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IFN-γ and TNF-α differentially regulate immunomodulation by murine mesenchymal stem cells

Karen English [a], Frank P. Barry [b], Ciara P. Field-Corbett [c], Bernard P. Mahon [a,*]

[a] *Mucosal Immunology Laboratory, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland*
[b] *Regenerative Medicine Institute (REMEDI), National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland*
[c] *Epithelial Immunobiology Laboratory, Institute of Immunology, National University of Ireland Maynooth, Maynooth, Co. Kildare, Ireland*

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Abstract

Murine mesenchymal stem cells (MSC) have the ability to inhibit allogeneic immune responses. Two different mechanisms, either cell contact-dependent or independent, have been proposed to account for this immunosuppression. The focus of this study was to elucidate the involvement of soluble suppressive factors secreted by murine MSC in an inflammatory setting, and their role in MSC immunomodulation. In a non-inflammatory environment, bone marrow derived murine MSC constitutively expressed low levels of COX-2, PGE-2, TGF-β1 and HGF, but not IL-10, PD-1, PD-L1 or PD-L2. These MSC were able to significantly reduce alloantigen driven proliferation in mixed lymphocyte reactions as well as mitogen driven proliferation. The pro-inflammatory cytokines IFN-γ and TNF-α did not ablate MSC mediated immunosuppression. MSC expression of PGE-2, IDO and PD-L1 was differentially regulated by these cytokines. COX-2 and PGE-2 expression by MSC were upregulated by both IFN-γ and TNF-α, and using a biochemical inhibitor this was shown to have an essential, non-redundant role in modulating alloantigen-driven proliferation. However, the surface expression of PD-L1 was induced by IFN-γ but not TNF-α and similarly functional IDO expression was only induced by IFN-γ stimulation. Blocking studies using neutralising antibodies and biochemical antagonists revealed that while PD-L1 induction was not essential, IDO expression was a prerequisite for IFN-γ mediated MSC immunomodulation. These data demonstrate that murine MSC expression of immunomodulatory factors dramatically changes in a pro-inflammatory environment and that IFN-γ in particular has an important role in regulating MSC immunomodulatory factor expression.

Keywords: Mesenchymal stem cell; Immunosuppression; IFN-γ; TNF-α; Prostaglandin; IDO

1. Introduction

Mesenchymal stem cells (MSC) are multipotent adult progenitor cells which have the ability to differentiate into a number of lineages [1–3]. These cells are primarily found in the bone marrow but have also been isolated from other sites in the body [4]. Recent advances in the isolation, culture and differentiation of bone marrow derived MSC have highlighted the potential use of these cells in regenerative medicine. A number of studies have shown beneficial effects of therapeutic MSC delivery in vivo. Horwitz et al. showed engraftment of donor MSC and the formation of new bone in a case of osteogenesis imperfecta [5]. HLA identical MSC were shown to be safe and associated with a reversal of pathology in a study of metachromatic leukodystrophy [6]. Surprisingly, engraftment of allogeneic human MSC resulted in improved bone marrow stroma reconstitution in a case of severe aplastic anaemia [7]. Mouse models have also proved useful in the development of regenerative medicine therapies and for probing immunological mechanisms. For example, administration of murine MSC before the onset of disease prevented pathology in EAE, a model of multiple sclerosis [8]. Similarly, administration of MSC before the onset of lung damage induced by bleomycin, ameliorated fibrotic effects and decreased inflammation [9]. Recently, MSC have also been shown to be anti-tumorigenic in a mouse model of Kaposi's sarcoma by inhibiting AKT activity [10].

A major caveat in the application of MSC based regenerative medicine therapies concerns the potential immune-mediated rejection of allogeneic cells. Intriguingly a growing body of evidence suggests that mismatched MSC evade regular immune

* Corresponding author. Tel.: +353 1 708 3835; fax: +353 1 708 3845.
E-mail address: bpmahon@nuim.ie (B.P. Mahon).

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allorecognition [2,11–14] and can inhibit immune responses [11,15–18]. The mechanisms by which MSC mediate these immunosuppressive effects have not been fully elucidated, however both contact dependent and independent mechanisms have been proposed [18–20]. Krampera and colleagues have shown that the suppressive activity of MSC was abrogated when cells were physically separated by a transwell membrane [18]. Other studies have shown that MSC may interfere directly with T cell, or antigen presenting cell phenotype, causing these cells to adopt regulatory functions [17,18,21,22,24,25]. MSC may interfere with ligand–receptor interactions required for T cell activation and proliferation. In contrast, other studies have proposed a role for soluble factors in MSC mediated immunosuppression using transwell cultures or MSC supernatant [20,26,27]. Several candidate cytokines have been implicated in MSC mediated suppression, including IL-10 and TGF-β1.

The therapeutic use of MSC may well require delivery to sites of inflammation and the potential possibility for immunomodulatory activity by MSC under these conditions is not clear. Previously, we have proposed that MSC mimic many of the immunomodulatory aspects of the fetal allograft [28]. The fetal allograft uses an array of soluble factors with immunomodulatory potential as well as contact dependent mechanisms to prevent fetal loss [29,30]. To address this hypothesis, several soluble factors known to be involved in immunosuppression by the fetal allograft were examined for their role in MSC mediated immunosuppression under inflammatory and non-inflammatory conditions.

In this study, we report the constitutive expression of low levels of immunomodulatory factors: COX-2, PGE-2, TGF-β1 and HGF by MSC. We demonstrate that high level expression of PD-L1, IDO and PGE-2 by MSC are differentially regulated by IFN-γ and TNF-α. Using blocking studies, the role of these factors was examined to show that murine MSC retain immunomodulatory capability under inflammatory conditions and that IFN-γ or TNF-α upregulation of PGE-2 may be of particular importance in maintaining this effect. Overall this study supports the potential therapeutic use of allogeneic MSC and provides support for a rational mechanism by which MSC evade allogeneic rejection despite the presence of inflammatory cytokines.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female BALB/c and C3H/HeN mice (Harlan, UK) were used for experiments under the guidelines of the Irish Department of Health and the research ethics committee of the National University of Ireland Maynooth.

