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Prostaglandin E_2 Potentiates Mesenchymal Stem Cell–Induced IL-10^+IFN-$\gamma^+$$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-$\gamma^+$$CD4^+$ regulatory T type 1 (T_R1)–like cells. In an MLR system using porcine PBMCs, MSC-induced IL-10^+IFN-$\gamma^+$$CD4^+$ cells, which confer resistance to allogeneic proliferation in an IL-10–dependent manner, resemble T_R1-like cells. Both cyclooxygenase-derived PGE_2 and IDO help to induce T_R1-like cells by MSCs. MSCs constitutively secrete PGE_2, which is augmented in allogeneic reactions. However, T_R1-like cells were deficient in PGE_2 and 4-fold less potent than were MSCs in suppressing MLR. PGE_2 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 PGE_2, but not either alone, significantly reduced TA at the end of 6 wk (percentage of luminal area stenosis: T_R1-like cells + PGE_2: 11 ± 10%; PGE_2 alone: 93 ± 8.7%; T_R1-like cells alone: 88 ± 2.4% versus untreated 94 ± 0.9%, $p < 0.001$). These findings indicate that PGE_2 helps MSC-induced IL-10^+IFN-$\gamma^+$$CD4^+$ T_R1-like cells inhibit TA. PGE_2 combined with MSC-induced T_R1-like cells represents a new approach for achieving immune tolerance. ***The Journal of Immunology*****, 2013, 190: 2372–2380.**

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-$\gamma^{+/-}$ (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 ascribed, in part, to cyclooxygenase (COX)-derived PGE_2 (10, 25, 26). The biosynthesis of PGE_2 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. PGE_2 was demonstrated to foster the outgrowth of

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Abbreviations used in this article: CE, collision energy; CM, conditioned medium; COX, cyclooxygenase; IDM, indomethacin; IELA, internal elastic lamina; KYN, kynurenine; L-1-MT, 1-methyl-L-tryptophan; MPA, misoprostol acid; MSC, mesenchymal stem cell; TA, transplant arteriosclerosis; T_R1, T regulatory type 1; Treg, regulatory T cell; Trp, tryptophan.

T_R1 cells in the tumor microenvironment (32, 33). IDO expression in APCs was suggested to induce IL-10–secreting Tregs (34). Thus, COX-PGE_2 and IDO may be significant molecules for the generation of T_R1 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-γ, 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_R1-like cells. In this study, we demonstrate the induction of IL-10^+IFN-$\gamma^+$$CD4^+$ cells by MSCs in an allogeneic MLR. Furthermore, MSCs render a population of low-proliferative $CD4^+$ cells to commit to T_R1-like cells. We also provide evidence that MSCs induce T_R1-like cells and mediate immunosuppression in vitro through activation of the COX-PGE_2 and IDO pathways. Finally, the results indicate that dual treatment with PGE_2 and T_R1-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 (FACSAria; 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-γ–PerCP–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), mIgG2b (eBioscience), mIgG1–PerCP–Cy5.5 (P3.6.2.1; eBioscience), rat IgG2a-allophycocyanin (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 10% heat-inactivated adult porcine serum (Invitrogen) and 10% KNOCKOUT SR (Invitrogen). After 72 h, cell culture supernatant was harvested and frozen as MSC CM.

One-way MLR

We used MLR as a cell model to assess the potential of MSCs to induce T_R1-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, 1×10^5 responder cells and 1×10^5 stimulator cells were plated in each well. MSCs (5×10^4 cells/well) treated with Mitomycin-C (25 μg/ml) or MSC CM (0.1 ml/well) were added to the coculture. On the fourth day of coculture, cells were pulsed with 1 μCi/well [^{3}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 [^{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 (1×10^7) 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_2.

Transwell experiments

Transwell experiments were conducted using HTS Transwell-96 Well Permeable Supports (Corning). Responder and stimulator PBMCs (10^5 cells/100 μl MLR medium/well) were cultivated in a 96-well round-bottom plate, and MSCs (5×10^4 cells/100 μl MLR medium/insert) were seeded onto the semipermeable membrane of Transwell inserts with a 1.0-μm pore size.

Isolation of MSC-primed responder $CD4^+$ cells

To investigate the regulatory characteristics of MSC-primed $CD4^+$ 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. $CD4^+$ T cells of small size, low granularity, and intensive CFSE fluorescence, corresponding to nondividing responder T cells ($FSC^{low}SSC^{low}CD4^+CFSE^{high}$, designated as $CD4^+$R), were gated and sorted out for subsequent intracellular staining and for the suppression of the MLR.

