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Immunomodulatory Properties of Induced Pluripotent Stem Cell–Derived Mesenchymal Cells

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ABSTRACT

MSC-like populations derived from induced pluripotent stem cells (iPSC-MSC) serve as an alternative stem cell source due to their high proliferative capacity. In this study, we assessed the immunomodulatory potential of iPSC-MSC generated from periodontal ligament (PDL) and gingival (GF) tissue. The iPSC-MSC lines exhibited a similar level of suppression of mitogen-stimulated peripheral blood mononuclear cells (PBMNC) proliferation compared to their respective parental fibroblast populations in vitro. Moreover, iPSC-MSC demonstrated the ability to suppress T-cells effector cells, Th1/Th2/Th17 populations, and increase levels of Treg cells. In order to investigate the mechanisms involved, expression of common MSC-derived soluble factors known to supress lymphocyte proliferation were assessed in iPSC-MSC cultured with PBMNC with direct cell–cell contact or separated in transwells. Real-time PCR analysis of factors known to be involved in MSC mediated immune regulation, found a general trend of elevated *IDO1* and *IL6* transcript levels in iPSC-MSC lines and their respective primary cells co-cultured with activated PBMNC, with a wide range of gene expression levels between the different mesenchymal cell types. The results suggest that different iPSC-MSC may be useful as a potential alternative source of cells for future clinical use in therapeutic applications because of their potent immunosuppressive properties. J. Cell. Biochem. 9999: 1–10, 2016.

KEY WORDS: MESENCHYMAL STEM CELL; GINGIVAL FIBROBLASTS; PERIODONTAL LIGAMENT CELL; iPSC; T-CELLS

Mesenchymal stem cells (MSC) derived from various tissue sources are well recognized for their multilineage potential [Kuznetsov et al., 1997; Pittenger et al., 1999; Gronthos et al., 2000, 2003; Seo et al., 2004] and ability to modulate allogeneic immune cells [Le Blanc et al., 2003, 2004; Wada et al., 2013]. Bone marrow derived MSC (BMSC) are the most extensively studied MSC population, which are currently being evaluated as a therapy in a range of immunological inflammatory conditions and autoimmune diseases [Uccelli et al., 2008; Shi et al., 2010; Atoui and Chiu, 2012]. They are also thought to play a part in the transplantation tolerance and foetal-maternal tolerance [Bartholomew et al., 2002; Rasmusson, 2006; Nauta and Fibbe, 2007]. The immunomodulatory properties of MSC have been shown to be mediated through the inhibition of cell proliferation and survival by depletion of tryptophan via increased activity of the tryptophan catabolic enzyme indoleamine-2,3-dioxygenase (IDO) [Meisel et al., 2004],

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induction of cytokine secretions through direct and indirect cell–cell interactions with immune cells like antigen presenting cells, T cells [Di Nicola et al., 2002], B cells [Deng et al., 2005], natural killer (NK) cells [Sotiropoulou et al., 2006], and dendritic cells (DCs) [Maccario et al., 2005]. However, the utility of BMSC for clinical scale production is hindered by their limited life-span in ex vivo culture (40–50 population doublings) and decline in differentiation potential by 25–30 population doublings following ex vivo expansion [Menicanin et al., 2010; Bright et al., 2015]. Therefore, it is important that alternative, non-invasive, sources of MSC are identified for utilization in clinical therapies [Hynes et al., 2014].

Induced pluripotent stem cells (iPSC) are being considered as a promising cellular source for the generation of MSC [Lian et al., 2010]. These unique cells were initially generated from adult mouse fibroblast cells using transcription factors, Oct3/4, Sox2, c-Myc, and Klf4 [Takahashi and Yamanaka, 2006]. Human iPSC were later successfully generated from adult fibroblast cells with similar properties to embryonic stem cells, including their ability for unlimited growth and the capacity to differentiate into cells types of all three germ layers, without the requirement of embryonic tissue [Takahashi et al., 2007]. Since their discovery, iPSC have been widely used in disease modelling and drug discovery by characterizing the in vitro phenotype of disease-relevant pathological cells from patients, with an increasing interest for their application in the field of regenerative medicine [Grskovic et al., 2011; Robinton and Daley, 2012].

To overcome issues surrounding the use of primary MSC preparations, numerous groups have attempted to generate large qualities of MSC-like populations from different pluripotent stem cell sources [Barberi et al., 2005; Olivier et al., 2006; Karlsson et al., 2009; Mahmood et al., 2010; Gruenloh et al., 2011; Liu et al., 2012; Villa-Diaz et al., 2012; Wei et al., 2012]. Comparative epigenetic analyses of iPSC derived MSC-like cells (iPSC-MSC) and BMSC identified similar characteristics and properties [Frobel et al., 2014]. Importantly, iPSC-MSC were found to exhibit a distinct proliferative advantage over primary bone marrow derived MSC, due to their increased life-span of up to 120 population doublings without the loss of their multi-differentiation potential [Lian et al., 2010]. Proof-of-principal studies have demonstrated the efficacy of iPSC-MSC for the treatment of periodontal disease and allergic airway inflammation in suppressing inflammatory responses and promoting tissue recovery [Sun et al., 2012; Hynes et al., 2013]. In light of this, the immunomodulatory properties of iPSC-MSC could make them a promising alternative to primary MSC for therapeutic applications for immune based clinical indications.

