Effect of hypoxic/ischemic environment on the immunoprivilege of allogeneic mesenchymal stem cells

By

Ejlal Abu-El-Rub

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Department of Physiology and Pathophysiology,

Max Rady College of Medicine,

Rady Faculty of Health Science,

University of Manitoba

Winnipeg, Manitoba

Abstract

Bone marrow derived allogeneic (donor derived) mesenchymal stem cells (MSCs) are immunoprivileged and are considered to be the prominent cell type for regenerative therapy for numerous diseases including cardiovascular disorders. Even though the outcome of initial allogeneic MSCs based clinical trials in cardiac patients was encouraging, the overall enthusiasm has diminished lately due to failure of long-term survival of transplanted cells in the recipient heart. In fact, recent analyses of allogeneic MSCs based studies demonstrated that transplanted cells in the ischemic heart become immunogenic and were subsequently rejected by the host immune system. We demonstrate that hypoxia, a common denominator of ischemic tissues including the infarcted heart, induces an immune shift in MSCs from immunoprivileged to immunogenic state. The immunoprivilege of MSCs is preserved by downregulation or absence of major histocompatibility complex class II (MHC-II) molecules. We found that in rat and human MSCs, 26S proteasome-mediated intracellular degradation of MHC-II helps maintain the absence of MHC-II expression on the cell surface in normoxic cells and preserves their immunoprivilege. The exposure to hypoxia leads to dissociation of 19S and 20S subunits, and inactivation of 26S proteasome. This dissociation prevents the degradation of MHC-II, and as a result the MSCs become immunogenic under hypoxic conditions. Furthermore, we found that exposure to hypoxia induces an increase in the levels of Sug1, which is the ATPase subunit of 19S proteasome. The upregulation of Sug1 in hypoxic MSCs was associated with the activation of Class II Transactivator "CIITA" which is a master regulator of transcription initiation of MHC-II. We found that knocking down Sug1 in MSCs preserved their immunoprivilege *in vitro*

(rat and human MSCs) as well as *in vivo* (rat MSCs) in a rat model of myocardial infarction. Interestingly, our investigations also revealed that after exposing MSCs to a hypoxic stress, 26S proteasome is converted into a highly immunogenic complex called the immunoproteasome. Hypoxia induced formation of the immunoproteasome in MSCs was further associated with maturation and activation of MHC-II and loss of immunoprivilege. Taken together these findings provide a novel insight into the molecular events responsible for hypoxia induced shift in the phenotype of MSCs from immunoprivileged to immunogenic state. More importantly these studies also provide targets to preserve immunoprivilege of MSCs under hypoxic or ischemic conditions.

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Contributions of authors:

The research work in this sandwich thesis includes multi-author manuscripts. Since, this thesis has multiple authors, an outline of author contributions in each manuscript is mentioned below in order of appearance in the thesis:

For the three manuscripts presented in the thesis, **Ejlal Abu-El-Rub** conceptualized the studies and designed the experiments, performed the majority of experiments, acquired and analyzed the data, interpreted the data, performed statistical analyses, designed figures, drafted the manuscript. **Dr Sanjiv Dhingra** conceptualized the studies and designed the experiments, corrected the drafted manuscript and approved the final version of each manuscript. All other author's contributions are listed below:

Manuscript 1: Hypoxia induced 26S proteasome dysfunction increases immunogenicity of mesenchymal stem cells. Cell Death & Disease January 2019. (Published)

- **Authors: Ejlal Abu-El-Rub**, Glen Lester Sequiera, Niketa Sareen, Meenal Moudgil, Mohammad Golam Sabbir and Sanjiv Dhingra.
  - **Glen Lester Sequiera**: carried out the experiments, acquired and analyzed the data, interpreted the data, performed statistical analyses and drafted the manuscript.
  - **Niketa Sareen**: carried out the experiments, acquired and analyzed the data, interpreted the data, performed statistical analyses.
  - **Meenal Moudgil**: carried out the experiments, acquired and analyzed the data.
  - **Mohammad Golam Sabbir**: designed 2D gel electrophoresis, acquired, analyzed and interpreted 2D gel data, prepared the 2D gel-based figure and wrote the 2D gel-based results.

All Authors approved the final version of the manuscript.

Manuscript 2: Hypoxia induced increase in Sug1 leads to poor post-transplantation survival of allogeneic mesenchymal stem cells. (Revisions in progress: FASEB Journal)

- **Authors: Ejlal Abu-El-Rub**, Niketa Sareen, Glen Lester Sequiera, Hania I. Ammar, Weiang Yan, Asmaa M. ShamsEldeen, Ilan Rubinchik, Heba S. Shokry, Laila A. Rashed, Meenal Moudgil, Sanjiv Dhingra.
  - **Niketa Sareen**: carried out the experiments, acquired and analyzed the data, interpreted the data, performed statistical analyses, and drafted the manuscript.
  - **Glen Lester Sequiera**: carried out the experiments, acquired and analyzed the data, interpreted the data, performed statistical analyses.
  - **Hania .I. Ammar**: designed the experiments, interpreted the data and performed statistical analyses.
  - **Weiang Yan**: carried out the experiments, acquired and analyzed the data.
  - **Asmaa M. ShamsEldeen**: carried out the experiments, acquired and analyzed the data.
  - **Ilan Rubinchik**: carried out the experiments, acquired and analyzed the data.
  - **Heba S. Shokry**: carried out the experiments, acquired and analyzed the data.
  - **Laila A. Rashed**: carried out the experiments, acquired and analyzed the data.
  - **Meenal Moudgil**: carried out the experiments, acquired and analyzed the data.

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- **Authors: Ejlal Abu-El-Rub**, Niketa Sareen, Weiang Yan, Keshav Narayan Alagarsamy, Alireza Rafieerad, Vincenzo Desiderio, Sanjiv Dhingra.
  - **Niketa Sareen**: carried out the experiments, acquired and analyzed the data, interpreted the data, performed statistical analyses, and drafted the manuscript.
  - **Weiang Yan**: carried out the experiments, acquired and analyzed the data, interpreted the data, performed statistical analyses, and drafted the manuscript.
  - **Keshav Narayan Alagarsamy**: carried out the experiments, acquired and analyzed the data.
  - **Alireza Rafieerad**: carried out the experiments, acquired and analyzed the data.
  - **Vincenzo Desiderio**: designed the experiments, drafted the manuscript.

All authors approved the final version of the manuscript.

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List of abbreviations:

MSCs: Mesenchymal stem cells

MHC-II: Major Histocompatability Complex II

HSP90α: Heat Shock protein 90 subclass "α"

HSP90β: Heat Shock protein 90 subclass "β"

BLM10: bleomycin resistance protein 10

NOB1: NIN1 binding protein 1

CIITA: Class II Transactivator

RFX5: Regulatory Factor X5

PCAF: P300/CBP-associated factor

LMP2: Low Molecular Mass Protein 2

LMP7: Low Molecular Mass Protein 7

MECL1: Multicatalytic Endopeptidase Complex Subunit MECl-1

HLA-DRα: Human Leukocyte Antigen DR alpha chain

PSMA1: Proteasome subunit alpha type-1

PSMA6: Proteasome subunit alpha type-6

HLA-DM: human leukocyte antigen DM

CD74: Cluster of Differentiation 74, HLA-DR antigens-associated invariant chain

Chapter I:

Literature Review:

Cardiovascular diseases (CVDs), mainly acute myocardial infarction (AMI), continue to be associated with a high rate of mortality and morbidity worldwide; 17.6 million deaths per year, and it is expected that mortality will reach more than 23.6 million deaths per year by 2030[1,2]. The majority of CVDs cause ischemic cell death which leads to subsequent adverse events, including ventricular remodeling and scarring, and they are often followed by a dramatic drop in the heart functions and ultimately pump failure[3–5]. Heart transplantation is a remedy but is associated with a high rate of rejection even with using extensive immune suppression regimens[6–8]. It has been well-known that cardiomyocytes (cells responsible for beating of heart) are terminally differentiated because these cells lose the ability to proliferate[9–11]. Therefore death of cardiomyocytes due to myocardial injury causes irreversible damage to the heart muscle, which eventually leads to heart failure [12–17] [18–20]. In this regard, stem cell therapy has been reported to promote cardiac repair and prevent adverse events leading to heart failure[21–28]. Several studies have demonstrated the presence of specialized stem cells niche or cardiac progenitors cells in mammalian myocardium[29–31]. These cells were reported to possess the properties of self-renewal and differentiation into cardiac cells and help in restoring mechanical and functional properties of the heart[29,30,32–38]. However, later on it was reported that the number of these endogenous cells is too low to adequately repair and regenerate the injured heart. Furthermore, the beneficial effects of cardiac progenitor cells are reported to deteriorate during stress or cardiac injury[32,34,39–41]. In this regard infusion of exogenous stem cell preparations into the infarcted myocardium was considered to be an option for cardiac repair and regeneration[42–50]. In the last 15-20 years different types of stem cells including cardiac progenitor cells, bone marrow or adipose tissue

derived stem cells, embryonic stem cells and induced pluripotent stem cells have been explored as sources of exogenous cardiac stem cell therapy [41,49,51]. However, bone marrow derived mesenchymal stem cells (MSCs) have received significant attention as a candidate cell type for cardiac repair. In different pre-clinical and clinical studies, after transplantation MSCs were able to significantly improve cardiac function[51,52]. However, poor survival of transplanted stem cells in the injured heart has dampened the enthusiasm regarding cardiac stem cell therapy[41,53]. Therefore, rigorous preclinical investigations are required to understand the mechanisms leading to the poor survival of transplanted stem cells in the injured heart before initiating further clinical trials.

1.1 Mesenchymal stem cells therapy for cardiac repair and regeneration:

MSCs are multipotent cells which have the ability to differentiate into various cell types including adipocytes, chondrocytes, osteocytes and ectoderm-type cells such as cardiomyocytes, endothelial cells or smooth muscle cells[54,55]. MSCs were initially thought to be present in the stem cell niche in the bone marrow and constitute around 0.001–0.1% of the total bone marrow nucleated cell population[56,57]. However, it has been found that MSCs can be isolated from other sources like adipose tissues and umbilical cord[52,58]. MSCs are the most studied stem cell type in field of cardiac regeneration for the following reasons; i) these cells are easily isolated from multiple sources and can be expanded for large number of passages without significant phenotype change; 2) can be simply modified and engineered;3) most importantly MSCs are considered to be immunoprivileged, therefore after transplantation, these cells can avoid the allo-immune response by the host tissue [55,59–64]. MSCs transplantation in rodents and larger animal models of MI showed significant improvement in heart function[65–67]. Similarly, intracoronary infusion of autologous bone marrow-derived MSCs to MI patients improved left ventricular (LV)

function and myocardial perfusion[68–73]. In heart failure models, transplantation of autologous or allogeneic MSCs significantly abrogated ventricular remodeling [74–78]. Intravenous injection of umbilical cord derived MSCs into patients with stable heart failure and reduced ejection fraction, induced noticeable improvements in left ventricular function and quality of life[74]. Teerlink et al, conducted the Congestive Heart Failure Cardiopoietic Regenerative Therapy study (Chart-1) that has demonstrated that intramyocardial administration of bone-marrow-derived, lineage-directed, autologous cardiopoietic mesenchymal stem cells in patients with advanced congestive heart failure, reversed ventricular remodeling and improved patient's functional status over 52 weeks of follow-up[78]. Adipose-derived MSCs infusion into remodeled myocardium in a rat model of chronic MI resulted in cardiac function restoration as well as reversal of the thinning of scarred myocardial walls [79,80]. Furthermore, clinical trial showed that both autologous and allogeneic MSCs were safe to be administered and effectively treated ischemic and non-ischemic heart diseases [81–83]. Hou et al found that the highest retention rate of MSCs at the injection site was achieved by intramyocardial infusion as compared to intravenous infusion [84]. Consistent with this, Karantalis et al. demonstrated that intramyocardial injection of MSCs into non-revascularized segments in patients undergoing CABG reduced fibrosis and improved myocardial perfusion and contraction[82]. Florea et al. reported that transendocardial injection of 100 million allogeneic MSCs in patients with ischemic cardiomyopathy increased ejection fraction and emphasized on the importance of determining the optimal cell dose to advance the field[85]. However, some studies described that transplanted MSCs do not actually differentiate into beating cardiomyocytes, rather their major therapeutic benefits observed after MSCs transplantation were due to paracrine factors secreted by MSCs [86–90].

1.2 Immunomodulatory properties of mesenchymal stem cells:

Mesenchymal stem cells are considered to be immunoprivileged because these cells do not express cell surface immune antigens- major histocompatibility complex (MHC) molecules[91–93]. Therefore, MSCs can avoid the host immune system after transplantation[92,94]. Furthermore, MSCs secrete several immunosuppressive soluble factors including prostaglandin E2 (PGE2), indoleamine 2,3- dioxygenase (IDO), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF) and interleukin-10 (IL-10)[95–100]. Therefore MSCs can downregulate the proliferation of infiltrating T- and B-cells, and regulate the balance of Th1/Th2 cells [101–103]. Further, MSCs can induce a state of immunotolerance by inducing a shift in the phenotype of T-cells toward regulatory T cells (Treg)[104,104]. Moreover, MSCs induce cell cycle arrest of B-cell and reduce their activity; in addition, MSCs can affect the release of antibodies and co-stimulatory molecules of B cells[105,106]. MSCs are also reported to regulate and control other arms of the immune system, such as inhibit interleukin-2 (IL-2) activation of natural killer (NK) cells [107,108], the antigen presentation of dendritic cells[109,110], and can potentiate the induction of immunosuppressive M2 macrophage (Figure 1.1)[111,112].

Interestingly, current evidence suggest that immunosuppressive functions of MSCs can vary depending on exposure to the local microenvironment or disease status[113,114]. For instance, MSCs can suppress Th1 response in patients with acute graft versus host disease (GvHD) and autoimmune diseases such as systemic lupus erythematosus (SLE)[115]. Further, MSCs can intensify Th1 responses in airway allergic inflammatory diseases, including allergic rhinitis and asthma[115]. In addition, certain micro-environmental conditions such as inflammatory stress can influence the ability of MSCs to induce Treg formation[116,117]. MSCs are primarily considered to be immunoprivileged due to the negligible expression of MHC-II or HLA-D on the cell surface which allows their transplantation without the risk of being rejected by the host immune

system[55,55,118]. The immunosuppressive potential of MSCs has been broadly validated in several animal models, including skin grafts, solid-organ transplants, graft - versus - host disease (GvHD) and various cardiovascular diseases[74,115]. Despite promising data on safety and beneficial effects of MSCs in different animal disease models, it is now known fact that transplanted cells do not survive in the host tissue for a long time, these discouraging results led to a significant drop in success rate of MSC therapy. Several studies have now reported that even though MSCs are innately immunoprivileged, the cells become immunogenic after transplantation into the damaged tissue and are rejected by the host immune system[119–124]. In this regard, Isakova et al. reported that allogeneic MSCs are immunogenic when transplanted to unrelated donor-recipient and negatively impacts their long term engraftment levels[125]. Further, Pezzanite et al. reported that allogeneic MSCs elicit intense antibody responses and massive inflammatory responses *in vivo* in MHC mismatch donor-recipient which could restrain their therapeutic effectiveness[126]. Gu et al. reported that MSCs after transplantation through the tail vein exhibited mild immune response, however, when cells were injected directly to the pancreas, they triggered a strong immune response [127]. Therefore, the mode of transplantation and host microenvironment play a very important role in immunological behavior of transplanted MSCs. The outcome of these studies suggests that it is very important to study post-transplantation immunological behavior, and understand the mechanisms of loss of immunoprivilege of MSCs before planning any future clinical trials. In this regard, the microenvironment at the site of cell implantation is reported to play a significant role in immunological behavior of transplanted MSCs[117,120,124]. Hypoxia is a common denominator of ischemic tissues [128–130]. Hypoxic conditions are reported to influence the biological characteristics of MSCs[131]. Antebi et al. reported that short term exposure of both porcine and

human MSCs to 2% hypoxia potentiates their therapeutic characteristics including proliferation rate and self-renewing capacity[132]. Another study by Xu et al. reported an increase in the chemotaxis behavior and migration of MSCs after being exposed to moderate hypoxia[133]. A study by Ho et al. exhibited that exposing MSCs to moderate hypoxia increases ability of cells to form a high density MSCs-spheroids that were more resistant to apoptosis and had improved osteogenic differentiation potential[134]. Contrary to that, Zhu et al. reported that MSCs transplantation to mouse model of limb ischemia was associated with poor retention and survival due to metabolic alterations resulting from glycogen synthesis inhibition[135]. Another interesting study by Westrich et al. observed that 1% of total intramyocardially injected allogeneic MSCs survived at day 2 in a rat subacute MI model[136]. Similarly, Tano et al. demonstrated in a rat model of MI that epicardial placement of allogeneic MSCs led to strong allo-immune response by the host tissue and rejection of implanted cells[137]. Therefore, these studies suggest that after transplantation in the hypoxic environment of the damaged tissue MSCs become immunogenic and are rejected. However, the underlying mechanisms of hypoxia-induced shift in the phenotype of MSCs from immunoprivileged to immunogenic state are unknown, it needs to be investigated to maintain immunoprivilege of MSCs, prevent rejection and preserve benefits of transplanted MSCs.

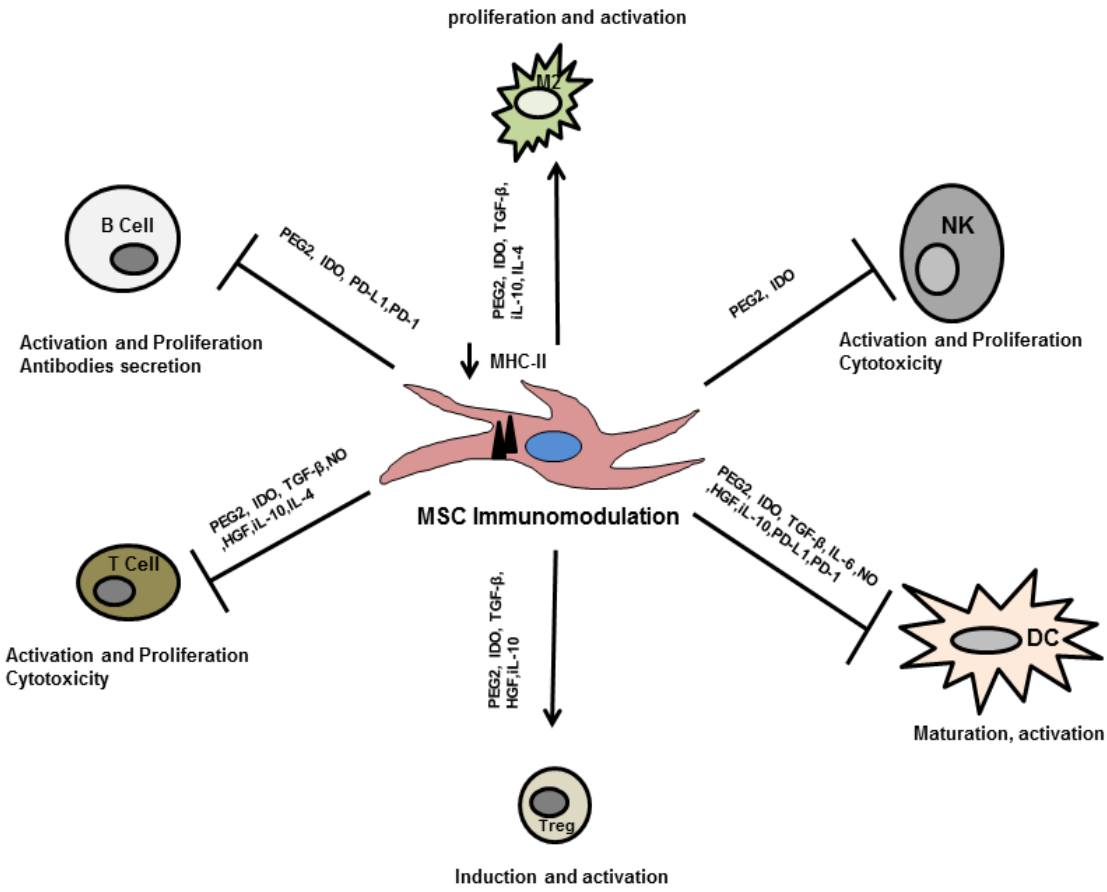


Figure 1.1: The figure depicts immunomodulatory and immunosuppressive properties of MSCs. MSCs exert immunomodulatory effects by secreting immunosuppressive soluble factors including, prostaglandin E2 (PGE2), transforming growth factor-β 1 (TGFβ 1), nitric oxide (NO), indoleamine 2, 3-dioxygenase (IDO), or hepatocyte growth factor (HGF), interleukin-10(IL-10). Due to the secretion of these factors, MSCs can suppress the proliferation of immune cells including $CD4^+$ and $CD8^+$ T-lymphocytes, natural killer (NK) cells, B-lymphocytes. MSCs are also reported to promote the induction of regulatory T-lymphocytes (Tregs).

1.3 Role of major histocompatibility complex class II (MHC-II) in MSC immunogenicity:

MSCs are immunoprivileged because of the absence or negligible expression of MHC-II (or HLA-D in case of humans) on the cell surface [105,138,139]. MHC-II plays a key role in initiating immune response against allo-antigens[140,141]. Unlike MHC-I, which is widely expressed in nucleated cells, MHC- II expression is generally restricted to a subset of antigen presenting cells, such as macrophages, dendritic cells, and B cells[142,143]. However, various stress signals can induce its expression on other cell types. It has been previously reported in different cells including MSCs that exposure to interferon γ and TNF-α stimulates upregulation of MHC-II [144–148]. Another study reported that differentiation of MSCs to myogenic cells, smooth muscle cells and endothelial cells was associated with upregulation of MHC-II levels[149]. MHC-II molecules are responsible for antigen presentation to $CD4^+$ T cells.

1.4 MHC-II regulation:

MHC-II or HLA-D genes encode three polymorphic molecules HLA-DR, -DQ, and -DP which are expressed as α- and β-chain heterodimers on the cell surface. MHC-II or HLA-D expression is regulated at the level of transcription by promoters which are characterized by the presence of conserved *Cis* –acting elements represented as the W (or S), X, X2 and Y boxes. Each box element is occupied by specific transcription factors, for instance, the W and X boxes are bound by RFX (regulatory factor X), a trimeric complex composed of RFX5, regulatory factor X-associated ankyrin-containing protein (RFXANK or RFX-B) and Regulatory Factor X Associated Protein (RFXAP). The X2 box is recognized by cyclic AMP response element binding protein (CREB). Finally, the remaining Y box is occupied by trimeric Nuclear Factor Y

(NF-Y) complex, composed of NF-YA, NF-YB and NF-YC. When the above mentioned transcription factors bind to *Cis*-elements of MHC-II promoters, the resulting structure is known as the MHC-II enhanceosome. The MHC-II enhanceosome assembly is a transient step, it requires binding of Class II Transactivator (CIITA) to stabilize the assembled enhanceosome and initiate the process of MHC-II transcription. Class II Transactivator (CIITA) is the master MHC-II transcriptional coactivator which is essential for MHC-II transcription initiation and completion. CIITA is constitutively expressed in antigen presenting cells. However, its expression can be induced under certain conditions such as IFN-γ, LPS, and IL-4 stimulation. The protein-protein interactions of CIITA with MHC-II enhanceosome lead to the formation of an active and stabilized MHC-II transcriptosome (Figure 1.2)[141,150–153].

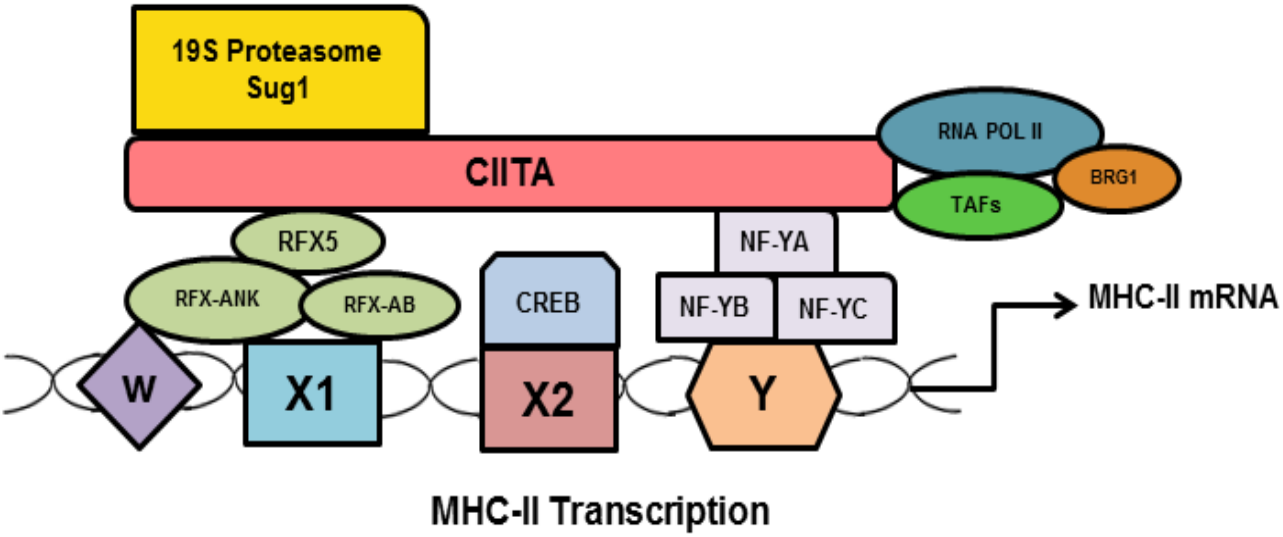
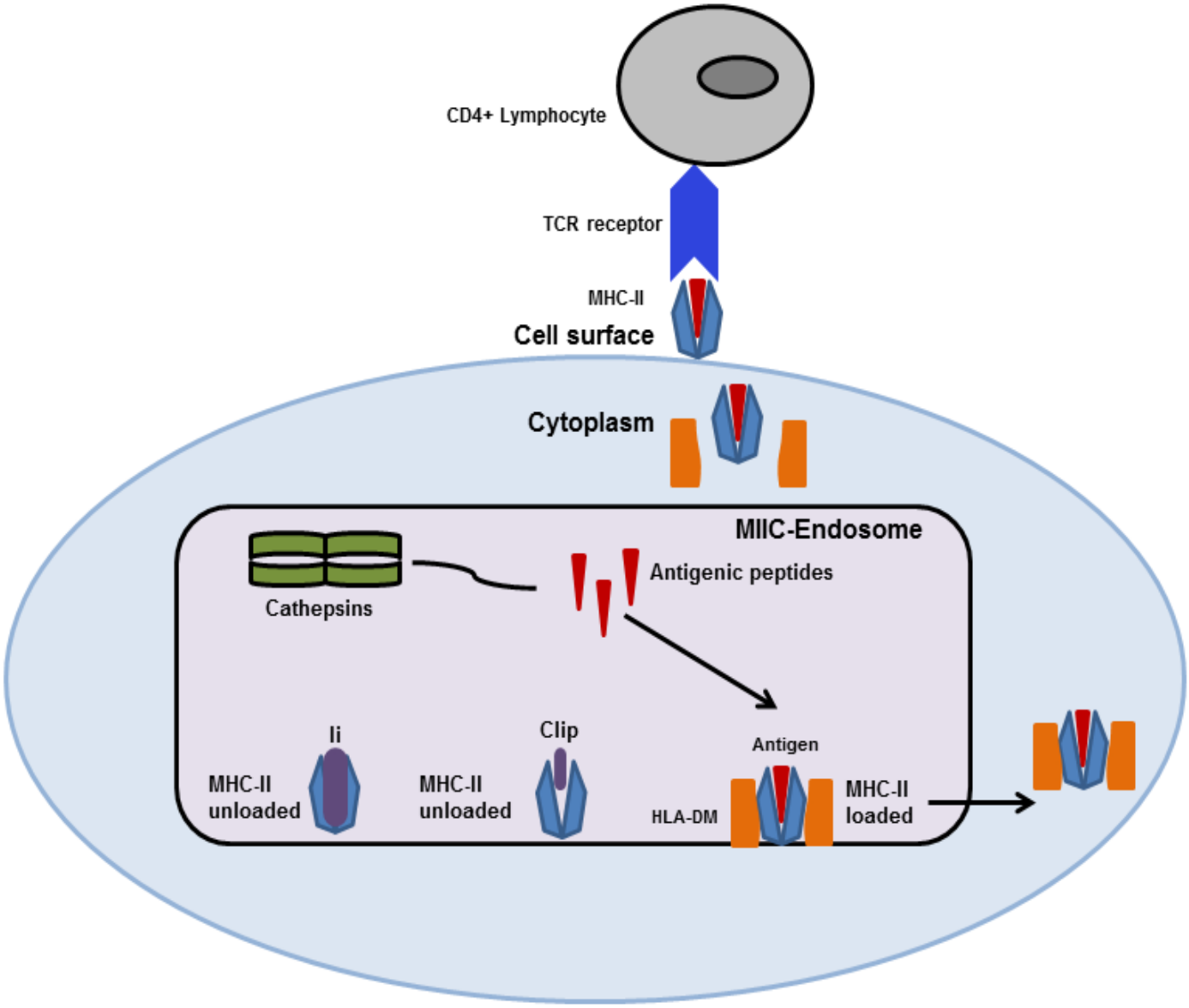


Figure 1.2: MHC-II transcription initiation and regulation. The figure describes the MHC class II enhanceosome representing the *Cis* –acting elements; W, X1, X2 and Y boxes within the proximal promoter region. Seven basal transcription factors bind these cis elements—the trimeric (Tri) RFX complex, cAMP-response element binding protein (CREB) and the trimeric (ABC) NF-Y complex. This complete structure is called MHC-II enhancesome and binds to the Class II

Transactivator (CIITA). Also, 19S proteasome ATPase "Sug1" is reported to activate CIITA to facilitate it's binding to MHC-II enhanceosome. CIITA then recruits basal transcription machinery (TAFs and POL II), the ATP-dependent chromatin remodeling component BRG-1 and several other coactivators to initiate MHC-II transcription.

1.5 MHC-II loading and expression on the cell surface:

In the endoplasmic reticulum (ER), nascent MHC-II molecules which are preassembled will bind to invariant chain (Ii or CD74). Ii is a dedicated chaperone protein that prevents premature peptide binding to MHC-II peptide binding groove and directs MHC-II through *Cis*-Golgi and the *trans*-Golgi network to the late-stage endosomal compartment, known as endosomal–lysosomal antigen-processing compartment or MHC-II compartment (MIIC). Within MIIC, proteases will fragment the Ii chain and leave a set of remaining Ii small fragments known as class II invariant chain-associated peptides (CLIP). CLIP plays a role in blocking the peptide binding groove of the MHC class II molecule similar to Ii. A non-classical HLA catalytic protein, HLA-DM, will substitute CLIP and binds MHC-II to allow processed peptides to be placed in MHC-II peptide binding groove. The MHC-II- peptide complexes will move to surface of antigen presenting cells where they interact with $CD4^+$ T cells through the T-cell receptor, which serves as first step toward activation of antigen-specific adaptive immune response (Figure 1.3)[141,151,154–156].



MHC-II loading dynamics where in the endoplasmic reticulum (ER), newly formed MHC-II molecules will bind the invariant chain (Ii or CD74) that prevents premature peptide binding to MHC-II peptide binding groove. MHC-II-Ii complex will be transported to the late-stage endosomal compartment, known as endosomal–lysosomal antigen-processing compartment or MHC-II compartment (MIIC). In the MIIC, Ii chain will be cleaved to a small fragment known as class II invariant chain-associated peptides (CLIP). A non -classical HLA-DM will assist in

the cleavage of CLIP and allows processed peptides to occupy MHC-II peptide binding groove. The MHC-II- peptide complexes will then move to the surface of antigen presenting cells to activate antigen-specific adaptive immune response.

1.6 MHC-II turnover:

Once MHC-II-peptide complexes are expressed on the cell surface and initiate the immune response, they have a very short half- life and ultimately their expression is reduced. It is well known that the majority of plasma membrane proteins are transported to lysosomes for eventual degradation; many proteins can also be recycled from the plasma membrane to early endosomes and back to the plasma membrane[157]. However, when it comes to the fate of MHC-II molecules, little is known about the molecular machinery that precisely regulates turn-over of this molecule. In immature antigen presenting cells which do not express MHC-II molecules, it is reported that these cells possess rapid MHC-II degradation mechanism, which is mediated mainly by E3 ubiquitin ligase; membrane associated ring-CH-type finger 1 (MARCH1) ubiquitylation[158–160]. Ubiquitylation is an important step for MHC-II endocytosis and lysosomal degradation[155,161]. MARCH1 E3 Ligase is ubiquitously expressed by inactive or immature antigen presenting cells[159,160]. MARCH1 expression can be boosted by many anti-inflammatory cytokines such as interleukin-10 (IL-10), which explains the observation that IL-10 potentially down regulates MHC-II expression and can help to suppress the overall immune system attack[162,163]. The expression of MARCH1 decreases rapidly after complete activation of antigen presenting cells. MARCH1 has a very short half-life (less than 30 minutes) and it is mainly regulated by auto-ubiquitylation[156,164]. MARCH1-MHC-II ubiquitylation process can be modulated by the action of many chaperones and markers. For example, CD83 marker expressed by active antigen presenting cells, stabilizes MHC-II expression by suppressing the interaction of MHC-II with

MARCH1[165,166]. Recent data found that toll-interacting protein (TOLLIP) reduces the expression of MARCH1 and upregulates MHC-II on the surface of antigen presenting cells[167,168]. The ubiquitylated MHC-II molecules interact with multiprotein endosomal sorting complex required for transport (ESCRT) which aids in their trafficking to multivesicular bodies (MVBs) in the lysosome for final degradation[169]. Despite available evidence about MARCH1 mediated ubiquitylation as a regulatory process of MHC-II degradation, it is still believed that MHC-II turnover and degradation are a multifaceted process and can involve nested mechanisms that need further investigation.

1.7 26S proteasome degradation machinery:

The 26S proteasome degradation system plays a key role in cellular homeostasis by removing mid-folded and damaged proteins [170–173]. Around 80% of the misfolded protein degradation in majority of mammalian cells is carried out by 26S proteasome[170,171,174–176]. The 26S proteasome consists of two distinct sub-complexes, 20S proteasome or core particle (CP) and 19S regulatory particle (RP/ PA700)[170,171,174–177]. The 20S core particle is composed of four stacked rings; the two outer rings called α-rings and each ring has seven α-subunits (called PSMAs), while the two inner rings are β-rings, with each ring comprises seven β-subunits (called PSMBs). The three out of seven β-subunits formed the proteolytic unit of the 26S proteasome; PSMB6 (β1), PSMB7 (β2) and PSMB5 (β5)[170,173,175,177,178]. The 19S regulatory complex (RP) functions by recognizing and binding the polyubiquitinated proteins, followed by deubiquitination and linearization of the detected proteins in order to translocate them to the proteolytic chamber of the 20S CP[178–182]. The 19S RP is further divided into two complementary sub-complexes, the "base" and "lid". The 19S base ring consists of six regulatory particles called AAA ATPase subunits named PSMCs or Rpts, as well as four other regulatory particles called non-ATPase

subunits named PSMDs or Rpns (Rpn1, Rpn2, Rpn10 and Rpn13) Rpn1, Rpn10 and Rpn13 serve as ubiquitin receptors and binding sites for the targeted proteins[170,179–184]. The horseshoe-shaped 19S lid consists of nine remaining PSMDs or Rpns subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15) (Figure 1.4). The main role of 19S lid subunits is de-ubiquitination and unfolding of the polyubiquitinated target proteins, which is mediated by de-ubiquitinating enzymes (DUBs) Rpn11, Uch37 and Ubp6/Usp14[178,179,181,184]. Efficient 26S proteasome degradation function requires the assembly of 19S and 20S sub-complexes together which is facilitated and regulated by the action of proteasome chaperones including bleomycin resistance protein 10 or Proteasome Activator Complex Subunit 4 (BLM10 or PA200), Ecm29 proteasome adaptor and scaffold (ECM29), heat shock protein 90α (HSP90), NIN1-binding protein 1 (NOB1) and Proteasome Inhibitor Subunit 1(PI31). The functionally assembled 26S proteasome is fundamental for protein homeostasis; therefore, 26S proteasome has become a therapeutic target in many preclinical disease models and clinical trials. Ubiquitination is the first step in initiating the 26S proteasome degradation process[170,173,182,185–187]. Ubiquitination comprises the attachment of ubiquitin molecules through an isopeptide linkage with lysine residues exposed on of the targeted protein. This conjugation is facilitated through the sequential actions of three enzymes; E1 ubiquitin-activating enzymes, the E2 ubiquitin-conjugating enzymes, and the E3 ubiquitin-protein ligases, that utilize ATP hydrolysis for isopeptide bond formation[172,174,183]. Once the protein is ubiquitinated, the 26S proteasome doesn't automatically degrade the ubiquitin tagged proteins, but initially it will determine whether the ubiquitinated protein is malfunctioning or misfolded and should go through complete degradation or intact and can escape from being degraded, which ensures efficient and selective degradation[170,174,175,182,188,189]. Based on the importance of protein degradation and homeostasis, 26S proteasome master

degradation system coordinates vital cellular functions including cell-cycle control, cell development, proliferation, cell differentiation, DNA repair, apoptosis and autophagy[172,176,180,187].