The immunosuppressive properties of $CD4^+$R were evaluated using a subsequent allogeneic MLR (second MLR), in which the responder and stimulator PBMCs are identical to those in the first MLR. $CD4^+$R sorted from the first MLR were treated with mitomycin-C (25 μg/ml) and added at a cell ratio of 1:1 ($CD4^+$R/responder PBMCs). To investigate the role of IL-10 in $CD4^+$R-mediated immunosuppression, neutralizing mAb against IL-10 (10 μg/ml; Invitrogen) or isotype control IgG1 (10 μg/ml; BioLegend) was added to the second MLR. To assess the allogeneic responsiveness of $CD4^+$R, 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 $CD4^+$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; eBiosciences) 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-γ.

ELISAs

Using commercially available kits, we analyzed the levels of IL-10, IFN-γ, and PGE_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 (Invitrogen), 39 pg/ml for IFN-γ (R&D Systems), and 7.8 pg/ml for PGE_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 (1×10^6 cells/ml), and vortexed for 2 h at room temperature. After centrifugation at $2300 \times g$ 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. Liquid chromatography coupled with tandem mass spectrometry was performed using an ultra-HPLC system (Agilent 1290) connected to a triple quadrupole tandem mass spectrometer (Agilent 6460) outfitted with an Agilent Jet Stream electrospray ionization source operating in the positive mode. An Agilent Poroshell EC-C18 (100 mm × 2.1 mm inner diameter column; 2.7 μm particle size) was used for reverse-phase chromatography. The mobile phase consisted of 0.1% (v/v) acetic acid in water (A) and 0.1% (v/v) acetic acid in acetonitrile (B). The

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→192 (CE = 5V) for KYN. The ion pair monitored for qualification was at m/z 205→118 (CE = 25V) and m/z 205→61 (CE = 60V) for Trp and at m/z 209→146 (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, PGE$_2$ group, MSC-primed CD4$^+$R cell group, and PGE$_2$ plus MSC-primed CD4$^+$R cell group. PGE$_2$ was administered in two stages. In the perioperative stage, dinoprostone (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 [^{3}H]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