2.2. Isolation and culture of bone marrow derived mesenchymal stem cells

Murine MSC were isolated and expanded using an in-house modification of the method of Peister and colleagues [31]. Murine MSC were obtained from 8- to 10-week old female BALB/c mice (Harlan, Oxon, UK). Mice were sacrificed by cervical dislocation and the femur and tibia removed, cleaned of all connective tissue, and placed in ice cold isolation medium consisting of RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum (Invitrogen-Gibco, Paisley, Scotland), 10% (v/v) equine serum (Hyclone laboratories, Logan, UT), 1% (v/v) penicillin/streptomycin (Invitrogen-Gibco,), and 1% (v/v) L-glutamine (Invitrogen-Gibco). The ends of the bones were cut to expose the marrow. Cells were then flushed out with isolation medium using a 5 ml syringe with a 27-gauge needle. Cell clumps were disaggregated using a 21-gauge needle and syringe followed by filtration through a 70-μm nylon mesh filter. Cells were then centrifuged ($600 \times g$, 5 min), resuspended in 15 ml isolation medium and cultured at 37 °C, 5% CO_2. Non-adherent cells were removed 24 h later by washing with sterile phosphate buffered saline, and cultures fed with isolation medium. This process was repeated every 3–4 days for 28 days. After 28 days, cells were removed from the flask by mild trypsinisation. Passage 2 cells were seeded at low density (50 cells/cm^2) and cultured in expansion medium (essentially isolation medium in which RPMI 1640 is replaced by α Minimum Essential Medium (Invitrogen-Gibco)). Contamination in MSC populations is a key confounding variable; therefore rigorous quality control was adopted at passage 3 and thereafter to ensure that MSC were not contaminated with hematopoietic or other cell contaminants, and that cells retained differentiation capacity as previously described [31]. MSC used in these studies were MHC class I$^+$, Sca-1$^+$, CD80$^+$, CD44^{low}, CD106^{low}, MHC class II$^-$, CD11b$^-$, CD34$^-$, CD45$^-$ and CD117$^-$. Stem cells were used between passages 3–10.

2.3. Characterisation of mesenchymal stem cells

The following antibodies were used to characterise murine MSC. MHC class I-FITC, MHC class II-PE, Sca-1-FITC, CD106-FITC, PD-1-FITC, PD-L1-PE, PD-L2, CD45 (eBiosciences, San Diego, CA), CD11b-PE, CD34-PE, CD45R-PE, CD44-FITC, CD80-PE, CD86-FITC, CD90-PE, CD117-FITC (ImmunoTools, Friesoythe, Germany). Unconjugated antibodies were detected with a secondary antibody conjugated to FITC (anti-biotin FITC, Miltenyi Biotech, Surrey UK) or Extravadin PE (Sigma–Aldrich, Dublin, Ireland). MSC were analysed for surface expression of these markers by flow cytometry. Briefly 1×10^5 cells were incubated with fluorochrome conjugated specifically or isotype control antibody at 4 °C for 30 min; cells were then washed and analysed using a FACScalibur™ with CellQuest software™ (Becton Dickinson, San Jose, CA).

2.4. Semi quantitative RT-PCR

MSC were seeded at 2×10^5 cells/ml in 24 well tissue culture plates (Nunc, Rorsklide, Denmark) overnight and harvested the next day for RNA isolation, carried out using TRI Reagent™ (Molecular Research Centre, Cincinnati, OH) according to manufacturer's instructions. cDNA were analyzed for the expression of TGF-β1, HGF, IL-10, PD-1, PD-L1, PD-L2, IDO and COX-2 by MSC. PCR primers for mTGF-β1 were forward 5′-TGA-

CGTCACTGGAGTTGTACGG-3′, reverse 5′-GGTTCATGTCATGGATGGTGC-3′; mHGF: forward 5′-CATTCAAGGCCAAGGAGAAG-3′, reverse 5′-AACTCGGATGTTTGGGTCAG-3′; mIL-10: forward 5′-TCCTTAATGCAGGACTTTAAGGGTTACTTG-3′, reverse 5′-GACACCTTGGTCTTGGAGCTTATTAAAATC-3′; mCOX-2: forward 5′-GGGTTGCTGGGGGAAGAAATGTG-3′, and reverse 5′-GGTGGCTGTTTTTGGTAGGCTGTG-3′.

PCR primers for mPD-1 and mPD-L1 were based on the sequence of Augello et al. [20]. mPD-1: forward 5′-TTCACCTGCAGCTTGTCCAA-3′, reverse 5′-TGGGCAGCTGTATGATCTGG-3′; mPD-L1: forward 5′-AAAGTCAATGCCCCATACCG-3′, reverse 5′-TTCTCTTCCCACTCACGGGT-3′; mPD-L2 forward 5′-CGTGACAGCCCCTAAAGAAG-3′, reverse 5′-GATGACCAGGCAACGGTACT-3′; mIDO: forward 5′-GGCTAGAAATCTGCCTGTGC-3′, reverse 5′-AGAGCTCGCAGTAGGGAACA-3′; GAPDH: forward 5′-GGTGAAGGTCGGAGTCAACG-3′ and reverse 5′-CAAAGTTGTCATGGATGACC-3′.

2.5. Quantitative real time RT-PCR

cDNA was analyzed for the expression of murine HGF by fluorogenic 5′-nuclease PCR assay (MJ Research Inc., Waltham, MA). Briefly, cDNA (500 ng) were amplified in the presence of SYBR® Green PCR mastermix. As an internal positive control GAPDH-specific forward and reverse primers were used in a similar reaction. Accumulation of gene-specific PCR products was measured continuously by means of fluorescence detection over 35 cycles. Standard curves for HGF expression were generated amplifying 10-fold serial dilutions of known quantities of HGF PCR product standards. Quantification of target gene expression was obtained using sequence detector system software (MJ Research Inc.).

2.6. In vitro stimulation of MSC

MSC were seeded at 2×10^5 cells/ml and cultured overnight in 1 ml volumes then stimulated with the pro-inflammatory cytokines TNF-α (10 ng/ml) (R&D systems, Abington, UK) and/or IFN-γ (10 or 200 ng/ml) (Peprotech, London, UK) for 6 h. Concentrations and times were chosen after optimization studies (data not shown). mRNA was isolated from 6 h cultures and expression of TGF-β1, HGF, IL-10, PD-1, PD-L1, PD-L2, IDO and COX-2 by MSC was measured by RT-PCR.

