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P2X7 Receptors Regulate NKT Cells in Autoimmune Hepatitis

Hiroki Kawamura, Fred Aswad, Masahiro Minagawa, Sugantha Govindarajan, and Gunther Dennert

Adenine nucleotides induce danger signals in T cells via purinergic receptors, raising the question whether they exert similar effects on innate immunity. Here we show that micromolar concentrations of nicotinamide adenine dinucleotide (NAD) induce a rapid increase of annexin V staining in NKT cells in vitro, a response that requires expression of P2X7Rs. Consistent with this result, treatment of mice with NAD causes a temporary decrease of NKT cells in the liver and protects from Con A- and α-galactosylceramide-induced hepatitis, both of which require functional NKT cells. Resistance to liver injury is associated with decreased cytokine production by NKT cells in NAD-treated mice. In contrast, when NAD is injected into Con A- or α-galactosylceramide-primed mice, liver injury is exacerbated and cytokine production by NKT cells is increased. This effect is caused by P2X7-R-mediated stimulation of activated NKT cells. In agreement, mice lacking P2X7Rs on lymphocytes suffer reduced liver injury, and animals lacking ADP-ribosyltransferase, the enzyme that uses NAD to attach ADP-ribosyl groups to cell surfaces, are also resistant to Con A-induced hepatitis. These results prompt the conclusion that engagement of P2X7Rs on NKT cells inhibits naive, while stimulating activated cells, resulting in suppression or stimulation of autoimmune hepatitis. The Journal of Immunology, 2006, 176: 2152–2160.

Incubation of murine T lymphocytes with cell metabolite nicotinamide adenine dinucleotide (NAD) increases annexin V staining within seconds (1–3). Treatment of T cells with NAD before injection into semiallogeneic hosts abrogates the ability to induce graft-vs-host disease, and T cells treated with NAD fail to proliferate in recipient mice (1). These results lead to the hypothesis that NAD released from cells during infection and trauma provides a “danger signal” that regulates adaptive immunity (3). Because the innate immune system plays a similarly important role in these responses, the question arises whether NAD can regulate innate immunity.

Among the many cell types that participate in innate immunity, NKT cells share many properties with conventional T cells, raising the question of whether they are regulated by similar mechanisms. NKT cells are abundant in the liver and, therefore, are believed to play a major role in innate immunity of this organ. In support, elimination of NKT cells expressing the invariant Vα14-Jα18 TCR protects from Con A-induced liver injury (5–9). This finding, as well as the abundance of hepatic NKT cells, makes the liver an organ of choice to study NKT cell regulation.

In the present study, we set out to investigate the effects of NAD on NKT cells. Our results demonstrate that NAD induces an inhibitory signal via stimulation of P2X7Rs on NKT cells of naive mice (“naive” NKT cells). Induction of this signal requires expression of the NKT cell surface enzyme ADP-ribosyltransferase 2 (ART-2), which attaches ADP-ribosyl groups to the cell surface (3). As a consequence of this reaction, injection of NAD into mice protects from Con A and α-galactosylceramide (α-GalCer)-induced liver injury. Consistent with this, NKT cells from NAD-injected mice respond with impaired cytokine production upon stimulation with α-GalCer. In contrast, when NAD is injected into Con A- or α-GalCer-primed mice, liver injury is exacerbated and many animals die. On the basis of these results, we propose that, while engagement of P2X7Rs on naive NKT cells induces an inhibitory signal and protects from liver injury, signaling through the receptor in primed NKT cells activates these cells, thereby increasing liver injury.

