Published in final edited form as:

Cytotherapy. 2016 September ; 18(9): 1114–1128. doi:10.1016/j.jcyt.2016.05.017.

In Vitro Cytokine Licensing Induces Persistent Permissive Chromatin at the *IDO1* Promoter

Yazmin I. Rovira Gonzalez, Patrick J. Lynch, Elaine E. Thompson, Brian G. Stultz, and Deborah A. Hursh

Cellular and Tissue Therapies Branch, Division of Cellular Gene Therapies, Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, Maryland, USA

Abstract

Background—Mesenchymal stromal cells (MSCs) are being investigated as therapies for inflammatory diseases due to their immunosuppressive capacity. IFN- γ treatment primes MSC immunosuppression partially through induction of Indoleamine 2,3-dioxygenase (*IDO1*), which depletes tryptophan necessary to support proliferation of activated T-cells. We investigated the role of histone modifications in the timing and maintenance of induced *IDO1* expression in MSCs under clinical manufacturing conditions, such as cryopreservation.

Methods—We used chromatin immunoprecipitation and quantitative polymerase chain reaction (PCR) to assay levels of transcriptionally permissive acetylated H3K9 and repressive trimethylated H3K9 histone modifications surrounding the transcriptional start site for *IDO1*, and reverse transcriptase PCR and immunoblotting to detect mRNA and protein.

Results—MSCs derived from three donors approached maximum *IDO1* mRNA levels following 24 hours of *in vitro* cytokine treatment. Induction of *IDO1* expression correlated with increased acetylation of H3K9 concomitant with reduction of trimethylated H3K9 modifications at the promoter. Examination of two additional donors confirmed this result. While induced *IDO1* levels declined within two days after cytokine removal and freeze thawing, the activated chromatin state was maintained. Upon re-exposure to cytokines, previously primed MSCs accumulated near-maximum *IDO1* mRNA levels within four to eight hours.

Discussion—Our data indicate that *in vitro* priming of MSCs causes chromatin remodeling at the *IDO1* promoter, that this alteration is maintained during processing commonly used to prepare MSCs for clinical use, and that once primed, MSCs are poised for *IDO1* expression even in the absence of cytokines.

Correspondence: **Deborah Hursh**, PhD, Division of Cellular and Gene Therapies, Center for Biological Evaluation and Research, US Food and Drug Administration, 10903 New Hampshire Ave. Bldg. 72, Rm. 3216, Silver Spring, Maryland 20993, USA. Telephone: 240-402-9597; Fax: 301-595-1093; Deborah.Hursh@fda.hhs.gov or **Patrick Lynch**, PhD, Division of Biotechnology Research and Review, Office of Biotechnology Products, Center for Drug Evaluation and Research, 10903 New Hampshire Ave. Bldg. 71, Rm. 2016, Silver Spring, Maryland 20993, USA. Telephone: 240-402-9713; Patrick.Lynch@fda.hhs.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure of Interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

Keywords

mesenchymal stromal cells; immunomodulation; indoleamine 2,3-dioxygenase; cytokine licensing; histone modifications; chromatin remodeling

Introduction

Mesenchymal stromal cells (MSCs), are plastic-adherent, non-hematopoietic cells, traditionally derived from bone marrow, and propagated *ex vivo* [1, 2]. They are defined by expression of cell surface markers CD105, CD73, and CD90, lack of expression of hematopoietic markers CD45, CD34, CD11b, CD19, and human leukocyte antigen (HLA)-DR, and their capacity to differentiate *in vitro* along osteogenic, adipogenic, and chondrogenic pathways [3, 4]. While they are being investigated for regenerative potential, their range of immunomodulatory activities has led to increased focus on this function [5–7].

Administered MSCs migrate to sites of injury and respond to inflammatory environments by producing factors that modulate host immune activity, a desirable function in immune cytotherapy [8]. However, MSCs immunomodulatory phenotype is not constitutive, but must be activated in response to environmental inflammatory cytokines such as interferon gamma (IFN- γ) through a priming process referred to as “licensing” (reviewed in Krampera 2011) [9]. For instance, when unlicensed allogenic MSCs are co-cultured with unstimulated lymphocytes, they support lymphocyte survival and may be pro-inflammatory [10, 11]. In contrast, when MSCs are primed *in vitro* through exposure to IFN- γ , they inhibit proliferation of activated T cells [8, 12–16]. Moreover, while IFN- γ is required for licensing of MSCs, addition of other cytokines such as tumor necrosis factor alpha (TNF- α), interleukin 1 alpha, or interleukin 1 beta enhance T-cell inhibitory effects of MSCs [8]. Immunosuppression is mediated in part by the expression of multiple factors including prostaglandin E2, TNF-stimulated gene 6 protein (TSG-6), indoleamine 2,3-dioxygenase (IDO), N-terminal quadruplicate cleaved CCL2, human leukocyte antigen-G5, interleukin 6, hepatocyte growth factor, and others from licensed MSCs [5–8]. Thus, the effects of MSCs depend on immunophenotype plasticity, which may affect their clinical function.

Evidence suggests that expression of IDO by MSCs plays a critical role in damping immune system activity [9, 17–19]. IDO is the first and rate-limiting enzyme responsible for tryptophan catabolism in the kynurenine pathway. Tryptophan depletion and kynurenine production limit proliferation of T cells [14, 19]. IDO activity correlates with the capacity of MSCs to suppress T-cell proliferation *in vitro* [17], leading to suggestions that it be monitored during immunotherapeutic applications [20].

Several factors may influence MSC immunophenotype plasticity and may contribute to clinical outcomes. Intrinsic heterogeneity of MSC batches in the timing and maintenance of the primed state may affect their ability to mediate immune responses [21–26]. Furthermore, generating allogeneic MSCs for cell-based immunotherapy typically requires *ex vivo* expansion, washing, and cryopreservation processing steps prior to patient administration. Indeed, recent studies suggest that MSC functions can be negatively impacted by cryopreservation, and would benefit from a culture recovery period following freeze-thaw to

restore impaired immunosuppressive properties [21, 23]. In addition, MSC manufacturing protocols for clinical use are not uniform, which may further affect their immunomodulatory capabilities [22]. The impact of these issues on MSC immunophenotype plasticity remains poorly understood. For these reasons, genes, such as IDO, that could assess cell quality for MSC immunonotherapeutic applications are of interest.

MSC licensing leads to the induction of *IDO1* mRNA expression [27, 28]. Chromatin remodeling of promoter elements often precedes increased gene transcription, allowing fine-tuned gene expression to occur in a cell-type specific manner. Hence, dynamic chromatin modifications can influence a cell's transcriptional, and ultimately phenotypic response to external factors. To this effect, post-translational histone modifications co-localize with regulatory elements in the genome and influence their activity [29]. Several histone modifications are associated with promoter activity, including acetylation at lysine residues, such as lysine 9 (acH3K9), which is correlated with transcriptionally active chromatin. In contrast, trimethylation at this same residue (H3K9me3) correlates with transcriptionally repressed chromatin [30–32]. While H3K9me3 is traditionally associated with regulating broad regions of the genome, it can also associate with and regulate euchromatic promoters [33]. At these promoters, the balance of acH3K9 and H3K9me3 can provide a relative measure of gene activity. For example, T helper 2 (T_H2) cells maintain high H3K9me3 to acH3K9 ratios at T_H1 lineage-specific promoters, which promotes transcriptional silencing of T_H1 genes and stabilizes the T_H2 phenotype [34]. Understanding the epigenetic processes governing MSC cytokine licensing may provide information on how MSCs behave in response to different inflammatory environments encountered *in vitro* or *in vivo*.

