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COMMUNICATION

Pannexin-1 influences peritoneal cavity cell population but is not involved in NLRP3 inflammasome activation

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ABSTRACT

Pannexin-1 (Panx1) forms nonselective large channel in cell plasma membrane and has been shown to be associated with NLRP3 inflammasome activation, ATP release and phagocytes recruitment. In the current study, by manipulation of Panx1 expression in human myeloid cells and application of Panx1 deficient mice, we failed to find a correlation between Panx1 and NLRP3 inflammasome activation, although an interaction between these two proteins was evident. However, in thioglycollate induced peritonitis, Panx1 deficient mice showed much more phagocytes infiltration. Further analyses showed that mice deficient for Panx1 exhibited enlarged F4/80^{low}Gr1^{-}Ly6C^{-} cell population in the peritonea. Our study thus reveals an important role for Panx1 in regulation of peritoneal cell population and peritonitis development.

KEYWORDS innate Immunity, inflammasome, NLRP3, Pannexin-1, peritonitis

NTRODUCTION

Pannexin-1 (Panx1) is a vertebrate homologue of invertebrate innexin gap junction proteins (Panchin et al., 2000). It is a widely expressed tetra-transmembrane channel protein with two loops outside of the plasma membrane and two intracellular terminals (Bruzzone et al., 2003). Upon activation, Panx1 forms a large nonselective channel and mediates the release of small molecules (Locovei et al., 2006; Pelegrin and Surprenant, 2006; Kienitz et al., 2011). This channel activity contributes to a variety of physiological or pathological functions of Panx1. For example, Panx1 mediated release of ATP and UTP from apoptotic cells is required for recruiting phagocytic cells (Chekeni et al., 2010). Moreover, activation of Panx1 in enteric neurons underlies neuronal death and the subsequent development of abnormal gut motility in inflammatory bowel diseases (Gulbransen et al., 2012).

In macrophages, Panx1 is reported to mediate ATP and bacterial toxin induced NLRP3 inflammasome activation and IL-1β release (Pelegrin and Surprenant, 2006; Kanneganti et al., 2007; Pelegrin and Surprenant, 2007). The NLRP3 inflammasome is a multi-protein complex containing NLRP3 (aka Cryopyrin and NALP3), ASC (Apoptosis-associated speck-like protein containing a CARD) and Caspase-1 (Agostini et al., 2004). Activation of the NLRP3 inflammasome leads to auto-activation of Caspase-1 which then cleaves the pro-inflammatory cytokine pro-IL-1β into mature IL-1β. Panx1 was shown to interact with the ATP receptor P2X7 to license NLRP3 inflammasome activation, but the detailed mechanism was not explored (Pelegrin and Surprenant, 2006). Thus we further studied the role of Panx1 in NLRP3 inflammasome activation. However, we found that Panx1 was not involved in NLRP3 inflammasome activation in several cell types including human monocytic THP-1 cells, mouse primary bone marrow cells, bone marrow derived macrophages (BMDMs), bone marrow derived dendritical cells (BMDCs) as well as peritoneal macrophages.

Thioglycollate induced peritonitis is a common technique for increasing yield of peritoneal macrophages. Monocytes were recruited to the peritoneum by thioglycollate and differentiated into mature macrophages (Zhang et al., 2008). Blood monocytes are divided into two subsets, the CCR2^{+} CX3CR1^{low}F4/80^{low}Gr1^{+}Ly6C^{+} proinflammatory monocytes and CCR2^{-}CX3CR1^{hi}F4/80^{low}Gr1^{-}Ly6C^{-} resident monocytes (Geissmann et al., 2003). Similarly, the peritoneal mac-

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rophages also consist of two subsets, the F4/80^{hi}MHC-II^{-} large macrophages and the F4/80^{low}MHC-II^{hi} small macrophages (Ghosn et al., 2010). In the present study, we found that after thioglycollate injection, Panx1$^{-/-}$ mice carried much more F4/80^{low}Gr1^{-}Ly6C^{-} cells in the peritoneal cavity, indicating that this population is neither inflammatory monocytes nor classical macrophages. Instead, they could be either resident monocytes or the small macrophages.