Materials and Methods

Mouse strains, induction of Con A hepatitis, injections, and serum alanine aminotransferase (ALT) assays

Pathogen-free female C57BL/6 (B6) and BALB/c mice, 6–8 wk of age, were obtained from The Jackson Laboratory. P2X7R-deficient (P2X7R–/–) mice on the B6 background (10) were provided by Dr. C. A. Gabel (Amgen, Seattle, WA) and Pfifer. ART-2-deficient (ART-2–/–) mice on the B6 background (11) were provided by Dr. F. Koch-Nolte (University of Hamburg, Hamburg, Germany). All gene-deleted mice were bred at the University of Southern California animal facility. Bone marrow chimeras were prepared by irradiating B6 or P2X7R–/– mice with 900 rad, immediately followed by i.v. injection of 2 × 107 bone marrow cells and 3 to 102 spleen cells from B6 or P2X7R–/– mice. Chimeric mice were used for experiments 2 wk later. To induce autoimmune hepatitis, animals were injected i.v. with 10 mg/kg Con A (Sigma-Aldrich) (6). NAD was injected at a dose of 1 mg per mouse (Sigma-Aldrich) i.v. in 200 μL of PBS. To stimulate NKT cells in vivo, animals were injected i.v. with 5 μg of α-GalCer (12). Serum ALT was assayed as described (12, 13) using a commercial assay kit (Sigma-Aldrich).

Isolation of liver mononuclear and spleen cells

Liver mononuclear cells (MNC) were isolated from exsanguinated mice (5, 8). This procedure yields cell populations indistinguishable from those prepared from perfused livers (12, 13). Livers and spleens were gently removed and passed through a 200-gauge stainless steel mesh in RPMI 1640, containing 2% FBS. The cell suspension was centrifuged 500 × g for 10 min.

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min, and the supernatant was discarded. Spleen cells were used for experiments after removal of erythrocytes. Cell pellets from the liver were re-suspended in osmolality- and pH-adjusted 33% Percoll, diluted with RPMI 1640 (15 ml/liver), containing 100 U/ml heparin. After centrifugation for 15 min at 500 × g at room temperature, the cell pellet containing MNC was harvested. To remove erythrocytes, cells were suspended in 10 ml of RBC lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA (pH 7.3)) and incubated for 5 min on ice. Cells were then washed twice in RPMI 1640 containing 2% FBS.

**In vitro cell death assays and culture of lymphocytes**

To assay induction of cell death, liver MNC or spleen cells were incubated for various times with or without NAD or ATP (Sigma-Aldrich) in RPMI 1640 without FBS at 37°C and assayed for cell recovery and annexin V staining. To inhibit P2X,R function, 20 μM KN-62 (Sigma-Aldrich) dissolved in 0.01% DMSO was added 10 min before addition of NAD (3, 14). To examine the potential action of ATP in cell death, ATPase in the form of potato apyrase (Sigma-Aldrich) was added to the culture in the absence or presence of exogenous NAD. For assay of NKT cell responsiveness, spleen cells were cultured in RPMI 1640 containing 10% FBS at 2.5 × 10⁶ cells per milliliter with 100 ng/ml α-GalCer or vehicle for 72 h (15). Supernatants were harvested and assayed for IL-4 and IFN-γ by ELISA.

**Flow cytometric analysis and ELISA**

For flow cytometric analysis, MNC were preincubated with anti-mouse CD16/CD32 (2.4G2) mAb from BD Biosciences to block FcγR, and then incubated with various mAbs for 30 min at 4°C (3). For phenotypic analysis, directly labeled anti-mouse CD3e (145-2C11) and anti-NK1.1 (PK-136) mAbs (BD Biosciences) were used. To monitor induction of cell death, cells were stained with the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences).

To quantify cell surface ADP-ribosylation, liver MNC (4 × 10⁶ cells/ml in RPMI 1640) were incubated with 300 μM etheno-NAD (Sigma-Aldrich), followed by incubation with etheno-ADP-ribose-specific Ab 1G4, provided by Dr. R. M. Santella (Columbia University, New York, NY) (3, 16), and a FITC goat anti-mouse Ig (BD Biosciences). To detect intracellular IFN-γ and IL-4, cells stained with anti-CD3e and anti-NK1.1 were fixed and permeabilized using the Cytotix/Cytoperm kit (BD Biosciences). Cells were analyzed by FACSCalibur (BD Biosciences). IFN-γ and IL-4 serum levels were assayed by an enzyme-linked immunosorbent assay kit by following the supplier’s protocol (eBioscience).

