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Conversion of Th17 into IL-17A^{neg} Regulatory T Cells: A Novel Mechanism in Prolonged Allograft Survival Promoted by Mesenchymal Stem Cell–Supported Minimized Immunosuppressive Therapy

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The ultimate goal in transplantation medicine is the promotion of operational tolerance. Although Th cells of the Th17 type have been predominantly associated with rejection of allogeneic solid organ grafts, regulatory T (T_{reg}) cells appear to foster operational tolerance. Induced T_{reg} and Th17 cells have a higher lineage plasticity than has been recognized thus far. We found that when mesenchymal stem cells (MSCs) were used to induce long-term acceptance of allogeneic heart grafts in mice, the induction of T_{reg} cells was preceded by development of a CD11b^{hi}Gr1^{int} myeloid–derived immunosuppressive cell–mediated Th17 response. Substantial suppression of Foxp3$^+$ T_{reg} cell generation from retinoic acid receptor–related orphan receptor $\gamma^{-/-}$ T cells by MSCs revealed that retinoic acid receptor–related orphan receptor γ is a common factor in the differentiation of T_{reg} and Th17 cells. Immunosuppressant mycophenolate mofetil treatment of enriched IL-17A$^+$ cells from MSC-primed allograft mouse recipients resulted in a reduction of IL-17A production and an increase in the Foxp3$^+$ T_{reg} cell fraction. Furthermore, identification of IL-17A$^+$ Foxp3$^+$ double-positive and ex–IL-17–producing IL-17A^{neg}Foxp3$^+$ T cells strongly argues for direct conversion of Th17 cells into T_{reg} cells as the underlying mechanism of immune regulation in MSC-mediated allograft survival. The Th17 into T_{reg} conversion identified in this study constitutes an important immunological mechanism by which MSC-induced myeloid-derived immunosuppressive cells mediate operational transplant tolerance. The possibility to create T_{reg} cell–regulated operational tolerance in the absence of complete immune suppression provides strong clinical implications for cell therapy–assisted minimization protocols. *The Journal of Immunology*, **2014, 193: 4988–4999.**

T cell activation and proliferation are prerequisites for allograft rejection. Although the capacity of Th17 cells to play a proinflammatory role and favor allograft rejection is becoming increasingly evident (1, 2), regulatory T cells (T_{regs}) have shown an anti-inflammatory activity that maintains transplant tolerance and prolongs graft survival (3, 4).

Unstable expression of Foxp3 or IL-17 and a high degree of flexibility in the differentiation of T_{reg} and Th17 cells suggest that CD4$^+$ T cells, particularly induced T_{reg} and Th17 cells, have a higher lineage plasticity than has been recognized thus far (5–10). Furthermore, T cells coexpressing Foxp3 and retinoic acid receptor–related orphan receptor γ (RORγ) have been identified in vivo (6, 11), implying that Foxp3$^+$RORγ$^+$ cells may exist as a transient population that differentiates into either T_{reg} or Th17 cells (6). In addition, T_{reg} cells have the propensity to differentiate into IL-17–producing cells in the absence of TGF-β_1 (12), after exposure to IL-6 (7), or IL-23–producing dendritic cells (13), and only Foxp3$^+$RORγ$^+$ T cells, but not Foxp3$^+$RORγ^{neg} T cells, can differentiate into Foxp3$^+$IL17$^+$ T cells (14). There has not been direct demonstration of the reciprocal conversion of T_{reg} cells from IL-17–expressing cells, but TGF-β_1 and retinoic acid have been found to reverse the Th17/T_{reg} balance toward T_{reg} cells (15, 16).

One aim in transplantation medicine is promotion of operational tolerance through immunosuppressive drugs that enhance immune regulation toward the donor. In association with moderate doses of certain immunosuppressive drugs, mesenchymal stem cells (MSCs) have shown unique immunoregulatory properties and promote long-term graft acceptance (17–20). Various studies, including our own, have shown that MSC-mediated tolerance in vivo can be maintained by T_{reg} cells (21–26). Combinatorial therapy synergistically promotes T_{reg} cell–mediated tolerance with a significant increase in intragraft CD4$^+$CD25$^+$Foxp3$^+$ T cell frequency, whereas MSC treatment in the absence of additional immunosuppression accelerates allograft rejection (22, 27).

In this study, we describe Th17/T_{reg} cell plasticity and Th17-into-T_{reg} cell conversion as novel phenomena that play an integral role in long-term acceptance of allogeneic heart grafts induced by MSC-based combinatorial therapy in mice. The synergistic effect of MSC and immunosuppressant combinatorial therapy relies on

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Abbreviations used in this article: B6, C57BL/6; COX, cyclooxygenase; MDSC, myeloid-derived immunosuppressive cell; MMF, mycophenolate mofetil; MSC, mesenchymal stem cell; p/s, penicillin/streptomycin; ROR, retinoic acid receptor–related orphan receptor; T_{reg}, regulatory T cell.

the conversion of MSC-induced Th17 cells into T_{reg} cells by immunosuppressant treatment. This effect is mediated through myeloid-derived immunosuppressive cells (MDSCs) as (previously identified and confirmed in this study) intermediates of MSCs. Our data provide an explanation for the inconsistent outcomes of allograft recipients treated with MSCs using different treatment protocols (28, 29). Moreover, we identify an additional mechanism of T_{reg} cell induction and highlight the principal importance of Th17/T_{reg} cell plasticity for allograft acceptance and suggest that the microenvironment driving this balance is essential for peripheral operational tolerance.

Materials and Methods

Mice

Sex-matched C57BL/6 (B6) and BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories (Sulzfeld, Germany). B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (Pep Boy), B6.129P2(Cg)-Rorc^{tm2Litt}/J (Ror$\gamma^{-/-}$), STOCK Il17a$^{tm1.1(icre)Stck}$/J, and B6.129 × 1-Gt(ROSA)26Sor$^{tm1(EYFP)cos}$/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were carried out in accordance with the regional regulations of the Upper Palatinate (Bavaria, Germany).

Heterotopic heart transplantation model

A well-established, fully allogeneic B6-into-BALB/c model of heterotopic heart transplantation was used as described previously (27). Mycophenolate mofetil (MMF; Roche, Grenzach-Wyhlen, Germany) was administered daily into the peritoneal cavity at a dose of 160 mg/kg body weight starting on the day of transplantation (day 0) until day 7 to achieve prolongation of heart allograft survival but not long-term graft acceptance (Fig. 1A). A total of 1×10^6 MSCs was injected into the tail vein at day −4 before heart transplantation. To deplete CD11b$^+$ cells in designated groups, clodronate-filled liposomes (1 ml/100 g body weight) were administered i.v. 24 h before and 48 h after MSC administration.

Expansion and cultivation of MSCs

MSCs were isolated from adipose tissue of B6 and BALB/c mice. Adipose tissue was minced with a scalpel knife and enzymatically digested with 0.5 mg/ml collagenase type IV (Life Technologies, Paisley, U.K.) in MEM-α supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (1% p/s) for 30 min at 37˚C under continuous shaking. After centrifugation at 1200 × *g* for 10 min, the cell pellet was resuspended in RBC lysis buffer (eBioscience, San Diego, CA) and incubated for 10 min at room temperature. The cells were then washed, resuspended in MSC culture medium consisting of 2 mM L-glutamine, 1% p/s, and 15% FBS (Lonza, Verviers, Belgium), and filtered through a 70-μm cell strainer (BD Biosciences, San Jose, CA). The cells were then transferred to a 175-cm^2 cell culture flask (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) and expanded in a humidified atmosphere with 5% CO_2 at 37˚C. Nonadherent cells were removed after 3–4 d. Culture medium was refreshed twice weekly. Cells were removed from culture flasks at subconfluency using 0.05% trypsin-EDTA (Life Technologies) and reseeded at 1000 cells/cm^2 to ensure optimal proliferation. MSCs were used for experiments between passages 6 and 12.

