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K^+ regulates relocation of Pellino-2 to the site of NLRP3 inflammasome activation in macrophages

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Pellino proteins are E3 ubiquitin ligases involved in the innate immune system. Recently, Pellino-2 was reported to modulate the activation of the mouse Nlrp3 inflammasome. We examined the intracellular localization of human Pellino-2 in THP1-derived macrophages during activation with LPS and ATP. We observed that Pellino-2 changed intracellular localization and colocalized with the inflammasome proteins NLRP3 and ASC late in the assembly of the inflammasome. Colocalization with NLRP3 and ASC was also seen in cells maintained in potassium-free medium. The colocalization and inflammasome activation were abrogated by several potassium channel inhibitors, supporting a role for potassium efflux in modulating intracellular localization of Pellino-2. The data suggest that Pellino-2 is essential for mediating the effect of potassium efflux on inflammasome activation.

Keywords: ASC; inflammasome; NLRP3; PELI2; potassium efflux

The four mammalian Pellinos (Pellino-1, Pellino-2, and the two isoforms Pellino-3a and Pellino-3b) have been described as key E3 ubiquitin ligases that mediate innate immune responses [1]. Pellino-2 associates with several signaling proteins involved in the Toll-like receptor (TLR) pathway and functions as a positive regulator of NF-kB activation, leading to the upregulation of pro-inflammatory transcripts IL-6, IL-8, and TNFα [2]. Although the Pellino proteins seem to play distinct roles in immune signaling cascades [3–7], the mechanisms that selectively recruit Pellino-2 remain elusive.

The NLRP3 inflammasome is a cytosolic multiprotein complex that regulates the innate inflammatory response [8]. Its activation is a two-step process, beginning with 'priming'. In this phase, TLRs are activated by extracellular ligands, leading to increased protein synthesis of components of the inflammasome and precursors of pro-inflammatory cytokines, among them pro-IL-1β [9]. In the second step, 'activation', the inflammasome is assembled, cleaving inactive precursors to active pro-inflammatory molecules, such as mature IL-1β [10]. A common intracellular response, potassium (K^+) efflux [11,12], triggered by a variety of

Abbreviations
ASC, apoptosis-associated speck-like protein; CO_2, carbon dioxide; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; fps, frames per second; HRP, horseradish peroxidase; IgG, immunoglobulin G; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; IRAK-1, interleukin 1 receptor-associated kinase 1; K^+, potassium ions; KCl, potassium chloride; LDS, lithium dodecyl sulfate; LPS, lipopolysaccharide; Lys, lysine; NaCl, sodium chloride; NF-kB, nuclear factor kappa B; NLRP3, NLR family pyrin domain containing 3; PB, phosphate buffer; PFA, paraformaldehyde; PMSF, phenylmethylsulfonyl fluoride; pro-IL-1β, precursor of interleukin 1 beta; s.e.m., standard error of the mean; TLR, Toll-like receptor; TNFα, tumor necrosis factor alpha.

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danger signals, like viral DNA, bacterial toxins, and nanoparticles [13,14], leads to the activation of the NLRP3 inflammasome. The effect of K^+ efflux is supported by studies showing that (a) depletion of cytosolic K^+ in response to ATP or nigericin mediates IL-1β maturation, (b) K^+ efflux alone can activate the NLRP3 inflammasome, and (c) high extracellular K^+ concentrations inhibit the activation of the NLRP3 inflammasome [11,15,16].

Recently, Pellino-2 was found to be involved in the activation of the mouse Nlrp3 inflammasome [17]. In a Pellino-2 constitutive knockout mouse model, Humphries and co-workers showed that Pellino-2 is essential in the priming step for the activation of the canonical pathway of the mouse Nlrp3 inflammasome. At the same time, they showed that Pellino-2 did not directly induce poly-ubiquitination of Nlrp3, thus suggesting that Pellino-2 may exert its effects on Nlrp3 in an indirect manner [17].

Although the function of Pellino-2 has been characterized to some extent, a detailed examination of the intracellular localization of Pellino-2 has not been reported. Therefore, in this study, we examined the intracellular localization of Pellino-2 in a human macrophage cell line, with particular emphasis on its interaction with the NLRP3 inflammasome. We show that Pellino-2 colocalizes with the inflammasome proteins NLRP3 and ASC in activated THP1-derived macrophages. This effect is abrogated when pharmacological K^+ channel blockers are used. Although NLRP3 appears to change localization for inflammasome activation, blocking of the K^+ channels prevents the relocation of Pellino-2 and thus the assembly and maturation of the inflammasome. Our findings indicate a significant effect of K^+ efflux on Pellino-2 localization and point to an essential role of Pellino-2 relocation in the activation of the NLRP3 inflammasome.

Materials and methods

Expression vectors

Human *PELI2* cDNA encoding full-length human Pellino-2 (UniProt ID: Q9HAT8) was subcloned from a pLenti-Peli2-myc-DDK vector (RC203409L1, OriGene, Rockville, USA) into a pQCXIH/pQCXIP retroviral expression vector (Clontech, California, USA), using BamHI and EcoRI restriction enzymes. An N-terminal GFP tag was inserted containing a linker between the GFP and Pellino-2, consisting of six amino acids (Ser-Gly-Leu-Arg-Ser-Ala) [18]. An additional plasmid was generated by inserting a C-terminal HA tag. All constructs were subsequently verified by Sanger sequencing.

