Localization and Functionality of the Inflammasome in Neutrophils

**Abstract**

Neutrophils represent the major fraction of circulating immune cells and are rapidly recruited to sites of infection and inflammation. The inflammasome is a multiprotein complex that regulates the generation of IL-1 family proteins. The precise subcellular localization and functionality of the inflammasome in human neutrophils are poorly defined. Here we demonstrate that highly purified human neutrophils express key components of the NOD-like receptor family, pyrin domain containing 3 (NLRP3), and absent in melanoma 2 (AIM2) inflammasomes, but not IL-1α or IL-33. Neurophilic inflammatory diseases, such as cystic fibrosis, rheumatoid arthritis, or sepsis.

Neutrophils are the major fraction of circulating immune cells in humans. Upon infection or sterile inflammation, these phagocytes are rapidly chemoattracted to sites of injury (2). Besides their beneficial role in antibacterial and antifungal host defense, neutrophils also drive inflammation and cause tissue injury in chronic disease through release of proteases, oxidants, and proinflammatory cytokines and chemokines (3). Whereas neutrophils are well known to release IL-1β protein (4), the precise mode of IL-1β activation and secretion by human neutrophils remains only partially understood.

The “inflammasomes” are multiprotein complexes that generate IL-1 family cytokines through caspase activities, prototypically the proinflammatory cytokines IL-1β and IL-18. Inflammasome-derived cytokines are involved in a variety of infectious and noninfectious diseases and therefore represent a key target of novel anti-inflammatory targeting strategies (5). Besides this inflammasome-mediated pathway, IL-1β can also be processed from pro-IL-1β to mature IL-1β protein in a caspase-1-independent fashion through the action of serine and other proteases (6–16). Given these two distinct IL-1 family cytokine generation mechanisms, an unresolved issue in the field remains whether neutrophil-released IL-1 family cytokines, prototypically IL-1β, derive mainly from inflammasome- or serine protease-dependent activities. Among different leukocyte subtypes, capable of producing IL-1β, this caspase versus protease question is of particular relevance for neutrophils, as these phagocytes represent the major source of serine proteases, stored in their primary/azurophilic granules (3, 17). Previous studies supported the notion that serine proteases are the key component in generating mature IL-1β protein in neutrophils (6, 11, 12, 16). However, this view has been challenged by a recent elegant study demonstrating that murine neutrophils are also capable of generating mature IL-1β protein...
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independent from serine proteases (18, 19). To translate these findings into the human situation, we systematically studied the expression, subcellular localization, and function of inflammasomes in human neutrophils and the contribution of caspases and proteases in generation of IL-1 family cytokines. Because traditional neutrophil density gradient preparations are commonly contaminated with monocytes (20, 21), which represent a major source of IL-1β, we utilized highly purified human neutrophils in our studies.

Here we demonstrate that highly purified human neutrophils express key components of the NLRP3 and AIM2 inflammasome complexes at mRNA and protein levels and show that inflammasome components are localized besides the cytoplasm in distinct subcellular compartments in human neutrophils. By studying inflammasome-associated IL-1 family cytokines, we found that highly purified human neutrophils expressed and secreted IL-1β and IL-18, whereas no protein release of IL-1α or IL-33 was found. IL-1β protein release by human neutrophils was substantially affected by cell purity and protease activity.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Human Neutrophils—Peripheral blood was obtained after informed consent and approval of the study by the Ethics Committee of the University of Tübingen from healthy volunteers (n = 13, mean age: 27 years) or age-matched patients with Muckle-Wells syndrome (MWS) (n = 4; mean age, 31 years), characterized by mutations in the NLRP3/cold-induced autoinflammatory syndrome 1 (CIAS1) gene featuring constitutively increased NLRP3 inflammasome activity as described previously in detail (22). Human neutrophils and autologous peripheral blood mononuclear cells (PBMCs) were prepared by Ficol-Hypaque density gradient centrifugation of heparinized blood from healthy volunteers. After erythrocyte lysis, neutrophils were additionally enriched to highly purified neutrophils using the EasySep human neutrophil enrichment kit (STEMCELL Technologies) by negative selection using a tetrameric antibody complex recognizing CD2, CD3, CD9, CD19, CD36, CD56, glycophorin A, and dextran-coated magnetic particles. The purity of neutrophils was 98.92% as assessed by flow cytometry. The remaining 1.08% contaminating cells were debris and did not contain monocytes (supplemental Fig. 1). Cells were cultured in RPMI (RPMI 1640 medium (Biochrom) supplemented with 10% FCS, 10 mM HEPES, 1.5 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Sigma-Aldrich or PAA Laboratories) in 96-well round-bottom plates for stimulation experiments.

