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Induced pluripotent stem cells have similar immunogenic and more potent immunomodulatory properties compared with bone marrow-derived stromal cells *in vitro*

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Abstract

Aim—To evaluate the *in vitro* immunogenic and immunomodulatory properties of induced pluripotent stem cells (iPSCs) compared with bone marrow-derived mesenchymal stromal cells (MSCs).

Materials & methods—Mouse embryonic fibroblasts (MEFs) were isolated from C3HeB/FeJ and C57BL/6J mice, and reprogrammed to generate iPSCs. Mixed leukocyte reactions were performed using MHC-matched and -mismatched responder leukocytes and stimulator leukocytes, iPSCs or MSCs. To assess immunogenic potential, iPSCs and MSCs were used as stimulator cells for responder leukocytes. To assess immunomodulatory properties, iPSCs and MSCs were cultured in the presence of stimulator and responder leukocytes. MEFs were used as a control.

Results—iPSCs had similar immunogenic properties but more potent immunomodulatory effects than MSCs. Co-culture of MHC-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T-cell proliferation than observed for MHC-mismatched leukocytes alone and at more responder leukocyte concentrations than with MSCs. In addition, MHC-mismatched iPSCs significantly reduced responder T-cell proliferation when co-cultured with MHC-mismatched leukocytes, while MHC-mismatched MSCs did not.

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Author contributions

All authors contributed to the study design. LV Schnabel, CM Abratte, JM Cassano and JA Cross performed the experiments. TL Southard carried out histologic assessments on the teratoma assays. All authors contributed to data analysis and interpretation. LV Schnabel and LA Fortier were responsible for drafting the manuscript. All authors revised the manuscript and approved the final version.

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Conclusion—These results provide important information when considering the use of iPSCs in place of MSCs in both regenerative and transplantation medicine.

Keywords

allogeneic stem cell therapy; immunogenicity; immunomodulatory; induced pluripotent stem cell; mesenchymal stromal cell; transplantation medicine

Induced pluripotent stem cells (iPSCs) were first generated in 2006 and regarded as the most promising stem cell candidate for the clinical application of regenerative therapies [1–3]. iPSCs are pluripotent, unlike mesenchymal stromal cells (MSCs), and can be used in an autologous manner, unlike pluripotent embryonic stem cells (ESCs). Additionally, iPSCs avoid the ethical concerns surrounding the isolation and use of human ESCs [4]. However, many concerns have been raised over the safety of iPSCs in terms of genetic instability, tumorigenic potential and immunogenic potential [4–10]. It has become evident that iPSC lines must be thoroughly screened for stability, safety and efficacy prior to clinical application [7,10]. Such screening, after an already lengthy generation process, makes autologous iPSC use impractical for many of the diseases that would potentially benefit from stem cell therapy. Furthermore, it has been demonstrated that genetic background affects generation of iPSCs, suggesting that autologous iPSC therapy may not be feasible for some patients regardless of timing issues [11]. For these reasons, the immunogenicity of iPSCs is of particular concern as the need for having a bank of previously screened cells has become a reality [7,10,12].

The immunogenic and immunomodulatory properties of MSCs continue to be investigated so that these cells can be available at the time of diagnosis for immediate treatment [13–19]. Both the genetic background and age of the patient affect proliferation and differentiation rates of MSCs, suggesting that allogeneic MSC therapy may be required for some patients as for iPSCs [20–22]. Adult MSCs have low immunogenicity when used in an autologous manner and possess significant immunomodulatory properties [14,16,23–26]. Many mechanisms for the immunosuppressive effects of MSCs have been described including inhibition of T-cell proliferation, alteration of dendritic cell maturation, induction of regulatory lymphocytes and apoptosis of CD8⁺ T cells [16,23,27–29]. Mesenchymal stem cells were initially believed to be immune privileged due to these immunosuppressive properties [30–33], but immune rejection of allogeneic MSCs has also been reported [34–39]. The finding that MSCs are capable of fluctuations in their MHC class I and II expression profiles is likely the cause of these conflicting results. MSCs from many species were originally described as having the phenotype of high MHC class I expression and low or negative MHC class II expression, but MSCs from mice, humans and more recently horses with high MHC II expression levels have also been described [34,35,40,41]. Additionally, both MHC class I and II expression levels on MSCs can be upregulated by proinflammatory cytokines such as IFN- γ [42,43]. These studies suggest that MSCs have a dynamic immunophenotype that can alter their immune status.

