Immunology Letters 205 (2019) 40–50

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Review

Functional insights into nucleotide-metabolizing ectoenzymes expressed by bone marrow-resident cells in patients with multiple myeloma

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ARTICLE INFO

Keywords:
Multiple myeloma
Tumor niche
Metabolic reprogramming
Nucleotides
Nucleosides
Immunosuppression

ABSTRACT

Human myeloma cells grow in a hypoxic acidic niche in the bone marrow. Cross talk among cellular components of this closed niche generates extracellular adenosine, which promotes tumor cell survival. This is achieved through the binding of adenosine to purinergic receptors into complexes that function as an autocrine/paracrine signal factor with immune regulatory activities that i) down-regulate the functions of most immune effector cells and ii) enhance the activity of cells that suppress anti-tumor immune responses, thus facilitating the escape of malignant myeloma cells from immune surveillance. Here we review recent findings confirming that the dominant phenotype for survival of tumor cells is that where the malignant cells have been metabolically reprogrammed for the generation of lactic acidosis in the bone marrow niche. Adenosine triphosphate and nicotinamide-adenine dinucleotide extruded from tumor cells, along with cyclic adenosine monophosphate, are the main intracellular energetic/messenger molecules that serve as leading substrates in the extracellular space for membrane-bound ectonucleotidases metabolizing purine nucleotides to signaling adenosine. Within this mechanistic framework, the adenosinergic substrate conversion can vary significantly according to the metabolic environment. Indeed, the neoplastic expansion of plasma cells exploits both enzymatic networks and hypoxic acidic conditions for migrating and homing to a protected niche and for evading the immune response. The expression of multiple specific adenosine receptors in the niche completes the profile of a complex regulatory framework whose signals modify multiple myeloma and host immune responses.

1. Multiple myeloma

Multiple myeloma (MM) is a hematological malignancy that originates from a clone of malignant plasma cells (mPCs) infiltrated in the bone marrow (BM) to establish deleterious interactions with the multicellular tumor microenvironment (TME) or niche, for survival and proliferation [1,2]. The symptomatic stage of MM is preceded by the indolent stages of monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM) [3,4]. The estimated risk of yearly progression to symptomatic MM disease ranges from < 1% to > 3% - 10%, respectively [5,6]. The progressive trend of the disease is influenced by i) sequential genetic events in the malignant clones and ii) alterations of the BM microenvironment in the composition of cells and surrounding molecular fluid [7,8].

2. Myeloma BM niche

The collective behavior of normal cells is regulated by an array of mechanisms ranging from physical cross talk among homo- or heterotypic cells to soluble substances that calibrate their communication. In turn, both mechanisms are regulated by the metabolic *status* of the cellular microenvironment. It is thus not difficult to imagine that closed environments (such as a tumor niche) may provide the setting for cell adhesion effects and plasmatic soluble factors to acquire new and different functions, sometime even going in opposite direction compared to the ones performed in open systems (e.g., as the blood stream).

Abbreviations: AC, adenylyl cyclase; ADA, adenosine deaminase; ADO, adenosine; ADK, adenosine kinase; ADP, adenosine diphosphate; ADPR, adenosine diphosphate ribose; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cADPR, cyclic ADPR; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; mPCs, malignant plasma cells; NAD^+, nicotinamide adenine dinucleotide; NAM, nicotinamide; NPP, nucleotide pyrophosphatase/phosphodiesterase; 5′-NT, 5′-nucleotidase; NTPDase, ectonucleoside triphosphate diphosphohydrolase; PDE, phosphodiesterase

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https://doi.org/10.1016/j.imlet.2018.11.007
Received 25 October 2018; Accepted 9 November 2018
Available online 15 November 2018

MM grows in a niche found in the BM, where mPCs are secluded in a physically constrained three-dimensional site containing different cells [(where osteoblasts (OBs), osteoclasts (OCs), stromal cells (SCs), and immune cells are dominant)] in all stages from asymptomatic MGUS and SMM to overt MM disease [9,10]. Cells are surrounded by plasma fluids, which act as a liquid communicator among the different cell components. Patients with MM undergo aspiration of the plasma and cells inside the BM for diagnostic and therapeutic purposes. This makes it possible to closely track the events occurring within the BM niche and assess their resulting products [11,12].

The cellular components are used and assembled differently by mPCs to elude immune surveillance and to ensure their survival and progressive expansion [13]. To do so, malignant cells overcome the reduced blood supply by exploring supplementary sources of energy. Metabolic reprogramming is the most common strategy for survival: this is obtained by hijacking and adapting molecules and mechanisms of normal cells for a private use. MM is an archetypical disease where tumor site sees a reversion of the local anti-tumor immunity derived by mPCs along with immune and non-immune cells inside the BM. The final consequence is the generation of a favorable niche simultaneously providing tumor cell growth and immune escape [14,15,12]. The suppressive mechanistic framework relies on immune cells [e.g., regulatory T (Treg) cells, myeloid-derived suppressor cells (MDSC), natural killer (NK) cells and dendritic cells (DC)], bone cells (OCs, OBs, BMSCs) along with soluble factors: the immunosuppressant nucleoside adenosine (ADO) is one of these in the BM niche [16–18]. Furthermore, the cellular component of the BM niche induces the overexpression of the hypoxia inducible factor (HIF)-1α, a transcriptional factor involved in malignant cell adaptation to hypoxic stress [19,20]. This complex process is being untangled by dissecting cellular and molecular elements of TME, making it possible to identify the mechanistic framework in which the metabolic pathways contribute to mPC survival. What is sufficiently clear, at the moment, is that mPCs exploit local metabolic reprogramming to survive (Fig. 1).

MM demands energy for rapid growth [21]. As a consequence, mPCs seeking to obtain all of the necessary nutrients from the TME become addicted to alternative metabolic pathways, including glucose metabolism for tumor cell survival [22–24]. Normal cells are characterized by a basal rate of glucose conversion to pyruvate, generating 36 ATPs by mitochondrial oxidative phosphorylation (Oxphos). Conversely, mPCs sacrify efficiency for speed, consuming glucose at a higher rate than normal cells and secreting most of the glucose-derived carbon as lactate rather than oxidizing it to generate ATP, a phenomenon known as the “Warburg Effect” [25–28]. This metabolic shift is paralleled by generation of hypoxic conditions, and leads to a decrease in ATP production and a concurrent increase in NAD^+ levels to sustain high-rate glycolysis [29]. (Fig. 1, upper frame).

