



NAMPT and NAPRT: Two Metabolic Enzymes With Key Roles in Inflammation

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Nicotinamide phosphoribosyltransferase (NAMPT) and nicotinate phosphoribosyltransferase (NAPRT) are two intracellular enzymes that catalyze the first step in the biosynthesis of NAD from nicotinamide and nicotinic acid, respectively. By fine tuning intracellular NAD levels, they are involved in the regulation/reprogramming of cellular metabolism and in the control of the activity of NAD-dependent enzymes, including sirtuins, PARPs, and NADases. However, during evolution they both acquired novel functions as extracellular endogenous mediators of inflammation. It is well-known that cellular stress and/or damage induce release in the extracellular milieu of endogenous molecules, called alarmins or damage-associated molecular patterns (DAMPs), which modulate immune functions through binding pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and activate inflammatory responses. Increasing evidence suggests that extracellular (e)NAMPT and eNAPRT are novel soluble factors with cytokine/adipokine/DAMP-like actions. Elevated eNAMPT were reported in several metabolic and inflammatory disorders, including obesity, diabetes, and cancer, while eNAPRT is emerging as a biomarker of sepsis and septic shock. This review will discuss available data concerning the dual role of this unique family of enzymes.

Keywords: inflammation, cancer, signaling, metabolism, DAMPs, NAMPT, NAPRT, TLRs

INTRODUCTION

One of the key roles of the innate immune system is to initiate immune responses against invasive pathogens. Pathogen-associated molecular patterns (PAMPs) include sugars/lipoproteins or nucleic acids [i.e., bacterial DNA as unmethylated repeats of dinucleotide CpG, double-stranded (ds) or single-stranded (ss) RNA] (1, 2). PAMPs can initiate immune responses through the activation of classical pattern recognition receptors (PRRs), among which there are toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), multiple intracellular DNA sensors, and other non-PRRs DAMPs receptors (2–4). However, these receptors can be engaged also by endogenous ligands. It is now largely accepted that cells in conditions of hypoxia, acidosis, redox imbalance, hypertonic/hypotonic stress, and intracellular ion or cytoskeleton perturbations, can release small endogenous molecules called damage-associated molecular patterns (DAMPs) or sometimes “danger signals” or “alarmins,” triggering immune responses through the activation of PRRs (4–6). Intriguingly, many of these DAMPs have a well-characterized intracellular function and have been serendipitously identified in the extracellular space where they initiate inflammatory responses, independently of pathogen

infection, a phenomenon referred to as sterile inflammation (4, 7, 8). Similar to pathogen-induced inflammation, DAMPs can prime neutrophils, macrophages, and dendritic cells (DCs), but also non-immune cells, including endothelial and epithelial cells and fibroblasts (7). Activation of these cells leads to the production of several cytokines and chemokines, which in turn recruit inflammatory elements and trigger adaptive immune responses. Although sterile inflammation plays an essential role in tissue repair and regeneration, unresolved chronic inflammation is deleterious to the host leading to the development of metabolic, neurodegenerative, autoimmune disorders, and cancer (4).

Since their original definition as DAMPs in 2003, the list of endogenous molecules are increased considerably (4) and now includes high-mobility group box 1 protein (HMGB-1), heat shock proteins (HSPs), histone and extracellular matrix components (for example, hyaluronic acid and biglycan). All these molecules exert pro-inflammatory functions through binding to TLRs. HMGB-1 is among the most studied DAMPs. It is a nuclear DNA binding protein that can be found in the extracellular space not only as a consequence of necrosis, but also through dedicated secretion mechanisms (9, 10). Extracellularly, HMGB-1 elicits pro-inflammatory effects linked to consequent TLR4 binding and activation of the nuclear-factor kappa B (NF- κ B) signaling pathway (11, 12). In animal models, HMGB-1 is as a late mediator of lethal systemic inflammation, involved in delayed endotoxin lethality (13). Others DAMPs include F-actin, Sin3A associated protein 130 (SAP130), β -glucosylceramide and N-glycans binding to CLRs; monosodium urate (MSU) crystals, cholesterol crystals, β -amyloid (A β), and adenosine 5'-triphosphate (ATP) that activate NLRP3 inflammasome (4). In addition, numerous cytokines [i.e., interleukin (IL)-1 β , tumor necrosis factor (TNF), and type I interferon (IFN-I)], pro-inflammatory proteins, such as interferon-induced protein 35, and bioactive lipids like lysophospholipids, are referred as "inducible DAMPs" or "conditional DAMPs" (14).

Nucleotides and nucleosides, for long time considered simply electron-shuttling agents involved in supporting energy metabolism, are gaining interest together with the network of enzymes that control their synthesis and degradation. Interestingly, while all these factors have a well-characterized intracellular function, they can be released in the extracellular space, where they bind and activate different sets of cellular receptors, including purinergic and PRRs. For example, ATP a key intracellular energy molecule, can be massively released by passive leakage when cells become injured, stressed, or even necrotic, acting as a DAMP (15). Extracellular ATP and its derivative nucleotides (adenosine, AMP, ADP) synthesized by endonucleotidases achieve many of their effects through purinergic receptors, via inflammatory cascades and the production of proinflammatory cytokines (16, 17). Among the enzymes involved in nicotinamide adenine dinucleotide (NAD) synthesis, nicotinamide phosphoribosyltransferase (NAMPT)—the focus of this review—emerges as new mediator of inflammation. Intracellularly, it catalyzes the first and rate-limiting step in the biosynthesis of NAD from nicotinamide (Nam) (18, 19). Increased eNAMPT levels are reported

in conditions of acute or chronic inflammation (18, 20–25). eNAMPT effects are mostly linked to the activation of an inflammatory signature mainly in macrophages, with recent data suggesting that it binds TLR4, therefore adding the enzyme to the number of "danger" signals activating this receptor (26). NAMPT is structurally and functionally related to a second NAD-biosynthetic enzyme (NBE), i.e., nicotinate phosphoribosyltransferase (NAPRT), which is rate-limiting in the NAD salvage pathway that starts from nicotinic acid (Na) (27–29). Our group recently discovered the presence of NAPRT in extracellular fluids (eNAPRT), highlighting a role also for this enzyme as a ligand for TLR4.

This review summarizes the current knowledge on NAMPT and NAPRT, as intracellular NBEs involved in the regulation/reprogramming of cellular metabolism, and as cytokines/DAMPs in the extracellular environment. Lastly, we will discuss the role of these enzymes especially in relation to the development of inflammatory conditions, including cancer, and their potential therapeutic values.

NAD LEVELS MODULATE CELLULAR TRANSCRIPTIONAL RESPONSES AND METABOLIC ADAPTATION

Our knowledge on NAD biology has grown exponentially over the past few years, including biosynthetic and degrading pathways. A general decrease in cellular NAD is described in many age-related diseases, whereas increased NAD levels are associated to inflammatory conditions, including cancer. **Figure 1** illustrates the main NAD-biosynthetic and -consuming pathways, as well as the crosstalk between intracellular (i)NAD and eNAD.