Investigation into the immunogenic properties and immune plasticity of iPSCs has just recently begun [7,12,44–47]. It is known that undifferentiated iPSCs, like ESCs, express low

or absent levels of MHC class I, and are negative for MHC class II expression [47,48]. Unlike MSCs, iPSC MHC class II expression is not upregulated by differentiation or by stimulation with IFN- γ [47–50]. The extent to which MHC class I expression can change upon iPSC differentiation or stimulation with proinflammatory cytokines, however, is not understood. Several studies have shown increased MHC class I expression in iPSCs with differentiation or IFN- γ stimulation, but often to a level still much less than that of somatic cells [45,47,48]. The consequence of such a change in MHC class I expression is complex as a high expression level of MHC class I could lead to T-cell activation while a continued lack of MHC class I expression could result in iPSCs being targeted by natural killer (NK) cells *in vivo* [6,47]. Conflicting results have been reported for ESCs on this subject, with some groups reporting ESCs as susceptible to NK cell lysis, and others reporting that ESCs are neither susceptible to NK cell lysis nor capable of eliciting T-cell responses [6,51]. It is likely that culture conditions or differences in ESC lines could have affected these results.

It is not surprising that conflicting results have also been reported on the immunogenicity of iPSCs, as iPSCs are in many ways more variable than ESCs, particularly with the discrepancies in reprogramming methods including viral versus nonviral and integrating versus nonintegrating [44–47,49,52,53]. The first report on immunogenicity of iPSCs revealed that undifferentiated autologous (syngeneic) mouse iPSCs were immune rejected in a teratoma model study [44]. Two other reports since then have shown that both undifferentiated and differentiated syngeneic mouse iPSCs are non-immunogenic *in vitro* and *in vivo* [45,46]. To date, no studies have examined the immunomodulatory properties of iPSCs even though it is known that ESCs are capable of immunosuppression through multiple mechanisms including expression of arginase I [49,54], prevention of dendritic cell maturation [55] and up-regulation of regulatory T cells [49,56]. When considering the use of iPSCs as an alternative for MSC therapy, this information is critical. The purpose of this study, therefore, was to evaluate the *in vitro* immunogenic and immunomodulatory properties of iPSCs compared with adult bone marrow-derived MSCs using modified mixed leukocyte reactions (MLRs). Our hypothesis, based on prior ESC knowledge, was that undifferentiated iPSCs would have similar immunogenic and immunomodulatory properties as MSCs.

Materials & methods

A schematic of the study design and methods is shown in Figure 1.

Mice

Male and female mice of the C3HeB/FeJ (MHC H2 haplotype *k*) and C57BL/6J (MHC H2 haplotype *b*) inbred strains were purchased from The Jackson Laboratory (ME, USA). For each strain, mice were bred to produce offspring needed to harvest different cell types and perform experiments. NOD.CB17-*Prkdc*^{scid}/J mice, used for teratoma formation assays, were also purchased from The Jackson Laboratory. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of Cornell University.

Mouse embryonic fibroblast isolation

Embryonic day 13.5 C3HeB/FeJ and C57BL/6J embryos were collected and processed to generate mouse embryonic fibroblasts (MEFs) from each strain, as previously described [11]. MEFs were cultured in MEF media (high-glucose Dulbecco's Modified Eagle's Medium [DMEM]), containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml) and cryopreserved at passage 1 (P1) for iPSC generation and at P2 for controls in MLR experiments. MEFs to be used as feeder cells from each strain were culture expanded, irradiated with 30 Gy from a Cs-137 source, and cryopreserved.

Lentiviral reprogramming of MEFs

Lentiviral supernatant generation and reprogramming of MEFs was performed as previously described by our laboratory [11] using vectors for doxycycline-inducible transgene expression of the mouse factors *Oct4*, *Sox2*, *Klf4* and *c-Myc*. All plasmids were purchased from Addgene (MA, USA). Briefly, P1 MEFs from each strain were thawed and cultured in MEF media for 24–48 h, after which they were trypsinized and counted. The P2 MEFs were seeded onto gelatin-coated six-well tissue culture plates at a density of 6.75×10^3 cells/cm² in MEF media and allowed to adhere for 24 h. Culture media was replaced with fresh MEF media supplemented with viral super-natant for an additional 24 h. Following incubation with viral media, MEFs were trypsinized and passaged onto 60 mm tissue culture plates seeded with feeder cells of the same strain. Culture media was changed to ESC media (KnockOut™ DMEM (Gibco, NY, USA) supplemented with 15% KnockOut™ Serum Replacement (Gibco), recombinant LIF, MEM non-essential amino acids solution (100 µm), 2 mM GlutaMAX™ (Gibco), 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 µg/ml) and doxycycline (2 µg/ml; Sigma, MO, USA). Media was refreshed daily during reprogramming.

