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Differential Regulation of P2X\textsubscript{7} Receptor Activation by Extracellular Nicotinamide Adenine Dinucleotide and Ecto-ADP-Ribosyltransferases in Murine Macrophages and T Cells

Shiyuan Hong,* Nicole Schwarz,† Anette Brass,‡ Michel Seman,† Friedrich Haag,† Friedrich Koch-Nolte,‡ William P. Schilling,* and George R. Dubyak*†

Extracellular NAD induces the ATP-independent activation of the ionotropic P2X\textsubscript{7} purinergic receptor (P2X\textsubscript{7},R) in murine T lymphocytes via a novel covalent pathway involving ADP-ribosylation of arginine residues on the P2X\textsubscript{7},R ectodomain. This modification is catalyzed by ART2.2, a GPI-anchored ADP-ribosyltransferase (ART) that is constitutively expressed in murine T cells. We previously reported that ART2.1, a related ecto-ART, is up-regulated in inflammatory murine macrophages that constitutively express P2X\textsubscript{7},R. Thus, we tested the hypothesis that extracellular NAD acts via ART2.1 to regulate P2X\textsubscript{7},R function in T cells, this P2X\textsubscript{7},R modification in macrophages does not gate the channel but decreases the threshold for gating in response to ATP binding. These findings indicate that extracellular NAD and ATP can act synergistically to regulate P2X\textsubscript{7},R signaling in murine macrophages and also suggest that the cellular context in which P2X\textsubscript{7},R signaling occurs differs between myeloid and lymphoid leukocytes. The Journal of Immunology, 2009, 183: 578–592.

The P2X\textsubscript{7}, purinergic receptor (P2X\textsubscript{7},R) is an ATP-gated, nonselective cation channel that is predominantly expressed in cells of hematopoietic origin, including macrophages and T lymphocytes. The subunit structure of this 595-aa receptor includes two transmembrane segments, an intracellular N terminus, an intracellular C terminus, and a large extracellular loop (47–329 aa) that contain a presumed site or sites for ATP binding (2). Three of these P2X\textsubscript{7} subunits assemble to form the trimeric P2X\textsubscript{7},R channel (3). Stimulation of the P2X\textsubscript{7},R with extracellular ATP rapidly triggers increased Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} fluxes across the plasma membrane (2), followed by the delayed induction of a nonselective pore that facilitates the permeation of molecules up to 900 Da in mass (4). Given its expression in myeloid and lymphoid leukocytes, many studies have identified roles for the P2X\textsubscript{7},R in the regulation of various proinflammatory and immune responses (reviewed in Refs. 5–7).

An unusual and defining feature of the P2X\textsubscript{7},R is its high threshold for activation by extracellular ATP (EC\textsubscript{50} ~ 500 μM); this contrasts with much lower activation thresholds for the other six members of the P2X family (EC\textsubscript{50} ~ 10 μM) (1, 8, 9). Intracellular ATP concentration is only 3–5 mM, and most cells express significant ecto-ATPase activities. Thus, it is unlikely that submillimolar levels of extracellular ATP can be sustained for significant durations within interstitial tissue compartments except perhaps during a massive lysis of host cells or the killing of invading pathogens. Clearly, the P2X\textsubscript{7},R is activated within situ inflammatory loci or during normal development as indicated by the reduced levels of cytokines that accumulate within the inflamed footpads of P2X\textsubscript{7},R knockout mice (10), as well as the marked changes in bone density during the aging of these mice (11). Autocrine activation of P2X\textsubscript{7},R via the release of endogenous ATP has been recently reported in human monocytes in response to LPS stimulation of TLR4 signaling (12, 13) and in T cells in response to Ag stimulation of TCR signaling cascades (14). This released ATP may accumulate within diffusion-restricted microdomains of the cell surface, such as caveolar or nascent endosomal invaginations not readily accessible to the bulk extracellular medium.

However, physiological P2X\textsubscript{7},R activation may involve modes of regulation in addition to autocrine stimulation. One of these modes is allosteric modulation of ATP affinity via conformational changes in P2X\textsubscript{7}, trimeric channels produced by local biophysical conditions such as pH, ionic composition (15–17), or membrane lipid composition (18). For example, lysophosphatidylcholine and other lysolipids reduce the threshold level of ATP required for...
P2X,R-dependent Ca\(^{2+}\) influx and pore formation in murine microglia (19). Another type of regulation involves the gating of P2X,R channels by mechanisms independent of reversible ATP binding. Notably, extracellular NAD induces the ATP-independent activation of P2X,R in murine T lymphocytes via a novel covalent pathway involving ADP-ribosylation of arginine residues on the P2X,R ectodomain (20–22).

The ADP-ribosylation of P2X,R is catalyzed by ART2, a GPI-anchored ADP-ribosyltransferase (ART) constitutively expressed on the cell surface of murine T cells (23–25). ART2 belongs to a family of ectoenzymes that use extracellular NAD to transfer the ADP-ribose (ADP-R) moiety to substrate proteins (26). ART2 mediates P2X,R transactivation via ADP-ribosylation of the R125 residue within the extracellular loop of the receptor (21). This covalent modification apparently mimics the conformational changes in P2X,R induced by noncovalent ATP binding and triggers both Ca\(^{2+}\) influx and the secondary nonselective pore permeable to fluorescent dyes. The NAD- and ART2-dependent activation of P2X,R consequently induces phosphatidylserine (PS) exposure on T cell surfaces, increased shedding of CD62L, and acceleration of T cell death. Other studies have shown that extracellular NAD induces P2X,R activation in in vivo models of immune and inflammatory responses (21, 27, 28).

ART2 includes two isoforms, ART2.1 and ART2.2, which are encoded by tandem genes (Art2a and Art2b) located on murine chromosome 7 (26). Although ART2.1 is functionally and structurally similar to ART2.2, it contains two additional cysteine residues (Cys\(^{300}\) and Cys\(^{301}\)) that readily form a disulfide bond that allosterically suppresses catalytic activity (29). This inhibited state of ART2.1 is reversed by extracellular thiol reductants, such as exogenous DTT or the endogenous cysteine and glutathione released by inflamed or damaged tissues (30, 31). T cells from most inbred mouse strains (e.g., BALB/c) natively express both ART2.1 and ART2.2 (25, 32–34). However, the latter isoform is sufficient for NAD-induced P2X,R activation and cell death, because these responses occur in the absence of extracellular thiol reductants and in T cells from C57BL/6 mice that express a mutated Art2a gene and no functional ART2.1 protein (20, 35).