Transient transfection and cell lysis

For transient transfection, 8 μL Lipofectamine 2000 reagent (Invitrogen, Waltham, Massachusetts, USA) and 2 μg plasmid DNA encoding HA-tagged or GFP-tagged human Pellino-2 were added to HEK293 cells upon 60–70% confluency in 10-cm plates. Cells were harvested after 40 h. The cells were lysed in 50 mM Tris–HCl pH 7.4, 200 mM NaCl, 5 mM EDTA, 0.1% NP-40, 0.5% Tween-20, 1 mM PMSF and supplemented with complete protease inhibitors (Roche Diagnostics, Mannheim, Germany, #11-836-145-001). Cell lysates were centrifuged to remove cell debris. Bolt LDS sample buffer (Thermo Fisher, Waltham, MA, USA, #B0007) and Bolt sample reducing agent (Thermo Fisher, #B0009) were added before immunoblot analysis.

Immunoblot analysis

Proteins were separated and transferred onto nitrocellulose membranes using the Bolt® Bis-Tris Plus electrophoresis and transfer system (Thermo Fisher Scientific). Membranes were incubated overnight at 4 °C with primary antibodies. The immunodetection of Pellino-2 (1 : 1000 dilution, Sigma-Aldrich, St. Louis, MO, USA, #HPA053182), the HA tag on Pellino-2 (1 : 500 dilution, Thermo Scientific, #71-5500), the GFP tag on Pellino-2 (1 : 500 dilution, Clontech, clone JL-8, #632381), and GAPDH (1 : 10 000 dilution, Sigma-Aldrich, #G9545) was performed according to Cell Signaling Technologies instructions (details in Supporting information). HRP-linked anti-rabbit IgG antibody (Cell Signaling, Danvers, MA, USA, #7074) at 1 : 2000 dilution was used as secondary antibody (detailed description in Supporting information).

To test for binding specificity of the Pellino-2 antibody, a blocking peptide PrEST antigen PELI2 (Sigma-Aldrich, #APREST83881) was used at 1 : 1000 dilution. Incubation with the blocking peptide and Pellino-2 antibody was performed in parallel with overnight incubation with the Pellino-2 antibody only.

THP-1 cell differentiation into macrophages

THP-1 cells (ATCC TIB-202™) were cultured in RPMI-1640 medium, supplemented with 10% FBS, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 2 mM L-glutamine, and 0.05 mM 2-mercapto-ethanol. Cells were cultured for no longer than 15 passages.

THP-1 monocytes were gradually transitioned for cultivation in Dulbecco's modified Eagle's medium (DMEM), high glucose (4500 $mg \cdot L^{-1}$), supplemented with 10% fetal bovine serum (FBS), 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 2 mM L-glutamine, and 0.05 mM 2-mercapto-ethanol.

THP-1 monocytes were differentiated into adherent macrophages, as previously described [19], using 200 nM phorbol-12-myristate-13-acetate (PMA, Cell Signaling, #4174S) for 3 days, followed by a 5-day resting period (THP1PMA cells).

Treatment of THP1PMA macrophages

Following differentiation into macrophages, the THP1PMA cells were primed with 100 ng·mL^{-1} LPS (*E. coli* O111 : B4, Sigma-Aldrich, #LPS25) for 4 h and activated with 5 mM ATP (Thermo Scientific, #R0441) for 15 min, before fixation.

In order to block the activation of the NLRP3 inflammasome, THP1PMA cells were treated with either 10 μM quinine (Abcam, Cambridge, England, #ab141247), 30 mM KCl (Sigma-Aldrich, #1049360500) or 50 μM glyburide (Cayman Chem, Ann Arbor, MI, USA, #15009) for 30 min, before priming with LPS and activation with ATP, as described above.

THP-1 monocytes were also gradually transitioned for cultivation in complete DMEM culture medium, for immunofluorescence experiments involving potassium-free (K^+-free) culture medium. Before fixation, the cell culture medium was changed for 3 h to DMEM without potassium chloride (KCl) (Cell Culture Technologies, custom-made).

Immunofluorescence

Cells were seeded at a density of 75 000 cells/well in 12-well dishes on 18-mm glass coverslips coated with 0.1 mg·mL^{-1} poly-L-lysine. The staining procedure was performed in accordance with Sannerud and co-workers (2008) (see Supporting information for details) [20]. The fixation step was performed at room temperature for 30 min using prewarmed 3% PFA in either PBS pH 7.4 or 0.1 M phosphate buffer (PB) pH 7.2.

Double-labeling immunofluorescence analysis was performed in THP1PMA macrophages, using antibodies against Pellino-2 (Sigma-Aldrich, #HPA053182), NLRP3 (Enzo, #ALX-804-819-C100), and ASC (Santa Cruz, #sc-514414). Goat anti-rabbit IgG Alexa Fluor® 488 and goat anti-mouse IgG Alexa Fluor® 594 (#111-546-144 and #115-586-146, both from Jackson Laboratories) were used as secondary antibodies.

Quantification of secreted IL-1β upon NLRP3 inflammasome activation

THP-1 cells were seeded in 96-well tissue-culture plates (Corning Costar, #9018) at a density of 50 000 cells/well in 100 μL of THP-1 culture medium, supplemented with 200 nM of phorbol-myristate acetate (PMA, Cell Signaling, #4174S), and incubated for 3 days. The medium was replaced with THP-1 culture medium without PMA, and the cultures were allowed to rest for 5 days in the CO_2 incubator.