piggyBac reprogramming of MEFs

Passage 2 MEFs were transfected with the Nucleofector® II electroporation device (Amaxa Biosystems, MD, USA) set on program A-023. Each electroporation was performed in a 2-mm cuvette (Amaxa Bio-systems) with 2×10^6 cells and a DNA mixture of 1 µg each of the *piggyBac* plasmids PB-TET-MKOS, PB-CAG-rtTA and PB-CAG-GFP (kindly provided by the laboratory of Dr Nagy [57]), as well as 1 µg of the transposase expression vector pCyL43 (Wellcome Trust Sanger Institute, Cambridge, UK) in a total volume of 100 µl Ingenio® electroporation solution (Mirus Bio, WI, USA). Following electroporation, cells from each cuvette were seeded onto a 100-mm tissue culture plate in MEF media. After 24 h, culture media was changed to ESC media.

iPSC line generation

Lentiviral and *piggyBac* iPSC colonies were picked with pipette tips and culture expanded on feeder cells in ESC media, as previously described [11]. Lentiviral iPSC colonies were picked on day 7–11 of reprogramming, while *piggyBac* iPSC colonies were picked on day 17–22 post-transfection. Doxycycline was removed from media around P7 and doxycycline-independent cell lines were then further expanded (P10-P12) in order to reach cell numbers necessary for teratoma formation assays and cryopreservation of stock from each strain. In

preparation for MLR experiments, iPSC cell lines from each strain were further cultured in modified RPMI 1640 media containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 µg/ml), and ESGRO® LIF (1 µl/ml; Millipore, MA, USA). Following transition to modified RPMI 1640 media, teratoma assays were again performed.

Teratoma formation & histological analysis

iPSC lines from each strain were trypsinized, pelleted and suspended at 1×10^7 cells/ml in a 1:3 solution of Matrigel™ (BD Biosciences, CA, USA) to MEF media. Of this cell suspension, 150 µl (1.5×10^6 cells) was injected subcutaneously into the flank of a NOD.CB17-*Prkdc^{scid}*/J mouse [11]. For each cell line, a total of two to four injections were performed. A total of 4–5 weeks post injection, tumors were surgically dissected, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. All histologic sections were reviewed by a board-certified veterinary pathologist (Teresa L Southard) for teratoma formation.

Bone marrow harvest & isolation of MSCs

Ten female mice 3–6 weeks of age from each strain were euthanized, prepared with ethanol and processed for bone marrow harvest according to a protocol kindly provided by the laboratory of Dr Rocky S Tuan [Pers. Comm.]. Hindlimbs were skinned, disarticulated from the pelvis, and placed in a petri dish with MEMα media with nucleosides and l-glutamine (Gibco) containing 10% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and Fungizone® (0.25 µg/ml; Gibco). All muscle and tissue was removed from the bone using a scalpel blade and placed in a second petri dish with phosphate-buffered saline (PBS). Next, the ends of the long bones were cut off so that the marrow cavity was exposed. The marrow cavity of each bone was flushed with MEMα media using a 27-g needle and 12-ml syringe into an empty petri dish. The bone marrow cell suspension was then passed through a 70-µm cell strainer (BD Biosciences, CA, USA), pelleted, resuspended in red blood cell lysis buffer (0.84% NH₄Cl), and incubated for 2 min on ice. Following the incubation, cells were washed with MEMα media, counted and seeded at 25×10^6 cells/ml onto 100-mm tissue culture plates with MEMα media. After 5 h of incubation, media was removed, plates were gently washed to remove non-adherent cells, and new MEMα media containing FGF-2 (5 ng/ml) added. For the next 72 h, media exchange occurred every 12 h; afterwards media exchange occurred every 72 h. When cells were approximately 80% confluent, they were trypsinized at room temperature for 2 min, counted, and seeded at 5000 cells/cm² onto T-75 flasks with MEMα media containing FGF-2 (5 ng/ml). Cells were expanded to P2 and P3, and stocks were cryopreserved for immunophenotyping and MLR experiments.

Immunophenotyping of MEFs, iPSCs & MSCs

Mouse embryonic fibroblasts (MEFs), iPSCs and MSCs were immunophenotyped for expression levels of MHC class I and II. MSCs were additionally phenotyped for a panel of positive (CD44, CD29) and negative (CD45, CD117) markers using flow cytometry [58–60]. Leukocytes were used as a control cell type. MHC class I (rat anti-mouse; PE-conjugated), MHC class II (rat anti-mouse; PE-Cy5-conjugated) and CD29 (hamster anti-

mouse; PE-conjugated) antibodies were purchased from eBioscience (Affymetrix, CA, USA). CD44 (rat anti-mouse; FITC-conjugated), CD45 (rat anti-mouse; PerCP-Cy5.5) and CD117 (rat anti-mouse; APC-conjugated) antibodies were purchased from BD Biosciences. Cells were pelleted in aliquots containing approximately 1×10^6 cells on 96-well V-bottom plates and treated with a 10-min blocking step using anti-mouse CD16/CD32 (Fc Block™; BD Biosciences) at 1:100 in PBS. Cells were pelleted and resuspended in conjugated primary antibody and incubated for 45 min at 4°C. Cells were then washed, resuspended in PBS and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, CA, USA) flow cytometer equipped with 488-µm argon and 635-µm red diode lasers and BD Cell Quest™ analysis software (BD Biosciences). Cells exposed to appropriately conjugated rat or hamster IgG were used as negative isotype controls. Data were collected on 2×10^4 cells for each sample.