Although the NAD-induced, ART2-dependent mechanism is clearly a major pathway for P2X,R activation in mouse T lymphocytes, it is unclear whether this mechanism is operative in macrophages, another class of leukocytes that natively express P2X,R at high levels. Relevant to this issue, we have reported the inducible expression of ART2.1, but not ART2.2, in murine bone marrow-derived macrophages (BMDM) stimulated by multiple inflammatory factors (36). This prompted us to examine the role of ART2.1 as a regulator of P2X,R receptors natively expressed in murine macrophages or heterologously expressed in HEK293 cells. We demonstrate that coexpression of the murine P2X,R with ART2.1 or ART2.2 in HEK293 cells facilitates similar NAD-driven ADP-ribosylation of the receptor. However, NAD stimulation of P2X,R in macrophages or HEK293 cells is not sufficient to activate the receptor. Rather, the NAD/ART2-dependent modification of the P2X,R potentiates the ability of ATP to activate the receptor as indicated by a left shift in the ATP dose-response relationship. Thus, extracellular NAD acts to regulate the ionotropic P2X,R in both macrophages and T cells but via distinct mechanisms on the gating of channel activity. These observations support a role for extracellular NAD in the regulation of P2X,R-dependent inflammatory responses in macrophages and additionally suggest that the cellular context (i.e., myeloid vs lymphoid cells) dictates the outcome of signaling through the P2X,R.

**Materials and Methods**

**Materials**

Recombinant mouse IFN-γ was from Boehringer Mannheim Biochemical and recombinant mouse IFN-β was from US Biologicals. LPS (Escherichia coli serotype 01101:B4) was from List Biological Laboratories. ATP, NAD, etheno-NAD (ε-NAD), ADP-R, reduced glutathione (GSH), and TRIZol were from Sigma-Aldrich. Oligo(dT) primer was from Promega. Provirun myeloblastosis virus reverse transcriptase was from Roche. Tag DNA polymerase was from New England Biolabs. The 1G4 mouse mAb (a generous gift from Dr. R. Santella, Columbia University, New York, NY) was prepared as previously described (36). Fura-2-AM, ethidium bromide, allophycocyanin-conjugated annexin V and YO-PRO-1 were from Molecular Probes. The anti-P2X,R mAb K1G Ab and mAbs directed against mouse ART2.1 or ART2.2 were generated and used as recently described (37). BALB/c, C57BL/6, and New Zealand White (NZW) mice were purchased from Taconic Farms. P2X,R\(^{-/-}\) mice were originally provided by Pfizer Global Research and Development and then backcrossed into a pure C57BL/6 background for >12 generations from a P2X,R\(^{-/-}\) mouse strain described previously (38). All experiments and procedures involving mice were approved by the Institutional Animal Use and Care Committees of Case Western Reserve University (Cleveland, OH) or Hamburg University Hospital (Hamburg, Germany).

**Cell culture and animals**

BMDM and splenocytes isolated from BALB/c, C57BL/6, NZW, or P2X,R\(^{-/-}\) mice were prepared as previously described (36). Bac1.2F5 murine macrophages were cultured in DMEM (Sigma-Aldrich) supplemented with 25% L cell-conditioned medium, 15% calf serum (HyClone Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) in the presence of 10% CO\(_2\). RAW264.7 macrophages were cultured in DMEM supplemented with 10% calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin in the presence of 10% CO\(_2\). Where indicated, BMDM or the macrophage cell lines (Bac1.2F5 and RAW264.7) were primed for 24 h with either LPS (100 ng/ml), IFN-γ (100 U/ml), or IFN-β (100 U/ml) to induce an inflammatory phenotype and the up-regulation of ART2.1 expression. Murine BW5147 T lymphoma cells were maintained in RPMI 1640 supplemented with 10% calf serum and 1% penicillin-streptomycin in the presence of 5% CO\(_2\). Wild-type (WT) HEK293 cells were cultured in DMEM supplemented with 10% calf serum and 1% penicillin-streptomycin in the presence of 10% CO\(_2\). HEK293 cells stably transfected with either the WT murine P2X,R (HEK-mP2X\(_7\) cells) or the mutant R276K P2X,R (HEK-mP2X,R\(_{276K}\) cells) were selected and maintained in DMEM supplemented with 400 μg/ml G418 or 10 μg/ml blasticidin. The ART2 plasmids were transiently transfected into HEK P2X,R cells using cells seeded at 6 × 10\(^{5}\) per 35-mm dish 24 h before transient transfection. The cells were transfected using PolyFect reagent (Qiagen) with 2.5 μg of plasmid DNA per dish followed by incubation at 37°C for 36 h before experiments.

**RT-PCR analyses**

Total RNA was extracted using TRIZol; all primers and PCR conditions for cto-ARTs, inducible NO synthase (iNOS), IFN-β, and GAPDH were prepared and used as previously described (36). The PCR amplicons were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining; the resulting fluorescence images were recorded with a BioRad Gel Doc 1000 system.

**1G4 mAb-based assay of ART2 activity**

ART activity in intact Bac1.2F5 macrophages, RAW264.7 macrophages, or BW5147 T lymphocytes was assayed using a Western blot protocol based on the 1G4 mAb as previously described (36). Briefly, intact cells were transferred to basic salt solution (BSS) containing 130 mM NaCl, 1.5 mM KCl, 1.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 25 mM HEPES (pH 7.5), 5 mM glucose, and 0.1% BSA. The cells were incubated at 37°C for 15 min with 50 μM ε-NAD and 1 mM ADP-R in the presence or absence of 1 mM DTT. Washed cells were lysed in PBS, 1% Triton-X100, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Sigma-Aldrich) for 20 min at 4°C. Insoluble material was pelleted by high-speed centrifugation (for 15 min at 13,000 × g). K1G Ab