Characterization of MSCs

We confirmed that MSC readily differentiated into adipocytes and osteoblasts and that their phenotype was in keeping with consensus guidelines of the International Society of Cellular Therapy. For immunophenotyping, MSCs were harvested, washed in FACSFlow (BD Biosciences) and stained with Sca-1, CD11c, CD11b, CD34, CD44 and CD45, MHC class II, CD117, CD105, CD106, CD29, CD31, CD19, CD73, and MHC class I (H2K^b and H2K^d) (all BD Biosciences), and measured on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

Adipogenic differentiation was induced by culturing confluent MSCs in MEM-α supplemented with 2 mM L-glutamine, 1% p/s, 15% heat-inactivated FBS, 50 μg/ml L-ascorbic acid (Sigma-Aldrich, Munich, Germany), 500 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 60 μM indomethacin, 2 μM insulin (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich) for 14 d. Lipid-filled vesicles were detected by Oil Red O staining. Cells were washed with PBS, fixed with 60% isopropanol for 1 min, and incubated with filtered 0.3% Oil Red O (in 60% isopropanol; Sigma-Aldrich) for 30 min. Following three washes with PBS, the cells were photographed. Osteogenic differentiation was induced by culturing confluent MSCs in MEM-α supplemented with 2 mM L-glutamine, 1% p/s, 15% heat-inactivated FBS, 5 mM β-glycerophosphate (Sigma-Aldrich), 50 μg/ml L-ascorbic acid, and 10 nM dexamethasone for 21 d. The deposition of calcified nodules was identified using von Kossa staining. Cells were washed with PBS and fixed with cold 4% paraformaldehyde for 5 min. Following an additional wash step with PBS and two wash steps with distilled water, cells were incubated with 1% silver nitrate (in water) on a light box until blackening occurred. Cells were washed three times with water, incubated with 2.5% sodium thiosulfate (in water) for 5 min, washed again twice with water, and photographed. For examination of the immunosuppressive capacity of MSCs, mouse splenocytes were stained with Fixable Viability Dye eFluor 450 (eBioscience), according to the manufacturer's protocol and stimulated with 4 μl/ml Dynabeads Mouse T-Activator CD3/CD28 (anti-CD3/CD28; Life Technologies, Darmstadt, Germany) in the absence or presence of MSCs added at ratios of 1:10–1:200 in 96-well plates. After 5–7 d, proliferation was measured on a FACSCanto II flow cytometer using FACSDiva software (BD Biosciences). Sample analysis was computed using FlowJo version 10 software (Tree Star, Ashland, OR).

Isolation of cells from blood, spleen, liver, and heart graft

GentleMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to prepare single-cell suspensions from heart grafts and livers according to the manufacturer's protocol. Briefly, tissues were homogenized using a GentleMACS C-tube (Miltenyi Biotec). Cardiac allograft tissues were additionally digested at 37˚C in buffer containing collagenase II (600 U/ml) and DNase I (60 U/ml) for 30 min. Single-cell suspensions from spleens were obtained by injecting PBS into the spleens over a 100-μm cell strainer. Lymphocytes were isolated following hypotonic RBC lysis. For RT-PCR analysis, cell pellets were resuspended in RNeasy Lysis Buffer buffer, and mRNA isolation was performed, according to the manufacturer's protocol (Qiagen, Hilden, Germany). For cytokine production analysis (ELISA, intracellular staining), cells were activated with 3.5 μl/ml Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) for 5–7 d (10^5 cells/200 μl in a 96-well U-bottom plates).

IL-17A$^+$ cells were enriched using a mouse IL-17 secretion assay–cell enrichment and detection kit (Miltenyi Biotec), according to the manufacturer's protocol.

Flow cytometry

Cells were harvested and stained with Fixable Viability Dye eFluor 780 and anti–CD3-PE-Cy7, anti–CD4-PerCP-Cy5.5, anti–CD8-Pacific Blue, anti–γδTCR-allophycocyanin, anti–NKp46-FITC, anti–CD11b-Pacific Blue, anti–F4/80-PE, anti–Ly6G/C (Gr1)-PE-Cy7, and anti–CD184 (CXCR4)-allophycocyanin for 30 min at 4˚C in 1% BSA in PBS (FACS buffer). IL17A$^+$-enriched cells were stained with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) and stained with CD4-allophycocyanin-Cy7. Intracellular staining was achieved using a Foxp3 staining Kit (BioLegend, Fell, Germany), according to the manufacturer's protocol. For intracellular cytokine production analysis, cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich), and after 1 h, brefeldin A (10 μg/ml) was added for an additional 3–4 h prior to staining. The following Abs were used: IFN-γ-FITC and IL-17A-PE (eBioscience) and Foxp3-allophycocyanin (BioLegend). Stainings were performed at 4˚C for 45 min and then washed and resuspended in FACS buffer prior to analysis. Flow cytometric analysis was performed on a FACSCanto (BD Biosciences, San Diego, CA) using FACSDiva software and analyzed using FlowJo version 10 software (Tree Star).

Cocultures of MSCs and lymphocytes

Cells from lymph nodes and the spleen were stimulated with 3.5 μl/ml Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) in a 12-well plate (1×10^5 cells/well) in the presence or absence of MSCs in direct culture or a Transwell system. Where indicated, Th17-inducing cytokines (25 ng/ml IL-23, 30 ng/ml IL-6, and 2 ng/ml TGF-β_1) were added to cultures. For T_{reg} cell–driving conditions, TGF-β_1 (2 ng/ml) was added to cultures. All experiments used 1×10^6 T cells/well at a concentration of 5×10^5 cells/ml. Cyclooxygenase (COX)2 inhibitor celecoxib was applied at 10 μM, anti–IL-10, anti–TGF-β_1, and anti–IL-6 inhibitory Abs at 0.25, 0.5, and 5 μg/ml, respectively, and MMF at a concentration of 0.1 μM. As an alternative to stimulation with Dynabeads Mouse T-Activator CD3/CD28, T cells were stimulated with an anti-CD3 Ab or irradiated allogeneic splenocytes at a stimulator:responder cell ratio of 1:2.

For experiments using Ror$\gamma^{-/-}$ mice, Ror$\gamma^{-/-}$ and Pep Boy T cells were isolated using the *Pan* T Cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer's protocol, with the ratio of the negative fraction adjusted to 1:1. Ror$\gamma^{-/-}$ and Pep Boy cells were cultured at a 1:1

