The Extracellular NADome Modulates Immune Responses

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The term NADome refers to the intricate network of intracellular and extracellular enzymes that regulate the synthesis or degradation of nicotinamide adenine dinucleotide (NAD) and to the receptors that engage it. Traditionally, NAD was linked to intracellular energy production through shuffling electrons between oxidized and reduced forms. However, recent data indicate that NAD, along with its biosynthetic and degrading enzymes, has a life outside of cells, possibly linked to immuno-modulating non-enzymatic activities. Extracellular NAD can engage purinergic receptors triggering an inflammatory response, similar - to a certain extent – to what described for adenosine triphosphate (ATP). Likewise, NAD biosynthetic and degrading enzymes have been amply reported in the extracellular space, where they possess both enzymatic and non-enzymatic functions. Modulation of these enzymes has been described in several acute and chronic conditions, including obesity, cancer, inflammatory bowel diseases and sepsis. In this review, the role of the extracellular NADome will be discussed, focusing on its proposed role in immunomodulation, together with the different strategies for its targeting and their potential therapeutic impact.

Keywords: nucleotides, NAD, signaling, DAMPs, immune cell regulation, immunometabolism, tumor microenvironment

INTRODUCTION: THE MANY FACES OF NAD, FROM ENERGETIC FACTOR TO DANGER SIGNAL

NAD is an essential intracellular metabolite with key roles in energy metabolism and electron transfer (1–7). In addition, NAD is a cofactor for different families of enzymes, including sirtuins and poly-ADP-ribose polymerases (PARPs). NAD can be present outside of cells, with levels fluctuating widely in response to extracellular signals (8–11). A firm observation is that under steady state extracellular (e)NAD levels are thousands of times lower (nM) compared to the intracellular ones (µM-mM) (7, 12–16).

However, during conditions of cellular stress, such as those observed in an inflamed microenvironment, or during hypoxia, or in conditions of shear stress due to physical distortion, plasma membrane damage, stress elicited by cytotoxic agents, NAD concentrations may rapidly spike. This observation, together with the finding that some purinergic receptors are activated by NAD suggested that cNAD serves as a “danger signal” that alerts the immune system to tissue damage (8–10, 12, 17–20). According to this view, eNAD could be considered as damage-associated molecular pattern molecule (DAMP), able to activate the innate immune system, like what has been...
shown for pathogen-associated molecular patterns (PAMPs) (18, 21–24). For example, released eNAD from active neuronal cells can serve as neurotransmitter and neuromodulator (25–27); or in a mouse model of inflammation, induced by injection of polyacrylamide beads, eNAD reached a concentration of 10mM acting as danger signal (28).

NAD release may occur by several mechanisms involving active exocytosis, or diffusion through transmembrane transporters (e.g., pannexin, connexin) in living cells, or passive leakage across the membrane from necrotic or injured cells (15, 29–32).

Homeostasis is rapidly restored through a scavenging circuit operated by nucleotide-catabolizing enzymes that produce the immunosuppressant adenosine (ADO) and inosine, which can re-enter the cell, reconstituting the nucleotide pool (5, 33–36). All these mechanisms of nucleotide/nucleoside release to alert or switch off the immune system, respectively, are enhanced during acute and chronic inflammation, including cancer (29, 37, 38). Even though very unlikely, eNAD synthesis has not been conclusively ruled out, also in consideration of the presence of several key NAD biosynthetic enzymes (NBEs) (16, 39).

### Intracellular and Extracellular NAD-Metabolizing Machinery

The biosynthesis of NAD takes place in different locations in the cell, through one de novo pathway starting from the catabolism of tryptophan, and via degradation of vitamin B3 precursors. The latter is considered a salvage pathway that occurs through the metabolism of three precursors [i.e. nicotinic acid (Na), nicotinamide (Nam) and nicotinamide riboside (NR)]. In the majority of tissues, intracellular NAD is generated mostly from Nam, which is the degradation product of all NAD-consuming signaling reactions (6, 40–42). Under normal conditions >70% of the cellular NAD content is stored and is utilized in the mitochondria primarily for metabolic purposes (16, 43). The cytosolic and nuclear NAD pools serve primarily to sustain activity of PARPs and sirtuins, which are NAD-dependent enzymes with key roles in regulating DNA repair and epigenetic controlling of gene transcription, respectively (Figure 1) (7, 44, 45). NAD levels can therefore restrict the activity of these two classes of NAD-metabolizing enzymes. Intriguingly, NAD can rapidly shuttle between different cellular locations.