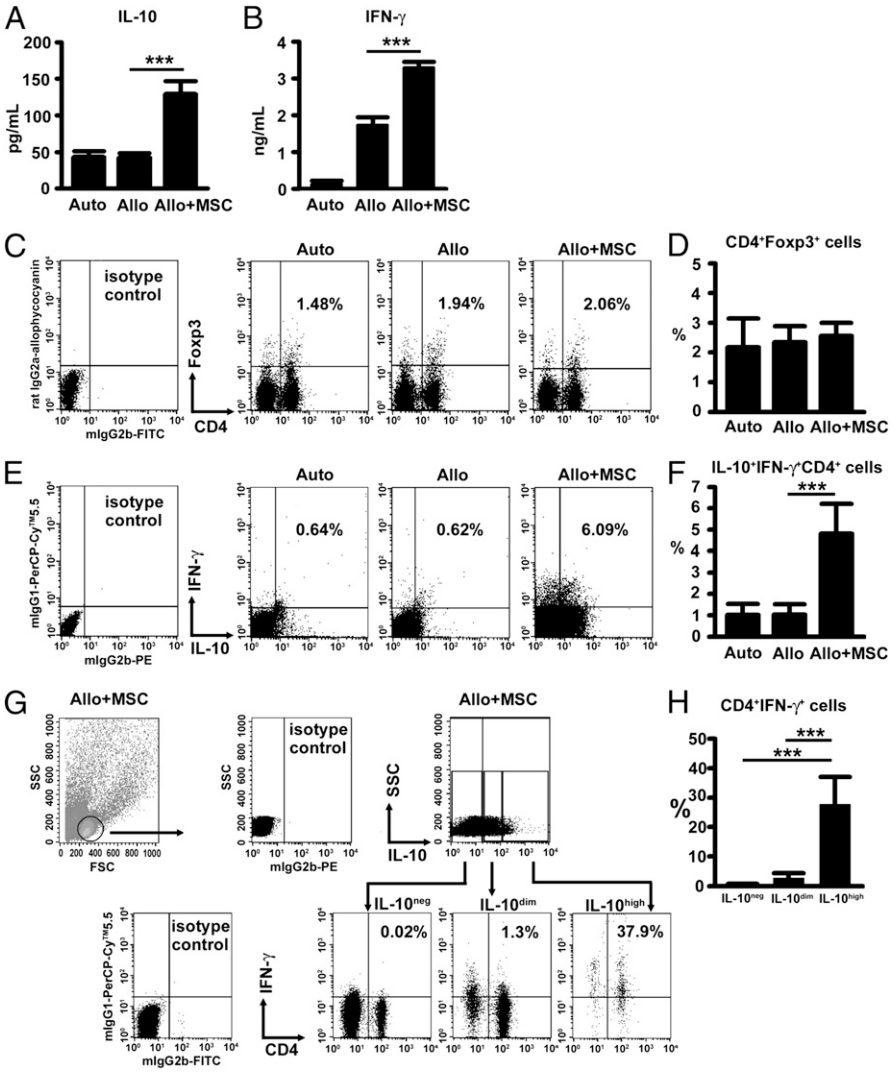

FIGURE 1. MSCs enhance levels of IL-10 and IFN-γ and induce IL-10$^+$IFN-γ$^+$CD4$^+$ cells, but not Foxp3, in allogeneic reactions. Responder PBMCs were cultured with autologous (Auto) or allogeneic stimulator PBMCs in the absence (Allo) or presence (Allo + MSC) of MSCs. Levels of IL-10 (**A**) and 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$^+$IFN-γ$^+$ 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-10^{high} cells ($n = 3$, $p < 0.001$, Fig. 1G, 1H). These findings suggest that MSCs induce IL-10$^+$IFN-γ$^+$CD4$^+$ T_R1-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.

PGE$_2$ 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 allostimulated PBMCs compared with MSCs or allostimulated 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). PGE$_2$ was reported to promote immune tolerance by regulating IDO expression (36, 37). To corroborate whether COX-derived PGE$_2$ acts as another potential humoral factor, we incubated MSC-treated PBMCs in the MLR with IDM, a nonselective COX inhibitor. IDM 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 PGE$_2$. To delineate the relationship between COX and IDO in the MSC-mediated induction of CD4$^+$IL-10$^+$IFN-γ$^+$ cells, we added IDM 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 IDM and L-1-MT alone (Fig. 2D, 2E). L-1-MT synergized with IDM to block the induction of IL-10$^+$IFN-γ$^+$CD4$^+$ cells by MSCs (Fig. 2D, 2E). Furthermore, neither IDM nor L-1-MT alone could completely abolish the effect of MSCs in suppressing allogeneic PBMC proliferation, whereas IDM combined with L-1-MT abrogated the antiproliferative effect of MSCs (Fig. 2F). These results suggest that COX-derived PGE$_2$ and IDO act together to induce CD4$^+$IL-10$^+$IFN-γ$^+$ cells to suppress allogeneic responses.