RESULTS

Interactions between Panx1 and NLRP3 inflammasome components

To identify if Panx1 is part of the NLRP3 inflammasome, we first checked whether Panx1 could interact with NLRP3. As shown in Fig. 1A, co-immunoprecipitation (co-IP) of indicated proteins expressed in HEK293T cells showed that NLRP3 and Panx1 interacted reciprocally with each other. Moreover, we found that NLRP3 immunoprecipitated both Panx1 and ASC (Fig. 1B), which indicated that Panx1 may form a complex with NLRP3 and ASC in the inflammasome. However, an interaction between Panx1 and ASC was not identified (Fig. 1C). Nonetheless, it is possible that NLRP3 could work as an adaptor to recruit Panx1 and ASC together. To test this possibility, we co-expressed NLRP3 protein with Panx1 and ASC for co-IP assays. However, even in the presence of NLRP3, Panx1 and ASC still failed to interact with each other (Fig. 1D), which indicated that there could be a steric effect that makes NLRP3 interact with either Panx1 or ASC but not both of them at the same time, and the latter two cannot interact with each other directly or indirectly in our experiment system mentioned above.

^{10}Panx1 peptide is not involved in the interaction between Panx1, P2X7 and NLRP3 inflammasome components

Previous report showed that a decapeptide called ^{10}Panx1 from the first outer membrane loop of Panx1 had an inhibitory effect on NLRP3 inflammasome activation (Pelegrin and Surprenant, 2006). We also confirmed that ^{10}Panx1 and a Panx1 specific inhibitor called Probenecid inhibited ATP or Alum induced NLRP3 inflammasome activation in LPS primed J774 cell line and bone marrow derived macrophages (BMDMs) (Fig. S1). Since the interaction between NLRP3 and Panx1 was evident, we reasoned that ^{10}Panx1 might interfere with this interaction through competitive binding to NLRP3. Therefore, we deleted this decapeptide from Panx1 protein via site directed mutagenesis. However, the shortened protein d10Panx1 still interacted with NLRP3 (Fig. 2A). In addition, the interaction between d10Panx1 and P2X7 receptor was also intact (Fig. 2B). Finally, direct addition of ^{10}Panx1 peptide into the co-IP system mentioned above did not interfere with the interaction between NLRP3 and Panx1, P2X7 receptor and Panx1 or between NLRP3 and ASC either (Fig. 2C).

Knockdown and over expression of Panx1 did not affect NLRP3 inflammasome activation in THP-1 cells

Next, we silenced Panx1 expression in THP-1 cells through shRNA. Knockdown efficiency was confirmed by 293T cell ectopic expression of Panx1 protein and THP-1endogenous Panx1 mRNA level (Fig. 3A). However, NLRP3 inflammasome activation was not affected when Panx1 was silenced (Fig. 3A). Meanwhile, overexpression of Panx1 in THP-1 cells also failed to increase the inflammasome activity as revealed by similar IL-1β and caspase-1 activity in comparison with normal THP-1 cells (Fig. 3B and 3C). In contrast, over expression of NLRP3 in THP-1 cells did cause a moderate elevation of IL-1β secretion upon LPS stimulation (Fig. 3B and 3C).

Inflammasome activation is intact in Panx1$^{-/-}$ mice

To further confirm the role of Panx1 in inflammasome activation, Panx1 deficient mice were utilized for experiments. Deletion of Panx1 in these mice was characterized in various tissues and BMDMs (Fig. S2). However, in functional assays, BMDMs from Panx1$^{-/-}$ mice exhibited a comparable IL-1β secretion with wild type BMDMs, indicating that this gene is not involved in inflammasome activation *in vivo* (Fig. 4A). Interesting enough, the NLRP3 inflammasome activation induced by LPS-ATP treatment was also inhibited by the ^{10}Panx1 peptide in the Panx1$^{-/-}$ cells (Fig. 4B), which indicated that the effect of this peptide on inflammasome activation reported before was very likely due to certain off-target effect. When we extended our assays to BMDCs, primary bone marrow cells and peritoneal macrophages from the same set of mice mentioned above, IL-1β secretion upon various stimulants again exhibited similar levels from Panx1 deficient or wild type mice (Fig. 4C–E). Moreover, in an NLRP3 inflammasome dependent MSU induced peritonitis model, neutrophil infiltration was not affected by Panx1 deficiency either (Fig. 4F).