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**FIGURE 1** | Schematic representation of the NADome. Schematic representation of the network of substrates/ligands, NAD-metabolizing cell surface and intracellular enzymes and their products in the extracellular and intracellular space. Biological functions regulated by NAD-related enzymes and products are listed. NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; eNAD, extracellular NAD; Nam, nicotinamide; NR, nicotinamide riboside; Na, nicotinic acid; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, nicotinate phosphoribosyltransferase; NRK, nicotinamide riboside kinase; ARTs, mono adenosine diphosphate (ADP)-ribose transferases; PARPs, poly ADP-ribose polymerases; ADPR, ADP ribose; cADPR, cyclic ADP ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; Ca²⁺, calcium; NMN, nicotinamide mononucleotide; ADO, adenosine; AMP, adenosine monophosphate; ENPP1; ectonucleotide pyrophosphatase/phosphodiesterases; TLR4, toll-like receptor 4.
compartments to reconstitute the pool that allows enzyme activation, as has recently been shown (46). When in the extracellular space, eNAD functions are linked to the modulation of cell surface P2X and P2Y purinergic receptor families, thereby acting in an apparently enzyme-independent way and eliciting pro-inflammatory immune responses (Figure 1). In addition, within the extracellular space, a complete network of different ectonucleotidases can rapidly hydrolyze eNAD generating intermediates that modulate signaling, cell metabolism, adhesion, migration and activate immunoregulatory circuits (14, 39, 47), as summarized in Figure 1. eNAD is degraded by different classes of ectoenzymes: the NADases CD38 and CD157 (48-50), the ADP-ribosyltransferases (ARTs) (51), the Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) and the ecto-5’-nucleotidase CD73 (34, 52, 53). NADase, ENPP1 and CD73 can lead to the formation of ADO, a potent natural immunosuppressive factor mediating the activation of the inhibitory P1 purinergic receptors (34, 54, 55). In addition, eNAD can be cleaved to nicotinamide mononucleotide (NMN) and subsequently dephosphorylated to NR by CD38 and CD73 (53, 56, 57). All these intermediates can enter the cell as NAD precursors and can be used by NBs, reconstituting the intracellular pool (57, 58) (Figure 1).

In the next sections of this review, we will summarize the role of eNAD, its derived-metabolites and a set of NAD-dependent enzymes, giving examples of their role in the regulation of specific immune responses.

**eNAD AND PURINERGIC RECEPTORS**

The idea of purinergic signaling, i.e., of nucleotides acting as extracellular signaling molecules, was initially put forward by the seminal work of Geoff Burnstock in 1972 (39, 60).

Since then, this complex network of receptors has progressively been unveiled to reveal seven evolutionarily conserved subtypes of the P2X ion channel receptors and eight subtypes of the P2Y G protein-coupled receptor, all with roles in immune cell activation (5, 24, 61). On the contrary, four subtypes of the ADO P1 receptors on effector T cells have immunosuppressive effects. Shifting the balance from pro-inflammatory P2R signaling to anti-inflammatory P1R signaling or vice versa, the purinergic signaling system fine-tunes immune cell functions (5). eNAD can bind different subtypes of purinergic P2 receptors as summarized in Figure 2.

For example, eNAD activates human granulocytes by binding P2Y11 and triggering: (i) overproduction of cyclic (c)AMP, (ii) activation of protein kinase A, (iii) stimulation of ADP-ribosyl cyclase and overproduction of cyclic ADP-ribose (cADPR), a universal calcium (Ca^{2+}) mobilizer, and (iv) influx of extracellular Ca^{2+}, ultimately causing increased proliferation and migration (62). eNAD can bind P2Y1 and P2Y11 in human monocytes activated with lipopolysaccharide (LPS), triggering a transient rise in intracellular Ca^{2+}, which is caused by a release of Ca^{2+} from IP (3)-responsive intracellular stores and an influx of extracellular Ca^{2+} (63). eNAD has also been identified as an agonist at P2Y1 receptors in human embryonic kidney (HEK) cells and mouse colonic muscle (27, 64). Moreover, binding to postsynaptic P2Y1 receptors, like ATP, eNAD also acts as a neurotransmitter, released by stimulated terminals of mammalian central nervous system and peripheral nervous system neurons (65). In addition, it has been shown that purinoceptors, including P2X1, P2X4, and P2X7, are engaged in eNAD-mediated signaling (27, 63, 66). However, more experimental data should be published to confirm this direct binding of NAD per se.