Here, we investigated the transcriptional response of *IDO1* to cytokine exposure, asked whether activation was accompanied by alterations in histone modifications at its promoter, and whether such modifications were affected by manufacturing steps, such as cryopreservation, used to prepare MSCs for clinical use. We characterized the kinetics of *IDO1* induction during cytokine treatment and processing of MSCs derived from multiple donors. Using previously described *in vitro* cytokine treatment [17, 35], we determined that it takes 24 hours for MSCs to fully induce *IDO1* expression. This occurs concomitantly with an increase in acH3K9 and loss of H3K9me3 histone modifications at the promoter, demonstrating the involvement of histone modifications in regulating *IDO1* transcripts. *IDO1* expression levels were reduced at the transcript and protein levels following cryopreservation and post-thaw recovery period without treatment; however, *IDO1* promoter chromatin remained transcriptionally permissive for at least 48 hours regardless of how MSCs were processed. For comparison, we also examined the expression kinetics of another gene involved in MSC-mediated immunosuppression, *TSG-6*. In contrast to *IDO1*, chromatin at the *TSG-6* promoter was not associated with the histone modifications investigated in this study. When primed MSCs were re-induced with cytokines after a 48 hour washout period, *IDO1* mRNA approached maximum levels at early four and eight hour time points, suggesting that primed cells were poised to re-induce *IDO1*. Our results suggest that priming MSCs may predispose them for a role in immunosuppression in part by establishing a permissive histone conformation that favors expression at *IDO1*.

Methods

MSC Culture Conditions

Bone marrow-derived MSCs were obtained from AllCells (Alameda, CA), Lonza (Allendale, NJ), and RoosterBio (Frederick, MD) at passage one (Supplemental Table 1). MSCs in this study were previously expanded, characterized, and cryopreserved to generate cell banks as described [36, 37]. Cells were cultured as described in Lynch 2015 [38]. Cells were passaged and collected at 80% confluence using 0.25% trypsin/EDTA (Life Technologies) and cryopreserved in CCM with 30% FBS and 5% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) at 1×10^6 cells/mL. Cryopreserved MSCs were thawed and washed with phosphate buffered saline pH 7.4 (PBS, Life Technologies) for experimentation, or re-plated at 5,000–6,000 cells per cm^2 for further expansion and treatment. Assays were performed on passage 2, 6, or 7 cells fed 24 hours before treatment. MSCs were cultured in CCM with cytokines (10 ng/mL IFN- γ , AF-300-02, and 3 ng/mL TNF- α , AF-300-01A, Peprotech, Rocky Hill, NJ). Cells were trypsinized, washed with PBS, and snap-frozen for subsequent experimentation.

Quantitative Reverse Transcription PCR

RNA was isolated using RNeasy columns (Qiagen, Hilden, Germany) and snap-frozen. RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA Integrity Number (RIN) values were > 9.4 . Complementary DNA (cDNA) was synthesized from 1 μg of RNA using QuantiTect Reverse Transcription kit (Qiagen), and stored at -20°C . Gene specific mRNA was quantified in 10 μl PCR reactions containing 1x SSoAdvanced universal probes supermix (172–5284, Bio-Rad, Hercules, CA), 10 μM oligonucleotides and probes (TaqMan, Life Technologies), and 4.5 μl of cDNA diluted 1:20 in water. Amplification was performed in a C100 Touch Thermal Cycler (Bio-Rad) with 40 cycles of denaturation (95°C for 30 seconds), annealing (95°C for 15 seconds), and extension (60°C for 30 seconds). TaqMan gene expression probes are listed in Supplemental Table 2. Gene expression assays were qualified using triplicate serial dilutions of cDNA, where assays met pre-defined criteria for amplification efficiency (90–110%) and correlation coefficients of $r^2 \geq 0.980$. Relative mRNA values were calculated using qBase software (Biogazelle, Zwijnaarde, Belgium) with normalization methods described previously [39–41]. Mean target gene mRNA values were normalized to mean values from five reference genes (*TBP*, *IPO8*, *HPRT1*, *YWHAZ*, and *UBC*). The geNorm algorithm was used to determine the optimal combination of reference genes to normalize target gene mRNA in MSCs before and after 48 hours of treatment with IFN- γ and TNF- α . Normalized target gene mRNA values were then scaled to mRNA values measured in a sample of human reference total RNA (Clontech, Palo Alto, CA). Reported values represent the averages of three experimental replicates quantified in duplicate PCR reactions.

Chromatin IP

Chromatin was prepared from cells treated with 20 mM sodium butyrate (NaB, Sigma-Aldrich) for 2 minutes to inhibit histone deacetylation. Cells were trypsinized and washed with cold PBS containing 20 mM NaB. Chromatin was cross-linked using truChIP Chromatin Shearing Kit (520154, Covaris, Woburn, MA) according to manufacturer's

instructions, washed twice with cold PBS, and snap-frozen. Cross-linked nuclei were lysed for 10 minutes on a rotator at 4°C, and washed with cold PBS. 1.0 mL aliquots were transferred to milliTUBEs (520132, Covaris) and sonicated for 16 minutes at 4–10°C according to manufacturer's instructions using a Covaris M220 focused ultrasonicator (500295, High Cell Protocol, Covaris) to achieve an average chromatin fragment length of 700 bp. Sheared chromatin was spun at $1,700 \times g$ for 5 minutes at 4°C, transferred to microcentrifuge tubes on ice and snap-frozen. DNA was quantitated following phenol:chloroform:isoamyl alcohol (PCI), pH 8.0, extraction using a nanodrop spectrophotometer (Thermo Scientific, Rockford, IL). Chromatin samples were diluted in RIPA buffer containing 10 mM Tris, pH 7.5, 1mM EDTA, 1% Triton X-100 (Sigma-Aldrich), 0.1% Na-Deoxycholate, 140 mM NaCl, 20 mM NaB, 1mM PMSF, 2µg/mL pepstatin A, 1 µg/mL aprotinin, and 1X complete protease inhibitors (Roche, Indianapolis, IN), to a final concentration of 8 µg per 500 µl for each IP. 4 µL of rabbit polyclonal antibodies to histone H3 acetyl K9 (ABE18; Millipore, Temecula, CA), histone H3 (05–928; Millipore), and histone H3 trimethyl-K9 (ab8898, Abcam, Cambridge, U.K.) were used per IP. Samples were incubated overnight at 4°C with antibodies and 25 µL magnetic Dynabeads Protein-A (Life Technologies), washed three times in RIPA buffer, once in Tris-EDTA, pH 8.0, and resuspended in 500 µL of elution buffer (20 mM tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% SDS, and 50 µg/mL protease K; Life Technologies). Samples were incubated for two hours at 1,300 rpm, at 68°C on a Thermomixer (Eppendorf AG, Hamburg, Germany) to reverse cross-links. DNA was extracted from supernatants with PCI, ethanol precipitated, then resuspended in 100 µL of 10 mM Tris-HCl, pH 8.0, and analyzed by quantitative real-time PCR as described [38]. Relative IP values were determined by taking the ratio of the mean chromatin IP value for each target locus to the mean value of an internal control locus located approximately 8 kb upstream from the transcriptional start site (TSS) of *SMARCA4* [38]. Unless otherwise indicated, values represent averages of four IPs quantified in duplicate PCR reactions. Sequences and locations of the oligonucleotide pairs used are listed in Supplemental Table 3 and Supplemental Figure 1.