Our group has previously generated MSC-like cells from human iPSC derived from different dental derived tissues, gingival fibroblasts (GF) and periodontal ligament (PDL) cells [Hynes et al., 2014] that meet the minimal criterion for classification of MSC [Dominici et al., 2006]. Given that somatic fibroblast cells from periodontal ligament and gingiva-derived MSC have been reported to have suppress immune cell responses [Wada et al., 2009; Zhang et al., 2009; Chen et al., 2013], we hypothesized that the iPSC-MSC derived from these tissues would also possess similar properties. Therefore, the aim of this study was to assess and compare the immunomodulatory properties and cytokine profile of different populations of human iPSC derived MSC to the primary cells from the respective tissues.

MATERIALS AND METHODS

CELL CULTURE

Primary gingival fibroblast (GF) and periodontal ligament (PDL) cells were prepared from healthy human premolars collected with informed consent from normal adult patients undergoing orthodontic therapy in the University of Adelaide Dental Hospital, in accordance with procedures approved by the Royal Adelaide Hospital Ethics Committee (University of Adelaide Human Research Ethics Committee number H-112-2008), and used to generate iPSC lines as previously described [Wada et al., 2011b]. Generation and characterization of iPSC-MSC derived from periodontal and gingival tissue has been previously described [Hynes et al., 2013, 2014]. The iPSC-MSC lines and their respective primary GF and PDL cells (University of Adelaide Human Research Ethics Committee number H-112-2008) were cultured and maintained in α-MEM with 10% fetal calf serum, 1× non-essential amino acids (Gibco, Thermo Fisher, Waltham, MA), 15 mM HEPES (Sigma–Aldrich), 10 mM sodium pyruvate, 100 μM L-ascorbate-2-phosphate, 2 mM L-Glutamine, and 50 U/ml penicillin/50 μg/ml streptomycin as previously described [Hynes et al., 2014].

Human peripheral blood mononuclear cells (PBMNC) were isolated by density gradient using heparinized blood collected from normal healthy adult volunteers, following informed consent in accordance with procedures approved by the Royal Adelaide Hospital Human Ethics Committee (protocol number 940911A) on Ficoll-Isopaque (Lymphoprep; Fresenius Kabi Norge AS, Olso, Norway) then cultured in RPMI-1640 medium as previously described [Wada et al., 2009].

PBMNC PROLIFERATION ASSAY

iPSC-MSC and primary GF and PDL cells were inactivated by γ-irradiation (30 Gy) and plated into a 96-well flat-bottom plate at a concentration of 1×10^5/well 24 h before the addition of PBMNC pre-labelled with 2 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Eugene, OR). PBMNC were cultured in the presence or absence of iPSC-MSC or primary cells, at a 1:1 (iPSC-MSC/primary cell: PBMNC) ratio, in 10 μg/ml of concanavalin A (Con A; Sigma–Aldrich), an inducer of PBMNC proliferation, for 5 days. Colcemid (Gibco), a cell-cycle arresting agent, was used as a positive control at a concentration of 100 ng/ml. PBMNC proliferation was analyzed by flow cytometry to detect green fluorescence (CFSE) and analysis of cell division and proliferation index (average fold-expansion) were achieved using FCS 4 express flow cytometry software (De Novo Software, Los Angeles, CA). Proliferation index was expressed as a percentage of PBMNC proliferation in the absence of iPSC-MSC, GF, and PDL cells.

TRANSWELL CO-CULTURES

Irradiated iPSC-MSC cells, GF or PDL cells (1×10^5/well for 96-well plate; 1.5×10^5/well for 24-well plate) were seeded into the bottom well, 24 h prior to the addition of PBMNC. An equal number of

CFSE-labelled PBMNC were then added to the 0.4 μM transwell membrane inserts (Corning, Corning, NY) in the presence of Con A (10 μg/ml) and cultured for 5 days.

ANNEXIN V STAINING OF APOPTOTIC CELLS

At day 5, PBMNC collected from direct co-culture or transwell plates were washed twice with 1× phosphate buffered solution (PBS), and stained with Annexin-V-APC (BD Bioscience, San Jose, CA) to detect apoptotic cells, according to manufacturer's instructions. Annexin V positive cells were analyzed using the Cytomics FC500 flow analyzer (Software version 2.2, Beckman Coulter, Miami, FL).

REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS (qPCR)

Total RNA of iPSC-MSC, GF, and PDL cells were collected from 6-well co-cultures and transwell plate using TRIzol (Invitrogen, Thermo Fisher) as per manufacturer's instructions. Complementary DNA (cDNA) was generated using SuperScript III Reverse Transcriptase Kit (Invitrogen) according to manufacturer's instructions. Transcript levels for *IL-6*, *TGF-β1*, and *IDO1* were assessed by CFX connect Real-Time system (Bio-Rad, Hercules, CA) using RT^2 SYBR Green/ROX qPCR Master mix (Qiagen, Hilden, Germany). Primer Sequences: *IL6* (NM_000600.3, Fwd: acagacagccactcacctctt, Rev: tttcaccaggcaagtctcct); *TGFB1* (NM_000660, Fwd: cacgtggagctgtaccagaa, Rev: gaacccgttgatgtccactt); *IDO1* (NM_002164.5, Fwd: agagtcaaatccctcagtcc, Rev: aaatcagtgcctccagttcc). *ACTB* (NM_001101.3, Fwd: gatcattgctcctcctgagc, Rev: gtcatagtccgcctagaagcat).

