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A1-Reprogrammed Mesenchymal Stromal Cells as a Therapeutic Vaccine Against Solid Tumors

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Résumé

L'efficacité de la réponse antitumorale repose sur l'activité des cellules T cytotoxiques, qui peut être stimulée par des vaccins contenant des antigènes spécifiques aux tumeurs. Malgré le fait d'être les principales cellules présentatrices d'antigènes (CPA) responsables de l'activation des cellules TCD8, les cellules dendritiques (CD) ont rencontré des défis dans le développement de vaccins contre le cancer, notamment en ce qui concerne leur fabrication et efficacité. Pour combler ces problèmes, cette étude propose d'utiliser des cellules stromales mésenchymateuses (CSM) comme plateforme de vaccination alternative, en exploitant les avantages des CSMs en matière de fabrication, de sécurité et de plasticité. La plasticité remarquable des CSMs leur permet d'acquérir une capacité de présentation croisée sous des stimuli spécifiques. Étant donné que la présentation croisée est essentielle pour induire l'activation des cellules T contre les antigènes tumoraux, cette étude vise à convertir les CSMs en cellules présentatrice d'antigènes en améliorant l'exportation des antigènes des endosomes vers le cytosol - une étape critique du processus. Dans cette démarche, nous avons examiné une librairie de molécules dérivés de l'Accum, une molécule initialement conçue pour favoriser la destruction de la membrane endosomale. Après avoir évalué leur potentiel à induire la présentation croisée, nous avons sélectionné la molécule A1 pour des investigations subséquentes. Les études mécanistiques ont démontré qu'A1 déclenchait des processus cellulaires essentiels favorisant une présentation croisée efficace, notamment une augmentation de la capture, dégradation et évasion des antigènes des endosomes ainsi que la production de d'espèces oxygénés réactifs. L'efficacité thérapeutique des CSMs reprogrammées par A1 (ARM) en tant que vaccin anticancéreux a été évaluée chez des souris ayant des tumeurs, en monothérapie et en combinaison avec l'anti-PD-1. La thérapie combinée ARM a induit une régression tumorale et a augmenté les taux de survie dans les modèles de tumeurs solides. En conclusion, cette étude présente une stratégie innovante pour transformer les CSM en cellules à présentation croisée en déclenchant l'échappement endosomal de l'antigène. Les cellules ARMs en association avec des inhibiteurs des points de contrôle immunitaire présentent un potentiel en tant que plateforme de vaccination contre les tumeurs solides. De plus, ces résultats soulignent l'importance de l'évasion des endosomes dans

la présentation croisée d'antigènes et ouvrent la voie à de nouvelles plateformes de vaccins contre le cancer.

Mots-clés: cellules stromales mésenchymateuses, présentation croisée, évasion endosomale, vaccin contre le cancer, immunothérapie

Abstract

The effectiveness of antitumoral response relies on cytotoxic T-cell activity, which can be stimulated through vaccines carrying tumor-specific antigens. Despite being the primary antigen-presenting cells (APCs) responsible for CD8 T-cell activation, dendritic cells (DCs) have encountered challenges in cancer vaccine development, particularly in manufacturing and efficiency. To address this gap, this study proposes mesenchymal stromal cells (MSCs) as an alternative vaccine platform, leveraging the advantages in manufacturing, safety profile, and plasticity of MSCs. The remarkable plasticity of MSCs enables them to acquire cross-presentation capacity under specific stimuli. Given that cross-presentation is pivotal for inducing T-cell activation against tumor antigens, this study aims to convert MSCs into antigen cross-presenting cells by enhancing the export of antigens from endosomes to the cytosol—a critical step in the process. In this pursuit, we screened a library of Accum and variant molecules designed to promote endosomal disruption. After evaluating their potential to induce cross-presentation, we selected the molecule A1 for further investigation. Mechanistic studies demonstrated that A1 triggers essential cellular processes supporting efficient cross-presentation, including enhanced antigen uptake, processing, endosomal escape, and reactive oxygen species production. The therapeutic efficacy of A1-reprogrammed MSCs (ARMs) as an anticancer vaccine was evaluated in tumor-bearing mice, as monotherapy and combined with anti-PD-1. In solid tumor models, ARMs combination therapy induced tumor regression and increased survival rates. In conclusion, this study presents an innovative strategy to transform MSCs into cross-presenting cells by triggering antigen endosomal escape. ARM cells in combination with immune checkpoint inhibitors hold potential as a vaccination platform against solid tumors. These findings underscore the importance of endosomal escape on antigen cross-presentation and pave the way for new cancer vaccine platforms.

Keywords: mesenchymal stromal cells, cross-presentation, endosomal escape, cancer vaccine, immunotherapy

Table of Contents

List of Figures

List of Acronyms and Abbreviations

A1: Cholic acid-hnRNAP1

ACT: Adoptive cell transfer

ADC: Antibody drug conjugate

AF647: Alexa Fluor 647

ALI: Acute lung injury

AMEM: Alpha Modification of Eagle's Medium

ANOVA: Analysis of variance

APA: Antigen cross presentation assay

APCs: Antigen-presenting cells

ARDS: Acute respiratory distress syndrome

ARM: A1-Reprogrammed MSCs

BA: Bile acid

BCR: B cell receptor

BM: Bone marrow

CA: Cholic acid

CAR-T cells: Chimeric Antigen Receptor T-cells

CD: Crohn’s disease

cDC: Conventional dendritic cell

CPRG: Chlorophenol red-β-D-galactopyranoside

COVID-19: Coronavirus disease 2019

COX-2: Cyclooxygenase 2

Cyt-C: Cytochrome C

DAMPs: Damage-associated molecular patterns

DHE: Dihydroethidium

DCs: Dendritic cells

DEGs: Differentially expressed genes

DPI: Diphenyleneiodonium chloride

E.G7: Ovalbumin-expressing T lymphoblasts

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic Reticulum

ERAD: Endoplasmic-reticulum-associated protein degradation

ESCRT: Endosomal sorting complex required for transport

FBS: Fetal bovine serum

FDA: Food and Drug Administration

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GvHD: Graft versus host disease

HLA: Human leukocyte antigen

HSC: Hematopoietic stem cell

HSF1: Heat Shock Factor 1

ICD: Immunogenic cell death

ICI: Immune-checkpoint inhibitors

IDO1: Indoleamine 2,3-dioxygenase

IFN: Interferon

IFN-α: Interferon-alpha

IFN- γ: Interferon-gamma

IL: Interleukin

IT: Intra-tumoral

mAb: Monoclonal antibody

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

ML171: 2-Acetylphenothiazine

Mo-DCs: Monocyte-derived dendritic cells

MS: Multiple sclerosis

MSCs: Mesenchymal stromal cells

NAC: N-acetyl cysteine

NK: Natural Killer cell

NOX: NADPH oxidases

NLS: Nuclear localization signal

OA: Osteoarthritis

OD: Optical density

OV: Oncolytic virus

OVA: Ovalbumin

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate-buffered saline

PD-1: Programmed cell death protein 1

PD-L1: Programmed cell death protein 1 ligand

PRR: Pattern recognition receptor

RNA-seq: RNA sequencing

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute

SC: Subcutaneous

SD: Standard deviation

TAA: Tumor-associated antigen

TIL: Tumor infiltrating lymphocyte

TLR: Toll-like receptor

TME: Tumor microenvironment

Treg: Regulatory T cell

TSA: Tumor-specific antigen

UPR: Unfolded protein response

To my parents and grandparents, whose love for their family and belief in the transformative power of education were a driving force behind their hard work to provide opportunities to their next generations.

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CHAPTER 1

1. Introduction

1.1. The immune system

The immune system is one of the most complex systems within the human body, comprising a myriad of molecules, cells, and organs. Its primary role involves identifying foreign agents (such as viruses, bacteria, fungi, parasites, and toxins) or abnormal events (i.e., tumor cells), mounting protective responses against them, and subsequently returning to a homeostatic state upon resolution (1). A fundamental characteristic of the immune system is its remarkable ability to discern between self and non-self, thus safeguarding the individual from targeting healthy self-cells and potentially detrimental events caused by foreign agents. These orchestrated processes are meticulously regulated, and any perturbation in this cascade can make an individual susceptible to a diverse range of infectious and non-infectious illnesses (2,3). The immune response follows a coordinated order, temporally and spatially, encompassing two types, the innate and adaptive response (1). Although comprised of different components, they share numerous connections, and an immune response is formed by the crosstalk among both.

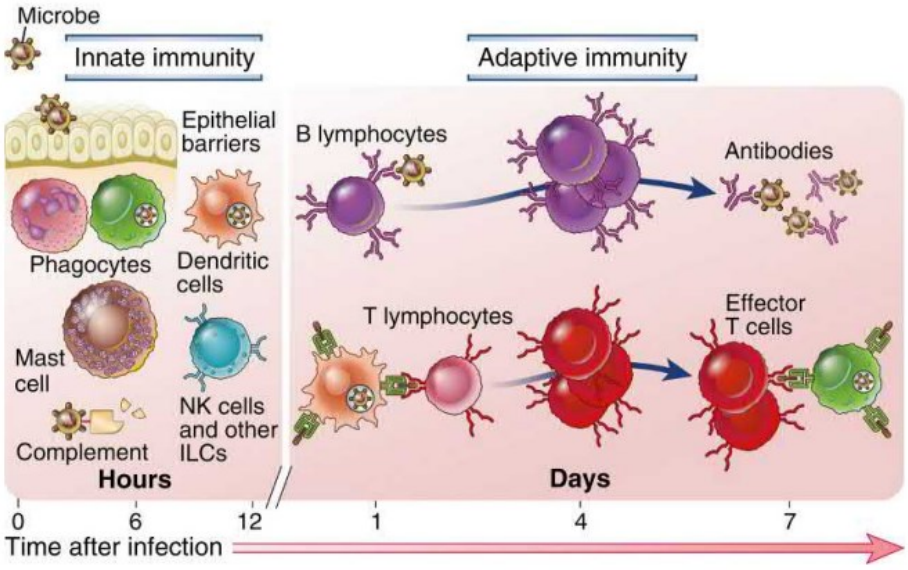

Figure 1 - Timeline of an immune response and components comprising the innate and adaptive immunity (1)**.**

The innate immune system act as the initial responders to infection, reacting promptly or within hours after exposure to pathogens. The adaptive immunity develops its effector mechanisms after recognizing the harmful agent, leading to the development of a tailored, antigen-specific response produced by activated T lymphocytes. This process may take days upon exposure.

1.1.1. The innate immune system

The innate immune system is responsible for the primary response against pathogens. It is phylogenetically the oldest segment of the immune system, having evolved alongside pathogens over time. This interaction is evident in certain components structurally similar to those found in plants and insects, such as antimicrobial peptides and receptors that recognize microbe patterns (4).

This system comprises physical barriers like the skin and mucosal epithelia, as well as secreted molecules like complement proteins and lectins. Alongside these, it includes various types of cells, such as phagocytes (macrophages, neutrophils, NK cells, and mast cells **(**

Figure 1**, left panel)** (1). These cells have limited specificity for individual pathogens, recognizing molecule patterns shared among groups of microbes (pathogen-associated molecular patterns or PAMPs) and those from damaged host cells (damage-associated molecular patterns or DAMPs) through pattern recognition receptors (PRRs). This limited specificity arises from the limited diversity of recognition molecules encoded by germline genes (5).

The innate response is triggered immediately or within a few hours of infection, relying on resident cells at the infection site and/or the recruitment of cells through cytokines, and complement protein activity. Molecules produced during innate immune responses stimulate adaptive immunity and influence the nature of adaptive responses (6).

1.1.2. The bridge between the innate and adaptive response

Specialized cells, known as professional antigen-presenting cells (APCs), play a pivotal role in bridging the innate and adaptive immune systems. The initiation of the adaptive response relies on these APCs, which capture antigens from target cells or microbes. Subsequently, they present these antigens to T lymphocytes to induce a tailored reaction against these immunogens (1).

The process of presentation occurs through the display of antigens derived from the target in a specific molecule called major histocompatibility complex (MHC) or human leukocyte antigen (HLA) (7). Additional stimuli are also required to fully activate T cells(8).

Different cell types are considered typical professional APCs, capable of activating naive T cells. Examples include macrophages and B lymphocytes (**Figure 2**) (9). However, dendritic cells (DCs) are considered the most effective APCs, as they harbor several specialized cellular processes that enhance their capacity to capture, process, present, and activate lymphocytes (10). The mechanisms and specificities of antigen presentation will be explored in more detail later in this chapter.

DCs are a highly heterogeneous group of myeloid cells, being present all over the body and performing various functions. They can be classified into distinct biological subsets based on their phenotype, ontogeny, and function. This classification, continuously refined with advancements in research technologies, primarily segregates DCs into three main subsets: 'conventional' DCs (cDCs) type 1 cDC (cDC1), type 2 cDC (cDC2), and plasmacytoid DC (pDC)1 (11). These subsets are functionally different, characterized by specialized roles and distinct interactions (12). For example, cDC1 is specialized in activating $CD8^+$ T cells through cross-presentation and is a significant producer of interferon-gamma (IFN-γ), which is crucial in directing T cell response towards the T_H1 arm. Notably, they are the only APCs capable of efficiently priming tumor specific $CD8^+$ T cells; their presence in the tumor microenvironment (TME) is positively linked to patient survival (13). The primary role of cDC2s is presenting exogenous antigens to $CD4^+$ T_H subsets, besides the high secretion of interleukin-12 (IL-12), a cytokine that prompts the expansion and survival of T and NK cells. pDCs are a rare subset characterized by their substantial secretion of type 1 IFNs (i.e., IFN-α), playing a pivotal role in antiviral immunity (12).

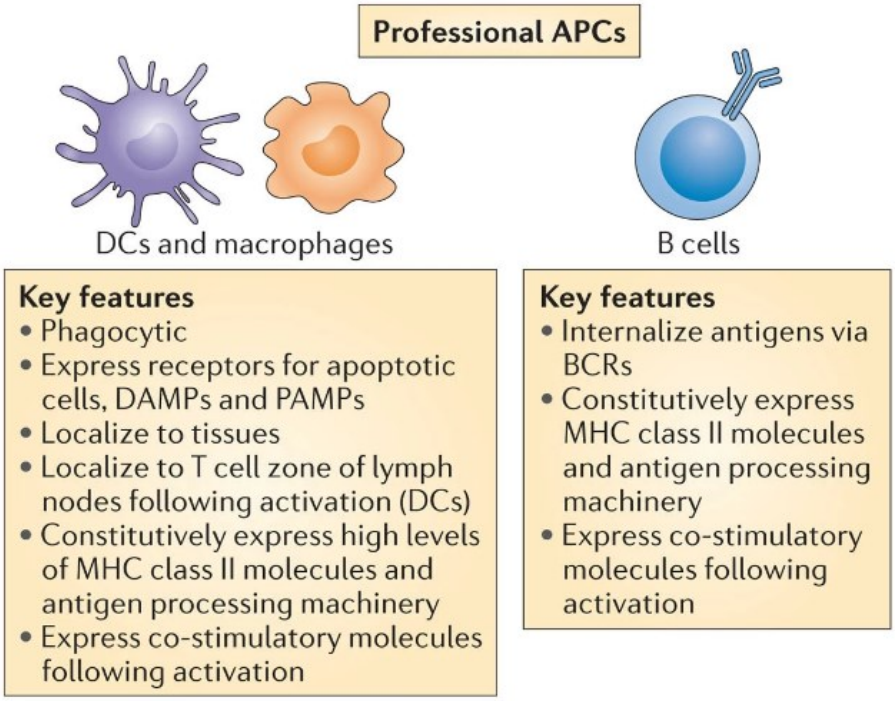

Figure 2 - Characteristics of professional antigen presenting cells (APCs). Adapted from (9)**.**

Different cell types function as professional APCs that can activate naive T cells. Professional APCs comprise cells with constitutive expression of MHC class II and associated proteins, along with the machinery required for antigen processing. These include DCs and, with a comparatively smaller role, macrophages, and B cells.

1.1.3. The adaptive immune system=

The adaptive immune system provides a specific and robust response against infectious agents, infected cells, and cancer cells. It is formed by cellular components - B and T lymphocytes - and humoral components - antibodies secreted by B cells (

Figure 1**, right panel**) (1). The specificity is achieved through receptors capable of recognizing a specific antigen presented on the surface of target cells. This capacity for precise recognition, extendable to virtually any existent antigen, is attainable through a remarkable diversity of antigen receptors generated via somatic recombination of gene segments (14).

Upon antigen recognition mediated by an APC, T lymphocytes undergo activation into effector T cells, a result of clonal expansion and differentiation, becoming effectively capable of eliminating the target antigen (15). After successful antigen clearance, the immune response contracts,

resulting in the apoptosis of effector cells. The increase and regression of the response magnitude along the days after antigen exposure are equally significant, safeguarding homeostasis and avoiding deleterious consequences (**Figure 3**) (1). Moreover, a reservoir of memory T cells is established, conferring long-lasting protective immunity against the stimulating antigen, yielding one of the most important features of adaptive immunity: immunological memory (16–18).

Each exposure to a foreign antigen enhances the immune system's ability to respond to that antigen due to immunological memory. Subsequent responses to the same antigen are typically faster and more robust, driven by memory cells that become numerous and more potent than the antigen-specific naive lymphocytes activated during the initial antigen exposure (19).

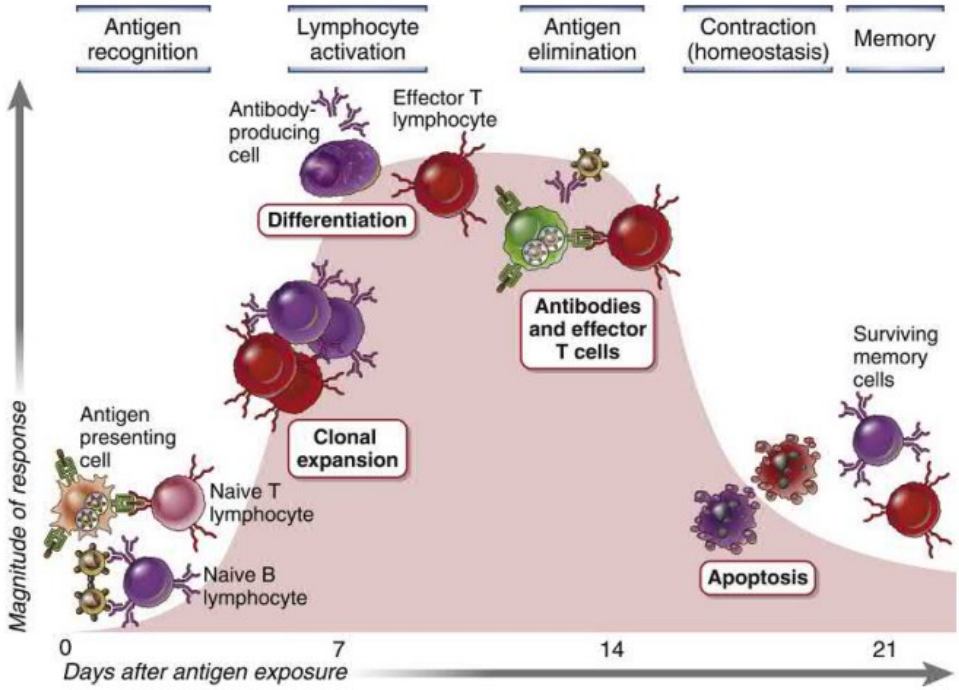

Figure 3 - Adaptive immune response progress upon antigen exposure (1)**.**

The adaptive response begins with an antigen recognition phase, where antigen presenting cells present processed antigens to naïve T lymphocytes. Subsequently, during the lymphocyte activation phase, clonal expansion and differentiation take place, leading to B cells becoming antibody-producing cells and T cells transforming into effectors. This effector phase orchestrates the elimination of antigens. As antigens are eliminated, the response contracts and stimulated lymphocytes undergo apoptosis, restoring homeostasis. The surviving antigen-specific cells constitute the memory components of the adaptive immune system.

1.1.4. The immune system against cancer

Cancer cells primarily arise from genome instability and mutations, generally involving activation of oncogenes and the loss of tumor suppressor genes. Several factors can act as triggers, such as carcinogenic molecules, radiation, viral infections, chronic inflammation, and inherited genetic mutations (20). Without effective elimination, cancer cells can progress to neoplastic growth states, characterized by a gradual multistep acquisition of functional capabilities further leading to malignant tumor formation (21). Various features are involved and necessary for cancer development (**Figure 4**) (22,23). Some well-established features include continuous proliferation coupled with resistance to cell death. This is facilitated by sustained activation of proliferation signals, insensitivity to antigrowth cues, loss of proper cell cycle regulation, and limitless replicative potential (23).

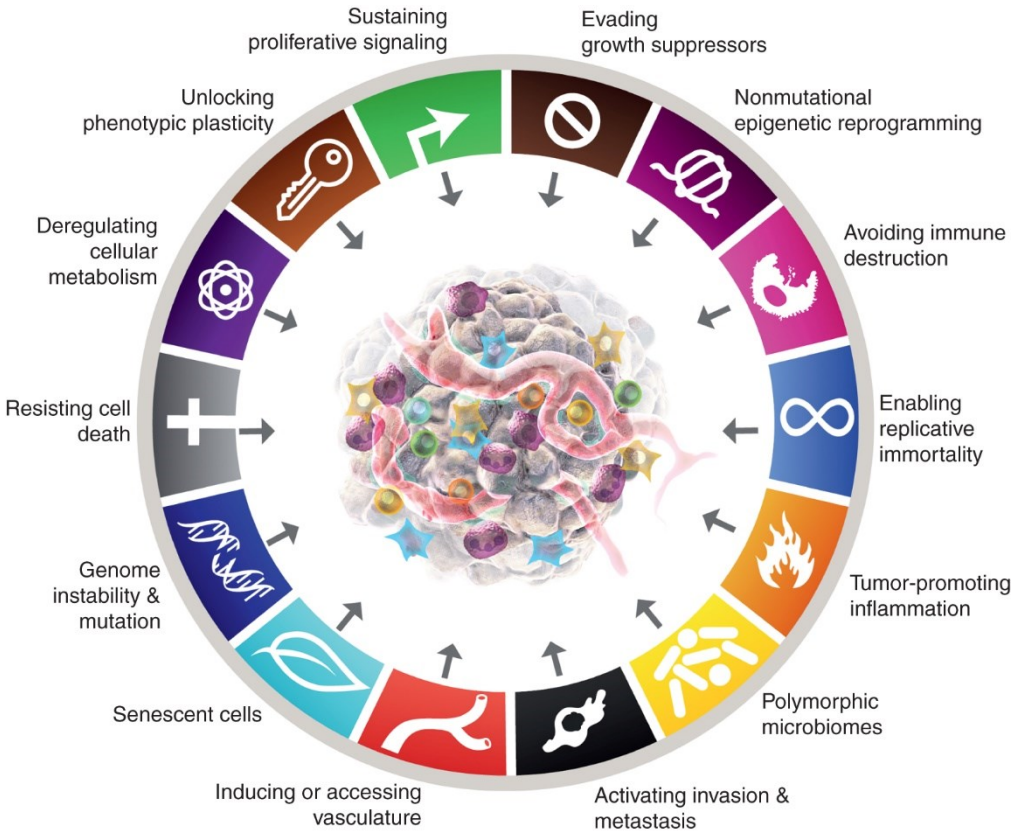

Figure 4 - Hallmarks of cancer (22)**.**

Tumor development is a multistep and complex process. Cancer cells acquire numerous biological capabilities that facilitate tumor formation and further advance

its progression. The hallmarks provide concepts to rationalize and comprehend the process of cancer development. These characteristics include events that enable the emergence of cancer cells, such as genome instability, mutations, and non-mutational epigenetic reprogramming. Additionally, they encompass traits that sustain and promote tumor progression, for example, sustained proliferative signaling, replicative immortality, and angiogenesis induction. Moreover, these hallmarks include features that empower cancer cells to resist and evade immune surveillance.

Due to their abnormal behavior and deleterious consequences on body homeostasis, it is imperative for the immune system to identify cancer cells as threats to be eliminated. As cancer cells develop new characteristics and acquire additional functions, they also express new antigens, named neoantigens, which distinguish them from normal cells (24). These neoantigens enable immune cells to identify and target tumor cells. However, the level of immunogenicity, reflecting the capacity to be recognized and incite an immune response, varies among cancer types and individuals (25). Immunogenicity also relates to the tumor microenvironment (TME). The TME comprises not only tumor cells but also a heterogeneous population of non-cancerous cells, including immune cells, cancer-associated fibroblasts, endothelial cells, tissue-resident cells, and the extracellular matrix in which they are embedded. The TME actively supports cancer growth by generating inflammation that promotes tumor development, providing access to blood vessels that facilitate metastasis, and avoiding immune destruction by immunosuppressive mechanisms (26).

In this scenario, the immune system and cancer cells have a dynamic and complex interaction, leading to paradoxical outcomes. This phenomenon, termed immunoediting (**Figure 5**), happens in three phases: elimination, equilibrium, and escape, referred as the “three Es”(27). The elimination phase corresponds to the successful detection and eradication of tumor cells through the combined efforts of the innate and adaptive immunity before any clinical manifestation arises. During the equilibrium phase, residual resistant cancer cells that survived the elimination phase become functionally dormant, coexisting under the immune surveillance, and having their growth controlled by the adaptive immune system. However, the immune response's continuous targeting and elimination of cancer apply a selective pressure, shaping the characteristics of tumor cells. This dynamic interaction contributes to a scenario in which resistant cells gain an advantage, facilitating immune escape. During the escape phase, the tumor surpasses the

immune system. Several factors can contribute to the tumor gaining an advantage over the immune system. For example, less immunogenic tumor cells can evade the immune surveillance, and cancer cells can develop immunosuppressive mechanisms that hinder the activity of cytotoxic lymphocytes and shift the profile of the immune response toward a more tolerant, immunosuppressant mode. External factors, such as environmental stress, immune system deterioration due to cytotoxic treatments, and even immunotherapies, can influence the trajectory of the tumor among the three Es (27,28).