On the day of the assay, cells were primed with 100 ng·mL^{-1} LPS (E. coli O111 : B4, #LPS25, Sigma-Aldrich) for 4 h to upregulate genes necessary for NLRP3 inflammasome activation and activated with 5 mM ATP (Thermo Scientific, #R0441) for 15 min. Wherever indicated, THP1PMA cells were also treated with either 10 μM quinine (Abcam, #ab141247), 30 mM KCl (Sigma-Aldrich, #1049360500), or 50 μM glyburide (Cayman Chem, #15009) for 30 min, before priming with LPS and activation with ATP, as described above.

After incubation, supernatants were collected and assayed for IL-1β release using a human IL-1β ELISA kit (Thermo Fisher, #88-7261-88) according to the manufacturer's protocol. Briefly, after precoating with a specific IL-1β antibody, the plates were blocked and incubated overnight with cell culture media from THP1PMA cells. After being washed, the plates were incubated with biotinylated detection antibody, avidin–HRP, and developed by chemiluminescence (see Supporting information for details).

Cell viability assay

Cell proliferation/viability following NLRP3 inflammasome activation in THP1PMA cells was assessed by WST-1 assay. Twenty-four hours after inflammasome activation, the WST-1 reagent (Roche, #5015944001) was added into the wells at a 1 : 10 dilution and incubated at 37 °C for 4 h. The optical density (OD) was measured at wavelength 440 nm with correction at 650 nm, using a BioTek Synergy™ HT microplate reader.

Image acquisition and analysis

Images were acquired using Leica SP5 and SP8 confocal laser scanning microscopes (Leica Microsystems). The Z-stack function was used to enhance resolution and allow for 3D examination, in order to confirm colocalization. Scale bar is 20 μm and the video frame rate 5 fps. Postprocessing of images was performed using the integrated adaptive deconvolution module Lightning (Leica Microsystems).

Statistical analysis and reproducibility

Two-way ANOVA, followed by Tukey's multiple comparisons test, was performed for statistical analysis. All results have been replicated in at least 3 independent experiments.

Results

Validating Pellino-2 antibody specificity

In order to validate the commercial rabbit polyclonal Pellino-2 antibody used in this paper (targeting a C-terminal amino acid sequence NRKEVVEEKQPWA

YLSCGHVHGYHN), we first examined HEK293 cells transiently transfected with plasmids containing Pellino-2 tagged with either an HA tag or a GFP tag. Cell lysates were analyzed by immunoblotting using anti-Pellino-2 antibody, as well as anti-HA, anti-GFP, and anti-GAPDH antibodies (Fig. S1A). The anti-Pellino-2 antibody revealed endogenous Pellino-2 (MW 46 kDa), as well as the overexpressed HA-tagged Pellino-2 (MW 50 kDa) and GFP-tagged Pellino-2 (MW 73 kDa) at expected molecular weights. The overexpressed protein was also identified with anti-HA and anti-GFP antibodies. Next, we used a blocking peptide against the Pellino-2 antibody, PrEST antigen PELI2 (Fig. S1B), and we showed that binding of the antibody was effectively blocked by this antigen.

In immunofluorescence experiments, the anti-Pellino-2 antibody recognized both endogenous and overexpressed FLAG-tagged Pellino-2 (Fig. S1C). We thereby concluded that the antibody was specific for Pellino-2.

Pellino-2 colocalization with NLRP3 and ASC in primed and activated THP-1 derived macrophages

Given the recent report indicating that Pellino-2 influences Nlrp3 inflammasome activation in mouse macrophages, we investigated the localization of Pellino-2 in the human-derived immune cell line THP-1. We differentiated THP-1 monocytes into adherent macrophages (THP1PMA) using phorbol-12-myristate-13-acetate (PMA), followed by priming and activation, to initiate inflammasome assembly and release of mature pro-inflammatory cytokines.

In THP1PMA cells under resting conditions (Fig. 1), Pellino-2 was constitutively expressed and

