Hypoxia impairs mesenchymal stromal cell-induced macrophage M1 to M2 transition

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The transition of macrophages from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype is crucial for the progression of normal wound healing. Persistent M1 macrophages within the injury site may lead to an uncontrolled macrophage-mediated inflammatory response and ultimately a failure of the wound healing cascade, leading to chronic wounds. Mesenchymal stromal cells (MSCs) have been widely reported to promote M1 to M2 macrophage transition; however, it is unclear whether MSCs can drive this transition in the hypoxic environment typically observed in chronic wounds. Here we report on the effect of hypoxia (1% O2) on MSCs’ ability to transition macrophages from the M1 to the M2 phenotype. While hypoxia had no effect on MSC secretion, it inhibited MSC-induced M1 to M2 macrophage transition, and suppressed macrophage expression and production of the anti-inflammatory mediator interleukin-10 (IL-10). These results suggest that hypoxic environments may impede the therapeutic effects of MSCs.

**Keywords:** Wound Healing; Mesenchymal Stromal Cells; Macrophages; Hypoxia; Classically Activated (M1) Macrophages; Alternatively Activated (M2) Macrophages.

**INNOVATION**
Mesenchymal stromal cells (MSCs) have been tested in a variety of therapeutic applications involving inflammation and tissue injury. A potentially promising area is to treat injuries that fail to heal using current therapies, thus leading to refractory chronic wounds. While pre-clinical studies have shown that MSCs have the ability to modulate inflammation and improve wound healing, MSC therapeutic ability has yet to be demonstrated in human chronic wounds. Pre-clinical studies often exclude conditions such as disrupted blood flow and hypoxia that are attributed to chronic wounds. Herein, we report on an assay to demonstrate the impact of the local hypoxic wound environment on the ability of MSCs to modulate inflammation. This study shows that hypoxia decreases macrophage polarization from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype, needed to promote wound healing. Thus, the local injury environment must be taken into account when evaluating the therapeutic potential of MSCs.

**INTRODUCTION**
Wounds heal by a series of events including an inflammatory phase to recruit immune cells that kill bacteria and remove dead cells, followed by a proliferation phase where the cells that reform the damaged tissue components proliferate and migrate. The mechanisms that control the transition from the inflammatory to the proliferative phases are complex and multi-faceted, and recent evidence suggests that macrophages play a key role in coordinating this process. For example, macrophages present at the wound site switch from a classically activated M1 to an alternatively activated M2 phenotype to resolve the inflammation. Macrophages polarized towards the pro-inflammatory M1 phenotype, in the presence of inflammatory stimuli such as LPS or IFN-γ, produce high levels of pro-inflammatory factors such as tumor necrosis factor (TNF)-α, interleukin (IL)-12 and IL-23. In contrast, anti-inflammatory stimuli such as IL-4 or prostaglandin-E2 (PGE2) drive macrophage polarization towards the anti-inflammatory M2 phenotype. These M2 macrophages express high levels of the mannose receptor (CD206) and produce high levels of IL-10 and transforming growth factor (TGF)-β1 and low levels of TNF-α and IL-12. Furthermore, M2 macrophages can be divided into subpopulations depending on their secretion and expression profiles, but in general they regulate cellular migration into the wounds and local cell proliferation.

Disruption of the wound healing process in skin leading to non-healing chronic wounds is a common occurrence in skin of older individuals who have advanced diabetes and/or vascular disease. Although chronic wounds may have different etiologies, a common denominator is impaired blood flow that causes tissue hypoxia, impaired cellular functions, and ultimately cell death. As a result, chronic wounds appear to be “stuck” at the inflammatory stage. Sindirilaru et al. showed that macrophages isolated from chronic venous ulcers fail to differentiate into the M2 phenotype to initiate the tissue repair process. Furthermore, ischemia impairs some of the bacterial killing mechanisms, such that ischemic chronic wounds are often infected. The continual presence of bacterial-derived products also promotes persistent secretion of pro-inflammatory factors by macrophages.
Recent studies have shown that adult bone marrow-derived mesenchymal stromal cells (MSCs) secrete soluble factors that promote macrophage differentiation from the M1 to the M2 phenotype \textit{in vitro}, including PGE2\textsuperscript{5,6}. Furthermore, there is evidence that MSCs may promote wound healing in experimental animals, albeit these studies were carried out in acute wounds that are expected to experience normoxic conditions\textsuperscript{8,9,10}. Herein, we explored if MSC-derived M2 macrophage polarization is impaired under conditions of hypoxia, as would be expected to occur in human chronic wounds.

