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Mesenchymal Stromal Cell Secretion of Programmed Death-1 Ligands Regulates T Cell Mediated Immunosuppression

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ABSTRACT

Mesenchymal stromal cells (MSCs) exert broad immunosuppressive potential, modulating the activity of cells of innate and adaptive immune systems. As MSCs become accepted as a therapeutic option for the treatment of immunological disorders such as Graft versus Host Disease, our need to understand the intricate details by which they exert their effects is crucial. Programmed death-1 (PD-1) is an important regulator in T cell activation and homeostatic control. It has been reported that this pathway may be important in contact-dependent mediated immunomodulation by MSCs. The aim of this study was to establish whether MSCs, in addition to their cell-surface expression, are able to secrete PD-1 ligands (PD-L1 and PD-L2) and their potential importance in modulating contact-independent mechanisms of MSC immunosuppression. Here we report that MSCs express and secrete PD-L1 and PD-L2 and that this is regulated by exposure to interferon γ and tumor necrosis factor α. MSCs, via their secretion of PD-1 ligands, suppress the activation of CD4+ T cells, downregulate interleukin-2 secretion and induce irreversible hyporesponsiveness and cell death. Suppressed T cells demonstrated a reduction in AKT phosphorylation at T308 and a subsequent increase in FOXO3 expression that could be reversed with blockade of PD-L1. In conclusion, we demonstrate for the first time, that MSCs are able to secrete PD-1 ligands, with this being the first known report of a biological role for PD-L2 in MSCs. These soluble factors play an important role in modulating immunosuppressive effects of MSCs directly on T cell behavior and induction of peripheral tolerance. STEM CELLS *2017;35:766–776*

SIGNIFICANCE STATEMENT

Mesenchymal stromal cells (MSCs) exert immunomodulatory effects via contact-dependent and independent mechanisms. Here we report a novel mechanism for MSC suppression of T cells, through the secretion of programmed death 1 ligands (PD-L) 1 and 2. Licensing of MSCs to an anti-inflammatory phenotype, by exposure to pro-inflammatory cytokines, upregulates cell surface and secreted forms of both ligands. Blocking experiments confirm their role in suppressing T cell proliferation, interleukin-2 secretion, inducing hyporesponsiveness and cell death. We conclude that soluble PD-1 ligands play an important role in modulating MSC effects on T cell behavior and peripheral tolerance.

INTRODUCTION

Mesenchymal stromal cells (MSCs) have been extensively reported to possess immunosuppressive properties via the modulation of immune cells within both the innate and adaptive systems [1–4]. Their mode of action is differential, with a plethora of soluble factors as well as contact-dependent mechanisms documented to influence their ultimate effect on immune cell function.

Delineating the mechanism of action and cell signaling pathways involved in mediating MSC immunomodulation is of critical importance as these cells become a therapeutic option for the treatment of various immunological disorders, such as Graft versus Host Disease (GvHD), diabetes, and amyotrophic lateral sclerosis (ALS) [5–7].

It is well documented that MSCs can both directly and indirectly alter the status of CD4+ T cells, suppressing their proliferation and skewing them toward a regulatory phenotype (Treg) [8, 9]. MSCs require licensing to an anti-inflammatory phenotype, via exposure to a pro-inflammatory cytokine rich milieu generated primarily by activated T cells, in order to convey their immunomodulatory effects. Interferon γ (IFNγ) is well reported to license MSCs, with its removal significantly reducing the anti-proliferative effects on T cells [10]. Tumor necrosis factor α (TNFα) is additionally an important T cell effector cytokine in MSC immunomodulation. Its effects are differential to that of IFNγ, with known roles in modulating heme-oxygenase 1 (HO-1) and insulin-like growth factor 1 (IGF-1) [11].

Complete activation of T cells requires a complex cascade of intracellular signaling events initiated through the T cell receptor (TCR) and enhanced by costimulatory signals such as CD28. These positive signals drive cellular metabolism, inhibit cell death and enhance proliferation [12]. The receptor programmed death-1 (PD-1) is expressed on the cell surface of activated T and B cells, in addition to myeloid cells and thymocytes [13]. Interaction of PD-1 with its known ligands, PD-L1/B7-H1 and PD-L2/B7-DC provides an inhibitory signal in regulating cellular activation and proliferation [14–16]. The importance of the PD-1 pathway in T cell homeostasis is evident, with PD-1 knockout mice demonstrating splenomegaly and an increased susceptibility to autoimmune diseases [17, 18]. Awareness and understanding of the importance of the PD-1 pathway and its regulation is increasing, with reports suggesting a pivotal role in mouse models of GvHD [19] and type I diabetes mellitus [20]. Further investigations into the potential interplay of MSCs with this pathway are essential in establishing mechanisms of action for MSC therapy.

PD-L1 is reported to be expressed on both non-hematopoietic (including MSCs) and some subsets of hematopoietic cells [21–23]. In contrast, PD-L2 has a more restricted expression profile to activated antigen presenting cells (APCs), with a few reports suggesting cell surface expression on MSCs [21, 22, 24]. Whilst the amino acid identity between PD-L1 and PD-L2 is approximately 40%, the affinity of PD-L2/PD-1 binding is reportedly two to six fold higher than that of PD-L1/PD-1. Therefore, it is expected that PD-L2, if expressed at the same level as PD-L1, would outcompete its rival. However, in general, PD-L2 is expressed at much lower levels than PD-L1 [25].

Soluble (s)PD-1 ligands arise from the proteolytic cleavage of the membrane bound form of the protein and, in the case of sPD-L2, the transcription of splice variants [26, 27]. No splice variants have been detected to date for PD-L1, but specific cleavage by matrix metalloproteinase (MMP) 13 to the soluble form has been described in foreskin fibroblasts [26]. Cell surface cleavage of PD-L2 has been reported to be less specific, with sensitivity to both MMP 9 and 13. Two splice variants of human PD-L2 have also been reported, with variant III created by splicing out of exon 3, resulting in loss of the transmembrane domain [27]. Conversion to the soluble form in foreskin fibroblasts has been linked to loss of immunosuppressive activity and exacerbation of inflammatory responses, suggesting that within fibroblasts this is not a mechanism of T cell suppression [26].

Previous studies have demonstrated a role for MSC inhibition of T cell proliferation and effector function via contact-dependent interactions of PD-1/PD-L1 with IFNγ licensing [21, 28]. However, little is understood about the mechanism of MSC interactions with the PD-1 pathway and ultimately how this interplay affects downstream signaling cascades and T cell function. To the authors' knowledge, no study has reported the secretion of sPD-1 ligands by human MSCs and whether these ligands can directly affect activated T cells. The aim of this study was therefore to elucidate whether MSCs, in addition to their contact-dependent effects, can produce sPD-1 ligands and the mechanism by which these soluble factors can suppress T cell proliferation and effector function.

Materials and Methods

Isolation and Expansion of MSCs

Bone marrow (BM) MSCs ($n = 5$ donors) were isolated, expanded, and characterized as described previously [6, 29, 30] in line with the guidelines of the MSC Consortium of the European Blood and Marrow Transplantation Group and approved by the Swedish National Board of Health and Welfare [6, 31]. The study was approved by the review board at Karolinska University Hospital, Huddinge with donors providing informed written consent in line with the Helsinki declaration.