2.7. Cytokine and soluble factor measurement

Cytokines and soluble factors present in MSC or MLR cell culture supernatants were measured after 4 days culture by standard sandwich ELISA techniques using commercially available ELISA kits: TGF-β1 (BD Biosciences, Oxford, UK), IL-10 and IFN-γ (R&D Systems, Abington, UK), PGE-2 (Cayman Chemical, Michigan, USA) according to manufacturers' instructions. Cytokine concentrations were calculated by comparison with known cytokine standards, all determinations were made in triplicate, results are presented as mean cytokine concentration (±S.E.)

2.8. Mixed lymphocyte reactions

MSC were seeded in 100 μl cultures in 96 well flat-bottom plates (Nunc, Rorsklide, Denmark) in complete RPMI (cRPMI) at 0.75×10^5 cells/ml and cultured for 24 h at 37 °C, 5% CO_2. 24 h later, spleenocytes were isolated from mice by disaggregation of spleens. Erythrocytes were lysed by NH_4Cl lysis and cells washed three times in cRPMI. Responder and stimulator spleenocytes were seeded in triplicate cultures (±MSC) at a concentration of 1×10^6 cells/ml in a total volume of 200 μl. In mitogen-driven proliferation assays, cultures contained 5 μg/ml concanavalin A (Con A) (Sigma–Aldrich). After 4 days incubation, cultures were pulsed with 5 μCi/ml ^{3}H-thymidine (Amersham, Buckinghamshire, UK) for 6 h. Cells were harvested and thymidine incorporation quantified as proliferation in counts per minute. Results are expressed as count per minute (±S.E.).

2.9. Statistical methods

Results are expressed as the mean ± (S.E.). A Student's *t*-test was used to determine significance among the groups. A value of $p < 0.05$ was considered significant. Analyses and graphical representation were performed using Graph-Pad Prism™ software (Graphpad, San Diego, CA).

3. Results

3.1. Murine MSC constitutively express low levels of COX-2 and the immunomodulatory mediators PGE-2, TGF-β1, and HGF

Murine MSC have the ability to suppress alloreactivity. The specific mechanisms by which this occurs have not yet been elucidated. Conflicting data report that this inhibitory effect could be mediated through either cell contact or through soluble factors [18–20,23]. Initially, this study sought to focus on and to characterise the immunomodulatory cytokines that murine MSC express. In line with previous studies, murine MSC did not stimulate but significantly inhibited alloantigen driven proliferation when co-cultured with MHC mis-matched donor spleen cells (Fig. 1A). Similarly, MSC significantly ($P < 0.001$) inhibited mitogen driven proliferation (Fig. 1B). Murine fibroblast cells showed no such effect (data not shown).

A number of candidate cytokines and immunomodulatory factors were chosen and investigated because of their known immunosuppressive functions. IL-10 and TGF-β1 are known immunosuppressive cytokines involved in immune regulatory processes [32]. Hepatocyte growth factor (HGF) or scatter factor has the ability to modulate dendritic cell function [33] and improve GVHD [34]. To investigate a possible role for TGF-β1, HGF or IL-10 in the immunosuppression observed above, allogeneic MLR were performed with these cytokines at physiologically relevant concentrations. TGF-β1 at a concentration

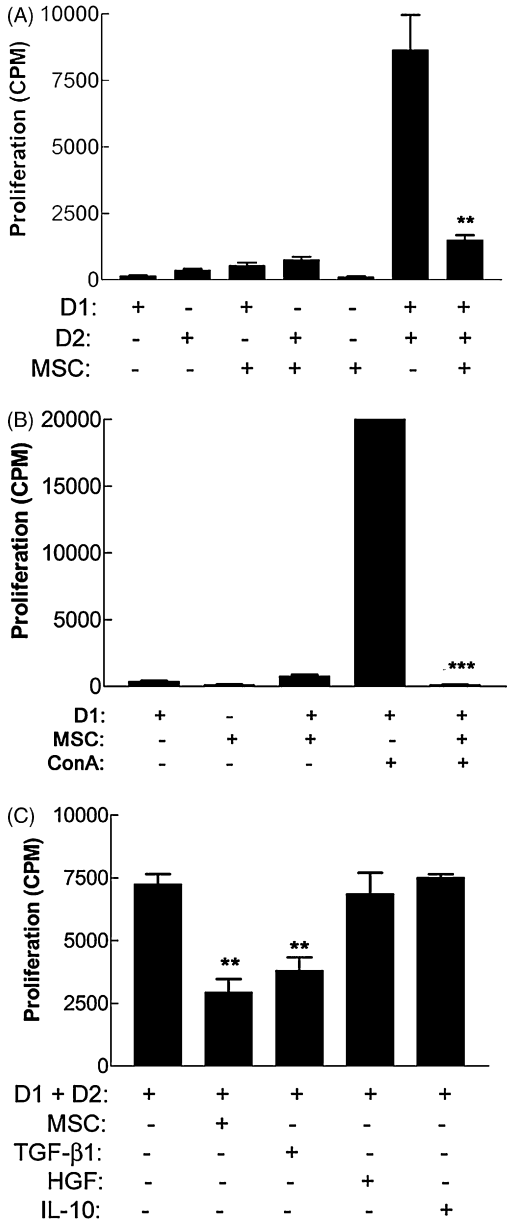

Fig. 1. MSC or immunosuppressive cytokines suppress alloantigen or mitogen driven proliferation. (A) MSC were co-cultured with spleen cells from MHC mismatched mice (D1 or D2) for 96 h and alloantigen driven proliferation measured by ^{3}H-thymidine incorporation expressed as mean cpm ± S.E. Significant reduction in proliferation by responder spleen cells (**$p < 0.01$) was determined by *t*-test analysis. (B) MSC also significantly suppressed Con A mitogen (5 μg/ml) driven proliferation (***$p < 0.001$). MSC or spleen cells cultured alone are shown for comparison. (C) Exogenous cytokines reduced alloantigen driven proliferation. Selected cultures contained MSC, recombinant mouse HGF (10 ng/ml), IL-10 (2000 pg/ml) or TGF-β1 (10 ng/ml). Results are representative of three experiments, each performed at least in duplicate. Statistical analysis by *t*-test showed that only TGF-β1 significantly decreased alloantigen driven proliferation comparable to MSC (**$p < 0.01$). No significant loss of viability or induction of apoptosis was detected in this or subsequent experiments.

