Mesenchymal stem cells and a vitamin D receptor agonist additively suppress T helper 17 cells and the related inflammatory response in the kidney

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Duffy MM, McNicholas BA, Monaghan DA, Hanley SA, McMahon JM, Pindjakova J, Alagesan S, Fearnhead HO, Griffin MD. Mesenchymal stem cells and a vitamin D receptor agonist additively suppress T helper 17 cells and the related inflammatory response in the kidney. Am J Physiol Renal Physiol 307: F1412–F1426, 2014. First published October 22, 2014; doi:10.1152/ajprenal.00024.2014.—Mesenchymal stem cells (MSCs) suppress T helper (Th)17 cell differentiation and are being clinically pursued for conditions associated with aberrant Th17 responses. Whether such immunomodulatory effects are enhanced by coadministration of MSCs with other agents is not well known. In the present study, individual and combined effects of MSCs and the vitamin D receptor (VDR) agonist paricalcitol on Th17 induction were investigated in vitro and in a mouse model of sterile kidney inflammation (unilateral ureteral obstruction). In vitro, MSCs and paricalcitol additively suppressed Th17 differentiation, although only MSCs suppressed expression of Th17-associated transcription factors. Combined administration of MSCs and paricalcitol resulted in an early (day 3) reduction of intrarenal CD4+ and CD8+ T cells, CD11b+/lymphocyte antigen 6G2+ neutrophils, and inflammatory (lymphocyte antigen 6C10+) monocytes as well as reduced transcript for IL-17 compared with untreated animals. Later (day 8), obstructed kidneys of MSC/paricalcitol double-treated mice, but not mice treated with either intervention alone, had reduced tubular injury and interstitial fibrosis as well as lower numbers of neutrophils and inflammatory monocytes and an increase in the ratio between M2 (CD206+) and M1 (CD206−) macrophages compared with control mice. Adjunctive therapy with VDR agonists may enhance the immunosuppressive properties of MSCs in the setting of pathogenic Th17-type immune responses and related inflammatory responses.

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Mesenchymal stem cells (MSCs) have unique modulatory effects on mononuclear phagocyte activation and phenotype as well as on T cell activation and differentiation (7, 9, 19, 30). However, the relative potency of MSCs as immune modulators has been variable among different experimental models and in the hands of different investigators (6, 12, 32). One strategy for improving the therapeutic efficacy of MSCs for inflammatory diseases that has not been extensively addressed to date is that of combining MSC administration with additional immune modulatory agents with a view to inducing additive or synergistic benefits. Ideally, such interventions would mediate their effects through mechanisms distinct from those associated with MSCs and would constitute a safe and practical regimen when combined with a cellular therapy. In this regard, proinflammatory phenotypes of mononuclear phagocytes and T helper (Th) cells are also known to be directly suppressed by vitamin D receptor (VDR) agonists such as calcitriol and synthetic vitamin D analogs (21, 28). Such immunomodulatory properties have raised substantial interest in the use of VDR agonists to treat and prevent a variety of immune-mediated diseases. Additionally, VDR agonist immune modulation has been shown to be additive to or synergistic with that of other therapeutic agents (8, 40).

The clinical relevance of MSC intervention for kidney disease has been well demonstrated in experimental models of both acute kidney injury (AKI) and chronic kidney disease. Variable degrees of renoprotection have been demonstrated in the setting of sepsis (30), ischemia-reperfusion injury (24, 44, 45), obstrucive nephropathy (25, 26), cisplatin-induced acute renal failure (2, 29), renal artery stenosis (48), rhabdomyolysis (15), and chronic renal failure (38) despite, in some cases, limited renal localization and in vivo survival of systemically administered MSCs (15, 30, 45). Indeed, MSC-specific modulation of kidney disease is currently being translated to clinical practice with reported successful use as an induction agent in renal transplantation (39). Furthermore, paricalcitol, a synthetic analog of calcitriol with reduced calcemic properties, displayed renoprotective effects in cyclosporine-induced kidney injury (33), gentamicin-induced kidney injury (34), diabetic nephropathy (8), and obstructive nephropathy (40, 42).

In preclinical models of AKI, initial activation of resident and rapidly infiltrating mononuclear phagocytes (dendritic cells, monocytes, and macrophages) results in rapid production of multiple proinflammatory mediators. This innate immune response is accompanied by influx and activation of effector memory CD4+ T cells with proinflammatory Th1 and Th17 phenotypes, which additionally contribute to tissue damage through the action of signature cytokines and chemokines (10). As an example of the link between mononuclear phagocyte activation and Th cell activation during AKI, we recently demonstrated that intrarenal dendritic cell- and monocyte-derived IL-1 enhances the activation of chemokine (C-C motif) receptor (CCR)6+ IL-17-producing effector memory phenotype Th17 cells in the mouse unilateral ureteral obstruction (UUO) model (36).
We also previously reported that MSCs suppress primary Th17 induction via PGE2 production induced by direct contact with activated CD4+ T cells (11).

Based on this background, the initial aim of the present study was to examine the individual and combined effects of MSCs and paricalcitol on the Th17 differentiation pathway in vitro. We then sought to determine the potential benefit of paricalcitol administration as an adjunct to MSC therapy in AKI by examining the effects of single and combined therapies on intrarenal mononuclear phagocytes, Th17 cells, and tissue injury in the mouse UUO model. Our results demonstrate the potential for a VDR analog to augment the effects of MSC therapy via complementary immunosuppressive/anti-inflammatory actions.