**MATERIALS AND METHODS**

**Cell culture maintenance**

Human bone marrow-derived MSCs were purchased from the Institute of Regenerative Medicine at Texas A&M at passage 1 and cultured as previously described\textsuperscript{11,12}. Briefly, MSCs were cultured in Roswell Park Memorial Institute (RPMI; Life Technologies, Grand Island, NY) 1640 medium, supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA), 1% v/v penicillin-streptomycin (pen-strep; Life Technologies) and 4 mM L-glutamine (Life Technologies). Passage 3 cells were cultured until 70% confluency and immobilized in alginate sheets as previously described\textsuperscript{12}. Briefly, 5 × 10\textsuperscript{4} MSCs were mixed with 3% soluble alginate, placed in a 2 × 2 cm PDMS mold, and submerged into a 500 mM CaCl\textsubscript{2} bath.

Primary peripheral blood-derived macrophages were isolated from peripheral blood as previously described\textsuperscript{11}. Briefly, human whole blood was purchased from the Blood Center of New Jersey (East Orange, NJ) and mononuclear cells were isolated by density gradient centrifugation. Following mononuclear cell separation, CD14\textsuperscript{+} monocytes were isolated using magnetic activated cell sorting. Ten million CD14\textsuperscript{+} monocytes were cultured in advanced RPMI 1640 medium supplemented with 10% v/v FBS, 1% v/v pen-strep and 4 mM L-glutamine for 2 hours to allow for cell attachment. Then cells were washed with phosphate buffered saline (PBS) to remove non-adherent cells and cultured in fresh medium supplemented with 5 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF; R&D, Minneapolis, MN) for 7 days. CD14\textsuperscript{+} monocytes stimulated with GM-CSF produce a subset of macrophages expressing the M1 phenotype\textsuperscript{13,14}. After 7 days in culture, cells were detached from the flasks using trypsin-EDTA (Life Technologies) and 1 × 10\textsuperscript{6} cells/cryovial were frozen in 1 ml tissue culture medium or sham conditioned medium with or without 1 μg/ml LPS.

**Cellular viability**

M1 macrophages (5 × 10\textsuperscript{4} cells/ml) were cultured in normoxia (5% CO\textsubscript{2}, 21% v/v O\textsubscript{2}, balance N\textsubscript{2}) or hypoxia (5% CO\textsubscript{2}, 1% v/v O\textsubscript{2}, balance N\textsubscript{2}) for 48 hours. The cells were then incubated at 37°C with 3 μM calcine-AM + 6 μM ethidium homodimer-1 and the nucleus counterstained with Hoechst 33342 (1:10000; Life Technologies) for 15 minutes in advanced RPMI 1640 medium with 10% v/v FBS, 1% pen-strep and 4 mM L-glutamine. The cells were washed briefly with medium and imaged on an Olympus fluorescent microscope using 10× objective. Slidebook software (Intelligent Imaging Innovations, Denver, CO) was used to quantify the number of live (green fluorescence), dead (red fluorescence), and total (blue fluorescence) cells to calculate the percent viability as described elsewhere\textsuperscript{12,15}.

**Cytokine secretion**

M1 macrophages (5 × 10\textsuperscript{4} cells/ml) or immobilized MSCs (2.5 × 10\textsuperscript{5} cells/ml) were cultured alone, with LPS or PGE2 for 48 hours in hypoxia or normoxia. The cell supernatant was collected and assayed for TNF-α (BioLegend, San Diego, CA), IL-10 (BioLegend), PGE2 (Cayman Chemicals, Ann Arbor, MI), and TGF-β1 (BioLegend) using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

**MSC-conditioned medium**

MSC-conditioned medium was made by culturing immobilized MSCs (2.5 × 10\textsuperscript{5} cells/ml) with LPS (MSC-CM + LPS) or without LPS (MSC-CM) for 48 hours in normoxia. For the control group (LPS), sham-conditioned medium was made by culturing cell-free alginate sheets with LPS for 48 hours in normoxia. Before M1 macrophages were cultured in the conditioned medium, 1 μg/ml LPS was added to the medium to stimulate the macrophages. The control group in this study is defined as macrophages cultured in sham-conditioned medium.