**Histological procedures**

For histological examination, liver tissue samples were fixed in 3% paraformaldehyde, and sections were stained with H&E for examination by light microscopy (12).

**Statistical analysis**

All data are shown as mean ± SEM. For comparisons of means between two experimental groups, a Student’s unpaired t test was used. A value of p < 0.05 was considered significant.

**Results**

**NKT cells express ART-2 activity and respond to NAD with an increase in annexin V staining**

Previous experiments had shown that rapid, NAD-induced T cell death requires expression of cell surface enzyme ART-2, which consumes NAD to attach ADP-ribosyl groups to cell surface proteins (3). Therefore, it was pertinent to examine expression of ART-2 activity on NKT cells. Liver MNC from normal and ART-2-deficient (ART-2−/−) mice (11) were isolated, incubated with ART-2 substrate etheno-NAD (3), reacted with etheno-ADP-ribose-specific Ab 1G4, and analyzed by FACS. Fig. 1A shows that NKT and T cells but not NK cells from B6 mice undergo significant etheno-ADP-ribosylation, whereas cells from ART-2−/− mice show no labeling. These results demonstrate that liver NKT cells express cell surface ART-2 activity and, therefore, could be sensitive to NAD-induced cell death.

To examine this, liver MNC were incubated with or without NAD for 2 h and assayed for phosphatidyl-serine (PS) exposure by annexin V staining. Fig. 1B shows that among cells from B6 mice cultured under normal conditions, 32.8% of NKT cells and 19.3% of T cells stain with annexin V, whereas NK cells show little staining. Addition of NAD increases annexin V staining to 64.4% in NKT cells and to 28.7% in T cells. No substantial annexin V staining is demonstrable in cells from ART-2−/− mice.

**Time-kinetic studies**

Time-kinetic studies at a high concentration of 500 μM NAD reveal a gradual increase of annexin V staining on NKT cells over 2 h, without a detectable loss in cell numbers (Fig. 2A). Titration of NAD shows a significant increase in annexin V-staining NKT cells at 1 μM, i.e., a concentration 1/1000 that inside cells (17) (Fig. 2B). These results demonstrate that in response to NAD, NKT but not NK cells expose PS on their cell surface in an ART-2-dependent reaction.
Induction of PS exposure by NAD requires expression of purinergic receptor P2X7

In conventional T cells, NAD-induced PS exposure requires expression of functional P2X7Rs (3). To examine whether the same is the case for NKT cells, liver MNC were incubated with NAD and a P2X7-R inhibitor. Among known inhibitors, KN-62 is a relatively specific competitive inhibitor of the human and mouse P2X7-R. However, higher concentrations of KN-62 are required to inhibit the mouse P2X7-R (14, 18, 19). Fig. 2C shows that, in liver MNC cultured under normal conditions, a high percentage (36.7%) of NKT cells stain with annexin V, and addition of NAD increases staining to 61.8%. Interestingly, KN-62 decreases annexin V staining to very low levels in NKT cells incubated under normal culture conditions as well as in the presence of added NAD (Fig. 2C). Moreover, NKT cells from P2X7-R-deficient (P2X7-R−/−) mice do not stain with annexin V, regardless of the addition of NAD (Fig. 2C). These data show that functional P2X7-Rs are required for NAD-induced PS exposure in NKT cells. Moreover, they show that components in the medium and/or released from cells during culture induce P2X7-R-dependent PS exposure in the absence of exogenously added NAD.

To test whether ATP, the well-established ligand of P2X7-Rs (20), plays a role in the NAD-induced effects, liver MNC were incubated with or without NAD in presence of ATP degrading enzyme apyrase. Fig. 2D shows that addition of 10 U of apyrase to BALB/c T cells, which are especially sensitive to ATP-induced cell death (3), inhibits induction of annexin V staining by ATP. However, this dose of apyrase does not interfere with NAD-induced annexin V staining of NKT cells (Fig. 2E), which suggests that NAD-induced P2X7-R signaling does require extracellular ATP.