Subcellular Fractionation of Neutrophils—Subcellular fractionation of neutrophils was performed by nitrogen cavitation and sedimentation of the postnuclear supernatant on a four-layer Percoll density gradient. This method has been established and described previously by us (23) and has been adapted in a modified form from the method described previously in detail by Clemmensen et al. (24, 25). In brief, neutrophils isolated from peripheral blood were resuspended in disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM Na₂ATP, 3.5 mM MgCl₂, 10 mM PIPES, pH 7.2) with a protease inhibitor mixture added as described by the manufacturer (Roche Applied Science). Neutrophils were disrupted by nitrogen cavitation at 380 p.s.i. for 5 min and collected in 1.5 ml EGTA. Further, the cavitate was centrifuged at 400 × g for 15 min to remove nuclei and unbroken cells, and the supernatant was added to a Percoll solution with a density of 1.11 g/ml at a ratio of 1:1, resulting in a final density of 1.055 g/ml. Nine milliliters of the lowest density Percoll solution (1.03 g/ml) was placed in a centrifuge tube. Next, 9 ml of the 1.09 g/ml solution was placed underneath the 1.03 g/ml Percoll solution followed by the 1.055 g/ml Percoll solution to separate plasma membranes/cytosol, secretory vesicles, and gelatinase granules. Furthermore, 9 ml of the 1.12 g/ml solution was placed underneath to create a flotation medium for separation of azurophil and specific granules. The top layer with the density of 1.03 g/ml was used as a flotation medium to separate plasma membranes and secretory vesicles (26). The pH was adjusted to 7.0 by HCl. The four-layer gradient was centrifuged 20,000 × g for 40 min, resulting in five major bands, the α-band enriched in primary/azurophil granules (marker, myeloperoxidase), the β₁-band enriched in secondary/specific granules (marker, neutrophil gelatinase-associated lipocalin), the β₂-band enriched in tertiary/gelatinase granules (marker, gelatinase/matrix metalloproteinase 9), a γ₁-band enriched in secretory vesicles (marker, albumin), and the γ₂-band containing plasma membranes (marker, human leukocyte antigen). Samples were subjected to ELISA analysis or to SDS-PAGE and Western blot analysis. Myeloperoxidase, neutrophil gelatinase-associated lipocalin, matrix metalloproteinase 9, albumin, and human leukocyte antigen were quantified in each fraction by ELISA and used as marker proteins for azurophil granules, specific granules, gelatinase granules, secretory vesicles, and plasma membrane, respectively. Where indicated, a three-layer Percoll gradient was used to analyze the cytosolic neutrophil compartment (27).

Western Blotting—Neutrophil fractions were separated on Bolt 4–12% Bis-Tris Plus gels and a Bolt Mini Gel Tank (Invitrogen), and immunoblotting was performed by standard procedures using the iBlot system (Invitrogen). After blocking, primary antibodies against caspase-1 (Abcam), AIM2 (Abnova), or ASC (AdipoGen/Biomol) were incubated overnight; HRP-conjugated secondary antibodies (all from Dako) were incubated for 1 h. Blots were processed by using ECL Plus Western blot Detection Reagents (GE Healthcare). Semiquantitative analysis was performed with the Quantity One software system (Bio-Rad).

