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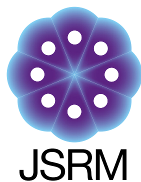

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Original Article

A novel role of CD73-IFNγ signalling axis in human mesenchymal stromal cell mediated inflammatory macrophage suppression

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A B S T R A C T

Introduction: Immunomodulation is the predominant mechanism via which Mesenchymal stromal cells (MSCs) mediate their therapeutic benefits. However, inconsistent success in numerous clinical trials warrants a better understating of the molecular mechanisms regulating their immunomodulatory properties. CD73, an ecto-5′-nucleotidase is abundantly expressed by MSCs, however its precise role in regulating their immunomodulatory properties is still elusive. The present study explored the role of CD73 in Interferon-gamma (IFNγ) sensing and in turn their ability to suppress "inflammatory" M1 macrophages.

Materials and methods: CD73 knockdown MSCs (CD73-KDN) were initially assessed for expression of immunoregulatory molecules and IFNγ sensing ability by analysing expression of IFNγ signalling downstream targets such as pSTAT-1, Interferon-Stimulated Genes (ISG) and Indoleamine 2,3-dioxygnease (IDO), a prototypic IFNγ-induced immunomodulator. Next CD73-KDN MSCs were co-cultured with inflammatory M1 macrophages and evaluated for their ability to suppress them. To delineate the contributory role of CD73 and IFNγ signalling downstream target IDO, they were over-expressed independently in CD73-KDN MSCs and re-evaluated for their ability to suppress M1 macrophages.

Results: CD73-KDN MSCs exhibited reduced expression of immunoregulatory molecules and were refractory to IFNγ signalling as indicated by attenuated expression of pSTAT-1, Interferon-Stimulated Genes (ISG) and Indoleamine 2,3-dioxygnease (IDO) upon IFNγ exposure. Since sensing of inflammation is critical for MSC mediated immunomodulation, CD73-KDN MSCs were functionally evaluated for their ability to immune-modulate "inflammatory" M1 macrophages wherein they failed to suppress M1 macrophages. Interestingly, ectopic expression of either CD73 or IFNγ signalling target IDO1 in CD73-KDN MSCs restored their ability to suppress M1 macrophages, establishing the importance of CD73-IFNγ signalling axis in MSC-mediated inflammatory macrophage suppression.

Conclusion: The present study uncovers the unexplored role of CD73-IFNγ axis in MSC-mediated M1 macrophage suppression. MSC-educated macrophages are the actual immune-modulators at MSC transplant sites, thus CD73 can serve as a key immune-potency marker for benchmarking therapeutically relevant MSCs.

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1. Introduction

Mesenchymal Stromal Cells (MSC) have garnered substantial attention as advanced therapy medicinal products owing to their reparative properties. However despite overwhelming clinical enthusiasm their actual therapeutic benefits have been unpredictable and there is lack of clarity on key "functional" markers for annotating therapeutically relevant MSCs within heterogenous

Abbreviations: NT-MSC, non targeting vector control MSC; CD73-KDN MSC, CD73 knockdown MSC; IDO-KDN MSC, IDO knockdown MSC; IDO-OE, IDO overexpression MSC; CD73-OE-CD73-KDN MSC, CD73 overexpression in CD73 knockdown MSC; IDO-OE-CD73-KDN MSC, IDO overexpression in CD73 knockdown MSC.

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populations. According to the first position statement published by International Society for Cell and Gene Therapy (ISCT) in 2005, a minimal criteria for identifying MSCs included adherence to plastic dishes, positive expression of CD73, CD90, CD105 and absence of haematopoietic markers such as CD11b, CD14, CD19, CD34, HLA-DR and trilineage differentiation potential [1]. However growing evidence indicated that stemness and therapeutic properties could be delinked and a revised MSC position statement which included a need for a matrix of assays to earmark therapeutically relevant cells was released [2].

Emerging evidence from *in vitro* and *in vivo* models led to the consensus that majority of the therapeutic benefits of MSCs are due to their immunomodulatory and trophic functions which are induced at sites of injury upon immune cell interface [3]. Sensing inflammatory cues is a key component of MSC mediated reparative effects and determines their immune plasticity. Licensing by inflammatory cytokines enhances their immunomodulatory mechanisms [4,5]. IFNγ$R^{-/-}$ MSCs when transplanted fail to confer protection against Graft-Versus-Host Disease (GvHD) [6]. Reiterated by such evidence, revised guidelines for MSC based therapy propose immunological characterization of inflammatory cytokine primed MSCs to be included as potency release criteria for advanced phase clinical trials [7,8]. However, the precise functional role of *bona fide* MSC markers such as CD73 and the impact of inflammatory-licensing on their expression and impact on downstream immune-mechanisms are elusive, thus motivating the present study.

CD73, a 5′-nucleotidase, is a rate-limiting enzyme in the adenosinergic pathway hydrolysing extracellular Adenosine monophosphate (AMP) to adenosine. Extracellular adenosine is a potent immunosuppressant. Deficiency of CD73 or its pharmacological inhibition exacerbates GvHD [9]. CD73 generated adenosine also induces T cell suppression [10–12].

Macrophages play a central role in both onset and resolution of inflammation at injury site and MSCs instruct macrophage reprogramming in both *in vitro* and *in vivo* contexts [13,14]. In fact, most MSC mediated regenerative benefits are speculated to be caused by MSC- educated macrophages [15]. Extracellular vesicles derived from inflammatory-cytokine pre-conditioned gingival MSCs exhibited enhanced CD73 expression and induced macrophage polarization shifts [16]. However, scientific evidence underscoring the importance of CD73 inputs in inflammatory licensing of MSCs are missing. The present study elucidates the importance of CD73 inputs in inflammatory cytokine sensing and licensing of human bone marrow MSCs immune-modulatory mechanisms using loss and gain of function approaches. Further the role of CD73-Interferon gamma (IFNγ) signalling axis in MSC mediated M1 macrophage suppression were studied in detail in MSC-macrophage co-culture setups. Experimental evidence provided in the study indicates that CD73, apart from being an MSC identifier could also serve as an important immune-potency marker predictive of its ability to suppress macrophage activation at sites of inflammation in turn influencing repair/immune outcomes.

2. Materials and methods

2.1. Cell culture

Human bone marrow MSCs (Lonza) were cultured and expanded in Knockout Dulbecco's Minimal Essential Medium (DMEM-KO, Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco), 2 mM glutamax (Gibco) and 2 ng/ml basic fibroblast growth factor (Sigma) and maintained in a 37 °C, 5% CO_2 incubator (Thermo Scientific). Human monocytic cell line, THP-1 was cultured in RPMI-1640 (Gibco) media supplemented with 10% (v/v) FBS (Gibco), 2 mM glutamax (Gibco), 1 mM sodium pyruvate (Gibco) and maintained in a 37 °C, 5% CO_2 humidified incubator (Thermo Scientific). The cells were seeded at an initial concentration of 2×10^5 cells/ml and maintained till they attained a concentration of 8×10^5 cells/ml following which they were subcultured.

2.2. MSC/M1 macrophage co-culture

For differentiation of human monocyte cell line, THP-1 to naïve macrophages, referred as M_0 in the manuscript, cells were seeded at a density of 7.5×10^4 cells/cm^2 in complete RPMI media supplemented with 20 nM Phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 h. Naïve M_0 macrophages obtained as above were polarized to M1 state by treating them with 100 ng/ml of Lipopolysaccharide (LPS, Sigma) and 15 ng/ml of Interferon-γ (IFNγ) for another 48 h. For deriving Primary human macrophages, PBMC's (Lonza) were plated in 12 well plates (2.5×10^5 cells/cm^2) in complete RPMI media supplemented with M-CSF (50 ng/ml) for 7 days. Naïve macrophages (M_0) thus obtained were subsequently treated with Lipopolysaccharide (LPS -50 ng/ml) and IFNγ (10 ng/ml) for another 48hrs to polarize them towards M1 state Immediately after initiation of M1 macrophage polarization, parallelly, MSCs were seeded separately (7.5×10^3 cells/cm^2) in 0.4 μm cell culture inserts in DMEM-KO complete media and allowed to adhere overnight. MSCs were seeded at the specified density in order to obtain an MSC: macrophage ratio of 1:10. To establish MSC-M1 macrophage co-cultures, inserts containing MSCs were placed with M1 macrophages 18 h post initiation of M1 macrophage polarization treatment and the co-culture system was maintained for the next 30 h to facilitate bi-directional communication between both the cell types (Refer Fig. 3A).

2.3. Lentiviral transduction

shRNA constructs for generating stable CD73 knockdown (CD73 shRNA: TRCN0000323281:sequence–ATTAACATAGGGCCCTATAAG) and IDO-1 knockdown (IDO-1 shRNA: TRCN0000056744: sequence – CCATCTGCAAATCGTGACTAA) MSCs were purchased from Sigma. Lentiviral constructs for expression of human CD73 (pHIV-dTomato-CD73) and IDO-1 (pHIV-dTomato-IDO1) were developed by cloning full length human CD73 and IDO cDNA into pHIV-dTomato vector (Addgene, 21374) at EcoRI and BamHI sites. The primer sequences have been mentioned in supplementary Table S2. pLKO vector containing scrambled shRNA and empty pHIV-dTomato vector were used as non-targeting (NT) vector control. HEK293T cells were used as a packaging cell line for preparing replication incompetent lentiviral particles which were used for transducing MSCs in order to knockdown or overexpress the gene of interest. Cells were cultured in Dulbecco's Minimal Essential Medium High glucose (DMEM-HG, Gibco) supplemented with 10% (v/v) FBS (Gibco), 2 mM glutamax (Gibco), non-essential amino acids (NEAA, Gibco) and maintained in a 37 °C, 5% CO_2 incubator (Thermo Scientific). One day prior to transfection cells were plated in 60 mm dishes at a seeding density of 1.5×10^5 cells/cm^2 in order to attain a confluency 80–90%. On the day of transfection HIV-1-gag-pol helper plasmid (psPAX2), VSVG envelope plasmid (pMD2.G), and lentiviral vectors were diluted in 0.5 ml serum free opti-MEM media (Gibco) to which XtremeGENE HP DNA transfection reagent (Roche) was added. This transfection mix was incubated at room temperature for 10–15 min after which it was added to HEK293T cells for transfecting them. The transfected cells were kept in 37 °C, 5% CO_2 incubator and were left undisturbed for the next 48 h. Supernatants containing lentiviral particles were harvested at 48 h and 72 h post transfection, filtered through 0.4 μm syringe filter and concentrated using amicon filters (Merck-Millipore) by

centrifuging at 3000 rpm for 15 min at room temperature. MSCs were transduced with either NT-vector control lentiviral particles or with knockdown or overexpression vector containing lentiviral particles after which they were subjected to puromcyin selection (1 μg/ml) for 72 h. The selected cells were then expanded, validated for knockdown and overexpression, and used for further experiments.