of 10 ng/ml (Fig. 1C), but not 200 pg/ml (data not shown) significantly reduced proliferation comparable to MSC, however neither HGF nor IL-10 significantly reduced proliferation when added into an MLR (Fig. 1C). The expression of the enzyme COX-2 was also examined as it is involved in the production

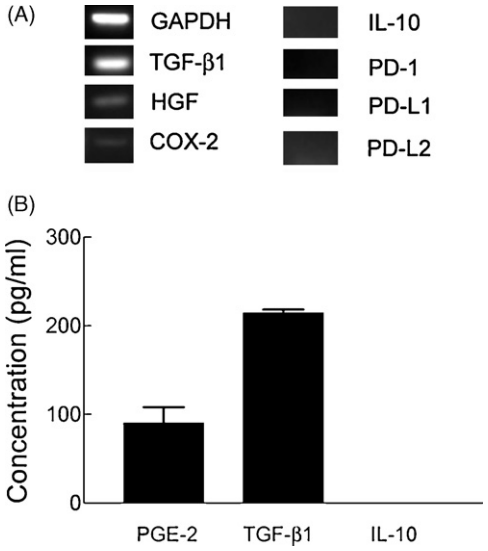

Fig. 2. MSC constitutively express immunomodulatory factors determined by RT-PCR (A) and ELISA (B). Six-hour cultures of unstimulated MSC were lysed, RNA isolated and subjected to semi-quantitative RT-PCR for expression of TGF-β1, HGF, COX-2, IL-10, PD-1, PD-L1 and PD-L2 as detailed in Section 2. GAPDH was the internal positive control (A). Supernatants from parallel cultures were also examined at 48 h for PGE-2, TGF-β1 and IL-10 protein expression by ELISA (B). Results are representative of three experiments, ELISA determinations were each performed in triplicate and expressed as mean concentration ± S.E.

of Prostaglandin E2 (PGE-2), known to be involved in immune regulation [35]. Murine MSC constitutively expressed COX-2, TGF-β1 and low levels of HGF mRNA, but not IL-10, PD-1, PD-L1 or PD-L2 (Fig. 2A). TGF-β1 and PGE-2 were detected in supernatants from 4 day cultures by ligand specific ELISA. Neither IL-10 protein or mRNA could not be detected above threshold levels using ELISA or RT-PCR respectively (Fig. 2B).

The role of PGE-2 in MSC mediated immunosuppression was further examined using biochemical antagonists. Addition of indomethacin, a specific COX-2 inhibitor [36] significantly reduced PGE-2 production by MSC (Fig. 3A). Furthermore, introduction of indomethacin into an allogeneic MLR cultured in the presence of MSC resulted in the significant restoration of cell proliferation (Fig. 3B) suggesting that PGE-2 plays an important role in the suppression by MSC of allogeneic responses under non-inflammatory conditions.

3.2. IFN-γ and TNF-α upregulate expression of COX-2, PGE-2, and HGF

In order to use allogeneic MSC for regenerative processes in vivo, it is desirable that MSC retain their immunosuppressive properties under conditions where pro-inflammatory cytokines are present. The site of inflammation in vivo is an environment rich in pro-inflammatory cytokines, in particular TNF-α and IFN-γ [37]. The effect of IFN-γ and TNF-α on MSC mediated inhibition of cell proliferation was therefore examined in vitro. An inflammatory environment was simulated by stimulating MSC in vitro with exogenous IFN-γ and TNF-α. MSC exposed to IFN-γ and cultured in an allogeneic MLR resulted

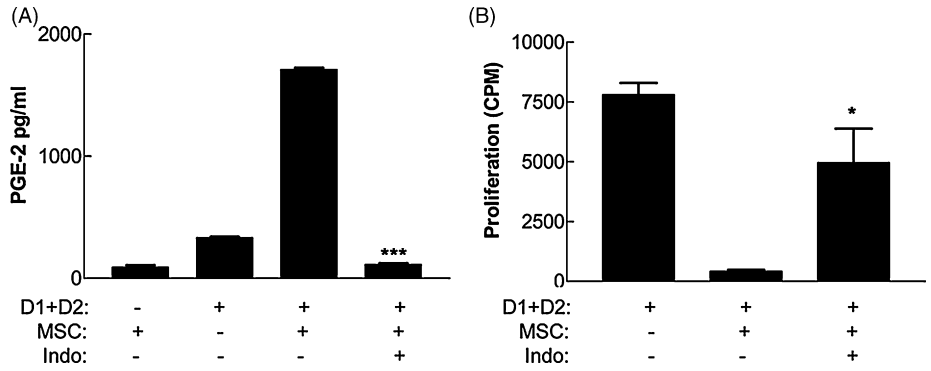

Fig. 3. Murine MSC modulated alloresponses through prostaglandin E-2 production. (A) Murine MSC constitutively expressed PGE-2 determined by specific ELISA and expression was upregulated in the presence of an allogeneic MLR (D1 + D2) from 48 h culture supernatants of MSC and allo-MLR. Addition of indomethacin (Indo, 20 μM) significantly reduced PGE-2 expression. (B) Likewise PGE-2 was involved in alloantigen driven proliferation. MSC were co-cultured in MLR with MHC mismatched donors (D1 + D2) as in Fig. 1, but some cultures contained indomethacin (Indo) as a an antagonist of PGE-2 production. Results are representative of three experiments, each performed at least in triplicate. *t*-test analysis showed that PGE-2 inhibition significantly restored alloantigen driven proliferation (*$p < 0.05$).

in consistent inhibition of cell proliferation; suggesting that this cytokine could not break MSC mediated immune suppression (Fig. 4A). TNF-α exposure to MSC produced a similar effect (Fig. 4A). Furthermore, the combination of IFN-γ and TNF-α did not break MSC mediated suppression (Fig. 4A). Interestingly, both IFN-γ and TNF-α significantly upregulated MSC production of PGE-2 as shown by ELISA (Fig. 4B). Consistent with this, COX-2 mRNA expression by MSC could be upregulated by either IFN-γ or TNF-α (Fig. 4C), although expression could not be enhanced by combining both cytokines (Fig. 4B and C). These data support retention of an immunosuppressive role for MSC at sites of inflammation.

