN-γ+CD4+ cells, which confer resistance to allogeneic proliferation in an IL-10–dependent manner, resemble T_{R1}–like cells. Both cyclooxygenase-derived PGE2 and IDO help to induce T_{R1}–like cells by MSCs. MSCs constitutively secrete PGE2, which is augmented in allogeneic reactions. However, T_{R1}–like cells were deficient in PGE2 and 4-fold less potent than were MSCs in suppressing MLR. PGE2 mimetic supplements can enhance the immunosuppressive potency of T_{R1}–like cells. In a porcine model of allogeneic femoral arterial transplantation, MSC-induced T_{R1}–like cells combined with PGE2, but not either alone, significantly reduced TA at the end of 6 wk (percentage of luminal area stenosis: T_{R1}–like cells + PGE2: 11 ± 10%; PGE2 alone: 93 ± 8.7%; T_{R1}–like cells alone: 88 ± 2.4% versus untreated 94 ± 0.9%, p < 0.001). These findings indicate that PGE2 helps MSC-induced IL-10+IFN-γ+CD4+ T_{R1}–like cells inhibit TA. PGE2 combined with MSC-induced T_{R1}–like cells represents a new approach for achieving immune tolerance. The Journal of Immunology, 2013, 190: 000–000.

Transplant arteriosclerosis (TA), with its distinctive concentric intimal hyperplasia, is characterized by the diffuse narrowing and occlusion of vessels in the allograft. The pathogenesis of TA involves a combination of peritransplant events, infections, alloimmunity, and conventional risk factors of atherosclerosis (1). TA develops specifically in allografts, which suggests the critical role of an immunological response. Using current immunosuppressive agents, the allograft may be relatively free of parenchymal cellular rejection but simultaneously exhibit TA (2). Therefore, novel therapeutic strategies for preventing TA are required to enhance the survival rate of solid-organ transplant recipients.

Mesenchymal stem cells (MSCs), which modulate various aspects of innate and adaptive immunity, have been applied to the treatment of autoimmune diseases (3, 4), allergic airway inflammation (5), and allograft rejection (6–8). In adaptive immunity, MSCs tilt T cell responses toward regulatory T cell (Treg) profiles both in vitro (9–13) and in vivo. Tregs are characterized by their suppressive and anergic properties. The two most relevant classes of CD4+ Tregs are CD4+CD25+Foxp3+ Tregs (14) and regulatory T type 1 (T_{R1}) cells (15). T_{R1} cells are defined by their suppression of Ag-specific immune responses in an IL-10–dependent manner (15), without Foxp3 expression (16). The canonical profile initially described for T_{R1} cells is IL-10+TGF-β+IL-4+IL-2−IFN-γ− (15). Subsequently, a subpopulation of the IL-10–secreting T_{R1} cells was found to produce substantial levels of IFN-γ (17–19), and they were designated “T_{R1}–like cells” (20–23). MSCs were demonstrated to drive the generation of Foxp3+ Tregs. Recently, MSCs were reported to expand T_{R1} cells bearing the CD4+CD25+IL-10+ phenotype (24). Whether MSCs achieve immune tolerance by inducing T_{R1}–like cells with a CD4+IL-10+IFN-γ− signature remains unknown.

The modulating effect of MSCs on allogeneic responses is described, in part, to cyclooxygenase (COX)-derived PGE2 (10, 25, 26). The biosynthesis of PGE2 by MSCs is not only constitutive, it also is augmented by inflammatory stimuli (10, 27). Certain studies suggested that inflammation, through the induction of COX-2 and IDO, is a prerequisite for licensing MSCs to become immunosuppressive (28–30). IDO catalyzes the rate-limiting step of tryptophan (Trp) degradation along the kynurenine (KYN) pathway (31), and both the reduction in local Trp concentration and the generation of Trp metabolites contribute to the regulatory effects of IDO. PGE2 was demonstrated to foster the outgrowth of...
T \textsubscript{reg} cells in the tumor microenvironment (32, 33). IDO expression in APCs was suggested to induce IL-10–secreting Tregs (34). Thus, COX-PGE\textsubscript{2} and IDO may be significant molecules for the generation of T \textsubscript{reg} cells.

We recently reported that bone marrow–derived MSCs are effective in suppressing TA and that they are associated with an enhanced expression of tissue levels of IL-10, IFN-\gamma, and IDO but not of Foxp3 (35). These results led us to propose that the control of TA by MSCs may be mediated by inducing a subtype of Tregs, such as T \textsubscript{reg}-like cells. In this study, we demonstrate the induction of IL-10\textsuperscript{+}IFN-\gamma\textsuperscript{+}CD4\textsuperscript{+} cells by MSCs in an allogeneic MLR. Furthermore, MSCs render a population of low-proliferative CD4\textsuperscript{+} cells to commit to T \textsubscript{reg}-like cells. We also provide evidence that MSCs induce T \textsubscript{reg}-like cells and mediate immunosuppression in vitro through activation of the COX-PGE\textsubscript{2} and IDO pathways. Finally, the results indicate that dual treatment with PGE\textsubscript{2} and T \textsubscript{reg}-like cells alleviates intimal proliferation in porcine models of allogeneic femoral arterial transplants.