Splenocyte isolation & leukocyte purification

Spleens were aseptically harvested from C3HeB/FeJ and C57BL/6J female mice 6–12 weeks of age and dissociated in RPMI 1640 media (Gibco) using a cell dissociation sieve equipped with a 40-mesh screen (Sigma-Aldrich). The resultant splenocyte suspension was passed through a 100-µm cell strainer (BD Biosciences), pelleted, resuspended in red blood cell lysis buffer (0.84% NH₄Cl) and incubated for 5 min at room temperature with rocking. Following red blood cell lysis, the suspension was washed with PBS, pelleted and purified using Lympholyte®-M density gradient centrifugation (Cedarlane Laboratories, NC, USA) according to the manufacturer's directions to obtain leukocytes. Cells destined to be stimulator leukocytes in MLRs were aliquoted at this time. The remaining leukocyte suspension was plated onto 100-mm tissue culture plates in RPMI 1640 medium (Gibco) containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 units/ml). After 2 h, nonadherent cells were removed from plates, pelleted and counted. Non-adherent leukocytes were then positive selected for CD62L, a naive T-cell marker [61–64], using MACS CD62L microbeads and LS columns (Miltenyi Biotec, CA, USA), according to manufacturer directions. Adherent leukocytes (containing antigen-presenting cells [APCs]) were dissociated using Accumax® cell dissociation solution (Innovative Cell Technologies Inc, CA, USA), counted and aliquoted. All leukocytes were used fresh in MLRs.

Modified one-way MLRs

Modified one-way MLRs were performed in duplicate in 24-well tissue culture plates using MHC-matched and -mismatched C3HeB/FeJ responder leukocytes and C3HeB/FeJ and C57BL/6J stimulator leukocytes, MEFs, iPSCs and MSCs. In order to assess immunogenic potential of cells, MEFs, iPSCs and MSCs were used as stimulator cells for responder leukocytes. In order to assess the immunomodulatory properties of cells, MEFs, iPSCs and MSCs were cultured in the presence of stimulator and responder leukocytes. MHC-matched stimulator leukocytes were used to establish baseline T-cell proliferation and MHC-mismatched stimulator leukocytes were used as positive MLR controls. MEFs were considered the negative control in immunomodulatory potential studies. C3HeB/FeJ responder leukocytes were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE [0.13 µg/ml], Sigma- Aldrich) and examined at four different concentrations (2

$\times 10^5$, 4×10^5 , 8×10^5 and 1.2×10^6 cells/well). The proliferative ability of responder cells was verified via mitogen stimulation with phytohemagglutinin (PHA-P [5 μ g/ml], Sigma-Aldrich). Stimulator MEFs, iPSCs and MSCs were plated 24 h prior to addition of responder leukocytes in their appropriate media such that all cells would be approximately 80% confluent by the end of the experiment. MEFs were seeded at 1×10^4 cells/well, iPSCs (removed from feeders) at 7.5×10^4 cells/well, and MSCs at 3×10^4 cells/well. Stimulator leukocytes were irradiated with 9 Gy from a Cs-137 source to inhibit proliferation and plated at 1.6×10^6 cells/well. Responder APCs (adherent cells during isolation) were plated at 1×10^5 cells/well. Importantly, responder leukocytes and APCs were mixed with stimulator leukocytes prior to plating due to concern that the 3D nature of the iPSC colonies could interfere with responder and stimulator cell contact. The resultant ratios of responder:stimulator cells was based on previously published experimental protocols and determined to be optimal for these studies in preliminary experiments [65–67]. Cultures were maintained for 5 days with modified RPMI 1640 media (1.5 ml/well) containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 μ g/ml), and ESGRO® LIF (1 ul/ml; Millipore, MA, USA). Media were not exchanged over the 5 days. Following culture, leukocytes were aspirated from wells and stained with a hamster anti-mouse APC-conjugated CD3 antibody (Abcam, MA, USA). The antibody staining process for flow cytometry analysis was performed as described above for immunophenotyping.

Proliferation of gated CFSE-labeled CD3⁺ responder T cells was evaluated via CFSE attenuation using flow cytometry. Cells were first gated on FL4 so that only CD3⁺ cells (T cells) were then examined on FL1 for CFSE attenuation. Nonstimulated responder T cells were used to set the boundary of nonproliferating cells such that all cells to the left (lower fluorescence intensity on FL1) of that boundary were determined to be proliferating. Because the number of cell counts in the proliferating T-cell gate was measured, data was collected on the entirety of each sample.

