NAD^+ and ATP Released from Injured Cells Induce P2X7-Dependent Shedding of CD62L and Externalization of Phosphatidylserine by Murine T Cells

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NAD$^+$ and ATP Released from Injured Cells Induce P2X$_7$-Dependent Shedding of CD62L and Externalization of Phosphatidylserine by Murine T Cells$^1$


Extracellular NAD$^+$ and ATP trigger the shedding of CD62L and the externalization of phosphatidylserine on murine T cells. These events depend on the P2X$_7$ ion channel. Although ATP acts as a soluble ligand to activate P2X$_7$, gating of P2X$_7$ by NAD$^+$ requires ecto-ADP-ribosyltransferase ART2.2-catalyzed transfer of the ADP-ribose moiety from NAD$^+$ onto Arg125 of P2X$_7$. Steady-state concentrations of NAD$^+$ and ATP in extracellular compartments are highly regulated and usually are well below the threshold required for activating P2X$_7$. The goal of this study was to identify possible endogenous sources of these nucleotides. We show that lysis of erythrocytes releases sufficient levels of NAD$^+$ and ATP to induce activation of P2X$_7$. Dilution of erythrocyte lysates or incubation of lysates at 37°C revealed that signaling by ATP fades more rapidly than that by NAD$^+$. We further show that the routine preparation of primary lymph node and spleen cells induces the release of NAD$^+$ in sufficient concentrations for ART2.2 to ADP-ribosylate P2X$_7$, even at 4°C. Gating of P2X$_7$ occurs when T cells are returned to 37°C, rapidly inducing CD62L-shedding and PS-externalization by a substantial fraction of the cells. The “spontaneous” activation of P2X$_7$ during preparation of primary T cells could be prevented by i.v. injection of either the surrogate ART substrate etheno-NAD or ART2.2-inhibitory single domain Abs 10 min before sacrificing mice. The Journal of Immunology, 2009, 182: 2898–2908.

NAD$^+$ and ATP are universal currencies of energy metabolism and are found in all cells in all kingdoms of life. Mounting evidence indicates that these nucleotides play important roles also as signaling molecules in the extracellular environment (1, 2). In the context of the immune system of higher organisms, it has been proposed that the release of NAD$^+$ and ATP from lysed cells may alert cells of the immune system to tissue damage (2–6). Indeed, lymphocytes and macrophages are equipped with numerous sensors for extracellular NAD$^+$ and ATP, including nucleotide-metabolizing ecto-enzymes and nucleotide receptors (7, 8).

Mono-ADP-ribosyltransferases (ARTs)$^4$ transfer the ADP-ribose moiety from NAD$^+$ to specific amino acids in target proteins (9, 10). This is the mechanism by which several bacterial toxins, like cholera- and pertussis-toxin, cause pathology after translocating into mammalian host cells (11). ART2.2 is a GPI-anchored, raft-associated ecto-enzyme prominently expressed by murine T cells (12–14). ART2.2 catalyzes ADP-ribosylation of CD8, the integrin LFA-1, the P2X$_7$ receptor, and several other target proteins (12–15). T cell activation induces the metalloprotease-mediated shedding of a soluble, enzymatically active isoform of ART2.2 (16). ART2-deficient mice (17) exhibit reduced sensitivities to Con-A-induced hepatitis (18).

The type II transmembrane protein CD38 is a potent ecto-NAD$^+$-glycohydrolase (ecto-NADase) (19, 20) expressed by lymphocytes, endothelial cells, and several other cell types. CD38-deficient mice show impaired humoral immune responses, neutrophil chemotaxis, and dendritic cell (DC) trafficking (21–23). Cells from CD38-deficient mice do not metabolize ecto-NAD$^+$ efficiently, and the resulting higher levels of ecto-NAD$^+$ lead to a higher level of ART-mediated cell surface protein ADP-ribosylation (24). CD38-deficient mice show enhanced sensitivity to insulin-dependent diabetes mellitus, which is dependent on the presence of ART2.2 (25). Likely, this reflects the enhanced activity of ART2.2 in these mice as a consequence of increased levels of extracellular NAD$^+$ (24).

The cytolytic P2X$_7$ receptor is a homo-trimeric, ligand-gated, nonspecific ion channel that has sparked interest because of its peculiar ability to induce the formation of a large nonsel ective membrane pore (4, 26–28). High concentrations of extracellular ATP (0.2–2 mM) are required to gate P2X$_7$. Much lower concentrations of extracellular NAD$^+$ (2–20 µM) suffice to gate P2X$_7$ on cells coexpressing ART2.2 (13). ART2.2-catalyzed ADP-ribosylation at residue R125 presumably positions the common nucleotide-diphosphate moiety into the ligand binding site at the interface of adjacent subunits of the homotrimeric receptor (29). Activation of P2X$_7$ on T cells by ATP or by NAD$^+$-dependent ADP-ribosylation initiates a cascade of events, including influx of calcium, the shedding of the L-selectin/CD62L homing receptor, and the externalization of phosphatidylserine (PS) on the outer leaflet of the cell membrane (13, 29, 30). Chronic
activation of P2X$_7$ induces apoptosis and cell lysis (13). The natural allelic P451L polymorphism in the C-terminal tail of murine P2X$_7$ distinguishes common strains of laboratory mice (31). Wild-type (WT) P451 is expressed by BALB/c and most other strains of mice, whereas the 451L variant is expressed by C57BL/6 and DBA mice (31). The 451L variant displays normal membrane currents, but impaired pore formation (31, 32). Naive BALB/c T cells are very sensitive to NAD$^+$ and ATP, whereas C57BL/6 T cells are less sensitive (13).