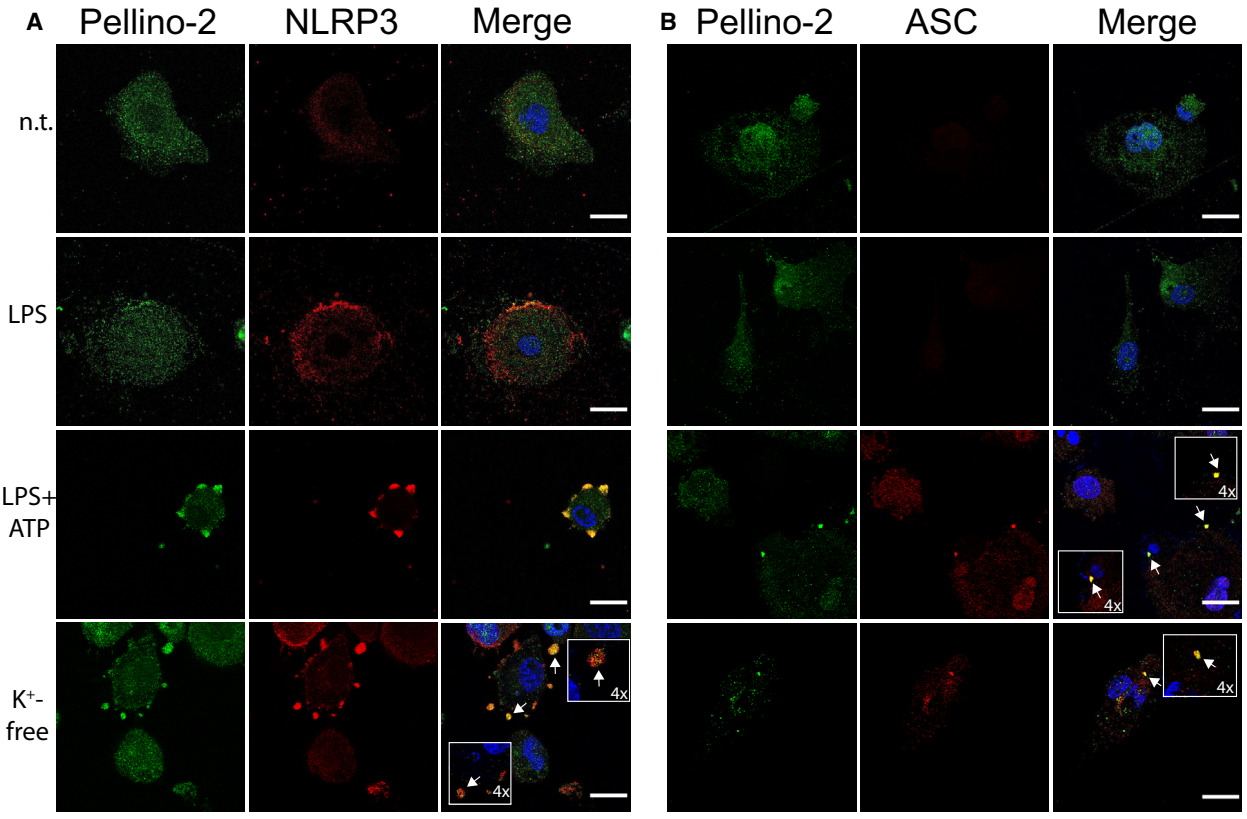

Fig. 1. Pellino-2 colocalizes with NLRP3 and ASC in primed and activated macrophages. THP-1 monocytes were differentiated into adherent macrophages using 200 nM phorbol-12-myristate-13-acetate (PMA) for 3 days, followed by a 5-day resting period. THP1PMA macrophages were subjected to indirect immunofluorescence, using PBS as fixation buffer for the fixative agent (3% PFA) without any prior treatment (n.t.), primed with 100 ng·mL^{-1} LPS for 4 h (LPS), primed with 100 ng·mL^{-1} LPS for 4 h and stimulated with 5 mM ATP for 15 min (LPS+ATP), or cultivated in potassium-free DMEM medium for 3 h (K^+-free). The fluorescent images show endogenous Pellino-2, endogenous NLRP3 (A) or endogenous ASC (B) and nuclei (DAPI). The insets represent a 4 times magnification of the original images, postprocessed using the deconvolution module Lightning. Scale bar: 20 μm.

located in the cytoplasm, while expression of the NLRP3 protein was minimal.

Upon priming with LPS, upregulation of NLRP3 protein expression was seen, with a more specific and restricted localization close to the plasma membrane, indicating early stages of inflammasome activation. This was confirmed by the levels of secreted IL-1β in LPS-primed THP1PMA cells (Fig. 2). After activation with ATP, Pellino-2 changed location and became colocalized with the inflammasome protein NLRP3 (Fig. 1A) and with the adaptor protein ASC (Fig. 1B).

Thus, Pellino-2 changed its intracellular localization, depending on the activation status of the macrophages: In resting macrophages, Pellino-2 was spread diffusely in the cytoplasm, whereas during activation with ATP after priming with LPS, Pellino-2 concentrated at the site of the inflammasome and colocalized with NLRP3 and ASC.

Extracellular K^+ ions influence localization of endogenous Pellino-2 and the activation of the inflammasome

Due to the role of K^+ efflux in NLRP3 inflammasome activation [11], we wanted to examine whether extracellular K^+ levels influence the localization of Pellino-2.

We first cultivated THP1PMA cells in K^+-free medium for 3h and performed immunofluorescence analysis using antibodies against Pellino-2, NLRP3, and ASC. In these cells, which had not been treated with LPS and ATP, restricted localization of the NLRP3 protein at the plasma membrane (Fig. 1A: K^+-free), as well as ASC speck formation (Fig. 1B: K^+-free), was seen, indicating activation of the inflammasome. In addition, relocation of Pellino-2 from the cytoplasm to the site of NLRP3 inflammasome activation (Fig. 1) and colocalization with the NLRP3 protein (Video S1)