eNAD may also engage P2X7R receptors, the main eATP receptor, extensively studied in the context of inflammation and immunity (24). P2X7R signaling is a major regulator of the intensity and duration of inflammatory responses (24, 67, 68). The receptor/channel is prominently expressed on all cells of innate and adaptive immunity and aberrant signaling has been linked to diverse inflammatory and autoimmune diseases, as recently reviewed in (5, 24). P2X7R signaling mediates NLR family pyrin domain containing 3 (NLPR3) inflammasome activation, cytokine, and chemokine release [i.e., interleukin (IL)-1β, tumor necrosis factor (TNF), IL-6, monocyte chemotactrant protein-1 (MCP-1/CCL2)], T lymphocyte survival and differentiation, transcription factor activation, and cell death (24, 69, 70). At inflammatory sites, P2X7R could also be bound directly by alternative ligands, including eNAD that accumulates at sites of inflammation and tissue damage (28). In murine T lymphocytes, eNAD serves as an ADP-ribose donor to ADP-ribosylate the P2X7R at arginine 125, close to the ATP-binding pocket (71). This reaction, catalyzed by the plasma membrane enzyme ART2.2, causes long-lasting activation of the inhibitory P1 purinergic receptors (72, 73, 74). eNAD is demonstrated only in mouse models: additional research is needed to determine whether it is relevant for human immune responses too.

**eNAD DEGRADATION-SIGNALING SYSTEM IN REGULATING IMMUNE RESPONSES**

One of the reasons why eNAD levels are generally low is that there are several extracellular enzymes that rapidly transform it, guaranteeing recycling of a high energy molecule through the generation of products that can be easily up-taken by cells. The intermediates, however, have a life of their own as signaling molecules, thereby modulating activity of immune cells.
The best-known NAD-degrading/signaling systems rely on the activity of CD38, an immunomodulatory enzyme (Figure 3).

Human CD38, the main member of the NADase/ADPR cyclase family that includes also CD157/BST-1, is a surface glycoprotein characterized by a relatively large extracellular domain that contains the catalytic site, a single transmembrane pass, and a short cytoplasmic tail (73, 74). CD38 is a multifunctional ectoenzyme, involved in the catabolism and degradation of eNAD (under normal pH) and NAD phosphate (NADP, under acidic pH), producing ADP ribose (ADPR) together with signaling metabolites involved in intracellular Ca\(^{2+}\) mobilization. The main catalytic activity is the NAD glycohydrolase that generates Nam and ADPR. CD38 can also act as NAD cyclase, producing cADPR, which is then hydrolyzed to ADPR. Lastly, in the presence of NADP and Na, under acidic pH levels, CD38 can generate nicotinic acid adenine dinucleotide phosphate (NAADP) (49). The finding of an extracellular enzymatic activity of CD38 leading to the generation of messengers that enter cells to induce intracellular Ca\(^{2+}\) fluxes remains an unsolved “topological paradox” (49, 75). More recent data have enriched the picture by showing that CD38 can also be found in the nucleus and mitochondrial membrane and that a soluble form of CD38 is most likely present in the cytoplasm, leading to the hypothesis of a compartmentalized generation of NAD-derived signaling metabolites (49, 76–78). ADPR, cADPR and NAADP share the ability to mobilize Ca\(^{2+}\) ions from intracellular stores: cADPR binds to ryanodine receptors (RyR) expressed on the endoplasmic reticulum, ADPR binds to membrane melastatin related transient receptor potential cation channels TRPM2 (49, 79) and NAADP binds to receptors expressed by acidic organelles, such as lysosomes, suggesting a role as Ca\(^{2+}\) messenger in the endocytic pathway (80). It is therefore likely that during an immune response, NAD, released outside of cells due to local conditions of inflammation and cellular damage is converted into Ca\(^{2+}\)-active metabolites through the action of CD38 expressed by activated lymphocytes,
which in turn contribute to lymphocyte activation through Ca\(^{2+}\) signaling (80–82).

