Vesicular nucleotide transporter mediates ATP release and migration in neutrophils

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Abbreviations: fMLP, formyl-Met-Leu-Phe; FPR L1, formyl peptide receptor-like 1; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; PMN, polymorphonuclear neutrophil; VNUT, vesicular nucleotide transporter
Abstract

Neutrophils migrate to sites infected by pathogenic microorganisms. This migration is regulated by neutrophil-secreted ATP, which stimulates neutrophils in an autocrine manner through purinergic receptors on the plasma membrane. Although previous studies have shown that ATP is released through channels at the plasma membrane of the neutrophil, it remains unknown whether it is also released through alternate secretory systems involving vesicular mechanisms. In this study, we investigated the possible involvement of vesicular nucleotide transporter (VNUT), a key molecule for vesicular storage and nucleotide release, in ATP secretion from neutrophils. RT-PCR and western blotting analysis indicated that VNUT is expressed in mouse neutrophils. Immunohistochemical analysis indicated that VNUT mainly co-localized with matrix metalloproteinase-9 (MMP-9), a marker of tertiary granules, which are secretory organelles. In mouse neutrophils, ATP release was inhibited by clodronate, which are potent VNUT inhibitors. Furthermore, neutrophils from VNUT−/− mice did not release ATP and exhibited significantly reduced migration in vitro and in vivo. These findings suggest that tertiary granule-localized VNUT is responsible for vesicular ATP release and subsequent neutrophil migration. Thus, these findings suggest an additional mechanism through which ATP is released by neutrophils.
Introduction

Purinergic chemical transmission is involved in regulating the function of many types of blood cells (1). Polymorphonuclear neutrophils (PMNs) are primary phagocytic cells that play a crucial role in defense against invading microorganisms such as bacteria, fungi, and protozoa. When these organisms invade, PMNs sense the infection and secrete nucleotides such as ATP, which act as autocrine or paracrine signals to initiate a series of responses including granular release, chemotaxis, the production of superoxide, and the regulation of apoptosis upon binding to purinoceptors in neutrophils (2-7). For example, during chemotaxis, ATP released from chemoattractant-stimulated neutrophils is involved in signal amplification, controlling gradient sensing, and controlling migration speed via the purinergic P2Y2 receptor and A3 receptor in an autocrine manner (3). Thus, purinergic chemical transmission plays a crucial role in neutrophil function.

Even though ATP signal reception by neutrophils is well understood, the mechanism through which neutrophils secrete ATP is relatively unknown. At least two major mechanisms have been postulated for the release of ATP; one is that ATP release is mediated by ATP channels at the plasma membrane, and the another is exocytosis of ATP (8, 9). Connexin-43 and pannexin-1 channels are involved in ATP release from neutrophils, leading to migration (10-12). However, the mechanism of ATP release is controversial; specifically, there are multiple ATP release pathways in neutrophils, including the secretion of granules that store ATP, as the amount of ATP released is dependent on the concentration of the chemoattractant (13). These studies suggest the existence of granule-mediated ATP exocytosis.

Vesicular nucleotide transporter (VNUT) is responsible for the vesicular storage of ATP and plays an essential role in the exocytosis of ATP upon stimulation (14). This protein is a member of the SLC17 anion transporter family, and transports nucleotides such as ATP and ADP into secretory vesicles using the membrane potential (Δψ; inside positive) across membranes, which is established by vacuolar H+-ATPase (14, 15). Recent studies indicated that VNUT is expressed and functions in neuroendocrine and immune cells, which have been reported to be associated with purinergic tissues or cells (14-22). VNUT−/− mice were shown to lose vesicular ATP contents and vesicular ATP release, resulting in loss of purinergic chemical transmission in vivo (19). VNUT is primarily responsible for vesicular ATP storage and release, and plays an essential role in purinergic chemical transmission.

In this study, we determined whether VNUT is expressed and functions in neutrophils. We showed that VNUT is expressed and localized in the tertiary granules of neutrophils,
and that neutrophils release ATP in a VNUT-dependent manner. In addition, VNUT-mediated ATP release was shown to be involved in neutrophil migration, which was blocked by the clinically available VNUT inhibitor clodronate.

