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NAD^+: A modulator of immune functions

Andreas Grahnert, Anja Grahnert, Carina Klein, Erik Schilling, Janine Wehrhahn, Sunna Hauschildt

Department of Immunobiology, Institute of Biology II, University of Leipzig, Leipzig, Germany

Latterly, nicotinamide adenine dinucleotide (NAD^+) has emerged as a molecule with versatile functions and of enormous impact on the maintenance of cell integrity. Besides playing key roles in almost all major aspects of energy metabolism, there is mounting evidence that NAD^+ and its degradation products affect various biological activities including calcium homeostasis, gene transcription, DNA repair, and intercellular communication. This review is aimed at giving a brief insight into the life cycle of NAD^+ in the cell, referring to synthesis, action and degradation aspects. With respect to their immunological relevance, the importance and function of the major NAD^+ metabolizing enzymes, namely CD38/CD157, ADP-ribosyltransferases (ARTs), poly-ADP-ribose-polymerases (PARPs), and sirtuins are summarized and roles of NAD^+ and its main degradation product adenosine 5′-diphosphoribose (ADPR) in cell signaling are discussed. In addition, an outline of the variety of immunological processes depending on the activity of nicotinamide phosphoribosyltransferase (Nampt), the key enzyme of the salvage pathway of NAD^+ synthesis, is presented. Taken together, an efficient supply of NAD^+ seems to be a crucial need for a multitude of cell functions, underlining the yet only partly revealed potency of this small molecule to influence cell fate.

Keywords: ADP-ribosylation, calcium channels, NAD hydrolases, Nampt, sirtuins, immune cells

Abbreviations: ADPR, adenosine 5′-diphosphoribose; $(ADPR)_2$, dimeric ADPR; AGEs, advanced glycation end-products; ARTs, ADP-ribosyltransferases; BCR, B-cell receptor; cADPR, cyclic ADPR; COX-2, cyclooxygenase-2; DCs, dendritic cells; eNampt, extracellular Nampt; ER, endoplasmic reticulum; fMLP, formyl-methionyl-leucyl-phenylalanine; GPI, glycosylphosphatidylinositol; HNPs, human neutrophil peptides; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL, interleukin; IP_3, inositol-1,4,5-trisphosphate; LPS, lipopolysaccharide; mARTs, mono-ARTs; MCP-1, monocyte chemo-attractant protein-1; MDDC, monocyte-derived dendritic cell; MVP, major vault protein; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD^+, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; Nampt, nicotinamide phosphoribosyltransferase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa B; NMN, nicotinamide mononucleotide; Nmnat, nicotinamide mononucleotide adenylyltransferase; NT, nucleotide transporter; OAADPR, 2′-*O*-acetyl-ADPR; PARGs, poly-ADPR-glycohydrolases; PARPs, poly-ADP-ribose-polymerases; PBMCs, peripheral mononuclear blood cells; PRPP, phosphoribosyl-pyrophosphate; RAGE, receptor for AGEs; ROS, reactive oxygen species; SIRT, silencing information regulator; TNF, tumor necrosis factor; TRPM2, transient receptor potential melastatin-related channel 2

Introduction

Nicotinamide adenine dinucleotide (NAD^+) has been classically known as a molecule involved in energy metabolism and electron transfer. As a redox co-enzyme, it shuttles between oxidized form (NAD^+) and reduced form ($NADH^+$) but the total concentration remains constant. However, it has been shown lately that there are several enzymes that constantly consume NAD^+. The enzymes that will be discussed in this review are the NAD^+ glycohydrolases CD38/CD157, ADP-ribosyltransferases (ARTs), poly-ADP-ribose-polymerases (PARPs), and sirtuins, which function as NAD^+-dependent protein deacetylases. Among these

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Correspondence to: Sunna Hauschildt, Department of Immunobiology, Institute of Biology II, University of Leipzig, Talstraße 33, 04103 Leipzig, Germany. Tel: +49 341 9736747; Fax: +49 341 9736789; E-mail: shaus@rz.uni-leipzig.de

enzymes, PARPs and sirtuins are localized within the cell, ARTs are expressed on the outer cell membrane surface or are secreted, whereas CD38 primarily functions as an ectoenzyme but has also been detected intracellularly.

All enzymes cleave NAD$^+$ resulting in the production of free nicotinamide. In order to sustain their activity, a salvage pathway that converts the inhibitory nicotinamide back to their substrate NAD$^+$ is essential. Only recently has the rate-limiting enzyme in this pathway, nicotinamide phosphoribosyltransferase (Nampt), been extensively studied.

The utilization of NAD$^+$ by the enzymes mentioned here leads to the formation of free NAD$^+$ metabolites like adenosine 5′-diphosphoribose (ADPR), cyclic ADPR (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP) or 2′-*O*-acetyl-ADPR (OAADPR), all of which were shown to have Ca^{2+} mobilizing properties. Adenosine 5′-diphosphoribose is described to be the endogenous activator of the non-selective cation channel TRPM2 (transient receptor potential melastatin-related channel 2) causing Ca^{2+} influx across the cell membrane and, in addition, ADPR seems to be a ligand for G-protein-coupled receptors. Ligand activity at the TRPM2 channel is also discussed for OAADPR. Cyclic ADPR and NAADP are highly effective in inducing the depletion of intracellular calcium stores, both by binding to ryanodine receptors on the endoplasmic reticulum (ER) or, in the case of NAADP, to a not yet fully identified receptor on acidic stores.

Thus, NAD$^+$ and its metabolites not only serve as substrates for nucleotide metabolizing enzymes. They also act as ligands for ion channels and extra- and intracellular receptors (Fig. 1).

Without extensively reviewing the properties and targets of NAD$^+$, here we intend to draw the attention to the potential of NAD$^+$ to regulate important immunological functions.

CD38 and CD157

CD38 is a type II glycosylated membrane protein with a short terminal cytoplasmic tail. In addition to its

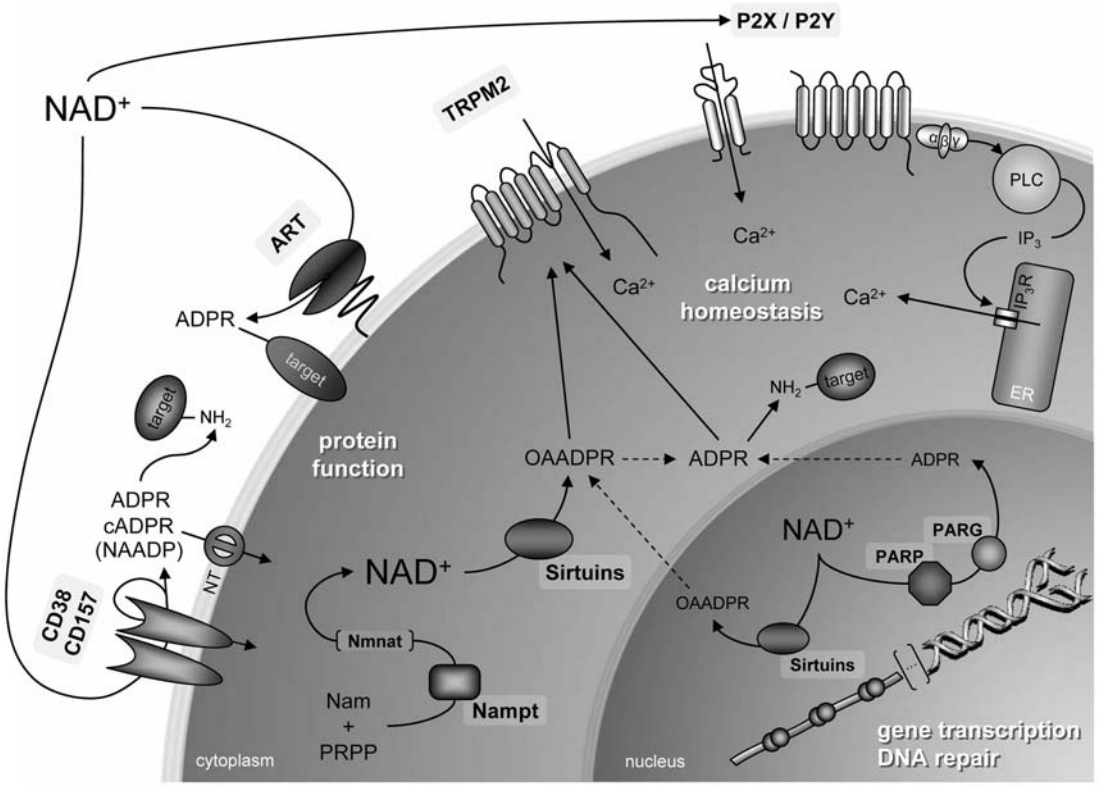

Fig. 1. Schematic presentation of NAD$^+$ and its metabolites in cell signaling. Extracellular NAD$^+$ can serve as a substrate for CD38/CD157 and ARTs or it can act as a ligand for P2X and P2Y receptors. CD38/CD157 catalyze the formation of the ADPR, cADPR, and NAADP and ARTs transfer an ADPR moiety to their target protein. The PARPs use intracellular NAD$^+$ to generate poly-ADP-ribosylated targets. The removal of the ADPR moieties from the targets is catalyzed by PARGs. The ADPR, cADPR, and NAADP as well as OAADPR, a reaction product of sirtuins, display Ca^{2+} mobilizing properties with the ADPR and OAADPR acting on TRPM2 channels. The ADPR can also serve as a substrate for non-enzymatic ADP-ribosylation (glycation) of free amino groups. Nampt plays an essential role in the salvage pathway of NAD$^+$.

