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Extracellular UDP and P2Y<sub>6</sub> Function as a Danger Signal To Protect Mice from Vesicular Stomatitis Virus Infection through an Increase in IFN-β Production

Ruimei Li,*† Binghe Tan,*† Yan Yan,* Xiaobin Ma,* Na Zhang,* Zhi Zhang,‡ Mingyao Liu,* Min Qian,* and Bing Du*

Extracellular nucleotides that constitute a “danger signal” play an important role in the regulation of immune responses. However, the function and mechanism of extracellular UDP and P2Y<sub>6</sub> in antiviral immunity remain unknown. In this study, we demonstrated the in vitro and in vivo protection of UDP/P2Y<sub>6</sub> signaling in vesicular stomatitis virus (VSV) infection. First, we demonstrated that VSV-infected cells secrete UDP from the cytoplasm as a danger signal to arouse surrounding cells. Meanwhile, expression of the UDP-specific receptor P2Y<sub>6</sub> was also enhanced by VSV. Consequently, UDP protects RAW 264.7 cells, murine embryonic fibroblasts, bone marrow-derived macrophages, and L929 cells from VSV and GFP lentivirus infection. This protection can be blocked by the P2Y<sub>6</sub> selective antagonist MR52578 or IFN-β receptor–blocking Ab. VSV-induced cell death and virus replication were both enhanced significantly by knocking down and knocking out P2Y<sub>6</sub> in different cells. Mechanistically, UDP facilitates IFN-β secretion through the p38/JNK- and ATF-2/c-Jun–signaling pathways, which are crucial in promoting antiviral immunity. Interestingly, UDP was released through a caspase-cleaved pannexin-1 channel in VSV-induced apoptotic cells and protected cells from infection through the p38/JNK- and ATF-2/c-Jun–signaling pathways, which are crucial in promoting antiviral immunity. Further, UDP also protected mice from VSV infection through P2Y<sub>6</sub> receptors in an acute neurotropic infection mouse model. Taken together, these results demonstrate the important role of extracellular UDP and P2Y<sub>6</sub> as a danger signal in antiviral immune responses and suggest a potential therapeutic role for UDP/P2Y<sub>6</sub> in preventing and controlling viral diseases.

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The “danger” hypothesis was first proposed by Matzinger in 1994 (1) and suggests that infection or other pathological processes that induce cell damage or death could be a universal signal of danger. Following this, more and more danger-associated molecular patterns have been found to activate immune responses to cell injury or stress. Among them, extracellular nucleotides released by injured cells can alert the immune system by binding to plasma membrane P2 receptors, which are widely expressed on immune cells (2, 3). For example, cell apoptosis can cause the release of ATP and UTP as a “find-me” signal through pannexin-1 channels (4). Pannexin-1 hemichannel-mediated ATP release can regulate T cell activation at the immune synapse through activation of P2X1 and P2X4 receptors (5). Recently, the P2 family was shown to participate in the regulation of immune responses in a variety of physiological stress conditions, such as hypoxia, trauma, inflammation, and infection (6, 7). Therefore, a basic understanding of how the immune system detects and responds to danger signals in viral infection will be important in the development of efficacious antiviral drugs.

Based on their structure and distinct signal-transduction mechanisms, P2 receptors can be divided into P2X (P2X1–7) and P2Y (P2Y<sub>1–14</sub>) subfamilies. P2X receptors are ligand-gated ionic channels, whereas P2Y receptors belong to the G protein–coupled receptor family and have seven transmembrane regions. ATP can activate all P2X receptors, whereas the ligands for each P2Y receptor are specific and even changed in different species (7, 8). In particular, P2Y<sub>6</sub>, which could be specifically activated by UDP, plays an important role in the regulation of cytokine and chemokine production in a variety of cells. Recently, several reports indicated that P2Y<sub>6</sub> participates in TLR1/2-induced neutrophil migration by regulating IL-8 secretion (9), inducing expression of CCL2 in microglia and astrocytes (10), and increasing the expression of CXCL8 in intestinal epithelial cells (11). Our previous study showed that P2Y<sub>6</sub> promotes host defense against bacterial infection via MCP-1–mediated monocyte/macrophage recruitment through the MEK/ERK/AP1 pathway (12). However, regulation of the type I IFN–signaling pathway by P2Y<sub>6</sub> during viral infection has not been well clarified.