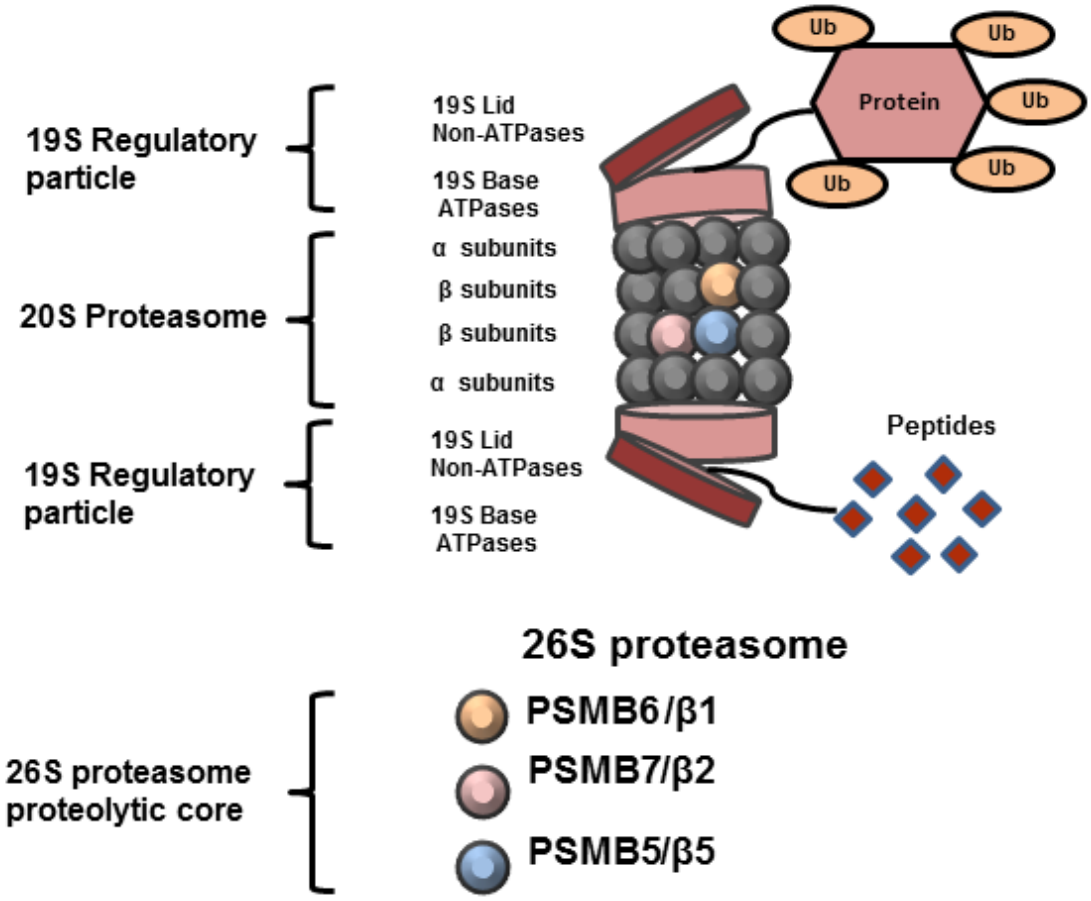


Figure 1.4: 26S proteasome structure. The figure depicts the structure of 26S proteasome with its subunits. 26S proteasome consists of 19S regulatory particle and 20S proteolytic core region. The 20S core consists of four stackable rings, where the two inner rings called "β "rings are flanked with the two outer rings called "α" rings (α-β-β-α). The 19S regulatory particle is composed of a 'Lid' which has the non-ATPases subunits and a 'Base' which contains the ATPase subunits. Once the targeted protein binds ubiquitin (ub), the 19S will receive the poly-ub protein and transfer it to the proteolytically active 20S subunits: β1, β2, and β5 to complete its degradation into small pepetides.

1.8 Non-proteolytic functions of 26S proteasome:

Several lines of investigation have revealed the involvement of the 26S proteasome in the regulation of gene transcription. Notably, the non-proteolytic role of 26S proteasome is carried out by 19S regulatory particle ATPases located in the 19S base sub-complex[190,191]. The non-proteolytic activity of 19S proteasome has been linked to the regulation of various aspects of gene transcription and translation, including initiation and elongation steps, chromatin remodeling and posttranslational repressive or suppressive modifications[191–194]. Recent biochemical and genetic studies have shown that the 19S proteasome can actually interact with transcription factors and co-activators to control gene transcription. Sug1/Rpt6 is the most studied 19S ATPase subunit that actively and physically interacts with transcription factors to control gene transcription in a degradation independent fashion [190,192,195]. The current notion is that Sug1/Rpt6 is recruited to the site of transcription for the subsequent deployment and recruitment of transcription machinery, including chromatin remodeling and stabilization of enhanceosomes at the site of transcription. The role of Sug1 in regulating transcription machinery is found to be crucial in controlling the immune response by modulating CIITA-MHC-II expression and antigen presentation function. It has been demonstrated that under inflammatory cytokine IFN-γ stimulation, Sug1 mediates CIITA inducible activation, which in turn drives MHC-II molecules expression to initiate an adaptive immune responses by binding and presenting antigenic peptides to $CD4^+$ T lymphocytes (Figure 1.2)[192,194–196]. These observations help in better understanding the nature of various immune related disorders such as autoimmune diseases like systemic lupus erythematosus (SLE), acquired and inherited immune-deficiencies including Bare Lymphocyte Syndrome (BLS) and Severe Combined Immune Deficiency (SCID)[197–200]. Furthermore, this non-proteolytic role of the 19S proteasome in

immune activation helps to explain the mechanism by which cancer cells are able to escape the attack by the immune system[190,192–194]. The contribution of 26S proteasome in regulating immune system functions has been highlighted in its prominence as a target for treating many diseases. Sug1 can regulate CIITA by inducing acetylation and methylation of histones H3 and H4 at pIV promoter[190,192,195,201]. For example, following IFN-γ stimulation, Sug1 bind to CIITApIV promoter causing promoter histone acetylation and co-factor binding. Reduced expression of Sug1 enhances histone H3 lysine 27 trimethylation at the CIITA promoter and results in repression of its transcription[192,195]. Additionally, Sug1 can also activate or suppress CIITA by undergoing post-translational modifications including phosphorylation, acetylation and monoubiquitination [195,196,201–204]. It has been shown that Sug1 knockdown diminishes the acetylation of lysines K9, K18, and K2 of CIITA and reduces its activity and half-life. Sug1 can promote CIITA activation via monoubiquitination while preventing CIITA polyubiquitination and its subsequent degradation[202–204].

1.9 Immunoproteasome:

The Immunoproteasome is an alternate form which is derived from constitutive proteasome and is expressed in immune cells under stress conditions. The process of switching the 26S proteasome toward the immunoproteasome is quite dynamic, β subunits of 20S proteasome (β1, β2 and β5) are replaced with specialized subunits. These subunits are identified as: β1i (also known as low molecular weight protein 2, PSMB9 and LMP2), β2i (also known as multicatalytic endopeptidase complex-like 1, PSMB10, LMP10, and MECL-1) and β5i (also known as low molecular weight protein 7, PSMB8 and LMP7). The 19S regulatory particle is replaced with 11S complex, also known as PA28 or PSME4. Unlike 19S subunit, 11S does not contain any ATPases, which compromises its degradation function for only small proteins (Figure 1.5)[205–208].

The immunoproteasome is reported to mainly degrade and prepare antigenic peptides to be carried by the MHC-I molecule to trigger $CD8^+$ cytotoxic lymphocytes [205,209–211]. The role of immunoproteasome in MHC-II mediated antigen presentation has not been reported yet. However, it is reported that the immunoproteasome is constitutively abundant in antigen presenting cells or can be induced in any cell type in cases of infections and inflammatory stress[210,212,213]. Spleen has the highest levels of immunoproteasome expression and activity compared to other organs that strongly suggests that immunoproteasome is involved in immune cell functions[210,212,214,215]. Furthermore, immature dendritic cells (DCs) have been reported to express immunoproteasome at equal levels to that of the standard 26S proteasome. Further, the high basal levels of immunoproteasome expression in immune cells are likely due to permanent activation of intracellular signaling pathways which are reported to be partially dependent on non-phosphorylated signal transducer and activator of transcription 1 (STAT1)[216–220]. While non-immune cells express standard 26S proteasomes exclusively, immunoproteasome expression is only induced under the influx of proinflammatory cytokines such as IFN-γ treatment[215–217,219,221,222]. Interestingly, it has been reported that the immunoprivileged sites such as the cornea and brain express standard 26S proteasome and minimally express immunoproteasome even if they have been subjected to persistent cytokine stimulation, suggesting the involvement of 26S proteasome system in regulating the immunoprivilege status of these sites[223–225]. In this regard, bone marrow derived MSCs are believed to be immunoprivileged, therefore, these cells can avoid host immune response after transplantation in the injured heart. However, recent analysis of preclinical and clinical studies confirmed that allogeneic MSCs after transplantation in the infarcted heart were safe, and were able to improve cardiac function[66,78,82,226]. However, late after implantation in the ischemic/hypoxic heart, MSCs became immunogenic, and were rejected by

recipient immune system[117,120,123,126]. These findings challenge the fact that MSCs are immunoprivileged and can be transplanted safely without the risk of immune rejection. Therefore, in order to improve survival and beneficial effects of allogeneic MSCs after transplantation in the infarcted heart, it is important to understand the mechanisms of loss of immunoprivilege of MSCs under hypoxic or ischemic conditions. Therefore, in this thesis, we performed *in vitro* (in rat and human cells) and *in vivo* (in a rat model) investigations to understand the mechanisms controlling the switch in the phenotype of MSCs from immunoprivileged to immunogenic state under hypoxic or ischemic conditions. Our studies also suggest that therapeutic interventions are possible through modifications of MSCs to maintain immunoprivilege and improve survival of transplanted allogeneic MSCs in the ischemic heart.

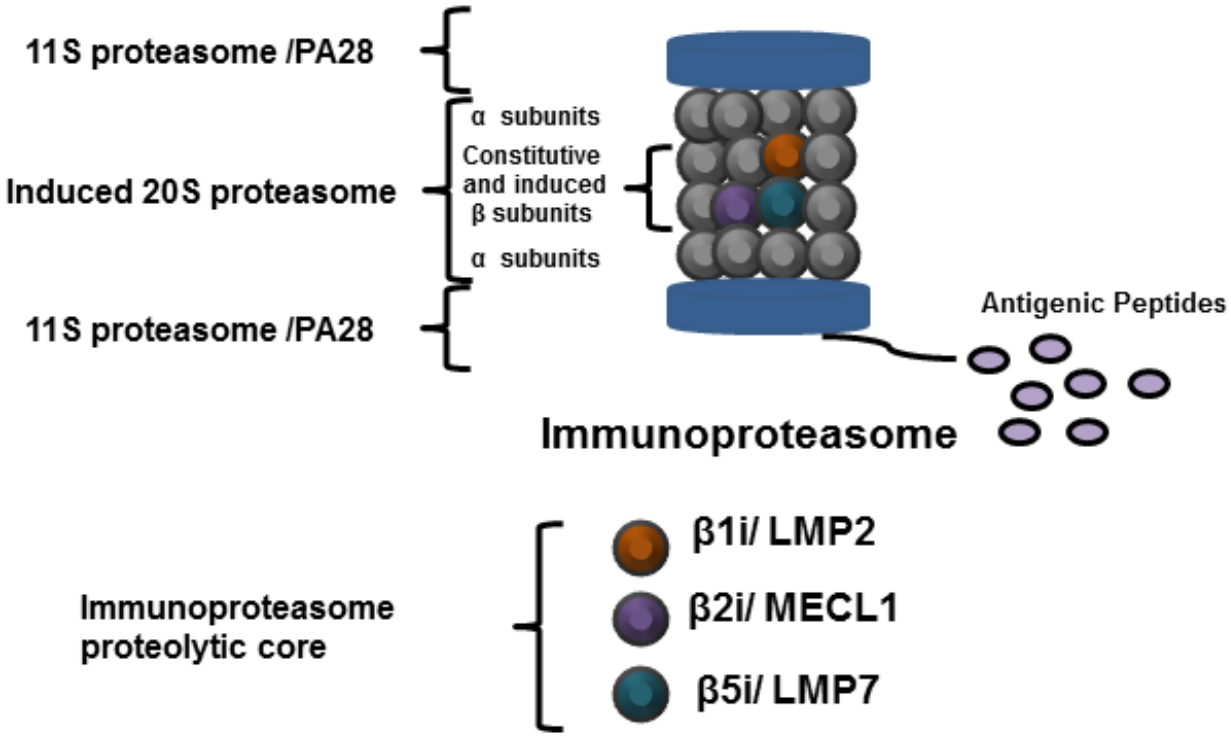


Figure 1.5: Immunoproteasome structure. The figure explains the detailed structure of the immunoproteasome. Exposure to stress stimulus such as interferon-γ (IFN-γ) triggers the replacement of the three proteolytic β-subunits of the constitutive 26S proteasome by inducible β counterparts: β 1i (also known as low molecular mass peptide 2 (LMP2); β 2i (also known as multicatalytic endopeptidase complex-like 1 (MECL-1) and β 5i (also known as LMP7) which will form the induced 20S proteolytic core (i20S). The i20S will then bind to PA28 α/β (11S proteasome) regulatory particle to form an immunogenic proteasome complex called the immunoproteasome.

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OVER ALL RATIONALE, HYPOTHESIS AND OBJECTIVES:

Rationale: Accumulated evidence reported that bone marrow derived MSCs are considered to be the most favorite cell type in treating various degenerative diseases and auto-immune disorders. However, there have been several challenges reported in clinical translation of MSCs based therapies and the post-transplantation poor survival of allogeneic MSCs is a major hurdle in this regard. Hypoxia/ischemia is a harsh hallmark of several pathological conditions including cardiovascular disorders. However, the effect of hypoxic environment or ischemia on the immunoprivilege of allogeneic MSCs is not studied yet. Therefore in the current study we performed extensive *in vitro* and *in vivo* investigations to understand the effect of hypoxia or ischemia on the immunoprivilege of allogeneic MSCs. The immunoprivilege of MSCs is preserved by negligible expression of MHC-II molecules on the cell surface. MHC-II plays an important role in rejection of transplanted allograft. Therefore, in order to investigate the mechanisms, we sought to understand the effect of hypoxic environment on MHC-II in rat as well as human MSCs.

Hypothesis: Exposure to hypoxic environment leads to loss of immunorpvilege of allogeneic MSCs which is further associated with an upregulation of MHC-II levels.

Objectives: Following are the overall objectives of our studies:

Objective 1: To investigate the effect of hypoxia on the immunoprivilege of allogeneic MSCs.

Objective 2: To investigate the mechanisms of hypoxia induced loss of immunoprivilege of allogeneic MSCs.

Chapter II: Role of 26S proteasome in preserving immunoprivilege of allogeneic MSCs

Rational and Hypothesis:

As previously reviewed, mesenchymal stem cells are considered to be the ideal cell type in the realm of regenerative medicine. However, cumulative evidence confirms that post-transplantation poor survival of allogeneic MSCs in the ischemic heart impacted the therapeutic potential of MSCs therapies. Furthermore, recent studies reported a shift in the phenotype of allogeneic MSCs from immunoprivileged to immunogenic state under hypoxic conditions. Therefore, it is imperative to investigate the mechanisms of MSCs immunogenicity under hypoxic conditions. The immunoprivilege of MSCs is preserved by absence of MHC-II molecules on cell surface. MHC-II plays a major role in initiating immune response against implanted allografts. In this chapter, we have investigated the role of 26S proteasome machinery in preserving immunoprivielge of MSCs by degrading MHC-II protein. We also studied the effects of hypoxia on 26S proteasome machinery, MHC-II levels and immunoprivilege of MSCs.

Hypothesis:

Hypoxia induced inactivation of 26S proteasome in mesenchymal stem cells leads to increase in MHC-II expression and loss of immunoprivilege of allogeneic MSCs.

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Hypoxia induced 26S proteasome dysfunction increases immunogenicity of mesenchymal stem cells

Ejlal Abu-El-Rub[1,2], Glen Lester Sequiera[1,2], Niketa Sareen[1,2], Meenal Moudgil[1,2], Mohammad Golam Sabbir[2,3], Sanjiv Dhingra[1,2]

[1]Regenerative Medicine Program, Institute of Cardiovascular Sciences, Department of Physiology and Pathophysiology, University of Manitoba, Winnipeg, Canada
[2]St. Boniface Hospital Albrechtsen Research Centre, Winnipeg, Canada
[3]Division of Neurodegenerative Disorders, University of Manitoba, Winnipeg, Canada

Short Title: Immunogenicity of mesenchymal stem cells

Correspondence:
Sanjiv Dhingra, PhD, FCVS
Regenerative Medicine Program
Institute of Cardiovascular Sciences,
St. Boniface Hospital Research Centre
R-3028-2, 351 Tache Avenue, Winnipeg, R2H2A6, Canada
Phone: 204-235-3454; Fax: 204-233-6723
Email: sdhingra@sbrc.ca

2.1 Abstract

Bone marrow derived allogeneic (donor derived) mesenchymal stem cells (MSCs) are immunoprivileged and are considered to be promising candidates for regenerative therapy for numerous degenerative diseases. Even though the outcome of initial allogeneic MSCs based clinical trials was encouraging, the overall enthusiasm of lately has dimmed down. This is due to failure of long-term survival of transplanted cells in the recipient. In fact, recent analyses of allogeneic MSCs based studies demonstrated that cells after transplantation became immunogenic and were subsequently rejected by the host immune system. The current study reveals a novel mechanism of immune switch in MSCs. We demonstrate that hypoxia, a common denominator of ischemic tissues, induces an immune shift in MSCs from immunoprivileged to immunogenic state. The immunoprivilege of MSCs is preserved by downregulation or absence of major histocompatibility complex class II (MHC-II) molecules. We found that 26S proteasome-mediated intracellular degradation of MHC-II helps maintain the absence of MHC-II expression on cell surface in normoxic MSCs and preserves their immunoprivilege. The exposure to hypoxia leads to dissociation of 19S and 20S subunits, and inactivation of 26S proteasome. This prevented the degradation of MHC-II, and as a result the MSCs became immunogenic. Furthermore, we found that hypoxia-induced decrease in the levels of a chaperon protein HSP90α is responsible for inactivation of 26S proteasome. Maintaining HSP90α levels in hypoxic MSCs preserved the immunoprivilege of MSCs. Therefore, hypoxia-induced inactivation of 26S proteasome assembly instigates loss of immunoprivilege of allogeneic mesenchymal stem cells. Maintaining 26S proteasome activity in mesenchymal stem cells preserves their immunoprivilege.

2.2 Introduction

Bone marrow derived mesenchymal stem cells (MSCs) are considered to be immunoprivileged because these cells do not express or have negligible expression of cell surface immune antigen-major histocompatibility complex class II (MHC-II) molecules[1,2]. The MHC-II molecules are cell surface immune antigens that act as signals to alert the host immune system to initiate immune response against transplanted cells[3]. Due to negligible expression or absence of MHC-II on the surface of MSCs, transplanted allogeneic MSCs (donor derived) are able to escape the recipient's immune system and survive in the host. These unique properties have made allogeneic MSCs the "flagbearer" for regenerative medicine. In several animal models of degenerative diseases including neurodegenerative, cardiovascular and autoimmune disorders, the transplanted allogeneic MSCs were able to initiate repair processes and improve function[4–7]. Based on the encouraging outcomes of preclinical studies, several clinical trials have been conducted to assess the safety and efficacy of allogeneic MSCs[8]. Even though the outcome of initial animal studies and clinical trials was positive, but the overall enthusiasm of lately has dimmed down. This is due to failure of long-term survival of transplanted cells and diminishing benefits over a period of time after transplantation. Infact, the recent data from pre-clinical studies and clinical trials indicate that allogeneic MSCs after transplantation provoke an immune response in the recipient[9–12]. In a pig model, allogeneic MSCs elicited immune responses after transplantation in the ischemic heart[10]. We recently reported in a rat model of myocardial infarction that allogeneic MSCs after 5 weeks of transplantation became immunogenic and were rejected in the infarcted/ischemic heart[12]. These findings strongly suggest that allogeneic MSCs become immunogenic after implantation in the ischemic tissues in recipient and are rejected by host immune system. Therefore, understanding the mechanisms of immune-switch in MSCs

from immunoprivileged to immunogenic state would help in planning strategies to prevent rejection and enhance benefits of allogeneic MSCs based therapy. Hypoxia (part of ischemic environment) is a harsh hallmark of many pathological diseases including cardiovascular diseases[13–16]. In this study, we examined the effect of hypoxic environment on the immunoprivilege of MSCs. Our studies reveal that exposure to hypoxic conditions instigates an immune switch in MSCs from immunoprivileged to immunogenic state. The current study also provides a novel mechanism of hypoxia induced immune switch in MSCs.

2.3 Results

2.3.1 Exposure to hypoxic environment triggers loss of immunoprivilige in MSCs: The immunoprivilege of MSCs is preserved by the downregulation or absence of MHC-II molecules[1,2]. We wanted to determine if there was any change in the expression of MHC-II in MSCs under hypoxic conditions. BM-MSCs were incubated in the hypoxia-chamber for 24 hours, MHC-II levels were assessed by Western blot and immunostaining. There was a significant increase in MHC-II levels in hypoxia exposed MSCs as compared to normoxic cells (Fig 2.1 a, b).

To investigate association between hypoxia induced MHC-II upregulation and immunogenicity of MSCs, the stem cells were co-cultured with allogeneic leukocytes for 72 hrs and the extent of leukocytes mediated cytotoxicity in MSCs was measured. The cytotoxicity was measured by determining the amount of lactate dehydrogenase (LDH) released and was found to be significantly greater in hypoxic MSCs as compared to normoxic cells (Fig 2.1c). Interestingly, siRNA mediated inhibition of MHC-II prevented leukocyte mediated cytotoxicity in hypoxic MSCs (Fig 2.1 a, c). Therefore, we infer that hypoxia induced increase in MHC-II levels is associated with the loss of immunoprivilege of MSCs. However, the presence of siRNA against

MHC-II did not change the level of cytotoxicity in normoxic MSCs after co-culture with allogeneic leukocytes (Supplementary Figure 2.1). MSCs are immunoprivileged and promote immune tolerance by enabling the phenotype change from cytotoxic T cells toward regulatory T (Treg) cell population[17,18]. Treg cells can suppress the proliferation of cytotoxic T cells and promote immune tolerance. In the current study, we counted the number of $CD4^+CD25^+$ Treg cells in a mixed leukocyte population after 72hrs of co-culture with allogeneic MSCs by flow cytometry. The Treg cell number decreased after co-culture with hypoxia-exposed MSCs compared to normoxic cells (Fig.2.1 d). MHC-II inhibited MSCs were able to promote Treg cell induction (Fig 2.1 d).

MSCs also have the ability to suppress leukocyte proliferation and promote immune tolerance[19]. The leukocyte activation and proliferation was measured by counting the number of cells entering S-phase and G_2/M phase from G_0/G_1 phase of the cell cycle and by a cell proliferation assay kit. There was a significant increase in leukocyte proliferation after co-culture with hypoxic MSCs compared to normoxic cells (Fig.2.1e, Supplementary Figure 2.2). The number of resting leukocytes in G_0/G_1 phase was greater after co-culture with normoxic MSCs compared to hypoxic MSCs (Fig. 2.1e). At the same time the number of leukocytes entering S phase (proliferating phase) and G_2/M phase increased after co-culture with hypoxic MSCs compared to normoxic stem cells (Fig. 2.1e). These results demonstrate that MSCs under normoxic conditions were able to suppress leukocyte proliferation, after exposure to hypoxia, MSCs lost this ability. The co-culture with MHC-II inhibited hypoxic MSCs decreased leukocyte proliferation, as there was an increase in the leukocyte number in G0/G1 phase and decrease in the leukocyte number in S phase (Fig.2.1e, Supplementary Figure 2.2). Therefore, hypoxia induced upregulation of

MHC-II was associated with increase in immunogenicity and a decrease in immune tolerance of allogeneic MSCs.

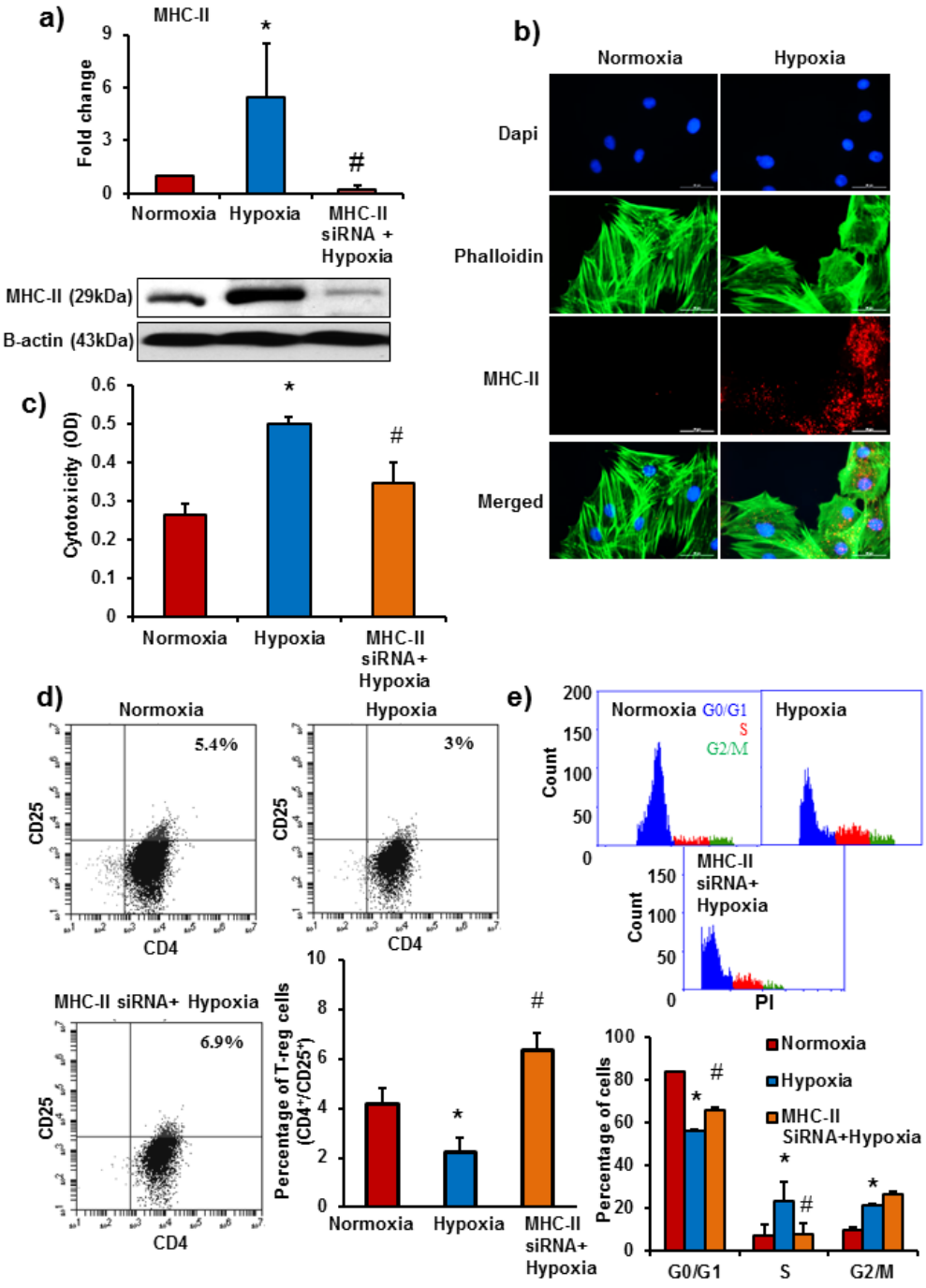

Fig 2.1: Exposure to hypoxia induces loss of immunoprivilege in MSCs. (a) Rat bone marrow derived MSCs were exposed to hypoxia for 24h. MHC-II levels as measured by Western Blot increased in hypoxic MSCs, which showed regression when inhibited by siRNA. n=3; (b) Immunofluorescence images showed a significant increase in the expression of MHC-II under hypoxia compared to normoxia. n=6; (c-e) To determine the immunogenicity of MSCs, normoxic and hypoxic rat MSCs (with or without siRNA) were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. (c) Leukocyte mediated cytotoxicity in MSCs (LDH release) increased significantly in hypoxic MSCs vs. normoxic cells, which was rescued by siRNA mediated inhibition of MHC-II. n=10; (d) The effect of MSCs on Treg cell (CD4+CD25+) induction in a mixed leukocyte population was assessed by flow cytometry. The number of Treg cells decreased after co-culture with hypoxic MSCs, siRNA mediated inhibition of MHC-II increased Treg cell number. n=3; (e) The effect of MSCs on leukocyte activation and proliferation was determined using PI staining, by assessing the number of cells present in different phases of cell cycle. The % of activated and proliferating leukocytes showed a significant increase under hypoxia. The number of activated and proliferating leukocytes decreased after siRNA mediated MHC-II inhibition in MSCs. n=3. *$p<0.05$ compared to normoxia group; #$p<0.05$ compared to hypoxia group. Each experiment was repeated 4-6 times.

2.3.2 26S proteasome degrades MHC-II in normoxic MSCs and preserves immunoprivilege: Next, we wanted to determine the mechanisms that lead to the absence of MHC-II in normoxic MSCs. Intracellular synthesis, activation, transport and storage of MHC-II have been studied extensively, but the turnover of MHC-II protein itself remains largely unexplored. In this regard, the 26S proteasome system is reported to mediate degradation of unwanted or damaged proteins by proteolysis[20]. Therefore, to explore the possibility of MHC-II degradation by 26S proteasome system in normoxic MSCs, we incubated the cells with 26S proteasome inhibitor, MG132 (2 µM and 5µM) for 24 hr followed by determination of MHC-II expression. There was a dose dependent increase in MHC-II protein levels in normoxic MSCs in the presence of 26S inhibitor (Fig 2.2 a, b). This dose and treatment protocol (for MG132 treatment) was optimal based on our pilot studies (Supplementary Figure 2.3).

To mediate degradation of unwanted proteins by 26S proteasome, the lysine residue of target protein (protein to be degraded) is conjugated with ubiquitin (a small protein, 8.5kDa), and this complex (ubiquitinated protein) is recognized by 26S proteasome that catalyses its degradation and clearance. We performed immunoprecipitation (IP) assay to monitor the levels of ubiquitinated MHC-II in normoxic MSCs before and after treating the cells with 26S inhibitor. There was a significant increase in the accumulation of ubiquitinated MHC-II in MG132 treated MSCs (Fig 2.2c). Interestingly, we also found a significant increase in the accumulation of ubiquitinated MHC-II in hypoxia treated MSCs compared to normal stem cells (Supplementary Figure 2.4). These data suggest that in normoxic MSCs, 26S proteasome facilitates degradation of MHC-II, and pharmacological inhibition of 26S proteasome or exposure to hypoxia lead to an increase in MHC-II levels in MSCs. Furthermore, in the MSCs and allogeneic leukocytes co-culture experiment, the presence of the 26S inhibitor increased leukocytes-mediated cytotoxicity

in normoxic MSCs (Fig. 2.2d). However, siRNA mediated inhibition of MHC-II in MG132 treated normoxic MSCs prevented this increased leukocyte mediated cytotoxicity (Supplementary Figure 2.1). Therefore, upregulation of MHC-II in 26S inhibitor treated MSCs is responsible for leukocyte mediated cytotoxicity. In our co-culture experiments we also found that the number of $CD4^{+}CD25^{+}$ Treg cells decreased and leukocyte proliferation increased after co-culture with 26S inhibited MSCs compared to normoxic cells (Fig 2.2e, Supplementary Figure 2.5). Therefore, 26S proteasome mediated degradation of MHC-II preserves immunoprivilege of normoxic MSCs.