T-CELL SELECTION AND SUBSET ANALYSIS

Magnetic activated cell sorting was used to isolate $CD3^+$ T cells from PBMNC preparations as per the manufacturer's instructions as described above. CD3+ T cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 μg/ml ionomycin, and 290 nM BD GolgiStop™ for 4 h then cultured in the presence or absence of irradiated PDL cells or PDL-iPSC-MSC like cells for 3 days. After the 3 days in co-culture cells were harvested and subjected to cytometric flow analysis, to identify the proportion of different T-cell populations using a T-cell panel analysis antibody kit (BD Pharmingen™): human naïve T-cells ($CD45RA^+CD197^+$), effector T-cells ($CD45RA^+CD197^-$), central memory T-cells ($CD45RA^-CD197^+$), effector memory T-cells ($CD45RA^-CD197^-$). The Human T helper cells (Th), were defined as: Th1 ($CD4^+IFN\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$), Th17 ($CD4^+IL\text{-}17A^+$). T-regulatory cells (T-regs) were assessed as previously described [Sivanathan et al., 2015]. Briefly, irradiated iPSC-MSC or PDL cells were seeded overnight into 24-well flat bottom plates. $CD3^+$ T cells were co-cultured with iPSC-MSC or PDL in the presence of absence of the mitogen, 10 μg/ml phytohemagglutinin [Tan et al., 2015] for 5 days. The percentage of Tregs was based on the phenotype $CD3^+CD4^+CD25^{high}CD127^{low}FoxP3^+$. All data were collected using a BD LSRFortessa™ X-20 (BD Biosciences) and analyzed with FCSExpress™4.

STATISTICAL ANALYSIS

Prism v6 (GraphPad software, La Jolla, CA) was used for statistical analysis. Ordinary one-way ANOVA with Dunnett's multiple comparisons test, with single pooled variance was used for annexin V staining. Ordinary one-way ANOVA, with Greenhouse–Geisser correction and Tukey's multiple comparison test, with individual variances computed for each comparison was used for comparison of proliferation suppression by all cell lines. A two-way ANOVA test with Sidak's multiple comparisons test was used to compare qPCR expression between MSC and iPSC-MSC co-cultured with PBMNC directly or separated in transwells, and comparison of proliferation suppression by PDL and GF iPSC-MSCs to primary PDL and GD primary lines. *P*-value ≤ 0.05 were considered significant.

RESULTS

iPSC-MSC SUPPRESS THE PROLIFERATION OF ACTIVATED PBMNC

The ability of iPSC-MSC to suppress PBMNC proliferation was examined under direct cell–cell contact (co-culture) and contact-independent (transwell) conditions. Con A-stimulated PBMNC were labelled with the fluorescence dye, CFSE, and then cultured in the presence or absence of iPSC-MSC or primary GF or PDL cells. PBMNC proliferation was assessed after 5 days of culture by flow cytometric analysis. Analysis of the proliferation index showed that that only PDL iPSC-MSC showed suppression of Con A-stimulated PBMNC proliferation from all five donors in contact-dependent co-culture conditions (Fig. 1). GF iPSC-MSC showed significant suppression of PBMNC proliferation in four out of five PBMNC donors (Fig. 1A). In the contact-independent transwell assays, all cell lines showed significant suppression of PBMNC proliferation with all four PBMNC donors with no significant difference between the two iPSC-MSC lines (Fig. 1B). The efficiency of the suppression of PBMNC proliferation by PDL iPSC-MSC and GF iPSC-MSC lines were also compared with the primary PDL and GF lines from which they were originally generated (Fig. 1C–F). Both PDL iPSC-MSC and GF iPSC-MSC displayed comparable levels of suppression of Con A-stimulated PBMNC in comparison to their respective primary cell lines both in co-culture (Fig. 1C and D) and in transwell (Fig. 1E and F) conditions.

To determine whether iPSC-MSC were inducing PBMNC to undergo apoptosis, annexin V cell surface staining of PBMNC was assessed in the presence or absence of iPSC-MSC or primary cells under co-culture or transwell conditions. While colcemid treatment induced a significant increase in PBMNC apoptosis, there was no significant increase in the percentage of annexin V positive PBMNC cultured with the iPSC-MSC lines or the primary PDL or GF cell lines in co-culture or transwell conditions compared with Con-A stimulated PBMNC alone (Fig. 2A and B).

SOLUBLE FACTORS INVOLVED IN THE SUPPRESSION OF CON A-STIMULATED PBMNC PROLIFERATION

We next employed qPCR to assess the gene expression levels of the cytokines TGF-β1, IL-6, and the tryptophan catabolic enzyme Indoleamine-pyrrole 2,3-dioxygenase (IDO1). These factors were previously shown to contribute to the immunosuppressive effects of bone marrow derived MSC [Wada et al., 2013; Sivanathan et al., 2014]. Increased levels of *IDO1* gene expression were observed in the different iPSC-MSC, PDL, and GF cells when co-cultured with activated PBMNC, albeit at different levels (Fig. 3A). Similarly, gene expression levels of *IL6* were found to be unregulated in the iPSC-MSC lines, GF, PDL cells, when co-cultured with activated PBMNC