transmembrane form, a 39-kDa soluble form was identified in normal and pathological biological fluids.[1–3] CD38 is widely known as a multifunctional enzyme since it transforms NAD^+ into ADPR (NAD^+ glycohydrolase activity) and cADPR (ADPR cyclase activity) and hydrolyzes cADPR into ADPR (cADPR hydrolase activity). Moreover, in the presence of high concentrations of free pyridines, this enzyme also catalyzes base-exchange reactions (transglycosidation activity), allowing for the formation of NAADP from $NADP^+$ (Fig. 2).[4]

Both cADPR and NAADP are powerful mobilizers of intracellular Ca^{2+}.[5,6] They seem to target separate Ca^{2+} stores and to be bound by distinct receptors. Cyclic ADPR mobilizes the endoplasmic Ca^{2+} stores[7,8] via ryanodine receptors while NAADP releases Ca^{2+} from lysosomes and endosomes.[9] However, NAADP has also been shown to release Ca^{2+} from the endoplasmic reticulum.[10,11] Adenosine 5′-diphosphoribose the main degradation product activates the TRPM2 channel and thus facilitates Ca^{2+} influx.[12]

Recently, CD38 has been described to catalyze the exchange of ADPR with the nicotinamide group of NAD^+ leading to the formation of a dimeric ADPR, $(ADPR)_2$.[13] As shown in sea urchin eggs, $(ADPR)_2$ itself did not release Ca^{2+} from microsomal vesicles, but potentiated the Ca^{2+} releasing activity of subthreshold concentrations of cADPR. Thus, $(ADPR)_2$ is a new product of CD38 that amplifies the Ca^{2+} mobilizing activity of cADPR.

Soon after the discovery that CD38 is an ectoenzyme, the question was raised how cADPR and ADPR generated by CD38 can function inside the cell. De Flora *et al.*[13] suggested that the extracellular messengers generated by CD38 are directly transported by CD38 or, more likely, by nucleotide transporters (NT) into the

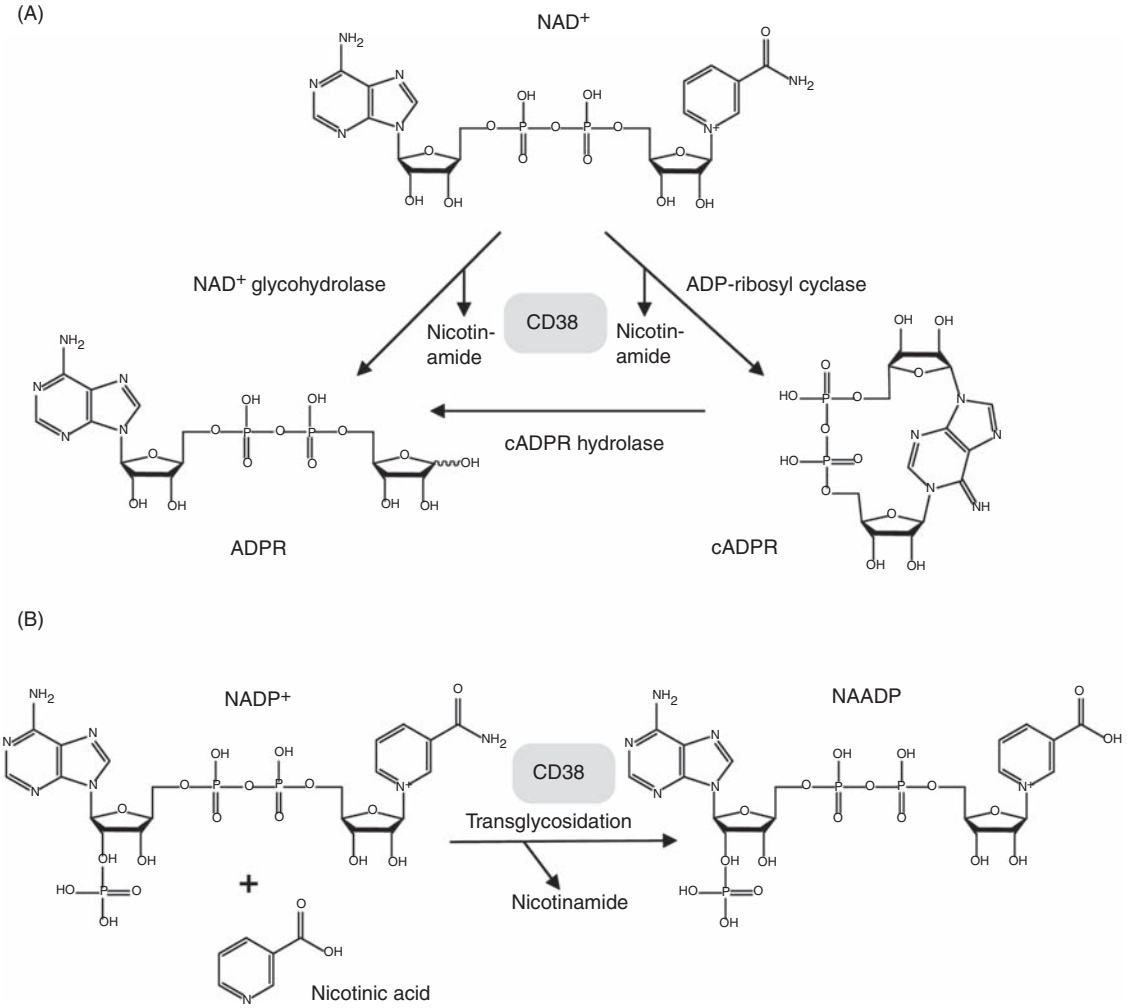

Fig. 2. CD38 catalyzed reactions. (A) The extracellular NAD^+ metabolizing enzyme CD38 catalyzes the formation of cADPR and ADPR by ADPR cyclase and NAD^+/cADPR hydrolase activity, respectively. (B) In addition, CD38 mediates the generation of NAADP from $NADP^+$ by a transglycosidation reaction.

cytosol (reviewed by De Flora *et al.*[14]). In addition, there are reports indicating that CD38 is localized in intracellular nuclear membranes[15,16] with potential access to intracellular NAD^+ allowing for the formation of ADPR and cADPR inside the cell.

Apart from its enzymatic properties, CD38 also signals as a receptor. In response to its ligand CD31 or agonistic CD38 antibodies, CD38 not only operates as an adhesion molecule involved in cell–cell interaction,[17] but also initiates transmembrane signaling.[18,19]

CD38 is present on a wide number of different tissues[20] but predominantly expressed on hemopoietic cells, where it shows a rather peculiar distribution pattern. It is strongly expressed on lymphocyte precursors, declines once the cells differentiate and is up-regulated again on mature lymphocytes and plasma cells.[20–23]

Ligation of CD38 on human peripheral T- and B-cells with agonistic anti-CD38 monoclonal antibodies which mimic the natural ligand, results in various responses including cell activation, proliferation, transcription of cytokine genes, and protein phosphorylation (for review, see Malavasi *et al.*[24]). Analysis of CD38-mediated signaling mechanisms revealed several interesting features. First, CD38 which cannot signal by itself shares steps in signal transduction pathways with the T-cell and B-cell receptor.[24] Second, CD38-mediated signal transduction resulting in biological effects occurs independently of its enzymatic activity.[24] Third, localization of CD38 to lipid rafts is essential for signaling.[19,25] In dendritic cells (DCs), CD38 also takes part in the regulation of immune responses.[26] The pattern of CD38 expression during the monocyte/mMDDC (mature monocyte-derived dendritic cell) transition is reminiscent of that found in lymphocytes.[27,28] CD38 is highly expressed by monocytes while down-regulated during differentiation into immature MDDCs and re-expressed by mature MDDCs.[29] Differentiation of monocytes to macrophages is accompanied by a down-regulation of CD38 expression.[30] This decrease correlates with a reduction in NADase activity, indicating that the amount of functional active CD38 molecules decreases during differentiation.[30] In monocytes, CD38 plays a role as a co-receptor in the activation of T-lymphocytes induced by superantigens.[31] Ligation of CD38 by an agonistic monoclonal antibody results in the release of interlukin (IL)-1β, IL-6, and IL-10.[32] Up-regulation of CD38 was observed upon treatment with interferon (IFN)-γ whereas other monocyte activators such as lipopolysaccharide (LPS) did not affect CD38 expression.[33]

In the last few years, CD38 has gained much interest as a marker in the diagnosis of leukemia and myeloma, B-CLL (chronic lymphocytic leukemia), HIV and NIDDM (type II non-insulin-dependent diabetes mellitus).[34–37]

Like CD38, CD157 displays a dual nature behaving both as an ectoenzyme and as a receptor.[4] It shows 36% amino acid identity with human CD38 and 33% with the soluble ADPR cyclase from *Aplysia californica*.[38] Because it lacks a cytoplasmic domain, CD157 is likely to be associated with some conventional receptors to compensate the structural limit to transduce signals. Recent results suggest that CD157 is functionally and structurally associated with the CD11b/CD18 complex on human neutrophils.[39]