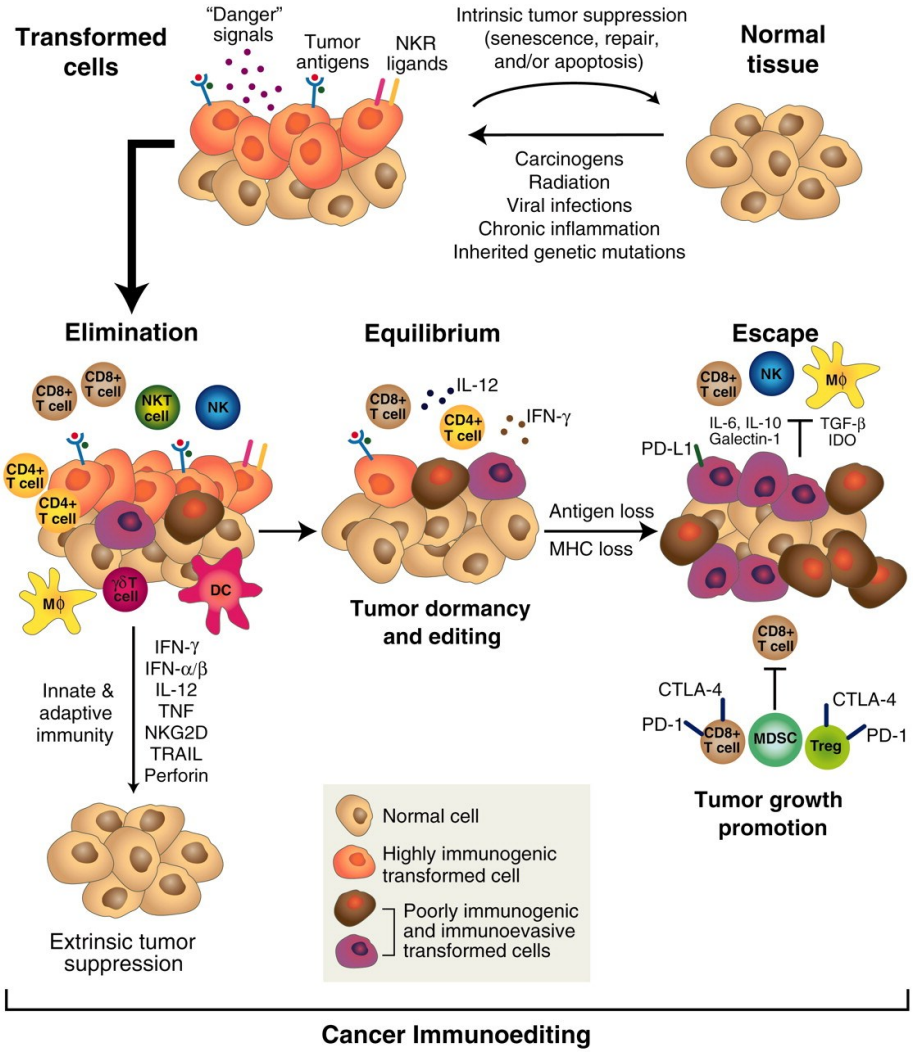

Figure 5 - Cancer immunoediting: the crosstalk between tumor cells and the immune system and how it contributes to tumor suppression and progression (28)

Throughout evolution, the human body has developed mechanisms to suppress the appearance of tumor cells. Intrinsic factors involve inducing senescence, repairing damage, or triggering apoptosis. These mechanisms come into play when oncogenes are activated or when tumor suppressor genes are deactivated. In cases where mutational events occur and intrinsic tumor suppressors fail, extrinsic tumor suppression mechanisms, including both innate and adaptive immunity, focus on identifying and eliminating transformed cells. If the immune system cannot completely eradicate all tumor cells, resistant cells (often poorly immunogenic) reach a state of equilibrium, remaining dormant to avoid immune surveillance. This equilibrium is facilitated by the immunosuppressive traits that the tumor acquires. During this phase, the tumor and the immune system coexist with equal advantages. However, as the immune system continuously exerts selective pressure, it creates an environment where transformed cells with reduced antigen and MHC expression, along with strong

immunosuppressive traits, can emerge and thrive. Consequently, tumor cells become less responsive to effector mechanisms. Reduced levels of proinflammatory cytokines, heightened anti-inflammatory cytokines, and the activation of immune regulatory mechanisms in the tumor microenvironment all contribute to the promotion of tumor growth. This ongoing battle between cancer cells and the immune system, and the resulting impact of the immune system on shaping tumor development, collectively form the concept of cancer immunoediting.

1.2. Cancer therapy

1.2.1. Cancer treatment modalities

Over the years, significant progress has been made in the development of cancer treatments. Typically, surgery is the first-line treatment for a variety of cancer diagnoses, either as a sole approach or to reduce local tumor burden before the application of another therapeutic approaches. Chemotherapy is one of the most widely used treatments against cancer, followed by radiation. The choice of treatment strategy depends on factors like the cancer type and its stage. As our comprehension of cancer biology and technological capabilities has progressed, these conventional methods have been refined (29). This refining process coincides with the emergence of targeted therapies, driven by insights into molecular processes and the mechanistic aspects of cancer from fundamental research. Substantial clinical benefits have arisen from the application of targeted therapies, such as through high throughput screening of small molecules to target receptor inhibitors (30). For instance, BCR-ABL1 tyrosine-kinase inhibitors induced long-term remission in patients with chronic myeloid leukemia (31).

However, owing to the significant side effects of approaches like chemotherapy, recurrence and resistance in all approaches, and the lack of treatments suitable for the vast array of cancer types, the pursuit of novel treatment approaches remains an ongoing and imperative necessity (32). In recent decades, the discoveries of the impact and influence of the immune system on cancer have put this system into the spotlight for exploration as a promising avenue in therapy development. This involves targeting immunological processes or harnessing immune cell functions, referred to as immunotherapies (33).

1.2.2. Immunotherapies

Cancer immunotherapies encompass approaches that take benefit from the highly skilled capabilities of the immune system to fight cancer, either by inducing, inhibiting, or boosting immune responses. Compared to conventional treatments, immunotherapies have shown substantial advancements in extending patient survival and improving their quality of life (33). In contrast to chemotherapy, that has relatively low tumor specificity and high toxicity to health cells, immunotherapy has reduced systemic side effects (34). However, it is important to acknowledge that they may still lead to other significant side effects (35) and are typically costlier and less accessible approaches. Cancer immunotherapy strategies can be generally classified into five main categories: I) cytokines, II) immune-checkpoint inhibitors (ICIs), III) adoptive cell transfer (ACT), IV) oncolytic virus, and V) cancer vaccines (**Figure 6**) (36).

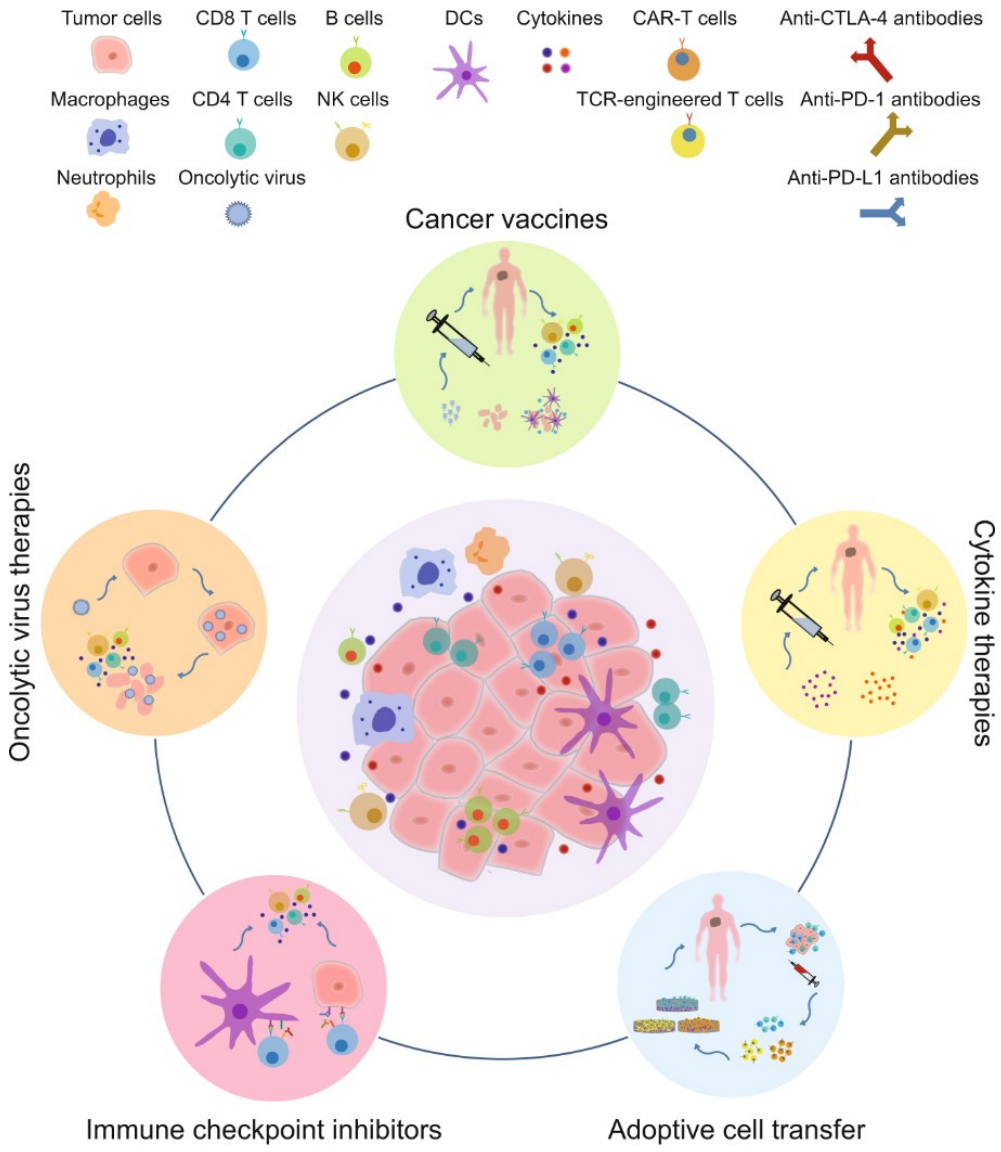

Figure 6 - Five major classifications of immunotherapies (36)**.**

The major categories of immunotherapy. Different types of cancer immunotherapy include oncolytic virus therapies, cancer vaccines, cytokine therapies, adoptive cell transfer, and immune checkpoint inhibitors. Their development has advanced, posing them as promising therapeutic approaches. The overall cellular components and molecular features involved in each category are illustrated within the graphical abstract.

1.2.2.1. Cytokines

Cytokines are administered to stimulate the growth and activation of immune cells, acting systemically, and propagating immune signaling. IFNs, ILs, and granulocyte-macrophage colony-

stimulating factor (GM-CSF) have been explored for their therapeutic potential (37). Up to now, only two cytokines have received approval from The Food and Drug Administration (FDA): interleukin-2 (IL-2) and IFN-α. IL-2 has the capacity to expand T cells, engaging effector and regulatory (Treg) cells, and has been approved for advanced renal carcinoma and metastatic melanoma (38). IFN-α is a pleiotropic molecule that can act through the induction of senescence and apoptosis, and by boosting the stimulation of DC maturation and of T-cell cytotoxicity. It received approval for the treatment of hairy cell leukemia, follicular non-Hodgkin lymphoma, melanoma, and acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma. However, the need for high doses to achieve therapeutic benefit results in severe toxicity and limited tolerability, hampering the use of cytokines as a monotherapy approach (39). For example, in a phase II trial for the use of IL-2, life-threatening cardiac and pulmonary effects were caused by the toxicity of IL-2, including a therapy-related death (40). The use of IFN-α has demonstrated toxicity with the occurrence of flu-like symptoms, nausea, fatigue, and hematological toxicity, due to high myelotoxicity and paradoxical immunosuppressive effects (41). However, several ongoing studies are investigating the use of cytokines in combination regimens with other immunotherapies and in association with adjuvants to improve their efficacy and diminish side-effects (37).

1.2.2.2. Immune checkpoint inhibitors (ICIs) / receptor agonists

Immune checkpoint inhibitors (ICIs) and receptor agonists are monoclonal antibodies (mAb) that target surface molecules, inducing blockade and stimulation, respectively. Among these, checkpoint inhibitors have garnered the most attention in immunotherapy research, targeting mechanisms that suppress the immune response (36). The most explored are PD-1/PD-L1 blockade and CTLA4 inhibition. PD-1 is a receptor expressed on the surface of T cells and plays a role in downregulating T cell activity. Cancer cells express PD-L1 and exploit the PD-1/PD-L1 pathway to evade the immune system, as the binding of PD-L1 to PD-1 triggers T cell inactivation. To counteract this process, mAbs can target either the receptor or the ligand, obstructing their interaction and the consequent PD-L1-mediated T-cell suppression (42). CTLA-4 is a receptor that is constitutively expressed by Tregs and binds to CD80 and CD86, which are expressed by APCs. This interaction with CD80/CD86 has high affinity and avidity, reducing the availability of

CD80/CD86 for other ligands, such as CD28. Since CD28 serves as a co-stimulatory molecule for T cells, CTLA-4, by limiting CD28-mediated signaling, hampers T cell activation, ultimately leading to immune regulation. In the TME, the abundance of Tregs contribute to immune response suppression. Anti–CTLA-4 antibodies bind to CTLA-4 molecules with high affinity, leading to Treg depletion or functional blockade, thereby resulting in enhanced T-cell, and bolstering immunological responses against cancer(42). FDA-approved ICIs for the aforementioned blockade strategies exist and are indicated for several types of cancer such as melanoma, renal carcinoma, lung cancer, colorectal cancer, and lymphoma, among others (43). ICIs have shown remarkable clinical impact. However, their therapeutic benefit requires the presence of tumor-infiltrating lymphocytes (TILs), limiting their effectiveness in some cancer types (i.e., cold cancers) (36). Additionally, acquired unresponsiveness in some patients might also be due to the tumor's capacity to circumvent the targeted blockade by activating alternative mechanisms of immune suppression or by evading immunosurveillance (42). This phenomenon can be exemplified by the mutations observed in a cohort of melanoma patients that acquired resistance to PD-1 blockade, related to defects in IFN-receptor signaling and impair in antigen presentation by loss of MHC I surface expression (44).

1.2.2.3. Adoptive cell transfer (ACT)

Adoptive cell transfer (ACT) employs autologous or allogenic T cells, which have some features augmented to enable a more efficient antitumoral activity, and these enhanced T cells are subsequently infused into the patient (33). T lymphocytes can be obtained from the patient via apheresis or from tumor surgical excisions. This is followed by ex vivo expansion through cytokine stimulation. One example of ACT approach is the isolation of TILs, which are isolated, and stimulated ex vivo with IL-2. The cytokine stimulus prevents T cell exhaustion, and death that could be caused by withdrawal of cytokines. This process enhances the quantity and activity of TILs before they are reinfused into the patient (45). Alternatively, isolated T cells can be genetically engineered to express either T cell receptors (TCRs) or chimeric antigen receptors (CARs) (46). TCR-engineered lymphocytes respond to cancer cells that present tumor antigens through MHC, whereas CAR-T cells can bypass this limiting step by targeting any type of surface molecule on cancer cells, not limited to MHC. This is an advantage of CAR-T cells, when compared

to TCR-engineered lymphocytes, as one of the features of cancer cells is the evasion of immune response by downregulation of MHC expression. However, a challenge associated with CAR-T cell is the tissue/cell type-specific antigen target selection. This choice is crucial to avoid adverse effects that may occur if the selected surface molecule is also present on healthy cells and off-target tissues. This limitation narrows the range of surface molecule candidates for CARs (47). Up to now, five generations of CARs have been developed, aimed at refining their specificity and potency as well as minimizing their off-target effects and T cell exhaustion (48). Notably, all currently available FDA-approved CAR-T cell therapies are designed for the treatment of B-cell malignancies. These CAR-T cells, designed to target the CD19 surface molecule expressed by B cells, have been indicated for different types of B-cell lymphoma and lymphoblastic leukemia. Furthermore, CAR-T cells targeting B-cell maturation antigen (BCMA) were approved for the treatment of multiple myeloma (49). While CAR-T cell therapy has demonstrated notable clinical success, challenges persist, including target antigen loss leading to treatment failure and relapse (47,49). Additionally, the exorbitant treatment costs and the demand for specialized care facilities and experts are noteworthy limiting considerations (50). For example, the CAR-T cell therapy tisagenlecleucel, indicated for B-cell acute lymphoblastic leukemia, can cost more than $1 million per patient (51); even though the product itself only costs between $400,000-500,000, treatment facilities, management of adverse events, specialized personnel, among others, add more than $500,000 to the final cost of the therapy.

1.2.2.4. Oncolytic viruses (OVs)

Oncolytic virus (OVs) therapy employs genetically modified viruses that efficiently infect and replicate within tumor cells while being non-productive in normal cells (52). This capacity stems from OVs exploiting pathways that are differently activated or inactivated in tumor cells. For instance, tumor cells possess impaired antiviral mechanisms, like the hijacked IFN-γ pathway, making them more vulnerable to viral infection. Once infected, the tumor cells undergo oncolysis, leading to immunogenic cell death (ICD). This selective replication in cancer cells provides them with distinct advantages, not only because of the specificity, but also due to the self-amplifying capacity of OV replication, which intensifies the treatment's impact. The promotion of antitumor immunity can be attributed to the direct cancer cell destruction and induction of the immune

response due to ICD (53). However, if the antiviral response becomes too robust, oncolytic viruses might be neutralized before effectively eradicating tumors. Furthermore, oncolytic viruses offer a favorable safety profile. Despite their benefits, challenges lie in optimizing clinical efficacy, regulatory aspects, and the clinical logistics demanded for practical application (52). FDA-approved OV includes a modified human herpes simplex virus 1 (HSV-1), attenuated and expressing granulocyte macrophage colony-stimulating factor (GM-CSF), indicated for melanoma (54).

1.2.2.5. Cancer vaccines

Cancer vaccines consist of an active immunotherapy approach designed to boost and/or initiate an antitumoral response. Their types, applications, and distinct features, such as their exceptional ability to stimulate immunological memory, will be elaborated on in the subsequent section.

1.2.3. The potential of cancer vaccines

The concept of cancer vaccines has been under exploration for decades, emerging from the development of vaccines against infectious diseases and the recognition of the role of antitumor immunity. This field has progressed in parallel with discoveries in fundamental research and the advancement of models and techniques enabling more intricate investigations (55).

Cancer vaccines can be explored as prophylactic and therapeutic approaches. In either scenario, their role is to stimulate the immune system to identify cancer cells as a target for attack, while also activating effector cells. Effective elimination of tumor cells involves multiple steps that enable the replication and expansion of the effector elements, known as the tumor-immune cycle (**Figure 7**Figure 8) (56). The remarkable potential of cancer vaccines comes from their role as initiators of the adaptive immune response. When functioning effectively, they not only target one step in cancer scenario, as some types of therapies, but they also subsequently activate all essential components of the immune response cascade required for eradicating cancer, like the activation of T cells and B cells that together mount a robust cellular and humoral response against the tumor, with the potential to induce complete tumor regression. Furthermore, cancer

vaccines hold the capacity to induce immunological memory, rendering them capable of protecting the patient from subsequent cancer relapse or secondary tumors (56,57).

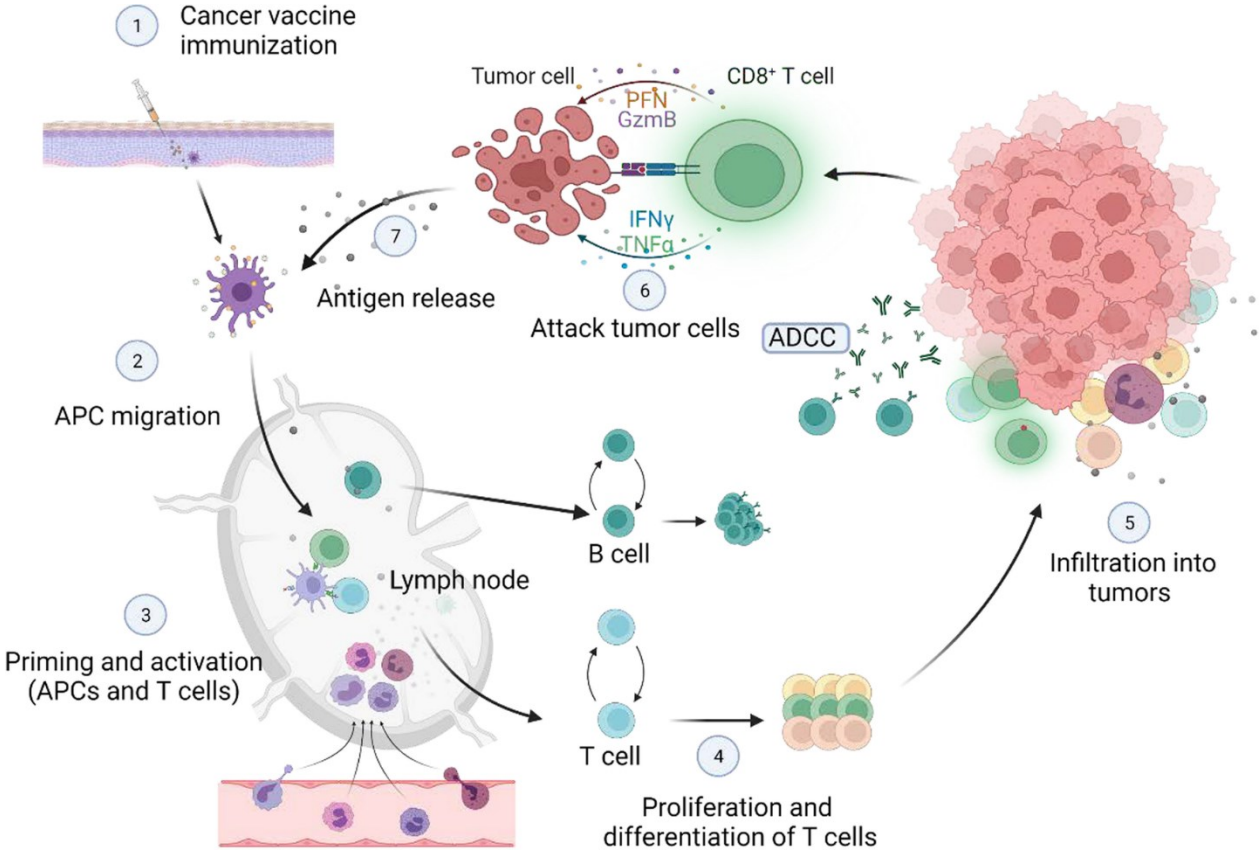

Figure 7 - Tumor-immune cycle induced by cancer vaccines (56)**.**

An effective antitumor response requires cycles of repetition and expansion. After immunization, DCs uptake and process tumor antigens, migrate towards the lymph nodes and activate T cells through presentation of peptides by MHC II or MHC I (cross-presentation). B cells (memory and plasmacytoid) are promoted by follicular DCs. Activated T cells proliferate and differentiate into cytotoxic effector cells, which infiltrate the tumor microenvironment and attack tumor cells. Dead tumor cells are immunogenic, shredding tumor antigens and danger signaling molecules that reinitiate the cycle.

Virus-targeted prophylactic vaccines have made significant progress against hepatitis B and human papillomavirus (HPV), leading to reduced incidences of hepatocellular carcinoma and cervical cancer, respectively (58,59). These vaccines rely on viral antigens, which, being foreign pathogens, elicit higher immunogenic responses. However, preventive vaccines using nonviral antigens encounter a constraint – the necessity for a tumoral antigen before the cancer's

establishment, which limits their development (55). In this scenario, the development of sophisticated tools for early diagnosis are critical, as pre-malignant lesions, harboring neoantigens, have an important role in research development of cancer preventive vaccines.

Therapeutic vaccines aim to treat established neoplastic cells, with their origins dating back to 1910 when William Coley administered inactivated Streptococcus and Serratia to established tumors as a treatment approach (60). Later on, the discovery of tumor-associated antigens (TAAs), which are antigens highly expressed on cancer cells in comparison with normal cells, laid the foundation for the development of therapeutic vaccines. The earliest approach, dating to the 1970s, involved patient-derived tumor cells combined with adjuvants or viruses to elicit a polyclonal immune response to TAAs (33).

Therapeutic vaccines can be categorized into four types based on three parameters: I) the previous definition of the stimulating antigen, II) if those antigens are unique to individual patients or shared among those with the same tumor type, and III) how the antigens interact with professional APCs, following the flowchart in **Figure 8a** (61). Regarding the first parameter, vaccines can be based on predefined or anonymous antigens. The second parameter applies if the antigen is predefined, then they can be classified as a shared antigen (expressed in a population with the same tumor type) or a predefined personalized antigen, meaning that it was determined from each patient. The third parameter stands for how the antigen encounters APCs, *in situ* this can occur through ICD or administration of antigens directly to the patient; they can also be loaded *ex vivo* onto APCs (either autologous or allogeneic), which are subsequently reintroduced into the patient's body (**Figure 8b**) (61).

Vaccines utilizing anonymous antigens have demonstrated a higher capacity to present the full spectrum of tumor antigens, in contrast to predefined antigen vaccines, with the potential to induce systemic tumor regression (17–19). Evidence also suggests that antigen delivery by APCs, particularly exemplified by DCs, offers greater effectiveness when compared to the use of whole tumor cells (10,20).

As DCs are the most effective APCs, numerous studies have investigated their utilization as a cell-based cancer vaccine. Trial watches (13,62,63) identified more than 100 peer-reviewed studies

reporting completed trials evaluating DC vaccines. Most of these studies involved autologous DCs pulsed with TAA or TAA-derived peptides, TAA-coding RNAs, and autologous cancer cell lysates. As of August 2023, there are over 220 entries of clinical trials with DC vaccines as the intervention/treatment. However, there has been a decline in the number of clinical trials with DCs over the years, attributed to the rapid rise of ICIs coupled with the unsatisfactory clinical performance of DC vaccines (13). To date, sipuleucel-T remains the only FDA-approved DC-based oncologic therapy, with its use indicated for castration-resistant prostate cancer (64). Considering these observations, concerns have emerged regarding the use of *ex vivo* monocyte derived DCs (moDCs) as a limiting factor, as they lack characteristics and performance exhibited by physiological DC subsets. However, generating substantial quantities of cDC1/2 from patients poses a major manufacturing obstacle, given their scarce presence within the body (65). To address these concerns, our research group has explored mesenchymal stromal cells (MSCs) as a potential platform for cancer vaccines, for either prophylactic or therapeutic application. Our preclinical studies have demonstrated a positive impact of MSC-based vaccines on the development of antitumoral immunity (66–69), through pharmacological stimulation and genetic engineering of MSCs.

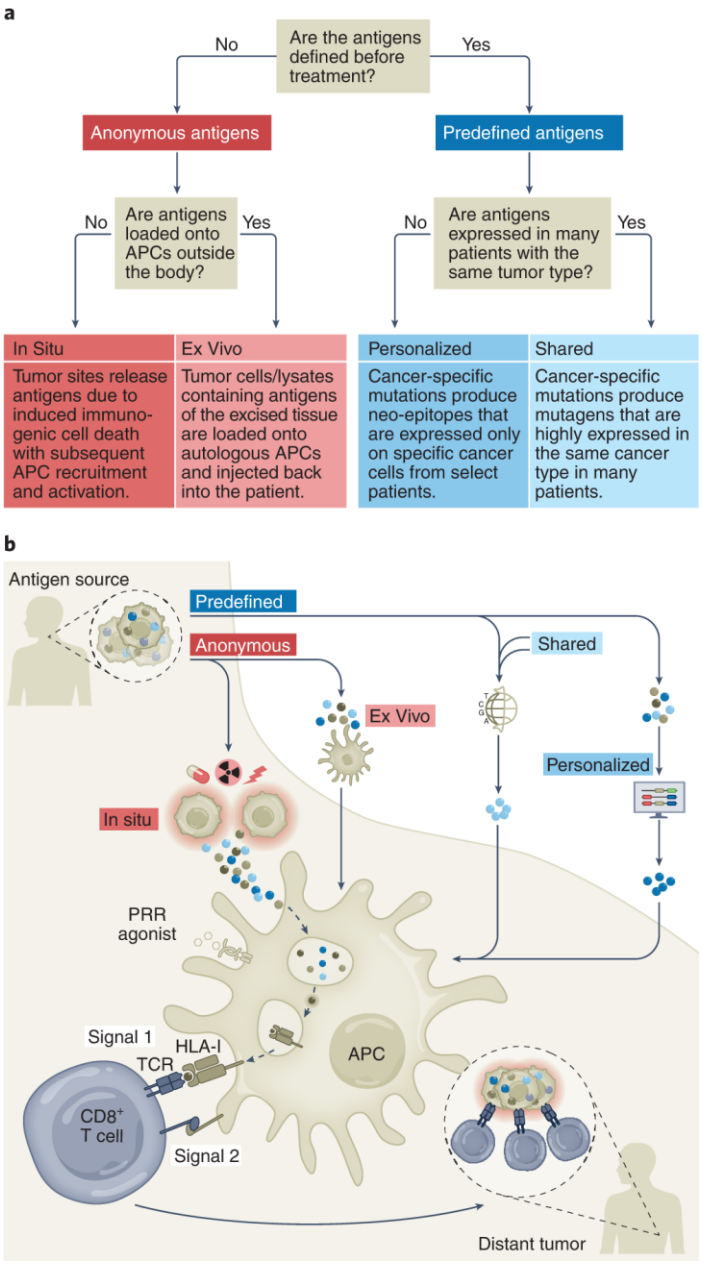

Figure 8 - Cancer vaccine classifications based on source of antigen, characteristics of antigen and loading site (61)**.**

a) Flowchart of vaccine types. Anonymous antigen vaccines can be loaded into APCs *ex vivo*, in laboratory conditions, or *in situ*, at the tumor site. Predefined vaccines demand antigen identification by tumor biopsy and computational analysis (personalized) or shared features among patients with the same tumor type (shared). **b)** Comprehensive illustration of proposed cancer vaccine definition.