NAD: Energy Cofactor

As energetic co-enzyme, NAD is essential as electron acceptor donor in various metabolic pathways including cytosolic glycolysis, serine biosynthesis, tricarboxylic acid cycle (TCA), oxidative phosphorylation, as well as cell redox state homeostasis redox reactions (30, 31). Cofactor of almost 300 dehydrogenase, NAD is primarily used during glycolysis in the sixth step of the enzymatic chain by glyceraldehyde phosphate dehydrogenase (GAPDH) and at the end of the process by lactate dehydrogenase (LDH), catalyzing the interconversion of pyruvate and lactate and simultaneously of NADH and NAD. The final glycolytic product pyruvate, can be metabolized to produce acetylCoA by the pyruvate dehydrogenase complex (PDC), a reaction accompanied by NAD reduction to NADH (32). During the TCA cycle, NAD is reduced to NADH moieties in several key steps by isocitrate dehydrogenase (IDH), oxoglutarate dehydrogenase (OGD), and malate dehydrogenase (MDH). NADH produced in all these reactions, working as electron equivalent redistributor, is used by the electron transport chain (ETC) to generate ATP (33).

The ratio between NAD/NADH and their relative phosphorylated form (NADP/NADPH), are also critical for enzymatic defense systems against oxidative stress, regulating

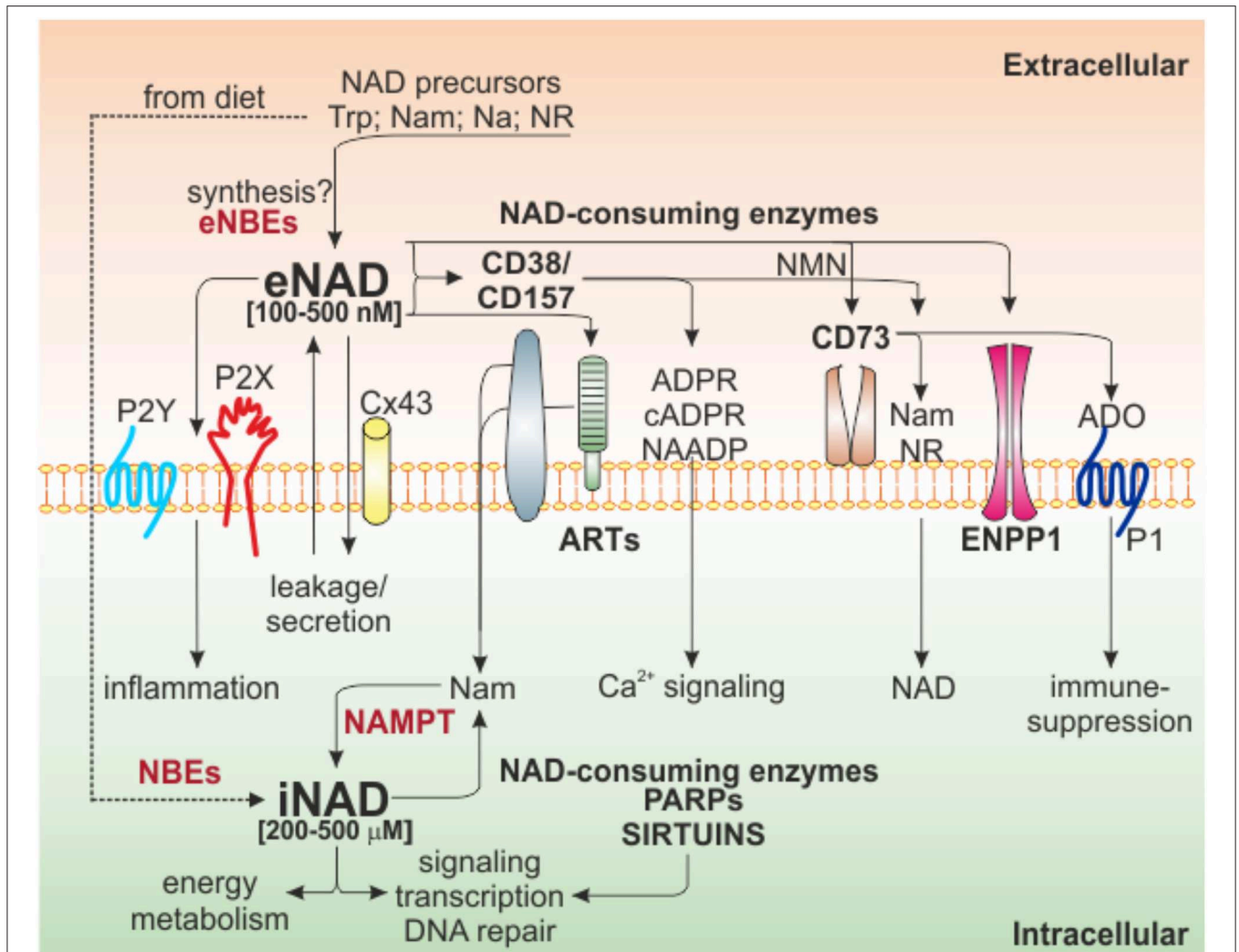


FIGURE 1 | Intra/extra NAD interconnections and NAD-metabolizing enzymes. Schematic representation of the network of NAD-metabolizing cell surface and intracellular enzymes and their products. Several NAD precursors derived from diet can be internalized to generate iNAD, via NBE activities, to support energy metabolism, signaling, and other biological processes through the activities of intracellular NAD-consuming enzymes (PARPs and Sirtuins). These enzymes release Nam that, in turn, via NAMPT-dependent salvage pathway, support NAD production. Once in the extracellular space because of secretion/leakage, via Cx43, or because of direct extracellular synthesis from precursors (not confirmed), eNAD can function by binding purinergic receptors (P2Y, P2X), an event that leads to intracellular signaling and inflammatory conditions. Alternatively, eNAD can also be metabolized by a series of ecto-enzymes of the cell surface (CD38/CD157, ARTs, CD73, ENPP1) generating different metabolites (cADPR, ADPR, and NAADP) involved mainly in Ca^{2+} -signaling. The end product of the reaction, adenosine, can modify signal transduction by acting on P1 purinergic receptors, generally leading to immunosuppression. In the square brackets are indicated the range of iNAD [200–500 μM] or eNAD [100–500 nM]. Trp, tryptophan; Nam, nicotinamide; NR, nicotinamide riboside; Na, nicotinic acid; NBEs, NAD-biosynthetic enzymes; NAMPT, nicotinamide phosphoribosyltransferase; ARTs, mono adenosine diphosphate (ADP)-ribose transferases; PARPs, poly ADP-ribose polymerases; Cx43, connexin 43; ADPR, ADP ribose; cADPR, cyclic ADP ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; NMN, nicotinamide mononucleotide; ADO, adenosine; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterases.

redox homeostasis through the main cellular scavenging systems which are the glutathione (GSH/GSSG) and the thioredoxin-mediated (Trx-SH/Trx-SS) mechanisms (34–37). In this context, NADPH is the indispensable reducing agent for ROS elimination and redox homeostasis, primarily produced by glucose-6-phosphate dehydrogenase (G6PD) and -phosphogluconate dehydrogenase (6PGD) in the pentose phosphate pathway (36).