This metabolic reprogramming is characterized by a switch of the energetic cell metabolism into glycolysis, and it occurs even under aerobic conditions [30]. This switch, regulated by genetic modulation of HIF-1α, leads to a metabolic accumulation of pyruvate that inhibits its enzymatic degradation [31]. The increased HIF-1α levels regulate glycolytic enzymes, followed by increased expression of the glucose transporter (GLUT-1) and thus of glucose consumed [32,33]. In contrast to Oxphos, the glycolytic metabolism of tumor cells engages lactate dehydrogenase A (LDH-A) to convert pyruvate into lactic acid [34]. Consequently, the metabolic reprogramming of mPCs produces (i) only 2 ATPs, but increases cytoplasmic NAD^+, (ii) lactic acid and iii) protons (H^+). The resulting acid accumulation (e.g., lactic acidosis), is neutralized by the overexpression of a monocarboxylate transporter (MCT), which co-transports the anion lactate and H^+ [35]. Malignant cells do respond in this way to hypoxia, with resulting catalysis of the proton-linked transport of lactic acid across the plasma membrane, consequently reducing intracellular acidity [36]. As shown in Fig. 1, lactate and H^+ accumulated overtime in the extracellular space because the defective blood perfusion provokes acidosis in the TME [37–39]. Hydrolysis of ATP is another source of H^+ in the TME.

Metabolic reprogramming creates a very harsh TME, but provides an efficient mechanism for malignant cells to escape the immune response [40,41]. Indeed, it favors tumor cell growth in different ways, such as blocking immunological reaction against malignant cells. Consequently, hypoxia plus lactic acidosis may contribute to MM progression and to the selection of resistant malignant clones able to survive the difficult environment created in the BM niche.

Thus portrayed, the tumor metabolism seems to be an inefficient means of energy production (generation of only 2 ATP and 2 lactate molecules vs. 36 molecules of ATP from each mole of glucose). Such a wasteful form of metabolism constitutes an apparent paradox that warrants some considerations.

First, it allows tumor cells to use the most abundant extracellular nutrient, glucose, to produce ATP, notwithstanding the low yield per glucose molecule metabolized. Secondly, glucose degradation provides tumor cells with metabolic byproducts needed for biosynthetic pathways, including ribose sugars for nucleotides, glycerol and citrate for lipids, and non-essential amino acids for proteins. These precursors are derived from the tricarboxylic acid cycle (TCA), a metabolic pathway that consumes rather than produces ATP. Thirdly, glycolysis increases enzymatic activities, such as that of the LDH-A to regenerate NAD^+ [34]. The consequence of the metabolic shift of the malignant cell is an acidification of the TME, leading immune cells unable to survive such stringent conditions to undergo apoptosis. The lactic acidosis and hypoxia induced by a Warburg phenotype can therefore be considered as a strategy adopted by mPCs to confer growth advantage to tumor cells in a complex BM milieu [42]. Furthermore, lactic acidosis leads to overconsumption of glucose, yielding another advantage to malignant cells. Since excessive glucose consumption leads to glucose starvation and consequently to a transition to a non-glycolytic phenotype, tumor cells can make more economic use of the scarce glucose. In this metabolic state, glucose is consumed much more slowly, so the progressive proliferation of tumor cells is maintained over a significantly longer period of time. In other words, tumor cells with a limited supply of glucose generally suffer a quick death due to exhausted glucose, but lactic acidosis adapt the malignant cells to metabolic environments with a limited supply of glucose. Further, tumor cells located near the blood vessels have a rich supply of oxygen and prefer anion lactate as their main energy source. More recently, it was proposed that glycolytic tumor cells can also symbiotically induce aerobic glycolysis in neighboring stromal and mesenchymal cells. Thus, these tumor-associated cells secrete lactic acid in the TME, which is up-taken by oxygenated tumor cells to use it in the mitochondrial Oxphos. This scenario, called “Reverse Warburg Effect”, envisages the normal stroma, which providing the necessary energy substrate, resulting in a higher biomass, increased proliferative capacity and tumor progression [43,44,29].

3. Immunological outcome of the metabolic reprogramming in the BM niche

Malignant plasma cells are recognized by immune cells infiltrating the tumor, expected to impair MM progression. However, mPCs can evade immune response by rendering the TME an environment hostile for the dedicated cells exploiting a set of biochemical reactions leading to hypoxia in acidic conditions. These events are indirectly supported by the observation that the metabolic reprogramming is paralleled by (i) phenotypic modifications of the cells surrounding the tumor, and (ii) by increased levels of purine nucleotides and nucleosides inside the BM niche.

3.1. Malignant plasma cells and extracellular purines: a complex connection

Purine nucleotides appeared very early in evolution [45,46] as the most ancient molecules with a biological activity [47–49]. Adenosine

