EphB and Ephrin-B Interactions Mediate Human Mesenchymal Stem Cell Suppression of Activated T-Cells

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Mesenchymal stromal/stem cells (MSC) express the contact-dependent erythropoietin-producing hepatocellular (Eph) receptor tyrosine kinase family and their cognate ephrin ligands, which are known to regulate thymocyte maturation and selection, T-cell transendothelial migration, activation, co-stimulation, and proliferation. However, the contribution of Eph/ephrin molecules in mediating human MSC suppression of activated T-cells remains to be determined. In the present study, we showed that EphB2 and ephrin-B2 are expressed by ex vivo expanded MSC, while the corresponding ligands, ephrin-B1 and EphB4, respectively, are highly expressed by T-cells. Initial studies demonstrated that EphB2-Fc and ephrin-B2-Fc molecules suppressed T-cell proliferation in allogeneic mixed lymphocyte reaction (MLR) assays compared with human IgG-treated controls. While the addition of a third-party MSC population demonstrated dramatic suppression of T-cell proliferation responses in the MLR, blocking the function of EphB2 or EphB4 receptors using inhibitor binding peptides significantly increased T-cell proliferation. Consistent with these observations, shRNA EphB2 or ephrin-B2 knockdown expression in MSC reduced their ability to inhibit T-cell proliferation. Importantly, the expression of immunosuppressive factors, indoleamine 2, 3-dioxygenase, transforming growth factor- β 1, and inducible nitric oxide synthase expressed by MSC, was up-regulated after stimulation with EphB4 and ephrin-B1 in the presence of interferon (IFN)- γ , compared with untreated controls. Conversely, key factors involved in T-cell activation and proliferation, such as interleukin (IL)-2, IFN- γ , tumor necrosis factor- α , and IL-17, were down-regulated by T-cells treated with EphB2 or ephrin-B2 compared with untreated controls. Studies utilizing signaling inhibitors revealed that inhibition of T-cell proliferation is partly mediated through EphB2-induced ephrin-B1 reverse signaling or ephrin-B2-mediated EphB4 forward signaling by activating Src, PI3Kinase, Abl, and JNK kinase pathways, activated by tyrosine phosphorylation. Taken together, these observations suggest that EphB/ephrin-B interactions play an important role in mediating human MSC inhibition of activated T cells.

Introduction

MULTIPOTENTIAL HUMAN BONE marrow-derived mesenchymal stromal/stem cells (MSC) exhibit immunomodulatory properties that are capable of restraining allogeneic reactions [1–3] due to lack of expression of MHC class II antigens and co-stimulatory molecules such as CD40, CD80, CD86, or CD40L [4–8]. As a result, MSC are unable to trigger T-cell activation but rather act as a third-party population to inhibit allostimulated T-cell proliferation [1,3]. These immunosuppressive properties have been reported to be mediated by different soluble factors such as hepatocyte growth factor (HGF), prostaglandin E2 (PGE₂), transforming

growth factor- β 1 (TGF- β 1), indoleamine 2,3-dioxygenase (IDO), interleukin-10 (IL-10), nitric oxide (NO), and the contact-dependent B7-H1/PD-1 pathway [1,2,9,10]. While some of these factors partially contribute to the immunomodulatory properties of MSC, the exact underlying mechanisms that regulate MSC-mediated immune cell action remain to be elucidated.

Erythropoietin-producing hepatocellular (Eph) receptors, the largest family of cell membrane-bound receptor tyrosine kinases, regulate many biological processes by interacting with their cognate ligands, termed ephrins [11–13]. Many reports have shown that Eph/ephrin molecules are involved in MSC-mediated cell attachment, migration, and

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differentiation [14–17]. The Eph receptor family is subdivided into two subclasses, A and B, based on their binding affinity to their cognate ephrin ligands. EphA receptors (A1–8) generally bind to ephrin-A ligands (A1–5) and EphB receptors (B1–6) bind to ephrin-B ligands (B1–3), with exceptions of EphA4, which can bind to ephrin-B ligands and ephrin-A5 binding to EphB2. It is known that Eph and ephrin molecules are highly redundant and their interactions are promiscuous [12,18,19]. Both the Eph receptor and the ephrin ligand can conduct downstream signaling on activation, where forward signaling refers to signaling through the Eph receptor while signalling via the ephrin ligand is termed reverse signaling. In many cases, both forward and reverse signaling can occur simultaneously, which is known as bidirectional signaling [12,20,21].

Studies have shown that Eph/ephrin molecules play an important role in the development and function of immune cells [22–26]. However, the contribution of Eph/ephrin molecules during T-cell activation and proliferation remains controversial. Many reports indicate that Eph/ephrin molecules of both subclasses suppress T-cell function. For instance, ephrin-A1 reverse signaling has been shown to suppress T-helper-2-cell activation and inhibit activated CD4⁺ T-cell proliferation [27]. This is potentially mediated by ephrin-A activation of Src-family kinases, Akt phosphorylation, and inhibition of antigen receptor-induced apoptosis of T-cells [28]. Under pathological conditions, ephrin-A1 suppresses T-cell activation and Th2 cytokine expression, while preventing activation-induced cell death in asthma patients [27]. Conversely, some reports demonstrate that Eph/ephrin molecules stimulate T-cell functions. For instance, the interaction between EphB6/ephrin-B2 enhances T-cell responses to antigens by *in vitro* TCR stimulation [29], as EphB6^{-/-} T-cells are defective in their response to TCR stimulation *in vitro* and *in vivo* [23,30,31]. Moreover, ephrin-B1 is crucial in T-cell/T-cell cooperation in response to antigen stimulation [32], while ephrin-B2 and ephrin-B3 play major roles in T-cell co-stimulation [33], by enhancing T-cell signaling [31]. In rheumatoid arthritis, EphB1/ephrin-B1 signaling affects the population and function of CD3⁺ T-cells, resulting in enhanced lymphocyte migration [34]. While the data relating to the contribution of Eph/ephrin interactions to the development of T-cell effector functions are conflicting, a recent study showed that the involvement of ephrin-B1 and ephrin-B2 in T-cell proliferation is dose dependent [35]. Here, it was shown that at a low dose, ephrin-B1 and ephrin-B2 enhanced CD3-mediated murine T-cell proliferation. However, they suppressed proliferation at a higher dose, thought to be by potentially phosphorylating EphB receptors, resulting in the recruitment of SH1P, a phosphatase that suppresses the Lck phosphorylation [35]. Therefore, substantial evidence suggests that Eph/ephrin signaling may regulate T-cell activation and proliferation. Given that EphB/ephrin-B molecules, involved in the regulation of MSC, play a critical role in T-cell development and function under normal and pathological conditions, it is plausible that EphB/ephrin-B interactions are important for MSC-mediated suppression of T-cell proliferation. In the present study, we examined the potential role of EphB/ephrin-B interactions in mediating human MSC inhibition of activated T-cells. We found that EphB2 and ephrin-B2, expressed by MSC, suppress T-cell proliferation.