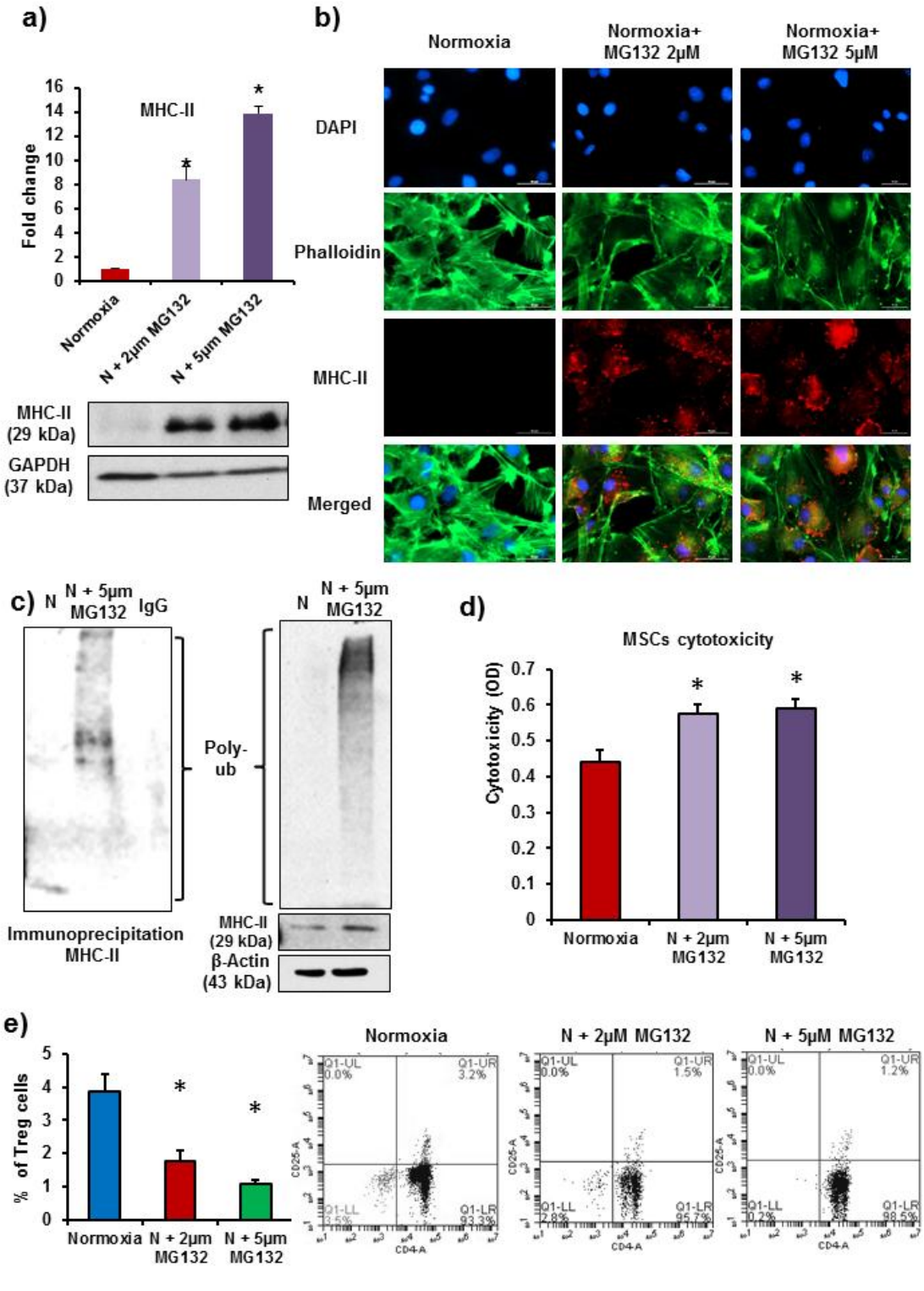
a)
MHC-II
Fold change
16
14
12
10
8
6
4
2
0
Normoxia
N + 2µm MG132
N + 5µm MG132
MHC-II
(29 kDa)
GAPDH
(37 kDa)
b)
Normoxia
Normoxia+
MG132 2µM
Normoxia+
MG132 5µM
DAPI
Phalloidin
MHC-II
Merged
c)
N
N + 5µm
MG132
IgG
Poly-
ub
Immunoprecipitation
MHC-II
MHC-II
(29 kDa)
β-Actin
(43 kDa)
d)
MSCs cytotoxicity
Cytotoxicity (OD)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Normoxia
N + 2µm
MG132
N + 5µm
MG132
e)
% of Treg cells
5
4
3
2
1
0
Normoxia
N + 2µm
MG132
N + 5µm
MG132
Normoxia
N + 2µM MG132
N + 5µM MG132
Q1-UL
0.0%
Q1-UR
3.2%
Q1-LL
3.5%
Q1-LR
93.3%
Q1-UL
0.0%
Q1-UR
1.5%
Q1-LL
2.8%
Q1-LR
95.7%
Q1-UL
0.2%
Q1-UR
1.2%
Q1-LL
0.2%
Q1-LR
98.5%
CD25-A
CD4-A

Fig 2.2: 26S proteasome regulates MHC-II levels and preserves immunoprivilege of MSCs. (a-b) Rat MSCs were treated with 26S proteasome inhibitor (MG132, 2μM and 5μM for 24 h), MHC-II levels determined by Western blot (a) and immunostaining (b) showed a dose-dependent increase. (n=3); (c) Immunoprecipitation (IP) analysis was performed in rat MSCs with or without 26S inhibitor to determine the involvement of 26S proteasome in the degradation of MHC-II. IP data revealed a significant accumulation of ubiquitinated MHC-II protein in 26S inhibited group. IP was performed with MHC-II antibody, and blotting was performed with polyubiquitin antibody. (n=4); (d-e) To determine the immunogenicity of MSCs, normoxic MSCs (with or without 26S inhibitor) were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. (d) LDH levels increased significantly in 26S inhibitor treated MSCs. (n=10); (e) Treg (CD4+CD25+) cell number in the mixed leukocyte population decreased significantly after co-culture with 26S inhibited MSCs. n=3. *$p<0.05$ compared to normoxia group. Each experiment was repeated 4-6 times.

2.3.3 Exposure to hypoxia leads to inactivation of 26S proteasome assembly in MSCs: The inhibition of 26S proteasome activity in normoxic MSCs was associated with loss of immunoprivilege. Also, there was a significant increase in the accumulation of ubiquitinated MHC-II in MSCs under hypoxia (Supplementary Figure 2.4). These findings prompted us to test that the observed MHC-II upregulation and loss of immunoprivilege during hypoxia might be related to decreased 26S function and activity. The 26S proteasome activity requires binding as well as coordinated action of 19S and 20S subunits for carrying out degradation and proteolysis of ubiquitinated proteins (Fig. 2.3a). We performed immunoprecipitation assay to assess the binding of 19S proteasome subunit and 20S proteasome subunit in normoxic and hypoxic MSCs. Our data demonstrate a dramatic decrease in the binding between 19S and 20S subunits in hypoxic MSCs (Fig. 2.3b). In order to further verify that exposure to hypoxia is associated with disassociation of 26S proteasome assembly, we performed two-dimensional (2D) blue native polyacrylamide gel electrophoresis (BN-PAGE/SDS-PAGE) assay to study protein-protein interaction between subunits of 26S proteasome. The cell lysates from normoxia and hypoxia exposed MSCs were subjected to 2D SDS-PAGE and immunoblotted using specific antibodies for Sug1 (one of the constituents of 19S subunit) and α3 (one of the constituents of 20S subunit). In 2D SDS-PAGE, the first dimension "native PAGE" separates whole multiprotein complexes (MPCs) and the 2nd dimension "denatured SDS-PAGE" separates interacting protein components within one MPC, which appears on a vertical line[21]. The Sug1 and α3 bind together only when these two proteins are part of respective 19S and 20S subunits of 26S proteasome complex. Further, the molecular weight of functional 26S proteasome complexes has been reported to be in the range of 1200-2000kDa[22–24]. Since we used specific antibodies for Sug1 and α3 proteins for immunoblotting, therefore the multiprotein complex appearing in the high molecular weight

range ~1200 kDa (Fig. 2.3c, white arrows in the lower panel) represent 26S proteasome (Molecular weight ~1200 kDa). The remaining low molecular weight complexes where Sug1 and α3 appeared partially overlapped may refer to other protein complexes involving Sug1 and α3 (Fig. 2.3c, red arrows). Interestingly, the dynamics of 26S proteasome complex were significantly different in hypoxic MSCs vs. normoxic cells, the amount of 26S complex was lesser in hypoxia exposed cells compared to normoxic group (Fig. 2.3c, white arrows). Furthermore, quantitative densitometric analysis of 2D immunoblots reveal that the fluorescence intensity (RFU) of 26S complex is stronger in normoxic MSCs compared to hypoxia exposed cells (Fig. 2.3d). Also, the ratio of bound vs. unbound fractions of Sug1 (19S subunit) and α3 (20S subunit) involved in the formation of 26S proteasome complex were significantly higher in normoxic cells compared to hypoxia exposed MSCs (Fig. 2.3e). These data confirm that binding between Sug1 and α3 subunits decreased in MSCs under hypoxia.

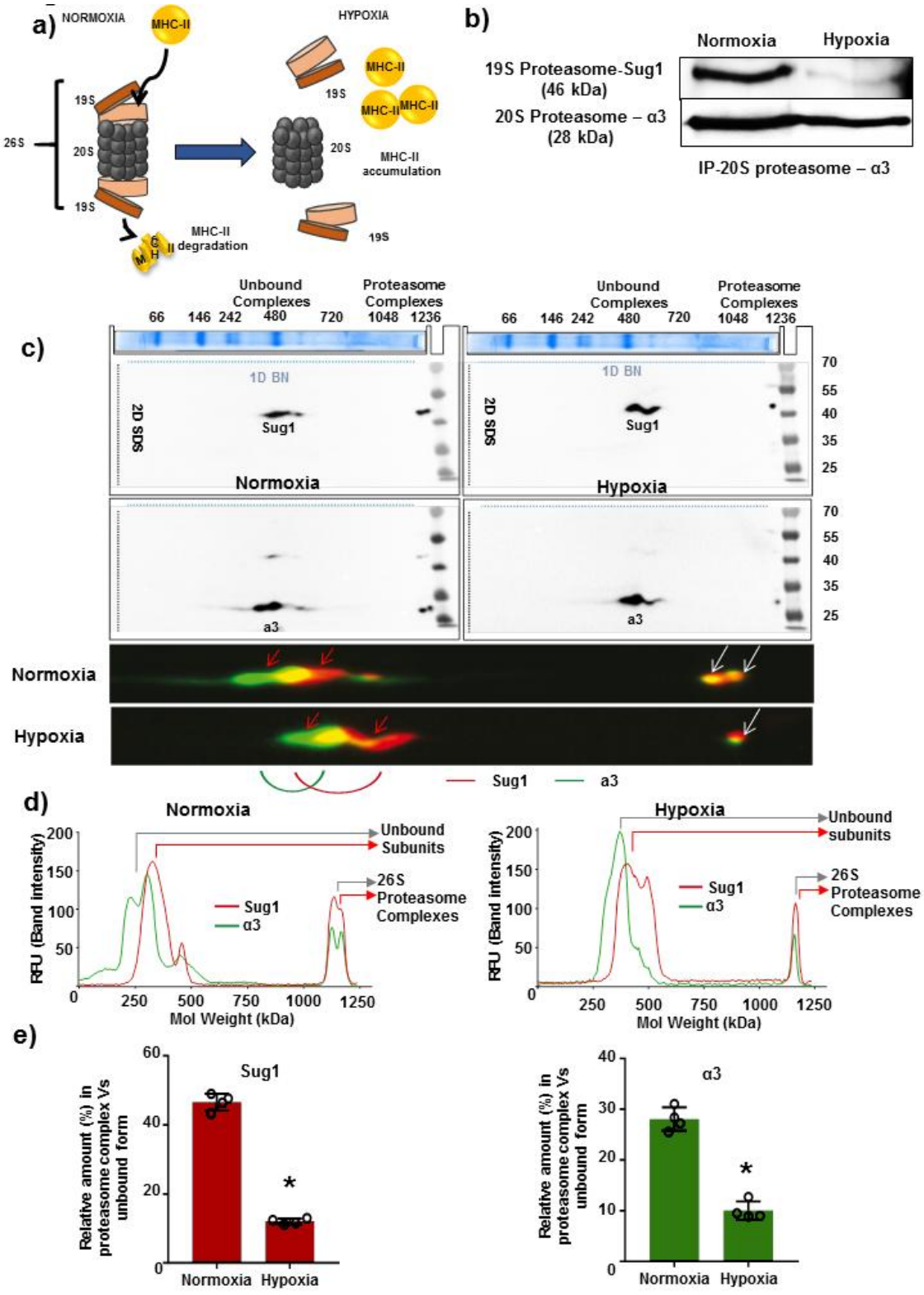
a)
NORMOXIA
MHC-II
HYPOXIA
19S
26S
20S
19S
MHC-II
MHC-II
MHC-II
20S
MHC-II accumulation
19S
MHC-II degradation
b)
Normoxia
Hypoxia
19S Proteasome-Sug1 (46 kDa)
20S Proteasome – α3 (28 kDa)
IP-20S proteasome – α3
c)
Unbound Complexes
Proteasome Complexes
66 146 242 480 720 1048 1236
1D BN
2D SDS
Sug1
Normoxia
Hypoxia
70 55 40 35 25
a3
Normoxia
Hypoxia
Sug1
a3
d)
Normoxia
Hypoxia
RFU (Band intensity)
Unbound Subunits
26S Proteasome Complexes
Sug1
α3
Mol Weight (kDa)
e)
Relative amount (%) in proteasome complex Vs unbound form
Sug1
α3
*
Normoxia Hypoxia

Fig 2.3: Exposure to hypoxia led to dissociation of 26S proteasome complex in rat MSCs. (a). Model depicts 26S proteasome structure, MHC-II degradation by 26S maintains absence of MHC-II in normoxic MSCs. Hypoxia induced dissociation of 26S proteasome (19S and 20S subunits) results in accumulation of MHC-II. (b) Immunoprecipitation (IP) assay was performed to monitor interaction between 19S and 20S subunits in normoxic and hypoxic MSCs. IP was performed with 20S antibody and blotted with antibodies for 19S and 20S. In normoxic MSCs 19S and 20S subunits bind to form functional 26S proteasome. The binding of two subunits decreased in hypoxic MSCs. (n=4); (c) The two dimensional (2D) blue native polyacrylamide gel electrophoresis (BN-PAGE)/SDS-PAGE assay was performed to study protein-protein interaction between subunits of 26S proteasome. The cell lysates from normoxia and hypoxia exposed MSCs were subjected to 2D SDS-PAGE and immunoblotted using specific antibodies for Sug1 (one of the constituents of 19S subunit) and α3 (one of the constituents of 20S subunit). The multiprotein complex appearing in the high molecular weight range ~1200-2000 kDa (white arrows) represent 26S proteasome. The amount of 26S complex was lesser in hypoxia exposed cells compared to normoxic group. (n=3); (d) Quantitative densitometric analysis of 2D immunoblots reveal that the fluorescence intensity (RFU) of 26S complex is stronger in normoxic MSCs compared to hypoxia exposed cells. (n=3); (e)The ratio of bound vs. unbound fractions of Sug1 (19S subunit) and α3 (20S subunit) involved in the formation of 26S proteasome complex were significantly higher in normoxic cells compared to hypoxia exposed MSCs. (n=3). *$p<0.05$ compared to normoxic MSC. Each experiment was repeated 3-4 times.

To mediate proteolytic actions of 26S proteasome, the 19S subunit recognizes ubiquitinated target proteins, unfolds and translocates them to the interior of 20S subunit, where proteins finally get proteolysed[25]. Both 19S and 20S subunits are able to perform deubiquitination and proteolysis of target proteins only when these two subunits are assembled as the intact 26S proteasome. Therefore, to precisely determine 26S activity, we measured deubiquitinating activity of 19S and proteolysing activity of 20S by fluorescence assays. There was a significant decrease in the activities of 19S and 20S proteasomes in hypoxic MSCs compared to normoxic cells (Fig. 2.4a). These studies demonstrate that exposure to hypoxia led to dissociation and inactivation of 26S proteasome assembly in MSCs compared to normoxic cells.

2.3.4 Hypoxia induced downregulation of a chaperone protein heat shock protein 90α leads to dissociation of 26S proteasome and loss of immunoprivilege of MSCs: Molecular chaperones including bleomycin resistance protein 10 (BLM10), heat shock protein90α (HSP90α), HSP90β, and NIN1 binding protein 1 (NOB1) are reported to play a role in assembling and maintenance of 26S proteasomal machinery[25,26]. Alterations in chaperone protein levels result in defective assembling or dissociation of 19S and 20S complex that affects the proteolytic function of 26S. In order to understand the mechanisms of hypoxia induced dissociation of 26S proteasome, we measured the levels of these chaperones in MSCs before and after hypoxia treatment. Interestingly, the mRNA and protein levels of BLM-10, HSP90β and NOB1 did not change significantly in MSCs under hypoxia (Fig. 2.4b, c). However, we found a significant decrease in both mRNA and protein levels of HSP90α in hypoxic MSC (Fig. 2.4b, c).

Fig

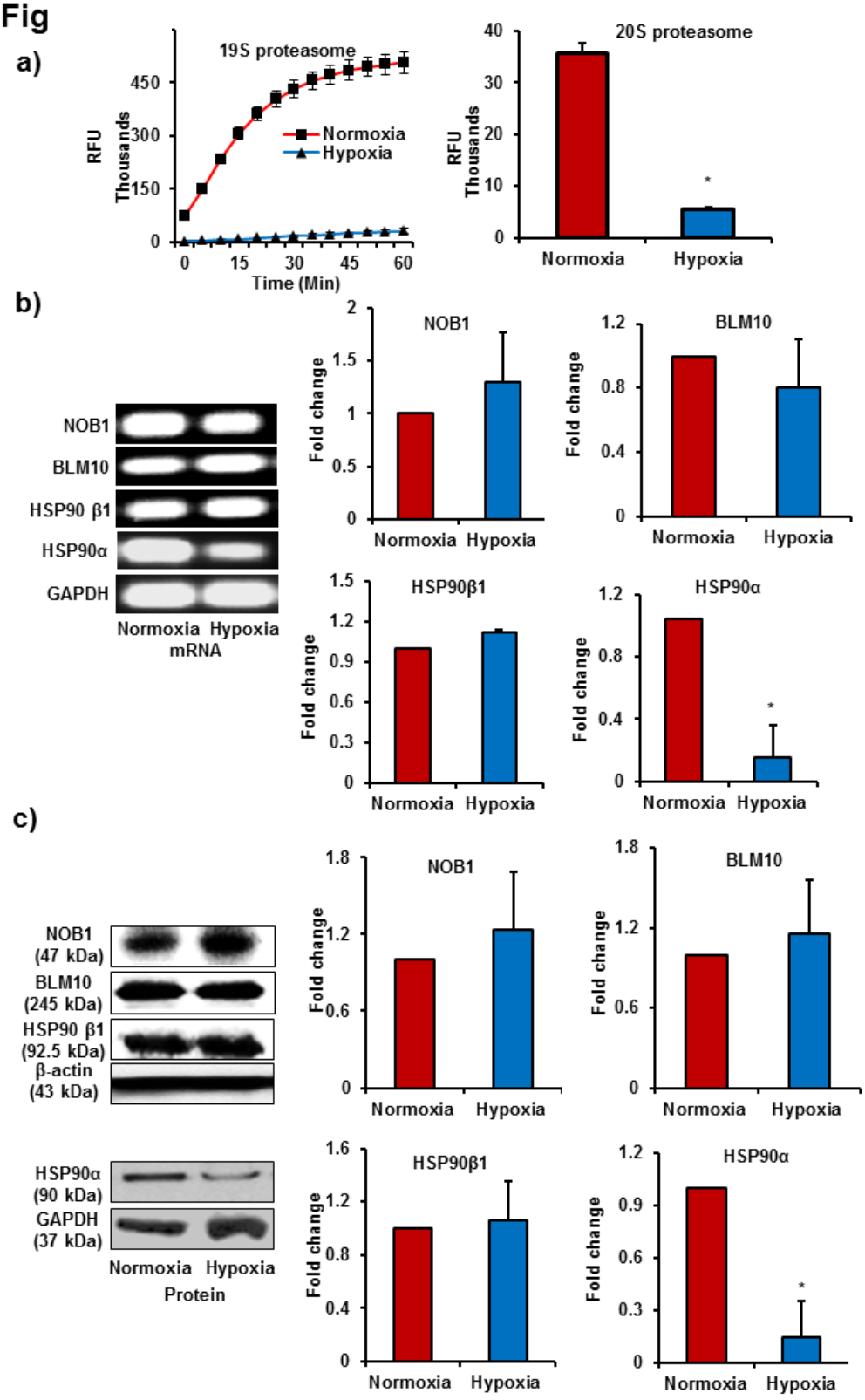
a)
19S proteasome
RFU Thousands
Normoxia
Hypoxia
Time (Min)
20S proteasome
b)
NOB1
BLM10
HSP90 β1
HSP90α
GAPDH
Normoxia Hypoxia
mRNA
Fold change
HSP90β1
c)
NOB1 (47 kDa)
BLM10 (245 kDa)
HSP90 β1 (92.5 kDa)
β-actin (43 kDa)
HSP90α (90 kDa)
GAPDH (37 kDa)
Protein

Fig 2.4: 26S proteasome activity and HSP90α levels were downregulated in hypoxic rat MSCs. (a) To measure 26S activity, the levels of both 19S (deubiquitinating activity) and 20S (proteolysing activity) were determined. The activities were measured by using flurogenic substrates: - U-555 for 19S and SUC-LLVY-AMC for 20S. Hypoxic MSCs were found to have a marked reduction in 26S activity. (n=3). (b-c) NOB1, BLM10, HSP90α and HSP90β mRNA and protein levels were determined by RT-PCR and Western blot. NOB1, BLM10 and HSP90β levels did not change in MSCs after exposure to hypoxia for 24 h. However, HSP90α mRNA and protein levels decreased in hypoxia exposed MSCs. (n=4); *$p<0.05$ compared to normoxic MSC. Each experiment was repeated 4-6 times.

In the next set of experiments, we wanted to investigate whether HSP90α regulates 26S proteasome activity in normoxic MSCs, and hypoxia induced decrease in HSP90α was associated with the inactivation of 26S proteasome system and increase in immunogenicity of MSCs. We blocked HSP90α in normoxic MSCs using a pharmacological inhibitor (SNX-2112) and measured deubiquitinating activity of 19S and proteolysing activity of 20S by fluorescence assays. There was a significant decrease in the activities of 19S and 20S subunits in HSP90α inhibited normoxic MSCs (Fig.2.5a). We also found a dose dependent increase in MHC-II levels in HSP90α inhibited normoxic MSCs (Fig. 2.5b).

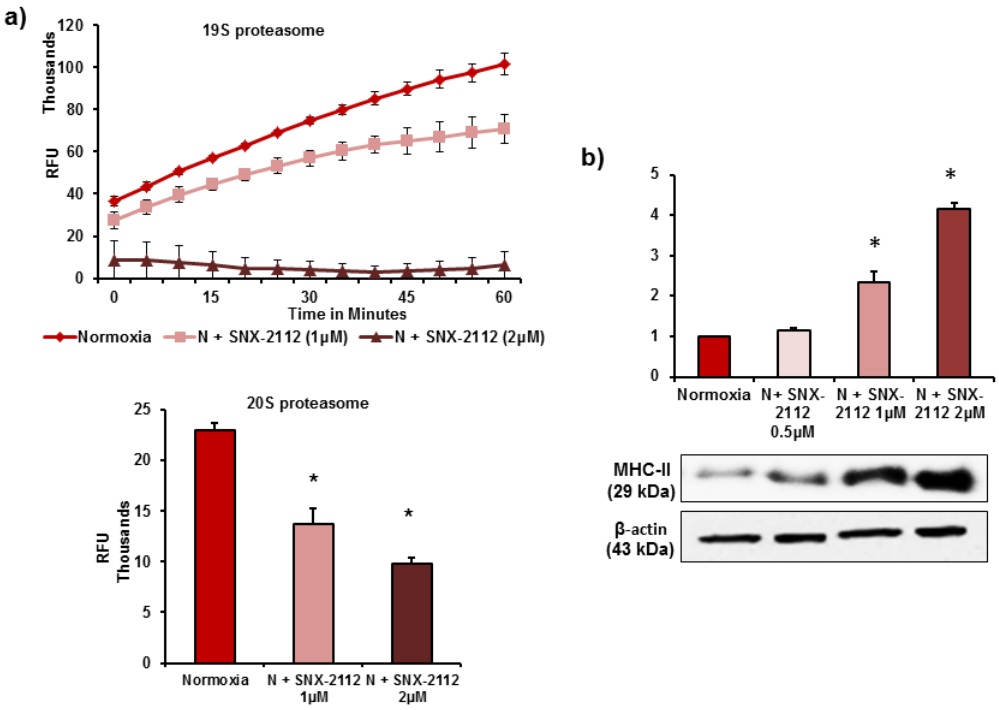

Fig 2.5: HSP90α regulates 26S activity and MHC-II levels in normoxic MSCs. (a-b) Rat MSCs were treated with HSP90α inhibitor (SNX-2112, 0.5μM, 1μM and 2μM for 24 h), 26S activity (19S and 20S activities) by fluorescence assay and MHC-II levels by Western blot were measured. (a) 26S activity decreased in HSP90α inhibited MSCs. (n=3). (b) MHC-II expression

increased in HSP90α inhibited MSCs in a dose dependent manner. (n=4). *$p<0.05$ compared to normoxic MSC. Each experiment was repeated 4-6 times.

In order to assess whether maintaining HSP90α in hypoxic MSCs would preserve immunoprivilege of stem cells, we used lentiviral particles to over express HSP90α in MSCs. The lentivirus mediated overexpression of HSP90α maintained the levels of HSP90α in hypoxic MSCs (Fig. 2.6a). We probed the same PVDF membrane to probe it with MHC-II antibody, our data demonstrate that maintaining HSP90α levels prevented hypoxia induced increase in MHC-II levels (Fig. 2.6a). We also found that maintaining HSP90α prevented hypoxia-induced downregulation of 26S activity (Fig. 2.6b). Furthermore, over expression of HSP90α decreased leukocytes mediated cytotoxicity in hypoxic MSCs (Fig. 2.6c). In allogeneic MSCs and leukocyte co-culture experiments, HSP90α overexpressing hypoxic MSCs were able to increase Treg cell numbers in mixed leukocyte population (Fig. 2.6d). Therefore, maintaining HSP90α levels in MSCs prevented hypoxia induced decrease in 26S activity and preserved their immunoprivilege.

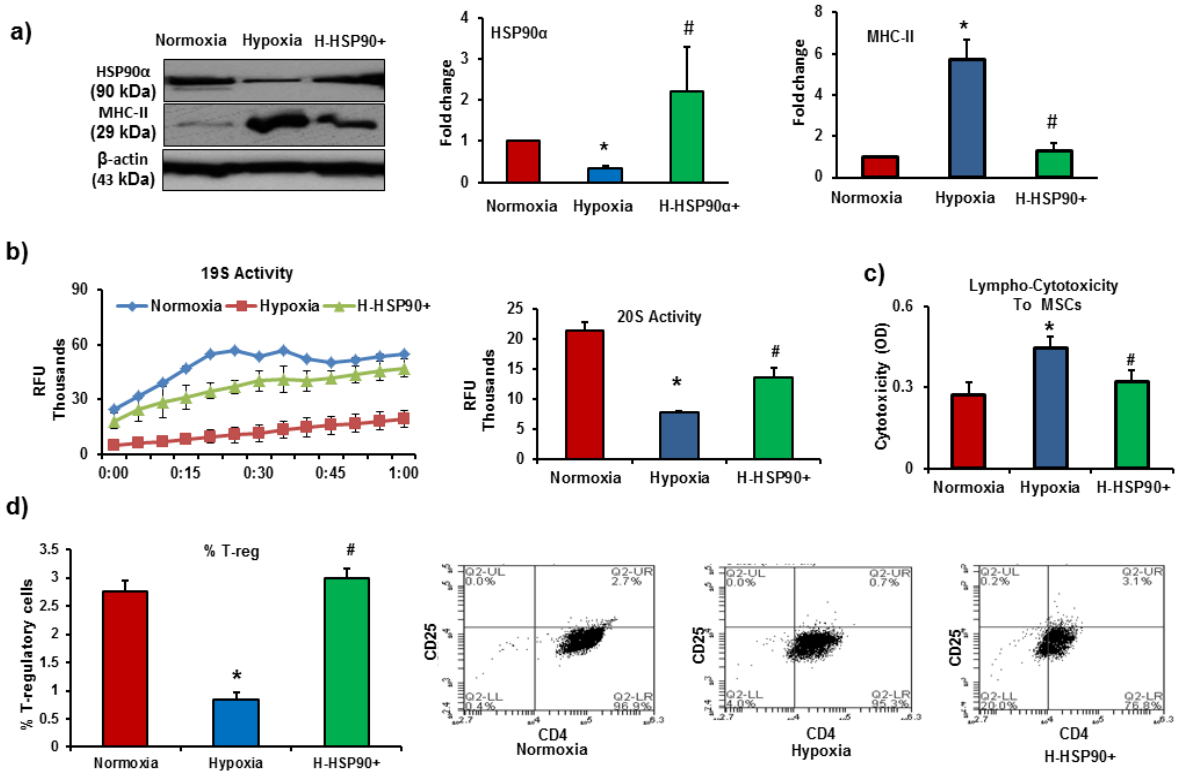

Fig 2.6: Maintaining HSP90α levels preserves immunoprivilege of MSCs under hypoxia. (a) Rat MSCs were transduced with lentiviral construct to over express HSP90α. HSP90α and MHC-II levels were measured by western blot in normoxic, hypoxic and HSP90α overexpressing hypoxic MSCs (n=4); (b) 26S proteasome activity by fluorescence assay in normoxic, hypoxic and HSP90α overexpressing hypoxic MSCs. HSP90α overexpression rescued 26S activity in hypoxic MSCs (n=4); (c-d) To determine the immunogenicity of MSCs; normoxic MSCs, hypoxic MSCs and HSP90α overexpressing hypoxic MSCs were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. (c) LDH levels increased significantly in hypoxic MSCs, HSP90α overexpression prevented hypoxia induced increase in LDH levels. (n=10). (d) Treg (CD4+CD25+) cell number in the mixed leukocyte population decreased significantly after co-culture with hypoxic MSCs, HSP90α overexpressing hypoxic MSCs were able to induce Treg

cell number. n=3. Each experiment was repeated 4-6 times. *p<0.05 compared to normoxia group; #p<0.05 compared to hypoxia group.

2.3.5 Exposure to hypoxia lead to loss of immunoprivilege in human BM-MSCs: To demonstrate translational potential of our studies, we also performed parallel experiments in human bone marrow derived MSCs (hMSCs). In rodents, MHC-II, and in humans the molecules of MHC-II complex, human leukocyte antigens-DR (HLA-DR), HLA-DP and HLA-DQ present antigens to $CD4^+$ T cells leading to activation and proliferation of T cells and allograft rejection[27,28]. Our data demonstrate that exposure to hypoxia led to loss of immunoprivilege in hMSCs. We found a significant increase in the levels of HLA-DR in hypoxia exposed hMSCs (Fig. 2.7a). In hMSCs and allogeneic leukocytes co-culture experiments, the level of cytotoxicity was significantly higher in hypoxia exposed hMSCs compared to normoxic cells (Fig. 2.7b). Also, there was a significant decrease in Treg cell number in the mixed leukocyte population after co-culture with hypoxic hMSCs compared to normoxic cells (Fig. 2.7c). Therefore, exposure to hypoxia was associated with loss of immunoprivilege in hMSCs. Furthermore, we found a significant decrease in 26S levels in hypoxia exposed hMSCs vs. normoxic cells (Fig. 2.7d). The inhibition of 26S activity in normoxic hMSCs led to an increase in HLA-DR expression in a dose dependent manner (Fig. 2.7e). Also, the inhibition of HSP90α (using pharmacological inhibitor SNX2112) in normoxic hMSCs resulted in a significantly decreased 26S activity, and an increase in HLA-DR protein levels (Fig. 2.8a,b). In the leukocytes and normoxic hMSCs co-culture experiments, the inhibition of 26S activity and HSP90α levels led to an increase in leukocyte mediated cytotoxicity in hMSCs (Fig. 2.8c). The number of Treg cells in the mixed leukocyte population decreased after co-culture with 26S and HSP90α inhibited normoxic hMSCs compared to control group (Fig. 2.8d). Therefore, in normoxic hMSCs,

HSP90α maintains 26S proteasome function and immunoprivilege of cells. The exposure to hypoxia leads to inactivation of 26S proteasome, increase in HLA-DR and loss of immunoprivilege.

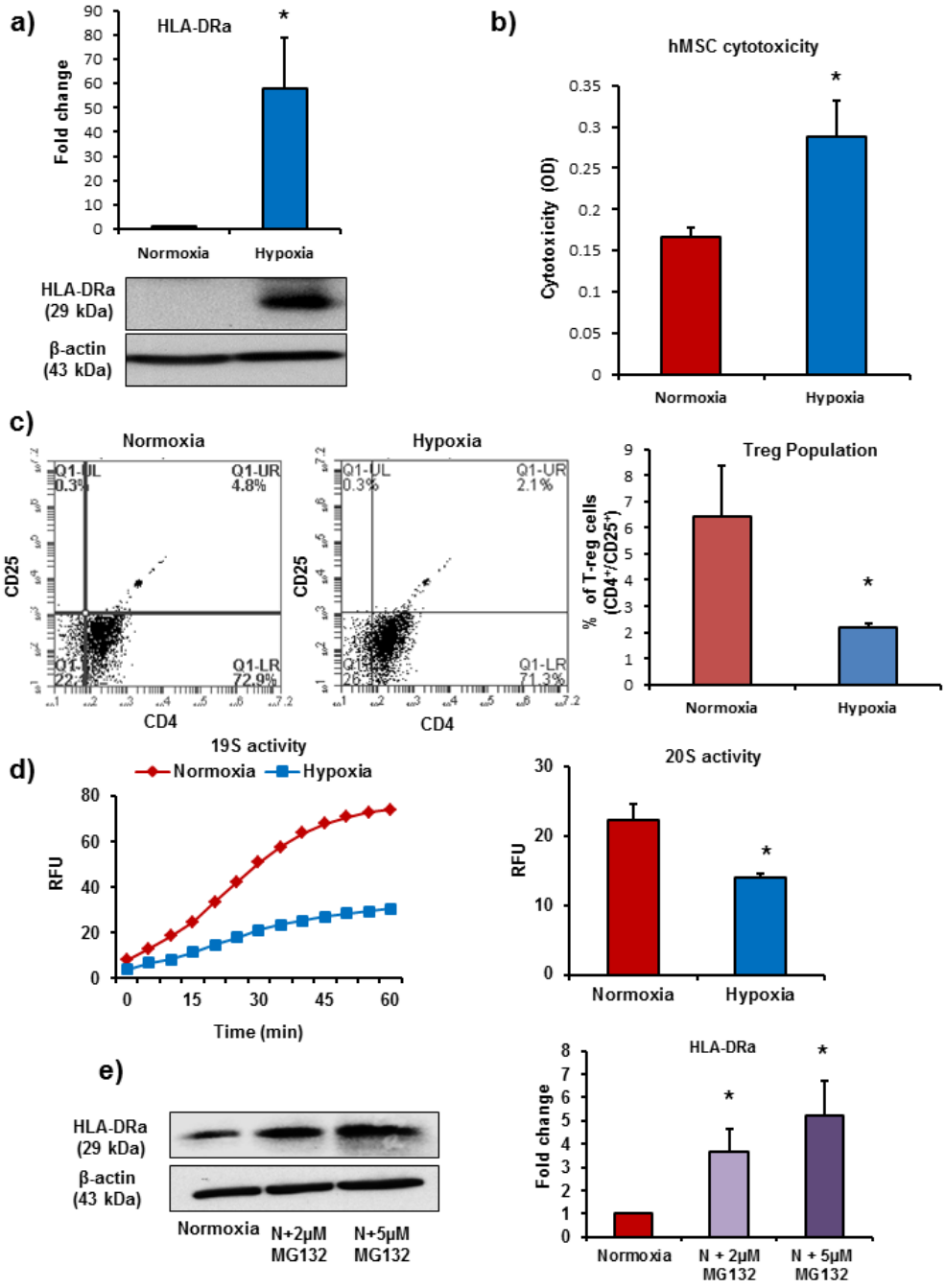

Fig 2.7: Loss of immunoprivilege of human MSCs after exposure to hypoxia. (a) Human bone marrow derived MSCs (hMSCs) were exposed to hypoxia for 24h. HLA-DRα levels as measured by Western Blot increased in hypoxic MSCs, (n=3). (b-c) To determine the immunogenicity of MSCs; normoxic and hypoxic hMSCs were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. (b) Leukocyte mediated cytotoxicity (LDH release) increased significantly in hypoxic hMSCs vs. normoxic cells. n=10; (c) The effect of hMSCs on Treg cell (CD4+CD25+) induction in a mixed leukocyte population was assessed by flow cytometry. The number of Treg cells decreased after co-culture with hypoxic hMSCs. n=4; (d) 26S proteasome activity was measured by determining the activities of both 19S (deubiquitinating activity) and 20S (proteolysing activity). The exposure to hypoxia led to a significant decrease in 26S activity in hMSCs. (n=4); (e) hMSCs were treated with 26S proteasome inhibitor (MG132, 2μM and 5μM for 24 h), HLA-DRα levels determined by Western blot showed a dose-dependent increase. (n=3); *$p<0.05$ compared to normoxia group. Each experiment was repeated 4-6 times.