Materials and Methods

Animals

Adult Taiwanese Lanyu minipigs (25–40 kg), procured from the Animal Propagation Station, Livestock Research Institute (Taitung, Taiwan), were maintained in the Laboratory Animal Center of National Taiwan University. All minipigs were used in the study according to the Guidelines for Animal Care. The experimental protocol was approved by the Institutional Animal Care and Use Committee (approval number 20060178).

Reagents

For flow cytometry analysis (FACSCalibur; BD) and FACS sorting (FACS-Aria; BD), the following Abs for positive staining were used: anti-CD4a-FITC (MIL17; Serotec), anti-CD4a-PE (74-12-4; BD), anti–IL-10 (262715; R&D Systems), anti–IFN-\gamma–Per-Cy5.5 (P2G10; BD), anti–Foxp3–allophycocyanin (FJK-16S; eBioscience), and anti-IDO (H-110; Santa Cruz). The Abs used for isotype-control staining were mIgG2b-FITC (MG2b-57; BioLegend), mIgG2b-PE (MG2b-57; BioLegend), mIgG1–Per-Cy5.5 (P3.6.2.1; eBioscience), and normal rabbit IgG (Santa Cruz).

Preparation of porcine bone marrow–derived MSCs and conditioned medium

MSCs were derived from the bone marrow aspirates of minipigs and grown in MSC medium consisting of αMEM (Invitrogen) and 10% FBS (Invitrogen) supplemented with basic fibroblast growth factor and epithelial cell growth factor (10 ng/ml each; R&D Systems). To prepare the MSC conditioned medium (CM), MSCs were initially plated in MSC medium to reach 50% confluence, followed by seeding in MLR medium composed of RPMI 1640 medium (Invitrogen) supplemented with basic fibroblast growth factor and epithelial cell growth factor (10 ng/ml each; R&D Systems). One-way MLR

We used MLR as a cell model to assess the potential of MSCs to induce T \textsubscript{reg}-like cells.

PBMCs were isolated from minipig blood using density-gradient centrifugation (Ficoll-Paque; GE Healthcare Bio-Sciences AB). The one-way MLR assay was performed in a 96-well round-bottom plate, as we described previously (35). In brief, 1 × 10\textsuperscript{4} responder cells and 1 × 10\textsuperscript{5} stimulator cells were plated in each well. MSCs (5 × 10\textsuperscript{3} cells/well) or MSC CM (0.1 ml/well) were added to the coculture. On the fourth day of coculture, cells were pulsed with 1 \muCi well\textsuperscript{\textsuperscript{\textsuperscript{-}}}[\textsuperscript{\textsuperscript{-}}]H-thymidine (Perkin Elmer) for 18 h. Radioactivity was counted using a MicroBeta Filter Mate-96 Harvester and a 1450 MicroBeta TriLux (both from PerkinElmer). Responder cell proliferation was measured by [\textsuperscript{\textsuperscript{3}}H]-thymidine uptake and expressed as cpm or calculated as the percentage of suppression. In parallel, the allospecific reactivity of responder PBMCs was determined by labeling responder cells with CFSE. Responder cells (1 × 10\textsuperscript{5}) were suspended in 1 ml PBS containing CFSE (5 μM; Sigma-Aldrich) and shaken at 10 rpm for 8 min at room temperature, to which cold PBS containing 2% FBS was added to stop the labeling process.

CSFE fluorescence intensity was measured by FACS analysis. To evaluate the influence of COX and/or IDO inhibitors on MSC action, indomethacin (IDM; 50 μM), celecoxib (25 μM), and/or 1-methyl-L-tryptophan (L-1-MT; 1 mM; all from Sigma-Aldrich) were added to the MSC-MLR coculture. Where indicated, dinoprostone or misoprostol acid (MPA; both from Sigma-Aldrich) at various concentrations was used to investigate the role of PGE\textsubscript{2}.

Transwell experiments

Transwell experiments were conducted using HTS Transwell-96 Well Permeable Supports (Corning).Responder and stimulator PBMCs (10\textsuperscript{5} cells/100 μl MLR medium/well) were cultivated in a 96-well round-bottom plate, and MSCs (5 × 10\textsuperscript{3} cells/100 μl MLR medium/well) were seeded onto the semipermeable membrane of Transwell inserts with a 1.0-μm pore size.

Isolation of MSC-primed responder CD4\textsuperscript{+} cells

To investigate the regulatory characteristics of MSC-primed CD4\textsuperscript{+} T cells, responder PBMCs were labeled with CFSE (5 μM) prior to coculture with allogeneic stimulator PBMCs, in the absence or presence of MSCs, with or without L-1-MT, plus IDM for 5 d (first MLR). Cell mixtures harvested from the first MLR were stained with an Ab against CD4 and analyzed by flow cytometry. CD4\textsuperscript{+} T cells of small size, low granularity, and intense CSFE fluorescence, corresponding to nondividing responder T cells (FSCK\textsuperscript{+}SSC\textsuperscript{MM}CFSE\textsuperscript{high}, designated as CD4\textsuperscript{R}), were gated and sorted out for subsequent intracellular staining and for the suppression of the MLR.