Western Blot

Protein was extracted from 1×10^6 MSCs using M-PER (78501; Thermo Scientific) supplemented with complete protease inhibitors (Roche), and stored at –80°C. Protein was boiled 10 minutes, size-separated on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride (162–0177; Bio-Rad) membranes, and blocked overnight at 4°C in 5% nonfat dried milk w/v, TBS-T (TBS, 0.5% Tween-20; Bio-Rad). Blots were incubated for an hour at room temperature with rabbit antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling, Beverly, MA), signal transducer and activator of transcription 1 (STAT-1, Cell Signaling), IDO (Millipore), and phosphorylated STAT-1 (p-STAT-1, Cell Signaling) diluted in 5% nonfat dried milk/TBS-T according to manufacturer's instructions. Blots were incubated for one hour at room temperature with goat anti-rabbit IgG-horseradish peroxidase diluted 1:10000 in 5% nonfat dried milk/TBS-T buffer (Santa Cruz Biotechnology; Santa Cruz, CA), and visualized by chemiluminescence (Thermo Scientific SuperSignal West Femto).

Kynurenine ELISA Assay

The concentration of kynurenine, which is produced by IDO activity in cytokine stimulated MSCs, was determined by an enzyme-linked immunosorbent assay (ELISA). Media from MSC culture was collected after 0 and 48 hours of cytokine treatment and stored frozen at -80°C . Frozen aliquots were thawed to room temperature, and an ELISA was performed as per kynurenine ELISA kit instructions (IBL-America, Minneapolis, MN). A standard curve was constructed from known concentrations: 100, 300, 1000, 3000 and 10,000 ng/ml kynurenine provided in the kit and the data for all experimental samples were interpolated from the standard curve. All samples were measured in duplicate.

Statistical Analyses

Experiments were analyzed with repeated measures ANOVAs to test for statistical significance across MSC lots, using a mixed linear model with compound symmetry in SAS software, version 9.4 (SAS Institute Inc., Cary, NC). Because there were multiple statistical analyses in the study, the significance cutoff was set to $p < 0.01$ and the study wide false discovery rate was limited to 0.05 using the method of Benjamini and Hochberg [42]. ChIP data analyses were performed on the averages of the technical replicates; qPCR data analyses were run on the log of the normalized data, and thus on the log-average of the technical replicates. Tukey post-hoc comparisons with a significance cutoff of 0.05 were used to compare means.

Results

In vitro priming of MSCs de-represses the IDO1 locus

To assess gene activation during cytokine pre-treatment of MSCs, we identified reference genes to serve as internal controls for quantitative RT-PCR analysis. The expression of twelve housekeeping genes was measured in eight MSC lots derived from different donors before and after treatment with $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$. We selected commonly-used housekeeping genes: beta glucuronidase (*GUSB*), actin beta (*ACTB*), ubiquitin C (*UBC*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein L13a (*RPL13A*), succinate dehydrogenase complex, subunit A (*SDHA*), TATA box binding protein (*TBP*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (*YWHAZ*), importin 8 (*IPO8*), Ribosomal protein S18 (*RPS18*), RNA polymerase II, polypeptide A (*POLR2A*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and determined those most unresponsive to cytokine treatment using geNorm software [39, 41]. This produced M values where *TBP* expression was identified as the most stable, followed by *IPO8*, *HPRT1*, *YWHAZ*, *UBC*, *ACTB*, *GAPDH*, *POLR2A*, *GUSB*, *RPL13A*, and *RPS18* (Supplemental Figure 2A). We used qBase pairwise variation (*V*) analysis to determine the optimal number of reference genes necessary to minimize signal variability [39, 41]. Incremental reductions in overall signal variability were achieved by stepwise addition of the five most stable genes (Supplemental Figure 2B). We therefore selected them to normalize all subsequent RT-PCR data for analyses.

We next assessed the kinetics of *IDO1* induction during *in vitro* priming of MSCs with pro-inflammatory cytokines. We selected an $\text{INF-}\gamma$ and $\text{TNF-}\alpha$ cytokine combination for *in vitro*

priming referenced by a recent ISCT working proposal using concentrations previously reported for *in vitro* priming assays [17, 20]. For comparison, we used *TSG-6* mRNA under the same conditions, because its expression is upregulated by *in vitro* priming, and it is thought to play an important role in MSC-mediated immunosuppression [22, 43]. We used three lots of previously characterized MSCs. The karyotypes, cell surface marker expression, transcriptomes, and proteomes of these lots have been examined, as well as their ability to differentiate along the adipogenic lineage [36, 37, 44–47]. These MSC lots were treated with cytokines for up to 72 hours. *IDO1* transcripts were undetectable in the untreated MSCs, but were measurable at 2 hours (Figure 1A). By 24 hours, *IDO1* transcripts increased significantly by two orders of magnitude across these MSC samples as compared to levels seen at 2 hours ($F(6,12)=190.6$, $p<0.0001$). By comparison, *TSG-6* transcripts were detectable in untreated MSCs and increased significantly by one order of magnitude during cytokine treatment (Figure 1B, $F(7,14)=64.59$, $p<0.0001$). It is important to note that kinetics of *IDO1* and *TSG-6* mRNA were evaluated at one dose-level combination of cytokines, and hence, different dose-levels may impact rates of *IDO1* and *TSG-6* inductions. However, under the selected assay conditions, the kinetics of *IDO1* and *TSG-6* transcript accumulation were similar, with a post-hoc comparison of means showing maximal expression at 24 hours and no further significant changes in mRNA levels at 48 or 72 hours. We examined IDO protein accumulation in parallel to see if it followed similar trends to *IDO1* mRNA. Following a delay of 12 hours, IDO protein was first detected in one lot of MSCs (PCBM1641), and in all lots by 24 hours (Figure 1C). We tested for evidence of IDO protein activity by using an ELISA assay to determine kynurenine concentration in the media, since IDO converts tryptophan to kynurenine. We found substantial increases in the kynurenine concentration in media from these lots of cells after 48 hours of cytokine stimulation, demonstrating the presence of active IDO (Figure 1D). We did not investigate the induction of TSG-6 protein because we were unsuccessful in qualifying an antibody to reliably detect TSG-6 by immunoblotting. We also analyzed the timing of IFN- γ induced STAT-1 phosphorylation which corresponds with nuclear translocation and subsequent activation of target genes, and thus reports on the earliest molecular response to IFN- γ . As expected, and consistent with mRNA results, p-STAT-1 was observed in the earliest samples collected after addition of INF- γ and TNF- α (Supplemental Figure 3). Collectively our results for IDO suggest that maximal de-repression of the *IDO1* gene occurs within 24 hours.

The chromatin structure at *IDO1* changes from a repressive to permissive state during *in vitro* priming

In a previous study, we determined that transcriptionally repressive H3K9me3 modifications associate with the promoter region for *IDO1* [38]. We therefore asked whether changes in H3K9me3 modifications in the promoter region of *IDO1* occur concomitantly with increased gene expression. Using antibodies specific for H3K9me3, we immunoprecipitated chromatin from MSCs collected at pre-treatment, 4, and 48 hours of exposure to IFN- γ and TNF- α , and quantified the co-precipitated DNA by RT-PCR using primer sequences located within 250 base pairs of transcriptional start sites (TSS, Supplemental Table 3). As expected, H3K9me3 poorly associated with the constitutively active *GAPDH* promoter in MSCs collected at all timepoints (Figure 2A). In contrast, all lots of untreated MSCs were enriched

by H3K9me3 modifications at the *IDO1* promoter region compared to *GAPDH*. The lowest level of H3K9me3 enrichment at *IDO1* in untreated MSCs occurred in cells from lot 1641, while H3K9me3 associated with the same region at approximately 3-fold higher levels in lots 110877 and 8F3560. These results suggest variability exists in basal H3K9me3 levels at *IDO1* between different lots of MSCs. After 48 hours of cytokine treatment, H3K9me3 levels at the *IDO1* promoter were significantly reduced across all donor lots ($F(2,4) = 27.79$, $p < 0.0045$). The reduction was 6-fold in MSCs from donor lot 1641, and 2-fold in cells from donor lots 110877 and 8F3560. Although maximum *IDO1* mRNA levels were achieved well before 48 hours of cytokine treatment, H3K9me3 signals remained considerably higher than those observed at *GAPDH* under the same conditions in two of the three MSC lots tested (8F3560 and 110877). In contrast to *IDO1*, no significant changes in the density of H3K9me3 histone marks were seen at the *TSG-6* promoter between untreated and cytokine treated MSCs, suggesting that this promoter is not subject to dynamic H3K9 methylation under these conditions.