NAD: A Pleiotropic Signaling Molecule

Independently of its redox properties, NAD is also the substrate of enzymes with fundamental roles in gene expression and cell signaling (38). In these reactions, NAD is cleaved at the glycosidic bond between Nam and ADP-ribose acquiring the characteristic of signaling molecule (27).

The large family of NAD consuming enzymes includes mono adenosine diphosphate (ADP)-ribose transferases

(ARTs) and poly ADP-ribose polymerases (PARPs), the NAD-dependent deacetylases, sirtuins (SIRT1-7), and the cyclic ADP-ribose hydrolases, NAD glycohydrolases, ectonucleotide pyrophosphatase/phosphodiesterases and ecto-5'-nucleotidase (CD38/CD157 and ENPP1/CD73) (19, 39, 40) (**Figure 1**).

Through their functional activities of post-translational modifications (ADP-ribosylation and deacetylation), or through the modulation of Ca^{2+} signaling, these enzymes regulate gene transcription, cell differentiation, cell cycle progression, circadian rhythm, DNA repair, chromatin stability, cell adaptation to stress signals, and immune responses (41, 42). Therefore, PARPs and sirtuins represent connecting elements between the metabolic state of a cell and its signaling and transcriptional activities (43).

Extracellular NAD and Its Biological Role

The eNAD concentration is in the range of 100–500 nM, considerably lower than its intracellular levels (200–500 μ M) (39, 44–46). eNAD and iNAD levels are highly linked, due to intra-extra membrane transport of NAD precursors, intermediates of reaction and NAD itself (47). The canonical view is that NAD is unable to cross lipid bilayers, but it enters the cell using dedicated NAD transporters, such as connexin 43 (Cx43) channels, or exits through exocytosis (45, 48–50). In addition, conditions of environmental stress can induce NAD release (51–53). On the other hand, whether there is direct eNAD synthesis remains controversial (39) (**Figure 1**), despite the presence of extracellular precursors and biosynthetic enzymes. Specifically, it is known that among the different forms of vitamin B3 (NAD precursor), transport of Na is mediated by membrane carrier systems potentially including either a pH-dependent anion antiporter or a proton cotransporter (54, 55). Nam is present extracellularly and its uptake is possible either as direct transport in intact form or converted to salvage pathway metabolites. However, NAMPT's substrates ATP and 5-phosphoribosyl-1-pyrophosphate (PRPP) were shown to be unavailable in sufficient quantities in the extracellular space (56) to support direct eNAD generation.

eNAD can bind different subtypes of purinergic P2 receptors, including P2Y11, leading to the opening of L-type Ca^{2+} channels and activation of a cAMP/cADPR/ $[Ca^{2+}]_i$ signaling cascade, ultimately causing increased proliferation and migration (57). In T cells and monocytes, P2X7 receptor activation generally results in Ca^{2+} internalization, opening a non-selective, large membrane pore, causing cell death (58, 59). eNAD also acts as a neurotransmitter, released by stimulated terminals of mammalian central nervous system and peripheral nervous system neurons, binding to post-synaptic P2Y1 receptors, similar to ATP (60).

The very low levels of eNAD are due to its rapid metabolism/degradation by NAD-catabolic enzymes present on the surface of the cell (61), suggesting that also NAD metabolites may mediate cellular responses in the extracellular environment.

eNAD is degraded by three main classes of specific ectoenzymes: CD38 and CD157 (62, 63), ARTs (64), ENPP1 and CD73 (61, 65, 66). NADase, ENPP1 and CD73 can lead to the formation of adenosine (ADO), a potent immunosuppressant factor, independently of the activity of CD39 (61, 67, 68). Beside generating ADO, eNAD can be degraded to nicotinamide

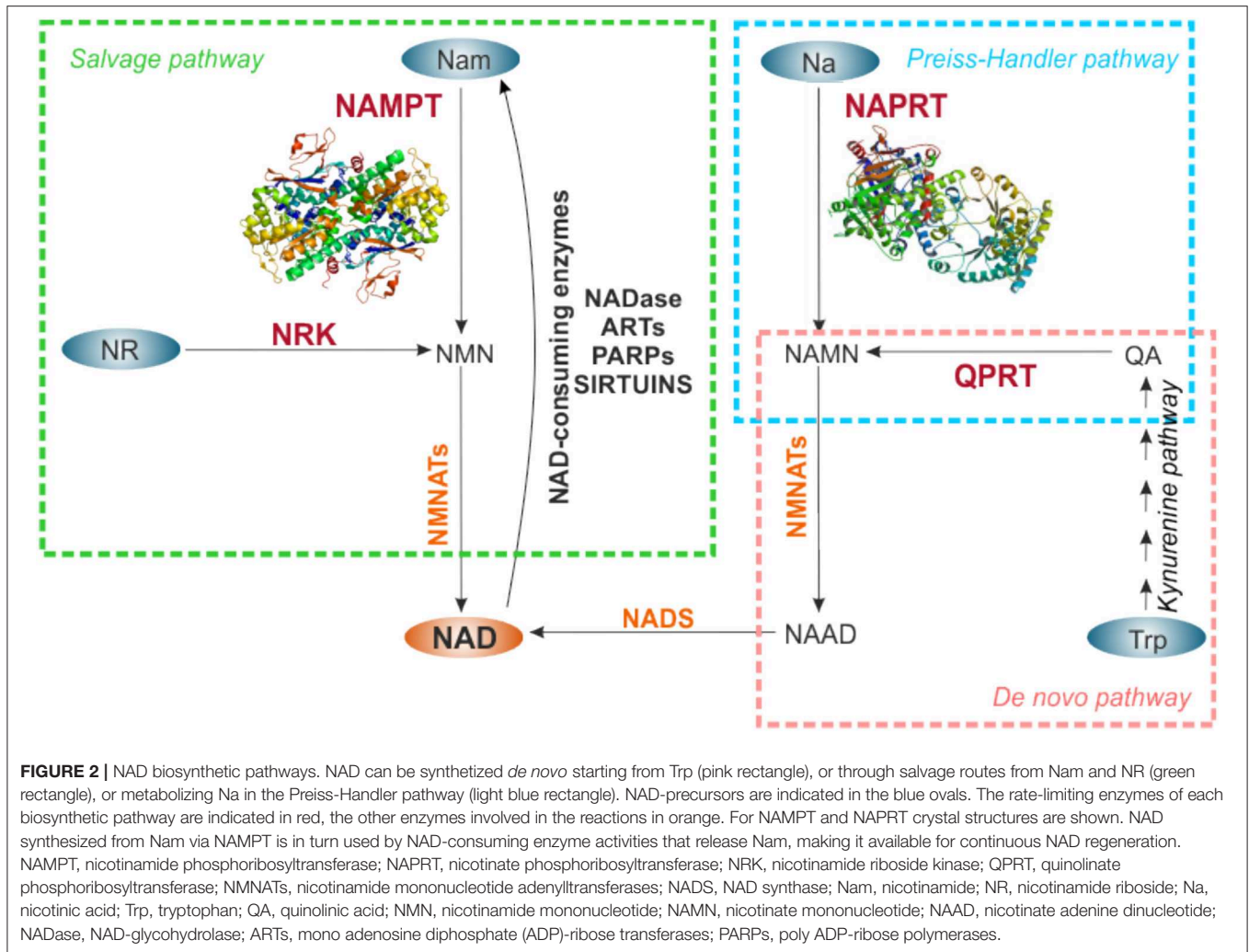
mononucleotide (NMN) by CD38, generating Nam which can cross plasma membranes and be re-converted to NAD through NAMPT and NMN adenylyltransferase (NMNAT) (69). On the other side, NMN can be also used by CD73, which generates nicotinamide riboside (NR) (66, 70), that, likely through equilibrative nucleoside transporters (ENTs), can be imported as NAD precursor (71, 72) (**Figure 1**). Recently, Slc12a8 was identified as specific NMN transporter (73), suggesting that NMN can be internalized without conversion to NR. Studies on cell type expression pattern of this transporter will clarify this possibility.