1.3. Mesenchymal stromal cells (MSCs)

1.3.1. Properties and characteristics

Mesenchymal stromal cells (MSCs) are multipotent cells that can be found in all supportive stromal tissues of mammals (70). Initially identified by Friedenstein in a series of studies in the 1960s and 1970s, isolated from guinea pig bone marrow, they are described as spindle-shaped, plastic-adherent cells, capable of developing fibroblast colony forming units (71). While bone marrow is the primary source for their isolation, MSCs can also be obtained from adipose tissue, umbilical cord blood, peripheral blood, hair follicles, muscles, and other sources (72). Defining MSCs has been challenging due to their heterogeneity and lack of exclusive phenotypic markers. To address this, the International Society for Cellular Therapy (ISCT) proposed minimum criteria to define MSCs (73) based on: **I)** their capacity of plastic adherence; **II)** phenotype (requiring the expression of CD73, CD90 and CD105, while lacking monocyte and macrophage markers (CD11b or CD14), hematopoietic progenitor and endothelial cell marker (CD34), a leukocyte marker (CD45), B cell markers (CD19 or CD79a) and HLA-DR); and **III)** trilineage differentiation capacity (into adipocytes, chondrocytes and osteoblasts) *in vitro* (**Figure 9**) (73). Although they harbor differentiation capacity, rare self-renewing populations were identified among MSCs. Hence, the term ‘stromal’ was proposed as an alternative to ‘stem’ (74,75).

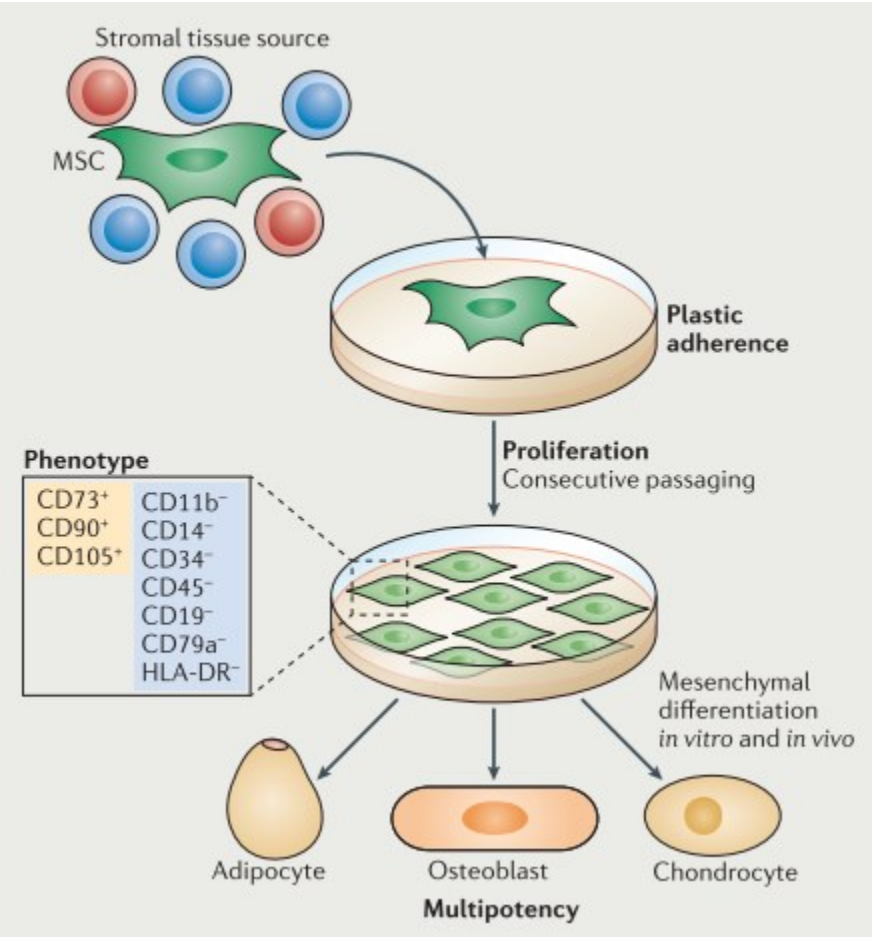

Figure 9 - Criteria for the definition of MSCs proposed by the ISCT (70)**.**

The International Society for Cellular Therapy (ISCT) has outlined essential criteria for defining mesenchymal stromal cells. These cells are derived from stromal tissue sources and display the capability to adhere to plastic surfaces. They are selected after successive passages, demonstrating proliferative capacity and expression of specific phenotypic surface markers. Additionally, these cells exhibit multipotency, manifested by their capacity to differentiate into adipocytes, osteoblasts, and chondrocytes both in vitro and in vivo.

MSCs exhibit a wide range of functions across various niches. In the bone marrow, they play a pivotal role in supporting hematopoiesis and safeguarding the hematopoietic stem cell (HSC) reservoir (76). Their protective microenvironment preserves HSCs by shielding them from differentiation and apoptotic stimuli, thus maintaining them in a quiescent state (77,78). Beyond the bone marrow, MSCs also contribute significantly to tissue regeneration. Guided by chemotactic signals, MSCs migrate to damaged tissue sites, where inflammatory cytokines trigger

the secretion of numerous growth factors such as EGF, FGF, PDGF, TGF-β, VEGF, HGF, Ang-1, KGF, SDF-1, IGF-1, and others (79). These growth factors coordinate endothelial cells, fibroblasts, and stem cells to drive tissue regeneration and repair by enhancing angiogenesis, suppressing leukocyte transmigration, and promoting cell differentiation (80).

Their capacity to engage with components from both the innate and adaptive immune system grants them robust immunomodulatory properties (81). MSC-mediated immunomodulation can be achieved through cell-cell contact and secretion of soluble factors. Within the context of innate immunity (70), MSCs control the polarization of macrophages. Through the expression of cyclooxygenase-2 (COX-2) and indoleamine-2,3-dioxygenase (IDO1), MSCs steer macrophages towards an M2 profile that expresses IL-10 along with increased PGE2, TSG-6 and IL-1RA (70). However, the diverse Toll-like receptors (TLRs) expressed by MSCs lead to distinct phenotypes in response to microenvironmental stimuli, potentially skewing MSCs into two distinct phenotypes: pro-inflammatory (MSC1) and anti-inflammatory (MSC2) (82). This dichotomy affects their biological functions related to differentiation, proliferation, migration, antioxidative repertoire, and suppressive potency. MSCs also influence neutrophil behavior by attraction, activation, and lifespan extension (83).

Regarding the adaptive immune response, MSCs interact with T cells, causing a reduction in inflammatory helper T cells (T_H)1 and an increase in Treg and T_H2 cells (84). This interaction leads to decreased IFN-γ and increased IL-10, IL-4, and IL-5 levels. Crosstalk with DCs leads to a reduction in proinflammatory mature DC1, along with a decrease in secretion of TNF-α and IL-12. In contrast, immature DC and DC2 show an increase in tandem with higher IL-10 expression. The interaction between MSCs and NK cells also results in a reduction in the secretion of IFN-γ by NK cells (84). Furthermore, MSCs can suppress antibody production from B cells and hinder bacterial growth through direct or indirect mechanisms (85). It is important to note that these properties emerge, and change based on the communication with the surrounding environment.

Beyond they pleiotropic effects, the manufacturing and safety attributes of MSCs make them suitable for clinical applications across various pathological scenarios (**Figure 10**). For instance, their possibility to be isolated from several sources facilitates their acquisition according to

different circumstances. Their strong proliferation capacity, plastic adherence, and ability to thrive in minimally demanding culture conditions enable cost-effective large-scale production (86). To date, clinical trials involving MSCs have established a robust safety profile, corroborated by two meta-analyses (87,88). These compelling attributes have positioned MSCs as the most extensively studied cell type in advanced therapies for various therapeutic objectives, which will be further explored in the subsequent section.

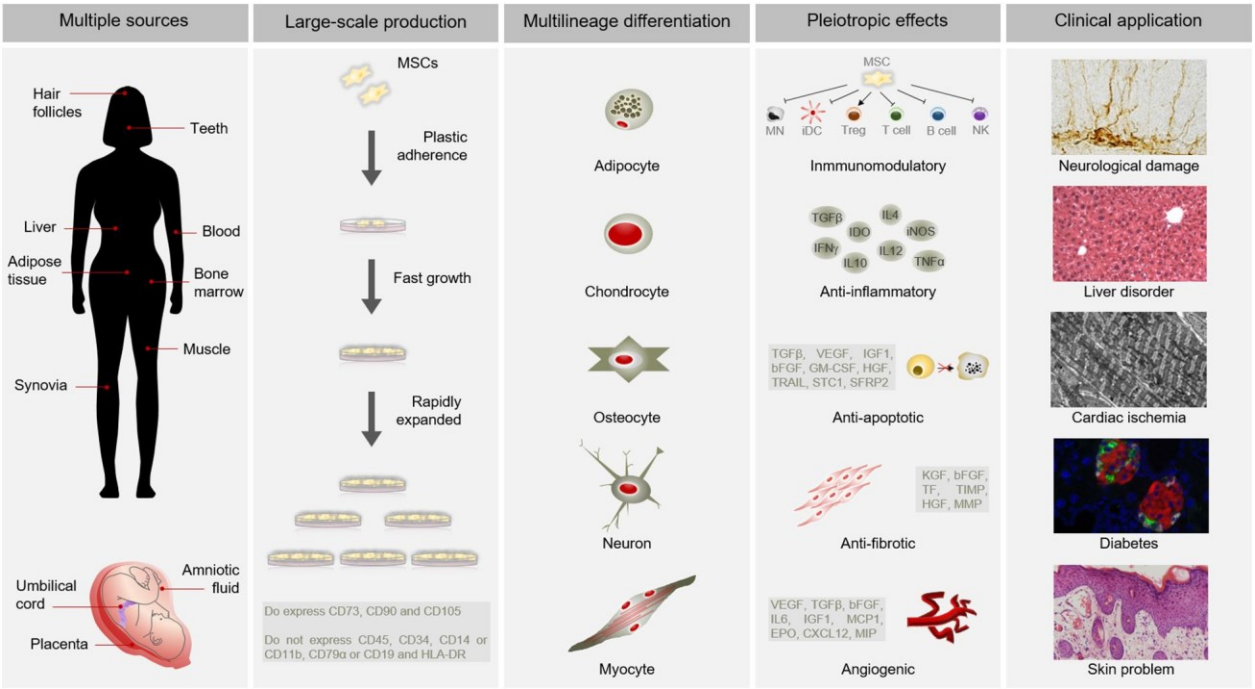

Figure 10 - Advantages of MSCs for clinical use (86)**.**

Mesenchymal stromal cells (MSCs) offer numerous advantages for clinical use, as they can be extracted from multiple sources, manufactured on a large scale, harboring multilineage differentiation capacity, and exerting pleiotropic effects. These attributes render MSCs highly suitable for clinical applications across a range of pathological conditions, encompassing neurological injuries, liver disorders, cardiac ischemia, diabetes, and skin problems.

1.3.2. Applications of MSCs

Regenerative medicine and wound healing

MSCs have been extensively explored in the context of repair and wound healing due to their noteworthy capacity in fostering a favorable environment for tissue regeneration. For example,

MSCs exhibit the ability to migrate toward injured tissues, facilitating immune response regulation through the secretion of anti-inflammatory agents. Furthermore, they play a role in collagen synthesis, angiogenesis, and re-epithelialization, contributing to tissue healing. Notably, their paracrine or autocrine roles, involving the production of growth factors, coupled with their capacity to differentiate into diverse cell types, reinforce their significance in these processes (89). In Amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease, where motor neuron degeneration and dopaminergic system impairment respectively cause significant distress, MSC-based interventions have displayed promising results (90,91). These interventions exhibit variability in treatment responses, with instances of disease progression slowdown and improvements in quality of life. Encouragingly, these studies reported minimal adverse effects. Explorations into liver regeneration, particularly in cases of acute kidney injury and autosomal dominant polycystic kidney disease (ADPKD), have yielded moderate symptom amelioration, alongside a favorable safety profile (80). In the context of atherosclerotic renovascular diseases (ARVD), MSC infusions have led to enhanced renal blood flow and mitigation of factors linked to disease progression (80). MSC-based therapies for heart injuries, encompassing acute myocardial infarction, hypoplastic left heart syndrome, cardiomyopathy, and chronic stroke, have showed positive outcomes. Improved cardiac function, heart remodeling, and overall improvement in quality of life have been observed in patients (92). Clinical studies involving MSCs for bone fractures, osteoporotic vertebral compression fractures, and osteoarthritis have demonstrated their viability and clinical benefit. MSCs stimulate bone architecture, fostering fracture healing as supportive agents for augmenting bone tissue formation, with minimal patient-side effects (93). Tissue injuries trigger skin wound healing that occurs in four stages: hemostasis, inflammation, proliferation, and maturation. MSCs and their exosomes can assist in all these steps as they migrate to damaged sites, suppress inflammation, and induce the growth and differentiation of fibroblasts, epidermal cells, and endothelial cells. Clinical studies indicate the potential of MSCs in enhancing wound healing without significant adverse effects (80,94).

Immune-based disorders

Leveraging their potent immunosuppressive capabilities, and their pleiotropic interaction with cells from the immune system (**Figure 11**), MSCs have been extensively explored in pathologies

characterized by heightened immune responses. For instance, MSC clinical application has been evaluated worldwide in graft versus host disease (GVHD), multiple sclerosis (MS), Crohn's disease (CD), osteoarthritis (OA), and, more recently, in the immune-dysregulating infectious disease coronavirus disease 2019 (COVID-19) (95). In the context of GVHD, clinical trials demonstrated the feasibility and effectiveness of in vitro-expanded MSCs, regardless of donor source, for steroid-resistant acute GVHD cases. Positive outcomes included a reduction in both the onset of chronic GVHD and symptom severity (96). MS is characterized by damage to the central nervous system (CNS), inducing physical, cognitive, and neurological dysfunction. CNS restoration and improvements in functional neurological signs were observed in certain patients a month post-injection (97). For CD, an inflammatory bowel disease impacting the entire gastrointestinal tract and disrupting immune tolerance to mucosal antigens, MSC administration, particularly when derived from adipose tissue, improved quality of life by symptom attenuation and clinical remission post-local or systemic injection (98). However, a few participants experienced adverse side-effects. MSCs have also been explored in the context of acute respiratory distress syndrome (ARDS) and its milder form, acute lung injury (ALI), characterized by respiratory failure caused by multiple invasions to the pulmonary parenchyma or vasculature. Allogeneic BM-MSCs were verified for safety and feasibility in transplanted patients, but their efficacy demands further investigation (99). COVID-19 is characterized by acute respiratory syndrome triggered by cytokine storm. Clinical trials demonstrated MSC safety and benefits, including symptom relief, decreased inflammatory cytokines, enhanced lung recovery, improved oxygenation, and better short-term survival in severe cases. However, substantial evidence on its efficacy in treating COVID-19 is yet to be established (100).

Collectively, the use of MSC therapy in an array of scenarios has been demonstrated to be safe and feasible. Yet, it lacks robust evidence on the actual effectiveness of MSCs. Studies suggest that enriching MSC cultures along with appropriate induction factors, strategies to enhance MSC homing post-transplantation, coupled with optimization of MSC delivery dosage and route, could potentiate beneficial therapeutic outcomes (101).

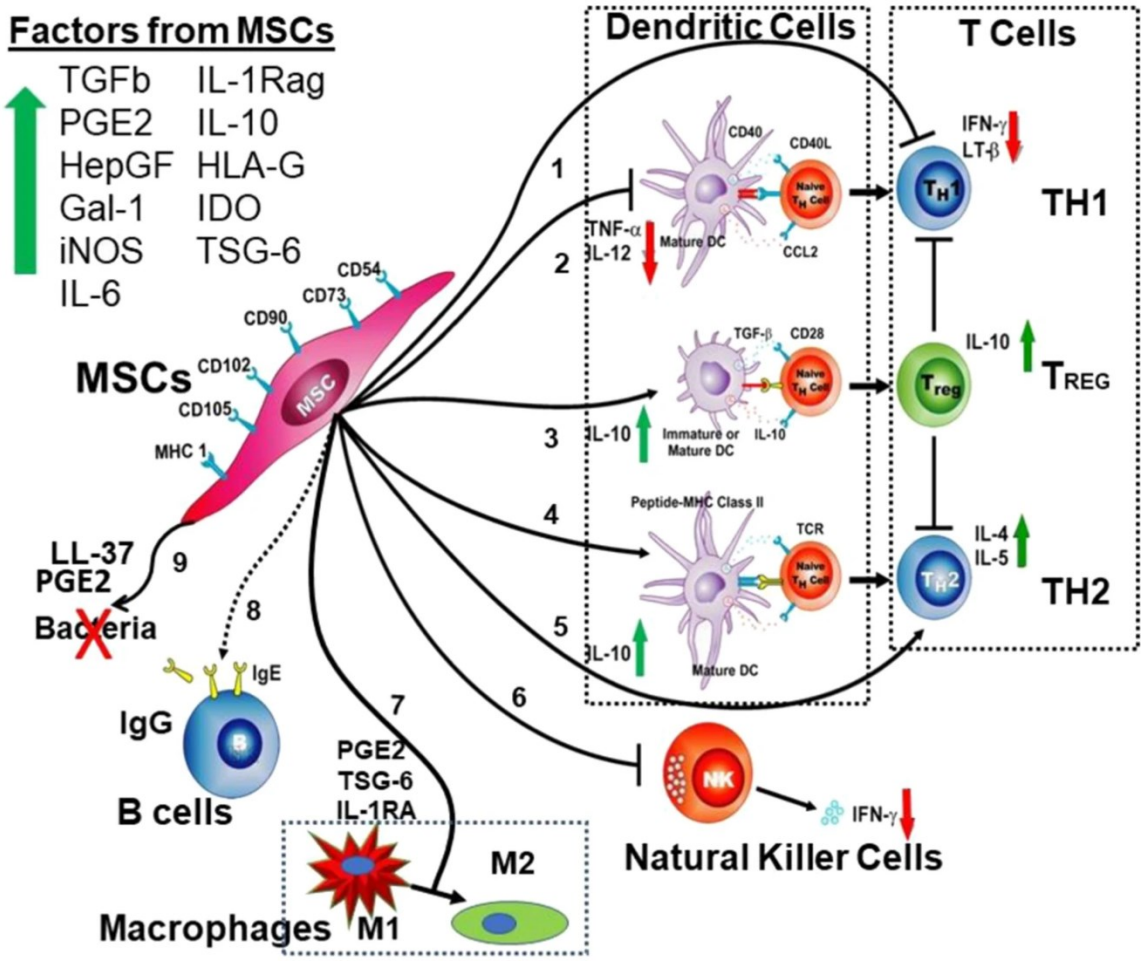

Figure 11 - MSC-immune cell interactions that support their therapeutic application against immune-based disorders (85)**.**

MSCs have the capacity to generate over 10 factors that play a role in immune cell modulation. These factors can either inhibit the function of DCs and diminish the activity of TH1 cells, or prompt the maturation of immature DCs, consequently fostering TH2 and Treg responses. Additionally, MSCs influence the secretion of IFN-γ by NK cells. Macrophage polarization toward a pro-inflammatory or anti-inflammatory profile hinges on the nature of stimuli that activate MSCs. MSCs also possess the ability to reduce antibody production by B cells. Besides their interaction with immune cells, MSCs secreted factors also possess antibacterial effect.

Cancer

Similar to damaged tissues, tumors release chemoattractants, prompting MSCs migration to tumor sites (102). In addition to this feature, high permissibility to genetic modification allowed MSC exploration as Trojan horses to deliver anti-cancer payloads to tumor cells selectively (86).

Genetically engineered MSCs have been equipped to express cytokines like IFN-β, IL-18, and TRAIL. Preclinical studies showed that IFN-β inhibited proliferation and metastasis of breast cancer cells (103), while TRAIL eradicated intracranial gliomas in mice (104). Beside cytokines, other tumor-suppressing proteins have been used. For instance, Bone Morphogenetic Protein 4 (BMP4)- and Phosphatidylinositol 3,4,5-Trisphosphate 3-Phosphatase (PTEN) were evaluated in glioma models. Another interesting use of MSCs as a Trojan horse for cancer treatment is loading them with oncolytic viruses and anti-cancer drugs (86). Most clinical trials involving the application of MSCs for cancer therapy to date are ongoing phase 1 or 2, evaluating their safety and efficacy. Interestingly, a completed phase I/II clinical trial investigated the use of bone marrow derived autologous MSCs infected with the oncolytic adenovirus to treat solid tumors revealed an excellent safety profile for multidose, along with beneficial anti-tumor effects. One pediatric case even achieved complete remission three years after treatment (105). Interestingly, MSCs use has been considered not only to target cancer cells but also an approach to mitigate the side effects of conventional cancer treatments (86).

1.3.3. MSCs as cell-based vaccines

Although MSCs are conventionally recognized for their immunosuppressive nature, they can acquire pro-inflammatory behavior upon specific stimuli. Exposure to a narrow concentration window of IFN-γ triggered a series of changes within MSCs, turning them capable to cross-present soluble antigens (106). Treated MSCs also exhibited increases in MHC I expression and *de novo* expression of MHC class II. These modifications enable them to activate CD4+ T cells via a CD80-dependent mechanism, resulting in the production of cytokines such as IL-2 (107). Furthermore, through cross-presentation, MSCs activated CD8+ T cells. The activation of a complete immune response was demonstrated when IFN-γ-stimulated MSCs, with the pulsing of the model antigen ovalbumin (OVA), provided complete protection against OVA-expressing E.G7 tumors (108). However, there are certain constraints associated with IFN-γ stimulation that could potentially impede effective T cell activation. Exposure to elevated IFN-γ levels induced a decrease in MHC class II expression (107), along with an increase in the expression and secretion of PD-L1 (109). Reduced MHC II levels was associated with limited allogeneic potential (107). Additionally, the interaction between PD-L1 and PD-1 on T cell surfaces triggers a cascade of immune suppressive

events. When active, the immune checkpoint PD-1 drives downregulation of T cell receptors (TCRs), modulates T-cell metabolism, inhibits T lymphocyte proliferation, suppresses T cell activation, and reduces IL-2 secretion, culminating in an overall suppression of pro-inflammatory behavior (110). These findings indicate that a robust inflammatory response, imperative for effective immunization, may compromise the APC function of IFN-γ-stimulated MSCs.

Yet, these studies indicate the promising potential that MSCs harbor to become pro-inflammatory cells if properly modulated and function as non-hematopoietic APC-like cells. The limitations posed by IFN-γ treatment create an opportunity for the exploration of alternative strategies to convert MSCs into pro-inflammatory antigen-presenting MSCs. In light of this potential, MSCs have been genetically engineered to express the immunoproteasome (IPr) (66) and thymoproteasome (TPr) (67), while also being pharmacologically stimulated using the small molecule UM171 (69) and an LSD1-inhibitor(68). These innovative approaches lay the groundwork for the use of MSCs as a cell-based platform in the development of cancer vaccines.

1.4. Antigen processing and presentation

1.4.1. Classical pathways

Antigen processing and presentation are the kick-starters and the foundation for the development of adaptive immune response (111). CD8+ T cells can only kill virus-infected and tumor cells if previously activated (19). High-affinity antibody production by B cells demands previous activation by CD4+ T cells (112). These vital immune mechanisms revolve around the activation of T lymphocytes, which is solely achieved via APCs subsequent to antigen encounter, processing, and presentation (8). A significant concept to understand from antigen presentation is that it provides a tool for the adaptive immune system to monitor the host proteome and possible alterations, which allows for the detection of changes that could signal undesirable events, such as pathogenic infection or malignant mutations (111). The complete antigen presentation process can be segmented into six distinct steps: I) acquisition of antigens, II) tagging antigens for proteolysis into peptides, III) proteolysis, IV) delivery of antigens to major histocompatibility complex (MHC) molecules, V) loading of peptides onto MHC molecules, and VI)

display of MHC molecules at the cell surface. These six steps are applicable to both classical pathways of antigen presentation: MHC class I (**Figure 12**) and MHC class II (**Figure 13**) (111).

1.4.1.1. MHC I

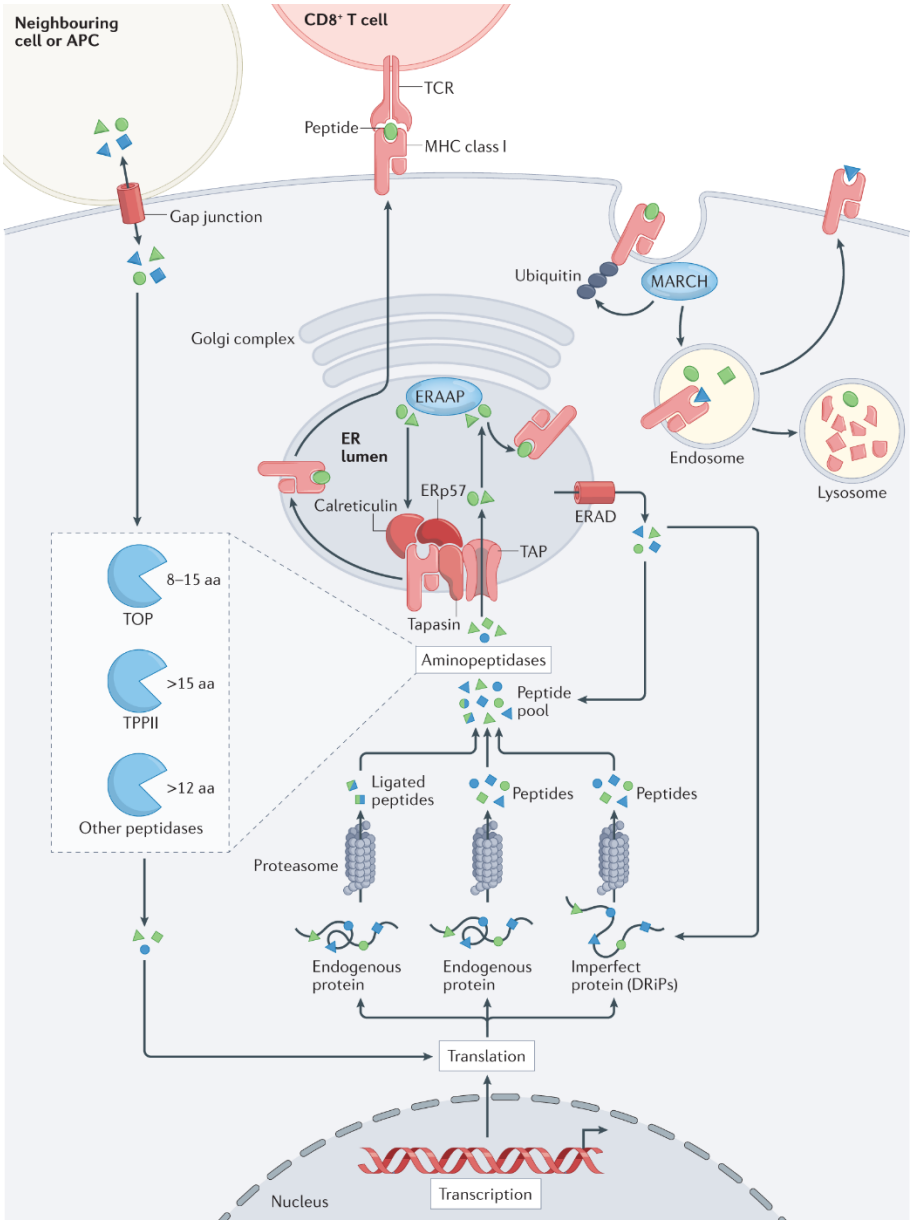

Figure 12 - Overview of MHC I antigen presentation (111)**.**

Endogenous antigens, including defective ribosomal products (DRiPs), are degraded by the proteasome in the cytosol. Proteasome-derived peptides can undergo additional processing, as, for example, by cytosolic aminopeptidases (i.e.: thimet oligopeptidase (TOP) and tripeptidyl peptidase II (TPPII)). These peptides can then be translocated into the ER via the transporter associated with antigen processing (TAP), which is part of

the peptide-loading complex. In the ER, peptides can either be further processed by ER aminopeptidase associated with antigen processing (ERAAP) and then bind to MHC I, or directly bind to MHC I via peptide-loading complex. Peptide–MHC class I complexes then are transferred to membrane surface, where they can serve for antigen presentation to CD8+ T cells. Neighboring APCs can receive cytosolic peptides, before going to the ER, through gap junctions. Later on, MHC I can be either internalized and degraded in a ubiquitin-dependent manner or recycled back to the surface, having the loaded peptide exchanged to another peptide within endosomes.

