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Characterization of the impact of senescent fibroblasts on the adenosine pathway in human NK cells

par

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Mémoire présenté à la Faculté de Médecine en vue de l’obtention du grade de M.Sc. en Microbiologie et Immunologie

Janvier, 2023

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RÉSUMÉ

Les fonctions immunitaires déclinent au cours du vieillissement, un phénomène qui pourrait être lié à l'accumulation de cellules sénescentes dans les tissus. La sénescence est un état irréversible d'arrêt de croissance qui s'engage principalement en réponse à des dommages irréparables de l'ADN. Les cellules sénescentes ont un phénotype sécrétoire pro-inflammatoire (SASP) qui affecte les tissus voisins. CD73 est une enzyme qui travaille en collaboration avec CD39 pour produire de l'adénosine à partir d'adénosine triphosphate (ATP). Il a été démontré que des concentrations plus élevées d'adénosine dans un microenvironnement tumoral nuisent aux fonctions des cellules immunitaires. L'objectif de ce projet est de déterminer si les fibroblastes sénescents ont la capacité d'induire l'expression de CD39/CD73 à la surface des cellules tueuses naturelles (NK) et d'inhiber la réponse immunitaire antitumorale. Nos résultats montrent que les cellules NK-92, NKAES (cellules tueuses naturelles amplifiées) et les cellules NK primaires expriment des niveaux plus élevés de CD39/CD73 lorsqu'elles sont cultivées avec des fibroblastes sénescents. De plus, nous avons observé que le marqueur CD73 est aussi augmenté dans les fibroblastes sénescents. L'augmentation était cependant plus prononcée lorsque la sénescence était induite en raison de la surexpression de l'oncogène $hRAS^{v12}$ plutôt que suite à l'exposition à des radiations ionisantes. En outre, la cytotoxicité des cellules NK diminue lorsque celles-ci sont exposées à un environnement sénescent et lorsqu'on traite les cellules avec 2-Chloro Adénosine (CADO), un analogue de l'adénosine. Nous supposons que l'augmentation de l'expression de CD39/CD73 conduira à une production accrue d'adenosine, créant ainsi un environnement immunosuppressif. La caractérisation de l'impact de la sénescence cellulaire sur les fonctions des cellules NK pourrait donner un aperçu du développement de stratégies visant à augmenter la capacité du système immunitaire à éliminer les cellules tumorales, améliorant potentiellement les résultats du traitement du cancer.

Mots-clés: Sénescence, adénosine, CD73, CD39, SASP, cellules NK, cancer, cytotoxicité, CADO, fonction des cellules immunitaires, système immunitaire, microenvironnement tumoral.

ABSTRACT

Immune functions decline during aging, a phenomenon that may be linked to the accumulation of senescent cells in tissues. Senescence is an irreversible state of cell growth arrest often in response to irreparable DNA damage. Senescent cells have a proinflammatory secretory phenotype (SASP) that affects nearby tissues. CD73 is an enzyme that works in collaboration with CD39 to produce adenosine from adenosine triphosphate (ATP). Higher concentrations of adenosine in a tumor microenvironment were shown to impair immune cell functions. The objective of this project is to determine whether senescent fibroblasts have the ability to induce CD39/CD73 expression at the surface of natural killer (NK) cells and inhibit the antitumoral immune response. Our results show that NK-92, NKAES and primary NK cells express higher levels of CD39/CD73 when grown in co-culture with senescent fibroblasts. Similarly, we also observed that the CD73 marker is increased in senescent fibroblasts. The effect was, however, more pronounced when fibroblasts were induced to senesce because of the overexpression of oncogenic $hRAS^{v12}$ compared to when induced to senesce following exposure to ionizing radiation. In addition, the cytotoxicity of NK cells decreases when NK cells are exposed to a senescent environment and when treated with 2-Chloroadenosine (CADO), an analog of adenosine. We hypothesize that increased CD39/CD73 expression will lead to an increased production of adenosine creating an immunosuppressive environment. Characterization of the impact of cellular senescence on the function of NK cells could provide insights into the development of strategies to increase the ability of the immune system to eliminate tumor cells, potentially improving cancer treatment outcomes.

Keywords: Senescence, adenosine, CD73, CD39, SASP, NK cells, cancer, cytotoxicity, immune cell function, CADO, immune system, tumor microenvironment.

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A

ADO: Adenosine

ADA: Adenosine Deaminase

ADCC: antibody-dependent cell-mediated cytotoxicity

A2AR: Adenosine A2a Receptor

ADP: Adenosine diphosphate

ADPR: Adenosine diphosphate ribose

AMP: Adenosine monophosphate

APC: Antigen-presenting cell

AR: Adenosine Receptor

ARF: Alternative reading frame

ARN: Ribonucleic acid

ATM: Ataxia-telangiectasia mutated

ATP: Adenosine triphosphate

ATR: Rad3-related

B

BaEv-Lv: Baboon Envelope Lentivirus

C

Ca2+ : Calcium

CADO: 2-chloroadenosine

CAF: Cancer associated fibroblasts

cAMP: Cyclic adenosine 3′,5′-monophosphate

CAR: Chimeric antigen receptor

CCL2: C-C Motif Chemokine Ligand 2

CDK: cyclin-dependent kinase

CDKI: cyclin-dependent kinase inhibitor

CTL: Cytotoxic T lymphocytes

D

DDR: DNA Damage Response

DNA: Deoxyribonucleic acid

DNA-SCARS: DNA segments with chromatin alterations reinforcing senescence

DPBS: Dulbecco's phosphate buffered saline

E

eADO: extracellular adenosine

ENT: Equilibrium nucleoside transporters

F

FAS-L: Fas Ligand

FBS: fetal bovine serum

G

GPI: Glycosylphosphatidylnositol proteins

Gy: Gray

GVHD: Graft-versus-host disease

H

HDF: Human Dermal Fibroblasts

HEK293T: Human embryonic kidney 293 T cell line

HIF-1alpha: Hypoxia-inducible factor-1α

hRas: Human Ras

hTERT: Human telomerase reverse transcriptase

HTS: High throughput system

I

IFN: Interferon

IFN-I: Type I interferon

IFN-γ: Interferon gamma
IL-13: Interleukin-13

IL-15: Interleukin-15

IL-2: Interleukin-2

IL-4: Interleukin-4

IR: Irradiated

K

KO: Knock out

L

L: Liter

LAK: Lymphokine-activated killer

M

M2: type 2 macrophages

MAPK: Mitogen-activated protein kinase

MDA-5: melanoma differentiation-associated protein 5

MDSC: Myeloid-derived suppressor cells

MFI: Mean fluorescence intensity

MHC: Major Histocompatibility Complex

mTOR: Mammalian target of rapamycin

N

NAD: Nicotinamide adenine dinucleotide

NF-kB : Nuclear factor kappa-light-chain-enhancer of activated B cells

NFkB: nuclear factor k-light-chain-enhancer of activated B-cells

NK: Natural Killer cell

NKT: Natural Killer T cell

NKAES: Expanded Natural Killer Cells

NKG2D: natural killer group 2 member D protein

O

OIS: Oncogene-induced senescence

P

PBMC: Peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PDGF: Plateled-derived growth factor

PI3K: Phosphatidylinositol 3-kinase

PKC: Protein kinase C

PRRs: Pattern recognition receptors

R

RB: Retinoblastoma protein

RIG-I: retinoic acid-inducible gene I

ROS: Reactive oxygen species

S

SA-β-gal: senescence-associated β-galactosidase

SAHF: Senescence-associated heterochromatin foci

SAHFs: Senescence-associated heterochromatic foci

SASP: Senescence-associated secretory phenotype

SLT: Secondary lymphoid tissue

SMAD: Suppressor of Mothers against Decapentaplegic

T

TAMs: Tumor-associated macrophages

TCR: T-cell receptor

TGF-β: Transforming growth factor β

TIS: Therapy Induced Senescence

TME: Tumor microenvironment

TNF: Tumour Necrosis Factor

TNF-α: Tumour Necrosis Factor alpha

TRAIL: TNF-related apoptosis-inducing ligand

Tregs: Regulatory T cells

U

uPAR: Urokinase-type plasminogen activator receptor

UV: Ultraviolet

53BP1: p53-binding protein 1

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ACKNOWLEGMENTS

First of all, I would like to thank my supervisor, Christian Beauséjour, for giving me the opportunity to be part of his laboratory and for being patient with me. Thank you for your positive attitude during these two years and for taking the time to explain to me whenever I had any doubts. I feel very fortunate to have worked in your lab, as it is an excellent place not only to acquire a lot of knowledge, but also to enjoy doing science and making good friends.

I also want to thank all the members of the lab, and especially my lab mates, Damien, Marie Lyn -thank you for the emotional talks we had-, thank you Josh for always encouraging me to keep going and for listening to me when I felt sad and for your advice, I am very grateful to all of you because besides being excellent scientists you are very good people and you were always willing to help me and solve my doubts. To Oanh, for being willing to teach me and I would especially like to thank Monica because since I came to the lab you have been a pillar in my development not only academically, but personally as well, thank you for your friendship, your advice and for being my mentor also in the lab. I would also like to thank Joana for all her support and invaluable friendship, because even though we were only together for 6 months, I felt that we created very strong bonds and I am grateful to have finished this stage of our lives together, dankeschön.

I also want to thank Hugo, Alice and Louise from Haddad's lab and Paulo, Leila and Emilie from Duval's lab, because whenever I had questions, you never hesitated to help me. I am also very grateful to have met Angela from Sinnet's lab, thank you for the talks about feminism and science and for your moral support when I arrived in Montreal, I miss you.

I would like to thank MITACS for giving me the opportunity to continue my master studies in Canada.

Thanks to all my friends in Mexico, especially Eduardo, Amin, Jorge (DNAwesome nerds) and to my friends in Montreal. To Naomi and Anna, I am very grateful to have you as roomates and I appreciate your support, the laughs and the controversial conversations we had, which throughout this process have been of great support. I want to thank my aunt Sonia and Emiliano because they have made me miss Mexico less, thank you for always making me laugh and for all your advice.

Finally, I want to thank my family now in Mexico and Germany, because even though we are far away, I have always felt their support and love. Andreas and Paty, thanks for your great support and for encouraging me to keep going. Pamela, thank you for being loyal, funny and believing always in me and thanks to my parents, because you're everything to me and without you I would not have made it this far. Los amo!

1 Introduction

1.1 Cellular Senescence

As the global population ages, the health and economic consequences of aging pose a significant burden on society. Aging is a major risk factor for the development of numerous chronic diseases, including cancer. According to the World Health Organization, the proportion of the world's population over 60 years old will nearly double between 2015 and 2050, rising from 12% to 22%[1]. In recent years, the scientific community has focused on studying the processes and factors associated with aging that can cause molecular and cellular damage over time. One promising area of research is cellular senescence, a process in which cells lose their functions, including the ability to divide and replicate, but continue to secrete molecules that are able to affect neighboring cells. Different studies have highlighted the importance of the impaired immune response linked to aging, the accumulation of senescent cells and the deleterious effects that might be caused by their phenotype, particularly the senescence-associated secretory phenotype (SASP). Therefore, it is crucial to understand the mechanisms by which immune cells interact with senescent cells and to develop interventions that delay, treat, and diminish age-related dysfunction and senescence-associated diseases.

Cellular senescence is a cellular state characterized by a durable cell cycle arrest of replication-competent cells in response to irreparable damage. In 1969, Hayflick and Moorhead first described the mechanism of senescence in fibroblasts in culture[2], which was characterized by a limited proliferation capacity and genomic instability. Despite this initial discovery, the presence of senescent cells *in vivo* was not confirmed for many years, so the question remained whether these changes in morphology and function were only observed *in vitro*. However, in 1995, the existence of senescent cells in the human dermis was demonstrated using a histochemical assay for the marker enzyme β-galactosidase[3].

The process of telomere shortening has been linked to replicative senescence (see below)[4], as observed in human fibroblasts where telomeric DNA length decreases with each round of cell division. Senescent cells remain metabolically active but fail to initiate DNA replication[5], acquiring a distinct set of phenotypic characteristics that define the senescent state[6]. These characteristics may vary depending on the cell type, the nature of the stress that caused senescence, and the cellular context, and will be discussed in further detail below.

1.1.1 Cellular characteristics of senescence

Permanent growth arrest of cells is one of the main features of cellular senescence (Figure 1). The process of growth arrest is characterized by the prolonged inhibition of the activity of cyclin-dependent kinases (CDKs) by p21 and p16. p21 inhibits CDK2, while p16 inhibits the CDK4/6 complex. This leads to a block in RB protein (pRB) inactivation and a continued repression of E2F target genes, which are required for the initiation of the cell cycle[7].

In addition to growth arrest, senescent cells also display morphological changes due to changes in their metabolism[8]. These changes include an irregular and enlarged shape, flattening, vacuolization, and loss of nuclear integrity, which is caused by the loss of Lamin B1 as a result of the activation of the p53 or pRB tumor suppressor pathways[9].

The increasing activity of the enzyme β-galactosidase has been used as a non-exclusive marker to detect senescent cells. The elevated lysosomal content of senescent cells allows the detection of lysosomal β-galactosidase at suboptimal pH (pH 6.0). However, inhibition of this enzyme does not reduce senescence, indicating that it has no physiological role in acquiring or establishing a senescent phenotype[10].

Moreover, it is well established that changes in epigenetics play a role in the initiation and progression of cellular senescence. Specifically, modifications to chromatin, such as DNA segments with chromatin alterations (DNA-SCARS) and Senescence-associated

heterochromatic foci (SAHFs), have been identified as contributing to the suppression of genes involved in cell proliferation, thereby promoting senescence[11,12].

The DNA damage response (DDR) is another characteristic of senescence which is activated in response to various forms of DNA damage, such as telomere dysfunction and mutations. This has been associated with senescence by inducing cell cycle arrest and activating the p53, p21, and p38 mitogen-activated protein kinase (MAPK) pathways. The latter pathway increases the activity of the transcription factor Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which will regulate the expression of different genes, including pro-inflammatory cytokines such as IL-6 and IL-8, two components of the SASP (discussed below)[8]. The response to DNA damage can be quantified using specific markers such as p53-binding protein 1(53BP1)and γH2AX foci[11].

One of the key characteristics of senescent cells is the resistance to certain apoptotic signals. This resistance is thought to be caused by growth factor deprivation and oxidative stress, and is linked to the tumor suppressor protein p53[5]. However, recent evidence suggests that senescent cells may counteract pro-apoptotic signals by upregulating the expression of anti-apoptotic proteins such as BCL-w and BCL-xL[13].

The SASP is a hallmark of senescence that refers to the ability of senescent cells to secrete a wide range of factors that can affect the surrounding microenvironment. These factors include cytokines, chemokines, growth factors, proteases, and reactive oxygen species[14]. The SASP has both beneficial and deleterious effects, including promoting tumor suppression and tissue repair, but can also promote tumor growth, aging and inhibition of the immune system[6]. Additionally, the SASP plays an important role in promoting a pro- or anti-inflammatory microenvironment during senescence. In chronic diseases, it promotes the accumulation of senescent cells and immune evasion against them due to persistent damage[15]. It has been demonstrated in a PTEN-null prostate cancer model that epithelial cells undergoing senescence express immunosuppressive SASP factors dependent on the JAK2/STAT3 pathway signaling. These factors lead to infiltration of myeloid-derived suppressor cells and promote tumor growth[16].

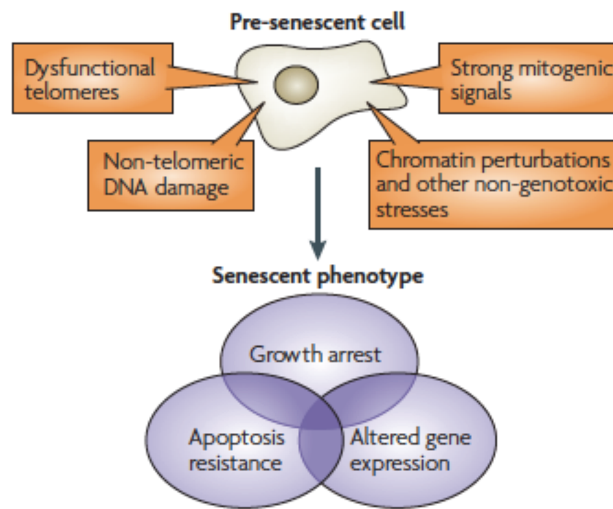


Figure 1. The senescent phenotype induced by multiple stimuli. (Campisi et al, 2007)

In order to define a senescent cell, it is necessary to consider different characteristics or markers. The presence of two or more of the hallmarks described below are sufficient to confirm the induction of cellular senescence *in vivo* and *in vitro* (Table 1).

Table 1. Senescence Markers
Lack of cell proliferation
Large and flat morphology of cells
Lack of response to growth factors
SA-B-Gal (Senescence-associated B-galactosidase)
SASP (senescence-associated secretory phenotype)
DDR (ATM, 53BP1, g-H2AX,)
Hetherochromatin markers (HP1, H3K9)
Increase expression of CDKIs (p16,p21)
Activation of p53
Lamin B1 reduction

Table 1. Senescence Markers. (Salama et al, 2014)

1.1.2 Types of Senescence

As previously described, **replicative senescence** is caused by multiples rounds of cell division and excessive telomere shortening. This mechanism serves to protect against uncontrolled cell proliferation and cancer. Telomeres, which act as a kind of "meter" for cell division, are responsible for regulating cell lifespan. When telomeres are lost or shortened, cells trigger a DDR and enter a state of senescence. The ectopic expression of telomerase, an enzyme that can extend telomeres, is sufficient to immortalize human fibroblasts, further emphasizing the importance of telomere shortening in replicative senescence[12]. Besides telomere shortening, a variety of non-telomeric stimuli have been shown to prematurely induce senescence.