We next investigated the levels of permissive acH3K9 precipitated from the same chromatin samples as H3K9me3. Because methylation and acetylation are mutually exclusive on the same H3K9 residue, we hypothesized that the observed loss of H3K9me3 at *IDO1* would coincide with a gain in acH3K9. Indeed, treatment with pro-inflammatory cytokines resulted in a significant increase in acH3K9 enrichment at *IDO1* by 8 to 12-fold in cells across all three MSC lots (Figure 2B, $F(2,4) = 46.62$, $p < 0.0017$). The highest levels of acH3K9 at 48 hours of treatment were observed in cells from lot 1641, which were approximately 3-fold higher than the levels observed in lots 110877 and 8F3560. In contrast to *IDO1*, the levels of acH3K9 at the *TSG-6* promoter were significantly reduced after cytokine treatment ($F(2,4) = 70.47$, $p < 0.0008$).

We also examined the levels of total histone H3 using an antibody recognizing the core protein. Changes in total H3 levels would suggest alterations in nucleosome occupancy at promoter regions. As expected, constant low levels of total H3 were associated with *GAPDH* throughout the time course (Figure 2C). Compared to total histone H3 levels seen in untreated MSCs, the amount of total H3 in primed cells was significantly reduced at the *IDO1* promoter site by 2 to 5-fold with the largest difference observed in cells from lot 1641 ($F(2,4) = 28.71$, $p < 0.0022$), which is consistent with a reduction in nucleosome density near the *IDO1* promoter. By comparison, levels of total H3 at the *TSG-6* promoter were not significantly affected by cytokine treatment. These results demonstrate that priming of MSCs results in loss of H3K9me3 enrichment, decreased nucleosome occupancy, and acetylation of H3K9 at the *IDO1* promoter. In contrast, these chromatin structures do not appear to impact changes in *TSG-6* gene expression induced under the same conditions.

In a confirmatory experiment, we extended these observations to two additional donors. Our previous donors were analyzed beginning with passage 5. To verify the results in earlier passage cells that are often targeted for clinical use, we analyzed cells at passage 2 from the additional donors. We limited our examination to untreated (0 hours) and fully primed (48 hours) cells. As seen before, strong induction of *IDO1* and *TSG-6* mRNA was observed at 48 hours (Supplemental Figure 4A, B). In contrast to our previous results, detectable amounts of *IDO1* transcript were observed in untreated cells from these donors. This low

level of *IDO1* transcription in unprimed passage 2 cells is difficult to interpret as measurements of this transcript are too close to the limit of quantitation in our qRT-PCR assay to be reliable.

We examined levels of repressive H3K9me3, permissive acH3K9 and total histone at the *IDO1* and *TSG-6* promoters in the same uninduced and 48 hour induced samples shown in Supplemental Figure 4A, B. We again saw reduction in repressive H3K9me3 histone marks and concomitant increase in permissive acH3K9 marks at the *IDO1* promoter as seen in our previous experiment. We also confirmed the same decrease in total histone H3 enrichment (Supplemental Figure 4C–E). These data provide strong support for the observations seen in Figure 1 and Figure 2, and suggest that chromatin remodeling of the *IDO1* promoter is a general phenomenon seen in MSCs after priming with TNF- α and IFN- γ . We again failed to see any changes in promoter chromatin at *TSG-6*, as observed in our first experiment. Our confirmatory experiment indicates that, even at early passages, there is no difference in the response of *IDO1* to cytokine treatment.

IDO expression is reduced following freeze-thaw and recovery periods in MSCs

Primed MSCs may require processing prior to patient administration, such as washing, cryopreservation, and post-thaw recovery. To better understand how these manufacturing steps might affect *IDO1* expression, we compared the levels of induced mRNA following each step. MSCs were primed with IFN- γ and TNF- α for 48 hours, and then analyzed following: a) freeze-thaw (FT), b) freeze-thaw with a 48 hour recovery period in the presence of cytokines (FT + IFN- γ /TNF- α Rec.), c) freeze-thaw with a 48 hour recovery period in the absence of cytokines (FT + Rec.), and d) continuous culture with a 48 hour culture washout period in the absence of cytokines (Washout, Figure 3A). To control for potential mRNA degradation due to freeze-thaw, we measured *IDO1* and *TSG-6* transcript levels in primed MSCs immediately after thawing. As anticipated, the relative levels of induced mRNA were slightly lower following one cycle of freeze-thaw for *IDO1* (Figure 3B, compare to Figure 1A) and *TSG-6* (Figure 3C, compare to Figure 1B), but remained well above values observed in untreated MSCs. We next measured mRNA levels in primed MSCs following a 48 hour post-thaw culture recovery period in the presence or absence of cytokines. When primed MSCs were thawed and cultured with cytokines for 48 hours, *IDO1* mRNA levels were comparable to those observed immediately after freeze-thaw. In contrast, when primed cells were thawed and cultured without cytokines for 48 hours, *IDO1* mRNA levels dropped approximately 3 orders of magnitude (Figure 3B). Similarly, when primed MSCs were transferred directly to standard culture conditions lacking cytokines and cultured for 48 hours (referred to as Washout), *IDO1* mRNA levels decreased by more than 2 orders of magnitude. However, this drop in *IDO1* transcripts was more pronounced in freshly-thawed cells compared to continuously-cultured cells (Figure 3B, compare columns 3 and 4). Changes in *TSG-6* mRNA levels were consistent with the trends observed for *IDO1* mRNA under the same experimental conditions, albeit with less dramatic differences in the total values (Figure 3C). These results indicate that most of the induced *IDO1* and *TSG-6* transcripts are down-regulated within 48 hours after removal from cytokines, and that freeze-thawing cells enhances this decline.

We also investigated induced IDO protein in post-thaw primed MSCs before and after 48 hours of post-thaw recovery in the absence of cytokines (Figure 3D). As expected, IDO protein was detected in all freshly-thawed MSC lots. Following a 48 hour post-thaw recovery period in cytokine-free culture, IDO proteins were reduced to levels undetectable by our immunoblots (Figure 3D, middle panel). We addressed whether freeze-thawing primed MSCs precipitated the decline in IDO protein levels by comparing IDO in freshly-thawed and continuously-cultured primed MSCs after return to cytokine-free culture conditions. In contrast to post-thaw cells, IDO proteins were detected in at least 2 of the 3 continuously-cultured MSC donor lots following 48 hour return to cytokine-free culture (Figure 3D, compare second and third panels). Initially, IDO protein was undetectable in MSCs from lot 8F3560 despite mRNA levels comparable to the other 2 MSC lots. Using the same protein samples, we repeated the immunoblot using a longer exposure time and determined that some IDO protein was in fact present in cells from this lot, albeit at considerably lower signal compared to other MSC lots tested (Figure 3D'). We also assessed STAT-1 phosphorylation in primed MSCs upon return to cytokine-free culture to determine whether signaling was diminished. Compared to primed cells (FT), p-STAT-1 was still detectable in all lots after 48 hours of cytokine-free culture (Supplemental Figure 5). These findings suggest that after processing steps, IDO levels become reduced to undetectable levels within 48 hours after removal from cytokines.

