Purinergic $P2Y_2$ Receptors Promote Neutrophil Infiltration and Hepatocyte Death in Mice With Acute Liver Injury

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BACKGROUND & AIMS: During progression of liver disease, inflammation affects survival of hepatocytes. Endogenous release of adenosine triphosphate (ATP) in the liver activates purinergic P2 receptors (P2R), which regulate inflammatory responses, but little is known about the roles of these processes in the development of acute hepatitis. **METHODS:** We induced acute hepatitis in C57BL/6 mice by intravenous injection of concanavalin A and then analyzed liver concentrations of ATP and expression of P2R. We assessed $P2Y_2R^{-/-}$ mice and C57BL/6 wild-type mice injected with suramin, a pharmacologic inhibitor of P2YR. Toxic liver failure was induced in mice by intraperitoneal injection of acetaminophen. Hepatocyte-specific functions of P2R signaling were analyzed in primary mouse hepatocytes. **RESULTS:** Induction of acute hepatitis in wild-type C57BL/6 mice released large amounts of ATP from livers and induced expression of $P2Y_2R$. Liver damage and necrosis were greatly reduced in $P2Y_2R^{-/-}$ mice and C57BL/6 mice given injections of suramin. Acetaminophen-induced liver damage was reduced in $P2Y_2R^{-/-}$ mice. Analysis of liver-infiltrating immune cells during acute hepatitis revealed that expression of $P2Y_2R$ in bone marrow–derived cells was required for liver infiltration by neutrophils and subsequent liver damage. Hepatic expression of $P2Y_2R$ interfered with expression of genes that regulate cell survival, and promoted tumor necrosis factor–α-mediated cell death, in a cell-autonomous manner. **CONCLUSIONS: Extracellular ATP and $P2Y_2R$ have cell-type specific, but synergistic functions during liver damage that regulate cellular immune responses and promote hepatocyte death. Reagents designed to target $P2Y_2R$ might be developed to treat inflammatory liver disease.**

Keywords: Liver Disease; Immune Regulation; Mouse Model; Apoptosis.

BASIC AND TRANSLATIONAL LIVER

Release of endogenous adenosine triphosphate (ATP) to the extracellular compartment and subsequent activation of purinergic nucleotide receptors (P2R) is increasingly recognized as a major signaling pathway involved in the regulation of inflammation and cellular stress responses.[1] Extracellular concentrations of ATP are very low under physiological conditions (in the nanomolar range) and tightly regulated by ectonucleotidases (eg, CD39 and CD73), which dephosphorylate ATP to adenosine diphosphate, adenosine monophosphate, and adenosine.[2] However, extracellular ATP concentrations can be markedly increased under cellular stress conditions, such as inflammation, infection, hypoxia, or trauma, due to active or passive release from a number of cell types, including hepatocytes and inflammatory cells.[3] In addition, ATP metabolism under these conditions can be altered by concomitant down-regulation of nucleotidases. The autocrine and paracrine effects of extracellular ATP are mediated through interaction with members of the P2R family consisting of ligand-gated ion channels ($P2X_1R$–$P2X_7R$) and G-protein–coupled P2Y receptors ($P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, $P2Y_{11}R$-$P2Y_{14}R$), and subsequent activation of diverse intracellular signaling pathways.[4]

Expression of several P2R subtypes has been reported in human and rat hepatocytes and activation of these receptors was linked to various cellular functions, such as hepatocyte metabolism and proliferation.[5–7] Interestingly, ATP also appears to be an important determinant of cell survival in the liver because hepatocyte apoptosis during fulminant hepatitis is strikingly reduced by the P2R antagonist suramin in mice.[8]

Besides these functions on hepatocyte fate, extracellular ATP is also an important danger signal and regulates the recruitment and function of inflammatory cells, such as dendritic cells, neutrophils, and T lymphocytes.[3,9] These findings suggest that extracellular ATP and P2R can serve as key regulators of inflammatory liver disease. In keeping with this notion, $P2X_7R^{-/-}$ mice are protected against concanavalin A (ConA)–mediated hepatitis, a well-established model of fulminant tumor necrosis factor (TNF)–α and T-cell–mediated liver inflammation.[10]

Abbreviations used in this paper: ATP, adenosine triphosphate; ConA, concanavalin A; FACS, fluorescence-activated cell sorter; IL, interleukin; JNK, Jun N-terminal kinase; NF-κB, nuclear factor-κB; NKT, natural killer T; P2R, purinergic P2 receptor; PMH, primary mouse hepatocyte; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor; *WT*, wild-type.

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$P2X_7R$ is an important mediator of interleukin (IL)-1β release and critically regulates many inflammatory cell responses.[11] It was proposed that $P2X_7R$ receptor function is required for the activation of natural killer T (NKT) cells during ConA-mediated hepatitis.[10] However, this function is likely concentration-dependent because increased ATP concentrations in *CD39*$^{-/-}$ mice result in increased NKT-cell apoptosis, thereby protecting against ConA hepatitis.[12] Besides $P2X_7R$, other P2 receptors are likely to play an important role in inflammation, especially in chemotaxis and release of inflammatory mediators. In this context, overwhelming evidence points to $P2Y_2R$ as a main chemotactic receptor for neutrophils.[9] These chemotactic functions of $P2Y_2R$ are of particular interest, given the key pathogenic role of neutrophils in acute hepatitis.[13]

Here we demonstrate that ConA-mediated hepatitis results in increased release of endogenous ATP and subsequent induction of *P2Y$_2$R* expression. Of note, these alterations appeared to be of major pathophysiological relevance because hepatitis severity was substantially alleviated in *P2Y$_2$R*$^{-/-}$ knockout mice. Mechanistically, $P2Y_2R$ mediated cell-type–specific but synergistic functions by regulating neutrophil infiltration as well as hepatocyte death.