Furthermore, cross-linking of CD157 by a polyclonal antibody induces tyrosine phosphorylation of a 130-kDa protein identified as focal adhesion kinase (FAK) in selected myeloid cell lines.[40,41] CD157 also regulates calcium homeostasis, promotes polarization in neutrophils, and mediates superoxide (O_2^-) production in the human U937 myeloid cell line.[42,43] Additionally, CD157-mediated signals control diapedesis and regulate chemotaxis induced by the high-affinity formyl-methionyl-leucyl-phenylalanine (fMLP) receptor.[43,44]

Similar to the *Aplysia* enzyme and to CD38, soluble CD157 incubated with NAD^+ produces cADPR and subsequently ADPR, indicating that this molecule has both ADPR cyclase and cADPR hydrolase activities.[45,46] But the enzymatic efficiency of CD157 is several hundred-fold lower than that of CD38. It requires an acidic pH and metal ions far beyond a physiological context.[41,45,47,48] These different properties may be useful in discriminating between CD157 and CD38 activities and suggest that the enzymatic activity of each molecule can play distinct roles in different environments.[20]

Like CD38, CD157 also seems to be associated with the pathogenesis of certain diseases. Thus, high serum concentrations of soluble CD157 have been reported in patients with severe rheumatoid arthritis characterized by joint destruction.[49]

ARTs and PARPs

Among its functions in cell metabolism, NAD^+ can serve as a substrate for a post-translational protein modification called ADP-ribosylation. This modification, which involves the transfer of the ADPR moiety of NAD^+ to an acceptor amino acid in target proteins, usually results in an alteration of the target protein's function.[50,51] The enzymes catalyzing the reaction are named ARTs. Depending on the number of ADPR moieties transferred, they are classified into two groups. The mono-ARTs (mARTs) transfer a single ADPR moiety to their specific target proteins whereas the PARPs catalyze the transfer of multiple moieties and branching of the ADPR chain. The number of ADPR

units within these negatively charged poly-ADPR-polymers can reach up to 400 *in vitro* even more *in vivo* (reviewed elsewhere[52–55]). The recent observation that PARP 10, expected to function as a PARP, acts as a mART, puts the latter remark into perspective and will be discussed below.[56]

More than 40 years ago, the mono-ADP-ribosylation was discovered as the mechanism by which the diphtheria toxin inhibits the mammalian protein synthesis via targeting the eukaryotic elongation factor 2.[57] Thus, it is not surprising that bacterial ARTs are the best characterized ARTs with regard to their molecular organization, function and substrate specificity.[58] However, toxin-related ARTs were also found in vertebrates. In mammals, six ART-family members were identified that are expressed as secreted or GPI-anchored extracellular enzymes (reviewed elsewhere[59–61]). They include four human (ART1, 3, 4, and 5) and six murine (ART1, 2.1, 2.2, 3, 4, and 5) paralogues. The avian group of ARTs encompasses five members designated as ART6.1, 6.2, 7.1, and 7.2 as well as the recently described avian ART4 orthologue.[62–64] Except for mammalian ART3 and ART4, all other ARTs transfer the ADPR to arginine residues in their target proteins, most likely due to the presence of conserved amino acid residues in the catalytic domain the so called R-S-EXE motif.[61,65]

Under physiological conditions, the concentration of NAD^+ in extracellular body fluids is in the submicromolar range, below the K_m of ARTs, whereas cytosolic NAD^+ amounts to 1 mM.[66] To reach high extracellular NAD^+ concentrations, NAD^+ can be released into the extracellular compartment from cells as a consequence of cell lysis during tissue injury and inflammatory immune reactions.[67–70] However, there is also some evidence that connexin 43, a component of intercellular gap junctions, can mediate the transport of NAD^+ across the cell membrane into the extracellular space and that mechanical stress and electric field stimulation can provoke specific release mechanisms.[71–73]

The ADP-ribosyltransferases are widely expressed in cells of the immune system including T-cells,[74] B-cells,[75] monocytes,[76] and granulocytes.[77] Amongst the ART family members, ART1 and ART2 are the best characterized so far. As the first mammalian ART, ART1 was identified after protein purification from skeletal muscle and cDNA cloning from rabbit,[78] human skeletal muscle,[79] and mouse lymphoma cells.[80]

The ARTs show a rather tissue-specific expression with ART1 predominantly expressed in skeletal and cardiac muscle.[65] In a search of proteins being ADP-ribosylated by ART1, α7-integrin was identified as a key protein.[81,82] Modification of α7-integrin has a positive effect on the interaction with its ligand laminin which may be of advantage in situations where enhanced interactions are required, such as muscle injuries or diseases.[69] The transcriptional regulation of ART1 seems to involve molecular mechanisms similar to those used to activate muscle-specific genes.[83]

When overexpressing ART1 in a mouse T-cell lymphoma, it ADP-ribosylates a couple of cell surface proteins, *e.g.* LFA1, CD27, CD43, CD44, and CD45. This causes an inhibition of T-cell receptor signaling, concomitant with a suppression of $p56^{lck}$ kinase activation.[84]

The ART1 protein has also been detected in human polymorphonuclear neutrophil granulocytes that participate in innate immune responses.[85] When the cells are stimulated with the chemotaxins fMLP, IL-8 or the platelet activating factor, ART1 cell surface expression increases, most likely due to the recruitment of ART1 from intracellular stores.[85] Furthermore, the stimulation of neutrophil leukocytes resulted in the secretion of antimicrobial and cytotoxic HNPs (human neutrophil peptides) that belong to the group of defensins.[86] These cationic peptides are arginine-rich and contain three disulfide bonds.[87] They can induce IL-8 synthesis in airway epithelial cells and function as T-cell chemoattractant proteins.[88,89] The high content of arginine residues in HNPs predisposes them for ADP-ribosylation by ART1. Indeed, ART1 has been shown to ADP-ribosylate HNP-1 *in vitro* thereby modulating its properties. Studies of Paone *et al.*[90,91] suggest that ADP-ribosylated HNP-1 may regulate the inflammatory response by inhibiting antimicrobial and cytotoxic activities of HNP-1, while retaining its T-cell chemoattractance and promoting neutrophil recruitment.[90] Not only *in vitro* but also *in vivo* ADP-ribosylated HNP-1 and even di-ADP-ribosylated HNP-1 have been found. The modified HNP-1 was isolated from the bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis, asthma, or from smokers.[90,91]

Nicotinamide adenine dinucleotide is an excellent substrate for ART2. There are two isoforms of ART2, designated ART2.1 and ART2.2. When cytotoxic T-cells are exposed to NAD^+, the cells proliferate less and show a diminished cytotoxicity. This inhibition is accompanied by an ADP-ribosylation of cell surface proteins, *e.g.* LFA-1, CD8, CD27, CD43, CD44, and CD45.[92] Furthermore, NAD^+, when added to T-cells or injected into mice, rapidly induces T-cell apoptosis, an effect which is mediated by the purinergic $P2X_7$ receptor. An ADP-ribosylation of $P2X_7$ Arg-125 results in Ca^{2+} influx, macropore formation, phosphatidylserine exposure, CD62L shedding, and accelerated cell death.[93–96] Activated T-cells shed the ART2 molecule and, therefore, reduce their susceptibility to the NAD^+-induced T-cell death.[97] According to Seman *et al.*[95] the T-cell death may represent a safeguard mechanism against the undesirable activation of irrelevant and

potentially autoreactive bystander T-cells during inflammatory responses where NAD^+ is thought to be released. Due to the presence of three premature stop codons in the corresponding gene, ART2 is not expressed in humans.[98] The ART4, another member of the ART family, shows a predominant expression in hemopoietic and lymphatic tissues similar to ART2.[99] As ART4 appears to have lost its enzyme activity, its functional role in mammals is unclear. However, the fact that it represents the Dombrock blood group antigen present on erythrocytes and that it is up-regulated in response to toll-like receptor (TLR)2 and TLR4-dependent stimuli in human monocytes and alveolar epithelial cells suggests that it may have acquired other biological functions.[76,100–102]

Similar to ART4, ART3 does not display any enzyme activity. It is predominantly expressed in human testis and, to a lesser extent, in heart and skeletal muscle.[99] This tissue distribution seems to be driven by two different promoters as the ART3 gene transcripts differs in the 5′-untranslated region in these tissues.[103] Its expression in human testis is restricted to spermatocytes, indicating that ART3 exerts a specific function required at a particular stage of spermatogenesis.[104] A weak expression of ART3 mRNA has also been observed in human monocytes and macrophages.[76,103]

The cDNA of ART5 was originally cloned from the Yac-1 murine lymphoma cell line.[105] It is most strongly expressed in mouse and human testis.[65,105] While the human ART5 possesses ART activity, mouse ART5 shows mainly NAD^+-glycohydrolase (NADase) activity. At high NAD^+ concentrations (1 mM), it loses its NADase activity and gains ART activity.[65,106] A role of ART5 in immune cells is not known.