**Results**

**VNUT is localized in tertiary granules of neutrophils.**

As the first step of the study, we isolated neutrophils from the bone marrow of wild-type and *VNUT*−/− mice and examined the status of VNUT. Regarding the form of neutrophils, there was no morphological difference between those from wild-type and *VNUT*−/− mice, based on Giemsa staining (Fig. 1A). Reverse transcriptase PCR (RT-PCR) analyses indicated that a 523-bp VNUT-specific transcript was amplified from mouse neutrophils, whereas no such transcript was detected in wild-type neutrophils without the RT reaction, or in neutrophils from *VNUT*−/− mice (Fig. 1B). Immunoblotting with specific antibodies against VNUT indicated that the membrane fraction of wild-type mouse neutrophils contained an immunoreactive polypeptide with an apparent molecular mass of 70 kDa (Fig. 1C). This was absent in neutrophils from *VNUT*−/− mice. The protein levels of other neutrophil granules or vesicle proteins, including V-ATPase, formyl peptide receptor-like 1 (FPRL1), and vesicle associated membrane protein 2 (VAMP2), were unaffected by the absence of VNUT (Fig. 1C). Furthermore, indirect immunofluorescence microscopy indicated that VNUT was present in mouse neutrophils and exhibited a punctate distribution, whereas control reactions with pre-absorbed antibodies resulted in only background levels of staining (Fig. 1D). In *VNUT*−/− mice, no VNUT immunoreactivity was observed, similar to that observed in the negative control (Fig. 1D).

Next, we examined whether VNUT immunoreactivity was associated with granular components in mouse neutrophils. Neutrophils harbor some secretory organelles such as azurophil granules, specific granules, tertiary granules, and secretory vesicles, which contain an abundance of enzymes or receptors (23). Double-labeling immunofluorescence microscopy revealed that VNUT immunoreactivity was co-localized with matrix metalloproteinase-9 (MMP-9), a tertiary granule marker (colocalization coefficients for VNUT: M1:0.703, M2:0.764), but not with myeloperoxidase (MPO), an azurophil granule marker (M1:0.170, M2:0.378); lactoferrin, a specific granule marker (M1:0.121, M2:0.449); or CD35, a secretory vesicle marker (M1:0.163, M2:0.400) in mouse neutrophils (Fig. 2A). Immunofluorescence microscopy also showed no apparent differences in the fluorescent staining images between wild-type and *VNUT*−/− mice (Fig. 2A). VNUT was not co-localized with GM130 (M1:0.079, M2:0.581),

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early endosome antigen 1 (EEA1; M1:0.229, M2:0.341), lysosomal-associated membrane protein 1 (LAMP1; M1:0.240, M2:0.396), or protein disulfide-isomerase (PDI; M1:0.257, M2:0.565), markers of the Golgi apparatus, early endosome, lysosome, and endoplasmic reticulum, respectively (Fig. 2B). Furthermore, VNUT was partially co-localized with VAMP2 (M1:0.659, M2:0.772), a marker of secretory granules (Fig. 2B). These results suggested that VNUT is localized to tertiary granules in mouse neutrophils.

**VNUT-dependent vesicular ATP release from mouse neutrophils**

Further, we examined whether the vesicular release of ATP from neutrophils occurs in a VNUT-dependent manner. ATP release was observed in isolated mouse neutrophils upon stimulation with 5 µM A23187, a Ca²⁺ ionophore, or 100 nM W-peptide, formyl peptide receptor ligand; however, this was almost completely abolished in cells from VNUT−/− mice (Fig. 3A and B, left). On the other hand, no differences were observed in the release of MPO after stimulation with 5 µM A23187 and 100 nM W-peptide, or in the release of MMP-9 upon stimulation with 5 µM A23187 in cells from wild-type and VNUT−/− mice; however, W-peptide-induced MMP-9 release was reduced in VNUT−/− cells (Fig. 3A and B, middle and right). ATP release was also blocked by 0.1 µM or 1 µM clodronate, a potent and selective VNUT inhibitor (24) (Fig. 3C). After adding 0.1 µM or 1 µM clodronate, neutrophil viability was as follows; 97.6% and 96.9%, respectively. Previous studies reported that vesicular ATP release is strongly inhibited by low temperatures or Ca²⁺ chelators (16, 24, 25).

In this study, we showed that A23187-stimulated ATP release is significantly reduced when neutrophils are incubated at 20 °C or 4 °C (Fig. 3D). In the presence of an extracellular and intracellular Ca²⁺ chelator EGTA and EGTA-AM, the release of ATP stimulated by 5 µM A23187 was also reduced (Fig. 3E). These results strongly suggested that ATP release from mouse neutrophils can occur via a VNUT-mediated exocytotic mechanism.

