Université de Montréal

UM171-INDUCED ROS PROMOTE ANTIGEN CROSS-PRESENTATION OF IMMUNOGENIC PEPTIDES BY BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS

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Résumé

En raison de leur multipotence considérable, les cellules stromales mésenchymateuses (CSM) ont été énormément utilisées en clinique dans le contexte de la médecine régénérative. Pourtant, la stimulation des CSM avec de faibles concentrations d'interféron-gamma (IFN-gamma) déclenche une augmentation du complexe majeur d'histocompatibilité de classe I et II, et surtout une capacité *de novo* de présentation croisée des antigènes. Ainsi, malgré leurs propriétés immunosuppressives naturelles, les CSM peuvent être modulées pour devenir pro-inflammatoires. Comme le dérivé pyrimidoindole UM171 induit l'augmentation de l'expression de plusieurs gènes impliqués dans la présentation antigénique dans les cellules souches hématopoïétiques humaines, nous avons étudié son potentiel pour le déclenchement de la présentation antigénique par les CSM. L'analyse par cytométrie en flux a montré une élévation des niveaux de H2-k^B après le traitement avec le médicament, en corrélation avec une augmentation de la présentation de l'antigène, démontrée par une activation plus importante de la lignée de cellules T B3Z spécifique au peptide SIINFEKL. Cette présentation croisée *de novo* d'un peptide immunogène ne résulte pas d'une augmentation de l'absorption ou de la digestion enzymatiques des protéines, mais plutôt de l'expression du gène *Psmb8* induit par le médicament. Comme le traitement avec plusieurs antioxydants et inhibiteurs des complexes de la chaîne de transport des électrons a réduit de manière significative les effets observés, nous concluons que la présentation croisée médiée par UM171 est dépendante des ROS. Dans le contexte de la vaccination thérapeutique, l'immunisation avec des CSM traitées par UM171 chez des souris présentant des tumeurs EG.7 préétablies a permis d'obtenir un taux de survie de 40%. Dans l'ensemble, notre étude révèle une nouvelle approche pharmacologique pour modifier les CSM afin qu'elles deviennent des cellules présentatrices d'antigènes, ce qui permet de développer de nouveaux vaccins anticancéreux innovants et puissants.

Mots-clés : cellules stromales mésenchymateuses, cross-présentation antigénique, UM171, espèces réactives d'oxygène, psmb8

Abstract

Due to their considerable multipotency, mesenchymal stromal cells (MSCs) have been tremendously employed in the clinic for regenerative medicine. Yet, stimulation of MSCs with low concentrations of interferon-gamma (IFN-gamma) triggers an increase in the major histocompatibility complex I and II, and most importantly, a *de novo* antigen cross-presentation capacity. Thus, despite their natural immunosuppressive properties, MSCs can be modulated to become pro-inflammatory. As the pyrimidoindole derivative UM171 induces the upregulation of several antigen presentation-involved genes in human hematopoietic stem cells, we investigated its potential for inducing antigen presentation by MSCs. Flow cytometry analysis showed an upregulation in H2-k^B levels after treatment with the drug, correlating with an increase in antigen presentation indicated by higher activation of the SIINFEKL-specific B3Z T cell line. This *de novo* cross-presentation of an immunogenic peptide did not result from an increase in protein uptake or processing, but rather stemmed from the drug-induced expression of the *Psmb8* gene. As treatment with multiple antioxidants and inhibitors of the electron transport chain complexes significantly reduced the observed effects, we conclude that UM171-mediated cross-presentation is ROS-dependent. In the context of therapeutic vaccination, immunization with UM171-treated MSCs in mice with pre-established EG.7 tumors resulted in 40% survival. Overall, our study reveals a new pharmacological approach in modifying MSCs to become antigen presenting cells, hence allowing the development of future innovative and potent anti-tumoral vaccines.

Keywords: mesenchymal stromal cells, antigen cross-presentation, UM171, reactive oxygen species, Psmb8

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List of abbreviations

ALL: Acute lymphoblastic leukemia

ANOVA: Analysis of variance

APC: Antigen presenting cell

β2M: Beta 2-microglobulin

Calr: Calreticulin

CAR T cells: Chimeric antigen receptor T cells

CPRG: Chlorophenol red-β-D-galactopyranoside

cTEC: Cortical thymic epithelial cell

DC: Dendritic cell

DEG: Differentially expressed gene

EPCR: Endothelial protein C receptor

ER: Endoplasmic reticulum

ERAD: Endoplasmic-reticulum-associated protein degradation

ERAP1: Endoplasmic reticulum-associated aminopeptidase 1

ETC: Electron transport chain

FBS: Fetal bovine serum

FDA: USA Food and Drug Administration

GM-CSF: Granulocyte macrophage-colony stimulating factor

GvHD: Graft-versus-host-disease

HLA: Human leukocyte antigen

HSC: Hematopoietic stem cell

ICI: Immune checkpoint inhibitor

IL: Interleukin

IFN: Interferon

IPr: Immunoproteasome

LT-HSC: Long term-hematopoietic stem cells

LIX: Lipopolysaccharide-induced CXC chemokine

MEF: Murine embryonic fibroblast

MHC: Major histocompatibility complex

MHCI: Major histocompatibility complex I

MHCII: Major histocompatibility complex II

MIIC: MHC class II compartment

MSC: Mesenchymal stromal cell

MTD: Maximum tolerated dose

NAC: N-acetylcysteine

NK: Natural killer cell

NRF2: Nuclear factor erythroid 2–related factor 2

OVA: Ovalbumin

PD-1: Programmed cell death protein 1

PD-L1: Programmed death ligand 1

PEG: Polyethylenglycol

PI3K: Phosphoinositide-3-kinase

PSMB8: Proteasome 20S Subunit Beta 8

ROS: Reactive oxygen species

SC: Sub-cutaneous

SD: Standard deviation

STAT3: Signal transducer and activator of transcription 3

TAA: Tumor-associated antigen

TAP: Transporters associated with antigen presentation

TCR: T cell receptor

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

TPr: Thymoproteasome

Treg: Regulatory T cell

UC: Umbilical cord

VEGF: Vascular endothelial growth factor

Wnt signaling: Wingless-type MMTV integration site

To my parents,

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Chapter 1 – Introduction

1.1 The Immune System

1.1.1 The Immune System's Role

The immune system is responsible for distinguishing self from non-self, hence protecting the body from endogenous- or exogenous-derived diseases. Constituted of white blood cells, tissues and organs including the thymus, spleen, lymph nodes and vessels, tonsils and bone marrow, the immune system is generally divided into two branches: innate and adaptive immunity(1).

1.1.2 The Innate Immune System

The innate immune system is the first barrier of defense, present since birth. It is composed of physical barriers, as the skin and mucous, physiological barriers and cellular non-specific responses mediated by macrophages, mast cells, dendritic cells, and polymorphonuclear cells (including neutrophils, basophils and eosinophils). Cytokine release and toll-like receptors play a major role in innate immunity. As an example, the toll-like receptor-4 recognizes LPS and triggers immunity against bacteria. However, the innate immune system's ability to fight remains limited, due to its lack of specificity. If this primary immune response does not suffice, adaptive immunity will be induced to generate a more specific response(1).

1.1.3 The Adaptive Immune System

The adaptive immune system is both specific and modulated with time and exposure to several stimuli. First, it involves antibody production by plasma cells that originate from B cells' differentiation. Also, T cells cytotoxicity is essential to the killing of target cells expressing non-self-antigens. T cells are activated following recognition of the antigen presented by antigen presenting cells (APCs). The central distinction between adaptive and innate immunity is the establishment of an immunological memory(1).

1.1.4 The Immune System's Fight Against Cancer

As cancer cells distinguish themselves from normal cells through their antigen and biochemical structure, they can be recognized by the host's immune system. This dynamic interaction between cancer cells and the immune system consists of three main phases: elimination, equilibrium, and escape.(1)

The elimination step refers to ongoing immunosurveillance, meaning that the immune cells identify and eliminate cancer cells. Then, an equilibrium stage takes place resulting in a co-existence between cancer and immune cells, where the tumor does not progress, yet is not completely destroyed by the host's defense mechanisms. Finally, when the escape phase is reached, cancer progresses and metastasizes. In this case, tumor cells escape immune recognition and elimination through various mechanisms, including the downmodulation of antigen processing and presentation machinery, the recruitment of suppressor immune cells and the secretion of immunosuppressive soluble factors. (1) **(Figure 1)**

1.2 Cancer Immunotherapy

Contrary to chemotherapy that directly kills tumor cells, cancer immunotherapy aims to stimulate the host's immune system to fight cancer(2). This approach is mainly divided into five categories: i) the use of immune-checkpoint inhibitors (ICI), ii) administration of lymphocyte-promoting cytokines, iii) delivering agonistic antibodies against co-stimulatory molecules, iv) engineering T cells and v) designing cell-based cancer vaccines.(2)

1.2.1 The Use of ICI

Immune checkpoints regulate immune responses to protect healthy tissues from damage caused by excess inflammation. Programmed cell death protein 1 (PD-1) and CTLA-4 consist of two extensively studied checkpoints for immunotherapy. In fact, activated T cells express the PD-1 in order to recognize abnormal cells(1, 2). However, to escape immune attack, cancer cells express programmed death ligand 1 (PD-L1), which interacts with PD-1 resulting in T-cell inactivation. On the other hand, CTLA-4 is a co-inhibitory molecule capable of binding to CD80 and CD86 at the surface of APCs, hence decreasing T-cell activity. The use of monoclonal antibodies against these

immune-checkpoints has shown an increase in anti-tumoral activity(1, 2). However, these ICIs can be patient- and organ-specific. In addition, numerous autoimmune side effects were reported following their use, including gastrointestinal, dermatologic, hepatic and endocrine toxicities(1, 2).

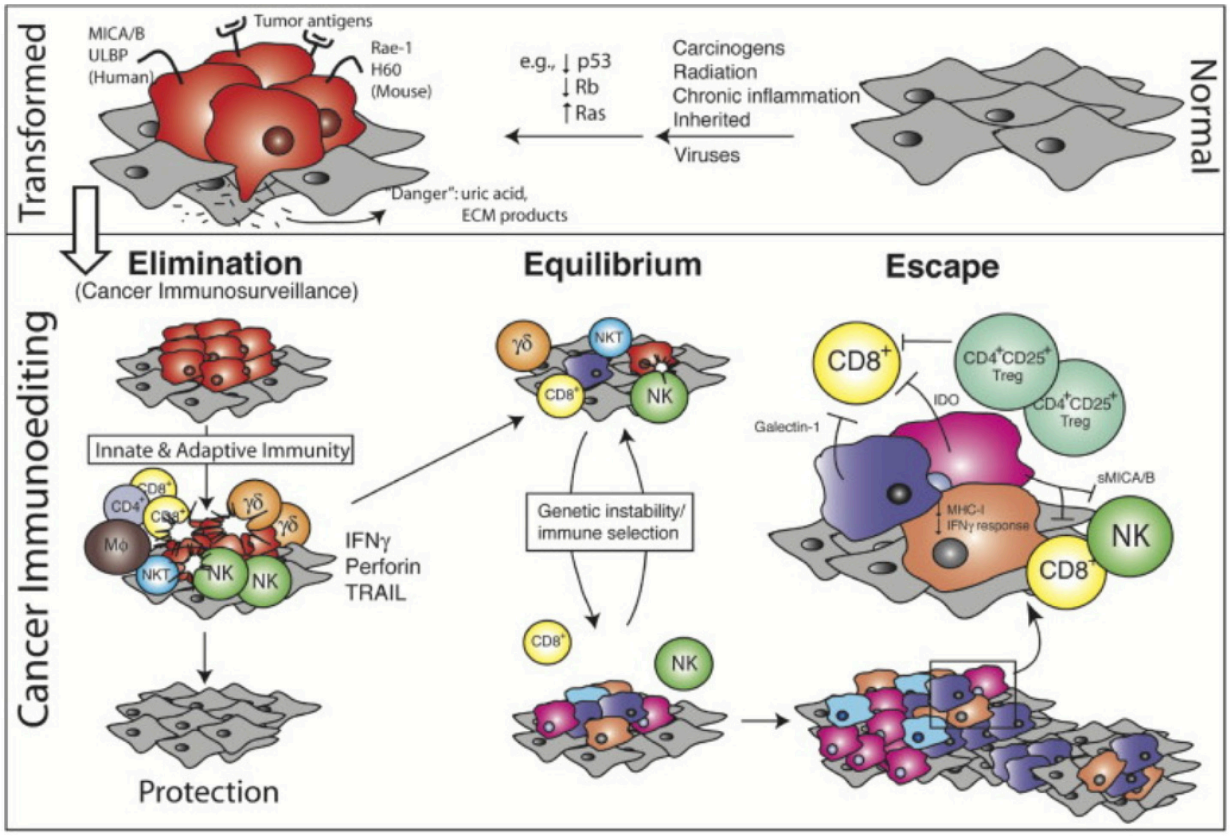


Figure 1. – Cancer Surveillance and Immunoediting (figure from (3))

Immunoediting describes the complex interactions between the immune system and the tumor cells. It can be divided into 3 phases, being elimination (immune cells eliminate tumor cells), equilibrium (co-existence between immune cells and tumor cells) and escape (tumor cells escape immune attack and the cancer metastasizes).

1.2.2 Administration of Lymphocyte-Promoting Cytokines

The first class of immunotherapy that made it to the clinic consists of using lymphocyte-promoting cytokines(1, 2). Those include interleukins (ILs), interferons (IFN-gamma), and granulocyte-macrophage colony-stimulating factor (GM-CSF)(1, 2). Indeed, recombinant IFN-alpha was approved for clinical use in 1986(1, 2). Since then, other recombinant cytokines were approved by the FDA, such as recombinant IL-2 used to treat melanoma and kidney cancer(2). Currently, there is great interest in the development of IL-15-based immunotherapies. The Food and Drug Administration (FDA) also approved the use of IL-2 (Proleukin) as a potent therapy for metastatic melanoma and renal cell carcinoma(4). Despite observed response rate of 15-20%, IL-2-based treatments require very high doses due to their short half-life. Consequently, important side effects are reported, including regulatory T-cell activation, vascular leak syndrome and cytokine release syndrome(1, 2). Efforts were deployed in engineering an IL-2 variant displaying a higher half-life, by covalently binding it to activated polyethylenglycol (PEG). When tested in human during a clinical trial, the pharmacokinetic profile of PEG-IL-2 showed increased plasma levels and lower clearance when compared to regular IL-2 administration(5).

1.2.3 Delivering Agonistic Antibodies Against Co-Stimulatory Molecules

Agonistic antibodies against specific T cell surface receptors can be used to induce T cell proliferation, survival and anti-tumoral activity(1, 2). Being one of the most characterized co-stimulatory molecules, CD28 plays a crucial role in the induction of T lymphocytes and regulatory T cells(6). This receptor binds to CD80/CD86 at the surface of the APC, activating T cells. However, CD28 competes with CTLA-4, a co-inhibitory molecule that displays higher affinity for its ligands, hence explaining the difficulties faced in phase I clinical trial(7). Furthermore, the use of anti-CD28 for several cancers has been associated with hyperinflammation(6). Another interesting target is CD137, a tumor necrosis factor receptor (TNFR) involved in the activation of T cells and production of IFN-gamma. Monoclonal antibodies against CD137 have shown impressive results stemming from higher levels of tumor antigen-specific memory T cells and activation of cytotoxic T cells, natural killer (NK) cells and macrophages. Anti-CD137 utomilumab is currently under clinical trial (NCT03971409) and is achieving astonishing outcomes as less than 10% of the patients report experiencing treatment-related side effects(8).

1.2.4 Engineering T-Cells

Another class of immunotherapies comprises chimeric antigen receptor T cells (CAR T cells). In this approach, a patient's T cells are isolated from the blood and modified genetically to express a CAR specific to a tumor antigen. When these modified immune cells are re-injected into the same patient, they quickly recognize their target antigen, hence the impressive tumor cell death observed. According to the different biomarkers selected and the structural complexity, different generations of CARs models exist, with current development reaching the fourth generation. Anti-CD19 CAR T cells have been extensively studied as a therapeutic approach for acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma(9). Since the first FDA approval for tisagenlecleucel-T in the context of B cell ALL, two other CAR T cell technologies have been approved. Yet, resistant B-cell ALL clones developed because of CD19-expression losses (known as CD19-negative relapse) and/or limited persistence of CAR T cells (referred to as CD19-positive relapse), explaining the observed cancer relapse in 7% to 25% of the patients(9, 10). Therefore, the need for novel CAR T cell approaches led to the emergence of anti-CD20 and anti-CD22 CAR T cells(9). A phase I clinical trial involving bispecific CD19/CD22 CARs supported the rationale for the combination of antigen-targeting models, as 75% of patients experiencing CD19-therapy relapse achieved complete response(11). Despite the outstanding results, this efficient technique remains limited by its complexity, high costs and time requirements(1, 2).

1.2.5 Designing Cellular-Based Anti-Cancer Vaccines

Cancer vaccines have been extensively tested in cancer immunotherapy. Indeed, they provide an interesting tool since they allow the establishment of an immune memory response. Nucleic acids-based vaccination requires the administration of DNA or RNA encoding tumor antigens. APCs uptake DNA or RNA, translate them into peptides or proteins and present them to responsive T cells. If successful, an anti-tumoral response is then mounted. Yet, this type of vaccines is significantly limited by nuclear delivery barriers and immunogenicity. On the other hand, cellular-based vaccines are commonly studied, with the highest emphasis on dendritic cells (DCs)-based vaccination(1, 2).