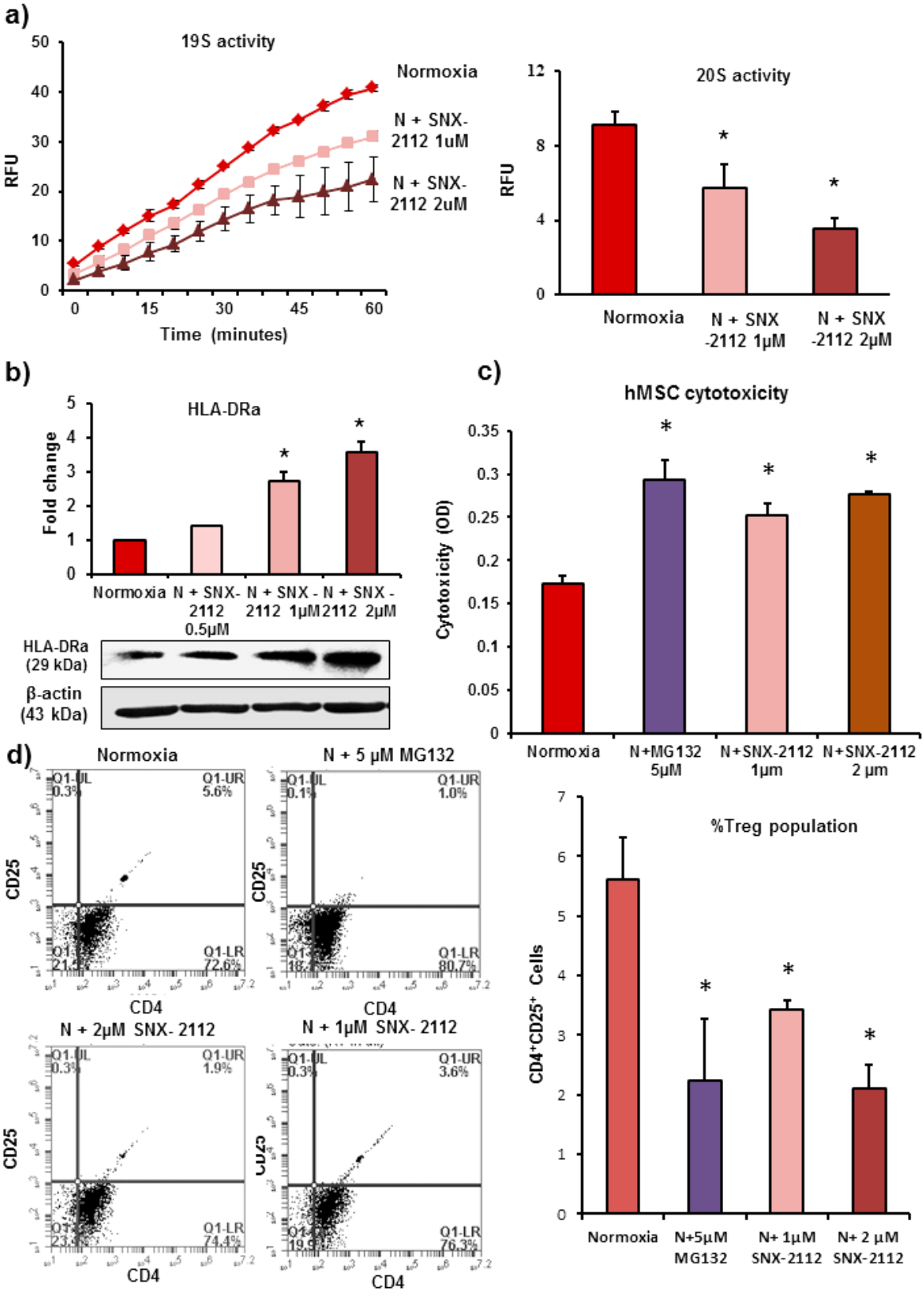
a)
19S activity
RFU
Time (minutes)
Normoxia
N + SNX-2112 1uM
N + SNX-2112 2uM
20S activity
RFU
Normoxia
N + SNX -2112 1µM
N + SNX -2112 2µM
b)
HLA-DRa
Fold change
Normoxia
N + SNX-2112 0.5µM
N + SNX - 2112 1µM
N + SNX - 2112 2µM
HLA-DRa (29 kDa)
β-actin (43 kDa)
c)
hMSC cytotoxicity
Cytotoxicity (OD)
Normoxia
N+MG132 5µM
N+SNX-2112 1µm
N+SNX-2112 2 µm
d)
Normoxia
N + 5 µM MG132
N + 2µM SNX- 2112
N + 1µM SNX- 2112
CD25
CD4
Q1-UL
Q1-UR
Q1-LR
%Treg population
CD4+CD25+ Cells
Normoxia
N+5µM MG132
N+ 1µM SNX-2112
N+ 2 µM SNX-2112

Fig 2.8: HSP90α maintains 26S activity and preserves immunoprivilege of hMSC. (a-b) hMSCs were treated with HSP90α inhibitor (SNX-2112, 0.5μM, 1μM and 2μM for 24 h), 26S activity (19S and 20S activities) by fluorescence assay and HLA-DRα levels by Western blot were measured. (a) 26S activity decreased in HSP90α inhibited MSCs. (n=3) (b) HLA-DRα expression increased in HSP90α inhibited MSCs. (n=3). (c-d) To determine the immunogenicity of hMSCs after HSP90α inhibition, hMSCs were treated with SNX-2112 (0.5μM, 1μM and 2μM for 24 h), and then co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. (c) Leukocyte mediated cytotoxicity (LDH levels) in hMSCs increased significantly in the presence of HSP90α inhibitor. (n=10). (d) Treg (CD4+CD25+) cell number in the mixed leukocyte population decreased significantly after co-culture with HSP90α inhibited hMSCs. n=10. *$p<0.05$ compared to normoxia group. Each experiment was repeated 4-6 times.

2.4 Discussion

The outcome of several allogeneic MSC based preclinical studies and initial clinical trials suggested that bone marrow derived MSCs have the potential to treat a number of degenerative diseases. However, the beneficial effects of transplanted allogeneic MSCs were short lived which has caused decline in the overall enthusiasm about MSC therapy. In fact, recent analyses of allogeneic MSCs based studies demonstrated that cells after transplantation turned immunogenic and were subsequently rejected by host immune system[29–31]. A number of studies have reported the mechanisms of immunoprivilege of mesenchymal stem cells, and MSCs mediated immune suppression[10,12]. However, the mechanisms of immune-switch in MSCs from immunoprivileged to immunogenic state have not yet been studied conclusively. Understanding the mechanisms of loss of immunoprivilege of allogeneic MSCs would help in planning strategies to prevent rejection and preserve the benefits of allogenic MSCs based therapies. Here we have demonstrated that immunoprivilege of MSCs is tightly mediated by absence of cell surface immune antigen MHC-II. We identified that MHC-II expression increased in both rat and human (HLA-DR) MSCs after exposure to hypoxia which was associated with loss of immunoprivilege. Investigating the mechanisms for absence of MHC-II on MSCs surface, we found that 26S proteasome mediated intracellular degradation of ubiquitinated MHC-II protein in normoxic MSCs prevented MHC-II expression on cell surface and preserved immunoprivilege of allogeneic MSCs. Our immunoprecipitation and 2D BN-PAGE/SDS-PAGE electrophoresis data demonstrate for the first time that hypoxic environment lead to inactivation of 26S proteasome and loss of immunoprivilege of MSCs. Function of the 26S proteasome in MSCs is maintained by a chaperone protein HSP90α, the levels of HSP90α decreased in hypoxic MSCs. Furthermore, maintaining HSP90α levels in MSCs prevented hypoxia induced inactivation of 26S proteasome

and preserved immunoprivlege of allogeneic MSCs. Hypoxia is the integral component of ischemic environment, which is associated with majority of pathological conditions at organ and tissue levels in the body. Several studies have investigated the effects of hypoxia on proliferation and differentiation potential of bone marrow derived MSCs[13,32,33], and reported that exposure to mild-to-moderate degree of hypoxia (3%-21% of oxygen) inhibits senescence, increases proliferation and differentiation potential of MSCs[34]. However, exposure to severe hypoxic conditions (1% or less than 1%) significantly decreases proliferation and differentiation potential of MSCs[35]. In the bone marrow, oxygen tension levels range between 4% and 7%, hence, bone marrow derived MSCs are adapted to moderate hypoxic conditions[36]. However, in the ischemic tissues (where stem cell transplantation is needed), the oxygen level drops below 1%, leading to severe hypoxia. The effects of such a low level of oxygen (severe hypoxia) on the immunoprivilege of MSCs are largely unknown and it requires thorough investigation to maximize the regenerative potential of MSCs. We have performed extensive investigations in this direction and our data demonstrate that exposure to severe hypoxic conditions lead to a transition from an immunoprivileged to immunogenic phenotype and rendered allogeneic MSCs susceptible to leukocyte mediated cytotoxicity and rejection.

Bone marrow derived MSCs are inherently considered to be immunoprivileged, mostly because they do not express MHC-II on the surface. Therefore, MSCs can escape allo-immune response after transplantation. MHC-II is expressed constitutively in antigen presenting cells including dendritic cells, mononuclear phagocytes and B cells[37,38]. However, this molecule can also be induced in other cell types by interferon- γ stimulation[3,39,40]. In the current study, normoxic MSCs expressed negligible amount of MHC-II, and the cells were originally immunoprivileged and induced negligible immune reaction when co-cultured with allogeneic leukocyte. However,

after exposure to hypoxia there was a sharp increase in MHC-II levels and the MSCs became immunogenic in the co-culture assays. Our results are in agreement with other studies where MSCs, when exposed to a certain stress or change of environment e.g. treatment with IFN-γ and IL-1β, led to noticeable upregulation of MHC-II which was associated with the increase in immunogenicity of MSCs[39–41].

The life cycle of MHC-II in the cells has been studied extensively – especially its synthesis, antigen loading, activation, transport and storage[42]. However, the turnover of MHC-II protein itself remains largely unexplored. In this regard, the 26S proteasome system is reported to mediate degradation of unwanted or damaged proteins by proteolysis. Previously the role of 26S proteasome in MHC-I antigen processing and presentation has been reported[43]. The inhibition of 26S proteasome can cause a decline in MHC-I antigen processing and presentation. Similarly, the antigen loading for MHC-II has been reported to be highly enhanced through poly-ubiquitination of MHC-II in the lyso-endosomal complexes[44]. However, to the best of our knowledge, the involvement of the 26S proteasome in MHC-II molecule turnover has not been investigated yet. In the current study, when we inhibited 26S proteasome function in normoxic MSCs, the levels of MHC-II increased and the cells became immunogenic. The 26S proteasome assembly is comprised of two subunits: 20S core subunit and 19S regulatory subunit. These two subunits bind together to form an active proteasome complex and degrade unwanted proteins. Our data demonstrate that exposure to hypoxic environment lead to dissociation of 19S and 20S subunits, and downregulation of 26S proteasome activity in MSCs. Previously, it has been reported that intracellular oxidative stress leads to dissociation of 20S core particle and 19S regulatory particle of 26S proteasome[45]. The dissociation of 26S proteasome system in the current study was associated with accumulation of ubiquitinated MHC-II and loss of

immunoprivilege of MSCs. Furthermore, molecular chaperones are essential for the binding of subunits as well as complex formation of the 26S system. Thence, we investigated various chaperones playing a direct role in the assembly of 26S system. HSP90α markedly decreased in MSCs under hypoxic conditions. HSP90α is among the most abundant proteins in the body, it constitutes 1-2% of cellular proteins[46]. The major role of HSP90α is to bind and fold other proteins into their functional three-dimensional structures. It is also reported to play a role in assembling the 26S proteasomal machinery[47,48]. HSP90α binds to 19S lid-subunits and mediates association of 19S and 20S to form a functional 26S proteasome assembly. However, the role of HSP90α in the immunoprivilege of MSCs has not been investigated yet. In the current study, when we blocked HSP90α in normoxic MSCs, the cells became immunogenic. On the other hand, overexpression of HSP90α in hypoxic MSCs maintained 26S activity and preserved immunoprivilege of MSCs. Therefore, HSP90α downregulation in hypoxic MSCs is associated with inactivation of 26S proteasome and loss of immunoprivilege of allogeneic MSCs. Interestingly; some studies have previously reported increases in HSP90α levels under hypoxic conditions. Almgren and Olson found upregulation of HSP90 in vascular tissue exposed to hypoxic environment[49]. In H9c2 cells, treatment with CoCl2 (a hypoxia mimetic agent) at 50 to 200 µM concentrations prevented serum and glucose deprivation induced decrease in HSP90[50]. Therefore, hypoxia induced alterations in HSP90α seem to be cell specific as well as dependent upon dose and duration of hypoxic conditions.

The present study suggests that 26S proteasome mediated degradation of MHC-II maintains absence of MHC-II on MSC's surface that preserves immunoprivilege of allogeneic MSCs, (Supplementary Figure 2.6). The exposure to hypoxic environment led to dissociation as well as inactivation of 26S proteasome assembly and loss of immunoprivilege of MSCs. These

observations provide unique insights into the mechanisms responsible for hypoxia induced loss of immunoprivilege of MSCs. Our data also suggest that maintaining the optimal level of HSP90α preserves the immunoprivilege of allogeneic MSCs under hypoxic conditions. More significantly our studies also reveal that hypoxia induced loss of immunoprivilege is not only limited to rodent MSCs, human bone marrow derived allogeneic MSCs are also susceptible to hypoxia induced immune switch from immunoprivileged to immunogenic state. Further, we have shown in a definitive manner, that therapeutic interventions are possible through genetic modification (overexpression) of HSP90α, which can be targeted to preserve immunoprivilege of MSCs under hypoxic conditions. Therefore, our study may help in increasing the success rates of ongoing allogeneic MSCs based clinical trials and allowing a better planning for future trials.

2.5 Material and methods

Experimental animals: Unrelated Sprague-Dawley rats were used for the isolation of MSCs from the bone marrow, and for the isolation of splenic leukocytes. The study protocols were approved by the Animal Care Committee of the University of Manitoba and conformed to the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Rat MSCs isolation and characterization: Rat MSCs were isolated from the femurs and tibias as previously described[4,12]. After connective tissue around the bones was removed and both ends snipped, the bone marrow plugs were flushed with Dulbecco's Modified Eagle's Medium supplemented with 15% fetal bovine serum (FBS), 100 units/ml penicillin G and 0.1 mg/ml streptomycin. Cells were plated and cultured in the same medium. Next day, the medium was changed and non-adherent cells were discarded. The medium was replaced every 3 days, and the cells were sub-cultured when confluency exceeded 90%. To characterize the cells, flow

cytometry was performed– the cells which were $CD44^+$ and $CD29^+$ (Santa Cruz) and negative for hematopoietic progenitors markers – $CD45^-$ and $CD34^-$ (Santa Cruz) were used for further experiments[4,12].

Human mesenchymal stem cells: Bone marrow derived human MSCs (hMSCs) were purchased from Lonza (PT 2501 CA10064-080). All the human MSCs related *in vitro* studies were approved by the University of Manitoba's Research Ethics Board.

Experimental treatments:

Hypoxia treatment was employed for 24 h, the culture plates were placed in hypoxia chamber (oxygen level regulated at 0.0%-0.1%) in the incubator (Biospherix hypoxia chamber). To block 26S proteasome MSCs were treated with its specific inhibitor MG132 (2μM and 5μM) for 24h. To inhibit HSP90α activity in normoxic MSCs, the cells were treated with SNX2112 (0.5μM, 1μM and 2μM) for 24h.

Western blot: The protein levels for MHC-II, NOB1, BLM10, HSP90α, HSP90β and HLA-DRα were measured by Western blot using species specific antibodies. Briefly, total protein levels were measured by Bradford method and 40 μg of protein was loaded onto SDS-PAGE. Following electrophoresis, proteins were transferred to PVDF membrane and incubated with appropriate primary and secondary antibodies. The membranes were developed using X-ray film and bands were quantified using Quantity One software for densitometry.

Immunoprecipitation: The immunoprecipitation procedures were carried out according to the manufacturers' guidelines (Santa Cruz Biotechnology). Briefly, total cell lysates were prepared from the cells in different groups. The lysates were then precleared using appropriate preclearing matrix. To form IP antibody-IP matrix complex, 40-50 μl of suspended (25% v/v) IP matrix, 1-5 μg of IP antibody in 500 μl of PBS were incubated overnight at 4° C. 300 μg of total cellular

protein was transferred to the pelleted matrix and incubated overnight at 4°C. The samples were then analyzed using electrophoresis as described for the Western Blotting procedure and probed with primary antibodies and secondary antibodies. The membranes were developed using X-ray film and bands were quantified using Quantity One software for densitometry.

Two dimensional blue native polyacrylamide gel electrophoresis assay: The two dimensional (2D) blue native polyacrylamide gel electrophoresis (BN-PAGE)/SDS-PAGE assay was performed to study the association of proteasome subunits 19S and 20S. The first dimension BN-PAGE and 2nd dimension SDS-PAGE were performed as described previously[21]. Briefly, the cell lysates were prepared by sonication in 20mM Bis-tris, 500 mM ε-aminocaproic acid 20 mM NaCl, 2 mM EDTA (pH 8.0) and Glycerol 10% supplemented with 1X Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and 1.5% *n*-Dodecyl β-D-maltoside (Sigma). The proteins were then separated in 4-15% gradient blue-native polyacrylamide gel. The gel strips (individual lanes) were carefully excised including the 3.2% stacking gel and immersed in the Laemmli sample buffer containing freshly prepared DTT (54mg/ml). The gel slices were incubated in sample buffer for 30mins at room temperature (RT) and then the proteins in the gel slices were separated in 2nd dimension SDS-PAGE and immunoblotted using specific antibodies for Sug1 (one of the constituents of 19S subunit) and α3 (one of the constituents of 20S subunit).

Immunocytochemistry: MSCs were seeded onto sterile coverslips, and allowed to grow till 60% confluency. The plated cells were fixed with 4%PFA and permeabilized using 0.2% Triton X in PBS at room temperature. The cells were then stained with respective primary and secondary antibodies and phalloidin (for F-actin, Invitrogen). Thereafter, the cells were counter stained with DAPI for nuclei. The cells were imaged using Cytation 5 imaging system (BioTek Instruments).

Reverse-Transcription PCR: Total RNA was isolated using high pure RNA isolation kit (Roche) and transcribed to cDNA using cDNA kit (Thermo scientific) for RT-PCR. The following PCR primers were used: BLM-10- forward primer: CGTGTGGATGGGAAGAAGTT, reverse primer: CAGAAGGCGGCTTGTTAAAG; HSP90α forward primer: CAACCAATGGAGGAAGAGGA, reverse primer: AGCGTCTGAGGAGTTGGAAA; NOB1- forward primer: GATGGGTCTGAGAACCTGGA, reverse primer: CTCCTCCCTTCCATCAATCA. HSP90β1- forward primer: GTCGGGAAGCAACAGAGAAG, reverse primer: CTGGTATGCTTGTGCCTTCA. The PCR products were loaded onto 1% agarose gel after mixing the samples with 6X DNA loading buffer. The gels were imaged using ChemiDoc system (BIO-RAD).

MHC-II siRNA inhibition in MSCs: We employed siRNA to block MHC-II in rat MSCs, for that we used siGENOME Rat RT1-Bb (Cat # M-102315-00-0005), and as a control siGENOME siRNA (Cat #1D-001206-13-050) from Dharmacon. We used FuGENE® HD Transfection Reagent from Promega. Briefly, 100µM stock solution of siRNA was prepared. One million MSCs were seeded per well and 80 pmoles of both targeting and non-targeting siRNA were added after incubating the siRNA with Fugene HD for 10 minutes. This was followed by addition of siRNA-Fugene complex to each well and incubation in the CO_2 incubator for 18 hours. Next day the cells were used to perform further experiments.

26S proteasome activity assay: To determine 26S proteasome activity, we measured deubiquitinating activity of 19S and proteolysing activity of 20S by fluorescent substrates. The deubiquitinating activity of 19S was measured by using Ubiquitin-Rhodamine 110 (Boston Biochem) at a concentration of 1µM. The fluorescent intensity of each well was read at 485 nm

(excitation) and 535 nm (emission) for 1 hour with reading interval of 5 minutes. The 20S subunit activity was determined by a kit purchased from Cayman Chemicals (10008041).

Mixed leukocyte mediated cytotoxicity: To measure leukocyte mediated cytotoxicity in rat MSCs, the leukocytes were isolated from spleen (SD rat) using HISTOPAQUE 1083 (Sigma-Aldrich) and co-cultured with allogeneic MSCs in the ratio of 10:1 as described in our previous studies[12,51]. After 72 h of co- culture, leukocyte-mediated cytotoxicity in the MSCs was assessed by measuring the lactate dehydrogenase (LDH) released from the damaged MSCs (LDH Cytotoxicity Detection Kit; Clontech).

To measure leukocyte mediated cytotoxicity in human MSCs, hMSCs were co-cultured with leukocytes isolated from peripheral blood derived from healthy individuals at a ratio 1:10 for 72 h.

Leukocyte proliferation: Leukocytes were co-cultured with allogeneic normoxic and hypoxic MSCs (10:1). The leukocyte proliferation was assessed after co-culture with MSCs by flow cytometric analysis (BD Accuri). Briefly, after 72 h of co-culture, the leukocytes in the supernatant were collected and centrifuged at 1000rpm for 5minutes. The pellet was washed three times using PBS, and suspended in 100µl of cold PBS. After fixing with 5ml of 70% ice cold ethanol, the cells were treated with RNase (20µg/ml) for 30 minutes. The leukocytes were then stained with propidium iodide (PI, 5µg/ml) for 5 minutes at room temperature, and analyzed using flow cytometry. To measure leukocyte proliferation cell cycle analysis was done by counting the number of cells entering S-phase (proliferating phase) and G2/M phase from G0/G1 phase (resting cells) of the cell cycle. The leukocyte proliferation was also measured by a Cell Proliferation Assay Kit (Biovision Inc. Cat # K301).

Regulatory T cells measurement: The number of $CD4^+CD25^+$ regulatory T (Treg) cells were counted in total leukocyte population after 72 hr of co-culture with allogeneic MSCs using BioRad Treg cell estimation kits for rat (Cat # DC040) and human (Cat # DC027) by flow cytometry.

HSP90α overexpression: Rat MSCs were transduced with lentiviral vector encoding the genes for Hsp90α and GFP (Vector builder, LVS-VB160907-1147sms) at 25 multiplicity of infection (MOI) for 24 h, followed by second induction dose of 25 MOI, the next day. The lentiviral vector encoding only GFP (empty vector: Vector Builder, LVL-VB160109-10005) was used as control. Transduction efficiency was assessed by fluorescence microscopy. To generate stable HSP90α overexpressing MSCs, cells were grown under selection media containing Puromycin at a dose of 2.5µg/ml. The HSP90α levels were assessed using western blot analysis.

Statistical analysis: Experimental values are expressed as mean ± SD. Comparison of mean values between various groups was performed by one-way-analysis of variance (one-way ANOVA) followed by multiple comparisons by Tukey test. P value <0.05 was considered to be significant.

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Conflict of interest

Authors declare no conflict of interest.

Author Contributions

E.A.R and S.D conceptualized the study; E.A.R and S.D designed the experiments; E.A.R, G.L.S, N.S and M.M carried out the experiments, acquired and analyzed the data; E.A.R, N.S, G.L.S and S.D. interpreted the data and performed statistical analyses; M.G.S designed 2D gel electrophoresis, acquired, analyzed and interpreted 2D gel data, prepared the 2D gel-based figure and wrote the 2D gel-based results; E.A.R, G.L.S, and S.D drafted the manuscript. All the authors have read and approved the final version of the manuscript.

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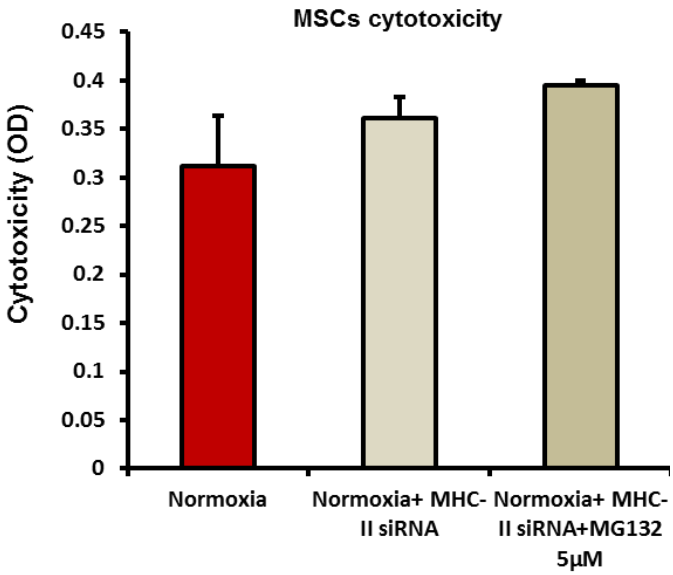
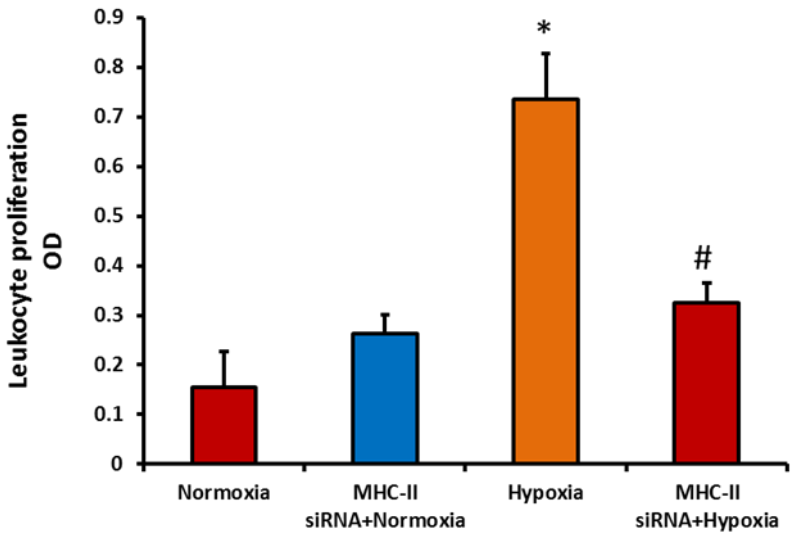
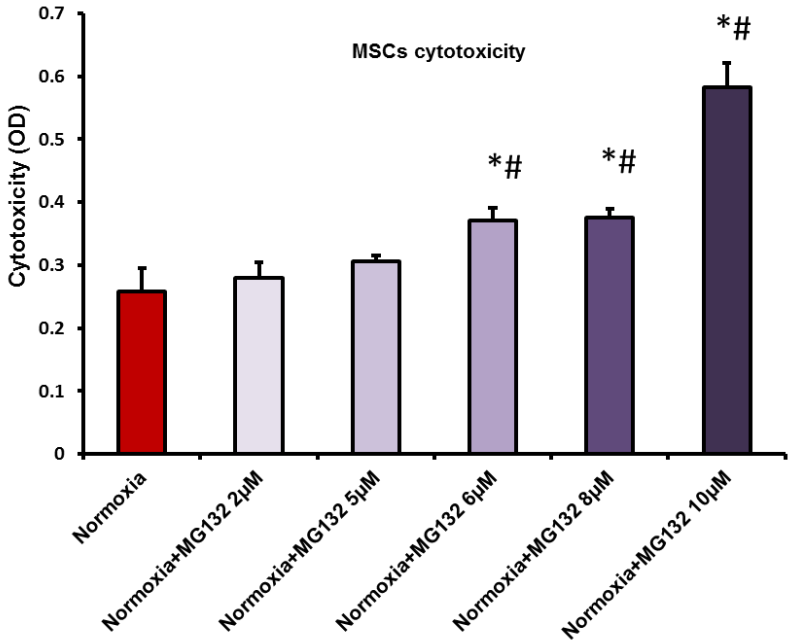


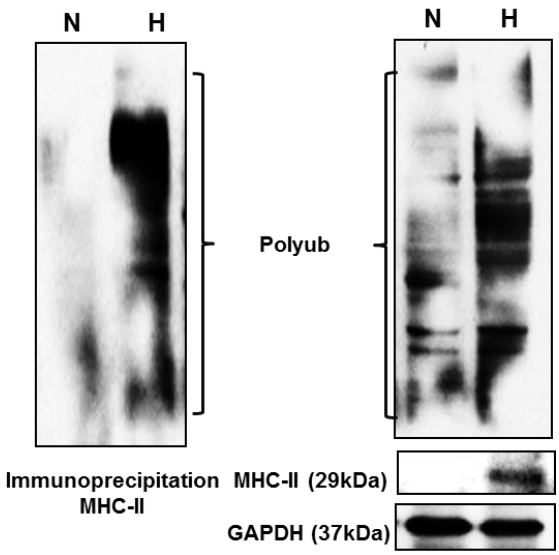
Supplementary figure 2.1: Blocking MHC-II (with or without 26S inhibition) in normoxic MSCs does not affect immunoprivilege. Rat bone marrow derived normoxic MSCs (with or without siRNA against MHC-II; and MG132 at 5 µM) were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. Leukocyte mediated cytotoxicity (LDH release) did not show any significant change in normoxic MSCs vs. MHC-II inhibited normoxic MSCs vs. MHC-II inhibited MG132 treated normoxic MSCs. (n=8).



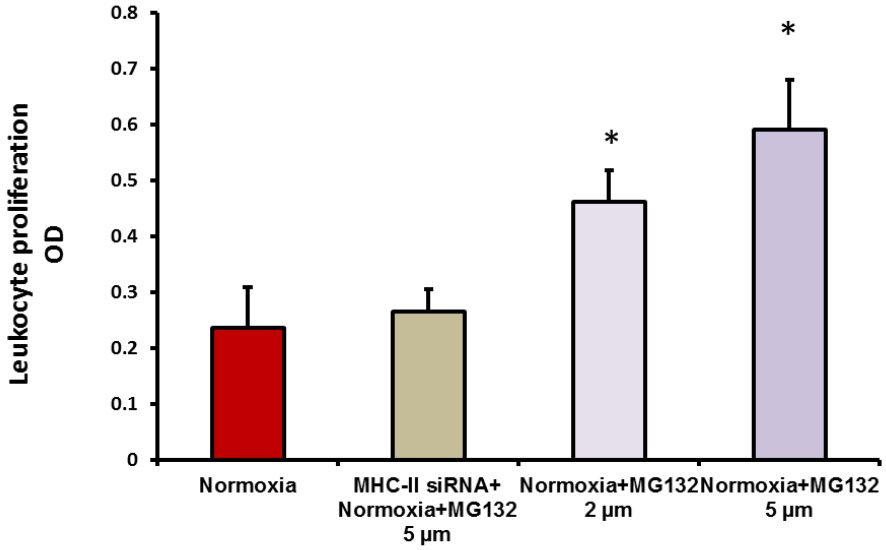
Supplementary figure 2.2: Blocking MHC-II in hypoxic MSCs downregulated allogeneic leukocyte proliferation. Rat bone marrow derived normoxic MSCs and hypoxic MSCs (with or without siRNA against MHC-II) were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. The effect of MSCs on leukocyte proliferation was measured by proliferation assay kit (Biovision Inc.). The rate of leukocyte proliferation increased after co-culture with hypoxic MSCs compared to normoxic cells, siRNA mediated inhibition of MHC-II in hypoxic MSCs decreased leukocyte proliferation after the co-culture. (n=10). *$p<0.05$ compared to leukocytes co-cultured with normoxic MSC; #$p<0.05$ compared to leukocytes co-cultured with hypoxic MSCs.



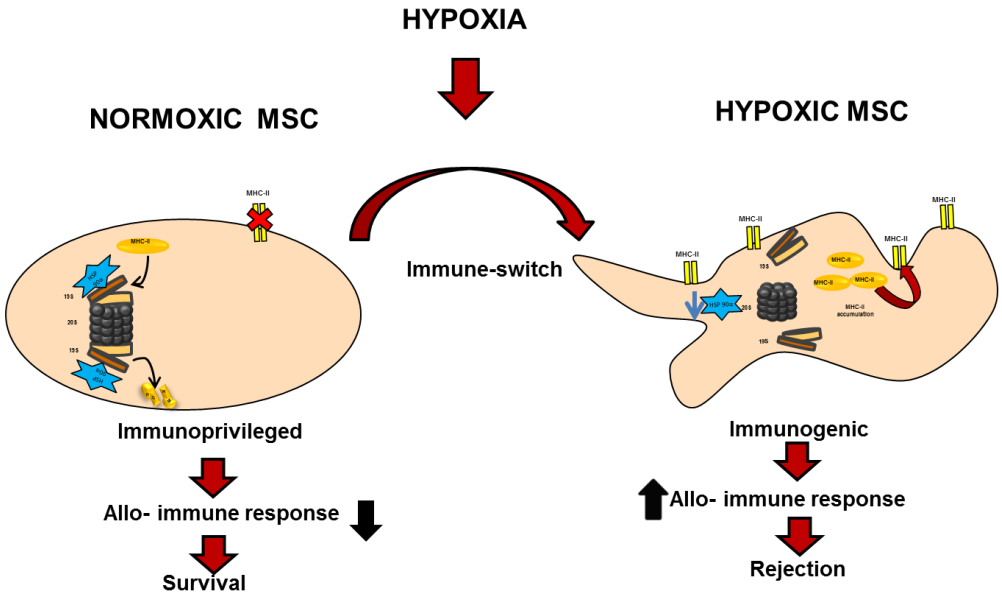
Supplementary figure 2.3: MG132 dose response in MSCs. MSCs were treated with MG132 (26S proteasome inhibitor) at different concentrations 2μM, 5μM, 6μM, 8μM and 10μM for 24h. LDH release was measured to assess cytotoxicity caused by MG132 in MSCs. MG132 at a dose of 2μM and 5μM was found to be safe and effective. These concentrations were used for further experiments (n=8). *$p<0.05$ compared to normoxic MSC; #$p<0.05$ compared to normoxia +5μM of MG132 group.



Supplementary figure 2.4: 26S proteasome regulates MHC-II levels and preserves immunoprivilege of MSCs. Immunoprecipitation (IP) analysis was performed in rat normoxic and hypoxic MSCs to determine the involvement of 26S proteasome in the degradation of MHC-II. IP data revealed a significant accumulation of ubiquitinated MHC-II protein in hypoxic MSCs. IP was performed with MHC-II antibody, and blotting was performed with polyubiquitin antibody. (n=3).



Supplementary figure 2.5: Blocking 26S proteasome in normoxic MSCs increased leukocyte proliferation. Rat bone marrow derived normoxic MSCs (with or without MG132) were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. The effect of MSCs on leukocyte proliferation was measured by proliferation assay kit (Biovision Inc.). The rate of leukocyte proliferation did not change after co-culture with MHC-II inhibited MG132 (5µM for 24 h) treated normoxic MSCs compared to normoxic cells. However, presence of only MG132 (2 µM and 5µM for 24 h) increased leukocyte proliferation after the co-culture. (n=10). *$p<0.05$ compared to leukocytes co-cultured with normoxic MSCs.



Supplementary figure 2.6: Schematic diagram depicts MHC-II regulation and immunoprivilege of MSCs. In normoxic MSCs MHC-II degradation by 26S preserves immunoprivilege of cells. Exposure to hypoxic environment leads to inactivation of 26S proteasome, and accumulation of MHC-II and loss of immunoprivilege of allogeneic MSCs

Chapter III: Role of 19S proteasome in the immunoprivilege of MSCs

Rational and Hypothesis:

In Chapter II, we reported that in normoxic MSCs, 26S proteasome mediated degradation of MHC-II preserves their immunoprivilege. We found that exposure to hypoxia disrupted binding between the19S regulatory subunit and the proteolytic 20S subunit, which is required to form functional 26S proteasome in MSCs. It has been previously reported that 19S proteasome ATPases, play non-proteolytic role by activating the expression of various genes. 19S proteasome has been reported to regulate transcription of several genes at different levels, including initiation and elongation steps, chromatin remodeling and posttranslational repressive or suppressive modifications. Further, Sug1 is the most studied 19S ATPase subunit that actively interacts with transcription factors to control gene transcription in a degradation independent fashion. In this chapter, we have investigated the role of Sug1 in regulating the expression of MHC-II in MSCs at the transcription level.

Hypothesis:

Hypoxia induced increase in Sug1 in MSCs leads to upregulation of MHC-II, loss of immunoprivilege and poor survival of transplanted cells in the ischemic heart.

