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Novel evidence that the P2X1 purinergic receptor–Nlrp3 inflammasome axis orchestrates optimal trafficking of hematopoietic stem progenitors cells

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Abstract

Introduction. Our previous research demonstrated P2X purinergic receptors as important extracellular adenosine triphosphate (eATP) sensing receptors promoting the trafficking of hematopoietic stem progenitor cells (HSPCs). Accordingly, mice deficient in expression of P2X4 and P2X7 receptors turned out to mobilize poorly HSPCs. Similarly, defective expression of these receptors on transplanted HSPCs or in the bone marrow (BM) microenvironment of graft recipient mice led to defective homing, engraftment, and delayed hematopoietic reconstitution. This correlated with decreased activation of intracellular pattern recognition receptor Nlrp3 inflammasome. The P2X receptor family consists of seven purinergic receptors (P2X1-7) and we noticed that in addition to P2X4 and P2X7, HSPCs also highly express rapidly signaling the P2X1 receptor. Therefore, we asked if P2X1 receptor is also involved in HSPCs trafficking.

Material and methods. We employed *in vitro* and *in vivo* murine models to study the role of P2X1 receptor blocked on HSPCs or bone marrow microenvironment cells by specific small molecular inhibitor NF499. First, we performed *in vitro* cell migration assays of bone marrow mononuclear cells (BMMNCs) isolated from normal mice that were exposed to NF499 and compared them to unexposed control cells. Next, in experiments *in vivo* we mobilized mice exposed to NF499 with G-CSF or AMD3100 and compared mobilization to control unexposed animals. Flow cytometry was employed to identify cell populations and clonogenic assays to enumerate the number of mobilized clonogenic progenitors. Similarly, in homing and engraftment experiments BMMNCs or recipient mice were exposed to NF499 and we evaluated homing and engraftment of transplanted cells by enumerating the number of cells labeled with fluorochromes in recipient mice BM and by evaluating the number of clonogenic progenitors in BM and spleen 24 hours and 12 days after transplantation. We also evaluated the potential involvement of Nlrp3 inflammasome in P2X1 receptor-mediated HSPCs trafficking.

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Results. We report that the functional P2X1 receptor is highly expressed on murine and human HSPCs. We could demonstrate that the P2X1 receptor promotes the trafficking of murine cells in Nlrp3 inflammasome--dependent manner. Mice after exposure to P2X1 receptor inhibitor poorly mobilized HSPCs from the bone marrow into the peripheral blood. Mice transplanted with BMNNCs exposed to NF499 or recipient mice pretreated with this inhibitor demonstrated defective homing and engraftment as compared to control animals transplanted with cells not exposed to P2X1 inhibitor. Similar effects were noticed for control recipient mice that were not exposed to NF499.

Conclusions. This study demonstrates for the first time the novel role of the P2X1 receptor in HSPCs trafficking in the mouse. Furthermore, it supports an important role of purinergic signaling engaging its downstream target Nlrp3 inflammasome in the mobilization, homing and engraftment of HSPCs. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 3, 280–290)*

Keywords: mouse; extracellular ATP; P2X1 receptor; NF499; Nlrp3 inflammasome; stem cell mobilization; homing and engraftment

Introduction

P2X1 receptor has been described as highly expressed at smooth muscle cells and neurons [1, 2]. For example, in response to eATP stimulation P2X1 mediates synaptic transmission between neurons and from neurons to smooth muscle and is responsible for sympathetic vasoconstriction in small arteries [2–4]. The P2X1 receptor is also expressed by blood platelets, where it contributes to their activation and thrombosis [5, 6] and by neutrophils and lymphocytes to promote chemotactic migration of these cells [7]. Based on the high sensitivity of this receptor to eATP with EC_{50} value ranging between 0.05 and 1 mM [2, 8, 9] and its positive effect on the migration of granulocytes and lymphocytes [7, 9] we decided to explore its potential role in the migration of normal HSPCs.

Evidence accumulated that purinergic signaling plays an important role in regulating normal and malignant hematopoiesis [10–18]. In particular, adenosine triphosphate (ATP) — a known intracellular energy transfer molecule, if secreted into extracellular space by pannexin-1 channel [19] or as part of exosome cargo [20], activates as extracellular ATP (eATP) both P2X and P2Y receptors expressed on the surface of hematopoietic cells [10, 11]. While P2X receptors are activated exclusively by eATP [21, 22], P2Y receptors in addition to eATP respond to other extracellular nucleotides (*e.g.*, eATP degradation products such as eADP and eAMP or other triphosphate nucleotides such as extracellular UTP) [23].

Purinergic ligand-gated ion channel P2X receptors are endowed with relatively high calcium permeability in response to eATP and consist of seven members (P2X1-P2X7) [12, 21, 22, 24]. The previous work from our laboratory demonstrated that P2X4$^{-/-}$ and P2X7$^{-/-}$ mice turned out to mobilize poorly HSPCs and HSPCs from P2X4$^{-/-}$ and P2X7$^{-/-}$ mice, display defective homing and engraftment after transplantation of bone marrow mononuclear cells (BMMNCs) [10, 11, 25]. Moreover, recipient P2X4$^{-/-}$ and P2X7$^{-/-}$ mice engraft worse with transplanted normal BMMNCs [10, 11]. In the current work, we confirmed that murine and human HSPCs highly express functional P2X1 receptor, that as reported, is the most sensitive member of the P2X receptor family in response to stimulation by eATP [2, 8, 9]. Moreover, we demonstrate for the first time that it is involved *in vitro* and *in vivo* in eATP--mediated trafficking of these cells. Furthermore, we noticed that P2X1-mediated effects on mobilization, homing, and engraftment of HSPCs are the result of activation of intracellular pattern recognition receptor known as Nlrp3 inflammasome [26, 27].