Stimulation of Cells—All inflammasome activation assays were performed according to previously published protocols for inflammasome stimulation (28). All preparatory steps were handled in a standardized manner with great caution to prevent unspecific neutrophil activation, which is a common cause of unspecific cytokine release and cell shape change. In brief, 1 × 10^6 cells of isolated PBMCs, 20 × 10^6 highly purified neutrophils (EasySep), or 20 × 10^6 partially purified neutrophils (Ficoll) were stimulated with ultrapure LPS (10 ng/ml; Invivo-
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gen) in cRPMI at 37 °C, 5% CO_2_. After 4 h, ATP (5 mM; Invivogen), nigericin (5 μM; Sigma-Aldrich) or poly(dA:dT) (50 μg/ml; Sigma-Aldrich) was added for another 2 h.

RNA Isolation, cDNA Synthesis, and Quantitative Real-time PCR—Quantitative RT-PCR was performed in isolated highly purified human peripheral blood neutrophils and isolated autologous CD14^+^ monocytes. mRNA was isolated with the NucleoSpin RNA II kit (Macherey and Nagel), cDNA was synthesized with the iScript Advanced Synthesis kit (Bio-Rad), and real-time RT-PCR was performed by using the Power SYBR Green Master Mix (Applied Biosystems) and a ViiA7 Real-time PCR cycler (Applied Biosystems) according to the manufacturer’s protocols. Results were calculated by the 2^(-ΔΔCT^) method and are given as relative expression related to the housekeeping gene β-actin. The following primers were utilized: AIM2 forward, 5′-gcatgttgacatcgtga-3′ and reverse, 5′-gcggcttctgcggactcgg-3′; ASC forward, 5′-gcggcttctgcggactcgg-3′ and reverse, 5′-tcctgacaacatgctgatgtga-3′; IL1B forward, 5′-aatctgtacctgtcctgcgtgtt-3′ and reverse, 5′-tgggacctcctccaaatgtt-3′; NLRC4 forward, 5′-tcctgacaacatgctgatgtga-3′ and reverse, 5′-tgggacctcctccaaatgtt-3′; IL1A forward, 5′-gilagccgcttgctgctgctg-3′ and reverse, 5′-ggaggcggggcttgctgctg-3′; NLRP3 forward, 5′-cagctttgctctgctc-3′ and reverse, 5′-tcttgagggctctgctc-3′; NAIP forward, 5′-aatctgtacctgtcctgcgtgtt-3′ and reverse, 5′-tgggacctcctccaaatgtt-3′; CASP1 forward, 5′-aatctgtacctgtcctgcgtgtt-3′ and reverse, 5′-tgggacctcctccaaatgtt-3′.

Confocal Microscopy—For visualization of AIM2, the samples were incubated with rabbit anti-human AIM2 (Abcam, polyclonal) and mouse anti-human CD35 (Abcam, monoclonal). The latter antibody was used as a marker for secretory vesicles. Primary antibodies were detected in confocal laser scanning microscopy by means of a secondary goat anti-rabbit immunolabeling. The specimens were analyzed with a confocal laser scanning microscope (Olympus IX 51).

Immunofluorescence Electron Microscopy—Isolated peripheral blood neutrophils were fixed in 3% paraformaldehyde and 0.01% glutaraldehyde. After centrifugation, the sediment was embedded in 3% agarose at 37 °C, and then cooled on ice. Small parts of the agarose blocks were embedded in Lowicryl (Polysciences Ltd.). Thirty-nanometer ultrathin sections were mounted on Formvar-coated nickel grids and incubated with rabbit anti-human AIM2 (Abcam) followed by 12 nm of gold-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). To increase the intensity of rabbit-anti ASC immunolabeling, the ultrathin sections were exposed to antigen retrieval in citrate buffer, pH 6.0 (Thermo), at 80 °C, incubated with rabbit anti-human ASC (AdipoGen), followed by 6 nm of gold-conjugated goat anti-rabbit IgG. Negative controls were obtained by omitting the primary antibody. Finally, the grids were counter-stained with 1% uranyl acetate (Sigma-Aldrich) and examined using a transmission electron microscope (Zeiss LIBRA 120).

ELISA—ELISA kits from R&D Systems (IL-1β, IL-1α, IL-18, IL-33) were used to quantify human protein levels in cell culture supernatants after inflammasome stimulation. Assays were performed according to the manufacturer’s recommendations.