TLRs involved in virus recognition include TLR3, TLR7, TLR8, and TLR9, which are mainly expressed in the endosomes of cells. TLR3 and TLR7 recognize viral RNA, whereas TLR9 recognizes viral DNA (13). Upon virus infection, the immune system recognizes the invading virus via germline-encoded pattern recognition receptors and releases IFNs with antiviral, immunomodulatory, and...
antiproliferative activities (14–16). The IFNs are classified into two major subtypes: type I (α, β) and type II (γ). Type I IFNs play critical roles in antiviral immunity, including regulating the immune response, inhibiting viral transcription and trafficking, inducing the cellular resistance to viral infection, and apoptosis of virus-infected cells (17–20). By contrast with bacterial infection, a virus requires the host cell machinery to replicate; thus, the elimination of infected cells would shut this machinery down and prevent the virus from spreading. As mentioned previously, the damaged or necrotic cells also can release a danger signal to regulate immune responses. Therefore, an improved understanding of the role of danger signals in cell-intrinsic antiviral defense would be beneficial.

In this study, we demonstrated that UDP can be released through plasma membrane channel pannexin-1 in vesicular stomatitis virus (VSV) infection and that extracellular UDP protects the host against the viral infection both in vitro and in vivo by increasing IFN-β production through ATF-2/c-Jun transcriptional activity. Accordingly, UDP-mediated protection from viral infection and VSV-induced cell death can be suppressed by P2Y6 mutations and the use of the P2Y6 antagonist MR52578 and an IFN-α/β receptor–blocking Ab. Therefore, we suggest that UDP/P2Y6, which also can release a danger signal to regulate immune responses.

Materials and Methods

Reagents

DMEM, RPMI 1640, FBS, penicillin-streptomycin, and TRIzol reagent were purchased from Invitrogen. SYBR Premix Ex Taq and PrimeScript RT Master Mix were from Takara, and the FuGENE HD Transfection reagent was from Roche Applied Science. Carbenoxolone (CBX), N-ethylmaleimide (NEM), UDP, and MR52578 were from Sigma-Aldrich, and SB203580 and SP600125 were from EMD Chemicals. The Mouse IFN-β ELISA Kit and Mouse IFN-alpha/beta receptor block/neutralize polyclonal Abs specific to c-Jun and phosphorylated c-Jun were from Epitomics. Indiana serotype of VSV was a gift from Cell Signaling Technology. Abs specific to c-Jun and phosphorylated c-Jun were from eBioscience. The Transcreener UDP2 FP Assay Kit was from Bellgoat. Goat IgG (AF1083) were from R&D Systems, and normal goat IgG was from cBiocalence. The Transcreener UDP2 FP Assay Kit was from Bellbrook Labs. Poly(I:C) LMW was acquired from InvivoGen, and Dual-Luciferase Reporter assay reagent was from Promega. Abs specific to β-actin, p38, phosphorylated p38, INK, phosphorylated INK, IRF3, phosphorylated IRF3, AKT, phosphorylated AKT, IκBα, phosphorylated IκBα, p65, phosphorylated p65, ATE-2, and phosphorylated ATF-2 were from Cell Signaling Technology. Abs specific to c-Jun and phosphorylated c-Jun were from Epitomics. Indiana serotype of VSV was a gift from Dr. Wenzheng Jiang (East China Normal University).

Cell culture

RAW 264.7, L929, and HEK-293T cells were obtained from the American Type Culture Collection. For cell cultures, RAW 264.7 and HEK-293T cells were grown in DMEM and L929 in RPMI 1640 containing 10% FBS. For the preparation of primary murine embryonic fibroblasts (MEFs), C57BL/6 mice were bred to produce embryos. Cells were prepared from embryos dissected from the same pregnant female at day 13.5 and were cultured in DMEM supplemented with 10% (v/v) FBS. For bone marrow–derived macrophages (BMMs), the femur and tibia were harvested from mice and washed with PBS, and bone marrow–derived monocytes were flushed out with PBS. The cell suspension was filtered through a 70-μm cell strainer to remove any cell clumps. The single-cell suspension was then cultured in RPMI 1640 medium containing 10% (v/v) FBS and M-CSF (20 ng/ml; R&D Systems). In some experiments, L929-conditioned medium was used as a source of M-CSF. To fully differentiate the BMMs, the cells were cultured for 7 d with fresh medium that was changed every other day. The generated BMMs were CD11b+ and F4/80+ (purity > 90%) (21). P2Y6-knockdown L929 cells were cultured in RPMI 1640 with 10% FBS and 2 μg/ml puromycin.

Generation of P2Y6-knockout mice

P2Y6-knockout mice were generated by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems. In brief, Cas9-encoding plasmids and guide RNA expression vectors that contained specific sequences targeting mouse P2Y6 were constructed and linearized by restriction enzymes. Linearized templates were then subjected to in vitro transcription using the In Vitro Transcription T7 Kit (TaKaRa).