MATERIALS AND METHODS

Experimental animals. Eight- to twelve-week-old female C57BL/6 mice were purchased from Harlan Laboratories UK (Bicester, UK) and Charles River Laboratories (Margate, UK). Experimental animals were housed in a specific pathogen-free facility and fed a standard mouse chow diet. All animal procedures were carried out under license from the Irish Department of Health and Children by procedures approved by the National University of Ireland, Galway Animal Care Research Ethics Committee.

MSC isolation, characterization, and culture conditions. C57BL/6 mouse MSCs were isolated from bone marrow according to the method of Peister and colleagues (35), and trilineage differentiation capacity was determined as previously described (11). All experiments were carried out with passage 5–9 MSCs grown to 80% confluence in supplemented Iscove’s modified Dulbecco’s medium (containing 9% FCS, 9% donor equine serum, 1% L-glutamine, and 1% penicillin-streptomycin).

Th17 differentiation cultures. In vitro Th17 differentiation was induced by activation of magnetic-activated cell sorting (MACS)-enriched mouse CD4+ T cells (1 x 10^6 cells/ml) or fluorescence-activated cell sorting (FACS)-purified CD4+CD25-CD62L+ naïve and CD4+CD25-CD62L+ memory responders as previously described (11) in the presence of IL-6 (25 ng/ml), transforming growth factor-β1 (5 ng/ml), anti-interferon-γ (5 µg/ml), and anti-IL-4 (4 µg/ml) with anti-CD3e (0.1 µg/ml) and autologous antigen-presenting cells (APCs) (2 x 10^6 cells/ml) or anti-CD3/CD28 T cell expander beads for 3 days in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% penicillin-streptomycin, 1% HEPES, 1% nonessential amino acids, and 0.1% 2-mercaptoethanol. Paricalcitol or vehicle (ethanol) was added in graded concentrations. MSCs were added at a T cell ratio of 1:400 MSC-to-CD4+ T cells (APCs) (2.5 x 10^5 cells/ml) or anti-CD3/CD28 T cell expander beads for 3 days in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% penicillin-streptomycin, 1% HEPES, 1% nonessential amino acids, and 0.1% 2-mercaptoethanol. Paricalcitol or vehicle (ethanol) was added in graded concentrations. MSCs were added at a T cell ratio of 1:400 MSC-to-CD4+ T cell ratio. Other reagents were added as described for individual experiments. CD4+ T cells were repurified by MACS after coculture and subjected to Western blot analysis or restimulated for 8 h with anti-CD3/CD28 beads in the presence of brefeldin A for intracellular flow cytometric staining.

Flow cytometry, intracellular staining, and FACS. For flow cytometry experiments, cells were suspended in FACS buffer (Dulbecco’s PBS (D-PBS), 2% FBS, and 0.05% NaN3) at 5 x 10^6 cells/ml and incubated with various combinations of fluorochrome-labeled antibodies for 20 min at 4°C. Cells were washed and resuspended in FACS buffer before being analyzed using a BD Biosciences FACSCanto cytometer (San Jose, CA) and FlowJo software (TreeStar, Olien, Switzerland). In some experiments, CD4+ T cells were labeled before culture using a CellTrace CFSE cell proliferation kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions for analysis of proliferation by flow cytometry. For intracellular staining, brefeldin A (Golgi Plug, 1 µM, BD Biosciences) was added to cultures for 8 h before analysis. Surface staining was performed before intracellular staining using Cytofix/Cytoperm reagents (BD Biosciences). For FACS experiments, magnetic column-enriched CD4+ T cells were incubated for 20 min in sorting buffer (Ca2+- and Mg2+-free D-PBS, 1% FCS, 25 mM HEPES, and 2 mM Na2EDTA) at 4°C with combinations of fluorochrome-labeled antibodies. Cells were washed and resuspended in sorting buffer before being sorted using a BD FACSariaII.

Measurement of analytes in supernatants. Supernatants from cultures and cocultures were analyzed for IL-17A by ELISA using Duoset ELISA Development Systems or for PGE2 using a Parameter PGE2 competitive assay (both from R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Western blot analysis. Equal amounts of protein (10 µg) were separated by electrophoresis in a Mini-Protean Tetra Cell (Bio-Rad, Hercules, CA) for ~1 h at 150 V. Electrottransfer to Immobilon P polyvinylidene difluoride membranes (Millipore, Billerica, MA), was performed and membranes were stained with Ponceau S solution to confirm equal loading. Membranes were blocked in 5% (wt/vol) milk powder in Tris-buffered saline-Tween (TBST) at 4°C overnight. Membranes were incubated with primary antibodies in 5% (wt/vol) milk powder in TBST overnight at 4°C followed by a wash in TBST. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-linked secondary antibody diluted in 5% (wt/vol) milk powder in TBST with streptactin-HRP (1:30,000) for detection of the chemiluminescent ladder. Membranes were washed in TBST. Development was achieved using Immobilon Western Chemiluminescent HRP substrate and imaged on a Fluorochrom chemiluminescent imaging system. Target bands were normalized to their respective β-actin bands for accurate spot densitometry, which was performed on a Fluorochrom chemiluminescent imaging system.