Panx1 deficient mice have more peritoneal F4/80^{low} cell population in thioglycollate-induced peritonitis

Although Panx1 was not involved in neutrophil infiltration in MSU induced peritonitis, we noticed that Panx1 deficient mice carried much more cells in peritonea than WT mice after thioglycollate injection (Fig. 5A). Cell population analysis showed that the total F4/80^{high} cell numbers were similar between WT and Panx1$^{-/-}$ mice, indicating that normal macrophage population was not affected (Fig. 5B). However, Panx1 deficient mice displayed much more abundant F4/80^{low} cell population in the peritonea (Fig. 5C). Further analysis showed that this population was both Gr1 and Ly6C negative, indicating that they are not neutrophils, not eosinophils, nor inflammatory monocytes (Fig. 5D). Therefore, according to previous reports (Geissmann et al., 2003; Ghosn et al., 2010), these cells should be the resident monocytes or the small macrophages and may contribute to the peritoneal homeostasis and inflammation regulation.

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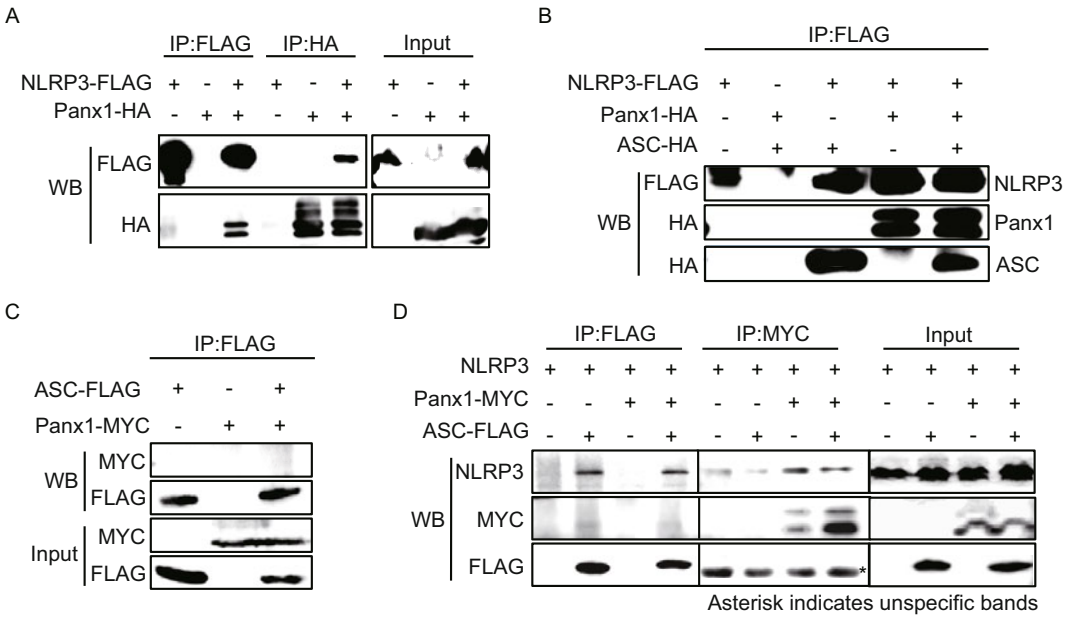

Figure 1. Panx1 interacts with NLRP3 but not ASC. Different combinations of tagged NLRP3, Panx1 and ASC expressing plasmids were transfected into HEK293T cells; (A) NLRP3-FLAG and Panx1-HA; (B) NLRP3-FLAG, Panx1-HA and ASC-HA; (C) ASC-FLAG and Panx1-MYC; (D) NLRP3, ASC-FLAG and Panx1-MYC. Cell extracts were subjected to immunoprecipitation for FLAG, HA or MYC tags. Data are representative of two (B and C) or three (A and D) independent experiments.