Materials and methods

Animals. Pathogen-free 6-8 weeks old C57BL/6J female mice (WT) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at least 2 weeks before experiments. Animal studies were approved by the Medical University of Warsaw (Warsaw, Poland) (WAW2/125/2019, WAW2/124/2019 and WAW2/119/2019).

Human umbilical cord blood. Clinical-grade umbilical cord blood (UCB) research units were obtained from the Cleveland Cord Blood Center (Cleveland, OH, USA). This study was performed in accordance with the guidelines and approval of the Institutional Review Board at the University of Louisville School of Medicine (Louisville, KY, USA).

Drug administration in animals. For mobilization studies, mice were injected subcutaneously with Granulocyte-Colony Stimulating Factor (G-CSF, 150 μg/kg daily; Amgen, Thousand Oaks, CA, USA) for 3 days or with a single dose of AMD3100 (SigmaAldrich, St. Louis, MO, USA) [5 mg/kg, intraperitoneally (*i.p.*)]. Some mice also received injections of P2X1 inhibitor — NF 449 (Tocris, Minneapolis, MN, USA) (10 mg/mouse/dose, *i.p.*, prior and during mobilization, 4 days

total). For transplantation studies (Homing, Early engraftment, Recovery) mice were injected with NF 449, for Homing — 5 doses (10 mg/kg), once a day, for Engraftment 5 doses (10 mg/kg), once a day, for Recovery studies 8 doses (10 mg/kg), every 2nd day.

Isolation of peripheral blood and bone marrow. Peripheral blood (PB) and bone marrow (BM) samples were collected from all groups of mice. Briefly, a 50 μL of PB was withdrawn from the retro-orbital plexus or tail vein into EDTA-coated microvette tubes (Sarstedt Inc., Newton, NC, USA) for hematologic analysis. The blood samples were run on an Exigo hematology analyzer (Boule Diagnostics, Spanga, Sweden) within 2 h of collection. PB was also collected after opening the abdomen under inhalation anesthesia (isoflurane 3% for induction; 1.5% for maintenance) from the posterior vena cava (with a 25-gauge needle and 1-mL syringe containing 250 U heparin). The red blood cells (RBCs) were lysed by hypotonic lysis buffer (BD Biosciences, San Jose, CA, USA) and PB mononuclear cells (PBMNCs) were obtained by centrifugation. BM mononuclear cells (BMMNCs) were also flushed from tibias and femurs with cold phosphate-buffered saline (PBS). RBCs present in BM were lysed, washed, and BMMNCs were resuspended in PBS or RPMI-1640 medium (Corning, New York, NY, USA) containing 2% fetal bovine serum (FBS, Corning) as necessary.

Migration assay. The RPMI-1640 medium containing 0.5% bovine serum albumin (BSA, SigmaAldrich) was used for the transwell migration assay. A 650-μL volume of medium with or without stromal-derived factor 1 (SDF-1, 5 ng/mL, PeproTech, Rocky Hill, NJ, USA), sphingosine-1-phosphate (S1P, 0.1 μM), ceramide-1-phosphate (C1P, 100 μM) all from SigmaAldrich. An aliquot (1×10^6 cells per 100 μL) of murine BMMNCs exposed (or not, control) to NF 449 (10 μM for 1 h) was loaded onto the upper chamber separated by 5-μm pore size insert. The plate was incubated for 3 h at 37°C in a 5% CO_2 incubator. Following incubation, an aliquot of cells from the lower chamber was harvested, split, and scored by FACS analysis. Briefly, the cells were gated according to their forward-scatter (FSC) and side-scatter (SSC) parameters and counted during a 30-s acquisition at a high flow rate. The rest of the BMMNCs from the lower chamber were resuspended in a human methylcellulose base medium provided by the manufacturer (R&D Systems, Minneapolis, MN, USA), supplemented with murine granulocyte/monocyte stimulating factor (mGM-CSF, 25 ng/mL) and murine interleukin 3 (mIL-3, 10 ng/mL) for determining the number of colony-forming unit-granulocyte/monocyte (CFU-GM) colonies. Next, the cultures were incubated for 7 days at 37°C in a 5% CO_2 incubator. The CFU-GM colonies were then counted under an inverted microscope.

Mobilization analysis. Peripheral blood withdrawn from the retro-orbital plexus was used for white blood cells (WBCs) counts. The blood samples were analyzed in an Exigo hematology analyzer (Boule Diagnostics). PB blood isolated from vena cava was lysed, and PBMNCs were isolated as motioned above. PBMNCs were resuspended in RPMI-1640 medium containing 2% FBS. The PBMNCs were cultured on a human methylcellulose base medium (R&D Systems) supplemented with 25 ng/mL recombinant murine granulocyte/macrophage colony-stimulating factor (mGM-CSF, PeproTech, Rocky Hill, NY, USA) and 10 ng/mL recombinant murine interleukin 3 (mIL-3, PeproTech). Cells were incubated for 7 days (37°C, 95% humidity, and 5% CO_2). After 7 days of incubation, the CFU-GM colonies were scored using a simple inverted microscope (Olympus, Center Valley, PA, USA). In addition, PBMNCs were also stained for the analysis of Sca-1^+c-Kit^+Lin^- (SKL) cells using the following monoclonal antibodies: FITC–anti-CD117 (also known as c-Kit, clone 2B8; BioLegend, San Diego, CA, USA), PE–Cy5–anti-mouse Ly-6 A/E (also known as Sca-1, clone D7; eBioscience, San Diego, CA, USA), and anti-mouse lineage-marker antibodies. All these antibodies were purchased from BD Biosciences anti-CD45R (also known as B220, clone RA3-6B2), anti-Ter-119 (clone TER-119), anti-CD11b (clone M1/70), anti-T cell receptor β (clone H57-597), anti-Gr-1 (clone RB6-8C5), and anti-TCRγδ (clone GL3) conjugated with PE (BD Biosciences). Staining was performed in RPMI-1640 medium containing 2% FBS. All monoclonal antibodies were added at saturating concentrations, and the cells were incubated for 30 min on ice, washed twice, and analyzed using the BD FACSVerse instrument (BD Biosciences).