Previous studies have demonstrated that normal cells respond to the activation of several oncogenes by undergoing cellular senescence. Serrano *et al*. described for the first time the characterization of a tumor suppressor response by the activation of the *RAS* oncogene in human fibroblasts referred to as **oncogene-induced senescence (OIS)**. They observed that the overexpression of hRAS leads to a definitive proliferative growth arrest accompanied by DNA damage and the accumulation of p19ARF, p53, pRb and p16INK4a[18]. The *RAS* gene is mutated in many types of cancer, however, it requires the cooperation of other oncogenes (*MYC*, *PTEN*, *BRAF*) to drive transformation, in which the p53/p21 and p16 pathways play an essential role in regulating the triggering of senescence through oncogenic signaling[4]. As an example, senescence induced by loss of the tumor suppressor PTEN is characterized by a DDR independent mechanism and mTOR/ARF pathway-dependent activation of p53 and also p16 induction via Ets2[19].

During aging, cells show increased levels of ROS (reactive oxygen species), primarily responsible for endogenous oxidative DNA damage[20]. Reactive oxygen species, whether produced endogenously by mitochondrial dysfunction or by extrinsic events (e.g. Ultraviolet [UV] radiation, smoke), are capable of inducing **DNA damage-induced senescence** through DDR and activation of the MAPK signaling pathway and its downstream effector p38[20,21].

Therapy-induced senescence (TIS) is a phenomenon that occurs when cells undergo a state of irreversible growth arrest in response to certain cancer therapies, including chemotherapy and radiation therapy. The first chemotherapeutics shown to induce senescence in tumor cells were cisplatin[22] and doxorubicin[23]. These two drugs also cause many hallmarks of senescence, such as prolonged growth arrest and expression of senescence-associated β-galactosidase (SA-β-gal), etc. On the other hand, it has been reported that irradiated cells arrest cycle in the G2/M phase followed by the restart of DNA replication, thereby triggering permanent activation of DDR signaling and induction of cellular senescence[24].

1.1.3 Molecular pathways leading to senescence

Several inducers can act to force cells into senescence, but there are two key pathways that are involved in this mechanism: p16INK4a/Rb and p53/p21 (Figure 2). Both p16 and p21 function as inhibitors of cyclin dependent kinases 1 (CDK1), CDK2, CDK4, and CDK6, leading to the hypophosphorylation of pRb and, consequently, to the suppression of S-phase genes and subsequent stable cell cycle arrest[25].

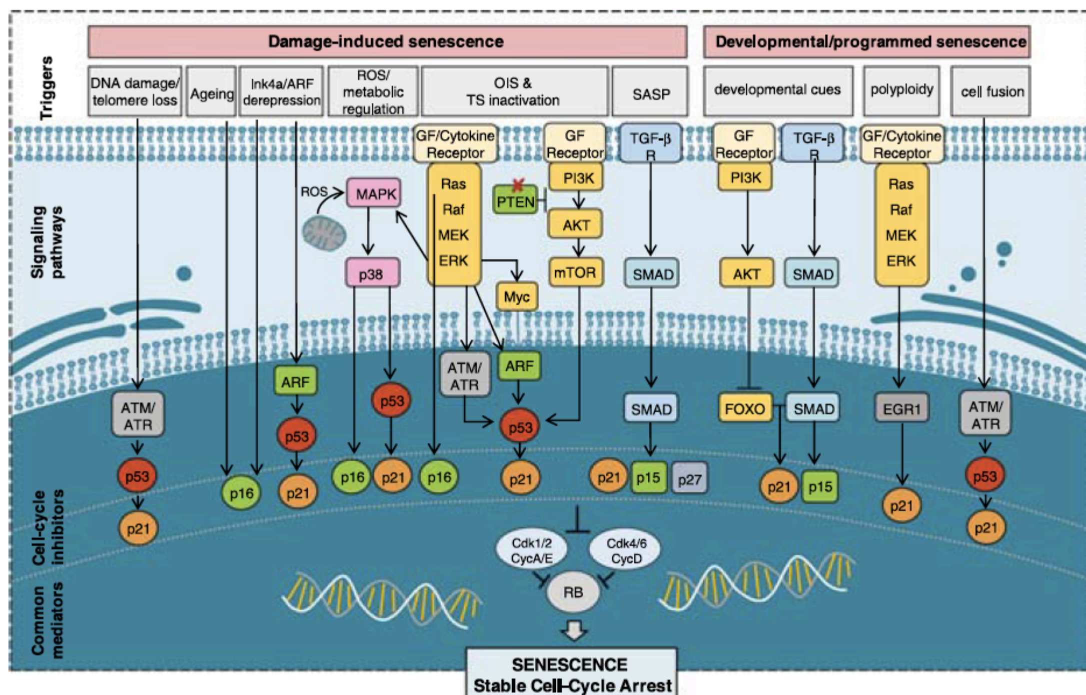


Figure 2. Molecular mechanisms of cellular senescence. Cellular Senescence in Disease (Serrano *et al.*, 2021)

Telomere loss or shortening and various DNA-damaging agents activate the DNA damage response, leading to the accumulation of ataxia-telangiectasia mutated (ATM) or ATM and Rad3-related (ATR) kinases, which block cell cycle progression by stabilizing the p53 protein. The activation of p53 leads to the upregulation of *cyclin-dependent kinase 1 (CDK1A) inhibitor,* coding for p21[19].

Furthermore, replicative senescence also results in the epigenetic derepression mechanisms of the *cyclin 2A-dependent kinase inhibitor* locus *CDKN2A,* which encodes the cell cycle inhibitor p16. Activation of MKK3 and MKK6 kinases and the downstream effector kinase p38, also lead to senescence (Figure 2). Similarly, oncogenic signaling or loss of tumor suppressors is responsible for the activation of p16 and p53, in collaboration with DDR and ARF[4].

On the other hand, the transforming growth factor-β (TGF-β), a notable component of the SASP, can induce senescence via p21 or p15/p27 through the SMAD and PI3K pathways (Figure 2)[4].

As previously discussed, activation of p53/p21 pathway results in cellular senescence via different ways, however, p16 activity may be a crucial factor for maintenance of the senescent-cell-cycle arrest[26]. Once p21 is activated, it triggers the onset of senescence-associated secretory phenotype (SASP), which becomes irreversible, even if the p53 or Rb proteins are inactivated [27]. While overregulation of p21 can result in cell cycle arrest, the proper maintenance of p21 requires the presence of p16 [28].

Both the p53 and p16 pathways are known to interact closely, but they are also capable of independently halting cell cycle progression and responding to various stimuli. Furthermore, the specific pathway used by a cell to induce senescence can differ depending on its type. Recent research has shown that p16-independent senescence can occur in a genetic mouse model[29], further highlighting the complex nature of these pathways and their regulation of cellular senescence.

1.1.4 Cellular Senescence and Cancer

Cellular senescence has largely been considered a pleiotropic mechanism in which, depending on the context, it will be beneficial or detrimental to cancer development (Figure 3). It has been observed that the SASP can directly or indirectly affect the behavior of nearby cells, including the induction of senescence in neighboring tumor cells. In the tumor microenvironment, senescent cells can promote tumor progression, stimulating cell proliferation and tumor vascularization via the SASP[14].

The SASP contains inflammatory chemokines, cytokines and growth factors that contribute to inflammation and modulate stromal and immune cells[30]. An example of this is TIS in cancer patients, where it may initially be beneficial in blocking tumor cell proliferation, but, in parallel, results in an impaired immune response to eliminate senescent tumor cells[19].

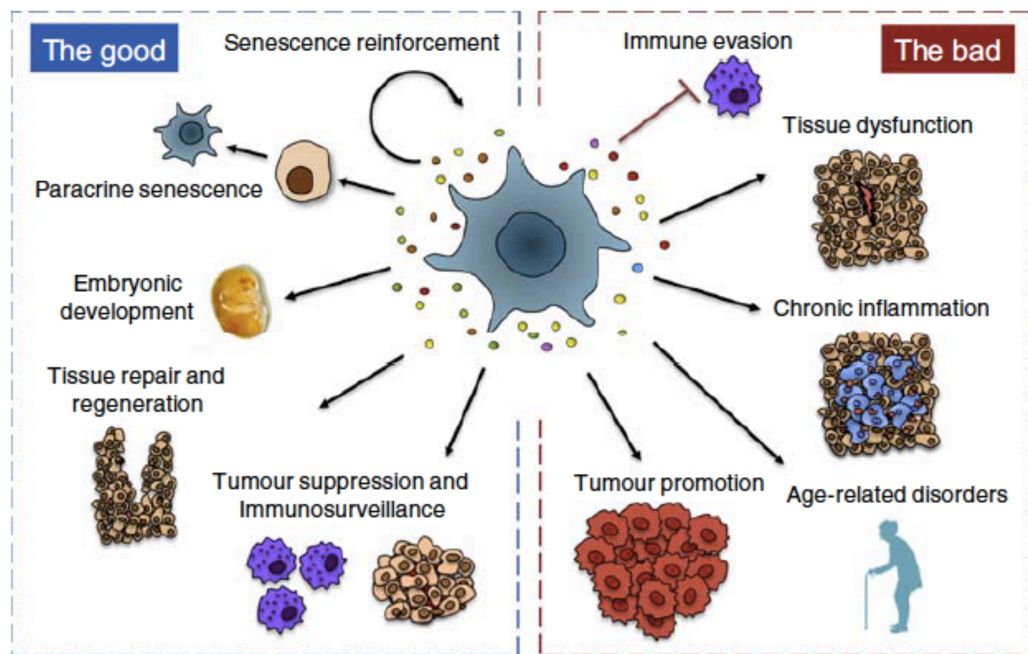


Figure 3. Antagonistic roles of cellular senescence in physiology and pathology. Cellular Senescence in Disease (Serrano et al, 2021)

Senescent cells have been identified as a potential contributor to tumor growth through the secretion of the SASP[31]. The secretion of cytokines, chemokines, and growth factors causes the recruitment of immune cells, including natural killer (NK) cells, macrophages, T lymphocytes, and neutrophils, contributing to an inflammatory environment[4,32]

The initial evidence of immune-mediated clearance of senescent cells was discovered in the context of liver tumors, where p53 reactivation played a key role[33]. p53 has been shown to promote the secretion of cytokines, such as CCL2, to attract NK cells to eliminate senescent cancer cells[34]. On the other hand, CD8+ T cells can interfere with senescent cell clearance through the same receptor that mediates granular exocytosis in NK cells, the NKG2A receptor[35].

As we age, the immune system undergoes changes that impair its response and result in various pathologies. One of the key challenges in aging is immune evasion by cancerous senescent cells, which is primarily attributed to the senescence-associated secretory phenotype (SASP)[31]. Therefore, there is a growing interest in developing therapies that target senescent cells and the SASP, with the goal of improving the immune response and reducing the incidence of age-related diseases. By targeting the SASP and senescent cells, it may be possible to develop new interventions that promote healthy aging and prevent the onset of disease.

1.1.5 Therapeutic approaches to eliminate senescent cells

Baker *et al.* demonstrated in a murine model with chronological aging that the removal of p16-positive cells contributes to the suppression of the senescent phenotype in different organs[36]. This report led to the conclusion that elimination of persistent senescent cells can ameliorate, delay, and reverse age-associated disorders. The findings of this study lend support to the idea that removal of senescent cells can have a positive impact on age-associated disorders, suggesting that interventions aimed at preventing senescent cell action may be beneficial.

Senomorphics are drugs that inhibit different pathways that lead to the SASP. Inhibitors such as rapamycin, metformin and p38/MAPK pathway inhibitors have been shown to reduce the aging process and cellular senescence associated effects[37]. Senomorphics appear to mitigate the negative effects of the SASP on surrounding cells without eliminating senescent cells through two primary mechanisms. The first mechanism involves modifying the secretome of senescent cells. The second involves the neutralization of factors released by SASP to improve the efficiency of cell immunosurveillance, for example, by blocking the activity of different cytokines with monoclonal antibodies such as IL-6 that is upregulated by the SASP. However, blocking these factors using senomorphics may also affect pathways that are important for other biological functions such as tissue homoeostasis[37,38]. Rapamycin has been shown to be a potent senomorphic of the mTOR pathway that suppresses the SASP[39]. In murine fibroblasts, treatment with rapamycin was found to increase the expression of Nrf2, a signaling pathway associated with senescence. This resulted in a reduced induction of SASP, as well as decreased levels of the senescence mediators p16 and p21[39].

Compared with senomorphics, senolytics are a promising approach for inducing apoptosis in senescent cells, thus promoting their elimination and attenuating inflammation in tissues. Senolytics transiently deactivate anti-apoptotic pathways, driving senescent cell apoptosis[40].

An additional approach that is being investigated involves the targeting of senescent cells through the use of NK cells. It has recently been reported that NK cells are the main cells of the immune system responsible for the clearance of senescent cells[41]. This is due to the broad expression of activating and inhibitory molecules present on their surface that allow them to recognize Major Histocompatibility Complex (MHC) class I and class I-like molecules that serve as indicators of cellular stress in damaged/senescent cells. In mouse models of liver carcinoma, restoration of p53 function allows senescent cells to be eliminated by NK cells, primarily through NKG2D recognition[42].

Immunotherapies

Different strategies have been developed to promote the removal of senescent cells by immune cells. For example, Chimeric antigen receptor (CAR) T cell therapy involves genetically modified autologous T cells expressing a chimeric antigen-binding receptor[43]. Once modified, the cells are injected into the patient in order to eliminate the target cells. The modification of T cells allows targeting of antigens expressed by senescent cells[32]. The urokinase-type plasminogen activator receptor (uPAR) has been discovered as a surface protein linked to senescence. In mice with liver fibrosis, the targeting of uPAR via CAR T cells proved successful in rejuvenating tissue function[44].

1.2 Immune System

Throughout history and evolution, multiple strategies have been developed by the immune system to counter invaders that get past the first barrier defenses. These strategies are aimed at reacting immediately after a pathogen overcomes the barriers as well as additional defenses that target established infections[45].

1.2.1 Immune response

The immune system protects the body from pathogens and other foreign molecules by recognizing and responding to them. This is accomplished through two types of immune responses: the innate response and the adaptive response[45]. The innate response is the first line of defense and is always present. It includes physical barriers such as the skin and immune cells that can quickly identify and respond to pathogens. If the innate response is unable to eliminate the pathogen, the adaptive response is activated [45]. The adaptive response involves specialized immune cells, called lymphocytes, that can recognize and remember specific pathogens. This response produces antibodies that can neutralize the pathogen or activate other immune cells to eliminate it. After the initial encounter with a pathogen, the adaptive response produces a memory response that leads to a stronger and faster response upon encountering the same pathogen in the future[45]. This immune memory prevents certain diseases from being contracted a second time. It is important to note that both the innate and adaptive responses work together to

provide effective immune responses against a wide range of pathogens. As an individual ages, the immune system may lose some of its effectiveness, making immune memory more crucial to counteract certain pathogen.

1.2.2 Innate Immunity

Innate immunity is the first line of defense against infection, consisting of physical and anatomical barriers. Cellular components and molecules of innate immunity are present before the onset of infection and constitute a group of resistance mechanisms against disease that are not specific to a particular pathogen but will recognize frequently encountered pathogens. NK cells, neutrophils, eosinophils, endothelial cells, macrophages, dendritic cells, mast cells and eosinophils are cellular components of the innate immune response[45]. These cells provide a nonantigen-specific response to viral, bacterial, and fungal pathogens. Moreover, the type I Interferon (IFN-I) response underlies the immune system first line of defense against pathogens by inhibiting viral replication and spread at the earliest stage of infection, mediated by pattern recognition receptors (PRRs) such as RIG-1 and MDA-5[46]. It has been shown that IFN production declines with age thus leading to impairment of the innate response of NK cells, macrophages and dendritic cells against diseases including cancer[47] and infectious diseases[48].

1.2.3 Adaptative Immunity

The adaptive immune system can recognize and selectively eliminate specific microorganisms and foreign molecules. Unlike innate immunity, the adaptive response is not the same in all members of the same species but will generate a response to specific antigens. The four characteristics of adaptive immunity are antigen specificity, memory, diversity, and self/non-self recognition[49].

The immune system can distinguish subtle differences between antigens because of its antigenic specificity and is also capable of generating a diversity of recognition molecules, which allows the identification of countless unique structures in rare antigens,

subsequently recognizing individual types of microorganisms and distinguishing between individuals of this species that present minimal variations in their MHC-I.

In addition to its role in identifying and responding to foreign antigens, the adaptive immune system has been linked to the process of aging and senescence. As individuals age, their adaptive immune response tends to become less effective, a phenomenon known as immunosenescence[50]. This decline in immune function can lead to increased susceptibility to infections, reduced vaccine efficacy, and higher risk of developing chronic diseases such as cancer and autoimmune disorders[51]. Furthermore, changes in the adaptive immune system with age have also been implicated in the development of inflammaging, a chronic low-grade inflammatory state that is thought to contribute to age-related disease[52].

It is important to note that the adaptive immune response is subdivided into two major components: humoral immunity and cellular immunity.

1.2.4 Humoral immunity

Humoral immunity involves the interaction of cells present in the blood and mucous secretions, called antibodies, produced by B cells. The interaction of B lymphocytes with the antigen and their proliferation and differentiation into antibody-secreting plasma cells are part of the humoral response of the immune system. The antibody acts as an effector of the humoral response, binds to an antigen, and neutralizes or facilitates its elimination[45]. Humoral immunity is the main defense mechanism against extracellular microbes and their toxins because the secreted antibodies can bind to them and contribute to their destruction (Figure 4). The antibodies themselves are specialized and each type can activate different effector mechanisms[49].

There is no evidence that humoral immune responses play an important role in the immune response to senescent cells.