**Expression of VNUT in human neutrophils**

To examine the function of VNUT in greater detail, we prepared human neutrophils from blood samples obtained from volunteers and determined whether VNUT was expressed in human neutrophils (Fig. 4A). Through RT-PCR analyses, a 115 bp VNUT-specific transcript was amplified in human neutrophils (Fig. 4B). Indirect immunofluorescence microscopy further indicated that VNUT immunoreactivity occurred in human neutrophils and exhibited a punctate distribution, whereas control staining with preabsorbed antibodies exhibited only background levels of staining (Fig. 4C). Furthermore, in agreement with our results in
mouse neutrophils, double-labeling immunofluorescence microscopy and digital image analysis (colocalization coefficients) indicated that VNUT immunoreactivity was colocalized with MMP-9 (M1:0.796, M2:0.639) (Fig. 4D). In contrast, this immunoreactivity was not colocalized with MPO (M1:0.191, M2:0.420), lactoferrin (M1:0.341, M2:0.354), or CD35 (M1:0.167, M2:0.407). Taken together, it can be suggested that VNUT is also localized in tertiary granules in human neutrophils.

**VNUT−/− mice and VNUT inhibitors result in impaired neutrophil migration**

Finally, we studied the role of VNUT-independent ATP release on neutrophil function. To assess neutrophil migration *in vitro* using cells isolated from the bone marrow of wild-type and *VNUT−/−* mice, we performed transwell assays consisting of upper wells with neutrophils and lower wells with 100 nM W-peptide separated by a filter with 3-µm pores. Consistent with the results of ATP release, we found that the W-peptide-induced migration of neutrophils from *VNUT−/−* mice was decreased by 54% compared to that of cells from wild-type mice (Fig. 5A). No effect on exogenous ATP was observed in the W-peptide-induced migration of neutrophils from *VNUT−/−* mice, whereas the addition of adenosine or IB-MECA, an A3 receptor agonist, increased the migration of neutrophils from *VNUT−/−* mice (Fig. 5A, B). The addition of 1 µM clodronate also inhibited W-peptide-induced neutrophil migration by 41% (Fig. 5C). To confirm the importance of VNUT for neutrophil migration *in vivo*, we assessed cell recruitment to the hindpaw of wild-type control and *VNUT−/−* mice after a subcutaneous injection of 20 µL 1 mg/mL complete Freund’s adjuvant (CFA). As shown in Fig. 5D and E, CFA-induced inflammatory and neutrophil recruitment was observed in the hindpaw of wild-type mice, but this was reduced in *VNUT−/−* mice. Compared to neutrophil numbers in wild-type mice, those in *VNUT−/−* mice were decreased by 73%, 46%, 38%, and 45% at 6, 12, 24, and 48 h, respectively, after injecting CFA (Fig. 5E). These results indicated that VNUT is important for neutrophil migration both *in vitro* and *in vivo*.

**Discussion**

How and where ATP is stored in neutrophil granules and how vesicular ATP release is regulated during neutrophil migration are unresolved questions. In the present study, we found that VNUT is involved in the vesicular release of ATP from neutrophils. VNUT is expressed and associated with granules in neutrophils. *In vitro*, neutrophils were found to release ATP and migrate upon W-peptide stimulation, which is abolished by a VNUT inhibitor and in *VNUT−/−* mice. *In vivo*, CFA induced neutrophil migration into the footpad of wild-type mice, whereas this migration was reduced in *VNUT−/−* mice. These observations
indicated that VNUT is involved in ATP release and migration in neutrophils.

Neutrophils possess several distinct granule subsets, namely azurophil granules, specific granules, and tertiary granules, and secretory vesicles (23). These granules and vesicles are secretory organelles that are classified based on their size, contents, and other parameters. It remains unknown whether ATP is stored in neutrophil granules and subsequently released through granule exocytosis. Our immunohistochemical analyses indicated that VNUT is co-localized with MMP-9, indicating that VNUT-containing granules are tertiary granules that contain V-ATPase as the driving force of ATP transport ($\Delta\psi$) (26). These granules also contain matrix-degrading enzymes (such as MMPs) and membrane receptors (23). Exocytosis of these enzymes from tertiary granules is essential for interstitial structures during neutrophil migration. Upon stimulation, the formyl-Met-Leu-Phe (fMLP) receptor, which is localized to tertiary granules, translocates to the plasma membrane through exocytosis (27). These results suggested that the translocation of fMLP receptor to the leading edge of the plasma membrane coincides with vesicular ATP release and promotes more efficient migration in neutrophils. Identification of VNUT-containing granules will be helpful for understanding the mechanisms that regulate ATP release and migration in neutrophils.