In chicken, two ARTs (ART6 and ART7) have been identified. The ART6, present in chicken polymorphonuclear leukocytes (so called heterophiles) is released from cytoplasmic granules after stimulating the cells with a calcium ionophore or serum-opsonized zymosan. Simultaneously, a target protein (p33) is set free from the same granules. In the presence of NAD^+, p33 becomes ADP-ribosylated, suggesting that ARTs may be involved in regulating neutrophil functions.[107] Besides p33, tuftsin has been identified as a target of ART6. This tetrapeptide present in the chicken plasma can stimulate phagocytosis of macrophages. When it becomes ADP-ribosylated, its biological activity is diminished.[108]

The ART7 enzyme consists of two isoforms (ART7.1 and ART7.2) which are differentially expressed in chicken tissues. Besides bone marrow and lung, ART7.1 mRNA is predominantly expressed in immune cells (*e.g.* T- and B-cells), whereas a strong expression of ART7.2 mRNA was found in heart and skeletal muscle.[75,109]

The isoform ART7.1 is expressed on chicken B-cells as a GPI-anchored molecule. The transfection of DT40 cells, an immature chicken B-cell line, with ART7.1 leads to long-lasting phosphorylations of ERK and Akt kinase as well as proliferation after cross-linking the B-cell receptor (BCR) with anti-IgM antibodies.[75] Terashima *et al.*[75] suggested that ART7.1 on B-cells might function as a B-cell co-receptor to augment BCR signaling and ultimately regulate B-cell maturation. The role of NAD^+ in this process has yet to be defined.

In summary, the present data point to different roles for ART subtypes during immune responses and the importance of ARTs in the immune system can be underlined by stimuli-induced mRNA and protein expression.[76,101,110]

Poly-ADP-ribose-polymerases in contrast to ARTs transfer more than one ADPR moiety to the target protein. The best studied function of this reaction is their involvement in the DNA damage repair. The synthesis and transfer of ADPR polymers occur immediately after DNA damage. This leads to a drastic decrease in the intracellular NAD^+ content within minutes.[111–113] The poly-ADP-ribosylation seems to be a very ancient cellular process since poly-ADP-ribosylation-like reactions and PARP-like protein encoding genes have been identified in higher and lower eukaryotes, Bacteria, and Archaea.[52,114,115]

Until now, 17 members of the PARP family have been described in humans. According to Till *et al.*[116] they can be divided into three subgroups. The first group contains PARP1–6 as 'classical' PARPs, the second group PARP7, 8, 10–12, and 14–16 which are likely to be mono-ADP-ribosyltransferases, and the third group includes PARP9 and 13, which lack enzyme activity. The recent finding that PARP10 acts as a mART rather than a PARP is possibly due to a mechanism designated as substrate-assisted catalysis. In this case, the catalytic glutamic acid residue is not present in the transferase itself but is localized in the target protein, leading to mono-ADP-ribosylation of acidic residues in this protein.[56] This strengthens the hypothesis that ART3 and ART4, obviously enzymatically inactive, exhibit such a narrow target specificity that it cannot be detected with usually applied ART-activity assays.[65]

The best characterized PARP so far is PARP1.[52] The protein becomes activated and increases its activity in response to DNA single-strand breaks as well as being involved in DNA repair mechanisms.[117] The PARP1 enzyme is cleaved by caspases in an early stage of apoptosis, indicating that the loss of PARP1 function is required for the efficient completion of apoptosis.[118] Besides its function in poly-ADP-ribosylation-mediated cell death and in modulation of chromatin structure, PARP1 can also act as co-activator or co-repressor of gene transcription (reviewed by Hassa and Hottiger[52]). For example, PARP1 binds to nuclear factor-kappa B (NF-κB), a key regulator in the expression of

inflammatory mediators such as cytokines, chemokines, and adhesion molecules.[119] Inhibitors of PARP1 can attenuate the severity of inflammatory diseases including colitis, asthma, and experimental allergic encephalomyelitis indicating that PARP1 accounts for deregulated inflammation.[120–122] Another report describes the influence of PARP1 on gene expression of CD3/CD28 activated T cells. The expression of more than 200 genes is significantly altered by PARP1-deficiency including genes associated with the immune response (chemokines) or genes involved in the Th1/Th2 balance.[123] This is in accordance with the induction of PARP activity in activated T-cells. Members of the nuclear factor of activated T cells (NFAT) family (NFATc1 and NFATc2) interact with PARP1 and are substrates for poly-ADP-ribosylation. Therefore, poly-ADP-ribosylation modulates the activation of NFAT in T cells.[124]

A closely related molecule to PARP1 is PARP2. They share about 43% identity in the catalytic domain. PARP2 binds to damaged DNA, synthesizes poly-ADPR-polymers in a DNA-dependent manner and displays automodification activity similar to PARP1.[125] The substrates and the physiological functions of PARP2 remain to be elucidated.[52] Interestingly, PARP1/PARP2 double knock-out mice are not viable and die at the onset of gastrulation, indicating that the expression of both proteins is essential during early embryogenesis.[126]

The least analyzed and smallest PARP is PARP3. It contains a small DNA binding domain with a potential targeting motif responsible for its centrosomal localization.[127,128]

Additional proteins that interact with PARP1–3 include kinases involved in signal transduction, histones, PARPs itself, or centromere binding proteins as summarized by Hassa and Hottiger.[52]

Another protein with PARP activity is PARP4 alias vault-PARP. This 193-kDa protein is a component of giant ribonucleoprotein particles (vaults) which besides PARP4 contain the major vault protein (MVP), the telomerase-associated protein (TEP-1), and small RNAs.[129–131] The MVP is expressed in cells of the monocytic lineage as well as in DCs. During maturation of DCs, an up-regulation of MVP could be observed. The treatment of DC cultures with polyclonal anti-MVP antibodies results in a reduced expression of differentiation and maturation markers including CD1a and CD86 and shows an inhibitory effect on antigen-specific T-cell proliferation and IFN-γ secretion.[132] This points to a role of vaults in supporting DC maturation and resulting immune responses.[129] Another innate immune response influenced by MVP is the clearance of pathogens during lung infection by *Pseudomonas aeruginosa*. Lung epithelial cells from MVP knock-out mice show a decreased internalization of the bacteria.[133] However, the role of PARP4 in these processes remains to be clarified.[52]

For the sake of completeness, we mention two other molecules known as PARP5 and PARP6 alias tankyrase-1 and tankyrase-2. Both proteins were identified as components of a telomeric complex and both display poly-ADP-ribosyltransferase activity.[134,135] They are involved in the regulation of telomere length requiring the catalytic activity of the PARP domain.[136]

Sirtuins

The sirtuins are a phylogenetically conserved family of enzymes found in diverse organisms, from Archaea to humans.[137] They all have a NAD^+-dependent catalytic core domain that may act as an ART and/or a NAD^+-dependent deacetylase.[138] In mammals there are seven sirtuins (SIRT1–7) with SIRT1 being the most extensively studied.[139] Human SIRT1 is homologous to the prototypical yeast sirtuin Sir2p, which is essential for maintaining silent chromatin via the deacetylation of histones.[140–142] NAD^+-dependent deacetylation involves the transfer of acetyl groups from lysine residues of the target protein to the ADPR moiety of NAD^+, generating nicotinamide and OAADPR (Fig. 3).[143] The latter compound has been implicated in activating the cytoplasmic domain of the TRPM2 channel, a non-selective cation channel, whose prolonged activation leads to cell death.[144]

Sirtuin1 resides primarily in the nucleus and deacetylates various proteins such as the transcriptional co-activators PGC1-α[145] and p300,[146] histones (H1, H3, H4)[147] and multiple transcription factors such as FOXOs,[148–152] p53,[153–155] and NF-κB.[156,157] These transcription factors mediate stress resistance, apoptosis and inflammatory responses that participate in physiological responses to toxicity. The SIRT1 molecule has varying effects on FOXO-induced gene expression, ranging from activation to repression.[150,152,158,159] Its ability to up-regulate stress-protective pathways and to inhibit apoptotic processes are of particular relevance for neutrophil granulocytes and monocytes/macrophages, cells that are exposed to high concentrations of reactive oxygen intermediates generated during the oxidative burst.

It has been shown that SIRT1 in situations of oxidative stress, for which extracellular application of H_2O_2 is an experimental model, deacetylates FOXO transcription factors,[148–152] leading to an increased transcription of enzymes being engaged in oxygen detoxification such as manganese superoxide dismutase.[150]

In addition, by deacetylating FOXO, SIRT1 suppresses transcription of the pro-apoptotic proteases

caspase 3 and caspase 7 as well as FAS and BIM (Bcl-2 interacting mediator of cell death).[150,160]

Of specific interest for immune cells is the role of SIRT1 in regulating NF-κB, which plays a central role in mediating innate and adaptive immune responses. Yeung *et al*.[157] were the first to show that SIRT1 physically interacts with the RelA/p65 subunit of NF-κB and specifically deacetylates lysine 310 suggesting that acetylated lysine 310 potentiates the transactivation capacity of the NF-κB complex.[161]

Suppression of the normal function of SIRT1 as a negative regulator of NF-κB has been described to occur during HIV infection.[162] For HIV to replicate, it requires activated T-cells, since T-cell activation enhances viral transcription through the activation of various transcription factors, notably NF-κB.[163] The viral transactivator Tat directly interacts with SIRT1 and blocks its ability to deacetylate NF-κB. As acetylated p65 is highly active, Tat potently activates NF-κB-dependent gene expression in infected T-cells, leading to T-cell hyperactivation and later on to a decline in T-cells.