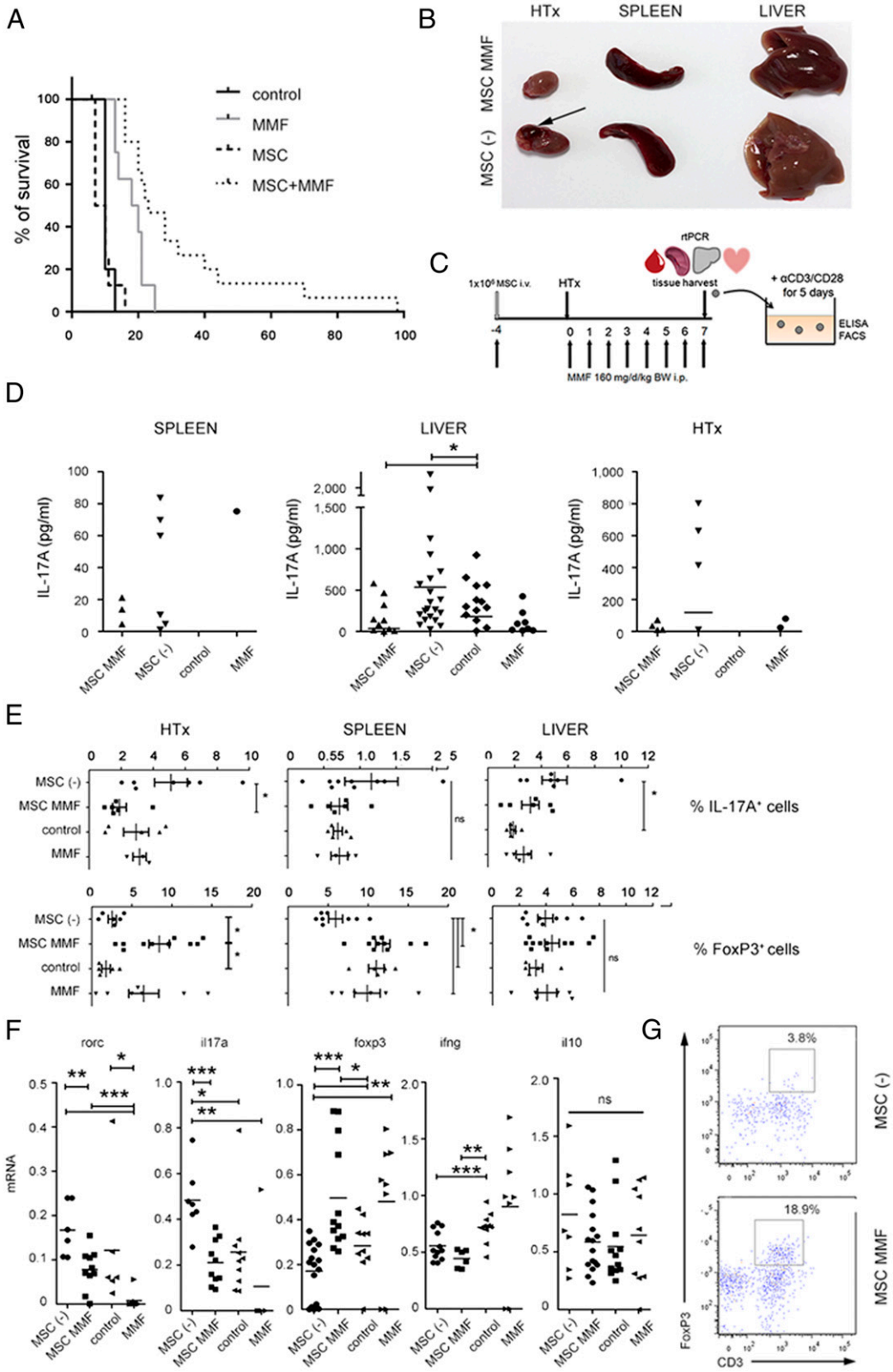

FIGURE 1. Prolonged survival of heart allografts is associated with reversal of the Th17/Foxp3 ratio. (**A**) Control mice receiving PBS experienced acute graft rejection (10.6 ± 1.34 d, $n = 5$). MMF alone slightly prolonged graft survival (16.8 ± 5.78 d, $n = 5$; $p = 0.0012$). The combination of donor-derived MSCs+MMF resulted in significant prolongation of allograft survival (33.9 ± 22.97 d, $n = 15$. $p < 0.0001$). MSCs alone tended to accelerate allograft rejection (9.4 ± 3.16 d, $n = 8$. $p = 0.5927$). (**B**) Comparison of the spleen, liver, and heart grafts of MSC+MMF- and MSC-treated recipient mice at day 7 posttransplantation. Arrow indicates the necrotic site of the rejected graft in MSC-treated mice. (**C**) Model overview. Allogeneic B6 hearts were transplanted into BALB/c recipients. Donor MSCs were injected at day −4 before transplantation. MMF was administered at a daily dose of 160 mg/kg for 7 d. Blood, spleen, liver, and transplanted hearts were removed at day 7 posttransplantation for further analyses. (**D**) Production of IL-17A in spleen, liver, and heart graft cells from control mice ($n = 8$) and mice that received MMF alone ($n = 5$), MSCs i.v.+MMF ($n = 5$), or MSC i.v. alone ($n = 6$). Blood cells did not produce IL-17A (Supplemental Fig. 2A). (**E**) The percentage of IL-17A$^+$ and Foxp3$^+$ cells in the heart grafts, spleen, and (*Figure legend continues*)

ratio. CD11b cells were depleted using CD11b MicroBeads (Miltenyi Biotec), according to the manufacturer's protocol.

ELISA

ELISAs were performed according to the manufacturer's protocols (R&D Systems) for IL-17A, IL-10, and IFN-γ at days 5–7 by plating isolated cells at 1×10^5 cells/well in a 96-well plate and stimulating with Dynabeads Mouse T-Activator CD3/CD28.

TaqMan assays of mRNA expression

mRNA expression was analyzed in freshly isolated cells from blood, liver, spleen, heart grafts, and/or 7-day Dynabeads Mouse T-Activator CD3/CD28–stimulated cell cultures. TaqMan assays were performed on a LightCycler using TaqMan-recommended inventoried or made-to-order assays: Il17a, QT00103278; rorc, QT00197722; Foxp3, QT00138369; Il10, QT00106169; and ifng, QT01038821 (Qiagen). The expression of each gene was normalized to the GADPH mRNA level and expressed as the relative expression, that is, fold increase ($2^{-\Delta CT}$), where $\Delta CT = CT_{(Target\ gene)} - CT_{(GADPH)}$.

Statistical analysis

All data were evaluated using GraphPad Prism Version 6 software (GraphPad Software, San Diego, CA) and analyzed by the Student *t* test (two-tailed) and one-way or two-way ANOVA as appropriate. Survival data were compared using the log-rank test. $p < 0.05$ was considered significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Results

Prolonged survival of heart allografts is associated with reversal of the Th17/Foxp3 ratio

In accordance with our previous observations in a rat model (26, 27), MSCs injected before transplantation (day −4) synergized with MMF when administered on days 0–7 but not when given as a single dose on day 1, resulting in modest, but significant prolongation of allogeneic heart graft acceptance in mice (MSC+MMF, mean: 33.9 d, $n = 15$; control, mean: 10.6 d, $n = 8$) compared with untreated control mice or mice that received MMF only. In contrast, MSCs alone did not affect the allogeneic response or even shortened the median survival time of allografts (MSCs, mean: 9.4 d, $n = 7$; MMF, mean: 16.8 d, $n = 8$) (Fig. 1A, 1B).

This unexplained dual effect of MSCs led us to use the mouse heart transplantation model to further study the features of MSC-mediated allograft acceptance (Fig. 1C). We observed marked intragraft lymphocyte infiltration at day 7 posttransplantation in mice treated with MSCs+MMF, but not in mice treated with MMF alone. The fraction of effector IFN-γ^+CD8^+ T cells was significantly lower in the spleen and heart grafts of mice treated with MSCs+MMF than in those that received MSCs only. Similarly, the fraction of NK cells was lower in the spleen of MSC+MMF-treated mice when compared with that of untreated control mice or mice that received MSCs only (Supplemental Fig. 1).

The recipient liver was identified as a prominent site of MSC-mediated regulation of the immune response (Fig. 1D, 1E, Supplemental Figs. 1, 2). Further analysis demonstrated that hepatic T cells mounted a strong Th17 response after MSC administration (Fig. 1D, 1E, Supplemental Fig. 2), whereas lower absolute numbers of Th17 cells were present in the heart grafts (Fig. 1D–F). Unexpectedly, the hepatic Th17 response was completely abrogated in mice that received MMF therapy (Fig. 1D, 1E, Supplemental Fig. 2) and replaced by potent induction of $Foxp3^+$ T_{reg} cells in the heart grafts and spleen (Fig. 1E–G, Supplemental Fig. 2). These findings demonstrated that the Th17/T_{reg} ratio was associated with allograft acceptance and could be reversed by MSC+MMF-based immunomodulation.

MMF-induced immune suppression reduces the Th17 response and increases the fraction of T_{reg} cells

Significant differences in the Th17/T_{reg} cell ratio observed in vivo (Fig. 1) prompted us to test the hypothesis of direct Th17-T_{reg} cell plasticity in the current transplantation model. To this end, we performed $IL17A^+$ cell enrichment of mononuclear cells from MSC-treated mice. As a result, the percentages of $IL17A^+$ cells were increased from 7.15, 7.73, and 2.38% IL-$17A^+CD3^+$ cells among heart-, liver-, and spleen-infiltrating cells to 65.5%, 18.7%, and 9.27% IL-$17A^+CD3^+$ cells, respectively (Fig. 2A, *left panel*). High percentages of $CD3^+$IL-$17A^+$ cells in the spleen and liver were γδ-T cells, NK cells, and $CD8^+$ T cells, whereas the predominant population in heart grafts was $CD4^+$ T cells (Fig. 2A, *right panel*). Exposure of IL-17A–producing cells to MMF (0.1 μg/ml, a concentration that does not affect cell viability/proliferation) resulted in a significant reduction of IL-17A production (Fig. 2B) and an increase in the fraction of $Foxp3^+$ T_{reg} cells isolated from the spleen and heart grafts (Fig. 2C).

MSCs promote Th17 differentiation via PGE_2-dependent induction of $Gr1^{int}CD11b^+F4/80^+$ MDSCs

Considering the apparent dual effect of MSCs and their need for an additional immunosuppressive trigger to switch immune responses from inflammatory to protective, we investigated their mechanism of action when cocultured with allogeneic splenocytes (Fig. 3A). MSCs were added as modulators to cocultures of irradiated syngeneic stimulatory splenocytes and allogeneic responder splenocytes. As expected, they suppressed the proliferation of responder T cells in a concentration-dependent manner (Supplemental Fig. 3A). MSCs in direct contact with splenocytes suppressed their Dynabeads Mouse T-Activator CD3/CD28-induced activation (Supplemental Fig. 3B) and abrogated the production of inflammatory cytokines IFN-γ and IL-17A. In addition, MSCs strongly suppressed the emergence of $CD4^+Foxp3^+$ T_{regs} (Supplemental Fig. 3C). Surprisingly, in a Transwell system in which interactions can be adapted and limited, MSCs did not affect the proliferation of activated splenocytes (Fig. 3B, Supplemental Fig. 3B). In addition, MSCs induced a strong Th17 response with an increased fraction of $IL\text{-}17A^+$ cells (Fig. 3C) and high production of IL-17A at mRNA and protein levels (Fig. 3B–3D and Supplemental Fig. 3C), but not IFN-γ or IL-10 (Fig. 3B; data not shown). Such effects were observed even with early removal of MSCs from the coculture. Furthermore, syngeneic MSCs exerted the same effects (Fig. 3B), indicating that the actions of MSCs were not merely the result of allogeneic stimulus but rather the result of specific effects of MSC-produced factors. The same effects were observed by applying MSC-conditioned medium, although to a lesser extent.