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Hypoxia induced increase in Sug1 leads to poor post-transplantation survival of allogeneic mesenchymal stem cells

Ejlal Abu-El-Rub[1], Niketa Sareen[1], Glen Lester Sequiera[1], Hania I. Ammar[2], Weiang Yan[1], Asmaa M. ShamsEldeen[2], Ilan Rubinchik[1], Heba S. Shokry[2], Laila A. Rashed[2], Meenal Moudgil[1], Sanjiv Dhingra[1]

[1]Regenerative Medicine Program, Institute of Cardiovascular Sciences, St. Boniface Hospital Albrechtsen Research Centre, Department of Physiology and Pathophysiology, College of Medicine, Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada

[2]Department of Physiology and Biochemistry, Faculty of Medicine, Cairo University, Egypt

Short Title: Immunogenicity of mesenchymal stem cells

Correspondence:
Sanjiv Dhingra, PhD, FCVS
Regenerative Medicine Program
Institute of Cardiovascular Sciences,
St. Boniface Hospital Research Centre
R-3028-2, 351 Tache Avenue, Winnipeg, R2H2A6, Canada
Phone: 204-235-3454; Fax: 204-233-6723Email: sdhingra@sbrc.ca

3.1 Abstract

Allogeneic mesenchymal stem cells (MSCs) from young and healthy donors have potential to treat degenerative diseases. However, recent reviews of clinical trials report poor survival rates of transplanted cells in the recipient that in turn dampened the enthusiasm regarding MSC therapies. Increasing evidence now confirm that though initially immunoprivileged, MSCs eventually become immunogenic after transplantation in the ischemic environment and are rejected by host immune system. We performed *in vitro* (in rat and human cells) and *in vivo* (in rat model) investigations to understand the mechanisms leading to immunogenicity of MSCs. The immunoprivilege of MSCs is preserved by the absence of the cell surface immune antigen, MHC-II molecule. Exposure to hypoxia upregulates Sug1 in MSCs and its binding to CIITA (a coactivator of MHC-II transcription) that promotes acetylation and K63 ubiquitination of CIITA leading to its translocation to the nucleus, and MHC-II up-regulation. In both rat and human MSCs, knocking down Sug1 inactivated MHC-II and preserved their immunoprivilege *in vitro* and *in vivo*. Therefore, the current study provides novel mechanisms of post-transplantation loss of immunoprivilege and poor survival of MSCs.

3.2 Introduction

Mesenchymal stem cells (MSCs) derived from bone marrow are considered to be a promising cell type for regenerative therapies for various acute and chronic disorders such as cardiovascular, neurodegenerative, hepatic and autoimmune conditions[1–5]. MSCs have long been reported to be immunoprivileged, and the immunoprivilege allowed transplantation of allogeneic (donor derived) MSCs between mismatched donors-recipients without the risk of immune rejection[6]. These properties of MSCs conceptualized the potential of "off-the-shelf" therapies consisting of universal donor derived young and healthy cells grown in culture[7,8]. In fact, several studies have reported the advantages of allogeneic MSCs from young and healthy donors over autologous MSCs from aged subjects, including their excellent regenerative potential and immediate availability for application [8,9]. However, the long-term follow-ups of animal studies and clinical trials revealed that allogeneic MSCs were able to exert therapeutic effects in the transplanted areas only for a short period of time, and ultimately the benefits were lost. A potential limitation of MSCs based therapies is that transplanted cells do not persist in the host tissue [10,11].

Increasing evidences now confirm that though initially immunoprivileged, the MSCs eventually become immunogenic after transplantation in the stressful environment of diseased tissues and are rejected by the host immune system [12–16]. The poor survival and decline in the MSC's mediated benefits over time has dampened the enthusiasm regarding allogeneic MSCs based regenerative therapies. In this regard, several studies have reported the mechanisms of immunoprivilege of MSCs and MSCs mediated beneficial effects. However, the mechanisms of

loss of immunoprivilege of MSCs after transplantation in stressful environment of diseased tissues or organs have not yet been established. Understanding these mechanisms would help in planning strategies to prevent rejection, improve survival of transplanted cells and preserve the benefits of allogenic MSCs based therapies for longer periods. Hypoxia or ischemia is a hallmark of diseased or injured tissues[17]. Previous studies have reported that allogeneic MSCs after exposure to hypoxia under *in vitro* conditions became immunogenic [18,19]. However, the mechanisms of hypoxia induced immunogenicity of MSCs after transplantation in the ischemic tissues of diseased organs have not yet been studied thoroughly. Therefore, in the current study, we performed *in vitro* (in rat and human cells) and *in vivo* (in a rat model) investigations to understand the mechanisms of immune switch in the phenotype of MSCs from immunoprivileged to immunogenic state under hypoxic or ischemic conditions. The current study also suggests that therapeutic interventions are possible through modifications of MSCs to maintain immunoprivilege and improve survival of transplanted allogeneic MSCs in the host tissue.

3.3 Results:

3.3.1 Exposure to hypoxia activates CIITA and increases immunogenicity of MSCs:

MSCs are inherently immunoprivileged due to the absence of cell surface immune antigen-major histocompatibility complex class II (MHC-II) molecules. MHC-II molecules expressed on the allograft provide signals to alert the host immune system to initiate immune response against transplanted organs or cells. MHC-II biosynthesis is regulated by class II transactivator (CIITA), a transcriptional co-activator[20,21]. CIITA is considered as a master regulator of MHC-II gene transcription[20–22]. In the current study, we found that exposure to hypoxia led to increased

CIITA levels (Fig. 3.1A). Under normal conditions CIITA is present in the cytoplasm, upon activation it translocates to nucleus and binds to transcription factor RFX5 and up regulates MHC-II transcription [23,24].We found a significant increase in nuclear accumulation of CIITA in hypoxic MSCs as compared to normoxic cells (Fig. 3.1B). The hypoxia induced CIITA activation and translocation to the nucleus was associated with increased MHC-II levels (Fig. 3.2A) and immunogenicity of MSCs. To investigate immunogenicity, we co-cultured MSCs with allogeneic leukocytes for 72 h and evaluated leukocytes mediated cytotoxicity in MSCs by measuring the amount of lactate dehydrogenase (LDH) released, the level of cytotoxicity was found to be significantly greater in hypoxic MSCs as compared to normoxic cells (Fig. 3.2B). MSCs are known to have the ability to downregulate leukocyte proliferation and suppress the immune response [25,26]. We measured leukocyte activation and proliferation by counting the number of cells entering S-phase and G2/M phase from G0/G1 phase of the cell cycle by flow cytometry. There was a significant increase in leukocyte proliferation after co-culture with hypoxic MSCs compared to normoxic cells (Fig. 3.2C). The number of resting leukocytes in G0/G1 phase significantly decreased after co-culture with hypoxic MSCs (Fig. 3.2C). At the same time the number of leukocytes entering S phase (proliferating phase) increased after co-culture with hypoxic MSCs (Fig. 3.2C).

MSCs are also reported to promote immune tolerance by inducing phenotype change from cytotoxic T cells toward immunosuppressive regulatory T (Treg) cells [27]. Treg cells can suppress the proliferation of cytotoxic T cells and promote immune suppression. We counted the number of $CD4^{+}CD25^{+}$ Treg cells in a mixed leukocyte population after co-culture with allogeneic MSCs by flow cytometry. The Treg cell number decreased after co-culture with hypoxia exposed MSCs

compared to normoxic cells (Fig. 3.2D). Therefore, these studies suggest that exposure to hypoxia lead to activation of CIITA and loss of immunoprivilege of allogeneic MSCs.

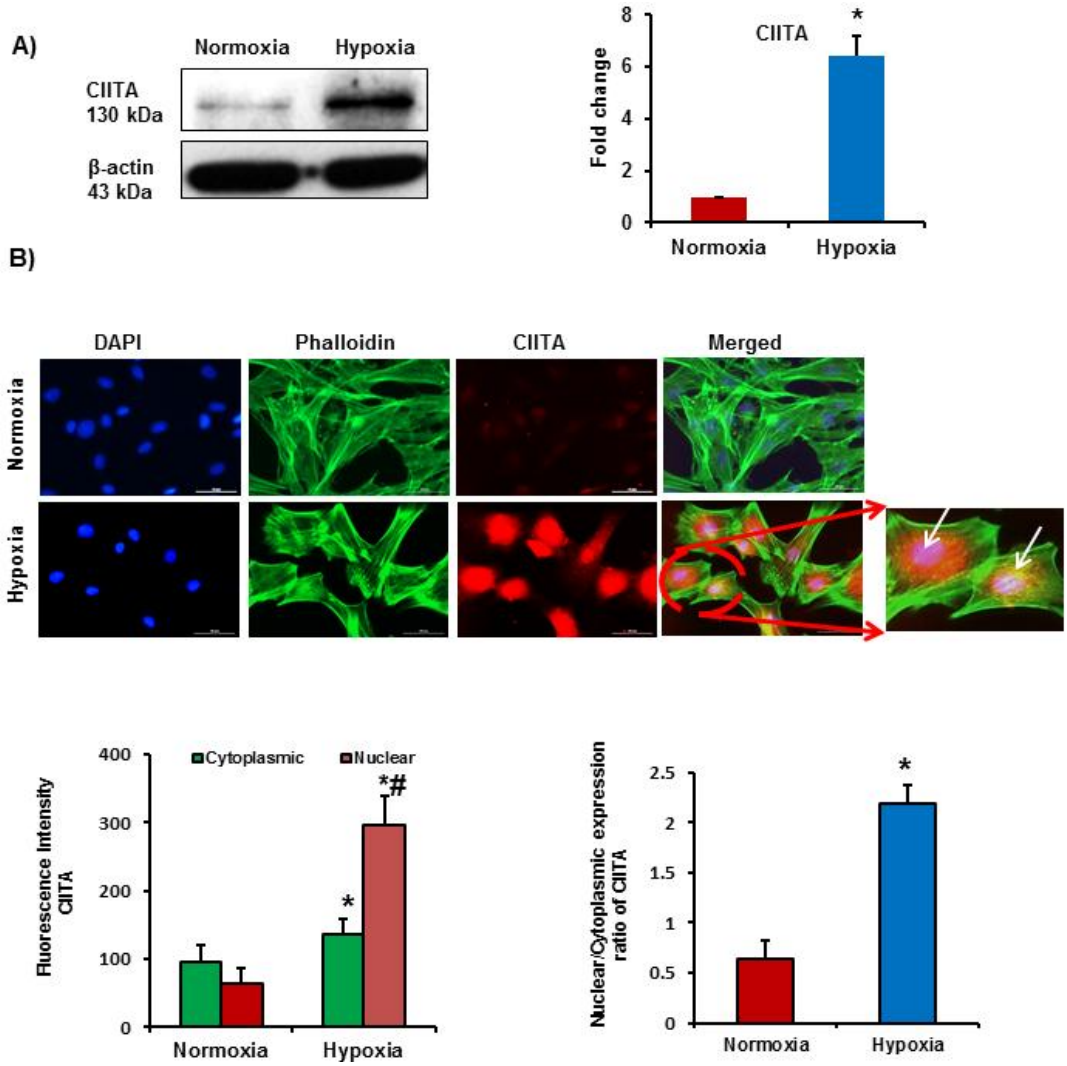

Figure 3.1: Exposure to hypoxia upregulates CIITA in MSCs. (A) Rat bone marrow derived MSCs were exposed to hypoxia for 24h. CIITA levels as measured by Western blot increased in hypoxic MSCs vs. normoxic MSCs. n=3. **(B)** Immunofluorescence images showed a significant increase in the expression of CIITA in hypoxic MSCs vs. normoxic cells. Also, the localization of CIITA increased in nucleus compared to cytoplasm in hypoxic MSC. n=6. *p<0.05 compared to normoxic MSC. #p<0.05 compared to cytoplasmic fraction. Each experiment was repeated 3-4 times.

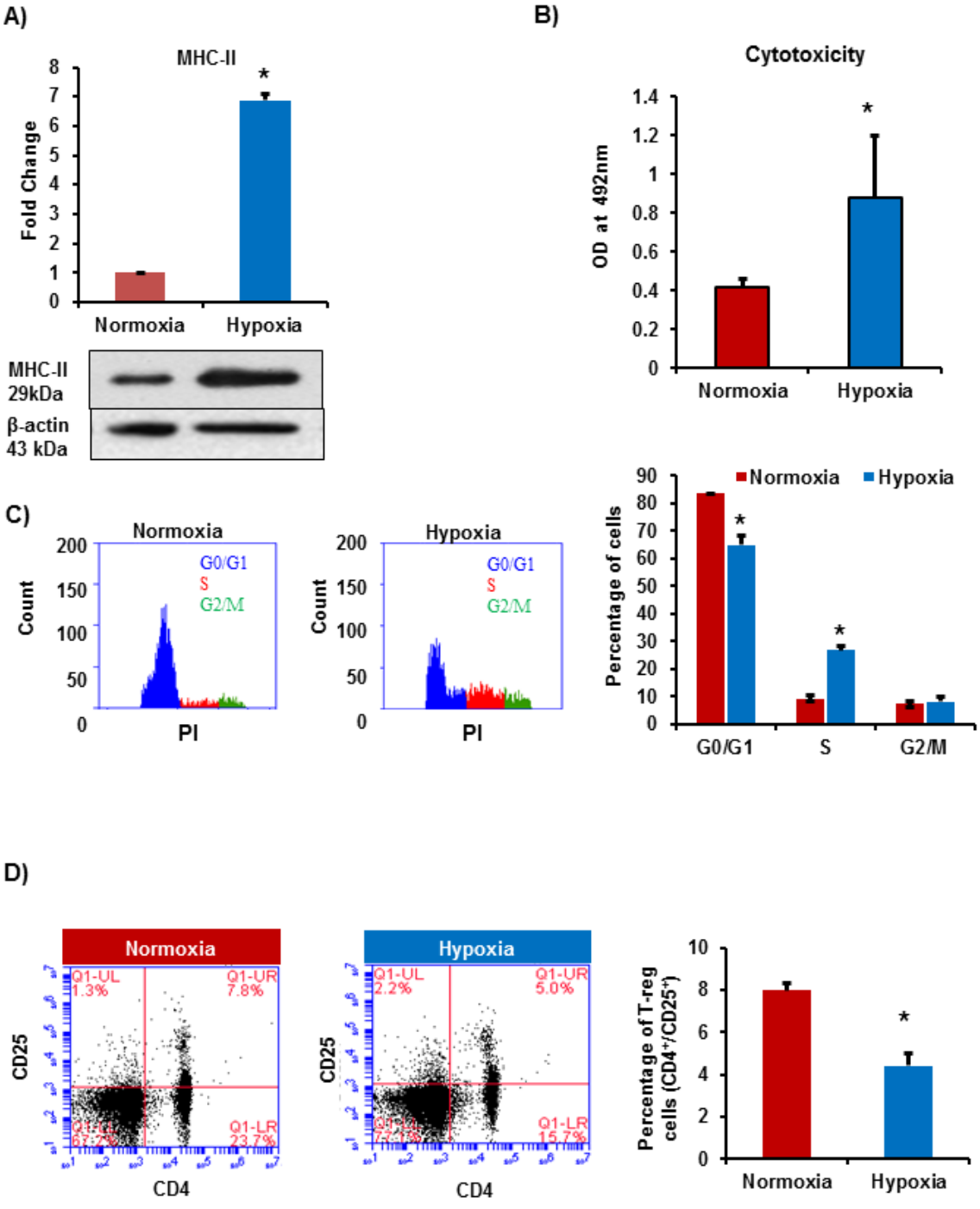
A)
MHC-II
Fold Change
Normoxia
Hypoxia
MHC-II
29kDa
β-actin
43 kDa
B)
Cytotoxicity
OD at 492nm
Normoxia
Hypoxia
C)
Normoxia
Hypoxia
Count
PI
G0/G1
S
G2/M
Percentage of cells
D)
Normoxia
Hypoxia
CD25
CD4
Q1-UL
1.3%
Q1-UR
7.8%
Q1-LR
23.7%
Q1-UL
2.2%
Q1-UR
5.0%
Q1-LR
15.7%
Percentage of T-reg cells (CD4+/CD25+)

Figure 3.2: Exposure to hypoxia upregulates immunogenicity of MSCs. (A) Rat bone marrow derived MSCs were exposed to hypoxia for 24h. MHC-II levels as measured by Western blot increased in hypoxic MSCs vs. normoxic cells. n=3. **(B-D)** To determine the immunogenicity of MSCs, normoxic and hypoxic MSCs were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h: **(B)** Leukocyte mediated cytotoxicity in MSCs (LDH release) increased significantly in hypoxic MSCs vs. normoxic cells, n=10. **(C)** The effect of MSCs on leukocyte activation and proliferation was determined using PI staining, by assessing the number of cells present in different phases of cell cycle. The % of activated and proliferating leukocytes showed a significant increase in hypoxic MSCs vs. normoxia. n=3. **(D)** The effect of MSCs on Treg cell ($CD4^{+}CD25^{+}$) induction in a mixed leukocyte population was assessed by flow cytometry. The number of Treg cells decreased after co-culture with hypoxic MSCs compared to normoxic MSCs. n=3. *$p<0.05$ compared to normoxic MSC. Each experiment was repeated 3-4 times.

3.3.2 Hypoxia up regulates 19S proteasome "Sug1" which in turn induces activation of CIITA in MSCs:

In the next set of experiments, we investigated the mechanism of hypoxia induced activation of CIITA and MHC-II upregulation. It has been reported that Sug1 which is an ATPase subunit of 19S proteasome also acts as a coactivator and upregulates the expression of several genes [28]. In the current study, we found a significant increase in mRNA and protein levels of Sug1 in hypoxic MSCs (Fig.3.3A). Also, our co-immunoprecipitation data suggest that binding of Sug1 to CIITA increased in hypoxic MSCs (Fig. 3.3B). Interestingly, inhibition of Sug1 using a pharmacological inhibitor (B-AP15, 10μm for 4hr) prevented hypoxia induced upregulation of CIITA and MHC-II (Fig.3.3C). To corroborate these findings, we generated Sug1-knockdown

(Sug1 KD) MSCs cell line using commercially available lentiviral vectors and conducted *in vitro* experiments for further validation. Our western blot and immunostaining data demonstrate that knocking down Sug1 prevented hypoxia induced increase in CIITA and MHC-II (Fig. 3.4, A and B). Therefore, hypoxia induced increase in Sug1 correlated with CIITA and MHC-II upregulation.

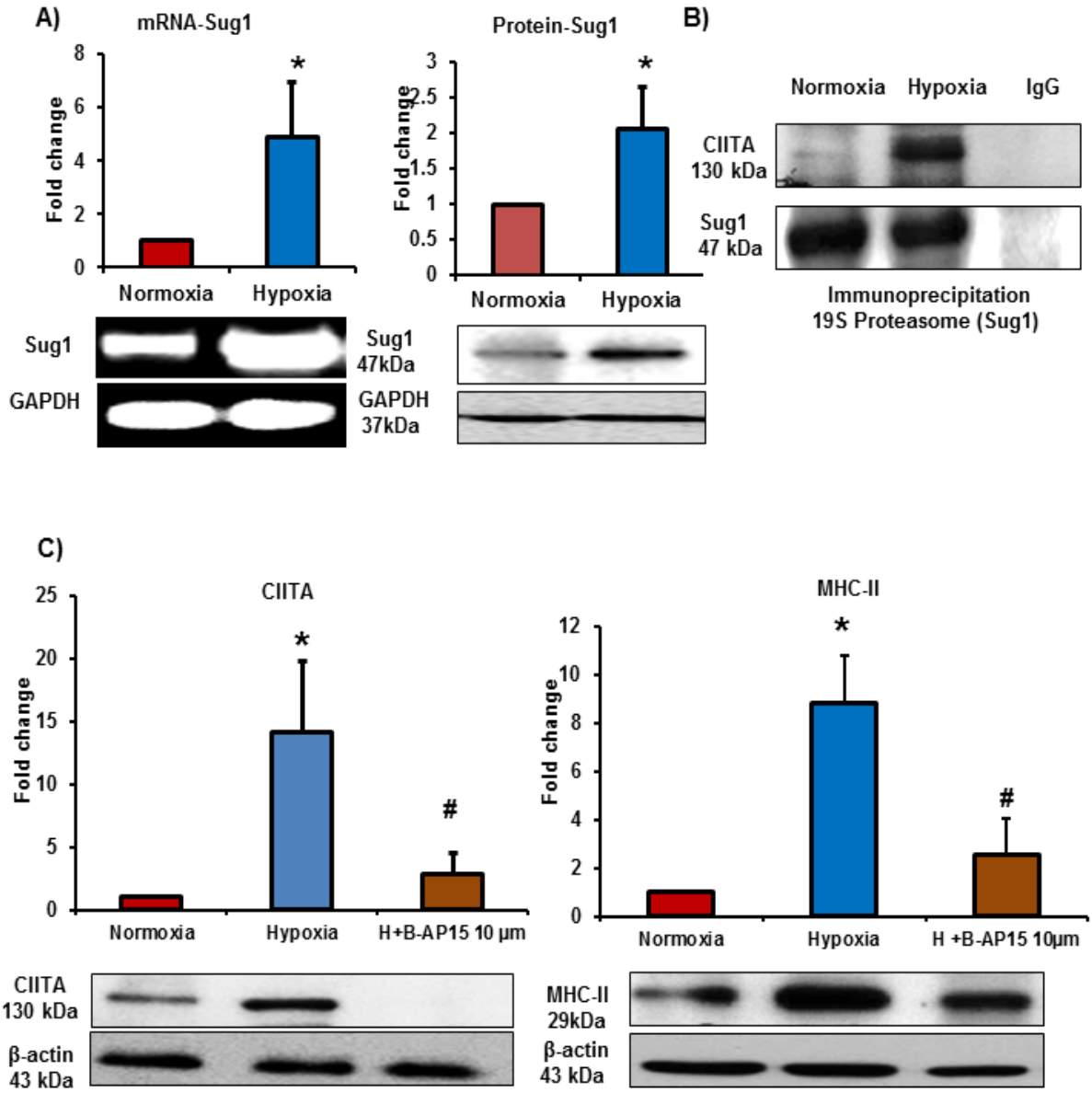

Figure 3.3: Treatment with hypoxia increases Sug1 mRNA and protein levels. (A) Rat MSCs were exposed to hypoxia for 24 h. Sug1 mRNA and protein levels as determined by RT-PCR and Western blot, elevated in hypoxic MSCs vs. normoxic cells. n=3. **(B)** Immunoprecipitation (IP) assay was performed in MSCs to determine the association of Sug1 with CIITA. IP data revealed a significant increase in the binding between Sug1 and CIITA in hypoxic MSCs vs. normoxic cells, (n=4). **(C)** MSCs were treated with Sug1 inhibitor (B-AP15, 10μm for 4h) and then exposed to hypoxia for 24 h. Treatment with B-AP15 prevented hypoxia induced increase in CIITA and MHC-II protein levels as measured by Western blot. n=3. *$p<0.05$ compared to normoxic MSC; #$p<0.05$ compared to hypoxic MSCs. Each experiment was repeated 2-4 times.

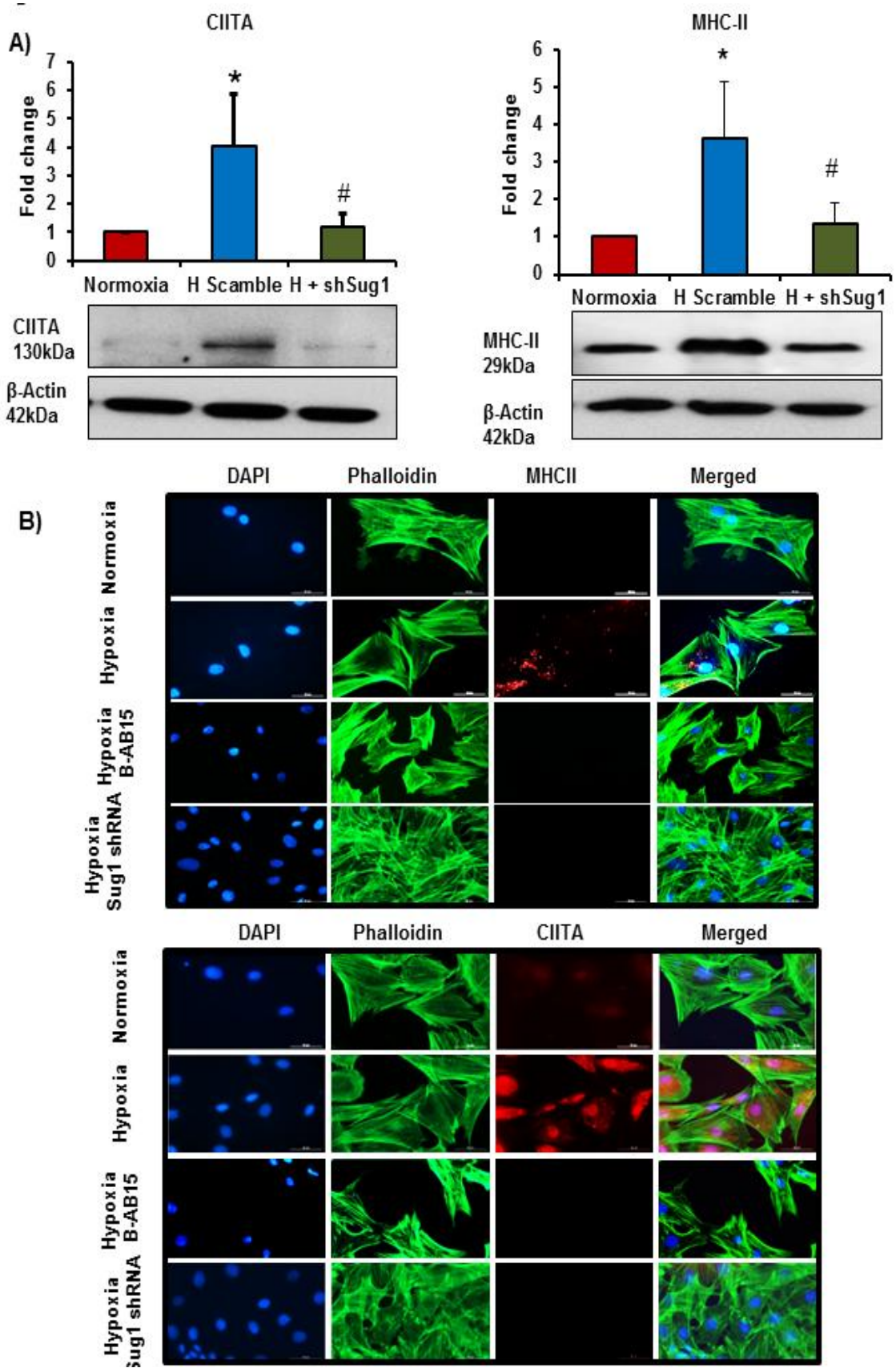
A)
CIITA
Fold change
Normoxia
H Scamble
H + shSug1
CIITA
130kDa
β-Actin
42kDa
MHC-II
MHC-II
29kDa
β-Actin
42kDa
B)
DAPI
Phalloidin
MHCII
Merged
Normoxia
Hypoxia
Hypoxia B-AB15
Hypoxia Sug1 shRNA
DAPI
Phalloidin
CIITA
Merged
Normoxia
Hypoxia
Hypoxia B-AB15
Hypoxia Sug1 shRNA

Figure 3.4: Hypoxia induced increase in Sug1 levels is responsible for CIITA upregulation. **(A)** Sug1 was knocked down in rat MSCs using shRNA lentiviral system and stable Sug1 knock out MSCs (Sug1KO) line was generated. The genetically modified MSCs were exposed to hypoxia for 24 hours. Knocking down Sug1 prevented hypoxia induced increase in CIITA and MHC-II protein levels as measured by Western blot, n=3. **(B)** Immunofluorescence images showed a significant increase in CIITA and MHC-II expression in hypoxic MSCs, and blocking Sug1 either by B-AP15 or shRNA Lentivirus prevented it. n=6. *$p<0.05$ compared to normoxic MSC; #$p<0.05$ compared to hypoxic MSCs. Each experiment was repeated 2-4 times.

3.3.3 Blocking Sug1 restores the immunoprivilege of allogenic MSCs:

To find out if blocking Sug1 would prevent hypoxia induced loss of immunoprivilege in MSCs, we co-cultured MSCs with allogeneic leukocytes for 72 h and assessed the level of cytotoxicity induced by leukocytes in MSCs. Both pharmacological and shRNA mediated inhibition of Sug1 prevented leukocytes mediated cytotoxicity in hypoxic MSCs (Fig. 3.5A). The ability of hypoxic MSCs to suppress the activation and proliferation of leukocytes was increased in Sug1 loss of function cells (Fig. 3.5B). Also, Sug1 KD MSCs were able to induce Treg cell number even following hypoxia (Fig. 3.5C). These findings collectively provide evidence that hypoxia induced upregulation of Sug1 plays a definitive role in upregulating immunogenicity of MSCs through the activation of CIITA.

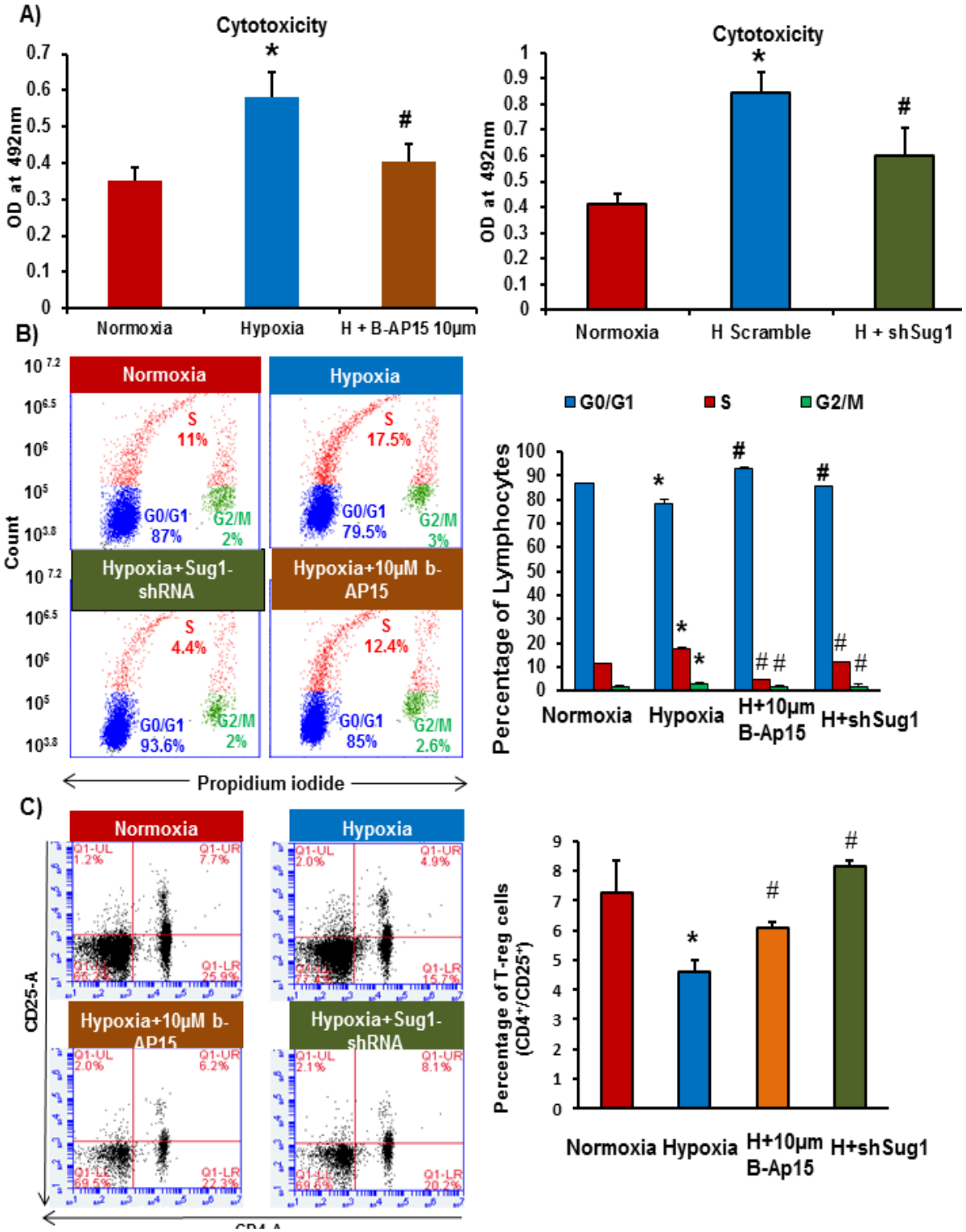

Figure 3.5: Blocking Sug1 preserved immunoprivilege of allogeneic MSCs under hypoxic conditions. (A-C) To determine the immunogenicity of MSCs, normoxic MSCs, hypoxic MSCs,

B-AP15 MSCs+hypoxia, and Sug1 KO+hypoxia were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. **(A)** LDH levels increased significantly in hypoxic MSCs, pharmacological and genetic inhibition of Sug1 prevented hypoxia induced increase in LDH levels, (n=10). **(B)** Leukocyte proliferation was determined using PI staining, by assessing the number of cells present in different phases of cell cycle. Exposure to hypoxia increased the % of proliferating leukocytes compared to normoxic group. Sug1 inhibition suppressed the hypoxia induced activation and proliferation of leukocytes, n=3. **(C)** Effect of MSCs on Treg cell ($CD4^+CD25^+$) induction in a mixed leukocyte population was assessed by flow cytometry. The number of Treg cells decreased after co-culture with hypoxic MSCs, blocking Sug1 with either B-AP15 or shRNA Lentivirus increased Treg cell number, n=3. *$p<0.05$ compared to normoxic MSC; #$p<0.05$ compared to hypoxic MSCs. Each experiment was repeated 2-3 times.

3.3.4 Sug1 activation of CIITA in hypoxic MSCs is mediated through posttranslational modifications:

The posttranslational modifications including acetylation, phosphorylation and ubiquitination promote CIITA activation and stabilize its nuclear localization to initiate MHC-II transcription [29,30]. We wanted to explore the role of hypoxia induced upregulation of Sug1 in acetylation, phosphorylation and/or monoubiquitination events leading to CIITA activation. Our immunoprecipitation data demonstrate that there was no change in K48 ubiquitination and phosphorylation of CIITA in normoxic vs. hypoxic MSCs (Fig. 3.6, A, B and E). However, acetylation and K63 ubiquitination increased in hypoxic MSCs as compared to normoxic cells (Fig. 3.6 C-E). Interestingly, knocking down Sug1 prevented hypoxia induced acetylation and K63 ubiquitination of CIITA (Fig. 3.6 C-E). Furthermore, p300/CBP associated factor (PCAF) is

a known histone acetyltransferase (HAT), which has also been recently reported to act as E3 ubiquitin ligase [31–33]. However, the role of PCAF in Sug1 mediated post-translational activation of CIITA has not been explored yet. In the current study, our co-immunoprecipitation data suggest that binding of PCAF to CIITA increased in hypoxic MSCs, interestingly it decreased in Sug1 KD cells (Fig. 3.6D). We also found a sharp increase in CIITA binding to RFX5 in hypoxic MSCs, and knocking down Sug1 prevented it (Fig. 3.6D). RFX5 is a transcription factor that binds to MHC-II promoter and upregulates its transcription. Therefore, PCAF –CIITA binding, acetylation and K63 ubiquitination of CIITA was elevated in hypoxic MSCs that subsequently enhanced the activation of CIITA and its binding with RFX5. On the contrary, in Sug1 KD MSCs there was a downregulation of PCAF-CIITA association that led to decrease in the level of acetylation and K63 ubiquitination of CIITA, prevented its activation and binding to RFX5 (Fig. 3.6, C-E). The immunostaining of MSCs with PCAF antibody showed a profound increase in its expression in hypoxic cells vs normoxic MSCs, whereas in Sug1 KO MSCs, PCAF expression was remarkably reduced (Fig. 3.6F).