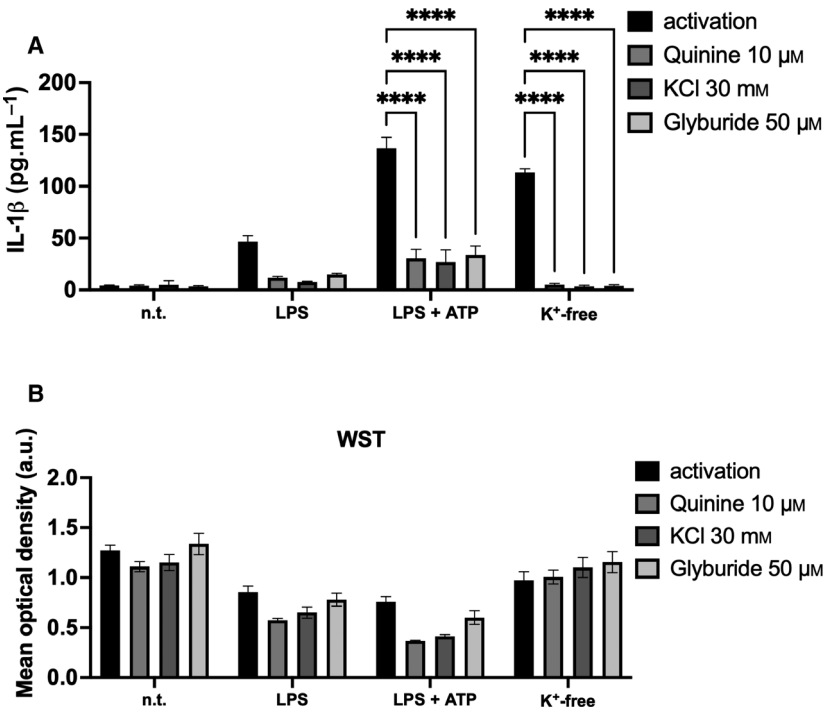

Fig. 2. NLRP3 inflammasome activation, measured in the levels of secreted IL-1β, followed by cell viability assay. (A) THP1PMA cells were left without any treatment (n.t.), or were primed with 100 ng·mL^{-1} LPS for 4 h (LPS), primed with 100 ng·mL^{-1} LPS for 4 h and stimulated with 5 mM ATP for 15 min (LPS+ATP) or cultivated in potassium-free DMEM medium for 3 h (K^+-free), to induce NLRP3 inflammasome activation. Additionally, the cells were incubated with either 10 μM quinine, 30 mM KCl, or 50 μM glyburide for 30 min, before priming with LPS and activation with ATP, as described above. Supernatants were harvested, and the measurement of secreted IL-1β was performed by ELISA analysis. Data are shown as mean ± s.e.m. of 3 biological replicates, each with 2 technical replicates. Statistical analysis was performed using two-way ANOVA, followed by Tukey's test. **** $P < 0.0001$. (B) The viability of the cells was measured 24 h after NLRP3 inflammasome activation, using the WST-1 assay. The WST-1 reagent was added in 1 : 10 dilution to the cells and further incubated for 4 h at 37 °C. Data are shown as mean ± s.e.m. of 3 biological replicates. The cell viability assay performed 24 h after activation of the THP1PMA macrophages showed that the NLRP3 inflammasome activation affects the cells' viability.

and ASC protein (Video S2) were seen. This was similar to our findings in cells activated with ATP at physiological K^+ concentration. We used the Lightning deconvolution tool to achieve confocal super-resolution down to 120 nm: Pellino-2 was detected in very close proximity to both NLRP3 and ASC. The data suggested that low levels of extracellular K^+ alone are sufficient to activate the NLRP3 inflammasome and also to initiate the interaction of Pellino-2 with NLRP3 and ASC.

We next proceeded to study several known pharmacological K^+ channel inhibitors. We blocked the efflux of K^+ ions from THP1PMA-derived macrophages by incubating them with quinine (Fig. 3A), KCl (Fig. 3B), or glyburide (Fig. 3C) prior to their activation. Upon treatment with these compounds, Pellino-2 did not change localization even upon activation with LPS plus ATP or by cultivation in K^+-free medium. Pellino-2 continued to show only a punctate cytoplasmic localization, indicating that Pellino-2 localization is directly sensitive to K^+ efflux from the cells.

In contrast, in cells treated with quinine, KCl, or glyburide, activation with LPS plus ATP or cultivation in K^+-free medium resulted in a change of NLRP3 localization (Fig. 3). However, while in activated cells, the NLRP3 inflammasome is normally present as large aggregates close to the plasma membrane, in cells treated with K^+ channel blockers, NLRP3 was seen as multiple, small protein collections along the plasma membrane. We also found significantly reduced levels of IL-1β secretion (Fig. 2), suggesting that the final stages of NLRP3 inflammasome assembly had not been fully completed.

The effect of K^+ ions in the fixation buffer

Since we observed an effect of K^+ efflux on Pellino-2 intracellular localization, it was of interest to examine the consequences of removing K^+ from the fixation buffer. The fixation buffer used in the immunofluorescence experiments was PBS (Figs 1 and 3), which contains 2.7 mM KCl and 1.8 mM KH_2PO_4. In immunofluorescence experiments, PBS is frequently interchanged with PB, which does not contain K^+.

In PB-fixed THP1PMA cells, Pellino-2 was localized to the cytoplasm (Fig. 4). However, in THP1PMA cells primed with LPS before PB-fixation, Pellino-2 presented in the nucleus. Otherwise, priming and activation of the NLRP3 inflammasome, as well as relocation of Pellino-2 intracellularly and its colocalization with NLRP3 after culturing the cells in K^+-free culture medium, were similar in PB- and PBS-fixed cells (Figs 1 and 4). Thus, the absence of K^+ in the fixation buffer may influence the localization of Pelino-2 under certain circumstances.