Antigen presentation via MHC I primarily involves intracellular proteins, such as self-proteins, tumoral or viral antigens. Proteins available in the cytosol are processed by proteasome into immunogenic peptides (113). These peptides are translocated into the endoplasmic reticulum (ER) lumen through the transporter associated with antigen presentation (TAP). These peptides can undergo additional trimming by ER aminopeptidase associated with antigen processing (ERAAP) before binding to the MHC I molecule. MHC I molecules form the peptide loading complex (PLC) with β2-microglobulin (B2M), tapasin, ERp57 and calreticulin, which ensure proper MHC I folding. Then, antigens are loaded into the peptide-binding groove of the MHC I complex. Stable peptide–MHC I complex dissociate from the PLC and are directed to the cell surface via Golgi complex and transport vesicles, this process is stimulated by IFN-γ signaling. On the cell surface, loaded MHC molecules present antigens to CD8+ T cells. These MHC I molecules can undergo ubiquitylation and degradation, or they can be endocytosed and recycled (114).

1.4.1.2. MHC II

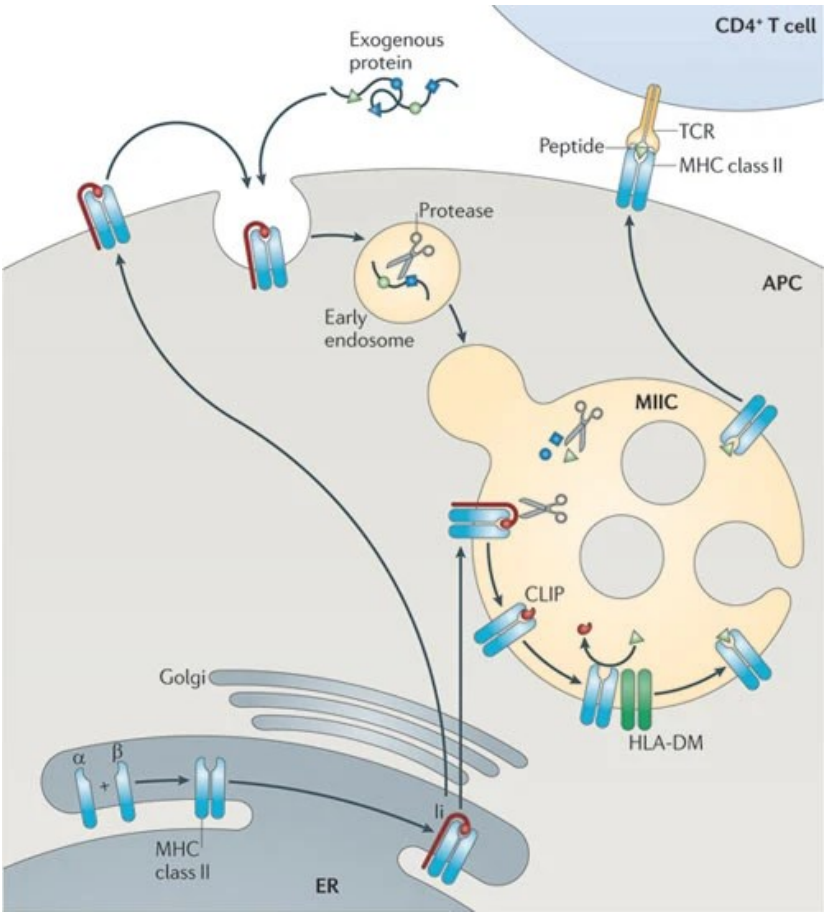

Figure 13 - Overview of MHC II antigen presentation (114)**.**

Antigen presentation through MHC II molecules involves processing exogenous proteins into peptides by endosomal proteases. MHC class II is formed in the ER by the formation of a complex comprised by α- and β-chains, and the invariant chain (Ii). When formed, this complex moves to the MHC class II compartment (MIIC), where Ii is cleaved into class II-associated invariant chain peptide (CLIP). CLIP is later replaced by an antigenic peptide previously processed by proteases, aided by HLA-DM. Finally, the loaded MHC class II molecules reach the cell surface, presenting antigens to activate CD4+ T cells.

Antigen presentation via MHC II molecules involves antigens from extracellular sources, such as bacterial antigens, which are processed by endolysosomal enzymes into peptides. In the ER, MHC class II α- and β-chains assemble and form a complex with the invariant chain (Ii). The Ii–MHC class II heterotrimer is then transported from the ER to the MHC class II compartment (MIIC) via Golgi, either directly and/or through the plasma membrane. Proteins internalized through

endocytosis and Ii are degraded by resident proteases within the MIIC, producing a fragment of Ii called class II-associated invariant chain peptide (CLIP), that remains in the peptide-binding groove of MHC class II. Subsequently, CLIP is replaced in exchange of an antigenic peptide facilitated by the chaperone HLA-DM (known as H2-M in mice). Assembled and loaded MHC class II molecules are then transported to the plasma membrane. Peptide-MHC class II complexes on the cell surface present antigens to CD4+ T cells, resulting in their activation (**Figure 13**) (114).

1.4.2. Cross-presentation

All nucleated cells present antigens in MHC class I, a pivotal feature that enables the adaptive immune system to surveil self-antigens from altered and foreign proteins (111). However, while most cells can only present endogenous proteins on MHC class I, a unique process known as cross-presentation permits specialized APCs to display exogenous antigens in MHC class I *in vivo* (115). DCs are the main cross-presenting APCs, with subsets specialized in this function. The biology of cross-presentation in DCs is still not fully understood (116). Differences among DC subsets in terms of specialization, whether attributed to inherent characteristics or activation within the inflammatory environment, remain uncertain. Nevertheless, observations have shown that the ability for cross-presentation in CD8+ DCs is acquired in the later stages of development and differentiation (117).

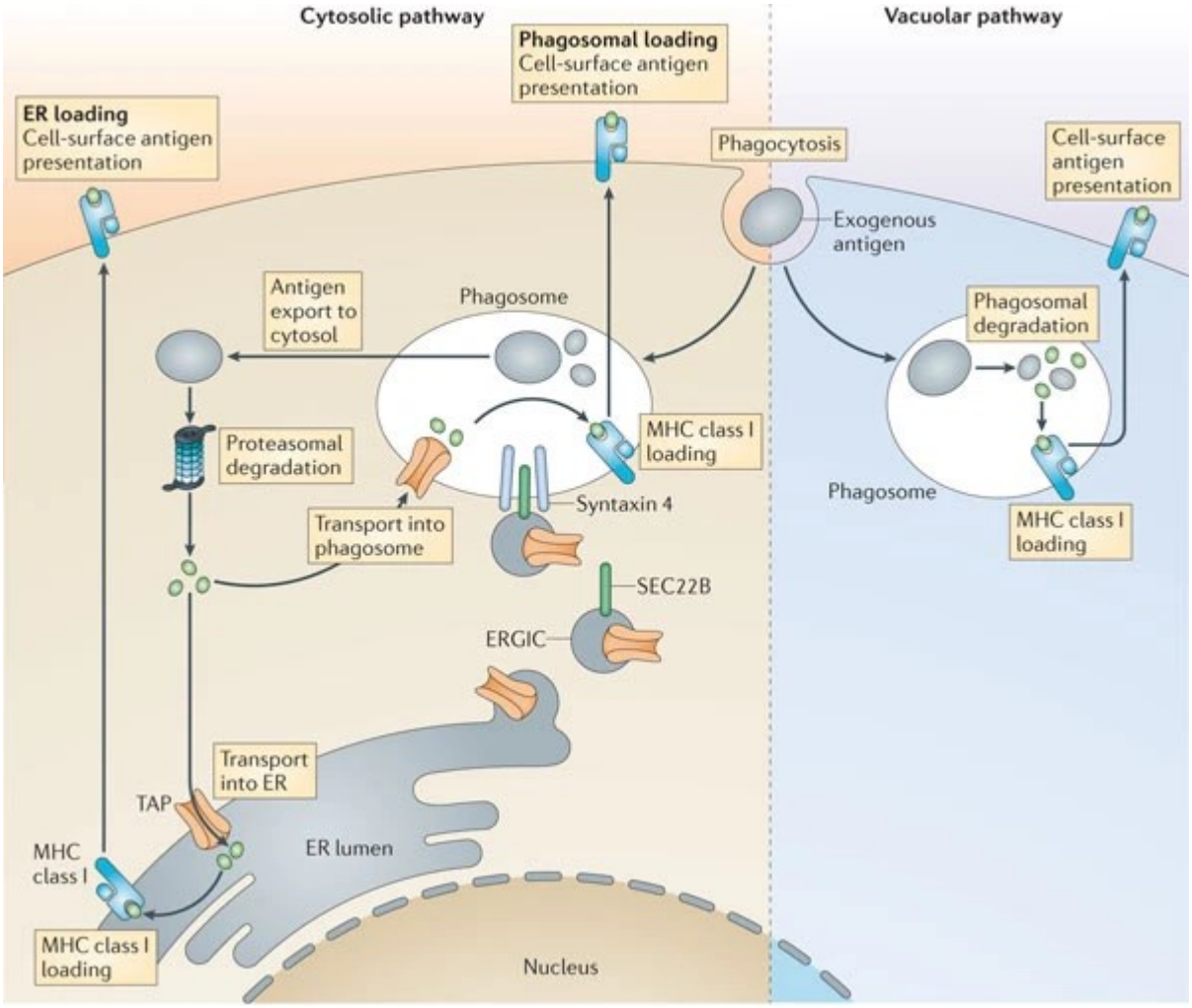

Figure 14 - Schematic overview of cytosolic and vacuolar cross-presentation pathways (116)**.**

Cross-presentation can occur in two different pathways: the cytosolic and the vacuolar. In the cytosolic pathway, extracellular antigens are phagocytosed and exported to the cytosol, where they are degraded by the proteasome. These processed peptide antigens can subsequently be loaded in MHC class I molecules in the ER, after being translocated by TAP protein, or in phagosomes. After loading, the MHC I-antigen complex is presented on the surface of the cell. In the vacuolar pathway, the extracellular antigen processing, and the load of peptides in MHC I occurs within the phagosome.

Two main pathways for cross-presentation have been identified, the 'vacuolar' and 'cytosolic' pathways (**Figure 14**) (116). In the vacuolar pathway, the antigen processing and loading into MHC I occurs within the endocytic compartments. The antigen processing is attributed to lysosomal protases, as the use of cathepsin S inhibitors impaired the pathway, while data suggests that TAP

and MHC class-I loading complex can be recruited to endosomes. Cross-presentation through the cytosolic pathway denotes antigen processing within the cytosol, regardless of where the peptide is loaded in MHC I, given the lack of direct evidence for peptide loading occurring in the ER. Upon internalization through endocytosis, exogenous antigens reach the cytosol, where they are degraded via the proteasome. The peptides generated by the proteasome then proceed through the classical MHC class I antigen presentation pathway, discussed in the previous section. Some key attributes distinguish specialized DCs enabling their cross-presentation efficiency, including the regulation of endocytic pH and proteolytic activity, the ER-phagosome connections, and the antigen export to the cytosol (115).

The capacity to modulate endocytic pH prevents the acidification and activation of lysozymes. Consequently, the endocytosed antigens have their degradation limited within the endosome, which was demonstrated to be correlated with more efficient cross-presentation (118). Similarly, antigens directed to later-stage degradative endosomes/lysosomes predominantly presented epitopes on MHC class II molecules (119). Reduced proteolysis is attributed to diminished levels and activity of lysosomal proteases. This decline in activity is linked to higher pH as a result of low levels of V-ATPase and elevated NADPH oxidase 2 (NOX2) activity in phago/endosomes. NOX2 deficiency correlates with impaired cross-presentation, underscoring the need for limiting unspecific protein degradation in endosomes to preserve potential immunogenic MHC class-I binding epitopes (120).

Interestingly, cross-presenting DCs contain ER-resident proteins in phagosomes, implying direct intercommunication between the two compartments (121). An ER-resident SNARE protein, SEC22B, is required for the recruitment of ER proteins to phagosomes. SNAREs are central proteins to membrane fusion machinery, and SEC22B silencing impaired cross-presentation while sparing classical MHC I and II pathways (122). Therefore, SEC22B and ER are thought to contribute to phagosome maturation delay and antigen transfer to cytosol.

The export of antigens from the endosome to the cytosol is a core step for cross-presentation and will be explored further in the following section.

1.4.2.1. Antigen export from the endosome to the cytosol

The core factor of cross-presentation is the access to the cytosol that exogenous proteins achieve, allowing their access to proceed to MHC I pathway. The biology of antigen export from the endosome to the cytosol remains incompletely elucidated, at both the molecular and cellular levels. Nevertheless, some hypotheses have emerged regarding the molecular mechanisms mediating antigen export to the cytosol (123).

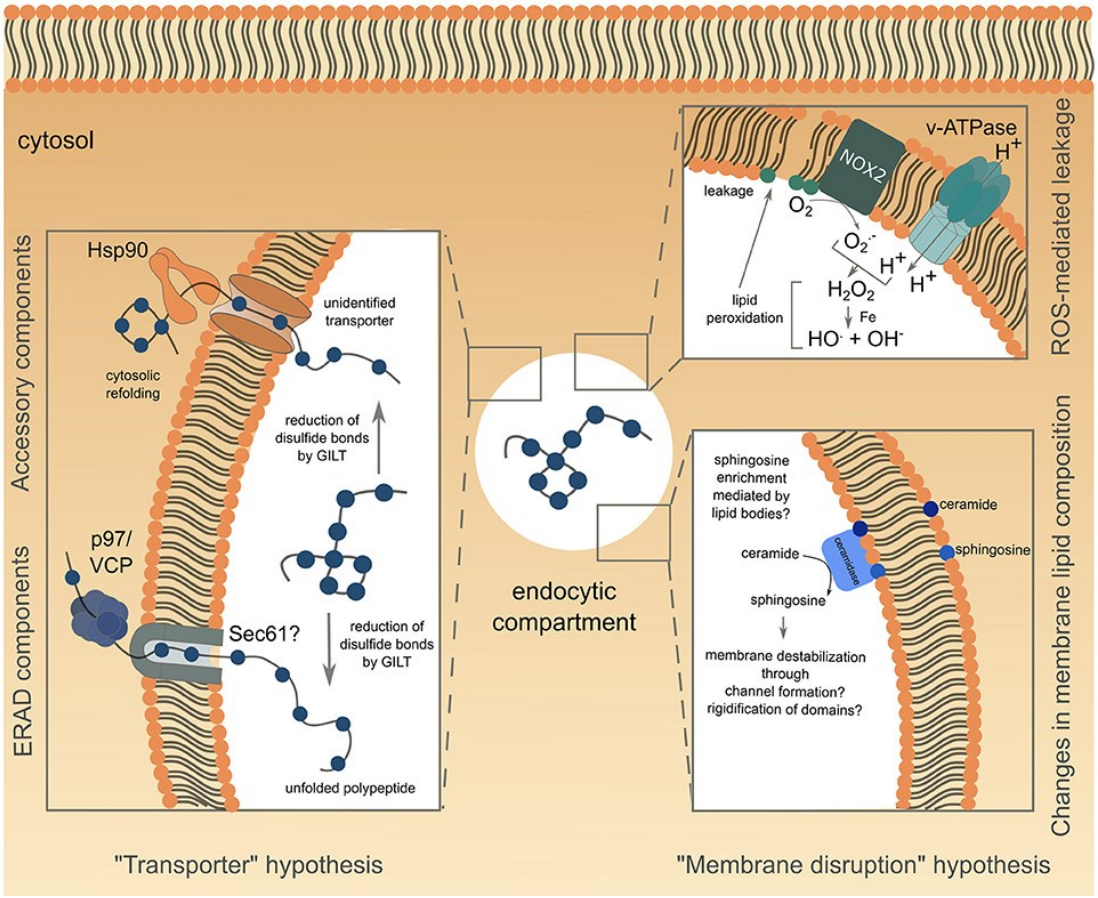

Figure 15 - Schematic representation of the "transporter" and "membrane disruption" hypothesis of antigen export to the cytosol during cross-presentation (123)**.**

In the current comprehension of antigen export to the cytosol during cross-presentation, two main hypotheses are considered. The "transporter" hypothesis proposes that antigens are transported via ERAD and/or associated components. The "membrane disruption" hypothesis suggests that antigens gain access to the cytosol by disrupting the endocytic membrane, either through lipid peroxidation mediated by ROS or by altering the membrane's lipid composition.

The “transporter” hypothesis suggests that the antigens are transported from the endosome to the cytosol mediated by endoplasmic-reticulum-associated protein degradation (ERAD) components, along with accessory proteins (**left panel, Figure 15**) (123). Given the limited diameter of described transporters, antigens might have their native structure modified to be able to be translocated. This notion gains support from studies demonstrating the decrease in transporting efficiency of structurally fixed antigens, when compared to flexible ones (124). Besides, GILT, a constitutive phagolysosomal thiol reductase in APCs, is essential for cross-presentation (125). Studies point to Sec61, a channel that mediates retro translocation of misfolded proteins from the ER to the cytosol, as a potentially responsible for translocation of antigens to the cytosol (126). However, its precise role is still unclear as it could also be involved in the transport of other important components like MHC I. The AAA ATPase p97, a key ERAD component, is indicated as the energy provider for the passage of proteins through the channel (127). Curiously, the chaperone Hsp90 potentially aids not only in mediating antigen transport (128) but also in refolding antigens upon cytosolic arrival (129). Collectively, studies propose that ERAD contributes to antigen export but likely in addition to other mechanisms (130). Just recently, a new mechanism has come to light involving the channel-forming perforin-2 (131). While perforin-1 is well-established in the cytotoxicity of CD8+ T cells—facilitating pore formation for granzyme access—perforin-2, initially recognized for its antibacterial function in innate immunity, has been recently detected within cross-presenting DCs. To become an effective channel-forming protein, perforin-2 is recruited to antigen-containing compartments and requires maturation, which is pH-dependent. Interestingly, perforin-2 induces pore formation without apparent disruption of the endosomal membrane, maintaining endocytic vesicle integrity through mechanisms yet to be studied (131).

Initially, the "membrane disruption" hypothesis emerged from the observation of heightened cross-presentation efficiency for larger and particulate particles, implying potential membrane leakage giving antigen access to the cytosol (132). Recent evidence underscores two possible mechanisms: ROS-mediated leakage and changes in the lipid composition of the membrane **(right panel, Figure 15)** (123). NOX2 activity, which captures protons and generates hydrogen peroxide within endosomes, has been proposed to, besides controlling the pH, induce lipid peroxidation

(133). Hydrogen peroxide-induced lipid peroxidation destabilizes and disrupts endosomal membrane integrity, allowing antigen entry to the cytosol. This mechanism was supported by elegant studies (133,134) and by mechanistic biophysical demonstrations (135) Besides lipid peroxidation, ceramide enrichment that could lead to changes in lipid composition has been implicated in triggering increase in membrane permeability. Ceramide can be converted to sphingosine, which could destabilize the membrane either through the formation of larger channels or the creation of structural defects via rigidification. Interestingly, lipid droplets potentially mediate sphingosines and correlate with cross-presentation efficiency (136). However, the exact role of lipid bodies and the conversion of ceramide into membrane-disrupting sphingosine necessitates further exploration (123).

It is still unclear what are the compensatory mechanisms for endocytic membrane rupture. For example, how do APCs prevent inflammasome-induced cell death that could be caused by the release of cathepsins? The ESCRT machinery, known for its role in viral budding, in cell division abscission, and as a core component of biological membrane repair after damage, has been proposed as a potential player, but it has not been investigated so far (123).

In sum, the exact roles, scope and to what extent these mechanisms contribute to the export of antigens from the endosome to the cytosol remain uncertain. However, its significance as a rate-limiting step in cross-presentation, closely linked to its efficiency, highlights its potential as a therapeutic target to enhance cross-presentation as an approach for therapy.

1.5. Accum technology

1.5.1. Definition and applications

Accum is a technology developed to improve the intracellular accumulation of a molecule of interest (137). It was originally designed to overcome limitations of existing antibody-drug-conjugate (ADCs) approaches, which have their efficacy limited by endosomal entrapment (138). Hence, the original goal of Accum was to avoid endosomal entrapment by disrupting the endosome while preserving the integrity of the conjugate and the cell, resulting in enhanced intracellular retention and target cell selectivity. This technology combines a cholic acid (CA)

moiety and a nuclear localization signal (NLS) moiety (**Figure 16**). CA, a bile acid, was inspired by the escape mechanism used by nonenveloped viruses, which, through ceramide formation, disrupt the endosome and reach the cytosol without killing the host cell (139,140). The NLS directs the molecule/conjugate to the nucleus and increases its accumulation.

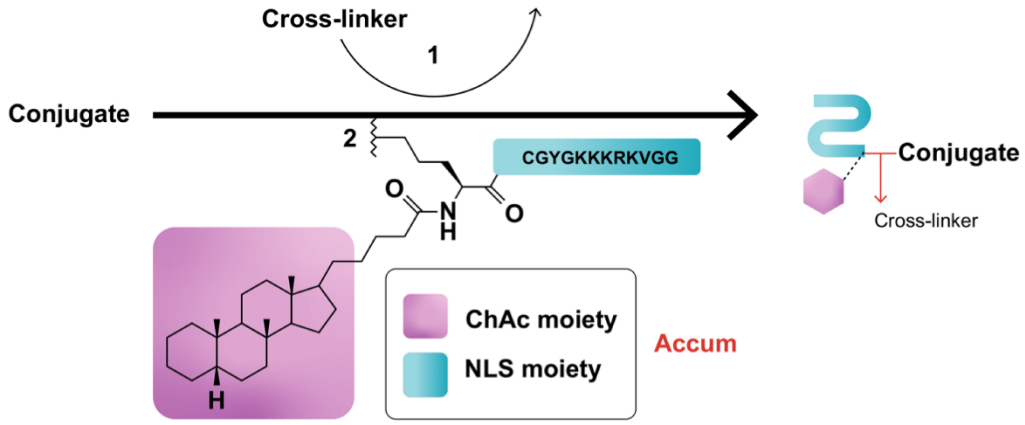

Figure 16 - Schematic representation of Accum molecule and conjugation process (137)**.**

Accum is formed by a ChAc moiety (bile acid highlighted in purple) and an NLS moiety (peptide sequence highlighted in blue). The conjugation between Accum and proteins/conjugates is held through a cross-linking process. In step 1, the protein/conjugate is activated by the cross-linker. In step 2, the activated conjugate reacts with the N-terminus cysteine cap present on the NLS, yielding an Accum-conjugated molecule.

Accum has demonstrated broad applicability for various therapeutic purposes (137). For example, when conjugated with a specific monoclonal antibody (mAb) targeting the interleukin-3 receptor-α (IL-3Rα), it produced 7G3-Accum. IL-3Rα is an antigen found on the surface of leukemic blasts and leukemic stem cells, making it a suitable target for immunotherapy. The creation of 7G3-Accum resulted in enhanced intracellular antibody retention (141,142). Another application involved the conjugation of Accum with a mAb against IL-5Rα, resulting in A14-Accum, which targets a marker overexpressed in Muscle Invasive Bladder Cancer (MIBC) cells. A14-Accum showed improved accumulation within and enhanced target selectivity for IL-5Rα-positive invasive bladder cancer cells (142,143). Furthermore, Accum was used in combination with T-DM1, an ADC targeting epidermal growth factor receptor 2 (HER2)-positive breast cancer. The

resulting T-DM1-Accum exhibited stronger cytotoxicity against tumoral cells while maintaining the integrity and specificity of T-DM1 against HER2-positive cancer cells (144).

The ability of Accum to disrupt the endosome led to another application in the field of DCs and cancer vaccines (145). DCs use endosome-to-cytosol antigen translocation to enhance the presentation of exogenous antigens and activate T cells, in a process denominated cross-presentation. By conjugating Accum with soluble antigens, our research group investigated whether Accum could enhance antigen export from the endosome to the cytosol and cross-presentation properties of $CD8^-$ DCs, which have their cross-presentation capacity underdeveloped during ex vivo generation. Ovalbumin (OVA) was used as the model antigen, and studies demonstrated that OVA-Accum induced endosomal rupture, leading to enhanced immune cell activation, and heightened inflammatory cytokine secretion. Accum-modified proteins derived from lymphoma lysate also showed increased immunogenicity in DCs against tumors in a murine model (145), besides providing protection against tumor growth when used as a prophylactic vaccine, with 96-day survival post-immunization, and induced 50% overall survival as a therapeutic vaccine in tumor-bearing mice. Additionally, OVA-Accum was tested as a prophylactic protein vaccine and, when combined with adjuvants, provided complete protection for over 42 days post-immunization (145).

1.5.2. Variants

Aiming to enhance Accum's capabilities of endosomal disruption, intracellular accumulation, and target cell selectivity, a library of variants was created. Given the modular design of Accum, comprising a BA moiety and an NLS moiety, the variants were generated, each with distinct combinations of BA-NLS modules. This approach aimed to refine the molecule's potential and explore its broader applicability as an onco-immunotherapy strategy.

Thirty-four variants were generated, featuring diverse types of BAs, including cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), glycoursodeoxycholic acid (GUDCA), glycocholic acid (GCA), CDCAC, LCAC. The NLS moiety was modified either with alternative types of NLS or peptides sourced from proteins such as Simian virus 40 (SV40, GWG-SV40), RRRR (NLS1),

RRKR (NLS2, NLS2-RG), RRAR (NLS3), Human antigen R (HuR), terminus utilization substance (Tus), Ribosomal Protein S17 (NLS1-RPS17, NLS2-RSP17, NLS3-RPS17), cMyc, different proteins from the family of heterogeneous nuclear ribonucleoprotein D (hnRNPD, hnRPNPM, and hnRNPA1), Polyglutamine Binding Protein 1 (PQBP1) and Nucleoplasmin.

1.6. Project hypothesis

An effective antitumoral response heavily relies on the activity of cytotoxic T-cells, which can be triggered and boosted by cancer vaccines presenting tumor-specific antigens. Despite extensive testing, DC vaccines have encountered manufacturing and efficacy challenges. To address these limitations, our study proposes a novel approach to treating cancer using cross-presenting MSCs as an alternative vaccine platform due to their versatile plasticity, safety profile and manufacturing advantages. Cross-presentation is a crucial mechanism for activating T cells against tumor antigens, and it depends on the activation of specific cellular events, such as the export of antigens from the endosome to the cytosol. Therefore, we hypothesize that a molecule designed to disrupt the endosome could induce cross-presentation properties in MSCs turning them into a potential cancer vaccine.

1.6.1. Objective 1: Screening a library of molecules designed to disrupt the endosome for the capacity to induce cross-presentation in MSCs.

Our first goal was to screen a library of Accum-derived molecules, designed to disrupt the endosome, and assess their potential at inducing cross-presentation in MSCs. To achieve this, an antigen presentation assay (APA) was used to screen molecules capable of inducing cross-presentation in MSCs. The MSCs were incubated with the Accum variants and the model antigen ovalbumin. After incubation, we added B3Z (SIINFEKL/H2-K^b-specific T-cell line) to the MSCs. A colorimetric assay then measured B3Z activation. After the screening, we selected the most potent molecule for inducing cross-presentation. We optimized MSC treatment regarding the time and concentration of the drug treatment, the duration, concentration of antigen pulsing, and the formulation to reduce the toxicity of the variant. We also evaluated if the MSCs maintained the same surface marker phenotype after treatment through flow cytometry analysis.