Transplantation of BMMNCs. The short-term transplant homing, early engraftment, and hematopoietic recovery analysis were performed in C57BL/6J mice irradiated with a lethal dose of γ-irradiation (1000 cGy). C57BL/6J recipient mice were irradiated 24 h before the transplantation procedure. Mice were divided into 2 experimental groups. The first experimental group was transplanted *i.v.* with BMMNCs incubated with P2X1 inhibitor NF 499 (1 *m*M for 1 h) or BMMNCs non-exposed to P2X1 inhibitor (control cells). The second group of BM recipient mice was injected before BMMNCs transplantation with NF 449 (10 mg/mouse for 5 consecutive days) or injected with PBS (control), and subsequently, mice were transplanted with BMMNCs. For the short-term homing experiment, BMMNCs (5×10^6/100 μL) from WT mice were labeled with PKH-67 green fluorescent dye (SigmaAldrich) and transplanted into the recipient mice. The next day, BMMNCs were obtained from femurs by the aid of Ficoll-Paque density-gradient centrifugation. The cells were divided into two aliquots, one of which was analyzed by flow cytometry for PKH-67-positive cells, and the other aliquot was resuspended in a human methylcellulose base medium supplemented with mGM-CSF (25 ng/mL) and mIL-3 (10 ng/mL). The cultures were incubated for 7 days at 37°C in a 5% CO_2 incubator. The CFU-GM colonies were then counted using an simple inverted microscope. Similarly, early engraftment and hematopoietic recovery analysis were performed in irradiated recipient mice using unlabeled BMMNCs transplants. Mice in the engraftment assay groups were transplanted with 1.5×10^5 (100 μL) BMMNCs. Twelve days after the transplantation, femora were flushed with RPMI-1640 medium containing L-glutamine (GE

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Healthcare, South Logan, UT, USA), then treated and cultured for 7 days at 37°C in a 5% CO2 incubator for CFU-GM colonies as described above. The spleen was removed from all mice and immediately fixed in Telesyniczky's solution. CFU-S colonies on the surface of the spleen were counted using a magnifying glass. To perform a hematopoietic recovery study, recipient mice were transplanted with 7.5×10^5 (in 100 μL PBS) BMMNCs. At the time intervals (day 0, 3, 7, 14, 21 and 28) a 50 μL of PB was drawn into EDTA-coated Microvette tubes (Sarstedt Inc., Newton, NC, USA) to count WBCs and platelets (PLT).

Adhesion assay. The adhesion assay was formed in a 96 well plate coated with fibronectin (10 μg/mL; SigmaAldrich) at 4°C for overnight and treated with 0.05% BSA blocking medium for 2 h at 37°C. BMMNCs from WT mice were suspended in RPMI-1640 medium with 0.5% BSA at a density of $5\times10^4/100\,\mu$L. The cells were maintained in a quiescence state for 3 h at 37°C and then were incubated in the presence or absence of NF449 (10 μM) for 1 h at 37°C. Cells were placed in the fibronectin pre-coated plates. After 5 min incubation at 37°C, non-adherent cells were washed with PBS, and the adherent cells were counted using an inverted microscope.

RQ-PCR. MNCs and HSCs from the BM of WT mice or human UCB were harvested and the total RNA was isolated using RNeasy Mini kit (Qiagen Inc., Hilden, Germany), and reverse transcribed with iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA). Evaluation of the target genes was then performed using iTaq Universal SYBR Green Supermix (Bio--Rad) and specific primers. The samples were run on Bio-Rad CFX96 qPCR Instruments detection system (Bio-Rad). The PCR cycling conditions were 95°C (30 s), 40 cycles at 95°C (5 s), and 60°C (30 s). A melting curve was created to emphasize the specificity of the primer and avoid the possibility of amplifying DNA contamination. Quantification was calculated using the comparative ΔCT method where mRNA levels of target receptors were normalized to the β-2microglobulin mRNA level. The receptors with the lowest mRNA level were set as controls (P2X5 for human MNCs and P2X2 for murine MNCs). According to melting point analysis, only one PCR product was amplified under these conditions. PCR products were visualized on 2% agarose gels. The following primer pairs were used for analysis:

Human:

P2X1 — F:GGTGGAGTGGTTGGCATCACCA; R:CTGAAGTTGAAGCCTGGGGAGAGATTTTTC; *P2X2* — F:GTGGAAATGAAAGACATCATCGTGCTGGT; R:AGGCCCAGGAGGAATCTGAATGGG; *P2X3* — F:GGTTTTCTTGCACGAGAAGGCTTACCA; R:TGAGGTGGCGTCACGTAATCAGACA; *P2X4* — F:GTGGGTGTTTGTGTGGGAAAAGGG; R:CTGAGCTGGTATCACATAATCCGCCA; *P2X5* — F:ACACCTCCCTGCAGAGTGCTGT; R:TTGGGGGTCACAATCAGGTTGGTGA; *P2X6* — F:AGGCCAGTGTGTGGTGTTCA; R:TCTCCACGGGGCACCAACTC; *P2X7* — F:CTTTGCTCTGGTGAGTGACAAGCTGT; R: CTGCAAAGGGAAGGTGTAGTCTGCG; *β-2microglobulin* — F:TGACTTTGTCACAGCCCAAGATA; R: AATGCGGCATCTTCAAACCT.