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were detected by autoradiography. P2X7 was detected with a rabbit 
samples were loaded onto 15% SDS-polyacrylamide gels and proteins 
ished with a rabbit anti-P2X7 C-terminal peptide Ab (1/1000) (Alomone Labs) and perox-
FACS analysis
Staining for the P2X7R was performed with Alexa Fluor 488-con jugated 
ats were centrifuged and the cell pellets were washed twice with BSS. Primary spleen lymphocytes or 
ulation of ART2.1 protein and ADP-ribosyltransferase activity in 
results
We confirmed the ability of extracellular NAD to induce P2X7R 
ally leukocyte cell lines from separate cultures. All data, unless otherwise stated, represent mean ± 

Results
NAD induces activation of P2X7-R in murine lymphocytes but 
not murine macrophages
We confirmed the ability of extracellular NAD to induce P2X7-R 
activation (as assayed by Ca2+ influx) in freshly isolated splenic 
lymphocytes from BALB/c or C57BL/6 mice (Fig. 1, A and B), but 
in lymphocytes from P2X7−/− mice (Fig. 1C). (Although splenic 
lymphocytes include both T cells and B cells, P2X7-R is not 
expressed by murine B cells; see Ref. 46). We tested lymphocytes 
from the BALB/c and C57BL/6 mouse strains because they express 
polyomorphic variants of the P2X7-R (P451 for BALB/c and 
L451 for C57BL/6) (47). Additionally, T lymphocytes from 
BALB/c mice express both ART2.1 and ART2.2 as functional 
enzymes, whereas leukocytes from C57BL/6 mice express only 
ART2.2 due to a premature stop codon in ART2.1 mRNA that 
prevents translation of a functional protein (34, 35).

In contrast to its actions on T cells, NAD did not trigger Ca2+ 
influx in naive BMDM isolated from either BALB/c or C57BL/6 
mice (Fig. 1, D and E) even though ATP stimulated robust in-
creases in Ca2+ in both WT BMDM populations, but not in 
BMDMs from P2X7-R-deficient mice (Fig. 1F). This absence of an 
NAD effect in naive macrophages was consistent with our previous 
findings that murine macrophages express only low levels of ecto-
ARTs in the absence of proinflammatory activation by IFNs or 
LPS. Likewise, C57BL/6 BMDM were unresponsive to NAD even 
after proinflammatory activation by LPS (Fig. 1H) or IFNs (not 
shown); this is consistent with the general lack of ART2.2 expres-
sion in murine myeloid leukocytes and the specific lack of ART2.1 
expression in C57BL/6 myeloid leukocytes (35). As expected, nei-
ther NAD nor ATP elicited a Ca2+ influx response in P2X7−/− 
BMDM (Fig. 1I). Surprisingly, however, inflammatory 
BALB/c BMDM primed with IFN-γ (Fig. 1G) up-regulate 
ART2.1 were also unresponsive to extracellular NAD but retained 
a robust response to ATP. We have previously described the use of 
anti-ART2.1, anti-ART2.2, and anti-eADP- mAbs in FACS 
analyses to confirm that LPS and IFN-γ induced the specific up-reg-
ulation of ART2.1 protein and ADP-ribosyltransferase activity in 
BMDM from WT BALB/c mice (36), but not in BMDM from an 
ART2.1 knockout BALB/c strain (data not shown). Consistent with the thiol 
dependence of ART2.1 enzyme activity, the inclu-
sion of DTT markedly increases ADP-ribosylation of cell surface 
proteins in intact BALB/c BMDM (36). However, the inclusion of 
extracellular DTT did not facilitate NAD-induced Ca2+ influx in 
these IFN-primed BMDM (Fig. 1G).

The murine P2X7-R is a substrate for ADP-ribosylation and 
gating by the thiol-sensitive ART2.1 ectoenzyme
The ability of extracellular NAD to activate the P2X7-R in BALB/c 
T cells that express ART2.1 and ART2.2, but not in BALB/c macro-
phages that express only ART2.1, raised the critical question of 
whether ART2.1 (similarly as ART2.2) can recognize the P2X7-R 
as a substrate. To test this, the murine P2X7-R was coexpressed 

HPLC analysis of extracellular NAD metabolism
Monolayers of Bac1.2F5 macrophages (106 cells/well in 6-well dishes) 
were incubated in 1 ml of BSS at 37°C, supplemented with 100 μM NAD 
in the presence or absence of 1 mM ADP-R. At selected times (0–60 min), 
100-μl aliquots of the extracellular medium were removed, boiled for 5 
min, and centrifuged to sediment any precipitated protein. NAD and its 
principle metabolite, ADP-R, were separated and quantified using a re-
verse-phase HPLC protocol. Briefly, 50-μl aliquots were injected onto an 
Alltech C18 Adsorbosphere column that was isocratically eluted at 1.3 
ml/min with a running buffer of 0.1 M KH2PO4 and 5% methanol (pH 6). 
NAD (elution time 8.2 min) and ADP-R (elution time 4 min) were detected 
by absorbance at 254 nm.

Statistics
All experiments were repeated 2–6 times using different preparations of 
primary leukocytes isolated from different mice or with leukocyte cell 
lines from separate cultures. All data, unless otherwise stated, represent mean ± 
SEM. A two-tailed, one-variable Student’s t test was used to analyze these 
data with statistical significance defined as p < 0.05.
with ART2.1 or ART2.2 in HEK293 cells. The ART2-expressing cells were briefly incubated with [32P]NAD in the presence or absence of DTT before extraction, immunoprecipitation of the P2X7R, SDS-PAGE, and detection of [32P]ADP-ribosylated P2X7R by autoradiography. Fig. 2A illustrates the extracellular domain of the mP2X7R including the relative positions of seven (of 18 total) arginine residues within this domain. Previous studies identified R125 and R133 as the critical sites for ART2.2-catalyzed ADP-ribosylation of the P2X7R (21). Thus, we compared WT P2X7R with saP2X7R construct (R125K/R133K) wherein the two target arginines for ART2.2 within the cysteine-rich loop were replaced by lysine and, as a consequence, cannot function as an acceptor for ADP-ribosylation by ART2.2. The expression of ART2.1 in HEK293 cells facilitated the robust ADP-ribosylation of the WT P2X7R, but not the double arginine mutant variant as assayed by incorporation of [32P]ADP (Fig. 2B). Notably, the ability of ART2.1 to ADP-ribosylate the WT P2X7R was highly dependent on exogenously added DTT. In contrast, HEK293 cells coexpressing ART2.2 and WT P2X7R showed strong and equivalent [32P]ADP-ribosylation of P2X7R in the absence or presence of DTT; mutants of P2X7R in which the R125 and R133 residues were changed to lysine did not function as a substrate for ART2.2 regardless of the presence or absence of DTT (data not shown).