There is a second alternative possibility that is gaining momentum in the context of tumor immunosuppression. According to this hypothesis, ADPR could also be short-circuited to ADO via the action of CD203, which generates AMP from ADPR and CD73 (53, 83, 84), which cleaves the last phosphate, generating ADO (49). In this way, CD38 could contribute to the generation of a tumor-favorable environment, as recently demonstrated in tumors characterized by a large T cell infiltrate (85). Therefore, it seems that according to the environment, CD38 can generate both immune-boosting and immune-suppressive metabolites, thereby activating or suppressing immune responses.

These at times opposing roles of CD38 in defining immune responses are in part confirmed by studies on CD38-deficient mice. Interestingly, when the animals are kept in clean facilities without infectious challenges, they grow and develop normally, without major defects (86). On the other hand, during infections they show impaired lymphocyte activation and homing and are ultimately more susceptible to death due to sepsis (87, 88). CD38-deficient animals also show reduced tumor formation, attributed to the lack CD38-mediated immunosuppression.

In the human system, CD38 is widely expressed on the surface of immune cells, particularly in conditions of cellular activation. On the cell surface, CD38 is part of the immunological synapse, forming lateral associations with critical receptors on T, B, and myeloid cells, thereby positioning itself at the center of action (48, 49). In fact, it was reported that CD38 localizes in close contact with T cell receptor (TCR), the B cell receptor (BCR), and key chemokine receptors, among other molecules (89). Perhaps the best understood function of CD38 is in the regulation of T lymphocyte functions, where the enzyme works again different ways (90–93).

First, CD38-dependent-Ca\(^{2+}\) signaling directly contributes to T cell activation, likely providing an essential second signal that drives gene expression and consequently differentiation, development, and cytotoxicity (93–95).

As a second level of T cell regulation, the NAD/CD38 axis was proposed to control T cell metabolic reprogramming needed for full T lymphocyte activation through the modulation of sirtuin activity (90, 96). Several studies are shedding light on this
molecular circuit as an important metabolic checkpoint contributing to several aspects of cellular energy metabolisms, including glycolysis, oxidative phosphorylation (OXPHOS), glutaminolysis, which are strictly associated with T cell functional fate (90, 93, 97, 98). According to the models proposed, expression of CD38 on the cell surface would limit intracellular NAD levels, negatively impacting on the activities of the NAD-dependent enzymes SIRT1 and SIRT3, which are deacetylases with fundamental roles in epigenetic regulation (93). Lastly, recent data indicate that CD38 is highly expressed by specific subsets of immunosuppressive tumor infiltrating lymphocytes, including regulatory T cells and T helper 17 cells (90, 99–101). Expression of the molecule occurs often in association with exhaustion markers, such as programmed cell death protein 1 (PD-1), pointing to an active role of CD38 in modulating T cell fate toward the generation of an immune tolerant landscape in tumors, likely through the generation of ADO (90) (Figure 3).

What remains unclear so far is what are the factors that tip the balance in favor of Ca\(^{2+}\)-active metabolites and hence immune activation or in favor of ADO and hence immunosuppression (93, 94, 102–104). Therefore, inhibition of CD38 is a valid therapeutic strategy to reestablish a functional immune surveillance (105), open the way to combination therapies with immune checkpoint inhibitors, as discussed in a separate paragraph.

**eNAD BIOSYNTHETIC-SIGNALING SYSTEM IN REGULATING IMMUNE RESPONSES**

Beside NAD-consuming, also NBEs were reported in the extracellular compartment. The best known and characterized among them is nicotinamide phosphoribosyltransferase (NAMPT), which catalyzes the conversion of Nam to NMN in the presence of phosphoribosyl pyrophosphate (PRPP) and ATP (7, 44).

The presence of NAMPT in biological fluids is now well established: several years were needed before realizing that a cytokine promoting B cell differentiation and originally described in mid-nineties (106), and an extracellular adipokine called visfatin were in fact the same protein as NAMPT (11, 107, 108). Of note, different cell types, including neutrophils, monocytes, macrophages, and cancer cells secrete eNAMPT in the extracellular space in response to inflammation, cellular stress, infections, and hypoxic conditions, among others. In human plasma eNAMPT normal levels are in the low nanomolar range (2-4 ng/ml), but it is over-expressed in several inflammatory and metabolic disorders, including cancer, where concentrations can increase 10-20 times (11, 108).