The RNA product was purified and injected into mice (C57BL/6) zygotes. Founders of F0 mice were identified by TTEI mismatch–sensitive assays. TA cloning and sequencing were conducted using a TA cloning kit (TaKaRa), according to the manufacturer’s instructions. Sequences of CRISPR target sites for mouse P2Y6 in the genome were 5′-GGTATACCTGCCTGGTCG-GG-3′ (site 1) and 5′-GGTCCGCAGTGCCAGCTT-GGG-3′ (site 2). The Cas9 enzyme–mediated genome editing led to a random deletion of 151 bases between these two sites, which contributed to the loss of function of mouse P2Y6. Sequences for primers used for the identification of mutations are 5′-CACGT-TATGGCAAGGACAAA3′ (sense) and 5′-AAGCCAGTAAAGCCTTGAAG-3′ (antisense). All animal experiments conformed to the regulations drafted by the Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by the East China Normal University Center for Animal Research.

MTS assay

MTS assays were performed using MTS reagent (Promega), according to the manufacturer’s instructions. Briefly, cells were seeded at a density of 1 × 10⁶ cells/well in 96-well plates and allowed to grow overnight. The cells were then treated as indicated in the figure legends. After 24 h of incubation, 20 μl MTS was added to each well and incubated for 2 h at 37°C. The absorbance was measured at a wavelength of 490 nm using a microplate reader. Cell viability of the untreated group was normalized to 100%.

RNA isolation and PCR

Total RNA was isolated from cultured cells using TRIzol reagent, according to the manufacturer’s instructions. cDNA was then synthesized using the PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa) as per the manufacturer’s instructions, and 500 ng cDNA was used as a template and subjected to semiquantitative RT-PCR or quantitative PCR (qPCR). Sequence-specific primers of mouse P2Y6 receptors were described previously (12). The following primers were used: IFN-α, 5′-CAGCGAGGAAGGG- TGGAFGCCCAACG-3′ (forward) and 5′-CAGACACCTTCCGAAGAAGG- CGAG-3′ (reverse); β-actin, 5′-GTACCCAAACACATGCTG-3′ (forward) and 5′-CGTCTACATCTGGCCTGGTCT-3′ (reverse); and VSV Indiana serotype, 5′-ACGGCGTACTCCTCACAGTTG-3′ (forward) and 5′-CTCGGTTCTA- AGATCACCGT-3′ (reverse). Data were normalized to the level of β-actin expression in each sample.

ELISA for IFN-β detection

RAW 264.7 cells were seeded into 24-well plates at 5 × 10⁵ cells/well and incubated overnight. Cells were treated with the indicated reagent or infected with VSV for the indicated amount of time (see figures). The concentration of IFN-β in the supernatant was measured using the Mouse IFN-β ELISA Kit, as recommended by the manufacturer (R&D Systems).

Western blot analysis

After treatment or stimulation, cells were washed twice with cold PBS and lysed by RIPA buffer (Cell Signaling Technology). The concentration of protein was measured by BCA assay (Pierce) and equalized to the same concentration with the extraction reagent. Samples were loaded and heated for 10 min at 100°C, subjected to 8% SDS-PAGE, transferred onto nitrocellulose membranes, and blocked with 5% BSA. Following incubation with primary Abs and incubation with the appropriate fluorescent secondary Abs, the immunoreactive proteins were detected using the Odyssey laser digital imaging system (Gene Company).

Vector construct

To obtain the pIFNβ200-luc construct, mouse IFN-β core promoter regions from −223 to −24 were amplified by PCR with sequence-specific primers: 5′-GCCGCTGAGCAGGCAAGCTGAGTTAATAAATTG-3′ (forward) and 5′-GCCCTCGAAGCTGCCTAGCTAAGGAG-3′ (reverse). Mouse genomic DNA derived from RAW264.7 cells was used as a template. PCR products were purified, protection bases were removed, and PCR products were cloned into the pGL3-basic vector. After sequencing, the plasmid was extracted and purified using the Plasmid Mini Kit (Omega) for cell transfection. To obtain the mutant pIFNβ200-luc construct (with ATF-2/c-Jun binding site mutation), site-directed mutagenesis was carried out on the pIFNβ200-luc vector. The mutagenesis primers were 5′-GAAGCCTG- TTAGATTTTAAAGAGAATGCCCAGTGGAGAAACTC-5′ (forward) and 5′-CTCCCTTTAGTTCCTCTGACCTCTCTTCC- TAACAGCTTC-3′ (reverse). pIFNβ200-luc was used as a template. Following the first PCR and DNA digestion of the parental template, the plasmids were transformed into Escherichia coli DH5α. The sequenced construct was then extracted and purified using the Plasmid Mini Kit for cell transfection.
For the construction of the shRNA-mediated knockdown of P2Y6 (shP2Y6) vector and screening for stable knockdown of P2Y6 in L929 cells, the pLKO.1-TRC vector was used according to the manufacturer’s instructions. The shP2Y6 forward oligonucleotides was 5′-CCGGCA-TCAACACGAGGCTTTCCGAAAGATGTCGCGTGGATGTTTTTGG-3′, and the reverse oligonucleotides was 5′-AATCTACAAATCCACCAAGACACGCTCAGGAAGTAGGATGCCTTGTTGATGTTTTG-3′, and the reverse oligonucleotides was 5′-AATTCAAA ACTAGGAAA GGGCCTGCCGTTGAGGGTGATCGCTTCTCTTCTCTAGTGG-3′.