DC-Based Vaccines

DCs can bridge innate and adaptive immunity. Discovered by Steinman in 1973, those stellate-shaped immune cells express high levels of major histocompatibility complex (MHC) molecules, chemokine receptors (CCR7, CXCR4 and CXCR5), antigen uptake receptors and co-stimulatory molecules (CD40, CD70, CD80 and CD86)(12). Thus, they can efficiently uptake, process and present tumor-associated antigens (TAAs) in addition for being capable of modulating inflammatory cytokines and chemokines' levels and migrating between lymphoid and non-lymphoid tissues(13). DC-based anti-cancer vaccines can take various forms. First, non-targeted analog antigens optimized for MHC-I binding can be uptaken by DCs *in vivo,* resulting in MHC class I-restricted antigen-specific CD8+ T cell activation(14-16). Also, when antigens coupled to anti-DC-antibodies are injected, antigen-specific tolerance is observed, hence their potent use as autoimmune diseases' therapy. Finally, *ex vivo* generated DCs can be loaded with antigens, resulting in higher numbers of circulating tumor antigen-specific CD4+ T cells and CD8+ T cells(14). Despite their numerous benefits and being the best APC known so far, clinical responses to DCs vaccines have been, to a great extent, disappointing. First, no standardized protocols for the *ex vivo* generation of DCs exist so far. Discrepancies in the source, characterization, maturation stimulus and *in vivo* administration route have therefore been observed(17). Furthermore, DC's survival post-injection is too short for them to efficiently migrate towards secondary lymphoid organs for the transport and presentation of their immunogenic cargo, an essential process in the activation of the host's immune system(18). In fact, less than 5% of the injected DCs end up reaching the lymph nodes(19). Also, the DC subsets (cDC1 or XCR1+ DCs) capable of effective antigen cross-presentation represent a small proportion of the blood's composition, therefore complicating their use as a therapeutic vaccine(20). All the above-stated factors greatly limit the use of DCs in immunotherapy and emphasize the importance of discovering innovative alternatives.

1.3 Mesenchymal Stromal Cells (MSCs)

1.3.1 Origin and Terminology

MSCs were first discovered in 1976 by Friedenstein and colleagues, who isolated them from a guinea pig's bone marrow(21). They characterized them as spindle-shaped, plastic-adherent cells capable of developing fibroblastic colony-forming units(22). Few years later, Owen suggested the existence of a hierarchical system, in which stromal cells arise from an initial stromal stem cell(23). Further studies led to the isolation and expansion of human bone marrow cells with osteochondrogenic potential(23). Although the principal and most characterized source of MSCs remains the bone marrow, these cells can be isolated from a variety of different adult tissues, including the adipose tissue, blood vessels and inner organs. MSCs could also origin from the umbilical cord, the amniotic fluid and membrane, and the placenta(24, 25).

Complications in the characterization of MSCs were raised, due to the heterogeneity observed between cells from different donors, tissues or culture methods(26). MSCs also manifest astonishing levels of plasticity in response to their microenvironment and external stimuli(26). Debates regarding what the MSC acronym stands for have been at the core of the scientific community for years. Indeed, MSCs has come to take various significations, between mesenchymal stem cells, multipotent stromal cells or mesenchymal stromal cells. To resolve this issue, the International Society for Cellular Therapy has established *multipotent mesenchymal stromal cell* as the correct term to employ(27). Furthermore, they have established three criteria to categorize cells as MSCs : i) they must be plastic-adherent ; ii) they must express (>95%) a distinct family of surface antigens, being CD44, CD73, CD90 and CD105 while being negative (<2%) for hematopoietic cell markers (CD34 and CD45); iii) upon stimulation by specific agents, they ought to differentiate into cells of the mesoderm lineage, being chondrocytes, osteoblasts and adipocytes, hence their mesenchymal properties(27, 28). On the other hand, MSCs have a tri-lineage differentiation capacity, meaning that they can transform into cells of the endoderm and ectoderm as well, through a process known as trans-differentiation(29).

1.3.2 The Advantages of Using MSCs

MSCs have been widely used in the clinic. In fact, current evidence supports their use for diverse clinical applications. First of all, MSCs can be easily isolated from the bone marrow or other tissues, with accessible and affordable culture media and methods(30).

They have also tremendous expansion potential *in vitro*, hence reducing the number of donors needed to generate a single vaccine(30). Due to their MHCI expression level, and their absent expression of MHC class II as well as co-stimulatory molecules, MSCs exhibit low immunogenicity. Yet, recent studies question this property, suggesting that the use of an autologous MSC source could be preferable(31). The aforementioned advantages for the use of MSCs explain the interest they raised in clinical studies. Indeed, the number of registered MSC-based clinical trials has more than quadrupled between 2007 and 2012 and reached 1,138 by July 2020(32, 33).

1.3.3 MSC Versatility

MSCs in Tissue Repair and Wound Healing

MSCs's differentiation is a two-steps procedure, involving first the transformation of MSCs to lineage-specific progenitors, known as the lineage commitment(34). Then, the progenitor differentiates into a specific cell type, completing the process. Several studies have shown that signaling pathways such as wingless-type MMTV integration site (Wnt) signaling or fibroblast growth factors, are critical in regulating the fate of MSCs differentiation(34).

Due to their multipotency, MSCs have been largely used in the field of regenerative medicine, which consists in the regrowth, repair or replacing of damaged cells, tissues or organs(35). In fact, MSCs can be used to reconstruct tissues of the musculoskeletal system, nervous system, liver, myocardium and skin(36) **(Figure 2)**. Although autologous bone grafting is considered so far, the best therapeutic strategy to cure bone defects, this approach is accompanied by several drawbacks, being the lack of supply for autologous bones, the increased time required by the surgery and bone morbidity at the donor site(36). As bone marrow-derived MSCs have a tremendous osteoblast-differentiation capacity, they serve as an interesting alternative. Their

transformation can be induced *in vitro* by several agents, the most common one being bone morphogenetic protein 2(36).

MSCs have also been used in several clinical trials targeting cartilage reconstruction(37). As a matter of fact, the cartilage displays poor self-repair capacity and therefore consists of a medical challenge when it comes to its defects. MSC's differentiation into chondrocytes can be achieved by multiple specific stimulating factors, such as BMP-2, BMP-4, TGF-β or insulin-like growth factor-1; co-culture of the cells with chondrocytes would further lead to better applications(38). MSCs could also generate other musculoskeletal tissues, including tendons, ligaments, and the meniscus(36).

Injuries to the central nervous systems are irreversible since neurons can't be repaired. MSCs can resolve this issue in multiple ways. First of all, MSCs derived from the bone marrow have the ability to induce the regeneration of axons and to reduce scar formation in the context of spinal cord injuries(39). Furthermore, umbilical-cord-derived MSCs can differentiate into neuron-like cells(40). As a cure for neurological diseases, MSCs have been extensively used for the treatment of multiple sclerosis, ischemic stroke, and Parkinson's disease(41-43).

When stimulated with hepatocyte growth factor or oncostatin M, bone marrow-derived and umbilical cord-derived MSCs can differentiate into hepatocytes, providing an alternative to transplantation in the context of liver failure(44). In addition, the immunomodulatory capacity of MSCs is accompanied by mechanisms mediating the cells' curative potential, such as the inhibition of Col deposition, neoangiogenesis, vascular support or paracrine effects(45). Chances of liver failure following MSCs injection also decrease, following inhibition of T cell and B cell proliferation, as well as an increase in regulatory T cell levels (Tregs) (46). MSCs have therefore been associated with improved liver function in alcoholic and autoimmune cirrhosis (47, 48).

MSCs from different sources have been associated with an ability to transform into cardiomyocytes, hence their use in myocardium regenerative medicine(49). Moreover, MSCs

promote vasculogenesis and angiogenesis through the secretion of vascular endothelial growth factor (VEGF), processes involved in cardiac repair(50). Wound healing is an elaborate process in which soluble factors play a crucial role(51). MSCs secrete several molecules improving both wound repair and angiogenesis. As a matter of fact, MSCs produce tumor necrosis factor-alpha-stimulated protein 6, protein associated with tissue protection and anti-inflammation(52). Furthermore, not only does MSCs-secreted VEGF induce angiogenesis, but it also triggers wound repair through a keratinocyte-dependent process(53). Transplantation of both allogeneic and autologous MSCs have led to regeneration of skin defects, diabetes-induced ulcers and radiation-caused skin lesions(36).

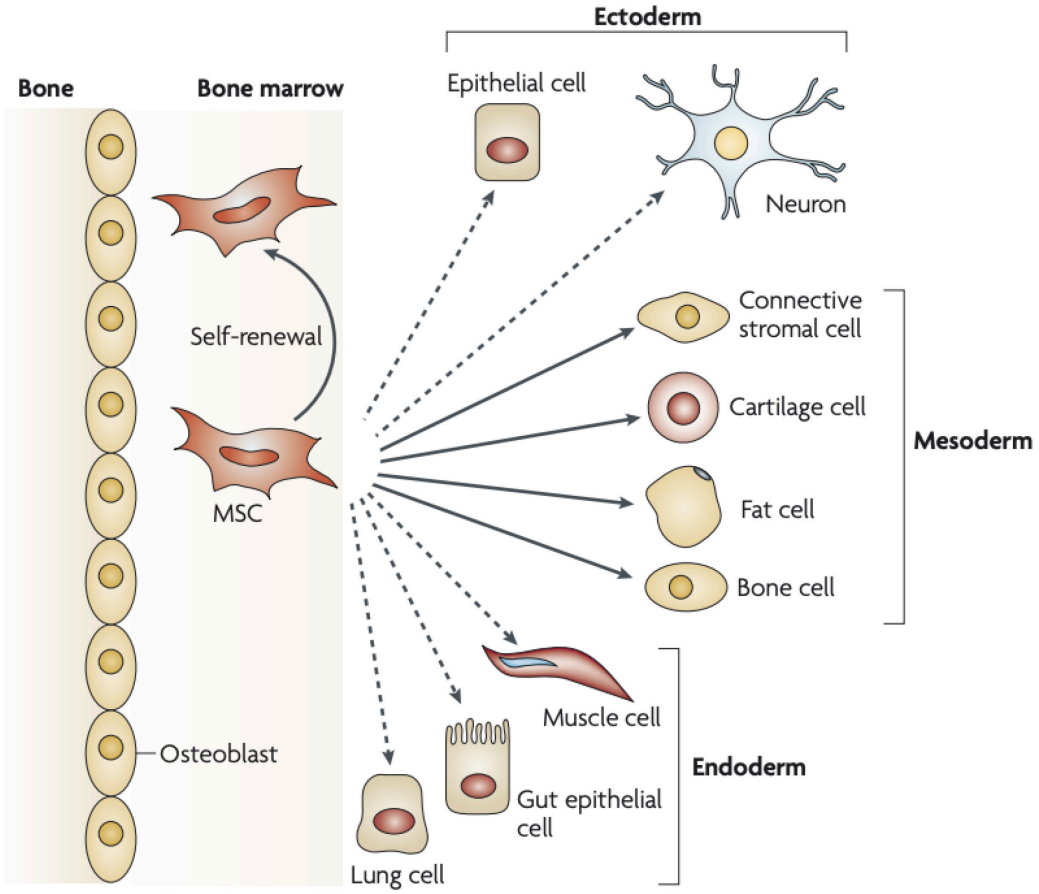


Figure 2. – The Multipotency of MSCs (figure from (29))

The most common source for MSCs is the bone marrow. MSCs can self-renew and have a tremendous differentiation capacity. They can transform into cells of the mesoderm, being connective stromal cells, cartilage cells, fat cells and bone cells. Furthermore, MSCs have a trilineage differentiation capacity, meaning that they can transform into cells of the ectoderm and endoderm via a process known as trans-differentiation.

Genetically-Modified MSCs for Delivery of Pharmacologically-Relevant Molecules

Although MSC-based therapy has shown promising results, some progress can still be achieved. Therefore, efforts in genetically modifying the stromal cells have raised, for them to exert specific functions. In fact, the advancement of cellular therapy has contributed dramatically to science, providing innovative utility in regenerative medicine, as well as successful disease therapies(54-57).

Genetic modification occurs through the transfection or viral transduction of MSCs with a gene of interest to enhance the cells' adhesion capacity, migration to sites of injuries, survival, or to modulate MSCs with new properties(58) **(Figure3)**. De Becker *et al*. overexpressed the CXC chemokine receptors 4 and 7 as a method to improve MSC's migration. In fact, CXCR4/CXCR7 are two receptors for the stromal cell-derived factor 1, a powerful migration chemokine. Results have shown an increase in MSCs' relocation ability, along with more powerful paracrine and proliferative capacities(59). Song *et al.* upregulated MSCs's expression of intregrin-linked kinase, which plays a crucial role in the adhesion process. Survival of the cells increased by 50%, and adhesion by 32% after transplantation in an ischemic myocardium model(60). Mao *& al*. confirmed the above results, demonstrating that an overexpression of integrin-linked kinases under hypoxic conditions triggers IL-6 secretion, which activates several signaling pathways including JAK2/STAT3 and Wnt(61).

As MSCs secrete multiple soluble factors, they can be further modified to produce a cytokine of interest. As an example, Eliopoulos *et al.* engineered bone marrow-derived MSCs to produce IL-12. This pro-inflammatory cytokine is normally produced by DCs, monocytes, macrophages and B cells. It plays a crucial role in regulating the activity of T-helper 1 cells. Furthermore, it promotes the proliferation and survival of activated T cells and NK cells. When embedded in a matrix, the IL-12-secreting MSCs displayed impressive anti-cancer properties in syngeneic hosts, with the mice showing increased IL-12 and IFN-gamma blood levels. The genetically-modified cells displayed impressive *in vivo* results, as there was a significant decrease in the development of 4T1 breast cancer and B16 melanoma pre-established in C57BL/6 mice. The observed effects are not

related to the systemic secretion of IL-12, but it is rather immune-mediated as the therapeutic outcome was absent in immunodeficient mice(62).

Bikorimana *& al*. have used bone marrow-derived MSCs to express the thymoproteasome (TPr) as an anti-tumoral therapeutic vaccine. The TPr is encoded by the genes B1i, B2i and B5t and its expression is limited to cortical thymic epithelial cells (cTECs). It plays a major role in the positive selection of CD8 thymocytes. TPr-expressing MSCs (MSC-TPr) show lower levels of MHC class I molecules while they secrete higher levels of chemokines such as CCL2, CCL9, CXCL1, LIX and CX3CL1. Administration of MSC-TPr to immunocompetent mice led to important levels of memory CD4 and CD8 T cells, yet with moderate anti-tumoral response. However, when depletion of macrophages was conducted through the injection of clodronate pre-immunization, the anti-tumoral response of MSC-TPr increased significantly, hence suggesting that phagocytic cells reduce MSC-TPr-mediated protection(63). Thus, the therapeutic effect of MSC-TPr relies on the interplay with macrophages and DCs through the secretion of several chemokines stated above. More specifically, recruitment of macrophages leads to efferocytosis- macrophages phagocytosing MSCs- preventing them to act as APC *in vivo*. Yet, the anti-tumoral response of MSC-TPr requires DC cross-priming as anti-CD11c treatment in mice results in the absence of therapeutic effects. (63). In fact, the integrin CD11c, classical DC marker, has been shown to play a critical role in the DC-mediated capture of missing-self CD47 cells(64).

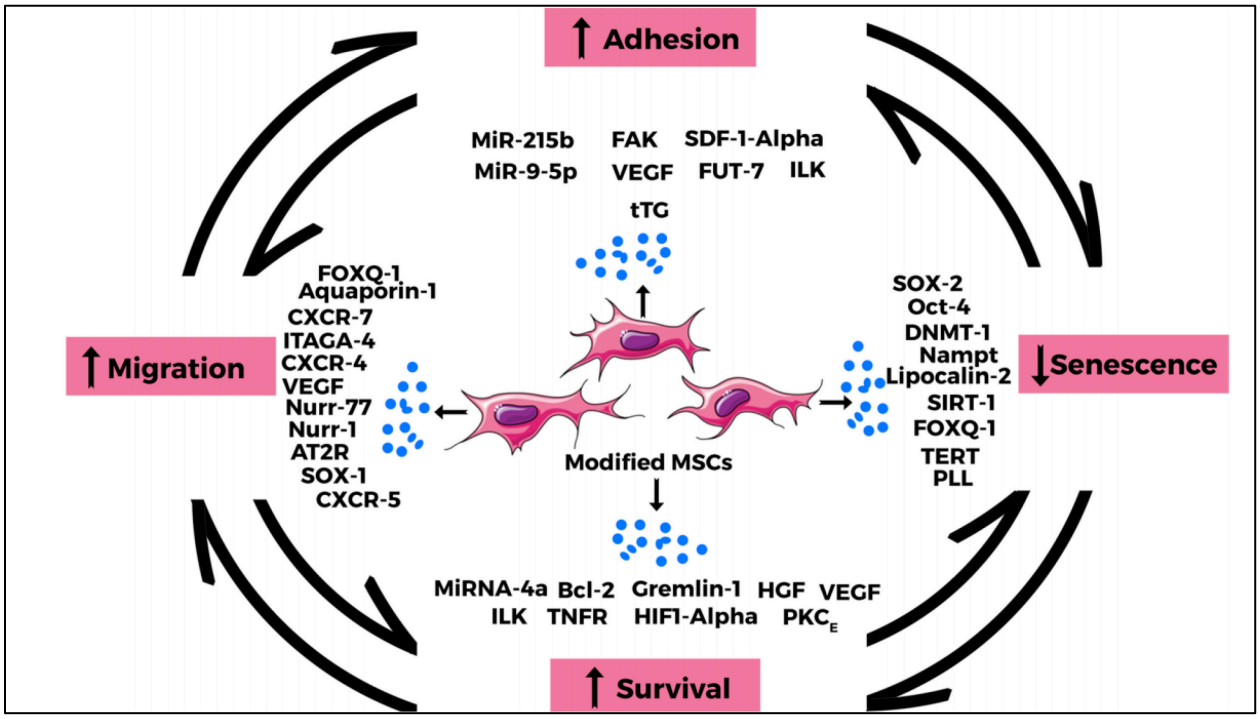


Figure 3. – Enhanced MSCs Properties Through Genetic Modification (figure from (58))

Genetic modification occurs through the transfection or viral transduction of MSCs with a gene of interest to enhance certain MSCs properties, or to make them gain new functions. Overall, genetic alterations aim to increase the cells' adhesion capacity, migration to sites of injuries, survival, or to slow down senescence.

MSCs-Mediated Immunosuppression

Recently, other features of MSCs have been identified, therefore widening their scope of applications. In fact, this multipotent cell type has been associated with strong immunosuppressive properties, which have been exploited for the treatment of several diseases(29). DCs, NK cells and neutrophils are crucial to the host's innate immunity(29). MSCs can modulate their activity, resulting in a decrease in inflammation. Mature myeloid DCs play a critical role in the activation of T cells through antigen presentation. Their maturation is induced by various pro-inflammatory cytokines or chemokines, and results in an up-regulation of MHC class I and class II, along with the expression of co-stimulatory molecules (CD80 and CD86)(29). MSCs can inhibit the differentiation of hematopoietic progenitor cells or monocytes into immature DCs(65-67). Moreover, DCs co-cultured with MSCs display lower levels of MHC class II, CD11c, CD83 and co-stimulatory molecules, as well as lower secretion levels of the pro-inflammatory cytokine IL-12 and tumor necrosis factors (TNFs)(65, 67-69). Thus, an important decrease in DCs antigen presentation capacity is observed.

