Endometrial mesenchymal stem/stromal cell modulation of T cell proliferation

Xiaoqing Yang^{1,2,*}, Meivita Devianti^{1,*}, Yuan H Yang³, Yih Rue Ong¹, Ker Sin Tan¹, Shanti Gurung⁴, Jean L Tan¹, Dandan Zhu¹, Rebecca Lim^{1,5}, Caroline E Gargett^{1,5} and James A Deane^{1,5}

¹The Ritchie Centre, Hudson Institute of Medical Research, Clayton, Victoria, Australia, ²Department of Obstetrics and Gynecology, Affiliated Hospital of Nantong University, NanTong, Jiangsu, China, ³Centre for Inflammatory Diseases, Department of Medicine, Monash University, Monash Medical Centre, Clayton, Victoria, Australia, ⁴Centre for Reproductive Health, Hudson Institute of Medical Research, Clayton, Victoria, Australia and ⁵Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia

Correspondence should be addressed to J A Deane; Email: james.deane@hudson.org.au

*(X Yang and M Devianti contributed equally to this work)

Abstract

Perivascular mesenchymal stem/stromal cells can be isolated from the human endometrium using the surface marker SUSD2 and are being investigated for use in tissue repair. Mesenchymal stem/stromal cells from other tissues modulate T cell responses via mechanisms including interleukin-10, prostaglandin E2, TGF- β 1 and regulatory T cells. Animal studies demonstrate that endometrial mesenchymal stem/stromal cells can also modify immune responses to implanted mesh, but the mechanism/s they employ have not been explored. We examined the immunomodulatory properties of human endometrial mesenchymal stem/stromal cells on lymphocyte proliferation using mouse splenocyte cultures. Endometrial mesenchymal stem/stromal cells inhibited mitogen-induced lymphocyte proliferation *in vitro* in a dose-dependent manner. Inhibition of lymphocyte proliferation was not affected by blocking the mouse interleukin-10 receptor or inhibiting prostaglandin production. Endometrial mesenchymal stem/stromal cells continued to restrain lymphocyte proliferation in the presence of an inhibitor of TGF- β receptors, despite a reduction in regulatory T cells. Thus, the *in vitro* inhibition of mitogen-induced lymphocyte proliferation by endometrial mesenchymal stem/stromal cells occurs by a mechanism distinct from the interleukin-10, prostaglandin E2, TGF- β 1 and regulatory T cell-mediated mechanisms employed by MSC from other tissues. eMSCs were shown to produce interleukin-17A and Dickkopf-1 which may contribute to their immunomodulatory properties. In contrast to MSC from other sources, systemic administration of endometrial mesenchymal stem/stromal cells did not inhibit swelling in a T cell-mediated model of skin inflammation. We conclude that, while endometrial mesenchymal stem/stromal cells can modify immune responses, their immunomodulatory repertoire may not be sufficient to restrain some T cell-mediated inflammatory events.

Reproduction (2019) 157 43–52

Introduction

Mesenchymal stem/stromal cells (MSCs) are plastic adherent, colony-forming cells with adipogenic, chondrogenic and osteogenic differentiation potential (Phinney & Prockop 2007). They have a characteristic combination of cell-surface markers including CD29, CD44, CD73 and CD105, and lack the haematopoietic markers CD34 and CD45 (Dominici *et al.* 2006, Lv *et al.* 2014). MSC were originally isolated from bone marrow, but have more recently been identified in many other tissues including the human endometrium (Schwab & Gargett 2007, Crisan *et al.* 2008, Gargett *et al.* 2009).

An important feature of MSC is their ability to reduce inflammation or modify immune responses

resulting in reduced fibrosis (De Miguel *et al.* 2012). These properties have been harnessed to treat disease and injury. The mechanisms by which MSCs modulate immune responses include producing anti-inflammatory factors such as interleukin-10 (IL-10), prostaglandin E2 (PGE2), transforming growth factor- β 1 (TGF- β 1) and hepatocyte growth factor (HGF) (Soleymaninejadian *et al.* 2012). These factors and contact-mediated mechanisms suppress the proliferation and activity of T cells, B-cells, natural killer cells and dendritic cells and promote regulatory T cell (Treg) differentiation (Augello *et al.* 2005, English *et al.* 2009, Zhang *et al.* 2009, Ghannam *et al.* 2010, Duffy *et al.* 2011, Gebler *et al.* 2012).