A decrease in SIRT1 levels associated with an increased acetylation of RelA/p65 NF-κB has also been described in monocytes when exposed to cigarette smoke.[164] Furthermore, the authors showed that cigarette smoke-induced IL-8 production was inversely correlated with SIRT1 levels. These data, together with the finding that SIRT1 levels were diminished in lungs of smokers and patients with chronic obstructive pulmonary disease,[164] points to a pivotal role of SIRT1 in regulating NF-κB-dependent pro-inflammatory mediators in inflammatory lung diseases.

To reveal important physiological functions affected by SIRT1, Sequeira *et al*.[165] created SIRT1-null mice and studied their phenotypes. Among a variety of defects, mice lacking SIRT1 suffer from a mild

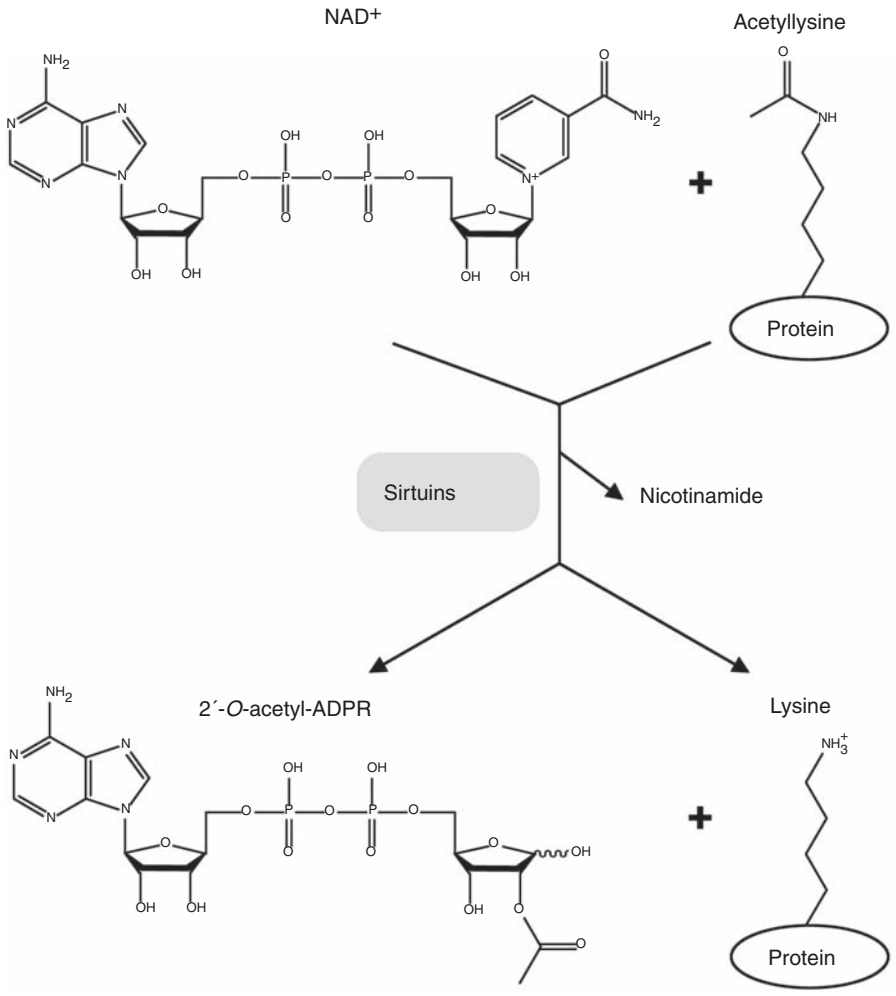

Fig. 3. Sirtuin-mediated deacetylation reaction. Sirtuins catalyze the NAD^+-dependent deacetylation of an acetylated lysine side chain, resulting in the formation of the OAADPR.

autoimmune condition that is manifested by the deposition of immune complexes in the liver and kidneys. The autoimmune condition in which antinuclear antibodies are made, resembles that of systemic lupus erythematosus. Although the molecular mechanisms underlying this defect are not clear, it has been suggested that besides hyperactivation of NF-κB in B- or T-cells, a missing autophagy, *i.e*. clearance of apoptotic bodies, may contribute to the disease.[165]

Recently Van Gool *et al*.[166] showed that another member of the sirtuin family, SIRT6, is able to regulate TNF production by acting at a post-transcriptional step. These studies reveal a previously undescribed relationship between metabolism and the inflammatory response.

NAD^+ and its metabolites as ligands of nucleotide receptors

As already described above, the NAD^+ degradation products cADPR and NAADP act on ligand-gated Ca^{2+} stores, and ADPR and OAADPR can bind to intracellular binding sites of the cation channel TRPM2, as will be discussed later. Furthermore, extracellular NAD^+ can be utilized to activate ion channels on the cell surface, promoting Ca^{2+} influx across the cell membrane into the cell. This is the case for ART-mediated ADP-ribosylation of the purinergic receptor $P2X_7$.[95,96] All of these ligand activities depend on the metabolization of NAD^+ by different enzymes, thereby generating Ca^{2+}-active degradation products.

Not only NAD^+ degradation products, but also the intact NAD^+ molecule *per se* is able to induce calcium signals via receptor activation.[167–174]

In 2001, Bortell *et al*.[167] reported that extracellular NAD^+ inhibits the proliferation of mitogen-activated rat T-cells. This effect was independent of the transferase activity of ART enzymes, since they found ADPR to inhibit proliferation to the same extent as NAD^+. The authors hypothesized that NAD^+ and its metabolites may act via purinergic receptors. Indeed, they found the inhibitory effect of the P2 receptor agonist ADP being similar to that of NAD^+ and ADPR, whereas P1 receptor (adenosine receptor) agonists were less potent. Probing the type of P2 receptor responsible for the signaling, they assumed that a G_s-protein-coupled P2Y receptor could play a role, since an increase in intracellular cAMP concentrations ($[cAMP]_i$) caused by cholera toxin also inhibited T-cell proliferation.[167]

Three years later, a role of NAD^+ as a paracrine signaling molecule received further support by the study of Gerth *et al*.[168] showing that NAD^+ triggers a concentration-dependent increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in freshly isolated, resting human monocytes. This Ca^{2+} signal was elicited by the intact NAD^+ molecule, since neither inhibition of CD38 nor the use of a hydrolysis-resistant NAD^+ derivative abolished the calcium response. The NAD^+-induced transient increase in $[Ca^{2+}]_i$ interfered with ATP-induced Ca^{2+} signaling, again suggesting the involvement of P2 receptors. As the NAD^+-induced rise in $[Ca^{2+}]_i$ solely depended on extracellular Ca^{2+}, ionotropic P2X receptors were considered the more likely candidates exerting the observed effects in resting human monocytes.[168] In a later study, the authors confirmed the participation of P2X receptors and found $P2X_1$, $P2X_4$, and $P2X_7$ subtypes to mediate the NAD^+-induced effects on $[Ca^{2+}]_i$.[174] Furthermore, the group showed, that ADPR-triggered Ca^{2+} changes similar to that observed with NAD^+, but no specific receptor has been identified so far.[174]

In 2006, Ishii *et al*.[169] also reported Ca^{2+} mobilizing activity of ADPR in the rat β-cell line RIN-5F. Here, the Ca^{2+} signal was created by Ca^{2+} release from intracellular stores via phospholipase C activation and IP_3 generation, but again no specific receptor was identified.[169]

The results of this group were confirmed by Lange *et al*.[171] who also found ADPR-induced Ca^{2+} signals in the rat β-cell line INS-1 as well as in primary mouse β-cells. They demonstrated that ADPR can act as a primary ligand at G-protein-coupled P2Y receptors, thereby promoting Ca^{2+} release from intracellular stores-of the endoplasmic reticulum. In primary β-cells, NAD^+ functioned as a competitive antagonist of ADPR at the P2Y receptor subtypes but, however, displayed no Ca^{2+} mobilizing effect.[171]

In contrast to these results, Bruzzone *et al*.[67] reported a direct agonism of NAD^+ at the $P2Y_{11}$ receptor subtype in human granulocytes. In a previous study, the authors detected a slow, sustained Ca^{2+} increase upon addition of extracellular NAD^+ in low micromolar concentrations, which caused superoxide and nitric oxide generation and enhanced chemotaxis. This Ca^{2+} increase was preceded by a transient elevation of the intracellular cAMP concentration and subsequent production of cADPR, which initiated Ca^{2+} release, followed by store-operated Ca^{2+} influx.[172] In the subsequent study, the authors reported an additional rapid and transient Ca^{2+} peak previous to the sustained increase in $[Ca^{2+}]_i$ when millimolar concentrations of NAD^+ were applied. In a convincing line of experiments, they identified the dually coupled purinergic receptor $P2Y_{11}$ to mediate the NAD^+-induced effects. This P2Y receptor subtype is coupled to G_q-proteins as well as to G_s-proteins accounting for both the rapid transient rise in $[Ca^{2+}]_i$ and the transient rise in $[cAMP]_i$, which triggered cADPR production and promoted the slow sustained Ca^{2+}

increase responsible for the functional activation of granulocytes.[67]