NK cells are best known for their anti-viral and anti-tumoral activities(29). In contrast with cytotoxic T cells, NK cells do not require prior activation through MHC-peptide complexes to trigger their cytolytic activity(70). Virally-infected cells and tumor cells tend to lose their MHC class I molecule as a mechanism to hide from the adaptive immune system. However, NK cells manage to recognize them through multiple cell-surface receptors interactions(29). In fact, NK cells display inhibitory receptors to identify cells expressing low to absent MHC class I levels(71, 72). Other NK-specific cell-surface receptors interact with the target cells, leading to lysis activity(71, 72). Inducing the downregulation of activation receptors (NKp30 and natural-killer group 2, member D), MSCs inhibit the cytotoxic activity of the NK cells(71, 72). NK cells also secrete IL-2 and IFN-gamma. Following co-culture with MSCs, the levels of these pro-inflammatory cytokines significantly decreased(73, 74).

Finally, neutrophils mobilize and kill microorganisms in the context of bacterial infections. Their mechanism of action is mainly via the binding to bacterial products followed by a ROS-releasing

process, known as the respiratory burst(75). MSCs dampen the respiratory burst and delay the apoptosis of resting and activated neutrophils via an IL-6-dependent-mechanism(76).

MSCs also affect cells of the adaptive immune system, being the T cells and the B cells.
After T-cell receptor activation, T cells proliferate and exert pro-inflammatory functions, including the release of specific cytokines. MSCs inhibit the proliferation of T cells through a non-MHC-restricted process(77-80). This inhibition is mediated by an arrest of the cells in the G0/G1 phase of the cell cycle(81). Both *in vitro* and *in vivo*, MSCs induce a shift in T cells from a pro-inflammatory state (associated with IFN-gamma production) to an anti-inflammatory state (resulting in IL-4 production)(82). Furthermore, MSCs increase IL-10 release by plasmacytoid DCs, itself inducing the generation of Tregs(82). Tregs consist of a subpopulation of T cells responsible for the suppression of the immune system's activity, in order to maintain homeostasis and tolerance to self-antigens(29).

B cells are the second type of immune cells mediating the adaptive immunity. They are responsible for the production of antibodies. Conflicting results have been obtained *in vitro* regarding the interaction between MSCs and B cells(81, 83). Although most studies show that MSCs inhibit their proliferation and differentiation capacity, others notice an MSC-mediated induction of differentiation from B cells to antibody-secreting cells(81, 83, 84). These discrepancies could be explained due to different experimental conditions used. Yet, one should keep in mind that B cells's activity is mostly driven by T cells. Therefore, through the inhibition of T cells's proliferation, MSCs might be involved in decreasing B cells's activity(85). For instance, MSCs play a role in inhibition of antibody production by plasma cells and IL-10-mediated immunosuppression. In fact, MSCs secrete the chemokines CCL2 and CCL7, which are further processed into metalloproteinases, leading to an antagonistic CCL2 variant. This latter suppresses signal transducer and activator of transcription 3 (STAT3) in plasma cells, hence the observed effects(86).

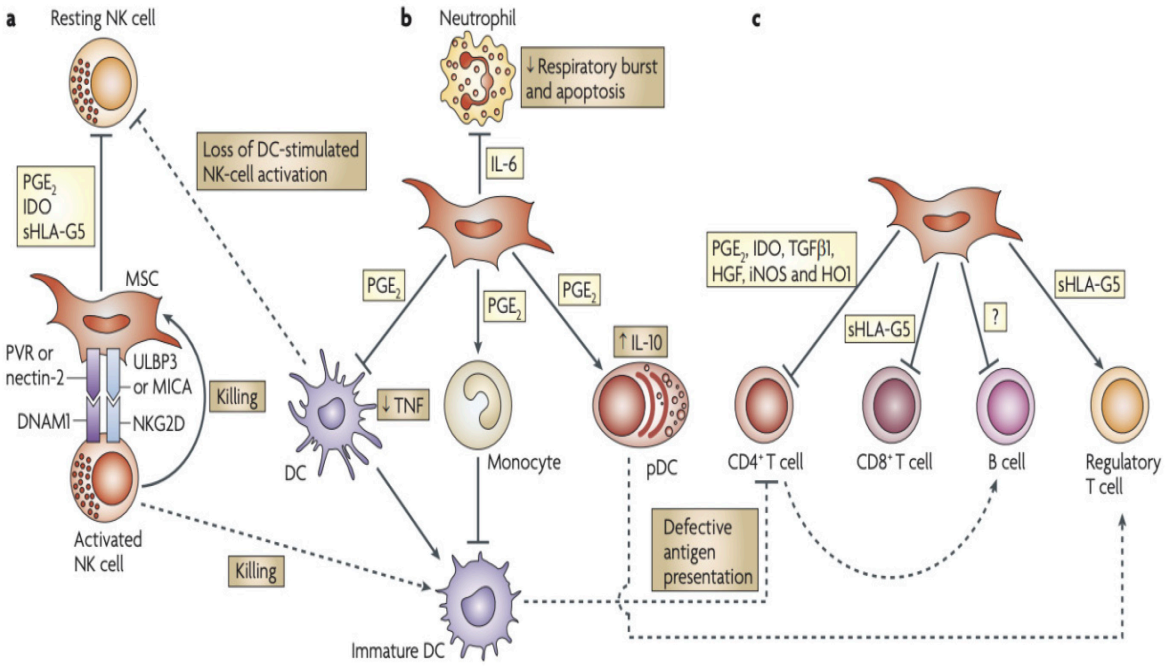


Figure 4. – The Effect of MSCs on Immune Cells (figure from (29))

MSCs are characterized as immunosuppressive cells, as they interact with immune cells to induce their inhibition. These interactions are at the levels of both the innate and adaptive immune system. The mechanisms employed are diverse, including the release of cytokines and chemokines or direct cell-cell interactions.

So far, the most intensively researched disease in the context of MSC-built therapies is graft-versus-host-disease (GvHD). There are currently 76 clinical trials in that matter. The fierce interest in this field is due to encouraging success through intravenous injection in the clinic(30). An elegant study by Leblanc *et al.* involved the transplantation of haploidentical MSCs in a 9-year-old patient with severe grade IV acute GvHD in his gut and liver. In fact, the young boy suffered from acute lymphoblastic leukaemia. He received a transplant of blood stem cells along with cyclophosphamide and fractionated total body irradiation. 11 days following the transplant, the patient started developing symptoms such as a rash on his thorax, abdominal pain and loss of appetite. Progressively, the symptoms evolved to become important adverse effects including strong diarrhea (up to 20 times per day) and repeated bacterial, viral, and fungal infections. The mother was chosen as a third-party donor of haploidentical MSCs since she was readily available and MSCs compatibility is not necessary for immunosuppression functions. 2 x 10^6 MSCs /kg of the patient's weight were administered intravenously. No toxicity was detected, and symptoms of GvHD decreased significantly starting 4 days post-injection. 77 days after the first injection, the patient started experiencing moderate diarrhea, and biopsy of his caecum revealed mild GvHD. Therefore, he received 1 x 10^6 cells MSCs /kg of the patient's weight from the same batch previously used. 50 days after the second injection, he was able to go back home. The patient was initially unresponsive to all available therapies. Yet, MSC-built approach delivered astonishing results, as he is doing well one year post-treatment(87). Despite the beautiful outcome of the study, other researchers have shown little or no difference in the risk of GvHD development between patients receiving MSCs and their respective controls(88-90). The observed discrepancies suggest the need for further investigation regarding the matter.

MSCs as Pro-Inflammatory APCs

Conversely, when stimulated with low concentrations of IFN-gamma, MSCs can become pro-inflammatory as they behave as non-professional APC. The pro-inflammatory cytokine induces an increase in MHC class I and a *de novo* expression of MHC class II at the surface of stromal cells. Treated MSCs can then cross-present immunogenic peptides to CD8+ T cells, triggering a potent cytotoxic immune response with significant levels of IL-2 secreted by T cells. Similar results were

obtained with influenza matrix protein 1-pulsed human DR1-positive MSCs when co-cultured with DR1-restricted influenza specific humanized T cell hybridoma. These studies suggest that despite their strong immunosuppressive properties, MSCs can be modulated to stimulate inflammation(91, 92).

Yet, IFN-gamma-stimulated-MSCs display several drawbacks that could interfere with T-cell activation. First of all, when exposed to higher concentrations of IFN-gamma, a decrease in the expression of MHC class II is observed at the surface of MSCs, correlating with the deprivation in allogeneic potential, as shown via results obtained from mixed lymphocyte reactions. Thus, low endogenous levels of IFN-gamma are required to induce APC-like behavior in stromal cells(91). This implies that a strong inflammatory response could inhibit APC functions in treated MSCs. Furthermore, when exposed to IFN-gamma, MSCs respond by upregulating PD-L1(93). PD-L1 interacts with a T-cell surface marker known as PD-1, resulting in the inhibition of the immune cell activation through various mechanisms. First of all, the immune checkpoint results in the downregulation of the T cell receptor (TCR), as interfering with the PD-L1/PD1 interface inhibits TCR downmodulation. Also, this interaction modulates T-cell metabolism, hence decreasing inflammation. In fact, T-cells require glycolysis for proper differentiation. A refined study by Patsoukis *et al.* has shown that PD-1 signaling prevents phosphoinositide-3-kinase (PI3K) activation, therefore preventing Akt phosphorylation. Akt signaling pathway plays a crucial role in stimulating cell growth and proliferation. As a result, there is blockage of glucose and amino acid metabolism, whereas lipolysis and fatty acid oxidation are upregulated(94-96).

Overall, these studies show that MSCs have tremendous potential as pro-inflammatory cells when modulated accordingly. Despite its ability to trigger APC functions in the stromal cells, IFN-gamma-stimulated MSCs fail to maintain proper inflammatory responses due to PD-L1 induction and immunosuppression at high IFN-gamma doses. Other approaches are needed to convert naturally immunosuppressive MSCs to pro-inflammatory tools.

1.4 Antigen Presentation to Responsive T Cells

1.4.1 Classical Pathways

There are two essential ways through which antigens can be presented to T cells. Mainly, peptides derived from endogenous proteins are presented via MHC class I molecules to CD8 + T cells(97). On the other hand, peptides derived from exogenous proteins are presented to CD4 + T cells through MHC class II molecules(97) **(Figure 5)**.

All nucleated cells express MHC class I molecules at their surface, allowing for presentation of endogenous proteins-derived peptides to CD8+ T cells(97). This process occurs in the cytosol, where the protein is degraded by the proteasome. The resulting peptides are delivered to the endoplasmic reticulum by transporters associated with antigen presentation (TAP) for loading onto the MHC class I molecules(97). Several chaperones and proteins interact with the MHC class I molecule in order to stabilize it, such as beta-2-microglobulin, calreticulin or protein disulphide isomerase. The stable MHC-I-peptide complex is transported to the cell surface through the Golgi apparatus. Yet, if the complex fails to associate properly, they find their way back to the cytosol for degradation(97, 98).

Antigenic presentation through the MHC class II differs on several points. First, MHC class II molecules expression is not ubiquitous. In fact, they are usually expressed by professional APCs, such as DCs, macrophages, and B cells(97). The formation of the MHC class II molecule occurs in the ER, where its transmembrane chains are associated with an invariant chain termed as li(97). The assembled complex is translocated to a late endosomal compartment known as MHC class II compartment (MIIC). There, the li chain is digested, leaving a peptide called CLIP at the MHC class II binding groove. Meanwhile, exogenous proteins are endocytosed by the cell for degradation by proteases in the early endosome. The resulting peptides enter the MIIC compartment, where they are exchanged with the CLIP peptide. The final immunogenic complex is transported to the plasma membrane for presentation to CD4 + T cells(97).

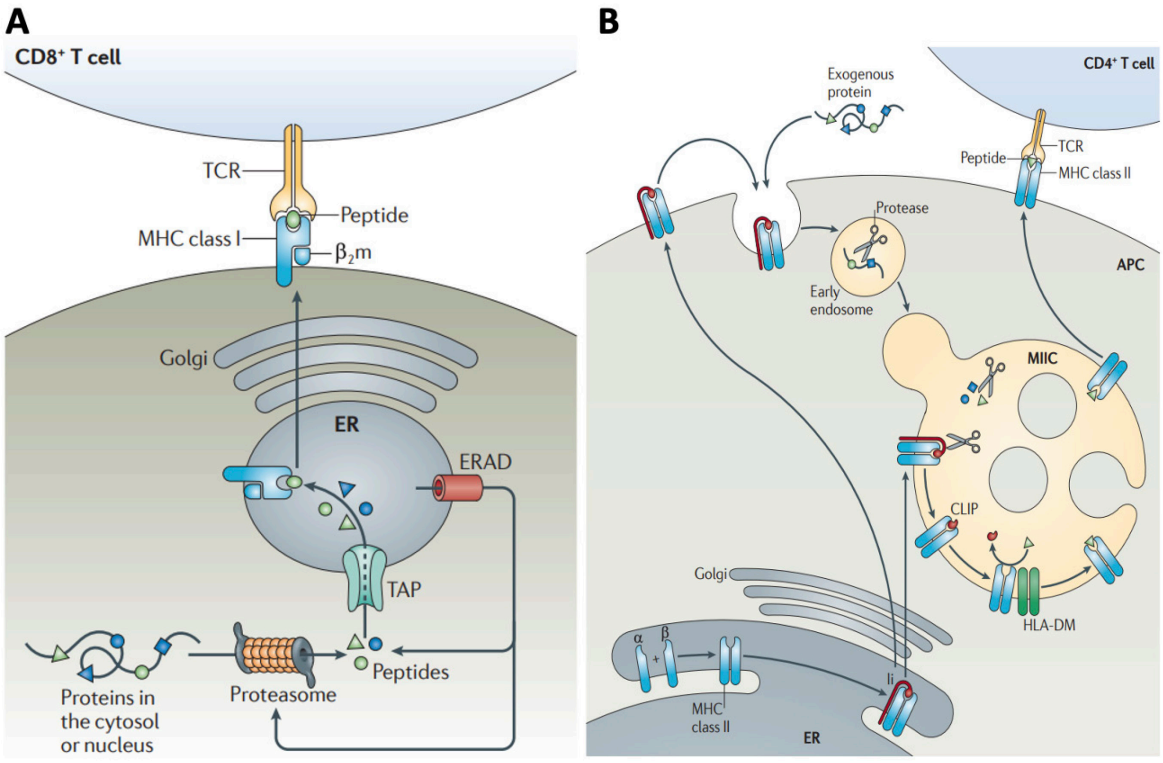


Figure 5. – Simplified Overview of Antigen Presentation Through the A) MHC Class I Pathway and B) MHC Class II Pathway (figure adapted from (97))

Antigen presentation can occur via two pathways, each one involving a different MHC complex molecule. A) When antigen presentation occurs via the MHC-I molecule, endogenous proteins are degraded by the proteasome in the cytosol, after which the resulting peptides are transported to the ER. There, they are loaded onto the MHC-I molecules for presentation to CD8+ T cells at the surface of the APC. B) Exogenous proteins are uptaken for presentation via MHC-II molecules. Once in the endosome, they are degraded by proteases and the resulting peptides enter the MIIC compartment. There, they are exchanged with the CLIP peptide and transported to the surface for presentation to CD4+ T cells.

1.4.2 Antigen Cross-Presentation for Anti-Tumoral Activity

An interesting bridge exists between the two pathways for antigen presentation, known as antigen cross-presentation. Cross-presentation occurs when peptides derived from exogenous proteins are presented via MHC class I molecules to CD8 + T cells(97). So far, this process occurs through either one of two main intracellular pathways described, being the cytosolic or vacuolar pathways. The cytosolic pathway was shown to be sensitive to proteasome inhibitors and TAP proteins, suggesting that endocytosed exogenous proteins are released in the cytosol for degradation by the proteasome(99). After degradation, the resulting peptides are further trimmed by amino-terminal enzymes such as the endoplasmic reticulum-associated aminopeptidase 1 (ERAP1)(100). The final peptides follow the classical MHC class I pathway, meaning that TAP proteins translocation to the ER occurs. There, the MHC-I-peptide complex is assembled before transportation to the cell surface via the Golgi apparatus(100). Indeed, there is direct evidence that TAP proteins are involved in the cytosolic pathway, but no direct evidence for the role of the ER(101, 102). On the other hand, the vacuolar pathway displays resistance to proteasome and TAP proteins inhibitors(103). Yet, this process is sensitive to lysosomal proteases inhibitors, advocating that both the antigen processing and loading onto MHC class I molecules occur in endo/lysosomal compartments(100). The lysosomal protease cathepsin S plays a critical role in antigen cross-presentation via the vacuolar pathway(104).

All in all, both intracellular pathways remain partly unresolved by the scientific community, despite several studies over the past decade(100). For instance, some studies brought to light TAP-independent cross-presentation via the cytosolic pathway, further complicating our general understanding of the exact processes involved(105). Both *in vitro* and *in vivo*, the relative involvement of either one of the aforementioned pathways is hard to ascertain(100). So far, evidence suggests an overriding employment of the cytosolic pathway. Indeed, a unique form of proteasome, known as the immunoproteasome, is associated with potent activation of CD8+ T cells following cross-presentation of immunogenic peptides(106).