Quantitative Real Time PCR confirmed significant HGF mRNA upregulation by both IFN-γ and TNF-α (Fig. 5A). Fur-

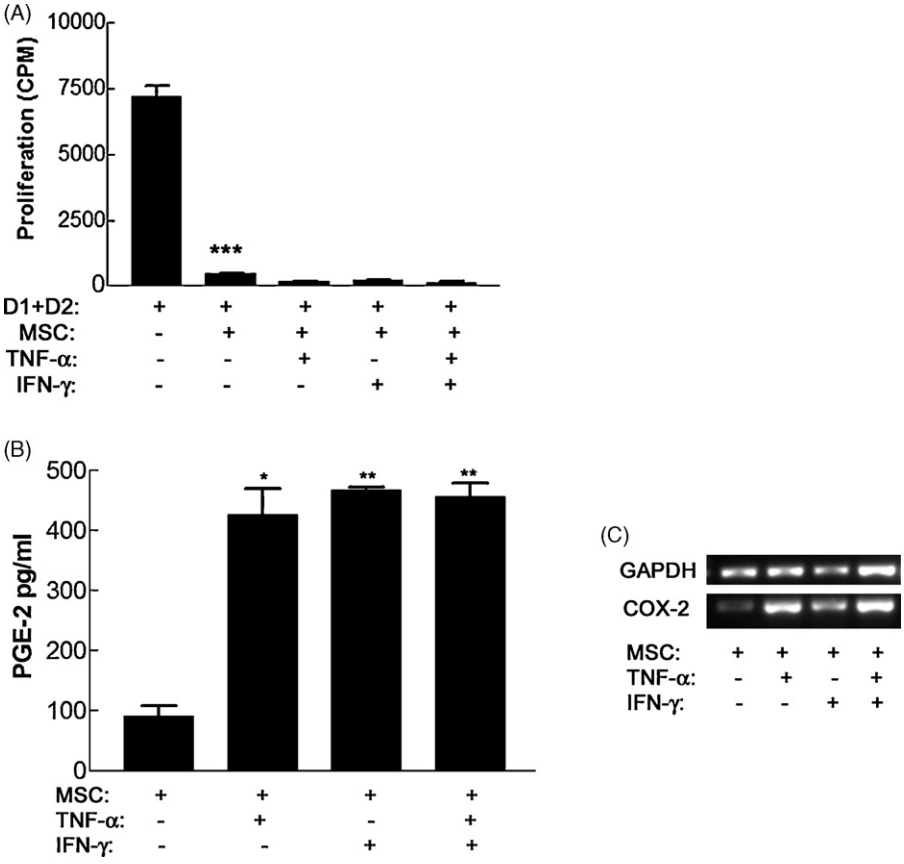

Fig. 4. IFN-γ or TNF-α did not ablate MSC suppression of alloresponsiveness. (A) MLR were performed in the presence or absence of MSC as described in legend to Fig. 1. Additionally selected wells contained IFN-γ (200 ng/ml) and/or TNF-α (10 ng/ml) for the duration of the culture. Results are representative of three experiments, each performed in triplicate; *t*-test analysis showed a significant reduction in proliferation by responder spleen cells even in the presence of IFN-γ and TNF-α (***$p < 0.001$). (B) MSC production of PGE-2 was significantly upregulated by both IFN-γ and TNF-α as detected by ELISA. (C) Constitutive expression and upregulation of COX-2 mRNA by MSC was also determined by RT-PCR.

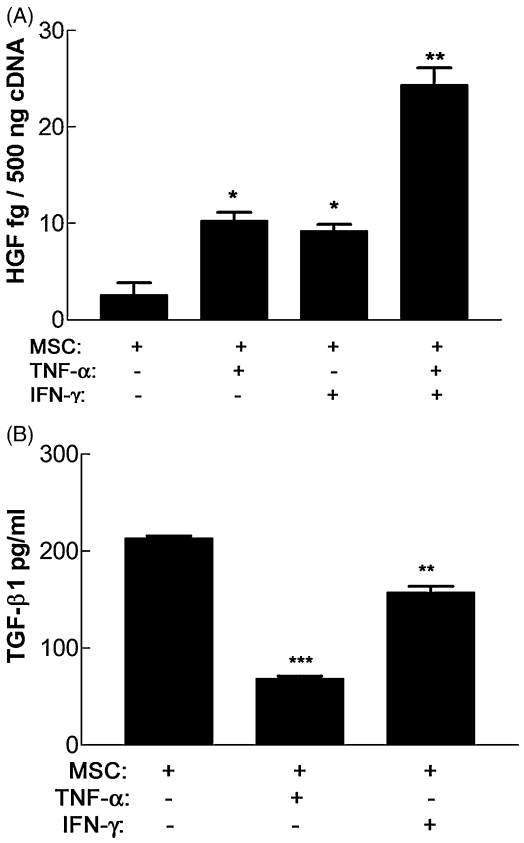

Fig. 5. IFN-γ and TNF-α upregulated HGF, but decreased TGF-β1 expression by MSC. Levels of HGF mRNA (6 h) were determined by quantitative real time PCR from unstimulated or IFN-γ and/or TNF-α stimulated murine MSC. (A) IFN-γ and TNF-α significantly upregulated HGF mRNA expression by MSC (*$p < 0.05$). (B) In contrast TGF-β1 protein expression by MSC was reduced in the presence of IFN-γ and TNF-α measured by cytokine specific ELISA (**$p < 0.01$, ***$p < 0.001$).

thermore, this upregulation was significantly greater when both cytokines were used in combination (Fig. 5A, $p < 0.05$ compared to either TNF-α or IFN-γ alone, and $p < 0.01$ compared to control). In contrast TGF-β1 protein expression by MSC was significantly reduced by IFN-γ and also by TNF-α (Fig. 5B) but expression was not further reduced by a combination of both cytokines (data not shown). These data suggest that in the presence of IFN-γ or TNF-α, it is COX-2, PGE-2 and possibly HGF expression, rather than TGF-β1 that supports the immunomodulatory capacity of MSC.