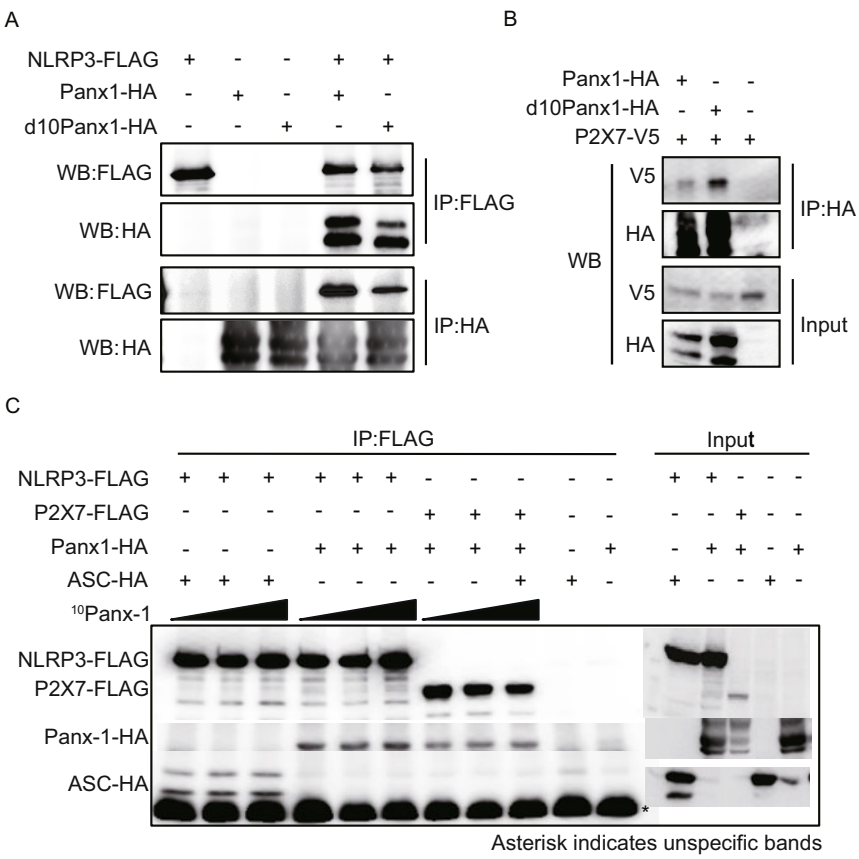

Figure 2. Absence or presence of ^{10}Panx1 peptide failed to disrupt interactions between Panx1, P2X7 and NLRP3 inflammasome components. Different combinations of NLRP3-FLAG, Panx1-HA, d10Panx1-HA, P2X7-V5 and ASC-FLAG plasmids were transfected into HEK293T cells. Cell extracts were subjected to immunoprecipitation of FLAG and HA (A), HA (B) and FLAG (C). In (C), increased concentration of ^{10}Panx1 peptide (DMSO, 2 mmol/L and 5 mmol/L) was added into the IP system.

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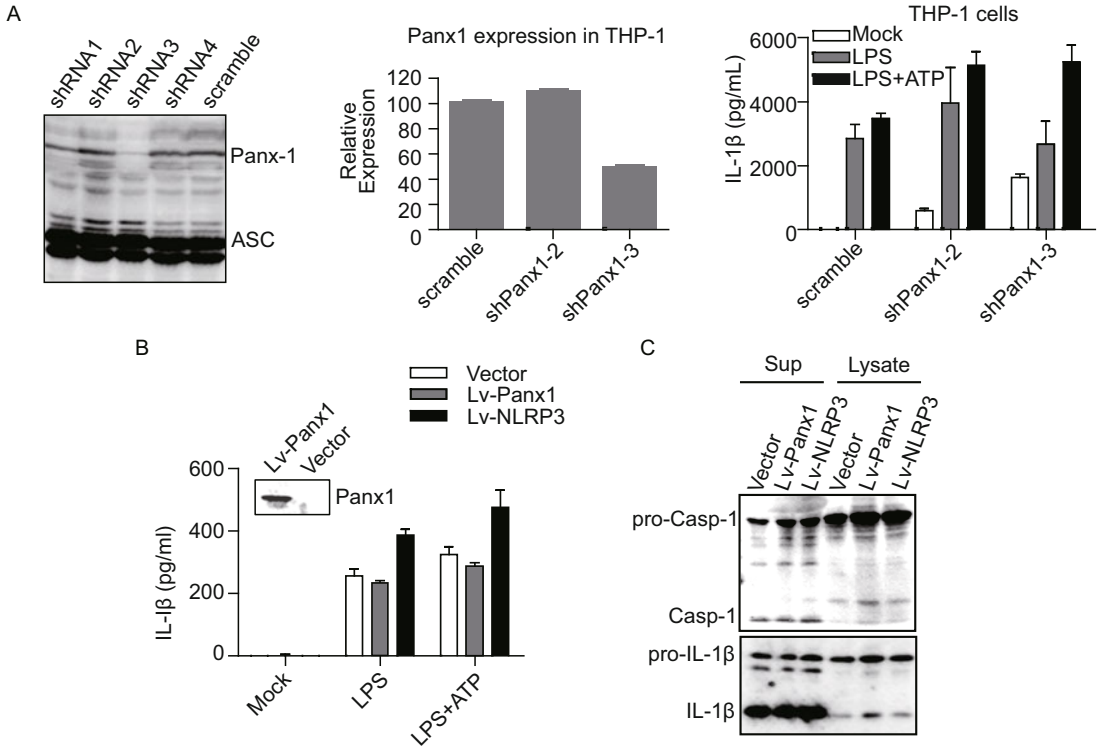