1.4.3 The Proteasomal Machinery and Protein Degradation

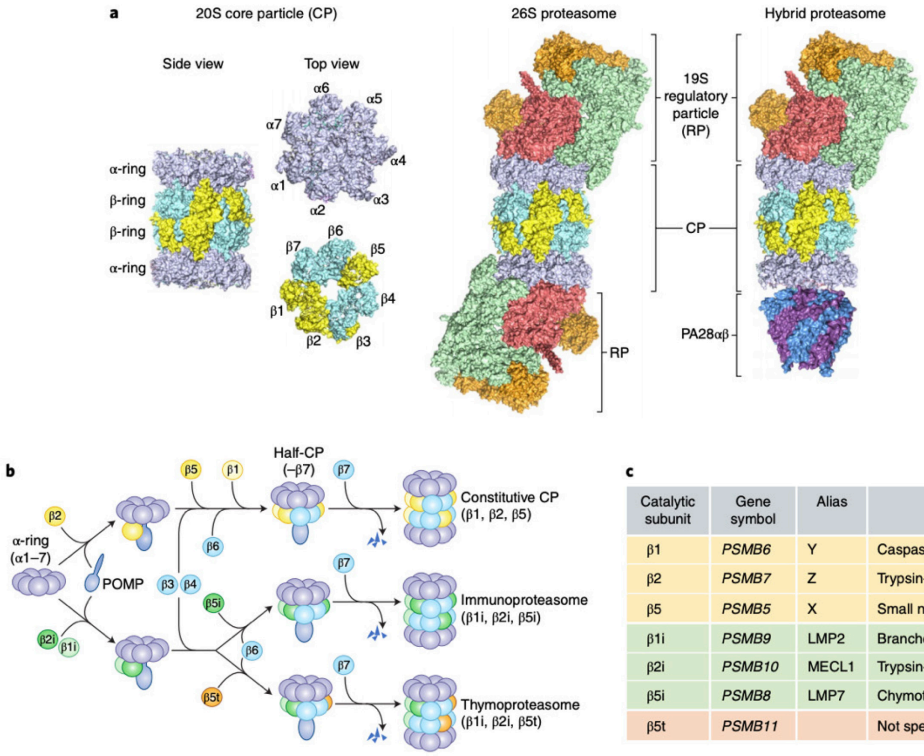
Proteasomes are ATP-dependent proteases responsible for selective proteolysis of ubiquitinated proteins(107). This complex is relatively sophisticated, with a size of approximately 2.5 MDa(108). The proteasome is constituted of a core particle 20S and regulatory particles 19S(108). The core particle is associated with peptide-cleavage properties, functional after binding of the regulatory particles. The proteasome is comprised of four rings, each further composed of 7 subunits. The two outer rings are constituted of alpha subunits, whereas the inner ones are made of beta subunits. It is at the level of the beta subunits 1, 2 and 5 that gene duplication occurred, resulting in the evolution of two specialized proteasomes fulfilling immune functions: the TPr and the immunoproteasome (IPr)(108).

While the constitutive proteasome expresses the B1, B2 and B5 subunits (encoded by the PSMB6, PSMB7 and PSMB5 genes respectively), the TPr expresses modified B1i, B2i and B5t subunits (encoded by the genes PSMB9, PSMB10 and PSMB11 respectively). As briefly discussed in an earlier section, the TPr is abundant in cTECs. The B5t subunit contains mainly hydrophilic amino acids in its substrate-binding pocket, feature unique to the TPr. Thus, this proteasome can generate unique sets of peptides associated with MHC class I molecules. It is well known that B5t plays a significant role in the positive selection of CD8+ T cells, even though the mechanism through which this happens remains unresolved. In fact, B5t-deficient mice develop CD8+ T cells with altered TCR repertoire, while CD4+ T cells and regulatory T cells remain unaffected(107).

The IPr, on the other hand, is composed of the B1i, B2i and B5i subunits, encoded by the PSMB9, PSMB10 and PSMB8 genes respectively. It is constitutively expressed by hematopoietic cells. It can also be induced in non-immune cells following stimulation with pro-inflammatory cytokines, with the best results following IFN-gamma treatment(109, 110). The IPr generates immunogenic peptides compared to the constitutive proteasome, due to the differences in the cleavage activity of its subunits. Indeed, The B1 subunit displays caspase-like activity whereas the B1i binds hydrophobic P1 residues (branched-chain amino acid-preferring activity). On the other hand, the B5 subunit is associated with small neutral amino acids, whereas the B5i binds bulky hydrophobic

P1 residues (chymotrypsin-like activity)(111). Overall, the immunoproteasome generates more antigenic peptides that suit better the MHC class I binding groove. Additional functions are attributed to the IPr. In fact, its expression has been reported in T cells, where it plays an essential role in the maintenance and proliferation of T cells(112, 113). It also promotes differentiation of helper T cells of type 1 and 17, while it inhibits the proliferation of Tregs(114). Finally, the IPr induces the secretion of pro-inflammatory cytokines IL-2, IFN-gamma and TNF-α in T cells(115).

Antigen cross-presentation is ideal for personalized cancer immunotherapy(116). As tumor cells constantly mutate their target antigens and display low immunogenicity, they can escape the immune system very efficiently, hence the difficulty in finding potent therapies(116). DCs play a major role in fighting cancer, part of it since they are considered the best antigen cross-presenting cells(117). In fact, through antigen cross-presentation, CD8+ T cells are activated following recognition of a peptide derived from exogenous proteins. Cytotoxic T lymphocytes are essential for their anti-tumoral capacity(117). Furthermore, following treatment with a given lysate, antigen cross-presentation allows effective presentation of the target immunogenic antigens without prior knowledge of the target TAA. Thus, modulating antigen cross-presentation constitutes an interesting strategy for the design of new cellular-based cancer vaccines.



Catalytic subunit	Gene symbol	Alias	Peptidase activity
β1	*PSMB6*	Y	Caspase-like
β2	*PSMB7*	Z	Trypsin-like
β5	*PSMB5*	X	Small neutral amino acid preferring
β1i	*PSMB9*	LMP2	Branched-chain amino acid preferring
β2i	*PSMB10*	MECL1	Trypsin-like
β5i	*PSMB8*	LMP7	Chymotrypsin-like
β5t	*PSMB11*		Not specified

Figure 6. – Structure and Forms of the Proteasome (figure adapted from (107))

Model arrangement of the proteasome. A) The proteasome is made of four rings, each further composed of 7 subunits. The two outer rings are constituted of alpha subunits; the inner ones are made of beta subunits. B) Structure of the several proteasomal forms. It is at the level of the beta subunits 1, 2 and 5 that evolved two specialized proteasomes fulfilling immune functions: the TPr and the IPr. C) Catalytic subunits of the proteasomes. The table displays each subunit's function.

1.5 UM171: A Hematopoietic Stem Cell (HSC) Self-Renewal-Promoting Agent with a Potential Pro-Inflammatory Function

HSCs can self-renew and differentiate into all blood cells lineages, hence preserving blood homeostasis(118). Suitably, transplantation of these pluripotent cells serves as a potent therapy for hematological diseases. Although HSCs can be isolated from various tissue types, cord blood sources display interesting features for their use in clinic, such as greater tolerance of mismatched-human leukocyte antigen (HLA) leukocytes, higher availability, and lower rates of chronic GvHD(119). An important challenge encountered with HSC transplantation is the limited number of cells, resulting in delay of treatment as well as increased rate of infections and mortality(119). Great emphasis is put on developing ways to enhance HSC's expansion *ex vivo*. The pyrimidoindole derivative UM171 promotes *ex vivo* expansion of long-term HSCs with high multilineage reconstitution potential(120). Indeed, the molecule suppresses differentiation and promotes CD34+ cord blood cells. The drug is also more effective on HSCs with primitive phenotype (CD34+ CD45A-). UM171 can trigger the expansion of stem cells by 10- to 80-folds and has demonstrated the best results so far relative to other expansion procedures(120). From a cohort of 22 patients with advanced blood cancer, not even one needed immunosuppressive treatment up to 13 months post-trial. This is impressive as after usual transplants, at least 50% of the patients would require such interventions. With phase I completed, UM171 is currently under several phase II clinical trial. Cohen *et al.* have run a phase I-II clinical trial, in which 27 patients were enrolled. 96% of cord blood units (26 patients out of 27) were successfully expanded using UM171. No unusual adverse effects were observed following injection. Overall, the study suggests a feasible and safe UM171 cord blood stem cell expansion that might overcome the drawbacks of other transplant methods while maintaining low risks of GvHD and relapse(121).

At the molecular level, UM171 enables the preservation of epigenetic marks that would normally be downregulated under *ex vivo* culture of HSCs. Briefly, under regular conditions, the transcriptional repressor LSD1-coREST complex is stable. It therefore inhibits the expression of stem cell genes. However, in UM171-treated HSCs, ubiquitination of the complex is observed,

targeting it for proteasomal degradation. As the repressor can't fulfill its functions, the stem cell genes are turned on and *ex vivo* expansion is achieved(122). **(Figure 6)**

Furthermore, UM171-mediated expansion of HSCs is the result of a balance between key pro-inflammatory and anti-inflammatory effects via the NF-kB pathway and the endothelial protein C receptor (EPCR), respectively. Levels of reactive oxygen species (ROS) elevated dramatically as early as 6 hours post-treatment with the drug, reaching its peak between 48 and 72 hours. Several pro-inflammatory genes were induced by UM171, such as IL-1b and CD86. An increase in the expression of HLA molecules and beta-2-microglobulin were also noticed, providing further evidence that UM171 triggers inflammation in HSCs. Furthermore, the pro-inflammatory signaling is essential to UM171-induced expansion, as the use of NF-kB inhibitors and other anti-inflammatory inhibitors reversed the drug's effects. Conversely, UM171 induces the expression of EPCR, a previously suggested inhibitor for the NF-kB pathway. It is proposed that the anti-inflammatory receptor reduces the pro-inflammatory stress imposed on the cells, therefore enhancing the response(123). **(Figures 7-8)**

The increase in CD86 attracts attention in the field of immunology since it consists of a co-stimulatory molecule for the activation of T cells. In fact, T cells require three signals upon contact with an APC for them to get activated and promote a potent inflammatory response. The first signal involves the recognition of a peptide presented via MHC molecules of the APC to the TCR of the immune cell. Then, the second signal follows, being the interaction of co-stimulatory molecules of the APC (CD80 or CD86) with their respective ligands at the surface of the T-cell (CD28 and CD152). Finally, the third signal mainly consists in the release of pro-inflammatory cytokines by the APC, such as IL-12 and IFN- α/β. This enhances cytotoxic T cells expansion and activation, resulting in IFN-gamma production(124). Finally, activated T cell will produce several molecules, driving further proliferation and differentiation of neighboring immune cells, hence the activation of the host's defense system. Several studies have shown that in the absence of co-stimulation (signal 2), anergy or tolerance is achieved(125).

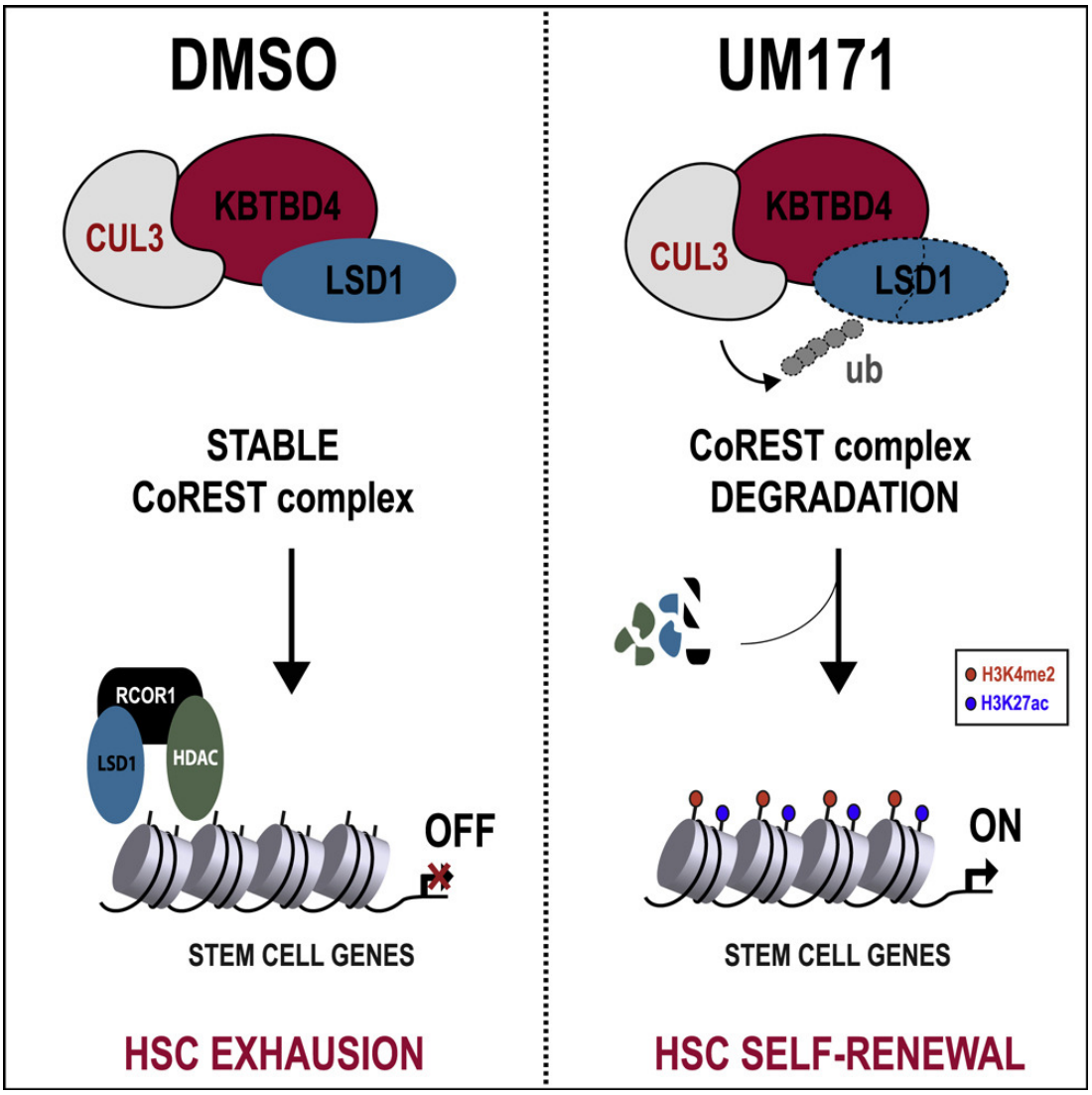


Figure 7. – Graphical Abstract of UM171's Mode of Action (figure from (122))

UM171 promotes the degradation of the CoREST complex, which acts as a repressor complex for the expression of several stem cell genes. Under normal conditions, the coREST complex is stable and inhibits the expression of the stem cell genes. Therefore, there is hematopoietic stem cell exhaustion. However, upon UM171 treatment, the coREST complex is degraded by the proteasome, thus allowing for the expression of the stem cell genes, explaining the hematopoietic stem cell self-renewal capacity.

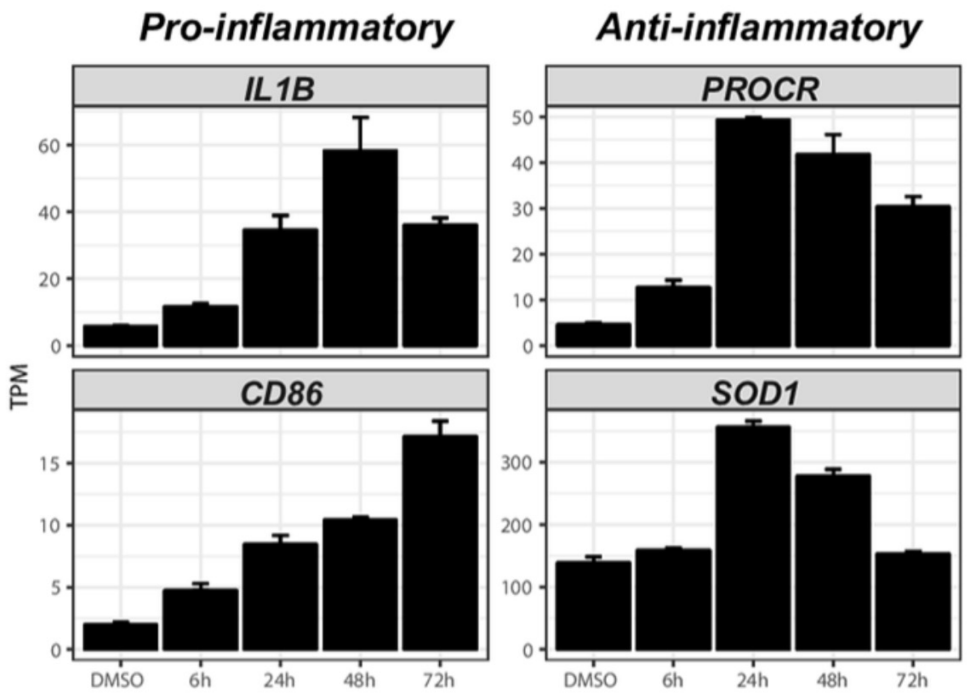


Figure 8. – Examples of Pro-Inflammatory and Anti-Inflammatory Genes Expression Changes Following UM171 Treatment in OCI-AML5 Cells (figure from (123))

UM171 triggers simultaneous activation of pro-inflammatory (IL1B and CD86) and anti-inflammatory (PROCR and SOD1) gene expression in hematopoietic cell lines. The changes are observed as early as 6h post-treatment, with 48h-72h being the peak.

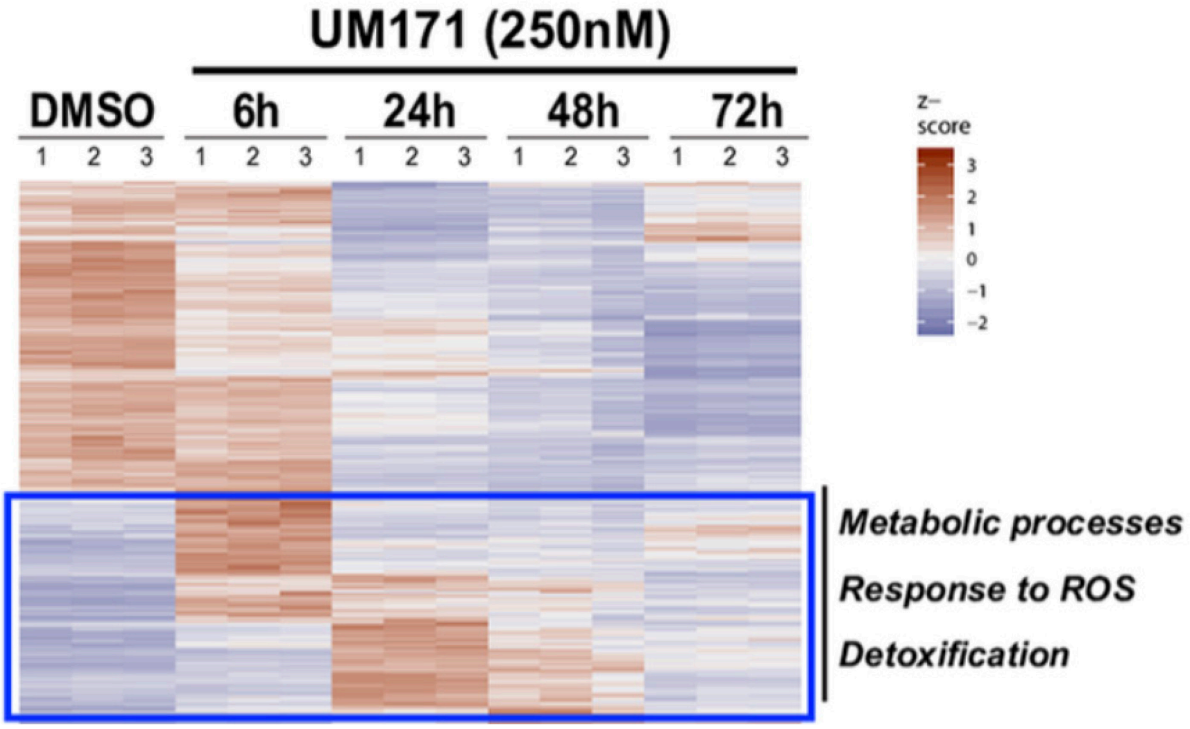


Figure 9. – Heat Map of Differently Expressed Transcripts Following UM171 Treatment After 6h, 24h, 48h and 72h in OCI-AML5 Cells (figure from (123))

UM171 triggers a detoxification response in hematopoietic cell lines. This process starts already 6h post-treatment with the drug and reaches its peak around 48h-72h after UM171 treatment.