3.3. IFN-γ and TNF-α induce MSC surface marker expression of PD-L1

The programmed death-1 (PD-1) pathway has emerged as a co-inhibitory pathway with immune regulatory properties [38,39]. This pathway has been implicated in murine MSC mediated immune suppression [19]. Murine MSC were therefore stimulated with pro-inflammatory cytokines to examine their effect on expression of immunomodulatory factors. In particular the effect exerted by TNF-α and IFN-γ on PD-1 pathway members by MSC was studied. Murine MSC did not constitutively express PD-1 or its ligands PD-L1/PD-L2 (Fig. 6). However PD-L1, but not PD-1 or PD-L2 mRNA expression could be induced by IFN-γ (Fig. 6A), and this was mirrored by surface marker expression (Fig. 6B), supporting a role for IFN-γ in PD-L1 signalling. In contrast to IFN-γ, TNF-α did not induce PD-L1 protein expression detectable by FACS. Typically mRNA profiles matched this although weak expression of PD-L1 mRNA was observed occasionally (two from six times) from MSC stimulated with high concentrations (10 ng/ml) of TNF-α. This expression was transient and was never accompanied by surface expression in MSC ($n = 6$). Likewise combining TNF-α with IFN-γ did not reduce PD-L1 protein expression supporting the use of different mechanisms by the two cytokines, with the IFN-γ response dominant. Interestingly, blockade of PD-1, PD-L1 or PD-L2 by neutralising antibodies did not restore alloantigen driven cell proliferation in MLR containing MSC (Fig. 6C). Taken together these data show that IFN-γ plays a role in upregulation of PD-L1 by MSC. However, some redundancy may exist with regard to the role of PD-L1 in MSC immunomodulation as this could not be blocked through neutralisation.

3.4. IFN-γ but not TNF-α differentially induced IDO expression by murine MSC

Indolamine 2,3-dioxygenase (IDO) is an enzyme which catabolises L-tryptophan and is required for T cell proliferation, and has a defined role in fetal tolerance [30,40]. We have previously hypothesised that MSC may have retained some aspects of fetal tolerogenic capacity [28]. Unstimulated murine MSC did not constitutively express mRNA for IDO but this could be induced by IFN-γ (Fig. 7A). Expression of IDO mRNA was not induced by TNF-α and TNF-α did not counter IFN-γ induced expression (Fig. 7A); supporting independent mechanisms for these cytokines in supporting MSC mediated immunosuppression. To correlate mRNA expression with functional IDO activity, blocking studies were performed using an inhibitor of the IDO pathway, 1-methyl-L-tryptophan (1-MT). The addition of 1-methyl-L-tryptophan to an allogeneic MLR containing murine MSC resulted in significant restoration of cell proliferation (Fig. 7B). Taken together, these data support a non-redundant role for IDO in inhibiting alloantigen driven proliferation by MSC exposed to IFN-γ.

4. Discussion

In order to clarify the utility of using mesenchymal stem for regenerative medicine, the soluble and surface immunomodulatory molecules expressed by MSC were characterised under regular and inflammatory conditions. MSC retained the ability to suppress alloantigen driven proliferation in MLR in the presence of IFN-γ and TNF-α, however these cytokines used different mechanisms to achieve this effect. Both IFN-γ and TNF-α upregulated COX-2 and PGE-2 expression and using indomethacin we show that this had an essential, non-redundant role in modulating alloantigen-driven proliferation. However, IFN-γ but not TNF-α induced surface expression of PD-L1 by MSC and similarly IFN-γ but not TNF-α induced expression of functional IDO, an enzyme which catabolises L-tryptophan.