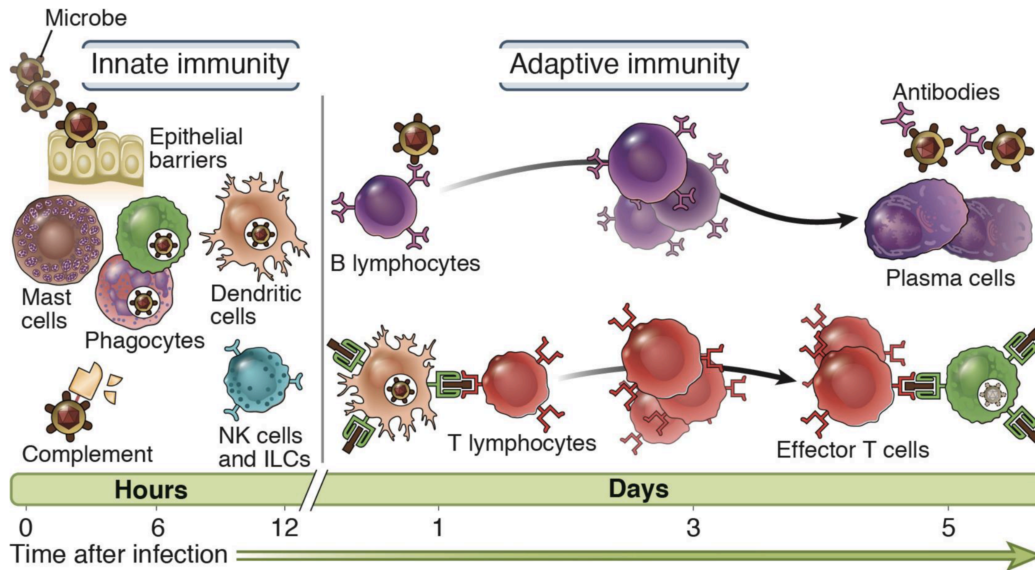


Figure 4. Kinetics of innate and adaptive immune responses. *Basic Immunology, 5thedition (* Abbas et al, *2016)*

1.2.5 Cellular Immunity

T cells mediate cellular immune responses. They recognize antigens presented on MHC complexes by professional antigen-presenting cells (APCs) or specific target cells. This recognition activates the T cell, leading to the production of a primary immune response against cells that present the antigen on MHC. Following repeated cycles of cell division, the T cell undergoes blast transformation. T lymphocytes are categorized into CD4+ and CD8+ cells based on their surface markers and function, with mature cells expressing either of these two proteins depending on the type of MHC proteins they recognize. Autologous but altered (e.g., transformed) cells can also be recognized by cellular immunity. Alterations in the presentation of neoantigens on MHC molecules can make them more visible to the immune system, leading to recognition and elimination of cancer cells. Different studies have shown that cytotoxic CD8+ T cells are powerful effectors in the anticancer immune response[53], and recent evidence has shown that senescent cancer cells trigger enhanced CD8+ T cell-mediated antitumor protection evoked primarily by senescence-associated peptide antigens[54].

While the primary activity of T cells is to recognize peptides presented by MHC molecules, NK cells will display receptors that recognize autologous stress-induced proteins on cancer cells [55].

1.2.6 Natural Killer Cells

NK cells are lymphocytes that belong to the innate immune system and do not require any stimulation or priming to conduct their effector functions. These cells are characterized as CD56+CD3- and are present in two populations in the human body. $CD56^{bright}$ NK cells are more dominant in the secondary lymphoid tissues (SLT) and their counterpart $CD56^{dim}$ NK cells are less abundant and found in blood, spleen and bone marrow[56]. The major difference between these two populations is that $CD56^{bright}$ cells produce higher levels of cytokines and chemokines upon activation but lack lytic capability, whereas $CD56^{dim}$ NK cells possess cytotoxic ability and produce lower levels of cytokines[55].

The cytotoxic activity of NK cells begins with the activation of different families of NK receptors that will recognize the altered expression of target cells. Loss or deficiency of MHC class-I expression in virus infected cells or tumor cells can lower the inhibitory signal of NK cells, resulting in the activation of NK cells and their cytolytic activity against the target cell. Recognition of the target cell by NK cells depends on a balance between the activating and inhibitory signals delivered to NK cells[55]. In the context of senescence, NK cells play a critical role in the clearance of senescent cells[41], yet a subset of these cells may evade NK cell-mediated lysis through immunoevasion mechanisms.

NK cells can mediate their cytotoxic activity in two different ways. The first is the release of cytotoxic granules containing perforin and granzymes. Perforins are proteins that integrate into the cell membrane and induce the formation of pores that cause osmotic pressure and allows the granzymes to enter the cell and induce apoptosis by activating the apoptosis-inducing caspase cascade[57]. NK cell activation causes CD107a externalization as a marker of lytic granule release. Thus, degranulation of cells can be

determined by measuring the expression of this maker of NK cells by flow cytometry[57]. The second mechanism by which NK cells exert their cytotoxic activity is through the expression and engagement of death receptors, such as TNF-related apoptosis-inducing ligand (TRAIL) and FAS ligand, on the surface of target cells. These ligands bind to their cognate receptors, leading to the initiation of intracellular signaling cascades that result in the induction of apoptosis in the targeted cells[57].

NK cells have emerged as a promising option for allogenic immunotherapy, owing to their potent cytotoxic capabilities and ability to rapidly target and eliminate a wide range of cancer and hematological malignancies[58]. Additionally, the lack of graft-versus-host disease (GVHD) associated with NK cell-based therapies[59], and the feasibility of off-the-shelf manufacturing[60], make them an attractive choice for immunotherapy.

As described above, senescent cells can interact with other cells and shape their surrounding environment by affecting different functions. Recent studies suggest that NK cells can recognize and eliminate senescent cells through several mechanisms, including the expression of activating receptors and the secretion of cytokines and chemokines [41]. One mechanism by which NK cells eliminate senescent cells is through the recognition of stress-induced ligands that are upregulated on the surface of senescent cells. These ligands can activate NK cells through various receptors, including NKG2D, DNAM-1 and NKp30, leading to the secretion of cytokines such as IFN-γ and TNF-α and the induction of apoptosis in senescent cells[41,61,62].

The interaction between senescent cells and NK cells is crucial for maintaining tissue homeostasis and preventing age-related diseases. A decline in NK cell function or an increase in the number of senescent cells can impair this interaction, leading to various age-related pathologies such as cancer and chronic inflammation[61,62].

1.2.7 Immune evasion and antitumor response

As previously discussed, the aging process is accompanied by a decline in the efficiency of the immune system, and cellular senescence has been identified as a major contributor

to the development of various diseases, including cancer[63]. This decline in immune function makes individuals more susceptible to infections and malignancies, highlighting the need for further research to understand the underlying mechanisms and potential interventions to improve immune function in older adults.

Schereiber *et al.* proposed the immunosurveillance theory at the beginning of the 21st century[64]. This theory complemented the idea of cancer immunoediting proposed by Sir Macfarlane Burnet (1959), relying on several mechanisms that may cause tumor escape from immune surveillance[65]. They proposed three phases that occur in the process of cancer immunoediting stating that the immune system not only protects the host against tumor development, but also promotes tumor growth by selecting different tumor escape variants with reduced immunogenicity. The phases that comprise cancer immunoediting are elimination, which refers to cancer immunosurveillance in which cells and molecules that compose the innate and adaptive response (e.g., NK, T cells, IFN-γ, perforin, etc.) recognize and destroy developing tumors. Tumor cells then enter a stage of equilibrium, a period in which the tumor and immune system present a dynamic balance. Finally, in the last phase, denominated as escape, tumor variants that emerge from immunoselection become tumor clones with reduced immunogenicity[66]. Whether these three phases also occur ultimately resulting in the accumulation of senescent cells remains largely unknown.

Tumor escape results from changes that occur at the tumor level and directly inhibits cancer cell recognition or cytolysis by immune effector cells. Tumor cells can evade detection either by a lack of immunological recognition or by induction of central or peripheral tolerance. Central tolerance is a process by which self-reactive T cells are eliminated on the thymus. Due to this process and the absence of neoantigen expression, tumors remain invisible to the adaptive immune system and grow unabated [67]. Peripheral tolerance is a mechanism that helps to prevent autoimmune reactions by eliminating or suppressing T cells that recognize self-antigens that are not expressed in the thymus. However, this process may also temporarily allow for an immune response against tumors

to occur before tolerance is established, as the development of tolerance could potentially contribute to the advancement of the tumor.[67]

A wide variety of immunosuppressive regulatory leukocytes can suppress immune function, leading to tumor escape. Regulatory T cells (Tregs) express CD25, CD4, and FOXP3, inhibiting cytotoxic T lymphocytes (CTLs) function[68]. Natural killer T (NKT) and B cells also inhibit effector responses against transformed cells through several mechanisms involving IL-13/TGF-β production and accompanied by the activation of the IL-4R-STAT6 pathway[69]. On the other hand, myeloid-derived suppressor cells (MDSCs), a heterogeneous group of myeloid cells, can inhibit lymphocyte functions by different mechanisms, including the production of immunosuppressive cytokines and growth factors, such as TGF-β. Similarly, the secretion of IL-4 and IL-13 leads to the polarization of type 2 macrophages (M2), inhibiting antitumor immunity through the production of TGF-β and IL-10 and promote angiogenesis through the platelet-derived growth factor (PDGF)[70].

An important immunosuppressive mechanism that has emerged recently is the adenosine pathway, in which high levels of this metabolite are present and generate immune evasion by tumor cells within the tumor microenvironment (TME)[71]. The adenosine pathway has also been shown to play an important role in modulating the activity of NK cells through the activation of different receptors[72,73], which can suppress the function of these immune cells and contribute to the negative impact of chronic inflammation and susceptibility to cancer.

1.3 Adenosine Pathway

Adenosine is a nucleoside that is composed of an adenine-group attached to a ribose sugar. This compound is implicated in a wide variety of basic biological functions, including biosynthesis, cellular energy metabolism, wound healing and angiogenesis[74]. Adenosine operates primarily as a signaling molecule outside the cell, where it is activated by adenosine receptors (ARs) on the extracellular surface of cell membranes[74].

The extracellular formation of adenosine from Adenosine 5′-triphosphate (ATP) occurs due to a combination of processes that are triggered by inflammatory conditions in the tumor microenvironment. Adenosine is exported from the cell by various nucleoside transport proteins and can cross the cell membrane via concentrative or equilibrium nucleoside transporters (ENTs). ENT1 and ENT2 are functionally the most relevant adenosine transporters and represent channels that allow adenosine to pass the cell membrane following a concentration gradient[75]. Under normal physiological conditions, the intracellular and extracellular adenosine concentrations are very low[74]. Changes in the levels of extracellular adenosine can occur through two distinct mechanisms: passive diffusion or active transport of intracellular adenosine utilizing ATP. The passive flux of adenosine under normal conditions is significantly relevant and has been observed in tissue injury, necrosis, and ischemia[74]. This passive flux could also lead to an increased concentration of extracellular adenosine which may contribute to tumor growth[76].

Adenosine produced under conditions of inflammation, stress, or hypoxia is derived from phosphodiester hydrolysis of precursor molecules, such as ATP, 5'-adenosine diphosphate (ADP) or 5'-adenosine monophosphate (AMP)[77]. These nucleotides comprise a nucleoside adenosine bound to a variable number of phospho-esters attached to a 5' atom of its sugar ribose ring. Extracellular ATP, ADP, and AMP are relatively unstable due to the catalytic ability of the enzymes that hydrolyze them. Most cells express enzymes on their cell surface that catalyze the phosphohydrolysis of nucleotides.

The main pathway leading to extracellular adenosine production is extracellular ATP dephosphorylation by CD39 and CD73 ectonucleotidases[78] (Figure 5).The degradation from ATP to adenosine starts with the dephosphorylation of ATP (or ADP) by the ectonucleotidase enzyme CD39, then ATP and ADP are converted to AMP, followed by the hydrolysis to adenosine by CD73. The conversion of ATP to AMP by CD39 is reversible by the action of extracellular kinases such as adenylate kinase. In contrast, the conversion of AMP to adenosine by CD73 is only reversible after the transport of adenosine into the cells, where it can be converted to AMP by adenosine kinase[79,80].

Adenosine production occurs in three main steps (Figure 5). This mechanism begins with the release of intracellular nucleotides that can be secreted by multiple cell types and are normally stored in the form of ATP and ADP[77]. However, it is important to emphasize that the intracellular ATP levels are very high (5-8 mM). Therefore, release from intracellular sources occurs during cellular damage or death (e.g., lysis or apoptosis) or through specific gradient-driven channels[81]. As a second step, CD39 hydrolyzes ATP and ADP to their respective nucleotides, and, during the last step of extracellular adenosine generation, CD73 hydrolyzes extracellular monophosphate nucleotides to bioactive nucleoside intermediates, thus metabolizing AMP to adenosine (Figure 5)[82,83]. Adenosine Deaminase (ADA) converts adenosine to inosine and controls the adenosine levels in the body. ADA binds to CD26, reducing the local concentration of adenosine on the cell surface, and prevents adenosine from binding to its receptors, primarily A2AR[84].

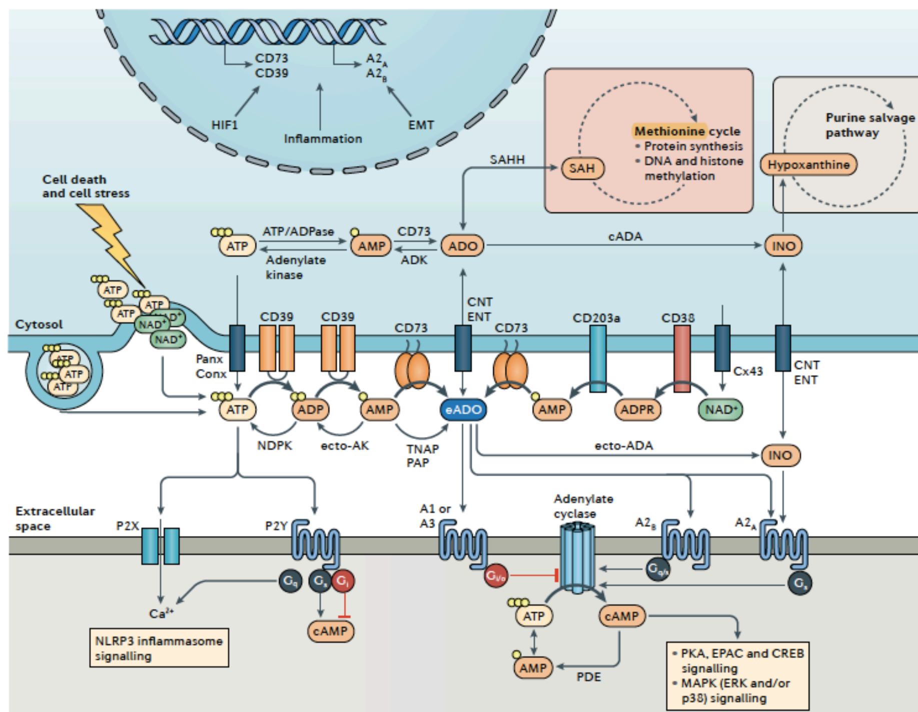


Figure. 5. Extracellular and intracellular pathways regulating extra cellular adenosine eADO production, clearance and signaling (Allard *et al*, 2020)

In 1975, it was first shown that extracellular adenosine suppressed the cytotoxic activity of T cells against EL4 lymphoma cells[85], and later studies demonstrated the importance

of this compound within a tumor microenvironment[86]. Adenosine signaling is a key metabolic pathway that regulates tumor immunity and generates an immunosuppressive response. Adenosine is an immunosuppressive metabolite produced at higher concentrations in the TME[87] compared to basal conditions, and contributes to the pathogenesis of solid tumors.

1.3.1 CD39 (E-NTPDase 1)

CD39 is a member of the ectonucleoside triphosphate diphosphohydrolase family. It is an integral membrane protein that efficiently phosphohydrolyzes ATP and ADP and is dependent on Ca^{2+} and Mg^{2+} to produce AMP. CD39 is a 510 amino acid protein with seven N-linked glycosylation sites, 11 cysteine residues, and two transmembrane regions[88]. Structurally, this protein is characterized by two transmembrane domains, a small cytoplasmic domain comprising the NH2- and COOH-terminal segments, and a long extracellular hydrophobic domain, including five highly conserved domains better known as apyrase domains, which are pivotal for the catabolic activity of this enzyme. Glycosylation of CD39 plays a crucial role in correct protein folding, membrane targeting, and enzymatic activity[88]. In addition to Mg^{2+}, Ca^{2+} and Zn^{2+} ions can generate high activation and enzymatic activity in CD39 ectodomains[89]. This enzyme is predominantly expressed in the lung, placenta and spleen[88], and it is present on both endothelial cells and various immune cells including B cells, NK cells, dendritic cells, monocytes, macrophages, neutrophils, and regulatory T cells[90].

1.3.2 Role of CD39 in the Immune response

CD39 plays a significant role in macrophage differentiation by controlling the balance between inflammatory and regulatory activity[91]. However, in the ovarian tumor microenvironment, CD39 has been shown to drive an immunosuppressive phenotype[92].

In leukocytes, CD39 is indispensable for adenosine generation and consequently dictates its immunosuppressive function[78,93]. CD39 has been found to be highly expressed in two populations of regulatory CD4 T cells, FOXP3+ and FOXP3-. Through the hydrolysis of

pro-inflammatory extracellular ATP, CD39 activity represents a mechanism of regulatory and suppresses the activity of memory of CD4+ T cells[94].

Under specific circumstances, CD39 is regulated by different cytokines in immune cells and can also modulate cytokine expression. *In vivo* studies have shown that a combination of T cell receptor (TCR) engagement and IL-6 can increase CD39 expression on CD8+ T cells[93 95]. There has been evidence that CD39 is the most prominent ectonucleotidase expressed by NK and NK-T cells and impacts cytokine production[93,96], but the relevance of this remains unclear.

1.3.3 CD73 (Ecto-5-nucleotidase-NT5E)

CD73, known as the 5'- ectonucleotidase, is a glycoprotein linked to membrane-anchored protein called glycosylphosphatidylnositol (GPI) protein that converts extracellular AMP into adenosine. The mature form of CD73 consists of 548 amino acids and 2 identical subunits of 70 kDA. Two catalytic divalent metal ions bind to the N-terminal domain, and AMP binds to the C-terminal domain[88,97]. In 1978, it was first demonstrated that purified CD73 was found in a soluble form released from the plasma membrane of various tissues, and, at the same time, could be a viable marker of maturation in T and B lymphocytes[98]. CD73 was first identified as a lymphocyte differentiation and maturation antigen, but it was soon realized that it acts as a relevant adhesion molecule that allows lymphocytes to bind to the endothelium[99]. CD73 has recently been shown to be involved in the control of a variety of physiological responses, including tissue injury, platelet function, and vascular leakage[83]. Previous studies have shown that this protein participates in cell-cell and cell-matrix interactions and is involved in tumor promotion[82].