The W5C5 mouse monoclonal antibody recognising the human SUSD2 surface protein (Sivasubramanian *et al.* 2013) identifies an oestrogen receptor- α (ER- α)-negative population of perivascular MSC in the human endometrium (eMSC) with colony-forming and multilineage differentiation properties (Masuda *et al.* 2012). SUSD2⁺ eMSCs are distinct from endometrial fibroblasts, which are sometimes also referred to as MSC, but have a more limited differentiation capacity, are not clonogenic and express ER- α (Gargett *et al.* 2016).

SUSD2⁺ eMSC can be isolated from an endometrial biopsy obtained in an anaesthetic-free, office-based procedure and are easily expanded in culture (Ulrich *et al.* 2013). In a rat fascial defect model, eMSC delivered on a mesh scaffold reduced immune cell infiltration, skewed macrophages toward a reparative M2 phenotype and reduced the stiffness of tissue repair (Ulrich *et al.* 2014). In a mouse model of subcutaneous mesh implantation, eMSC reduced inflammatory mediators and promoted M2 polarisation of macrophages (Darzi *et al.* 2018). Thus, eMSCs are readily accessible and have many potential therapeutic applications (Ulrich *et al.* 2013); however, the immunomodulatory mediators they employ have not been defined. Here, we use *in vitro* and *in vivo* assays to investigate the immunomodulatory mechanisms of human SUSD2⁺ eMSC.

Materials and methods

Human endometrial tissue collection

Human endometrium was obtained from six women with regular menstrual cycles undergoing endometrial biopsy or a hysterectomy, who had not taken exogenous hormones for 3 months prior to tissue collection. Informed written consent was obtained from each patient and the study protocol was approved by Monash Health (09270B) and Monash University (CF/10/2080 – 2010001150) Human Research Ethics Committees. Samples were collected in Hepes-buffered Dulbecco Modified Eagle Medium/Hams F-12 (DMEM/F-12; Invitrogen) with antibiotics and 5% fetal bovine serum (FBS) (CSL, Parkville, Australia), stored at 4°C and processed within 18 h.

Endometrial MSC purification and expansion

Endometrial tissue was diced and dissociated into a single-cell suspension by digestion in Ca²⁺ and Mg²⁺-free phosphate buffered saline (PBS) (Gibco/ThermoFisher Scientific) containing 50 µg/mL collagenase type I (Worthington Biochemical Corporation, Freehold, NJ, USA) and 40 µg/mL deoxyribonuclease type I (Roche Diagnostics) for 60 min on a rotating MacsMix (Miltenyi Biotec) at 37°C in a 5% CO₂ humidified incubator (Ulrich *et al.* 2014). Cells were filtered through a 40 µm sieve (Merck Millipore) to separate dissociated stromal cells from collagenase-resistant epithelial structures and undigested fragments. Ficoll-Paque

(GE Healthcare Biosciences) was used to separate stromal single-cell suspensions from red blood cells.

Dissociated endometrial stromal cells were incubated with phycoerythrin (PE)-conjugated SUSD2 antibody (50 ng/10⁷ cells, Biolegend, San Diego, CA, USA) for 20 min at 4°C in 0.5% FBS in PBS (100 µL/10⁷ cells) (Bead Medium), washed with Bead Medium and incubated with anti-PE magnetic-activated cell sorting (MACS) Microbeads (Miltenyi Biotec) for 20 min at 4°C in Bead Medium (80 µL/10⁷ cells). Cells were washed with Bead Medium and applied to a Miltenyi Column (Miltenyi Biotec) in a magnetic field to purify magnetically labelled SUSD2⁺ eMSC.

Purified SUSD2⁺ cells were seeded at 1.25 × 10⁵ cells/cm² on fibronectin-coated culture flasks in stromal medium, containing DMEM/F-12 medium, 10% FBS, 1% glutamine (Invitrogen) and 0.2% primocin antibiotic (InvivoGen, San Diego, CA, USA), with 10 ng/mL basic fibroblast growth factor. Medium was changed every two days and cells were grown until 70–90% confluent. TrypLE Express (Life Technologies/ThermoFisher Scientific) containing a trypsin substitute was used to harvest cells at each passage and eMSCs were used in assays at passage 1–3.