Importantly, we used the K1G anti-P2X7 Ab and FACS analyses to confirm similar cell surface expression levels of all WT and mutant P2X7R constructs (Fig. 2).

We next asked whether ADP-ribosylation of P2X7R by ART2.1 also induces the gating of P2X7R. To this end, we examined NAD-induced and P2X7R-dependent pore formation in T lymphocytes from BALB/c mice, which express both ART2.1 and ART2.2, or corresponding cells from NZW mice, which lack ART2.2 and express only ART2.1 (Fig. 2C). The formation of membrane pores that allow the incorporation of DNA-staining dyes like YO-PRO-1 is considered to be a typical hallmark of P2X7 activation. Fig. 2D shows that T lymphocytes from BALB/c mice, which express high levels of ART2.2, incorporate YO-PRO-1 in response to micromolar NAD regardless of the presence or absence of DTT. In contrast, NAD-induced YO-PRO-1 uptake into T lymphocytes from NZW mice, which express only ART2.1, is strictly dependent on DTT and also requires higher concentrations of NAD.

**NAD potentiates ATP-induced P2X7R activation in HEK293 cells coexpressing ART2 and P2X7R**

The data in Fig. 2 verified the ability of NAD to effectively ADP-ribosylate the WT murine P2X7R when heterologously expressed
with ART2.1 in the HEK293 cell background. However, we have reported that this covalent modification of the WT P2X7R expressed in HEK293 cells is not sufficient for functional activation of the receptor (21). Fig. 3A shows this by comparing NAD
 with ATP as stimuli for Ca$^{2+}$/H$^{+}$ influx in a HEK293 line (HEK-mP2X7 cells) stably transfected with murine P2X7R cDNA before transient transfection with an ART2.2 expression plasmid. Western blot analysis confirmed the expression of functional ART activity in ART2.2-transfected, but not parental, HEK293 cells (Fig. 3C). This assay involves incubation of intact cells with NAD (an NAD analog) to covalently ADP-ribosylate cell surface proteins followed by cell extraction, SDS-PAGE, and probing with the anti-ADP-R 1G4 mAb. Fig. 3C also shows that inclusion of extracellular ADP-R potentiates the accumulation of ADP-ribosylated proteins by attenuating the metabolism of NAD (or NAD) by ectonucleotidases. Despite the robust ART activity in the cotransfected HEK-mP2X7 cells, extracellular NAD did not mimic the ability of ATP to trigger P2X7R-dependent Ca$^{2+}$/H$^{+}$ influx.

We have described another P2X7R arginine residue (R276) that is not an ecto-ADP-ribosylation site but is a critical modulator of ATP potency (21). Fig. 3B shows that the HEK293 line (HEK-mP2X7-R276K), stably transfected with the R276K gain of function mutant of P2X7R, exhibited a maximal Ca$^{2+}$/H$^{+}$ influx response to 50 μM ATP, which is a subthreshold concentration in cells expressing WT P2X7R (see Fig. 4A). Notably, this R276K mutation also facilitated the gating of P2X7R channel activity in response to NAD in HEK293 cells cotransfected with ART2.2 (Fig. 3B).

Fig. 3D demonstrates that ART2.1 also mediates the NAD-dependent activation of the hypersensitive R276K mutant P2X7R in transiently cotransfected HEK293 cells. These experiments used two FACS-based readouts of P2X7R function: 1) the transfer of PS to the external leaflet of the plasma membrane bilayer (“PS-flip”) as measured by increased binding of fluorochrome-conjugated annexin V; and 2) induction of the nonselective permeability pore as measured by the influx of YO-PRO-1 dye. In the absence of either ATP or NAD stimuli, the cells exhibited little if any surface annexin V staining or YO-PRO-1 accumulation. When stimulated by ATP, the majority of the ART2.1-mP2X7-R276K-cotransfected HEK cells showed strongly increased annexin V binding and YO-PRO-1 uptake. Treatment with NAD in the presence of DTT caused a similar stimulation of annexin V binding but a somewhat lower induction of YO-PRO-1 uptake. Treatment with NAD in the presence of DTT caused a similar stimulation of annexin V binding but a somewhat lower induction of YO-PRO-1 uptake. In contrast, cells treated with ADP-R plus DTT exhibited control levels of annexin binding and dye accumulation. Notably, these PS-flip and YO-PRO-1 influx responses to NAD were absent when the R276K mutation was combined with the double R125K/R133K substitutions that
eliminate the P2X7 ADP-ribosylation sites targeted by ART2.1. In contrast, and in accord with our previous report (21), the triple R276K/R125K/R133K mutant of P2X7 retained robust responses to ATP.

The differential ability of NAD/ART2 to activate the R276K-mutated P2X7R (Fig. 3, B and D), but not the WT P2X7R (Fig. 3A), in an HEK293 background is similar to the observations in Fig. 1 regarding the differential ability of NAD to activate P2X7R in murine T lymphocytes, but not in murine macrophages. These differences indicate that the consequences of ADP-ribosylation on P2X7R function are cell type specific, perhaps due to the differential expression of cell-specific accessory signaling molecules or of variant forms of P2X7R. We tested the hypothesis that ADP-ribosylation of WT P2X7R in HEK293 cells, although insufficient to activate the receptor per se, might modulate the ATP activation threshold. HEK293 cells coexpressing ART2.2 and WT P2X7R were prestimulated with or without 100 μM NAD for 3–5 min before being challenged with increasing concentrations of ATP to activate P2X7R-mediated Ca2+ influx. The NAD pretreatment decreased the threshold concentration of ATP required to stimulate P2X7R in cells that coexpressed ART2 (Fig. 4, A and B). In Fig. 4, C–F compare the quantification of ATP-induced changes in Ca2+ (with or without NAD pretreatment) at 30 s (Fig. 4, C and E) and 3 min (Fig. 4, D and F) in ART2-transfected or -nontransfected HEK-mP2X7 cells. The NAD-induced shift in ATP sensitivity was most apparent with [ATP] in the 200–600 μM range. NAD did not further increase the Ca2+ influx response to [ATP] ≥ 1 mM, which maximally activates P2X7R function. Notably, NAD pretreatment did not shift the ATP dose-response relationship in