Figure 3. Knockdown and over expression of Panx1 have no effect on NLRP3 inflammasome activation in THP-1 cells. (A) Panx1 knockdown THP-1 monocytes were treated with LPS (500 ng/mL) for 5 h; ATP (5 mmoL) was added 30 min before harvest (left). ShRNA knockdown efficiency was confirmed by Western blot in HEK 293T cells through overexpression of Panx1 and ASC (middle) and Q-PCR in THP-1 cells (right), (B and C) THP-1 cells were transducted with lentiviral vectors expressing Panx1 or NLRP3 (Lv-Panx1 or Lv-NLRP3). Panx1 expression was confirmed by Western blot. After LPS and ATP treatment, IL-1β cleavage and Caspase-1 activation were detected by ELISA (B) and Western blot (C).

Discussion

Previous reports about the role of Panx1 in NLRP3 inflammasome activation are controversial. While Pelegrin and colleagues showed that Panx1 is necessary for NLRP3 inflammasome activation (Pelegrin and Surprenant, 2006, 2007), Qu et al. recently showed that Panx1 is dispensable for the activation of NLRP3, NLRC4 and AIM2 inflammasomes (Qu et al., 2011). Pelegrin and colleagues mainly used mimetic peptide and inhibitory drugs, whereas Qu et al. generated Panx1 deficient mice for experiments, which is more compelling. Consistent with Qu's results, we also found that Panx1 is dispensable for NLRP3 inflammasome activation. Interestingly, we found that Panx1 interacts with NLRP3, indicating undefined non-inflammasome associated roles for Panx1 and/or NLRP3 may exist.

Although Panx1 is not involved in NLRP3 inflammasome activation in our study, we fortuitously found that thioglycollate injected Panx1$^{-/-}$ mice have much higher peritoneal cell count than WT mice. Under microscope, there are relatively more monocyte-like 'small cells'. Flow cytometry results showed that these cells are F4/80^{low}Gr1^{-}Ly6C^{-} indicating that they are not neutrophils, not eosinophils, nor inflammatory monocytes. According to previous reports, they could be resident monocytes migrated from blood or the so called 'small peritoneal macrophages' (SPM) which are differentiated from the migrated monocytes (Geissmann et al., 2003; Ghosn et al., 2010). Further experiments are needed to identify this population and clarify whether there is an enhancement of cell infiltration into the peritoneal cavity or whether there is a cell differentiation defect in the Panx1 deficient mice. Because Panx1 mediates the release of ATP from apoptotic cells, thus the peritoneal cavity ATP concentration could be changed in the absence of Panx1, which may be responsible for the cell population change as we observed. Since Panx1 is involved in cell apoptosis (Gulbransen et al., 2012), it is also possible that Panx1 deficient cells survive longer than WT cells in the peritonea. In any case, these cells may contribute to the development of peritonitis and the roles of these cells need to be further explored. With more experimental findings coming out, the roles of Panx1 in inflammation and infection will be further defined.

MATERIALS AND METHODS

Reagents, plasmids and antibodies

pRK5-Panx1-HA was a courtesy of Dr. Rolf Dermietzel. pcDNA3.1(-)-Panx1-c-myc was kindly provided by Dr. Pablo Pelegrín. pRK5-