The second NBE dosed in biological fluids is nicotinic acid phosphoribosyltransferase (NAPRT), which controls the NAD generation pathway starting from Na. While the NAMPT pathway is probably the predominant one in most cells and tissues, considering that all NAD-consuming enzymes generate Nam, the activity of NAPRT is believed to boost NAD levels in stress conditions (44, 109–111). Information on eNAPRT is far more limited, even though concentration data indicate again a physiological level in low nanomolar range (1-2 ng/ml), raising sometimes dramatically, particularly during sepsis (112).

Whether these enzymes are active in the extracellular compartment remains uncertain, mainly because of the absence of detectable PRPP levels, an essential co-factor to produce NMN and nicotinic acid mononucleotide (NaMN). In addition, the rest of the enzymatic cascade producing NAD has never been reported in the extracellular space (109). From data present in the literature, we can exclude a direct eNAD synthesis in physiological conditions, but we cannot exclude a site-specific and transient eNAD synthesis in inflammatory conditions, due to release of intracellular molecules (ATP, PRPP) and enzymes. In favor of a compartment-specific function, the active forms of these enzymes are in a dimeric conformation, but within the extracellular compartment they should be in a monomeric, and hence inactive, form (113). Lastly, functional studies have shown that eNAMPT and eNAPRT, genetically modified to be enzymatically inactive, retain their pro-inflammatory properties (112) (Figure 3).

A second area of investigation concerns the mechanisms of trafficking of these enzymes from the intracellular to the extracellular space, which appear “non-classical”, as secretion is unaffected by monensin and brefeldin A, two inhibitors of the classical endoplasmatic reticulum (ER)–Golgi secretory pathway (114–117). An interesting finding indicates that NAMPT secretion could be regulated through SIRT1- and SIRT6-deacetylation, thereby linking NAD-biosynthetic and -consuming enzymes, and potentially suggesting eNAMPT secretion as regulatory mechanism to decrease its intracellular concentrations (118, 119). Recent evidence showed that eNAMPT is carried in extracellular vesicles (EVs) through systemic circulation in mice and humans. EV-contained-eNAMPT is internalized into cells, enhancing NMN and hence NAD synthesis (120). eNAPRT is actively secreted via exosomes also from microglia during neuroinflammation due to ischemic injury (121). These findings support the possibility of metabolic exchange between tumor/inflammatory and immune cells and vice versa within the site of inflammation or the tumor microenvironment (TME), as previously described for other cytokines and metabolic molecules (122, 123).

The conclusion from these data is that outside of cells it is unlikely that NAMPT and NAPRT function as NAD-producing enzymes, raising the alternative possibility that they possess different functions. In fact, eNAMPT can directly bind Toll-like receptor 4 (TLR4) (112, 124) and C–C chemokine receptor type 5 (CCR5) (125), which might explain how the protein is involved in the activation of an inflammatory signature. The binding with TLR4 was demonstrated in different cellular models, leading to activation of specific intracellular signaling pathways (e.g., STAT3, NF-xB, Akt, P38) within minutes, and activation of inflammasome in few hours (112, 124).

Less recently, it was reported that eNAMPT can selectively inhibit infection of monocytes by human immunodeficiency virus (HIV) and this activity was linked to a direct interaction with CCR5, shown using surface plasmon resonance (SPR) (126). More recently, Torretta et al. suggested that eNAMPT...
acts as a natural antagonist of CCR5 in cancer cells (125). Within the cancer microenvironment, eNAMPT seems to contribute to shape an immunotolerant environment, mostly acting on the myeloid component. We described a role for eNAMPT in the differentiation of circulating monocytes from chronic lymphocytic leukemia (CLL) patients toward tumor-supporting M2 macrophages (127). Recently, it was demonstrated that iNAMPT acts also on myeloid-derived suppressor cells (MDSCs) via a SIRT1/hypoxia-inducible factor (HIF)-1α axis, promoting their mobilization (128). The activation of these circuits creates an immunosuppressive and tumor-promoting microenvironment (Figure 3).

Much less is known on eNAPRT, even though from early information it seems to possess properties similar to NAMPT when in extracellular fluids. Managò et al. demonstrated that eNAPRT binds TLR4 on macrophages triggering NF-kB activation and pro-inflammatory cytokines secretion (112). Moreover, eNAPRT shares with eNAMPT the activation of a transcriptional program, maybe mediated by the induction of macrophage colony-stimulating factor (M-CFS), to force monocyte differentiation into macrophages. In turn, macrophages are a source of eNAMPT and eNAPRT in vivo (112). Even if several issues remain to be investigated, a functional role of these enzymes in primary innate immunity responses is clearly emerging, opening the way to target these enzymes to modulate inflammation.