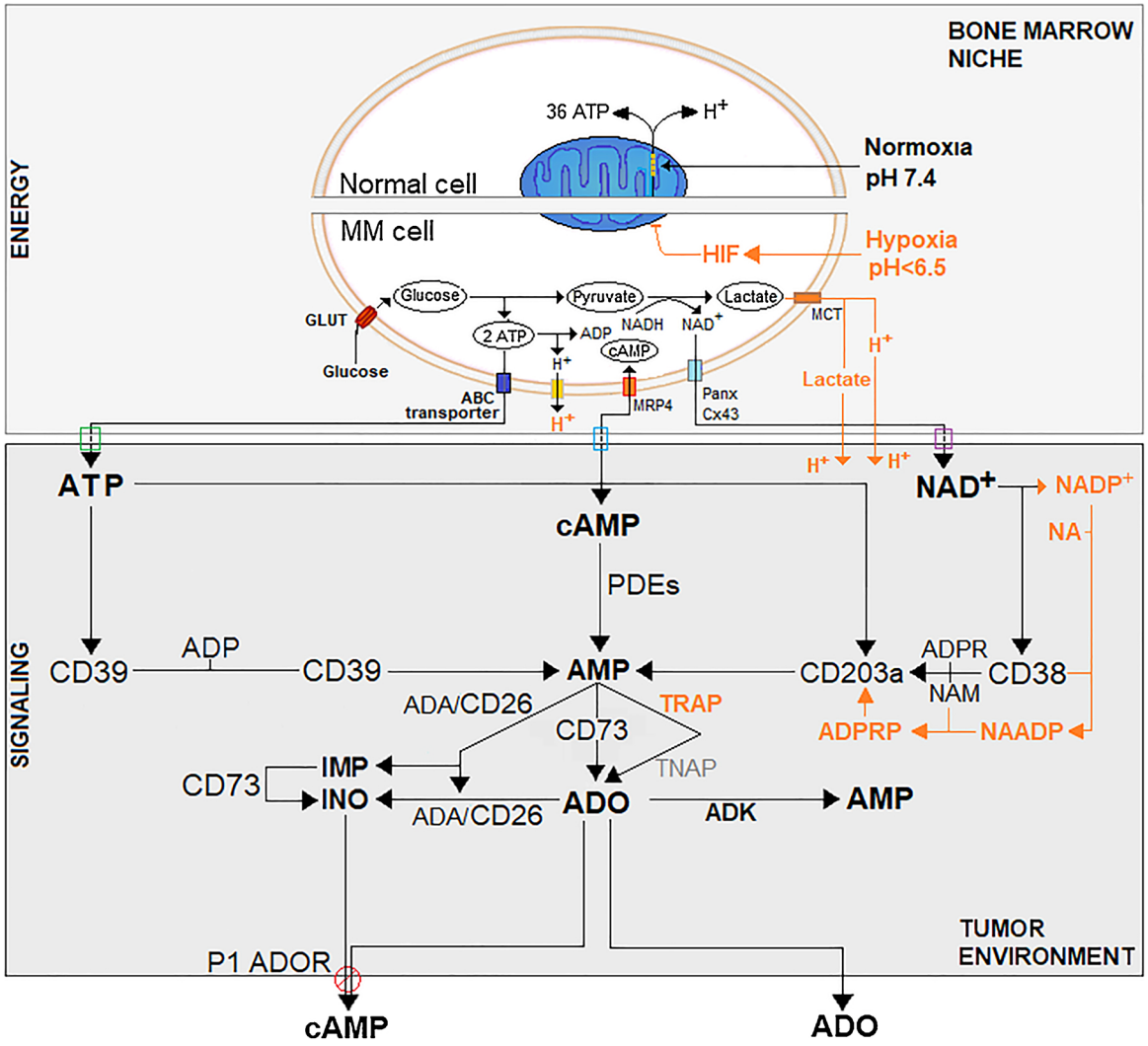

Fig. 1. The link between metabolic reprogramming and adenosinergic pathways in the MM microenvironment.
A) Metabolic reprogramming of malignant plasma cells (upper frame). Normal cells are characterized by a basal rate of glucose conversion to pyruvate, generating 36 ATPs per mole of glucose by mitochondrial oxidative phosphorylation (Oxphos). Tumor cells utilize glycolysis instead of Oxphos: most of the resulting pyruvate is catalyzed by lactate dehydrogenase A (LDH-A) to lactate simultaneously producing protons (H^+), cytoplasmic NAD^+, and 2 ATPs. **B)** Extracellular nucleotides metabolism (lower frame). Nucleotide transporters contribute to the release of intracellular ATP, NAD^+ and cAMP, which are metabolized in the hypoxic acidic TME to ADO. First, cytoplasmic ATP, cAMP and NAD^+ are actively secreted across Pannexin/Connexin channels (among others) or passively after cell lysis, are then metabolized to ADO using classical (CD39/CD73) and/or salvage (CD38/CD203a/CD73) pathways, flanked by an alternative (PDE/CD73) pathway that converts cAMP to ADO. An acidic TME (pH $<$ 6.5) increased cellular egress of cAMP via multidrug resistance-related proteins (MRP) transporter and also contributes to the activation of enzymes catalytically active under such acidic conditions, to create a very efficient mechanism of tumor immune escape generating ADO using NADP/NA as substrate and a tartrate-resistant acid phosphatase (TRAP) as CD73 surrogate. Generated immunosuppressive ADO signals after binding to P_1 purinergic receptors (A2A/A2B) and activates adenylyl cyclase (AC), which catalyzes the formation of the intracellular second messenger cyclic adenosine monophosphate (cAMP). Because most of the nucleoside receptors are expressed by immune or bone cells inside the BM niche, the outcome is either i) a block of the effectiveness of immune cells (Teff, NK, DC) that are capable of destroying tumor cells or ii) an increase in the number of regulatory T cells (Tregs) and myeloid derived stromal cells (MDSC), which suppress immune cells from responding to the tumor (not shown). Eventually, ADO can also be inactivated at the cell surface by an ADA/CD26 complex that converts it into inosine (INO) or internalized by nucleoside transporters.

(ADO), a purine nucleoside composed of adenine and D-ribose, is a building block of the genetic code and also a core molecule of the mononucleotide adenosine triphosphate (ATP) and the dinucleotide of adenine (NAD^+). Both share structural and functional characteristics as universal energy sources of the biological reactions [50]. More recently, purines have been attributed a second life in the extracellular environment as intercellular communicators and signal transducers [51,12].

Evolutionary pressure selected ATP and Ca^{2+} as a molecular tandem controlling intracellular energy for life and extracellular signals *via* purinergic P_2 receptors [52]. The processes of generating energy and signals were later acquired by the glycolytic H^+ acceptor NAD^+, a cellular metabolite involved in energy production, reductive biosynthesis, and calcium homeostasis [53,49]. NAD^+ successively evolved as a substrate for developmentally conserved ectoenzymes (e.g., NAD^+-glycohydrolase/CD38) [54]. An essential constant supply of NAD^+, a molecule insoluble in the plasma cell membrane due to its polar chemical nature, is provided by four different precursors: (i) tryptophan as the *de novo* pathway, and (ii) nicotinamide (NAM), (iii) nicotinic acid (NA), and (iv) nicotinamide riboside (NR) as elements of salvage pathways [55,56].