We found that W-peptide-stimulated ATP release is abolished in neutrophils derived from $VNUT^{-/-}$ mice, whereas non-stimulated cells constantly release ATP (Fig. 3B). Previous inhibitor-based studies have shown that ATP release is also dependent on pannexin 1 (PANX1) hemichannels and connexin 43 (Cx43) from leukotriene B4 (LTB4)-stimulated neutrophils, as well as vesicular release (8-12). However, we previously found that disruption of ATP release using inhibitors of hemichannels also inhibits VNUT (28). In addition, the amount of ATP released varies according to the stimulation intensity (13), suggesting that neutrophils have multiple ATP release pathways. Neutrophils might use multiple ATP release pathways depending on the type and concentration of chemoattractant. Therefore, the characterization of ATP release pathways might help to elucidate how VNUT-mediated and channel-mediated ATP release is involved in the pathogenic mechanisms of inflammatory diseases.

In the present study, it was determined that A23187-stimulated MMP-9 release from $VNUT^{-/-}$ mice was the same as that from wild-type mice (Fig. 3A). This suggests that VNUT is involved in the vesicular storage and release of ATP, but not granular accumulation or the release of other contents. On the other hand, W-peptide-stimulated MMP-9 release was reduced in neutrophils derived from $VNUT^{-/-}$ mice (Fig. 3B). Extracellular ATP caused an increase in intracellular $Ca^{2+}$ through the P2Y2
receptor, leading to degranuation (11). These results suggested that MMP-9 release is dependent on physiological stimuli, and is positively controlled by VNUT-mediated ATP release. A similar mechanism of autocrine regulation of ATP has been reported in connection with the release of adrenaline from chromaffin cells (19). VNUT might be a key molecule for autocrine regulation of ATP in neutrophils. Other possibility of this observation is that VNUT regulates tertiary granules secretion independent of ATP secretion under physiological stimulation. We also found that neutrophil migration was not rescued by the addition of ATP, but migration was rescued by adding adenosine and IB-MECA, an A3 agonist, in VNUT−/− cells in vitro. (Fig. 5A, B). Previous reports showed that A3 receptors promote neutrophil migration and P2Y2 receptors are involved in signal amplification, gradient sensing and cell motility (29). Our results suggest that exogenous ATP access P2Y2 receptors omnidirectionally, result in a loss of gradient sensing and polarity in neutrophils.

Recently, we identified a potent allosteric inhibitor of VNUT, clodronate (24). VNUT mediated-ATP transport activity is allosterically regulated by Cl−, and inhibited competitively and reversibly by keto acids such as acetoacetate and glyoxylate (22, 30). A previous study indicated that clodronate inhibits vesicular ATP release, and decreases the release of inflammatory mediators, thereby attenuating chronic inflammation (24). In the present study, we found that clodronate also inhibits vesicular ATP release from neutrophils, and reduces neutrophil migration, indicating a novel mechanism through which VNUT mediates its anti-inflammatory effects (Fig. 5C).

Neutrophils migrate to the sites of pathogenic invasion or inflammation and release cytokines in response to a concentration gradient of chemoattractants from microbial pathogens or injured cells. Interestingly, purinergic chemical transmission is involved not only in migration, but also in phagocytosis and the production and release of inflammatory mediators (31, 32). It has been reported that adenosine, which is a product of ATP degradation, affects phagocytosis via the A1 or A2A receptor, and modulates inflammatory mediators such as tumor necrosis factor (TNF) via the A2A receptor (31, 32). Neutrophils generate reactive oxygen species (ROS) to kill pathogens targeted for phagocytosis; however, the release of such compounds from over-activated neutrophils can also damage host tissues in inflammatory diseases. Purinergic chemical transmission also affects the generation of ROS in neutrophils (31, 33). VNUT can be a major source of extracellular ATP and is involved in neutrophil activation; it might also be the cause of some inflammatory diseases.

It is noteworthy that VNUT could be a good target for the treatment of chronic inflammatory diseases such as chronic
obstructive pulmonary disease and cystic fibrosis. In these diseases, controlling of neutrophil function including recruitment would be an efficient therapy to treat inflammation (34). Clodronate is a specific inhibitor of VNUT, and is currently used as an anti-osteoporotic drug (24, 35). Our previous study on the biochemistry and pharmacology of clodronate showed that the compound has an anti-inflammatory effect. Therefore, our findings support the clinical significance of clodronate for the treatment of acute sepsis and chronic inflammatory diseases. Further pathological studies regarding the inhibitory effects of clodronate on neutrophil migration are currently in progress in our laboratories.