The immunosuppressive properties of CD4\textsuperscript{R} cells were evaluated using a subsequent allogeneic MLR (second MLR), in which the responder and stimulator PBMCs are identical to those in the first MLR. CD4\textsuperscript{R} sorted from the first MLR were treated with mitomycin-C (25 μg/ml) and added at a cell ratio of 1:1 (CD4\textsuperscript{R}/responder PBMCs). To investigate the role of IL-10 in CD4\textsuperscript{R}-mediated immunosuppression, neutralizing mAb against IL-10 (10 μg/ml; Invitrogen) or isotype control IgG1 (10 μg/ml; Bio-Legend) was added to the second MLR. To assess the allogeneic responsiveness of CD4\textsuperscript{R} cells, cells were left to rest in the MLR medium for 72 h after being sorted from the first MLR and then restimulated with PBMCs either from the original minipig (original S) used to induce CD4\textsuperscript{R} cells or from different minipigs (third-party S).

Intracellular staining

Cells were stained for Foxp3 and IDO using an intracellular staining buffer set (eBioscience), following the manufacturer’s instructions. For cytokine staining, the protein transport inhibitor monensin (1 μl/ml; eBioscience) was added for the final 6 h of the MLR. In selected experiments, cells were restimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml; both from Sigma-Aldrich). After fixation with 4% paraformaldehyde, cells were permeabilized with 0.1% saponin (eBioscience) and stained for IL-10 and IFN-\gamma.

ELISAs

Using commercially available kits, we analyzed the levels of IL-10, IFN-\gamma, and PGE\textsubscript{2} in supernatants harvested at specified time points in the MLR. The detection limits of the ELISA kits were 7.8 pg/ml for IL-10, 39 pg/ml for IFN-\gamma (R&D Systems), and 7.8 pg/ml for PGE\textsubscript{2} (R&D Systems).

Preparation of cell lysates and analyte extraction

Cell pellets prepared from the MLR were washed twice with PBS, resuspended in 50% (v/v) methanol (1 × 10\textsuperscript{5} cells/ml), and vortexed for 2 h at room temperature. After centrifugation at 2300 × g for 5 min, the supernatants were concentrated to dry under a nitrogen stream. The residues were reconstituted in deionized water for further analysis.

Liquid chromatography coupled with tandem mass spectrometry

Standards (500 ng/ml) for Trp and KYN (both Sigma-Aldrich) were prepared in 50% (v/v) methanol (1 × 10\textsuperscript{5} cells/ml), and subjected to the mobile phase for liquid chromatography coupled with tandem mass spectrometry. The mobile phase consisted of 0.1% (v/v) acetic acid in water (A) and 0.1% (v/v) acetic acid in acetonitrile (B)
compounds were separated using a linear gradient that began at A/B = 99/1 for 0.5 min and ended at 10 min with A/B = 20/80. The flow rate was 0.3 ml/min, and the injection volume was 10 μl. The ion pair monitored for quantitation was at m/z 205→188 (collision energy [CE] = 5V) for Trp and at m/z 209→196 (CE = 17V) and m/z 209→94 (CE = 13V) for KYN. The multiple-reaction monitoring mode dwell time was 20 ms. The fragmentor voltage setting was 70 and 93 V for Trp and KYN, respectively. MassHunter Qualitative Analysis software version B.03.01 (Agilent) was used for data acquisition and processing. IDO activity was expressed as the ratio of KYN/Trp integrated from ion current peaks at the appropriate retention time.

**Pig model of femoral arterial transplantation**

Femoral arterial transplantation was performed as described (35). Minipigs undergoing femoral arterial transplantation were divided into four experimental groups (n = 3 for each group): untreated group, PGE2 group, MSC-primed CD4+R cell group, and PGE2 plus MSC-primed CD4+R cell group. PGE2 was administered in two stages. In the perioperative stage, dinitroprostone (100 μg) was infused i.v. for 30 min just at the time of graft anastomosis, and misoprostol (200 μg; Pfizer) was administered orally in the postoperative stage four times daily until graft harvest. MSC-primed CD4+ R cells (2×10^6 cells in 1 ml divided into 10 injections for each graft) were injected into the skeletal muscle surrounding the vascular grafts.

**Histological analysis**

Formalin-fixed vascular grafts were embedded in paraffin, cross-sectioned, deparaffinized, rehydrated, and subjected to H&E or orcein staining. Intimal hyperplasia of vascular grafts was determined from computer images of orcein-stained cross-sections. The area surrounded by internal elastic lamina (IELA) and the luminal area (LA) were calculated by an image-analysis program (Image J, Version 1.46r, NIH Image). The severity of intimal hyperplasia was calculated by the formula: [(IELA − LA)/IELA] × 100%.

**Statistical analysis**

All data are expressed as mean ± SD. Statistical analysis was performed using Prism 5 software (GraphPad Software, San Diego, CA). Group differences were assessed by the t test, and three or more data sets were compared using one-way ANOVA with Bonferroni multiple comparison for measuring significance. A p value <0.05 was considered statistically significant.