2.4. mRNA expression analysis

Total RNA was isolated using TriReagent (Sigma) and 500 ng was reverse transcribed for synthesizing cDNA using PrimeScript™ RT Reagent Kit (Perfect Real Time) [Takara Bio – RR037A]. Semi-quantitative PCR and qPCR were performed using EmeraldAmp GT and TB Green Premium ExTaq (Tli RNase H plus master mix (Takara) in a nexus gradient (Eppendorf) and Quantstudio 5 thermal cycler (Thermo Fisher Scientific) respectively. GAPDH was used for normalization and relative expression for qPCR analysis was calculated according to $2^{-\Delta\Delta Ct}$ method and depicted as relative fold change. Primers used in the study are listed in supplementary table S1.

2.5. Flow cytometry

The procedure for sample preparation was different for MSCs and macrophages as detailed below. MSC monolayer was rinsed twice with ice cold PBS, trypsinized and centrifuged at 1500 rpm for 5 min at 4 °C to pellet the cells. After removing the supernatant, cells were resuspended in ice cold FACS buffer (PBS with 5% FBS and 0.1% sodium azide), counted, distributed equally into tubes, and stained with mouse anti-human CD73 PE monoclonal antibody (BD pharmingen) or isotype control on ice for 30 min in dark. Macrophages in culture were rinsed twice with ice-cold PBS and then carefully harvested on ice by gentle scraping in the presence of FACS buffer supplemented with 2 mM EDTA. The cells were then centrifuged at 1500 rpm for 5 min at 4 °C to pellet them. To prevent non-specific binding of antibodies to macrophages, cells were pre-blocked with human FcR blocker (Miltenyi Biotec) and incubated on ice for 15 min. The cells were then washed with FACS buffer to remove unbound FcR blocker by centrifuging at 1500 rpm for 5 min at 4 °C. After discarding the supernatant, cells were resuspended in FACS buffer, a count was taken after which they were distributed equally into tubes and stained with mouse anti-human HLA-DR FITC (BD pharmingen) or isotype control on ice for 30 min in dark. After staining, MSCs or macrophages were washed with FACS buffer to remove excess unbound antibody and gently resuspended in FACS buffer to get a single cell suspension. Cells were acquired on LSRII flow cytometer (BD biosciences) and a total of 10,000 events were recorded for each sample. Data was analyzed using FCS express 6.0 flow cytometry analysis software (De Novo software).

2.6. Immunoblotting

Cells were lysed in ice cold RIPA buffer containing protease inhibitor cocktail with phosphatase inhibitors. Lysates were collected in an eppendorf tube and centrifuged at 15,000 rpm for 25 min at 4 °C to pellet cell debris. Protein concentration in cleared lysates were determined by BCA method. Protein extract (10–20 μg) was separated on a 10% SDS PAGE and transferred to a pre-activated PVDF membrane, following which immunoblotting was performed with specific antibodies (refer supplementary table 3) after prior blocking with 3% BSA or 3 % skimmed milk in TBST for 1.5 h. Membranes were washed with TBST and secondary antibody diluted in TBST was added to the membranes for 2 h. After multiple washes with TBST, immunoreactive proteins were visualized after developing with WesternSure PREMIUM Chemiluminescent substrate kit (Li-COR) and captured using a ChemiDoc imaging system (BioRad). Band intensities were quantitated using Image Lab Software (Bio-Rad) and lane normalization factor was calculated as per housekeeping gene signal intensity which is further used to normalize signal intensity of the experimental genes. Ratio of normalized gene intensity of the test sample with control sample (NT-MSC) was plotted as the fold difference. Since β-actin (42 KDa) and IDO1 (45 KDa) have similar molecular weights β-actin blots were stripped and probed for IDO1 for all immunoblotting experiments. Details of antibodies used are mentioned in supplementary table S3.

2.7. CD73 activity assay

CD73 enzymatic activity was determined using malachite green assay. Spent media was removed and MSCs were washed thrice with phosphate free buffer (2 mM $MgCl_2$, 1 mM KCl, 10 mM Glucose, 10 mM HEPES and 125 mM NaCl) to remove any residual inorganic phosphate (Pi). AMP (25 μM) was added to MSCs in the presence of phosphate-free buffer and incubated for 15 min in a 37 °C, 5% CO_2 incubator during which CD73 hydrolyses AMP to Adenosine and Pi. The reaction is terminated by completely removing the supernatant and collecting it in a separate tube. RIPA is immediately added to MSCs, and the lysate is collected for estimating the protein content using BCA kit as per the manufacturer's instructions (Takara-Bio). The Pi concentration in the supernatant is determined using malachite green kit as per manufacturer's instructions (Sigma). Pi in the supernatant reacts with malachite green molybdate to form a green colored complex which was measured at 620 nm using Ensight spectrophotometer (PerkinElmer). The amount of colored complex formed is directly proportional to the concentration of Pi in the supernatant and thus the degree of enzymatic activity. The absolute amount of Pi in the supernatant is calculated using a standard curve and the value obtained is normalized with protein content and by duration of the assay (15 min) to obtain the actual enzymatic activity which is expressed in units of pmol of Pi/min/μg.

2.8. IDO activity assay

Cell culture supernatant was collected and centrifuged at 5000 rpm for 5 min to remove cell debris. 150 μl of cleared supernatant was collected in a separate tube to which 75 μl of 30% Trichloro acetic acid was added, vortexed and centrifuged at 10,000 rpm for 5 min at room temperature. Next, 50 μl of the supernatant was transferred to a 96 well plate to which equal volume of 2% Ehrlich reagent was added and incubated at room temperature for 10 min. Absorbance was measured at 490 nm on PerkinElmer Ensight spectrophotometer and kynurenine levels were determined using a kynurenine standard curve.

2.9. Enzyme-linked immunosorbent assay

Cell culture supernatants were collected and centrifuged at 5000 rpm for 5 min at 4 °C to remove cell debris. The cleared supernatant was then used for quantitation of IL–10, TNF-α, IL-6 and IL-2 using cytokine ELISA kits (OptEIA Human ELISA sets; BD Biosciences) as per manufacturer instructions. Concentrations of cytokines in supernatants were calculated using a standard curve and depicted as pg/ml.

2.10. Growth factor array

Cell culture supernatants were collected and centrifuged at 5000 rpm for 5 min at 4 °C to remove cell debris. The cleared supernatant was then used for quantitation of various growth factors

using human growth factor antibody-based array (RayBio® Human Growth Factor Array AAH-GF-1-8; Ray Biotech, Norcross GA, USA) as per the manufacturer's instructions. Chemiluminescence was captured using ChemiDoc (Bio-Rad) instrument and analyzed by Image Lab software version 6.1 (Bio-Rad). Initially spot signal intensities were calculated for background, positive control and growth factor specific spots. A global background subtraction was then performed using the background spot signal intensities. Next, signal intensities of individual growth factor spots were normalized with signal intensities of positive control spots for each array. The normalized spot intensities obtained for arrays incubated with supernatants derived from NT-MSC and CD73-KDN MSC were then compared to determine differences in expression levels of various growth factors. To ensure that the signal intensities obtained were specifically due to growth factors secreted by MSC's and not from FBS present in the media, arrays were incubated with just complete media.

2.11. PHA induced lymphoproliferation assay

BM-MSC's seeded (20×10^3 cells/cm^2) in 48 well plate were allowed to adhere overnight following which they were treated with Mitomycin C (10 μg/ml) for 2 h. Post incubation the cells were thoroughly washed with PBS to remove any traces of mitomycin C. In order to stimulate T cell blast formation, PBMCs were suspended in complete RPMI media supplemented with T-cell mitogen, Phytohemagglutinin (PHA, 20 μg/ml) and added to MSC's, 1×10^5/well, to obtain an MSC: PBMC ratio of 1:5. The co-culture was continued for 72 h and 5-bromo-2-deoxyurdine (BrdU) priming was performed 12 h prior to lymphoproliferation assessment. After 72 h, photomicrographs of blasts were captured using Nikon TE-2000S microscope. The cell culture supernatant was collected for IL-2 ELISA while the cells were used for performing BrdU incorporation assay (BrdU Cell proliferation kit, Calbiochem) as per the manufacturer's instructions. PBMC alone served as negative controls whereas PHA treated PBMC's served as positive controls. The extent of T cell blast suppression was calculated by determining the level of BrdU incorporation compared to the PHA treated PBMCs which was considered as 100%.