Immunofluorescence microscopy

P1 SUSD2⁺ endometrial cells were grown to confluence in stromal medium on round plastic coverslips in six-well plates, fixed for 10 min with 4% paraformaldehyde, washed with PBS and then 0.2% Triton X-100 in PBS, blocked using serum-free protein blocking reagent (Prod #X0909 – Dako, North Sydney NSW, Australia), incubated for 1 h at 25°C with PE-conjugated SUSD2 antibody (1:100) or a PE-conjugated Mouse IgG-1 (Invitrogen/ThermoFisher Scientific) isotype control and then washed. Nuclei were stained with Hoechst 33258. Plastic coverslips and attached cells were mounted under a glass coverslip and examined using a Nikon C1 confocal microscope.

Flow cytometry

To assess whether MACS-purified eMSC maintained their identity when expanded in culture, 1 × 10⁵ P2 SUSD2⁺ cells in 100 µL of 2% FBS in PBS (Flow Cytometry Buffer) were blocked with 5 µL of mouse serum for 5 min on ice, washed in Flow Cytometry Buffer, incubated with PE-SUSD2 antibody or a PE-IgG1 isotype control at a 1:100 dilution for 20 min on ice, washed and analysed on a FACS Canto II flow cytometer with FACS Diva Software (BD Biosciences, Le Pont-de-Claix, France).

Mouse splenocytes

Mouse splenocytes were obtained from spleens scavenged from adult C57BL/6J background mice or FoxP3-GFP mice euthanised for colony maintenance or training purposes. Mice were from breeding colonies or projects approved by a Monash Medical Centre Animal Ethics Committee.

[³H]-thymidine lymphocyte proliferation assay

Irradiated P1 eMSC (30 Gy) were seeded into a flat-bottom 96-well plate in 200 µL of stromal medium per well. Mouse splenocytes (5×10^5 cells/well) were plated into wells seeded with eMSC at eMSC:mouse splenocyte ratios spanning 1:3250 to 1:10. An IL-10 receptor blocking antibody specific for mouse (clone 1B1.3A, BioXCell, West Lebanon, NH, USA) (25 µg/mL) (O'Farrell *et al.* 1998) or a Rat IgG1 (25 µg/mL) isotype control, were added to cocultures of eMSC and splenocytes at a 1:10 ratio.

Concanavalin A (ConA) (Sigma-Aldrich) at 0.1 µg/µL was used to induce lymphocyte proliferation and cocultures were incubated for 48 h in 5% CO₂ at 37°C. Two mCi [³H]-thymidine was added 16–18 h prior to culture termination. This assay was performed in triplicate, harvested and proliferation was gauged by measuring radioactivity incorporation using a liquid scintillation analyzer. Assays with clear ConA-mediated splenocyte proliferation in the absence of eMSC (cpm > three times unstimulated control) were included in analyses.

Measuring lymphocyte proliferation using a CFSE dilution assay

1×10^5 P2–3 eMSC were plated into a flat-bottom 24-well plate, cultured for 24 h and a cell count was performed on a representative well. Mouse splenocytes were labelled with 5 µM carboxyfluorescein succinimidyl ester (CFSE) in PBS for 5 min at room temperature and washed three times with PBS + 5% FBS and added to eMSC at a 1:5 ratio of eMSC to splenocytes. ConA at 0.5 µg/µL was used to induce lymphocyte proliferation. Indomethacin (Sigma-Aldrich) was used at 30 µM or 60 µM to inhibit prostaglandin synthesis. A83-01 (Tocris Bioscience) was used at 1 µM to inhibit TGF-β signalling via the ALK4, ALK5 and ALK7 receptors.

Cocultures were incubated for 72 h in 5% CO₂ at 37°C. Cultures were harvested and CD4⁺ T cells analysed for CFSE on a BD LSR II flow cytometer using anti-CD4-APC at 1:200 (clone GK1.5, eBioscience) and 4',6-diamidino-2-phenylindole (DAPI) staining to exclude dead cells. Peaks of CFSE labelling corresponding to undivided cells with the highest CFSE intensity, and weaker peaks corresponding to divided cells were identified in CD4⁺ CFSE peaks. The proliferation modelling feature of FlowJo 10.3 software (FlowJo LLC, Ashland, Oregon) was used to calculate expansion index (fold-expansion of original population) and replication index (fold-expansion of responding cells) (Roederer 2011).