To isolate MSCs, BM mononuclear cells were separated over a gradient of Percoll (Redigrad, GE Healthcare, Uppsala, Sweden), washed and resuspended in DMEM low-glucose medium (Invitrogen, Stockholm, Sweden) supplemented with 5% pooled human platelet rich plasma and 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml Fungizone (Invitrogen; herein termed as culture media) and plated at a density of 1.6×10^5 cells/cm^2. When the cultures reached confluence (>80%), the cells were detached with 0.05% Trypsin-EDTA (Invitrogen), replated at a density of 4×10^3 cells/cm^2 and cultured for up to five passages.

Co-Culture of MSCs and Purified T Cells

Peripheral blood mononuclear cells were prepared by centrifugation of heparinized blood on Ficoll-Isopaque (Lymphoprep, Oslo, Norway) and untouched CD3+ T cells were isolated by magnetic activated cell sorting (MACS; Human Pan T Cell Isolation Kit; Miltenyi Biotec Norden AB, Lund, Sweden). T cells were activated using anti-CD2/CD3/CD28 microbeads (Miltenyi Biotec) at a 1:2 bead to cell ratio and cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), L-glutamine (2 mM; Invitrogen) and 10% heat-inactivated pooled human blood type AB serum (herein termed T cell media). MSCs (Passage 2 [P2]; $n = 5$ donors) were added at a 1:10 ratio to T cells either in direct contact or in 0.4 μm PET transwell membrane inserts and cultured for 3 days. Where relevant anti-PD-L1 (250 ng/ml polyclonal goat anti-human PD-L1, #AF156; R&D Systems, Abingdon, U.K. [32]), anti-PD-L2 (250 ng/ml polyclonal goat anti-human PD-L2, #AF1224; R&D Systems) or anti-PD-1 (50 ng/ml monoclonal mouse anti-human PD-1, #329911; Biolegend, San Diego, CA) were added to co-cultures.

Evaluating the Hyporesponsive State of T Cells Induced by MSCs

T cells from the above transwell co-cultures were counted and transferred to 96-well plates (1×10^5 cells/well in triplicate) in fresh T cell media ± fresh activation microbeads. Where appropriate 15 ng/ml interleukin-2 (IL-2; Peprotech Nordic, Stockholm, Sweden) was added to cultures to evaluate potential for reverting to a responsive state. Cells were cultured at 37°C/5%CO_2 for 3 days before the addition of 5Ci/mM ^{3}H thymidine. Cultures were maintained for a further 16 hours at 37°C before harvesting. Cells were harvested onto a glass fiber filter (Harvester 96, Tomtec Inc., Hamden, CT) and radioactivity quantified using a micro-β scintillation counter (Perkinelmer Sverige AB, Upplands Väsby, Sweden).

Flow Cytometry Analysis for Surface and Phosphorylated Molecules

MSC Cell Surface Expression of PD-L1 and PD-L2. MSCs (P2; $n = 4$ donors) were cultured in culture media ± 100 IU/ml IFNγ and/or 10 ng/ml TNFα for 3 days before trypsinizing and staining with anti-PD-L1 PE (#12-5983) or anti-PD-L2 APC (#17-5888) as per the manufacturer's instructions (eBioscience Inc., San Diego, CA). Cells were acquired on a FACSCalibur (BD Biosciences, Stockholm, Sweden).

T Cell Expression Profile After MSC Co-Culture. T cells were stained with anti-CD3 V450 (#560365; BD), anti-CD4 PerCP-Cy5.5 (#560650; BD), anti-CD25 PE (#555432; BD) and anti-PD-1 eVolve655 (#86-2799; eBioscience) and run on a BD LSRFortessa (BD).

T Cell Viability. For the assessment of T cell survival, cells were stained with 5 μl of 7-AAD (BD) and 5 μl of annexin V (Av) PE (#640907; Biolegend) in Av binding buffer (0.1 M HEPES [pH 7.4], 1.4 M NaCl, and 25 mM $CaCl_2$). Cells were acquired on a FACSCalibur (BD).

Phospho-AKT Detection. After 3 days of transwell co-culture, T cells were isolated and restimulated with plate-bound anti-CD3 (2 ug/ml; BD) for 10 minutes in a water bath at 37°C. The stimulation was attenuated by fixation with BD cytofix-fixation buffer (BD) for 10 minutes at 37°C. Cells were then permeabilized with Perm Buffer III (BD) for 30 minutes on ice before staining with anti-p-AKT (pT308) antibody (#562465; BD) for 1 hour. For intracellular FOXO3 staining, cells were fixed using fixation buffer (eBioscience) for 30 minutes. Cells were washed twice before permeabilizing with permeabilizing buffer (eBioscience) for 30 minutes at room temperature. Permeabilized cells were treated with anti-FOXO3 (#2497; Cell Signaling Technology Europe BV, Leiden, The Netherlands) for 20 minutes, followed by 20 ug/ml goat anti-rabbit Alexa Fluor 647 secondary antibody (#4414; Cell Signaling Technology) for 20 minutes. Cells were washed twice with FACS buffer before acquiring on a BD LSRFortessa (BD).

Fifty thousand gated events were recorded per sample and analyzed using FlowJo Version 7.6 (FlowJo, Ashland, OH).

Enzyme-Linked Immunosorbent Assay (ELISA)

MSCs ($n = 5$ donors) were stimulated with 100 IU/ml IFNγ and/or 10 ng/ml TNFα for 3 days. Secretion of sPD-L1 and sPD-L2 was measured using ELISA as per the manufacturer's instructions (R&D Systems). For sPD-L1 measurement, conditioned media samples were concentrated approximately 2.5-fold (degree of concentration measured for individual samples) using 10K cut-off Amicon Ultra centrifugal filters (Millipore AB, Solna, Sweden).

Suppression of T cell effector function was assessed by measurement of TNFα, IL-2, and IFNγ within the co-culture supernatants by ELISA as per the manufacturer's instructions (R&D Systems).

Gene Expression Analysis Using Real-Time Quantitative PCR

Total RNA was extracted from MSCs ± IFNγ and/or TNFα stimulation ($n = 4$ donors) and T cells post-transwell culture ($n = 4$ donors) using the RNeasy Mini Kit (QIAGEN AB, Sollentuna, Sweden). cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Stockholm, Sweden) and qRT-PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems). The amplification was performed on a CFX384 C1000 Touch Real-time system (Bio-Rad Laboratories AB. Sundbyberg, Sweden). Expression was normalized to β-actin levels. The primers used were: β-actin; FW: AGCTACGAGCTGCCTGAC, REV: AAGGTAGTTTCGTGGATGC; PD-L1; FW: GGCATCCAAGATACAAACTCAA, REV: CAGAAGTTC CAATGCTGGATTA; PD-L2; FW: GAGCTGTGGCAAGTCCTCAT, REV: GCAATTCCAGGCTCAACATTA; AKT; FW: GGCTATTGTGAAGGAG GGTTG, REV: TCCTTGTAGCCAATGAAGGTG. PD-L2 primers detect total PD-L2, including the splice variant III form.

Statistics

Comparisons were statistically analyzed using Student's *t* test or Mann–Whitney *U* test where data did not fulfill requirements for parametric testing (normal distribution and equal variances). Significance was assumed at $p < .05$ (Prism 5.0; Graphpad Software Inc., La Jolla, CA and SPSS Statistics 24.0; IBM, Armonk, NY).