1.6.2. Objective 2: Investigating the mechanism of action of the cross-presentation-inducer-Accum variant.

Our second objective was to understand the mechanism by which the selected Accum variant induces MSC cross-presentation. First, we assessed whether the Accum variant induces endosomal breakage by conducting an endosomal disruption assay. The assay consisted of pulsing MSCs with exogenous cytochrome C and monitoring changes in cell death levels. Intact cytochrome C can only reach the cytosol if the endosome is disrupted early on, leading to irreversible apoptosis, which was measured by assessing cell death through flow cytometry. Flow-cytometry-based assays were also used to evaluate other vital events related to cross-presentation. APAs in association with drug inhibitors were performed to analyze specific cellular processes. We also conducted RNA sequencing to explore differential gene expression induced by the Accum variant used throughout the study. Complementary studies were conducted to explore cellular events found to be upregulated in transcriptomic analysis.

1.6.3. Objective 3: Evaluating the therapeutic potency of cross-presenting MSCs as a cancer vaccine.

Our final objective was to assess the therapeutic effectiveness of cross-presenting MSCs, activated by an endosomal disrupting molecule, as a potential cancer vaccine. To achieve this, we evaluated the response against solid tumors in a murine model (C57BL/6). We tested the vaccination as a monotherapy and as a combination with the immune-checkpoint inhibitor anti-PD-1. We also evaluated the vaccine in syngeneic and allogeneic (with BALB/c MSCs) regimens using a T-cell lymphoma model expressing ovalbumin. To refine the vaccination protocol, we tested different administration routes (intratumoral and subcutaneous) and vaccine doses (varying the number of cells per shot). Moreover, we sought to investigate the translational potential of the vaccine by using a melanoma model and its tumor lysate as the stimulating antigen load. The antitumoral response was evaluated by tumor volume measurement over 40 days, considering ulceration, tumor volumes ≥ 1000 mm^3, and weight loss as endpoints.

CHAPTER 2

A1-Reprogrammed Mesenchymal Stromal Cells Prime Potent Anti-Tumoral Responses

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2.1. Abstract

Background: Mesenchymal stromal cells (MSCs) are largely known for their innate capacity to repair ischemic tissues or suppress unwanted immune reactions. However, they can be genetically or pharmacologically converted into potent antigen-presenting cells (APCs) capable of priming responding CD8 T cells. Along this line of thought, we recently identified a novel molecule (A1) capable of eliciting antigen cross-presentation properties in MSCs. The use of A1-reprogrammed MSCs (ARM) as a cellular vaccine, triggering regression of pre-established solid tumors.

Methods: A series of in vitro assays were first used to identify the A1 variant and to investigate its ability to enhance soluble antigen uptake and processing. Mechanistically, cross-presentation assays and molecular profiling studies were used to decipher the A1 mode of action, including its effect on endosomal escape, reactive oxygen species (ROS) production, and cytokine secretion. Finally, the therapeutic potency of the ARM vaccine was evaluated as a monotherapy or in combination with the anti-PD-1 immune-checkpoint inhibitor in both lymphoma and melanoma animal models.

Results: Treatment of MSCs with A1 admixed with soluble antigens enhances antigen uptake, processing, and cross-presentation. The ability of ARM cells to cross-present relies on endosomal disruption, most likely caused by intra-endosomal ROS production, subsequently resulting in lipid peroxidation. Consistent with these observations, transcriptomic analysis of A1-treated cells revealed up-regulation of the unfolded protein response due to A1-mediated protein aggregation. Administration of the ARM vaccine, pulsed with a defined antigen, in combination with the anti-PD-1 immune-checkpoint inhibitor to animals with pre-established solid lymphoma, effectively controlled tumor growth in a syngeneic setting. This effect was further enhanced with the use of allogeneic ARM cells. Similar results were obtained in melanoma using tumor lysate-pulsed allogeneic ARM cells.

Conclusions: Overall, we describe in this study a pioneering strategy aimed at transforming MSCs into efficient non-hematopoietic APCs. ARM cells could therefore be used as a vaccination

platform and synergize with immune-checkpoint blockers to trigger tumor regression. These findings not only highlight the importance of endosomal escape on antigen cross-presentation, but they pave the way for the design of novel and adaptable anti-cancer vaccines.

KEYWORDS

Mesenchymal Stromal Cells; Antigen Cross-Presentation; Accum; Endosomal Escape; Cancer Vaccine.

2.2. Background

Cancer vaccines offer promising alternatives to current immunotherapy strategies as they can potentially cure established tumors while imprinting a long-lasting response through immunological memory (146). Cancer vaccines can also boost pre-existing effector cells while priming a fresh cohort of tumor-reactive T cells (61) if designed to target a broader range of antigens (147,148). Different types of cancer vaccines are categorized by antigen type (predefined or anonymous) or delivery method (direct or via the use of antigen-presenting cells - APCs) (61). Several studies suggest that antigen delivery by APCs, such as dendritic cells (DCs), offers greater effectiveness compared to whole tumor cells (61,149). These observations, combined with the fact that DCs are professional APCs, make them a logical choice for cancer vaccination (10). However, the natural bloodstream scarcity of DCs combined with the hurdles associated with the use of *ex-vivo* generated monocyte-derived (Mo)-DCs pose several limitations to the effectiveness of DC-based cancer vaccines (150,151).

To address these concerns, we have previously explored mesenchymal stromal cells (MSCs) as an alternative vaccine platform (66–69). MSCs are versatile, can be acquired from various sources, and are highly proliferative in cell culture (85). Their isolation is simple and cost-effective (101,152), and their safety has been extensively demonstrated in clinical studies (87). Although their inherent immunosuppressive and regenerative profiles make them an ideal treatment modality for the induction of tolerance, graft survival, suppression of immune-based disorders, and regenerative medicine (153), MSCs harbor a unique plasticity allowing them to acquire a pro-inflammatory phenotype under certain stimuli (82,154,155). The latter characteristic makes them potential candidates for cell-based vaccines. For instance, our group has previously demonstrated how MSCs can be genetically engineered or pharmacologically reprogrammed to behave as APCs capable of cross-presenting antigens resulting in tumor control (66,67,69).

Cross-presentation is an indispensable process for antitumoral immunity as it is the sole mechanism by which exogenous antigens (like those shed by cancer cells) can be processed and presented through major histocompatibility complex (MHC) class I molecules (111). A crucial process in cross-presentation is antigen export from endosomes to the cytosol (126,133,156).

This step may involve the activation of NADPH oxidases (NOX) (120,157), which generate intra-endosomal reactive oxygen species (ROS), helping to create an alkaline environment (120,157,158). Such alkalization prevents endosomal maturation and unspecific antigen degradation that could occur upon lysosomal protease digestion. In addition, ROS triggers lipid peroxidation leading to endosomal membrane disruption (133,159). As a result, intact antigens can reach the cytosol enabling more efficient processing by the proteasomal machinery to generate a larger pool of immunogenic peptides to be presented on the cell surface (116). We thus focused on endosomal antigen export as a primary target in our endeavor to pharmacologically convert MSCs into potent APCs. To do so, we explored the use of Accum, a technology initially designed to enhance intracellular drug delivery and accumulation by disrupting endosomal membranes (145,160). The Accum structure can be modulated and further optimized, a feature enabling the generation of a variant library to be screened to identify molecules capable of inducing antigen cross-presentation. We herein present a new way of utilizing an Accum variant (named A1) as a means to reprogram MSCs into powerful cross-presenting cells capable of inducing potent antitumoral responses.

2.3. Materials and methods

2.3.1. Animals and Ethics

All female BALB/c and C57BL/6 (6–8 weeks old) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed in a pathogen-free environment at the animal facility located at the Institute for Research in Immunology and Cancer. All experimental procedures and protocols were approved by the Animal Ethics Committee of Université de Montréal.

2.3.2. Antibodies and Reagents

The flow-cytometry antibodies (CD44, CD45, CD73, CD90, H2-K^b, and I-A^b) were purchased from BD Biosciences (San Jose, CA, USA). The Accum variants were synthesized as previously described (160). Cytochrome (Cyt)-C, Dp44mT, N-acetyl-Cysteine (NAC), MitoTEMPO, alpha-tocopherol, Diphenyleneiodonium chloride (DPI), the ovalbumin (OVA) protein, the SIINFEKL peptide, and Accutase® were purchased from Sigma Aldrich (Oakville, ON, CANADA). The OVA-AF647 and OVA-DQ® reagents were purchased from Life Technologies (Waltham, MA, USA) and used according to the manufacturer's instructions. The Annexin-V staining kit and human IFN-γ ELISA were purchased from Cedarlane (Burlington, ON, CANADA). The superoxide indicator dihydroethidium (DHE) was purchased from ThermoFisher (St-Laurent, QC, Canada) and used according to the manufacturer's instructions. The NOX1 inhibitor ML171 was purchased from Millipore Corp (Burlington, MA, EUA). The Bradford reagent for protein quantification was purchased from Bio-Rad (Montreal, QC, Canada) and used according to the manufacturer's instructions.

2.3.3. Cell lines

The E.G7 and B16 cell lines used in this study were obtained from ATCC. The B3Z cells were a generous gift from Dr. Etienne Gagnon (Université de Montréal, Montreal, QC, Canada). E.G7 cells were cultured in RPMI 1460 supplemented with 10% FBS, 50 U/mL Penicillin-Streptomycin, 2 mM L-glutamine, 10mM HEPES, 1mM Sodium Pyruvate, and 0.5 mM β-Mercaptoethanol, and kept under selection using 0.4 mg/ml of G418. The B16 line was cultured in DMEM supplemented with 10% FBS and 50 U/mL Penicillin-Streptomycin. All cells were maintained at 37°C in a 5%

CO_2 incubator. B3Z cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM Sodium Pyruvate, 2 mM L-glutamine, 1X MEM Nonessential Amino Acid Solution, 50 U/mL Penicillin-Streptomycin, and 0.5 mM β-Mercaptoethanol. All cell culture media and reagents were purchased from Wisent Bioproducts (St-Bruno, QC, Canada). The human derived MSCs were purchased from RoosterBio (Frederick, MD, USA) and cultured according to the manufacturer's instructions.

2.3.4. Generation of murine BM-Derived MSCs

Generation of murine bone marrow (BM)-derived MSCs was done as previously described(66). Briefly, the femurs of 6–8-week-old female BALB/c or C57BL/6 mice were isolated and flushed with Alpha Modification of Eagle's Medium (AMEM) supplemented with 10% FBS and 50 U/mL Penicillin–Streptomycin in a 10 cm cell culture dish, then incubated at 37 °C. Two days later, non-adherent cells were removed, and the media was replaced every 3 to 4 days until plastic-adherent cells reached 80% confluency. The generated cells were detached using 0.05% trypsin and expanded until a uniform MSC population was obtained. The generated MSCs were validated for their innate phenotype by flow cytometry prior to their freezing in liquid nitrogen.

2.3.5. Antigen cross-presentation assay

To screen the different Accum constructs, a cross-presentation assay was used where 25 x 10^3 cells MSCs were seeded per well in a 24-well plate prior to pulsing with different Accum variants (at 50 µM) for 6 h. At the end of the pulsing period, the cells were washed to remove excess antigen, then 5 x 10^5 B3Z cells were added. The co-culture was incubated for 17-19 hours before cell lysis and incubation for another 4-6 hours at 37°C with a CPRG solution. The optical density signal was detected at wavelength 570 using a SynergyH1 microplate reader (Biotek, Winooski, VT, United States).

2.3.6. Monitoring antigen uptake and processing

To evaluate OVA uptake, MSCs were first treated with 1 µg/ml of OVA-AF647 admixed with the Accum variant A1 for 3 hours at 37°C then assessed for their fluorescence intensity by flow

cytometry. To evaluate antigen processing, MSCs were incubated with 10 µg/mL OVA-DQ® admixed with A1 at 37°C. One hour later, cells were washed, and regular media was added for 3 hours. At the end of the indicated incubation, cells were collected and washed with cold PBS containing 2% FBS. Fluorescence was monitored by flow cytometry.

2.3.7. Assessing Endosomal Escape

Endosomal leakage was assessed using an apoptosis assay as previously described (133). Briefly, 10^5 MSCs were first supplemented with 10 mg/ml of exogenous Cyt-C for 6 h at 37°C in the presence or absence of A1 (50 µM). Once the incubation period completed, the cells were collected using Accutase®, washed with ice-cold PBS, then stained for Annexin-V according to the manufacturer's instructions prior to analysis using BD FACS Diva on CANTO II.

2.3.8. Evaluating ROS production and ROS and NOX neutralization

ROS production was evaluated by DHE staining. Briefly, 2 x 10^4 cells were plated for 24 h on a 12-well plate on day 0. On day 1, cells used as a positive control were treated with 2.5 µM of Dp44mT, a ROS-inducing agent, for 24h. On day 2, correspondent wells were treated with A1 +/- OVA for 6h and 20 mM of NAC, a general cysteine donor used as the negative control, for 1h. After incubation, cells were collected, washed, and stained with DHE 10 µM for 30 minutes at 37°C. After staining, cells were washed and analyzed by flow cytometry within 1 hour. To evaluate the effects of ROS neutralization on A1-induced cross-presentation, the same antigen cross-presentation assay described above was performed. Briefly, the selected inhibitors were pulsed for the same period of time as the A1 molecule (6 h), followed by a washing step and the addition of B3Z cells. In addition to using NAC (5 mM) as a general ROS inhibitor, MitoTEMPO (10 µM) was used as a specific mitochondrial ROS inhibitor and α-tocopherol (2000 µM) was tested as a blocker for lipid peroxidation. To evaluate the effect of NOX inhibition on A1-induced cross-presentation, the antigen cross-presentation assay was performed as described above in association with NOX inhibitors. DPI (20 µM), a general blocker of flavoproteins, was used as a general NOX inhibitor, while ML171 (20 µM) was used for specific NOX1 inhibition.

2.3.9. Turbidity Assay

For the turbidity assay, OVA (1 mg/mL) and A1 (50 µM) were admixed in serum-free AMEM, and 100 µL of each sample was added to a polystyrene flat bottom 96-well plate (Corning). The wavelength for measurement was defined according to the examination of the absorbance spectra of the buffer (serum-free AMEM), in which no significant peak was observed. Thus, turbidity was assessed at 420 nm using a Synergy H1 microplate reader (BioTek). Plates were incubated at 37°C and shaken for 5 seconds before each reading, taken every 15 minutes. The experiment was conducted 4 times with 6 technical replicates for each condition.

2.3.10. Cytokine and Chemokine Analysis

For cytokine and chemokine profiling, 15 cm cell culture dishes containing 80-90% confluent MSCs were grown in serum-free AMEM for 24 h at 37 °C and 5% CO_2. MSCs were then treated with 50 µM of A1 in serum-free AMEM for 6h. The supernatant was collected, and fresh serum-free AMEM was replenished without A1. After 24h of the initial A1 treatment, the supernatant was collected and added to the previous collection prior to their concentration using the Amicon Ultra-4 centrifugal filters (3000 NMWL) for 1 h at 4 °C. Collected concentrates (80×) were then frozen at -80 °C until shipped to EveTechnologies (Calgary, AB, Canada) for cytokine/chemokine assessment by Luminex.

2.3.11. Generation of B16 Tumor lysate

B16 cells were cultured until reaching 80-90% confluency and collected using 0.05% trypsin. Collected cells were washed 3 times with PBS in centrifugation cycles of 1000 rpm x 10 min. Washed cells were kept at -80°C until lysis. For the lysis procedure, the cell pellet was subjected to 5 freezing (liquid nitrogen)/thawing (room temperature) cycles, with complete homogenization conducted before every freezing/thawing step. The final solution was centrifuged for 10 min at 5000 x g at 4ºC and the protein lysate supernatant was collected and kept at -80°C until further use. Protein quantification was performed using Bio-Rad Protein Assay (Bio-Rad).

2.3.12. Therapeutic vaccination

For therapeutic vaccination, female C57BL/6 mice (n=5-10/group) received a subcutaneous (SC) injection of 5 x 10^5 EG.7 cells at day 0. Three days later (appearance of palpable tumors ~ 35-50 mm^3), mice were intratumorally (IT) or SC-injected with 5 x 10^5 A1/OVA-pulsed MSCs (two injections one week apart). Control animals received 5 x 10^5 tumor cells alone. Treated animals were followed thereafter for tumor growth. For therapeutic vaccination in combination with the anti-PD-1 immune-checkpoint inhibitor, mice received intraperitoneal (IP) injections of the antibody or its isotype at 200 µg/per dose every 2 days for a total of 6 doses over two weeks. A similar approach was conducted for allogeneic dosing and A1 dimer-related vaccination in C57BL/6 mice but using BALB/c-derived MSCs or B16 lysate-treated MSCs.

2.3.13. RNA-seq and Bioinformatic analysis

For RNA-seq, MSCs were treated with A1 alone or A1 + OVA for 6h. Their RNA was extracted using the RNeasy Mini Kit (QIAGEN). Quantification of total RNA was made by QuBit (ABI), and 500 ng of total RNA was used for library preparation. The quality of total RNA was assessed with the BioAnalyzer Nano (Agilent), and all samples had a RIN above 8. Library preparation was done with the KAPA mRNAseq stranded kit (KAPA, Cat no. KK8420). Ligation was made with 9 nM final concentration of Illumina index, and 10 PCR cycles were required to amplify cDNA libraries. Libraries were quantified by QuBit and BioAnalyzer. All libraries were diluted to 10 nM and normalized by qPCR using the KAPA library quantification kit. Libraries were pooled to equimolar concentration. Sequencing was performed with the Illumina Hiseq2000 using the Hiseq Reagent Kit v3 (200 cycles, paired-end) using 1.7 nM of the pooled library. All Fastq files (strand-specific sequencing, N=4 per group) were aligned to GRCm38 (mouse genome Ensembl release 102) with STAR (v2.7). Raw reads mapping to genomic features (summarized per gene) were extracted with featureCounts (strand-specific option). Expression matrices were filtered, genes with very low counts were removed, and protein-coding genes were kept for further analyses. Gene expression in both Accum-A1- and A1 + OVA-treated MSCs was compared to BM-derived MSC controls with DESeq2 to generate a ranked list of differentially expressed genes based on the log2 fold change. Gene set enrichment on either ranked lists of genes or a number of significantly up-or down-unregulated genes perturbed by A1 alone or admixed with Accum A1 variant compared to MSC

controls were performed using the Reactome collection of pathways. The variance stabilizing transformation was applied to gene expression matrices prior to visualization. If not mentioned in the text, the significance threshold is set to 5% after p-value adjustment with the Benjamini–Hochberg method to control for false positives among differentially expressed genes (DEGs). All custom scripts, including the prediction of putative targets, were written in R programming and statistical language. Data visualization was made with ggplot2, enrichplot, Upset plots, and Pheatmap R functions.

2.3.14. Statistical Analysis

p-values were calculated using one-way analysis of variance (ANOVA) or Log-rank test for animal survival experiments. 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. Identification of A1, a molecule inducing cross-presentation in MSCs.

Accum is a technology initially designed to enhance drug delivery and intracellular accumulation when conjugated to biomedicines (137). Its unique structure consists of a bile acid linked to a peptide-based nuclear localization signal (NLS). Inspired by the infection mechanism of non-enveloped viruses, the Accum composition destabilizes the endosomal membrane, provoking the escape of captured cargo to the cytosol (145,160). In fact, we previously demonstrated how OVA-linked Accum potentiates the antitumoral effect induced by DCs in the context of cancer vaccination (145), which prompted us to assess whether similar effects could be instilled in MSCs. To test this hypothesis, we screened a library of 34 Accum variants (with different bile acids and/or NLS sequences) using two different cross-presentation assays. In the first assay (**Figure 17A**), the variants were chemically linked to the OVA protein as opposed to the second assay, where the variants were admixed with OVA (**Figure 17B**). Although no signal could be detected in any of the experimental conditions tested in the chemically linked assay (**Figure 17C**), one Accum variant (cholic acid-hnRNPA1 - hereafter referred to as A1) triggered B3Z activation in the admixed condition (Figure 17**D**). Despite sharing a bile acid similar to other variants, including the original Accum molecule (Figure 17**E**), our data indicate that the A1 molecule **(Figure 17F**) exhibits properties that are distinct from all tested variants.

2.4.2. A1 pulsing enhances antigen uptake and processing while triggering endosomal escape.

Following A1 identification, we next tested various parameters to determine the best condition yielding a balance between maximal antigen cross-presentation and absent/minimal cell death. To do so, we first compared the impact of PBS versus distilled water as diluents for A1. Although the B3Z T cells responded to MSCs treated with both 25 and 50 µM of A1 diluted in PBS (**Figure 18A**), only the 50 µM dose worked in the water condition, with no apparent cell death induced according to Annexin-V staining on treated MSCs (**Supplementary Figure 1A**). Based on these data, we next determined the optimal pulsing duration to be 6 h (**Figure 18B**) with 0.5 mg/ml

being the minimal and 1 mg/ml being the optimal protein concentration required to obtain a detectable B3Z response (**Supplementary Figure 1B**). So far, all conducted tests were centered on antigen cross-presentation with no assessment of A1 potential impact on the process of antigen presentation. As shown in **Figure 18C**, robust activation of B3Z occurred upon exposure to A1+OVA-treated MSCs, when compared with OVA-only pulsed MSCs, while the presence of A1 did not induce significant changes when the cells were pulsed with the SIINFEKL peptide. To investigate the mechanistic basis of A1-induced cross-presentation, we next examined if A1 affects antigen uptake and processing, which are both critical for cross-presentation. When monitored for their capacity to capture fluorescent OVA (OVA-AF647), a significant increase in fluorescence emission was detected in MSCs treated with the antigen/A1 mix compared to control groups (**Figure 18D**). Similar results were obtained with respect to antigen processing using OVA-DQ®, a self-quenching fluorescent probe emitting fluorescence upon proteolysis (**Figure 18E**). Similar up-take and processing results were obtained when re-tested on human-derived MSCs (**Supplementary Figure 2A-D**). In line with these observations, an endosomal disruption assay was next conducted to assess whether A1 elicits antigen export from the endosome to the cytosol. The assay involved pulsing MSCs with exogenous Cyt-C and monitoring changes in cell death (133). Since Cyt-C is promptly endocytosed and cannot naturally cross the endosomal membrane, its ability to induce apoptosis relies on reaching the cytosol as an intact antigen (161). As shown in **Figure 18F**, Cyt-C only triggers cell death when combined with A1, an effect not observed with the parent Accum molecule. Furthermore, the observed effects did not change the overall H2-K^b or I-A^b cell surface levels (**Figure 18G**) nor the innate phenotype of MSCs as shown by CD44, CD45, CD73, and CD90 staining by flow-cytometry (**Supplementary Figure 3**). Overall, our data indicate a central role for A1 on various cross-presentation-related processes such as antigen uptake, processing, and escape to the cytosol.

2.4.3. A1 triggers intra-endosomal ROS production through NOX activation.

To explore other commonly observed events in DCs capable of cross-presenting, we next assessed ROS production using DHE, a cell-permeable superoxide indicator dye. Interestingly, A1 triggers similar ROS production levels compared to Dp44mt (**Figure 19A**), a ROS-inducing agent used as a positive control. To assess the importance of this observation, we next evaluated the neutralizing

impact of different antioxidants on antigen cross-presentation. Treatment with NAC, a general cysteine donor scavenging free radicals, completely abolished cross-presentation (**Figure 19B**). In contrast, treatment with MitoTEMPO, a mitochondria-targeted antioxidant, did not affect B3Z activation, therefore excluding a role for mitochondria-driven ROS (**Figure 19B**). Interestingly however, co-incubation with α-tocopherol, a vitamin-E derivative capable of blocking lipid peroxidation, significantly decreased MSCs ability to cross-present (**Figure 19B**). As a role for mitochondrial-derived ROS is inapparent, we next focused on the possible activation of NOX, which can be found within endosomes as reported in other specialized DCs subsets (120,157,158). To do so, A1-pulsed MSCS were co-treated with generic (DPI) versus NOX1-specific (ML171) inhibitors. Although ML171 partially decreased B3Z activation, the use of DPI completely suppressed antigen cross-presentation (**Figure 19C**). These findings indicate that A1 leads to intra-endosomal ROS production via NOX, which causes endosomal break via lipid peroxidation (**Figure 19D**).

2.4.4. A1 induces protein aggregation and drives the unfolded protein response (UPR).

To better understand the impact of A1 on MSCs, we conducted a whole transcriptome analysis by comparing control to A1- or A1/OVA-treated MSCs. There is a strong correlation between the two A1 conditions (**Supplementary Figure 4A**), indicating a similar expression profile; A1 treatment leads to more than 1,500 differentially expressed genes (DEGs - **Supplementary Figure 4B**). Amongst the up-regulated processes in A1-treated MSCs cells, we observe UPR, metabolism of nucleotides, glycolysis, and regulation of HSF-1-mediated heat shock response (**Figure 20A**) whereas pathways related to fatty acid or steroid metabolism, cholesterol biosynthesis as well as bile acid and bile salt metabolism were downregulated (**Figure 20B)**. Since misfolded, damaged, or aggregated proteins can all lead to ER stress and UPR up-regulation, including the expression of sensory pathways related to the endoplasmic reticulum (ER) stress such as ATF4, ATF6 and XBP1 (**Figure 20C**), a turbidity assay was conducted using OVA admixed with A1. As shown in **Figure 20D**, A1 on its own aggregates, an observation that was further enhanced when admixed with the OVA protein. Besides UPR-related changes (**Figure 20C**; **Supplementary Figure 4;** and **Supplementary Figure 5**), molecular profiling of A1-treated cells revealed activation of IFN-

stimulated genes and IL-12 signaling (**Figure 20A** and **Supplementary Figure 6A**). Consistent with these observations, secretome analysis using Luminex revealed increased secretion of pro-inflammatory mediators such as G-CSF, GM-CSF, IL-6, TNF-alpha, and IL-12 (**Supplementary Figure 6B**). Based on the extensive impact of A1 on the molecular and physiological profile of MSCs, we elected to designate these cells as A1-Reprogrammed-MSCs (ARMs).

2.4.5. Vaccination using ARM cells induces potent antitumoral responses.

To evaluate the antitumoral capacity of the ARM cells, we conducted a series of vaccination studies against solid tumors. As illustrated in the experimental design (**Figure 21A**), SC delivery of syngeneic ARM cells was performed 3 days post-EG.7 tumor implantation followed by a second dose a week later as a monotherapy or in combination with anti-PD-1. In contrast to all tested conditions, co-administrating the ARM vaccine with anti-PD-1 elicits a prominent tumor control response (**Figure 21B**), with almost all mice (80%) surviving by day 40 (**C**). The vaccination was also evaluated in allogeneic settings, aiming to investigate the potential of using MSCs from donors, instead of autologous cells. In this set up, ARM administration as a monotherapy triggered a superior response (**Figure 21D-E**) compared to the same treatment group under syngeneic settings. Nevertheless, combining the allogeneic ARM cells with anti-PD-1 cured all treated animals (**Figure 21D**) with complete survival obtained by day 40 (**Figure 21E**). No differences in antitumoral responses were observed when allogeneic ARM cells were delivered using the SC versus IT route (**Supplementary Figure 7A-B**). On the other hand, the ARM effector response was dose-dependent, with a loss in therapeutic potency observed using the 1×10^4 cell dose (red line) in contrast to using 5×10^5 cells (green line - **Supplementary Figure 7C-D**).

All proof-of-concept studies conducted so far used OVA as a single pre-defined antigen. This approach may not be clinically viable for two main reasons. First, there is currently no known tumor-specific antigens (TSA) shared by a large portion of the population and capable of inducing potent antitumoral responses (162). Second, past studies showed that targeting a single antigen is most likely prone to elicit tumor editing/escape over time (61). Since we have a cell type capable of effectively cross-presenting soluble antigens, we elected to overcome these limitations using tumor lysate preparations. This approach not only allows the presentation of multiple unknown

neoantigens or TSAs, but it also permits the generation of an immune response specific to each patient/cancer indication. When tested against the B16 melanoma model, for instance, the immune response generated following allogeneic ARM cell administration (monotherapy) effectively controlled tumor growth (**Figure 21F**) with 40% survival rate obtained over 40 days (**Figure 21G**). Co-administration with anti-PD-1, on the other hand, significantly improved the antitumoral response (**Figure 21F**), doubling therefore the survival rate (**Figure 21G**). Overall, our findings convey two important messages. First, the ARM vaccine can indeed induce potent antitumoral responses even when pulsed with tumor lysate. Second, the developed approach is versatile and can be adapted to different cancer types if given access to tumor tissues/biopsies.

