The NLRP3 inflammasome can be activated by pathogen-associated molecular patterns or endogenous danger-associated molecular patterns. The activation of the NLRP3 inflammasome results in proteolytic activation and secretion of cytokines of the interleukin-1 (IL-1) family. The precise mode of activation of the NLRP3 inflammasome is still elusive, but has been postulated to be mediated by reactive oxygen species (ROS) generated by an NADPH oxidase. Using primary cells from chronic granulomatous disease (CGD) patients lacking expression of p22phox, a protein that is required for the function of Nox1-4, we show that cells lacking NADPH oxidase activity are capable of secreting normal amounts of IL-1β. Thus, we provide evidence that activation of the NLRP3 inflammasome does not depend on ROS generated from an NADPH oxidase. (Blood. 2010;115(26):5398-5400)

Methods

Venous blood was collected from healthy donors and from patients, after obtaining informed consent. Blood studies had been approved by the Sanquin Research institutional medical ethical committees in accordance with the standards laid down in the 1964 Declaration of Helsinki. Heparinized peripheral blood of p22phox patients and controls was diluted 1:10 in HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) medium (132mM NaCl, 20mM HEPES, 6mM KCl, 1mM MgSO4, 1.2mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid 1:10 in HEPES (vol/vol) human serum albumin [Sanquin Reagents], pH 7.4) and cultured overnight in HEPES medium (100 μg/mL; InvivoGen), silica (100 μg/mL; Alfa Aesar), imiquimod (10 μg/mL; InvivoGen), or LPS (10 μg/mL; InvivoGen), after which the IL-1β concentration was determined in the supernatant by an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions (Sanquin Reagents). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood over isotonic Percoll, as described.6 Cells were cultured overnight in HEPES medium at a concentration of 5 x 10⁶/mL in the presence of uric acid (100 μg/mL) or silica (100 μg/mL), after which the IL-1β concentration was determined in the supernatant by payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

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ELISA. When treated with N-acetyl-L-cysteine (NAC; 25mM; Sigma-Aldrich) or diphenylene iodonium (DPI; 20 μM; Sigma-Aldrich), PBMCs were incubated for 6 hours in the presence of the indicated stimuli, after which the IL-1β concentration was determined in the supernatant by ELISA.

Results and discussion

Recently, we had the opportunity to test the secretion of IL-1β in 2 CGD patients carrying mutations in CYBA, the gene encoding the p22phox protein. These 2 patients carry different homozygous mutations in CYBA, an insertion of a C at position c.166_167, resulting in a premature stop codon in exon 3, and a missense mutation at position c.70G>A, resulting in an amino acid substitution of a glycine to an arginine at position 24.7 Expression of Nox2 and p22phox was undetectable in both patients, and ROS production in response to phorbol myristate acetate was reduced to below the detection limit and less than 5% of normal, respectively.7 We tested the secretion of IL-1β in blood samples of these patients after stimulation with NLRP3 activators, such as uric acid, silica, imiquimod, and LPS. Strikingly, we found normal secretion of IL-1β in the blood samples of these patients, despite their genetic defects in p22phox (Figure 1A). This proves that IL-1β can be effectively secreted in patient samples that lack expression of p22phox-containing NADPH oxidases and contradicts the hypothesis that ROS generation by Nox1-4 is important for NLRP3 inflammasome activation.

Furthermore, we found IL-1β secretion also to be unaffected in PBMCs isolated from the blood of 2 patients carrying a mutation in CYBB (the gene encoding Nox2), both mutations being substitutions (c.781-C>T and c.271C>T, respectively) that result in premature stop codons.8 In this report, the authors used primary cells from Nox2-deficient patients and found that NLRP3 inflammasome activation is ROS dependent in primary cells.8 In conclusion, these results exclude a role for Nox1-4 in NLRP3 inflammasome activation.

To determine whether the secretion of IL-1β was dependent on ROS from sources other than Nox1-4, we preincubated the PBMCs from healthy controls with NAC or DPI and subsequently incubated them with LPS. Collectively, our data indicate that ROS, although clearly not derived from Nox1-4 oxidase activity, do play an important role in NLRP3 inflammasome activation. A recent report by Zhou et al, which were all obtained from cell lines, are based on artefacts introduced by the short hairpin RNA (shRNA) method used to repress the expression of p22phox and/or on the use of aspecific inhibitors such as DPI.2,3

Figure 1. Normal IL-1β secretion in p22phox and Nox2-deficient patients. (A) Peripheral blood of p22phox patients and controls was diluted 1:10 and cultured overnight in the presence of uric acid (100 μg/mL), silica (100 μg/mL), imiquimod (10 μg/mL), or LPS (10 μg/mL). (B) PBMCs from Nox2-deficient patients (patients 3 and 4), a p47phox-deficient patient (patient 5), and a control were cultured overnight in the presence of uric acid (100 μg/mL) or silica (100 μg/mL).

Figure 2. IL-1β secretion is ROS dependent in primary cells. PBMCs from healthy controls were pretreated with NAC or DPI and subsequently incubated with LPS (10 μg/mL), uric acid (100 μg/mL) or imiquimod (10 μg/mL). Numbers represent the means of 3 individual donors.

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References

Human NLRP3 inflammasome activation is Nox1-4 independent

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