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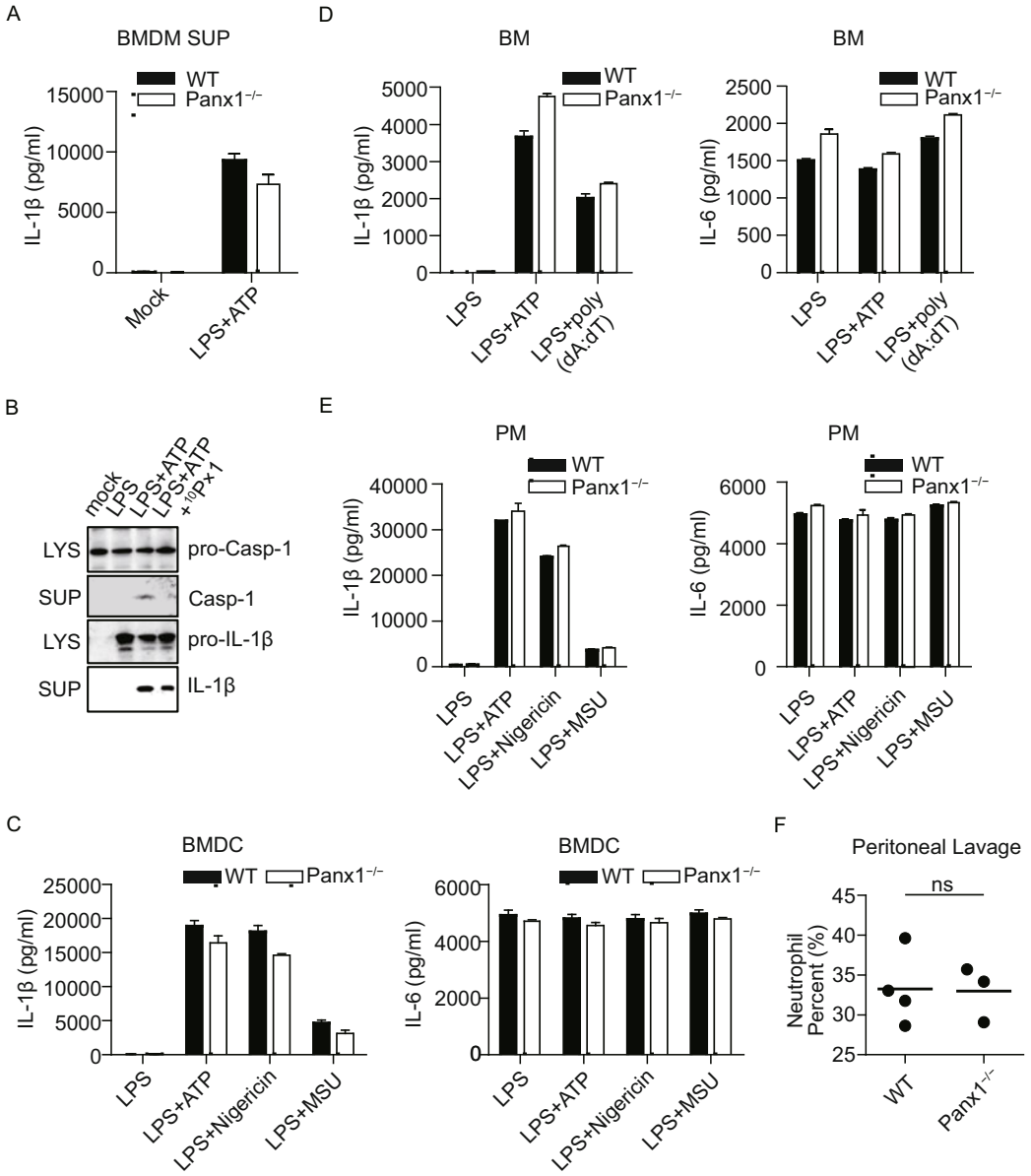

Figure 4. Endogenous Panx1 is not involved in NLRP3 inflammasome activation in vitro and *in vivo*. (A) BMDMs from WT and Panx1$^{-/-}$ mice were treated with LPS (500 ng/mL) for 6 h and ATP (5 mmol/L) for 30 min. IL-1β in supernatant was detected by ELISA. (B) Western blot of Caspase-1 and IL-1β from Panx1$^{-/-}$ BMDMs. 10Panx1 peptide (5 mmol/L) were added 30 min before ATP treatment. (C–E) BMDCs, Bone marrow cells and peritoneal macrophages were treated with LPS (500 ng/mL) for 5hr and then with ATP (5 mmol/L) for 30 min, nigericin (25 mmol/L) for 30 min, MSU (200 mg/mL) for 6 h or poly(dA:dT) (2 ng/mL) mixed with lipo2000 for 12 h. IL-1β and IL-6 in the supernatant were detected by ELISA. (F) Age and gender matched WT and Panx1 mice were i.p. injected with MSU (1 mg/mouse). 6 h later, peritoneal cells were lavaged for detection of Gr-1+ population percent by FACS. Data are representative of two (B–F) to three (A) independent experiments.

