Cancer and microenvironment : the functional interplay between intra- and extracellular nucleotide metabolisms

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Préambule : Réflexions autour du cancer

Avant de commencer une thèse, que ce soit pour sa lecture ou sa réalisation, il est sans doute nécessaire de la mettre en contexte, c'est-à-dire donner aux lecteurs les outils pour en comprendre l'intérêt. Pas uniquement sur le point scientifique, mais aussi dans les projets de son auteur.

La thèse est souvent la première longue aventure dans la vie professionnelle de celui qui la réalise. C'est une sorte de premier engagement, qui n'est que rarement pris à la légère. Au travers de cette version d'un travail de réflexion réalisé au cours du Master, j'aimerais partager avec le lecteur un peu de ce qui m'a poussée vers ce chemin. Pourquoi vouloir devenir Enseignante-Chercheure ? Et pourquoi la cancérologie ?

Les victimes du cancer par Louise MBENGUE et Octavia CADASSOU

La cellule, constitue l'unité fondamentale de la vie, puisqu'elle porte en elle la molécule d'ADN, qui est le support de l'information génétique nécessaire au développement et au fonctionnement de tout organisme, mais aussi le support de l'hérédité.

Les trois niveaux d'organisation que sont la cellule, les tissus et les organes sont chacun soumis à des contraintes provenant de leur milieu intérieur comme extérieur, et comme c'est très souvent le cas en biologie, tout est une question d'équilibre. De ce constat émerge la notion d'homéostasie, fondement de la biologie moderne que l'on doit aux travaux de Claude Bernard, médecin et physiologiste français du XIX^{e} siècle. A l'échelle de la cellule, ce concept d'homéostasie se manifeste au travers de la régulation de la balance entre vie et mort cellulaire. En effet dans un tissu sain, on compte autant de nouvelles cellules créées que de cellules qui meurent. On estime que le nombre total de cellules présentes dans un corps humain adulte est de l'ordre de 10^{14} et c'est la rupture de cette homéostasie cellulaire qui va conduire à l'apparition d'un cancer.

Le cancer fait partie de l'une des causes majeures de mortalité. Selon des données de l'OMS, 8.2 millions de décès dans le monde étaient dus à des cancers en 2012. En France, en 2010, les tumeurs (tous types confondus) sont devenues la première cause de mortalité chez les plus de 45 ans, place anciennement occupée par les maladies cardio-vasculaires. Toujours selon des estimations provenant du GLOBOCAN PROJECT mené par l'IARC (International Agency for Research on Cancer), une sous-agence de l'OMS, en 2012 le taux d'incidence des cancers dans le monde était de 14.1 millions de cas. Cette même année, on dénombrait près de 32.6 millions de personnes vivant avec un cancer, toujours à l'échelle mondiale. Face à de tels chiffres, force est de constater qu'à l'heure actuelle le cancer continue à faire de nombreuses victimes.

Puisque l'on parle de « victimes », il convient de définir ce terme qui constitue par ailleurs le fil conducteur de notre sujet : *une victime est définie comme étant une personne subissant un dommage (qu'il soit corporel ou non), un abus, en somme les conséquences néfastes d'un évènement extérieur. On trouve aussi cette définition donnée par le Larousse : « Qui est atteint d'une maladie, d'un mal subit ».*

Si par définition un malade est « une victime », ce terme peut être étendu à d'autres, comme nous le verrons par la suite. Mais qui sont ces « autres victimes » ?

Enfin, en dépit de ce qu'en disent les définitions, la place de l'Homme au sein de sa propre maladie se résume-t-elle nécessairement à celle de victime ?

Le patient

Je souffre d'un cancer du sein. J'en souffre depuis qu'on me l'a diagnostiqué. La première douleur ressentie à cause de ce cancer était morale. Je me considérais comme « bien portante » avant que le verdict ne tombe. Et en quelques minutes, je suis passée de « bien portante « » à « en sursis ». A ce moment-là, tout s'assombrit, on a l'impression d'être passé à côté de sa vie et de s'éteindre tout doucement sans pouvoir rien y faire. L'impression de faire son propre deuil. Mais après cette annonce, j'ai rapidement repris le dessus puisque d'après ce qu'on entend, le cancer du sein est le cancer le mieux soigné de nos jours. Je devrais donc avoir toutes mes chances de rémission et ensuite, je reprendrai le cours de ma vie, comme si rien ne s'était passé. Malheureusement pour moi, c'est un espoir qui s'est évaporé. Mon cancer est plus agressif que ce à quoi les médecins s'attendaient. J'ai besoin d'une mastectomie. En fait, pour survivre, je dois sacrifier ma féminité et le symbole de ma maternité. Pour rester en vie, je dois renoncer à être une femme ! Est-ce que cela vaut vraiment le coup ? Et après l'opération, je serai soumise à des chimiothérapies. Je vais perdre mes cheveux et être regardée de travers partout où je passerai. Je vais être affaiblie, fatiguée, amaigrie. Souffrir de vomissements, autres troubles digestifs et peut être même perdre la peau de mes mains et de mes pieds. Je vais souffrir de ne plus me sentir femme et je vais souffrir à cause de la douleur. Je vous le redemande, est-ce que cela vaut vraiment le coup ? Pour une simple éventuelle rémission ? Car je l'ai compris à présent, les médecins ne parlent jamais de guérison mais bien de rémission. Pour être claire, j'irai éventuellement mieux pour quelques temps mais la menace constante d'une rechute planera au-dessus de moi. Ma maladie, c'est l'image de la mort autant à mes yeux qu'à ceux de toute la société. Bien sûr, nous sommes tous condamnés à mourir. Mais la plupart des gens ont le luxe de se refuser à y penser pour se cacher de cette peur de la mort qui est ancrée en chacun. Le cancer, lui, agit à la manière d'un catalyseur, en ce sens qu'il accélère la réaction dont le résultat est la prise de conscience que notre propre finitude est inéluctable. Il me prive de cette naïveté. Il en prive aussi tous ceux qui m'entourent et qui sont spectateurs de mon extinction progressive. Par conséquent, pour s'en protéger, ils m'isolent. J'ai déjà perdu des amis, je perdrai peut-être

aussi mon travail, comme 19% des patients atteints de cancer. Je serai discriminée, même si certains essaient de faire en sorte que nous, les fameux « Cancéreux », soyons intégrés.

L'annonce de ma maladie ne m'a pas seulement dévastée moi. Elle a aussi choqué et traumatisé mes proches. Eux aussi sont victimes de ma maladie. J'entends les pleurs étouffés de ma fille le soir, je perçois le désespoir dans le regard de mon époux. Ils essaient de faire semblant, mais nos relations ont changé, ils se forcent à profiter de chaque moment comme s'il était le dernier mais ce n'est pas ce dont j'ai besoin. J'ai besoin que ma fille ne se sente pas coupable de sortir un samedi soir avec ses amis plutôt que de rester auprès de moi. Comme tous ses amis, comme avant. C'est elle qui me materne et qui est aux petits soins. Mon époux et elle sont devenus mes parents. Plus rien n'est à sa place. Je sens qu'ils ont peur. Peur de me perdre, peur de ma mort. Ils vivent dans la crainte, ils vivent mon cancer.

Le chercheur

Je me suis lancé dans la recherche pour qu'un jour on puisse guérir le cancer, par solidarité. Je crois qu'on peut le faire, je crois qu'on en a les moyens. Avec toutes ces technologies de plus en plus performantes et efficaces, avec les moyens que nous avons de mettre en commun toutes nos connaissances et avec les nouvelles façons d'aborder la maladie. Notre monde a toutes les cartes en mains, toutes les chances de notre côté.

Mon rôle en tant que chercheur est d'apporter ma pierre, ou peut-être mon grain de sable, à cet immense édifice qu'est la lutte contre le cancer. Ce n'est pas grand-chose pour une vie entière de travail mais les patients comptent sur moi. Sur mes succès, comme sur mes échecs. Pour moi c'est ça être chercheur. Mon objectif c'est d'obtenir des résultats fiables. Quel qu'en soit le prix. Je manipule, re-manipule, pense et repense mon protocole, modifie et nuance mes techniques pour être toujours plus proche de la vérité. Sans jamais pouvoir l'atteindre vraiment puisque tous les résultats sont biaisés. Je me bats contre le cancer. C'est une vraie guerre que je mène contre lui. Je l'attaque à coups de pipetage, de dissections, de colorations, de séquençages, de thérapies ciblées. Et il riposte sournoisement. Parfois, il change ses stratégies de combat pour s'adapter à mes assauts pour me pousser à la résignation, d'autres fois il me tend des leurres, me lance sur des mauvaises pistes.

Et même plus loin : le cancer m'envahit. Non pas de la même façon que pour les patients, il attaque au-delà de la matérialité de mon corps. Il se glisse parfois dans mes rêves, par petites touches, son ombre me menace dans chacun de mes gestes quotidiens. J'ai parfois la sensation qu'il est tapi dans l'ombre en train de m'observer et à l'affut de la moindre erreur de réplication au sein de mes cellules, de la cigarette ou de la minute d'exposition au soleil en trop pour m'achever et prendre l'emprise de mon corps. Et moi, je le surveille. Il est partout : dans mon environnement, dans mon subconscient, dans mon travail. Il ne me quitte pas. C'est une obsession. C'est une paranoïa. C'est une maladie.

Finalement, il persiste et cette lutte, je ne la gagnerai pas. En effet, je m'éteindrai avant lui. Je serai mort avant que le cancer ne disparaisse et il aura immiscé dans la vie une angoisse constante, j'en suis conscient. Mais je veux des résultats. Je veux apporter mon grain de sable pour qu'un jour on puisse le vaincre. Et finalement ma vocation va au-delà de la solidarité. Elle relève de l'ordre du sacrifice. Je dois me rendre à l'évidence, accepter l'inacceptable : je fais au mieux mais les patients continuent de mourir. Car la mort est une fatalité dont on ne peut, en dépit de tous ces efforts, s'affranchir en aucun cas.

La cellule saine

Je suis une cellule tout à fait normale. Je suis soumise à toutes les régulations de mon environnement. Je me suis toujours soumise aux règles de la biologie, aux règles de la vie. J'assure mes fonctions selon les besoins de mes voisines et même de tout l'organisme. Je suis née pour cela.

Et comme tout ce qui vit, je suis vouée à mourir un jour. Vous appelez cela le cycle de la vie, je l'appelle l'homéostasie. Je l'accepte, on ne peut pas être éternel. Pour le bon fonctionnement d'un tout, il faut savoir laisser sa place. Vous, êtres humains, le savez bien : ne pas laisser sa place, c'est devenir un fardeau. Vous le dites si bien : « il faut savoir laisser son poste et partir à la retraite pour garder une société dynamique, laisser leur chance aux jeunes ». Mon malheur, c'est que je suis située à proximité de cellules qui se sont dressées contre cette logique qui pourtant équilibre le monde depuis toujours. Elles n'obéissent à aucune règle, vivent égoïstement. Elles prennent ce dont elles ont besoin pour s'auto-suffire. Elles veulent s'abroger de l'organisme, le renient. Pourtant, nous disposons d'assez pour faire

vivre tout le monde, pour que chacune d'entre nous puisse s'accomplir. Tout est fait pour que nous ayons toutes juste ce qu'il faut d'oxygène, de chaînes carbonées, d'ions.

Mais ces cellules sont atteintes de la folie des grandeurs. Elles nous forcent à la restriction, nous vouent à la pénurie. Elles se servent dans ce qui aurait dû être nos ressources, ne nous donnent rien en retour, nous regardent nous affaiblir en mettant au point les stratégies qu'elles adopteront quand les temps deviendront difficiles pour elles. Sans chercher à se restreindre pour justement éviter cette catastrophe. Elles condamnent l'organisme au chaos. Quelles naïves ! Elles imaginent qu'elles survivront à leur hôte, qu'elles trouveront un moyen de vivre sans l'organisme.

Mais elles se trompent. Elles mourront. Ce ne sont que des cellules, elles ne se suffiront jamais à elles-mêmes. Elles ne peuvent survivre que si nous sommes là, si elles peuvent tirer profit de nous. Si nous ne sommes plus là, elles n'ont plus rien. Je suis en sursis. Je vous en prie, ne devenez pas tumoraux vous aussi, ne devenez pas des cancers. Vous l'Occident, vous qui sucez avidement les ressources de la Terre, qui épuisez tout ce qui est précieux au détriment des plus démunis. Vous qui n'avez plus de scrupules à vivre dans l'opulence, à ne penser qu'à votre propre intérêt, à gagner pour posséder plus.

Conclusion : Se replacer en tant qu'Homme au centre de sa pathologie

Aujourd'hui, on peut dire que la Science est à chacun d'entre nous ce que Dieu est à un croyant. C'est vrai, la science moderne parce qu'elle cherche à tout prix à vouloir tout expliquer, se veut omnisciente, omnipotente.

En réalité, l'essence même de toute science réside dans sa capacité à reconnaître ses limites et à se réfuter elle-même, ce qu'elle tend parfois à oublier. En effet, le problème de la science actuelle est qu'elle est fondamentalement objectaliste. De ce fait découle la vision du corps mécanique, et c'est bien là le problème : l'Homme est loin d'être une machine et le considérer comme tel serait très réducteur. C'est pourtant ce que font la science et en particulier la médecine dont l'objet est le symptôme.

On peut alors se demander, comment la médecine peut-elle prétendre soigner si elle ne considère pas l'Homme dans son entièreté ? La réponse est très simple : la médecine soigne le symptôme et avec lui la maladie, mais n'est-il pas envisageable qu'en réalité elle se trompe ? Et si le symptôme était la maladie elle-même, manifestation de la souffrance qui sommeille en chacun d'entre nous ? Il s'agit peut-être là de la question que nous devrions nous poser quand survient une maladie aussi grave que le cancer. Loin de la vision visant à diaboliser la maladie ou encore à la considérer comme « un accident », il ne semble pas impossible que nous devrions en fait allez explorer au plus profond de nous-même, afin de répondre à cette interrogation au premier abord banale : « Pourquoi moi ? ».

La réalité c'est qu'avoir un cancer ne fait pas de nous une victime pour autant, le cancer est peut-être simplement le reflet de la vérité qui se cache en chacun d'entre nous. Si nous sommes victimes ce n'est pas du fait de la pathologie, mais à cause de notre propre aveuglement.

C'est en cela que la science se veut toute puissante : elle tente de s'arroger de notre propre vérité. Mais nous ne devons pas la laisser faire, il nous faut nous replacer en tant que sujet et non en tant qu'objet, au centre de notre maladie, car pour reprendre ces mots très célèbres de Descartes c'est bien parce que nous pensons, que nous sommes. Nous sommes Hommes, dotés d'un corps et d'une âme qui selon l'hylémorphisme d'Aristote ne sont pas dissociables. Bien que cette idée soit discutable (preuve en est Platon défendait la thèse inverse), ce qui est intéressant, c'est la notion d'unité du vivant qui lui est sous-jacente : l'Homme et la Nature sont faits de la même matière, ils ne sont pas déconnectés l'un de l'autre. Tout comme la cellule qui interagit avec son milieu interne et son milieu externe, il existe une dialectique entre notre environnement externe et notre environnement interne. Il n'est donc pas dénué de sens de penser que la pathologie pourrait être la manifestation physique de cette dialectique.

Justement, puisque l'on parle de « sens » il se trouve que la vie n'en est elle-même pas dénuée, c'est pourquoi se questionner sur le sens même que peut avoir une pathologie, sa pathologie, est tout à fait justifié. Et si la pathologie a un sens, alors on pourrait lui attribuer celui de la nécessité du Mal pour accéder à la connaissance, comme le soutient Goethe dans son *Faust*.

Avoir un cancer, en dépit du traumatisme qu'il cause, peut s'avérer dans certain cas « formateur », en permettant une remise en question sur sa vie, ses actes quotidiens, afin de les appréhender différemment.

Ainsi, voici ce qu'écrit Fritz Zorn dans *Mars*, son essai autobiographique :

« Je ne veux pas prétendre ainsi que le cancer soit une maladie qui vous apporte beaucoup de joie. Cependant, du fait que la joie n'est pas une des principales caractéristiques de ma vie, une comparaison attentive m'amène à conclure que depuis que je suis malade, je vais beaucoup mieux qu'autrefois, avant de tomber malade. »

Dans cet ouvrage Fritz Zorn, alors âgé de 32 ans seulement et atteint d'un cancer dont il se sait condamné, décrit sa vie : de ses parents névrosés en passant par son incapacité à communiquer avec le monde. Il confirme cette idée selon laquelle, la perte de dialectique des cellules cancéreuses avec leur environnement pourrait être le reflet de notre propre perte de dialectique aussi bien le monde qu'avec nous-mêmes. Là est peut-être la clé : il y a nécessité de rétablir le dialogue, en commençant par écouter ce que la maladie a à nous apprendre sur nous-mêmes.

Mais ce n'est pas chose facile, car notre façon de penser la maladie est culturellement ancrée en nous. Ce qu'il faudrait ce n'est non pas une révolution, mais plutôt une évolution de la Science qui ne serait pas sans rappeler celle qui a eu lieu au cours du Siècle des Lumières, siècle au cours duquel est d'ailleurs née la science moderne. Replacer l'Homme au centre de la connaissance, telle était la devise sous les Lumières, mais c'est désormais une place occupée par la science. A l'instar de la cellule cancéreuse et du patient, peut-être les biosciences ont-elles aussi perdu leur capacité à dialoguer, notamment avec les autres sciences et en particulier les sciences humaines. Or la compréhension des pathologies humaines passe par une meilleure compréhension de l'Homme, et ce pas uniquement d'un point de vue anatomique.

Alors pourquoi pas une anthropologie du cancer ?

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Ce travail nous a permis à Louise et moi-même de se souvenir que derrière les études en laboratoire de recherche ou en clinique, au-delà des politiques et courses aux publications, il y a un enjeu réel et noble : l’Humain. Et toutes deux, nous nourrissons l’espoir que la recherche en soit constamment consciente, qu’elle soit désintéressée et que son seul objectif soit d’aider un ou des Humains atteints d’un mal. A l’issue de notre master, chacune a choisi une voie différente pour apporter son support à la lutte collective contre le cancer. Pour moi, ce sera (je l’espère) par la recherche, pour ses nouvelles idées, pour la beauté de sa rigueur, pour sa persévérance optimiste et créative, pour son dévouement. Mais ce sera aussi par l’enseignement, pour susciter de nouvelles vocations, pour transmette cet optimisme et que cet élan perdure.

Résumé

Les nucléotides jouent un rôle majeur dans une pléiade de processus biologiques comme la composition des acides nucléiques, la signalisation, ou la régulation de la balance énergétique. Les nucléotides extracellulaires exercent également des fonctions biologiques. Par conséquent, des dérégulations des pools de nucléotides impactent l'homéostasie de multiples façons, par exemple en promouvant l'instabilité génétique ou un environnement immunosuppresseur. Or, ces paramètres font partie des « Hallmarks du Cancer » décrits par Hanahan et Weinberg. Ces observations confirment l'éventualité d'un rôle clé des nucléotides dans le cadre du cancer.

cN-II et CD73 sont des 5'-nucléotidases impliquées respectivement dans les métabolismes nucléotidiques intra- et extracellulaire. Elles sont de nouvelles cibles thérapeutiques en oncologie. Cependant, leurs rôles dans la biologie de la cellule cancéreuse, ou le possible impact de leur utilisation en tant que cible thérapeutique sur le comportement des cellules tumorales sont peu connus. Considérant l'implication de ces enzymes dans les métabolismes nucléotidiques, nous avons enquêté sur les modifications de l'agressivité de la cellule cancéreuse ou sur sa capacité à interagir avec son microenvironnement, dans le cas d'une invalidation ou une diminution d'expression de cN-II et/ou CD73. cN-II semble donc impliquée dans l'adaptabilité métabolique et la combinaison des invalidations de cN-II et CD73 est associée à une modification d'expression d'enzymes du métabolisme du glucose. CD73 peut aussi moduler l'expression de gènes de la migration cellulaire. cN-II est impliquée dans la migration cellulaire, via l'axe COX-2/PGE2, et dans la sensibilité à des agents modulant ce paramètre. Ces caractéristiques sont plus marquées en association avec une invalidation de CD73. Ici, cN-II et CD73 ne semblent pas jouer de rôle dans la prolifération ou le dialogue avec une sous-population de cellule de l'immunité innée.

Summary

Nucleotides play a major role in nucleic acids constitution and are involved in various cell phenomena. Indeed, intracellular ATP, GTP, AMP, GMP and their cyclic forms are components of cell signaling and define the energetic balance. Extracellularly, they also play multiple roles. Thus, when nucleotide pools are deregulated various processes are impacted. For example, a low availability of nucleotides supports genetic instability and aberrant levels of extracellular adenosine can lead to an immunosuppressive microenvironment. Interestingly, the cited parameters are among the Cancer Hallmarks described by Hanahan and Weinberg. These observations confirm the possibility of a key role of these molecules in this pathology.

cN-II and CD73 are 5'-nucleotidases, involved in intra- and extracellular nucleotide metabolism respectively and have been identified as possible targets for new anti-cancer therapies. Nevertheless, very little is known about their biological roles on cancer cells and what parameters of cell biology could be impacted by such strategies. Considering the involvement of these purines in cell metabolism, we wondered what changes a decrease in cN-II and/orCD73 expressions or their silencing could trigger in cancer cells as well as in the interplay with their microenvironment.

We studied cancer cell aggressiveness and the interplay with innate immune cells under cN-II and CD73 modulations. We observed that cN-II is involved in metabolic adaptability. The association of cN-II and CD73 invalidations results in glucose-metabolism-related gene modifications. CD73 can regulate migration-related genes expression but does not affect the process. cN-II is also involved in cell migration, via the COX-2/PGE2 axis. Again, these characteristics are accentuated when associated with CD73 deficiency. Here, cN-II and CD73 do not seem to be involved in cancer cell proliferation or in their interplay with a subset of innate immune cells.

Table des matières

Glossary

2DG: 2-deoxyglucose

5-FU:5-Fluoro-Uracil

ADA: adenosine deaminase

ADCC: antibody-driven cell cytotoxicity

ADK: adenosine kinase

ADORA: adenosine receptor

ADP: adenosine 5'-diphosphate

AK: adenylate kinase

AMP: adenosine 5'-monophosphate

AMPK: AMP-dependent protein kinase

ATP : adenosine 5'-triphosphate

Bcl: B-cell lymphoma

CD73: 5'-ecto nucleotidase

CFSE: carboxyfluorescein diacetate succinimidyl ester

cN-II: cytosolic 5' -nucleotidase

CNT: concentrative nucleoside transporter

COX-2: cyclooxygenase 2

DNA: deoxyribonucleic acid

EMT: epithelial to mesenchymal transition

ENT : equilibrative nucleoside transporter

FAK : focal-adhesion kinase

G6PDH: glucose-6-phosphate dehydrogenase

GPCR: G protein-coupled receptor

GPI : Glycosylphosphatidylinositol

HIF-1: hypoxia induced factor 1

IAP: inhibitor of apoptosis protein

IFN: interferon

IL: interleukine

MMP: matrix metalloproteinase

NAD: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NK: natural killer lymphocyte

PGE2: prostaglandin E2

PKA : protein kinase A

PKC : protein kinase C

PLC : phospholipase C

PNP: purine nucleoside phosphorylase

ROS: reactive oxygen species

SAM: S-adenosyl-methionine

SAH: S-adenosyl-homocysteine

SAHH: SAH hydrolase

TAM: tumor-associated macrophage

TAN: tumor-associated neutrophil

TGF : transforming growth factor

TIMP: inhibitor of matrix metalloproteinase

TNF : transforming growth factor

VEGF: vascular-endothelial growth factor

General introduction

Introduction: Nucleotides and nucleosides in cell biology

Nucleotides are mono-, di- or triphosphorylated nucleosides. Nucleosides consist of a nitrogenous base that can be a purine, a pyrimidine or a pyridine, and a sugar moiety, which is a ribose or a deoxyribose (Figure 1). These molecules are widely studied in various biological and chemical domains, mainly because their polymerization constitute nucleic acids.

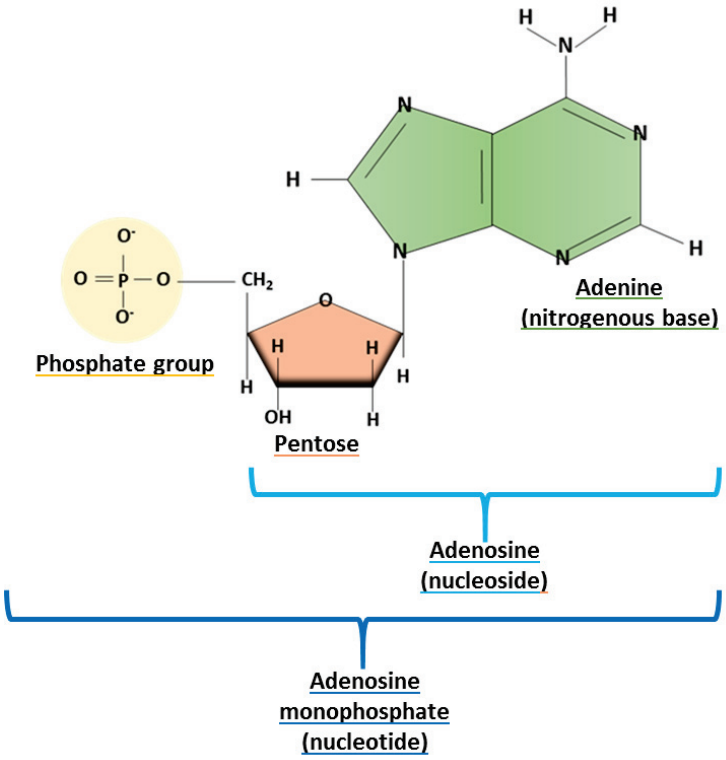

Figure 1: Adenine nucleosides and nucleotides structure. Nucleosides consist of a nucleobase associated with a sugar (ribose or deoxyribose). They can be mono-, di- or tri-phosphorylated to form nucleotides.

In addition to playing a major role in nucleic acids constitution, nucleotides are involved in various cell phenomena. Indeed, intracellular ATP, GTP, AMP, GMP and their cyclic forms are major secondary messengers in cell signaling as they play a role in the cascades downstream various receptors like GPCR (G protein-coupled receptors). As ATP is the main

energy source in cells, it and its derivatives (ADP and AMP) also define the energetic balance in cells. This reflects the cellular activity together with the efficiency of energy-producing pathways such as mitochondrial respiration or glycolysis. Nucleotides enter in the composition of co-factors (like NAD or coenzyme A) and biosynthetic intermediates. Extracellular nucleotides also play multiple roles in biology, and particularly in immune modulations or angiogenesis (Allard et al., 2014; Du et al., 2015; Ernens et al., 2015). Consequently, when nucleotide pools are deregulated, because of abnormal nucleotide release by damaged cells (therapy (Schneider et al., 2015)) or abnormal extracellular catabolism (overexpression of nucleotidases), various processes are impacted. For example, a low availability of nucleotides in newly transformed cells supports genetic instability (Bester et al., 2011), and aberrant levels of extracellular adenosine can lead to an immunosuppressive microenvironment, favoring cancer progression (Stagg and Smyth, 2010) Interestingly, the cited parameters are among the Cancer Hallmarks described by Hanahan and Weinberg in their seminal reviews (Hanahan and Weinberg, 2011). These observations confirm the possibility of a key role of these molecules in this pathology. Thus, hereafter, we will present purine metabolism with a particular focus on adenosine and its biological functions in cancer to introduce the lector to the aim of our project.

Nucleotides are divided into two families, purines and pyrimidines, differing by the nature of the nucleobase. Distinct pathways allow purines and pyrimidines formation. Nucleotide metabolism combines anabolic and catabolic processes (Figure 2) to constitute and maintain homeostatic pools. In this project, we will focus on purines and more precisely adenine derivatives. The following paragraph will then present purine synthesis pathways to understand how intracellular pools can be generated.

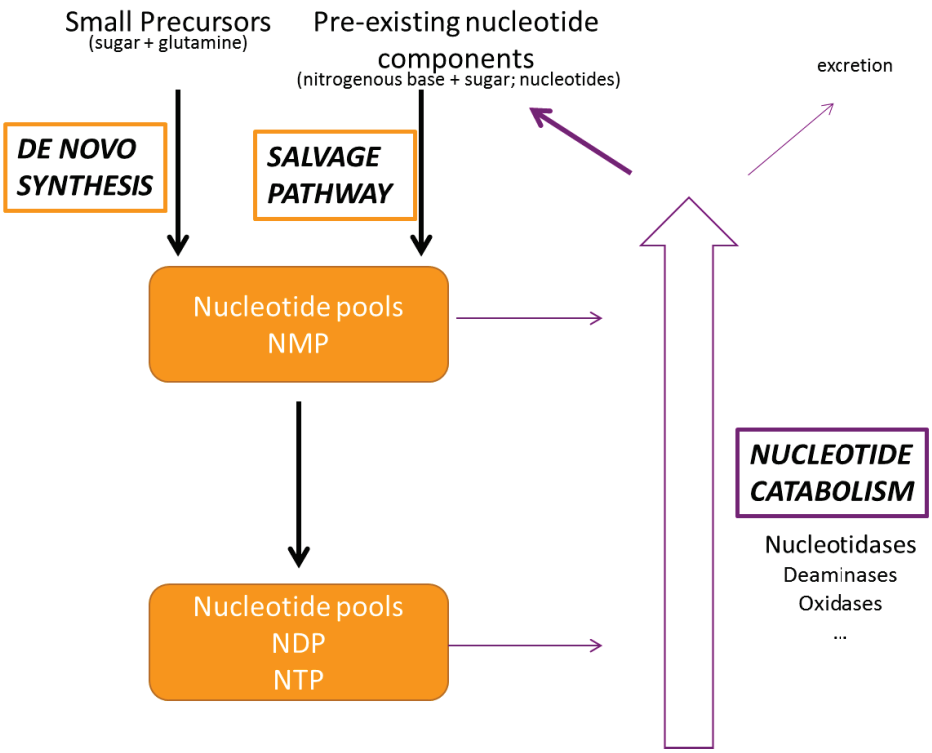

Figure 2: Nucleotide metabolism: Cells have two ways to constitute their nucleotide pools that are de novo synthesis and salvage pathway. Nucleotide degradation is realized by various enzymes. The subsequent products are then re-used for recycling or excreted.

Purine synthesis

Purines can be synthesized from two connected pathways: *de novo* synthesis that uses small precursors, and salvage pathway by which new nucleotides are generated from the degradation of previous ones. As *de novo* synthesis has a high energy cost, most cells usually recycle their nucleotides.

De novo *synthesis*

De novo synthesis mostly happens in the liver and consumes energy (under the form of ATP or GTP) and ends in the formation of AMP and GMP ribonucleotides via IMP (Figure 3). The latter are then converted into ribonucleotides by phosphorylations. Deoxyribonucleotides are generated from their dephosphorylated forms for DNA synthesis.

Both purine and pyrimidine synthesis require a common sugar precursor which is PRPP (5-phosphoribosyl-1-pyrophosphate). PRPP formation requires a ribose-5-phosphate molecule, that originates form the pentose phosphate pathway, rendering this metabolic way essential for nucleotide synthesis. Ribose-5-phosphate is activated by the ATP-consuming enzymes PRPP synthases to form the nucleotides precursor.

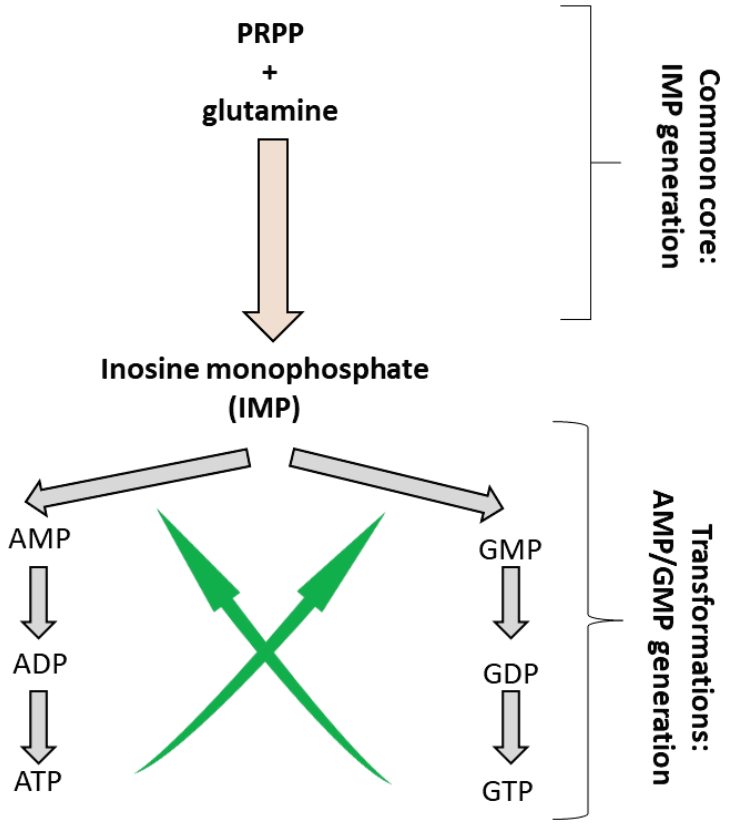

Figure 3: De novo purine synthesis, a multi-step pathway. De novo purine synthesis consists of two major phases: the common core (IMP formation) and the transformation (AMP and GMP generation).

The first part of the purine *de novo* synthesis, or common core uses the phosphorylated sugar PRPP and glutamine as first metabolic precursors allows to obtain IMP. This purine consists of a hypoxanthine base with a ribose and one phosphate group. A cascade of ten enzymatic reactions are necessary to form IMP (Figure 4 and Table 1).

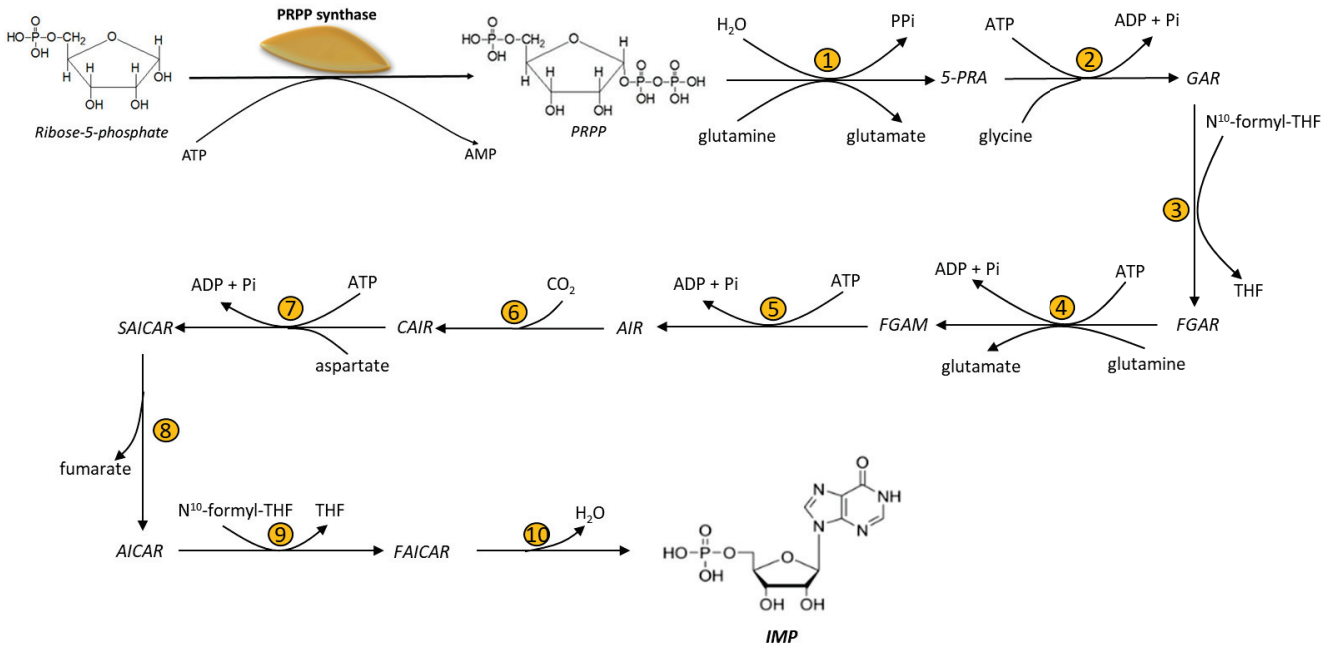

Figure 4: Common core of purine sunthesis: IMP generation. PPi =inorgaanic pyrophosphate; THF= tetrahydrofolate; 5-PRA=5-phospho-β-Dribosylamine; GAR= glycinamine ribonucleotide; FGAR= formylglycinamine ribonucleotide; FGAM= formylglycinamidine ribonucleotide; AIR= amino-imidazole ribonucleotide; CAIR= carboxyamino-imidazole ribonucleotide; SAICAR= amino-imidazo succinylcarboxamide ribonucleotide; AICAR= amino-imidazo carboxamide ribonucleotide; FAICAR= formamino-imidazo carboxamide ribonucleotide.