**Results**

Abortive alloresponse in the presence of MSCs is associated with elevation of IL-10 and IFN-γ but not Foxp3

The one-way MLR assay showed that MSCs at a ratio of 1:2 (MSC/responder PBMCs) significantly reduced [3H]thymidine incorporation of allostimulated responder PBMCs (n = 3, p < 0.001, Supplemental Fig. 1A). In parallel, MSCs restrained the division of the CFSE-labeled responder PBMCs over the entire culture period (Supplemental Fig. 1B, 1C). These findings suggest that MSCs attenuate allogeneic lymphocyte proliferation. IL-10 and IFN-γ in the culture supernatant of MSCs alone were nearly undetectable (data not shown). The level of IL-10 (n = 3, p < 0.001, Fig. 1A) and IFN-γ (n = 3, p < 0.001, Fig. 1B), but not that of intranuclear Foxp3

**FIGURE 1.** MSCs enhance levels of IL-10 and IFN-γ and induce IL-10+IFN-γ+CD4+ cells, but not Foxp3, in allogeneic reactions. Responder PBMCs were cultured with autologous (Auto) or allogeneic stimulator PBMCs in the absence (Allo) or presence (Allo + MSC) of MSCs. Levels of IL-10 (A) and IFN-γ (B) in cell culture supernatants. Cells were double labeled with FITC–anti-CD4 and allophycocyanin–anti-Foxp3 or triple labeled with FITC–anti-CD4, PerCP–Cy5.5–anti-IFN-γ, and anti-IL-10, followed by PE–anti-mouse IgG. Appropriate Abs were used as an isotype control. Representative FACS plots are shown for CD4 versus Foxp3 (C) and IL-10 versus IFN-γ (E) on gated CD4+ cells and are displayed as their corresponding quantitative analysis (D, F). Percentages of CD4+IL-10+ cells in subpopulations stratified by IL-10 expression levels in MSC-primed MLR are shown as representative FACS plots (G) and quantitative analysis (H). n = 3 for each experiment. ***p < 0.001.
(n = 3, p = 0.475, Fig. 1C, 1D), of the MLR were upregulated by MSCs. These results suggest that MSCs induce a subset of PBMCs to express IL-10 and IFN-γ but not Foxp3.

**MSCs induce IL-10*IFN-γ*CD4*+ cells**

To characterize the subset of PBMCs producing IL-10 and IFN-γ, we analyzed intracellular IL-10 and IFN-γ in gated CD4*+ cells by flow cytometry. IL-10*IFN-γ*CD4*+ cells in the MLR with MSCs increased 3-fold over those in the MLR without MSCs (4.8 ± 1.4% versus 1.0 ± 0.5%, n = 5, p < 0.001, Fig. 1E, 1F). Among the different subpopulations of CD4*+ cells stratified by intracellular IL-10 levels, IFN-γ was most prominently expressed in the IL-10high* cells (n = 3, p < 0.001, Fig. 1G, 1H). These findings suggest that MSCs induce IL-10*IFN-γ*CD4*+ T*reg-like cells.

**MSCs induce IL-10*IFN-γ*CD4*+ cells by releasing soluble factors**

We next determined in a Transwell culture system whether MSCs induce IL-10*IFN-γ*CD4*+ cells through the release of soluble factors. MSCs cultured in the upper chamber retained potent inhibition of allogeneic cell proliferation in the lower chamber (p < 0.001, Supplemental Fig. 2A). Furthermore, CM derived from MSCs significantly reduced allogeneic proliferation of responder PBMCs (n = 3, p < 0.001, Supplemental Fig. 2B). Flow cytometry analysis revealed that MSCs induced an ~5-fold increase in IL-10*IFN-γ*CD4*+ cells in the Transwell system (4.7 ± 0.2% versus 0.9 ± 0.5%, n = 3, p < 0.001, Supplemental Fig. 2C, 2D). Similarly, the CM of MSCs expanded IL-10*IFN-γ*CD4*+ cells >3-fold over the control MLR medium (3.6 ± 0.5% versus 0.9 ± 0.5%, n = 3, p < 0.001, Supplemental Fig. 2C, 2D). These results indicate that MSCs induce IL-10*IFN-γ*CD4*+ cells and modulate allogeneic immune response by releasing soluble factors into the extracellular milieu.

**PGE2 and IDO mediate the induction of IL-10*IFN-γ*CD4*+ cells and suppression of the MLR**

We next searched for soluble factors that induce IL-10*IFN-γ*CD4*+ cells. IDO was considered because we demonstrated in a previous study that MSCs induce IL-10*, IFN-γ*, and IDO* cells in the vascular allografts (35). MSCs induced a significant increase in IDO* cells when added to allogeneic PBMCs compared with MSCs or allogeneic PBMCs alone (Fig. 2A, 2B). Corresponding to the increase in IDO* cells, the enzyme activity of IDO was enhanced in MSC-treated PBMCs in the MLR (Fig. 2C). PGE2 was reported to promote immune tolerance by regulating IDO expression (36, 37). To corroborate whether COX-derived PGE2 acts as another potential humoral factor, we incubated MSC-treated PBMCs in the MLR with IDA, a nonselective COX inhibitor. IDA significantly reduced IDO* cells (Fig. 2A, 2B) and IDO activity (Fig. 2C) in MSC-treated PBMCs. These findings suggest that the induction of IDO expression by MSC-treated PBMCs in the MLR relies, in part, on COX-derived PGE2.