**IS THE NADome A THERAPEUTIC TARGET?**

Alterations in the NADome have been described in several human diseases, including inflammatory conditions (gastric and intestinal inflammatory diseases, graft-versus-host disease, sepsis and multiple organ failure, allergies particularly in the lungs, atherosclerosis, age-associated insulin resistance, neuroinflammation/degeneration), autoimmune diseases (multiple sclerosis, psoriasis, systemic lupus erythematosus), cardiovascular diseases and cancer (7, 55, 129).

In addition to their role in shaping the immune system and in creating immunosuppressive conditions, in some instances NAD-metabolizing enzymes are considered biological prognostic markers and therapeutic targets. Among them, the most promising are CD38, CD73 and NAMPT and the disease setting is cancer (Figure 4).

CD38 is expressed in hematological malignancies, including acute B lymphoblastic leukemia (B-ALL), acute myeloid leukemia (AML), mantle cell lymphoma (MCL), CLL, multiple myeloma (MM) and NK/T cell leukemia (T-ALL) (55, 94, 102, 105, 130–132). The role of CD38 has been widely explored and defined in CLL and in MM. On CLL B lymphocytes, CD38 associates with the BCR complex [BCR/CD81/CD19/CD21] and cooperates to amplify the signal transduction driving cell proliferation (55, 133, 134).

Patients with CLL with a higher proportion of leukemic cells expressing CD38 ≥30% experience a shorter time to first treatment and a more aggressive clinical course with inferior overall survival compared to patients who have <30% of CD38+ CLL cells, thus establishing surface CD38 as a marker of poor prognosis (135–137).

MM is a plasma cell neoplastic aggressive disease with a median overall survival of 4.4–7.1 years (138). CD38 is highly and ubiquitously expressed on MM cells and at low levels on normal lymphoid and myeloid cells (49, 139). Daratumumab is a first-in-class anti-CD38 therapeutic monoclonal antibody (mAb) approved in 2015 for the treatment of relapsed/refractory MM (140).
The documented mechanisms of action include antibody-dependent cell cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and inhibition of CD38 enzymatic activities and induction of apoptosis in a caspase-dependent manner (132, 141, 142). This Ab is now used in combination with other drugs; however, the density of CD38 molecules on MM cells is a predictive factor to the efficacy and durability of daratumumab treatment (143). In CLL, CD38 engagement by daratumumab modulates BCR signaling and enhances the anti-CLL activity of treatment (144). In addition, CD38 is highly expressed in different solid tumors (i.e., gliomas, pancreatic cancer, non-small cell lung cancer, melanoma, hepatocellular carcinoma), generally associated to increased aggressiveness and creating a tumor-supporting microenvironment (145), providing a rationale for the expansion of daratumumab’s field of action.

Targeting CD73 to interfere with the degradation of AMP into ADO, reducing the generation of an immunosuppressed and pro-angiogenic niche that promotes the onset and progression of cancer, is an attractive therapeutic option (146). CD73 expression is higher in the majority of human solid tumors. Its expression and activity are closely associated with tumor invasiveness and metastasis (147, 148).

Inhibition of CD73 using either mAb or small molecule inhibitors such as a,b-methylene-ADP (APCP) have demonstrated antitumor activities in preclinical tumor mouse models (148, 149). Furthermore, a number of anti-CD73 mAbs (MEDI9447, BMS986179, SRF373/NZV930, CPI-006/CXP-006, IPH5301, TJ004309) and selective inhibitors (LY3475070, TJ004309) are being tested in early phase clinical trials, as recently reviewed in (147, 150).

Therefore, combination therapies with CD73 blocking Abs or small molecule inhibitors and other therapeutic strategies including immune checkpoint blockade, adoptive T cell therapy, agonistic immunotherapy, chemotherapy, and radiation therapy, could have synergistic effects in various cancers boosting immune response to keep the tumor cells in control, as emerged by recent studies (148, 151).

The first NAMPT inhibitor FK866 (also known as APO866) was described in 2003 by Hasmann et al. (152) Since that, several specific NAMPT inhibitors were developed as recently reviewed in (7, 153, 154). The rationale was mainly supported by the over-expression of NAMPT in cancer cells, as extensively described by us and by several research groups (11, 108, 117, 127, 155–158).