1.6 Project Hypothesis

Antigen presentation is an essential process enabling for a specific adaptive immune response. Antigen-presenting cells uptake, process and present MHC-bound short peptides to cytotoxic and helper T-cells. Three signals must then follow for a proper T-cell activation, being antigen recognition, co-stimulation by specific surface proteins and finally cytokines, leading to T-cell differentiation. Along with the co-stimulatory molecule CD86, HLA molecules are crucial to antigen presentation properties, as they are orthologs of the murine MHC class I molecules H2-K and H2-D. As the compound UM171 increased the expression of CD86 and HLA molecules at the surface of human HSCs, we hypothesized that treatment of primary bone marrow-derived MSCs with UM171 could trigger similar upregulation of antigen presentation-associated genes. Under such circumstances, MSCs would acquire APC functions.

1.6.1 Objective 1

STUDY THE EFFECT OF UM171 ON MSC'S CAPACITY TO PRESENT/CROSS-PRESENT IMMUNOGENIC PEPTIDES

First of all, the effect of the compound on the cells will have to be established. Cells will therefore be treated with the drug at several concentrations and for different durations of treatment. Their characteristic family of markers (CD44+, CD45-, CD73+, CD90.1+, CD105+) and immune phenotype (MHCI, MHCII, CD80, CD86, PD-L1) will be assessed through flow cytometry and compared to a control sample. To assess antigen cross-presentation/presentation, UM171-treated MSCs versus DMSO-treated MSCs will be pulsed with 5mg/mL of OVA protein or 0.1 ug/mL of the SIINFEKL peptide to test for antigen cross-presentation or antigen presentation respectively. Treatment times for the OVA protein is 6-8h while it is between 2-3h for the SIINFEKL peptide. Once the pulsing is completed, cells will be co-cultured with 5 x 10^5 B3Z (SIINFEKL/H2-K^b-specific T-cell line) for 15-17 h. The following day, a colorimetric assay will allow for the readout.

1.6.2 Objective 2

UNDERSTAND THE MODE OF ACTION OF UM171 IN BONE-MARROW DERIVED MSCs

To understand how UM171 acts on MSCs to trigger antigen presentation or cross-presentation of immunogenic peptides, UM171- or DMSO-treated cells will be pulsed with OVA-AlexaFluor647 and OVA-DQ to test for protein uptake and processing respectively. We will also extract RNA from drug-treated cells versus control cells, after which qPCR will be conducted to look at the expression levels of several antigen presentation machinery-linked genes. To evaluate if the cross-presentation is ROS-mediated, MitoSox analysis using flow cytometry will be conducted. If ROS is detected, we will treat the cells with antioxidants (N-acetyl cysteine, alpha-tocopherol and mitotempo) to evaluate if the drug-mediated effects are ROS-dependent. As mitotempo inhibits mitochondrial-derived ROS, inhibitors of the electron transport chain complexes could also be tested in the context of antigen cross-presentation assay to assess if any of them are involved in UM171's action.

1.6.3 Objective 3

EVALUATE THE POTENCY OF THERAPEUTIC VACCINATION USING ANTIGEN-PULSED UM171-TREATED MSCs TO MEDIATE TUMOR REJECTION

We will investigate if vaccination using OVA-pulsed UM171-treated MSCs could mediate tumor rejection *in vivo*. To do so, we will be transplanting subcutaneously female mice with 5×10^5 EG.7. Once palpable tumors are established (size of approximately 55 mm^3), we will inject subcutaneously each one with 2.5×10^5 UM171- or DMSO-treated MSCs pulsed with 5mg/mL OVA protein for 6h. Injections will be done once weekly for two consecutive weeks. Control mice will receive the tumor cells alone. Assessment of mice survival and tumor growth will follow.

Chapter 2 – UM171-induced ROS Promote Antigen Cross-Presentation of Immunogenic Peptides by Bone-Marrow Derived Mesenchymal Stromal Cells

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2.1 Abstract

Background: Mesenchymal stromal cells (MSCs) have been extensively used in the clinic due to their exquisite tissue repair capacity. However, they also hold promise in the field of cellular vaccination as they can behave as conditional antigen presenting cells in response to interferon (IFN)-gamma treatment under a specific treatment regimen. This suggests that the immune function of MSCs can be pharmacologically modulated. Given the capacity of the agonist pyrimido-indole derivative UM171a to trigger the expression of various antigen presentation-related genes in human hematopoietic progenitor cells, we explored the potential use of UM171a as a means to pharmacologically instill and/or promote antigen presentation by MSCs.

Methods: Besides completing a series of flow cytometry-based phenotypic analyses, several functional antigen presentation assays were conducted using the SIINFEKL-specific T-cell clone B3Z. Anti-oxidants and electron-transport chain inhibitors were also used to decipher UM171a mode of action in MSCs. Finally, the potency of UM171a-treated MSCs was evaluated in the context of therapeutic vaccination using immunocompetent C57BL/6 mice with pre-established syngeneic EG.7 T-cell lymphoma.

Results: Treatment of MSCs with UM171a triggered potent increase in H2-K^b cell surface levels along with the acquisition of antigen cross-presentation abilities. Mechanistically, such effects occurred in response to UM171a-mediated production of mitochondrial-derived reactive oxygen species as their neutralization using anti-oxidants or Antimycin-A mitigated MSCs ability to cross-present antigens. Processing and presentation of the immunogenic ovalbumin-derived SIINFEKL peptide was caused by *de novo* expression of the *Psmb8* gene in response to UM171a-triggered oxidative stress. When evaluated for their anti-tumoral properties in the context of therapeutic vaccination, UM171a-treated MSC administration to immunocompetent mice with pre-established T-cell lymphoma controlled tumor growth resulting in 40% survival without the need of additional supportive therapy and/or standard-of-care.

Conclusions: Altogether, our findings reveal a new immune-related function for UM171a and clearly allude to a direct link between UM171a-mediated ROS induction and antigen cross-presentation by MSCs. The fact that UM171a treatment modulates MSCs to become antigen-presenting cells without the use of IFN-gamma opens-up a new line of investigation to search for

additional agents capable of converting immune-suppressive MSCs to a cellular tool easily adaptable to vaccination.

Keywords: Mesenchymal stromal cells; Antigen cross-presentation; UM171a; Reactive Oxygen Species; PSMB8; Electron transport chain; Antioxidants; Cellular vaccine; Anti-tumoral immunity.

2.2 Background

Several characteristics support the extensive use of culture-adapted mesenchymal stromal cells (MSCs) as cellular biopharmaceuticals.(126, 127) These include: **i)** simple isolation protocols from small bone marrow (**BM**) aspirates, **ii)** rapid *in vitro* proliferation, **iii**) standard and defined culture medium, **iv)** low senescence through multiple passages, **v)** gene modification easiness, and **vi)** distinct *in vivo* migration capabilities towards damaged or inflamed tissues.(86, 92, 126-130) The latter point combined with the mesenchymal differentiation plasticity of MSCs explains why these cells are extensively used for tissue repair and wound healing. Besides, MSCs can display remarkable immunomodulatory properties.(86, 92, 126-130) These immune functions are however influenced by the surrounding pro-inflammatory environment.[91] For instance, MSC stimulation with low interferon (IFN)-gamma doses (<25pg/ml) triggers antigen-presenting cell (APC)-like functions whereas higher and/or sustained IFN-gamma concentrations correlate with MSCs switching roles to immune-suppressor cells.(91) Physiologically, this means that MSCs could act as ''gatekeepers'' in the BM to preserve hematopoietic homeostasis during exacerbated inflammation.(91) From a therapeutic point of view however, high or sustained exposure of MSCs to IFN-gamma may not be suitable for cellular vaccination as it can halt their APC-like function 12 h post-priming.(91, 92, 94-96) Besides, IFN-gamma-primed MSCs express the immune checkpoint inhibitor PD-L1, which is known to impair metabolic and effector function of cytotoxic T lymphocytes.(91, 92, 94-96) Furthermore, IFN-gamma treatment of human MSCs did not promote antigen presentation. Instead, it enhanced their capacity to suppressive T-cell proliferation and graft-versus-host disease progression in humanized mice via production of indoleamine 2,3-dioxygenase.(131) Similar outcomes were observed when responding T cells were co-cultured with antigen pulsed IFN-gamma-stimulated human MSCs.(132) Thus, the design of novel pharmacological strategies capable of triggering consistent pro-inflammatory functions in both murine and human MSCs while bypassing the above-mentioned limitations remains a central goal for the development of immune-stimulatory MSC-based therapeutics.

Several stem cell "pharmaco-optimization" strategies were previously reported to enhance MSCs innate function as a means to ensure the development of a desirable therapeutic effect. For

instance, *ex vivo* pre-conditioning of MSCs with the anti-oxidant hormone melatonin was shown to improve implanted cell survival while decreasing their apoptosis rates.(133) Likewise, pre-conditioning of MSCs or other stem cell products with Celastrol, a natural compound known to promote anti-oxidant responses through activation of the nuclear factor erythroid 2-related factor 2 gene, represents another example showing how pharmacological stimulation enhances the endogenous protective effects of MSCs by increasing cell viability and therapeutic efficiency.(134, 135) Although these examples demonstrate that it is indeed feasible to pharmacologically modulate MSC function, no drug/compound besides IFN-gamma was ever reported to trigger APC-like properties in MSCs.

We show in this study how culture-adapted MSCs treated with the pyrimido-indole derivative UM171a, exhibit enhanced production of mitochondrial-derived superoxide anion. As a result, treated MSCs up-regulate their major histocompatibility complex (MHC) I expression and acquire the capacity to cross-present immunogenic peptides from captured soluble protein. When tested as a therapeutic cellular vaccine, antigen-pulsed UM171a-treated MSCs significantly interfered with the growth of pre-established solid T-cell lymphoma. Altogether, these studies indicate that UM171a-treated MSCs can indeed serve as a possible alternative to standard dendritic cells in the future design of cancer vaccines.

2.3 Methods

2.3.1 Animals and Ethics

All C57BL/6 female mice (6- to 8-week-old) used in this study were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in a pathogen-free environment at the animal facility of the Institute for Research in Immunology and Cancer (IRIC). Animal protocols were approved by the Animal Care Committee of Université de Montréal.

2.3.2 Cell Lines and Reagents

The EG.7 and B3Z cell lines were kindly provided by Dr. Jacques Galipeau (University of Wisconsin-Madison, WI, USA). Murine embryonic fibroblasts (MEFs) were kindly provided by Dr. John Stagg (CR-CHUM, QC, Canada). All cell culture media and reagents were purchased from Wisent Bioproducts (Saint-Jean-Baptiste, QC, CANADA). The anti-endothelial protein C receptor (EPCR) antibody, the anti-PD-L1 neutralizing antibody, and the IFN-gamma/IL-2 quantikine ELISAs were purchased from R&D Systems (Minneapolis, MN, USA). All remaining antibodies used for flow-cytometry were purchased from BD Biosciences (San Jose, CA, USA). The albumin from chicken egg white (OVA), Accutase®, Rotenone, Malonate, Antimycin A, Sodium Azide, Oligomycin, MitoTEMPO, α-tocopherol, N-acetylcysteine (NAC), and Chlorophenol red-β-D-galactopyranoside (CPRG) were purchased from Sigma-Aldrich (St-Louis, MI, USA). The SIINFEKL peptide was synthesized by GenScript (NJ, USA). Recombinant IFN-gamma was purchased from Peprotech (Rocky Hill, NJ, USA). The UM171a compound was provided by ExCellThera (Montreal, QC, CANADA). Alexa Fluor® 647-conjugated OVA, OVA-DQ® and MitoSox™ were purchased from ThermoFisher (Waltham, MA, USA). The CD8 T-cell isolation kit was purchased from STEMCELL Technologies (Vancouver, BC, CANADA). Amicon Ultra-4 centrifugal filters were purchased from Millipore-Sigma (Burlington, MA, USA).

2.3.3 Generation of Primary BM-Derived MSCs

To generate mouse primary MSCs, BM was flushed from femurs of a female C57BL/6 mouse and cultured in AMEM supplemented with 10% fetal bovine serum (FBS) and 50 U/mL Penicillin-Streptomycin. The media was changed every 2-3 days until MSC colonies were apparent. Following 2-3 passages, MSCs were phenotyped by flow-cytometry using antibodies against CD44, CD45, CD73, CD90.1, CD105, and H2-K^b diluted according to manufacturer's instructions. After washing using the staining buffer, cells were re-suspended in 400 µl of staining buffer. The samples were acquired by BD FACS Diva on CANTOII, then analyzed using FlowJoV10.

2.3.4 Assessment of the UM171a Maximum Tolerated Dose (MTD)

To identify the MSC MTD for UM171a, 5 x 10^4 plated MSCs were treated with ascending doses of the compound (35 to 8000 nM) for 72 h. Treated cells were then washed, detached and counted using trypan blue to differentiate between live and dead cells. The highest dose with no toxicity or decreased proliferation effects was selected for subsequent studies.

2.3.5 Antigen Cross-Presentation Assay

To assess antigen cross-presentation, MSCs or MEFs were first seeded in a 24-well plate at 1.5 x 10^4 cells per well. The following day, adherent cells were treated with 35, 250 or 1000 nM of UM171a or equivalent DMSO concentration for three days prior to pulsing with OVA at 5 mg/ml for 5-6 h. A similar approach was used for the SIINFEKL peptide (at 0.1 µg/ml) except that the pulsing period was 2-3 h. Once the pulsing is completed, cells were washed twice to remove excess antigen/peptide followed by the addition of 5 x 10^5 B3Z (SIINFEKL/H2-K^b-specific T-cell line) for 15-17 h. The following day, all cells were lysed then stained with a CPRG solution for 18 h at 37°C. The optical density signal was detected using a SynergyH1 microplate reader (Biotek, Winooski, VT, United States). For all experiments using inhibitors or antioxidants, the same assay was conducted and inhibitors/antioxidants were added as detailed elsewhere. A similar set-up was used for OT-I-based antigen presentation assays except that isolated OT-I-derived CD8 T cell were co-cultured for three days with MSCs prior to assessing IFN-gamma and IL-2 production by respective quantikines.

2.3.6 Monitoring Antigen Up-Take and Processing

To evaluate the effect of UM171a on OVA uptake, 1.5 x 10^4 cells were seeded per well in a 24 well plate. On the following day, cells were treated with DMSO or UM171a (1000 nM) for three days. Once the UM171 treatment period was completed, 1 µg/ml of Alexa Fluor® 647-conjugated OVA was added on cells for 2h prior to their trypsinization and washing with cold PBS containing 2% FBS. Fluorescence was then assessed by flow-cytometry. For OVA processing, UM171a- or DMSO-treated MSCs (as explained above) were incubated with 10 µg/mL OVA-DQ at 37°C. One hour later, cells were washed, and regular media added (pulse and chase). After 3 h, cells were collected and washed with cold PBS containing 2% FBS. Fluorescence was monitored by flow-cytometry.

2.3.7 Luminex Analysis

To screen and quantify cytokine production, UM171a- or DMSO-treated MSCs were cultured for three days in the absence of serum. Once the incubation period completed, supernatants were collected, centrifuged for 10 min at 750 x g to remove any floating cells or cell debris prior to concentrate the collected media using the Amicon Ultra-4 centrifugal filters (3000 NMWL) for 45 min at 4°C. Collected concentrate was then frozen at -80°C until shipped to EveTechnologies (Calgary, AB, CANADA) for luminex assessment.

2.3.8 Bioinformatics Analysis

Bulk RNA seq data was downloaded from GEO (GSE138487). Gene-level count data was imported and processed in DESeq2.(136) Expression data from OCI-AML5 cells treated with UM171a over 72 h was contrasted with data from DMSO-treated cells. The resulting differential analysis (DEG) generated a ranked list of genes using the Wald statistic, which was subsequently used for Gene set enrichment analyses.(137) The Biological process GO annotations are selected to identify gene sets up- or down-regulated by UM171a. Heatmaps were plotted in R statistical programming (using heatmap.2 function in gplots package).

2.3.9 Immunization and Tumor Challenge Studies

For therapeutic vaccination, female C57BL/6 mice (n=10/group) were first subcutaneously (SC) transplanted with 5 x 10^5 EG.7 cells at day 0. Four to five days later (e.g. following appearance of palpable tumors ~ 20-40 mm^3), mice were SC-injected with 2.5 x 10^5 UM171a- or DMSO-treated MSCs pulsed with 5 mg/ml OVA protein for 5-6h (detached using Accutase®). Two injections were given one week apart. Control animals received 5 x 10^5 tumor cells alone. Vaccinated animals were followed thereafter for tumor growth.

2.3.10 Statistical Analysis

p-values were calculated using the one-way analysis of variance (ANOVA), except for figure 3B, where a student *t*-test was used. Results are represented as average mean with standard deviation (S.D.) error bars, and statistical significance is represented with asterisks: *P<0.05, **P<0.01, ***P<0.001.