To delineate the relationship between COX and IDO in the MSC-mediated induction of CD4*+IL-10*+IFN-γ* cells, we added IDA and L-1-MT, an IDO inhibitor, to the MLR. The induction of IL-10*IFN-γ*CD4*+ cells by MSCs was partially blocked by IDA and L-1-MT alone (Fig. 2D, 2E). L-1-MT synergized with IDA to block the induction of IL-10*IFN-γ*CD4*+ cells by MSCs (Fig. 2D, 2E). Furthermore, neither IDA nor L-1-MT alone could completely abolish the effect of MSCs in suppressing allogeneic PBMC proliferation, whereas IDA combined with L-1-MT abrogated the antiproliferative effect of MSCs (Fig. 2F). These results suggest that COX-derived PGE2 and IDO act together to induce CD4*+IL-10*+IFN-γ* cells to suppress allogeneic responses.
MSCs convert CD4+ cells into immunosuppressive T_{R1}-like cells

To test the hypothesis that MSCs prime a subset of CD4+ cells in the MLR to IL-10+IFN-γ+CD4+ T_{R1}-like cells, we isolated, by cell sorting, nondividing small CD4+ responder lymphocytes (FSC_{low}SSC_{low}CD4^{+}CFSE_{high} designated as CD4^\text{R}) from allostimulated PBMCs that were treated or not with MSCs (Fig. 3A). CD4^\text{R} cells sorted from MSC-treated PBMCs showed a higher level of IL-10 and IFN-γ expression than did those from PBMCs not treated with MSCs (Fig. 3A). The expression of IL-10 and IFN-γ in the CD4^\text{R} cells sorted from MSC-treated PBMCs was abrogated by IDM plus L-1-MT (Fig. 3A). These results suggest that MSCs convert a fraction of CD4+ cells in the allostimulated PBMCs to T_{R1}-like cells.

To confirm that CD4^\text{R} cells possess immunosuppressive properties, we added MSC-treated and control CD4^\text{R} cells to the MLR and analyzed their proliferative activity. CD4^\text{R} cells from MSC-treated PBMCs significantly reduced cell proliferation, whereas CD4^\text{R} cells from control PBMCs had no effect (Fig. 3B). The suppressive effect of MSC-treated CD4^\text{R} cells was abrogated by IDM plus L-1-MT (Fig. 3B). Moreover, this suppression was blocked in the presence of neutralizing mAb against IL-10 (Fig. 3B). In correlation with the suppression of cell proliferation, IL-10+IFN-γ+CD4+ cells induced by MSC-primed CD4^\text{R} cells were abrogated by IDM plus L-1-MT (Fig. 3C, 3D). Furthermore, MSC-primed CD4^\text{R} cells increased IDO activity in alloactivated PBMCs (Fig. 3E). These results indicate that MSCs converted CD4+ cells, giving them immunosuppressive properties resembling those of MSCs in vitro.

We next determined whether CD4+R cells isolated from MSC-treated allostimulated PBMCs are anergic to allostimulation. In the restimulation assay, CD4^\text{R} cells served as responder cells, and the PBMCs from either the original minipig used to induce CD4^\text{R} cells (original S) or other minipigs (third-party S) served as the stimulator cells. CD4^\text{R} cells from the MLR without MSC treatment proliferated in response to the original S and the third-party S (Fig. 3F). In contrast, CD4^\text{R} cells isolated from MSC-treated PBMCs did not proliferate when stimulated with the original or the third-party S (Fig. 3F). However, CD4^\text{R} cells isolated from MSC-primed allostimulated PBMCs in the presence of IDM plus L-1-MT lost anergy and proliferated in response to the original and the third-party PBMC stimulation (Fig. 3F). These results suggest that MSCs prime the nondividing small CD4^\text{R} cells to suppress allostimulation by increasing T_{R1}-like cells, as well as to become anergic.

Expansion of IL-10+IFN-γ+CD4+ cells correlates temporally with IDO expression but lags behind PGE\textsubscript{2} production

To elucidate the relationship among PGE\textsubscript{2}, IDO, and IL-10+IFN-γ+CD4+ cells, we determined the temporal profile of the MSC-suppressed MLR over 5 d of culture. Allogeneic lymphocyte proliferation increased progressively and achieved a maximum at the end of the 5-d MLR (Fig. 4A). In the presence of MSCs, PGE\textsubscript{2} levels began to increase as early as 3 h after the MLR (data not shown) and reached a plateau on day 1 (Fig. 4B). However, IDO activity did not increase significantly until day 3 of the MLR (Fig. 4C). In parallel, IL-10+IFN-γ+CD4+ cells increased progressively from days 3 to 5 (Fig. 4D).