Enzyme	Reaction
Glutamine phosphoribosylpyrophosphate amidotransferase =GPAT	Step 1: Use of PRPP and glutamine as substrates
5'-phosphoribosylglycinamide transformylase = GART (or AIR synthase= AIRS). Trifunctional enzyme	Step 2: ATP-consuming step Step 3: tetrahydrofolate production Step 5: ATP-consuming step
Phosphoribosylformlglycinamidine synthase = PFAS	Step 4: Use of glutamine as substrate in an ATP-consuming reaction
Phosphoribosylaminoimidazole-succinocarboxamide synthase= PAICS (or AIR carboxylase = AIRC). Dual enzymatic activity	Step 6: Use of CO_2 as substrate Step 7: Use of aspartate in an ATP-consuming reaction
Adenylosuccinate lyase =ADSL	Step 8: Fumarate production
AICA transformylase= ATIC. Dual enzymatic activity	Step 9: tetrahydrofolate production Step 10: IMP formation

Table 1: Common core enzymes and major steps of IMP formation

IMP is then converted into AMP or GMP in a phase called transformation phase. There are two separated branches leading to adenosine and guanosine derivatives. These two branches mutually regulate as AMP synthesis is stimulated by GTP and requires energy from its hydrolysis, and vice versa.

For AMP production, two enzymes are required: ADSS (adenylosuccinate synthetase) and ADSL (adenoylosuccinate lyase). GTP regulates AMP production as is provides energy to ADSS to convert IMP and aspartate into adenylosuccinate. GMP production also happens in two steps, with two different enzymes: IMPDH (IMP dehydrogenase) and XMP-glutamine amidotransferase. It requires water, glutamine and ATP as a source of energy for the second enzyme. Thus, AMP and GMP synthesis pathways regulate each other, insuring a balanced production of these two purines. At the same time, these nucleotides exert a negative feedback on their own generation (Figure 3).

Once formed, AMP and GMP can further be phosphorylated by nucleoside monophosphate and nucleoside diphosphate kinases to form the di- and triphosohorylated pools.

Deoxyribonucleotides that enter in the composition of DNA are then formed from the corresponding ribonucleotide pools. RNR (ribonucleotide reductase) is the reducting enzyme complex that converts purine and pyrimidine NDPs into dNDPs. RNR complex consists of two subunits: RRM1 (ribonucleotide-diphosphate reductase large subunit) associated in tetramers with RRM2 (ribonucleotide-diphosphate reductase small subunit) and necessitates NADPH to

function. Owing to its function, RNR is crucial to maintain the balance between ribo- and deoxyribonucleotides in one hand, and the balance between the different deoxyribonucleotides in the other hand. Thus RNR is finely regulated by diverse mechanisms as reviewed in (Guarino et al., 2014).

De novo purine synthesis is regulated at several levels. The PRPP precursor production can be inhibited by ADP and GDP that exert an inhibition of phosphoribosyl pyrophosphate synthetase (Smith et al., 1994) (which role is to generate PRPP). The final products AMP and GMP also exert a negative feedback on their own formation, through inhibition of GPAT, the first enzyme of the common core. There also is an interplay between the purinosome enzymes (involved in purine formation) and deaminases and nucleotidases that participate in their catabolism (see following section).

Salvage pathway

The salvage pathway consists in recycling preexisting nucleobases or nucleosides produced from the degradation of nucleic acids or nucleotides. Consequently, nucleotide degradation can be considered as a part of the salvage pathway.

To be degraded into uric acid which can be excreted, AMP and GMP undergo a series of dephosphorylations, deaminations, phosphorolyses and oxidations (Figure 5). Several enzymes are involved, allowing nucleoside as well as nucleotide catabolism. During this catabolic process, free PRPP and nucleobases are released and can be recycled and reassembled. APRT (adenine phosphoribosyltransferase) and HGPRT (hypoxanthine-guanine phosphoribosylransferase) are respectively responsible for adenine and guanine/hypoxanthine reassembly with PRPP (Berg et al., 2002). The formed AMP and GMP can then be further phosphorylated.

Purine degradation can also free nucleosides such as adenosine, guanosine or inosine, that can be rephosphorylated by enzymes like adenosine kinase (ADK).

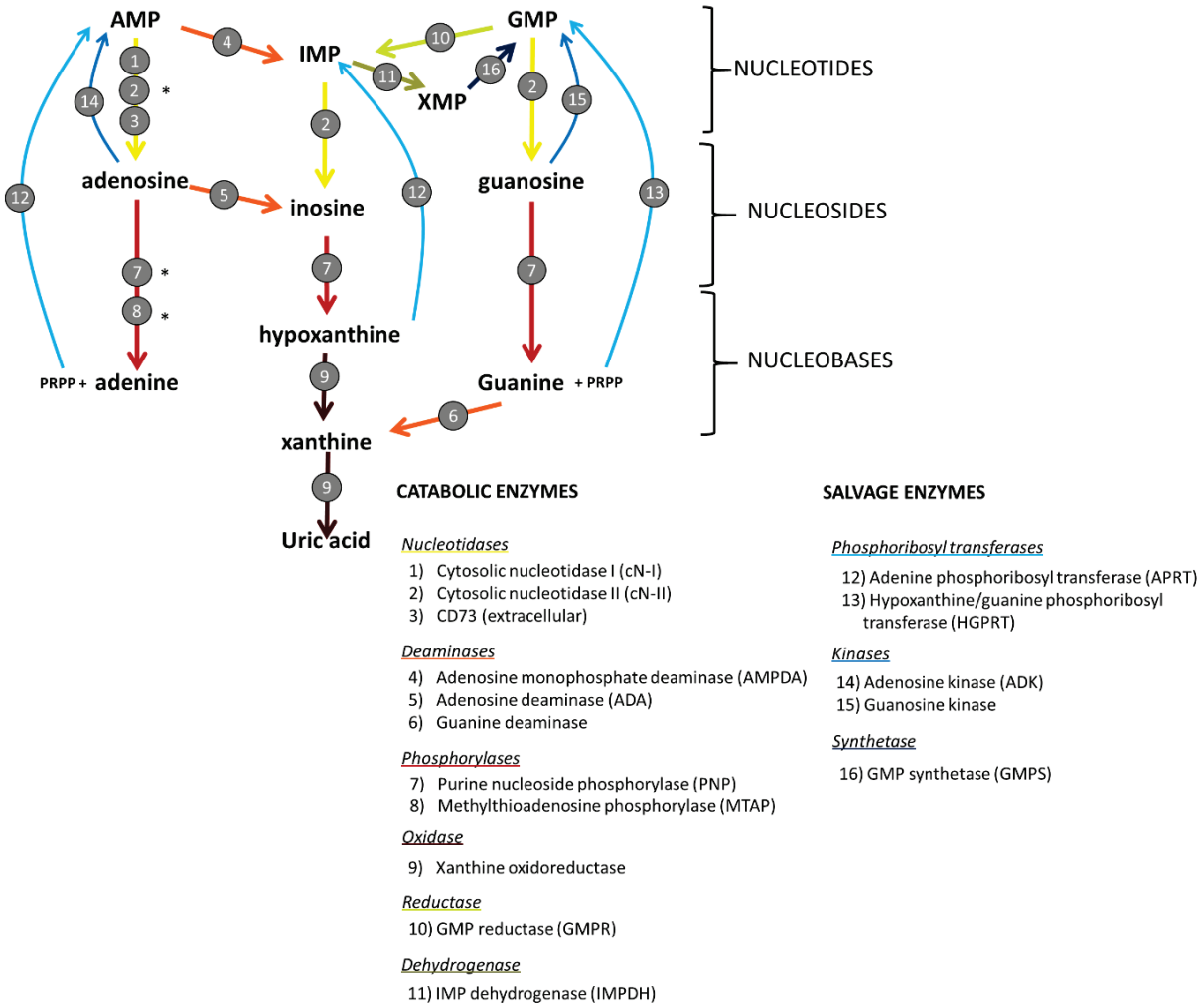

Figure 5: Purine catabolism and salvage. AMP, GMP and IMP catabolism provide precursors that can be converted to reform nucleotide pools. The stars indicate reactions that have been demonstrated as realizable but might not occur at relevant rates in vivo, due to the presence of substrates with higher affinities for the enzyme.

De novo synthesis, nucleotide catabolism and salvage pathway are intimately related in organisms. Indeed, the nucleotides that are formed in the liver need to be dephosphorylated or cleaved into the corresponding nucleobase or nucleoside and PRPP to be transported. Then, in distant organs, they will be reconstituted mostly using the salvage pathway.

Adenosine metabolism

After these general comments on purine metabolism, we will now focus on adenosine fates in cell biology (its conversion and biological functions), and the involved molecules.

Extracellular adenosine generation

Extracellular adenosine is mostly generated from the degradation of extracellular ATP that comes from cell lysis or secretion. Its catabolism is ensured by CD39 and CD73. These enzymes are ecto-nucleotidases respectively catalyzing the degradation of ATP and ADP into AMP and AMP into adenosine. Subsequently, the newly formed adenosine can have three fates: (i) degradation into inosine by adenosine deaminase (ADA), (ii) internalization through specific nucleoside receptors, or (iii) interaction with the specific receptors ADORA. These processes will be detailed in the following sections.

Intracellular adenosine generation and fates

Intracellular adenosine can form from the breakdown of AMP by cytosolic 5'-nucleotidases, similarly with its extracellular production by CD73. It can also originate from the transmethylation pathway. The latter relies on SAM (S-adenosyl-methionine) that is the main methyl donor in the organism and it participates in processes such as epigenetic regulations (by CpG methylations) or posts transcriptional modifications. During the methyl transfer by methyl transferases, SAM is converted into SAH (S-adenosyl-homocysteine) which is then hydrolyzed by SAHH (SAH hydrolase) into homocysteine and adenosine. Reversely, adenosine can reform SAH by association with homocysteine. These reactions are regulated by intracellular adenosine levels (Boison, 2016).

When present in the cytosol, adenosine can be transported to the extracellular space but the major fraction is rapidly phosphorylated to form ATP (Li et al., 2013) and during this process, AMP and ADP can also be generated. Adenosine kinase (ADK) and adenylate kinases are the major enzymes involved in adenosine phosphorylation. ADK is expressed in the cytosol, at the cell membrane, and can be released in the interstitial space. It has a higher affinity for adenosine than its degrading enzyme, ADA (adenosine deaminase), suggesting that

it is responsible for adenosine clearance in physiologic conditions, when adenosine concentrations are low, whereas ADA's role would be more important when adenosine concentrations are higher (Boison et al., 2010).

Adenosine degradation into inosine and its fate

Adenosine does not accumulate inside or outside the cells and has a very short half-life time, due to the fact that it is rapidly catabolized as mentioned previously. The nucleoside is first irreversibly deaminated into inosine by adenosine deaminase (ADA). Subsequently, inosine is reversibly degraded by a purine nucleoside phosphorylase (PNP) to form hypoxanthine, its corresponding nucleobase and PRPP or its precursor ribose-1-phosphate. Adenosine is believed to be circulating at less than 0.1 µg/mL in the blood (Ramakers et al., 2011; Traut, 1994). If its half-life time has been estimated to be 10 seconds in human blood (Moser et al., 1989), it is more difficult to estimate *in vitro* as it depends on the ADA availability in the culture conditions. ADA is present in the cytosol as well as at cell membrane, complexed with CD26 (Kameoka et al., 1993), and is responsible for both adenosine and deoxyadenosine degradations. Due to its activity, this enzyme indirectly regulates the adenosinergic signaling, by modulating the interaction between adenosine and its receptors. In addition to degrading adenosine, ADA is able to bind the receptors, changing their conformation and facilitating the signaling triggered by adenosine (Ciruela et al., 1996; Gracia et al., 2008, 2013).

Other enzymes such as PNP (purine nucleoside phosphorylase) and MTAP (methylthioadenosine phosphorylase) could participate in adenosine degradation by contenting it into adenine but these reactions are more likely not to occur *in vivo* due to the availability of substrates with higher affinity (Stoeckler et al., 1997; Toorchen and Miller, 1991).

Inosine results from the rapid degradation of adenosine and is more stable and abundant in the microenvironment than its precursor (Bell et al., 1998; John W. Phillis et al., 1987). This nucleoside is known to interact with adenosine receptors (ADORA) (Welihinda et al., 2016, 2018), and to exert immunomodulatory functions (Haskó et al., 2000; da Rocha Lapa et al., 2013). As for adenosine, inosine can be internalized trough nucleoside transporters, and found in the cytosol. Its intracellular function are not precisely known but it is believed that it could

strengthen intracellular adenosine roles (Collis et al., 1986). Moreover, the cytosolic 5'-nucleotidase cN-II has a phosphotransferase activity that allows the conversion of inosine into IMP, that can then be converted into AMP and GMP (Worku and Newby, 1982) .

Internalization by nucleoside transporters

Intracellular adenosine can originate from extracellular adenosine or from intracellular degradation of adenine nucleotides. The proportion of extracellular adenosine that is not degraded is rapidly internalized through nucleoside transporters. There are two families of nucleoside transporters, which can favor adenosine uptake: the ENT (equilibrative nucleoside transporters) and the CNT (concentrative nucleoside transporters) (Figure 6). The CNT family counts three members of cation-coupled channels (CNT1-3) that all can co-transport adenosine. They display a higher affinity for adenosine than the ENT and lead to adenosine flux against its concentration gradient. They are also involved in nucleoside-analogs transport (Young, 2016).

The ENT family counts four members (ENT1-4). Unlike CNTs, ENTs allow a bidirectional adenosine transport. As for the CNT family members, the specificity for the transported nucleosides varies between the four ENTs. ENT1 and -2 are the best described equilibrative transporters and considered as facilitated diffusion systems. ENT-3 and -4 on the other hand are cation-dependent transporters and are sensitive to the environing pH. ENT-3 has the particularity to be located at intracellular membranes, ensuring adenosine translocation between different cellular compartments (Young et al., 2008, 2013). Some equilibrative transporters can ensure nucleobases transport. ENTs are regulated by various mechanisms, in particular downstream the ADORA signaling, by PKA and PKC that can phosphorylate the transporters and affect their function and cellular localization (Hughes et al., 2015; Young et al., 2013). Hypoxia also negatively regulates ENT expression via HIF-1 (hypoxia-induced factor 1) that interacts with SLC29 genes promotors (Eltzschig et al., 2005).

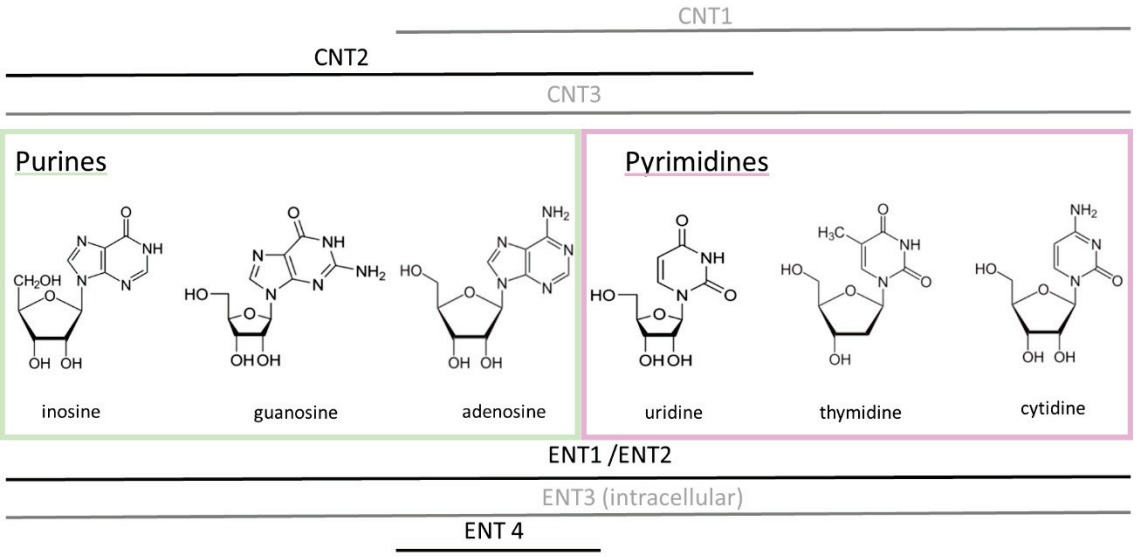

Figure 6: Concentrative and equilibrative nucleotide transporters specificity towards natural purine and pyrimidine nucleosides. All known nucleoside transporters can transport adenosine.

Thanks to their function, nucleoside transporters allow adenosine to enter the cell to be metabolized and play diverse roles on cell biology. Furthermore, ENTs and CNTs activities participate in the control of the extracellular adenosine available to interact with the ADORAs. Thus, adenosine transporters can be considered as true modulators of the adenosine signaling.

Extracellular adenosine fates

ADORA receptors

Extracellular adenosine can act as a signaling molecule. Adenosine interacts with four types of specific P1-receptors named ADORA: A1, A2A, A2B and A3 (Figure 8). All of them belong to the G protein-coupled receptor (GPCR) family and are involved in various physiological phenomena such as sleep, neural or cardiac functions. These receptors are expressed all over the body with higher expression levels in some organs, according to their type. They also vary by their affinity with adenosine: A1 and A2A are high affinity receptors (nanomolar) whereas A2B and A3 are low affinity receptors (micromolar).

A1 receptor

A1 receptor is mainly expressed in brain, eye, atria and adrenal gland but is also found at lower levels in skeletal muscle, liver, kidney, adipose tissue, salivary glands, colon, testes, esophagus and antrum (Fredholm et al., 2000). A1 is coupled to G_o or G_i proteins. Its activation inhibits adenylate cyclase activity, leading to a decrease of cytoplasmic cAMP (cyclic AMP) concentration, and activates phospholipase C (PLC), thus increasing cytoplasmic inositol-1,4,5-trisphosphate (IP3) and calcium concentrations (Chen et al., 2013). This receptor has a high affinity for adenosine and can also interact and respond to AMP (Rittiner et al., 2012).

A2A receptor

The cells that express the most this receptor are leukocytes, platelets, splenocytes, thymus cells and some GABAergic neurons. On immune cells, A2A is the main way by which adenosine exerts its anti-inflammatory effects (Fishman et al., 2009). As A2A is coupled to G_s or G_{olf} proteins, its interaction with adenosine leads to activation of the adenylate cyclase/cAMP/PKA (protein kinase A) axis.

A2B receptor

A2B receptor is widely expressed in organs and particularly on caecum, colon and bladder cells. This receptor is often overexpressed in tumors, compared to normal tissue (Mousavi et al., 2015; Zhou et al., 2017). This overexpression is believed to be due to hypoxic environment (Kong et al., 2006). A2B is the adenosine receptor that has the lowest affinity for its natural substrate, thus, in physiological conditions, this receptor is not activated. A2B is coupled to G_s or G_q proteins that allow PKA activation and increased intracellular Ca^{2+} concentrations.

A3 receptor

A3 receptor is preferentially expressed in the liver but is often overexpressed in cancer cells lines and patient samples (Madi et al., 2004). A3 is mainly coupled to G_0 or G_i proteins, thus leading to adenylate cyclase inhibition and intracellular Ca^{2+} release. G_q coupling also sustains high cytosolic Ca^{2+} concentrations and inositol-3-phosphate production. A3 transcription is favored by NFkB.

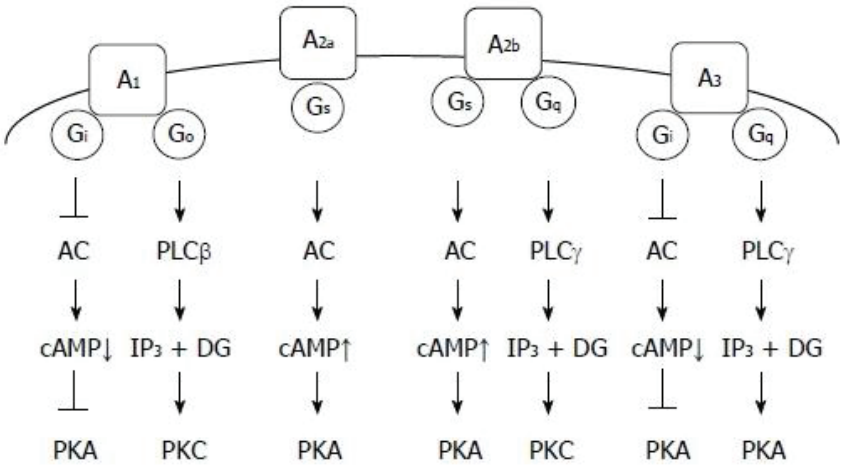

Figure 7: (Tsuchiya and Nishizaki, 2015)*: Adenosine receptors and their downstream canonical pathways. PLC = phospholipase C, IP3= inositol 1,4,5 triphosphate, DG= diacylglycerol, PKA= protein kinase A, PKC= protein kinase C.*

In cancer, A1 tends to have antitumor effects, in contrast to A2A that mediates pro-tumoral processes, mainly through immune system regulations. The roles of A2B and A3 remain controversial, according to the studied models. Interestingly, in tumor microenvironment, and in particular under hypoxic conditions, extracellular adenosine levels are elevated, which favors the low-affinity A2B receptor activation. This suggest that this receptor could play specific roles in cancer. As many other GPCR, ADORA can interact with alternative pathways such as MAP kinases (Crespo et al., 1994; Goldsmith and Dhanasekaran, 2007), PI3K/AKT (Murga et al., 1998) or GSK3β/β-catenin pathways. In the tumoral context, ADORA receptors are the main intermediates for adenosine effects. For example, immune-suppressive effects are observed downstream A2A receptor activation by mechanisms involving PKA and subsequent NFκB translocation inhibition that results in an overall reduction of pro-inflammatory cytokines production (Campo et al., 2012). It is however difficult to conclude or predict the overall effect of the stimulation of ADORA in cancer cells. Indeed, due to the differences in expression and affinity, and the multiple steps leading to PKA and PKC activation or inhibition, the effect of adenosine on a cell will depend on all these parameters as well as redundant signaling pathways.

The combination of adenosine receptors in cancer cells and in surrounding cells makes it difficult to conclude on their involvement in cancer progression. For example, when they are

co-expressed, A2A and A2B can associate in complexes where A2B inhibits A2A downstream cascade (Hinz et al., 2018).

Interplay between adenosine metabolism and signaling

Owing to the multiplicity of enzymes involved in adenosine metabolism, its extracellular and intracellular levels can be regulated more or less directly by several intermediates. A2A activation by extracellular adenosine enhances CD73 expression that is mediated by MAPK activation in colorectal cancer. Consequently, adenosine accumulates in the tumor microenvironment due to an increased production (Yu et al., 2018). Vannoni and colleagues observed that ADA and ADK, which participate in lowering the intra- and extracellular levels of adenosine, display higher activities in tumor tissues as compared to non-cancerous tissues. This observation was interpreted as a strategy to counterbalance the cytotoxic effects of adenosine on cancer cells (Vannoni et al., 2004). (Vannoni et al., 2004). Nevertheless, their inhibition leads to cell death, probably due to adenosine accumulation and reinforcement of its pro-apoptitic effects (see following paragraph). Thus in cancers, both adenosine synthesis and conversion are enhanced but end in an overall increase in adenosine concentrations. Consequently, to favor malignant cell elimination by the immune system or adenosine-induced death respectively, both processes could be relevant as targets, to deregulate the balance cancer cells rely on.

Physiological parameters can also impact adenosine concentrations. It is the case for hypoxia. Indeed, under hypoxic conditions, CD39 and CD73 expressions are increased (Hatfield et al., 2014; Synnestvedt et al., 2002), leading to a higher production of extracellular adenosine. This phenomenon is accompanied with a downregulation of adenosine-converting enzymes such as adenosine kinase and adenosine deaminase. (Decking et al., 1997; Fishman et al., 2009). Low oxygen availability also increases adenosine receptors expression in tumors, while repressing its transporters expression (Eltzschig et al., 2005; Kong et al., 2006). Hypoxia thereby results in an extracellular adenosine accumulation and amplification of ADORA-mediated effects.

To conclude this section, Figure 8 presents a summary of adenosine metabolism.

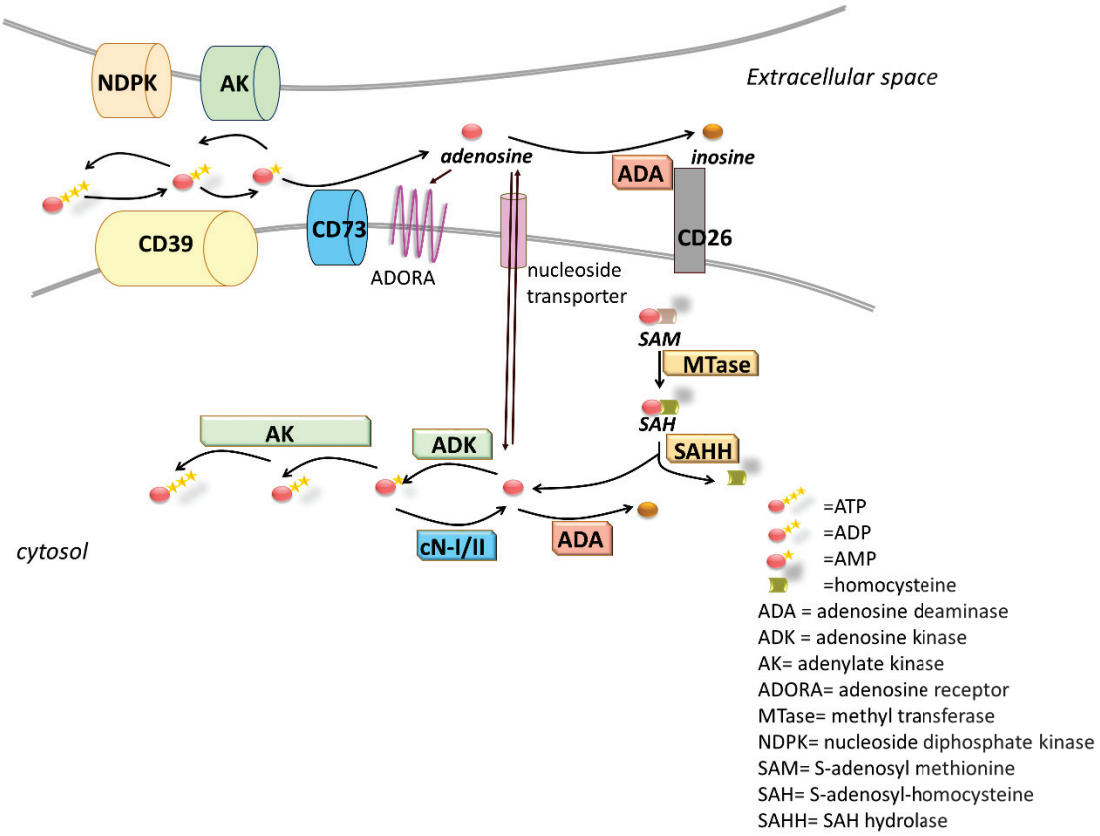

Figure 8: Intracellular and extracellular adenosine metabolism. Extracellular adenosine is mainly produced from ATP degradation. It can then be degraded, internalized or interact with specific receptors: ADORA. Intracellularly, adenosine is re-phosphorylated or degraded. Intracellular adenosine can be produced from adenine nucleotide catabolism or from th transmethylation pathway.

Adenosine in biology: roles

Adenosine plays various roles in biology. Cells can use adenosine as a precursor for nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADPH) and S-adenosyl methionine (SAM), via adenine nucleotide (ATP) use. Independently from the functions ensured by these derivatives, adenosine itself can act as a signaling molecule to play a role in multiple biological processes. For example, it is involved in neural and cardiac activity modulations (Borycz et al.; Butler et al.; Headrick et al., 2011; Quarta et

al.), and related pathologies like schizophrenia or migraine (Rial et al., 2014). Hereafter, we will focus on the impact of adenosine on cellular functions that can modify cancer cell biology.

Immune escape

In tumors, the immune system plays a central role in the control of cancer cell proliferation and is also responsible for immunoediting. These processes involve innate and adaptive immune cells. Adenosine is widely known as an immunomodulator because of its inhibiting role on effector T cells and its ability to orientate myeloid cells differentiation. Therefore, adenosine-mediated immune regulations are among the most relevant parameters to study in oncology (Young et al., 2014). As immune cells express adenosine receptors, most adenosine immunomodulating effects are mediated by ADORA signaling.

Innate immune cells

NK cells

Natural killer (NK) lymphocytes are described as the most competent cells for tumor immune surveillance. They recognize and eliminate "abnormal cells" through the perforin/granzyme or Fas/Fas-L systems. NK cells also participate in anti-tumor responses in established tumors, notably by cooperating with other immune cells (Deauvieau et al., 2014). In addition to their cytotoxic functions toward cancer cells, they secrete pro-inflammatory cytokines such as IFNγ and TNFα to recruit other immune cells. These lymphocytes express the A2A and A3 receptors whose activations are associated with less efficient NK cells maturation and proliferation, reduced cytotoxic functions and cytokine production (Baginska et al., 2013; Young et al., 2018). All these parameters render the NK cells less operative to control tumor initiation. Adenosine-mediated NK inhibition also promotes metastases occurrence (Beavis et al., 2013; Qin et al., 2014).

Macrophages

Macrophages are innate immune cells that are often recruited in tumors. In this case, they are called TAMs (tumor associated macrophages). Macrophages are antigen-presenting cells and digest apoptotic bodies from dead cancer cells in order to present peptides to the adaptative immune cells and generate a tumor-specific response. They also secrete inflammatory cytokines to support other immune functions. However, in the macrophage tumor infiltrate, two distinct subsets are identifiable: the "classically activated" anti-tumoral

M1 macrophages and “alternatively activated” pro-tumoral M2 macrophages. Most TAMs have a M2-like phenotype.

M1 macrophages secrete inflammatory cytokines and favor Th1 (cytotoxic) and Th17 responses, supporting direct and indirect anti-tumor responses. This branch of macrophage differentiation can be induced by purine signaling, and more precisely by the activation of the ATP receptor $P2Y_2R$ (Eun et al., 2014). Subsequently, the cells express the inducible nitric oxide synthase (iNOs) that metabolizes L-arginine to NO and is a marker of M1 lineage. On the contrary, M2 are believed to rather be pro-tumoral cells. Indeed, they secrete pro-angiogenic and pro-metastatic factors such as cytokines and matrix metalloproteinases, resulting in a microenvironment that supports cancer cell growth and metastatic processes (Condeelis and Pollard, 2006; Lewis and Pollard, 2006). M2 macrophages can be divided into four subsets: M2a, M2b, M2c and M2d. They have distinct markers and can be generated from different stimuli. Adenosine induces the M2d lineage and participate in increasing arginase 1 expression, that is a M2-marker (Csóka et al., 2012; Ferrante and Leibovich, 2012; Ferrante et al., 2013) . Moreover, adenosine favors the secretion of pro-angiogenic molecules (VEGF and matrix-remodeling proteins) (Ernens et al., 2010) while impeding the production of pro-inflammatory factors by M1 (Haskó et al., 1996).

An antigen presenting cell-dependent isoform of ADA (ADA2) is also involved in monocyte differentiation toward macrophages or dendritic cells *in vitro,* and indirectly favors T-cell proliferation (Zavialov et al., 2010). These processes involve the secrection of this enzyme and its interaction with a specific receptor. Nevertheless, it is not obvious that adenosine itself is involved in these phenomena, as ADA2 exerts its deaminase activity in an acidic an inflammatory context (Zavialov and Engström, 2005). The role of ADA2 on the tumor immune microenvironment remains to be better understood.

Neutrophils

Similarly as for macrophages, neutrophils are recruited to tumors and are associated with cancer cells. These tumor-associated neutrophils (TANs) can also adopt a pro- or an antitumor phenotype and impact tumor initiation, progression, or associated mechanisms. For example, N1 anti-tumoral neutrophils favor cancer cell death by a direct process or involving other immune cells (Mensurado et al., 2018; Zivkovic et al., 2007). N2 play their pro-tumoral role by

producing pro-angiogenic molecules such as VEGF or matrix modifying enzymes (Piccard et al., 2012) or can facilitate metastases (Wculek and Malanchi, 2015).

Adenosine binds the ADORA receptors present on neutrophils to regulate their function in a concentration-dependent manner. Indeed, low adenosine concentrations favor phagocytosis whereas elevated levels of extracellular adenosine impair this process (Barletta et al., 2012). At low physiological concentrations, adenosine favors neutrophils chemotaxis and recruitment but A2A stimulation with high concentrations of adenosine reduces ROS (reactive oxygen species) production (Bednarska et al., 2014; Fredholm et al., 1996) and thus the cytotoxicity toward tumor cells. In the context of cancer, extracellular adenosine levels increase, again favoring anti-inflammatory mechanisms and attenuating the anti-tumor response.

Dendritic cells

Dendritic cells are professional APCs. After their maturation, they overexpress major histocompatibility complex (MHC) molecules and co-stimulation molecules (CD80/86) that activate T lymphocytes, to generate an adaptive immune response.

Pro-inflammatory cytokines support T-cell differentiation for them to acquire their effective functions. In addition to reducing the production of these cytokines by dendritic cells (Challier et al., 2013; Schnurr et al., 2004), adenosine induces the production of pro-angiogenic and tolerogenic cytokines (Novitskiy et al., 2008). Moreover, dendritic cells that maturate under adenosine stimulation are not able to efficiently activate T lymphocytes (Challier et al., 2013).

Adaptive immune cells

In the tumor microenvironment, CD4+ and CD8+ T lymphocytes are crucial to drive anti-tumor immunity and kill cancer cells, respectively. These lymphocytes express A1, A2A and A2B adenosine receptors that mediate the nucleoside-induced effect. Adenosine can be considered a negative regulator for T cells as it inhibits their functions at different levels. First of all, adenosine signaling reduces T cell survival by impeding pro-survival signals (mediated by the IL7/IL7R axis), thus reducing the availability of cells that could fight the tumor (Cekic et

al., 2013). Moreover, extracellular adenosine interferes with T cells activation. Indeed, it can impede the activating phosphorylations downstream TCR/CD3 interaction with MHC/peptide complexes, impeding T cells activation levels (Hoskin et al., 2008). It also inhibits pro-inflammatory cytokines production (Lappas et al., 2005; Raskovalova et al., 2007) and T cell proliferation (Hoskin et al.) by diverse mechanisms, among which the negative regulation of IL-2 cascade (Butler et al.; Zhang et al., 2004) . Extracellular adenosine also alters cytotoxic functions of CD8+ lymphocytes by inhibiting their adhesion to target cells (MacKenzie et al., 1994, 2002) and interfering with cytotoxic-granule molecules production and excretion (Hoskin et al.; Raskovalova et al., 2007).

The interactions between B cells and adenosine are less known. A subset of CD39 and CD73 co-expressing B cells can release and produce adenosine from ATP. Then this adenosine can act in an autocrine or paracrine way. Adenosine production seems to be interrelated with commutation switch recombination in B cells (Schena et al., 2013). Conversely, the CD73 inhibitor APCP enhanced anti-tumor responses through mechanisms involving immunoglobulin-producing B cells (Forte et al., 2012).

Adenosine can also impact immune cells differentiation. For example, it promotes regulatory T cells lineage (Linden and Cekic, 2012), thus indirectly favoring the inhibition of the anti-tumor adaptive response.

All together, these observations designate adenosine as a factor that strongly promotes immune escape and thereby promotes cancer progression (Kaku et al., 2014) (Figure 9).