2.4.6. Dimerization improves the molecular stability of A1 while eliciting similar antitumoral effects.

Throughout our studies, we noticed a decrease in A1 stability over time, most likely due to the oxidation of exposed amino acid residues. In addition, the manufacturing of the monomeric A1 form can result in the generation of non-specific "contaminants" that can impair its overall activity. To bypass these limitations, we engineered a dimer of A1, where two molecules are linked together through the NLS peptide (**Figure 22A**). The dimer remained active, and its ability to induce cross-presentation by MSCs was observed at a much lower dose (20 µM in contrast to 50 µM with the monomeric form) with a decrease in ARM viability at higher concentrations (**Figure 22B**). When tested in the context of therapeutic vaccination against the E.G7 lymphoma model as outlined in the timeline in **Figure 22C**, the response induced by the ARM vaccine was improved (60% versus 40% survival with the A1 monomer - (**Figure 21E**) as a monotherapy and was further enhanced when combined with anti-PD-1 (**Figure 22D-E**). These results demonstrate that the A1 dimer is indeed active, requiring lower pulsing concentrations (20 µM compared to 50 µM) while improving the therapeutic potency of the allogeneic ARM vaccine.

2.5. Discussion

While DC-based vaccination showed moderate therapeutic effects against cancer, their clinical use is hindered by their limited availability and associated manufacturing hurdles (146,163). Our study proposes an alternative vaccination platform utilizing the ARM cells, which not only synergize with immune checkpoint inhibitors leading to tumor regression and increased survival rates, but also offers a compelling and versatile strategy adaptable to the generation of a broader scope of cancer vaccines. More specifically, our strategy consists of pharmacological reprogramming of MSCs to cross-present antigens. By admixing A1 with a defined or a set of mixed antigens (e.g., tumor lysate), protein aggregation takes place and is captured in endosomes by MSCs. At that stage, A1 stimulates NOX to produce ROS resulting in lipid peroxidation and cargo release in the cytosol. Since protein aggregation is seen as a "danger signal," the cell responds through UPR upregulation, which ends up targeting captured proteins for proteasomal degradation. Generated peptides are then presented on the cell surface to responding CD8 T cells while the pro-inflammatory cytokine profile of ARM cells supports the ongoing immune response (**Figure 23**).

Besides enhanced antigen uptake and processing, one of the most salient observations made in this study relates to ROS production. Surprisingly, the origin of ROS was found to be unrelated to the mitochondria but instead involves NOX activation, a process previously described in cross-presenting cDC1 (120,157,158). Intra-endosomal production of ROS in this context has two crucial advantages. First, it prevents endosomal acidification, which would impair protease activation, avoiding, therefore, non-specific degradation of the captured cargo (120,157,158). Second, ROS production triggers lipid peroxidation, which destabilizes and ruptures endosomal membranes, thereby releasing captured intact antigens into the cytosol (133). Intriguingly, the parent Accum molecule could not promote endosome-to-cytosol escape (**Figure 18G**) nor antigen cross-presentation in MSCs when admixed with the OVA antigen. This indicates a distinct gain of function for A1 related to ROS-induced endosomal disruption. Although our model provides direct links between A1, NOX activity, and ROS production, additional investigations are needed to confirm whether lipid peroxidation acts synergistically with an additional A1-induced membrane perturbation mechanism or if it is the sole inducer of endosomal disruption.

Transcriptomic analysis revealed that A1 triggers the upregulation of both the heat shock factor 1 (HSF1) and the unfolded protein response (UPR), two key players in cellular stress response and the re-establishment of protein homeostasis. Proteostasis is a vital cellular process and is tightly regulated to avoid problems that could lead to cellular dysfunction and/or death (164). The conducted turbidity assay, combined with UPR, corroborates the hypothesis that A1 induces protein aggregation (164). In consequence, UPR activation has two possible outcomes: restoring homeostasis or inducing apoptosis (165). Interestingly, the ubiquitin-proteasome system is tightly related to ER stress (166). Hence, stimulation of the proteasomal machinery could lead to the activation of antigen presentation machinery (7), contributing to the efficiency of ARM cells in cross-presenting antigens. In cross-presenting $CD8\alpha^+$ DCs, constitutive activation of ER genes was found to be essential for maintaining proper protein homeostasis, particularly during increased demands for cytokine and MHC production (167,168), which correlates to the efficiency in their role as professional APCs. Although we did not observe an increase in MHC expression, our secretome analysis unveiled an increase in pro-inflammatory cytokine secretion by ARM cells. This could suggest a connection between ER signaling and the antigen-presenting capabilities of the ARM cells, resembling professional APCs.

The crosstalk between ER stress, UPR, and lipid metabolism has been debated in the literature (169,170). Beyond the classical view that the UPR induces upregulation of lipogenesis to support ER membrane extension, recent studies demonstrated how the UPR could also have implications in lipid and sterol synthesis, promoting lipolysis by activation of intermediary sensors in the UPR pathway that alter lipid enzymes (170,171). Moreover, it is possible that A1-induced lipid peroxidation caused by A1 leads to cytosolic lipid accumulation, causing a state of lipid overload and downregulation of fatty acid metabolism. The structure of the A1 molecule, containing cholic acid, may also play a role in reducing cholesterol biosynthesis as a response to lipid overload since cholesterol is a precursor of cholic acid, potentially resulting in the impact observed in bile acid metabolism. As a result, various cellular events may have induced lipid-related pathways to reduce lipid accumulation and restore cellular homeostasis.

The MSC mode of action remains a highly debated topic within the field of cell therapy (85). Initially, the therapeutic function of MSCs as immunosuppressants was attributed to their

secretome (85,172). However, elegant studies have shown that administered MSCs undergo apoptosis, which attracts phagocytes to attack and capture their particles through a process called efferocytosis, even in the absence of host cytotoxic or alloreactive cells (173,174). In this case, it is logical to stipulate that phagocytes capture, process, and present peptides derived from apoptotic ARMs, subsequently interacting with host-derived responding T cells. Although the ARM cells may not directly activate cytotoxic cells, their ability to cross-present antigens make them effective vehicles for transporting processed tumor antigens, initiating a cascade of cell activation, and eliciting a robust immune response against solid tumors. The superior response observed in allogeneic versus syngeneic vaccination settings may be attributed to the more efficient mobilization of phagocytes due to allorecognition. The latter hypothesis is supported by the fact that allogeneic vaccination increases the immunogenicity of cancer vaccines (175,176). The use of allogeneic MSCs in therapeutic approaches has been extensively studied. While clinical data available do not decisively favor either autologous or allogeneic MSCs as the superior option, allogeneic cells have demonstrated a robust safety profile. Moreover, they facilitate the establishment of donor cell banks, which not only increases the availability for immediate use but also reduces the overall time required for treatment. As a result, the adoption of allogeneic cell-based vaccines offers advantages in terms of efficacy and manufacturing, positioning ARM cells as a versatile off-the-shelf vaccine candidate.

2.6. Conclusion

Utilizing cells from unrelated donors allows the establishment of a predefined "master" and/or "working" cell banks, readily available for patient use is an appealing cell therapy modality (177,178). This eliminates the need for obtaining and expanding MSCs for each patient, which would require specialized infrastructure and skilled personnel at every treatment center, thereby streamlining the treatment process and decreasing the number of invasive procedures (177). The personalized and simplified off-the-shelf approach proposed in this study holds promise for tailoring treatments to individual patients and tumor types while offering manufacturing advantages. It further lays the groundwork for further optimization and demands future investigations to unravel the precise mechanism of action, paving the way for potential advancements and applications in the future.

2.7. Declarations

2.7.1. Ethics approval and consent to participate

Animal protocols were approved by the Animal Care Committee of Université de Montréal (protocol # 22-065).

2.7.2. Consent for publication

Not applicable

2.7.3. Availability of data and materials

The accession number for RNA Seq sequence data reported in this paper are deposited in GenBank.

2.7.4. Competing interests

Daniela Stanga and Sebastien Plouffe are employees of Defence Therapeutics and declare competing financial interests.

2.7.5. Funding

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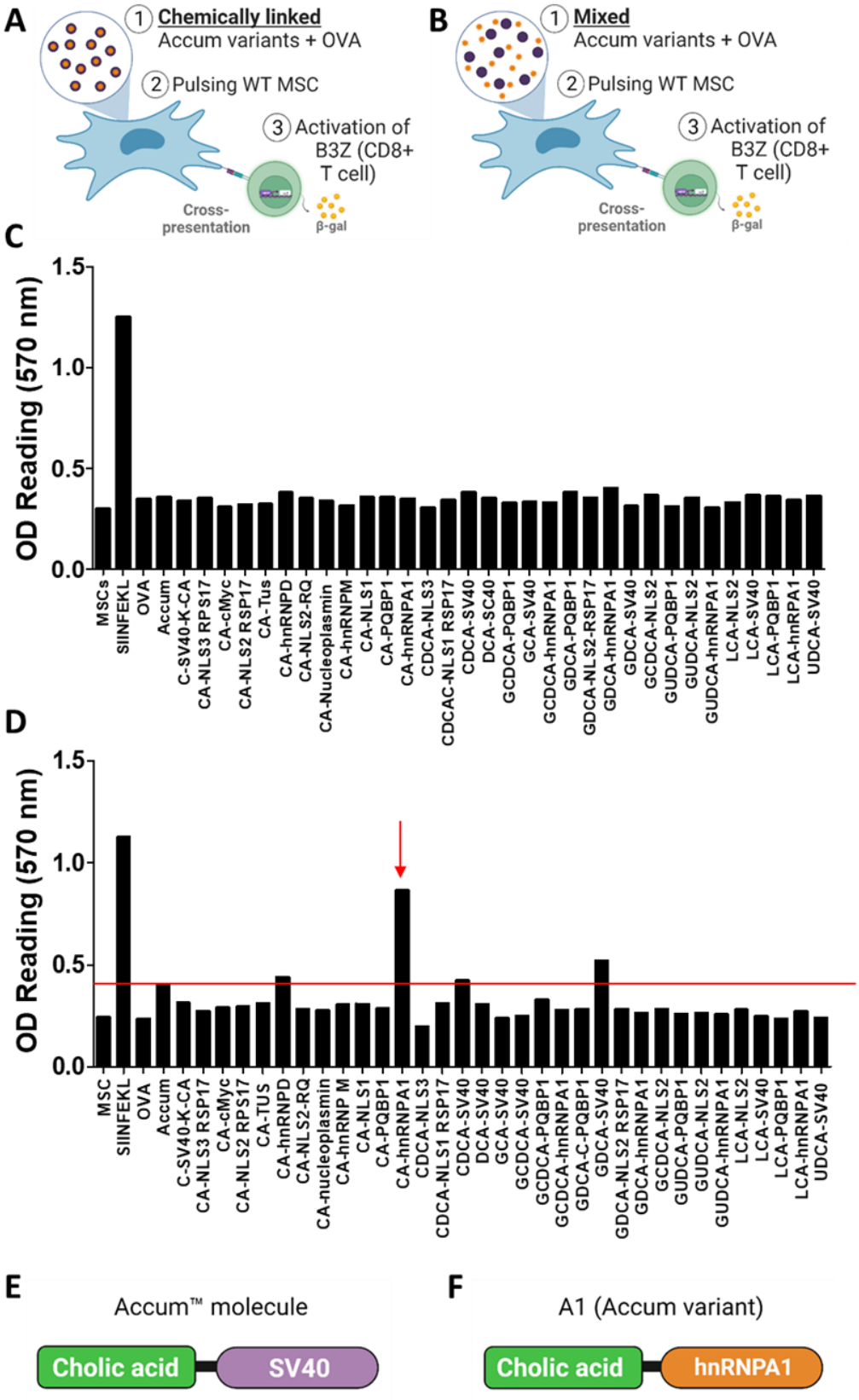
2.7.6. Authors' contributions

MPG designed and conducted most of the *in vitro* assays, analyzed data, and wrote the first draft of the manuscript. NH conducted the transcriptomic analysis. WS, RF, and JPB executed some *in vitro* experiments. JA, DS, SB, and SP contributed to data analysis. MR conceived the study design, supervised the project, conducted *in vivo* assays, and analyzed all collected data. All authors contributed to manuscript editing.

2.7.7. Acknowledgments

We wish to thank all animal technicians from the IRIC animal facility for their kind support regarding some of the *in vivo* experiments.

2.7.8. Figures


A
1 Chemically linked
Accum variants + OVA
2 Pulsing WT MSC
3 Activation of B3Z (CD8+ T cell)
Cross-presentation
β-gal
B
1 Mixed
Accum variants + OVA
2 Pulsing WT MSC
3 Activation of B3Z (CD8+ T cell)
Cross-presentation
β-gal
C
OD Reading (570 nm)
1.5
1.0
0.5
0.0
MSCs
SIINFEKL
OVA
Accum
C-SV40-K-CA
CA-NLS3 RPS17
CA-cMyc
CA-NLS2 RSP17
CA-Tus
CA-hnRNPD
CA-NLS2-RQ
CA-Nucleoplasmin
CA-hnRNPM
CA-NLS1
CA-PQBP1
CA-hnRNPA1
CDCA-NLS3
CDCAC-NLS1 RSP17
CDCA-SV40
DCA-SC40
GCDCA-PQBP1
GCA-SV40
GCDCA-hnRNPA1
GDCA-PQBP1
GDCA-NLS2-RSP17
GDCA-hnRNPA1
GDCA-SV40
GCDCA-NLS2
GUDCA-PQBP1
GUDCA-NLS2
GUDCA-hnRNPA1
LCA-NLS2
LCA-SV40
LCA-PQBP1
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D
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1.5
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MSC
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C-SV40-K-CA
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CA-cMyc
CA-NLS2 RPS17
CA-TUS
CA-hnRNPD
CA-NLS2-RQ
CA-nucleoplasmin
CA-hnRNP M
CA-NLS1
CA-PQBP1
CA-hnRNPA1
CDCA-NLS3
CDCA-NLS1 RSP17
CDCA-SV40
DCA-SV40
GCA-SV40
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GCDCA-PQBP1
GCDCA-hnRNPA1
GDCA-C-PQBP1
GDCA-SV40
GDCA-NLS2 RSP17
GDCA-hnRNPA1
GCDCA-NLS2
GUDCA-PQBP1
GUDCA-NLS2
GUDCA-hnRNPA1
LCA-NLS2
LCA-SV40
LCA-PQBP1
LCA-hnRNPA1
UDCA-SV40
E
Accum™ molecule
Cholic acid
SV40
F
A1 (Accum variant)
Cholic acid
hnRNPA1

Figure 17 - Screening Accum variants capable of inducing cross-presentation in MSCs.

A) Schematic diagram of the antigen cross-presentation assay in which MSC are pulsed using different AccumTM-linked OVA variants. Treated MSCs are co-cultured with B3Z, a T cell hybridoma expressing a TCR recognizing the OVA-derived SIINFEKL peptide in the context of MHC I. B3Z express beta-galactosidase (lacZ) driven by NF-AT elements activated after recognition and activation. **B)** Same as in (A), except that the Accum variants were admixed**. C)** Antigen presentation assay screening the AccumTM-linked OVA constructs. SIINFEKL is used as a positive technical control for B3Z, MSCs (untreated), OVA, and Accum are used as negative controls. **D)** Same as in (C), except that the antigen presentation assay is screening the Accum variants admixed with OVA. **E)** Cartoon structure of the original Accum molecule, comprising Cholic acid (CA) as the bile acid module and SV40 as the NLS moiety**. F)** Cartoon structure of the A1 Accum variant, which maintains the CA, but is linked to the NLS hnRNPA1.

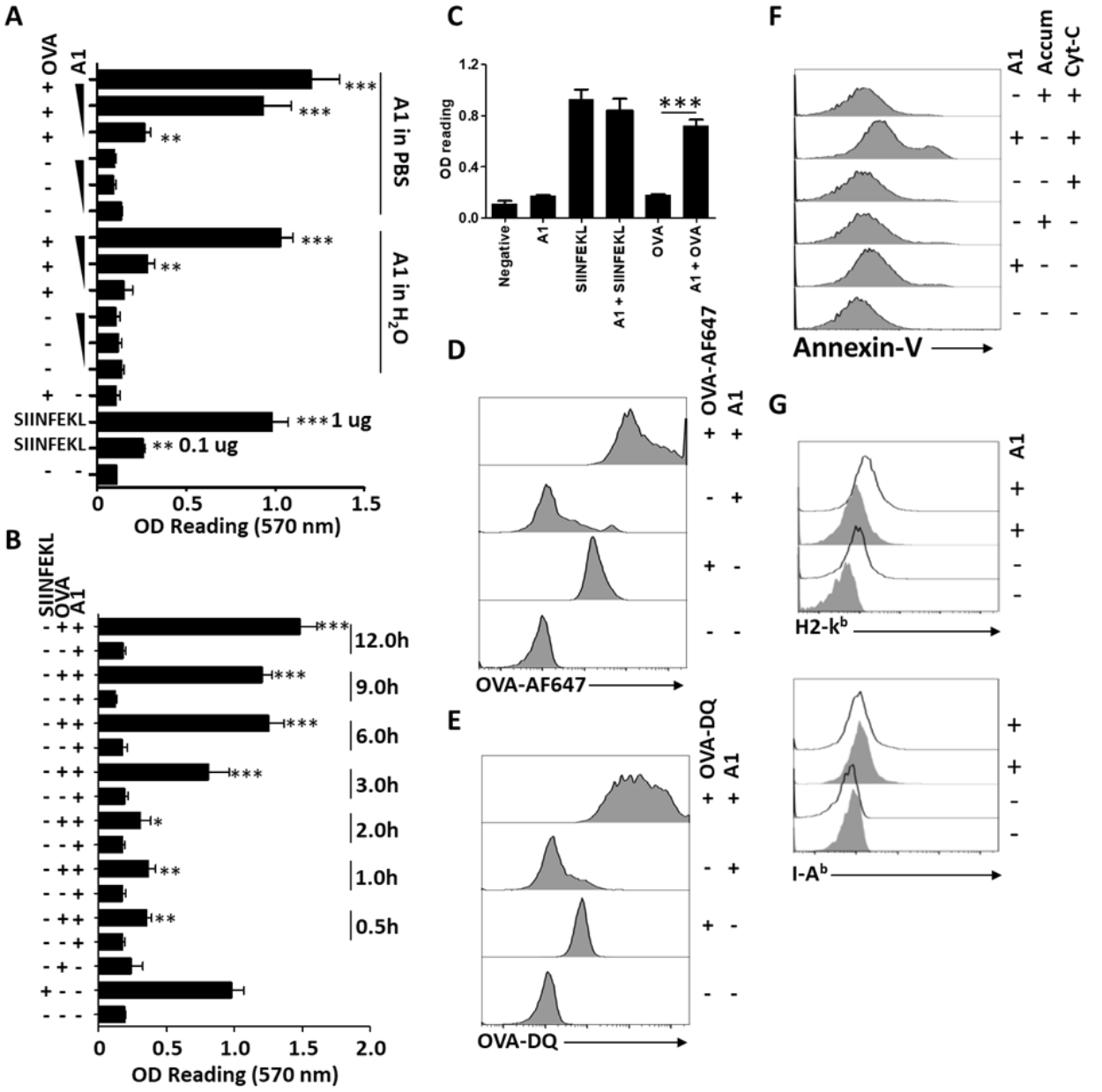


Figure 18 - Characterizing the cross-presentation capacity of the A1 variant.

A) Antigen cross-presentation assay conducted using A1 diluted in PBS or water to compare the impact of A1 formulations in the capacity to induce cross-presentation. **B)** Antigen cross-presentation assay conducted using different pulsing time points to optimize the treatment period. **C)** Antigen cross-presentation assay conducted using A1 admixed with SIINFEKL to assess the impact on antigen presentation and A1 admixed with OVA to confirm induction of cross-presentation. **D)** Evaluating the effect of A1 on antigen uptake by MSCs using OVA-AF647. **E)**

Assessing the effect of A1 on antigen processing by MSCs using OVA-DQ. **F)** Assessing the endosomal damaging properties of A1 on MSCs co-treated with Cyt-C. After endocytosis, Cyt-C can induce apoptosis if reaching the cytosol upon endosomal break. Annexin-V staining was used to analyze changes in cell death. **G)** Representative flow-cytometry analysis of H2-Kb (top panel) and I-Ab (lower panel). Gray histograms show isotype controls, whereas test-stained samples are in white. For panels A-C, n=4/group with *P<0.05, **P<0.01 and ***P<0.001.

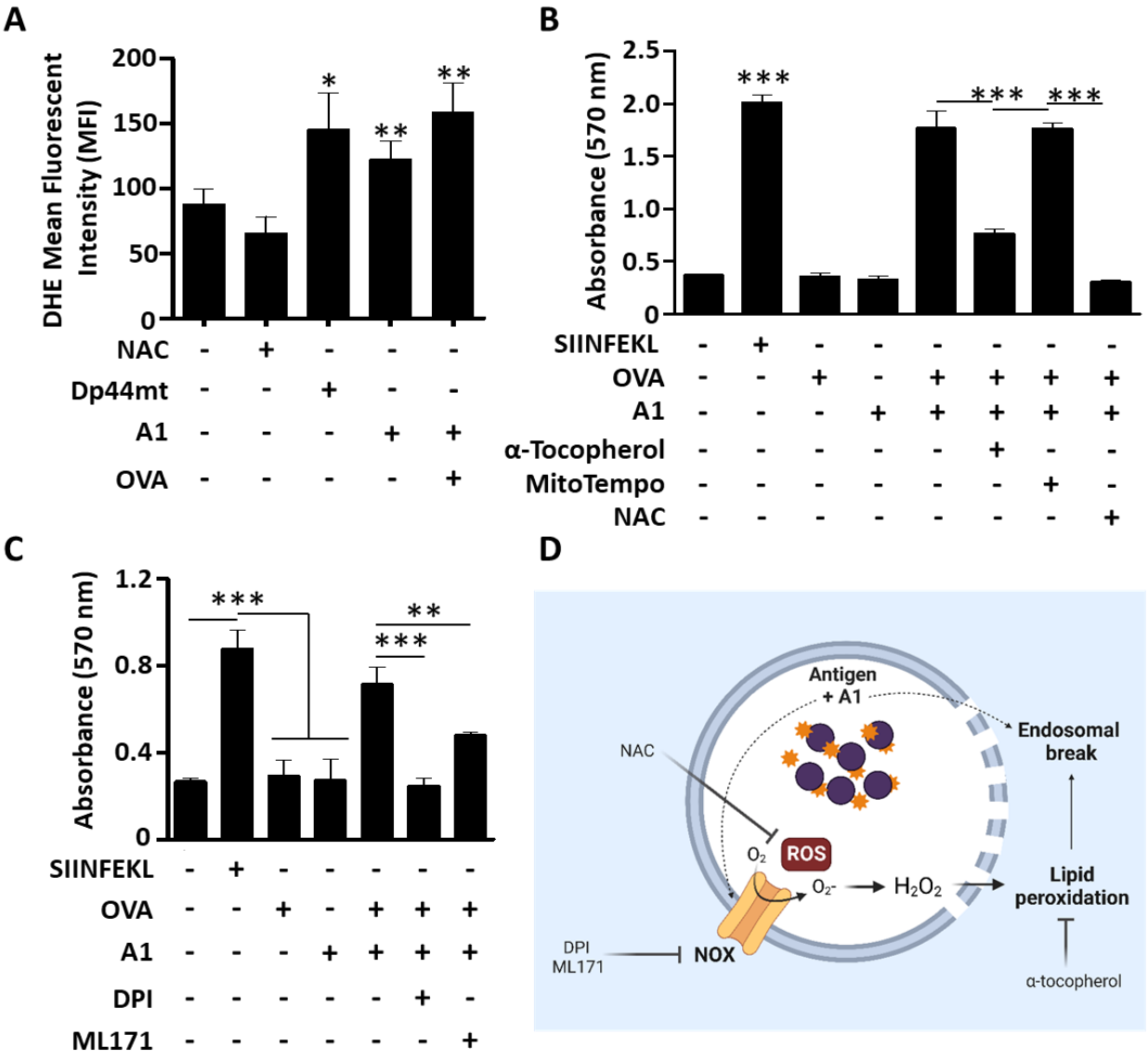


Figure 19 - The A1-induced antigen cross-presentation capacity increases and requires ROS production.

A) Flow-cytometry assessment of ROS production by MSCs in response to A1 using the dye DHE. NAC was used as the negative control whereas Dp44mt was used as a positive control**. B)** Antigen cross-presentation assay performed to investigate the effect of neutralizing ROS on A1-related activity. **C)** Same as (B) except that it was conducted using NOX inhibitors. **D)** A graphical abstract summarizing the mechanistic insights of A1-driven endosomal disruption obtained from the data above. For panels A-C, n=4/group with *P<0.05, **P<0.01 and ***P<0.001.

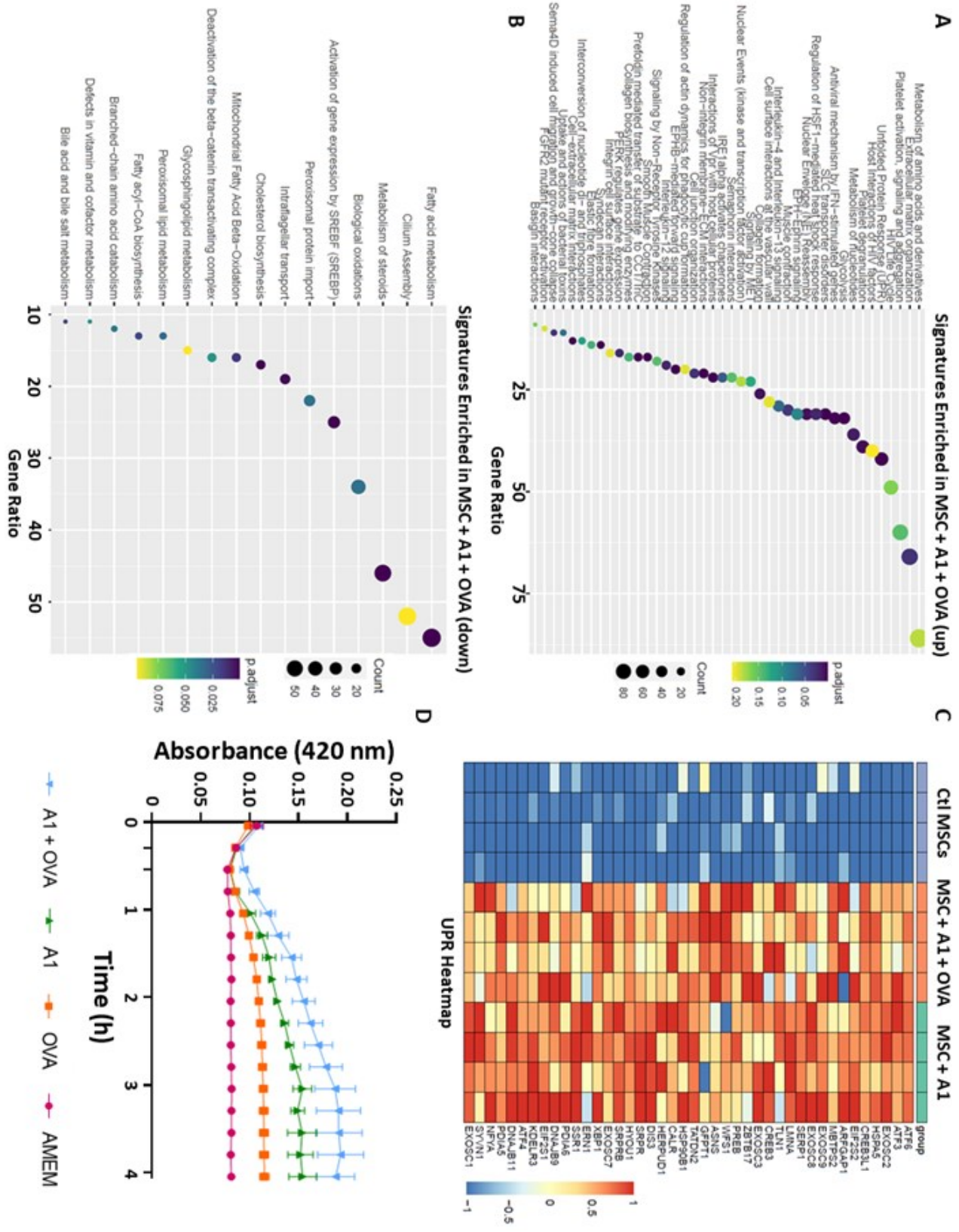


Figure 20 - Molecular characterization of the ARMs.