PGE\textsubscript{2} enhances the antiproliferative action of MSC-primed CD4+R cells

We compared the antiproliferative activity of MSC-primed CD4+R cells with MSCs by mixing those cells at various cell concentration ratios with allogeneic PBMCs and analyzing [\textsuperscript{3}H]thymidine uptake by PBMCs. MSCs achieved maximal suppression of cell proliferation when added at 1:2 ratio of MSC:PBMC. To test the anergic potential of CD4+R cells, cells were restimulated with stimulators identical to those in the first MLR (original S) and third-party PBMCs (third-party S).

**FIGURE 3.** MSC-primed nondividing CD4+ T cells are immunosuppressive to alloreactive cells and anergic to allostimulation. CFSE-labeled responder PBMCs were activated with allogeneic stimulator (first MLR) in the absence (Allo) or presence of MSCs without (Allo + MSC) or with IDM plus L-1-MT (Allo + MSC + IDM + L-1-MT). (A) After a 5-d coculture period, cells were labeled with PE–anti-CD4, and CD4+ cells were added to a 5-d coculture period, cells were labeled with PE–anti-CD4, and CD4+ cells were added to the restimulation assay, CD4+ cells served as responder cells, whereas CD4+ cells sorted from MSC-treated PBMCs showed a higher level of IL-10 and IFN-γ expression than did those from PBMCs not treated with MSCs (Fig. 3A). The expression of IL-10 and IFN-γ in the CD4+ cells sorted from MSC-treated PBMCs was abrogated by IDM plus L-1-MT (Fig. 3A). These results suggest that MSCs convert a fraction of CD4+ cells in the allostimulated PBMCs to T_{R1}-like cells.

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To elucidate the relationship among PGE\textsubscript{2}, IDO, and IL-10+IFN-γ+CD4+ cells, we determined the temporal profile of the MSC-suppressed MLR over 5 d of culture. Allogeneic lymphocyte proliferation increased progressively and achieved a maximum at the end of the 5-d MLR (Fig. 4A). In the presence of MSCs, PGE\textsubscript{2} levels began to increase as early as 3 h after the MLR (data not shown) and reached a plateau on day 1 (Fig. 4B). However, IDO activity did not increase significantly until day 3 of the MLR (Fig. 4C). In parallel, IL-10+IFN-γ+CD4+ cells increased progressively from days 3 to 5 (Fig. 4D).

PGE\textsubscript{2} enhances the antiproliferative action of MSC-primed CD4+R cells

We compared the antiproliferative activity of MSC-primed CD4+R cells with MSCs by mixing those cells at various cell concentration ratios with allogeneic PBMCs and analyzing [\textsuperscript{3}H]thymidine uptake by PBMCs. MSCs achieved maximal suppression of cell proliferation when added at 1:2 ratio of MSC:PBMC. To test the anergic potential of CD4+R cells, cells were restimulated with stimulators identical to those in the first MLR (original S) and third-party PBMCs (third-party S).
proliferation at an MSC/responder PBMC ratio of 1:2 (Fig. 5A). In contrast, MSC-primed CD4+R cells did not achieve maximal suppression until the ratio of 2:1 was reached. At a 1:2 ratio, MSC-primed CD4+R cells exerted 40% suppression of [3H] thymidine uptake. These results indicate that MSC-primed CD4+R cells are 4-fold less potent than MSCs in controlling the allogeneic stimulation of lymphocyte proliferation. One hypothesis for the higher antiproliferative action of MSCs is the production of PGE2 by MSCs but not by MSC-primed CD4+R cells. To confirm this hypothesis, we investigated the dependence of PGE2 in immunosuppression mediated by MSC-primed CD4+R cells. The PGE2 level was enhanced significantly in the MSC-treated MLR but was low in the MSC CM (Fig. 5B). In contrast, PGE2 was nearly undetectable in the culture of allogeneic MLR treated with MSC-primed CD4+R cells (Fig. 5B). Moreover, the addition of IDM attenuated half of the immunosuppressive potency of MSCs, whereas it did not repress the immunosuppressive effects of MSC-primed CD4+R cells (Fig. 5C). We further determined whether the exogenous addition of dinoprostone or MPA, an active metabolite of misoprostol (PGE2 mimetic), enhances the action of MSC-primed CD4+R cells. Dinoprostone and MPA suppressed lymphocyte proliferation equipotently (Fig. 5D). A suboptimal concentration (1 ng/ml) of MPA enhanced the antiproliferative effect of MSC-primed CD4+R cells at a cell ratio of 1:1 (Fig. 5E). However, MPA exerted a lesser effect on MSC-primed CD4+R cells at a cell ratio of 1:2 (Fig. 5E). These results suggest that MSC-primed CD4+R cells are intrinsically deficient in COX-derived PGE2 and that their immunosuppressive potency can be augmented by PGE2 supplements.