NAD causes a temporary decrease in the percentage of liver NKT cells and inhibits Con A-induced hepatitis

The finding that low concentrations of NAD trigger P2X7-R-dependent PS exposure in NKT cells in vitro raises the possibility that similar effects can occur in vivo. To examine this, B6 and P2X7-R−/− mice were injected with 1 mg of NAD, which, in the absence of NAD hydrolyzing enzymes such as CD38, should result in a circulating concentration of 1 mM NAD (21). Liver MNC were isolated and assayed for presence of NKT cells. Fig. 3A documents a decrease in the percentage of NKT cells 30 min after NAD injection in livers of B6, but not P2X7-R−/− mice. Ten-times higher NAD doses caused similar decreases as well as increased annexin V staining of the remaining NKT cells (data not shown). Therefore, NAD can exert effects on NKT cells in vivo, provided P2X7-Rs are expressed. However, these effects are short lived; 60 and 120 min after NAD injection, NKT cell percentages revert to normal levels (Fig. 3A).

Given these results, we asked whether the temporary decrease in NKT cell percentage is associated with suppression of their function. To examine this, normal and NAD-treated mice were injected with Con A to induce activation of NKT cells and autoimmune hepatitis (5–7). Fig. 3B shows that B6 mice injected with NAD, followed by Con A 2 h later, express significantly lower levels of liver enzyme ALT in the serum than mice injected with Con A.
only. These results demonstrate that injection of NAD before Con A protects against liver injury.

**Mice lacking P2X7Rs are relatively resistant to Con A-induced liver injury**

The observation in Fig. 3A that the decrease in percentage of liver NKT cells inducible by NAD requires expression of P2X7Rs, predicts that mice lacking P2X7Rs should not be protected from Con A-induced liver injury by injection of NAD. To test this, P2X7R−/− mice were given a dose of NAD followed by ConA 2 h later. Results in Fig. 3B show that, as expected, treatment of P2X7R−/− mice with NAD before Con A does not result in lower ALT levels. However, unexpectedly, ALT levels in Con A-injected P2X7R−/− mice are much lower than in B6 mice (Fig. 3B). In agreement, liver sections from Con A-injected P2X7R−/− mice show little if any injury, whereas those from B6 mice show heavy mid-zonal necrosis (Fig. 3C). Although these experiments are consistent with the notion that P2X7Rs protect mice from Con A-induced hepatitis when NAD is injected before Con A, they also argue that these receptors can exert stimulatory effects and increase liver injury.

**P2X7Rs on lymphoid cells stimulate Con A-induced liver injury**

The observation that P2X7Rs can stimulate liver injury raises the question where these receptors need to be expressed to cause these effects. To find out, bone marrow chimeras were prepared in which either the liver parenchyma or the lymphoid compartment lacks P2X7Rs. Fig. 4A shows that absence of P2X7Rs from the liver parenchyma leads to a small and insignificant decrease of serum ALT values in Con A-injected mice. In contrast, absence from lymphoid cells results in significantly lower ALT levels. Therefore, expression of P2X7Rs on lymphoid cells stimulates Con A-induced liver injury.

**The principal component activating P2X7Rs in Con A-induced liver injury appears to be NAD**

The demonstration that expression of P2X7Rs increases the severity of hepatitis (Fig. 3 and 4A) suggests that, concomitant to Con A-induced lymphocyte activation, P2X7R agonists increase in the extracellular compartment and interact with P2X7Rs. Because ATP is the canonical ligand for P2X7Rs (20), an expectation is that among components released from cells, ATP rather than NAD should provide the principal agonist for P2X7R stimulation. To examine this, ART-2−/− mice were tested for sensitivity to Con A-induced liver injury because they are not able to attach ADP-ribosyl groups to the cell surface of NKT cells (Fig. 1). Although T cells from ART-2−/− mice are sensitive to ATP-induced cell death in vitro (data not shown), injection of Con A does not induce high ALT values (Fig. 4B). Therefore, an ADP-ribosylation reaction is required for maximal liver injury induced by Con A. In conclusion, NAD rather than ATP provides the principal ligand for P2X7Rs in vivo.