RESULTS

MSCs Constitutively Express and Secrete PD-1 Ligands

MSCs were cultured ± the pro-inflammatory T cell effector cytokines and known MSC licensing factors, IFNγ and/or TNFα. MSCs were subsequently assessed for PD-L1 and PD-L2 expression at mRNA, cell surface and secreted levels. Our results demonstrate that MSCs constitutively express both PD-L1 and PD-L2 on their cell surface (Fig. 1A, 1B) and actively secrete these immunomodulatory molecules (Fig. 1C, 1D). Here the secretome of MSCs includes both free and vesicle bound proteins. Furthermore, we report differential responses to IFNγ and TNFα, with IFNγ inducing a 5.5-fold upregulation of PD-L1 (Fig. 1A; $p < .05$) but not PD-L2 (Fig. 1B) at the cell surface. These data are supported by a significant upregulation in mRNA levels of PD-L1, confirming response at the transcriptional level (Fig. 1E; $p < .05$). In contrast, TNFα induced an upregulation of both PD-L1 and PD-L2 (Fig. 1A, 1B; $p < .05$ in 1A and $p < .01$ in 1B) at the cell surface, although the effect was higher on PD-L2, with expression increasing 3.4-fold compared to resting controls. These findings were also supported at the transcriptional level (Fig. 1F; $p < .05$). A synergistic effect of IFNγ and TNFα, when used in combination, was evident on PD-L1 cell surface expression, resulting in a 5.6-fold increase over controls (Fig. 1A; $p < .01$) and a further 2.4-fold increase over TNFα stimulation alone (Fig. 1A; $p < .01$).

Effects of pro-inflammatory stimuli on the secreted levels did not map to the previously described modulation of cell surface expression. PD-L1 secretion was specifically upregulated in response to IFNγ and TNFα in combination (Fig. 1C; $p < .05$), whereas PD-L2 secretion levels increased in response to both cytokines, 4-fold by IFNγ and 3.3-fold by TNFα compared to

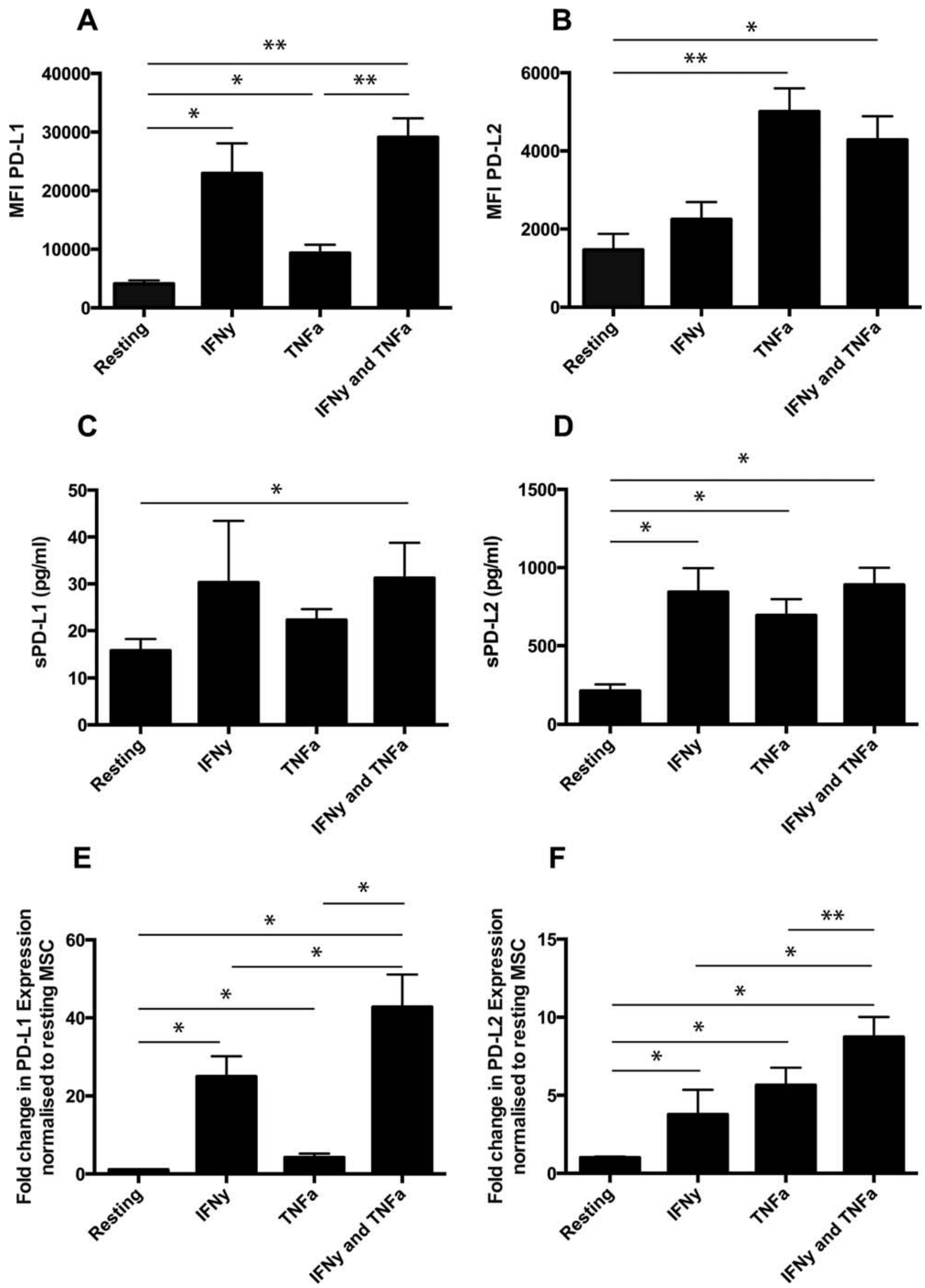

Figure 1. Mesenchymal stromal cell (MSC) cell surface expression and secretion of PD-L1 and PD-L2 are potentiated by pro-inflammatory cytokines, IFNγ and TNFα. MSCs ($n = 4$) were exposed to 100 U/ml IFNγ and 10 ng/ml TNFα for 3 days in culture. Cell surface expression (MFI) of **(A)** PD-L1 and **(B)** PD-L2 was assessed by flow cytometry. Secretion of **(C)** soluble (s)PD-L1 and **(D)** sPD-L2 within the conditioned media of stimulated cells was assessed by ELISA. Bar charts indicate mean $\pm$ SEM. Transcriptional regulation of **(E)** PD-L1 and **(F)** PD-L2 were assessed by qRT-PCR. mRNA data are expressed as fold change compared to unstimulated, resting MSCs $\pm$ SEM. *, $p < .05$; **, $p < .01$. Abbreviations: IFNγ, Interferon γ; MFI, mean fluorescence intensity; PD-L1 and PD-L2, programmed death 1 ligands 1 and 2; TNFα, tumor necrosis factor α.

controls (Fig. 1D; $p < .05$). It is noteworthy to comment that sPD-L2 levels were markedly higher than sPD-L1 in resting MSCs, with further dramatic upregulation upon licensing.