Materials and Methods

Cell culture of human MSC

Human MSC were purified by STRO-1 antibody immunoselection from bone marrow mononuclear cells prepared from normal human bone marrow aspirates as previously described [4,5,36]. Primary cultures were established by culturing $5 \times 10^4/\text{cm}^2$ STRO-1⁺ mononuclear cells in alpha modification of Eagle's medium (α MEM), containing 20% (v/v) fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 μ M L-ascorbate-2-phosphate and penicillin (50 i.u./mL)/streptomycin sulfate (50 μ g/mL), at a 37°C humidified atmosphere in the presence of 5% CO₂. On achieving 90% confluence, adherent cells were detached by a treatment with trypsin/EDTA (JRH Biosciences), counted, and then replated at a density of $8 \times 10^3/\text{cm}^2$, in α MEM growth medium supplemented with 10% fetal calf serum (FCS).

Isolation of peripheral blood mononuclear cells and T-cells

Human peripheral blood mononuclear cells (PBMNC) were isolated as previously described [3]. Human T-cells were prepared from PBMNC suspensions by positive magnetic-activated cell sorting using anti-CD3 magnetic beads (CD3 microbead kit; Miltenyi Biotec) as per manufacturer's instructions. Purity of CD3⁺ T-cells was always >95% as determined by flow cytometry using FITC-conjugated anti-CD3 antibody (BD BioSciences).

Real-time-polymerase chain reaction

Gene expression analysis from human culture-expanded MSC and CD3⁺ T-cells using real-time (RT) quantitative polymerase chain reaction (PCR) were performed as previously described [14]. Briefly, total RNA was isolated using the Trizol (Invitrogen Life Technologies) according to the manufacturer's instruction. Total RNA was then subject to reverse transcription using Oligo dT primer and Superscript III reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's protocol. Synthesized cDNA (1:100 dilution) was used for quantitative RT-PCR. RT-PCRs were performed using SyberGreen PCR Master Mix (SA-Biosciences) and Rotorgene 3000 series (Corbett Life Sciences). EphB, ephrin-B, INF γ , IDO, HGF, and TGF- β 1 primer sets were used as previously described [14,37]. Other primer sequences (0.05 μ M; Geneworks) are indicated in Table 1. The reactions for each sample were performed in triplicate. Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method relative to β -actin CT values.

Immunoblotting and immunoprecipitation

Cell lysates were prepared as previously described [14], and equivalent amounts of protein lysates (40 μ g) were resolved on 8% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequently transferred to polyvinylidene difluoride membranes. Immunoblotting was performed as previously described [14] with the following antibodies: Goat anti-EphB2, goat anti-ephrin-B1, goat anti-EphB4 (R&D Systems), or rabbit anti-ephrin-B2 (Santa Cruz Biotech), followed by alkaline phosphatase-conjugated secondary antibodies (Millipore) of

TABLE 1. PRIMER SEQUENCE

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
IL-10	TCAAGGCGCATGTGAACTC	GATGTCAAACCTCACTCATGGCT
iNOS	CCTTACGAGGCGAAGAAGGACAG	CAGTTTGAGAGAGGAGGCTCCG
PGE ₂	CCTCTTCCCGAAAGGAAAAAT	GACTGAACGCATTAGTCTCAGAAC
IL-2	CCTGTCTTGCATTGCACTAAG	CATCCTGGTGAGTTTGGGATT
IL-17	AGATTACTACAACCGATCCACCTC	GGGGACAGAGTTTCATGTGGTA
IL-4	ATGGGTCTCACCTCCCAACT	GATGTCTGTTACGGTCAACTCG
TNF- α	TGCTTGTTCCCTCAGCCTCTT	GCTGGTTATCTCTCAGCTCCA
EphA1	CAAGGACGCAGAGACACTGA	TCTCGAATGGTGAAGCTCTG
EphA2	TGGTACTGCTGGACTTTTGCTG	GTCGCCAGACATCACGTTG
EphA3	TTGCCAAGGAATTGGATGC	AGCCAACCTTCAGGGTCTTAATG
EphA4	GGCTCAGAGGGTGTATATTGAGA	TGAAACGCTCTTTGTCGTTGT
EphA5	TGGGAAGAGATTGGTGAAGTG	CATCGGCAGCAATGGTATC
EphA6	AGGTGTGGTAAGGAAGGACTGG	GCTTTGGACCTTGTGGAAGAGT
EphA7	TACCCCGATACGAACATACCAG	AGGAAGACTGTTACAATCCCTCA
EphA8	CCTGGCAATGATTGTGACTG	CATAGCCCAGGTCTGAGAGGTA
Ephrin-A1	CCCCAGTCCAAGGACCAA	GATTTTGCCACTGACAGTCACC
Ephrin-A2	AGAAGTTCCAGCTCTTCACGC	CGGGCTGCTACACGAGTTAT
Ephrin-A3	TCTCTGGGCTACGAGTTCCAC	CAGCACGTTGATCTTCACATTG
Ephrin-A4	GAGTTCTGGCCAGTGCTTGA	AATTCGCAGAAGACGAAGAATC
Ephrin-A5	GTTGACGCTGGTGTCTTCTGG	ATGGTAGTCACCCCTCTGGAAT

IL, interleukin; PGE₂, prostaglandin E₂; TNF- α , tumor necrosis factor- α ; iNOS, inducible nitric oxide synthase.

either anti-goat IgG (Millipore) or anti-mouse IgG (Chemicon) or anti-rabbit IgG (Chemicon). Membranes were developed with enhanced chemifluorescence substrate (GE Healthcare). Quantitative analysis was performed using ImageQuant software (GE Healthcare). To control for protein loading, the blots were stripped in stripping buffer (Western Blot Recycling Kit; Alpha Diagnostic Intl. Inc) for 15 min at room temperature and re-probed with mouse anti-human β -actin (Sigma).