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**FIGURE 3.** Differential effects of NAD/ART2 on activation of the murine P2X7R vs R276K-mutated P2X7R in HEK293 cells. A and B, HEK293 cells were stably transfected with either WT murine P2X7R (HEK-mP2X7 cells) in A or the R276K mutant of murine P2X7R (HEK-mP2X7-R276K cells) in B. The stably transfected lines were transiently cotransfected with murine ART2.2 24 h before an analysis of P2X7R-dependent Ca2+ influx was conducted. Cytosolic Ca2+ was measured in fura-2-loaded HEK cell suspensions as described in Materials and Methods and Fig. 1. The cell suspensions were pretreated with a mixture of 50 μM ADP and 50 μM UTP to activate and desensitize P2Y receptors 5 min before P2X7R stimulation by the indicated concentrations of ATP or NAD. NAD was added together with 1 mM ADP-R to attenuate metabolism of the NAD. These traces are representative of observations from three to four experiments with each cell line. Digi, Digitonin. C, HEK293 cells were transiently mock transfected (left lane) or transfected with ART2.2 (middle and right lanes). After 36 h, the intact cells were acutely incubated for 15 min with 50 μM e-NAD with or without 1 mM ADP-R before extraction, SDS-PAGE, and Western blotting with the 1G4 mAb that detects e-ADP-ribosylated proteins. IB, Immunoblotting. D, HEK cells were transiently cotransfected with murine ART2.1 and the indicated murine P2X7R mutants (R276K single mutant or R125K/R133K/R276K (R276K125K133K) triple mutant) before experiments. At 20 h posttransfection, the cells were harvested by trypsinization without further treatment (control) or following 10-min incubations with 1 mM ADP-R, 50 μM NAD, or 500 μM ATP separately in the presence of 1 mM DTT. The cells were then treated with 1 μM YO-PRO-1 for 1 h. Aliquots of the cotransfected cells were stained with allophycocyanin-conjugated annexin V before FACS analysis. All results are representative of observations from two or more independent experiments. The numbers in each panel represent the percentages of cells in the respective quadrants.
the HEK293 cells expressing P2X$_7$R but not ART2.2 (Fig. 4, B, E, and F).

These experiments focused on defining changes in P2X$_7$R activation by ATP in the threshold-to-EC$_{50}$ range because we (16) and others (2, 15) have noted that the conventional analysis of ATP concentration-response relationships for recombinant or native P2X7R is complicated by the following: 1) the unusually high ATP EC$_{50}$ (~1 mM); 2) the allosteric effects of extracellular Mg$^{2+}$ and Ca$^{2+}$ on P2X$_7$R activity; and 3) the strong chelation of divalent cations by ATP (a divalent anion at physiological pH and ionic strength), such that the extracellular concentrations of free Mg$^{2+}$ and free Ca$^{2+}$ are decreased as the concentration of added ATP is increased to supramillimolar levels. The assay of Ca$^{2+}$ influx as a sensitive and convenient readout of P2X$_7$R channel gating is further convoluted by the decreased extracellular [Ca$^{2+}$] and the consequent reduction in the chemical driving force at the supramillimolar [ATP]. Because the contribution of these various complicating factors is minimized at submillimolar [ATP], we assayed changes in threshold ATP concentrations rather than changes in the ATP EC$_{50}$ as the simplest index of increased ATP potency at the ADP-ribosylated P2X$_7$R.
The observation that NAD treatment decreased the threshold ATP concentration for heterologously expressed P2X7R in HEK293 suggested that NAD/ART2.1 might similarly regulate natively expressed P2X7R in murine macrophages. To facilitate these studies, we first determined that established murine macrophage and T cell lines were appropriate models for mechanistic analysis of the differential effects of NAD/ART2 on P2X7R function in primary murine macrophages vs T cells. We tested two murine macrophage cell lines (Bac1.2F5 and RAW264.7) and a murine T lymphoma cell line (BW5147). As in primary BMDM (36), the Bac1.2F5 macrophages (Fig. 5A) and RAW264.7 macrophages (data not shown) lacked basal expression of any ecto-ART subtypes at the mRNA level (heart or spleen extracts from BALB/c mice were used as positive control sources of ART1, ART2, ART3, ART4, and ART5 transcripts). However, Bac1.2F5 cells stimulated with 100 ng/ml LPS for 2–24 h (priming incubation) were then stimulated with 50 μM L-NAD and 1 mM ADP-R in the presence or absence of 2 mM DTT at 37°C for 15 min (test incubation) before extraction for SDS-PAGE and Western blot analysis of 1G4-reactive, εADP-ribosylated proteins. IB, Immuno blotting. E, Bac1.2F5 macrophages were stimulated with or without IFN-γ (100 U/ml) for 24 h. The primed cells were then stimulated with 50 μM ε-NAD and 1 mM ADP-R in the presence or absence of the indicated concentrations of GSH or DTT at 37°C for 15 min. F and G, ATP- or NAD-induced changes in Ca2+ were assayed in fura-2-loaded suspensions of untreated BW5147 T lymphocytes (F) or Bac1.2F5 macrophages primed for 24 h with IFN-γ (G). For Bac1 cells, the 100 μM NAD was added together 1 mM ADP-R and 1 mM DTT. These results are representative of observations from three or more experiments.