NAD BIOSYNTHESIS: THE ENZYMATIC FUNCTIONS OF NAMPT AND NAPRT

NAD turnover within the cell is dynamic, displaying circadian oscillations that are regulated by the core clock machinery CLOCK:BMAL1 (74, 75). Total intracellular levels are maintained between 200 and 500 μ M, depending on the cell type or tissue, increasing in response to different stimuli (43). NAD homeostasis is the result of the balance between a number of NAD-consuming reactions and NAD-biosynthetic routes, via three distinct pathways: the *de novo* biosynthetic pathway, the Preiss-Handler pathway, and the salvage pathway, as reviewed in Houtkooper et al. (27), Ruggieri et al. (29), and Audrito et al. (42) and illustrated in **Figure 2**.

Specifically, *de novo* NAD biosynthesis starts with the catabolism of the amino acid tryptophan to kynurenine by indoleamine-2,3-dioxygenase. Kynurenine is then metabolized through the kynurenine pathway to quinolinic acid (QA), which is converted by quinolate phosphoribosyltransferase (QPRT), rate-limiting enzyme, to Na mononucleotide (NaMN). The Preiss-Handler pathway metabolizes kynurenine pathway-derived NaMN or diet-derived Na, or Na as a product of Nam deamidation by intestinal flora (76) to NAD, via NAPRT rate-limiting activity. In the salvage pathway, NAMPT metabolizes Nam and PRPP to NMN in a rate limiting step, which is then converted into NAD. In a further salvage route, NR, derived from diet, can be used by nicotinamide riboside kinase (NRK), to generate NAD (**Figure 2**).

Quantitatively, the Nam salvage pathway is the most relevant in mammalian cells. Several lines of evidence support this observation. First, Nam is the most abundant NAD precursor in the bloodstream (39), and can be easily introduced by diet (vitamin B3). Second, Nam is a by-product of all NAD-metabolizing enzymes activity, increasing its availability (77). Third, the rate limiting enzyme NAMPT (EC 2.4.2.12) is expressed in all mammalian tissues (78), as detailed below. Linked to this, *NAMPT* gene deletion in mice is embryonically lethal (79), suggesting the importance of this route to regenerate NAD. In this pathway, Nam N-methyltransferase (NNMT) recently emerged as an evolutionarily conserved regulator of Nam availability. In fact, NNMT N-methylates Nam preventing its accumulation and inhibition of NAD-consuming enzymes, while on the other side, limiting its availability to NAMPT (80, 81).



The functional NAMPT forms a homodimer to catalyze the conversion of Nam and PRPP to NMN. Structural and site-directed mutagenesis studies by Khan et al. demonstrated that Asp219 is fundamental in defining the substrate specificity of NAMPT (82). Wang et al. showed that NAMPT has an autophosphorylation activity and hydrolyzes ATP. Autophosphorylation can increase its enzymatic activity (83). Recently, NAMPT was found to be a direct substrate of SIRT6 deacetylation, a post-translational mechanism that up-regulates its enzymatic activity (84). On the contrary, mutations of His247, a central conserved residue in the active site of the enzyme, significantly decreases or abolishes NAMPT enzymatic activity (83).

NAPRT (EC 2.4.2.11) catalyzes the conversion of Na and PRPP to NaMN and pyrophosphate (PPi). The enzyme, originally named NaMN pyrophosphorylase, was first described by Handler in human erythrocytes, where it increases NAD levels (85).

NAPRT activity is more tissue-specific. Although enzyme activity can be detected in most mouse tissues (86), Na acts as a more efficient precursor than Nam in mice liver, intestine, heart

and kidney (87). Furthermore, Na is more efficient than Nam in raising NAD levels in cells exposed to oxidative stress (56, 85, 88).

Contrary to NAMPT, NAPRT is not inhibited by NAD, which explains its significantly higher efficiency in raising NAD levels *in vivo* (56, 89). Moreover, NAPRT is strongly activated by phosphate (85), while ATP behaves as an allosteric modulator of the enzyme (29, 85, 90).

In 2015 Marletta et al. resolved the structure of human (h)NAPRT, highlighting a high degree of structural homology between the human and the bacterial NaPRTases due to evolutionary adaptation (91). As with NAMPT, the functional NAPRT enzyme works as dimers, and despite sharing very limited sequence similarity, hNAPRT shows a molecular fold that closely resembles that firstly described for hNAMPT (83). This opened new hypotheses of shared motifs in NAMPT and NAPRT involved in the binding of extracellular proteins to the receptor, as described in section eNAMPT Functions.

The presence of these multiple NAD biosynthetic routes most likely reflects differences in tissue distribution and/or intracellular compartmentalization of NBEs (39, 46, 76, 92, 93). Our group recently showed that NAMPT and NAPRT are mainly

located in cytoplasm and nucleus, while NRK is more expressed in mitochondria, impacting on iNAD levels and response to NAMPT inhibitors (39, 46, 76, 92, 93).

Identification, Characterization, and Expression of NAMPT and NAPRT

NAMPT

The enzyme NAMPT is highly conserved with orthologs in bacteria (94), invertebrate sponges (95), amphibians (96), birds and mammals (97). Not long after its discovery in 1994 by Samal et al. as a pre-B-cell colony enhancing factor (PBEF) secreted by activated lymphocytes and bone marrow stromal cells, Rongvaux et al. (98) showed that murine PBEF could catalyze the conversion of Nam to NMN, a rate-limiting step in NAD biosynthesis. These authors also showed that *Actinobacillus pleuropneumonia*, a bacterium lacking the *NadV* gene, transformed with murine PBEF acquires NAD independence, confirming that the enzymatic activity is evolutionarily conserved from bacteria to mammals (98).

In recent years, NAMPT has received increasing attention due to new evidence indicating that it is a pleiotropic protein that may function as NBE, as well as growth factor, cytokine and adipokine [reviewed in (18, 25)]. Although NAMPT lacks the typical signal peptide needed for extracellular secretion, the mature protein can be found in the medium of many cellular cultures due to an active secretion mechanism (99, 100). However, in conditions of cell damage eNAMPT can be released as passive diffusion across cell membranes, as usual for other DAMPS. In addition, the 3' untranslated region (UTR) contains multiple TATT motifs that are characteristic of cytokines (99).