MSCs have previously been shown to suppress innate and adaptive immune responses in vitro as well as the production of proinflammatory cytokines (14, 30–36). Our findings demonstrate that these effects are due to nonspecific strong inhibition of $CD3^+$

liver of control mice and mice that received MSCs i.v.+MMF, MSCs i.v. alone, or MMF alone. (**F**) Relative gene expression of RORc, IL-17A, Foxp3, IFN-γ, and IL-10 in cells isolated from heart grafts of control mice and mice that received MSCs i.v.+MMF, MSCs i.v. alone, or MMF alone. (**G**) Representative staining of $Foxp3^+CD3^+$ T cells isolated from heart grafts of control mice and mice that received MSCs i.v.+MMF, or MSCs i.v. alone. The graphs in (D)–(F) show aggregate data from *n* mice, expressed as the means ± SD from five independent experiments (triplicate cultures for ELISAs and duplicates for RT-PCR and flow cytometry). ns, $p > 0.05$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

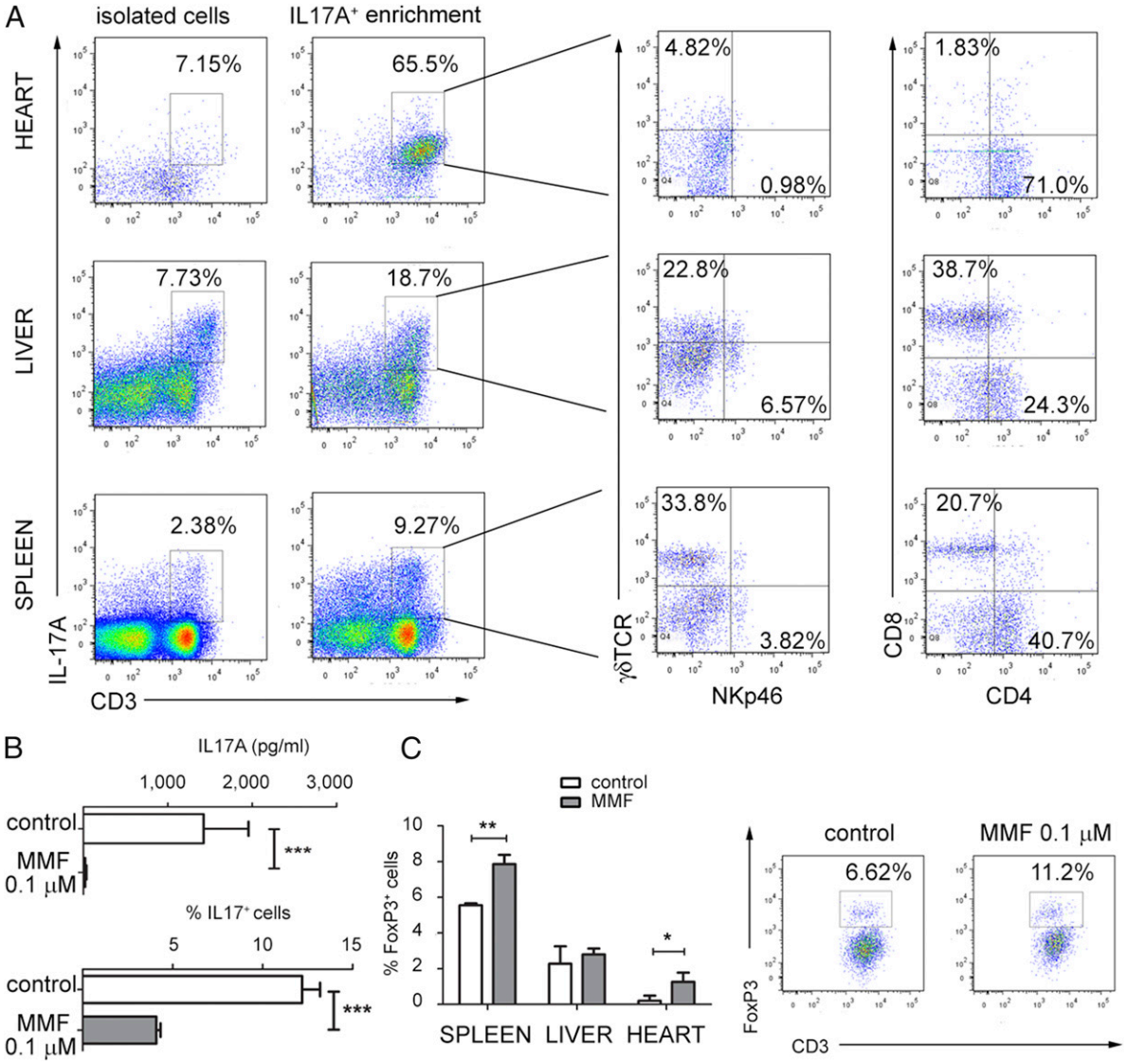

FIGURE 2. MMF-induced immune suppression reduces the Th17 response and increases the fraction of T_{reg} cells. (**A**) *Left panel*, IL17A$^+$ cell-enrichment of cells isolated from the spleen, liver, and heart grafts of mice treated with MSCs. As a result, the percentages of IL17A$^+$ cells were increased from 7.15, 7.73, and 2.38% IL-17A$^+$CD3$^+$ cells among heart-, liver-, and spleen-infiltrating cells to 65.5, 18.7, and 9.27% IL-17A$^+$CD3$^+$ cells, respectively. *Right panel*, The percentage of γδ-TCR$^+$, NKp46$^+$, CD4$^+$, and CD8$^+$ IL-17A–producing T cells isolated and enriched from respective organs. The results were confirmed in three independent experiments. (**B**) IL-17A production (*top panel*) and the percentage of IL-17A$^+$ cells (*bottom panel*) in 5-d cultures of isolated and enriched cells from the liver of MSC-treated mice stimulated with Dynabeads Mouse T-Activator CD3/CD28 in the absence or presence of MMF. The graphs show the mean ± SD from one representative experiment (triplicate cultures). (**C**) Percentages of Foxp3$^+$ cells (mean ± SD from $n = 3$ mice in duplicate) and representative intracellular staining (Foxp3 versus CD3, right) in 5-d cultures of isolated and enriched cells from the liver of MSC-treated mice stimulated with Dynabeads Mouse T-Activator CD3/CD28 in the absence or presence of MMF.

T cells in direct contact with MSCs (Supplemental Fig. 3) rather than modulation of Th immune responses. Similar to our in vivo observations in the transplantation model, the presence of low-dose MMF in conjunction with MSCs in the Transwell system resulted in inhibition of the Th17 response and an increase in the percentage of T_{reg} cells as well as Foxp3 mRNA expression (Fig. 3C–F, Supplemental Fig. 3C).

When applying the Transwell system or MSC-conditioned medium, we were intrigued by the emergence of adherent cells that markedly expanded from days 3 to 5 onwards and expressed a Gr1^{int}CD11b^{hi} MDSC–associated phenotype (Fig. 4A, 4B). Depletion of these CD11b$^+$ cells before the coculture resulted in abrogation of IL-17A induction by MSCs (Fig. 4C). Although MSC-associated soluble factors were involved in Gr1^{int}CD11b^{hi} F4/80$^+$CXCR4$^+$ MDSC induction, readdition of CD11b$^+$ cells to either the culture of splenocytes or MSCs demonstrated that the contact of MDSCs with lymphocytes was required to promote IL-17A production (Fig. 4C). Moreover, inhibition of MDSC-associated factors IL-6, TGF-β_1, and COX2/PGE_2 significantly reduced IL-17A production by MSCs (Fig. 4D).

Next, we injected clodronate-filled liposomes and observed strong suppression of the MSC-induced Th17 liver response by removal of F4/80$^+$Gr1$^+$ liver cells (Fig. 4E, Supplemental Fig. 4), confirming our in vitro data. Additional development of Foxp3$^+$ T_{reg} cells in the presence of MMF (Fig. 4F) confirmed the requirement of MDSCs for induction of the IL-17A/T_{reg} axis in vivo.