MSCs Suppress T Cell Activation via the Secretion of PD-1 Ligands

MSCs in direct contact or transwell co-culture with T cells suppressed T cell activation (CD25+) and PD-1 expression to a comparable level (Fig. 2Aii; $p < .05$). Further experiments to delineate the mechanism of the PD-1 pathway in MSC-mediated immunosuppression were therefore performed in transwell cocultures. Addition of an anti-PD-1 blocking antibody to the MSC/T cell transwell co-culture reversed the inhibition of CD25 expression within CD4+ T cells back to that of controls (Fig. 2B; $p < .05$). A partial reversal of CD25 inhibition was seen where each of the PD-1 ligands were blocked

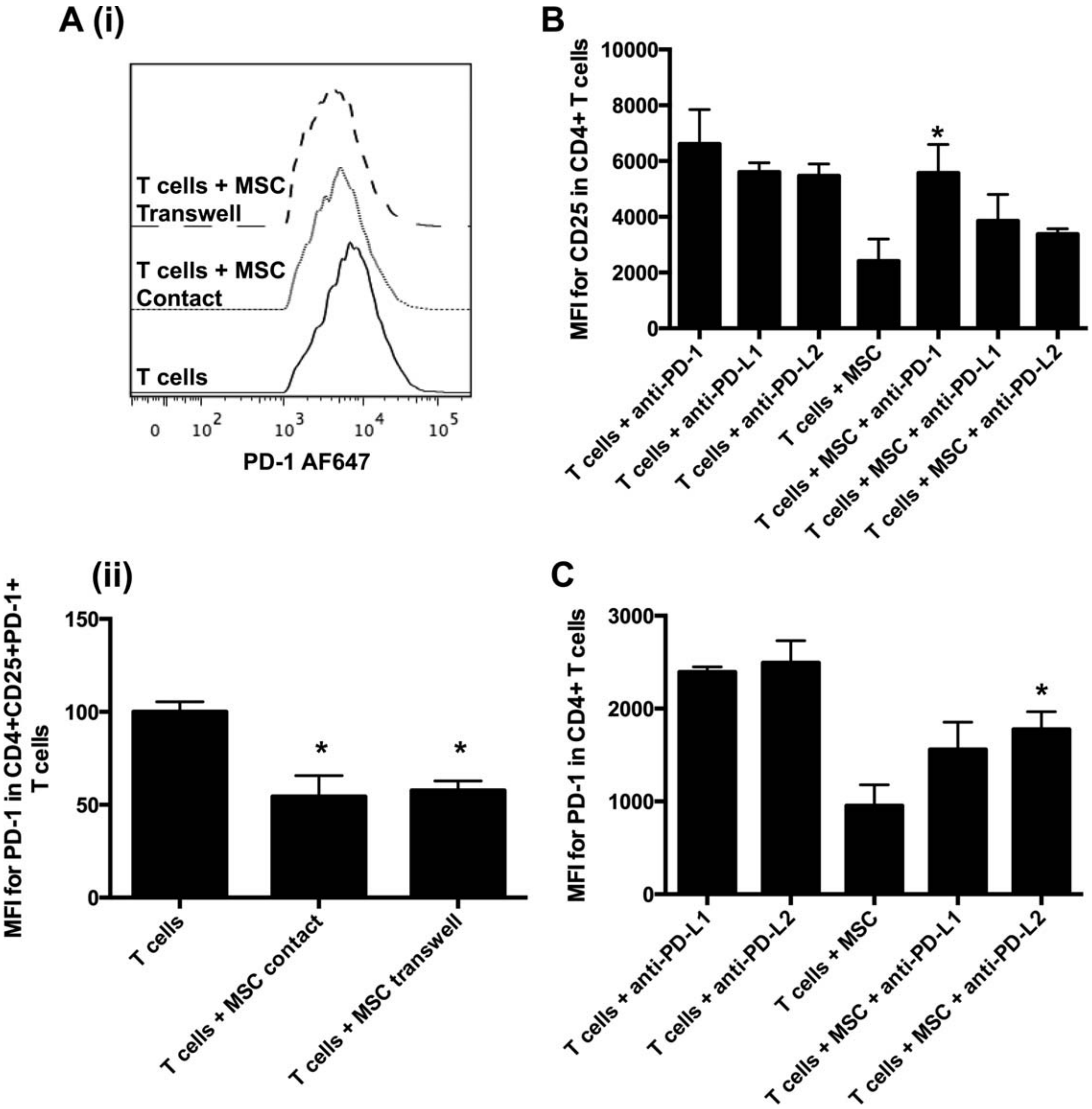

Figure 2. MSCs suppress CD4+ T cell activation and downregulate T cell surface PD-1 expression via the secretion of PD-1 ligands. **(A)**: MSCs ($n = 5$) were co-cultured with purified CD3+ T cells in direct contact and under transwell conditions. Cell surface expression of PD-1 was downregulated in CD4 + CD25+ activated T cells by MSCs to the same extent under both contact and transwell conditions. Further experiments were therefore conducted in transwell only to assess the role of MSC secreted soluble PD-1 ligands on CD4+ T cell function. **(Ai)**: Representative histograms are shown for one donor. **(Aii)**: MFI was used to quantify changes in expression between treatment groups. Data are normalized to activated T cell controls. **(B)**: Blocking of the PD-1 pathway inhibited MSC-mediated suppression of T cell activation (CD25+) via the secretion of PD-L1 and PD-L2, whereas (**C**) specific blockade of soluble PD-L2 inhibited suppression of PD-1 cell surface expression by MSCs. Bar charts indicate mean ± SEM. *, $p < .05$. Abbreviations: IFNγ, interferon γ; MSC, mesenchymal stromal cell; MFI, median fluorescence Intensity; PD-L1 and PD-L2, programmed death 1 ligands 1 and 2; TNFα, tumor necrosis factor α.

separately, suggesting a combinatory effect in down-regulating CD25 expression by the MSCs. In contrast down-regulation of PD-1 expression by the MSCs in CD4+ T cells could only be significantly restored by blockade of sPD-L2 (Fig. 2C; $p < .05$), although blocking sPD-L1 demonstrated a trend toward a similar effect.

MSCs Suppress IL-2 Secretion Through Soluble PD-1 Ligands

Transwell co-culture of MSCs with T cells directly suppressed the secretion of IFNγ (Fig. 3A; $p < .05$), TNFα (Fig. 3B; $p < .05$), and IL-2 (Fig. 3C; $p < .05$). Blockade of the individual PD ligands did not reverse the suppression of IFNγ or TNFα by the MSCs (Fig. 3A, 3B). However, both neutralization of sPD-L1 and sPD-L2 significantly restored the secretion of IL-2 by the activated T cells compared to MSCs alone (Fig. 3C; $p < .01$).

MSCs Induce Tolerance Through the Induction of T Cell Hyporesponsiveness and Apoptosis

MSCs were cultured in transwell with activated T cells ± PD-1 blocker, before removal of the MSCs and their conditioned media. The primed T cells were then cultured with fresh media and activation stimuli ± IL-2 to assess whether the secretion of PD-1 ligands by the MSCs could suppress T cell proliferation and induce a hyporesponsive state.

As expected, exposure of the T cells to the PD-1 blocker in combination with the activation stimuli induced proliferation compared to the activated T cells alone (Fig. 4A; $p < .01$).