d10Panx1-HA plasmid was produced based on pRK5-Panx1-HA using QuikChange lightning Site-Directed Mutagenesis Kit (Stratagen). The primers are: forward, 5′-CTTCTCCCCGAGTTCTTTCTCCTGCTGGGCTGCTGTACAGC-3′; reverse, 5′-GCTGTACAGCAGCCCAGCAGGAGAAAGAACTCGGGGAGAAG-3′. ^{10}Panx1 peptide, Nigericin, LPS and ATP were obtained from Sigma. poly(dA:dT) (tlrl-patn-1) was from Invivogen. Alum was from Pierce. MSU was prepared as described before (Hu et al., 2010). Anti-flag, anti-HA, anti-myc, anti-V5, anti-ASC (sc-22514) anti-Casp-1 (sc-514), anti-IL-1β (sc-7884) and secondary antibodies were from Santa cruz. Cryo2 anti-NLRP3 (alx-804-881) was from Enzo Life Sciences. IL-1β and IL-6 ELISA kits were purchased from BD Pharmingen.

Cells and mice

HEK293T cells were grown in DMEM supplemented with 10% FBS. THP-1 cells were grown in RPMI1640 medium with 10%FBS.

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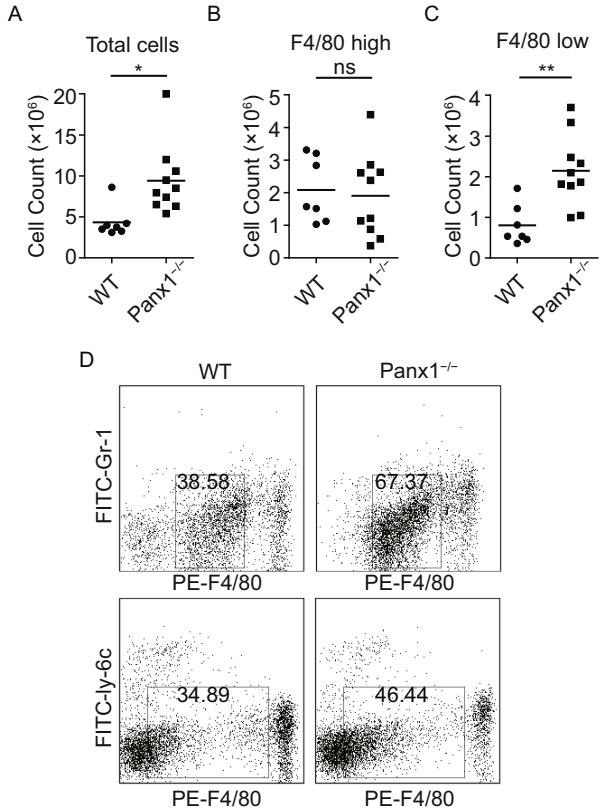

Figure 5. Panx1$^{-/-}$ mice have more peritoneal F4/80^{low} cells in thioglycollate induced peritonitis. 12–16 week old mice were i.p. injected with 1mL 4% thioglycollate broth. 4 days after injection, peritoneal cells were collected, enumerate and stained for FACS. (A) Total cell number count of peritoneal lavage from WT and Panx1$^{-/-}$ mice. (B and C) F4/80 high or low cell population was calculated according to total cell number and population percentage. (D) Representative image of Gr1, Ly6C and F4/80 defined populations from WT and Panx1$^{-/-}$ peritoneal lavage cells, the number in the defined area is percentage of F4/80^{low} population of analyzed cells.

C57BL/6 mice were purchased from Shanghai Laboratory Animal Center (SLAC). Panx1 deficient mice were purchased from KOMP. BMDMs and BMDCs were differentiated from fresh bone marrow cells in IMDM medium supplemented with 10% FBS in the presence of L cell supernatant or 1640 medium supplemented with 10% FBS in the presence of AG8653 cell supernatant, respectively. Peritoneal macrophages were lavaged from mice 4-day post 4% thioglycollate injection. After 6h in culture, unattached cells were washed away and attached cells were harvested for experiment.