RORγ is required for the development of Foxp3$^+$ T_{reg} cells

Although the majority of CD3$^+$IL-17A$^+$ T cells were CD4$^+$ and CD8$^+$ T cells and, to a lesser extent, unconventional γδ-T cells, detailed analysis of the MSC-induced Th17 cells revealed that a distinct fraction was IL17A$^+$Foxp3$^+$. This finding demonstrated

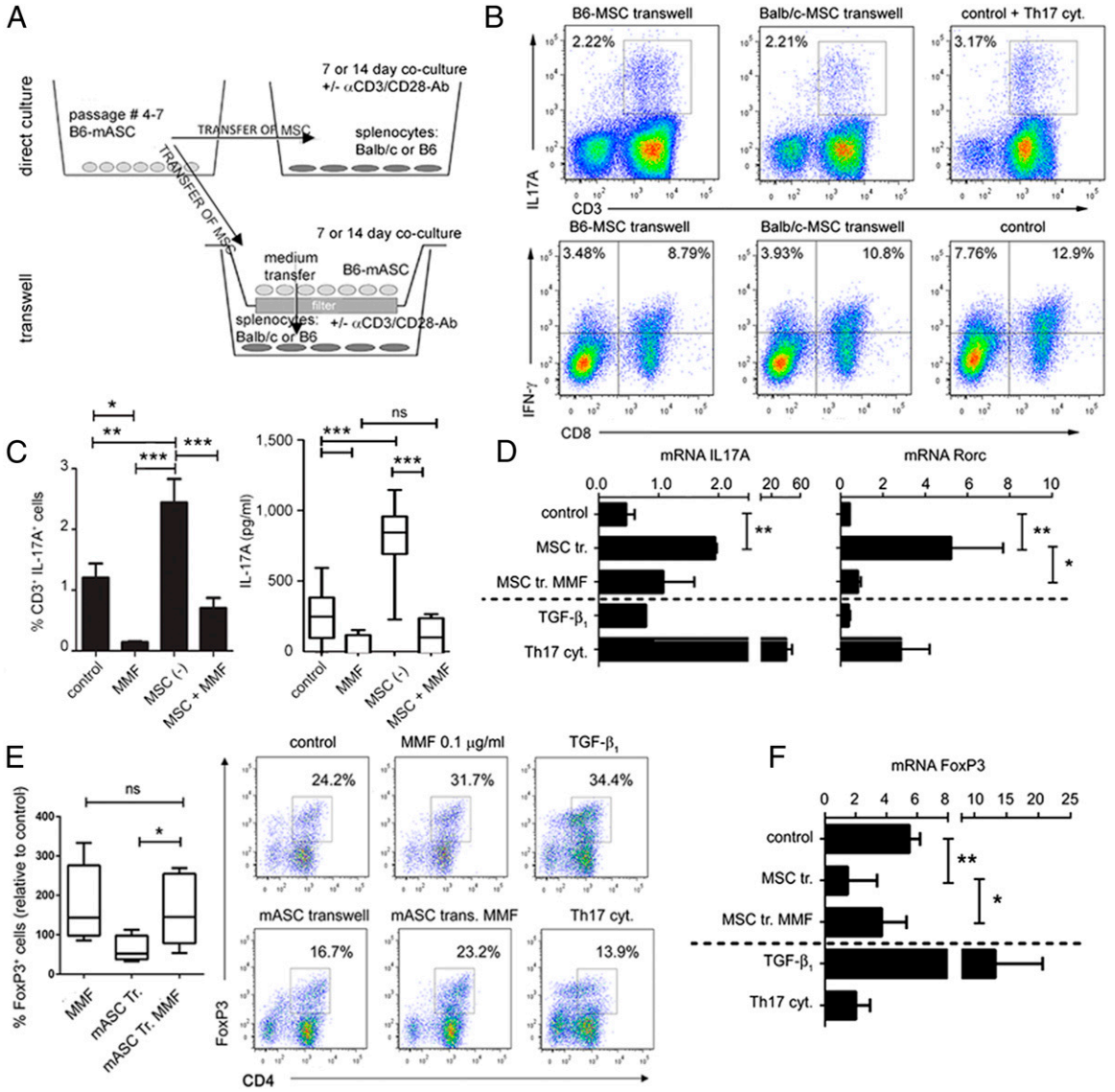

FIGURE 3. Limited MSC-lymphocyte contact and an immunosuppressive environment determine the Th17/T_{reg} balance. (**A**) Experimental model. MSCs were grown to confluency, and then, conditioned medium was harvested on day 7. As indicated, the cells were transferred into either direct coculture or a Transwell system, or their conditioned medium was added to cultures of lymphocytes activated with anti-CD3/CD28 Abs for 5–7 d (see *Materials and Methods*). (**B**) Representative staining of IL-17A$^+$ (*top panel*) and IFN-γ$^+$ (*bottom panel*) T cells in a Transwell system of syngeneic or allogeneic MSC cocultures compared with that in control or Th17-driving (IL-6, IL-23, and TGF-β_1) conditions in the absence of MSCs. (**C** and **D**) The percentage of IL-17A$^+$ cells (C, *left panel*), IL-17A production levels (C, *right panel*), and expression (D) of IL-17A and RORc (aggregate data of five independent experiments in triplicates [ELISA] and duplicates [RT-PCR]; mean ± SD) in lymphocytes stimulated with Dynabeads Mouse T-Activator CD3/CD28 in the absence or presence of allogeneic MSCs in a Transwell system and/or MMF. (**E** and **F**) The percentage of Foxp3$^+$ cells and representative staining (E) and expression (F) of Foxp3 (aggregate data of five independent experiments in duplicates; mean ± SD) in lymphocytes stimulated with Dynabeads Mouse T-Activator CD3/CD28 in the absence or presence of MSCs in a Transwell system and/or MMF. ns, $p > 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the existence of a possible intermediary in the conversion of Th17 into T_{reg} cells (Fig. 5A). To further study this phenotype, we cocultured congenic CD45.1$^+$ T cells and CD45.2/ROR$\gamma^{-/-}$ cells (the CD3$^+$/CD3^{neg} ratio was matched to 1:1 for both strains) in Transwells with MSCs in the presence of MMF. Remarkably, the majority of induced Foxp3$^+$ cells were CD45.1$^+$, indicating that RORγ was required for effective T_{reg} cell induction (Fig. 5B). Moreover, Foxp3 mRNA expression was not induced in RORγ $^{-/-}$ cells (Fig. 5C), and TGF-β_1 strongly induced Foxp3 expression in wild-type cells (Fig. 3F) but did not significantly upregulate Foxp3 expression in ROR$\gamma^{-/-}$ cells (Fig. 5C, 5D).

An in vivo model further confirmed the importance of ROR$\gamma^{-/-}$ in the development of T_{reg} cells through MSC+MMF cotherapy. Although MSCs in combination with MMF induced an increase of Foxp3$^+$ T_{reg} cells in wild-type mice (Fig. 1E–G), ROR-γ*t*-deficient mice treated with MSCs+MMF had a smaller fraction of Foxp3$^+$ cells that was comparable with the percentage of Foxp3$^+$ cells in untreated wild-type mice (Fig. 5E). In addition, ROR-γ*t*–deficient mice treated with MMF alone did not show an increase of Foxp3$^+$ cells compared with that in MMF only–treated wild-type mice (Fig. 5E). These data indicate that ROR-γt is essential for MSC+MMF-mediated Foxp3$^+$ T cell induction but is most likely not the sole mechanism of T_{reg} cell induction.

A subset of Foxp3$^+$ T_{reg} cells are ex–IL-17A–producing cells

An IL-17a^{Cre}Rosa^{eYFP} reporter mouse strain has been introduced recently, which enables fate mapping of cells with highly activated IL-17A expression, regardless of their IL-17A production at the