Immunoprecipitation assays were performed as previously described [35]. Briefly, T-cells (1×10^6) were seeded with preclustered EphB2-Fc or ephrin-B2-Fc or human-Fc control ($1 \mu\text{g}/\text{mL}$; R&D Systems) onto precoated tubes, centrifuged at 350 rpm for 3 min, and incubated at 37°C and 5% CO₂ for 2 h. Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and lysed in RIPA lysing buffer (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 150 mM NaCl, 1% NP40, 2 mM sodium vanadate, 2 mM sodium fluoride, 0.1% SDS, 10 mM sodium pyrophosphate, 10% glycerol, and 0.5% deoxycholate; Roche inhibitors). Total cell lysates (100 μg) were incubated with anti-PY antibody (clone 4G10; Millipore) and ChIP-Grade Protein-G magnetic beads (Cell Signalling Technology) for 18 h at 4°C. The immunoprecipitates were washed with immunoprecipitation buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 150 mM NaCl, 1% NP40, 2 mM sodium vanadate, 2 mM sodium fluoride, 10 mM sodium pyrophosphate, and 0.5% deoxycholate; Roche inhibitors). Mouse monoclonal IgG₁ (Millipore) was used as the negative control. Membranes were incubated with goat anti-EphB4 or goat anti-ephrin-B1 ($0.1 \mu\text{g}/\text{mL}$; R&D Systems) to detect phosphorylation.

T-cell proliferation assay

T-cell proliferation experiments were performed using allogeneic mixed lymphocyte reaction (MLR) assay in vitro

[3]. T-cells (5×10^4 cells/well) were incubated with allogeneic PBMNC (5×10^4 cells/well), which were inactivated by γ -irradiation (30 Gy, blood irradiator with a ¹³⁷Cs γ -irradiation source at 378 cGy/min) in the presence of Fc-fusion proteins ($1 \mu\text{g}/\text{mL}$): Human-IgG-Fc (Rockland Immunochemicals), EphB2-Fc, or ephrin-B2-Fc (R&D Systems). The fusion proteins were preclustered with a 10-fold concentration of Human anti-Goat IgG ($10 \mu\text{g}/\text{mL}$) in PBS for 1 h at room temperature. Ninety-six-well round-bottomed plates were coated with poly-L-lysine (0.01% solution; Sigma) for 5 min at room temperature. The preclustered Fc-fusion proteins were added to the coated wells for 2 h at 37°C in a 5% CO₂ incubator, then washed twice with PBS. The cells were co-cultured at a total of 200 μL culture medium per well, for 5 days at 37°C humidified atmosphere in the presence of 5% CO₂. T-cell proliferation was evaluated by incubating cells with [³H]-thymidine (Perkin Elmer) of $0.4 \mu\text{Ci}/\text{well}$ for the final 16 h. Cells were then harvested onto glass filter membranes (Packard) using a cell harvester (Filtermate; Packard). Thymidine incorporation was measured using the scintillation counter (Top Count NXT; Perkin Elmer) Top Count NTX program (v 2.53). KaryoMax colcemid solution ($0.1 \mu\text{g}/\text{mL}$; Gibco Invitrogen), an inhibitor of T-cell proliferation, was used as a negative control. All experiments were performed in five replicates.

In experiments, to determine the gene expression level of soluble factors produced by MSC, irradiated MSC (2.5×10^5 cells, 30 Gy) were seeded in a 24-well flat-bottomed plate and incubated at 37°C and 5% CO₂ for 24 h. Cells were then stimulated with or without interferon (IFN)- γ ($25 \text{ ng}/\text{mL}$; Peprotech, Inc.) and EphB4-Fc or ephrin-B1-Fc or human-Fc control ($5 \mu\text{g}/\text{mL}$; R&D Systems) and incubated at 37°C and 5% CO₂ for 24, 48, or 72 h. Cells were then harvested for RT-PCR analysis. For experiments to determine soluble factors produced by T-cells, T-cells were allo-stimulated in MLR assay in 96-well round-bottomed plates precoated with

preclustered EphB2-Fc or ephrin-B2 or human-Fc control (1 µg/mL; R&D Systems) and incubated at 37°C and 5% CO₂ for 24 or 48 h. Cells were then harvested for RT-PCR analysis.

For EphB receptor blocking experiments, allogeneic human MSC (5×10^4 cells/well) were added to the MLR as a third-party population. These MSCs were liberated by Trypsin-EDTA treatment (JRH Biosciences), resuspended in culture medium, inactivated (30 Gy, γ -irradiation), plated onto 96-well flat-bottomed plates, and incubated overnight at 37°C in 5% CO₂ before being used in the MLR. To block the function of EphB2 or ephrin-B2, expressed by MSC or EphA4, expressed by T-cells, cells were preincubated with 100 µM blocking peptides that were specific for EphB2 (SNEWILPRLPQH), EphB4 (TNYLFSPNGPIARAW), EphA4 (VTMEAINLAFFG), or scramble control peptide (RTVAHHGGLYHTNAEVK) (Mimotopes) for 1 h at 37°C in a 5% CO₂ incubator, before being added to the MLR. To block the function of ephrin-B1, T-cells were preincubated with 50 µM ephrin-B1 blocking peptide (PTD-EFNB1-C; GRKKRRQRRRPPQGGGVQEMPPQSPANIYYKV) or scramble control peptide (PTD-Scram; GRKKRRQRRRPPBGGG-EISKPMYPQVQVYPNA) (Mimotopes) for 1 h at 37°C in 5% CO₂. The cells were then added to the MLR in the presence of plates precoated with EphB2-Fc or human-Fc control (1 µg/mL).

Inhibitor assays

T-cells were incubated for 30 min with the following pharmacological signaling inhibitors: PD184352 (an MEK inhibitor, 1 µM, obtained from Phillip Cohen's laboratory); PP2 (a Src-family kinases inhibitor, 10 µM; CalBiochem); SB203580 (a p38 MAPK inhibitor, 10 µM; CalBiochem); LY294002 (a PI3Kinase inhibitor, 10 µM; Sigma); SP600125 (a JNK inhibitor, 10 µM; Sigma); Imatinib mesylate (an Abl kinase inhibitor, 3 µM, kindly provided by Novartis); or 0.1% dimethyl sulfoxide (DMSO) before being added to the MLR.