List of top Reactome pathways that are enriched for both up-regulated **(A)** and down-regulated **(B)** genes in the A1 treated group versus control MSCs. The circle's color corresponds to adjusted p-values; the size of the circles corresponds to the ratio count of genes in the tested set. **C)** A representative unfolded-protein response (UPR) heatmap displaying the genes that contribute the most to the pathway enrichment and modulated in response to A1 treatment (FDR < 5%); gene expression is scaled between -1 and +1, followed by color code indicated in the figure. **D)** A turbidity assay to evaluate the A1 capacity to form protein aggregation when mixed with the OVA protein. The groups and controls are depicted according to the color code.

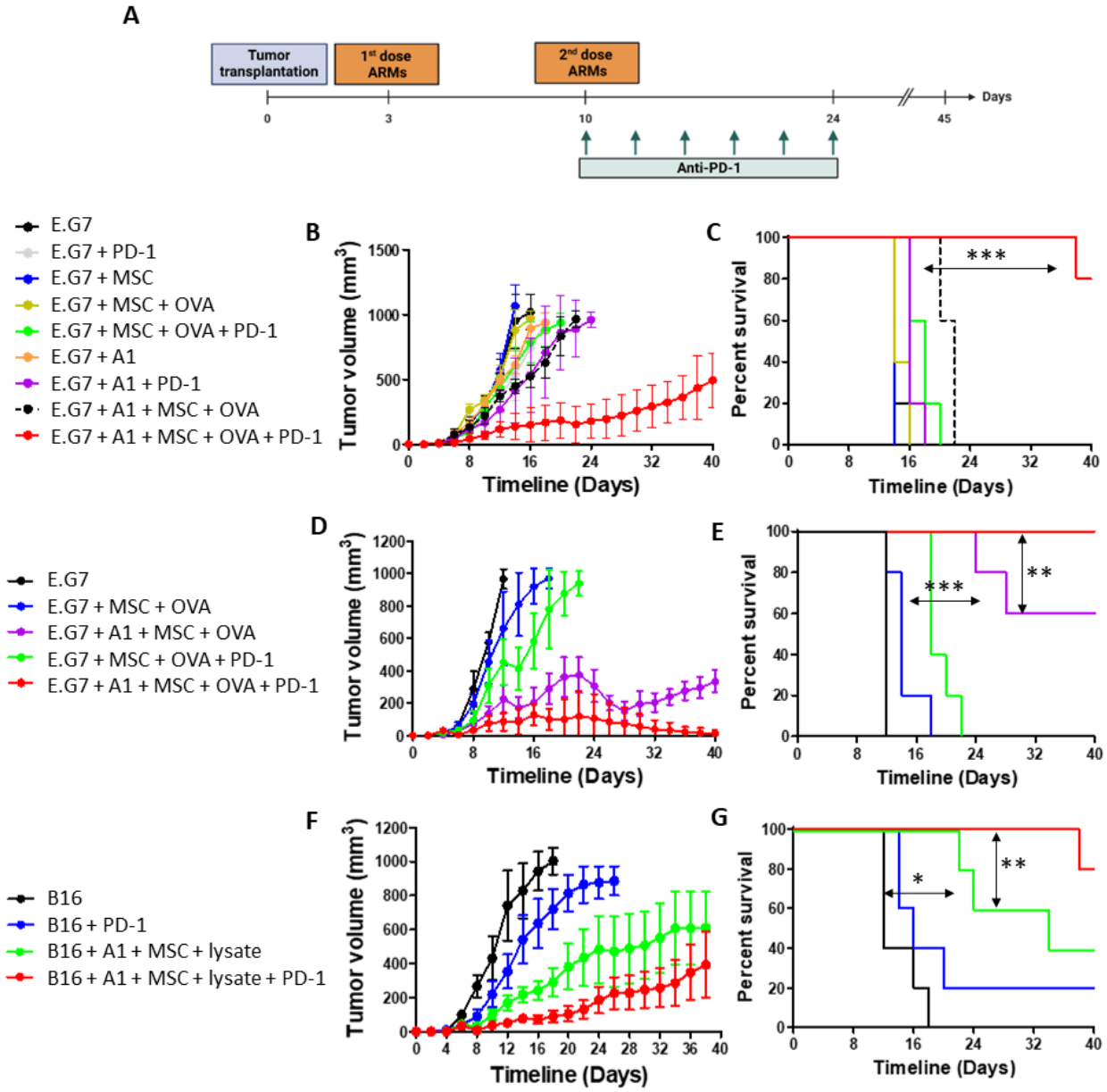


Figure 21 - Therapeutic vaccination using the ARM vaccine can induce regression of established tumors.

A) Experimental design represented by the timeline of the ARM therapeutic vaccination as a monotherapy or combination treatment approach with the ICI anti-PD-1. **B)** Evaluation of E.G7 tumor growth in response to syngeneic ARM vaccination (MSCs were obtained from and administered to C57BL/6 mice). The group conditions are indicated by the color code. E.G7 is an OVA-expressing T cell lymphoma, and the protein OVA is used as the stimulating antigen. **C)**

Kaplan-Meier survival curve of the experiment shown in panel B. **D)** Evaluation of E.G7 tumor growth in response to allogeneic ARM vaccination (MSCs were obtained from BALB/c mice and administered to tumor-bearing C57BL/6 mice), using OVA as the stimulating antigen **E)** Kaplan-Meier survival curve of the experiment shown in panel D. **F)** Evaluation of B116 tumor growth in response to allogeneic ARM vaccination using B16 lysate as stimulating antigen. B16 is a melanoma cell line, and the protein lysate of B16 cells was used as the pulsing antigen. **G)** Kaplan-Meier survival curve of the experiment shown in panel F. For panels B-E, n=5/group. PD-1 refers to the antibody anti-PD-1.

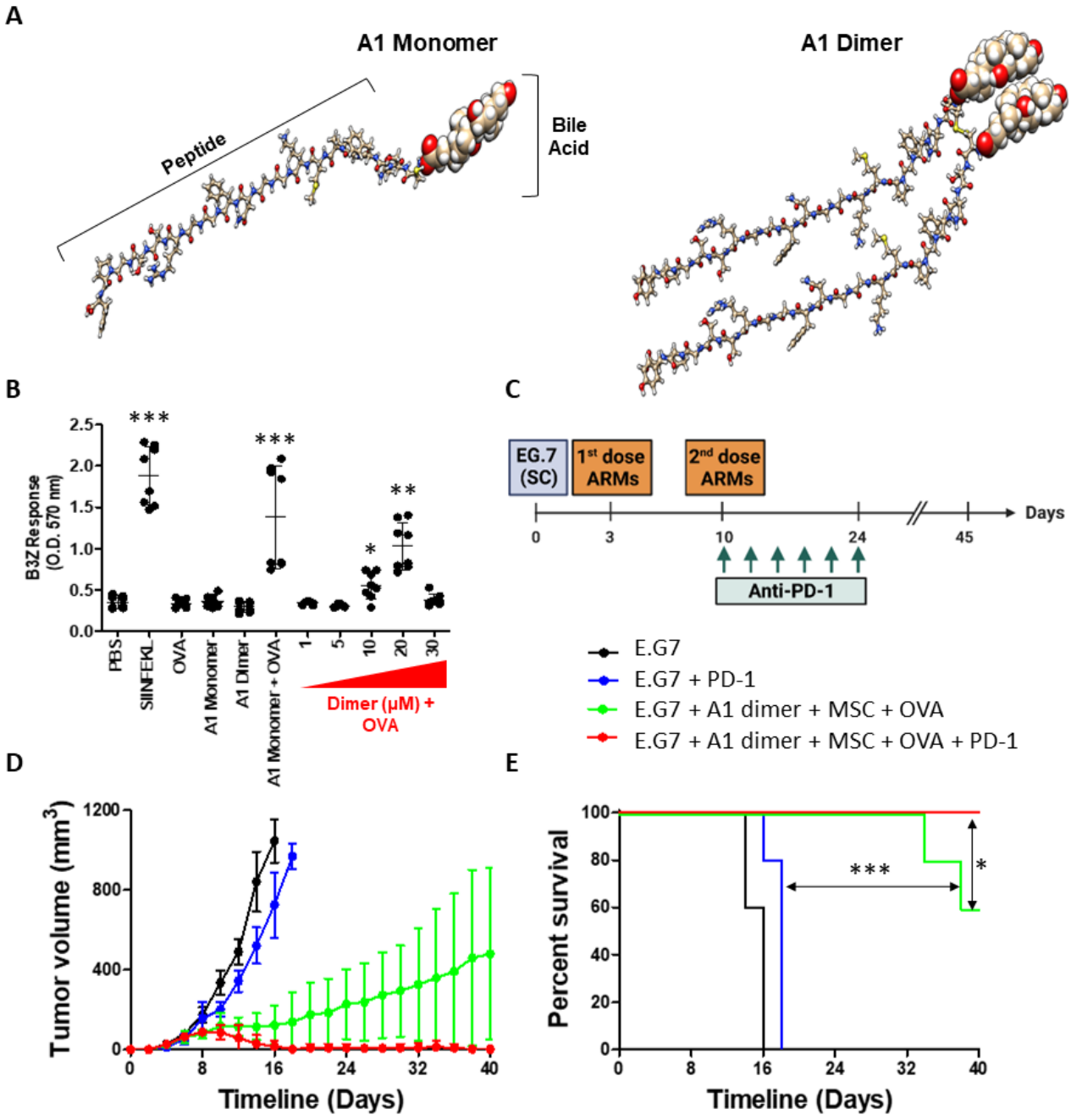


Figure 22 - Optimizing the therapeutic potency of the ARM vaccine using a dimeric form of A1.

A) Structure of the A1 monomer (left) versus dimer (right). **B)** Comparing the antigen cross-presentation capacities of ARM cells generated in response to the monomer versus different concentrations of dimer form of A1. **C)** Experimental design used for the therapeutic vaccination

used to test the ARM cells generated using the A1 dimer, as a monotherapy or combination treatment approach with the ICI anti-PD-1. **D)** EG.7, an OVA-expressing T cell lymphoma, tumor growth in response to allogeneic ARM vaccination. The vaccination consisted of BALB/c MSCs pulsed with A1 dimer and the antigen OVA, administered to E.G7-bearing C57BL/6 mice. **E)** Kaplan-Meier survival curve of the experiment shown in panel G. For panels B, n=6/group. For panels D and E, n=10/group with *P<0.05, **P<0.01 and ***P<0.001. PD-1 refers to the antibody anti-PD-1.

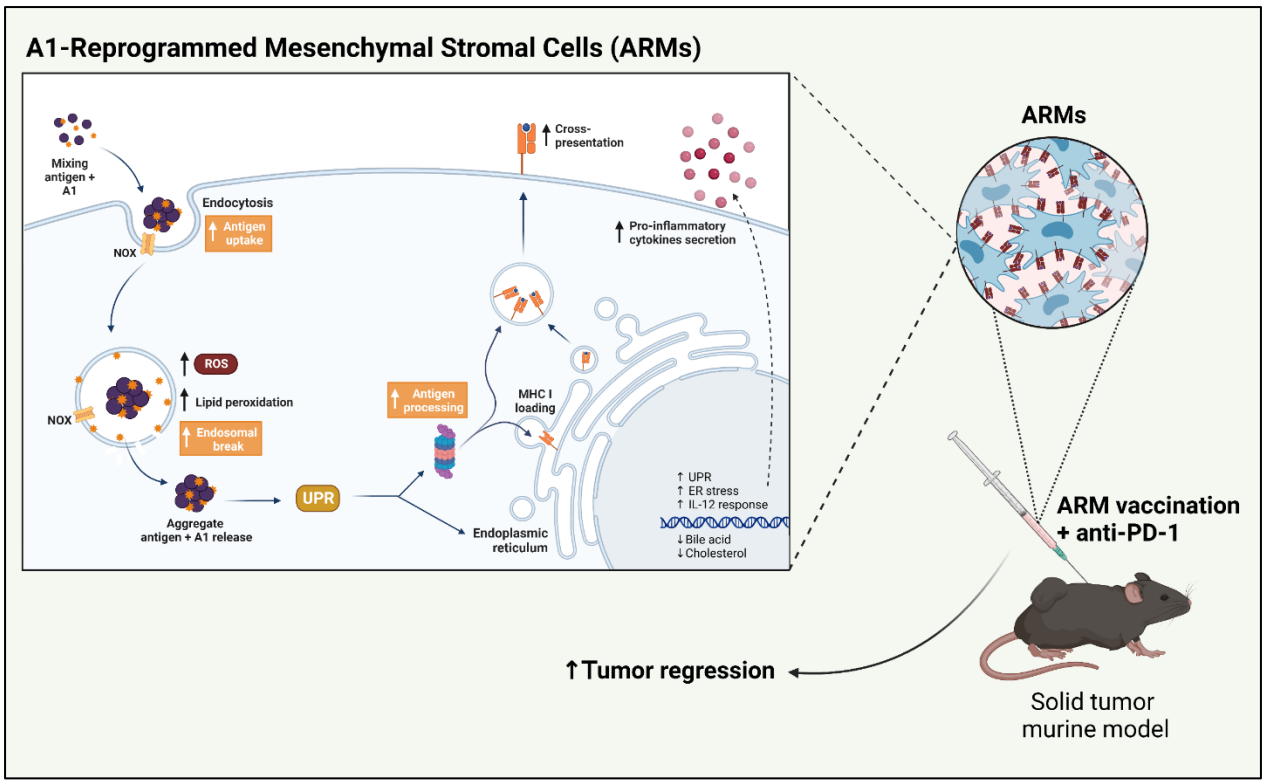
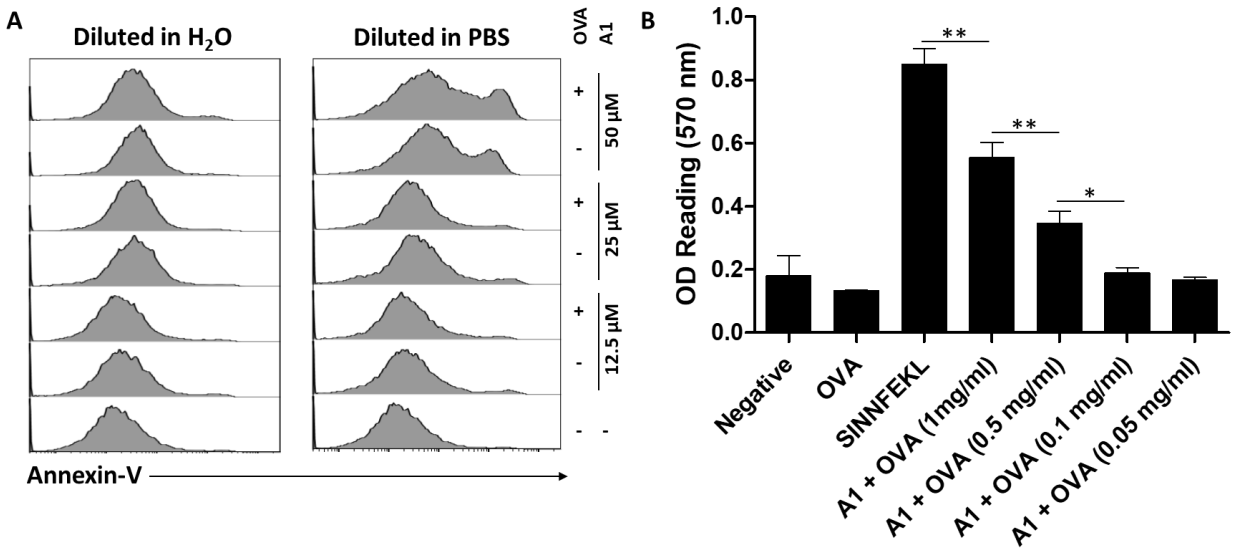


Figure 23 - Graphical abstract suggesting the mode of action of ARM cells.

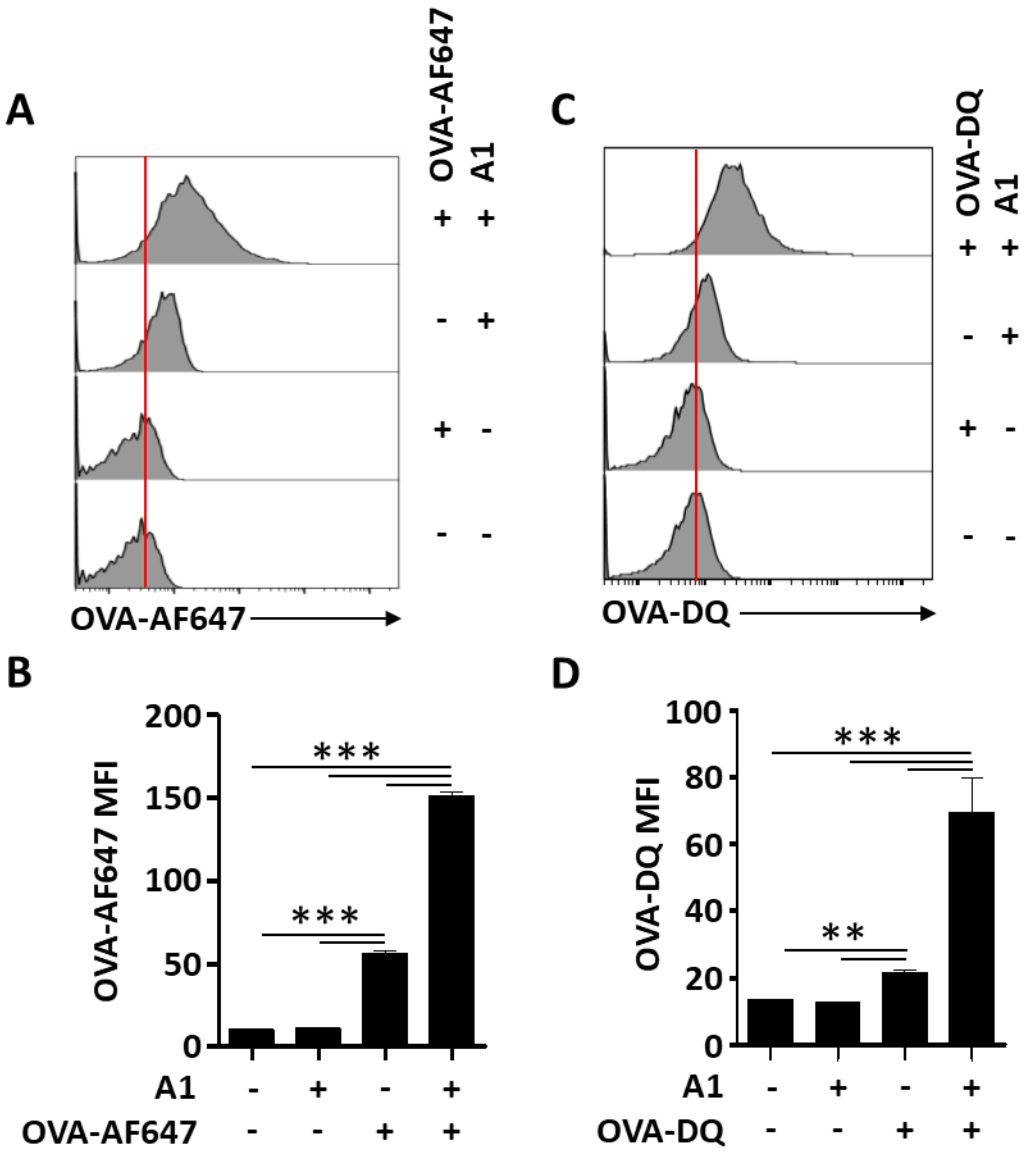
Upon treating MSCs with an A1/antigen mix, A1 induces protein aggregation, which is captured and entrapped into endosomes. At that point, NOX is activated and produces ROS, which causes lipid peroxidation, disrupting the membrane, therefore, releasing the cargo into the cytosol. Protein aggregates are then sensed by the cells triggering a UPR response, which enhances the proteasomal activity and degradation of antigens. The generated peptides are then loaded onto MHC I molecules and presented on the cell surface. A1 treatment also induced A1 downregulation of bile acid and cholesterol metabolism, while it upregulates, besides the UPR, ER stress, and production of pro-inflammatory cytokines including IL-12. Administration of these generated ARM cells prime potent antitumoral activity against solid tumor models.

2.7.9. Supplementary figures



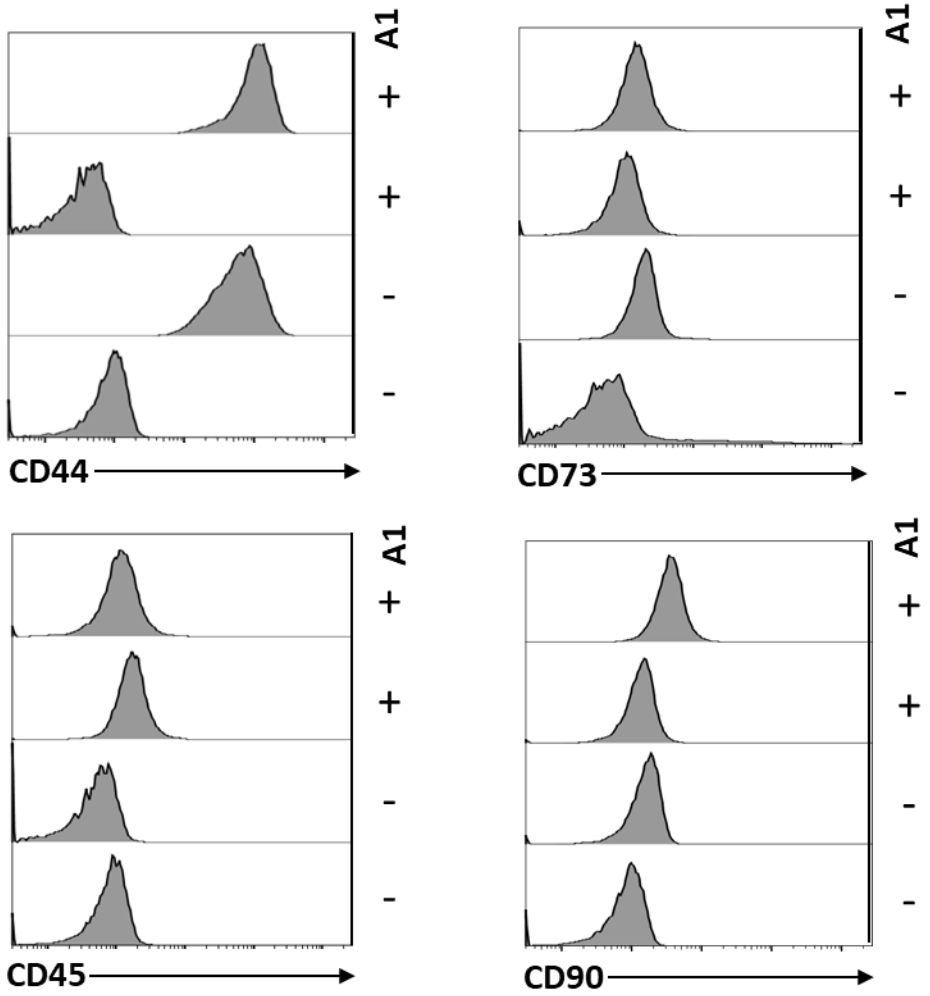
Supplementary Figure 1 - Optimizing the A1 treatment conditions to tune cross-presentation induction in MSCs.

A) Flow-cytometry assessment of apoptosis in response to ascending A1 doses diluted in water versus PBS. **B)** Antigen cross-presentation assay conducted using different OVA concentrations to find the minimal concentration needed to induce cross-presentation in MSCs.



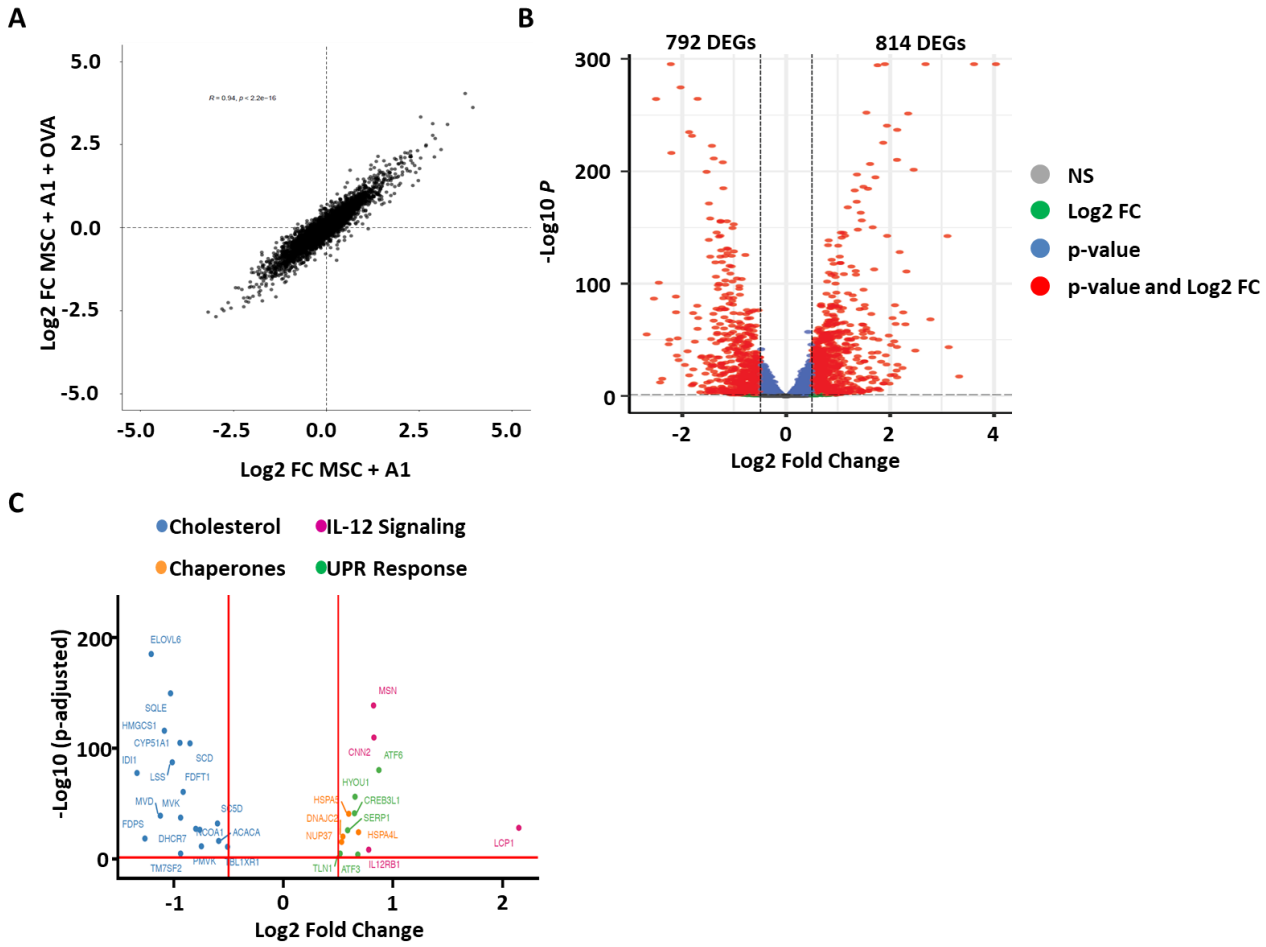
Supplementary Figure 2 - Validating the antigen cross-presentation properties of A1 on human MSCs.