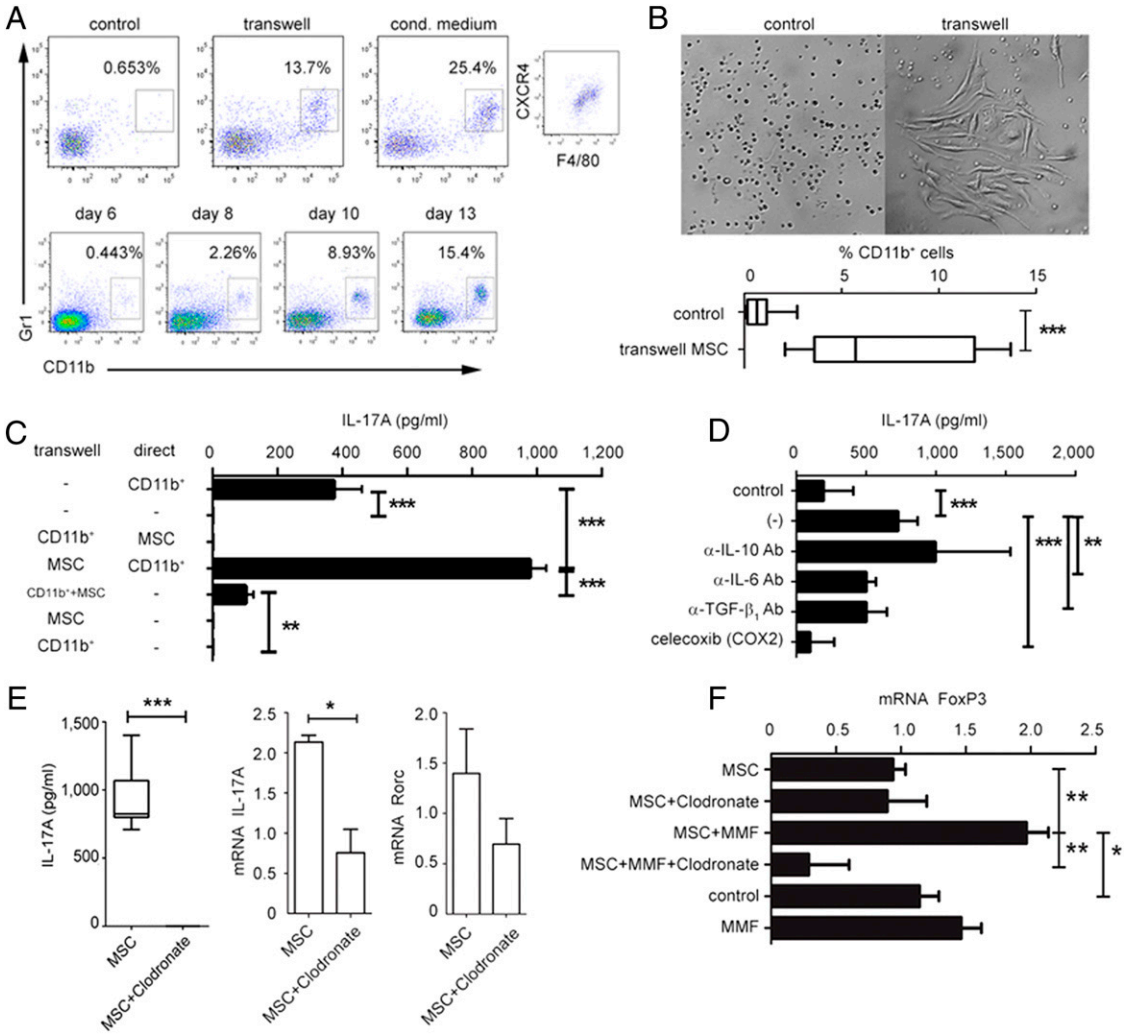

FIGURE 4. MSCs promote Th17 differentiation via PGE_2-dependent induction of $Gr1^{int}CD11b^+F4/80^+$ MDSCs. (**A** and **B**) Representative staining (A), photomicrographs (original magnification ×40) (B, *top panel*), and statistical analysis (B, *bottom panel*) of $CD11b^+Gr1^+$ $CXCR4^+F4/80^+$ cell induction in an MSC coculture Transwell system compared with that in control conditions in the absence of MSCs. (**C**) IL-17A production of $CD11b^+$-depleted lymphocytes in a Transwell coculture system without or with $CD11b^+$ cell replenishment as indicated. (**D**) IL-17A secretion from Dynabeads Mouse T-Activator CD3/CD28/MSC-expanded lymphocytes in the presence of specific inhibitory Abs against programmed death-1 (PD-1), IL-10, IL-6, TGF-β_1, or COX2 inhibitor celecoxib. Data are the mean ± SD derived from one representative experiment performed as triplicate cultures. The results were confirmed in three independent experiments. (**E**) IL-17A production levels and expression of IL-17A and RORc (aggregate data of two independent experiments in triplicates [ELISA] and duplicates [RT-PCR]; mean ± SD) in liver cells isolated from mice treated with MSCs alone or in combination with myeloid cell depletion by clodronate-filled liposomes. (**F**) Foxp3 expression (aggregate data of two independent experiments in duplicates; mean ± SD) in cells isolated from mice treated with MSCs alone or in combination with myeloid cell depletion by clodronate-filled liposomes. ns, $p > 0.05$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

time of analysis (37). When treated with MSCs+MMF or MSCs only, $IL\text{-}17a^{Cre}Rosa^{eYFP}$ mice exhibited profound induction of eYFP expression in heart allografts compared with that in MMF only–treated mice (Fig. 6A). We then analyzed $eYFP^+$ cells isolated from the heart allografts, liver, and spleen of MSC+MMF-treated mice. A significant percentage of $eYFP^+$ cells did produce IL-17A at the time of analysis. However, another distinct population of $eYFP^+$ cells from the heart and spleen, but not the liver, did not produce IL-17A and expressed Foxp3 (Fig. 6B), indicating that these cells were ex–IL-17A–producing $Foxp3^+$ T_{reg} cells.

Furthermore, using these cells in culture experiments in vitro demonstrated that a significant proportion of $Foxp3^+$ T cells expressed eYFP when cultured under T_{reg}-driving conditions (TGF-β_1 or MMF) (Fig. 6C). Among $eYFP^+Foxp3^+$ cells, the majority of cells did not express IL-17A at the time of analysis, indicating that these T_{reg} cells expressed IL-17A during their development before shifting to their current state of activation (Fig. 6D). A proportion of cells cultured under Th17-inducing conditions were double-positive for eYFP and Foxp3 and expressed IL-17A at the time of analysis, suggesting that they were an intermediary that had not yet switched to the T_{reg} phenotype (Fig. 6D). Furthermore, addition of MMF to cells that had been primed for the Th17 phenotype (day 3, $MMF^{day\ 3}$) revealed that MMF not only skewed differentiation from Th17 cells toward T_{reg} cells (day 0, $MMF^{day\ 0}$) but also converted a proportion of Th17 cells into $Foxp3^+IL\text{-}17A^{neg}$ T_{reg} cells (Fig. 6E). This result suggested the existence of ex–IL-17A–producing T cells and demonstrated that $IL\text{-}17A^+$ Th17 cells directly differentiated into T_{reg} cells in the present model system.

Discussion

MSC-based cell therapy has emerged as a promising immunomodulative tool for solid organ transplantation allowing for advantageous prolongation of graft acceptance. However, the immunology