Discussion

Relocation of the Pellino protein has been reported in smaller organisms such as *Drosophila* [21]. In this study, we aimed to characterize the intracellular localization of Pellino-2 in a human macrophage cell line, with particular emphasis on its relation to the NLRP3 inflammasome. We showed that Pellino-2 colocalizes with the inflammasome proteins NLRP3 and ASC in activated THP1-derived macrophages. In addition to the activation status of the macrophages, the absence or presence of K^+ in the extracellular environment also determined the intracellular localization of Pellino-2, suggesting that Pellino-2 may translocate between various intracellular compartments in response to K^+ efflux, a common intracellular mechanism that leads to NLRP3 inflammasome activation.

The colocalization of Pellino-2 with the NLRP3 inflammasome is in line with previous reports of Pellino-2 assisting in NLRP3 inflammasome activation [17]. Humphries and co-workers suggested that Pellino-2 participates in the inflammasome activation, by indirectly promoting Lys-63-linked poly-ubiquitination of NLRP3 in the priming step. They demonstrated that LPS induces a time-dependent interaction of Pellino-2 with NLRP3 [17]. While they did not see a direct binding of Pellino-2 to NLRP3 in immunoprecipitation experiments, Pellino-2 coimmunoprecipitated with IRAK1. This suggested that ubiquitination could occur by counteracting the inhibitory effect of the Pellino-2 interaction partner IRAK1 on NLRP3. Our observation, that Pellino-2 colocalized with the inflammasome proteins NLRP3 and ASC after activation with ATP, is consistent with an indirect effect of Pellino-2 on the NLRP3 inflammasome during the priming phase, but also suggests that Pellino-2 could also have a more direct effect during the activation phase.

The presence of activated NLRP3 inflammasomes was indicated by the restricted localization of the NLRP3 protein (Fig. 1A) and the speck formation of ASC (Fig. 1B), as well as the increased secretion of IL-1β in the supernatants of the cell culture (Fig. 2A). While resting or primed macrophages displayed typical morphological characteristics of macrophages, such as increased cytoplasmic volume, activated macrophages by LPS plus ATP treatment had a smaller cellular volume, presumably, at least in part, due to K^+ ions efflux. It is in these smaller cells that we observed colocalization between Pellino-2 and NLRP3 and ASC (Fig. 1).