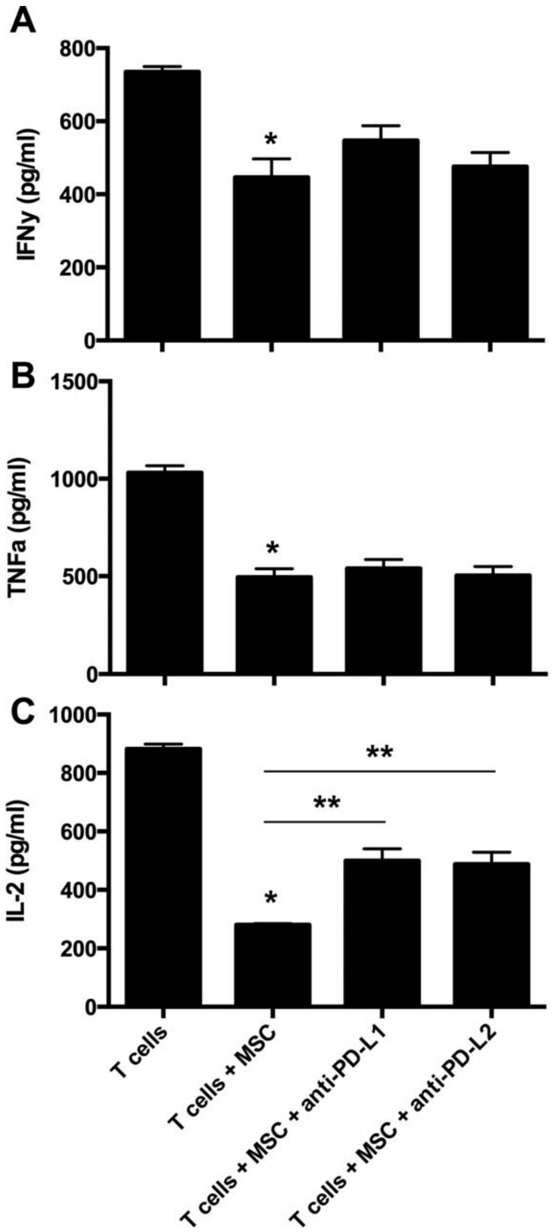

Figure 3. MSCs suppress IL-2 secretion in activated T cells via the secretion of PD-1 ligands. Levels of T cell effector cytokines were assessed within conditioned media derived from transwell co-culture of MSCs and activated T cells ($n = 5$) ± blockers for PD-L1 and PD-L2 by ELISA. MSCs suppressed the secretion of (**A**) IFNγ, (**B**) TNFα, and (**C**) IL-2 via the secretion of soluble factors. Blockade of soluble PD-L1 and PD-L2 significantly restored the production of (C) IL-2, but not (A) IFNγ or (B) TNFα. Data are shown as mean ± SEM. Data are compared to activated T cell controls unless otherwise indicated. *, $p < .05$; **, $p < .01$. Abbreviations: IFNγ, interferon γ; IL-2, interleukin-2; MSC, mesenchymal stromal cell; PD-L1 and PD-L2, programmed death 1 ligands 1 and 2; TNFα, tumor necrosis factor α.

Exposure to the MSC secretome induced a suppression of proliferation in the T cells that could not be reversed with removal of the conditioned media (containing MSC derived immunomodulatory soluble factors) and addition of fresh stimuli (Fig. 4A; $p < .01$). Further supplementation of IL-2 did not reverse this suppressive effect, suggesting the T cells had been rendered unresponsive (Fig. 4A). The presence of the PD-1 blocker during co-culture of the MSCs/T cells prevented this phenomenon, allowing the T cells to proliferate once more in response to new activation stimuli (Fig. 4A; $p < .01$).

Flow cytometry confirmed this lack of responsiveness was accompanied by a suppressed ability for the T cells to upregulate their cell surface CD25 and PD-1 expression in response to fresh stimulus and removal of the MSCs (Fig. 4B; $p < .01$). Further supplementation with IL-2 did not alter this response (Fig. 4B; $p < .001$).

T cell survival after MSC exposure was assessed by Av staining in combination with 7-AAD. Levels of apoptotic (Av + 7-AAD+) T cells were reduced with blocking of the PD-1 receptor and specifically sPD-L1 compared to MSCs alone (Fig. 4C; $p < .01$). Total T cell death (7-AAD+) was increased with exposure to the MSC secretome compared to T cell only controls (Fig. 4C; $p < .05$). This could be prevented by blocking of PD-1 or sPD-L1 to a similar extent, confirming the specific induction of T cell death via MSC secretion of sPD-L1 (Fig. 4C; $p < .01$). Blocking of PD-L2 had no effect on levels of T cell death (7-AAD+) compared to MSCs alone.

T Cell Hyporesponsiveness is Induced by MSCs Through PD-1-Mediated Blockade of the AKT Pathway

To ascertain the intracellular mediated mechanisms of T cell behavior affected by exposure to MSC secreted soluble factors, the expression of AKT and downstream targets was investigated. qRT-PCR of the T cells after co-culture with MSCs confirmed no change in mRNA expression of AKT (Fig. 5A). Phospho-flow was therefore utilized to confirm whether the phosphorylation status of signaling molecules downstream of the PD-1 receptor had been altered in response to MSC exposure. T cells previously co-cultured with MSCs in transwell were restimulated with CD3 after removal of the MSCs and their conditioned media. Prior exposure to MSCs induced a hyporesponsive state, suppressing the ability of the T cells to respond to CD3 stimulation, with a reduced phosphorylation of AKT at T308 (Fig. 5B; $p < .05$) and a consequential upregulation of the AKT regulated transcription factor FOXO3 (Fig. 5C; $p < .05$), but not FOXO1 (data not shown). Blockade of sPD-L1 secreted by the MSCs was able to partially restore levels of AKT phosphorylation (Fig. 5B; $p < .05$) and completely suppress the FOXO3 upregulation seen (Fig. 5C; $p < .05$), back to that of control levels.

Discussion

The immunosuppressive qualities of MSCs have long been appreciated and studied [29, 33]. Multiple mechanisms of action have been proposed, both through contact-dependent induction of suppression and the release of soluble factors such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2, and HO-1 [9, 34]. As cellular therapy with MSCs becomes an accepted therapeutic option, focus has shifted toward delineating the intricate mechanisms of action by which MSCs mediate their effects on individual immune cell subsets.