In conclusion, we demonstrated that VNUT is localized to tertiary granules and is responsible for vesicular ATP release from neutrophils, which is blocked by a clinically available VNUT inhibitor, clodronate. These results strongly suggest that VNUT represents the missing link in the ATP release pathway of neutrophils.

**Experimental procedures**

**Materials**

Nucleotides, dichloromethylenediphosphonic acid disodium salt (clodronate), CFA, 3,3′-diaminobenzidine (DAB), A23187, and DMSO were obtained from Sigma Aldrich (St. Louis, MO, USA). W-peptide (Trp-Lys-Tyr-Met-Val-Met-NH₂) was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Giemsa’s azur eosin methylene blue solution was obtained from Merck (Darmstadt, Germany). EGTA was obtained from Wako (Tokyo, Japan). IB-MECA was obtained from Abcam (Cambridge, UK). EGTA-tetraacetoxymethyl ester (AM) was obtained from AnaSpec Inc. (Fremont, CA, USA).

**Animals**

C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). VNUT⁻/⁻ mice were generated as previously described (19). All animal procedures and care were approved by the Institutional Animal Care and Use Committee, and were performed according to the guidelines of the Okayama University. For the in vivo experiment, 20 µL 1 mg/mL CFA was injected subcutaneously into the plantar region of the left hindpaw of mice (males, 9–13 weeks old) using a 100-µL Hamilton microsyringe with a 27-gauge needle.

**Isolation of mouse PMNs from bone marrow and of human PMNs from peripheral blood**

Neutrophils were isolated from the bone marrow of mice as previously described (36). Briefly, tibias and femurs were removed and stripped of their muscles. The bone marrow was flushed using neutrophil isolation buffer containing 0.4% sodium citrate in Hanks’ balanced salt solution (HBSS). Neutrophils were separated by
density centrifugation using a Percoll (62% v/v in neutrophil isolation buffer, GE Healthcare, Little Chalfont, UK) gradient at 1,000 × g for 30 min at 4 °C. PMNs were recovered as a pellet at the bottom of the 62% Percoll gradient, and washed with phosphate-buffered saline (PBS). Erythrocytes were removed by hypotonic lysis. Human PMNs were isolated from peripheral blood samples using Histopaque-1119 and Histopaque-1077 (density: 1.119 g/mL and 1.077 g/mL, respectively, Sigma Aldrich) double-density gradient centrifugation. Peripheral blood was obtained from healthy volunteer donors in compliance with the guidelines of the ethics committee of Okayama University, (permit number: 1388). Blood samples (36 mL) were mixed with 4 mL 3.8% sodium citrate immediately after drawing, layered on a Histopaque gradient and centrifuged at 700 × g for 30 min at room temperature. The PMN fraction was collected and washed with PBS. The purity of mouse and human neutrophil populations was >90% and >93%, respectively, and cell viability was >97% and >93%, respectively, as determined by Giemsa and Trypan blue staining. The specimens were observed using an Olympus IX83 microscope and Olympus DP80 camera.

**RT-PCR analysis**

An RNeasy mini kit (Qiagen, Hilden, Germany) was used for RNA extraction from neutrophils, according to the manufacturer’s instructions. Complementary DNA (cDNA) was generated using the PrimeScript RT Master Mix (Takara Bio, Shiga, Japan) and total RNA as the template. RT-PCR was performed as previously described (37). For PCR amplification, cDNA was added to the reaction buffer containing 0.2 mM total dNTP (50.0 µM each dNTP), 10 pmol primers, and 1.0 U Ex Taq (Takara Bio). The reaction was conducted over 35 cycles as follows: for human neutrophils: denaturation at 95 °C for 30 s, annealing at 62 °C for 35 s, and extension at 72 °C for 20 s; for mouse neutrophils: denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. The amplification products were analyzed using a 10% polyacrylamide gel electrophoresis.

qPCR was carried out with 400 nM specific forward and reverse primers and SYBR Premix Ex Taq II (Takara Bio). qPCR conditions included an initial denaturation step of 95 °C for 30 s, followed by 35 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. The primer sets used were as follows: for human VNUT (115 bp):

5’-TGGTCTTTGCATCAGCCTCCATCGG-3’ and 5’-GTGTTGGCCACACAAACAGAAAGC-3’;

and for mouse VNUT (523 bp):

5’-GGTCTGCTCCAAGGTGTCTAC-3’ and 5’-GACTGATAAGGCGGTCGGAG-3’; for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH; 115 bp):

5’-GGTGAAGGTCGGAGTCAACGG-3’ and
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5’-GGTTGAGGTCAATGAAGGGGTC-3’; for mouse G3PDH (150 bp): 5’-TGTGTCCGTCGTGGATCTG-3’ and 5’-TTGCTGTTGAAGTCGCAGG-3’.