**Con A-primed mice suffer severe liver injury and death when injected with NAD**

The demonstration that severe liver injury requires expression of ART-2 suggests that NAD plays a major role in Con A-induced hepatitis. To further substantiate this, the effects of NAD injection into Con A-primed mice were assayed. Fig. 5, A and C, show that injection of NAD at the time of Con A injection and 3 h later...
causes decreased ALT levels and little, if any, liver injury. In contrast, when animals were first injected with Con A and given NAD 3 h later, serum ALT levels increased by 6-fold (Fig. 5B) and 40% of the animals died within 24 h (data not shown). Histological examination of liver tissue from surviving mice revealed massive focal necrosis in animals that had received NAD 3 h after Con A priming (Fig. 5C). Injection of NAD into Con A-primed P2X7R−/− mice did not increase ALT levels (Fig. 5B). These experiments show that administration of NAD into Con A-primed B6 but not P2X7R−/− mice induces severe liver injury, pointing to a mechanism involving activation of NKT cells via stimulation of P2X7Rs by NAD derived ligands.

Injection of NAD into Con A-primed mice activates NKT cells

Injection of Con A into mice activates NKT as well as other effector cells, which in turn results in an increase of IFN-γ and IL-4 in the serum (22, 23). Therefore, the observation that injection of NAD into Con A-primed mice enhances liver injury (Fig. 5), predicts that this should be associated with an increase in IFN-γ and IL-4 production. Fig. 6A shows that, when compared with Con A-injected controls, mice injected with NAD 3 h after Con A priming express increased levels of the two cytokines, whereas mice that were given NAD at the time of Con A injection and 3 h later, express decreased levels.

To show that NKT cells are responsible for the increase in cytokine levels, liver MNC and spleen cells were stained for intracellular IFN-γ and IL-4. Because activation of NKT cells causes their rapid disappearance from the liver (24), assay of these cells can be performed only shortly after Con A injection. Animals were primed with Con A and injected with NAD 1 h later, and cells were collected 2 h after Con A injection. Fig. 6B shows that liver and spleen NKT cells from Con A-primed mice express significant levels of IFN-γ and IL-4 compared with unprimed controls. There is also some IFN-γ staining in NK but not in T cells. In liver NKT cells from Con A-primed mice injected with NAD, IFN-γ staining increases from 34.6 to 51.6% and IL-4 staining increases from 30.3 to 40.2%. Similarly, in spleen NKT cells, IFN-γ staining increases from 19.9 to 28.1% and IL-4 staining increases from 12.5 to 35.1%. No effects were demonstrable in NKT cells from mice injected with NAD only (data not shown). These data show that in Con A-primed mice injection of NAD stimulates NKT cells resulting in increased production of IFN-γ and IL-4.

NAD decreases responsiveness of NKT cells in naive mice, while increasing responsiveness in primed mice

The finding that NAD injection stimulates NKT cell cytokine production and the severity of liver injury, contradicts our observation that NAD stimulates annexin V staining of NKT cells in vitro and can protect from Con A-induced liver injury. A plausible explanation for these discordant observations is that, in primed NKT cells, NAD induces an activation signal, whereas in naive NKT cells, it induces an inhibitory signal.

To examine whether NAD inhibits the function of naive NKT cells, mice were injected with NAD and were injected 2 h later with α-GalCer, which activates the majority of liver NKT cells (25). Fig. 7A shows that mice treated with NAD express significantly lower IFN-γ and IL-4 serum levels than mice injected with α-GalCer only. Because the effect of NAD injection on liver NKT cells is short lived (Fig. 3A), the lower cytokine levels in Fig. 7A are likely due to lower NKT cell responsiveness rather than decreased NKT cell numbers. To directly show this, NKT cells from spleens were analyzed because isolation of liver MNC involves extensive tissue destruction and NAD release, which could interfere with the effects of injected NAD. Fig. 7B shows that the percentage of NKT cells in spleen 2 h after NAD injection is unchanged, compared with PBS controls. Culture of these cells with α-GalCer reveals lower IFN-γ and IL-4 production by cells from NAD-injected mice, compared with controls (Fig. 7C). These results indicate that NKT cells from NAD-treated mice are functionally impaired. Consistent with this, Fig. 7D shows that NAD-injected mice challenged with α-GalCer express lower serum ALT levels than mice injected with α-GalCer only. In contrast, mice in which NKT cells had been primed by α-GalCer 3 h before injection of NAD suffer much increased liver injury, indicated by high ALT values, and a 40% mortality rate (Fig. 7D and data not shown). These results show that activated NKT cells are stimulated upon contact with NAD, whereas naive NKT cells are inhibited.