Cytosolic [Ca2+]i measurements

A total of 1 × 10^5 L929 cells was transfected with P2Y6 shRNA plasmids, and the corresponding control plasmids were seeded into the dish and cultured overnight in RPMI 1640 medium supplemented with 10% FBS and penicillin (100 U/ml) and streptomycin (100 μg/ml). After washing twice with HBSS buffer (Life Technologies), cells were loaded with 10 μM the [Ca2+]i-sensitive dye Fluo-2 AM (Beotime) in HBSS and incubated in the dark at room temperature for 30 min. Following this, the incubation buffer was removed, the cells were washed twice with HBSS, and fresh buffer was added to each dish. The cells were stimulated or not with UDP (100 μM), and the fluorescence signal of the intracellular [Ca2+]i was recorded and analyzed with a digital fluorescence imaging system (OLYMPUS). Data are represented as the ratio of fluorescence excitation at 340/380 nm at different time points.

Transient transfection and luciferase assay

RAW 264.7 cells were seeded into 24-well plates at 6 × 10^4 cells/well overnight and transfected with 250 ng pFNβ200-luc or μ-pFNβ200-luc constructs using FuGENE HD for 24 h, according to the manufacturer’s instruction. As an internal control, 50 ng Renilla was used per well. Next, the cells were treated with UDP or LPS. After 12 h of stimulation, luciferase activity was measured with the Dual-Luciferase Assay System, and the results were normalized with Renilla activity.

GFP lentivirus infection assay

The GFP lentivirus was packaged with the pL3.7 system, as described (22). After transfecting HEK-293T cells with pL3.7, pMD2.G, and psPAX2 for 24 h, the viral supernatant was collected and stored at −80°C. RAW 264.7 cells were seeded into a 12-well plate at a density of 1 × 10^5 cells/well and allowed to grow overnight. Cells were pretreated or not with 10 μM MRS2578 for 1 h and then stimulated with 100 μM UDP for 12 h prior to GFP lentivirus infection. Cells were photographed using a fluorescence microscope 24 h later, and the GFP+ cell ratio was analyzed by FACS.

Measurement of UDP-mediated antiviral activity by crystal violet staining

Cells were seeded into 96-well plates at a density of 1 × 10^5 cells/well overnight and pretreated or not with 10 μM MRS2578 for 1 h. The cells were stimulated with 100 μM UDP for 6 h prior to VSV infection. The medium was removed and washed with PBS 24 h later, and the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Paraformaldehyde was aspirated, and 50 μl crystal violet stain was added to each well. The plates were incubated for 2 h at room temperature and washed with PBS five times prior to the photographs being taken.

UDP detected by Transcreener UDP2 FP Assay

RAW 264.7 cells were seeded into 24-well plates at 6 × 10^4 cells/well overnight and then changed to phenol red-free DMEM. Subsequently, cells were infected with VSV at a multiplicity of infection (MOI) of 1 for the indicated amount of time (see figures) after treating them or not with CXB and NEM for 1 h. As recommended by the manufacturer (BellBrook Labs), 15 μl the supernatant was used for the fluorescence polarization assay.

FACS for cell apoptosis

RAW 264.7 or MEFs were seeded at a density of 2 × 10^5 cells/well into six-well plates 1 d prior to treatment. Cells were then switched to fresh medium and treated or not with UDP (100 μM) or MRS2578 (10 μM) overnight. VSV was added to each well at an MOI of 1 in fresh medium. After 24 h, apoptotic and dead cells were determined by Annexin V and propidium iodide double staining using a flow cytometer (all from BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences).

Intranasal VSV infection and immunofluorescence staining

For intranasal infection, mice were anesthetized with ketamine/Rompun and pretreated or not with 100 μM UDP or 10 μM MRS2578 for 1 h. A total of 50 μl PBS buffer containing 10^7 PFU VSV in PBS was pipetted into both nostrils for 12 h. Mice were perfused with 5 ml PBS and with an additional 8 ml 4% formaldehyde-supplemented saline for subsequent immunohistological staining. For immunofluorescence, brains of the VSV-infected mice were fixed with formaldehyde-supplemented saline and cut into 8-μm slices. Tissue was stained with DAPI.

Results

VSV infection increases UDP release and P2Y6 expression

Previous studies showed that P2Y6 is the most abundant member of the P2Y family in murine macrophages (12). To investigate the role of UDP/P2Y6 in viral infection, we assessed the release of endogenous UDP and the expression of P2Y6 in VSV-infected cells. As shown in Fig. 1A, extracellular UDP, which is the endogenous agonist of P2Y6, was increased in VSV-infected cells in a time-dependent manner, whereas little change was observed in uninfected cells. Expression of P2Y6 also was significantly increased by VSV (Fig. 1B) and polyinosinic-polycytidylic acid [poly(I:C)] (Fig. 1C) in RAW 264.7 cells. These data indicate that the expression of P2Y6 is induced in virus-infected cells.