Murine:

P2X1 — F:CCAGACCTCAAGTGGCCTTATCAGC; R:CTGGGAAGACATAGTCAGCCACGTC; *P2X2* — F:GGCAACATTGCAAGCCAGAAGAGTG; R:CTGTGAAGTTCTCTCCTGCTTGCTCTAC; *P2X3* — F:TTCTTGCACGAGAAGGCCTACCAAG; R:CATCCATGACTCTGTTGGCATAGCGT; *P2X4* — F:CATTTATAATGCGCGGACGGATCCC; R:CTCCACTGCCATCTCCTGAAAGCTG; *P2X5* — F:GGCGGTGTGATAGGAATCCACATTGAAT; R:CCCAGAGGAGATGGATTGTGTGTGTTTG; *P2X6* — F:GTGGGCCCTCTTGGCCAAAAAAG; R:GTGATGGCTTCACAAAGTCAGCCAC; *P2X7* — F:CAGTATGAGACAAACAAAGTCACCCGGA; R:ATGTAGGAAAAGACGATCATGTGCAAGACC; *β-2microglobulin* — F:TGCTGCTTGTCTCACTGAC; R:GGATTTCAATGTGAGGCGG.

Glow assay to measure activation of Nlrp3 inflammasome. To measure the activity of caspase-1 in cells Caspase-Glo® 1 Inflammasome Assay (Promega, Madison, WI, USA) was employed, and analyses were performed according to the manufacturer's protocols. Samples of control and cells exposed to ATP or ATP + P2X1 inhibitor NF 449 (10 μM) were collected and 1 x 10^6 of BMMNCs were plated in 96 wells plates. Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent were added (100 μL/well), and luminescence was measured using a GloMax 9301 Multi Detection System after 90 minutes.

Statistical analysis. All results are presented as mean ± SEM. Statistical analysis of the data was done using Student's *t*-test for unpaired samples, with $p \leq 0.05$ considered significant.

Results

Murine and human HSPCs express functional P2X1 receptor

First, we phenotyped by employing PCR murine bone marrow and human umbilical cord blood (UCB)-derived mononuclear cells (MNCs) and HSPCs for expression of P2X receptors. Fig. 1 demonstrates that these cells express mRNA for all P2X receptors, including P2X1. To address if this receptor is functional on these cells, we performed a Transwell chemotactic assay of BM-derived MNCs to gradients of HSPC chemoattractants such as stromal-derived factor-1 (SDF-1), and to two bioactive phosphospingolipids — sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P). Fig. 2A shows that murine BM-derived HSPCs exposed to a small molecular specific inhibitor of P2X1, that is, NF449 displayed decreased migration from upper to lower chamber in Transwells, as measured by a number of migrating clonogenic CFU-GM progenitors. At the same time, as expected, inhibition of P2X1 by NF449 resulted in enhanced adhesion of these cells to fibronectin-covered plates (Fig. 2B). This

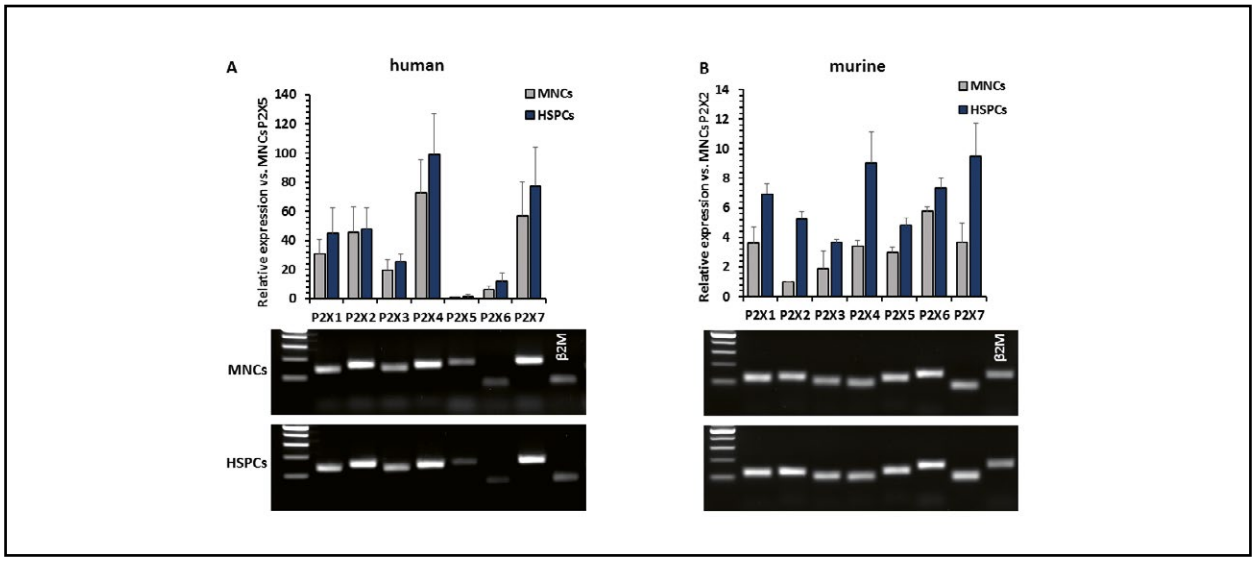

Figure 1. Expression of P2X receptors in human and murine mononuclear cells (MNCs) and hematopoietioc stem/progenitor cells (HSPCs). Expression of P2X receptors was evaluated in human umbilical cord blood-derived (**A**) and murine bone marrow-derived MNCs and HSPCs (**B**) were isolated as described in Methods. Isolated total RNA was reverse-transcribed and amplified with the use of iTaq Universal SYBR Green Supermix and specific primers. The quantification of the mRNA levels of the studied purinergic receptors was calculated using the comparative ΔCT method. The expression was evaluated in the material of three different cell isolations which were analyzed two times with similar results. Products of the reaction were visualized on 2% agarose gel. Representative gels pictures are shown.