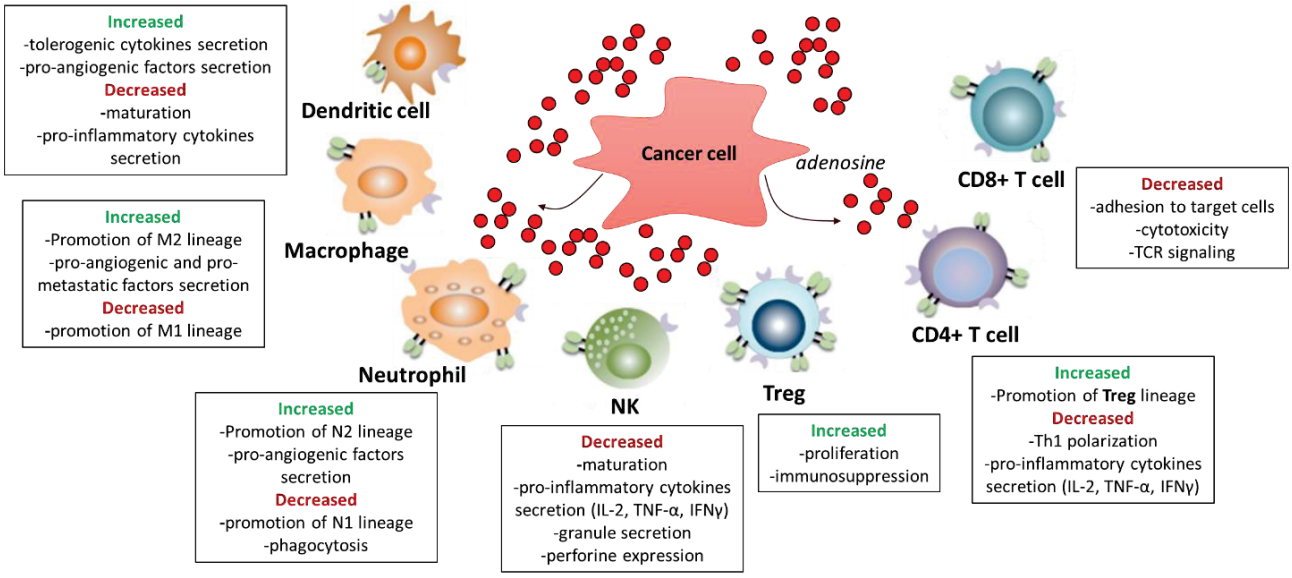

Figure 9: Adenosine and immune cells in the tumor microenvironment. High concentrations of adenosine impact innate and adaptive cells and result in an overall pro-tumoral behavior of immune cells. Adapted from "Targeting cancer-derived adenosine: New therapeutic approaches. (Young et al., 2014)

Cell proliferation and DNA stability

Nucleotide pools

Cells need to incorporate dNTPs to replicate their DNA. Deoxynucleotide pools imbalance thus increases the risk of mistakes in DNA synthesis, and favors genome instability (Bester et al., 2011; Kunz et al., 1994). As mentioned earlier, these dNTP pools originate from their corresponding ribonucleotides. Thus, to keep dNTP pools balanced, it is necessary to maintain the homeostasis in the activity of enzymes involved in their conversion and their degradation (Menezes et al., 2012; Rampazzo et al., 2010).

Adenosine and cell proliferation

Multiple roles of adenosine on cell proliferation have been described. Depending on the studied cell type and the nucleoside concentration among other factors, it can be either pro or anti-proliferative (Aghaei et al., 2011; Ethier and Dobson, 1997). These effects are either mediated by cellular uptake and subsequent metabolism of adenosine in the cells (Brown et

al., 2000), or through the ADORA signaling. Indeed, the activation of A1, A2A, A2B or A3 receptors on cancer cells can lead to signaling cascades that modulate cell proliferation (Fernandez-Gallardo et al., 2016; Gessi et al., 2017). Whether each receptor is rather associated with pro- or anti-proliferative effects remains in debate and seems to be cell or tissue specific. Some other studies suggest that adenosine internalization and receptor-independent mechanisms also play a role in cell proliferation (Brown et al., 2000).

These effects of adenosine on cell proliferation opens to the possibility to use adenosine metabolism-related enzymes modulators, in order to decrease *in vivo* tumor growth. For example, Nakajima *et al.* used an ADA inhibitor, to accumulate adenosine and this reduces tumorigenicity in mice (Nakajima et al., 2015).

Adenosine induced cell death

Adenosine inhibitory effects on cell proliferation are often associated with apoptotic effects. High concentrations of adenosine trigger cell death in several cancer cell lines, through intrinsic or extrinsic mechanisms. The extrinsic pathways involve the ADORA receptors and their downstream cascades whereas the intrinsic ones depend on adenosine internalization and, most of the time, its conversion into AMP (Tanaka et al., 1994; Tsuchiya and Nishizaki, 2015).

Intrinsic effects have been identified thanks to the fact that inhibiting nucleoside transporters reduces adenosine-induced cell death, meaning that the nucleoside can trigger apoptosis through its intracellular fates. Several studies showed that phosphorylation of adenosine into AMP is necessary to induce cell death(Li et al., 2013; Nogi et al., 2012; Schrier et al., 2001; Wakade et al., 1995; Yang et al., 2007). Indeed, when it is internalized and converted by adenosine kinase, the generated AMP can activate AMPK-dependent kinase (AMPK), reinforcing the intrinsic activation of apoptosis that is activated downstream this sensor (Mello et al., 2014; Saitoh et al., 2004; Yang et al., 2011a). Indeed, different caspase-activating mechanisms were identified following adenosine internalization. For example, in the hepatocarcinoma Huh-7 cell line, activation of caspase 8 was induced by downregulation of its inhibitor c-FLIP ((FADD)-like interleukin-1β-converting enzyme inhibitory protein)(Yang

et al., 2007). Adenosine is also able to modify the expression of apoptosis modulators such as DIABLO-S, Bcl-XL, IAP2 and IAP3, leading to caspase-9-independent caspase-3 activation in Huh-7 hepatoma cells (Yang et al., 2010).

In different models, adenosine is described as triggering apoptosis in a caspase-independent way, by upregulating an apoptosis-inducing factor-homologous mitochondrion-associated inducer of death (AMID) in the nucleus (Yang et al., 2007, 2011b, 2011b). Also, in mesothelioma, adenosine upregulates p53 expression, triggering caspase-dependent or -independent cell death (Nogi et al., 2012)

Several teams described cytotoxic effects of adenosine that were mediated by ADORAs only, or in association with the intrinsic pathways, and thereby modulated by ligands to these receptors. The A3 receptor is the one that displays pro-apoptotic activity in a wider range of cells. It can trigger cell death in mesothelioma, lung, hepatocellular, breast, thyroid and colon cancer cell lines (Kanno et al., 2012a, 2012b). A1 and A2A (Hardie et al., 2012; Merighi et al., 2002) have also been identified as participating in this process. Downstream their activation, ADORA can activate caspases (Kamiya et al., 2012) or generate cell death through caspase-independent (Kanno et al., 2012a, 2012b; Nogi et al., 2012) processes like AMID accumulation.

Energetic status and metabolism

Intracellular adenosine is mostly phosphorylated to corresponding nucleotides. The generation of di- or tri-phosphorylated nucleotides may have an impact on the energetic balance defined as [(ATP+ 1/2.ADP)/(ATP+ADP+AMP)] (Atkinson, 1968). Downstream, the global cell metabolism can change by influencing the activation status of AMP-dependent protein kinase (AMPK). Indeed, the latter is a key enzyme that allows cells to switch between anabolic and catabolic metabolisms according to the energetic status, particularly when ATP levels are low and AMP levels are high (Hardie et al., 2012). Under these conditions, AMPK will facilitate the activation of catabolic pathways such as autophagy, lipid β-oxidation or glycolysis that provide cells with macromolecules to ensure their growth while saving energy. On the contrary, anabolic high-energy consuming processes such as protein synthesis are

downregulated by AMPK. Thus, adenosine, through AMP and AMPK, is a potential regulator of major cellular metabolisms.

Migration and invasion

Extracellular adenosine plays a role in cell migration and invasion. Indeed, this purine nucleoside acts in an autocrine/paracrine way and is able to trigger cytoskeleton reorganization, thus indirectly impacting cell motility capabilities (Abbracchio et al., 1997).

It has been shown that cancer cell migration could be reduced with low micromolar to low millimolar concentrations of adenosine and involving all the ADORA receptors. According to the cell type and the concentrations used, receptor-dependent and receptor-independent mechanisms can be involved (Virtanen et al., 2014). Various signaling pathway can impact cell migration but not all of them can be affected by adenosine. For example, CD44 and FAK are known to be pro-migration molecules. Nevertheless, in PC3 cancer cells, adenosine can slow migration and invasion down without affecting these molecules (Virtanen et al., 2014). On the contrary, in other cancer cell lines, adenosine can interact with several migration and EMT-related pathways (Martínez-Ramírez et al., 2017) like in glioblastoma in which adenosine exposure is associated with the reduction of the pro-migration MMP-9 gelatinase expression and overall activity by modulating the ERK/AKT pathway (Gessi et al., 2010).

Conversely, other studies suggest a pro-migration receptor-mediated effect on adenosine (Fernandez-Gallardo et al., 2016; Schneider et al., 2015). Therefore, the nucleoside effects on cell migration can be cell-dependent. Virtanen *et al.* mentioned in their work that adenosine signaling tends to stimulate pro-migration behavior but the sub localization of migration-related molecules to determine the leading edge of the cells is important to induce cell movement.

Nucleosides and cancer therapies

Nucleotide analogs are common as chemotherapeutical agents used against cancer. These drugs are in competition with endogenous nucleotides to be incorporated in neo-synthesized DNA during cell proliferation. Nucleotide analogs such as 5-FU (5-Fluoro-Uracil) are synthesized under an unphosphorylated form, they need the target cell machinery to get tri-phosphorylated and be incorporated in neo-synthesized DNA. Other mechanisms are also involved in their toxicity. For example, 5-FU interfere with pyrimidine metabolism by inhibiting thymidylate synthase. Considering the importance of modifying nucleoside analogs for their incorporation in DNA , nucleotidases (Sk ładanowski, 2013) and nucleoside transporters (Koczor et al., 2012) play crucial roles in their metabolism and efficiency. Consequently, efforts are made to target these molecules to optimize treatment responses, as it is the case for the cytosolic 5'-nucleotidase cN-II (see following section). Other purine metabolism-related enzymes are also explored in therapy. For example, a PNP-Annexin-V fusion protein is explored for its ability to enhance the conversion of chemotherapeutical agents into cytotoxic metabolites, specifically in cancer cells (Krais et al., 2013). RNR is often overexpressed in cancers and confers resistance to treatments. It is thus targeted by chemotherapeutical agents, to impede these effects (Aye et al., 2015).

Owing to its multiple roles in cancer biology, adenosine metabolism is widely studied for innovative therapies. The following table (Table 2) shows some of the promising adenosine-metabolism-targeting molecules and their interest. This includes for example CD73-targeting and ADORA-targeting therapies. These strategies aim to inhibit tumor CD73 activity or adenosine receptors activation with antagonist antibodies or small molecules. Consequently, less adenosine is produced in the tumor microenvironment, or its interaction with the receptors on immune cells is reduced thus favoring immune anti-tumor responses.

CD73-targeting strategies can be coupled with CD39-inhibiting agents. Indeed, this allows to block an upstream step of adenosine production, which is ATP degradation into AMP. Although current CD73-targeting therapies mostly focus on inhibiting its

enzymatic activity, it is also relevant to develop strategies that could also limit its pro-tumoral non-enzymatic functions.

MEDI9447 or Oleclumab is a monoclonal antibody targeting CD73. It is a CD73 non-competitive inhibitor that is able to target the membrane-anchored and soluble forms of the enzyme by maintaining it under an inactive conformation (Geoghegan et al., 2016). This antibody showed the ability to reduce AMP hydrolyze *in vitro* and an antitumor activity *in vivo* (Hay et al., 2016). These promising preliminary data lead to ongoing clinical studies to test MEDI9447 toxicity and efficiency alone, or in association with other therapies in cancer. Other antibodies or small molecules are currently being developed, *in silico, in vitro* and *in vivo*, as it is the case for sulfonic acid compounds / pyrazolopyridines/benzofuropyrimidines, or the AD2 monoclonal antibody (Iqbal et al., 2013; Miliutina et al., 2018; Rahimova et al., 2018; Terp et al., 2013).

PFB-509 (a monoclonal antibody) and CPI-444 (a small molecule) are A2A receptor inhibitors that aim to reduce adenosine-induced immune suppression in the tumor microenvironment by binding the receptor on immune cells. These compounds were able to restore T cell and NK cell activities while reducing metastases occurrence, sensitizing cancer cells to immune checkpoint inhibitors and improving survival in mouse models. Both therapies are currently being tested in clinical trials with the aim to combine their use with immune checkpoint inhibitors (Emens et al., 2017; Leone et al., 2018; Mediavilla-Varela et al., 2017). As A2B receptor activation is strongly involved in immune suppression in cancers, efforts are made to generate A2B inhibitors as well as A2A/A2B dual antagonists (Galezowski et al., 2018).

Target	Strategy	molecule	references
CD73	Reduce tumor CD73-mediated extracellular adenosine generation, mostly to restore a context that favors immune cells anticancer functions	APCP and derivates	(Bhattarai et al., 2015)
		Anti-CD73 monoclonal antibodies (MEDI9447; AD2 monoclonal antibody)	(Geoghegan et al., 2016; Hay et al., 2016) Clinical trials ID: NCT02503774, NCT03381274, NCT03267589 (Terp et al., 2013)
		Sulfonic acid compounds	(Iqbal et al., 2013)
		Pyrazolopyridines and benzofuropyridines	(Miliutina et al., 2018)
A2 adenosine receptors	Inhibit A2A and A2B signaling in immune cells to restore their antitumor functions	A2A receptor antagonists (PBF-509, CPI-444, AB928)	(Allard et al., 2016) PFB-509 clinical trial ID: NCT02403193 (Mediavilla-Varela et al., 2017) CPI-444 clinical trial ID: NCT02655822; NCT03454451; NCT03337 98 (Emens et al., 2017; Leone et al., 2018) AB928: (Walters et al., 2017)
		Dual A2A and A2B antagonists	(Galezowski et al., 2018; Vijayan et al., 2017)
		A2B antagonist PSB1115	(Iannone et al., 2013; Mittal et al., 2016)

Table 2: Non-exhaustive list of therapeutic adenosine-targeting strategies. Ongoing studies aim to reduce extracellular adenosine in tumors or to block its cellular effect on

ADORA. The adenosine-metabolism targeting molecules are tested alone or in combination with other treatments such as immune checkpoint inhibitors.

5'-nucleotidases cN-II and CD73

5'-nucleotidases participate in nucleotide catabolism by converting mono-phosphorylated nucleotides into their corresponding nucleoside (Zimmermann, 1992). Thus, they regulate nucleotide/nucleoside pools in the cells. There are seven human 5'-nucleotidases that vary by their substrate and tissue specificities. Six of the known 5'-nucleotidases are intracellular (cN-IA, cN-IB, cN-II, cN-III, cdN and mdN) and one is anchored to the membrane by a GPI (glycosylphosphatidylinositol) anchor and can be liberated in the interstitial space (CD73). Due to their proven roles in cancer, we are interested in cN-II and CD73.

cN-II

cN-II is encoded by *NT5C2* gene situated on chromosome 10 and is highly conserved among species (Cividini et al., 2015a). It is one of the five cytosolic (but not mitochondrial) 5'-nucleotidases and has a phosphatase and a purine-specific-phosphotransferase activity. It differs from its counterparts because of its preferential substrates. cN-I preferentially dephosphorylate AMP, cN-III has UMP ad CMP pyrimidines as substrates and cdN is deoxyribonucleotides-specific, whereas cN-II has a higher affinity for IMP and GMP (Bianchi and Spychala, 2003; Ipata and Balestri, 2013; Zimmermann, 1992). Nevertheless, the latter can also metabolize AMP with a lower activity. ATP, other tri-phosphorylated purines and inosine are major positive regulators of cN-II. In rat and bovine cells, it has been demonstrated that the energy charge can also stimulate its activity (Pesi et al., 1994).

The physiological roles of this enzyme are not fully understood yet, but it has been demonstrated that cN-II is involved in the maintenance of the balance in intracellular purine pools (Allegrini et al., 2013; Cividini et al., 2015b), impacting the energy status and consequently AMPK activation, as demonstrated in skeletal muscle (Kulkarni et al., 2011; Kviklyte et al., 2017).

cN-II also participates in cancer cell biology. In a study from Filoni and colleagues, cN-II downregulation was associated with enhanced cell death (Filoni et al., 2011) and another study highlighted a link between cN-II activity and astrocytoma cells survival (Careddu et al., 2008). This enzyme has also been associated with cell proliferation in ADF cell line (Cividini et al., 2015c). cN-II has the ability to interact with other intracellular proteins as exemplified by the inflammasome protein IPAF (NRLC4), suggesting further roles for this protein, potentially independent from its enzymatic activities (Cividini et al., 2015a).

In cancer, cN-II is involved in prodrugs metabolism. More specifically, its phosphotransferase activity can help to activate nucleoside analogs for them to be incorporated to DNA. On the contrary, its phosphatase activity can dephosphorylate the chemotherapeutic agents, rendering them nontoxic for the cancer cell (Jordheim et al., 2006, 2015; Mazzon et al., 2003). Consistently, mutations associated with increased enzymatic activity of cN-II have been associated with bad response to nucleobase-analogue-based treatment or rapid relapse in ALL (acute lymphoblastic leukemia) (Meyer et al., 2013a, 2013b; Tzoneva et al., 2013), and patients with high cN-II expression levels show a worse prognosis than those with lower expression levels when treated with nucleoside analogues (Galmarini et al., 2001). Therefore, as cN-II can represent an interesting target in anti-cancer therapies, efforts are made in the direction to finding inhibitors for this enzyme (Gallier et al., 2011; Jordheim et al., 2013; Marton et al., 2015). As its high activity and high expression are associated with bad prognosis in acute myeloid leukemia and chronic lymphocytic leukemia, the innovative strategies would aim to inhibit is activity.

CD73

CD73 is encoded by *NT5E* gene located on chromosome 6 and is a GPI (glycosylphosphatidyl inositol)-anchored enzyme that exposes its catalytic site outside the cytoplasm. It is mostly located at lipid rafts, associated as homodimers (Bianchi and Spychala, 2003). CD73 is a key enzyme of extracellular purine catabolism due to its hydrolase activity that allows the conversion of mono-phosphorylated nucleotides such as AMP, IMP or NMN into their corresponding nucleosides.

It is common that ovarian, breast (Wang et al., 2008), bladder, thyroid, esophageal, head and neck squamous carcinoma (Ren et al., 2016), prostate, melanoma, glioma glioblastoma and leukemic cells over express or present a high CD73 activity. CD73 expression can be induced in already established tumors and promote a more aggressive phenotype (Monteiro et al., 2018; Reinhardt et al., 2017). Indeed, hypoxia is one of the major parameters that promote this ecto-5'-nucleotidase expression and neoangiogenesis, which is a way to correlate CD73 with bad prognosis (Ren et al., 2016). In addition, the subsequent higher overall CD73 activity increases extracellular adenosine concentrations leading to the enhancement of its pro-tumoral effects (described in previous sections). Moreover, enzymatic activity-independent functions of CD73 have been described in cancer (Gao et al., 2017). For example, it was shown that CD73 expression was associated with enhanced migration and proliferation. This phenomenon was not reversed by using enzymatic inhibitors of CD73, indicating alternative ways for this protein to favor cancer progression, independently from adenosine production. These alternative ways involved overexpression of growth factors receptors (EGFR) or growth factors (VEGF).

Considering these effects, it can be considered that CD73 controls tumor progression and immune escape, making it a relevant target for therapeutic strategies. Consistently, many studies aim to find and evaluate molecules and antibodies targeting CD73 activity. Most of them focus on re-establishing an anti-tumor immune response. Therefore, these studies sometimes combine anti-CD73 strategies and block the ADORA signaling in immune cells to inhibit overall adenosine effects in the tumor.

Aims of the PhD project

cN-II and CD73 have been identified as possible targets for new anti-cancer therapies. Nevertheless, very little is known about the biological roles of cN-II and what parameters of cell biology could be impacted by such strategies. In parallel to the development of inhibitors, we are interested in increasing the knowledge about the roles of this enzyme in cancer. As cN-II activity impacts intracellular adenine nucleosides/nucleotides pools (Tzoneva et al., 2018) and considering the involvement of these purines in cell metabolism, we wondered what

changes a decrease in cN-II expression or its silencing could trigger in cancer cells as well as in the microenvironment.

Similarly, although CD73 is widely studied in cancer because it favors immune escape by producing adenosine, little is known about its role on cancer cells themselves. CD73 participates in the regulation of extracellular adenine nucleosides/nucleotides pools. As these molecules are able to be translocated toward the intracellular compartment, we can wonder about the consequences on the cell biology as well as on the intracellular nucleotide pools.

Thus, the aims of our current project were to (i) Increase knowledge about cN-II and CD73 in cancer cell biology, in physiological conditions and nucleotide stress, (ii) Understand the ability of cancer cells to modify their microenvironment through nucleotides and according to cN-II and CD73 expressions. Here, we more precisely focused on cancer cells metabolic plasticity, migration and their possible interplay with the innate immune microenvironment.

Part I: cN-II, CD73 and metabolic adaptability of cancer cells

The cytosolic 5'-nucleotidase cN-II lowers the adaptability to glucose deprivation in human breast cancer cells

The first work concerned the investigation of the roles of cN-II in metabolic adaptability. To do so, we generated models in which the cytosolic 5'-nucleotidase expression was decreased with stable transfection of shRNA-encoding plasmids. We then evaluated the impact on glucose metabolism and ROS defense. The results are presented in the following article, published in Oncotarget in 2017 (Bricard et al., 2017)

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Research Paper

The cytosolic 5'-nucleotidase cN-II lowers the adaptability to glucose deprivation in human breast cancer cells

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ABSTRACT

The cytosolic 5'-nucleotidase cN-II is a highly conserved enzyme implicated in nucleotide metabolism. Based on recent observations suggesting additional roles not directly associated to its enzymatic activity, we studied human cancer cell models with basal or decreased cN-II expression. We developed cancer cells with stable inhibition of cN-II expression by transfection of shRNA-coding plasmids, and studied their biology. We show that human breast cancer cells MDA-MB-231 with decreased cN-II expression better adapt to the disappearance of glucose in growth medium under normoxic conditions than cells with a baseline expression level. This is associated with enhanced *in vivo* growth and a lower content of ROS in cells cultivated in absence of glucose due to more efficient mechanisms of elimination of ROS. Conversely, cells with low cN-II expression are more sensitive to glucose deprivation in hypoxic conditions. Overall, our results show that cN-II regulates the cellular response to glucose deprivation through a mechanism related to ROS metabolism and defence.

INTRODUCTION

The hallmarks of cancer include genome instability and mutations, leading to deregulated energetic homeostasis, sustained proliferative signaling and escape from immune surveillance [1]. These major characteristics are all influenced by nucleoside metabolism as shown by: i) increased rate of genomic modifications when nucleotide pools are deregulated [2], ii) the important role of nucleotide derivatives as sources of energy for the cell and intracellular signaling effectors, and iii) the tumor- and immuno-modulating roles of adenosine and ATP [3, 4]. Therefore, nucleotide metabolism has become a subject of major interest in cancer research and constitutes a potential target for anticancer therapy.

5'-nucleotidases are involved in nucleotide metabolism by dephosphorylating nucleoside monophosphates into nucleosides and inorganic phosphate. There are today eight different human 5'-nucleotidases described, and they differ by their subcellular localization, substrate affinities and regulatory mechanisms [5, 6]. The cytosolic enzyme cN-II has a preference for IMP and GMP and has also been described as being capable of phosphorylating nucleosides through a phosphotransferase activity [7]. We have previously shown that this enzyme is involved in the sensitivity of cancer cells to nucleoside

analogue-based chemotherapy [8, 9], and developed and studied enzymatic inhibitors [10–14]. The clinical relevance of this approach has been confirmed by the observation of hyperactive cN-II mutants in relapsed pediatric acute lymphoblastic leukemia patients associated with a resistance to purine analogues [15, 16]. However, very little is known about the overall physiological role of cN-II in cells, and especially in cancer cells from solid tumors. Transient inhibition of its expression in neuroblastoma cells by siRNA indicated a role in cell survival as this was associated with induction of apoptosis [17], whereas a similar decrease in skeletal muscle cells induced activation of AMPK (which regulates lipid and glucose metabolism) as well as modified lipid metabolism and glucose transport [18]. In addition, stable up- or down-regulation of cN-II expression in various cancer cells has shown its implication in cell proliferation even though this is not the case for all cell lines [9, 19, 20]. Finally, the recently demonstrated interaction between cN-II and the inflammasome-protein NLRC4/Ipaf suggests other and still unknown properties of this enzyme in cell biology that could be independent of its enzymatic activity [21].

In this study, we show that cN-II decreases the capacity to manage intracellular levels of reactive oxygen species (ROS), suggesting an important role of this protein in cell biology.

RESULTS

Transfected cells have decreased cN-II expression and enzymatic activity

The pScN-II cell models used in this study have previously been shown to have decreased cN-II protein expression [19]. This modification in protein expression was associated with a 1.3-2.2-fold decrease in specific enzymatic activity in all cell lines. Indeed, the specific enzymatic activity (nmol of inosine produced by minute per milligram of protein) in presence of ATP was 2.49 ± 0.20 for MDA-MB-231-pScont vs. 1.15 ± 0.04 for MDA-MB-231-pScN-II; 5.49 ± 0.75 for HCT-116-pScont vs. 2.89 ± 0.50 for HCT-116-pScN-II; 1.85 ± 0.25 for NCI-H292-pScont vs. 1.46 ± 0.16 for NCI-H292-pScN-II; 1.98 ± 0.14 for MIA PaCa-2-pScont vs. 1.33 ± 0.02 for MIA PaCa-2-pScN-II.

Decreased cN-II expression is associated with enhanced *in vivo* xenograft growth

Initial experiments of *in vitro* proliferation of the transfected models by CFSE titration did not show any differences between pScont and pScN-II cells [19]. We here continued the characterization with the evaluation of tumor growth in scid mice after the injection of 5 million cells subcutaneously. As indicated in Figure 1, the growth of pScN-II cells was consistently more rapid than for pScont cells in the four different models evaluated. This difference was modest and statistically significant for MIA PaCa-2 cells at day 27, suggesting that stably reduced content of cN-II in these cell models can favor tumor growth. Whereas tumors with NCI-H292, MIA PaCA-2 and HCT-116 cells reached a volume of approximately 1000 mm^3 after 28 days, MDA-MB-231 cells grew more slowly.

pScN-II cells have modified *in vitro* growth as compared to pScont cells

To investigate the proliferation and behavior of the transfected cells *in vitro*, we performed long-term cell culture with real-time assessment of proliferation and adherence capacity using the xCELLigence technology. In these experiments, the cell culture media was not changed during the culture. As indicated in Figure 2 for MDA-MB-231 cells, there is a clear shift in the cell index appearing after approximately 7 days of culture in media containing initially 25 mM glucose. These variations in growth curves are much less pronounced for pScN-II cells as compared to pScont cells, indicating a major difference in the behavior between the two cell lines in response to the modifications appearing at this moment. When the initial concentrations of glucose in the media were lower, the same event appeared earlier (5 days with 10 mM, 4 days with 5 mM) and was always less pronounced for pScN-II cells. This suggested that the shift in cell index was associated with the disappearance of glucose in the culture medium. No interpretable growth curves were obtained with long term culture of any of the other cell models probably due to the rapid growth of these cells, and thus no glucose-dependent variations were observed. We therefore focused most of the further experiments on the MDA-MB-231 cell model.

Decreased cN-II expression does not modify glucose uptake or lactate secretion *in vitro*

As the MDA-MB-231-pScont and -pScN-II cells displayed different behavior in terms of cell index that was dependent on initial glucose concentration, we compared the glucose uptake and associated lactate secretion between the two cell models. When cells were seeded at the same ratio of cells/volume of media as in previous experiments and with 10 mM glucose, the glucose disappeared from the medium after 6 days of culture both for pScN-II and pScont cells (Figure 3). No notable differences in extracellular glucose concentration (reflecting glucose uptake) or in extracellular lactate concentrations (reflecting lactate secretion) were observed between the two cell lines when the cell number, as determined by direct counting, was taken into account. This suggested that the difference in cell behavior observed in Figure 2 was rather due to the

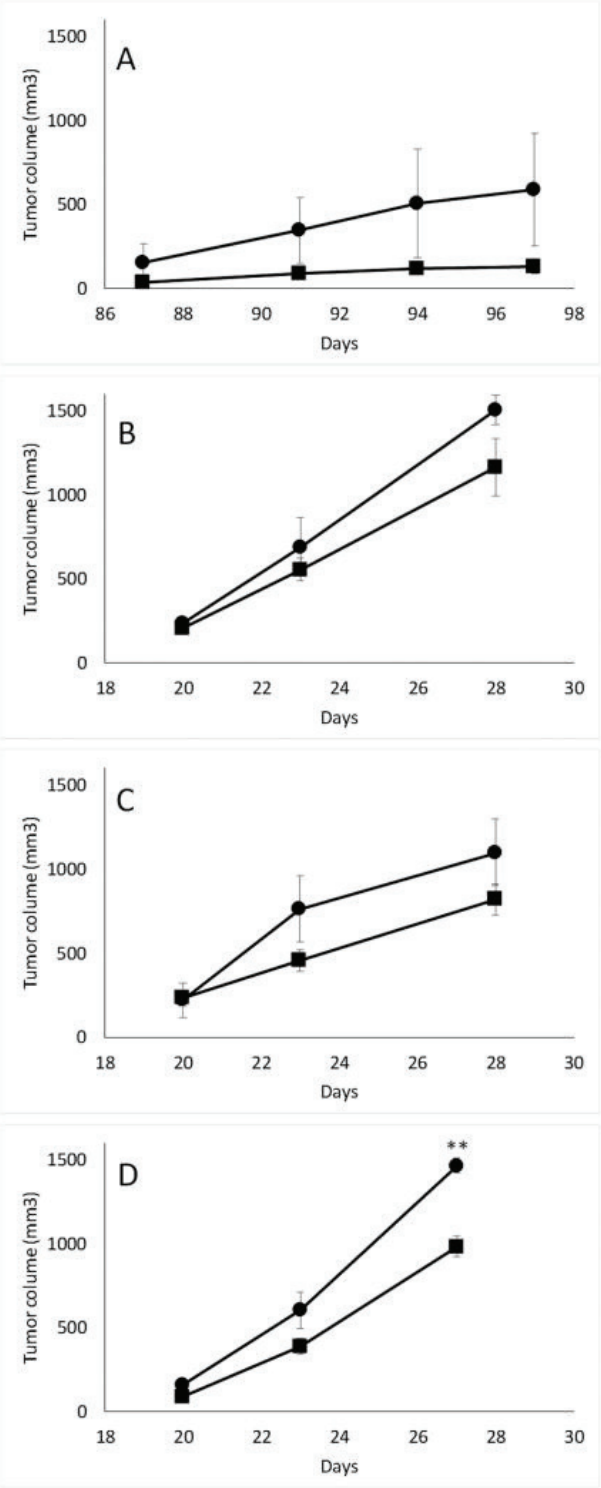

Figure 1: *In vivo* tumorigenesis of MDA-MB-231 **(A)**, HCT-116 **(B)**, NCI-H292 **(C)** and MIA PaCa-2 **(D)** pScont (■) and pScN-II cells (●). Tumor volumes are mean values from 3 mice per group and error bars are standard deviation. **: $p<0.005$ with Student's *t*-test.

ability of the different cells to adapt to culture media without glucose rather than to their use of glucose. In addition, as extracellular lactate levels reached 20 mM for an initial concentration of 10 mM glucose, it seems that the cells metabolized glucose preferentially through glycolysis rather than through oxidative phosphorylation.

pScN-II cells have lower content of ROS during long-term *in vitro* growth

When glucose is completely consumed, cells have to switch their metabolism towards the use of extracellular lactate as a carbon source or to beta-oxidation of fatty

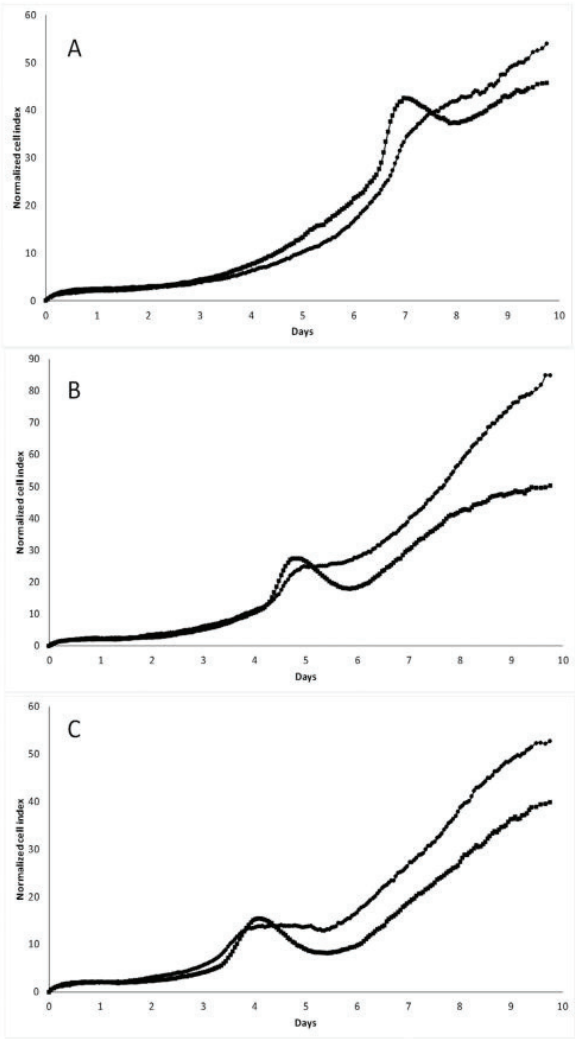

Figure 2: Long term *in vitro* cell growth of MDA-MB-231-pScont (■) and -pScN-II (●) cells in presence of 25 mM **(A)**, 10 mM **(B)** or 5 mM **(C)** glucose**.** Cells were seeded at 3000 cells per well in a final volume of 250 µl. Graphs show the normalized cell index during time (normalized on 5 hours).

acids. Glutamine is another potential substrate but is highly unstable under our experimental conditions and is rapidly cleared from the culture medium. Lactate is transformed into pyruvate and acetyl-CoA while fatty acids release acetyl-CoA, which is further processed through the tricarboxylic acid cycle and oxidative phosphorylation in the mitochondrion. It has been shown that ROS-induced activation of AMPK further induces activation of pyruvate dehydrogenase kinase (PDK) and phosphorylation of pyruvate dehydrogenase (PDH) that stimulates lactate processing [22], and that AMPK stimulates beta-oxidation by ACC phosphorylation [23]. We propose that MDA-MB-231-pScN-II cells are more prone to perform this shift from glucose metabolism to lactate metabolism or to beta-oxidation. However, the oxidative phosphorylation is reported to be associated with enhanced levels of reactive

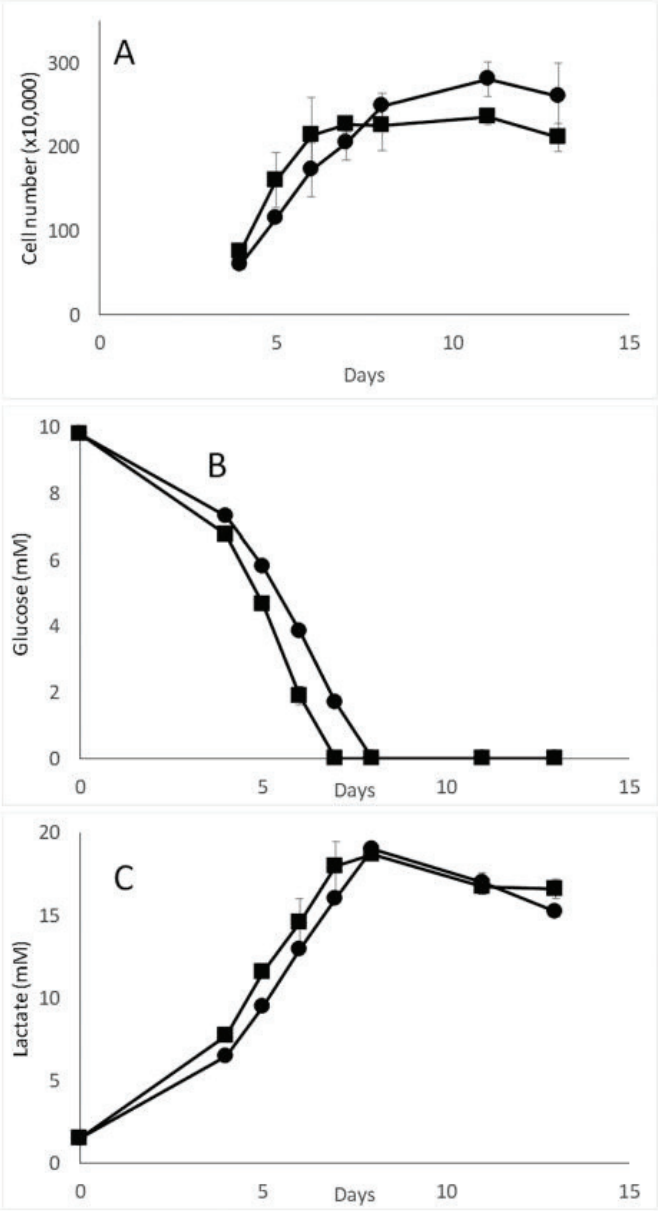

Figure 3: Cell number **(A)**, glucose **(B)** and lactate **(C)** concentration in media during long term *in vitro* culture of MDA-MB-231-pScont (■) and -pScN-II (●) cells. Cells were seeded in 6-well plates (90 000 cells per plate) in media containing 10 mM glucose. Values are mean results of duplicates from a representative experiment and error bars are standard deviation.