IDO1 promoter chromatin remains transcriptionally permissive after manufacturing steps in primed MSCs

Our analysis of transcript and protein accumulation indicated that steps used to process MSCs for clinical use altered *IDO1* gene expression. Since priming MSCs with cytokines resulted in increased acH3K9 and reduced H3K9me3 at the *IDO1* promoter, we next asked if post-priming processing steps also impacted dynamic H3K9 modifications. To ensure experimental consistency, samples for chromatin IP were collected in parallel to those described in Figure 3. Consistent with our previous results in continuously-cultured cells (Figure 2A), H3K9me3 remained weakly associated with *IDO1* in primed MSC lots after freeze-thaw (Figure 4A). However, upon returning MSCs to culture without cytokines for 48 hours (FT + Rec.), which resulted in significantly lower mRNA ($F(3,6) = 1693$, $p < 0.0001$), we did not observe a significant change in the levels of repressive H3K9me3 modifications at *IDO1* (Figure 4A). Low levels of H3K9me3 were similarly observed in MSCs under washout conditions. Furthermore, no significant changes in either acH3K9 or total H3 associated with the *IDO1* promoter were observed after returning MSCs to cytokine-free culture conditions for 48 hours, (Figure 4B and 4C). Thus, despite the observed reduction in gene expression, activated chromatin was maintained at *IDO1* after cytokine removal. We also saw no significant changes in H3K9me3 at *TSG-6* across MSC donor lots that would correlate with the significant reduction in gene expression seen ($F(3,6) = 16.64$, $p < 0.0026$). However, relative to primed MSCs post-thaw (FT), a small but significant increase in *TSG-6* promoter acH3K9 levels after both 48-hour treatments ($F(2,4) = 19.79$, $p < 0.0084$) was consistently observed across donor lots. Nonetheless, both promoters maintained a permissive chromatin state based on H3K9me3 and acH3K9 levels regardless of the processing conditions. These results indicate that the reduction in gene expression observed in our processing treatments is not facilitated by changes in H3K9 modifications at the *IDO1*

and *TSG-6* promoters. Moreover, these experiments show that the permissive chromatin state induced by cytokine treatment at the promoter of *IDO1* persists for at least 48 hours in the absence of cytokine stimuli, regardless of processing method.

Primed MSCs accumulate IDO1 transcripts at early timepoints upon re-exposure to cytokines

Due to the maintenance of a transcriptionally permissive chromatin conformation at *IDO1* in primed MSCs during our post-treatment processing steps, we hypothesized that priming MSCs would sensitize them to downstream cytokine stimuli as may occur when infusing cells *in vivo*. We thus sought to test whether primed MSCs accumulate *IDO1* mRNA more rapidly during a second exposure to IFN- γ and TNF- α as compared to their untreated counterparts. To compare *IDO1* mRNA between primed and untreated MSCs, we freeze-thawed MSCs from a single donor lot (1641) that had either been pretreated with IFN- γ and TNF- α or cultured in cytokine-free medium for 48 hours, then cultured them in cytokine-free medium for an additional 48 hours and re-introduced IFN- γ and TNF- α to the cultures. Samples were collected for quantitative RT-PCR analysis at baseline (0 time point taken immediately prior to re-introducing cytokines) and at the indicated time points during cytokine treatment (Figure 5A). At baseline, *IDO1* transcripts were detected in the primed MSCs but not the untreated cells, which precluded objective comparison of mRNA measurements from the starting timepoint (Figure 5B). However, after 4 hours of cytokine exposure, *IDO1* mRNA levels were an order of magnitude higher in primed MSCs compared to untreated MSCs. Within 8 hours of a second exposure to IFN- γ and TNF- α , primed MSCs accumulated *IDO1* mRNA at levels not observed until 24 hours after the first exposure. Primed and untreated cells demonstrated comparable levels of mRNA by 24 hours of exposure. *TSG-6* mRNA levels between 4 and 8 hours of treatment showed differences of approximately 3-fold when comparing primed and untreated MSCs (Figure 5C). Differences in *TSG-6* mRNA accumulation were modest between the first and second rounds of cytokine exposures. *TSG-6* transcripts were comparable in the two conditions following 24 hours of cytokine stimulation regardless of the treatment state. Overall, these results suggest that priming MSCs with IFN- γ and TNF- α can shorten the time it takes cells to fully re-induce *IDO1* expression by several hours.

Discussion

MSCs are attractive candidates for cell therapy to treat immune disorders as they migrate to sites of injury and produce factors to locally inhibit activated T-cells. Furthermore, allogenic MSCs have relatively low immunogenicity, and thus are amenable to use as “off-the-shelf” therapies. The successful incorporation of MSCs into autoimmune cell therapies requires infused MSCs to respond to the *in vivo* environment appropriately. In an effort to understand the factors that influence the MSC immunosuppressive plasticity, our study aimed to characterize environmental events and resulting epigenetic changes that influence regulation of *IDO1*.

Inflammatory stimuli prime MSCs towards an immunomodulatory phenotype, characterized in part by the upregulation of IDO [9]. IFN- γ is required to license the MSC

immunomodulatory phenotype, while TNF- α and other cytokines enhance this phenomenon [8, 13]. Several proteins affecting immune system activity are expressed by primed MSCs. Among these, IDO levels inversely correlate with activated T-cell proliferation, and its activity is hypothesized to play the key role in the mechanism of action for MSC-mediated immunosuppression [17, 35]. For these reasons, IDO is a candidate marker for evaluating therapeutic potential of MSC lots. It is important to understand the capacity of MSCs to upregulate *IDO1*, and the effect that processing steps may have on its expression. For instance, it was recently hypothesized that MSC performance in clinical studies may be negatively affected by cryopreservation [48]. We therefore studied the kinetics of *IDO1* mRNA activation upon cytokine priming and preparation of MSCs.

In our kinetics experiment, we found that after 24 hours of treatment, most MSCs achieved full expression of *IDO1* mRNA. *TSG-6* mRNA also reached its maximal accumulation after 24 hours of cytokine exposure, although the magnitude of induction was not as large as for *IDO1*. Additionally, full IDO protein expression was observed within 24 to 48 hours. A 34-fold increase in kynurenine concentration in the media was also observed after 48 hours, clearly confirming that the IDO protein was active. These data are consistent with ISCT recommendations for licensing MSCs via a 40 hour pre-treatment period [20]. Notably, we observed some variation in *IDO1* and *TSG-6* mRNA levels among the three donor lots of MSCs. Such variability was expected as MSCs are heterogeneous and known for exhibiting phenotypic differences depending on donor source and culture conditions [25, 49]. However, all three donor lots examined in the kinetic experiment reached similar levels of maximal gene expression within 24 hours and full protein expression within 48 hours of cytokine treatment. Additionally, we tested two other donor lots at a lower passage and confirmed strong mRNA induction at 48 hours. Thus, our data imply that two days of *in vitro* priming is sufficient to achieve full upregulation of IDO in MSCs, regardless of lot-to-lot differences in the starting material.