UDP promotes antiviral activity in RAW 264.7 cells, MEFs, and L929 cells

To elucidate the influence of P2Y6 signaling on cell proliferation, we treated RAW 264.7 and L929 cells with UDP (100 μM) and MRS2578 (10 μM). Our data showed that both UDP and MRS2578 have little effect on the viability of RAW 264.7 and L929 cells in 24 h (Fig. 1D), indicating that neither the agonist nor the antagonist of P2Y6 regulates cell proliferation. Next, we infected RAW 264.7 cells with VSV at an MOI of 1 for 24 h. The results showed that cell survival in VSV infection was increased dramatically by UDP, and this activation could be blocked by the P2Y6 selective antagonist MRS2578 (Fig. 2A). Similar results were observed in L929 cells where cell cytopathic effects were induced by VSV, as determined using a crystal violet staining assay (Fig. 2B). These data suggested that UDP/P2Y6 could protect cells from virus-induced damage, but this does not occur through increased cell viability alone. Furthermore, we also assessed the ability of UDP to protect MEFs from VSV-induced cell apoptosis. As shown in Fig. 2C, VSV-induced cell death or apoptosis was reduced by UDP, and this reduction could be blocked by MRS2578. Furthermore, the quantity of intracellular virus in MEFs was reduced significantly by UDP and increased by MRS2578 (Fig. 2D). Meanwhile, the infection efficiency of GFP lentivirus to RAW 264.7 cells was notably reduced by UDP in FACS, and this reduction could be lessened by MRS2578 (Fig. 2E). These results showed the positive regulation of antiviral immunity by UDP/P2Y6 in viral infection.

Knockdown of P2Y6 promotes VSV infection and replication

To confirm the antiviral activity of P2Y6, we knocked down the expression of P2Y6 in L929 cells by short hairpin RNA (Fig. 3A). Meanwhile, we also measured UDP-activated cytosolic [Ca2+]i in P2Y6-knockdown L929 cells (cells transfected with shP2Y6-2). The cytosolic [Ca2+]i increased in control cells treated with UDP in a very short time (20 s), whereas the cytosolic [Ca2+]i in shP2Y6 cells showed little change. This suggested that the UDP-activated signaling pathway was blocked significantly in P2Y6-knockdown cells (Fig. 3B). As shown in Fig. 3C and 3D, knockdown of P2Y6 reduced cell viability with (Fig. 3D) or without (Fig. 3C) UDP after VSV infection at a low MOI in the MTS assay. Meanwhile, VSV RNA replication in infected cells also increased in P2Y6-knockdown cells (Fig. 3E). Knockdown of P2Y6 eliminated the...
Thus, we generated P2Y6-mutant mice using CRISPR/Cas9 to in-gene loci, CRISPR has been widely used in gene editing (23). As a highly effective technology in inducing mutations at specific manner

Expression of P2Y6 was measured by quantitative RT-PCR

IRF3/IRF7, NF-

cooperative activation of different transcription factors, such as increase in p38, JNK, ATF-2, and c-Jun phosphorylation, whereas

 UDP stimulation resulted in a time- and concentration-dependent increase in p38, JNK, ATF-2, and c-Jun phosphorylation, whereas

 minimal changes were seen for IRF3, IκBα, and p65. UDP-activated ATF-2 and c-Jun can be inhibited by pretreating the cells with SP600125 (a JNK inhibitor; Fig. 5C), suggesting that UDP/P2Y6-enhanced IFN-β production occurs primarily through the JNK/ATF-2/c-Jun–signaling pathway. Although SB203580 (a p38 inhibitor) cannot block UDP-induced phosphorylation of ATF-2, it was suggested that p38 could be involved in UDP-mediated IFN production through an ATF-2–independent pathway.

UDP facilitates IFN-β secretion through p38/JNK- and ATF-2/c-Jun–signaling pathways

To further determine the key role of MAPKs and ATF-2/c-Jun in UDP-induced IFN-β production, specific inhibitors of p38 (SB203580) and JNK (SP600125) were added. As shown in Fig. 6A and 6B, RAW 264.7 cells pretreated with SB203580 or SP600125 showed attenuated UDP-enhanced IFN-β expression (Fig. 6A, 6B), suggesting that P2Y6-induced IFN-β expression occurs through phosphorylation of p38 and JNK.