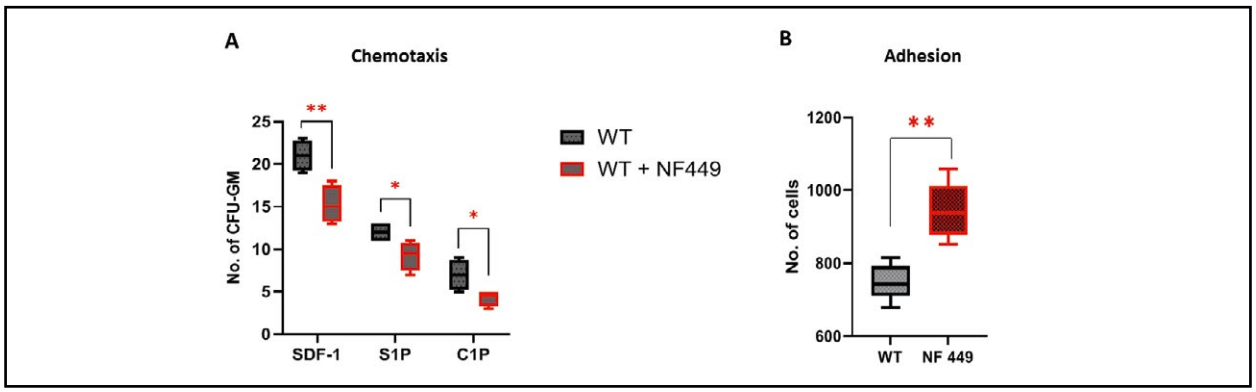

Figure 2. Impact of purinergic P2X1 receptor on chemotaxis and adhesion of murine bone marrow mononuclear cells (mBMMNCs). Cells were isolated from murine femora as described in Methods section. The chemotactic responsiveness of cells exposed to NF 449 (10 μM for 1 h) and run in chemotaxis assay against stromal-derived factor 1 (SDF-1, 5 ng/mL) or sphingosine-1-phosphate (S1P, 0.1 μM) or ceramide-1-phosphate (C1P, 100 μM) gradients in comparison with parental control cells. Results represent mean ± SD of three separate BMMNCs isolations *P < 0.05. **P < 0.01. Adhesion of mBMMNCs to fibronectin was described in Methods section. Data represent means ± SD. The number of adherent cells is indicated, and data from three separate experiments are pooled together. **P < 0.01. The numbers on Y axes refer to well area.

data revealed an important role of P2X1 in HSPCs migration.

The functional P2X1 receptor is crucial for optimal pharmacological mobilization of HSPCs

After we learned that the P2X1 receptor is important in regulating the migration of HSPCs, we asked if it can affect the mobilization of these cells *in vivo*. To address this question, mice were mobilized by G-CSF or AMD3100 in the absence or presence of NF449, a specific P2X1 inhibitor. Fig. 3A shows that mice exposed to NF449 mobilized poorly in response to G-CSF as compared to control animals — white blood cells (WBC), Sca-1$^+$c-kit$^+$lin$^-$ stem/progenitor cells (SKL), and clonogenic *in vitro* colony forming units of granulocyte/monocytes progenitors (CFU-GM). Similar effect we observed in mice mobilized with AMD3100 (Fig. 3B).

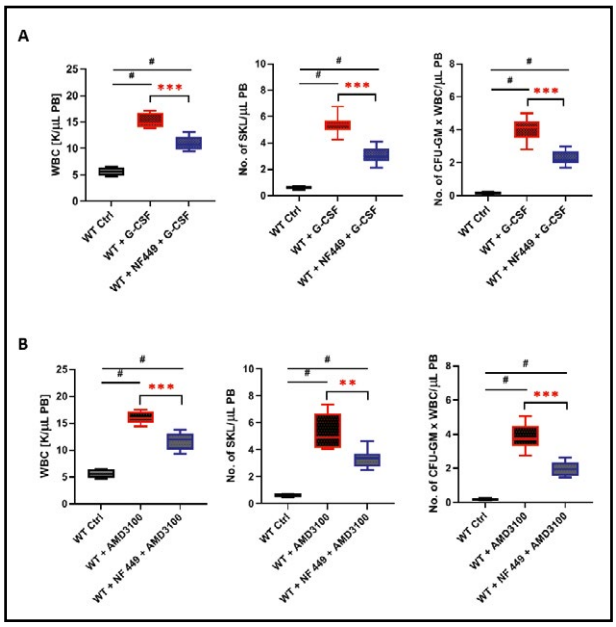

Figure 3. NF449 decreases mobilization of hematopoietic stem progenitor cells (HSPCs) induced by granulocyte-colony stimulating factor (G-CSF) or AMD3100. Mononuclear cells were isolated from peripheral blood (PB) of wild type (WT) mice after 3 days of G-CSF (150 mg/kg per day) mobilization (**A**) or after AMD3100-induced (5 mg/kg, single dose) mobilization (**B**), moreover, some mice received also NF449 (10 mg/mouse, for 4 days) prior and during mobilization. The numbers of WBCs, SKL (Sca-1+cKit+Lin−) cells, hematopoietic stem cells (HSCs) and CFU-GM clonogenic progenitors were evaluated. Results from two separate experiments (each with n = 4) are pooled together. The data are presented as means ± SE, and an unpaired Student's *t*-test was used for the statistical analysis of differences, where *P ≤ 0.05, ** P ≤ 0.001 ***, P ≤ 0.0001.

The P2X1 receptor is required for proper navigation of transplanted HSPCs to BM niches as well as primes BM microenvironment of mice conditioned for transplantation by lethal irradiation for proper homing and engraftment

Homing and engraftment of transplanted cells is an inverse phenomenon to mobilization. Therefore, we asked if the P2X1 receptor is also involved in the hematopoietic reconstitution of transplanted mice. First, we exposed *ex vivo* normal BMMNCs to NF449 before transplantation and transplanted these cells to mice conditioned for transplantation by lethal irradiation. Fig. 4 shows that BMMNCs exposed to P2X1 inhibitor had defective homing, early engraftment and slightly delayed recovery or peripheral blood counts after transplantation of BMMNCs. Early homing was evaluated by counting donor cells derived from $PKH76^{+}$ fluorochrome-labeled cells and clonogenic CFU-GM progenitors recovered from the bone marrow of transplanted mice at 24 hours after transplantation. Early engraftment was measured at day 12 by evaluating the number of donor-derived CFU-GM progenitors in BM by colony forming units in the spleen (CFU-S) assay in transplanted animals. Hematopoietic recovery of peripheral blood counts has been evaluated by counting recovery of WBC and platelets in the peripheral blood of transplanted animals. This data indicates that the P2X1 receptor is involved not only in the responsiveness of BM chemoattractants in vitro, as shown in Fig. 2A, but also in their navigation to BM niches *in vivo*.