We note that the specific normalization method used for investigating the kinetics of *IDO1* mRNA was critical for our experiments. Expression levels of individual housekeeping genes commonly used to normalize quantitative RT-PCR data can vary widely between cell populations and treatment conditions [41]. Therefore, we used a more robust normalization strategy by incorporating multiple reference genes from independent pathways [50, 51]. Using the geNorm algorithm, we qualified reference genes for use by analyzing their stability across multiple MSC donor lots treated with cytokines. We identified *TBP*, *IPO8*, *HPRT1*, *YWHAZ*, and *UBC* as the most appropriate stable endogenous controls for MSC priming experiments [39–41], which allowed for an accurate estimation of mRNA levels for *IDO1* and *TSG-6* during our kinetic analysis.

Epigenetic modifications influence gene activation and prolonged changes in gene expression. Characterizing histone modifications upon cytokine stimulation advances our understanding of the molecular mechanisms that govern the plasticity of the MSC immunophenotype. In particular, histone post-translational modifications affect gene expression by regulating DNA accessibility and by recruiting proteins that interpret the underlying genetic information. For instance, acetylation of H3K9 at gene promoters results in more accessible, transcriptionally permissive chromatin, while trimethylation of H3K9 is

associated with chromatin condensation, rendering the DNA less permissive to transcription [30–32]. To assess the role of histone modifications on *IDO1* expression, MSCs were primed with cytokine treatment, followed by processing steps commonly used to prepare MSCs for clinical use. Our data demonstrated that the repressive H3K9me3 modifications known to occupy the *IDO1* promoter region in untreated MSCs [38], were significantly reduced upon cytokine treatment. We also found a concomitant significant gain of permissive acH3K9 marks. In addition, the total levels of histone H3 are significantly reduced at the *IDO1* promoter region upon priming, indicating nucleosomes shift away from start site of transcription. This result was observed in both our initial kinetics experiment using three donors at passage 5, and in an additional confirmatory experiment using two donors examined at passage 2, suggesting that dynamic histone modifications at the *IDO1* promoter are a general response in MSCs treated with these cytokine conditions.

We observed residual levels of repressive H3K9me3 in two of the three MSC donor lots after 48 hours of cytokine treatment in our initial kinetic experiment, but not in either of the two MSC donor lots in our confirmatory exercise. These persistent H3K9me3 marks were maintained even in the presence of maximum expression of mRNA. One explanation for this result is that the surrounding nucleosomes upstream of the *IDO1* promoter remain methylated at the H3K9me3 site within the resolution of our chromatin IP assay. However, it is also plausible that MSC subpopulations exist that fail to remove H3K9me3 marks from the *IDO1* promoter.

Our results demonstrate a role for dynamic acetylation and methylation of H3K9 at the *IDO1* promoter. *IDO1* transcription is upregulated by IFN- γ induced phosphorylation of STAT-1. Dynamic effects on chromatin have been seen in other IFN- γ responsive genes. In human fibrosarcoma cells, higher order chromatin changes are seen in the human *Major Histocompatibility Complex (MHC)* upon IFN- γ stimulation and are accompanied by binding of the ATP-dependent chromatin remodeling enzyme, *Brahma-Related Gene 1* (BRG-1) [52]. In several cancer cell lines, BRG-1 activity is also required for the IFN- γ inducible gene *Class II Transactivator* (CIITA), the master regulatory protein responsible for MHC class II gene activation. In addition, increases in the acetylation level of both H3 and H4 are also seen at the CIITA promoter after IFN- γ stimulation [53], but notably, this promoter did not display H3K9, indicating that this modification was not used to silence CIITA. Dynamic histone modifications have also been observed at positions known to correlate with induction of Interferon Regulatory Factor 1 (IRF1) gene expression, which like *IDO1* is upregulated through IFN- γ activation of STAT-1 signaling [54]. In human MSCs, IFN- γ induces expression of many genes, including MHC I, MHC II, and PD-L1 [20]. Dynamic histone modifications could be involved in transcriptional activation of these genes, either through the H3K9me3 and acH3K9 modifications interrogated here, or other types of histone modifications. However, the genome contains thousands of potential STAT-1 target genes [55], and it is unlikely they all use identical regulatory hierarchies to regulate transcription. Determining the contribution of dynamic chromatin modifications to cytokine licensing of MSCs may help to clarify the complex pro- and anti-inflammatory properties displayed by these cells.

A prior study comparing MSC-mediated inhibition of activated T-cell proliferation reports that untreated, freeze-thawed MSCs require a culture recovery period of 24 hours to inhibit *in vitro* T-cell proliferation at the same levels as freshly harvested MSC cultures [21]. We found that although protein and mRNA levels dropped upon freeze-thaw and subsequent culture recovery, active chromatin at the *IDO1* promoter was maintained throughout washing, cryopreservation, and post-thaw recovery procedures. These results suggest that *in vitro* priming establishes a persistent chromatin state permissive to transcription. Consistent with this view, we determined that previously primed MSCs accumulate near-maximum *IDO1* mRNA levels at early time points when re-exposed to cytokine treatment as compared to untreated cells from the same donor. The permissive chromatin state at *IDO1* might explain why primed MSCs outperform unstimulated cells at suppressing proliferation of activated T-cells *in vitro* [35]. Indeed, one might speculate that *in vitro* primed MSCs are poised to respond more efficiently to inflammatory surroundings after administration due to permissive chromatin structures at important immunomodulatory genes such as *IDO1*, although other factors, such as the persistence of phosphorylated STAT-1 may also contribute. These may result in more robust immunomodulation and enhanced therapeutic effect.

Our studies focused on *IDO1* expression because of the critical importance this gene product plays in the MSC mechanism of action. We also investigated dynamic *TSG-6* expression. *TSG-6* is secreted from MSCs and plays an immunoregulatory role in a mouse model of myocardial infarction [43] and in human corneal injury [56]. Although *TSG-6* transcription is upregulated upon cytokine treatment, we found that the *TSG-6* promoter was not associated with the same dynamic modifications at H3K9 after cytokine stimulation. This does not preclude other histone marks present in this region or that a different mechanism, such as DNA methylation, is responsible for *TSG-6* upregulation. These results demonstrate, as suggested [9], the intricacy of the multi-leveled MSC licensing process, where chromatin remodeling events arise within the first steps.

To our knowledge, this is the first study to link cytokine licensing to occupancy and maintenance of specific histone modifications at the *IDO1* promoter in MSCs. This is noteworthy because removal of repressive histone marks at the promoter of *IDO1* in primed MSCs might aid in sensitizing these cells to cytokine re-exposure upon systemic administration and reduce the response time for MSCs to inhibit immune activity *in vivo*. Our results also suggest that monitoring the epigenetic states at genes associated with critical attributes in MSCs may provide information on the effect of culture conditions and other processing steps on the immunosuppressive function of MSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the FDA Modernizing Science program, the FDA Medical Countermeasures Initiative and the Division of Cellular and Gene Therapies. Yazmin Rovira Gonzalez and Elaine Thompson were supported by fellowships administered by the Oak Ridge Institute for Science and Education. We also thank Drs. Cheng Hong

Wei, Steven Bauer, Jeanie Prescott, Ross Marklein and Mark Mortin for critically reviewing the manuscript and members of the FDA's MSC Consortium for valuable discussions.