The involvement of P2Y receptors in NAD^+-induced signaling was supported by Mutafova-Yambolieva *et al*.[173] in 2007 showing the direct agonism of NAD^+ at $P2Y_1$ receptor subtypes. They found that NAD^+ released by stimulation of enteric nerves in gastrointestinal smooth muscles provokes inhibitory junction potentials in the colonic muscles, causing cessation of action potentials, repolarization, and relaxation from contraction. By the use of a $P2Y_1$ receptor-specific antagonist and $P2Y_1$-transfected cells, participation of the $P2Y_1$ subtype was confirmed. Furthermore, the authors defined NAD^+ as a new inhibitory neurotransmitter, since it meets all classical criteria for this classification.[173]

Only recently, Klein *et al*.[170] supplied further evidence for the agonism of NAD^+ at P2Y receptors. They found that LPS-activated human monocytes respond to micromolar concentrations of NAD^+ with a rapid and transient Ca^{2+} signal, triggered by the release of Ca^{2+} from intracellular stores. By the use of P2Y subtype-specific antagonists and by cAMP measurements, they showed a dual response of both the $P2Y_1$ and $P2Y_{11}$ receptors upon stimulation with extracellular NAD^+.[170]

Overall, the findings reviewed here suggest that the pyridine-purine dinucleotide NAD^+ and its main metabolite ADPR are recognized by purinergic receptors via their purine moiety and can act as primary agonists. The specific P2 receptor subtypes responsible for the signal perception and transduction seem to depend on the respective species, cell type and/or the stage of differentiation. Since P2 nucleotide receptors comprise ligand-gated ion channels (P2X) and G-protein-coupled receptors (P2Y), extracellular NAD^+ can trigger signaling cascades that either mobilize Ca^{2+} from internal stores or induce Ca^{2+} influx across the cell membrane and, in addition, can interfere with cAMP-modulated pathways. The intracellular Ca^{2+} concentration is tightly controled to maintain a level of about 100 nM, as Ca^{2+} signals are the initiating impulse to start major cellular processes (reviewed by Clapham[175]). The fact that NAD^+ and ADPR display ligand activity on receptors affecting the Ca^{2+} and cAMP homeostasis implies a strong immunomodulatory impact in scenarios where extracellular NAD^+ concentrations are elevated. Whereas normal extracellular NAD^+ concentrations are in the submicromolar range,[176] at sites of infection and inflammation extensive cell destruction can lead to NAD^+ concentrations exceeding normal levels for up to 10-fold.[66] These elevated NAD^+ levels may act as a tissue injury signal, leading to anti-inflammatory or immunosuppressive effects to limit tissue destruction of the host.

TRPM2

In 2001, a new role for ADPR as an intracellular signaling molecule was assigned by the discovery that ADPR is able to activate the cation channel TRPM2 specifically.[5,12]

The Ca^{2+}-permeable TRPM2 is a member of the melastatin-related TRP channels, which represent the most diverse group within the TRP superfamily of cation channels.[177–182] The TRPM2 channel is found in a variety of tissues, with highest expression levels in brain, lung, and spleen.[182,183] It has been reported to be ubiquitously expressed in immunocytes, including cells of the monocytic lineage, granulocytes and T lymphocytes.[5,12,183–186] Sharing the general structure of the TRP superfamily, TRPM2 has six membrane-spanning α-helices, a pore, which is located between the fifth and the sixth transmembrane span, and two cytosolic tails.[5,182] What makes TRPM2 unique is a C-terminal structure, which shows 39% homology to the human nucleoside diphosphate-linked moiety X-type motif 9 (NUDT9), an ADPR pyrophosphatase that hydrolyzes ADPR to AMP and ribose-5′-phosphate.[187] It has been shown that the enzymatic activity of the NUDT9 homology region, which could be detected at marginal levels, is not required for channel opening. This domain rather seems to act as a binding region for free intracellular ADPR, that directly initiates conformational changes causing the TRPM2 pore opening.[188,189] Functional TRPM2 channels are predicted to work as homotetramers including four NUDT9 homology regions attached to a single channel structure.[190] The importance of the NUDT9 homology domain for ADPR binding and resultant gating of TRPM2 was confirmed by the finding that a TRPM2 splice variant lacking a portion of this domain (TRPM2-ΔC) did not respond to ADPR, although the catalytic site of the ADPR pyrophosphatase was not affected by the deletion.[191]

Shortly after the first characterization of TRPM2, H_2O_2 was reported as a potent extracellular activator for this channel.[183,191,192] Although a direct binding of the oxidant cannot be excluded, it is more likely that the activation by H_2O_2 is due to an indirect, stress-induced mechanism, that requires ADPR as a second messenger.[188,193]

Hydrogen peroxide is known to initiate a vast range of cellular processes and serves as an experimental paradigm of oxidative stress.[194] It reflects the situation of the respiratory burst, an important function of granulocytes and cells of the monocytic lineage to eliminate invading pathogens.[195] During this process, reactive oxygen species (ROS) and particularly H_2O_2 accumulate in high concentrations around the cells,[196] accompanied by an increase in $[Ca^{2+}]_i$, which is mediated by intracellular store depletion and Ca^{2+}

entry from the extracellular space.[197,198] Heiner *et al*.[199] speculated that the Ca^{2+}-dependent release of H_2O_2 during the respiratory burst of neutrophil granulocytes is enhanced through Ca^{2+} entry via TRPM2, thus establishing a positive feedback loop by an autocrine/paracrine pathway. The addition of sublethal doses of H_2O_2 to human monocytic U937 cells was shown to induce IL-8 production depending on TRPM2-mediated Ca^{2+} influx. The H_2O_2-evoked Ca^{2+} influx was demonstrated to amplify ERK signaling, finally resulting in nuclear translocation of NF-κB essential for the production of IL-8.[200] Activation of TRPM2 by H_2O_2 has also been reported to occur in activated microglia, the host macrophages of the central nervous system.[185]

The H_2O_2-induced gating of TRPM2 has often been associated with cell death.[183,192,201,202] When transfecting U937 monocytic cells with TRPM2, Zhang *et al*.[202] observed a diminished cell viability and increased apoptosis in response to H_2O_2. Furthermore, suppression of endogenous TRPM2 by the use of siRNA protected cells from cell death. Cell death was accompanied by a cleavage of caspases-8, -9, -3, and -7, which was inhibited by the intracellular Ca^{2+} chelator BAPTA, confirming the critical role of $[Ca^{2+}]_i$ in mediating these effects.[202] However, in an earlier study using TRPM2 transfected HEK cells, Hara *et al*.[183] suggested that the oxidative stress-induced cell death was caspase-independent, since no DNA-fragmentation or caspase-3 activation could be observed.

As a source of free ADPR the PARP/PARG pathway has been widely discussed. It is generally believed that the production of free ADPR through the activation of PARP 1 and poly-ADPR-glycohydrolases (PARGs) or other oxidative stress-mediated pathways enables the physiological activation of TRPM2.[189,193,203,204] Based on the finding that H_2O_2-induced death in U937 monocytes is accompanied by PARP cleavage[202], Miller[205] suggested that, once TRPM2 is activated by PARP, TRPM2 activation in turn results in PARP cleavage and inactivation. This negative feedback loop may partially protect the cells from death, depending on the extent of the activation of other cell death-inducing pathways.

Activation of TRPM2 has also been described to occur in neutrophil granulocytes, that do not express PARP 1.[206] When stimulating these cells with the chemo-attractant peptide fMLP, Heiner *et al*.[208] observed an increase in Ca^{2+} influx but no change in the intracellular ADPR concentration, which was shown to be about 5 μM. The authors postulate that at an elevated $[Ca^{2+}]_i$ of 1 μM, as may be physiologically achieved by fMLP through IP_3-mediated mobilization of intracellular calcium stores, considerably lower ADPR concentrations are needed to activate TRPM2.[207,208] The finding that intracellular Ca^{2+} sensitizes TRPM2 to gating by ADPR has also been shown by other groups.[209–211]

Another enzyme which catalyzes the production of ADPR and which seems to be associated with TRPM2 activity is the transmembrane glycoprotein CD38. Not only ADPR, but also cADPR was shown to enable TRPM2 gating, either directly or, in lower and physiological concentrations, by synergizing with ADPR.[212] According to Partida-Sanchez *et al*. ADPR and cADPR generated by CD38 together with elevated Ca^{2+} concentrations are needed to control chemotaxis in mouse neutrophils induced by fMLP.[213,214] The crucial role of TRPM2 during chemotaxis has been confirmed by Yamamoto *et al*.[200] who showed that the fMLP-induced Ca^{2+} response and *in vitro* migration are suppressed in TRPM2-deficient neutrophils.

Besides its degradation products, NAD^+ itself has been discussed to activate TRPM2.[12,183,184,215] However, its gating ability seems to be restricted to certain cell types and experimental conditions. Furthermore, NAD^+ degradation products could not be excluded from being responsible for the observed effects. Indeed, NAADP, another CD38 reaction product, and OAADPR, generated by the sirtuin deacetylation reaction have been identified as TRPM2 activators.[144,216] However, NAADP most likely represents a synergistic co-activator rather than a primary activator for TRPM2.[216]

The physiological function of TRPM2 is far from being clear. Nevertheless, the numerous studies carried out during the last decade point to an essential role of TRPM2 in the immunogenic context. Pathways involving TRPM2 could offer potent targets for therapeutic intervention in inflammatory diseases.