Western blot analysis

For immunoblotting, mouse neutrophils were suspended in 25 mL 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose, 5 mM EDTA, 10 µg/mL pepstatin A, and 10 µg/mL leupeptin, and then pressurized with N2 for 20 min at 350 psi with constant stirring at 4 °C (38). The cavitate was centrifuged at 500 × g (mouse neutrophils) for 10 min. The resulting supernatant was centrifuged at 250,000 × g for 1 h. The pellet (membrane fraction) was suspended in the same buffer, and then denatured with sample buffer containing 1% sodium dodecyl sulfate and 10% β-mercaptoethanol. Polyacrylamide gel electrophoresis and western blotting were performed as described previously (39). Immunoreactivity was visualized by enhanced chemiluminescence (ECL) amplification according to the manufacturer (GE Healthcare). Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

Immunofluorescence microscopy

Cells on poly-L-lysine-coated coverslips were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were washed with PBS and incubated with the same buffer containing 0.1% triton X-100 for 20 min, and then further incubated with 2% goat serum and 0.5% BSA in the same buffer for 30 min at room temperature. Primary antibody treatment was performed using antibodies specific for VNUT (1:100), MPO (1:100, 1:50), lactoferrin (1:100, 1:50), MMP-9 (1:50), and CD35 (1:50) in PBS containing 0.5% BSA for 1 h at room temperature. The secondary antibodies used were Alexa Fluor 488-labeled anti-rabbit IgG (1:500) and Alexa Fluor568-labeled anti-mouse IgG (1:1000) (Molecular Probes, Eugene, OR, USA). The specimens were observed using an Olympus FV300 confocal laser microscope. Quantitative analysis of colocalization was performed with M1 and M2 coefficients using the JACoP plugin in ImageJ.

Antibodies

The specificity of rabbit polyclonal antibodies against human and mouse VNUT and V-ATPase prepared in-house were determined as described previously (14, 39). The following antibodies were obtained commercially: anti-human MPO mouse monoclonal (MS-1439; NeoMarkers, Portsmouth, NH, USA), anti-mouse MPO mouse monoclonal (GTX16886; GeneTex, Irvine, CA, USA), anti-lactoferrin mouse monoclonal (ab166803; Abcam), anti-MMP-9 mouse monoclonal (ab119906; Abcam), anti-CD35 mouse monoclonal (MA5-13122; Thermo Fisher Scientific), anti-FPRL1 rabbit polyclonal (bs-3654R; Bioss Antibodies, Woburn, MA,
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USA), anti-GM130 mouse monoclonal (610822; BD Biosciences, San Jose, CA, USA), anti-EEA1 mouse monoclonal (610456; BD Biosciences), anti-LAMP1 mouse monoclonal (SMC-140C/D; StressMarq Biosciences, Victoria, Canada), anti-PDI mouse monoclonal (ab2792; Abcam), anti-VAMP2 mouse monoclonal (104211; Synaptic Systems, Göttingen, Germany), and anti-mouse Ly-6G/Ly-6c (Gr-1) rat monoclonal (MAB1037; R&D Systems, Minneapolis, MN, USA).

ATP release assay
Mouse neutrophils (1.0 × 10⁶ cells) were preincubated in Krebs-Ringer solution consisting of 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 26 mM NaHCO₃, 2.4 mM CaCl₂, 10 mM D-glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-Tris (pH 7.4), and 0.2% BSA in the absence or presence of inhibitors for 30 min at 37 °C. The cells were stimulated through the addition of 5 µM A23187 (Ca²⁺ ionophore) or 100 nM W-peptide at 37 °C. After aliquots were taken at the indicated times, the amount of ATP was measured using an ATP Bioluminescent Assay Kit (Sigma Aldrich) based on a luciferin-luciferase reaction, and with Varioskan Flash Microplate Readers (Thermo Scientific).

MPO assay and MMP-9 assay
MPO and MMP-9 supernatants were analyzed by an enzyme-linked immunosorbent assay (ELISA) using the mouse MPO ELISA kit (Abcam) and the mouse total MMP-9 quantikine ELISA kit (R&D Systems), according to the manufacturer’s instructions.