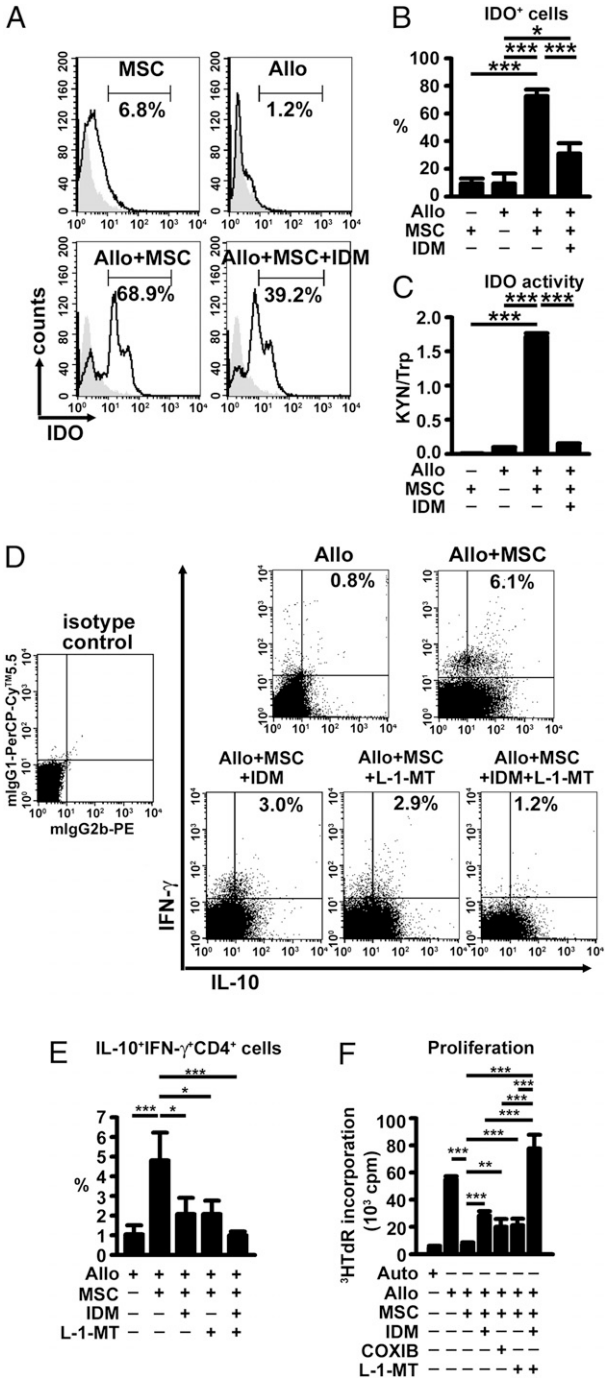

FIGURE 2. MSCs induce IL-10$^+$IFN-γ$^+$CD4$^+$ cells and suppress allogeneic reaction by PGE$_2$ and IDO metabolites. Cells were labeled with anti-IDO, followed by FITC-goat anti-rabbit IgG, or staining with isotype-matched Abs. Cytoplasmic IDO Ag expression was determined in cultures of MSCs alone (MSC) or in the allogeneic MLR in the absence (Allo) or presence of MSCs (Allo + MSC), or treated with indomethacin (IDM, 50 μM) (Allo + MSC + IDM). Percentages of IDO-expressing cells are displayed as representative FACS graphs (**A**) and quantitative analysis (**B**) ($n = 3$ independent experiments). In (A), open areas indicate staining with IDO Abs, and shaded areas represent isotype controls. (**C**) IDO activity, expressed as KYN/Trp ratio, was analyzed using liquid chromatography coupled with tandem mass spectrometry. Representative FACS plots of IL-10 and IFN-γ expression in the CD4$^+$ gate (**D**), statistical evaluation of the percentage of IL-10$^+$IFN-γ$^+$CD4$^+$ cells (**E**), and alloactivated PBMC proliferation (**F**) are shown in the absence or presence of MSCs, with or without indomethacin and/or L-1-MT (1 μM); $n = 3$ for each experiment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

MSCs convert CD4$^+$ cells into immunosuppressive T$_R$1-like cells

To test the hypothesis that MSCs prime a subset of CD4$^+$ cells in the MLR to IL-10$^+$IFN-γ^+CD4$^+$ T$_R$1-like cells, we isolated, by cell sorting, nondividing small CD4$^+$ responder lymphocytes (FSC^{low} SSC^{low}CD4$^+$CFSE^{high} designated as CD4$^+$R) from allostimulated PBMCs that were treated or not with MSCs (Fig. 3A). CD4$^+$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$^+$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$_R$1-like cells.

To confirm that CD4$^+$R cells possess immunosuppressive properties, we added MSC-treated and control CD4$^+$R cells to the MLR and analyzed their proliferative activity. CD4$^+$R cells from MSC-treated PBMCs significantly reduced cell proliferation, whereas CD4$^+$R cells from control PBMCs had no effect (Fig. 3B). The suppressive effect of MSC-treated CD4$^+$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$^+$R cells were abrogated by IDM plus L-1MT (Fig. 3C, 3D). Furthermore, MSC-primed CD4$^+$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$^+$R cells served as responder cells, and the PBMCs from either the original minipig used to induce CD4$^+$R cells (original S) or other minipigs (third-party S) served as the stimulator cells. CD4$^+$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$^+$R cells isolated from MSC-treated PBMCs did not proliferate when stimulated with the original or the third-party S (Fig. 3F). However, CD4$^+$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$^+$R cells to suppress allostimulation by increasing T$_R$1-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$_2$ production

To elucidate the relationship among PGE$_2$, 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$_2$ 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$_2$ 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 [^{3}H]thymidine uptake by PBMCs. MSCs achieved maximal suppression of cell