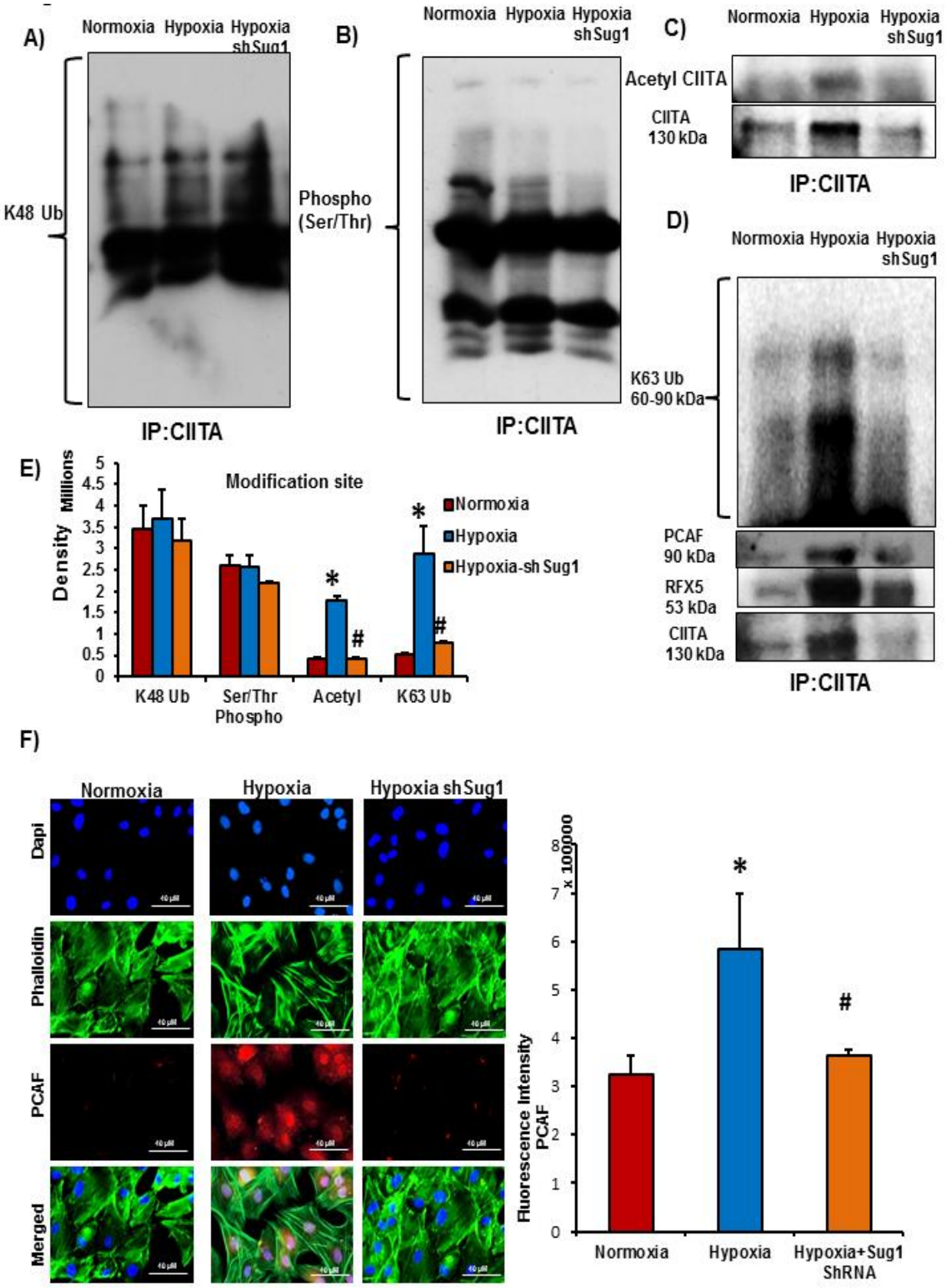
A)
Normoxia Hypoxia Hypoxia shSug1
K48 Ub
IP:CIITA
B)
Normoxia Hypoxia Hypoxia shSug1
Phospho (Ser/Thr)
IP:CIITA
C)
Normoxia Hypoxia Hypoxia shSug1
Acetyl CIITA
CIITA 130 kDa
IP:CIITA
D)
Normoxia Hypoxia Hypoxia shSug1
K63 Ub 60-90 kDa
PCAF 90 kDa
RFX5 53 kDa
CIITA 130 kDa
IP:CIITA
E)
Density Millions
Modification site
Normoxia
Hypoxia
Hypoxia-sh Sug1
K48 Ub
Ser/Thr Phospho
Acetyl
K63 Ub
F)
Normoxia
Hypoxia
Hypoxia shSug1
Dapi
Phalloidin
PCAF
Merged
Fluorescence Intensity PCAF x 100000
Normoxia
Hypoxia
Hypoxia+Sug1 ShRNA

Figure 3.6: Hypoxia induced activation of CIITA by Sug1 is mediated through posttranslational modifications. (A-E) Immunoprecipitation (IP) analysis was performed in rat MSCs to determine the mechanism of Sug1 mediated activation of CIITA through posttranslational modifications. **(A,B, E)** IP data demonstrate that there was no change in K48 ubiquitination and phosphorylation of CIITA in normoxic vs. hypoxic MSCs. (n=4). **(C-E)** IP data demonstrate that acetylation and K63 ubiquitination increased in hypoxic MSCs compared to normoxic cells. Knocking down Sug1 prevented hypoxia induced acetylation and K63 ubiquitination of CIITA. **(D)** Binding of PCAF to CIITA increased in hypoxic MSCs, it was prevented by Sug1 knock down. There was also a significant increase in CIITA binding to RFX5 in hypoxic MSCs, and knocking down Sug1 prevented it. (n=4). **(E)** Histograms depict densitometric analysis and quantification of ubiquitination, phosphorylation and acetylation. (n=4). **(F)** Immunostaining of MSCs with PCAF antibody showed a profound increase in PCAF expression in hypoxic cells vs normoxic MSCs, and Sug1 knockdown prevented it. (n=3). Each experiment was repeated 2-4 times. *$p<0.05$ compared to normoxic MSC; #$p<0.05$ compared to hypoxic MSCs.

3.3.5 Knocking down Sug1 mitigated host immune response and improved survival of transplanted MSCs in the ischemic heart *in vivo*:

In the current study, *in vitro* experiments suggest that allogeneic MSCs undergo an immune shift from immunoprivileged to immunogenic phenotype under hypoxic conditions, and knocking down Sug1 preserved the immunoprivilege of MSCs. Next, we wanted to examine whether knocking down Sug1 would preserve immunoprivilege *in vivo* and prevent rejection of transplanted allogeneic MSCs under ischemic conditions. We transplanted wild type MSCs and Sug1 KD MSCs in a rat model of myocardial infarction, and assessed host immune response, MHC-II expression in transplanted MSCs, and survival of cells in the myocardium. Since hypoxia/ischemia prevail early (the first 1-3 days) after a myocardial infarction [34,35]. Therefore, MSCs were transplanted in the infarcted area, 24 hr after performing the left coronary artery ligation (myocardial infarction). After 5 weeks of cell transplantation host immune response was determined by assessing the infiltration of cytotoxic T cells (Fig. 3.7A). There was a significant increase in the number of $CD4^+$ and $CD8^+$ T cells in MSCs transplanted hearts compared to sham and MI group. However, the number of $CD4^+$ and $CD8^+$ T cells decreased significantly in Sug1 KD MSCs transplanted hearts (Fig. 3.7B). Another well-established indicator of allograft rejection is complement component C4d, which is a specific marker for humoral immunity and the presence of donor-specific antibodies (DSA) against allograft [36]. In the current study, there was a significant increase in myocardial C4d expression in MSC group compared to sham and MI animals (Fig.3.7 C) Interestingly, C4d expression decreased significantly in Sug1 KD MSCs transplanted hearts (Fig. 3.7C). Therefore, knocking down Sug1 mitigated host immune response against transplanted MSCs.

The tracking of transplanted MSCs was performed by staining the cells with a fluorescent cell tracker PKH26 (the half-life of PKH26 is greater than 100 days). After 5 weeks of cell transplantation, we found that most of the cells were rejected in MSCs group, whereas as knocking down Sug1 in MSCs significantly increased the survival of transplanted cells in the ischemic hearts (Fig. 3.7D). In order to correlate MHC-II expression in transplanted MSCs *in vivo* with cell retention in the myocardial sections, immunohistochemistry was performed to investigate co-localization of PKH2 and MHC-II expression in infarcted myocardium. There was a significant decrease in MHC-II signal in surviving transplanted Sug1 KD MSCs compared to wild type MSCs (Fig. 3.7E).

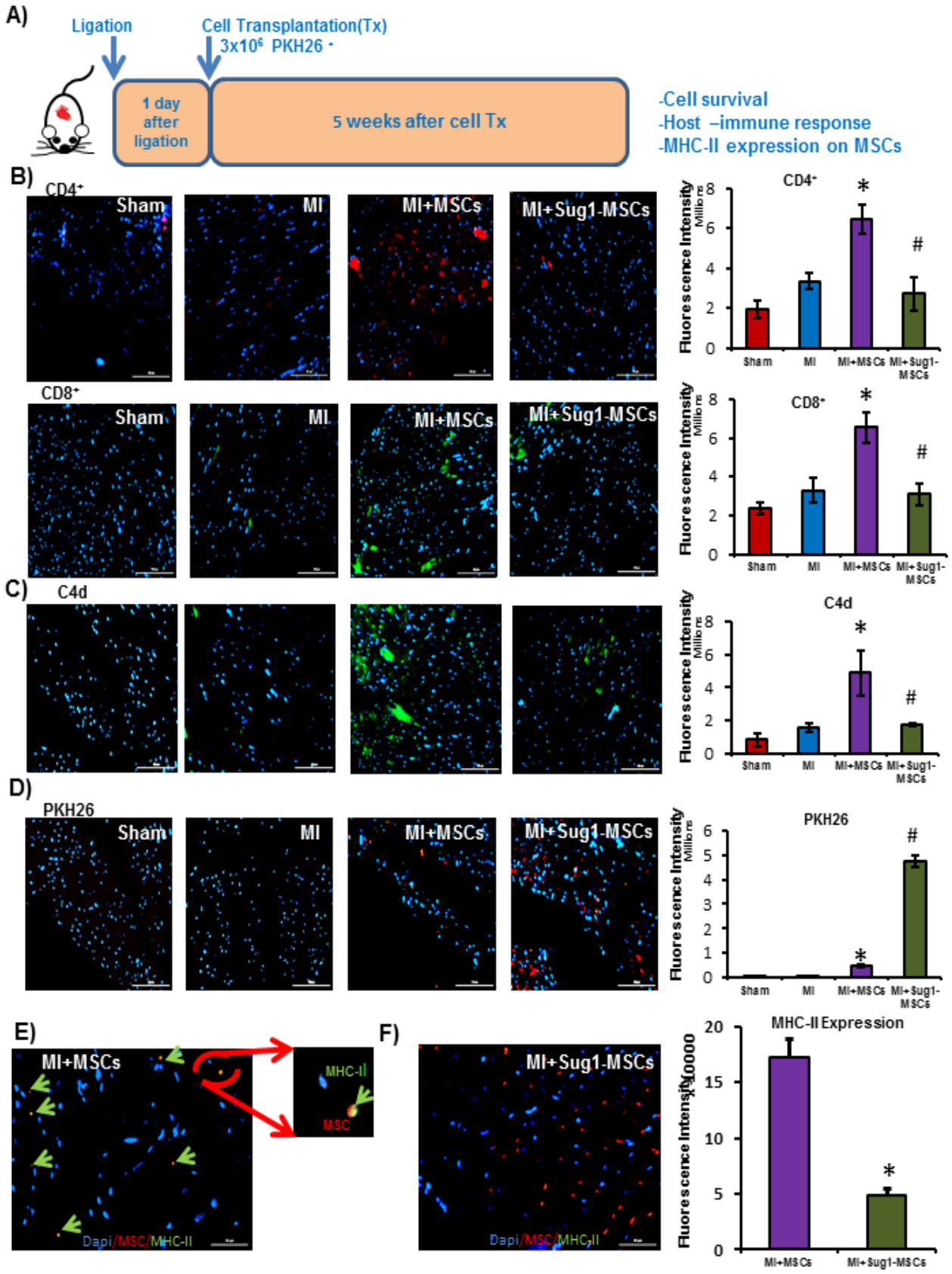
A)
Ligation
Cell Transplantation(Tx)
3x10⁶ PKH26 +
1 day after ligation
5 weeks after cell Tx
-Cell survival
-Host –immune response
-MHC-II expression on MSCs
B)
CD4+
Sham
MI
MI+MSCs
MI+Sug1-MSCs
Fluorescence Intensity Millions
CD8+
C)
C4d
D)
PKH26
E)
MHC-II
MSC
Dapi/MSC/MHC-II
F)
MHC-II Expression
Fluorescence Intensity x10000
MI+MSCs
MI+Sug1-MSCs

Figure 3.7: Knocking down Sug1 mitigated host immune response and improved survival of transplanted MSCs in the ischemic heart *in vivo*. (A) Schematic diagram to depict time-line for *in vivo* experiments. MSCs were transplanted 24 h after coronary artery ligation. Host immune response, cell survival and MHC-II expression was measured after 5 weeks of cell transplantation. **(B,C)** Immunohistochemistry was performed in myocardial sections to measure $CD4^+$ and $CD8^+$ T cells infiltration, and C4d expression in the heart. There was a significant increase in $CD4^+$ and $CD8^+$ T cells infiltration, and C4d expression in MSC group, Sug1 knock down prevented it, n=5. **(D)** Cell survival as measured by tracking PKH26 positive cells (red fluorescent tracking dye) increased significantly in Sug1 KO group compared to MSC group, n=5. **(E)** MHC-II expression in transplanted MSCs as measured by immunohistochemistry decreased significantly in Sug1 KO group compared to MSC group, n=3. *$p<0.05$ compared to MI group; #$p<0.05$ compared to MI- MSCs group.

3.3.6 Exposure to hypoxia upregulates CIITA levels and immunogenicity in human BM-MSCs:

To explore translational potential of our findings, we conducted *in vitro* experiments to investigate the effects of hypoxic environment on immunogenicity in human BM-MSCs (hMSCs). In rodents, MHC-II and in humans, human leukocyte antigens-DR (HLA-DR) synthesis is regulated by CIITA. HLA-DR presents antigens to $CD4^+$ T cells leading to activation and proliferation of T cells and allograft rejection. We found a significant increase in CIITA, HLA-DRα and Sug1 in hypoxic hMSCs as compared to normal cells (Fig. 3.8 A-C). Interestingly, the addition of Sug1 inhibitor prevented hypoxia induced increase in CIITA and HLA-DRα levels in hypoxic hMSCs (Fig. 3.8 A and B).

In MSCs and leukocytes co-culture experiments, there was a significant increase in the level of leukocyte mediated cytotoxicity in hypoxic MSC, and treatment with a Sug1 inhibitor decreased the hypoxia induced cytotoxicity in hMSCs (Fig.3.8D). Also, there was a significant decrease in the percentage $CD4^+CD25^+$ Treg cell number after co-culture with hypoxic hMSCs vs normoxic cells, the presence of Sug1 inhibitor increased the number of Treg cells (Fig.3.8 E and F). These results confirm that hypoxia induced upregulation of Sug1 and loss of immunoprivilege is not only limited to rodent cells, human MSCs are also susceptible to this immune switch. We also found that blocking Sug1 preserves immunoprivilege of allogeneic hMSCs under hypoxic conditions.

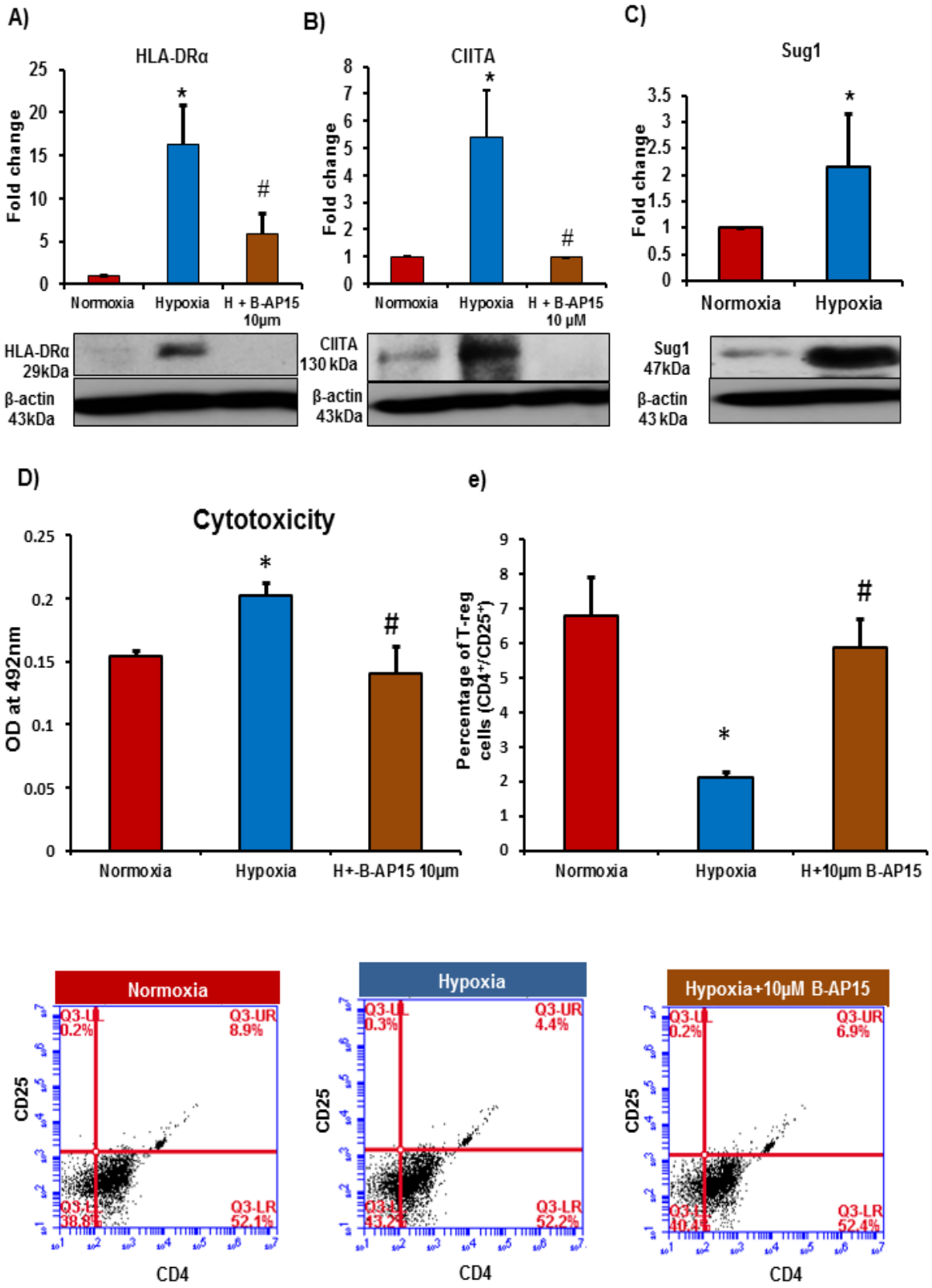
A)
HLA-DRα
Fold change
Normoxia
Hypoxia
H + B-AP15 10μm
HLA-DRα 29kDa
β-actin 43kDa
B)
CIITA
Fold change
Normoxia
Hypoxia
H + B-AP15 10 μM
CIITA 130 kDa
β-actin 43kDa
C)
Sug1
Fold change
Normoxia
Hypoxia
Sug1 47kDa
β-actin 43 kDa
D)
Cytotoxicity
OD at 492nm
Normoxia
Hypoxia
H+-B-AP15 10μm
e)
Percentage of T-reg cells (CD4+/CD25+)
Normoxia
Hypoxia
H+10μm B-AP15
Normoxia
Q3-UL 0.2%
Q3-UR 8.9%
Q3-LR 52.1%
CD25
CD4
Hypoxia
Q3-UL 0.3%
Q3-UR 4.4%
Q3-LR 52.2%
CD25
CD4
Hypoxia+10μM B-AP15
Q3-UL 0.2%
Q3-UR 6.9%
Q3-LR 52.4%
CD25
CD4

Figure 3.8: Exposure to hypoxia upregulates CIITA levels and immunogenicity in human MSCs.(A-C) Human MSCs (hMSCs) were exposed to hypoxia for 24h. HLA-DRα, CIITA and Sug1 levels as measured by Western blot increased in hypoxic MSCs vs. normoxic cells. Sug1 knock down (B-AP15, 10μM) prevented hypoxia induced increase in HLA-DRα and CIITA, n=3. **(D)** To determine the immunogenicity of hMSCs, normoxic and hypoxic MSCs were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 h. Leukocyte mediated cytotoxicity in MSCs (LDH release) increased significantly in hypoxic MSCs vs. normoxic cells, knocking down Sug1 prevented it, n=10. **(E,F)** The effect of hMSCs on Treg cell ($CD4^+CD25^+$) induction in a mixed leukocyte population was assessed by flow cytometry. The number of Treg cells decreased after co-culture with hypoxic hMSCs compared to normoxic cells, inhibition of Sug1 prevented it, n=3. *$p<0.05$ compared to normoxic hMSC; #$p<0.05$ compared to hypoxic hMSCs.

3.4 Discussion:

Bone marrow derived MSCs were initially reported to possess unique immunoprivilege characteristics, therefore transplantation of allogeneic MSCs from young and healthy donors to old and debilitated patients is possible without the risk of allograft rejection [26,37]. This "universal donor phenomena" of allogeneic MSCs allured stem cell biologists and clinicians to conduct numerous preclinical and clinical studies using the "one size fits all" trend. However, the outcome of these studies was not as predicted due to the poor survival of transplanted MSCs, which caused a decline in the overall enthusiasm about MSC based regenerative therapies [38,39]. Recent analyses of allogeneic MSCs based studies demonstrated that the transplanted cells undergo an immune-switch from immuniprivileged into immunogenic state, and are subsequently recognized by host immune system and are rejected [19]. The notion of MSCs becoming immunogenic not immunoprvileged after being placed in ischemic environment of diseased tissues or organs demanded to be highlighted and tackled in order to extend the beneficial effects of allogeneic MSCs and make it feasible to employ these cells therapeutically [14,17]. The mechanisms of MSCs-mediated immune suppression through the secretion of several potent inhibitory molecules have been well addressed. However, the mechanisms of post-transplantation immune-switch in MSCs from an immunoprivileged to immunogenic state have not been studied thoroughly [37,40]. Hypoxia is a stress signal that accompanies the ischemic environment, and is associated with majority of pathological conditions at organ and tissue levels in the body [41–44]. In the current study, we found that exposure to hypoxia was associated with the loss of immunoprivilege of allogeneic MSCs. The immunoprivilege of MSCs is preserved by the absence of MHC-II expression in cells. MHC-II is a cell surface immune antigen that alerts host immune system against transplanted cells [30,45]. The constitutive and inducible expression of

MHC-II is regulated globally by CIITA, which is considered as a master regulator of MHC-II gene [20,21,23]. CIITA was initially discovered in patients with a rare inborn disorder called bare lymphocyte syndrome characterized by total loss of MHC-II expression and function as well as deficiency in $CD4^+$ T cell mediated adaptive immunity. CIITA is required for the activation of MHC-II promoter through interactions with transcription factor RFX5 [20,46]. In the current study, there was a significant increase in CIITA mRNA and protein levels in hypoxia exposed MSCs. CIITA is present in the cytoplasm under basal conditions, it translocates to nucleus upon activation and binds to RFX5. We found a significant accumulation of CIITA in the nucleus in hypoxic MSCs. Therefore, hypoxic environment leads to activation and translocation of CIITA to the nucleus that further upregulates MHC-II biosynthesis and loss of immunoprivilege of allogeneic MSCs.

In the current study, we found a significant increase in the protein and mRNA levels of Sug1 in hypoxic MSCs. Sug1 is ATPase subunit of 26S proteasome, apart from its proteolytic role, Sug1 is also reported to function independently and regulate the activity of several proteins and genes [28,47,48]. Here, we report for the first time that there was significant increase in the binding of Sug1 to CIITA in hypoxic MSCs. Furthermore, our data demonstrate that pharmacological as well as genetic inhibition of Sug1 in MSCs prevented hypoxia induced upregulation of CIITA and MHC-II, also Sug1 knock down restored immunoprivilege of MSCs under hypoxic conditions. Previous studies in cancer cells have reported that post translational modifications of CIITA promote its activation and stabilization in the nucleus to initiate MHC-II transcription [49,50]. However, posttranslational modifications of CIITA in MSCs and its effect on MSCs immunoprivilege under hypoxic conditions have not been investigated yet. In the current study, we found that hypoxia induced upregulation of Sug1 was responsible for acetylation and K63

ubiquitination of CIITA. Furthermore, PCAF, which is a known histone acetyltransferase has also been recently reported to act as E3 ubiquitin ligase [31,33]. Our co-immunoprecipitation data in wild type and Sug1 KD MSCs reveal that hypoxia induced upregulation of Sug1 promotes binding of PCAF to CIITA. We also found a sharp increase in CIITA binding to RFX5 in hypoxic MSCs, which was prevented by Sug1 knock down. RFX5 is a transcription factor that binds to proximal regulatory region of MHC-II promoter and plays a critical role in the activation of MHC-II transcription. Therefore, we found that in allogeneic MSCs, exposure to hypoxia or ischemic environment increases Sug1 levels and its binding to CIITA. Sug1 activation of CIITA is mediated by the recruitment of acetyltransferase PCAF that facilitates acetylation and K63 ubiquitination of CIITA leading to upregulation and translocation of CIITA to the nucleus and it's binding to RFX5, and ultimately MHC-II up-regulation. Collectively, this is the first study reporting non-proteolytic role of Sug1 in MSCs immunoprivilege under hypoxic conditions. Furthermore, these observations provide unique insight into the mechanism of hypoxia induced switch in the phenotype of MSCs from immunoprivileged to immunogenic state.

Allogeneic MSCs from bone marrow are increasingly being investigated for treating a wide range of clinical diseases [1,2,51]. The data from preclinical studies and several industry-sponsored or academic-investigator driven clinical trials have reported positive outcome and beneficial effects of MSCs [52,53]. However, a potential limitation acknowledged by experts in the field of MSC therapy is that transplanted cells do not persist following transplantation, and the majority of the cells die immediately after transplantation. The initial assumptions that allogeneic MSC preparations represent "one-size-fits-all" and "off-the-shelf" products were originated from the facts that MSCs were immunoprivileged. However, recent data from preclinical studies and

clinical trials strongly suggest that even though allogeneic MSCs display immunoprivlege under *in vitro* conditions, late after transplantation in the host micro-environment MSCs become immunogenic and provoke an immune response resulting in rejection [12,25,54]. We found that hypoxic or ischemic environment in the host tissue leads to upregulation of CIITA and cell surface immune antigen MHC-II in rat and human MSCs. In the current study, we also found that preventing hypoxia induced MHC-II upregulation in MSCs by knocking down Sug1 preserved immunoprivilege of MSCs *in vivo* in the ischemic heart, mitigated host immune response and improved survival of transplanted cells in a rat model of myocardial infarction. Therefore, preserving the immunoprivilege of MSCs in hypoxic conditions would prevent their rejection and prolong their survival in the ischemic host tissue. More significantly our studies also reveal that the hypoxia induced immune switch is not only limited to rodent cells, but also applies to human MSCs. Therefore, our study may help in increasing the success rates of ongoing allogeneic MSCs based clinical trials and allowing a better planning for future trials. The strategies to prolong post-transplantation persistence of allogeneic MSCs can be divided into two categories: modification of MSCs or improving immune tolerance within the host tissue using immunosuppressive drugs. The later approach seems to be cumbersome as immunosuppressive drugs have several side effects. However, modification of cells seems to be simple and easy to handle approach. Therefore, the outcome of current study may help in development of alternate therapeutic interventions away from using the immunosuppressive drugs which are harmful in the long run and do not provide complete protection from rejection.

3.5 Material and Methods:

Animals: All animal study protocols were approved by the Animal Care Committee of the University of Manitoba and conformed to the ‘Guide for the Care and Use of Laboratory

Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

To isolate bone marrow MSCs and leukocytes from spleen, we used unrelated Sprague-Dawley rats. Wistar rats were used for all the *in vivo* cell transplantation studies.

Isolation and culture of MSCs: MSCs were isolated from the femurs and tibias derived from Sprague-Dawley rats as described in our recent studies [12,52,55]. Bone marrow was flushed with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum, 100 units/ml penicillin G and 0.1 mg/ml streptomycin. The cells were plated and cultured in DMEM over-night. Next day the non-adherent cells were discarded and medium was replaced every 3 days. The cells were sub-cultured when confluency exceeded 90%. MSCs were characterized using flow cytometry– the cells which were $CD44^+$ and $CD29^+$ (Santa Cruz) and $CD45^-$ and $CD34^-$ (Santa Cruz) were used for further experiments

Human bone marrow MSCs (hMSCs) were purchased from Lonza group (PT 2501 CA10064-080).

MSCs treatment protocols: Both rat and human MSCs in different groups were treated with hypoxia for 24 h. The culture plates were placed in hypoxia chamber (oxygen level regulated at 0.0%-0.1%) in the incubator (Biospherix hypoxia chamber).

To block 19S proteasome (Sug1) in normoxic MSCs, the cells were treated with B-AP15 (10 μM) for 4 hours, and treated MSCs were exposed to hypoxia for 24 hours.

Western blot: Western blotting was performed to measure protein levels for MHC-II (Cat# ab23990, Abcam), CIITA (Cat #NBP1-76296, Novus Bilogicals), Sug1 (Cat# ab178681,Abcam) , and HLA-DRα(Cat# sc-53499, Santa Cruz Biotechnology) using species specific antibodies. Briefly, cell lysates were prepared from the cells in different groups and total protein levels were

measured by Bradford method. 50 µg of protein samples were loaded onto SDS-PAGE. The proteins on the gel were transferred to PVDF membrane. The membranes were incubated with appropriate primary and secondary antibodies, and developed using X-ray film. The bands were quantified using Quantity One software for densitometry.

Immunoprecipitation assay: The protein-protein interactions were studied by immunoprecipitation (IP) assay following manufacturers' instructions (Santa Cruz biotechnology). Briefly, the lysates from different groups were precleared using preclearing matrix. To form IP antibody-IP matrix complex, 50 µl of IP matrix (25% v/v), 1-5 µg of IP antibody in 500 µl of phosphate buffer saline (PBS) were incubated overnight at 4° C. The protein samples (300 µg) were then transferred to the pelleted matrix and left for overnight incubation at 4°C. Then SDS-PAGE electrophoresis was performed as described for the Western Blotting procedure and probed with primary antibodies and secondary antibodies. The membranes were developed using X-ray film and bands were quantified using Quantity One software for densitometry.

Immunocytochemistry: Immunocytochemistry was performed to measure expression and localization of CIITA, MHC-II and Sug1 in MSCs. The cells were plated onto sterile coverslips, and allowed to grow to reach 70% confluency. The plated MSCs were fixed with 4% PFA and permeabilized using 0.2% Triton X in PBS at room temperature. The cells were then stained with primary and secondary antibodies as well as phalloidin (for F-actin, Invitrogen). Thereafter, the MSCs were counter stained with DAPI for nuclei. Finally, Cytation 5 system (BioTek Instruments) was used for imaging the cells.

Reverse-Transcription PCR: Total RNA was isolated using high pure RNA isolation kit (Roche) and cDNA was formed using cDNA kit (Thermo Scientific) for RT-PCR. The following

PCR primers were used: Sug1 - Forward Primer: AAGAGGGAAGATGGCGCTTGATG; Reverse Primer: TTGAGGGATCCACACAAAGGACAC; GAPDH - Forward Primer: GATGGTGAAGGTCGGTGTGAAC; Reverse Primer: CCCATTCTCAGCCTTGACTGTG. The PCR product samples were mixed with 6X DNA loading buffer and loaded onto 1% agarose gel. The BIORAD chemidoc system was used to image the gels.

Sug1 shRNA knockdown and stable MSCs line generation: shRNA lentiviral particles *viz.* TL711271VA, TL711271VB, TL711271VC and TL711271VD packaged from pGFP-C-shLenti vector (Origene, Rockville, MD) were pooled together at an MOI of 6 to infect MSCs (Supplementary Fig 3.1). To enhance infection efficiency, Polybrene (Sigma-Aldrich, Oakville, ON) was used at a concentration of 8 μg/ml. The infected cells were selected by using 2.5 μg/ml Puromycin (Sigma-Aldrich, Oakville, ON). Sug1 knockdown in cells was confirmed using Western Blot. The sequences used for the shRNA knockdown are:

Scrambled Control: 5' GCACTACCAGAGCTAACTCAGATAGTACT 3
TL711271VA: AAGGTGCCAGACTCAACCTACGAGATGAT
TL711271VB: AAGAACTCCAGTTGATTGTGAATGACAAG
TL711271VC: CGACTCTATCGGTTCTTCACGGCTGGAGG
TL711271VD: GGACTTTGAGATGGCAGTAGCCAAGGTCA

Mixed leukocyte mediated cytotoxicity: The immunogenicity of MSCs was assessed by performing mixed leukocyte reaction assay. Mixed peripheral blood leukocytes were isolated from rat spleens using HISTOPAQUE 1083 (Sigma-Aldrich) and co-cultured with allogeneic MSCs from different treatment groups in the ratio of 10:1 for 72 h. After the co- culture, leukocyte-mediated cytotoxicity in the MSCs was assessed by measuring the lactate dehydrogenase (LDH) released from the damaged MSCs (LDH Cytotoxicity Detection Kit; Clontech).

To measure leukocyte mediated cytotoxicity in human MSCs, hMSCs were co-cultured with leukocytes isolated from peripheral blood derived from healthy individuals. All the human cells related *in vitro* studies were approved by the University of Manitoba's Research Ethics Board.

Leukocyte proliferation: MSCs promote immune tolerance by suppressing the proliferation of leukocytes. Leukocyte proliferation was measured after 72 h of co-culture with normoxic and hypoxic allogeneic MSCs (10:1) by flow cytometric analysis (BD Accuri). Briefly, after co-culture with MSCs, the supernatant containing leukocytes was collected and centrifuged at 1000rpm for 5minutes. After washing three times with PBS, leukocytes were fixed with 70% ice cold ethanol, and then treated with RNase (20µg/ml) for 30 minutes. The leukocytes were then stained with propidium iodide (PI, 5µg/ml) for 5 minutes and analyzed using flow cytometry. To measure leukocyte proliferation cell cycle analysis was done by counting the number of cells entering S-phase (proliferating phase) and G2/M phase from G0/G1 phase (resting cells) of the cell cycle.

Regulatory T cells measurement: Flow cytometric analysis was performed to count the number of $CD4^{+}CD25^{+}$ regulatory T (Treg) cells in total leukocyte population after 72 h of co-culture with allogeneic MSCs. We used BioRad Treg cell estimation kits for rat (Cat. no. DC040) and human (Cat. No. DC027) Treg cell counting.

***In vivo* studies**

Acute myocardial infarction model and Sug1 knockout MSCs transplantation*:* Myocardial infarction (MI) was induced surgically in Wistar rats. Animals were placed in right decubitus position over a heating blanket. Left lateral thoracotomy was performed and hearts were visualized using self-retaining retractor, the pericardium was gently removed. Myocardial infarction (MI) was induced by ligating the left anterior descending coronary artery. After 1 day

of ligation, a suspension (50 µl/rat) of both regular MSCs as well as Sug1 KO MSCs derived from SD rats were transplanted (3×10^{6} cells/rat) in the infarct area.

After five weeks of cell transplantation, the hearts were rapidly excised, and fixed in 10% formaldehyde for 4 to 7 days. Hearts were cut into 5 µm thick sections and immunohistochemistry was performed as described in the following sections to measure host immune response, transplanted stem cell survival and MHC-II expression in the myocardial sections.

Assessment of host immune response: Host immune response against transplanted MSCs was assessed by measuring the infiltration of $CD4^{+}$ and $CD8^{+}$ T cells in the heart. We also measured the expression of C4d in the myocardium. Immunohistochemistry was performed in the myocardial sections at 5 weeks after cell transplantation. Briefly, heart tissue samples were fixed in formaldehyde and cut into 5-µm thick sections on poly-lyisne coated slides. After removing the wax, sections were rehydrated using different concentrations of ethanol (100%, 95%, 70%, 50%) and washed with 1X PBS for 10 min. After blocking with 1% horse serum for 30 min, sections were incubated with anti-CD4 (CL003AP, Cedarlane) or anti-CD8 antibody (Cedarlane, CL004AP) or C4d **(**HP8038, Hycult) at 1:100 dilution overnight at 4°C. After staining with secondary antibody, the slides were mounted with anti-fade mounting media containing DAPI (Abcam, CA). The images were recorded using Cytation 5 imaging reader and quantified using Image J software.

Tracking of transplanted stem cells and MHC-II expression *in vivo*: Before, transplantation MSCs were tagged with a red fluorescent tracking dye PKH26, using manufacturer's protocol (Sigma-Aldrich). After 5 weeks of cell implantation sections were assessed using Cytation 5 imaging system and cell number was quantified using Image J software.