2.4 Results

2.4.1 UM171a is Well Tolerated by Primary MSCs and Triggers MHCI Up-Regulation.

The parent UM171a compound was initially discovered by a high-throughput screening assay designed for the identification of compounds capable of triggering primary CD34+ cell proliferation.(138) A series of chemical modification was then conducted to create the final UM171a product, which effectively promotes *ex vivo* expansion of long term (LT)-hematopoietic stem cells (HSCs).(139) When further studied to decipher its potential mode of action on human $CD34^+$ stem cells, UM171a was found to trigger a marked increase in the expression of several immune-related genes including human leukocyte antigens (HLA)-A and B - ortholog of the murine MHCI (aka H2-K/H2-D)-, beta 2-microglobulin (β2M) as well as the co-stimulatory molecule CD86.(123) Since these specific genes are central to antigen presentation, we posited that treatment of primary murine MSCs with UM171a would trigger or enhance the expression of these genes resulting in the acquisition of antigen presentation properties. Prior to testing this hypothesis, we first identified the working concentration of UM171a by conducting MTD experiments on murine MSCs over three consecutive days. We elected to work with a UM171a concentration of 1000 nM as higher doses impair cell proliferation (**Fig. 9A**). Further characterizations revealed that UM171a treatment did not alter the innate MSC phenotype as the cells remained CD45 negative while expressing CD44, CD73, CD90 and CD105 (**Fig. 9B**). Although no increase in cell surface expression of the co-stimulatory molecules CD86 nor its homolog CD80 was detected on murine MSCs (**Fig. 9B**), a sharp increase in H2-K^b expression was observed (**Fig. 9B**). To see if this H2-K^b increase requires a 72h treatment and/or a dose as high as 1000 nM, we evaluated the effects of multiple UM171a doses (35, 250 or 1000 nM) in a timely manner. Indeed, H2-K^b levels were only enhanced following a three-day treatment with 1000 nM of UM171 (**Fig. 9C**), and remained steady up to a dose of 8000 nM (**Fig. 9D**). Interestingly, assessment of EPCR expression, a marker of UM171-induced activation, followed an expression profile kinetic akin to H2-K^b (**Fig. 9E**) indicating a direct correlation between H2-K^b increase and enhanced EPCR expression. To ensure that these observations can be replicated using human

cells, human umbilical cord (UC)-derived MSCs were treated with 1000 nM UM171a and showed a similar increase pattern in HLA-A/B/C expression (**Fig. 9F)**. Altogether, these results indicate that UM171a is well tolerated by MSCs and can trigger potent increase in MHCI/HLA cell surface expression.

2.4.2 Treatment of Murine MSCs with UM171a Instills Antigen Cross-Presentation Abilities with No Protagonist Effect on Antigen Uptake and Processing.

The observed increase in MHCI/HLA levels on the surface of UM171a-treated MSCs suggests that these cells may exhibit enhanced antigen presentation or the capacity to cross-present captured soluble antigens to responding CD8 T cells. We thus tested whether the identified dosing and treatment regimen affects antigen cross-/presentation by MSCs following soluble OVA protein or SIINFEKL peptide pulsing respectively (**Fig. 10A**). Besides exhibiting enhanced antigen presentation (as shown by the SIINFEKL response), UM171a-treated MSCs were also able to cross-present the immunogenic OVA-derived SIINFEKL peptide (**Fig. 10B**) with a comparable T-cell response following longer (7 instead of 3 days) treatment regimen (**Fig. 10C-D**). When tested on MEFs - another non-hematopoietic cell - UM171a treatment failed to trigger antigen cross-presentation despite improved antigen presentation (**Fig. 10E**) and increased EPCR expression (**Fig. 10F**). Since the observed antigen cross-presentation effect mediated by UM171a can be potentially enhanced by increased extracellular antigen capturing and driven by differential intracellular processing, MSCs were first treated with UM171a for three days then pulsed with either fluorescent OVA-AF647 (to assess antigen capturing) or OVA-DQ (to evaluate OVA processing). Compared to DMSO-treated MSCs, no increase in antigen uptake (**Fig. 10G**) nor antigen processing (**Fig. 10H**) was observed. The sum of these observations stipulates that UM171a can trigger *de novo* antigen cross-presentation by MSCs in a mechanism(s) independent of enhanced antigen uptake or processing.

2.4.3 Reactive Oxygen Species (ROS) Production Drives Antigen Cross-Presentation in UM171-Treated MSCs

Since antigen up-take and processing could not explain the induced cross-presentation ability of UM171a-treated MSCs, we next wondered whether such treatment affects the endoplasmic-

reticulum (ER)-associated protein degradation (ERAD) machinery. ERAD is a cellular pathway responsible for targeting misfolded proteins for ubiquitination and subsequent degradation by the proteasomal complex.(140) Analysis of publicly-available transcriptomic data conducted on human HSCs revealed UM171-mediated changes in the expression of several ERAD-related genes such as *Erap1/2*, *β2m*, *Tap1/2* as well as *H2-t* and *H2-q* molecules (**Fig. 11A**). Although expression of murine homolog of these genes remained steady in UM171-treated murine MSCs, a noticeable increase in the expression of other tested genes, *Psmb8* and *Calr*, was observed (**Fig. 11B**). This is a salient observation for three main reasons. First, *Psmb8* - the β5i-subunit of the immunoproteasome - possesses a strong chymotryptic- and tryptic-like processing activity capable of generating 8-9 amino-acid peptide fragments that can efficiently fit within MHCI grooves.(141, 142) Second, *Calr* plays an important role in capturing misfolded proteins preventing their migration from ER to the golgi apparatus.(143) Third, the expression of these two genes can be induced in response to misfolded proteins that accumulate intracellularly due to aggregations or damages inflicted by elevated ROS production.(144-147) This is in line with the previous observation that treatment of human $CD34^{+}$HSCs with UM171a induces detoxification pathways as a defense mechanism to counteract the toxic effects mediated by elevated ROS levels.(123) When we investigated UM171a-triggered ROS (more specifically superoxide anion) production in both murine MSCs and MEFs following a 72h treatment condition, a signal was only detected in MSCs (**Fig. 11C**). Production of superoxide anion production was however completely abolished in MSCs following MitoTEMPO (an inhibitor of mitochondrial-derived ROS), vitamin E derivative α-tocopherol (inhibitor of lipid peroxidation), or NAC (a general antioxidant and cysteine donor) co-treatments (**Fig. 11D**). These observations prompted us to further explore whether ROS production predisposes MSCs to acquire antigen cross-presentation abilities. We thus, co-treated UM171a-pulsed MSCs with the same antioxidants listed above prior to conducting an antigen presentation assay. As shown in **Fig. 11E**, addition of MitoTEMPO or α-tocopherol completely blunted antigen cross-presentation by UM171a-treated MSCs whereas significant inhibition was observed with the use of NAC. Antigen presentation (e.g. SIINFEKL pulsing), on the other hand, remained unchanged between anti-oxidants and control treatments. To further re-enforce this hypothesis, we next compared the transcript levels of *Psmb8* in

UM171a-treated MSCs co-treated with antioxidants. As expected, *Psmb8* expression was impaired in response to α-tocopherol, MitoTEMPO or NAC (**Fig. 11F**) clearly indicating a central role played by ROS in mediating antigen cross-presentation via *de novo* expression of *Psmb8*.

ROS are generally produced by mitochondria during the process of oxidative phosphorylation.(148) More specifically, electron transfer between complexes of the electron transport chain (ETC) lead to partial reduction of oxygen to form superoxide anion.(149) Since UM171a triggers both ROS and their cognate detoxification mechanisms, it is logical to stipulate that it may act either directly or indirectly onto ETC complex(es). We thus tested the effect of various ETC inhibitors (ETCi - **Fig. 12A**) on MSC-mediated antigen cross-presentation as a co-treatment strategy with UM171a (e.g. since day 1) or during the antigen pulsing step (after the 3 day treatment period with UM171a - **Fig. 12B**). Surprisingly, antigen cross-presentation by UM171a-treated MSCs was unaffected by ETCi during the co-treatment regimen (**Fig. 12C - upper panel**), whereas a significant decrease in B3Z activation was only observed when Antimycin-A (inhibitor of complex III) was co-treated with OVA (**Fig. 12C - lower panel**). Interestingly, UM171a-pulsed MSCs co-treated with Antimycin-A showed very low or absent superoxide anion production (**Fig. 12D**) with the absence of major effects on OVA uptake or processing (**Fig. 12E**). These results clearly indicate that mitochondrial-derived ROS production is the main factor driving antigen cross-presentation by UM171a-treated MSCs.

2.4.4 UM171a Treatment Does Not Induce PD-L1 Expression on MSCs.

We know so far that MSC treatment with both UM171a or IFN-gamma leads to enhanced MHCI expression (**Fig. 13A**) and promote antigen cross-presentation (**Fig. 13B**).(92) We thus decided to compare the functional potency of both treatments in an antigen presentation assay. Since the OVA pulsing protocols for UM171a- and IFN-gamma-treatment are different (8 versus 18h respectively), we tested both conditions and noted a significantly higher T-cell response with the IFN-gamma treatment (**Fig. 13B**) most likely owing to the elevated H2-K^b levels following IFN-gamma treatment (**Fig. 13A**). Interestingly however, UM171a did not induce *de novo* expression of PD-L1 on the surface of MSCs compared to IFN-gamma treatment (**Fig. 13C**). Since the B3Z cell

line may not be highly responsive/sensitive to PD-1/PD-L1 interaction due to its low/absent PD-1 expression profile (**small panel in Fig. 13C**), we repeated the antigen presentation assay using primary OT-I-derived CD8 T cells and assessed their responsiveness by quantifying both IFN-gamma and IL-2 production. Although the T-cell response to SIINFEKL presentation by IFN-gamma-treated MSCs was substantially higher compared to the UM171a-treated group (**Fig. 13D-E**), the antigen cross-presentation ability of UM171a-treated MSCs was superior to IFN-gamma treatment (**Fig. 13D-E**), but became comparable to the IFN-gamma group in the presence of PD-L1 neutralizing antibodies (**Fig. 13D-E**). These results clearly highlight a therapeutic advantage for the use of UM171a as it precludes the negative role played by PD-L1 expression normally induced in response to IFN-gamma stimulation.

2.4.5 Therapeutic Vaccination Using UM171a-Treated MSCs Significantly Delays Tumor Growth.

Given the potent *in vitro* cross-presentation ability of UM171a-treated MSCs, we finally assessed the ability of these cells to trigger anti-tumoral immune response in immunocompetent animals with pre-established EG.7 T-cell lymphomas (**Fig. 14A**). The SC delivery of OVA-pulsed MSCs treated with UM171a significantly delayed tumor growth compared to OVA-pulsed MSCs or untreated control mice (**Fig. 14B**) with a 40% survival rate reached up to 40 days post-tumor transplantation (**Fig. 14C**). Although this therapeutic effect can be explained by the immunogenic potential of the vaccine, MSCs can further modulate immunity via their capacity to secrete various immune soluble mediators.(150, 151) We thus evaluated whether UM171a affects the secretome of MSCs, hence amplifying their anti-tumoral properties. Indeed, a three-day treatment with UM171a led to significant increases in various pro-inflammatory cytokines (M-CSF, GM-CSF, IL-6, and IP-10), chemokines (KC, LIX, MIP-2) as well as VEGF (**Fig. 14D**), which are all known for their ability to recruit and modulate the activity of host-derived innate and adaptive immune cells. Altogether, these findings indicate that UM171a-treated MSCs can be effectively exploited in the design of cellular vaccines capable of triggering potent anti-tumoral responses.

2.5 Discussion

The idea of testing the effect of UM171a on antigen presentation by culture-adapted primary MSCs stems from the initial observation that UM171a-treated human LT-HSCs up-regulate several immune-related genes such as CD86.(123) Although *de novo* expression of this costimulatory molecule was undetected on the surface of treated MSCs, the expression levels of MHCI were significantly increased following UM171a treatment and correlated directly with enhanced antigen presentation. Interestingly, UM171a-treated MSCs also acquired the ability to cross-present peptides from soluble antigens without exhibiting enhanced uptake or intracellular processing of captured proteins. Instead, the cross-presentation ability of UM171a-treated MSCs requires mitochondrial-mediated ROS production. Although ROS play important physiological roles in eukaryotic cells, they are also known for their ability to disrupt proteostasis by causing protein damages and aggregation resulting in ER stress.(123) This can explain the induced *de novo* expression of *Psmb8* in response to UM171a treatment as a means to initiate protein processing/degradation in order to "clean-up" the cell and re-establish protein homeostasis. Under such context, we can presume that any exogenous protein (example OVA) captured by UM171a-treated MSCs is subjected to ROS-mediated oxidations/damages, which ends-up targeting the protein for degradation by β5i-containing proteasomes consequently resulting in the generation of stable and immunogenic peptides (**Graphical abstract**).

The most salient observation in this study is the direct link between UM171a-mediated ROS production and antigen cross-presentation. This is supported by the blunting effect of MitoTempo and α-tocopherol treatments as they directly neutralized mitochondrial-derived ROS and lipid peroxidation respectively, impairing MSCs ability to activate responding T cells. Their neutralizing effect did not however inhibit antigen presentation as reflected by the sustained SIINFEKL stimulation, suggesting another mechanism at play for MHCI enhanced up-regulation by UM171a. This is not surprising for two reasons. First, UM171a was suggested to share a common molecular pathway with tranylcypromine and potentially other LSD1 inhibitors, which can regulate the expression of both stem cell as well as classical and non-classical MHCI-related genes.(152, 153) This may explain the functional discrepancy observed between MEF and MSC responses. More

specifically, the inability of UM171-treated MEFs to cross-present can be due to the absence of ROS induction, but the enhanced antigen presentation observed along with the increased EPCR expression following UM171a treatment suggests an additional mechanism at play. Second, our data allude to an important role for ETC complex III in this context as co-treatment of UM171a-treated MSCs with Antimycin-A, but not other ETCi (e.g. rotenone, malonate, oligomycin and sodium azide), during OVA pulsing impaired antigen cross-presentation. It is not clear so far if UM171a directly binds complex III or supports an indirect function associated with: i) oxidative phosphorylation, ii) TCA cycle activity, iii) regulating the expression of genes associated with complex III, or even iv) inducing hypoxia. However, complex III is the only ETC component capable of releasing superoxide anion to both sides of the inner mitochondrial membrane (e.g. matrix and cytoplasm).(154) Although the exact Antimycin-A mode of action remains ill-defined, this ETCi was proposed to regulate ROS flow away from the mitochondrial matrix into the cytoplasm.(154) This suggests that matrix-resident ROS are central to UM171a-related cross-presentation activity as their diminished production in response to Antimycin-A impairs T-cell activation. Further studies are therefore warranted to decipher the exact UM171a mode of action alone or in concert with Antimycin-A to understand the implication of this molecule at the mitochondrial level.

2.6 Conclusion

The use of IFN-gamma to promote antigen cross-presentation by MSCs highlights the pleotropic function that can be mediated by this non-hematopoietic cell following its pharmacological stimulation. However, the IFN-gamma stimulation approach is hampered by several factors including the long-term negative effect of IFN-gamma stimulation, the use of an appropriate dosing, which cannot be controlled once the cells are administered *in vivo*, as well as T-cell inhibition via *de novo* expression of the PD-L1 immune-checkpoint. UM171a has the advantage of bypassing most of these limitations as it does not seem to be negatively modulated once a pharmacological effect has taken place while triggering a pro-inflammatory phenotype without inducing PD-L1. The sum of these attributes explains the remarkable effect observed on tumor growth following therapeutic vaccination. As such, the concept of using UM171a to drive ROS production as a means to trigger components of the immunoproteasome complex pave the path for the search of additional compounds that may act on this pathway for the future design of cancer cell vaccines as an alternative to the use of standard dendritic cells.

2.7 Declarations

2.7.1 Ethics Approval and Consent to Participate

All animals used in the study were housed in a pathogen-free environment at the animal facility of the Institute for Research in Immunology and Cancer (IRIC) and maintained in accordance with the guidelines approved by the Animal Care Committee of Université de Montréal.

2.7.2 Consent for Publication

All authors consent to the publication of provided data.

2.7.3 Availability of Data and Material

Data sets and material/reagents analyzed and/or used in this study are available upon reasonable request.

2.7.4 Competing Interests

The authors declare no competing financial interest.

2.7.5 Funding

Studies were funded by an operating grant from the Cancer Research Society (OG24054).

2.7.6 Authors' Contributions

NS, JPB, NEH and WS conducted most of the *in vitro* and *in vivo* assays. MK contributed to the study design. GS developed and provided the UM171a compound and contributed to study design along with JZ and NE. MR conceived and supervised the project, analyzed data and wrote the first draft of the manuscript. All authors contributed to the editing of the manuscript.

2.7.7 Acknowledgements

We wish to thank the personnel at the IRIC animal facility for their kind help with some of the *in vivo* experiments.

Figure 10. – Characterizing the pharmacological effect of UM171a on MSCs.

A) Assessment of various UM171a doses on the proliferation of MSCs over a period of 72h. For this panel, n=3/group. B) Phenotypic analysis of MSCs treated with 1000 nM UM171a for 72 h. C) Timeline comparison of the effect of three UM171a doses on H2-Kb induction. D) Testing the effect of UM171 on H2-Kb using doses higher than 1000 nM. E) Flow analysis of EPCR cell surface expression on MSC treated with 1000 nM UM171a for 72h. F) Representative flow-cytometry analysis of HLA-A/B/C on the surface of human UC-derived MSCs treated with UM171a with 35, 250 and 1000 nM. All experiments were repeated at least three times.