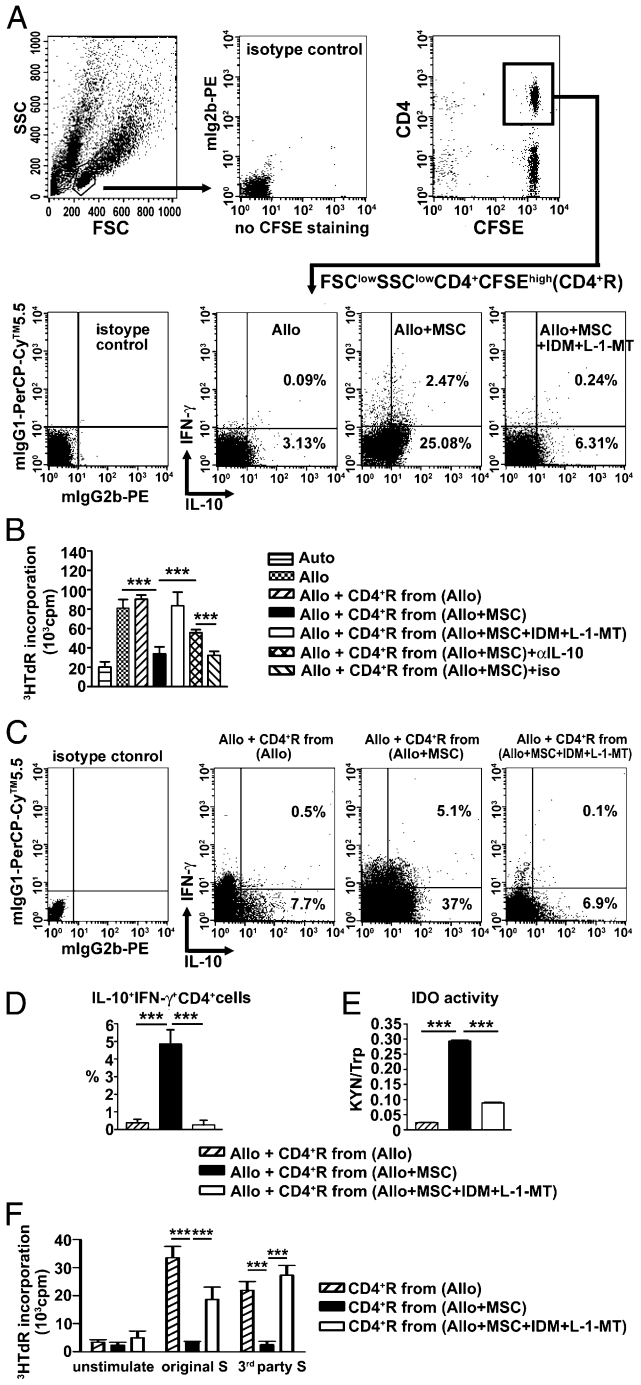

FIGURE 3. MSC-primed nondividing CD4$^+$ T cells are immunosuppressive to alloreactive cells and anergic to allostimulation. CFSE-labeled responder PBMCs were activated with allogeneic stimulator (first MLR) in the absence (Allo) or presence of MSCs without (Allo + MSC) or with IDM plus L-1-MT (Allo + MSC + IDM + L-1-MT). (**A**) After a 5-d coculture period, cells were labeled with PE–anti-CD4, and CD4$^+$ T cells of small size, low granularity, and intensive CFSE fluorescence, corresponding to nondividing responder T cells (FSC^{low}SSC^{low}CD4$^+$ CFSE^{high} designated as CD4$^+$R), were sorted out and assessed for the intracellular expression of IL-10 and IFN-γ. CD4$^+$R cells were added to a subsequent allogeneic MLR and analyzed for [^{3}H]thymidine incorporation in the absence or presence of neutralizing anti–IL-10 mAb (αIL) or isotype control IgG1 (iso) (**B**), intracellular IL-10 and IFN-γ profiles gated on CD4$^+$ T cells (**C**), percentage of IL-10$^+$IFN-γ$^+$CD4$^+$ cells (**D**), and IDO activity (KYN/Trp) (**E**). (**F**) To test the anergic potential of CD4$^+$R, cells were restimulated with stimulators identical to those in the first MLR (original S) and third-party PBMCs (third party S). ***$p < 0.001$.