ATP and NAD^+ have distinctive intra- and extra-cellular properties which represent dual-face Janus activities. Intracellular concentrations are very high, spanning from 1 to 10 mM for ATP and 0.3–3 mM for

NAD^+ [57,52]. The presence of plasma-membrane transporters [(i.e., ATP-binding cassette (ABC), connexin hemichannels and pannexin channels)] allow the release of both nucleotides into the extracellular environment. Here the physiological concentrations (0.4-0.7 μM) generate a homeostatic gradient [58–60]. The concentration of ATP and NAD^+ in the extracellular space (the purinergic agonists) are maintained by the enzymatic activities of membrane-bound ectonucleotidases. The active sites of these enzymes are oriented toward the extracellular space: their catalytic activities make it possible a rapid disassembling of these nucleotides to ADO, ending with ATP and NAD^+ extracellular signaling mediated by specific P_2 purinergic receptors. A second effect is the reutilization of nucleosides scavenged to be reused in the intracellular nucleotide pool [61,62]. Both are *sine-qua-non* conditions to maintain the proper qualitative and quantitative equilibrium of the nucleotide pool, with no significant influence immunologic functions.

The homeostatic gradient may be perturbed under several conditions, including hypoxia and a acidification characteristic of the TME [63]. In this context, the presence of nucleotides at a higher concentration is lead to activation of immune molecules and as well as cells [64–66]. The consequence is a shift in the balance between these metabolites acting in inflammation (high ATP-NAD^+ concentration) or immunosuppression (high ADO concentration). This opposite connection induces nucleotides to boost the immune system by creating a pro-inflammatory environment, and nucleosides to blunt this response. In physiological conditions this prevents tissue damage while in pathology wards off immunosuppression. The conclusion is that mPCs trigger a complex connection with non-immune silent purine molecules in the extracellular space.

In pathological conditions, such as MM, tumor lactic acidosis is associated with the inefficient production of ATP, but with an increase of NAD^+ in the intracellular space [56]. Cell plasma membranes are impermeable to NAD^+ and ATP, but they can be released from the intracellular space by lytic and nonlytic mechanisms [67,68]. Among them, the multidrug resistance-associated proteins (MRPs) family, also known as the ABC transporter subfamily C induce the excretion of intracellular cyclic AMP (cAMP) [69,70]. The latter operates with ATP and NAD^+ by influencing extracellular nucleotide signaling (Fig. 1, lower frame). Consequently, the extracellular milieu surrounding mPCs contains elevated concentration of ATP, NAD^+, cAMP reaching a 100-μM range [71,72], as result of a balance between the process of nucleotide release and of its enzymatic degradation. Indeed, extracellular purine concentrations are modulated by ectonucleotidases controlling extracellular levels of signal transmitters by means of multiple specific receptors (Fig. 1, lower frame).

Another relevant link in a closed environment is the molecular characteristic of both short-lived ATP and the more-resistant NAD^+. The freed ATP is likely unable to reach sites distant from the tumor niche, a notion which is compatible with its shorter half-life in biological fluids and local pericellular action [73,74]. However, NAD^+ could also diffuse and reach cells outside the tumor niche [56]. The duration of extracellular signaling via NAD^+ is controlled under physiological conditions by CD38 NAD^+-glycohydrolase/ADP-ribosyl cyclase that hydrolyze NAD^+ to ADP-ribose (ADPR) and nicotinamide (NAM). CD203a, a second element of control is represented by a phosphatase/phosphodiesterase which hydrolyzes NAD^+ to nicotinamide mononucleotide (NMN) and ADPR to AMP. Concentration of ATP and the duration of signaling via ATP in the extracellular compartment follow a similar approach. Indeed, they are controlled by the CD39 ecto-nucleotidase and CD203a phosphodiesterase, which hydrolyzes ATP to AMP via ADP or directly to AMP, respectively [75–78] (Fig. 1).

3.2. Adenosinergic network: mechanical frame in the BM niche

A speculation is that ADO in primitive organisms covers the two roles of purine nucleotides (e.g., energy and signals) to adapt the metabolic demand of cells in function of the available sources of energy as well as of new functions [79]. Indeed, ADO was originally conserved as a molecule with central roles in biochemical pathways exerting intracellular functions (e.g., transmethylation process common to both post-translational protein modification and gene regulation) or extracellular ones (e.g., purinergic activation) [80]. Active metabolization in biological fluids in the context of open (blood stream) and closed (tumor niche) systems, leads as a consequence that ADO developed as a more sophisticated regulatory system. *in vivo* this made it possible a fine-tuning of key functions in normal and pathological physiology [81].

Indeed, pathways generating ADO are endowed with the ability to modulate the immune response, shaping the course and severity of disease progression. The objective of this review is to demonstrate how this complex network of molecules, (continuously or discontinuously associated in the extracellular space), are a finely-tuned machinery, which acquire further relevance in the MM context [14,82].

The adenosinergic ectonucleotidases operating in MM rely on a chain of discontinuously associated ectoenzymes, as demonstrated by Horenstein et al. [17]. Indeed, a peculiar feature of the adenosinergic axis inside the BM niche is that it can operate with two modalities. The individual components of the chain are located on different cells and their interactions are facilitated by a spatially restricted system (or niches). The conclusions are that ADO on one side is able to influence the local immune response of the area and that such action is facilitated by the physical constriction of the niche, but it is apparently unable to sustain long-lasting effects, due to its short *in vivo* half-life.

Cells producing ADO in MM and the conditions and synergies that lead to the production of the nucleoside were recently reported [11,17]. Preliminary answers were provided by co-culturing twin combinations of mPCs with bone marrow SCs, OBs, and OCs, respectively. The results indicated that ADO production appears to be the consequence of interactions taking place between mPCs, on the one hand, and SCs and OCs on the other.

The metabolic reprogramming occurring in the TME leads to the extracellular accumulation of diverse nucleotides released by cells and the subsequent production and accumulation of ADO. Whether nucleotide/nucleoside mass ratio in the TME proves beneficial or detrimental for the host depends on (i) the expression and catalytic activity of the adenosinergic ectonucleotidases, and (ii) the panel of purinergic receptors expressed by mPCs, bone cells and infiltrating immune cells.