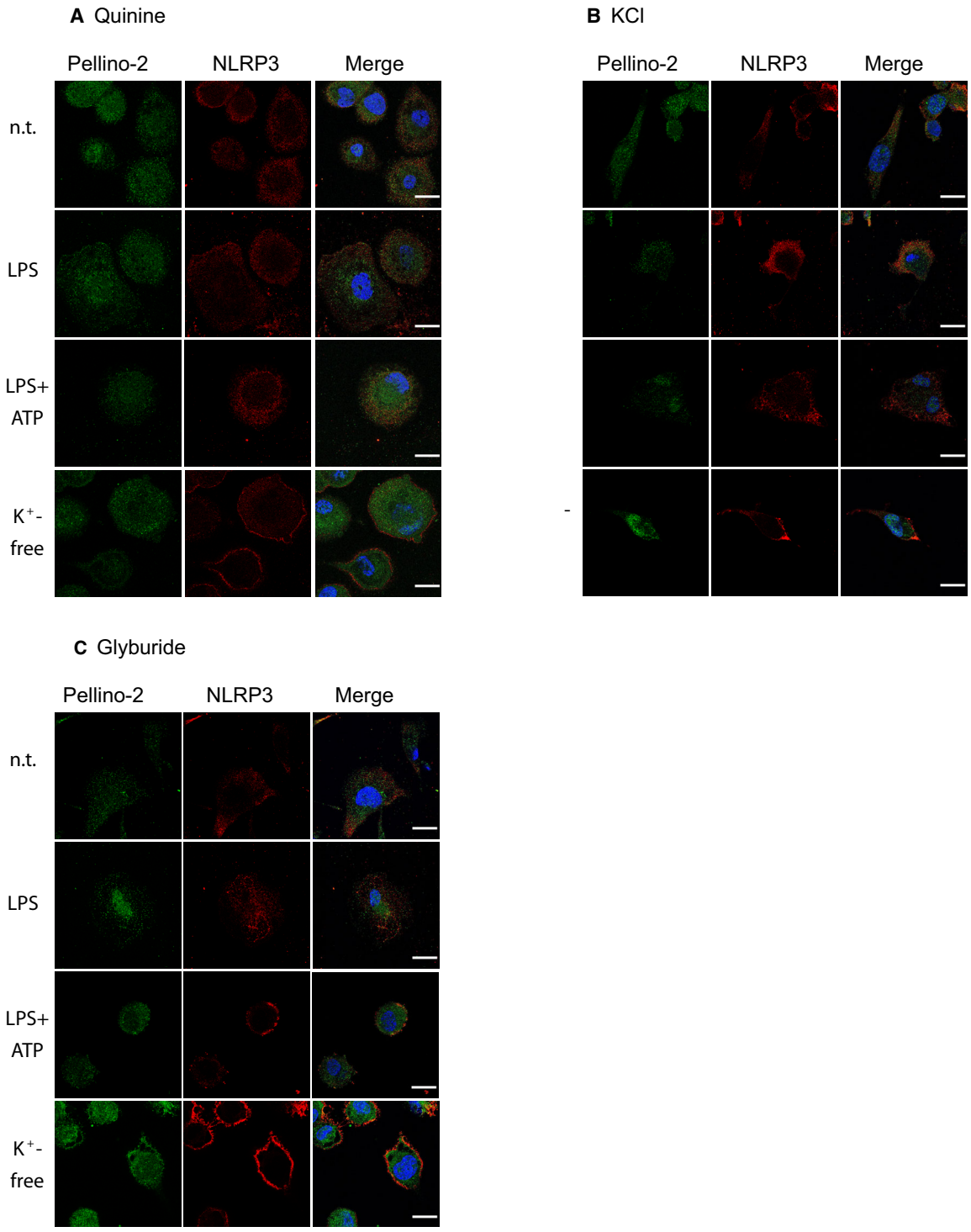

Fig. 3. Blocking the activation of the NLRP3 inflammasome abrogates colocalization between Pellino-2 and NLRP3. THP-1 monocytes were differentiated into adherent macrophages using 200 nM phorbol-12-myristate-13-acetate (PMA) for 3 days, followed by a 5-day resting period. THP1PMA macrophages were subjected to indirect immunofluorescence, using PBS as fixation buffer for the fixative agent (3% PFA). The cells were incubated for 30 min with 10 μM quinine (A), 30 mM KCl (B), or 50 μM glyburide (C), followed by no treatment (n.t.), priming with 100 $ng{\cdot}mL^{-1}$ LPS for 4 h (LPS), priming with 100 $ng{\cdot}mL^{-1}$ LPS for 4 h and stimulation with 5 mM ATP for 15 min (LPS+ATP), or cultivation in potassium-free DMEM medium for 3 h (K^+-free). The fluorescent images show endogenous Pellino-2, endogenous NLRP3, and nuclei (DAPI). Scale bar: 20 μm.

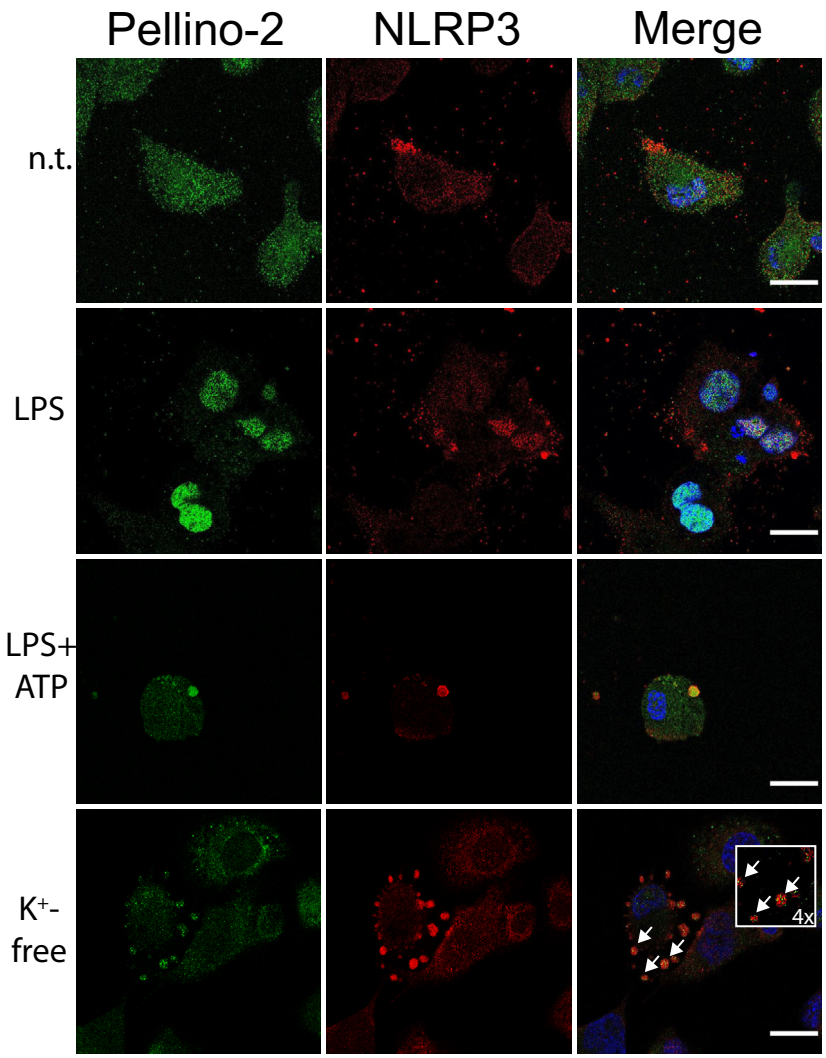

Fig. 4. The fixation buffer influences Pellino-2 localization in primed and activated macrophages. THP-1 monocyte cells were differentiated into adherent macrophages using 200 nM PMA for 3 days, followed by a 5-day resting period. THP1PMA macrophages (A) without any prior treatment (n.t.), (B) primed with 100 ng·mL^{-1} LPS for 4 h (LPS), (C) primed with 100 ng·mL^{-1} LPS for 4 h and stimulated with 5 mM ATP for 15 min (LPS+ATP), or (D) cultivated in potassium-free DMEM medium for 3 h (K^+-free) were fixed using 3% PFA in 0.1 M phosphate buffer, pH = 7.2 and subjected to indirect immunofluorescence. The fluorescent images show endogenous Pellino-2, endogenous NLRP3, and nuclei (DAPI). The inset represents a 4 times magnification of the original image, postprocessed using the deconvolution module Lightning. Scale bar: 20 μm.