2.12. Statistical analysis

All statistical analyses were performed using graph pad prism 8.2.1 version (GraphPad Inc, San Diego, CA) with data obtained from at least 3 independent experiments with different biological replicates. Results are represented as mean ± standard error of the mean (SEM), except for antibody based growth factor array where data is indicative of 2 biological replicates. An unpaired student's *t*-test was used when comparing two groups while for multiple group comparison one-way analysis of variance test was used. Significance for all experiments is determined as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

3.1. CD73 expression on MSCs is critical for expression of immunoregulatory molecules

Immunosuppression mediated by adenosine signalling is one of the less well studied paracrine mechanisms via which MSCs immunomodulate. Generation of adenosine in the extracellular space is facilitated by concerted action of ecto-nucleotidases such as CD39 [hydrolyses extracellular Adenosine Triphosphate (ATP)/Adenosine Diphosphate (ADP) to AMP] and CD73 (hydrolyses AMP to Adenosine). Since CD73 is the predominant enzyme which hydrolyses AMP to adenosine, its expression levels dictate the extracellular concentration of adenosine and therefore the threshold of adenosine signalling. However in addition to MSCs, CD73 is ubiquitously expressed by multiple cell types including immune cells as a result of which it is difficult to precisely decipher the exact role of CD73 expressed by MSCs towards adenosine mediated immunosuppression. Therefore to precisely determine the exact role of CD73 on immunomodulatory properties of MSCs, we knocked down its expression in them using a short hairpin RNA (shRNA) based approach which are hereafter referred to as CD73-KDN MSCs. Knockdown of CD73 was initially assessed by analyzing mRNA and protein and as seen in Fig. 1A and B, a significant abrogation in its expression was seen in CD73-KDN MSCs when compared to non-targeting vector transduced MSCs (NT-MSCs). A substantial reduction in its cell surface expression (Fig. 1C) as well as ecto-5′-nucleotidase activity (Fig. 1D) further validated knockdown of CD73 in MSCs. Three independent CD73-KDN MSC clones (~ 90% reduction in CD73) were generated and used for further experimentation (Figure S1). Since MSCs predominantly immunomodulate via secreting a broad array of paracrine factors, CD73-KDN MSCs were characterized for expression of known immunoregulatory molecules, prostaglandin-endoperoxide synthase 2 (*PTGS2*), Hepatocyte growth factor (*HGF*), Transforming growth factor β1 (*TGF−β1*), Interleukin-10 (*IL-10*) and Heme oxygenase - 1 (*HO-1*). As seen in Fig. 1E a significant reduction in their mRNA levels was noted in CD73-KDN MSCs when compared to NT-MSCs with greatest reduction being observed in *PTGS2, HGF* and *IL-10* followed by *TGF−β1* and *HO-1.* To further validate the impact of CD73-KDN on MSC secretome, supernatants were probed for 41 representative growth actors using a human growth factor array and as observed in Fig. 1F a marked reduction in levels of HGF, Insulin-like growth factor binding protein 2 (IGFBP2) and IGFBP6 were noted in CD73-KDN MSCs as compared to NT-MSCs in two independent biological replicates of MSCs (Fig. 1F and Figure S2). Levels of HGF, a known MSC-secreted angiopoietic/immunoregulatory factor were strikingly compromised in CD73-KDN MSCs. In consensus with this result, in a mouse model of myocardial infarction sorted mouse CD73+MSCs, which exhibited increased expression of pro-angiogenic factors including HGF, were more competent at cardiac recovery [17]. IGFBP family members such as IGFBP4 and IGFBP7 have also been implicated in MSC-immunomodulation [18,19]. Similarly a significant reduction in IL-10 secretion (Fig. 1G) as well as HO-1 protein levels (Fig. 1H and S3) was noted in CD73-KDN MSCs when compared to NT-MSCs. Put together these results show that CD73 is essential for expression of key immunoregulatory molecules by MSCs.

3.2. CD73-KDN MSCs exhibit attenuated IFNγ signalling

Immunomodulatory functions of MSCs are not constitutive but are instead induced upon exposure to an inflammatory microenvironment such as those prevalent during tissue injury. The endowment of immune modulatory behaviour to MSCs upon exposure to pro-inflammatory cytokines is termed as inflammatory licensing. IFNγ, either alone or in combination with other pro-inflammatory cytokines, immune licenses MSCs *in vitro* and enhances their therapeutic outcomes [3,21]. In fact, blocking IFNγ reception or deficiency of IFNγR1 in MSCs has been shown to annul MSC mediated immunosuppression [6,20,21]. To evaluate whether CD73-KDN MSCs are receptive to IFNγ-mediated immune-licensing, CD73-KDN cells were treated with IFNγ and induction of IDO, a prototypic interferon gamma stimulated gene (ISG), and an immunomodulatory molecule was assessed as a readout of IFNγ-sensing ability. In the absence of IFNγ, NT-MSCs and CD73-KDN MSCs do not show any basal IDO expression (Fig. 2B).

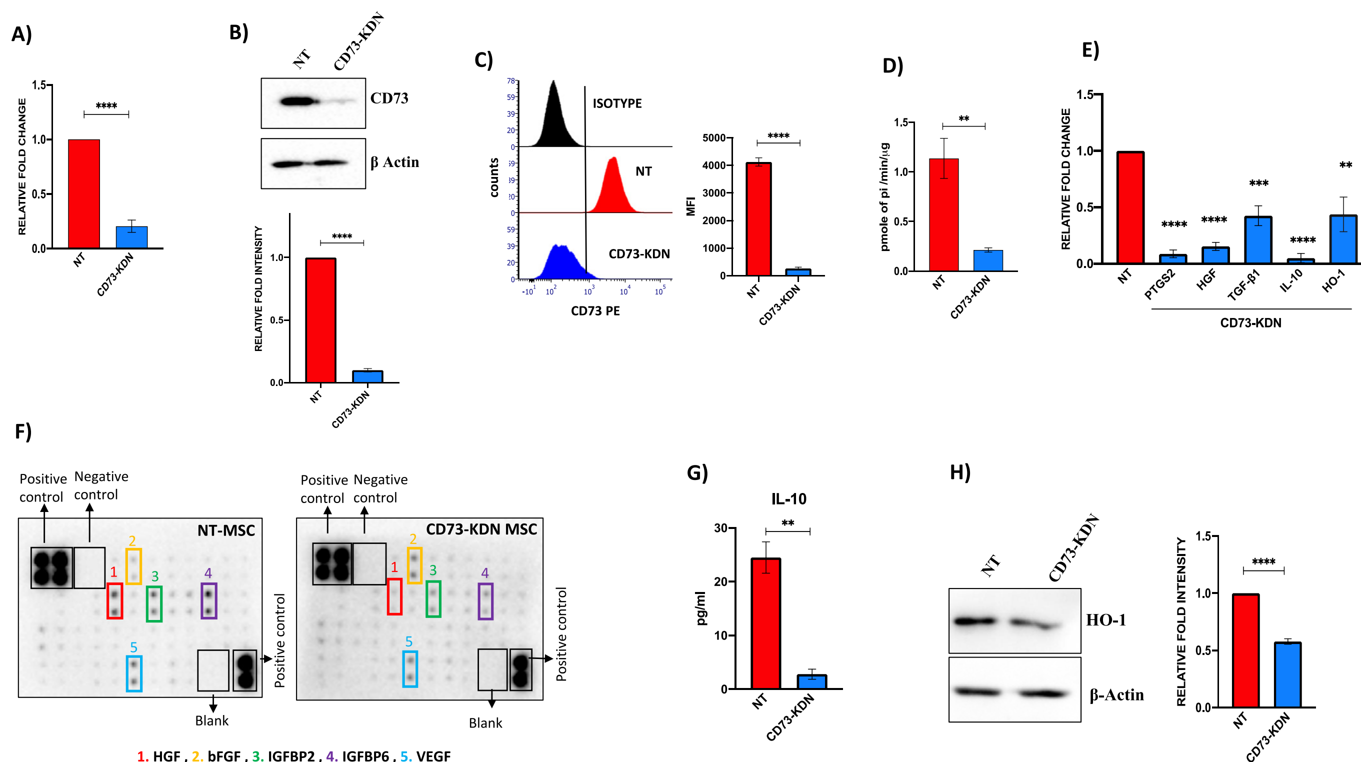

Fig. 1. CD73 knockdown (CD73-KDN) Mesenchymal stromal cells (MSCs) exhibit compromised expression of immunoregulatory molecules. Confirmation of loss of CD73 in CD73-KDN MSCs by assessing its A) mRNA expression, B) protein expression, C) cell surface expression and D) ecto-5′-nucleotidase activity. E) Bar graph depicting relative fold decrease in mRNA expression of critical immunomodulatory genes Prostaglandin-endoperoxide synthase 2 (*PTGS2*), Hepatocyte growth factor (*HGF*), Transforming growth Factor-β1 (*TGF-β1*), Interleukin − 10 (*IL-10*), and Heme oxygenase *-1 (HO-1)* in CD73-KDN MSCs compared to vector transduced MSCs (NT-MSCs). F) Antibody based growth factor array depicting differences in levels of growth factors secreted by NT-MSCs and CD73-KDN MSCs. G) ELISA based quantification of IL-10 secretion in CD73-KDN MSCs in comparison to NT-MSCs. H) Immunoblot of HO-1 in NT-MSCs and CD73-KDN MSCs along with corresponding quantification depicting reduction in expression levels in CD73-KDN MSCs. Results (A–H) are represented as mean ± SEM and obtained from 3 independent experiments (n = 3) except for growth factor array which is representative of two independent biological replicates (n = 2). Significance was determined as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

However, upon IFNγ treatment, NT-MSCs exhibited robust induction of IDO as evidenced by increase in its mRNA and protein expression as well as by increase in kynurenine levels indicative of heightened IDO activity. In striking contrast to NT-MSCs, IDO induction was severely compromised in CD73-KDN MSCs as seen by drastic reduction in its expression at mRNA, protein, and activity (Fig. 2A, B, C). A modest enhancement of CD73 protein was noted in IFNγ treated NT-MSCs (Fig. 4D) which is concordant with results obtained in a previous study wherein exosomes derived from inflammatory cytokine primed gingival MSCs also depicted enhanced CD73 expression [16].

IFNγ on binding to its receptors induces dimerization of IFNγR1 and IFNγR2 resulting in activation of Janus kinases (JAKs) which phosphorylate both IFNγR1/R2 creating a signal transducer and activator of transcription 1 (STAT1) docking site. IFNγR recruited STAT1 undergoes Tyr^{701} phosphorylation. Phosphorylated STAT1 (pSTAT1) dimerizes, undergoes nuclear translocation to bind to GAS (Gamma Interferon Activated Sites) sequences on promoters of ISGs inducing their expression. Since IDO expression is downstream of IFNγ signalling, a compromised IDO induction observed in CD73-KDN MSCs could be indicative of a perturbed IFNγ signalling pathway. Therefore to investigate the activation status of IFNγ signaling pathway we assessed STAT1 and pSTAT1 levels in both NT-MSCs and CD73-KDN MSCs in the presence of IFNγ. As seen in Fig. 2D, in response to IFNγ, total STAT1 was induced to comparable levels in both NT-MSCs and CD73-KDN MSCs, however pSTAT1 induction was drastically reduced in CD73-KDN MSCs when compared to NT-MSCs. Therefore, reduced activation of IFNγ signaling pathway in CD73-KDN MSCs might be responsible for their inability to upregulate immune-regulatory IDO in response to IFNγ. In order to ascertain this aspect, we further analyzed the expression of direct downstream targets of IFNγ-pSTAT1 signaling such as Interferon regulatory factor 1 (*IRF1*), Class II major histocompatibility complex transactivator (*CIITA)* and Suppressor of cytokine signaling 1 (*SOCS1)*. As seen in Fig. 2E, CD73-KDN MSCs depicted reduced expression of all these genes when compared to NT-MSCs thus substantiating attenuated IFNγ signaling in CD73-KDN MSCs. On further analysing the expression pattern of Interferon gamma receptor 1 (*IFNGR1)* and *IFNGR2*, a significant reduction in their expression was seen in CD73-KDN MSCs when compared to NT-MSCs (Fig. 2F) indicating attenuated receptivity to IFNγ signalling. Therefore overall, these results demonstrate that CD73 is critical for IFNγ mediated licensing of MSCs. To the best of our knowledge, this the first report of CD73 influencing IFNγ responsiveness in human bone marrow MSCs.