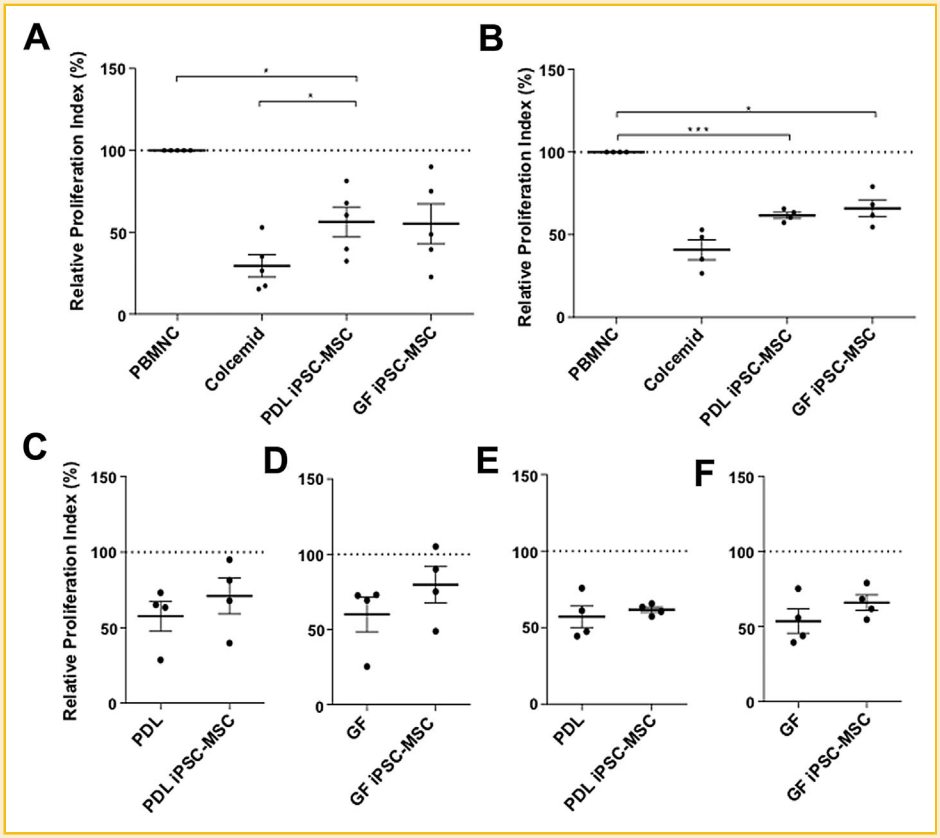

Fig. 1. iPSC-MSC mediated suppression of activated PBMNC. The graphs represent relative proliferation index of Con A-stimulated PBMNC co-cultured (A) or cultured in transwell (B) with different iPSC-MSC and primary fibroblasts. The data were normalized to that of PBMNC alone. Colcemid was used as a positive control for suppression of Con A-stimulated PBMNC proliferation. (C–F) Showed the relative proliferation suppression of con-A stimulated PBMNC by dental iPSC-MSC cells compared with their respective parental primary fibroblast lines. Results show mean ± SEM data from 5 (A) or 4 (B–F) PBMNC donors, each performed in triplicate. * = $P \leq 0.05$, ordinary one-way ANOVA, with Greenhouse–Geisser correction and Tukey's multiple comparison test (A and B), and two-way ANOVA with Sidak's multiple comparisons test (C–F).

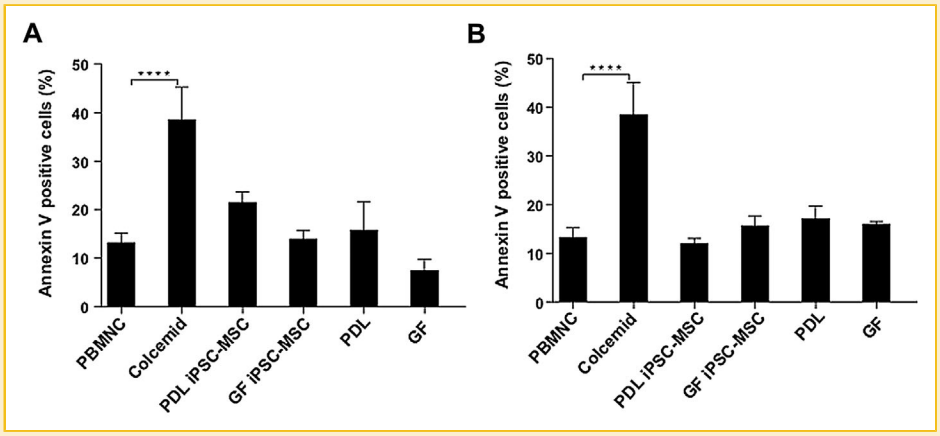

Fig. 2. iPSC-MSC do not induce apoptosis in PBMNC co-cultures. Annexin V staining of con-A stimulated PBMNC in co-culture (A) or in transwell (B) in the presence or absence of immunomodulatory cells (iPSC-MSC, primary PDL and GF fibroblasts). All experiments were performed in triplicates and values are expressed as mean ± SD. * = $P \leq 0.05$, ordinary one-way ANOVA with Dunnett's multiple comparisons test, with a single pooled variance was used.