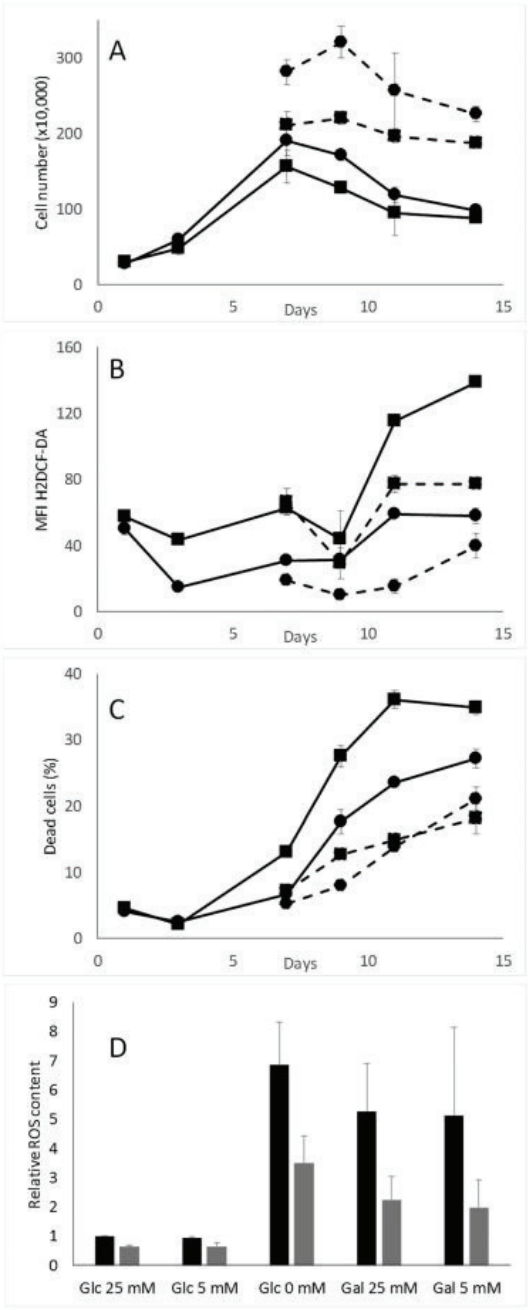

Figure 4: Cell number **(A)**, ROS content **(B)** and cell death **(C)** in MDA-MB-231-pScont (■) and -pScN-II (●) cells cultivated long-term without (full lines) or with renewed glucose (dotted lines). Cells were seeded in 6-well plates (180 000 cells per well in 3 mL of media with 5 mM glucose) and glucose (5 mM) was renewed three times a week. In A-C, results are means of duplicates from a representative experiment and error bars are standard deviation. **(D)** ROS content in MDA-MB-231-pScont (black) and -pScN-II (grey) cells cultivated for 3 days in media containing either 5 or 25 mM glucose or galactose or no sugar. Cells were seeded in 6-well plates (200 000 cells per well, 3 mL of media) and adhered overnight before culture in indicated media. In **(D)**, results are mean values of the ratio of MFI in each condition and the glucose 25 mM condition in three independent experiments and error bars are standard deviation. For both cell lines, Glc 0 mM and Gal 25 mM and Gal 5 mM were statistically significant ($p<0.05$, Student's *t*-test) from Glc 25 mM, and for Glc 25 mM, Glc 5 mM, Glc 0 mM and Gal 25 mM differences between pScont and pScN-II were statistically significant ($p<0.05$, Student's *t*-test).

oxygen species [24], which would rather be detrimental than beneficial for pScN-II cells. We therefore evaluated ROS levels in cells during cell culture simulating the conditions used during xCELLigence experiments. As shown in Figure 4A-4C, the ROS level increased in MDA-MB-231-pScont cells some days after the disappearance of glucose in the cell culture media (approximately when cell growth reaches a plateau), whereas ROS levels remained lower in pScN-II cells. The increase in ROS levels was associated with enhanced cell death as determined by Annexin V/PI staining, and both phenomena were delayed when glucose deprivation was avoided by adding 5 mM glucose to the media twice a week. A similar decrease in the ROS content was obtained by N-acetylcysteine instead of glucose during the experiment (data not shown). The influence of glucose starvation on ROS accumulation was confirmed in a 3-day experiment where pScont cells cultivated in absence of glucose accumulated much more ROS than pScN-II cells (Figure 4D). The replacement of glucose by galactose, which forces cells to perform oxidative phosphorylation, yielded similar results as for cells without glucose. Similar experiments performed on NCI-H292, MIA PaCa-2 and HCT-116 cell models did not show any differences between pScont and pScN-II cells (data not shown).

Cells with low cN-II expression are sensitive to combined hypoxia and glucose deprivation

We further performed xCELLigence experiments in hypoxic conditions (1% O_2), and observed that pScN-II cells were clearly more sensitive and died earlier than pScont cells (Figure 5). The time of cell death was here also dependent on the initial concentration of glucose in the culture medium suggesting that tolerance to reduced glucose was different under normoxic and hypoxic conditions.

If the difference observed in cell survival in hypoxic conditions is due to a difference in remaining oxidative phosphorylation, the cells could have been expected to

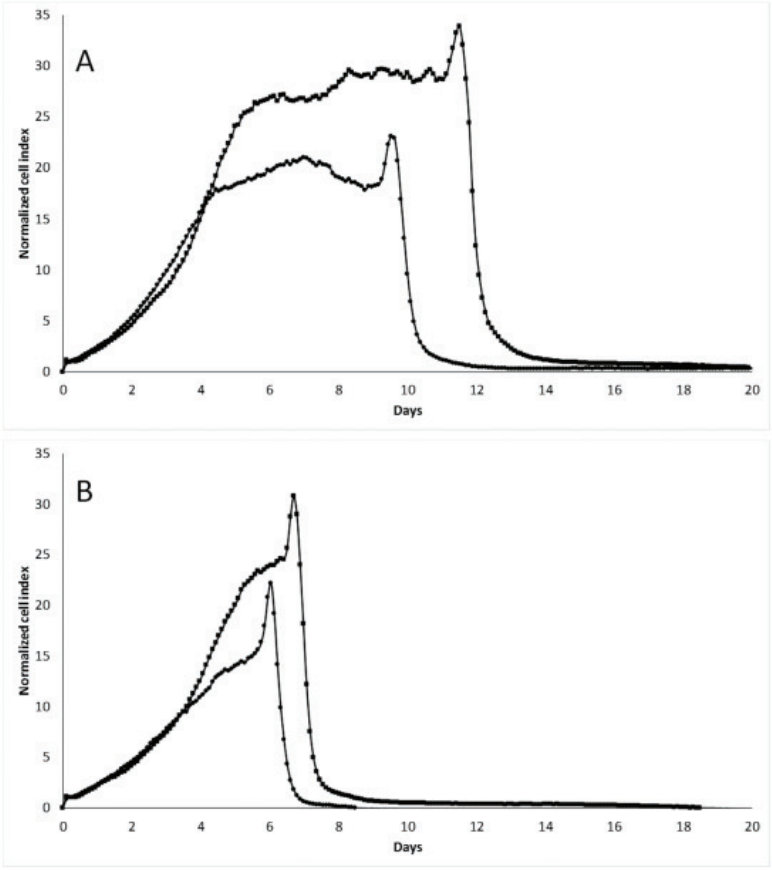

Figure 5: *In vitro* cell growth of MDA-MB-231-pScont (■) and -pScN-II (●) cells in hypoxic (1% O_2) conditions in presence of 10 mM **(A)** or 5 mM **(B)** glucose. Cells were seeded at 3000 cells per well in a final volume of 250 µl. Graphs show the normalized cell index during time (normalized on 5 hours).

display different sensitivities to inhibitors of oxidative phosphorylation. This was however not the case in our MDA-MB-231 models, as the percentage of dead cells was similar in pScN-II cells as compared to pScont cells after 48 hour exposures to 5 or 50 μM rotenone, a mitochondrial complex I inhibitor (Figure 6A).

Finally, we evaluated the ROS content in pScont cells and pScN-II cells cultured in hypoxic conditions with or without glucose. We observed similar results as in normoxia, *i.e.* pScont cells accumulating higher levels of ROS than pScN-II cells in absence of glucose, and lower ROS contents for both cell lines when glucose was added to the medium during cell growth (Figure 6B). These results are consistent with the fact that pScN-II cells would have a lower induction of hypoxia-inducible factor-1 (HIF-1) as this is dependent both on hypoxia and on ROS content [25, 26]. Indeed, if pScN-II cells have less ROS in hypoxic conditions, they would have a lower induction of HIF-1, and thus a worse adaptability to the hypoxic condition as compared to pScont cells.

cN-II downregulated cells have a better defense against ROS

As ROS levels were higher in pScont cells despite an apparently similar glucose consumption and metabolism, we assumed that the antioxidative defense mechanisms could be more abundant or more efficient in pScN-II cells. We first quantified the relative gene expression of NAD(P)H quinone dehydrogenase 1 (*NQO1*), thioredoxin-2

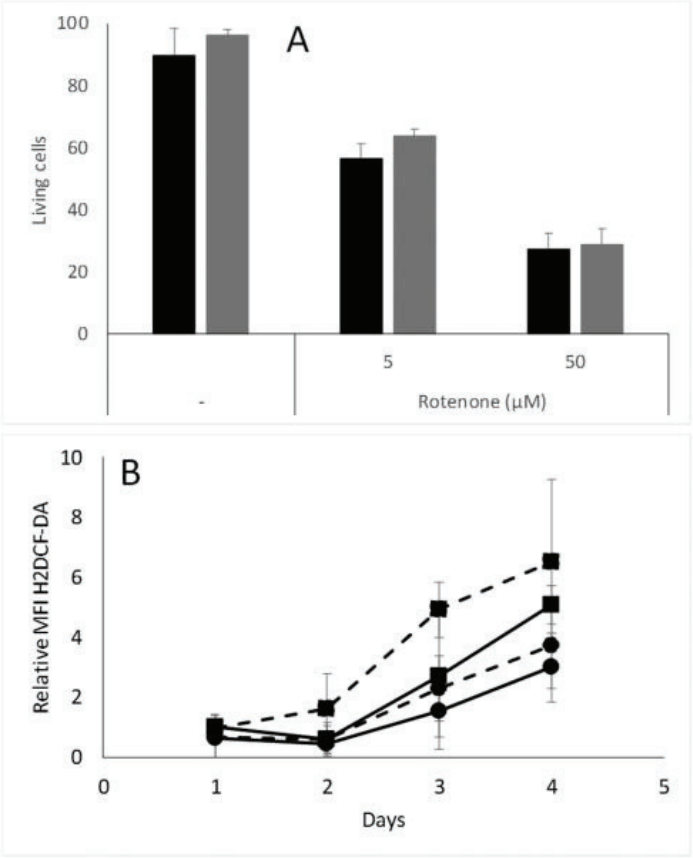

Figure 6: **(A)** Percentage of living MDA-MB-231-pScont (black bars) and -pScN-II (grey bars) cells incubated alone or in presence of indicated concentrations of rotenone and menadione. Cells were seeded in 6-well plates at 200 000 cells per well in 3 mL of culture media, and living cells are AnnexinV and propidium iodide negative. Results are mean values and error bars are standard deviation of three independent experiments. **(B)** Relative ROS content in MDA-MB-231-pScont (■) and -pScN-II (●) cells incubated with 1 mM glucose (filled lines) or without glucose (dotted lines) in hypoxic conditions (1% O_2). Results are expressed as relative to MFI on day 1 for pScont cells incubated with glucose. Cells were seeded at 200 000 cells per well in 6-well plates with 3 mL culture media. Results are mean values and error bars are standard deviations of four independent experiments. Means were compared with Student's *t*-test and differences were statistically significant at day 3 between pScont and pScN-II without glucose, and at day 4 between pScont and pScN-II with 1 mM glucose.

(*TXN2*), superoxide dismutase 1 and 2 (*SOD1* and *SOD2*) and glutathione S-transferase π (*GSTP1*). As shown in Figure 7A, these genes were expressed either at the same level or slightly less in pScN-II cells when cultured in presence of glucose. However, after 5 hours of culture in absence of glucose, these genes were all found to be more expressed in pScN-II cells as compared to pScont cells. Similar results were obtained when cells were exposed to the positive control menadione, a well-described ROS and anti-ROS defense inducer. This is in line with our hypothesis that pScN-II cells have a better overall capacity to respond to ROS after glucose deprivation.

This increase in gene expression was confirmed at the protein level for TXN2 but not for GSTP1 after an 8 hour incubation in the absence of glucose (Figure 7B). Indeed, still no difference was observed when cells were cultured in presence of glucose, but TXN2 expression increased 2-fold in pScN-II cells cultured in absence of glucose. Again, similar results were obtained with exposure to menadione.

cN-II downregulation increases the autophagy flux

Autophagy can be induced by energy deprivation, and contributes to the regeneration of ATP and other nutrients in the cells. Therefore, autophagy could also explain the metabolic advantages of pScN-II cells as

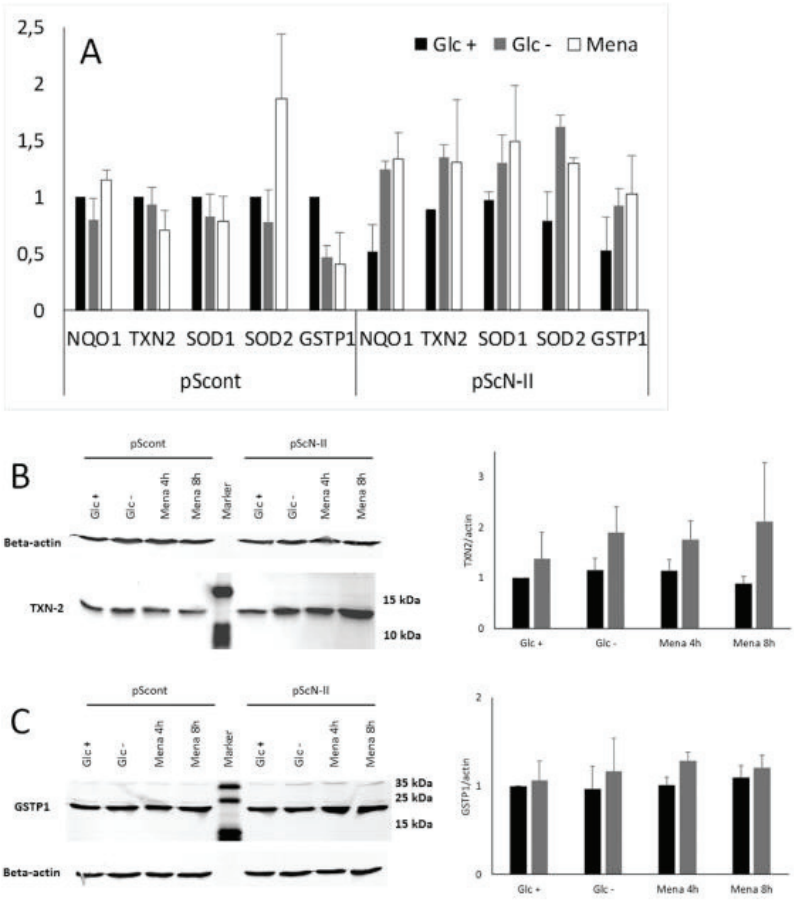

Figure 7: (A) Relative gene expression of anti-ROS genes in MDA-MB-231-pScont (left) and -pScN-II (right) cells incubated in presence (black bars) or in absence (grey bars) of 25 mM glucose, or in presence of glucose and 20 μM menadione (white bars) for 5 hours**.** Results are expressed as mean values of two independent experiments using pScont cells in presence of glucose as reference, and error bars are standard deviation. **(B** and **C)** Protein expression of TXN-2 (**B**, size 12 kDa) and GSTP1 (**C**, size 24 kDa) and beta-actin (size 42 kDa) in MDA-MB-231-pScont and -pScN-II cells cultured in presence (Glc +) or absence (Glc -) of glucose or in presence of glucose and 20 μM menadione (Mena) for 8 hours. The gels show results from a representative experiment and for the quantification, all samples were standardized to the ratio of protein/actin in pScont cells with glucose. The graph shows the mean values ± standard deviation of the quantification of bands obtained in three independent experiments. Means were compared with Student's *t*-test but not found statistically significantly different.

compared to pScont cells. We investigated whether autophagy markers were differentially expressed between the two cell lines. As shown in Figure 8, pScN-II cells had higher expression of LC3-II, a marker for autophagy flux, than pScont cells when cultured in normal culture media. As expected, its expression increased after incubation with 25 mM of 2-deoxyglucose for 16 hours in pScont cells whereas this was not the case in pScN-II cells suggesting that the autophagy flux was already at its highest level in the basal conditions. It is however unclear whether autophagy is involved in the differences observed in hypoxic conditions.

DISCUSSION

During our work on cN-II over the last 15 years, several observations have suggested that this enzyme might play major roles in human cells, independently from its activity in purine metabolism and sensitivity to nucleoside analogues [27]. First, transient inhibition by siRNA or by enzymatic inhibitors induce cell death in certain cancer cell models [12, 17]. Second, modulation of cN-II expression was associated with variations in cell growth rate and intracellular energy charge [20, 28]. Third, the highly conserved structure of cN-II among species, which is not limited to active and regulatory sites, suggested interactions with other cellular proteins [21]. Using our stable cell models for decreased cN-II expression, we here show consistent results on the implication of this enzyme in the regulation of cellular defense to oxidative stress.

Our results from the *in vivo* experiments show that the decrease of cN-II expression favors tumor growth. This is to our knowledge the first published data on tumor growth of cells with modified cN-II expression. However, this is not consistent with the previously reported results showing no difference in *in vitro* proliferation on these same cell lines [19] or decreased *in vitro* cell growth in a neuroblastoma model [20]. Enhanced tumor growth *in vivo* is thus not simply explained by enhanced proliferation of

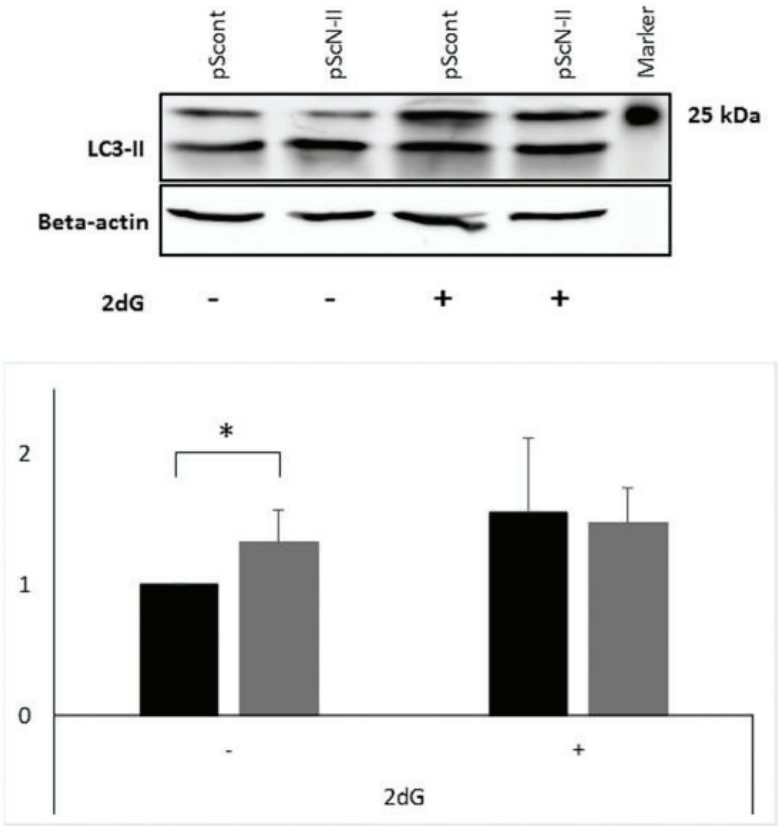

Figure 8: Protein expression of LC3-II (size 17 kDa) and beta-actin (size 42 kDa) in MDA-MB-231-pScont and -pScN-II cells cultured in normal media or exposed 16 hours to 2-deoxyglucose. The gel shows result from a representative experiment and the graph shows the mean values ± standard deviation of the quantification of bands obtained in four independent experiments. *: $p<0.005$ with Student's *t*-test.

the cell lines in short term *in vitro* cultures. This difference in *in vivo* growth was however at least partially explained by our long term *in vitro* cell growth experiments. Our xCELLigence experiments were performed without renewal of culture media, and we showed that glucose was a limiting factor in this culture, since the differences in growth curves were observed earlier when cells were seeded in lower glucose concentrations (Figure 2). As this was not due to a difference in the glucose consumption between our cell lines, we proposed that the cN-II downregulation had induced a better capacity to respond to this low glucose environment. This could indeed be the case in the tumors as both cell lines grow similarly in the beginning, but diverge once they have reached a certain volume, eventually corresponding to a stage at which the tumor is poorly irrigated and the microenvironment is depleted of certain metabolites. However, such tumors would also be rather hypoxic, and thus more prone to cell death as suggested by our *in vitro* data. Unfortunately, due to technical issues, we were not able to obtain interpretable results on the xCELLigence apparatus for the glucose-dependency of NCI-H292, MIA-PaCa 2 and HCT-116 cells.

We also showed that the ROS content was much higher in control cells than in pScN-II cells when cells lacked glucose in the media either after long term culture or by incubating them directly without glucose. ROS-production by NADPH oxidase and mitochondrion has been shown to be induced by glucose deprivation in cancer cells and associated to subsequent cell death [29]. As, in our model, the highest ROS content was associated with a higher cell death, we conclude that this ROS content can directly explain the observed differences in cell death between our two cell lines. Furthermore, pScN-II cells displayed a better defense against ROS with both an enhanced induction of TXN2-expression and more autophagy.

One explanation to the observed glucose-deprivation-related differences between MDA-MB-231-pScont and -pScN-II cells could be the action of cN-II on metabolites of nicotinamide adenine dinucleotide (NAD). Indeed, it has been shown first in yeasts [30] and later in humans [31] that the mononucleotides of nicotinamide (NMN) and nicotinic acid (NAMN) are substrates for both cytosolic pyrimidine preferring 5'-nucleotidase (human cN-III and yeast Sdt1) and the purine preferring 5'-nucleotidase (human cN-II and yeast Isn1). Thus, it is possible that cN-II expressing cells have overall a lower level of NAD and its reduced form NADH (and thus of NADP) due to a higher degradation of their precursors, and consequently a poorer defense towards ROS that are produced in the presence of low glucose. The recent observation of an association between oxidative stress and an IMP/GMP-preferring 5'-nucleotidase (*yktC*) in *Bacillus subtilis*, strengthens this hypothesis [32].

When cells were grown under hypoxic conditions, the decrease of cN-II expression was shown to be associated with a higher sensitivity to the disappearance of glucose in the media. This could be explained by a higher ROS content in these cells. Indeed, it has been proposed that in hypoxic conditions, the ROS production is higher in cells with high NADH level [33]. This would again increase the glucose consumption by regulating GLUT-4 expression on the cell membrane as described in skeletal muscle cells [34]. This would be consistent with our hypothesis stating that pScN-II cells have a higher level of NADH because of a lower catabolism of its precursors. However, this was not the case in our ROS content quantification assays in cells grown in hypoxic conditions, suggesting another and yet unknown mechanism for this increased sensitivity to low glucose concentrations in hypoxia. It has been shown in a particular model that concomitant hypoxia and low glucose induced unfolded protein response [35]. This could indeed be the case also in our models, but additional experiments would be needed to confirm this.

The fact that pScN-II cells do not accumulate more ROS than pScont cells in hypoxic conditions whereas they die earlier (as shown by xCELLigence experiments in hypoxic conditions), could indicate that in normoxic conditions, a difference in ROS content is involved in the differential behavior between the two cell lines, whereas in hypoxic conditions, this difference is ROS-independent. We showed here that the pScN-II cells have a better overall anti-ROS defense, with a particular involvement of thioredoxin 2 and autophagy. The detailed mechanism by which these cells acquire this phenotype is not yet understood, but we believe that cells with downregulated cN-II expression will adapt through an increase of phosphorylated nicotinamide derivatives or through an adapted kinetics in AMPK activation. Ongoing studies should help us decipher this particular mechanism. Our results might have clinical relevance in settings where cancer patients are treated with metabolic inhibitors targeting directly or indirectly glucose metabolism. Indeed, such therapeutic approaches will be more efficient on cancer cells expressing high levels of cN-II than on cells with low levels of cN-II, making this protein a potential marker for response to treatment as already described in other clinical settings [8].

MATERIALS AND METHODS

Cells and culture

In this study we used human cancer cell lines from lung (NCI-H292), pancreas (MIA PaCa-2), colon (HCT-116) and breast (MDA-MB-231). Cells were transfected to express either a shRNA against cN-II (pScN-II cells) or control shRNA (pScont). General cell culture conditions

and the development of transfected cell lines have been previously described [9, 19].

cN-II activity assessment

cN-II activity in transfected models was assessed using a validated non-radioactive method of liquid chromatography coupled to a tandem mass spectrometry as described elsewhere [9].

In vivo tumor growth

Female severe combined immunodeficiency CB17 mice (2-4 months old, approximately 20 g, Charles River, L'Arbresle, France) were injected with indicated amount of cells subcutaneously on day 1. Tumor size was measured twice a week and mice were euthanized when the tumor volume was >1500 mm^3. The protocol for experiments in mice was approved by the University of Lyon Animal Ethics Committee. Mean tumor volumes were compared with Student's *t*-test.

Cell proliferation and adherence

The xCELLigence system (ACEA Biosciences) was used to concomitantly determine cell proliferation and adherence in real-time analysis. Cells were seeded (3000 cells per well, 250 µl of media) in 16-wells E-plates as indicated by the manufacturer and the cell index was recorded every 15 minutes for 24 hours then every 30 minutes up to 20 days. Cell proliferation and survival were also assessed by direct counting of cells both adhered to the flask and in the cell culture media using a Cellometer Auto T4 (Nexcelom Bioscience).

Quantification of glucose and lactate in cell media

Cells were seeded in 6-well plates (90 000 cells per plate) in media containing 10 mM glucose and incubated at 37 °C for indicated times. At each time point, cells were trypsinized and counted, and the supernatant was recovered for dosage of glucose and lactate. Glucose was quantified by the measurement of NADPH produced during enzymatic reactions, and lactate by its conversion to pyruvate and hydrogen peroxide followed by the conversion of ABTS into a chromogen as described earlier [36].

Cell survival assay

Cell survival was assessed by the quantification of Annexin V / propidium iodide negative cells on a FACScalibur flow cytometer using Annexin-V-FLUO Staining kit (Roche) as indicated by the manufacturer and after culture in indicated conditions. Adherent and spontaneously detached cells were pooled for this analysis.

Quantification of intracellular reactive oxygen species

The content of reactive oxygen species (ROS) in cells was determined using an oxidation sensitive fluorescent dye (H_2DCFDA, Life Technologies). Cells were cultured as indicated and for ROS quantification, cells were washed with PBS, incubated 30 minutes at 37 °C with 5 µM H_2DCFDA, washed twice with PBS and incubated with complete media for 10 minutes at 37 °C. Finally, cells were washed twice with PBS, trypsinized, centrifuged (5 minutes, 300 g) and resuspended in 200 µl PBS. ROS were measured by flow cytometry on a FACScalibur ($\lambda_{ex/em}$: 490 / 530 nm) and compared to the autofluorescence of cells incubated with PBS instead of H_2DCFDA.

Western blot analyses

Proteins were extracted from cell pellets with cold RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 M DTT, 1 M NaF, protease inhibitor cocktail, phosphatase inhibitors buffer and 100 mM sodium orthovanadate) or buffer A (20 mM Tris-HCl pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP40, 2% protease inhibitor cocktail) on ice for 60 (RIPA) or 15 (buffer A) minutes followed by centrifugation (15 minutes, 12 000 g, 4 °C). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane using the iBlot® system (Life Technologies). Membranes were incubated with specific antibodies for LC3-II (NB100-2220, 1/500; Novus Biologicals), GSTP1 (A5691, 1/500; NeoBioLab), TXN2 (A6782, 1/500; NeoBioLab) and beta-actin (clone AC-15, 1/5000; Sigma) and anti-murine antibody (IRDye® 800CW, 1/5000; LI-COR Biosciences) or anti-rabbit antibody (IRDye® 680, 1/5000; LI-COR Biosciences), and protein expression was visualized using the Odyssey infrared system (LI-COR Biosciences). Bands were quantified using the Odyssey system, and the results are presented as ratio of the expression of proteins of interest to beta-actin expression.

Quantitative RT-PCR

Total RNA was extracted from cells using RNeasy mini kit (Qiagen) as described by the manufacturer. Reverse transcription was performed with Moloney leukemia virus reverse transcriptase and quantitative PCR on a LightCycler thermal cycler (Roche) in the following conditions: 5 minutes initial denaturation at 95 °C follow by 40 cycles of 10 seconds at 95 °C, 10 seconds at 60 °C and 10 seconds at 72 °C and terminated by a melting curve from 70 °C to 95 °C. Primers were: NQO1 forward:

5'-ATGTATGACAAAGGACCCTTCC-3'; NQO1 reverse 5'-TCCCTTGCAGAGAGTACATGG-3'; TXN forward 5'-TTACAGCCGCTCGTCAGA-3'; TXN reverse 5'-AAGGCTTCCTGAAAAGCAGTC-3'; SOD1 forward 5'-TCATCAATTTCGAGCAGAAGG-3'; SOD1 reverse 5'-GCAGGCCTTCAGTCAGTCC-3'; SOD2 forward 5'-AAGTACCAGGAGGCGTTGG-3'; SOD2 reverse 5'-TGAACTTCAGTGCAGGCTGA-3'; GSTP1 forward 5'-GGCAACTGAAGCCTTTTGAG-3' and GSTP1 reverse 5'-GGCTAGGACCTCATGGATCA-3'. Relative quantification was calculated using the ΔΔCT-method and human ribosomal 28S RNA as reference gene.

Autophagy flux assessment

Cells (3.10^6 per dish) were plated in 10 cm culture dishes and exposed or not to 25 mM 2-deoxyglucose (Sigma) for 16 hours before cells were subjected to protein extraction and Western blot analysis as indicated above.

Abbreviations

pScN-II: pSuperior-cN-II; pScont: pSuperior-control; ROS: reactive oxygen species; NQO1: NAD(P)H quinone dehydrogenase 1; TXN2: thioredoxin-2; SOD1: superoxide dismutase 1; SOD2: superoxide dismutase 2; GSTP1: glutathione S-transferase π.

Author contributions

GB, OC, LEC and ECP prepared and performed biological experiments. LPG performed glucose and lactate dosages. JYP and ILT prepared and performed LC-MSMS experiments for cN-II activity assessment. GB, OC, LEC, MGT, CD and LPJ designed the study and experiments and analyzed results. CD and LPJ assured funding. LPJ was the major contributor in writing the manuscript. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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cN-II and CD73 in global cell metabolism

In continuation with this work, we wanted to further understand the roles of 5'-nucleotidases in cancer cell metabolic plasticity by comparing and combining modulation of both cN-II and CD73. This would indeed give us particularly interesting models to study the interplay between intracellular and extracellular nucleotide pools and the role of cN-II and CD73 in this phenomenon. To do so, we used MDA-MB-231, human triple negative breast cancer cells and decreased cN-II and/or CD73 expressions with stable shRNAs. These models were used to identify possible metabolic parameter that could be co-regulated by cN-II and CD73.

Material and methods

Cell transfection

Wild type MDA-MB-231 cells were transfected with pSuperior.Neo or pSuperior.Puro containing a coding sequence for shRNAs targeting either CD73 or cN-II RNA or a non-targeting control shRNA (see sequences hereafter). Each cell line received two pSuperior plasmids, conferring a double resistance to geneticin (0.8 mg/mL) and puromycin (0.5 µg/mL). We generated four models: cN-II^{high}CD73^{high} (that contains two control shRNAs), cN-II^{high}CD73^{low} (with one control shRNA sequence and one targeting CD73), cN-II^{low}CD73^{high} (with one control shRNA sequence and one targeting cN-II) and cN-II^{low}CD73^{low} (with one shRNA sequence targeting cN-II and one targeting CD73).

Plasmid sequence for cN-II shRNA: The target sequences are underlined (oligomers were purchased from Dharmacon):

5'-GATCCCCAACCTCTTGGTCTGTGCACATTTCAAGAGAATGTGCACAGACCAAGAGGTTTTTTTGGAAA-3'

5'-TCGATTTCCAAAAAAACCTCTTGGTCTGTGCACATTCTCTTGAAATGTGCACAGACCAAGAGGTTGGG-3'

Plasmid sequence for CD73 shRNA: The target sequences are underlined (oligomers were purchased from Dharmacon):

5'-GATCCCCGCCACTAGCATCTCAAATATTCAAGAGATATTTGAGATGCTAGTGGCTTTTTA-3'

5’-TCGATAAAAAGCCACTAGCATCTCAAATATCTCTTGAATATTTGAGATGCTAGTGGCGGG-3’

Plasmid sequence for scrambled shRNA (oligomers were purchased from Dharmacon):
5’-GATCCCCAACCTCTTGGTCTGTGCACATTTCAAGAGAATGTGCACAGACCAAGAGGTTTTTTTGGAAA-3’
5’-TCGATTTCCAAAAAAACCTCTTGGTCTGTGCACATTCTCTTGAAATGTGCACAGACCAAGAGGTTGGG-3’

CD73 activity evaluation

MDA-MB-231 cells were plated on day 1 in 24 well plates (2.10^5 cells per well) with 1 mL of complete culture medium. On day 2, the medium was removed, and the cells were washed twice with 1 mL of phosphate free buffer, pH7,4 (2 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES buffer pH 7,4 in H_2O). Then, MDA-MB-231 were incubated with 500 µL of phosphate free buffer or of phosphate free buffer + 1 mM AMP or phosphate free buffer or of phosphate free buffer + 1 mM AMP+ 30 µM APCP for 30 minutes at 37 °C, 5% CO_2. Pi (inorganic phosphate) production was revealed by allowing 80 µL of supernatant to react with 20 µL of Working reagent from Genesaur kit (Bioassay) systems for 30 minutes at room temperature. Absorbance was read at 595 and 690 nm. The experiment was performed with biological triplicate and technical duplicates.

For radioactive adenosine production from AMP, we incubated our models with radioactive labeled [^{3}H] AMP (400 µM) for 30 minutes. Then, cell supernatant was collected and underwent a thin layer chromatography to separate nucleotide derivatives (ATP, ADP, AMP, adenosine, inosine, hypoxanthine). The extracellular radioactive adenosine fraction was quantified as a CD73 activity indicator.

Extracellular [^{3}H] adenosine follow-up

We incubated our models with radioactive labeled [^{3}H] adenosine (25 µM) for one hour. Then the medium was removed and the cells washed and lysed. We realized a thin layer chromatography with the obtained lysates to separate and quantify nucleotide derivatives (ATP, ADP, AMP, adenosine, inosine, hypoxanthine).

Anoikis resistance assessment

Cells (1.10^6 per well in 6 well plates) were seeded in Corning® Costar® Ultra-Low Attachment culture dishes, not to allow them to adhere. After 24 hours, they were harvested, and cell death was measured with annexin-V/PI labelling and flow cytometry.

Other experiments

The material and methods for other experiments are described in part II.

Results

Characterization of cell models

cN-II and CD73 expression and activity

The cN-II targeting shRNA sequence almost completely abolished the cytosolic 5'-nucleotidase expression at the mRNA and protein level, but the CD73-targeting sequence only resulted in a slight diminution of CD73 surface expression (Figure 10 and 11). Therefore, to fully validate this model, we tested CD73 activity in these cells with two techniques. A malachite green assay allowed us to indirectly measure CD73 activity by evaluating inorganic phosphate production, from AMP dephosphorylation. cN-II^{high}CD73^{high} and cN-II^{low}CD73^{high} cells were able to produce high quantities of inorganic phosphate when incubated with AMP, whereas cN-II^{high}CD73^{low} and cN-II^{low}CD73^{low} cells produced 25 to 30% less inorganic phosphate in the same conditions (Figure 12-A). This production was reversed if the cells were co-incubated with the CD73 inhibitor APCP, indicating that this phosphate production originates from CD73 activity. Using tritium (^{3}H) labelled AMP, we also directly measured adenosine production from AMP by these cells. The cells that received the CD73-targettinng mRNA produced only 40 to 50 % of the adenosine that was produced by cN-II^{high}CD73^{high} and

cN-II^{low}CD73^{high} cells, confirming that the slight decrease in surface CD73 expression in our models was enough to decrease the overall enzyme activity (Figure 12-B).