Transwell migration assay
Migration assays using a transwell system were performed with a 96-well Multi-Screen-MIC plate (Millipore, Billerica, MA, USA) with a pore size of 3.0 µm, as described previously (3). A 100-µL suspension of 1 × 10⁶ cells in HBSS, with or without inhibitors, with nucleotides or agonists at the indicated concentrations, was added to each well of the upper filter plate. Chemoattractants in HBSS (150 µL) were added to each of the lower wells. After incubation at 37 °C for 50 min, the upper plate was removed and cells in the lower plate were counted.

Assessment of neutrophil migration into footpads
CFA-treated footpads were decalcified with 19% EDTA, fixed in 4% paraformaldehyde in PBS, and cut into 10-µm-thick sections. Immunohistochemical analysis was performed using the horseradish peroxidase-DAB method, as described previously (37). In brief, samples were quenched of endogenous peroxidase activity with 0.3% H₂O₂ and incubated in PBS containing 1.5% goat serum for 30 min. The samples were then incubated with anti-mouse Gr-1 antibodies (1:500) in PBS containing 0.1% BSA and 0.05% Tween 20 for 1 h at room
temperature. Samples were washed four times with PBS containing 0.05% Tween 20, and then incubated with biotinylated-labeled anti-rat IgG (1:200; Vector Laboratories, Burlingame, CA, USA) as the secondary antibody for 30 min at room temperature. Samples were washed four times with PBS containing 0.05% Tween 20, incubated with Vectastain ABC reagent (Vector Laboratories) for 30 min, and then incubated with a peroxidase substrate solution consisting of 0.02% DAB and 0.005% H$_2$O$_2$. Finally, samples were mounted with Mount-Quick (Daido Sangyo, Tokyo, Japan) and observed using a BZ-X700 microscope (Keyence, Osaka, Japan). Stained and migrated neutrophils in the footpads were quantified using BZ-X Analyzer software (Keyence).

Data analysis
All numerical values are shown as the mean ± standard error of the mean (SEM) unless otherwise specified. Statistical significance was determined by performing a two-tailed paired Student's $t$ test, or Dunnett's test for multiple comparisons after analysis of variance (ANOVA). These tests were performed using GraphPad Prism version 6 software (GraphPad Software, La Jolla, CA, USA). Differences were considered significant at $p < 0.05$.

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Conflict of interest
The authors declare that they have no conflicting financial interests.

Author contributions
Y.H. performed the majority of the experiments, designed the experiments, analyzed the data, and wrote the paper. Y.K. performed and designed the experiments. T.M., H.O., Y.M., and M.H. designed the experiments, analyzed the data, and wrote the paper.
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Figure Legends

Figure 1. Expression of vesicular nucleotide transporter (VNUT) in mouse neutrophils.
A, neutrophils from the bone marrow of wild-type (WT) and VNUT−/− (KO) mice were stained with a Giemsa stain. Segmented nuclei were stained purple. Bar, 10 µm. B, reverse transcriptase PCR (RT-PCR) analysis was performed using total RNA isolated from bone marrow-derived neutrophils of WT and KO mice (523 bp) after RT reaction (+RT) and without RT reaction (-RT). The PCR product from VNUT cDNA was shown as a positive control. The expression of mouse glyceraldehyde-3-phosphate dehydrogenase (mG3PDH) was also shown for internal RNA quality control (150 bp). C, western blot of bone marrow-derived neutrophil membrane vesicles (100 µg) prepared from wild-type and VNUT+/− mice and probed using anti-VNUT antibody. The position of VNUT (70 kDa) is marked with an arrow. The expressions of V-ATPase subunit A, formyl peptide receptor-like 1 (FPRL1), and vesicle associated membrane protein 2 (VAMP2) are also shown. D, Indirect immunofluorescence microscopy revealed that VNUT is expressed in wild-type mouse bone marrow-derived neutrophils. No VNUT immunoreactivity was observed in neutrophils from VNUT−/− mice. Inset, background signal with pre-absorbed anti-mouse VNUT antibody. Bars, 10 µm.

Figure 2. Localization of vesicular nucleotide transporter (VNUT) in mouse neutrophils.
A–B, mouse neutrophils derived from bone marrow were double-immunostained with antibodies against VNUT (green, left), granule markers (myeloperoxidase (MPO), lactoferrin, matrix metalloproteinase (MMP)-9, and CD35) (A); GM130, endosome antigen 1 (EEA1), lysosomal-associated membrane protein 1 (LAMP1), protein disulfide isomerase (PDI), and vesicle associated membrane protein 2 (VAMP2) (B) (red, middle). Merged images (right) are also shown. Images of neutrophils from VNUT−/− mice which immunostained with antibodies of granule markers also shown. Areas surrounded by dotted lines are enlarged in insets. Arrowheads, merged regions. Bars, 10 µm. C, Summary of digital image analysis (colocalization coefficients) in A–B. M1 (upper) and M2 (lower) were shown. Data are shown as the mean ± SEM. n = 3.