Statistical Analysis—Data are depicted as mean ± S.E. Statistical significance of differences was determined by one-way analysis of variance followed by Bonferroni post hoc testing unless stated otherwise. In all tests, differences were considered significant at p < 0.05.

RESULTS

Human Neutrophils Express Key Inflammasome Components at mRNA and Protein Level and Store Them in Distinct Intracellular Compartments—To investigate the inflammasome machinery in human neutrophils, we quantified mRNA expression levels in highly purified neutrophils (supplemental Fig. 1). We used negatively selected highly purified neutrophils because traditionally density gradient-only (Ficoll/Percoll) isolated neutrophil fractions are known to contain a substantial proportion of contaminating monocytes, which have been found previously to modulate neutrophil homeostasis and responsiveness to inflammatory stimuli at several levels (20, 21). Using quantitative RT-PCR, we found that highly purified neutrophils expressed higher mRNA levels of caspase-1, AIM2, ASC, IL1B, and NLRP3 compared with peripheral blood monocytes, whereas IL1B, IL1A, IL18, and NLRP3/CIAS were expressed at a lower mRNA level (Fig. 1). Priming of neutrophils with LPS increased gene expression levels of IL1A and NLRP3. At the protein level, Western blot analyses confirmed
the expression of ASC, AIM2, and caspase-1 in neutrophils, whereas we were unable, using different antibodies, to detect protein expression of NLRP3 in human neutrophils (Fig. 2A and data not shown). To characterize the subcellular localization of these proteins in neutrophils precisely, we utilized subcellular fractionation. These studies demonstrated that ASC, AIM2, and caspase-1 were expressed in the cytoplasm, as already well known for other cell types, but were also co-localized noncanonically in secretory vesicle fractions, in fractions reflecting the plasma membrane and tertiary granule fractions of neutrophils (Fig. 2A). Whereas ASC and caspase-1 showed co-localization with secretory vesicle and plasma membrane fractions, AIM2 expression was observed mainly in the cytoplasm with only a low expression found in secretory vesicle fractions. ASC and, to a lesser degree, caspase-1 protein, were detected in fractions characteristic for tertiary granules (Fig.
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2A). Priming of neutrophils indicated subcellular redistribution/mobilization of inflammasome components upon LPS stimulation (supplemental Fig. 2). Immuno-transmission electron and confocal laser scanning microscopy also supported the vesicular/granular localizations of ASC and AIM2 in human neutrophils (Fig. 2, B and C).

Human Neutrophils Release IL-1β and IL-18, but Not IL-1α or IL-33—Next, we performed studies to assess whether the inflammasome machinery expressed in highly purified neutrophils is functional and mediates IL-1 family cytokine release. Therefore, we stimulated highly purified neutrophils and autologous PBMCs in parallel with canonical inflammasome activators. Because we observed in preliminary experiments already a substantial interdonor variability in IL-1β production, we included a representative number of age-matched healthy control subjects without any signs of infection, allergy, or recent medication history and performed blood drawing at a standardized time of day. These studies demonstrated that canonical inflammasome activation (priming with LPS, followed by the secondary licensing stimuli ATP, nigericin, or DNA) in highly purified human neutrophils triggered IL-1β (Fig. 3A, left panel), but not IL-18, IL-1α, or IL-33 protein secretion (Fig. 3B and supplemental Figs. 3 and 4). Autologous PBMCs were capable of secreting IL-1β, IL-18, and IL-1α in an inflammasome-dependent manner (Fig. 3, C–E), with the exception of IL-33 that was undetectable in PBMC cell culture supernatants under baseline conditions or after inflammasome activation in our assays (data not shown). IL-18 protein levels were already secreted by highly purified neutrophils at baseline conditions and could not be enhanced further through any inflammasome stimulation (Fig. 3B and supplemental Fig. 3). The amount of IL-1β protein secreted by highly purified human neutrophils was substantially lower compared with autologous PBMCs and also strikingly lower compared with traditionally partially purified neutrophils (Fig. 3F). These studies suggest that monocyte contaminations bias and overestimate IL-1β protein levels quantified in neutrophil cell culture supernatants if traditional density gradient isolations are applied (Fig. 3F). Interestingly, whereas ATP and nigericin were equally potent in triggering IL-1β release after LPS priming in PBMCs, nigericin was approximately 2-fold more potent in triggering IL-1β release by both partially and highly purified neutrophils compared with ATP. When viewed in combination, these studies demonstrate that highly purified human neutrophils are capable of releasing IL-1β, but not IL-1α or IL-33 protein, upon canonical inflammasome activation and are distinct from PBMCs in their inflammasome-mediated cytokine release characteristics.