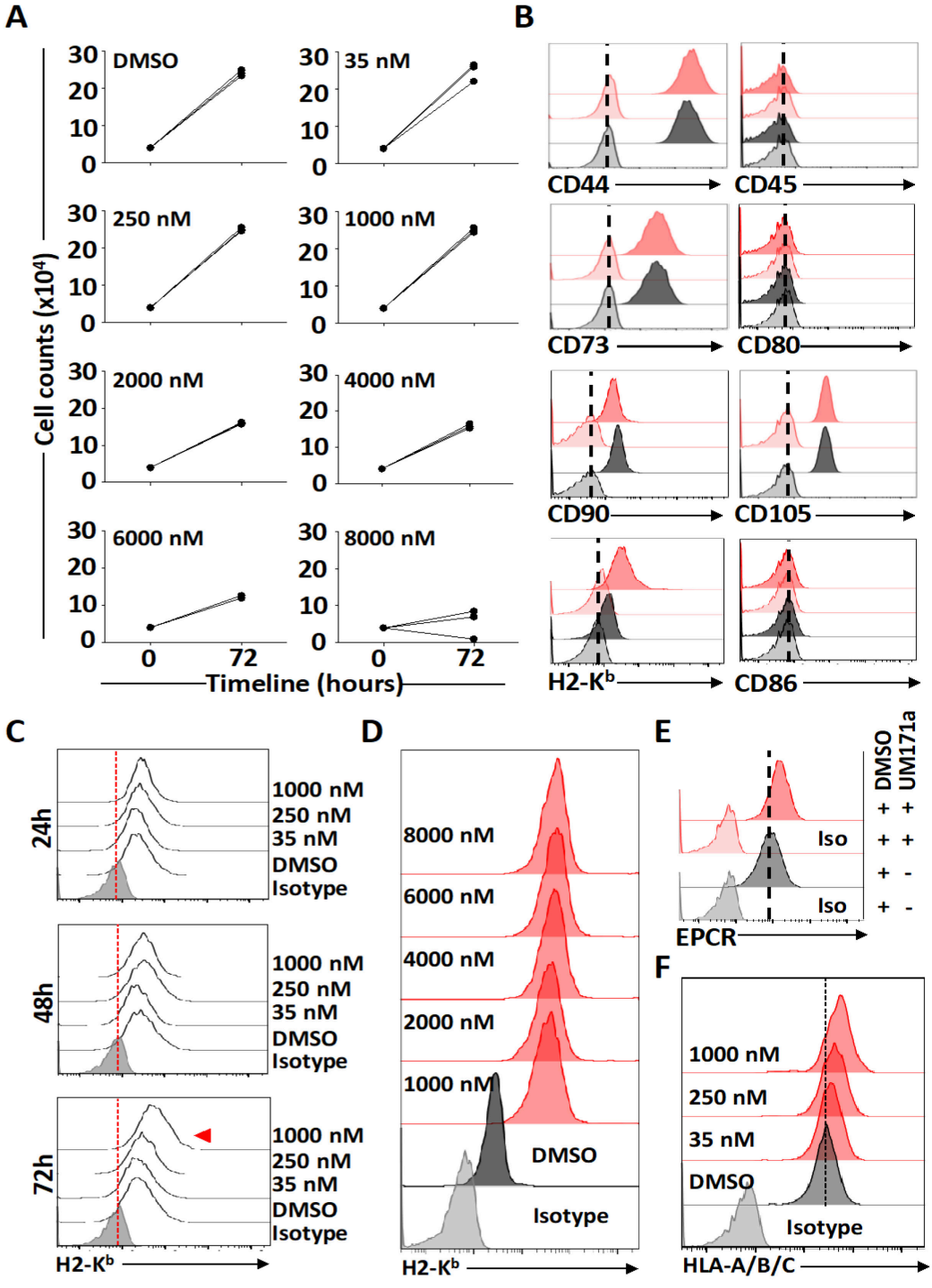

A
DMSO
35 nM
250 nM
1000 nM
2000 nM
4000 nM
6000 nM
8000 nM
Cell counts (x10⁴)
Timeline (hours)
B
CD44
CD45
CD73
CD80
CD90
CD105
H2-Kᵇ
CD86
C
24h
48h
72h
1000 nM
250 nM
35 nM
DMSO
Isotype
H2-Kᵇ
D
8000 nM
6000 nM
4000 nM
2000 nM
1000 nM
DMSO
Isotype
H2-Kᵇ
E
DMSO
UM171a
Iso
EPCR
F
1000 nM
250 nM
35 nM
DMSO
Isotype
HLA-A/B/C

Figure 11. – UM171a-Treated MSCs Can Cross-Present Soluble Antigen.

A) Schematic diagram showing the design of the antigen cross-presentation assay. **B)** UM171a triggers *de novo* cross-presentation by MSCs and enhances antigen presentation. **C**) Schematic diagram showing the design of the antigen cross-presentation assay in response to 3- or 7-day treatment. **D**) OVA cross-presentation response following a 3- or 7-day treatment. **E**) Antigen presentation assay conducted on primary MEFs treated with UM171a. **F)** Assessment of EPCR expression by flow-cytometry on the surface of UM171-treated MEFs. **G)** Flow-cytometry assessment of fluorescent OVA uptake by UM171a-treated MSCs. DMSO treated cells are shown by grey histograms whereas UM171a-treated cells are depicted in red. **H)** Evaluating OVA processing as in panel G. All experiments were repeated at least three times. For panels B, D and E, n=6/group with *P<0.05 and ***P<0.001.

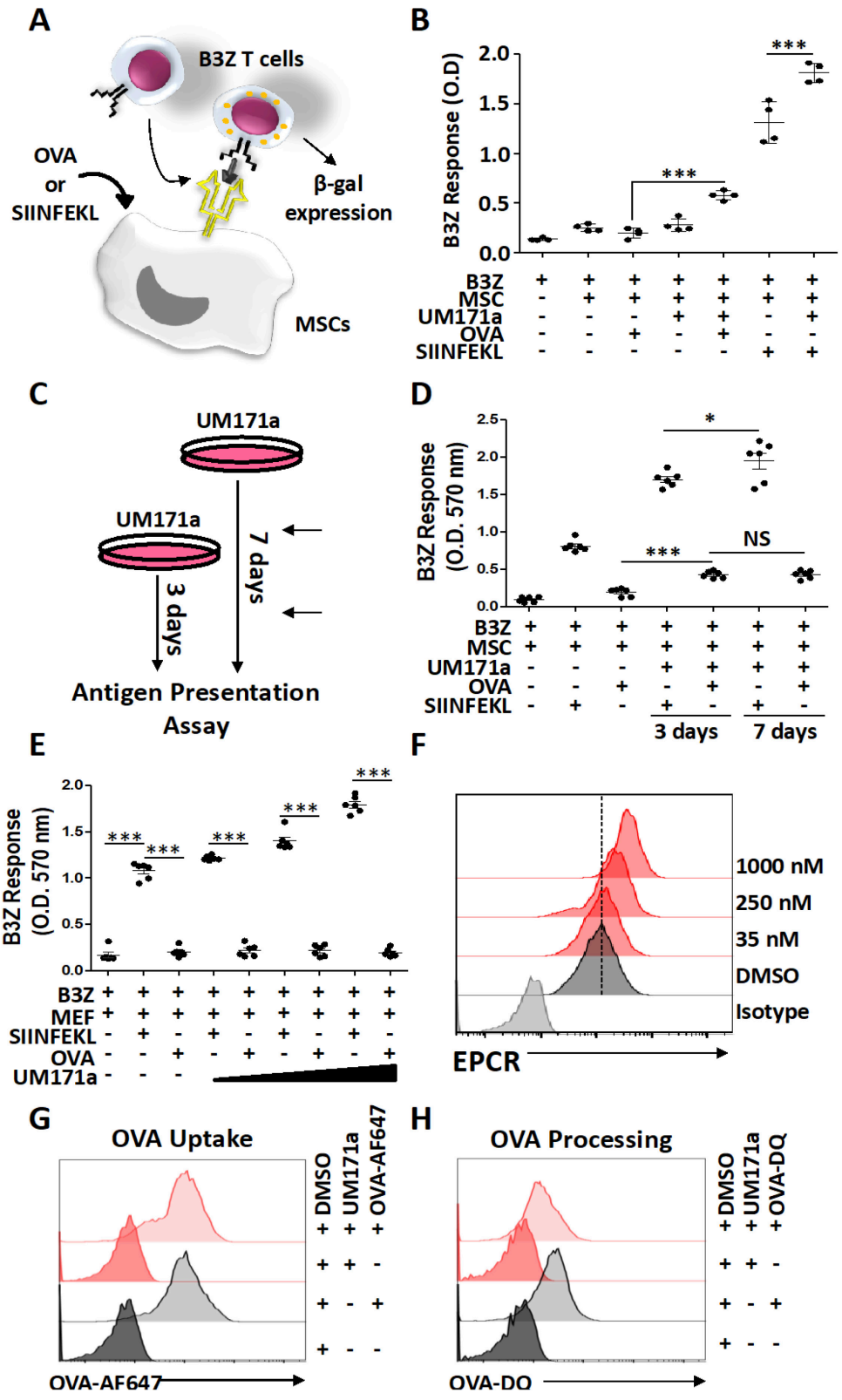

A
B3Z T cells
OVA
or
SIINFEKL
β-gal
expression
MSCs
B
B3Z Response (O.D)
2.0
1.5
1.0
0.5
0.0
***
***
B3Z + + + + + + +
MSC - + + + + + +
UM171a - - - + + - +
OVA - - + - + - -
SIINFEKL - - - - - + +
C
UM171a
UM171a
7 days
3 days
Antigen Presentation
Assay
D
B3Z Response
(O.D. 570 nm)
2.5
2.0
1.5
1.0
0.5
0.0
*
***
NS
B3Z + + + + + + +
MSC + + + + + + +
UM171a - - - + + + +
OVA - - + - + - +
SIINFEKL - + - + - + -
3 days
7 days
E
B3Z Response
(O.D. 570 nm)
2.0
1.5
1.0
0.5
0.0
***
***
***
***
***
B3Z + + + + + + + + +
MEF + + + + + + + + +
SIINFEKL - + - + - + - + -
OVA - - + - + - + - +
UM171a - - -
F
1000 nM
250 nM
35 nM
DMSO
Isotype
EPCR
G
OVA Uptake
+ DMSO
+ UM171a
+ OVA-AF647
+ + -
+ - +
+ - -
OVA-AF647
H
OVA Processing
+ DMSO
+ UM171a
+ OVA-DQ
+ + -
+ - +
+ - -
OVA-DQ

Figure 12. – UM171a Treatment Leads to ROS Production.

A) A heatmap showing differentially expressed genes in GO:0019883 (Antigen processing and presentation). This process is substantially up-regulated in UM171a 72h treated group (normalized enrichment score = 2.1; FDR < 0.01). **B)** Transcript quantification of genes involved in the ERAD pathway. **C)** Representative MitoSOX staining of MSCs or MEFs treated with UM171a. **D)** Representative experiment of MitoSOX staining of UM171a-treated MSCs following antioxidant treatment. **E)** Antigen cross-presentation assay using the antioxidants MitoTEMPO (10 µM), α-tocopherol (800 µM), and NAC (5 mM) added at day 0 with UM171a for 72h. Red arrows highlight the inhibitory effect of the antioxidants on antigen cross-presentation. **F**) Quantification of *Psmb8* transcript in UM171a-treated MSCs undergoing co-treatment with antioxidants over 72h. The UM171a group (positive control) is displayed in red. For panels B, E and F, n=6/group with *P<0.05, **P<0.01 and ***P<0.001.

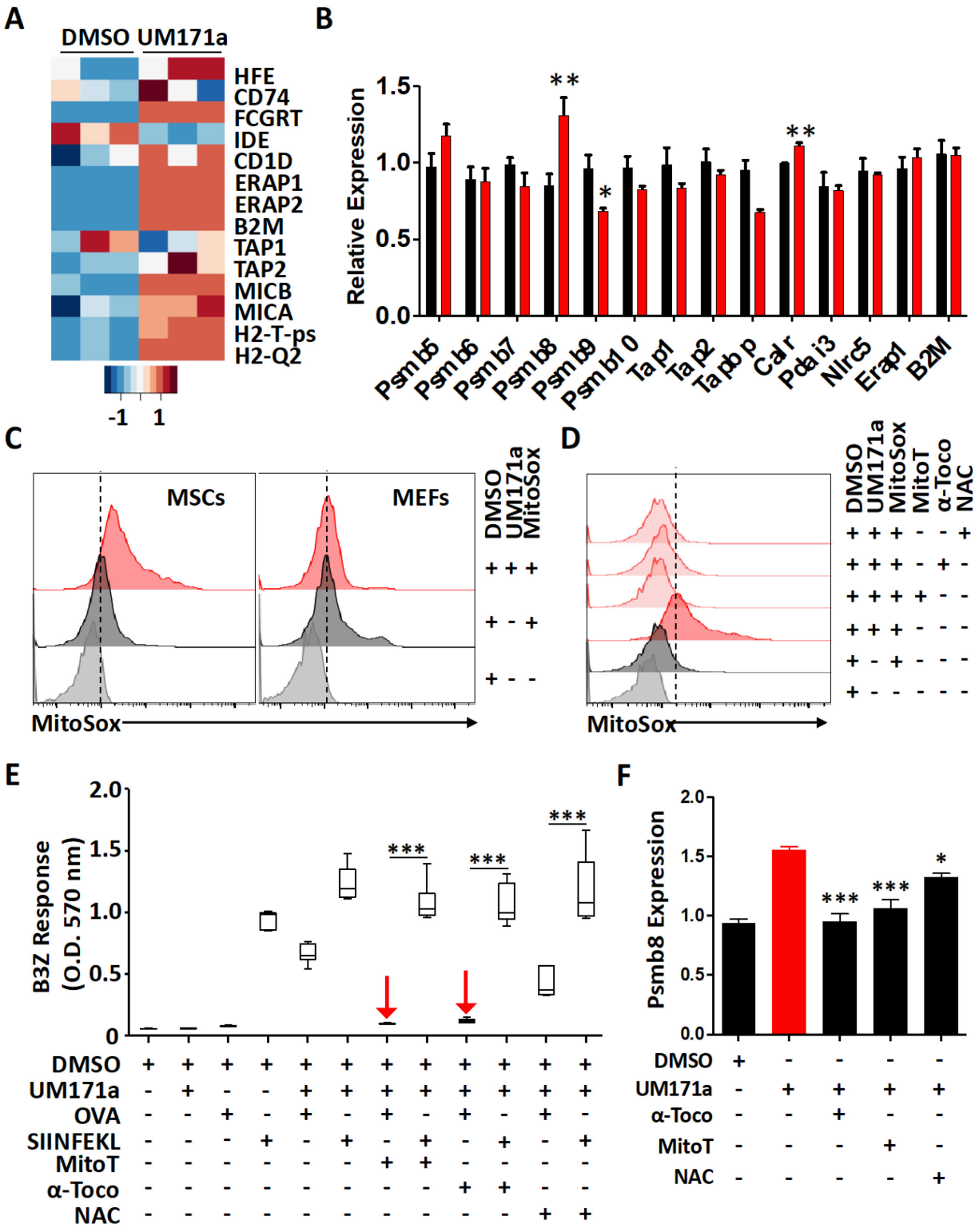

A
DMSO UM171a
HFE
CD74
FCGRT
IDE
CD1D
ERAP1
ERAP2
B2M
TAP1
TAP2
MICB
MICA
H2-T-ps
H2-Q2
-1 1
B
Relative Expression
1.5
1.0
0.5
0.0
**
*
**
Psmb5 Psmb6 Psmb7 Psmb8 Psmb9 Psmb10 Tap1 Tap2 Tapbp Calr Pdia3 Nlrc5 Erap1 B2M
C
MSCs
MEFs
DMSO
UM171a
MitoSox
MitoSox
D
DMSO
UM171a
MitoSox
MitoT
α-Toco
NAC
MitoSox
E
B3Z Response (O.D. 570 nm)
2.0
1.5
1.0
0.5
0
***
***
***
DMSO
UM171a
OVA
SIINFEKL
MitoT
α-Toco
NAC
F
Psmb8 Expression
2.0
1.5
1.0
0.5
0.0
***
***
*
DMSO
UM171a
α-Toco
MitoT
NAC

Figure 13. – UM171a-Triggered Cross-Presentation Requires ROS Production.

A) Representative diagram displaying the ETC complexes and their respective inhibitors. **B)** Schematic diagram representing the experimental design of ETC inhibitor use along with UM171a. **C)** Antigen presentation assay using ETCi co-treated with UM171a (upper panel) or added at day 3 during OVA pulsing. **D)** Representative flow cytometry analysis of MitoSOX in MSCs co-treated with UM171a and Antimycin-A. The ETCi was added during the OVA pulsing period. The dashed line represents basal ROS levels before treatments. **E)** Representative flow cytometry analysis of OVA uptake (left panel) versus OVA processing (right panel) in the absence or presence of Antimycin-A co-treatment. All experiments were repeated at least three times. For panels in C, n=6/group with *P<0.05 and ***P<0.001.