3.3. CD73 expression on MSCs is essential for suppression of inflammatory M1 macrophages

Macrophages are key cellular intermediaries through which MSCs mediate their therapeutic benefits and they do so by altering their polarization states via paracrine secretions [15]. Since compromised expression of immune-regulatory molecules as well as receptivity to IFNγ was noted in MSCs upon CD73 knockdown, MSC-M1-macrophage co-cultures were setup to evaluate effectiveness of CD73-KDN MSCs to suppress M1 macrophages. M1 macrophages are generated from naïve macrophages upon exposure to inflammatory cues and secrete inflammatory cytokines such

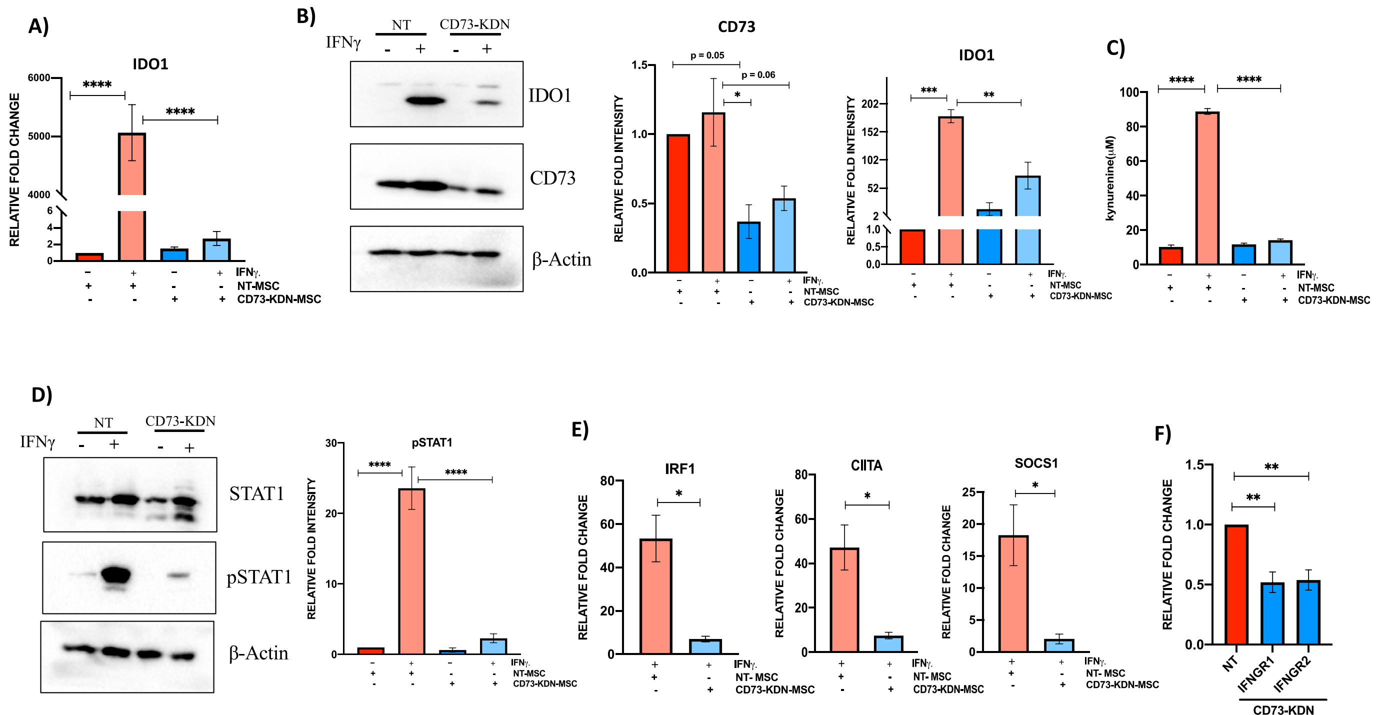

Fig. 2. CD73-KDN MSCs are refractory to Interferon gamma (IFNγ) signaling. Impaired Indoleamine 2,3-dioxygenase (IDO) induction in CD73-KDN MSCs as depicted by downregulation in IDO A) mRNA levels, B) protein expression and corresponding quantification and C) activity as demonstrated by lower kynurenine levels. D) Representative immunoblot of total Signal transducer and activator of transcription 1 (STAT1) and pSTAT1 in NT-MSCs and CD73-KDN MSCs along with corresponding quantification depicting dramatic reduction in pSTAT1 levels in CD73-KDN MSCs. Reduction in mRNA levels of E) IFNγ target genes; Interferon regulatory factor 1 (*IRF1*), Class II major histocompatibility complex transactivator *(CIITA)*, Suppressor of cytokine signaling *(SOCS1)* and F) *IFNGR1* and *IFNGR2* in CD73-KDN MSCs indicative of attenuated IFNγ signaling. MSCs were exposed to 15 ng/ml of IFNγ for 48 h before analysis. Results are represented as mean ± SEM and obtained from 3 independent experiments (n = 3). Significance was determined as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

as TNFα. A co-culture system was established using transwell inserts wherein NT-MSCs or CD73-KDN MSCs were cultured in the insert while M1 macrophages were cultured in the well thus facilitating reciprocal interaction between both the cell types (Fig. 3A). To obtain M1 macrophages, human monocytic cell line THP-1 were initially treated with Phorbol 12-myristate 13-acetate (PMA) for 48hrs to obtain naïve macrophages (M_0) which were subsequently treated with Lipopolysaccharide (LPS -100 ng/ml) and IFNγ (15 ng/ml) for another 48hrs to polarize them towards M1 state. To establish MSC-M1 macrophage co-cultures, inserts containing MSCs were placed with macrophages pre-primed for 18 h with M1 stimuli and the co-culture system was maintained for the next 30 h with LPS and IFNγ (Fig. 3A). In comparison to naïve macrophages (M_0), M1 macrophages exhibited enhanced mRNA expression of Tumour necrosis factor α (*TNFα*), Interleukin 6 *(IL-6)*, Chemokine receptor 7 *(CCR7)*, Human leukocyte antigen-DR isotype (*HLA-DR)* and CD86 (Fig. 3B). Similar results were obtained on analyzing secreted TNFα and IL-6 levels (Fig. 3C) as well as cell surface HLA-DR expression (Fig. 3D) thus indicating successful acquisition of M1 state. As expected, and as previously reported by our group [13], NT-MSCs successfully supressed M1 macrophages as evidenced by substantial reduction in mRNA levels of M1 macrophage associated markers such as *TNFα*, *IL-6*, *CD86* and *CCR7* (Fig. 3B). Similarly, co-culture with NT-MSCs resulted in significant reduction in secretory levels of TNF-α and IL-6 (Fig. 3C). However, a modest downregulation in HLA-DR expression at both transcript and cell surface level was observed in M1 macrophages co-cultured with NT-MSCs (Fig. 3B and D). Surprisingly, unlike NT-MSCs, CD73-KDN MSCs failed to suppress M1 macrophages as evidenced by lack of reduction in expression of M1 associated transcripts *TNFα*, *IL-6*, *CCR7*, *HLA-DR* and *CD86*, secreted levels of TNF-α and IL-6 as well as surface expression of HLA-DR (Fig. 3B, C and D). In fact, with respect to pro-inflammatory cytokines TNF-α and IL-6, co-culture with CD73-KDN MSCs seems to exacerbate their expression to levels which are beyond that of M1 macrophages at both mRNA and secretory level (Fig. 3B and C). A similar trend was observed with respect to HLA-DR expression wherein M1 macrophages co-cultured with CD73-KDN-MSCs exhibited slightly higher cell surface expression than M1 macrophages ($p = 0.31$) (Fig. 3D). Put together, the results obtained emphasize that CD73 is essential for MSCs to mediate their immune-modulatory functions as its deficiency impairs their ability to suppress M1 macrophages. It also seems to result in a shift in the immune plasticity of MSCs from M1 macrophage-suppressing to M1-activating.

3.4. CD73 overexpression in CD73-KDN MSCs restores expression of immune-regulatory genes and responsiveness to IFNγ signalling

To confirm that CD73 deficiency was specifically responsible for compromised immunosuppressive abilities observed in CD73-KDN MSCs, gain-of-function studies were performed by overexpressing CD73 in CD73-KDN MSCs (CD73-OE-CD73-KDN-MSC) following which expression of immunoregulatory genes and IFNγ receptivity were reassessed. As depicted in Fig. 4A and B, overexpression of CD73 in CD73-KDN MSCs resulted in notable increase in mRNA levels as well as enzymatic activity when compared to CD73-KDN MSCs. As shown previously in Fig. 1E, genetic knockdown of CD73 led to substantial downregulation in expression of important immunomodulatory molecules such as *PTGS2*, *HGF*, *TGF-β1*, *HO-1* and *IL-10*. However, overexpression of CD73 in CD73-KDN MSCs reinstated the expression of all these genes (Fig. 4C), with mRNA levels of most of them, except *IL-10*, being significantly higher than that of NT-MSCs. This was commensurate to enhanced expression and activity of CD73 observed in CD73-OE-CD73-KDN MSCs which