4. Expression of adenosinergic ectonucleotidases and specific receptors of their products

The purinergic machinery in the TME is controlled by several specific ectonucleotidases associated with the plasma membrane, including: i) nucleoside triphosphate diphosphohydrolases (NTPDases), ii) nucleotide pyrophosphatases/phosphodiesterases (NPPs), iii) alkaline and acid phosphatases [tissue non-specific alkaline phosphatase (TNAP) and tartrate-resistant acid phosphatase (TRAP)], iv) phosphodiesterases (PDEs) and v) 5′-nucleotidase (5′-NT). These ectonucleotidases operate in the TME according to a discontinuous spatial arrangement and rapidly degrade nucleotides and nucleosides, adding another level of complexity to purinergic signaling. In fact, the by-products of the metabolization of nucleotides (ATP, NAD^+, cAMP) are chemical transmitters on their own. In detail, metabolization of nucleotides released in the hypoxic niche generate an accumulation of extracellular ADO, which in normal tissues scores 10–100 nM range. ADO is an immunosuppressant molecule that contributes to the MM-supportive microenvironment thanks to the presence of: P_2 for nucleotides and P_1 for ADO [83]. These purinergic receptors are co-expressed by mPCs as well as by immune and non-immune cells [13,78,82], and act as keepers of the nucleotide/ADO mass balance in the BM niche.

4.1. Adenosine-generating pathways

The BM niche of mPCs contains high levels of extracellular nucleotides, such as ATP and NAD^+, which are metabolized to ADO by the action of ectoenzymes [56]. ADO is a nucleoside produced under metabolic stress (e.g., hypoxia), which modulates inflammation and immune responses [13]. The same molecule in melanoma models suppresses T cell proliferation and their ability to kill cancer cells [84]. Nucleotides and their degradation products are also believed to modulate communication between mPCs and normal cells, contributing to the immunocompromised state of MM patients [17,12].

4.1.1. ATP as the classical substrate for adenosine production

Intracellular ATP is actively released (about 5–500 μM) *via* transporters and vesicle exocytosis or passively leaked out from damaged cells into the pericellular space. It is first sensed by P_2 purinergic receptors on the same cell or on the surrounding cells [85], opening up the possibility that the nucleotide acts as a warning signal, playing a key role in immune cell activation as alarmins [86,87]. Thus, pro-inflammatory extracellular ATP regulates immune responses [88]. ATP can also be metabolized to ADO in the extracellular space using different redundant pathways. According to the metabolic reprogramming of TME and calibration of the ATP/ADO mass ratio, by the catalytic activities of ectonucleotidases mediating the step-wise phosphohydrolysis of the nucleotide [89], ATP is first hydrolyzed to adenosine diphosphate (ADP) and then to adenosine monophosphate (AMP) by nucleoside triphosphate diphosphohydrolase (NTPDase-1/CD39) or directly by the low-affinity nucleotide pyrophosphatase/phosphodiesterase (NPP/CD203a). The final phosphate group from AMP is cleaved by the 5′-nucleotidase (5′-NT/CD73), thereby generating ADO [90]. While the conversion of ATP to AMP can be reverted by an extracellular kinase activity, the conversion of AMP to ADO is irreversible and can only be offset upon transport of ADO into cells. This condition positions CD73 at a crucial control point in the metabolization of extracellular ATP (Fig. 1).

ATP is the primary substrate for ectonucleotidases to generate immunosuppressive ADO, thus implicating the classic CD39/CD73 tandem in the inception of an anergic TME [91]. However, such canonical pathway has some points of weakness. For instance, NTPDase-1/CD39 ectonucleotidase is responsible for the inefficient effector T cell responses in patients with chronic HIV-1 infection. However, blocking the enzymatic function of CD39 does not recover T-cell effector functions to overcome the immunosuppression associated with retroviral infections. Similar findings come from cancer patients, where inhibition of the CD39 enzymatic functions on surface tumor cells only partially relieves the suppressive effects of Treg on effector T cells [92,93]. On the other hand, recent evidence shows that CD39 has functions highly reminiscent of those attributed to Th17 cells, γδ T cells, and Breg cells. The neoteric functions are added to the notion that, CD39 is an isoprenoid diphosphate phosphohydrolase which abrogates γδ T cell activation, a first line of defense against tumors [94,95]. The old and new characteristics of CD39 raise intriguing questions, such as i) which was the first substrate of CD39 and ii) secondly, considering that CD39 may not be the exclusive switch of the immune system to trigger ADO-mediated immunosuppression, which alternative(s) are operative for an ADO-generating axis?

The extracellular ATP breakdown in aerobic conditions follows the classical adenosinergic pathway (ATP/ADP/AMP/ADO). ADO arises from dephosphorylation of AMP, travels in the blood at a concentration of about 0.5 μM [58]. However, due to its metabolic instability and limited half-life, ADO is generally converted to inosine (INO) by adenosine deaminase (ADA), which leads to the generation of the stable end product uric acid by xanthine oxidase [96]. However, the K_m of ADA for ADO is still high enough (25–150 μM) to allow for ADO metabolization [97,88]. The conclusion is that the nucleoside cannot accumulate in normal cells at very high concentrations without being deaminated by ADA into INO.

Fig. 1 schematize what happens in pathological conditions. ATP degradation mainly produces INO, since high ATP concentration in the TME lead AMP to be deaminated into inosine monophosphate (IMP), in turn dephosphorylated into INO. Indeed, ADA and 5′-NT/CD73 are both allosterically activated by ATP [98,99]. The IMP pathway (ATP/ADP/AMP/IMP/INO), originally described as prevalent in the cytosolic cell compartment [100], was recently reported as apparent in the environment of tumor cells [e.g., multiple myeloma (MM) and neuroblastoma (NB), as well as in cell lines (BF01 myeloma and LAN-1 and SHSY-5Y neuroblastoma cell lines)]. These findings were inferred by using BM plasma from MM and NB patients and cell supernatants, respectively [101].

Notwithstanding these observations, the enzymatic kinetics of the CD39 molecule also raise some concerns about the possible functionality of the classical adenosinergic system *in vivo*. Indeed, the optimal pH for the CD39 enzyme is in the alkaline range of 8–8.3 [102]. They might preclude its enzymatic activity in a hypoxic TME, where its acidic pH is secondary to the production of lactic acid and generation of protons. Further, the conversion of extracellular ATP to ADO as catalyzed by CD39 is kinetically complex, with the upstream ADP metabolite acting as a feed-forward inhibitor of the 5′-NT/CD73. The consequence is that AMP tends to accumulate [103]. This ADP-dependent feed-forward inhibition does not appear to significantly modulate purinergic signaling, as human cell surfaces are normally exposed to low ATP (< 1 μM) [104]. However, higher ATP levels of the TME might induce NPP/CD203a to blunt the signals mediated by P_2 receptors through an ATP conversion step that bypasses the formation of ADP. The even lower affinity displayed by ATP for CD203a as referred to CD39 indirectly supports another view. Described ectoenzymatic CD38/CD203a tandem using NAD^+ as an alternative substrate for ADO production may become a relevant producer of AMP when high levels of ATP are present in the BM niche [56]. In other words, normal and pathological observations indicate that high ATP metabolization in the TME tends to compensate for a lack of ADO by activating alternative adenosinergic pathways, independently from CD39.