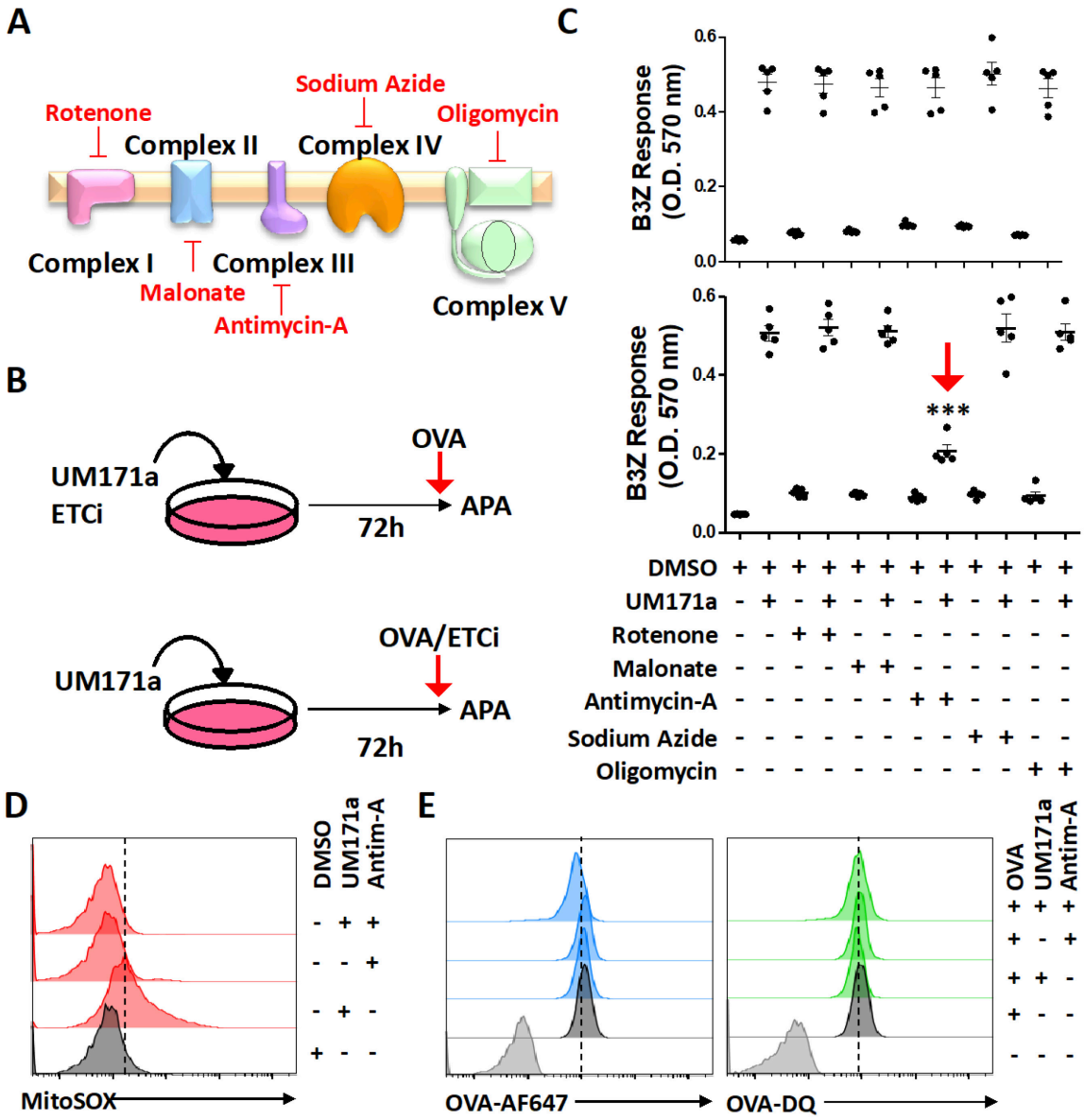

A
Rotenone
Sodium Azide
Oligomycin
Complex II
Complex IV
Complex I
Complex III
Malonate
Antimycin-A
Complex V
B
UM171a
ETCi
OVA
APA
72h
UM171a
OVA/ETCi
APA
72h
C
B3Z Response (O.D. 570 nm)
0.6
0.4
0.2
0.0
***
DMSO + + + + + + + + + + + +
UM171a - + - + - + - + - + - +
Rotenone - - + + - - - - - - - -
Malonate - - - - + + - - - - - -
Antimycin-A - - - - - - + + - - - -
Sodium Azide - - - - - - - - + + - -
Oligomycin - - - - - - - - - - + +
D
DMSO UM171a Antim-A
- + +
- - +
- + -
+ - -
MitoSOX
E
+ OVA + UM171a + Antim-A
+ - +
+ + -
+ - -
- - -
OVA-AF647
OVA-DQ

Figure 14. – UM171a Instills Antigen Cross-Presentation Properties Without PD-L1 Induction on the Surface of MSCs.

A) Representative flow-cytometry analysis of H2-K^b on MSCs treated with IFN-gamma or UM171a. The dashed line represents the basal expression level of H2-K^b before treatments. **B**) An antigen cross-presentation experiment comparing MSCs treated with UM171a versus IFN-gamma. OVA pulsing was conducting for both 8 or 18h. **C)** Representative flow-cytometry experiment assessing the expression of PD-L1 on both UM171a- or IFN-gamma-treated MSCs. The small integrated histogram represents PD-1 expression (in orange) on the B3Z cell line. **D-E)** IFN-gamma and IL-2 quantification by OT-I CD8 T cells in response to UM171a- or IFN-gamma-treated MSCs. The PD-L1 neutralizing antibodies were used at 0.5µg/ml. For panels A, C and D, n=5/group with *$p<0.05$ and ***$p<0.001$.

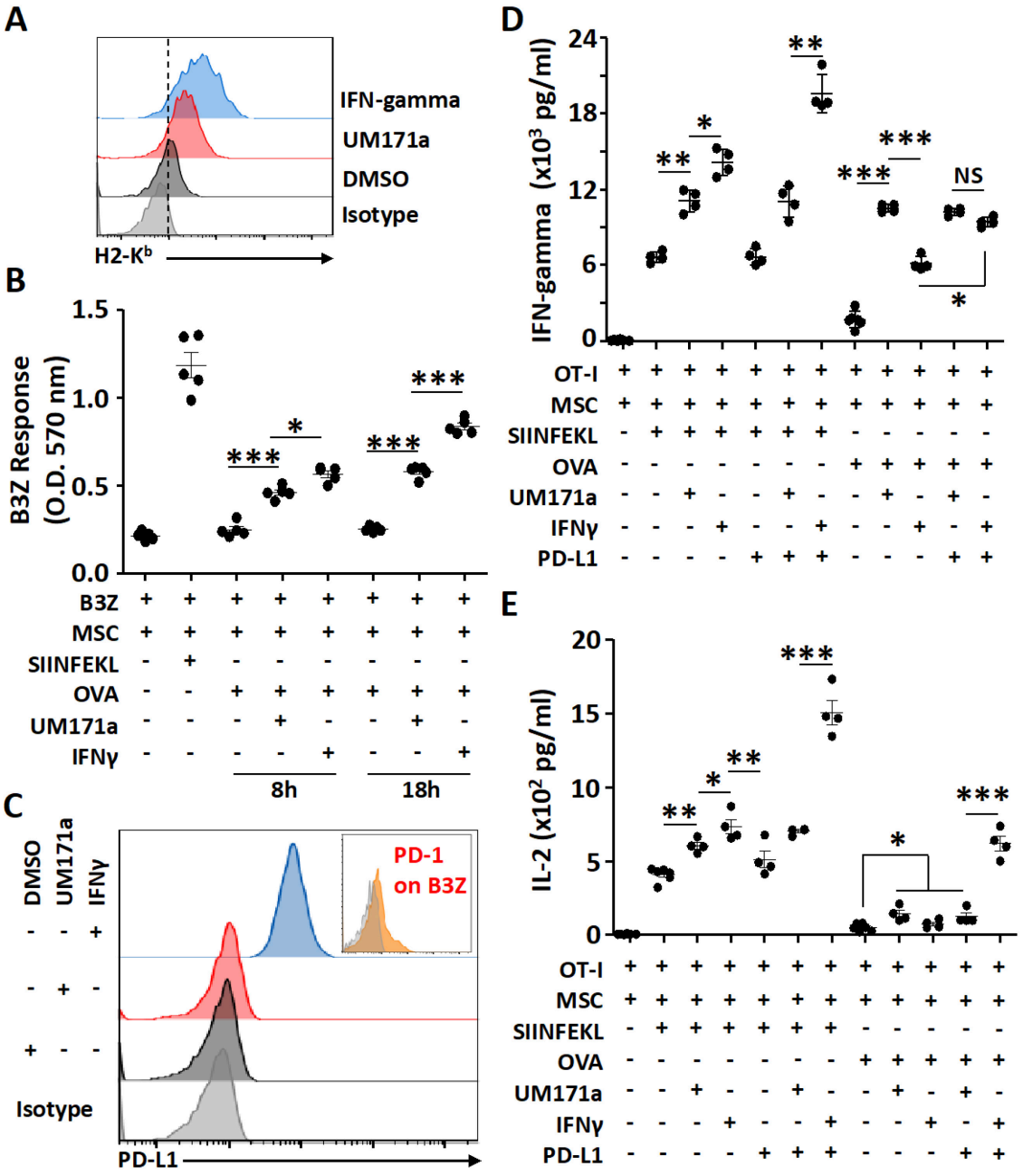

A
IFN-gamma
UM171a
DMSO
Isotype
H2-K^b
B
B3Z Response (O.D. 570 nm)
1.5
1.0
0.5
0.0
***
*
***
***
B3Z + + + + + + + +
MSC + + + + + + + +
SIINFEKL - + - - - - - -
OVA - - + + + + + +
UM171a - - - + - - + -
IFNγ - - - - + - - +
8h
18h
C
DMSO
UM171a
IFNγ
PD-1 on B3Z
Isotype
PD-L1
D
IFN-gamma (x10^3 pg/ml)
24
18
12
6
0
**
*
**
***
***
NS
*
OT-I + + + + + + + + + + + +
MSC + + + + + + + + + + + +
SIINFEKL - + + + + + + - - - - -
OVA - - - - - - - + + + + +
UM171a - - + - - + - - + - + -
IFNγ - - - + - - + - - + - +
PD-L1 - - - - + + + - - - + +
E
IL-2 (x10^2 pg/ml)
20
15
10
5
0
***
**
*
**
*
***
OT-I + + + + + + + + + + + +
MSC + + + + + + + + + + + +
SIINFEKL - + + + + + + - - - - -
OVA - - - - - - - + + + + +
UM171a - - + - - + - - + - + -
IFNγ - - - + - - + - - + - +
PD-L1 - - - - + + + - - - + +

Figure 15. – Anti-Tumoral Response Induced by Therapeutic Vaccination Using UM171a-Treated MSCs.

A) Schematic diagram showing the experimental design used for therapeutic vaccination. **B)** Assessment of tumor growth overtime following administration of DMSO-treated MSCs (green) or UM171-treated MSCs (red) pulsed with OVA. Mice with injected EG.7 tumors are depicted in black. **C)** Kaplan-Maier survival curve of the experiment shown in panel B. **D)** Secretome profiling conducted on DMSO- (black) versus UM171-treated MSCs (blue) cultured for 72h. For this panel, n=6/group with ***P<0.001.

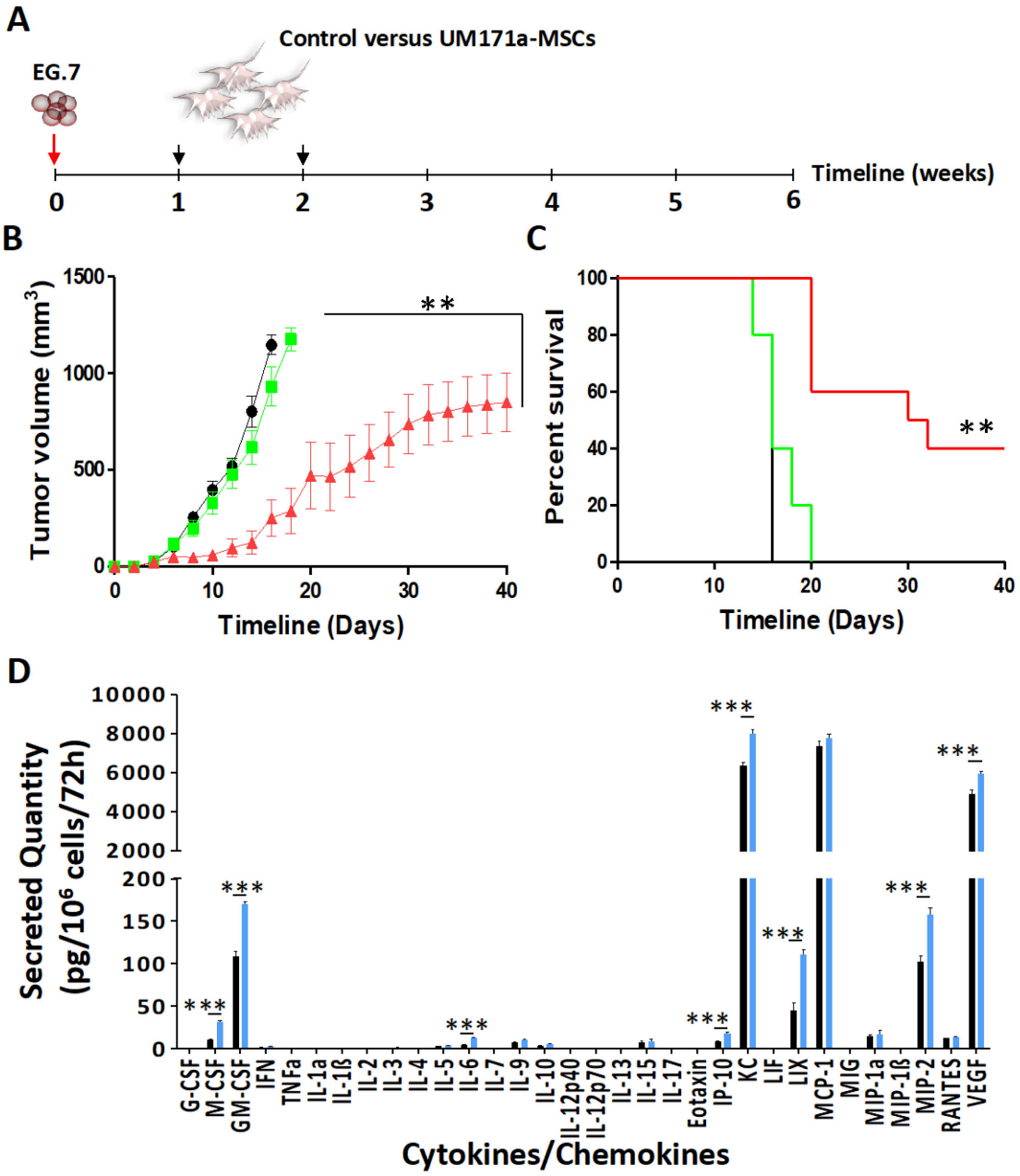
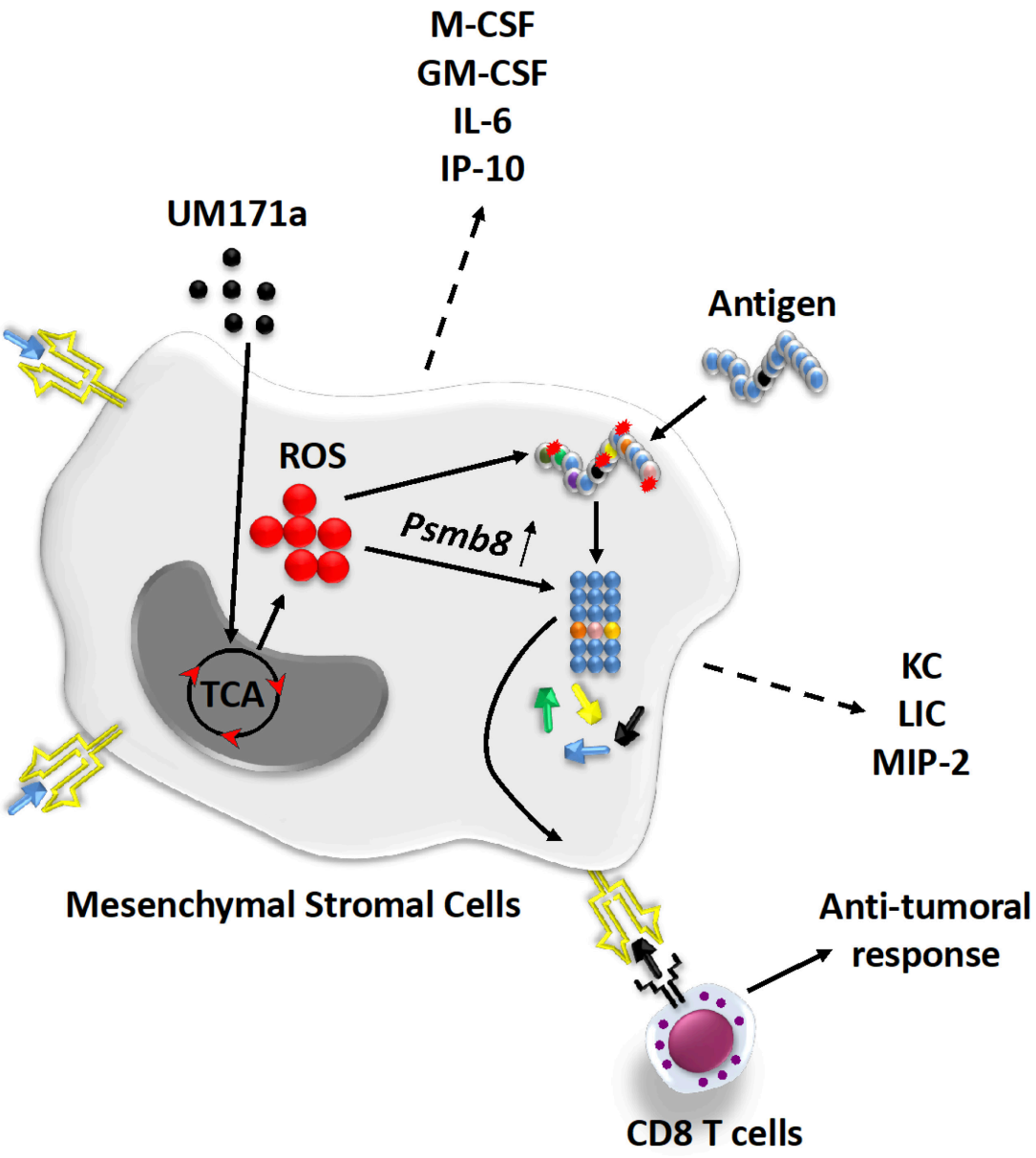

A
Control versus UM171a-MSCs
EG.7
Timeline (weeks)
0
1
2
3
4
5
6
B
Tumor volume (mm3)
1500
1000
500
0
**
0
10
20
30
40
Timeline (Days)
C
Percent survival
100
80
60
40
20
0
**
0
10
20
30
40
Timeline (Days)
D
Secreted Quantity (pg/10^6 cells/72h)
10000
8000
6000
4000
2000
200
150
100
50
0
***
G-CSF
M-CSF
GM-CSF
IFN
TNFa
IL-1a
IL-1ß
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-12p40
IL-12p70
IL-13
IL-15
IL-17
Eotaxin
IP-10
KC
LIF
LIX
MCP-1
MIG
MIP-1a
MIP-1ß
MIP-2
RANTES
VEGF
Cytokines/Chemokines

Figure 16. – GRAPHICAL ABSTRACT

Besides modulating the secretion of several pro-inflammatory cytokines and chemokines, MSC treatment with UM171a leads to enhanced production of mitochondrial-derived ROS. This ROS effect eventually results in protein stress and/or misfolding consequently triggering *de novo Psmb8* expression. Captured OVA protein may therefore be subjected to ROS-mediated damages prior for its targeting for Psmb8-mediated proteasomal processing as a collateral effect resulting in immunogenic peptide presentation and activation of responding CD8 T cells.


M-CSF
GM-CSF
IL-6
IP-10
UM171a
Antigen
ROS
Psmb8
TCA
KC
LIC
MIP-2
Mesenchymal Stromal Cells
Anti-tumoral
response
CD8 T cells

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