PGE2 enhances the competency of MSC-primed CD4+R cells in control of TA

To investigate whether the PGE2-augmented immunosuppressive potency of MSC-primed CD4+R cells occurs in vivo, we treated minipigs undergoing allogeneic femoral arterial transplantation with PGE2 plus MSC-primed CD4+R cells or with PGE2 and MSC-primed CD4+R cells alone, whereas others received no treatment. Histological examination of the vascular grafts 6 wk after transplantation showed that the untreated allograft was occluded by neointima (Fig. 6). Administering either PGE2 or MSC-primed CD4+R cells alone did not exert a significant suppression of intimal hyperplasia. In contrast, TA was abrogated through concomitant treatment with PGE2 and MSC-primed CD4+R cells (Fig. 6). We next determined the temporal effect of PGE2 on potentiating anti-TA by MSC-primed CD4+R cells by administering PGE2 for the first 14 d only or beginning on day 15. Compared with the group in which PGE2 was given for 42 d, intimal hyperplasia was evident in the group in which PGE2 was either given for the first 14 d or administered starting on day 15 (Fig. 6 versus Supplemental Fig. 3). These results, in concordance with the in vitro findings, suggest that administering PGE2 throughout the allogeneic response is required to potentiate MSC-primed CD4+R cells to protect the transplanted arterial allografts from TA.
FIGURE 6. PGE2 enhances the ability of MSC-primed CD4+R cells to inhibit TA. (A) Representative cross-sectional images of allografts from the untreated group (a, e), PGE2 group (b, f), MSC-primed CD4+R cell group (c, g), and PGE2 plus MSC-primed CD4+R cell group (d, h) 6 wk posttransplantation [H&E (a–d), orcein stain (e–h)]. Delivery of PGE2 included perioperative i.v. infusion of dinoprostone (100 μg for 30 min), followed by oral administration of misoprostol (200 μg, four times daily). A total of 2 × 10^6 of MSC-primed CD4+R cells was used for local injection. Arrows indicate IELA. Scale bars, 200 μm. (B) Severity of intimal hyperplasia determined from orcein-stained histological sections by an image-analysis program (Image J, Version 1.46r, NIH Image); n = 3 for each group. ***p < 0.001.

Discussion

Our findings elucidate a new paradigm of immunomodulation by MSCs. We provided evidence that MSCs control immune reactions in an allogeneic MLR cell system by inducing T_R1-like cells bearing the CD4+IL-10–IFN-γ+ signature. MSCs generate PGE2 through the COX pathway, which, in turn, activates IDO. COX-PGE2 and IDO act together to induce T_R1-like cells and suppress allostimulated immune responses. Consistent with the in vitro data, administering PGE2 with a local injection of MSC-induced T_R1-like cells attenuated intimal hyperplasia significantly in a porcine femoral arterial transplantation model. Results from this study provide a unified concept for explaining the broad immunomodulatory actions of MSCs on innate and adaptive immunity, as well as their diverse therapeutic effects on a wide variety of diseases caused by autoimmunity, allergies, and allograft rejection in solid organ transplantation (3, 5, 7, 8).

Our results provide evidence that, by cell sorting, MSC-primed CD4+R cells are phenotypically equivalent to IL-10–IFN-γ–CD4+ cells. We provide additional evidence that shows that MSC-primed CD4+R cells are functional T_R1-like cells. First, they are immunosuppressors. Although MSC-primed CD4+R cells represent only a small subset coexpressing IL-10 and IFN-γ (2.47%, Fig. 3A), they suppressed the proliferation of allogeneic PBMCs by 50% in an IL-10–dependent manner (Fig. 3B). Second, they are hyporesponsive to alloantigens (Fig. 3F). This anergic character-

istic correlates with previous studies wherein T_R1 was reported to have an intrinsically low proliferative capacity (20). In most recent studies, T_R1-like cells are defined by the coexpression of IL-10 and IFN-γ, associated with functional competence in immunomodulation (21, 22). Therefore, we conclude that the MSC-primed CD4+R cells in this study are IL-10–IFN-γ–CD4+ T_R1-like cells.

Although IL-10 produced by T_R1 cells is known for its immunosuppressive activity, the role of coexpressed IFN-γ in the MSC-induced IL-10–IFN-γ–CD4+ cells remains elusive. In a rat model of MHC-mismatched cardiac transplantation, IFN-γ expressed by Foxp3+CD4+ T cells of tolerant recipients may induce IDO expression in an allograft endothelial cell (38). Furthermore, IL-10 and IFN-γ were found to cooperatively regulate dendritic cell functions toward immune tolerance through the augmentation of IDO (39). In our MLR model, IDO activity was enhanced by the addition of MSC-primed CD4+R cells (Fig. 3E). This finding reinforces the biological significance of the coproduction of IL-10 and IFN-γ in immunologic tolerance mediated by T_R1-like cells.