MLRs were performed in a total of three separate experiments. MEFs from two different embryos of each strain were tested in addition to three iPSC lines (two lentiviral and one *piggyBac*) from each strain and batched MSCs from each strain. Due to naturally occurring variation in leukocyte responses between mice and experiments, the relative T-cell proliferation was reported as the fold change from that of MHC-matched MLR for the immunogenic potential experiments (i.e., looking for an increase from baseline T-cell proliferation potential if immunogenic) and as the percentage proliferation of MHC-mismatched MLR for the immunomodulatory potential experiments (i.e., looking for a decrease from positive control T-cell proliferation if immunomodulatory).

Measurement of cytokine concentrations in MLR media

Media from immunomodulatory MLR experiments were harvested after centrifugation to pellet leukocytes for flow cytometry and stored at -80°C in aliquots with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche, IN, USA). Media from control MHC-mismatched MLRs and MHC-mismatched MLRs cultured in the presence of matched or mismatched MCSs or iPSCs were pooled according to experimental group and assayed for active IFN- γ , prostaglandin E₂ (PGE₂), and TGF- β 1 concentrations using the IFN- γ

Quantikine ELISA kit (R&D Systems, MN, USA), the Prostaglandin E2 ELISA kit (Abcam, MA, USA) and the TGF β 1 Quantikine ELISA kit (R&D Systems), respectively.

Statistical analyses

Mixed leukocyte reaction (MLR) data for lentiviral (average of the two lines tested) and *piggyBac* iPSC lines were first compared using two-sample t-tests. All MLR data were normalized by log transformation and analyzed with analysis of covariance (ANCOVA), with experiment as a covariate, followed by a Tukey multiple comparisons test. All ELISA data were determined to be normally distributed via the Shapiro–Wilk test and were analyzed by analysis of variance (ANOVA) followed by a LSD multiple comparisons test. Analyses were performed using Statistix 9 software (Analytical Software, FL, USA) and significance was set at $p = 0.05$.

Results

iPSC line generation & validation

Multiple doxycycline-independent lentiviral and *piggyBac* iPSC lines were established from each strain and early passage stocks were cryopreserved. Two doxycycline-independent lentiviral iPSC lines and one doxycycline *piggyBac* iPSC line from each strain was tested after expansion in ESC media and then again after expansion in modified RPMI 1640 media. These lines were capable of producing teratomas in NOD.CB17-*Prkdc*^{scid}/J mice by 5 weeks post injection (Supplementary Figure 1; please see online at www.futuremedicine.com/doi/full/10.2217/rme.14.29), thereby confirming pluripotency and lack of alteration due to the change in media.

Immunophenotyping

MEFs had a phenotype of MHC class I positive (low) and MHC class II negative, while iPSCs had a phenotype of both MHC class I and II negative (Supplementary Table 1). Mesenchymal stromal cells (MSCs) were positive for expression of MHC class I, CD44 and CD29, and negative for expression of MHC class II, CD45 and CD117 (Supplementary Figure 1).

Modified one-way MLRs

There were no significant differences in responder T-cell proliferation when stimulated by lentiviral iPSCs or *piggyBac* iPSCs, or when stimulated by MHC-mismatched leukocytes in the presence of lentiviral iPSCs or *piggyBac* iPSCs. Cells were therefore considered one group and are collectively referred to as iPSCs for the remainder of the results.

As predicted based on MHC class II expression, all cell types tested (MEFs, iPSCs and MSCs) had low immunogenicity when either MHC-matched or MHC -mismatched with responder leukocytes (Figures 2 & 3). MHC-mismatched MSCs resulted in the highest levels of responder T-cell proliferation compared with MEFs and iPSCs, but these levels still did not reach those of the positive control of MHC-mismatched leukocytes (Figure 3E–H).

At the majority of responder leukocyte concentrations tested, both iPSCs and MSCs cultured in the presence of MHC-mismatched responder and stimulator leukocytes (MHC-mismatched MLR) resulted in a reduction of responder T-cell proliferation from that observed for the MHC-mismatched MLR baseline value (Figures 4 & 5). Importantly, MEFs cultured in the presence of MHC-mismatched responder and stimulator leukocytes were unable to reduce responder T-cell proliferation in any of responder leukocyte concentrations tested. Reduction of responder T-cell proliferation was greatest when iPSCs and MSCs were MHC-matched with responder leukocytes compared with when MHC-mismatched with responder leukocytes. MHC-matched iPSCs resulted in significantly decreased responder T-cell proliferation compared with both MHC-mismatched MLR and the negative control of MEFs at the three highest responder leukocyte concentrations (Figure 5B–D). While MHC-matched MSCs resulted in statistically equivalent responder T-cell proliferation compared with iPSCs for the same three responder leukocyte concentrations, mean T-cell proliferations were greater than for iPSCs and in some cases also equivalent to MHC-mismatched MLR and/or the negative control of MEFs (Figure 5B–D). When MHC-mismatched cell types were evaluated, only iPSCs were able to reduce responder T-cell proliferation significantly from MHC-mismatched MLR at the responder leukocyte concentration of 8×10^5 cells (Figure 5G). Once again, while MHC-mismatched MSCs resulted in statistically equivalent responder T-cell proliferation compared with iPSCs at this concentration, the mean T-cell proliferation was greater than for iPSCs and also equivalent to that of MEFs and the positive control of MHC-mismatched leukocytes (Figure 5G).