Abbreviations

MSCs	mesenchymal stromal cells
IFN-γ	Interferon gamma
TNF-α	tumor necrosis factor alpha
IDO	indoleamine 2,3-dioxygenase
TNFAIP6 (TSG-6)	tumor necrosis factor, alpha induced protein 6

References

1. Friedenstein AJ, Chailakhyan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet.* 1970; 3:393–403. [PubMed: 5523063]
2. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation.* 1974; 17:331–340. [PubMed: 4150881]
3. Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006; 8:315–317. [PubMed: 16923606]
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999; 284:143–147. [PubMed: 10102814]
5. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell.* 2013; 13:392–402. [PubMed: 24094322]
6. English K. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol Cell Biol.* 2013; 91:19–26. [PubMed: 23090487]
7. Le BK, Davies LC. Mesenchymal stromal cells and the innate immune response. *Immunol Lett.* 2015;10. [PubMed: 25794633]
8. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell.* 2008; 2:141–150. [PubMed: 18371435]
9. Krampera M. Mesenchymal stromal cell 'licensing': a multistep process. *Leukemia.* 2011; 25:1408–1414. [PubMed: 21617697]
10. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. *Stem Cells.* 2007; 25:1753–1760. [PubMed: 17395776]
11. Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood.* 2006; 107:2570–2577. [PubMed: 16293599]
12. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett.* 2007; 110:91–100. [PubMed: 17507101]
13. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells.* 2006; 24:386–398. [PubMed: 16123384]
14. Plumas J, Chaperot L, Richard MJ, Molens JP, Bensa JC, Favrot MC. Mesenchymal stem cells induce apoptosis of activated T cells. *Leukemia.* 2005; 19:1597–1604. [PubMed: 16049516]

15. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp. Immunol.* 2007; 149:353–363. [PubMed: 17521318]
16. Sheng H, Wang Y, Jin Y, Zhang Q, Zhang Y, Wang L, et al. A critical role of IFN γ in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Res.* 2008; 18:846–857. [PubMed: 18607390]
17. Francois M, Romieu-Mourez R, Li M, Galipeau J. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther.* 2012; 20:187–195. [PubMed: 21934657]
18. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood.* 2004; 103:4619–4621. [PubMed: 15001472]
19. Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? *Immunol Today.* 1999; 20:469–473. [PubMed: 10500295]
20. Krampera M, Galipeau J, Shi Y, Tarte K, Sensebe L. Immunological characterization of multipotent mesenchymal stromal cells--The International Society for Cellular Therapy (ISCT) working proposal. *Cytotherapy.* 2013; 15:1054–1061. [PubMed: 23602578]
21. Francois M, Copland IB, Yuan S, Romieu-Mourez R, Waller EK, Galipeau J. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-gamma licensing. *Cytotherapy.* 2012; 14:147–152. [PubMed: 22029655]
22. Menard C, Pacelli L, Bassi G, Dulong J, Bifari F, Bezier I, et al. Clinical-grade mesenchymal stromal cells produced under various good manufacturing practice processes differ in their immunomodulatory properties: standardization of immune quality controls. *Stem Cells Dev.* 2013; 22:1789–1801. [PubMed: 23339531]
23. Moll G, Alm JJ, Davies LC, von BL, Heldring N, Stenbeck-Funke L, et al. Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem Cells.* 2014; 32:2430–2442. [PubMed: 24805247]
24. Pollock K, Sumstad D, Kadidlo D, McKenna DH, Hubel A. Clinical mesenchymal stromal cell products undergo functional changes in response to freezing. *Cytotherapy.* 2015; 17:38–45. [PubMed: 25457275]
25. Phinney DG. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle.* 2007; 6:2884–2889. [PubMed: 18000405]
26. Luetzkendorf J, Nerger K, Hering J, Moegel A, Hoffmann K, Hoefers C, et al. Cryopreservation does not alter main characteristics of Good Manufacturing Process-grade human multipotent mesenchymal stromal cells including immunomodulating potential and lack of malignant transformation. *Cytotherapy.* 2015; 17:186–198. [PubMed: 25593077]
27. Ren G, Su J, Zhang L, Zhao X, Ling W, L'huillie A, et al. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells.* 2009; 27:1954–1962. [PubMed: 19544427]
28. Robinson CM, Hale PT, Carlin JM. The role of IFN- γ and TNF- α -responsive regulatory elements in the synergistic induction of indoleamine dioxygenase. *J Interferon Cytokine Res.* 2005; 25:20–30. [PubMed: 15684619]
29. Rada-Iglesias A, Wysocka J. Epigenomics of human embryonic stem cells and induced pluripotent stem cells: insights into pluripotency and implications for disease. *Genome Med.* 2011; 3:36. [PubMed: 21658297]
30. Bernstein BE, Kamal M, Lindblad-Toh K, Bekiranov S, Bailey DK, Huebert DJ, et al. Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell.* 2005; 120:169–181. [PubMed: 15680324]
31. Ernst J, Kheradpour P, Mikkelsen TS, Shores N, Ward LD, Epstein CB, et al. Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature.* 2011; 473:43–49. [PubMed: 21441907]

32. Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA. A chromatin landmark and transcription initiation at most promoters in human cells. *Cell*. 2007; 130:77–88. [PubMed: 17632057]
33. Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, et al. Rb targets histone H3 methylation and HP1 to promoters. *Nature*. 2001; 412:561–565. [PubMed: 11484059]
34. Allan RS, Zueva E, Cammas F, Schreiber HA, Masson V, Belz GT, et al. An epigenetic silencing pathway controlling T helper 2 cell lineage commitment. *Nature*. 2012; 487:249–253. [PubMed: 22763435]
35. Cuerquis J, Romieu-Mourez R, Francois M, Routy JP, Young YK, Zhao J, et al. Human mesenchymal stromal cells transiently increase cytokine production by activated T cells before suppressing T-cell proliferation: effect of interferon-gamma and tumor necrosis factor-alpha stimulation. *Cytotherapy*. 2014; 16:191–202. [PubMed: 24438900]
36. Lo Surdo JL, Millis BA, Bauer SR. Automated microscopy as a quantitative method to measure differences in adipogenic differentiation in preparations of human mesenchymal stromal cells. *Cytotherapy*. 2013; 15:1527–1540. [PubMed: 23992827]
37. Lo Surdo J, Bauer SR. Quantitative approaches to detect donor passage differences in adipogenic potential clonogenicity in human bone marrow-derived mesenchymal stem cells. *Tissue Eng Part C. Methods*. 2012; 18:877–889. [PubMed: 22563812]
38. Lynch PJ, Thompson EE, McGinnis K, Rovira Gonzalez YI, Lo Surdo J, Bauer SR, et al. Chromatin Changes at the PPAR-gamma2 Promoter During Bone Marrow-Derived Multipotent Stromal Cell Culture Correlate with Loss of Gene Activation Potential. *Stem Cells*. 2015:10.
39. Hellemans J, Mortier G, De PA, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol*. 2007; 8:R19. [PubMed: 17291332]
40. Hellemans J, Vandesompele J. Selection of reliable reference genes for RT-qPCR analysis. *Methods Mol. Biol*. 2014; 1160:19–26. [PubMed: 24740218]
41. Vandesompele J, De PK, Pattyn F, Poppe B, Van RN, De PA, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002; 3 RESEARCH0034.
42. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc B Met*. 1995; 57:289–300.
43. Lee RH, Pulin AA, Seo MJ, Kota DJ, Ylostalo J, Larson BL, et al. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem. Cell*. 2009; 5:54–63. [PubMed: 19570514]
44. Stultz BG, McGinnis K, Thompson EE, Lo Surdo JL, Bauer SR, Hursh DA. Chromosomal stability of mesenchymal stromal cells during in vitro culture. *Cytotherapy*. 2016; 18:336–343. [PubMed: 26780865]
45. Bellayr IH, Catalano JG, Lababidi S, Yang AX, Lo Surdo JL, Bauer SR, et al. Gene markers of cellular aging in human multipotent stromal cells in culture. *Stem Cell Res Ther*. 2014; 5:59. [PubMed: 24780490]
46. Mindaye ST, Ra M, Lo Surdo JL, Bauer SR, Alterman MA. global proteomic signature of undifferentiated human bone marrow stromal cells: evidence for donor-to-donor proteome heterogeneity. *Stem. Cell Res*. 2013; 11:793–805. [PubMed: 23792435]
47. Mindaye ST, Ra M, Lo Surdo JL, Bauer SR, Alterman MA. Improved proteomic profiling of the cell surface of culture-expanded human bone marrow multipotent stromal cells. *J Proteomics*. 2013; 78:1–14. [PubMed: 23153793]
48. Galipeau J. The mesenchymal stromal cells dilemma--does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? *Cytotherapy*. 2013; 15:2–8. [PubMed: 23260081]
49. Ikebe C, Suzuki K. Mesenchymal stem cells for regenerative therapy: optimization of cell preparation protocols. *Biomed Res Int*. 2014; 2014:951512. [PubMed: 24511552]
50. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009; 55:611–622. [PubMed: 19246619]

51. Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, et al. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Mol. Biol.* 2010; 11:74. [PubMed: 20858237]
52. Christova R, Jones T, Wu PJ, Bolzer A, Costa-Pereira AP, Watling D, et al. P-STAT1 mediates higher-order chromatin remodelling of the human MHC in response to IFN γ . *J Cell Sci.* 2007; 120:3262–3270. [PubMed: 17726060]
53. Ni Z, Karaskov E, Yu T, Callaghan SM, Der S, Park DS, et al. Apical role for BRG1 in cytokine-induced promoter assembly. *Proc Natl Acad Sci U S A.* 2005; 102:14611–14616. [PubMed: 16195385]
54. Buro LJ, Chipumuro E, Henriksen MA. Menin RNF20 recruitment is associated with dynamic histone modifications that regulate signal transducer activator of transcription 1 (STAT1)-activated transcription of the interferon regulatory factor 1 gene (IRF1). *Epigenetics. Chromatin.* 2010; 3:16–13. [PubMed: 20825659]
55. Satoh J, Tabunoki H. A Comprehensive Profile of ChIP-Seq-Based STAT1 Target Genes Suggests the Complexity of STAT1-Mediated Gene Regulatory Mechanisms. *Gene Regul Syst Bio.* 2013; 7:41–56.
56. Roddy GW, Oh JY, Lee RH, Bartosh TJ, Ylostalo J, Coble K, et al. Action at a distance: systemically administered adult stem/progenitor cells (MSCs) reduce inflammatory damage to the cornea without engraftment primarily by secretion of TNF-alpha stimulated gene/protein 6. *Stem. Cells.* 2011; 29:1572–1579. [PubMed: 21837654]

Figure 1. Time course for gene expression and protein induction in response to cytokine treatment. (A) *IDO1* mRNA quantitated by RT-PCR analysis on RNA samples isolated from MSCs before and during treatment with INF- γ (10 ng/mL) and TNF- α (3 ng/mL) for the indicated times. Levels of *IDO1* mRNA were first normalized to the mean expression of five internal reference genes (*TBP*, *IPO8*, *HPRT1*, *YWHAZ*, *UBC*) and then scaled to levels from a human reference RNA standard. Values represent the averages and standard deviations of three experimental replicates obtained in duplicate PCR reactions. (B) *TSG-6* mRNAs

analyzed as described in A. (C) Protein expression analyzed by immunoblot in MSCs collected from the same treatment described in A. Blots were probed with antibodies recognizing IDO and GAPDH (loading control). Treatments and donor identification numbers are indicated at top. (D) Kynurenine concentration in cell culture media after 48 hours of cytokine treatment as compared to untreated cells, measured by ELISA. The average from duplicate samples was used to calculate the kynurenine concentration from a standard curve.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2.

Histone acetylation and methylation patterns change at *IDO1* and *TSG-6* promoters during cytokine treatment of MSCs. (A) Relative enrichment of H3K9me3 near the *GAPDH* (control locus), *IDO1*, and *TSG-6* promoters before and during treatment of MSCs with INF- γ (10 ng/mL) and TNF- α (3 ng/mL) for the indicated times. Chromatin IP DNA was isolated and quantitated by real-time PCR using primers specific for sequences less than 300 base pairs away from transcription start sites (TSS), or at the TSS (*GAPDH*). Values on the y-axis represent the average of four immunoprecipitations from two independent

experiments, after normalization to an internal control site located more than 8 kilobases upstream from the *SMARCA4* gene. (B) Enrichment of acH3K9 and (C) total histone H3 were assessed in the same samples and analyzed as described in A. An asterick (*) indicates a significant change over time across the three lots of MSCs ($p < 0.01$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Effect of post-priming processing steps on induced gene expression in MSCs. (A) Schematic of experimental design. Cryopreserved MSCs were expanded and treated *in vitro* with IFN- γ (10 ng/mL) and TNF- α (3 ng/mL) for 48 hours. The cells were separated into four sets and treated as follows: set 1 was cryopreserved and analyzed immediately after thawing (FT), set 2 was cryopreserved, thawed, and re-treated with cytokines for 2 days (FT + IFN- γ / TNF- α Rec.), set 3 was cryopreserved, thawed, and recovered without cytokine treatment (FT + Rec.), and set 4 was continuously cultured without cytokines after trypsinization (Washout). (B–C) RNA was isolated from MSCs after the steps described in panel A and *IDO1* and *TSG-6* mRNAs were analyzed as described in Figure 1A. Values represent the averages and standard deviations of three experimental replicates quantitated in duplicate PCR reactions. All four treatments in panel B were significantly different. In panel C, an

asterisk (*) indicates a significant pairwise difference in treatments ($p < 0.05$). (D) Protein expression as assessed by immunoblot in MSCs collected from the same experiment described in panel A and probed with antibodies recognizing the indicated proteins. (D') A second immunoblot of the same samples as in the "Washout" panel in D was simultaneously probed with antibodies recognizing GAPDH and IDO, and then exposed for an extended period of time. Note low level expression of IDO detected on long exposure in the 8F3560 sample.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4.

Histone acetylation and methylation patterns at *IDO1* and *TSG-6* promoters in primed MSCs after return to cytokine-free conditions. Relative associations of (A) H3K9me3, (B) acH3K9, and (C) total histone H3 at indicated promoters in cytokine-treated MSCs following freeze-thaw (FT), freeze-thaw with 48 hour recovery in cytokine-free media (FT + Rec.), or 48 hour culture in cytokine-free medium (Washout). Values on the y-axis represent the average of 4 chromatin immunoprecipitations from 2 independent experiments, and were

analyzed as described in Figure 2A. An asterisk (*) indicates a significant change vs. FT ($p<0.01$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

Time course for gene expression in response to sequential cytokine treatments. (A) Schematic of experimental design. MSCs from donor 1641 were grown to sub-confluence and treated *in vitro* with cytokines for 48 hours (primed) or kept in culture for 48 hours without cytokines (untreated), and cryopreserved. Primed and untreated MSCs were freeze-thawed and recovered in the absence of cytokines for 48 hours, and then induced with cytokines for 4, 8, and 24 hours. (B–C) *IDO1* and *TSG6* mRNA were quantitated by RT-PCR as described in Figure 1A. Values represent the average and standard deviation of three experimental replicates quantitated in duplicate PCR reactions. ND = not detected.