The plasma membrane of living cells is impermeable to NAD$^+$ and ATP, but they can be released from cells by lytic and nonlytic mechanisms (33, 34). Intracellular levels of NAD$^+$ and ATP are in the upper micromolar and lower millimolar range, whereas serum concentrations are two to three orders of magnitude lower and are kept low by ecto-nucleotidases. The duration of extracellular signaling via NAD$^+$ is controlled by the CD38 family of ecto-NAD$^+$-glycolydrolases/ADP-ribosylcycas that hydrolyze NAD$^+$ to ADP-ribose and the CD203 family of phosphodiesterases that hydrolyze NAD$^+$ to nicotinamide mononucleotide and AMP (20, 35, 36). Similarly, the concentration of ATP and the duration of signaling via ATP in the extracellular compartment are controlled by the CD39 family of ecto-nucleotidases and the CD203 family of phosphodiesterases which hydrolyze ATP to ADP and/or AMP (37–39).

The principle aim of this study was to determine whether endogenous sources of NAD$^+$ and/or ATP can activate P2X$_7$. To address this question, we used two model systems, hemolysis and mechanization. The mechanical manipulation of cells as during the routine preparation of primary cells from lymph nodes and spleen. Our results show that both scenarios, indeed, result in the release of endogenous nucleotides in sufficient concentration to activate P2X$_7$, with ATP-mediated effects dissipating more rapidly than those mediated by NAD-dependent ADP-ribosylation.

**Materials and Methods**

**Chemicals and Abs**

ADP-ribose, ATP, NAD$^+$, etheno-NAD$^+$, and KN-62 (1-(N,N-bis(5-isouquinolinesulfonfyl)-N-methyl-1-tyrosyl)-4-phenylpiperazine) were obtained from Sigma-Aldrich. PE- and FITC-conjugated mAbs and Annexin-V were purchased from BD Pharmingen, including anti-CD3e (145-2C11), anti-CD4 (RM4–5), anti-CD8 (53–5.8), anti-CD38 (90), and anti-CD62L (ME1–14). 32P-P-NAD$^+$ was obtained from Amersham Biosciences. mAbs Nika102 (anti-ART2.2) pAb K1G (anti-P2X$_7$), and single domain Abs s1-10a and l1-17 (anti-ART2.2) were prepared as described previously (12, 40, 41).

**Animals and cells**

BALB/c mice were obtained from The Jackson Laboratory or Charles River Laboratories. CD38-deficient mice (21) and ART2-deficient mice (17) were backcrossed onto the BALB/c background for 12 generations. The ART2$^{−/−}$ lines are deficient in both ART2.1 and ART2.2 (17). P2X$_7$–/– mice are from the twelfth backcross generation to C57BL/6 mice (3). Where indicated, mice received i.v. injections of PBS, single domain Abs in PBS, or etheno-NAD$^+$ in PBS (200 µl into the tail vein). Mice were sacrifed by exposure to O$_2$/CO$_2$. All animal experiments were performed in accord with the German animal-protection law. Single-cell suspensions were prepared from lymph nodes and spleens in cold (4°C) RPMI 1640 medium by gentle dissection and passage through Nitex mesh, Tetko). Alternatively, single cell suspensions were prepared by two other techniques: 1) from spleen by gentle perfusion with 10 ml ice cold medium using a 1.0-gauge needle and 10 ml syringe or 2) from lymph nodes and spleen by gentle pipetting following preincubation in medium containing 10 mg/ml collagenase (Life Technologies) for 60 min at 4°C. B cells were depleted using magnetic cell separation with Dynabead-immobilized goat anti-mouse IgG (Dynal Biotech) at 4°C as described (13). Purity of T cells was always >95% as verified by FACS analyses using PE-conjugated anti-B220 and FITC-conjugated anti-CD3.

**Preparation of erythrocyte lysates**

Blood was collected from mice by retroorbital puncture into ice cold heparinized Eppendorf tubes. Erythrocytes were pelleted by centrifugation at 4°C, washed once in ice cold PBS, and were resuspended in 1.5 volumes of ice cold PBS. Cells were ruptured on ice by ultrasonication in an MK2 ultrasonic disintegration machine (MSE Scientific Instruments) by two 10-second sonifications at a power setting of 12 micron. Cellular debris was removed by centrifugation (15,000 × g) for 5 min at 4°C. Clarified lysates were diluted in ice cold PBS. Aliquots of lysates (50 µl) were added to cell suspensions in RPMI 1640 medium (50 µl, 4 × 10$^6$ cells). NAD$^+$ concentrations in cell lysates were determined by a sensitive cycling assay (42). ATP concentrations were measured with a luciferase-based assay system according to the manufacturer’s instructions (Sigma-Aldrich). In some cases, lysates were incubated at 37°C for the indicated times before addition to T cells.