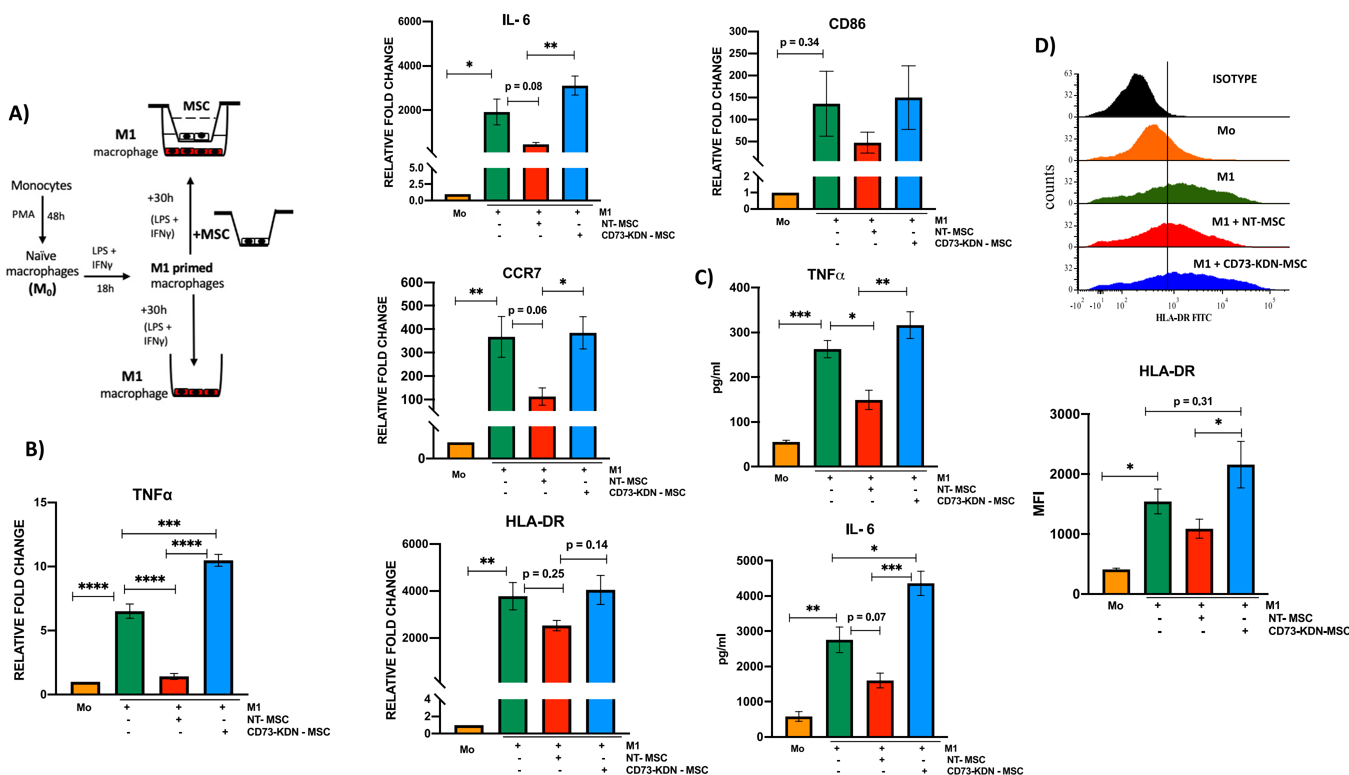

Fig. 3. CD73-KDN MSCs fail to suppress M1 macrophage activation. A) Schematic represents MSC-M1 macrophage co-culture setup. B) Relative fold change in mRNA levels of M1 macrophage associated markers; Tumor necrosis factor α (*TNFα*), Interleukin 6 (*IL-6*), Chemokine receptor 7 (*CCR7*), Human leukocyte antigen–DR isotype (*HLA-DR*), *CD86* in M_0, M1 and M1 macrophages co-cultured with NT-MSCs or CD73-KDN MSCs. C) ELISA based quantitation of pro-inflammatory cytokines, TNFα and IL-6 in supernatants derived from M_0, M1 and M1 macrophages co-cultured with NT-MSC or CD73-KDN MSCs. D) Flow cytometry analysis of HLA-DR expression on M_0, M1 and M1 macrophages co-cultured with NT-MSC or CD73-KDN MSCs. MFI = Median Fluorescence Intensity. Results are represented as mean ± SEM and obtained from 3 independent experiments (n = 3). Significance was determined as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

was over and above NT-MSCs (Fig. 4A, B, C). The extent to which expression levels of *PTGS2* and *HGF* were upregulated on overexpression of CD73 in CD73-KDN MSCs is noteworthy. This result thus indicates that CD73 is directly involved in regulating the expression of these genes thereby playing a pro-active role in maintaining immune-suppressive mechanisms in MSCs. As depicted in Fig. 2A, B, C, in contrast to NT-MSC, CD73-KDN MSCs exhibited attenuated IDO induction when treated with IFNγ. Therefore CD73-OE-CD73-KDN-MSCs were re-evaluated for their ability to effectively sense IFNγ signals akin to NT-MSCs by assessing IDO expression. Overexpression of CD73 in CD73-KDN MSCs increased IDO inducibility upon IFNγ exposure as compared to IFNγ–treated CD73-KDN MSCs thus indicating restoration of IFNγ responsiveness through IFNγR signalling (Fig. 4D). Increase in IFNγ mediated IDO induction on overexpression of CD73 in CD73-KDN MSCs was noted in three independent CD73-KDN clones tested (Fig. 4D and Figure S4) substantiating re-sensitization of IFNγ signalling responses.

Ectoenzyme activity of CD73 results in generation of extracellular adenosine, thus *5′-N-ethylcarboxamide adenosine* (NECA), a non-hydrolysable adenosine analog was added to activate adenosine receptor signalling in CD73-KDN MSCs to evaluate if it restores IFNγ responsiveness. Unlike CD73 overexpression, NECA was incompetent at increasing IFNγ mediated IDO induction in CD73-KDN MSCs (Fig. 4D). This was surprising as CD73-generated adenosine has been implicated in impacting immunosuppression in MSC-T cell co-cultures [10]. MSC derived exosomes were also reported to induce M2 polarization in an adenosine dependent mechanism via using adenosine receptor blockers [22]. However, till date cell autonomous role of CD73 in impacting isolated MSC immune properties had not been explored using precise loss-of-function approaches as in the present study. Thus, the above results underscore the importance of downstream signalling inputs emerging from CD73 receptor signalling/associations in MSC-immunomodulation in a cell-autocrine manner which cannot be mimicked by mere addition of extraneous adenosine mimetics.

3.5. Generation of IDO-knockdown and -overexpression MSCs

IDO is an enzyme which catalyzes the initial rate-limiting step of tryptophan catabolism in the kynurenine pathway. Tryptophan is an essential amino acid and its depletion by IDO activity creates metabolic stress. Moreover, local accumulation of tryptophan catabolites like kynurenine, kynurenic acid, 3-hydroxyanthranilic acid are also known to modulate behavior of immune cells. Put together, metabolic stress due to tryptophan depletion and simultaneous accumulation of its breakdown products are the key mechanisms via which IDO mediates immunosuppression [23]. In fact, IDO inhibitors are being considered for cancer immunotherapies to promote tumour immune effector responses [24]. Based on this rationale, IDO-1 gain and loss of function experiments were performed in MSCs to understand its role in mediating M1 macrophage suppression in MSC-macrophage co-cultures.

IDO-1 expression was silenced in MSCs using an shRNA-based approach following which the transduced cells were treated with IFNγ and IDO expression was assessed at mRNA and protein level to determine the knockdown efficiency. As seen in Fig. 5A, in the presence of IFNγ, IDO-KDN MSCs exhibit almost 90% reduction in

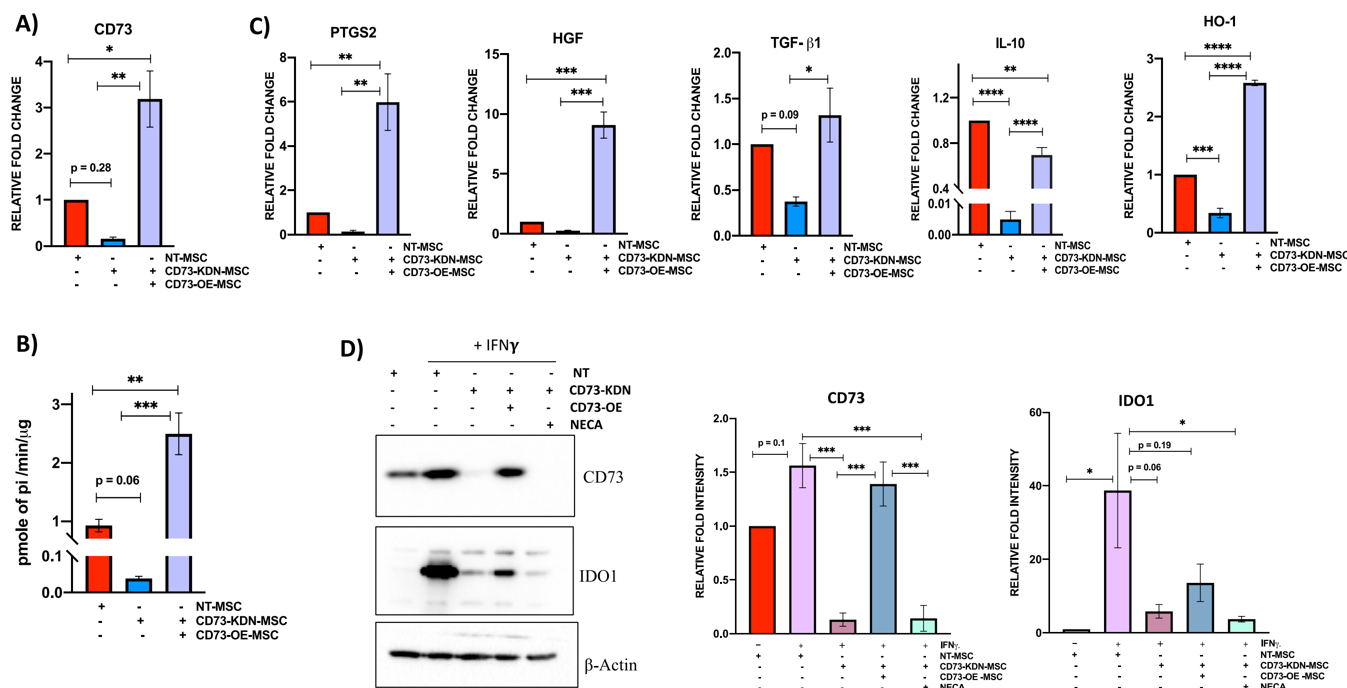

Fig. 4. Ectopic expression of CD73 in CD73-KDN MSCs restores expression of immunomodulatory genes and improves responsiveness to IFNγ signaling. Bar graphs depicting increase in A) CD73 mRNA expression and B) ecto-5′-nucleotidase activity on overexpressing CD73 in CD73-KDN MSCs. C) Bar graph showing rescue in expression of immunoregulatory genes on overexpressing CD73 in CD73-KDN MSCs as evidenced by increase in mRNA levels of *PTGS2*, *HGF*, *TGF-β1*, *IL-10 and HO-1* when compared to CD73-KDN MSCs. D) Representative immunoblot and corresponding quantification depicting rescue of IDO expression on overexpressing CD73 in CD73-KDN MSCs. Transduced MSCs were treated with 15 ng/ml of IFNγ for 48 h with or without 100 μM *5′-N-ethylcarboxamide adenosine* (NECA). Results are represented as mean ± SEM and obtained from 3 independent experiments (n = 3). Significance was determined as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