Differential Effects of Intrinsic Inflammasome Activation on IL-1 Family Cytokine Secretion in Human Neutrophils—To investigate the effect of intrinsic inflammasome activation on neutrophils, we studied patients with MWS, a rare autosomal dominant disorder belonging to the family of diseases called cryopyrin-associated periodic syndromes, characterized by mutations in the NLRP3/CIA51 gene, featuring constitutively increased NLRP3 inflammasome activity in the absence of infection (29–31). Highly purified peripheral blood-isolated neutrophils from patients with MWS showed a tendency toward higher baseline secretion of IL-1β compared with healthy control cells, which was, however, statistically not significant (Fig. 4A, left panel), whereas IL-1α levels were undetectable in cell culture supernatants obtained from MSW or healthy control neutrophils (data not shown). After extrinsic NLRP3/NALP3 inflammasome stimulation with LPS/ATP or LPS/nigericin, highly purified neutrophils from patients with MWS released significantly lower levels of IL-1β compared with healthy control neutrophils. Release of IL-18 protein by neutrophils in these assays appeared to be inflammasome-independent and tended to be higher in healthy control than in MWS neutrophils (Fig. 4, right panel). In combination, these studies show that intrinsic inflammasome activation at baseline in MWS drives IL-1β, but not IL-1α, IL-18, or IL-33 generation in highly purified neutrophils and suggests that constitutive inflammasome activation limits exogenous ligand-induced inflammasome responsiveness.

Caspases and Serine Proteases Are Differentially Involved in IL-1β Generation in Human Neutrophils—Because serine proteases are abundantly present in human neutrophils and have been involved in IL-1β generation by cleaving pro-IL-1β, we analyzed the contribution of serine proteases for IL-1β generation in highly purified neutrophils compared with autologous PBMCs by using chemical inhibitors. In parallel, we applied the pan-caspase inhibitor Z-VAD to block the proteolytic activity of caspases in these assays. These studies demonstrated that the caspase inhibitor Z-VAD completely abrogated IL-1β generation upon LPS/ATP or LPS/nigericin stimulation in PBMCs, whereas inhibition of serine proteases had no effect on PBMC-derived IL-1β release (Fig. 5A). In contrast, in highly purified neutrophils, the inhibition of serine proteases largely abrogated LPS/ATP-induced IL-1β generation, whereas it had a weaker effect on LPS/nigericin-induced release of IL-1β protein (Fig. 5B). Chemical caspase inhibition in highly purified neutrophils almost completely abrogated LPS/nigericin-triggered IL-1β generation, whereas the effect on LPS/ATP was less substantial. Because serine proteases seemed to play no or only a minor role in these assays, IL-18 and IL-1α protein secretion by PBMCs were only analyzed with or without caspase inhibitor pretreatment. These results demonstrated that Z-VAD significantly inhibited IL-18 and IL-1α protein release by PBMCs (supplemental Fig. 4). In line with our observation that nigericin- and ATP-triggered IL-1β releasing pathways differed between highly purified neutrophils and autologous PBMCs, these studies imply that these two inflammasome inducers act differentially depending on (i) the cell types and (ii) their downstream requirement for serine proteases versus caspases for neutrophilic IL-1β generation.

DISCUSSION

Previous studies provided evidence that neutrophils represent a substantial source of IL-1β in vitro and in vivo, both in the murine and the human system (4, 6–9, 32–34). However, the subcellular localization of the inflammasome machinery and the contribution of neutrophils to the release of the IL-1 family cytokines IL-1α, IL-18 and IL-33 remained incompletely understood.