Next, we asked if the P2X1 receptor expressed in the BM microenvironment of mice conditioned for transplantation by lethal irradiation is involved in facilitating homing and engraftment of transplanted normal BMMMNCs. To address this, normal mice were exposed or not before transplantation to P2X1 inhibitor. Fig. 5 shows that indeed P2X1 receptor is important in proper homing, early engraftment, and hematopoietic recovery of transplanted animals. These parameters were evaluated as described above.

The P2X1 receptor contributes to activation of Nlrp3 inflammasome required for optimal trafficking of HSPCs

On the basis of previously published studies [2, 3, 26, 27] we decided to check whether stimulation of BMMNCs by extracellular ATP may contribute to the activation of the Nlrp3 inflammasome. To address this issue, we stimulated murine BMMNC with eATP in the presence or absence of P2X1 inhibitor NF449. As expected, cells exposed to P2X1 inhibitor display a significant decrease in Nlrp3 activation in response to eATP as demonstrated by Caspase-Glo® 1 Inflammasome Assay (Fig. 6).

Discussion

The silent novel observation of this report is that the P2X1 purinergic receptor is involved in regulating migration, mobilization, homing, and engraftment of HSPCs. Moreover, activation of the P2X1 receptor in hematopoietic cells activated intracellular innate immunity pattern recognition receptor — Nlrp3 inflammasome, that as demonstrated by, is required for optimal trafficking of these cells during pharmacological mobilization out of bone marrow into peripheral blood and after hematopoietic transplantation from peripheral blood into bone marrow microenvironment [26, 27, 31–33]. Nlrp3 inflammasome plays also an important role in priming and facilitating the BM microenvironment of recipient mice for proper homing and engraftment of HSPCs [31, 32].

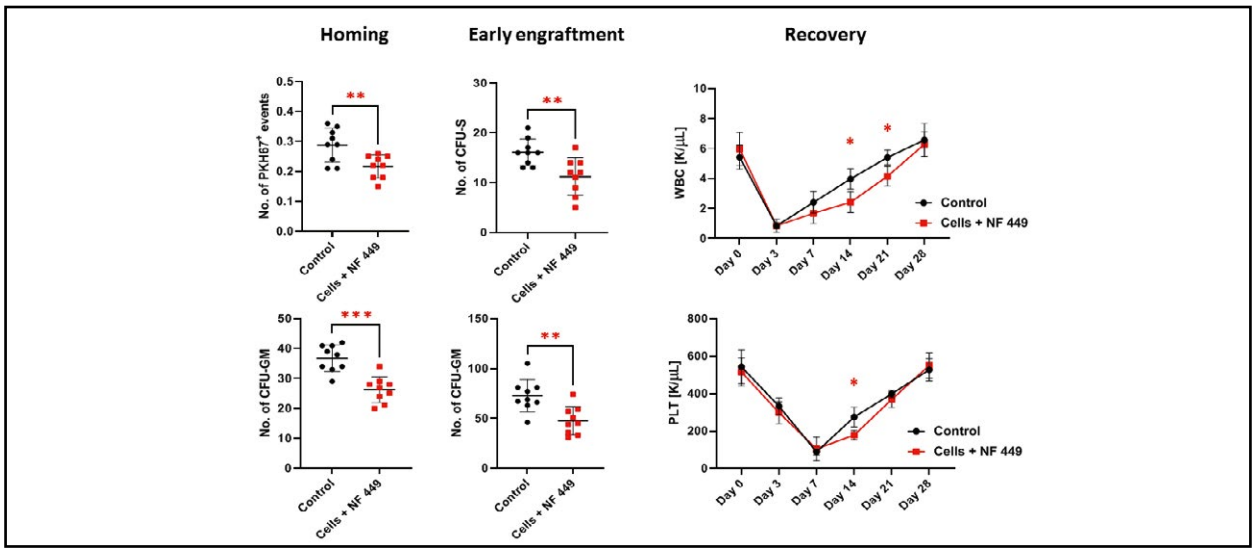

Figure 4. P2X1 blockage on BMMNC by NF 449 decreases homing and engraftment of these cells. **Left panel — Homing.** Lethally irradiated C57BL/6J mice were transplanted with bone marrow mononuclear cells (BMMNCs) from C57BL/6J mice alone or incubated with NF 449, which had been previously labeled with a PKH67 immunofluorescence cell linker as described in Methods section. Twenty-four hours after transplantation, the femoral BMMNCs were harvested, the number of PKH67$^+$ cells evaluated by FACS, and the CFU-GM clonogenic progenitors enumerated in an *in vitro* colony assay. **Middle panel — Early engraftment.** Lethally irradiated C57BL/6J mice were transplanted with BMMNCs alone or incubated with NF 449 from C57BL/6J, and 12 days after transplantation femoral BMMNCs were harvested and plated to count the number of CFU-GM colonies and the spleens removed for counting the number of CFU-S colonies. No colonies were formed in lethally irradiated, untransplanted mice (irradiation control). *P < 0.05. **Right panel — Recovery of peripheral blood counts**. Lethally irradiated C57BL/6J mice were transplanted with BMMNCs from C57BL/6J mice alone or incubated with NF 449. White blood cells (WBC) (left) and platelets (PLT) (right) were counted at intervals (at 0, 3, 7, 14, 21, and 28 days after transplantation). *P < 0.05, **P < 0.01.