Assessment of apoptosis

The experiments were performed as previously described [37]. Briefly, the MLR assay was employed; T-cells (5×10^5 /well) and irradiated PBMNC (5×10^5 /well) were seeded in the 96-well round-bottomed plates precoated with EphB2-Fc or ephrin-B2-Fc or human-Fc control (1 µg/mL; R&D Systems), incubated at 37°C and 5% CO₂ for 96 h. The cells were harvested, blocked with blocking buffer (Hanks' balanced salt solution containing 5% [v/v] fetal calf serum, 5% human serum, 10% BSA, and penicillin [50 i.u./mL]/streptomycin sulfate [50 µg/mL]) for 30 min on ice, washed (ice-cold Hanks' balanced salt solution [Sigma]) supplemented with 5% FCS, and incubated with CD3-PECy7 (BioLegend) and CD25-PE (BD Biosciences) for 30 min on ice. Cells were incubated with Annexin-V-Alexa Fluor 488 (Invitrogen) for 15 min following viability dye 7-AAD (Beckman Coulter) as per the manufacturer's protocol. Samples were analyzed using a Becton Dickinson Aria flow cytometer (BD Biosciences).

shRNA knockdown

RNA duplexes of human EphB2 and ephrin-B2 were cloned into the pFIV-H1-copGFP lentiviral vector as per the

manufacturer's instructions (System Biosciences) [14]. The customized shRNA oligonucleotide sequences used in this study, as previously described [14], were EphB2 shRNA_1; EphB2 shRNA_2; ephrin-B2 shRNA_1; ephrin-B2 shRNA_2; and shRNA scramble nonsilencing control. The cloned EphB2 or ephrin-B2 shRNA vectors were transfected into HEK-293T-cells, and viral supernatants were used to infect primary human MSC. The top 10% of GFP-expressing cells were selected using FACS (BD BioSciences) as previously described [38]. MSC cell lines that exhibited greater than 60% reduction in EphB2 and ephrin-B2 gene expression, compared with corresponding shRNA scramble MSC control lines, were used in this study.

Statistical analysis

Multiple treated groups were compared with the control group using one-way ANOVA, dunnett post hoc test. Treated groups were compared with their corresponding control using a two-tailed unpaired Student's *t*-test. *P* values of less than 0.05 were considered as indicating statistical significance. The results are expressed as the mean $\pm$ SEM of three to four independent experiments from three to four donors.

Results

Human primary T-cells express high levels of ephrin-B1 ligand and EphB4 receptor

The present study examined the expression profile of EphB/ephrin-B molecules on the human T-cells. RT-PCR analysis showed that CD3+ selected human peripheral blood T-lymphocytes expressed high levels of ephrin-B1 and EphB4 (Fig. 1A). These molecules bind with highest specificity to EphB2 and ephrin-B2, respectively [18], which are highly expressed by human bone marrow-derived MSC (Fig. 2) as previously described [14]. Supportive western blot analysis identified a 45 and a 120 kDa bands in human T-cell lysate extracts representing ephrin-B1 and EphB4 protein, respectively (Fig. 1B). These data indicate that ephrin-B1 and EphB4 are highly expressed by human T-cells, and they may potentially interact with EphB2 and ephrin-B2 present in human MSC, respectively.

EphB/ephrin-B interactions suppress proliferation of allostimulated T-cells

We performed allogeneic MLR to determine whether suppression of activated T-cells could be mediated through EphB2/ephrin-B1 and ephrin-B2/EphB4 interactions, in the presence of EphB2-Fc, ephrin-B2-Fc, or human-Fc control fusion proteins. The data showed that EphB2-Fc and ephrin-B2-Fc significantly inhibited T-cell proliferation compared with the human-Fc control (Fig. 3A, $P < 0.05$, one-way ANOVA, dunnett post-test, data from three independent experiments).

To examine whether blocking the function of EphB2 and ephrin-B2 present in MSC could affect T-cell proliferation, allogeneic MLR were performed with a third-party MSC population, in the presence of either EphB receptor specific blocking peptides: EphB2 (SNEW) or EphB4 (TNYL) or the control peptide (RTVA). The addition of EphB2 blocking peptide (SNEW) or EphB4 blocking peptide (TNYL) resulted in a significant increase in T-cell proliferation compared with

FIG. 1. Erythropoietin-producing hepatocellular B (EphB)/ephrin-B expression by human T-cells. Human primary T-cells were purified by CD3⁺ magnetic-activated cell sorting from peripheral blood mononuclear cells of healthy donors. (A) Gene expression data was obtained using real-time polymerase chain reaction (RT-PCR) and normalized to β -actin. Data represent the mean $\pm$ SEM of three independent donors. (B) The data represent western blot analyses of EphB4 and ephrin-B1 protein expressed by human primary T-cells from three donors and the corresponding β -actin controls.

the control peptide (RTVA) (Fig. 3B, $P < 0.001$, one-way ANOVA, dunnett post-test, data represent three independent experiments of three MSC donors). These observations suggest that blocking EphB2/ephrin-B1 and EphB4/ephrin-B2 interactions between MSC and T-cells partially reversed MSC-mediated suppression of activated T-cell proliferation.

To confirm that EphB2 could signal via ephrin-B1 expressed in T-cells, function inhibitor studies were performed using an ephrin-B1 specific blocking peptide in an MLR [39]. The results showed that the addition of ephrin-B1 blocking peptide (PTD-EFNB1-C) completely reversed the EphB2-Fc-mediated T-cell suppression compared with the scramble control peptide (PTD-Scram) (Fig. 3C, $P < 0.01$, one-way ANOVA, dunnett post-test).

In addition to EphB2 and ephrin-B2, MSC were also shown to express EphB4, ephrin-B1 (Fig. 2), EphA2, and ephrin-A5 (Supplementary Fig. S1C; Supplementary Data are available online at www.liebertpub.com/scd); while T-cells were found to express different EphA/ephrin-A molecules (Supplementary Fig. S1B). Due to the promiscuous binding between Eph/ephrin molecules, we next examined the functional role of EphB4, ephrin-B1, EphA2, and ephrin-

FIG. 2. EphB/ephrin-B expression by human mesenchymal stromal/stem cells (MSC). Human ex vivo expanded MSC were purified by STRO-1⁺ magnetic-activated cell sorting from bone marrow mononuclear cells of healthy donors. (A) Gene expression analysis obtained using RT-PCR and normalized to β -actin. Data represent the mean $\pm$ SEM of three independent donors. (B) Supportive western blot data of EphB2 and ephrin-B2 protein expressed by human MSC from four donors and the corresponding β -actin controls. RT-PCR and western blot data are consistent with our previously published data.¹⁴

A5 in regulating T-cell proliferation using Eph-Fc and ephrin-Fc fusion molecules in an allogeneic MLR assay. These studies demonstrated that T-cell proliferation was not significantly suppressed in the presence of EphB4-Fc, ephrin-B1-Fc, EphA2-Fc, or ephrin-A5-Fc when compared with the human-Fc controls (Supplementary Fig. S2A). Furthermore, since T-cells also express high levels of EphA4, known to bind promiscuously to ephrin-B2 [18,20], pretreatment of T-cells with EphA4 blocking peptide before performing an MLR assay confirmed that there were no differences in T-cell proliferation in the presence of EphA4 blocking peptide (VTM) compared with scramble control (RTVA) (Supplementary Fig. S2B).