1.3.4 Role of CD73 in the Immune response

CD73 is expressed on different subsets of T cells, but it is most abundant in Tregs[100]. Recent studies have also demonstrated the expression of this ectoenzyme in mice where it is expressed in 60% of the CD4+ cells and 80% of the CD8+ T cells [101]. Other studies

have also shown that the synergy between the two CD39-CD73 ectoenzymes upon binding to the A2A receptor elicits an immunosuppressive mechanism utilized by regulatory T cells. The effect of the adenosine pathway on these cells is characterized by strong enzymatic activity that promotes the immunosuppression of effector T cells via the A2A receptor. In addition, CD73 was shown to act as a marker of Tregs[101].

It has been observed that the expression of CD73 in macrophages is variable according to the activation state of these cells and a low expression and activity of both CD39 and CD73 enzymes was observed in M1 macrophages compared to M2 macrophages[102]. On the other hand, CD73 has an impact on dendritic cells since its expression is relevant for the adhesive interaction between these cells and germinal center B cells. This suggests a regulatory role in the maturation process of B cells[103]. CD73 expression on NK cells is very low (~1%), but recent studies have shown that exposure of NK cells to mesenchymal stem cells can induce overexpression of this marker on peripheral blood NK cells, and, similarly, an increase in adenosine levels was detected[104]. It has been found that NK cells expressing CD73 exhibit a hyperfunctional state of activation compared to those without CD73 expression [105]. These CD73+ NK cells show higher levels of functional markers of NK cell activation such as IFN-γ and NKG2D, and increased levels of receptors TIM-3, PD-1, and TIGIT[105].

1.3.5 Expression of CD73 and CD39 in cancer

Evidence suggests that the interaction between immune cells and the tumor microenvironment may generate an immunosuppressive response, leading to tumor growth and poor prognosis. Adenosine is present in cancer at higher concentrations under hypoxic conditions because of the increased intracellular adenosine production and subsequent release by the cells[106]. Damage to endothelial cells leading to disruption of normal blood and oxygen supply has been associated with a decrease in intracellular ATP, an increase in intracellular AMP, and intracellular adenosine dysfunction. However, outside the cell, CD73 serves primarily as a signaling molecule, and under conditions of cellular stress (e.g., inflammation, injury, acute hypoxia), it accumulates in the extracellular space. CD73 activity requires prior production of AMP; therefore, CD39

expression is also increased in several types of cancer, including melanoma, ovarian, and head cancer (Table 2).

Table 2. CD73 and CD39 Predictive and Prognostic Biomarkers

	Tumor Type	Findings	Author
CD73 Negatively prognostic	NSCLC (STAGE I-III)	High CD73 expression was an independent risk factor for decreased overall survival and decreased recurrence-free survival	Inoue, et al. [107]
	Prostate Cancer	CD73 expression in normal tissue was a negative prognostic factor for prostateinfiltrating CD8(+) cells. However, high expression of CD73 in tumor stroma was associated with longer recurrencefree survival	Leclerc, et al.[108]
	Breast Cancer (Triple Negative)	CD73 expression is associated with anthracycline resistance and poor prognosis	Loi, et al. [109]
	High-Grade Serous Ovarian Cancer	High levels of CD73 are associated with shorter disease-free survival and overall survival	Gaudreau, et al.[110]
	Colorectal cancer (stage I-IV)	High expression of CD73 predicts poor survival	Wu, et al. [111]
	Gastric Cancer (stage I-IV)	High expression CD73 is associated with lowered overall survival	Lu, et al. [112]
	Melanoma (stage IV)	High soluble CD73 activity was associated with poor overall survival and poor progression-free survival	Morello, et al. [113]
	Head and Neck (stage I-IV)	High levels of CD73 are associated with reduced overall survival	Ren, et al.[114]
	Renal Cell Cancer (stage I-IV)	High expression of CD73 is associated with disease progression and shortened overall survival	Yu, et al. [115]

CD39 Negatively prognostic	Gastric Cancer (stage I-IV)	High CD39 expression is a predictor of poor outcome following radical resection	Cai X,et al.[[116]]
	Hepatocellular carcinoma	High CD39 expression is an independent indicator of decreased overall suvival after radical resection	Cai X,et al.[[117]]
	Chronic lymphocytic leukemia	CD39 expression on CD4+ lymphocytes are increased in the peripheral blood of patients with CLL and correlates with advanced stage of disease	Perry C, et al.[[118]]

Table 2. CD73 and CD39 Predictive and Prognostic Biomarkers, modified from Leone and Emens Journal for ImmunoTherapy of Cancer (2018)

Several studies have pointed out the immunosuppressive effect of the adenosine pathway, emphasizing the collaboration of CD39 and CD73 and the increase in adenosine levels that promotes tumor progression[119]. The overexpression and activity of these enzymes have been observed not only in solid tumors but also in peripheral blood. The CD39-CD73 complex participates in the process of tumor escape by inhibiting the activation, clonal expansion, and migration of tumor-specific T cells (CD8+ cytotoxic), decreasing cytotoxicity mainly through adenosine binding to A2AR, followed by an alteration in the process of tumor cell elimination by cytotoxic T lymphocytes (CTL) and NK cells[88,119]. Studies have shown that TGF-β can induce CD39/CD73 expression on tumor-infiltrating MDSCs via the TGF-β-mTOR-HIF-1 pathway. The upregulation of CD39/CD73 impedes the cytotoxic activity of both NK and T cells, ultimately promoting tumor progression[120]. Furthermore, the expression of both markers in cancer exosomes can upregulate adenosine levels in the tumor microenvironment, inhibiting the response of T cells[121].

1.3.6 Adenosine and Immunosuppression

Purinergic signaling is an important peripheral immune regulator[119]. T cells are inhibited by adenosine in response to antigenic stimulation, including the synthesis of IL-2 and proinflammatory cytokines such as IFN-γ and TNF-α[122,123]. It also affects the expression of cytotoxic effector molecules, such as perforin and FAS ligand, CTL adhesion to tumor target cells, granule exocytosis by CTLs, and T-cell activation, a mechanism associated with signal transduction through the TCR and co-stimulatory CD28 molecules[124].

NK cells exhibit lytic activity independent of prior activation, and they produce inflammatory cytokines such as IL-2, IL-15, and IL-21, as well as lysing MHC class I-deficient cancer cells. Miller *et al.* [125] were the first to provide comprehensive insight into the effect of adenosine on NK cells. Adenosine acts as a suppressor of cytokine production and cytolytic activity in NK by activating A2A receptors and stimulating the downstream cascade of cAMP/PKA-dependent pathway[126] (Figure 6).

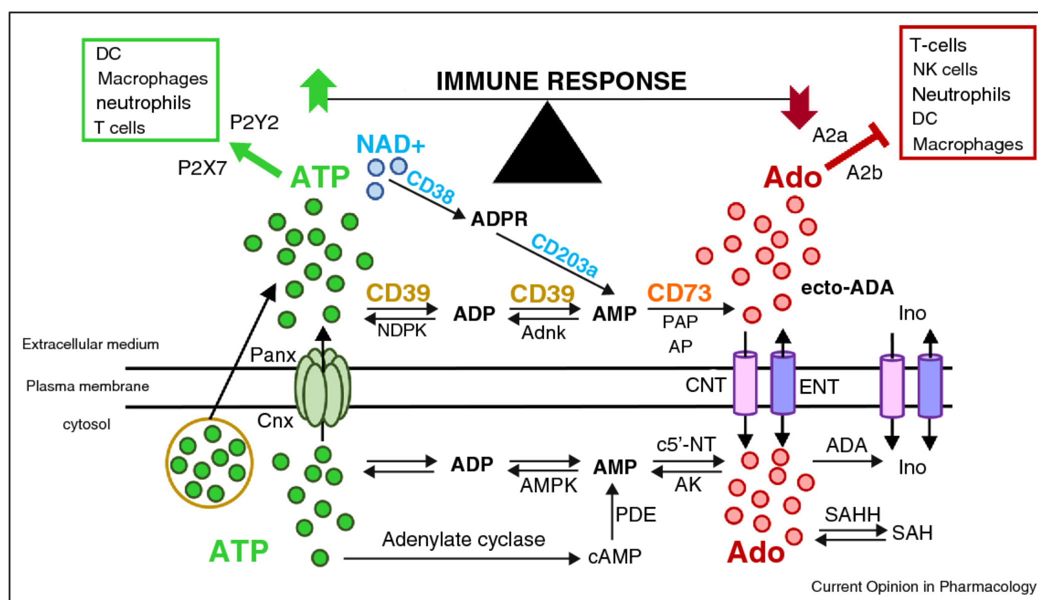


Figure 6. The balance between extracellular ATP and adenosine regulates immune responses (Allard et al, 2016*)*

Recent studies have shown that hypoxia is an important factor in immune evasion, as it transcriptionally upregulates CD39 and CD73 as well as A2A and A2B receptors via H1FI-α, leading to adenosine accumulation and signaling in hypoxic tumors[127]. TGF-β is also an important driver of extracellular adenosine production because it upregulates CD39/CD73 in different cell types, e.g., T cells, NK, tumor cells, fibroblasts, and endothelial cells. It has been shown in mice that TGFβ receptor 2 deficiency downregulates CD39 and CD73 expression in tumor-infiltrating MDSCs[128,129]. NK cells have a higher transcriptional activity of A2AR[130]. Furthermore, it has been shown that adenosine and specific A2AR agonists inhibit the functions of NK cells isolated from wild-

type mice. However, inhibition of cytotoxic function and cytokine production was not observed in NK cells obtained from A2AR KO mice[72].

1.3.7 Adenosine receptors

Extracellular adenosine induces its potent immunosuppressive effects via four specific receptors (ARs) that have been characterized as A1, A2A, A2B and A2[131,132](Figure 7). ARs have seven transmembrane domains that couple to intracellular GTP-binding proteins (G-proteins) using intracellular cyclic AMP (cAMP) as a second messenger[133]. High-affinity A1 and low-affinity A3 receptors are coupled to Gi proteins; A2 receptors are subdivided into high-affinity A2A and low-affinity A2B, which are coupled to G proteins[132,134].

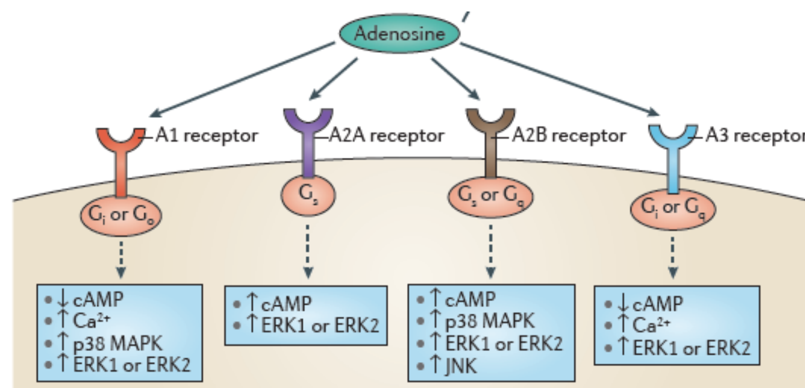


Figure 7. Adenosine receptors. Figure modified from (*Antonioli et al, 2013.)*

Adenosine receptor signaling is regulated by the level of extracellular adenosine[135] and occurs through the inhibition or stimulation of adenylyl cyclase and increased or decreased cyclic AMP levels, respectively[136].

A1 and A3 receptors induce a decrease in intracellular cAMP levels, whereas A2A and A2B receptors induce the activation of adenylate cyclase, leading to an increase in intracellular cAMP levels. A1 and A3 receptors also induce the activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC). The high-affinity A1, A2A, and A3 receptors participate at low adenosine concentrations, whereas at high

concentrations, notably in the tumor microenvironment, the low-affinity A2B receptor is involved in the signaling process[78].

The adenosine receptor signaling exerts modulatory effects on biological functions in immune cells, such as differentiation, maturation, and activation[136]. In T cells, TCR signaling causes a rapid increase in A2AR mRNA levels, which is related to a significant increase in cAMP[78]. In addition, eADO inhibits NF-κB signaling pathway-dependent activation of T cells[137]. This activation is dependent on the A2A receptor, which causes a decrease in the cytolytic and proliferative activity on T lymphocytes[138].

Adenosine is able to promote cancer cell proliferation[139], stimulates angiogenesis[140] and inhibits the anti-tumor immune response through direct binding of its specific receptors expressed on tumor cell surface[141]. A1 is one of the main receptors that contributes to tumor cell proliferation and induces activation of the cell cycle[78,142]. The A3 receptor also appears to be overexpressed in many types of cancers and primarily promotes angiogenesis. It has been shown that the A3-dependent mechanism of adenosine leads to an increase in hypoxia-inducible factor-1α (HIF-1α) in response to hypoxia in human melanoma, colon cancer, and glioblastoma[78]. Among the adenosine receptors, the importance of the A2AR signaling pathway has recently been highlighted because it is directly involved in the cytotoxic activity of immune cells such as cytotoxic T cells, NK cells, macrophages, etc. It was shown that the A2AR pathway can generate immune evasion against solid tumors followed by adenosine accumulation in the tumor microenvironment[143].

1.3.8 CD73-CD39 and immunosuppression as a therapeutic target

Immunosuppression within the tumor microenvironment is emerging as a promising therapeutic target for cancer treatment. As previously discussed, adenosine promotes tumor proliferation and neoangiogenesis; it can inhibit the anti-tumor effector activity of CD8+ T and NK cells, and both ectoenzymes are highly expressed in tumor and immune cells within the tumor microenvironment. Hence, the importance of blocking different

pathways and inhibiting adenosine receptors on tumor cells to promote the antitumor response is of particular interest.

In recent years researchers have tried to develop new treatment approaches, either by blocking the signaling pathway by CD39/CD73 ectonucleases or by inhibiting ARs to improve the antitumor response and treatment. Several CD39/CD73 inhibitors and A2AR antagonists have been developed in recent decades and have been tested in clinical trials. The therapeutic effect of anti-CD73 monoclonal antibodies (mAbs) has been demonstrated to promote antitumor immunity by stimulating dendritic cells and macrophages and by restoring the activation of T cells isolated from cancer patients[144]. Small molecule inhibitors of CD73 in combination with anti-PD-1 in patients with advanced stage solid cancer are superior to monotherapy for controlling tumor growth and metastasis[129]. CD39 blocking mAbs not only reduce the production of ADO, but also increase eATP levels, which generates an antitumor response[129 145]. Furthermore, in preliminary results of a prospective trial with cancer patients, adenosine pathway inhibitors were shown to have an effect on the target in addition to an increased activation of immune cells in the blood and T cell infiltration[129]. Preclinical studies indicate that combining A2AR and CD39/CD73 inhibitors synergistically enhances the efficacy of chemotherapy by alleviating adenosine-mediated immunosuppression[129,146]. Overall, these results highlight the potential utility of adenosine pathway inhibitors as a promising therapeutic approach for cancer treatment.

1.4 Hypothesis

The importance of the adenosinergic pathway characterized by the co-expression of CD39/CD73 ectoenzymes and adenosine production has recently been reported to be an important mechanism of immunosuppression exerted by tumor cells on the activation and effector function of NK cells[147]. Notably, prior research has revealed that the interaction of mesenchymal cells with NK induces the expression of CD73[148], which is typically expressed at low levels in normal conditions. The tumor microenvironment comprises a heterogeneous population of cells, including mesenchymal, immune cells, and fibroblast,

along with senescent cells that are known to modulate inflammatory responses. In this context, we hypothesize that the presence of senescent cells may primarily contribute to the induction and upregulation of CD39/CD73 expression on NK cells, leading to the production of adenosine and consequent attenuation of the cytotoxic activity of NK cells.

1.5 Objectives

Based on this hypothesis, three major objectives were defined:

1.Evaluation of the impact of different senescent inducers (ionizing radiation and Ras transduction) on CD39/73 expression by NK cells (NK-92, primary NK and NKAES).

2. Characterization of the impact of the increased expression of CD39/CD73 on the cytotoxic potential of NK.

3. Generation a KO of the adenosine receptor A2AR in NK to evaluate whether it makes them insensitive to the negative effect of adenosine.

2 Materials and Methods

2.1 Cell culture

Human dermal fibroblasts (HDF) were isolated from healthy donors. Tissues were obtained from consented donors in accordance with the Comité d’éthique de la recherche du CHU Sainte-Justine. HDFs were transduced with a vector encoding the human telomerase (hTERT) protein and named HDF-hTERT. The culture medium used for HDF-hTERT is Dulbecco's modified Eagle medium (DMEM), with 10% fetal bovine serum (FBS) and 1% primocin. The skin fibroblasts were maintained at low passage and subsequently passaged once a week.

Three NK cell sources were used: NK-92 (American Type Culture Collection, ATCC), NK cells freshly isolated from peripheral blood mononuclear cells (PBMCs) (see Isolation of NK cells from PBMCs) and activated and expanded NK cells (NKAES) (see isolation and expansion of NKs from PBMCs from healthy donors). NK-92 cells were cultured in Alpha Minimum Essential medium (AMEM), without ribonucleosides or deoxyribonucleosides and containing 1% L-glutamine, 12.5% HBS 'horse serum', and 12.5% FBS. NK-92 cells were cultured in a T25 Corning® flask and passaged every two days. Isolated NK and NKAES cells were directly cocultured with fibroblasts in Roswell Park Memorial Institute medium (RPMI-1640) containing 10% FBS.

K562 cells (a human erythroleukemia cell line) were used as NK activating targets and were cultured in RPMI-1640 containing 10% FBS, 1% primocin, and 1% glutamine. The K562 cells were cultured in a Corning® T25 flask and passaged every two days at a confluence of 70-80%.

For co-culture conditions, NK cells were incubated with HDF-hTERT, in a ratio of 1 NK cell for 3 dermal fibroblasts, overnight in 24-well plates in DMEM containing 10% FBS and supplemented with IL-2 (200 IU/mL). The 24-well plates were incubated under normoxic conditions of 5% CO_2 and 5% O_2 at 37°C.

For lentivirus production, 293 human embryonic kidney cells expressing a mutant SV40 large T antigen (HEK 293T) were used. These cells were cultured in DMEM with 10% FBS and 1% primocin at 37°C and 5% CO_2.