4.1.2. Emerging substrates for adenosine production in the BM microenvironment

4.1.2.1. NAD^+ as the alternative salvage substrate. While the expression of CD38 by human mPCs is known since long [105,106], its role in the production of ADO is however less studied. Our Laboratory confirmed that the classical CD39/CD73 pathway for converting ATP is flanked by another set of surface molecules leading to the same result, but using NAD^+ as a starting substrate [107]. This salvage pathway occurs independently of ATP and bypass CD39. Components of the second pathway are the NAD^+-glycohydrolase/CD38, the ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1)/CD203a and the 5′-ectonucleotidase (5′-NT)/CD73. As shown in Fig. 1, CD38 generates nicotinamide (NAM) and adenosine diphosphate ribose (ADPR) that is further hydrolyzed by CD203a to produce AMP. The conversion from AMP to ADO is regulated by CD73. The key player of the network and responsible for the conversion of extracellular NAD^+, is CD38, as inferred by i) its topological features (i.e., accessibility to exogenous NAD^+) [108]; ii) the enzymatic functions as a primary regulator of extracellular NAD^+ levels [109,110], and iii) its high expression levels in mPCs [111–113]. The metabolic halo formed following intracellular NAD^+ efflux influences the action of CD38 located on the mPCs surface and adjacent non-tumor cells catalyzing the conversion of NAD^+ to cyclic ADP-ribose (cADPR) *via* cyclase activity and cADPR to ADPR *via* hydrolase activity [109,76]. The stoichiometry of the reaction catalyzed by CD38 involves a massive amount of NAD^+ (~ 100 molecules) to yield a single cADPR [114].

In the acidic conditions of the TME after metabolic reprogramming, CD38 may additionally convert NAD^+ phosphate (NADP) to nicotinic acid adenine dinucleotide phosphate (NAADP) [56]. The consequence

is that of extracellular NAD^+ (or NADP) are depleted and cADPR, ADPR, and NAADP mediators are produced. These promote intracellular Ca^{2+} increase, forming intracellular NAM for NAD^+ resynthesis. NAD^+ hydrolysis by CD38 is also capable to generate nicotinamide mononucleotide (NMN) for intracellular NAD^+ supply [115].

The ability to use NAD^+ as a substrate led to hypothesize that CD38 acts as a metabolic sensor, which limits the duration of NAD^+-signaling in the extracellular compartment. The activity of CD38 for a given extracellular NAD^+ level is defined by the Michaelis constant (K_m) for the reaction. This constant describes the NAD^+ concentration, when the reaction rate is half of the maximum during NAD^+ excess. CD38 displays a K_m for NAD^+ in the low micromolar range (1–5 μM) [75]. Under normal homeostatic conditions, CD38 is expressed at low levels, whereas extracellular NAD^+ is in limited amount (~ 0.1 μM): therefore, small quantities of metabolites derived are produced after NAD^+ disassembling by CD38. However, tumoral growth increases the concentrations of extracellular NAD^+, which may reach concentrations of 5–10 μM. This outreach the K_m of CD38. In conclusion, CD38 protein expression, (hence, its ectoenzymatic activity) is up-regulated in the presence of increased extracellular NAD^+, either generating Ca^{2+} second messengers (cADPR, ADPR) or transformating it in ADO, when the CD203a/CD73 enzymatic tandem is expressed.

The pyrophosphatase/phosphodiesterase CD203a is capable of hydrolyzing ADPR to produce AMP. The latter byproduct converges at the cross-roads with other known adenosinergic pathways and is then metabolized to ADO by 5′-NT/CD73. (Fig. 1).

The functions of the CD38/CD203a/CD73 pathway were revealed in T cell leukemia [107] and melanoma [84], as well as in natural- and cytokine induced-killer cells [116,117]. The functions of this salvage pathway were also confirmed in the MM niche [11,17,113].

Hypoxia in the MM niche synergizes with selected cytokines to modulate the adenosinergic pathways, giving advantage to the salvage NAD^+ dependent pathway of ADO production [118,12]. The above conditions induce the CD203a and CD73 ectonucleotidases to be expressed or up-modulated by cells involved in NAD^+ degradation. Acidification of the TME [119,41] is another conditional parameter of the MM niche: for this reason, our group analyzed a set of conditions able of controlling the *in situ* production of ADO. The generation of extracellular ADO from nucleotides occurs through the sequential enzymatic activities of the membrane-bound ectonucleotidases. The enzymatic activity of CD38 is highly dependent on pH: it is reasonable to assume that the *in vivo* activity of the enzyme may change according to the environment (Fig. 1. Lower frame). Indeed, ADPR are formed by CD38 under physiological conditions at a neutral pH. The phosphorylated form of NAD^+ (NADP) is originated from NAD^+ by the catalytic activity of nucleoside disphosphate kinase (NADK) and transported through the connexin 43 hemi-channel [67] to the extracellular compartment. CD38 is the only molecule that catalyzes a base-exchange reaction of the nicotinamide group of the substrate NADP with nicotinic acid (NA), to produce the Ca^{2+}-mobilizing second messenger NAADP [120,121]. The shift from catalysis to the exchange reaction requires an acidic pH, because of the electrostatic repulsion between the negatively charged NA and acidic residues of CD38 [122]. Under the same conditions, CD38 converts NAADP to ADP-ribose 2′-phosphate (ADPRP), a product of cyclic ADPR 2′-phosphate hydrolysis. Finally, ADPRP can be dephosphorylated by CD203a into AMP [56].

Hypoxia and pH may shift the balance from one pathway to the other. For instance, the expression of CD73, which dephosphorylates AMP to ADO, is up-regulated by HIF-1α [123]. However, the fall in pH which occurs during aerobic glycolysis is followed by a marked inhibition of 5′-NT/CD73 [124,91]. An effect is the reduction of ADO generation. It thus seems reasonable to speculate that a Tartrate-Resistant Acid Phosphatase (TRAP) - a pH dependent nucleotidase active in acid environment - may efficiently cooperate in ADO production [125]. Indeed, it was observed that ADO production under acidic conditions, i.e., such as in a bone resorptive environment typical of the MM niche [126], increases when mPCs are cultured in the presence of OCs and after the addition of the substrate NADP at pH 5.5 in the presence of high concentrations of NA. The last step of the reaction showed the production of NAADP, ADPRP, AMP, and ADO [121,56,122].