ShRNA mediated gene silencing in THP-1 cells

ShPanx1 sequences were constructed into pLKO.1 and transfected into HEK293T cells with VSVG and Δ8.91 plasmids. The packaged virons were transducted into THP-1 cells. Puromycin was used for screening. Panx1 shRNA sequences are shown below (forward and reverse): shRNA1, 5′-CCGGTGCATTTCAAATCTGAAGGGTATTCAAGAGATACCCTTCAGATTTGAAATGCTTTTTG-3′, 5′-AATTCAAAAAGCATTTCAAATCTGAAGGGTATCTCTTGAATACCCTTCAGATTTGAAATGCA-3′;shRNA2, 5′-CCGGTGGAGCAGTACTTGAAGACATTCAAGAGATTGTCTTCAAGATACTGCTCCTTTTTG-3′, 5′-AATTCAAAAAGGAGCAGTACTTGAAGACATCTCTTGAATGTCTTCAAGTACTGCTCCA-3′; shRNA3, 5′-CCGGTCCCAATGCTACTCCTGACAAATTCAAGAGATTTTGTCAGGAGTAGCATTGGGTTTTTG-3′, 5′AATTCAAAAACCCAATGCTACTCCTGACAAATCTCTTGAATTTGTCAGGAGTAGCATTGGGCA 3′; shRNA4, 5′-CCGGTGCATCAAATCAGGGATCCTTTCAAGAGATAGGATCCCTGATTTGATGCTTTTTG-3′, 5′-AATTCAAAAAGCATCAAATCAGGGATCCTTCTCTTGAAAGGATCCCTGATTTGATGCA-3′.

Co-immunoprecipitation assays

HEK 293T cells were seeded into 6-well plates and transfected using lipo2000 (Invitrogen). After incubation for 48 h, the transfected cells were lysed by lysis buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% NP-40, Complete Protease Inhibitor Cocktail (Roche)) and were centrifuged at 10,000 g for 15 min at 4ºC. The supernatant was pre-cleared using Protein A agarose beads (Invitrogen) and then incubated with Protein A agarose beads and 0.5–2 μg anti-FLAG or anti-HA or anti-MYC antibody at 4ºC overnight. The beads were then washed three times with lysis buffer and boiled with western blotloading buffer for SDS-PAGE and Western blot.

ELISA

THP-1 cells or mouse cells were seeded into 96-well plates. After being treated with indicated stimulations or drugs, the culture media were collected for measuring the IL-1β and IL-6 level using the IL-1β and IL-6 ELISA kits (BD Pharmingen).

Quantitative real-time PCR

Isolation of mRNA from mouse tissues or cultured cells with Trizol (Invitrogen), synthesis of cDNA with reverse transcription kits (Applied Biosystems) and amplification by PCR with the SYBR Green method with the ABI 7500 Fast PCR detection system (Applied Biosystems) were carried out according to manufacturer's instructions. The human Panx1 primers are 5′-GCTCTTTGCGATCCTCCTGTA-3′ and 5′-TTGACAAAGTTTACAACCGTGCA-3′. The cycling threshold (C_T) value was defined as the number of PCR cycles in which the fluorescence signal exceeded the detection threshold value. For each sample, the normalized amount of target mRNA (N_T) was calculated from the obtained C_T values for both target and GAPDH mRNA with the following equation: $NT = 2^{CT\ of\ GAPDH\ –\ CT\ of\ target}$.

FACS

Peritoneal cell suspensions were preincubated with anti–CD16/CD32 mAb to block FcγRII/III receptors and stained on ice for 15 min with the different fluorochrome conjugated mAb combination: FITC-labeled anti-Ly-6C, Gr-1, PE-labeled anti-F4/80. Antibodies were purchased from BD Pharmingen. Cells were analyzed and sorted on Becton Dickinson LSRII. Data were collected for 1–3 × 10^5 cells. Data were analyzed with FlowJo software (TreeStar).

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Statistics

Statistical evaluation between control and experimental samples was performed using the two-tailed Student's *t*-test. $P < 0.05$ were considered to indicate significance (*), $P < 0.01$ were considered to indicate great significance (**).

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ABBREVIATIONS

ASC, Apoptosis-associated speck-like protein containing a CARD; BMDM, bone marrow derived macrophages; BM, bone marrow cells; BMDC, bone marrow derived dendritic cells; IL-1β, interleukin 1 beta; NLRP3, NLR family, pyrin domain containing 3; PM, peritoneal macrophages

DISCLOSURES

The authors have no financial conflicts of interest.

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