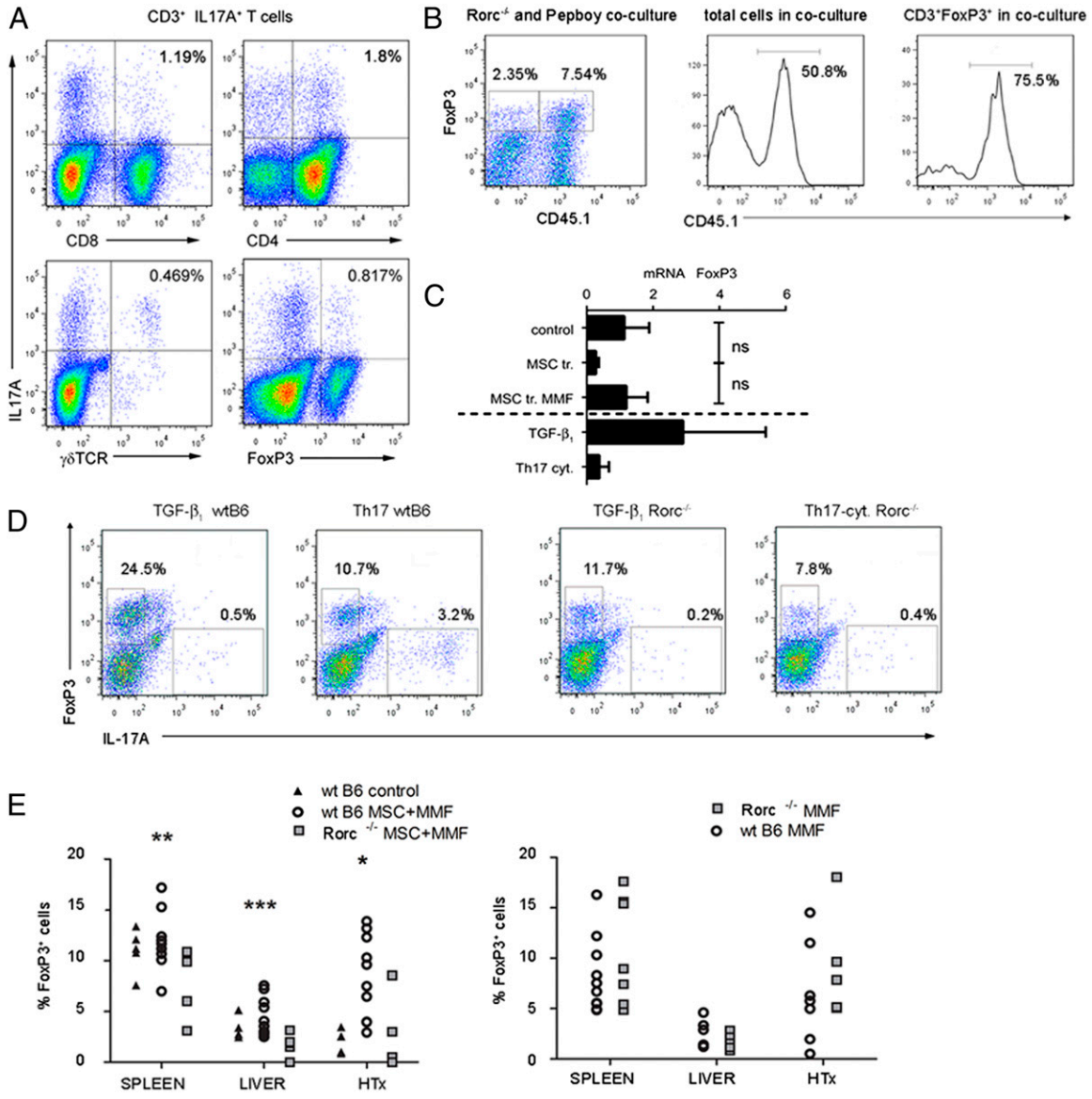

FIGURE 5. Identical features Foxp3$^+$ T_{reg} and Th17 cell development. (**A**) Representative staining of IL-17A versus γδ-TCR, CD4 and CD8 T cells in a MSC coculture Transwell system. (**B**) Representative staining of Foxp3 versus CD45.1 (B, *left panel*) and the percentage of CD45.1$^+$ cells in a 1:1 coculture or Foxp3$^+$ cells (B, *right panel*). (**C**) Foxp3 expression in ROR$\gamma^{-/-}$ cells in a MSC coculture Transwell system (aggregate data of three independent experiments in duplicates; mean ± SD). (**D**) Representative staining of Foxp3 versus IL-17A in wild-type (wt) and ROR$\gamma^{-/-}$ cells cultured in the presence of Th17 (IL-6, IL-23, and TGF-β_1)- or T_{reg} (TGF-β_1)-driving cytokines. The results were confirmed in three independent experiments. (**E**) Percentage of Foxp3$^+$ cells in the heart grafts, spleen, and liver of ROR$\gamma^{-/-}$ and wild-type mice that received MSCs i.v.+MMF (*left panel*) or MMF alone (*right panel*) compared with that in control wild-type mice (aggregate data of three independent experiments in duplicates; mean ± SD). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate the difference between ROR$\gamma^{-/-}$ and wild-type mice. ns, $p > 0.05$ for ROR$\gamma^{-/-}$ mice compared with control wild-type mice.

behind the emerging clinical successes needs to be examined in more detail. Various studies have shown inconsistent and highly context-dependent immune functions of MSCs. Although allogeneic MSCs can cause relative acceleration of transplant rejection (27), they prolong graft acceptance when combined with tailored immunosuppression (19, 27, 38, 39), demonstrating the requirement for adequate T cell activation to achieve synergism. In addition, MSC effects are highly dependent on the timing of MSC administration with MSC infusions prolonging graft survival and increasing levels of Foxp3$^+$ T_{reg} cells only when administered before graft transplantation in some models (25, 38). It is also unclear how various preparations and subpopulations of MSC-like stem cells function in comparison with true MSCs.

In this study, we identified a novel immunomodulative pathway involving direct Th17-to-T_{reg} plasticity as the in vivo mechanism of MSC cotherapy. We further demonstrated that MSC-driven immunomodulation features MDSC-mediated Th17 cell induction during the early phase, followed by conversion of the Th17 cell–dominated response into a T_{reg} cell–dominated response that sustains allograft acceptance.

Our data outline a scenario in which MSCs administered before transplantation, besides potentially resulting in a degree of allosensitization, induce MDSCs in an Ag-nonspecific manner. MDSCs then induce a potent Th17 response, both in the liver and manifesting in the graft, which is further converted into a regulatory pattern by MMF. Direct progeny of the Th17 cells appear to be intragraft localized ex–Th17 IL-17^{neg}Foxp3$^+$ T_{reg} cells. Subsequently, CD8$^+$ T and NK cell infiltration (typically accompanying the Th17 response) is reduced in the graft together with the development of long-term allograft protection (Fig. 7). The indicated

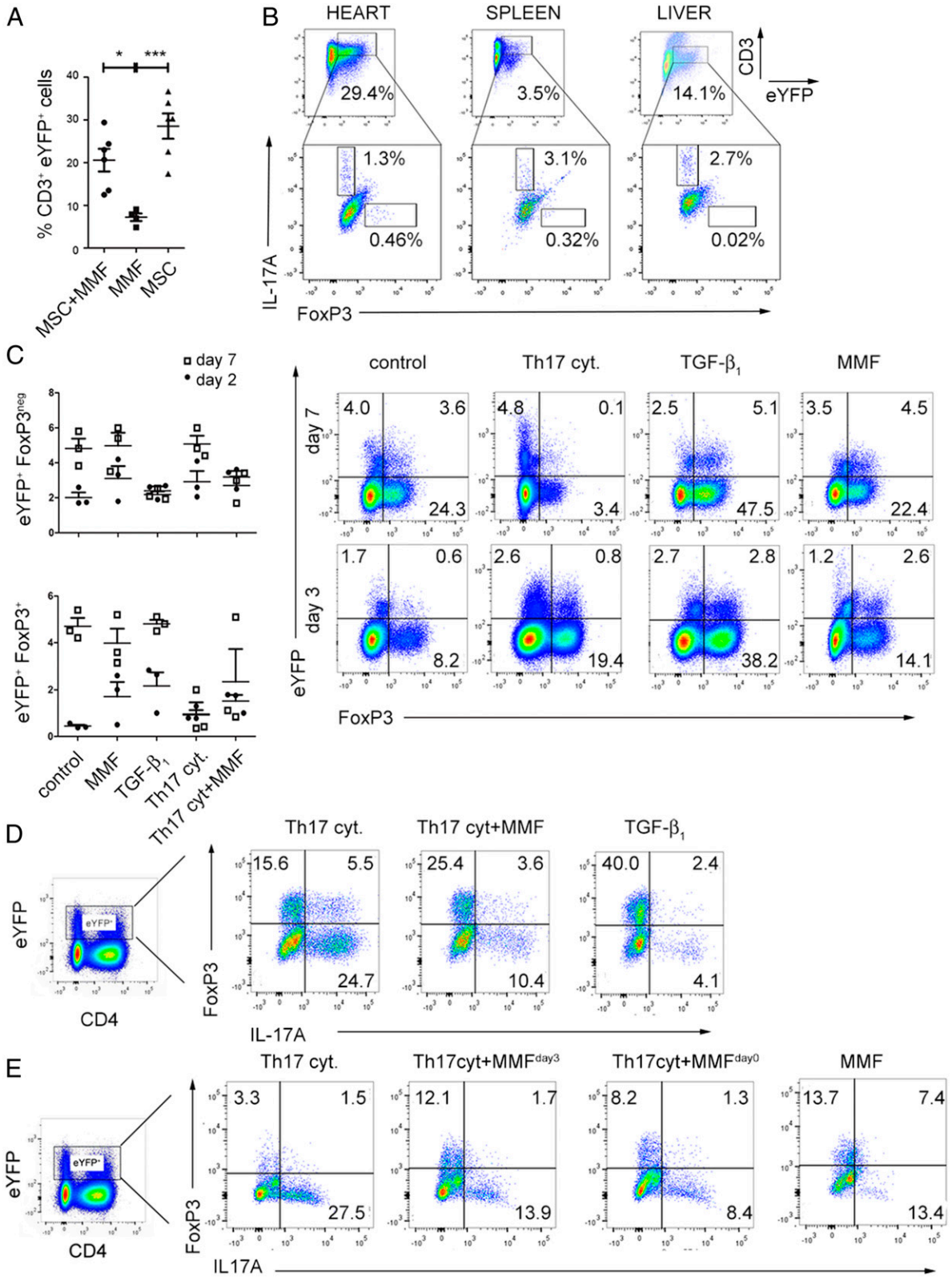

FIGURE 6. IL-17A–producing cells convert to IL17A^{+}Foxp3^{+} and IL17A^{neg}Foxp3^{+} T_{reg} cells. (**A**) The percentages of CD3^{+}eYFP^{+} cells in heart grafts of Il17a^{Cre}R26R^{eYFP} mice that received MSCs i.v.+MMF, MSCs i.v. alone, or MMF alone. (**B**) Representative staining of Foxp3 versus IL-17A in cells (eYFP^{+}CD3^{+} gated) from Il17a^{Cre}R26R^{eYFP} mice that received MSCs i.v.+MMF. Similar results were obtained in three independent experiments. (**C**) Statistical analysis (*left panel*, aggregate data of three independent experiments; mean ± SD) and representative staining (*right panel*) of Il17a^{Cre}R26R^{eYFP} reporter cells (CD3^{+} gated) cultured in the presence of Th17 (IL-6, IL-23, and TGF-β_1)- or T_{reg} (TGF-β_1)-driving cytokines or MMF and assessed for eYFP and Foxp3 expression on days 3 and 7. (**D**) Representative staining of Il17a^{Cre}R26R^{eYFP} reporter cells (eYFP^{+}CD3^{+} gated) cultured in the presence of T_{reg} (TGF-β_1)- or Th17 (IL-6, IL-23, and TGF-β_1)-driving cytokines and MMF and assessed for Foxp3 and intracellular IL-17A expression on day 3. (**E**) Representative staining of Il17a^{Cre}R26R^{eYFP} reporter cells (eYFP^{+}CD3^{+} gated) cultured in the presence of Th17 (IL-6, IL-23, and TGF-β_1)-driving cytokines and MMF supplemented on day 3 or at the beginning of the cultures (day 0) and assessed for Foxp3 and intracellular IL-17A expression on day 7. The results were confirmed in three independent experiments.

mechanism of tolerance induction may be present in other therapeutic approaches where monocytic MDSC-mediated T_{reg} induction plays an essential role, for example, donor-specific transfusion (40) inducing analogous prolongation of allograft acceptance (26).