Neutrophils isolated by density gradient centrifugations are commonly contaminated with monocytes (21), which repre-
sent a major source of IL-1β, necessitating studies with highly purified human neutrophils to assess their relative quantitative role in IL-1β generation. By applying high purity human neutrophil isolation procedures, cytokine protein quantification, and subcellular nitrogen cavitation approaches (35), we are the first to demonstrate that human neutrophils store components of the inflammasome machinery both in the cytoplasm and in fractions co-localizing with secretory vesicles. Further studies demonstrate that, in contrast to PBMCs, highly purified neutrophils are unable to release IL-1α or IL-33 protein, at least under our experimental conditions. IL-18 protein was released by highly purified neutrophils, but did not show any association to inflammasome-specific stimulations, which could be due to the stimulation protocols used in our study. Based on these results, we hypothesize that in contrast to other canonical inflammasome-expressing cell types, such as monocytes/macrophages/dendritic cells, IL-1β and IL-18 protein release is differentially regulated in human neutrophils, with IL-1β secretion being largely inflammasome dependent and IL-18 secretion seeming to be, at least partially, inflammasome-independent.
Comparing partially and highly purified neutrophil fractions, our studies further demonstrated that the abundance of IL-1β protein measured in conditioned cell culture supernatants from traditional neutrophil preparations probably derives from contaminating monocytes. This could have functional relevance, because monocyte-neutrophil interactions may amplify paracrine IL-1β production and modulate other cellular cross-talk mechanisms, an issue that requires further research.

IL-1β is synthesized as a 33-kDa precursor protein, which lacks biologic activity and requires processing into the mature 18-kDa protein, a process involving proteolytic cleavage of the N-terminal part of the precursor protein. Beyond IL-1β-converting enzyme (caspase-1), previous studies demonstrated that IL-1β can be generated in a caspase-1-independent fashion (6–8). In particular, proteases capable of cleaving the pro-IL-1β protein include neutrophil serine proteases (6, 11, 12, 16),
bacterial cysteine proteases (15), granzyme A (14), matrix metalloproteinases (13), and others. Most relevant for neutrophils in this cellular context are presumably the serine proteases proteinase 3 and elastase (6, 11, 16), which are stored at high amounts in primary/azurophilic granules of neutrophils. However, murine bone marrow-derived neutrophils were recently shown to express inflammasome components and to generate IL-1β in a serine protease-independent manner (18). In our studies using highly purified human neutrophils, serine proteases were found to play a role in neutrophil-derived, but not in PBMC-derived IL-1β generation, with a stronger effect of serine protease inhibition in LPS/ATP-induced compared with LPS/nigericin-induced IL-1β generation. Further studies in highly purified human neutrophils are required to dissect the intracellular pathways that are caspase- and/or serine protease-dependent in processing IL-1 family cytokines in neutrophils.

Our studies confirm a previous study in mice by showing that also human neutrophils express inflammasome components and are capable of generating IL-1β protein (18). Moreover, this study extends the view on inflammasome-mediated cytokine elaboration in neutrophils by demonstrating that this mechanism seems to be somewhat selective for IL-1β, at least in highly purified neutrophils, because other IL-1 family cytokines, namely IL-18, IL-1α, or IL-33, were not clearly linked to in vitro inflammasome activation (IL-18) or were not detectable in conditioned cell culture supernatants (IL-1α or IL-33). These results may be explained by (i) previous reports of caspase-independent IL-18 processing in neutrophils, mediated through serine proteases (36), and/or by (ii) intracellular or cell surface-associated localizations of these cytokines in neutrophils, which are not detectable by ELISA read-out approaches in the conditioned cell culture supernatants. Methodologically, our studies also raise the point that contaminating monocytes have to be considered when interpreting IL-1β quantification results from traditional density gradient-derived human neutrophil culture supernatants.