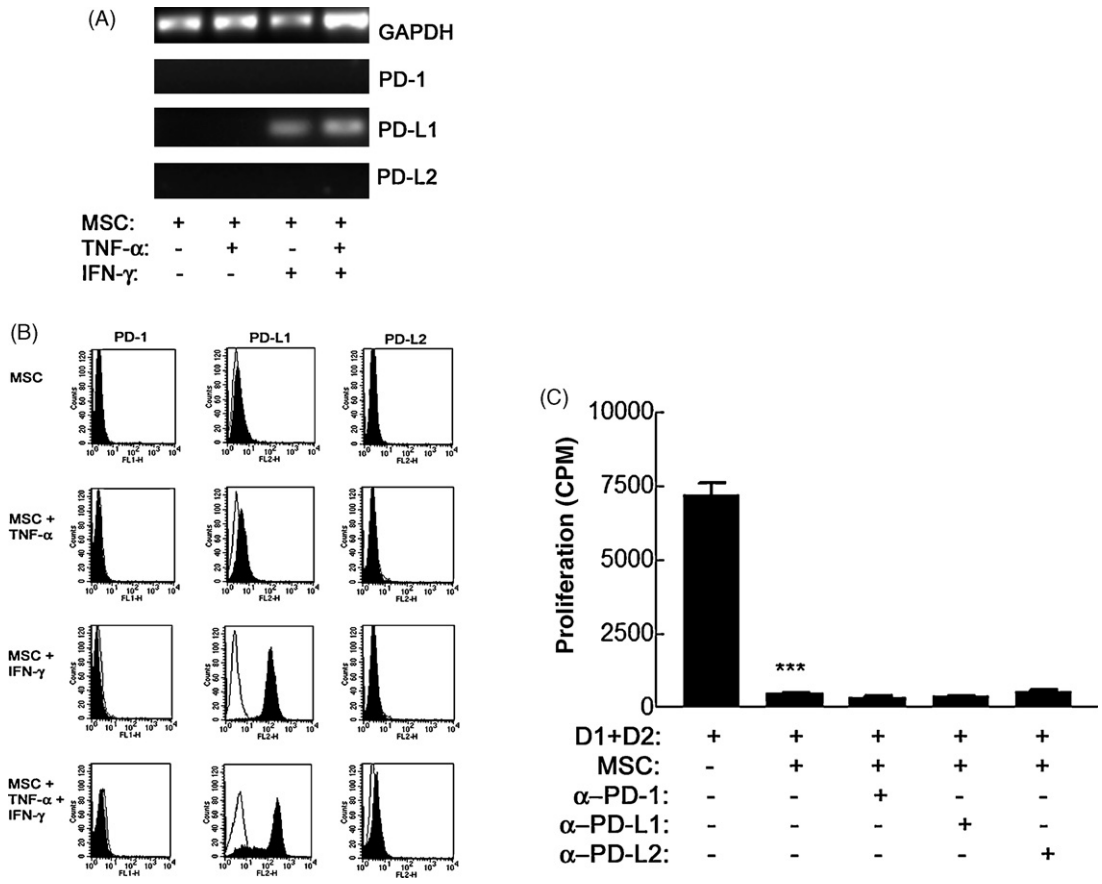

Fig. 6. PD-L1, but not PD-1 or PD-L2 expression by MSC is upregulated by IFN-γ. (A) mRNA from 6 h cultures of unstimulated, IFN-γ and/or TNF-α stimulated MSC were assayed by semi-quantitative RT-PCR for PD-1, PD-L1 or PD-L2 expression as detailed in Section 2. (B) FACS analysis for surface protein expression of PD-1, PD-L1 or PD-L2 (solid shading) or isotype controls (open shading) was performed on corresponding 24 h cultures. (C) Proliferative responses were examined for MSC co-cultured in MLR as in Fig. 1, but additionally cultures were established containing neutralizing antibodies to PD-1, PD-L1 and PD-L2 (5 μg/ml). Results are representative of three experiments, each performed in triplicate. Although MSC ablated alloantigen driven proliferation, *t*-test analysis showed that neutralizing antibodies to PD-1 or its ligands did not significantly restore alloantigen driven proliferation.

Blocking studies using neutralising antibodies and biochemical antagonists revealed that while PD-L1 induction was not essential, IDO expression was a requirement for IFN-γ mediated MSC immunomodulation. Taken together these data clarify the mechanisms used by MSC in immunomodulation, and support the possible use of MSC in regenerative therapy during inflammatory conditions.

Under regular culture conditions, bone marrow derived murine MSC constitutively expressed low levels of COX-2, PGE-2, TGF-β1 and HGF, but not IL-10, PD-1, PD-L1 or PD-L2. These MSC were able to significantly reduce alloantigen driven proliferation in mixed lymphocyte reactions as well as mitogen driven proliferation. As MSC may be required for use in inflammatory environments such as the synovium, it is interesting to note that neither IFN-γ nor TNF-α broke MSC mediated immunosuppression. Our findings with directly isolated MSC, conflict with MSC cell line derived data of Djouad et al. [14], suggesting heterogeneity between MSC from different sources. TNF-α or IFN-γ addition to in vitro cultures of MSC resulted in increased production of PGE-2 by MSC and subsequent blocking of PGE-2 production through prior exposure of MSC to indomethacin, restored alloantigen driven proliferation in MLR, emphasising that both cytokines require eicosanoid production as part of the mechanism of suppression, and that combination of the factors could not further enhance expression. However, IFN-γ and TNF-α do not use identical mechanisms to achieve the suppressive effect, and differ in their capacity to induce PD-L1 and IDO expression by MSC. IDO could be induced by IFN-γ but not TNF-α. The addition of a specific IDO inhibitor, the Tryptophan antagonist 1-methyl-L-tryptophan, restored alloantigen driven proliferation indicating an essential role for this mechanism in IFN-γ but not TNF-α mediated suppression. Whilst IFN-γ had a similar influence on PD-L1 surface expression by MSC, neutralizing antibodies to PD-1 or the ligands PD-L1 and PD-L2 did not ameliorate suppression. This does not mean that PD-L1 is not involved in suppression but may indicate either redundancy in this pathway or the existence of an alternative receptor for PD-L1.

This study shows an important role for IFN-γ in supporting immunosuppresssion mediated through MSC by induction of soluble immunomodulatory factors. The role of cytokines in MSC mediated suppression has been investigated by a number of groups, although findings have been confused by the variety of model systems, MSC purification protocols and readouts. Di

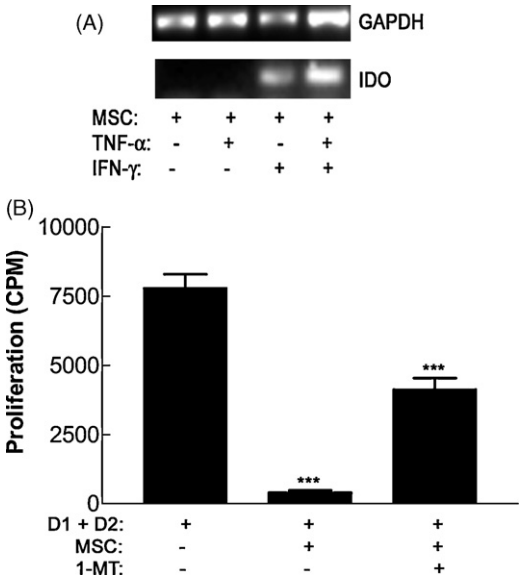

Fig. 7. IDO plays a role in MSC mediated suppression of allo-responsiveness. Constitutive or inducible mRNA expression of IDO by MSC was examined by semi-quantitative RT-PCR. (A) Six-hour cultures of unstimulated, IFN-γ and/or TNF-α stimulated MSC were lysed and assayed for IDO mRNA expression. (B) MLR were performed with mismatched donors (D1 and D2) as described in Fig. 1, in the presence or absence of MSC and the IDO antagonist 1-methyl-L-tryptophan (1-MT) (40 μM). *t*-test analysis showed significant restoration of proliferation when IDO was blocked (***$p < 0.001$).