2.1.1 Induction of senescence

Induction of senescence was conducted in two different ways. Fibroblasts were transduced with a lentivirus carrying the oncogenic Ras ($hRAS^{V12}$) gene and named hRAS-transduced HDF. To induce overexpression of oncogenic hRAS-V12 and thus cellular senescence in human fibroblasts, cells were seeded one day prior to treatment. Medium was removed and unconcentrated, sterile-filtrated hRas-LV produced in

HEK293T cells was added in a dilution of 1:10-1:20 to the fresh medium containing 8 µg/mL polybrene. After incubation overnight, the virus-containing medium was removed, and fresh medium was added to cells. Cells were used for experiments 7-10 days post-transduction.

The second method involved irradiating HDF-hTERT at 12 Gy (Gray) (HDF-hTERT IR). For irradiation, cells were seeded one day before the treatment. Cells were irradiated at 12 Gy and used for experiments 7-10 days post-irradiation.

Senescence was confirmed by cells staining positive for senescence-associated β-galactosidase (SAβ-gal).

2.1.2 Lentivirus Production

Lentiviral vectors were used to insert genes into the genome of human cells *in vitro*. HEK 293T cells were seeded in a 150 mm Petri dish to reach a confluency of 70-90% the next day. On the day of transfection, cells were incubated in OptiMEM supplemented with 10% FBS for 30 min prior to the transfection. For each transfection, a solution of 1.61 mL serum-free OptiMEM and 140 µL Polyethylenimine (PEI) was mixed with 1.75 mL serum-free OptiMEM containing 6 µg REV plasmid, 7.8 µg VSVG plasmid, 15 µg pMDL (gag-pol) plasmid and 9 g plasmid carrying the gene of interest, $hRAS^{V12}$. After 15 min of incubation at room temperature, the mix was added to the cells and incubated overnight at 37°C. The next day, the medium was replaced by culture medium followed by 30 h of incubation at 37°C allowing virus production and extracellular release. Lentivirus-containing culture medium was collected after a short centrifugation at 1500 rpm at room temperature and filtration (0.22 µm) to remove cells and debris prior to storage at -80 °C.

2.1.3 BaEv-LV production

In order to investigate the role of the A2AR in NK cells, we employed the Baboon envelope pseudotyped lentiviral vector (BaEv-LV) engineered to express Cas9 and a guide RNA targeting A2AR in NK cells grown *in vitro* (hereafter referred as **BaEv-LV A2AR KO**).

HEK 293T cells (2.4x10^6 cells) were seeded in a 100 mm Petri dish. On the day of transfection, the cells were incubated in Reduced Serum Medium OptiMEM GlutaMAX for 30 min prior to transfection. For the transfection of 10 plates, a solution of 10.7 mL of OptiMEM GlutaMAX with reduced serum and 484 µL of PEI was mixed drop by drop while gently vortexing with 11.6 mL of OptiMEM GlutaMAX medium with reduced serum, containing 86 µg of the PAX2 plasmid, 70 µg of the BaEv plasmid, and 39.24 µL of plasmid with the genetic modification. After 20 minutes of incubation at room temperature, the mixture was added to the cells dropwise and incubated overnight at 37°C. The next day, the medium was replaced with culture medium and incubated for 25 hours at 37°C, allowing for virus production and extracellular release. The BaEV-LV-containing culture medium was collected 2 days after the medium was changed and centrifuged at 500g for 8 minutes at room temperature. The supernatant was transferred to an ultracentrifugation tube and centrifuged at 22,000 rpm for 1 hour and 30 minutes at 4°C. The supernatant was discarded, and the virus was resuspended in OptiMEM GlutaMAX and stored at -80°.

2.1.3.1 Viral transduction

HEK293 T cells were seeded one day prior to treatment. Medium was removed and, sterile-BaEv-LV with the A2AR KO was added using serial virus dilutions to the fresh medium containing 8 µg/mL polybrene. After incubation overnight, the virus-containing medium was removed, and fresh medium was added to cells. Selection with puromycin was performed one day after transduction by adding 2 µg/mL puromycin to fresh medium and was changed every 2-3 days.

2.1.4 Isolation of PBMCs

Peripheral blood was collected from healthy donors. Blood was collected in heparinized tubes and diluted in PBS. PBMCs were collected using Stemcell SepMate™ tubes with 15ml of Ficoll-Paque solution at the bottom of the tube. Following centrifugation, the supernatant was collected and washed twice with PBS. PBMCs were counted and

resuspended in RoboSepTM Separation Buffer (StemCell Technologies) to obtain a concentration of 6 x 10^7cells/ml.

2.1.5 Isolation of NK cells from PBMCs

Fresh NK cells were isolated by negative selection from peripheral blood after density gradient separation Ficoll-Paque (GE Healthcare) using the EasySep™ Human NK Cell Enrichment kit (StemCell Technologies). Cell purity was assessed by flow cytometry and it ranged from 66% to 90%, after staining with CD56-APC (Biolegend) and CD3-FITC (BD Biosciences). After isolation and purification, NK cells were co-cultured with pre-senescent and senescent fibroblasts.

2.1.6 Expansion of NK cells (NKAES)

NK cells were enriched from PBMC using a CD56-positive selection kit (StemCell Technologies). NK cells were expanded using the Amplification and Expansion System (NKAES) with irradiated K562mbIL21 feeder cells as described below[149].

NK cells were enriched from PBMC using a CD56-negative selection kit (Stemcell Technologies, Canada). NK cells were incubated in a 24-well tissue culture plate with irradiated (100 Gy) K562-mb15-41BBL cells and 100 IU/mL human IL-2 (Preprotech) in RPMI 1640 and 10% fetal bovine serum. K562-mb15-41BBL cells were added at a ratio of 1 CD56+CD3- cell to 10 K562-mb15-41BBL cells. Cells were cultured in a T75 flask (Thermofisher Scientific) in a humidified incubator at 37 C, 5% CO_2, with an initial total culture volume of 20 mL/flask. After 48 and 120 h, each cell culture flask was fed by the addition of 20 mL fresh complete medium and 100 IU/mL human IL-2. Cells were harvested after 7 days. Residual T cells were removed with Dynabeads CD3 (Invitrogen).

2.2 Flow cytometry

2.2.1 Analysis of expression of CD39/CD73 by flow cytometry

Following co-culture of NK cells with and without fibroblasts, the supernatant containing NK cells was collected and transferred into FACS tubes, centrifuged for 5 min at 1200 rpm and resuspended in ice-cold PBS containing 2% FBS. After centrifugation for 5 min at 1200 rpm, cells were resuspended and incubated for 10 min at room temperature in PBS containing 1:1000 Zombie NIR^{TM} protected from light to stain dead cells. Subsequently, the staining was disrupted by cold PBS 2% FBS, and cells were washed by centrifugation for 5 min at 1200 rpm and resuspension in cold PBS 2% FBS. CD73 and CD39 on the cell surfaces were stained using antibodies from Biolegend, and they are as follows: Anti-CD56/PE/APC/BV786, Anti-CD73 PE/APC, Anti-CD39 BV421/PE (1:100 in PBS 2% FBS). To confirm that the CD73 and CD39 antibodies were specific, isotype control antibodies were used at an equal concentration. These isotype control antibodies were Biolegend mouse PE anti-IgG2a/ mouse APC anti-IgG1 for CD73 and Biolegend mouse BV421 anti-IgG1 for CD39. After 15 min incubation on ice protected from light, cells were washed twice by centrifugation for 5 min at 1200 rpm and resuspension in PBS 2% FBS. Flow cytometry was performed using the BD Fortessa™ and BD™ High Throughput Sampler (HTS) instrument (BD Biosciences). Data analysis was done using FlowJo software V.10 (FlowJo, LLC).

Fibroblasts were plated in 24-well plates in the same ratio as the co-culture with NK cells 30,000 and cells detached with 0.25% trypsin (Wisent) for 3 minutes following the addition of DMEM media and Zombie NIR™ stain. CD39/CD73 expression was assessed by flow cytometry using Anti-CD39 BV421/PE and Anti-CD73 PE/APC antibodies using an HTS-enabled LSRFortessa (BD Biosciences).

2.2.2 Degranulation assay

NK cells previously cocultured with pre-senescent or senescent fibroblasts and supplemented with IL-2 (200 IU/mL), were put in contact with their targets K562 cells, in a ratio of 10(NK)/1(K562) and the CD107 antibody was added at the start of the incubation. Cells were centrifuged for 30 seconds at 1100 rpm RT followed by 1 h of incubation at 37°C. After 1h, BD GolgiStopTM reagent was added in a dilution of 1:10 in

RPMI medium, and cells were incubated for 3 h. The expression of the CD107a/b degranulation marker was assessed by flow cytometry using an anti-CD107a/FITC conjugated antibody.

2.3 Adenosine production and nucleotide consumption

10,000 NK cells were cultured with 30,000 pre-senescent and senescent HDF in a (1 NK / 3 HDF) ratio in RPMI-1640 (no serum) at 37°C and 5% CO_2 in 24-well plates (Costar Corning) in the presence or absence of AMP (20 mM). Supernatants were collected after 1 h, and a solution of acetonitrile (40%), methanol (40%), and RPMI media (20%) previously chilled at 4°C was added to stabilize adenosine. Samples were then centrifuged at 1,200 rpm at room temperature for 5 min and supernatants were collected and stored at -80°C until use. The presence of adenosine, AMP, ATP, and NAD+ was investigated by HPLC at Centre de Recherche du CHUM (CRCHUM).

2.4 Statistical Analysis.

Statistical analyses were performed with GraphPad PRISM 9.0 software (GraphPad Software, La Jolla, USA) using one-way or 2-way ANOVA test with multiple testing to determine statistical significance.

3 Results

3.1 Characterization of adenosine pathway markers CD39 and CD73 in pre-senescent and senescent HDF

CD73 is a surface marker that in normal conditions is expressed in different tissues and cell types such as mesenchymal cells, endothelial cells, and fibroblasts. CD73 is overexpressed in different types of cancer and, consequently, excess adenosine is known to contribute to immune evasion by promoting an immunosuppressive environment. In the present work, we analyzed the expression of the CD39/CD73 markers (or enzyme) in normal or senescent HDF (Figure 1, panels A-C). We also analyzed the expression in NK

cells, the NK-92 cell line, and NKAES, both in co-culture or not with senescent HDF, as shown in Figure 2, panels A-E, Figure 3, panels A-E, and Figure 4, panels A-E, respectively.

Figure 1A outlines the protocol for inducing senescence and analyzing CD73/CD39 expression. The proportion of HDF expressing CD73 is close to 100% for all cell types analyzed (Figure 1B). However, the induction of senescence resulted in a significant increase in the expression of CD73; this effect was more pronounced in $hRAS^{v12}$-transduced HDFs (Mean fluorescence intensity, MFI= 142,971) than in irradiated HDF (MFI= 85,478) (Figure 1C). Of note, the expression of CD39 was not detected in either senescent or pre-senescent HDFs (data not shown). Our data demonstrate that only the expression of CD73 is upregulated in a senescent context, especially in HDF induced to senesce with the $hRAS^{v12}$ oncogene.

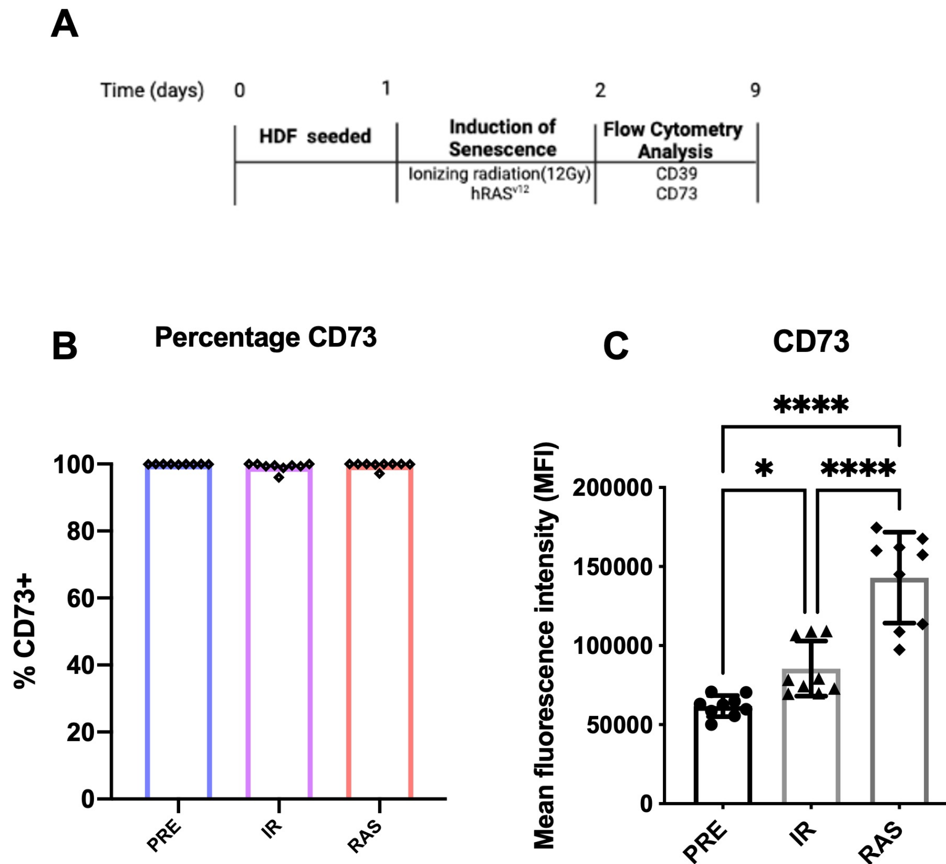


Figure 1. Expression of CD73 on HDF. (A) Schematic approach of cell culture and senescence induction. (B) HDF were induced to senesce and the proportion of cells expressing CD73 (left panel) and (C) the level of expression (right panel) was measured by flow cytometry. The MFI was calculated for the CD73 and

CD39-positive cell populations after subtracting the MFI of their respective isotype controls. HDFs were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior to flow cytometry analysis. Graphs show the data of three independent experiments (n=3). One-way ANOVA test with multiple testing was performed. The p values are indicated when differences are significant (****$p \leq 0.0001$, *$p \leq 0.05$).

3.2 Senescent HDF induce CD73 expression in primary NK, NKAES and NK-92 cells.

Here we wanted to determine if the co-culture of HDF with NK cells would affect the expression of CD39/CD73 at the surface of NK cells. As shown in Figure 2A, we established a co-culture protocol of primary NK cells with hRas-transduced HDF. Our results demonstrate a significant increase in both the proportion and expression levels of CD73 on individual cells (Figure 2B and 2C). Conversely, co-culturing with pre-senescent and irradiated HDF did not lead to a comparable increase in CD73 expression (Figure 2C). Similarly, the proportion of primary NK cells expressing CD39 was more pronounced only when cells were placed in co-culture with hRas-transduced HDF (Figure 2D and 2E).

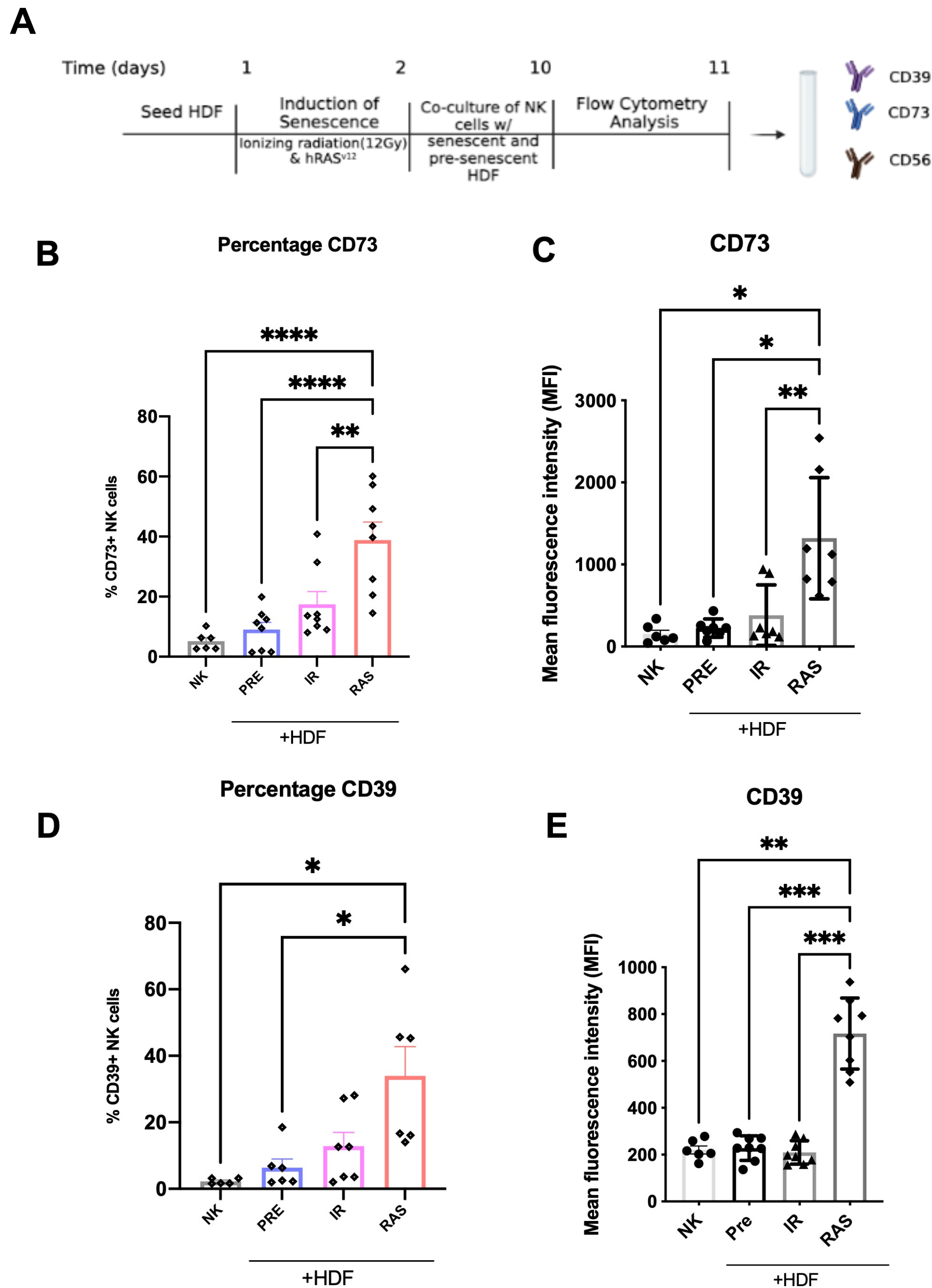


Figure 2. Expression of CD73 and CD39 on primary NK in co-culture with HDF. (A) Schematic approach to evaluate CD73 and CD39 Expression on NK Cells**.** NK cells were cultured overnight and stimulated with IL-2 (200 IU/mL), in the presence and absence of senescent and pre-senescent HDF. HDFs were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior the co-culture with NK cells. Proportion of cells expressing (B) CD73+ and (D) CD39+ and the level of expression (C) CD73+ and (E) CD39+ was measured by flow cytometry. The MFI was calculated for the CD73 and CD39-positive cell populations after subtracting the MFI of their respective isotype controls. Graphs show the data of three independent experiments from one donor (n=3). One-way ANOVA test with multiple testing was performed. The p values are indicated when differences are significant (****p ≤ 0.0001, ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05).