In experiments using K^+-depleted culture medium, we showed that low extracellular level of K^+ alone can activate the inflammasome protein NLRP3 and initiate the interaction of Pellino-2 with NLRP3, pointing to an essential role of Pellino-2 relocation in the activation of the inflammasome. Similar observations of the effect of K^+ efflux on NLRP3 inflammasome activation have been reported by Muñoz-Planillo and coworkers [11]. They showed that a reduction in cytosolic levels of K^+ is both a necessary and sufficient step for activating the NLRP3 inflammasome in mice. However, the K^+ response of Pellino-2 in our hands was different from that of NLRP3, in that it occurred later in the inflammasome assembly and was completely blocked by several known blockers of the NLRP3 inflammasome.

While the canonical pathway for activating the NLRP3 inflammasome consists of two signals, priming and activation, in cells cultured in K^+-free medium this process seems to be reduced to a single step consisting only of K^+ efflux. Our findings suggest that Pellino-2 is a sensor for K^+ efflux that can subsequently trigger intracellular signaling downstream of TLR in the absence of a ligand.

NLRP3 inflammasome activation can be blocked by various chemicals. Quinine has been shown to inhibit K^+ efflux via the two-pore domain potassium channel TWIK2 [22]; high concentrations of extracellular K^+ ions inhibit the K^+ efflux necessary for activation of the NLRP3 inflammasome [11]; glyburide inhibits LPS plus ATP-induced caspase-1 activation, IL-1β secretion, and macrophage death [23]. We found that upon treatment with any of these blockers, signs of early activation of the NLRP3 inflammasome were present, albeit to a much lesser extent than in untreated cells. While NLRP3 relocated to the edge of the cells (Fig. 3), the levels of secreted IL-1β were significantly reduced (Fig. 2). Pellino-2, on the other hand, did not change intracellular localization when K^+ efflux was blocked with quinine, KCl, or glyburide (Fig. 3). It appears that NLRP3 can be partially activated, even in the presence of NLRP3 inflammasome blockers, suggesting that the blockers do not directly affect the NLRP3 protein. In contrast, blocking K^+ efflux prevents Pellino-2 relocation, and this could be the reason full inflammasome activation does not occur.

Paraformaldehyde is a common fixative used in immunofluorescence experiments and both PB and PBS are used as fixation buffers. Since Pellino-2 was observed to relocate in the cells in the absence of extracellular K^+, we compared Pellino-2 localization using the two buffers. When using K^+-free fixation buffer in primed immune cells, Pellino-2 surprisingly relocated to the nucleus (Fig. 4: LPS). While the significance of this finding for physiological conditions is not clear, it is important in future studies to use a K^+-containing fixation buffer to maintain physiological conditions and avoid inconsistent results.

In summary, we show that Pellino-2 colocalizes with the inflammasome proteins NLRP3 and ASC in THP1-derived activated macrophages at a late stage of the inflammasome assembly. In immunofluorescence experiments, Pellino-2 is found in different intracellular compartments, depending on the absence or presence of K^+ in the extracellular environment, indicating a role for K^+ efflux in modulating intracellular transport of Pellino-2. When blocking K^+ efflux, the relocation of Pellino-2 is abrogated, as well as the activation of the NLRP3 inflammasome. Thus, our results indicate that Pellino-2 localization is directly sensitive to K^+ efflux from the cells and modulates the activation of the NLRP3 inflammasome.

Acknowledgments

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Data accessibility

The data that support the findings of this study are available in the supplementary material of this article.

Author contributions

ER and CB conceived and supervised the study. IC and OB performed the experiments. IC, ER, and CB wrote the manuscript. IC, OB, ER, and CB designed experiments, analyzed data, and reviewed the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Video S1. Pellino-2 colocalizes with NLRP3 protein in activated macrophages. THP-1 monocytes were differentiated into adherent macrophages using 200 nM phorbol-12-myristate-13-acetate (PMA) for 3 days, followed by a 5-day resting period. THP1PMA macrophages were primed with 100 ng·mL LPS for 4 h and stimulated with 5 mM ATP for 15 min. The cells were subjected to indirect immunofluorescence, using PBS as fixation buffer for the fixative agent (3% PFA). The fluorescent images show endogenous Pellino-2, endogenous NLRP3 and nuclei (DAPI). Scale bar is 20 μm and the video frame rate 5 fps.

Video S2. Pellino-2 colocalizes with ASC protein in activated macrophages. THP-1 monocytes were differentiated into adherent macrophages using 200 nM phorbol-12-myristate-13-acetate (PMA) for 3 days, followed by a 5-day resting period. THP1PMA macrophages were primed with 100 ng·mL LPS for 4 h and stimulated with 5 mM ATP for 15 min. The cells were subjected to indirect immunofluorescence, using PBS as fixation buffer for the fixative agent (3% PFA). The fluorescent images show endogenous Pellino-2, endogenous ASC and nuclei (DAPI). Scale bar is 20 μm and the video frame rate 5 fps.

Supplementary Material Effect of K^+ ions on Pellino-2 intracellular localization.

Fig. S1. Anti-Pellino-2 antibody specifically recognizes endogenous and overexpressed Pellino-2 and is blocked by a specific blocking antigen.