Discussion

The suggestion that danger signals play an important role as regulators of adaptive immunity and the experimental evidence supporting this (4) have raised the question whether similar mechanisms might regulate the innate immune system. In this study, we report that, among liver resident lymphocytes, NKT and T cells express ADP-ribosyltransferase activity and undergo rapid ADP-ribosylation, when incubated with substrate etheno-NAD. As a consequence of this reaction, incubation of NKT cells with NAD induces PS exposure, demonstrable by staining with annexin V. We show that concentrations as low as 1 μM induce annexin V staining, consistent with the suggestion that NAD released during trauma modulates NKT cell function. The finding that NKT cells from ART-2−/− mice do not respond to NAD provides strong evidence for a mechanism in which ADP-ribosyl groups attached to the cell surface induce a signal. This reaction could serve to

FIGURE 4. Con A-induced ALT levels in P2X7R−/− bone marrow chimeras and ART-2−/− mice. A, Groups of three bone marrow chimeras, lacking P2X7Rs in the liver parenchyma (P2X7R−/− recipient) or in the lymphoid compartment (P2X7R−/− donor), were injected with Con A as indicated. Serum ALT levels were tested after 24 h. The data shown represent means ± SEM of triplicate samples. **, p < 0.01. B, Groups of three B6 or ART-2−/− mice were injected with Con A, and serum ALT values were determined after 24 h. The data shown represent means ± SEM of triplicate samples. **, p < 0.01. Shown is one of two representative experiments.
sensitize the P2X7R to signaling by ATP. Although this is not strictly excluded by the experiments reported in this study, the finding that presence of ATPase does not inhibit NAD-induced PS exposure favors a mechanism of signaling in which ADP-ribosyl groups attached close to the P2X7R react with the receptor-ligand-binding site. In this regard, it is noteworthy that ATP is not a very efficient agonist of mouse P2X7Rs. In contrast to rat and human P2X7Rs, which respond to just one application of 1 mM ATP, mouse P2X7Rs expressed on HEK 293 cells require repeat applications to elicit maximal electrical currents in patch clamp experiments (26). In contrast, ATP can induce annexin V staining and cell death of mouse NKT cells in vitro, presumably because more sustained contact of the receptor with ATP takes place under these conditions (data not shown).

Time kinetic studies reveal that NAD-induced signals do not cause rapid death of mouse NKT cells in vitro, presumably because more sustained contact of the receptor with ATP takes place under these conditions (data not shown). NAD causes only a short-lived decrease in the percentage of NKT cells in liver and spleen (Fig. 3A and data not shown). Whether this decrease is caused by cell migration or cell death remains to be determined.

We show that NKT cells in NAD-injected mice are functionally impaired. Challenge of NAD-treated mice with Con A or α-GalCer induces low serum ALT levels and mild liver injury. Consistent with this, circulating IFN-γ and IL-4 levels are also reduced, pointing to impaired NKT cell responses. In support, stimulation of

**FIGURE 5.** Stimulatory effects of NAD on Con A-induced liver injury. A, Groups of three B6 mice were injected with 1 mg of NAD and Con A at the times indicated. Controls received PBS injections. ALT values were determined after 24 h. The data shown represent means ± SEM of triplicate samples. *, p < 0.05; **, p < 0.01. B, Groups of three B6 or P2X7R−/− mice were injected with Con A, followed by 1 mg of NAD 3 h later. Controls received PBS, and ALT values were determined after 24 h. The data shown represent means ± SEM of triplicate samples. ***, p < 0.01. C, B6 mice were injected with Con A and 1 mg of NAD at the times indicated. Livers were harvested after 24 h, and sections were prepared for histological evaluation. Experiments were repeated three times.