Our data identified the liver and graft as prominent sites of the MSC-induced Th17 response after i.v. administration, while we were unable to detect Th17 cell induction in the spleen or blood (Supplemental Fig. 2A). (Ex)IL-17A–producing (eYFP$^+$) cells were however present in the liver, heart graft and spleen of MSC-treated mice, demonstrating that MSC-induced Th17 cells migrate into the graft and lymphoid organs. Whereas previous reports have demonstrated that MSCs—not pertaining to their antigenicity—are present in the spleen and liver when administered in the absence or before transplantation (25, 39, 41) and preferentially migrate to injury sites and localize in the graft after post-transplantation administration (25, 42), we recently showed that MSCs are short-lived and fail to migrate beyond the lungs after i.v. infusion, although their debris is detectable in the liver (43). This led us to believe that the immunomodulatory effects of infused MSCs must be mediated via a secondary cell type. To identify this secondary cell, we carried out a series of in vitro experiments that demonstrated a prominent induction of adherent F4/80$^+$Gr1$^+$CD11b$^+$ MDSCs by MSCs. Abrogation of IL-17A production in these cultures by predepletion of CD11b$^+$ cells confirmed the crucial role of MDSCs for Th17 induction. Clodronate depletion of F4/80$^+$Gr1$^+$ MDSCs in vivo prevented Th17 cell induction in the liver, confirming that Th17 cells were induced by MSC-primed MDSCs. This current observation is in line with our recent finding that clodronate treatment in vivo abrogates allogeneic transplant survival induced by multipotent adult progenitor cells, a related cell type with comparable functional and immunological features (44). This intermediary role of MDSCs is also in keeping with recent results from our group and others, demonstrating that MDSCs effectively promote Th17 cell differentiation and IL-17A production in various other models (45–47).

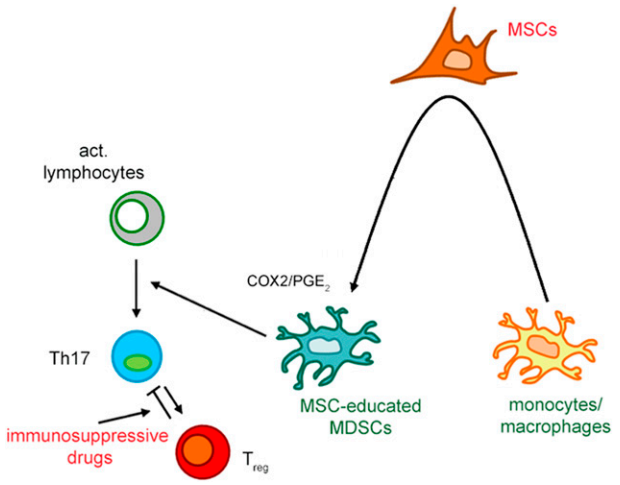

FIGURE 7. Schematic model for MSC cotherapy–mediated prolongation of allograft survival. MSC cotherapy for solid organ transplantation uses MDSCs and Th17/Treg plasticity. MSCs administered before transplantation induce the surrogate cell type, MDSCs. These cells induce a potent Th17 response found in the liver and within the graft. By MMF, Th17 cells directly convert into localized intragraft ex–IL-17–producing IL-17A$^-$Foxp3$^+$ T_{reg} cells. Although IL-6 and TGF-β play a role in MSC-mediated Th17 induction, PGE_2 produced by MSC and/or MDSCs represents the key mediator and may be involved in the induction of MDSCs (54, 58) and direct Th17 cell differentiation (59). This model outlines the Th17/Treg balance as a novel target in stem cell transplantation.

MSCs and MDSCs spontaneously produce significant amounts of IL-6 and TGF-β_1 (48, 49), the factors implicated in the induction of Th17 cells (5, 50–53) and blockage of either of the two factors significantly reduced IL-17A production instigated by MSCs. Furthermore, inhibition of COX-2/PGE_2, a typical MDSC-inducing and MDSC-derived regulatory factor (54), strongly suppressed IL-17A production, indicating the importance of PGE_2 among the various immunomodulatory factors produced by MDSCs and MSCs in the development of Th17 immunity (26, 31, 33, 55–57).

The ability of syngeneic and allogeneic MSCs to induce MDSC-dependent IL-17A production in a Transwell model demonstrates that a soluble factor produced by MSCs rather than presentation of an alloantigen by MSCs is needed for induction of Th17 cells. These data support the hypothesis that the effect of MSCs is not a mere product of donor Ag processing, but a result of specific interaction of MSCs with the host immune system (i.e., the induction of MDSCs). In line, previous observations demonstrate that recipient-derived MSCs, although not as efficient as donor-derived MSC, do promote the graft survival (21, 26). Although the conversion of cytokine-induced Th17 cells into T_{reg} cells promoted by MMF in vitro is related to general lineage commitment shift and independent of the allospecificity of Th17 cells, the allogeneic autonomy of MSC-induced Th17 cells and Th17-converted T_{reg} cells for their suppressive functions will need to be confirmed further.

Cotherapy with MSCs and MMF, a state-of-the-art clinical immunosuppressive drug, successfully shifted the Th17/T_{reg} balance toward the T_{reg} cell lineage, thereby creating an immunosuppressive microenvironment in the graft, which supports its long-term acceptance. Our results strongly suggest that T_{reg} cells may be a direct progeny of Th17 cells. However, the current data indicate that this is not the sole mechanism of T_{reg} cell induction but rather a competing pathway—for which relevance will need to be established in forthcoming clinical trials in humans. In support of Th17/T_{reg} conversion, in vitro cocultures with MSCs in the presence of low-dose MMF demonstrated reduced RORγ and induced Foxp3 expression. Furthermore, culture of in vivo–induced and fully differentiated IL-17A–producing Th17 cells in the presence of MMF resulted in a decrease in IL-17A production with concomitant induction of Foxp3 expression. These observations confirmed the plasticity of the two subsets and possible interconversion of Th17 cells into Foxp3$^+$ T cells by MMF. Interestingly, the absence of Foxp3$^+$ cell induction in RORγ$^{-/-}$ mice by MSC combination therapy with MMF implies a role of RORγ in the generation of both Th17 and T_{reg} cells. This was confirmed with RORγ$^{-/-}$ cell cultures, having impaired generation of T_{reg} cells induced by MSC in the presence of MMF or addition of TGF-β_1.

Pursuing the possibility of direct conversion of Th17 into T_{reg}, we identified CD3$^+$eYFP$^+$Foxp3$^+$IL17A^{neg} cells in heart grafts and spleen but not liver of IL-17a^{Cre}Rosa^{eYFP} reporter recipient mice treated with MSC combination therapy, demonstrating that Th17 cells, which are being induced by MSC in the liver, can convert into IL17A^{neg}CD3$^+$Foxp3$^+$ cells in the grafts. The importance of this conversion was further strengthened by in vitro studies showing that IL-17A$^+$Foxp3$^+$ T cells developed as an intermediary cell population in cocultures with MSCs+MMF and that under both T_{reg}- and Th17-driving conditions a fraction of induced Foxp3$^+$ T cells expressed eYFP. These results demonstrated that these cells were in fact ex–IL-17A–producing cells. Furthermore, addition of MMF to Th17$^{day\ 3}$-primed cells led to a decrease in IL-17A production and conversion to Foxp3 expression in eYFP$^+$ (ex)-Th17 cells, whereas the subset of Foxp3$^+$eYFP$^+$ cells ceased IL-17 production. These data not only conclusively argue the previously recognized high plasticity of Th17 and T_{reg} cell subsets but also that

IL-17A$^+$ cells directly convert into IL-17A^{neg}Foxp3$^+$ ex-Th17-T_{reg} cells.

Previous reports have tried to explain the anti-graft immune response in solid organ transplantation based on one-way Th cell lineage differentiation leading to end-stage commitment of T cells. Our study introduces a novel perspective for the plasticity of Th17 and T_{reg} cell subsets. This plasticity constitutes one important immunological mechanism by which MSCs mediate operational transplant tolerance under favorable conditions. The possibility to create T_{reg} cell–regulated operational tolerance in the absence of complete immune suppression through induction of Th17 immunity may have clinical implications for cell therapy and minimization protocols. These aspects will need to be carefully analyzed in ongoing first-in-man clinical studies in this intriguing field of MSC therapy for solid organ transplant recipients.

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Disclosures

The authors have no financial conflicts of interest.

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