Knockdown of EphB2 and ephrin-B2 expression in MSC decreases their capacity to suppress activated T-cell proliferation

To further confirm the suppression effect of EphB2 and ephrin-B2 expressed by MSC, the T-cell response in allogeneic MLR was assessed in the presence of third-party MSC using stably transduced shRNA-mediated EphB2 or ephrin-B2 knockdown or scramble control human MSC lines. RT-PCR data demonstrated that EphB2 expression was

FIG. 3. EphB/ephrin-B interactions suppress T-cell proliferation. (A) T-cell proliferation was significantly suppressed in the presence of EphB2-Fc (1 μ g/mL) or ephrin-B2-Fc (1 μ g/mL) compared with human-Fc control. T-cell proliferation counts are expressed relative to human-Fc control (data represent the mean $\pm$ SEM of three independent experiments from three T-cells, one-way ANOVA, dunnett post-test, $*P < 0.05$; $***P < 0.001$). (B) Alternatively, blocking the function of EphB2 or ephrin-B2, expressed by MSC, using EphB2 receptor blocking peptide (SNEW, 100 μ M) or EphB4, the highest ephrin-B2 binding receptor, blocking peptide (TNYL, 100 μ M), respectively, increases T-cell proliferation significantly compared with the control peptide (RTVA). Proliferation counts are expressed relative to "No MSC" control ($n = 3$, one-way ANOVA, dunnett post-test, $***P < 0.001$). (C) Blocking the function of ephrin-B1 expressed by T-cells using blocking peptide (PTD-EFNB1-C, 50 μ M) also significantly increases T-cell proliferation in the presence of EphB2-Fc compared with human-Fc control (representative data from $n = 3$, one-way ANOVA, dunnett post-test, $**P < 0.01$).

significantly reduced in the EphB2 knockdown MSC lines compared with the corresponding scramble control MSC (Fig. 4A). Similarly, ephrin-B2 expression was reduced in the ephrin-B2 knockdown MSC lines compared with the scramble control MSC (Fig. 4B). The knockdown of EphB2 in MSC resulted in a reduced capacity to suppress activated T-cell proliferation compared with the corresponding shRNA scramble control MSC lines (Fig. 4C, $P < 0.05$, Student's t -test, $n = 4$ independent experiments). Similarly, shRNA knockdown of ephrin-B2 in MSC reduced their inhibitory effect on T-cell proliferation compared with the nonsilencing scramble control MSC (Fig. 4D, $P < 0.05$, Student's t -test, $n = 4$).

To determine whether EphB2 and ephrin-B2 mediate inhibition of T-cell proliferation or cause cell death, the viability of T-cells was measured using trypan blue uptake method and flow cytometry analysis using cell surface staining of the early apoptotic marker, Annexin-V, and the DNA dye, 7AAD, gating in activated T-cells (CD3+CD25+). We observed that there were no significant differences in the percentage of trypan blue positive or Annexin-V positive or 7AAD positive activated T-cells in MLR cultured with EphB2-Fc or ephrin-B2-Fc compared with untreated controls (Supplementary Fig. S3). The data support the notion that the suppression of activated T-cells is due to the inhibitory effect of EphB2 and ephrin-B2 on proliferation rather than through apoptosis.

EphB4 and ephrin-B1 induce IFN- γ dependent IDO, TGF- β 1, and inducible nitric oxide synthase expression in MSC

We next examined the contribution of Eph/ephrin in the expression of molecules involved in MSC-mediated T-cell suppression. Previous studies have demonstrated that MSC, on being stimulated by IFN- γ produced by activated T-cells, secrete soluble factors that play important roles in mediating MSC immunosuppressive properties such as IDO, TGF- β 1, and NO [1,2,40]. In the present study, gene expression analyses were performed on MSC after incubation with or without IFN- γ in the presence of EphB4-Fc, ephrin-B1-Fc, or human-Fc control. We found that the transcript levels of IDO in MSC were significantly elevated after 24 h stimulation with EphB4-Fc or ephrin-B1-Fc compared with human-Fc controls (Fig. 5A, $P < 0.01$, one-way ANOVA, dunnett post-test). In addition, the gene expression levels of TGF- β 1 and inducible nitric oxide synthase (iNOS), a molecule required for the synthesis of NO, were significantly up-regulated after 48 h stimulation with EphB4-Fc compared with human-Fc controls (Fig. 5B, C, $P < 0.001$, one-way ANOVA, dunnett post-test).

EphB2 and ephrin-B2 suppress IFN- γ , tumor necrosis factor- α , IL-2, and IL-17 expression in T-cells

We investigated the contribution of Eph/ephrin in the expression level of molecules by lymphocytes known to play vital roles in T-cell activation and proliferation such as IFN- γ , tumor necrosis factor- α (TNF- α), IL-2, and IL-17. RT-PCR was performed to determine the gene expression analysis of these molecules on T-cells after stimulation with EphB2-Fc or ephrin-B2-Fc or human-Fc controls. The data showed that

FIG. 4. shRNA knockdown of EphB2 or ephrin-B2 expression in MSC reduces their inhibitory function on T-cell proliferation. **(A)** EphB2 gene expression is reduced in EphB2 knockdown MSC lines compared with nonsilencing scramble MSC control. **(B)** ephrin-B2 gene expression is decreased in ephrin-B2 knockdown MSC lines compared with nonsilencing scramble MSC control. Gene expression analysis obtained using RT-PCR and normalized to β -actin. **(C)** T-cell proliferation was significantly suppressed in a dose-dependent manner (*open bars*) in the presence of MSC compared with a culture without MSC (*gray bar*). However, MSC-mediated T-cell suppression was significantly reversed in the presence of shRNA knockdown EphB2 MSC compared with the nonsilencing scramble MSC control. **(D)** MSC-induced T-cell suppression was significantly reduced in the presence of shRNA ephrin-B2 MSC compared with the nonsilencing scramble MSC control. Data represent the mean $\pm$ SEM of four independent experiments; * $P < 0.05$, unpaired Student's *t*-test.

the transcript levels of IFN- γ , TNF- α , and IL-17 were significantly reduced in T-cells after 24 h stimulation with EphB2-Fc or ephrin-B2-Fc compared with human-Fc controls (Fig. 5D–F, $P < 0.001$, one-way ANOVA, dunnett post-test). In addition, the gene expression level of IL-2 was significantly suppressed in T-cells after 48 h stimulation with EphB2-Fc or ephrin-B2-Fc compared with human-Fc controls (Fig. 5G, $P < 0.05$, one-way ANOVA, dunnett post-test).