Measurement of cytokine concentrations in MLR media

Based on the immunomodulatory results described above, only media from the immunomodulatory MLR experiments at the responder leukocyte concentrations of 4×10^5 and 8×10^5 cells were assayed. At both responder leukocyte concentrations tested, there was a significant reduction in the concentration of IFN- γ in the MLR media when MHC-mismatched leukocytes were cultured in the presence of matched MSCs, matched iPSCs or mismatched iPSCs. While the presence of matched MSCs resulted in the lowest mean IFN- γ for all groups tested, the presence of mismatched MSCs had a significant but lesser effect at the responder leukocyte concentration of 4×10^5 cells and no significant effect at the responder leukocyte concentration of 8×10^5 cells (Figure 6A & D). At both responder leukocyte concentrations tested, the presence of either matched or mismatched MSCs resulted in the highest mean PGE₂ concentrations compared with control and both matched and mismatched iPSCs. For mismatched MSCs, however, such concentrations were only significantly greater than control at responder leukocyte concentration of 4×10^5 cells. Although mean PGE₂ concentrations were higher in media from MHC-mismatched MLRs cultured in the presence of either matched or mismatched iPSCs compared with control MHC-mismatched MLRs, these results were not statistically significant (Figure 6B & E). No significant differences in media TGF- β 1 concentrations were found for any of the cell types tested (Figure 6C & F).

Discussion

In this study we directly compared iPSCs to MSCs in terms of immunogenicity and immunomodulatory capability using MLRs. Our comparisons revealed that iPSCs generated through both lentiviral and *piggyBac* reprogramming methods have similar immunogenic properties as MSCs and may possess more potent immunomodulatory properties than MSCs *in vitro*. Co-culture of MHC-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T-cell proliferation than observed for MHC-mismatched leukocytes alone at more responder leukocyte concentrations tested than was observed for co-culture of MHC-mismatched leukocytes with MHC-matched MSCs. In addition, MHC-mismatched iPSCs were able to significantly reduce responder T-cell proliferation at the responder leukocyte concentration of 8×10^5 cells when co-cultured with MHC-mismatched leukocytes while MHC-mismatched MSCs were not.

None of the cells (MEFs, iPSCs or MSCs) tested in this study were irradiated for use in MLRs due to the fact that iPSCs died following even very low doses (100–200 rads) of gamma irradiation. This finding suggests that iPSCs undergo p53-independent apoptosis in response to DNA damage as described for ESCs [68–70] rather than p53-mediated cell cycle arrest as is well described for somatic cells [71]. For this reason, preliminary experiments were performed to determine the proper seeding density of all cell types such that they were approximately 80% confluent on the 5th (final) day of MLR culture. This method is different than most previously described for MSC immunology studies examining MSC effects in MLRs in which MSCs are irradiated and then plated at different ratios to responder leukocytes [26,42]. It is possible that because the cells were growing that they could have either not reached or surpassed the optimal cell:leukocyte ratio for immunomodulation in some instances. The fact that both MHC-matched iPSCs and MSCs were able to significantly downregulate responder T-cell proliferation to as much as 25–30% of that of the MHC-mismatched MLR, however, suggests a broad enough range of responder leukocyte cell concentrations was covered to confidently determine whether the cells were causing immunomodulation.

Due to the nature of MLRs, it must also be considered that T-cell proliferation may have been falsely diminished due to T-cell competition with growing cells for nutrients in the media. At no point during the experiments, however, did the media appear exhausted in color. More importantly, co-culture with MEFs, which were rapidly growing and just as confluent as the other cell types by the end of the experiments, did not result in reduced T-cell proliferation. This argues against nutrient competition or depletion as a reason for the reduced responder T-cell proliferation observed in MLR co-cultures with iPSC and ESCs. Thus, MEFs were a critical experimental control for these immunomodulatory experiments. Another potential concern is that the leukocyte media used included leukemia inhibitory factor (LIF), which has been shown to have a role in MSC-mediated immunosuppression [24]. LIF is commonly used in iPSC media to maintain pluripotency and prevent differentiation [1,2,11]. It was used in these experiments for that reason and also to avoid use of feeder cells with iPSCs culture, which would have further complicated experimental design and interpretation [72]. The same leukocyte media with LIF was used for all MLR experiments, which should have prevented any biases between cell type comparisons and

against MHC-matched and mismatched MLR controls. Again, the finding that control MEFs did not cause significant downregulation of responder T-cell proliferation argues against this concern.