**Differential effects of NAD/ART2 on P2X7R function in established murine macrophage and T lymphocyte cell lines**

The observation that NAD treatment decreased the threshold ATP concentration for heterologously expressed P2X7R in HEK293 suggested that NAD/ART2.1 might similarly regulate natively expressed P2X7R in murine macrophages. To facilitate these studies, we first determined that established murine macrophage and T cell lines were appropriate models for mechanistic analysis of the differential effects of NAD/ART2 on P2X7R function in primary murine macrophages vs T cells. We tested two murine macrophage cell lines (Bac1.2F5 and RAW264.7) and a murine T lymphoma cell line (BW5147). As in primary BMDM (36), the Bac1.2F5 macrophages (Fig. 5A) and RAW264.7 macrophages (data not shown) lacked basal expression of any ecto-ART subtypes at the mRNA level (heart or spleen extracts from BALB/c mice were used as positive control sources of ART1, ART2, ART3, ART4, and ART5 transcripts). However, Bac1.2F5 cells stimulated with 100 ng/ml LPS for 2–24 h (priming incubation) were then stimulated with 50 μM ε-NAD and 1 mM ADP-R in the presence or absence of 2 mM DTT at 37°C for 15 min (test incubation) before extraction for SDS-PAGE and Western blot analysis of 1G4-reactive, εADP-ribosylated proteins. IB, Immuno blotting. E, Bac1.2F5 macrophages were stimulated with or without IFN-γ (100 U/ml) for 24 h. The primed cells were then stimulated with 50 μM ε-NAD and 1 mM ADP-R in the presence or absence of the indicated concentrations of GSH or DTT at 37°C for 15 min. F and G, ATP- or NAD-induced changes in Ca2+ were assayed in fura-2-loaded suspensions of untreated BW5147 T lymphocytes (F) or Bac1.2F5 macrophages primed for 24 h with IFN-γ (G). For Bac1 cells, the 100 μM NAD was added together 1 mM ADP-R and 1 mM DTT. These results are representative of observations from three or more experiments.

![Figure 5](http://www.jimmunol.org/DownloadedFrom)
thiol reductant, also supported the ADP-ribosylation of multiple surface proteins in the IFN-primed macrophages (Fig. 5E). Similarly as primary spleen T cells from BALB/c mice, BW5147 T lymphoma cells constitutively expressed both ART2.1 and ART2.2 mRNA (Fig. 5A and B) and also expressed functional ART2 activity as detected by the 1G4 mAb assay (data not shown). Splenic T cells from C57BL/6 mice expressed ART2.2, but not ART2.1, at significant levels. Notably, the BW5147 T cells also exhibited robust Ca\(^{2+}\)/H\(_{1001}\) influx responses to either ATP or NAD (Fig. 5F) as observed in primary splenic lymphocytes (Fig. 1A and B). In contrast, IFN-\(\gamma\)/H9253-primed Bac1.2F5 macrophages responded to exogenous ATP, but not to NAD alone (Fig. 5G), similarly as IFN- or LPS-primed primary BMDM (Fig. 1D).

Recent studies have indicated that extracellular NAD also activates some subtypes of G protein-coupled P2Y nucleotide receptors that can trigger mobilization of intracellular Ca\(^{2+}\)/H\(_{1001}\) stores (48). The Ca\(^{2+}\)/H\(_{1001}\) influx-based assay used to monitor P2X7R activity required prestimulation of the macrophages with a mixture of ADP and UTP to first activate and desensitize the Ca\(^{2+}\)-mobilizing P2Y1, P2Y2, and P2Y6 receptors expressed in these cells (41, 42). We tested the possibility that this protocol also desensitized NAD-reactive P2Y receptors that might be expressed in T cells and macrophages. BW5147 and Bac1.2F5 macrophages were stimulated with only single-nucleotide agonists with no prior desensitization incubation. Although murine T cells have been reported to express mRNA for various P2Y receptor subtypes (14), we observed no Ca\(^{2+}\)/H\(_{1001}\)-mobilizing responses to micromolar concentrations of ADP (P2Y1 agonist), UTP (P2Y2/P2Y4 agonist), or UDP (P2Y6 agonist) in the BW5147 T cells (Fig. 6A). Moreover, primary T cells from P2X7R-knockout mice showed no Ca\(^{2+}\)/H\(_{1001}\) response to ATP or NAD (Fig. 1). Thus, naive murine T cells do not express functionally significant levels of Ca\(^{2+}\)/H\(_{1001}\)-mobilizing P2Y receptors that can be activated by ATP, ADP, UTP, UDP, or NAD. In contrast, Bac1 macrophages, like primary murine BMDM (36), exhibited strong Ca\(^{2+}\) mobilization responses to ADP, ATP, UTP, and UDP, but not to NAD (Fig. 6B). Thus, the differential responses of T cells vs macrophages to NAD do not involve obvious roles for NAD-sensitive P2Y receptor subtypes.

Other ectoenzymes that modulate the efficiency of NAD-dependent ADP-ribosylation of cell surface proteins can also be differentially expressed in leukocyte subsets. These include the CD38 NAD-glycohydrolases and CD203 nucleotide pyrophosphatases that metabolize extracellular NAD and thereby reduce

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**FIGURE 6.** P2Y receptor-based Ca\(^{2+}\)/H\(_{1001}\) signaling and extracellular NAD metabolism in murine macrophage and T lymphocyte cell lines. A and B, Cytosolic Ca\(^{2+}\) was assayed in fura-2-loaded suspensions of BW5147 T cells (A) or IFN-\(\gamma\)-primed Bac1.2F5 macrophages (B) challenged with the indicated concentrations of ADP, UTP, UDP, NAD, or ATP. The results are representative of observations from two experiments. Dig, Digitonin. C and D, Monolayers of Bac1.2F5 macrophages (10\(^6\) cells/well in 6-well dishes) were incubated in 1 ml of BSS at 37°C supplemented with 100 \(\mu\)M NAD in the presence or absence of 1 mM ADP-R. At selected times (0–60 min), 100-\(\mu\)l aliquots of the extracellular medium were removed and processed for HPLC analysis. C, Stacked HPLC chromatograms of extracellular samples taken at the 0-, 10-, 30-, and 60-min time points. D, NAD concentrations at the indicated times were calculated from the HPLC chromatograms.
substrate drive to the ARTs (26). We used HPLC analyses to test whether the inability of NAD per se to trigger \( \text{Ca}^{2+} \) influx or mobilization in murine macrophages was due to very rapid metabolism of the added extracellular NAD. Although Bac1 macrophages metabolized 100 \( \mu \text{M} \) NAD to ADP-R, the \( t_{1/2} \) for this reaction was \( \approx 20 \) min, such that \( >50 \) \( \mu \text{M} \) NAD was present throughout the 1–10 min test periods used to assay \( \text{Ca}^{2+} \) influx/mobilization responses (Fig. 6C). Inclusion of exogenous ADP-R (routinely added to maintain ADP-ribosylation of target proteins) further slowed the rate of NAD clearance. Thus, excessive NAD catabolism is an unlikely reason for the differential responses of macrophages vs T cells to extracellular NAD.