Wild-type (WT) and mutant (ATF-2 binding site mutation) mouse IFN-β core promoter regions (from −223 to −24) were cloned into pGL3-basic constructs to obtain pIFNβ-WT and pIFNβ-mutant. After transfection of those plasmids into the RAW 264.7 cells for 24 h, the cells were stimulated with 100 μM UDP for 12 h to detect luciferase activity. As shown in Fig. 6C, transcriptional activity of the IFN-β core promoter was increased by UDP, and the activation was eliminated by a mutation at the ATF-2 binding site (Fig. 6D). Furthermore, if the cells were treated with an anti–IFN-α/β receptor Ab, the protection that UDP provides against VSV-induced cell death apoptosis was reduced (Fig. 6E). These data demonstrated that UDP-enhanced IFN-β production occurs mainly through p38/JNK- and ATF-2/c-Jun–signaling pathways, which can protect cells from VSV-induced cell death and apoptosis.

VSV triggers UDP secretion through pannexin-mediated gap junctions

As shown in Fig. 1A, VSV-infected cells release UDP as an intracellular danger signal to alert the immune system to invading pathogens. Therefore, it is interesting to explore why and how the danger signal is released from injured cells. Thus, we pretreated RAW 264.7 cells with different inhibitors to nucleotide-release
FIGURE 2. UDP reduces cell apoptosis and virus infection in RAW 264.7 and L929 cells and MEFs. (A) RAW 264.7 cells were treated or not with MRS2578 1 h before being exposed to 100 μM UDP for 6 h and subsequently were infected with VSV at an MOI of 1 for 24 h. Cell viability was detected by MTS assay. (B) L929 cells were treated or not with 10 μM MRS2578 for 1 h before being exposed to 100 μM UDP for 6 h. The cells were then infected with VSV at an MOI of 1 for 24 h. Then the cells was stained by crystal violet and observed under microscope. Original magnification ×400. (C) MEFs were treated or not with MRS2578 for 30 min before being exposed to 100 μM UDP for 12 h. The cells were then infected with VSV at an MOI of 1 for the indicated amount of time in fresh medium. Cell apoptosis was detected through annexin V–FITC by FACS. (D) MEFs were treated or not with MRS2578 30 min before being exposed to 100 μM UDP for 12 h and then were infected with VSV at an MOI of 1 for 12 h. Intracellular VSV RNA replicates were measured by qRT-PCR (n = 3). (E) RAW 264.7 cells were treated or not with MRS2578 1 h before being exposed to 100 μM UDP for 24 h. The cells were then infected with GFP lentivirus for 24 h. The GFP+ cells were analyzed by FACS. *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 3. Knockdown and knockout of P2Y6 reduces cell survival and promotes VSV replication. (A) Identification of P2Y6 interference efficiency in L929 cells by qRT-PCR. (B) A total of $1 \times 10^5$ L929 cells in each dish was loaded with Fluo-2 AM and treated or not with 50 μM UDP, and the changes in cytosolic [Ca^{2+}] were assessed. Each curve represents the typical [Ca^{2+}] signal of cells in a microscope field. shNC and shP2Y6-2 L929 cells were not treated (C) or were treated with (D) 100 μM UDP for 6 h and then infected with VSV, as indicated, for 24 h. Cell viability was detected by MTS assay, and results represent a percentage of the control. (E) shNC and shP2Y6-2 L929 cells were infected or not with VSV at an MOI of 10 for 12 or 24 h. Intracellular VSV RNA replicates were measured by qRT-PCR. (F) shNC and shP2Y6-2 L929 cells were treated or not with UDP (100 μM) for 6 h and then infected with VSV at an MOI of 1 for 12 h. Intracellular VSV RNA replicates were measured by qRT-PCR. (G) P2Y6 WT and mutant BMMs were treated or not with 100 μM UDP for 12 h and then infected with VSV at an MOI of 1 for 12 h. Intracellular VSV RNA replicates were measured by qRT-PCR. $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 
FIGURE 4. P2Y6 upregulates IFN-β expression. (A) RAW 264.7 cells were stimulated with 100 μM UDP for 1–6 h or left unstimulated. IFN-α and IFN-β were measured by qPCR after the cells were harvested, and 100 ng/ml LPS was added as a positive control. (B) RAW 264.7 cells were treated with 10 μM MRS2578 for 1 h or left untreated and then exposed to 0–100 μM UDP for 1 h. IFN-β was measured by RT-PCR. (C–E) RAW 264.7 and L929 cells were treated with 10 μM MRS2578 for 1 h or left untreated and then exposed to 100 μM UDP for 6 h. The cells were then infected with VSV at an MOI of 1 for 15 h (C and E) or stimulated with 1 μg/ml poly(I:C) for 4 h (D). IFN-β was measured by qRT-PCR. (F) RAW 264.7 cells were treated with 10 μM MRS2578 for 1 h or left untreated and then exposed to 100 μM UDP 6 h before, simultaneously, or up to 3 h after they were infected with VSV at an MOI of 1 (24 h later). IFN-β was detected by ELISA. n = 3. *p < 0.05, **p < 0.01, ***p < 0.001.
channels, including CBX (a pannexin channel inhibitor) and NEM (inhibits vesicular exocytosis), prior to VSV infection. Subsequently, UDP release was detected using fluorescence polarization assays. VSV-triggered UDP release was inhibited by CBX, indicating that VSV-induced UDP release occurs through pannexin channels (4). Apoptosis was detected in VSV-infected cells by Western blotting and FACS. As shown in Fig. 7B, VSV induced cell apoptosis and cleaved caspase 3 in a time-dependent manner, which could result in the cleavage of the pannexin-1 channel and release of UDP. The released UDP protects cells from VSV-induced apoptosis and functions as a negative-feedback loop (Fig. 7C). Taken together, virus-induced cell apoptosis activates pannexin-1 channels through caspase cleavage that could then enable the release of UDP as a danger signal in viral infection.