Thus, we used these models to study the roles of cN-II and CD73 in cell biology and its metabolic plasticity.

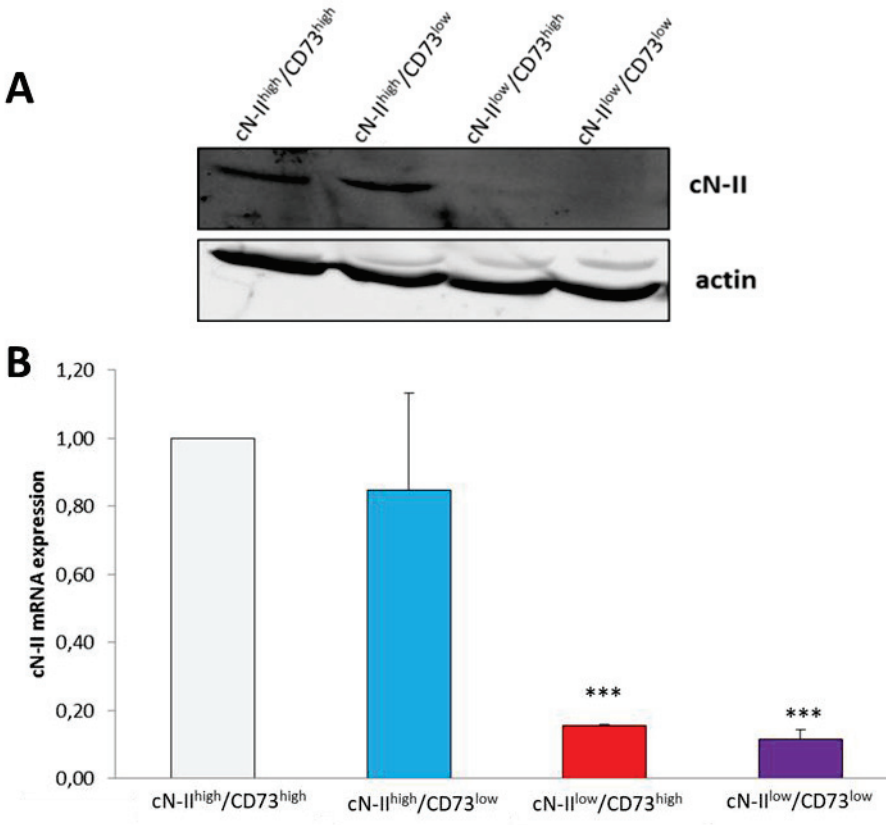

*Figure 10: cN-II expression was evaluated at protein (A) and mRNA (B) levels, by westrn blot and RT q-PCR respectively. Bars represent means ± sd of 3 independent experiments. *** =p< 0.001 with a Student's t-test, in comparison with the cN-I ^{high}/CD73^{high} cell line.*

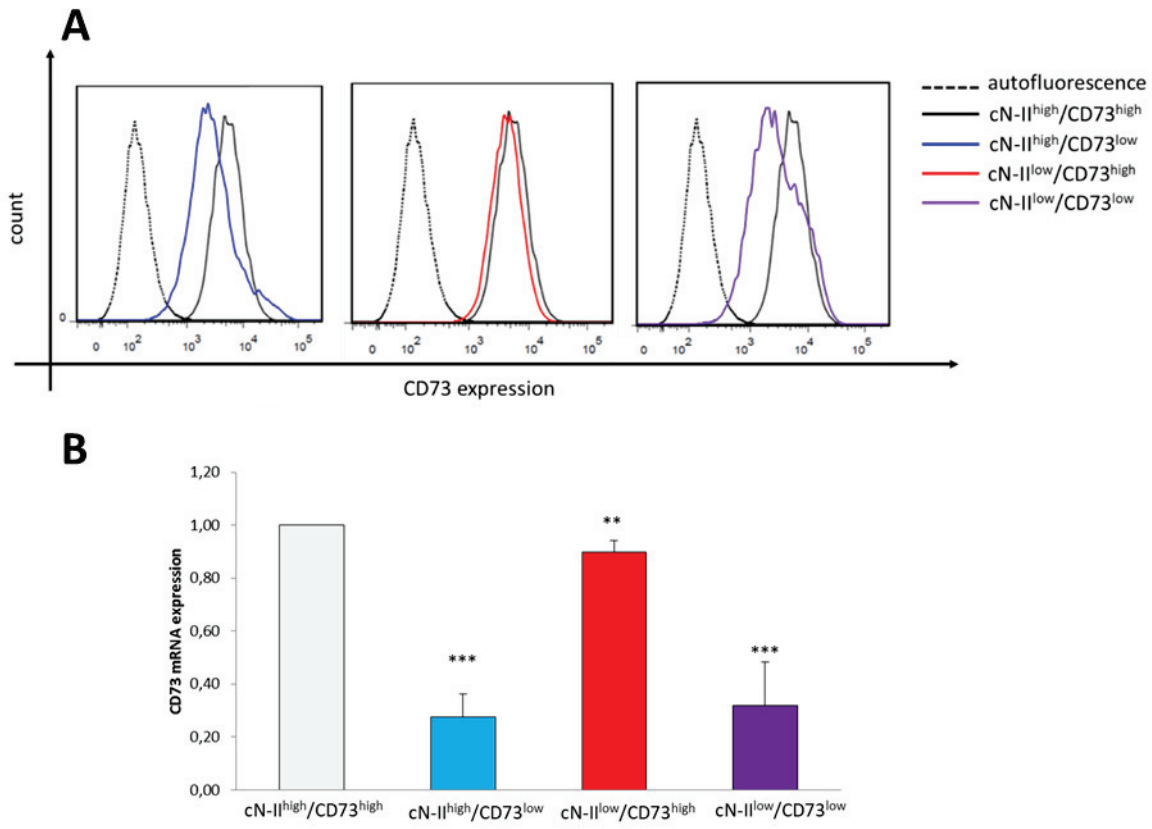

*Figure 11: CD73 surface expression was assessed by flow cytometry (A) and its mRNA expression evaluated by RT-qPCR (B). Bars represent means ± sd of 3 independent experiments. ** =p< 0.01; *** =p< 0.001 with a Student's t-test, in comparison with the cN-II^{high}/CD73^{high} cell line.*

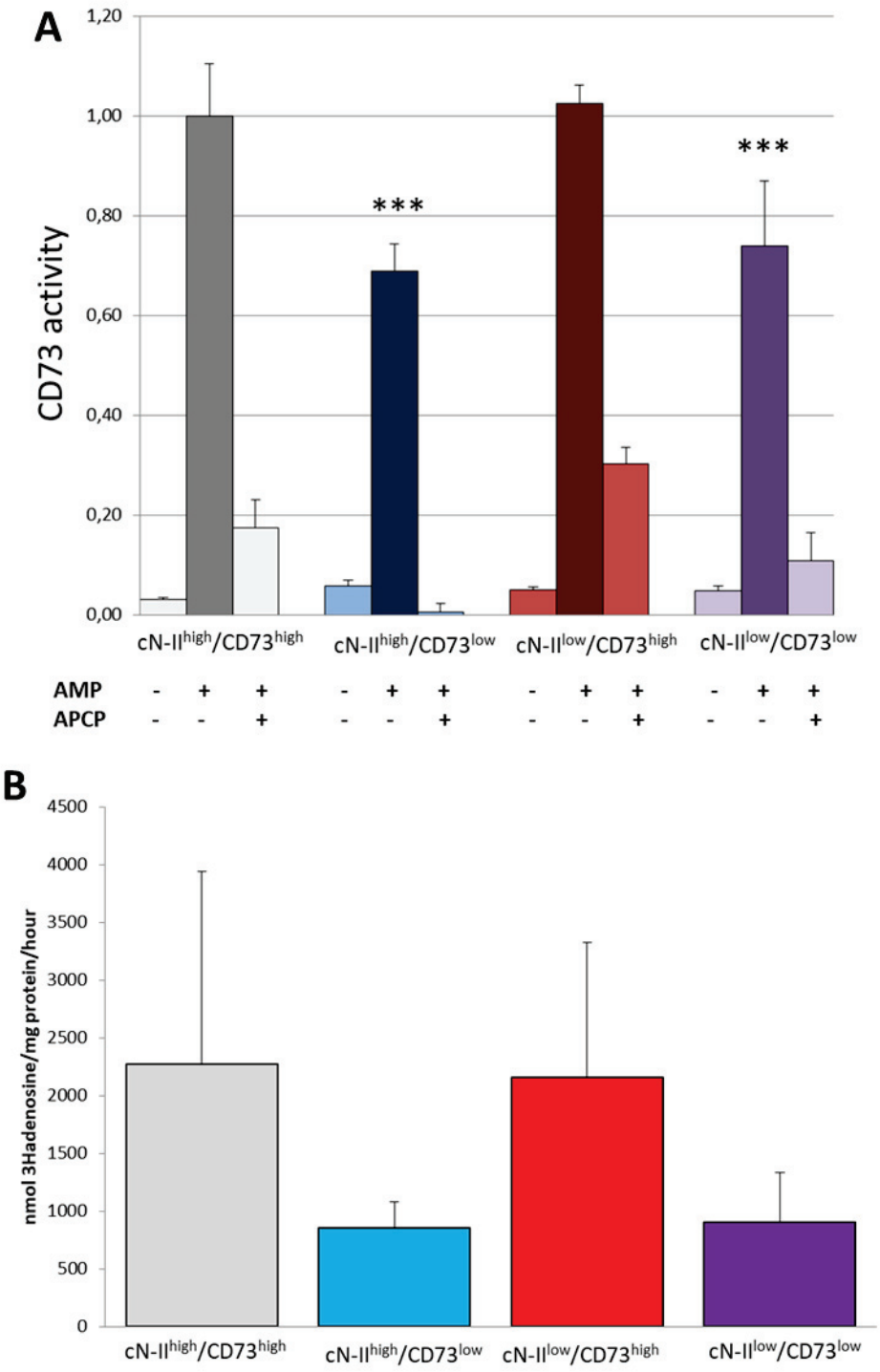

*Figure 12: CD73 activity was indirectly evaluated with a malachite green assay (Pi production measurement) (A) and directly by measuring extracellular adenosine formation from extracellular AMP (B). Bars represent means ± sd of 3 independent experiments. *** =p< 0.001 with a Student's t-test, in comparison with the cN-I ^{high}/CD73^{high} cell line.*

Roles of cN-II and CD73 in extracellular adenosine intracellular fates

As indicated in the introduction, extracellular adenosine can interact with surface receptors or be internalized through nucleoside transporters. In the cytoplasm, the nucleoside is either re-phosphorylated to form adenine nucleotides or degraded into inosine and hypoxanthine. Here we were interested in how adenosine was intracellularly metabolized, according to cN-II and CD73 expressions. To study possible differences in adenosine metabolism between our models, they were incubated with 25 µM extracellular radioactively labelled adenosine for one hour. Then, we collected intracellular lysates to separate and quantify the adenosine derivatives in our four cell lines. intracellular radioactive ATP, ADP, AMP, adenosine, inosine and hypoxanthine were quantified (Figure 13). We did not notice any differences in the quantity of total intracellular labelled adenosine derivatives, suggesting that the same amount of extracellular adenosine was internalized in all the models (Figure 13-A). Thus, cN-II and CD73 are not involved in adenosine transport modulations. Therefore, we can hypothesize that these two 5'-nucleotidases do not regulate nucleoside transporters expression or activity in MDA-MB-231 cells. This should be confirmed by comparing ENT and CNT expression patterns in these models.

Once in the cytoplasm, most of the internalized adenosine was re-phosphorylated, essentially to form ATP (60 to 80% of radioactive pools corresponded to the ATP fraction). When cN-II expression was downregulated, radioactive AMP and ADP tended to be more important than in cN-II^{high}CD73^{high} cells, but the difference was not statistically significant (Figure 13-B). Thus, extracellular adenosine does not seem to be differently metabolized according to cN-II or CD73 expressions.

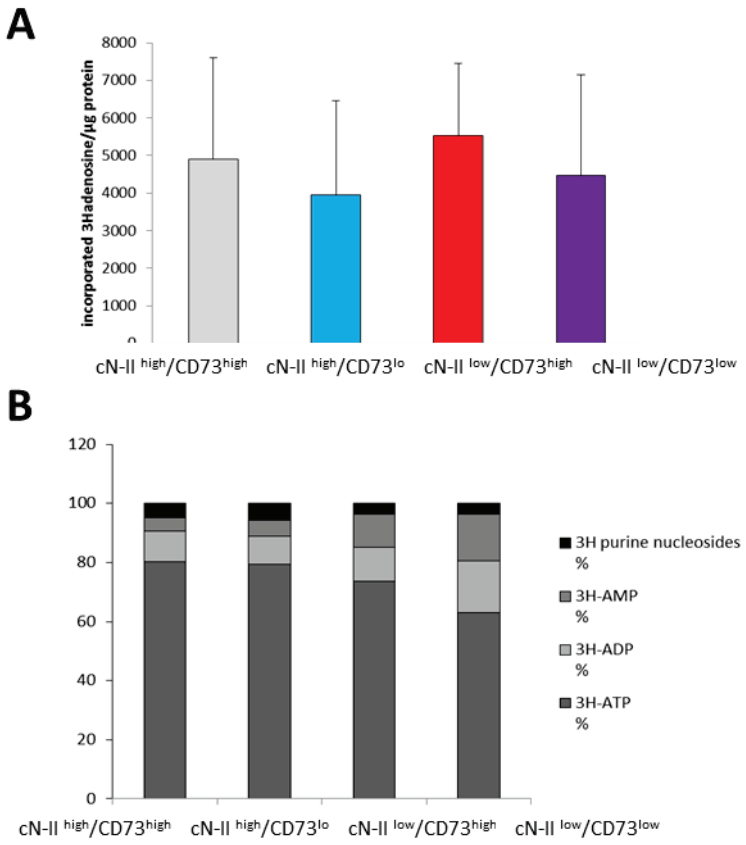

Figure 13: [^{3}H] adenosine internalization follow-up. We measured the quantity of [^{3}H] adenosine that was internalized in our four models (A) and identified what fraction of intracellular adenosine derivates were obtained from its internalization (B). Bars represent the means ± sd of 3 independent experiments.

Cell proliferation under a metabolic stress

We evaluated the cell proliferation rates of our models under a metabolic stress. cN-II and CD73 downregulations did not impact cell proliferation in classic culture conditions (Figure 14). We then inhibited cell glycolysis with 25 mM 2DG (2-deoxyglucose), as a competitor with glucose for this pathway, or autophagy with 30 µM chloroquine, that impedes autophagosome and lysosome fusion. 2DG and chloroquine decreased cell proliferation rate in all our models. cN-II^{high}CD73^{high} cells seemed to be slightly less sensitive to 2GD than the others. Nevertheless, no significant difference was observed according to cN-II or CD73 expression levels. cN-II^{high}CD73^{low} cell proliferation was more sensitive to chloroquine than the others. Surprisingly, this increased sensitivity was not present in cN-II^{low}CD73^{low} cells. This suggests that CD73 downregulation alone can render the cells more dependent on autophagy to provide macromolecules to grow, but this could be rescued by cN-II downregulation. Interestingly, cN-II downregulation alone does not seem to impact this parameter.

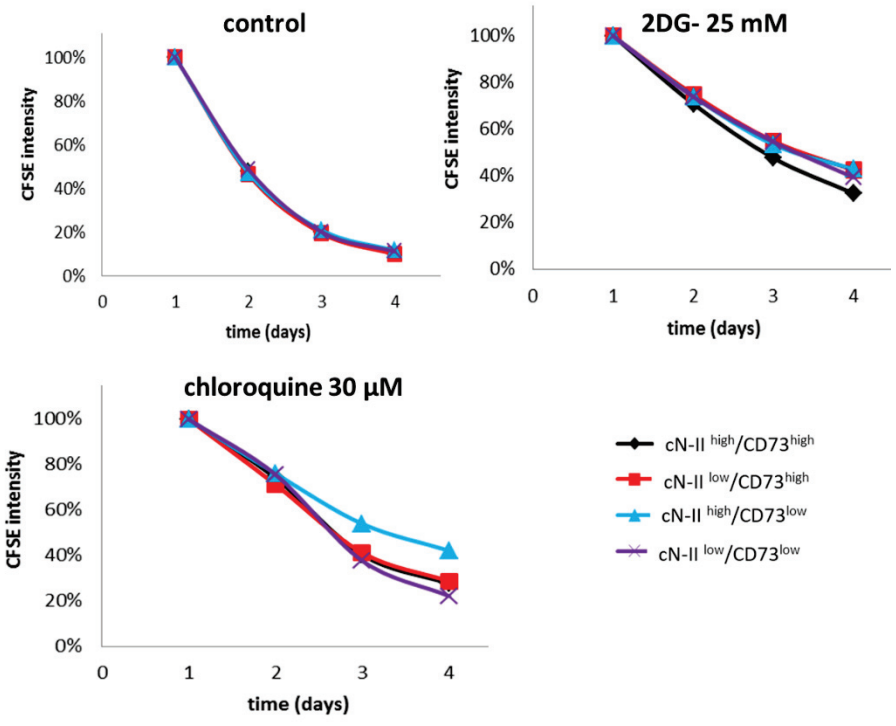

Figure 14: Cell proliferation was monitored with a CFSE assay, over 4 days, in classic culture conditions (A) or in presence of 2DG (2-deoxyglucose, 25 mM) as a glycolysis inhibitor or chloroquine (30 µM) as an autophagy inhibitor.

Cell death induction by metabolic modulators

In order to study the roles of cN-II and CD73 in cell adaptability to metabolic stress, we incubated our four models with metabolic drugs. We also used MDA-MB-231 in which cN-II and/or CD73 expressions were invalidated with a knockout realized thanks to the CRISPR/Cas9 technique (These models are described in Part II and the submitted article). We thus obtained four more models: cN-II$^+$/CD73$^+$ cells (expressing both cN-II and CD73), cN-II$^+$/CD73$^-$ cells (expressing cN-II but not CD73), cN-II$^-$/CD73$^+$ (deficient for cN-II but not for CD73) and cN-II$^-$/CD73$^-$ (deficient for both cN-II and CD73).

We used 2DG, chloroquine and etomoxir to respectively inhibit glycolysis, autophagy and lipid β-oxidation, that are catabolic pathways. These metabolic pathways allow the cells to provide macromolecules under nutrient deprivation, by degrading and recycling pre-existing components of the cells, thus allowing growth while saving energy. After 48 hours of incubation with the MDA-MB-231, 2DG and chloroquine but not etomoxir tended to generate cell death in all models, but the differences with the control condition were never statistically significant (Figure 15-A and B). It suggests that MDA-MB-231 do not rely on one metabolic pathway but they can switch between these cells metabolism, according to their needs. Moreover, no difference was observed between the cell lines, according to cN-II and CD73 expressions, meaning that cN-II and CD73 do not modify cell dependence to catabolic pathways. However, when the cells were simultaneously incubated with the three inhibitors, cN-II and CD73 down regulations seemed to favor survival. This was not observed in the models with 5'-nucleotidases silencing. We then inhibited oxidative phosphorylation with rotenone in the knockout models, again no modification in cell survival was observed (Figure 15-C). Thus, these metastatic cells seem to be able to switch between catabolic strategies and oxidative phosphorylation, according to the pressure they undergo. cN-II and CD73 do not seem to play a role in this metabolic adaptability.

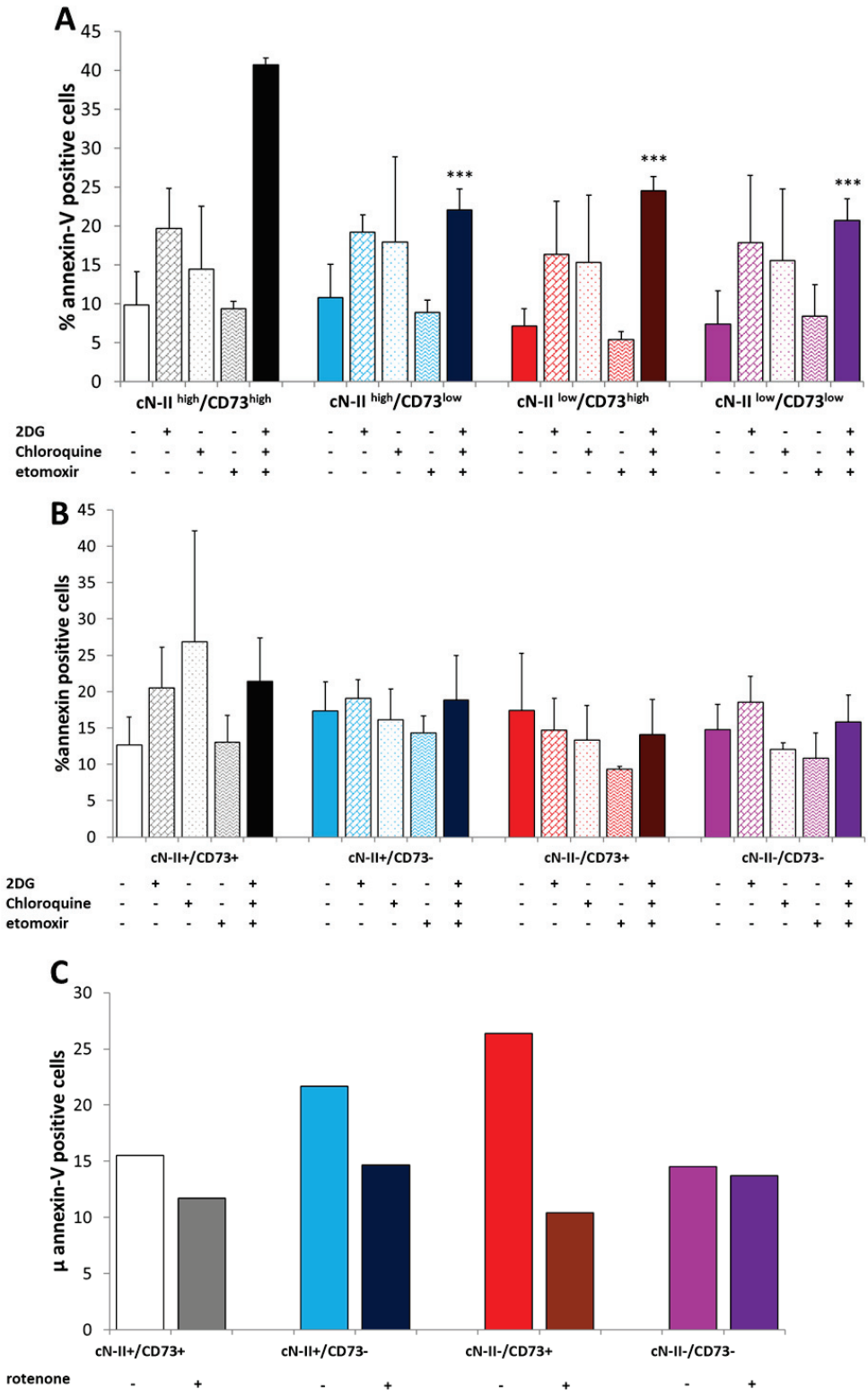

Figure 15: Cells with deceased cN-II and/or CD73 expression (A) or deficient for these 5'-nucleotidases (B) were exposed to 2DG (25 mM) as a glycolysis inhibitor, chloroquine (30 µM) as an autophagy inhibitor, etomoxir (30 µM) as a lipid ß-oxidation inhibitor, or these three compounds for 48 hours. Cell death was then assessed by annexin-V/PI labelling and flow

*cytometry analysis. Bars represent the means of 5 independent experiments. *** =p< 0.001 with a Student's t-test, in comparison with the cN-II$^+$/CD73$^+$ cell line. In (C), cells with cN-II and/or CD73 invalidation were incubated with rotenone (30µM) and their survival was evaluated after 48 hours. Bars represent the man of technical duplicates of one experiment.*

Glucose metabolism-related enzymes

We then investigated the mRNA expression of glucose metabolism-related enzymes in the knockout models (Figure 16). Hexokinases phosphorylate glucose to activate it and render it available for catabolism. These enzymes thus indirectly reflect glucose availability for cells. In the cell, glucose can be metabolized into pyruvate for glycolysis or serve as a substrate for the pentose phosphate pathway.

In our models, hexokinase IV (also known as glucokinase) was expressed at higher levels in cN-II$^-$/CD73$^-$ cells than in the other models (3.5-fold higher expression in cN-II$^-$/CD73$^-$ compared to cN-II$^+$/CD73$^+$ cells). We observed that glucose-6-phosphate dehydrogenase (G6PDH) and glucose-6-phosphate isomerase that participate in the pentose phosphate pathway and glycolysis respectively, are differentially expressed in cN-II$^-$/CD73$^-$ cells compared to the others (1.7-fold higher expression in cN-II$^-$/CD73$^-$ compared to cN-II$^+$/CD73$^+$ cells for glucose-6-phosphate isomerase and G6PDH was 1.9-fold less expressed in cN-II$^-$/CD73$^-$ than in cN-II$^+$/CD73$^+$ cells).

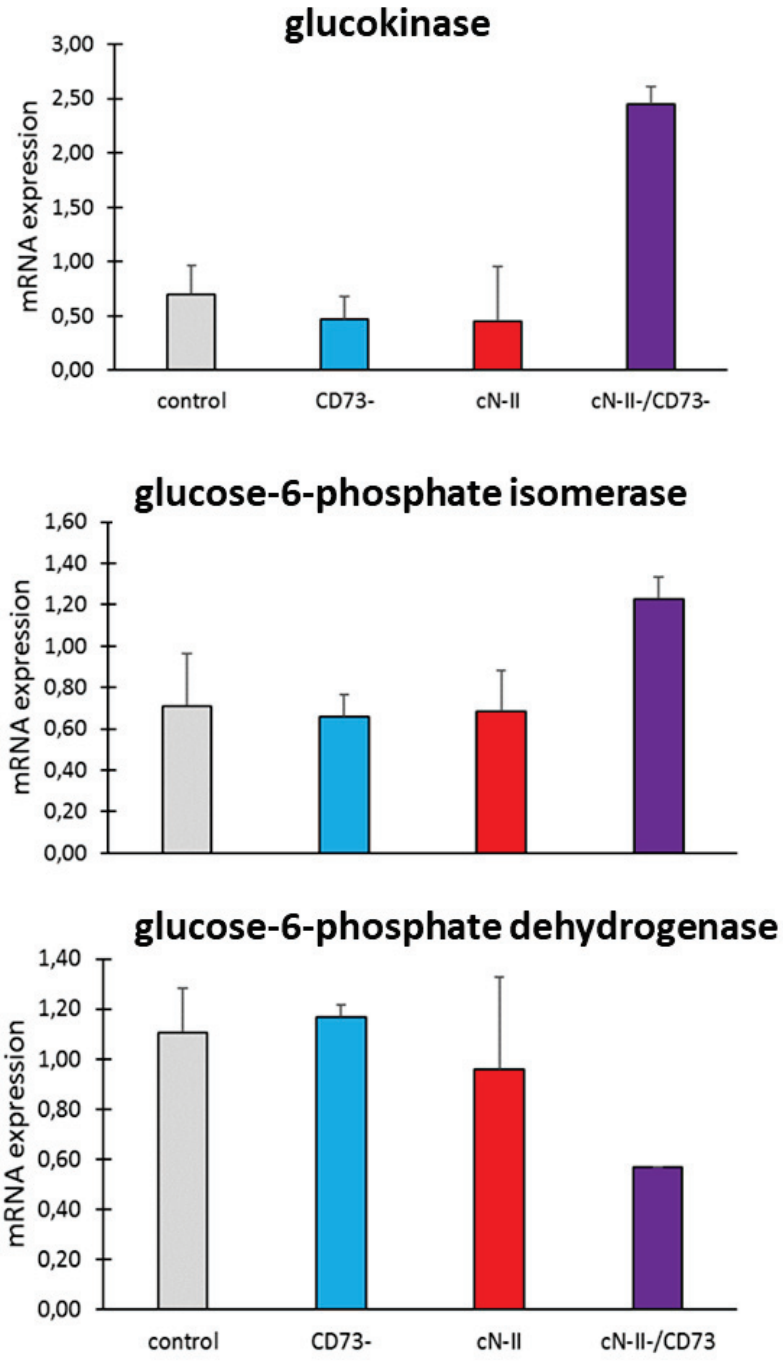

Figure 16: Glucose metabolism-related enzymes mRNA expression was evaluated in MDA-MB-231 expressing or not cN-II and CD73, by RT q-PCR. Bars represent means ± sd of 3 independent experiments with technical triplicates, except for cN-II$^-$/CD73$^-$ cells that are represented from two experiments with technical triplicates.

Autophagy flux

We assessed autophagy activation in our models containing shRNAs and in our models with cN-II and/or CD73 knock outs, by evaluating the expression levels of LC3-II (microtubule-associated protein 1A/1B-light chain 3). LC3 undergoes a conjugation to phosphatidylethanolamine to form LC3-II that is incorporated in autophagosome membrane. LC3-II is thus widely used as an autophagy marker (Tanida et al., 2008). In MDA-MB-231, cN-II downregulation or deficiency was associated with a slightly enhanced autophagy flux, reflected by a higher LC3-II expression than their cN-II-highly-expressing counterparts (Figure 17), similarly as what was observed in our previous study (Bricard et al., 2017). In the absence of glucose, the contrast between cN-II expressing and cN-II deficient cell is even more marked. Indeed, glucose deprivation activates autophagy in a more efficient way when cN-II is absent.

This suggests that when the cytosolic 5'-nucleotidase is not expressed in the cells, they might be more sensitive to metabolic changes that favor autophagy induction.

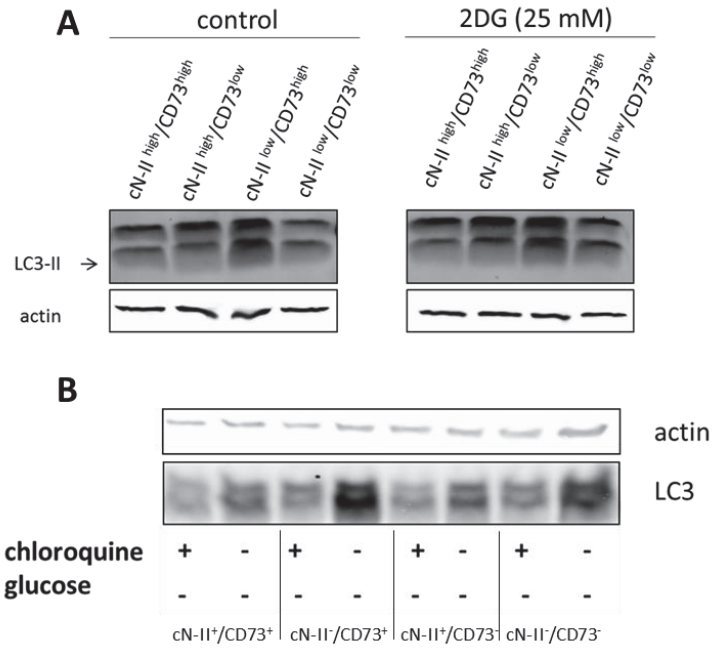

Figure 17: Cells were incubated or not with 2DG (25 mM) (A); or without glucose, in presence or not of chloroquine (30 µM), and their autophagy flux was assessed by evaluating LC3-II expression by western blot after 24 hours.

Dependency on glucose for cell survival

Considering the high expression levels of hexokinase IV in cN-II$^-$/CD73$^-$ cells and the differential autophagy levels between the models, we assessed their glucose dependency. We cultured the cells in glucose-free medium and cell survival was assessed after 48 hours. Glucose deprivation tended to slightly trigger cell death, but this was statistically significant only when CD73 was not expressed in the cells, suggesting that MDA-MB-231 can rely on metabolic pathways that are not glucose-dependent to keep on surviving, and CD73 expression could interfere with this plasticity.

As cN-II-deficient cells present a higher autophagy flux than their cN-II-proficient counterparts, we assessed their sensitivity to chloroquine, an autophagy inhibitor, in the presence or absence of glucose. After a 48-hour exposure to chloroquine, in the presence of glucose, we observed more cell death than in control conditions, but the difference induced by chloroquine was not statistically significant. As mentioned earlier, glucose deprivation generated cell death in a statistically significant manner and promoted autophagy induction in cN-II-deficient cells, but the addition of chloroquine did not enhance glucose deprivation-induced cell death in any of the tested models. Thus, although cN-II-deficient cells display higher autophagy rates, they do not seem to need this process for survival, in presence or absence of glucose.

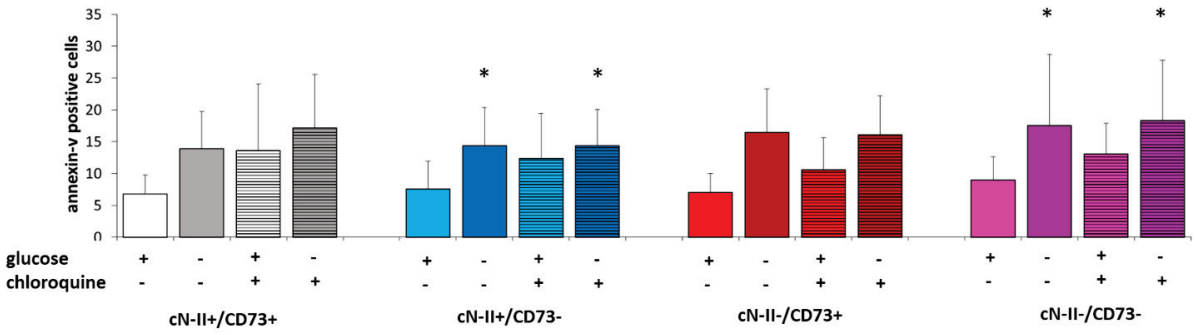

*Figure 18: Cells with cN-II and/or CD73 invalidation were cultured in presence or absence of glucose (25 mM), in presence or not of chloroquine (30 µM). Their survival was assessed after 48 hours. Mean of 4 independent experiments are represented ± sd. * = p> 0.05 with Student's t test in comparison with the condition with glucose only.*

Anoikis resistance

Anoikis is a type of cell death generated when the cells are not allowed to interact with an extracellular matrix. To detach from the original tumor and form metastases, cancer cells need to resist anoikis. Such ability requires major metabolic changes (Caneba et al., 2012; Lu et al., 2015). Due to their metastatic phenotype, MDA-MB-231 can survive when they are not allowed to adhere, compared with non-metastatic cell lines. In order to investigate to what extent cN-II and CD73 can be involved in anoikis resistance, we cultured our cell models in low adhesion culture plates for 24 h. Their survival was then assessed with an Annexin-V/PI staining.

In the models that under-expressed CD73 by shRNA transfection, cells tended to survive better than those with unmodified CD73 expression levels (Figure 19-A). Nevertheless, the observed difference was not significant. Interestingly, complete knock out of CD73 was not associated with a better survival (Figure 19-B). On the contrary, we observed that neither CD73 or cN-II complete knock out was associated with an accentuated anoikis resistance. These results indicate that the metabolic pathways that are regulated by cN-II and/or CD73 do not interfere with those that are required for anoikis resistance.