**Assay for phosphatidylserine exposure and propidium iodide (PI) staining**

Following treatment with exogenous NAD$^+$, ATP, or erythrocyte lysates for the indicated times at 4 or 37°C, cells were washed in RPMI 1640 medium adjusted to 2 mM CaCl$_2$, and were stained for 20 min on ice with FITC-conjugated Annexin-V (1 µg/ml) (BD Biosciences) and PI (10 µg/ml) before flow cytometry. In some cases, KN-62 was added during or after treatment with NAD$^+$ and ATP. Relative high concentrations (10 µM) were used because KN-62 exhibits low potency at mouse P2X$_7$ receptors (43, 44). For temperature response analyses, cells were incubated in the absence or presence of ATP for 30 min at the indicated temperature in a PCR machine. For pulse chase analyses, cells were treated with NAD$^+$ or ATP for 30 min at 4 or 37°C. Cells were then washed and incubated further for 60 min at 4°C before staining with Annexin-V/PI as above.

**Immunoprecipitation and Western blot analyses**

T cells (1 × 10$^6$/ml) were incubated for 20 min at 4 or 37°C in the presence of radiolabeled 32P-NAD$^+$ (1 µM, 5aCi/ml) in RPMI 1640 medium containing 1 mM ADP-ribose. Cells were then washed in cold PBS to remove free NAD$^+$. Cells were lysed in 250 µl PBS, 1% Triton X-100, 1 mM AEBSF at 4°C for 30 min on ice. Cell lysates were precleared by centrifugation (14,000 × g at 4°C for 30 min) followed by incubation with 20 µl Protein-G-Sepharose (Pharmacia Biotech) for 60 min at 4°C. Immunoprecipitation was performed in parallel with K1G anti-P2X$_7$ immune serum (1 µg) (40), LFA-1-specific mAb M17/4 (1 µg), or CD8-specific mAb 53–6.7 (1 µg) each immobilized on 20 µl Protein-G-Sepharose. Proteins were size fractionated by SDS-PAGE on precast Nupage (10%) gels (InVitrogen) and blotted onto nitrocellulose membranes. Radioactivity was detected by autoradiography by exposing the membrane to Kodak X-omat Films at −80°C for 48 h.

**Results**

**Exposure of murine T cells to exogenous NAD$^+$ or ATP causes shedding of CD62L and externalization of phosphatidylserine**

Treatment of purified murine T cells with exogenous NAD$^+$ or ATP at 37°C induced, within minutes, the externalization of phosphatidylserine on the outer leaflet of the plasma membrane (Fig. 1A, panels 2 and 4) and the shedding of L-selectin/CD62L (Fig. 1B, panels 2 and 4). T cells from P2X$_7$–/– deficient mice (3) were completely resistant to the effects of NAD$^+$ and ATP (Fig. 1, C and D), indicating that these effects are dependent on P2X$_7$. Consistently, WT cells incubated with NAD$^+$ or ATP in the presence of KN-62, a specific inhibitor of P2X$_7$ (45), neither shed CD62L nor exposed phosphatidylserine (Fig. 1, A and B, panels 3 and 5). In the case of T cells obtained from C57BL/6 mice that express the 451L P2X$_7$ variant (31), much higher concentrations of NAD$^+$ and ATP were required to induce PS exposure and CD62L shedding than by T cells from BALB/c mice that express WT P2X$_7$ (Fig. 1, A and B, panel 9), but were resistant to NAD-induced activation of P2X$_7$ (Fig. 1, A and B, panel 7), which requires ART2-catalyzed ADP-ribosylation of P2X$_7$ (29).
NAD⁺ and ATP released from lysed erythrocytes activate P2X7 on murine T cells

The membrane of living cells is impermeable to NAD⁺ or ATP. Cells lysed during tissue injury or in the course of necrotic cell death present a potential source of extracellular nucleotides. To test whether nucleotides released from lysed cells can activate P2X7, we exposed purified T cells to crude cell lysates generated by ultrasonication of mouse erythrocytes (Fig. 2). We chose erythrocytes for these analyses because the lysis of erythrocytes occurs under various pathological conditions in vivo (46–48) and because erythrocyte lysis is commonly used to deplete these cells during the preparation of primary lymphocytes (49).

The results shown in Fig. 2 demonstrate that T cells, indeed, respond in a dose-dependent manner to erythrocyte lysates with externalization of PS and shedding of CD62L (Fig. 2, A and B, panels 1 and 3). As in case of exogenously added nucleotides (Fig. 1), these responses were suppressed by the P2X7 antagonist KN-62 (Fig. 2, A and B, panels 2 and 4). Note the lower intensity of Annexin-V staining in cells treated with concentrated vs dilute lysates (Fig. 2A, panels 1 and 3). This likely reflects the presence

![Figure 1](http://www.jimmunol.org/Downloadedfrom)
of endogenous annexins released from lysed erythrocytes that compete with the fluorochrome-conjugated Annexin-V for binding to externalized PS on T cells. Note further that ART2-deficient cells responded only to concentrated lysates (Fig. 2, panels 5 and 7) whereas WT T cells responded to both concentrated and diluted lysates (Fig. 2, panels A and B, panels 1 and 3). These findings indicate that diluted lysates contain NAD\(^+\) but not ATP in sufficient concentration to activate P2X\(_7\), whereas concentrated lysates also contain sufficient ATP.

To better assess the relative contributions of NAD\(^+\) and ATP from erythrocyte lysates on l-selectin shedding and PS exposure by T cells, we performed comparative dose response analyses with erythrocyte lysates diluted 1/7.5, 1/20 in PBS for 30 min at 37°C. Parallel incubations were performed in the presence of 10 \(\mu\)M KN-62 (panels 2, 4, 6, and 8). Cells were washed and stained with Annexin-V-FITC and PI (A) or with anti-CD62L-PE and anti-CD3\(^\text{FITC}\) (B) before FACS analysis. Numbers indicate the percentage of cells in each quadrant (in B those in the upper left quadrant). Results are representative of four independent experiments.