**Macrophage co-culture with MSCs**

M1 macrophages (5 × 10\textsuperscript{4} cells/ml) were plated on 24 mm and 3 μm-pore size transwell inserts (Corning; Corning, NY) in RPMI 1640 medium with 10% FBS, 1% pen-strep and 4 mM L-glutamine and allowed to attach overnight. The following day, the medium in the inserts was replaced with medium supplemented with 1 μg/ml LPS and immobilized MSCs (2.5 × 10\textsuperscript{5} cells/ml) with LPS (MSC + LPS) or without LPS (MSC) or alginate sheets alone with LPS (LPS) was added to the bottom well. The cells were cultured in this configuration for 48 hours in normoxia or hypoxia and the medium was collected from the transwell and bottom well and assayed for TNF-α, IL-10, and TGF-β1. The control group in this study is defined as macrophages co-cultured with alginate sheets and LPS alone.

**Western blot**

M1 macrophages (2 × 10\textsuperscript{5} cells) cultured in normoxia or hypoxia and with or without LPS were treated with 1X RIPA Buffer (Thermo Fisher Scientific; Waltham, MA) with 1% protease inhibitor, 1% EDTA, and then detached from the well using a cell scraper. Total protein content was determined using a bichinoninic acid protein assay (Thermo Fisher). Equal amounts of total protein were separated by 10% SDS-PAGE gels (Bio-Rad, Hercules, CA) followed by blotting to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk (Bio-Rad) or 5% bovine serum albumin (BSA; Sigma) in tris-buffered saline and tween-20 for 2 hours and then incubated overnight at 4°C with polyclonal hypoxia inducible factor (HIF)-1α antibody (Abcam, Cambridge, MA). After washing, the membrane was incubated with the secondary antibody for 1 hour at room temperature. Signals were detected by staining the membrane with SuperSignal\textsuperscript{TM} West Pico chemiluminescent (Thermo Fisher) for 5 minutes and bands digitized with a scanner.

**Immunocytochemistry**

M1 macrophages (5 × 10\textsuperscript{4} cells/ml) were cultured on glass bottom 24-well plates in normoxia or hypoxia for 48 hours with MSC-conditioned medium or sham conditioned medium with or without 1 μg/ml LPS. Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes. Cells were washed 3 times and incubated in 1% w/v BSA (Sigma) + 10% v/v goat serum (Sigma) + 0.1% v/v Tween-20 (Sigma) + 0.1% v/v Triton X-100 in PBS for 1 hour to permeabilize the cell membranes and to block non-specific protein-protein binding. Cells were washed again and incubated with the anti-mannose receptor antibody (CD206; 1:100 dilution; Abcam) overnight at 4°C. Then, the cells were incubated with the secondary antibody (1:500 dilution; Abcam) and the nucleus counterstained with Hoechst 33342 (1:5000 dilution; Life Technologies) for 1 hour. Cells were imaged on an Olympus fluorescent microscope using 10X objective. Slidebook software (Intelligent Imaging Innovations) was used to quantify the total number of cells (blue fluorescence) and CD206\textsuperscript{+} (green fluorescence) cells and calculate the percent of CD206\textsuperscript{+} cells.
measured from these M1 macrophages cultured without LPS. The effect of macrophages cultured without LPS. In addition, very little IL-10 was secreted by macrophages cultured without LPS augments macrophage and MSC secretion in the absence of a stimulus. Therefore, we first cultured M1 macrophages from human whole blood and treated with GM-CSF to acquire the M1 phenotype. In general, macrophages secrete very little cytokine in the absence of a stimulus. Hence, we first cultured M1 macrophages and MSCs under hypoxia, at a level similar to that found in chronic wounds (1% v/v O₂), to assess any changes in cell function and their ability to transition into the M2 phenotype. After 48 hours under hypoxia there was an increase in protein levels of HIF-1α (Fig. 2a), a marker for cell adaptation to low oxygen tension. We also observed that after 48 hours under normoxia or hypoxia, macrophages remained essentially viable in any significant difference in viability between the two groups (Fig. 2b). Hypoxia had no effect on LPS-stimulated MSC secretion of PGE2 (Fig. 2c) or macrophage expression of TNF-α (Fig. 2d). However, Fig. 2d shows that hypoxia down-regulated macrophage expressions of IL-10 and indoleamine 2,3-dioxigenase (IDO). The down-regulation of IL-10 and IDO expression suggests that hypoxia increased the M1 phenotype.