Non-enzymatic ADP-ribosylation

In contrast to cADPR, NAADP and OAADPR, ADPR is generated by a multitude of enzymes and thus can be considered the main NAD^+ degradation product besides nicotinamide.

The removal of ADPR moieties from proteins by ADPR hydrolases or PARGs and the hydrolysis of cADPR by CD38/CD157 and of OAADPR by esterases or acetyltransferases cause an elevation of the available pool of free ADPR in the nucleus, cytoplasm and extracellular space.[217]

Since ADPR contains a reactive reducing sugar moiety, it holds the potential for non-enzymatic modifications of target molecules like proteins, lipids or nucleic acids. This non-enzymatic ADP-ribosylation, similar to enzymatic ADP-ribosylations, is a post-translational modification that usually causes the alteration or the loss of function of the modified molecule.

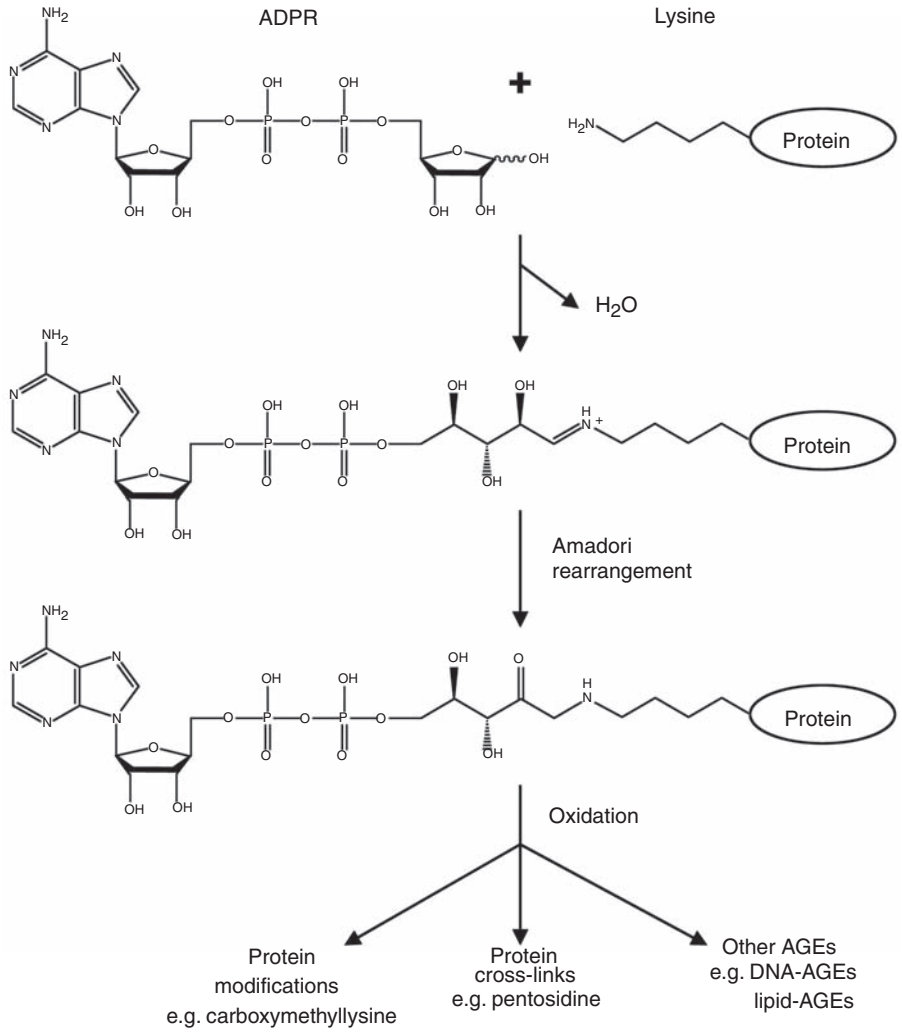

Fig. 4. Non-enzymatic glycosylation reaction (glycation) between the ADPR and the amino group of a lysine residue. The ADPR binds to the lysine amino group to form a protonated Schiff-base, which is subsequently re-arranged to a ketoamine product. Secondary modifications like oxidation lead to the production of advanced glycation end-products (AGEs).

When free ADPR is present in excess, non-enzymatic glycosylation (glycation) of free amino groups followed by glycoxidation and formation of high molecular weight cross-linked products might be the consequence. This process of non-enzymatic browning (Maillard reaction) was known to occur in stored and heated food and has already been described to happen *in vivo* since the 1950s.[218,219] Glycation starts with the initial binding of the northern ribose moiety of ADPR to a free amino group of the target forming a reversible Schiff-base adduct, followed by Amadori re-arrangement resulting in a ketoamine product.[220] This primary glycation product can undergo extensive secondary reactions like oxidation/dehydration, re-arrangement, degradation or can cause cross-linking between proteins proceeding from middle stage products like dicarbonyl compounds (*e.g.* glyoxal, methylglyoxal) to a broad range of complex, heterogeneous structures termed advanced glycation end-products (AGEs; Fig. 4).[221]

Many of the AGEs described so far were generated *in vitro* but the relevance and extent of their production *in vivo* has not been fully elucidated. The dominant and thus best analyzed AGEs found *in vitro* and *in vivo* are the fluorescent protein cross-linking product pentosidine and carboxymethyllysine, which consists of a C2 fragment remaining at the lysine residue after ADP-glycerate was released from ADPR-lysine.[222] Advanced glycation end-products mainly accumulate on long-lived proteins like skin collagen and lens crystallins or on protein deposits like amyloids, due to their slow turn over.[223,224]

Glycated metabolites are recognized by numerous receptors and binding proteins, *e.g.* AGE-R1–3, FEEL1–2, SCR-II, CD36.[225] The best studied receptor among

them is a pattern recognition receptor called RAGE (receptor for AGEs), existing in a membrane bound and a soluble form. This receptor is a multiligand-binding member of the immunoglobulin superfamily, that interacts with diverse endogenous ligands.[226] It is expressed by different cell types, including immune cells like monocytes/macrophages, neutrophils, lymphocytes and dendritic cells,[227] as well as by endothelial, neuronal and smooth muscle cells.[228] Activation of RAGE promotes the generation of ROS via NAD(P)H oxidase and this oxidative stress, in turn, facilitates AGE formation in a positive feedback manner.[229] The major impact of RAGE signaling is the activation of NF-κB, leading to the production of pro-inflammatory cytokines. Additionally, ROS, MAP-kinases like ERK1/2, JNK, p38 and the PI3K/Akt pathway seem to be involved in the signaling cascades.[226] In addition to signaling, ligand-activated RAGE also functions as an adhesion molecule in leukocytes to facilitate their extravasation across the endothelial barrier. RAGE-mediated leukocyte adhesion to endothelial cells *in vitro* is caused by a direct interaction between RAGE and the β2-integrin subunit Mac-1.[230]

Beyond the receptor-mediated signaling which seems to account for most of the AGE-induced effects, AGEs can bind to proteins, lipids and nucleic acids in a receptor-independent manner and cause dysfunction or immunogenicity of the respective molecule.

The formation and action of AGEs represents a somehow unbalanced physiological state; consequently, they are associated with, for example, cardiovascular, renal, ophthalmic or neurodegenerative disorders and accumulate in aging processes in general.[231–234] An implication in the pathophysiology of inflammation and aging-related diseases has already been proposed by different scientists since the late 1970s[223,235] and was confirmed by many groups. Some examples with relevance for innate immunity and inflammation are given below.

Dan *et al*.[236] showed that AGE-BSA enhances smooth muscle cell migration and elevates mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemo-attractant protein-1 (MCP-1) in smooth muscle cells, thereby increasing THP-1 monocytic cell adhesion to smooth muscle cells. Such events are thought to be part of the development of atherosclerosis. Analyzing the effects of AGE-BSA and methylglyoxal in peripheral mononuclear blood cells (PBMCs), Gawlowski *et al*.[237] found a dose- and time-dependent increase of tissue factor expression by monocytes, enhanced expression of the activation marker P-selectin on platelets and increased Mac-1 expression on neutrophils. These alterations together with an increased neutrophil apoptosis resulted in a higher number of platelet–neutrophil aggregates promoting a procoagulant state.[237] Yeh *et al*.[238] reported, that carboxymethyllysine-modified human serum albumin induced secretion of TNF-α, IL-1β and MCP-1. This pro-inflammatory action is supported by findings of Shanmugam *et al*.[239] who showed an AGE-induced increased expression of cyclooxygenase-2 (COX-2) mRNA in human monocytes *in vitro* and in diabetic patients, respectively. The COX-2 enzyme generates prostaglandins, which are potent mediators of inflammation.[239]

In summary, AGE formation and RAGE activation lead to the generation of oxidative stress and the up-regulation of pro-inflammatory molecules, thereby increasing inflammatory conditions.