MSC suppression of T-cell proliferation is mediated through both EphB4 forward and ephrin-B1 reverse signaling

To elucidate EphB4 forward signaling and ephrin-B1 reverse signaling pathways in ephrin-B2 and EphB2-mediated inhibition of T-cell proliferation, respectively, a pharmacological approach was employed by utilizing chemical signaling pathway inhibitors. The inhibitor assays were performed using allogeneic MLR in the presence of pre-clustered EphB2-Fc, ephrin-B2-Fc, or human-Fc control, immobilized to the bottom of the wells. Signal inhibitors

were incubated with CD3+ purified T-cells before being added to the MLR cultures. The data showed that ephrin-B2-Fc-mediated suppression of T-cell proliferation was reversed in the presence of PP2, LY294002, Imatinib, or SP600125 (Fig. 6A, $P < 0.001$, one-way ANOVA, dunnett post-test, data represent four independent experiments). Conversely, ephrin-B2-Fc-mediated suppression of T-cell proliferation was similar to that of the vehicle control (DMSO) in the presence of PD184352 or SB2035. In parallel experiments, EphB2-Fc-mediated suppression of activated T-cells was partially blocked in the presence of the ephrin-B1 reverse signaling pathway inhibitors to PP2, LY294002, Imatinib, or SP600125, compared with the corresponding human-Fc controls (Fig. 6B, $P < 0.01$, one-way ANOVA, dunnett post-test, data represent four independent experiments). Conversely, EphB2-Fc-mediated T-cell suppression was unaffected in the presence of PD184352 or SB2035. Collectively, the signaling inhibitor studies indicated that MSC-mediated T-cell suppression was occurring through the Src, JNK, PI3-Kinase, and Abl kinase pathways for both EphB4 forward signaling and ephrin-B1 reverse signaling.

FIG. 5. Gene expression of soluble factors in MSC and T-cells. (A–C) The expression of immunosuppressive factors: indoleamine 2,3-dioxygenase (IDO) (A) in MSC were induced after 24 h stimulation with INF- γ (25 ng/mL) and EphB4-Fc (5 μ g/mL) or ephrin-B1-Fc (5 μ g/mL) compared with human-Fc control; while transforming growth factor (TGF- β 1) (B) and inducible nitric oxide synthase (iNOS) (C) expression was up-regulated after 48 h stimulation with interferon (IFN)- γ (25 ng/mL) and EphB4-Fc (5 μ g/mL) compared with human-Fc control. (D–G) The expression of key molecules involved in T-cell activation and proliferation: IFN- γ (D), tumor necrosis factor- α (TNF- α) (E) and interleukin (IL)-17 (F) are down-regulated after 24 h stimulation with EphB2-Fc (1 μ g/mL) or ephrin-B2-Fc (1 μ g/mL) compared with human-Fc control, whereas IL-2 (G) expression was reduced after 48 h stimulation with EphB2-Fc (1 μ g/mL) or ephrin-B2-Fc (1 μ g/mL) compared with human-Fc control. Gene expression analysis was performed by RT-PCR. Representative data of three independent experiments, expression is relative to β -actin control. One-way ANOVA, dunnett post-test, * P < 0.05, ** P < 0.01; *** P < 0.001.

FIG. 6. MSC inhibitory effect of T-cell proliferation is mediated through EphB4 forward and/or ephrin-B1 reverse signaling. **(A)** Inhibitors of EphB4 forward signaling pathways were used in the mixed lymphocyte reaction (MLR) assay in the presence of ephrin-B2-Fc (1 μ g/mL) or human-Fc control. In the vehicle control (dimethyl sulfoxide), ephrin-B2-Fc suppresses T-cell proliferation significantly compared with human-Fc control. However, in the presence of PP2, LY294002, Imatinib, or SP600125, which are inhibitors of Src, PI3K, Abl, and JNK, respectively, ephrin-B2-mediated T-cell suppression was prevented compared with human-Fc control. However, PD184352 or SB2035, which are inhibitors of MEK or p38 MAPK pathways, respectively, failed to elicit any significant changes in EphB4 forward signaling-mediated suppression of T-cell proliferation. Data represent the mean $\pm$ SEM of four independent experiments from four T-cell donors, *** P < 0.001, unpaired Student's t -test. **(B)** Similarly, EphB2 suppresses T-cell proliferation through Src, PI3K, Abl, and JNK pathways downstream of ephrin-B1 reverse signaling but not through MEK or p38 MAPK pathways. Data represent the mean $\pm$ SEM of four independent experiments from four T-cell donors. ** P < 0.01, *** P < 0.001 unpaired Student's t -test. **(C, D)** Immunoprecipitation of tyrosine phosphorylation (anti-pY) after immunoblotting of EphB4 **(C)** or ephrin-B1 **(D)** in T-cells. T-cells were stimulated with allogeneic cells in MLR and ephrin-B2-Fc (1 μ g/mL) or EphB2-Fc (1 μ g/mL) or human-Fc accordingly. A greater intensity band of 120 kDa representing EphB4 was observed in T-cell lysates treated with ephrin-B2-Fc compared with human-Fc. Similarly, a greater intensity band was found at 45 kDa, indicating ephrin-B1 in T-cell lysates treated with EphB2-Fc compared with human-Fc.