We conducted a second iteration of the experiment, following the protocol outlined in Figure 3A and utilizing the NK-92 cell line. The expression of NK-92 exposed to pre-senescent and irradiated HDFs remained relatively stable, but, as depicted in Figures 3B and 3C, the expression and proportion of CD73 was significantly increased in NK-92 cells exposed to hRAS-transduced HDF (MFI=13,237) compared to pre-senescent human dermal fibroblasts (MFI= 1,546).

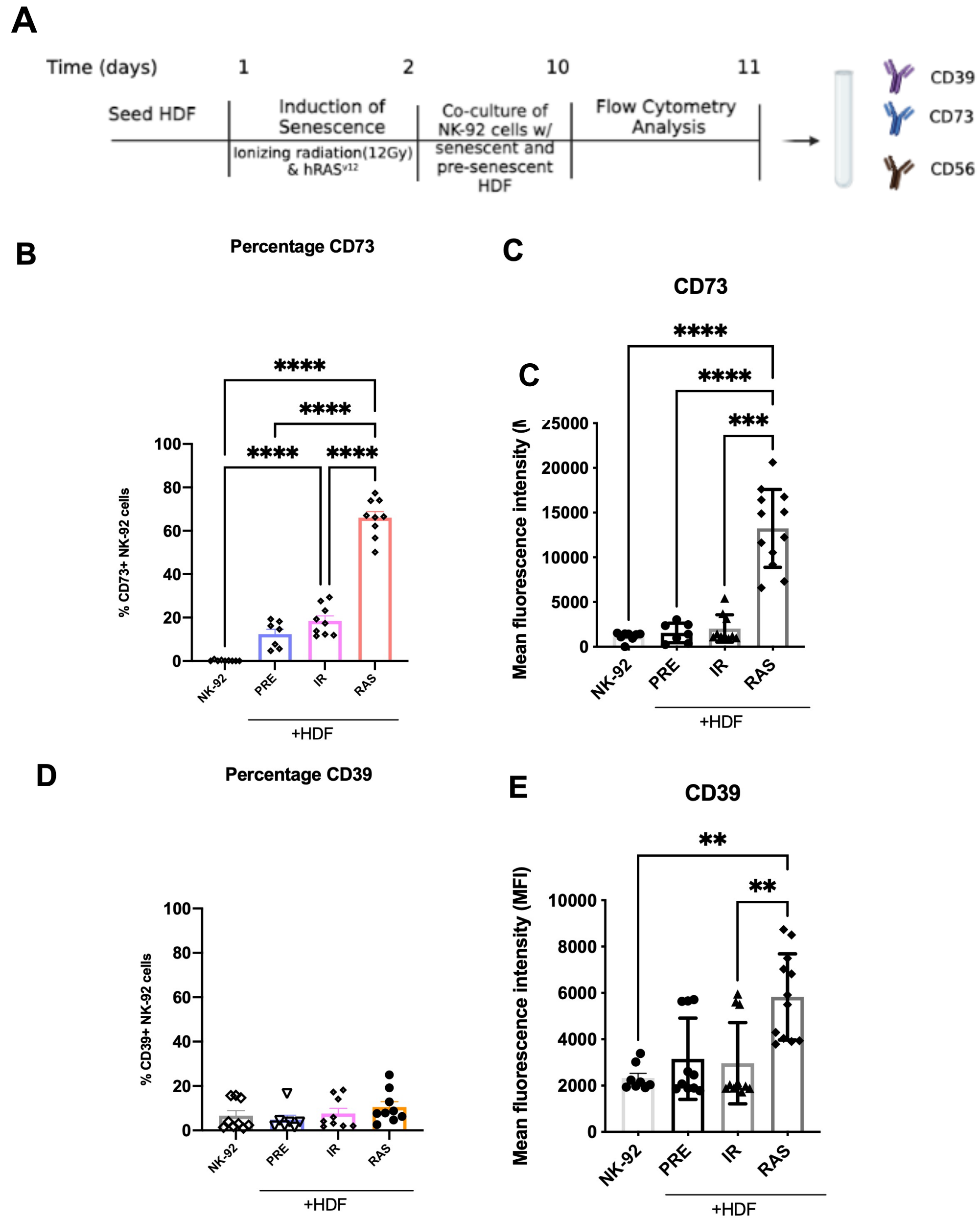


Figure 3. Expression of CD73 and CD39 on NK-92 cells in co-culture with HDF. (A) Schematic approach to evaluate CD73 and CD39 expression on NK-92 cells. NK-92 cells were cultured overnight and stimulated with IL-2 (200 IU/mL), in the presence and absence of senescent and pre-senescent HDF. HDFs

were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior the co-culture with NK-92 cells Proportion of cells expressing (B) CD73+ and (D) CD39+ and the level of expression (C) CD73+ and (E) CD39+ was measured by flow cytometry. The MFI was calculated for the CD73 and CD39-positive cell populations after subtracting the MFI of their respective isotype controls. Graphs show the data of three independent experiments (n=3). One-way ANOVA test with multiple testing was performed. The p values are indicated when differences are significant (****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$).

The percentage of CD73+ cells was also higher in NK-92 cells in co-culture with hRAS-transduced HDF (66%) compared to pre-senescent HDF (12%) (Figure 3B). The percentage of CD39-positive cells did not differ significantly among the different conditions, as shown in Figure 3D. However, as depicted in Figure 3E, the expression of CD39 on NK-92 cells was not significantly different between pre-senescent HDFs and hRAS-transduced HDFs.

Following the same protocol outlined previously, we analyzed the expression of CD39 and CD73 markers on NKAES cells, as depicted in Figure 4A. Our results demonstrated a slight variation in the percentage of CD73-positive cells between NKAES cells exposed to pre-senescent HDF and hRAS-transduced HDF for donor 1, as shown in Figure 4B. Similarly, the expression of CD73 was higher in NKAES cells exposed to HDF, with almost a 2-fold increase in CD73 expression in cells exposed to hRAS-transduced HDFs (MFI=14,153) compared to pre-senescent HDFs (MFI=7,045) and a 6-fold increase compared to NKAES alone (MFI=2,048) for donor 1 (Figure 4C).

Figure 4C shows a slight increase in CD73 expression on NKAES cells exposed to irradiated HDFs (MFI=11,492) compared to pre-senescent HDFs, although this difference was not statistically significant. We did not observe any difference in the percentage of CD73-positive cells in the different conditions for donor 2 (Figure 4D). However, as shown in Figure 4E, we noticed a slight increase in CD73 expression on NKAES between pre-senescent and hRAS-transduced HDF, but this difference was not statistically significant.

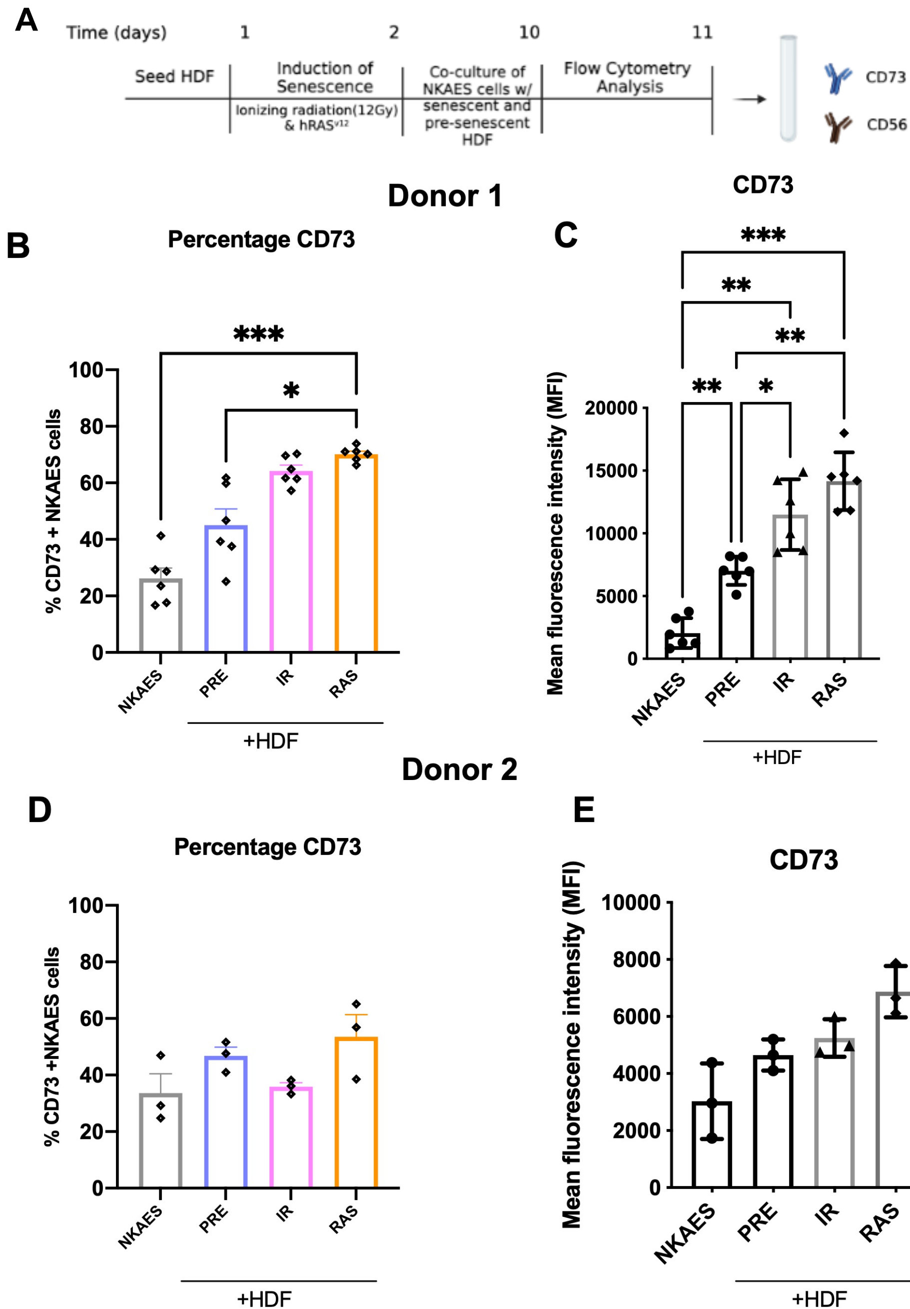
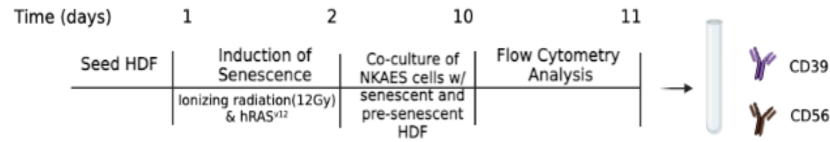


Figure 4. Expression of CD73 on expanded NK cells (NKAES) in co-culture with HDF. (A) Schematic approach to evaluate CD73 Expression on NKAES. NKAES cells were cultured overnight and stimulated with IL-2 (200 IU/mL), in the presence and absence of senescent and pre-senescent HDF. HDFs were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior the co-culture with NKAES cells. Proportion of cells expressing CD73+ panel (B) and (D) and the level of expression CD73+ panel (C) and (E), was measured by flow cytometry. The MFI was calculated for the CD73 and CD39-positive cell populations after subtracting the MFI of their respective isotype controls. (B) and (C) Donor 1 and (D) and (E) Donor 2. Graphs show the data of two independent experiments for Donor 1 (n=2) and one experiment for Donor 2 (n=1). One-way ANOVA test with multiple testing was performed. The p values are indicated when differences are significant (****p ≤ 0.0001, ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05).



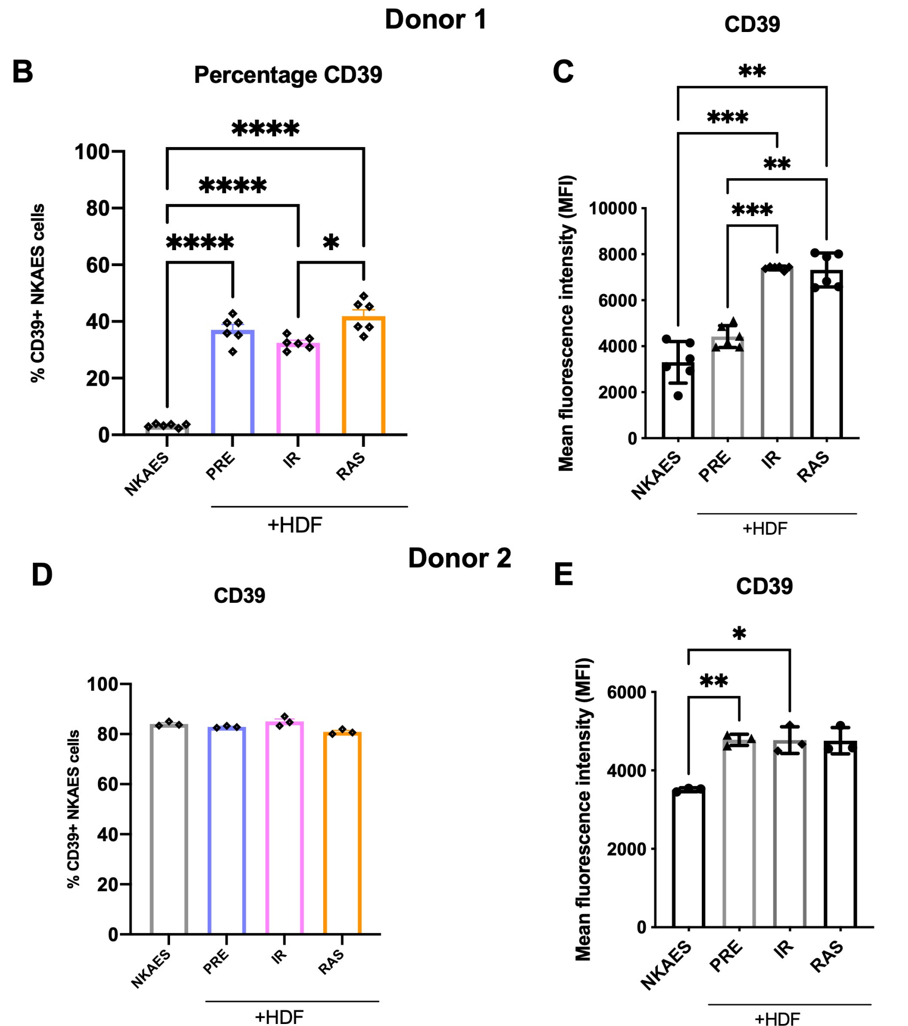


Figure 5. Expression of CD39 on expanded NK cells (NKAES) in co culture with HDF. (A) Schematic approach to evaluate CD39 expression on NKAES. NKAES cells were cultured overnight and stimulated with IL-2 (200 IU/mL), in the presence and absence of senescent and pre-senescent HDF. HDFs were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior the co-culture with NKAES cells. Proportion of cells expressing CD73+ panel (B) and CD39+ (D) and the level of expression CD73+ panel (C) and CD39+ (E), was measured by flow cytometry. The MFI was calculated for the CD73 and CD39-positive cell populations after subtracting the MFI of their respective isotype controls. (B)-(C) Donor 1 and (D)-(E) Donor 2. Graphs show the data of two independent experiments for Donor 1 (n=2) and one for Donor 2 (n=1). One-way ANOVA test with multiple testing was performed. The p values are indicated when differences are significant (****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$).

To evaluate the expression of CD39 on NKAES cells, we followed the same protocol as the previous experiment (Figure 5A). Our results revealed that the percentage of CD39-positive cells showed a slight increase in NKAES exposed to hRAS-transduced HDFs, compared to NKAES co-cultured with irradiated HDFs, as illustrated in Figure 5B. A significant increase in CD39 expression was observed on NKAES exposed to both irradiated HDFs (MFI=7,403) and HDFs transduced with hRAS, compared to pre-senescent HDFs (MFI=4,421), as shown in Figure 5C. However, in Donor 2, we did not observe any variation in the percentage of CD39-positive cells (Figure 5D), and there was no significant difference in NKAES expression levels following co-culture with pre-senescent or senescent HDFs (Figure 5E).

According to the literature, NK cells express CD39, but less than 1% of CD73[104]. Our data demonstrates that CD39 and CD73 are highly expressed on the surface of primary NK cells, NK-92 and NKAES, when exposed to HDF. However, the highest expression of both markers was observed when NK cells were exposed to hRAS-transduced cells. This suggest that both ectoenzymes may be enzymatically active and able to hydrolyze ATP to adenosine primarily in a senescent environment and promote NK cell inhibition. It would be interesting to further investigate the mechanisms that allow CD39/CD73 expression in NK cells.

3.3 Adenosine production by NK-92 cells and HDF.

The adenosine pathway involves the collaboration of CD39 and CD73 ectoenzymes to generate extracellular adenosine. First, CD39 phosphohydrolysis of ATP and ADP and then CD73 converts AMP into adenosine. Here, we wanted to analyze the functional activity of both enzymes by measuring the generation of extracellular adenosine under different conditions: pre-senescent and senescent HDF in presence and absence of AMP and NK-92 cells co-cultured with pre-senescent and senescent HDF and treated with AMP.