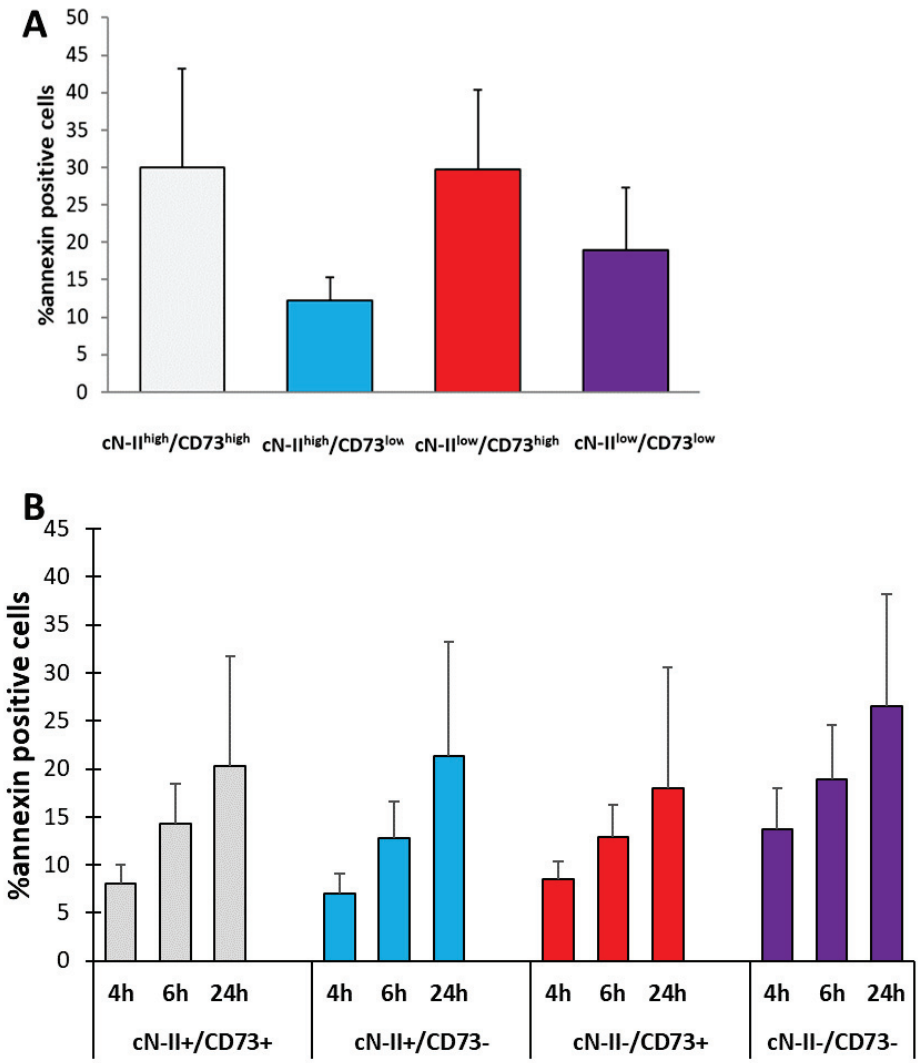

Figure 19: Cells were seeded in classic culture medium in plates that do not allow adherence. Their survival was assessed after 24 hours (for the models with decreased cN-II and CD73 in A), or after 4, 6 and 24 hours (for the models with cN-II and CD73 knockout in B).

Discussion

So far, our models allowed us to observe some differences in metabolic plasticity that depend on cN-II or cN-II and CD73 expression. Nevertheless, these trends do not seem to affect cancer cell survival or proliferation. At this point, it is thus difficult to confirm whether cN-II and CD73 represent relevant targets to deregulate cancer cell metabolism and favor cell death. The experiments we performed revealed a link between cN-II and autophagy. This

could be due to an imbalance in intracellular nucleotide pools, caused by the absence of the enzyme. Indeed, as cN-II modulates intracellular nucleotide pools, variations in its overall activity could impact these nucleotide pools and thus the energy charge (Cividini et al., 2015c). As a result, this can end in AMPK activation and modulations of downstream pathways (such as autophagy). Autophagy is not only a recycling but also a clearance pathway, that favors intracellular ROS elimination. Therefore, to better understand how autophagy overactivation could benefit cN-II-deficient cells, it is necessary to study AMPK activation and ROS managing in our models. To study this question, it is also important to keep in mind that the chemical autophagy inhibitor, chloroquine can affect various factors in cells. Thus, silencing autophagy-related genes can be a more relevant and specific strategy in this context.

We observed that cN-II$^-$/CD73$^-$ cells express high levels of hexokinase IV and glucose-6-phosphate isomerase mRNAs, contrary to G6PDH mRNA. This could be the reflection of an enhanced glucose entry in these cells, in response to ATP deprivation and aiming to enhance the glycolytic flux to provide energy. Although these observations suggest that cN-II$^-$/CD73$^-$ cells might use the glycolytic pathway better than the other models, further experiments are needed to know if these differences are functional. Indeed, it could be interesting to assess hexokinase I, II and III expressions in these models, to measure glucose consumption and to evaluate if cN-II and CD73 expression are associated with any differences in glucose transporters. Combined with the study of specific metabolites of glycolysis and pentose-phosphate pathways, this could help us to better understand the glucose metabolism-related enzymes expression variation we observed. Indeed, because of is numerous levels of regulation, glucose-6-phosphate dehydrogenase expression is not enough to reflect the pentose phosphate pathway activation. In the cells, this enzyme can be monomeric and latent or dimeric and active. Glucose-6-phosphate dehydrogenase activity is also negatively regulated by NADPH, the product of the reaction catalyzed by this enzyme (Au et al., 2000). In addition, is has been described that cN-II can metabolize NAD-related nucleotides (Kulikova et al., 2015). We can then imagine that this function interacts with AMP dephosphorylation to impact global cell metabolism. Very interestingly, G6PDH expression and activity can be regulated by cAMP, that is generated downstream adenosine signaling, confirming the necessity to measure extracellular adenosine pools variations according to cN-II and CD73 expressions.

When we assessed anoikis resistance in the metastatic cell line MDA-MB-231, we observed that these cells survive well when they don't adhere to the culture dishes in an optimal way, and we did not observe any striking role for cN-II or CD73 in this characteristic. Nevertheless, this does not exclude a role for these enzymes in the acquisition of anoikis resistance. Indeed, with their metastatic phenotype, MDA-MB-231 might not be as sensitive to metabolic changes involved in anoikis resistance as non-metastatic models. During our work on cell migration, we noticed that cN-II-deficiency was associated with TIMP-1 downregulation (see part III). Ricca and colleagues demonstrated that TIMP-1 expression is associated with anoikis resistance acquirement during melanoma genesis (Ricca et al., 2009). This observation confirms that anoikis resistance study might be more relevant in non-metastatic models, to establish a possible link with cN-II and /or CD73. Thus, it would be interesting to repeat this assay in non-metastatic models. Also, Lu and colleagues demonstrated that ROS favor anoikis resistance (Lu et al., 2015). As we previously showed, decreased cN-II expression is associated with a better cellular equipment against ROS accumulation. Considering these points, it remains relevant to expect cN-II expression to impact anoikis resistance.

Part II: cN-II, CD73 and cancer cell migration

In cancer, enhanced migration is one of the parameters that promotes metastases occurrence, and thus participates in cancer aggressiveness. Considering that adenosine can affect cell migration in several cancer cell lines, we wanted to evaluate a possible role for cN-II and CD73 in this process, in MDA-MB-231.

We realized preliminary assays on MDA-MB-231 cells with cN-II and/or CD73 downregulation, in classic culture conditions or in presence of AMP (1 mM) (Figure 20). These experiments revealed that cN-II^{low}CD73^{high} and cN-II^{low}CD73^{low} cells migrated faster than the models with high cN-II expression, suggesting that this enzyme could negatively regulate migration in this cell line. In cN-II^{high}CD73^{high} cells, exposure to AMP (1 mM) reduced cell migration. We then hypothesized that extracellular nucleotide stress can also impact and regulate this process.

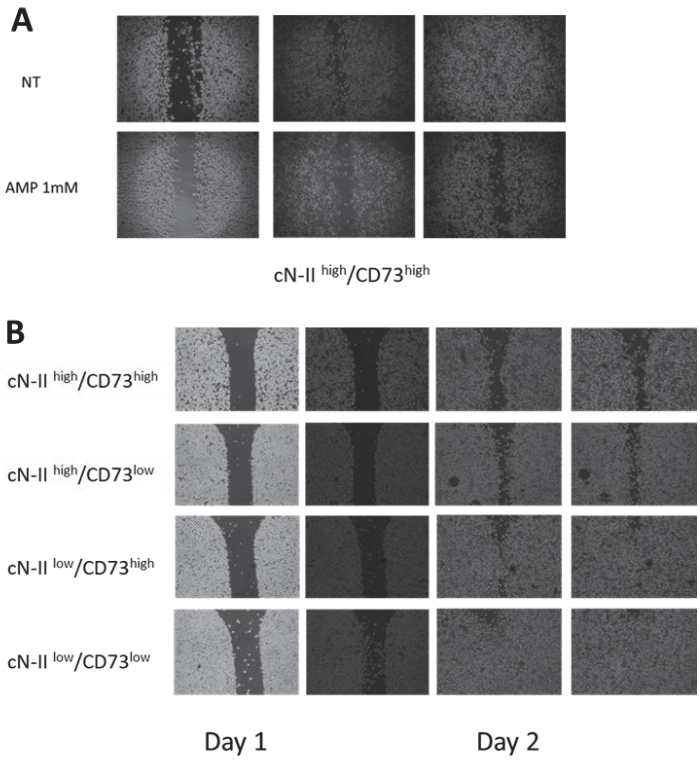

Figure 20: Cell migration in MDA-MB-231 with decreased cN-II and/or CD73 expressions (A), or in presence of extracellular AMP (B). The wound was created with an insert that was present in culture dishes until the cells become confluent. Then the insert was removed, and the medium replaced. We monitored wound healing by taking pictures of the wound twice a day (0, 5, 24 and 29 hours after insert removal).

We further continued to explore this migration using the knock-out models for cN-II and CD73. This work corresponds to the major part of this PhD as to a manuscript submitted for publication. I therefore present this part with the submitted paper (see following pages).

Upregulation of migration capacities and COX-2/PGE2/Akt axis in breast cancer cells deficient for 5'-nucleotidases cN-II and CD73

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Running title: cN-II and CD73 in cancer

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Tables: 1

Supplemental material: 1 figure and 2 tables

Abstract

Purine metabolism involves various intracellular and extracellular enzymes including cN-II and CD73 that dephosphorylate intracellular and extracellular nucleoside monophosphates into their corresponding nucleosides. Given nucleotide and nucleoside trafficking and their roles in cell biology, it is possible that cN-II and CD73 are involved together in processes that regulate cancer cell aggressiveness. We abolished cN-II and/or CD73 expression in the triple negative human breast cancer cells MDA-MB-231, using the CRISPR/Cas9 technique, and evaluated the impact of knock-out of cN-II and CD73 on intracellular nucleotide pools, nucleotide metabolism-related gene expression and cell migration under an extracellular nucleotide stress. Intracellular nucleotide contents were altered in the modified cell models both at the basal level and after exposure to adenosine or AMP, in particular for the intracellular contents of AMP, adenosine, IMP and inosine. Altered cN-II and CD73 contents were also associated with modifications in cell migration, and we show that decreased TIMP-2 expression, increased expression of MMP-2 and MMP-9, as well as an increase in the COX-2/PGE2/Akt pathway, are involved in this process. Our results show new and important roles of cN-II and CD73 in cancer cell biology and provide insight into the interactions between different intracellular pathways. These observations are expected to be associated to the interplay between intracellular and extracellular nucleotide metabolisms and contents.

Introduction

The purine nucleoside adenosine and its phosphorylated metabolites play major roles in human physiological and pathological conditions. Within a tumor, ATP can for example stimulate the anti-tumoral response of the infiltrated immune system as well as inhibit the growth of cancer cells, whereas adenosine inhibits the immune system and induces apoptotic cell death of cancer cells at higher concentrations (1). These effects are somewhat tissue- and cell-specific and depend on the cellular expression of ATP (P2) and adenosine (P1, ADORA) receptors, as well as the associated intracellular signal transduction machinery. Adenosine can also enter the cell and exert its biological properties after phosphorylation to AMP or ATP (2).

As effects of adenosine are dependent on its concentration, the expression and activities of purine metabolism enzymes within the tumor are expected to play a role cellular processes. In the extracellular compartment, adenosine is produced from the degradation of ATP by the subsequent actions of CD39 and CD73 (3). It can thereafter either interact with its receptors, enter the cell through nucleoside transporters or be transformed to inosine by adenosine deaminase (ADA) (3). Intracellularly, adenosine, that can either come from the extracellular compartment or the hydrolysis of intracellular AMP or S-adenosyl-methionine, is rapidly phosphorylated to AMP by adenosine kinase (ADK), and thereafter to ADP and ATP. Intracellular purine metabolism involves a number of enzymes including ADA and nucleotide degrading enzymes such as cN-I and cN-II (2).

Several of the aforem{Citation}entioned proteins have gained increased attention over the last decade, and we are particularly interested in the 5'-nucleotidases CD73 (NT5E) and cN-II (NT5C2). The latter is an IMP/GMP-preferring enzyme for which a phosphotransferase activity has also been described (4), and both enzymes have demonstrated roles in cancer biology. Indeed, the proliferation of astrocytoma cells (ADF) was increased in case of enhanced cN-II expression (5) whereas its down-regulation did not influence the proliferation of other cell lines of various origins transfected with cN-II-targeting shRNA (6,7). However, in the breast cancer cell line MDA-MB-231, downregulation of cN-II by stable shRNA was associated with an increased adaptability to glucose starvation, indicating an important role in cancer cell biology (8). cN-II is also involved in the response to cancer treatments (5,7,9). Concerning CD73, the modulation of its activity by enzymatic inhibitors or of its expression level in cancer cells, has allowed to show its involvement in cell proliferation (10–12), cell migration (13,14)

and sensitivity to radiation based or targeted cancer treatments (15,16). Both cN-II (17) and CD73 (18–20) are currently considered as potential targets in oncology.

In the current work, we studied the implication of CD73 and cN-II in breast cancer cell biology using an original set of MDA-MB-231 cell models expressing both CD73 and cN-II, only CD73 or cN-II or none of these. In addition to the study of the models in classical cell culture conditions, we evaluated their response to high concentrations of AMP and adenosine that correspond to those which can be observed in case of extracellular nucleotide stress.

Material and methods

Cell culture and transfection

Human triple negative breast cancer cell line MDA-MB-231 was obtained from ATCC and cultivated in RMPI medium (Roswell Park Memorial Institute, RPMI 1640, Gibco) supplemented with bovin fetal serum and penicillin/streptomycin (Thermofisher Scientific). Cells were routinely tested for *Mycoplasma* every two weeks. CD73 and/or cNII knockout MDA-MB-231 cells were generated using CRISPR/Cas9 technology. Oligonucleotides were inserted into pLentiCRISPRv2-blast or pLentiCRISPRv2-puro plasmids (Addgene) using BsmBI (ThermoFisher-Fermentas). Virus were produced using HEK 293T cells and a 24-hours incubation and used for the infection of cells, and stable models were selected with puromycin and/or blasticidine. Target RNA sequences for CD73 and cN-II plasmids were: 5'-CCACTAGCATCTCAAATATC-3' and 5'- CTCCGTCTTTGACACACTGTA-3' respectively. Cells were cultured in complete Roswell Park Memorial Institute (RPMI 1640, Gibco) medium supplemented with 10% (v/v) fetal bovine serum (FBS), and Fungizone (2 µg/mL), 100 U/mL penicillin, 100 mg/mL streptomycin on collagen-coated flasks in a humidified atmosphere containing 5% CO_2 at 37 °C.

Protein expression

Cells (0.5-1.10^6 per flask) were seeded and allowed to adhere before exposed to experimental conditions for indicated times. Cells were then rinsed with PBS and lysed with RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 M DTT, 1 M NaF, protease inhibitor cocktail, phosphatase inhibitors buffer and 100 mM sodium orthovanadate). After centrifugation (15 minutes, 12,000 g, 4 °C), the supernatant was

collected and proteins (60 µg per condition) were separated by electrophoresis and transferred to a nitrocellulose membrane with the iBlot™2. TBS or PBS Odyssey Blocking buffer were used to block the membranes and dilute the primary antibodies, and PBS or TBS to rinse the membranes and dilute the secondary antibodies. The following antibodies were used: anti-cN-II (H00022978-M02, Novus Biologicals: 1/500), anti-pAkt (4060, Cell Signaling: 1/500), anti-Akt (5239, Cell Signaling: 1/500), anti-COX-2 (12282S, Cell Signaling: 1/500), anti-actin (A5441, Sigma: 1/5000), anti-murine antibody (IRDye® 800CW, 1/5000; LI-COR Biosciences) or anti-rabbit antibody (IRDye® 680, 1/5000; LI-COR Biosciences). Protein expression was visualized using the Odyssey infrared system (LI-COR Biosciences).

Surface CD73 and CD44 expression were evaluated by flow cytometry using anti CD73 FITC-labeled (561245, BD Biosciences: 1:100) and anti CD44 APC-labeled (A10193, BD Biosciences: 1/100) antibodies. Cells were harvested, pelleted and washed in PBS before a 30-minute staining with the antibodies or control isotypes (IgG1 FITC-labeled: 555748, BD Biosciences: 1:100 and IgG1 APC-labeled: 555751, BD Biosciences: 1:100).

Gene expression

The mRNA extraction was performed on pellets of 10^6 cells using the Qiagen column extraction kit, following the manufacturer's protocol. One microgram of mRNA was used for reverse transcription with M-MLV reverse transcriptase (InVitrogen). The cDNA was then diluted, and relative gene expression was determined by PCR in a final volume of 5 µL with Takyon NO ROX SYBR Mmix dTTP blue mix (Eurogentec). Runs were performed on a Lightcycler (LC480, Roche Life Science). Quantification was performed by the ΔΔCT method using 28S mRNA expression as a housekeeping gene. Primers used for each gene are indicated in supplemental table 1.

Intracellular nucleotides

Cells (2.10^6 per flask in 25 mm² flasks) were seeded, adhered for 24 hours and incubated 1 hour in presence or absence of 400 or 1600 µM adenosine (Sigma) or AMP (Adenosine 5'-monophosphate sodium salt, Sigma). Then, cells were rinsed three times with cold PBS and nucleotides were extracted with a cold mixture of methanol/water (70/30, v/v). Extracted

nucleosides and nucleotides were quantified using a validated on-line extraction coupled with LC-MS/MS method as described elsewhere (21). The nucleotide content of each sample was calculated as the peak area of the compound of interest divided by the peak area of the corresponding internal standard, further divided by the number of cells as determined in a flask containing cells cultured under the same conditions. For each nucleoside and nucleotide, internal standards were corresponding labelled nucleotides except for IMP for which we used GTP.

Proliferation assay

Cells were trypsinized and rinsed with PBS to be stained with a solution containing 10 µM Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) in PBS-0.1% BSA. After labeling, the cells were rinsed with culture medium and seeded in 6-well plates (200,000 cells/well) and allowed to adhere before being exposed to adenosine or AMP. Cells from one well were scratched for every time point, to evaluate CFSE fluorescence by flow cytometry on the BD FACSCalibur.

Cell survival

Cells (50,000 per well in 24 well-plates) were cultured in presence or absence of the indicated compounds. At indicated times, cells were trypsinized, washed and stained with an anti-Annexin V-FITC labeled antibody and propidium iodide, from the Annexin-V FLUOS kit (11 988 549 001, Roche). The associated fluorescence was measured by flow cytometry on the BD LSR-II Flow Cytometer. Annexin-V and/or PI-positive cells were considered as dead or dying cells.

Cell migration

Cells (50,000 per well) were seeded in an ImageLock 96-well plate (Essen BioScience) and cultured to confluence. A wound was generated, using a Woundmaker 96 (Essen BioSCience). The cells were then rinsed with PBS and incubated under the indicated conditions. The wound closure was monitored every 2 hours with the IncuCyte® device.

PGE2 quantification

Cells (500,000 per well) were seeded and allowed to adhere before being exposed to fresh culture medium with or without 15 µM arachidonic acid (Abcam). PGE2 quantification was performed on cells supernatants after a 24-hour incubation, using the Parameter™ Prostaglandin E_2 assay from R&D Systems®, according to the manufacturer's protocol.

Statistical analysis

The statistical analysis was performed using unpaired Student's t-test with Microsoft Excel. $p < 0.05$ was considered statistically significant.

Results

Characterization of cell models

Using the CRISPR/Cas9 technique, we abolished cN-II and/or CD73 expressions in the triple-negative breast cancer cells MDA-MB-231. These were validated for their cN-II and CD73 protein expression and hereafter referred to as cN-II$^+$/CD73$^+$ cells (expressing both cN-II and CD73), cN-II$^+$/CD73$^-$ cells (expressing cN-II but not CD73), cN-II$^-$/CD73$^+$ (deficient for cN-II but not for CD73) and cN-II$^-$/CD73$^-$ (deficient for both cN-II and CD73) (Figure X). We determined the expression profiles of selected genes coding adenosine receptors or proteins involved in purine metabolism (Table 1). Among the notable differences, we observed a 6-8-fold decrease in the expression of adenosine receptor A1 in cells without cN-II, a 11-fold increase of ADSL in cN-II$^+$/CD73$^-$ cells as well as a 1.5-1.7-fold increase in SAMHD1 in cN-II negative cells as compared to their respective controls.

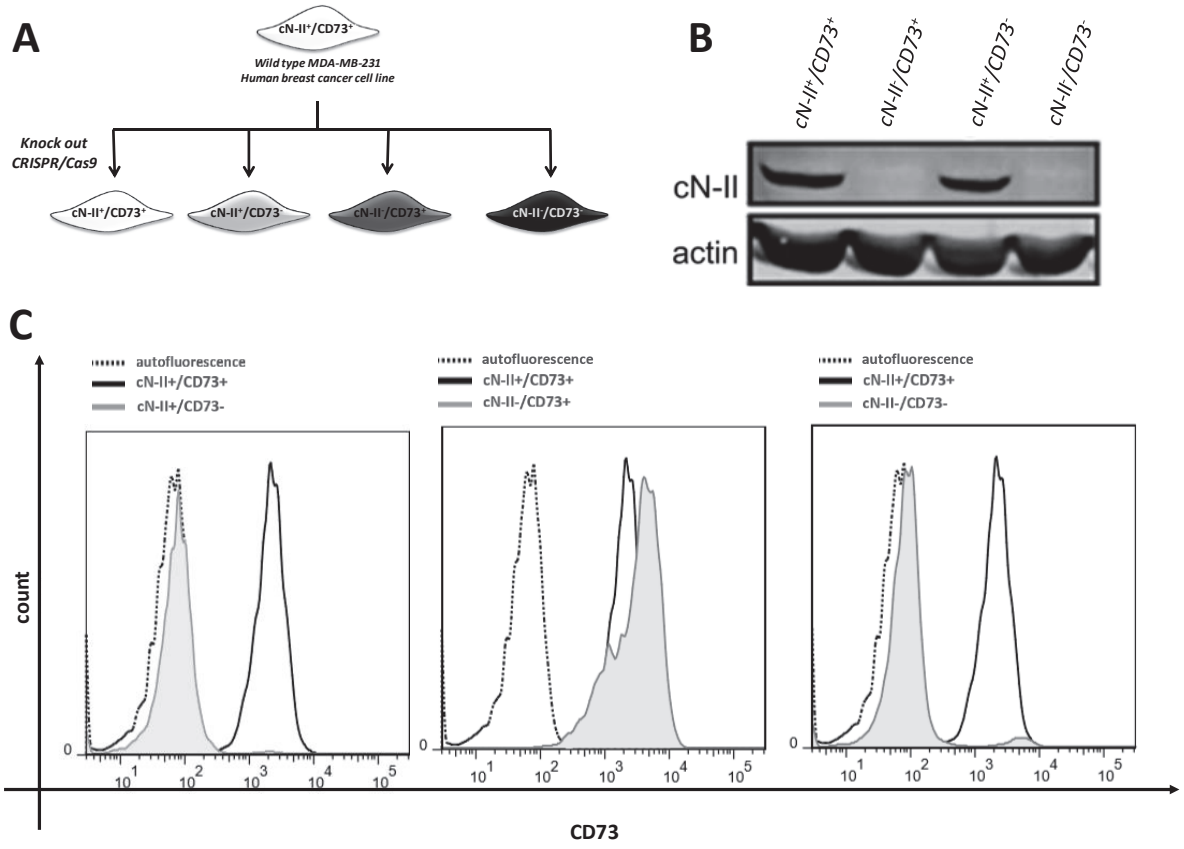

*Figure 1. Characterization of cell models. (**A**): The CD73 and/or cN-II knockout of MDA-MB-231 cells were obtained by using CRISPR/Cas9 technology as described in material and methods. (**B**): cN-II expression in the different cell models as determined by Western blot. Representative image of 3 similar Western blots. (**C**): CD73 expression in the different cell models as determined by flow cytometry. The image is representative of 4 analyses.*

Gene	cN-II$^+$/CD73$^+$	cN-II$^+$/CD73$^-$	cN-II$^-$/CD73$^+$	cN-II$^-$/CD73$^-$
A1	0.74 ± 0.19	0.98 ± 0.13	0.12 ± 0.03 ***	0.16 ± 0.07 ***
A2A	0.84 ± 0.17	0.78 ± 0.04	0.69 ± 0.22	0.78 ± 0.34
A2B	5.40 ± 3.93	5.92 ± 4.08	9.58 ± 6.51	6.37 ± 3.26
ADA	0.85 ± 0.12	0.90 ± 0.06	0.72 ± 0.12	1.19 ± 0.11 **
AdK	0.97 ± 0.09	1.03 ± 0.10	0.88 ± 0.12	0.82 ± 0.07 *
AMPD3	1.14 ± 0.26	1.21 ± 0.27	1.56 ± 0.36	1.55 ± 0.28
ADSL	0.95 ± 0.04	11.01 ± 0.02 *	0.85 ± 0.07	0.92 ± 0.10
ENT-1	1.94 ± 0.68	2.02 ± 0.82	1.59 ± 0.98	1.61 ± 0.47
dCTPPP1	1.06 ± 0.11	1.10 ± 0.10	1.14 ± 0.06	1.35 ± 0.20 *
GMPR	1.12 ± 0.08	1.57 ± 0.02 ***	0.82 ± 0.1 **	1.09 ± 0.20
GMPS	1.11 ± 0.10	1.37 ± 0.17 *	1.16 ± 0.11	1.12 ± 0.15
IMPDH1	1.12 ± 0.13	1.24 ± 0.10	1.16 ± 0.22	1.46 ± 0.48
IMPDH2	1.13 ± 0.18	1.16 ± 0.09	1.03 ± 0.06	1.01 ± 0.09
ITPA	0.96 ± 0.04	1.06 ± 0.17	1.18 ± 0.24	1.59 ± 0.21 ***
PNP	1.05 ± 0.14	0.72 ± 0.06 **	1.19 ± 0.21	1.55 ± 0.26 *
PPAT	2.13 ± 0.79	1.72 ± 0.41	2.19 ± 0.40	2.40 ± 0.64
RRM1	1.19 ± 0.17	1.12 ± 0.44	0.87 ± 0.60	0.92 ± 0.76
SAMHD1	1.43 ± 0.36	1.61 ± 0.15	2.10 ± 0.24 *	2.70 ± 0.36 **

*Table 1. mRNA expression of nucleotide metabolism-related genes and ADORA receptors. The mRNA expressions of nucleotide metabolism-related genes and ADORA receptors were assessed in the four models. Values are means ± standard deviation of four independent experiments, performed in triplicate. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ using Students t-test in comparison with the corresponding cN-II$^+$/CD73$^+$ cell line. See legend of supplemental table 1 for gene names.*

Extracellular adenosine affects cell proliferation and survival, independently from cN-II and CD73 expression levels

Considering the involvement of nucleotide pools in cell biology, we investigated whether cN-II and/or CD73 invalidation impacted cell proliferation and viability. Under classical culture conditions, these knockouts did not impact cell proliferation. As showed in figure 2A, CSFE intensity decreased to 23.5-25.4% of the initial value after 3 days for all the cell lines. In addition, cell viability did not vary between the cells lines after 72 h in culture (16.2 ± 7.9% annexin-V positive cells for cN-II$^+$/CD73$^+$, versus 17.6 ± 3.5%, 15.7 ± 8.0% and 16.7 ± 8.3% for cN-II$^-$/CD73$^+$, cN-II$^+$/CD73$^-$ and cN-II$^-$/CD73$^-$ respectively) (Figure 2B). These results indicate that cN-II and CD73 are not involved in cell proliferation or survival in these cells under these conditions.

As cN-II and CD73 are involved in nucleotide metabolism, we investigated to what extent these enzymes could modify cellular response to an extracellular nucleotide stress. To do so, we exposed the cells to high concentrations of AMP or adenosine (1600 µM) and evaluated their survival after 48 hours. AMP exposure resulted in a strong increase of Annexin-V cells in CD73-expressing cells (from 16.2 ± 7.9% annexin-V positive cells to 41.6 ± 12.2% for cN-II$^+$/CD73$^+$, and from 15.7 ± 8.0% to 32.2 ± 10.4% for cN-II$^-$/CD73$^+$ cells). This AMP-induced cell death was totally inhibited by the CD73 inhibitor APCP (adenosine 5'-(α,β-methylene)diphosphate), indicating that CD73 activity, and thus adenosine generation, is required for this effect in these models. Interestingly, cN-II$^-$/CD73$^-$ cells also showed a slight sensitivity to AMP (27.8 ± 4.2% vs 16.7 ± 8.3% for cN-II$^+$/CD73$^+$, $p<0.05$), and this was insensitive to APCP. Overall, exposure to adenosine induced cell death in all cell models. This effect was not significantly different according to cN-II or CD73 expressions, suggesting that these 5'-nucleotidases do not modulate cell survival in the presence of high concentrations of adenosine. When the cells were incubated with adenosine and A2A and A2B antagonists, we did not observe any rescue. Thus, A2A and A2B receptors do not appear to mediate adenosine-induced cell death.

Similarly, we evaluated cell proliferation in the presence of initial high concentrations of AMP or adenosine (Figure 2A). The two purines slowed proliferation down, but adenosine had a stronger effect than AMP ($p<0.05$ for adenosine, $p>0.05$ for AMP). Indeed, in presence of this nucleotide, CFSE intensity after 3 days reached 34.9-43.0 % of the initial value, versus

23.5-25.4% without adenosine. Here again, no difference was observed according to cN-II and CD73 expression between our cell lines.

These results show that an extracellular nucleotide stress can affect cell proliferation and cell survival, independently from cN-II or CD73.

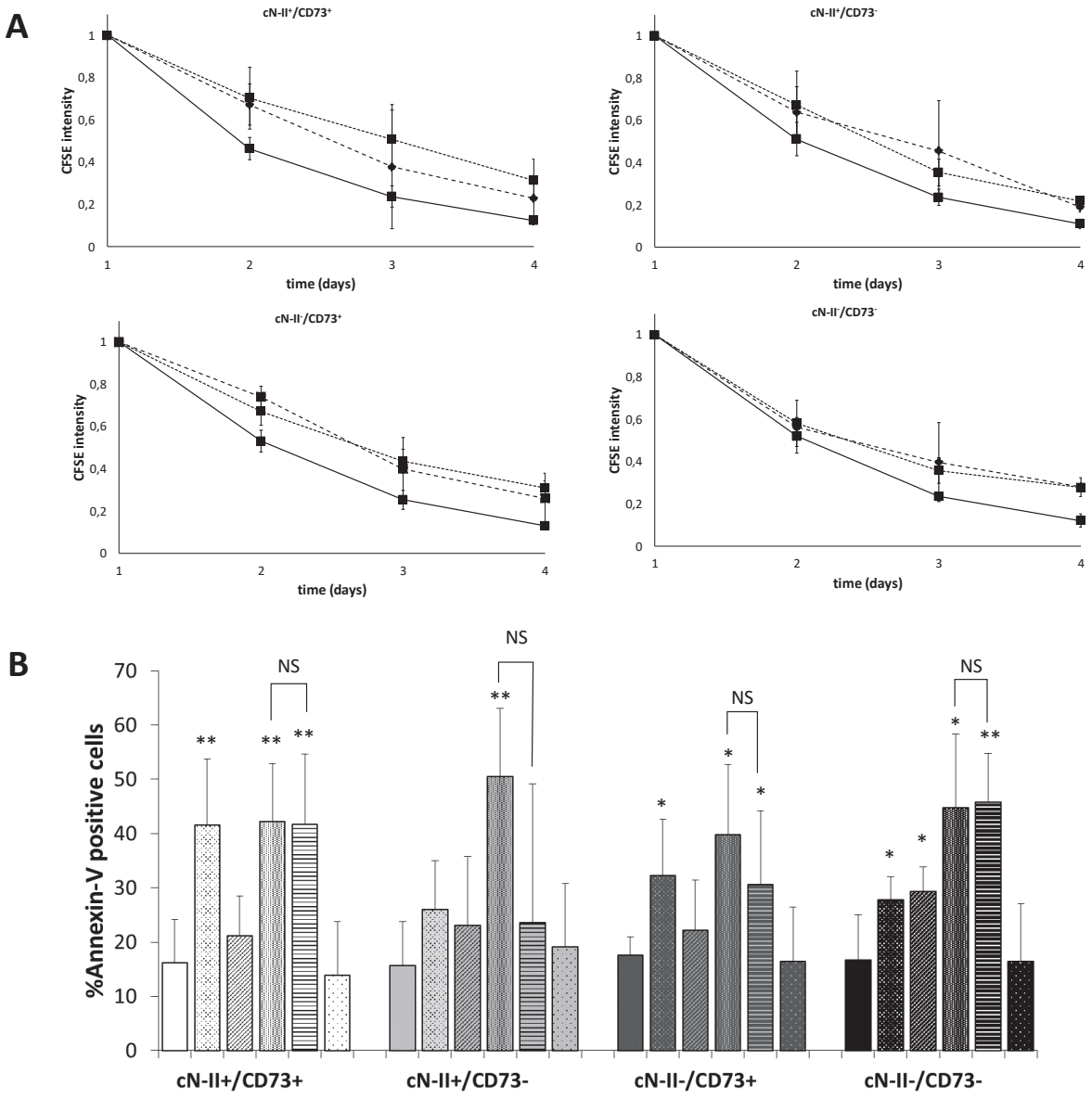

*Figure 2. Cell proliferation (**A**) and survival (**B**) of cell models. For cell proliferation (**A**), MDA-MB-231 were stained with CFSE to monitor cell proliferation over 4 days by flow cytometry in the presence or not of 1600 µM adenosine (•••••) or AMP (- - - - -). The graphs show mean values of 4 independent experiments and error bars are standard deviations. * = p*

*< 0.05; *** = p < 0.005 using Students t-test for comparison between adenosine and the control condition. (**B**): Cell death was evaluated for the four cell models in presence or not of adenosine (1600 µM), AMP (1600 µM) or in presence of a combination of AMP (1600 µM) + APCP (100 µM) or adenosine (1600 µM) + the A2A antagonist ZM 241385 (100 nM) + the A2B antagonist PSB 1115 (10 µM). APCP alone did not trigger any cell death. The graphs show mean values of 5 independent experiments and error bars are standard deviations. * = p < 0.05; ** = p < 0.01 using Students t-test in comparison with the control condition.*

Intracellular nucleotide pools in cell models

As indicated in the introduction, both cN-II and CD73 are expected to regulate intracellular nucleotide pools. We measured the pools, with a particular interest in purines, in the four models both under baseline conditions and after 1 hour exposure to 400 or 1600 µM adenosine or AMP (Figure 3 and table S1). Exposure to 2-deoxyglucose was used as a control condition inducing major modifications in NTP content. Both ATP and AMP were more abundant in cN-II$^-$/CD73$^+$ and cN-II$^-$/CD73$^-$ cells (1.4-1.6-fold) and unmodified in cN-II$^+$/CD73$^-$ cells as compared to control cells, whereas adenosine and inosine were decreased in both models lacking CD73 (1.4-3.2-fold) and increased in cN-II$^-$/CD73$^+$ cells (4.6- and 2.1-fold, respectively). Finally, IMP was decreased in cN-II$^-$/CD73$^+$ (3.4-fold) and increased in cN-II$^-$/CD73$^-$ cells (5.7-fold). After exposure to adenosine or AMP, control cells had increased content of ATP, AMP, inosine and adenosine. In modified cells, the most striking modifications were the increases of AMP and adenosine in cN-II$^-$/CD73$^+$ cells and of IMP in cN-II$^-$/CD73$^-$ cells. The lack of precision in samples exposed to adenosine or AMP resides in the important matrix effect during the LC-MS/MS analysis.