This led to a first wave of molecules that entered clinical trials for cancer therapy; however, no molecules reported to have progressed to later stages [www.clinicaltrials.gov (7, 153)].

Toxicity of old inhibitors and rescue mechanisms by the activation of other NBEs following NAMPT block, have limited the use of NAMPT inhibitors as single agents. However, increasing evidence suggests that a better selection of tumor subtype rely exclusively on NAMPT activity to generate NAD, as well as novel drugs less toxic, could open a second life for NAMPT inhibition strategy. Moreover, a combination between NAMPT inhibitors and selective inhibitors of oncogenic signaling driving cancer progression could be therapeutically exploited as suggested (11, 117, 159).

An unknown notion is whether these inhibitors could also affect eNAMPT activity, even if, as mentioned before, the enzymatic activity of eNAMPT is controversial. Travelli et al. developed novel inhibitors that can’t cross the plasma membrane and have more activity to block eNAMPT form, demonstrating reduced growth of triple negative mammary carcinoma in mice (160). On the other hand, there is also intense research to develop a blocking antibody to neutralize eNAMPT and reduce its “cytokine-like activity” within the TME. The group of Garcia firstly has devised a polyclonal eNAMPT neutralizing antibody (pAb) (161). They used this Ab in different models of inflammation and cancer, including lung injury and prostate cancer. Recently, in acute respiratory distress syndrome (ARDS) they demonstrated the highly significant contribution of endothelial cell (EC)-derived NAMPT to the severity of inflammatory lung injury in preclinical ARDS models. Intravenous delivery of either eNAMPT-neutralizing pAb/mAb significantly attenuated inflammatory lung injury in mouse model. In vitro studies on EC demonstrated that eNAMPT-neutralizing antibodies strongly abrogate eNAMPT-induced TLR4 pathway activation (162). In invasive prostate cancer (PCa) Sun et al. proved the activity of eNAMPT in supporting the invasive features of PCa, and the tumor blocking activity of the anti-eNAMPT neutralizing antibody in a pre-clinical in vivo model of PCa invasion (163). In parallel, the group of Prof. Genazzani in Italy is developing a novel monoclonal antibody (C269) that neutralizes in vitro the cytokine-like action of eNAMPT and that reduces its serum levels in rodents. This Ab is able to significantly reduce acute and chronic colitis in two models of induced-colitis (164), suggesting a role of eNAMPT in the pathogenesis of inflammatory bowel disease (IBD) and the therapeutic potential of its neutralization in this pathology.

The general idea of targeting eNAMPT in tumors and in inflammatory diseases is increasing to counteract the extracellular functions of this protein, mainly linked to the activation of TLR4 and modulation of immune responses. The best option could be to combine i/eNAMPT targeting with immunomodulatory agents to obtain a tumor growth regression and a concomitant reversion of immunosuppressive conditions, acting on the immune system. In support of this, two papers demonstrated that NAMPT inhibitors enhance the anti-tumor efficacy of immune checkpoint inhibitors, i.e. antibody against PD-1 (128, 165).

CONCLUSIONS AND FUTURE PERSPECTIVES

Since the discovery of the presence of extracellular nucleotides such as ATP and NAD released from intracellular stores in conditions of cell stress or inflammation, they are considered “danger signals” to alert the immune system, participating in the recruitment, activation, and differentiation of immune cells, and promoting the production and release of pro-inflammatory
cytokines. Within the TME, extracellular nucleotides create pro-tumor conditions acting directly on tumor aggressive features but also on immune cells promoting a general immunosuppression.

The extracellular machinery that regulates eNAD functions is complex, as we summarized in this review several eNAD-metabolizing enzymes rapidly degrade it into metabolites that in turn can function as signaling messengers or can be internalized and used to reconstitute the intracellular NAD pool. Directly, eNAD can bind purinergic receptors and activate signaling. The effects of eNAD are therefore dependent on the presence of receptors, metabolizing enzymes and cellular stress conditions within the microenvironment. Understanding this intricate machinery remains the most important challenge to develop therapeutic strategies to modulate expression of these extracellular nucleotides, relative enzymes, and receptors to re-educate the immune system in different diseases, including cancer.

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AUTHOR CONTRIBUTIONS

SD designed and reviewed the work, which was assembled by VA, with contribution of VM and LB. All authors contributed to the article and approved the submitted version.

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