Mouse UUO and treatment regime. UUO was performed as previously described (36). Mice were randomly assigned into one of the following four treatment groups (n = 5 mice/group): 1) no treatment, 2) MSCs alone, 3) paricalcitol alone, and 4) MSCs and paricalcitol (Fig. 1). Autologous MSCs (2.5 x 10^6 cells/ml) were administered in 200 µl of sterile saline via tail vein injection at 18 and 48 h relative to ureteral ligation. Paricalcitol (0.3 µg·kg^-1·day^-1) was administered subcutaneously in a volume of 100 µl of saline daily starting on day 0 relative to ureteral ligation. Mice were euthanized 3 or 8 days after UUO. Kidneys were subjected to RT-PCR, histology, and immunohistochemistry. Preparation of kidney single cell suspensions for flow cytometry was performed by collagenase digestion as previously described (11).

RNA isolation from kidney tissue and quantitative RT-PCR. Tissue samples were homogenized using a pestle before RNA isolation using the TRIzol method. RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer. Equal quantities of RNA were DNase treated before remeasurement of RNA content. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor and a GeneAmp 9700 A Thermal Cycler from Applied Biosystems (Carlsbad, CA). cDNA was equalized between samples and subjected to quantitative RT-PCR using inventoried TaqMan gene expression assays and reagents and a StepOne Plus Real Time PCR System (Applied Biosystems). The comparative threshold cycle method was used to determine relative quantification. Target genes were normalized to GAPDH and expressed as fold changes relative to the appropriate reference sample.

Histology. Kidneys were dissected and placed in 10% neutral buffered formalin for ~24 h before being processed in a Leica ASP 300 tissue processor (Wetzlar, Germany). Tissues were wax embedded in a Leica EG1151H1 wax embedder, and 2-µm sections were cut using a Leica RM2235 microtome. Sections were transferred to Superfrost Plus microscope slides (Fisher Scientific Ireland) and dried overnight at 55°C. Tissue sections were subjected to the following staining protocols.

For hematoxylin and eosin (H&E) staining, sections were dewaxed in xylene, rehydrated, stained in Mayer’s hematoxylin followed by eosin before being dehydrated through graded alcohols, cleared in xylene, and then covered with DPX mounting medium and coverslips.

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For Masson’s trichrome staining with Gomori’s aldehyde fuchsin staining, sections were dewaxed in xylene and rehydrated. Sections were oxidized in equal parts 0.5% potassium permanganate-0.5% sulfuric acid and bleached in 2% sodium metabisulphite. Sections were stained in Gomori’s aldehyde fuchsin before celestine blue and then Mayer’s hematoxylin. Sections were washed and differentiated in acid alcohol before staining in Masson’s cytoplasmic stain and differentiation in 1% dodeca-molybdophosphoric acid. Sections were then counterstained in fastgreen, differentiated in 1% acetic acid, and dehydrated before being cleared in xylene and mounted in DPX medium.

For periodic acid-Schiff (PAS) staining, sections were dewaxed in xylene, rehydrated, and washed in PBS followed by oxidation in 2% periodic acid solution. Sections were then washed before being incubated in Schiff reagent, counterstained in Mayer’s hematoxylin, dehydrated, cleared in xylene, and mounted in DPX medium.

Semiquantitative scoring of kidney tissue injury. Stained sections of control and obstructed kidneys were analyzed in blinded fashion by light microscopy at ×20 magnification using an Olympus BX51 brightfield microscope (Olympus, Center Valley, PA) and Improvisation Volocity software (Perkin-Elmer, Waltham, MA). For each kidney, 10 nonoverlapping fields of a stained section were scored by a blinded observer for tubular dilatation and atrophy (H&E), interstitial inflammatory cell infiltration (PAS), and interstitial fibrosis (trichrome). Scoring was carried out according the following semiquantitative scoring scales: for tubular dilatation and atrophy, 1 = <10%, 2 = 10–25%, 3 = 25–50%, and 4 = >50%; and for interstitial fibrosis and interstitial inflammatory cell infiltration, 1 = <3%, 2 = 3–10%, 3 = 10–25%, and 4 = >25%. The total interstitial injury score consisted of the sum of the scores for the three histological abnormalities. Mean scores were calculated for each individual kidney, and final results for control and obstructed kidneys were expressed as group means ± SD.

In vivo localization of intravenously administered MSCs. MSCs were labeled with the fluorescent dye PKH26 (Sigma-Aldrich) according to the manufacturer’s protocol. Groups of mice were subjected to UUO and administered 0.5 × 10^6 PKH26-labeled MSCs in 200 μl saline or saline alone at 18 and again at 48 h as described above. At 72 h after UUO, animals were euthanized, and the obstructed kidney, control kidney, lungs, liver, heart, and spleen were dissected. Portions of the dissected organs were subjected to collagenase dissection and flow cytometric analysis (all organs) or 2 fixed in paraformaldehyde, frozen in liquid nitrogen-cooled 2-methylbutane, cryosectioned, counterstained with 4′,6-diamidino-2-phenylindole, and examined by immunofluorescence microscopy on an Olympus BX-51 polarising fluorescence microscope using Image Pro-plus software (Media Cybernetics, Rockville, MD). All composite images were made by overlaying individual images in FIJI (ImageJ).

Statistical analysis. Results are expressed as means ± SD, and differences between conditions were tested statistically by ANOVA and post hoc tests where indicated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Significance was assigned at P < 0.05.