Primary mPCs isolated from BM aspirates do not produce detectable levels of ADO [11]. This may be because of the low to nil levels of CD73, the nucleotidase responsible for the final production of ADO from AMP. The experimental plan to identify which cells producing ADO and the conditions and synergies leading to the production of the nucleoside, relied on co-cultures of twin combinations of myeloma cells with BMSC, OCs, and OBs, respectively. The results obtained after treating with different adenosinergic substrates (ATP, NAD^+, ADPR and AMP) indicate that ADO production appears mainly to be the consequence of interactions taking place between mPCs, on the one hand, and BMSCs and OCs (PBMCs isolated from BM samples of MM) on the other. A further consequence is that ADO increases due of cell-to-cell contacts supporting the growth of malignant cells in the niche [11].

mPCs and bone cells are indeed equipped with the ectoenzymatic machinery that produces ADO in the BM niche [17]. Specifically, mPC cell lines established from patients with MM express CD38 and its non-substrate ligand CD31 [127,128], whereas CD203a, CD39 and CD73 expression is undetectable [13]. The expression of all these ectonucleotidases by BMSCs during OB differentiation shows CD38 as decreasing during CD203a increases. CD39 appears as undetectable in differentiated and non-differentiated cells. Surface CD73 remains stable during OB differentiation [11].

The analysis and distribution of ectonucleotidases in the MM niche was recently observed in bone biopsies, primary mPCs from BM aspirate and osteogenic cells [17,113]. In line with the above report mPCs are characterized by high levels of CD38 while CD39 and CD73 levels displays inter-patients differences. Lastly, CD203a, (also known as Plasma Cell-1, PC-1), is expressed at low levels by primary mPCs. The expression profile of BMSCs and OBs reveals that both cell types are $CD38^{-ve}/CD39^{-ve,}$ while expressing CD73 and CD203a [113]. This data indicates that the components of the adenosinergic salvage pathway (CD38, CD203a and CD73) and those of the classical pathway (CD39 and CD73), are expressed by cells of the BM niche even if at various levels. This means that not all the molecules has to be expressed by an individual cell, provided that they are operating in a closed environment (such as mPCs and non-immune cells in the BM niche). It is not defined whether both the salvage CD38/CD203a/CD73 and the classical CD39/CD73 pathways may harmonize or the relative expression of CD38, CD203a and CD39 ectonucleotidases determines which pathway is more active in the TME. However, the metabolic reprogramming in the BM niche leads to an acidic environment: for this reason, it is reasonable to assume that the CD38-dependent salvage pathway assumes a compensatory role for CD39 activity in the acidic locus.

4.1.2.2. The cyclic nucleotide cAMP signaling pathway as an adenosine precursor. An enzymatic pathway previously unexplored was recently proposed as an alternative route to producing extracellular ADO. This axis hinges around the cyclic AMP (cAMP) nucleotide-metabolizing membrane-targeted ectoenzyme phosphodiesterase (PDE) and the 5′-NT/CD73 [129,130]. Indeed, extracellular cAMP is sequentially dephosphorylated to AMP and then to ADO by an ecto-PDE and 5′-NT/CD73, respectively [131–133]. This pathway may flank or synergize the known ATP/NAD^+-catabolic pathways. The substrate cAMP, one of the oldest signaling molecules known, is produced from ATP by adenylyl cyclases (AC) [129]. Most AC are membrane-bound and are activated by heterotrimeric G protein-coupled receptors, such as ADO receptors [134,135].

Intracellular cAMP nucleotide levels are regulated by its efflux to the extracellular compartment by means of MRP-transporters [136]. Furthermore, cAMP efflux is attributed to play a key role in the

differentiation and proliferation of human myeloid leukaemia cells [70]. Further, an acidic TME improves the egression of cAMP *via* MRP4 [137,138]. The transport of cAMP into the extracellular milieu in mammalian cells is believed to regulate intracellular levels of cAMP. However, it seems uneconomical for the cells to reduce intracellular cAMP levels by shuttling cAMP into the TME when a cytoplasmic synthesis is already operative. Alternatively, cAMP efflux might regulate extracellular ADO levels and thus optimize the autocrine and paracrine immunosuppressive effects of the nucleoside. Simplifying, an ADO stimulus mediated by PDE/CD73 pathway may be further processed to modulate cAMP contents in the target cell. This would increase the quantitative efficiency of cAMP efflux in a positive feed-back loop [139] (Fig. 1). The ability of NPP1/CD203a to hydrolyze cAMP was reported [140], adding further a bit of complexity to this adenosinergic pathway. However, the advantage is reported by the notion that cAMP is stable in biological fluids, thus potentially acting at distant sites. In fact, in the TME, ADO is rapidly up-taken by red blood cells, limiting its half-life to < 10 s. The existence of cAMP transporters, PDE, CD73 and AC yields a functional axis (Fig. 1). To support this is the observation that NB lines (e.g., LAN-1 and SHSY-5Y) are now included in the list of cells that can use extracellular cAMP/ADO pathway to generate ADO [101]. This happens by flanking the use of ATP and NAD^+ by neuroblastoma and immune cells in a tumor niche [141].

5. Adenosine in the BM niche and its regulation

ADO is mainly generated by the activity of ectonucleotidases operating on surface cell membrane as functional tandems (CD39/CD73 and CD203a/CD73) or triads (CD38/CD203a/CD73). ADO accumulation is further sustained through the reduction of internalization by a HIF-dependent inhibition of the concentration based transporters (CNTs) and equilibrium-dependent transporters (ENTs) [142]. ADO levels in the TME is also enzymatically balanced by i) adenosine deaminase (ADA) [which converts ADO into inosine (INO)] ii) purine nucleoside phosphorylase (PNP) [which converts INO into hypoxanthine (not shown)] and iii) ADO kinase [which forms AMP from ADO] (Fig. 1). Soluble ADA becomes an ectoenzyme on the surface of different cells with the serine protease dipeptidase IV (CD26/DIPPIV) providing an anchorage. ADA is multifunctional, by performing as a catalyst [e.g., degrades ADO at the CIK cell surface preventing autocrine immunosuppression [117] and as a costimulatory molecule [e.g., preventing proliferation of effector T cells by interacting with CD26] [143,144].