IDO-1 mRNA expression when compared to NT-MSCs. On assessing expression at protein level, immunoblot analysis showed that IDO protein was almost completely absent in IDO-KDN MSCs when compared to NT-MSC (Fig. 5B). These results were corroborated by IDO activity as well wherein IDO-KDN MSCs exhibited significantly lower kynurenine levels as opposed to NT-MSCs (Fig. 5C). Since CD73 deficient MSCs depicted markedly reduced IDO induction upon IFNγ treatment (Fig. 2A, B, C), IDO1 was overexpressed in CD73-KDN MSCs and IDO overexpressing CD73-KDN MSCs were subsequently re-evaluated for their ability to supress M1 macrophages (Fig. 6, lane 6). Towards this, human IDO overexpression construct was cloned and its overexpression was confirmed by assessing IDO1 mRNA expression and enzymatic activity. As seen in Fig. 5D and E, a significant increase in IDO1 expression at mRNA level as well as enzymatic activity can be appreciated in IDO-OE MSCs compared to NT-MSCs.

3.6. Ectopic expression of CD73 and IDO1 individually in CD73 knockdown MSCs restores THP-1 derived M1 macrophage suppression ability

Either IDO-KDN MSCs (Fig. 6, **lane 4**) or CD73-KDN MSCs with ectopic expression of IDO1 (IDO-OE-CD73-KDN MSCs, Fig. 6, **lane 6**) were evaluated alongside CD73-KDN MSCs for their ability to suppress M1 macrophages by establishing MSC/M1 macrophage co-cultures as described in Fig. 3A. As seen in Fig. 6A and B, when compared to naïve M_0 macrophages, M1 polarized macrophages (**lane 2 vs 1**) exhibited significantly higher expression of all M1 associated markers thus indicating successful acquisition of M1 state. As shown previously, NT-MSCs successfully suppressed M1 macrophages as demonstrated by downregulation in expression of M1 associated markers (Fig. 6A and B, **lane 3 vs 2**). However, IDO-KDN MSCs exhibited slightly lower suppression of M1 macrophages as depicted by a subtle but consistently higher expression of several M1 markers *TNFα* (p = 0.99), *IL-6* (p = 0.8*), CCR7* (p = 0.91), and *CD86* (p = 0.67*)* in comparison to NT-MSCs (Fig. 6A, **lane 4 vs 3**). P values are obtained by comparing NT-MSC with IDO-KDN MSCs. Similarly M1 macrophages co-cultured with IDO-KDN MSCs secreted slightly higher levels of TNFα and IL-6 when compared to those cultured with NT-MSCs in concordance with the mRNA expression analysis (Fig. 6B, **lane 4 vs 3**). However, overexpression of IDO in CD73-KDN MSCs re-established their ability to suppress M1 macrophages as seen by reduction in mRNA levels of M1 associated markers. The greatest reduction was observed in *TNFα* followed by *CCR7*, *CD86* and *IL-6* (Fig. 6A, **lane 6 vs 5**). Similarly IDO-OE-CD73-KDN MSCs significantly supressed TNFα and IL-6 secreted by M1 macrophages as compared to CD73-KDN MSCs (Fig. 6B, **lane 6 vs 5**). These observations thus indicate that IDO secreted by MSCs plays an important role in suppression of M1 macrophages in co-cultures. In fact, kynurenine generated as a result of IDO catabolism has been implicated in driving macrophage polarization switch to an anti-inflammatory phenotype through Kyneurine-AhR-Nrf2 activation [25].

Since overexpression of CD73 in CD73-KDN MSCs restored expression of immunoregulatory molecules as well as IDO, they were re-assessed for their ability to supress M1 macrophages. Akin to previously obtained results, when compared to NT-MSCs, CD73-KDN MSCs failed to suppress M1 macrophages (Fig. 6A and B, **lane 5 vs 3**). However, overexpression of CD73 in CD73-KDN MSCs rescued their ability to suppress M1 macrophages as depicted by reduction in expression of M1 associated markers (Fig. 6A and B, **lane 7 vs 5**). M1 macrophages co-cultured with CD73-OE-CD73-KDN MSCs exhibited substantial reduction in mRNA levels of *TNFα*, *CD86* followed by *IL-6* and *CCR7* when compared to those co-cultured with CD73-KDN MSCs (Fig. 6A, **lane 7 vs 5**). Similar results were obtained on analyzing secreted levels of TNF-α, IL-6 wherein M1

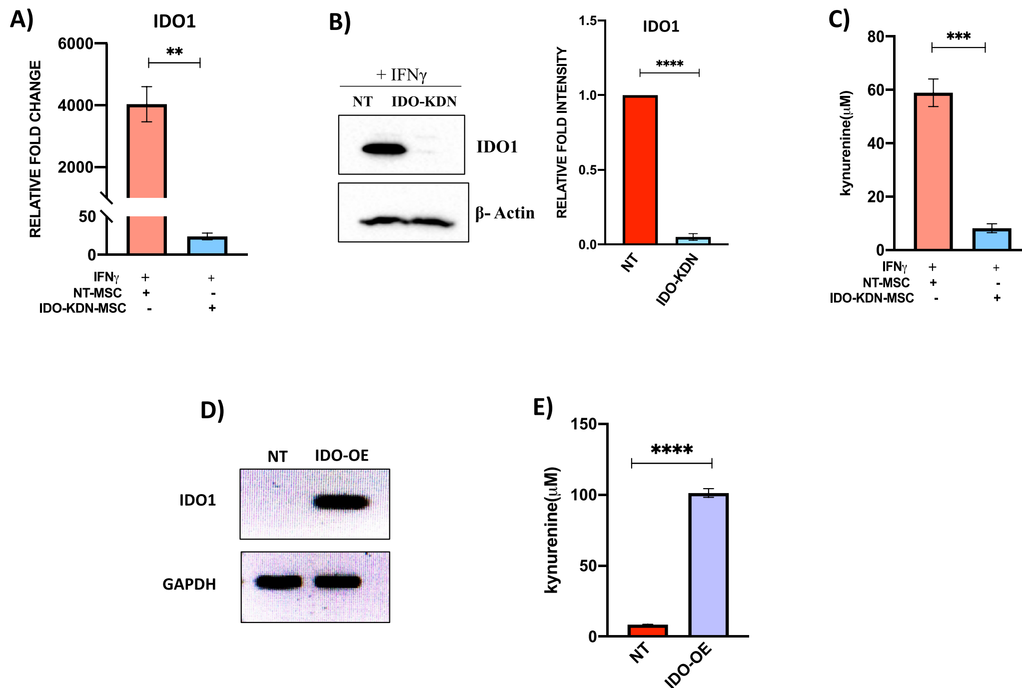

Fig. 5. Validation of IDO-knockdown and -overexpression MSCs. Validation of loss of IDO expression at A) mRNA, B) protein and C) Activity level in IDO-KDN MSCs treated with 15 ng/ml of IFNγ for 48 h. Authentication of IDO overexpression at D) mRNA and E) activity level upon ectopic expression of IDO1 in CD73-KDN-MSCs. Results are represented as mean ± SEM and obtained from 3 independent experiments (n = 3). Significance was determined as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

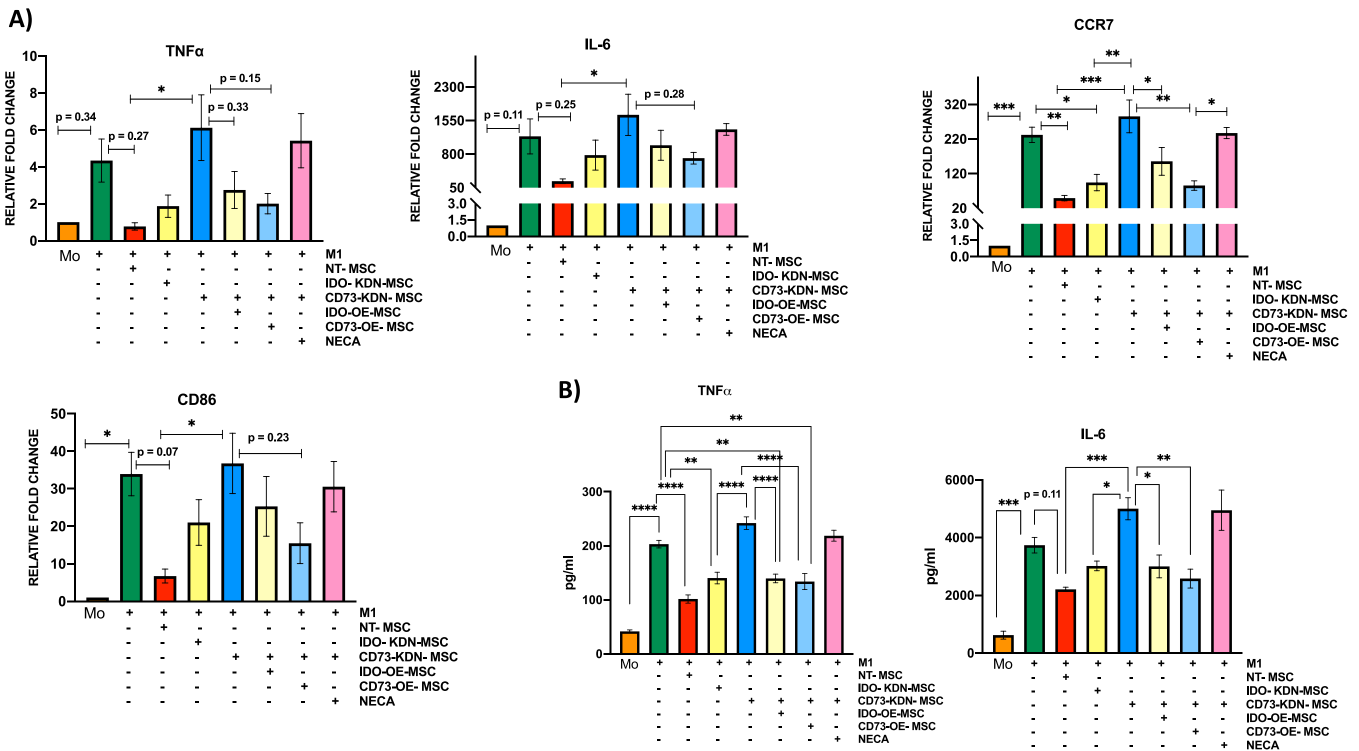

Fig. 6. CD73-IFNγ-IDO1 axis in MSCs enables THP-1 derived M1-macrophage suppression in MSC-macrophage co-cultures. Bar graphs depicting comparative A) mRNA expression analysis of M1 associated markers *TNFα*, *IL-6*, *CCR7*, *CD86* and B) ELISA based quantification of pro-inflammatory cytokines TNFα and IL-6 in M_0, M1 and M1 macrophages co-cultured with differentially transduced MSCs as depicted. Results are represented as mean ± SEM and obtained from 3 independent experiments (n = 3). Significance was determined as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