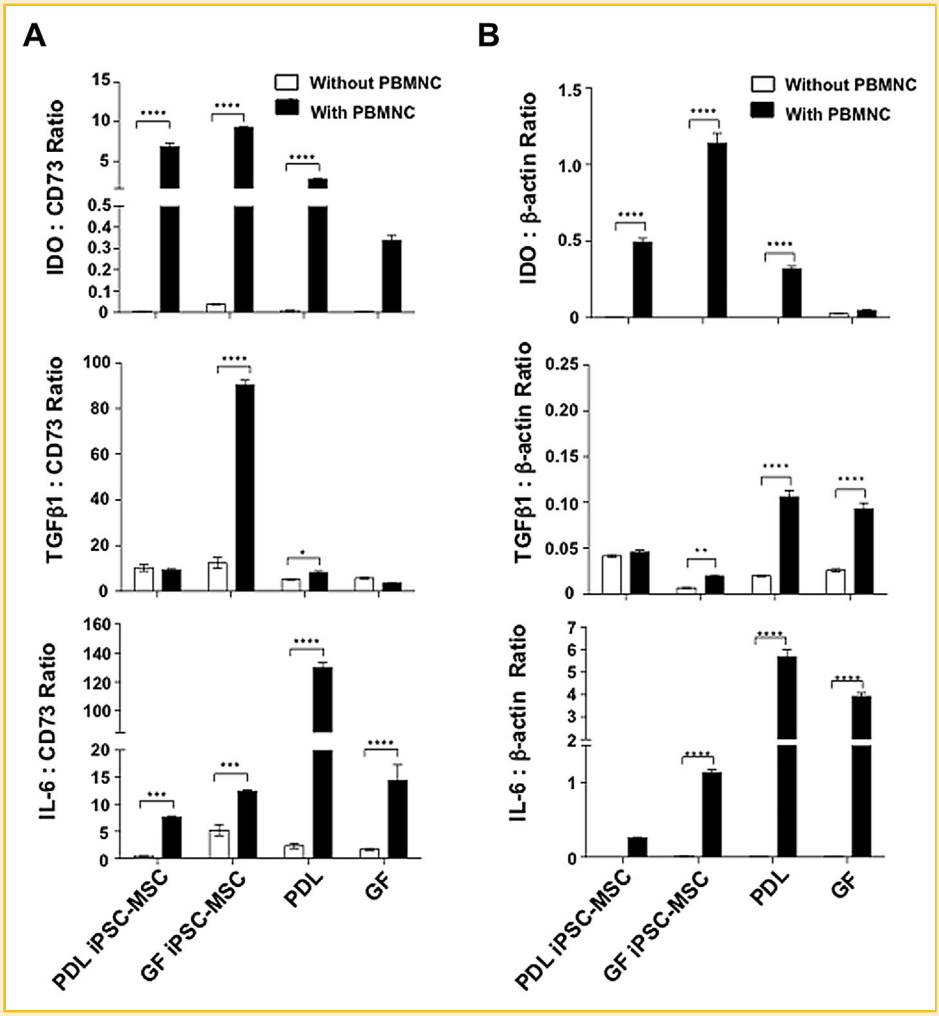

Fig. 3. Differential cytokine expression patterns in iPSC-MSC and primary fibroblasts co-culture with PBMNC. (A) *IDO1*, *TGFB1*, and *IL6* gene expression levels by iPSC-MSC and primary PDL or GF cells co-cultured alone or with activated PBMNC were measured by qPCR. Gene expression levels were normalized to the MSC marker CD73. All experiments were performed in triplicates and values are expressed as mean ± SD from a representative experiment. (B) Gene expression levels of *IDO1*, *TGFB1*, and *IL6* in iPSC-MSC, PDL, or GF primary cells cultured in the presence or absence of activated PBMNC in transwell conditions were measured by qPCR. Gene expression levels were normalized to β-actin. All experiments were performed in triplicates and values are expressed as mean ± SD from a representative experiment. * = $P \leq 0.05$, two-way ANOVA with Sidak's multiple comparisons test, compared with MSCs without PBMNC, ^ = $P \leq 0.05$, two-way ANOVA with Sidak's multiple comparisons test.

(Fig. 3A). In contrast, differential *TGFB1* gene expression patterns were observed between the various iPSC-MSC populations and primary fibroblasts. All data were normalized to the MSC-associated marker, CD73.

Parallel experiments examined *IDO1*, *TGFB1*, and *IL6* gene expression levels in the iPSC-MSC lines and primary cell populations, when cultured with activated PBMNC in a contact-independent transwell setting (Fig. 3B). A general elevation in the transcript levels of *IDO1*, *TGFB1*, and *IL6* was observed for the different iPSC-MSC lines and primary fibroblasts, in the presence of activated PBMNC, with varying levels of expression between the different mesenchymal populations (Fig. 3B).

iPSC-MSC REGULATION OF T-CELL SUBSETS

We next investigated the effect of iPSC-MSC and primary lines on subsets of helper T-cells in co-cultures by assessing the proportions of naïve T-cells, effector cells, central memory cells, effector memory cells. These experiments were conducted with the PDL iPSC-MSC and primary PDL cells as the PDL iPSC-MSC had demonstrated the most significant suppression of PBMNC proliferation in earlier experiments, when compared to GF iPSC-MSC. Flow cytometric analysis of the proportions of T-cell populations in the co-culture assays showed that PDL iPSC-MSC and PDL cells had no effect on the proportion of naïve, central memory, and effector memory $CD3^+CD4^+$ T-cell populations (data not shown). However, PDL iPSC-MSC and primary PDL cells significantly decreased the proportion of $CD3^-CD4^+$ effector cells ($CD45RA^+CD197^-$), compared to that of stimulated T-cells (Fig. 4). Assessment of the effects of PDL iPSC-MSC and primary PDL cells on Th1 ($CD4^+CD8^-IFN\gamma^+$), Th2 ($CD4^+CD8^-IL\text{-}4^+$) and Th17 ($CD4^+CD8^-IL\text{-}17^+$) cells demonstrated that PDL iPSC-MSC and primary PDL cells significantly reduced the proportion of Th1 and Th2 cells present (Fig. 5).