Monitoring regulatory T cells

eMSCs were plated with splenocytes isolated from mice with GFP⁺ Tregs (FoxP3-GFP) (Fontenot *et al.* 2005) at a 1:5 ratio of eMSC to splenocytes and cultured with 0.5 µg/µL ConA for 72 h plus/minus A83-01 as described for the CFSE dilution assay. Tregs were identified as a CD4⁺FoxP3-GFP⁺ population by flow cytometry using CD4-APC at 1:200 (clone GK1.5, eBioscience) and DAPI staining to exclude dead cells.

eMSC cytokine, chemokine and growth factor profile

Supernatant was collected from CFSE dilution assays at 72 h, centrifuged to remove debris and stored at –20°C. 500 µL of supernatant from two independent eMSC/splenocyte cocultures plus or minus ConA was used on a membrane-based antibody array (Human XL Cytokine Array Kit, Cat #ARY022B, R&D Systems) according to the manufacturer's instructions. The antibodies used in this array recognise human proteins with little or no cross reactivity to mouse. Results were collected using a ChemiDoc XRS+ imaging system (BIO RAD). FIJI software (Schindelin *et al.* 2012) was used to subtract background and measure integrated pixel density.

Mouse contact sensitivity model of T cell-mediated skin inflammation

Mouse experiments were approved in advance by a Monash Medical Centre Animal Ethics Committee and conducted in accordance with the National Health and Medical Research Council of Australia guidelines for the use of animals in research. Mice were sensitised by the application of 50 µL 5% oxazolone (Sigma-Aldrich) in an acetone/olive oil vehicle (4:1) to a shaved patch on the back. Five days after sensitisation 1×10^6 eMSC in PBS were injected via the tail vein. Six days after sensitisation 20 µL of 0.5% oxazolone in vehicle was applied to the right ear, and vehicle applied to the left ear as a control. Ear thickness was measured using a micrometre.

Statistical analysis

GraphPad Prism was used for data analysis and plotting graphs. [³H]-thymidine incorporation lymphocyte proliferation data frequently did not pass the Shapiro–Wilk normality test and was analysed using Friedman's test and Dunn's multiple comparison test to identify statistically significant differences between groups. CFSE dilution and ear swelling data passed the Shapiro–Wilk normality test and was analysed using one-way ANOVA and Tukey's multiple comparisons test.

Results

Perivascular eMSC isolated using the W5C5 antibody retain a SUSD2⁺ phenotype after expansion in culture

The W5C5 antibody recognised SUSD2⁺ perivascular cells in the human endometrium (Fig. 1A). Magnetic bead sorting of dissociated endometrium with the W5C5 antibody yielded cells that grew vigorously to confluence for all samples used. After 1–2 passages in culture most cells continued to express SUSD2 (>60%) as judged by immunofluorescence microscopy (Fig. 1B) and flow cytometry (Fig. 1C and D). A broad range of labelling intensities were observed indicating variable levels of SUSD2 expression in cultured eMSC (Fig. 1B and D) as described previously (Masuda *et al.* 2012). Isotype controls verified the specificity of immunofluorescence

Figure 1 Purification and expansion of human perivascular SUSD2⁺ endometrial MSC. (A) The W5C5 antibody recognises SUSD2⁺ perivascular cells (red, arrows) in the human endometrium. g, gland. Inset shows an isotype control to demonstrate antibody specificity. (B, C and D) SUSD2 antibody-purified and cultured eMSC retain SUSD2 expression as detected by immunostaining (B, red), or by flow cytometry (D). An isotype control (C) demonstrates antibody specificity. Nuclei are stained with Hoechst in A and B. Scale bars = 50 μ m.

(Fig. 1A) and flow cytometric (Fig. 1C) detection of SUSD2⁺ cells using the W5C5 antibody.

eMSC inhibit lymphocyte proliferation in a dose-dependent manner independent of IL-10 signalling

The ability of human eMSC to suppress lymphocyte proliferation was tested by coculture with ConA-stimulated mouse splenocytes. As shown in Fig. 2A, eMSC suppressed lymphocyte proliferation at a high eMSC:lymphocyte ratio of 1:10, but suppression diminished in a dose-dependent manner as the relative number of eMSC was reduced. Baseline lymphocyte proliferation in the absence of ConA was not affected by eMSC at the ratios tested (Fig. 2A).

We investigated whether the anti-inflammatory cytokine IL-10 was responsible for inhibiting ConA-mediated lymphocyte proliferation by blocking the mouse IL-10 receptor on splenocytes with an antibody. Blocking the IL-10 receptor did not prevent the inhibitory effect of eMSC on lymphocyte proliferation (Fig. 2A). A lymphocyte proliferation dataset was compiled from five patient-derived eMSC samples using a ratio of cpm for ConA⁺ vs ConA⁻ to control for inter-assay and sample variation (Fig. 2B). Dose-dependent inhibition of ConA-mediated lymphocyte proliferation was a consistent feature of eMSC that did not require splenocyte IL-10 receptor function.