### RESULTS

**LPS augmented macrophage and MSC secretion**

Primary macrophages were differentiated from monocytes isolated from human whole blood and treated with GM-CSF to acquire the M1 phenotype. In general, macrophages secrete very little cytokine in the absence of a stimulus. Therefore, we first cultured M1 macrophages alone with 1 μg/ml LPS under normoxia for 48 hours and examined their secretion of TNF-α and IL-10. As shown in Fig. 1a, LPS-stimulated M1 macrophages secreted significantly more TNF-α and IL-10 than M1 macrophages cultured without LPS. In addition, very little IL-10 was measured from these M1 macrophages cultured without LPS. The effect of LPS on MSC secretion of PGE2 was also examined because MSCs induce the M2 phenotype in macrophages by secretion of PGE2. 

### Statistical analysis

All numerical results are presented as means ± standard error of the mean (SEM). Statistical analysis of three or more independent experiments were assessed using two-tailed Student’s t-test or one-way analysis of variance (ANOVA) followed by Fischer post hoc analysis where p < 0.05 represents statistical significance.

### DISCUSSION

There is a plethora of evidence demonstrating that MSCs can induce macrophage transition from the M1 phenotype to the anti-inflammatory M2 phenotype. However, these studies were carried out under normoxic conditions, which is not the typical condition in infected tissues or chronic wounds. In chronic wounds, prolonged hypoxia due to impaired blood flow to the tissue significantly impairs cell function. Specifically, wound macrophages fail to resolve inflammation and instead perpetuate the chronic inflammatory state. This study investigated the interplay between classically activated or pro-inflammatory M1 macrophages and bone marrow-derived mesenchymal stromal cells (MSCs) under hypoxia in vitro.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>IL-10</td>
<td>5′ CATCGATTCTTCTGCCGTGA 3′</td>
<td>5′ TCTTTGAGCTTTATTAAGGCATT 3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′ ATGACACGTAAGAGATGC 3′</td>
<td>5′ GAGGGCTGATTAGAGAGGT 3′</td>
</tr>
<tr>
<td>IDO</td>
<td>5′ CAAATGGATGAGAATGAGG 3′</td>
<td>5′ CCACCAATAGAGACACGG 3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′ ACAACCTTGGTATCGTGAAA 3′</td>
<td>5′ AAATTGTTGTCATACCCAGG 3′</td>
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**MSCs transition M1 macrophages towards the M2 phenotype**

To assess the immunomodulatory properties of MSCs, LPS-stimulated M1 macrophages were cultured in MSC-conditioned medium for 48 hours under normoxia or hypoxia, and macrophage expression of CD206, a well-accepted M2 phenotype marker, was determined by immunocytochemistry. Our results showed that in comparison to the control group (macrophages cultured without MSCs), MSC-conditioned media led to a significant increase in CD206+ macrophages under both normoxia and hypoxia (Fig. 3). Under normoxia, there was a 72% increase in CD206 expression in the MSC group and an 81% increase in the MSC + LPS group compared to the control. However, hypoxia alone unexpectedly led to a significant increase in CD206 expression compared to normoxia, there was a 30% increase in CD206 expression in the MSC group and a 19% increase in the MSC + LPS group compared to the control. We also found no significant differences in macrophage expression of CD206 between the MSC and MSC + LPS groups. Additionally, in both MSC groups, there was no significant difference in CD206 expression between cells cultured under normoxia or hypoxia.