The human *NAMPT* gene spans over 34.7kb on the long arm of chromosome 7 (7q22) and contains 11 exons and 10 introns (101, 102). Two distinct promoter sites are present in the 5'-flanking region, suggesting the possibility of tissue specific differential expression (101). The region proximal to the promoter is GC-rich and contains 12 binding sites for specificity protein 1 (SP-1), multiple activating protein 2 (AP-2), lymphoid enhancer-binding factor 1 (LF-1), cAMP response element-binding protein (CREB), and signal transducer and activator of transcription (STAT) binding sites (101, 103). Furthermore, the presence of two hypoxia inducible factor (HIF) response elements (HREs) suggest that the gene is upregulated under hypoxic conditions (104). The distal promoter region contains several CAAT boxes and TATA-like sequences, as well as binding sites for nuclear factor 1 (NF-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), CCAAT/enhancer binding protein (C/EBP β), the glucocorticoid receptor (GR), and activating protein 1 (AP-1) (101). The majority of these transcription factors, including NF-1, AP-1, AP-2, NF- κ B, and STAT, regulate cytokine expression and their presence in the promoter region of *NAMPT* suggests a role for this enzyme in immunity (42, 105).

Recently, 65 kb downstream of the *NAMPT* transcription start site on chromosome 7 (hg19: 105,856,018–105,860,658), a putative *NAMPT* enhancer was identified as specifically marked by H3K27ac and/or an accessible DNase I hypersensitive (DHS)

signal (106). Fine-mapping of the 4.6-kb putative enhancer by stepwise 1-kb deletions or insertions identified the 1-kb enhancer "B" region as responsible for (i) the control of expression of the *NAMPT* gene through c-MYC and MAX activities. (ii) In addition, it is the target of H3K27 acetylation; (iii) it regulates iNAD levels; and (iv) it is required for cell survival in NAMPT-dependent tumors (106). Some genetic polymorphisms were identified in the human *NAMPT* gene, potentially responsible for NAMPT expression. Different representation of these Single Nucleotide Polymorphisms (SNPs) were described in patients with acute respiratory distress syndrome, type 2 diabetes, glucose and lipid metabolism alterations, diastolic blood pressure and hypertensive disorders compared to controls (107).

In the cell, NAMPT is abundant in the cytosol and present in the nucleus (108–110). Recently, Svoboda et al. showed that nuclear NAMPT translocation is a regulated process induced by genotoxic, oxidative, or dicarbonyl stress, mainly to finance NAD production for increased PARP and sirtuin activity (111). Moreover, NAMPT cytosol/nucleus localization changes according to cell cycle phases: it is excluded from the nucleus immediately after mitosis and it migrates back into it as the cell cycle progresses (111). These data were confirmed also by Grolla et al. that demonstrated a transport of NAMPT into the nucleus, GAPDH-mediated, in response to DNA damage (112). On the contrary, the presence of NAMPT in mitochondria remains controversial (30, 46, 109).

Furthermore, an increasing number of cell types have been shown to release eNAMPT, including adipocytes, hepatocytes, cardiomyocytes, activated immune cells and several tumor cells (100, 113–117). While it was shown that a regulated positive secretory process exists (79), the exact mechanisms of release are presently under investigation. The most accredited hypothesis, yet to be confirmed in most cell types, is that eNAMPT is secreted through a "non-classical" secretory pathway, which is not blocked by monensin and brefeldin A, two inhibitors of the classical endoplasmic reticulum (ER)–Golgi secretory pathway (79, 113, 118, 119). A recent paper showed that eNAMPT is carried in extracellular vesicles (EVs) through systemic circulation in mice and humans. EV-contained-eNAMPT is internalized into cells, enhancing NAD synthesis (120). The same conclusion was obtained by another group identifying that eNAMPT is actively secreted via exosomes from microglia during neuroinflammation due to ischemic injury (121). However, this mechanism of secretion could be context dependent: in fact in 3T3-L1 adipocytes eNAMPT release and secretion do not appear to occur through microvesicles (113).

Whether the extracellular form possesses specific differences in terms of truncations or post-translational modifications is presently unclear. Different groups suggested that deacetylation by sirtuins can impact eNAMPT secretion (84, 122), adding a new layer of complexity.

NAPRT

Highly conserved across species, the human *NAPRT* gene is located at chromosome 8q24.3, containing 12 exons. Similar to NAMPT, intracellular NAPRT is located in both the nucleus and the cytoplasm, but not detected in mitochondria (46, 71, 123).

Our group firstly reported the presence of an extracellular form of NAPRT in biological fluids in physiological (healthy donor's blood) and inflammatory conditions opening a new field of investigations (124).

Several information about NAPRT expression and regulation emerged in tumors, in relation to the efficacy of NAMPT inhibitors (NAMPTi) as potential anti-cancer agents (125, 126), as described in the dedicated section NAMPT and NAPRT in Tumors.

EXTRACELLULAR NAMPT AND NAPRT: ADIPOCYTOKINES AND DAMPS

In addition to a direct effect on NAD and its metabolites, the enzymes involved in synthesis of NAD also have important extracellular functions, as summarized in **Figure 3**.

eNAMPT Functions

eNAMPT/PBEF was first identified as an immunomodulatory cytokine able to synergize with interleukin 7 (IL-7) and stem cell factor (SCF) to promote pre-B cell colony formation (99). It is now well-established that eNAMPT is a soluble factor that is up-regulated upon activation in innate and adaptive immune cells, including neutrophils, monocytes, and macrophages, and in epithelial and endothelial cells (18, 127). NAMPT expression can be rapidly induced by inflammatory signals, in particular both pathogen-derived lipopolysaccharide (LPS) and host-derived inflammatory stimuli (TNF- α , IL-1 β , IL-6, leptin) in amniotic epithelial cells, macrophages, human osteoarthritic chondrocytes and a synovial fibroblast cell line (101, 103, 128, 129).

eNAMPT has a variety of biological functions (**Figure 3**): (i) it is an important mediator of inflammatory programs (18, 130) and (ii) it acts as a cytokine that modulates the