Materials and Methods

Cell Culture

Primary mouse hepatocytes (PMH) were obtained and cultured as described.[14] PMH viability was determined by Trypan blue staining and exceeded 75%–80%. To study inflammation-associated hepatocyte damage in vitro, PMH were incubated with recombinant TNFα (20 ng/mL; R&D Systems, Wiesbaden, Germany) and galactosamine (5 mM; Sigma, Schnelldorf, Germany) or antibody against CD95 (Jo-2, 0.5 μg/mL; BD, Heidelberg, Germany). P2YR function was also inhibited by co-incubation with suramin (200 μM; Tocris, Ellisville, MO).

Animal Experiments

P2Y$_2$R$^{-/-}$ knockout mice have been described[15] and were backcrossed for more than 8 generations on a C57BL/6 genetic background and housed under specific pathogen-free conditions at Freiburg University Hospital. Age- and sex-matched C57BL/6 wild-type (*WT*) mice were used as controls. Acute hepatitis was induced in 6- to 12-week-old mice by intravenous injection of ConA (type IV; Sigma, 15 μg/g body weight) and mice were sacrificed at the indicated time points. To inhibit P2Y receptors, animals were co-injected with suramin (200 μg/g body weight intraperitoneally). Bone marrow chimeric mice were generated by injection of 5×10^6 bone marrow cells into sublethally irradiated recipients (2× 4.5 Gy), followed by ConA injection 4–6 weeks later. Toxic liver failure was induced by intraperitoneal injection of acetaminophen (Sigma, 450 mg/kg body weight). All animals received humane care and experiments were performed in accordance with local and institutional regulations.

Determination of Extracellular ATP in Vivo

Extracellular ATP was determined by tail-vein injection of pmeLuc cells (5×10^6)[16] with plasma membrane-targeted luciferase 30 minutes before ConA. Animals were intraperitoneally injected with luciferin at the indicated time points and imaging was performed for 5 minutes using an IVIS100 system and Living Image Software v4.0 (both Xenogen, Caliper Life Sciences, Mainz, Germany) upon anesthesia with isoflurane.

Cytotoxicity Assays and Enzyme-Linked Immunosorbent Assay

Hepatocyte damage was determined by analysis of serum transaminases in cell culture supernatant or mouse sera using semi-automated clinical routine methods. Enzyme-linked immunosorbent assay for CXCL1, CXCL2, IL-1β, IL-2, and IL-6 (all R&D Systems) was performed according to manufacturer's instructions.

Isolation of Intrahepatic Immune Cells

Livers were minced into small pieces and forced gently through a 70-μm cell strainer (BD) using a sterile syringe plunger with addition of Dulbecco's modified Eagle medium (Gibco, Karlsruhe, Germany) containing 5% fetal calf serum. The preparation was centrifuged at 1500 rpm for 7 minutes at 4°C. The resulting pellet was resuspended in 40% Percoll (Sigma) and layered over a 60% Percoll solution and centrifuged at 2500 rpm (900*g*) with the no-brake setting for 20 minutes at room temperature. After centrifugation, intrahepatic immune cells were collected from the inter-phase, transferred to a new tube, and centrifuged to discard residual Percoll. Next, erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 μM EDTA [pH 8.0]) was applied to the pellet. Cells were washed once with fluorescence-activated cell sorter (FACS) buffer before counting and flow cytometric analysis.

Flow Cytometric Analysis of Intrahepatic Immune Cells

Cells were washed and incubated for 15 minutes with unlabeled CD16/32 antibody (2.4G2) to block Fc receptors. For the identification of different immune cell populations, cells were washed and extracellular marker proteins have been stained with fluorophore-conjugated antibodies for 30 minutes on ice: CD19 (1D3), CD11b (M1/70), CD11c (N418), NKp46 (29A1.4), NK1.1 (PK136), Ly6-C (HK1.4), Ly6-G (1A8), Gr-1 (RB6-8C5), T-cell receptor β (H57-597), T-cell receptor $\gamma\delta$ (GL3), and major histocompatibility complex class II (M5/114.5.2; all from eBioscience, Frankfurt, Germany). Staining with fluorescently labeled murine CD1d-tetramer loaded with PBS57 (National Institutes of Health tetramer facility) diluted in FACS buffer was performed for 1 hour at room temperature. Cells were washed twice and acquisition was performed on a FACS Canto II flow cytometer (BD). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Histology, Immunohistochemistry, and Immunofluorescence

For histology, livers were fixed in 3.7% neutral-buffered formaldehyde at 4°C and embedded in paraffin. Confluent liver necrosis was quantified on H&E-stained liver sections using ImageJ software (National Institutes of Health, Bethesda, MD). Immunohistochemistry was performed using antibodies for neutrophils (sc-71674; Santa Cruz, Heidelberg, Germany) and CD3 (sm1754pt; Acris, Herford, Germany). Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assays were performed using the in situ cell death detection kit (Roche, Mannheim, Germany).