The EC\(_{50}\) values obtained with exogenous NAD\(^+\) or ATP correspond well to our measurements of NAD\(^+\) and ATP in erythrocyte lysates and those of previous studies (50–52). Experiments performed with ART2-deficient T cells, sensitive only to ATP, show that the effects obtained with the EC\(_{50}\) of ATP (\(\sim 120\) \(\mu\)M) are reproduced with erythrocyte lysates diluted \(\sim 10\)-fold, consistent with the interpolated ATP concentration in undiluted erythrocyte lysates of 1.0 mM (\(\pm 400\) \(\mu\)M, \(n = 13\)). Similarly, experiments performed with WT T cells show that the effects obtained with the EC\(_{50}\) of NAD\(^+\) (\(\sim 2.6\) \(\mu\)M) are reproduced with erythrocyte lysates diluted 100-fold, consistent with the interpolated concentration of NAD\(^+\) in undiluted erythrocyte lysates of 260 \(\mu\)M (\(\pm 80\) \(\mu\)M, \(n = 13\)).

In these experiments, we consistently noted a slightly higher proportion of cells “spontaneously” exposing PS in preparations obtained from WT mice vs ART2-deficient mice (i.e., compare control values for WT and ART2-deficient cells in the absence of exogenously added nucleotides or lysates in Fig. 3, A–D). The hypothesis that this observation reflects the exposure of cells to NAD\(^+\) before or during cell preparation will be addressed further below.

**The P2X\(_7\)-inducing activities in erythrocyte lysates fade upon incubation at 37°C**

The fate and half-life of extracellular nucleotides is determined by numerous nucleotide-metabolizing ecto-enzymes such as CD38 and CD39, many of which are expressed by erythrocytes (53, 54). One should, therefore, expect that ultrasoundication of erythrocytes would expose the released NAD\(^+\) and ATP to nucleotide-degrading enzymes. To determine whether nucleotide turnover affects the P2X\(_7\)-inducing potential of erythrocyte lysates, we incubated these lysates for various times at 37°C and then assayed the capacity of the lysates to induce PS exposure by T cells (Fig. 4). A substantial reduction in the capacity of lysates to induce PS exposure was seen already after 10 min of preincubation (Fig. 4A, panels 2 and 5 and Fig. 4B) and all PS-inducing activity was lost within 60 min (Fig. 4A, panels 3 and 6 and Fig. 4B). Taken together, these results indicate that erythrocyte lysates contain both, NAD\(^+\) and ATP, in sufficient concentrations to activate P2X\(_7\) on T cells, and further that both nucleotides are degraded with a half-life of less than 10 min upon incubation at 37°C. The results again pinpoint a clear difference in the background proportion of freshly prepared T cells from WT mice vs ART2-deficient mice, exposing PS on their surface (Fig. 4B). The following experiments were designed to further explore this phenomenon.
In vivo blockade of ART2.2 prevents the spontaneous PS flashing and shedding of CD62L by freshly prepared lymph node cells

The “spontaneous” externalization of PS and shedding of CD62L by a fraction of primary WT T cells from freshly prepared lymph nodes (Fig. 1 and 4B) might reflect their prior encounter with extracellular nucleotides released from endogenous sources. The spontaneous PS exposure and shedding of CD62L evidently depend on functional ART2 and P2X7, as these phenomena are not observed in T cell preparations from ART2-deficient (Fig. 1, A and B, panel 6) or P2X7-deficient (Fig. 1, C and D, panel 6) mice. Spontaneous PS exposure and shedding of CD62L was specific for T cells, consistent with the notion that the effect is dependent on ART2.2, which is expressed by T cells, but not by B cells, NK cells, or macrophages (12). The finding that ART2-deficient cells do not spontaneously expose PS (Fig. 1A, panel 6) or shed CD62L (Fig. 1B, panel 6) indicates that these effects are caused by endogenous NAD+ rather than by endogenous ATP because ART2-deficient T cells retain unabated sensitivity to exogenous ATP (Fig. 1A, panel 9). Consistently, T cells from CD38-deficient mice which lack the major NAD-hydrolyzing ecto-enzyme (21, 24) show markedly increased levels of spontaneous PS externalization (Fig. 5A, panel 4) and CD62L shedding (Fig. 5A, panel 3).

In principle, the spontaneous PS exposure and CD62L shedding could reflect exposure of cells to extracellular NAD+ either before, i.e., in vivo, or during cell preparation, i.e., after sacrificing of the mouse. To distinguish between these possibilities, we sought conditions that would prevent these reactions either by blocking ART2.2 or by blocking the activation of P2X7. To this end, we first used two distinct, recently described single domain Abs (sdAbs) that block the enzymatic and cytotoxic activities of ART2.2 (41). As illustrated in Fig. 5, a single i.v. injection of either sdAbs s16a or 1-17 10 min before sacrificing effectively blocked both PS externalization (Fig. 5A, panel 2 and Fig. 5B) and shedding of CD62L (Fig. 5A, panel 4 and Fig. 5B), even in case of the highly susceptible cells from CD38-deficient mice. As a complementary approach, we used etheno-NAD+ as ART-substrate (55). Etheno-ADP-ribosylation does not activate P2X7 and, in addition, prevents activation of P2X7 by subsequent ADP-ribosylation (13, 29). Indeed, as in the case of the sdAbs, a single i.v. injection of etheno-NAD+ 10 min before sacrificing effectively blocked both PS externalization and shedding of CD62L (Fig. 5B) by freshly prepared cells. These results strongly suggest that exposure of cells to endogenous NAD+ occurred during sacrificing and/or cell preparation.