The finding that iPSCs and MSCs that were MHC-matched with respect to the responder leukocytes resulted in a greater reduction in responder T-cell proliferation compared with iPSCs and MSCs that were MHC mismatched is interesting. Engraftment studies evaluating the effect of MSCs have previously demonstrated similar findings, with only syngeneic (MHC-matched) MSCs resulting in enhanced engraftment [17,38]. Previous MLR studies evaluating the immunosuppressive effects of ESCs, however, have found no difference in using MHC-matched or mismatched ESCs with the responder leukocytes [73]. The reasons for this discrepancy between iPSCs and MSCs is unclear, but could be due to specific immunosuppressive mechanisms employed by cells or due to differences in MHC antigen expression between cell types with iPSCs expressing very low or negligible levels of MHC class I and MSCs expressing high levels of MHC I. Of note is the fact that although MLR co-cultures with mismatched MSCs resulted in high levels of PGE₂ in the media that were fairly consistent with that of matched MSCs, mismatched MSCs were unable to produce the same decrease in IFN- γ concentration as was observed in media from MLR co-cultures with matched MSCs. This result suggests that perhaps the immune stimulus of mismatched MSCs was strong enough to cause responder T-cell IFN- γ secretion despite the expected downregulation or inhibition of IFN- γ by PGE₂ secreted from the MSCs [16]. Future studies evaluating the kinetics of responder T-cell proliferation during such MLR co-cultures may prove useful for distinguishing between increased immunogenicity and decreased immunomodulatory potential of the cells.

It is also an important finding that the significant decrease in media IFN- γ concentrations in MLRs co-cultured with iPSCs did not correlate well with the very modest increases in PGE₂ concentrations in the same media. This suggests that iPSCs are inhibiting responder T-cell IFN- γ secretion via a mechanism other than PGE₂. Further studies must be performed to elucidate this result, including examination of other soluble factors potentially expressed by iPSCs and MSCs into the media during MLRs such as TGF- β 2, IL-6, IL-10, IDO and LIF [13,14,16,24,32,47,74], as well as examination of arginase-I expression by iPSCs, which could be responsible for the inhibition of responder T-cell IFN- γ secretion as previously described for ESCs [49,54]. Evaluation of the gene expression levels of these soluble factors by different cell types would also be of great interest because media additives such as LIF could be affecting protein expression and because proteins could be rapidly degrading in the culture system. While this would be difficult to perform in the MLR co-culture system described in this study in which all cell types are mixed together within a well, it is possible that the cell types could be sorted at the end of the culture system. Once suspect factors have been identified, the next essential study would be to inhibit these factors and determine whether or not responder T-cell proliferation is restored in the MLR co-culture system.

Perhaps the greatest question raised by this study is whether or not iPSCs can retain their immunogenic and immunomodulatory properties upon differentiation as it is unlikely that undifferentiated iPSCs will be used in human clinical applications due to concerns of teratoma formation. Follow-up studies must focus on differentiating iPSC lines into specific

cell types and then re-evaluating their MHC class I and II expression in addition to their immunogenic and immunomodulatory properties in MLRs. Soluble factor release into the media by these cells during MLRs must also be re-assessed and compared with levels pre-differentiation. Once these *in vitro* studies have been completed, *in vivo* studies evaluating the immunogenic and immunomodulatory effect of iPSCs outside the controlled environment of the MLR must be performed. Such studies will be critical for consideration of iPSC use in the place of MSCs for both regenerative medicine and transplant medicine [7,12,52].

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• of interest

•• of considerable interest

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Executive summary

Introduction (rationale & aim)

- The immunologic properties of induced pluripotent stem cells (iPSCs) require investigation as it has become evident that banked iPSCs will be needed for most clinical applications due to cell generation time and time associated with screening for both efficacy and safety.
- The aim of this study was to evaluate the *in vitro* immunogenic and immunomodulatory properties of iPSCs compared with adult bone marrow-derived MSCs using modified mixed leukocyte reactions.

Materials & methods

- In order to assess immunogenic potential, iPSCs and MSCs were used as stimulator cells for responder leukocytes. In order to assess immunomodulatory properties, iPSCs and MSCs were cultured in the presence of stimulator and responder leukocytes. MEFs were used as a control.