Within this study we report, for the first time, the secretion of both sPD-L1 and sPD-L2 by BMMSCs. The previous report of PD-L1 within the microvesicles of murine MSCs suggests that these ligands may exist as free entities and/or vesicle bound within the MSC secretome [35]. As previously published, exposure to pro-inflammatory cytokines resulted in

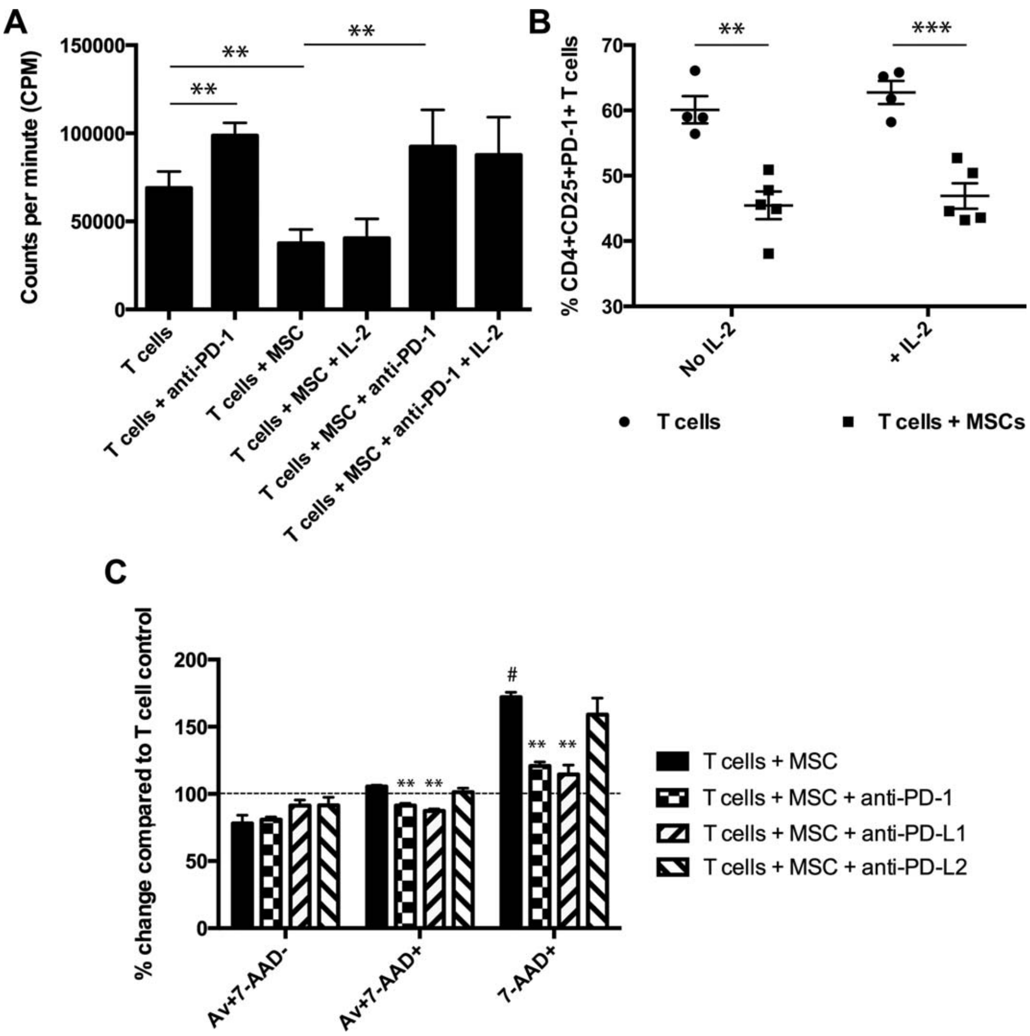

Figure 4. MSCs induce hyporesponsivess and apoptosis in T cells via their secretome. MSCs ($n = 4$) were co-cultured in transwell with activated T cells for 3 days. T cells were subsequently separated from the transwell MSC co-cultures and placed in fresh culture media and restimulated with anti-CD3, anti-CD2 and anti-CD28 microbeads. **(A)**: T cell proliferation was significantly inhibited in those cells previously exposed to MSCs and could not be restored by IL-2 supplementation, indicative of irreversible hyporesponsiveness. Presence of a PD-1 blocker at the point of initial MSC/T cell co-culture prevented induction of this hyporesponsive state, with T cells proliferating to levels comparable to controls. Data are expressed as counts per minute ± SEM **, $p < .01$ **(B)**: Flow cytometry ($n = 5$ MSC donors) confirmed the proliferation data, demonstrating a reduced ability for T cells to upregulate CD25 and PD-1 in response to fresh stimulus (±IL-2) after MSC exposure. Data are expressed as percentage CD4 + CD25 + PD-1+ T cells ± SEM **, $p < .01$; ***, $p < .001$ **(C)**: Annexin V and 7-AAD staining of T cells post-MSC co-culture confirmed induction of apoptosis by exposure to the MSC secretome. Cells are defined as pre-apoptotic (Av + 7-AAD-), apoptotic (Av + 7-AAD+) or total dead cells (7-AAD+). Data are expressed as a percentage compared to T cell only controls ± SEM. Dotted line indicates 100%. #, $p < .05$ compared to T cell only control *, $p < .05$ **, $p < .01$ compared to T cells + MSC. Abbreviations: IFNγ, interferon γ; IL-2, interleukin-2; MSC, mesenchymal stromal cell; PD-1, programmed death-1; PD-L1 and PD-L2, programmed death 1 ligands 1 and 2; TNFα, tumor necrosis factor α.

upregulation of both these ligands on the MSC cell surface [21, 36], but we also report that this induction extends to their secreted levels. Furthermore, we demonstrate the differential effects of IFNγ and TNFα, with the former upregulating cell surface expression of PD-L1 but not PD-L2. In contrast, TNFα upregulated both ligands at the cell surface. PD-L2 has a 2-6-fold higher affinity for the PD-1 receptor than PD-L1, but its low expression in comparison on APCs has led to the opinion that PD-L1 is the more important ligand in vivo [25]. Here we report that MSCs secrete significantly higher levels of sPD-L2 compared to sPD-L1 in both unstimulated/resting and licensed MSCs, indicating the potential importance of sPD-L2 in MSC immunomodulation.

Upregulation of cell surface PD-L1 on MSCs and subsequent inhibition of T cell proliferation via a contact-dependent mechanism has been previously reported [36–38]. In contrast, English et al. [39] reported that PD-L1 played no role in MSC-mediated immunosuppression. The differences in these reports have been attributed to the use of different immune cell subsets and species variation. Therefore, this study was designed to specifically investigate the effects of human BMMSCs on purified human CD3+ T cells.

Here we demonstrate that cell surface levels of PD-1 on T cells are dramatically upregulated in response to activation via CD3/CD28 costimulation. In order for MSCs to initiate inhibitory effects via the PD-1 pathway, they require T cells to be