T cells prepared at 4°C do not externalize PS, but do so when returned to 37°C

To further test the hypothesis that NAD+ is released during cell preparation, we next analyzed the effects of temperature during and after cell preparation on spontaneous PS externalization and CD62L shedding (Figs. 6 and 7). Cells that had been prepared and kept at 4°C did not spontaneously externalize PS (Fig. 6A, panels 1 and 5). In contrast, cells prepared and kept at 37°C for 30 min contained substantial numbers of spontaneously PS-exposing cells, with higher proportions of such cells in preparations from CD38-deficient vs WT mice (Fig. 6A, panels 2 and 6). Remarkably, cells prepared at 4°C did expose PS when subsequently incubated at 37°C, with cells from CD38-deficient mice again showing stronger responses than WT cells (Fig. 6A, panels 3 and 7). Further, cells prepared at 37°C did not re-internalize PS when subsequently...
Purified lymph node T cells from BALB/c WT, ART2 and CD38 
NAD from cells during preparation rather than by exposure of cells to 
the spontaneous activation of P2X 7 is caused by NAD 
Temperature dependency of ATP and 

**FIGURE 7.** Temperature dependency of ATP and NAD\(^+\) induced PS externalization and CD62L shedding. Purified lymph node T cells from BALB/c WT, ART2\(^{-/-}\), and CD38\(^{-/-}\) mice were incubated for 30 min at the indicated temperatures in the absence or presence of exogenous NAD\(^+\) (25 \(\mu\)M) (A and C) or ATP (250 \(\mu\)M) (B and D). Washed cells were stained with Annexin-V\(^{FITC}\) and PI (A and B), or with anti-CD62L\(^{PE}\) and anti-CD3\(^{FITC}\) (C and D) before FACS analyses. Results are representative of three independent experiments.

**FIGURE 6.** T cells prepared at 4°C do not externalize PS but do so when returned to 37°C. Purified lymph node T cells from BALB/c WT (+/+) and CD38-deficient mice (−/−) were incubated for 30 min at 4 or 37°C in the absence (A) or presence (B) of exogenous NAD\(^+\) (25 \(\mu\)M). Cells were washed and stained with Annexin-V\(^{FITC}\) and PI for FACS analyses either directly or following a further incubation for 60 min at 4 or 37°C as indicated. Numbers in A and B indicate the percentage of cells in each quadrant. Results are representative of three independent experiments.

ADP-ribosylation of cell surface proteins proceeds more efficiently at 4°C than at 37°C

To assess the extent of cell surface protein ADP-ribosylation at different temperatures, we used a previously described FACS-based assay using the NAD\(^+\) analog etheno-NAD\(^+\) and the

and 5), while cells prepared at 37°C (panels 2 and 6) responded vividly with PS externalization as did cells that were returned to 37°C for 30 min after preparation at 4°C (panels 3 and 7). Note that addition of exogenous NAD\(^+\) to WT cells resulted in a response of similar magnitude as the spontaneous response of CD38-deficient cells (to endogenous NAD\(^+\)) (Fig. 6B, panel 2 vs Fig. 6A, panel 6). Note further that the level of PS exposure by CD38KO cells in response to endogenously released NAD\(^+\) already was near maximal, i.e., was enhanced only slightly by the addition of exogenous NAD\(^+\) (panel 6 in Fig. 6A vs panel 6 in Fig. 6B). This indicates that P2X\(_7\) is already ADP ribosylated to a large extent on CD38KO cells during cell preparation.

Fig. 7 illustrates more detailed analyses of the temperature-dependency of PS-externalization (Fig. 7, A and B) and CD62L-shedding (Fig. 7, C and D) by T cells induced by exogenously added ATP and NAD\(^+\). The results reveal that T cells do not externalize PS or shed CD62L at 4°C, even when exposed to relatively high concentrations of ATP (250 \(\mu\)M) or NAD\(^+\) (25 \(\mu\)M). Cells exposed to ATP did respond vividly at room temperature (20°C) (Fig. 7, B and D), while cells exposed to NAD\(^+\) showed maximal shedding only at 37°C (Fig. 7, A and C). These results indicate that the soluble ligand ATP is a better agonist for P2X\(_7\) than the covalently attached ADP-ribose. Consistent with this interpretation, the temperature response curves were shifted even further to the left when T cells were treated with the highly potent P2X\(_7\) agonist benzoyl-ATP (results not shown).