MHC-II expression in myocardial sections was measured by immunohistochemistry as described in the above section. Briefly, after blocking with 1% horse serum for 30 min, sections were incubated with primary antibody (Santa Cruz Inc.) at 1:50 dilution overnight at 4°C. After staining with secondary antibody, the slides were mounted with anti-fade mounting media containing DAPI (Abcam, CA). The images were recorded using Cytation 5 imaging reader and quantified using Image J software.

Statistical analysis: Experimental values are expressed as mean ± SD. Comparison of mean values between various groups was performed by one-way-analysis of variance (one-way ANOVA) followed by multiple comparisons by Tukey test. P value <0.05 was considered to be significant.

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Conflict of interest

Authors declare no conflict of interest.

Author Contributions

E.A.R and S.D conceptualized the study; E.A.R, H.I.A and S.D designed the experiments; E.A.R, N.S, G.L.S, W.Y., A.M.S, I.R, H.S.S and L.A.R carried out the experiments, acquired and analyzed the data; E.A.R, N.S, G.L.S, H.I.A and S.D. interpreted the data and performed

statistical analyses, E.A.R, N.S and S.D drafted the manuscript. All the authors have read and approved the final version of the manuscript.

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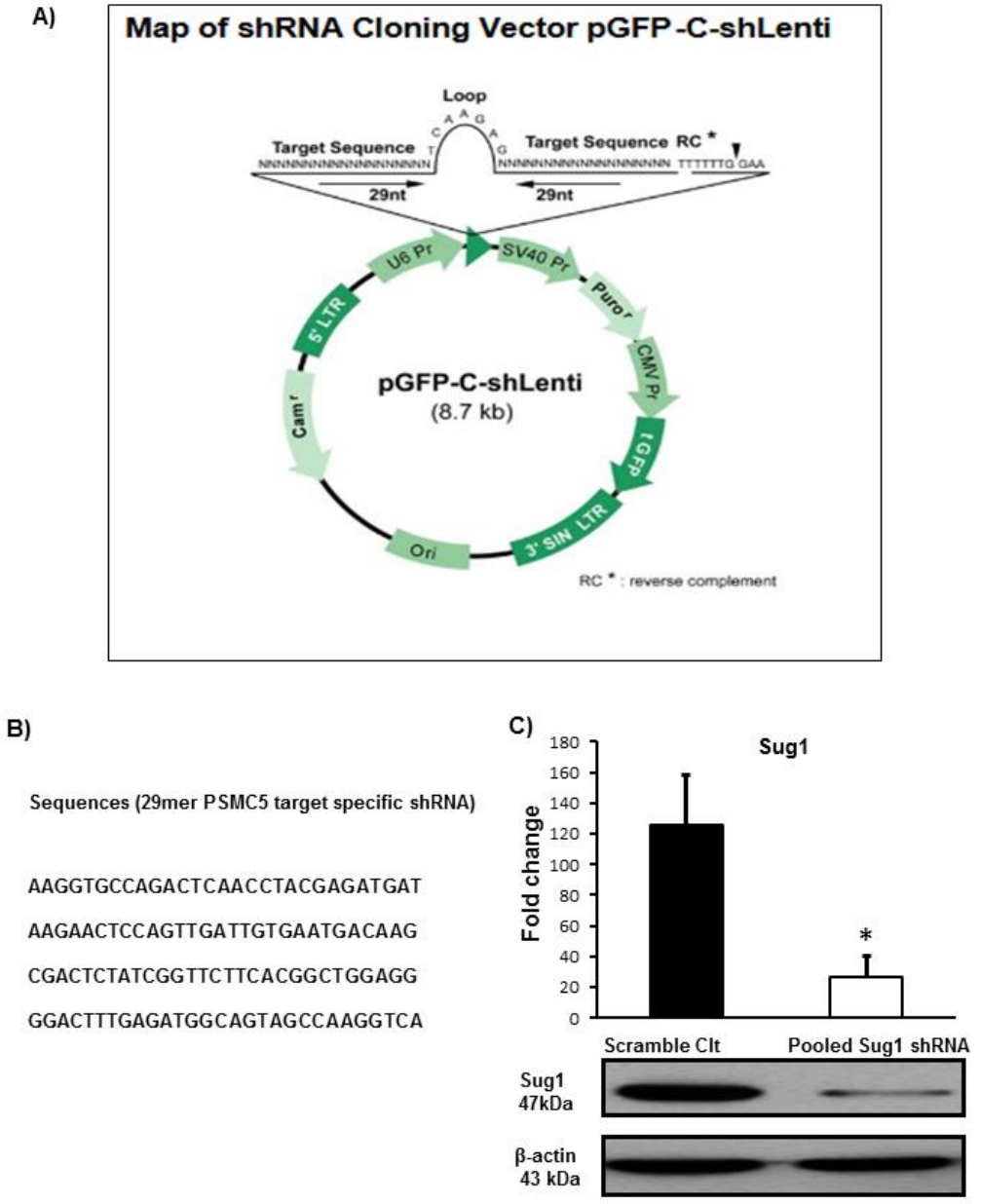

Supplementary Figure 3.1: Lentiviral shRNA system used to knock down Sug1 (a) The map of Lentiviral shRNA system used to knock down Sug1 in MSCs. (b) The shRNA sequences used to knock down Sug1. (c) Western blot analysis showed that Sug1 was profoundly knocked down in MSCs, n=4. *$p<0.05$ compared to scrambled control group.

Chapter IV: Role of immunoproteasome in the immunogenicity of MSCs

Rational and Hypothesis:

In Chapters II and III, we found that exposure to a hypoxic environment led to the upregulation of MHC-II and loss of immunoprivilege of MSCs. We reported that 26S proteasome degrades MHC-II in normoxic MSCs and preserves their immunoprivilege. Exposure to hypoxia causes inactivation of 26S proteasome and loss of immunoprivilege of MSCs. Next, we also reported that the levels of Sug1 (one of the subunits of 19S proteasome) increased in MSCs after exposure to hypoxia, which was associated with upregulation of MHC-II transcription and loss of immunoprivilege of MSCs. Knocking down Sug1 in MSCs preserved their immunoprivilege *in vitro* and *in vivo*. Interestingly, we also found that when MSCs were exposed to hypoxia, there was a significant increase in the levels of 11S, and it's binding to 20S proteolytic subunit to form the immunoproteasome, which is an alternate form of proteasome, and is expressed under stress conditions. The immunoproteasome is reported to process antigenic peptides for binding to MHC-I molecule to trigger $CD8^+$ T cells. The role of immunoproteasome in MHC-II mediated antigen presentation has not been reported yet. In this chapter we investigated the role of immunoproteasome in regulating MHC-II expression and immunogenicity of MSCs under hypoxic conditions.

Hypothesis:

Hypoxia induced shift in the phenotype of 26S proteasome toward immunoproteasome upregulates immunogenicity of mesenchymal stem cells.

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Hypoxia induced shift in the phenotype of proteasome from 26S toward immunoproteasome triggers loss of immunoprivilege of mesenchymal stem cells

Ejlal Abu-El-Rub[1], Niketa Sareen[1], Weiang Yan[1], Keshav Narayan Alagarsamy[1], Alireza Rafieerad[1], Abhay Srivastava[1], Vincenzo Desiderio[2], Sanjiv Dhingra[1]

1. Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, 9 Regenerative Medicine Program, Department of Physiology and Pathophysiology, Rady 10 Faculty of Health Sciences, University of Manitoba

2. Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", 80138, Naples, Italy.

Short Title: Immunoprivilege of mesenchymal stem cells

Correspondence:

Sanjiv Dhingra, PhD, FCVS

Regenerative Medicine Program
Institute of Cardiovascular Sciences,
St. Boniface Hospital Research Centre
R-3028-2, 351 Tache Avenue, Winnipeg, R2H2A6, Canada
Phone: 204-235-3454; Fax: 204-233-6723
Email: sdhingra@sbrc.ca

4.1 Abstract

Allogeneic mesenchymal stem cells (MSCs) are immunoprivileged and are being investigated in phase I and phase II clinical trials as potential treatments for different degenerative and autoimmune diseases. In spite of encouraging outcome of initial trials, the poor survival of transplanted cells long- term in the host tissue has reduced the overall enthusiasm for the field. Recent analyses of allogeneic MSCs based studies confirms that after transplantation in the hypoxic or ischemic microenvironment of diseased tissues, MSCs become immunogenic and are rejected by the recipient immune system. The immunoprivilege of MSCs is preserved by the absence of cell surface antigen, human leukocyte antigen (HLA) – DRα. We found that in normoxic MSCs, the 26S proteasome degrades HLA-DRα and maintains immunoprivilege of MSCs. The exposure to hypoxia leads to inactivation of 26S proteasome and formation of the immunoproteasome in MSCs, which is associated with upregulation and activation of HLA-DRα, and as a result MSCs become immunogenic. Furthermore, inhibition of immunoproteasome formation in hypoxic MSCs preserves their immunoprivilege. Therefore, hypoxia induced shift in the phenotype of proteasome from 26S toward immunoproteasome triggers loss of immunoprivilege of allogeneic MSCs. The outcome of current study may provide molecular targets to plan interventions to preserve immunoprivilege of allogeneic MSCs in the hypoxic or ischemic environment.

4.2 Introduction

Bone marrow derived allogeneic (donor derived) mesenchymal stem cells (MSCs) are considered to be a potential cell type for the treatment of degenerative diseases and autoimmune disorders(1–5). MSCs are reported to be immunoprivileged, that allowed transplantation of allogeneic MSCs without the risk of being rejected by host immune system (1,6–11). These properties of MSCs promoted the concepts of universal young and healthy donor derived "off-the-shelf" allogeneic cell based products for older and debilitated patients (12,13). Infact, in the last 10-15 years several clinical trials have tested the safety and efficacy of allogeneic MSCs based products in phase I and II clinical trials (14–19). The outcome of most of these trials confirmed the safety of transplanted cells (20–22). However, the long-term follow-ups of many of these clinical trials revealed that allogeneic MSCs were only able to exert beneficial effects in the transplanted areas for a short period of time, ultimately the benefits were lost (19,23,24). One of the major limitations of allogeneic MSCs based therapies is poor survival of transplanted cells in the host tissue (25–28) . Furthermore, the outcome of several studies now confirms that allogeneic MSCs after transplantation in stressful micro-environment of the host tissue, become immunogenic and are rejected by the host immune system that results in poor survival of transplanted cells (28–32). Therefore, in order to maintain therapeutic benefits of allogeneic MSCs, there is a need to preserve immunoprivilege of transplanted cells in the host tissue.

The immunoprivilege of MSCs is preserved by absence of immune antigen- human leukocyte antigen (HLA) – DR(9,10,31,33). The HLA-DR molecules are cell surface immune antigens that alert the host immune system to initiate an immune response against transplanted cells or tissues. HLA-DR plays a critical role in T-cell-dependent allo- immune responses by presenting the

processed exogenous antigens to T helper (Th) cells (31,34,35). Therefore, HLA-DR has been implicated as the major contributing factor in allograft rejection. Although HLA-DR is expressed constitutively on antigen-presenting cells (monocytes/macrophages, B cells, and dendritic cells), this molecule can be induced in most cell types and tissues in the presence of pro-inflammatory cytokines e.g. IFN-γ or under stressful conditions (31,36–38). We recently reported in rat and human MSCs that exposure to hypoxia or ischemic conditions was associated with upregulation of HLA-DRα or MHC-II and loss of immunoprivilege of allogeneic MSCs (31). Hypoxia or ischemic environment is a common underlying condition of many diseased or injured tissues. In this study, we examined the mechanisms of hypoxia induced upregulation and activation of HLA-DRα in allogeneic human MSCs. We report for the first time that exposure to hypoxic environment led to formation of the immunoproteasome in MSCs which is responsible for activation of HLA-DRα and loss of immunoprivilege of allogeneic MSCs.

4.3 Results

4.3.1 Hypoxia causes downregulation of 19S regulatory subunits and 20S proteolytic core subunits of 26S proteasome: We recently reported in human MSCs that 26S proteasome mediated degradation of HLA-DRα maintains absence of this molecule on MSCs surface and preserves immunoprivilege of allogeneic MSCs (31). Exposure to hypoxic environment was responsible for upregulation of HLA-DRα and immunogenicity of MSCs. These exciting findings prompted us to investigate the fate of 26S proteasome in MSCs under hypoxic conditions and its effects on immunoprivilege of MSCs. The 26S proteasome is composed of a regulatory unit 19S and proteolytic core containing 20S. The 19S regulatory unit receives ubiquitinated target protein and transfers it to the proteolytic core of 20S where the target protein is processed and degraded (39,40). The deubiquitination proteins PSMD11 and PSMD4 (or

Rpn10), which are present in 19S unit, play an important role in processing of target protein(41,42). In the current study, we found a significant decrease in the expression of these two subunits (Fig. 4.1a and b). The 20S particle contains three subunits β1 (or PSMB6), β2 (or PSMB7) and β5 (or PSMB5) which are responsible for mediating proteolytic activities of 20S(39,43). We found a significant decrease in the expression of these three proteins (Fig. 4.1a and c). These findings demonstrate that exposure to hypoxia leads to downregulation of proteasome regulatory subunits and proteolytic subunits. Interestingly, the "α" subunits which form the outer ring of 20S proteasome- α3 (PSMA4) and α6 (or PSMA1) did not change significantly in hypoxic hMSCs compared to normoxic cells (Fig. 4.2 a and b). The "α" subunits interact with regulatory subunits to mediate proteolytic activities of the proteasome.

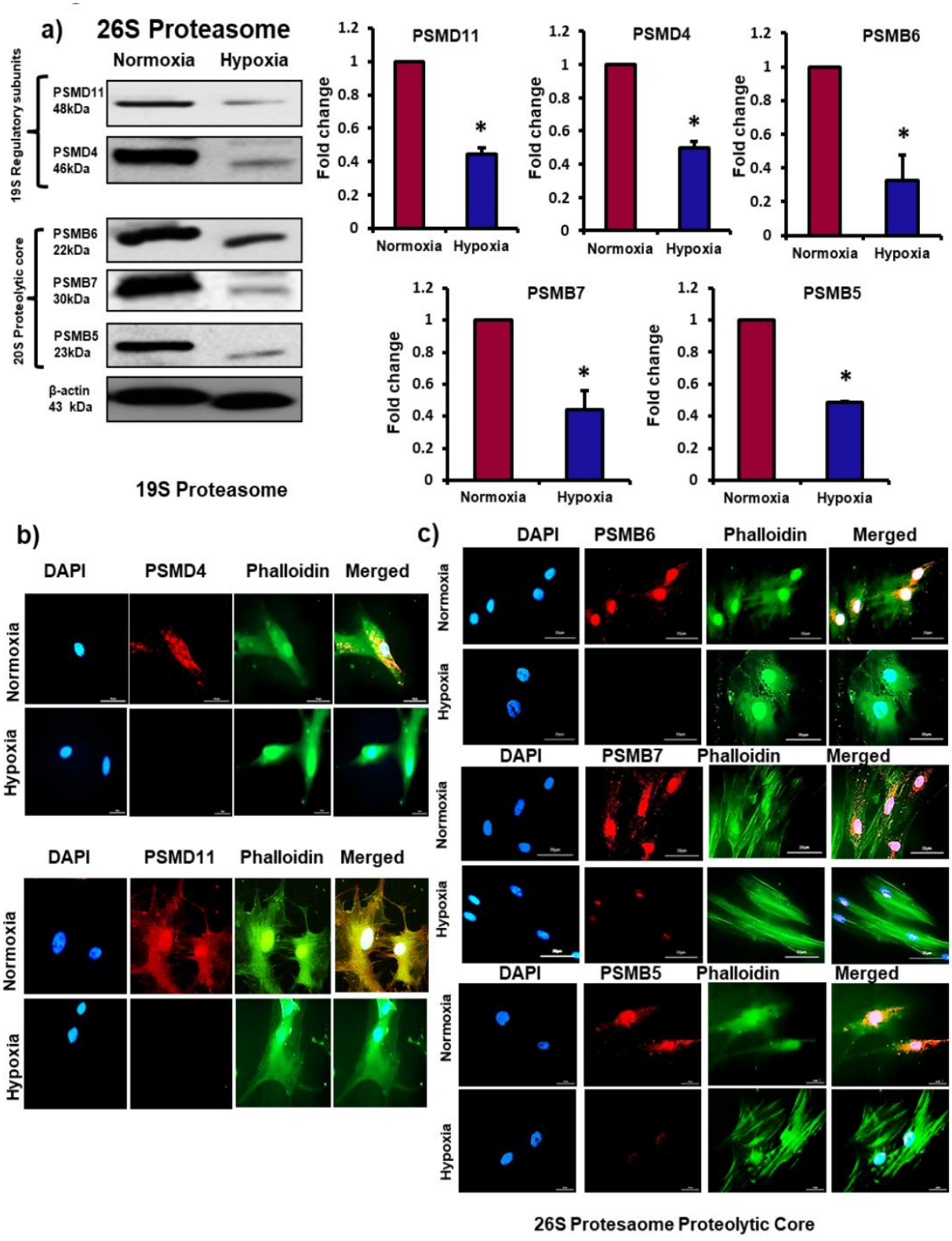

a)
26S Proteasome
Normoxia
Hypoxia
19S Regulatory subunits
PSMD11
48kDa
PSMD4
46kDa
20S Proteolytic core
PSMB6
22kDa
PSMB7
30kDa
PSMB5
23kDa
β-actin
43 kDa
19S Proteasome
PSMD11
PSMD4
PSMB6
PSMB7
PSMB5
Fold change
Normoxia
Hypoxia
b)
DAPI
PSMD4
Phalloidin
Merged
DAPI
PSMD11
Phalloidin
Merged
c)
DAPI
PSMB6
Phalloidin
Merged
DAPI
PSMB7
Phalloidin
Merged
DAPI
PSMB5
Phalloidin
Merged
26S Protesaome Proteolytic Core

Figure 4.1: Exposure to hypoxia causes downregulation of regulatory subunits and proteolytic core subunits of 26S proteasome in MSCs. Human bone marrow-derived MSCs were incubated in hypoxia chamber for 24hr. (a) Protein levels of PSMD11, PSMD4 (Rpn10), PSMB6 (β1), PSMB7 (β2), and PSMB5 (β5) as measured by Western blot showed a significant decrease in hypoxic MSCs compared to normoxic cells; n=3. (b & c) Immunofluorescence images exhibited a significant decrease in the expression of PSMD4 (Rpn10), PSMD11, PSMB6 (β1), PSMB7 (β2) and PSMB5 (β5) in hypoxic MSCs compared to normoxic cells; n=4. *$p<0.05$ compared to normoxic MSCs. Each experiment was repeated 3-4 times.

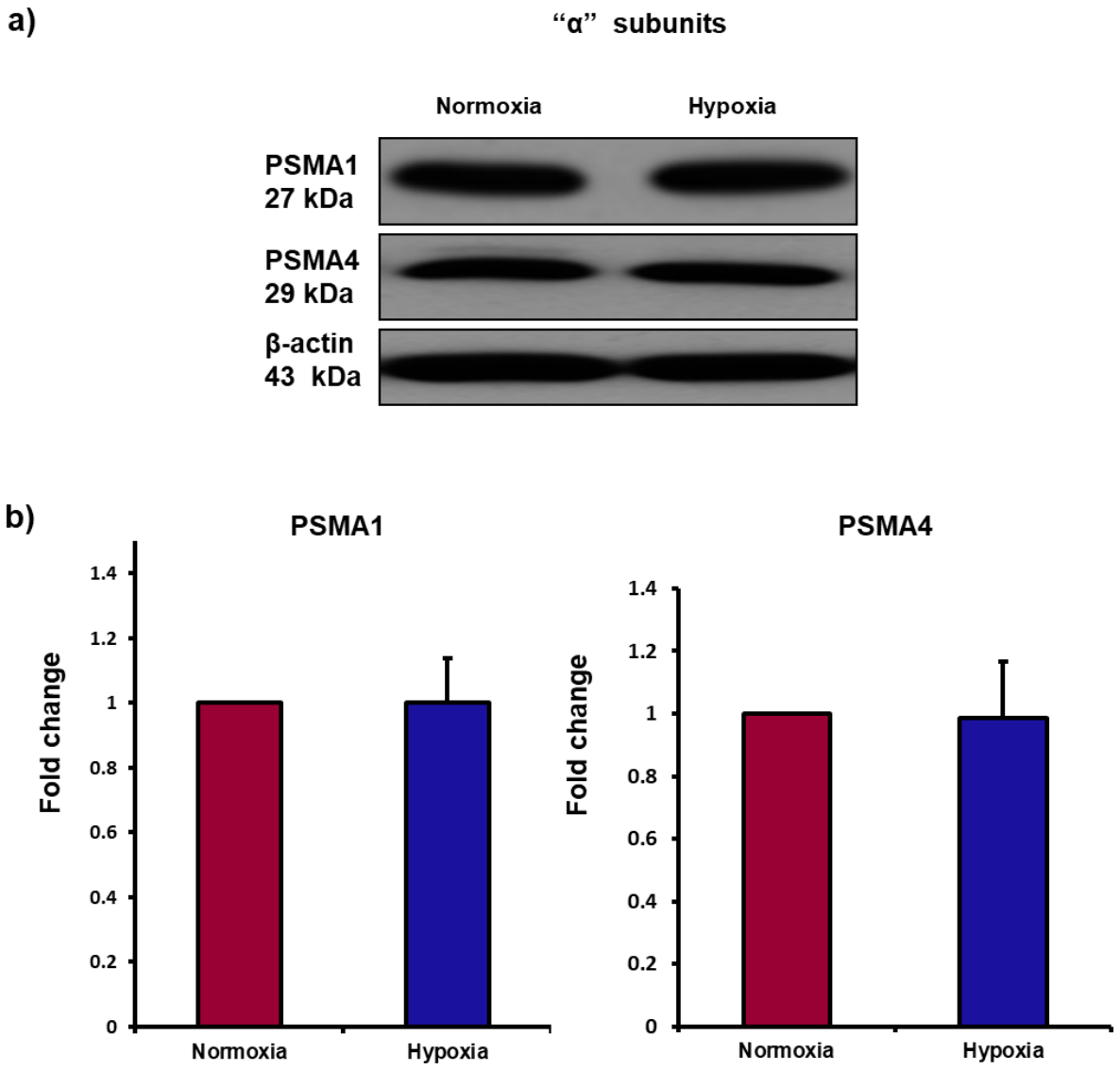


Figure 4.2: Expression of "α" subunits of 26S proteasome did not change in MSCs after exposure to hypoxia. Human bone marrow-derived MSCs were incubated in a hypoxia chamber for 24 hr. The protein levels of 26S proteasome "α" subunits, PSMA1 (α6) and PSMA4 (α3) were detected by Western blot. (a & b) Protein levels of PSMA1 (α6) and PSMA4 (α3) did not change in normoxic vs. hypoxic MSCs; n=3. Each experiment was repeated 3 times.

4.3.2 Exposure to hypoxia causes formation of immunoproteasome in MSCs: Interestingly, we found that in addition to downregulation 26S subunits, exposure to hypoxia in MSCs was associated with the upregulation of the 11S particle (or proteasome activator 28α- PA28α). 11S is a regulatory subunit which is reported to replace 19S and bind to 20S particle to form an alternative proteasome termed the "immunoproteasome" (44). The immunoproteasome is an inducible form of proteasome which is derived from the constitutive 26S proteasome under stress conditions (45,46). The formation of functional proteasome is a complex process it involves multiple subunits to bind together to form an active complex. Furthermore, the process of switching the 26S proteasome toward the immunoproteasome is quite dynamic, β1 is replaced with iβ1 (large multifunctional peptidase 2, LMP2 or PSMB9), β2 is replaced with iβ2 (multi-catalytic endopeptidase complex-like-1, MECL-1, or PSMB10), and β5 is replaced with iβ5 (large multifunctional peptidase 7, LMP7 or PSMB8), which collectively form the proteolytic core of immunoproteasome (47,48). In the current study, we measured the expression of LMP2, MECL1 and LMP7 in MSCs after exposure to hypoxia. There was a dramatic increase in the level of these proteins in hypoxic MSCs as compared to normoxic cells (Fig. 4.3 a - c).

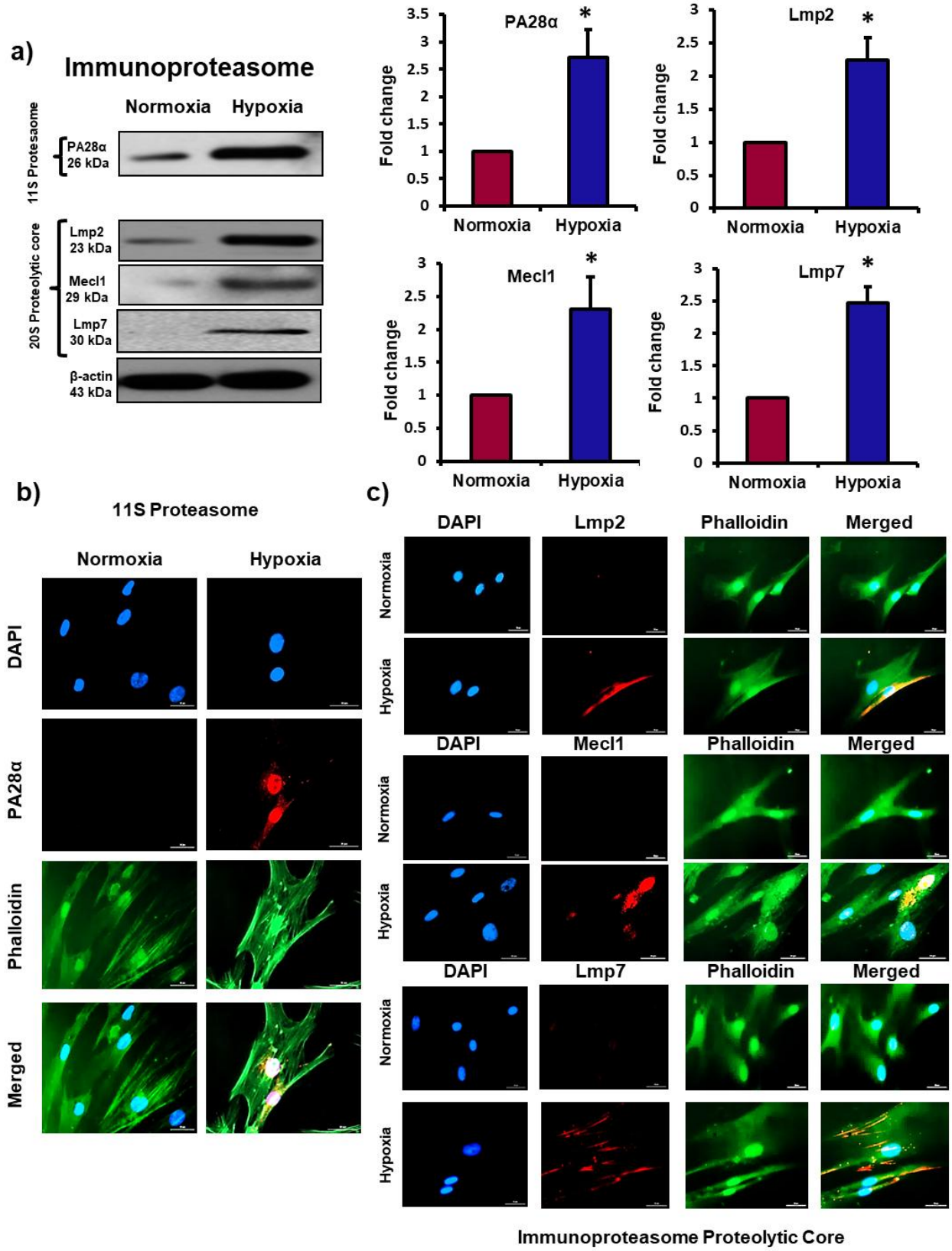
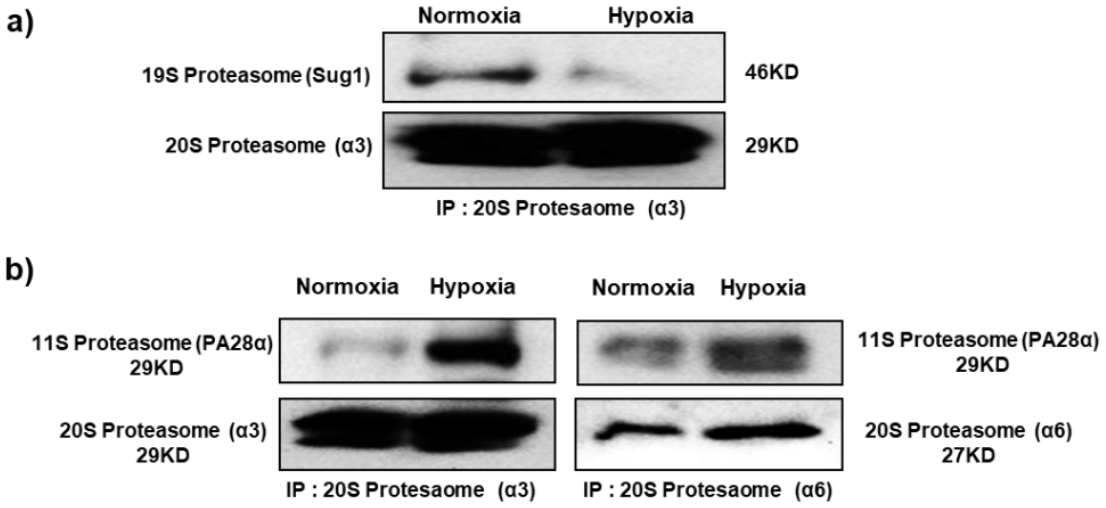

a)
Immunoproteasome
Normoxia
Hypoxia
11S Protesaome
PA28α
26 kDa
20S Proteolytic core
Lmp2
23 kDa
Mecl1
29 kDa
Lmp7
30 kDa
β-actin
43 kDa
PA28α
Lmp2
Mecl1
Lmp7
Fold change
*
b)
11S Proteasome
DAPI
PA28α
Phalloidin
Merged
c)
DAPI
Lmp2
Phalloidin
Merged
Mecl1
Lmp7
Immunoproteasome Proteolytic Core

Figure 4.3: Exposure to hypoxia causes formation of immunoproteasome in MSCs. Human bone marrow- derived MSCs were incubated in a hypoxia chamber for 24hr. (a) Western Blot analysis of PA28α (11S), LMP2 (β1i), MECL1 (β2i), and LMP7 (β5i) showed a significant increase in protein levels in hypoxic MSCs compared to normoxic cells; n=3. (b & c) Immunofluorescence images displayed a significant increase in the expression of PA28α (11S), LMP2 (β1i), MECL1 (β2i), and LMP7 (β5i) in hypoxic MSCs compared to normoxic cells; n=4. *$p<0.05$ compared to normoxic MSCs. Each experiment was repeated 3-4 times.

To further verify that exposure to hypoxia in MSCs lead to formation of immunoproteasome, we performed co-immunoprecipitation (IP) analysis to determine binding between 19S regulatory particle (Sug1 or PSMC5) and the 20S "α" subunits or PSMA4. Our data displayed a notable downregulation in the binding between Sug1 and α3 in hypoxic MSCs in comparison to the normoxic MSCs (Fig. 4.4a). On the other hand, the co-IP binding assay between 11S regulatory particle (PA28α) and 20S "α" subunits (both α3 and α6) elucidated a remarkable increase in binding between 11S proteasome and α3 (PSMA4) as well as α6 (PSMA1) in hypoxic MSCs compared to normoxic cells (Fig. 4.4b). These data confirm that in human MSC exposure to hypoxia lead to disassembly of 26S proteasome and formation of immunoproteasome.

Furthermore, in order to validate these data, we analyzed the degradative activities of 26S proteasome and immunoproteasome using specific substrates for these two proteasomes: SUC-LLVY-AMC (specific substrate for 26S proteolytic function), and Ac-PAL-AM as well as Ac-ANW-AMC (specific substrates for proteolytic activities of immunoproteasome subunits β1i/LMP2 and β5i/LMP7) (49,50). The degradation activity of 26S proteasome decreased significantly in hypoxic MSCs compared to normoxic cells (Fig 4.4c). However, the proteolytic

activity of immunoproteasome in terms of β1i/LMP2 and β5i/LMP7 activities was remarkably elevated in hypoxic MSCs in comparison to normoxic cells (Fig. 4.4c). These proof of concept data further validated hypoxia induced switch in the phenotype of proteasome degradation machinery from 26S proteasome to immunoproteasome.



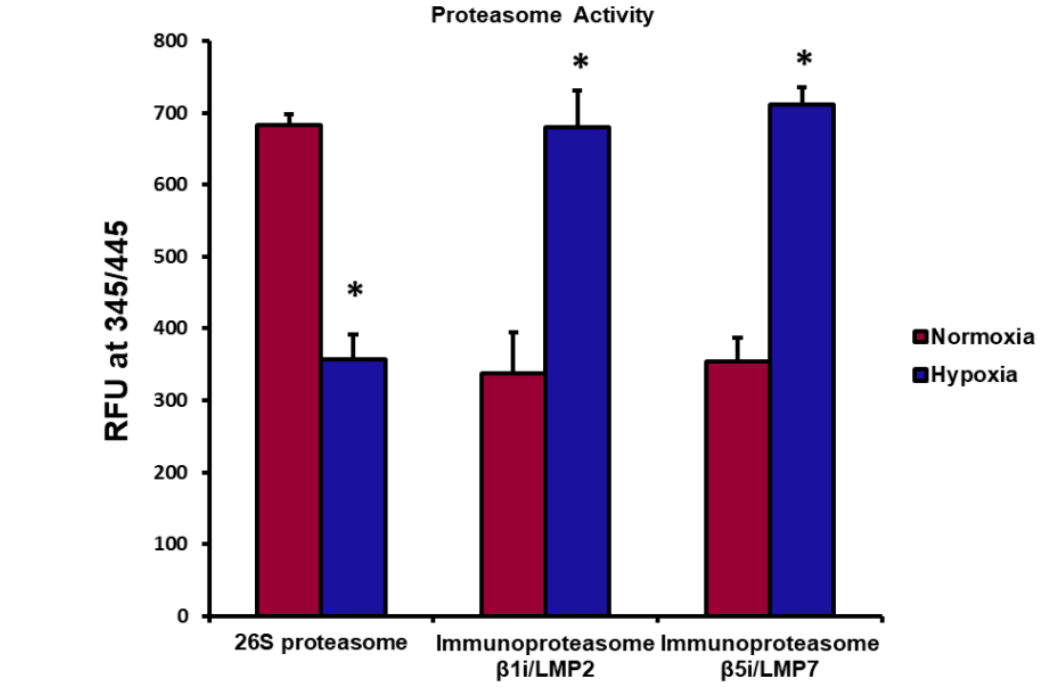


Figure 4.4: Exposure to hypoxia causes conversion of 26S proteasome to immunoproteasome in MSCs. Human bone marrow-derived MSCs were incubated in a hypoxia chamber for 24 hr. (a & b) Co-immunoprecipitation analysis was performed in cell lysates to study binding between 19S and 20S subunits; as well as binding between 11S and 20S subunits. (a) The binding affinity between 19S proteasome (Sug1) and 20S proteasome α3 (PSMA4) decreased significantly in hypoxic MSCs compared to normoxic cells. (b) The binding between 11S subunit (PA28α) and α3 (PSMA4); as well as 11S subunit (PA28α) and α6 (PSMA1) increased in hypoxic MSCs vs. normoxic MSCs. (c) Proteasome degradation activities of 26S proteasome and immunoproteasome were measured using specific substrates for these two proteasomes: SUC-LLVY-AMC (specific for 26S proteolytic function), and Ac-PAL-AM as well as Ac-ANW-AMC (specific substrates for proteolytic activities of immunoproteasome subunits β1i/LMP2 and β5i/LMP7). There was significant decrease in 26S proteasome activity in hypoxic MSCs compared to normoxic MSCs. However, immunoproteasome activity significantly increased in hypoxic MSCs compared to normoxic cells; n=5. *$p<0.05$ compared to normoxic MSC. Each experiment was repeated 3-4 times.