Figure 2 Dose-dependent inhibition of lymphocyte proliferation *in vitro* by endometrial MSC. (A) Inhibition of lymphocyte proliferation by eMSC from a representative patient-derived culture. Black columns show ConA-mediated lymphocyte proliferation measured by incorporation of [³H]-thymidine. Basal levels of proliferation in the absence of ConA are shown by white columns. α IL-10R indicates the inclusion of an IL-10 receptor-blocking antibody and isotype indicates a control antibody. (B) Proliferation index (ConA⁺ cpm / ConA⁻ cpm) measured from five patient-derived eMSC cultures. Boxes show median and interquartile range; whiskers show minimum to maximum. * $P < 0.05$ and ** $P < 0.01$ using Friedman's test and Dunn's *post hoc* test.

eMSC inhibition of CD4⁺ T cell proliferation does not require PGE synthesis or TGF- β signalling

A CFSE dilution assay was optimised to measure CD4⁺ T cell proliferation history in splenocyte/eMSC cocultures over 72 h and allow proliferation modelling. In this assay, CFSE-loaded splenocytes produced only small T cell colonies in the absence of ConA, while ConA induced the growth of larger colonies (Fig. 3). The inclusion of eMSC at a ratio of 1:5 inhibited ConA-induced colony growth in a manner that was unaffected by the inhibitor of prostaglandin synthesis indomethacin, but colony growth was restored by the TGF- β receptor inhibitor A83-01 (Fig. 3).

Flow cytometry for CD4⁺ T cells in cultures without ConA detected a single bright peak for CFSE

Figure 3 Lymphocyte colonies from cultured mouse splenocytes and the effects of eMSC, inhibition of prostaglandin synthesis and TGF- β signalling. Indomethacin was used to inhibit prostaglandin synthesis and A83-01 to inhibit TGF- β signalling. Mouse splenocytes cultured for 72 h without ConA (No ConA); with ConA (+ConA); with ConA and eMSC (+ConA +eMSC); with ConA, eMSC and indomethacin (+ConA +eMSC +indometh); with ConA, eMSC and A83-01 (+ConA +eMSC +A83-01). Images are representative of $n=7$ for No ConA and +ConA; $n=5$ for +ConA +eMSC and +ConA +indometh 30 μ M; $n=4$ for +ConA +eMSC +indometh 60 μ M, and $n=3$ for +ConA +eMSC +A83-01.

corresponding to non-divided CD4⁺ T cells (Fig. 4A). Cultures stimulated with ConA had multiple additional weaker CFSE peaks corresponding to CD4⁺ T cells that had undergone one or more rounds of division (Fig. 4A). The inclusion of eMSC increased the relative size of the peak corresponding to non-divided lymphocytes (Fig. 4A). The effect of eMSC was not altered by indomethacin, but was reversed by A83-01 (Fig. 4B).

The relative magnitude of CFSE peaks corresponding to non-divided and dividing cells was used to model proliferation (Fig. 5). Control cultures lacking eMSC were used to examine the potential for eMSC-independent effects of indomethacin and A83-01 on ConA-mediated lymphocyte proliferation (Fig. 5). eMSC reduced the extent of ConA-induced lymphocyte proliferation as measured by the expansion index (fold-expansion of original population) and the replication index (fold-expansion of responding cells) for CD4⁺ T cells (Fig. 5). The addition of indomethacin at two concentrations (30 μ M and 60 μ M) failed to reverse the inhibition of CD4⁺ T cell proliferation by eMSC, but A83-01 restored CD4⁺ T cell proliferation to levels that were not significantly different to control cultures lacking eMSC (Fig. 5). Controls lacking eMSC indicated that indomethacin did not significantly change ConA-mediated lymphocyte proliferation as measured by expansion or replication indices. A83-01 in the absence

Figure 4 CD4⁺ T cell proliferation and the effects of eMSC measured by CFSE dilution. (A and B) A gate for CFSE-labelled CD4⁺ T cells (rectangle in dot plot panels) was used to generate histograms of CD4⁺ counts vs CFSE intensity (lower panels). Arrows in histograms show peaks of undivided cells. (A) Splenocyte cultures without ConA (no ConA), with ConA (+ConA), or with ConA and eMSC (+ConA +eMSC 1:5). (B) Splenocyte/eMSC cocultures with ConA plus indomethacin or A83-01. Plots are representative of $n=7$ for No ConA and +ConA; $n=5$ for +ConA +eMSC 1:5 and +ConA +eMSC 1:5 +30 μ M indometh; $n=4$ for +ConA +eMSC 1:5 +60 μ M indomethacin, and $n=3$ for +ConA +eMSC 1:5 +A83-01.

of eMSC resulted in significantly higher lymphocyte proliferation than eMSC plus A83-01 (Fig. 5), indicating that eMSC restrain lymphocyte proliferation even when TGF- β signalling is inhibited.