Note that the temperature response curves of CD38-deficient cells in the absence of exogenous nucleotides resemble those of WT cells exposed to exogenously added NAD\(^+\) (Fig. 7, A and C). Moreover, ART2-deficient cells, which respond normally to exogenous ATP, neither spontaneously expose PS or shed CD62L nor do so in response to exogenous NAD\(^+\) at any temperature. These results substantiate the interpretation that NAD\(^+\) rather than ATP is the effective signaling molecule inducing spontaneous PS exposure and CD62L shedding during T cell preparation.
etheno-adenosine-specific mAb 1G4 to detect etheno-ADP-ribo-
bsylation of cell surface proteins (Fig. 8) (55). In accord with
previous reports, ART2-deficient cells did not show any detect-
able etheno-ADP-ribosylation of cell surface proteins (Fig. 8A).
Remarkably, on WT T cells, the extent of cell surface protein
ADP-ribosylation decreased with increasing temperatures (Fig.
8A), i.e., was much higher on cells incubated at 4°C than on
those incubated at 37°C (Fig. 8B, panels 1 and 2). Further,
subjecting cells that had been labeled at 4°C to a subsequent
incubation at 37°C resulted in a marked decrease in the level of
protein ADP-ribosylation (Fig. 8A, panel 3 vs panel 1). In con-
trast, subjecting cells that had been labeled at 37°C to a subse-
quent incubation at 4°C resulted in little if any detectable
changes in the level of protein ADP-ribosylation (Fig. 8A, panel
4 vs panel 2). Similar results were obtained with CD38-defi-
cient T cells (Fig. 8A, panels 5–8). These results show that
ADP-ribosylation of cell surface proteins proceeds efficiently at
4°C and, further, suggest that labeling is reversed at 37°C, e.g.,
by enzymatic removal of the etheno-ADP-ribose group and/or
internalization or shedding of labeled proteins.

**Differential radiolabeling of P2X7, LFA-1, and CD8 in cells
prepared from WT vs CD38-deficient mice**

ART2.2 is known to ADP-ribosylate several distinct T cell surface
proteins (14, 29, 56). ADP-ribosylation sites on cell surface pro-
teins already occupied during cell preparation would not be avail-
able to subsequent ADP-ribosylation, e.g., upon addition of exog-
enously added NAD\(^+\). To determine to which extent the exposure
to NAD\(^+\) released during cell preparation affects the ADP-
ribosylation of cell surface proteins to a subsequent exposure to
exogenous NAD\(^+\), we incubated freshly prepared T cells from WT
and CD38-deficient mice at 4 or 37°C with exogenously added
radioactive NAD\(^+\) (1 \(\mu\)M), followed by lysis of cells and immu-
noprecipitation of known ART2-target proteins with specific Abs
(Fig. 9).

Incubation of cells with radiolabeled NAD\(^+\) at 4°C leads to
covalent radiolabeling of numerous cell surface proteins in T cells
from both, WT and CD38KO mice (Fig. 9A, lanes 1 and 2). Cells
from ART2KO mice do not incorporate any radiolabel under these
conditions (data not shown). The results confirm the efficient ac-
tivity of ART2 at 4°C and indicate that numerous binding sites are
still available for ADP-ribosylation. The reduced radiolabeling of
P2X7 in cells from CD38KO vs WT mice (Fig. 9A, lanes 5 and 6)
is consistent with the notion that most ADP-ribosylation sites on
P2X7 are already occupied on cells from CD38KO mice as a result
of prior ADP-ribosylation by NAD\(^+\) released during cell prepara-
tion. Incubation of cells with radiolabeled NAD\(^+\) at 37°C results
in much lower radiolabeling of proteins than incubation with
NAD\(^+\) at 4°C (Fig. 9B vs Fig. 9A), as in case of labeling with
etheno-NAD\(^+\) (Fig. 8A), consistent with reversion of ADP-ribo-
sylation at 37°C. Note further that at 37°C, overall radiolabeling of
proteins is lower in WT than CD38KO cells (Fig. 9B, lanes 1 and
2), presumably due to CD38-mediated NAD\(^+\) hydrolysis by WT
cells (24).

**Discussion**

The results of this study demonstrate that P2X7, on T cells can be
activated by endogenous sources of NAD\(^+\) and ATP released from
lysed cells. We show that massive lysis of erythrocytes can result
in the release of sufficient quantities of these nucleotides to activate
P2X7. Our results further indicate that techniques routinely used in
immunology laboratories to prepare primary lymphocytes from
spleen and lymph nodes cause the release of NAD\(^+\) in sufficient
quantity to gate P2X7, and to phenotypically and functionally alter
a substantial fraction of cells.

In accord with previous studies (13, 57, 58), we show in this
study that exposure of T cells to exogenous ATP or NAD\(^+\) triggers
the P2X7-dependent externalization of phosphatidylserine and
R125 seems to be a less potent agonist for P2X7 than ATP; even ATP suffice to activate P2X7, the ADP-ribosyl group linked to cells (29). Even though much lower concentrations of NAD than ADP-ribosylation of R125 on WT cells but not on ART2-deficient cells (29). Even though much lower concentrations of NAD than ADP-ribosylation of R125 on WT cells but not on ART2-deficient cells (29). Even though much lower concentrations of NAD than ADP-ribosylation of R125 on WT cells but not on ART2-deficient cells (29). Even though much lower concentrations of NAD than ADP-ribosylation of R125 on WT cells but not on ART2-deficient cells (29).

Interestingly, shedding of CD62L (Fig. 1). Using T cells from ART2-deficient and WT mice as biological indicators, we can distinguish the effects of ATP on P2X7, from those of NAD+ on P2X7, in case of exogenously added nucleotides (Fig. 1) as well as in case of endogenous nucleotides released from lysed cells (Fig. 2); ATP acts as a soluble ligand that gates P2X7 on both WT and ART2-deficient cells, whereas NAD+ gates P2X7 via ART2.2-catalyzed ADP-ribosylation of R125 on WT cells but not on ART2-deficient cells (29). Even though much lower concentrations of NAD than ATP suffice to activate P2X7, the ADP-ribosyl group linked to cells (29). Moreover, because ART2KO cells do not ADP-ribosylate P2X7, activation of P2X7 in these cells is entirely dependent on ATP. Consistently, ART2KO cells respond more vividly than WT cells to exogenous ATP (Fig. 1A, panels 2 and 4) and to concentrated cell lysates (Fig. 2A, panels 1 and 5, Fig. 4, panels 1 and 4). Evidently, partial ADP-ribosylation of P2X7 on WT cells (in response to NAD released from cells) retards ATP-induced conversion to the double-positive stage.