Considering the crucial role of free ADPR in pathophysiological conditions, controlling ADPR amounts is a critical issue. In general, ADPR can be degraded by ADPRase, a ADPR pyrophosphatase cleaving the phosphodiester bond, creating AMP and ribose-5′-phosphate.[186] AMP can subsequently be converted by 5′-nucleotidase (CD73) into adenosine and phosphate, followed by adenosine elimination through adenosine deaminase.[240] The remaining ribose-5′-phosphate can be recycled into phosphoribosyl-pyrophosphate (PRPP) by PRPP-synthetase[241] and, together with the released nicotinamide, NAD^+ can be recreated from its degradation products by Nampt.

Nampt (visfatin/PBEF)

Nicotinamide phosphoribosyltransferase is the key enzyme in the conversion of nicotinamide to NAD^+ (Fig. 5).[242] It catalyzes the generation of nicotinamide mononucleotide (NMN) from nicotinamide and PRPP. The product NMN is then converted to NAD^+ by nicotinamide mononucleotide adenylyltransferase (Nmnat).[243] Besides this endogenous salvage pathway, mammals can also use the *de novo* pathway from tryptophan in the liver to partially meet the NAD^+ requirements. The Nampt gene was first discovered in 1994 as pre-B-cell colony-enhancing factor (PBEF) by Samal *et al*.[244] when screening a cDNA library of activated peripheral blood monocytes. It was originally described as a putative cytokine, which in the presence of IL-7 and stem cell factor enhances the maturation of B-cell precursors.[244] Recently, Nampt was discovered to be a visceral fat-derived adipokine and also named visfatin.[245] Its potential function as a novel adipokine has been discussed elsewhere.[246]

The expression of Nampt is widely induced by inflammatory stimuli in cells involved in innate immunity, specifically neutrophils, monocytes, macrophages and dendritic cells.[247,248] However, the stimuli which include LPS and inflammatory cytokines have also been shown to up-regulate Nampt expression in other cell

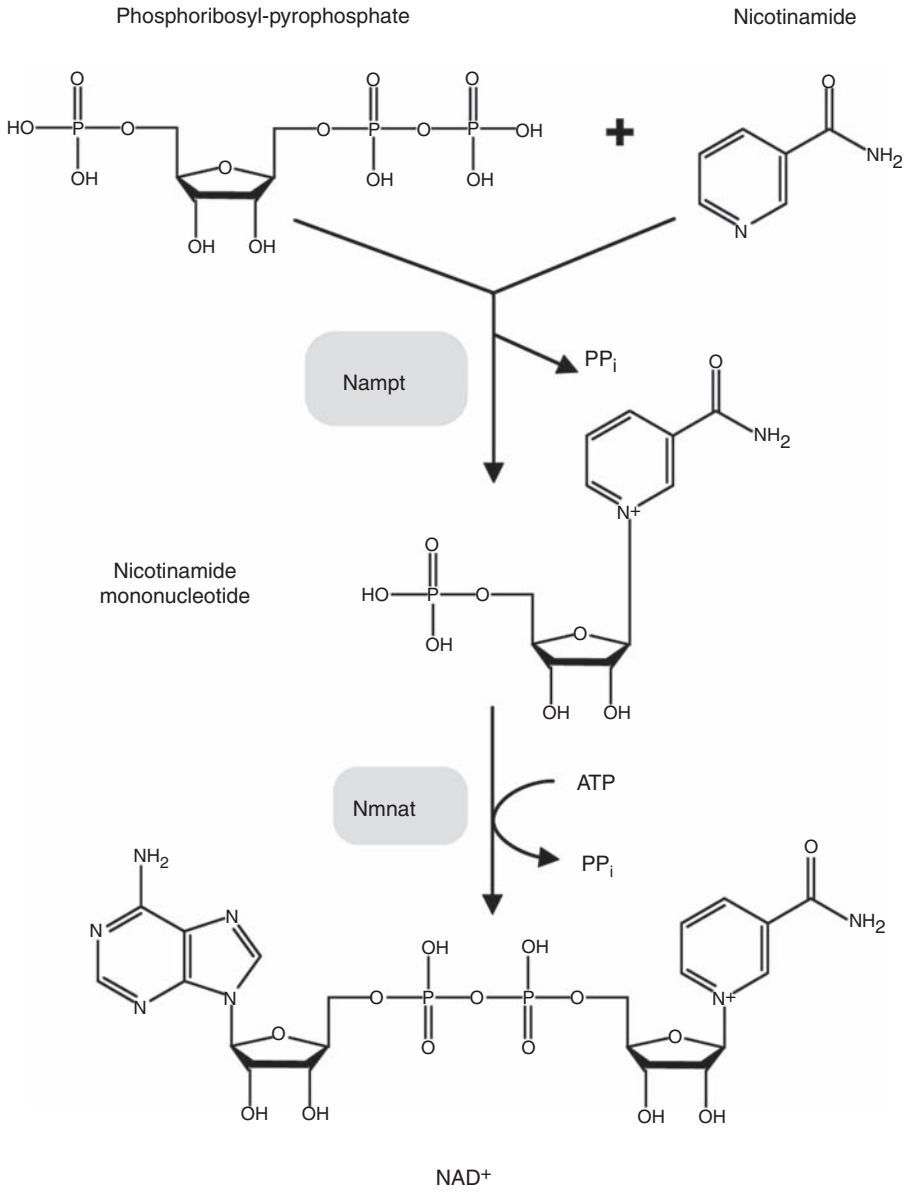

Fig. 5. Synthesis of NAD^+ from nicotinamide and phosphoribosyl-pyrophosphate. Nampt catalyzes the conversion of nicotinamide and phosphoribosyl-pyrophosphate to nicotinamide mononucleotide and pyrophosphate. This rate-limiting step in NAD^+ biosynthesis via the endogenous salvage pathway is followed by the ATP-dependent production of NAD^+-mediated by Nmnat.

types such as epithelial cells,[249] endothelial cells,[250] and synovial fibroblasts.[251]

According to Revollo *et al*.[252] who characterized the biochemical nature of the mammalian NAD^+ biosynthetic pathway with the purified proteins Nampt and Nmnat, Nampt is the rate-limiting component in this pathway. Therefore, activation of Nampt seems to result in increased intracellular NAD^+ concentrations which, in turn, could regulate the function of NAD^+-dependent enzymes such as sirtuins or PARPs.

In addition to its intracellular location, Nampt has also been found in serum and culture supernatants.[242,244,253–257] The question whether Nampt is secreted or simply released because of cell death is still a matter of debate. Recent studies have shown that extracellular Nampt (eNampt) functions as a pro-inflammatory cytokine. Jia *et al*.[257] reported that Nampt is detectable in culture supernatants from LPS-stimulated neutrophils and that the supernatant exerts an anti-apoptotic effect in neutrophils which can be blocked by anti-Nampt antibodies. However, eNampt only displayed its suppressive activity on apoptosis in the presence of endogenous Nampt.[257]

The Nampt protein is also expressed in neutrophils from sepsis patients, in whom rates of apoptosis are profoundly delayed. The prevention of Nampt

translation in septic neutrophils through the use of a Nampt antisense oligonucleotide restores the normal kinetics of apoptosis. Therefore, the authors conclude that Nampt plays a requisite role in delayed neutrophil apoptosis in clinical experimental sepsis.[257]

An anti-apoptotic effect of Nampt was also observed by Li *et al*.[258] who subjected macrophages to ER stress after having incubated them with eNampt. The authors show that eNampt blocks macrophage apoptosis and that the cell survival pathway is mediated by STAT3, which is activated in an autocrine/paracrine manner by eNampt-induced IL-6. They addressed the critical question whether eNampt-mediated effects depend on Nampt's enzymatic properties and found that Nampt does not require enzyme activity to activate STAT3.[258]

Moschen *et al*.[259] reported that recombinant Nampt activates human leukocytes and induces cytokine production in $CD14^+$ monocytes. The authors showed that the surface expression of co-stimulation molecules CD54, CD40 and CD80 was increased in response to eNampt and that IL-6 serum concentrations were elevated in mice injected with recombinant Nampt. Similar to Moschen *et al*.[259] Dahl *et al*.[260] observed a Nampt-induced secretion of cytokines from peripheral blood mononuclear cells as well as an increased metalloproteinase-9 activity in THP-1 monocytes. The same group discovered an up-regulation of Nampt in plaques from the carotid artery of patients with symptoms of stroke and at the sites of plaque rupture in patients with acute myocardial infarction.

Considering that increased Nampt expression has been described in a variety of chronic inflammatory diseases, including inflammatory bowel disease,[259] psoriasis,[261] and rheumatoid arthritis,[248,251,262] Nampt could have a role as a new player in inflammatory disorders.

Conclusions

While in recent years there have been many significant discoveries regarding the metabolism and biological functions of NAD^+, many questions remain to be answered to understand fully the multiple roles NAD^+ plays in cell biology. It will be exciting to understand the interaction of NAD^+-generating and NAD^+-consuming enzymes, to identify new targets of ARTs and PARPs, to determine NAD^+ transport systems as well as to determine the functions of NAD^+ degradation products in cell metabolism. The answer to all these questions is likely to result in new discoveries, which may help to understand poorly understood regulatory mechanisms of the immune system.

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