As shown in Figure 6, adenosine was detected in all samples. hRAS-transduced HDF produce 2.59-fold more adenosine than pre-senescent HDF.

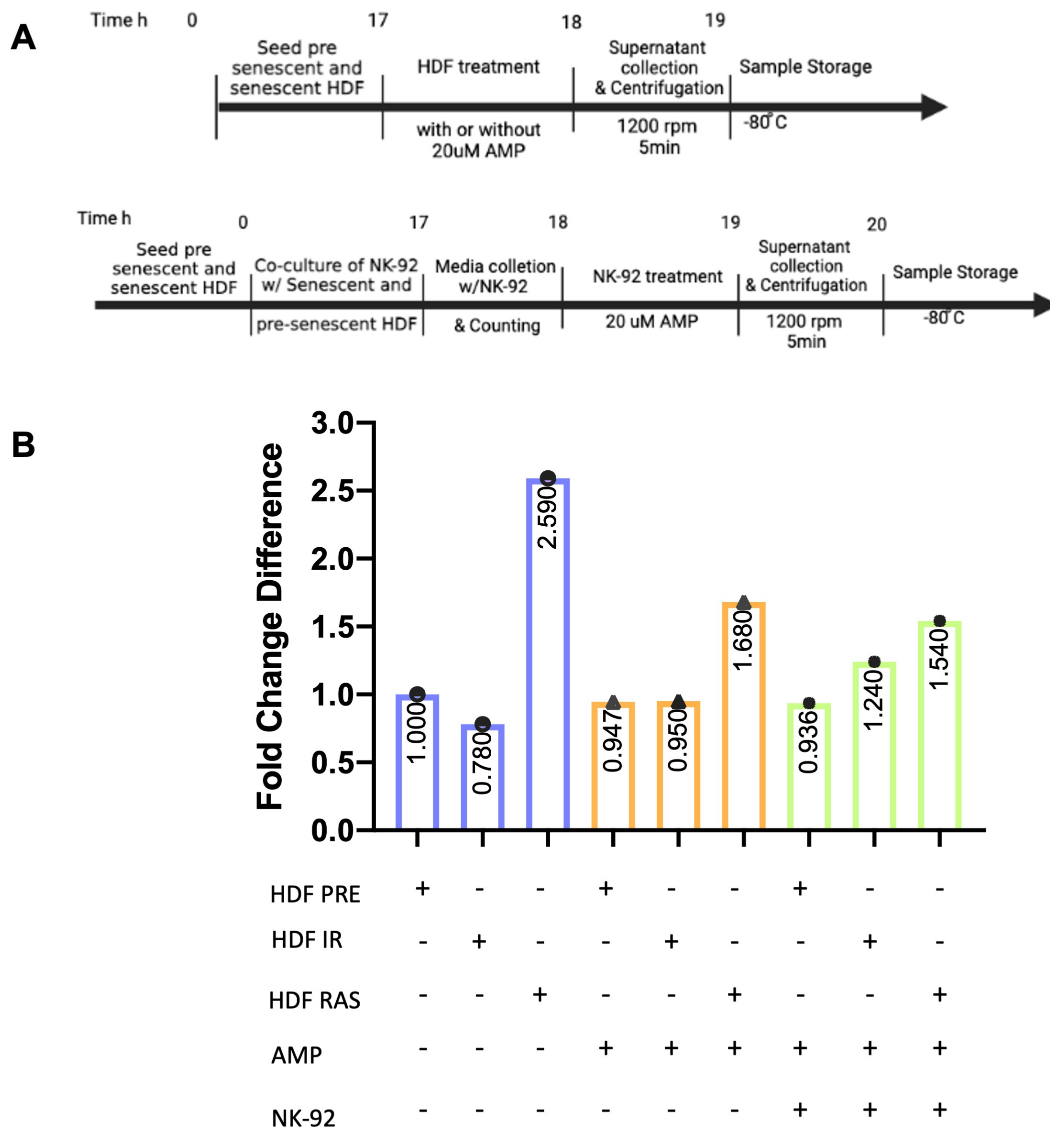


Figure 6. Adenosine quantification in culture media collected from pre-senescent and senescent HDF alone or upon AMP treatment and co-culture with NK-92 cells. (A) Schematic timeline of cell treatment and sample storage for HPLC analysis. (B) Production of adenosine was assessed in pre-senescent and senescent HDF and on NK-92 cells previously co-cultured overnight with senescent and pre-senescent HDF. HDFs were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior the co-culture with NK-92 cells. NK-92 and HDFs cells were cultured for 1 h in the presence or absence of AMP 20μM. Adenosine generation was analyzed using HPLC. Graphs show the mean of two different experiments for HDFs cultured with AMP and co-cultured with NK-92 cells (n=2) and one for HDFs cultured without any treatment (n=1). Values were normalized to untreated pre-senescent HDFs for each condition.

Importantly, addition of AMP to pre-senescent HDFs or co-culture of HDFs with NK-92 cells did not significantly alter the production of adenosine. These preliminary results

suggest that only hRAS-transduced HDFs have the potential to generate a higher amount of adenosine (Figure 6). The fact that the addition of AMP did not lead to an increased production of adenosine suggests that a sufficient amount of AMP was present in the media likely produced from the hydrolysis of ATP or direct release by the cells.

3.4 Adenosine inhibits NK cell degranulation upon exposure to different HDFs

Once the effect of senescent HDFs on CD73 expression and adenosine production was established and since adenosine was shown to strongly inhibit the cytotoxicity capacity of NK cells by binding to the A2AR[150], we wanted to determine the effect of senescent HDF on NK cell cytotoxicity. To do so, we measured NK-92 cell activity by investigating expression CD107a at the cell surface. CD107a is a marker specific to activation and degranulation of NK-92 cells. It has been described as one of the main pathways by which these cells carry out their effector functions.

NK-92 cytotoxic activity was measured by performing degranulation assay against K562, a cancer cell line highly sensitive to NK cells. NK-92 cells were co-cultured overnight in the presence or absence of pre-senescent and senescent HDF and in the presence/absence of 20uM AMP. NK cells were also cultured in the presence of CADO (20uM), an analog of adenosine used as a positive control to observe the effect of adenosine on the cytotoxicity abilities of NK-92 cells against K562 (Figure 7A).

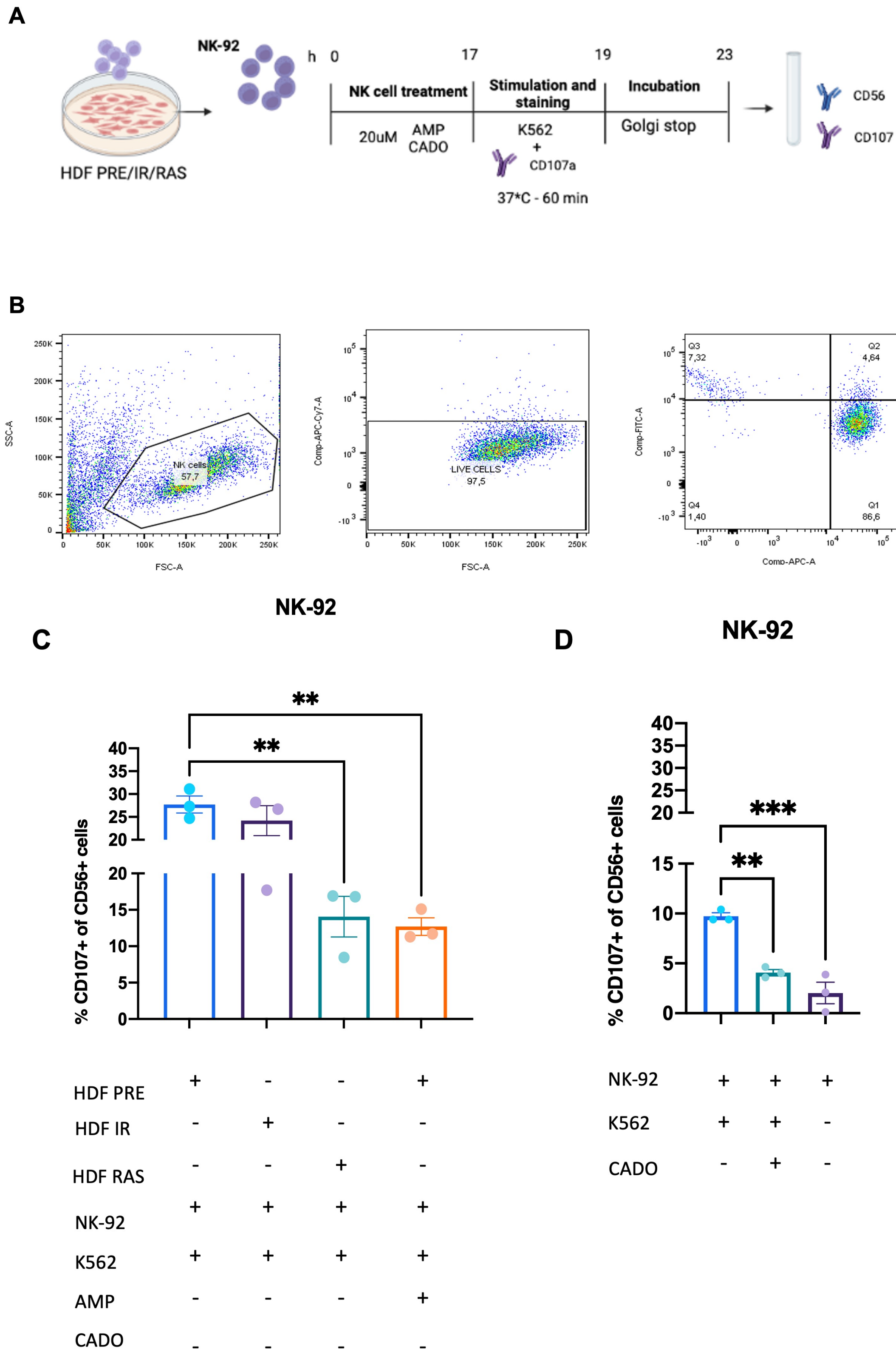


Figure 7. Degranulation of NK-92 exposed to HDFs. (A) Schematic approach to evaluate degranulation activity on NK-92 cells. (B) Flow cytometry plot representative of 107a expression after NK-cell degranulation assay against K562 cells. The plot also displays APC-Cy7-Live/Dead staining for cell

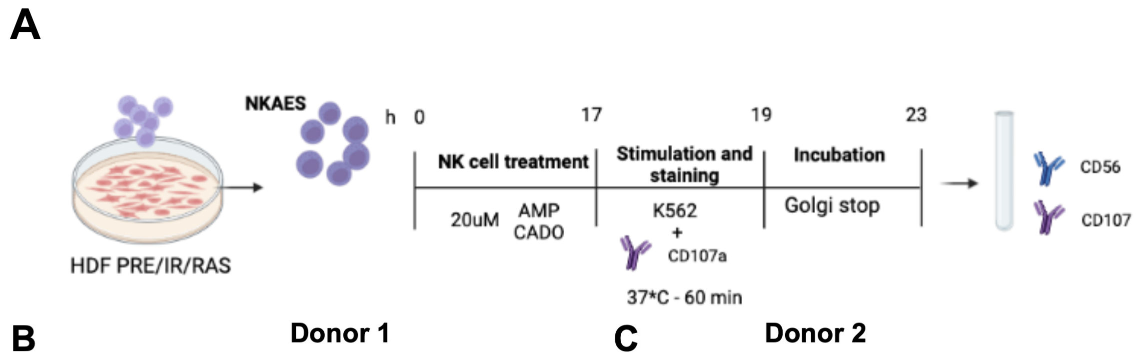
viability, APC for CD56+ cells, and FITC for CD107a+ cells. (C)-(D) Degranulation of NK-92 in presence or not of K562 was investigated after coculture of pre-senescent irradiation-induced senescent or hRAS-induced senescent fibroblasts in the absence or presence of AMP and CADO (20uM). HDFs were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior the co-culture with NK-92 cells. NK-92 were stained and CD107a/b expression on the surface of NK cells was analyzed by flow cytometry. Graphs show the mean of three different experiments (n=3). 2-way ANOVA test with multiple testing was performed. The p values are indicated when differences are significant (***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05).

The percentage of CD107a+ cells was evaluated by flow cytometry (Figure 7B). Our results in Figure 7C indicate that NK-92 cells exposed to hRAS-induced senescent fibroblasts exhibited a lower degranulation level compared to those exposed to pre-senescent fibroblasts. Additionally, when we treated NK-92 cells with pre-senescent fibroblasts with AMP, we surprisingly observed a lower degranulation level compared to NK-92 cells alone co-cultured with pre-senescent fibroblasts (12% and 27% CD107a+, respectively). These findings suggest that AMP could inhibit the cytolytic activity of NK cells. In Figure 7D, we can observe that NK-92 cells cultured alone and exposed to CADO exhibit a significantly reduced level of degranulation compared to cells that were not exposed to it.

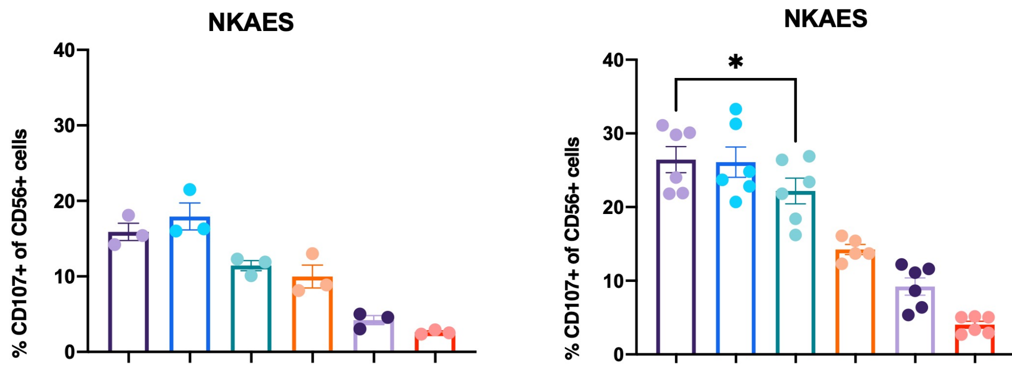
Similarly, to what we did with NK-92 cells, we repeated the degranulation assay using NKAES cells. The number of cells required to perform the experiment prevented us from using primary NK cells in the absence of in vitro expansion. We also decided to treat the cells with AMP so as not to have a limited amount of adenosine generated by fibroblasts. Figure 8A illustrates the protocol followed to perform the degranulation assay. Our results showed no significant decrease in the degranulation of NKAEAS for donor 1 (Figure 8B), while there was a slight decrease in degranulation of cells exposed to HDF for donor 2 (Figure 8C), nevertheless this difference is not statistically significant. This may be due to the limited number of replicates. However, CADO-treated cells demonstrate a marked reduction in degranulation, with a decrease of almost 50% compared to cells without CADO treatment (5.0% versus 10.2%; see Figure 8B). This suggests that NKAES cells are also sensitive to the effects of adenosine. Although not statistically significant, a slight decrease in the percentage of degranulation was observed in hRAS-transduced HDF-exposed cells compared to pre-senescent control cells (11.4% versus 15.9% for Donor 1 and 22.8% versus 26.4% for Donor 2; see Figure 8B and 8C).

Overall, these results indicate that the percentage of CD107a expression was slightly decreased in NK-92 and NKAES cells co-cultured with senescent HDFs, particularly hRAS-transduced HDFs. In addition, NK cells pre-cultured with pre-senescent HDFs and treated with CADO exhibited a lower level of degranulation compared to pre-senescent HDFs alone.

A



B **Donor 1** **C** **Donor 2**



	B1	B2	B3	B4	B5	B6	C1	C2	C3	C4	C5	C6
HDF PRE	+	-	-	-	-	-	+	-	-	-	-	-
HDF IR	-	+	-	-	-	-	-	+	-	-	-	-
HDF RAS	-	-	+	-	-	-	-	-	+	-	-	-
NKAES	+	+	+	+	+	+	+	+	+	+	+	+
K562	+	+	+	+	+	-	+	+	+	+	+	-
AMP	+	+	+	-	-	-	+	+	+	-	-	-
CADO	-	-	-	-	+	-	-	-	-	-	+	-

Figure 8. Degranulation of expanded NK cells (NKAES) exposed to HDFs. A) Schematic approach to evaluate degranulation activity on NKAES. (B)-(C) NKAES cell degranulation in presence or not of K562

was investigated after coculture of pre-senescent, irradiation-induced senescent or hRAS-induced senescent fibroblasts in the absence or presence of AMP and CADO (20uM). HDFs were irradiated at 12 Gray or transduced with an hRas lentivirus 8 days prior the co-culture with NK-92 cells. Cells were stained and the expression of the CD107a marker on the surface of NKAES cells was analyzed by flow cytometry. Graphs show the data from three independent experiments: (B) Donor 1 (n=1), (C) Donor 2 (n=2). 2-way ANOVA test with multiple testing was performed. The p values are indicated when differences are significant (*p ≤ 0.05).

3.5 CD39 and CD73 expression on T cells and monocytes upon exposure to pre senescent and senescent HDF

Given we previously observed that exposure of NK cells to HDF has an impact on CD73 expression, we next wanted to investigate CD39 and CD73 expression in other immune cell types such as T cells and monocytes. PBMCs were obtained from healthy donors and co-cultured with senescent and pre-senescent HDFs. CD3+, CD14+, and CD56+ cells were gated by flow cytometry and expression of CD39/73 was analyzed. We decided to look at the expression of both markers again in CD56+ cells within the context of full PBMCs to investigate whether the interaction with other immune cells affected the expression of CD39 and CD73.

As observed with NK cells alone, CD73 expression was also found higher in gated CD3+ and CD56+ cells in the presence of HDFs induced to senescence with $hRAS^{v12}$ (Figure 9A and 9B). However, CD39 expression does not seem to be significantly different in any conditions (Figure 9).