The results of dose response analyses permit an estimation of the EC50 values for nucleotide-induced activation of P2X7, i.e., 120 µM for ATP and 2.6 µM for NAD+ (Fig. 3). Our results indicate that such concentrations are reached in the vicinity of lysed cells. In physiological settings, the effects of extracellular nucleotides released from lysed cells on T cells would depend on both time and distance from the lysis event. Using dilution of erythrocyte lysates to mimic increasing distance, we show that ATP-mediated effects dominate only in the short range (i.e., at high concentrations of lysates), whereas NAD-mediated effects are effective also at longer ranges (i.e., at high dilutions of lysates) (Fig. 2). Preincubating erythrocyte lysates at 37°C before T cell exposure indicates that the effects of ATP dissipate faster than those mediated by NAD+ (Fig. 4). The relative duration of signaling by these extracellular nucleotides is determined largely by nucleotide degrading ecto-enzymes such as the transmembrane ecto-enzymes CD38 and CD39 (21, 39, 59).

Massive lysis of erythrocytes is observed in a number of pathological conditions, e.g., during malaria infection, genetically inherited hemolytic diseases, and adverse reactions to blood transfusion (46–48). It is likely that the mechanisms described in this study with mechanically disrupted erythrocytes act also in these and other settings in vivo. Indeed, recent results from three different mouse models of inflammation support the notion that nucleotides released during tissue injury induce P2X7 activation on T cells in vivo (6, 18, 25). Firstly, injection of Con A induces T cell-dependent hepatitis that is accompanied by fulminant liver cell damage as evidenced by the release of cytosolic enzymes into the circulation. Mice genetically deficient in ART2 or P2X7 develop a milder form of the disease, correlating with decreased sensitivities of liver-resident INK-T cells in these mice to apoptosis induced by extracellular nucleotides (18). Secondly, genetic ablation of the major ecto-NAD-glycohydrolase CD38 results in elevated tissue NAD levels and enhanced levels of T cell surface ADP-ribosylation (24). Transfer of the deficient CD38 allele into the autoimmune diabetes-prone NOD/Lt background resulted in accelerated disease progression, correlating with an enhanced sensitivity of regulatory T cells to ART2-dependent NAD-induced cell death in these mice (25). Indeed, these changes were corrected when CD38 deficiency was combined with ART2 deficiency. Thirdly, local inflammatory responses induced by s.c. injection of Biogel lead to release of NAD into the inflammatory pouch, causing shedding of CD62L by T cells in the draining but not in the nondraining lymph nodes (6).

The results obtained in this study with the second experimental system, i.e., the mechanical manipulations during routine preparation of cells from lymphatic organs, are of special pertinence to immunologists working with primary lymphocytes. Our results show that routine cell preparation techniques can lead to the gating of P2X7 on a fraction of cells, and subsequently to the shedding of CD62L and externalization of PS. Similar degrees of P2X7 activation were observed whether cells were prepared from lymph nodes or spleen and when cells were prepared by gentle passage through nytex membranes, collagenase digestion, or perfusion with medium (results not shown). This has important implications for experiments designed to study lymphocyte functions both, in vitro and after adoptive transfer in vivo. Our results show that cells prepared and kept strictly at 4°C do not externalize PS or shed CD62L (Fig. 6). However, when cells are prepared at 37°C or when cells are returned to 37°C subsequent to a preparation at 4°C, a substantial fraction of the cells do externalize PS and shed CD62L. In the case of WT mice, a relatively small fraction of T cells (5–10%) was affected (Fig. 6A, panels 6 and 7), whereas the majority of T cells was affected in case of CD38-deficient mice (Fig. 6A, panels 6 and 7), which lack the major NAD-hydrólyzing ecto-enzyme (21, 24). This finding, together with the observation that ART2-deficient mice which lack the major T cell ecto-ADP-ribosyltransferase (17) do not spontaneously shed CD62L or expose PS (Fig. 7) imply that NAD+ but not ATP is released in sufficient quantity to induce activation of P2X7 during cell preparation.

Consistently, blocking the ADP-ribosylation of cell surface proteins by injection of an ART2.2-inhibitory sdAb 10 min before killing of the animal, completely prevented the subsequent shedding of CD62L and externalization of PS by WT and CD38-deficient cells (Fig. 5). Similar effects were achieved by injecting etheno-NAD+ before sacrifice, which results in the etheno-ADP-ribosylation of P2X7, thereby blocking the subsequent activation by ADP-ribosylation (Fig. 5). ART2.2-inhibitory sdAbs are not expected to have any adverse side effects. Because sdAbs lack the Fc domain, they cannot activate complement or Ab-dependent cytotoxicity. Moreover the small (15kd) sdAbs are rapidly eliminated via the kidney, with a serum half life of <5 min. Blockade of ART2 by sdAbs is reversible and ART2.2 activity on lymph node cells is largely restored 24 h after injection (41). However, systemic administration of etheno-NAD+ could have unwanted side effects as this would provide other members of the ART-family with substrate, leading to the etheno-ADP-ribosylation of other cell surface proteins.