Fig. 2. Paricalcitol dose dependently inhibits IL-17A production. CD4^+ T cells were cultured for 3 days under T helper (Th)17-skewed conditions in the presence of graded concentrations of paricalcitol (10^-1 to 10^-11 M) or equivalent volumes of vehicle (ethanol) before 8 h restimulation with anti-CD3/anti-CD28-coated beads. A: concentration of IL-17A in day 3 culture supernatants. B: flow cytometric analysis of the percentage of IL-17A^+ cells among cells gated on CD4^+ after restimulation. C: representative examples of contour plots of intracellular IL-17A staining of restimulated CD4^+ T cells. D: graphical representation of the percentage of CD4^+ T cells that underwent three or more cell divisions. E: surface expression of CD25 on CD4^+ T cells after restimulation. Data are representative of two individual experiments. *P ≤ 0.05 and ***P ≤ 0.001 compared with the equivalent vehicle control group (by Bonferroni posttest).
Re-activated Th17 culture:

- **A** Day 3 Th17 culture: IL-17A concentration
- **B** Re-activated Th17 culture: % IL-17A+ among CD4+ T-cells
- **C** Re-activated Th17 culture: CD25 surface expression
- **D** Re-activated Th17 culture: rate of proliferation
- **E** Re-activated Th17 culture: CD25 surface expression
RESULTS

Dose-dependent inhibition of IL-17A production by paricalcitol. CD4⁺ T cells were cultured in the presence of varying concentrations of paricalcitol under Th17-skewed conditions. Paricalcitol was associated with dose-dependent inhibition of IL-17A production (Fig. 2A). After restimulation for 8 h under non-Th17-skewed conditions, CD4⁺ T cells originally cultured in the presence of paricalcitol had a lower frequency of IL-17A-producing cells as measured by intracellular IL-17A flow cytometric staining (Fig. 2, B and C). The optimal concentration of paricalcitol chosen for further experimentation was 10⁻⁸ M (10 nM). In contrast to the potent suppression of IL-17A, paricalcitol had a minimal effect on the proliferation of CD4⁺ T cells (Fig. 2D) and did not suppress CD25 surface expression (Fig. 2E).

Enhanced suppression of Th17 responses by combining MSCs with paricalcitol. Combined effects of paricalcitol (10 nM) and C57BL/6 MSCs (1:400 MSC-to-CD4⁺ T cell ratio) were examined on FACS-purified naïve and memory phenotype CD4⁺ T cells undergoing primary Th17 differentiation. This low T cell-to-MSC ratio was selected based on our previously reported results for varying T cell-to-MSC ratios in Th17 differentiation cultures (11) and on the rationale that a low density of MSCs would better reflect in vivo MSC/T cell encounters after systemic administration. As expected, memory phenotype responders produced more IL-17A under Th17-skewed conditions than their naïve phenotype counterparts (Fig. 3, A and D). Paricalcitol alone significantly inhibited IL-17A production in primary and restimulated cultures of naïve and memory phenotype responders; however, suppression of memory phenotype responders was less potent than that of naïve phenotype responders (Fig. 3). Qualitatively similar results were observed in a total of three similar experiments with median proportionate inhibition of IL-17A production after restimulation of 9% (range: 2–17%) for memory phenotype responders and 45% (range: 34–44%) for naïve phenotype responders.

The combination of MSCs and paricalcitol further suppressed IL-17A production in both naïve (Fig. 3, A–C) and memory (Fig. 3, D–F) phenotype cultures. Surface expression of CD25 on CD4⁺ T cells was significantly suppressed in the presence of MSCs, but paricalcitol did not affect the expression of CD25 (Fig. 4A). CCR6 is highly expressed by Th17 cells and is an important determinant of Th17 migration in vivo in response to chemokine (C-C motif) ligand 20 (36). Paricalcitol additively suppressed CCR6 surface expression on CD4⁺ T cells in the presence of MSCs (Fig. 4B). In addition to the suppressive effects on T cells, both MSCs and calcitriol have been reported to inhibit APC maturation, antigen presentation, and cytokine production (20, 21). To confirm direct effects on T cells, MACS-enriched CD4⁺ T cells were cultured under Th17-skewed conditions for 3 days in the presence of anti-CD3/anti-CD28-coated beads in an APC-free culture system. Both MSCs and paricalcitol significantly inhibited IL-17A production in the absence of APCs. Furthermore, the combination of MSCs and paricalcitol further suppressed IL-17A production (data not shown). Therefore, both MSC- and paricalcitol-mediated inhibition of primary Th17 differentiation occurred independently of APCs.

Paricalcitol and MSCs suppress Th17 cells, but only MSCs inhibit expression of Th17-associated transcription factors via PGE2. We recently demonstrated the requirement for cyclooxygenase (COX)2 induction and PGE2 production in MSC-mediated suppression of primary Th17 differentiation (11). As previously observed, the selective COX2 inhibitor NS-398 (Sigma-Aldrich) reversed MSC-induced inhibition of IL-17A in day 3 culture supernatants but had minor or no effects on paricalcitol-induced inhibition of IL-17A production (Fig. 5A). Restimulation of T cells for 8 h in the presence of anti-CD3/anti-CD28-coated beads showed that addition of NS-398 to MSC/Th17 cocultures reversed the suppressive effect of MSCs (Fig. 5, B and C). NS-398 was not associated with a reversal of IL-17A suppression when CD4⁺ T cells were cultured with paricalcitol alone (Fig. 5, B and C). Similar experiments were performed using L-161,982, a selective EPa antagonist (Cayman Chemicals), which has been previously shown to prevent MSC-mediated inhibition of primary Th17 differentiation (11), with comparable results (data not shown), confirming that paricalcitol-induced suppression of Th17 differentiation occurs independently of COX2/PGE2.