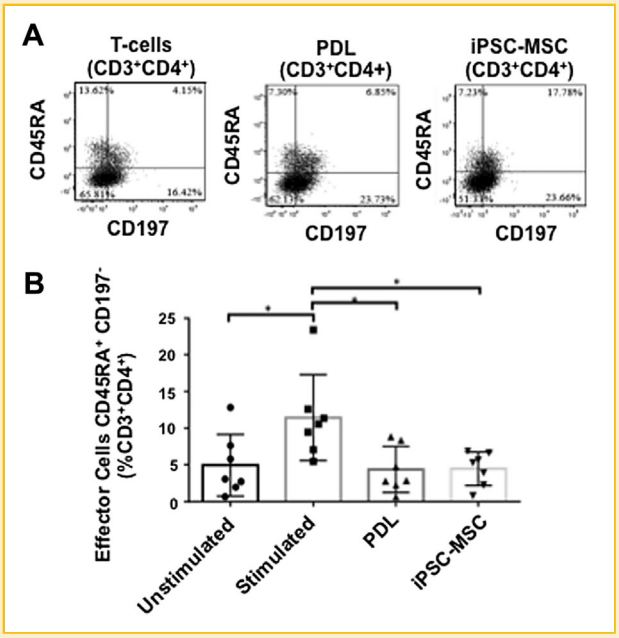

Fig. 4. PDLSC and iPSC-MSC like cells supress effector T-cells. CD3+ T-cells were stimulated with PMA/Ionomycin and co-cultured with PDLSC or PDLSC-iPSC-MSC like cells for 3 days, after which time the proportion of various CD4+ T-cell populations were assessed by flow cytometric analysis. (A) Representative histogram plots of CD45RA and CD197 cell surface staining on stimulated T-cells alone or in the presence of PDL cells or iPSC-MSC gated on the $CD3^+CD4^+$ population. (B) The proportion of $CD45RA^+$ $CD197^-$ effector cells present within the $CD3^+CD4^+$ T-cell population. Values represent mean % fluorescence SD, n = 6–7 donors ($^* = P<0.05$ as determined by one-way ANOVA with Turkey's multiple comparisons).

Furthermore, co-culture with PDL iPSC-MSC and primary PDL cells also resulted in a reduction in the proportion of Th17 cells present, however; only primary PDL cells achieved a significant reduction in Th17 cells (Fig. 5).

Similar experiments were conducted to assess the proportion of Tregs in activated $CD3^+$ T-cells following co-culture with either PDL derived iPSC-MSC or primary PDL cells. The data showed that primary PDL cells exhibited a significant increase in the percentage of Tregs ($CD4 + FoxP3^+$), compared to unstimulated and stimulated T-cells alone (Fig. 6). However, as seen above with the Th17 cells, co-culture of activated $CD3^+$ T-cells with PDL iPSC-MSC resulted in an increased trend in the proportion of Treg cells, which was not statistically significant when compared to the stimulated $CD3^+$ T-cell population alone (Fig. 6).

DISCUSSION

In this study, we compared the immunomodulatory properties of different human iPSC-MSC lines [Hynes et al., 2014] to those of primary GF and PDL cells. Our study demonstrated that iPSC-MSC exhibited the ability to regulate the proliferation of mitogen activated lymphocytes similar to that reported for MSC-like populations generated from embryonic stem cells [Kimbrel et al., 2014]. All human derived iPSC-MSC and primary cell lines