**NAD potentiates ATP-induced P2X7R activation in murine macrophages and T lymphocytes**

IFN-\( \gamma \)-primed Bac1.2F5 macrophages were briefly pretreated with NAD in the presence of DTT to allow ADP-ribosylation of cell surface proteins and then challenged with various doses of ATP to trigger P2X7R-dependent \( \text{Ca}^{2+} \) influx. Similar to its effects in ART2/P2X7-transfected HEK293 cells, NAD pretreatment increased the sensitivity of the IFN-primed Bac1 macrophages to submillimolar ATP (Figs. 7, A–C). In contrast, naive Bac1.2F5 cells, which were not primed with IFN-\( \gamma \) and thus lacked expression of ART2.1, did not exhibit the NAD-dependent increase in ATP sensitivity (Figs. 7, D and E). Additional experiments demonstrated that NAD pretreatment produced similar increases in ATP sensitivity in other murine macrophage models, including IFN-\( \beta \)-primed RAW264.7 macrophages (Fig. 8A) and IFN-\( \gamma \)-primed BALB/c primary BMDM (Fig. 8B). Similarly as Bac1.2F cells (Fig. 5) and primary BMDM (36), the RAW264.7 macrophage line exhibited up-regulation of ART2.1 mRNA and thiol-dependent ecto-ART activity in response to IFN-\( \beta \) stimulation (data not shown).

NAD pretreatment also increased the sensitivity to ATP in the BW5147 T lymphocytes. These experiments involved stimulation of these cells with submaximally active concentrations of NAD (10 \( \mu \text{M} \)) and/or ATP (300 \( \mu \text{M} \)). Notably, when combined, 10 \( \mu \text{M} \) NAD and 300 \( \mu \text{M} \) ATP acted synergistically to increase P2X7R-mediated \( \text{Ca}^{2+} \) influx (Fig. 8C).

ART2 can use dinucleotide substrates other than NAD to covalently ribosylate arginine residues in target proteins; these alternative substrates include \( e \)-NAD and nicotinamide guanine dinucleotide (NGD). We previously reported that ribosylation of the P2X7R by \( e \)-NAD or NGD in primary murine T cells does not stimulate the receptor but rather antagonizes NAD-induced receptor activation (20). We observed similar effects of \( e \)-NAD on P2X7R function in the BW5147 T cell line (Figs. 9, A and B). Notably, P2X7 receptors ribosylated by \( e \)-NAD also have decreased sensitivity to activation by submaximal ATP as shown in primary...
murine T cells (20), BW5147 T lymphoma cells (Figs. 9, A and B), IFN-γ-primed Bac1.2F5 macrophages (Fig. 9C), and IFN-β-primed RAW264.7 macrophages (Fig. 9D).

NAD potentiates ATP-induced P2X7R-dependent pore formation in murine macrophages

The previous data showing that ADP-ribosylation of P2X7R potentiates ATP activation of these receptors in murine macrophages and HEK293 cells used increases in cytosolic Ca²⁺ as a sensitive readout of P2X7R channel activity. We considered the possibility that the P2Y receptors (triggers G protein-coupled signaling pathways that modulate the functional interactions between ART2 and P2X7R in macrophages and HEK293 cells. Thus, we measured the effects of NAD/ART2 on nonselective pore formation as an alternative readout of P2X7R activation that does not require prestimulation and desensitization of P2Y receptors. Pore formation was assayed by ethidium accumulation in IFN-γ-primed (Fig. 10A) vs control (Fig. 10B) Bac1.2F5 macrophages, similar to the YO-PRO-1 accumulation assay previously described for HEK293 cells (Fig. 3D). NAD (plus DTT) by itself did not stimulate ethidium accumulation but did increase the rate of accumulation triggered by submillimolar ATP in the IFN-γ primed, but not control, Bac1 macrophages. Notably, the potentiating effects of NAD on ethidium influx were observed at a higher range of extracellular ATP concentrations (0.5–1 mM) than the effects on Ca²⁺ influx (0.1–0.5 mM ATP; Fig. 7). This is consistent with the fact that ethidium influx is a secondary response to P2X7R stimulation (49) that involves gating of pannexin-1 hemichannels (4). Previous studies have indicated that the ATP concentration-response relationship describing this secondary response is right shifted relative to the concentration-response relationship describing the primary gating of P2X7R channels (2, 50).

Discussion

From the initial functional characterization of the permeabilizing “P2Z” ATP receptor 30 years ago (51–53), through the molecular identification of the P2Z receptor phenotype as the product of the P2X7 gene (2), and up to the most recent analyses of in vivo functional deficits in P2X7-knockout mice (10, 11, 54), two fundamental and perplexing questions regarding the P2X7R have been repeatedly considered. First, why does this particular ATP receptor, in sharp contrast to the six other P2X receptor subtypes, require millimolar levels of extracellular ATP for activation when studied in isolated cells? This unusual characteristic suggests that low affinity variants of an ancestral P2X7R were favored by positive selection as the receptor acquired its physiological roles as a regulator of proinflammatory signaling and cell death. Low ATP affinity prevents inadvertent activation of these highly consequential but poorly reversible responses until leukocytes accumulate at sites of tissue damage or microbial invasion. However, this raises a second and corollary question: how does the P2X7R become activated in leukocytes within these latter tissue compartments given the receptor’s low affinity of ATP? Recent studies support three possible mechanisms that are not mutually exclusive: 1)
highly localized accumulation of ATP for autocrine activation of P2X7R within diffusion-restricted cell surface compartments (12–14); 2) allosteric modulation of ATP affinity via conformational changes in P2X7 trimeric channels produced by local biophysical conditions or covalent modification of the P2X7R protein itself (15, 16, 18, 19, 55, 56); and 3) the ATP-independent activation of P2X7R via conformational changes produced by ADP-ribosylation of key arginines within the extracellular loop of the P2X7R (20, 21, 57). The experiments described in this report provide new insights into the latter two regulatory mechanisms and additionally suggest that a fourth mechanism, one involving tissue/cell-selective expression of accessory molecules and/or of P2X7R splice variants, contributes to the regulation of P2X7R function.