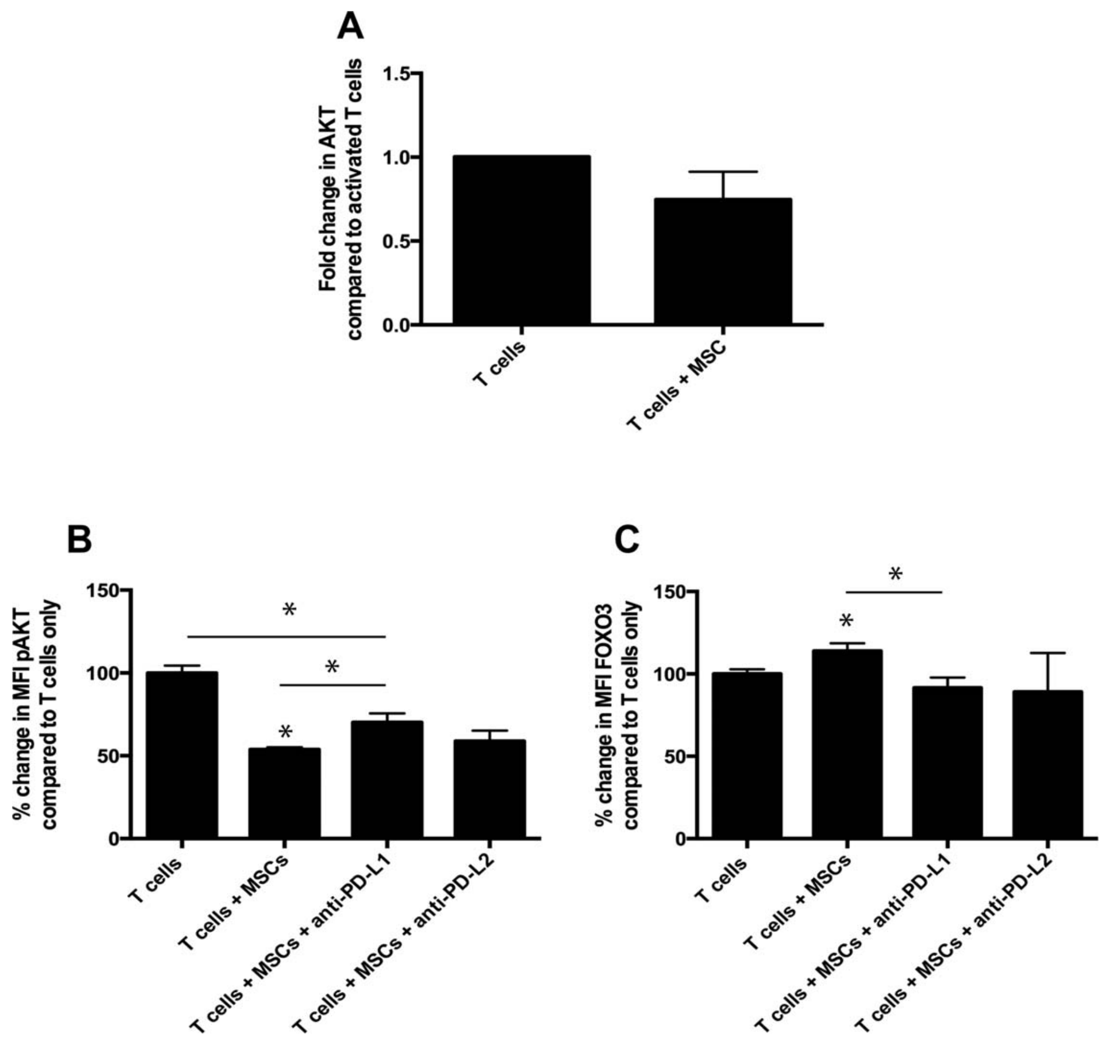

Figure 5. MSC-mediated induction of the PD-1 pathway results in reduced AKT phosphorylation and increased FOXO3 expression in T cells. **(A)**: T cells co-cultured with MSCs ($n = 4$) in transwell conditions were assessed for mRNA expression of the PD-1 pathway downstream signaling molecule AKT by qRT-PCR. No change in the transcriptional level of AKT in response to exposure to MSCs was evident. Data are expressed as fold change compared to activated T cell controls. **(B)**: Phospho-flow confirmed that prior exposure of the T cells to MSCs render the cells unable to respond to T cell receptor (TCR) simulation with plate bound CD3, with a decrease in AKT phosphorylation at position T308. This ultimately increased the expression of transcription factor (**C**) FOXO3 within the MSC exposed T cells. MFI was used to quantify changes in expression between treatment groups. Bar charts indicate percentage change in MFI compared to T cell only controls ± SEM. *, $p < .05$. Abbreviations: MSC, mesenchymal stromal cell; MFI, mean fluorescence Intensity; PD-L1 and PD-L2, programmed death 1 ligands 1 and 2.

expressing PD-1 on their cell surface (data not shown). MSCs can subsequently suppress the level of activated CD25 + PD-1+ T cells both in direct contact and in transwell to the same extent. In contrast to the published literature, stating the need for direct cell contact for triggering of the PD-1 pathway, this suppression in T cell activation occurs directly through the interaction of sPD-1 ligands, secreted by MSCs, with blockade of the PD-1 receptor abolishing this effect. Neutralising the individual PD-1 ligands demonstrated a trend toward restoring CD25 expression in T cells suppressed by MSCs alone, but this was not statistically significant, indicating that both sPD-L1 and sPD-L2 play a combinatory role in exerting the suppression of T cell activation. In contrast, blockade of sPD-L2 alone was sufficient to prevent downregulation of PD-1 on the cell surface of the T cells, supporting its superior ability to bind to PD-1 compared to PD-L1. This shift in activation status may also represent a skewing toward a more regulatory phenotype. Francisco et al. [15] proposed that PD-L1 activation of the PD-1 pathway was sufficient for conversion of naive CD4+ T cells into FoxP3+ inducible Tregs to induce peripheral tolerance, a mechanism, which has already been reported for MSCs [9]. Further studies to delineate the effects of sPD-1 ligands on skewing of the immune cell repertoire would be needed to validate this hypothesis.

Effector cytokine analysis confirmed that exposure to the MSC secretome directly suppresses the production of IFNγ, TNFα, and IL-2 in activated T cells, but that only IL-2 suppression can be attributed to sPD-1 ligand secretion by the MSCs. Blocking experiments confirmed that this suppressive effect is through both sPD-L1 and sPD-L2. These data support the above findings, that both sPD-L1 and sPD-L2 work in unison to exert their effects on activated T cells. In contrast to