Next, CD4⁺ T cells were cocultured for 3 days in the presence of APCs under Th17-skewed conditions with or without MSCs (1:200) and/or paricalcitol (10 nM). CD4⁺ T cells were repurified and analyzed by immunoblot analysis for the level of expression of VDR and known intracellular mediators of the Th17 differentiation pathway. Predictably, VDR expression was increased in the presence of paricalcitol (Fig. 6A). Consistent with current understanding, phosphorylated STAT3, retinoic acid receptor-related orphan receptor-γ (RORγt), interferon regulatory factor 4 (IRF4), and unphospho-related transcription factor 1 (Runx1) were highly induced during Th17 differentiation (Fig. 6, B–F). Of interest, neither MSCs, paricalcitol, nor the combination of the two affected total STAT3 or STAT3 phosphorylation. In contrast, expression of the transcription factors RORγt, IRF4, and Runx1 were modestly but reproducibly lower in the presence of MSCs (Fig. 6, C–F), whereas paricalcitol, either alone or in combination with MSCs, had no effect on expression of these transcription factors. Thus, while T cell contact with MSCs resulted in reduced expression of multiple transcription factors required for Th17 differentiation, the additional Th17 suppression mediated by paricalcitol occurred independently of this effect.

Combined administration of MSCs and paricalcitol attenuates early T cell accumulation in the obstructed kidney. To determine the combined effect of MSCs and paricalcitol to

Fig. 3. Paricalcitol and MSCs additively suppress IL-17A production by naïve and memory T cells. Magnetic-activated cell sorting (MACS)-enriched CD4⁺ T cells were further purified by fluorescence-activated cell sorting into naïve (CD25⁻/CD62L⁻) and memory (CD25⁻/CD62L⁺) T cells. T cells were separately activated under Th17-skewed conditions for 3 days in the presence of paricalcitol (10 nM) or an equivalent volume of vehicle with or without MSCs (1:400 ratio). Cells were restimulated for 8 h with anti-CD3/anti-CD28-coated beads. A and D: concentrations of IL-17A in day 3 culture supernatants. B and E: flow cytometric analysis of the percentage of IL-17A⁺ cells among cells gated on CD4⁺ after restimulation. C and F: representative examples of contour plots of intracellular IL-17A staining by restimulated CD4⁺ T cells. Data are representative of three individual experiments. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 compared with the equivalent vehicle control group (by Bonferroni posttest).
Naive-phenotype responders:

Day 3 Th17 culture:
IL-17A concentration

![Graph showing IL-17A concentration for day 3 Th17 culture with and without MSCs and Paricalcitol.](image)

Memory-phenotype responders:

Day 3 Th17 culture:
IL-17A concentration

![Graph showing IL-17A concentration for day 3 Th17 culture with and without MSCs and Paricalcitol.](image)

Re-activated Th17 culture:
% IL-17A+ among CD4+ T-cells

![Graph showing % IL-17A+ among CD4+ T-cells for re-activated Th17 culture with and without MSCs and Paricalcitol.](image)

Re-activated Th17 culture:
% IL-17A+ among CD4+ T-cells

![Graph showing % IL-17A+ among CD4+ T-cells for re-activated Th17 culture with and without MSCs and Paricalcitol.](image)

Naive-phenotype responders:

![Flow cytometry plots for IL-17A expression among CD4+ T-cells with and without MSCs and Paricalcitol.](image)

Memory-phenotype responders:

![Flow cytometry plots for IL-17A expression among CD4+ T-cells with and without MSCs and Paricalcitol.](image)
Re-activated Th17 culture: CD25 surface expression

A

B

Vehicle
Paricalcitol

Vehicle
Paricalcitol

Fig. 4. Combined effects of MSCs and paricalcitol. CD4+ T cells were cultured for 3 days under Th17-skewed conditions in the presence of paricalcitol (10^{-8} M) or an equivalent volume of vehicle with or without MSCs before 8 h restimulation with anti-CD3/anti-CD28-coated beads. A: surface expression of CD25 on CD4+ T cells after restimulation. B: surface expression of (C-C motif) receptor (CCR)6 on CD4+ T cells after restimulation. Data are representative of four individual experiments. **P ≤ 0.01 and ***P ≤ 0.001 compared with equivalent vehicle control group (by Bonferroni post-test).

Control and obstructed kidneys were dissected and digested, and single cell suspensions prepared before multicolor flow cytometry on day 3 after UUO. Total leukocytes (CD45+) were reduced in obstructed kidneys of MSC and paricalcitol double-treated mice (group 4) on day 3 compared with untreated animals (Fig. 7A). Obstructed kidneys from group 4 also contained lower proportions of CD4+T cell receptor (TCR)-β+ , CCR6+/CD4+, and CD8+/TCR-β+ T cells (Fig. 7, B–D). Mice that received both MSCs and paricalcitol had a significant reduction in detectable IL-17A transcripts in obstructed kidneys compared with the no treatment group (Fig. 7E). Neither of the two groups that received single treatment alone was statistically different from the untreated group (Fig. 7). Thus, coadministration of MSCs and paricalcitol attenuated overall early T cell accumulation, including CCR6+/CD4+ T cells, in the obstructed kidney after UUO and was associated with reduced Th17 activity within the kidney at the same time point.