macrophages co-cultured with CD73-KDN MSCs secreted high levels of TNF-α, IL-6 (Fig. 6B, **lane 5 vs 3**) which were substantially reduced on co-culture with CD73-OE-CD73-KDN MSCs (Fig. 6B, **lane 7 vs 5**). However in contrast to CD73 overexpression, application of adenosine mimetic, NECA, failed to rescue the M1 suppressive potential of CD73-KDN MSCs in co-cultures (Fig. 6A and B, **lane 8 vs 5**) which is in consensus with our previous observation (Fig. 4D). This re-emphasizes the seminal role of signals downstream of “surface CD73” as well as IFNγ sensing in MSC-mediated immune suppression, which are not compensated by extraneous adenosine receptor activation in instructing macrophage activation switches.

3.7. Ectopic expression of CD73 and IDO1 individually in CD73 knockdown MSCs restores primary M1 macrophage suppression ability similar to THP-1 derived M1 macrophages

THP-1 cell line is often used as a surrogate for human macrophage activation studies. However, differences in polarization methodologies and extent of cytokine secretions in human peripheral blood monocyte derived macrophages *versus* THP-1 derived macrophages could impact responses upon MSC interface. Thus human peripheral blood monocyte derived macrophage-MSC co-cultures were established to check the validity of CD73 mediated regulation in primary human macrophage context to derive clinical relevance. For deriving M1 macrophages, PBMCs were initially treated with MCSF (50 ng/ml) for 7 days and the naïve macrophages (M_0) thus obtained were subsequently treated with Lipopolysaccharide (LPS -50 ng/ml) and IFNγ (10 ng/ml) for another 48hrs to polarize them towards M1 state. MSC-M1 macrophage co-cultures were set up in a similar way as that of THP-1 derived macrophages (Fig. 3A). In comparison to naïve macrophages (M_0), those polarised to M1 state exhibited enhanced expression of M1 associated transcripts *TNFa, IL-6, CCR7, CD86* (Fig. 7A, **lane 2 vs 1**) as well as secreted greater levels of TNFα and IL-6 (Fig. 7B, **lane 2 vs 1**) thus indicating successful acquisition of M1 state. Co-culture with NT-MSCs resulted in a significant reduction in mRNA levels of *TNFα, IL-6, CD86* and *CCR7* (Fig. 7A, **lane 3 vs 2**) as well as secreted levels of TNFα and IL-6 (Fig. 7B, **lane 3 vs 2**)thus indicating successful suppression of M1 macrophages. However co-culture with CD73-KDN MSCs failed to suppress primary M1 macrophages as evidenced by lack of reduction in expression of M1 associated transcripts *TNFa, IL-6, CCR7, CD86* (Fig. 7A, **lane 4 vs 3**) as well as secreted levels of TNF-α and IL-6 (Fig. 7B, **lane 5 vs 3**).

Overexpression of IDO1 in CD73-KDN MSCs significantly restored their ability to suppress M1 macrophages as demonstrated by substantial reduction in expression levels of M1 associated transcripts (Fig. 7A, **lane 5 vs 4**) as well as secreted TNF-α and IL-6 (Fig. 7B, **lane 6 vs 5**). The significance of IDO secreted by MSCs in mediating M1 macrophage suppression is further corroborated by the observation that M1 macrophages co-cultured with IDO-KDN MSCs secrete higher levels of TNF-α and IL-6 when compared to those co-cultured with NT-MSCs (Fig. 7B, **lane 4 vs 3**) thus indicating that IDO-KDN MSCs have diminished potential to suppress M1 macrophages. Analogous to IDO, overexpression of CD73 in CD73-KDN MSCs restored their ability to suppress M1 macrophages as shown by a significant reduction in expression of M1 associated genes (Fig. 7A, **lane 6 vs 4**) and M1-cytokine secretion (Fig. 7B, **lane 7 vs 5**). In fact, the extent of suppression was greater in CD73-OE-CD73-KDN MSCs when compared to IDO-OE-CD73-KDN MSCs (Fig. 7B, **lane 7 vs 6**) and on par with that of NT-MSCs (Fig. 7A, **lane 6 vs 3** and Fig. 7B, **lane 7 vs 3**). Substitution of adenosine mimetic, NECA, failed to rescue the M1 suppressive potential of CD73-KDN MSCs in co-cultures (Fig. 7B, **lane 8 vs 5**), an observation consistent with previously obtained results (Fig. 6A and B, **lane 8 vs 5** and Fig. 4D). Overall, the results obtained from primary M1

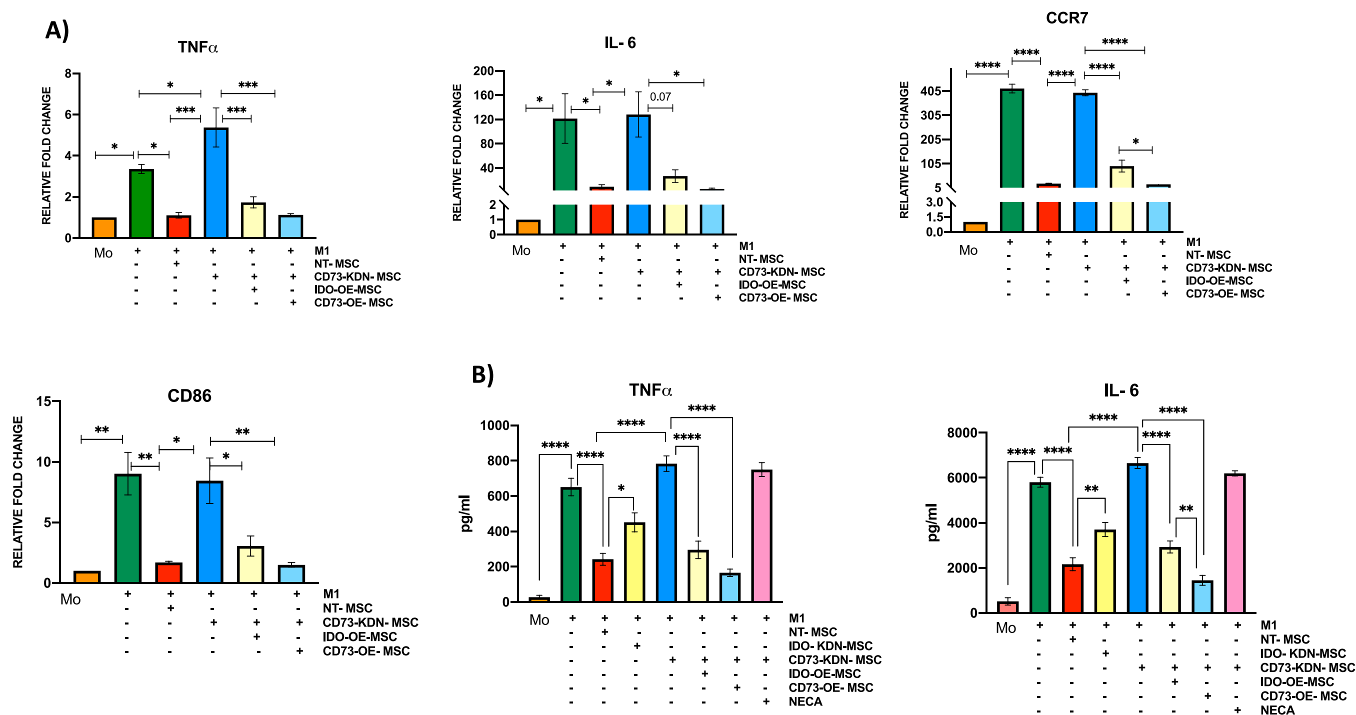

Fig. 7. CD73-IFNγ-IDO1 axis in MSCs enables primary M1-macrophage suppression in MSC-macrophage co-cultures. Bar graphs depicting comparative A) mRNA expression analysis of M1 associated markers *TNFα, IL-6, CCR7, CD86* and B) ELISA based quantification of pro-inflammatory cytokines TNFα and IL-6 in M_0, M1 and M1 macrophages co-cultured with differentially transduced MSCs as depicted. Results are represented as mean ± SEM and obtained from 3 independent experiments (n = 3). Significance was determined as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

macrophages are consistent with those obtained from THP-1 derived macrophages despite differences in extent of pro-inflammatory cytokine secretion by THP-1 derived M1 *versus* primary macrophage derived M1 macrophages.

4. Discussion

For the past two decades MSCs have been widely investigated as potential cell based therapeutics for treating various inflammatory and degenerative diseases. The key feature which makes them a promising candidate for cell based therapy is their ability to sense injured microenvironments and tailoring their immune-modulatory responses to facilitate tissue repair [26]. Despite astounding success in preclinical studies similar results were not recapitulated in clinical settings thereby hindering bench to bedside transition. Heterogeneity in MSC populations with respect to their immunomodulatory properties is considered to be one of the reasons for the lack of consistency in therapeutic outcomes observed in clinical trials. Further differences in immune system of mice and human could also account for the mechanistic differences upon transplantation in mice vs human. Thus there is a need for a reliable marker for identifying therapeutically relevant human MSC populations. CD73, a 5′ ecto-nucleotidase which hydrolyses extracellular AMP to adenosine, is abundantly expressed by MSCs, however its precise functional role in regulating their immunomodulatory properties is still unexplored. Further CD73 has been implicated in generating immunosuppressive adenosine in tumorigenic contexts. However its relevance with respect to MSC immunomodulation and human macrophage reprogramming is elusive.