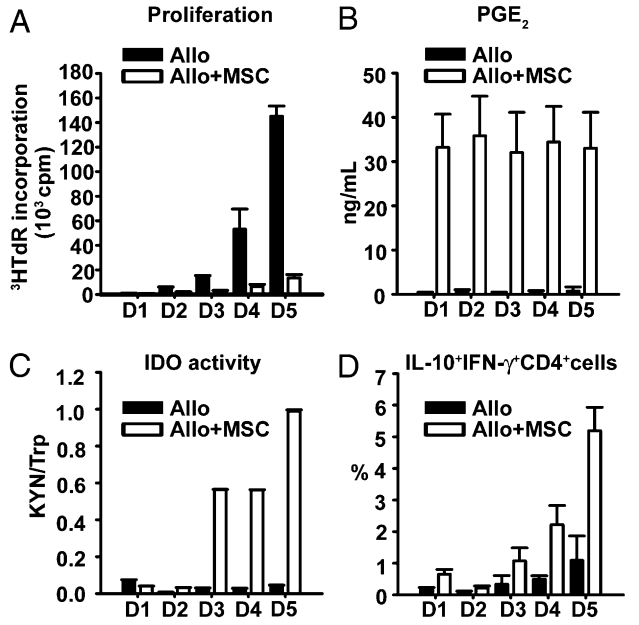

FIGURE 4. Temporal profiling of PGE_2, IDO activity, and IL-10^+IFN-γ^+CD4^+ cells in MSC-suppressed MLR. Responder PBMCs were cultured with allogeneic stimulator PBMCs in the absence or presence of MSCs. Cells or cell supernatants were harvested daily over a 5-d culture period. (**A**) Proliferation of responder PBMCs. (**B**) PGE_2 levels in cell culture supernatants. (**C**) IDO activity, expressed as a KYN/Trp ratio. (**D**) Percentage of $CD4^+$IL-10^+IFN-γ^+ cells; $n = 3$ for each experiment.

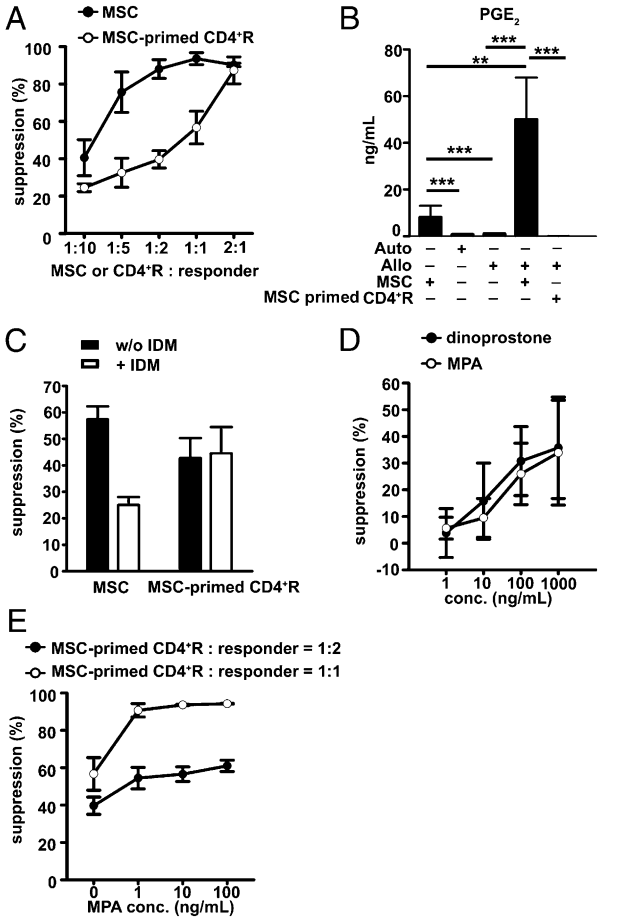

FIGURE 5. Effect of PGE_2 mimetic on the immunosuppressive potency of MSC-primed $CD4^+$R cells. (**A**) A 5-d MLR was established in the presence of MSCs or $CD4^+$R cells sorted from MSC-treated MLR (designated MSC-primed $CD4^+$R) and then analyzed for suppressive potency (A) and PGE_2 levels in cell supernatants (**B**). (**C**) MSCs/responder PBMCs at a ratio of 1:4 or MSC-primed $CD4^+$R cells/responder PBMCs at a ratio of 1:1 were added to MLR in the absence (w/o IDM) or presence (+IDM) of IDM. Dinoprostone, misoprostol acid (MPA) (**D**), or MSC-primed $CD4^+$R cells plus MPA (**E**) were analyzed for their suppressive potency. Suppression (%) of alloactivated lymphocyte proliferation was calculated as (1 − [cpm {Allo + MSC or MSC-primed $CD4^+$R cells or dinoprostone or MPA}/cpm {Allo}]) × 100. $^{**}p < 0.01$, $^{***}p < 0.001$.