Monitoring cell surface protein ADP-ribosylation using exogenously added etheno-NAD+ (Fig. 8) or 32P-NAD+ (Fig. 9) confirmed that ART2.2-catalyzed ADP-ribosylation of cell surface proteins proceeds efficiently at 4°C. In contrast, the gating of P2X7 requires elevated temperatures (Fig. 7). These findings imply that unwanted activation of P2X7 by ATP released from cells during cell preparation can be prevented simply by keeping cells at 4°C during preparation until the soluble ligand ATP is washed away. However, ADP-ribosylation of P2X7 in response to NAD+ released from cells during cell preparation cannot be prevented by keeping cells at 4°C because the covalently attached ADP-ribose moiety cannot be removed by washing and thus will activate P2X7 when cells are returned to 37°C. To prevent Ab-induced modulation of cell surface proteins, immunologists routinely perform staining of lymphocytes for FACS-analyses at 4°C, whereas cells...
are returned to 37°C for functional assays, e.g., in vitro TCR-ligation and proliferation assays or in vivo migration studies. Under such conditions, NAD-induced PS externalization and CD62L shedding due to activation of P2X7 by ADP-ribosylation could escape detection.

Both the externalization of PS and the shedding of CD62L can profoundly affect T cell functions. Externalization of PS is a common eat-me signal for macrophages, and such cells are equipped with adapter proteins and cell surface receptors for binding PS-exposing cells (60–62). Externalization of PS by T cells could thus lead to enhanced binding to and/or phagocytic clearance by macrophages. Moreover, PS exposure is associated with increased cellular adhesion to endothelium and might promote the extravasation of T cells. CD62L is the major homing receptor for lymph nodes, and cells lacking CD62L show impaired migration to peripheral lymph nodes (63–65). Metalloproteinase-mediated shedding of CD62L is triggered also upon activation of T cells by engagement of the TCR or by mitogenic stimulation (66, 67). Our results indicate that a substantial fraction of cells in primary lymphocyte preparations may exhibit a CD62L-negative phenotype as a consequence of P2X7-ADP-ribosylation during cell preparation rather than as a sign of conventional T cell activation. Moreover, it is possible that the constitutive externalization of PS described for CD4+/CD45RBhi cells is a consequence of exposure to NAD+ during cell preparation (68).

In this context it is important to note that common strains of laboratory mice carry allelic variants of both P2X7 and ART2 which affect the sensitivity to endogenously released nucleotides (12, 31, 69). BALB/c mice express WT P2X7 and both copies of ART2.2 depend on its association with lipid rafts. When expressed at much higher levels than BALB/c mice (12, 69). Whether the reported high sensitivity of both the externalization of PS and the shedding of CD62L can be attributed to the presence of the ART2.2 locus at much higher levels than BALB/c mice, and whether the high sensitivity is a consequence of exposure to NAD+ during cell preparation will be an important subject of future investigations.

The results of our experiments testing the temperature-dependency of T cell surface ADP-ribosylation reactions indicate that ADP-ribosylation of cell membrane proteins is reversible (Figs. 8 and 9), in accord with previous studies (56, 71). Enzymes capable of reversing ADP-ribosylation include ADP-ribosylhydrolases which can remove the entire ADP-ribose moiety (72, 73) and phosphodiesterases which remove only AMP, leaving ribose phosphate attached to the target protein (74). To date ADP-ribosylhydrolases have been described only as intracellular proteins (75), whereas phosphodiesterase isoforms have been cloned and characterized that function as membrane bound and secretory ecto-enzymes (76, 77). Because both labels used in this study (etheno-adenosine) and (α-32P) would be removed by phosphodiesterases and ADP-ribosylhydrolases, other tools will be required to determine the relative contributions of these two enzyme families to reversion of protein ADP-ribosylation. The differential labeling of P2X7 vs LFA-1 and CD8 at 4°C vs 37°C (Fig. 9) indicate that ADP-ribose moieties buried in the lipid-binding pocket of P2X7 may be better protected against de-ADP-ribosylating enzymes than ADP-ribose moieties linked in a more exposed manner to other cell-membrane proteins. That activation of P2X7 by ADP-ribosylation can play a role in physiological settings has been demonstrated previously by our finding that NAD+ released at inflammatory sites induces ART2-dependent shedding of CD62L and T cell death in draining lymph nodes (6). The results of the present study provide an additional plausible scenario for the activation of P2X7 in vivo, i.e., by NAD+ and ATP released during hemolysis. Malaria, for example, is associated with periodic hemolysis and complex changes in T cell function and apoptosis (47, 78). It will, therefore, be of interest to determine whether and to what extent the genetic ablation or pharmacological inhibition of ART2 and/or P2X7 affects disease progression in murine malaria models. Our results further demonstrate that techniques routinely used in immunology laboratories to prepare primary lymphocytes cause the release of sufficient quantities of NAD+ for ART2.2-catalyzed activation of P2X7. When cells are returned to 37°C, this induces the externalization of PS and shedding of CD62L, thereby likely altering T cell functions. An efficient means to prevent the activation of P2X7 during cell preparation is an i.v. injection of ART2.2-blocking sAbs shortly before sacrificing.

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Disclosures

The authors have no financial conflict of interest.

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