Thus, CD73 expression was silenced in MSCs and the impact of its absence on their ability to immunomodulate was investigated. MSCs predominantly immunomodulate via secreting a broad array of paracrine factors which alter the fate of immune cells thus modifying their functions to create an anti-inflammatory environment [27]. Absence of CD73 severely compromised the expression of several immunoregulatory molecules in MSCs such as PTGS2, HGF, IL-10, TGF-β1and HO-1 previously implicated in MSC mediated immune-modulation. PGE2 secreted by MSCs was shown to provide protection in an animal model of colitis by stimulating macrophages to secrete IL-10, a potent anti-inflammatory cytokine [28]. A previous study from our group has illustrated the role of MSC secreted PGE2 in macrophage re-programming via altering their metabolic activity [13]. HGF, a pro-angiogenic growth factor abundantly secreted by MSCs plays an important role in tissue repair and remodeling by promoting vascularization of damaged tissues. Further HGF confers protection in animal models of various diseases such as Alzheimer's disease [29], acute kidney injury [30], pulmonary fibrosis [31] as well as multiple sclerosis [32]. In fact, in a mouse model of myocardial infraction, murine MSCs expressing higher levels of CD73 expressed greater levels of HGF and were more competent at cardiac recovery when compared to MSCs expressing lower levels of CD73 and HGF [17]. Our study reestablishes this regulation as CD73KDN human MSCs express and secrete lower levels of HGF than NT-MSCs (Fig. 1E and F). IL-10 is an anti-inflammatory cytokine which supresses inflammation by regulating activity of various immune cells such as macrophages, T cells and neutrophils [33] and provided beneficial effects in animal models of GvHD [34], acute ischemic stroke [35] and collagen induced arthritis [36]. TGFβ secreted by BM-MSCs provided therapeutic benefits in a murine model of ragweed induced asthma by reducing eosinophil infiltration, mucus production, Th2 associated cytokines and IgG1, IgE antibodies [37]. It also aided in generation of Tregs from $CD4^+$ T cells by inducing expression of Foxp3 and CD25 in a contact dependent manner [38]. HO-1 plays an important role in neutralizing oxidative cellular damage observed in situations associated with abnormal oxygen saturation levels such as hypoxia and hyperoxia. MSCs overexpressing HO-1 were shown to provide protection in animal models of pulmonary arterial hypertension and ischemia reperfusion injury [39,40]. Observations in the present study establish CD73 as an important upstream molecule in effecting the expression and secretion of several immunoregulatory molecules implicated to influence MSC immune modulation in mice preclinical models. Further thresholds of CD73 surface expression seems to influence MSC immune-plasticity, as over expression of CD73 in CD73KDN MSCs resulted in upregulation of key immune-suppressive molecules beyond control MSCs imploring its role as a key indicator of MSC immune-regulatory behaviour.

Macrophages are principal cellular intermediaries through which MSCs mediate majority of their therapeutic benefits. In fact experimental evidence from numerous investigations show that MSCs fail to provide protection when transplanted into animal models depleted of macrophages. This reiterates the importance of MSC-educated macrophages as the actual players in mediating regenerative/repair benefits of MSCs at transplant sites. The two most common polarization states in which macrophages are known to exist include the classically activated M1 state and the alternatively activated M2 state. M1 macrophages are highly inflammatory and tissue damaging in nature whereas M2 macrophages are anti-inflammatory and pro-regenerative in nature and facilitate tissue repair [41]. MSCs modulate macrophage behaviour by suppressing their M1 state and polarising them towards an M2 phenotype thus facilitating efficient tissue repair and regeneration. Since loss of CD73 impaired the secretion of critical immunomodulatory molecules, we wanted to assess the functional significance of this observation in context of macrophage programming. A previous study from our group had depicted activation state based manipulation of macrophage plasticity by MSCs with MSCs attenuating M1 macrophage activation but further enhancing M2 polarized states [13]. Thus CD73KDN MSCs were studied for their ability to suppress M1 macrophages in particular.

Interestingly, CD73-deficient MSCs failed to suppress M1 macrophages upon interface. CD73/CD39 pathway mediated production of Adenosine at the MSC-T cell interface was implicated in suppression of T cell activation [10]. However addition of non-hydrolysable adenosine mimetic NECA was incapable of re-establishing M1 suppression in CD73-KDN MSC-M1 macrophage co-cultures highlighting additional roles of CD73 downstream signalling targets apart from adenosine in MSC-immunomodulation. Hence, the ability of CD73-KDN MSCs to attenuate polyclonal T cell activation responses were investigated in a parallel study. In consensus with results obtained with M1 macrophages, CD73-KDN MSCs failed to suppress T cell blast responses as demonstrated by similar BrdU incorporation as well as IL-2 secretion in PHA-induced control T cell blasts cultures and T cell blasts co-cultured with CD73-KDN MSCs [Figure S5] reiterating a general compromise in immune-modulatory abilities of CD73-KDN MSCs.

Immunomodulatory functions of MSCs are not constitutively expressed but are instead induced upon exposure to an inflammatory microenvironment, a process termed as inflammatory licensing. Sensing of inflammation is essential for MSCs as it enables them to attune their responses accordingly [26]. Licensing by pro-inflammatory cytokine IFNγ plays a pivotal role in enhancing the immunosuppressive functions of MSCs. This is highlighted in the study performed by Ren et al. wherein MSCs derived from $IFN\gamma R1^{-/-}$ mice when transplanted into an animal model of GvHD failed to alleviate the pathological symptoms [6]. Similar results were obtained in another study wherein MSCs co-transplanted with $IFN\gamma^{-/-}$ T cells failed to suppress GvHD resulting in 100%

mortality [42]. One of the key effector molecules which is upregulated by IFNγ in MSCs is IDO, an enzyme which catalyzes the initial rate-limiting step of tryptophan catabolism in the kynurenine pathway. Depletion of tryptophan, an essential amino acid, creates metabolic stress which affects the viability of immune cells. Moreover, local accumulation of tryptophan catabolites like kynurenine, kynurenic acid, 3-hydroxyanthranilic acid also modulate immune cell behavior [23]. Since CD73-KDN MSCs could not suppress M1 macrophages despite being exposed to an inflammatory milieu prevalent during M1/MSC co-cultures, we speculated that loss of CD73 might have impacted their ability to sense inflammation which in turn affected their immune-modulatory abilities. To investigate this aspect we initially evaluated IDO expression and observed that CD73-KDN MSCs failed to upregulate it in response to IFNγ suggesting perturbations in IFNγ signaling pathway. Further analysis showed decline in expression of pSTAT1 levels, its downstream target genes IRF1, SOCS1, CIITA as well as IFNγ receptors thus confirming that CD73 is critical for IFNγ licensing of MSCs as its deficiency attenuates IFNγ signaling in turn resulting in downmodulation of immune-modulatory IDO and its metabolites.

IDO has been majorly studied with respect to modulating T cell functions but its influence on macrophages is only being recently appreciated. Overexpression of IDO in naïve macrophages has been shown to polarize them towards an M2 phenotype with increased expression of M2 associated markers such as IL-10 and CXCR4 with concomitant reduction in expression of M1 associated markers such as CCR7 and IL-12p35 [43]. Similar results were obtained by Francois et al. [44] who showed that IDO released by MSCs induced monocytes to differentiate and attain an M2 macrophage phenotype. It is to be noted that similar suppression of TNFα and IL-6 secretion was noted when M1 macrophages were co-cultured with either IDO overexpressing CD73-KDN MSCs or CD73 overexpressing CD73-KDN MSCs (Fig. 6B and 7B). Overexpression of IDO1 in CD73-KDN MSCs significantly rescued their ability to suppress M1 macrophages despite loss of IFNγ sensitivity reiterating the importance of IDO1 downstream of CD73 and IFNγ sensing in MSC-mediated inflammatory macrophage suppression.

Overall the present study highlights the unexplored role of CD73-IFNγ signalling axis in MSC mediated inflammatory macrophage suppression. Metabolic reprogramming of macrophages has been reported in pathological niches such as tumours. Macrophage metabolic programming can influence immune landscapes in pathological niches, thus therapeutic targeting of immune metabolism has garnered significant interest [45]. In this context, we propose that IDO generated by exogenously transplanted CD73+MSCs and endogenous CD73+MSCs could instruct macrophage reprogramming in inflammatory microenvironments and could prove beneficial in curtailing inflammation associated damage. *In consensus* with the study, exosomes from IDO-overexpressing MSCs were reported to accelerate recovery in a mice model of ischemia/reperfusion-induced kidney injury by regulating macrophage polarization [46]. The link between CD73 and IFNγ responsiveness and the ability of IDO1 to rescue M1 suppression even by CD73-KDN MSCs (as evidenced in the study) has major implications in usage of IDO OE-MSCs in conditions of immune incompetence such as Type 2 diabetes where CD73 is compromised [47].

5. Conclusion

MSCs secrete a plethora of immunomodulatory molecules and dominance of a specific immune-suppressive mechanism is context dependent. Further the type and threshold of inflammatory stimuli and activation status of immune cells on interface influences MSC-immune plasticity. Though enough evidence exists on key MSC-mediated immune mechanisms, knowledge regarding key molecular sensors on MSCs which integrate microenvironmental stimuli to determine MSC-immune plasticity are missing. In this context, the present study underscores CD73 as a key molecular indicator of MSC-immune suppression abilities and a major molecular integrator of inflammatory cytokine licensing. Reconstituting CD73 or IDO1, a downstream target of IFNγ signalling, in CD73-deficient MSCs restores macrophage immune suppression reiterating the importance of MSC derived immune-metabolic inputs in directing macrophage functional states. In a nutshell, CD73 expression on MSCs could be of utmost functional relevance in determining their translational potential as cell-based immune-therapeutics and to devise accurate potency assays for MSCs before clinical use.

Author contributions

Methodology, C.S., G.M., D-L.M.; investigation, C.S., G.M., D-L.M., J.P.; formal analysis, C.S.; data curation, C.S.; Writing-original draft, J.P.; Writing-reviewing and editing, C.S., A.K., J.P.; Funding, J.P.; Conceptualization, J.P.; Supervision, J.P. All authors have read and agreed to the published version of the manuscript.

Data availability statement

Data is contained within the article and supplementary material.

Declaration of competing interest

All the authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.05.011.

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