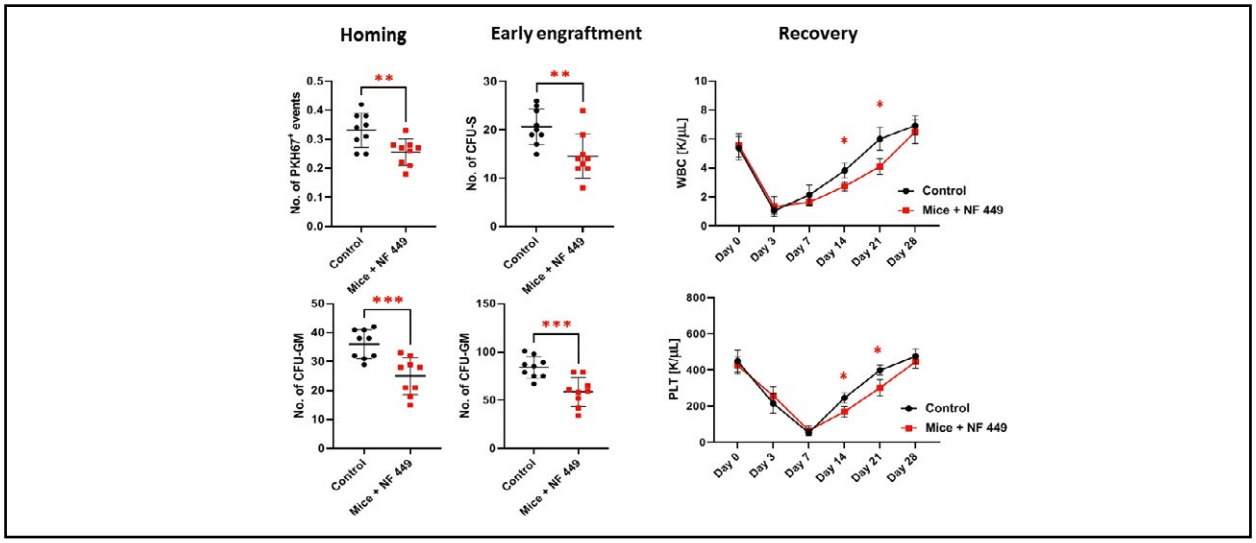

Figure 5. Mice exposed to P2X1 inhibitor NF 449 engraft worse with transplanted normal bone marrow mononuclear cells (BMMNCs). **Left panel — Homing.** Lethally irradiated C57BL/6J mice injected with NF 449 were transplanted with BMMNC from C57BL/6J mice, which had been previously labeled with a PKH67 cell linker. Twenty-four hours after transplantation, the femoral BMMNCs were harvested, the number of PKH67$^+$ cells evaluated by FACS, and the CFU-GM clonogenic progenitors were enumerated in an *in vitro* colony assay. **Middle panel — Early engraftment.** Lethally irradiated C57BL/6J mice were injected with NF 449 as described in Methods section and then were transplanted with BMMNC from C57BL/6J mice. Twelve days after transplantation femoral BMMNCs were harvested and plated to count the number of CFU-GM colonies, and spleens were removed for counting the number of CFU-S colonies. No colonies were formed in lethally irradiated, untransplanted mice (irradiation control). *P < 0.05. **Right panel — Recovery of peripheral blood counts**. Lethally irradiated C57BL/6J mice injected with NF 449 were transplanted with BMMNCs from C57BL/6J mice. White blood cells (left) and platelets (right) were counted at intervals (at 0, 3, 7, 14, 21, and 28 days after transplantation). *P < 0.05, **P < 0.01.

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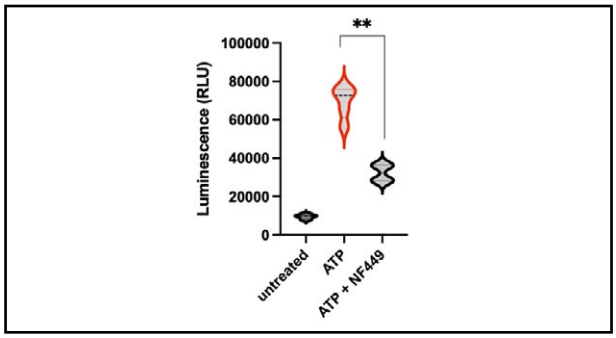

Figure 6. P2X1 inhibition by NF 449 decreases level of Nlrp3 inflammasome activation in response to eATP. Murine Sca-1$^+$ cells enriched in HSPCs were exposed to eATP (0.25 ng/mL, 1.5 h) or eATP + P2X1 inhibitor NF 449 (10 μM). Activation of Nlrp3 inflammasome was measured after exposure of cells to Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent by employing luminescence assay a GloMax 9301 Multi Detection System as described in Methods.*$P < 0.05$.

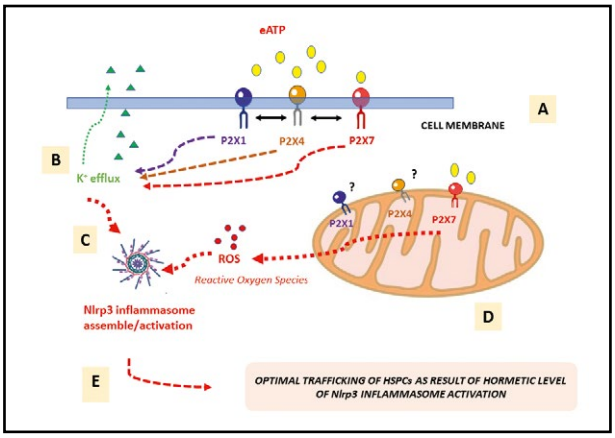

Figure 7. Identified and hypothetical effects of P2X receptors on Nlrp3 inflammasome- mediated trafficking of HSPCs. All three receptors from P2X receptor family promote Nlrp3-inflammasome mediated trafficking of HSPCs. To explain this activation by eATP of P2X1, P2X4 and P2X7 receptors expressed on cell surface membrane (**A**) led to K$^+$ efflux (**B**) that activates Nlrp3 inflammasome (**C**). An open question for further research is a mutual interaction of these receptors in signaling as well as if in addition to P2X7, also P2X1 and P2X4 are expressed on mitochondrial membranes (**D**). It is known that in response to eATP signaling mitochondria release reactive oxygen species (ROS) that similarly as K$^+$ efflux (**C**) promote Nlrp3 inflammasome assemble and activation. The coordinated signaling of all these purinergic receptors is required for optimal Nlrp3 inflammasome mediated (**E**) trafficking of HSPCs.