Nicola et al. and others showed a role for TGF-β1 and HGF in human MSC immunosuppression using neutralizing antibodies [20,27]. In contrast other groups have suggested that TGF-β1 does not play a central role in MSC mediated suppression of alloantigen driven proliferative responses [18]. Although our data confirms that TGF-β1 reduces alloantigen driven proliferation in MLR, it is unlikely that this cytokine plays a central role in MSC mediated immune suppression at inflammatory sites, given that TNF-α and IFN-γ reduced expression of TGF-β1 by murine MSC (Fig. 5B).

IL-10 was not constitutively expressed by murine MSC in this study and was not induced by pro-inflammatory cytokines. Consistent with this finding Krampera et al. did not find IL-10 expression by murine MSC [18]. This is in contrast to findings from our own laboratory using human MSC, which constitutively express IL-10 (Ryan et al. manuscript submitted), and studies from Rasmusson et al. and Beyth et al. who only found IL-10 in human MSC co-cultures [26,42]. A possible role for IL-10 in human MSC mediated immunosuppression has been indicated by studies showing that the suppressive effect of MSC was partially ablated by neutralising IL-10 activity in MLR [26]. Given that IL-10 is downregulated by inflammation, these results suggest that IL-10 is unlikely to be mediating IFN-γ or TNF-α driven effects on MSC. Nevertheless these findings highlight that species differences and differences in isolation procedure may have a profound influence on the characteristics of MSC. Murine and human MSC constitutively express COX-2 (Fig. 4A, and Ryan et al. manuscript submitted). COX-2 is an inducible enzyme involved in the production of PGE-2 [28] and PGE-2 production is upregulated after co-culture of human MSC with PBMC [13,41], suggesting communication between MSC and lymphocytes that leads to the increased production of immunosuppressive factors such as PGE-2. Using an inhibitor of COX-2 and therefore PGE-2 biosynthesis, we have demonstrated that PGE-2 plays a role in the suppression of alloresponsiveness. These findings are consistent with those of Rasmusson et al. who demonstrated a partial effect using indomethacin but only in MLR cultures stimulated with PHA [42]. These data are also supported by Aggarwal but conflict with data from Tse who saw no such effect [13,41]. However, despite the differences in source and purification protocols, results from three different laboratories including our own, have demonstrated that PGE-2 is a significant factor mediating suppression of alloresponsiveness [13,42]. Furthermore, the present study showed that IFN-γ and TNF-α upregulation of COX-2 is an essential, non-redundant, component of MSC immunomodulation. This observation provides a rational mechanism explaining the allosuppressive capacity of MSC and suggests that their therapeutic use may be feasible under inflammatory conditions.

PD-1 and its ligands PD-L1 and PD-L2 are known to regulate immune activity [43–45]. The PD-1 pathway appears to promote survival of cardiac allografts [46], and PD-L1 has a role in mediating peripheral T cell tolerance [43]. PD-1 engagement is known to inhibit the progression of the cell cycle in some cell types [47]. This point is noteworthy because MSC mediated inhibition of T cell proliferation has been linked to control of the cell division cycle [48]. Although the present study found no essential role for the PD-1 pathway in MSC mediated suppression, Augello et al. have previously shown that bone marrow mesenchymal progenitor cells inhibited lymphocyte proliferation through activation of this pathway [19]. In contrast to the data herein, that study showed constitutive expression of PD-1, PD-L1 and PD-L2 by bone marrow mesenchymal progenitor cells, and upregulation of PD-1 and PD-L1 upon stimulation with PHA. The reasons for these differences are most likely due to the very different stem cell populations studied. The cell population used by Augello et al. expressed CD28, CD13 and CD34, markers which are not usually associated with MSC but more characteristic of cells known to constitutively express PD-1 [43]. Although a direct effect for this pathway was not observed in this study, it is intriguing that pro-inflammatory cytokines induce PD-L1 and it may be that this pathway has some involvement but that either a degree of redundancy is present in the system or an alternative receptor exists for PD-L1.

Recent data has shown a role for IDO in human MSC mediated immune suppression [29]. IDO is the rate-limiting enzyme involved in the catabolism of the essential amino acid tryptophan into its breakdown product Kynurenine [49]. IDO is involved in the inhibition of T cell proliferation by dendritic cells [50] and the expression of IDO by dendritic cells can be induced by IFN-γ [49]. MSC do not constitutively express IDO, however when stimulated with IFN-γ, MSC can be induced to express IDO [29]. We and others have suggested a role for this enzyme in human MSC suppression [28,29,51,52]. Addition of a specific inhibitor of IDO, resulted in reversal of MSC mediated inhibition of proliferation, supporting the findings of Meisel et al. [29]. However, in direct contrast to the present study, Tse et al. did

not find a significant restoration of proliferation after addition of 1 mM 1-methyl-L-tryptophan [41]. The observation herein that IFN-γ but not TNF-α induced IDO expression by MSC study clarifies that discrepancy. That finding, and the associated antagonist studies (Fig. 7B), indicates that whilst IDO is not an exclusive mechanism for MSC immunomodulation, it is essential for that mediated in the presence of IFN-γ. This complexity may explain the apparently conflicting data, in that the role of IDO is either redundant or essential depending on the nature of the inflammatory stimulus. Although IDO expression by MSC may induce T cell apoptosis [53], we observed no significant increase in apoptosis over the time course of these studies (data not shown).

This study sheds light on the immunomodulatory factors that murine MSC constitutively express and subsequently delineates how pro-inflammatory cytokines affected the expression of these. This study demonstrates that murine MSC share many but not all features of human MSC immunomodulation. Although there are species differences in the role of IL-10, the immunosuppressive effects of murine MSC mediated through production of PGE-2 and IDO, are consistent with data seen in human MSC. Immunosuppressive activity was not ablated by stimulation with inflammatory cytokines but IFN-γ upregulated expression of COX-2, PGE-2 and IDO suggesting an important role for IFN-γ in murine MSC mediated immunomodulation. Taken together these data indicate that murine MSC model a number of important aspects of human MSC interaction with the immune system, highlight the significance of COX-2, PGE-2 and IDO expression and offer rational mechanisms supporting the therapeutic use of allogeneic MSC.

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