Given the apparent lack of intrarenal T cell-suppressive effect of MSCs alone, the localization of MSCs to the obstructed kidney and other organs 24 h after the second intravenous administration in this model was examined using flow cytometry and immunofluorescence microscopy to detect PKH26-labeled MSCs. In keeping with the results reported in other models of AKI (15, 30, 45), labeled MSCs were detected in the lungs but were not clearly detectable within the obstructed kidneys by flow cytometry (Fig. 8A). In addition, no PKH26+ MSCs were detected in the liver, heart, spleen, or unobstructed kidney (data not shown). By immunofluorescence microscopy, occasional PKH26+ cells were observed in the interstitial space of the obstructed kidney of MSC-treated mice (Fig. 8B). No such cells were seen in control or obstructed kidneys of vehicle-treated mice.

Combined MSC and paricalcitol administration is associated with reduced tubular injury and fibrosis and with predominance of CD206+ (M2) macrophages. The impact of MSCs and/or paricalcitol on overall kidney structure was examined on day 8 after UUO. Histologically (PAS stain), interstitial cellular infiltration was not suppressed in obstructed kidneys by MSC and/or paricalcitol administration (Fig. 9A). However, tubular atrophy and dilation (H&E stain; Fig. 9B), interstitial fibrosis (Masson’s trichrome stain; Fig. 9C) and overall kidney injury scores (Fig. 9D) were reduced in obstructed kidneys from double-treated mice compared with the no treatment group. Neither of the two groups that received single treatment alone had significantly reduced tubular atrophy and dilation, interstitial fibrosis, or overall injury score compared with the no treatment group (Fig. 9).

Using flow cytometry, the pan myeloid marker CD11b was then used to enumerate total myeloid cells as well as myeloid cell subsets, including neutrophils [CD11b+/lymphocyte antigen (Ly)6G+], dendritic cells (CD11b+/CD11c+), inflammatory monocytes (CD11b+/Ly6C+/F4/80+), M1 proinflammatory macrophages (CD11b+/Ly6C+/F4/80+/CD206−), and M2 anti-inflammatory macrophages (CD11b+/Ly6C+/F4/80+/CD206+). All three treatment groups had significantly lower proportions of neutrophils (Fig. 10A) compared with the no treatment group. Inflammatory monocytes were significantly reduced in group 4 (Fig. 10B). Obstructed kidneys from group 4 also had increased CD206+ (M2) and decreased CD206− (M1) macrophages (Fig. 10, C and D). Paricalcitol alone treatment also significantly reduced the percentage of infiltrating M1 macrophages compared with the no treatment group (Fig. 10C). The proportions of dendritic cells in obstructed kidneys were not reduced by MSCs and/or paricalcitol on day 8 after UUO (data not shown). Thus, compared with no treatment or single-agent treatments, combined MSC and paricalcitol administration significantly altered the proportionate representation of individual myeloid cell phenotypes within CD11b+ populations of obstructed kidneys. This included reduced proportions of neutrophils and inflammatory monocytes with a concomitant increase in CD206+ (M2) macrophages in association with reduced interstitial fibrosis and tubular atrophy and dilation.

**DISCUSSION**

In the present study, our initial in vitro experiments provided proof-of-principle evidence that suppression of Th17 differentiation by MSCs (a known immunomodulatory property of these therapeutic stem cells) is further enhanced in the presence of the clinically available vitamin D analog paricalcitol. Subsequently, in the well-established UUO model of acute, kidney-
specific sterile inflammation, we observed that the combined administration of MSCs and paricalcitol resulted in reduced intrarenal Th17 activity along with other potentially beneficial modulations to T cell and myeloid cell infiltrates of obstructed kidneys. These immune modulatory effects of combined treatment were not observed in vivo for either MSCs or paricalcitol alone.

From a mechanistic perspective, we and others have previously reported that induced production of PGE2 by MSCs in direct contact with CD4+ T cells undergoing primary activation is chiefly responsible for the suppressive effects on Th17 cells in vitro (3, 11, 16, 43). In the present study, consistent with reports that have examined the immunomodulatory properties of multiple vitamin D analogs (4, 22, 27, 31), paricalcitol was also associated with dose-dependent inhibition of IL-17A production by CD4+ T cells undergoing primary Th17 induction and restimulation, although this effect was independent of the PGE2/COX2 pathway. Of interest, while paricalcitol ex-