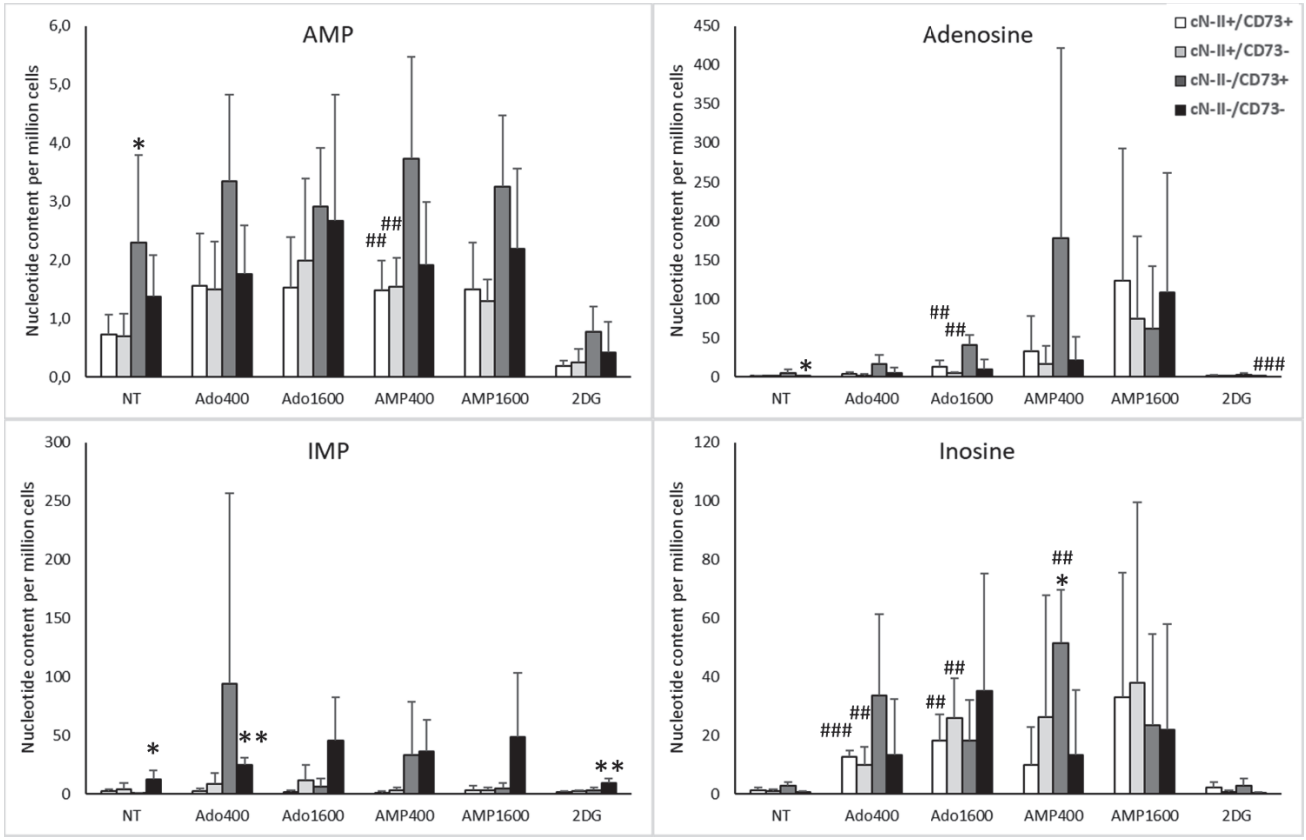

*Figure 3. Relative content of intracellular adenosine, inosine, AMP and IMP in the studied cell models, cN-II$^+$/CD73$^+$ in white, cN-II$^+$/CD73$^-$ in bright grey, cN-II$^-$/CD73$^+$ in dark grey and cN-II$^-$/CD73$^-$ in black. Nucleotides and nucleosides were quantified as indicated in material and methods and are expressed as mean values of the ratios of the surface of the compound of interest / surface of internal standard. The results where further normalized for one million cells and expressed as relative content as compared to unexposed cN-II$^+$/CD73$^+$ cells in each experiment. Values shown are from three independent experiments. For complete data, see supplemental table 2. . * = p < 0.05,** = p < 0.01, in comparison with the control cell line, # = p < 0.05; ## = p < 0.01 ; ### = p < 0.001 in comparison with the control condition..*

cN-II expression and extracellular adenosine modulate cell migration

Cell migration was determined by a wound healing assay and monitored by the IncuCyte® device (Figure 4A). Under normal cell culture conditions, reduced cN-II expression was associated with enhanced migration (Figure 4B). Indeed, 10 hours after injury, the wound confluence reached 70 to 77% confluence for cN-II$^-$/CD73$^+$ and cN-II$^-$/CD73$^-$ cells, whereas it was only 49 to 50% in cN-II$^+$/CD73$^-$ and cN-II$^+$/CD73$^+$ cells. We also monitored cell migration in the presence of high initial concentrations of AMP. In cN-II$^+$/CD73$^+$ cells and cN-II$^-$/CD73$^+$ cells, AMP delayed migration. This effect was reversed by co-incubation with APCP, suggesting that its conversion into adenosine might be necessary to impact this biological process. When we realized this assay in the presence of high initial concentrations of adenosine, the nucleoside reduced cell migration in all our cell models, confirming the importance of adenosine production to affect migration (Figure 4C). Interestingly, we observed that cell migration was more affected by adenosine in cN-II-deficient cells when we compared them to their cN-II-proficient counterparts (Δ wound confluence = 9.9 ± 3.6 in cN-II$^+$/CD73$^+$ cells versus 36.5 ± 14.8 in cN-II$^-$/CD73$^+$ cells and Δ wound confluence = 13.0 ± 2.9 in cN-II$^+$/CD73$^-$ cells versus 29.5 ± 12.3 in cN-II$^-$/CD73$^-$ cells) (Figure 4D). Again, we observed that AMP could also impact migration in cN-II$^-$/CD73$^-$ cells, suggesting a CD73-independent AMP effect. The observed effects were not due to adenosine or AMP-induced cell death as these purines do not significantly affect cell survival at early times (Figure S1).

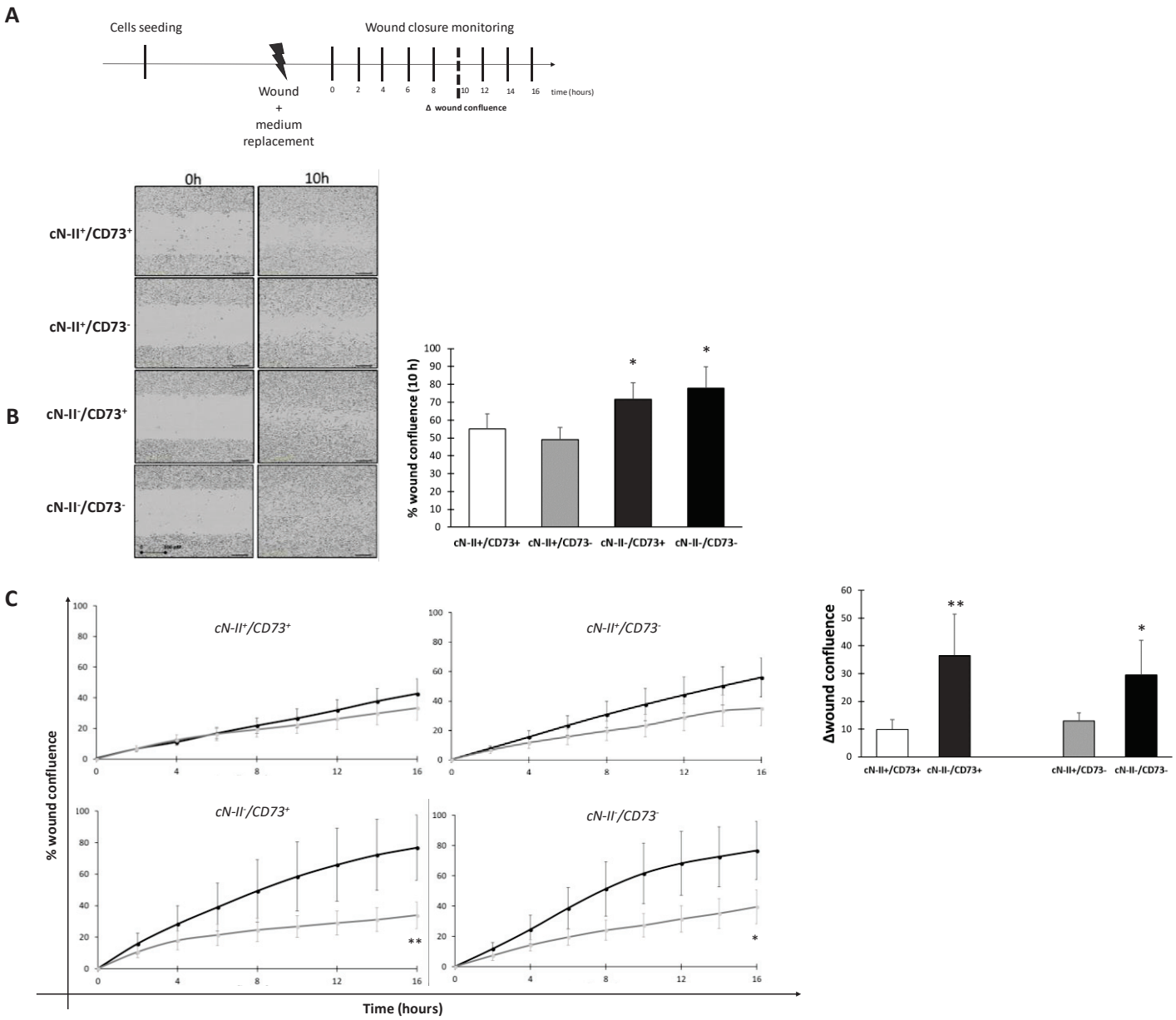

*Figure 4. Cell migration of studied cell models. Cell migration assay was performed as indicated in material and methods and in (**A**). (**B**): Images of cell confluence just after (0h) and 10h after injury (left panel), and the quantification of confluence at 10h after injury (right panel). Graphs are mean values of 4 independent experiments performed in triplicate, and error bars are standard deviation. * = p < 0.05 using Students t-test in comparison with the cN-II⁺/CD73⁺ cells. (**C**): Effect of adenosine (light curves) as compared to unexposed cells (dark curves) over 16 hours after injury. Graphs show mean values of 4 independent experiments performed in triplicate, and error bars are standard deviation. * = p < 0.05; ** = p < 0.01 using Students t-test in comparison with the control conditions 16 hours after injury. (**D**): Quantification of the effect of adenosine on cell migration. Δ wound confluence was calculated*

*10 hours after injury. For each cell line we used Δ wound confluence = | % wound confluence $_{control}$ - % wound confluence $_{adenosine}$|. Graphs show mean values of 4 independent experiments performed in triplicate, and error bars are standard deviation. * = p < 0.05 using Students t-test in comparison with the corresponding cN-II-proficient cell line.*

cN-II knock-out is associated with migration-related molecular modifications

In order to find molecular modifications explaining the differences in cell migration between the cN-II-deficient and cN-II-proficient cells, we studied the gene expression of the migration-related genes TIMP-1, TIMP-2, MMP-2 and MMP-9. In accordance with the migration experiments, at the mRNA level, cN-II-proficient cells expressed high levels of TIMP-2, but not TIMP-1, when compared to their cN-II-deficient counterparts (Figure 5A). Indeed, cN-II knock out was accompanied with a reduction of 34.0 % and 47.9 % of TIMP-2 mRNA expression for cN-II$^-$/CD73$^+$ and cN-II$^-$/CD73$^-$ respectively. MMP-2 and MMP-9 were more expressed in the CD73-deficient cells but did not vary according to cN-II expression. This MMP upregulation was not associated with any significant modification in cell migration.

CD44 is a glycoprotein that promotes migration in cancer cells, in association with MMPs (22). Thus, we determined its expression at the surface of our cell models and observed that cN-II-deficient cells express more CD44 than their cN-II-proficient counterparts (Figure 5B). CD73 deficiency is also associated with CD44 overexpression as the pro-migration glycoprotein is more expressed on CD73-deficient cells than on their CD73-proficient counterparts. Nevertheless, cN-II$^+$/CD73$^-$ cells expressed more CD44 than and cN-II$^-$/CD73$^+$ cells but this was not associated with a better migration. This suggests that high CD44 expression is not sufficient to mediate migration enhancement in these models, similarly as for MMP-2 and MMP-9 mRNA expressions.

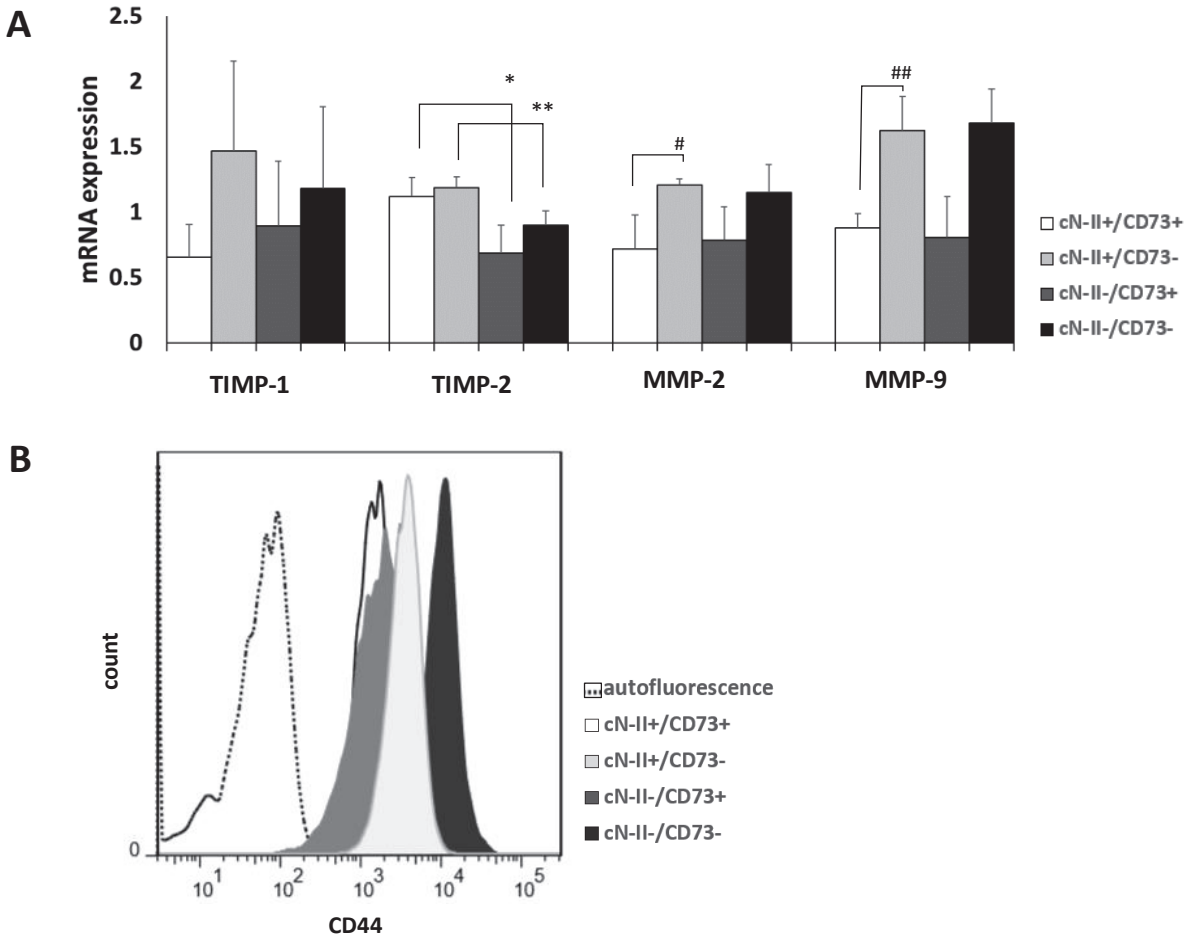

*Figure 5. Expression of migration-related proteins in cell models. (**A**): mRNA expression of TIMP-1, TIMP-2, MMP-2 and MMP-9. Graphs are mean values of at least three independent experiments performed in triplicate and error bars are standard deviation. * = p < 0.05; ** = p < 0.01 using Students t-test in comparison with the corresponding cN-II-proficient cell line. # = p < 0.05; ## = p < 0.01 using Students t-test in comparison with the corresponding CD73-proficient cell line. (**B**): CD44 protein expression at cell surface was evaluated by flow cytometry as indicated in material and methods. Autofluorescence is similar for all models and we used cN-II$^+$/CD73$^+$ to represent autofluorescence in the figure.*

cN-II modulates the COX-2/PGE2/Akt axis

COX-2 is involved in breast cancer cell lines migration (23–25) and TIMP-2 expression as well as cell migration can be regulated by the COX-2/PGE2/Akt axis (26). Indeed, cyclooxygenase 2 (COX-2) participates in prostaglandin E2 (PGE2) production from arachidonic acid in cells. The latter can bind surface G-protein-coupled receptors, leading to the activating

phosphorylation of Akt thus promoting cell migration and regulating gene expression. Therefore, we investigated the involvement of this axis in our models.

cN-II deficiency was associated with a drastically increased COX-2 expression both at the mRNA and protein levels (Figure 6A and B). This was accompanied with a higher PGE2 secretion in the cell supernatants (Figure 6C). Interestingly, CD73 silencing alone was not associated with any significant changes but when associated with cN-II silencing in MDA-MB-231 cN-II$^-$/CD73$^-$, it accentuated COX-2 expression (25.2-fold mRNA expression in cN-II$^-$/CD73$^+$ and 55.2-fold in cN-II$^-$/CD73$^-$, as compared to cN-II$^+$/CD73$^+$) and PGE2 production.

In the presence of the COX-2 inhibitor celecoxib, cell migration tended to slow down in the four cell lines (cN-II$^+$/CD73$^+$ cells were limited to 19.5 ± 9.4% wound confluence after 10 hours, cN-II$^+$/CD73$^-$ to 21.1 ± 11.7% wound confluence, cN-II$^-$/CD73$^+$ to 30.6 ± 8.7% wound confluence and cN-II$^-$/CD73$^-$ to 31.2 ± 14.5% wound confluence under 60 µM celecoxib exposure) (Figures 6D and E). On the contrary, arachidonic acid-induced PGE2 production slightly enhanced cell migration in cN-II-proficient models, thus confirming that COX-2 activity indeed is involved in this process in these models. Interestingly and similarly as for adenosine, celecoxib was able to more efficiently reduce cell migration in the cN-II-deficient cells, and particularly when it was associated with a CD73-deficiency. Consistently, arachidonic acid enhanced migration less efficiently in cN-II deficient cells, suggesting that cN-II can be involved in COX-2/PGE2 pathway modulations of cell migration.

AKT activation occurs downstream of prostaglandin receptor activation and is known to promote cell migration (27–30). Therefore, we evaluated its phosphorylation status, and observed that cN-II-negative cells, and particularly cN-II$^-$/CD73$^-$ cells showed a stronger basal activation of AKT (Figure 6F). In addition, incubation of the cells with 1600 µM adenosine for 1 hour reduced both COX-2 expression and AKT phosphorylation, which could explain its impact on cell migration. Taken together, these results suggest that the COX-2/PGE2/AKT axis is reinforced when cN-II is not expressed, and that adenosine can inhibit this axis.

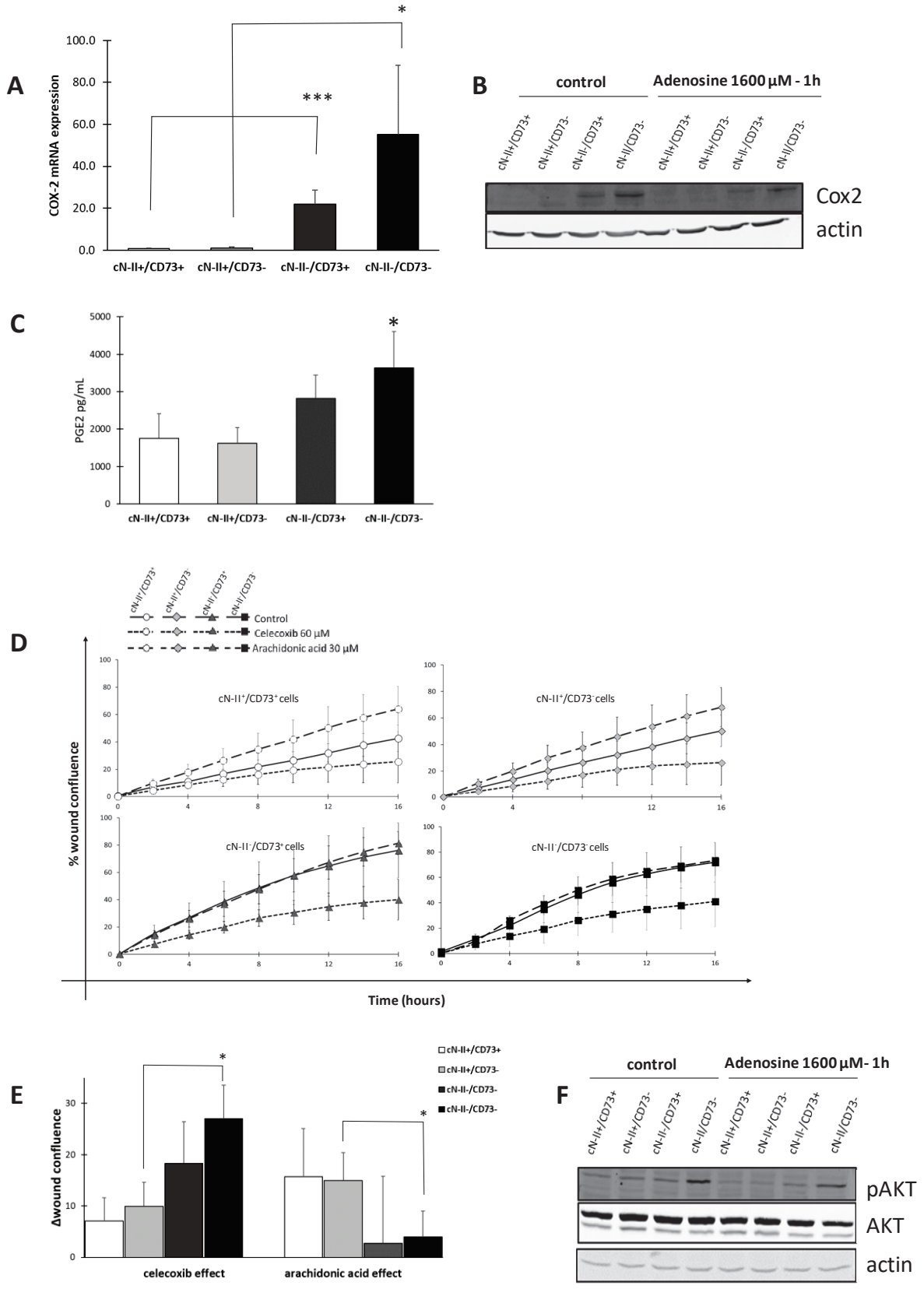

*Figure 6. Study of the COX-2/PGE2/Akt axis in cell models. COX-2 mRNA (**A**) and protein (**B**) expression were determined as indicated in material and methods after one-hour exposure*

*to adenosine (1600 µM). In (**A**), graphs are mean values of four independent experiments performed in triplicate and error bars are standard deviation, and (**B**) shows a representative image of 3 experiments. * = p < 0.05 and *** = p < 0.001 using Students t-test in comparison with the corresponding cN-II-proficient cell line. PGE2 secretion (**C**) was quantified in cell supernatants after a 24-hour stimulation with arachidonic acid (15 µM). Graphs are mean values of three independent experiments performed in triplicate and error bars are standard deviation. * = p < 0.05 using Students t-test in comparison with the corresponding cN-II^{+}/CD73^{+} cell line. (**D**): Wound healing curves of cells exposed or not to celecoxib 60 µM or arachidonic acid (30 µM). Graphs are mean values of three independent experiments performed in triplicate and error bars are standard deviation. (**E**): quantification of the effect of celecoxib and arachidonic acid on cell migration 10 hours after injury. For each cell line and each condition, we used Δ wound confluence = | % wound confluence $_{control}$ - % wound confluence $_{celecoxib\ or\ arachidonic\ acid}$ |. Graphs are mean values of four independent experiments performed in triplicate and error bars are standard deviation * = p < 0.05 using Students t-test in comparison with the corresponding cN-II-proficient cell line. (**F**): AKT expression and phosphorylation in cells exposed or not to adenosine (1600 µM, 1 hour).*

Discussion

In this study, we established new cell models that allow a better understanding of the role of 5'-nucleotidases in cancer cell biology. We showed that in the triple negative human breast cancer cell line MDA-MB-231, cN-II and CD73 are not necessary for cell proliferation or survival in optimal conditions or under a nucleotide stress. We also confirmed that adenosine impacts cancer cell biology. In the tumor microenvironment, extracellular adenosine concentrations increase because of the inflammatory environment and damaged cells. In this case, the concentrations can reach the micromolar range whereas it is in the nanomolar range under physiological conditions (31). It is then rapidly degraded by ADA or internalized by the nucleoside transporters. Because of its very short half-life time, we worked with very elevated initial concentrations of adenosine (1600 µM), but these experimental conditions do not allow us to know what concentrations of adenosine remain in the medium at the time points we studied. Although considered as pro-tumoral due to its immunomodulatory effects, we confirmed that adenosine also affects cancer cells themselves by slowing their proliferation and migration down and triggering cell death when it is present at very high concentrations, as suggested in previous studies (32,33). Nevertheless, cN-II and CD73 do not seem to be involved in these effects.

Little is known about the intracellular nucleoside and nucleotide pools in cells with modified expression of cN-II and CD73. In non-cancerous cells, the overexpression of cN-II was shown to only slightly decrease the NTP (34–36). No major differences were observed in stably transfected cells with cN-II targeting shRNA in four different models (6), and to our knowledge, no similar data exist for CD73-modified cancer cells. In our models, ATP was increased when either or both cN-II and CD73 were knocked out. We also observed increased IMP and decreased inosine in cN-II$^-$/CD73$^-$ cells, consistent with the fact that these cells will degrade less IMP into inosine. Whether these differences in nucleotide pools have a role in the phenotypic differences observed between our cell models remains unclear.

Previous studies suggested that CD73 is involved in cancer cell migration by adenosine-dependent and by adenosine-independent mechanisms. Our results support the predominant importance of this nucleoside in MDA-MB-231 cells, as adenosine could affect cell migration independently from CD73 expression. cN-II$^-$/CD73$^-$ cell migration and survival were also sensitive to AMP, independently from its conversion to adenosine. This could be the result of

a direct effect of the nucleotide on these cells. Indeed, it has been described that the adenosine receptor A1 can be activated by AMP (37) and can mediate cell death (38). In our models, A1 seems to be downregulated in cN-II$^-$/CD73$^-$ cells at the mRNA level and might not be responsible for this effect. To our knowledge, other enzymes such as prostatic acid phosphatase can hydrolyze AMP, independently from CD73 and mediate cell death by generating adenosine (3). Thus, their expression could be studied in cN-II$^-$/CD73$^-$ cells compared to the other models.

Metalloproteinases 2 and 9 are gelatinases that are highly expressed in MDA-MB-231 and regulate cell migration. Their activity depends on their regulators TIMP-1 and TIMP-2. High TIMPs expression being associated with low MMP maturation and reduced migration, and low TIMPs expression with enhanced MMP activity and migration (26), we evaluated their expression level in our models. We observed higher levels of MMP-2 and MMP-9 mRNA expression under CD73 knockout. This phenomenon is possibly related to purine-dependent signaling. Indeed, by degrading AMP, CD73 promotes high concentrations of adenosine which reduce AKT activation. Downstream of AKT, different gene expressions, including MMPs, are enhanced (39–41). Thus, in the absence of CD73, we expected reduced pools of adenosine, leading to a better AKT phosphorylation and a higher expression of MMPs, consistently with previous studies that show an inhibiting effect of adenosine on MMPs expression (42,43). However, our experiments did not reveal any detectable difference in AKT activation or in migration in the absence of CD73 alone. Notably, in association with cN-II deficiency this AKT activation was more striking. This suggests that MMP-2 and MMP-9 expression modulation upon CD73 silencing is not sufficient to significantly impact cell migration but these alterations interact with cN-II-relative modifications to accentuate the pro-migration phenotype induced by cN-II silencing.

Similarly, COX-2 expression can also be promoted by AKT activation (44,45) and regulated by adenine nucleotides/nucleosides. Lin *et al*. showed that ATP promotes COX-2 expression through NADHP oxidase activity and an increase in ROS production (44,46). The triphosphate nucleotide and adenosine tend to have opposite effects on cells and consistently with this notion, in our models, the latter triggered a lower AKT activation and a lower COX-2 expression.

Furthermore, in the models we generated, cN-II repression was associated with an enhanced constitutive AKT activation that can mediate the observed COX-2 expression. As mentioned above, cN-II can impact intracellular nucleotide pools and its knock-out could lead to an accumulation of nucleotides and a decrease of adenosine levels in the cytoplasm. As adenosine is transferred to the extracellular space through nucleoside transporters, this decrease can also impact extracellular adenosine pools thus resulting in a weaker activation of ADORA receptors, which is not intuitively in accordance with the observed increase in AKT-activation in our models. Nevertheless, we must also consider the expression and activation state of the four PGE2 receptors (EP1, EP2, EP3 and EP4 receptors) on our models. Indeed, these receptors also belong to the GPCR family and are expressed in MDA-MB-231 cells (47). These receptors share intracellular mediators with ADORA signaling involving cAMP generation and Akt activation. Simultaneous activation of these pathways can thus lead to different cellular responses according to the panel of receptors expressed on the cells. In addition, our team previously demonstrated that cN-II downregulation with stable shRNAs is accompanied with lower ROS contents (8). If a complete knockout of this 5'-nucleotidase impacts the cellular ROS contents, that should also act towards a downregulation of COX-2. Adenosine also has dual roles on phospholipase A2 expression and activity, which releases arachidonic acid, the PGE2 precursor (48,49), thus revealing another link between nucleotide metabolism and COX-2/PGE2 axis. Further studies are needed to decipher whether COX-2 is directly impacted by cN-II and nucleotide metabolism. A recent study demonstrated that cN-II interacts with cytoplasmic proteins, opening the possibility of enzymatic activity-independent effect of the cytosolic 5'-nuceotidase. Indeed, cN-II interacts with the inflammasome protein Ipaf, through its leucin-rich region (LRR) (50), and the absence of this interaction could result in Ipaf oligomerization and thus inflammation. Other members of the inflammasome such as NLRP3 also contain a LLR region and could interact with cN-II, and NLRP3 has been described as regulating COX-2 expression and PGE2 production (51). With a similar mechanism as for Ipaf, an interaction between cN-II and NLRP3 could consequently impact COX-2 expression as observed in our models.

We showed that the COX-2/PGE2/AKT axis is involved in MDA-MB-231 cell migration and that this axis is reinforced when cN-II is absent. Indeed, the COX-2 inhibitor celecoxib reduced cell migration and, inversely, stimulating PGE2 production with arachidonic acid enhanced cell

migration. Nevertheless, cN-II-deficient cells are more sensitive to these effects than their cN-II-proficient counterparts. These observations suggest that cN-II could play a role in migration regulation. The way cN-II is involved in cell sensitivity to agents that can impact on cell migration remains to be studied.

In conclusion, the generation of cN-II and/or CD73 knockout cells provides new tools that will serve to better understand nucleotide metabolism in cancer, and more precisely, the possible interaction between intra- and extracellular compartments of purines. Indeed, until recently, these compartments have been studied independently and in various pathologies, whereas we know that both direct (nucleoside and nucleotide transports) and indirect (transcriptional regulation through receptors) interplays exist. The model characterization provides further arguments to consider these 5'-nucleotidases as targets to disturb cancer cell biology and reduce their aggressiveness.

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Supplementary material

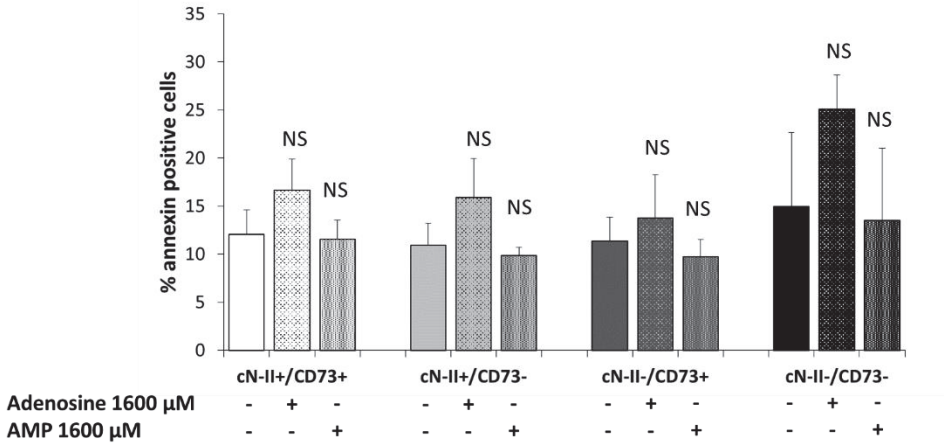

Figure S1. Cell death after a 10-hour exposure to adenosine (1600 µM) or AMP (1600 µM). Cell death was assessed by flow cytometry with Annexin-V and PI labelling. Graphs are mean values of three independent experiments performed in duplicate and error bars are standard deviation. Students t-test was used to compare exposed cells to controls. NS: p>0.05.

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
28S	CGATCCATCATCCGCAATG	AGCCAAGCTCAGCGCAAC
COX-2	TTATCTACACGGCCCCCTCC	CCAGGGCACGATGAAGTCAC
MMP-2	CCCATGAAGCCCTGTTCACC	CGGTCGTAGTCCTCAGTGGT
MMP-9	GGAGGCGCTCATGTACCCTA	GGACCATAGAGGTGCCGGAT
TIMP-1	GCTGGAAAACTGCAGGATGGA	GTCCGTCCACAAGCAATGAGT
TIMP-2	TTATCTACACGGCCCCCTCC	CCAGGGCACGATGAAGTCAC
A1	GAGCCGGAGGACTATGAGC	CCTGGAAAGCTGAGATGGAG
A2A	TCTTCAGTCTCCTGGCCATC	TCCAACCTAGCATGGGAGTC
A2B	CTCCATCTTCAGCCTTCTGG	CATGCACAGGTAACCAGCAC
ADA	AGCCCAAAGTAGAACTGCAT	CAAACTTGGCCAGGAAGTCT
ADK	TCACCCAAGGGAGAGATGAC	ATAGTGGCCAGCACGGATAC
AMPD3	CACATCCTGGCTCTCATCAC	GGATGTGTGTGTCCACCTTT
ADSL	ACAGCTACCGCTCACCTCTT	GCCGCCATGTCCGGAATTTA
ENT-1	GCTGGGTCTGACCGTTGTAT	CTGTACAGGGTGCATGATGG
dCTPP1	AAATGGACATCAACCGGCGA	AGTCACAGGGAATGTCCGCA
GMPR	ACGGAGTGTGCTGGAGAAGT	CCTCCTGCGTGCTTGTTCAT
GMPS	AAGGTTGTGGCACGTTCTGG	AGGGTGGAACTGTGCTCCAT
IMPDH 1	GCCCTAGATTGGACCTCGCT	ATCAGGTAGTCCGCCATGCT
IMPDH 2	GAGGCAATGTGGTCACTGCT	GATGGAGCCACTTCCCATGC
ITPA	TCCCCGGCCCCTACATAAAG	GCTGACTTGTCCTCGAACCC
PNP	TGAAATCCCCAACTTTCCCC	AATGTCACCTTCCAGAGTGGG
PPAT	ATCACACAAGGGAATGGGTC	ACAGACCAATACCATG ACGC
RRM1	GCAGCTGAGAGAGGTGCTTT	CAGGATCCACACATCAGACA
SAMHD1	CCCAAAGTTTGCTCGACGTGA	TGCATTCCATAATCCATGTTG

Supplemental table 1. Primers for gene expression analysis.