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 [^{3}H] 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 PGE_2 by MSCs but not by MSC-primed $CD4^+$R cells. To confirm this hypothesis, we investigated the dependence of PGE_2 in immunosuppression mediated by MSC-primed $CD4^+$R cells. The PGE_2 level was enhanced significantly in the MSC-treated MLR but was low in the MSC CM (Fig. 5B). In contrast, PGE_2 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 (PGE_2 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, suppression was comparable to that of MSCs 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 PGE_2 and that their immunosuppressive potency can be augmented by PGE_2 supplements.

PGE_2 enhances the competency of MSC-primed $CD4^+$R cells in control of TA

To investigate whether the PGE_2-augmented immunosuppressive potency of MSC-primed $CD4^+$R cells occurs in vivo, we treated minipigs undergoing allogeneic femoral arterial transplantation with PGE_2 plus MSC-primed $CD4^+$R cells or with PGE_2 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 PGE_2 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 PGE_2 and MSC-primed $CD4^+$R cells (Fig. 6). We next determined the temporal effect of PGE_2 on potentiating anti-TA by MSC-primed $CD4^+$R cells by administering PGE_2 for the first 14 d only or beginning on day 15. Compared with the group in which PGE_2 was given for 42 d, intimal hyperplasia was evident in the group in which PGE_2 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 PGE_2 throughout the allogeneic response is required to potentiate MSC-primed $CD4^+$R cells to protect the transplanted arterial allografts from TA.

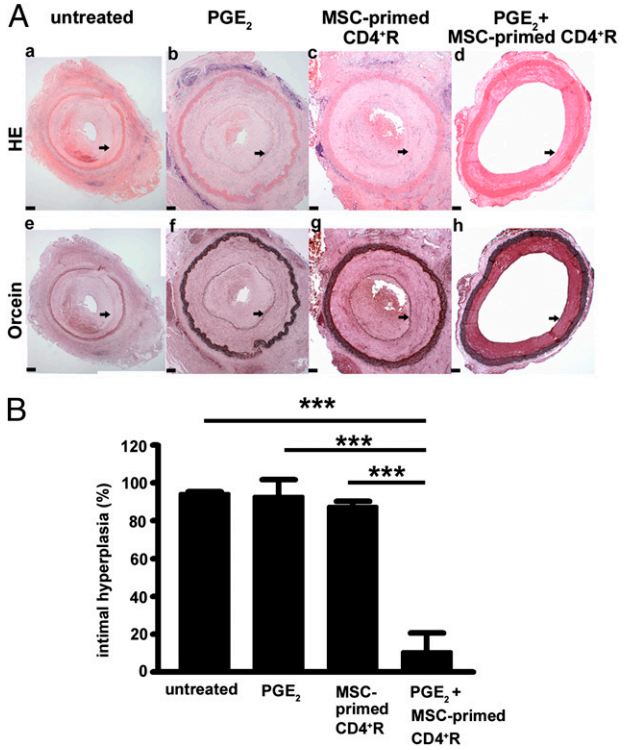

FIGURE 6. PGE$_2$ enhances the ability of MSC-primed CD4$^+$R cells to inhibit TA. (**A**) Representative cross-sectional images of allografts from the untreated group (**a**, **e**), PGE$_2$ group (**b**, **f**), MSC-primed CD4$^+$R cell group (**c**, **g**), and PGE$_2$ plus MSC-primed CD4$^+$R cell group (**d**, **h**) 6 wk posttransplantation [H&E (a–d), orcein stain (e–h)]. Delivery of PGE$_2$ 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$_R$1-like cells bearing the CD4$^+$IL-10$^+$IFN-γ$^+$ signature. MSCs generate PGE$_2$ through the COX pathway, which, in turn, activates IDO. COX-PGE$_2$ and IDO act together to induce T$_R$1-like cells and suppress allostimulated immune responses. Consistent with the in vitro data, administering PGE$_2$ with a local injection of MSC-induced T$_R$1-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$_R$1-like cells. First, they are immunosuppressors. Although MSC-primed CD4$^+$R cells represent only a small subset coexpressing IL-10 and IFN-γ (2.47%, Fig. 3A), they suppressed the proliferation of allodriven PBMCs by 50% in an IL-10–dependent manner (Fig. 3B). Second, they are hyporesponsive to alloantigens (Fig. 3F). This anergic characteristic correlates with previous studies wherein T$_R$1 was reported to have an intrinsically low proliferative capacity (20). In most recent studies, T$_R$1-like cells are defined by the coexpression of IL-10 and IFN-γ, associated with functional competence in immunomodulation (21, 22). Therefore, we conclude that the MSC-primed CD4$^+$R cells in this study are IL-10$^+$IFN-γ$^+$CD4$^+$ T$_R$1-like cells.