Both PGE2 (10, 26, 27) and IDO (28, 29) were demonstrated to participate in MSC-mediated immunomodulation. PGE2 (32) and IDO (34) were suggested to play a role in the induction of IL-10–secreting Tregs. Therefore, it is reasonable to propose that MSCs can induce T_R1 or T_R1-like cells to combat deviated immune responses in a PGE2- or IDO-dependent manner. Following this speculation, we found that MSCs blunted allogeneic reactions and expanded IL-10–IFN-γ–CD4+ cells mainly through soluble factors (Supplemental Fig. 2). However, inhibitors targeting either PGE2 production or IDO activity could attenuate the MSC-induced immunomodulation only partially (Fig. 2F). Only when PGE2 and IDO were concomitantly blocked could the MSC-expanded IL-10–IFN-γ–CD4+ subpopulation be completely diminished (Fig. 2D, 2E) and the allogeneic response be restored (Fig. 2F).

Although PGE2 was reported to promote immune tolerance through the enhancement of downstream IDO expression and activity (40–42), this redundancy suggests that MSC-secreted PGE2 promotes the ontogeny of T_R1-like cells either directly or through IDO. However, in this study, the role of IDO in concerted immunomodulation of MSCs was investigated only by adding an IDO inhibitor in allogeneic MLR in vitro. The significance of IDO-induced KYN pathway metabolites in vivo cannot be confirmed until we demonstrate that IDO inhibition can abrogate MSC-induced long-term allograft patency in our femoral arterial transplantation model.

The clinical use of PGE2 has been limited by poor oral bioavailability, short half-lives, and significant toxicity profiles. Misoprostol, a synthetic analog of PGE1, binds to and activates each of the four heptahelical G protein–coupled E prostanoid receptors normally ligated by the endogenous PGE2 (43). The regulatory effects of misoprostol on both innate and adaptive immune systems were proved to be similar to those of natural PGE2 (44, 45). Misoprostol, given in the dosage used in this study (200 μg, 4 times daily) and in the presence of conventional immunosuppressants, was reported to reduce acute rejection in renal allografts (46). Therefore, oral misoprostol can reasonably substitute for PGE2 in our TA model.

We previously demonstrated that bone marrow–derived MSCs are effective in suppressing TA in a porcine model of femoral arterial transplantation (35). In this study, we showed that PGE2 combined with MSC-primed T_R1-like cells may replace MSCs to achieve immune tolerance in TA. This finding raises several implications. First, MSC-primed T_R1-like cells represent only one subset of immunomodulatory cells induced by MSCs. In our study, the potency of MSC-induced T_R1-like cells was 4-fold less...
than that of MSCs in suppressing the allogeneic MLR (Fig. 5A). Because we applied the same cell numbers for MSC-induced Tr1-like cells as we did previously for MSCs in the porcine femoral arterial transplantation model, Tr1-like cells might require the assistance of PGE2 to inhibit TA. Second, PGE2 and Tr1-like cells may act through independent mechanisms to modulate the immune system. PGE2 is known to regulate dendritic cell functions and may act through independent mechanisms to modulate the downstream effects and promote the de novo induction of more Tr1-like cells. Finally, in the allogeneic MLR modulated by MSCs, the early production of PGE2 and the expansion of Tr1-like cells at later stages (Fig. 4) suggest that each immunomodulatory mediator must exert its regulatory effects at the appropriate time in vivo to prevent TA. Because Tr1-like cells alone (Fig. 5A) and PGE2 alone (Fig. 5D) partially suppress the allogeneic MLR, both are expected to partially dampen TA when given independently. However, neither Tr1-like cell transfers alone nor PGE2 administration alone is effective in preventing TA (Fig. 6). We speculate that PGE2 is efficient in regulating allogeneic priming. When Tr1-like cells were provided in the absence of PGE2 during femoral arterial transplantation, the allogeneic Ag was recognized, and it set in motion an ever-widening immune response that ultimately led to TA. However, the observation that PGE2 alone is partially effective at alleviating the allogeneic MLR may only be applicable to an in vitro culture with a short duration (5-d coculture period of the MLR). When Tr1-like cells did not act in turn with PGE2 to modulate allogeneic responses in the femoral arterial transplantation model, the protective effects of PGE2 were unable to last for 6 wk, and TA ensued. Therefore, it is reasonable to propose that both PGE2 and Tr1-like cells are indispensable mediators generated by MSCs to maintain long-term immune tolerance. Further studies are required to elucidate the temporal interaction between PGE2 and Tr1-like cells and to identify their specific targets in the immune system.

We have provided evidence that MSCs induce IL-10^INF_g+CD4^Tr1-like cells during allogeneic reactions. Furthermore, MSCs prime a subpopulation of low-proliferative CD4^+ cells to commit to coproducing IL-10 and INF-γ and to suppress allogeneic proliferation. We also identified COX-derived PGE2 and IDO metabolites as soluble factors that induce the generation of Tr1-like cells and mediate the immunomodulatory effects of MSCs synergistically in vitro. Moreover, neither PGE2 nor Tr1-like cells alone can account for the full suppressive potency of MSCs in vitro; only when both were applied concomitantly in vivo could TA be alleviated. Together, PGE2 and Tr1-like cells may play nonredundant roles in MSC-mediated immunomodulation against TA. Our findings provide a new approach for the prevention of TA in cardiac transplantation.

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Disclosures

The authors have no financial conflicts of interest.

References


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