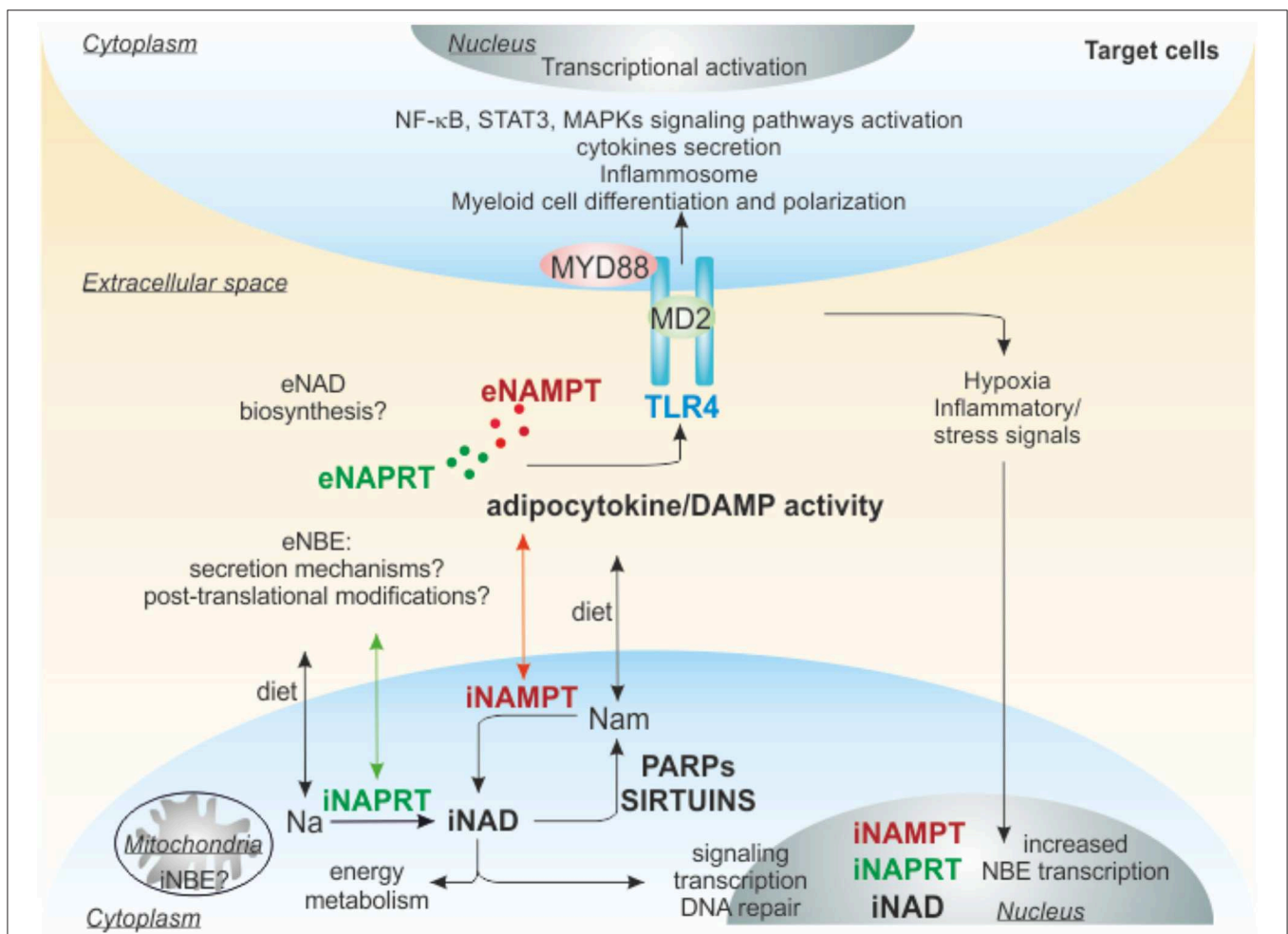


FIGURE 3 | Extracellular functions of NAMPT and NAPRT. iNAMPT and iNAPRT are involved in NAD generation inside of cells, but can be also secreted, through unknown mechanisms, in the extracellular space due to cellular stresses (damage/inflammation/pathological conditions). Extracellularly, they can act as adipocytokine/DAMP binding to TLR4 and triggering intracellular signaling promoting differentiation/polarization of myeloid cells, activation of inflammasome, secretion of pro or anti-inflammatory cytokines. The final outcome depends on the cellular context, for example in tumors eNAMPT creates an immunosuppressive microenvironment, favoring cancer progression, while eNAPRT in sepsis amplifies the inflammatory response. TLR4, Toll-like receptor 4; MD2, myeloid differentiation 2; MYD88, myeloid differentiation primary response gene 88; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, nicotinate phosphoribosyltransferase; NBEs, NAD-biosynthetic enzymes; Nam, nicotinamide; Na, nicotinic acid; DAMP, damage-associated molecular pattern; PARPs, poly ADP-ribose polymerases.

immune response (42). Notably, the cytokine-like functions appear, at least in part, independent of the protein catalytic activity, as inferred by the use of an enzymatically inactive NAMPT H247E mutant that retains the ability to activate signaling pathways (26, 83, 131, 132). In keeping with this view, NAMPT's substrates PRPP and ATP are apparently unavailable extracellularly to sustain its enzymatic activity (56). Following NAMPT treatment, interleukins IL-1 β , IL-6, IL-10, and tumor necrosis factor- α (TNF- α) are up-regulated and secreted by peripheral blood mononuclear cells (PBMCs) and CD14⁺ monocytes (133). Co-stimulatory molecules such as CD40, CD54, and CD80 are also up-regulated in response to eNAMPT exposure, an effect mediated through the activation of PI3-kinase and MAPKs pathways (133). Furthermore, in macrophages NAMPT increases matrix metalloproteinases (MMPs) expression and activity (134). In addition, (iii) eNAMPT has anti-apoptotic effects on immune cells, including neutrophils and macrophages, for example it promotes macrophage survival after induction of endoplasmic reticulum (ER) stress triggering IL-6 secretion and phosphorylation of STAT3 (103). (iv) eNAMPT is also reported as an adipokine, also known as visfatin, playing a critical role in the regulation of glucose-stimulated insulin secretion in pancreatic β cells (21, 135). While a direct role for insulin receptor in eNAMPT-mediated cytokine release was discarded (18, 133), eNAMPT is up-regulated in obese and diabetic patients: it is enriched in visceral fat and secreted by adipocytes (113, 136, 137). The role as adipokine seems more related to the extracellular generation of NMN: in fact systemic administration of NMN to aged mice or mice subjected to a high-fat diet restores normal NAD levels in white adipose tissue and liver, and ameliorates glucose intolerance and type II diabetic syndrome (138). (v) eNAMPT can also act as a pro-angiogenic factor, promoting endothelial cell proliferation, migration, and capillary tube formation in a concentration-dependent manner in human umbilical vein endothelial cells (HUVEC) (139–142). These proliferative effects of eNAMPT seem to be mediated, or at least partially mediated by vascular endothelial cell growth factor (VEGF), the master regulator of endothelial cell program (139). Thus, eNAMPT upregulates VEGF synthesis and secretion, as well as the expression of the VEGF receptor 2, which has been proposed to mediate the angiogenic actions of VEGF (139). Beside VEGF, in endothelial cells eNAMPT upregulates production of other pro-angiogenic soluble factors, such as fibroblast growth factor-2 (FGF-2), monocyte chemoattractant protein-1 (MCP-1) and IL-6 (143, 144). Indeed, both MCP-1 and FGF-2 have also been identified as mediators of eNAMPT-induced angiogenesis (143). Beyond *in vitro* studies, the angiogenic activities of eNAMPT were demonstrated in *ex vivo* and *in vivo* approaches (139, 140).

eNAMPT Functions

Starting from the structural and functional similarity between human NAMPT and NAPRT (91), our group for the first time investigated whether NAPRT exists in an extracellular form, thus sharing with NAMPT its moonlighting abilities (124). By setting up a new luminex/ELISA assay, we dosed eNAMPT in a cohort of > 100 plasma from normal blood donors (HD), highlighting

a mean concentration similar to that recorded for eNAMPT (in the range of 1.5–2.0 ng/ml), with no differences according to gender or age. We used mass spectrometry to confirm the presence of NAPRT peptides in human plasma. Moreover, we demonstrated that endogenous eNAMPT is enzymatically active (86, 124). Analyzing eNAMPT in sera from patients with acute or chronic inflammatory conditions, we demonstrated that this enzyme strongly increased in acute inflammatory diseases such as sepsis and septic shock, driving inflammatory responses related to the activation of macrophages (Figure 3). We also observed that cellular stress [i.e., treatment with TNF- α and cycloheximide to trigger apoptosis, or with ionomycin and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to trigger necrosis] is accompanied by marked increase of eNAMPT in macrophage culture media (124), as previously described also for HMGB-1 (9) and others DAMPs (145).

eNAMPT/eNAMPT in Myeloid Cells Function: the Role of TLR4

NAD synthesis has a driving role in myeloid differentiation and in supporting macrophage inflammatory responses (42, 146, 147), prompting investigation on the function of NAMPT and NAPRT in these cells.