Blocking TGF- β signalling reduces regulatory T cell abundance

A CD4⁺FoxP3-GFP⁺ population of Tregs and a CD4⁺FoxP3-GFP⁻ population of conventional T cells were identified in splenocyte cultures by flow cytometry (Fig. 6A). Treg abundance as a percentage of total CD4⁺ T cells was significantly higher in eMSC/splenocyte cultures relative to A83-01-treated splenocytes without

Figure 5 Quantification of CD4⁺ T cell proliferation. CD4⁺ T cell expansion index (A) and replication index (B) were calculated from CFSE dilution experiments. Data are shown as average ± S.E.M. and open white columns show cocultures containing eMSC, 30μM indomethacin. $n=7$ for untreated control without eMSC; $n=5$ for eMSC, eMSC + 30μM indomethacin; $n=4$ for eMSC + 60μM indomethacin, eMSC + A83-01, 60μM indomethacin; $n=3$ for A83-01. * $P<0.05$, ** $P<0.01$, *** $P<0.005$, **** $P<0.001$ using one-way ANOVA and Tukey's multiple comparisons test.

Figure 6 Quantification of regulatory T cells in splenocyte cultures. (A) CD4⁺FoxP3-GFP⁺ regulatory T cells (Treg) and CD4⁺FoxP3-GFP⁻ conventional T cells (Tconv) were identified by flow cytometry. Spl, splenocytes; eMSC, endometrial mesenchymal stem cells. Plots shown are representative of $n=4$ for each group. (B) The relative abundance of CD4⁺FoxP3⁺ Tregs is expressed as a percentage of the total CD4⁺ population including conventional T cells. Data are shown as average ± S.E.M. and open white columns show cocultures containing eMSC. $n=4$ for all groups. Data were analysed using one-way ANOVA and Tukey's multiple comparisons test. (C) eMSC vs eMSC + A83-01 analysed using a paired t test.

eMSC (Fig. 6B). A83-01 reduced Treg abundance in eMSC/splenocyte cocultures compared with paired untreated cocultures (Fig. 6C).

eMSC cytokine, chemokine and growth factor profile

Secreted human products detected in the supernatant from two eMSC/mouse splenocyte cocultures are shown in Fig. 7. Factors produced were similar for both eMSC cocultures, with the exception of chitinase 3-like 1 and IGFBP-2 that were prominent in supernatant from only one eMSC coculture (Fig. 7A and B). Factors detected readily for both eMSC cocultures were angiogenin, angiopoietin-1, Dickkopf-1 (Dkk-1), fibroblast growth factor-19 (FGF-19), growth/differentiation factor-15 (GDF-15), interleukin-8 (IL-8), interleukin-17A (IL-17A), monocyte chemoattractant protein-1 (MCP-1), stromal cell-derived factor 1α (SDF-1α), serpin E1 and thrombospondin-1 (Fig. 7B). The anti-inflammatory/immunomodulatory factors HGF and IL-10 were not readily detected (Fig. 7B). Stimulating mouse lymphocyte proliferation in cocultures using ConA did not change the profile of factors produced by eMSC (Fig. 7B).

eMSCs do not reduce skin contact sensitivity

Oxazolone challenge of sensitised mice resulted in ear reddening and swelling that peaked after 24h (Fig. 8A and B). The course of swelling as measured by ear

Figure 7 Profiling eMSC cytokines, chemokines and growth factors. (A) A human-specific membrane-based antibody array assay was used to assess factors secreted by two independent human eMSC lines (samples 1 and 2) cocultured with mouse splenocytes. Membrane arrays comparing eMSC/splenocyte cocultures from sample 1 without (left) or with ConA (right) are shown. Each factor is represented by a pair of antibody spots at a designated position on the array as labelled on the left panel. The pairs of spots on the top left, top right and bottom left are reference spots. (B) Spots from eMSC/splenocyte cocultures using samples 1 and 2 were quantified by measuring their integrated pixel density and each bar is an average of duplicate spots.

thickness was not significantly modified at 8, 24 or 48 h in mice treated with intravenous eMSC 24 h prior to challenge relative to vehicle-treated controls (Fig. 8C).