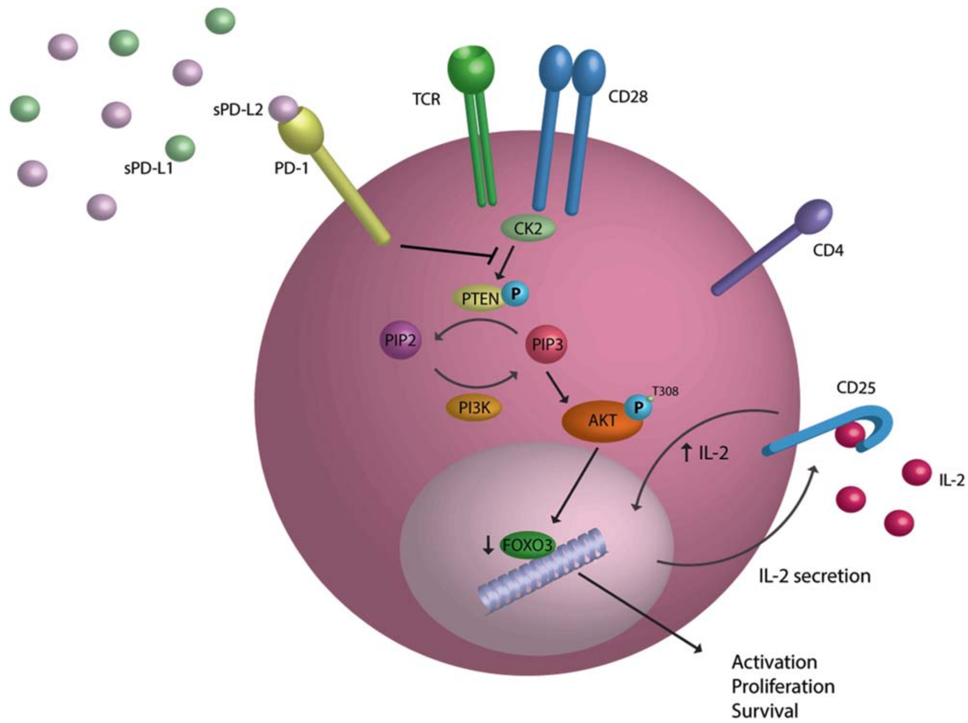

Figure 6. Proposed model by which mesenchymal stromal cell (MSC) derived soluble PD-1 ligands modulate the activation status and effector function of CD4+ T cells. Within this study we have demonstrated that MSCs can respond to pro-inflammatory cytokines secreted by activated T cells. MSCs upregulate their secretion of both PD-L1 and PD-L2 in response to this stimulus. These ligands bind directly to the PD-1 receptor on the surface of activated (CD25+) CD4+ T cells resulting in a downregulation of CD25 and the PD-1 receptor, with repression of the AKT pathway. A decreased phosphorylation of AKT is seen at T308, leading to an upregulation of the transcription factor FOXO3. Decreased CD25 expression and IL-2 secretion interferes with the positive auto- and paracrine feedback loops maintaining the activated state of the T cell. These downstream effects ultimately culminate in a decrease in T cell activation and PD-1 cell surface expression, IL-2 production, proliferation and survival. Abbreviations: IL-2, interleukin-2; sPD-L1 and sPD-L2, soluble programmed death ligands 1 and 2; TCR, T cell receptor.

Chinnadurai et al. [21], we demonstrate that MSCs can disrupt IL-2 cytokine secretion via a contact-independent mechanism through the PD-1 pathway. Differential results could be potentially attributed to the different methods of detection. Secreted levels were detected here, in contrast to intracellular IL-2 levels, perhaps indicating a blockade in secretion but not intracellular accumulation of the cytokine. However, in agreement with Chinnadurai et al. [21], we report evidence for multiple suppressive pathways for MSCs, acting in both contact and contact-independent pathways through the PD-1 receptor on activated T cells, as well as other non-redundant mechanisms such as IDO. These findings demonstrate the importance of the multiple immunosuppressive pathways that MSCs exert, with each mode of action contributing to the complete overall effect observed.

It has previously been demonstrated that the downregulation of IL-2 via the PD-1 pathway is linked to T cell anergy [40, 41]. Here we demonstrate that, in addition to blocking the activation and effector function of T cells, MSCs can suppress T cell proliferation and induce irreversible hyporesponsiveness through sPD-1 ligands. This anergic state was not responsive to IL-2 supplementation. Furthermore, blockade of the PD-1 receptor completely prevented both suppression of proliferation and anergy. Anergic responses to MSCs have been previously reported within the literature [42]. This reported divisional arrest, purely suppressed T cell proliferation however and cells remained responsive through an upregulation of CD25 upon stimulation. In contrast, we demonstrate that the anergic response induced by MSCs through the PD-1 pathway affects both proliferation and response to stimulation via activation. Flow cytometry for CD25 and PD-1 confirmed that prior MSC exposure significantly suppressed T cell activation upon restimulation.

This induction of peripheral tolerance through anergy appears to be further supported by a second mechanism acting through T cell death. Av and 7-AAD staining of the cells established that a significant proportion of T cells die following exposure to the MSC secretome. Blocking of MSC secreted sPD-L1 could prevent this effect, whereas sPD-L2 had no effect on inducing T cell death. Induction of apoptosis in T cells has been established as a key mechanism in inducing peripheral tolerance through triggering transforming growth factor beta in macrophages and directly upregulating Treg induction [43].

It is well established that CD3/CD28 costimulation leads to tyrosine phosphorylation of CD28, recruiting and activating phosphoinositide 3-kinase (PI3K) [44]. The lipid products of PI3K bind proteins such as AKT. The AKT pathway has been directly linked to regulation of the FOXO family of transcription factors, mediating numerous cellular processes such as proliferation, apoptosis and cytokine production [45]. Of this family, FOXO3 has been shown to be central to inflammatory processes, with global deletion resulting in organ inflammation and dysregulation of T cell activation and proliferation

[46]. Using phospho-flow, we demonstrate that binding of MSC secreted sPD-L1 to the PD-1 receptor results in a decrease in AKT phosphorylation. This inhibitory effect leads to a downstream upregulation of FOXO3 expression. This effect on FOXO3 can be reversed back to control levels with the addition of a PD-L1 blocker. As envisaged, blockade of PD-L1 only partially restored AKT phosphorylation, demonstrating the effects of multiple immunomodulatory soluble factors secreted by MSCs on this signaling molecule, including IDO [47].

Importantly, within this study we demonstrate that MSC secretion of sPD-L1 and L2 both have direct effects on T cell behavior and effector function, but that these effects are differential. It has been previously demonstrated that PD-L1 and PD-L2 directly compete for the PD-1 receptor, however, the nature of the binding interaction is unique, with PD-L1 changing its conformation on binding to PD-1 [25]. Here, these interactions are simplified in this pure co-culture system to delineate direct effects, but within the in vivo context, where PD-L2 has been shown to have direct activating effects on dendritic cells enhancing immune responses [48], we hypothesis that MSCs could utilize their secretion of both of these soluble ligands and their relative frequency to orchestrate adaptive and innate responses.

Combining the knowledge gleamed from this study with the literature, we propose a model (Fig. 6) by which CD3/CD28 costimulation leads to an increased activation of the protein kinase casein kinase 2 (CK2). CK2 leads to phosphorylation of phosphatase and tensin homolog (PTEN) in the C-terminal region, inhibiting its activity. This allows sustained phosphorylation of AKT via PI3K pathway [49]. Phosphorylation of AKT leads to a decrease in FOXO3, ultimately resulting in increased activation, proliferation and effector cytokine production. We demonstrate within this study that MSC derived sPD-1 ligands can directly inhibit this pathway, with sPD-L1 leading to a decrease in AKT phosphorylation at T308 and increased levels of FOXO3. These pathway interruptions lead to a decrease in proliferation, anergy, cell death (via sPD-L1 binding) and a decreased production of IL-2, modulated by both ligands. Decreased CD25 expression in combination with a dampened secretion of IL-2 additionally blocks the auto- and paracrine positive feedback loop on the T cell activation status. As exogenous IL-2 supplementation did not affect CD25 expression, even after MSC removal, we suggest that these effects are separate actions by the MSCs and not a knock-on effect of one another.

Conclusion

In summary, this study reports, for the first time, the secretion and function of sPD-1 ligands by MSCs, in addition to reporting the first known biological function of sPD-L2. We demonstrate that both PD-L1 and PD-L2 are secreted by MSCs and that this is significantly upregulated in response to pro-inflammatory cytokines. These soluble ligands are secreted at levels sufficiently high enough to dampen the AKT pathway, thereby suppressing the activation status and effector function of T cells, in addition to inducing an irreversible hyporesponsive state.

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Author Contributions

L.C.D.: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; N.H.: Conception and design, collection of data, data analysis and interpretation, final approval of manuscript; N.K.: Collection of data, data analysis and interpretation, final approval of manuscript; K.L.B.: Conception and design, financial support, provision of study material, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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