We next determined the phosphorylation status of EphB4 and ephrin-B1 expressed by T-cells, using an allogeneic MLR assay in the presence of preclustered EphB2-Fc, ephrin-B2-Fc, or human-Fc controls. Immunoprecipitation of tyrosine residue phosphorylation, followed by immunoblotting with an anti-EphB4 antibody identified an expected 120 kDa band, with greater intensity in human T-cell lysate extracts treated with ephrin-B2-Fc compared with human-Fc control (Fig. 6C), indicating phosphorylation of EphB4 receptor by ephrin-B2. Similarly, there was a marked increase in the intensity of the expected 45 kDa band representing ephrin-B1 observed in T-cells lysate extracts treated with EphB2-Fc compared with human-Fc controls (Fig. 6D).

Discussion

Here, we have demonstrated a functional role for EphB/ephrin-B interactions in MSC-mediated suppression of T-cell proliferation. We found that human primary T-cells express high levels of ephrin-B1 and EphB4, which are known to bind at highest affinity to EphB2 and ephrin-B2

respectively, that has previously been shown to be highly expressed by human MSC [14]. While the expression of ephrin-B1 has been reported in murine T-cells [22,32,41–43] and in peripheral blood lymphocytes of patients associated with rheumatoid arthritis [34], here we show, for the first time, that ephrin-B1 and EphB4 are expressed by human primary T-cells.

Initially, Eph receptors and ephrin ligands were identified as mediators of growing axons, at the boundary formation of neural crest cells and in angiogenesis [44–46]. It is now clear that their functions extend beyond these developmental processes, to the regulation of stem cells, immune function, and particularly T-cell proliferation. Previous studies have reported that ephrin-B1 and ephrin-B2 suppressed murine T-cell proliferation [35], and EphB6/ephrin-B2 interaction enhances T-cell responses to antigens by in vitro TCR stimulation [29]. In the present study, we examined the role of EphB2/ephrin-B1 and EphB4/ephrin-B2 interactions in MSC-mediated suppression of T-cell proliferation. Since Eph/ephrin interactions are highly promiscuous [13], we employed Eph- and ephrin-Fc fusion molecules to examine

the specific effects of EphB2 and ephrin-B2 activation in T-cell proliferation. We found that, EphB2-Fc and ephrin-B2-Fc fusion proteins significantly suppressed human T-cell proliferation, in allogeneic mixed lymphocyte culture. While our findings are in contrast to previous studies reporting that ephrin-B ligands act as co-stimulatory molecules for murine T-cell proliferation [32,33] [31,35], Kawano and colleagues have demonstrated a switch from stimulation to suppression of proliferative responses in the presence of Eph/ephrin-Fc fusion molecules at concentrations of 5 µg/mL or greater. In the present study, we observed a consistent suppression of activated human T-cells in the presence of either EphB2-Fc or ephrin-B2-Fc over the concentration range tested (1–10 µg/mL, data not shown). Our observations that EphB2 or ephrin-B2 inhibits T-cell proliferation is in agreement with other studies which found that EphA/ephrin-A interactions inhibit activated CD4⁺ T-cell proliferation [27]; while ephrin-B1 and ephrin-B2 were also reported to suppress murine T-cell proliferation [35]. Similarly, ephrin-B2 signaling has been associated with the inhibition of endothelial cell proliferation [47,48] and breast tumor growth [49].

In addition to EphB2 and ephrin-B2, MSC also express EphB4, ephrin-B1, EphA2, and ephrin-A5 that can promiscuously bind to various Eph and ephrin molecules expressed by T-cells. Studies examining the functional role of these Eph/ephrin molecules in T-cell proliferation demonstrated that T-cell proliferation was unaffected in the presence of these molecules. These observations confirm the specific role of EphB2 and ephrin-B2 in T-cell suppression, possibly by interacting with ephrin-B2 and EphB4, which are highly expressed by human T-cells. The functional role of EphB2 and ephrin-B2 in inhibiting T-cell proliferation was further substantiated using blocking peptides, which have previously been shown to effectively block the ligand-binding site of specific Eph receptors [14,50]. In addition, these blocking peptides not only act to block stabilized activation of the EphB receptor, but can also inhibit simultaneous activation of ephrin-B reverse signaling [14,49–54]. The present study demonstrated that T-cells exhibited increased proliferation in the presence of EphB2 or EphB4 blocking peptides compared with the scrambled peptide control. Therefore, the addition of EphB2 and EphB4 blocking peptides, to disrupt the EphB2/ephrin-B1 and ephrin-B2/EphB4 interactions respectively, reversed MSC-mediated T-cell suppression.

Furthermore, T-cells were also found to express high gene expression levels of EphA4, which is known to promiscuously bind to ephrin-B2 [13,18,20]. Our results showed that there were no differences in T-cell proliferation in the presence of EphA4 blocking peptide compared with scramble control. This observation further supported our findings that ephrin-A5, the highest-binding affinity ligand to EphA4 and expressed by MSC, played no functional role in T-cell suppression. Taken together, these observations support the notion that EphA and ephrin-A molecules are not directly involved in MSC-mediated suppression of activated T-cells.

In addition to ephrin-B1, we showed that human T-cells also express ephrin-B3, which is known to bind to EphB2 [18]. We, therefore, examined the role of EphB2 in T-cell suppression in the presence of an ephrin-B1 blocking peptide in an MLR assay. Our results showed that EphB2-Fc-mediated suppression of T-cell proliferation was completely

reversed in the presence of ephrin-B1 blocking peptide compared with scramble control, suggesting that ephrin-B3 is unlikely to be involved in EphB2-mediated inhibition of T-cell proliferation. Our results are in agreement with a previous report that ephrin-B3 plays a stimulatory role in murine T-cell proliferation induced by anti-CD3, while ephrin-B1 strongly inhibits murine-activated T-cells [35].

Consistent with the EphB2- or ephrin-B2-Fc and EphB or ephrin-B1 blocking experiments, shRNA knockdown of either EphB2 or ephrin-B2 in human MSC resulted in a decreased capacity to suppress T-cell proliferation, compared with nonsilencing shRNA scrambled control MSC. Notably, the suppressive effect of EphB2 and ephrin-B2 was independent of T-cell apoptosis. This result is consistent with a previous report showing that EphB2 and ephrin-B1 modulated the anti-CD3 antibody-induced apoptosis of T-cells [41]. In addition, substantial evidence from our group and others have demonstrated that MSC inhibition of T-cell proliferation is not a consequence of induced apoptosis [37] [1,2,6,10,40,55–57]. These results indicated a direct contribution of EphB2 and ephrin-B2 in the function of MSC mediating the suppression of T-cell proliferation, which is also known to affect MSC attachment and migration [14]. In accordance with our findings, the role of Eph/ephrin molecules in T-cell proliferation has previously been demonstrated using transgenic mouse models. A recent study showed that double knockout of ephrin-B1 and ephrin-B2 in the T-cell compartment of mice (through loxP-mediated gene deletion) caused reduced thymus and spleen size and cellularity, where the T-cells exhibited a compromised homeostatic expansion [22]. Furthermore, a previous study using EphB2-deficient mice revealed that thymic hypocellularity was associated with altered survival and proliferation of the differentiating lymphocytes [58]. Therefore, it appears that EphB/ephrin-B molecules are involved in T-cell proliferation, where our findings implicate a role for EphB2/ephrin-B1 and ephrin-B2/EphB4 interactions in human MSC-mediated suppression of activated human T-cells.