### Quantitative real-time PCR

M1 macrophages (2 x 10^5 cells) were cultured in medium with 1 μg/ml LPS in normoxia or hypoxia for 48 hours. After which, the medium was removed, cells were washed with PBS and lysed with QIAshredder (Qiagen, Valencia, CA). RNA was isolated and purified with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Equal amounts of RNA were used to synthesize cDNA using the Reverse Transcription System (Promega Corporation, Madison, WI) according to the manufacturer’s protocol. For qualitative PCR, the cDNA was combined with SYBR green master mix containing distilled water and primers to target genes and then polymerized using the PikoReal Real Time PCR System (Thermo Fisher). Specific primer sequences utilized for target genes are listed in Table 1. Relative fold induction was calculated via the ΔΔCT method and values were normalized with GAPDH as the endogenous control.
Conventionally, monocytes that migrate into the wound differentiate into the pro-inflammatory M1 macrophages due to the presence of interferon (IFN)-γ, LPS or GM-CSF. These M1 macrophages are pro-inflammatory; they clear the wound from debris and pathogens and produce high levels of the anti-inflammatory IL-10 and IL-6 and produce low levels of the anti-inflammatory IL-10 and IL-6. As wound healing progress, they transition into the M2 phenotype. M2 macrophages can be divided into 4 subsets and are characterized by their expression of specific markers and their production of specific cytokines. Even so, M2 macrophages generally secrete high levels of anti-inflammatory mediators such as TGF-β and IL-10, and are involved in tumor progression, resolution of inflammation, and tissue repair. However, macrophages in chronic wounds fail to transition into the M2 phenotype and experience a prolonged M1 phenotype.

Therefore, a therapeutic strategy for chronic wounds is to drive macrophage polarization towards the M2 phenotype. However, it is important to first understand the impact of hypoxia, a major feature of the chronic wound environment, on the M1 phenotype. As result, we evaluated the expressions of IDO, IL-10, and TNF-α in LPS-stimulated M1 macrophages cultured under normoxia (21% O₂) or hypoxia (1% O₂). The enzyme IDO is a key regulator of the innate immune system; IDO catalyzes tryptophan degradation, thus limiting the growth of bacteria, parasites and viruses. While macrophages do not constitutively express IDO, IDO expression can be induced by a variety of inflammatory stimuli such as IFN-γ. Our study showed that similar to IFN-γ, LPS induced IDO expression in macrophages cultured under normoxia. However, IDO expression was significantly suppressed by hypoxia.
Several studies have shown that bone marrow-derived MSCs can promote macrophage transition from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype by PGE2 secretion. PGE2 binds to EP4 receptors on macrophages and activate the MAPK/Erk signaling pathways, which lead to an increase in macrophage production of the anti-inflammatory cytokine IL-10. Our results showed that, on the one hand, macrophages co-cultured with MSCs increased the number of M2 macrophages by increasing the level of M2-cytokine IL-10 and the growth factor TGF-β1, the expression of the M2-marker CD206, and decreased the level of M1-cytokine TNF-α. On the other hand, the MSC-induced increase in IL-10 production observed under normoxia was suppressed under hypoxia. Our results also showed that stimulating M1 macrophages with exogenous PGE2 at a concentration 10 times greater than concentration secreted by MSCs did not reverse the hypoxia-mediated suppression of IL-10 production by macrophages. However, both exogenous PGE2 and MSC treatment significantly increased macrophage secretion of TGF-β1 both in normoxia and hypoxia. The upregulation of TGF-β1 secretion by macrophages also suggest an upregulation in M2 transition.

Unlike, IL-10, there was no significant difference in TGF-β1 secretion in hypoxia as compared to normoxia. Other authors also found that hypoxia reduced basal and induced IL-10 protein and gene expressions. Taken together, these data suggest that hypoxia significantly impairs IL-10 expression in macrophages, and this impairment is not ameliorated by

in LPS-stimulated M1 macrophages cultured under hypoxia. This is consistent with Schmidt et al. who also showed that hypoxia diminished both IDO expression and activity in fibroblasts and tumor cells. Modulation of IDO expression, in this manner, has been implicated in macrophage polarization. Recently, Wang et al. demonstrated that overexpression of IDO in macrophages resulted in increased M2 markers and decreased M1 markers. Their data is consistent with other authors who showed that IDO is overexpressed in tumor-associated macrophages (TAMs), which are a subset of M2 macrophages. Wang et al. also showed that the knockdown of IDO expression was associated with increased M1 markers and decreased M2 markers. From these studies, we can exclude the notion that hypoxia would induce an M2 phenotype similar to TAMs. Our results show that along with a decrease in IDO expression and no change in TNF-α expression are indicative of M1 phenotype persistence as a result of hypoxia.