**FIGURE 6.** Effects of NAD on Con A-induced cytokine production by NKT cells. A, Groups of three B6 mice were injected with Con A and 1 mg of NAD at the times indicated. Blood was harvested 4 and 8 h after Con A injection and assayed by ELISA for IL-4 (4-h sample) and IFN-γ (8-h sample). The data shown represent means ± SEM of triplicate samples. *, p < 0.05; **, p < 0.01. B, B6 mice were injected with Con A, and 1 h later with 1 mg of NAD. Liver MNC and spleen cells were isolated 2 h after Con A injection and stained for CD3, NK1.1, IL-4, and IFN-γ, and analyzed by FACS. Numbers in scatter grams and histograms indicate the percentage of staining cells. Shown is one of two representative experiments.
were harvested 2 h after NAD injection and cultured with 100 ng/ml three B6 mice shown in
assayed by FACS for presence of NKT cells. C injected with 1 mg of NAD, and spleens were harvested 2 h later and
duce decreased IFN-$\gamma$/H9253
represents means
16 h after the last injection and tested for ALT levels. The data shown represent means
Cer for 72 h. Culture supernatants were harvested and assayed for IFN-$\gamma$/H11569/H11569
A
NKT cells, and liver injury.

FIGURE 7. Effect of NAD on $\alpha$-GalCer-induced cytokine production. NKT cells, and liver injury. A. Groups of three B6 mice were injected with
1 mg of NAD, followed by 5 $\mu$g of $\alpha$-GalCer 2 h later. Blood was harvested 3 and 16 h after $\alpha$-GalCer injection and assayed by ELISA for IL-4
(3-h sample) and IFN-$\gamma$ (16-h sample). The data shown represent means $\pm$ SEM of triplicate samples. *, $p < 0.05$; **, $p < 0.01$. B. B6 mice were
injected with 1 mg of NAD, and spleens were harvested 2 h later and assayed by FACS for presence of NKT cells. C. Spleens from groups of
three B6 mice shown in B, which had been injected with 1 mg of NAD, were harvested 2 h after NAD injection and cultured with 100 ng/ml $\alpha$-GalCer
for 72 h. Culture supernatants were harvested and assayed for IFN-$\gamma$ and IL-4 levels by ELISA. The data shown represent means $\pm$ SEM of triplicate samples. **, $p < 0.01$. D. Groups of three B6 mice were injected with
1 mg of NAD and 5 $\mu$g of $\alpha$-GalCer as indicated. Blood was harvested 16 h after the last injection and tested for ALT levels. The data shown
represent means $\pm$ SEM of triplicate samples. **, $p < 0.01$. Experiments were repeated twice with similar results.

NKT cells from NAD-injected mice with $\alpha$-GalCer in vitro induces decreased IFN-$\gamma$ and IL-4 levels. These results provide strong evidence for a mechanism in which NAD inhibits $\alpha$14-Jo18 TCR expressing NKT cells by signaling through P2X-Rs.

Data in the literature are in agreement with these observations. Binding of ATP to P2X-Rs opens nonselective ion channels, which can dilate to pores that allow passage of 900-Da molecules (28, 29). Depending on the cell type, membrane injury by pore formation for a short period of time may be tolerated as it permits resealing, whereas permeabilization for an extended time causes death by apoptosis or an osmotic colloidal mechanism (3, 30, 31). Our results with naive NKT cells point to transient or slow effects, which cause inhibition of cell functions rather than rapid cell death.