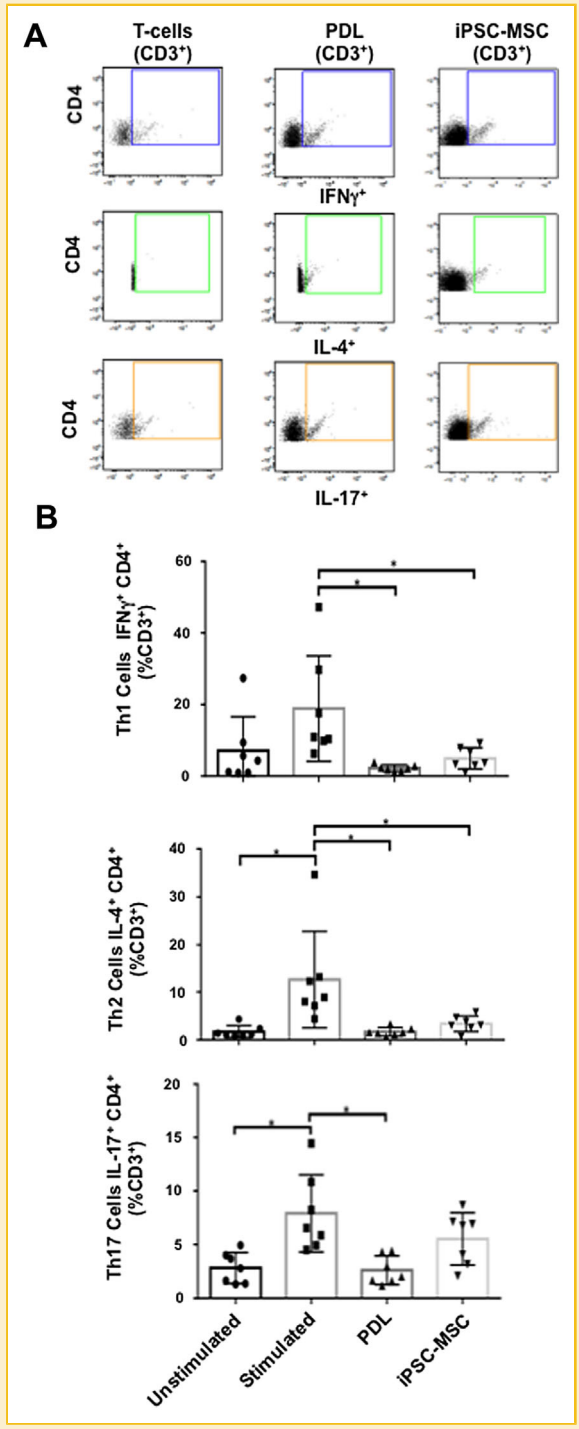

Fig. 5. PDLSC and iPSC-MSC like cells supress helper T-cells. CD3+ T-cells were stimulated with PMA/Ionomycin and co-cultured with PDLSC or PDLSC-iPSC-MSC like cells for 3 days, after which time the proportion of various CD4+ T-cell populations were assessed by flow cytometric analysis. (A) Representative histogram plots of CD4 and IFNγ, IL-4, or IL-17 cell surface staining on stimulated T-cells alone or in the presence of PDL cells or iPSC-MSC gated on the $CD3^+$ population. (B) The proportion of Th1 ($CD4^+IFN\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$), and Th17 ($CD4^+IL\text{-}17^+$) cells present within the $CD3^+$ T-cell population. Values represent mean percent fluorescence ± SD, n = 6–7 donors ($^* = P< 0.05$ as determined by one-way ANOVA with Turkey's multiple comparisons).

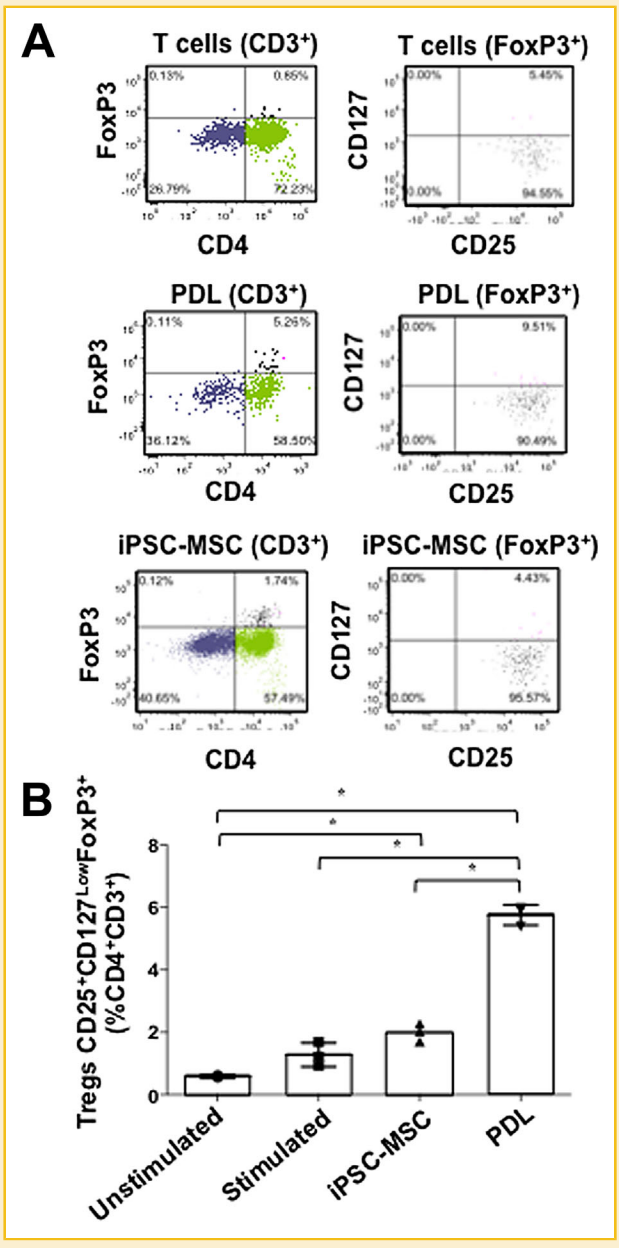

Fig. 6. PDLSC and iPSC-MSC like cells induce regulatory T-cells. CD3+ T-cells were stimulated with PHA and co-cultured with PDLSC or PDLSC-iPSC-MSC like cells for 5 days, after which time the proportion of various CD4+ T-cell populations were assessed by flow cytometric analysis. (A) Representative histogram plots of CD4 and Foxp3 expression on stimulated T-cells alone or in the presence of PDL cells or iPSC-MSC gated on the $CD3^+$ population, and CD25 bright and CD127 low population gated on $CD4^+FoxP3^+$ cells. (B) Proportion of Tregs in unstimulated, stimulated T cells alone or co-cultured with PDLSC or their iPSC-MSC. Values represent mean percent fluorescence ± SD, n = 3–4 donors (* = $P<0.05$ as determined by one-way ANOVA with Turkey's multiple comparisons).

demonstrated the capacity to suppress activated PBMNC through direct cell–cell interactions in co-cultures and through cell contact-independent mechanisms in transwell conditions. While the two different iPSC lines assessed displayed no significant differences in their ability to suppress allogenic PBMNC proliferation compared to one another, some variability between different iPSC-MSC lines was observed. These data concur with our previous findings demonstrating that the induction of somatic cells to iPSC and subsequent differentiation to MSC-like populations [Hynes et al., 2014], has varying effects on their functional properties. Similarly, other studies have also shown that the differences in the immunomodulatory properties between various iPSC-MSC lines may be attributed to the tissue of origin [Polo et al., 2010]. Of note, direct comparisons between PDL- and GF-derived iPSC-MSC with primary PDL and GF fibroblasts, respectively [Wada et al., 2009], suggested that the conversion of primary dental fibroblasts into iPSC lines followed by induction into iPSC-MSC had no major consequences on the immunosuppressive properties of the re-derived MSC populations.