**UDP/P2Y6 protects mice from VSV-mediated acute neurotropic infections**

Acute neurotropic infections are often studied in mice treated with VSV because this virus was shown to infect neuroepithelial cells (24). Therefore, both P2Y6 WT and mutant mice were infected with VSV through intranasal injection. Following intranasal virus inoculation, we used immunofluorescence assays to measure VSV infection by VSV-G–specific Ab in the olfactory bulb. As shown in Fig. 7D, there were fewer FITC + (VSV-infected) cells in the UDP-treated mice than in the PBS-treated mice, whereas UDP-mediated protection was reduced by the P2Y6-specific inhibitor MRS2578. Furthermore, VSV infection in the olfactory bulb was more severe in P2Y6-knockout mice, even when they were pretreated with UDP. Our data further confirmed that UDP/P2Y6 signaling plays a crucial role in VSV infection in mice and showed the potential of UDP/P2Y6 in curing and preventing viral infection (Fig. 8).

**Discussion**

Various immune cells, including CD4+ T cells and macrophages, are involved in viral infection (25). In our previous study, P2Y6 was found to be highly expressed in macrophages compared with other P2Y receptors, but the function and mechanism of P2Y6 in macrophage-mediated antiviral immunity are poorly understood. In this study, we showed that UDP was secreted as a danger signal in an autocrine or paracrine manner by injured cells. The released UDP subsequently facilitated virus-induced IFN-β production to suppress virus replication and virus-induced cell damage both in vitro and in vivo. These data could advance and broaden our understanding of UDP/P2Y6 signaling as a self-regulator in host defense against virus.

VSV is an enveloped negative-stranded RNA virus that belongs to the *Vesiculovirus* genus of the Rhabdoviridae family. Although VSV infects cattle, horses, deer, and pigs, it seldom causes severe disease in humans. Nevertheless, VSV is similar to viruses such as Ebola and Marburg hemorrhagic fever, as well as rabies. Thus, VSV may be a good and safe experimental model of viral infection (26). In this study, the function of UDP in VSV infection was evaluated both in vitro and in vivo. As shown in Fig. 1, the expression of P2Y6 is induced by VSV, and UDP release (as a specific agonist to P2Y6) from infected cells also is increased dramatically. As a classic G protein–coupled receptor, activation by extracellular agonists is a key step in P2Y6-mediated physiological functions. Thus, we treated RAW 264.7 cells, MEFs, BMMs, and L929 cells with UDP to explore the protection of UDP in viral infection. Although previous studies showed that UDP can promote the survival of osteoclasts through NF-κB signaling (27), the survival of both RAW 264.7 and L929 cells was minimally affected by UDP or MRS2578, suggesting that protection against viral infection does not only occur by increasing cell viability. Subsequent experiments confirmed this hypothesis, showing that both
VSV-induced cell death and damage and the quantity of infected virus could be significantly reduced by UDP. Consistent results also were observed in P2Y6-knockdown and -knockout cells and mice, which further defines the positive regulation of UDP on antiviral immunity.