Results

- iPSCs generated through both lentiviral and *piggyBac* reprogramming methods had similar immunogenic properties and more potent immunomodulatory properties than MSCs *in vitro*.
- Co-culture of major histocompatibility complex (MHC)-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T-cell proliferation than MHC-mismatched leukocytes alone at more responder leukocyte concentrations tested than was observed for co-culture of MHC-matched leukocytes with MHC-matched MSCs.
- MHC-mismatched iPSCs were able to significantly reduce responder T-cell proliferation at the responder leukocyte concentration of 8×10^5 cells when co-cultured with MHC-mismatched leukocytes, while MHC-mismatched MSCs were not.
- A significant decrease was found in media IFN- γ concentrations in MLRs co-cultured with iPSCs; however, this decrease did not correlate well with the modest increase in prostaglandin E₂ concentration in the same media.

Discussion & conclusion

- iPSCs are presumably inhibiting responder T-cell IFN- γ secretion via a mechanism other than prostaglandin E₂ as for MSCs.
- Further studies must be performed in order to determine whether iPSCs retain their immunogenic and immunomodulatory properties upon differentiation into specific cell or tissue types.
- This information is critical when considering the use of iPSCs in the place of MSCs for both regenerative medicine and transplant medicine.

Figure 1. Schematic of the study design and methods used

iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stem cell.

Figure 2. Immunogenicity (A) of MHC-matched, and (B) MHC-mismatched MEFs, MSCs and iPSCs as determined by responder T-cell proliferation in modified one-way mixed leukocyte reactions

Data are presented as the log fold change of MHC-matched MLR, which was considered the baseline responder T-cell proliferation value. Bars represent mean $\pm$ SD from a total of three separate experiments performed with multiple cell lines.

MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell.

Figure 3. Immunogenicity of mouse embryonic fibroblasts, mesenchymal stem cells and induced pluripotent stem cell as determined using modified one-way mixed leukocyte reactions and with data reported as the relative T-cell proliferation, which was calculated as the log fold change from MHC-matched MLR

MLR results as shown in Figure 2 are displayed here for MHC-matched cells (A-D) and MHC-mismatched cells (E-H) at the different responder leukocyte concentrations tested. Bars represent mean $\pm$ SD from a total of three separate experiments performed with multiple cell lines. Superscript letters indicate significant differences between groups by analysis of covariance, with experiment as a covariate, followed by a Tukey multiple comparisons test, $p < 0.05$.

iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell

Figure 4. Immunomodulatory potential of (A) MHC-matched and (B) MHC-mismatched MEFs, MSCs and iPSCs as determined by responder T-cell proliferation in modified one-way mixed leukocyte reactions in which MHC-mismatched leukocytes were cultured in the presence or absence of these cells

Data are presented as the percentage proliferation of the control MHC-mismatched MLR as a decrease in proliferation is indicative of immunomodulation. Bars represent mean $\pm$ SD from a total of three separate experiments performed with multiple cell lines. A reference line has been placed at 100% to denote the positive control of the MHC-mismatched MLR. iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell.

Figure 5. Immunomodulatory potential of mouse embryonic fibroblasts, mesenchymal stem cells and induced pluripotent stem cells as determined using modified one-way mixed leukocyte reactions in which MHC-mismatched leukocytes were cultured in the presence or absence of these cells

Data are reported as the relative T-cell proliferation, which was calculated as the percentage proliferation of the MHC-mismatched MLR alone. MLR results as shown in **Figure 4** are displayed here for MHC-matched cells (**A–D**) and MHC-mismatched cells (**E–H**) at the different responder leukocyte concentrations tested. Bars represent mean $\pm$ SD from a total of three separate experiments performed with multiple cell lines. Reference lines have been placed at 100% to denote the positive control of the MHC-mismatched MLR. Superscript letters indicate significant differences between groups by ANCOVA, with experiment as a covariate, followed by a Tukey multiple comparisons test, $p < 0.05$. iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell.

Figure 6. Immunomodulatory mixed leukocyte reaction experiment media concentrations of (A & D) IFN- γ , (B & E) PGE $_2$ and (C & F) TGF- β 1 for control MHC-mismatched MLRs and for MHC-mismatched MLRs cultured with matched or mismatched MSCs or iPSCs

Media from experiments using responder leukocyte concentrations of 4×10^5 cells (A–C) and 8×10^5 cells (D–F) were examined based on the significant immunomodulatory results found at these concentrations as shown in Figures 4 & 5. Bars represent mean $\pm$ SD from a total of three pooled samples of each cell type from each MLR experiment. Superscript letters indicate significant differences between groups by ANOVA, followed by a LSD multiple comparisons test; $p < 0.05$.

iPSC: Induced pluripotent stem cell; MEF: Mouse embryonic fibroblast; MHC-M: MHC-matched; MHC-MM: MHC-mismatched;

MLR: Mixed leukocyte reaction; MSC: Mesenchymal stromal cell; PGE $_2$: Prostaglandin E $_2$.