A) Representative flow-cytometry analysis of OVA uptake by A1-treated human MSCs. B) Signal quantification of the results presented in panel A. **C)** Representative flow-cytometry analysis of OVA processing by A1-treated human MSCs. **D)** Signal quantification of the results presented in panel C.



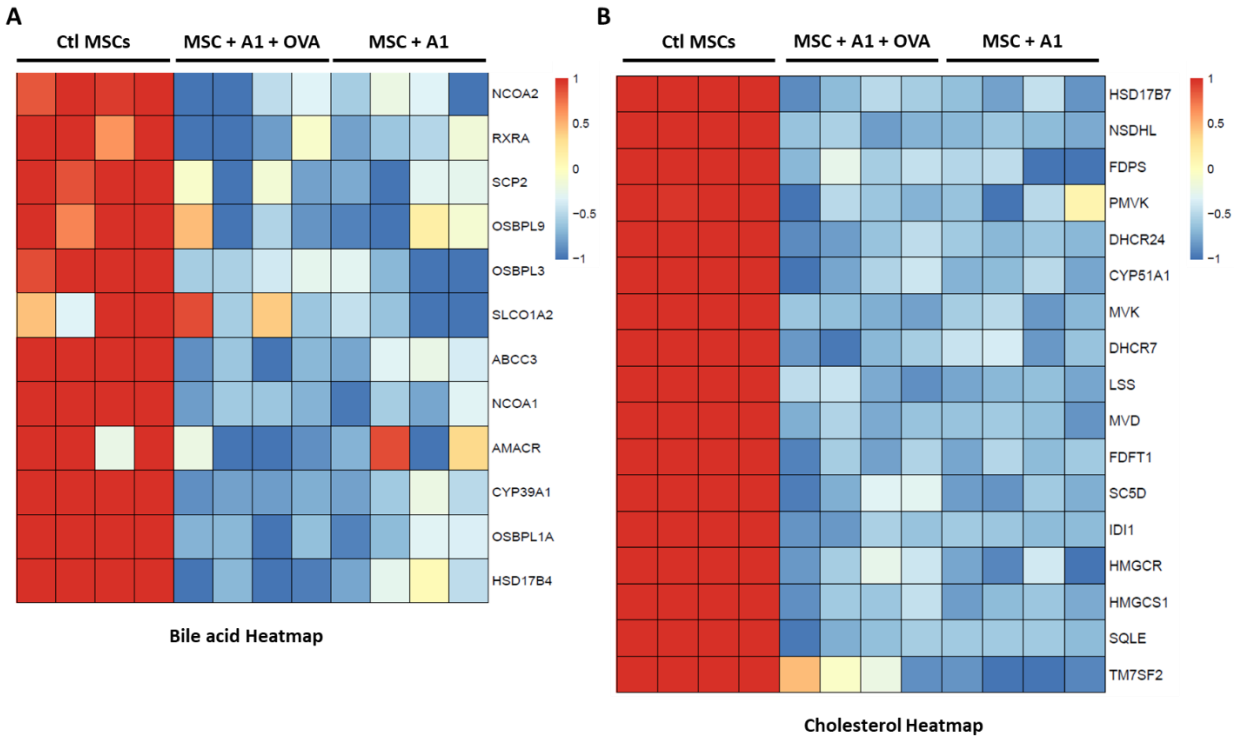
Supplementary Figure 3 - Phenotype characterization of A1-treated MSCs.

Control versus A1-treated MSCs were stained by flow cytometry to assess the expression of CD44, CD45, CD73, and CD90. For each surface marker, from the bottom to the top, the first and third rows comprise the isotype, and the second and forth row comprise the targeted surface marker.



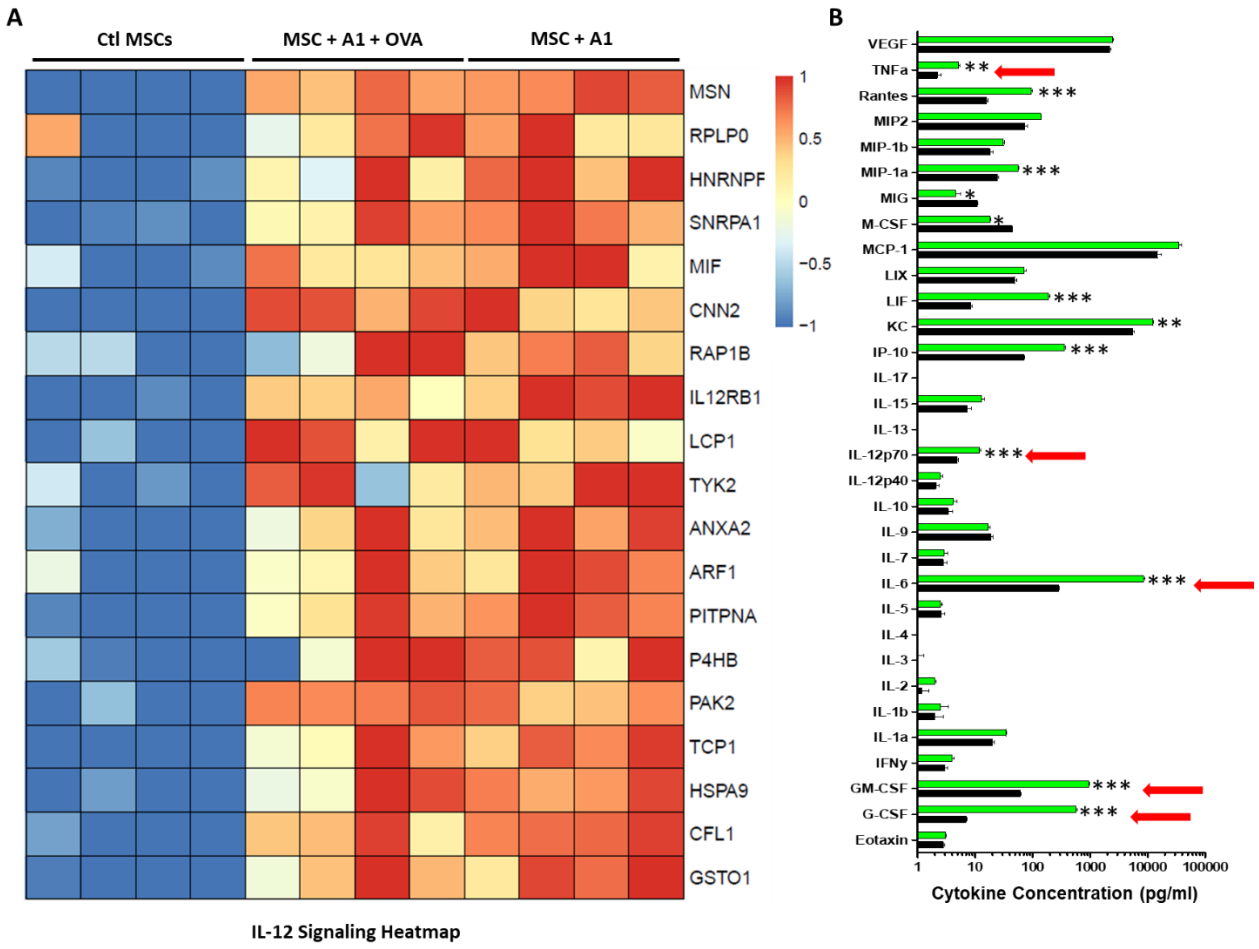
Supplementary Figure 4 - Characterizing the molecular response of A1-treated MSCs.

A) Correlation plot showing Spearman's rank correlation coefficient of DEGs (log2 fold changes) shows the significant similarity of gene expression patterns between the A1 and A1+ OVA groups. **B)** Volcano plot representing differentially expressed genes in response to A1. **C)** Volcano plot depicting some important biological processes modulated in MSCs in response to A1. All genes from corresponding reactome analyses and showing a log2FC greater or equal to 0.5 are labeled for further investigation.



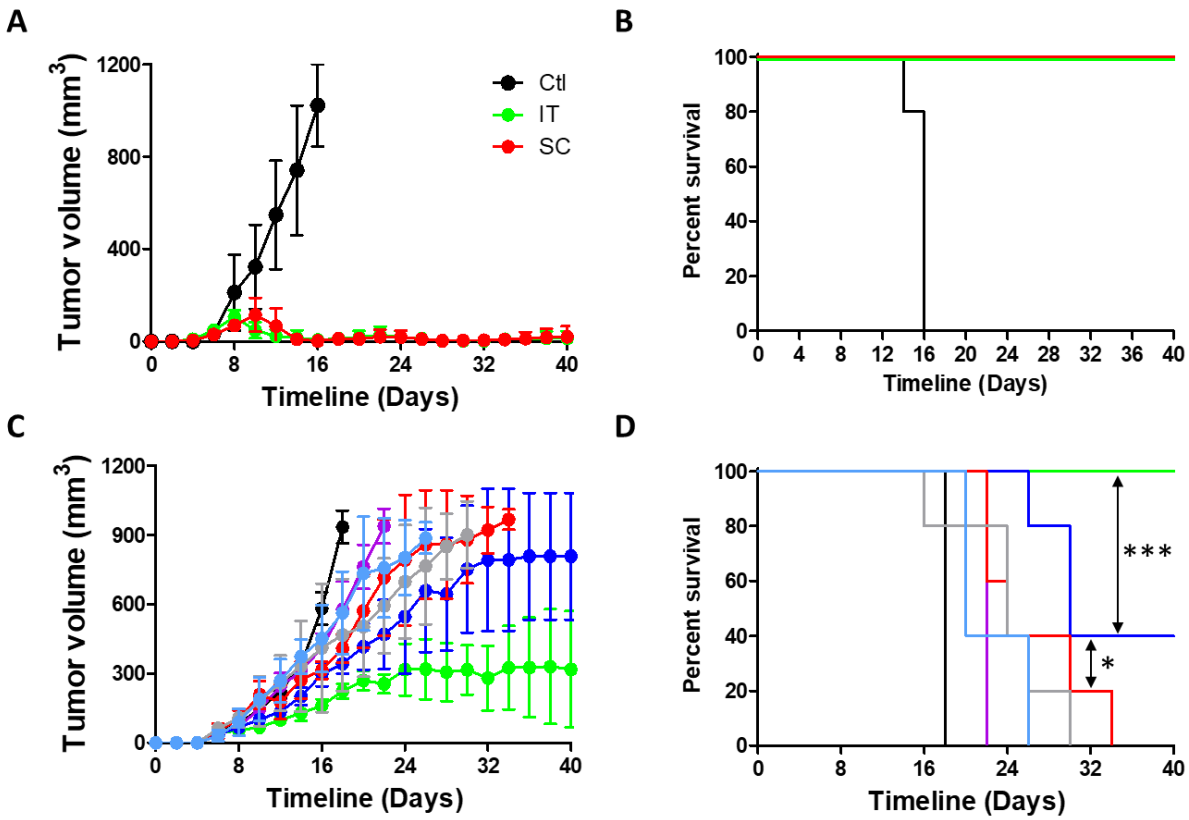
Supplementary Figure 5 – Downregulated cellular processes highlighted during characterization of the molecular response of A1-treated MSCs.

A) Heatmap depicting genes related to bile acid metabolism that are downregulated by A1 treatment. **B)** Same as in A, except that represents genes involved in cholesterol biosynthesis. Genes showed in heatmaps A and B were also contributing to significant statistics from both differential expression and pathway analyses (FDR < 5%).



Supplementary Figure 6 - Upregulated genes related to IL-12 signaling and cytokine secretion profile.

A) The IL-12 response heatmap depicting genes that are modulated by A1 treatment. The same description is in Supplementary Figure 4, and gene expression is scaled from -1 to 1 range. **B)** Luminex analysis of various cytokines in response to A1 treatment (in green). The red arrows highlighted pro-inflammatory cytokines of interest with significant changes. For this panel, n=6/group.



Supplementary Figure 7 - Comparing the routing and dosing of the allogeneic ARM vaccine.

A) EG.7 tumor growth in response to IT or SC allogeneic ARM vaccination pulsed with OVA and co-delivered with anti-PD-1. The black line represents EG.7 growth (control group). **B)** Kaplan-Meier survival curve of the experiment shown in panel A. **C)** EG.7 tumor growth in response to OVA-pulsed allogeneic ARM delivered at different doses. The black line represents EG.7 growth (control group). As for the doses: Green (5 x 10^5), dark blue (2.5 x 10^5), red (1 x 10^5), gray (5 x 10^4), light blue (1 x 10^4), purple (5 x 10^3). D) Kaplan-Meier survival curve of the experiment shown in panel C. For this experiment, n=5/group.

CHAPTER 3

3.1. Discussion

As the field of immunotherapy expands, new challenges emerge in the production of effective treatments. While DC vaccination shows potential for cancer therapy (163), the clinical application of mature DCs is hindered by their limited availability within the human body. Instead, *ex vivo* monocyte-derived-DCs are used because of acquisition and expansion feasibility (179). However, the variability in monocyte stimulation protocols and the *ex vivo* maturation process, which cannot reproduce entirely the cellular maturation process that occurs *in vivo*, pose obstacles in the manufacturing process, and limit the ultimate efficacy of these treatments (65,180). Hence, our study proposes an alternative vaccine platform utilizing ARMs with cross-presentation capabilities to address manufacturing gaps. We demonstrate that ARMs synergize with immune checkpoint inhibitors, leading to tumor regression and increased survival rates, offering a compelling strategy for cancer treatment.

MSCs offer several manufacturing advantages. They can be acquired from various sources, have a favorable safety profile, and their generation and expansion protocol are simple, resulting in a high cell yield(86). These characteristics have the potential to overcome manufacturing hurdles faced by DC therapy. Although MSCs do not possess inherent cross-presenting capacity, they have the potential to acquire this ability when exposed to appropriate stimuli (69,181), owing to their remarkable plasticity. The present study explores a pharmacological strategy to stimulate cross-presenting capacity in MSCs, enabling their utilization as a vaccine platform. The approach employed in this study was inspired by the advancing knowledge of APCs and T-cell activation (33,61). Specialized subsets of DCs possess specific activation pathways that make them highly efficient in their role. Accordingly, we targeted a crucial step in cross-presentation, which involves the transport of antigens from the endosome to the cytosol (115). This process reduces nonspecific antigen degradation that could occur during endosomal maturation, which could result in the loss of immunogenic epitopes. Moreover, it facilitates antigen access to the

proteasomal machinery, leading to the generation of more immunogenic peptides that can subsequently be presented by MHC I molecules (123)

We screened a library of molecules rationally designed to disrupt the endosome. We identified a variant that, by mixing with an antigen, effectively broke the endosome and induced cross-presentation in MSCs, as confirmed by antigen presentation assays. These results were both unexpected and encouraging, as the original method of Accum technology involved and required a chemical link with the drug/antigen of interest (137), introducing complexities that increased the time and cost involved in molecule production. Furthermore, the development of a dimeric form of the A1 molecule enabled the reduction of “contaminants” (byproducts) in the synthesis process, as reported by the manufacturer, and has improved the molecule's stability. Treating MSCs with the dimeric form requires a lower stimulating concentration, which in turn reduces production costs, all while maintaining the capacity to induce cross-presentation in MSCs. These discoveries offer a more straightforward and cost-effective approach, facilitating process standardization.

Mechanistic investigations have revealed that the molecule A1 not only disrupts endosomes but also triggers other critical events for cross-presentation. These include increased antigen uptake, processing, and production of ROS. Further exploration of ROS production unveiled its crucial role in cross-presentation. In contrast with previous studies conducted by our research group, ROS was found not to be derived from the mitochondria. This led us to investigate a source known to be associated with cross-presentation: NOX, which is activated in cross-presenting DCs (120). Activation of NOX generates ROS within the endosome, leading to two crucial events: preventing the acidification of the endosome, which would activate nonspecific proteases, and inducing lipid peroxidation. Lipid peroxidation is an essential process for efficient cross-presentation in DCs, and it involves a cascade of lipid degradation triggered by the conversion of ROS into hydrogen peroxide, ultimately leading to endosomal rupture (133). Remarkably, our findings demonstrated that NOX activity was critical in A1-induced cross-presentation, suggesting that NOX might be the source of ROS increase. Moreover, blocking lipid peroxidation resulted in a decrease in the cross-presentation capacity of A1-treated MSCs.

The discovery of these mechanisms has raised questions about the direct impact of A1 on endosomal rupture, the connection between NOX activity and A1, and whether lipid peroxidation induced by ROS is the sole inducer of endosome disruption or acts synergistically with A1, which would induce a direct membrane perturbation. Further investigations are required to address these inquiries and gain a deeper understanding of the interplay between A1, NOX, and lipid peroxidation in cross-presentation. Another intriguing finding was that Accum could not break the endosome in MSCs when only admixed to the antigen, unlike A1, which demonstrated the ability to do so. This observation suggests that Accum's inability to induce cross-presentation might be related to its failure in disrupting the endosome in MSCs when not linked to an antigen, while its variant, A1, exhibits the capacity.

Transcriptomic analysis revealed that A1 triggers the upregulation of HSF1 and UPR, key players in cellular stress response and re-establishment of protein homeostasis. A disbalance in protein homeostasis can be caused by unfolded or misfolded proteins, protein overload, and protein aggregation (164). Indeed, the turbidity assay performed suggests the occurrence of aggregation upon A1 addition. The activation of HSF1, a crucial transcription factor that drives the expression of several molecular chaperones (heat shock proteins) and other proteostasis network components (182), corroborates with the hypothesis that A1 induces protein aggregation. Besides it, protein aggregates can also induce ER stress, which can activate the UPR as a rescue and protection mechanism (183).

The UPR responds by activating mechanisms to restore cell homeostasis, and if it fails to do so apoptosis is induced (165). ER stress also crosstalks with the ubiquitin-proteasome system (166), activating the proteasomal machinery to handle disbalanced proteins, which can ultimately lead to a downstream effect of stimulation of antigen presentation machinery (7). Hence, we propose that this relation between UPR-ER stress-proteasome-antigen presentation contributes with the cross-presentation capacity of A1-induced ARMs. The ER activation also plays another role in protein homeostasis related to antigen presentation. As observed in cross-presenting DCs, constitutive activation of ER genes is pivotal during cell activation and their role as APCs, they maintain equilibrium during high demands of cytokines and MHC expression (167,168,184). While we did not detect an upregulation in MHC expression, our examination of cytokines revealed

heightened cytokine secretion by ARMs. This may indicate a potential link between ER signaling and the antigen-presenting abilities of ARMs, resembling those of APCs. Changes in the secretome pattern of ARMs, as observed by increase in pro-inflammatory cytokines secretion, suggest their potential to create a pro-inflammatory environment, which could stimulate the activation of effector cells (7,8) in addition to antigen presentation, supporting ARMs function acquisition as APC-like cells.

Transcriptomic analysis also detected downregulation of lipid metabolism-related pathways. The relation among ER stress, UPR, and lipid metabolism has been explored in the perspective of upregulation of lipogenesis induced by UPR to enable ER membrane extension (169,170). But in contrast to that, studies also demonstrated that UPR can induce a different outcome, by activating sensors that ultimately promotes lipolysis by lipid enzymes alteration (170,171). In addition to that, it is possible that A1-induced lipid peroxidation could lead to cytosolic lipid accumulation, causing a state of lipid overload that activates the downregulation of fatty acid metabolism. The structure of the A1 molecule, containing cholic acid, may also play a role in reducing cholesterol biosynthesis as a response to lipid overload since cholesterol is a precursor of cholic acid, potentially resulting in the impact observed in bile acid metabolism. As a result, various cellular events may have induced lipid-related pathways to reduce lipid accumulation and restore cellular homeostasis.

The evaluation of ARMs as a cell-based therapeutic vaccine against solid tumors resulted in encouraging outcomes. In syngeneic settings, ARMs used as monotherapy did not generate a significant alleviation in tumor burden. However, when combined with the immune-checkpoint inhibitor anti-PD-1, they not only delayed tumor growth but also increased overall survival. Although immune-checkpoint inhibitors have gotten attention for efficacy in some settings, their use faces tumor resistance in specific cancer types and populations (185), as any known antitumoral solo therapy approach. Therefore, it is imperative for researchers to consider the importance of associating therapies that target different cancer hallmarks simultaneously to cope with the ways out of tumor resistance (186,187).

The efficacy of ARMs in an allogeneic setting was also evaluated, aiming to develop an off-the-shelf cell vaccine. Remarkably, as a single therapy, ARMs induced temporary tumor regression and increased overall survival. When combined with anti-PDI-1, they impressively induced total tumor regression followed by complete survival in mice. These findings suggest that antigen presentation, along with the suppression of T-cell inhibitory factors, enabled an effective response against the solid tumor model. However, direct activation of CD8+ cytotoxic T cells demands three simultaneous signals: antigen presentation by MHC I, pro-inflammatory cytokines, and costimulatory receptors (8). Despite harboring two of the necessary signals through stimulation by A1, when evaluated for their immunophenotype, the ARMs did not exhibit an increase in the surface expression of co-activators. Therefore, despite the features stimulated by A1, it is unlikely that ARMs directly activate cytotoxic T cells.

This instigates a highly debated topic within the field of MSC-based cell therapy: their actual mechanism of action (188). Initially, the therapeutic function of MSCs as immunosuppressants was attributed to secretion (189,190). However, studies have shown that MSCs apoptosis is required for their effect, even in the absence of host cytotoxic or alloreactive cells (173,174). Potentially, macrophages handle apoptotic ARMs through efferocytosis and are likely responsible for activating T cells, or ARMs could also be processed by DCs, which could be responsible for TCD8 activation. As we face the first of its kind, the nature of its therapeutic effects demands further investigation. Although even ARMs may not directly activate cytotoxic cells, their ability to cross-present makes them effective vehicles for transporting tumor antigens, initiating a cascade of cell activation and eliciting a robust immune response against solid tumors.

The superior response observed in allogeneic vaccination compared to syngeneic may be attributed to more efficient mobilization of macrophages due to allorecognition. Some studies demonstrated that allogeneic settings increase the immunogenicity of cancer vaccines (175,176). Given recent studies highlighting the crucial role of efferocytosis in the therapeutic effects of MSCs, the activation of an anti-tumoral response suggests that ARMs may predominantly attract M1 macrophages. M1 macrophages have the capacity to activate and draw T cells, as opposed to M2 macrophages, which typically foster an immunosuppressive response that would potentially support the tumor microenvironment and cancer progression. Furthermore, compared to the

syngeneic vaccine, the allogeneic vaccine offers manufacturing advantages, positioning ARMs as a potential off-the-shelf vaccine. Utilizing cells from unrelated donors allows for establishing a predefined bank of donor cells, readily available for patient use and applicable on a larger scale (177,178). This removes the barrier of specialized infrastructure and human resources required in treatment locations, in addition to reducing the frequency of invasive interventions (177).

In our melanoma model, the ARM vaccine exhibited significant antitumoral activity when targeting predefined overexpressed antigens and using tumor lysate, which contains a broader range of antigens without prior knowledge or specific targeting. While predefined antigens offer certain advantages for some cancer types, such as ones with shared mutations (191,192), even with the advance of technologies, the process of defining neoepitopes common to various cancer types and patient cohorts remains time-consuming, laborious, and costly, limiting their clinical application (61). In contrast, tumor lysates offer a broader spectrum of tumor antigens, including novel antigen types that are technically challenging to identify and are not included in most neo-epitope pipelines (61). Resected tumor masses can be used to stimulate ARMs, allowing a straightforward stimulation approach, even in cases of recurrence. Besides, studies suggest that autologous tumors are a better source of antigens and that *ex vivo* antigen presenters are more effective than whole tumor cells in inducing systemic tumor regressions (193–195). Therefore, this personalized, yet, simplified off-the-shelf approach, shows potential for customizing therapies for specific patients and cancer varieties while offering manufacturing advantages that could allow scaling up to reach a more significant number of patients.

3.2. Limitations of the study and future directions

Although cross-presentation assays have demonstrated the capacity of A1 to induce the presentation of exogenous antigens and activate CD8 T cells, it is important to note that current models for studying cross-presentation have their limitations, as acknowledged by DC researchers (116). Cross-presentation is typically quantified using indirect methods, such as measuring the activation of TCR in transgenic or hybridoma CD8+ T cells. However, these methods cannot provide a definitive conclusion that T cell activation would occur *in vivo* if they interact with the

studied cross-presenting cells, as critical factors for T cell activation, such as co-stimulation and cytokines, are not adequately represented in these models. Furthermore, these models do not directly confirm that the analyzed cells are indeed cross-presenting proteins. The direct detection of the presence of peptide-MHC I complex can be achieved using specific antibodies, but few groups have succeeded in detecting cross-presentation through this methodology. To address these concerns, future immunopeptidomic studies could be conducted, also allowing the identification of the repertoire of antigens that ARMs present.

Despite unveiling various mechanisms underlying A1 activity, specific gaps that remain unsolved could further explored. For example, investigating whether the capacity of A1 to induce endosomal breaks arises from lipid peroxidation only, or through induction of ceramide formation, or a contribution from both. Some approaches to investigate ceramide formation, such as Liquid Chromatography Mass Spectrometry (LC-MS) and inhibition of lipid peroxidation through inhibitors or knocking out NOX followed by endosomal break assay, for example, could be performed. The knockout of NOX could also clarify contributions from other elements, such as confirming ROS origin and role. The presence of protein aggregates could also be investigated with structural biology approaches, which are more refined, such as X-ray crystallography, NMR spectroscopy, and cryo-electron microscopy (cryo-EM) or dynamic light scattering (DLS). Besides its impact on UPR activation.

The potent tumor regression induced by ARM vaccination and immune-checkpoint inhibitor could also be further assessed within other tumor types. Studies to evaluate ARMs in ovarian cancer and pancreatic cancer are in progress. If recommended and approved by the animal ethics committee, it would also be valuable to consider increasing the sample size per study group. Doing so could help reduce the margin of error and potentially mitigate the significant variability observed under different conditions. Additionally, optimization of the tumor lysate process holds promise for optimizing ARM efficiency. Given its clinical implications, validating the use of A1 in human MSCs through further investigation represents a crucial and potential avenue for future research.

3.3. Conclusion

The development of cancer vaccines has experienced ups and downs over time, with initial enthusiasm dampened by unimpressive clinical outcomes and the rise of alternative therapies. However, recent advances in our understanding of cancer biology, the tumor microenvironment, and the potential for combination therapies with other immunotherapies have reinvigorated interest in cancer vaccines. CAR-T cells required decades of troubleshooting and refinement to evolve into an efficient technology suitable for clinical use, bringing substantial improvements in patient lives. This transformation was made possible due to the solid rationale behind their development, new discoveries in the biology of cancer and the immune system, and the persistent efforts of scientists in addressing challenges. In a similar manner, cancer vaccines have exhibited encouraging preclinical data and are grounded in a strong rationale, which supports the importance of further exploring their potential.

In this study, we proposed a novel cancer vaccine utilizing a well-characterized and clinically tested cell type, mesenchymal stromal cells. Leveraging the manufacturing benefits of MSCs, these cells serve as a promising cell-based platform. Additionally, the A1 molecule derived from the Accum technology offers a simple method to reprogram MSCs into potent cross-presenting cells capable of carrying antigens. As our understanding of immunology deepens, the significance of proper tumor cell recognition and effector activation remains a critical foundation, while reversing the suppressive tumor environment emerges as another crucial aspect.

By combining ARMs with ICIs, we have identified a potential therapeutic approach for solid tumors. This initial study lays the groundwork for further optimization and demands future investigations to unravel the precise mechanism of action, paving the way for potential advancements and applications in the future. The potential of cancer vaccines to bolster our arsenal against cancer warrants further exploration and holds promise for more effective and targeted treatments in the fight against this devastating disease.

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