In contrast to the inhibitory effects P2X-Rs induce in naive NKT cells, they can also induce cell activation. Thus, injection of NAD into Con A- or $\alpha$-GalCer-sensitized mice causes severe liver injury and death. We demonstrate that this is associated with increased IL-4 and IFN-$\gamma$ levels in the serum and higher intracellular IL-4 and IFN-$\gamma$ staining in NKT cells. The possibility that this effect is caused indirectly by removal of regulatory T cells (27) was excluded in experiments with animals depleted of CD4$^+$CD25$^+$ Treg cells (data not shown). Therefore, the expression of P2X-Rs on effector cells regulates disease outcome, a result that is in agreement with findings in an anti-collagen-induced autoimmunity model, in which expression of P2X-Rs on macrophages is required for induction of arthritis (32).

Although the stimulatory effect of NAD on NKT cells was unexpected, it is consistent with reports in the literature. Transfection of P2X-Rs into a T cell line stimulates cell growth under limiting serum conditions (33). Moreover, engagement of P2X-Rs on Jurkat cells activates p56$^{lck}$, ERK, and JNK, and induces production of IL-2 (34). In mast cells, signaling through P2X-Rs was reported to cause phosphorylation of STAT6, ERK, and Jak2 and production of IL-4, IL-6, IL-13, and TNF-$\alpha$ (35). Therefore, it is well documented that engagement of P2X-Rs can induce cell activation. Importantly, the stimulatory effect of P2X-Rs demonstrable in activated NKT cells is in agreement with the aforementioned cited results in tumor cell lines, because they are in a state of permanent activation. Nevertheless, why P2X-Rs transmit an inhibitory signal in naive cells, while stimulating activated cells remains to be investigated; however, both signals are likely caused by receptor-mediated increases in intracellular calcium levels (36–38).

We show that ART-2$^{-/-}$ mice are resistant to Con A-induced hepatitis. Although this constitutes the first demonstration that absence of ART-2 determines disease outcome, it also provides strong support for the notion that NAD, as substrate for ART-2, can regulate autoimmune liver injury. The demonstration in vitro that micromolar NAD concentrations activate P2X-Rs in NKT cells also supports the contention that in Con A-induced liver injury, local concentrations of this molecule can be reached, which suffice to trigger receptor signaling.

Although these results discount an involvement of ATP, a role for this molecule as a danger-transmitting messenger in general, is by no means excluded. Human and rat P2X-Rs express higher ATP sensitivity than mouse P2X-Rs (26, 39). Moreover, T cells from these two species undergo little or no cell surface ADP-ribosylation when incubated with NAD (data not shown). Therefore, in these two species, the principal P2X$_{R}$ ligand may be ATP. In addition, ADP-ribose has been reported to modulate intracellular free calcium concentrations in human monocytes and to induce annexin V staining in rat T cells (40, 41). Thus, in humans and rats, P2X$_{R}$ transmitted danger signals may be triggered by soluble, rather than cell surface attached ligands.

Based on these results, we propose a model for action of P2X-Rs in Con A-induced hepatitis (Fig. 8). NAD is released from injured cells, providing a danger signal (4). When naive NKT cells
encounter this molecule, it serves as substrate for ART-2, which attaches ADP-ribosyl groups close to the P2X-R receptor-binding site. The ADP-ribosyl groups engage P2X-Rs, which induces energy. This mechanism may serve to inhibit autoreactive effector cells in cases of tissue trauma, not involving infections by pathogens. In contrast, during infections NKT cells encounter NAD as activated effector cells, simulated by injection of Con A or α-GaICer. In this case, the NAD danger signal causes cell activation, resulting in increased hepatocyte lysis and increased release of NAD. Although this model depicts NKT cells as primarily responsible for the increase in liver injury, it is entirely possible that NK cells, T cells, and perhaps even APCs, which all express P2X-Rs (3, 42, 43), may also be activated by this mechanism and thereby increase liver injury. Thus, suppression of hepatitis in Con A-injected P2X-R−/− mice may not be solely due to absence of P2X-Rs on NKT cells.

In summary, our data demonstrate the ability of purinergic receptors to tip the balance between immune response and immune suppression, which makes these receptors promising targets for the treatment of infections, cancer, and autoimmune disease. Therefore, it is of much interest to understand the pathways by which P2X-Rs and perhaps other purinergic receptors modulate immunity.

Disclosures

The authors have no financial conflict of interest.

References