The present study demonstrated that the suppression of PBMNC proliferation by iPSC-MSC and primary PDL cells and GF was not due to an increase in lymphocyte apoptosis, but rather through the inhibition of activated PBMNC cell division, as we and others have previously reported for different primary MSC-like populations [Glennie et al., 2005; Wada et al., 2009; Fu et al., 2012]. These findings suggest the involvement of other mechanisms at play, where MSC-like populations have been found to modulate immune cells by various mechanisms, including secretion of different cytokines and soluble factors [Di Nicola et al., 2002; Tse et al., 2003; Meisel et al., 2004; Beyth et al., 2005; Jiang et al., 2005; Wada et al., 2009; Wada et al., 2013; Xishan et al., 2013; Sivanathan et al., 2014], to suppress the proliferation of B cells and T cells, inhibit maturation of monocytes, and induce the generation of T regulatory cell and M2 macrophages [Rasmusson, 2006; Nauta and Fibbe, 2007; Nguyen et al., 2013]. Previous reports suggested that MSC-like populations derived from pluripotent populations use similar cytokines and soluble factors as bone marrow derived MSC to suppress T lymphocyte proliferation in allogenic mixed lymphocytes reaction assays [Fu et al., 2015; Schnabel et al., 2014]. As expected, *IDO1* transcript levels were upregulated in the different iPSC-MSC, PDL and GF cells albeit at varying levels. Similar trends of upregulated IL6 transcript, over a range of gene expression levels, were identified for the majority of iPSC-MSC populations and primary fibroblasts under the two co-culture conditions. These data imply that IDO-1 and IL-6 may be common factors utilized by different mesenchymal cell populations to induce their immunomodulatory affects. While *TGFB1* gene expression patterns were found to increase in the various iPSC-MSC populations and primary fibroblasts under transwell conditions, differential gene expression levels were observed in direct co-cultures with activated PBMNC. Therefore, different mechanisms are likely to dictate the efficiency of immunosuppression exhibited by the different iPSC-MSC and primary fibroblasts during direct cell-cell contact or distally, as we have previously reported between BMSC and skin fibroblasts [Wada et al., 2011a]. Whilst the few factors examined in the present study present a limited repertoire of the many mechanisms utilized by immunomodulatory cells, these analyses support the notion that iPSC-MSC and primary fibroblasts, modulate a cocktail of different pro- and anti-inflammatory cytokines, together with soluble factors to regulate different immune cell populations [Ranganath et al., 2012; Ankrum et al., 2014].

In the present study, PDL derived iPSC-MSC demonstrated the ability to significantly suppress effector T-cells and Th1/Th2 cells, and had a moderate affect on T_H17 cell proliferation, compared to primary PDL cells. Moreover, PDL derived iPSC-MSC exhibited a moderate capacity to stimulate the proliferation of Treg cells, via factors such as IDO and TGFβ, previously shown to be a key feature of primary MSC populations [Sivanathan et al., 2015]. Therefore, variations in the immune regulatory mechanisms used for each mesenchymal cell type could influence the overall immune/inflammatory response on different immune cell types and their subsets [Bettelli et al., 2008], that may ultimately impact on the therapeutic outcome for specific autoimmune/inflammatory based diseases [O′Connor et al., 2009].

With the emerging interest in the use of mesenchymal stem cell-like populations as potential therapies for immunological diseases, a stable and reliable source of stem cells capable of modulating the immune system would be highly beneficial [Klyushnenkova et al., 2005]. MSC derived from different pluripotent stem cells have shown promising results in potent immounomodulatory and therapeutic properties [Fu et al., 2015], and in some instances superior to the immunomodulatory properties of primary bone marrow derived MSC in vitro [Schnabel et al., 2014] and in vivo [Wang et al., 2014]. Published studies utilizing iPSC-MSC in pre-clinical animal models have also supported the immunomodulatory potential for these cells as treatment for multiple diseases including, periodontal disease [Hynes et al., 2013] myocardial infarction [Miao et al., 2014], lupus nephritis [Kimbrel et al., 2014], multiple sclerosis [Wang et al., 2014], and allergic airway inflammation [Sun et al., 2012]. Although previous studies have suggested the possibility of immunogenicity of iPSC-derived cells due to the presence of major histocompatibility complex (MHC) antigen class I expression [Okita et al., 2011], other studies have failed to demonstrate any significant immunogenicity affect [Liu et al., 2013; Schnabel et al., 2014]. This is probably due to a lack of expression of immune helper antigen such as HLA-DR (MHC Class II), CD40, CD80, and CD86 by different MSC-populations [Wada et al., 2009], including the PDL and GF derived iPSC-MSC used in this study (data not shown), which did express HLA-ABC class1 antigen akin to primary MSC. Other studies have proposed that the establishment of a MHC-typed bank of pluripotent stem cells may service a wide range of clinical indications as an alternative source of therapeutic grade stem cells [Tsuji et al., 2010], but may only be applicable to homogenous populations.

In conclusion, our results suggest that different tissue sources of iPSC-MSC may be useful as a potential alternative source to MSC-like populations for future clinical use in therapeutic applications because of their comparable immunosuppressive properties. Further elucidation of immunomodulatory properties of iPSC-MSC and their immunogenicity is required to determine the safety and efficacy of these populations for different clinical indications using pre-clinical animal models.

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