COX-2: cyclooxygenase 2; MMP-2: matrix metalloproteinase 2 ; MMP-9 : matrix metalloproteinase 9 ; TIMP-1 : tissue inhibitor of metalloproteinases 1 ; TIMP-2 : tissue inhibitor of metalloproteinases 2; A1: adenosine receptor 1; A2A: adenosine receptor 2A; A2B: adenosine receptor 2B; ADA: adenosine deaminase; ADK: adenosine kinase; AMPD3: AMP (adenosine monophosphate) deaminase 3; ADSL: adenylosuccinate lyase; ENT-1: equilibrative nucleoside transporter 1; dCTPP1: dCTP (deoxycytidine triphosphate) pyrophosphatase 1; GMPR: GMP (guanosine monophosphate) reductase; GMPS: GMP synthetase; IMPDH 1: IMP (inosine monophosphate) dehydrogenase 1; IMPDH 2: IMP (inosine monophosphate) dehydrogenase 2; ITPA: Inosine triphosphate pyrophosphatase; PNP: purine nucleoside phosphorylase; PPAT: amidophosphoribosyltransferase; RRM1: ribonucleoside-diphosphate reductase large subunit; SAMHD1: deoxynucleoside triphosphate triphosphohydrolase

Cell model	Experimental condition	ATP	AMP	Adenosine	GTP	IMP	dATP	dGTP	UTP	CTP	Inosine
cN-II$^+$/CD73$^+$	-	93 ± 9	72 ± 34	123 ± 52	97 ± 4	202 ± 202	95 ± 6	91 ± 11	93 ± 13	96 ± 7	132 ± 85
	Adenosine 400 µM	143 ± 23	156 ± 90	410 ± 243	113 ± 32	252 ± 229	129 ± 14	96 ± 12	103 ± 26	126 ± 31	1255 ± 239
	Adenosine 1600 µM	126 ± 17	152 ± 86	1320 ± 864	113 ± 7	154 ± 146	104 ± 13	98 ± 15	94 ± 9	138 ± 19	1805 ± 920
	AMP 400 µM	162 ± 32	147 ± 51	3342 ± 4450	105 ± 3	115 ± 92	155 ± 33	97 ± 7	86 ± 7	104 ± 24	999 ± 1293
	AMP 1600 µM	149 ± 20	150 ± 79	12310 ± 16966	118 ± 10	304 ± 375	135 ± 10	104 ± 5	107 ± 9	130 ± 23	3311 ± 4229
	2-deoxy-glucose	38 ± 3	19 ± 10	172 ± 86	51 ± 12	154 ± 115	16 ± 4	21 ± 9	32 ± 22	29 ± 18	223 ± 166
cN-II$^+$/CD73$^-$	-	77 ± 11*	69 ± 40	75 ± 28	88 ± 15	417 ± 150	73 ± 20*	57 ± 18**	72 ± 10*	77 ± 8**	92 ± 49
	Adenosine 400 µM	116 ± 18	149 ± 83	184 ± 181	111 ± 39	845 ± 902	102 ± 7*	75 ± 12	75 ± 6	106 ± 22	981 ± 609
	Adenosine 1600 µM	125 ± 29	198 ± 141	480 ± 197	111 ± 39	1155 ± 1284	94 ± 21	52 ± 46	73 ± 21	117 ± 44	2596 ± 1343
	AMP 400 µM	111 ± 27	153 ± 51	1688 ± 2295	88 ± 24	278 ± 291	102 ± 39	59 ± 12**	64 ± 26	79 ± 29	2633 ± 4146
	AMP 1600 µM	99 ± 16*	129 ± 38	7512 ± 10505	90 ± 18	317 ± 251	92 ± 18*	60 ± 7*	70 ± 26	88 ± 35	3805 ± 6165
	2-deoxy-glucose	25 ± 10	25 ± 22	48 ± 1	40 ± 18	207 ± 64	10 ± 7	14 ± 10	19 ± 14	21 ± 15	76 ± 48
cN-II$^-$/CD73$^+$	-	146 ± 35**	230 ± 149*	568 ± 384	144 ± 23*	60 ± 52	114 ± 33	73 ± 28	128 ± 40	153 ± 49*	276 ± 137
	Adenosine 400 µM	204 ± 61	334 ± 148	1629 ± 1163	173 ± 59	9409 ± 16236	143 ± 52	89 ± 28	144 ± 68	171 ± 56	3363 ± 2757
	Adenosine 1600 µM	212 ± 22**	291 ± 101	4160 ± 1175	188 ± 19**	627 ± 659	133 ± 23	73 ± 17	135 ± 34*	214 ± 20	1827 ± 1378
	AMP 400 µM	204 ± 29	373 ± 174	17812 ± 24391	204 ± 45*	3316 ± 4537	155 ± 52	83 ± 24	119 ± 52	164 ± 74	5158 ± 1799**
	AMP 1600 µM	208 ± 6**	326 ± 121	6231 ± 7962	174 ± 8**	449 ± 496	157 ± 51	80 ± 24	138 ± 19	178 ± 40	2354 ± 3093
	2-deoxy-glucose	71 ± 16*	77 ± 44	336 ± 140	94 ± 35	289 ± 35	13 ± 5	19 ± 19	57 ± 43	63 ± 42	278 ± 249
cN-II$^-$/CD73$^-$	-	127 ± 31*	138 ± 70	38 ± 1*	142 ± 53	1206 ± 835*	107 ± 47	77 ± 39	103 ± 24	114 ± 23	55 ± 43*
	Adenosine 400 µM	157 ± 33	176 ± 83	527 ± 677	176 ± 79	2453 ± 667*	105 ± 44	71 ± 48	94 ± 13	133 ± 42	1335 ± 1907
	Adenosine 1600 µM	163 ± 25	266 ± 216	981 ± 1258	182 ± 90	4528 ± 3702	84 ± 31	75 ± 47	94 ± 26	162 ± 76	3512 ± 4013
	AMP 400 µM	140 ± 34	192 ± 108	2136 ± 2972	152 ± 49	3600 ± 2686	101 ± 40	69 ± 32	94 ± 10	119 ± 16	1329 ± 2200
	AMP 1600 µM	153 ± 36	219 ± 137	10889 ± 15279	167 ± 62	4836 ± 5475	118 ± 56	67 ± 33	84 ± 17	118 ± 14	2199 ± 3582
	2-deoxy-glucose	62 ± 23	42 ± 52	30 ± 1	111 ± 63	902 ± 401	18 ± 13	15 ± 19	29 ± 16	35 ± 22	35 ± 24

*Supplemental table 2. Complete data for intracellular nucleotides and nucleosides in studied cell models. Nucleotides and nucleosides were quantified as indicated in material and methods and are expressed as mean values of the ratios of the surface of the compound of interest / surface of internal standard. The results where further normalized for one million cells and expressed as relative content as compared to unexposed cN-II$^+$/CD73$^+$ cells in each experiment. Values are means of three independent experiments ± standard deviations. * = $p < 0.05$; ** = $p < 0.01$ using Students t-test in comparison with unexposed cN-II$^+$/CD73$^+$ cells.*

Part III: tumor cN-II and CD73 roles in the interplay with NK cells.

Material and methods

NK lymphocytes and MDA-MB-231 co-culture

NK cells were maintained in culture in RMPI medium, supplemented with bovine fetal serum, penicillin/streptomycin and IL-2(100 UI/mL).

For survival and spontaneous cytotoxicity assessments, NK cells were stained with PKH26 red, a fluorescent marker. We cultured them with MDA-MB-231 in RMPI medium, supplemented with bovine fetal serum and penicillin/streptomycin. NK and MDA-MB-231 cells survival was evaluated by flow cytometry, with an Annexin-V labeling. The PKH26 red labelling allowed to discriminate the two populations.

Antibody-driven cell cytotoxicity assessment

We tested NK cells ADCC, targeting RL cells (Non-Hodgkin's lymphoma CD20 positive cell line as target cells) in the presence or absence of MDA-MB-231. The target cell cytoplasm was marked with calcein a luminescent marker. We then incubated the target cells with the effector NK in a medium containing 10 mg/mL Rituximab for 4 hours. The cells were present at the following ratio 2:1 (effector:target), in the presence or not of MDA-MB-231 expressing or not cN-II and CD73. Calcein release was then measured as a marker of target cells lysis. The experiment comprised a negative control, without Rituximab, to take in account the antibody-independent calcein release in our analysis.

Extracellular purine nucleotides regulate immune cells biology by directly interacting with the specific receptors expressed on their surface. Therefore, purine metabolism is widely

studied for new therapies in oncology, in order to promote immune cells activation and tumor-specific responses. Nevertheless, little is known about how cancer cells can impact the immune system under a nucleotide stress. We showed that nucleotide stress can modify cell biology and gene expression. Our study on cell migration revealed that there is a link between nucleotide metabolism actors and secreted factors. Thus, we can hypothesize that nucleotide stress or nucleotide metabolism-related enzymes can alter cancer cells secretome, thus orientating immune cells fate. Here, we investigated whether our cell models can impact NK lymphocytes survival and function.

NK cells participate in antitumor responses by direct cytotoxicity (perforine/granzyme or TRAIL/TRAIL-receptor systems), or indirectly by promoting anti-tumor responses through cytokine production mainly. In the tumor context, they are often inhibited and therapeutic strategies aim to reactivate them to improve treatment efficiency (Li and Sun, 2018). They are also implicated in the antitumor activity of monoclonal antibodies via the antibody-dependent cell cytotoxicity (ADCC), in which an antibody linked to an antigen on the cancer cell can activate NK cells through interaction with FcR.

Results

Effect of cancer cells on NK survival

NK cells need to be supplied with IL-2 to be maintained in culture. Considering this point, we evaluated their survival in co-culture with MDA-MB-231 without any additional IL-2 addition, in order not to impact the breast cancer cells biology and evaluate NK cells survival, independently from what is triggered by IL-2 absence. A preliminary experiment allowed us to observe that IL-2 deprivation does not impact NK cells survival at early times (Figure 21). Consequently, we measured NK survival in presence of MDA-MB-231 before 24 hours of culture.

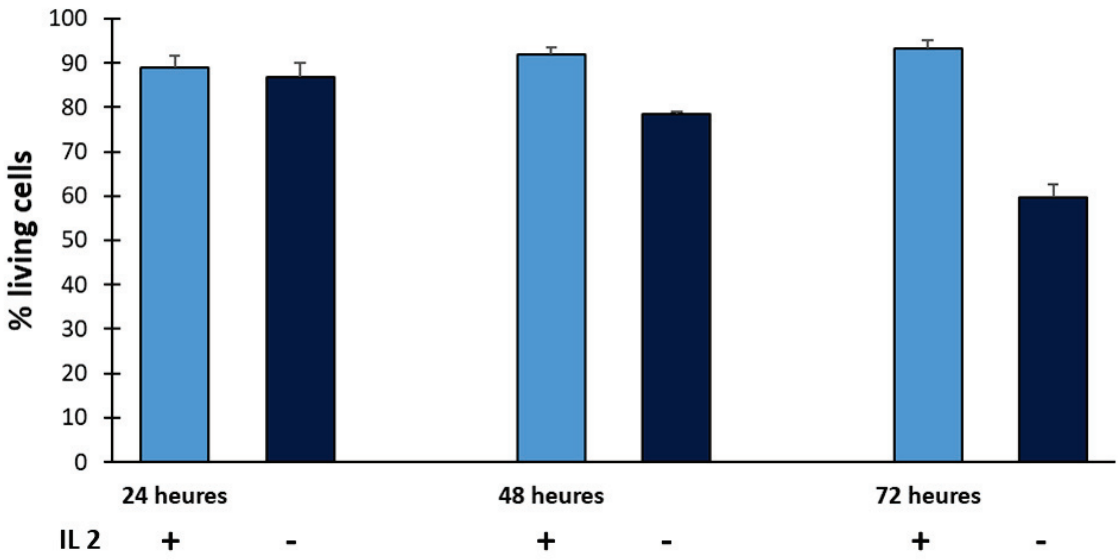

Figure 21: NK cells survival was assessed after 24, 48 or 72 hours in culture with or without IL-2. Bars represent means ± sd of 2 independent experiments.

MDA-MB-231 have a protective effect on NK cells. Indeed, the latter survive better when they are in presence of the triple negative breast cancer cells than when they are cultured alone (Figure 22). This effect is not dependent on cN-II or CD73 expressions but is related to the proportion of MDA-MB-231 versus NK cells. Indeed, the less the malignant cells are present, the more this effect seems to be marked, confirming the possibility of a complex interplay between these two cell types. This ratio-dependent effect could also be due to the ability of MDA-MB-231 to secrete IL-2 (Yiu and Toker, 2006).

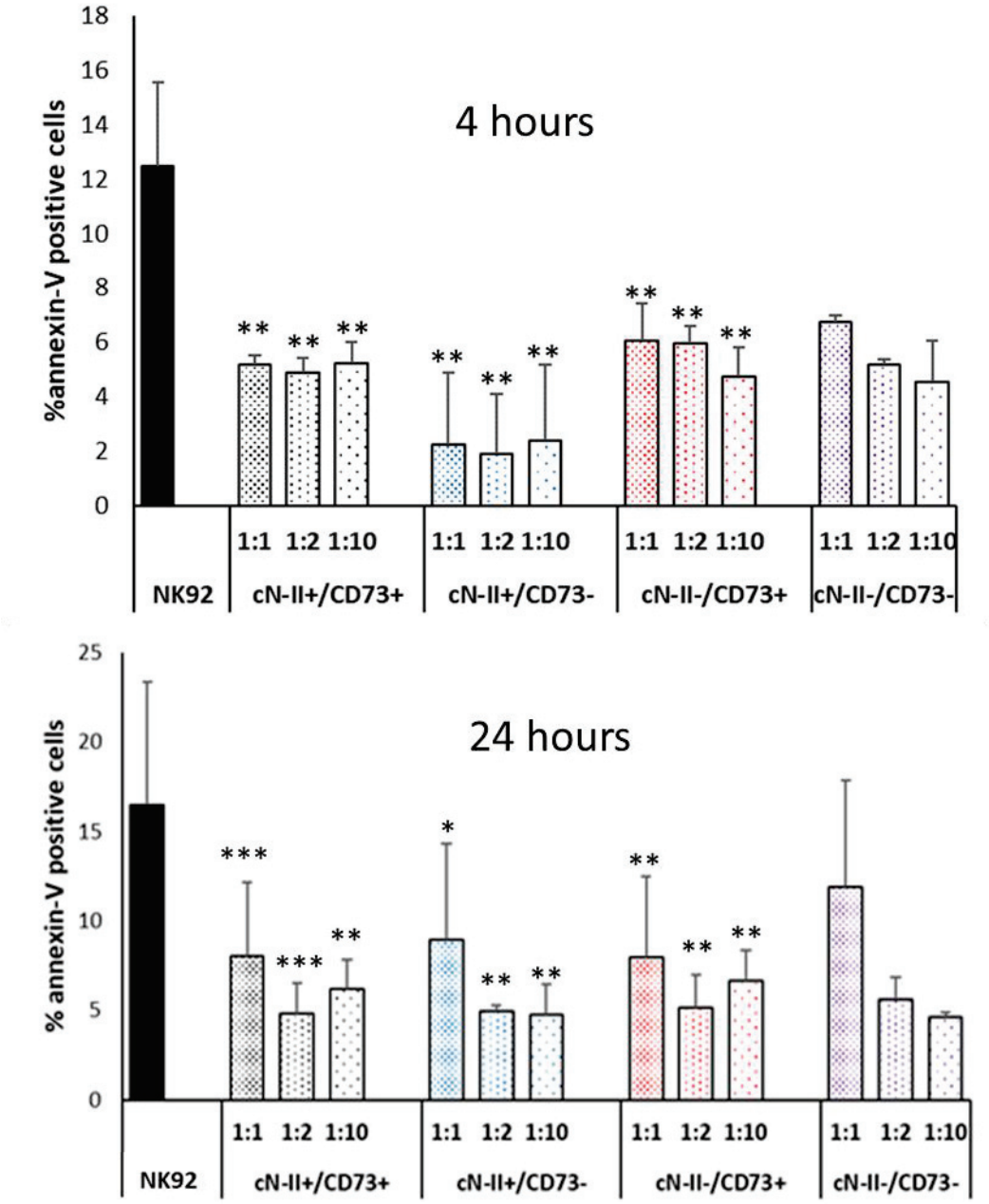

*Figure: NK cells survival was assessed after 4 or 24 hours in co-culture with different ratios of MDA-MB-231 expressing or not cN-II and CD73.Ratios are noted as NK:MDA-MB-231 Bars represent mean ±sd. * = p < 0.05; ** = p < 0.01 and *** = p < 0.005 with a Student's t test, in comparison with NK cells alone. For cN-II$^-$/CD73$^-$ cells, n=2*

Effect of cancer cells on NK cells cytotoxic functions

Oppositely to the protective effect of MDA-MB-231 on NK lymphocytes, when they are in sufficient ratio, NK cells display a slight spontaneous toxicity toward breast cancer cells (Figure 23). Interestingly, it seems to affect them more when cN-II is absent, indicating that cN-II might play a role in cancer cell vulnerability (from 14.9 ± 7.3% dead cells, without NK to 18.9 ± 10.0

% dead cells in presence of NK in cN-II$^+$/CD73$^+$ cells versus 17.0 ± 7.7% dead cells, without NK to 28.6 ± 9.28 % dead cells in presence of NK in cN-II$^-$/CD73$^+$ cells; and from 18.0 ± 10.5% dead cells, without NK to 20.0 ± 7.2 % dead cells in presence of NK in cN-II$^+$/CD73$^-$ cells versus 18.5 ± 8.7% dead cells, without NK to 27.9 ± 11.9 % dead cells in presence of NK in cN-II$^-$/CD73$^-$ cells.

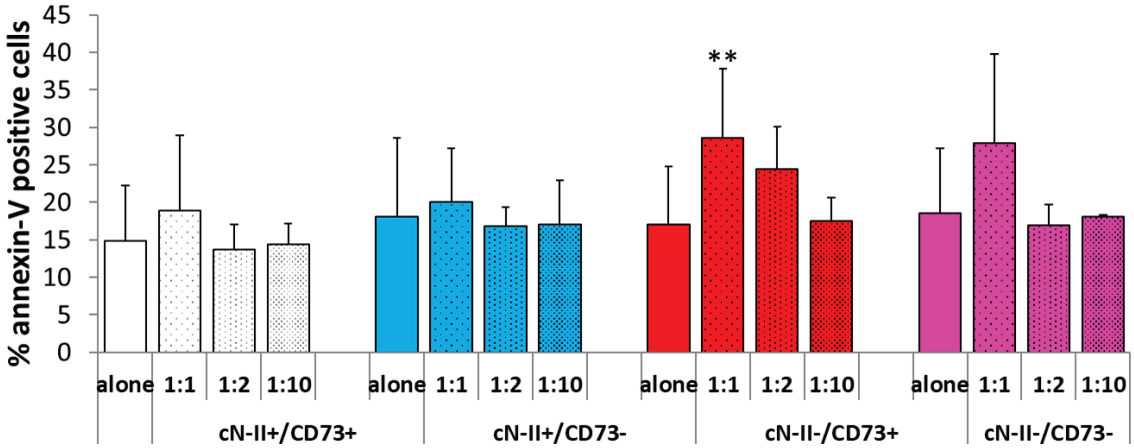

*Figure 23: MDA-MB-231 cells survival was assessed after 24 hours in co-culture with NK cells. Different ratios were used and noted as NK:MDA-MB-231 Bars represent mean ±sd. * = p < 0.05; ** = p < 0.01 and *** = p < 0.005 with a Student's t test, in comparison with NK cells alone. For cN-II$^-$/CD73$^-$ cells, n=2*

We assessed MDA-MB-231 ability to modifiy NK cell ADCC (antibody dependent cytotoxic activity). We used CD20-expressing RL malignant cells as targets. In the presence of rituximab, an anti-CD20 antibody, NK cells were able to induce RL lysis. However, in presence of MDA-MB-231, this ADCC was almost 50% less efficient (57.5% cell lysis in absence of MDA-MB-231 versus 26.8%, 32.4%, 28.1% and 32.9% in coincubation with cN-II$^+$/CD73$^+$, cN-II$^+$/CD73$^-$, cN-II$^-$/CD73$^+$ and cN-II$^-$/CD73$^-$ cells repectively). No significant difference was noticed according to cN-II and CD73 expression in the breast cancer cells. Thus, MDA-MB-231 reduce NK cells cytotoxicity but this phenomenon is not dependent on cN-II or CD73 expression. To decipher whether this effect was mediated by a secreted factor, we incubated NK cells in MDA-MB-231 supernatants to assess their cytotoxic activity. Here, no reduction of ADCC was observed, and consistently wih the previous observation, this was true independently from cN-II and CD73 expressions on MDA-MB-231. Thus, these triple-negative breast cancer cells are able to

diminish NK cell cytotoxicity by a process that does not invove a secreted factor or cN-II and CD73 in the conditions we tested.

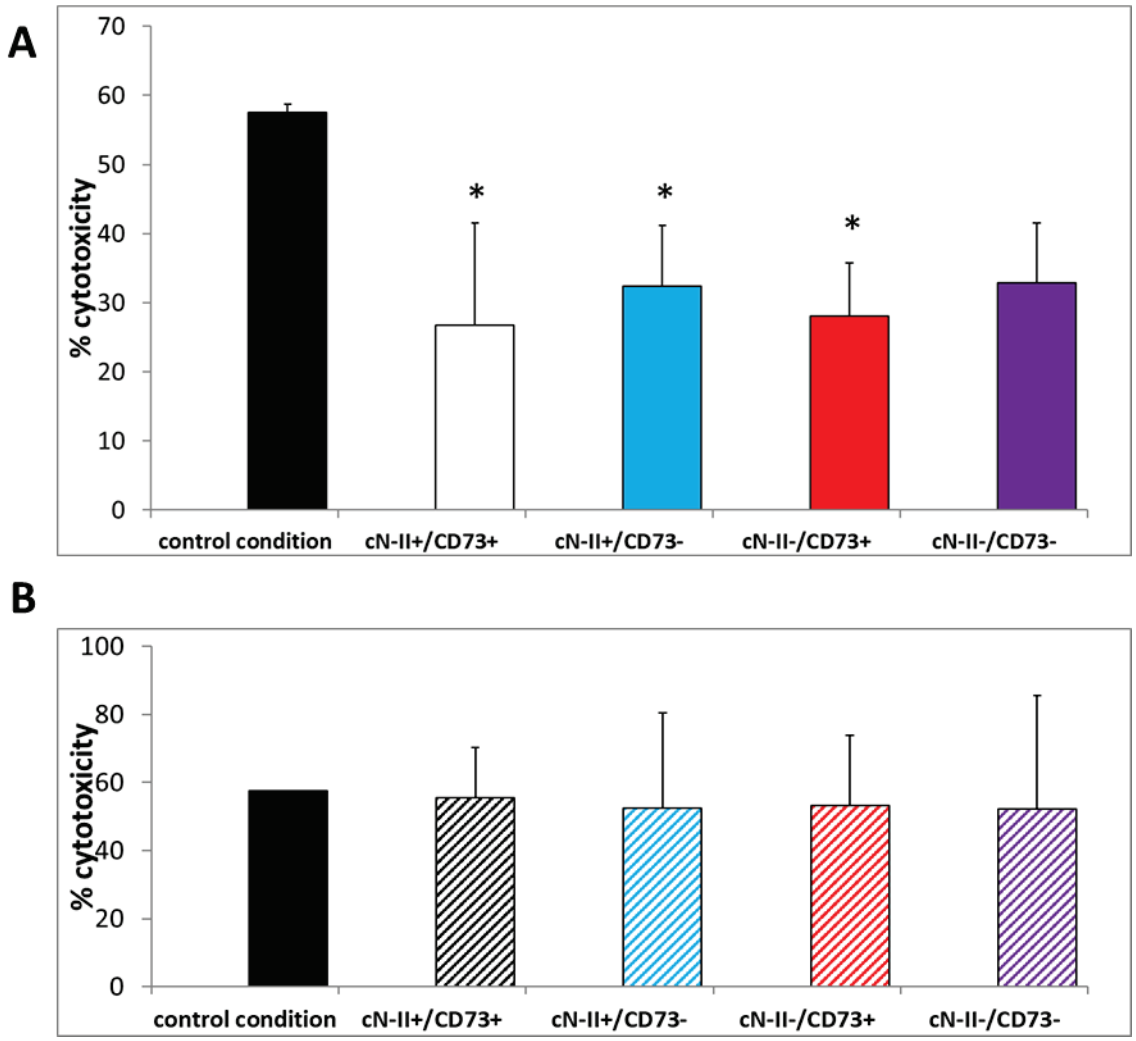

*Figure 24: NK cells ADCC on RL cells was assessed in presence of MDA-MB-231 expressing or not cN-II and/or CD73 (A), or in these cells' supernatant (B). Bars represent mean of 3 independent experiments ±sd. * = $p < 0.05$.*

Discussion

Here, we showed that MDA-MB-231 can modulate NK cells survival and functions. In the conditions we tested, we could not observe any involvement of cN-II and CD73 in these processes. It is not clear whether the protective effect of MDA-MB-231 is mediated by secreted factors or if it requires a direct contact between the two cell types.

Nevertheless, we previously demonstrated that cN-II expression can modulate secreted factors production such as PGE2 (see Part II). Previous studies demonstrated that NK cells express PGE2 receptors and that this prostaglandin reduced NK functions in breast cancers (Holt et al., 2011, 2012). Thus, in experimental conditions that would allow an optimal interaction between these innate immune cells and our models, we could observe cN-II dependent effects on NK cells. Moreover, these lymphocytes express adenosine receptors at their surface. Consequently, if cN-II and CD73 expressions on tumor cells impact microenvironment adenosine pools, it constitutes another way by which NK lymphocytes can be affected by tumor cells, by mechanisms involving nucleotide metabolism.

Here, we started to study the interplay between cancer cells and its immune microenvironment by focusing on the signals sent by tumor cells and how these signals can be modulated by cN-II and CD73 expressions. This strategy represents a tool to predict immune cell behavior according to the biological characteristic of cancer cells within the tumor.

Further *in vitro* studies are needed to quantify polarizing cytokine production by tumor cells to determine if they favor the constitution of an immunotolerant microenvironment. For example, TGFβ and IFN-β are interesting to quantify as they orientate neutrophils polarization toward a pro- or anti-tumor phenotype (Fridlender et al., 2009). PGE2 and IFNγ play similar roles for macrophage polarization (Ferrante and Leibovich, 2012).

We also need to generate immunocompetent *in vivo* models to study the effects of a paracrine interplay between cancer and NK cells. Histological studies will allow us to identify what immune cells are recruited in tumors with versus without cN-II or CD73 expressions. To go further, *ex vivo* experiments can help to evaluate how their function is impacted.

Upcoming experiments aim to focus on neutrophils and macrophages, in a similar way as we did for NK cells. By evaluating their survival and function (phagocytosis mainly), in proximity with tumor cells with altered cN-II and CD73 expressions will provide preliminary data for *in vivo* analyses of these immune cells in the tumor infiltrate.

General discussion and perspectives

Metabolism and migration

We showed that cN-II downregulation or suppression can be associated with metabolic changes. More precisely, cells without cN-II have modified glucose metabolism-related enzyme expression and we suggest that it could be accompanied with modified glucose uptake, lactate production and medium acidification, even though it is not the case shRNA-mediated cN-II down-regulation (Bricard et al., 2017). Such modifications can represent an interesting link between metabolic adaptability and cell migration. Indeed, lactate can act as a signaling molecule and trigger hypoxia-induced factor 1 (HIF-1) expression. The latter can induce CD73 and pro-migration molecules expression such as MMPs (Baumann et al., 2009; Goetze et al., 2011; Guedes et al., 2016; Kato et al.). Thus, in our models, it is relevant to evaluate migration, CD73 and MMPs expression and sensitivity to lactate and to associate this with an evaluation of lactate production according to cN-II and CD73 expressions. This axis could complete the PGE2/COX-2 axis that is involved in migration in our cell lines, as we demonstrated.

Moreover, glucose-6-phosphate isomerase mRNA expression is increased under simultaneous invalidation of cN-II and CD73. This glycolysis enzyme can be secreted in cancers, and act as a pro-migration autocrine factor (Ahmad et al., 2011; Funasaka et al., 2009) by stimulating the expression of pro-migration factors such as IL-8 (Araki et al., 2009). This particularity could also represent a link between metabolic regulations and migration. Indeed, glucose-6-phosphate isomerase overexpression correlates with increased cell migration. It should be reminded that cN-II$^-$/CD73$^-$ cells and cN-II$^-$/CD73$^+$ cell both display enhanced motility abilities, but this is accentuated in the cN-II$^-$/CD73$^-$ model. By its interaction with migration pathways, glucose-6-phosphate isomerase overexpression could participate in this difference. We also need to further investigate the activation state of pentose phosphate pathway in our

models and in non-metastatic cell lines with suppressed cN-II and/or CD73 expression, as the non-oxidative branch activation of this pathway is considered as a characteristic of metastatic tumors. Other metabolic pathway can also interact with migration processes and therefore could be related with cN-II expression (Han et al., 2013).

To go further in metabolic studies, we realized an analysis of intracellular metabolites in our models expressing or deficient for cN-II and/or CD73, in basal conditions or under a nucleotide stress (high adenosine or AMP concentrations). The analysis of these data is currently ongoing and will hopefully provide us new insights to unravel the involvement of 5'-nucleotidases in global cell metabolism.

Migration and immunity

We mentioned the importance of quantifying cytokine secretion by our models, to evaluate what signals the cancer cell sends to its immune microenvironment, according to its cN-II or CD73 status. Among these cytokines, several can impact cancer cell migration. It is the case for PGE2 in our cN-II-deficient models, and that was shown to reduce cytotoxic functions and phagocytosis while favoring cancer cell migration (Kalinski, 2012). TGFβ is another example of cytokine that can orientate immune cells polarization, as it favors the differentiation of tumor-associated neutrophils toward their pro-tumoral phenotype, N2 (Fridlender et al., 2009), and influences on cell migration according to the context (Matise et al., 2012; Zhao et al., 2018). In response to these signals, immune cells not only modify their function but also acquire the ability to modify the extracellular matrix, which potentially facilitates tumor cell migration. Consequently, it is interesting to study the interaction between these pathways in immunocompetent mice with cN-II or CD73-expressing tumors. To so do, we plan to invalidate cN-II and/or CD73 in 4T1 cells that are murine breast cancer cells. We will then develop corresponding tumors in BALB/c immunocompetent mice in order to analyze if the immune infiltrate varies according to the models. From these, we will be able or determine whether cN-II and CD73 expressions in tumor cells makes immune cells secretome vary or if the secreted cytokines belong to different phenotypes or activation states. Among the secreted factors, it will be important to focus on matrix-remodeling

proteins in order to initiate histological analysis of tumors to evaluate to what extent the matrix could favor or not tumor spreading.

Tumor 5'-nucleotidases and adipose tissue

Similarly to immune cells, adipose tissue is a major component of microenvironment. Indeed, adipose tissue favors tumor aggressiveness by diverse processes. For example, it provides energy for cancer cells by modifying their metabolism (Nieman et al., 2011), or protects them from therapeutic agents (Duong et al., 2015). Little is known about the direct link between white adipose tissue and nucleotides/nucleosides but it has been described that brown and beige adipose tissue are sensitive to adenosine signaling (Gnad et al., 2014; Rines et al., 2015). In mouse adipose tissue, it has been described that adenosine impacts adipocytes metabolism by interfering with lipolysis. (Johansson et al., 2008). Such interactions can alter the interplay between adipocytes and cancer cells in a way that could render cancer cells more vulnerable. Moreover, adipose tissue metabolism can indirectly be impacted by extracellular adenosine, via immune cells (Csóka et al., 2014). Thus, because of these interplays, studying the roles of tumor cN-II and CD73 in the relationship between tumor cell and adipose tissue is a major parameter to focus on.

In our team a project focuses on the protective effect of adipocytes on cancer cells toward chemotherapies (Duong et al., 2017). Therefore, we possess *in vivo* and *in vitro* tools to study the interaction between adipose tissue and cancer cells. For example, we could assess whether differentiated adipocytes still display these protective properties in presence (or after an exposure) to an extracellular nucleotide stress. Moreover, the roles of cN-II and CD73 in this adipocyte-induced resistance could be investigated thanks to our models.

General conclusion

This project allowed us to increase our knowledge about the roles of 5'-nucleotidases and particularly cN-II in cancer cell biology. Indeed, we showed how they can be involved in processes that impact cancer cell aggressiveness and plasticity. So far, we have focused on one cancer cell line (MDA-MB-231, which is a metastatic breast cancer cell line) that allowed us to reveal these new roles for cN-II and CD73. Nevertheless, in a different model, we might observe different behaviors as both adenosine receptors and nucleotide pools regulations might be different. In order to answer this, we already engineered cN-II and CD73 knockout in NCI-H292, a lung adenocarcinoma cell line with a less metastatic phenotype than MDA-MB-231, and we are currently using these models to investigate the same points that we did with MDA-MB-231 (PhD project of MZ Raza). Also, to confirm our data about cN-II involvement in metabolic adaptability and migration, we will re-express cN-II in our cN-II-deficient models using a plasmid coding a cN-II sequence insensitive to the guide RNA. In addition, these two 5'-nucleotidases could also be involved in tumor initiation or cell transformation. Thus, further studies based on non-cancerous cells and non-metastatic cells are needed to complete the knowledge about cN-II and CD73 in cancer. This work confirmed the relevance of emerging strategies to target nucleotide metabolism in cancer therapy, but such models will help to better understand at what point of cancer development they can be the most relevant, and to refine the future therapeutic strategies to avoid selection pressure that favor relapses in patients.

We also confirmed an important role of extracellular nucleotide pools on cell behavior, by altering it with high concentrations of adenosine or AMP. To obtain more precise information and potentially more physiologically relevant conditions, we are trying to use a pump-based system allowing to control or have a constant concentration in extracellular purines. Also, the quantification of extracellular nucleotides and nucleosides in cell culture experiments will have a major importance for the understanding of the interplay between extracellular and intracellular nucleotide metabolisms and pools. The set-up of a UHPLC-based technique for this is ongoing in our laboratory.

From the observations we made, new perspectives arise to better understand cN-II, CD73 and nucleotide metabolism in cancer cells, and ongoing research should allow answering

these questions. To open the field, we realized a RNA sequencing and relative quantification in our models. The resulting analysis will reveal other genes and pathways that can be modified by cN-II and CD73 and thereby can be relevant subject for future studies.

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Annex 1 - Scientific communications

Scientific articles

- **Cadassou O**, Raza MZ, Cros-Perrial E, Machon C, Gudefin L, Armanet C, Chettab K, Guitton J, Tozzi MG, Dumontet C & Jordheim LP. Upregulation of migration capacities and COX-2/PGE2/Akt axis in breast cancer cells deficient for 5'-nucleotidases cN-II and CD73. Submitted.
- Bricard G, **Cadassou O**, Cassagnes LE, Cros-Perrial E, Payen L, Puy JY, Lefevbre-Tournier I, Tozzi MG, Dumontet C & Jordheim LP. The cytosolic 5'-nucleotidase cN-II lowers the adaptability to glucose deprivation in human breast cancer cells. *Oncotarget*, 2017; 8 (40): 67380-67393.

- Other projects: Delaney JR, Patel CB, McCabe Willis K, Haghighiabyaneh M, Axelrod J, Tancioni I, Lu D, Bapat J, Young S, **Cadassou O**, Bartakova A, Sheth P, Haft C, Hui S, Saenz C, Schlaepfer DD, Harismendy O, and Stupack DG. Haploinsufficiency networks identify targetable patterns of allelic deficiency in low mutation ovarian cancer, *Nature Communications*. 2017 Feb 15;8:14423.

Oral presentations (the speaker's name is underlined)

- **Cadassou O**, Raza MZ, Cros-Perrial E, Armanet C, Gudefin L, Chettab K, Dumontet C & Jordheim LP. *5ème Journée Scientifique du CRCL*, Lyon, France, Juin 2018.
- **Cadassou O**, Cros-Perrial E, Armanet C, Balde M, Chettab K, Dumontet C & Jordheim LP. The involvement of 5'-nucleotidases cN-II and CD73 in breast cancer cell aggressiveness. *17th International Symposium on Purine and Pyrimidine Metbolism in Man*, Gdansk, Pologne, Septembre 2017.

Posters

- Raza MZ, Cros-Perrial E, **Cadassou O**, Dumontet C & Jordheim LP. 5'-nucleotidases are involved in the biology of human lung cancer cell lines. *5ème Journée Scientifique du CRCL*, Lyon, France, Juin 2018.
- **Cadassou O**, Raza MZ, Cros-Perrial E, Armanet C, Gudefin L, Chettab K, Manié S, Dumontet C & Jordheim LP. Impact of cN-II and CD73 inhibition on cancer cell migration. *109th Annual Meeting of the American Association for Cancer Research*, Chicago, Il, USA, Avril 2018.
- Raza MZ, Cros-Perrial E, **Cadassou O**, Dumontet C & Jordheim LP. 5'-nucleotidases are involved in the biology of human lung cancer cell lines. *Forum de la recherche en Cancérologie Auvergne-Rhône-Alpes*, Lyon, France, Avril 2018.
- **Cadassou O**, Raza MZ, Cros-Perrial E, Armanet C, Gudefin L, Chettab K, Dumontet C & Jordheim LP. Nucleotide metabolism and cancer cell aggressiveness. *Forum de la recherche en Cancérologie Auvergne-Rhône-Alpes*, Lyon, France, Avril 2018.
- **Cadassou O**, Cros-Perrial E, Armanet C, Balde M, Chettab K, Dumontet C & Jordheim LP. 5'-nucleotidases and cancer cell aggressiveness. *3rd Symposium of the Cancer Research Center of Lyon*, Lyon, France, Septembre 2017.
- **Cadassou O**, Cros-Perrial E, Gudefin L, Dumontet C & Jordheim LP. The 5'-nucléotidases cN-II and CD73 impact on the metabolic adaptability and cell/matrix interactions in breast cancer model. *Forum de la recherche en Cancérologie Auvergne-Rhône-Alpes*, Lyon, France, Avril 2017.