Discussion

SUSD2⁺ eMSC have therapeutic potential because they are easily obtained and purified, can be expanded in culture and modulate inflammatory responses (Masuda *et al.* 2012, Rajaraman *et al.* 2013, Ulrich *et al.* 2014, Darzi *et al.* 2018). Lymphocyte proliferation is an important part of most immune responses and the ability of MSC to suppress lymphocyte proliferation is used as a measure of their immunosuppressive capacity. In this study, we show that eMSCs suppress lymphocyte proliferation induced by the mitogen ConA in a dose-dependent manner as demonstrated for MSC from other tissues (Krampera *et al.* 2013). However, eMSC did not employ common immunomodulatory mechanisms described for MSC from other human tissues and were unable to restrain swelling in a mouse model of T cell-mediated skin inflammation.

IL-10 can mediate the anti-proliferative effects of bone marrow MSC (Beyth *et al.* 2005, Gao *et al.* 2008, Yang *et al.* 2009) and mouse cells are receptive to human IL-10 (Moore *et al.* 1993). In our *in vitro* model, blocking the IL-10 receptor on splenocytes with a mouse-specific antibody did not reduce eMSC suppression of lymphocyte proliferation. Prostaglandin E2 is a common MSC-derived inhibitor of lymphocyte proliferation (Najar *et al.* 2010, Zafranskaya *et al.* 2013). Inhibition of prostaglandin synthesis with indomethacin failed to prevent eMSC-mediated inhibition of CD4⁺ T cell proliferation. These results argue against a role

for IL-10 and prostaglandins in the inhibition of T cell proliferation by eMSC in the *in vitro* context studied.

TGF- β 1 derived from bone marrow MSC restrains lymphocyte proliferation (Di Nicola *et al.* 2002). In our experiments inhibiting TGF- β signalling with A83-01 increased lymphocyte proliferation in splenocyte cultures containing eMSC. Taken in isolation, this result could be interpreted as an indication that MSCs inhibit lymphocyte proliferation via TGF- β signalling. However, A83-01 also increased lymphocyte proliferation in control A83-01 treated cultures lacking eMSC, suggesting that a basal level of TGF- β signalling restrains lymphocyte proliferation in ConA-stimulated splenocyte cultures. Lymphocyte proliferation in A83-01-treated cultures containing eMSC did not reach the elevated levels observed in A83-01 treated cultures lacking eMSC, indicating that eMSCs continue to inhibit lymphocyte proliferation via mechanisms independent of TGF- β signalling. TGF- β signalling promotes the differentiation of anti-inflammatory Tregs by TGF- β 1 via the ALK5 receptor and synergistic effects of Activin A via the ALK4 receptor (Chen *et al.* 2003, Huber *et al.* 2009). We confirmed that A83-01 reduces Treg abundance in both the presence and absence of eMSC. Tregs inhibit lymphocyte proliferation by multiple mechanisms (Schmidt *et al.* 2012). It is likely that reduced Treg differentiation in the presence of A83-01 accounts for observed increases in lymphocyte proliferation. This result also demonstrates that the anti-proliferative effect of eMSC is not linked to the abundance of Tregs. The concepts considered here are summarised in Fig. 9.

Potential factors involved in eMSC inhibition of lymphocyte proliferation were further explored by examining the cytokines, chemokines and growth

Figure 8 eMSC do not inhibit T cell-mediated ear inflammation. (A) A timeline of the mouse contact sensitivity model of ear swelling. (B) An uninflamed vehicle-challenged ear compared to an inflamed oxazolone-challenged ear. (C) Measurement of ear thickness difference between oxazolone and vehicle-treated ears. Data are shown as average $\pm$ s.e.m. and were analysed using an unpaired *t* test with *P* value shown for control vs eMSC. *n* = 13 for vehicle control and 11 for eMSC at 0–24 h. *n* = 11 for vehicle control and 8 for eMSC at 48 h.

factors produced by eMSC cocultured with mouse splenocytes. IL-17A and Dkk-1 are the most likely candidates identified because they have previously been implicated in the ability of MSC to inhibit T cell proliferation (Sun *et al.* 2011, Han *et al.* 2014, Sivanathan *et al.* 2015). Thrombospondin-1 was also produced by eMSC may activate TGF- β to promote Treg differentiation (Futagami *et al.* 2007). HGF is involved in the inhibition of lymphocyte proliferation by bone marrow MSC (Di Nicola *et al.* 2002), but its production by eMSC was minimal in the context studied. IL-10 is a notable MSC anti-inflammatory factor that was not readily detected. This is in keeping with our observation that blocking the IL-10 receptor did not reverse eMSC