Increasing evidence demonstrated a direct role of NAMPT in regulating the differentiation program and the metabolic phenotypes of myeloid cells. Both iNAMPT and eNAMPT influence monocyte/macrophages differentiation, polarization and migration (132, 146, 148). We described a role for eNAMPT in creating an immunosuppressive and tumor-promoting microenvironment in chronic lymphocytic leukemia, where eNAMPT is important for the differentiation of monocytes toward tumor-supporting M2 macrophages (132). Recently, it was demonstrated that iNAMPT acts also on myeloid-derived suppressor cells (MDSCs), where NAMPT blocks *CXCR4* transcription, via a SIRT1/HIF-1 α axis. The activation of this circuit, in turn, leads to MDSCs mobilization and enhances the production of nitric oxide, promoting immunosuppression (149).

The NAMPT/NAD/SIRT1 axis seems to play a relevant role in myeloid cell activation. NAMPT-dependent NAD generation is crucial in the metabolic switch characterizing the transition from the early stage of acute inflammation, primarily relies on glycolysis, to the later adaptation phase more dependent on fatty acid oxidation (FAO) for energy production (150–152). Moreover, NAMPT/NAD levels significantly increased during activation of pro-inflammatory M1 macrophages (153). In a further feedback loop some cytokines, including IL-6 and TNF- α , induced during monocyte activation, are able to promote NAMPT expression via HIF-1 α . In turn, NAMPT, triggering NF- κ B signaling pathway, sustains *IL6* and *TNFA* transcription forcing myeloid cell activation (131). It has been also shown that NAMPT/NAD/SIRT1 axis can regulate neutrophilic granulocyte differentiation via CCAAT/enhancer-binding protein α/β (C/EBP α/β) induction, ultimately, up-regulating granulocyte colony-stimulating factor (G-CSF). In turn, G-CSF further increases NAMPT levels (148). NAMPT inhibition, reducing

NAD levels, thereby decreasing SIRT1 activity, leads to the dramatic elevation of acetylated C/EBP α levels and reduces amounts of total C/EBP α protein, accompanied by diminished mRNA expression of C/EBP α target genes (G-CSF, G-CSFR, and ELANE) (148, 154). Moreover, exposure of the acute myeloid leukemia cell line HL-60 to recombinant NAMPT or NAMPT overexpression induced myeloid differentiation of these cells *per se* (154).

A controversial issue in NAMPT biology is whether its cytokine-like properties are all linked to its enzymatic activity or are mediated by the binding to a cell surface receptor. In 2015, Camp et al. showed that eNAMPT produces robust TLR4-mediated NF- κ B signaling activation, by directly binding TLR4-MD2 (26) (**Figure 3**). However, due to possible contamination of LPS, the natural ligand of TLR4, in the recombinant NAMPT preparations used to treat cells *in vitro*, the interpretation of these results remains controversial. Our group recently confirmed the binding of eNAMPT to TLR4 in macrophage cellular model (124), performing surface plasmon resonance (SPR) under the same conditions previously established for the NAMPT-TLR4 interaction (26). More recently, the same group that firstly identified TLR4 as NAMPT receptor published new details about this interaction (155). At the same time, a direct role of NAD in activating the inflammasome was recently reported by Yang et al. The authors demonstrated that NAD manipulation, using NAMPT inhibitors or the treatment with NAD precursors, affects TLR4-mediated NF- κ B activation and PYD-domain 3 (NLRP3) inflammasome activity connecting intracellular NAD levels and inflammation (156).

Similar properties were attributed to eNAPRT. By using a surface coated with an anti-NAPRT antibody, we showed that a pre-mixed solution of recombinant (r)NAPRT and rTLR4 resulted in increased binding when compared to rNAPRT alone, indicating that a direct molecular interaction was occurring between the proteins (124). TLR4 triggering by rNAPRT activates an inflammatory signature in human macrophages differentiated from PBMC of healthy donors, as observed also using rNAMPT, promoting robust activation of NF- κ B signaling, transcription and secretion of pro-inflammatory cytokines, including IL-1 β , IL-8, TNF- α , CCL3, and inflammatory mediators such as caspase-1 (CASP1) and P2X purinoreceptor (124) (**Figure 3**). These effects are lost in TLR4-silenced macrophages. Accordingly, in macrophages, derived from TLR4^{-/-} mice, rNAPRT exposure was not able to activate NF- κ B signaling and cytokine production. Lastly, we demonstrated that the rNAPRT enzymatic deficient mutant is still able to trigger inflammasome in macrophages, indicating that the enzymatic activity is irrelevant to the pro-inflammatory functions of eNAPRT.

rNAPRT, as previously observed for eNAMPT (132, 146, 148), is also able to force monocyte differentiation into macrophages, up-regulating macrophage colony-stimulating factor (M-CSF). This function in triggering macrophage differentiation is a unique feature of eNAMPT/eNAPRT and not shared by LPS, suggesting that even though TLR4 is a receptor for multiple soluble factors and proteins, each specific ligand has a peculiar role. Notably, eNAPRT could be detected in macrophage culture

supernatants, suggesting that macrophages are a source of eNAPRT *in vivo*.

Lastly, in this paper, we demonstrated that the signaling functions of hNAMPT and hNAPRT are not an evolutionarily conserved trait. In fact, the bacterial rNAPRT (PncB) or the bacterial rNAMPT (NadV) invariably failed to activate NF- κ B signaling in macrophages. Furthermore, a comparison of the surface properties of the bacterial and hNAPRT proteins revealed the presence in hNAPRT of an arginine-rich stretch (65RFLRAFRLR) forming a large mouth-like positively charged area on the top of the dimer, which is absent in the bacterial ortholog, but is present in a similar form in NAMPT, and could be involved in the binding to TLR4 (124). Even if several issues remain to be investigated, these data support the notion of another NBE acting as extracellular mediator with a direct role in macrophage functions, binding TLR4.