The extracellular nucleotides and purinergic signaling were for many years somehow understudied and recently emerged to be important modulators of HSPCs trafficking [1–3, 7, 34]. Recent evidence also shows that hematopoietic cells express several receptors involved in regulating immune responses, such as, for example, intracellular pattern recognition receptor Nlrp3 inflammasome [27, 35]. The regulation of hematopoiesis by some components of innate immunity should not surprise as both hematopoiesis and innate immunity possess the common stem precursor cell [36]. Moreover, cells involved in innate immune responses, including, *e.g.*, macrophages, neutrophils, and dendritic cells, are endowed with robust responsiveness to chemoattractants similarly as HSPCs and the downstream molecular machinery regulating cell migration and adhesion seems to be similar [37]. These processes require energy and mitochondria generate ATP and, in addition, release reactive oxygen species (ROS) that are potent activators of the Nlrp3 inflammasome [18, 38].

Nlrp3 inflammasome plays an important role not only in regulating metabolism [38] and aging [40] of BM cells but, as our recent research indicates, is also a crucial element orchestrating cell migration [31, 32]. Nlrp3 inflammasome is activated in response to all HSPCs chemoattractants including a-chemokine stromal-derived factor-1 (SDF-1) [41], as well as to the other chemoattractants such bioactive phosphosphingolipids sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P) [42] and what is highly relevant to current report also to eATP [27]. This latter chemoattractant as an extracellular mediator is mainly released from activated cells in a Pannexin-1 channel-dependent manner [10], acts in an autocrine/paracrine manner and activates P2X purinergic receptors [2, 3]. These receptors are ligand-gated ion channels and consist of seven members P2X1-7 [1, 7, 12, 13]. Our previous research demonstrated that two of these receptors, P2X4 and P2X7, are required for optimal trafficking of HSPCs [2, 3]. Mice deficient in expression of these receptors are poor mobilizers in response to G-CSF or AMD3100 and show defective homing and engraftment [2, 3]. Based on our observations that HSPCs highly express P2X1 member of this receptor family and published data that it is highly sensitive to eATP [17–19] and plays a role in the migration of more differentiated hematopoietic and lymphopoietic cells [1, 43] we become interested if this receptor similarly to P2X4 and P2X7 also affects migration of HSPCs. Herein, we confirmed the expression of this receptor by highly purified human and murine HSPCs and demonstrated by *in vitro* Transwell migration assays that this receptor is functional.

Moreover, our data shows that despite some redundancy in P2X receptor signaling, a proper function of P2X1, similarly as P2X4 and P2X7, is required for optimal trafficking of HSPCs, and the lack of one of these receptors is not fully compensated by the presence of other receptors and impacts on overall biological

responsiveness of HSPCs to eATP. This intriguing phenomenon requires further studies. Interestingly, it has been hypothesized that for example P2X4 and P2X7 receptors may dimerize [44]. However, if P2X1 is also involved in such interactions requires further studies. Another possibility to consider is that these receptors are expressed not only on cell membranes but also on intracellular organelles. In such a case their potential expression at various intracellular compartments may be required for final biological effects. To support this possibility, it has been recently demonstrated that P2X7 receptor in addition to cell membranes is also expressed on mitochondria [18, 43, 45]. Interestingly, as mentioned above mitochondria are a source of reactive oxygen species (ROS) that activate directly intracellular Nlrp3 inflammasome [18, 37]. Therefore, eATP-mediated purinergic signaling in HSPCs involving P2X7, P2X4 [2, 3] and as shown in our current work P2X1 receptor, leads to Nlrp3 inflammasome activation. Therefore, the intracellular distribution of P2X receptors and their interplay with other receptors of this family expressed on cell membranes as shown in the summarizing figure, requires further studies and may explain mutual functional dependence.

P2X1 receptor has been so far mostly studied in non-hematopoietic cells including smooth muscle cells, neurons and blood platelets [22–24]. P2X1 receptor expressed on blood platelets is involved in the pathogenesis of thrombosis [24] and as mentioned above expressed on neutrophils is involved in the chemotactic migration of these cells [25]. It has been also reported to be expressed at mRNA level along with P2X4 and P2X7 receptors on human lymphocytes, monocytes and $CD34^+$ HSPCs [46, 47]. In this work we demonstrate that both human and murine purified HSPCs express mRNAs of all seven members of this family Therefore, further studies are needed to determine if these other receptors play a role in the trafficking of HSPCs. In addition since P2X1 receptor has been reported to be expressed by leukemic cells [4, 48] further studies are needed to address its role in hematopoietic malignancies.

In conclusion, our data demonstrate a novel role of the P2X1 receptor in the trafficking of HSPCs that occurs in eATP-Nlrp3 inflammasome-dependent manner. Therefore, this study sheds not only more light on the underappreciated role of the P2X1 receptor in the mobilization, homing, and engraftment of HSPCs. It also demonstrates a regulatory link between purinergic signaling and innate immunity and the role of Nlrp3 inflammasome being an important orchestrator in these processes.

Author's contributions

MZR planned experiments, MZR and MK wrote a paper, KB, MA, AA, AT, NI, JR, MK performed experiments. All authors approved the submission.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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