Type I IFN induction is primarily controlled at the gene transcriptional level by IFN regulatory factors (IRFs). The core promoter region of IFN-β includes at least four positive regulatory domains (PRDs): PRD I, PRD II, PRD III, and PRD IV (28). PRD I and PRD II
FIGURE 7. VSV induces UDP release through pannexin-mediated gap junctions and protects mice from VSV infection. (A) RAW 264.7 cells were pretreated or not with 10 μM CBX and 5 μM NEM 1 h before being infected with VSV at an MOI of 1 for the indicated amount of time. UDP release was measured by Transcreener UDP FP Assay. (B) RAW 264.7 cells were infected with VSV at an MOI of 1 for the indicated amount of time. Caspase 3, cleaved caspase 3, and β-actin were detected by Western blot. (C) RAW 264.7 cells were treated or not with MRS2578 for 30 min before being exposed to 100 μM UDP for 12 h. The cells were then infected with VSV at an MOI of 1 for the indicated amount of time with fresh medium, and cell apoptosis was detected through annexin V–FITC by FACS. (D) P2Y₆ WT or mutant mice were pretreated or not with 100 μM UDP or 10 μM MRS2578 before being intranasally infected with VSV for 12 h. The mice were sacrificed to detect invaded VSV by immunofluorescence. Scale bars, 5 μm.
primarily binds with NF-κB and PRD IV specifically binds with AP-1 (ATF-2 with c-Jun). After viral infection, PRD I-IV direct the assembly of ATF-2, c-Jun, IRFs, NF-κB, and HMG-I (Y) to initiate the transcription of IFN-β (29). Many genes have been found to positively regulate type I IFN signaling to improve the antiviral activity of cells; however, the molecular mechanisms for full activation of virus-induced production of type I IFN remain unclear. In this study, the expression of IFN-β was significantly enhanced by UDP both at the protein and RNA levels (Fig. 4), suggesting a potential mechanism of UDP-induced antiviral immunity. Therefore, we treated RAW cells with UDP in a time- and dose-dependent manner to determine which signaling is influenced by UDP. To our surprise, neither IRF3 nor NF-κB signaling, both of which are crucial in type I IFN induction, was influenced by UDP. However, phosphorylation to p38, JNK, and ATF-2 was increased dramatically by UDP. qPCR and ELISA showed that inhibition of JNK- and p38-associated signaling attenuated P2Y6-induced IFN-β production. Additionally, UDP increased the transcriptional activity of the WT IFN-β promoter but not the ATF-2/c-Jun binding site mutant IFN-β promoter. This suggested that UDP regulates IFN-β production mainly through the activation of transcriptional activity of ATF-2/c-Jun but not IRF3 or NF-κB. Furthermore, anti-IFNR Ab, which can block the function of IFN-β, rescued UDP/P2Y6-mediated protection. Taken together, these data implied that the JNK-p38/ATF-2-c-Jun pathway in UDP/P2Y6 signaling has a key role in the regulation of IFN-β and, thus, plays an important role in antiviral activity. Our findings further highlight the positive role of ATF-2 in the regulation of IFN production and extend the function of UDP/P2Y6 in IFN-mediated antiviral immune responses.

Extracellular nucleotides have been regarded as danger signals for a long time, but the mechanism of how and why they are released from injured cells has not been clarified. Generally, extracellular nucleotides, such as ATP, UTP, and UDP, can be released through three potential mechanisms: exocytosis, blebbing, or passage via a plasma membrane channel (4). In this study, although a small amount of UDP release from VSV-infected cells was observed in the early stages (1 h postinfection), the secretion of UDP was dramatically enhanced in the later stages (24 h postinfection). It seems that the release of UDP from VSV-infected cells is mainly through a long-term mechanism that is not consistent with the involvement of vesicular exocytosis. Therefore, we treated the infected cells with different inhibitors to explore the potential mechanism of VSV-induced UDP release. As shown in Fig. 7A, NEM, an inhibitor of vesicular exocytosis, has minimal influence on VSV-induced UDP release, whereas UDP release was inhibited notably by CBX (an inhibitor of pannexin channels). Pannexins belong to a family of gap junction proteins that are composed of intercellular channels, which provide ionic and metabolic coupling, and nonjunctional channels, which can function as paracrine-signaling pathways (30). A previous study showed that pannexin-1 is required for ATP release during apoptosis in LPS-primed BMMs (31). Therefore, we postulated whether VSV-induced UDP release occurred through a specific caspase cleavage site within pannexin-1 during VSV-induced apoptosis. To examine the activity of caspase, cleaved caspase 3 in VSV-infected cells was investigated. As shown in Fig. 7B, cleaved caspase 3 was detected at 20 h postinfection, which was consistent with the time when UDP release was high (Fig. 1A). Furthermore, apoptosis in VSV-infected cells was evaluated using annexin V-FITC/propidium iodide double staining. The number of apoptotic and dead cells increased in VSV-infected cells as time passed, and this was consistent with the release time of UDP. Thus, we hypothesized that virus-induced cell apoptosis eliminates viral replication, as well as activates pannexin-1-mediated plasma membrane channels to release UDP as a danger signal to evoke an antiviral immune response. Interestingly, UDP also can protect cells from TNF-induced apoptosis by suppressing the activation of caspase 3 and 8 in 1321N1 astrocytoma cells (32). Therefore, the release of UDP can be regarded as a feedback regulatory loop that could be important in reducing virus-induced cell death and protecting the host from viral infection. Taken together, our study extends the novel function of UDP/P2Y6 as a danger signal in antiviral immune responses through extracellular nucleotides.

Disclosures
The authors have no financial conflicts of interest.

References
UDP AND P2Y\(_6\) PROTECT MICE FROM VSV INFECTION

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