NAMPT AND NAPRT AS BIOMARKERS OF CHRONIC AND ACUTE INFLAMMATORY DISEASES

iNAMPT over-expression as well as increased circulating levels of eNAMPT were documented in metabolic/inflammatory conditions including obesity, type 2 diabetes, metabolic syndromes, atherogenic inflammatory diseases, therefore supporting a role for eNAMPT as a potential biomarker of cardio- cerebro-vascular disorders (157–160). Enhanced eNAMPT levels are also described in kidney transplantation recipients (161), polycystic ovary syndrome (162), preeclampsia (163), and acute coronary syndrome (158, 164). Increased eNAMPT levels were additionally reported in non-metabolic chronic inflammatory diseases [i.e., osteoarthritis (103) and acute lung injury (ALI) (165, 166)], characterized by systemic inflammation. eNAMPT also seems to play a role in several types of infections like sepsis (167, 168) or intrauterine infection (chorioamnionitis) (169), and in autoimmune inflammatory diseases including psoriasis (170), rheumatoid arthritis (RA) (171) Crohn's disease (CD) and ulcerative colitis (UC) (172). **Table 1** summarized main activities of i/eNAMPT in these pathological conditions.

The first indication that NAPRT can be present in the extracellular space was published by our group in 2019. We dosed eNAPRT in sera from patients with sepsis or septic shock due to bacterial infections. Our results indicated that median eNAPRT levels picked-up to ~25 ng/ml in septic individuals (compared to a median of about 2 ng/ml in HD), underlying high levels of this enzyme in this acute inflammatory condition (124). Circulating NAPRT has a role in mediating endotoxin tolerance at low/physiological doses, in fact the highest plasmatic eNAPRT levels were dosed in patients who died because of septic shock, while those with low concentrations survived. We confirmed a significant association between high levels of eNAPRT and mortality, suggesting that eNAPRT is a novel risk factor in sepsis (124). Even if the biological explanation behind this observation is still partly missing, findings in our work are significant starting points to evaluate the functional

TABLE 1 | NAMPT and NAPRT functions in chronic and acute inflammatory diseases.

Type of disease	Main findings	Therapeutic options	References
e/iNAMPT			
Type 2 diabetes	-Higher eNAMPT levels in cases than controls -eNAMPT induces a diabetic phenotype in pancreatic islets	Blocking Ab?	(157, 173)
Obesity	-Higher eNAMPT levels in cases than controls -iNAMPT supports adipose plasticity and the pathological progression to obesity.	Blocking Ab? Pharmacological inhibitors?	(157, 174)
Atherogenic inflammatory diseases; cardio- cerebro-vascular disorders; stroke; acute coronary syndrome	-eNAMPT prognostic marker of atherosclerosis, endothelial dysfunction, and vascular damage -Active player promoting vascular inflammation -Deregulated NAD metabolism	Blocking Ab? Pharmacological inhibitors (pre-clinical)	(158–160, 164, 175)
Kidney transplant recipients	-eNAMPT was significantly higher in kidney allograft recipients than in HD -Associated with endothelial damage	Blocking Ab?	(161)
Polycystic ovary syndrome; preeclampsia	-Higher eNAMPT levels in cases than controls - eNAMPT induces the expression of pro-angiogenic factors	Blocking Ab?	(162, 163)
Acute lung injury (ALI); acute respiratory distress syndrome (ARDS)	-Higher eNAMPT levels in cases than controls -eNAMPT induces the secretion of inflammatory cytokines and activation of signaling pathways -iNAMPT supports NAD metabolism, inhibiting apoptosis	Blocking Ab (pre-clinical) Pharmacological inhibitors (pre-clinical)	(165, 166)
Sepsis; septic shock	-Higher eNAMPT levels in cases than controls -eNAMPT induces the secretion of inflammatory cytokines and activation of signaling pathways, supporting inflammation -Diagnostic and prognostic biomarkers (risk factor)	Blocking Ab?	(167, 168)
Intrauterine infection (chorioamnionitis)	-Higher eNAMPT levels in cases than controls	Blocking Ab?	(163, 169)
Psoriasis	-Higher eNAMPT levels in cases than controls -Positive correlation with disease severity	Blocking Ab?	(170)
Rheumatoid arthritis (RA); osteoarthritis	-Higher eNAMPT levels in cases than controls -eNAMPT induces the secretion of inflammatory cytokines and activation of signaling pathways -Block NAMPT have reduced RA progression and inflammatory markers	Blocking Ab? Pharmacological inhibitors (pre-clinical)	(103, 171, 176)
Inflammatory bowel disease (IBD); Crohn's disease (CD); and ulcerative colitis (UC)	-iNAMPT/eNAMPT overexpression/secretion -Association with inflammation, hypoxia (active) and tissue repair (inactive disease)	Blocking Ab? Pharmacological inhibitors (pre-clinical)	(172, 177)
Solid tumors: colorectal, ovarian, breast, gastric, prostate, thyroid, pancreatic cancers, melanoma, gliomas, sarcoma, endometrial =carcinomas, and hematological malignancies	-iNAMPT/eNAMPT over expression -Negative prognostic marker - Regulates metabolic adaptation, DNA repair, gene expression, signaling pathways, cell growth, invasion, stemness, epithelial to mesenchymal transition program, metastatization, angiogenesis, secretion of both pro-inflammatory and immunosuppressive cytokines, resistance to genotoxic stress	Blocking Ab (pre-clinical) Pharmacological inhibitors (pre-clinical and phase I-II-III)	(18, 25, 106, 178, 179)
e/iNAPRT			
Sepsis/septic shock	-Higher eNAPRT levels in cases than controls -eNAPRT activates inflammasome -Risk factor for patient survival	Blocking Ab?	(124)
Prostate, ovarian, colorectal, and pancreatic cancers	-NAPRT gene amplification -iNAPRT overexpression; -correlation with a BRCAness gene expression signature -NAPRT silencing reduced energy status, protein synthesis, and cell size	Pharmacological inhibitors (pre-clinical)	(88, 106, 123, 180)

HD, healthy donors; Ab, antibody.

appears as a promising strategy to overcome NAPRT-mediated resistance to NAMPT inhibitors in patients (Table 1).

Blocking the extracellular cytokine-like function of eNAMPT and eNAPRT would be very useful to restore immune competence in cancer, as well as, infection setting (Table 1). In the tumor microenvironment, neutralization of eNAMPT using blocking antibodies could be effective to repolarize myeloid cells (TAMs/MDSCs) against tumor. Some groups/companies are working on the production of these antibodies (193), hypothesizing a combination strategy with immunotherapy, or a double inhibition of i/eNAMPT. Blocking eNAPRT in acute inflammatory conditions, such as in septic patients, could be an important strategy to prevent the damaging action of a massive secretion of eNAPRT leading to decreased survival of patients, but this remains, at this moment, only a speculative hypothesis.

In conclusion, in this review we summarized current knowledge on these two old enzymes involved in NAD biosynthesis that can powerfully modulate immune responses. If NAMPT has now an acknowledged role in regulating several cellular processes in physiological and pathological conditions,

and as biomarker in several diseases, the biology of NAPRT, especially as new soluble factor, acting as DAMP in acute inflammation, needs to be extensively studied to determine potential pharmacological settings.

AUTHOR CONTRIBUTIONS

VA and SD have made a substantial, direct and intellectual contribution to the work, contributed equally to writing the manuscript, and approved it for publication. VM contributed to writing the final version of the manuscript and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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