Although IL-10 produced by T$_R$1 cells is known for its immunosuppressive activity, the role of coexpressed IFN-γ in the MSC-induced IL-10$^+$IFN-γ$^+$CD4$^+$ 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$_R$1-like cells.

Both PGE$_2$ (10, 26, 27) and IDO (28, 29) were demonstrated to participate in MSC-mediated immunomodulation. PGE$_2$ (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$_R$1 or T$_R$1-like cells to combat deviated immune responses in a PGE$_2$- or IDO-dependent manner. Following this speculation, we found that MSCs blunted allogeneic reactions and expanded IL-10$^+$IFN-γ$^+$CD4$^+$ cells mainly through soluble factors (Supplemental Fig. 2). However, inhibitors targeting either PGE$_2$ production or IDO activity could attenuate the MSC-induced immunomodulation only partially (Fig. 2F). Only when PGE$_2$ 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 PGE$_2$ was reported to promote immune tolerance through the enhancement of downstream IDO expression and activity (40–42), this redundancy suggests that MSC-secreted PGE$_2$ promotes the ontogeny of T$_R$1-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 PGE$_2$ has been limited by poor oral bioavailability, short half-lives, and significant toxicity profiles. Misoprostol, a synthetic analog of PGE$_1$, binds to and activates each of the four heptahelical G protein–coupled E prostanoid receptors normally ligated by the endogenous PGE$_2$ (43). The regulatory effects of misoprostol on both innate and adaptive immune systems were proved to be similar to those of natural PGE$_2$ (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 PGE$_2$ 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 PGE$_2$ combined with MSC-primed T$_R$1-like cells may replace MSCs to achieve immune tolerance in TA. This finding raises several implications. First, MSC-primed T$_R$1-like cells represent only one subset of immunomodulatory cells induced by MSCs. In our study, the potency of MSC-induced T$_R$1-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 T_R1-like cells as we did previously for MSCs in the porcine femoral arterial transplantation model, T_R1-like cells might require the assistance of PGE_2 to inhibit TA. Second, PGE_2 and T_R1-like cells may act through independent mechanisms to modulate the immune system. PGE_2 is known to regulate dendritic cell functions in the early priming stage of the allogeneic response (47), whereas T_R1-like cells are more competent in blocking the proliferation of effector T cells and their migration to the target organ (48). Third, a functional cross-talk probably exists between PGE_2 and T_R1-like cells in immune regulation. PGE_2 was reported to augment IL-10 signaling and biological activity (49). Under the influence of PGE_2, IL-10 secreted by pre-existing T_R1-like cells may enhance its downstream effects and promote the de novo induction of more T_R1-like cells. Finally, in the allogeneic MLR modulated by MSCs, the early production of PGE_2 and the expansion of T_R1-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 T_R1-like cells alone (Fig. 5A) and PGE_2 alone (Fig. 5D) partially suppress the allogeneic MLR, both are expected to partially dampen TA when given independently. However, neither T_R1-like cell transfers alone nor PGE_2 administration alone is effective in preventing TA (Fig. 6). We speculate that PGE_2 is efficient in regulating allogeneic priming. When T_R1-like cells were provided in the absence of PGE_2 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 PGE_2 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 T_R1-like cells did not act in turn with PGE_2 to modulate allogeneic responses in the femoral arterial transplantation model, the protective effects of PGE_2 were unable to last for 6 wk, and TA ensued. Therefore, it is reasonable to propose that both PGE_2 and T_R1-like cells are indispensable mediators generated by MSCs to maintain long-term immune tolerance. Further studies are required to elucidate the temporal interaction between PGE_2 and T_R1-like cells and to identify their specific targets in the immune system.

We have provided evidence that MSCs induce IL-10^+IFNγ^+ $CD4^+$ T_R1-like cells during allogeneic reactions. Furthermore, MSCs prime a subpopulation of low-proliferative $CD4^+$ cells to commit to coproducing IL-10 and IFN-γ and to suppress allogeneic proliferation. We also identified COX-derived PGE_2 and IDO metabolites as soluble factors that induce the generation of T_R1-like cells and mediate the immunomodulatory effects of MSCs synergistically in vitro. Moreover, neither PGE_2 nor T_R1-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, PGE_2 and T_R1-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.

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