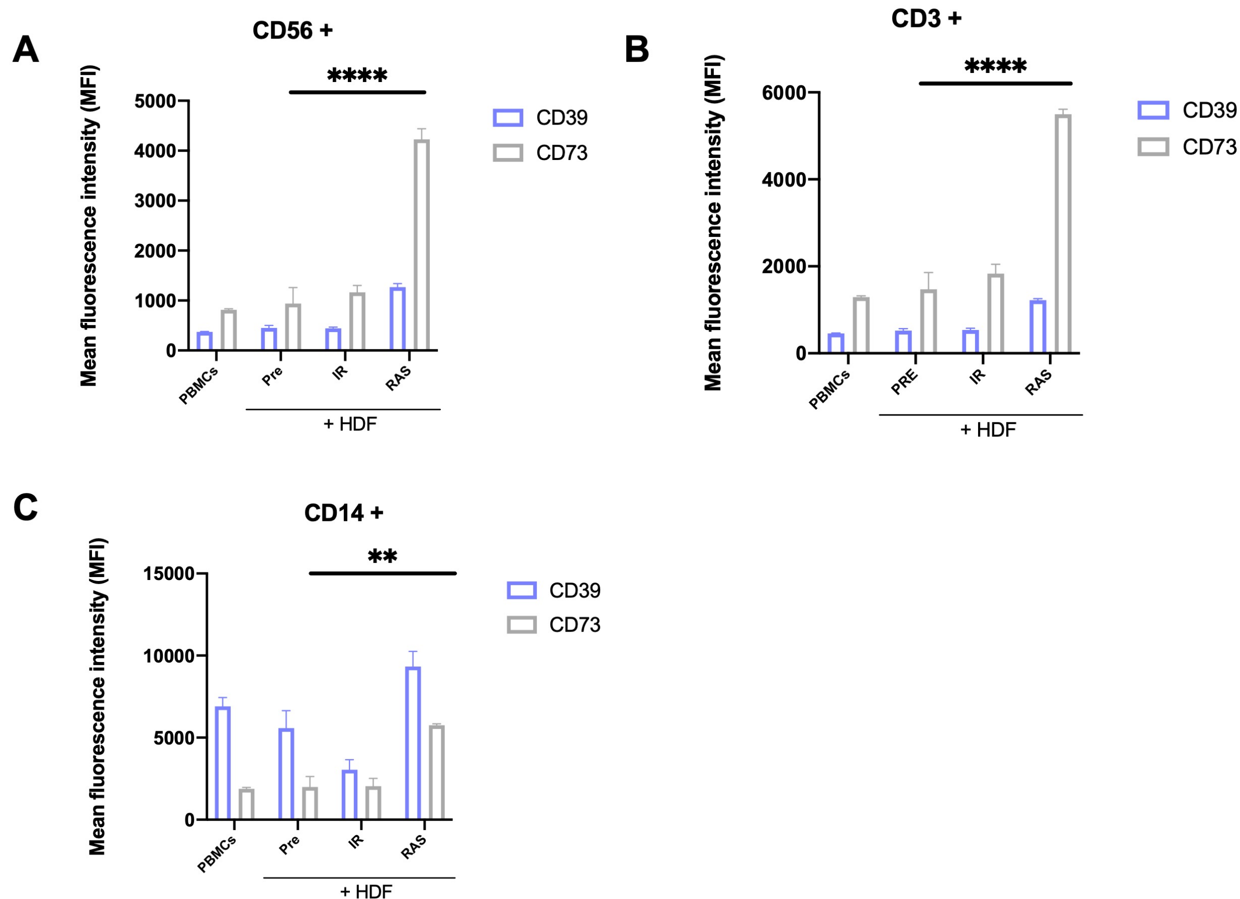


Figure 9. CD39 and CD73 expression on CD3+, CD14+ and CD56+ cells exposed to HDFs. CD73 and CD39 expression was analyzed using flow cytometry (A) CD56+, (B) CD3+, (C) CD14+. The MFI is shown after subtraction of the MFI of the respective isotype. Graphs shown the data of three independent experiments (n=3) from the same donor. One-way ANOVA test with multiple testing was performed.

3.6 Transduction of BaEv-LV A2AR KO on HEK 293T cells.

It was shown that binding of A2AR to adenosine provides a checkpoint that negatively regulates the development of functionally mature NK cells[150]. To further investigate this mechanism, we generated a BaEv-LV A2AR KO virus and successfully transduced 293 T cells with the virus, followed by antibiotic selection with puromycin. However, due to time constraints, we were unable to confirm the genetic modification via DNA sequencing. In future studies, it is planned to perform transduction in primary NK cells and NKAES to verify the impact of A2AR deletion on their effector functions.

4 Discussion

The ability of the immune system to clear senescent cells declines with age, leading to the accumulation of these cells in tissues possibly contributing to the development of age-related diseases, such as cancer. The adenosine pathway has emerged as an important mechanism involved in immune evasion. Indeed, adenosine can act as an immunosuppressive molecule promoting tumor growth and progression[129].

CD73 expression has been investigated in multiple cell types, including endothelial cells, cancer cells, mesenchymal stem cells, and immune cells such as T and B cells. The main function of CD73 is to produce adenosine in cooperation with CD39. Adenosine has been shown to impair the effector activity of immune cells such as CD8+ T cells[151]. Moreover, co-culture of mesenchymal cells with NK cells was shown to increase the expression of CD39/73 at the surface of NK cells affecting their cytolytic function[104].

In this study, we aimed to examine the interaction between NK cells and senescent cells through the expression of CD39/73 and adenosine production. To achieve these objectives, we used a model of HDF with a well-defined senescence response, in combination with various sources of NK cells, that is primary NK cells isolated from peripheral blood, primary NK cells following in vitro expansion (NKAES) and a well-defined NK-92 cell line.

4.1 Adenosine production coincides with CD73 expression in senescent HDF

Tumor cells in the TME are often enclosed by fibroblasts, which are a predominant cell type responsible for producing most extracellular components in the TME. In addition, some cells within this environment overexpress CD73. Therefore, to better understand the role of fibroblasts in the TME, we sought to investigate the expression of CD39 and CD73 in both pre-senescent and senescent fibroblasts.

We aimed to induce senescence in HDFs by allowing for an 8-day induction period before co-culture with NK. Our results indicated the successful induction of a senescent phenotype in the HDFs, as exposure to either 12 Gy or transduction of HDFs with $hRAS^{v12}$ resulted in the expression of SAβ-gal in HDFs (data not shown).

Our findings demonstrate that CD73 expression is significantly increased in senescent fibroblasts induced by $hRAS^{v12}$ compared to pre-senescent fibroblasts or fibroblasts that underwent senescence following exposure to ionizing irradiation. In contrast, CD39 expression was not detected in either senescent or pre-senescent HDFs. Although there was a trend towards higher adenosine production in senescent fibroblasts compared to non-senescent fibroblasts, this difference was not significant. The increased production of adenosine in senescent fibroblasts and upregulation of CD73 may be due to faster conversion of its precursor, AMP into adenosine[152].

Previous research has shown that overexpression of CD73 is associated with chronic inflammation in various models[153 88]. Therefore, we hypothesize that the pro-inflammatory phenotype of senescent cells, known as the SASP, may be responsible for the upregulation of CD73. SASP is characterized by the production of various pro- and anti-inflammatory cytokines and chemokines, which may contribute to this mechanism[104,154,155]. To assess the impact of the SASP on CD39/73 expression, it would be pertinent to analyze the supernatant of senescent HDFs by luminex assay in order to identify the specific SASP components, such as cytokines, chemokines, and microvesicles, that may be involved in this mechanism. Transforming growth factor beta (TGFB) is a key component of the SASP and plays an important role in the regulation of immune response[156 157] . Recent studies have demonstrated that TGFB can upregulate the expression of CD73 on both CD4+ and CD8+ T cells[158]. This finding is of significance as it highlights the crucial role of the TGF-β-CD73 axis in regulating the expression and function of immune cells, as well as in modulating immune response and inflammation.

4.2 Impact of senescent HDFs on the induction of CD39 and CD73 on NK cells

Our results show that senescent HDFs have a higher ability to induce CD73 expression on the surface of NK cells compared to non-senescent HDFs. However, hRAS-transduced HDFs appear to be more effective at inducing CD73 expression in NK-92, physiological NK cells, and NKAES. Notably, the expression levels of both CD39 and CD73 markers were significantly higher in NK-92 and NKAES cells than in primary NK, potentially attributed to the genetic modifications of NK-92 cell line and molecular changes in NKAES during the expansion process, in addition to the cytokines that were added to the medium, such as IL-2[159]. Importantly, co-culture of NK cells with HDF was performed overnight, with additional experiments performed for 48 hours to assess possible variations in NK expression of CD39 and CD73 markers (data not shown), yet no significant differences in CD39 and CD73 expression were observed between these two time points.

Several studies have indeed described the acquisition of CD73 expression in immune cells, including monocytes, CD8+ T cells and NK cells upon interaction with MSCs and cancer cells[104,160,108]. It has been demonstrated that in an inflammatory environment, such as in cancer, the overexpression of CD73 on immune cells like CD8+ T cells can lead to the conversion of effector CD8 T cells into immunosuppressive CD8+ T cells[108]. A previous study has shown that NK cells are the primary immune cells responsible for eliminating senescent cells, helping to reduce their accumulation and the negative effects they can have on tissues[41]. However, our findings suggest that senescent HDFs may upregulate CD73 expression on NK cells, potentially enabling senescent cells to evade immune surveillance as it is well established that extracellular adenosine can be produced through the canonical pathway involving CD39 and CD73.

Additionally, an alternative pathway for adenosine production, independent of CD39, involves the CD38 and CD203a enzymes[161]. CD38, also known as nicotinamide adenine dinucleotidase (NAD), metabolizes extracellular NAD+ to produce ADPR, which is then converted to AMP by CD203a. AMP is then further converted to adenosine by CD73. This alternative pathway highlights the complexity of extracellular adenosine production and

underscores the importance of understanding the various mechanisms involved in this process. It would be of interest to investigate whether the induction of CD73 expression on the surface of NK cells requires direct cell-cell contact with HDFs or if there is some other transcriptional mechanism involved in the interaction between these two cell types and the pathways that trigger the expression of CD73 and subsequent generation of adenosine. For example, to confirm whether the expression of CD73 is generated at the transcriptional level, we could perform qPCR or western blot analysis.

4.3 Senescence induction reduce degranulation activity in NK-92 and NKAES cells

As previously discussed, adenosine has been demonstrated to modulate the activity of various immune cells and previous studies have shown that CADO, an analogue of adenosine, can inhibit the function of immune cells including NK and B cells[162,104]. We investigated the impact the increased expression of CD73 and adenosine production may have on NK cell cytotoxicity. We exposed NK cells to senescent and pre-senescent HDFs and proceeded to measure their degranulation capacity. To ensure adenosine production by pre-senescent and senescent HDFs was not limited, we choose to supplement HDFs with AMP. We observed that NK cells exposed to pre-senescent HDFs alone were not able to reduce the frequency of the CD107a expression compared to NK treated with CADO.

Our findings also suggest that pre-incubation with HDF may activate NK cells and contribute to their ability to kill target cells. This is supported by the increased percentage of NK cell degranulation observed when cells are exposed to fibroblasts, compared to the percentage of degranulation in NK cells cultured alone against their target K562. Although the data presented in Figure 6 do not demonstrate significant adenosine production when pre-senescent fibroblasts are treated with AMP, it seems that during the degranulation assay there is an inhibition in the cytolytic capacity of the cells. It would be interesting to investigate whether K562 target cells are involved in this phenomenon or what mechanism during the co-culture/incubation may generate this inhibition.

In conclusion, our data show a decrease in degranulation upon exposition of NK cells to senescent fibroblasts, particularly hRAS-transduced HDFs compared to pre-senescent fibroblasts. These findings are consistent with previous evidence that adenosine binding to the A2AR leads to an increase in intracellular cAMP, which in turn diminishes the cytotoxic activity of NK cells.

4.4 Increased CD73 expression in T cells and monocytes upon exposure to senescent HDF

Since several immune cell types interact in the tumor microenvironment, we wanted to investigate the expression of CD39 and CD73 in other immune cells. PBMCs were isolated from healthy donors and exposed to pre-senescent and senescent fibroblasts. We performed an analysis of T cells, monocytes, and NK cells expression to investigate whether interactions with pre-senescent and senescent fibroblasts resulted in any changes in CD39 and CD73 expression. Our results showed that CD3 and CD14 cells had increased CD73 expression when exposed to hRAS-transduced HDFs. The expression of both markers in CD56+ NK cells was consistent with our previous results obtained using purified NK cells alone.

To summarize, our research showed that a senescent microenvironment can induce the expression of CD73 ectoenzyme in immune cells, particularly in NK cells. This leads to adenosine production which reduces the cytolytic capacity of NK cells. If such a mechanism occurs in vivo it could potentially contribute to immunoevasion and thus provide mechanistic insights into how the accumulation of senescent cells may favor tumor growth.

4.5 Perspectives

The results we obtained in this project are important as they shed light on the intricate interactions between senescent cells, immune cells, and cancer cells. Indeed, senescence induction in normal cells (for example stromal or endothelial cells) or cancer cells themselves during the course of therapy could enhance overall tumor progression

by inhibiting immune cells using a mechanism implicating adenosine production. Therefore, it is essential to conduct additional experiments that will allow us to better understand the mechanisms underlying the expression of CD39/CD73 enzymes and adenosine production by immune cells in a senescent tumor microenvironment.

Although we were unable to fully explore all aspects of our project due to time constraints, one possible hypothesis for CD39 and CD73 expression on immune cells, particularly on NK cells, in a senescent context, is that the expression of these two ectoenzymes may be regulated by the SASP. Previous research has shown that the SASP can release exosomes in addition to various pro-inflammatory molecules, such as cytokines, chemokines, and growth factors[163]. These exosomes have the ability to mediate intercellular communication and influence the responses of nearby cells, potentially impacting their fate[163]. Some studies have suggested that exosomes released by certain types of cancer cells can stimulate CD73 on immune cells leading to suppression of immune responses[164]. On the other hand, it has been shown that tumor-derived exosomes can bind sequestering antibodies, leading to a reduction in NK-dependent ADCC[165]. No link between this property and CD73 activity in senescent cells has been explored. We hypothesis that by analyzing the components of the SASP and exosomes, we could gain a better understanding of how the expression of CD39/CD73 is induced and how it may have an impact on NK cell functions.

One of the main limitations of this study was the use of NKAES from a limited number of donors. To further validate our findings, it would be beneficial to include cells from an additional donor and to conduct at least three replicates for each donor to determine if the observed reduction in degranulation and CD39/CD73 expression by NKAES cells is donor dependent. Additionally, while NKAES cells have high cytolytic capacity and NK-92 cells have many activating receptors which can enhance their cytotoxicity upon stimulation with IL-2, it would be interesting to investigate if primary NK cells show a similar behavior.

As previously discussed, it is highly interesting to investigate the impact of the A2AR KO on the cytotoxic activity of NK cells in a senescent context. This can be assessed by

transducing primary NK or NKAES cells with a lentiviral vector that has been engineered to express both Cas9 and a guide RNA that specifically targets A2AR, and subsequently treating them with CADO. By confirming their sensitivity to adenosine and performing cytotoxicity assays, we can assess the effects of the A2AR on the function of NK cells. It will also be informative to determine whether senescent fibroblasts require direct cell-to-cell contact in order to induce CD73 expression in NK cells, and which mechanisms are involved in this process.

For our last objective, our research aimed to understand the role of adenosine in inhibiting NK cell activity. To achieve this, we wanted to generate NK cells that were deficient in A2AR expression, as we hypothesized that these cells would not be inhibited by adenosine. We started by generating A2AR-deficient induced pluripotent stem cells (iPSCs) and differentiating them into NK cells (referred to as iNKs), using a protocol developed by the iPSC platform at CHU Sainte-Justine. We achieved the deletion of A2AR in the iPSCs by using nucleofection to transfect a guide RNA and a recombinant Cas9 protein. Unfortunately, we encountered difficulties in amplifying the iNKs from the knockout iPSC clone, so we had to try an alternative strategy. Our next step was to transduce NKAES cells directly using a Baboon envelope pseudotyped lentiviral vector (BaEV-LVs) expressing the A2AR-targeting gRNA and Cas9 protein. Unfortunately, due to time constraints and difficulties in generating an efficient viral titer, we were unable to complete this experiment. In the future, we plan to use this alternative approach to generate A2AR-deficient NK cells and investigate the effects of adenosine on their cytolytic activity.

Finally, upon confirmation that A2AR-deficient NK cells exhibit enhanced cytotoxic activity against cancer cells in a senescent environment *in vitro*, the next step would be to investigate their ability to clear cancer cells *in vivo* using a mouse model containing senescent tumor cells. Such models have been developed in the laboratory of Dr Beauséjour[166]. However, evaluating the efficacy of NK cells *in vivo* is challenging as multiple injections of NK cells are required, and these cells require recombinant IL-15 to

survive in mice. Additionally, IL-15 has been shown to enhance the cytotoxicity activity and ADCC of NK[167].

4.6 Conclusions

Senescent cells have been shown to accumulate in the tumor microenvironment and have been linked to cancer progression and resistance to therapy. These cells secrete a variety of factors that can inhibit or activate immune cells, promote tissue remodeling, and enhance angiogenesis, which can support the growth and survival of cancer cells. Adenosine has been shown to act as an immunosuppressive agent and allow cancer cells to evade immune surveillance, contributing to tumor growth and progression.

This project allowed us to confirm that human NK cells could acquire the expression of CD73 when exposed to pre-senescent and senescent HDFS. We found that NK cells exposed to senescent HDFs, specifically h-RAS-transduced fibroblasts, had significantly higher levels of CD73 expression than NK cells co-cultured with pre-senescent HDFs. Increased adenosine produced in a senescent environment is likely the reason we observed reduced NK cell cytotoxicity against K562 cells. Our findings also demonstrate that CD73 is highly upregulated in senescent fibroblasts, suggesting its potential importance in the tumor microenvironment.

Expression of CD39 and CD73 by immune cells may have a crucial role in their ability to modulate the tumor microenvironment, potentially affecting the elimination of senescent cells and enabling cancer cells to evade immune surveillance. Exploring the mechanisms behind the induction of CD73 and CD39 expression on NK cells could provide insights into the development of strategies to boost the immune system ability to eliminate both senescent and tumor cells, potentially improving cancer treatment outcomes.

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