4.3.3 Hypoxia induced switch in the phenotype of proteasome from 26S to immunoproteasome leads to upregulation and activation of HLA-DRα in MSCs: In the next experiments, we wanted to investigate the effect of hypoxia induced change in the phenotype of proteasome from 26S to immunoproteasome on HLA-DRα expression in MSCs. Therefore, we blocked 26S and immunoproteasome in MSCs using specific inhibitors and assessed for HLA-DRα expression. The inactivation of 26S proteasome upregulated HLA-DRα expression in normoxic MSCs (Fig. 4.5a). Previously, the immunoproteasome is reported to play a crucial role in MHC-I antigen presentation by degrading immunogenic peptides which help in loading of MHC-I. However, the role of immunoproteasome in HLA-DRα or MHC-II regulation and activation in MSCs has not been investigated yet. HLA-DRα activation requires its conversion from immature state to mature antigen. Immature HLA-DRα binds to invariant chain- Ii/CD74, that masks the antigen binding groove, and antigenic peptides cannot bind to HLA-DRα (51,52). On the other hand, for maturation and activation of HLA-DRα, the Ii/CD74 is replaced with antigenic peptide HLA-DM(53). To explore the role of immunoproteasome in HLA-DRα activation in hypoxic MSCs, we performed co-immunoprecipitation assay. Our data revealed that the binding between HLA-DRα and Ii/CD74 in hypoxic MSCs increased in the presence of immunoproteasome inhibitor (Fig. 4.5 b and c), on the other hand the binding between HLA-DRα and HLA-DM decreased in hypoxic MSCs when immunoproteasome activity was blocked (Fig. 4.5 b and c). Therefore, hypoxia induced formation of immunoproteasome is responsible for maturation and activation of HLA-DRα in MSCs.

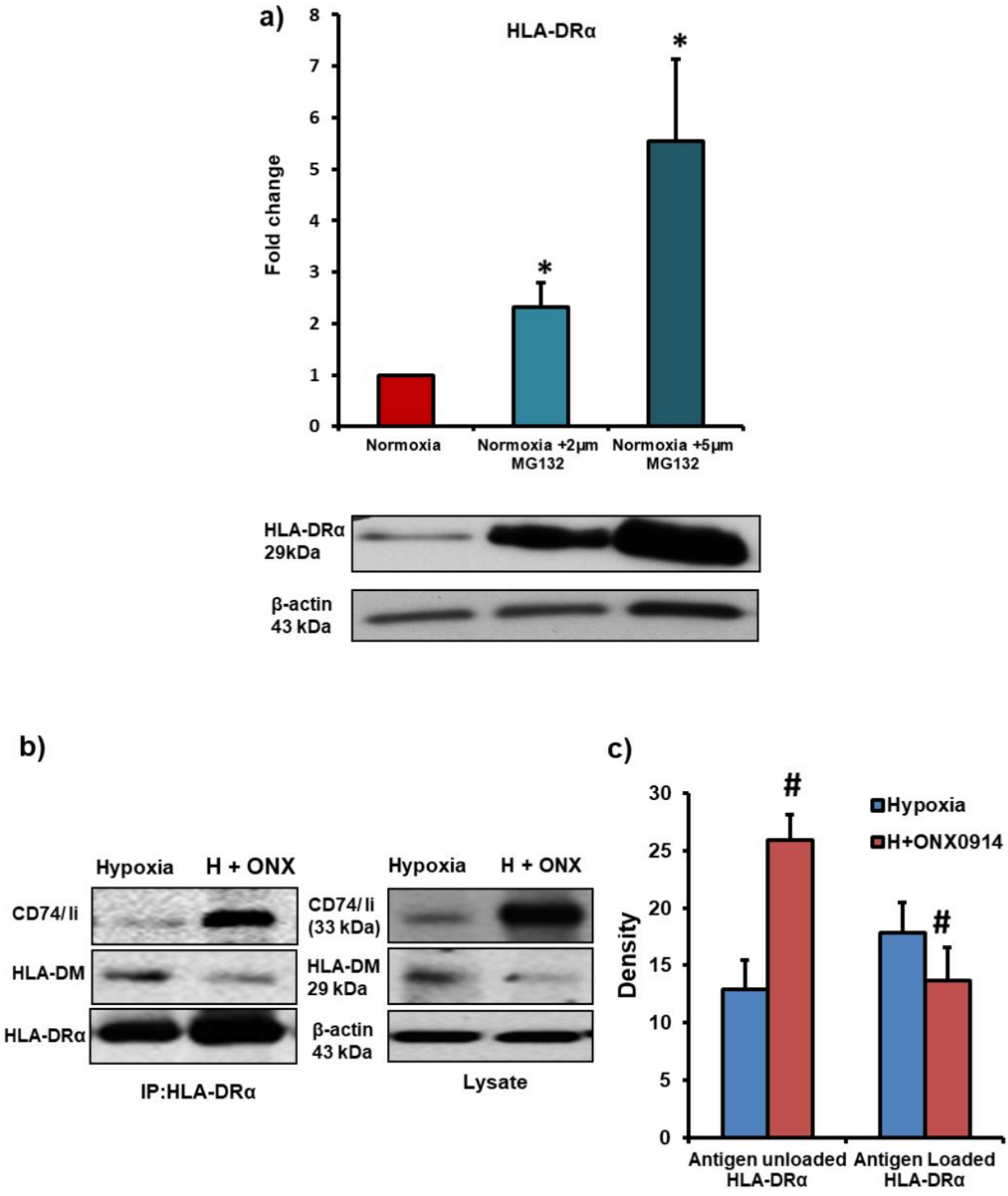


Figure 4.5: Hypoxia induced switch in the phenotype of proteasome from 26S to immunoproteasome leads to upregulation and activation of HLA-DRα in MSCs. (a) Human

bone marrow-derived MSCs were treated with 26S proteasome inhibitor (MG132, 2μM and 5μM) for 24 hr. Western blot analysis revealed a significant increase in HLA-DRα protein levels in 26S proteasome inhibited normoxic MSCs; n=3. (b & c) MSCs were incubated in hypoxia chamber with or without immunoproteasome inhibitor (Onx0914 1μM for 4 hr). Next, co-immunoprecipitation assay was performed in cell lysates to measure- the binding between HLA-DRα and invariant chain- Ii/CD74 (to measure the levels of immature HLA-DRα, or antigen unloaded HLA-DRα); and the binding between HLA-DRα and HLA-DM (to measure levels of activated HLA-DRα, or antigen loaded HLA-DRα). Inhibition of immunoproteasome increased the binding between HLA-DRα and Ii/CD74; and decreased the binding between HLA-DRα and HLA-DM in hypoxic MSCs; n=3. *$p<0.05$ compared to normoxic MSCs, # $p<0.05$ compared to hypoxic MSCs. Each experiment was repeated 3-4 times.

4.3.4 Hypoxia induced formation of immunoproteasome is responsible for loss of immunoprivilege of MSCs: In order to explore the effect of hypoxia induced formation of immunoproteasome on immunoprivilege of MSCs, the cells were treated with immunoproteasome inhibitor (Onx0914, 1 μm for 4hr), and immunogenicity of MSCs was studied under normoxic and hypoxic conditions. To assess immunogenicity, human MSCs were co-cultured with allogenic leukocytes for 72 hr and leukocyte mediated cytotoxicity in MSCs was evaluated by measuring the amount of lactate dehydrogenase released. The level of cytotoxicity increased significantly in hypoxic MSCs compared to normoxic cells (Fig. 4.6a). Treatment with the immunoproteasome inhibitor prevented hypoxia induced increase in leukocyte mediated cytotoxicity in MSCs (Fig. 4.6a). Interestingly, the presence of the immunoproteasome inhibitor had no effect on the level of cytotoxicity in normoxic MSC (Fig. 4.6a). This observation further verified the formation of immunoproteasome in hypoxic MSCs.

Bone marrow MSCs have the ability to downregulate leukocyte proliferation and suppress allo-immune responses(10,33). In the current study, we found that after 72h of co-culture with leukocytes, normoxic MSCs were able to suppress leukocyte proliferation (Fig. 4.6b). Hypoxia exposed MSCs were unable to suppress leukocyte proliferation (Fig. 4.6b). However, immunoproteasome inhibited hypoxic MSCs were able to suppress leukocyte proliferation (Fig. 4.6b).

MSCs are also reported to suppress allo-immune responses by promoting phenotype change from cytotoxic T cells toward immunosuppressive regulatory T (Treg) cells (54,55). Treg cells are known to suppress the proliferation of cytotoxic T cells and promote immune tolerance. We counted the number of $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells in a mixed leukocyte population after co-culture with allogeneic MSCs by flow cytometry. The Treg cell number decreased after co-culture with hypoxia exposed MSCs compared to normoxic MSCs (Fig. 4.6c). However, immunoproteasome inhibited hypoxic MSCs were able to upregulate Treg cell number in a mixed leukocyte population (Fig. 4.6c). Therefore hypoxia induced formation of immunoproteasome leads to upregulation and activation of HLA-DRα and loss of immunoprivilege of MSCs.

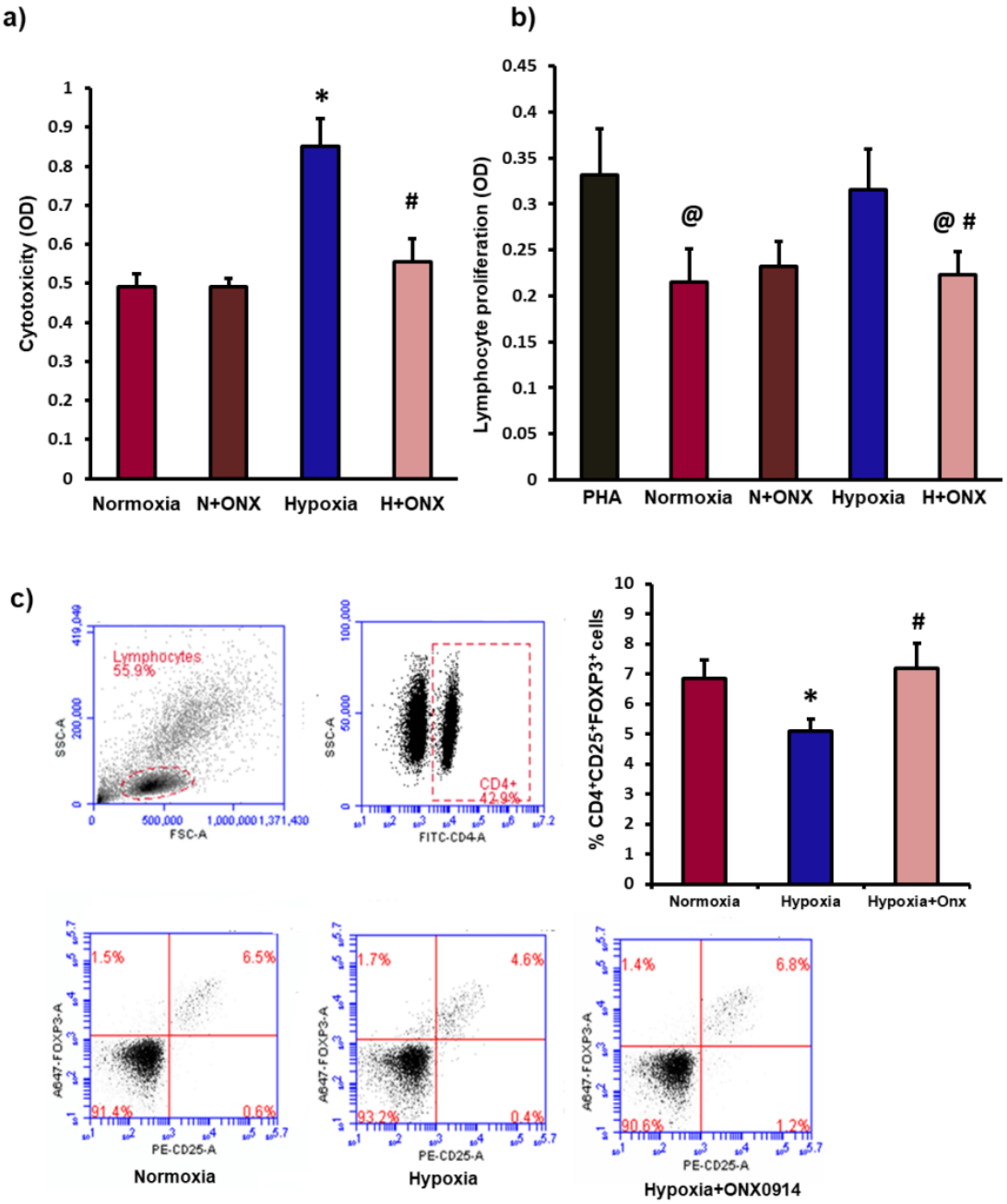


Figure 4.6: Hypoxia induced formation of immunoproteasome is responsible for loss of immunoprivilege of MSCs. To determine the immunogenicity of MSCs, normoxic and hypoxic human MSCs (with or without immunoproteasome inhibitor- Onx0914 1µM for 4 hr) were co-cultured with allogeneic leukocytes at a ratio 1:10 for 72 hr. (a) Leukocyte mediated cytotoxicity in MSCs (LDH release) increased significantly in hypoxic MSCs vs. normoxic cells, which was

rescued by inhibition of immunoproteasome. n=5; (b) The effect of MSCs on leukocyte proliferation was measured using WST1 proliferation assay kit. After 72 h of co-culture, normoxic MSCs were able to decrease leukocyte proliferation compared to control (PHA treated leukocytes). However, hypoxia treated MSCs had no effect on leukocyte proliferation, immunoproteasome inhibited hypoxic MSCs significantly decreased leukocytes proliferation. n=10; (c) After 72 h of co-culture, the effect of MSCs on $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cell induction in a mixed leukocyte population was assessed by flow cytometry. The number of Treg cells decreased after co-culture with hypoxic MSCs. However, co-culture with immunoproteasome inhibited hypoxic MSCs increased the number of Treg cells. n=3. *$p<0.05$ compared to normoxic MSC; @$p<0.05$ compared to PHA group; #$p<0.05$ compared to hypoxic MSCs, each experiment was repeated 3-4 times.

4.4 Discussion

Bone marrow derived allogeneic MSCs are being investigated in phase I and phase II clinical trials as potential therapies for several degenerative diseases (3,18,56). Despite encouraging therapeutic benefits after MSCs transplantation in patients, overall excitement of MSCs based therapies of lately has diminshed. A Major hurdle in bringing allogeneic MSCs to the clinic is the poor survival of cells after transplantation in the host tissue (24,28). MSCs were considered to be a preferred cell type to treat degenerative diseases because these cells were initially reported to be immunoprivileged (9,33). Therefore, the universal belief was that allogeneic transplants would be possible without MSCs being rejected by the host immune system. However, the outcome of recent allogeneic MSCs based preclinical studies and clinical trials suggested that cells after transplantation in the host microenvironment become immunogenic and are rejected by host immune system (30,31). Immunoprivilege of MSCs is preserved by absence

of cell surface immune antigen HLA-DRα that allows escape of transplanted MSCs from host immune system. HLA-DRα is responsible for antigen presentation which is a critical step to alert host $CD4^+$ T-cells to initiate allo- immune response against allograft. We have previously reported that 26S proteasome mediated degradation of HLA-DRα maintains absence of this molecule on MSC surface and preserves immunoprivilege of cells (31). We have also reported that exposure to hypoxic or ischemic environments is responsible for loss of the immunoprivilege (31). Hypoxic or ischemic injury is related to several pathological conditions at organ or tissue level such as myocardial infarction, stroke and peripheral vascular disease. Therefore, effect of hypoxic or ischemic environment on immunological behavior of MSCs needs to be studied in more detail in order to discover approaches to preserve immunoprivilege of transplanted cells under pathological conditions. In the current study, we investigated the effect of hypoxic environment on proteasome complex and immunoprivilege of allogeneic MSCs. The 26S proteasome is a master degradation system inside a cell; it contains a 19S regulatory particle and a 20S core particle. The 26S proteasome activity requires coordinated action of 19S and 20S assembly for carrying out degradation and proteolysis of ubiquitinated proteins. Basically, 19S particle receives ubiquitinated target protein and PSMD11 and PSMD4 subunits of 19S regulatory unit deubiquitinate the target protein and transfer it to the proteolytic core of 20S where the target protein is processed and degraded (39,43). The 20S particle contains three subunits β1 (or PSMB6), β2 (or PSMB7) and β5 (or PSMB5) which are responsible for proteolytic activities of 26S proteasome (45,48). In the current study, there was a significant decrease in the expression of PSMD11, PSMD4 and proteolytic subunits β1, β2 and β5. Therefore, exposure to hypoxic environment leads to dysfunction of 26S proteasome degradation machinery in allogeneic MSCs.

Furthermore, in the current study we found that hypoxic stress leads to formation of immunoproteasome in MSCs. Immunoproteasome is an inducible form of proteasome which is found in immune cells under stress conditions or after exposure to pro-inflammatory cytokines (44,46). Current study is the first to report formation of immunoproteasome in MSCs under hypoxic conditions. Interestingly, we found that blocking immunoproteasome formation prevented hypoxia induced increase in immunogenicity of allogeneic MSCs. Therefore, hypoxia induced inactivation of 26S proteasome and upregulation of HLA-DRα is not sufficient to induce immunogenicity of MSCs, instead formation of immunoproteasome in response to treatment with hypoxia is a crucial step toward increasing MSCs immunogenicity. Immunoproteasome is large proteolytic machinery, it is abundantly expressed in immune cells, such as antigen presenting cells (57,58) .The immunoproteasome has been involved in the pathogenesis of several inflammatory diseases such as autoimmune diseases (47,57). Therefore, blocking immunoproteasome is believed to be a clinically relevant strategy to treat inflammatory diseases in the future. Immunoproteasome has also been reported to play a role in skeletal muscle differentiation (59,60). However, the role of immunoproteasome in HLA-DRα activation and immunogenicity of mesenchymal stem cells has not been reported yet. HLA-DRα molecule is assembled in endoplasmic reticulum, where it associates with invariant chain- Ii/CD74, that masks the antigen binding groove, and antigenic peptides cannot bind to HLA-DRα (51,52). On the other hand, cleavage of HLA-DRα from Ii/CD74, and its association with HLA-DM facilitates activation of HLA-DRα and it's binding to antigenic peptides that promote antigen processing. In the current study, we found that blocking immunoproteasome formation in hypoxic MSCs prevented association between HLA-DRα and HLA-DM, it rather promoted binding between HLA-DRα and Ii/CD74 (51). These data confirm that hypoxia induced

formation of immunoproteasome is responsible for maturation and activation of HLA-DRα in MSCs. In this regard, previous studies have reported that immunoproteasome mediates antigen presentation role of MHC-I in immune cells by preparing and loading antigenic peptides on MHC-I to alert host $CD8^+$ T cells (46). However, the current study is the first to demonstrate the role of immunoproteasome in HLA-DRα activation. Furthermore, our findings also confirm that blocking immunoproteasome formation in hypoxic MSCs preserves their immunoprivilege. Therefore, these observations provide unique insight into the mechanisms responsible for hypoxia or ischemia induced increase in the immunogenicity of allogeneic human MSCs. Bone marrow derived allogeneic MSCs are in clinical trials for treating wide range of inflammatory and degenerative diseases (20,61). The outcome of initial clinical trials reported positive outcome and beneficial effects of transplanted MSCs (19,20,56). However, a major limitation acknowledged by experts in the field is poor survival of transplanted MSCs in the recipient. In fact, it is now established that even though MSCs are immunoprivileged under *in vitro* conditions, after transplantation in the host tissue MSCs become immunogenic and are rejected by host immune system (29,30,62). Therefore, the outcome of current study may provide molecular targets to plan interventions to prevent rejection of transplanted MSCs in the hypoxic or ischemic environment.

4.6 Material and methods

Human mesenchymal stem cells: Bone marrow-derived-human MSCs were purchased from Lonza (PT 2501 CA10064-080). All the human MSCs based studies were approved by the University of Manitoba's Research Ethics Board.

Experimental treatments: Hypoxia treatment was employed for 24 hr, the culture plates were placed in hypoxia chamber (oxygen level regulated at 0.2-0.4%) in the incubator (Biospherix

hypoxia chamber). To block 26S proteasome, MSCs were treated with a specific inhibitor MG132 (2μM and 5μM) for 24 hr. To inhibit immunoproteasome activity in MSCs, the cells were treated with Onx0914 (1μM) for 4 hr.

Western blot: The protein levels for HLA-DRα, PSMD11, PSMD4, β1, β2, β5, PSMA1, PSMA4 PA28α, LMP2, LMP7, and MECL-1 were measured by Western blot using specific antibodies as described in our previous studies (10,31). Briefly, total protein levels were measured by Bradford method and 35μg of protein was loaded onto SDS-PAGE. After separation with SDS-PAGE, proteins were transferred to PVDF membrane and incubated with appropriate primary and secondary antibodies. The membranes were developed using X-ray film, and bands were quantified using Quantity One software for densitometry.

Immunoprecipitation: To study protein-protein interactions, immunoprecipitation was performed using manufacturers' guidelines (Santa Cruz Biotechnology). Briefly, cell lysates were prepared from different treatment groups and precleared using appropriate preclearing matrix. To form IP antibody-IP matrix complex, 20 μl of suspended (25% v/v) IP matrix, 1-5 μg of IP antibody in 500 μl of PBS were incubated overnight at 4°C. Total cellular protein (300 μg) was transferred to the pelleted matrix and incubated overnight at 4°C. The protein samples were then analyzed using SDS-PAGE as described for the Western Blotting procedure and were probed with primary antibodies and secondary ones. The membranes were developed using X-ray film, and quantification of bands was performed using Quantity One software.

Immunocytochemistry: Immunohistochemistry was performed to measure expression of PSMD11, PSMD4, β1, β2, β5, PA28α, LMP2, LMP7, and MECL-1 in MSCs as described in our previous studies (11,31). The cells were seeded onto sterile coverslips, and allowed to expand until 60% confluency. MSCs were fixed with 4% PFA and permeabilized using 0.2% Triton X in

PBS at room temperature. The cells were then stained with primary and secondary antibodies as well as phalloidin (for F-actin). Nuclei were stained with DAPI. Finally MSCs were imaged using Cytation 5 system (BioTek Instruments).

Measurement of 26S proteasome activity: 26S proteasome activity was measured by determining the proteolytic activity of 20S subunit using a kit purchased from Cayman Chemicals (10008041). The fluorescent substrate (SUC-LLVY-AMC) in the kit was used to determine the activity and fluorescent intensity of each well was read at 350 nm (excitation) and 480nm (emission).

Immunoproteasome activity assay: To determine immunoproteasome activity, we measured proteolytic activities of β1i/LMP2 and β5i/LMP7 subunits, using fluorescent substrates- S310 (Ac-PAL-AMC) and S-320 (Ac-ANW-AMC) respectively. The fluorescence intensity of each well was read at 345 nm (excitation) and 445 nm (emission).

Leukocyte mediated cytotoxicity: To measure leukocyte mediated cytotoxicity in MSCs, commercially available human leukocytes were purchased from Stem Cell Technologies (Cat# 70025) and were co-cultured with allogeneic human MSCs in the ratio of 10:1 as described in our previous studies (10,31). After 72 hr of co-culture, leukocyte-mediated cytotoxicity in MSCs was determined by measuring the lactate dehydrogenase (LDH) which was released from the damaged MSCs (LDH Cytotoxicity Detection Kit; Clontech).

Leukocyte proliferation assay: Leukocyte proliferation after 72 hr of co-culture with MSCs was measured using the commercial kit (Biovision, Catalog #K301. Briefly, MSCs were seeded in 96 well plate ($5x10^4$ cells/well) and incubated for 24 hr. Then cells in different groups were co-cultured with allogenic human leukocytes (Stem Cell Technologies, Cat# 70025) at a ratio of 1:10. Leukocytes were pre-activated in the presence of 10 μg/ml phytohemagglutinin (PHA) for

24 hr. After 72 hr of co-culture, leukocytes suspended in the media were transferred to another 96 well plate, followed by the addition of 10µl of WST1 solution to each well. After incubation for 2 hr, the absorbance values were taken at 450 nm using Cytation 5 system (BioTek Instruments).

Regulatory T cell measurement assay: The number of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) were counted using flow cytometry (BD Accuri^{TM} C6) in the total leukocyte population after 72 hr of co-culture with allogeneic MSCs as described in our previous studies (11,31). Leukocytes were washed and subsequently stained with following monoclonal antibodies: FITC anti-rat CD4 (W3/25, BioLegend 201505), PE anti-rat CD25 (OX-39, BioLegend 202105), and Alexa Flour® 647 anti-mouse/rat/human FOXP3 (150D, BioLegend 320014). Appropriate isotype controls and a viability stain (BD Horizon$^{TM.}$Fixable Viability Stain 620, BD Biosciences 564996) were used. Lymphocytes were identified by their forward and side-scatter profiles and subsequently were gated on the $CD4^+$ T-cells from which the $CD25^+FOXP3^+$ subpopulation was identified.

Statistical analysis:

Data were reported as mean ± SD. Comparison of data between multiple groups was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test, and analysis between two groups was made using Student's t-test (two-tailed). Statistical significance is determined as $p<0.05$.

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Conflict of interest

Authors declare no conflict of interest.

Author Contributions

E.A.R, V.D and S.D conceptualized the study; E.A.R, V.D and S.D designed the experiments; E.A.R, N.S, W.Y, K.N.A, A.R and A.S carried out the experiments, acquired and analyzed the data; E.A.R, N.S, W.Y., and S.D. interpreted the data and performed statistical analyses. E.A.R, V.D and S.D drafted the manuscript. All the authors have read and approved the final version of the manuscript.

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Chapter V: Conclusions and Future directions:

Stem cells based regenerative therapies have the potential to treat numerous degenerative diseases including cardiovascular diseases. It is known that conventional medications can simply manage symptoms of disease. On the other hand, regenerative medicine including cell therapy and tissue engineering seeks to replenish and reestablish the function of damaged tissues or organs. This can be achieved by replacing damaged cells with new functional ones, along with modulating the internal microenvironment by regulating the action of trophic, anti-inflammatory, and immunomodulatory factors. In this regard, mesenchymal stem cells (MSCs) are clinically the most preferred cell type for regenerative therapies due to their unique biological properties, including ease of isolation, culture, maintenance, expansion and self-renewal capacity. Furthermore, MSCs are considered to be immunoprivileged and possess immunomodulatory properties that allow for allogeneic and xenogeneic transplantations. Due to these properties, MSCs are being tested in clinical trials to treat different diseases. To date, more than 900 MSCs based clinical trials have been reported and registered in the database "ClinicalTrials.gov." The outcome of many of these clinical trials reported that MSCs are safe to inject and are able to improve function and promote regeneration after transplantation. However, in spite of this encouraging outcome, there have been several challenges or obstacles that are acting as limiting factors in implementing MSCs based therapies in the treatment guidelines. One of the major hurdles is the rapid rejection of transplanted stem cells by the host immune system that leads to poor survival of cells in the recipient tissue. The host microenvironment in the diseased tissues plays a very important role in deciding the fate of implanted cells. We found that exposure to hypoxia and ischemic environment in the infarcted heart leads to change in the phenotype of MSCs from immunoprivileged to immunogenic state and accelerates their rejection. The

immunoprivilege of MSCs is preserved by negligible expression or absence of the cell surface immune antigen MHC-II (HLA-DR in humans). MHC-II is expressed on the surface of cells, and it plays a key role in alerting the host immune system against transplanted cells or organs by presenting antigens to cytotoxic $CD4^+$ T lymphocytes. We performed further experiments to understand the mechanisms of hypoxia induced increase in MHC-II, our studies demonstrate that 26S proteasome-mediated degradation of ubiquitinated MHC-II protein is essential to maintain its low levels and preserve immunoprivilege of MSCs. Our data demonstrate that exposure to hypoxia leads to inactivation of the 26S proteasome that results in an increase in MHC-II expression and loss of immunoprivilege of MSCs. The binding of 19S and 20S subunits to form a functional 26S proteasome, is mediated by a chaperone protein HSP90α; in our studies the levels of HSP90α decreased in hypoxic MSCs. We also found that overexpression of HSP90α in MSCs maintained 26S proteasome activity and immunoprivilege of cells even following hypoxia treatment.

Furthermore, it has been reported that 19S proteasome ATPase "Sug1" has a non-proteolytic function as an activator of transcription of several genes. In our studies, there was a significant increase in Sug1 in hypoxia exposed MSCs. Also our immunoprecipitation data suggest that there was an upregulation in the binding of Sug1 to CIITA, which is a master regulator of MHC-II gene. CIITA binds to the Cis-factors of MHC-II enhanceosome including RFX5 and recruits the transcription machinery to initiate the transcription of MHC-II. We observed a significant increase in CIITA protein levels that further led to MHC-II increase in hypoxia exposed MSCs. Furthermore, our data demonstrated that upregulation of Sug1-CIITA binding in hypoxic MSCs induced post-translational activation of CIITA through the recruitment of histone acetyltransferase PCAF. PCAF is recently reported to act as an E3 Ligase. It enhances the

acetylation and K63 ubiquitination of CIITA causing its activation. Inhibiting Sug1 preserved immunoprivilege of MSCs even following hypoxia. Furthermore, we found a significant increase in the levels of 11S particle in hypoxic MSCs. 11S is a regulatory subunit, it is reported to bind to 20S proteolytic core to form an alternate proteasome, "immunoproteasome". As mentioned previously, in our studies there was a significant decrease in 26S activity in hypoxic MSCs. These findings prompted us to examine the possibility of a shift in the phenotype of 26S proteasome toward immunoproteasome. Interestingly, in hypoxia treated MSCs the binding between 20S and 11S increased to form immunoproteasome. Furthermore, our data demonstrate that formation of the immunoproteasome in hypoxic MSCs was associated with maturation and activation of HLA-DR. Therefore our studies uncover the novel pathways responsible for hypoxia induced increase in immunogenicity of MSCs. Furthermore, these studies also reveal that hypoxia induced changes in the immunological behavior of MSCs are not limited to rat cells, human MSCs also become immunogenic under hypoxic conditions. Importantly, our studies also show that therapeutic interventions are possible through pharmacological or genetic modifications of MSCs to preserve their immunoprivilege. Therefore, these studies may help in interpreting the outcome of ongoing allogeneic MSCs based clinical trials and allow a better planning for future trials.

Limitations of our studies*:* Although MHC-II molecules are reported to have major contribution in triggering allo-graft rejection, however, the role of other immunogenic surface markers such as CD80 and CD86 should be examined in future in hypoxic MSCs to comprehensively understand the role of hypoxia induced switch in the phenotype of MSCs from immunoprivileged to immunogenic state. In this thesis report we systematically tested the effect of hypoxia on immunoprivilege of bone marrow derived MSCs. However, in future these the

studies can be performed in other cell types e.g. adipose tissue derived stem cells and umbilical cord derived stem cells. Furthermore, our findings can be further validated by different ischemic disease models such as limbal ischemia and ischemic acute renal injury.

Future Directions:

The current study confirms that exposure to hypoxic environment leads to activation of MHC-II and loss of immunoprivilege of allogeneic MSCs. We also found that transplantation of allogeneic MSCs in the ischemic heart in a rat model leads to activation of host immune response and rejection of implanted cells. Therefore in order to improve the efficacy of allogeneic MSC therapy for cardiac repair, future studies should be directed toward validation of these findings in large animal models and approaches to prevent upregulation of MHC-II in hypoxic or ischemic environment, and prevent rejection of transplanted MSCs in the injured heart.

Graphical summary of the thesis:

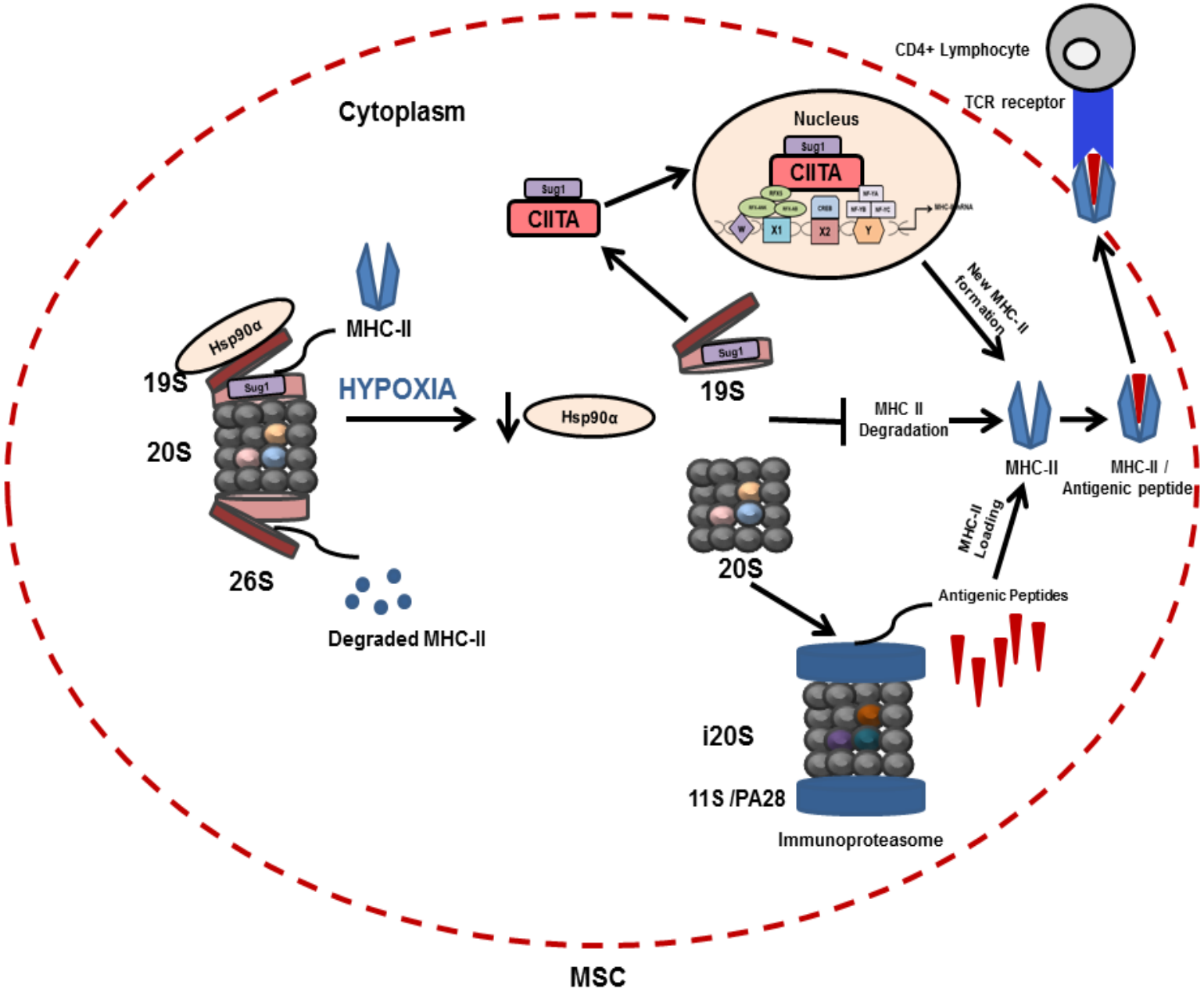

Figure 5: Graphical Summary of the thesis. The figure is a diagrammatic snapshot of the thesis. In normoxic MSCs 26S proteasome mediated degradation of MHC-II preserves immunoprivilege of allogeneic MSCs. Exposure to hypoxia leads to inactivation of 26S proteasome, upregulation of MHC-II and loss of immunoprivilege of MSCs. Furthermore, the disassembled 19S proteasome ATPase subunit "Sug1" activates CIITA to initiate the process of MHC-II transcription producing new MHC-II molecules. Also in hypoxic MSCs, the constitutive 20S proteolytic core is converted to an induced 20S proteolytic core (i20S) which binds to the 11S/ PA28 regulatory particle to form an immunogenic proteasome complex called immunoproteasome. Immunoproteasome processes antigenic peptides to be loaded on MHC-II molecules, which are required for MHC-II full expression and antigen dependent immune system activation.