ADO concentrations found in the MM BM niche (25–100 μM) [17] are > 1000 times superior to those of normal tissues (10–100 nM). ADO accumulated extracellularly may mediate its regulatory functions by binding to P_1-G protein-coupled purinergic (A1, A2A, A2B and A3) ADO receptors (ADORs) expressed by different cells, including immune effectors [83,145]. A1 and A2A ADOR (K_m for ADO, $\sim 10^{-8}$ to 10^{-7} M) exhibit relative affinities for ADO higher than A2B and A3 ADOR (K_m for ADO, $\sim 10^{-6}$ to 10^{-5} M). ADOR subtypes are coupled to different combinations of G-protein family members, namely A1 ADOR to Gi/Go, A2A ADOR to Gs/Go, A2B ADOR to Gs/Gq, and A3 ADOR to Gi/Gq. Once these receptors are engaged ADO activates (or inactivates) AC and modulates cAMP levels. A2A and A2B ADORs stimulate AC and increase cAMP levels, while A1 and A3 ADORs inhibit AC and down-regulate cAMP [145,146]. Signaling *via* cAMP is typically associated with significant immunosuppression [147], while inhibition of cAMP generation after P_1 engagement is generally viewed as an immuno-stimulatory mechanism. All ADO receptors are coupled to mitogen-activated protein kinase (MAPK) pathways.

The activity of extracellular ADO in the BM niche is mediated upon binding to the A2A ADOR on immune cells and on mPCs as well as the low-affinity A2B ADOR, when kinetic conditions permit. This means when MM tumor accumulates ADO in concentrations high enough (i.e., > K_m value) to stimulate even low-affinity A2B ADORs and dampen spontaneous anti-tumor immune responses.

Pericellularly produced ADO is partially transformed *in vivo* by tumor cells expressing CD26. A consequence is a lock of the paracrine immunosuppressive effects of the nucleoside, at the same time preventing the autocrine effects of ADO [148]. Pertinent to this is that A2A ADOR activation is self-inhibited by the action of A1 ADOR [117]. Reasonable to postulate that ADO binding to A1 expressed by mPCs induces autocrine inhibition of the nucleoside immunosuppressive effects. In this case, a high expression of the ADA/CD26 complex would be a *sine-qua-non* condition for self-safeguard with ADA providing a fine-tuning ADO concentrations which protect tumor cells from ADO-mediated inhibition of proliferation in the immunosuppressing TME [117].

In addition to ADO production, its degradation to INO further contributes to suppression of functions mediated by immune cells (e.g. Treg). Indeed, Treg cells do not express CD26 [149]. The absence of ADA anchorage in Treg suggests that pericellular ADO levels driving autocrine ADO signaling are high in these cells, potentially contributing to upregulation of the suppressive activity mediated by Treg. Conversely, newer evidence suggests that INO, a product of hydrolytic ADO deamination, binds A2A ADOR on T lymphocytes and functions as a potent agonist of this receptor. The outcome is the production of cAMP and functional immunosuppression [150]. Unlike ADO, characterized by a short half-life, INO is a stable metabolite. Its prolonged occupation of the A2A ADOR results in strong and sustained signaling and cAMP accumulation. Downstream INO signaling is linked to ERK1/2-, while ADO produces cAMP-based effects. Thus, INO emerges as a functional A2A ADOR agonist, that can mediate immunosuppressive effects long after ADO is catabolized [151]. The final step of the adenosinergic activity is focused on re-use: indeed, the main products ADO, NAM and phosphate can be taken up by cells and exploited for the regeneration of nucleotidic substrates, closing the adenosinergic loops.

6. Conclusion

BM niche is a regulatory microenvironment mainly composed of osteoblasts, osteoclasts, bone marrow endothelial cells, stromal cells, and extracellular matrix proteins. These elements not only play a role in the survival, growth and differentiation of blood cells, but also provide optimal growth environment for multiple hematological malignancies including MM. The BM niche aids the growth and spreading of mPCs by a complex interplay of ectoenzymes, cytokines, chemokines, and adhesion molecules. Moreover, the MM BM microenvironment was shown to be metabolic reprogrammed confering survival and chemoresistance of MM cells to current therapies. Many details of these processes are unknown. Therefore, there is a strong need to further dissect the MM BM niche and understand the process of how the complex interactions with BM milieu influence MM growth and survival. Therefore, in the present review, we have focused principally on i) the basic metabolic features and functions of the BM niche and have highlighted ii) its interaction with MM cells to start up an adenosinergic mechanistic framework leading to accumulate extracellular ADO, a biomolecule with pleiotropic effects on the host and the tumor. The process analyzed for bone marrow-resident cells in patients with MM, employed a purinergic complex consisting of nucleotide cell channels and transporters, nucleotide catabolizing ectoenzymes, a molecular network of nucleotide and nucleoside receptor proteins, originally formulated as purinome [152]. Functional insights lead to extracellular ADO (and possibly INO), which are considered as the first partner of this biomolecular interactive ectoenzymatic network. Here we also briefly reviewed metabolic pathways generating lactic acidosis, and discussed its function in the MM tumor niche. Conclusions showed i) how novel and recycled metabolic reprogramming provide important knowledge for a deeper understanding of the mechanisms through which the complex network of nucleotidic substrates, ectonucleotidases, signaling byproducts, and purinergic receptors functions in MM, and ii) that metabolism/

immunity tandem are taking center stage in our understanding of pathways that modulate MM disease progression.

Conflict of interest disclosure

F.M.: Research support from Janssen Pharmaceutical, Celgene, Tusk Therapeutics and Centrose, and serves on Advisory Boards for Centrose and Tusk Therapeutics.

Funding

The work done in Turin was supported by the University of Torino by an “Assegno di Ricerca” (ALH) and by grants from the Compagnia SanPaolo (Turin, Italy) (FM) and the “Associazione Italiana per la Ricerca sul Cancro” IG 17273 (VP).

Author contributions

ALH and FM analyzed data present in the literature and wrote the manuscript. CB contributes to unpublished experiments. FMo and VP contributed to the writing of the final version of the manuscript.

Acknowledgments

This paper is dedicated to the memory of Dr. Vito Pistoia, who recently passed away.

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