While EphB2/ephrin-B1 and ephrin-B2/EphB4 interactions suppress T-cell proliferation, the underlying mechanisms are still not fully understood. A number of studies have reported that soluble factors secreted by MSC, such as TGF-β1, IDO, NO, HGF, PGE₂ and IL-10, play important roles in immunosuppressive effects [1,2,40,55,59]. In the present study, we showed, for the first time, that IDO expression was induced by MSC after stimulation with EphB4-Fc or ephrin-B1-Fc in the presence of IFN-γ [37]; whereas TGF-β1 and iNOS expression was up-regulated after stimulation with EphB4-Fc alone. Induction of iNOS expression correlated with elevated levels of NO, as iNOS is induced by cytokines for the synthesis of NO [60]. However, the expression of HGF, PGE₂, and IL-10 was not affected by EphB4 or ephrin-B1 stimulation at 24 h, 48 h, or 72 h (data not shown). Our findings that EphB4 induced iNOS and TGF-β1 expression is in agreement with previous studies describing that EphB4 stimulated endothelial cell proliferation, by increasing nitrite production in these cells [61,62]; while loss of EphB receptor expression was associated with deficiency of the intracellular mediator of TGF-β, Smad3.

Soluble factors produced by T-cells that are vital stimulatory factors in T-cell activation and proliferation, such as

IFN- γ , TNF- α , IL-2, IL-17, and IL-4, were also examined. We found that IFN- γ , TNF- α , IL-2, and IL-17, but not IL-4 (data not shown), expression was down-regulated in T-cells after stimulation with EphB2 and ephrin-B2, expressed by MSC. Our data are consistent with the findings of Kawano et al., which stimulated murine T-cells with ephrin-B2 [35]. While MSC have been demonstrated to suppress T-cell proliferation by reducing IFN- γ and IL-17 production [2,55,63], our study contributes to the current understanding, demonstrating that TNF- α and IL-2 may also play an important role during this process.

It is known that Eph/ephrin interactions trigger numerous signaling pathways to mediate cellular functions such as cell attachment, migration, proliferation, and differentiation [14,19,21,64]. We further elucidated the underlying mechanisms of MSC-mediated suppression of human T-cell proliferation by investigating EphB2-induced ephrin-B1 reverse signaling and ephrin-B2-induced EphB4 forward signaling in activated T-cells. Substantial evidence suggests that Eph/ephrin signaling involves a complex array of signaling pathways [28,65–67], in which Src family kinase, PI3Kinase, JNK, Abl kinases, MEK, and p38 MAPK have previously been described to be involved in contributing to T-cell activation and proliferation [28,30,48,66,68]. In the present study, chemical inhibitors of these pathways were employed to demonstrate that suppression of activated T-cell proliferation through EphB4 forward signaling or ephrin-B1 reverse signaling appeared to be dependent on Src kinase, PI3Kinase, Abl, and JNK kinase signaling pathways. Conversely, chemical inhibitors to the MEK, p38 MAPK pathways failed to elicit any significant changes in EphB4 forward signaling or ephrin-B1 reverse signaling-mediated suppression of T-cell proliferation. Our findings are consistent with a previous study showing that the EphB4 receptor, when stimulated by its cognate ligand ephrin-B2, suppressed breast cancer cell growth in a mouse xenograft model [69]. This effect is potentially due to the activation of Abl family tyrosine kinases, leading to the inhibition of breast cancer cell proliferation, motility and invasion. Furthermore, our observations extend our current understanding on ephrin-B reverse signaling, which has previously been described to be mediated predominantly via the Src molecule, Grb4, the PDZ domain, or through the JNK pathway independent of tyrosine kinase phosphorylation [70,71]. Our findings are also supported by a recent study showing that reverse ephrin-B signaling inhibited MSC attachment and spreading by activating Src-, PI3Kinase- and JNK-dependent signaling pathways [14]. However, it should be mentioned that the general effect observed in T-cell suppression through the inhibition of Src, PI3K, JNK, and Abl pathways has previously been reported for a variety of other cell types unrelated to Eph/ephrin signaling [65,72–80]. Nevertheless, we observed a consistent suppression of T-cell proliferation after treatment with either EphB2-Fc or ephrin-B2-Fc compared with human-Fc control in the presence of Src, Abl, PI3Kinase, or JNK inhibitors.

Previous studies reported that Eph receptor repulsive/inhibitory function requires tyrosine kinase activity and receptor phosphorylation [67,81]. Since we have shown that ephrin-B2 suppressed T-cell proliferation by interacting with EphB4 receptor expressed by T-cells, it may be possible that ephrin-B2 stimulation is essential for EphB4 phosphoryla-

tion. In the present study, immunoblot analyses revealed that ephrin-B2 clearly promoted phosphorylation of EphB4 in T-cells activated by allogeneic MLR. This is consistent with the finding of Kawano et al. describing that ephrin-B2 inhibits primary murine T-cell proliferation by inducing EphB4 phosphorylation [35]. Similarly, we observed that EphB2-mediated suppression of T-cell proliferation was also dependent on tyrosine phosphorylation during ephrin-B1 reverse signalling. Therefore, our study shows that the interactions of EphB2/ephrin-B1 and/or ephrin-B2/EphB4 between MSC and T-cells play a role in MSC-mediated T-cell suppression by activating PI3Kinase, Src, Abl kinase, or JNK pathways and by modulating immunosuppressive factors secreted by MSC such as IDO, TGF- β 1, iNOS, and T-cell activation/proliferation factors, including INF- γ , TNF- α , IL-2, and IL-17. Our study contributes to the current understanding of how MSC exert their immunosuppressive effects on activated T-cells, and may offer a unique therapeutic drug target to facilitate the regulation of T-cell populations in immune related conditions.

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Author Disclosure Statement

No competing financial interests exist.

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