Figure 3. Vesicular nucleotide transporter (VNUT) is involved in ATP release from mouse neutrophils.
Data are shown as the mean ± SEM. N.D., not detected. N.S., not significant. **, p < 0.01. A–B, the release of ATP (left), myeloperoxidase (MPO) (middle), and matrix metalloproteinase-9 (MMP-9) (right) from bone marrow-derived neutrophils from wild-type (open bars) and VNUT+/− mice (filled bars) was assessed 3 min (ATP) or 20 min (MPO and MMP-9) after the addition of (A) 5 µM A23187
and (B) 100 nM W-peptide. n = 4–10. C, effects of 0.1 μM or 1 μM clodronate on A23187-induced ATP release from mouse neutrophils. D, temperature dependence for A23187-induced ATP release. n = 8–9. E, Calcium dependency for A23187-induced ATP release. Mouse neutrophils were incubated in the presence or absence of 1 mM EGTA (left) and 50 μM EGTA-AM (right). n = 7–10.

Figure 4. Expression and localization of VNUT in human neutrophils.
A, neutrophils from human peripheral blood samples were stained with Giemsa stain. Segmented nuclei were stained purple. Bar, 10 μm. B, reverse transcriptase PCR (RT-PCR) analysis was performed using total RNA isolated from neutrophils of human peripheral blood (115 bp) after RT reaction (+RT) and without RT reaction (-RT). The PCR product from VNUT cDNA is shown as a positive control. Expression of the human glyceraldehyde-3-phosphate dehydrogenase (hG3PDH) gene was also shown as an internal RNA quality control (115 bp). C, indirect immunofluorescence microscopy revealed that VNUT was expressed in human neutrophils. Inset: background signal with preabsorbed anti-human VNUT antibody. Bars, 10 μm. D, human neutrophils were double-immunostained with antibodies against human VNUT (green, left), granule markers (myeloperoxidase (MPO), lactoferrin, matrix metalloproteinase (MMP)-9, and CD35). Merged images (right) are also shown. Areas surrounded by dotted lines are enlarged in insets. Arrowheads, merged regions. Bars, 10 μm. E, Summary of digital image analysis (colocalization coefficients) in D. M1 (upper) and M2 (lower) were shown. Data are shown as the mean ± SEM. n = 3.

Figure 5. Vesicular nucleotide transporter (VNUT) is involved in mouse neutrophil migration.
All data are shown as the mean ± SEM. *, p < 0.05. **, p < 0.01. N.S., not significant. A-B, transwell migration assays, toward 100 nM W-peptide, were performed with neutrophils from wild-type (open bars) and VNUT-/- mice (filled bars). Migration was expressed as a percent of the response to W-peptide in wild-type mice. Effects of (A) 100 μM ATP, (B) 1 μM adenosine, and 1 μM IB-MECA on W-peptide-induced migration of wild-type and VNUT-/- mice neutrophils. n = 4 and n= 4, respectively. C, transwell migration assays were performed in the presence of 1 μM clodronate. n = 8–10. D, complete Freund’s adjuvant (CFA)-induced neutrophil recruitment into hindpaws of wild-type and VNUT-/- mice. The typical dorsa of hindpaw sections from wild-type and VNUT-/- mice at 24 h and 48 h after subcutaneous injection of CFA are shown. Photographs were analyzed by BZ-X Analyzer software for counting. The original image of Gr-1 immunohistochemistry staining (upper) and markup image after analysis with red (lower) are shown. Bars, 10 μm. E, neutrophil migration into the dorsa of wild-type (open squares) and VNUT-/- mouse (filled squares) hindpaws 3, 6, 12, 24,
and 48 h after subcutaneous injection of CFA was assessed. n = 3–6 animals (two sections per animal) for each group.
FIGURE 1. Expression of VNUT in mouse neutrophils
FIGURE 2. Localization of VNUT in mouse neutrophils.
FIGURE 3. VNUT is involved in release of ATP from mouse neutrophils.
FIGURE 4. Expression and Localization of Vnut in human neutrophils
FIGURE 5. VNUT is involved in migration of mouse neutrophil.
Vesicular nucleotide transporter mediates ATP release and migration in neutrophils
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