Fig. 5. Paricalcitol-mediated inhibition of primary Th17 differentiation does not require cyclooxygenase-2 enzymes. CD4+ T cells were cultured for 3 days under Th17-skewed conditions with or without MSCs and/or paricalcitol. NS-398 (1 μM) was added to the primary culture or equivalent volume of DMSO vehicle. A: concentration of IL-17A in the supernatant on day 3 (the minor reversal of paricalcitol-induced IL-17A suppression by NS-398 shown here was not observed in replicate experiments). B: graphical representation showing the frequency of IL-17A+ cells among cells gated on CD4+. C: representative contour plots of intracellular IL-17A staining after restimulation for 8 h. Data are representative of two individual experiments. *P < 0.05 and ***P < 0.001 compared with the equivalent vehicle (DMSO) control group (by Bonferroni posttest).
Fig. 6. Distinct effects of MSCs and paricalcitol on intracellular T cell mediators. CD4+ T cells were cultured in the presence or absence of MSCs and/or paricalcitol or ethanol vehicle for 3 days under Th17-skewed conditions. CD4+ T cells were repurified by MACS and subjected to Western blot analysis. MSCs cultured alone for 3 days and primary CD4+ splenic T cells were also analyzed. A–E: data are representative of two individual Western blots for the vitamin D receptor (VDR) and phosphorylated (p)STAT3, in which protein from two individual culture experiments were examined, and three Western blots for retinoic acid receptor-related orphan receptor-γ (RORγt), interferon regulatory factor 4 (IRF4), and runt-related transcription factor 1 (Runx1). Spot densitometry was performed on a Fluorochem chemiluminescent imaging system. Target bands were normalized to their respective β-actin bands. F: all samples were compared relative to the Th17 vehicle control group, which was appointed a spot densitometry value of 1.
hibited mild antiproliferative effects on T cells during Th17 differentiation, it potently suppressed their CCR6 expression, an effect that may be of clinical relevance in Th17-mediated diseases as Th17 cells require CCR6 for recruitment to sites of inflammation (14, 46). In these in vitro experiments, we observed that Th17 suppression begins to be manifest between concentrations of $10^{-9}$ and $10^{-8}$ M (equivalent to 0.42–4.2 ng/ml). Available information from human subjects receiving parenteral administration of clinically relevant doses of paricalcitol indicates that peak plasma concentrations are in the range of 0.25–1.25 ng/ml (17). Thus, the Th17-suppressive effects occur within a concentration range close to that achievable as a peak with intravenous bolus therapy in humans (although safe achievement of a level of $10^{-8}$ M is questionable). While we did not directly measure plasma paricalcitol concentrations in the treated mice in our experiments, the dosing regimen used has been documented by others not to be associated with hypercalcemia or other toxicity (43) and did, clearly, have specific immunomodulatory effects alone and in combination with MSC administration.

In the case of MSC-mediated inhibition of primary Th17 differentiation, we now show that protein levels of IRF4, ROR$^\gamma t$, and Runx1 were reduced in Th17 cells in the presence of MSCs, suggesting that PGE2/EP4 signaling is associated with the suppression of multiple key transcription factors. This is consistent with the findings of Valdez et al. (47), who demonstrated suppression of mRNA levels of IRF4 and downstream ROR$^\gamma t$ and IL-17A in mouse CD4$^+$ T cells undergoing primary Th17 differentiation in the presence of PGE2. In contrast to the influence of MSCs, paricalcitol-mediated suppression of Th17 differentiation was associated with increased expression of VDR, but this was not accompanied by detectable suppression of Th17 differentiation pathway transcription factors or the STAT3 signaling pathway. Other researchers have similarly ruled out a role for IL-2, STAT1, IL-10, apoptosis, STAT4, IL-21, and T-bet in vitamin D-mediated inhib-
bition of primary Th17 responses (5, 27, 31), and Joshi et al. (22) showed that Runx1 is sequestered by VDR, thus preventing it from binding as a coactivator to the IL-17A promoter. Significantly, paricalcitol and MSC-mediated inhibition of Th17 differentiation was additive for both naïve and memory phenotype responders and was independent of APCs. Taken together with previously published mechanistic studies (5, 11, 22, 27, 31, 47), our results are consistent with separate, direct effects of MSCs and paricalcitol on responder T cells. Nevertheless, we cannot rule out the possibility that paricalcitol may also indirectly affect Th17 differentiation by modulating MSCs, which, as we have shown, also express VDR. To date, however, the only reported effects of active vitamin D compounds on MSCs relate to osteogenic differentiation (37).

In regard to in vivo effects, combined administration of MSCs and paricalcitol in UUO attenuated overall early T cell accumulation and was associated with reduced intrarenal CD8+ T cells, CD4+ T cells, CCR6+/CD4+ T cells, and IL-17A transcript. This was accompanied by reduced proportions of neutrophils in the obstructed kidney, a finding that is
10-day UUO, in which collagen deposition and have been reported to play a protective role after reversal of day 3 significant differences between these two groups at both time and paricalcitol groups, statistical comparisons (not included in individual animals in the paricalcitol alone and combined MSC indexes examined, there was an overlap between the results for citol. Although we must acknowledge that, for the various double-treated group could be entirely attributable to paricalcitol. Despite this seemingly unfavorable distribution, however, there is clear convincing precedent for MSCs, delivered intravenously in similar numbers to those we have used in this study, to mediate tissue protective effects from a distant site (particularly the lungs) via their own secreted factors and/or via reprogramming of immune cells encountered there (19). For example, in the studies cited above, intravenously administered MSCs have been shown to induce the systemic production of the anti-inflammatory cytokine IL-10 by lung-resident monocyte/macrophages in models of sepsis (30) and renal ischemia-reperfusion also consistent with inhibition of Th17-type response (46). At a later time point, additional modulatory effects on intrarenal myeloid cell infiltration were observed in the combined treatment group, which included both a reduction of potentially destructive populations, including neutrophils, inflammatory monocytes, and M1 macrophages, and enhancement of CD206+ M2 macrophages. Significantly, M2 macrophages have been reported to play a protective role after reversal of 10-day UUO, in which collagen deposition and α-smooth muscle actin expression reduced over time in association with increased ratios of M2 markers (CD206 and CD204) to CD68 (23).