We found that neutrophils isolated from peripheral blood of patients with MWS (cryopyrin-associated periodic syndromes), characterized by constitutively increased NLRP3 inflammasome activity, showed only minor changes in their baseline production of IL-1 family cytokines compared with age-matched healthy control cells. Upon inflammasome activation, the protein release of IL-1β and IL-18 in neutrophils was significantly lower in patients with cryopyrin-associated periodic syndromes compared with controls. We currently have no mechanistic explanation for this phenomenon, but are tempted to speculate that the mutations in the NLRP3/CIAS1 gene, leading to constitutive overactivation of the inflammasome at baseline conditions, render neutrophils unresponsive toward exogenous inflammasome activators due to consumption of pro-IL-1β protein, an issue that could not be addressed in depth in our study due to limited patient sample material. This observation may also point toward an inside-out counteracting mechanism and so far unappreciated autoregulatory feedback loop between intrinsic and extrinsic inflammasome activation, which may restrict inflammasome responsiveness in conditions when the inflammasome is already fully activated. Although being speculative, such regulatory loops could involve a down-regulation of LPS/TLR4 and/or ATP receptors (that are expressed on neutrophils) on the cell surface of MWS neutrophils, a hypothesis that should be addressed in further studies.

Neutrophils are unique in their versatility and capacity to mobilize proteins from intracellular stores to the cell surface or fuse granules with pathogen-cargo-containing phagosomes (3, 35). This flexibility provides the neutrophil with a tool to rapidly adapt to its changing microenvironment. Granules and vesicles are released in a hierarchic manner, with secretory vesicles, containing integrins and albumin, being the first released, followed by tertiary granules, containing gelatinase (matrix metalloproteinase 9) to facilitate migration through the extracellular matrix, followed by secondary/specific granules, containing lactoferrin to combat pathogens and, finally primary/azurophilic granules that contain the most harmful and toxic weapons of the phagocyte, particularly serine proteases and peroxidases (3). Accordingly, the fine-tuned balance among granule release, migration, and antimicrobial host defense orchestrates the outcome of neutrophilic inflammation. Our observation that human neutrophils express components of the inflammasome machinery, in addition to well known cytoplasmic stores (as described for a variety of cell types (37)), in subcellular fractions characteristics for secretory vesicles and tertiary granules, may implicate that neutrophils are able to dynamically regulate their inflammasomes between intracellular stores (secretory vesicles) and surface localization (fusion of secretory vesicles with plasma membrane). Among the inflammasome components we studied in human neutrophils at protein level, ASC showed the most pronounced expression and was localized in different subcellular compartments, which is in line with a previously published proteomics approach (38). These findings were confirmed by microscopic methods, indicating the vesicular/granular localizations of ASC and AIM2 in neutrophils. The subcellular mechanisms by which inflammasome components in secretory vesicles and tertiary granules could interact with and process pro-IL-1β remain elusive, but given the role of secretory vesicles as shuttle compartment between intracellular sites and the plasma membrane, it is tempting to speculate, that, based on our previous findings on RIP-G (23), microbial stimuli in the neutrophil surrounding microenvironment, such as formulated peptides (fMLP/fMLF), may trigger the translocation of inflammasome components to the neutrophil surface, where the inflammasome could participate in pathogen recognition and uptake (39, 40). The potential functional role of inflammasome components in intracellular neutrophil compartments is further supported by a recent publication showing that the NLRP3 inflammasome controls phagosomal functionality in macrophages upon infection with Gram-positive bacteria (41), a concept that remains to be studied in neutrophils. Whether neutrophils are capable of releasing vesicle/granule-stored inflammasome components into the extracellular microenvironment remains to be established.

When viewed in combination, these studies demonstrate that (i) human neutrophils express key components of the inflammasome machinery at noncanonical intracellular sites; (ii) activation of the neutrophil inflammasome mediates IL-1β, but not IL-1α, IL-18, or IL-33 release; and (iii) IL-1β secretion in human neutrophils is cell purity- and partially protease-de-
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dependent. Targeting the neutrophil inflammasome or interfering with the subcellular trafficking of inflammasome components may present a future therapeutic strategy to modulate innate immunity and inflammation in neutrophilic disease conditions such as cystic fibrosis, rheumatoid arthritis, or sepsis.

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