Figure 9 Summary of the effects of eMSC and TGF- β signalling on lymphocyte proliferation. (A) TGF- β promotes the differentiation of regulatory T cells (Tregs) that inhibit T-lymphocyte (Tconv) proliferation. (B) eMSCs further inhibit lymphocyte proliferation via a mechanism independent of TGF- β signalling. (C) A83-01 increases lymphocyte proliferation by reducing TGF- β signalling-dependent Treg differentiation, but eMSCs continue to inhibit lymphocyte proliferation via a mechanism independent of TGF- β signalling. (D) Maximum lymphocyte proliferation occurs in the absence of TGF- β signalling-dependent Treg differentiation, and the absence of inhibition by eMSC.

inhibition of lymphocyte proliferation. Profiling also revealed that eMSCs produce immune cell chemo-attractants (IL-8 and MCP-1), angiogenic (angiogenin and angiopoietin-1) and profibrotic (serpin E1) factors. Stimulating T cell expansion with ConA did not change the eMSC factors produced, indicating that the profile observed is a basal repertoire, rather than a response to actively expanding T cells. Further work is required to test the effects of eMSC-derived IL-17A and Dkk-1 on lymphocyte proliferation. Studies of MSC from other sources also suggest that indoleamine 2,3-dioxygenase warrants further investigation (Meisel *et al.* 2004, Soleymanninejadian *et al.* 2012). It will also be important to confirm that the T cell-directed immunomodulatory properties of human eMSC identified in mouse splenocyte assays apply to human T cells.

Mouse skin contact sensitivity models are commonly used to investigate T cell-mediated inflammation. These models involve an initial application of chemical sensitizer, followed by a challenge with the same chemical several days later to elicit a robust T cell-mediated inflammatory response featuring immune cell recruitment and swelling (Deane & Hickey 2009, Deane *et al.* 2012). MSC from bone marrow (Chen *et al.* 2018), adipose tissue (Kikuchi *et al.* 2017) and gingiva (Su *et al.* 2011, Li *et al.* 2016) have the ability to inhibit ear swelling in contact sensitivity when delivered via and intravenous route. In contrast, eMSCs did not inhibit ear swelling in an oxazolone-induced model of contact sensitivity. eMSCs may be unable to suppress

inflammation in this setting because they do not employ the T cell-directed immunomodulatory mechanisms used by other MSC types, particularly prostaglandin E2. This is consistent with observations that the phenotype and function of MSC varies greatly depending on tissue of origin (Le Blanc & Davies 2018). Another pertinent difference may be the perivascular origin of eMSC used in our study, a feature that was not confirmed for the MSC used in previous studies of contact sensitivity. The molecular signature of perivascular MSC is different to those from a non-perivascular origin (Rohart *et al.* 2016) and may indicate functional differences. Cross species incompatibility has also been reported to limit the immunomodulatory properties of MSC in some instances (Lohan *et al.* 2018), a factor that may be relevant in the xenogeneic *in vivo* setting we examined.

We conclude that SUSD2⁺ eMSCs have a distinct immunomodulatory repertoire that lacks mechanisms commonly described for MSC from other tissues. The precise mechanism by which eMSC inhibit lymphocyte proliferation remains to be determined, although our analysis suggest IL-17A and Dkk-1 may be involved. Understanding the immunomodulatory repertoire of eMSC will inform the development of eMSC-based therapies and clarify the contribution of eMSC to the development of an endometrium that can support implantation and pregnancy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was funded by National Health and Medical Research Council (NHMRC) of Australia grants (1085235, C E G, J A D), 1081944 (C E G), NHMRC Senior Research Fellowship (1042298, C E G) and the Victorian Government Operational Infrastructure Support Scheme.

Acknowledgements

The authors acknowledge the assistance of Dr Courtney McDonald with proliferation assays, Dr Luke Larmour with interpreting pathology reports and patient data, Prof. Michael Hickey and Pamela Hall for providing FoxP3-GFP spleens and IL-10 receptor blocking antibody and the Monash Micro Imaging and Histology Facilities at the Hudson Institute of Medical Research. C E Gargett and J A Deane: equal senior authors.

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Received 24 May 2018

First decision 4 July 2018

Revised manuscript received 11 October 2018

Accepted 25 October 2018