The ability of NAD to drive the covalent modification of extracellular residues of the P2X7R comprises a novel mechanism to produce relatively long-lasting changes in the conformational state of these ligand-gated ion channels during transient increases in extracellular NAD, ATP, and other normally intracellular metabolites, such as lysolipids, that can regulate P2X7R function (22, 57, 58). Previous studies demonstrated that NAD can trigger P2X7R activation in murine T lymphocytes even when these cells were incubated in the presence of apyrase to scavenge any released ATP (20). Moreover, P2X7R activation was sustained in T cells briefly treated with NAD and then washed free of this nucleotide. In contrast, ATP-stimulated P2X7R rapidly deactivated when T cells were transferred to ATP-free medium. These findings indicate that ADP-ribosylation of P2X7R subunits in murine T lymphocytes induces a conformational change sufficient to gate the opening of these trimeric channels even in the absence of ATP binding. However, our studies of P2X7R function in murine macrophages and HEK293 cells indicate that this ATP-independent activation of P2X7R by ADP-ribosylation is not a general mode of P2X7R regulation but rather reflects the specialized conditions present in murine T lymphocytes.

Notably, although NAD by itself was able to gate P2X7R in NZW T lymphocytes expressing solely ART2.1, it failed to activate P2X7R either in murine macrophages that coexpress native P2X7R and ART2.1 or in HEK293 cells engineered to coexpress...
urine P2X-R and murine ART2 ectoenzymes. However, NAD acted synergistically with ATP to regulate P2X-R in both the macrophages and the engineered HEK cells, and this effect of NAD was strictly dependent on the expression of ART2.1 or ART2.2 in both cell models. How can these regulatory effects of NAD/ART2 on ATP-dependent P2X-R activation observed in murine macrophages and HEK293 cells be reconciled with the robust ATP-independent activation by NAD/ART2 in murine T cells? Possible explanations for the difference in P2X-R signaling observed between myeloid and lymphoid cells are that T cells, but not macrophages or HEK cells, express other regulatory proteins that facilitate the ATP-independent gating of P2X-R in response to ADP-ribosylation, or that the local membrane microenvironments containing P2X-R and ART2 are different in the two cell types. Another possible explanation might be the expression of different, recently identified splice variants of rodent P2X-R in T cells vs macrophages. Indeed, Taylor et al. have recently reported that P2X-R function is preserved in the T lymphocytes, but not in macrophages, from one strain of P2X-γ null mice that was generated by lacZ insertion into exon 1 of the p2rx7 gene (59). Determining whether different murine tissues and cells, particularly hematopoietic cell types, express splice variants of the P2X-R with altered functional responses to ART2-mediated modification is an important goal for future experiments.

It is important to consider how ADP-ribosylation of key Arg residues may affect the conformation of these trimeric channels. Electrophysiological analyses of P2X-family channels at the whole cell and single-channel levels indicate that at least two, and probably three, molecules of ATP need to be bound per channel for optimal gating (9, 60). Moreover, the critical ATP binding sites appear to be formed at the interfaces between the extracellular loops of individual subunits, rather than within each subunit loop as initially hypothesized (61, 62). In this regard, the covalently modified subunits or whether only one or two subunits per channel may interact with the key interfacial amino residues that form the ATP-binding site. However, ADP-R is larger than ATP per se, and it is unclear whether the P2X-R channel complex can accommodate ADP-ribosylation of all three subunits or whether only one or two subunits per channel can be efficiently modified. Differences in the number of covalently modified subunits per channel, due possibly to steric hindrance, may underlie the distinctive consequences of NAD/ART2 action on P2X-R function in macrophages vs T cells.

ADP-ribosylation of these receptors in macrophages may be limited to only one or two subunits per channel, which is insufficient for gating but sufficient for positive allosteric action at the remaining interfacial ATP-binding sites. This would be consistent with the observed increase in potency of ATP at P2X-R in ART2.1-expressing macrophages (or HEK293 cells) pretreated with NAD. In contrast, the predominant P2X-R channels in T cells or the mutant P2X-R-R276K channels in HEK cells may have conformations that accommodate and permit ADP-ribosylation of all three subunits.

It is currently unclear whether ADP-ribosylation is a common mechanism for the activation or sensitization of P2X-R signaling in other tissues or organisms. Notably, the Arg125 and Arg133 residues are conserved in the human P2X-R (63), but the human ART2A and ART2B loci are transcriptionally silent pseudogenes (26, 64). Thus, human T cells and macrophages lack the capacity for cis-regulation of P2X-R by a coexpressed ecto-ART. However, human ART1 is constitutively expressed in neutrophils, and this GPI-anchored enzyme is rapidly shed during neutrophil activation in response to bacterial infection (65). ART1 is also expressed by human airway epithelial cells basally and at increased levels in response to bacterial mediators (66–68). Thus, the P2X-R in human macrophages and T cells might be trans-regulated by shed ART1 that accumulates at sites of bacterial infection and neutrophil recruitment. Such a mechanism may also be operative in mice that also express ART1 in other tissues such as cardiac and skeletal muscle (69).

NAD is released to extracellular environments during the early stage of inflammatory response (57). Besides its ability to trigger P2X-R-dependent T cell death (20), extracellular NAD has been reported as an agonist for P2Y11 receptors in human granulocytes (48). Our study now shows that NAD also increases the sensitivity of the P2X-R to ATP gating in macrophages. This action of NAD requires expression of the thiol-sensitive ART2.1 enzymes and reduced thiols, such as glutathione and cysteine, that can accumulate at inflammatory loci due to release from activated macrophages and the hypoxia that often characterizes such loci (31). We have found that ART2.1 is widely expressed in other leukocytes, such as dendritic cells and B lymphocytes (70). Thus, the sensitization of ATP-dependent P2X-R activation by NAD/ART2.1 may provide an additional layer of regulatory control in multiple phases of innate and adaptive immunity.