The lack of efficacy of MSCs and paricalcitol when administered alone in UUO stands in contrast to some previously published studies (1, 25, 26, 40–42). The explanation for this may partly lie in rodent strain variability and/or heterogeneity of cultured mouse MSCs (19). Given that we observed primary localization of intravenously administered MSCs to the lungs with minimal migration to the obstructed kidney, it is reasonable to ask whether the intrarenal effects observed in the double-treated group could be entirely attributable to paricalcitol. Although we must acknowledge that, for the various indexed examined, there was an overlap between the results for individual animals in the paricalcitol alone and combined MSC and paricalcitol groups, statistical comparisons (not included in the relevant graphs for the purposes of clarity) indicated significant differences between these two groups at both time points [day 3 CD4+ T cells (P < 0.01), day 8 total histological score (P < 0.01), and day 8 CD206+ (M2) macrophages (P < 0.05)]. Overall, trends were in favor of a stronger immunomodulatory/anti-inflammatory effect in the double-treated group compared with the paricalcitol alone group for 11 of 14 flow cytometric/histological indexes that were quantified. Furthermore, for 10 of 14 indexes, the result for the double-treated group was the only one that proved to be significantly different from the untreated control group. Thus, we believe it is reasonable to conclude that the combination of MSCs and paricalcitol exerted immunomodulatory effects that did not occur with paricalcitol alone.

In this regard, the mechanism by which MSCs may have exerted an additive effect on intrarenal inflammation despite minimal localization to the obstructed kidney also merits discussion. MSC entrapment in the lung has been well described in rodent disease models (19) and has been recently reported in mouse and rat models of AKI (15, 30, 45). Furthermore, in keeping with our own results, tracking studies (13, 30) have indicated that MSCs trapped in the lung after intravenous injection have a short life span (≤24 h). Despite this seemingly unfavorable distribution, however, there is clear convincing precedent for MSCs, delivered intravenously in similar numbers to those we have used in this study, to mediate tissue protective effects from a distant site (particularly the lungs) via their own secreted factors and/or via reprogramming of immune cells encountered there (19). For example, in the studies cited above, intravenously administered MSCs have been shown to induce the systemic production of the anti-inflammatory cytokine IL-10 by lung-resident monocyte/macrophages in models of sepsis (30) and renal ischemia-reperfusion

Fig. 9. Histological analysis of control and obstructed kidneys after 8 days of UUO. A–D: graphical representation of cellular infiltration scores by periodic acid-Schiff (PAS) staining (A), atrophy and dilation scores by hematoxylin and eosin (H&E) staining (B), interstitial fibrosis scores by Masson’s trichrome staining (C), and total cumulative histological scores (D). Scores were as follows: 1 = <3% of the field, 2 = 3–10% of the field, 3 = 10–25% of the field, and 4 = >25% of the field in A and C and 1 = <10% of the field, 2 = 10–25% of the field, 3 = 25–50% of the field, and 4 = >50% of the field in B. Data are presented as control and obstructed kidneys from individual animals with n = 5 mice/group. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 compared with the equivalent control kidneys; †P ≤ 0.05 and ††P ≤ 0.01 compared with the equivalent no treatment group (by Bonferroni posttest).
injury (45) and have recently been shown to promote anti-inflammatory (M2) macrophage accumulation in the kidney during rhabdomyolysis despite the lack of MSC localization within the kidney (15). Although not directly investigated, we posit that a similar in vivo mechanism, albeit of limited potency in isolation, explains the additive immunomodulatory effect of intravenous MSCs in combination with paricalcitol in the experiments reported here.

Our primary rationale for studying the UUO model was that we and others have extensively documented the occurrence of a localized influx and activation of Th17 cells in the obstructed kidney along with recruitment/activation of other proinflammatory T cell and myeloid cell populations (10, 18, 42). From a clinical perspective, obstructive nephropathy is the most common reason for renal failure in children and is a frequent cause of acute and chronic renal failure in adults (18). The most significant disadvantage of the UUO model with regard to assessing the therapeutic value of novel interventions in human AKI is the lack of direct information on renal clearance function. Thus, our results cannot be extrapolated to a conclusion regarding the preservation or recovery of glomerular filtration rate after obstructive nephropathy. Nonetheless, others have demonstrated beneficial effects of MSCs and vitamin D on renal functional indexes in various other models of AKI, including ischemia-reperfusion injury (24, 44), cisplatin-induced acute renal failure (2, 29), glycerol-induced AKI (15), cyclosporine-induced kidney injury (33), and gentamicin-induced kidney injury (34).

In conclusion, as combination therapy with MSCs and paricalcitol was more efficacious than either of the individual treatments in specifically inhibiting the proinflammatory Th17 differentiation pathway as well as broadly modulating a complex destructive inflammatory response in vivo, we conclude that adjunctive therapy with VDR agonists may represent a strategy for safely consolidating and enhancing the immunosuppressive properties of MSCs in the setting of diverse inflammatory diseases.

DISCLOSURES

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AUTHOR CONTRIBUTIONS


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